Photosynthetic performance and biomass composition of *Chlorella fusca* (Chlorophyta) in thin-layer cascades:

Possible biotechnological applications

Doctoral Thesis

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Publicaciones y
Divulgación Científica

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EDITA: Publicaciones y Divulgación Científica. Universidad de Málaga

UNIVERSIDAD DE MÁLAGA
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Área de Ecología

Photosynthetic performance and biomass composition of *Chlorella fusca* (Chlorophyta) in thin-layer cascades: possible biotechnological applications

Memoria presentada para optar al Grado
de Doctor en
Ciencias Ambientales por
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Dirigida por Félix López Figueroa
Defendida en Málaga, el 15 de Mayo de 2015

Esta tesis doctoral se ha llevado a cabo en el Departamento de Ecología y Geología (Área de Ecología) y en la Unidad de Fotobiología de los Servicios Centrales de Apoyo a la Investigación (SCAI) de la Universidad de Málaga y ha sido financiada por el Ministerio de Educación mediante una beca de formación de profesorado universitario FPU (referencia AP2009-2770) y por fondos del grupo investigación RNM-295 "Fotobiología y Biotecnología de Organismos acuáticos" (FYBOA).

Cover design and layout: Ana Díaz Ruíz

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Abstract

We have not yet acquire the necessary physiological knowledge nor developed the required technology to take advantage of the potential of microalgae to produce energy, novel foodstuffs and renewable non-food commodities in a sustainable way. This doctoral thesis aimed to contribute the overcoming of the problems that obstacle the scaling-up of microalgal cultures by filling some of these gaps related to the photosynthetic performance and biochemistry of outdoor microalgal cultures.

In order to estimate microalgal carbon assimilation or production of *Chlorella fusca* cultures based on electron transport rate (ETR) as *in vivo* chlorophyll *a* fluorescence, it is necessary to determine the photosynthetic yield and the absorbed quanta by measuring the incident irradiance and the fraction of absorbed light, i.e. absorptance or absorption coefficient of photosynthetic active radiation (PAR). Due to difficulties associated to the determination of light absorption, the ETR is commonly expressed as relative units (rETR), which only considers the incident irradiance and not the absorbed light. Thus, the rETR is not a good estimator of the photosynthetic production since photobiological responses depend on the absorbed light. The Quantitative Filter Technique (QFT) is commonly used to measure the absorbed quanta of cells retained on a filter (AbQ_f) as estimator of the absorbed quanta of cell suspensions (AbQ_s). In this study, light attenuation of a thin layer of cell suspensions was determined by using a measuring system designed to reduce the scattering. Light attenuation was related to the absorption coefficient of both in-door and outdoor *C. fusca* cultures of different cell densities. A linear relation between AbQ_f and AbQ_s (R^2=0.99, $p<0.01$) was obtained, $AbQ_f = 1.98 \cdot AbQ_s$, which can be used to convert AbQ_s values into AbQ_f ones. Thus, the present study showed the usefulness of a procedure to determine light absorption of *C. fusca*, simply and rapidly, by measuring the light attenuation of a thin layer of cell suspension in a measuring system with reduced scattering. In addition, different expressions

of ETR i.e. surface, volume or chlorophyll units, which can be used according to the characteristics of the culture system, are presented. In thin-layer cascade systems or flat panel photobioreactors it would be appropriate to express ETR per unit area (μmol e^- m^{-2} s^{-1}) whereas in raceways or tubular photobioreactors the expression per unit volume would have more photobiological sense (μmol e^- m^{-3} s^{-1}). In both culture systems, ETR can be expressed per chlorophyll unit, what would represent the specific productivity (ETR*, μmol e^- mg $Chla^{-1}$ h^{-1}) as it is an expression of the production per unit of pigment or cell.

The thin-layer cascade (TLC) is an open system for microalgae cultivation composed of different compartments: a retention tank connected by pump and pipes to a horizontal exposed area that consists of an upper basin and a TLC. Light and hydrodynamics are different among compartments, so overall photosynthetic activity can be influenced by the retention time of the cells in each compartment. Two settings with different retention times in the cascade and tank were established to compare the photosynthetic activity of *Chlorella fusca* (Chlorophyta) among compartments. Changes in the retention time were achieved using 2 layer thicknesses in the cascade: 8 and 18 mm. Retention time in the cascade represented about 16 and 34% of the duration of a whole system cycle when H1 (8 mm thickness) and H2 (18 mm thickness) units, respectively, were used. These retention periods were lower than those in the tank (67 and 49%, respectively) but higher than those in the basin (12% for both H1 and H2). *In vivo* chlorophyll *a* fluorescence was used to monitor the photosynthetic performance of *C. fusca* cultivated in thin-layer cascades revealing useful information of the photosynthetic activity of the culture according to the hydrodynamics characteristics of each compartment of the system. Photosynthetic activity was measured *in situ* as relative electron transport rate (rETR) using a pulse-amplitude modulated fluorometer (Diving-PAM). In both setups, the highest rETR was reached in the cascade. The increase in the optical path resulted a good option to avoid photoinhibition. Estimating the mean rETR of the whole system considering the retention time is suggested since it can better reflect overall growth because it takes into

account the time that the cells spend in each compartment. These results are useful for optimization of photosynthetic activity and growth of outdoor microalgae mass cultures in TLCs for biotechnological purposes.

The photosynthetic performance, biomass composition and productivity of three cultures of *Chlorella fusca* BEA1005B grown in thin-layer cascades (TLCs) in different locations and time of the year were evaluated. The first (E1) and second (E2) experiments were conducted in Southern Spain in July and October 2012, respectively in a TLC with a ratio of exposed surface to volume (S/V) ratio of 20-27 m^{-1}. The third experiment (E3) was conducted in Czech Republic in July 2013 in a TLC with S/V ratio of 105-140 m^{-1}. *C. fusca* showed high capacity for sustained culture and storage compounds production in thin-layer cascades, in which the S/V ratio of the system resulted to be an essential factor regarding productivity of biomass and storage products. Growth was higher in E3, in which *C. fusca* achieved a biomass density of 11.4 g DW L^{-1} whereas it was 1.22 and 0.94 g DW L^{-1} in E1 and E2 (summer and autumn, respectively). Photosynthetic activity increased in all experiments although it was higher in E3 ($rETR_{max}$=345 μmol e^- m^{-2} s^{-1}). Final biomass composition was similar in E1 and E3, showing high lipid and protein content (~35-37% and 30-34%, respectively) and lower starch content (16-19% DW). Lower accumulation was found in E2, ~23, 28 and 13% DW for lipid, protein and starch content, respectively. Biomass productivity was higher in E3 (3.65 g L^{-1} d^{-1}). In E1 and E3, lipid productivity (0.09 and 0.66 g L^{-1} d^{-1}, respectively) was higher than that of starch and protein (~0.03-0.04 and 0.43-0.47 g L^{-1} d^{-1}). On the contrary, in E2 the three productivities were similar (0.02-0.03 g L^{-1} d^{-1}). The S/V ratio of the system was a key factor to obtain high biomass and storage product productivity. This strain was able to grow outdoors, exhibiting good photosynthetic performance and accumulating high lipid and protein content. To our knowledge, the biomass, lipid and protein productivity are among the highest ever reported in outdoor conditions in open systems.

In addition, online monitoring of *in vivo* chlorophyll *a* fluorescence provided data with high temporal resolution that revealed essential

information about the photosynthetic performance of the culture. Instantaneous and simultaneous measurements of incident PAR irradiance and effective quantum yield of PSII ($\Delta F/F_m'$) were recorded every 5 minutes using Junior-PAM fluorometer. From these data, daily-integrated electron transport rate (ETR) was calculated and transformed into biomass productivity according to several assumptions. Oxygen evolution rate was estimated from daily-integrated ETR assuming that the quantum requirement (QR) was 8 or 10 absorbed photons to produce a molecule of oxygen. From these, carbon assimilation rate was calculated assuming the number of mol of C produced per mol of O_2 evolved, the so-called photosynthetic quotient (PQ), which considering that nitrate was the N source was ranged from 1.2 to 1.4 mol O_2/mol CO_2. Since in microalgal biotechnology the measurement of biomass productivity is usually carried out through the assay of dry weight in time, biomass productivity estimated from ETR records was compared with biomass productivity measured by daily differences in dry weight. The best correlation was found when QR=1/8 mol O_2 (mol photons)$^{-1}$ and PQ=1.2 mol O_2 (mol CO_2)$^{-1}$. It was found that low dense cultures (<0.15 g DW L^{-1}, s=0.90) showed lower slope (s=0.42) than high dense cultures (0.15-11.4 g DW L^{-1}), which showed estimated values only 18% lower than the measured biomass productivity. To date, this is the first study presenting estimates of biomass productivity derived from ETR measurements in microalgal mass cultures that, in addition, agreed with measured values.

Short laboratory and outdoor controlled experiments were conducted to evaluate the combined effect nutrient and light stress on the accumulation and productivity of storage compounds in *Chlorella fusca*. The effect of nitrogen and sulphur starvation under high irradiance (PAR) showed a significant decrease of $rETR_{max}$, which caused a substantial drop in biomass production, cell number, biovolume and induction of lipid and starch accumulation. High starch content (45-50% of DW) was found at the initial stage in full nutrient culture and at the stationary phase in nutrient starved cultures. By the end of the trial all treatments showed high lipid content (~30% of DW). The full nutrient culture had higher biomass yield than starved treatments although starch (~0.2 g L^1 d^{-1}) and lipid (~0.15 g L^1 d^{-1})

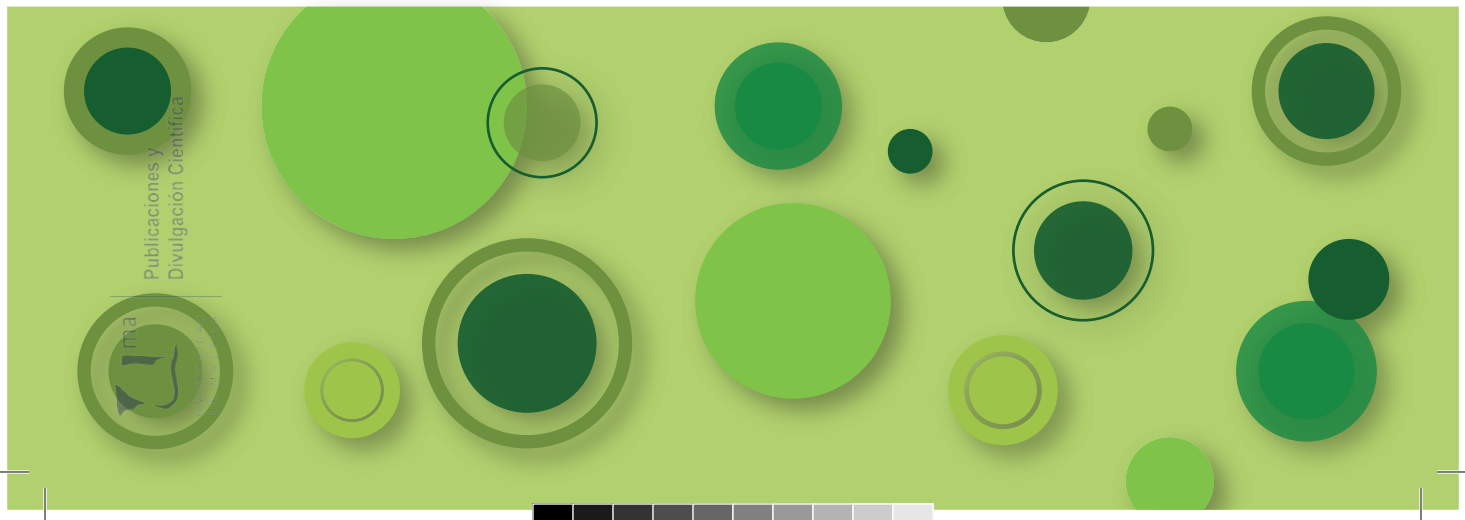
productivities were fairly similar in all the cultures. These results showed that we could enrich biomass of *C. fusca* (% DW) by lipids using a two-stage strategy (a nutrient replete stage followed by gradual nutrient limitation) while under either procedure, N- or S-starvation, both high lipid and starch contents could be achieved.

The interactive effects of UVR and nutrient depletion caused changes in productivity and cell number in a manner that affected biochemical composition. After 3 days, the percentage of lipids in the cultures under N deprivation reached values appropriate for being used as feed or food additives or for energy applications (35% of lipid content), regardless of the light conditions. A longer exposure (5 d) resulted in interactive effects of light and nutrient conditions. Specifically, PAR+UVR increased lipid content in all cases (1.3- to 2.3-fold), but particularly under S deprivation. Longer exposure to PAB also increased oxidative stress in UVR and nutrient-limited treatments (−N and −S). These results showed that the benefits expected from nutrient depletion (increase in biomolecule content e.g. lipids, carbohydrates and pigments) were modulated by the negative effects of algal UVR acclimation costs.

As conclusion, this Thesis demonstrated that although microalgal biotechnology still presents several obstacles related to biological and engineering limitations that need to be overcome, microalgae and specifically *Chlorella fusca*, present great potential to become a real alternative that contributes to satisfy the global demand for energy, food and non-food commodities. Chlorophyll *a* fluorescence resulted a useful tool to better understand the physiological processes involved in growth and photosynthetic activity, which might lead to achieve higher biomass productivity. On the other hand, the thin-layer cascade stands out as a promising cultivation system for a cost-effective mass microalgal production.

1 chapter

Introduction



The sustainability challenge

In a crowded world with population still rising and changing consumption patterns, planning and managing the future development of land and water resources is essential. By 2050, the Earth's population is expected to increase from 7 to over 9 billion people that will demand about 70% more food (FAO 2011; OECD 2012). Such increase in the world population will entail an increase in the overall water demand by 55% due to growing demand from manufacturing, thermal electricity generation and domestic use. Besides increasing water needs, to satisfy future food demand, agricultural production should also be increased, what could be achieved by higher yields from existing arable lands or by expanding agricultural land (Bruinsma 2003).

Only about 30% of Earth's surface corresponds to land, being the remaining 70% covered by water, from which only 0.6% corresponds to freshwater. In addition, at present, agriculture makes use of 70 percent of all water withdrawn from aquifers, streams and lakes. A map of the global land area would show that, at present, the majority of the available arable land is already in use (12%), 28% is under forest and 35% comprises grasslands and woodland ecosystems (FAO 2011). In addition, improvements in agricultural productivity have usually been associated with degradation of land and water resources, and the deterioration of related ecosystem including biomass, carbon storage, soil health, water storage and supply, biodiversity, and social and cultural services. To satisfy the higher food demand, future agricultural production will need to be more productive and sustainable at the same time since both land and water resources, the basis of our actual food and energy production, are finite and already under heavy stress. Therefore, scarcity of water resources, unavailability of free arable land and unsustainability of recent developed improvements in agricultural productivity would compromise food supply for the next 50 years.

With about 2.3 more billion people by 2050, the world economy will be four times larger than today and will use about 80% more energy. Considering the actual energy sources, by that time, fossil fuels will still account for 84% of the primary energy demand, what would entail an

intensification of greenhouse gasses emissions by more than 50% primarily due to a 70% increase in CO_2 (Zarrilli 2008). Considering the dramatic consequences of higher global temperature and climate change, society should find alternative energy sources, for what renewable energy reveals as the main option.

Currently, bioenergy represents about 10% of global energy, being used mainly in the form of vegetable biomass for traditional cooking and heating in developing countries (FAO 2011). The production of liquid biofuels (bioethanol and biodiesel) from crops is expected to increase and accounts for 5% of the total road transport energy use by 2030. The European Academies Science Advisary Council (2012) analyzed the current status of biofuels in the European Union. From this report, present situation and future prospects can be figured out. At present, biofuels contribute about 10 Mt of oil equivalent (Mtoe, equivalent to about 20 x 10^6 GJ or 11.7 TWh) to the EU road transport energy, of which about 80% come from biodiesel, mostly derived from rape seed, and 20% from bioethanol, mostly derived from wheat, maize, beet and sugar cane. From these, about 40% is imported from USA and Brazil. In addition, it is not clear if biofuels from crops will be environmentally sustainable when deforestation precedes the acquisition of arable land. To end with this controversy, microalgae have been proposed as the new source of food, energy and nonfood commodities to accomplish the sustainability challenge that human kind will face as a result of the demands of the growing world population.

Why such interest in microalgae?

Microalgae are unicellular aquatic organisms that convert sunlight, water and carbon dioxide into biomass via photosynthesis. The photosynthetic mechanism of microalgae is similar to that of terrestrial plants but a more simple cellular organization together with the absence of non-supporting structures such as stems or roots make them very efficient solar energy converters. The autotrophic microalgal biomass production is advantageous compared to other heterotrophic microorganisms (yeasts, bacteria…) because

the latter need organic molecules to produce these metabolites (Jin and Melis 2003). On the other hand, microalgae can present very high growth rates since most of the species divide every 1-2 days under favorable conditions, what gives them high growth capacity.

The biodiversity of microalgae is enormous and represents an almost unexploited resource. It has been estimated that there are between 200 000 and several million species (Norton et al. 1996). This huge diversity also means a great diversity in the chemical composition, what makes them extremely attractive for bioprospecting and potential exploitation as commercial sources of a wide range of biomolecules (Borowitzka 2013). A successful microalgal biotechnology process necessarily requires the selection of the proper species for specific culture conditions and product of interest. Given the huge number of species, a basic knowledge of the photosynthetic performance, physiology and ecology of the selected species is essential.

Biochemical composition of microalgae

Microalgae have high nutritional content because as product of photosynthesis they accumulate proteins, carbohydrates and lipids (many of the essential polyunsaturated fatty acids) for metabolic and structural functions and secondary metabolites like vitamins, antioxidants and polysaccharides. Many analysis of the biochemical composition of microalgae have been published in the literature. Becker (2013) gave a general overview of these estimations from which it can be highlighted that, although differences among species and strains, proteins is always the major organic constituent usually followed by lipids and then by carbohydrates.

Under different conditions microalgae can accumulate high content of proteins, showing an amino acid profile that in most species compares favorably with that of other food proteins such as egg, soy, wheat protein and FAO requirements (Becker 2007). In addition, since microalgae are capable of synthesizing all amino acids, they can provide the essential ones to humans and animals (Spolaore et al. 2006). The lipid content of microalgae varies markedly within the species and the strain ranging from 1 to 40%, although

some studies reported higher values under certain conditions (Sharma et al. 2012). Algal lipids are composed of glycerol, sugars or bases esterified to saturated or unsaturated fatty acids (12-22 carbon atoms). Microalgae can store carbohydrates in the form of starch, polysaccharides or sugars. The production of starch can be as high as 30-40% under certain stress conditions (Brányiková et al. 2011; Dragone et al. 2011; Takeshita et al. 2014). Moreover, it has been confirmed that the overall digestibility of the carbohydrates of the microalgae currently commercialized is good (Becker 2013).

Taking advantage of stress conditions

The metabolic flexibility is the most interesting characteristic of microalgae for biotechnological purposes as it allows them for the production of a wide variety of metabolites under very different environmental conditions. In large-scale outdoor microalgal cultivation, the proportion of the constituents of the cultivated microalgae can be modified to a certain extent by varying one or more growth parameters such as the irradiance, temperature or the composition of the nutrient medium.

Light regime in outdoor cultures could be varied by changing the optical path (culture depth). It has been observed that under high light conditions some species increase their content in carbohydrates (Friedman et al. 1991; Tredici et al. 1991), total lipids (Carvalho and Malcata 2005) or polyunsaturated fatty acids (PUFAs) (Brown et al. 1996). Stress due to exposition to UV radiation also enhanced PUFAs accumulation (Forján et al. 2011; Srinivas and Ochs 2012).

Temperature directly affects lipid content and fatty acid composition. Whereas generally an increase in temperature is related to higher total lipid content (Boussiba and Richmond 1979; Zhu et al. 1997; Renaud et al. 2002; Converti et al. 2009), lowering the temperature of the culture results in highest yields of PUFAs (Joh et al. 1993; Jiang and Gao 2004). Chlorophyll *a* and carotenoid concentration also increase at higher temperature (Thompson et al. 1992; Tjahjono et al. 1994). In addition, optimal growth temperatures result in smaller cells with lower carbon and nitrogen contents while sub- or

supra-optimal temperatures lead to higher cell volume and biochemical content (Harris 1986).

Nutrient availability has a significant impact on growth and biomass composition. Limitation or starvation of a particular nutrient would enhance specific metabolic pathways that might favor the accumulation of certain storage compounds. Low nitrogen availability is usually related to increase in the lipid content in several species (Illman et al. 2000; Yeh and Chang 2012; Mujtaba et al. 2012; Griffiths et al. 2014a) whereas nutrient replete conditions usually entail accumulation of proteins and favor growth rate (Geider et al. 1998). On the contrary, sulphur deficiency results in the accumulation of starch (Brányiková et al. 2011; Mizuno et al. 2013; Takeshita et al. 2014). However, nutrient limitation usually entails a decrease of growth and photosynthetic activity and therefore, attention should also be paid to the productivity of the target metabolite.

Microalgal aquaculture and biotechnology

Microalgae are the base of the aquatic food chain and therefore, due to their high growth rates and nutritional value, have traditionally been used in aquaculture as starting feeds for larvae of many species of mollusks, crustaceans and fish.

However, the potential use of the products of microalgal photosynthesis for human food raised the interest on microalgal production, starting to be a focus of research in 1948 (Burlew 1953). Commercial large-scale culture of microalgae began in the early 1960s in Japan with the culture of *Chlorella* (Tsukada et al. 1977). In the following two decades *Spirulina* culturing facilities were established in Mexico and Thailand; *Dunaliella salina* as a source of β-carotene became the third major microalgae industry in Australia and *Haematococcus pluvialis* began to be produced as a source of astaxanthin in USA, Chile and India.

Microalgae have also been used for bionenergy applications. In the early 1950s, several studies were conducted on the use of microalgae as gas

exchangers for space travel (Borowitzka 1999). In the 1960s, the USA investigated the use of microalgae for wastewater treatment and methane production with the resulting biomass. During the energy crisis in the 1970s, the production of biogas from microalgae received big attention. In 1990, a new crisis in the price of petroleum prompted the need to move away from the dependence upon fossil fuels as main source of energy. At the same time, the concern about global warming associate to CO_2 emissions mainly produced by burning of fossil fuels emphasized the unsustainability of this energy source. Attention was then paid to microalgae, which were considered as a promising alternative source for biodiesel production (Chisti 2007).

Therefore, in a period about 50 years, the industry of microalgal biotechnology has grown and diversified significantly. Several high-value products are already well established in the marketplace, producing about 5000 t of dry biomass per year that generate a turnover of approximately US$ 1.25 10^9 (Pulz and Gross 2004). However, there are still numerous opportunities for additional new products and applications.

Commercial applications of microalgae

Human nutrition The main product of microalgae cultivation is just the algal biomass, either as powder after a drying process or in compressed form as pastilles. The human health market is the main receptor of this biomass mainly due to the promoted immune-modulating effect of microalgal biomass on human health (Belay et al. 1993; Osinga et al. 1999). Food supplemented with microalgal biomass shows very promising prospective in the nutraceuticals market since countries such as Germany, Japan or USA have started to commercialize products like pasta, bread or yogurt supplemented with microalgae (Pulz and Gross 2004). Recently, the Company Fitoplancton Marino in Cádiz (Spain) has registred the microalga *Nanochloropsis gaditana* grown in horizontal tubular photobioreactors as novel food for marine microalgae in Europe and GRAS (Generally Recognized Save) in USA.

Animal feed and aquaculture For decades, microalgae biomass has been used as animal feed (up to 50% of the total feed) due to its high protein content. In fact, 30% of the current world microalgal production is sold for animal feed applications (Becker 2013). However, in the last years, a non-specific immune response was observed after adding only small amounts of microalgae (Belay et al. 1993). As pointed out previously, microalgal biomass has been traditionally used as starting feed in aquaculture. In 1999, the production of microalgae for aquaculture reached 1000 t (62% for mollusks, 21% for shrimps and 16% for fish) (Spolaore et al. 2006) but nowadays it is also being used as a source of pigments to color the flesh of salmonids or the skin of fancy fish (Muller-Feuga 2013). Currently, more than 40 species of microalgae are used in aquaculture worldwide, depending on the nutrient requirements of the produced species (Pulz and Gross 2004).

Pigments Carotenoids are used as natural food colorants and due to their very high antioxidant properties as food and feed additive, health supplements and cosmetics (Markou and Nerantzis 2013). The natural production of β-Carotene from *Dunalliella salina* and astaxanthin from *Haematococcus pluvialis* represents a major part of the total carotenoids market since these species contain the highest amounts of these carotenoids of any natural source (Borowitzka 2013). Microalgae can be a potential source of other carotenoids such as lutein astaxanthin or canthaxanthin from *Chlorella* sp. (Del Campo et al. 2004; Pelah et al. 2004) or lutein from *Muriellopsis* sp. and *Scenedesmus almeriensis* (Sanchez et al. 2008; Fernández-Sevilla et al. 2010). Phycobiliproteins are exclusive from cyanobacteria, criptophyceae and red algae and are commercially produced from *Arthrospira* and *Porphyridium* to be used as natural dyes for food and cosmetics.

Fatty acids Polyunsaturated fatty acids (PUFAs) of more than 18 carbons are essential for human and animal nutrition and the common source are fish and fish oil. As PUFAs found in fish come from the microalgae they consume, it is logical to consider microalgae as a potential source of PUFAs. However, although many microalgal species accumulate high PUFAs content, only heterotrophicaly grown algae are commercially produced since the

production of PUFAs from phototrophic microalgae is still an expensive process (Borowitzka 2013). Among PUFAs, omega-3 fatty acids and specifically eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6), have high nutritional value due to their benefits in reducing cardiac diseases among others. Consequently, microalgae are being intensely investigated as a commercial source of EPA and DHA (Adarme-Vega et al. 2012).

Other compounds Microalgae can accumulate high content of different products offering unexplored opportunities as sources of: sterols, which have pharmaceutical applications and are currently produced from pine and soy; polysaccharides, with wide applications in food industry and health market and produced from macroalgae and higher plants; vitamins, at the present mainly produced synthetically or polyhydroxyalkanoate to be used as bioplastics (Borowitzka 2013). Acid polysaccharides extracted from the red microalga *Porphyridium cruentum* present inmunostimulant activity (Abdala-Diaz et al. 2010).

Environmental applications

To date, it is generally accepted that technologies based on natural biological processes have many advantages over traditionally used physico-chemical techniques. The use of microalgae for wastewater treatment or heavy metal bioremediation is a promising field in microalgal biotechnology and is being extensively investigated (Brenner and Abeliovich 2013). Species from the genera *Chlorella* and *Scenedesmus* have been proven to be efficient in removing nitrogen, phosphorus and toxic metals from a wide variety of wastewater (Cai et al. 2013). However, although pilot studies have been conducted with good results, this technology is not commercialized yet.

Microalgae for biofuel production

Systems to produce biodiesel and bioethanol from crop plants, the so-called first generation of biofuels, were already being run as profitable business when the crisis of petroleum in 1990s lead to the search of renewable energy

sources to replace fossil fuels. Since then, commercialized biodiesel has been produced from canola palm, corn, jatropha and waste cooking oil (Chisti 2007). Nevertheless, the occupation of arable lands and the effect of this industry on food commodity prices were strong reasons not to consider biofuels from crops as a substitute of fossil fuels. In addition, the general concern about the environmental and economic sustainability of fossil fuels lead to the adoption of new energy policies during the last decade. Directive 2009/28/EC, on renewable energy established a target for transport energy of 20% derived from renewable energy in all EU Member States by 2020. In 2014, the European Commission established a new target of 27% of renewable energy by 2030. Based on the current European bioenergy situation, to meet 2020 target, production should increase to 30 Mt of oil equivalent. According to the current land used for biofuel production (7% of the total European arable land), to achieve this requirement an increase of land use of 14% would be needed, what would establish a competition for arable land with food production. Therefore, the major challenge is to make second generation biofuels, those not coming from crops, competitive alternatives to fossil fuels while guarantying the sustainability of the process (Barbosa and Wijffels 2013). Much attention was then centered in microalgae since they do not compete with traditional crops for arable land, they present high growth rates and some strains can achieve high lipid and/or starch content.

Several type of biofuels can be produced from microalgal biomass such as bioethanol from starch-rich strains (Brányiková et al. 2011), methane produced by anaerobic digestion of microalgal biomass (Spolaore et al. 2006) or photobiologically produced biohydrogen (Melis et al. 2000) although biodiesel from microalgal oil is the most popular and studied due to the high lipid content of many species. A lot of effort has been made in order to determine the optimal conditions for a cost-effective production process of biodiesel from microalgae. Several reports (Tredici 2010; Williams and Laurens 2010; Gouveia 2011) agree on the high potential of microalgal biofuels although the production process is still not feasible. Wijffels et al. (2010) made several assumptions and estimated the prices of biodiesel to be

0.50€/liter, which would be competitive considering actual diesel price but the costs of extracting the lipids and converting them to biodiesel were not taken into account. Chisti (2007) gave a value of 2.80$/liter of biodiesel when the recovering process of oil from microalgal biomass was considered. Thus, a significant reduction of the production costs of biodiesel from microalgae would be needed to compete with fossil fuels. To achieve lower prices of biodiesel, several strategies have been proposed such as maximizing biomass and lipid yields, improving the harvesting process or developing new advances in the cultivation system design and engineering. However, even when the implementation of these strategies decreases the cost of biodiesel, the production process is still not feasible.

The biorefinery concept

One of the main inputs of microalgal production is related to the nutrients, water and CO_2 required by cells to grow, from which only a part is used for lipid production. The rest of supplied inputs are used in the synthesis of other essential compounds, mainly proteins and carbohydrates, which are discarded during the lipid extraction process. For this reason, the idea of a biorefinery process in which all microalgal products are used has become the main strategy for the production of biodiesel from microalgae. The biorefinery is an integrated facility, analogous to modern petroleum refineries, that combines various processes to produce biofuels, energy and high-value compounds from microalgal biomass aiming to maximize biomass value in a sustainable process. Integrated biorefineries are already being operated in Canada, the US and Germany for producing biofuels and other products from crops such as corn and soybean (Chisti 2007).

In a general biorefinery process (Fig. 1.1), CO_2 would be provided by flue gas from industry microalgal biomass can be directly used as health food for human consumption, as animal feed or in aquaculture. The rest of the algal biomass from different process stages can be used either as a fertilizer, production of industrial materials or bioenergy applications such as biodiesel production. Extraction of other high-value products could be feasible depending on the biochemical composition of the species and strain used.

For example, proteins and other value biomolecules can be extracted for pharmaceutical and industrial purposes and high-value omega-3 fatty acids, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) could be separated to be used for food applications. This approach would allow a cost-effective production of microalgal biodiesel.

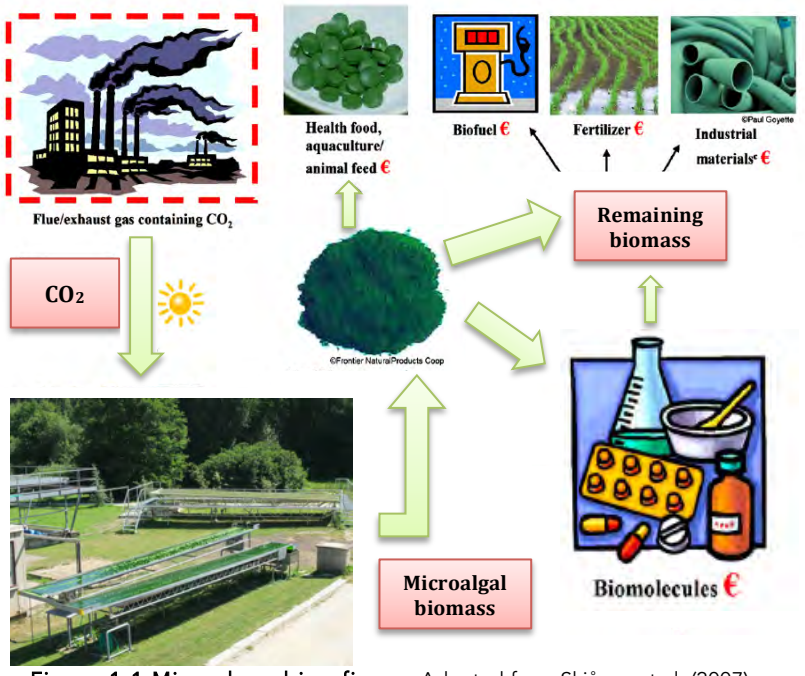


Figure 1.1 Microalgae biorefinery. Adapted from Skjånes et al. (2007)

To be able to exploit the complete produced biomass, the different cell fractions i.e. proteins, lipids and carbohydrates should remain available and undamaged after cell disruption, what seems to be the main bottleneck of the biorefinery approach. Common used methods to extract any of these fractions (heating, high pressure, osmotic shock…) usually entail damaging of the other cell components. Therefore, in a biorefinery process it is required a first step in which cells are disrupted by using mild techniques. These techniques comprehend pulsed electric field, supersonic flow fluid processing and ultrasound, which are quite new and consequently their application is being widely studied (Vanthoor-Koopmans et al. 2013). After mild cell disruption, separation of cell fractions without losing any product should be

guarantied. For this aim, new methods such as the use of ionic liquids and surfactants have been proposed. Finally, further separation of the different fractions would be needed to obtain specific products.

Cultivation systems: open versus closed

Several types of cultivation systems have been used since the late 1950s, although their design and engineering is still a very active field of research. Nowadays, large-scale commercial production of microalgae includes open systems such as ponds, raceways and thin-layer cascades and closed systems, mainly tubular and serpentine photobioreactors.

Closed systems imply that there is no direct contact between the culture and the atmosphere and thus, light does not impinge directly on the culture surface but on the transparent walls of the reactor and exchange of gases, liquid and particles is avoided (Tredici 2010). This confinement of the culture is the main advantage of closed systems, since it allows for the cultivation of certain species that present contamination problems in open systems. However, closed systems are more expensive than open systems. Therefore, only the cultivation of species unable to be cultured in open systems but which produce high-value compounds is recommended at commercial large-scale since the high market price of these compounds allows a cost-effective production process. Besides this, taking advantage of their low contamination risks, the use of closed systems to produce biomass to be used as inocula for large-scale production in open systems is a common practice. At the Arizona State University (USA), a small plant made of 32-column type PBRs, 40 L volume each, is in operation to provide the inoculum for a feedstock production in raceway ponds. A pilot-scale plant consisting of ten 2.8 m^3 vertical serpentine units was operated under a greenhouse at the Estación Experimental de Cajamar "Las Palmerillas" (Almería, Spain) for the production of *Scenedesmus almeriensis*, which is rich in lutein (Fernández-Sevilla et al. 2010). The company Fitoplancton Marino (Cádiz, Spain) produces *Nannochloropsis gaditana* in tubular photobioreactors to be commercialized as animal feed and in the cosmetics industry. HR BioPetroleum, Inc. (Kailua-Kona, Hawaii, USA) uses a 25 000 L serpentine reactor (38 cm diameter) to

produce the green stage of *Haematococcus pluvialis* to later inoculate raceway ponds in which astaxanthin and oil accumulation is induced (Huntley and Redalje 2007). Vertical flat panel reactors represent very promising culture devices and several prototypes have been used during the last decade to evaluate the productivity of different species (Aflalo et al. 2007; Hu et al. 2008; Rodolfi et al. 2009). Among these prototypes, the Green Wall Panel reactor (GWP) was scaled-up and is currently being used by Archimede Ricerche Srl (Imperia, Italy) in a plant made operated under a greenhouse covering 1000 m^2 to produce *Nannochloropsis, Tetraselmis* and *Isochrysis* to be used in cosmetic, aquaculture and pharmaceutical sectors (Zittelli et al. 2013).

Despite the advantages of closed systems, the low construction and operation costs of open systems make them the main type of cultivation system currently used at commercial scale (Borowitzka 1999; Zittelli et al. 2013). Open systems take advantage of the high growth rate or the selective growth medium of certain species and in consequence commercial production is limited only to a few species mainly of the genera *Arthrospira*, *Dunaliella* and *Chlorella*. Betatene Ltd (Whyalla, South Australia) produces *Dunaliella salina* in unmixed very large ponds (up to 250 ha) (Tredici 2004; Borowitzka 2005). Raceway ponds are the most common system in use. DIC LIFETEC Co. Ltd. (Japan) produces more than 700 t annually of *Arthrospira* in California (USA) and China. Cyanotech Co. (Hawaii, USA) produces about 300 t of *Haematococcus pluvialis* as a source of astaxanthin. Small production plants are located in Australia also for the production of *Arthrospira* and in Israel for the production of *Dunaliella*. *Chlorella* is produced in big circular ponds of about 10 000 m^2 by different companies in Japan and Taiwan. Yaemama Shokusan Co., Ltd (Japan) produces *Chlorella* in pools with a total area of 27 000 m^3 and a production capacity of 35 t per month. Tawian Chlorella Manufacturing Company (Taiwan) has two production plants of ~25 000 m^2 and an annual production capacity of 150-180 t each.

Thin-Layer Cascade systems (TLCs)

Chlorella has also been used by the Institute of Microbiology of the Academy of Sciences of the Czech Republic in the development and later scaling-up of the thin-layer cascade system (Šetlík et al. 1970; Doucha and Lívanský, 1999). In the TLC, the microalgal suspension flows in a thin layer (6-8 mm) over a sloping surface exposed southwards to solar irradiance (Masojídek et al. 2011). This short optical path allows an efficient light utilization that leads to high biomass densities (40-50 g L-1). Circulation of the suspension takes place during the day, and at night the algal culture is kept in an aerated tank. In relation to the cultivation area, the suspension volume is significantly lower and the culture density at harvest is much higher compared with raceway ponds, what enables higher yields and cheaper cultivation and harvesting technology reducing the costs of biomass production to about 15–20% of that associated with ponds (Doucha and Lívanský 2009).

The building costs at initial stages of development was higher than that of raceways but a new prototype made of cheaper materials is being under evaluation (Masojídek, personal communication, 2013). However, biomass productivity is much higher than in raceways (<1 g L^{-1}, Borowitzka 2005) and similar to that of closed systems, which still have higher production costs. In addition, the culture can be quickly stored in the retention tank in case of accidents or unfavorable weather conditions. Table 1.1 shows the differences among open (ponds and thin-layer cascades) and closed systems.

Photosynthesis in microalgae

Photosynthesis is a process of sunlight energy conversion in which inorganic compounds and light energy are converted to organic matter in the chloroplast of photoautotrophic cells (Masojídek et al. 2013). It is a redox reaction in which light absorption by the chlorophyll molecule results in the conversion of water into carbon dioxide and carbohydrates and synthesis of ATP and NADPH and O_2 release. The process comprehends a first stage driven by light that takes place in the thylakoid membrane and a second stage, the dark phase, which occurs in the stroma.

Table 1.1 Main characteristics of open (ponds and thin-layer cascades) and closed systems (adapted from Grobbelaar 2009; Gouveia 2011).

Feature	Open systems		Closed systems
	Ponds	Thin-Layer Cascades	
Algal species	Restricted		Flexible
Species selection	Growth competition		Shear-resistance
Contamination	Possible		Unlikely
Evaporation	High		Low
Control of growth	Not optimized		Optimized
Maintenance	Easy		Difficult
Construction costs	Low		High
Light utilization efficiency	Low	High	High
Gas transfer	Poor	Fair/high[a]	Fair/high
Overheating problems	Low	Low	High
Cleaning	None	Moderate[b]	Required
Biomass productivity	Low	High	High
Mixing efficiency	Poor	High[a]	High
CO_2 transfer rate	Poor	High[b]	High
Land required	High	Medium	Low
Periodical maintenance	Low	Medium	High
Capital investment	Small	Medium	High
Operating cost	Low	Medium	High
Scale up	Easy	Medium	Low

[a]Turbulent regime in the cascade (Masojídek et al. 2011)

[b]Injected directly in the pipe

The energy necessary to fix one molecule of carbon dioxide during the dark phase is produced during the light reaction in the form of NADPH (two molecules) and ATP (three molecules). On the other hand, the reduction of a molecule of nitrate to glutamate requires one molecule of ATP and per eight electrons provided by NADPH and reduced ferredoxin. Since the reaction centers of both photosystems must work in series for continuous oxygen evolution, meaning that it is necessary equal number of charge separations, and since four electrons (four photons in PSII) are required to oxidize two water molecules to evolve one molecule of oxygen, a minimum of eight

photons are required for the production of each O_2. For each carbon fixed, two molecules of NADPH (four electrons) are required. Thus, the minimum quantum requirement for the fixation of one carbon atom is also eight photons.

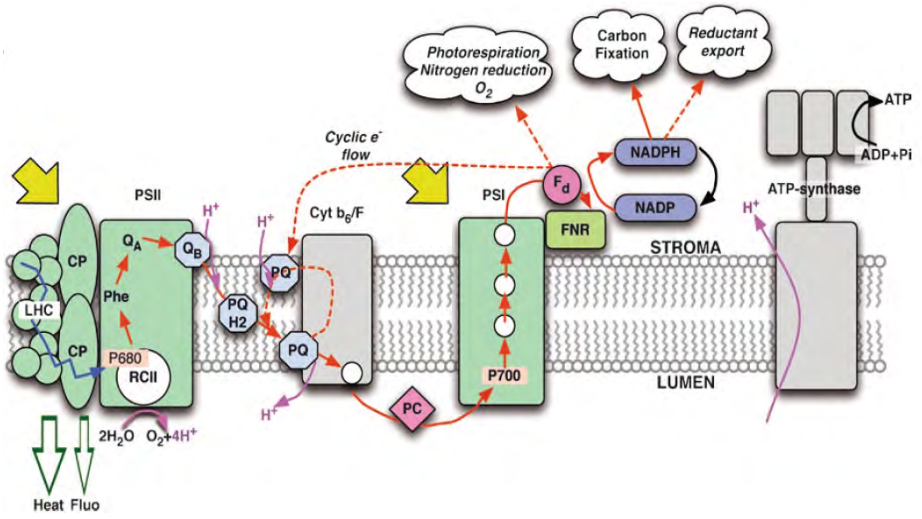


Figure 1.2 Schematic representation of the light reactions of photosynthesis. From left to right are represented four protein complexes: photosystem II (PSII), cytochromes b_6/f (Cyt b_6/F), photosystem I (PSI) and ATP synthase. The continuous red arrows indicate the linear electron flow while the dashed red arrows represent alternative paths. The energy absorbed in the light-harvesting antenna of PSII (LHC) is funneled to the chlorophyll complex P680, which transfer the electron to the pheaophytin (Phe) in a process commonly known as charge separation. The electron hole in $P680^+$ is filled by an electron from tyrosine, which obtains an electron from water via the manganese cluster. Phe reduces the first quinone acceptor, Q_A, which needs two electrons to reduce the secondary acceptor, Q_B. Electrons are transferred from Q_B to the reaction center of PSI, the chlorophyll P700, by the subsequent reduction of the plastoquinone pool (PQ), Cyt b_6/F and plastocyanin (PC). For this transfer to occur, the P700 is previously oxidized through a second light reaction where the absorbed energy is transferred through a series of carriers to ferredoxin (F_d), which finally reduces NADP to NADPH. Alternative sinks for the electrons from F_d and NADPH are shown in the white clouds. The electron transport chain between PSII and PSI leads to the formation of a proton gradient (purple arrows) that provides energy for the synthesis of ATP by the ATP-synthase complex. Taken from Huot and Babin (2010).

Linearity between PSII electron transport and oxygen evolution is usually found (Suggett et al. 2010), but non-linear behavior has also been observed (Masojidek 2001; Kromkamp et al. 2009), especially under conditions when, due to high irradiance, photosynthesis was over-saturated (Ralph et al. 2010). Several explanations have been suggested for these alternative electron pathways but the most accepted are cyclic electron flow around PSII (Prasil et al. 1996; Lavaud et al. 2002) or PSI (Bendall and Manasse 1995) or presence of alternative electron acceptors, especially the Mehler reaction, which entails the formation of hydrogen peroxide by donation of electrons to O_2 from PSI via ferredoxin (Asada 2000).

Measuring the photosynthetic activity

The photosynthetic activity can be measured by following changes in the rate of production or consumption of any of the involved inputs or resulting outputs of the process. Traditionally, gas measurements of either O_2 evolution or CO_2 fixation have been the most accepted methods to estimate photosynthetic activity. O_2 production has been measured by estimating the concentration of dissolved O_2 in water samples using colorimetric methods such as the Winkler method or electrochemically by using Clark-type electrodes. The later commercialization of oxygen optodes sensors allowed for the optical estimation of oxygen evolution based on a luminescence reaction that is quenched in the presence of oxygen. Carbon dioxide fixation has been widely measured by infrared gas analyzers (IRGA) and more recently by following the incorporation of inorganic radio-labelled $^{14}CO_2$ or $NaH^{13}CO_3$. However, photosynthetic activity can also be estimated by measuring the fluorescence emission or heat dissipation of PSII, which are directly related to photochemistry. The absorbed light energy in PSII is transferred to the reaction center to be used in photochemistry, but alternatively, the excitation energy can be dissipated as heat or re-emitted as fluorescence by the chlorophyll a molecule. Any change in one of these competing processes will result in a change on the other two processes since the sum of the energy entering the three processes is equal to the absorbed light energy. Therefore, chlorophyll *a* fluorescence directly reflects the performance of photochemical

processes in PSII since in microalgae the contribution of PSI is usually so small at ambient temperature that for practical purposes can be neglected.

On the other hand, photoacustics methods are being developed during the last years, based on the conversion of light energy emitted from a laser pulse absorbed by photosynthetic pigments into heat, what rises the temperature and thus, the pressure increases (Grinblat and Dubinsky 2010).

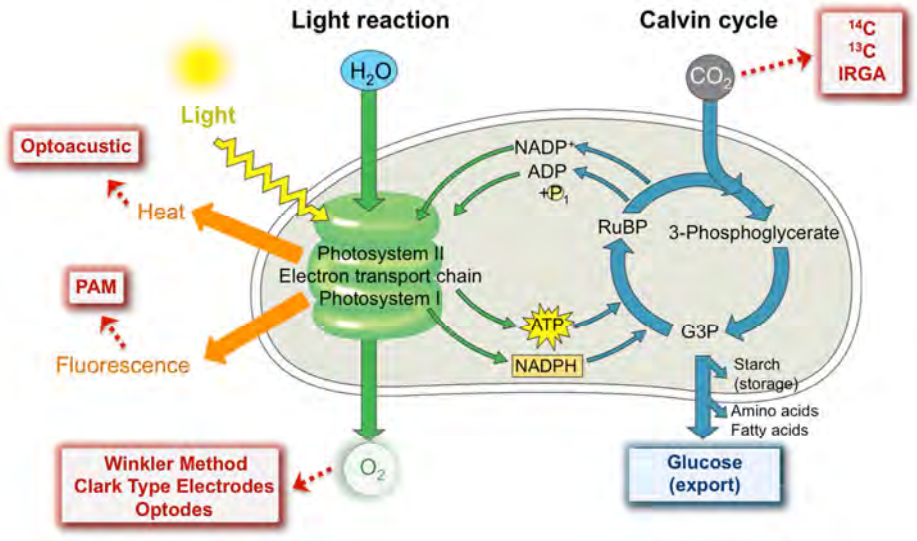


Figure 1.3 Overview of the photosynthetic processes. The absorbed light energy is used in photochemistry during the light reactions. The excess energy not entering this process is re-emitted as heat or fluorescence. The light energy converted into ATP and NADPH is used in the Calvin cycle to fix CO_2 and synthetize glyceraldehyde 3-phosphate (G3P), which is the precursor of glucose and is involved in the synthesis of starch, amino acids and fatty acids. The methods by which the different processes involved in photosynthesis can be measured are indicated in the red boxes.

In vivo chlorophyll a fluorescence - The saturation pulse method

Pulse-amplitude modulated fluorometers (PAM) are extensively used to measure chlorophyll *a* fluorescence of PSII by the saturation pulse method. In these instruments the measuring light is modulated, it is switched on and off at high frequency, and the detector only detects the fluorescence excited by this measuring light.

The saturation pulse method involves the analysis of the photochemical and non-photochemical quenching components. It is based on the redox state of the reaction centers before and after giving a saturating flash of light sufficiently intense as to maximally reduce Q_A. In the dark, the reaction centers are "open", indicating that Q_A is oxidized, and thus, the reaction centers are capable of performing photochemical reduction of Q_A. Exposure of a dark-adapted cell to the measuring light, which is non-actinic to ensure that Q_A remains fully oxidized, results in the minimal level of fluorescence, F_0. After applying a saturating pulse of light, Q_A will be maximally reduced so that photochemistry is reduced to zero, non-photochemical quenching will be negligible and thus, the maximal level of fluorescence, F_m, is reached. The difference between the minimum and maximum fluorescence level is called variable fluorescence, F_v.

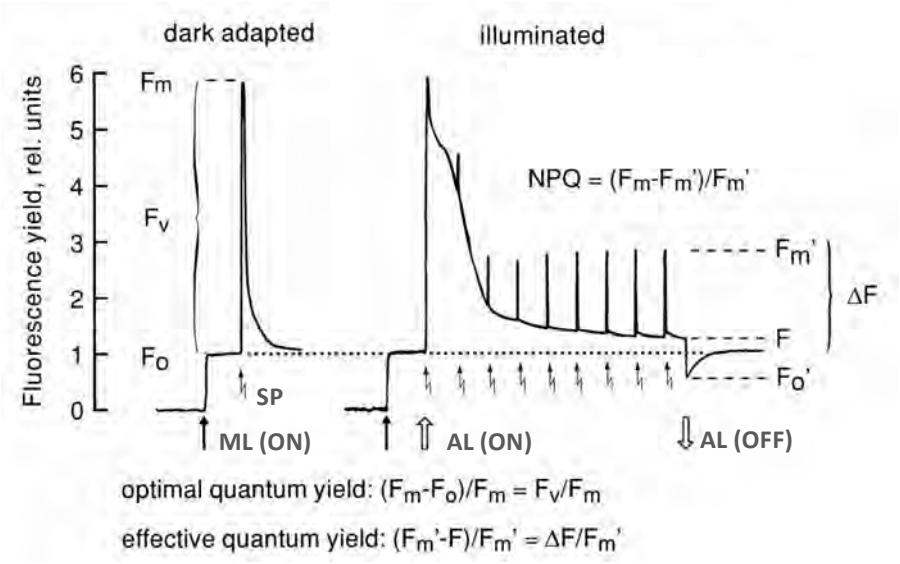


Figure 1.4 The saturation pulse method. Under dim measuring light (ML) the minimum fluorescence level (F_0) is measured. After applying a saturating light pulse (SP), the maximum fluorescence is achieved (F_m). Next, the sample is illuminated with actinic light (AL) and a series of saturating pulses in order to reach the steady state (F') and the maximum fluorescence (F_m'). Finally, the actinic light is switched off to measure the minimum fluorescence after illumination (F_0').

The reaction centers of light-adapted cells have reduced Q_A and are referred to as "closed". Under light conditions non-photochemical quenching (NPQ) is induced. It is inversely related to photochemistry and is considered a safety valve protecting PSII reaction centers from damage by excess irradiance (Masojídek et al. 2013). In this state there is a minimum level of fluorescence termed F' (the ' is indicative of parameters measured in light-adapted cells) and NPQ will reduce the fluorescence yield. Therefore, after a saturating light pulse, the achieved maximum fluorescence value will be lower, F_m'.

The saturation pulse analysis can be used to estimate the quantum yield of PSII, which be understand as the efficiency by which absorbed light is used in photochemistry. In the dark, it can be estimated by normalizing the variable fluorescence to the maximum fluorescence yield, F_v/F_m (Cosgrove and Borowitzka 2010). In light conditions maximum efficiency is not achieved but the effective quantum yield can be calculated as $\Delta F/F_m'$.

Table 1.2 Parameters calculated from chlorophyll fluorescence measurements. F_0, F_v, F_m – minimum variable and maximum fluorescence in dark-adapted state; F_0', F', F_v', F_m' – minimum, steady-state, variable and maximum fluorescence in light-adapted state; E_{PAR} – photosynthetic active radiation; fAQ_{PSII} – fraction of absorbed quanta to PSII, A – absorptance and a – light absorption coefficient

Parameter	Symbol	Formula
Optimal quantum yield	F_v/F_m	$(F_m - F_0)/F_m$
Effective quantum yield	Y(II) or $\Delta F/F_m'$	$(F_m' - F')/F_m'$
Relative electron transport rate	rETR	$\Delta F/F_m' \times E_{PAR}$
Absolute electron transport rate (correlated with GPP)	ETR	$\Delta F/F_m' \times E_{PAR} \times fAQ_{PSII} \times A$ or a
Non-photochemical quenching	NPQ	$(F_m - F_m')/F_m'$ =Y(NPQ)/Y(NO)
Regulated NPQ	Y(NPQ)	$F'/F_m' - F'/F_m$
Non-regulated NPQ	Y(NO)	F'/F_m'

[a]GPP gross photosynthetic productivity (net photosynthetic productivity plus respiration)

Electron transport rate as estimator of productivity

The relative electron transport rate (rETR) through PSII can be calculated as the product of the photosynthetic quantum yield $\Delta F/F_m'$ and the incident photosynthetically active radiation (PAR). The electron transport rate can be used as a proxy of photosynthetic capacity or productivity (Kromkamp et al. 2008; Figueroa et al. 2013). However, photosynthetic activity is directly related to the absorbed light and not to the incident irradiance. ETR values calculated using the incident (relative ETR, rETR) instead of the absorbed irradiance would correspond to relative conditions in which all the incident light is absorbed by PSII, what in fact is not common in most of microalgal cultures. Moreover, changes in environmental conditions can lead to variations in morphology, pigmentation and cell size that influence the pigment packaging effect and may entail changes in the optical properties of the cells, what would result in differences among cells with different optical properties when the absorbed light is not considered. Thus, the determination of absolute ETR values rely on independent measurements of light absorption that will allow for the comparison between different species or species under different environmental conditions.

Despite the importance of considering light absorption for ETR determination, no standardized method is currently available and different approaches are being used. In macroalgae, light absorption has been estimated by measuring the thallus absorptance, the fraction of incident light that is absorbed (Longstaff et al. 2002; Figueroa et al. 2003; Enríquez and Borowitzka 2010). In oceanographic studies, the absorption coefficient is usually determined spectrophotometrically from discrete water samples and related to the chlorophyll concentration (Flameling and Kromkamp 1998). For this, two approaches have been used: measurement of the absorptance of a cell suspension by the determination of both transmittance and reflectance by an integrating sphere (Bricaud et al. 1983; Maske and Haardt 1987) and the quantitative filter technique by which an amplification factor is obtained to correlate the absorption of the cell suspension and the absorption of cells retained in a glass fiber filter (Kishino et al. 1985; Arbones et al. 1996). In addition, it is not always possible to determine the cellular optical properties

under field conditions and on the other hand, the need of measuring the light absorption of discrete water samples constraints the scales at which fluorometric measurements can be made (Suggett et al. 2004). Moreover, in microalgal biotechnology light absorption is not usually considered when determining ETR values and relative approaches are common. Therefore, the consideration of light absorption in ETR determinations is a need if we aim to have real values of the photosynthetic activity but the lack of a reliable and easy methodology to measure light absorption should be undertaken.

ETR has been extensively used to estimate photosynthetic productivity since a linear relationship between fluorescence-based measures of photochemical efficiency of PSII and independent measures of the quantum yield of CO_2 fixation in maize were first described by Genty et al. (1989). This study was followed by others that confirmed the same linearity in microalgae (Gilbert et al. 2000; Kromkamp and Forster 2003; Wagner et al. 2012). However, a linear relationship between ETR, O_2 evolution and CO_2 fixation is not always found (Flameling and Kromkamp 1998; Kromkamp et al. 2008). Cyclic electron flow around PSII (Prasil et al. 1996) can uncouple ETR from the rate of O_2 production by PSII whereas alternative electron pathways related to the water-water cycle, donation of electrons from PSI to O_2 with the formation of hydrogen peroxide (Asada 2000), or to nitrate assimilation (Holmes et al. 1989), can lead to uncoupling with CO_2 fixation.

Application of chlorophyll a fluorescence to microalgal biotechnology

In outdoor microalgal cultures, environmental changes and unexpected unfavorable conditions may cause response of microalgal cells sometimes within only several seconds or minutes. Monitoring of the culture performance in order to detect warning signals that may entail decrease of productivity or culture loss is extremely desired. Dry weight and photosynthetic measurements carried out in laboratory have been the methods most commonly used to evaluate culture health and biomass productivity. However, in situ monitoring of chlorophyll a fluorescence began to be used in the 1990s to examine the photosynthetic performance of microalgal mass cultures (Vonshak et al. 1994; Torzillo et al. 1996). In situ monitoring can be

made by simply pointing a fluorometer fiber-optics at a photobioreactor or by submerging it into the suspension using ambient irradiance as actinic light (Masojídek et al. 2010) and as a result, the effective quantum yield would be obtained. In this way, monitoring of chlorophyll *a* fluorescence allows the evaluation of the effect of changing environmental conditions such as CO_2 supply, pH, temperature, mixing, nutrients, etc. on the physiology of the culture. The acquisition of photosynthetic data with high-temporal resolution and its relation to changes in culture conditions would make possible to optimize the photosynthetic performance of the culture.

In addition, if a PAR sensor is coupled to the chlorophyll fluorescence sensor so that the irradiance is measured simultaneously, rETR values could also be monitored. When light absorption data is available, the determination of absolute ETR might lead to the estimation of productivity in terms of oxygen evolution, carbon fixation or even biomass productivity if the carbon content is known. Such approach would provide valuable information to better understand the process involved in photosynthesis and consequently affecting the productivity of the culture.

Photosynthetic efficiency of microalgae

It is a common belief that microalgae are more efficient in terms of photosynthesis than higher plants, what would mean that microalgae are more efficient in converting light energy into chemical energy of carbohydrate or biomass. Nevertheless, this is still a matter of debate and several authors state that the advantages of microalgae over terrestrial plants do not rely on higher photosynthetic efficiencies (Tredici 2010; Wilhelm and Jakob 2011) but on their higher versatility, lower ecological impact, lower respiration losses, higher content in storage compounds and higher diversity in fatty acid composition (Williams and Laurens 2010).

On the other hand, both higher plants (C3 metabolism) and microalgae share the maximal theoretical value for photosynthetic efficiency, which has being calculated and assumed to be ~12% but when losses due to respiration, photorespiration, photosaturation and photoinhibition are

considered, this maximal value decrease to the commonly accepted 4.6% (Tredici 2007; Walker 2009; Williams and Laurens 2010). In the practice, the yields achieved in outdoor microalgal cultures decrease to one third to one tenth of this theoretical maximum since several factors such as temperature, carbon dioxide concentration, turbulence or nutrient availability are many times far from the optimal conditions. To date, long-term efficiencies of 4-5% are rarely reached with algal cultures under natural conditions (Tredici 2010). In the case of plant crops, the measured photosynthetic efficiency in the field is also lower than the theoretical 4.6% mainly because the growing season is reduced and only during a fraction of this period the canopy cover is maximum, among other stressing factors such as nutrients and temperature (Zhu et al. 2010).

The maximal photosynthetic efficiency is limited by stoichiometry thermodynamics reasons and therefore it is considered unbeatable but, as Williams and Laurens (2010) pointed out, there is no fundamental barrier to achieve it. According to this, some strategies such as light dilution, rapid mixing or genetic modification have been proposed to achieve photosynthetic efficiencies closer to the theoretical maximum.

Commercial production of *Chlorella*

The genus *Chlorella* (Chlorophyta, Trebouxiohyceae) comprehend species from wide variety of habitats, from marine to freshwater environments, with high temperature tolerance ranging from 15°C to even 35-40°C. Cells are globular or ellipsoidal with variable diameter, from 3 to 9 μm. It grows autotrophically in an inorganic medium although it also can grow in mixotrophic or heterotrophic conditions.

At present, *Chlorella* is being commercially cultivated by more than 70 companies all over the world (Spolaore et al. 2006). Its high growth rate prevents contamination and thus, it is widely produced photoautrophically in open systems such as ponds or thin-layer cascades. The largest *Chlorella* producer is Chlorella Manufacturing and Co. (Taipei, Taiwan), with 400 t of dried biomass produced in open ponds per year although it is also produced

in closed systems such as the tubular photobioreactors located in Klötze (Germany), which produces about 100 t dry biomass per year for the health food market (Pulz et al. 2013).

Chlorella is widely produced to be used as health food or feed supplement in Germany, Japan, China and other Asian countries because of its high content in proteins with a balanced amino acid composition (Becker 2007), fatty acids ,including oleic and linoleic acids (Petkov and Garcia 2007), carotenoids, vitamins and minerals (Masojídek et al. 2010). *Chlorella* extract have proven to prevent against tumors and cancers, enhance hypoglycemic effects, attenuate cognitive decline of age-dependent dementia and lower blood pressure (Liu and Hu 2013). High-value products can be also obtained from *Chlorella* biomass like some immunostimulators such as β-1,3-glucan, which can also act as a free-radical scavenger and a reducer of blood lipids (Iwamoto 2003). It also has different applications in the nutraceutical and cosmetics industry due to the high accumulation of lutein and astaxanthin of some species (Liu and Hu 2013) or as food additive owing to the taste and flavor of its coloring agent (Yamaguchi 1996). Some other applications have been proposed such as the production of recombinant proteins, but the production of *Chlorella* for high-value products is still limited due to high costs associated to production systems and processes. New advances in the cultivation technology and in the knowledge of the metabolism and physiology of this genus would lead to a future expansion of the market. Besides, *Chlorella* is among the microalgae that has arisen more interest for biofuel production since under certain stress conditions some strains can accumulate large amounts of lipids or starch.

Among *Chlorella* species, *C. fusca* has been widely used in the past decades in physiological studies that evaluated the effect of CO_2 on potassium transport (Tromballa 1983), the cell wall lytic activity (Loos and Meindl 1984) or the non-hydrolysable macromolecular constituents from outer walls (Derenne et al. 1992). In addition to the interesting characteristics already known of species of the genus *Chlorella* such as high growth rate, large size compared to other microalgae and high nutritional content, *C. fusca* has shown high content of a variety of high-value compounds. It has shown

high carotenoid content, specifically lutein (Becker 2013); it has been proposed as a source of sporopollenin, a UV screening compound (Priyadarshani and Rath 2012) and it can accumulate high content of essential fatty acids such as the omega-3 α-linolenic acid (ALA) or linoleic and oleic acids (Pohl et al. 1971). Moreover, it has been proven its efficiency in the biosorption of cadmium and copper (Wehrheim and Wettern 1994). Therefore, *C. fusca* represents a good candidate for to evaluate the accumulation of high-value compounds for possible biotechnological applications.

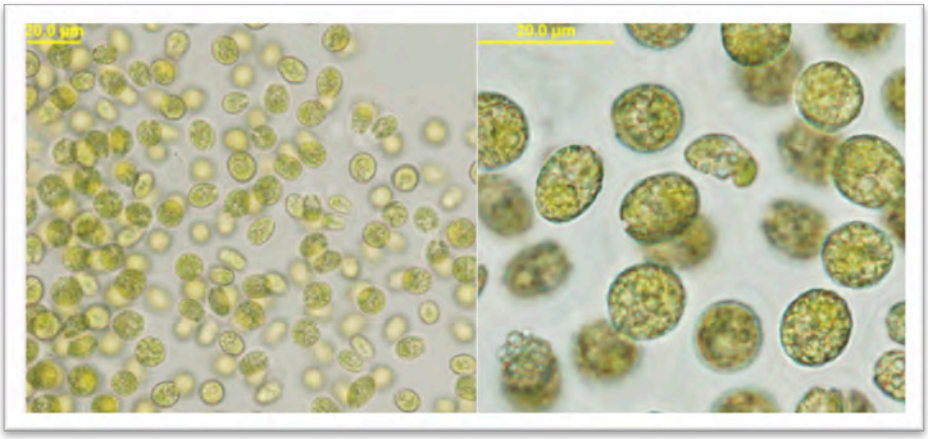


Figure 1.5. *Chlorella fusca* #UTEX 343. Taken from The Culture Collection of Algae (UTEX, Texas, US)

Thesis outline

Researchers have not yet acquire the necessary physiological knowledge nor developed the required technology to take advantage of the potential of microalgae to produce energy, novel foodstuffs and renewable non-food commodities in a sustainable way. Therefore, the present thesis aimed to contribute the overcoming of the problems that obstacle the scaling-up of microalgal cultures by filling some of these gaps related to the photosynthetic performance and biochemistry of outdoors cultures. To that end, the present study addressed the following questions:

1. Could the absorptance method currently used in macroalgae be adapted for its application for the measurement of light absorption in microalgal cultures?

   Hypothesis: by using an appropriate measuring system and optimal cell density, the absorptance of a thin layer of cell suspension could be well correlated to the absorption coefficient determined by the quantitative filter technique.

2. Could *in vivo* chlorophyll *a* fluorescence be used to monitor the photosynthetic performance of microalgal cultures in a new thin-layer cascade system? How can it be applied to evaluate the spatial-temporal variation of the photosynthetic activity in this system?

   Hypothesis: *in vivo* chlorophyll *a* fluorescence can be monitored *in situ* (solar radiation) in thin-layer cascades but the hydrodynamics of the system should be previously studied to optimize the measuring set-up.

3. Is *Chlorella fusca* able to grow in outdoor thin-layer cascades? How does its photosynthetic performance and biomass composition change in systems with different surface to volume ratio?

   Hypothesis: higher S/V ratio of the cultivation system is related to higher photosynthetic production.

4. Does the biomass productivity estimated from monitoring of *in vivo* chlorophyll a fluorescence correlates with the measured productivity determined by difference in dry weight?

   Hypothesis: biomass productivity can be estimated from daily-integrated electron transport determined *in situ* and correlated to measured biomass productivity. Discrete measurements of electron transport rate measured under artificial actinic light (different to solar radiation) are lower correlated to measured biomass productivity compared to the electron transport rate determined under solar radiation

5. Does the combination of nutrient and light stress have any effect on the accumulation and productivity of storage compounds in *Chlorella fusca* cultivated under controlled conditions?

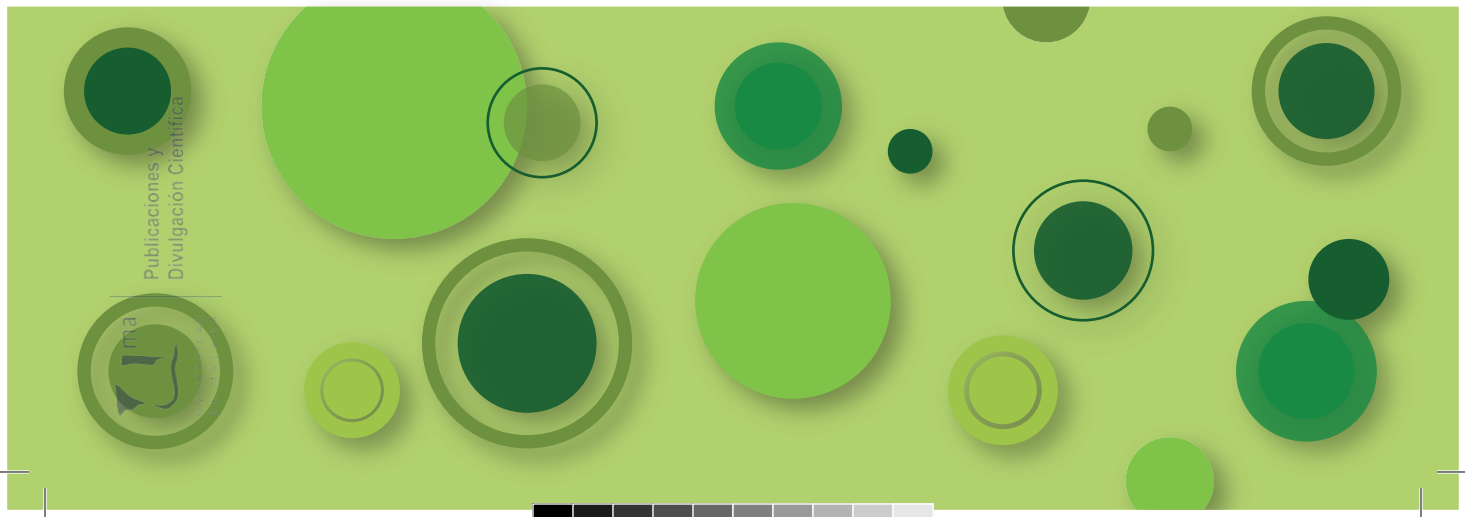
   Hypothesis: nutrient depletion or starvation and light stress (high PAR irradiance and UV radiation, λ=280-400 nm) are related to higher content of storage compounds.

2 chapter

Relation between light absorption measured by the quantitative filter technique and the attenuation of *Chlorella fusca* cultures of different cell densities: application to estimate the absolute electron transport rate (ETR)

Celia G. Jerez, Carolina B. García, Agustín Rearte and Félix L. Figueroa

Submitted to Journal of Applied Phycology (February 2015)



Abstract

In order to estimate microalgal carbon assimilation or production of *Chlorella fusca* cultures based on electron transport rate (ETR) as *in vivo* chlorophyll *a* fluorescence, it is necessary to determine the photosynthetic yield and the absorbed quanta by measuring the incident irradiance and the fraction of absorbed light, i.e. absorptance or absorption coefficient of photosynthetic active radiation (PAR). Due to difficulties associated to the determination of light absorption, ETR is commonly expressed as relative units (rETR) although this it is not a good estimator of the photosynthetic production since photobiological responses depend on the absorbed light. The Quantitative Filter Technique (QFT) is commonly used to measure the absorbed quanta of cells retained on a filter (AbQ_f) as estimator of the absorbed quanta of cell suspensions (AbQ_s) by using integrating spheres. In this study, light attenuation of a thin layer of cell suspensions is was determined by using a measuring system designed to reduce the scattering. Light attenuation was related to the absorptance of both in-door and out-door C. fusca cultures of C. fusca of different cell densities. A linear relation between AbQ_f and AbQ_s (R^2=0.99, $p<0.01$) was obtained, $AbQ_f = 1.98 \cdot AbQ_s$, which can be used to convert AbQ_s values into AbQ_f ones. On the other hand, it was discussed the use of the absorptance, light absorption or specific light absorption coefficients to calculate ETR values expressed per area (thin layer cascade or flat panel cultivators), volume (cylindrical and tubular photobioreactors) or chlorophyll units (any type of cultivation system) depending on the culture system. Simplification of the measurement of the light absorption coefficient presented in this study for *C. fusca* can be tested in other phytoplankton species and would promote the determination of absolute ETR, which provide relevant photobiological information of microalgal cultures.

chapter 3

Chlorella fusca grown outdoors in Thin-Layer Cascades

Publicaciones y Divulgación Científica

3.1 chapter

Hydrodynamics and photosynthesis performance of *Chlorella fusca* grown in a Thin-Layer Cascade (TLC) system

Celia G. Jerez, Enrique Navarro, Irene Malpartida, Rosa M. Rico, Jíri Masojídek, Roberto Abdala and Félix L. Figueroa

Aquatic Biology 22:111–122 (2014)

Abstract

The thin-layer cascade (TLC) system is an open system for microalgae cultivation composed of a retention tank connected by pump and pipes to a horizontal exposed area that consists of an upper basin and a TLC. Light and hydrodynamics are different among compartments, so overall photosynthetic activity can be influenced by the retention time of the cells in each compartment. We established two settings with different retention times in the cascade and tank to compare the photosynthetic activity of *Chlorella fusca* (Chlorophyta) among compartments. Changes in the retention time were achieved using two layer thicknesses in the cascade: 8 and 18 mm. Retention time in the cascade represented about 16 and 34% of the duration of a whole system cycle when H1 (8 mm thickness) and H2 (18 mm thickness) units, respectively, were used. These retention periods were lower than those in the tank (67 and 49%, respectively) but higher than those in the basin (12% for both H1 and H2). Photosynthetic activity was measured *in situ* as relative electron transport rate (rETR) using a pulse-amplitude modulated fluorometer. In both setups, the highest rETR was reached in the cascade. The increase of the layer thickness was a good option to avoid photoinhibition. We suggest estimating the mean rETR of the whole system considering the retention time, since it can better reflect overall growth because it takes into account the time that the cells spend in each compartment. These results are useful for optimization of photosynthetic activity and growth of outdoor microalgae mass cultures in TLCs for biotechnological purposes.

3.2 chapter

Chlorella fusca (Chlorophyta) grown in outdoor thin-layer cascades: growth, photosynthetic performance and biomass composition

To be submitted to Algal Research (Companion Paper-A)

Abstract

The photosynthetic performance, biomass composition and productivity of three cultures of *Chlorella fusca* BEA1005B grown in thin-layer cascades (TLCs) were studied in two locations with the aim to test this microalga as a production strain. The first (E1) and second (E2) experiments were conducted in the south of-Spain in July and October 2012, respectively in a TLC with exposed surface to total volume ratio (S/V) of 20-27 m^{-1}. The third experiment (E3) was conducted in the Czech Republic in July 2013 in a TLC with S/V ratio of 105-140 m^{-1}. Growth was higher in E3, in which *C. fusca* achieved a biomass density of 11.4 g DW L^{-1} whereas it was 1.22 and 0.94 g DW L^{-1} in E1 and E2 (summer and autumn, respectively). Photosynthetic activity increased in all experiments although it was higher in E3 ($rETR_{max}$=345 µmol e^- m^{-2} s^{-1}). Final biomass composition was similar in E1 and E3, showing high lipid and protein content (~35-37% and 30-34%, respectively) and lower starch content (16-19% DW). Lower accumulation was found in E2, ~23, 28 and 13% DW for lipid, protein and starch content, respectively. Biomass productivity was higher in E3 (3.65 g L^{-1} d^{-1}). In E1 and E3, lipid productivity (0.09 and 0.66 g L^{-1} d^{-1}, respectively) was higher than that of starch and protein (~0.03-0.04 and 0.43-0.47 g L^{-1} d^{-1}). On the contrary, in E2 the three productivities were similar (0.02-0.03 g L^{-1} d^{-1}). The S/V ratio of the system was a key factor to obtain high biomass and storage product productivity. This strain was able to grow outdoors, exhibiting good photosynthetic performance and accumulating high lipid and protein content. To our knowledge, the biomass, lipid and protein productivity are among the highest ever reported in outdoor conditions in open systems.

3.3 chapter

Chlorella fusca (Chlorophyta) grown in thin-layer cascades: estimation of biomass productivity by continuous monitoring of in vivo chlorophyll a fluorescence

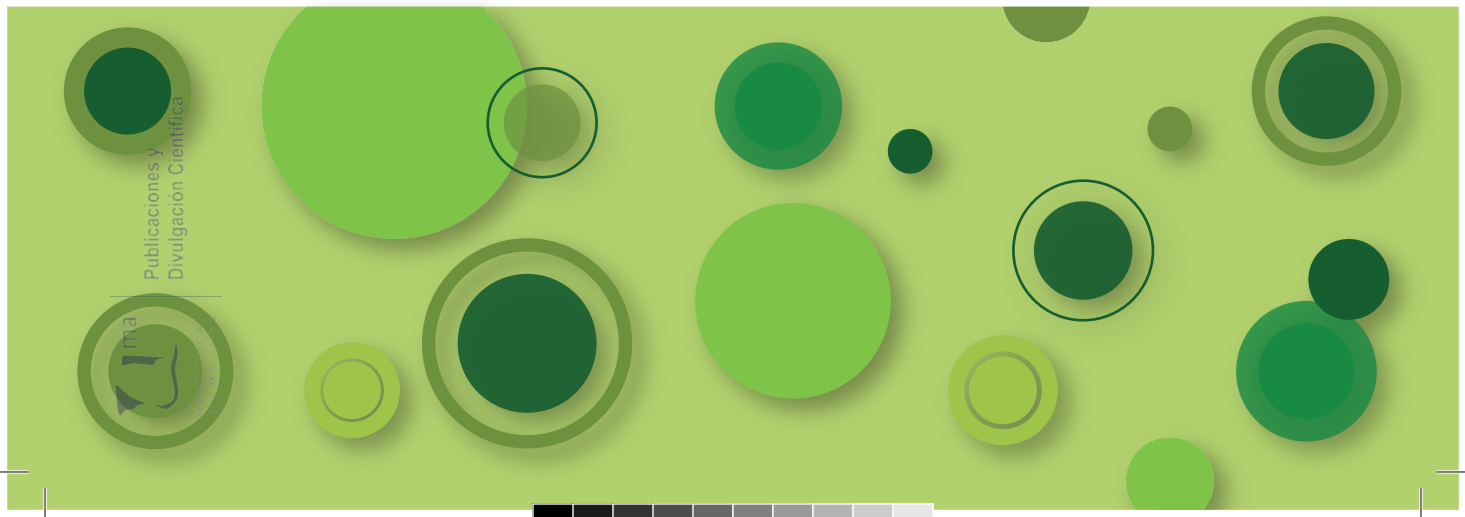
To be submitted to Algal Research (Companion Paper-B)

Abstract

In vivo chlorophyll *a* fluorescence was used to monitor the photosynthetic performance of three cultures of *Chlorella fusca* BEA1005B grown in thin-layer cascades (TLCs) in different locations and time of the year. The first (E1) and second (E2) experiments were conducted in South-Spain in July and October 2012, respectively in a TLC with S/V ratio of 20-27 m^{-1}. The third experiment (E3) was conducted in the Republic in July 2013 in a TLC with S/V ratio of 105-140 m^{-1}. Instantaneous and simultaneous measurements of incident PAR irradiance and effective quantum yield of PSII ($\Delta F/F_m'$) were recorded every 5 minutes using Junior-PAM fluorometer. From these data, daily-integrated electron transport rate (ETR) was calculated and transformed into biomass productivity according to several assumptions. Oxygen evolution rate was estimated from daily-integrated ETR assuming that the quantum requirement (QR) was 8 or 10 absorbed photons to produce a molecule of oxygen. From these, carbon assimilation rate was calculated assuming the number of mol of C produced per mol of O_2 evolved, the so-called photosynthetic quotient (PQ), which considering that nitrate was the N source ranged from 1.2 to 1.4 mol O_2/mol CO_2. Since in microalgal biotechnology the measurement of biomass productivity is usually carried out through the assay of dry weight in time, biomass productivity estimated from ETR records was compared with biomass productivity measured by differences in dry weight. The best correlation was found when QR=1/8 mol O_2 (mol photons)$^{-1}$ and PQ=1.2 mol O_2 (mol CO_2)$^{-1}$. It was found that low dense cultures (<0.15 g DW L^{-1}, s=0.90) showed lower slope (s=0.42) than high dense cultures (0.15-11.4 g DW L^{-1}), which showed estimated values only 18% lower than the measured biomass productivity closer to the measured biomass productivity. Online monitoring of *in vivo* chlorophyll fluorescence provided data with high temporal resolution that revealed essential information about the photosynthetic performance of the culture. To our knowledge, this is the first study presenting estimates of biomass productivity derived from ETR measurements in microalgal mass cultures that, in addition, agreed with measured values.

4 chapter

Chlorella fusca under stress conditions



4.1 chapter

Effect of nutrient starvation under high irradiance on lipid and starch accumulation in *Chlorella fusca* (Chlorophyta)

Celia G. Jerez, José R. Malapascua, Félix L. Figueroa and Jíri Masojídek

Marine Biotechnology, Submitted December 2014,
Resubmitted February 2015

Abstract

The effect of nitrogen and sulphur starvation under high irradiance (PAR) was studied in the green microalga *Chlorella fusca* (Chlorophyta) in order to follow lipid and/or starch accumulation. Growth, biomass composition and the changes in photosynthetic activity (*in vivo* chlorophyll *a* fluorescence) were followed in the trials. The full nutrient culture showed high biomass production and starch accumulation at Day 1, when photosynthetic activity was high. Gradual deprivation (no nutrients added) became evident when photosynthesis was significantly suppressed (Day 3 onwards), which entailed a decrease of maximum relative electron transport rate ($rETR_{max}$) and increase of non-photochemical quenching (NPQ), accompanied by the onset of lipid accumulation and decline in starch content. In N- and S-starved cultures, $rETR_{max}$ significantly decreased by Day 3, which caused a substantial drop in biomass production, cell number, biovolume and induction of lipid and starch accumulation. High starch content (45-50% of DW) was found at the initial stage in full nutrient culture and at the stationary phase in nutrient starved cultures. By the end of the trial all treatments showed high lipid content (~30% of DW). The full nutrient culture had higher biomass yield than starved treatments although starch (~0.2 g L^1 d^{-1}) and lipid (~0.15 g L^1 d^{-1}) productivities were fairly similar in all the cultures. The present results showed that biomass of *C. fusca* could be enriched (% DW) by lipids using a two-stage strategy (a nutrient replete stage followed by gradual nutrient limitation) while under either procedure, N- or S-starvation, both high lipid and starch contents could be achieved.

4.2 chapter

Synergistic effect of UV radiation and nutrient limitation on *Chlorella fusca* (Chlorophyta) cultures grown in outdoor cylindrical photobioreactors

Irene Malpartida, Celia.G. Jerez, M.D.M. Morales, Pedro Nascimiento, Isabel Freire, Joao Ezequiel, Rosa M. Rico, Eduardo Peralta, Yuly Florez, Jíri Masojídek, Roberto Abdala, Félix L. Figueroa and Enrique Navarro

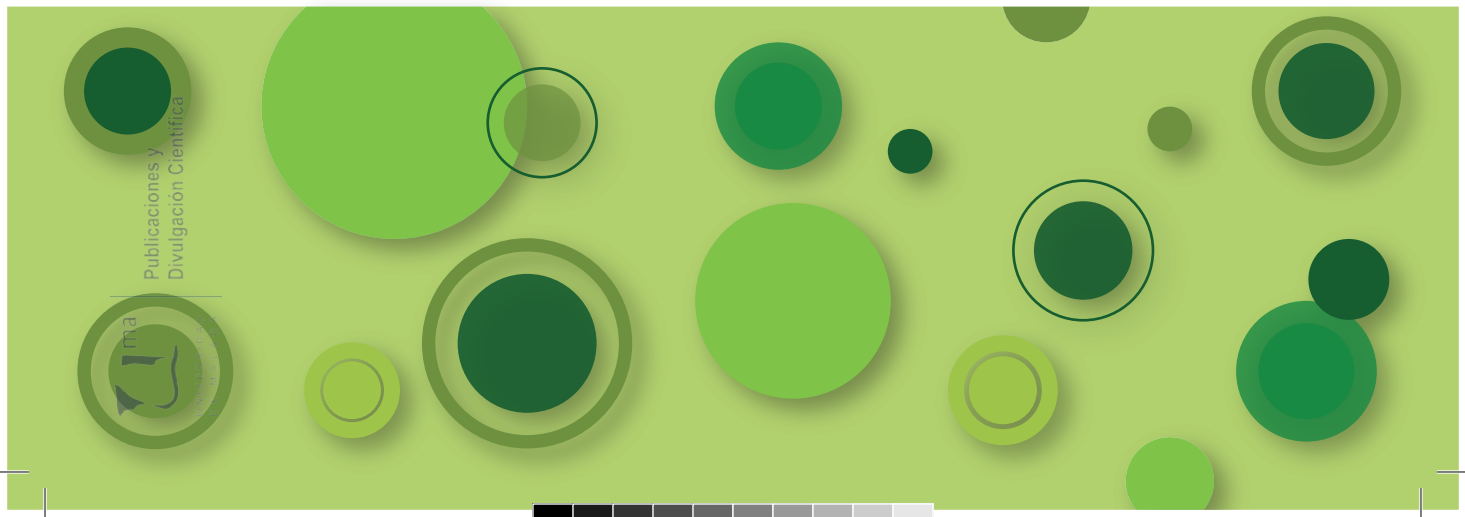
Aquatic Biology 22:1–40 (2014)

Abstract:

This study assessed the interactive effects of UVR and nutrient depletion on *Chlorella fusca* cultures on the production and accumulation of particular biomolecules. To accomplish this, algae were grown for 5 days in outdoor thin-layer cascade systems under three nutrient treatments: full nutrient, nitrogen (−N) and sulphur limitation (−S). Then, the cultures were transferred to outdoor cylindrical photobioreactors for another 5 days and exposed to full solar radiation (PAB) and solar radiation with decreased UVR (P(AB-). During the last 5 days, bio-optical properties, photosynthetic activity, pigments, biochemical composition and oxidative stress were assessed. Initially, nutrient limitation caused changes in productivity and cell number in a manner that affected biochemical composition. After 3 days, the percentage of lipids in the cultures under N limitation reached values appropriate for being used as feed or food additives or for energy applications (35% of lipid content), regardless of the light conditions. A longer exposure (5 days) resulted in interactive effects of light and nutrient regime. Specifically, PAB increased lipid content in all cases (1.3- to 2.3-fold), but particularly under S deprivation. Longer exposure to PAB also increased oxidative stress in UVR and nutrient-limited treatments (−N and −S). These results showed that the benefits expected from nutrient depletion (increase in biomolecule content e.g. lipids, carbohydrates and pigments) were modulated by the negative effects of algal UVR acclimation costs.

5 chapter

General discussion



The present world energy situation has raised the need to find alternative energy resources. Global energy demand is increasing due to rise of human population and our economy is still relies upon fossil fuels (petrol, gas and coal). Although new methods of extraction such as fracking are being used, the era of fossil fuels is coming to an end because of shortage of reserves and associated negative impacts. Microalgae meet many of the requirements necessary to be proposed as the alternative renewable source for energy, foodstuff and high-value compounds (Tredici 2010; Gouveia 2011; Draaisma et al. 2013). Microalgal production does not require arable land or drinking water since it can use non-fertile soils and reuse water from industry or domestic use. However, to achieve this goal, microalgal biotechnology needs to resolve several limitations to ensure the economical feasibility of the process. Among these limiting factors are the achievement of higher yields of biomass and its products, successful scaling-up and reducing harvesting costs. This thesis aimed to contribute to the overcoming of these constraints by:

- Assessing the potential productivity of *Chlorella fusca* in an open cultivation system still not commercially used, the thin-layer cascade, by comparing different units and defining optimal growth conditions.
- Evaluating the use of *in vivo* chlorophyll *a* fluorescence to monitor the photosynthetic performance of microalgal cultures and to obtain values of the electron transport rate that might be used to estimate the biomass productivity.
- Analyzing the photosynthetic and biochemical processes that might increase microalgal productivity and enhancing certain metabolic pathways that lead to the accumulation of products of interest such as proteins, lipids or starch.

Chlorella fusca in thin-layer cascades

To evaluate the potential of *Chlorella fusca* for biotechnological purposes, this species was cultivated outdoors in two thin-layer cascade systems with different surface to volume (S/V) ratio: a smaller unit located in Southern Spain (TLC_{ES}) with S/V ratio of 22-27 m^{-1} and a higher unit located in the Republic (TLC_{CZ}) with S/V ratio of 105-140 m^{-1}.

The smaller unit had not been previously used in any study, so first, it was conducted an experiment to describe the hydrodynamics and photosynthetic performance of the culture in the different compartments of the system (Chapter 3.1). The analysis revealed a low retention time of the culture in the illuminated area (basin and cascade) since microalgae spent more than half time of the cycle (67%) in the tank, which is considered a dark compartment due to its depth (10-15 cm). From a biotechnological point of view, this fact was one of the major drawbacks of this thin-layer cascade system for microalgal production compared to different units (Doucha and Lívanský 2009; Masojídek et al. 2011). PAM fluorometry was used to describe the photosynthetic performance of the culture in each compartment of the system. Discrete measurements of *in situ* PAR irradiance and effective quantum yield and later calculation of rETR allowed to detect the commonly known "saturation effect" in the cascade, a decrease of the $\Delta F/F_m'$ at midday that could be related to both closure of PSII reaction centers and photoinhibition processes as previously reported in microalgal cultures (Vonshak and Torzillo 2013, Tredici 2010). Photosynthetic activity also decreased at noon, mirrored as a decrease of rETR. This phenomenon is caused by the higher incident irradiance of the central day-light hours, which can exceed 2000 μmol photons m^{-2} s^{-1}, and entails that the irradiance inside the culture often surpasses that at which photosynthetic activity of microalgae usually becomes saturated (~200 μmol photons m^{-2} s^{-1}) (Burlew 1953; Tredici 2010).

Two strategies have been proposed to decrease the saturation effect to maintain photosynthetic efficiency and thus to prevent a decrease in the photosynthetic activity: firstly to operate at the highest biomass concentration

and mixing rate (Wilhelm and Jakob 2011) and secondly the design of closed photobioreactors that enhance light dilution (Tredici 2010) by increasing the S/V of the system. The increase of algal biomass entails an increase in the photosynthetic yield but consequently a decrease in the light available because of self-shading. Therefore, the productivity could decrease since it is the product of the photosynthetic yield and the absorbed photosynthetic radiation. The thin-layer cascade is among the open cultivation systems with higher light penetration and biomass productivity (Grobbelaar et al. 1995; Masojídek et al. 2011) but in spite of this, it can be affected by photoinhibition due to the high daily irradiance available at midday. Regarding optically thin cultures (~1.65 g L^{-1}) in thin-layer cascades, this thesis showed that the increase in the path length from 8 to 18 mm alleviated the photoinhibition effect observed at midday (Chapter 3.1). In addition, the increase in the optical path of the cascade entailed higher retention time of the culture in the illuminated area, what was mirrored as an increase of ~15% in the rETR. These results led to propose a strategy in which a higher optical path would be used in the cascade in the first stages of the growth period when the culture is not still dense enough to avoid photoinhibition whereas lower optical path would be suitable as biomass density increases. It should be highlighted that in cultivation systems with different compartments in which cells showed very different photosynthetic performance among each one, the retention time of the culture in each compartment should be considered to correct the rETR values measured in each one, as it was reported in Chapter 3.1.

Chlorella fusca was suitable for sustained outdoor cultivation in thin-layer cascade systems (Chapter 3.2) showing comparable growth rates (0.23-0.28 d^{-1} in the summer period) to other green microalgae cultivated outdoors in similar latitudes. The S/V ratio of the system was a key factor regarding biomass productivity and accumulation of storage compounds. It determines the amount of light that penetrates in the system per unit volume and consequently, higher S/V ratios have been related to higher volumetric productivities (Masojídek et al. 2011; Enzing et al. 2014). According to this, the highest cell weight, biomass density, $rETR_{max}$ and biomass productivity

were reached in the cultures grown in the TLC_{CZ}, a cultivation system with S/V ratio 5 times higher than that of the TLC_{ES}. Doucha and Lívanský (2006) found biomass productivities about 30 and 40% higher in summer and autumn, respectively when *Chlorella* sp. was cultured in a TLC with similar S/V ratio than that of the TLC_{CZ} but at lower latitude (Greece), what would indicate that *C. fusca* could achieve higher biomass productivities than those showed in the present study in Southern Spain but in a system with higher S/V ratio.

C. fusca also showed different light acclimation patterns between both TLC systems (Chapter 3.3). Cells cultured in the TLC_{CZ} presented typical parameters of cells exposed to high light i.e. higher $rETR_{max}$ and E_k and lower αETR than that of shade-acclimated pattern i.e. lower $rETR_{max}$ and E_k and higher αETR showed by the culture grown in the TLC_{ES}. In addition, the chlorophyll content per gram of biomass (Chapter 3.2) was higher in the experiments conducted in the TLC_{ES}, also in agreement with the shade-acclimation pattern of these cultures. It was concluded the relation of these acclimation patterns with the S/V ratio of the system (Chapter 3.2, 3.3). The TLC_{CZ} was operated so that there was no culture in the retention tank or its volume was not significant (~10% of the total volume), what would mean that practically all the culture was exposed to high irradiance in the cascade. On the contrary, in the TLC_{ES} the culture spent about half of the time in the retention tank (dark phase) (Chapter 3.1), what means that it was exposed for short periods of time to strong light in the cascade and then recovered from this stress in the tank. However, despite the light-dark cycles to which cultures were exposed in the TLC_{ES}, the low biomass density together with the high average daylight temperature, 36 and 27 °C in July and October, respectively, probably made the cells needed to dissipate more energy.

Different mechanisms for photoprotection and energy dissipation were found according to the S/V ratio of the culture system and the seasonal period. In the TLC_{ES}, the main fate of the excitation energy absorbed by PSII was Y(NPQ) and Y(NO) in July and October, respectively. Y(NPQ) has been related to regulated energy dissipation via xanthophyll cycle or pH gradient whereas Y(NO) is related to thermal energy dissipation (Klughammer and Schreiber 2008). Since in July the culture showed lower carotenoid content

per gram of biomass than in October, as García-Mendoza et al. (2002) also reported in *C. fusca*, it seems be a higher dependence of NPQ formation on the pH gradient, probably due to the higher temperature registered in July (Chapter 3.2). On the other hand, in the TLC_{CZ}, the culture showed a two-stage mechanism for energy dissipation by which it shifted from Y(NPQ), more energetically costly (Raven 2011), to Y(NO) in order to maintain the photosynthetic activity (ETR).

The $rETR_{max}$ achieved under solar irradiance (Chapter 3.3) was 75% higher in the culture grown in the TLC_{CZ} compared to those grown in the TLC_{ES}, what agreed with the difference in the S/V ratio (~80% higher in the TLC_{CZ} than in the TLC_{ES}). On the other hand, it would have been expected that the differences observed in the $rETR_{max}$ measured under solar irradiance corresponded with those obtained under actinic light (Chapter 3.2), but in the latter case the $rETR_{max}$ achieved in the TLC_{CZ} was only ~30-37% higher than that of TLC_{ES}. The comparison of rETR measured at similar irradiance under the blue actinic light provided by Junior-PAM and *in situ* under solar radiation resulted in a linear correlation (Chapter 3.3). However, *in situ* values were about two-fold higher than those obtained from RLCs (blue actinic light). The difference in the spectral quality of both light sources is obvious. The main accessory pigments present in the light harvesting antenna of green microalgae are carotenoids, which maximum absorption wavelength corresponds to blue light, and chlorophyll *b*, which absorbs both blue and red light. Thus, it is understandably that the rETR measured under blue actinic light was lower than that measured under solar radiation since in the first case there was no red light that can cause excitation of chlorophyll *b*. In addition, the ratio chlorophyll *b* to chlorophyll *a* has been reported to be an indirect indicator of the antenna size (Gordillo et al. 2001, Dubinsky 1992). In *C. fusca* this ratio was about 0.5, the same as the ratio total carotenoid to chlorophyll *b* (data not shown), what would mean a high antenna size and content of chlorophyll *b* compared to that of carotenoids. Therefore, the measurement in actinic blue light would have led to significantly underestimate the light absorption potential of PSII due to non-excitation of the red-light absorbing pigments while solar irradiance does have these excitation wavelengths.

In addition to the different S/V of both cultivation systems, differences in other variables affecting photosynthetic rate could also explain the discrepancies in biomass productivity among the two TLCs; the highest value achieved in the TLC_{CZ} was 3.65 g DW m^{-2} d^{-1} whereas in the TLC_{ES} it was only 0.15 g DW m^{-2} d^{-1}. Besides, it is not excluded that differences in the control of pH had an effect on biomass productivity. In the TLC_{ES} the pH was controlled manually between 7.5 and 8.5 with addition of pure CO_2 whereas the TLC_{CZ} had an automatic pH control system that maintained the pH between 7.4 and 7.8. On the other hand, alternative process should have taken place in cells cultured in TLC_{ES} so that not all the energy of photochemistry led to biomass production. It has been observed that under high temperature the respiration rate increases while photosynthetic activity can decrease (Turpin et al. 1988; Geider and Osborne 1989; Kliphuis et al. 2011).

Therefore, the highest daily temperatures registered in the TLC_{ES} in July might have a negative effect on biomass productivity. Moreover, the energy dissipation mechanism via xanthophyll cycle observed in July in the TLC_{ES} would used energy that otherwise would have been invested in growth. Consequently, biomass productivity estimated from ETR values was lowly correlated to the measured biomass productivity in those experiments conducted in the TLC_{ES} whereas data from TLC_{CZ} were highly correlated (Chapter 3.3).

Monitoring of chlorophyll *a* fluorescence

The use of PAM fluorometry in microalgal biotechnology has great potential for the evaluation of the photosynthetic performance of outdoor cultures (Torzillo et al.. 1998; Lippemeier et al.. 2001; Masojídek et al. 2011). In Chapter 3.3, it has been demonstrated the suitability of the use of PAM fluorometry to obtain instantaneous values of *in situ* PAR irradiance, effective quantum yield and ETR (derived from these data) at high temporal resolution (every 5 minutes) continuously during all the culture period. With this information, daily cycles of incident irradiance inside the culture, photochemical efficiency ($\Delta F/F_m'$) and photosynthetic activity (ETR) could be

obtained and related to changes in the environmental conditions i.e solar irradiance, temperature, hydrodynamics among others. Monitoring of these variables revealed important physiological information of processes occurring in PSII that otherwise is difficult to obtain. This information can be completed if nighttime processes are also monitored (Figueroa et al. 2014a) or if an incubation chamber is installed in-line so that processes after dark adaptation could be evaluated as reported by Masojídek et al. (2011).

Monitoring of chlorophyll *a* fluorescence has recently gained great interest since some studies reported the correlation of ETR with photosynthetic productivity measured by oxygen evolution or carbon assimilation methods in different conditions and species (Kromkamp et al. 2009; Obata et al. 2009; Blache et al. 2011). According to several authors (Figueroa et al. 2003; Kromkamp and Forster 2003), ETR values obtained from *in vivo* chlorophyll *a* fluorescence PAM fluorometry) can be used as a proxy of photosynthetic production. However, for this purpose, it is a requisite to consider light absorption, what means determining absolute rates instead of the commonly reported relative rates. In microalgal biotechnology studies, absolute ETR values are rarely reported mainly due to the lack of information on the convenience of the determination of absolute rates. Nevertheless, when such knowledge exists, the need of an appropriate method to measure light absorption in microalgal mass cultures arises. In oceanographic studies, light absorption is commonly measured spectrophotometrically by concentrating phytoplankton according to the quantitative filter technique (QFT) (Cleveland and Weidemann 1993; Arbones et al. 1996; Lohrenz 2000). It is worth to mention the incoherence of using this method in microalgal cultures, which are various orders of magnitude thicker than phytoplankton samples and thus, would require strong dilution before filtration. Therefore, in Chapter 2 it is proposed an appropriate method to measure light absorption in microalgal mass cultures. This method is based in the measurement of the absorptance of the microalgal culture exposed to an halogen lamp (similar light quality to that of spectrophotometers) in a thin layer using a set-up adapted from measurements reported in macroalgae (Longstaff et al. 2002; Gordillo et al. 2003a; Figueroa et al. 2009b; Enríquez and Borowitzka 2010).

To evaluate the suitability of the method, the absorptance was measured in laboratory and outdoor cultures of *C. fusca* and compared with values of the absorption coefficient of the same samples according to the QFT method. A good linear correlation was found for both laboratory and outdoor samples between filtered (QFT) and suspension (absorptance) measurements. Nevertheless, light absorption in filtered samples was two-fold higher than in the cell suspension, as previously discussed, mainly due to the amplification effect of filtered samples although the β correction is widely accepted (Mitchell and Kiefer 1984; Arbones et al. 1996; Lohrenz 2000) Both light absorption methods were later used to calculate the absolute daily-integrated ETR from high-temporal resolution data provided by the monitoring of the irradiance and the *in vivo* chlorophyll *a* fluorescence (Chapter 3.3).

Traditionally, ETR values have been used to estimate the rate of oxygen evolution or carbon fixation to later correlate them with the corresponding gas measurement (see summary table in Suggett et al. 2010). Going one step further, this Thesis aimed to convert *in situ* ETR to biomass productivity and correlate it with the biomass productivity measured by difference in dry weight. The estimation of biomass productivity from daily-integrated ETR values compared well with traditional measurements of biomass productivity conducted by daily difference in dry weight. Several values of quantum requirement (QR, mol e^- per mol O_2) and photosynthetic quotient (PQ, mol CO_2 per mol O_2) previously used by other authors (Ley and Mauzerall 1982; Laws 1991; Flameling and Kromkamp 1998; Figueroa et al. 2003; Kromkamp et al. 2008; Kromkamp et al. 2009; Tredici 2010) were assumed. The best correlation was found in high dense cultures (TLC_{CZ}) when QR=8 mol e^- per mol O_2 and PQ=1.2 mol CO_2 per mol O_2, being the estimated biomass productivity (g DW m^{-3} d^{-1}) very close to the measured values, only ~10% lower. Thus, in this case the quantum requirement for carbon fixation was ~9.6 mol e^-. Compared to other studies that reported values higher than 50 mol e^-/mol C (Lawrenz et al. 2013), this value is close to what should be obtained under optimal growth conditions (4-6 mol e^-/mol C), where net and gross primary productivity are similar (Genty et al. 1989). Under natural conditions net primary productivity (NPP) differs from gross primary

productivity (GPP) by a factor of 2 to 2.5 (Halsey et al. 2010; Halsey et al. 2011), but under optimal growth conditions, usually achieved in controlled systems, both productivities should be similar, meaning that biomass losses, mainly associated to nighttime respiration, are minimized. It is widely accepted that productivity estimated from ETR values accounts for GPP since it provides a direct measure of the photochemical operation of PSII (Suggett et al. 2010). On the other hand, the measurement of biomass productivity by daily difference (24-h) in dry weight would give NPP values. Therefore, it can be assumed that in Chapter 3.3 estimated GPP was correlated to measured NPP, showing both similar values as it should be expected under optimal growth conditions.

On the contrary, estimated biomass productivity of low dense cultures (TLC_{ES}) was significantly lower than measured values. In this culture system, uncoupling of electron transport and growth has been demonstrated (Chapter 3.2, 3.3) as shown by their shade acclimation pattern and high yield of regulated mechanisms for energy dissipation, Y(NPQ) among other processes already discussed. In addition, other processes have been suggested to act as uncouplers. Alternative electron cycling can act as mechanism that compliments other photoprotective processes (Ralph et al. 2010). Moreover, it has been observed that under stress conditions, *Chlorella* and other green microalgae can release organic carbon compounds to the medium (Hellebust 1958; Malinsky-Rushansky and Legrand 1996; Gordillo et al. 2003b; Watanabe et al. 2005). This process has been proposed as an alternative sink when fixed carbon cannot be invested in growth or stored therefore uncoupling photosynthetic activity from growth (Dubinsky and Berman-Frank 2001). The release of organic carbon under stress conditions can be up to 50% of the carbon previously assimilated (Carrillo et al. 2002; Korbee et al. 2012). According to this, the uncoupling between growth and photosynthesis evidenced by a poor correlation between estimated and measured productivity could have been affected by excretion of carbon compounds since this fraction was not considered. To estimate the biomass productivity from ETR values, the total content of internal C was used and therefore, the fraction of dissolved organic carbon (DOC) was omitted. On the other hand,

in low dense cultures, estimated values of biomass productivity would be closer to the measured ones if a lower quantum requirement for carbon fixation had been assumed (<9.6 mol e^-/mol CO_2). Lawrenz et al. (2013) established that quantum requirements lower than 5 mol e^-/mol CO_2 are more difficult to reconcile with physiological processes and would indicate the magnitude of possible methodological discrepancies, that in the present study would be related to the low biomass density of the cultures.

The results presented in Chapter 3.3 showed that it is possible to predict biomass productivity from chlorophyll *a* fluorescence measurements under optimal growth conditions, opening the way to elucidate the effect of changing environmental conditions and different stressors on the photosynthetic performance, growth and biomass productivity of outdoor microalgal cultures. In addition, chlorophyll *a* fluorescence was revealed as a very useful technique to have immediate evidence of the health of the culture, what would make possible taking decisions in case of sudden adverse conditions to avoid culture loss. Furthermore, having continuous recordings of the photosynthetic performance would allow the possibility of photo-optimize the cultivation regime in order to always obtain the highest productivity according to weather conditions. The experiments conducted in a thin-layer cascade unit that due to its hydrodynamics characteristics has low potential for biomass production (TLC_{ES}), allowed for obtaining valuable information to better understand key physiological process such as dissipation energy mechanisms, decoupling process or alternative metabolic routes that are essential to achieve high productivities in microalgal biotechnology. However, further studies aiming to obtain values of quantum requirement and photosynthetic quotient under different culture conditions are still necessary to increase the accuracy of estimates of biomass productivity from ETR values.

Effect of stress conditions on *Chlorella fusca*

Chlorella fusca showed great potential for outdoor growth in thin-layer cascades (Chapter 3.2) and higher capacity to accumulate proteins, lipids and starch. In a thin-layer cascade system under solar radiation and nutrient

replete conditions, the biomass composition was 37% lipids, 16% starch and 35% proteins in thin-layer cascade with high S/V ratio (TLC_{CZ}). When the S/V ratio was lower (TLC_{ES}), lipid, starch and protein content was higher in the summer period (31, 19 and 30%, respectively) compared to that achieved in autumn (23% lipid, 13% starch and 28% protein).

According to the high capacity showed by *C. fusca* to accumulate proteins, lipids and starch under "optimal" growth conditions, next step was aimed to evaluate if accumulation or productivity of these products could be enhanced under stress conditions. Many studies have reported the effect of UV radiation or nutrient starvation or limitation on the accumulation and production of storage compounds (Sharma et al. 2012; Procházková et al. 2013). However, several of them evaluated the combined effect of different stressors (Brown et al. 1996; Carvalho and Malcata 2005; Widjaja et al. 2009; Converti et al. 2009; Forján et al. 2011; Yeh and Chang 2012; Srinivas and Ochs 2012). The combination of high irradiance and nutrient starvation (nitrogen or sulphur) (Chapter 4.1) and limitation of these nutrients under full solar radiation (Chapter 4.2) revealed that the accumulation and productivity of these products could be enhanced under certain culture conditions. Under continuous high irradiance and nitrogen or sulphur starvation, *C. fusca* preferentially accumulated starch as storage compound although the accumulation of lipids was noteworthy (Chapter 4.1). Maximum lipid and starch productivities were achieved after three days of exposure to high irradiance in all nutrient treatments (replete medium and starved conditions), what made possible to suggest different cultivation strategies according to different biochemical composition of biomass. *C. fusca* would be enriched in lipids if culture in replete medium or in starch if sulphur starvation is applied. On the contrary, a balanced composition of lipid and starch would be obtained under nitrogen starvation. Although these results gave important information on the degree of flexibility of the metabolism of *C. fusca* under stress conditions, the experiment was conducted under artificial light and laboratory controlled conditions. In outdoor culture in thin-layer cascades, the culture is subjected to several conditions that are difficult to be reproduced in laboratory, being the spectral quality of solar irradiance i.e. ratio of

UVB:UVA:PAR one of the hardest to simulate (Aphalo et al. 2012). The effects of UVR on biomass productivity and composition are widely known (Buma et al. 1996; Germ et al. 2002; Beardall and Raven 2004; Gu et al. 2005; Figueroa et al. 2009a; Adarme-Vega et al. 2012). Therefore, the effect of UVR under nutrient limitation was tested in *C. fusca* cultures under solar irradiance (Chapter 4.2). The combined effect of nutrient limitation and full solar radiation enhanced lipid accumulation. After the third day of cultivation, lipid content increased in sulphur-limited cultures and maintained under nitrogen-limitation whereas it only maintained or decreased, respectively, under reduced UV radiation. Thus, outdoor experiments at small scale are of great utility to simultaneously compare the effect of different stressors under solar radiation. In addition, these data could be used as a proxy of the effect of UVR and nutrient limitation in outdoor cultures in higher scale culture systems (see table 4.1.).

Protein content has long been estimated from total nitrogen using a factor of 6.25 based on the assumption that proteins contain 16% nitrogen and that the concentration of nonproteinaceous nitrogen (NPN) is negligible (Jones 1931). However, this factor tend to overestimate the protein content of microalgae since they have significant amounts of NPN such as free amino acids, DNA and chlorophyll and therefore deviate from the established N-content of 16% in total protein (Mossé 1990). Lourenço et al. (2004) determined the content of total NPN of different microalgal species and proposed specific factors for each species and growth phase. The mentioned study showed a NPN content in *Chlorella minutissima* between 29.9 and 35.3% varying according to the growth phase of the culture. Therefore, to estimate the protein content more accurately in the present Thesis it was used the average nitrogen-to-protein conversion factor across all growth phases given for *Chlorella*-type species by Lourenço et al. (2004), which was 4.22 instead of the 6.25 traditionally used.

Future perspectives of microalgal biotechnology

In the recent years, there has been a "green bubble" fostered by numerous reports claiming that biodiesel from microalgae was the alternative energy source that would help to meet the targets for reducing greenhouse gas emissions. Even big energy companies invested in research and advertising about the immense advantages of biodiesel from microalgae. Therefore, if now people know something about microalgae, it will be probably because of biodiesel. Those reports were based on percentages of oil content in microalgae sometimes between 60-80% of dry biomass (Banerjee et al. 2002; Meng et al. 2009; Verma et al. 2010; Sobczuk and Chisti 2010), that although achievable, are not always associated with high lipid productivity or to high content of suitable oils for biodiesel production. The selected strain should be carefully tested not only focusing in its capacity for lipid accumulation. In addition, it was also claimed that microalgae have higher photosynthetic efficiency than higher plants, what later was also found to be wrong (Tredici 2010; Norsker et al. 2011).

Several studies concluded that cultivating microalgae only for biodiesel production is not economically feasible. One of the most cited reports concerning economics of biodiesel production is that by Chisti (2007). The author estimated that a liter of microalgal oil would cost 2.80$ if an oil content of 30% of dry weight is assumed in biomass cultivated in photobioreactors and that this price should be reduced to 0.48$ per liter to ensure competitiveness with petroleum. However, inputs related to costs of building the facility, nutrient or water requirement and power consumption were not considered. On the contrary, these essential inputs were included by Acién et al. (2012), who calculated the production costs of biomass based on experimental data of *Scenedesmus almeriensis* cultured in tubular photobioreactors. The production cost was 69€/kg although it was significantly reduced by simplification of the technology, increase of the production capacity and decrease of power consumption to 12.6€/kg. However, this price is still very high. Therefore, based on a precise feasibility analysis and in order to achieve the minimum costs, the authors proposed

reducing the costs of photobioreactors, reducing nutrient and CO_2 inputs by using wastewater and flue gases to decrease the costs to 1.8 €/kg. In this study, Acién et al. (2012) agreed with Norsker et al. (2011) in that the key strategies to reduce the production costs are selecting an appropriate location that allows maximum daily and annual solar irradiance; improving photosynthetic efficiency of microalgae by optimizing and controlling culture conditions and using free sources for nutrients and CO_2. In their analysis, Norsker et al. (2011) showed minimum production costs of 1.28, 0.70 and 0.68 €/kg for raceway ponds, tubular photobioreactors and flat panel photobioreactors, respectively, estimated for the island of Bonaire in the Caribbean. Nevertheless, a cost of ~0.70 € per kilogram of dried produced biomass is not yet economically feasible considering a value of biodiesel of 0.5 €/L (Chisti 2007; Wijffels et al.. 2010).

To date, there are no enough data available to make such economical analysis applied to thin-layer cascade. However, as noticed by Norsker et al. (2011, supplementary material) and regarding the biomass productivity values showed in the present study, it should be highlighted that in terms of biomass productivity and operating costs, the cost of biomass production in thin-layer cascade systems should resemble more to those of closed photobioreactors than to those of raceway ponds. In addition, it has been demonstrated that daily net productivity of *Chlorella* sp. cultured in thin-layer cascades was not affected by supplying CO_2 from flue gas containing ~5.5-7.5% CO_2, ~8-9.5% O_2, ~20-30% NO_X and ~1-3.5% CO. Furthermore, it has also been demonstrated the capacity of *C. fusca* to grow in different wastewater effluents, secondary-treated wastewater (Gómez et al. 2013) and lecheate resulting from the centrifugation of the sludge (Peralta López 2014), in which it was observed an increase in lipid accumulation and a clean effluent coming out. Although neither of the studies reported the starch content and productivity, a significant increase in both of them would have been expected as shown in Chapter 4 under nutrient stress conditions. In both studies a clean effluent was obtained. These results emphasize the that the use of flue gas and wastewater effluents as CO_2 and nutrient sources, respectively would

have not only economical but also environmental benefits, what in turn might contribute to the progress of microalgal biotechnology.

The previous estimations do not mean that biodiesel from microalgae is not possible, but the idea of biorefining microalgal biomass gets even more realistic. Biofiltration of wastewater or effluents from different sources as sewage (urban) or from farms (pig slurry, aquaculture) and the use of the available nutrients of these media to produce algal biomass is one of the best strategies to decrease the cost of algal production. The algal biomass can be biorefined (Fig. 5.1) to obtain high value compounds for medical, cosmetics, food and feed uses. The rest of the biomass could be used for energetic purposes since microalgae not only offer the possibility of producing biodiesel as energy source but also other types of biofuels such as bioethanol, biogas or hydrogen can be obtained.

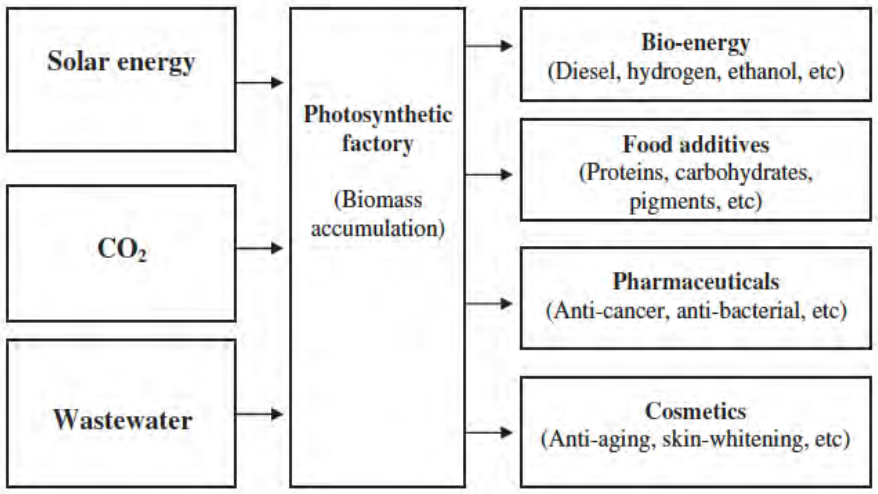


Figure 5.1 Schematic description of photosynthetic conversion of CO_2, solar energy, and wastewater into a variety of valuable end products (e.g., bioenergy, food additives,pharmaceuticals, and cosmetics) by using microalgae.

Wijffels et al. (2010) looked at the possibility of refining algal biomass into different products and analyzed the total value of biomass. They did not assume a combination of high-value products in niche markets because the market volumes of these products and biodiesel are incompatible, so they assumed biorefining of algal biomass into products for bulk markets according to the functionality of the products. Using the estimated prices of the products reported by Wijffels et al. (2010) and according to the biochemical composition of *C. fusca* grown in a thin-layer cascade of S/V= 105-140 m^{-1} in a Central European location (Chapter 3.2), the biorefinery analysis would be as subsequently detailed. *C. fusca* showed a biomass composition consisting of ~35% proteins, ~35% lipids and ~18% starch.

The protein fraction would be used as high-protein animal feed with an estimated value of 0.75€/kg whereas the starch value would be 0.6€/kg to be used as bioethanol. Although in the present thesis the fatty acid profile has not been assessed, it has been reported to be quite constant in species of the genera *Chlorella* (Petkov and Garcia 2007), being characterized by the presence of palmitic, oleic, linoleic and linolenic acids as the main fatty acids. Pohl et al. (1971) analyzed the fatty acid composition of *C. fusca*: palmitic acid (C16:0, 21.2%), oleic acid (C18:1, 25.4%), linoleic acid (C18:2, 17.4%) and linolenic acid (C18:3, 21.2%) being this composition later confirmed by Weber et al. (1989). This fatty acid composition is suitable for biodiesel production since fatty acids of 14-18 carbons are desired (Huang et al. 2010). However, the European Standard EN 14214 limits the content of linolenic acid to 12% for a quality biodiesel. Therefore, not all the lipid fraction would be used for biodiesel production (78.8% lipids, 0.5€/kg) but linolenic acid would be used as feedstock for the chemical industry since it is an omega-3 essential fatty acid (21.2% lipids, 2€/kg). Besides these main products, costs of biomass production could still be reduced taking advantage of the biorefinery process. *C. fusca* contained 8% of nitrogen and removal of nitrogen in wastewater treatment approximately costs 2€/kg. Thus, the use of wastewater would entail a cost reduction of 0.16€/kg.

If the value of all these products is added up, the total value of microalgal biomass would be 0.86€/kg. The aim of these estimations is just to

illustrate that biorefinery of microalgae is a suitable strategy to achieve a cost-effective production of microalgal biomass. However, it should be highlighted that following the analysis by Wijffels et al. (2010), the biorefining of the different microalgal fractions has not been considered. The extraction and separation is one of the major challenges of the biorefinery concept since the different fractions should be separated without damaging any of the products (Molina Grima et al. 2003; Gouveia 2011; Vanthoor-Koopmans et al. 2013). Mild, inexpensive and low energy consumption processes that are applicable for a variety of end products at large quantities should be investigated but, as Vanthoor-Koopmans et al. (2013) extensively described, new technological advances are being developed. Novel extraction techniques such as supercritical fluid extraction or assisted extraction by microwave, ultrasound or enzymes currently used in the food and pharmaceutical industries are strongly recommended (Michalak and Chojnacka 2014) since they allow obtaining products in a solvent-free environment that will be safe to plants, animals and humans. The use of these techniques in microalgal biotechnology would allow production and commercialization of high-value products in the food, nutraceutical and cosmetics industries.

Life cycle analysis (LCA) attempts to quantify the environmental impact of a selected process and it is usually used to find the directions towards which technology should develop to improve sustainability. As microalgae production systems are still at an early stage of development, it relies on extrapolation of laboratory or pilot-scale data and usually LCA information is limited and protected by private agreements (Barbosa and Wijffels 2013). Most of LCA applied to microalgal production agree that the environmental impacts most relevant to microalgal biorefinery include water consumption, eutrophication and global warming potential and land use (Lardon et al. 2009, Campbell et al. 2010, Brentner et al. 2011, Yang et al. 2011). On the other hand, the key points to improve economical feasibility and sustainability would be: better knowledge of microalgal biology, availability of genome-based metabolic flux models, improvement of culture system design and operation and careful strain selection.

Microalgal biotechnology presents several obstacles that need to be overcome to become a real alternative that contributes to satisfy the global demand for energy, food and non-food commodities. It might not be the absolute solution, but it can definitely help to decrease environmental impacts such as degradation of fertile soils, impoverishment of freshwater resources and endanger of natural ecosystems. Fortunately, solutions for biological and engineering limitations can be achieved, as there are no practical reasons that impede an overcoming associated to more research effort. The potential of microalgae for energy, food and non-food commodities production could be a reality, only time is required to develop the necessary technology to achieve it.

Conclusions

1. The absorptance method currently used in macroalgae was adapted and successfully used for the measurement of the absorptance of *C. fusca* cultures and later calculation of ETR values. A linear relation between the absorbed quanta of cells retained in a filter, AbQ_f, and that of the cell suspension, AbQ_s, was obtained (AbQ_f=1.98 AbQ_s; R^2=0.99, $p<0.01$), which can be used to convert AbQ_s values into AbQ_f ones.

2. Chlorophyll *a* fluorescence was used to monitor the photosynthetic performance of *C. fusca* cultivated in thin-layer cascades revealing useful information of the photosynthetic activity of the culture according to the hydrodynamics characteristics of each compartment of the system. The increase of optical path was a good option to avoid photoinhibition.

3. *C. fusca* showed high capacity for sustained culture and storage compounds production in thin-layer cascades, in which the S/V ratio of the system resulted to be an essential factor regarding productivity of biomass and storage products. The achieved biomass, lipid and protein productivities are among the highest reported in outdoor open systems: 3.65, 0.66 and 0.47 g L^{-1} d^{-1}, respectively.

4. Online monitoring of *in vivo* chlorophyll *a* fluorescence provided data with high temporal resolution that revealed essential information about the photosynthetic performance of the culture. Areal and volumetric biomass productivities were estimated from daily ETR values. In high-dense cultures the estimated volumetric productivity was highly correlated ($y=0.90x$, $R^2=0.94$) with measured productivity by difference in dry weight.

5. Short laboratory and outdoor controlled experiments demonstrated that accumulation and productivity of storage compounds could be enhanced in *C. fusca* under certain combination of nutrient and light stress conditions. N- or S-starvation under high irradiance enhanced lipid and starch accumulation (~30 and 45-50% of DW, respectively) whereas interactive effects of UVR and nutrient limitation increased lipid content (1.2- to 2.3-fold).

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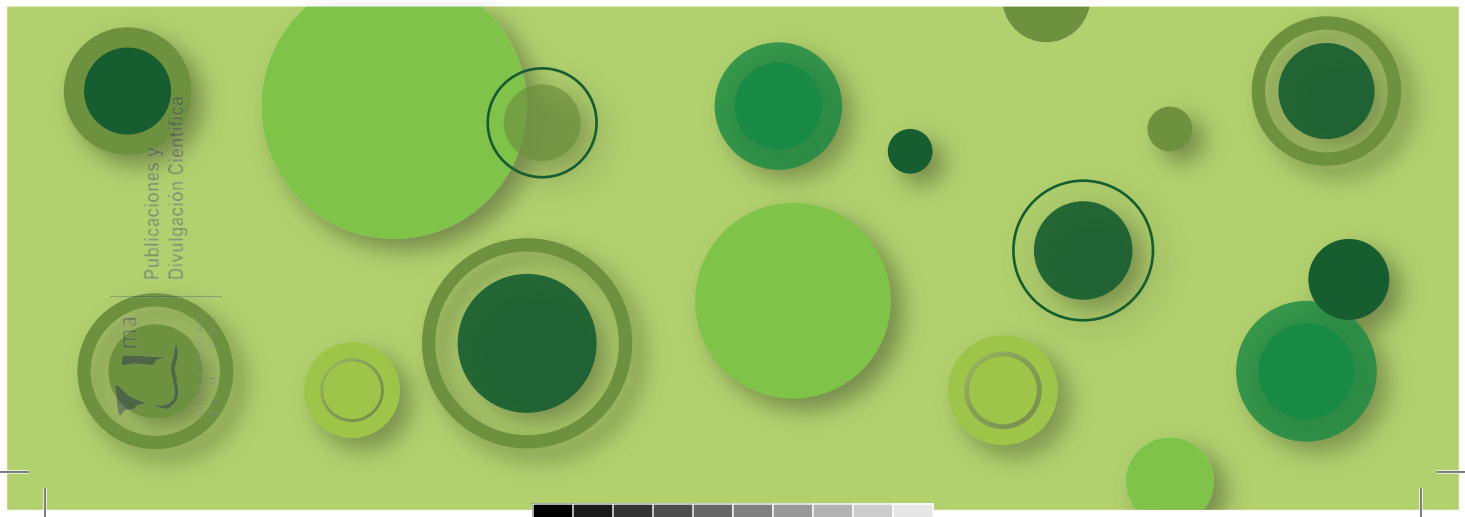

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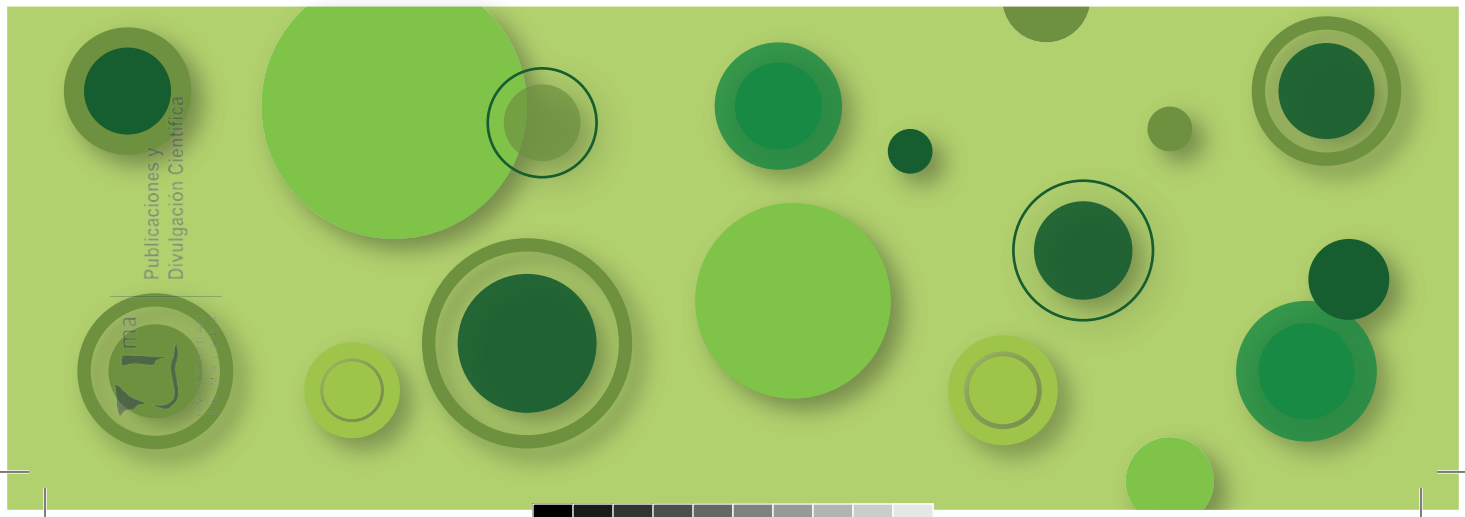
Resumen



Todavía no se tiene el conocimiento fisiológico ni la tecnología necesaria para aprovechar las potenciales ventajas de las microalgas para producir energía y nuevos productos alimentarios y de diversa índole. Esta Tesis Doctoral tiene como objetivo contribuir a superar los problemas que obstaculizan el escalado de los cultivos de microalgas ampliando la información relacionada con la actividad fotosintética y procesos bioquímicos de los cultivos de microalgas en exterior.

Para estimar la asimilación de carbono o producción de biomasa de *Chlorella fusca* a partir de la tasa de transporte electrónico (ETR, del inglés "electron transport rate") obtenida a partir de medidas de la fluorescencia *in vivo* de la clorofila *a*, es necesario determinar la eficiencia fotosintética y la cantidad de luz absorbida. Esta última se obtiene a través de medidas de la irradiancia incidente y del coeficiente de absorción de la radiación fotosintéticamente activa (PAR, del inglés "photosynthetically active radiation"). Debido a dificultades asociadas a la determinación de la luz absorbida, el ETR es comúnmente expresado en unidades relativas (rETR), aunque esta expresión no es un buen estimador de la producción fotosintética ya que las respuestas fotobiológicas dependen de la luz absorbida. La técnica cuantitativa en filtro es ampliamente utilizada para medir la absorción de luz de células retenidas en un filtro, para posteriormente y mediante factores de corrección, relacionarla con la absorción de células en suspensión. En este trabajo se propone un método para medir la atenuación de la luz por parte de una fina capa de células en suspensión a través de un sistema con reducida dispersión de la luz. Para ello se relacionó la atenuación de la luz de cultivos de *C. fusca*, tanto en condiciones de laboratorio como en exterior, con el coeficiente de absorción de cultivos de distinta densidad celular. Se encontró una relación lineal (R^2=0.99, $p<0.01$) según la cual $AbQ_f = 1.98 \cdot AbQ_s$, siendo AbQ_f los cuantos de luz absorbidos por células retenidas en un filtro y AbQ_s los cuantos de luz absorbidos por células en suspensión ambos expresados en µmol fotones m^{-3} s^{-1}. Dicha relación puede ser utilizada para convertir valores medidos directamente en la suspensión a valores medidos en el filtro (más comúnmente encontrados en la literatura). De esta forma, este estudio

muestra la utilidad de un método para determinar la absorción de la luz de forma rápida y sencilla en cultivos de *C. fusca* utilizando un sistema de medida que favorece una baja dispersión de la luz. Además, se propone el uso de las distintas expresiones de la tasa de transporte electrónico (ETR) en función del sistema de cultivo utilizado. En sistemas de cultivo en capa fina o en fotobioreactores verticales se recomienda la expresión del ETR por unidad de área (μmol e^- m^{-2} s^{-1}) mientras que en el caso de sistemas tipo raceway o fotobioreactores tubulares la expresión por unidad de volumen (μmol e^- m^3 d^{-1}) sería más apropiada. La expresión del ETR por unidad de clorofila (μmol e^- $Chla^{-1}$ h^{-1}), llamado ETR específico, sería de aplicación en todos los sistemas de cultivo ya que representa la productividad fotosintética específica al ser la expresión de la producción por unidad de pigmento o célula.

El sistema de cultivo de cascada en capa fina (TLC, del inglés "thin-layer cascade") es un sistema abierto para el cultivo de microalgas compuesto por distintos compartimentos: un tanque se conecta por medio de una bomba y un sistema de tuberías a una superficie horizontal expuesta a la radiación solar formada por una cubeta superior y una plataforma sobre la que fluye una capa fina de cultivo. Las características lumínicas e hidrodinámicas varían entre los distintos compartimentos de forma que la actividad fotosintética puede verse influenciada por el tiempo de retención de las células en cada compartimento. Se establecieron dos condiciones con distinto espesor de cultivo en la cascada, 8 y 18 mm, con la finalidad de modificar el tiempo de retención del cultivo en los dos compartimentos principales: tanque y cascada. Se comparó la actividad fotosintética de *Chlorella fusca* en cada uno de los compartimentos en ambas condiciones. El tiempo de retención de la cascada representó un 16 y un 34% de la duración de un ciclo completo cuando el espesor del cultivo fue 8 y 18 mm respectivamente. Por el contrario, en el tanque el tiempo de retención fue mayor en ambos casos: 67 y 49% para un espesor del cultivo en la cascada de 8 y 18 mm respectivamente. Se midió la fluorescencia *in vivo* de la clorofila *a* para evaluar la actividad fotosintética a través de la medida del ETR mediante fluorometría de pulso de amplitud modulada (PAM) utilizando el fluorímetro Diving-PAM. En ambos casos, 8 y 18 mm, el rETR más alto tuvo lugar en la

cascada. El aumento del camino óptico resultó ser una buena opción para disminuir la fotoinhibición del cultivo. Además, se propone la estimación del rETR promedio del sistema ponderado con el tiempo de retención del cultivo en cada compartimento ya que refleja mejor el crecimiento promedio del cultivo en el sistema al considerar el tiempo que las células pasan en cada uno de los compartimentos. Estos resultados son útiles para la optimización del crecimiento y la actividad fotosintética de cultivos de microalgas en sistemas exteriores de cascada en capa fina con fines biotecnológicos.

Se evaluó la actividad fotosintética, composición bioquímica y productividad de tres cultivos de *Chlorella fusca* BEA1005B crecidos en sistemas de cascada en capa fina (TLCs) en diferentes localidades y épocas del año. El primer (E1) y segundo experimento se llevaron a cabo en el sur de España durante los meses de Julio y Octubre de 2012 respectivamente en un TLC con una relación entre la superficie expuesta y el volumen de cultivo (S/V) de 20-27 m^{-1}. El tercer experimento (E3) tuvo lugar en la República Checa durante el mes de Julio de 2013. *C. fusca* mostró alta capacidad para ser cultivada a largo plazo en sistemas de cascada en capa fina además de una elevada tendencia a acumular compuestos de almacenamiento como lípidos, proteínas o almidón. En estos sistemas de cultivo, la relación S/V resultó ser un factor clave relacionado con alta producción tanto de biomasa como de productos de interés. El crecimiento fue mayor en el experimento E3, en el que se alcanzó una densidad de biomasa de 11.4 g PS L^{-1} mientras que durante los experimentos E1 y E2 la densidad alcanzada fue 1.22 y 0.94 g PS L^{-1} respectivamente. La actividad fotosintética aumentó en los tres experimentos aunque fue mayor durante el experimento E3 ($rETR_{max}$=345 µmol e^- m^{-2} s^{-1}). La composición final de la biomasa fue similar en los experimentos E1 y E3, mostrando un alto contenido en lípidos y proteínas (~35-37% and 30-34%, respectivamente) y un menor contenido en almidón (16-19%). Por el contrario, durante el experimento E2 se encontró una menor acumulación de estos compuestos: ~23, 28 y 13% de lípidos, proteínas y almidón, respectivamente. La productividad de la biomasa fue mayor en el experimento E3 (3.65 g L^{-1} d^{-1}). Durante los experimentos E1 y E3, la productividad de lípidos (0.09 y 0.66 g L^{-1} d^{-1}) fue mayor que la de almidón y

proteínas (~0.03-0.04 and 0.43-0.47 g L^{-1} d^{-1}). Por el contrario, durante el experimento E2 las tres productividades fueron similares en torno a 0.02-0.03 g L^{-1} d^{-1}. La relación S/V resultó ser un factor clave para conseguir alta productividad de biomasa y compuestos de reserve. La cepa de *C. fusca* utilizada mostró una alta capacidad para el crecimiento en exterior, mostrando a su vez una alta actividad fotosintética y un elevado contenido en lípidos y proteínas. Hasta la fecha, las productividades de biomasa, lípidos y proteínas presentadas en este estudio se encuentran entre las más altas de las publicadas en exterior en sistemas de cultivo abiertos.

Además, la monitorización continua de la fluorescencia *in vivo* de la clorofila *a* aportó datos con alta resolución temporal que revelaron información esencial sobre la actividad fotosintética de los cultivos. Se tomaron medidas medidas instantáneas y simultáneas de la radiación PAR incidente y del rendimiento cuántico efectivo del fotosistema II (PSII) cada cinco minutos utilizando el fluorímetro Junior-PAM. La obtención de estos datos permitió calcular la tasa diaria integrada de transporte de electrones (ETR), que posteriormente fue convertida a valores de productividad de biomasa considerando distintas asunciones. La tasa de producción de oxígeno se estimó a partir de los valores de ETR diario asumiendo un requerimiento cuántico (QR) de 8 o 10 fotones absorbidos necesarios para producir unas molécula de oxígeno. A partir de esta tasa se calculó la tasa de fijación de carbono asumiendo que el número de moles de oxígeno necesario para fijar un mol de carbono (cociente fotosintético, PQ) toma valores entre 1.2 y 1.4, considerando que la fuente de nitrógeno de la célula fue nitrato. Ya que en biotecnología de microalgas la medida de la productividad de la biomasa es normalmente determinada a través de medidas de diferencia de peso seco, en este trabajo, la productividad de la biomasa estimada a partir de los datos de ETR diario fue comparada con la productividad de biomasa medida a partir de diferencias diarias de peso seco. La mejor relación se encontró cuando QR=1/8 mol O_2 (mol fotones)$^{-1}$ y PQ=1.2 mol O_2 (mol CO_2)$^{-1}$. Se observó que los cultivos de menor densidad (0.15-11.4 g PS L^{-1}) mostraron una menor correlación (pendiente más alejada de 1) que los cultivos densos (>11.4 g PS L^{-1}), los cuales presentaron valores estimados solo un 18%

menores que los valores medidos. Este es el primer estudio que presenta valores de productividad de la biomasa estimados a partir de medidas de ETR *in situ* que además, concuerdan con los valores de productividad de biomasa medida.

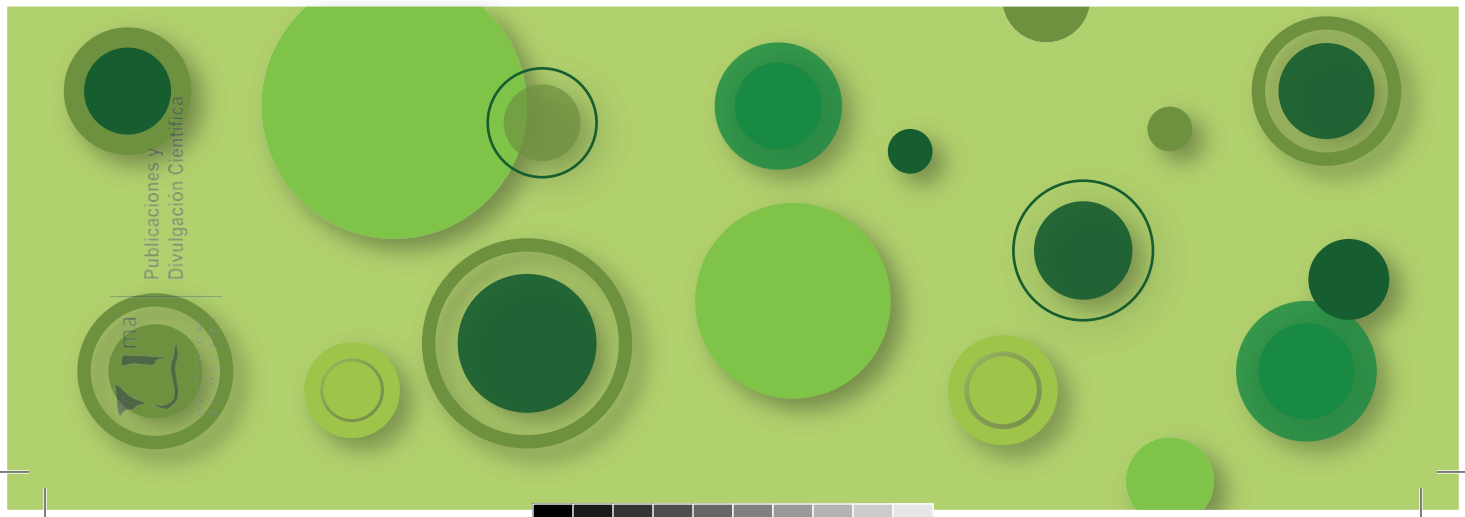
Se llevaron a cabo experimentos en condiciones controladas en laboratorio o en exterior con el objeto de evaluar el efecto combinado del estrés por nutrientes y luz en la acumulación y productividad de productos de reserva en *Chlorella fusca*. El efecto del hambre de nitrógeno o azufre bajo alta irradiancia (PAR) mostró una disminución significativa del $rETR_{max}$, lo que provocó una caída sustancial de la producción de biomasa, densidad celular, biovolumen e inducción de la acumulación de lípidos y almidón. Se encontró un alto contenido de almidón (45-50% PS) en la etapa inicial en el cultivo en medio repleto de nutrientes. Al final del experimento, todos los tratamientos mostraro nun alto contenido en lípidos (~30%). Sin embargo, el cultivo en medio repleto de nutrientes presentó una productividad de biomasa más alta aunque la productividad de almidón y lípidos fue parecida a la de los cultivos en hambre de nutrientes. Estos resultados mostraron que la biomasa de *C. fusca* puede ser enriquecida en lípidos utilizando una estrategia de cultivo en dos fases (una primera fase con condiciones de nutrientes óptima seguida de una limitación gradual). Por el contrario, tanto el hambre de nitrógeno como de azufre mostró que favorecen la acumulación de alto contenido en lípidos y almidón.

Los efectos interactivos del UVR y la limitación de nutrientes causaron cambios en la productividad y la densidad celular, lo que afectó directamente la composición bioquímica de la biomasa. Tras tres días, el porcentaje de lípidos en los cultivos en limitación de nitrógeno alcanzó valores adecuados para la utilización de la biomasa como alimento animal, nutracéutico o usos energéticos (35% lípidos). Tras cinco días de exposición a las condiciones de estrés, se observaron efectos interactivos entre el estrés por radiación UV y el estrés por limitación de nutrientes. En concreto, la presencia de radiación PAR y UV provocó el incremento del contenido de lípidos del orden del 1.3 a 2.3 veces en todos los casos, aunque en particular este aumento fue más significativo en los cultivos bajo limitación de azufre. La exposición a radiación

PAR y UV también provocó un incremento del estrés oxidativo en aquellos tratamientos sujetos a limitación de nutrientes. Estos resultados mostraron que las ventajas de la limitación de nutrientes (aumento en el contenido de biomoléculas tales como lípidos o carbohidratos) fue modulado por los efectos negativos de la aclimatación a la radiación UV.

Como conclusión, esta Tesis Doctoral demuestra que aunque la biotecnología de microalgas aún presenta algunos obstáculos relacionados con limitaciones biológicas y tecnológicas que necesitan ser superadas, las microalgas y específicamente *Chlorella fusca* presenta un gran potencial para convertirse en una alternativa real que contribuya a satisfacer la demanda global de energía y productos alimenticios y no alimenticios. Se ha comprobado que la fluorescencia *in vivo* de la clorofila *a* es una técnica muy útil para contribuir al mejor entendimiento de los procesos fisiológicos involucrados en el crecimiento y actividad fotosintética relacionados con cultivos con alta productividad. Por otro lado, el sistema de cascada en capa fina se presenta como un sistema de cultivo prometedor para una producción de microalgas viable en términos económicos y ambientales.

Publications



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Jerez CG, Malapascua JR, Masojídek J, Figueroa FL *Chlorella fusca* (Chlorophyta) grown in thin-layer cascades: estimation of biomass productivity by continuous monitoring of *in vivo* chlorophyll *a* fluorescence. Submitted to Algal Research (Companion Paper-B)

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