



HAL
open science

Investigation of lipid production by nitrogen-starved *Parachlorella kessleri* under continuous illumination and day/night cycles for biodiesel application

Ahmed Taleb, J. Legrand, H. Takache, S. Taha, Jeremy Pruvost

► To cite this version:

Ahmed Taleb, J. Legrand, H. Takache, S. Taha, Jeremy Pruvost. Investigation of lipid production by nitrogen-starved *Parachlorella kessleri* under continuous illumination and day/night cycles for biodiesel application. *Journal of Applied Phycology*, 2018, 30 (2), pp.761-772. 10.1007/s10811-017-1286-0 . hal-01951952

HAL Id: hal-01951952

<https://hal.science/hal-01951952>

Submitted on 20 Apr 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Investigation of lipid production by nitrogen-starved *Parachlorella kessleri* under continuous illumination and day/night cycles for biodiesel application

A. Taleb^{1,2,3} · J. Legrand¹ · H. Takache² · S. Taha³ · J. Pruvost¹

Abstract This study aims to investigate the triacylglycerol (TAG) productivity of *Parachlorella kessleri* grown under continuous illumination and to investigate its metabolism in simulated day/night cycles in order to estimate the feasibility of a large-scale production in outdoor solar photobioreactors. The strain was chosen for its ability to accumulate large amounts of triacylglycerol during nitrogen starvation. Several protocols of nitrogen starvation were tested in continuous illumination as well as in simulated day/night cycles. Sudden and progressive nitrogen starvation conditions have enhanced the TAG concentration and productivity of *P. kessleri* reaching up to 48 dry wt% and $4.4 \times 10^{-3} \text{ kg m}^{-2} \text{ day}^{-1}$, respectively. Microalgal cell metabolism was significantly affected by the day/night illumination cycles. The energy-rich compounds (TAGs and carbohydrates) were accumulated by *P. kessleri* during the photoperiods and partly consumed during the dark to sustain the microalgae vitality. This TAG oxidation ultimately led to a 26% decrease in TAG productivity in cultures exposed to day/night cycles compared to ones exposed to continuous illumination of equal 24-h average photon flux density. The results can dictate the optimal time for harvesting cells for recovering the largest amount of TAGs.

Keywords Chlorophyceae · Photobioreactors · Nitrogen starvation · TAG productivity · Day/night cycles · Biodiesel

Introduction

Seeking alternative resources of liquid fuels has become a pressing issue in the light of fears over depleting natural petroleum reserves and consequently the political and economic instability due to energy insecurity. In addition, climate change and its dramatic consequences on human persistence make this an existential matter. In this context, considerable research efforts were initiated over the last three decades to find renewable alternatives to conventional fossil fuels culminating in numerous research projects aimed to develop cost-effective biomass-based biofuel production platforms (Greenwell et al. 2010; Naik et al. 2010; Demirbas, 2011).

Microalgal biomass appears to be a sustainable feedstock for producing alternative fuels to petroleum owing to their efficient photosynthetic activity, their fast growth, and therefore their large biomass productivity (Chisti, 2007; Elsey et al. 2007; Hu et al. 2008; Li et al. 2008a, b). Moreover, these photosynthetic microorganisms exhibit higher lipid productivity with respect to terrestrial plants, due to their metabolism plasticity, since it is possible to control their metabolism and enhance their intracellular lipid accumulation by varying the culture conditions. Recently, the freshwater species *Parachlorella kessleri* was determined to be an efficient lipid producer (Přibyl et al. 2012; Li et al. 2013; Taleb et al. 2016). This freshwater species was previously named *Chlorella kessleri* Fott & Nováková (Huss et al. 1999). However, based on molecular data, Krienitz et al. (2004) revealed the existence of two sister genera *Chlorella* and *Parachlorella* in the Chlorellaceae and Trebouxiophyceae. Thus, *Chlorella kessleri* was transferred in 2004 to a new genus, *Parachlorella*, as

✉ J. Pruvost
jeremy.pruvost@univ-nantes.fr

¹ GEPEA, Université de Nantes, CNRS, UMR6144, bd de l'Université, CRTT - BP 406, 44602 Saint-Nazaire Cedex, France

² Department of Food Science and Technology, Faculty of Agricultural and Veterinary Medicine, Lebanese University, Dekwaneh, Lebanon

³ AZM Center for Biotechnology Research and Its Applications, Laboratory of Applied Biotechnology, Lebanese University, EDST, Tripoli, Lebanon

Parachlorella kessleri. In our recent work (Taleb et al. 2016), *P. kessleri* UTEX2229 was selected from 15 investigated microalgae strains, as the best candidate for biodiesel production thanks to its high triacylglycerol (TAG) accumulation potential (up to 40% DW), its aptitude to sustain a large biomass growth rate in nitrogen starvation conditions, and its large areal TAG productivity ($2.7 g_{TAG} m^{-2} day^{-1}$ or $10 t_{TAG} ha^{-1} year^{-1}$). Moreover, this species has shown great potential for allowing TAG extraction and recovery from wet biomass leading to significant energy savings associated with drying the biomass prior to TAG extraction (Angles et al. 2017). Under favorable growth conditions, *P. kessleri* cells contain low amounts of structural lipids such as glycolipids and phospholipids (1–10% DW) which are the main constituents of cell membranes. However, when the growth conditions become unfavorable, for example, when the cells are subjected to light or nutrient stress, the culture can accumulate large amounts of energy-rich compounds such as triglycerides and starch. It was also noted that in nitrogen-limited conditions, *P. kessleri* initially accumulated large amounts of starch and subsequently simultaneous lipid accumulation and starch consumption was initiated (Brányiková et al. 2011; Fernandes et al. 2013; Mizuno et al. 2013; Takeshita et al. 2014). Oleaginous microalgal strains often have a lower growth rate than non-oleaginous ones (Hu et al. 2008); nevertheless, *P. kessleri* has the ability to maintain a relatively large growth rate even in nitrogen deprivation conditions resulting in large lipid productivity (Taleb et al. 2016).

Nitrogen starvation is the most efficient strategy to trigger large amounts of neutral lipid accumulation in microalgae (Tornabene et al. 1983; Hu et al. 2008; Van Vooren et al. 2012; Kandilian et al. 2014; Taleb et al. 2015, 2016). In batch mode, nitrogen starvation can be performed via two methods: sudden starvation and progressive starvation. The former is achieved by separating the cells from a nitrogen-replete culture medium, and then re-suspending them in a nitrogen-free medium, whereas the latter is realized by inoculating the cells in a medium containing limited nitrogen concentration. Therefore, in progressive nitrogen starvation, microalgae cells become progressively nitrogen starved through the consumption of the initial nitrogen in the medium (Fernandes et al. 2013). In terms of energy consumption, progressive starvation technology has the advantage of avoiding the separation step which can be energy intensive (Pruvost et al. 2009; Van Vooren et al. 2012).

Light intensity and photoperiod also affect the growth rate as well as the lipid metabolism of microalgal cells (Pruvost et al. 2009, 2011; Van Vooren et al. 2012; Kandilian et al. 2014). Van Vooren et al. (2012) and Kandilian et al. (2014) have demonstrated that light absorption rate is a fundamental factor affecting lipid metabolism and subsequently lipid productivity of microalgae. Their results showed that fatty acid synthesis pathways were significantly enhanced by the combination of two fundamental

parameters: nitrogen concentration in the medium and the rate of photons absorption by the culture. Thus, for a given microalgal strain, maximizing TAG production entails the optimization of these two parameters. Note that light availability in microalgae culture systems mainly depends on the culture cells density, affecting light attenuation in the photobioreactor (PBR), as well as the radiative and optical properties of the strains (Kandilian et al. 2014, 2016).

Many studies have focused on microalgal TAG productivity in nitrogen-deficient conditions under continuous illumination (Van Vooren et al. 2012; Kandilian et al. 2014; Taleb et al. 2015). However, outdoor PBRs are exposed to solar radiation where the light intensity continuously changes during the day highly affecting cell physiology, growth, and biochemical composition (Sukenik and Carmeli, 1990; Fábregas et al. 2002; Khoeyi et al. 2012; Lacour et al. 2012; Gris et al. 2014). Therefore, it is essential to investigate TAG production under diurnal light cycles to estimate biodiesel production more accurately in industrial outdoor solar microalgae cultures. In diurnal regimes, light periods are characterized by high photosynthetic activity, elevated CO₂ fixation rate, and large biomass productivity (Sukenik and Carmeli, 1990; Fábregas et al. 2002; Gris et al. 2014). Moreover, the high rate of photon or energy absorption by the cells over this period enhances the intracellular accumulation of energy-rich compounds such as carbohydrates and TAGs. On the other hand, microalgae cells respire during the dark periods and resort to oxidization of their intracellular energy-rich compounds to support their growth (Sukenik and Carmeli, 1990; Fábregas et al. 2002). This typically leads to the loss of overall biomass productivity compared to the same culture grown in continuous illumination (Le Borgne and Pruvost, 2013; Michels et al. 2014).

Furthermore, day/night cycles and the duration of photoperiod not only affect lipid productivity, but also fatty acid composition of the cells (Fábregas et al. 2002; Perez-Pazos and Fernandez-Izquierdo, 2011; Khoeyi et al. 2012). Note that carbohydrates are also recognized as high-energy storage compounds and their synthesis is strongly influenced by the day/night cycles and the photoperiod. In addition, the metabolism of these energy-rich compounds under the dark/light regime is species dependent. Fábregas et al. (2002) reported that *Nannochloropsis* strains consume lipids rather than carbohydrates during the dark period. Moreover, Brányiková et al. (2011) and Fernandes et al. (2013) have examined the effects of light and dark periods on the energy-rich compounds of *Chlorella vulgaris* in both normal and nutrient limited cultures. These authors have reported that compared to control cultures, starved cells did not show a marked decrease of their energy stores compounds in the dark because their energy-requiring growth and reproductive processes were slowed due to nutrient restrictions. However, to the best of

our knowledge, no study has reported the effects of day/night cycles and nitrogen starvation on the biochemical composition and the growth rate of *P. kessleri*.

This work aimed to investigate the production of TAG and carbohydrates by the freshwater strain *P. kessleri* grown under continuous illumination or day/night cycles in nitrogen starvation conditions. Two different methods of nitrogen starvation were used and several culture protocols were applied to batch cultures of *P. kessleri* under continuous illumination. The strain was also exposed to simulated day/night cycles that represent solar conditions in Nantes, France.

Material and methods

Microalgal strain, photobioreactors, and culture medium

Parachlorella kessleri, strain UTEX2229, was obtained from the culture collection of algae at the University of Texas at Austin (UTEX). The cells were grown in nitrogen-replete modified Bold Basal medium (BBM) for nutrient-replete experiments and in nitrogen-free BBM for the nitrogen starvation experiments. The composition of the BBM medium was previously described by Pruvost et al. (2009). The cells were cultivated in 1 L airlift-type flat-panel PBR with thickness of 3 cm (specific illuminated area $a_{\text{light}} = 33 \text{ m}^{-1}$) for cultures grown in batch or continuous mode. The PBR was described in more detail in Pruvost et al. (2009) and Pruvost et al. (2011). The temperature and pH were regulated at 25 °C and 8, by forced air convection on the back of the PBR and continuous injection of sterile air with 2 vol.% CO₂ at a flow rate of 3 mL min⁻¹ (Taleb et al. 2016). Illumination was provided on one face of the PBR by a white LED light panel (NSPW500CS, NICHIA) with adjustable photon flux density (PFD) allowing the application of different light intensities and controlled day/night cycles during the cultivation. The incident PFD was measured over the PAR region at 12 different locations on the inside illuminated surface of the PBR which was made of transparent polymethyl-methacrylate (PMMA) using a quantum light sensor (LI-250A, LICOR, USA). Here, the reported incident light intensities represented the mean value of the PFDs measured at 12 locations.

Cultivation conditions under continuous conditions

Optimal growth conditions

Parachlorella kessleri was first grown in nitrogen-replete conditions in 1 L airlift-type PBR operating in continuous mode, with a low dilution rate $D = 0.01 \text{ h}^{-1}$, in order to produce seed biomass having identical physiological state for all the batch experiments. The continuous culture was exposed to an incident photon flux density of $250 \mu\text{mol}_{\text{h}\nu} \text{ m}^{-2} \text{ s}^{-1}$. Upon

reaching steady-state, some volume of the culture was harvested and used to supply biomass to inoculate the nitrogen starvation experiments.

Nitrogen deprivation conditions

Two sudden and two progressive nitrogen starvation experiments were carried out in batch mode exposed to continuous light. Cultivation conditions were varied in order to highlight the effect of light attenuation conditions on lipid metabolism. For sudden nitrogen starvation experiments, the PBRs were exposed to an incident PFD of $250 \mu\text{mol}_{\text{h}\nu} \text{ m}^{-2} \text{ s}^{-1}$ and two different initial biomass concentrations were chosen, namely $X_0 = 0.2$ and $X_0 = 0.76 \text{ kg m}^{-3}$. To prepare the inoculum, a given culture volume, depending on the desired initial biomass concentration of the experiment, was harvested from the continuous culture and then centrifuged for 5 min at 6000×g. Afterwards, the supernatant was eliminated and the pellet was transferred to another PBR containing nitrogen-free medium. For the two progressive starvation experiments, the initial biomass concentration X_0 and the nitrogen concentration $[N]_0$ were fixed at 0.05 kg m^{-3} and 2.3 mM, respectively, while the two different PFDs tested were 250 and $160 \mu\text{mol}_{\text{h}\nu} \text{ m}^{-2} \text{ s}^{-1}$.

Cultivation conditions under (16:8 h) day/night cycles

Finally, *P. kessleri* was cultivated in sudden starvation mode exposed to day/night illumination cycles for 7 days. Figure 1 shows the average annual day/night cycle in Nantes, France, obtained using the software Meteonorm (Meteonorm, Meteotest, Switzerland) (Pruvost et al. 2012). It shows that the day extends over 16 h, while the night duration was 8 h. During the day, the PFD progressively increases and reaches a maximum value of $850 \mu\text{mol}_{\text{h}\nu} \text{ m}^{-2} \text{ s}^{-1}$ after 9.25 h and subsequently decreases to zero. The PFD averaged over 24 h was

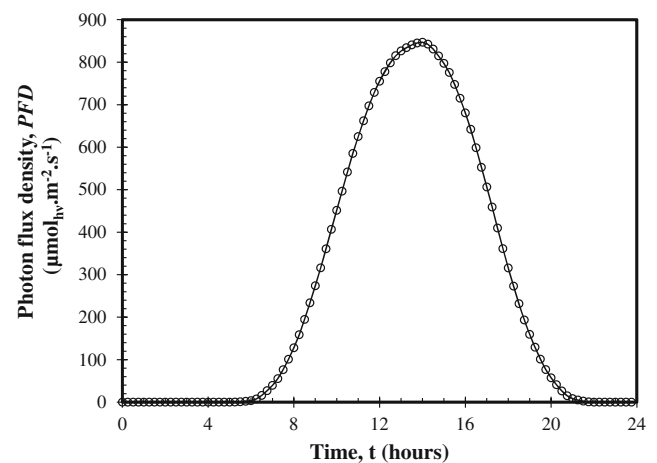


Fig. 1 Time evolution of PFD applied to the PBR during the simulated day/night cycles. This cycle represents the PFD received by a horizontal PBR during an average day in Nantes, France

equal to $270 \mu\text{mol}_{\text{hv}} \text{m}^{-2} \text{s}^{-1}$. The culture had an initial biomass concentration X_0 equal to 0.2 kg m^{-3} . The PBR was sampled twice daily on the first, fourth, and seventh days of the experiment. The cell proteins, carbohydrates, total lipids, and TAG concentrations were determined after 13.5 h of illumination each day/night cycle, and at the end of the dark period, i.e., 24 h after the start of the previous illumination cycle in order to investigate the metabolic alterations that occurred overnight.

Analytical methods

Cell count

Cell concentration was determined by counting the number of cells under an optical microscope using the Malassez counting cell. Cell concentration, N , was expressed as the number of cells per milliliter of culture (cells mL^{-1}).

Kinetics growth determination in nitrogen starvation conditions

In order to assess the kinetics growth of *P. kessleri*, the dry biomass concentration namely X (in kg m^{-3}) of each culture was monitored every 2 days for duration of 2 weeks for the nitrogen starvation experiments performed in continuous illumination conditions. Nevertheless, for the sudden nitrogen starvation experiment in day/night cycles, the biomass concentration was measured at the end of the illumination period (after 13.5 h of illumination), and at the end of the dark period on the first, fourth, and seventh day of the culture.

To determine X , a given culture volume was filtered through a pre-dried and pre-weighed glass fiber filter (Whatman GF/F). The filters were dried again at $105 \text{ }^\circ\text{C}$ for 24 h, cooled down in a desiccator for 10 min and then weighed again. The biomass concentration was then calculated from the filter weight difference after and before the culture filtration, divided by the filtered volume. Given results are the arithmetic means of three measurements. Biomass concentration was used to determine volumetric biomass productivity. In batch mode, it is defined as the biomass growth over the culture period " T ":

$$r_X = \frac{X_f - X_i}{T} \quad (1)$$

Here, X_i and X_f denote the dry biomass concentration at the beginning and at the end of the culture period, respectively.

Chlorophyll concentration

Chlorophyll was extracted in pure methanol (99.8%) for duration of 30 min at $44 \text{ }^\circ\text{C}$, in the dark to avoid pigments

degradation. Afterward, the extract was centrifuged, and the optical density of the supernatant was measured at 750, 665, and 652 nm using an UV-vis spectrophotometer. Subsequently, the obtained optical density OD_{665} , OD_{652} , and OD_{750} were used in Eqs. 2 and 3 according to Ritchie (2006) in order to estimate the concentrations of chlorophyll *a* (**Chl-a**) and chlorophyll *b* (**Chl-b**). All extractions were performed in triplicates.

$$\begin{aligned} [\text{Chl-a}] \text{ (mg L}^{-1}\text{)} \\ = [-8.0962 (OD_{652} - OD_{750}) + 16.5169 (OD_{665} - OD_{750})] \end{aligned} \quad (2)$$

$$\begin{aligned} [\text{Chl-b}] \text{ (mg L}^{-1}\text{)} \\ = [27.4405(OD_{652} - OD_{750}) - 12.1688 (OD_{665} - OD_{750})] \end{aligned} \quad (3)$$

Lipid extraction and TAG productivity

The total lipids were extracted from the dried biomass with 2:1 (v/v) organic solvent mixture of chloroform: methanol. Triglycerides were then separated from other lipid classes by solid-phase extraction (SPE). In continuous illumination, the nitrogen-starved cultures were sampled every 2 days over 2 weeks of cultivation. On the other hand, the nitrogen-starved culture exposed to the day/night cycles, was sampled at the end of the illumination period and at the end of the dark period of the first, fourth, and seventh day of the experiment. Total lipids and TAG concentrations were determined by gas chromatography with a flame ionization detector (GC-FID) after their transesterification with methanol. The protocol is described in more detail by Van Vooren et al. (2012). Volumetric TAG productivity, P_{TAG} (in $\text{kg m}^{-3} \text{ day}^{-1}$) was calculated as the product of the biomass production rate r_X and the TAG concentration by dry weight w_{TAG} . Likewise, the areal TAG productivity namely S_{TAG} (in $\text{kg m}^{-2} \text{ day}^{-1}$), was calculated from the volumetric TAG productivity and the PBR specific illuminated area by

$$S_{\text{TAG}} = P_{\text{TAG}} \times \frac{V}{S_{\text{light}}} = \frac{P_{\text{TAG}}}{a_{\text{light}}} \quad (4)$$

Here, V (m^3) and S_{light} (m^2), respectively, correspond to the volume and the illuminated surface area of the PBR while a_{light} (m^{-1}) denotes the illuminated specific area.

Total sugar measurement

Total sugar concentration was measured by the phenol-sulfuric method (Dubois et al. 1956). An appropriate dilution of microalgae suspension was first made to ensure that its optical density fit the calibration curve relating optical density to glucose standard concentrations $OD_{484} = f(\text{glucose})$; 0.5 mL of the diluted mixture was then placed in 15-mL tube, and 0.5 mL of

phenol (50 g L^{-1}) as well as 2.5 mL of sulfuric acid (98%) were added without touching the tube wall. After 10 min, the tubes were vortexed for 10 s each and incubated at $35 \text{ }^\circ\text{C}$ for 30 min. The sulfuric acid hydrolyzes polysaccharides into monosaccharides such as pentose and hexose that dehydrated and rearranged into furfural and hydroxymethylfurfural, respectively. These unstable products condensate with the phenol and a yellow coloration appears as result. The optical density **OD** was then measured at 484 nm, using UV-vis spectrophotometer. Total sugar concentration was finally determined from the glucose calibration curve.

Protein measurement

The procedure for measuring proteins was followed according to Lowry et al. (1951), using bovine serum albumin (BSA) as standard. First, microalgae suspension was diluted with distilled water so that the protein concentration in the mixture was in the range of 0 and 1000 mg L^{-1} . To enable protein extraction, the cells were hydrolyzed by NaOH for 15 min at $95 \text{ }^\circ\text{C}$. The mixture was then neutralized by adding HCl and centrifuged for 5 min at $6000\times g$ in order to remove the cell debris. Afterwards, 0.1 mL of the hydrolysate was placed in a tube and 1 mL of the reagent [10 mL (Na_2CO_3 20 g L^{-1} , NaOH 0.1 N) + 200 μL (CuSO_4 5 g L^{-1})] added. The tube was then vortexed and after 10 min, 0.1 mL of Folin reagent was added. This was immediately followed by vortex mixing. After 30 min, the optical density OD of the samples was measured at 750 nm in a UV-vis spectrophotometer. All samples were prepared in triplicates and measured.

Statistical analysis

Dry biomass and chlorophylls concentrations, in addition to total sugars and proteins measurements were performed in three replicates for all the samples, whereas the cells number of each sample was counted five times. Given results are the arithmetic means of the measurements and the error bars correspond to the standard deviation. However, TAG quantification measurements were not duplicated due to the PBR volume and the frequency of sampling.

Results and discussion

Growth kinetics assessment in sudden and progressive nitrogen starvations

Figure 2 illustrates the biomass and total chlorophyll concentration of *P. kessleri* in (a) sudden and (b) progressive nitrogen starvation experiments performed under continuous illumination. In all cases, the cultures displayed biomass concentration

increase despite the unfavorable conditions of nitrogen limitation. Indeed, Fig. 2a shows that the biomass concentration of *P. kessleri* cultures with X_0 equal to 0.2 and 0.76 kg m^{-3} grown in sudden nitrogen starvation conditions increased 14 and 7 times over the 14 days of cultivation, respectively. This was accompanied by a sharp decrease in cell total chlorophyll (Chl-a and Chl-b) concentration. Similar observations were made by Van Vooren et al. (2012) for *Nannochloropsis oculata* grown in sudden nitrogen starvation mode in continuously illuminated PBRs. Cells typically reduce their pigment concentration in response to the presence of large amounts of illumination. This is a mechanism for cells to prevent photoinhibition by reducing the amount of light absorbed (Dubinsky and Stambler, 2000). Indeed, at the end of the experiment, the culture with initial biomass concentration $X_0 = 0.2 \text{ kg m}^{-3}$ contained 18 times lower total chlorophyll concentration than the culture with $X_0 = 0.76 \text{ kg m}^{-3}$. These respectively corresponded to chl_{a+b} equal to 124 and 7 pg cell^{-1} . The difference in the total chlorophyll concentration between the two cultures may be explained by the fact that the culture with $X_0 = 0.2 \text{ kg m}^{-3}$ had a larger rate of light absorption resulting in more pigment degradation. Moreover, chlorophylls may also act as internal nitrogen storage. Their degradation in nitrogen starvation conditions may have supplied the cells the necessary nitrogen to sustain their growth.

Figure 2b shows that in both progressive nitrogen starvation cultures the total chlorophyll concentration increased until the nitrate added to the medium was consumed. Then, the sharp decrease in total chlorophyll concentration was similar to that observed in the sudden starvation cultures. On the other hand, biomass concentration increased 54-fold from $X_0 = 0.05 \text{ kg m}^{-3}$ at the beginning of the experiment to $X = 2.7 \text{ kg m}^{-3}$ after 11 days for both experiments. The two cultures displayed similar biomass growth despite the facts that they were exposed to two different PFDs. In contrast, the culture exposed to $250 \mu\text{mol}_{\text{hv}} \text{ m}^{-2} \text{ s}^{-1}$ had lower pigment concentration than that exposed to $160 \mu\text{mol}_{\text{hv}} \text{ m}^{-2} \text{ s}^{-1}$ after 11 days of cultivation. This may be due to cells' synthesis of chlorophyll in order to maximize their ability to absorb light in a well-known process called photoadaptation as described by Falkowski and LaRoche (1991), Hu et al. (2008), and Finazzi et al. (2010).

Investigation of TAG production in sudden and progressive nitrogen starvations

Figure 3 shows the TAG concentration and areal TAG productivity S_{TAG} for the four nitrogen starvation experiments. In general, the cells accumulated large amounts of TAG during both sudden and progressive nitrogen starvation cultures reaching a maximum TAG concentration of 48 and 46% by dry mass in sudden and progressive nitrogen starvation cultures, respectively.

Fig. 2 Time evolution of biomass and total chlorophyll concentration (*chl a* and *b*) under continuous illumination in **a** sudden and **b** progressive nitrogen starvations. Error bars show the standard deviation of the mean values of three replicates

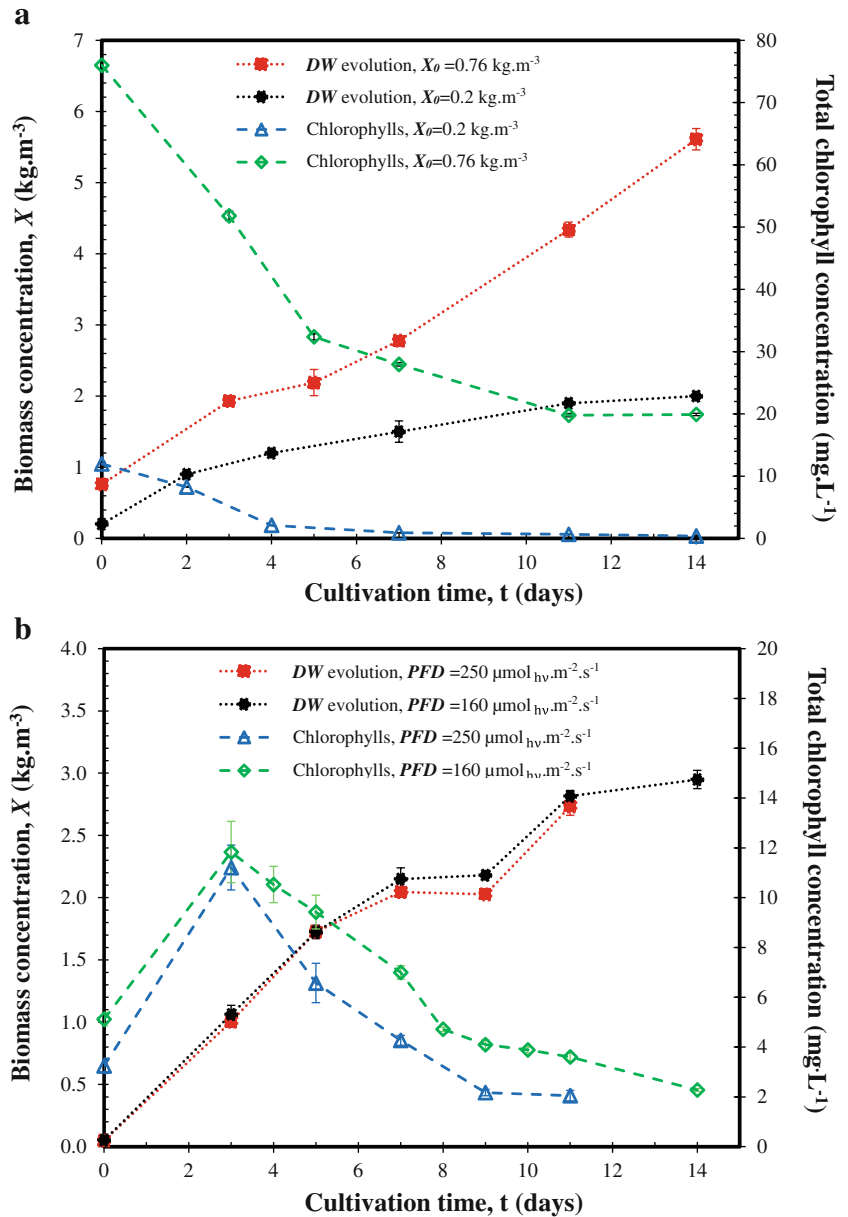


Figure 3a, b, respectively, show the temporal evolution of TAG concentration and TAG productivity of *P. kessleri* during the sudden nitrogen starvation experiments. They illustrate that TAG accumulation was directly initiated after transferring the cells to nitrogen-free medium. Moreover, the TAG accumulation rate was largest during the first 7 days of the experiment. The cultures with $X_0 = 0.2$ and 0.76 kg m^{-3} featured maximum TAG concentration of 45.6 and 37.6% DW after 2 weeks of cultivation. The larger maximum TAG concentration of the culture with $X_0 = 0.2 \text{ kg m}^{-3}$ may be attributed to the larger rate of energy absorption of the culture compared to the one with initial biomass concentration of 0.76 kg m^{-3} . These results highlight the fundamental effects of light on *P. kessleri* lipid metabolism and TAG

accumulation and agree with observations by Van Vooren et al. (2012) and analysis by Kandilian et al. (2014) for the marine eustigmatophyceae *Na. oculata*, where dilute microalgae suspensions accumulated larger concentration of TAGs compared to dense suspensions grown in identical conditions. Similar observations were reported by Brányiková et al. (2011) for *C. vulgaris*. Moreover, Fig. 3b shows that the culture with initial biomass concentration $X_0 = 0.76 \text{ kg m}^{-3}$ featured areal TAG productivity of $4.4 \times 10^{-3} \text{ kg}_{\text{TAG}} \text{ m}^{-2} \text{ day}^{-1}$ which was 63% larger than that of the culture with $X_0 = 0.2 \text{ kg m}^{-3}$. Note that despite the larger TAG concentration of the latter culture, it featured smaller TAG productivity due to its smaller biomass concentration (Fig. 3a).

Fig. 3 Time evolution of TAG concentration (a, c) and areal TAG productivity (b, d) in sudden (a, b) and progressive (c, d) nitrogen starvation

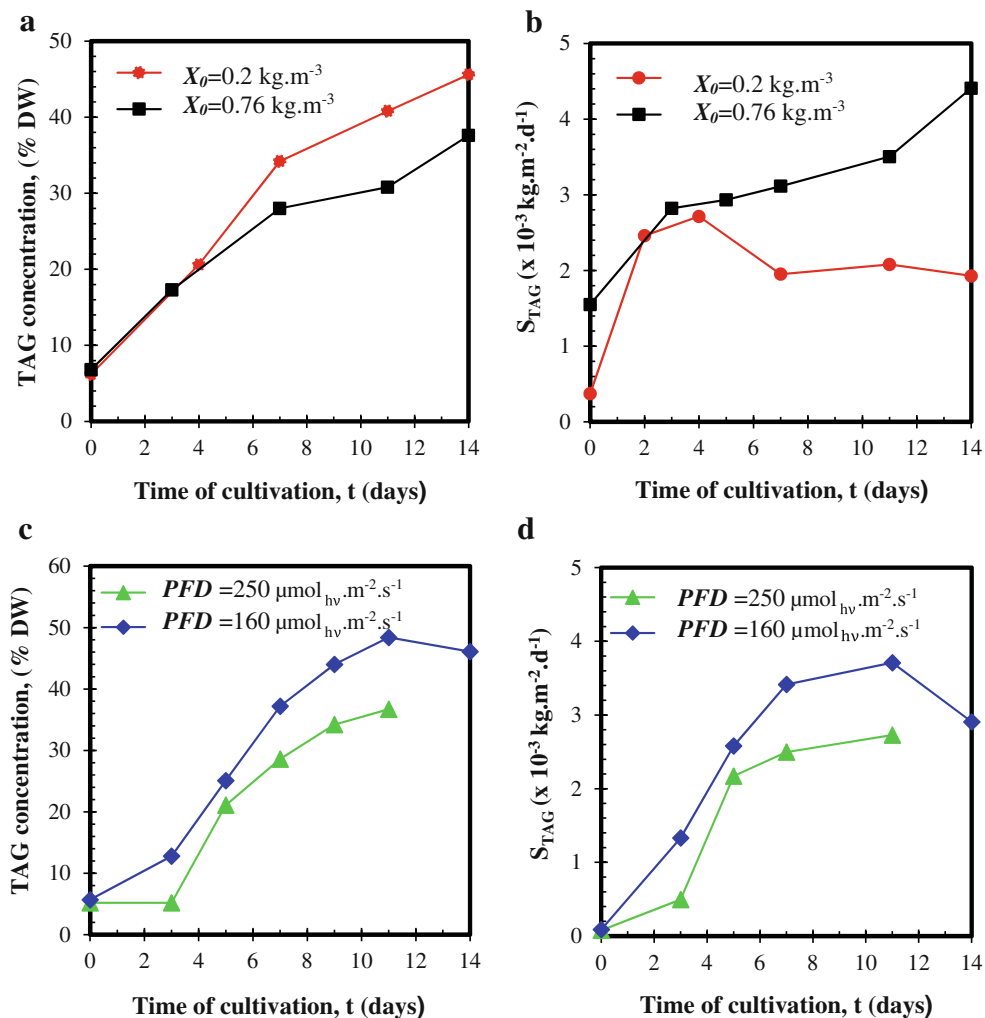


Figure 3c, d, respectively, show the temporal evolution of TAG concentration and TAG productivity of *P. kessleri* during the progressive nitrogen starvation experiments. It illustrates that the intracellular TAG accumulation in cells began 3 days after inoculating the PBRs. The cells consumed the initial nitrogen added to the medium during this period. Once the cells were starved of nitrogen, TAG productivity sharply increased. In addition, the culture exposed to $160 \mu\text{mol}_{hv}.\text{m}^{-2}.\text{s}^{-1}$ had larger intracellular TAG concentration and subsequently featured larger areal TAG productivity than the culture exposed to $250 \mu\text{mol}_{hv}.\text{m}^{-2}.\text{s}^{-1}$ despite the fact that both cultures featured identical initial biomass and nitrogen concentration. These respectively corresponded to 48.4% and 36.7% by dry weight on the eleventh day of the experiment equaling to maximum areal TAG productivity of $3.7 \times 10^{-3} \text{ kg}_{TAG} \text{ m}^{-2} \text{ day}^{-1}$ and $2.7 \text{ kg}_{TAG} \text{ m}^{-2} \text{ day}^{-1}$, respectively. The higher productivity of the culture exposed to $160 \mu\text{mol}_{hv} \text{ m}^{-2} \text{ s}^{-1}$ can be explained by its higher chlorophyll concentration. The latter led to a larger absorption cross section of the cells and therefore a larger rate of photon absorption ultimately leading to its larger TAG accumulation and productivity (Kandilian et al. 2014). Nevertheless,

the cells' regulation of their pigment concentration in response to the illumination conditions and the rate of light absorption may be strain dependent. Indeed, Van Vooren et al. (2012) grew *N. oculata* in identical conditions to that reported here and showed that TAG concentration of the cells increased with increasing light intensity. The latter is contradictory to the results obtained here with *P. kessleri*. Future studies can perform detailed light transfer analysis to quantitatively determine each culture's rate of photon absorption as demonstrated by Kandilian et al. (2014) for *N. oculata*.

The *P. kessleri* strain used in this study featured larger TAG concentration than the *N. oculata* strain grown by Van Vooren et al. (2012) in identical conditions. Moreover, *P. kessleri* had a larger biomass growth rate in nitrogen starvation conditions and subsequently featured 30% larger maximum areal TAG productivity compared to *N. oculata* (Table 1). Thus, *P. kessleri* UTEX2229 appears as a more attractive option than *N. oculata* for industrial biodiesel production. Nevertheless, to determine the feasibility of a large-scale production using *P. kessleri* UTEX2229, it is necessary to investigate its TAG

Table 1 Comparison of *P. kessleri* TAG content and areal TAG productivity to *N. oculata* Alphabiotec data reported by Van Vooren et al. (2012) in the same nitrogen starvation conditions

tbcolw60ptStrain	tbcolw60ptCulture system	tbcolw60ptNitrogen starvation method	tbcolw52ptMaximum total lipids content (% DW)	Maximum TAG content (% DW)	$S_{TAG_{max}}$ ($10^{-3} \text{ kg m}^{-2} \text{ day}^{-1}$)	Biomass productivity ($\text{kg m}^{-3} \text{ day}^{-1}$)	References
<i>P. kessleri</i> UTEX2229	Airlift PBR (1 L)	Sudden starvation	41.5 ^b –65 ^a	38 ^b –46 ^a	2.7 ^a –4.4 ^b	0.28 ^a –0.45 ^b	Present study
<i>N. oculata</i> Alphabiotec	Airlift PBR (1 L)	Sudden starvation	19 ^b –50 ^a	14.7 ^b –43 ^a	1.4 ^b –2.6 ^a	0.18 ^a –0.31 ^b	(Van Vooren et al. 2012)
<i>P. kessleri</i> UTEX2229	Airlift PBR (1 L)	Progressive starvation	45 ^c –55 ^d	37 ^c –48 ^d	2.7 ^c –3.7 ^d	0.3 ^{c, d}	Present study
<i>N. oculata</i> Alphabiotec	Airlift PBR (1 L)	Progressive starvation	48 ^d –50 ^c	32 ^d –42 ^c	2.6 ^d –2.9 ^c	0.2 ^d –0.24 ^c	(Van Vooren et al. 2012)

^a $X_0 = 0.2 \text{ kg m}^{-3}$

^b $X_0 = 0.76 \text{ kg m}^{-3}$

^c $PF D = 250 \mu\text{mol}_{\text{hv}} \text{ m}^{-2} \text{ s}^{-1}$

^d $PF D = 160 \mu\text{mol}_{\text{hv}} \text{ m}^{-2} \text{ s}^{-1}$

^e $PF D = 500 \mu\text{mol}_{\text{hv}} \text{ m}^{-2} \text{ s}^{-1}$

production in day/night cycles, i.e., in conditions more representative of outdoor solar production conditions.

Investigation of the strain's behavior in day/night cycles

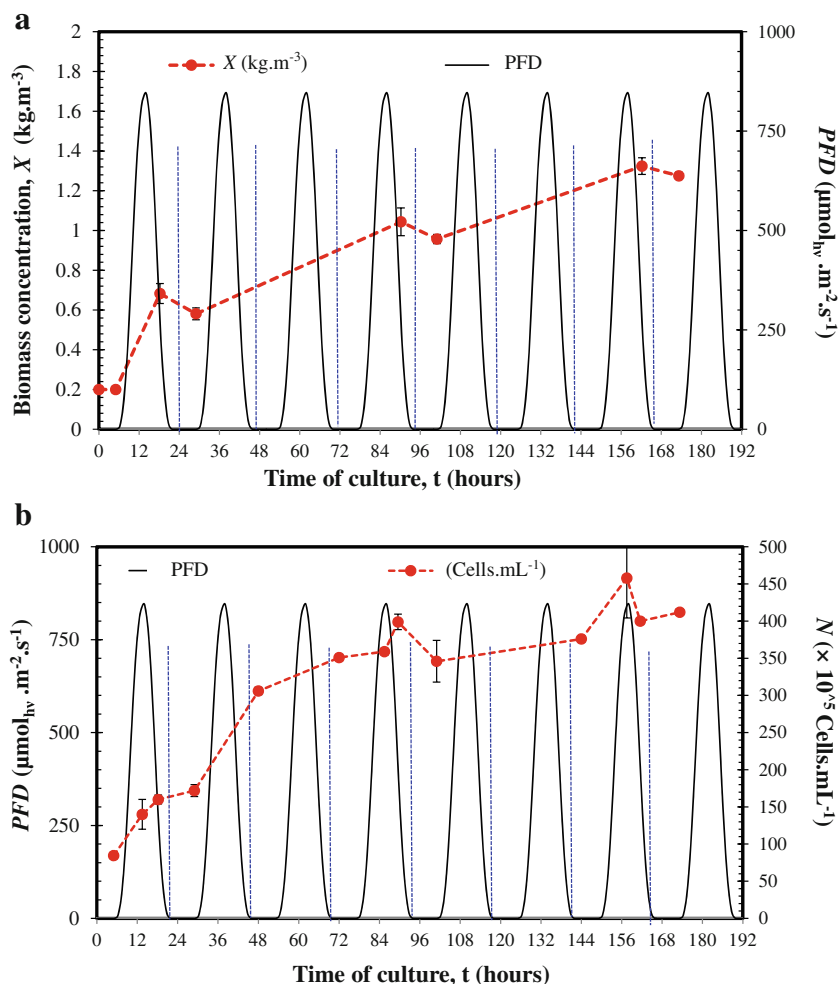
Effects of day/night cycles on growth and cell metabolism

Figures 4 and 5, respectively, show the biomass concentration and the biochemical composition of *P. kessleri* grown in sudden nitrogen starvation and under day/night illumination cycles. These figures illustrate the growth alteration along with dynamics variation of the cell metabolism in response to light fluctuations.

Figure 4a illustrates the increase in biomass concentration of the culture from 0.2 to 1.34 kg m^{-3} during 192 h of cultivation in day/night illumination cycles. The biomass concentration decreased at the end of the night period corresponding to days one, four, and seven of the batch culture. The culture respectively lost 15, 8, and 4% of its biomass concentration during night period on these days. The results were in accordance with observation by Le Borgne and Pruvost (2013) regarding biomass loss during dark periods. Note that the loss of biomass concentration during the night did not coincide with decrease in cell concentration. In fact, Fig. 4b shows a slight increase in cell concentration during the dark periods on the first and seventh day of cultivation. This may be explained by the fact that cell division occurred at the beginning of the dark period (Sukenik and Carmeli 1990; Fábregas et al. 2002). However, the loss of dry biomass concentration during the

night might be due to the consumption of carbohydrates and TAG that were accumulated by the cells during the light period. Figure 5 shows that the cells' total sugar and TAG concentration, respectively, decreased from 15.7 and 7.2 to 12.1 and 5.7 pg cell^{-1} on the fourth day of starvation. These corresponded to the consumption of 23 and 21% of the total sugar and TAG accumulated during the light period. Similar behavior was observed on the seventh day of cultivation where the intracellular content of total sugar decreased by 19% from 15 to 12.5 pg cell^{-1} and the TAG concentration by 19% from 11.6 to 9.3 pg cell^{-1} . These results demonstrate that *P. kessleri* did not show preferential consumption of one type of carbon reserve to sustain its growth in the absence of light. Moreover, the loss of TAG during the dark period was also accompanied by a slight increase in the total lipids concentration. This increase was larger on the fourth day of nitrogen starvation. These observations may indicate that during the dark periods, structural polar lipids were synthesized from TAG remobilization and not from de novo fatty acids synthesis pathways. Finally, intracellular protein concentration did not significantly change during the dark periods. However, the total protein concentration decreased from 16 pg cell^{-1} at the beginning of the experiment to less than 3 pg cell^{-1} on the fourth day of nitrogen starvation. This decrease was accompanied by simultaneous increase of both carbohydrate and lipid concentrations. The degradation of these nitrogenous compounds in response to nitrogen starvation resulted in significant change in the C/N ratio of the cells (Geider and La Roche 2002). In general, both low and high

Fig. 4 Time evolution of **a** dry biomass concentration, X (kg m^{-3}) and **b** cellular concentration, N (cells mL^{-1}) of *P. kessleri* batch culture in nitrogen starvation conditions and under day/night cycles. Error bars show the standard deviation of the mean values of three replicates



C/N ratios increase the synthesis of C storage compounds. Here, the degradation of proteins in nitrogen-limited conditions increased the C/N ratio and consequently promoting the synthesis of lipids and carbohydrate as a carbon storage mechanism in the excess of carbon supply (Chen and Johns 1991; Li et al. 2008a, b).

Effects of day/night cycles on TAG accumulation and productivity

Figure 6 shows the TAG concentration and areal TAG productivity of *P. kessleri* over 7 days of cultivation in sudden nitrogen starvation conditions under both continuous illumination and day/night cycles. Here, the incident photon flux density averaged over 24 h was equal ($\text{PFD} \approx 270 \mu\text{mol}_{\text{hv}} \text{m}^{-2} \text{s}^{-1}$) across the experiments and the culture conditions were identical in both cases. The continuously illuminated *P. kessleri* cells accumulated a larger concentration of TAG and subsequently displayed larger TAG productivity. This may be explained by the long duration of photoperiods in the case of continuously illuminated cultures. Figure 6a shows that TAG concentration in both cases increased from 5.2% to respectively reach

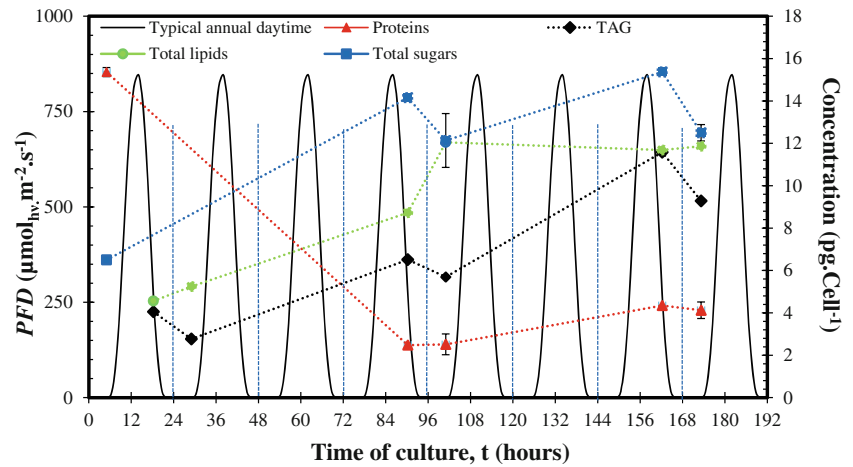
maximum TAG concentration of 35 and 40% after 7 days of cultivation for day/night cycles and continuous illumination experiments corresponding to maximum areal TAG productivity of 1.9 and $2.7 \times 10^{-3} \text{ kg m}^{-2} \text{ day}^{-1}$ (Fig. 6b).

Finally, the culture exposed to day/night illumination cycles featured 26% lower overall TAG productivity compared to the culture exposed to continuous illumination of equal mean photon flux density. This was due to the consumption of TAGs and sugars during the dark periods in order to sustain the cells.

Conclusion

A deep relationship between culture conditions in PBR and the TAG productivity of *P. kessleri* was demonstrated in this study. Nitrogen concentration in the medium and light absorption rate per cell were found to highly affect the microalgae cell metabolism. TAG concentration of *P. kessleri* cultures increased up to eight times in both sudden and progressive nitrogen starvation conditions compared to normal growth conditions in PBRs exposed to continuous illumination. The strain's large TAG concentration (up to 48% DW)

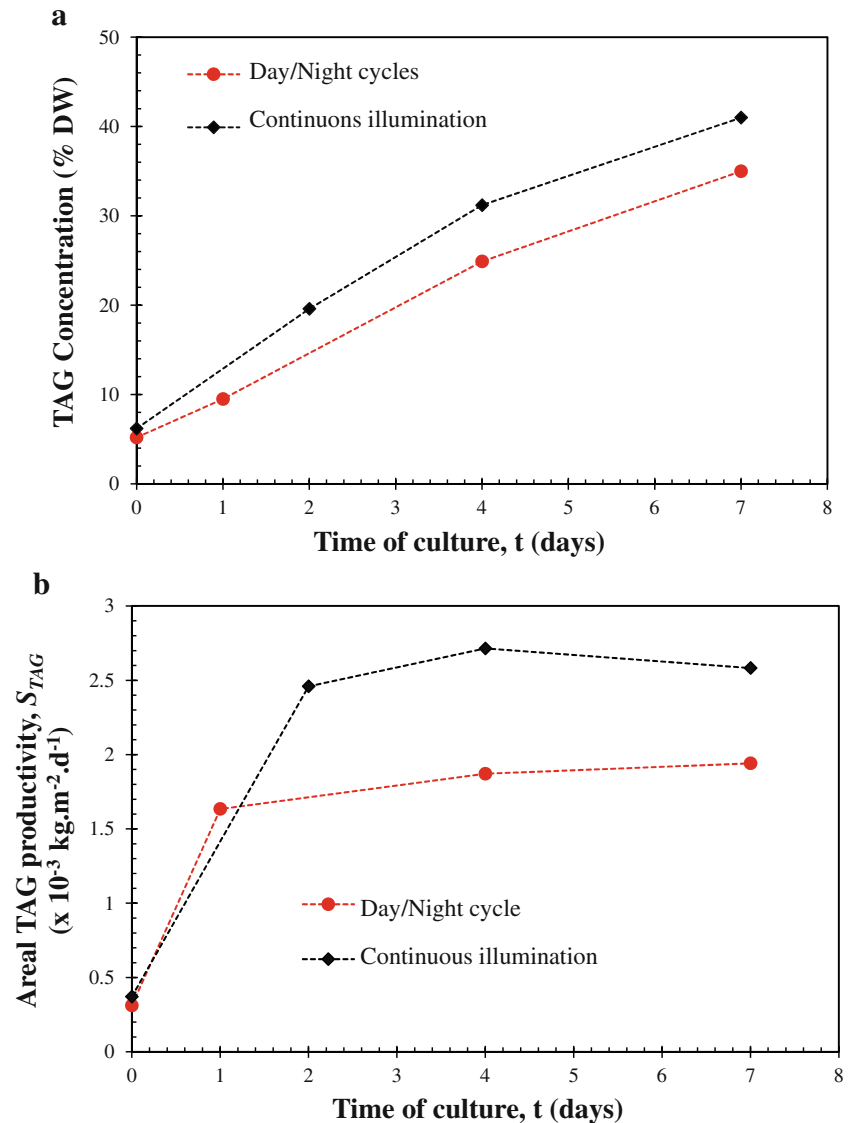
Fig. 5 Dynamic evolution of *P. kessleri* cellular components in day/night cycles and under nitrogen starvation conditions. Error bars show the standard deviation of the mean values of three replicates



along with a large biomass growth rate in nitrogen starvation conditions led to $4.4 \times 10^{-3} \text{ kg m}^{-2} \text{ day}^{-1}$ areal TAG

productivity, equivalent to an annual productivity of 16.4 tons of TAG per hectare.

Fig. 6 Comparison of the kinetics of **a** TAG concentration and **b** TAG areal productivity of *P. kessleri* nitrogen-starved culture in continuous illumination and day/night cycles. Note: In both experiments, the mean incident photon flux density over 24 h was approximately the same ($\text{PFD} \approx 270 \mu\text{mol}_{\text{hv}} \text{m}^{-2} \text{s}^{-1}$). For day/night cycles experiment, TAG concentrations and productivities were measured at the end of illumination periods



Cell metabolism and particularly intracellular lipid and carbohydrates pools were affected by the exposure of the culture to day/night illumination cycles. TAG and carbohydrates were accumulated by the cell during the illumination period and partly consumed during the dark period to generate the required energy to sustain the cells. Unlike other strains, *P. kessleri* did not show a preference for consuming sugars or TAG during the dark periods. Indeed, equal amounts of sugars and TAG were consumed by the cells during the dark periods to sustain their vitality. Moreover, TAG productivity was 26% smaller in cultures exposed to day/night illumination cycles compared to continuously illuminated cultures due to TAG consumption during the dark periods. The results presented here are encouraging for the cultivation of *P. kessleri* in outdoor solar PBRs. Future studies should investigate the effects of temperature fluctuations on lipid metabolism and TAG productivity to estimate more accurately biodiesel production in outdoor *P. kessleri* cultures.

Acknowledgements The authors thank H el ene MAREC for PBR's data acquisition and control and Delphine KUCMA and Candice PERRIER for lipid analysis.

Funding This research was supported by the French National Research Agency project DIENSALG (ANR-12-BIME-0001-02). AT is grateful to the AUF (Francophone University Agency) and the Lebanese National Council for Scientific Research (CNRS) for their financial support.

References

- Angles E, Jaouen P, Pruvost J, Marchal L (2017) Wet lipid extraction from the microalga *Nannochloropsis* sp.: disruption, physiological effects and solvent screening. *Algal Res* 21:27–34
- Br nyikov  I, Mar alkov  B, Doucha J, Br nyik T, Bi ov  K, Zachleder V, Vitov  M (2011) Microalgae—novel highly efficient starch producers. *Biotechnol Bioeng* 108:766–776
- Chen F, Johns MR (1991) Effect of C/N ratio and aeration on the fatty acid composition of heterotrophic *Chlorella sorokiniana*. *J Appl Phycol* 3:203–209
- Chisti Y (2007) Biodiesel from microalgae. *Biotechnol Adv* 25:294–306
- Demirbas MF (2011) Biofuels from algae for sustainable development. *Appl Energ* 88:3473–3480
- Dubinsky Z, Stambler N (2000) Photoacclimation processes in phytoplankton: mechanisms, consequences, and applications. *Aquat Microb Ecol* 56:163–176
- Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. *Anal Chem* 28:350–356
- Elsley D, Jameson D, Raleigh B, Cooney MJ (2007) Fluorescent measurement of microalgal neutral lipids. *J Microbiol Meth* 68:639–642
- F bregas J, Maseda A, Dom nguez A, Ferreira M, Otero A (2002) Changes in the cell composition of the marine microalga, *Nannochloropsis gaditana*, during a light:dark cycle. *Biotechnol Lett* 24:1699–1703
- Falkowski PG, LaRoche J (1991) Acclimation to spectral irradiance in algae. *J Phycol* 27:8–14
- Fernandes B, Teixeira J, Dragone G, Vicente AA, Kawano S, Bi ov  K, P ribyl P, Zachleder V, Vitov  M (2013) Relationship between starch and lipid accumulation induced by nutrient depletion and replenishment in the microalga *Parachlorella kessleri*. *Bioresour Technol* 144:268–274
- Finazzi G, Moreau H, Bowler C (2010) Genomic insights into photosynthesis in eukaryotic phytoplankton. *Trends in Plant Sci* 15:565–572
- Geider R, La Roche J (2002) Redfield revisited: variability of C:N:P in marine microalgae and its biochemical basis. *Eur J Phycol* 37:1–17
- Greenwell HC, Laurens LML, Shields RJ, Lovitt RW, Flynn KJ (2010) Placing microalgae on the biofuels priority list: a review of the technological challenges. *J Roy Soc Interface* 46:703–726
- Gris B, Morosinotto T, Giacometti GM, Bertucco A, Sforza E (2014) Cultivation of *Scenedesmus obliquus* in photobioreactors: effects of light intensities and light-dark cycles on growth, productivity, and biochemical composition. *Appl Biochem Biotechnol* 172:2377–2389
- Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant J* 54:621–639
- Huss VAR, Frank C, Hartmann EC, Hirmer M, Kloboucek A, Seidel BM, Wenzeler P, Kessler E (1999) Biochemical taxonomy and molecular phylogeny of the genus *Chlorella sensu lato* (Chlorophyta). *J Phycol* 35:587–598
- Kandilian R, Pruvost J, Legrand J, Pilon L (2014) Influence of light absorption rate by *Nannochloropsis oculata* on triglyceride production during nitrogen starvation. *Bioresour Technol* 163:308–319
- Kandilian R, Pruvost J, Artu A, Lemasson C, Legrand J, Pilon L (2016) Comparison of experimentally and theoretically determined radiation characteristics of photosynthetic microorganisms. *J Quant Spectrosc Ra* 175:30–45
- Khoeyi ZA, Seyfabadi J, Ramezani Z (2012) Effect of light intensity and photoperiod on biomass and fatty acid composition of the microalgae, *Chlorella vulgaris*. *Aquacult Int* 20:41–49
- Krienitz L, Hegewald EH, Hepperle D, Huss VAR, Rohr T, Wolf M (2004) Phylogenetic relationship of *Chlorella* and *Parachlorella* gen. nov. (Chlorophyta, Trebouxiophyceae). *Phycologia* 43:529–542
- Lacour T, Sciandra A, Talec A, Mayzaud P, Bernard O (2012) Diel variations of carbohydrates and neutral lipids in nitrogen-sufficient and nitrogen-starved cyclostat cultures of *Isochrysis* sp. *J Phycol* 48:966–975
- Le Borgne F, Pruvost J (2013) Investigation and modeling of biomass decay rate in the dark and its potential influence on net productivity of solar photobioreactors for microalga *Chlamydomonas reinhardtii* and cyanobacterium *Arthrospira platensis*. *Bioresour Technol* 138:271–276
- Li Y, Horsman M, Wang B, Wu N, Lan CQ (2008a) Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris Oleoabundans*. *Appl Microbiol Biotech* 81:629–636
- Li Y, Horsman M, Wu N, Lan CQ, Dubois-Calero N (2008b) Biofuels from microalgae. *Biotechnol Prog* 24:815–820
- Li X, P ribyl P, Bi ov  K, Kawano S, Cep k V, Zachleder V,  izkov  M, Br nyikov  I, Vitov  M (2013) The microalga *Parachlorella kessleri*—a novel highly efficient lipid producer. *Biotechnol Bioeng* 110:97–107
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275
- Michels MHA, Camacho-Rodr guez J, Vermu  MH, Wijff ls RH (2014) Effect of cooling in the night on the productivity and biochemical composition of *Tetraselmis suecica*. *Algal Res* 6:145–151
- Mizuno Y, Sato A, Watanabe K, Hirata A, Takeshita T, Ota S, Sato N, Zachleder V, Tsuzuki M, Kawano S (2013) Sequential accumulation of starch and lipid induced by sulfur deficiency in *Chlorella* and *Parachlorella* species. *Bioresour Technol* 129:150–155
- Naik SN, Goud VV, Rout PK, Dalai AK (2010) Production of first and second generation biofuels: a comprehensive review. *Renew Sust Energ Rev* 14:578–597

- Perez-Pazos J-V, Fernandez-Izquierdo P (2011) Synthesis of neutral lipids in *Chlorella* sp. under different light and carbonate conditions. *Ciencia, Tecnología y Futuro* 4:47–57
- Ritchie RJ (2006) Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. *Photosynth Res* 89:27–41
- Příbyl P, Cepák V, Zachleder V (2012) Production of lipids in 10 strains of *Chlorella* and *Parachlorella*, and enhanced lipid productivity in *Chlorella vulgaris*. *Appl Microbiol Biotechnol* 94:549–561
- Pruvost J, Van Vooren G, Cogne G, Legrand J (2009) Investigation of biomass and lipids production with *Neochloris oleoabundans* in photobioreactor. *Bioresour Technol* 100:5988–5995
- Pruvost J, Van Vooren G, Le Gouic B, Couzinet-Mossion A, Legrand J (2011) Systematic investigation of biomass and lipid productivity by microalgae in photobioreactors for biodiesel application. *Bioresour Technol* 102:150–158
- Pruvost J, Cornet JF, Goetz V, Legrand J (2012) Theoretical investigation of biomass productivities achievable in solar rectangular photobioreactors for the cyanobacterium *Arthrospira platensis*. *Biotechnol Prog* 28:699–614
- Sukenik A, Carmeli Y (1990) Lipid synthesis and fatty acid composition in *Nannochloropsis* sp. (Eustigmatophyceae) grown in a light-dark cycle. *J Phycol* 26:463–469
- Takeshita T, Ota S, Yamazaki T, Hirata A, Zachleder V, Kawano S (2014) Starch and lipid accumulation in eight strains of six *Chlorella* species under comparatively high light intensity and aeration culture conditions. *Bioresour Technol* 158:127–134
- Taleb A, Pruvost J, Legrand J, Marec H, Le-Gouic B, Mirabella B, Legeret B, Bouvet S, Peltier G, Li-Beisson Y, Taha S, Takache H (2015) Development and validation of a screening procedure of microalgae for biodiesel production: application to the genus of marine microalgae *Nannochloropsis*. *Bioresour Technol* 177:224–232
- Taleb A, Kandilian R, Touchard R, Montalescot V, Rinaldi T, Taha S, Takache H, Marchal L, Legrand J, Pruvost J (2016) Screening of freshwater and seawater microalgae strains in fully controlled photobioreactors for biodiesel production. *Bioresour Technol* 218:480–490
- Tomabene TG, Holzer G, Lien S, Burris N (1983) Lipid composition of the nitrogen starved green alga *Neochloris oleoabundans*. *Enz Microb Technol* 5:435–440
- Van Vooren G, Le Grand F, Legrand J, Cuiné S, Peltier G, Pruvost J (2012) Investigation of fatty acids accumulation in *Nannochloropsis oculata* for biodiesel application. *Bioresour Technol* 124:421–432