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Analisi della crescita microalgale mediante selezione artificiale

Analysis of microalgal growth by artificial selection

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Chapter 1 Sommario

Le microalghe sono considerate fonti di energia rinnovabili, sostenibili ed economiche e il loro uso risulta essere promettente al fine di ridurre l'impatto ambientale in molti settori, portando ad annullare le emissioni di diossido di carbonio (CO_2). Per sviluppare un metodo efficace di produzione della biomassa algale, molti ricercatori si impegnano nell'ottimizzazione delle condizioni di crescita.

Il presente lavoro analizza il processo di coltivazione di microalghe nella fase esponenziale prima di procedere all'implementazione su larga scala. Per tale scopo sono state coltivate fotoautotroficamente e studiate sei diverse specie di *Dunaliella* su piccola scala per determinarne il tasso di crescita (μ) al variare di salinità, forma chimica e quantità di carbonio inorganico. Le specie *D. bioculata* e *D. tertiolecta* sono state selezionate perché più veloci nella crescita e quindi cresciute in fotobioreattori. Anche in questo caso i tassi di crescita ottenuti a diverse temperature di coltivazione sono stati utilizzati per effettuare un'ulteriore selezione tra le specie. L'alga *D. bioculata* è risultata la più promettente per il valore di μ ed è stata scelta per un confronto con altre specie biotecnologicamente importanti, considerando anche l'efficacia di mantenere le colture in agitazione.

D. bioculata ha mostrato nuovamente un valore di μ performante, confermandola come migliore scelta tra le sei specie di *Dunaliella* considerate.

Chapter 2 Introduction

Microalgae and cyanobacteria are a vast and ubiquitous group of oxygenic phototrophic microorganisms that have been considered for a long time as a biological resource with considerable potential by the scientific community and the biotechnology industry. They are recognised to be as one of the oldest life-forms appeared on the Earth about 3.5 billion years ago. They are considered as ancestors of primitive plants (Thallophytes), vegetable organisms lacking roots, stems, leaves and made up of somatic cells around reproductive cells; more importantly, microalgae and cyanobacteria have Chlorophyll (Chl) *a* as their primary photosynthetic pigment (Falkowski & Raven, 2007). Algal structures are primarily used for energy conversion and their simple development allows them to adapt to prevailing environmental conditions and prosper in the long term (Falkowski & Raven, 2007; Lee, 1980). Cyanobacteria are prokaryotic cells lacking membrane-bound, they are improperly incorporated into the microalgal group due to their ability to perform oxygenic photosynthesis. Microalgae are considered eukaryotic cells, containing organelles (plastids, mitochondria, nuclei, Golgi bodies and flagella) that control the functions of the cells, allowing them to survive and reproduce. The vast diversity of existing microalgae originated from a

complicated evolutionary history, due to the primary endosymbiosis of a cyanobacterium with a heterotrophic eukaryotic host, a process that allowed to acquire the ability to make photosynthesis in different taxonomic groups (Archibald, 2015).

Studies carried out to enhance the use of microalgae, as an energy crop in various regions in the world, have highlighted their flexibility to grow both in marine and freshwater biotopes. It has been shown that the growth of microalgae is significantly faster than that terrestrial plants, if properly supplied with nutrients, light and carbon dioxide (CO_2); and they are able to survive under extreme conditions like high salt concentration or low pH (Hallmann, 2007; Pulz, 2001).

The culturing of microalgae in the laboratory is only about 140 years old, and the commercial farming of microalgae less than 60 years (Borowitzka & Moheimani, 2013). The microalgal biomass market has a size of about 5,000 t/year of dry matter and generates a turnover of ca. 40million USD/yr (De Carvalho *et al.*, 2020). Successful algal biotechnology mainly depends on choosing the right strain with relevant properties for specific culture conditions and products. There are probably million species of microalgae, only a few hundred of which are cultured in laboratories, and very few of

which have been characterized in detail and studied for their economic potential (Pulz & Gross, 2004).

2.1 Classification of major commercial microalgae and their biotechnological applications

The diversity of microalgae is wide and represents an intact resource in the biosphere. The scientific literature indicates the existence of 200,000 to several million species of microalgae when compared to about 250,000 species of higher plants. Genetic analysis and identification of all types of microalgae is still ongoing and there is still no complete and consistent classification (Norton *et al.*, 1996). Nowadays taxonomists distinguished several thousand species belonging to the main phyla Chlorophyta; Rhodophyta; Stramenopiles or Henterokontophyta; Haptophyta, Dinophyta (Heimann & Huerlimann, 2015; Pulz & Gross, 2004) and Euglenophyta (Gissibl *et al.*,2019). Concerning Chlorophyta, widely used in the biotechnologies and also in this work, the members of this phylum are able to grow in freshwater, marine or even terrestrial environments; Chlorophyta include unicellular and multicellular members possessing Chl *a* and *b* in a single chloroplast surrounded by two envelope membranes, which they use to capture light energy and produce sugar. In addition, under stress conditions, they accumulate starch and/or oil inside the cell. The best known microalgal genera, such as *Chlorella*, *Chlamydomonas*, *Dunaliella* and *Haematococcus*,

belong to this taxonomic group. *Euglena gracilis* is another interesting species from a biotechnological point of view; it possesses chlorophyll *a* and *b* but belongs to Euglenophyta phylum (Gissibl *et al.* 2019). *Euglena gracilis* is a source of dietary protein, pro(vitamins), lipids and the β-1,3-glucan paramylon (Shibakami *et al.*, 2014).

In the table 1 other main species used for their biotechnological potential are listed (Giordano & Wang, 2018).

Table 1 - Main algal species used in biotechnological applications (Giordano & Wang, 2018).

Species	Phylum and class	Product	Production status
Artrospira platensis	Cyanophyta, Cyanophyceae	Biomass as dietary supplement	Mass production
Chaetoceros muellerii	Bacillariophyta, Bacillariophyceae	Biomass as dietary supplement	Small scale
Chlorella vulgaris	Chlorophyta, Chlorophyceae	Canthaxanthin, astaxanthin, β-carotene, biomass as dietary supplement	Mass production
Crypthecodinium cohnii	Miozoa, Dinophyceae	Docosahexanoic acid	Mass production
Dunaliella salina	Chlorophyta, Chlorophyceae	β-Carotene, glycerol	Mass production
Hematococcus pluvialis	Chlorophyta, Chlorophyceae	Astaxanthin, cantaxanthin, lutein	Mass production
Isochrysis spp.	Chlorophta, Chrysophyceae	Biomass as dietary supplement	Mass production
Nannochloropsis spp.	Ochrophyta, Eustigmatophyceae	Eicosapentanoic acid	Small scale
Nitzschia closterium	Bacillariophyta, Bacillariophyceae	Eicosapentanoic acid	Research
Nostoc commune	Cyanophyta, Cyanophyceae	Biomass as dietary supplement	Collected, not cultivated
Nostoc flagelliforme	Cyanophyta, Cyanophyceae	Biomass as dietary supplement	Collected, not cultivated
Nostoc sphaeroids	Cyanophyta, Cyanophyceae	Biomass as dietary supplement	Collected, not cultivated
Odontella	Bacillariophyta, Mediophyceae	Eicosapentanoic acid, docosahexanoic acid	Small scale
Pavlova lutherii	Haptophyta, Pavlovophyceae	Biomass as dietary supplement	Research
Phaeodactylum tricornutum	Bacillariophyta, Bacillariophyta incertae sedis	Eicosapentanoic acid	Small scale
Porphyridium cruentum	Rhodophyta, Protoflorideae	Biomass as dietary supplement, arachidonic acid, triacylglycerols	Small scale
Scenedesmus almeriensis	Chlorophyta, Chlorophyceae	Lutein, β-carotene	Research
Skeletonema spp.	Bacillariophyta, Bacillariophyceae	Biomass as dietary supplement	Small scale
Tetraselmis spp.	Chlorophta, Prasinophyceae	Biomass as dietary supplement	Research

Generally, microalgae have higher photosynthetic efficiency than land plants because of their greater abilities to capture light and convert it to usable chemical energy. They are considered renewable, sustainable, and economical sources feedstock, and their use is promising since they allow reducing the environmental impact on many industries, removing greenhouse gas emissions (Sayre, 2010).

The process to exploit algal biomass can be divided into three main steps: growing the algal biomass, called Green phase in *Dunaliella* cultivation by Ben-Amotz (1995), harvesting and processing the biomass. In order to achieve a successful development of biomass production technologies robust algal strains that grow rapidly, are adapted to the planned culture-system environment and produce high levels of the desired products must be available. Numerous screenings of strain collections and isolation strategies have been employed to identify suitable strains (Barclay & Apt, 2013; Ben-Amotz, 1995; Venkatesan *et al.,*2015; Vonshak,1990).

When growing algae, environmental parameters such as light, temperature, salinity, nutrient availability and pH must be optimized; grazers and contamination by other algae must be avoided (Ben-Amotz & Avron, 1989; Vonshak, 1990).

Then algal cells are harvested. As described by Vonshak (1990), this phase consists of different techniques, depending on the species used and their subsequent applications. Starting by water removal, there are three main methods:

1. **-Filtration***:* This is the most used method whenever filamentous alga (such as Spirulina) are being harvested and involves different designs of either vibrating or static nets. As long as no problems in clogging of the filters are encountered, this is a relatively inexpensive and efficient harvesting method.
2. **-Centrifugation:** This technique has been widely used in the harvesting of microalgae, mainly unicellular, such as *Chlorella*. It is a very efficient, but relatively expensive, process due to the high investment cost and high energy inputs.
3. **-Flocculation and Sedimentation***:* These techniques are used mainly in the removal of algal biomass in wastewater treatment and are usually induced by the addition of chemicals such as aluminium sulphate, ferric sulphate or by the modification of growth conditions such as pH. These procedures are relatively inexpensive, and the main problem is that the flocculated product cannot be directly used as a food since complete removal of the flocculant is required.

Once algal cells are harvested and dried, they are processed according to the kind of desired product and its marketing. Species-specific extraction processes are developed according to the microalgae characteristics: e.g. presence or absence of a cell wall, its composition, strength and structure when present. *Dunaliella salina* lacks cell wall and it is more susceptible to shear breakage than other microalgae that possess a thick cell wall, which has to be broken down in order to allow access to the lipids (Borowitzka, 1998; Mata *et al.*, 2010). This is the case of *Haematococcus pluvialis*, which needs physical and chemical processes to promote the disruption of the rigid wall in order to extract the carotenoid astaxanthin, a pigment used in the aquaculture industry (Mendes-Pinto *et al.*, 2001). These differences will have an impact on the applicability of the extraction method, its efficiency and the rate at which the target components can be extracted.

The Figure 1 showed some of microalgal extensive applications.

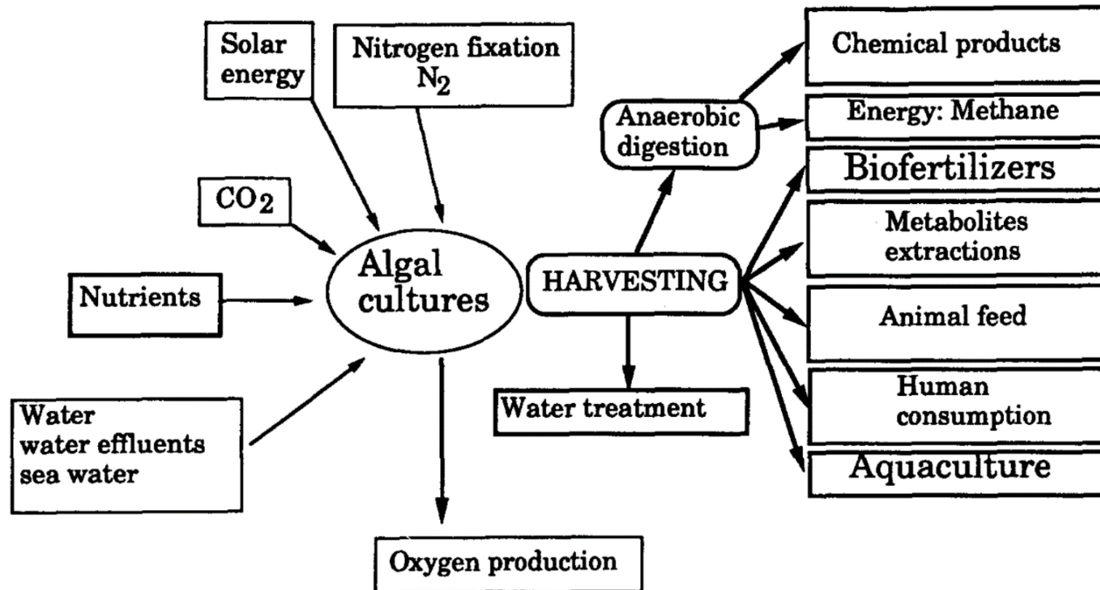


Figure 1 - Algal Biotechnology Input and Potential Outputs (Vonshak, 1990).

Several species are employed in animal feed and nutraceutical, cosmeceutical and pharmaceutical industries as good sources of antioxidant and intracellular signalling-pathway modulators. The bioactive molecules are polyphenols, chlorophyll, β-carotene, ascorbic acid, lycopene, α-tocopherol, xanthophylls, and PUFAs, which are useful for human and animal health and development. The cyanobacterium Spirulina is rich in linolenic acid and a good source for polyunsaturated fatty acid. The biomass produced is mainly sold to the health food market in the form of powder or pills (Mahajan & Kamat, 1995; Vonshak,1990). Other genera that have become useful for human and animal health and development are *Nostoc, Botryococcus, Anabaena,*

Chlamydomonas, Scenedesmus, Synechococcus, Parietochloris, Porphyridium (Simoons, 1990), *Chlorella* (Bishop & Zubeck, 2012; Muller-Fuega, 2000)*, Chaetoceros, Isochrysis, Skeletonema, Tetraselmis (*Muller-Fuega, 2000*), Nannochloropsis, Nostoc, Pavlova, Phaeodactylum, Thalassiosira* (Brown *et al.*, 1996) and *Dunaliella* (Borowitzka, 1998; Pulz & Gross, 2004) because they contain vitamins and essential elements such as potassium, zinc, iodine, selenium, iron, manganese, copper, phosphorus, sodium, nitrogen, magnesium, cobalt, molybdenum, sulfur, and calcium. In the animal feed industry microalgae as *Gymnodinium, Nannochloropsis* and some diatoms have a particular importance for the fry breeding, bivalve molluscs, and for the formulation of livestock feed because they are rich in ω6 (arachidonic acid) and ω3 (docosahexaenoic acid, eicosapentaenoic acid) fatty acids (Bishop & Zubeck, 2012; Pulz & Gross, 2004).

Another microalgal biomass application converts lipids into biodiesel; carbohydrates into ethanol and biosolar hydrogen (H_2), and proteins into feedstock of biofertilizer (Lee & Lavoie, 2013). Bioethanol is an alternative biofuel to gasoline which can be produced through yeast fermentation of complex carbohydrates, specifically from sugary and starchy feedstock, such as sugar cane, sugar beets, corn, and wheat (Chen *et al.*, 2011; Mussatto *et al.*, 2010; Naik *et al.*, 2010). Is possible to demonstrate that the biogas potential is

strongly dependent on the species and on the pre-treatment. For example, the anaerobic fermentation of the green alga *Chlamydomonas reinhardtii* was efficient in methane content of biogas 7–13% higher compared to biogas from maize silage; moreover, it has the capability to produce H_2 under anaerobic conditions (Mussgnug *et al.*, 2010; Klassen *et al.*, 2015). Other industrially relevant microalgal species which can also be tested in continuous fermentation mode are *Parachlorella kessleri* and *Scenedesmus obliquus*, which exhibit very similar properties to *C. reinhardtii* (Klassen *et al.*, 2015). The important role of microalgae in the soil ecosystem has often been neglected. One application as biofertilizer is described by Megharaj *et al.* (1994), who isolated two species of microalgae, *Chlorella vulgaris* and *Scenedesmus bijugatus*, and four of cyanobacteria, *Nostoc linckia*, *Nostoc muscorum*, *Oscillatoria animalis* and *Phormidium foveolare*. These microalgae allowed researchers to degrade methyl parathion, an insecticide which is commonly used in rice cultivation to control pests, within a 30-day period.

Several researchers have attempted to isolate strains with a high tolerance for the unique conditions of a wastewater environment with a goal of using these strains to participate in the wastewater treatment process. This relies on the ability of phototrophic microorganisms to supply oxygen to aerobic organic

pollutant degraders and enhance the removal of nutrients and pathogens. Conventional methods for the removal of heavy metals from wastewater include chemical precipitation, coagulation, ion exchange, membrane processing, electrochemical techniques, adsorption on activated carbon. To prove this, Craggs *et al.* (1997) reviewed that the strains *Phaeodactylum* spp. and *Oscillatoria* spp., when inoculated into the corrugated raceways system, could remove 100% of the ammonium and orthophosphate from the wastewaters over a 4-month period. *Dunaliella tertiolecta* has potential for use in wastewater management programmes, because of their ability to remove, accumulate compounds such as ammonium (NH_4^+) and phosphate (PO_4^{3-}) and heavy metals like copper and arsenic from the wastewater and effluents (Takimura *et al.* 1996; Yamaoka *et al.* 1999). It contains the heavy metal binding peptides, phytochelatins, and can be used in bioremediation for removing heavy metals from the environment (Hosseini Tafreshi & Shariati, 2009; Tsuji *et al.*, 2003). *Dunaliella tertiolecta* is also used as bioindicators for the evaluation of ecotoxicity of anthropogenic compounds on environments (Lewis *et al.*, 1998).

2.2 Cultivation models

As showed in Figure 2, microalgae can be grown in open systems (tanks) or closed systems (photobioreactors), depending on the species and the cultivation conditions.

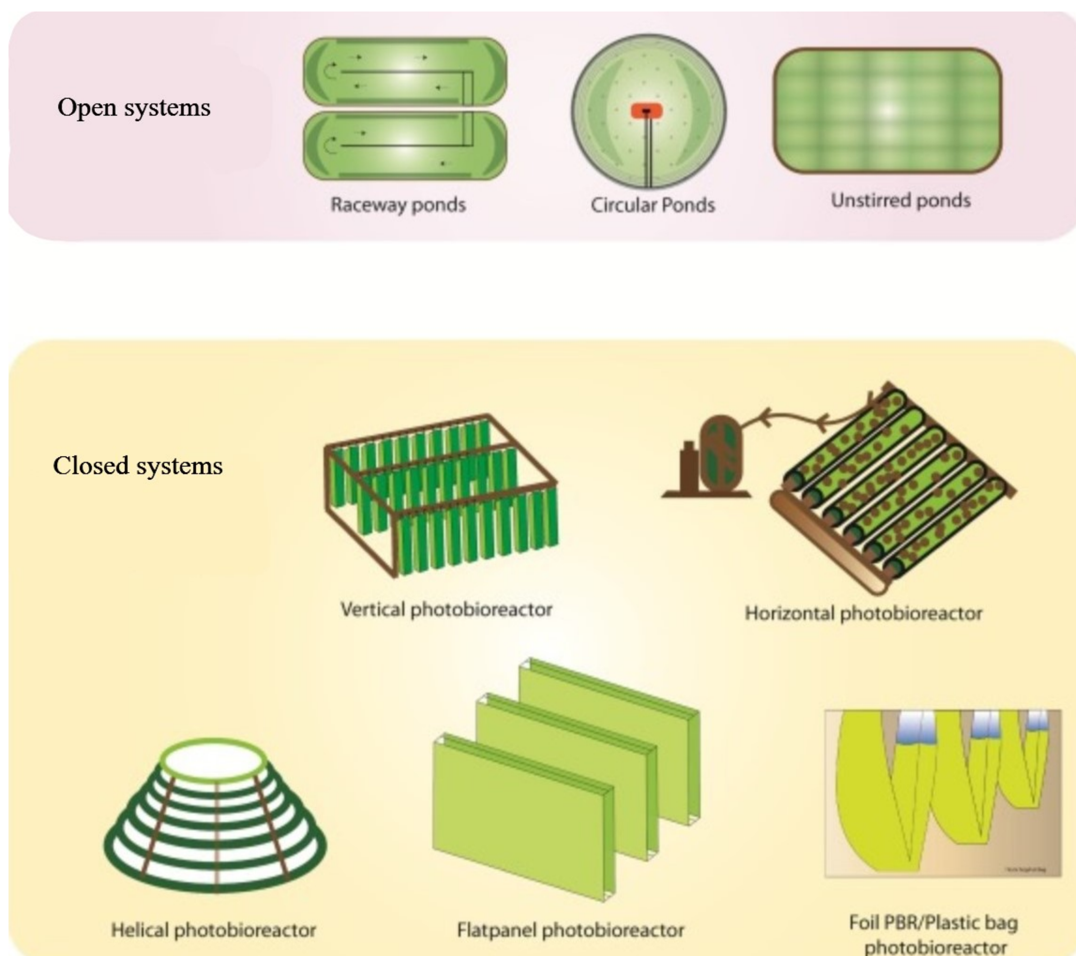


Figure 2 - Examples of different types of pond systems for microalgae cultivation.

Biotic and abiotic factors to be taken into account are pH, light intensity, the presence of contaminants, temperature, the presence of bicarbonate ions (Voshank *et al.*, 1982), nitrogen source, bioreactor type and initial biomass concentration (Costa *et al.*, 2000). These factors are more considered in closed cultivation than industrial scale because of the time and expense involved in loading, discharging and cleaning the ponds (Radmann *et al.*, 2007).

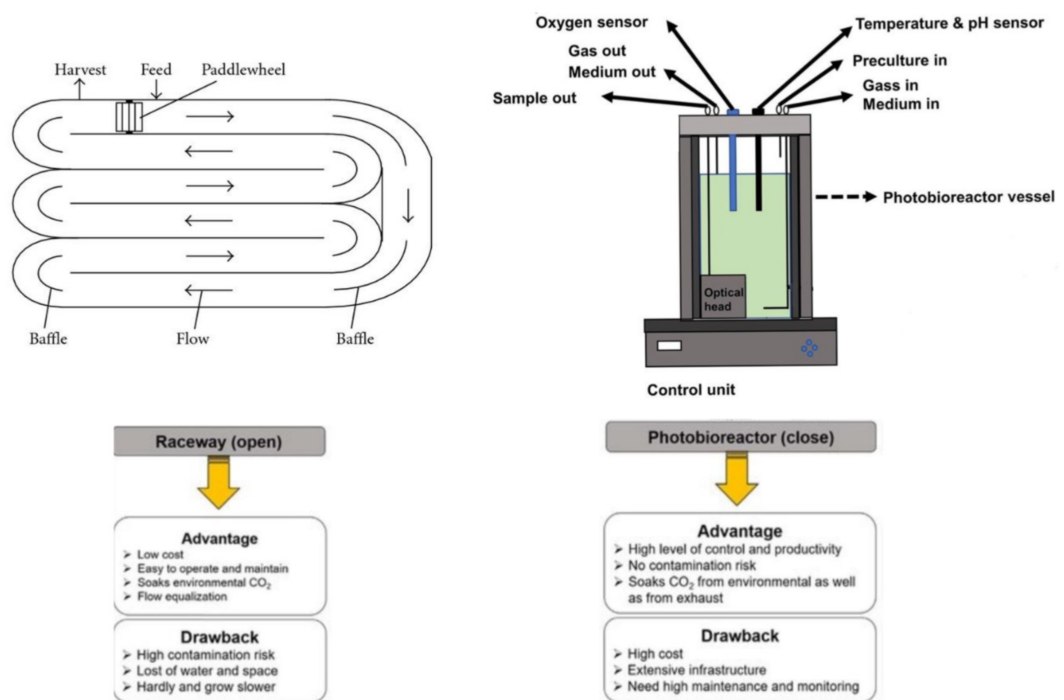


Figure 3 - Advantages and disadvantages of microalgal cultivation systems.

Ponds shapes can be raceway, circular with a rotating arm and unstirred types. To date, the raceway pond appears to be the most feasible method for mass cultivation of microalgae biomass. Those consist in a channel with a closed recirculation circuit about 30 cm deep (usually made of concrete and covered with white plastic) and a paddle wheel for mixing and circulation. The depth of the crops is inversely related to cell concentration and the amount of light available. Usually depths of 15-40 cm represent a compromise between energy expenditure for agitation, collection (rotating blades, water pumps, airlift) and daytime temperature range (Lam *et al.*, 2019) (Fig. 3).

Open systems are less expensive to build, have longer life and greater production capacity than closed systems, even if they occupy a larger area. Compared to closed systems, however, the tanks have a greater need for energy (to homogenize the nutrients) and water (at least 150 L/m^2); besides they are more susceptible to environmental conditions, like the variation of water temperature, evaporation and lighting that will depend on the season, climatic events and geographical location, and contamination by other unwanted microalgae, yeasts, fungi, molds and bacteria (Mata *et al.*, 2010; Lam *et al.*, 2019) (Fig. 3).

There exists a wide variety of photobioreactors (PBRs) types such as tubular, cylindrical, or flat panel systems, as illustrated in Fig. 2. This diversity of

PBR designs results from various attempts to optimize light capture while satisfying other practical constraints related to engineering design, including system integration, scale of production, materials selection, and cost. Moreover, this variety permits to optimize system operation concerned with CO_2 bubbling, oxygen removals, temperature and pH regulation, nutrient delivery, etc (Pruvost *et al.*, 2016). A PBR consists in a series of straight and transparent tubes, of plastic or glass, with a diameter not exceeding 0.1 m and a length less than 80 cm, in which sunlight is captured. To allow greater storage of solar energy, the best orientation of rows aimed at harnessing the highest amount of solar irradiance depends on the latitude (Richmond & Hu, 2013). PBRs have been used extensively for the cultivation of microalgae for a variety of applications from biofuels to high value products (Bux & Chisti, 2016). Those allow to have a better control of growth conditions and reduce risks of external contamination. The culture confinement increases the risk of biofilm formation on the PBR walls. It leads to oxygen accumulation in the culture which can have possible toxic effects on photosynthetic growth. It may also cause overheating of the culture especially under solar radiation due to the large amount of infrared radiation absorbed by the culture medium. The most limiting factor is the amount of light received and its use by the culture, which will determine the productivity of the system. Current industrial scale

biomass production is mainly performed in large open systems because they are easier to build and operate than PBRs. The critical design requirement to build PBR is the illumination surface area per unit volume and a high surface area to volume ratio (S/V ratio) is required to have an efficient PBR. These problems limit the size of photobioreactors, which currently consist mainly of small coils of limited length and limited volumes. Other limitations concern the high costs of construction and operation of the plant (Pruvost *et al.*, 2016) (Fig. 3).

The comparison of the two culture systems is not easy as there are several parameters to consider. PBRs represent a valid alternative for speed up the production of data and describe the influence of multiple parameters on microalgal growth, maintaining high experimental reliability and at the same time high performance, is the evaluation of growth parameters in different environmental conditions carried out in microscale devices. The advantages offered by microsystems include a reduced expenditure of time, cost and work, thanks to the possibility of conducting more parallel analysis and faster processes with lower consumption and greater automation. For this project we made use different microscale devices such as a multicultivator and photobioreactors, in order to be able to control more variables at the same time and optimize the algal growth conditions as much as possible.

2.3 Consideration on cultivating microalgae

Microalgae can convert light energy into valuable biomass with an interesting biochemical composition. Is possible to place cultivation tanks almost anywhere, like in areas not used for agricultural purposes and therefore not competing with land intended for food consumption, given their predisposition to live in hostile environments and requiring reduced amounts of nutrients. They need only light, water, carbon dioxide (CO_2) and some macro- and micronutrients, such as nitrogen (N), phosphorus (P) and their compounds. Most of the biotechnological potential of microalgae comes from the production of important materials starting from their biomass. Several species can be adapted to live in a wide variety of environmental conditions, they grow faster and have higher productivity in terms of oil content in the biomass compared to other terrestrial or aquatic plants, requiring a much smaller land area than other raw materials for biodiesel of agricultural origin (Mata *et al.*, 2010; Vonshak, 1990). Cultivation requirements are species specific, therefore culturing medium and environmental conditions need to satisfy the requirements of algae for optimal growth. Switching to a large-scale process, a premature collapse may occur when up-scaling algal cultures to volumes of hundreds of cubic meters, in an artificially protected

environment of semi-sterility, or other species better adapted to outdoor conditions can take-over. As a result, outdoor cultures of several m^3 usually last but for short periods of time which rarely exceed a few weeks. Another drawback of an open system is its susceptibility to weather conditions, with little control of water temperatures, evaporation, and lighting. Because of its open nature, this system is also more prone to contamination from other algae and bacteria. Although open culture systems have the potential to produce large quantities of microalgae, extensive land area is required. Furthermore, mass transfer of carbon dioxide to the culture is low due to the low levels of carbon dioxide in the atmosphere (0.03-0.06%), and this limitation may result in the slow growth of microalgae (Kim, 2015; Mata *et al.*, 2010). However, it is suggested to analyze the associated risks before starting large-scale production, focusing on the chance that organisms released from the cultivation system would be able to outcompete wild strains. It is unlikely that genetically modified algae and cyanobacteria will outcompete wild strains as these organisms probably are poor in competition at conditions occurring in nature. Regulations for large-scale outdoor production of genetically modified microalgae are not yet in place, however, experimental pilots are in progress (Pulz & Gross, 2004; Wijffels *et al.*, 2013).

2.4 The batch culture, the Growth Kinetics and Measurement of Algal Growth.

The batch culture is the most common commercial method for growing microalgae and produce high biomass. In a batch culture system, a limited amount of culture medium and inoculum are placed in a culture vessel and incubated in a sterilized, controlled environment for growth. A simple agitator or aeration pump is fitted for mixing the nutrients and proper gaseous exchange throughout the system. Structurally the culture vessel can be a simple conical flask or an environment-controlled fermenter. The CO_2 can be supplied continuously with CO_2 enriched air (aeration pump) and illuminated with natural or artificial light sources. This cultivation method is often used in laboratory as a pre-culture for other fermentation processes, retaining a portion of the inoculums for the next culture batch. Different growth phases in the culture system may reflect changes in the biomass and in its environment (Mata *et al.*, 2010; Satpati & Pal, 2018).

Talking about the microalgal growth in a batch culture, it occurs in four steps as illustrated in Richmond & Hu (2013) and Lee (2016) (Fig. 4):

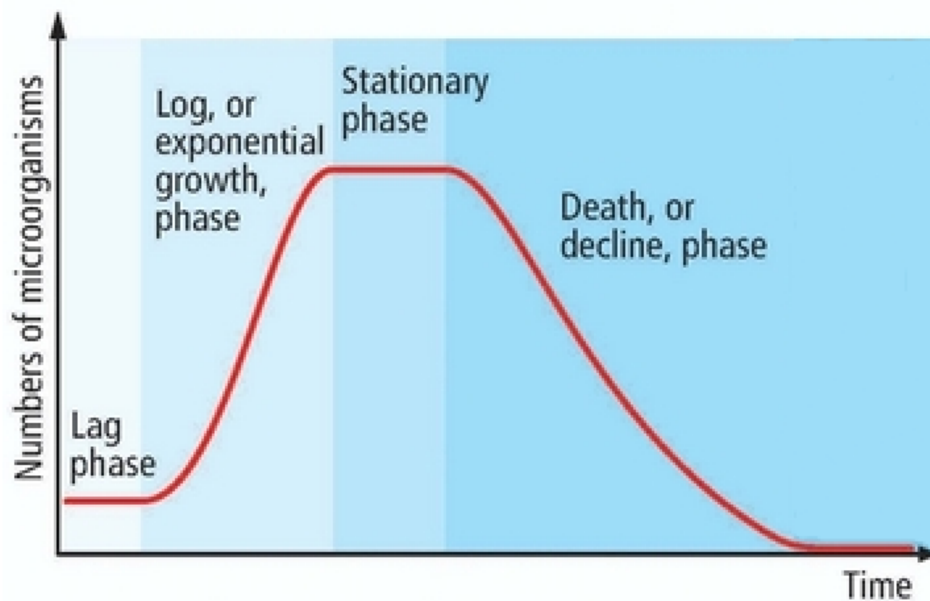


Figure 4 - Microbial growth curve in a closed system.

1. **Lag phase**, in which the growth delay could be due to the presence of non-viable cells in the inoculum or be the period of physiological regulation due to changes in nutrients or in culture conditions. For example, growth retardation can be observed when cells adapted to the shade are exposed to higher radiation. The induction phase can be prevented or reduced by using cells as an inoculum in the final phase of exponential growth, grown in the same culture medium and with the same growing conditions. At the end of the latency phase the cells have adapted to the new environment and begin to grow and multiply.
2. **Log or Exponential phase**, in which the population growth rate remains always positive over time and dependent on nutrients,

temperature and lighting available in the environment (initially the low number of cells in culture minimizes mutual shadowing and each cell is in luminous saturation). The time required to double the number of viable cells is called duplication time (T_d)(equation [1]) or generation time, as it represents the time taken to grow and produce a generation of cells. The number of cells in exponentially growing microbial culture could be mathematically described by the equation [2] (Levasseur *et al.*, 1993); where: N_1= cells concentration (units of OD); N_0= initial cells concentration at the beginning of the phase (units of OD); t =time; t_0 =time when the phase starts (normally corresponds to the first time point within a growth phase) and μ = the growth rate with units of 1/h or OD/h for exponential and linear growth, respectively.

$$[1]\, T_d = \frac{\ln 2}{\mu}$$

$$[2]\, \mu = \frac{(\ln N_1 - \ln N_0)}{t_1 - t_0}$$

3. **Stationary phase**, in which the growth rate of the population is very slowed down or zeroed and the algal concentration reaches a high value. For extensive crops it is advisable to maintain the growth curve at this stage, ensuring a proper supply of nutrients, regulating the algal concentration and ensuring enough light to the metabolically active cells.

4. **Death or Decline phase,** in which the cells tend to die, both the division phase and the metabolic phase being suspended. It generally coincides with excessive algal concentration, depletion of nutrients in the culture medium or with the onset of adverse growth conditions (unsuitable temperatures, presence of toxic substances, inadequate lighting).

The repeated batch cultivation is an alternative form of operation for microalgae production. In repeated batch cultivation the reactor is initially filled with the cultivation medium and incubated under ideal conditions. After certain period a specific cultivation volume is removed and replaced with an equal amount of fresh medium. Consequently, a part of cultivation medium is kept in reactor as starting inoculum. Repeated batch mode of operation provides an excellent means of regulating the nutrients feed rate to optimize the productivity while at the same time preventing the over and underfeeding of nutrients. Repeated batch cultivation presents several operational advantages, the most important of which are the maintenance of a constant inoculum and high growth rates. It is curious that although repeated batch cultivation is very often used for growing microalgae and cyanobacteria relatively few papers have been published on the dynamics of this type of

system involving variables such as blend concentration and renewal rate (Radmann *et al.*, 2007).

2.5 Grow condition and limiting factors

There are several biological mechanisms by which microalgae autonomously produce the energy necessary for their growth and sustenance and each organism have different types of metabolisms in response to climate and environmental changes. In the literature are described four main processes, also employed in biotechnologies for the cultivation, namely: photoautotrophy, heterotrophy, mixotrophy, and photoheterotrophy.

1. -**Photoautotrophy**: in which light (energy source) and carbon dioxide (carbon source) are used to produce chemical energy through photosynthesis. This is the most commonly mechanism used for microalgae cultivation. The main benefit of the process is the use of carbon dioxide as a source for the growth or production of fatty acids. Since carbon dioxide is the only source of carbon, it's possible to take advantage of the positioning of the cultivation plants near factories or companies that discharge CO_2 into the air. If compared to other types of growth, the photoautotrophic mechanism has the lowest risk of contamination. The major problem of photoautotrophy lies in the difficulty of light penetration when the density of the culture begins to increase (Chen *et al.*, 2011; Mata *et al.*, 2010).

2. -**Heterotrophy**: some microalgae are able to grow under dark conditions, only using organic carbon both as a carbon and energy source, like bacteria. The microalgae can use different types of sugars as a source of organic carbon: glucose, acetate, glycerol, fructose, sucrose, lactose, galactose and mannose. The advantage in this case is not needing light, but the risk of contamination is higher than in the photoautotrophy. This method is defined as more practical and economical than the photoautotrophic method and would ensure greater productivity, oil yield and efficiency (Chen *et al.*, 2011).
3. -**Mixotrophy:** in which growth is a combination of photoautotrophy and heterotrophy. Mixotrophy simultaneously uses sources of organic and inorganic carbon (CO_2) in the presence of light. Therefore, microalgae can live in both conditions, depending on the concentration of organic compounds and the available light intensity (Chen *et al.*, 2011; Mata *et al.*, 2010).
4. **Photoheterotrophy**: when the microalgae require light when using organic compounds as the carbon source. The main difference between mixotrophic and photoheterotrophic cultivation is that the latter requires light as the energy source, while mixotrophic cultivation can use organic compounds to serve this purpose. Hence,

photoheterotrophic cultivation needs both sugars and light at the same time. Although the production of some light-regulated useful metabolites can be enhanced by using photoheterotrophic cultivation, using this approach to produce biodiesel is very rare, as is the case with mixotrophic cultivation (Chen *et al.*, 2011).

In a microalgal cultivation system, for commercial production, it is essential to keep under control some parameters that regulate the growth of these organisms. It should be considered the algal life cycle, the growth rate, the productivity of the desired product, the genetic stability, the nutritional requirement and the tolerance to shear stress (Suh & Lee, 2003). In the following paragraphs the main factors that achieving high-density productive algal cultures are discussed.

2.5.1 Nutrients requirement and their effect on the growth

The nutrients availability is an important factor controlling the levels of the primary productivity of photosynthetic organisms. The culture media are supplied to the cells in the form of carbon dioxide, water and mineral salts in macro- and microelements. Macronutrients are those needed in greater quantities and are the structural components of cells or are important elements

for osmoregulation: carbon (C), hydrogen (H), oxygen (O), nitrogen (N), phosphorus (P), sulphur (S), magnesium (Mg), calcium (Ca), sodium (Na), potassium (K), chlorine (Cl) and silicon (Si). Micronutrients are needed in minimal quantities and usually act as catalysts of enzymatic reactions. They are represented by copper (Cu), manganese (Mn), iron (Fe), zinc (Zn), cobalt (Co), molybdenum (Mo), some trace elements (metals and chelating agents, such as EDTA or ethylenediaminetetraacetic acid) and vitamins (Mandalam & Palsson, 1998; Suh & Lee, 2003).

In order to obtain an optimum microalgal biomass and CO_2 fixation, it's important to develop a balanced medium: C, H and O are obtained from water and air and N, P and K must be absorbed from the culture medium. During the development, N and P will become limiting. They both play a role in controlling the growth ratio and lipid production of microalgae. The N:P ratio is often used as an important indicator: too high value meaning P restriction and too low value showing that the supply of N is falling short. Usually nitrogen and sulfur are assimilated to the cells at the lowest oxidation number, as NH_4^+ and S^{2-}, although they can (in the case of sulfur, usually must) have assimilation rates with their highest oxidation number, as nitrate (NO_3^-) and sulfate (SO_4^{2-}) (Falkowski & Raven, 2007; Giordano & Raven, 2014).

Nitrogen is one of the essential elements for the growth, development, reproduction, other physiological activities. When N is limiting, is observed: the specific degradation of phycobilisomes, anchored to thylakoid membranes, which causes a cell discoloration; an accumulation of organic carbon due to an increase in polysaccharides and fatty acids; and an accumulation of lipids. Phosphorus is another essential macronutrient that plays an important role in cellular metabolic processes through the formation of various functional and structural components required for the growth of microalgae such as nucleic acids, ATP and NADPH. With P-deficiency the chlorophyll content tends to decrease while the carbohydrate content increases. Compared to the lack of nitrogen, a reduction in phycobilisomes is observed because of a stop of their production while the cell division continues and not due to the degradation of photosynthetic pigments (Falkowski & Raven, 2007; Giordano & Raven, 2014). The exhaustion of phosphorus in the culture medium leads to an accumulation of ß-carotene in *Dunaliella* (Ben-Amotz *et al.*, 1995) and the accumulation of astaxanthin in *Haematococcus* (Boussiba *et al.*, 1992).

Nutrient uptake is related to all those factors that regulate algal growth such as light, temperature, and crop mixing. Organisms grow at the expense of a substrate and, as this is used, there is an exponential growth that continues

until the balanced availability of nutrients in the culture medium is compromised. With the exhaustion of one of the nutrients in the culture medium, could happened that each cell will continue to grow thanks to the secretion of autoinhibitory compounds. At the same time, they will progressively start to decline and to prevent this and allow the culture keep on rising, the spent medium is removed and replenished by perfusion with fresh medium (Suh & Lee, 2003).

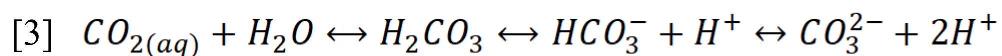
2.5.2 Salinity

Microalgae can be grown in a wide range of salinity, through their inherent defensive osmoregulation abilities such as rigid cell walls, regulation of salt uptake, synthesis of uncharged low molecular-weight compounds, and by excretion of water and salts. It is possible to distinguish hypotonic, usually freshwater species, which can sustain only low salt concentrations, and hypertonic marine species, which sustain high salt concentrations. About the latter, *Dunaliella* strains are among the largest representatives (Ben-Amotz, 2009). A substantial increase of the salt concentration in growth medium beyond the threshold of any species leads to inhibition of photosynthesis and growth; in extreme conditions, plasmolysis or cell bursting can occur.

Salinities in the range of 20-24 g/L have been found to be optimal for most of the microalgae (Singh *et al.*, 2015).

2.5.3 CO_2 Enrichment

As suggested by Suh & Lee (2003), carbon dioxide (or bicarbonate dissolves into the culture medium) is the carbon source in photoautotrophic cells. For a microalga growing photoautotrophically, approximately 95% of NADPH and more than 60% of the photosynthetically generated ATP are used to assimilate and reduce inorganic carbon. In oxygenic photoautotrophs, the pathway for inorganic carbon fixation involves the enzyme ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCo), which can use only CO_2 as a substrate (Falkowski & Raven, 2007). There is a complex relationship between CO_2 concentration and pH due to the chemical balance between the species CO_2, carbonic acid (H_2CO_3), hydrogencarbonate or bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}), as illustrated in the balance equation [3]:



$$[3] \quad CO_{2(aq)} + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+$$

Free CO_2 is found rarely as the major species of inorganic carbon in aquatic systems, predominant in acidic lakes; while CO_3 in alkaline lakes (Falkowski & Raven, 2007).

In the ocean, with an average pH of approximately 8.2, 95% of the inorganic carbon is present in the form of bicarbonate ions. Various unicellular green algae, when grown or adapted to air levels of CO_2, develop a dissolved inorganic carbon (DIC) concentrating mechanism (CCM), or a DIC pump. The latter is usually characterized by the presence of extracellular carbonic anhydrase (CA) to increase the rate of HCO_3 conversion to CO_2, which in turn is the main DIC form absorbed by the cells, with several other proteins intracellular isozymes of CA. An active DIC pump increases the rate of photosynthesis and thus productivity. *Chlamydomonas reinhardtii*, *Dunaliella* and *Chlorella* species, which contain DIC pumps, are usually characterized by the presence of extracellular carbonic anhydrase (CA) to increase the rate of HCO_3 conversion to CO_2 (Goyal *et al.*, 1992) As *Dunaliella* species can be adapted at various salinities, was found that when salinity of growth medium was increased there were consequences on the CA affinity, but is still not clear if the external or internal one. For example, Booth & Beardall (1991) found in *D. salina* a six-fold increase in external CA activity when salinity of growth medium was increased from 0.44 to 3.5 M NaC1, but almost no

change in a low level (less than five units) of internal CA activity. Goyal and co-workers (1992), instead, found with their *D. salina* there was no significant changes in the external CA activity, but a high internal CA activity was severely inhibited when salt was present in the assay.

During cultivating microalgae, sources of CO_2 include atmospheric CO_2; CO_2 from industrial exhaust gases (e.g. flue gas and flaring gas); and CO_2 chemically fixed in the form of soluble carbonates (e.g. $NaHCO_3$ and Na_2CO_3). The tolerance of various microalgal species to the concentration of CO_2 is variable. However, the CO_2 concentration in the gaseous phase does not necessarily reflect the CO_2 concentration to which organisms are exposed during dynamic liquid suspension, which depends on the pH and the CO_2 concentration gradient created by the resistance to mass transfer. Atmospheric CO_2 levels [~0.0387% (v/v)] are not enough to support the high microalgal growth rates and productivities needed for full-scale biotechnology production. Waste gases from combustion processes, however, typically contain >15% (v/v) CO_2; this percentage indicates that combustion processes will provide enough amounts of CO_2 for large-scale production of microalgae. Owing to the cost of upstream separation of CO_2 gas, direct utilization of power plant flue gas has been considered in microalgal biofuel production systems. Flue gases that contain CO_2 at concentrations ranging from 5 to 15%

(v/v) have indeed been introduced directly into ponds and bioreactors of various configurations that contain several microalgal species (Kumar *et al.,* 2010). In PBRs microalgae are generally CO_2-limited and additional CO_2 must be supplied to ensure satisfactory growth. In order to establish a basic environment monitoring algal growth, CO_2 is most introduced in the form of bubbles of enriched CO_2 gas mixture. The CO_2 concentration is in the range 1-5% balanced with air and it is continuously supply in PBRs (Suh & Lee, 2003).

2.5.4 pH

The pH of the algal culture media plays a very important role in regulating the uptake of essential nutrients including nitrate and phosphate. The speciation of inorganic carbon sources such as CO_2, HCO_3^- or CO_3^{2-}, ionization of biochemical metabolites, precipitation of the phosphates, solubility and availability of trace elements, etc. are pH dependent (Azov, 1982; Meseck, 2007). Moreover, various algal species are pH sensitive and have preferences over the various inorganic carbon sources, thus pH also regulates the species dominance within the mixed populations (Goldman *et al.*, 1982; Hansen, 2002). Increasing the pH will increase the salinity of the culture media and it

is very harmful for cells (Khan *et al.*, 2018). There is a risk of precipitation by several calcium salts and flocculation of the algal biomass at higher pH. This can lead to reduction in algal growth and hence it is necessary to avoid an increase of the pH above 8 in cultures (Ben- Amotz & Avron, 1989). Most microalgae species have a favourite pH range between 6 and 8.76 (Ben-Amotz, 2009; Khan *et al.*, 2018). It is still crucial to keep the pH of the culture within the optimal value to prevent the destruction of cellular processes.

2.5.5 Temperature

Temperature is another important factor in the growth of microalgae and directly influences the biochemical processes, changing the growth rate. Low temperatures affect photosynthesis by reducing carbon assimilation activity, whereas too-high temperatures reduce photosynthesis by inactivating the photosynthetic proteins and disturbing the balance of energy in the cell. Too high temperature also reduces cell size and respiration (Atkinson *et al.*, 2003). Each species has its own optimal growth temperature. The optimum temperature range for most algal species is 20–30°C (Singh & Singh, 2015), although thermophile algae such as *Anacystis nidulans* and *Chaetoceros* can

endure temperatures up to 40 °C (Khan *et al.*, 2018). Keeping in view *Dunaliella* strains, as described by Ben-Amotz (2009), the optimum temperature range varies with the species: for *D. salina* is 20-40°C, for *D. viridis* is 14-30°C, *D. bioculata* 25°C and for *D. tertiolecta* 20°C. These intervals, however, may vary depending on environmental factors such as salinity, pH and carbon dioxide.

2.5.6 Light intensity and Photoperiod

In all photoautotrophic organisms, all metabolic activities depend directly on the light intensity and photoperiod. The latter influence the growth and the circadian rhythm of the photosynthesis in algal cells and is specie specific (Singh *et al.*, 2015). Higher light intensities will increase photosynthetic rate to some maximum point, after the photosynthetic rate is balanced by photorespiration and photoinhibition. Thus, optimal light intensity needs to be determined experimentally to maximize CO_2 assimilation with a minimum rate of photorespiration and as little photoinhibition as possible (Ye *et al.*, 2012). A specific duration of light/dark periods is required for algal photosynthesis. Light is required for synthesis of ATP and NADPH, which drive the dark reactions of photosynthesis that will produce carbon skeletons

(Cheirsilp & Torpee, 2012). Increased photoperiod generally corresponds to increase in the growth of algae (Meseck *et al.*, 2005). Low L/D frequencies may lower growth productivity, and very high L/D frequencies may damage the cells. Exposure to prolonged periods of high irradiance increases the biomass production and carotenoid contents in algal cells but decreases the light-harvesting pigments such as chlorophyll (Singh *et al.*, 2015).

Most studies have shown that 16 h light/8 h dark is most suitable for algae growth. Appropriate light intensity and duration is necessary in bioreactors for microalgae to avoid photooxidation and growth inhibition. Appropriate penetration and uniform distribution of light is also needed to avoid photoinhibition, also called the self-shading effect, in which algae at lower layers are shaded from the light by upper layers. LED lights are a good choice for this purpose, although fluorescent tubes can also be used (Khan *et al.,* 2018). The optimum level of light intensities for most of the microalgae species are about 200–400 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Schuurmans *et al.*, 2015). Increasing the light intensity or by thoroughly mixing the culture continuously could prevent the photoinhibition (Singh *et al.*, 2015).

2.5.6.1 Photomovement

Many motile algae have groups of tightly packed carotenoid lipid-globules that constitute an orange-red eyespot or stigma that is involved in response to external physical factor, like light, temperature, pH, pesticide and heavy metals (Posudin *et al.*, 2010). Motile algae exhibit three types of responses: phototaxis, photophobia, and gliding. In phototaxis, the orientation of cell movement is effected by the direction and intensity of light. The cells move toward the light in positive phototaxis and away from the light in negative phototaxis. Eyespots are usually single structures in peripheral positions, most often oriented perpendicular to the axis of the swimming path, and usually have carotenoid-rich lipid globules packed in a highly ordered hexagonal arrangement (Lee, 1980). Phototaxis in *Chlamydomonas* is controlled by the beating of each flagellum. The flagellum closest to the eyespot is the cis flagellum while the trans flagellum is furthest from the eyespot. The light is received by the photoreceptor (chlemyrhodopsin) which controls the opening and closing of calcium (Ca) channels, and the level of intraflagellar Ca concentration (Mitchell, 2000). The transition of *Dunaliella* species from positive to negative phototopotaxis differs from that in *Chlamydomonas*,

infact the change in flagellar beating from a ciliary to an undulate mode was not observed in *Dunaliella* species. The beating of only one flagellum was observed, which caused turning of the cell followed by its subsequent movement in the opposite direction to the light source. Maximum values for motility, velocity, and positive and negative phototopotaxis were observed for the temperature range between 20 and 30°C and a pH range of 6.50 to 8.47. Also *Euglena gracilis* has the ability to move toward the direction of the light (Jennings, 1906; Mast, 1911).

Photophobia is a change in direction of movement of the cell caused by a rapid change in light intensity, irrespective of the direction of the light. Swimming cells stop and change the beat pattern from the normal asymmetric flagellar stroke to a symmetrical stroke that propels the cell backward. At the end of the photophobic response, the cells tumble and resume swimming in a new direction. Photophobic reactions in genus *Dunaliella* are not observed, though they are present in *Chlamydomonas* and *Euglena*. Since the cells of *Dunaliella* are not capable of photophobic reactions, the undulate mode of flagella beating has not been observed in the genus. *Chlamydomonas* cells change direction by using unequal beating frequencies between the cis- and trans-flagella. In contrast, *Dunaliella* cells temporarily stop the beating of one flagellum (Jennings, 1906; Mast, 1911, Mitchell, 2000, Posudin *et al.*, 2010).

In gliding motility, the flagella stop beating and adhere to a surface or an air/water interface (Mitchell, 2000). The cells can glide over the surface with one flagellum actively leading and the other passively trailing. Cells may switch direction by changing which flagellum is active. Gliding motility may be a common phenomenon among organisms that live in the thin film of water on soil particles (Lee, 1980). Both *Chlamydomonas* (Bloodgood, 1981) and *Euglena* (Häder, 1983) genus can used this photomovement.

2.5.7 Mixing and Turbulence

In high cell density cultures has been observed mutual shading as limiting factor. Therefore, mixing and aerating the cultures is very important about efficient gas exchange, equal distribution of the nutrients and metabolites as well as light energies, and to prevent gravitational sedimentation of the algal cells (Richmond & Hu, 2013). It is important to consider the mixing velocity in order to avoid the cells broken. Various kinds of mixing such as mechanical stirring, aeration with air pumps, paddle wheels, and jet pumps, etc. are used depending on the type and scale of the culture system. Based on their tolerance to not be ruptured, major microalgal groups can be arranged as green algae < blue-green algae < diatoms < dinoflagellates (Sing, 2010).

2.5.8 Sterility

The contamination of microalgal cultures is another factor, which could determine an unstable growth. Fungi, bacteria, viruses, protozoa, zooplankton, and even unwanted algae are considered as potent biological contaminants. Open pond systems are more inclined to both chemical and biological contamination. Microalgae tend to bioaccumulate various chemical contaminants from the growth medium (Harris, 1970). It should also be considered that monocultures are more susceptible to contamination than mixed cultures, especially in conditions that intrinsically do not allow a tight control of the microbiota (e.g., wastewater) (Chen *et al.* 2015; Giordano & Wang, 2018).

Anyway, it should be noted that is possible the interaction between microalgae and bacteria. The latter release stimulatory products that enhance the growth of the former, and vice versa, creating a mutualistic relationship. Knowledge on the importance of associations between bacteria and microalgae in aquatic ecosystems is rather limited at the moment. These consortia could have significant importance in different context, such as aquaculture (Natrah *et al.*, 2014; Fuentes *et al.,* 2016) or large-scale

cultivation aimed to biodiesel production (Santos & Reis, 2014). Another important aspect of using the symbiotic bacteria in microalgae cultures, is the competitive exclusion of other bacterial contaminants, as that niche is already occupied. This is called synthetic ecology as the microbial community has been chosen to solve a specific problem. (Kazamia *et al.*, 2012).

Chapter 3 Aim of the thesis

In order to meet population growth, reduce dependence on fossil fuels and invest in the circular economy (by cancelling CO_2 emissions), our society needs new resources. Microalgae are considered one of the most promising feedstocks for sustainable production of food, feed, chemicals, materials and fuels. Limited knowledge about costs on microalgal cultivation and processing at commercial scale is available, particularly concerning closed photobioreactors. Nevertheless, high costs associated to algal biomass production could be due to the request for water supply, macro- and micronutrient provisioning, electricity (to provide light to the culture and to keep it constantly moving), temperature control and cooling systems (if temperature gets too high). In order to lower production costs, the biotechnological industries require cultures that grow at maximum rates and achieve the highest possible concentration of cells per liter of cultivation medium, able to thrive in the presence of pests, predators and pathogens.

The main objective of microalgae biotechnology is to obtain a significant biomass to be converted into the bioactive compounds, required by the market, or into fuel. In order to accumulate a significant concentration of the desired compound, microalgae are grown using abiotic stress, compromising

the quality and health of the biomass. This research thesis firstly aims at identifying the most suitable *Dunaliella* species and its cultivation protocol to be used in biotechnological applications. For this purpose, growth rates of six species belonging to this genus have been determined by changing several growth parameters and interactions among those variables have been examined. *Dunaliella* genus was chosen for the extraordinary adaptability to survive in environmental stress conditions caused by changes in pH, temperature, salinity (intended as a change in NaCl concentration), light and nutrients. Focus also on phototactic movements of single cells, the cultivation of these species was made without mixing the cultures, trying to find an alternative way of saving production costs. In view of future mass cultivation, with this selection we wanted to investigate the existence of a species to be added to those already existing, which can be defined as a valid resource for its swimming potentiality.

Chapter 4 Materials & Methods

The experimentation was made at the Centre of Biotechnology (CeBiTec), Bielefeld University in Germany and this proposal was divided in three main part. In the first one, six different strains of genus *Dunaliella* were investigated in order to select the best species with the best adaptability to the growing conditions considered. The choice was taken according to the optimal μ per day obtained in the shortest time, processing all microalgae at different salinity values and investigating the best way to supply carbon source to the culture. Therefore, on strains selected, was tested the optimal temperature and made a new selection for the best organism. In the third step, the best species chosen was compared, with other famous biotechnological species, to prove its potentiality.

4.1 Experimental models

4.1.1 *Dunaliella* strains

The green microalgae own to genus *Dunaliella* were first described by Teodoresco (1905), based on material collected from a Rumanian salty lake, with *Dunaliella salina* as wild type species. The genus was named in honor of F. Dunal, who was the first to observe that in the salterns of Montpellier, on the Mediterranean coast of France (Dunal, 1838). Borowitzka & Siva (2007) placed the genus in a separate order of the Chlorophyceae (Dunaliellales), but, except lacking a rigid wall, *Dunaliella* has many characteristics in common with members of the Chlamydomonadales (Avron & Ben-Amotz, 1992). The cell shape in *Dunaliella* species varies from ellipsoid, ovoid, cylindrical and pyriform to almost spherical, under unfavorable condictions (Borowitzka & Siva, 2007; Ben-Amotz*,* 2009). Usually cell size ranges from 8-25 µm long and 5-15 µm wide, but may also vary to some degree with growth conditions and light intensity. The symmetry of *Dunaliella* cells could be radial, bilateral or slightly asymmetrical, flattened and dorsiventrally curved. As mentioned above, the cells lack a rigid wall, but is possible to observed, in older cells, a

mucilaginous cell coat covering of varying thickness (Oliveira *et al.,* 1980).The cells have two flagella, apically inserted, equal in length, and usually exhibit a homodynamic pattern of beating. Each cells have a single chloroplast occupies most of the cell body. It is cup-, dish-, or bell-shaped and has a thickened basal portion containing a pyrenoid. Thylakoids inside the chloroplast are sometimes arranged in dense stacks of up to 10 units. Usually around the pyrenoid there are starch grains, but may also be found at other places of the chloroplast (Borowitzka & Siva, 2007). Each organisms have a eyespot (stigma) located anterior peripheral location in the chloroplast, hardly visible with the light microscope (Ben-Amotz & Avron, 1989). The nucleus has a porous envelope and a single prominent nucleolus, which is often surrounded by clumped heterochromatin. It also is generally obscured by many granules. Mitochondrial profiles can be seen in various parts of the cell in thin sections, and the number and size of mitochondria may vary among cells at different stages of growth. The Golgi bodies occur in numbers of 2 to 4, each consisting of 10 to 15 cisternae. The endoplasmic reticulum (ER) typically underlies the plasmalemma over most parts of the cell. It appears that ER serves as a temporary reservoir for membrane material in temporary excess during stress periods when major cellular compartments shrink. Prominent ultrastructural constituents of the cytoplasm are vacuoles,

containing portions of membrane and vesicles as well as granular or thread-like material (Fig. 5).

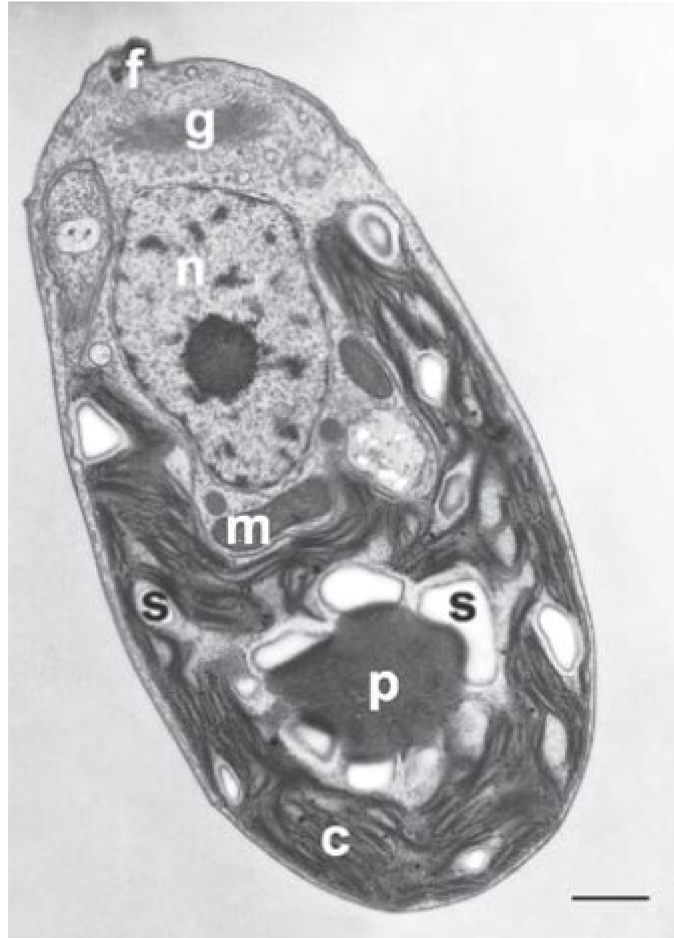


Figure 5: Electron micrograph (longitudinal section) of a green cell of Dunaliella tertiolecta showing the cup-shaped chloroplast (c), pyrenoid (p), starch grains (s), mitochondrion (m), nucleus (n), golgi apparatus (g), and flagella base (f). Scale bar = 5 µm. (Ben-Amotz & Avron, 1989).

The life cycles of *Dunaliella* genus encompass, in addition to division of motile vegetative cells, the possibility of sexual reproduction. Vegetative cell division has been described in detail by Borowitzka & Siva (2007). It begins with nuclear division followed, almost immediately, by an infurrowing of the cell usually first observed at the flagellar (anterior) end of the cell between the

flagella and, soon after, at the opposite (posterior) end of the cell. The infurrowing generally proceeds faster at the flagellar end. The posterior furrowing proceeds concomitantly with division of the chloroplast and pyrenoid until the daughter cells remain connected only by a thin colorless cytoplasmic bridge located about halfway between the anterior and posterior ends of the cells. During that process, the two daughter cells each regrow a second flagellum. Once this flagellum has reached near full length the two daughter cells begin to spin in opposite directions until the cytoplasmic bridge breaks and the two daughter cells separate. Some *Dunaliella* species can also develop a vegetative palmelloid stage consisting of round non-motile cells. The cells lose their flagellae and eyespot, become more rounded, and excrete a slime layer within which they divide repeatedly forming accumulations of cells within a gelatinous matrix. On returning to "normal" salinity, the cells usually reform their flagellae and return to the motile, free swimming state. *Dunaliella* genus organisms may also form vegetative cysts (aplanospores) under reduced salinity, diluition of the medium, nitrogen deficiency, and in cooler and low light conditions (Loeblich, 1969; Borowitzka & Huisman, 1993). The aplanospores have an extremely resistant, thick, two-layered, rugose wall (Leonardi & Cáceres, 1997). Instead, the sexual reproduction is by isogamy, with gametic fusion proceeding similarly to that in

Chlamydomonas. The gametes have the same size and the same structural features as growing cells of the same species. Several species of *Dunaliella* appear to be homothallic, whereas *D. salina* has been reported to be heterothallic. Meiosis takes place during the germination of the zygote. This latter is green or red and is surrounded by a thick, smooth wall. After a resting stage, the zygote nucleus divides forming up to 32 cells, which are liberated through a rupture in the mother cell wall. Cell morphology may be influenced to some degree by environmental growth conditions. Conspicuous changes in salt concentration, light intensity, and temperature may have some effects on, e.g., thylakoid structure, appearance of pyrenoid, and proliferation of the endoplasmic reticulum (Ben-Amotz & Avron, 1989)(Fig. 6).

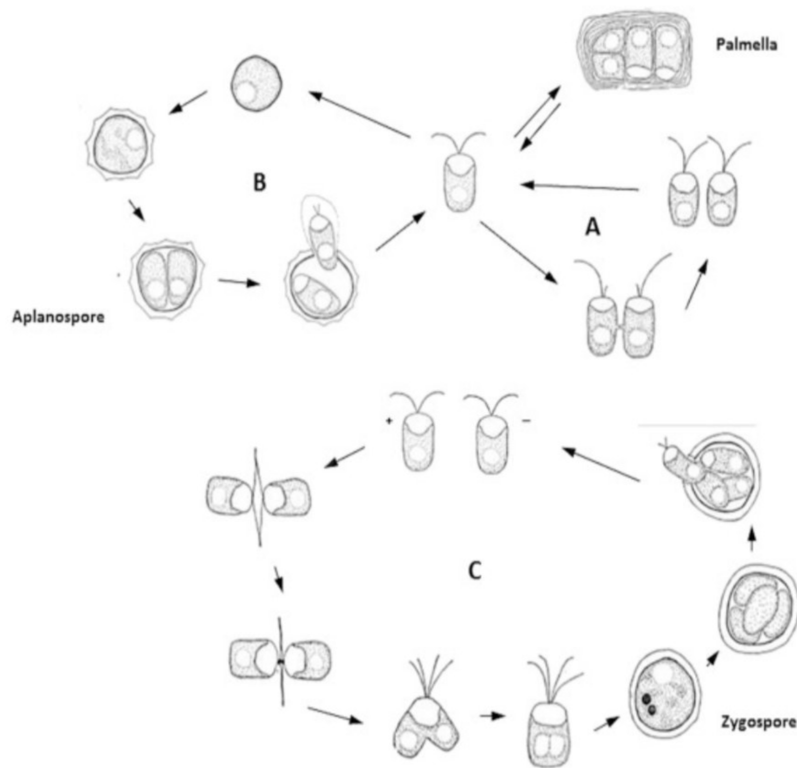


Figure 6 The life cycle of Dunaliella. (A) vegetative reproduction of the haploid motile cells by mitosis followed by cytokinesis. The formation of the new second flagellum is indicated. The formation of the palmella stage is also shown; (B) formation of asexual resting cysts, the aplanospores. These cysts are haploid and under favorable conditions one or more mitotic division will occur within the cyst and the daughter flagellated cells will be released; (C) sexual cycle starting with the mating of sexually two compatible (+ & −) strains. Following plasmogamy and karyogamy, the diploid zygospore is formed. Under favorable conditions meiosis will occur and the haploid daughter cells will be released from the mother spore (Borowitzka & Siva, 2007).

The *Dunaliella* genus is widely used because of the ease culturing of most of the species. It is known the ability of several species to grow over extremely salinities (around 5 M NaCl), and they are used as feedstock for several biotechnological applications, for example due to their production of glycerol.

Is also important to consider the accumulation of extremely high levels of β-carotene in *D. salina*, and the wide tolerance to heavy metals and pesticides by some strains (Borowitzka & Siva, 2007; Shariati & Hadi, 2011). *D. parva* has been intensively studied in relation to osmoregulation, photosynthesis and glycerol synthesis pathway (Ben-Amotz & Avron, 1973; Ginzburg, 1991); *D. viridis* is a species associated with *D. salina* in nature and it has been used in ecological studies (Post, 1977) as well as in mass cultivation experiments (Moulton & Burford, 1990) *D. tertiolecta* has been used for osmoregulation and photosynthesis studies (Gilmour *et al.,* 1985; Aoki *et al.*, 1986)), study of the carbon dioxide concentrating mechanism (Amoroso *et al.*, 1998), a promising feedstock for production of biodiesel and renewable diesel (Lakaniemi *et al.*, 2012) and used as feed in aquaculture (Fábregas *et al.* , 1986); *D. bioculata* has been used to study the plasma membrane ATPase ((Wolf *et al.*, 1995; Smahel *et al.*, 1990; Raschke & Wolf, 1996) and for comparative studies of ultrastructure (Marano *et al.,* 1985). In this work was made a first investigation on six *Dunaliella* strains, obtained from the Sammlung von Algenkulturen at the University of Göttingen (SAG), Germany. The species used are: *Dunaliella bioculata* (SAG19-4); *Dunaliella parva* (SAG19-1); *Dunaliella salina* (SAG184.80); *Dunaliella sp.* (SAG19-5); *Dunaliella tertiolecta* (SAG13.86) and *Dunaliella viridis* (SAG44.89). At

a first observation with the light microscope (Motic BA 310 Series) to the original stock species, they appeared axenic, except the latest two nominated which were contaminated by fungi and bacteria, as described in SAG culture informations. At the beginning stock cultures were propagated and maintained in SAG *Dunaliella* medium, which recipe is described in Figure 7.

	stock solution [g/100 ml]	nutrient solution [ml]
KNO_3	1	20
K_2HPO_4	0.1	20
soil extract *	30	
artificial seawater **	930	

* Preparation of soil extract (as in medium 1):
Fill a 6 litre flask one third with garden or leaf soil of medium, but not too great humus content which does not contain fertilizers or plant protective agents. Success of soil extract depends on selection of suitable soils. Those with high clay content are usually less satisfactory. Add de-ionized water until it stands 5 cm above the soil and sterilize by heating in a steamer for one hour twice in a 24 h interval. Separate the decanted extract from particles by centrifugation. Fill into small containers of stock solution each of a size appropriate to making a batch of media, autoclave for 20 min at 121 deg; C and store in the refrigerator.

** Preparation of artificial seawater:
Dissolve in 1000 ml of de-ionized or distilled water 60.0 g NaCl, 10.0 g $MgSO_4 .7H_2O$, 1.5 g KCl, and 2.0 g $CaSO_4$.

Figure 7 SAG Dunaliella medium recipe.

4.2 Salinity tests

For the salinity test, the six *Dunaliella* species were autotrophically grown in 24-well CytoOne® plates, carried out in a fume cupboard. The cultures were inoculated to an OD of ca.0.5 at 680 nm (measured with a spectrophotometer) and left to grow. In each well there were an initial concentration of 200 μl of stock culture and 1.8 ml of Provasoli *et al.* (1957) artificial growth medium respectively. The following components and concentrations were applied for 1L solution: K_2HPO_4 0.57 mM; H_3BO3_3 0.16 mM; $MgSO_4$*$7H_2O$ 4.87 mM; KCl 21.46 mM; $NaNO_3$ 11.77 mM; $CaCl_2$*$2H_2O$ 2.72 mM; $FeCl_3$*$6H_2O$ 12.2 μM; Na_2-EDTA Dihydrate 12.5 μM; TRIS 8.26 mM [initial salinity = 5.317part per thousand (ppt)]. The multiwells plate were oriented in order to have 6 columns, one for species, with 4 wells containing the new medium at NaCl concentrations of 1g, 0.25M, 0.5M, 1M, 1.5M and 2M respectively (Figure 8).

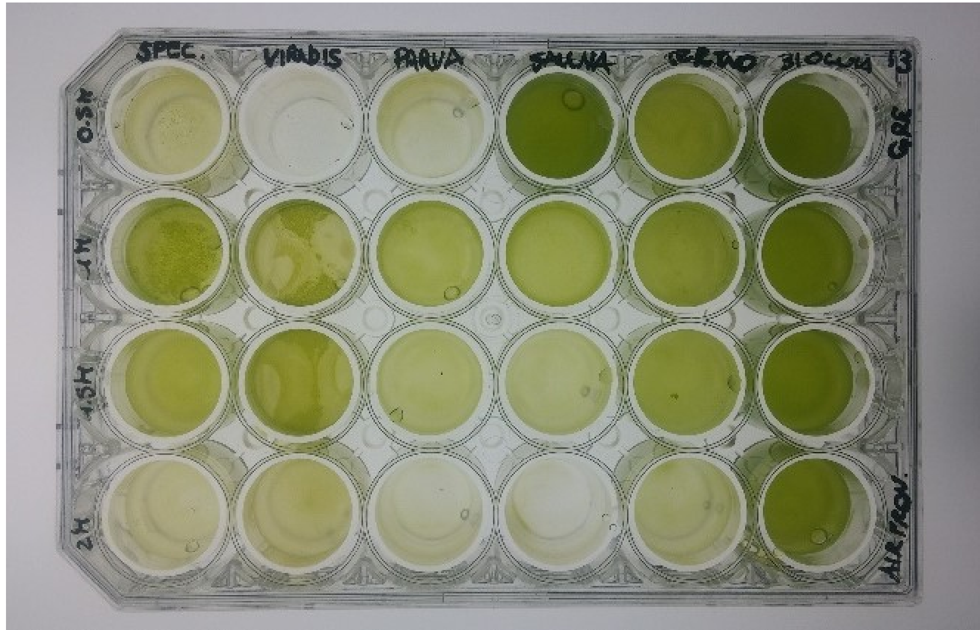


Figure 8: 24-well CytoOne® plates used for testing in this project. The columns represent the different species examined, from left to right: Dunaliella sp., D. viridis, D. salina, D. parva, D. tertiolecta and D. bioculata. In this case, the rows were the new culture medium prepared for salinity tests. The NaCl concentrations were 0.5 M, 1 M, 1.5 M and 2M respectively.

Algae were supplied with no filtered low CO_2 (standard air) or no filtered CO_2-enriched air (4%, v/v) in the gas phase of a plexiglass box containing the samples. Growth media did not contain any supplemental source of dissolved inorganic carbon (DIC). Other culture conditions were temperature of 26°C, a continuous photon flux density of 250 $\mu E \cdot m^{-2} \cdot s^{-1}$ provided by white fluorescent tubes, and no mixing movements. The health of microalgae was monitored by light microscopy observations (data not shown) and the growth was checked through daily measurements of the culture optical density (OD),

by using the Tecan Infinite® M200 Plate Reader, approximately at the same time. The measured absorbance wavelength was at 680 nm, associated to the pigment Chl-α associated with the pigment in live microalgae, and at 750 nm, to confirm the absence of cyanobacterial contaminations (Chen & Blankenship, 2011; Chen, 2014). When cultures reached logarithmic phase, the test was replicated by exploiting the cells at this stage to fill new multiwell plates. The concentrations, of culture strain and medium, were kept as close as possible to the initial quantities.

4.3 DIC tests

Concerning these tests, the six *Dunaliella* species were autotrophically grown in 24-well CytoOne® plates, executed in a fume cupboard. The cultures were inoculated to an OD of ca. 0.5 at 680nm and left growing. In each well there were an initial concentration of 200 µl of stock culture and 1.8 ml of different media. The wells preparation was made by using a modified Sorokin *et al.* (2000) artificial growth medium and by mixing it with Provasoli *et al.* (1957) medium, containing 0.5 M of NaCl. Therefore, final media mix contained the concentrations of 100%, 80%, 50% and 20% respectively of modified Sorokin *et al.* (2000) medium added to Provasoli *et al.* (1957) medium; Provasoli medium was considered as control medium. Sorokin and co-workers' (2000) medium was firstly used to grow the alkaliphilic bacterium *Roseinatronobacter thiooxidans*, isolated from a Soda Lake, and for this proposal was chosen in order to prevent contaminations because the high pH (around 10). The latter was reached by the presence of sodium, carbonate and bicarbonate in solution and it was supposed that *Dunaliella* genus was able to grow well to such conditions. Also considering the huge concentration of carbonate and bicarbonate, might make a useful alternative to provision carbon source and thereby avoid suppling CO_2, through bubbling. Below were

described components and relating elements concentrations in the Sorokin *et al.* (2000) medium: Na_2CO_3 537.79 mM, $NaHCO_3$ 214.27mM, $MgSO_4*7H_2O$ 4.87mM, KNO_3 11.78mM, K_2HPO_4 57.4µM, EDTA 17.11µM, $FeSO_4*7H_2O$ 7.19µM, $ZnSO_4*7H_2O$ 0.35µM, $MnCl_2*4H_2O$ 0.15µM, H_3BO_3 4.85µM, $CoCl_2*6H_2O$ 0.84µM, $CuCl_2*2H_2O$ 0.06µM, $NiCl_2*2H_2O$ 0.12µM, $Na_2MoO_4*2H_2O$ 0.08µM. The preparation of the medium wasn't required sterile condition, because of the high pH. The multiwell plates were equally oriented like salinity tests, and the other culture conditions were the same: temperature of 26°C, a continuous photon flux density of 250 $\mu E \cdot m^{-2} \cdot s^{-1}$ and no mixing movements. The tests were performed both under no filtered standard air and in a plexiglass box supplied no filtered 4% CO_2 enriched air (v/v). The growth of microalgae was monitored through daily measurements of the culture optical density (OD), at 680nm and 750nm, by using the Tecan Infinite® M200 Plate Reader, approximately at the same time. When cultures reached logarithmic phase, the test was replicated by exploiting the cells at this stage to fill new multiwell plates. The concentrations, of culture strain and medium, were kept as close as possible to the initial quantities.

4.4 Temperature tests

D. bioculata and *D. tertiolecta* were photoautotrophically cultivated for temperature tests. Two photobioreactors (PBR) FMT 150 were used for this experiment. They consisted in flat, rectangular glass cultivation vessel, with a volume of ca. 400ml. The first preparation of the experiment was made under fume cupboard, using cultures of previous tests. The initial cultures inocula was to an O.D. of 0.1 at 680nm. Them were placed in each photobioreactor and the remaining vessels were filled with Provasoli *et al.* (1957) medium, with 0.5M NaCl into solution. In this case, the preparation of the medium was under unsterile condition and without been autoclaved. Before starting the experimentation, cells weren't pre-adapted. This choice was made in order to see the answer, simulating the transfer from a laboratory condition to a more intensive cultivation. Other conditions were supplying no filtered 4% CO_2 enriched air and settled light intensity between 0-500 $\mu E \cdot m^{-2} \cdot s^{-1}$ because of the 16:8 hours photoperiod. The temperature measurements were 20°C, 25°C, 30°C, 35°C, 37.5°C, 40°C and 42.5°C. The OD 680 and OD 720 were periodically measured by machines, and the data were stored in the PBRs memory. The machine was settled to perform each single test within a median duration of 10 days. In proximity to the logarithmic phase of the growth

curves, the test was replicated filled PBRs with the cells at this stage and the medium, again prepared. The conditions of replicates were unsterile and was important to bear in mind that the O.D. culture value, at the beginning of each experimentations, was 0.1. Before starting a new experiment, each PBRs were washed with ethanol and tap water and cleaned with nylon brush. A deep cleaning was done only at the end of the project, also autoclaving PBRs.

4.5 Mixing tests

In the third part of this work, *D. bioculata* was compared with other species, which has biotechnological importance. The species used were *Chlamydomonas reinhardtii* (CC-1690) from *Chlamydomonas* Center, Duke University, Durham, NC, USA; *Chlorella sorokiniana* (SAG 211-8k); *Parachlorella kessleri* (SAG 211-11h) and *Euglena gracilis* (SAG 1224-5/25), and their main characteristics are reported in the table below (Table 2).

Table 2: Strains used for comparative tests

Strain numer	Taxonomy	Locality	Habitat	Diameter	Some biotechnological applications
SAG 19-4	*D. bioculata* (Butcher, 1959)	Salt lake, Russia	marine	10-15µm	Molecular analysis (Wolf et al., 1995; Smahel et al., 1990; Raschke & Wolf, 1996; Marano *et al.,* 1985)
CC1690	*C. reinhardtii* (Dangeard, 1888)		freshwater	ca. 10µm	Hydrogen-production and Biofuel (Mussnung et al.,2010; Klassen *et al.,* 2015)
SAG 211-8k	*C. sorokininana* (Shihira & Krauss, 1965)	Austin, Texas, USA	freshwater	2-10µm	Biofuel (Cazzaniga *et al.,* 2014)
SAG 211-11h	*P. kessleri* (Fott & Nováková, 1969)	New York city, USA	freshwater	2.5-8.9 µm	Biofuel (Klassen *et al.,* 2015); Oil-bearing (Rathod *et al.,* 2013)
SAG 1224-5/25	*E.gracilis* (Klebs, 1883)		freshwater	8-20µm	Paramylon (Gissibl *et al.,* 2019)

All the strains were photoautotrophically grown in 24-well CytoOne® plates, carried out under a fume cupboard. Before filled the wells, all the strains have undergone to a measurement of the absorbance at 680nm, for the purpose of obtain an initial culture value of ca.0.5. Then the strains were placed in the wells with their own culture medium and left to grow. For *D. bioculata* was prepared Provasoli *et al.* (1957) medium adding 0.5M of NaCl; for *C. reinhardtii, C. sorokiniana* and P. *kessleri* Provasoli *et al.* (1957) medium with 1g NaCl and for *E.gracilis* the modified (Cramer & Myers, 1952)

medium (renamed EG6) was used. The composition and concentration of the elements in the last-named medium were: $MgSO_4*7H_2O$ 0.81mM, $(NH_4)_2HPO_4$ 7.57mM, $(NH_4)_2SO_4$ 2.57mM, $CaCl_2*2H_2O$ 6.80mM, $EDTA*2Na*2H_2O$ 26.8Mm and K_2HPO_4 0.73mM. The tests were conducted both with and without shaking movements, with a velocity of 120 rpm. In both cases the multiwell plates were placed in plexiglass boxes, where no filtered 4% CO_2 enriched air was blowing inside. Other culture conditions were temperature of 30°C, and a continuous photon flux density of 250 $\mu E \cdot m^{-2} \cdot s^{-1}$ provided by white fluorescent tubes. The growth of microalgae was monitored through daily measurements of the culture OD 680 and OD 750, by using the Tecan Infinite® M200 Plate Reader, approximately at the same time. When cultures reached logarithmic phase, the test was replicated by exploiting the cells at this stage to fill new multiwell plates. The concentrations, of culture strain and medium, were kept as close as possible to the initial quantities.

4.6 Statistical tests

Every experiment was reproduced from 3 to 7 times to give it statistical significance. The measured growths rates from each test and its replicates were utilized to calculate the averages ± standard error of each log phases, considering the O.D. at 680nm. Statistical analysis was made by t-test in Microsoft Excel software; the significance was set at $p\text{-value}<0.05$ and all values were presented in tables at the end of the thesis.

Chapter 5 Results

5.1 Salinity tests

The effects of salinity on the growth kinetics of six *Dunaliella* species were quantitatively evaluated in this work by cultivating those strains, both under standard air and 4% CO_2 enriched air, continuously bubbled, without mixing the cultures. Viewing the cells by the microscope, some of them responded to culture medium by morphological change. They have expanded to a spherical shape and high amount of chloroplast granule was visible inside (data not shown).

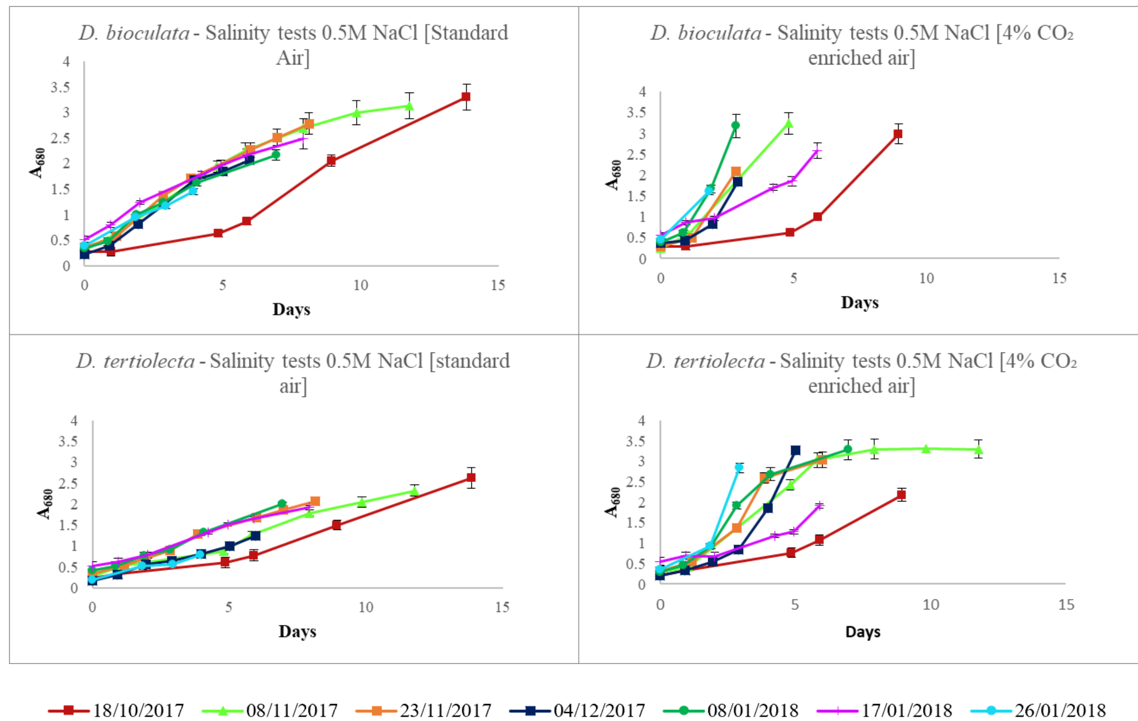


Figura 9 - Daily absorbance 680nm for salinity test. The different colours represent the replication of each tests. The error bars represent the standard errors.

Figure 9 was an example of growth curves of two species grew with a concentration of NaCl of 0.5M (29,2 g/L), measuring the absorbance 680nm at daily time points. Like for *D. bioculata* and *D. tertiolecta*, also for the other four species were made the same measurements and repeated the experimentation 7 times, selected artificially the new inoculum from the culture before. *D. bioculata* cultivations, under standard air, started with an OD_{680} of $0.23 \pm 0.05 < x < 0.52 \pm 0.07$ in the whole culture; while under CO_2 enriched air with CO_2 an initial OD_{680} of $0.29 \pm 0.07 < x < 0.55 \pm 0.07$ was applied. Instead, for *D. tertiolecta* cultivations, under standard air, the initial OD_{680} were $0.18 \pm 0.03 < x < 0.53 \pm 0.09$, and under CO_2 enriched air were

$0.22 \pm 0.059 < x < 0.56 \pm 0.1$ (Fig. 9). The biomass accumulation didn't occur in linear way, but like a logarithmic curve. The highest productivity was achieved in 10 days in the case of standard air cultivation, and in less than 5 days for the cultivation with CO_2 enriched air. After that time, the tests were repeated (Fig. 9).

Figure 10 showed the comparison among the different salinity, performed in terms of growth rate per day of the six strains, cultivating with standard air (dark grey bars) and CO_2 enriched air (light grey bars), and without mixing them. The mean value of the growth rate per day (μ), at the logarithmic phase of the all species replicates, is reported in this figure.

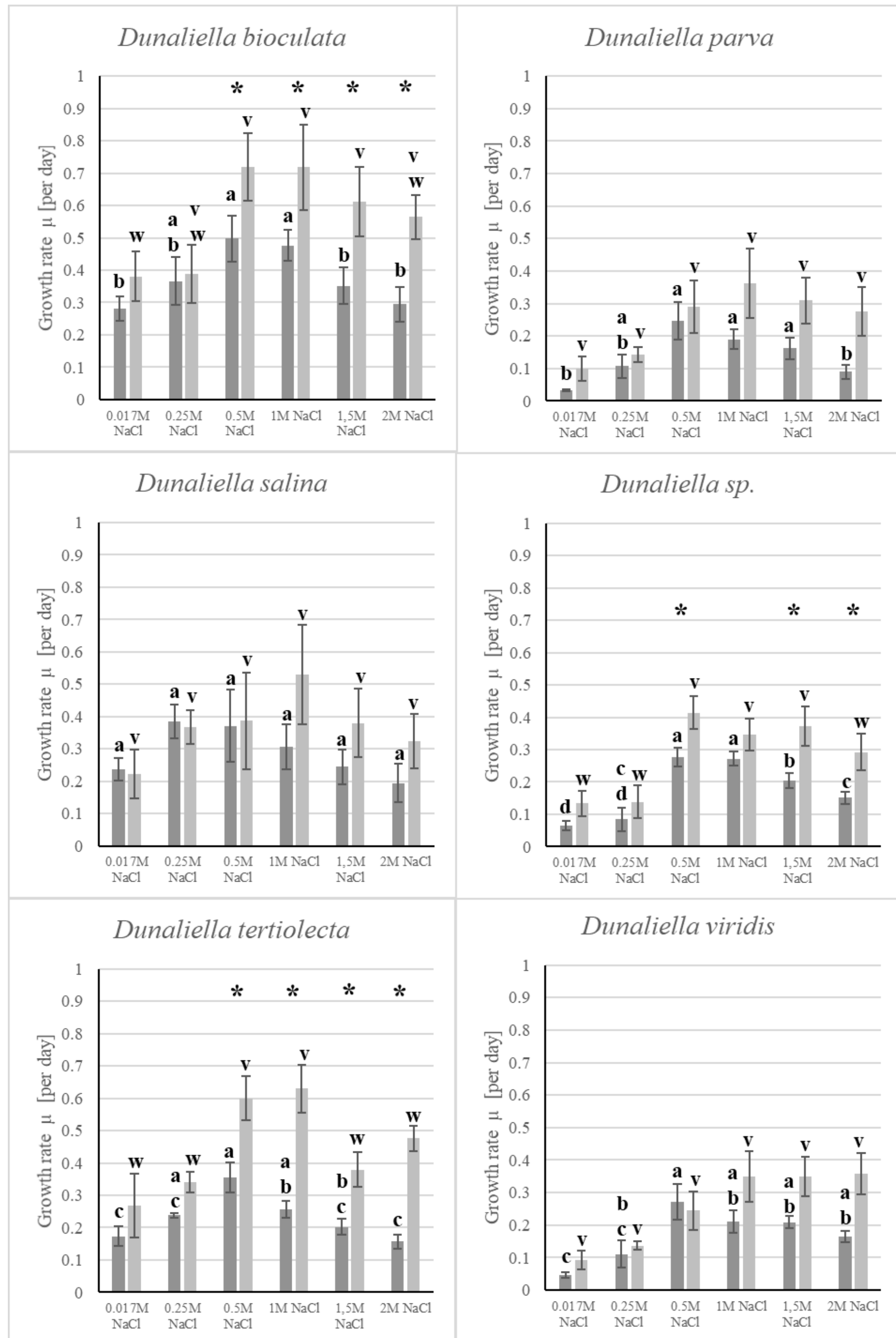


*Figura 10 - Mean value of the growth rate per day, at the logarithmic phase of salinity tests. Dark grey bars correspond to the standard air experiments, light grey bars to the 4%CO_2 enriched air tests, and with the error bars are represented the standard errors. n≥3. a, b, c correspond to statistical significance between standard air tests; v and w to statistical significance to CO_2 enriched air tests. Statistical significance, between the two air conditions, at each NaCl concentrations, was marked with *.*

The evaluation showed that species responded differently, to salinities in the culture medium. In this study the minimum applied salinity was set at 0.017M NaCl and maximum at 2M NaCl. Looking at cultures under standard air supplementation (Fig. 10), the growth rate was observed to be not very high at extreme concentrations, in fact bell curves were observed in all species graphs. For *D. bioculata* significant differences were noted between growth rates of cultures grown with 0.5M-1M NaCl and the other NaCl concentrations, apart from cells grown with 0.25M NaCl which didn't report statistical significance (Tab. 3). Significant differences were reported in *D. parva* cells cultivated with a NaCl concentrations from 0.5M to 1.5M and both 0.017M and 2M; instead cells grown with 0.25M NaCl didn't report statistical significance (Tab. 6). *D. salina* growth rates didn't report statistical significance (Tab. 9). In *D. sp.* were distinguishable statistical equality in cells grown with 0.5M NaCl and 1M NaCl (Tab. 12). In *D. tertiolecta* was observed statistical equality between cells grown with 0.25M NaCl, 0.5M NaCl and 1M NaCl; between cells grown with 1M NaCl and 1.5M NaCl; and between cells grown with 0.017M NaCl, 0.25M NaCl, 1.5M NaCl and 2M NaCl (Tab. 15). In *D. viridis* was observed statistical equality between cells grown with NaCl concentrations from 0.5M to 2M; between cells grown with

0.25M NaCl and a sodium chloride concentration from 1M to 2M; and between cells grown with 0.017M NaCl and 0.25M NaCl (Tab. 18).

Always in figure 10, but concerning cultures cultivated under CO_2 enriched air, also these growth rates were shown as bell curves. In *D. bioculata* significant differences were reported between cells grown with sodium chloride concentration from 0.5M to 1.5M and 0.017M, instead cells growth rates with a NaCl concentration of 0.25M and 2M didn't report statistical significance (Tab. 4). In *D. sp.* significant differences were shown between cultures cultivated with a NaCl concentration from 0.5M to 1.5M and the other concentrations (Tab. 13). In *D. tertiolecta* statistical equality were seen between cultures cultivated with a sodium chloride concentration from 0.5M to 1M and the others (Tab. 16). Both *D. parva, D. salina and D. viridis* cultures were reported statistical equality (Fig. 10; Tab 7, 10, 19).

Comparing the two air conditions, were obtained significant differences in *D. bioculata* and *D. tertiolecta* cultures where cells were cultivated with a concentration of NaCl from 0.5M to 2M (Fig. 10; Tab. 5, 17). Statistic differences were reported also in *D. sp.* cultures grown with a NaCl concentration of 0.5M, 1.5M and 2M (Fig. 10; Tab. 14).

Between all species the highest μ-values per day were obtained by *D. bioculata* (μ=0.5±0.07), *D. salina* (μ=0.371±0.112) and *D. tertiolecta*

(μ=0.355±0.046), grown with 05M NaCl at standard air (Fig. 10). While, cultures, cultivated with CO_2 enriched air, have found more optimal both the salinity of 0.5M NaCl and 1M NaCl. This latest was preferred by *D. parva, D. salina* and *D. viridis*. In this case, the highest μ-values were achieved by *D. bioculata* (μ=0.734±0.080), *D. salina* (μ=0.53±0.155) and *D. tertiolecta* (μ=0.62±0.089) (Fig. 10). According to the findings presented above, only results obtained using medium at the salinity of 0.5M and species, *D. bioculata* and *D. tertiolecta*, will be considered favourite species.

5.2 DIC tests

It was made tests in order to try a new medium for growing the six *Dunaliella* strains. In figure 11 are visible new modified Sorokin *et al.* (2000) medium mixed at percentages of 100% (A), 80% (B), 50% (C) and 20% (D) with the medium used for salinity tests, at the concentration of 0.5M NaCl (control). Cultures were grown under standard air (dark grey bars) and 4% CO_2 enriched air (light grey bars), without mixing them. All graphs represented the kinetic growth of each *Dunaliella* species, shown as the mean value of the growth rate per day at the logarithmic phase of all replicates. The highest productivity was achieved in less than 4 days in the case of standard air cultivation, and in less than 3 days for the cultivation with CO_2 enriched air. After that time, the tests were repeated.

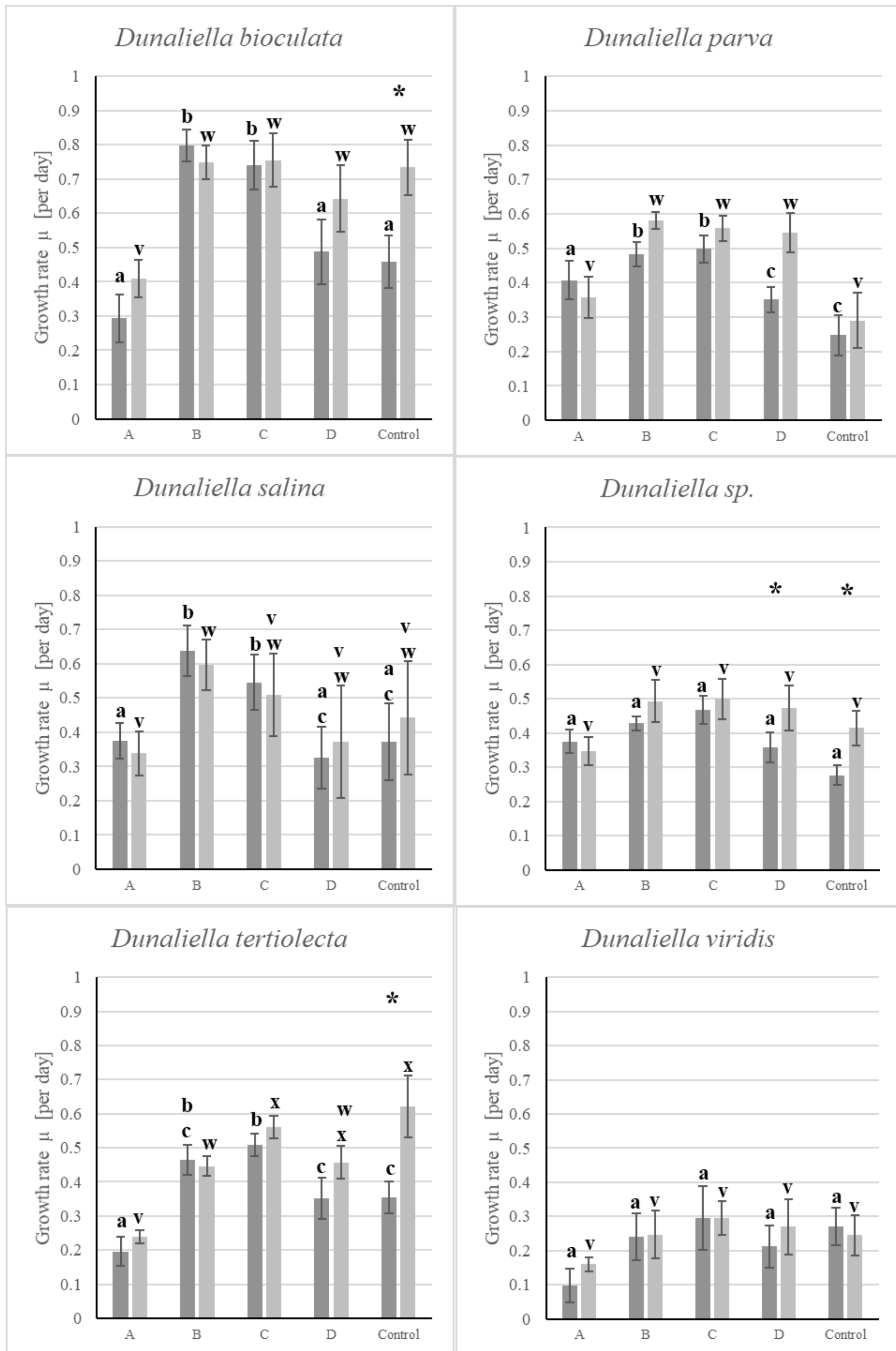


*Figure 11 - Mean value of the growth rate per day, at the logarithmic phase of provisioning carbon source tests. Dark grey bars correspond to the standard air experiments, light grey bars to the 4%CO_2 enriched air tests, and with the error bars are represented the standard errors. n≥3. a, b, c correspond to statistical significance between standard air tests; v, w and x to statistical significance to CO_2 enriched air tests. Statistical significance, between the two air conditions was marked with *.*

Considering cultures grown under air conditions (dark grey bars in Fig. 11) and under CO_2 enriched air (light grey bars in Fig. 11), growth rates were observed to be not very high at extreme media concentrations. An exception concerns species *D. sp.* and *D. viridis*, which didn't show statistical significance (Fig. 11; Tab. 32, 33, 38, 39). Statistical equalities were obtained cultivating *D. bioculata*, *D. parva* and *D. tertiolecta* in media B and C (Fig. 11; Tab. 21, 24, 33), under standard air. Under CO_2 enriched air, *D. bioculata* reported only with medium A significative differences between other media (Fig. 11; Tab. 22). In *D. parva*, cultivated with CO_2 enriched air, statistical similarity was observed with media B, C and D (Fig. 11; Tab. 25). Suppling CO_2 enriched air, *D. salina* reached in all media statistical equality to control medium, but media A and B didn't show statistical similarity (Fig. 11; Tab. 28). Suppling CO_2 enriched air, *D. tertiolecta* cultures lastly were reported statistical significance between media B and D; and between the media C, D and control (Fig. 11; Tab. 34).

In addiction, is important to consider that all species respond in equal way comparing both air conditions, referred to each single percentages of the new modified medium (Fig. 11; Tab. 23, 26, 29, 35, 38). An exception concerns *D. sp.* which reported statistical differences in cultivations in medium D (Fig. 11; Tab. 32).

Without consider control medium and focusing on growths under standard air, the highest μ-values per day were obtained by following species: *D. bioculata* in medium B was achieved a growth rate of μ=0.797±0.047 and in medium C of μ=0.741±0.07; *D. salina* was gained in medium B a μ=0.637±0.074 and *D. tertiolecta* in medium C was reached a μ=0.507±0.033 (Fig. 11). In cultures cultivated with CO_2 enriched air, the same species were obtained the highest growth rates per day in the same media (Fig. 11). *D. bioculata* have found more optimal both medium B, where the μ was 0.748±0.05 and medium C (μ=0.755±0.078) (Fig. 11). *D. salina* obtained a μ=0.596±0.073 in medium B and *D. tertiolecta* reached a μ=0.561±0.034 in medium C (Fig. 11). Since the aim of the study was to be able to use existing sources, it was decided to proceed the experimentation with the medium chosen in the previous experiment, supplying CO_2 as carbon source. *D. bioculata* and *D. tertiolecta* were confirmed as favoured species and were therefore selected for subsequent test.

5.3 Temperature tests

The effect of temperature on the growth kinetics, of *D. bioculata* and *D. tertiolecta* species, was quantitatively evaluated in this project by cultivating these strains in PBRs, with a photoperiod of 16 hours of light and 8 of dark. The light settled, gradually reached the intensity of 500 μE·m^{-2}·s^{-1}, in order to imitate sunrise and sunset.

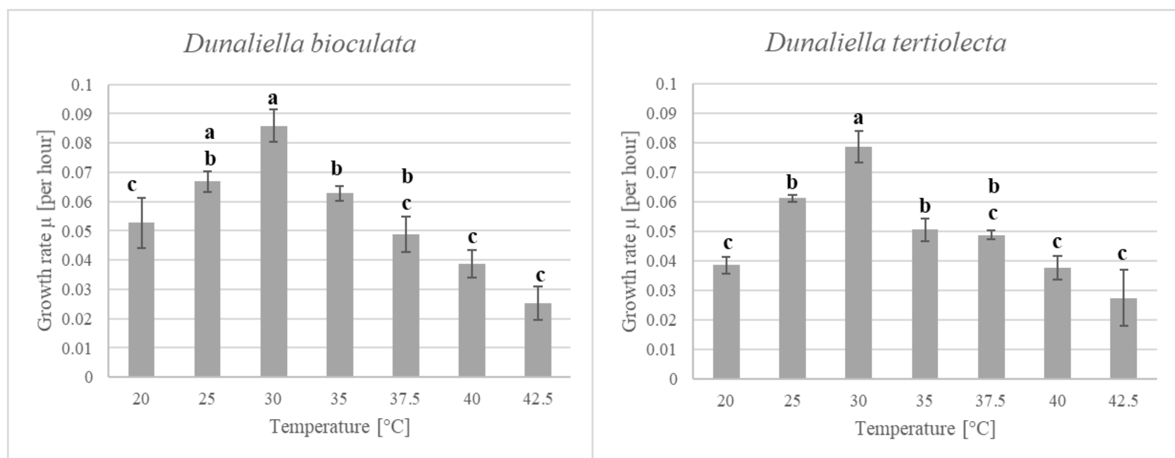


Figure 12 - Mean value of the growth rate per day, at the logarithmic phase of temperature tests. n≥3. a, b, c correspond to statistical significance between tests.

Figure 12 denote the mean value of the μ per hours, obtained at the logarithmic phase of all replicates of the two species. Both species, from an initial biomass in the whole culture of ca. 0.1 (OD_{680}), reached the logarithmic phase in about 4 days (100 hours), corresponding to the time between one

rerun and another. Minimum temperature was set at 20°C and the growth rate was observed to be not very high. The same was seen at the maximum temperatures (42.5°C), which led to death of microalgal cells in few days. In this experimentation bell curves are visible in both graphs. Statistical equalities were obtained cultivating *D. bioculata* cells at 25°C and 30°C; at 25°C, 35°C and 37.5°C; and at 20°C, 37.5°C, 40°C and 42.5°C (Fig. 12, Tab. 39). Regarding *D. tertiolecta* cultivations, cells grown at 30°C have reached a different statistical significance than the other cultures in temperature tests, where only cultivations at 37.5°C were statistically equal to the other experiments (Fig. 12; Tab 40).

Both species gained highest growth rates at 30°C, more precisely *D. bioculata* obtained a μ per hours of 0.086±0.002 and *D. tertiolecta* of 0.078±0.005 (Fig. 12). Considering results presented now and in previous tests, only *D. bioculata* will be investigated in next experiments. In order to try to decrease production costs, in mixing tests the temperature was maintained as in previous experimentations

5.4 Mixing tests

This experiment was designed and conducted in order to evaluate the effect of culture mixing on algae growth (especially on microalgae growth rates). For better comparison *D. bioculata* and other flagellated (*C. reinhardii* and *E. gracils*) and not flagellated (*P. kessleri* and *C. sorokiniana*) freshwater strains were tested in this setup.

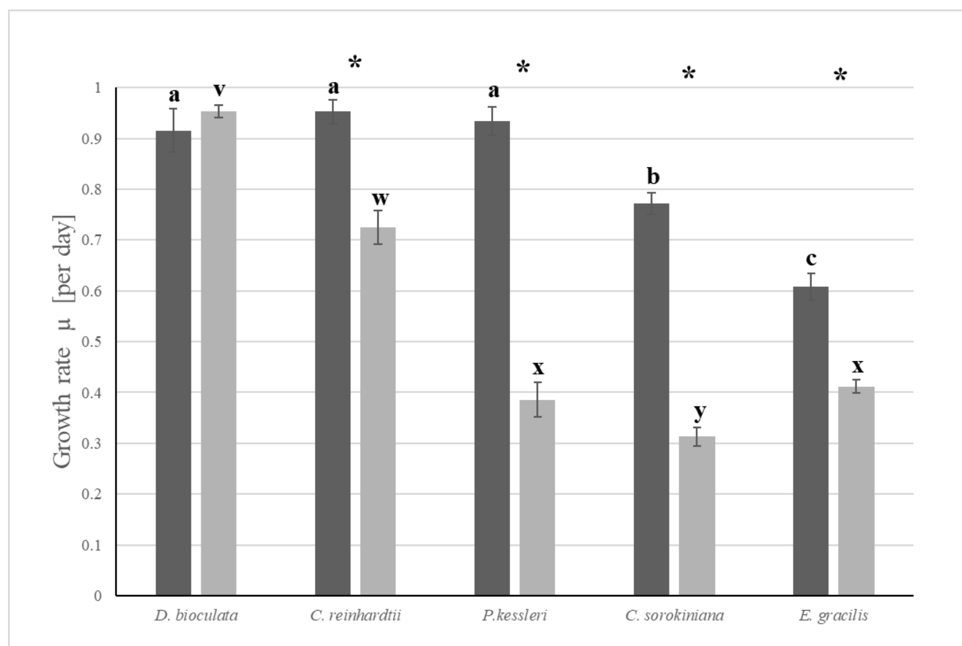


*Figure 13 - Mean value of the growth rate per day, at the logarithmic phase of mixing tests. Dark grey bars correspond to mixed experiments, light grey bars to no-mixed tests, and with the error bars are represented the standard errors. n≥3. a, b, c correspond to statistical significance between mixed tests; v, w, x and y to statistical significance to no-mixed tests. Statistical significance, between the two conditions was marked with *.*

Graphs in figure 13 represented the mean value of the μ per day, reached during the logarithmic phase by all species. The dark grey bars were mingled experiments, instead light grey bars the one not mixed. The highest productivity of *D. bioculata*, *C. sorokiniana* and *E. gracilis* was achieved in 3 days, growing under CO_2 enriched air, instead the other species took at 2 days. Considering mixed experiments, *D. bioculata*, *C. reinhardtii* and *P.kessleri* have achieved statistically equal growth rates (Fig. 13; Tab. 41). Regarding no mixed tests were reported significative differences between all species, except between *P. kessleri* and *E. gracilis* (Fig. 13; Tab. 42). Comparing both experimentations in each single species, only *D. bioculata* didn't report statistical differences (Fig. 13; Tab. 43). About the latter, mixed cultures achieved a μ=0.915±0.043, while the ones raised without be mixed achieved a μ=0.953±0.011 (Fig. 13). Regarding the other mixed cultures, the growth rates by species are as follows: *C. reinhardtii* μ=0.953±0.024; *P. kessleri* μ=0.934±0.028; *C. sorokiniana* μ=0.772±0.02 and *E. gracilis* μ=0.608±0.026 (Fig. 13). Considering the non-mixed cultures, the species obtained the following growth rates: *C. reinhardtii* μ=0.725±0.034; *P. kessleri* μ=0.386±0.035; *C. sorokiniana* μ=0.313±0.018 and *E. gracilis* μ=0.412±0.013 (Fig. 13).

Chapter 6 Discussion

Algae are multipurpose biomass feedstock for different industrial application such as food, medicine, feed and biofuel production. Current research efforts and business investments are driving attention on achieving the highest biomass in the shortest possible time. Both in small-scale and large-scale systems, algal growth conditions need to be carefully controlled and optimum nurturing environment must be provided. Such processes are most economical when combined with sequestration of CO_2 from flue gas emissions, with wastewater remediation processes, and/or with the extraction of high value compounds for application in other process industries. Also, light, nutrients, temperature, turbulence, contamination, CO_2 and O_2 levels need to be adjusted carefully to provide optimum conditions for biomass yield. It is therefore clear that a considerable investment in technological development and technical expertise is still needed before microalgal cultivation becomes economically viable and a reality.

Many authors have recognized the extraordinary capacity of *Dunaliella* genus to grow under a wide range of salt concentrations (Borowitzka *et al.*, 1977; Ginzburg & Ginzburg, 1985; Ginzburg, 1988), and this is well visible in the results (Fig. 10). Further, it was realized that this ability is related to the

intrinsic characteristics of the genus and to its culture history (Brown & Borowitzka 1979; Latorella & Vadas, 1973; Borowitzka *et al.*, 1977). The difficulty, encountered in establishing optimal growth conditions for all species, could be due to interactions between environmental factors: salinity, temperature, CO_2 level, light, ionic and nutrient composition of the medium. At the beginning all strains were maintained in the same SAG *Dunaliella* medium, and when they were transferred to the experimental salinity concentrations didn't adapt to a determined salt concentration, but rather tolerate the wide range of salinity. This was clear and visible in figure 9 that, as said by Brown & Borowitzka (1979), the microalgae of *Dunaliella* genus can be trained to grow in higher or lower salt concentration media by serial transfers. The high chloroplast granule accumulation, observed by optic microscope (data not shown), could be explained both as the osmotic pressure balancing (via glycerol production), or as a response to high level of nutrients, irradiance and temperature (Brown & Borowitzka, 1979; Liska *et al.*, 2004; Borovkov *et al.*, 2019); further studies are needed on. Authors in Ben-Amotz *et al.* (2009) argued that there are different salt growth optima between oligo/euryhaline and hyperhaline strains. In this study, there were only differences by their growth rates and this is confirmed by statistical analysis. Oligo/euryhaline species (*D. bioculata, D. salina* and *D. tertiolecta*)

grew faster than the other hyperhaline species studied in this work. Interesting to consider is the bell curve, which can be seen in salinity graphs (Fig. 10). Since growth rates were a measure of the response of cells to nutrients and environmental conditions, the maximum growth rates reached by cells suggest that the experimental growth parameters were optimal for each species, although differences among conditions were not always significant by statistical analysis (Table t-test salinity test). A possible explanation may be that the optimal growth condition for single species was limited, at low salinity, by the NaCl concentration and, at high salinity, by suppling CO_2. Considering the latter, there was a sharp increase in growth rates at high salinity when 4% CO_2 enriched air was supplied to cultures (Fig. 10). Ginzburg (1988) showed few *Dunaliella* strains have increased tolerance to NaCl when a carbon source was supplied to cultures.

The results obtained by studying a different way of administering carbon source led to several considerations. Carbonates and bicarbonates added to the solution allowed cells to grow in standard air (Fig. 11). As argued by Ginzburg (1988), higher carbonate and bicarbonate concentration could cause facilitated diffusion or active transport of DIC into cells. In *Dunaliella*, carbonic anhydrase enzyme is produced in response not only to low CO_2

concentrations in the medium, but also to increased salinity and pH (Ginzburg, 1988).

Another factor to comment is the high pH (between 7 and 10) in the latter experiment mentioned and the salt concentration of culture media, which were enough to deter growth of most bacteria likely to occur as contaminant. However, it should be remembered that some of the strains examined were already contaminated and that survival between microalgae and bacteria didn't affect the achievement of important growth rates, as in the case of *D. tertiolecta*. The obtained result could suggest the possibility to exploit the mutualistic relationship between microalgae and bacteria for biotechnological purposes, as also supported by Kazamia *et al.* (2012); Natrah *et al.* (2014); Fuentes *et al.* (2016) and Santos & Reis (2014).

Several temperature tests were carried out with the two selected species *D. bioculata* and *D. tertiolecta* (Fig. 12). Like Ben-Amotz (1995) and Hosseini Tafreshi & Shariati (2009) claimed, the optimum growth rate by both species, in the temperatures experiment, was reached at 30°C. Goldman (1977) studied the effects of temperature on marine planktonic species, including *D. tertiolecta*. All species reached maximum cell numbers at 20°C, but only *D. tertiolecta* still divided at a significant rate at 30°C. In his study, Eppley (1972) asserted that *D. tertiolecta* reached the maximum growth rate with a

salt concentration of 0.5M NaCl, but at a temperature a little less than 35°C. Additional analyses between 30°C and 35°C would therefore be advisable, but it can be said that *Dunuliella* strains had a tolerance to high temperature.

Another factor to consider was again unsterile condition, which didn't interfere with a successful result, considering growth rates of the two species in the range 25-35°C. This was probably because growth conditions for the microalgae were optimal or tolerable, such as not allow the bacteria to meddle with the growth. Since experiments were conducted with a dark-light cycle and Borovkov *et al.* (2019) stated that *Dunaliella* species, like *D. salina*, made reproduction in dark period, growth rates per hour could be defined as consistent with the growth rates found in continuous light as carried out in this project.

The favoured species *D. bioculata* was compared with four freshwater strains in order to evaluate their phototactic capabilities for a possible future biotechnological application (Fig. 13). When microalgae were shaked, best performances of all species were reached, proving optimal growth conditions. Growth rates obtained in no-mixed experiments support the initial thesis. *C. reinhardtii* probably didn't re-join the high μ due to a lack of time to adapt to new cultivation method. Posudin and co-workes (2010) stated that there were different sets of photoreceptor pigments, when comparing phototaxis

absorption spectrum between *Dunaliella* species and *C. reinhardtii. Dunaliella* photoreceptor pigments were carotenoids and carotenoproteins, instead rhodopsin was the basic photoreceptor pigment in *C. reinhardtii* (Posudin et al., 2010). *P. kessleri* and *C. sorokiniana* didn't reach high growth rate when grown without mixing cultures (Fig. 13). A possible explanation may be because these two species were devoid of flagella, therefore unable to swim towards the light source. It cannot be excluded that for *C. sorokiniana* the temperature was limiting, since Morita *et al.* (2000) claimed that the maximum growth rate was reached around 35°C. As well, optimal growth condition for *P. kessleri* were 30°C, 2% CO_2 and incident light intensity of 780 $\mu E \cdot m^{-2} \cdot s^{-1}$ as described by Li *et al*. (2013). *Euglena gracilis*, whether grown by mixing or not by mixing culture, didn't achieved performing growth rates (Fig. 13). As reported by Ogbonna *et al.* (2002), this species prefers growing in heterotrophic and mixotrophic conditions, rather than in photoautotrophic conditions. Another factor is light limitation caused by cells self-shading (Wang *et al.*, 2018), but further studies are needed.

Important to keep in mind is that growth rates didn't give a real estimation about culture biomass and factors like cell dimension, cell-shading or asynchronized life cycle, which could compromise the real cells number in

cultures. Further studies are necessary on that also to confirm the real potential of *D. bioculata*, whose data are scarce.

Chapter 7 Conclusion

Dunaliella genus is one of the most studied for mass culture and is commercially important for β-carotene and biofuel production. With this study, through the analysis of salinity, carbon sources, temperature and mixing movements, a selection was made in order to confirm or obtain a new valid species for biotechnological applications and try to minimize energy wastage. *D. bioculata* was found to be a good candidate for this purpose. This species obtained the highest growth rates in all tests, performed by taking advantage of its phototactic ability. *D. bioculata* performed highest μ grown with 0.5NaCl, 4% CO_2 air enriched, 26°C and cultures weren't mixed.

Regarding phototactic aspect, the ability of some microalgae to swim toward the light source, could be exploited in order to further reduce cultivation costs. This work should be a preliminary study in order to optimize cultivation conditions during green phase in a future large-scale culture. Further trials are also necessary in order to validate this proposal, first the measurement of algal biomass (e.g. dry weight). Analyses concerning glycerol, starch and lipids inside *D. bioculata* cells are advisable to better understand the potential

of this species, given that information concerning this microalga are scarce in literature.

T-test: Salinity tests

D. bioculata

Table 3

AIR	0.017M	0.25M	0.5M	1M	1.5M
2M	0.5905326	0.3753066	**0.002135**	**0.008125**	0.1660216
1.5M	0.9148729	0.6623926	**0.023067**	**0.050574**	
1M	**0.044927**	0.2548615	0.6586413		
0.5M	**0.042024**	0.4850704			
0.25M	0.4698152				

Table 4

4%CO_2	0.017M	0.25M	0.5M	1M	1.5M
2M	0.105212194	0.2258738	0.4743577	0.3511083	0.766252
1.5M	**0.02131699**	**0.039918**	0.3180885	0.5967789	
1M	**0.01212034**	**0.042144**	0.804315		
0.5M	**0.04890761**	**0.047049**			
0.25M	0.949446943				

Table 5

	0.017M	0.25M	0.5M	1M	1.5M	2M
AIR vs 4%CO_2	0.170912	0.8427633	**0.003944**	**0.02745518**	**0.08189**	**0.025595**

D. parva

Table 6

AIR	0.017M	0.25M	0.5M	1M	1.5M
2M	0.2718414	0.6976258	**0.02138**	**0.004673**	**0.006486**
1.5M	**0.006818**	0.0661333	0.0892905	0.1650556	
1M	**0.025363**	0.1342492	0.2724078		
0.5M	**0.025819**	0.1941251			
0.25M	0.172439				

Table 7

$4\%CO_2$	0.017M	0.25M	0.5M	1M	1.5M
2M	0.237357345	0.2472505	0.6665067	0.3967436	0.3436583
1.5M	0.066964965	0.1445362	0.3645147	0.5349258	
1M	0.112285858	0.1186295	0.3645147		
0.5M	0.233590468	0.2355153			
0.25M	0.229838016				

Table 8

	0.017M	0.25M	0.5M	1M	1.5M	2M
AIR vs $4\%CO_2$	0.2093778	0.5707202	0.5519238	0.148406864	0.0841859	0.067807

D. salina

Table 9

AIR	0.017M	0.25M	0.5M	1M	1.5M
2M	0.3110207	0.5717588	0.1167056	0.057481	0.1287236
1.5M	0.4317676	0.6439639	0.2556732	0.0981056	
1M	0.2608704	0.7268361	0.0981056		
0.5M	0.0637149	0.5107121			
0.25M	0.2035697				

Table 10

$4\%CO_2$	0.017M	0.25M	0.5M	1M	1.5M
2M	0.310848069	0.5125517	0.7067185	0.1703312	0.2094291
1.5M	0.236485639	0.3860571	0.46793	0.314837	
1M	0.245842989	0.2295026	0.4376999		
0.5M	0.622998776	0.6342345			
0.25M	0.295594842				

Table 11

	0.017M	0.25M	0.5M	1M	1.5M	2M
AIR vs $3\%CO_2$	0.38779	0.8605517	0.7892457	0.169395539	0.288256	0.1396142

D. sp.

Table 12

AIR	0.017M	0.25M	0.5M	1M	1.5M
2M	**0.028519**	0.2809326	**0.000899**	**0.000425**	**0.003836**
1.5M	**0.003994**	**0.03299**	**0.012979**	**0.000454**	
1M	**0.007507**	**0.030078**	0.8864699		
0.5M	**0.007903**	**0.019929**			
0.25M	0.712256				

Table 13

4%CO_2	0.017M	0.25M	0.5M	1M	1.5M
2M	**0.04129343**	**0.009592**	0.1905498	0.164333	0.0777251
1.5M	**0.04931865**	**0.038068**	0.5386802	0.5434296	
1M	**0.01637103**	**0.038068**	0.2390971		
0.5M	**0.04931865**	**0.005442**			
0.25M	0.901305519				

Table 14

	0.017M	0.25M	0.5M	1M	1.5M	2M
AIR vs 4%CO_2	0.2225916	0.1966751	**0.006119**	0.093147335	**0.006739**	**0.023162**

D. tertiolecta

Table 15

AIR	0.017M	0.25M	0.5M	1M	1.5M
2M	0.9674864	0.104233	**0.006053**	**0.014807**	0.0782169
1.5M	0.8768972	0.3145875	**0.011433**	0.1080712	
1M	**0.021788**	0.6576744	0.0714135		
0.5M	**0.013672**	0.1123167			
0.25M	0.1873769				

Table 16

$4\%CO_2$	0.017M	0.25M	0.5M	1M	1.5M
2M	0.141153184	0.2374804	**0.048491**	**0.03114**	0.0733398
1.5M	0.109213241	0.0375706	**0.011433**	**0.045019**	
1M	**0.02016429**	**0.013495**	0.090384		
0.5M	**0.03355229**	**0.011871**			
0.25M	0.590236731				

Table 17

	0.017M	0.25M	0.5M	1M	1.5M	2M
AIR vs $4\%CO_2$	0.4389998	0.0673107	**0.015064**	**0.00713727**	**0.003605**	**0.000257**

D. viridis

Table 18

AIR	0.017M	0.25M	0.5M	1M	1.5M
2M	**0.011633**	0.3912565	0.0774552	0.9557673	0.1001474
1.5M	**0.01658**	0.1950436	0.305884	0.9557673	
1M	**0.025305**	0.2965853	0.2327432		
0.5M	**0.013456**	**0.003449**			
0.25M	0.3312662				

Table 19

$4\%CO_2$	0.017M	0.25M	0.5M	1M	1.5M
2M	0.090023402	0.1047921	0.5922682	0.835657	0.3212001
1.5M	0.071624062	0.177165	0.431059	0.7753058	
1M	0.128273198	0.1412318	0.9597558		
0.5M	0.128297734	0.2539164			
0.25M	0.384003869				

Table 20

	0.017M	0.25M	0.5M	1M	1.5M	2M
AIR vs $4\%CO_2$	0.1624957	0.4583193	0.7776993	0.231742489	0.3919234	0.1588403

T-test: DIC tests

D. bioculata

Table 21

AIR	A	B	C	D
Control	0.1160008	**0.001774**	**0.006395**	0.927051
D	0.2869416	**0.010169**	**0.015244**	
C	**0.017608**	0.1367171		
B	**0.003323**			

Table 22

4%CO_2	A	B	C	D
Control	**0.01596373**	0.6852053	0.7071223	0.4910686
D	**0.00808564**	0.3955902	0.4215169	
C	**0.01158837**	0.8777064		
B	**0.00192674**			

Table 23

	A	B	C	D	Control
AIR vs 4%CO_2	0.078489	0.370803	0.8523335	0.2848926	**0.003944**

D. parva

Table 24

AIR	A	B	C	D
Control	**0.033698**	**0.023126**	**0.014558**	0.148787
D	**0.036406**	**0.005958**	**0.00042**	
C	**0.017172**	0.3871508		
B	**0.0263**			

Table 25

4%CO_2	A	B	C	D
Control	0.083131692	**0.011246**	**0.016566**	**0.02321**
D	0.093777096	0.4259099	0.6763171	
C	**0.01885624**	0.2639129		
B	**0.00780971**			

Table 26

	A	B	C	D	Control
AIR vs 4%CO_2	0.4651153	**0.023118**	0.0948961	**0.00362848**	0.5519238

D. salina

Table 27

AIR	A	B	C	D
Control	0.6328104	**0.022927**	0.1314908	0.6471941
D	0.2820123	**0.006749**	0.1156858	
C	**0.001126**	0.1886364		
B	**0.014663**			

Table 28

4%CO_2	A	B	C	D
Control	0.644726094	0.7067185	0.3121906	0.5384985
D	0.576438392	0.096395	0.1075454	
C	0.318426581	0.1884932		
B	**0.04114151**			

Table 29

	A	B	C	D	Control
AIR vs 4%CO_2	0.4051673	0.4709116	0.5727003	0.916517377	0.7892457

D. sp.

Table 30

AIR	A	B	C	D
Control	0.0712848	0.1374627	0.2051182	0.1293847
D	0.1264473	0.1897125	0.2147305	
C	0.4539761	0.2250496		
B	0.7863387			

Table 31

$4\%CO_2$	A	B	C	D
Control	0.408914178	0.7501155	0.9601995	0.8180505
D	0.334889723	0.1308573	0.5823273	
C	0.214922589	0.3194486		
B	0.741184575			

Table 32

	A	B	C	D	Control
AIR vs $4\%CO_2$	0.4732213	0.6799275	0.3526406	**0.00723608**	**0.006119**

<u>*D. tertiolecta*</u>

Table 33

AIR	A	B	C	D
Control	**0.03613**	**0.022842**	**0.00517**	0.9667216
D	**0.041795**	0.2490889	**0.014575**	
C	**0.000319**	0.3801302		
B	**0.000466**			

Table 34

$4\%CO_2$	A	B	C	D
Control	**0.01543477**	**0.025897**	0.2605697	0.1241835
D	**0.00868362**	0.6112799	0.2368983	
C	**0.00038359**	**0.002775**		
B	**0.00275688**			

Table 35

	A	B	C	D	Control
AIR vs $4\%CO_2$	0.2814292	0.6515037	0.2541013	0.265701871	**0.015064**

D. viridis

Table 36

AIR	A	B	C	D
Control	0.0697558	0.7433167	0.8292519	0.4731385
D	0.1528722	0.2891689	0.1385832	
C	0.1394587	0.1186567		
B	0.1423364			

Table 37

3%CO_2	A	B	C	D
Control	0.092400585	0.1151644	0.6505029	0.8826372
D	0.323765729	0.8148745	0.7023218	
C	0.110391278	0.7442634		
B	0.226646399			

Table 38

	A	B	C	D	Control
AIR vs 4%CO_2	0.3132549	0.8130551	0.5761797	0.36001611	0.7776993

T-test: Tempertaure tests

D. bioculata

Table 39

Temperature	20°C	25°C	30°C	35°C	37.5°C	40°C
42.5°C	0.0916635	**0.048125**	**0.008412**	**0.022811**	0.2369975	0.6433049
40°C	0.8925345	**0.006048**	**0.000143**	**0.004386**	0.2177839	
37.5°C	0.3969374	0.1004565	**0.001638**	0.1641315		
35°C	**0.002933**	0.6926135	**0.00453**			
30°C	**0.001089**	0.0807985				
25°C	**0.051048**					

D. tertiolecta

Table 40

Temperature	20°C	25°C	30°C	35°C	37.5°C	40°C
42.5°C	0.3629463	**0.034621**	**0.008094**	**0.029337**	0.254706	0.8886799
40°C	0.3011185	**0.008233**	**0.003662**	**0.022118**	0.1185741	
37.5°C	0.3360649	**0.001349**	**0.043264**	0.5941779		
35°C	**0.015452**	0.106117	**0.001248**			
30°C	**0.000607**	**0.053372**				
25°C	**0.015079**					

T-test: Mixed tests

Table 41

mixing	*D. bioculata*	*C. reinhardtii*	*P. kessleri*	*C. sorokiniana*
E.gracilis	**0.000146**	**0.000917**	**0.014442**	**0.003757**
C. sorokiniana	**0.048186**	**0.003998**	**0.004367**	
P. kessleri	0.5601719	0.2944453		
C. reinhardtii	0.5168449			

Table 42

no mixing	*D. bioculata*	*C. reinhardtii*	*P. kessleri*	*C. sorokiniana*
E.gracilis	**1.0363E-06**	**0.019335**	0.4186582	**0.004577**
C. sorokiniana	**7.3472E-08**	**0.011471**	**0.028714**	
P. kessleri	**6.494E-06**	**0.030051**		
C. reinhardtii	**0.00159203**			

Table 43

	D. bioculata	*C. reinhardtii*	*P. kessleri*	*C. sorokiniana*	*E. gracilis*
mixing vs no mixing	0.4212297	**0.035353**	**0.000248**	**1.1128E-05**	**0.000647**

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