Cultivation of microalgae in a high irradiance area

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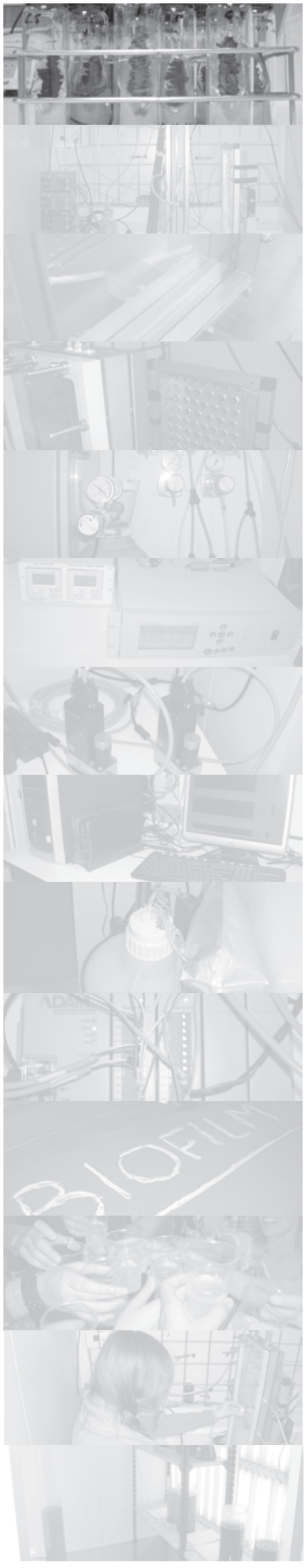
Chapter 1

Introduction and thesis outline



Microalgae

Microalgae are unicellular eukaryotic microorganisms (between 1 and 50 μm) (Figure 1.1), which can form colonies or live as individual cells. They are photoautotrophic organisms capable of using light to metabolize carbon dioxide inside energy-rich organic compounds.

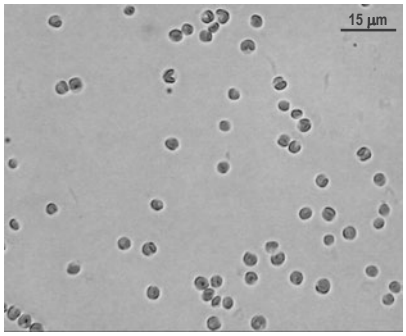


Figure 1.1: Example of a *Chlorophyceae*: the fresh water microalgae *Chlorella sorokiniana.*

The biodiversity of microalgae is enormous, two hundred thousand species has been estimated to exist but only about thirty five thousand species have been described (Norton et al. 1996). They can be found in different habitats, ranging from fresh to hyper-saline environments. Even acidic and alkaline environments can be optimal for extremophile microalgae.

Commercial interest in microalgae so far is related to their use as aquaculture feedstock and their potential as producers of specific compounds which can be used as nutraceuticals. Value-added molecules can be obtained from these phototrophic microorganisms, such as carotenoids and other vitamins and antioxidants, fatty acids, and specifically poly-unsaturated fatty acids.

However, large scale commercial production processes only date back to the 1950s, when *Chlorella* and *Arthrospira* started to be produced as an alternative nutritious source (Belasco, W., 1997; Grobbelaar, 2010; Schmidt et al. 2010; Spolaore et al., 2006).

Nowadays, microalgae are also considered a promising source of renewable energy and suitable for the fixation of carbon dioxide, although the interest in using microalgae for renewable energy already started in 1970s during the first oil crisis (Spolaore et al., 2006).

Several aspects are hindering the industrial exploitation of microalgae. Basically cost of production is too high. Construction of cultivations systems, gassing and mixing the microalgae cultures, harvesting the microalgae and supplying nutrients are still too expensive and require too much energy to make commercial production feasible. Despite these limitations, microalgae are considered one of the most attractive sources of feed, food and next-generation biofuels since they can grow in seawater, on non-arable land, and they have a higher oil and protein yield and a higher growth rate in comparison to traditional crops (Tredici, 2010).

Photosynthetic efficiency

Light energy is usually limiting the productivity of microalgae cultivations systems. For this reason it is important to use the light energy as efficiently as possible. Photosynthesis is the process by which biomass is produced from carbon dioxide and this process is driven by sunlight energy. The photosynthetic process is divided into light and dark reactions. In the light reactions, light energy is absorbed by the photosynthetic machinery, O_2 is released and chemical energy (NADPH and ATP) is produced, which is used in the dark reactions to reduce carbon dioxide to the level of carbohydrates (sugars). Minimally 8 moles of photons are needed to produce one mole of O_2 and incorporate one mole of CO_2 into carbohydrates (biomass). New microalgae biomass is built up from these carbohydrates.

In Figure 1.2, a typical photosynthesis-irradiance (PI) curve is shown. This curve shows the gross specific oxygen production rate (P_{O2}) of microalgae as a function of light intensity (PAR photon flux density, PFD). In the same curve the quantum yield of oxygen evolution (QY_{O2}) is shown, which is the amount (mol) of oxygen which can be produced per amount (mol) of PAR photons absorbed. When the level of irradiance is low there is photolimitation; the photosynthetic machinery receives less photons than it can process. At these light intensities the algal growth is light-limited and the efficiency of photosynthesis is high since the larger

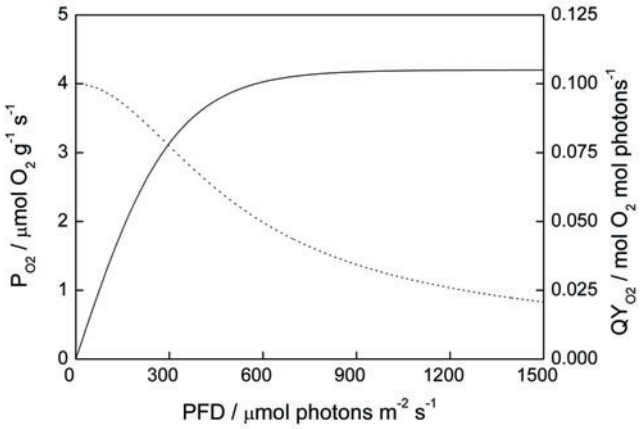


Figure 1.2: Typical PI-curve which shows the gross rate of photosynthesis (i.e. oxygen evolution, P_{O2}) as a function of light intensity (PFD) [-]. Photosynthetic efficiency is represented as the oxygen quantum yield (QY_{O2}) and also plotted versus PFD [..]. Numbers are based on the photosynthetic capacity of the green microalgae *Chlorella sorokiniana*.

part of the light energy absorbed by the algae can be allocated to biomass growth. The rate of photosynthesis (i.e. the oxygen production rate, P_{O2}) increases until the level of irradiance is saturating, where growth becomes limited by the dark reactions of photosynthesis (MacIntyre et al., 2002). At the same time the photosynthetic efficiency (i.e. the quantum yield of oxygen evolution, QY_{O2}) decreases because the rate of light absorption increases linearly with the light intensity while the rate of photosynthesis saturates. The excess of light energy absorbed will be dissipated as heat via a number of dedicated processes which are usually referred to as non-photochemical quenching (NPQ) (Huner et al., 1998; Maxwell and Jonhnson, 2000). Thanks to these processes microalgae are able to acclimate to high (sun)light levels preventing photoinhibition. But, consequently, not all the incoming light is used for growth at high light intensities and part is 'wasted' as heat (Huisman et al., 2002). This automatically leads to a drop in photosynthetic efficiency.

Except for light also temperature affects the photosynthetic activity and the growth of microalgae (Coles and Jones, 2000). Although the photochemical 'light' reactions are highly affected by irradiance and not sensitive to temperature, the biochemical 'dark' processes are temperature sensitive. At low temperatures the

metabolic rate is reduced and consequently less absorbed light energy can be converted into carbohydrates (Coles and Jones, 2000; Dauta et al., 1990; Huner et al., 1998). The slow biochemical reactions then can cause accumulation of light energy and over-excitation of the photosynthetic machinery. In this sense, suboptimal temperatures lead to an imbalance between the light absorbed through photochemistry versus the energy utilized through metabolism and algae use the same defense mechanism to over-excitation of the photosynthetic machinery (NPQ) at low temperatures as at high irradiances (Huner et al., 1998; Maxwell et al., 1994).

Outdoor production

Outdoors the algae are continuously exposed to changing irradiation and temperature conditions. In this sense, microalgae are not only exposed to the daily irradiance cycles, but also experience different irradiance levels due to seasonal changes, which are depending on the location of cultivation.

Next to irradiance, also the outdoor temperature affects productivity, as already explained. Though in most algae cultivation systems the temperature is remained at its optimal value for microalgae growth, it is also forming a major burden on production costs. When cooling in summer or heating in winter can be avoided, this will be of great benefit for the overall feasibility of the production process.

As explained above, photosynthesis is not a perfect process and the photosynthetic efficiency achieved under sunlight is much lower when compared with the maximal levels which can be achieved under limiting light levels. Losses due to reflection on the photobioreactor surface, photorespiration, cellular maintenance, will further diminish the photosynthetic efficiency. The effect of photosaturation though is the most dominant process limiting light use efficiently under high light intensities. Understanding and minimization of photosaturation is the only way to

achieve an efficient microalgae production.

Geographical areas with high irradiance conditions along the year are considered optimal for microalgae cultivation. However, evaluation of photosynthetic efficiency under real irradiance conditions is needed in order to develop an optimal production process. This thesis presents an overview on productivity and photosynthetic efficiency of *Chlorella sorokiniana* under simulated irradiance conditions. Different strategies leading to a higher photosynthetic efficiency by avoiding or reducing photosaturation were evaluated. Finally a production process for biomass and other added-value molecules with *Chlorella sorokiniana* is proposed for Huelva, south Spain, the selected study site.

Chlorella sorokiniana

In this thesis *Chlorella sorokiniana* was used as a model organism to evaluate the photobioreactor performance because of its high growth rate, robustness and the availability of comparable studies which could be used as reference. In this sense, *Chlorella* species are the most widely used microalgal strains for biomass production due to their high growth rate. The great variety of chemical components in different *Chlorella* strains makes them a good source of carbohydrates, proteins and vitamins (Matsukawa et al., 2000). Also in biofuel production they may play a role, because of their beneficial cellular composition and high growth rates (Qiao and Wang, 2009; Wahlen et al., 2011).

Specifically, *Chlorella sorokiniana* is a freshwater microalga with a high maximal specific growth rate (μ_{max} = 0.27 h^{-1}) and a mesophilic character (T_{opt} = 37 ºC). In fact it is the fastest growing eukaryotic microalgae known at the moment. Moreover, *C. sorokiniana* is tolerant to high irradiation levels (Matsukawa et al., 2000; Sorokin, 1959). Altogether this makes *C. sorokiniana* a suitable strain for cultivation in a high irradiance area.

Thesis outline

The potential for algal biomass production in a high irradiance area (Huelva, southern Spain) is studied in this thesis. The productivity and photosynthetic efficiency of *Chlorella sorokiniana* were studied under simulated outdoor irradiance conditions in a lab-scale panel photobioreactor (Figure 1.3).

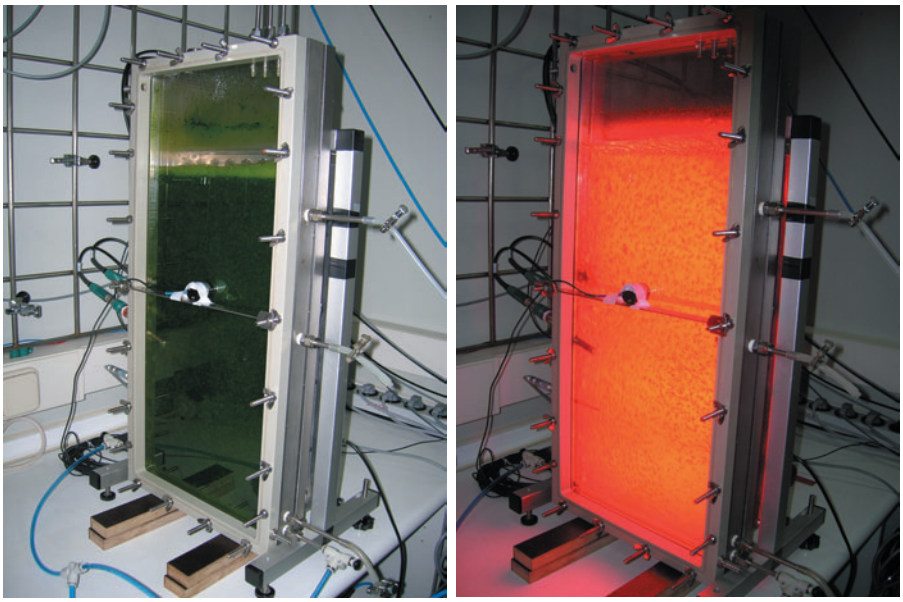


Figure 1.3: Panelar lab-scale photobioreactor used in the experiments.

The effect of extreme winter conditions is discussed in **Chapter 2**, where the strong influence of temperature on algal productivity during winter cultivation was shown. Due to the actual winter irradiance conditions, temperature control might be necessary in order to minimize photosaturation and/or photoinhibition. **Chapter 3** deals with the influence of extreme summer conditions on productivity and photosynthetic efficiency. Around noon the cells are exposed to over-saturating irradiance conditions, as high as 2100 μmol photons m^{-2} s^{-1}. *Chlorella sorokiniana*, due to its mesophilic character and resistance to high irradiance, showed a high growth rate under the conditions assayed. The photosynthetic efficiency and

productivity, even under continuous illumination, were high considering the oversaturating irradiance applied. Photoinhibition was not observed, and *C. sorokiniana* could become a good alternative for biomass production in a high irradiance area in short light path photobioreactors fully exposed to sunlight (i.e. placed horizontally).

The aim of the work described in **Chapter 4** and **Chapter 5** was to maximize the photosynthetic efficiency of *C. sorokiniana* under summer irradiance conditions. It was tested whether the photosynthetic efficiency could be increased by minimizing photosaturation by reducing the incoming light intensity on the surface of panel photobioreactors. This can be achieved by placing the light exposed panel surface of photobioreactors in a vertical orientation, for example a system of panels as shown in Figure 1.4. It was demonstrated in **Chapter 4** that this light dilution greatly enhances the photosynthetic efficiency and productivity per unit of ground area.

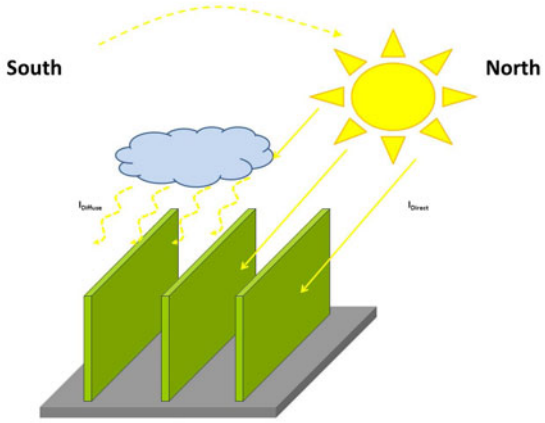
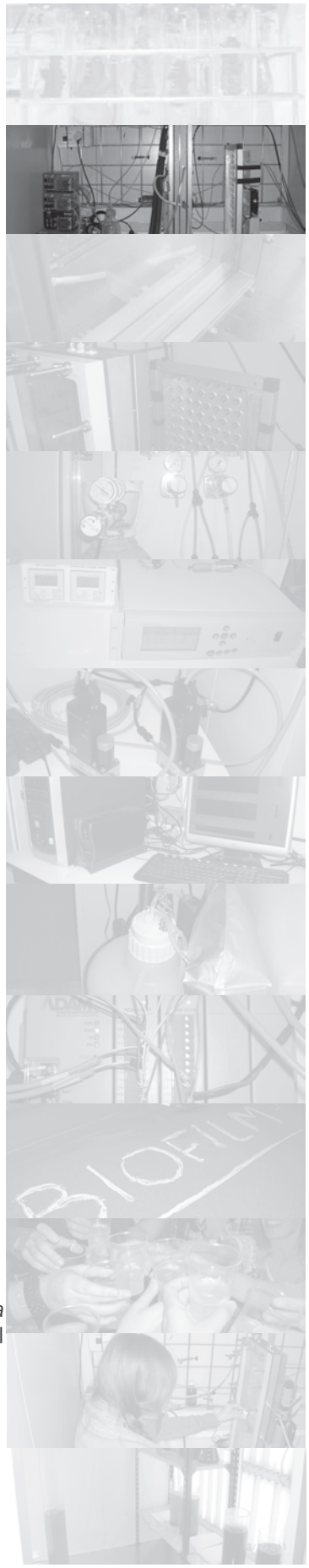


Figure 1.4: Scheme of the light dilution effect

Vertical photobioreactors therefore might be considered as optimal production systems to produce *C. sorokiniana* in Huelva when maximal areal productivity is the aim.

In **Chapter 5** the influence of luminostat operation on photosynthetic efficiency is evaluated. The biomass concentration inside the photobioreactor was continuously adapted to the irradiance conditions in order to maximize light absorption and prevent dark zones within the algae culture. Despite the initial hypothesis, luminostat operation did not lead to any improvement in productivity and photosynthetic efficiency when compared with a traditional chemostat operation. However, both operational conditions, chemostat and luminostat operation, avoided photoinhibition in a vertical photobioreactor, even under saturating irradiance conditions, yielding a high photosynthetic efficiency: 1.2 – 1.3 grams of biomass per mol of photons supplied.

Chapter 6 is a general discussion about the potential to grow microalgae in a high irradiance area. The biotechnological potential of *Chlorella sorokiniana* for commercial production of lutein in Huelva is also discussed.



Chapter 2

Chlorella sorokiniana's performance under simulated extreme winter conditions

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Abstract

High annual microalgae productivities can only be achieved if solar light is efficiently used through the different seasons. During winter time the productivity is low because of the light and temperature conditions.

The productivity and photosynthetic efficiency of *Chlorella sorokiniana* were assessed under the worst case-scenario during winter time in Huelva, south of Spain. The maximal light intensity (800 μmol m^{-2} s^{-1}) and temperature (20 ºC) during winter was supplied to a 14 mm short light-path photobioreactor. Chemostat conditions were applied and the results were compared with a temperature controlled situation at 38 ºC (optimal growth temperature for *C. sorokiniana*).

When temperature was optimal, the highest productivity was found at a dilution rate of 0.18 h^{-1} (P_v = 0.28 g Kg^{-1} h^{-1}) and the biomass yield on light energy was high ($Y_{x,E}$ = 1.2 g mol photons $supplied^{-1}$). However, at suboptimal temperature the specific growth rate of *C. sorokiniana* was surprisingly low, not being able to support continuous operation at a dilution rate higher than 0.02 h^{-1}. Therefore, also low productivity and biomass yield were found. At suboptimal temperature *C. sorokiniana* might experience the maximal winter intensity as excessive, yielding a very low photosynthetic efficiency. Temperature control and/or light dilution during winter time will clearly enhance the productivity.

Introduction

In outdoor cultivation microalgae are not only exposed to the daily irradiance cycles, but also experience different irradiance levels due to seasonal changes, which are depending on the location of cultivation (Eriksen, 2008; Jacob-Lopes et al., 2009; Zittelli et al., 2006). In order to reach high yearly productivities, light should be efficiently converted into biomass throughout the entire year. In another study we assessed the productivity of *Chlorella sorokiniana* under summer conditions (Cuaresma et al., 2009). In the current paper the performance of *C. sorokiniana* under the most extreme winter circumstances is studied.

To some extent algae can adapt their photosynthetic machinery to changing environmental conditions, this is called (photo)acclimation. It can lead to stable long-term adjustment to the new environmental condition on a phenotypic level (Huner et al., 1998; MacIntyre et al., 2002). But at high light or low temperature there is an imbalance between the light absorbed through photochemistry versus the energy utilized through metabolism, which leads to over-excitation of Photosystem II (PSII) (Huner et al., 1998). The excitation pressure of PSII can be decreased by reducing energy transfer to PSII (photoacclimation), or by dissipating the excess energy as heat (called non-photochemical quenching, NPQ). NPQ is a defense mechanism against excessive light and oxidative damage. Though the photosynthetic machinery is somehow able to acclimate to excessive light energy, all incoming light 'wasted' as heat in NPQ is not used for growth (Huisman et al., 2002) which automatically leads to a drop in photosynthetic efficiency.

Except for light, also temperature affects the photosynthetic activity and the growth of microalgae (Coles and Jones, 2000). The photochemical reactions are highly affected by irradiance and are insensitive to temperature (Raven and Geider, 1988). In contrast, the biochemical processes are temperature sensitive, and the absorbed light energy cannot be converted into carbohydrates efficiently at temperatures deviating from the optimum (Coles and Jones, 2000; Dauta et al.,

1990; Huner et al., 1998). At low temperatures the turnover of enzymes is reduced and the metabolic activity of the algae becomes lower. This leads to a lower growth rate and a reduced need of substrate. In the case of light-limited algal growth this substrate is light; therefore moderate irradiance can already be excessive at low temperatures (Coles and Jones, 2000; Dauta et al.,1990). Algae use the same defense mechanism to over-excitation of PSII at low temperatures as at high irradiances (Huner et al., 1998; Maxwell et al., 1994).

Though in most algae cultivation systems the temperature is maintained at its optimal value, it has an influence on production costs. Avoiding cooling in summer, and heating in winter, will result in a reduction in process costs.

The effect of the most extreme winter conditions on the productivity and photosynthetic efficiency of *Chlorella sorokiniana* in a short light-path flat panel photobioreactor under light limited chemostat conditions was assessed in this work. Maximal irradiance and temperature during winter time in a horizontal surface (800 µmol m^{-2} s^{-1}, 20 ºC) in Huelva (Spain, 37º15´N 6º57´W, PVGIS) were selected to grow *C. sorokiniana*. The influence of temperature control was also tested by applying the optimal growth temperature of *C. sorokiniana.*

Materials and Methods

Algae maintenance

Chlorella sorokiniana CCAP 211/8K (UTEX Culture Collection) was maintained under sterile conditions in Roux flasks containing modified M-8a medium (Cuaresma et al., 2009). The maintenance culture was kept inside a growth chamber at 25 ºC with continuous illumination (150 µmol photons m^{-2} s^{-1}). The pH was adjusted to 6.7 and the cultures were bubbled with 5%$_v$ CO_2-enriched air.

Urea ($60 \cdot 10^{-3}$ M) was used as nitrogen source during the photobioreactor experiments, and 3-fold concentrated medium was used to avoid nutrient limitation.

Experimental conditions

A flat panel reactor of 1.7 L and a light path of 14 mm was used in the experiments (Cuaresma et al., 2009). The culture was homogeneously mixed by air bubbling at a flow of 1.5 L per L of culture per minute. The pH was maintained at 6.7 by adding CO_2 via a separate mass flow controller (MFC). The gas outflow was leaving the reactor via a condenser to prevent evaporation of the culture broth. Temperature was kept constant by a water jacket connected to a cryostat. Temperature, pH and dissolved oxygen were measured online inside the reactor.

The reactor was illuminated by red light emitting diodes (LED) distributed homogeneously over the all reactor surface. To simulate winter irradiance at midday in Huelva (Spain, 37º15´N 6º57´W), data from PVGIS (see references) were used. January was selected as reference month, and the maximal irradiance on a horizontal surface was applied to the photobioreactor (800 µmol m^{-2} s^{-1}). The average PFD inside the culture chamber was calculated by measuring the PFD inside the empty reactor with a Licor SA190 quantum sensor at 45 spots distributed homogeneously over the reactor surface. Another Licor SA190 quantum sensor was placed on a reference position in the reactor outer surface, facing the lamps, to continuously measure the received PFD. A correlation factor between the average PFD inside the reactor and the reference PFD could be calculated. With this correlation factor, the PFD was automatically adapted to the desired intensity inside the photobioreactor.

After inoculation, batch cultivation was needed to adapt the microalgae to the light conditions. When biomass density was around 2 g Kg^{-1} (approx. 2 g L^{-1}) chemostat conditions were applied increasing the dilution rate stepwise. At suboptimal growth temperature adaptation first to the temperature, and then to

the dilution rate was needed.

During the steady state 4 samples were taken daily from the outflow when operating at optimal growth temperature. Due to the low dilution rate supported at suboptimal growth temperature and the technical pump limitations to supply the corresponding flow rate, the dilution was applied in 2-hour cycles of 50 minutes dilution. In order to have reproducible data, 3 samples were taken every day at the same time, corresponding with the end of a dilution cycle.

Biomass concentration

Dry weight was determined by filtration through glass microfiber filters (0.7 µm pore size). Filters were pre-washed with pre-filtered demineralized water and after filtering the samples they were washed again to remove salts from the filter retentate. After drying for minimal 16 hours at 80 ºC, and cooling to room temperature for minimal 2 hours in a desiccator, the weight of the full filters was determined. Dry weight was determined by differential weight in g Kg^{-1} (Cuaresma et al., 2009).

PSII maximum quantum yield: biomass viability

Maximum PSII quantum yield (F_v/F_m) was determined by pulse amplitude modulation (PAM) fluorometry with the saturating-pulse technique. The measurement was done by adapting the samples to dark conditions first during 15 minutes, to open all reaction centers and avoid measuring non-photochemical quenching. A chlorophyll fluorometer (PAM-210, Walz, Germany) was used. The measuring light (0.04 µmol photons m^{-2} s^{-1} was used to measure the zero fluorescence level (F_0) and a saturating light pulse (1850 µmol photons m^{-2} s^{-1}) was applied to measure the maximum fluorescence (F_m). The yield was

calculated by difference between the background fluorescence and the maximal fluorescence after applying a pulse of saturating light ($(F_m-F_0)/F_m = F_v/F_m$) (Maxwell and Johnson, 2000).

Chlorophyll and carotenoids

The chlorophyll and carotenoids content was determined by methanol extraction and spectrophotometry. After a centrifugation step, methanol was added to the pellet and the mixture was placed in an ultrasound bath to disregard the pellet. Subsequently, incubation at 60 ºC first and then at 0 ºC took place to break the cells. After another centrifugation step the supernatant was collected and analyzed by UV/Visible spectrophotometry. Modified Arnon's equations (Liechtenthaler, 1987) were used to calculate the chlorophyll and carotenoids concentrations in the extracts:

$$Chl_a = \left(16.72 \cdot A_{665} - 9.16 \cdot A_{652}\right) \cdot \text{ dilution factor} \qquad [\text{mg L}^{-1}]$$

$$Chl_b = \left(34.09 \cdot A_{652} - 15.28 \cdot A_{665}\right) \cdot \text{ dilution factor} \qquad [\text{mg L}^{-1}]$$

$$Chl_{tot} = Chl_a + Chl_b \qquad [\text{mg L}^{-1}]$$

$$\text{Car}_{tot} = \frac{\text{dilution factor} \cdot 1000 \cdot A_{470} - 1.63 \cdot Chl_a - 104.96 \cdot Chl_b}{221} \qquad [\text{mg L}^{-1}]$$

The cellular content of chlorophyll and carotenoids were expressed per gram of biomass and these were calculated based on the dry weight concentration in the samples used.

Calculations

Under chemostat conditions, and during the steady state, the specific growth rate is equal to the dilution rate applied.

The volumetric productivity (P_v) can be calculated per total culture weight ($M_{reactor}$) and is the product of the dilution rate (D) and biomass density (C_x), where dilution rate equals the flow of medium entering the reactor per unit of culture broth.

$$P_v = D \cdot C_x = \frac{F}{M_{reactor}} \cdot C_x \qquad [g \cdot Kg^{-1} \cdot h^{-1}]$$

The photosynthetic efficiency of the algae in the reactor is given by the biomass yield on light energy ($Y_{x,E}$), expressed as the amount of light energy that is converted into biomass per mol of photons supplied in the PAR range.

$$Y_{x,E} = \frac{M_{reactor} \cdot D \cdot C_x}{PFD_{in} \cdot 3600 \cdot A \cdot 10^{-6}} \qquad [g \cdot \text{ mol photons}^{-1}]$$

Results and Discussion

Cultivation of *Chlorella sorokiniana* in a flat panel photobioreactor with a light path of 14 mm was assayed in terms of biomass productivity and photosynthetic efficiency. Extreme winter conditions (irradiance and temperature) at midday in Huelva, southern Spain, were simulated and compared in terms of productivity and photosynthetic efficiency with optimal growth temperature (T_{opt}). When the temperature was below 20 ºC continuous cultivation was not possible at 800 mmol photons m^{-2} s^{-1}, probably related to the mesophilic character of *C. sorokiniana*. Only at maximal winter temperature, 20 ºC (called T_{sub}), the algae showed significant growth.

Before the steady state was reached, some days were needed to allow the cells to acclimate. This period was significantly longer at T_{sub} compared to T_{opt}. At T_{sub} the biomass concentration decreased drastically upon application of the dilution rate (D = 0.02 h^{-1}) and the stabilization of biomass was much slower.

Productivity and biomass yield

At optimal growth temperature (T_{opt} = 38 ºC), and when dilution rate was lower than 0.18 h^{-1}, the productivity increased with increasing dilution rate. However, a higher dilution rate (0.24 h^{-1}) led to a productivity decrease (Figure 2.1). Maximal productivity (P_v = 0.3 g Kg^{-1} h^{-1}) was therefore found at a dilution rate of 0.18 h^{-1}.

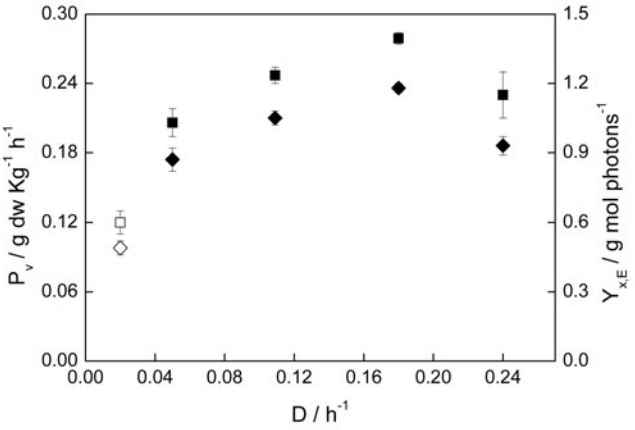


Figure 2.1: Volumetric productivity (P_v, ■) and biomass yield on light energy ($Y_{x,E}$, ◆) during chemostat cultivation at 800 μ mol photons m^{-2} s^{-1} and optimal growth temperature of *C. sorokiniana* (38 ºC). Open symbols correspond to suboptimal growth temperature (20 ºC).

The biomass yield obviously showed the same trend as productivity, with a maximal value of 1.2 g mol $photons^{-1}$ supplied when D = 0.18 h^{-1}. At high dilution rates, the cells experience more light availability since biomass density is low. It leads to a higher photosynthetic efficiency, and therefore productivity. However, when the dilution rate was 0.24 h^{-1}, close to the maximal specific growth rate of *C. sorokiniana* (0.27 h^{-1}), the low biomass density associated to that dilution rate resulted in a bigger loss of photons through the photobioreactor. It leads to a lower photosynthetic efficiency when it is defined as the amount of biomass produced by amount of photons supplied to the system. These results can be related to the findings in our previous work (Cuaresma et al., 2009) where *C. sorokiniana* was cultivated under continuous irradiance simulating summer conditions

(2100 µmol m^{-2} s^{-1}). The optimal dilution rate under these conditions was 0.24 h^{-1}, but the biomass density associated to that dilution rate was higher when compared with the experiments at 800 µmol m^{-2} s^{-1}. Nevertheless, the photosynthetic efficiency yielded at maximal winter irradiance was higher when compared with the summer experiments (1.2 versus 1.0 g biomass mol photons^{-1}). The exposition to a non-oversaturating irradiance during the winter experiments resulted in a higher photosynthetic efficiency.

At suboptimal growth temperature (T_{sub} = 20 ºC) the only successful dilution rate applied was 0.02 h^{-1}, due to the much lower specific growth rate of *C. sorokiniana*. It resulted in a productivity of 0.1 g Kg^{-1} h^{-1}, a 60% lower than the highest productivity found at T_{opt}. The biomass yield was also relatively low, 0.5 g mol photons^{-1} supplied. At suboptimal temperatures the irradiance applied might be experienced as over-saturating, resulting in a higher need of heat dissipation (NPQ).

The negative effect of suboptimal growth temperature on algal metabolism and efficiency is strengthened when low temperatures are accompanied by high irradiances, as already stated by different authors (Sorokin and Krauss, 1962; Spearing and Karlander, 1979; Vonshak and Torzillo, 2004; Vonshak et al., 2001). The irradiance tested in this study, 800 µmol m^{-2} s^{-1}, will be only reached around noon during outdoor conditions. Because *C. sorokiniana* mainly seemed photolimited, the irradiance tested would be the most beneficial situation at T_{opt}, but the absolute worst case at T_{sub}. In this sense, the negative effect of T_{sub} might be less at other times of the day, because of a lower irradiance relative to temperature. Bosma et al. (2007) also assessed the influence of temperature on productivity. They found a positive effect when controlling the temperature when irradiance was relatively high. In this case photoinhibition could be prevented. Heating the culture in the morning also diminishes the negative effect of temperature, leading to a 60% higher daily productivity as has been already observed by Vonshak et al. (2001).

Biomass density and cell viability

The lower residence time of the cells inside the photobioreactor when increasing the dilution rate led to a lower biomass density, as can be seen in Figure 2.2. At optimal temperature the biomass concentration was ranging from 1 to 4.1 g Kg^{-1}, and it was equal to 6.4 g Kg^{-1} when applying the suboptimal temperature.

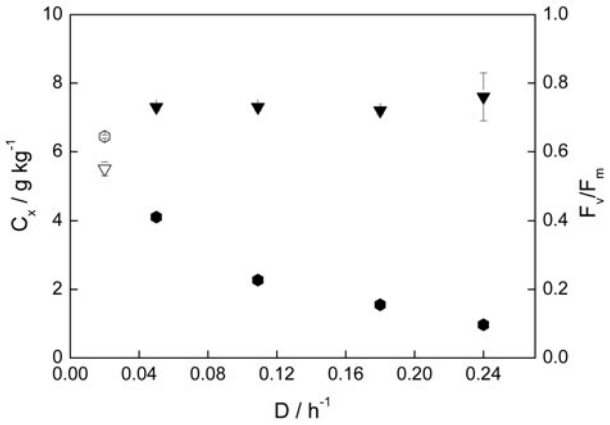


Figure 2.2: Biomass concentration (C_x, ⬢) and maximal quantum efficiency of PSII (F_v/F_m,▼) during chemostat cultivation at 800 μmol photons m^{-2} s^{-1} and optimal growth temperature of *C. sorokiniana* (38 ºC). Open symbols correspond to suboptimal growth temperature (20 ºC).

According to our previous work, Cuaresma et al. (2009), the biomass concentration during maximal winter irradiance was twice lower if compared with maximal summer irradiance at 0.24 h^{-1}. Since light was the only limiting factor at T_{opt}, and no significant photodamage was observed at 800 μmol photons m^{-2} s^{-1}, it can be concluded that the culture was light limited under winter conditions. On the other hand, under both simulations (winter and summer) the optimal biomass density was low if compared to the high biomass densities found at high irradiance for other algal strains (Hu et al., 1998; Meiser et al., 2004; Qiang and Richmond, 1996; Qiang et al., 1998b). In this sense, it has been suggested before that high temperature strains as *C. sorokiniana* require higher light intensity than other strains with a lower optimal growth temperature (Myers and Graham, 1961; Sorokin, 1960). Also Kliphuis et al. (2010) found a low optimal biomass density for

C. sorokiniana at high light intensities. All together this indicates that *Chlorella sorokiniana* is not only able to withstand high irradiance levels, but might actually need a high irradiance level to result in high productivities.

The maximal photosynthetic yield of PSII (F_v/F_m) at the optimal growth temperature remained above 0.7 for all dilution rates applied (Figure 2.2), indicating well-functioning of the photosynthetic machinery and absence of photoinhibition. The absence of significant photodamage at the irradiance tested, 800 μmol m^{-2} s^{-1},was in agreement with the results of Cuaresma et al. (2009), where no photoinhibition of *C. sorokiniana* was observed even at a much higher irradiance (2100 μmol m^{-2} s^{-1}).

The high biomass concentration found at suboptimal temperature is related to the low specific growth rate, and therefore the low dilution rate applied. As commented before, the slow growth at suboptimal temperatures could be related to the slow algal metabolism, which will result in a decline in substrate requirements, therefore meaning less light in the case of a light-limited chemostat. In this sense, the same amount of light energy supplied to the system can support a more dense culture at suboptimal temperature since the individual cells cannot grow fast anyway due to the temperature limitation.

From a photosynthetic point of view, at suboptimal temperatures the electron sinks will be continuously over-reduced due to the slow metabolism. The incoming light will be experienced then as over-saturating and it will result in more over-excitation of PSII, being photodamage more likely to occur. In this sense, at suboptimal temperatures a moderate irradiance level can already be counteracting as addressed before. Algae are able to defend themselves against excessive irradiance by dissipating the extra light energy as heat via non-photochemical quenching (NPQ). It can be observed in the low maximal quantum yield of PSII (F_v/F_m = 0.5), which was below a typical value for healthy cells (F_v/F_m = 0.7). This low maximum PSII quantum yield was also reflected in the low biomass yield and productivity reached at T_{sub}.

In this study a large deviation from T_{opt} was applied (ΔT = 18 ºC), which led to a 30% decrease in F_v/F_m compared to T_{opt}. This is in accordance with Grobbelaar (2007), who also reported a 30 % drop in F_v/F_m at midday when cultivating *Spirulina platensis* 10 ºC below the optimum growth temperature. Moreover, Maxwell et al. (1994) also found that growth of *Chlorella vulgaris* had the same photosynthetic response to low temperature as to high irradiances. Apparently suboptimal temperatures under (over)saturating light conditions leads to photoinhibition and a decrease in the photosynthetic efficiency.

Cell pigmentation

At optimal temperature (T_{opt}) the total chlorophyll content ranged from 35.4 mg g^{-1} to 19.4 mg g^{-1}, and the total carotenoids content from 6.7 to 3.7 mg g^{-1}. The total pigment content per unit of biomass decreased with increasing dilution rate (Figure 2.3), when the culture experienced a higher average irradiance due to the lower biomass concentration. At higher biomass concentrations higher pigment content is expected to compensate for the increase in photolimitation, a process called photoacclimation.

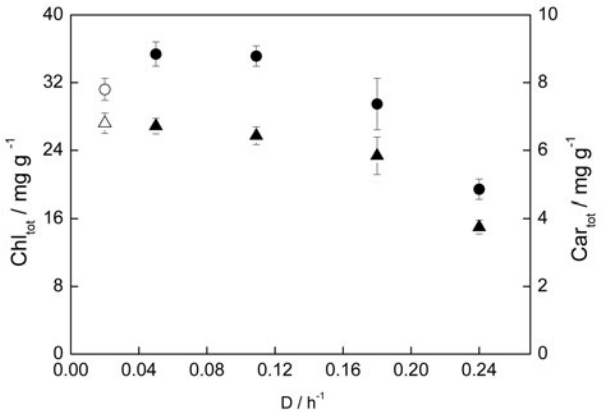


Figure 2.3: Total chlorophyll (Chl_{tot}, ●) and carotenoids content (Car_{tot}, ▲) during chemostat cultivation at 800 μmol photons m^{-2} s^{-1} and optimal growth temperature of *C. sorokiniana* (38 ºC). Open symbols correspond to suboptimal growth temperature (20 ºC).

The pigment content at suboptimal temperature (31.2 mg chlorophyll g dw^{-1} and 6.8 mg carotenoids g dw^{-1}) was lower than expected considering the biomass concentration was higher than the highest biomass concentration achieved at T_{opt}. The low metabolic activity, and thereby the lower requirement of light energy at suboptimal temperature could explain that observation.

In contrast to the chlorophyll content, the carotenoids content at suboptimal temperature was the same when compared with T_{opt}. This resulted in a slightly higher ratio of carotenoids to chlorophyll at T_{sub} (16% higher). This is probably related with the higher NPQ since the carotenoids also function as antioxidant and play a role in dissipation of excessive light as heat. At optimal growth temperature, the carotenoids/chlorophyll ratio was similar under all the dilution rates assayed, which seems to indicate that no extra photoprotective activity from the carotenoids was needed.

Conclusions

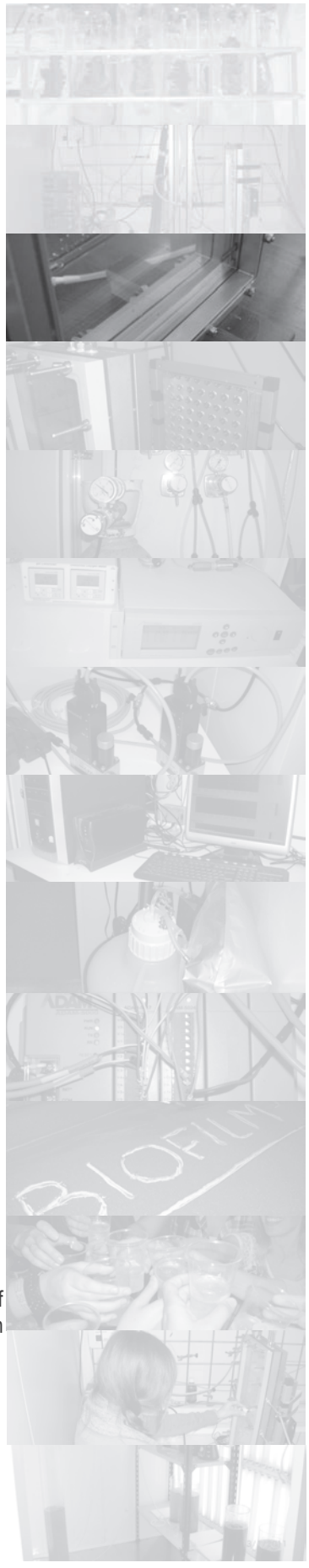
The strong influence of temperature on algal productivity during winter cultivation was confirmed by this study. Suboptimal growth temperatures lead to a low specific growth rate. Therefore, a low productivity was found at 20 ºC, the maximal winter temperature, for *C. sorokiniana* (0.12 g Kg^{-1} h^{-1}). Biomass yield was also low, 0.5 g mol $photons^{-1}$. The slow metabolism and the higher need of NPQ to dissipate the excessive light absorbed resulted in a lower photosynthetic efficiency of *C. sorokiniana* at suboptimal temperatures.

Due to the over-saturating irradiance experienced by the algae cells at suboptimal temperature, a higher carotenoids to chlorophyll ratio was observed, which was probably related to the induction of heat dissipative processes (NPQ).

Temperature control during winter time could clearly enhance the productivity and photosynthetic efficiency of *C. sorokiniana*. At optimal growth temperature the

productivity reached at maximal winter irradiance (800 μmol photons m^{-2} s^{-1}) was high when compared with suboptimal temperature, but about half of the productivity found with the same cultivation system at maximal summer irradiance (Cuaresma et al., 2009). Nevertheless, the biomass yield was higher at maximal winter irradiance ($Y_{x,E}$ = 1.2 g dw mol photons^{-1} supplied) than at summer irradiance ($Y_{x,E}$ = 1.0 g dw mol photons^{-1} supplied). The low winter irradiance resulted in a more efficient conversion of the available light biomass (higher $Y_{x,E}$) by *C. sorokiniana* when temperature is controlled. However, the lower photons availability during winter time led to a lower volumetric productivity when compared with summer irradiance, suggesting that the photobioreactor was operated in the photolimited regime.

During winter time the productivity is limited by low light and temperature conditions, being the suboptimal temperature the main factor reducing metabolic rates and, consequently, photosynthetic efficiency at relatively high irradiance (around noon). Temperature control will lead to a higher winter productivity. Reduction of irradiance at the photobioreactor surface, on the other hand, might be an attractive alternative to temperature control in outdoor cultivation during winter time.



Chapter 3

Productivity of Chlorella sorokiniana in a short light-path (SLP) panel photobioreactor under high irradiance

This chapter has been published as:
Cuaresma, M., Janssen. M., Vílchez, C., Wijffels, R.H., 2009. Productivity of *Chlorella sorokiniana* in a short light-path (SLP) photobioreactor under high irradiance. Biotechnology and Bioengineering, 104 (2): 352-359

3

Abstract

Maximal productivity of a 14 mm light-path panel photobioreactor under high irradiance was determined. Under continuous illumination of 2100 µmol photons m^{-2} s^{-1} with red LEDs (light emitting diodes) the effect of dilution rate on photobioreactor productivity was studied. The light intensity used in this work is similar to the maximal irradiance on a horizontal surface at latitudes lower than 37 º.

Chlorella sorokiniana, a fast-growing green microalga, was used as a reference strain in this study. The dilution rate was varied from 0.06 h^{-1} to 0.26 h^{-1}. The maximal productivity was reached at a dilution rate of 0.24 h^{-1}, with a value of 7.7 g of dry weight m^{-2} h^{-1} (m^2 of illuminated photobioreactor surface) and a volumetric productivity of 0.5 g of dry weight L^{-1} h^{-1}. At this dilution rate the biomass concentration inside the reactor was 2.1 g L^{-1} and the photosynthetic efficiency was 1.0 g dry weight per mol photons. This biomass yield on light energy is high but still lower than the theoretical maximal yield of 1.8 g mol photons^{-1} which must be related to photosaturation and thermal dissipation of absorbed light energy.

Introduction

The use of microalgae for production of high value compounds and biofuels, as well as their use in bioremediation and in animal and human feeding, is currently catching the attention from investors. However, the production cost for microalgae is still one of the main bottlenecks limiting large scale production. Since microalgae are photosynthetic organisms, the efficient use of light is a prerequisite for successful industrial production processes.

Under outdoor conditions, the daily solar cycles determine the main algae growth conditions in the photobioreactors: light and temperature regimens. While temperature can be controlled, light availability becomes the dominant factor determining the productivity. Since during the central daylight hours the solar irradiance can exceed 2000 µmol photons m^{-2} s^{-1} the light saturation effect imposes a serious limitation on the efficiency with which solar energy can be utilized in outdoor algal cultures.

Different principles to overcome this have been proposed, such as reactors maximally exposed to sunlight, a narrow light-path, and a mixing system designed to move the algal cells in and out of the photic volume at maximal frequency. Reactors designed along these principles may support ultrahigh cell densities resulting in high volumetric and areal yields, expanding thereby the economic basics of microalgal biotechnology (Richmond, 1997).

In this work, the biomass yield on light energy has been studied in a short light-path (SLP) panel photobioreactor. The biomass yields obtained have been compared with the theoretical maximal value to determine the magnitude of photosaturation under real production conditions under high photon irradiance. The maximum yield is based on the stoichiometric reaction equation for biomass formation on carbon dioxide, water and nitrogen (urea) and was calculated to be 1.8 g of biomass produced per mol of photons (PAR, 400-700 nm) absorbed (see Appendix).

Chlorella sorokiniana was selected as reference strain due to its high specific growth rate, 0.27 h^{-1} and its tolerance to high irradiance, high temperature and high CO_2 concentrations (Matsukawa et al., 2000; Sorokin, 1959). The lamps used to simulate high photon flux densities (PFD) were red light emitting diodes (LEDs). Compared with conventional lamps LEDs provide a narrow band wavelength with low power consumption and can be used to simulate high and homogeneous photon flux densities on controlled lab-scale photobioreactors. Red LED have been used before in several studies on microalgal physiology and microalgae production and it was demonstrated to provide reliable information on the relationship between microalgal growth and photon flux density (Lee and Palsson, 1995, 1996; Matthijs et al., 1996; Tennessen et al., 1994; Wang et al., 2007).

This paper describes the effect of dilution rate on biomass productivity of *Chlorella sorokiniana* in a flat panel photobioreactor under high irradiance conditions. During these experiments also the biomass yield on light energy was calculated. Chemostat operation was used because it allows full adjustment of the cells' physiology to the prevailing culture conditions and the specific growth rates can be maintained at pre-determined values for a prolonged time (Huisman et al., 2002). In this way, culture parameters such as biomass concentration, productivity and biomass yield could be readily adjusted and studied at fixed specific growth rates.

Materials and Methods

Microalgae and growth medium

Chlorella sorokiniana CCAP 211/8K was obtained from the UTEX culture collection. It was maintained in modified M-8a medium (Mandalam and Palsson, 1998) in Erlenmeyer flasks at 25 ºC and 165 µmol photons m^{-2} s^{-1}. The culture

medium was prepared as follows (composition expressed in mol L^{-1}): KH_2PO_4, $5.4 \cdot 10^{-3}$; $Na_2HPO_4 \cdot 2H_2O$, $1.5 \cdot 10^{-3}$; $MgSO_4 \cdot 7H_2O$, $1.6 \cdot 10^{-3}$; $CaCl_2 \cdot 2H_2O$, $0.9 \cdot 10^{-4}$; KNO_3, $30 \cdot 10^{-3}$; EDTA ferric sodium salt, $0.3 \cdot 10^{-3}$; $Na_2EDTA \cdot 2H_2O$, $0.1 \cdot 10^{-3}$; H_3BO_3, $1.0 \cdot 10^{-6}$; $MnCl_2 \cdot 4H_2O$, $0.7 \cdot 10^{-4}$; $ZnSO_4 \cdot 7H_2O$, $0.1 \cdot 10^{-4}$; $CuSO_4 \cdot 5H_2O$, $0.7 \cdot 10^{-5}$; $NaHCO_3$, $5 \cdot 10^{-3}$. The pH was adjusted to 6.7 with a concentrated solution of NaOH. During chemostat experiments in the photobioreactor nitrate was replaced by urea $60 \cdot 10^{-3}$ M and 3-fold concentrated medium was used to avoid nutrient limitation.

Photobioreactor

The flat panel reactor used has a light-path of 14 mm giving a surface to volume ratio of 71 m^{-1}. Liquid height applied was 47.7 cm (total reactor height is 60 cm) and reactor width is 24.9 cm, giving a height-to-width ratio of 1.9. It is illuminated with red LEDs (red Luxeon III Emitters, Philips Lumileds) and light intensity at the reactor surface could be varied between 0 and 3000 µmol m^{-2} s^{-1} in the PAR range (400-700 nm).

The culture suspension is mixed by bubbling air through a silicone tube with small holes placed horizontally in the bottom of the culture chamber. The air flow rate is continuously measured and controlled using a mass flow controller (Brooks-Emerson, Hatfield, USA). The carbon dioxide is added separately via a micro-sparger (sintered stainless steel, 10 micron pore size) in order to provide a very high mass transfer rate. The rate of CO_2 supply is controlled via a separate mass flow controller and is used to control pH. The pH and dissolved oxygen (DO) concentration were measured using Applisens sensors (Applisens, Schiedam, The Netherlands) connected to Liquisys M control modules (Endress-Hauser, Reinach/BL, Switzerland). Temperature is measured directly inside the culture broth. The photobioreactor is equipped with a thermostatized water bath (Lauda, Königshofen, Germany) connected to the cooling jacket of the reactor in order to

keep reactor temperature constant. To prevent water evaporation from the culture pre-humidified air is used for mixing. Values for pH, DO, reactor temperature and gas flows were recorded using an ADAM-5510/TCP data acquisition and control system (Advantech, USA) connected to a PC running a dedicated LabView 7.1 (National Instruments, Texas, USA) virtual instrument to register these data and control pH and power of the lamps (see later).

The reactor is equipped with different ports for addition of fresh medium (influent) and the culture's inoculum. Ports for continuous culture removal (effluent) and for sampling are present (Figure 3.1). For Chemostat experiments, a calibrated peristaltic pump (Watson Marlow, Cheltenham, UK) was used to provide the reactor with a constant flow of fresh medium. Outflow from the reactor was

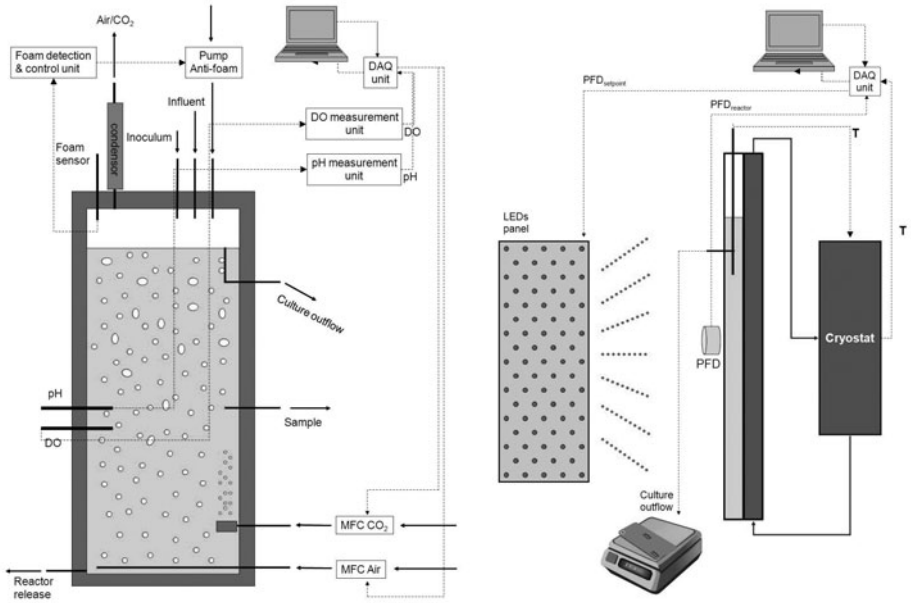


Figure 3.1: Schematic view of the flat panel photobioreactor configuration. Temperature and light control are also indicated. To ensure the culture level into the reactor remains constant, a fixed culture outflow port is provided. Fresh medium is pumped in via the influent port. An additional port is included in the bottom part of the reactor to be able to empty the reactor (reactor release) after every experiment.

weighed continuously on a balance (Gram precision, Barcelona, Spain) to determine the actual dilution rate by dividing the flow rate (g h^{-1}) by the total culture weight in the reactor (g).

Experimental conditions

During all the chemostat experiments temperature was set at 37 ºC ± 1 ºC and pH maintained at 6.7. Culture was continuously mixed with compressed air at a flow rate of 1.5 L L^{-1} of culture min^{-1}, corresponding to a superficial gas velocity of 0.013 m s^{-1}.

Prior to chemostat conditions, a batch cultivation with an intensity of 200 µmol photons m^{-2} s^{-1} was needed until a sufficient biomass density was reached. Intensity was then increased till 800 µmol photons m^{-2} s^{-1}. When the OD_{750} was about 1.0, maximal intensity was applied to the culture (2100 µmol photons m^{-2} s^{-1}). During the batch cultivation period (48 hours in total) urea was used as nitrogen source to allow the cells to acclimate to the imposed culture conditions. Chemostat cultivations were started up with a sufficient biomass concentration (OD_{750} ~ 10.0) as to support the applied dilution rate, according to previous experiments. The dilution range assayed varied from 0.06 h^{-1} to 0.26 h^{-1}. After each experiment, culture weight inside the reactor was measured to calculate the dilution rate. Table 3.1 shows the cultivation conditions during the different experiments.

Experiments	Dilution (h^{-1})	Culture weight (g)	Aeration flow rate (L min^{-1})
1	0.06	1774	2.8
2	0.11	1750	2.7
3	0.16	1750	2.7
4	0.2	1800	2.7
5	0.24	1800	2.7
6	0.26	1718	2.7

Table 3.1: Different operational conditions during the chemostat experiments under a photon flux density of 2100 µmol photons m^{-2} s^{-1}.

Illumination

The reactor was illuminated with a red LED (light emitting diodes) panel composed by 128 red LEDs (Luxeon III, emitter, Philips-Lumileds) distributed homogeneously over its surface. PAR photon flux density (PFD, 400-700 nm) was measured prior to each experiment using a LI-190 quantum sensor (LI-COR, USA) on the outer reactor surface as well as inside the empty culture chamber. Inside the culture chamber the PFD was measured on 45 homogeneously distributed spots along the illuminated surface and an averaged PFD was calculated. A correlation factor between both the external PFD and the average internal PFD was determined. During each experiment the output of the LED panel was automatically adapted using the LabView virtual instrument according to the external PFD measured and this correlation factor.

Figure 3.2 shows the spectral composition of the light source (LEDs) determined by a spectroradiometer (IRRAD 2000 fiber-optic spectroradiometer, TOP sensor

systems, Eerbeek, The Netherlands). As shown in Figure 3.2, the light emitted is confined to a narrow peak around 637 nm at the power applied during these experiments.

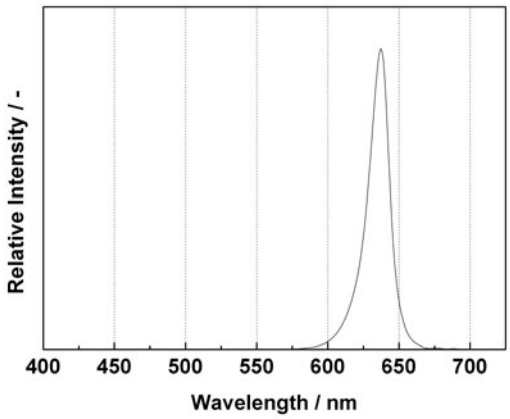


Figure 3.2: Relative spectral composition of the red Luxeon III Emitters (Philips-Lumileds) used in this study.

Dry weight and optical density determination

Biomass concentration inside the reactor was determined by dry weight and optical density measurements. Dry weight was determined by filtration of the culture broth over glass fibre filters with a pore size of 0.7 µm (Millipore APFF04700). The filter weight was determined on a 0.01 mg precision balance (Sartorius CP225D, Sartorius AG, Germany). Aliquots of 5 ml of culture broth, diluted 15 times with prefiltered demineralized water, were filtered through pre-washed, pre-dried and pre-weighed filters. After filtration, filters were washed again with 50 mL of prefiltered demineralized water to remove adhering inorganic salts. Filters were then dried at 80 ºC during at least 16 h and cooled down in a dessicator for at least 2 h. Dry weight, expressed as mg g^{-1} of culture broth and g L^{-1}, was calculated by differential weight.

Optical density was determined spectrophotometrically at 530 nm, 680 nm and 750 nm in an UV/Visible spectrophotometer (Ultrospec 3100pro, Amersham Pharmacia Biotech, Sweden). A 1 cm light path cuvette was used.

Specific growth rate during continuous operation

Cultures were grown in batch mode until significant development of biomass. Then, continuous dilution was started. While operating as a chemostat, the steady-state biomass concentration attained is determined by the imposed dilution rate as the only limiting growth factor is light availability. When the dilution rate is lower than the maximal specific growth rate the cells can be maintained at a constant specific growth rate for a prolonged time called steady-state. In this condition, the specific growth rate (μ, h^{-1}) equals the dilution rate (culture flow rate to culture volume rate) (Huisman et al., 2002).

The dilution rate was determined on daily measurements of the culture outflow. After each experimental run at a specific dilution rate the reactor was emptied, cleaned and inoculated for a new run. Every run was operated non-aseptically for two or three weeks without any contamination problem. Under these conditions biomass productivity and photosynthetic efficiency were calculated.

Productivity and biomass yield on light energy

Volumetric productivity is the product of the biomass density and the dilution rate. It was calculated during steady state for every chemostat experiment.

The efficiency of light utilization for photoautotrophic growth can be expressed in several ways. Biomass yield on light energy, expressed as dry weight produced per amount of quanta (photons) absorbed in the PAR range ($Y_{x,E}$) (Janssen et

al., 2003), can be easily measured and can be compared to theoretical yields. For each experiment this biomass yield was calculated by the following equation during the steady state.

$$Y_{x,E} = \frac{C_x \cdot \mu \cdot V}{PFD_{in} \cdot A_r \cdot 3600 \cdot 10^{-6}} \qquad [\text{g mol photons}^{-1}]$$

Statistic

Every measurement was done in duplicate unless otherwise indicated. Figures show means and standard deviations of the results.

Results and discussion

For maximal outdoor production of microalgae, either biomass or specific products, the optimization of the dilution rate becomes an essential technological target. In this study, the evaluation of the productivity of a SLP photobioreactor was carried out under high irradiance conditions, similar to those irradiances occurring when culturing microalgae outdoor at noon in the south of Spain (37 º North). Different dilution rates were applied in order to study the optimal biomass concentration and the maximal productivity of *C. sorokiniana* under these conditions.

Biomass concentration

To determine the optimum conditions for the photoautotrophic production of *C. sorokiniana*, continuous cultures were carried out by modifying the dilution rate under nutrient-replete conditions which means that light was the limiting substrate. As result, during the steady state the biomass concentration in the reactor ranged from 5.7 to 1.5 g L^{-1} (Figure 3.3).

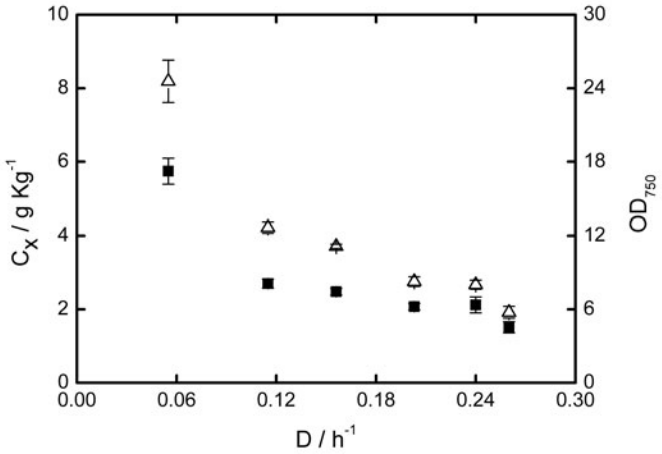


Figure 3.3: Influence of dilution rate (D) on the mean value of biomass concentration during the steady state under high irradiance (2100 µmol photons m^{-2} s^{-1}): [■] Biomass concentration (C_x); [△] Optical density at 750 nm (OD_{750}).

The highest biomass concentration was found at the lowest dilution rate (0.05 h^{-1}), with a value of 5.7 g L^{-1}. The biomass concentration decreased to 2.8 g L^{-1} when increasing the dilution rate from 0.05 h^{-1} to 0.10 h^{-1}. At higher dilution rates biomass concentration only decreased slightly when increasing dilution from 0.10 h^{-1} to 0.26 h^{-1}.

According to Molina Grima et al. (1996), during chemostat operation, high dilution rates must be supported by fast-growing cells whose illumination requirements can only be met at low biomass concentrations. Also in our work, the high dilution rates imposed were only supported by a low cell density of about 2 g L^{-1}. Masojidek et al. (2003) found that low biomass cultures of *Spirulina platensis* (cyanobacterium) in a novel tubular photobioreactor were able to acclimate to irradiance value as high as 7 mmol photon m^{-2} s^{-1}. In this case, low biomass cultures adapted to these high irradiance conditions by developing a high level of non-photochemical quenching, the optimal biomass concentration of the culture ranging from 1.2 to 2.2 g L^{-1}. As it will be discussed later in our study, also diluted cultures of *Chlorella sorokiniana* showed high productivity and photosynthetic efficiency under high irradiance conditions.

Microalgae productivity

Productivity values under high irradiance conditions are shown in Figure 3.4. From the lowest dilution rate applied, higher dilution rates correspond to higher culture outflow (kg of culture per day) and productivity values. Productivity peaks at a dilution rate of 0.24 h^{-1}. Higher dilution rates led to a drop in productivity. Apparently the microalgae could not keep up with this dilution rate because it approached the maximal specific growth rate of *C. sorokiniana,* which is reported to be 0.27 h^{-1} (Sorokin, 1959).

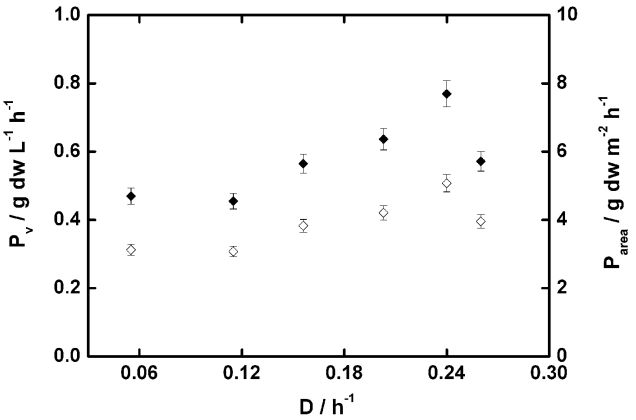


Figure 3.4: Influence of dilution rate (D) on the productivity of *C. sorokiniana* under high irradiance (2100 µmol photons m^{-2} s^{-1}): [◇] Volumetric productivity in gram of dry weight per liter per hour (P_v); [◆] Areal productivity in gram of dry weight per square meter of illuminated surface per hour (P_{area}).

The maximal productivity value was 185 g of dry weight per square meter of illuminated surface per day under continuous illumination, which corresponds to an areal productivity of 7.7 g dw m^{-2} h^{-1}. The maximal volumetric productivity was 12.2 g dw L^{-1} day^{-1} or 0.5 g dw L^{-1} h^{-1}. These high values were obtained under high dilution rate and low biomass concentration.

The productivities reached in our experiments were quite high compared with others reported for microalgae. For example, Doucha and Lívanský (2006) found a maximal productivity of 32.2 g of dw m^{-2} day^{-1} for *Chlorella* during outdoor culti-

vation, and Morita et al. (2000) reached a maximal productivity of 34.4 g dw per square meter of installation area per day (light/dark cycle of 12 h, 980 µmol photons m^{-2} s^{-1}). Also a closed tubular photobioreactor based on solar concentrators, which lead to high irradiance conditions, showed a net productivity of 32.5 g m^{-2} day^{-1} for *Spirulina platensis* (based on the minimum illuminated surface area) (Masojídek et al., 2003). These productivity values are lower than our data, where the lowest productivity is 109 g dw m^{-2} of illuminated surface day^{-1} under continuous illumination. Partly this is caused by the fact that these studies have been carried out under real day-night cycles and light input over a 24 hours period is lower. Nevertheless, the data presented in our study demonstrate the potential of this fast-growing *Chlorella* strain even under over-saturating photon flux densities.

Biomass yield on light energy

Together with the productivity, the biomass yield on light energy (gram of biomass produced per mol of photons absorbed) of *C. sorokiniana* increased as the dilution rate was increased from 0.06 h^{-1} to 0.24 h^{-1} (Figure 3.5). Dilution rates higher than 0.24 h^{-1} led to a decrease of the biomass yield. Nevertheless, for the different dilution rates assayed the biomass yield remained within the range of 0.6 to 1.0 g mol $photons^{-1}$.

The maximal biomass yield on light energy achieved was 1.0 g of biomass mol $photons^{-1}$. Comparing this value to the theoretical maximal one calculated based on urea as nitrogen source (see Appendix), 1.8 g per mol of photons, the biomass yield accounts for 57% of the maximal one. In other words, less than half of the light energy is absorbed but not used by photosynthesis. This unused light energy must be dissipated by the cells as heat.

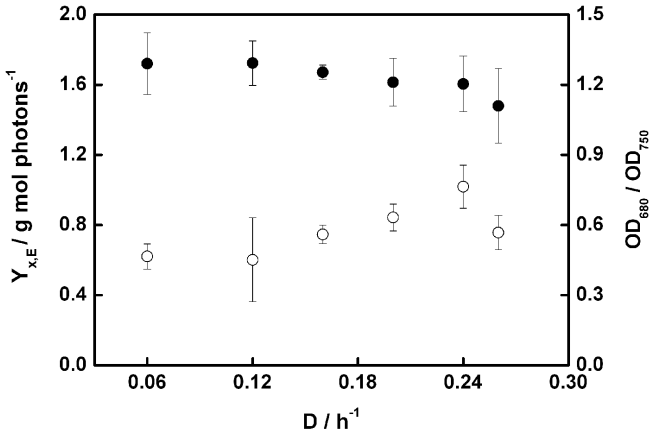


Figure 3.5: Influence of dilution rate (D) on the biomass yield and relative chlorophyll content of *C. sorokiniana* under high irradiance (2100 µmol photons m^{-2} s^{-1}): [○] Biomass yield in g of biomass produced per mol of photons absorbed ($Y_{x,E}$); [●] Relative cellular chlorophyll-*a* content expressed as the ratio between optical density at 680nm and 750 nm.

The maximal biomass yield obtained is high considering the fact *C. sorokiniana* was cultivated under over-saturating light and at a low biomass concentration. Under such conditions photoinhibition is likely to occur (Vonshak and Torzillo, 2004). In case significant photodamage occurs the specific growth rate will drop as has been shown by several independent studies (Han et al., 2000; Qiang and Richmond, 1994; Vonshak and Guy, 1992). However, during our study the biomass could be maintained at a dilution rate close to the maximal specific growth rate: 0.24 h^{-1} versus 0.27 h^{-1}. Therefore, productivity and photosynthetic efficiency were highest at 0.24 h^{-1}.

In order to indirectly assess the likely photoinhibition state of the cells, the ratio between optical density at 680 nm and 750 nm has been measured and is presented in Figure 3.5. This ratio can be use*d* as a relative measurement of the chlorophyll-*a* content of microalgae cells. When photoinhibition occurs, cells are bleached, which can be seen as a severe drop in this ratio. During our experiments, only the highest dilution applied led to a decrease in the ratio, with a value below 1.2. At all other dilution rates, the chlorophyll content was pretty much the same and above 1.2. It only decreased slightly when going from a low dilution

rate (i.e. high biomass concentration) to a high dilution rate (low biomass concentration). This general trend is probably related to the well-described process of photoacclimation, i.e. the decrease in pigment content when switching from a light-limited to a light saturated phase.

Considering both, the high specific growth rate and the absence of bleaching, indicate that photoinhibition is not a dominant process and probably dissipative processes, collectively called non-photochemical quenching (Muller et al., 2001), protect these cells from photodamage as was already reported by Masojídek et al. (2003). At over-saturating light intensities even higher photosynthetic efficiencies have been found in dense cultures of *Spirulina*: Qiang et al. (1998b) found a maximal productivity of 16.8 g dw L^{-1} h^{-1} under 8000 µmol photons m^{-2} s^{-1} with a cell density of 30 g L^{-1}; and Qiang and Richmond (1996) reported a productivity of 0.4 g dw L^{-1} h^{-1} under 1800 µmol photons m^{-2} s^{-1} with a cell density of 18 g L^{-1}. These high efficiencies were explained by the combination of a good mixing rate and the shading effect at high cell densities leading to only short exposure times to the over-saturating light at the light-exposed reactor surface.

Other studies on the cultivation of green microalgae, however, showed lower photosynthetic efficiencies under high irradiance conditions: Meiser et al. (2004) reported a productivity for *Phaeodactylum tricornutum* of 1.38 g dw L^{-1} day^{-1} under 1000 µmol photons m^{-2} s^{-1} with a cell density of 7.3 g L^{-1}; and Hu et al. (1998) reached a productivity for *Chlorococcum littorale* of 0.4 g dw L^{-1} h^{-1} under 2000 µmol photons m^{-2} s^{-1} with a cell density of 22 g L^{-1}. In this respect, the photosynthetic efficiencies at over-saturating light conditions in our study are the highest for green algae reported so far. Besides, the productivity was maximum at rather low biomass concentration in comparison with the ones found by Richmond and coworkers (concentrations up to 18 g L^{-1}) and cannot be explained in terms of cell density as they proposed in their studies. Richmond and coworkers suggested that the high productivity reached during their *Spirulina* cultivations was related to favourable light/dark cycling induced by high cell densities and turbulent mixing

in short light-path reactors (Richmond, 2000).

Dilute microalgae cultures with a high biomass yield also present advantages in terms of mass production. No medium refreshment is needed under these conditions since nutrient depletion or growth inhibition can easily be avoided at these low biomass concentrations as was shown for *Chlorella* cultures by Mandalam and Palsson (1998). The high productivities reported for *Spirulina* (Richmond, 2000), on the other hand, were only supported by daily medium refreshment. This leads to a complicated process in which a cell retention system for the biomass has to be developed. In this sense, diluted cultures of *Chlorella sorokiniana,* which also show high biomass yields under over-saturating light conditions, could also lead to a continuous production processes with a high productivity.

Conclusions

The results presented in this work are of interest since *Chlorella sorokiniana* may be efficiently produced in short light path panel photobioreactors at irradiance conditions as high as 2100 µmol photons m^{-2} s^{-1}. The productivity of *C. sorokiniana* was very high, 7.7 g of dw m^{-2} h^{-1} under continuous illumination. This maximal productivity was reached at a high dilution rate of 0.24 h^{-1} and a low biomass concentration of 2.1 g L^{-1}.

The high productivity reached can be explained on the basis of the following facts: (1) photobioreactor configuration with narrow light path and good mixing rate, which improves light distribution inside the reactor and allows cells to move from saturating light zones to dark zones; and (2) *Chlorella sorokiniana*, a strain that tolerates high irradiance and temperature conditions and has a high specific growth rate.

A biomass yield of 1.0 g mol photons^{-1} at an over-saturating light intensity of 2100 µmol photons m^{-2} s^{-1} and a dilution rate close to the maximal specific growth rate suggests that photoinhibition was not a dominant process during our study. The difference between observed yield and the maximal theoretical value of 1.8 g mol photons^{-1} must be due to thermal dissipation of excess light energy absorbed.

Appendix

Using the stoichiometry of photoautotrophic growth we can calculate the maximal biomass yield on light energy for growth on urea. The stoichiometry is given below:

$$0.94 \cdot CO_2(g) + 0.77 \cdot H_2O(l) + 0.06 \cdot CH_4ON(aq) \rightarrow CH_{1.78}O_{0.36}N_{0.12}(s)$$

Other information needed:

- According to equation, 1.18 moles of oxygen are produced per C-mol biomass produced.

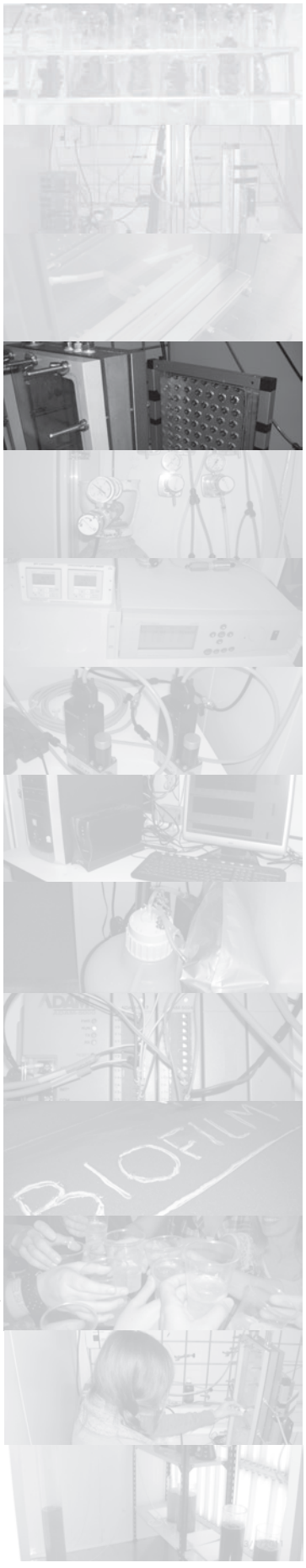
- Assuming an elemental composition of $CH_{1.78}O_{0.36}N_{0.12}$ for *Chlorella* (Duboc et al., 1999) the molecular mass of a C-mol biomass is 21.25 g mol^{-1}.

- The quantum yield (QY) of the light reactions is about 0.1 moles of oxygen per mol of photons averaged over the range of PAR. This value has been experimentally determined by a number of independent authors under low light for both higher plants and microalgae.

The biomass yield on light energy ($Y_{x,E}$) is defined as the amount of biomass in C-moles (or grams of dry weight) produced per mol of photons absorbed in the PAR range. Based on this information the maximal biomass yield on light energy, when using urea as nitrogen source, is 1.8 g mol^{-1}photons.

Chapter 4

Horizontal or Vertical photobioreactors? How to improve microalgae photosynthetic efficiency



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4

Abstract

The productivity of a vertical outdoor photobioreactor was quantitatively assessed and compared to a horizontal reactor. Daily light cycles in southern Spain were simulated and applied to grow the microalga *Chlorella sorokiniana* in a flat panel photobioreactor.

The maximal irradiance around noon differs from 400 μmol photons m^{-2} s^{-1} in the vertical position to 1800 μmol photons m^{-2} s^{-1} in the horizontal position. The highest volumetric productivity was achieved in the simulated horizontal position, 4 g Kg $culture^{-1}$ d^{-1}. The highest photosynthetic efficiency was found for the vertical simulation, 1.3 g of biomass produced per mol of PAR photons supplied, which compares favourably to the horizontal position (0.85 g mol^{-1}) and to the theoretical maximal yield (1.8 g mol^{-1}). These results prove that productivity per unit of ground area could be greatly enhanced by placing the photobioreactors vertically.

Introduction

The commercial achievements on microalgal biotechnology have so far been modest despite the increasing interest in microalgae for the production of biofuels or high added-value compounds. Although microalgae are not yet produced at large scale for bulk applications, recent advances -particularly in systems biology, material science, genetic engineering, and biorefining- present opportunities to develop this process in a sustainable and economical way within the next 10 to 15 years (Wijffels and Barbosa, 2010).

Outdoor cultures are exposed to a changing environment where temperature and nutrient availability can be controlled. Using CO_2-rich combustion gasses also CO_2 limitation can be prevented and productivities can be boosted to much higher levels than those achievable with higher plants. Consequently, light availability is by far considered the limiting nutrient when growing photosynthetic microorganisms, and therefore light use becomes the main factor affecting microalgae productivity.

Along the day, due to the natural light cycle, microalgae cells are exposed to limiting, saturating and over-saturating light conditions. During spring and summer photosynthesis will already saturate early in the morning, and during solar noon, sunlight levels at the reactor surface become over-saturating and could even lead to photoinhibition. Similar to well-described processes in higher plants, the exposure to excess light leads to cellular and molecular responses in algae to avoid photodamage and photoinhibition. One of the most important regulatory processes is called non-photochemical quenching (NPQ) by which excess light energy is dissipated as heat within the photosynthetic antennae complexes (Li et al., 2009). Integrated over the whole day, the over-absorbed and dissipated sunlight could account for about 60 % of the daily irradiance, as estimated by Melis (2009).

Geographical areas with high irradiances along the year and moderate temperatures are optimal for microalgae cultivation. Because of the average amount of

sunlight hours per day (10-12 h), and the mean solar irradiance ranging from 400 μmol photons m^{-2} s^{-1} (winter time) to 1800 μmol photons m^{-2} s^{-1} (summer time), southern Spain is considered especially suitable for outdoor cultivation of microalgae (García-González et al., 2003). Under these favourable light conditions, the calculated maximal theoretical algae biomass productivity is 220 tonnes ha^{-1} $year^{-1}$, or 60 g m^{-2} d^{-1} averaged over the year, if we assume maximal photosynthetic efficiency corresponding to 1.8 g dry matter per mol of PAR photons (Appendix A).

Different authors reported on the areal productivity of microalgae in outdoor conditions, and the maximal productivity ranges from 20 to 30 g m^{-2} d^{-1} (Blanco et al., 2007; Del Campo et al., 2001; García-Malea et al., 2006; Rebolloso et al., 1999). Clearly, there is still room for improvement if we compare these practical data with the maximal theoretical productivity. Optimizing photobioreactor orientation has been proposed to enhance productivity by avoiding or reducing the photosaturation, as can be inferred from the work of Hu and coworkers (Hu et al., 1996; Qiang et al., 1998a). By placing the photobioreactors vertical the sunlight falling on a given ground area is spread over a larger reactor surface area. As a result, more algae are exposed to lower intensities, being able to maximize their photosynthetic efficiency (Posten, 2009). This light dilution effect is implemented nowadays within different new photobioreactor designs developed by, for example, Subitec (Germany), Solix Biofuels (USA) or Proviron (Belgium) (Morweiser et al., 2010). Unfortunately, the main factor limiting the productivity in these examples cannot be addressed yet since no production data are available and multiple variables affect the productivity at the same time. In order to assess the productivity as a function of light regime and, as such reactor orientation, laboratory experiments where all cultivation parameters can be defined and controlled are still needed.

In this work, the influence of a vertical reactor position under completely defined conditions on the productivity and photosynthetic efficiency of the green microal-

gae *Chlorella sorokiniana* was assessed. The horizontal reactor position was used as reference, and real summer irradiance conditions in southern-Europe were simulated with red light emitting diodes (LEDs). A panel photobioreactor with a light path of 14 mm was operated under chemostat conditions, both under a low and high dilution rate in order to determine the influence of the biomass concentration.

Materials and Methods

Microalgae maintenance and culture medium

Chlorella sorokiniana CCAP 211/8k (UTEX culture collection), was maintained in M-8a medium ($3 \cdot 10^{-2}$ M KNO_3; $5.4 \cdot 10^{-3}$ M KH_2PO_4;$1.5 \cdot 10^{-3}$ M Na_2HPO_4;$1.6 \cdot 10^{-3}$ M $MgSO_4$; $0.9 \cdot 10^{-4}$ M $CaCl_2$;$0.3 \cdot 10^{-3}$ M Fe-EDTA; $0.1 \cdot 10^{-3}$ M Na_2-EDTA; $1 \cdot 10^{-6}$ M H_3BO_3; $0.6 \cdot 10^{-4}$ M $MnCl_2$; $0.1 \cdot 10^{-4}$ M $ZnSO_4$; $7.3 \cdot 10^{-6}$ M $CuSO_4$) in Roux flasks at 25 ºC and 165 μmol photons m^{-2} s^{-1} (continuous illumination) inside a growth chamber with coolwhite lamps (Philips TL-D 30W/33-640, The Netherlands). The pH was adjusted to 6.7 and the culture was bubbled with 5%$_v$ CO_2-enriched air.

For the experiments in the photobioreactor, urea ($60 \cdot 10^{-3}$ M) was used as nitrogen source, and 3-fold concentrated medium was used to avoid nutrient limitation. The final concentration of phosphate buffer in the medium was also increased to 10 mM (instead of 6.9 mM) to ensure a final bicarbonate concentration in the medium of 0.94 mM, which is in equilibrium with 0.31 mM of dissolved CO_2 (see Appendix B).

Experimental conditions

A flat panel reactor, with a light path of 14 mm and a working volume of 1.7 L, was used in the experiments. Temperature was kept constant at 37 ºC ± 1 ºC by a water jacket (optimal temperature for *Chlorella sorokiniana* (Sorokin, 1959)). Cultures were continuously mixed at a flow rate of 1.5 L per liter of culture per minute with a mixture composed of compressed air and CO_2, which was partly recirculated through the reactor (Figure 4.1). The combined flow rate of fresh air and CO_2 was 250 ml min^{-1}, giving a recycle ratio of 1:10. The outlet gas, which leaves the reactor through a condenser to avoid evaporatory water losses, was analyzed on-line for oxygen using a Servomex paramagnetic transducer (Gas Purity Analyser) placed in a Servomex 4100 unit (Servomex,UK). The ratio of compressed air and/or CO_2 was automatically adjusted to maintain the pH at 6.7.

Illumination was provided by a red LED panel composed of red Luxeon III emitters (Lumileds, California, USA) peaking at 637 nm under operating conditions. The illuminated surface was 0.119 m^2 (A_r), and the illuminated volume 1.7 dm^3 (V_r). A Licor SA190 quantum sensor (Li-COR, Lincoln, NE) was placed on the front surface of the reactor (facing the lamps) and was used to continuously monitor and adapt the photon flux density (PFD) at the reactor surface. The PFD measured at this reference position was correlated to the average PFD on the light-exposed inner surface of the culture chamber by a correlation factor. To obtain this factor, the PFD inside the empty culture chamber was measured prior to each experiment at 45 different points distributed equally over the light-exposed surface. A graphical programming environment called LabView Virtual Instrument (Labview 7.1, National Instruments, Texas, USA) was used to monitor and control the entire system.

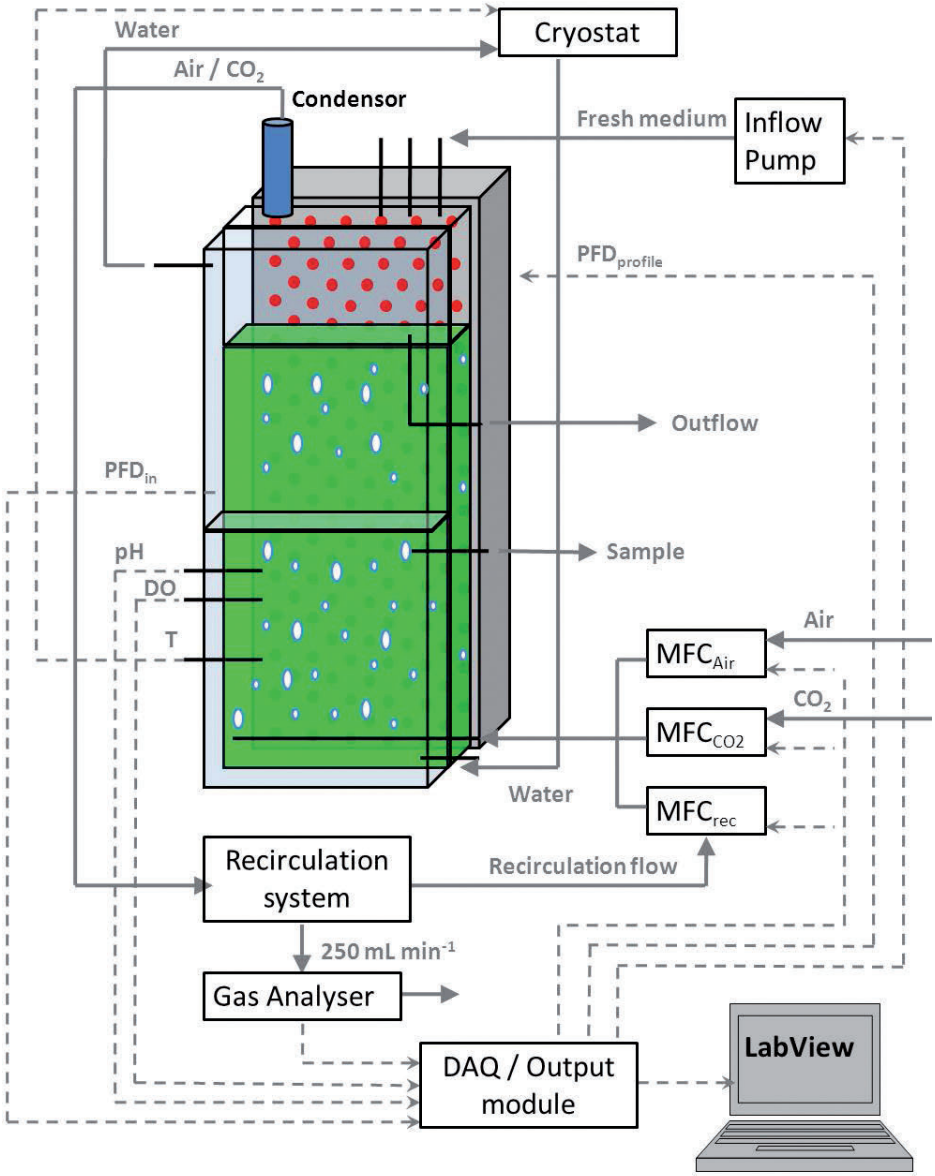

Figure 4.1: *Schematic view of the flat panel photobioreactor configuration. Temperature, pH and light control are indicated. A PAR quantum sensor is placed on the outer reactor surface facing the LED panel to record on-line the incident light intensity.*

In separate experiments the daily light cycle over a vertical and a horizontal surface was simulated and used to assess microalgae productivity. Instead of changing the position of the lab-scale photobioreactor, two different light profiles, where real outdoor irradiance data over a vertical and a horizontal surface were used, were simulated and applied by a red LED panel. During the illumination (day) period chemostat conditions were applied at two different dilution rates: 0.08 and 0.17 h^{-1}. Prior to reaching chemostat conditions a gradual cell acclimation to the light and dilution cycles was needed. First the algae were allowed to grow in batch at a fixed intensity of 500 μmol photons m^{-2} s^{-1}. When the biomass concentration was around 1 g Kg^{-1}, the light cycle was activated. When the biomass density reached 1.3 g Kg^{-1}, a constant dilution, at a lower rate than the one desired, was applied to the culture. Finally, when the biomass reached 2 g Kg^{-1}, the dilution rate was increased to the desired rate. In the end, the microalgae were exposed to a photoperiod of 14:10 (L:D), being diluted only during the light period (from 7 to 21 h) to avoid biomass wash out. The different cultivation conditions studied are summarized in Table 4.1.

Trial N°	Reactor orientation	PFD_d (mol photons m^{-2} d^{-1})	D (h^{-1})	
			Light period	Daily average
1	Horizontal	57.1	0.084	0.049
2			0.166	0.097
3	Vertical	12.2	0.079	0.046
4			0.165	0.096

Trial N°	C_x (g Kg^{-1})	Fluorescence (F_v/F_m)	Chl_{tot} (mg g^{-1})	Car_{ot} (mg g^{-1})	P_v (g Kg^{-1} d^{-1})	$Y_{x,E}$ (g mol^{-1})
1	2.41±0.08	0.70±0.02	41.1±1.8	7.4±0.7	2.8 ±0.11	0.60±0.09
2	1.71±0.05	0.69±0.03	25.9±1.5	5.1±0.2	3.97±0.09	0.85±0.02
3	1.21±0.10	0.75±0.03	45.1±2.3	7.9±0.8	1.33±0.13	1.29±0.13
4	0.55±0.04	0.75±0.11	32.9±3.2	5.9±0.7	1.26±0.06	1.23±0.06

Table 4.1: *Resume of the conditions and results of the chemostat experiments under simulated outdoor conditions. Data correspond to the analysis of the culture broth harvested daily during steady state for at least 6 days. Biomass concentration and volumetric productivity are expressed per kilogram of culture broth; Chlorophyll and carotenoids, per gram of dry matter; Biomass yield, per mol of PAR photons supplied.*

Light cycle simulation

The PVGIS database (see references, PVGIS) provides averaged solar irradiation data for 15 minutes intervals worldwide. June was chosen as a reference month, and the monthly-averaged irradiance data over a vertical and a horizontal surface under real sky-conditions in the province of Huelva, south of Spain (37º15'0" North, 6º57'0" West, 2 meters above sea level), were used in our simulation. Sigmaplot, statistic software, was used to interpolate the minute-by-minute irradiance data in order to apply a smooth light cycle to the photobioreactor.

Global real-sky irradiance data (including diffuse + beam irradiance) were used in the simulation of the light cycle over a horizontal photobioreactor. The vertical system was chosen to be oriented east-west in order to maximize the productivity (Zhang et al., 1999). With respect to the beam irradiance on the surface, it was assumed that the reactor was not shaded by other units in a hypothetical field of many panel photobioreactors. The final light cycle was the result of summing the different irradiance data: diffuse and beam irradiance on the south facing surface, and diffuse and beam irradiance on the north facing surface. Based on this ap-

proach, all light was assumed to enter the vertical reactor from the same side. The simulated irradiance profiles are shown in Figure 4.2.

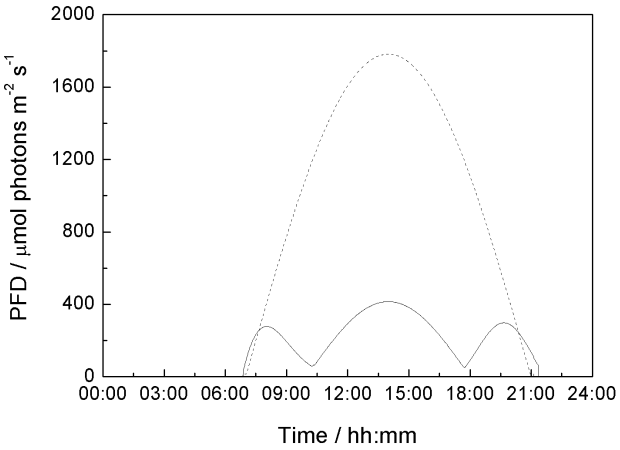

Figure 4.2: *The simulated irradiance profiles over a horizontal [..] and a vertical [-] surface are represented as the photon flux density in the PAR range (PFD).*

The light period covered 6:54 h to 21:23h in the vertical photobioreactor, and 6:53 h to 21:08 h in the horizontal photobioreactor. The profile in the vertical photobioreactor shows three peaks. The smaller ones in early morning and late afternoon reflect the beam irradiance falling on the north side of the panel. Outdoors, the larger fraction of irradiance (beam irradiance) will fall on the north photobioreactor side in the early morning and late afternoon. Around noon, the irradiance will fall on the south side. Based on this physical separation in time, in the lab-scale system the light could be safely applied from one side only without influencing the outcome of the experiments with respect to productivity or photosynthetic efficiency.

On-line gas analysis

In order to evaluate the oxygen production rate inside the photobioreactor, the part of the reactor outlet gas flow which was not recirculated but purged out

(250 mL min^{-1}, $F_{g,in}$) was first led through the gas analyser to monitor its oxygen content (X_{O2}).

To correct for the moisture content of the experimental gas data, before every chemostat experiment a dry and a wet baseline were measured. The dry baseline was performed by leading 250 mL min^{-1} of air over the gas analyser. For the wet baseline, the same air stream was sparged through the reactor, containing medium at the same temperature as during the experiments. The difference between the oxygen volume fraction in the dry and wet baseline ($X_{O2,db}$ and $X_{O2,wb}$ respectively) allowed us to correct the molar gas flow leaving the reactor for its water vapour content ($F^*_{g,out}$).

$$F^*_{g,out} = F_{g,in} \cdot \frac{X_{O_2,db}}{X_{O_2,wb}} \qquad [\text{mmol h}^{-1}]$$

The oxygen production rate (OPR, mmol h^{-1}) can then be calculated as follows:

$$OPR = \left(F^*_{g,out} \cdot \frac{X_{O_2}}{100} \right) - \left(F_{g,in} \cdot \frac{X_{O_2,db}}{100} \right) \qquad [\text{mmol h}^{-1}]$$

Dry weight and optical density determination

C. Sorokiniana samples, diluted 15 times with prefiltered demineralised water, were filtered through pre-washed, pre-dried and pre-weighed filters (glass fibre filters with a pore size of 0.7 μm) (Whatman GF/F, GE Healthcare UK Ltd, UK). Filters were then dried at 80 ºC during at least 16 h and cooled down in a dessicator for at least 2 h. The filter weight was determined on a 0.01 mg precision balance (Sartorius CP225D, Sartorius AG, Germany). The dry weight concentration (C_x), expressed as g Kg^{-1}, was calculated by differential weight.

The optical density was determined spectrophotometrically at 530 nm, 680 nm and 750 nm in a 1 cm light-path cuvette in an UV/Visible spectrophotometer (Ultrospec 3100pro, Amersham Pharmacia Biotech, Sweden).

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PSII maximum quantum yield

PSII fluorescence was used to measure the efficiency of Photosystem II of dark-adapted cells giving the maximum PSII quantum yield. This maximum quantum yield is widely accepted as a relative measure of photoinhibitory damage (Maxwell and Johnson, 2000). Samples were stored in a dark place at 0 ºC during 15 minutes and then transferred to the measurement cuvette of a Chlorophyll Fluorometer (PAM-210, Walz, Effeltrich, Germany). The measuring light (0.04 μmol photons m^{-2} s^{-1}) was switched on to measure the zero fluorescence level (F_0), and then a saturating light pulse (1850 μmol photons m^{-2} s^{-1}) was applied to measure the maximum fluorescence level (F_m). The maximum quantum yield of PSII (F_v/F_m) is then calculated as $(F_m-F_0)/F_m$.

Chlorophyll and Carotenoids content

Cell disruption for chlorophyll and carotenoids extraction was done according to Leu and Hsu, 2005. Two millilitres of culture were centrifuged (4400 rpm, 6 minutes) and pure methanol was added to the pellet. Samples were placed in an ultrasound bath for 5 minutes to disrupt the pellet and incubated at 60 ºC for 40 minutes. A temperature shock was then applied by transferring the samples to 0 ºC for 15 minutes. After a new centrifugation step, the absorbance of the supernatant was measured in the UV/Visible spectrophotometer. Modified Arnon's equations (Liechtenthaler, 1987) were used to calculate the chlorophyll and carotenoids concentrations in the extracts:

$$Chl_a = (16.72 \cdot A_{665} - 9.16 \cdot A_{652}) \cdot \text{dilution factor} \quad [\text{mg L}^{-1}]$$

$$Chl_b = (34.09 \cdot A_{652} - 15.28 \cdot A_{665}) \cdot \text{dilution factor} \quad [\text{mg L}^{-1}]$$

$$Chl_{tot} = Chl_a + Chl_b \quad [\text{mg L}^{-1}]$$

$$Car_{tot} = \frac{\text{dilution factor} \cdot 1000 \cdot A_{470} - 1.63 \cdot Chl_a - 104.96 \cdot Chl_b}{221} \quad [\text{mg L}^{-1}]$$

The cellular content of chlorophyll and carotenoids were expressed per gram of biomass and these were calculated based on the dry weight concentration in the samples used.

Statistics

Every measurement was done in duplicate unless otherwise indicated. Figures show means of the replicates.

Calculations

Productivity and biomass yield on light energy

The culture harvested during exactly 1 day (t_d) was collected on ice, weighed ($M_{harvest}$ in Kg) and its biomass concentration measured (C_x in g Kg^{-1}). Combining these data with the daily light input (PFD_d in mol m^{-2} d^{-1}) and illuminated surface (A_r) gives the biomass yield on light energy in grams of dry matter per mol of PAR photons supplied ($Y_{x,E}$).

$$Y_{x,E} = \frac{M_{harvest} \cdot C_x}{PFD_{in} \cdot A_r \cdot t_d} \quad [\text{g} \cdot \text{mol photons}^{-1}]$$

Taking into account the weight of the culture broth inside the reactor ($M_{reactor}$ in Kg) also the volumetric productivity in grams of dry matter per kilogram of culture broth (P_v) could be calculated as follows:

$$P_v = \frac{M_{harvest} \cdot C_x}{M_{reactor} \cdot t_d} \qquad [g \cdot Kg^{-1} \cdot d^{-1}]$$

Results and Discussion

Diurnal variations in PAR irradiance during the experiments are presented in Figures 4.2, showing typical summer radiation profile at the study site (Huelva, Spain), with a maximum PAR of 1785 μmol photons m^{-2} s^{-1} at 14:00 h when the photobioreactor is oriented horizontally, and 420 μmol photons m^{-2} s^{-1} for the vertical photobioreactor.

Chemostat conditions were applied under nutrient-replete conditions, light being the only limiting substrate. Before steady-state was reached, biomass acclimation to the light and dilution cycles was required as described in the materials and methods section. This acclimation period took around 8 days. At that moment, the sampling period started, which took another 8 days, during which the system was in steady state. The continuous culture outflow was collected on ice for every 24 hours interval and stored in the dark at 0 ºC for the daily analysis of culture parameters. The results of the daily collected biomass from the vertical and horizontal simulation were compared in terms of volumetric productivity, biomass yield on light energy, and biomass concentration (Table 4.1). In addition, punctual samples were taken every two hours from the photobioreactor along the day to study the algae behaviour during the light period. This procedure was repeated every day, being the sampling time different in order to cover the all light period.

Productivity and biomass yield

Volumetric productivity during the light period is the result of the product of the dilution rate imposed and the biomass concentration. The biomass concentration depends on the balance between specific growth rate of the microalgae and the dilution rate. The specific growth rate depends on the light exposure (dependent again on biomass concentration) and the photosynthetic efficiency, which will change along the day. All these dependencies make it very difficult to predict productivity when growing microalgae in a changing outdoor environment. In order to correlate productivity with the daily irradiance profile, laboratory experiments where real irradiance conditions are simulated while controlling the rest of the parameters are needed.

The maximal volumetric productivity was found for the horizontal photobioreactor (4.0 g Kg^{-1} d^{-1}) at the highest dilution rate (30% higher when compared with the low dilution rate). In the vertical photobioreactor, the maximal productivity (1.3 g Kg^{-1} d^{-1}) was reached at the lowest dilution rate, although the difference with the high dilution rate was modest (5% higher) (Table 4.1).

The biomass yield on light energy, on the other hand, was highest for the vertical photobioreactor. In this case, the biomass yield was 50% higher (1.3 g mol $photons^{-1}$) when compared with the horizontal positioning (0.85 g mol $photons^{-1}$), and maximal at the lowest dilution rate (0.08 h^{-1}). On the contrary, in the horizontal photobioreactor the maximal yield was found at the highest dilution rate (0.17 h^{-1}).

When cells were acclimated to low irradiance (vertical photobioreactor), the maximal productivity and biomass yield were found at the lowest dilution rate. However, when cells were acclimated to high light conditions (horizontal photobioreactor), the maximal biomass yield, and therefore the productivity, was found at the highest dilution rate. As the algae were exposed to a lower irradiance in the vertical position, cells were experiencing considerable light limitation and their specific

growth rate was affected. Under these conditions, the lowest dilution rate led to a higher biomass yield.

In the horizontal photobioreactor, on the other hand, the higher PFD resulted in a higher specific growth rate. The results of the horizontal position are in accordance with the results of our previous work (Cuaresma et al., 2009), where the maximal biomass yield under constant and over-saturating irradiance was found at a high specific growth rate of 0.24 h^{-1}. This work clearly showed the existence of an optimal combination of dilution rate and cell density leading to maximal productivity and biomass yield on light energy. In this sense, the productivity could be further improved by accurate assessment of the productivity and biomass yield as a function of dilution rate applied. Moreover, the dilution rate could be adjusted during the day to further optimize the productivity, a topic beyond the scope of this study.

Biomass concentration

The biomass concentration was higher for the horizontal positioning, and when the photobioreactor was operated at low dilution rate. During all the experiments, the maximal biomass concentration reached in the photobioreactor was 2.4 g Kg^{-1} and the minimal 0.5 g Kg^{-1} (Table 4.1).

Considering the daily biomass concentration evolution, the biomass concentration remained constant all light period along for the vertical photobioreactor: around 0.6 g Kg^{-1} at the high dilution rate (0.17 h^{-1}), and around 1.4 g Kg^{-1} at the low dilution rate (0.08 h^{-1}) (Figure 4.3). In the horizontal photobioreactor, a certain degree of cell wash out was observed at the beginning of the day (Figure 4.4). This was related to the dilution commence, together with the exposure of the cells to a fast-increasing light intensity (from 0 to almost 800 μmol photons m^{-2} s^{-1} in 2 hours). At the highest dilution rate, the biomass drop was more pronounced due to the

lower biomass concentration. But once the minimal biomass concentration was reached (around 9:00 h) it followed the same trend as the irradiance, with a maximal biomass concentration at 15:00 h. In the vertical photobioreactor the biomass concentration was not really affected due to the lower light intensity to which the cells were exposed at the beginning of the day.

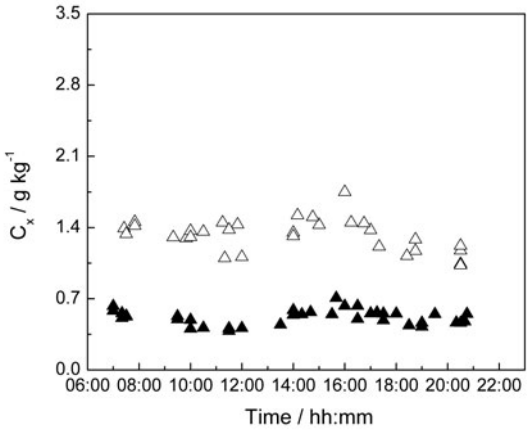


Figure 4.3: *Biomass concentration (C_x) of C. Sorokiniana along the day inside the vertical photobioreactor. Full symbols correspond to the highest dilution rate applied (0.17 h^{-1}) and open symbols to the lowest dilution rate (0.08 h^{-1}).*

In both photobioreactor orientations, biomass concentration was higher at the beginning of the day than at the end of the day. Since biomass accumulation during the dark period is by definition rejected, only the overestimation of the dry weight when sampling directly from the photobioreactor for the first time every day (covering the first two hours of the light cycle during the steady state) could explain that trend. We found that the biomass density of the daily harvested culture outflow was always lower than the calculated average biomass density if we take into account all punctual samples taken during the light period. But disregarding the samples collected before 9:00 h the calculated average biomass density (from punctual samples) compares well with the biomass concentration of the daily collected culture outflow. This finding does not have any implications for the measured photobioreactor productivity and photosynthetic efficiency since these were based on solely the daily collected culture outflow.

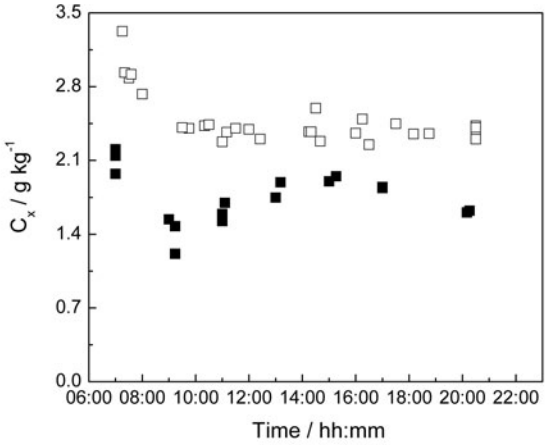


Figure 4.4: *Biomass concentration (C_x) of C. Sorokiniana along the day inside the horizontal photobioreactor. Full symbols correspond to the highest dilution rate applied (0.17 h^{-1}) and open symbols to the lowest dilution rate (0.08 h^{-1}).*

The biomass density which led to the maximal productivity and biomass yield in the vertical photobioreactor was 1.2 g Kg^{-1} (achieved at the lowest dilution rate), while in the horizontal it was 1.7 g Kg^{-1} (achieved at the highest dilution rate). These results are also in accordance with another work (Cuaresma et al., 2009), where maximal biomass yield was found at a cell density of 2.1 g Kg^{-1} at a constant light intensity (no day/night cycle) of 2100 mmol photons m^{-2} $s^{-1.}$ The results are also in agreement with the previous work of Zijffers et al. (2010), where the maximal biomass yields of *C. Sorokiniana* were found at biomass concentrations lower than 2 g Kg^{-1} when growing *Chlorella* at a constant light intensity of 900 μmol photons m^{-2} s^{-1} in panel photobioreactors of 1.25 and 2.15 cm deep. According to the work of Zijffers, at high biomass densities the biomass yield decreases because of the maintenance requirements. Takache and coworkers (Takache et al., 2010) also demonstrated the influence of biomass density on the final productivity, i.e. the productivity being maximal when the maximal amount of light is absorbed while there is still enough light deep inside microalgae cultures to compensate for respiration. In this sense, dark zones inside photobioreactors have to be prevented in order to achieve maximal photosynthetic efficiency, and therefore productivity. In the end, this results in an optimal biomass density which

is lower under lower irradiance.

Cell viability

In terms of cell viability, the maximal quantum efficiency of PSII was similar in both photobioreactors, and around 0.75, which is a typical value for non-stressed microalgae cells. It suggests that photoinhibition was not playing an important role during *C. Sorokiniana* cultivation. Nevertheless, a more detailed view to the fluorescence evolution along the day reveals two different profiles.

In the vertical photobioreactor, photoinhibition does not seem to occur since F_v/F_m remains constant all over the day for both dilution rate assayed (Figure 4.5).

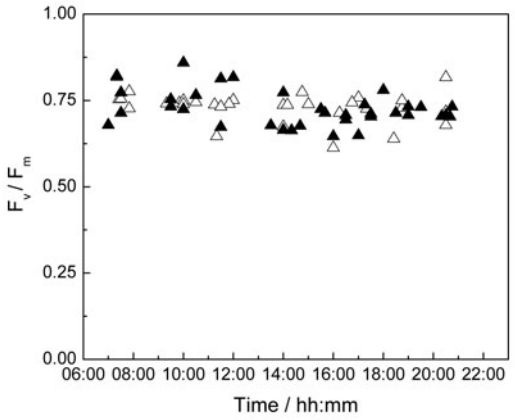


Figure 4.5: *Maximum PSII quantum yield (F_v/F_m) of C. Sorokiniana along the day inside the horizontal photobioreactor. Full symbols correspond to the highest dilution rate applied (0.17 h^{-1}) and open symbols to the lowest dilution rate (0.08 h^{-1}).*

In the horizontal photobioreactor, however, the maximal efficiency of PSII was affected by the high irradiance conditions and decreased from 9:00 h in the early morning to 15:00 h at solar noon (Figure 4.6). This effect was higher at the lowest biomass concentration. Nevertheless, the decrease was modest and the cells were able to fully recover at the end of the day under both dilution rates.

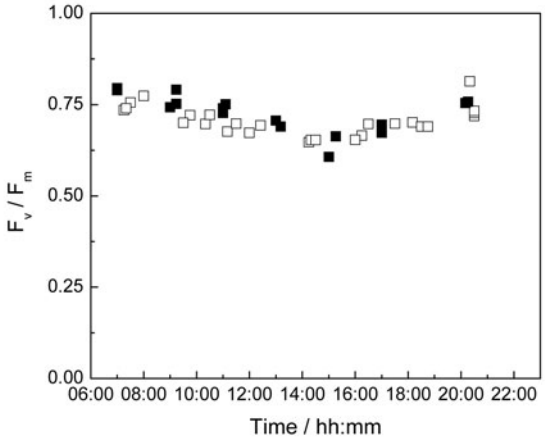


Figure 4.6: *Maximum PSII quantum yield (F_v/F_m) of C. Sorokiniana along the day inside the horizontal photobioreactor. Full symbols correspond to the highest dilution rate applied (0.17 h^{-1}) and open symbols to the lowest dilution rate (0.08 h^{-1}).*

The lower irradiance experienced by the cells and the high aeration rate (1.5 L L^{-1} min^{-1}) supplied to the photobioreactor, which allows the cells for moving through the light gradient inside the photobioreactor, might contribute to reduce the photoinhibition in the vertical position. However, in the horizontal photobioreactor a small drop of F_v/F_m when irradiance peaks might indicate photoinhibitory damage in response to excess photon flux (Maxwell and Johnson, 2000). Modified PSII centers, which are inactive in the electron transportation, can provide protection against high irradiance by dissipating the absorbed light as heat (Chow, et al., 2002). Moreover, as Jensen and Knutsen (1993) proposed, photoinhibition can be reversible and the degradation and regeneration of key components of photosynthetic apparatus coexist. Indeed in our experiments we see that *C. Sorokiniana* is able to fully recover its photosynthetic activity at the end of the day.

Figure 4.7 and Figure 4.8 show the oxygen production rate (OPR) at the lowest dilution rate (0.08 h^{-1}) for both the horizontal and vertical photobioreactor positioning. During the dark period cell respiration (negative OPR) is expected to occur.

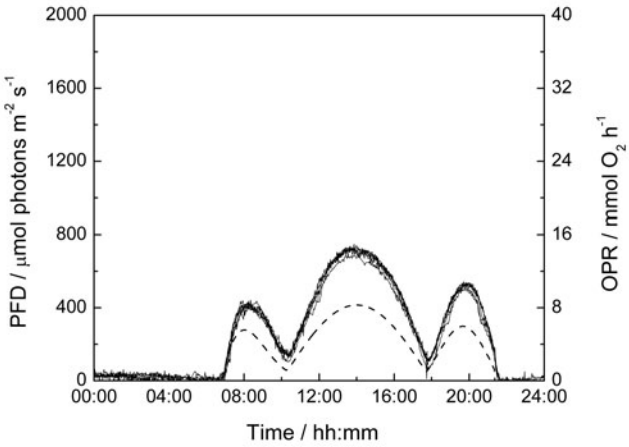


Figure 4.7: *Oxygen production rate (OPR) during the day under vertical positioning. The OPR during different days at the lowest dilution rate (0.08 h^{-1}) in the steady-state is represented [-]. The simulated irradiance profile, expressed as the photon flux density in the PAR range (PFD), is also represented [--].*

But due to the drift in the baseline during the dark period, related to the varying humidity of the gas, the magnitude of the respiration could not be accurately assessed. For this reason the (negative) OPR during the previous night was subtracted from the OPR during daytime. In this way the effect of the drifting baseline was removed and it can be clearly seen that the corrected OPR shows a reproducible trend for every day of the steady state period.

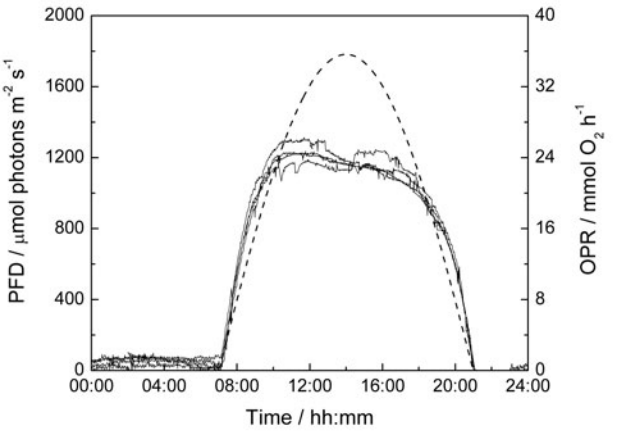


Figure 4.8: *Oxygen production rate (OPR) during the day under horizontal positioning. The OPR during different days at the lowest dilution rate (0.08 h^{-1}) in the steady-state is represented [-]. The simulated irradiance profile, expressed as the photon flux density in the PAR range (PFD), is also represented [--].*

The OPR was much higher when the photobioreactor was placed horizontal, and in both simulations the oxygen production followed the same trend as the irradiance. Nevertheless, in contrast to the vertical photobioreactor, in the horizontal position the photosynthetic activity saturated between 10:00 h and 16:00 h, when the irradiance was above 1200 μmol photons m^{-2} s^{-1}.

Based on the observation that photoinhibition was modest we conclude that the photosynthesis saturation observed in the horizontal photobioreactor (Figure 4.8) must be related to the activation of NPQ processes in order to handle the over-saturating light conditions (PFD above 1200 μmol photons m^{-2} s^{-1}).

Chlorophyll and carotenoids

The chlorophyll and carotenoids content of the microalgae was higher in the vertical photobioreactor (45 mg of Chl_{tot} per gram of dry biomass and 8 mg of Car_{tot} per gram of dry biomass respectively) (Figures 4.9, 4.10, 4.11 and 4.12).

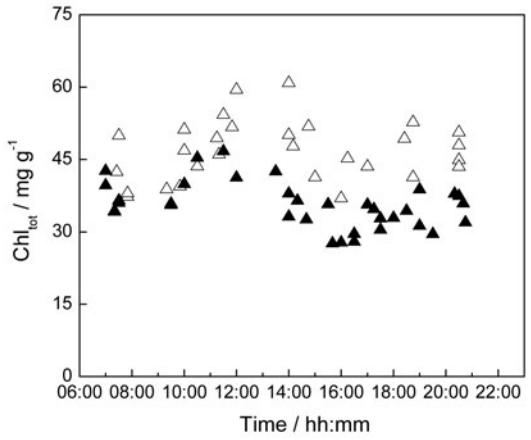


Figure 4.9: *Chlorophyll content (Chl_{tot}) of C. Sorokiniana along the day inside the vertical photobioreactor. Full symbols correspond to the highest dilution rate applied (0.17 h^{-1}) and open symbols to the lowest dilution rate (0.08 h^{-1}).*

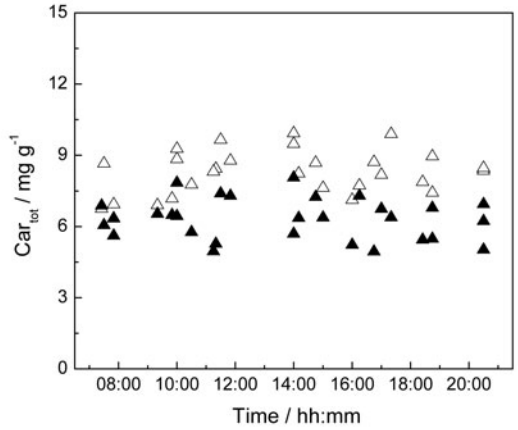


Figure 4.10: *Carotenoids content (Car_{tot}) of C. Sorokiniana along the day inside the vertical photobioreactor. Full symbols correspond to the highest dilution rate applied (0.17 h^{-1}) and open symbols to the lowest dilution rate (0.08 h^{-1}).*

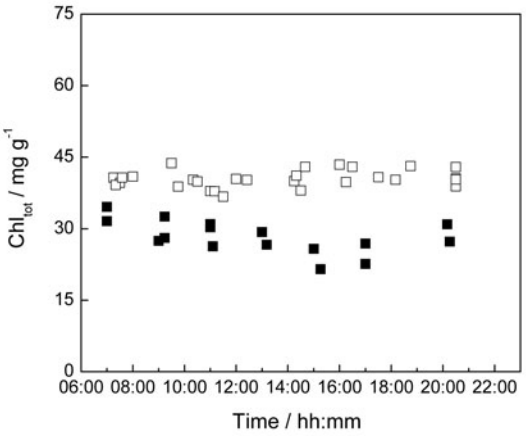


Figure 4.11: *Chlorophyll content (Chl_{tot}) of C. Sorokiniana along the day inside the horizontal photobioreactor. Full symbols correspond to the highest dilution rate applied (0.17 h^{-1}) and open symbols to the lowest dilution rate (0.08 h^{-1}).*

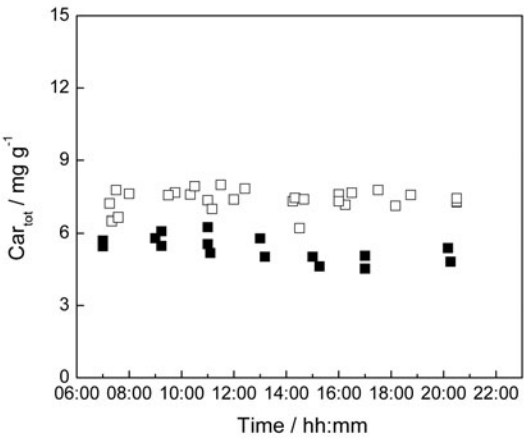


Figure 4.12: *Carotenoids content of C. Sorokiniana along the day inside the horizontal photobioreactor. Full symbols correspond to the highest dilution rate applied (0.17 h^{-1}) and open symbols to the lowest dilution rate (0.08 h^{-1}).*

In both simulations the cellular pigment content was maximal at the highest biomass concentration, achieved at the lower dilution rate (0.08 h^{-1}), and it remained constant all day along. Only a slight increase around midday was found in the case of the vertical photobioreactor (Figure 4.9).

Carotenoids per chlorophyll ratio remained constant along the day but it was slightly higher in the horizontal position (0.20 versus 0.18 in the vertical photobioreactor).

The higher chlorophyll and carotenoids content in the vertical photobioreactor is in accordance with the fact that the microalgae cells are acclimated to low light conditions. Light acclimation includes physiological changes in order to capture more light when light becomes limiting. Increasing intracellular concentration of chlorophyll and accessory pigments are among the most important changes. The content of the light-harvesting pigments decreases when the cultures are cultivated under high light (horizontal position) (Dubinsky and Stambler, 2009; Kromkamp et al., 2009).

Implications of photobioreactor position

The limitation imposed on bioproductivity by the light-saturation effect has long been recognized (Masojídek et al., 1999; Melis, 2009). In our case, the vertical photobioreactor resulted in a more efficient configuration where only 30% of the daily supplied light was dissipated as heat (assuming a maximal biomass yield of 1.8 g mol^{-1}). Nevertheless, in the horizontal photobioreactor these losses were close to 60% on daily basis. As commented before, this can also be inferred from the oxygen production data (Figure 4.8), where it can be seen that photosynthesis saturated at 10:00 for the horizontal photobioreactor when irradiance was above 1200 μmol photons m^{-2} s^{-1}, and only after 16:00 h the rate of photosynthesis (oxygen production) correlated again with the decreasing PFD.

Cuaresma et al., (2009) calculated a maximal biomass yield on light energy of 1.8 g dry matter per mol of PAR photons. This calculation is based on a number of assumptions among which neglecting energy requirements for maintenance purposes and biomass assembly are critical. Consequently, this "maximal" efficiency will be lower in practice. Taking into account the fact that energy requirements for maintenance and biomass assembly are not included in the calculation of the maximal biomass yield on light energy, the photosynthetic efficiency of *Chlorella sorokiniana* obtained in the vertical photobioreactor is very high (1.3 g mol^{-1} versus 1.8 g mol^{-1}). Even more so when considering that also night biomass loss and maintenance requirements were included in this overall efficiency factor.

Extrapolation from laboratory data to large scale outdoor production is very complicated because it requires optimization of operational parameters. Nevertheless, a rough estimation of the annual areal productivity at the study site has been made for a system of vertical panel photobioreactors. For this we used the biomass yield on light energy obtained (1.3 g mol^{-1}) and the yearly averaged solar irradiance at the study site (PVGIS, 4.79 kWh m^2 d^{-1}). Based on these results

we can extrapolate that 160 tons of dry matter could be produced per ha per year in southern-Spain.

In this calculation we thus assumed that our red LED light is representative for sunlight, that all solar irradiance on the ground surface can be collected by an optimized field of panel photobioreactors, that there is no negative effect of panel shading on photosynthetic efficiency, and that the photosynthetic efficiency obtained for June can be extrapolated to the whole year. Although red LEDs were used to simulate solar irradiance in our study, this extrapolation to sunlight seems to be possible according to the old action spectra of photosynthesis, in which any photon within the PAR range will be used in photosynthesis approximately at the same efficiency (Emerson and Lewis, 1943). In terms of annual biomass production, a higher photosynthetic efficiency during spring and autumn (where algae are exposed to lower light levels) will be expected, however it will be lower during winter time. Extrapolation of the photosynthetic efficiency obtained during June to the all year can thus be considered as a reference value. Another example of a productivity estimation can be found in Grobbelaar (2010), where a maximal areal productivity on average for the earth of 50 tons ha^{-1} $year^{-1}$ was suggested. Nevertheless, maximal photosynthetic efficiency and the average solar radiation reaching the earth's surface were used in the calculations.

Despite the lower volumetric productivity obtained in the vertical disposition (related to the lower volumetric light supply rate), we show that the productivity per ground area could be enhanced by placing more vertical photobioreactor-units in the same ground area instead of using a single horizontal system. However, it must be addressed that in a real system of vertical panel reactors the distance between the panel rows, the orientation of the rows, as well as panel height have to be optimized in terms of shading and sunlight collection. This is nicely illustrated by Slegers et al. (2011), where the influence of varying light regimes (location) and photobioreactor layout on biomass productivity was theoretically predicted.

Conclusions

It was demonstrated that the photosynthetic efficiency of microalgae cultures can be greatly improved by placing outdoor photobioreactors vertically and optimizing the dilution rate of the system. Further optimization of vertical photobioreactors disposition is needed in order to maximize areal productivity.

Biomass concentration plays an important role in algae acclimation to light conditions. The control of biomass density and/or dilution rate can be considered to be the most important tool to maximize productivity of outdoor photobioreactors.

Appendix A. Calculation maximal productivity in the study site

Based on solar irradiation data from PVGIS (see references) an estimation of the theoretical maximal biomass productivity in the south of Spain has been made.

In the calculation, several data and assumptions have been considered:

- averaged yearly global irradiance data in Huelva (37º15'N, 6º57'W) was used (4790 Watt hours per square meter per day)
- 43 % of global irradiance corresponds to the PAR range
- the average energy content of PAR photons is 218 KJ mol^{-1}
- 1.8 g of biomass per mol of PAR photons absorbed was calculated to be the maximal biomass yield on light energy for *Chlorella* grown on ammonium or urea as explained by Cuaresma et al., 2009. Energy requirements for maintenance and biomass assembly are neglected in this calculation.

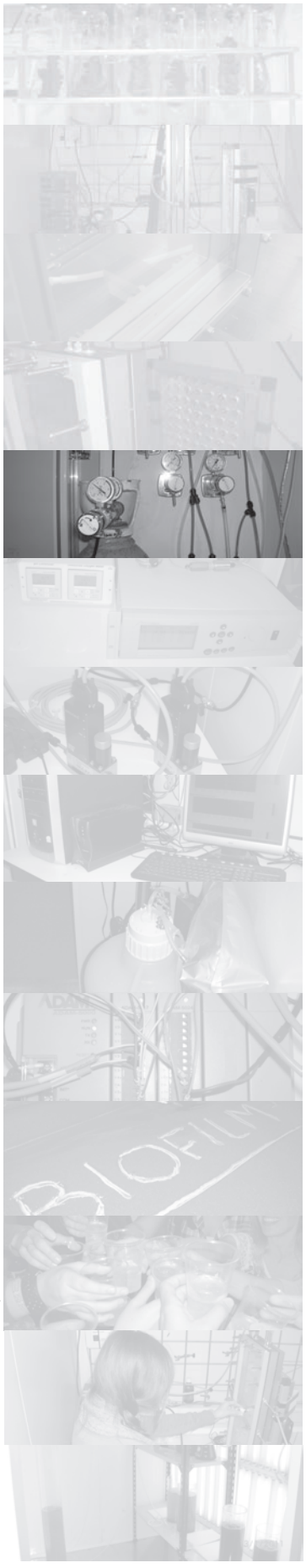
According to these calculations, a maximal amount of 220 tonnes of biomass per hectare per year can be produced in the study site.

Appendix B. Adaptation of M8-a medium for the experiments in the photobioreactor

The photobioreactor was operated as chemostat, where a constant dilution rate was applied during the light period. To ensure the presence of dissolved CO_2 in the medium stored in the inflow vessel, the final concentration of phosphate buffer in the culture medium M8-a was modified. In this sense, a concentration of 10 mM of phosphate was used instead of 6.9 mM (concentration in the original medium used for algae maintenance) and the pH was set at 6.9. When this new medium enters in the reactor, part of the CO_2 added to adjust the pH reacts with water forming carbonic acid. The protons of the acid react then with the base part of the phosphate buffer (HPO_4) and bicarbonate is formed until the pH is 6.7. Under these settings, 0.94 mM of bicarbonate is formed, which is in equilibrium with 0.31 mM of dissolved CO_2, which is finally in equilibrium with 1.20% v/v in the gas phase.

Chapter 5

Luminostat operation: a tool to maximize microalgae photosynthetic efficiency in photobioreactors during the daily light cycle?



This chapter has been submitted as:
Cuaresma, M., Janssen. M., van den End, F., Vílchez, C., Wijffels, R.H., Luminostat operation: a tool to maximize microalgae photosynthetic efficiency in photobioreactors during the daily light cycle?

5

Abstract

Continuous adaptation of biomass concentration inside photobioreactors to maximize light absorption and prevent the development of a dark zone, the luminostat regime, could lead to a higher photosynthetic efficiency.

Outdoor light conditions in Huelva (Spain) in June were applied to a lab-scale photobioreactor operated as a luminostat. The PFD transmitted (PFD_{out}) was maintained constant by automatic adjustment of the dilution rate. Different settings for PFD_{out}, ranging from 4 to 20 μmol photons m^{-2} s^{-1}, were used to assess the productivity and photosynthetic efficiency of *Chlorella sorokiniana*.

Maximal volumetric productivity (1.22 g Kg^{-1} d^{-1}) and biomass yield on PAR photons absorbed (1.27 g mol^{-1}) were found when PFD_{out} was maintained between 4 and 6 μmol photons m^{-2} s^{-1}. The luminostat resulted in a comparable photosynthetic efficiency as a chemostat control reported previously. Adaptation of the biomass density to the irradiance did not lead to any improvement as it was hypothesized.

Introduction

Microalgae production for fuels or bulk products is close to being economically feasible nowadays. A price of 0.68 € per kilogram of biomass has been calculated to be feasible optimizing the biomass production in terms of irradiation, mixing, photosynthetic efficiency, and medium and carbon dioxide supply (Norsker et al., 2011).

Currently, much effort is focused on increasing photosynthetic efficiency of microalgae. For example, Norsker et al. (2011) showed that the cost price of production of algal biomass in a 100 ha plant of flat panel photobioreactors will decrease by 37.5% when the photosynthetic efficiency (PE) increases from 5 to 8%. Different strategies have been adopted to increase the PE in photobioreactors: minimizing the antenna size (Melis, 2009; Neidhardt et al., 1998), decreasing the light path of photobioreactors while increasing turbulence in high cell density cultures (Kliphuis et al., 2010; Qiang et al., 1998b), and light dilution (Cuaresma et al., 2011b; Pulz and Scheibenbogen, 1998).

The advantage of light dilution is that no energy is required to increase PE, while increase of turbulence could cost more energy than gained. Also genetic engineering can not be considered an option nowadays due to the European perception of genetically modified organisms (GMOs) and the very strict regulations surrounding the application of these GMOs. Moreover, genetic modifications are not stable in time. The “light dilution effect” therefore, can be considered one of the best and simpler strategies to increase the PE (Wijffels and Barbosa, 2010). By light dilution the light intensity at the reactor surface is reduced and with that the effect of (over)-saturating light conditions is minimized. Light dilution by placing the reactor units vertically already proved to increase the photosynthetic efficiency (Cuaresma et al., 2011b).

Solar irradiance varies from zero to saturating or over-saturating light levels during a single day. The outdoor production of microalgae therefore is also restricted

by light limitation at the beginning, and end, of the day, and during the night period. Moreover, mutual shading of the cells at low sunlight levels or high biomass concentration will result in a dark zone inside the culture with negative rates of photosynthesis (respiration). This leads to a lower biomass productivity and lower overall photosynthetic efficiency.

Besides the daily variation in light intensity, irradiance also varies throughout the year and outdoor cultivation clearly leads to a more complex operation process than during continuous cultivation based on artificial light. Luminostat conditions, where the biomass concentration is continuously adapted to the irradiance, could lead to a reduction of photosaturation and photolimitation along the day and the year. Such a luminostat regime should ensure maximal absorption of the sunlight received without allowing a dark zone to develop inside the photobioreactor (Pruvost et al., 2011; Slegers et al., 2011). In this sense a higher photosynthetic efficiency and productivity could be expected.

Takache et al. (2010) also stated that controlling the light transmission during continuous cultivation and, as such the biomass concentration, will lead to maximal volumetric productivity. They showed that the light intensity at the rear of the photobioreactor should be just sufficient for photosynthesis to compensate respiration, the so-called compensation point for photosynthesis. This optimal light intensity at the back of the photobioreactor (PFD_{out}) can be easily, and automatically, controlled resulting in an optimal biomass concentration throughout the day. Nevertheless, experimentally this has not been tested yet under real daily light cycles.

In this study the continuous adaptation of biomass density to changing light conditions according to the so-called luminostat regime was tested as a tool to improve photosynthetic efficiency and volumetric productivity of *Chlorella sorokiniana*. Red light emitting diodes (LEDs) were used to simulate real summer irradiance conditions in southern-Europe (Huelva, Spain) on a vertical panel photobioreactor with a light-path of 14 mm. The light intensity at the photobioreactor front

surface (PFD_{in}) was controlled according to the daily light cycle and the light intensity at the back of the photobioreactor (PFD_{out}) was varied from 4 to 20 μmol photons m^{-2} s^{-1}.

Materials and Methods

Microalgae and culture medium

Modified M-8a medium ($3 \cdot 10^{-2}$ M KNO_3; $5.4 \cdot 10^{-3}$ M KH_2PO_4;$1.5 \cdot 10^{-3}$ M Na_2HPO_4;$1.6 \cdot 10^{-3}$ M $MgSO_4$; $0.9 \cdot 10^{-4}$ M $CaCl_2$;$0.3 \cdot 10^{-3}$ M Fe-EDTA; $0.1 \cdot 10^{-3}$ M Na_2-EDTA; $1 \cdot 10^{-6}$ M H_3BO_3; $0.6 \cdot 10^{-4}$ M $MnCl_2$; $0.1 \cdot 10^{-4}$ M $ZnSO_4$; $7.3 \cdot 10^{-6}$ M $CuSO_4$) was used to maintain *Chlorella sorokiniana* CCAP 211/8k (UTEX Culture Collection) in Roux flasks inside a growth chamber at 25 ºC. The pH was adjusted to 6.7 and the cultures were bubbled with 5%$_v$ CO_2-enriched air. Continuous illumination (165 μmol photons m^{-2} s^{-1}) was provided with cool white lamps.

Urea ($60 \cdot 10^{-3}$ M) was used as nitrogen source during the photobioreactor experiments, and 3-fold concentrated medium was used to avoid nutrient limitation.

Photobioreactor set-up and operation

A 14 mm light-path panel photobioreactor with a working volume of 1.7 L was used (see Figure 5.1 and Cuaresma et al., 2011b). The illuminated area was 0.119 m^2. Temperature was maintained at 37 ºC (optimal growth temperature of *C. sorokiniana*) by a temperature-controlled water jacket. The microalgae culture was continuously mixed at a flow rate of 1.5 L per L of culture per minute (1.5 vvm) with a mixture composed of compressed air or nitrogen (N_2) and carbon dioxide (CO_2).

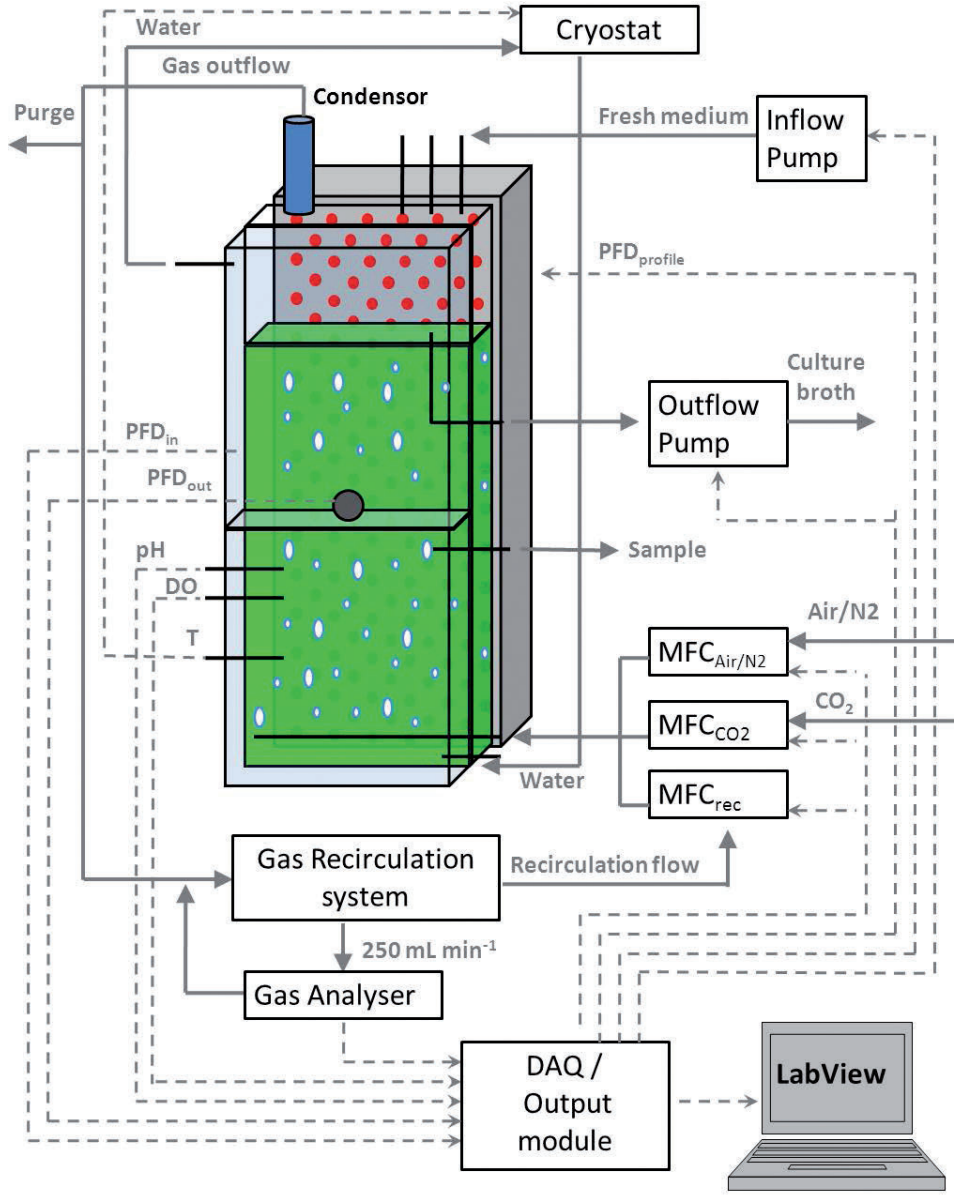


Figure 5.1: *Schematic view of the flat panel photobioreactor configuration. The reactor growth chamber is placed between the LED panel and the waterjacket. The solid lines represent the material flows (gas, water, medium and culture broth) and the dash lines the information flows (temperature, pH, DO, PFD, gas composition). A PAR quantum sensor is placed on the reactor surface facing the LED panel to record on-line the applied light intensity. This value was used to continuously adapt the light input to the desired light profile inside the photobioreactor. Another PAR quantum sensor is placed on the outer waterjacket surface to record on-line the transmitted light (PFD_{out}). This value was used to continuously adapt the biomass concentration inside the photobioreactor according to the desired PFD_{out} setpoint*

The gas was continuously re-circulated through the reactor (Figure 5.1) via a membrane pump and mass flow controller (MFC). The outlet gas left the reactor through a condenser to avoid evaporation. This gas was continuously analysed by a Servomex unit (Xentra 4100C, Servomex, UK) equipped with an oxygen paramagnetic transducer and a CO_2 infrared transducer and led back into the photobioreactor again. The concentration of O_2 and CO_2 inside the photobioreactor was controlled at 21% v/v and 2% v/v respectively by the automatic addition of N_2 or CO_2 via separate mass flow controllers. N_2 was added to remove photosynthetically produced O_2, and CO_2 to compensate for the CO_2 consumed (or the CO_2 lost from the system by N_2 addition). The surplus of gas was automatically purged from the system via an overflow valve. During night time there was no photosynthesis, solely respiration, leading to a lower O_2 concentration. For this reason a constant air flow of 50 mL min^{-1} was added to the re-circulated gas stream. Also during night the CO_2 level was controlled at 2% v/v and pure CO_2 had to be added to compensate for the CO_2 lost via the air bleed.

Illumination was provided by a panel of red LEDs (637 nm wavelength, see Cuaresma et al., 2009 for more details). The intensity inside the photobioreactor was automatically adapted according to the desired light cycle. A LiCor LI190 2π PAR quantum sensor was placed on the front surface of the reactor (facing the lamps) to monitor the PFD at the reactor surface (Figure 5.1). A correlation factor was used to continuously adapt the output of the lamps according to the desired intensity inside the photobioreactor, directly behind the transparent front plate. Another LI190 quantum sensor was placed behind the back surface of the reactor, after the water jacket, to continuously monitor and control the photon flux density leaving the photobioreactor (PFD_{out}). The sensor was placed at a position on the back surface such that the actual value corresponded to the surface-average of the PFD leaving the reactor. When PFD_{out} dropped below the set-point the excess of biomass was automatically removed by adding fresh medium with a peristaltic pump and at the same time microalgae culture was removed via the overflow to keep the culture volume constant.

The daily light cycle (PAR irradiance) on an east-west oriented vertical panel surface in June in Huelva (Spain, 37º15'0" North, 6º57'0" West) (Figure 5.2) was simulated and applied to the photobioreactor front side (see Cuaresma et al., 2011b for details on the light cycle simulation).

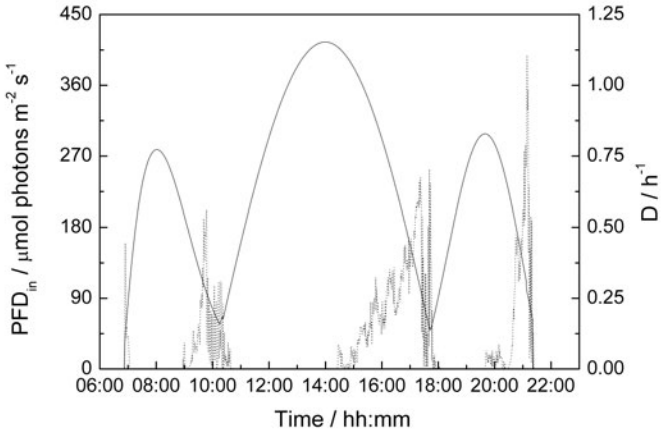


Figure 5.2: *Simulated irradiance profile (PFD, [-]), expressed as the photon flux density in the PAR range, and daily evolution of dilution rate (D, [..]), expressed per hour during the optimal settings (PFD_{out} = 6 μmol photons m^{-2} s^{-1}).*

Four different levels of PFD_{out} were tested: 20, 12, 6 and 4 μmol photons m^{-2} s^{-1}. As can be seen in the light profile, at the beginning and at the end of the day the PFD drops below 100 μmol photons m^{-2} s^{-1}. To prevent substantial biomass losses in this period, the PFD_{out} criterium was modified. Instead of using a fixed PFD_{out} during the all light period, the transmitted irradiance was set to only a fraction of the incident PFD when it was below 100 μmol photons m^{-2} s^{-1}. Moreover, the dilution control was completely stopped when PFD_{in} was below 30 μmol photons m^{-2} s^{-1} and during the night to avoid biomass wash out. Table 5.1 summarizes the different settings applied. The difference between the real PFD_{out} measured and the PFD_{out} desired (setpoint) will be discussed later.

Trial N°	Desired PFDout (µmol photons m^{-2} s^{-1})			Real PFD_{out} (µmol photons m^{-2} s^{-1})
	PFDout (PFD_{in} < 30)	PFD_{out} (30 < PFDin < 100)	PFD_{out} (PFDin>100)	
1	no dilution	15% of PFD_{in}	15	20
2	no dilution	10% of PFD_{in}	10	12
3	no dilution	5% of PFD_{in}	5	6
4	no dilution	2.5% of PFD_{in}	2.5	4

Table 5.1: *Resume of the main transmittance settings applied during the different experiments. The real transmittance is also included .*

Biomass analysis

Dry weight, optical density, PSII maximum quantum yield and cellular chlorophyll and carotenoids content were determined according to Cuaresma et al. (2011b).

Diluted *C. Sorokiniana* samples were filtered through Whatman GF/F glass fibre filters with a pore size of 0.7 µm and dried at 80 ºC during at least 16 h. Once the filters were cooled down, the dry weight (C_x, in grams of biomass per kilogram of culture broth) was calculated by differential weight using a 0.01 mg precision balance.

PSII fluorescence of dark-adapted cells was measured in a Chlorophyll Fluorometer (PAM-210, Walz, Germany) to evaluate the maximum PSII quantum yield. The maximum quantum yield of PSII (F_v/F_m) can be calculated as (F_m-F_0)/F_m.

Chlorophyll and carotenoids were extracted with pure methanol. Pellet disruption was done by placing the samples in an ultrasound bath and applying a temperature shock after (see Leu and Hsu, 2005 for more details). The extract absorbance was measured at specific wavelengths in an UV/Visible spectrophotometer and

modified Arnon's equations were used to calculate the pigment cellular content (Liechtenthaler, 1987). Based on the dry weight data, cell content was expressed per gram of dry biomass.

Statistics

Every measurement was done in duplicate unless otherwise indicated. Figures show means of the results.

Calculations

Productivity and biomass yield on light energy

The culture broth harvested every 24 hours (t_d, in 1 day) was collected on ice, weighed ($M_{harvest}$, in Kg) and its biomass concentration measured (C_x, in g Kg^{-1}). Combining these data with the daily light supplied to the photobioreactor (PFD = 12.52 mol m^{-2} d^{-1}) and the illuminated surface (A_r = 0.119 m^2) gives the biomass yield on light energy in g dry matter per mol of PAR photons supplied ($Y_{x,E}$).

$$Y_{x,E} = \frac{M_{harvest} \cdot C_x}{PFD_{in} \cdot A_r \cdot t_d} \qquad [g \cdot \text{ mol photons}^{-1}]$$

Considering the amount of light absorbed by the culture broth (PFD_{abs} = PFD_{in}-PFD_{out}) we can also calculate the amount of biomass produced per mol of PAR photons absorbed.

Taking into account the real culture broth weight inside the reactor ($M_{reactor}$, in Kg) also volumetric productivity (P_v) per day can be calculated.

$$P_v = \frac{M_{harvest} \cdot C_x}{M_{reactor} \cdot t_d} \qquad [g \cdot Kg^{-1} \cdot d^{-1}]$$

Results and Discussion

The light cycle simulated during the different experiments is presented in Figure 5.2. It represents the daily irradiance on a vertical surface oriented east-west during summertime (June) in Huelva, southern Spain. This irradiance profile was applied on one side of the photobioreactor. The maximum PFD_{in} was reached around 14:00 h and was 420 μmol photons m^{-2} s^{-1}. The final light cycle was the result of summing the diffuse and beam irradiance on the south facing photobioreactor surface, and diffuse and beam irradiance on the north facing photobioreactor surface. The smaller irradiance peaks found in the early morning and the late afternoon reflect the beam irradiance falling on the north side of the panel (see Cuaresma et al., 2011b for more information).

The reactor experiments were performed under nutrient-replete conditions and light was the sole factor limiting growth. Nutrients were supplied in excess and the carbon dioxide concentration in the outgoing gas stream was always maintained at 2% v/v. This, in combination with a gas flow rate of 1.5 vvm, resulted in a high CO_2 transfer capacity which prevented CO_2 limitation (calculation not shown). Moreover, oxygen was not allowed to accumulate, and the oxygen concentration in the outgoing gas maintained at the air level of 21% v/v. The dissolved oxygen concentration in the liquid phase was also monitored and varied from 130% air-saturation at peak irradiance and 100% air saturation during the night period (data not shown).

Before steady-state was reached, biomass acclimation to the light cycle and dilution was required. This acclimation period took around 8 days. At that moment, the sampling period started, which took another 10 - 14 days, during which the system was in steady state. The culture outflow was collected on ice for every 24 hours interval and stored in the dark at 0 ºC prior the daily analysis of culture parameters. The results of the luminostat experiments were compared in terms of volumetric productivity, biomass yield on light energy, and biomass concentration

(Table 5.2). In addition, punctual samples were taken from the photobioreactor during the light period to study the culture evolution along the day.

Trial N°	PFD_{out} (µmol photons m^{-2} s^{-1})	D (h^{-1})		C_x (g Kg^{-1})
		Light period	Daily average	
1	20	0.118	0.071	0.59±0.03
2	12	0.109	0.066	0.75±0.05
3	6	0.093	0.056	0.87±0.04
4	4	0.076	0.046	1.11±0.09

Trial N°	Fluorescence (F_v/F_m)	Chl_{tot} (mg g^{-1})	Car_{ot} (mg g^{-1})	Pv (g Kg^{-1} d^{-1})	$Y_{x,E}$ (g mol^{-1})	
					(g mol photons^{-1} absorbed)	(g mol photons^{-1} supplied)
1	0.78±0.04	35.5±1.3	5.6±0.9	1.04±0.05	1.14 ±0.05	1.05±0.05
2	0.71±0.05	37.4±2.7	6.7±0.6	1.18±0.07	1.26±0.08	1.19±0.07
3	0.75±0.03	40.6±0.9	6.8±0.3	1.22±0.09	1.27±0.10	1.23±0.10
4	0.75±0.02	43.3±2.7	6.8±0.4	1.22±0.06	1.25±0.06	1.23±0.06

Table 5.2: *Results of the luminostat experiments under simulated outdoor conditions in a vertical photobioreactor. Data correspond to the analysis of the culture broth harvested daily during steady state for at least 6 days. The average over this period is shown together with the standard deviation over the daily measurements. Biomass concentration and volumetric productivity are expressed per kilogram of culture broth; Chlorophyll and carotenoids, per gram of dry matter; Biomass yield, per mol of PAR photons absorbed and per mol of PAR photons applied.*

The luminostat control did not work perfectly when irradiance quickly increased in time (Figure 5.3) and the average PFD_{out} during the whole light period was slightly higher than the setpoint fixed (Table 5.1). The presence of a quantum sensor on the back surface of the photobioreactor allowed us to correct for the real transmittance data. For the PFD_{out} we always used this actual measured PFD_{out} averaged over the day. The deviation in the PFD_{out} therefore does not have any implication on the calculation of the biomass yield on absorbed light.

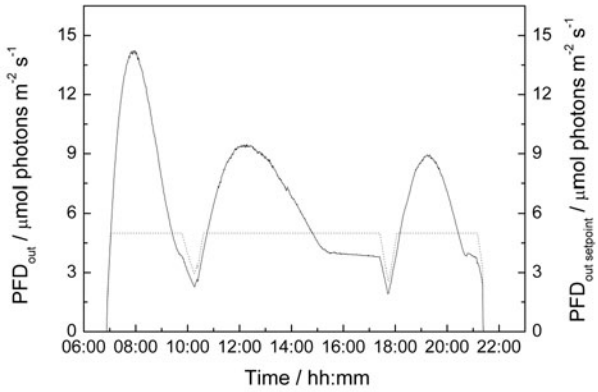


Figure 5.3: *Resultant transmittance (PFD_{out}, [-]), and desired transmittance ($PFD_{out\ setpoint}$, [..]) expressed as the photon flux density not absorbed through the photobioreactor during the optimal settings (PFD_{out} = 6 µmol photons m^{-2} s^{-1}).*

Nevertheless it is important to discuss the fact that it was not possible to exactly maintain the set-point throughput the day. As can be seen in Figure 5.3, the slow biomass growth when irradiance increased rapidly in early morning led to a transmittance higher than the setpoint. In other words, biomass growth could not keep up with the increase in irradiance. In early afternoon and late afternoon when irradiance levels increased again this effect was less and probably this is related to the fact that the biomass specific growth rate was higher at that time.

According to the different PFD_{out} settings, the averaged dilution rate during the light period ranged from 0.08 h^{-1} to 0.12 h^{-1}. A similar daily profile in the dilution rate was observed during all the conditions assayed. Figure 5.2 shows the evolution of the dilution rate at the optimal PFD_{out} (6 µmol photons m^{-2} s^{-1}). As can be observed, dilution was only needed early in the morning (from 9:00 h to 11:00 h), during the central hours (from 14:00 h to 18:00 h) and at the end of the day (from 20:30 h to 21:30 h). Dilution always starts when irradiance peaks, being maximal few hours after. The dilution is related to the microalgae growth, which is directly related to the irradiance. In addition, culture dilution is needed in the declining phase of the irradiance since the biomass concentration must be decreased in

order to maintain the PFD_{out} at a constant level while PFD_{in} decreases.

The oxygen and carbon dioxide concentration in the outgoing gas was kept constant at 21% v/v and 2% v/v respectively. Evolution of photosynthetic oxygen will lead to O_2 accumulation during the day in the microalgae culture broth and the recirculated gas stream. This was continuously compensated by refreshing the gas stream with pure nitrogen. The N_2 flow supplied is also given in Figure 5.4. It can be clearly seen that N_2 addition and, as such O_2 evolution, directly follow changes in irradiance. At the same time the carbon dioxide is consumed by the microalgae and this was compensated by adding pure CO_2 to the recirculated gas stream. Also the CO_2 addition directly correlates with irradiance and O_2 evolution (Figure 5.4).

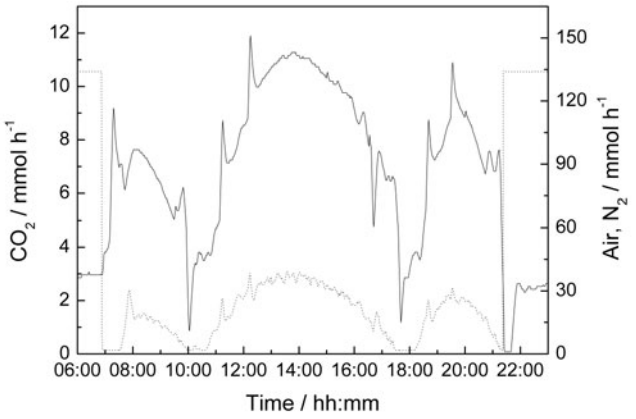


Figure 5.4: *Carbon dioxide (CO_2, [-]), and nitrogen or air supply (Air/N_2, [..]), expressed as mmol of gas per hour during the optimal settings (PFD_{out} = 6 μmol photons m^{-2} s^{-1}).*

During the night period the addition of compressed air was needed due to algae respiration. For this reason the re-circulated gas stream (2.6 L min^{-1}) was refreshed with a constant air flow of 50 mL min^{-1}. The surplus gas was purged out of the system and CO_2 was lost with this bleed. This was compensated by the automatic addition of fresh CO_2, even during the night period (Figure 5.4), to maintain the CO_2 level at 2% v/v.

Based on the photobioreactor configuration pH control was done indirectly via the control of the CO_2 concentration in the gas phase. The pH remained constant along the day around 6.4, despite the changes in CO_2 addition related to algal growth. This shows that there was sufficient buffer capacity in the medium based on the phosphate buffer, as well as the carbon dioxide-bicarbonate buffer. The resultant pH was in the range of that considered optimal for *Chlorella sorokiniana* (pH between 6.0 and 7.0) (Yoshida et al., 2006).

Biomass concentration and cell viability

Similar trends in the analysed biomass parameters have been found for the different levels of PFD_{out} assayed. As an example, data from the highest and the lowest PFD_{out} applied (20 and 4 μmol m^{-2} s^{-1}) are shown. As can be seen in Figure 5.5, the biomass concentration during the day followed the same trend as the irradiance, with a maximum around 15:00 – 16:00 h.

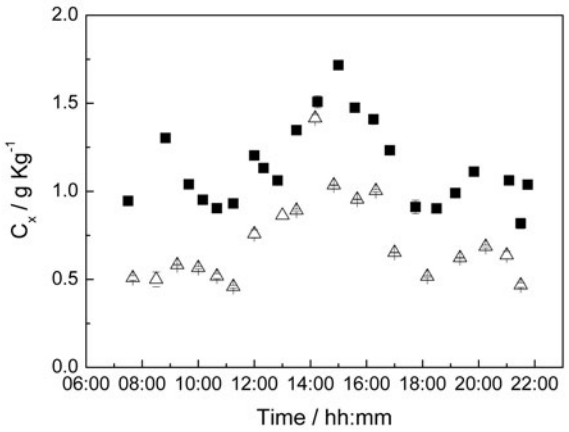


Figure 5.5: *Biomass concentration (C_x) of C. Sorokiniana along the day. In steady-state, and during the light period, punctual samples from the photobioreactor were analysed every two hours. This procedure was repeated every day, being the sampling time different in order to cover the all light period. Data represent the maximal [△] and minimal [■] PFD_{out} applied.*

The biomass evolution along the day was also assessed in another work (Cuaresma et al., 2011b), where chemostat conditions were applied under the same light cycle. During chemostat operation the biomass density remained constant over the day at 1.2 g kg^{-1}. However, when applying the luminostat strategy in the present study, the biomass concentration followed the irradiance trend, showing a steep profile with a maximum around 1.6 g kg^{-1} and a minimum around 0.7 g kg^{-1} (data corresponding to the optimal PFD_{out} of 6 μmol m^{-2} s^{-1} which are not shown in Figure 5.5).

The average biomass density during a complete day/night cycle was 0.9 g kg^{-1} in case of PFD_{out} maintained at 6 μmol photons m^{-2} s^{-1}. The average biomass concentration for all the experiments are given in Table 5.2 and these ranged from 0.6 to 1.1 g Kg^{-1} for the different PFD_{out} settings. The maximal biomass density was found at the lowest PFD_{out} (4 μmol photons m^{-2} s^{-1}), and the minimal one at the highest PFD_{out} (20 μmol photons m^{-2} s^{-1}).

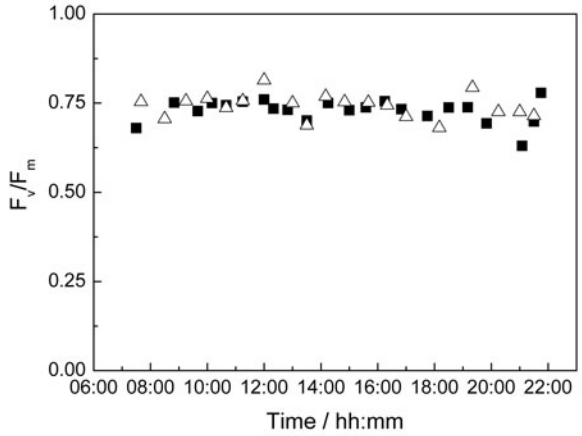


Figure 5.6: *Maximum PSII quantum yield (F_v/F_v) of C. Sorokiniana along the day. In steady-state, and during the light period, punctual samples from the photobioreactor were analysed every two hours. This procedure was repeated every day, being the sampling time different in order to cover the all light period. Data represent the maximal* [△] *and minimal* [■] *PFD_{out} applied.*

No differences were found in the maximal quantum efficiency of PSII during the different settings, remaining constant between 0.7 and 0.8 (Table 5.2), which are typical values for healthy microalgal cells. During the daily evolution, the absence of a drop in the fluorescence yield (F_v/F_m) when irradiance peaked shows that photoinhibition was not present (Figure 5.6).

Productivity and biomass yield

The maximal volumetric productivity, 1.22 g kg^{-1} d^{-1}, was found when the light transmission was around 6 μmol photons m^{-2} s^{-1}. Under these conditions, the biomass yield was also maximal, 1.27 grams of biomass produced per mol of photons absorbed (1.23 g per mol of photons supplied). However, no big differences in productivity or photosynthetic efficiency were found between the different settings applied. Productivity was improved about 17% and photosynthetic efficiency about 11% when decreasing PFD_{out} from 20 to 6 μmol photons m^{-2} s^{-1} (Table 5.2). It suggests we were actually working close to the optimal operational conditions.

The high biomass yield found for *Chlorella sorokiniana* under summer irradiance conditions suggests that only 30% of the daily irradiance was dissipated as heat. Assuming a theoretical maximal biomass yield of *Chlorella sorokiniana* of 1.8 g per mol of photons absorbed (Cuaresma et al., 2009), the maximal biomass yield reached during our experiments was high considering that maintenance and night biomass loss were included in the measured productivity, and that we were growing *C. Sorokiniana* under summer irradiance conditions. The light dilution effect, imposed by placing the photobioreactor vertical, and the luminostat control prevented over-saturating light conditions.

In both studies, also the optimal daily biomass concentration was around 1 g Kg^{-1}, although the daily evolution of the biomass concentration was different (see section on biomass concentration and viability). Surprisingly, also the maximal photo-

synthetic efficiency during luminostat operation is comparable to that achieved in chemostat mode in our previous study, where we found a $Y_{x,E}$ of 1.29 g mol^{-1}. In the case of those chemostat experiments all the light energy supplied was also absorbed because the photobioreactor back plate was made of stainless steel which reflects back the light transmitted through the culture. In the case of the luminostat experiments with a transparent lexan back plate we had to make a distinction between the biomass yield on supplied light energy and that on the light energy actually absorbed as described in the materials and methods.

Although a higher biomass yield was expected when optimizing the biomass concentration along the day, according to the luminostat operation, it was not observed during our experiments. A fixed compensation point of photosynthesis was assumed at the beginning of each experiment which was equal to the PFD_{out} which should be maintained. Possibly the compensation point is not a constant throughout the day and it could depend on the biomass growth rate and the light regime. Maintaining a fixed PFD_{out} in our system therefore might not be the most ideal solution. To come to an optimal control strategy more knowledge on the actual compensation point of photosynthesis is needed, and especially how it depends on the actual light regime and light history (i.e. acclimation state) of the microalgal cells. Also the interaction between the cell cycle and the day/night cycle is of interest. Different phases of the cell cycle might require different light regimes.

Chlorophyll and carotenoids

The chlorophyll and carotenoids content of the microalgae was maximal at the highest biomass concentration, achieved at the lowest PFD_{out} (43.3 mg of Chl_{tot} per gram of dry biomass and 6.8 mg of Car_{tot} per gram of dry biomass respectively). These results are in accordance with the general algae response when cells are acclimated to low light conditions. Under these conditions, an increment in the

light-harvesting pigments is expected in order to capture as much light as possible during light limitation (Dubinsky and Stambler, 2009; Kromkamp et al., 2009).

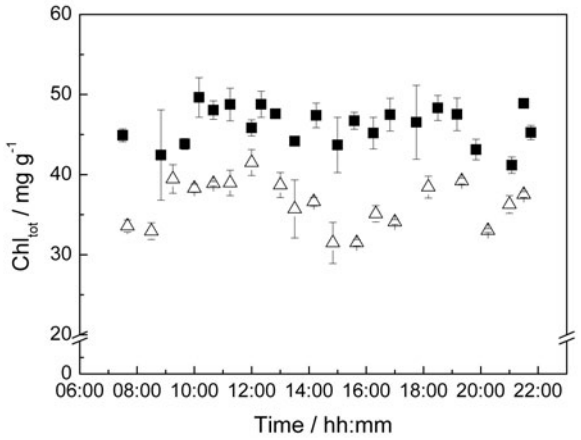


Figure 5.7: *Chlorophyll content (Chl_{tot}) of C. Sorokiniana along the day. In steady-state, and during the light period, punctual samples from the photobioreactor were analysed every two hours. This procedure was repeated every day, being the sampling time different in order to cover the all light period. Data represent the maximal [△] and minimal [■] PFD_{out} applied.*

The chlorophyll cell content showed two different trends during the different settings applied (Figure 5.7). When the average biomass density was maximal (1.1 g Kg^{-1}), at the lowest PFD_{out}, the chlorophyll content was also maximal and it remained constant along the day, despite the changes in irradiance. However, during the rest of the experiments at a higher PFD_{out}, the chlorophyll content showed the opposite trend when compared with the irradiance. Chlorophyll content was maximal when irradiance was minimal (around 11:00 h and 18:00 h), and minimal when irradiance was maximal (around 15:00 h). The higher photon availability, and the faster specific growth rate when PFD_{out} was higher, resulted in a continuous acclimation of pigmentation to the light conditions. However, when PFD_{out} was minimal, cells suffered a higher degree of light limitation, showing a higher and constant pigmentation along the day period.

Carotenoids cellular content showed a slightly descendent trend along the light period (Figure 5.8). The absence of carotenoids accumulation during the central

hours is another indication of the absence of over-saturation and photoinhibition (Dubinsky and Stambler, 2009).

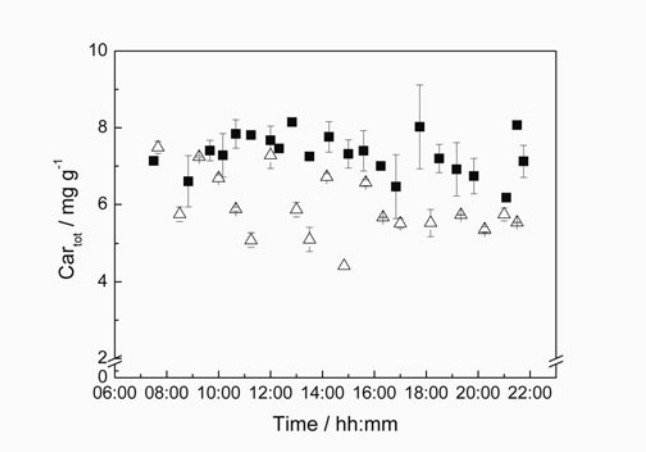


Figure 5.8: Total *carotenoids content (Car_{tot}) of C. Sorokiniana along the day. In steady-state, and during the light period, punctual samples from the photobioreactor were analysed every two hours. This procedure was repeated every day, being the sampling time different in order to cover the all light period. Data represent the maximal* [△] *and minimal* [■] *PFD_{out} applied.*

Implications of luminostat control on photosynthetic efficiency

Photosaturation and photolimitation are the main factors affecting the photosynthetic efficiency along the day. Luminostat operation, which continuously controls the light transmission and the corresponding biomass density in order to prevent the development of a dark zone and, at the same time, maximizing light capture, might lead to a higher efficiency.

However, the biomass yield obtained in this work (1.27 g mol^{-1} photons absorbed) is comparable, but not higher, than the yield obtained under chemostat conditions (Cuaresma et al., 2011b), where the dilution rate was kept constant over the day. Apparently adaptation of the biomass concentration to the irradiance is not so important as was suggested by Takache et al. (2010) and Pruvost

et al. (2011) . This finding could be related to microalgae growth kinetics. Possible the compensation point of photosynthesis and the level of respiration vary over the day. In this sense the PFD_{out} should not be fixed at a single value, as we did during our study to optimize productivity, but a more complex biomass density control procedure should be used.

The fact that similar biomass yields on light energy were obtained during both luminostat and chemostat operation is an indication that we were operating close to the maximal practical efficiency when growing *Chlorella sorokiniana* outdoors under summer irradiance conditions. Although the efficiency is lower than the theoretical maximal value, the biomass yield obtained during luminostat operation is very high: 1.3 g mol^{-1} versus 1.8 g mol^{-1}. Even more so when considering that energy requirements for cellular maintenance and biosynthesis, as well as night biomass loss, are not included in this theoretical maximal value but were included in the values we experimentally obtained.

Conclusions

Luminostat operation, together with the light dilution effect imposed by the vertical photobioreactor positioning, allows for efficient growth of *C. sorokiniana* during summer time in a high irradiance area, avoiding photoinhibition and excessive photosaturation. The maximal biomass yield found was 1.27 g of biomass per mol of photons absorbed.

The small differences in photosynthetic efficiency found for different PFD_{out} settings suggests that we were operating close to the optimal conditions.

The luminostat control, however, did not result in higher productivity and photosynthetic efficiency when compared with a traditional chemostat control (Cuaresma et al., 2011b).

Chapter 6

Cultivation of microalgae in a high irradiance area: biotechnological potential of Chlorella sorokiniana

6

Abstract

Microalgae are considered nowadays a promising source of high-value products as well as a source of feedstock for biofuels. Microalgae are photosynthetic organisms, the efficient use of light is one of the main prerequisites for successful industrial production processes.

During outdoor microalgae production the major factor limiting solar conversion efficiency in photosynthesis is the so-called light saturation effect. Maximization of photosynthetic efficiency in high irradiance areas, by avoiding or minimizing the photosaturation effect, will lead to higher productivities.

The potential biomass production of *Chlorella sorokiniana* in Huelva, southern Spain, is discussed based on experimental data. Summer irradiance conditions, as well as extreme winter conditions, were simulated in a lab scale photobioreactor to assess the effect on photosynthetic efficiency and productivity. The analysis of biomass composition under the different conditions applied revealed the potential of *Chlorella sorokiniana* as lutein producer.

Introduction

Added value molecules such as carotenoids, fatty acids, polysaccharides, vitamins and proteins can be obtained from microalgae. Algae are capable of accumulating heavy metals, fixing carbon dioxide, and are also considered a promising source of renewable energy. Algae-derived hydrogen, methane, triacylglycerols, and ethanol are potential materials for biofuels (Chisti, 2007; Hu et al., 2008; Rupprecht, 2009; Schenk et al., 2008; Wijffels et al., 2010). So far commercial production has only been developed in niche markets for high-value products. The world microalgal production is about 10 million kg of dry biomass per year and the biomass is mainly produced in China, Japan, Taiwan, USA, Australia and India (Benneman, 2008).

Potentially the productivity of culturing photoautotrophic microorganisms in high irradiance areas is higher because the availability of light. However, the efficiency of photosynthesis can be low due to the exposure to saturating or over-saturating light intensities. For most algal groups the saturation light intensity of photosynthesis (I_s) varies between 50 and 200 μmol photons m^{-2} s^{-1} (Goldman, 1979). Since during the central daylight hours the solar irradiance can exceed 2000 μmol photons m^{-2} s^{-1} the light saturation effect imposes a serious limitation on the efficiency with which solar energy can be utilized in outdoor algal cultures. In addition to that, it is difficult to control temperatures inside photobioreactors. In this sense, thermal-tolerant species with a higher optimal growth temperature should be considered a good alternative for outdoor production (Ono and Cuello, 2007; Sorokin and Myers, 1953).

Chlorella sorokiniana has potential as biomass producer in a high irradiance area as Huelva, southern Spain, because of its high growth rate, 0.27 h^{-1} and its tolerance to high irradiance, high temperature and high CO_2 concentrations (Matsukawa et al., 2000; Sorokin, 1959). Real outdoor irradiance conditions were simulated in a lab-scale panelar photobioreactor in order to assess the productivi-

ty and photosynthetic efficiency while controlling the rest of the operational parameters. Mainly over-saturating irradiance conditions were applied in order to find out the best strategy to minimize or avoid effects of photosaturation and photoinhibition. But according to the mesophilic character of *C. sorokiniana*, the influence of winter irradiance and temperature was also evaluated. The potential production of *C. sorokiniana* in Huelva, the study site, is discussed.

In parallel, the biomass composition under the different conditions applied was analyzed, which revealed the potential of *Chlorella sorokiniana* as lutein producer. The potential of commercial production of lutein with *C. sorokiniana* in Andalucía, southern Spain, will be discussed.

Photobioreactor design and microalgae strain

Laboratory experiments where all cultivation parameters can be defined and controlled have been used to study the main factor limiting the photosynthetic efficiency and productivity of microalgae; i.e. the varying light conditions over the day and over the year. In our studies a lab-scale panel photobioreactor (1.7 L of working volume) with a light path of only 14 mm was used. It allowed us to directly correlate the effect of the irradiance conditions on the photosynthetic efficiency and productivity of *Chlorella sorokiniana*, while keeping the rest of the operational parameters controlled (see Cuaresma et al., 2009, 2011b, Chapter 1 and Chapter 5 for more details). Red light emitting diodes (LEDs) were used to simulate the different irradiance conditions. Although LEDs could not be considered representative of the solar spectrum, none of the commercial lamps used to grow microalgae nowadays are. Moreover, according to the action spectra of photosynthesis any photon within the PAR range will be used in photosynthesis at roughly the same efficiency (Emerson and Lewis, 1943). This fact, in combination with the technical advantages of using LEDs (long lifetime, low power consumption, high

and homogeneous PFD, no heat radiation and ability to quickly modify the PFD), make LEDs a versatile and reliable light source.

Chlorella sorokiniana plays a role in a wide range of biotechnological applications. It is known as an interesting source for proteins (González et al., 2010) and carbohydrates (Watanabe et al., 2008), and more recently it has been used in wastewater treatment (Godos et al., 2009, 2010; González et al., 2008; de-Bashan et al., 2008), CO_2 mitigation (Morita et al., 2000; Ho et al., 2010), metal removal (Akhtar, et al., 2003, 2004; Chong, et al., 2000; Yoshida et al., 2006) and biofuel production (Amaro et al., 2010; Chen et al., 2011; Wahlen et al., 2011). We selected *Chlorella sorokiniana* as model microorganism because it allows us to perform experiments in a short time. Moreover, because of its tolerance to high irradiance and temperature conditions *C. sorokiniana* is a promising organism for commercial biomass production in the south of Spain.

Algal biomass production in a high irradiance area

Geographical areas with high irradiances along the year and moderate temperatures are optimal for microalgae cultivation. Because of the amount of sunlight hours per day, and the mean solar irradiance, Southern Spain is considered one of the best location for outdoor cultivation of microalgae in Europe (García-González et al., 2003).

Photosynthesis, the process where sunlight energy drives the fixation of inorganic carbon dioxide and its conversion into sugars and biomass unfortunately is not a perfect process. The photosynthetic efficiency (PE) achieved under sunlight is much lower when compared with the theoretical maximum due to losses related to photosaturation, photorespiration, respiration and photoinhibition.

The light saturation effect is considered the main factor limiting photosynthetic efficiency when growing microalgae outdoors. High annual microalgae productivi-

ties can only be achieved if solar light is efficiently used through the different seasons.

During winter time, microalgae productivity can be low due to the irradiance conditions. But also temperature has a strong effect on photosynthetic efficiency, and therefore on productivity. The maximal irradiance during winter time, combined with maximal winter temperature clearly lead to photoinhibition when producing microalgae in a high irradiance area (Chapter 2). Temperature mainly affects the algae metabolism, resulting in a decline in substrate requirements. In this sense, the winter irradiance is already experienced as over-saturating. However, when temperature is controlled at its optimal value for algae growth, a higher photosynthetic efficiency is found. At optimal temperature 1.2 g of biomass are produced per mol of photons supplied, while only 0.5 g of biomass mol photons^{-1} are produced when temperature is suboptimal. Therefore, when producing microalgae in a high irradiance area, temperature control has to be considered in the photobioreactor design in order to maximize photosynthetic efficiency.

During summer time, the effect of light saturation is more pronounced; cells can be exposed to irradiances as high as 2100 µmol photons m^{-2} s^{-1}. Nevertheless, *Chlorella sorokiniana* is able to grow under such irradiance conditions, and it shows a high photosynthetic efficiency (1.02 g of biomass produced per mol of photons supplied) (Cuaresma et al., 2009). Surprisingly also under these conditions we found a high volumetric productivity, which suggests that photoinhibition effects were minimal. Apparently the combination of the photobioreactor configuration and the microalgae used allowed a high productivity (P_v = 0.5 g kg^{-1} h^{-1}) under summer conditions. A narrow light path and a good mixing rate improves light distribution and allows cells to move from saturating light zones to dark zones. The robustness of *C. sorokiniana*, which tolerates high irradiance and has a high specific growth rate, also contributes to the high productivity.

Once the production of *Chlorella sorokiniana* in a panelar photobioreactor is demonstrated to be feasible in a high irradiance area, we looked at strategies to

maximize PE. Optimizing photobioreactor orientation to avoid or reduce photosaturation has been proposed (Qiang et al., 1998a). By placing the photobioreactors vertically, 30% of the daily supplied light is dissipated as heat, while heat dissipation accounts for 60% in horizontal systems (Cuaresma et al., 2011b). As a result the photosynthetic efficiency of *C. sorokiniana* in a vertical photobioreactor is very high, 1.3 g mol^{-1} versus the maximal 1.8 g mol^{-1}. This value includes night biomass loss and maintenance requirements. Despite the lower volumetric productivity associated, the productivity per ground area can be enhanced by placing more vertical photobioreactor-units in the same ground area (Figure 6.1). In this sense, only the photobioreactor layout should be improved in terms of shading and sunlight collection (Slegers et al., 2011). A rough estimation based on the PE obtained in the vertical photobioreactor, and the yearly averaged solar irradiance at the study site, proposes that 160 tons of dry matter could be produced per ha per year in southern-Spain (see Cuaresma et al., 2011b for more details).

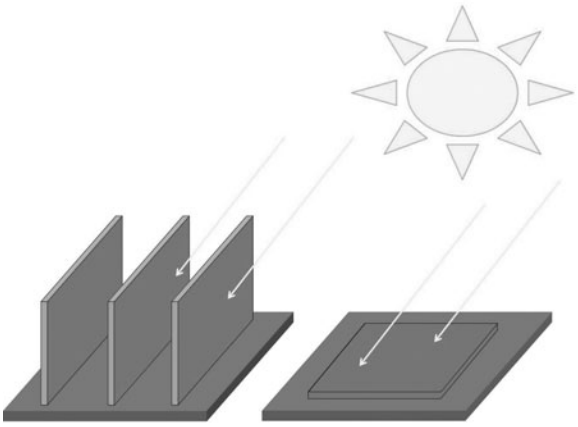


Figure 6.1: *Improving microalgae areal productivity: the light dilution effect.*

Luminostat operation, which continuously controls the light transmission in order to prevent the development of a dark zone and, at the same time, maximizing light capture, has been proposed to improve photosynthetic efficiency (Pruvost et al., (2011); Takache et al., (2010)). However, the biomass yield obtained is comparable, but not higher, with the yield obtained under traditional chemostat opera-

tion (Cuaresma et al., 2011b; Chapter 5).

According to the main findings discussed above, a biomass production process based on vertical panelar photobioreactors with a narrow light path, a good mixing rate, temperature control, chemostat operation and *Chlorella sorokiniana* as microalgae strain could be efficiently used in south Spain.

Cost estimation for a flat panel photobioreactor production plant can be found in Norsker et al., (2011). In this work, the flat panel photobioreactors are considered the best option to produce microalgae in a 100 ha plant. Considering a 5% of photosynthetic efficiency, and a productivity of 64 tons of dry matter per ha per year (according to the irradiance conditions in the Netherlands), a biomass production cost of 5.96 € per kilogram is expected. However, a sensitivity analysis on the final cost is also presented in the paper of Norsker and coworkers. A final price of 0.68 € kg^{-1} could be achieved optimizing the biomass production in terms of irradiation, mixing, photosynthetic efficiency, and medium and carbon dioxide supply. Considering the irradiance conditions of Huelva, the study site, a maximal productivity of 160 tons per ha per year could be expected, considering an average photosynthetic efficiency of 6 % (Cuaresma et al., 2011b). The higher productivity and PE expected in that area will have a positive influence on the biomass production costs, which should be lower than the reported 5.96 € kg^{-1}.

The costs allowed for biomass production depends on the value of the final product produced. For biofuel production the product value is low and so production costs should be really low as well (< 0.50 €/kg). For the production of valuable products the biomass production costs can be higher.

Biotechnological potential of *Chlorella sorokiniana*

Chlorella sorokiniana is normally produced for proteins and carbohydrates, but also fatty acids and antioxidants can be produced with this strain. Its ability to fix carbon dioxide from flue gas has been proved (Miller et al., 1971; Matsukawa et al., 2000) and more recently *C. sorokiniana* has been considered for biofuel production because of its lipid content (around 20 %) and high growth rate (Qiao and Wang, 2009; Wahlen et al., 2011). Also its potential for waste water treatment and phytoremediation has been demonstrated (Akhtar, et al., 2003, 2004; Godos et al., 2009, 2010; Yoshida et al., 2006). Apart from the environmental benefit of heavy metal removal, *C. sorokiniana* is able to incorporate metals as Selenium, which has antioxidant properties. Selenium plays an important role in prevention of a number of degenerative pathologies including cancer and inflammatory, cardiovascular and neurological diseases (Brown and Arthur, 2001; Patrick, 2004; Thomson, 2004; Rayman, 2005). *C. sorokiniana* incorporates inorganic selenite into intracellular macromolecules, including carbohydrates, proteins, lipids and selenoaminoacids. Organic forms of selenium are much more bioavailable than inorganic forms, which reveals the high potential of *C. sorokiniana* as nutraceutical.

Our studies (Cuaresma et al., 2009, 2011b, Chapter 1, Chapter 5) revealed the great potential of *C. sorokiniana* as lutein producer. *C. sorokiniana* has a high content of carotenoids, with lutein, neoxanthin and b-carotene as the most abundant ones. The potential of lutein production for the Huelva region will be presented.

Lutein production

Lutein ((3R,3′R,6′R)-β,ε-carotene-3,3′-diol) (Figure 6.2) is a xanthophyll (carotenoids which contain hydroxyl or carbonyl groups) that helps to prevent or diminish the effects of degenerative human diseases, such as age-related macular degeneration (AMD) (Carpentier et al., 2009; Chiu and Taylor, 2007; Granado et al., 2003) or cataracts (Arnal et al., 2009). It is largely consumed as food colorant in the United States and as feed additive in Europe and the market volume in 2010 accounted for about 190 million dollars (Vílchez et al., 2011).

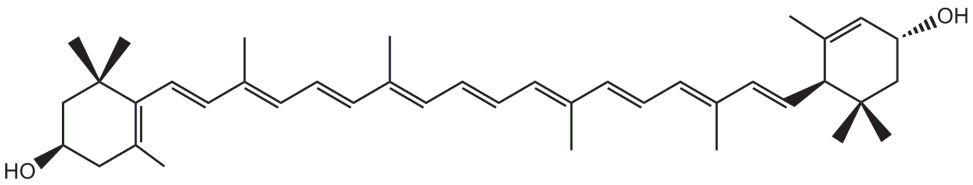


Figure 6.2: *Lutein structure.*

Currently lutein is obtained from marigold, where the lutein content is as low as 0.03% w/w (Piccaglia et al., 1998). The production is therefore a labor-intensive land-demanding process. Although it has also been produced synthetically, the prices of chemical synthesis (10,000 $ kg^{-1}) are not competitive in comparison to production in marigold nowadays.

Microalgae are considered an alternative source of lutein because of their high cellular content (0.5 – 1.2% dry weight), the higher biomass productivity, the potential to process the whole biomass, the ease to develop a continuous process, and the presence of valuable by-products (as proteins, other pigments and lipids) (Fernández-Sevilla, et al., 2010). *Muriellopsis* and *Scenedesmus* are among several microalgae with a reported large scale production system outdoors and their lutein content ranges from 4 to 6 mg g^{-1} (0.4 - 0.6% w/w). (Fernández-Sevilla, et al., 2010). Other algae as *Chlorococcum citriforme, Neospongiococcus gelatinosum* or *Chlamydomonas acidophila* have also been proposed as lutein producers

with a cellular content ranging from 4.6 to 10 mg g^{-1} (0.46 - 1.0 % w/w) (Cuaresma et al., 2011a; Del Campo et al., 2000). However, only batch experiments under continuous illumination have been carried out with that strains making it difficult to extrapolate their potential under real outdoor cultivation conditions.

Based on the biomass analysis realized during our studies (Cuaresma et al., 2009, 2011b, Chapter 1, Chapter 5), the lutein content of *C. sorokiniana* ranges from 2.4 to 6.0 mg g^{-1} (0. 24 – 0.6% w/w) (Table 6.1). That concentration is similar to the cellular content of *Muriellopsis* and *Scenedesmus*. However, the higher biomass productivities yielded by *C. sorokiniana* under simulated summer conditions might lead to a more profitable production system, with daily productivities ranging from 4 to 10 mg L^{-1} d^{-1} (Table 6.1). According to Fernández-Sevilla et al. (2010), a maximal productivity of 7.2 mg L^{-1} d^{-1} has been found when growing *Muriellopsis* outdoors in a horizontal tubular system (2.4 cm inner diameter). The production process of *Muriellopsis* is considered as a profitable process.

C. sorokiniana also showed a high neoxanthin content, ranging from 0.6 to 2.0 mg g^{-1} (Table 6.1). Neoxanthin (Figure 6.3) has been shown to induce apoptosis in prostate cancer cells (Kotake-Nara et al., 2005). As commented before, the presence of valuable by-products rebounds positively from a commercial point of view.

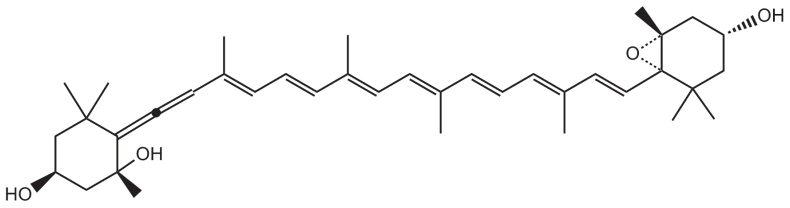


Figure 6.3: *Neoxanthin structure.*

Culture conditions	Lutein		Neoxanthin		β-carotene		References
	mg g^{-1} dw	mg L^1 d^{-1}	mg g^{-1} dw	mg L^1 d^{-1}	mg g^{-1} dw	mg L^1 d^{-1}	
Batch cultivation, Roux flasks 0.8 L, 25 °C, continuous illumination: 165 µmol photons m^{-2} s^{-1}	5.1	-	2.0	-	1.2	-	This chapter
Chemostat operation, SLP photobioreactor, 1.8 L, 38 °C, continuous illumination: 800 µmol photons m^{-2} s^{-1}	2.4-5.1	0.6-1.2*	0.6-1.8	0.1-0.4*	0.5-1.5	0.1-0.4*	Chapter 2
Chemostat operation, SLP photobioreactor, 1.8 L, 20 °C, continuous illumination: 800 µmol photons m^{-2} s^{-1}	5.0	0.6*	1.1	0.1*	0.85	0.1*	Chapter 2
Chemostat operation, SLP photobioreactor, 1.8 L, 38 °C, Simulated summer irradiance in a horizontal photobioreactor in Huelva	6.0	10.1-13.1	1.7	4.0-4.5	1.6	3.5-4.0	Cuaresma et al. 2011b
Chemostat operation, SLP photobioreactor, 1.8 L, 38 °C, Simulated summer irradiance in a vertical photobioreactor in Huelva	4.8	3.7-5.8	1.9	1.7-2.5	1.7	1.5-2.2	Cuaresma et al. 2011b
Luminostat operation, SLP photobioreactor, 1.8 L, 38 °C, Simulated summer irradiance in a vertical photobioreactor in Huelva	6.0	4.2-7.3	1.9	1.7-2.4	1.7	1.3-2.1	Chapter 5

Table 6.1: *Resume of the main carotenoids content of C. sorokiniana, per gram of dry matter, under different conditions. Productivity is expressed per liter of culture broth per day. When continuous illumination was applied the productivity is expressed per liter of culture broth per hour (data marked with *).*

Potential production of lutein in Huelva, Spain

In Cuaresma et al. (2011b) a rough estimation of maximal productivity in Huelva, south Spain, can be found. According to it, 160 tons of biomass per ha per year could be reached in vertical photobioreactors considering the yearly average irradiance conditions of the study site. Assuming an average cellular lutein content in *C. sorokiniana* of 5 mg g^{-1}, a productivity of 219 mg of lutein per square meter per day could be reached. It will lead to a maximal yearly productivity of 800 kg ha^{-1} $year^{-1}$. Considering an average cellular content of neoxanthin of 2 mg g^{-1}, the maximal productivity reached could be 88 mg m^{-2} d^{-1} (320 kg ha^{-1} $year^{-1}$). Comparing the data estimated in this paper with the productivities reported in Fernández-Sevilla et al., (2010), ranging from 100 to 290 mg m^{-2} d^{-1}, also a profitable production process might be expected in Huelva.

Nomenclature

A_{470}	measured absorbance at 470 nm
A_{652}	measured absorbance at 652 nm
A_{665}	measured absorbance at 665 nm
A_r	reactor illuminated surface, m^2
AMD	age-related macular degeneration
ATP	adenosine-5'-triphosphate
CCAP	Culture Collection of Algae and Protozoa, UK
Car_{tot}	cellular total carotenoids content, mg L^{-1}, mg g^{-1}
Chl_a	cellular chlorophyll a content, mg L^{-1}, mg g^{-1}
Chl_b	cellular chlorophyll b content, mg L^{-1}, mg g^{-1}
Chl_{tot}	cellular total chlorophyll content, mg L^{-1}, mg g^{-1}
C_x	biomass concentration, g Kg^{-1}
D	dilution rate, h^{-1}
DAQ	data acquisition module
DO	dissolved oxygen, %
dw	dry weight, mg g^{-1}, g Kg^{-1}
F	flow rate, Kg h^{-1}
F_0	zero fluorescence level
$F_{g,in}$	gas flow entering the reactor, mmol h^{-1}

$F^*_{g,out}$	corrected gas flow leaving the reactor, mmol h^{-1}
F_m	maximal fluorescence level
F_v/F_m	maximum PSII quantum yield
GMO	genetically modified organism
I_s	saturation light intensity
(L:D)	duration of the light:dark cycle, (h:h)
LED	light emitting diodes
$M_{harvest}$	culture broth harvested daily, Kg
$M_{reactor}$	culture broth weight inside the photobioreactor, Kg
MFC	mass flow controller
NADPH	nicotinamide adenine dinucleotide phosphate
NPQ	non photochemical quenching
OD_{680}	optical density at 680 nm
OD_{750}	optical density at 750 nm
OPR	oxygen production rate, mmol h^{-1}
P_{area}	areal productivity, g dw m^{-2} day^{-1} , g dw m^{-2} h^{-1}
P_{O2}	gross specific oxygen production rate, μmol O_2 g^{-1} s^{-1}
P_v	volumetric productivity, g dw Kg^{-1} d^{-1}, g dw Kg^{-1} h^{-1}
PAM	pulse amplitude modulation

PAR	photosynthetically active radiation (400 – 700 nm)
PE	photosynthetic efficiency, %
PFD	photon flux density, μmol m^{-2} s^{-1}
PFD_{abs}	light absorbed by the culture broth, μmol m^{-2} s^{-1}
PFD_{d}	daily light input, mol m^{-2} d^{-1}
PFD_{in}	light input on reactor surface, μmol m^{-2} s^{-1}
PFD_{out}	light output leaving the photobioreactor (not absorbed), μmol m^{-2} s^{-1}
$PFD_{out\ setpoint}$	desired light output leaving the photobioreactor, μmol m^{-2} s^{-1}
$PFD_{profile}$	simulated irradiance profile, μmol m^{-2} s^{-1}
PI-curve	photosynthesis-irradiance curve
PSII	photosystem II
QY	quantum yield
QY_{O2}	quantum yield of oxygen evolution, mol O_2 mol photons^{-1}
SLP	short light path
T	temperature
t_d	time, day
T_{opt}	optimal growth temperature, 38 ºC
T_{sub}	suboptimal growth temperature, 20 ºC
μ	specific growth rate, h^{-1}

μ_{max}	maximal specific growth rate, h^{-1}
V	liquid volume reactor, L
V_r	reactor illuminated volume, dm^3
X_{O2}	molar fraction of oxygen in the outflow gas, %
$X_{O2,db}$	molar fraction of oxygen in the dry baseline, %
$X_{O2,wb}$	molar fraction of oxygen in the wet baseline, %
$Y_{x,E}$	biomass yield on light energy, grams of biomass produced per mol of PAR photons supplied or absorbed

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Summary

Microalgal biomass production seems to be most attractive in regions with high irradiance and moderate temperatures along the year because light is usually the limiting substrate. However, photosynthesis is not a perfect process and the low photosynthetic efficiency reached in outdoor production systems, especially under high irradiance, restricts the development of commercially feasible microalgal production processes.

More knowledge on the light saturation effect, the main factor limiting microalgae photosynthetic efficiency outdoors, is needed. In laboratory experiments where real irradiance conditions can be simulated, while maintaining all other process conditions at the optimal levels, a better insight in microalgae cultivation can be obtained.

In this thesis, the simulation of real summer and winter irradiation conditions in Huelva, southern Spain, is realized in order to assess the potential productivity and photosynthetic efficiency of *Chlorella sorokiniana* in that region. Different strategies to minimize or avoid effects of photosaturation and photoinhibition are described and the potential production of *C. sorokiniana* in the study site is presented.

High annual productivities are limited by the lower microalgae productivity associated to winter time. The lower irradiance and temperature conditions are mainly affecting the photosynthetic efficiency of microalgae during that season. While reported algal productivity data are usually based on spring or summer time, also insight into microalgae performance under winter conditions is needed to optimize microalgae production over the whole year. In **Chapter 2** the quantitative assessment of productivity and photosynthetic efficiency of *Chlorella sorokiniana* is described under extreme winter conditions at the study site. As it is proven in this chapter, the combination of maximal winter irradiance in a horizontal photobioreactor and suboptimal growth temperature resulted in the microalgae cells experiencing over-saturating light conditions. The slow algae metabolism found at suboptimal growth temperature resulted in a decline in substrate requirements,

among others the requirement for light. The maximal winter irradiance therefore was experienced as over-saturating and photodamage occurred. Dissipation of excessive absorbed light through non-photochemical quenching (NPQ) was needed, resulting in a low photosynthetic efficiency. When temperature was controlled at its optimal value for growth of *C. sorokiniana*, a higher photosynthetic efficiency was found, which resulted in a higher winter productivity. However, the lower light availability during winter time still leads to a lower volumetric productivity when compared with summer irradiance, showing that the photobioreactor is operated in the photolimited regime during that season.

When moving to summer conditions, the high light intensity associated to that season will directly result in a reduced photosynthetic efficiency because of light saturation and possibly also because of photoinhibition. **Chapter 3** deals with the effect of light saturation during summer time on photosynthetic efficiency and productivity of *C. sorokiniana*. Maximal irradiance over a horizontal photobioreactor was applied while controlling temperature at its optimal value for growth. It is shown that a short light path panel photobioreactor placed horizontally can be used to grow *C. sorokiniana* efficiently even under over-saturating light conditions. A high productivity and photosynthetic efficiency were found at a low biomass concentration and a dilution rate close to the maximal specific growth rate. Based on these findings we concluded that photoinhibition effects were minimal. However, it must be addressed that these results are based on the narrow light path and good mixing rate of the photobioreactor used, which improves light distribution and allows cells to quickly move from saturating light zones to dark zones. Moreover, the microalgae used has a high specific growth rate. The experiments described in Chapter 2 and 3 were based on continuous illumination, which should not be considered in a real outdoor production process. The assessment of photosynthetic efficiency and productivity under daily light cycles is therefore described in the following chapters.

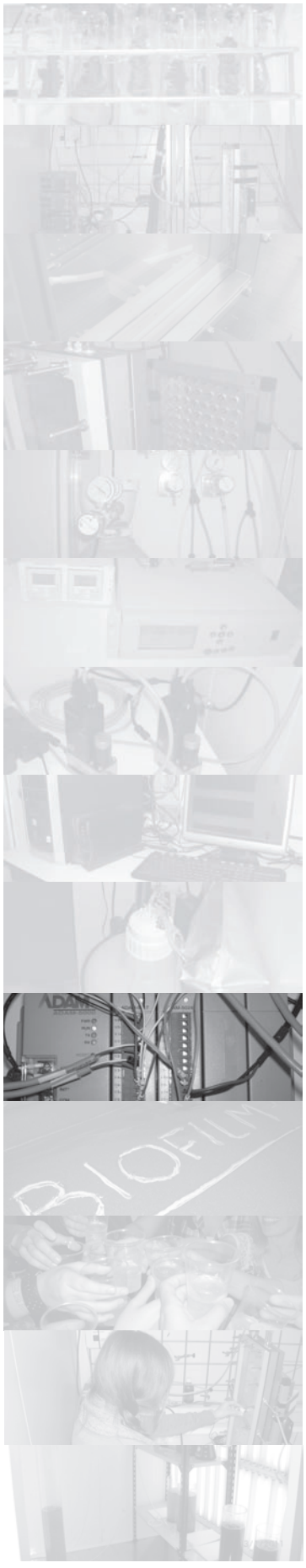
In **Chapter 4** one of the most popular strategies to minimize photosaturation in outdoors microalgae cultivation is evaluated: light dilution by vertical reactor orientation. The light dilution effect on productivity and photosynthetic efficiency of *Chlorella sorokiniana* was quantitatively assessed during simulated summer irradiance conditions at the study site. Under the same daily light cycle, only 30% of the supplied light was dissipated as heat by placing the photobioreactor units vertically, while it accounted for 60% in horizontal systems. Saturation of photosynthesis was already found halfway in the morning for the horizontal system, and it remained saturated until halfway the afternoon. Photosynthetic efficiency therefore was higher in the vertical system, and the value reached in this work was very high considering that also night biomass loss and maintenance requirements were included in the calculation. Moreover, productivity per ground area can be enhanced by placing more vertical photobioreactor-units in the same ground area despite the lower volumetric productivity obtained in the vertical systems. A rough estimation of the annual areal productivity in southern Spain is also presented in this chapter. It is based on the extrapolation to the whole year of the photosynthetic efficiency obtained in the vertical system and the yearly averaged solar irradiance at the study site. However, in a real system of vertical panel reactors the photobioreactor layout needs to be further optimized to maximize sunlight collection and minimize panel shading.

Luminostat operation, which continuously adapts the corresponding biomass density to the irradiance, in order to prevent dark zones and maximize light capture, has been proposed to lead to higher photosynthetic efficiencies. In **Chapter 5** the photobioreactor is operated as a luminostat while simulating a vertical position during summer time. Productivity and photosynthetic efficiency were evaluated and compared to traditional chemostat operation (Chapter 4). The photosynthetic efficiencies found during luminostat and chemostat operation were equal, indicating that we were already operating very close to the maximal efficiency to be expected. Apparently, adaptation of the biomass concentration to the irradiance does not lead to any further improvement in productivity as it is suggested. A

more advanced biomass control strategy based on a luminostat with a varying set -point could lead to a further improvement. But the photosynthetic efficiencies found in Chapters 4 and 5 must be very close to the maximal efficiency when growing *C. sorokiniana* outdoors under summer irradiance conditions in a vertical photobioreactor and it shows what we might maximally expect for large-scale microalgae production a high irradiance area.

Chapter 6 is a general discussion about the main findings of this thesis. The potential biomass production of *Chlorella sorokiniana* in a high irradiance area is presented based on the experimental data obtained. The ability of *Chlorella sorokiniana* to handle high irradiance, together with the photobioreactor configuration, makes *C. sorokiniana* an ideal candidate for biomass production in Southern Spain as well as many other locations. Moreover, it has been shown that *C. sorokiniana* contains high levels of lutein. Similar or higher lutein productivities to those already reported for commercial scale processes with other microalgae have been calculated. The potential role of *Chlorella sorokiniana* as a lutein producer is therefore discussed in this chapter.

Samenvatting



Productie van algen is veelbelovend in gebieden met veel zon en gematigde temperaturen gedurende het gehele jaar, omdat de hoeveelheid beschikbaar licht meestal de beperkende factor is. Fotosynthese is echter geen perfect proces en de lage fotosynthetische efficiëntie die onder buitenomstandigheden in de huidige productiesystemen wordt behaald, vooral wanneer de algen blootgesteld worden aan hoge lichtintensiteiten, remt de ontwikkeling van commerciële productieprocessen.

Om deze reden is meer kennis op het gebied van lichtverzadiging, de voornaamste reden voor de lage fotosynthetische efficiëntie die onder buitenomstandigheden met microalgen wordt behaald, belangrijk. Inzicht op dit gebied kan worden verkregen door microalgen te groeien in het laboratorium, waarbij werkelijke lichtintensiteiten gesimuleerd worden terwijl alle andere procesomstandigheden gecontroleerd worden op optimale waardes.

In dit proefschrift, werden de werkelijke lichtomstandigheden in Huelva, Zuid-Spanje, gedurende de zomer en winter gesimuleerd om de potentiële productie en de fotosynthetische efficiëntie van *Chlorella sorokiniana* in dit gebied te onderzoeken. Verschillende strategieën om fotosaturatie en foto inhibitie te minimaliseren of te voorkomen worden beschreven en de potentiële productie van *C. sorokiniana* in dit studiegebied wordt beschreven.

De jaarlijkse productiviteit van microalgen wordt gelimiteerd door de lage productiviteit gedurende de winterperiode. De lage lichtintensiteit en temperatuur zijn de belangrijkste oorzaken voor de lage fotosynthetische efficiëntie van microalgen in dit seizoen. Gerapporteerde productiviteiten zijn meestal gebaseerd op data verkregen tijdens de lente of zomer. Inzicht in de cultivatie van microalgen onder winterconditites is noodzakelijk om de jaarlijkse productie te kunnen optimaliseren. In **Hoofdstuk 2** wordt een kwantitatieve bepaling van de productiviteit en de fotosynthetische efficiëntie van *Chlorella sorokiniana* beschreven onder extreme winteromstandigheden in het studielocatie. In dit hoofdstuk wordt bewezen dat de combinatie van de maximale lichtintensiteit en de suboptimale groeitemperatuur

in de winter ertoe leidt dat de microalgen oververzadigde lichtintensiteiten ondervinden. Het langzame metabolisme van de algen, welke gevonden werd bij deze suboptimale temperaturen, leidde tot een lagere substraatbehoefte, onder andere de hoeveelheid licht. De maximale hoeveelheid licht gedurende het winterseizoen werd door de algen ervaren als oververzadigd en schade werd veroorzaakt door de te grote hoeveelheid licht. Dissipatie van het overmatig geabsorbeerde licht door middel van niet-fotochemische doving (non-photochemical quencing, NPQ) was nodig, wat leidde tot een lage fotosynthetische efficiëntie. Wanneer de temperatuur op de optimale groeitemperatuur van *C. sorokiniana* werd gereguleerd, werd een hogere fotosynthetische efficiëntie behaald wat leidde tot een hogere productiviteit gedurende het winterseizoen. Hoe dan ook, de lagere hoeveelheid beschikbaar licht gedurende dit seizoen, leidt nog steeds tot een lagere productiviteit per volume in vergelijking met de productiviteit per volume in de zomer, wat aantoont dat de fotobioreactor gedurende het winterseizoen licht gelimiteerd is.

Tijdens het zomerseizoen, zorgen de hoge lichtintensiteiten, die geassocieerd worden met dit seizoen, voor een gereduceerde fotosynthetische efficiëntie door fotosaturatie en waarschijnlijk ook foto inhibitie. **Hoofdstuk 3** behandelt het effect van lichtverzadiging gedurende de zomer en de invloed hiervan op de fotosynthetische efficiëntie en de productiviteit van *C. sorokiniana*. Algen werden blootgesteld aan de maximale lichtintensiteit die op een horizontale fotobioreactor valt terwijl de temperatuur optimaal voor groei werd gehouden. In dit hoofdstuk wordt bewezen dat een horizontaal geplaatste vlakke plaat fotobioreactor, welke een korte optische weg bezit, gebruikt kan worden om *C. sorokiniana* efficiënt te groeien, zelfs bij oververzadigde lichtintensiteiten. Een hoge productiviteit en fotosynthetische efficiëntie werden behaald bij lage biomassaconcentraties en een verdunningssnelheid die dichtbij de maximale specifieke groeisnelheid lag. Gebaseerd op deze bevindingen concluderen we dat foto inhibitie nauwelijks voorkwam. Hoe dan ook, het is belangrijk om te onthouden dat deze resultaten zijn behaald met een fotobioreactor met een korte optische weg en goede mengingskarakteristieken. Deze factoren zorgen voor een verbeterde lichtdistributie en cel-

len worden afwisselend blootgesteld aan korte perioden van verzadigde licht en donker condities. Bovendien zijn de behaalde resultaten ook gebaseerd op de gebruikte microalg welke een hoge specifieke groeisnelheid bezit. De experimenten die in Hoofdstuk 2 en 3 beschreven staan zijn gebaseerd op continue belichting, welke niet toegepast zal worden in een werkelijk productieproces dat buiten plaats vindt. Daarom wordt in de volgende hoofdstukken de fotosynthetische efficiëntie en productiviteit onder dagelijkse licht/donker ritmes bestudeerd.

In **Hoofdstuk 4** wordt één van de meest populaire strategieën om fotosaturatie bij het groeien van algen onder buitenomstandigheden te voorkomen geëvalueerd: het verdunnen van het invallende licht door de reactoren verticaal te plaatsen. Het effect van deze lichtverdunning op de productiviteit en fotosynthetische efficiëntie van *Chlorella sorokiniana* werd kwantitatief bepaald onder gesimuleerde lichtintensiteiten welke tijdens het zomerseizoen op de studielocatie voor zouden komen. Uit onze resultaten bleek dat onder dezelfde licht/donker ritme, slechts 30% van de ingestraalde licht werd gedissipeerd als warmte bij een verticaal geplaatste fotobioreactor , terwijl dit bij horizontale systemen 60% was. Verzadiging van het fotosysteem werd halverwege de morgen al gevonden bij de horizontale systemen en het fotosysteem bleef verzadigd tot halverwege de middag. Daardoor was de fotosynthetische efficiëntie hoger in het verticale systeem. De bereikte fotosynthetische efficiëntie was erg hoog, zeker omdat in de berekening van deze efficiëntie ook nog het biomassa verlies gedurende de nacht en de hoeveelheid energie die nodig is voor onderhoud werden meegenomen. Dit betekent dat de productiviteit per grondoppervlak verbeterd kan worden door meerdere fotobioreactoren op ditzelfde grondoppervlak verticaal te plaatsen ondanks de lagere productiviteit per volume in deze verticale fotobioreactoren. Dit hoofdstuk bevat ook een ruwe berekening van de jaarlijkse productiviteit van microalgen in Zuid-Spanje gebaseerd op extrapolatie van de behaalde fotosynthetische efficiëntie in het verticale systeem naar het hele jaar toe en de jaarlijkse gemiddelde zonlichtwaardes op de studielocatie. Maar, in een echt systeem van verticale vlakke plaatreactoren moet de indeling van de productiefaciliteit worden geoptimaliseerd

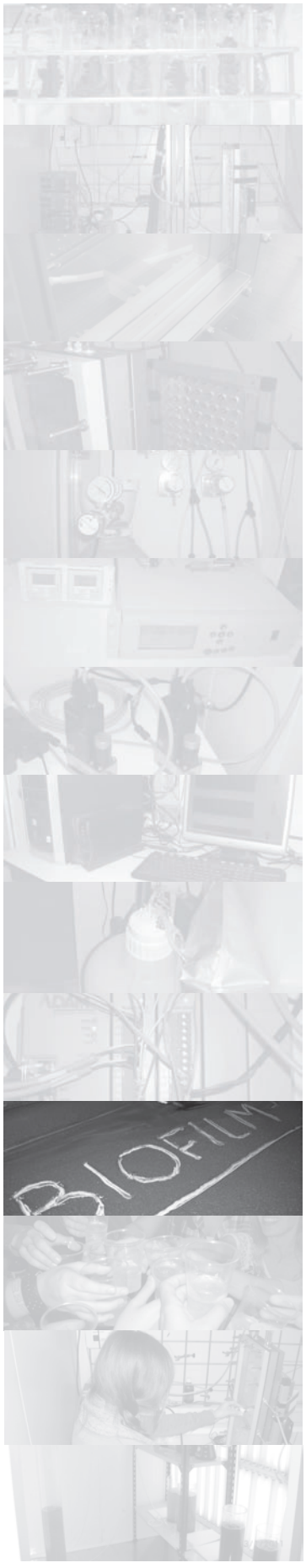
om zoveel mogelijk zonlicht op te vangen en om overschaduwing te minimaliseren.

Het wordt gesuggereerd, dat het bedrijven van reactoren via de luminostaat methode, waarbij de hoeveelheid biomassa continu wordt aangepast aan de hoeveelheid licht om donkere zones te voorkomen en zo veel mogelijk licht in te vangen, gebruikt kan worden om hogere fotosynthetische efficiënties te verkrijgen. In **Hoofdstuk 5** werd de fotobioreactor bedreven via deze luminostaat methode terwijl de hoeveelheid licht tijdens het zomerseizoen werd gesimuleerd voor een verticale gepositioneerd systeem. De productiviteit en de fotosynthetische efficiëntie werden geëvalueerd en vergeleken met traditionele bedrijving van het systeem volgens de chemostaat methode (Hoofdstuk 4). De gevonden fotosynthetische efficiënties van de microalgen voor beide methodes (luminostaat en chemostaat) waren identiek. Dit betekent dat de reactor bedreven werd tegen de maximale efficiëntie aan die behaald kan worden. Blijkbaar leidt de adaptatie van de biomassaconcentratie aan de hoeveelheid licht niet tot een verdere verbetering van de productiviteit zoals eerder gesuggereerd werd. Hoe dan ook, een meer geavanceerde controlestrategie voor biomassa, gebaseerd op de luminostaat methode met een variërende waarde, zou wellicht wel kunnen leiden tot een verdere verbetering. De in hoofdstuk 4 en 5 gevonden fotosynthetische efficiënties, moeten dus al dicht tegen de maximum efficiëntie aan liggen als *C. sorokiniana* gegroeid wordt onder zomerse lichtcondities in een verticale fotobioreactor. Dit laat zien welke maximale productie we mogelijk kunnen verwachten van een grootschalige algenproductie faciliteit op een locatie met veel zonlicht.

Hoofdstuk 6 behandelt de algemene discussie over de belangrijkste bevindingen in dit proefschrift. De potentiële commerciële productie van *Chlorella sorokiniana* in een gebied met veel zonlicht, gebaseerd op verkregen experimentele data, word in dit hoofdstuk besproken. Het vermogen van *Chlorella sorokiniana* om met hoge lichtintensiteiten om te gaan en de gebruikte fotobioreactorconfiguratie, maken *C. sorokiniana* een ideale kandidaat voor de productie van biomas-

sa in Zuid-Spanje. Daarnaast wordt aangetoond dat *C. sorokiniana* veel luteïne bevat. Gelijkwaardige of zelfs hogere luteïneproductiviteiten werden gevonden in vergelijking met eerder gerapporteerde productiviteiten voor productieprocessen met andere microalgen op commerciële schaal. De mogelijkheden van commerciële luteïne productie door middel van *Chlorella sorokiniana* worden besproken.

Resumen



La luz es el principal substrato para la producción de microalgas. Regiones con alta irradiancia y temperaturas moderadas a lo largo de todo el año son, por lo tanto, consideradas idóneas para la producción de biomasa de microalgas. Sin embargo, la fotosíntesis no es un proceso perfecto, y las bajas eficiencias fotosintéticas alcanzadas en cultivos en el exterior, especialmente bajo condiciones de alta irradiancia, limitan el desarrollo de sistemas de producción de microalgas a escala comercial.

Por tanto, debido a que el exceso de luz reduce la eficiencia fotosintética en el exterior, se requiere un mayor conocimiento sobre el efecto de la fotosaturación en el proceso fotosintético con objeto de mejorar la productividad de los cultivos. En este sentido, experimentos a nivel de laboratorio donde pueden simularse condiciones reales de irradiancia, a la vez que se mantienen el resto de parámetros operacionales en sus valores óptimos, proporcionan una mejor comprensión sobre el cultivo de microalgas.

Con el objeto de evaluar la productividad y eficiencia fotosintética potenciales de *Chlorella sorokiniana* en Huelva (suroeste de España), en esta Tesis se simulan condiciones de irradiancia propias del periodo estival, así como del invierno, en dicha región. Se evalúan diferentes estrategias para minimizar o evitar los efectos de la fotosaturación y de la fotoinhibición y se discute la producción de biomasa de *C. sorokiniana* en el lugar geográfico del estudio.

La obtención de elevadas productividades anuales está limitada por la baja productividad alcanzada durante el invierno. La baja irradiancia, junto con las bajas temperaturas típicas de este periodo, afecta drásticamente a la eficiencia fotosintética. Es por ello que la mayoría de los datos publicados sobre productividades se restringen a estaciones más cálidas, como la primavera o el verano. No obstante, es necesario comprender el comportamiento de las microalgas bajo condiciones típicas de invierno para poder optimizar la producción a lo largo de todo el año. En el **Capítulo 2** se evalúa cuantitativamente la productividad y eficiencia fotosintética de *Chlorella sorokiniana* bajo condiciones extremas de invierno típi-

cas de la región de estudio. Como se muestra en este Capítulo, la combinación de la máxima irradiancia experimentada durante el invierno, en un fotobiorreactor horizontal, y una temperatura de cultivo sub-óptima, hace que las células experimenten la irradiancia como sobresaturante. La temperatura de cultivo inferior a la óptima deriva en una menor tasa metabólica, lo que hace que se requiera menos substrato (luz). La irradiancia máxima de invierno bajo estas condiciones es, por lo tanto, experimentada como sobresaturante. Bajo estas condiciones el exceso de energía absorbida es disipado mediante un proceso llamado "quenching no fotoquímico" (NPQ), resultando en una menor eficiencia fotosintética. En cambio, cuando la temperatura de cultivo se mantiene en su valor óptimo para el crecimiento de *C. sorokiniana*, se alcanza una mayor eficiencia fotosintética, la cual da lugar a una mayor productividad durante el invierno. Sin embargo, la menor disponibilidad de luz durante esta época aún deriva en una menor productividad volumétrica cuando se compara con condiciones de irradiancia estivales, lo que sugiere que el fotobiorreactor estaría operando en condiciones de fotolimitación durante el invierno.

Durante el periodo estival, la elevada intensidad lumínica asociada a esta estación da lugar a una menor eficiencia fotosintética debido al efecto de la fotosaturación, y posiblemente de la fotoinhibición, sobre el proceso fotosintético. El **Capítulo 3** evalúa el efecto de la fotosaturación estival en la eficiencia fotosintética y productividad de *C. sorokiniana*. Para ello, se simula la máxima irradiancia solar experimentada sobre un fotobiorreactor horizontal mientras que la temperatura se controla en su valor óptimo para el crecimiento. Los resultados muestran que un fotobiorreactor panelar con un estrecho paso óptico y en posición horizontal podría utilizarse eficientemente para cultivar *C. sorokiniana* incluso bajo condiciones de luz sobresaturante. A una baja concentración celular y a una tasa de dilución próxima a la máxima tasa específica de crecimiento de esta microalga se obtienen elevadas productividades y eficiencia fotosintética. En base a ello, se concluye que los efectos de la fotoinhibición bajo las condiciones operacionales probadas son mínimos. Sin embargo, debe remarcarse que los resultados están funda-

mentados en el diseño del fotobiorreactor en sí, así como en la microalga utilizada, la cual presenta una tasa específica de crecimiento muy elevada. El estrecho paso óptico y la tasa de aireación/mezclado del fotobiorreactor hacen que se mejore la distribución de la luz en el interior del cultivo y permiten a las células moverse desde zonas donde perciben la luz como saturante hasta zonas donde experimentan una total oscuridad. Los experimentos descritos en el Capítulo 2 y 3 fueron realizados bajo iluminación continua, la cual obviamente no tiene lugar en un proceso de producción en exterior. La eficiencia fotosintética y la productividad bajo ciclos reales de día/noche son, por lo tanto, evaluadas y descritas en los siguientes capítulos.

En el **Capítulo 4** se estudia una de las estrategias más populares para minimizar la fotosaturación en cultivos de microalgas en el exterior: la dilución de la luz mediante disposición vertical de los fotobiorreactores. El efecto de la dilución de la luz en la productividad y eficiencia fotosintética de *C. sorokiniana* se evalúa cuantitativamente bajo condiciones de irradiancia estivales propias de la región de estudio, simulando el ciclo de luz/obscuridad propio de un día de verano en dicha región. Bajo este perfil de irradiancia, solo el 30% de la luz recibida por el cultivo es disipada en forma de calor cuando el fotobiorreactor se coloca en posición vertical, mientras que en sistemas horizontales la disipación alcanza el 60%. Además, cuando el fotobiorreactor se coloca en posición horizontal el proceso fotosintético se satura a media mañana, permaneciendo así hasta media tarde. Esto hace que la eficiencia fotosintética sea mayor en los sistemas verticales. La eficiencia alcanzada en este trabajo es además muy elevada, considerando que las pérdidas de biomasa durante la noche y los requerimientos propios del mantenimiento celular se han incluido en los cálculos. A pesar de obtener una menor productividad por unidad de volumen en los fotobiorreactores verticales, la posibilidad de colocar varias unidades en la misma superficie hace que la productividad final por unidad de área sea mayor. En este Capítulo se presenta, además, una estimación aproximada de la producción anual de *C. sorokiniana* en el suroeste de España. Dicha estimación se ha realizado en base a la irradiancia solar anual

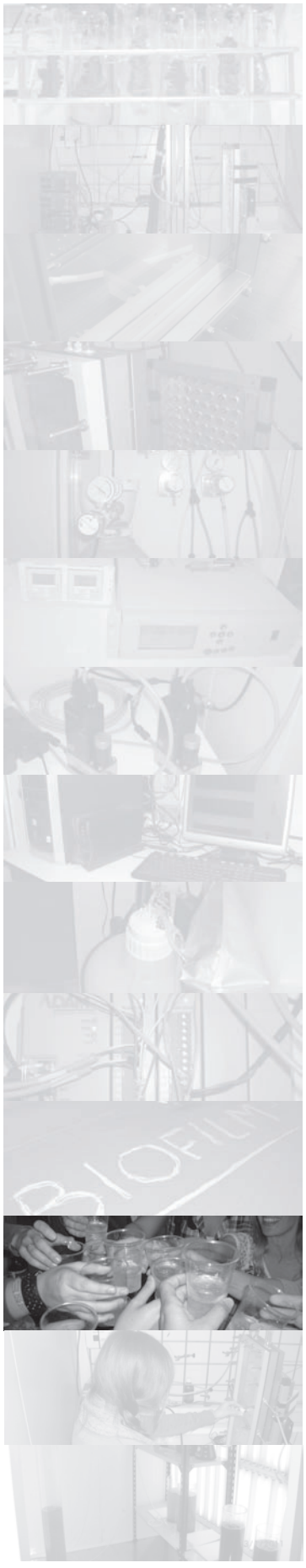
promedio en el sitio geográfico del estudio y en la extrapolación, a todo el año, de la eficiencia fotosintética obtenida en el fotobiorreactor vertical. Sin embargo, en un sistema real de reactores panelares verticales la disposición de los fotobiorreactores ha de ser optimizada para poder maximizar la captura de la luz y minimizar el efecto de sombreado entre las diferentes unidades.

La operación de fotobiorreactores en modo luminostato, el cual adapta continuamente la densidad celular a los valores de irradiancia reales, para prevenir zonas no iluminadas y maximizar la captura de la luz dentro del cultivo, se ha propuesto como medio para obtener mayores eficiencias fotosintéticas. Por ello, los experimentos mostrados en el **Capítulo 5** se realizaron operando el fotobiorreactor como luminostato, simulando de nuevo las condiciones de irradiancia estivales en un fotobiorreactor vertical ya estudiadas anteriormente. La productividad y eficiencia fotosintética se evalúan y comparan con las obtenidas durante la operación del fotobiorreactor en modo quimiostato (Capítulo 4). Las eficiencias fotosintéticas obtenidas durante la operación como luminostato y quimiostato fueron similares, sugiriendo proximidad a la máxima eficiencia que podría esperarse en la práctica. Aparentemente, la continua adaptación de la densidad celular a la irradiancia no conduce a una mejora significativa de la productividad, como se había sugerido. Sin embargo, un control más avanzado de la densidad celular basado en un luminostato dinámico (con un punto de compensación de la fotosíntesis variable a lo largo del día) podría conducir a una futura mejora. No obstante, las eficiencias fotosintéticas obtenidas en los Capítulos 4 y 5 deben estar cercanas a la máxima eficiencia esperable cuando se cultiva *C. sorokiniana* en el exterior bajo condiciones de irradiancia estivales en un fotobiorreactor vertical.

El **Capítulo 6** es una discusión general sobre los principales resultados de esta Tesis. Se discute la producción potencial de biomasa de *Chlorella sorokiniana* en una zona de alta irradiancia en base a los datos obtenidos experimentalmente. La habilidad de *Chlorella sorokiniana* para soportar condiciones de alta irradiancia, junto con la configuración del fotobiorreactor, hace que *C. sorokiniana* sea

considerada una candidata ideal para la producción de biomasa en el suroeste de España así como en localizaciones similares. Además, *C. sorokiniana* contiene elevadas cantidades de luteína. En esta Tesis se han obtenido productividades de luteína similares o mayores a las ya publicadas para otros procesos a escala comercial con otras microalgas. Por tanto, en este Capítulo se discute también el posible potencial de *Chlorella sorokiniana* como productora de luteína.

Acknowledgements



Once I read that to write the acknowledgments was delightful. Yes, it is. But it is also difficult. It is difficult to not forget someone who was also part of this work during this almost 5 years. And it is even more difficult when you have found people always ready to help you, no matter the country where you were neither the language you were speaking.

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Along this PhD I have had the pleasure of being part of two different groups. En Huelva he encontrado un grupo en el que desarrollarme como "científica" pero también como persona. Edu fue de las primeras personas que conocí en el laboratorio, junto con Inés, Mª José y Alonso, y esas primeras tardes juntos en el laboratorio hicieron que se despertara mi interés en este mundillo de las algas. Gracias **Edu** por tu buen humor, por estar siempre dispuesto a ayudar desinteresadamente y por ser tan humilde cuando en realidad todos sabemos que aprendemos muchísimo de ti, y contigo. De **Inés** admiro la fidelidad que tiene con el grupo y la voluntad que tiene siempre para colabo-

rar en la medida de lo posible. Para mí eres ejemplo de trabajo honesto y bien hecho. De **Mª José** admiro la fuerza de voluntad que tiene para seguir involucrada con el grupo, con nosotros, aun cuando no le quedan horas en el día. Y gracias a **Alonso**, quien a su vez me presentó a **Fernando** ("el gallego"), Holanda dejó de ser un país extraño para mí. Gracias por ese magnífico viaje en coche; nunca olvidaré la experiencia de comer una barra de salami a mordiscos en el coche, en medio de un atasco en una autopista alemana...

Al poco tiempo aparecieron Marta e Inma. **Marta**, aunque nuestras líneas de investigación iban por caminos distintos siempre ha habido tiempo para un café, una tarde de tiendas o un almuerzo en la playa. Hemos compartido mucho juntas y poco a poco hemos aprendido a valorarnos como personas, más que como compañeras de trabajo. Ante todo gracias por seguir ahí. Y a ti, **Inma**, gracias por tu humor ácido, que tantas veces nos ha hecho reír y nos hace plantearnos las cosas de otra manera. Después llegaron Carlete, Beni, Mayca y algo más tarde Ana Carolina. **Carlete**, el día a día nos ha hecho trabajar codo con codo y eso me ha enseñado a valorar tu precisión a la hora de hacer las cosas. Además, siempre ha sido un placer pasar algún tiempo juntos, ya sea discutiendo sobre el PAM, jugando un partidito de pádel, o cazando gamusinos… **Beni**, tu llegada al grupo aportó una visión más ingenieril de las cosas y nos ha permitido evolucionar mucho. Para mí eres ejemplo de arrojo y superación, aun cuando las cosas no siempre salen bien a la primera. **Mayca,** nos hemos peleado con los cortes eléctricos en el "labo" y con los pedales en la clase de spinning. Siempre tienes una sonrisa que ofrecer y para mí eres ejemplo de dedicación y de trabajo bien hecho. **Ana Carolina**, quizás hayamos aprendido más la una de la otra durante nuestros desayunos juntas que en el labo pero siempre es un placer compartir dudas y experiencias contigo.

Ahora llega el tiempo de la última generación. **Mª Carmen**, **Isa y Rocío**, vuestra energía y entusiasmo aportan savia nueva al grupo. Los ratitos y desayunos compartidos en Ciecem han sido inmejorables y siempre ha sido un placer trabajar con vosotras. **Encarni** y **Marta V.**, aunque en el trabajo diario no hayamos compartido mucho siempre habéis estado ahí. Y los ratos pasados juntas, ya sea tapeando, comiendo pizzas o en un concierto, han sido geniales; pero vaya "Marys" que estáis hechas… jeje…

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In 2007 other people arrived at the small town of Wageningen. Carsten and Francisco, together we founded the PRELBIT and at that time we really enjoyed to stay together. Although sometimes cultural differences are present we have learnt to respect each other. **Carsten**, there was always time for spontaneous things: a beer in the Zaaier, a dinner at your or my place. The time spent together made us feel confident and although we don't see too often lately, I enjoy a lot doing things with you and **Ana**. **Francisco**, it was a honour to be your paranymph, and it's always a pleasure to spend time with you; talking, singing, playing the guitar... Together with **Lena**, thanks for offering your house, for all the support during the first months, after moving here, and for making us feel at home... And I shouldn't forget **Taia**, the "chicken girl", with whom the most boring thing is experienced as amazing, and who has always a big smile to offer. Thanks for all the good moments together!.

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Dutch conversations. Thanks **Sina** for always being "un caballero", for offering yourself to pick me up when needed, for taking me ice-skating at Kinderdijk (even though your skills were not so good) and for enjoying sharing thoughts and feelings. It's always a pleasure to nag you… **Floor**, **Annette**, **Sayam, Jan-Willem**, **Packo, Mathieu, Caterina, Elsbeth, Niels, David**, **Marian, Gerrit, Pieter, Anne, Bas, Claudia,** we have travelled around Huelva together; we have spent some time at conferences, sharing the nerves before the first oral presentation in Galway; in the lab, enjoying the experience of lipid extractions in the evening; discovering Shibuya or singing in a karaoke in Japan; barbequing at the Rijn or dancing in a PhD party; driving along the Grand Canyon in the USA or sharing a leaking room in California ... It was a pleasure to find people who didn't mind to help you and with whom I've shared experiences that I won't forget. I also would like to thank **Marieke Buffing** for her dedication during her internship in Huelva.

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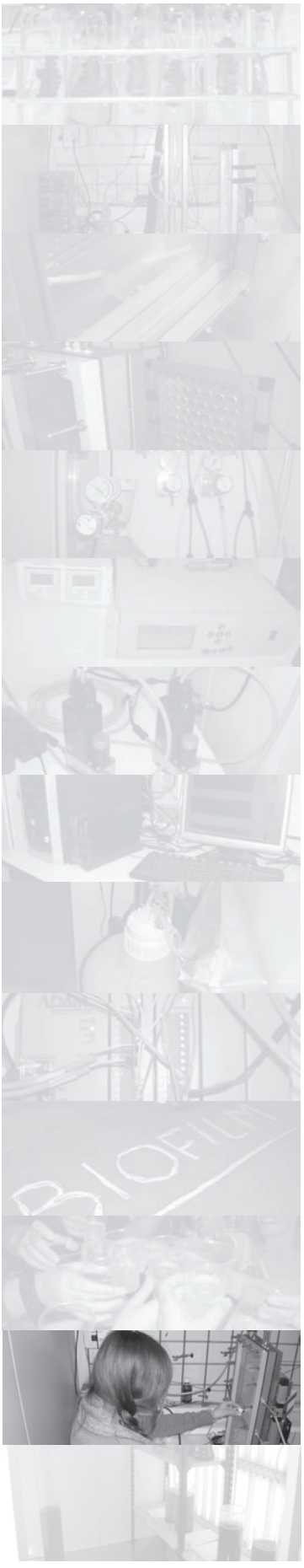
Por ultimo me toca agradecer a la gente que siempre ha estado apoyándome, que siempre ha creído en mí y a quien no les ha importado estar a miles de kilómetros de distancia. Entre ellas tengo que destacar a mis amigas, a quienes no les han importado mis ausencias cuando he estado liada y quienes siempre han sabido sacarme tiempo para un cafelito o unas tapas. Y en especial tengo que nombrar a mis padres, **Pepi** y **Marcelino**, y a mi hermana, **Araceli**. Al principio todo esto parecía una locura, dejar un trabajo por una beca no parecía lo más lógico. Aun así siempre habéis tenido palabras de aliento y habéis aceptado mis decisiones. Y a ti, **Ángel**, he de agradecerte más que a nadie tu paciencia infinita, que sí que la tienes. Soy consciente de que has sido de las perso-

nas que más han sufrido con esta tesis, pero aun así has sido siempre el primero en empujarme a dar el paso. Gracias por tu apoyo incondicional, por tu comprensión y sobre todo por seguir ahí.

También quiero recordar a esa gente que hoy día no están cerca, pero que en su día fueron también punto de apoyo importante y que siempre tenían una sonrisa en la cara cuando se hablaba con “la niña que estaba en Holanda”. Esta tesis es tan vuestra como mía, es algo que juntos hemos compartido a lo largo de estos casi cinco años y por ello no puedo más que daros las gracias.

Os quiere, María.

Curriculum vitae

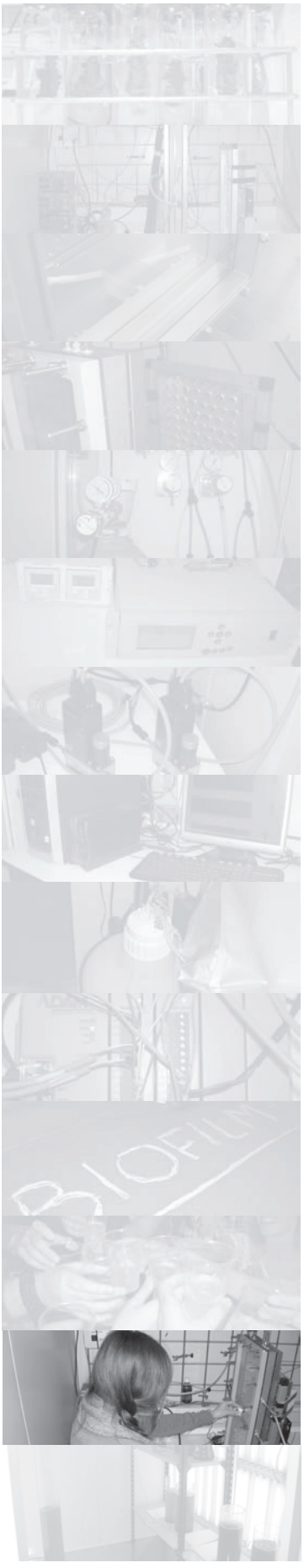


María Cuaresma Franco was born in Huelva, Spain, on April 23rd. In 1999 she started her studies on "Environmental Sciences" in the Faculty of Experimental Sciences, University of Huelva, Spain. During her studies she realized two internships both at the chemical company "FMC Foret S.L." in the department of "Quality, Security and Environmental Policy". During the last year of her studies she started to collaborate inside the group "Biotechnology of Microalgae", within the Biochemistry and Molecular Biology Area of the department of Chemical and Material Sciences, University of Huelva. In 2003 she graduated and started to develop her own research line inside the same group, which was related to isolation and cultivation of extremophilic microalgae from a local acidic river. At the same time, on May 2005, she started to work at "FMC Foret, S.L." inside the department of Technology (R+D).

In 2006 she obtained the Advanced Research Certificate from University of Huelva with the project entitled "Isolation, cultivation and biotechnological potential of *Chlamydomonas acidophila* isolated from Tinto River". In May 2006 she quit her job at FMC Foret, S.L. to start with a PhD project co-founded by University of Huelva in Spain and Wageningen University in the Netherlands. She focused on the maximization of microalgae photosynthetic efficiency under high irradiance conditions in a lab-scale photobioreactor.

Since March 2011 she is working as a postdoctoral researcher at AlgaePARC in Wageningen. She is leading the screening process for microalgae for biofuel production. In addition, she will also work on the optimization of operational conditions for microalgae production in lab-scale photobioreactors, as well as in pilot systems outdoors (2.4 m^2).

Publications



Cuaresma, M., Buffing, M.F., Janssen. M., Vílchez, C., Wijffels, R.H. *Chlorella sorokiniana's* performance under simulated extreme winter conditions. Journal of Applied Phycology. DOI: 10.1007/s10811-011-9687-y

Vílchez, C., Forján, E., **Cuaresma, M**., Bédmar, F., Garbayo, I. (2011) Marine carotenoids: biological functions and commercial applications. Marine Drugs 9(3), 319-333

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Garbayo, I., Cuaresma, M., Vilchez, C., Vega, J.M. (2008) Effect of abiotic stress on the production of lutein and b-carotene by *Chlamydomonas acidophila*. Process Biochemistry 43: 1158-1161.

Cuaresma, M., Garbayo, I., Vega, JM., Vílchez, C. (2006) Growth and photosynthetic utilization of inorganic carbon of the microalga *Chlamydomonas acidophila* isolated from Tinto river. Enzyme and Microbial Technology 40: 158-162.

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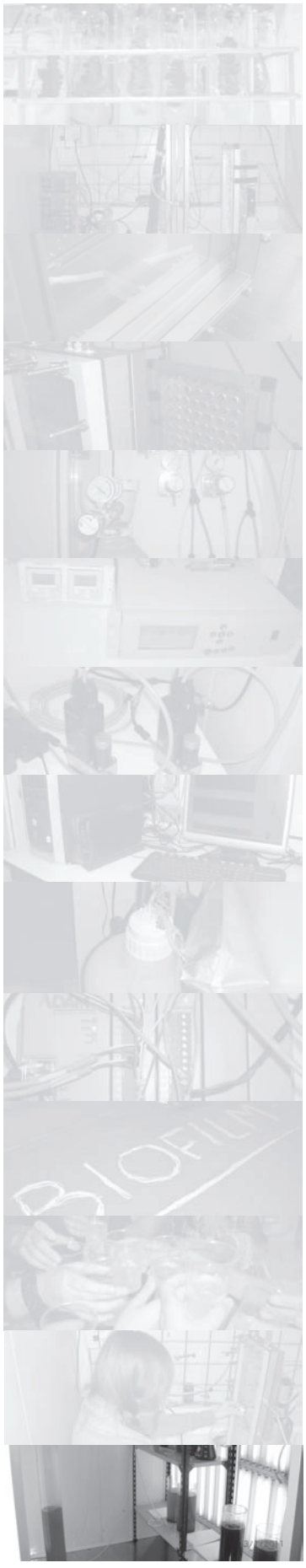
Garbayo, I., Domínguez, M.J., **Cuaresma, M**., Vílchez, C. & Vigara, J. (2005) Influencia de condiciones nutricionales en el consumo y metabolismo del nitrógeno en microalgas extremófilas. (Vega et al., eds.) Avances en el metabolismo del nitrógeno: de los microorganismos a las plantas. Publications service of University of Huelva, pps. 435-441. ISBN: 84-96373-55-X.

Training activities



Overview of completed training activities

Discipline specific courses

Advanced course on Molecular Biotechnology (2010)
Advanced course on Microbial Physiology and Fermentation Technology (2009)
Thermodynamics in Biochemical Engineering (2008)
Bioreactor Design and Operation (2006)
Basic Technics on Biotechnology (2005)
Introduction to Liquid Chromatography (2005)
Introduction to Gas Chromatography (2005)
Introduction to Infrared (FITR) and UV/visible Spectroscopy (2005)
In vitro Propagation of Vegetal Material (2005)
Environmental stress, Mutagenesis and Cancer (2005)

General courses

VLAG PhD week (2007)
English course, Level B.2.4 (2008)
English course, Level B.2.3 (2007)

Conferences

4th Annual Algae Biomass Summit, Arizona, USA (2010)
13th Netherlands Biotechnology Congress, Ede, The Netherlands (2010)
III Int. Conference on Environmental, Industrial and Applied Microbiology, Lisbon, Portugal (2009)
XXXII Congress of Spanish Society of Biochemistry and Molecular Biology, Oviedo, Spain (2009)
1st International Algal Conference, Amsterdam, The Netherlands (2008)
11th International Conference on Applied Phycology, Galway, Ireland (2008)
XXXI Congress of Spanish Society of Biochemistry and Molecular Biology, Bilbao, Spain (2008)
II Int. Conference on Environmental, Industrial and Applied Microbiology, Sevilla, Spain (2007)
7th European Workshop on Biotechnology of Microalgae, Nuthetal, Germany (2007)
1st Mediterranean Congress on Biotechnology, Tunisia (2006)
I Int. Conference on Environmental, Industrial and Applied Microbiology, Badajoz, Spain (2005)

Optionals

Preparation PhD research proposal (2010)
Bioprocess Engineering PhD study tour to USA (2010)
Bioprocess Engineering PhD study tour to Japan (2008)
Advanced Research Certificate (2006)

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