Photosynthesis of *Scenedesmus obliquus* in outdoor open thin-layer cascade system in high and low CO_2 in Belgium

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Abstract

Two outdoor open thin-layer cascade systems operated as batch cultures with the alga *Scenedesmus obliquus* were used to compare the productivity and photosynthetic acclimations in control and CO_2 supplemented cultures in relation with the outdoor light irradiance. We found that the culture productivity was limited by CO_2 availability. In the CO_2 supplemented culture, we obtained a productivity of up to 24 g dw.m^{-2}.day^{-1} and found a photosynthetic efficiency (value based on the PAR solar radiation energy) of up to 5%. In the CO_2 limited culture, we obtained a productivity of up to 10 g dw.m^{-2}.day^{-1} while the photosynthetic efficiency was up to 3.3% and decreased to 2.1% when the integrated daily PAR increased. Fluorescence and oxygen evolution measurements showed that ETR and oxygen evolution light saturation curves, as well as light-dependent O_2 uptake were similar in algal samples from both cultures when the CO_2 limitation was removed. In contrast, we found that CO_2 limitation conducted to a decreased PSII photochemical efficiency and an increased light-induced heat-dissipation in the control culture compared to the CO_2 supplemented culture. These features are in line with a lower light use efficiency and may therefore contribute to the lower productivity observed in absence of CO_2 supplementation in outdoor mass cultures of *Scenedesmus obliquus*.

1 Introduction

Over the last decades, microalgae have been increasingly studied because of their potential applications in the industry. Because of their great biodiversity, microalgae can produce a lot of valuable compounds for biofuels, food and feed, pharmaceutical and cosmetic industry. Despite a growing interest in microalgae mass cultures, the majority of studies on microalgae have been carried out at the laboratory scale and only few studies have addressed the question of photosynthetic acclimations in mass cultures.

Different mass culture systems are used in the world to produce microalgae biomass. Although some industry use closed tubular photobioreactors, most of microalgae biomass production units rely on horizontal open raceways systems (Zittelli *et al.*, 2013). The advantage of raceways are a relatively low building cost as well as a simple design permitting a rapid development of the installation. The culture thickness of these systems is usually high (15-30 cm), implying a low biomass density because of the reduced penetration of light in the suspension. Another drawback of these systems is the relatively poor mixing of the culture, which do not permit an efficient CO_2 and O_2 exchange with the atmosphere.

In this study, we used a thin-layer culture system similar to the one designed by Dr. Ivan Šetlík in the 1960s (Šetlík *et al.*, 1970). This system is characterised by an inclined surface exposed to sunlight in which the algal suspension flows by gravity (fig 1). At the end of the inclined surface, the suspension falls in a tank and is then pumped to the upper part of the inclined surface. Transverse laths are placed on the inclined surface in order to increase the mixing of the suspension, to ensure good gas exchange with the atmosphere and to favour fast light-dark cycle. The tank serves as a buffer to cushion the volume changes due to high evaporation during hot days or to heavy rainfalls. The productivity, the CO_2/O_2 exchange properties and the different variants of this system have been well characterised in the past using *Chlorella* and *Scenedesmus* species (Kajan *et al.*, 1994; Grobbelaar *et al.*, 1995; Doucha and Lívanský, 1995; Lívanský and Doucha, 1996; Doucha and Livansky, 1999; Livansky, 2000; Doucha *et al.*, 2005; Doucha and Lívanský, 2006; Doucha and Lívanský, 2009; Masojídek *et al.*, 2011; Jerez *et al.*, 2014). This system is currently used in a modified version for commercial production of *Spirulina* by the Biorigin farm in Ecuador.

Chlorophyll fluorescence has become one of the most common technique used to assess the photochemistry of photosynthetic organisms due to its non-invasiveness, sensitivity and to the wide availability of measuring instruments (Masojídek *et al.*, 2010). Chlorophyll fluorescence reflects the performance of PSII and is thus influenced by processes occurring downstream of PSII. One of the most used chlorophyll fluorescence approach is the saturation-pulse (PAM) method. With this method, fluorescence can be recorded continuously without being affected by ambient light and photochemical quenching and non-photochemical quenching can be easily separated (for a review, see Baker, 2008). Several parameters have been developed to account for the photosynthetic performances of sample. Maximal photochemical efficiency of PSII (F_V/F_M) is a parameter characterizing the proportion of absorbed light quanta which can be used by PSII to drive photosynthesis. The optimal value of this parameter is about 0.7-0.8, meaning

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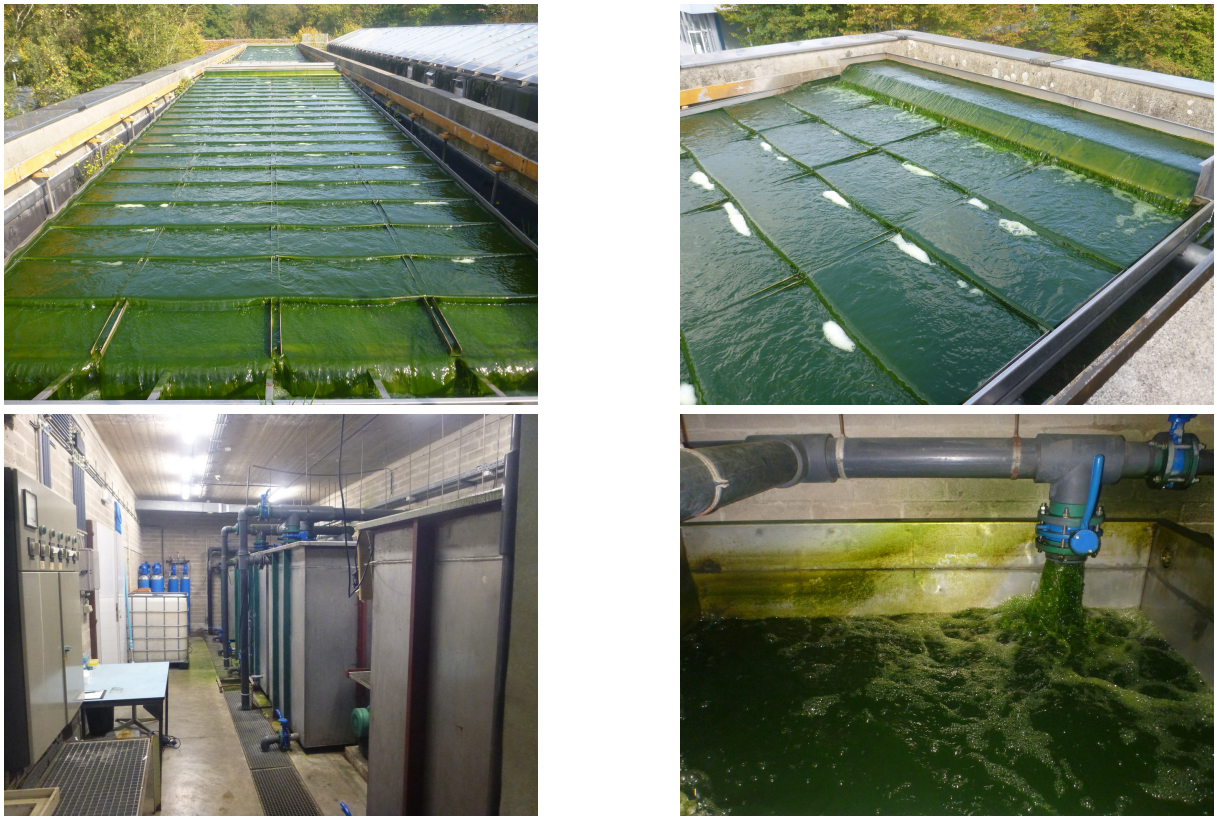

Figure 1: Top-left: light exposed part of the thin-layer cascade system on the roof. Top-right: zoom on the overflow tank. Bottom-left: engine room located downstairs. Bottom-right: zoom on a tank.

that in optimal conditions, about 70 to 80% of the absorbed light quanta are used for photochemistry while the rest is wasted as heat and fluorescence. This parameter is determined after dark-adaptation in order to permit the relaxation of dissipation and photoprotective processes. Another useful parameter is the PSII operating photochemical efficiency (ΦPSII) measured under actinic light, which is a measure of the actual photochemical capacity of PSII when photosynthesis is active. This means that any stress affecting a component of the photosynthetic apparatus will be reflected by a decreased ΦPSII. Finally, non-photochemical quenching (NPQ) is a third parameter which is often calculated to determine if photoprotective mechanisms are activated to deal with excessive absorbed light energy. NPQ is reflected as a general decrease of the fluorescence level and is composed of three components: energy-dependent quenching qE, which reflects an increased heat dissipation in the antennas, state transition qT which reflects a dissociation of light-harvesting complexes from PSII and photoinhibition qI, which reflect photodamages to PSII. These different components have different relaxation times ranging from a few seconds (qE) to several minutes (qT and qI).

In photoautotrophic atmospheric conditions, microalgae often have to deal with limited CO_2 availability, which prevents an efficient photosynthesis and a rapid growth. CO_2 limitation is usually associated with a reduced productivity, with the development of the carbon concentrating mechanism (Moroney *et al.*, 2011) and with a reduced PSII antenna size (Berger *et al.*, 2014). In lab-scale experiments, electron transfer to O_2 (most probably Mehler-type) has also been found to be very effective in condition of reduced CO_2 availability in *Chlamydomonas reinhardtii* (Sueltemeyer *et al.*, 1986; Bassi *et al.*, 2012) and in *Scenedesmus* (Radmer and Kok, 1976; Radmer and Ollinger, 1980; Flameling and Kromkamp, 1998). Among the studies on microalgae mass culture, as far as we know, none directly compared high CO_2 (CO_2 supplemented) and low CO_2 (non CO_2 supplemented) conditions. The aim of this study was to use chlorophyll fluorescence to analyse the possible photosynthetic acclimations of the culture in response to CO_2 availability in outdoor mass culture. For this, we performed two simultaneous microalgae cultures with or without CO_2 addition. Experiments were realised with *Scenedesmus obliquus*, which is known to have a high growth rate and a strong cell wall making it resistant for cultivation in various cultivation systems.

2 Material and methods

Organism and culture medium The *Scenedesmus obliquus 276.10* strain was used for cultivation (SAG culture collection). The medium was made of FloraGro and FloraMicro (GHE) diluted in tap water. FloraGro and FloraMicro were added in a ratio 1:1000 and 1:1000, respectively, for each 4kg of biomass accumulated. N content of the cultures was regularly checked to ensure that it was available and assimilated. This was taken as a indicator for the absence of nutrient limitation. FloraMicro and FloraGro composition can be found in Tocquin *et al.* (2012). In one culture, pH of the medium was stabilized to a value close to 7.5 by injecting pure CO_2 while in the other, pH was not stabilized.

Outdoor culture system The two outdoor open culture systems used for cultivation are similar to the one designed by Dr. Ivan Šetlík in the 1960s (Šetlík *et al.*, 1970). They consists of a 35m^2 inclined surface (inclination 2.5°) exposed to sunlight. The suspension flows on the surface due to the gravity before falling in a tank located downstairs. The suspension is then pumped to the roof by a hydraulic pump (1000 liters.h^{-1}) to ensure a continuous cycle. Transverse laths are placed on the inclined surface in order to increase the thickness and the mixing of the suspension. Layer thickness increases from 26mm behind the lath to 44mm in front of the next lath. The suspension volume was 4000l at the beginning of the culture. The volume on the inclined surface was 1900l while the volume in the tank varied around a value of 2100l, depending of evaporation and rain.

Supply of carbon dioxide In the CO_2 supplemented culture, pure CO_2 was added in the suction pipe of the circulation pump. CO_2 injection was regulated by a pH-meter to stabilize the pH at a value close to 7.5.

Outdoor light intensity measurement The light intensity was measured every 6 minutes by a brightness transmitter 7.1414.51.150 from Thies Clima (Göttingen, Germany). The light intensity was measured in lux and was converted to PAR (µmol PAR.m^{-2}.s^{-1}) by dividing lux by 82. This correction factor was determined by comparing the values of simultaneously measured lux and PAR at the Montrigi weather station (Belgium).

Culture temperature The culture temperature was recorded every 20 minutes by an immersed probe DS1922L from Waranet solutions (Auch, France).

Analytical methods Biomass concentration (g.l^{-1}) was determined daily by measuring the optical density at 750nm (A750). After having established the relationship between A750 and dry weight ($dw[g.l^{-1}] = A750 * 0.35$, R^2 =0.99), total biomass in the suspension was calculated, taking into account the suspension volume variations due to evaporation and rain. Net daily algal productivity, including night biomass loss, was estimated from the difference between successive morning values of total biomass divided by the exposed surface (35 m^2) of the photobioreactor.

Chlorophyll concentration determination Pigments were extracted from whole cells in ethanol. Extracts were incubated 4 hours on a shaker in presence of small beads and debris were removed by centrifugation at 10,000g for 5 min. The Chl (a + b) concentration was determined according to Lichtenthaler, 1987 with a lambda 20 UV/Vis spectrophotometer (Perkin Elmer, Norwalk, CT).

Chlorophyll fluorescence and O_2 evolution measurements Chlorophyll fluorescence emission measurements were made using either a PAM (pulse amplitude modulated) chlorophyll fluorimeter FMS1 from Hansatech instruments (UK) or using an Aquapen AP-C 100 fluorimeter from PSI (Czech Republic).

F_V/F_M was determined as $(F_M - F_O)/F_M$, where F_M is the maximal fluorescence level determined by applying a saturating pulse under darkness and F_O is the basal fluorescence level. ΦPSII was determined as $(F'_M - F_t)/F'_M$ where F'_M is the maximal fluorescence level determined by applying a saturating pulse under a particular actinic light intensity and F_t is the steady-state fluorescence level under this light intensity. NPQ was calculated as $(F_M/F'_M) - 1$ where F'_M is the maximal fluorescence level obtained by applying a saturating pulse under a particular actinic light intensity and F_M is the maximal fluorescence level when photoprotective mechanisms are not active.

For the dark-adapted measurements, we used the FMS1 fluorimeter. Cultures were dark-adapted for 30 minutes prior to each measurement and the chlorophyll concentration was adjusted to 8 µg.ml^{-1}. The analytical light was provided by light-emitting diodes with an emission maximum at 594 nm. The frequency of measuring flashes was 1500 per second and their integral light intensity was less than 0.1 µmol PAR.m^{-2}.s^{-1}. F_M and F'_M levels were obtained by applying a pulse of saturating light (6000 µmol PAR.m^{-2}.s^{-1}) provided by a halogen light source. rETR was determined by multiplying the photochemical efficiency of PSII by the light intensity and by 0.5 (assuming that light is equally absorbed by PSII and PSI). Oxygen evolution was simultaneously recorded using a Clark electrode system from Hansatech (UK). The protocol consisted of 6 light periods of 150 sec with light intensities of 50, 160, 300, 550, 750 and 1000 µmol PAR.m^{-2}.s^{-1} during which we recorded net oxygen evolution. Because mitochondrial respiration is known to increase with light intensity, successive light periods were separated by dark periods of 120 sec during which we recorded the respiration rate. Gross oxygen evolution was defined as $P_{gross} = P_{net} - R_{dark}$ (when using real values of P_{net} and R_{dark}). Light saturating pulses were given every 60 sec.

For the light-adapted measurements, we used the Aquapen fluorimeter. These measurements were always made at noon. Cultures were directly taken from the culture (dark-adaptation of ≈ 15 seconds) and diluted in the cell-free medium (obtained by centrifugation of the culture). Actinic light and saturating pulse were given at 455 nm. The protocol for NPQ and ΦPSII measurements consisted of a saturating pulse given in darkness followed by 5 different periods of 30 sec with light intensities of 50, 100, 200, 450 and 750 µmol PAR.m^{-2}.s^{-1}. Saturating pulses were given at the end of each period. For the DCMU-fluorescence rise curves, DCMU was added at a final concentration of 20µM and the light intensity was 2000 µmol PAR.m^{-2}.s^{-1}.

Typical fluorescence and oxygen evolution measurements curves are shown in Fig. S1.

pH measurement The pH of the control culture was recorded by a pH-meter BL931700 from Hanna instruments (USA). The pH of the CO_2 supplemented culture was regulated by a pH-meter Evolution deluxe from Dennerle (Germany).

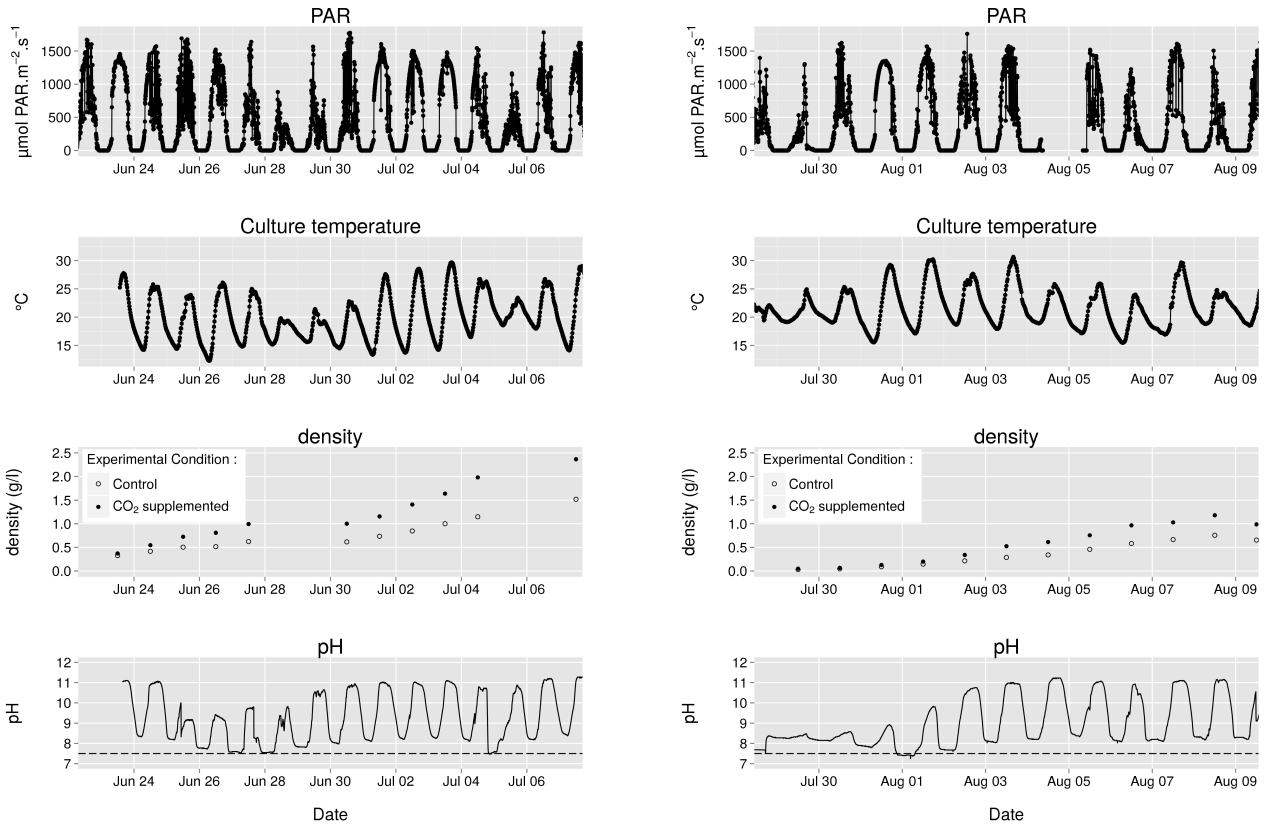

Figure 2: Summary of the data acquired during the two successive experiments. PAR: photosynthetic active radiation measurements from the weather station. The culture temperature was recorded using a probe immersed in the culture. The density of the culture was determined by dividing the total dry biomass by the volume of the culture. The pH of the control culture was recorded every 5 minutes. The pH of the CO_2 supplemented culture was not recorded but was set to 7.5 by injecting pure CO_2.

3 Results and discussion

3.1 Productivity and photosynthetic efficiency of *Scenedesmus obliquus* in high and low CO_2 conditions

In outdoor conditions, irradiance and temperature vary continuously, depending on the weather. During cloudy days, the light intensity and the air temperature are lower than during sunny days. This has an impact on the growth of the culture. We thus continuously recorded irradiance and culture temperature during the whole experiment. A fist experiment was started in June 24 2014 at a density of 0.4 g.l^{-1} and lasted 15 days. In order to obtain data from exponential-phase cultures, a second experiment was started in July 29 2014 at a lower density of 0.05 g.l^{-1} and lasted 12 days. Fig. 2 summarizes the irradiance, the culture temperature, the culture density and the pH during these two experiments. In previous studies on thin-layer cascade systems, cultures were usually operated at much higher biomass concentrations (e.g. 1-50 g.l^{-1} for Masojídek *et al.* (2011) and Doucha and Lívanský (2006)). The reason why we operated our cultures at lower biomass densities is that the layer thickness of the algal suspension in our system is higher than in other systems (26-44mm in this study in contrast to 6-8mm in the two previous studies). All the light being absorbed after a light path of 1cm at a density of 1 g.l^{-1} (see Fig. S2), operating the system at higher densities would reduce the productivity in our case because the major part of the culture thickness would be in darkness.

Despite a starting density similar at the beginning of the experiments, it is clear that it increased faster in the CO_2 supplemented culture than in the control culture. This shows that CO_2 availability is a limiting factor in microalgae mass cultures in open thin-layer cascade system.

The pH varied extensively in the control culture. It increased during the day because of carbon assimilation by photosynthesis. This is explained by the carbon dioxide equilibrium:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons CO_3^{2-} + 2H^+$$

When CO_2 is consumed by photosynthesis, H^+ is released in the medium, thus increasing the pH. During the night, photosynthesis is stopped and CO_2 concentration increases due to mitochondrial respiration and to equilibration with atmospheric CO_2, leading to a decrease of the pH. At low density (<0.25 g.l^{-1}), the pH shift during the day gradually increased with cell concentration whereas at higher density (>0.25 g.l^{-1}), the maximal pH attained during the day reached a plateau at a value of 11. The fact that the plateau of 11 was not reached at very low density implies that DIC availability was sufficient to drive photosynthesis and thus we can conclude that CO_2 supplementation is needed only at densities higher than 0.2 g.l^{-1}.

At pH higher than 9, it is known that there is virtually no CO_2 species and the totality of DIC is present in the form of HCO_3^- and CO_3^{2-} (Knud-Hansen *et al.*, 1998). The fact

that *Scenedesmus obliquus* can grow at such high pH, in contrast with other species like *Chlamydomonas reinhardtii*, is probably due to the existence of an additional alkaline HCO_3^- pump in this species (Thielmann *et al.*, 1990). The ability of this species to grow at high pH is interesting because the majority of contaminants cannot grow and rapidly die at such high pH. The pH shift could then be used to control contamination in outdoor microalgae mass cultures.

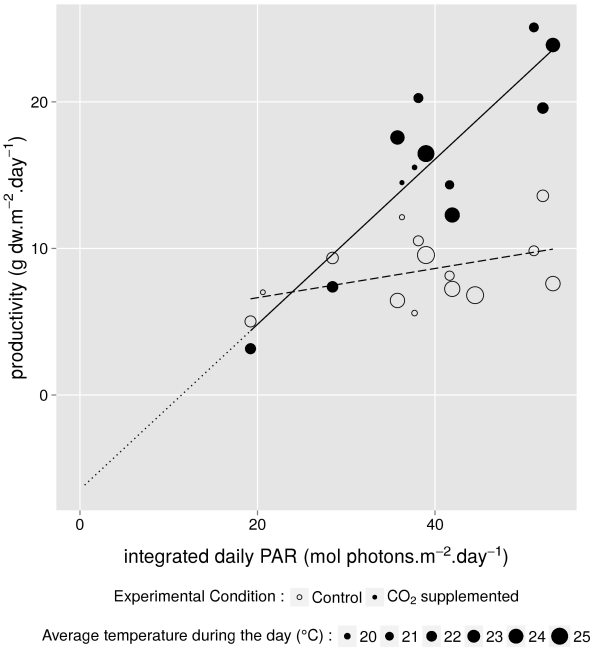

Figure 3: Correlation between productivity and daily integrated PAR for control and CO_2 supplemented conditions. The point size represents the average temperature during the day. Regression equation for control condition: y=4.65+0.099x, R^2=0.18. Regression equation for CO_2 supplemented condition: y=-6.44+0.56x, R^2=0.77. The data at very low density in the exponential phase (<0.2 g.l^{-1}, when the incident light is not limiting) have been considered.

In order to compare the growth of the control and CO_2 supplemented cultures, we made a correlation between daily areal productivity (g dry weight.m^{-2}.day^{-1}) and integrated daily photosynthetic active radiations (mol PAR.m^{-2}.day^{-1}). Fig 3 shows that during cloudy days, at integrated daily PAR around 20 mol PAR.m^{-2}.day^{-1}, the productivity of both cultures is similar. This indicates that CO_2 supplementation is not necessary when light availability is that low. However, when light supply increases, the productivity of the cultures differs with a near doubled productivity for the CO_2 supplemented culture during full sunny days (integrated daily PAR of about 55 mol PAR.m^{-2}.day^{-1}). The correlations between productivity and integrated daily PAR were fitted with a linear equation. The correlation coefficients were good for the CO_2 supplemented culture but not for the control culture (0.77 and 0.18, respectively). Two reasons may explain this fact. First, the relation is probably not linear in the control condition. The relation would probably be linear at low irradiance but would saturate at high irradiance due to limited DIC availability which would prevent an efficient photosynthesis. The second reason is the great variability observed in this condition (i.e. for the same irradiation, the productivity vary extensively). Possible explanations for this variability could have been the light history of the culture or the culture temperature. The latter parameter greatly influences the metabolic reactions of the cell. However, we did not find evidence for a relation between productivity and temperature (shown as the point size in Fig. 3) with the available data.

By extrapolation of the productivity/integrated PAR relationship of the CO_2 supplemented culture, the critical irradiance value for zero algal productivity was found to be 11.5 mol PAR.m^{-2}.day^{-1}. At this irradiance, a compensation point is reached at which an algal culture would not be productive due to a loss of biomass caused by respiration.

We then calculated the photosynthetic efficiencies (PE) of the cultures. PE are usually calculated from the heat of combustion of biomass and the energy content of the photons. Platt and Irwin (1973) developed a relationship between biomass calories on the one hand, and carbon and nitrogen contents on the other hands. As the C/N ratio is 8.9 and the carbon content accounts for 52.6% in the dry weight biomass in *Scenedesmus obliquus* (Zelibor *et al.*, 1988), the energy of the total biomass was estimated to be 24.6 kJ.g dry weight^{-1}. From the productivity/integrated PAR relationships of Fig. 3, we derived that at integrated daily PAR of 24 mol PAR.m^{-2}.day^{-1}, the productivities and the effective biomass yields of both cultures were similar (7 ± 2.4 g dw.m^{-2}.day^{-1} and 0.29 ± 0.1 g dw.mol photons^{-1}, respectively). Taking into account that the average energy content in the PAR region is 218 kJ.mol photons^{-1} (Tilzer *et al.*, 1985), we calculated a PE of 3.3 ± 1.1 % for both cultures at this low daily PAR value. The situation is completely different at the highest integrated daily PAR values (55 mol PAR.m^{-2}.day^{-1}) because the productivity saturates in the control culture while it does not in the CO_2 supplemented culture. At this high irradiance, the productivities were 10 ± 2.6 and 24 ± 3.9 g dw.m^{-2}.day^{-1} and the effective biomass yields were 0.18 ± 0.05 and 0.44 ± 0.07 g dw.mol photons^{-1} for the control and the CO_2 supplemented cultures, respectively. This leads to a PE of 2.1 ± 0.5 and 5 ± 0.8 % for the control and the CO_2 supplemented cultures, respectively.

The efficiency of light utilization can also be calculated with another approach, as done in Zijffers *et al.* (2010), on the basis of the biomass yield on light energy, expressed in g biomass per mol PAR. By expressing the observed value of this yield as % of its maximum theoretical value, obtained when the quantum yield of photosynthesis is maximal, an estimation of the actual photosynthetic efficiency is obtained. The maximum theoretical biomass yield is obtained from the stoichiometric reaction equation for formation of biomass and O_2 from carbon dioxide, water and the nitrogen source used for cultivation, by admitting that 10 mol PAR are necessary for the evolution of one mol O_2. Zijffers *et al.* found a value of 1.5 g.mol photons^{-1} for *Chlorella* species when grown on nitrate media. In practice, the actual yield is always lower because: 1) Some light is reflected at the culture surface. 2) Part of the absorbed energy is dissipated as heat due to exposure of algae to supersaturating light levels (typically close to the surface of the culture). 3) Respiration causes some biomass loss, especially during the night in outdoor condition. Night

Table 1: Summary of the productivities, photosynthetic efficiencies (PE based on biomass heat content), effective biomass yields on light energy and light utilization efficiencies. Low integrated daily PAR = 24 mol PAR.m^{-2}.day^{-1}. High integrated daily PAR = 55 mol PAR.m^{-2}.day^{-1}. Error ranges represent 95% confidence intervals around the mean.

	low integrated daily PAR	high integrated daily PAR	
	control and CO_2 supplemented	control	CO_2 supplemented
Productivity (g dw.m^{-2}.day^{-1})	7 ± 2.4	10 ± 2.6	24 ± 3.9
PE based on the heat content of biomass (%)	3.3 ± 1.1	2.1 ± 0.5	5 ± 0.8
Biomass yield on light energy (g dw.mol photons^{-1})	0.29 ± 0.1	0.18 ± 0.05	0.44 ± 0.07
Light utilization efficiency (%)	16 ± 5.4	10 ± 2.6	24 ± 3.9

respiratory loss has been estimated to represent about 6% of biomass accumulated during the day with *Scenedesmus obliquus* (Hindersin *et al.*, 2014). In an extreme case, it attained up to 64% with a *Chlorella* species (Masojídek *et al.*, 2011).

The maximum theoretical value of biomass yield on light energy calculated this way depends on the elemental composition of the microalgal species and on the nature of the nitrogen source (ammonium, nitrate or urea). The value calculated by Zijffers *et al.* was for *Chlorella* sp. grown on nitrate. However, the *Scenedesmus obliquus* elemental composition differs from that of *Chlorella* species (Zelibor *et al.*, 1988; Duboc *et al.*, 1999). Moreover, the nitrogen source of our medium is made of 4/5 of nitrate and 1/5 of ammonium. We thus recalculated the theoretical biomass yield for *Scenedesmus obliquus* for our medium composition (appendix). Maximum theoretical biomass yield in our culture conditions was 1.82 g.mol photons^{-1}. Using this reference value and the effective biomass yields obtained in this study (table 1), we obtained values of light utilization efficiencies of 16 ± 5.4 % under low integrated daily PAR (24 mol PAR.m^{-2}.day^{-1}) for both cultures and of 10 ± 2.6 and 24 ± 3.9 % under high integrated daily PAR (55 mol PAR.m^{-2}.day^{-1}) for the control and the CO_2 supplemented cultures, respectively.

The productivities, PE, effective biomass yields on light energy and light utilization efficiencies under low and high integrated daily PAR for the control and the CO_2 supplemented cultures are summarized in Table 1. The lower productivities, PE and light utilization efficiencies observed in the control culture at high integrated daily PAR indicate that outdoor mass cultures of *Scenedesmus obliquus* are limited by the CO_2 availability in open thin-layer cultivation systems for biomass densities higher than 0.25 g.l^{-1}. It may be argued that the high pH in the control culture (pH 11 compared to pH 7.5 for the CO_2 supplemented culture) may have been responsible for the low performances of this culture. However, both cultures had similar productivities under low integrated daily PAR (points below $\simeq$ 30 mol PAR.m^{-2}.day^{-1} in Fig. 3) while a high pH ($\simeq$11) was attained in the control culture. This indicates that high pH was not detrimental and suggests that the lower productivity (and thereby lower PE and light utilization efficiency) of the control culture under higher daily PAR was caused by the limitation in CO_2 availability.

Table 2 makes a review of published performances of thin-layer culture systems in terms of areal productivities and photosynthetic efficiencies, with *Chlorella* sp. or *Scenedesmus* sp. (mostly obtained in Czech Republic). Our data for *Scenedesmus* cultures in Liège are in the lower range, likely due both to the relatively high suspension thickness and to the large volume of the tank.

3.2 Characterisation of the photosynthetic apparatus in high and low CO_2 conditions

We then wanted to determine if the productivity differences were accompanied by adaptations of the photosynthetic apparatus to high and low CO_2 conditions. For this, we performed fluorescence measurements either on dark-adapted or on light-adapted samples. The dark-adaptation time was 40 min in order to permit the relaxation of dissipation and photoprotective processes. Measurements were carried in presence of saturating DIC concentrations (10mM $NaHCO_3$) in order to measure the photosynthetic performances of the samples without limitations. In this manner, we characterised the structural adaptations of the photosynthetic apparatus. In order to characterise the photosynthetic apparatus of the cells as they were in the culture units, we also performed fluorescence measurements on light-adapted samples without $NaHCO_3$ addition. For this, samples were directly taken from the mass culture systems and submitted to the measurements. These measurements were always made at noon. Due to the high sensitivity of the fluorimeter used for light-adapted measurements, samples had to be diluted. This was done in the cell-free medium obtained by centrifugation of the culture in order to keep the DIC concentration unchanged.

3.2.1 Fluorescence and oxygen evolution measurements on dark-adapted samples

We began our analysis by determining if the two conditions led to differences in the light responses of the photosynthetic apparatus. For this, we established light-response curves for simultaneously measured relative electron transport rate at PSII (rETR) and gross oxygen evolution (VO_2) in presence of saturating DIC on 40 min dark-adapted samples. No differences in electron transport rate or gross oxygen evolution could be found between the control and the CO_2 supplemented cultures for samples taken during the linear growth phase (Fig. 4). This demonstrates that the capacity of high density cultures to realise photosynthesis was not different once the CO_2 availability limitation was removed. It is thus tempting to conclude that the structural organisation of the photosynthetic apparatus of *Scenedesmus obliquus* is not modified by CO_2 availability in our mass cultivation system.

Table 2: Summary of published performances of thin-layer culture systems in terms of areal productivities and photosynthetic efficiencies. [a]: the way this value was derived is unknown.

Location	Species	Period of the year	CO_2 addition	Culture thickness (mm)	Surface (m^2)	Net areal productivity ($g.m^2.d^{-1}$)	Photosynthetic efficiency based on PAR radiations (%)	Reference
Czech Republic	*Chlorella* sp.	August	Yes	6	55	22.6 ± 3.9 19.1 ± 9.8	5.49 ± 0.89 6.88 ± 2.94	Doucha *et al.* (2005)
Czech Republic	*S. obliquus* and *chlorella* sp. mix	July and August	Yes	5-7 5-15	224	18.36 21.09	-	Grobbelaar *et al.* (1995)
Czech Republic	*S. obliquus*	August and September	Yes	6 10 40	224	14.9 ± 6.4 17.8 ± 6.2 13.5 ± 8	5.4 6.5 4.9	Doucha and Lívanský (1995)
Czech Republic	*Chlorella* sp.	Sunny days in July	Yes	6-7	224	38.2	7.05	Doucha and Lívanský (2009), see also Doucha and Lívanský (2015)
Czech Republic	*Chlorella* sp.	Sunny days in July, August and September	Yes	6 6	24 224	18.7 ± 5.14 14.3 ± 4.11	± 10[a]	Masojídek *et al.* (2011)
Czech Republic	*Chlorella* sp.	July September	Yes	6	224	23.5 11.1	6.48 5.98	Doucha and Lívanský (2006)
Southern Greece		July October		8	100	32.2 18.1	5.42 6.07	
Belgium	*S. obliquus*	July and August	Yes No	26-44	35	15.8 ± 6.3 8.5 ± 2.5	4.4 ± 1.2 2.6 ± 0.8	This study

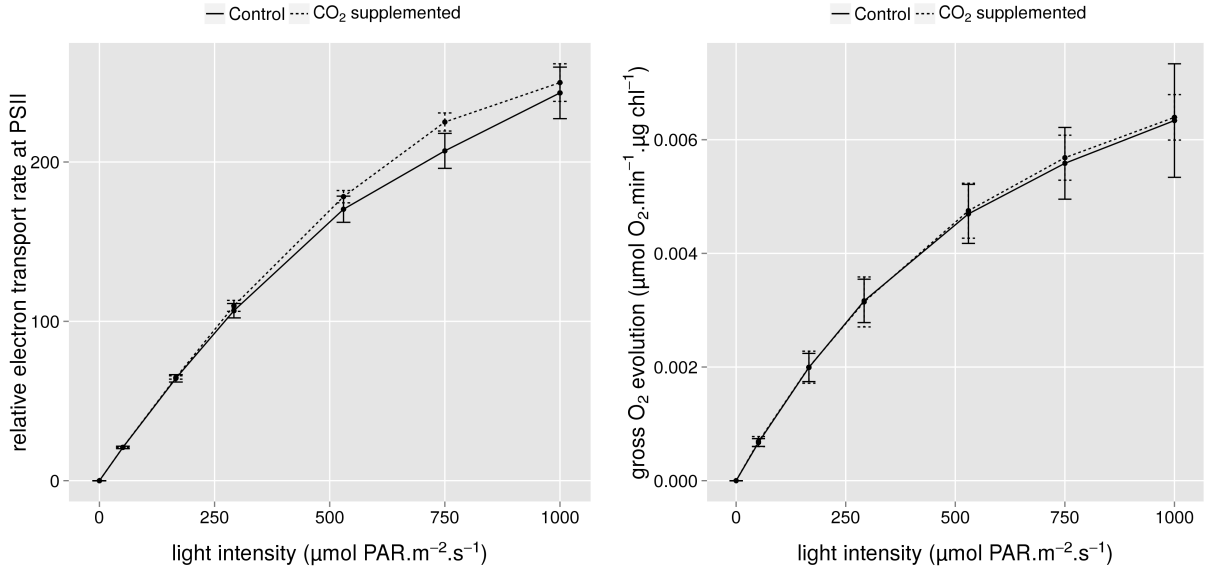

Figure 4: Light-response curves of the relative electron transport rate at PSII (rETR) and of the gross oxygen evolution under different light intensities in high and low CO_2 cells. Measurements were performed in presence of 10 mM $NaHCO_3$ after 40 min of dark-adaptation. Data are average of 5 measurements (±SD) obtained during the linear growth phase (beyond 0.2 $g.l^{-1}$ biomass density) of the two experiments shown in Fig. 2.

It has been shown that ΦPSII (or ETR) measured in the light was linearly correlated to the quantum yield of oxygen or CO_2 uptake in C3 higher plants when photorespiration was avoided (Genty *et al.*, 1989; Krall and Edwards, 1990; Cornic and Ghashghaie, 1991; Genty and Meyer, 1995; Hymus *et al.*, 1999). This finding permitted the use of simple fluorescence measurements to estimate the CO_2 uptake and the productivity of plants.

However, the photosynthetic electron transport chain of microalgae is endowed with great flexibility, due to the existence of auxiliary electron transfer pathways (Peltier *et al.*, 2010), such as electron transfer to O_2 at PSI (known as the Mehler reaction) or through PTOX (the plastidial oxidase). Electron transfer to O_2 (most probably Mehler-type) has been found earlier to be very effective in the green microalga *Chlamydomonas reinhardtii* (Sueltemeyer *et al.*, 1986; Bassi *et al.*, 2012). In *Scenedesmus*, a significant light-dependent O_2 uptake has been shown to occur (Radmer and Kok, 1976; Radmer and Ollinger, 1980; Flameling and Kromkamp, 1998) although it was not always observed (Heinze *et al.*, 1996). The function of this O_2-dependent electron flux is not clear. It could be a way to dissipate reducing power in DIC-limited condition in order to prevent reactive oxygen species (ROS) production which would lead to damages. It could also be a way to produce the ATP needed to concentrate CO_2 when its availability is restricted. It follows that electron transport rate estimations, performed fluorimetrically (using ETR as a basis), should not necessarily match photosynthetic rate but may include the rate of electron transport to O_2 as sink, even in the absence of significant photorespiration.

This was first investigated here in lab-grown *Scenedesmus obliquus* (grown in flasks at 200 µmol $PAR.m^{-2}.s^{-1}$) by plotting ETR and gross oxygen production measured at different light intensities. Measurements were carried out in presence of $NaHCO_3$ to avoid CO_2 limitations and pho-

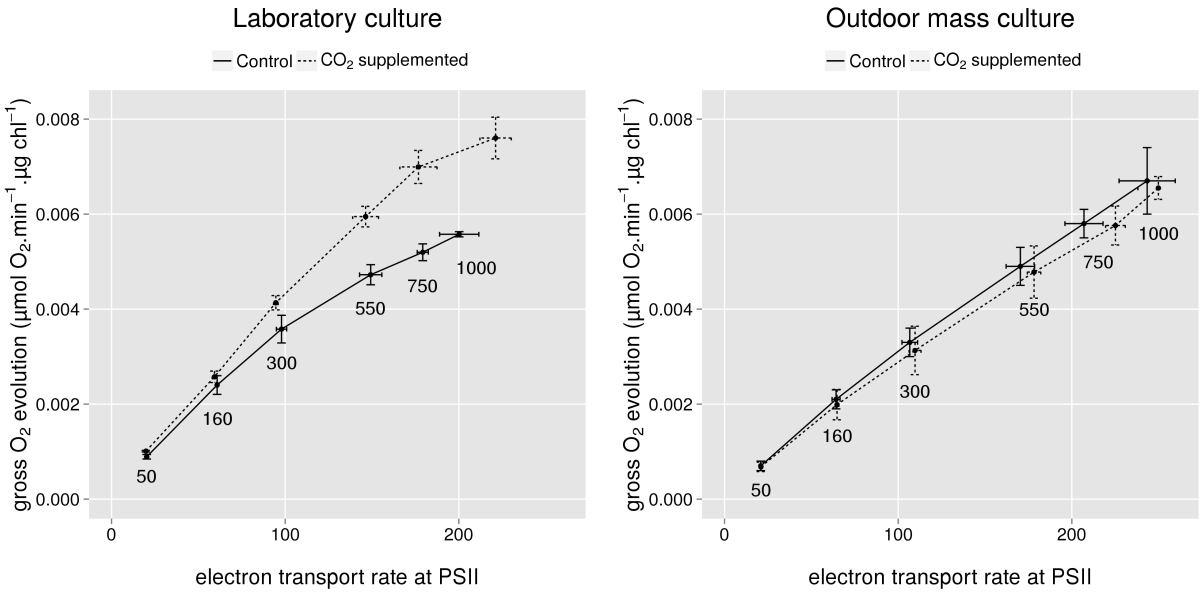

Figure 5: Relationship between relative electron transport rate and gross oxygen evolution measured at different light intensities. Left: Cultures grown in flasks in the laboratory under 200 µmol PAR.m^{-2}.s^{-1} continuous illumination in bold 3N medium. Cell were grown either with air or with CO_2 5% as sparging gas. Data are averages of 3 biological replicates (±SD). Right: Outdoor mass cultures. Data are average of 5 measurements (±SD) obtained during the linear growth phase of the cultures. Light intensities (µmol PAR.m^{-2}.s^{-1}) at which measurements were realised are indicated in the figure.

torespiration during the measurements. Fig. 5 shows that the ETR/VO_2 relationship in the control and the CO_2 supplemented cultures was different, even though the CO_2 limitation was removed during measurements.

In both conditions, the relationship was characterized by a curvature which indicates that the flux of electrons is gradually redirected to O_2 with increasing irradiance. However, in CO_2 supplemented culture, the ETR/VO_2 ratio became higher than in the control culture as the light intensity increased. This indicates that the proportion of electron flux directed to O_2 is higher in low CO_2 than in high CO_2 cells. Absence of CO_2 limitation during the measurement indicates that this O_2-dependent electron flux is structurally active. It can be concluded that alternative electron flow to O_2 occurs in both conditions at high light intensity but is most active after growth in low CO_2 condition.

However, this was not the case for the outdoor mass cultures for which the ETR/VO_2 relationships were characterised by a straight line for both conditions. This observation indicates that, if we except the photorespiration which was not assessed in this study, alternative electron flow to O_2 probably either did not occur in outdoor mass culture or was independent of CO_2 supply.

The reason for the absence of differences in the ETR/VO_2 relationship for the outdoor mass culture compared to the cultures grown in the laboratory must be due to the culture conditions which were very different. While the culture grown in the laboratory was cultivated at a low density and under continuous illumination, the outdoor mass culture was characterised by a higher density, day/night cycles and light/dark cycles during the day. The effect of these factors on the ETR/VO_2 relationship remains to be investigated.

3.2.2 Fluorescence measurements on light-adapted samples

We then measured the evolution of the PSII operating photochemical efficiency (ΦPSII) under different light intensities on light-adapted samples, i.e. after a dark period as short as 15 s which was necessary for transfer to the fluorimeter (Fig. 6). At low density during the first four days, there was no difference between the two cultures, suggesting that the control culture was not limited by DIC availability. This observation is confirmed by the small pH shift observed during the first four days (Fig. 2, second experiment). The fact that the maximal pH 11 was not attained at these days indicates that DIC availability was sufficient to sustain photosynthesis.

From day 5 to day 11, the general trend was that ΦPSII was always lower in the control culture than in the CO_2 supplemented culture, suggesting that DIC availability became too low to sustain an efficient photosynthesis in the former culture. Day to day variations in ΦPSII responses were observed, but we could not correlate them with the measured variables (temperature, daily PAR or productivities). More extended data sets would be necessary in order to analyse further the relationship between ΦPSII and the culture history.

Noteworthy, Fig. 6 also shows that the maximal ΦPSII values (F_V/F_M obtained in the dark) became lower in the control culture compared to the CO_2-supplemented culture starting from day 5, with this difference varying from one day to another. Low F_V/F_M values in microalgae mass cultures are generally taken as indicating some degree of photoinhibition. Midday PSII photoinhibition of $\approx$ 30% has been described in outdoor mass culture with *Arthrospira platensis* (Vonshak *et al.*, 1994; Vonshak *et al.*, 1996; Torzillo *et al.*, 1996; Torzillo *et al.*, 1998) and *Chlorella* (Masojídek *et al.*, 2011). Most photoinhibition

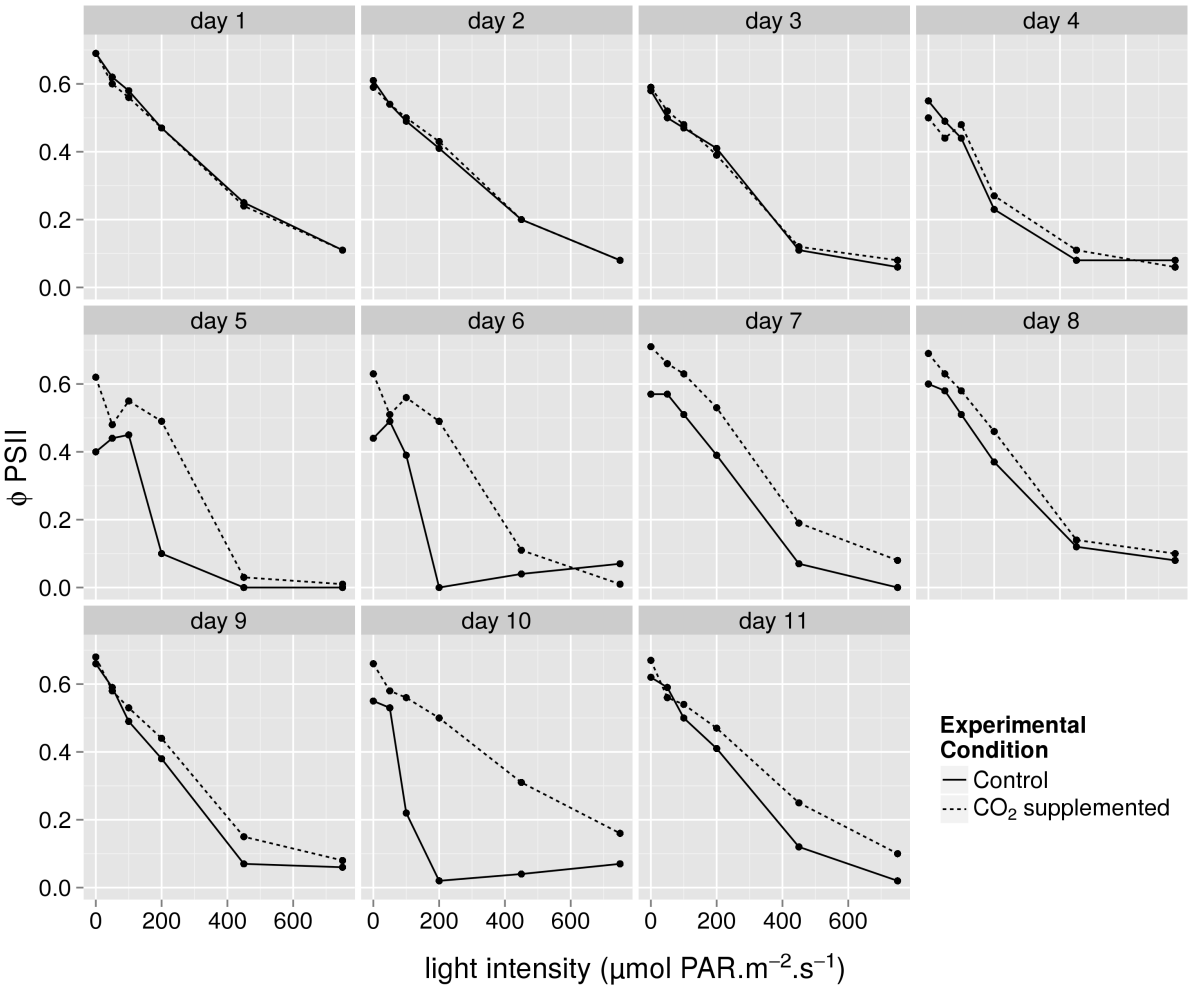

Figure 6: Evolution of the PSII photochemical efficiency (ΦPSII) under different light intensities during the course of the culture. Data shown are from experiment 2. A similar trend was observed for experiment 1.

studies conducted on *Scenedesmus obliquus* have been performed on cultures grown in the laboratory (Flameling and Kromkamp, 1997; Yang and Gao, 2003). Here, we show that a decreased F_V/F_M also occurs in Scenedesmus obliquus grown in outdoor mass cultures and that this photoinhibition is influenced by DIC availability. It must be noted, however, that differences in F_V/F_M and ΦPSII found here in relation to CO_2 supply were suppressed after dark-adaptation (40 min) followed by $NaHCO_3$ addition in the measurement cuvette (see Fig. 4).

A decreased photochemical efficiency (ΦPSII) in the control culture has to be compensated by dissipative mechanisms such as increased heat dissipation in the antennas (or energy dependent quenching qE NPQ). This process can be highlighted by monitoring the decrease of the F_M fluorescence level (NPQ) following illumination. We thus measured NPQ of samples directly taken from the cultures and exposed to different light intensities (Fig. 7). We checked that the fluorescence quenching observed here was relaxed in a few seconds, indicating that this quenching was due to qE NPQ and not to photoinhibition or state transitions. Fig. 7 shows that this NPQ was higher in the control culture than in the CO_2 supplemented culture. This demonstrates that a part of the absorbed light energy which could not be used for CO_2 assimilation due to its reduced availability in the control culture was dissipated as heat.

These measurements showed that heat dissipation for a particular light intensity is higher in the control culture compared to the CO_2 supplemented culture. However, although the cultures were inoculated at the same density, the density became different as the time elapses due to the different growth rates of the cultures (i.e. CO_2 supplemented culture density was higher than control culture density for each particular day). It follows that the average light intensity in the thickness of the cultivation system was higher in the control culture than for the CO_2 supplemented culture for the same incident light intensity and thus, differences in effective qE NPQ between the two culture must have been even higher than experimentally estimated on the basis of equal excitation.

A decreased PSII antenna size has been described in low CO_2 condition compared to high CO_2 condition in *Chlamydomonas reinhardtii* cultures grown in laboratory (Berger *et al.*, 2014). This phenomenon can be interpreted as a way to avoid on over-reduction of the electron transport chain in condition of restricted DIC availability. We thus determined if the control culture had a reduced PSII antenna size compared to the CO_2 supplemented culture. For this, we measured the functional PSII antenna size by measuring the halftime of the time course of the DCMU fluorescence rise. In presence of this inhibitor, the fluo-

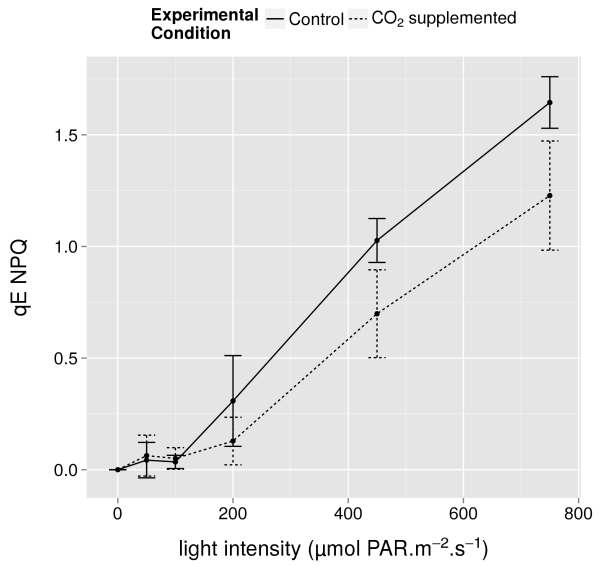

Figure 7: Evolution of the energy-dependent non-photochemical quenching (qE NPQ) under different light intensities showing a higher qE NPQ in the control culture than in the CO_2 supplemented culture at light intensities higher than 200 µmol PAR.m^{-2}.s^{-1}. Data are averages of 6 days (±SD) during the linear growth phase of the second culture, when DIC availability was limiting for the control culture.

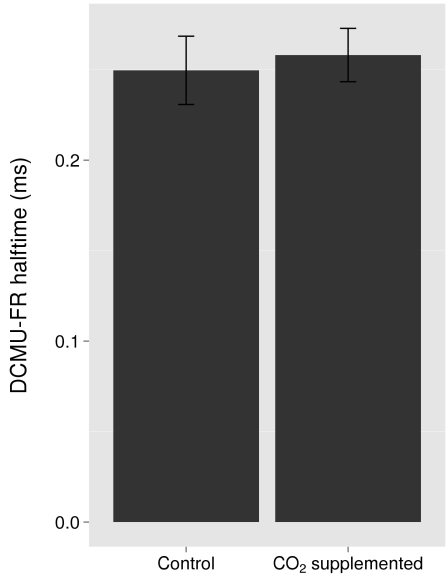

Figure 8: Average of the halftime of the DCMU-FR curve. The halftime is the time when the normalised variable fluorescence level reaches 0.5. Data are averages of 14 measurements (±SD) from experiments 1 and 2 during linear growth phase.

rescence rise represents the photochemical reduction of Q_A without influence of its reoxidation by plastoquinones. This measurement has been shown to be a good indicator of the PSII antenna size in *Chlamydomonas reinhardtii* (de Marchin *et al.*, 2014). We couldn't notice significant difference in the halftime between the control and the CO_2 supplemented culture during linear growth phase (Fig. 8). This indicates that the PSII functional antenna size is not modulated by CO_2 availability in *Scenedesmus obliquus* in outdoor mass culture conditions.

4 Conclusion

In this study, we showed that the productivity of outdoor mass cultures of *Scenedesmus obliquus* is limited by the CO_2 availability in open thin-layer cultivation systems at high light irradiance for biomass densities higher than 0.25 g.l^{-1}. This limitation is suppressed by injecting carbon dioxide in the culture. Under low integrated daily PAR (around 20-25 mol PAR.m^{-2}.day^{-1}), both cultures were characterised by a productivity of 7 ± 2.4 g dw.m^{-2}.day^{-1} and a PE of 3.3 ± 1.1 % (value based on the PAR solar radiation energy). In contrast, under the highest integrated daily PAR (55 mol PAR.m^{-2}.day^{-1}), the productivities and PE were very different with values of 10 ± 2.6 and 24 ± 3.9 g dw.m^{-2}.day^{-1} and 2.1 ± 0.5 and 5 ± 0.8 % for the control and the CO_2 supplemented cultures, respectively. From fluorescence and oxygen evolution measurements on dark-adapted samples, we couldn't find significant differences in the ETR and oxygen evolution light saturation curves between high and low CO_2 cultures. In contrast to cultures grown in the laboratory under constant light, there was no difference in the ETR/VO_2 relationship between the two cultures. This suggests that light-dependent O_2 uptake is not enhanced by DIC limitation in outdoor mass cultures. We conclude that the structural organisation of the photosynthetic apparatus is not affected by the CO_2 availability in outdoor mass cultures in this species. From fluorescence measurements on light-adapted samples, we found a lowest PSII photosynthetic efficiency and a higher NPQ for the low CO_2 culture which could be explained by an increased heat-dissipation and photoinhibition in this culture. In contrast, we couldn't find any differences in the PSII antenna size between the two conditions. The lowest productivity of the low CO_2 culture is paralleled by a decreased PSII photochemical efficiency and an increased heat-dissipation. These results exemplify the fact that high density microalgae mass cultures should be CO_2 supplemented to ensure efficient light utilisation and biomass productivity.

5 Appendix

5.1 Maximal theoretical biomass yield

The maximum theoretical biomass yield on light energy was calculated following Zijffers *et al.* (2010). Assuming an elemental composition of $CH_{1.64}O_{0.49}N_{0.10}$ for *Scenedesmus obliquus* (Zelibor *et al.*, 1988), the stoichiometric reaction equation for the formation of biomass on carbon dioxide, water and nitrogen source is the following:

- When grown on nitrate:

$$1\ CO_2(g) + 0.87\ H_2O(l) + 0.10\ NO_3^-(aq) \rightarrow CH_{1.64}O_{0.49}N_{0.10}(s) + 1.29\ O_2(g) + 0.10\ OH^-(aq) \quad (1)$$

- When grown on ammonium:

$$1\ CO_2(g) + 0.67\ H_2O(l) + 0.10\ NH_4^+(aq) \rightarrow CH_{1.64}O_{0.49}N_{0.10}(s) + 1.09\ O_2(g) + 0.10\ H^+(aq) \quad (2)$$

Assuming that the quantum yield of the light reactions is 0.1 mol O_2 evolved per mol of photons and that the molecular mass of a C-mol biomass of Scenedesmus obliquus is 22.88 g.mol^{-1}, 12.9 and 10.9 mol of photons are needed to produce one C-mol of biomass when grown on nitrate and ammonium, respectively. This leads to a theoretical biomass yield of 1.77 and 2.06 g.mol photons^{-1} when grown on nitrate and ammonium, respectively.

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7 Supplemental data

7.1 Example of typical fluorescence and oxygen evolution measurements

Figure S1.

7.2 Light attenuation in the thickness of the suspension

Figure S2.

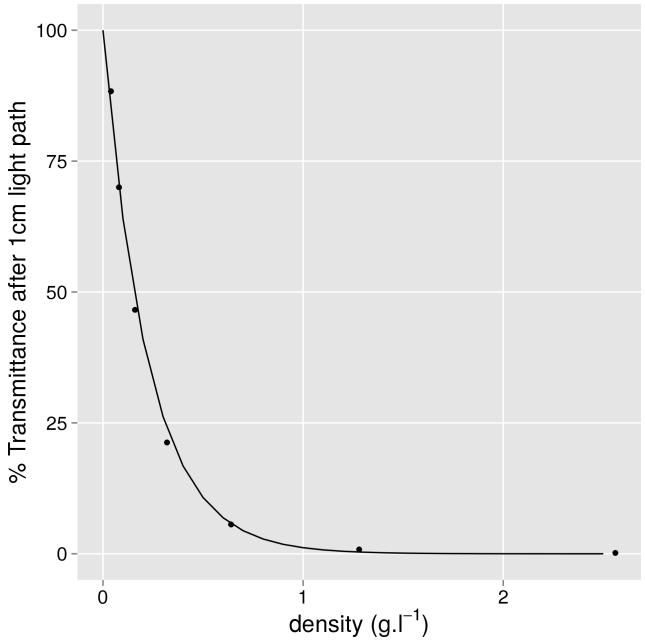
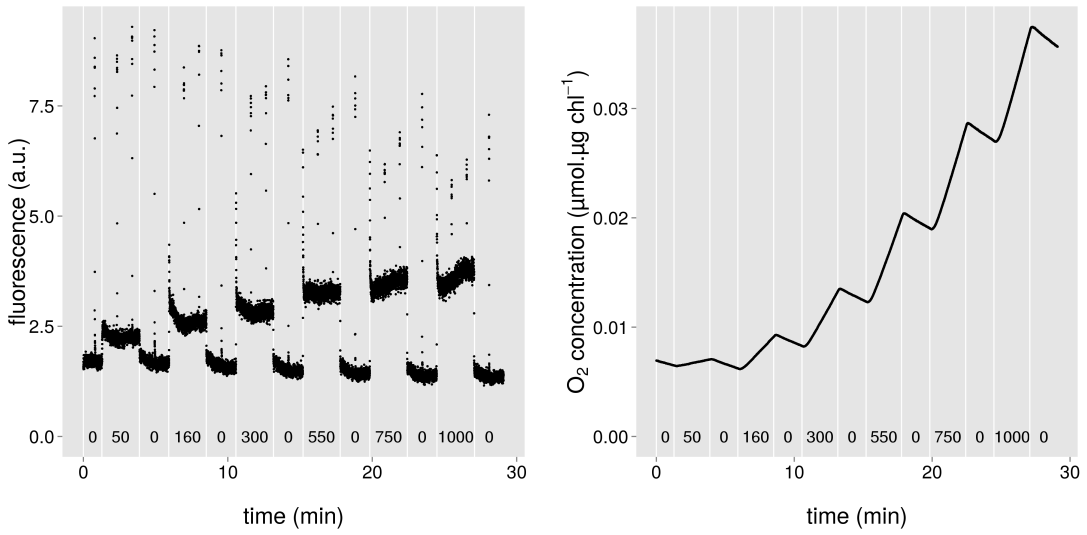
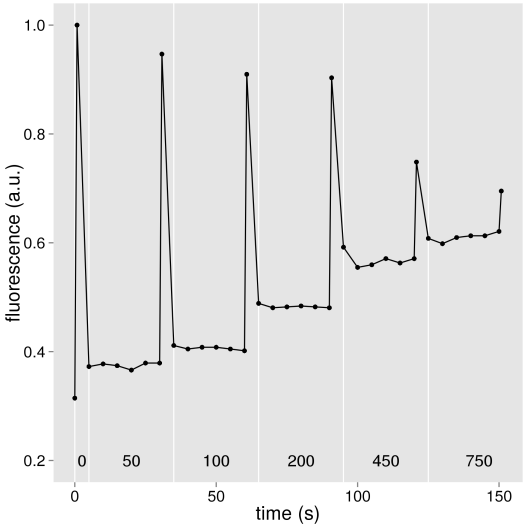


Figure S2: Transmittance (%) after 1 cm light path. Transmittance was determined by averaging the light transmission in the PAR region (400-700nm) of culture samples at densities ranging from 0.05 to 2.5 g.l^{-1}. Line: fit of the hyperbolic Beer-Lambert law (Yun and Park, 2003; Béchet *et al.*, 2013).



(a)

(b)



(c)

Figure S1: (a): Fluorescence trace during a typical dark-adapted experiment. (b): Variation in oxygen concentration during a typical dark-adapted experiment. Fluorescence and oxygen evolution measurements on dark-adapted samples were simultaneously recorded. (c): Fluorescence trace during a typical light-adapted experiment. Light intensities (µmol PAR.m^{-2}.s^{-1}) at which measurements were realised are indicated in the figure.