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Mention Microbiology, Plant Biology and Biotechnologies

Respirometric Balance and Analysis of four microalgae: *Dunaliella tertiolecta*, *Chlorella vulgaris*, *Spirulina platensis* and *Botryococcus braunii*

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Abstract

As a consequence of global warming many technologies are being developed in order to remove carbon dioxide from air. Biological carbon fixation through microalgae cultivation seems to be the most interesting one, since they allow the use of residual waters as media for growth, and industrial gases as carbon sources. Growth kinetics, metabolic behavior, nutrient requirement, biomass composition and carbon fixation capacity are essential data for industrial processes development. Such data were evaluated in four microalga of industrial interest cultivated in artificial medium with CO_2 as carbon source: *Spirulina platensis*, *Dunaliella tertiolecta*, *Chlorella vulgaris* and *Botryococcus braunii*. A respirometric balance based on gases analysis was used to evaluate carbon dioxide fixation rate and ion chromatography was employed to determine rate of consumption of nitrogen, phosphorus, potassium and magnesium for each microalga. The destination of carbon fixed was evaluated in terms of biomass and exopolyssaccharides production and as dissolved carbon, while nitrogen consumption was evaluated for protein and magnesium for chlorophyll production. *B. braunii* presented the highest CO_2 fixation rate, followed by, *S. platensis*, *D. tertiolecta* and *C. vulgaris* (496.98, 318.16, 272.40, and 251.64 mg L^{-1} day^{-1}, respectively). Nitrogen, potassium, magnesium and phosphorus (for *D. tertiolecta* calcium was analyzed instead of phosphorous) consumption rates (mg gX^{-1}) were 49.35, 32.18, 2.85 and 314.4 for *Chlorella vulgaris*; 40.72, 15.02, 2.60 and 175.9 for *B. braunii*; 61.80, 24.09, 4.17 and 247.4 for *S. platensis*; and 26.05, 59.71, 58.45 and 375.46 for *D. tertiolecta*.

Microalgae. *Dunaliella tertiolecta*. *Chlorella vulgaris*. *Botryococcus braunii*. *Spirulina platensis*. CO_2 fixation. Respirometry. Nutrients.

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1.0 INTRODUCTION

The Framework Convention on Climate Change, signed in Rio de Janeiro in 1992, made global warming a major focus and the development of technologies for reducing/absorbing greenhouse gases (GhG) gained importance.

Rubin et al (1992) divided the GhG reduction alternatives into three groups: conservation, direct mitigation and indirect mitigation. Conservation measures reduce electricity consumption and thus GhG emissions; direct mitigation techniques capture and remove CO_2 emitted by specific emissions sources; and indirect mitigation involve offsetting actions in which GhG producers support reductions in GhG emission.

The concept behind most disposal methods is to offset the immediate effect on the levels of carbon dioxide in the atmosphere by relocation, i.e., by injection into either geologic or oceanic sinks (Stewart et al, 2005). The problem with relocation that it is not the best solution; the sinks would be soon saturated once the major capacity is in ocean and deep saline formations amounting to 10^{12} ton of CO_2 while the global carbon dioxide emissions in 2009 were $31,5x10^6$ tons.

Therefore, other technologies for CO_2 and other GhG gases removal became to be developed. For Benemann (1996) GhG mitigation might promote the removal of CO_2 followed by its long-term sequestration. Various CO_2 mitigation strategies have been thus investigated, which can be generally classified into two categories: (1) chemical reaction-based and (2) biological CO_2 mitigation.

Chemical reaction-based CO_2 mitigation approaches are energy-consuming and costly processes (Lin et al. 2003), and the only economical incentive for CO_2 mitigation using the chemical reaction-based approach is the CO_2 credits to be generated under the Kyoto Protocol (Wang, 2008). Biological CO_2 mitigation has attracted much attention as a strategic alternative.

Microalgae cultivation gained importance because it associates CO_2 mitigation and production of commercial bioproducts.

Microalgae are microscopic organisms that typically grow suspended in a liquid medium and are able to use the solar energy to combine water with carbon dioxide to create biomass. In other words, basically they need a source of carbon (especially CO_2) and sunlight for growing.

Despite microalgae existence has been known for a long time, studies are relatively recent. The initial studies about microalgae cultivation began in the late 40's and early 50's as a potential source of food. Concerns about water pollution in the 1960's increased the interest in the use of microalgae in wastewater treatment. The perception in 1970's that fossil fuel would run out made these microorganisms a focus of renewable fuel production. In the 1980's microalgae were used as a source of value products, specifically nutriceuticals. In the 90's, global warming (described above) focused microalgae as an alternative.

There have been extensive studies on process optimization (media and physico-chemical parameters optimization, screening and isolation of high CO_2 tolerants, search for new valuable products, optimization and development of new vessels and systems for cultivation, for example) in order to try to overcome the economical issues faced in industrial scale production of microalgae. In addition, two other aspects are gaining importance: the use of industrial residues (in order to reduce media costs) and the carbon market (carbon credits as an additional element in the economic evaluation of the process).

The evaluation of nutrients needs in microalgal cultures is an important tool in process development using residues and the quantification of carbon dioxide fixation is of great industrial interest since it can be traded in the international market and used as a marketing move by companies.

The rate of carbon uptake is limited by the metabolic activity of microalgae, which is in turn limited by photosynthesis. The ability to identify rates of consumption of nutrients is thus of considerable importance to the understanding of the metabolism of microalgae and to avoid problems in industrial cultivation of such microorganisms.

The main objective of this study is to evaluate growth, metabolic behavior and consumption of nutrients and to quantify the carbon dioxide assimilation by four industrially important microalgae: *Spirulina platensis*, *Chlorella vulgaris*, *Dunaliella tertiolecta* and *Botryococcus braunii*, cultivated under autotrophic condition.

2.0 BIBLIOGRAPHIC REVIEW

2.1 *Microalgal Metabolism*

Microalgae are a very heterogeneous group of microorganisms. The term “microalgae” includes prokaryotes and eukaryotes. Cyanobacteria (blue-green algae) are frequently unicellular and some species forming filaments or aggregates. The internal organization of a cyanobacterial cell is prokaryotic, where a central region (nucleoplasm) is rich in DNA and a peripheral region (chromoplast) contains photosynthetic membranes. The sheets of the photosynthetic membranes are usually arranged in parallel, close to the cell surface. Eukaryotic autotrophic microorganisms are usually divided according to their light-harvesting photosynthetic pigments: Rhodophyta (red algae), Chrysophyceae (golden algae), Phaeophyceae (brown algae) and Chlorophyta (green algae). Their photosynthetic apparatus are organized in special organelles, the chloroplasts, which contain alternating layers of lipoprotein membranes (thylakoids) and aqueous phases, the stroma (Staehelin, 1986).

All photosynthetic organisms contain organic pigments for harvesting light energy. There are three major classes of pigments: chlorophylls (Chl), carotenoids and phycobilins. The chlorophylls (green pigments) and carotenoids (yellow or orange pigments) are lipophilic and associated in Chl-protein complexes, while phycobilins are hydrophilic. Chlorophyll molecules consist of a tetrapyrrole ring (polar *head,* chromophore) containing a central magnesium atom, and a long-chain terpenoid alcohol. Structurally, the various types of Chl molecules designated *a, b,* c and *d* differ in their side-group substituent on the tetrapyrrole ring. All Chl have two major absorption bands: blue or blue-green

(450-475 nm) and red (630-675 nm). Chl *a* is present in all oxygenic photoautotrophs.

Photoautotrophic cultures seldom reach very high cell densities; they are more than an order of magnitude less productive than many heterotrophic microbial cultures. However, microalgal photosynthetic mechanism is simpler than in higher plants, providing more efficient solar energy conversion. This makes microalgae the most important carbon fixative group (around 50%) and oxygen producer of the planet. Microalgae cultures have some advantages over vascular plants (Benemann, 1996): all physiological functions are carried out in a single cell, they don't differentiate into specialized cells and they multiply much faster.

Photosynthesis can be defined as a redox reaction driven by light energy, in which carbon dioxide and water are converted into carbohydrates and oxygen. The conversion is traditionally divided into two stages, the so-called *light* reactions and *dark* reactions (fig 1). The first process is the Light Dependent Process (Light Reactions), which occurs in the grana and requires the direct energy of light to make energy carrier molecules that are used in the second process. The Light Independent Process (or Dark Reactions) occurs in the stroma of the chloroplasts, where the products accumulated in the products of the Light Reaction are used to form C-C covalent bonds of carbohydrates. The Dark Reactions can usually occur if the energy carriers from the light process are present.

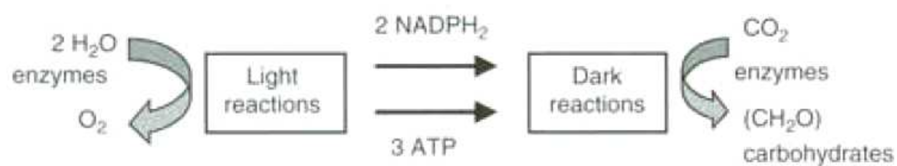


Figure 1. An overview on photosynthesis' Light and Dark reactions (Masojídek, 2004).

In the Light Dependent Processes (Light Reactions) light strikes chlorophyll *a* in such a way as to excite electrons to a higher energy state. In a series of reactions the energy is converted (along an electron transport process) into ATP and NADPH. Water is split in the process, releasing oxygen as a by-

product of the reaction. The ATP and NADPH are used to make C-C bonds in the Light Independent Process (Dark Reactions).

In the Light Independent Process, carbon dioxide from the atmosphere (or water for aquatic/marine organisms) is captured and modified by the addition of Hydrogen to form carbohydrates ($[CH_2O]_n$). The incorporation of carbon dioxide into organic compounds is known as carbon fixation. The energy comes from the first phase of the photosynthetic process (fig 2). Living systems cannot directly utilize light energy, but can, through a complicated series of reactions, convert it into C-C bond energy that can be released by glycolysis and other metabolic processes.

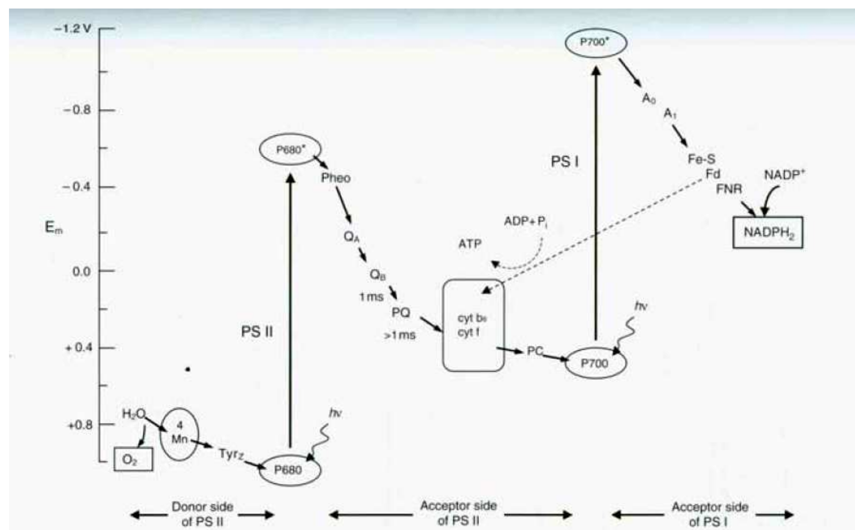
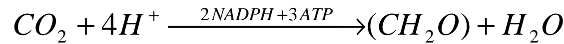


Figure 2. Electron flux and energy status over the photosynthetic process (Masojídek, 2004).

So, we can say that the main role of the light reactions is to provide the biochemical reducing agent $NADPH_2$ and the chemical energy carrier (ATP) for the assimilation of inorganic carbon, as the following reaction:

$$2\ NADP + 3\ H_2O + 2\ ADP + 2\ P_i \xrightarrow{Light, Chlorophyll} 2\ NADPH_2 + 3\ ATP + O_2$$

The fixation of carbon dioxide happens in the dark (in the stroma of chloroplasts) using the $NADPH_2$ and ATP produced in the light reaction of photosynthesis. The reaction can be expressed as:



$$CO_2 + 4H^+ \xrightarrow{2NADPH+3ATP} (CH_2O) + H_2O$$

Carbon dioxide can be available in water in three different forms: CO_2, bicarbonate (HCO_3^-) or carbonate (CO_3^{2-}) (fig 3). The relative amounts of each form are pH-dependent. CO_2 diffuse through the cell and is captured by the enzyme ribulose biphosphate (Rubisco) (Figure 4).

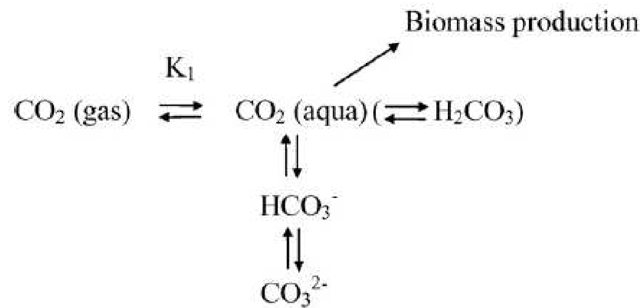


Figure 3. Different forms in which carbon dioxide is available in water.

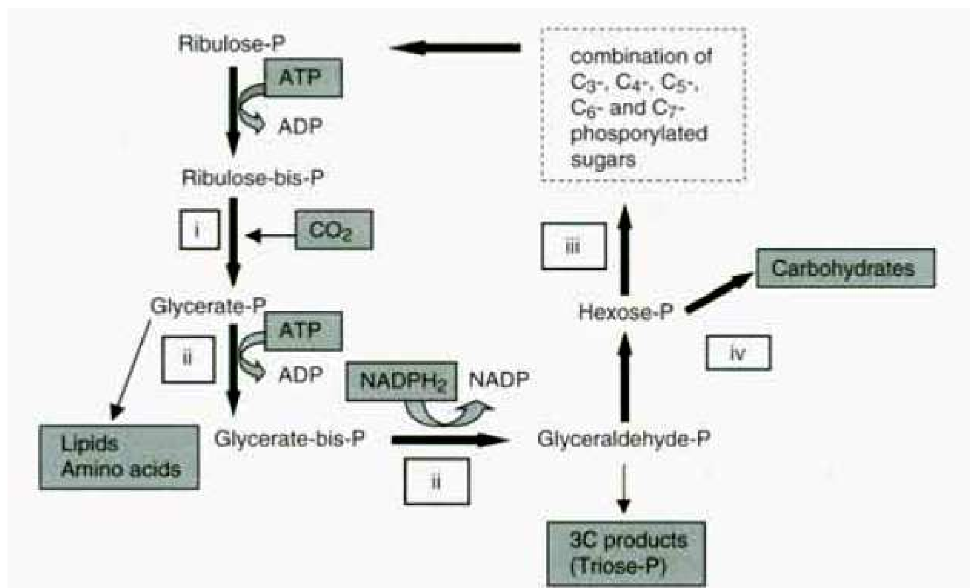


Figure 4. The dark process of CO_2 capture and transformation through metabolism of photosyntethic microalgae (Masojídek, 2004).

The fixation of CO_2 in form of sugar can be considered to occur in four distinct phases (Masojídek, 2004):

(i) *Carboxylation*: reaction whereby CO_2 is added to the 5 carbon sugar ribulose bisphosphate (Ribulose-bis-P) to form two

molecules of phosphoglycerate (Glycerate-P). This reaction is catalyzed by the enzyme ribulose biphosphate carboxylase/oxygenase (Rubisco);

(ii) *Reduction*: to convert Glycerate-P to 3-carbon products (Triose-P), energy must be added in the form of ATP and $NADPH_2$ in two steps, which are the phosphorylation of Glycerate-P to form diphosphoglycerate (Glycerate-bis-P) and the reduction of Glycerate-bis-P to phosphoglyceraldehyde (Glyceraldehyde-P) by $NADPH_2$;

(iii) *Regeneration*: Ribulose-P is regenerated for further CO_2 fixation in a complex series of reactions combining 3-, 4-, 5-, 6- and 7-carbon sugar phosphates, which are not explicitly shown in the diagram;

(iv) *Production*: primary end-products of photosynthesis are considered to be carbohydrates, but fatty acids, amino acids and organic acids are also synthesized in photosynthetic CO_2 fixation.

Photorespiration represents a competing process to carbon fixation, where the organic carbon is converted into CO_2 without any metabolic gain. Photorespiration depends on the relative concentrations of oxygen and CO_2 where a high O_2/CO_2 ratio stimulates this process, whereas a low O_2/CO_2 ratio favours carboxylation. Rubisco has low affinity to CO_2; its Km (half saturation) being roughly equal to the level of CO_2 in air. Thus, under high irradiance, high oxygen level and reduced CO_2, the reaction equilibrium is shifted towards photorespiration. For optimal yields in microalgal mass cultures, it is necessary to minimize the effects of photorespiration, achieved by an effective stripping of oxygen and by CO_2 enrichment. For this reason, microalgal mass cultures are typically grown at a much higher CO_2/O_2 ratio than that found in air.

The source of nitrogen in cultivation of microalgae seems to cause changes in oxygen production during photosynthesis. The ratio between O_2 evolution rate and CO_2 uptake rate (the photosynthetic quotient, PQ) depends on the composition of the produced biomass and the substrates that are

utilized. Especially oxidized nitrogen sources, which must be reduced before they are incorporated into the biomass, affect the PQ. When nitrate is used, it is expected a evolution of 1.3 mol O_2 per mol of CO_2 assimilated, while nitrite promotes a release of 1.2 mol O_2 and ammonia 1.0 mol O_2 (Eriksen et al, 2007). Approximately 20% of O_2 evolution equivalents can be accounted for by NO_3^- uptake and assimilation under N-replete conditions (Turpin, 1991). PQ can also be predicted from the degree of reduction of the biomass (Roels, 1980). Lipids and proteins accumulation result in a degree of reduction higher than carbohydrates.

2.2 ***Microalgae Mass Culture***

The first commercial production systems of microalgae were developed in Japan (1960's), where *Chlorella* was cultivated and sold as "health food". In the 70's *Spirulina*, a filamentous blue-green alga began to be cultivated in US and used as food supplement, aquaculture feed and food coloring. *Dunaliella* is another microalga whose industrial cultivation is well established. This alga is being used for beta-carotene production, food colorant and antioxidant/vitamin food supplement. The figure 5 illustrates an example of microalgae mass culture.

Figure 5. 90 acres commercial microalgae production facility, Kona, Hawaii. Note: green ponds culturing *Spirulina* and red ponds with *Haematococcus pluvialis*.

Latest developments have shown the potential of microalgae for the production of a variety of chemical and biologically active compounds, such as vitamins, pigments, carotenoids, proteins, aminoacids, lipids, polysaccharides,

antioxidants, antitumorals, antibiotics, antifungal agents, biofuels, and others are in progress (Becker, 1994). Table 1 shows some products produced by microalgae.

Table 1. Products produced by microalgae.

Food	Protein supplement/fortification in diets for malnourished children and adults.
Feed	Protein and vitamins supplement in feeds for poultry, cattle, pigs, fish and bivalves.
Health Food	Algal powder as ingredient and supplement in health food and products
Therapeutics	β-Carotene as possible anti-skin-cancer treatment. Algal antibiotics as wound treatment, enzymatic hydrolyzates to promote skin metabolism. Prostaglandin stimulation by γ–linolenic acid. Regulation of cholesterol synthesis. Isotopic compounds in medical researchers.
Pigments	β-Carotene as food color and food supplement (provitamin A). Xanthophylls in chicken and fish feeds. Phycobilins as food color, in diagnostics, cosmetics and analytical reagents.
Fine chemicals	Glycerol used in foods, beverages, cosmetics, pharmaceuticals. Fatty acids, lipids, waxes, sterols, hydrocarbons, amino acids, enzymes, vitamins C and E. Polysaccharides as gums, viscocifiers and ion exchangers.
Fuel	Long-chain hydrocarbons and esterified lipids as combustible oil. Hydrogen, biogas.
Hormones	Auxins, gibberellins and cytokines.
Others	Biofertilizer, soil conditioners, waste treatment.

Besides the commercial coverage of microalgae is very large, many production processes are still not feasible in industrial scale. More information about the products obtained from microalgae and their industrial uses are described in the next pages.

2.2.1 Food and Feed

The most common biotechnological application of microalgae is in aquiculture, as feed for some species of fishes and a variety of forage organisms (Derner et al, 2006). Nowadays microalgae are commercialized as natural supplement food in form of tablets or extracts (Becker, 2004).

2.2.2 Polysaccharides

Sulfonated polysaccharides are used commercially as thickening and flocculating agents. Carrageenans of red microalgae are an example. Other types include laminarin, starch, inulin, fucoidin and those comprised of xylose, arabinose, rhamnose and glucuronic acid.

2.2.3 Surfactants and fatty acids

Microalgae are a unique source of fatty acids. Much of interest in their application in aquaculture is due to unsaturated C18 and C22 fatty acids. The change in human diet in the last years associated with the increasing number of sicknesses as a consequence of low consume of polyunsaturated fatty acids, as well the therapeutical properties associated (Jiang, 1999), turns much attention to the production of fatty acids by microalgae (Simopoulus, 2002)..

Biodegradable biosurfactants from microalgae are glycolipids or long-chain fatty acids, such as phosphatydylglycerol, phosphatydylcholine, etc. Essential fatty acids found on microalgae include linolenic acid (18:2), gamma-linolenic (18:3) acid (GLA), eicosapentaeonic acid (20:5) (EPA), arachidonic acid (20:4) (ARA) and DHA (22:6) (Becker, 1994).

2.2.4 Aminoacids and vitamins

Several aminoacids could be potentially produced by microalgae. Under high salinity some species accumulate proline; blue-green algae can contain high concentration of cyanophycin bodies.

2.2.5 Pigments

Industrial interest in microalgae pigments is mainly due to the therapeutical properties associated, but includes use as food additive. As light absorbing systems, microalgae have high concentration and a great variety of pigments. As examples, beta-carotene is produced by *Dunaliella*, phycocyanin by *Spirulina*, lutein by *Neospongiococcus*, astaxanthin by *Haematococcus*.

2.2.6 Pharmaceutical and diagnostics

Some microalgal species show biological activity, such as antibacterial, antiviral, antifungal and even anti cancer. There is not yet industrial production of any of these pharmaceutical agents, since the way between finding a biological activity and the commercial final product is very long. But there is already some diagnostic reagents produced by these microorganisms, specifically phycobiliproteins of blue-green and red microalgae used as fluorescent labels in research and diagnostic kits (Europa Bioproducts - http://www.europa-bioproducts.com/).

2.2.7 Water treatment

Nowadays many investigations are being made about the metal absorption capacity of microalgae and about phosphorus and nitrogen consumption. Figure 6 is a example of large scale cultivation of microalgae for wastewater treatment.

Figure 6. Microalgae wastewater treatment ponds at Hollister, California (USA).

2.3 ***Microalgae Culture Fundamentals***

Studies on microalgae are preferably done under controlled conditions. Microalgae bioreactors are often designed differently from bioreactors used to grow other microorganisms. Two parameters are very useful to characterize the efficiency of microalgae cultivation systems: the volumetric productivity and the efficiency of light utilization.

The volumetric productivity is the product of the biomass density and the specific growth rate, which is enhanced by providing carbon (as CO_2, for example) in the media. A high volumetric productivity is beneficial because this means a smaller cultivation system. Moreover, a high volumetric productivity usually is accompanied with a high biomass density, which is more attractive with respect to downstream processing. However, working with photosynthetic microorganisms, the efficiency of light utilization should be considered too. Thus, supply, distribution and utilization of light in microalgal cultures are therefore central aspects.

Mixing, process monitoring and control, and exploration of heterotrophic and recombinant microalgae are other aspects of microalgal culturing that have seen novel developments in recent years.

2.3.1 Media for microalgae

As any other microorganism, microalgae have nutritional needs: carbon source, energy, water and inorganic nutrients. In the case of microalgae, the carbon source can be CO_2 and the energy comes from sunlight. As microalgae grow in aqueous suspension, the manipulation and control of culture conditions makes their cultivation feasible, thus the productivity is limited mostly by the available of light. Reponses by algal cells to nutrients and cultivation environment can be used to manipulate the processes to favor the production of algal biomass (Benemann et al, 2002).

The development of media for microalgae cultivation involves: a sufficient carbon source (up to 50% of the algal biomass); salt concentration (depending on the original biotope of the alga); nitrogen (represents about 5-10% of microalgae dry weight); phosphorus (DNA, RNA, ATP, cell membrane); sulfur (constituent of aminoacids, vitamins, sulfolipids, is involved in protein biosynthesis); potassium (cofactor for several enzymes and is involved in protein synthesis and osmotic regulation); magnesium (the central atom of the chlorophyll molecule); iron (constituent of cytrochromes and important in nitrogen assimilation); pH of the medium; temperature; trace elements and addition of organic compounds and growth promoters.

Carbon is important because it is the source of energy for many cellular events (such as metabolites production), reproduction and is part of the physical structure of the cell. In conditions of low dissolved inorganic carbon (DIC), a DIC transport is induced in most microalgae (Matsuda and Colman, 1995), allowing normal cell growth.

Nitrogen is used for the production of a wide range of organic molecules, such as protein, chlorophyll, vitamins, ficobilins and aminoacids. Nitrogen can also be accumulated and stored in the intracellular medium in the form of inorganic substances, especially under stress conditions. In N-sufficient cells amino acid synthesis depends on recent photosynthesis to provide carbon skeletons. Although nitrogen is assimilated by microalgae in form of ammonium, Lourenco and collaborators (2002) observed toxic effects on microalgal growth.

Nutrient-limited algae exhibit perturbations to their physiology, and this is often reflected in, and determined by, parameters such as C:N ratio, protein: carbohydrate ratios, and nutrient-uptake characteristics (Roberts et al, 2008).

During nutrient depleted growth phases, starch and other carbon and energy storage compounds may accumulate and constitute a major part of the biomass production in green algae (Zhila et al, 2005). In nitrogen depleted and carbon sufficient, accumulation of carbon and energy storage compounds may account for all the produced biomass (Stenholm et al, 1998). Depletion of nutrients, in particular the nitrogen source, also results in break-down of the photosynthetic apparatus (Coleman et al, 1988), including the photosynthetic pigments (Eriksen et al, 1995).

Depending on the material used in cultivation of microalgae and the utilization of biomass, three different systems can be distinguished (Becker, 1994):

I. Systems in which a selected algal strain is grown in a so called clean process, using fresh water, mineral nutrients and carbon sources. The algae in such systems are intended to be utilized mainly as food supplement.
II. Systems using sewage or industrial waste waters as the culture medium. The cultivation of the microalgae involves secondary (BOD removal) and tertiary (nutrient removal) treatments and production of commercially interesting products.
III. Cultivation of algae in enclosed systems under sunlight or artificial light, with cells being grown preferably in autotrophic media.

Microalgae are microorganisms that are capable of producing many different compounds of industrial interest, some with high and some with low aggregated value. The final value of the product and its destination influence directly the conditions of cultivation. Therapeutical compounds produced by microalgae, for example, must be produced through a totally controlled and clean process, while for fuels industry it can be used residues and the control of the process can be less accurate.

The utilization of complex media (those whose composition is not determinated, such as industrial residues) in the cultivation of microalgae is one alternative to turn the production of some microalgae's metabolites economically feasible. Associated to residue composition and microalgae

metabolic operation, the knowledge of the needs of the microalgae might save time (and money) and might help in the development of a process. It is very important to supply all microalgae chemical needs because it is known that variations in the chemical composition of phytoplankton are also tightly coupled to changes in growth rate (Goldman, 1979). To a large degree, this growth rate dependence provides a good description of the nutritional state of a cell population in response to different degrees of nutrient limitation (Rhee, 1973).

2.3.2 Cultivation vessels

Many different configurations of photobioreactors are possible: from simple unmixed open pounds to highly complex enclosed ones (Fig 7). Most of the recent research in microalgal culturing has been carried out in photobioreactors with external light supplies, with large surface areas, short internal light paths, and small dark zones. Examples include open ponds (the cheaper ones), tubular reactors, flat panel reactors, column reactors (stirred tank reactors, bubble columns, airlift).

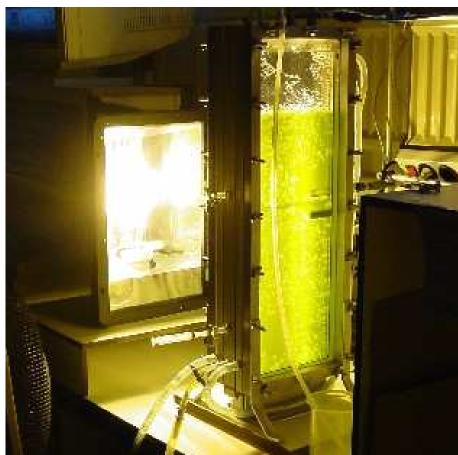


Figure 7. Some of the many types of reactors used for microalgal cells cultivation: (a) open ponds (raceway), (b) tubular reactor and (c) flat panel.

The applications of such systems range from the small-scale production of high value products to the large-scale and very low cost culture of algae, so the choice between the different designs of photobioreactors must be specific to the application intended and local circumstances.

Open ponds can be an important and cost-effective component of large-scale cultivation technology, and optimal design parameters have been known for many years. The elongated "raceway-type" of open pond, using paddlewheels for recirculation and mixing, was developed in the 1950s by the Kohlenbiologische Forschungsstation in Dortmund, Germany. However, sustained open pond production proved to be feasible for only three microalgae: *Spirulina platensis*, *Dunaliella salina* and fast grow *Chlorella*, in all cases because contamination by other species can be avoided.

2.3.2.1 Light diffusion

The most important parameter considered for the development and utilization of a kind of reactor for microalgae cultivation is the light diffusion. The productivity of photoautotrophic cultures is primarily limited by the supply of light and suffers from low energy conversion efficiencies caused by inhomogeneous distribution of light inside the cultures (Grobbelaar 2000). At culture surfaces, light intensities are high but absorption and scattering result in decreasing light intensities and complex photosynthetic productivity profiles inside the cultures (Ogbonna and Tanaka 2000). High light intensities at culture surfaces may cause photoinhibition, and the efficiency of light energy conversion into biomass, the photosynthetic efficiency (PE) is low. An overdose of excitation energy can lead to production of toxic species (e.g. singlet oxygen) and to photosynthesis damage (Janssen, 2002)

By minimizing depth, volume is reduced, light diffusion is maximized, and so is cell concentration (Fig 8). From the cited types of photobioreactors, open ponds are usually 10-30 cm depth, tubular reactors 1 to 5 cm, flat panel reactors 2 to 5 cm.

Reactor type light source	PFFD $\mu mol\ m^{-2}\ s^{-1}$	E $MJ\ m^{-2}\ day^{-1}$	Light path cm	Volume l	Algal strain	x $g\ l^{-1}$	P_{volume} $g\ l^{-1}\ day^{-1}$	P_{area} $g\ m^{-2}\ day^{-1}$	PE %	Ref.
Tubular photobioreactors										
Artificial	80	–	1.2	5.5	*Spirulina*	–	0.42		8.1	A
Sun	–	13.5	10	*146*	*Arthrospira*	2.37	1.15	25.4	*4.7*[b]	B
Sun	1,126	*21.2*[a]	6	200	*Phaeodactylum*	2.29	1.15	19.1	2.3[b]	C
Sun	2,690	*50.5*[a]	6	200	*Phaeodactylum*	4.1	1.52	25.3	*1.3*[b]	C
Flat panel photobioreactors										
Sun[c]	–	*9.4*	0.6	400	*Chlorella*	–	*3.8*	22.8	5.6	D
Sun[c]	–	*6.4*	0.6	400	*Chlorella*	–	*3.2*	19.4	6.9	D
Column photobioreactors										
Sun	ca. 1,000	9.6	5	120	*Tetraselmis*	1.7	0.42	38.2	9.6	E
Alternative reactor designs										
Parabola/sun	–	–	–	70	*Chlorococcum*	1–2	0.09	14.9	–	F
Dome/sun	–	–	–	130	*Chlorococcum*	1–2	0.1	11.0	–	F

Figure 8. Biomass densities (x), volumetric and areal productivities (P_{volume} and P_{area}), and photosynthetic efficiencies (PE) of selected photoautotrophic cultures grown in different types of enclosed photobioreactors and under various photosynthetic photon flux densities (PFFD) or light energy supplies (E) (see Eriksen, 2008).

The light regimen itself is influenced by incident light intensity, reactor design and dimension, cell density, pigmentation of the cells, mixing pattern, and more. In outdoor photobioreactors the light regimen is also influenced by geographical location, time of the day, and weather conditions. Nowadays, open paddle wheel-mixed pound is the most commonly used photobioreactor.

Some studies discuss the effect of mixing and productivity due to the “flashing light” effect: a few milliseconds flashes of high light intensity followed by a several-fold longer period of darkness do not reduce culture productivity from those under constant illumination (Kok, 1953). This effect is not observed in ponds, where the light/dark period is longer. As an example, while light/dark cycles of 94/94 ms were sufficiently short to increase the photosynthesis efficiency (PE) in cultures of *Dunaliella tertiolecta*, light/dark cycles of 3/3 s were too long and the PE decreased in comparison to continuously illuminated cultures (Janssen et al. 2001).

2.3.2.2 Mixing

To optimize the photosynthesis rate and the gases solubility in the media, mixing is very important. Besides that, mixing is important for homogeneous distribution of cells, metabolites, and heat and to transfer of gasses across gas–

liquid interfaces. Mixing can be done mechanically by paddle wheel in raceways (fig 9) or by gas flow in bubble columns.

Figure 9. Paddle wheel mixing of raceway ponds in Earthrise Farms Inc.

2.3.3 Carbon dioxide

Another issue in most photobioreactors is the CO_2 diffusion to the culture media. The solubility of CO_2 in the culture media depends on the depth of the pound, the mixing velocity, the productivity of the system, the alkalinity and the outgassing. It has been reported (Becker, 1994) that only 13–20% of the supplied CO_2 was absorbed in raceway ponds when CO_2 gas was sparged into the culture fluid as a carbon source. Gas–liquid contact time and gas–liquid interfacial area are, therefore, two key factors to enhance the gas–liquid mass transfer. In addition, high oxygen tension is problematic, since it promotes CO_2 outgassing and competes with CO_2 for the CO_2 fixing enzyme (RUBISCO).

Weissman and Goebel (1987) investigated different alternatives for introducing and storing carbon dioxide in a microalgal growth medium. The capacity for carbon dioxide storage in a growth medium is important because it determines the number of carbonation stations required for a pond of specified size, depth, and liquid velocity or, alternatively, the maximum pond size

serviceable with one CO_2 station. The first observation is that water chemistry will have a significant impact on the amount of carbon dioxide stored. pH is the major determinant of the relative concentrations of the carbonaceous system species in water and affects the availability of carbon for algal photosynthesis in intensive cultures (Azov, 1982).

The absorption of CO_2 into alkaline waters may be accelerated by one of two major uncatalyzed reaction paths: the hydration of CO_2 and subsequent acid-base reaction to form bicarbonate ion, and the direct reaction of CO_2 with the hydroxyl ion to form bicarbonate. The rate of the former reaction is faster at pH values below 8, while the latter dominates above pH 10. Between pH 8 and 10, both can be important (see figure 10).

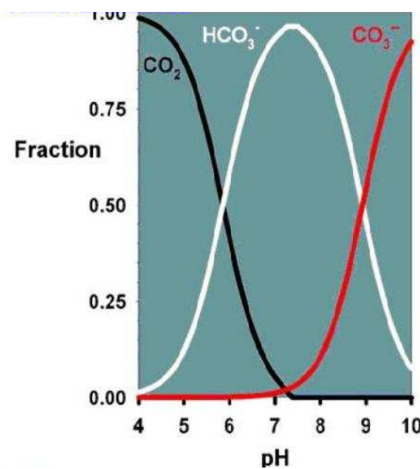


Figure 10. The dependence of pH of the media and the form that carbon dioxide is present.

2.3.4 Scaling Up

Scale up is one of the most difficult tasks in outdoor mass culture. At this point contamination poses the greatest problem, due to the dilute inoculum (Richmond, 1990).

There are two methods of scaling up to the production ponds. The first is by scaling up following a dilution ratio of 1:10 through successive volumes, the second is to derive the inocullum from an existing culture pond (Andersen, 2005). The latter method is preferable because the first one require a longer

time, and thus became more expensive. During the first steps of scaling up, data on the effect of environmental factors (light, contamination, temperature) must be collected and the performance of agitation, aeration and medium composition studied. Even in established large scale cultivation, a small pond must be maintained to serve as fresh inocullum.

2.3.5 Culture Monitoring

Successful culture maintenance requires continuous monitoring. The most basic kind and the most used is microscopic examination, enabling the detection of contamination and abnormal morphological changes. Early detection allows control measures to be undertaken to guarantee the stability of the system.

Routine tests on nutrient composition are used to avoid unexpected nutrient deficiencies. Regular monitoring of changes in pH, O_2 and CO_2 levels are essential. Healthy cultures show a regular diurnal pattern in these parameters; any significant variation is usually sign of problems in the culture.

2.4 *Carbon Uptake by Microalgae*

Microalgae can fixate carbon dioxide from different sources including CO_2 from the atmosphere, from industrial exhaust gases (e.g. furnaces flue gases) and in form of soluble carbonates. Traditionally microalgae are cultivated in open or closed reactors and aerated with air or air enriched with CO_2. Industrial exhaust gases contain up to 15% of carbon dioxide in its composition, being a rich source of carbon for microalgae growth. It is also possible to fixate CO_2 by chemical reactions to produce mineralized source of carbon.

Since outdoor sunlight cannot be controlled, carbon fixation by microalgae is usually studied indoors under artificial illumination. Several authors have studied the microalgae CO_2 fixation capacity. Most of them focused the fixation into biomass (Chae et al 2006, Jacob-Lopes et al 2008, Kajiwara et al 1997). However, these studies did not quantified the total carbon

dioxide fixed effectively by microalgae (Jacob-Lopes et al 2008, Fan et al 2007), since there are innumerous routes for carbon destination besides biomass generation. Other researches focused on the determination of global rates of carbon dioxide sequestration through mass balances for this component in the liquid or gas phase of the systems (Eriksen et al 2007). Carbon dioxide fixation, described in the literature includes, among others, mineralization (formation of soluble bicarbonate and carbonate) and production of extracellular products such as polysaccharides, volatile organic compounds (Shaw et al 2003), fuels, organohalogens (Scarratt et al 1996), hormones.

This indicates that carbon fixation by microalgae is a complex process where biomass production might be only part of the total carbon destination. In addition, little information is available with respect to the simultaneous research of both the global rates of carbon dioxide sequestration and the rates of incorporation of carbon into the microalgae biomass (Chiu et al 2008).

In microalgae cultivation, usually, higher concentration of CO_2 is not used because it may result in decreasing the pH since unutilized CO_2 will be converted to HCO_3^-. Shiraiwa et al (1991) and Aizawa et al (1986) reported that an increase in CO_2 concentration of several percent resulted in the loss of carbon concentration mechanism (CCM) and any further increase was always disadvantageous to cell growth.

If there is not enough CO_2 gas supply, algae will utilize (bi)carbonate to maintain its growth. When algae use CO_2 from bicarbonate an increase of pH is observed. To overcome this pH fluctuation, the CO_2 gas injection might be controlled in a way that photosynthesis rate are balanced with enough and continuous availability of dissolved carbon. Maintaining constant the CO_2 free concentration in the media will keep constant the carbon uptake.

The ability to accumulate dissolved inorganic carbon (DIC) has been shown to occur in many algae and cyanobacteria (Williams and Colman, 1995). Whilst CO_2 can diffuse into algal cells and is the substrate for carbon fixation by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), it forms a small proportion of the total available inorganic carbon. The largest proportion of total dissolved inorganic carbon (DIC) available to microalgae consists of ionic HCO_3^-, which has a low capacity for diffusion across cell membranes (Young et

al, 2001). A number of eukaryotic microalgae have developed mechanisms which permit the use of HCO_3^- for photosynthesis (Miller and Canvin, 1985). Access to the larger pool of HCO_3^- is assumed to involve one or both of two basic processes :

(i) In some green algae the use of HCO_3^-, has been correlated with the presence of external carbonic anhydrase (CA) activity (Aizawa and Miyachi et al, 1986). In these cases external CA is thought to facilitate the use of HCO_3^-, by maintaining equilibrium between HCO_3^-, and CO_2, and thereby maintaining the supply of CO_2, to a CO_2, transporter (Aizawa and Miyachi, 1986).

(ii) Direct HCO_3^- transport via a transmembrane bicarbonate transporter, which has been demonstrated even in cells that have external CA activity (Williams and Turpin, 1987). The involvement of transmembrane ATPase proteins was also reported in DIC uptake by chlorophytes (Ramazanov et al. 1995).

Kajiwara et al. (1997) found that *Synechococcus* achieved a maximum CO_2 uptake rate of 0.6 g L^{-1} day^{-1} at a cell mass concentration of 0.286 g L^{-1}. Hirata et al. (1996) used *Chlorella sp*. UK001, and achieved a mean rate of CO_2 fixation was 0.0318 g_{CO2} L^{-1} day^{-1} with an efficiency of conversion of energy to biomass equal to 4.3%. Marukami et al (1997) using *Synechocystis aquatilis* in a 5 l bioreactor and optimized conditions, obtained a maximum CO_2 fixation rate of 1.5 g_{CO2} L^{-1} day^{-1}. They isolated more than 10 strains with high capability of CO_2 fixation. Further experimentation using *Botryococcus braunii* gave a growth rate of around 0.5 g L^{-1} day^{-1}.

The table 2 (from Wang et al, 2008) gives rates for carbon dioxide fixation by microalgae.

Table 2. Data from the literature for cultivation parameters (%CO_2 and T), productivity (P) and CO_2 fixation rate (P_{CO2}) for different microalgae (see Wang et al, 2008)

Microalga	CO_2%	T °C	P g l^{-1} per day	P_{CO2} g l^{-1} per day
Chlorococcum littorale	40	30	N/A	1.0
Chlorella kessleri	18	30	0.087	0.163[a]
Chlorella sp. UK001	15	35	N/A	>1
Chlorella vulgaris	15		N/A	0.624
Chlorella vulgaris	air	25	0.040	0.075[a]
Chlorella vulgaris	air	25	0.024	0.045[a]
Chlorella sp.	40	42	N/A	1.0
Dunaliella	3	27	0.17	0.313[a]
Haematococcus pluvialis	16–34	20	0.076	0.143
Scenedesmus obliquus	Air	-	0.009	0.016
Scenedesmus obliquus	Air	-	0.016	0.031
Botryococcus braunii	–	25–30	1.1	>1.0
Scenedesmus obliquus	18	30	0.14	0.26
[b]*Spirulina* sp.	12	30	0.22	0.413[a]

2.4.1 Carbon market

In January 2005 the EU-wide CO_2 emissions trading system (EU-ETS) has formally entered into operation. The EU-ETS requires a cap-and-trade program whereby the right to emit a particular amount of CO_2 becomes a tradable commodity (Benz et al, 2009).

The carbon market jumped from 63 billion dollars in 2007 to 126 billion in 2008, which means almost 12 times the value of 2005, according to a World Bank report. Credits were sold for 4.8 billion tons of carbon dioxide, a value 61% higher than that of the previous year.

The mechanism of carbon credits in the European Union grew 87% last year, reaching 92 billion dollars, according to the World Bank. The numbers for the year of 2009 have not yet been announced. At the same time, the cuts in emissions actually made and sold by clean energy projects registered (at United Nations) in developing countries fell 30% in 2008.

Brazil is the third country in carbon credit generation, and the leader in Latin America. The most common type of CDM (Clean Development mechanism) in Brazil is the transformation of sugar cane bagasse into sugar and alcohol.

2.5 ***Microalgae Description and Specific Carbon Uptake Review***

2.5.1 *Chlorella vulgaris*

The first photosynthetic microbe to be isolated and grown in pure culture was the freshwater microalga, *Chlorella vulgaris*. It is a spherical unicellular eukaryotic green algae which presents a thick cell wall (100 to 200 nm) as the main characteristic. This cell wall provides mechanical and chemical protection and it is reported its relation with heavy metals resistance, which explains why *C. vulgaris* is one of the most used microorganisms for waste treatment. The chemical composition of *Chlorella* can be dramatically affected by cultivation conditions, from 8.7% protein and 86% lipid (oil) to 58% protein and 4.5% lipid (Spoehr and Milner, 1949).

The uptake of carbon by *C. vulgaris* cells is done through the enzyme carbonic anhydrase, which catalyzes the hydration of CO_2 to form HCO_3^- and a proton. Hirata and collaborators studies upon carbon dioxide fixation in 1996 by this microalga showed important variations comparing cultivation under fluorescent lamps and sunlight. In the first case the estimated rate of carbon dioxide fixation was 865 mg CO_2 L^{-1} d^{-1}; while in sunlight regimen the estimated rate achieves 31.8 mg CO_2 L^{-1} d^{-1}. Winajarko et al (2008) achieved a transferred rate of 441.6 g CO_2 L^{-1} d^{-1} under the same cultivation conditions of Hirata et al (1996).

Carbon fixation by *Chlorella vulgaris* is variable and depends, among other factors, on the concentration of CO_2 in the gaseous source. While Yun et al (1997) cultivated *C. vulgaris* in 15% of carbon dioxide and achieved a fixation of 624 mg L^{-1} day^{-1}; Scragg et al (2002) achieved a fixation of 75 mg L^{-1} day^{-1} under CO_2 concentration of 0.03%. In the same study, Scragg tested a medium with low nitrogen and the fixation rate was 45 mg L^{-1} day^{-1}, suggesting that nitrogen also influences carbon uptake rate.

Some studies (Chinassamy et al 2009; Morais and Costa 2007) indicate that the best concentration of CO_2 for *C. vulgaris* growth is near to 6%.

2.5.2 *Botryococcus braunii*

Botryococcus is a colonial microalga which is widespread in fresh and brackish waters of all continents. It is characterized by its slow growth and by containing up to 50% by weight of hydrocarbons. *B. braunii* is classified into A, B and L races (table 3) based on the difference between the hydrocarbons produced (Metzger and Largeau, 2005).

Table 3. Races of B. braunii and their different characteristics (from Banerjee et al, 2002).

B. braunii			
	Race-A	Race-B	Race-L
Nature of hydrocarbon	C_{25}–C_{31} odd numbered n-alkadienes/trienes	Botryococcenes (triterpenes) C_nH_{2n-10}, n = 30–37	Lycopadienes (tetra-Terpene) $C_{40}H_{78}$
Colony color in stationary phase	Pale yellow or green	Orange-reddish or orange-brownish due to accumulation of carotenoids	
Long chain alkenyl phenols	Present	Absent	Absent
Nature of biopolymers	Very long aliphatic chains cross-linked by ether bridges and bearing fatty esters		Tetraterpenoid cross-linked by ether bridges

The cells of *B. braunii* are embedded in a communal extracellular matrix (or "cup"), which is impregnated with oils and cellular exudates (Blackburn, 1936). Cells are attached to each other by a refringent material that sometimes links two or more distinct clumps of cell. The wall of each cell possesses an internal fibrillar layer made of polysaccharide (Largeau et al, 1980). *B. braunii* is capable of synthesizing exopolyssaccharides, as was first reported for the A race (Casadevall et al., 1985). The yield of EPS ranges from 250 g m^{-3} for A and B races to 1 kg m^{-3} for the L race. Higher growth and production of EPS occur when nitrate is the nitrogen source instead of urea or ammonium salts (Banerjee et al, 2002). Phosphorus and nitrogen are also important factors in accumulation of hydrocarbons by the microorganism (Jun et al, 2003).

The metabolic energy devoted to produce such large amounts of hydrocarbons makes this species noncompetitive in open mass cultures, since strains not so burdened can grow much faster and soon dominate an outdoor pond culture (Benemann, 2003). *B. braunii* has been reported to convert 3% of

the solar energy to hydrocarbons (Gudin et al., 1984). Being synthesized by a photosynthetic organism, hydrocarbons from algae can be burned without contributing to the CO_2 concentration in the atmosphere.

Dayananda et al (2005) cultivated Botryococcus braunii strain SAG 30.81 in shake flasks and obtained a maximum cell concentration of 0,65 g L^{-1} under 16:8 light dark cycle. Experiments with different strains of *B. Braunii* indicate that the biomass yield is inversely proportional to lipids accumulation. The maximum biomass yield achieved was 2 g L^{-1} (with 40% of lipids) and the lower was 0.2 g L^{-1} (with 60% of lipids). Outdoor experiments with this microalga achieved a high biomass yield of 1.8 g L^{-1} but a very low lipid accumulation. It was also showed by Dayananda and collaborators that exopolyssaccharides production by *Botryococcus braunii* SAG 30.81 is not affected by light regimen in MBM media, differently than from lipids and proteins production.

Marukami et al (1997) achieved a carbon dioxide fixation greater than 1 gram per liter by *Botryococcus braunii* cultivated for hydrocarbon accumulation.

2.5.3 Spirulina platensis

Spirulina are multicellular and filamentous blue-green microalgae belonging to two separate genera *Spirulina* and *Arthrospira* and consists of about 15 species (Habib et al, 2008). It grows in water, reproduce by binary fission and can be harvested and processed easily, having significantly high macro- and micronutrient contents. Their main photosynthetic pigment is phycocyanin, which is blue in color. The helical shape of the filaments (or trichomes) is characteristic of the genus and is maintained only in a liquid environment or culture medium.

Spirulina is found in soil, marshes, freshwater, brackish water, seawater and thermal springs. Alkaline, saline water (>30 g/l) with high pH (8.5–11.0) favor good production of *Spirulina*, especially where there is a high level of solar radiation. It predominates in higher pH and water conductivity. Like most cyanobacteria, *Spirulina* is an obligate photoautotroph, i.e. it cannot grow in the

dark on media containing organic carbon compounds. It reduces carbon dioxide in the light and assimilates mainly nitrates.

Spirulina contains unusually high amounts of protein, between 55 and 70 percent by dry weight, depending upon the source (Phang *et al.*, 2000). It has a high amount of polyunsaturated fatty acids (PUFAs), 1.5–2.0 percent of 5–6 percent total lipid and is a good source of vitamins (B_1, B_2, B_3, B_6, B_9, B_{12}, C, D, E). Spirulina is a rich source of potassium, and also contains calcium, chromium, copper, iron, magnesium, manganese, phosphorus, selenium, sodium and zinc. These bacteria also contain chlorophyll a and carotenoids.

The optimum pH of the Spirulina sp. culture is between 8.5 and 9.5 (Watanabe et al, 1995). Cyanobacteria possess a CO_2-concentating mechanism that involves active CO_2 uptake and HCO_3^- transport. In experiments conducted by Morais and Costa (2007), carbon fixation in terms of biomass by *Spirulina platensis* was estimated in 413 mg L^{-1} d^{-1} (Wang, 2008).

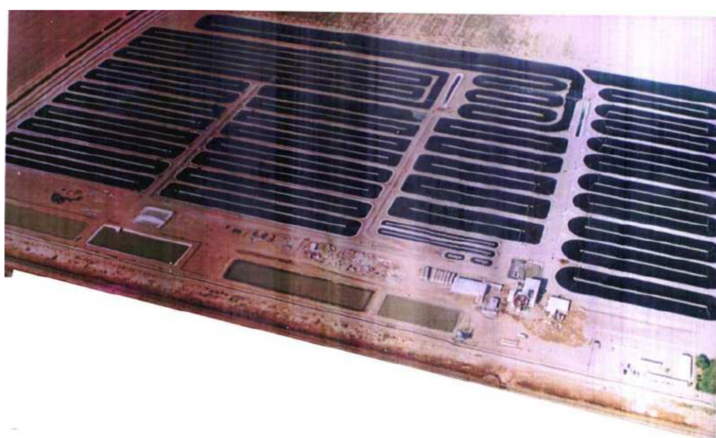


Figure 11. *Spirulina* production facility of Earthrise Farms, Inc., in California.

2.5.4 Dunaliella tertiolecta

Dunaliella tertiolecta is a marine unicellular green algal species. This round-shaped algal species is native to New Zealand and is found in brackish environments; it is a motile species and has a high tolerance for salt, temperature, and light. *Dunaliella tertiolecta* is relatively easy to culture. The cell divides by simple binary fission and no evidence of cell lysis, encystment, or

spore formation is observed (Segovia et al., 2003). Motion of cells is important since it facilitates nutrient transport, specially when in poor-nutrient waters.

Nitrate assimilation system in *Dunaliella tertiolecta* was studied by Grant in 1967 and 1968. The system is located completely within the chloroplast and is dependent on CO_2 and light. *D. tertiolecta,* although showing a large increase in both nitrate and nitrite assimilation in light, did not show the stoichiometric evolution of extra oxygen found in other algae, as described in pages 18 and19. It was also noticed that the CO_2 evolved in a 2:1 ratio to NO_3 assimilated and a 1.5:1 ratio to nitrite assimilated (Grant, 1968).

It is generally accepted that *D. tertiolecta* changes the internal concentration of glycerol in order to survive to different NaCl concentrations (the osmotic balance achieved with glycerol). The calvin-benson cycle normally functions under constant NaCl extracellular concentration, and under such conditions it is needless to produce glycerol at a high rate. Internal pH and salt shock could trigger starch degradation and formation of glycerol (Goyal et al, 1989). *D. tertiolecta* require Na^+ for the uptake of phosphate. It is also suggested that there is a Na^+/K^+ pump in these cells (Avron et al, 1992).

Dunaliella tertiolecta thrives over a wide pH range and expresses a capacity for extremely efficient DIC accumulation, incorporating a capacity to use HCO_3^- in addition to CO_2 (Aizawa *et al*., 1986; Young et al, 2001). Kishimoto et al (1994) cultivated a *Dunaliella* strain for pigment production with 3% of CO_2 and achieved a carbon uptake of 313 mg L^{-1} day^{-1}.

Dunaliella is an important microalgae for industrial processes since it produces a wide variety of commercial products (mainly pigments) and the rupture of the cells is very easy.

3.0 MATERIAL AND METHODS

3.1 *Microorganisms and Culture Conditions*

C. vulgaris LEB-104 was obtained from Federal University of Santa Maria (UFSM, Brazil) and cultivated in Modified Bristol Medium (Watanabe, 1960).

D. tertiolecta SAG 13.86 was obtained from the Culture Collection of Algae at Gottingen and cultivated in artificial sea water (DUN medium).

Botryococcus braunii SAG 30.81 was obtained from the Culture Collection of Algae at Gottingen, Germany, and grown at 25ºC in 3N-MBM medium.

Spirulina platensis strain LEB 52 was obtained from Federal University of Santa Maria (UFSM) and grown in Zarrouk (Zarrouk 1966) modified medium. As carbon dioxide was used as carbon source, the amount of bicarbonate on the recipe was reduced to 2.8 g L^{-1}, the lowest concentration needed to achieve buffering effect (Andrade et al, 2008).

Table 4 summarizes the specific conditions used in each of the microalga cultivation. To all of experiments nitrate was added at 1 g L^{-1}.

Table 4. The conditions of growing for *S. platensis*, *D. tertiolecta*, *C. vulgaris* and *B. braunii*.

Parameter	*C. vulgaris*	*D. tertiolecta*	*B. braunii*	*S. platensis*
Temperature (ºC)	30	25	25	30
pH	7.2±0.2	7.2±0.2	7.2±0.2	9.0±0.2
Aeration (L/min)	1,0	1,0	1,0	1,0
Carbon dioxide (ml/min)	50	50	50	50
Light (lux)	3500	3500	3500	3500
Mechanical Agitation (rpm)	200	200	150	150
Volume (liters)	8	8	8	8

3.2 ***Cultivation conditions***

The main cultivations were performed in a 11L BioFlo Fermentor (New Brunswick Sci) (fig 12) with working volume of 8 Liters. For pH measurement a pH sensor was used and the pH was controlled by automatic injection of specific acid and/or base as required.

Air enriched with CO_2 (White Martins, Curitiba, Brazil) was sparged through a ring sparger and the gases concentration in exhaust gas measured as described below. Illumination of culture was provided by eight cool white 32W fluorescent lamps (providing 3500 lux) in 12:12 (light:dark) hours photoperiod. Mechanical agitation was also provided as indicated in table 4. Temperature was measured by a thermocouple and controlled. Experiments duration was 15 days for all microalgae tested.

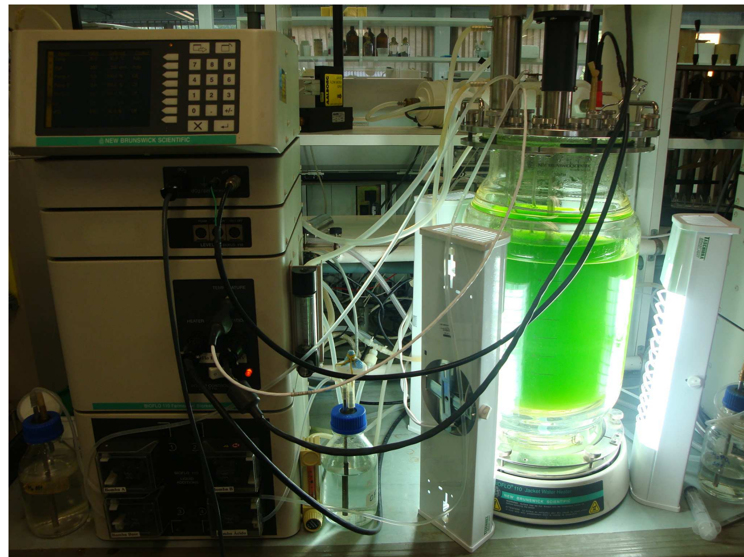


Figure 12. New Brunswick reactor and control cabinet used for the cultivation of microalgae. Eight cool white lamps were used for illumination.

3.3 ***Kinetic Parameters Calculation***

In all experiments the growth kinetics parameters was calculated. Based on the growth curve the exponential phase was identified as the linear interval plotting the biomass concentration logarithmic versus time. In the exponential phase (maximum) specific growth rate was obtained by equation 1 and biomass doubling time by equation 2. Maximum cell productivity was determined by the maximum difference between biomass concentration in a day and the day before.

$$\mu = \frac{(\log N - \log N_i) \cdot 2{,}303}{t - t_i} \qquad \text{Equation 1}$$

where N and N_i are, respectively, biomass concentration at the end and beginning of exponential phase, t and ti are the time in days.

$$td = \frac{\ln 2}{\mu} \qquad \text{Equation 2}$$

3.4 ***Analytical Determinations***

Samples were withdrawn each 24 hours and centrifuged in a Sorvall Legend Mach 1.6 R centrifuge (Sorvall, Germany) at 246xg for 15 minutes. Cells were washed once and dried at 60ºC, while the cell-free medium was used for further analysis of nitrate, alkalinity, phosphorus and cations concentration.

3.4.1 *Nitrate*

Nitrate determinations were done daily by the colorimetric method proposed by Cataldo (1975).

The method is based on the formation of color by the addition of 800 µl of a solution of 5% salicylic acid in pure H_2SO_4 to 200 µl of the nitrate solution (in this case the cell-free medium) completing the volume to 20 ml with NaOH 2M after 20 minutes. The nitrate absorbance is read in a spectrophotometer at 410 nm. The nitrate concentration is determined using the previously prepared standard curve (fig 13).

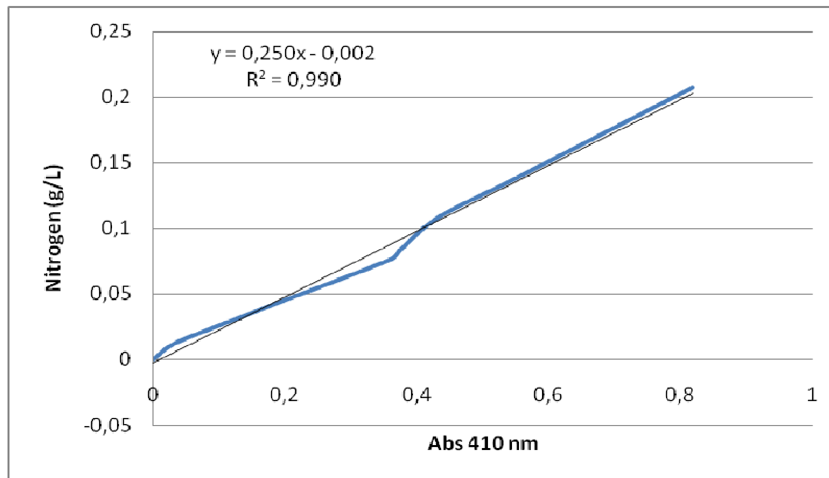


Figure 13. Standard curve of nitrogen for analysis of Nitrate in the growth media.

3.4.2 Alkalinity

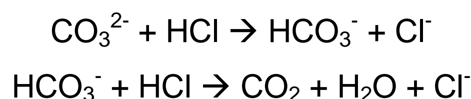
Alkalinity was also measured daily by titration of 10 ml of the cell-free medium with 1.0 N HCl using as dye indicators phenolphthalein (0.2 g L^{-1} in ethanol 95%) for carbonate and methylorange (0.5 g L^{-1} in water) for bicarbonate.

Phenolphthalein is added to 10 milliliters of the cell-free medium and titration is conducted with HCl 0.1N until disappearance of color. To this sample methylorange is added and titrated until formation of pink/red color.

The alkalinity depends on the composition and pH of the solution, with:

- pH > 9.4 → carbonate and hydroxide;
- 9.4>pH>8.3 → carbonate and bicarbonate
- pH>4.4 → CO_2

Alkalinity is thus calculated considering the volume of acid solution used and the following reactions:



$$CO_3^{2-} + HCl \rightarrow HCO_3^- + Cl^-$$
$$HCO_3^- + HCl \rightarrow CO_2 + H_2O + Cl^-$$

In all cases, except for *Spirulina*, the main form (and the only that was considerate in calculations) of dissolved CO_2 was HCO_3^-. For *Spirulina* the

quantification was done firstly by titration of CO_3^{2-} (v_1) and then of HCO_3^- (v_2), considering the amount of bicarbonate as v_1- v_2.

3.4.3 Cations Analysis

The determination of cations was done with a 761 Compact IC 817 Bioscan chromatograph. The column used was METROSEP C3 250/4.0 (Metrohm), 250 mL x 4.0 mmID. Analytical conditions were: 3.5 mM HNO_3, 1.0 mL/min, 40ºC, 20 µL sample volume, 11.2 MPa. A standard chromatogram was prepared with the following salts: $CaCl_2.2H_2O$, $MgCl_2.6H_2O$, KCl, Na_2SO_4, $ZnSO_4.7H_2O$, NH_4Cl e $FeSO_4.7H_2O$.

3.4.4 Phosphorus Analysis

Phosphorus consumption was assessed during the experiment in 5 days intervals by the quantification of soluble phosphorus in the media by the phospho-molybdate method. To 400 µl of cell-free medium was added 1.5 ml of a solution containing ammonium molybdate (1M), pure acetone and 5N sulfuric acid 1:2:1 (in volume). After homogenization, 100 µl of citric acid 100 mM was added. In presence of phosphorus a yellow color is developed, and read in 410 nm against a standard curve.

3.4.5 Total carbohydrate analysis

The cell-free medium from the last day was analyzed for total sugars by the phenol-sulfuric method (Dubois et al, 1956). To 0.5 ml of supernatant, 0.5 ml of a solution of 5% phenol was added, followed by the addition of 2.5 ml of concentrated sulfuric acid. The quantification was done by reading the samples in spectrophotometer at 490 nm and comparing with a standard curve (figure 14) made using glucose.

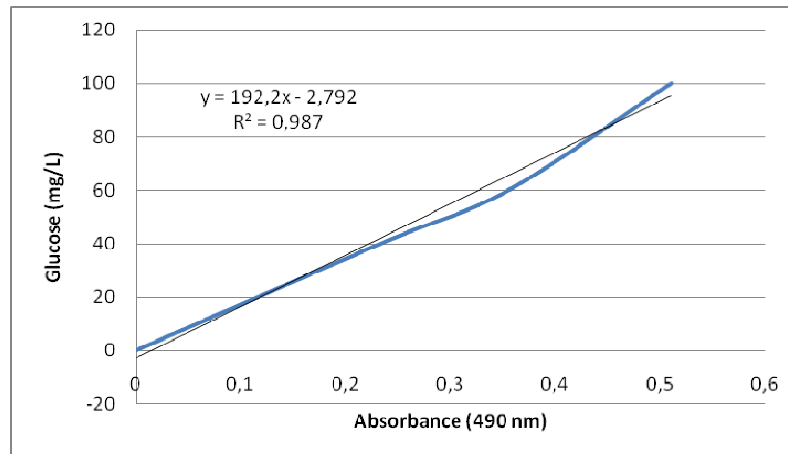


Figure 14. Standard curve for total carbohydrate analysis by the Phenol-Sulfuric method.

3.5 *Carbon Dioxide Data Acquisition*

The cultivation vessel was coupled with sensors for the measurement of carbon dioxide and oxygen in the gas inlet and outlet (figure 15). In the inlet, carbon dioxide flow was monitored by a rotameter and measured by a thermal dispersion mass flow sensor (Aalborg GFM) (figure 16); while oxygen concentration in air was measured by an electrochemical sensor (Alphasense O2-A2) and monitored by a rotameter. In the outlet, total flow was measured by a mass flow sensor (Aalborg model GFM), the percentage of carbon dioxide in the air was measured by an infrared sensor (Vaisala GMT) and the percentage of oxygen by the O2-A2 sensor. Data acquisition occurred at 15 minutes intervals by Laquis software (Laquis, 2009). These sensors were all connected to Novus model N1100 controllers (figure 17) to achieve an industrial net requirement (STURM et al. 2008). To perform the calculations, this industrial net was connected to a personal computer running the Laquis software (figure 17).

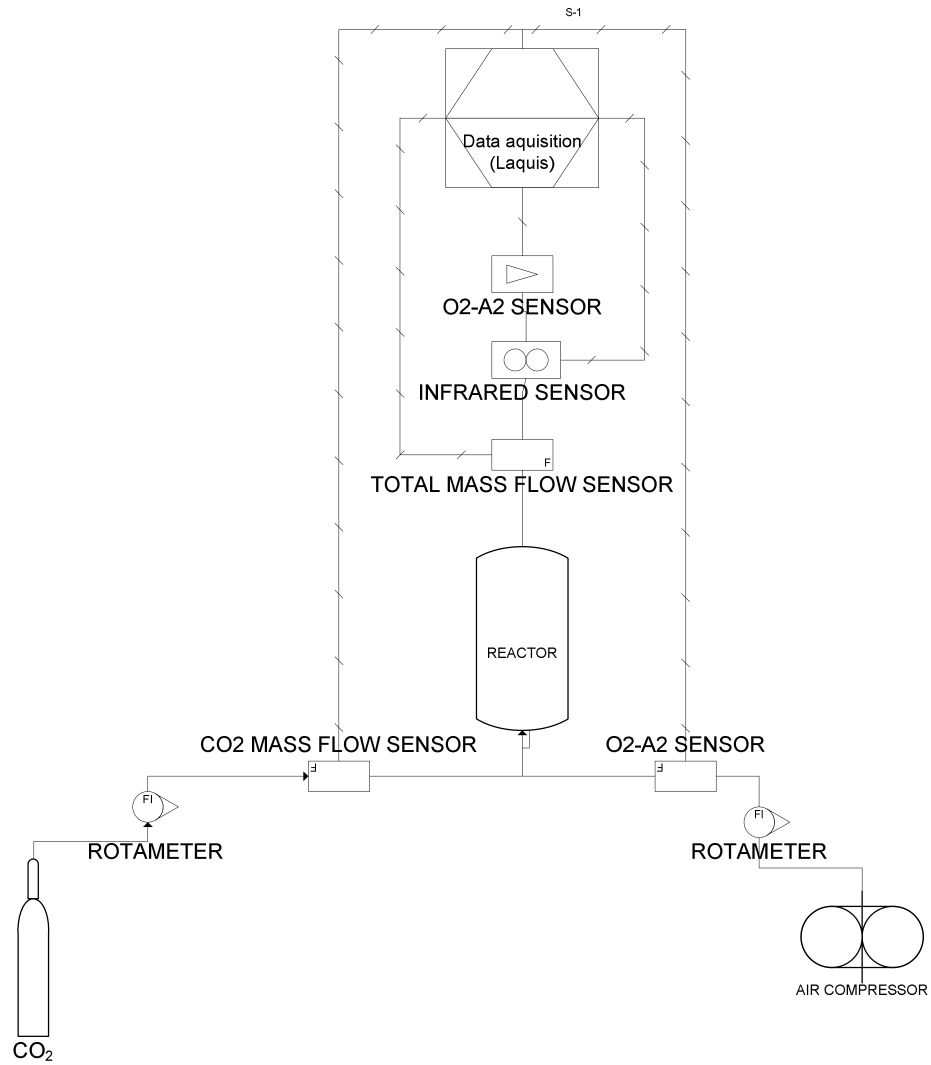


Figure 15. Scheme of the instrumentation used in carbon dioxide fixation quantification by microalgae.

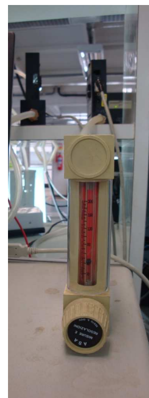


Figure 16. Rotameter used in CO_2 flow measurement in first plan. On the top at right there is the thermal dispersion mass flow sensor Aalborg GFM. On the top at left there is the other mass flow sensor (used for total flow measurement).

Figure 17. The Novus model N1100 controllers and the data acquisition by a personal computer with Laquis software.

A blank trial, using only media in the vessel, was run for 5 days with data acquisition to obtain total saturation of the media with carbon dioxide and to be used as basis to further calculations of carbon dioxide consumption.

In the Annex I the calculations made from the data acquired by the software are presented. The data obtained for carbon dioxide fixation (grams per hour) was plotted against time and the area was obtained by integration by the trapezoidal method as described by Etter and Ingber (2007).

3.6 **Biomass Analysis**

After 15 days of experiment, the cells removed by centrifugation were dried at 60ºC until constant weight. The dried biomass was analyzed for chlorophyll, carbohydrate, protein, lipids and ash.

3.6.1 Pigments

The biomass was firstly extracted with 90% acetone at 4ºC overnight. For each gram of dry biomass, 40 ml of the solvent was used. After centrifugation (120xg, 10 min), the cell-free medium was analyzed for total pigments and for chlorophyll *a* content.

The quantification of total chlorophyll (Chl) was based in the somatory of the equations proposed by Strickland & Parsons (1968):

$$Chla\left(\frac{mg}{m^3}\right) = \frac{11{,}6 \cdot D_{665} - (1{,}31 \cdot D_{645} + 0{,}14 \cdot D_{630} + D_{750})}{V_2 \cdot L} \cdot V_1$$

$$Chlb\left(\frac{mb}{m^3}\right) = \frac{(20{,}7 \cdot D_{645} - 4{,}34 \cdot D_{665} - 4{,}42 \cdot D_{630})}{V_2 \cdot L} \cdot V_1$$

$$Chlc\left(\frac{mg}{m^3}\right) = \frac{(55 \cdot D_{630} - 4{,}64 \cdot D_{665} - 16{,}3 \cdot D_{645})}{V_2 \cdot L} \cdot V_1$$

where: D = absorbance reading in the specific wavelength,

V1 = acetone 90% volume (ml),

V2 = volume of sample (liter),

L = optical path of the cuvette (cm).

After the spectrophotometric analysis, the acetone extract was dried at 60ºC until constant weight. The difference between chlorophyll a calculation and the dry weight was called “other pigments”.

3.6.2 Lipids Extraction

The acetone-extracted dried biomass was extracted with methanol: chloroform 1:1 (Bligh and Dyer, 1978) for lipid quantification. The extraction was realized in a Soxhlet extractor, which improves the removal of lipids from solid material promoting heated solvent recirculation. To the chloroform:methanol extract was added hexane (liquid-liquid extraction). The nonpolar phase was considered as lipid.

3.6.3 Total Carbohydrates Quantification

For total carbohydrate quantification by the Phenol-Sulfuric method (Dubois et al, 1956), the biomass was hydrolysed with 0.1N NaOH overnight at 4ºC.

3.6.4 Protein Quantification

For proteins quantification the biomass was hydrolysed with 0,1N NaOH overnight at 4ºC. The Lowry protein assay method (Lowry, 1951) combines the reactions of cupric ions with the peptide bonds under alkaline conditions with the oxidation of aromatic protein residues.

A standard curve with bovine serum albumin (figure 18) was used for the determination of protein concentration in the biomass.

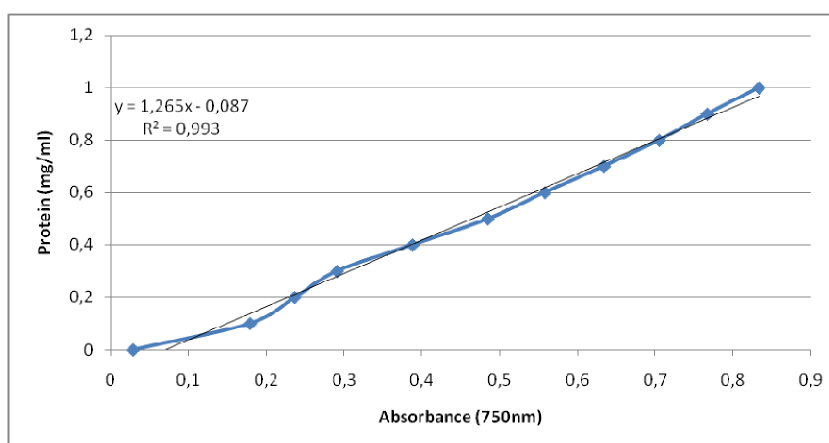


Figure 18. Standard curve used for protein quantification in microalgae biomass by the method of Lowry (1951).

3.6.5 Ash

The biomass obtained from the bioreactor was analyzed for ash content by the AOAC 941.12 method with some modifications. A sample of three grams of biomass was weighted and incinerated so that the sample fumes off without catching fire. Then incinerated samples were heated at 550ºC in furnace for 4 hours and distilled water was added. After careful evaporation of the water, the

samples were put again in a furnace (Quimis, Brazil). The samples were weighted after cooling in dessicator to room temperature until constant weight.

4.0 RESULTS

4.1 *Chlorella vulgaris LEB 104*

4.1.1 Growth Parameters

Figure 19 shows the growth profile of *Chlorella vulgaris* LEB 104. Maximum cell concentration (1.94 g L^{-1}) was reached on the last day (15th) of cultivation. Maximum specific growth rate of 0.29 d^{-1} was determinated during the exponential growth (96 to 168 hours), the biomass doubling time was 2.39 days, and the maximum productivity was 0.31 g L^{-1} d^{-1}.

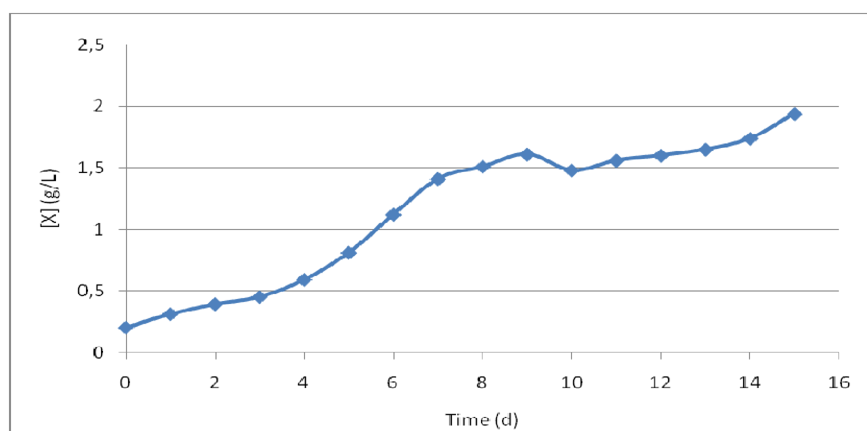


Figure 19. *C. vulgaris* LEB 104 growth profile in MBM media.

4.1.2 Media Analysis

Carbon solubility (C_{SOL}) was determinated daily during cultivation of *C. vulgaris* and compared with the biomass production (X_t – X_0, where X_0 is the

biomass concentration in the beginning of the experiment and X_t is the concentrationin a given time) (figure 20).

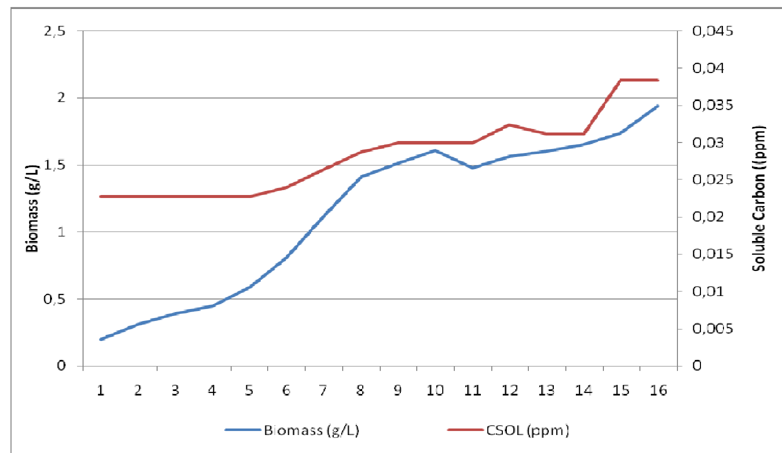


Figure 20. Dissolved carbon in form of bicarbonate in MBM during growth of *C. vulgaris*.

The profile of cations dissolved in MBM media is shown in fig 21 (the chromatograms can be seen in Annex II) . The rates of consumption of nitrogen, potassium and magnesium were calculated by divinding the difference between the initial and final concentration by total biomass produced (X) and resulted in, respectively, 49.35, 32.18 and 2.85 gX^{-1}.

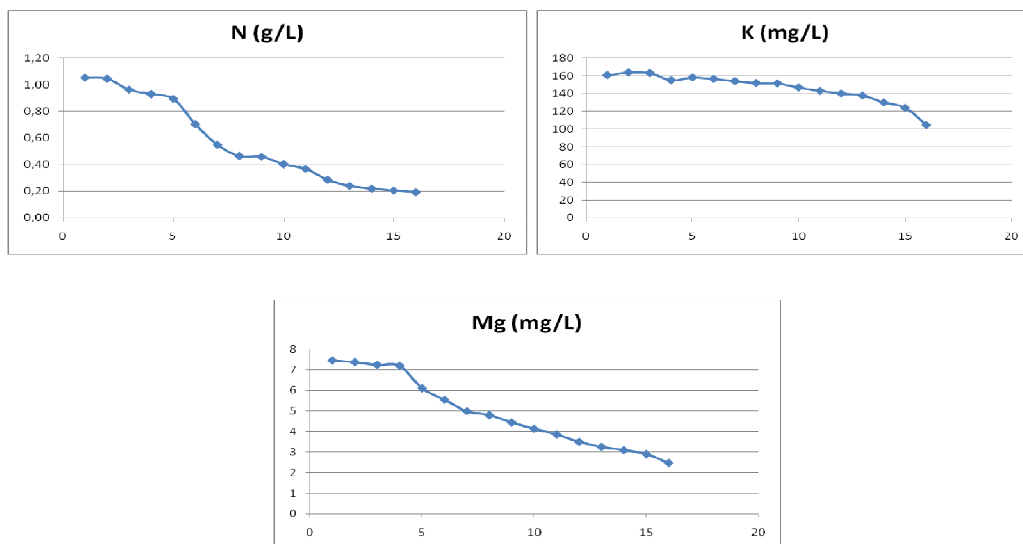


Figure 21. The profile of consumption of nitrogen, potassium, magnesium obtained by ion cromatography.

The accumulated production of biomass was plotted against the accumulated consumption of each cation. These are showed in figure 22.

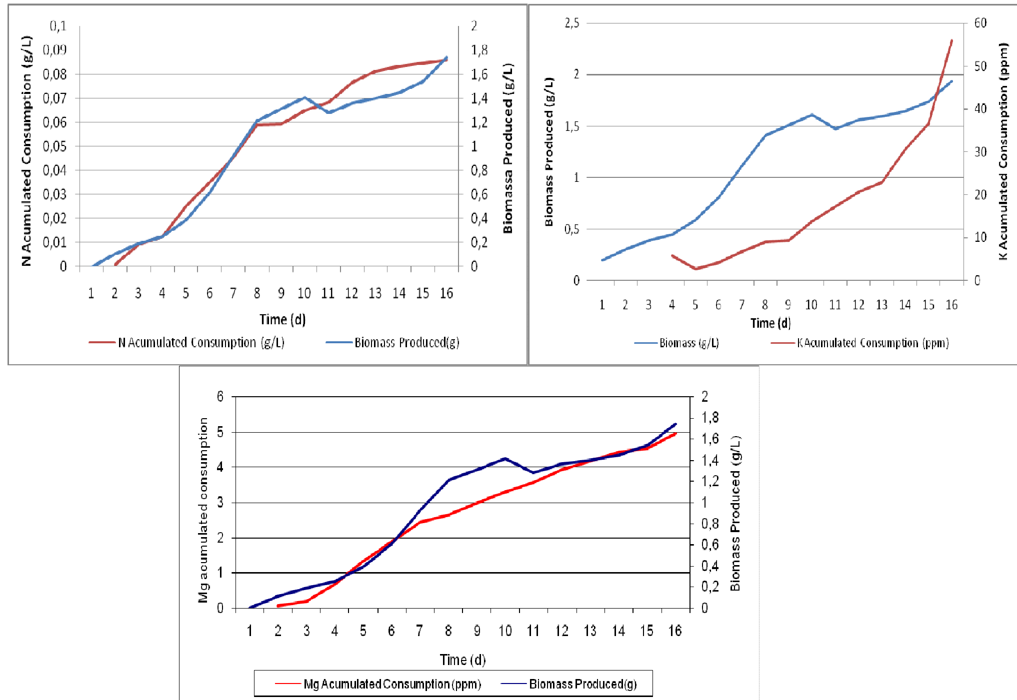


Figure 22. Relation between accumulated biomass production and accumulated consumption of each cation analyzed.

Dissolved phosphorus was also analyzed at 5 days intervals. The rate of this anion consumption was determinated as 314.4 mg gX^{-1}. The profile of dissolved phosphorus during the experiment is presented in figure 23.

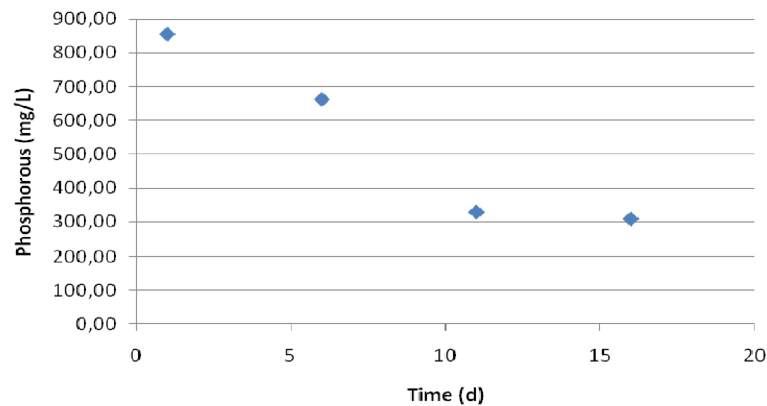


Figure 23. Dissolved phosphorus during cultivation of *C. vulgaris* in MBM media.

4.1.3 Carbon Dioxide Fixation

Figure 24 shows the profile of carbon dioxide and oxigen consumption in comparison with the blank trial during the 15 days of experiment.

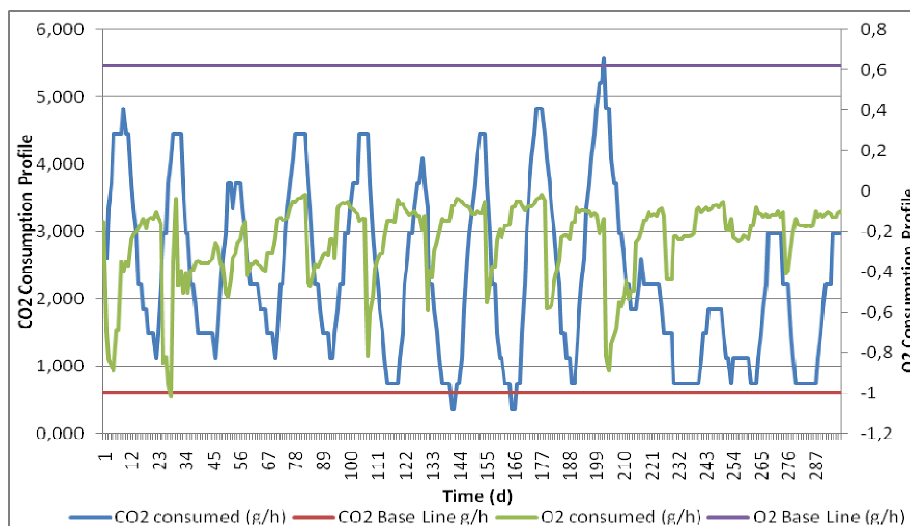


Figure 24. Carbon dioxide and oxygen profiles plotted togheter presenting symmetry and accordance to photossynthesis and respiration processes.

For carbon dioxide fixation quantification the area above (CO_2 consumption) and under (CO_2 production) the blank line was integrated and subtracted, resulting in total carbon dioxide transfer rate of 251.64 mg L^{-1} day^{-1}.

The amount of carbon dioxide mineralized in soluble form of bicarbonate in the medium was estimated in 0.038 mg_{CO2} L^{-1}. Total extracellular concentration of carbohydrates at the end of the experiment was 238.86 mg L^{-1}.

By the same integration method used for CO_2 calculation, oxygen production was estimated in 982.48 mg L^{-1} day^{-1}.

4.1.4 Biomass Composition

Table 5 shows the estimated composition of the *C. vulgaris* strain LEB 104 biomass.

Table 5. Estimated composition of *C. vulgaris* LEB 104.

Proteins	Sugars	Chlorophylls	Lipids	Ash
40,95%	16,74%	9,09%	9,95%	13,35%

4.2 ***Botryococcus braunii***

4.2.1 Growth Parameters

The growth profile of *Botryococcus braunii* is showed in figure 25. It may be observed the growth is linear throughout almost the whole experiment. The maximum biomass concentration, 3.11 g L^{-1}, was observed in the last day.

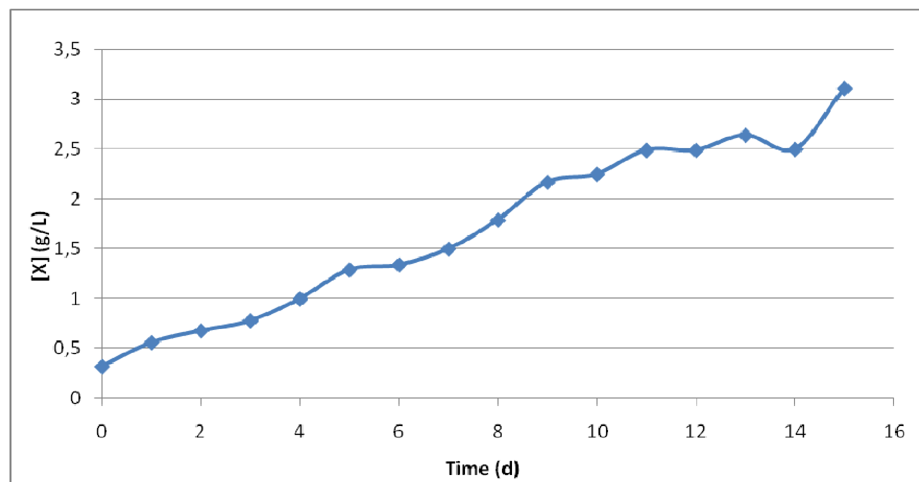


Figure 25. Growth profile of *B. braunii* cultivated in 3N-MBM media.

By plotting the log of biomass concentration versus time, it was noticed that exponential growth was taken until 144 hours. Maximum specific growth

rate, productivity and the biomass doubling time were calculated and resulted in 0.24 d^{-1}, 0.61 g L^{-1} and 2.9 days, respectively.

4.2.2 Medium Analysis

Carbon solubility (C_{SOL}) during the cultivation of *B. braunii* in 3N-MBM medium in comparison with biomass growth profile can be observed in figure 26.

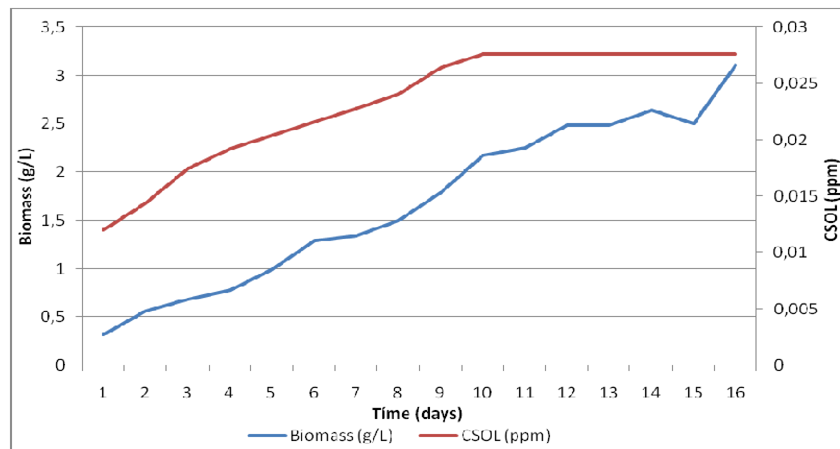
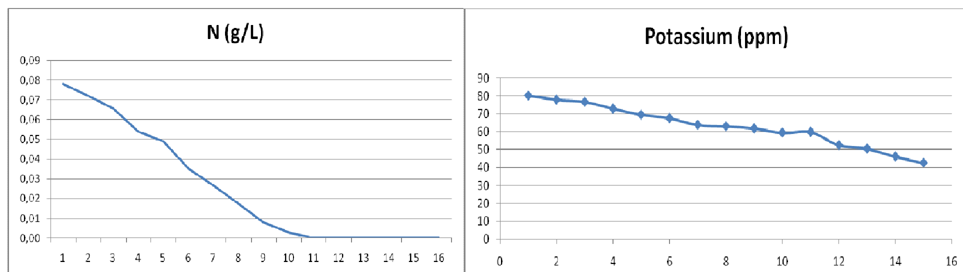


Figure 26. Comparison between dissolved carbon and growth of *B. braunii*.

Consumption of nitrogen, magnesium and potassium during growth was determinated through the dissolved concentration in the medium (figure 27). The averae rates of consumption for each of the cations was calculated and resulted in 40.72 mg gX^{-1} for nitrogen, 15.02 mg gX^{-1} for potassium and 2.60 mg gX^{-1} for magnesium.



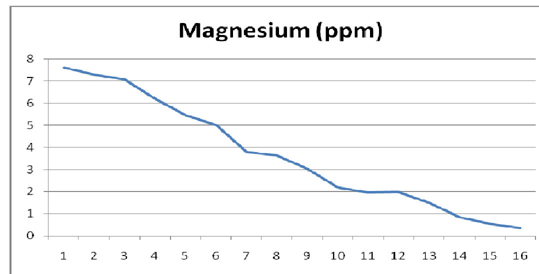


Figure 27. Dissolved salt profiles during cultivation of *B. braunii* in 3N-MBM media.

The accumulated production of biomass was plotted against the accumulated production of each cation. These graphics are showed in figure 28.

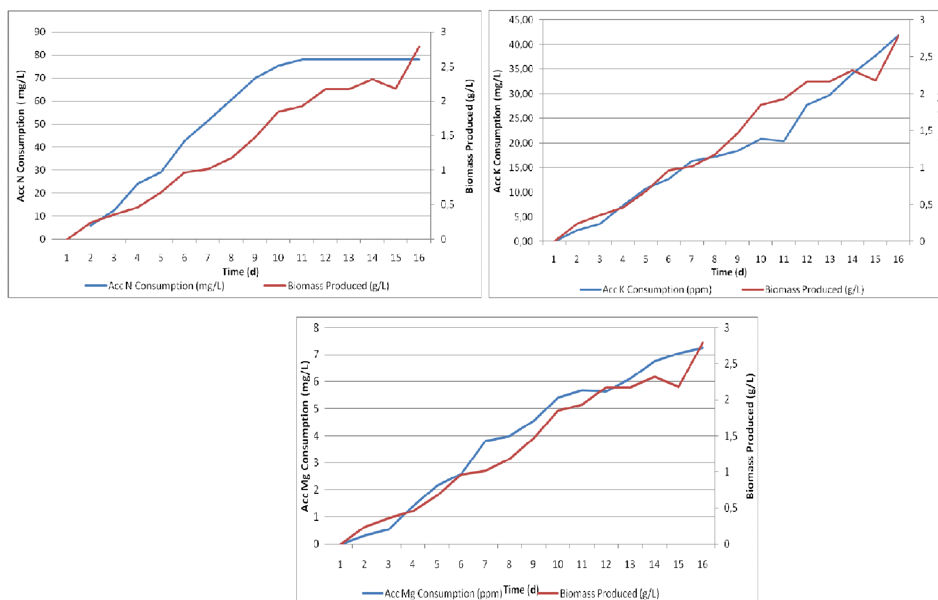


Figure 28. Relation between accumulated biomass production and accumulated consumption of each cation analyzed.

Dissolved phosphorus was analyzed during growth (figure 29) and its rate of consumption was 175.9 mg gX^{-1}.

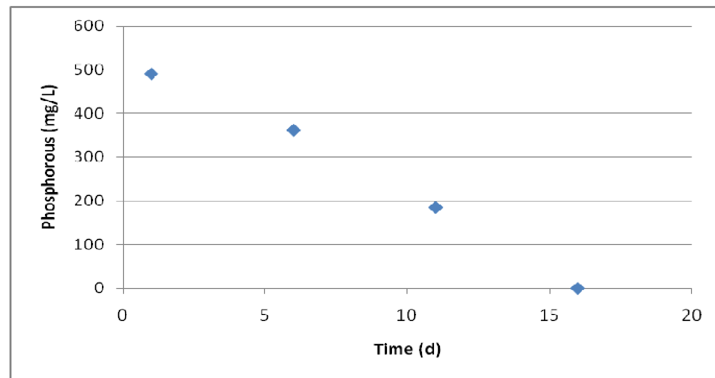


Figure 29. Dissolved phosphorus during cultivation of *B. braunii* in 3 N- MBM media.

4.2.3 Carbon Dioxide Fixation

Figure 30 show the pattern of consumption of carbon dioxide and oxigen in comparison with the blank trial.

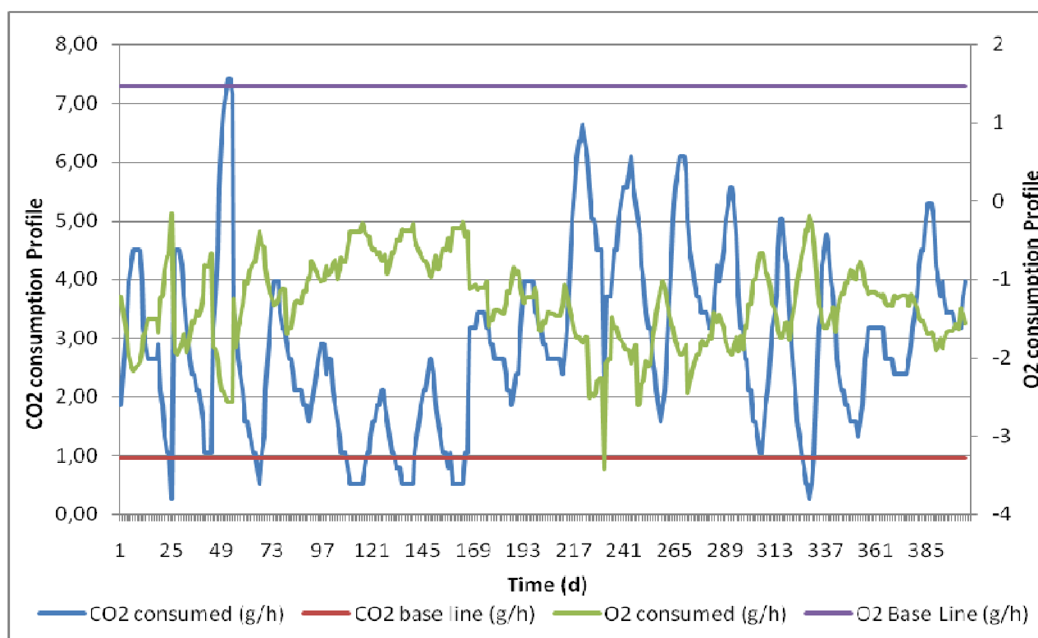


Figure 30. Carbon uptake and Oxygen production during growth of *Botryococcus braunii*.

Carbon dioxide fixation was quantified by the same method described for *C. vulgaris*, and totalized 496.98 mg L^{-1} day^{-1} (41,94 mg CO_2 h^{-1} $g_{biomassa}{}^{-1}$). In exponential growth almost half of total carbon dioxide was fixed. Oxygen production was estimated in 1192.39 mg L^{-1} day^{-1}.

The amount of carbon dioxide mineralized in soluble form of bicarbonate in the medium was estimated as 0.028 mg_{CO2} L^{-1} (see fig 9). Total extracellular carbohydrates concentration at the end of the experiment was 35.85 mg L^{-1}.

4.2.4 Biomass Composition

The biomass composition of *Botryococcus braunii* cultivated at the described conditions is indicated in table 6.

Table 6. Composition of *B. braunii* cultivated in 3N-MBM media with carbon dioxide as carbon source.

Proteins	Sugars	Chlorophyll	Lipids	Ash
39,61%	2,38%	6.50%	33%	7,54%

4.3 ***Spirulina platensis***

4.3.1 Growth Parameters

The growth profile of the microalga *Spirulina platensis* LEB 52 is show in figure 31. Spirulina platensis presented two phases of exponential growth: one between 96 and 168 hours and other from 240 to 264 hours.

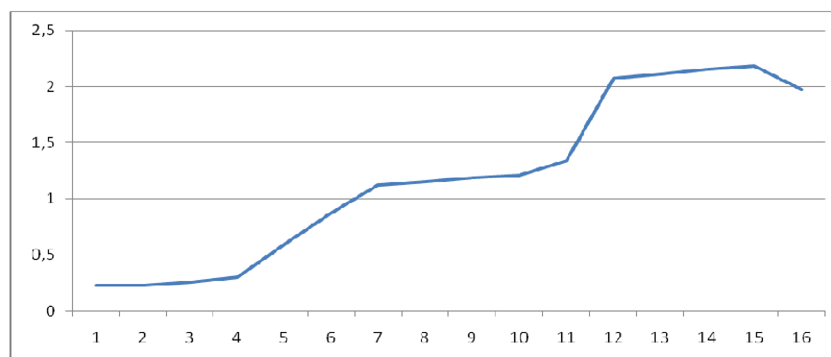


Figure 31. Growth profile of *Spirulina platensis* grown in Zarrouk media.

Maximum cell concentration was observed in day 14th (2.18 g L^{-1}). Specific growth rate and doubling time were calculated in the exponential growth phase and resulted in 0.22 d^{-1} and 3.12 days, respectively. Maximum cell productivity was 0.73 g L^{-1} d^{-1}.

4.3.2 Medium Analysis

Dissolved carbon (C_{SOL}) in Zarrouk media during cultivation of *S. platensis* is presented in figure 32.

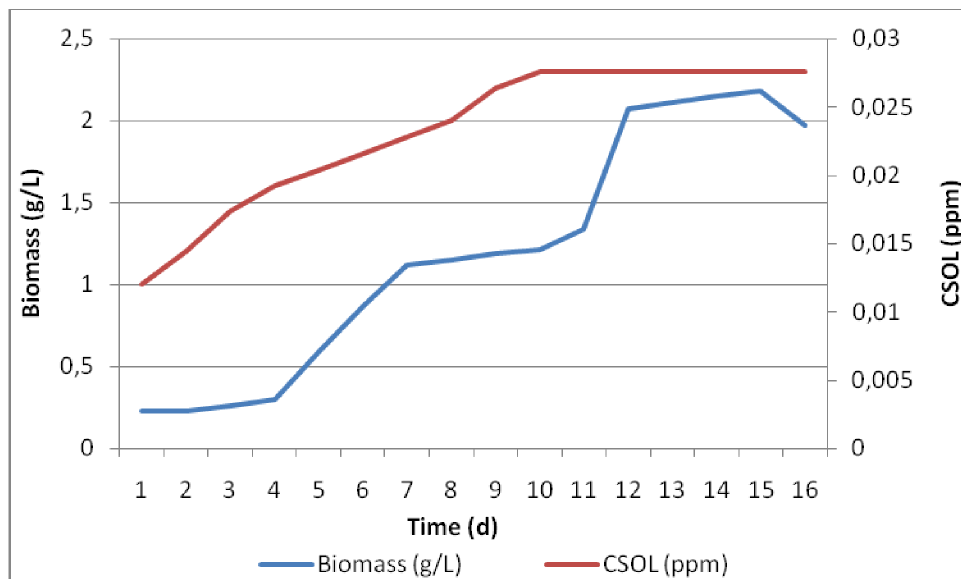


Figure 32. Carbon solubility during growth of *S. platensis*.

The profiles of consumption of nitrogen, magnesium and potassium during growth are showed in figure 33. The average rate of consumption for these cations was calculated and resulted in 61.8 mg gX^{-1} for nitrogen, 24.09 mg X^{-1} for potassium and 4.17 mg X^{-1} for magnesium.

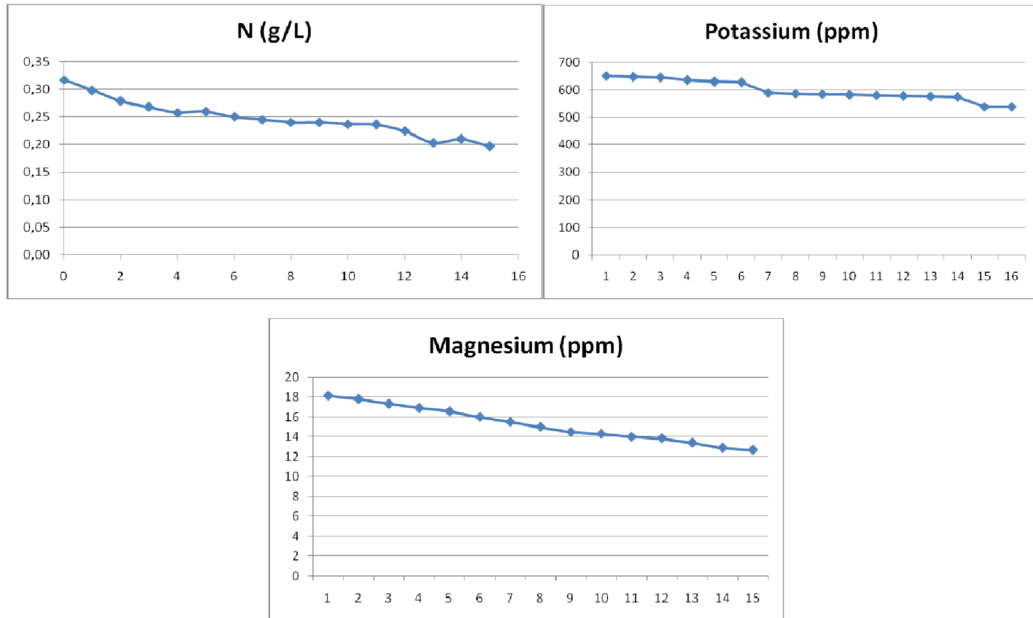


Figure 33. Profile of consumption of nitrogen, potassium and magnesium.

The accumulated production (C_f – C_0, where zero is the biomass concentration in the beginning of the experiment and f is in a determinated day) of biomass was plotted against the accumulated production of each cation. These graphics is showed in figure 34.

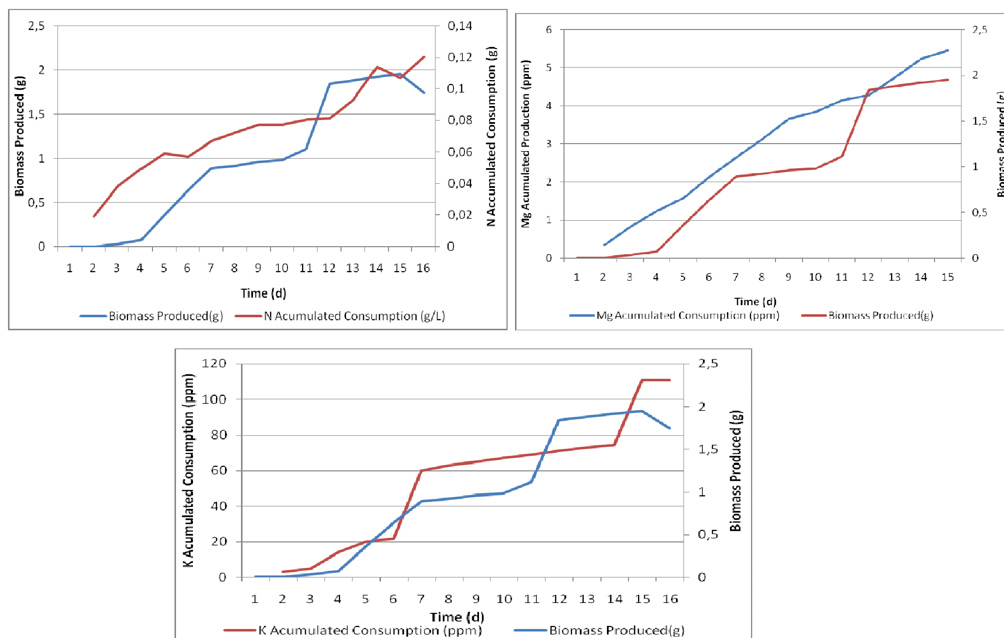


Figure 34. Profiles of accumulated biomass production versus accumulated consumption of each cation analyzed.

Dissolved phosphorus was analyzed during growth (figure 35) and its rate of consumption was 247.4 mg gX^{-1}.

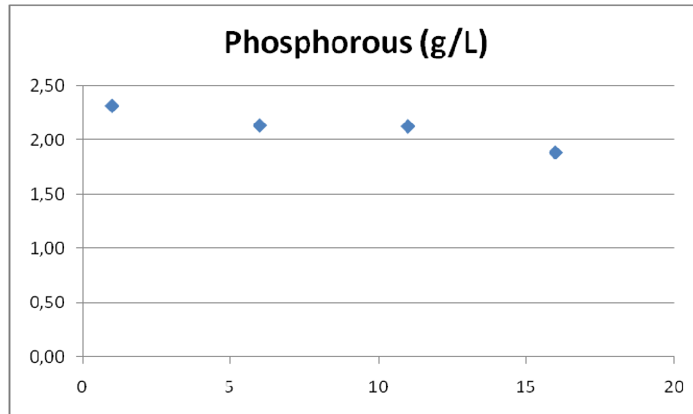


Figure 35. Dissolved phosphorus during cultivation of *S. platensis* in Zarrouk media.

4.3.3 Carbon Dioxide Fixation

Figure 36 shows the pattern of consumption of carbon dioxide and oxigen in comparison with the blank trial.

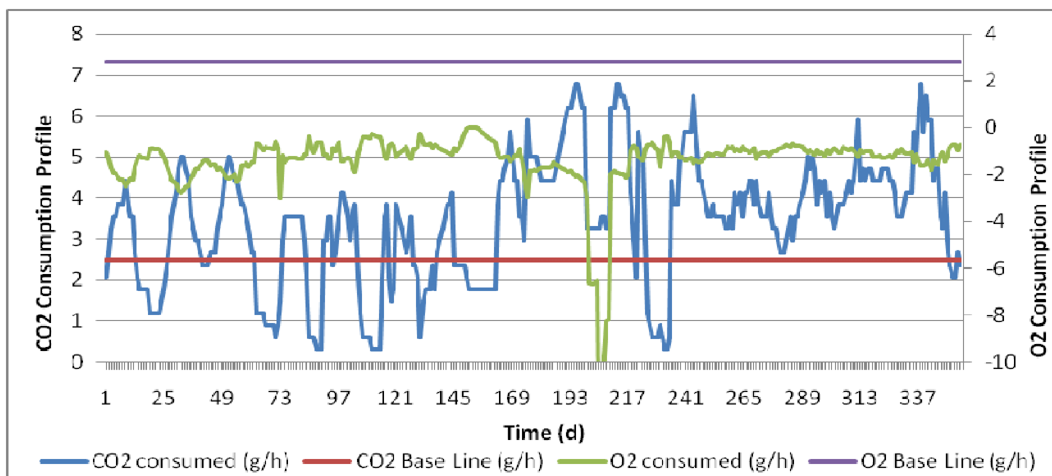


Figure 36. Carbon uptake and Oxygen production during growth of *Spirulina platensis*.

Carbon dioxide fixation was quantified by the same method described before and totalized 318.16 mg L^{-1} day^{-1}. Oxygen production was estimated in 1389.85 mg L^{-1} day^{-1}.

The amount of carbon dioxide mineralized in soluble form of bicarbonate in the medium was estimated in 0.028 mg_{CO2} L^{-1}. Total dissolved extracellular carbohydrates concentration at the end of the experiment was 114.33 mg L^{-1}.

4.3.4 Biomass Composition

Table 7 indicates the composition of *Spirulina plate*nsis biomass cultivated.

Table 7. Composition of *S. platensis* cultivated in modified Zarrouk media with carbon dioxide as carbon source.

Proteins	Sugars	Chlorophyll a	Lipids	Ash	Other Pigments
42.33%	11%	12.13%	11%	7.11%	3.99%

4.4 ***Dunaliella tertiolecta***

4.4.1 Growth Parameters

Dunaliella tertiolecta results was considered here as a sum of a duplicate. Problems related to carbon dioxide fixation quantification (due to electrical outages) in one experiment and in growth profile analysis in other were faced. As both experiments was done in the same conditions, with very similar proportion of inoculum (initial biomass equal to 0.2 in one and 0.24 g L^{-1} in the other) and almost identical profiles of nitrogen consumption and dissolved carbon was obtained, the results was mixed.

The growth profile of the microalga *Dunaliella tertiolecta* is shown in figure 37. The microalga has presented an exponential growth from 0 to 360 hours.

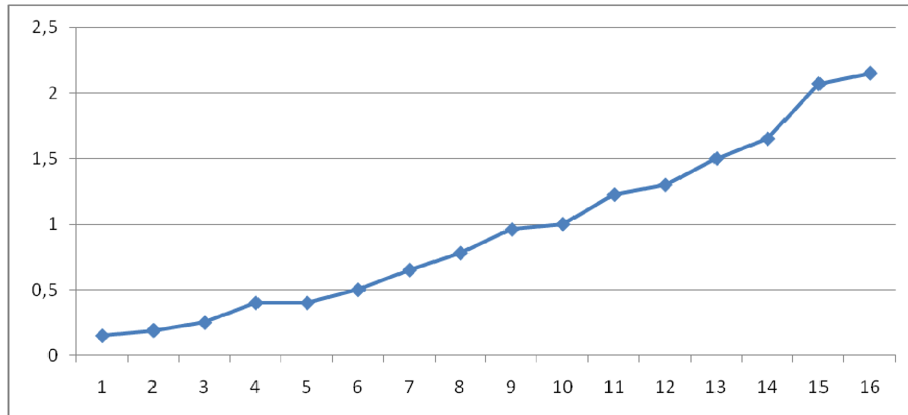


Figure 37. Growth profile of *D. tertiolecta* grown in Dun media.

Maximum cell concentration was observed in day 15th (2.15 g L^{-1}). Specific growth rate and doubling time was calculated at the exponential growth phase and resulted in 0.21 d^{-1} and 3.29 days, respectively. Maximum cell productivity was 0.42 g L^{-1} d^{-1}.

4.4.2 Media Analysis

Dissolved carbon (C_{SOL}) in Dun media during cultivation is presented in figure 38.

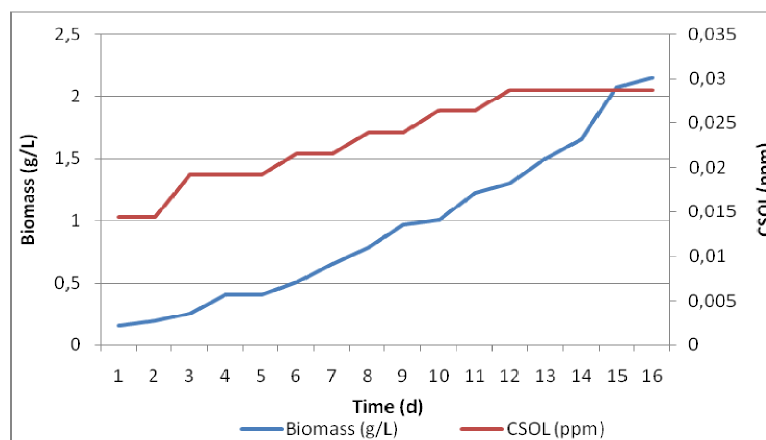


Figure 38. Carbon solubility during growth of *D. tertiolecta*.

The profiles of consumption of nitrogen, magnesium, calcium and potassium during growth are showed in figure 39.

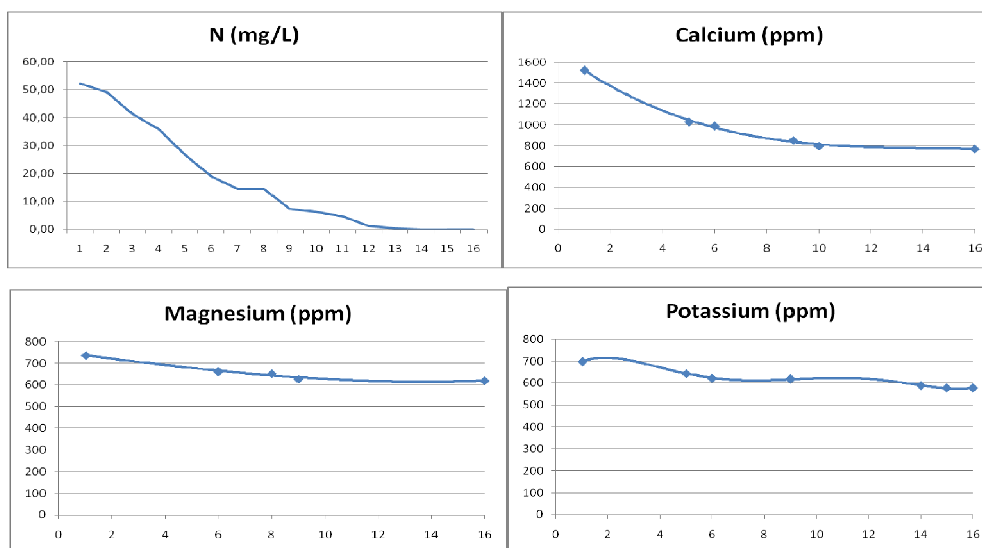
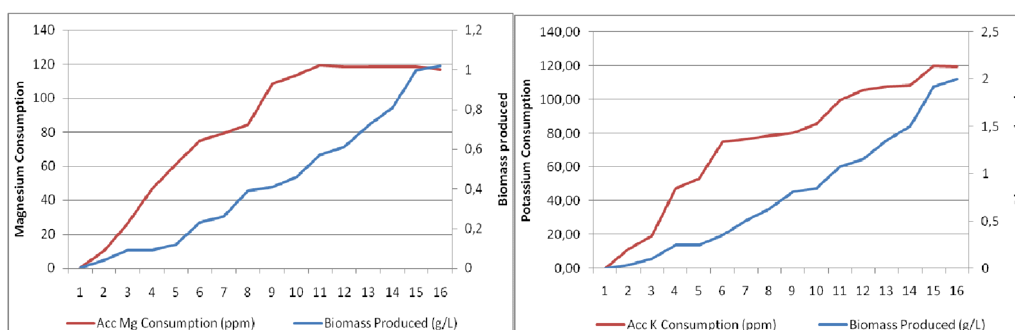


Figure 39. Profile of consumption of nitrogen, potassium and magnesium.

The average rate of consumption for each of the salts analyzed was calculated and resulted in 26.05 mg X^{-1} for nitrogen, 59.71 mg X^{-1} for potassium, 375.46 mg X^{-1} for calcium and 58.45 mg X^{-1} for magnesium. The profiles of consumption versus biomass production is show in figure 40.



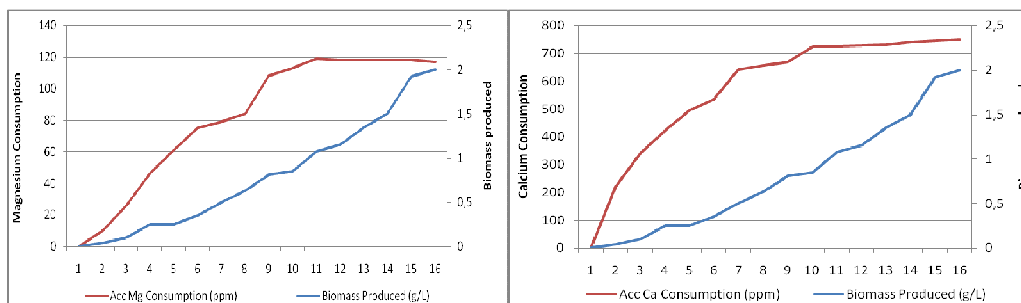


Figure 40. Profiles of cations consumption versus *Dunaliella tertiolecta* biomass production.

4.4.3 Carbon Dioxide Fixation

Figure 41 show the pattern of consumption of carbon dioxide and oxigen in comparison with the blank trial.

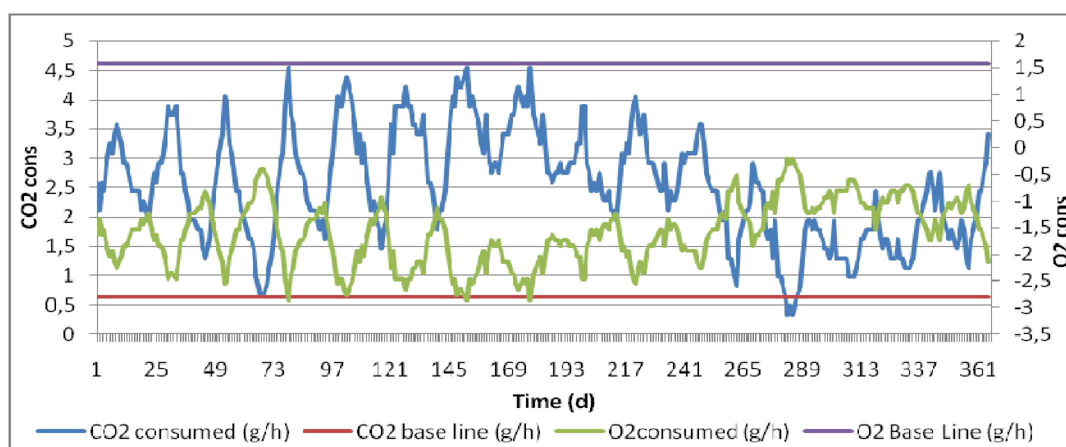


Figure 41. Carbon dioxide and oxygen consumption pattern.

Carbon dioxide fixation was quantified by the same method described earlier and totalized 272.40 mg L^{-1} day^{-1}. The amount of carbon dioxide mineralized in soluble form of bicarbonate in the medium was estimated in 0.028 mg_{CO2} L^{-1} (see fig 9). The concentration of total extracellular carbohydrates at the end of the experiment was 280.1 mg L^{-1}.

4.4.4 Biomass Composition

The composition of *Dunaliella tertiolecta* biomass is indicated in table 8.

Table 8. Composition of *D. tertiolecta* cultivated in 3N-MBM media with carbon dioxide as carbon source.

Proteins	Sugars	Chlorophyll	Lipids	Ash
29.41%	13.95%	7.61%	11.44%	33.35%

5.0 DISCUSSION

This section is divided into general and specific discussion.

5.1 General discussion

Ash analysis of the microalgae presented visual differences (figure 42). The different colors observed indicate a variation in microalgae biomass composition, a consequence of the absorbed substances, probably due to specific metabolism characteristics.

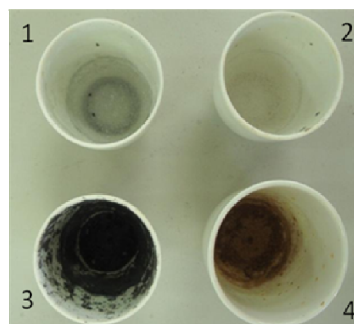


Figure 42. Visual differences among ash of the microalgae. 1- *B. braunii*, 2- *D. tertiolecta*, 3- *S. platensis*, 4- *C. vulgaris*.

Carbon solubility in media for the microalgae studied increases in all cases. Eriksen et al (2007) related that the concentration of HCO_3^- in the growth medium increased in proportion to the decrease in the concentration of the nitrogen source, which was also observed during our experimentations. This is a consequence of the nitrogen source used. The reduction of 1 mol of NO_3^- consumes 1 proton, which comes from the dissociation of carbonic acid.

Through the analysis of dissolved cations and anions in the media, it was possible to calculate the rates of consumption of phosphorus, magnesium, nitrogen and potassium. These data are of great importance in the utilization of residues for microalgae growth because they can be used to determine the need of supplementation and the capacity of removal of such nutrients by the microalgae. It was observed that for all microalgae nitrogen and magnesium consumption was associated with growth. Potassium consumption presented different behavior for the microalgae studied.

The profiles of carbon dioxide and oxygen presented (figures 24, 30, 37, 41) showed clearly the complementary behavior of photosynthesis and respiration during microalgal growth. Under lighting regimem the increase in carbon dioxide consumption is simultaneously accompanied by a decrease in oxygen consumption (photossynthesis process); and the opposite was observed under dark regimem (respiration process). The distances between peaks and valleys of carbon dioxide consumption line is aproximately 12 hours, which is in accordance with the duration of photosynthesis and respiration under the light photoperiodicity.

The equipment developed in our laboratory (and that is in process of patenting) has the potential for measuring total carbon fixation by microalgae, becoming a important tool for microalgal clean development mechanisms, specially those that use closed photobioreactors.

5.2 **Specific Discussion**

5.2.1 Growth Profiles

The analysis of growth pattern and parameters for the strain LEB 104 of Chlorella vulgaris is in accordance with Morais and Costa (2007) who obtained a specific growth rate of 0.31 d^{-1}, a doubling time of 2.27 days and a maximum productivity of 0.28 g L^{-1} d^{-1} for the same strain.

Our results of *B. braunii* growth kinetics are in accordance to Vovola et al (1998), who achieved a biomass concentration of 3.9 g L^{-1} and generation time of 3 to 4 days in modified Prat medium. The specific growth rate during exponential phase obtained by Vovola was very close to that obtained in this study (0.235 and 0.24 d^{-1}, respectively). Other authors also presented very similar results (Órpez et al, 2009 and Qin,2005).

Dunaliella tertiolecta presented a specific growth rate of 0.21 d^{-1} during the exponential phase, which occurs during 360 hours of cultivation. This data is considerably low comparing to those obtained by Roberts et al (2008) which achieved 1.25 d^{-1} and Sciandra et al (1997) 0.5 d^{-1}.

Spirulina platensis maximum growth rate was 0.223 d^{-1} and a biomass doubling time of 3.12 days. These data are in accordance with Binaghi (2003) and Morais and Costa (2007).

5.2.2 Media Analysis

The main difference between all the experiments taken was related to potassium consumption. It was observed that for *C. vulgaris* and *S. platensis* the major potassium consumption was observed from the end of the exponential growth, while in *B. braunii* and in *D. tertiolecta* it accompanied growth. Calcium consumption analysis in *D. tertiolecta* growth indicates major consumption in the beginning of the growth, what may be a consequence of adsorption to the cell membrane.

From the nitrogen consumed by *Chlorella vulgaris* (49.35 mg gX^{-1}) and considering the composition of the biomass (table 4), it was possible to determine the destination of the nitrogen during growth. Approximately 76% was transformed into proteins, while 1.45% was used for chlorophyll production and 1.93% remained soluble in the medium. Almost all of the magnesium (97.84%) destine was to chlorophyll production.

B. braunii presented a rate of consumption of 40.72 mg gX^{-1} for nitrogen, 15.02 mg gX^{-1} for potassium and 2.60 mg gX^{-1} for magnesium. Phosphorus removal was equal to 175.90 mg gX^{-1}.

Around 65% of the nitrogen consumed by cells of *B. braunii* was used in protein production, while 1.68% was used for chlorophyll. Nitrate was totally consumed in 9 days of cultivation. Around 70% of the magnesium was destinated to chlorophyll.

Protein was the destination of more than 56% of the nitrogen consumed by *Dunaliella tertiolecta* cells, while almost 50% of the magnesium was used in chlorophyll production.

In Spirulina, the fate of 91% of the nitrogen consumed was the production of proteins and 1.1% in chlorophyll. Magnesium was almost all (97%) used in the production of chlorophyll.

5.2.3 Carbon Dioxide Assimilation

The total amount of carbon dioxide fixed by *Chlorella vulgaris* LEB 104 estimated in 251.64 mg L^{-1} d^{-1}. Through the biomass compositions the biomass composition in terms of elementar carbon was estimated. It was obseved that around 87% of the carbon fixated was used for the generation of microalgal biomass. Taking into account the amount of carbon dioxide mineralized in soluble form of bicarbonate in the medium (0.038 mg_{CO2} L^{-1}) and considering the produced extracellular carbohydrates (238.86 mg L^{-1}) as glicose (carbon represents 40%), we reach 91% of the known carbon destination.

The same calculations presented in the preceding paragraphs were done for the other algae:

Botryococcus braunii carbon dioxide fixation was estimated in 496.98 mg L^{-1} day^{-1}, representing an annual CO_2 fixation of 110 tons per acre. The amount fixed in form of biomass is estimated as 88%. Extracellular carbohydrates (35.85 mg L^{-1}) and the mineralized carbon (0.0276 mg L^{-1}) was not significant in terms of CO_2 fixation (less than 0.7% of total fixated).

Spirulina platensis carbon dioxide fixation rate was estimated in 318.16 mg L^{-1} day^{-1}. This represents 82.6 tons of CO_2. CO_2 destination included around 80.40% for biomass production and 3% for other known destination (114.33 mg L^{-1} as extracellular carbohydrates and 0.115 mg L^{-1} as mineralized carbon).

Dunaliella tertiolecta was able to fixate 272.40 mg L^{-1} day^{-1} of carbon dioxide, which represents 60.36 tons $acre^{-1}$ $year^{-1}$. About 70% was used in biomass production and 9% for extracellular carbohydrates.

5.2.4 Biomass Composition

Biomass composition (table 5) indicates that *C. vulgaris* is a great source of proteins. Furthermore, the microalga presented a great production of extracellular polysaccharides (238.86 mg L^{-1}, which is in accordance to Chinassamy et al, 2009), which was the destination of about 60% of the carbon dioxide fixed.

The biomass yield of *B. braunii* obtained in this experiment was very high (3.11 g L^{-1} in 15 days) associated with a high lipid accumulation (33%) comparable to the literature. In terms of accumulation of hydrocarbons, Dayananda et al (2005) cultivated the same SAG 30.81 strain in shake flask and obtained a maximum cell concentration of 0.65 g L^{-1} under 16:8 light dark cycle with 50% of lipids accumulation. Further experiments achieved a maximum biomass yield of 2 g L^{-1} with 40% of lipid and 1.8 g L^{-1} in outdoor cultivation with 12% of lipids.

Spirulina platensis biomass is a good source of proteins, which represents 42% of the biomass, confirming its capacity of use in food and feed industry.

Dunaliella tertiolecta stood out as a great exopolyssaccharides producer. It was achieved a high amount of ash in biomass, which might be a consequence of the high saline medium.

6.0 CONCLUSIONS

- The destination of carbon dioxide in C*hlorella vulgaris* experiment was around 90.8% to biomass production. CO_2 fixation rate was estimated in 251.64 mg L^{-1} day^{-1}. Nitrogen, potassium, magnesium and phosphorus consumption rates (mg gX^{-1}) were 49.35, 32.18, 2.85 and 314.4.
- *Botryococcus braunii* presented a carbon fixation capacity of 496.98 mg L^{-1} day^{-1}. Almost all of the fixed CO_2 was used in biomass production (88%). *B. braunii* biomass presented high growth rates associated with a high lipid production. Nitrogen, potassium, magnesium and phosphorus consumption rates (mg gX^{-1}) were 40.72, 15.02, 2.60 and 175.9.
- Carbon dioxide fixation rate by *Spirulina platensis* was estimated in 318.16 mg L^{-1} day^{-1}. Major carbon dioxide fixed was used in biomass production (80.40%), which presented 68% of proteins. Nitrogen, potassium, magnesium and phosphorus consumption rates (mg gX^{-1}) were 61.8, 24.09, 4.17 and 247.4.
- *Dunaliella tertiolecta* produced the higher amount of exopolyssaccharides (280,1 mg L^{-1} day^{-1}). CO_2 fixation rate was estimated in 272.40 mg L^{-1} day^{-1}, being almost 70% used for biomass production. Nitrogen, potassium, magnesium and calcium consumption rates (mg gX^{-1}) were 26.05, 59.71, 58.45 and 375.46.

- Biomass seems to represent only a small part of carbon metabolism and fixation of CO_2 might not be quantified considering only biomass production in microalgae cultivation.
- The strategy used for quantification of CO_2 by the microalgae presented good results in comparison with the literature and seems to be useful for closed systems of microalgae production.

7.0 FUTURE WORKS

- Evaluate the effect of nutrients (excess or depletion) on carbon dioxide fixation and biomass composition of microalgae.
- Evaluate carbon fixation, nutrients consumption and biomass composition in cultures containing liquid and/or gaseous industrial wastes.
- Evaluate other microalgae in terms of carbon fixation capacity.

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ANNEX

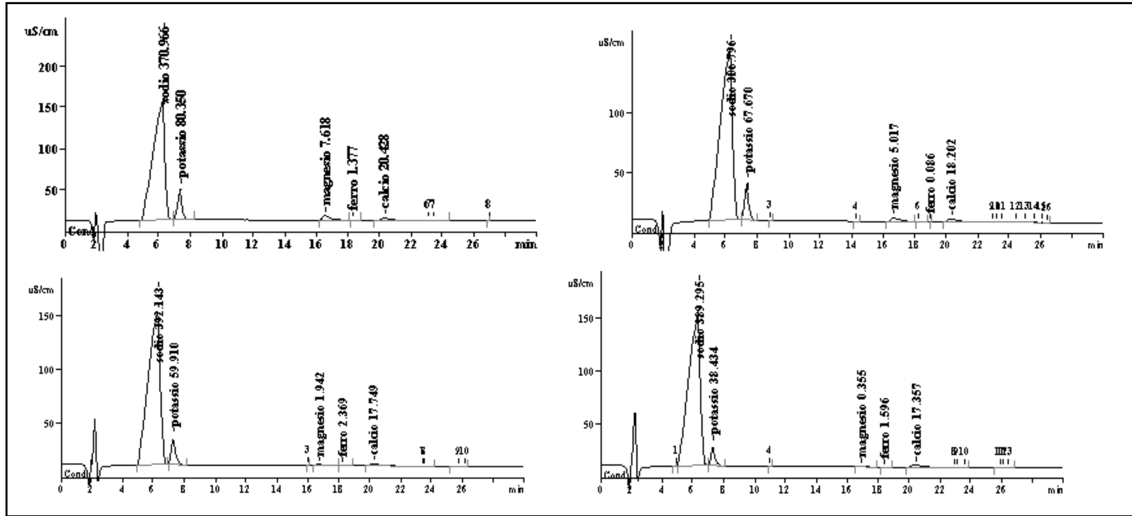
Annex I

The data acquired by the software (*) was used for further calculations to obtain the carbon fixated.

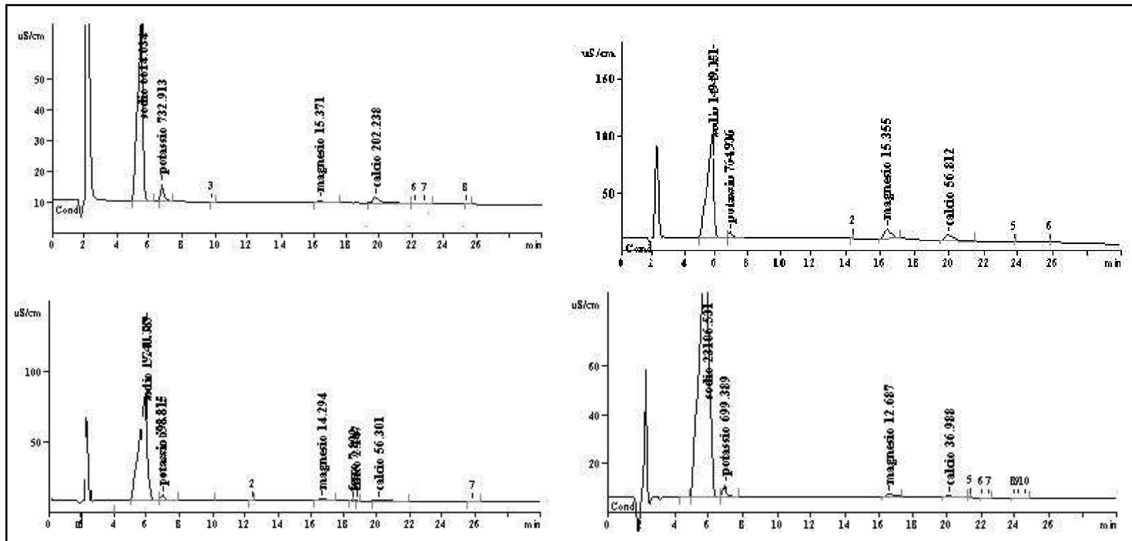
Time*	CO_2 in* (ml/min)	CO_2 out* (%)	O_2 in* (%)	O_2 out* (%)	Gas flow* (L/min)
11/3 11:01	38,38	2,88	20,51	20,08	1
12/3 12:01	36,17	2,87	20,50	20,11	1
13/3 13:01	38,39	2,87	20,48	20,12	0,9
14/3 14:01	35.43	2,84	20,47	20,14	1
15/3 15:01	37,65	2,78	20,47	20,17	1
16/3 16:01	36,91	2,74	20,48	20,19	1
17/3 17:01	36,91	2,70	20,48	20,21	0,9

CO_2 in (%) = (CO2in/GasFlow)*100	CO_2 cons (%) = CO_2in-CO_2out	CO_2 cons (g/h) = (CO2 cons)*(44/22,4)*60*GasFlow
3,84	0,96	1,13
3,62	0,75	0,88
4,27	1,40	1,48
3,54	0,70	0,83
3,76	0,98	1,16
3,69	0,95	1,12
4,10	1,40	1,49

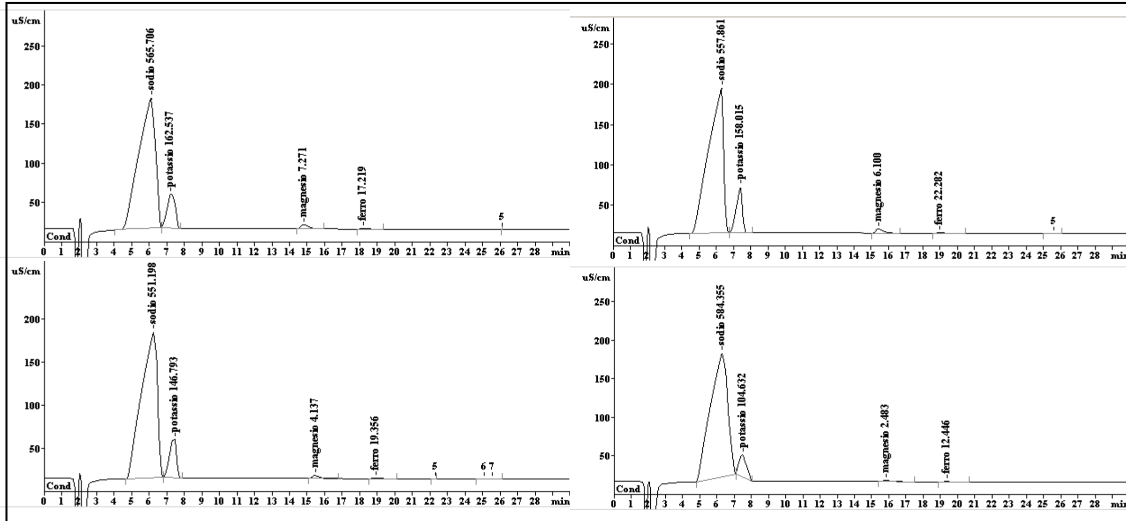
Annex II



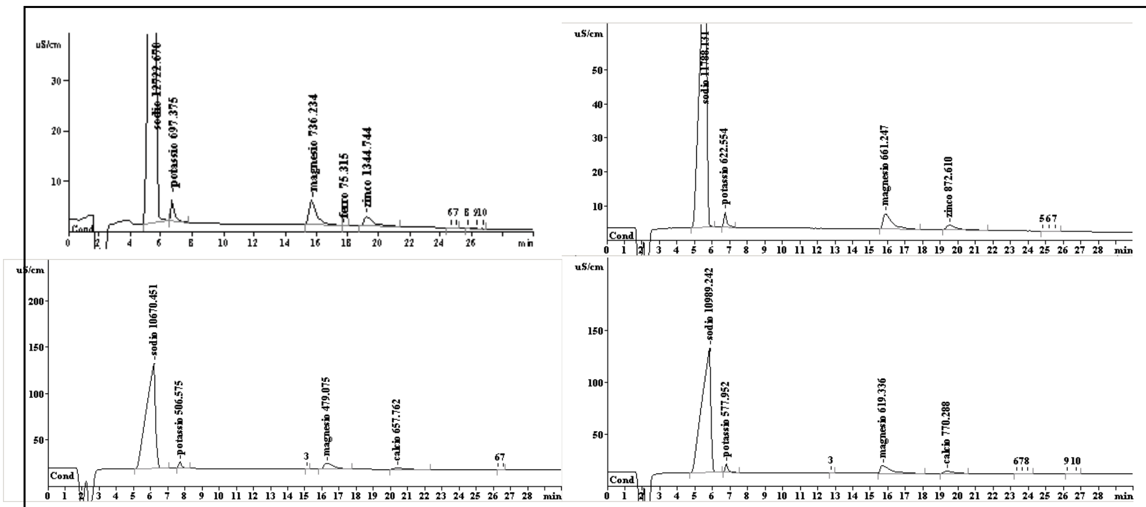
Five-day interval chromatograms of dissolved cations during growth of *Botryococcus braunii* in 3N-MBM media.



Five-day interval chromatograms of dissolved cations during growth of *Spirulina platensis* in modified Zarrouk media.



Five-day interval chromatograms of dissolved cations during growth of *Chlorella vulgaris* in MBM media.



Five-day interval chromatograms of dissolved cations during growth of *Dunaliella tertiolecta* in Artificial Sea Water media.