Algal Research 25 (2017) 160–167

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

The role of starch as transient energy buffer in synchronized microalgal growth in *Acutodesmus obliquus*

G. Mitsue León-Saiki[a,*,1], Ilse M. Remmers[a,1], Dirk E. Martens[a], Packo P. Lamers[a], René H. Wijffels[a,b], Douwe van der Veen[a]

[a] Bioprocess Engineering and AlgaePARC, Wageningen University and Research, P.O. Box 16, 6700 AA Wageningen, The Netherlands
[b] Faculty of Biosciences and Aquaculture, Nord University, N-8049, Bodø, Norway

ARTICLE INFO

Keywords:
Microalgae
Scenedesmus obliquus
Starchless mutant
Diurnal biochemical changes
Photosynthetic efficiency
Day/night cycles

ABSTRACT

Photosynthetic organisms have evolved to use light efficiently by scheduling their cellular processes, such as growth and cell division, at specific times of the day. During the day, fixed carbon is used for growth and is partially stored as carbohydrates (e.g., starch). It is commonly assumed that this accumulated starch is essential for fuelling up cell division at night. To test this hypothesis, this study investigates growth, cell division and presence of a transitory energy storage (TES) in both the wild-type and starchless mutant strain of *Acutodesmus obliquus* under light/dark (LD) cycles and nitrogen replete conditions.

A. obliquus (formerly known as *Scenedesmus obliquus*) wild-type utilized light 20% more efficiently under LD regimes compared with continuous light. When exposed to LD regimes, the wild-type scheduled cell division in a 4-hour period starting 2 h before 'sunset'. Starch acted as the major transitory energy storage (TES) compound: it was accumulated during the last part of the light period and was consumed throughout the entire dark period. The slm1 mutant, with a blocked starch synthesis pathway, showed diurnal rhythms in growth and cell division. However, no other carbohydrates nor triacylglycerols took over the role of TES compound in slm1. Therefore, in contrast to what is generally acknowledged, this study shows that neither starch nor any other major alternative TES is required for synchronized growth and cell division in *A. obliquus*. The starchless mutant did show a reduced growth and cell division rate compared to the wild-type. Starch, thus, plays a major role in efficient harnessing of light energy over LD cycles, likely because the ability to accumulate starch enhances biomass production capacity and accelerates cell division rate in *A. obliquus*.

1. Introduction

Diurnal cycles of biological activities are ubiquitous and allow many organisms to anticipate and adapt to changing environmental conditions [1–4]. In plants, it has previously been reported that numerous endogenous and environmental factors can regulate cell growth, with the light period being the major agent for entrainment of the diurnal cycle [4–6]. Nowadays, microalgae receive attention as promising sustainable sources for both commodity products (e.g., biofuels and proteins) and high value compounds (e.g., polyunsaturated fatty acids and pigments). Although some microalgae-derived high-value products are already commercially available, large scale production is hampered by high costs of production and downstream processing [7–10]. One key bottleneck in reducing these costs is to improve biomass growth and yield [9]. In both terrestrial plants and microalgae, it has been shown that growth is influenced by the presence of a diurnal energy storage system like starch, and that a lack of such a system often results in reduced growth rates [11,12]. Therefore, a thorough understanding of the role of storage metabolites under diurnal, outdoor conditions might contribute to commercialization of large-scale microalgal production systems [13].

In most photosynthetic organisms, carbon is fixed in storage compounds (e.g., carbohydrates or lipids) during the light period, which are subsequently consumed during the dark period to support different nocturnal metabolic processes [4,14–17]. Specifically for microalgae, some strains use starch as the primary storage metabolite [18–22], while others use non-starch carbohydrates (e.g., chrysolaminarin) or lipids [23–25]. The presence of such transitory energy storage

Abbreviations: LD, light/dark; TES, transitory energy storage; OD_{750}, optical density at 750 nm; μ_t, time-specific cell division rate; D, dilution rate; DW, dry weight; $Y_{x,ph}$, biomass yield on light; CHO, carbohydrates; NSC, non-starch carbohydrates; TAG, triacylglycerols

* Corresponding author at: Bioprocess Engineering and AlgaePARC, Wageningen University and Research Centre, P.O. Box 16, 6700 AA Wageningen, The Netherlands.
E-mail address: graciela.leonsaiki@wur.nl (G.M. León-Saiki).

[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.algal.2017.05.018
Received 10 January 2017; Received in revised form 12 April 2017; Accepted 20 May 2017
2211-9264/ © 2017 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/BY/4.0/).

(TES) compounds allows efficient use of sunlight under diurnal light/dark (LD) conditions, as was shown for *Neochloris oleoabundans* [4]. The role of TES compounds and the regulation of the cell cycle have been studied extensively [22,26–30]. Many studies imply that the energy required for synchronized cell division at night is supplied by TES compounds. However, the mechanisms for carbon partitioning with regard to TES remain poorly understood [18,27,31–34].

In recent years, many microalgal strains have been suggested as promising candidates for the production of food, fuel, or chemicals [9,35–37]. Among them, *Acutodesmus obliquus* (formerly known as *Scenedesmus obliquus* [38]) is considered as an industrially relevant strain for food and fuel production [39]. To further improve triacylglycerols (TAG) productivity in *A. obliquus*, a starchless mutant was created using random mutagenesis [40]. This mutant showed a 51% higher TAG yield on light compared to the wild-type (0.144 ± 0.004 in the wild-type to 0.217 ± 0.011 g TAG·mol_{ph}^{-1} in the starchless mutant) under batch wise nitrogen starvation, while maintaining its photosynthetic efficiency [41], showing the potential of blocking the starch pathway for TAG production. This starchless mutant shows potential for large scale food and biofuel production. Similar mutations have been generated in other microalgal strains and have been studied intensively for biomass productivity, lipid content, carbon partitioning and photosynthetic efficiency [42–48]. However, the impact of starch deficiency on synchronized growth and cell division in microalgae remains unknown.

This study aims to understand the role of starch in synchronized growth and cell division in *A. obliquus* wild-type under nitrogen replete and different diurnal light conditions (continuous and day/night). A starchless mutant of *A. obliquus* was used to explore the consequences of the absence of starch and if there are other compounds, such as TAG, that can serve as alternative TES under diurnal LD cycles and nitrogen replete conditions.

2. Materials and methods

2.1. Strains, pre-culture conditions and cultivation medium

Wild-type *Acutodesmus obliquus* UTEX 393 was obtained from the Culture Collection of Algae, University of Texas. *A. obliquus* was recently reclassified from *Scenedesmus obliquus* [38]. The starchless mutant of *A. obliquus* (slm1) was generated as described by De Jaeger et al. [40]. Liquid cultures were maintained in a culture chamber with shaker (25 °C, 100 rpm, air in headspace, continuous illumination at 30–40 μmol·m^{-2}·s^{-1}) in 250 mL Erlenmeyer flasks containing 100 mL of filter sterilized (pore size 0.2 μm) defined medium, as described in [49]. Prior to the start of the experiments, cultures were placed in a shake incubator operating at 23 °C with a light intensity of 180 μmol·m^{-2}·s^{-1}, a 16:8 h (light:dark, LD) block cycle and a headspace enriched with 2.5% CO_2 to reach the desired inoculation cell density.

2.2. Reactor set-up and experimental conditions

A. obliquus was continuously cultivated in an aseptic flat panel airlift-loop reactor with a 1.7 L working volume and a 0.02 m light path (Labfors 5 Lux, Infors HT, Switzerland). Cultures were continuously sparged with air containing 2% CO_2 at 1 L·min^{-1}. The temperature was controlled at 27.5 °C and the pH was maintained at 7.0 ± 0.1 by automatic supply of 5% v/v H_2SO_4. Several drops of a sterile 1% v/v solution of antifoam (Antifoam B, Baker, The Netherlands) were manually added to the culture when foam was observed (0–1 mL·day^{-1}). The reactors were illuminated by 260 LED lamps with a warm white spectrum (450–620 nm) spread evenly on the reactor's culture side. A black cover was placed on the back of the reactor to ensure that environmental light could not enter the reactor. Before inoculation, the incident photon flux density was calibrated and set at 500 μmol·m^{-2}·s^{-1}. Light was provided either in continuous mode or in a 16:8 h LD block cycle. After inoculation, the light intensity at the back side of the culture was continuously measured by a light meter (LI-250, Licor, USA). All cultivations were turbidostat controlled, which ensured automatic dilution of the culture with fresh medium when the light intensity at the back of the reactor dropped below the setpoint (10 μmol·m^{-2}·s^{-1}). During all the cultivations, cultures were exposed to light limitation only and nitrogen was continuously measured to ensure that there was no nitrogen limitation.

The reactor was inoculated to an optical density (OD_{750}) of 0.1. Duplicate independent experiments were run to check reproducibility. Systems were operated in batch mode until the light at the back of the reactor reached the set point. At this moment, the turbidostat control was started and the system was allowed to reach steady state. Cultures exposed to diurnal light conditions showed identical repetitive 24 h oscillations in dilution rate. Therefore, steady state was defined as a constant biomass concentration and dilution rate for a period of at least 3 residence times. The overflow was collected on ice for 24 h periods and used determine the 24 h average dilution rate (D_{24h} in day^{-1}, and biomass composition according to de Winter et al. [50].

The total amount of light absorbed is kept constant by the turbidostat control. This means that changes in biomass concentration in the reactor are possible when light absorbing and scattering properties of the biomass change (e.g., due to cell division or changes in pigmentation) over short time intervals. Therefore, growth rate does not equal the dilution rate over small time intervals (D_t in h^{-1}) during the day. The time-specific cell division rate (μ_t in h^{-1}) can be calculated using a balance for cell counts (C_{cells} in number of cells·mL^{-1}) over short time intervals (< 15 min), as shown with Eq. (1):

$$\frac{dC_{cells}}{dt} = -D_t \cdot C_{cells} + \mu_t \cdot C_{cells} \quad (1)$$

Sampling was done every hour for the experiments with a LD cycle. For all the other experiments, sampling was done every 3 h. Liquid samples were freshly taken from the reactor and either immediately used for wet analysis or centrifuged for 5 min at 1200 × *g* for biochemical analysis. For biochemical analysis, the resulting pellet was transferred to bead beating tubes (Lysing Matrix E; MP Biomedicals Europe) or glass tubes (for total carbohydrates analysis), freeze dried overnight and stored at − 20 °C until further analysis.

2.3. Wet biomass analysis

2.3.1. Biomass concentration

Optical density was measured at 750 nm (OD_{750}). The dry weight (DW) concentration was determined by filtrating a known volume of culture broth over pre-weighted glass fibre filters (Whatman™, GE Healthcare, UK) and measuring the weight increase of the filters after drying, as described by Kliphuis et al. [51].

2.4. Biochemical analysis

2.4.1. Total carbohydrate and starch content

Total carbohydrates were extracted and quantified according to Dubois et al. [52] and Herbert et al. [53]. A phenol-sulphuric acid solution was added to 5–10 mg freeze dried algae. The absorbance was measured at 483 nm using glucose monohydrate as standard. Samples were analysed in duplicate. Starch content was determined using a total starch kit (Megazyme, Ireland) with modifications as described by de Jaeger et al. [40] with the difference that 5 mg of freeze dried biomass was used for the analysis. A known amount of starch was analysed as positive control and glucose monohydrate was used as standard on each assay. Starch productivity was calculated using a balance for starch over short time intervals (Eq. (2)). With the change over 1 or 3 h time intervals in starch content ($\frac{dC_{starch}}{dt}$), dilution rate (D_t), starch content

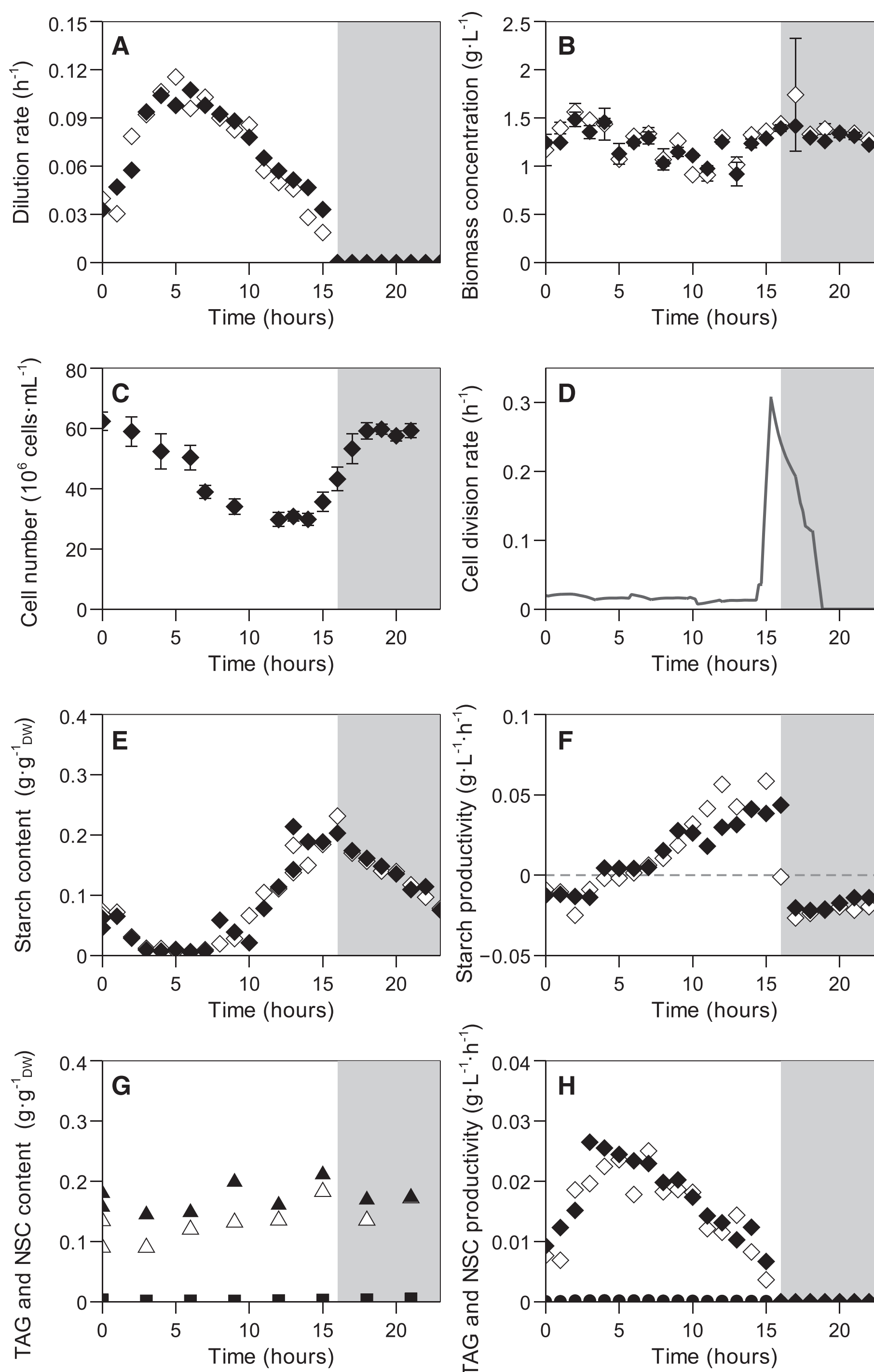

Fig. 1. Daily variation in (A) dilution rate, (B) biomass concentration, (C) cell number, (D) cell division rate, (E) starch content, (F) starch productivity, (G) triacylglycerols (TAG, squares) and non-starch carbohydrates (NSC, triangles) content, and (H) TAG (circles) and NSC (diamonds) productivity for *A. obliquus* wild-type under light/dark cycles. Open and closed symbols represent independent duplicate cultures. The x axis shows hours after 'sunrise'. Shaded areas indicate the dark period. Error bars show the standard deviation on technical variability (n = 2). Error bars for starch, TAG and NSC are not shown because they were smaller than 3% compared to the average value.

($C_{starch,t}$) and the starch productivity ($r_{starch,t}$):

$$\frac{dC_{starch}}{dt} = -D_t \cdot C_{starch,t} + r_{starch,t} \tag{2}$$

2.4.2. Lipid analysis

Triacylglycerol (TAG) content, total fatty acid (TFA) content, and fatty acid composition were determined as described by Breuer et al. [54] with the following modifications. Lipids were extracted from 5 mg of lyophilized biomass in the presence of a chloroform:methanol (1:1.25 v/v) solution containing 180 μg·mL^{-1} glyceryl trinonadecanoate (T4632; Sigma-Aldrich) and 300 μg·mL^{-1} 1,2-dipentadecanoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt) (840446, Avanti Polar Lipids Inc) as internal standards. Chloroform and methanol were evaporated and the lipid extract was dissolved in 1 mL hexane:diethylether (7:1 v/v). The lipid extract was separated in neutral and polar lipids using a Sep-Pak Vac silica cartridge (6 cm^3, 1 g; Waters). Silica cartridges were prewashed with 10 mL of hexane before loading the sample onto the column. The neutral lipid fraction, which contains TAGs, was eluted with 10 mL of hexane:diethylether (7:1 v/v). The

polar lipid fraction, which contains mainly membrane lipids, was eluted with 10 mL of methanol:acetone:hexane (2:2:1 v/v/v). Both extracts were separately methylated and quantified using gas chromatography (GC-FID) as described by Breuer et al. [54]. The TFA profile and content were calculated as the sum of each individual fatty acid in the neutral and polar lipid fraction.

2.4.3. Protein

The total protein concentration was determined using the colorimetric assay (Bio-Rad DC protein assay) as described by Postma et al. [55] with the difference that 10–15 mg of freeze dried biomass was used for analysis.

2.5. Cell number, diameter and division rate

A. obliquus is characterized by the formation of coenobia [27]. Cells were separated, but not disintegrated, by sonication for 30 s at 30% amplitude using a probe sonicator (Sonics vibra-cell, USA). Single cell presence was verified under the microscope. Cell number and diameter were determined using a Beckman Coulter Multisizer 3 (Beckman Coulter Inc., USA). The sonicated culture was diluted 200 times in Isoton® II diluent solution. Duplicate manual cell counts were done using a Neubauer improved counting chamber (DHC-N01, C-Chip, INCYTO, Republic of Korea).

2.6. Biomass yield on light

Biomass yield on light ($Y_{x,ph}$ in g_{DW}·mol photons^{-1}) was calculated using the daily dilution rate (D_{24h} in day^{-1}), the biomass concentration in the overflow ($C_{x,24h}$ in g·L^{-1}) and the volumetric photon supply rate (r_{ph} in mol photons·L^{-1}·day^{-1}) using Eq. (3):

$$Y_{x,ph} = \frac{D_{24\ h} \cdot C_X}{r_{ph}} \tag{3}$$

2.7. Reproducibility and statistical analysis

Biological replicate ($n = 2$) cultivations were run to check reproducibility under all experimental conditions. Unless stated differently, biochemical analysis was performed with technical duplicates ($n = 2$). Student's t-test with a significance level of $p < 0.05$ was used to support the results and conclusions in this study.

3. Results and discussion

3.1. A. obliquus wild-type shows synchronized growth under LD conditions

To investigate the role of starch as transitory energy storage (TES) mechanism in synchronized cell division in microalgae, we first characterized the growth of *A. obliquus* wild-type under 16 h:8 h light/dark (LD) cycles. Duplicate reactors were subjected to LD cycles at an incident light intensity of 500 μmol·m^{-2}·s^{-1}. All cultures were maintained at a fixed level of absorbed light in turbidostat-operated reactors, as the outgoing light intensity was measured and controlled at a predetermined setpoint (10 μmol·m^{-2}·s^{-1}). During the light period, constant turbidity was maintained by automatically adding fresh media.

In steady state, the average biomass concentration over 24 h is constant and the daily dilution rate is equal to the average specific growth rate over a repetitive 24 h LD period. *A. obliquus* wild-type exhibited an average dilution rate (D_{24h}) of 1.12 ± 0.01 day^{-1}.

The time specific dilution rate fluctuates clearly in a cyclic pattern (Fig. 1A), when calculated over 15 min time intervals. The rate initially increases and reaches a maximum of 0.11 h^{-1} after 5 h of light. Thereafter it decreases to 0 when the dark period starts (16 h after 'sunrise'). This fluctuating dilution rate pattern repeats itself every 24 h, indicating a synchronization to LD cycles. The cyclic pattern in dilution rate is due to variations in growth rate and light absorption (e.g., pigmentation or scattering due to alterations in biomass composition) [21]. The biomass concentration remained constant at 1.25 ± 0.25 g·L^{-1} (Fig. 1B) and, therefore, biomass productivity is proportional to the dilution rate.

To explain the change in dilution pattern, we studied the changes in cell number and diameter throughout the diurnal cycle. During each cycle, the cell number doubled from 28.4 ± 0.4 million cells·mL^{-1} to 61 ± 2.8 million cells·mL^{-1} (Fig. 1C) during the last 2 h of light (14 h after 'sunrise') and the first 2 h of dark (16–17 h after 'sunrise'). Total cell number remained stable afterwards until the end of the dark period. When the dark period was over, the culture was diluted and cells were washed out of the reactor during the first 12 h of light (Fig. 1C).

By calculating the cell division rate (Fig. 1D) based on dilution rates and cell numbers, we observed that the cell division rate increased sharply 2 h before the start of the dark period (14 h after 'sunrise') and decreased to zero approximately 2 h after the dark period started (18 h after 'sunrise'). In accordance with this, the cell diameter increased during the light period (from 3 to 14 h after 'sunrise') and thereafter decreased (from 4.2 to 3.3 μm) due to cell division (Supplementary Fig. S2). These results show that LD cycles result in synchronization of cell division in *A. obliquus* wild-type. Similar results have been described previously [56,57]. Bongers [57] also found increasing cell volume during a 14 h light period with a simultaneous decrease in cell number for continuous cultures. When the dark period started, mature cells divided within 2 h into new cells and the cell diameter decreased to the original value.

3.2. Biomass composition of A. obliquus wild-type fluctuates throughout diurnal light/dark cycles

In order to further study the role of transitory energy storage (TES) compounds on synchronized cell division, we examined the diurnal changes in biomass composition at 1 h intervals. When *A. obliquus* was grown under diurnal LD cycles, it was observed that starch metabolism was tightly synchronized to LD cycles (Fig. 1E). Starch was accumulated during the second half of the light period and consumed throughout the entire dark period. Starch started accumulating 7 h after 'sunrise', simultaneous with a decrease in biomass productivity, and reached its maximum content of 0.20 g·$g_{DW}$$^{-1}$ at the end of the light period. In the last hours of light, the change in biomass productivity was solely caused by starch accumulation. Therefore, the biomass productivity was equal to the starch productivity. Overnight losses in biomass and starch are similar (Supplementary Table 2). This supports the hypothesis that starch is respired overnight, as also observed in other diurnal studies on photosynthetic organisms [58]. Interestingly, although starch is consumed throughout the entire dark period, complete degradation was only observed 3 h after 'sunrise'.

Based on the diurnal accumulation and consumption, starch seems to act as the TES compound in *A. obliquus* wild-type. Previous work suggested that other storage metabolites such as triacylglycerols (TAG) or non-starch carbohydrates (NSC) could also act as TES [21,32]. However, these metabolite concentrations remained stable throughout the LD cycle (Fig. 1). The NSC fraction, which is defined as the total carbohydrate content minus the starch content, was stable at approximately 0.20 g·$g_{DW}$$^{-1}$. In addition, the TAG content was lower than 0.01 g·$g_{DW}$$^{-1}$ (Fig. 1G) and did not change throughout the diurnal cycle. As a consequence, both the NSC and TAG productivity followed the diurnal trend of biomass production rate (Fig. 1H). This observation suggests that starch acts as the sole TES compound, while the rate of NSC and TAG only fluctuates as a result of the production of functional biomass. We conclude that solely starch is used as the TES compound in *A. obliquus* wild-type.

3.3. Continuous illumination abolishes synchronized growth in A. obliquus wild-type

To further study the impact of LD cycles on cell physiology, we also cultivated *A. obliquus* wild-type under continuous light conditions. Here, as with the LD cycles, the biomass concentration remained stable due to turbidostat control (Supplementary Fig. 3A). In contrast to the cultures exposed to LD cycles, no cyclic diurnal behaviour was observed in the cultures adapted to continuous light. All measured parameters were constant over time: total carbohydrates 0.33 g/g_{DW}, proteins 0.47 $g \cdot g_{DW}^{-1}$, TAG 0.002 $g \cdot g_{DW}^{-1}$ and a dilution rate of 1.54 ± 0.03 day^{-1} (Supplementary Fig. 3C). In addition, no apparent fluctuations in the biomass productivity and composition (protein, total carbohydrates, and TAG content) were observed (Supplementary Fig. 3E and G). This indicates that *A. obliquus* does not synchronize its cell division under constant light conditions. Similar observations have been reported in terrestrial plants, where transcripts involved in starch metabolism were tightly light regulated; when plants were placed in dark conditions the oscillations diminished almost immediately [59,60]. In addition, also no growth rhythms were found in maize (*Zea mays*), rice (*Oryza sativa*) [61], and the microalga *N. oleoabundans* [4] when grown under constant light conditions.

3.4. The starchless mutant A. obliquus slm1 shows synchronized growth

It is often hypothesized that starch accumulation in plants acts as overflow metabolism during the light period and as a transitory carbon storage to fuel physiological processes (such as scheduled cell division) during the dark period [4,12,62,63]. In *A. obliquus* wild-type, we showed that starch acts as the sole transitory energy storage (TES) molecule under nitrogen replete conditions (Fig. 1E and G). The starch and TAG biosynthesis pathways are competing for carbon precursors [64]. Thus, it was hypothesized that when the pathway to starch synthesis is blocked, the carbon and energy flux was automatically redirected towards TAG. As TAG could also be used as diurnal TES compound in other microalgae [25], we hypothesized that these starchless mutants could potentially use TAG as the TES compound. To test this hypothesis, we examined growth characteristics of *A. obliquus* starchless mutant slm1 [40] under continuous light and diurnal light/dark (LD) cycles. Slm1 is not capable of producing starch due to a mutation in the small subunit of ADP-glucose pyrophosphorylase [65], a regulatory enzyme responsible for the first committed step in starch biosynthesis (i.e. synthesis of ADP-Glucose from ATP and D-Glucose-1-phosphate).

When slm1 was subjected to a 16:8 h LD cycle, an average dilution rate of 0.90 ± 0.01 day^{-1} was found. Dilution rate patterns of slm1 were similar to those observed for the wild-type (Fig. 2A) but absolute values were approximately 20% lower (Fig. 2A). As for the wild-type, biomass concentration remained constant over a 24 h cycle (Fig. 2B); therefore, biomass productivity is proportional to dilution rate. Also here, the biomass productivity of the slm1 were approximately 20% lower compared to the wild-type under LD cycles.

As for the wild-type, the slm1 was also grown under continuous light. However, under continuous light the dilution rate was even lower for slm1 when compared to the wild-type (1.03 ± 0.06 day^{-1} for slm1 compared with 1.50 ± 0.10 day^{-1} for the wild-type, Supplementary Fig. 3D). Similar reduced growth rates were also observed for starchless mutants of *Chlamydomonas reinhardtii* [66].

To obtain insight into the scheduling of cell division and possible interaction with an alternative TES, we also studied cell diameter and division rate throughout a 24 h LD cycle (with 16 h of light, followed by 8 h of darkness). As shown in Fig. 2C and D, *A. obliquus* slm1 shows cyclic behaviour in cell division rate and cell diameter (data available in Supplementary Fig. S2) throughout the LD cycle. During each cycle, cell number doubled from 22.0 ± 0.7 million $cells \cdot mL^{-1}$ to 41.4 ± 0.5 million $cells \cdot mL^{-1}$ (Fig. 2C). Cell division started 2 h before the dark period (14 h after 'sunrise') as for the wild-type. However, while for the wild-type cell division was confined to a 4-h period, the mutant continued cell division until the end of the dark period. Subsequently cell division stopped and the cell number decreased during the light period due to dilution of the culture. In accordance with this, average cell diameter increased from 3.8 to 5.1 μm during the light period (Supplementary Fig. S2) and decreased due to cell division afterwards (from 14 h after 'sunrise'). Interestingly, the mutant slm1 and the wild-type show different subpopulation patterns in cell diameter. The wild-type shows three subpopulations, while the mutant is more uniform. We do not know the cause for this. However, it can be seen that in the case of the wild-type all three subpopulations increase in size during the day and decrease in size due to division in the night (Supplementary Fig. S2). Based on our results, we can therefore conclude that starch is not necessary for synchronized cell division. However, presence of starch seems to enhance the rate of cell division and seems to confine it to a shorter time period.

Many studies speculate on the need for a TES compound to support cell division and maintenance during the night. To determine if other energy dense molecules (TAG or non-starch carbohydrates) could act as TES, we also analysed the biomass composition with intervals of 1 h. TAG content remained below the detection limit (Fig. 2E). Although the non-starch carbohydrate content was slightly higher in the starchless mutant (22% versus 27% for the wild-type and starchless mutant, respectively), there was no diurnal pattern observed under either LD cycles or continuous light (Fig. 2E and Supplementary Fig. 3F). Therefore, in contrast to our expectations, these findings indicate that neither TAG nor any alternative carbohydrates (Fig. 2E) are used in slm1 to replace starch as TES agent.

3.5. A starch knockdown reduces energy conversion efficiency

During photosynthesis, light energy is converted into chemical energy. This energy is subsequently used to produce biomass. Under LD cycles, the presence of a TES, such as starch, can improve the light-to-biomass conversion efficiency during nitrogen replete growth [18–25]. To elaborate on this, we calculated the biomass yield on light (Eq. (2)). *A. obliquus* wild-type uses light more efficiently under LD cycles compared to continuous light (Fig. 3), as it produces approximately 20% more biomass per mole photon absorbed when grown under LD cycles (0.98 ± 0.00 $g \cdot mol^{-1}$ photons for continuous light compared to 1.18 ± 0.03 $g \cdot mol^{-1}$ photons for LD cycles). In contrast, the slm1 showed similar biomass yields on light for both continuous and LD conditions (0.72 ± 0.04 $g \cdot mol^{-1}$ photons for continuous light compared to 0.80 ± 0.04 $g \cdot mol^{-1}$ photons for LD cycles). As the biomass composition was different for the two strains under the different light regimes, we corrected for the actual energy harnessed in the biomass (section 2.6). Similar trends as for biomass yield on light were found for the energy conversion efficiencies under the different light regimes (Supplementary File 1). We can therefore conclude that *A. obliquus* wild-type is able to retain more of the supplied energy in biomass under LD cycles compared to continuous light conditions and less energy is dissipated.

The benefit observed for the wild-type under LD cycles as compared to continuous light is lost for the starchless mutant slm1. We, thus, conclude that starch plays a role in efficient harnessing of light energy. Possibly, this is because the accumulation of starch allows the algae to fix extra light energy during the day. In addition, starch could supply energy and carbon and electrons for processes at night that prepare the algae for efficient light harvesting, or reduce photo damage, at the start of the day. This statement is supported by the increased dilution rate of the wild-type immediately after switching on the light (Fig. 1A).

Also, when comparing the two strains, we showed that the starchless mutant has reduced energy conversion efficiencies under both continuous and LD conditions compared to the wild-type (Supplementary File 1) under nitrogen replete conditions.

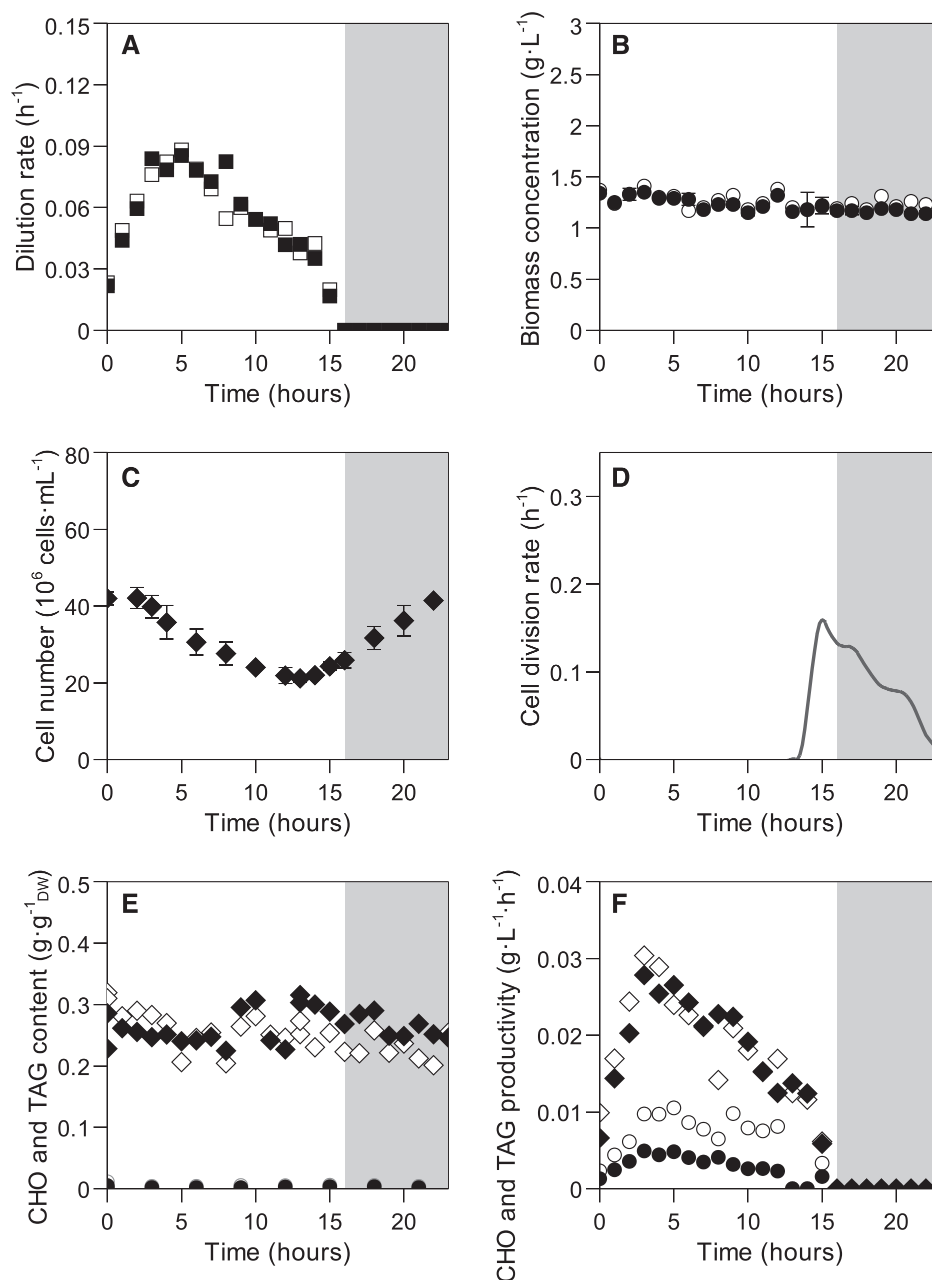

Fig. 2. Daily variation in (A) dilution rate, (B) biomass concentration, (C) cell number, (D) cell division rate, (E) carbohydrates (CHO, diamonds) and triacylglycerols (TAG, circle) content (E), and (F) CHO and TAG (circle) productivity for *A. obliquus* slm1 under light/dark cycles. Open and closed symbols represent independent duplicate cultures. The x axis indices hours after 'sunrise'. Shaded areas indicate the night period for the LD cultures. Error bars show the standard deviation of technical replicates (n = 2).

Interestingly, under nitrogen starvation and continuous light, Breuer et al. [41] observed that the mutant slm1 has a similar energy conversion efficiency compared with the wild-type. Nitrogen starvation is often concomitant with arrest of cell growth. Apparently the capability to accumulate starch only gives an advantage with respect to energy efficiency in growing algae. More research is needed to develop a better understanding in role of starch and the limitations in metabolism under diurnal LD cycles. Eventually, this knowledge may support new strategies to overcome the reduced photosynthetic efficiencies in for example starch less mutants.

4. Conclusions

A. obliquus synchronizes to LD cycles. In this study we found diurnal cyclic behaviour in growth rate, cell division and starch content for *A. obliquus* wild-type. Our results show that starch acts as the only major transitory energy storage (TES) metabolite during LD cycles for *A. obliquus*. By accumulating starch during the day and respiring it during the night, the wild-type maximizes its overall light energy conversion efficiency.

When starch synthesis is blocked, as in the starchless mutant slm1, we also observed diurnal rhythms in growth and cell division. Storage compounds, however, did not show diurnal changes. This indicates that none of the measured metabolites (TAG and carbohydrates) took over the role of starch as a TES compound. Transient storage of energy is, therefore, not required for synchronized cell division and growth. The starchless mutant did show a reduced growth rate compared to the wild-type, as well as an increased time necessary to perform cell division. It is, therefore, possible that the presence and nightly respiration/degradation of starch enhances the rate of cell division.

In addition, the lack of a TES resulted in a lower energy conversion efficiency compared to the wild-type. Therefore, by removing the

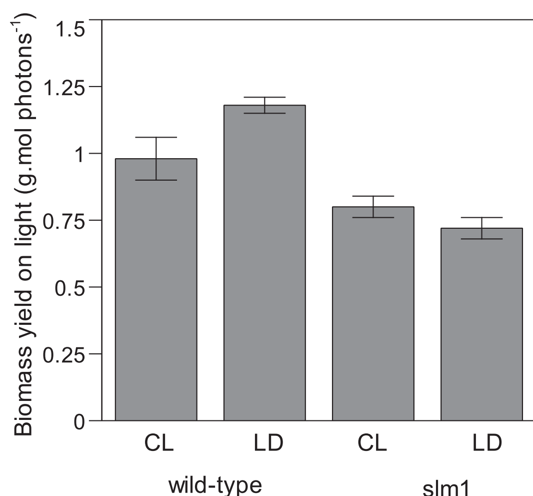

Fig. 3. Biomass yield on light. Values represent averages of at least 3 daily overflows for two replicate cultivation runs. The error bars show the standard deviation. CL: continuous light, LD: Light/dark cycles.

pathway for starch synthesis, the benefit of LD cycles in energy conversion efficiency is lost. A deeper insight into the role of starch in light harnessing efficiency is required to fully benefit from the LD cycles.

Author contributions

GMLS, IMR, DEM, PPL and DV conceived the research and designed the experiments; GMLS and IMR performed the experiments, analysed and interpreted the data. GMLS and IMR wrote the article. DEM, PPL, RHW and DV supervised and edited the manuscript. GMLS and IMR contributed equally to this work. All authors read and approved the final manuscript.

Funding information

This work was supported by the Consejo Nacional de Ciencia y Tecnología – CONACyT, Mexico, Scholar 218586/Scholarship 314173 and the EU FP7 Fuel4Me project under grant agreement No. 308983. In addition, G.M.L.S. is part of the program “Doctores Jóvenes para el Desarrollo Estratégico Institucional” by the Universidad Autónoma de Sinaloa.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors would like to thank Snežana Gegić for her help with the carbohydrate analysis and Wendy Evers with the TAG analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2017.05.018.

References

[1] E.M. Farré, The regulation of plant growth by the circadian clock, Plant Biol. 14 (2012) 401–410, http://dx.doi.org/10.1111/j.1438-8677.2011.00548.x.
[2] A. Goldbeter, C. Gérard, D. Gonze, J.-C. Leloup, G. Dupont, Systems biology of cellular rhythms, FEBS Lett. 586 (2012) 2955–2965, http://dx.doi.org/10.1016/j.febslet.2012.07.041.
[3] C.R. McClung, Plant circadian rhythms, Plant Cell 18 (2006) 792–803, http://dx.doi.org/10.1105/tpc.106.040980.
[4] L. de Winter, A.J. Klok, M. Cuaresma Franco, M.J. Barbosa, R.H. Wijffels, The synchronized cell cycle of *Neochloris oleoabundans* and its influence on biomass composition under constant light conditions, Algal Res. 2 (2013) 313–320, http://dx.doi.org/10.1016/j.algal.2013.09.001.
[5] N.I. Bishop, H. Senger, Preparation and photosynthetic properties of synchronous cultures of *Scenedesmus*, in: B.-M (Ed.), Enzymology, Academic Press, 1971, pp. 53–66.
[6] A. Graf, A. Schlereth, M. Stitt, A.M. Smith, Circadian control of carbohydrate availability for growth in *Arabidopsis* plants at night, Proc. Natl. Acad. Sci. 107 (2010) 9458–9463, http://dx.doi.org/10.1073/pnas.0914299107.
[7] O. Pulz, W. Gross, Valuable products from biotechnology of microalgae, Appl. Microbiol. Biotechnol. 65 (2004) 635–648, http://dx.doi.org/10.1007/s00253-004-1647-x.
[8] P. Spolaore, C. Joannis-Cassan, E. Duran, A. Isambert, Commercial applications of microalgae, J. Biosci. Bioeng. 101 (2006) 87–96, http://dx.doi.org/10.1263/jbb.101.87.
[9] R.H. Wijffels, M.J. Barbosa, An outlook on microalgal biofuels, Science 329 (2010) 796–799, http://dx.doi.org/10.1126/science.1189003.
[10] J. Ruiz, G. Olivieri, J. de Vree, R. Bosma, P. Willems, J.H. Reith, M.H.M. Eppink, D.M.M. Kleinegris, R.H. Wijffels, M.J. Barbosa, Towards industrial products from microalgae, Energy Environ. Sci. 9 (2016) 3036–3043, http://dx.doi.org/10.1039/C6EE01493C.
[11] M. Stitt, S.C. Zeeman, Starch turnover: pathways, regulation and role in growth, Curr. Opin. Plant Biol. 15 (2012) 282–292, http://dx.doi.org/10.1016/j.pbi.2012.03.016.
[12] T. Caspar, S.C. Huber, C. Somerville, Alterations in growth, photosynthesis, and respiration in a starchless mutant of *Arabidopsis thaliana* (L.) deficient in chloroplast phosphoglucomutase activity, Plant Physiol. 79 (1985) 11–17, http://dx.doi.org/10.1104/pp.79.1.11.
[13] L. de Winter, Circadian Rhythms in Microalgae Production, Bioprocess Engineering, Wageningen UR, 2015.
[14] M.H.A. Michels, J. Camacho-Rodríguez, M.H. Vermuë, R.H. Wijffels, Effect of cooling in the night on the productivity and biochemical composition of *Tetraselmis suecica*, Algal Res. 6 (2014) 145–151 (Part B), http://dx.doi.org/10.1016/j.algal.2014.11.002.
[15] S.J. Edmundson, M.H. Huesemann, The dark side of algae cultivation: characterizing night biomass loss in three photosynthetic algae, *Chlorella sorokiniana*, *Nannochloropsis salina* and *Picochlorum* sp, Algal Res. 12 (2015) 470–476, http://dx.doi.org/10.1016/j.algal.2015.10.012.
[16] J. Fábregas, A. Maseda, A. Domínguez, M. Ferreira, A. Otero, Changes in the cell composition of the marine microalga, *Nannochloropsis gaditana*, during a light:dark cycle, Biotechnol. Lett. 24 (2002) 1699–1703, http://dx.doi.org/10.1023/A:1020661719272.
[17] S.S. Nikaido, C.H. Johnson, Daily and circadian variation in survival from ultraviolet radiation in *Chlamydomonas reinhardtii*, Photochem. Photobiol. 71 (2000) 758–765, http://dx.doi.org/10.1562/0031-8655(2000)0710758DACVIS2.0.CO2.
[18] U. Klein, Intracellular carbon partitioning in *Chlamydomonas reinhardtii*, Plant Physiol. 85 (1987) 892–897, http://dx.doi.org/10.1104/pp.85.4.892.
[19] I. Brányiková, B. Maršálková, J. Doucha, T. Brányik, K. Bišová, V. Zachleder, M. Vítová, Microalgae—novel highly efficient starch producers, Biotechnol. Bioeng. 108 (2011) 766–776, http://dx.doi.org/10.1002/bit.23016.
[20] G. Ballin, J. Doucha, V. Zachleder, I. Šetlík, Macromolecular syntheses and the course of cell cycle events in the chlorococcal alga *Scenedesmus quadricauda* under nutrient starvation: effect of nitrogen starvation, Biol. Plant. 30 (1988) 81–91, http://dx.doi.org/10.1007/BF02878472.
[21] L. de Winter, L.W. Schepers, M. Cuaresma, M.J. Barbosa, D.E. Martens, R.H. Wijffels, Circadian rhythms in the cell cycle and biomass composition of *Neochloris oleoabundans* under nitrogen limitation, J. Biotechnol. 187 (2014) 25–33, http://dx.doi.org/10.1016/j.jbiotec.2014.07.016.
[22] M. Vitova, K. Bisova, S. Kawano, V. Zachleder, Accumulation of energy reserves in algae: from cell cycles to biotechnological applications, Biotechnol. Adv. 33 (2015) 1204–1218, http://dx.doi.org/10.1016/j.biotechadv.2015.04.012.
[23] T. Lacour, A. Sciandra, A. Talec, P. Mayzaud, O. Bernard, Diel variations of carbohydrates and neutral lipids in nitrogen-sufficient and nitrogen-starved cyclostat cultures of *Isochrysis* Sp.1, J. Phycol. 48 (2012) 966–975, http://dx.doi.org/10.1111/j.1529-8817.2012.01177.x.
[24] M.S. Chauton, P. Winge, T. Brembu, O. Vadstein, A.M. Bones, Gene regulation of carbon fixation, storage, and utilization in the diatom *Phaeodactylum tricornutum* acclimated to light/dark cycles, Plant Physiol. 161 (2013) 1034–1048, http://dx.doi.org/10.1104/pp.112.206177.
[25] E. Poliner, N. Panchy, L. Newton, G. Wu, A. Lapinsky, B. Bullard, A. Zienkiewicz, C. Benning, S.-H. Shiu, E.M. Farré, Transcriptional coordination of physiological responses in *Nannochloropsis oceanica* CCMP1779 under light/dark cycles, Plant J. 83 (2015) 1097–1113, http://dx.doi.org/10.1111/tpj.12944.
[26] A. Garz, M. Sandmann, M. Rading, S. Ramm, R. Menzel, M. Steup, Cell-to-cell diversity in a synchronized *Chlamydomonas* culture as revealed by single-cell analyses, Biophys. J. 103 (2012) 1078–1086, http://dx.doi.org/10.1016/j.bpj.2012.07.026.
[27] K. Bišová, V. Zachleder, Cell-cycle regulation in green algae dividing by multiple fission, J. Exp. Bot. (2014) ert466, , http://dx.doi.org/10.1093/jxb/ert466.
[28] F. Wanka, M.M.J. Joppen, C.M.A. Kuyper, Starch-degrading enzymes in synchronous cultures of *Chlorella*, Z. Pflanzenphysiol. (1970) (http://agris.fao.org/agris-search/search.do?recordID = US201301171279, accessed October 18, 2016).
[29] W. Schulze, M. Stitt, E.-D. Schulze, H.E. Neuhaus, K. Fichtner, A quantification of

the significance of assimilatory starch for growth of *Arabidopsis thaliana* L. Heynh 1, Plant Physiol. 95 (1991) 890–895.
[30] R. Sulpice, E.-T. Pyl, H. Ishihara, S. Trenkamp, M. Steinfath, H. Witucka-Wall, Y. Gibon, B. Usadel, F. Poree, M.C. Piques, M.V. Korff, M.C. Steinhauser, J.J.B. Keurentjes, M. Guenther, M. Hoehne, J. Selbig, A.R. Fernie, T. Altmann, M. Stitt, Starch as a major integrator in the regulation of plant growth, Proc. Natl. Acad. Sci. 106 (2009) 10348–10353, http://dx.doi.org/10.1073/pnas.0903478106.
[31] S.S. Merchant, J. Kropat, B. Liu, J. Shaw, J. Warakanont, TAG, You're it! *Chlamydomonas* as a reference organism for understanding algal triacylglycerol accumulation, Curr. Opin. Biotechnol. 23 (2012) 352–363, http://dx.doi.org/10.1016/j.copbio.2011.12.001.
[32] J. Jia, D. Han, H.G. Gerken, Y. Li, M. Sommerfeld, Q. Hu, J. Xu, Molecular mechanisms for photosynthetic carbon partitioning into storage neutral lipids in *Nannochloropsis oceanica* under nitrogen-depletion conditions, Algal Res. 7 (2015) 66–77, http://dx.doi.org/10.1016/j.algal.2014.11.005.
[33] X. Ma, J. Liu, B. Liu, T. Chen, B. Yang, F. Chen, Physiological and biochemical changes reveal stress-associated photosynthetic carbon partitioning into triacylglycerol in the oleaginous marine alga *Nannochloropsis oculata*, Algal Res. 16 (2016) 28–35, http://dx.doi.org/10.1016/j.algal.2016.03.005.
[34] Y. Li, D. Han, M. Sommerfeld, Q. Hu, Photosynthetic carbon partitioning and lipid production in the oleaginous microalga *Pseudochlorococcum* sp. (Chlorophyceae) under nitrogen-limited conditions, Bioresour. Technol. 102 (2011) 123–129, http://dx.doi.org/10.1016/j.biortech.2010.06.036.
[35] L. Rodolfi, G. Chini Zittelli, N. Bassi, G. Padovani, N. Biondi, G. Bonini, M.R. Tredici, Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor, Biotechnol. Bioeng. 102 (2009) 100–112, http://dx.doi.org/10.1002/bit.22033.
[36] T.M. Mata, A.A. Martins, N.S. Caetano, Microalgae for biodiesel production and other applications: a review, Renew. Sust. Energ. Rev. 14 (2010) 217–232, http://dx.doi.org/10.1016/j.rser.2009.07.020.
[37] J.N. Rosenberg, G.A. Oyler, L. Wilkinson, M.J. Betenbaugh, A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution, Curr. Opin. Biotechnol. 19 (2008) 430–436, http://dx.doi.org/10.1016/j.copbio.2008.07.008.
[38] L. Krienitz, C. Bock, Present state of the systematics of planktonic coccoid green algae of inland waters, Hydrobiologia 698 (2012) 295–326, http://dx.doi.org/10.1007/s10750-012-1079-z.
[39] G. Breuer, P.P. Lamers, D.E. Martens, R.B. Draaisma, R.H. Wijffels, The impact of nitrogen starvation on the dynamics of triacylglycerol accumulation in nine microalgae strains, Bioresour. Technol. 124 (2012) 217–226, http://dx.doi.org/10.1016/j.biortech.2012.08.003.
[40] L. de Jaeger, R.E. Verbeek, R.B. Draaisma, D.E. Martens, J. Springer, G. Eggink, R.H. Wijffels, Superior triacylglycerol (TAG) accumulation in starchless mutants of *Scenedesmus obliquus*: (I) mutant generation and characterization, Biotechnol. Biofuels 7 (2014) 69, http://dx.doi.org/10.1186/1754-6834-7-69.
[41] G. Breuer, L. de Jaeger, V.P.G. Artus, D.E. Martens, J. Springer, R.B. Draaisma, G. Eggink, R.H. Wijffels, P.P. Lamers, Superior triacylglycerol (TAG) accumulation in starchless mutants of *Scenedesmus obliquus*: (II) evaluation of TAG yield and productivity in controlled photobioreactors, Biotechnol. Biofuels 7 (2014) 1–11, http://dx.doi.org/10.1186/1754-6834-7-70.
[42] Y. Li, D. Han, G. Hu, D. Dauvillee, M. Sommerfeld, S. Ball, Q. Hu, *Chlamydomonas* starchless mutant defective in ADP-glucose pyrophosphorylase hyper-accumulates triacylglycerol, Metab. Eng. 12 (2010) 387–391, http://dx.doi.org/10.1016/j.ymben.2010.02.002.
[43] A. Sirikhachornkit, S. Vuttipongchaikij, A. Suttangkakul, K. Yokthongwattana, P. Juntawong, P. Pokethitiyook, K. Kangvansaichol, M. Meetam, Increasing the triacylglycerol content in *Dunaliella tertiolecta* through isolation of starch-deficient mutants, J. Microbiol. Biotechnol. 26 (2016) 854–866, http://dx.doi.org/10.4014/jmb.1510.10022.
[44] S. Vonlanthen, D. Dauvillée, S. Purton, Evaluation of novel starch-deficient mutants of *Chlorella sorokiniana* for hyper-accumulation of lipids, Algal Res. 12 (2015) 109–118, http://dx.doi.org/10.1016/j.algal.2015.08.008.
[45] A. Ramazanov, Z. Ramazanov, Isolation and characterization of a starchless mutant of *Chlorella pyrenoidosa* STL-PI with a high growth rate, and high protein and polyunsaturated fatty acid content, Phycol. Res. 54 (2006) 255–259, http://dx.doi.org/10.1111/j.1440-1835.2006.00416.x.
[46] V.H. Work, R. Radakovits, R.E. Jinkerson, J.E. Meuser, L.G. Elliott, D.J. Vinyard, L.M.L. Laurens, G.C. Dismukes, M.C. Posewitz, Increased lipid accumulation in the *Chlamydomonas reinhardtii* sta7-10 starchless isoamylase mutant and increased carbohydrate synthesis in complemented strains, Eukaryot. Cell 9 (2010) 1251–1261, http://dx.doi.org/10.1128/EC.00075-10.
[47] Z.T. Wang, N. Ullrich, S. Joo, S. Waffenschmidt, U. Goodenough, Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starchless *Chlamydomonas reinhardtii*, Eukaryot. Cell 8 (2009) 1856–1868, http://dx.doi.org/10.1128/EC.00272-09.
[48] M. Siaut, M. Heijde, M. Mangogna, A. Montsant, S. Coesel, A. Allen, A. Manfredonia, A. Falciatore, C. Bowler, Molecular toolbox for studying diatom biology in *Phaeodactylum tricornutum*, Gene 406 (2007) 23–35, http://dx.doi.org/10.1016/j.gene.2007.05.022.
[49] G. Breuer, P.P. Lamers, D.E. Martens, R.B. Draaisma, R.H. Wijffels, Effect of light intensity, pH, and temperature on triacylglycerol (TAG) accumulation induced by nitrogen starvation in *Scenedesmus obliquus*, Bioresour. Technol. 143 (2013) 1–9, http://dx.doi.org/10.1016/j.biortech.2013.05.105.
[50] L. de Winter, I.T.D. Cabanelas, A.N. Órfão, E. Vaessen, D.E. Martens, R.H. Wijffels, M.J. Barbosa, The influence of day length on circadian rhythms of *Neochloris oleoabundans*, Algal Res. 22 (2017) 31–38, http://dx.doi.org/10.1016/j.algal.2016.12.001.
[51] A.M.J. Kliphuis, A.J. Klok, D.E. Martens, P.P. Lamers, M. Janssen, R.H. Wijffels, Metabolic modeling of *Chlamydomonas reinhardtii*: energy requirements for photoautotrophic growth and maintenance, J. Appl. Phycol. 24 (2012) 253–266, http://dx.doi.org/10.1007/s10811-011-9674-3.
[52] M. DuBois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356, http://dx.doi.org/10.1021/ac60111a017.
[53] D. Herbert, P.J. Phipps, R.E. Strange, Chemical analysis of microbial cells, Methods Microbiol, Academic Press, London and New York, 1971, pp. 209–344.
[54] G. Breuer, W.A.C. Evers, J.H. de Vree, D.M.M. Kleinegris, D.E. Martens, R.H. Wijffels, P.P. Lamers, Analysis of Fatty Acid Content and Composition in Microalgae, J. Vis, Exp, 2013, http://dx.doi.org/10.3791/50628.
[55] P.R. Postma, T.L. Miron, G. Olivieri, M.J. Barbosa, R.H. Wijffels, M.H.M. Eppink, Mild disintegration of the green microalgae *Chlorella vulgaris* using bead milling, Bioresour. Technol. 184 (2015) 297–304, http://dx.doi.org/10.1016/j.biortech.2014.09.033.
[56] H. Senger, Charakterisierung einer Synchronkultur von *Scenedesmus obliquus*, ihrer potentiellen Photosyntheseleistung und des Photosynthese-Quotienten während des Entwicklungscyclus, Planta 90 (1970) 243–266, http://dx.doi.org/10.1007/BF00387177.
[57] L. Bongers, Changes in photosynthetic activity during algal growth and multiplication, Meded. Landbouwhogesch. Wagening. 58 (1958) 1–10.
[58] W. Blanken, A. Magalhães, P. Sebestyén, A. Rinzema, R.H. Wijffels, M. Janssen, Microalgal biofilm growth under day-night cycles, Algal Res. 21 (2017) 16–26, http://dx.doi.org/10.1016/j.algal.2016.11.006.
[59] S.M. Smith, Diurnal changes in the transcriptome encoding enzymes of starch metabolism provide evidence for both transcriptional and posttranscriptional regulation of starch metabolism in *Arabidopsis* leaves, Plant Physiol. 136 (2004) 2687–2699, http://dx.doi.org/10.1104/pp.104.044347.
[60] Y. Lu, Daylength and circadian effects on starch degradation and maltose metabolism, Plant Physiol. 138 (2005) 2280–2291, http://dx.doi.org/10.1104/pp.105.061903.
[61] R. Poiré, A. Wiese-Klinkenberg, B. Parent, M. Mielewczik, U. Schurr, F. Tardieu, A. Walter, Diel time-courses of leaf growth in monocot and dicot species: endogenous rhythms and temperature effects, J. Exp. Bot. (2010) erq049, , http://dx.doi.org/10.1093/jxb/erq049.
[62] A.A.R. Webb, A. Satake, Understanding circadian regulation of carbohydrate metabolism in *Arabidopsis* using mathematical models, Plant Cell Physiol. 56 (2015) 586–593, http://dx.doi.org/10.1093/pcp/pcv033.
[63] S.E. Weise, K.J. van Wijk, T.D. Sharkey, The role of transitory starch in C3, CAM, and C4 metabolism and opportunities for engineering leaf starch accumulation, J. Exp. Bot. (2011) err035, , http://dx.doi.org/10.1093/jxb/err035.
[64] J. Fan, C. Yan, C. Andre, J. Shanklin, J. Schwender, C. Xu, Oil accumulation is controlled by carbon precursor supply for fatty acid synthesis in *Chlamydomonas reinhardtii*, Plant Cell Physiol. 53 (2012) 1380–1390, http://dx.doi.org/10.1093/pcp/pcs082.
[65] L. de Jaeger, Strain Improvement of Oleaginous Microalgae, Bioprocess Engineering, Wageningen UR, 2015.
[66] Y. Li, D. Han, G. Hu, M. Sommerfeld, Q. Hu, Inhibition of starch synthesis results in overproduction of lipids in *Chlamydomonas reinhardtii*, Biotechnol. Bioeng. 107 (2010) 258–268, http://dx.doi.org/10.1002/bit.22807.