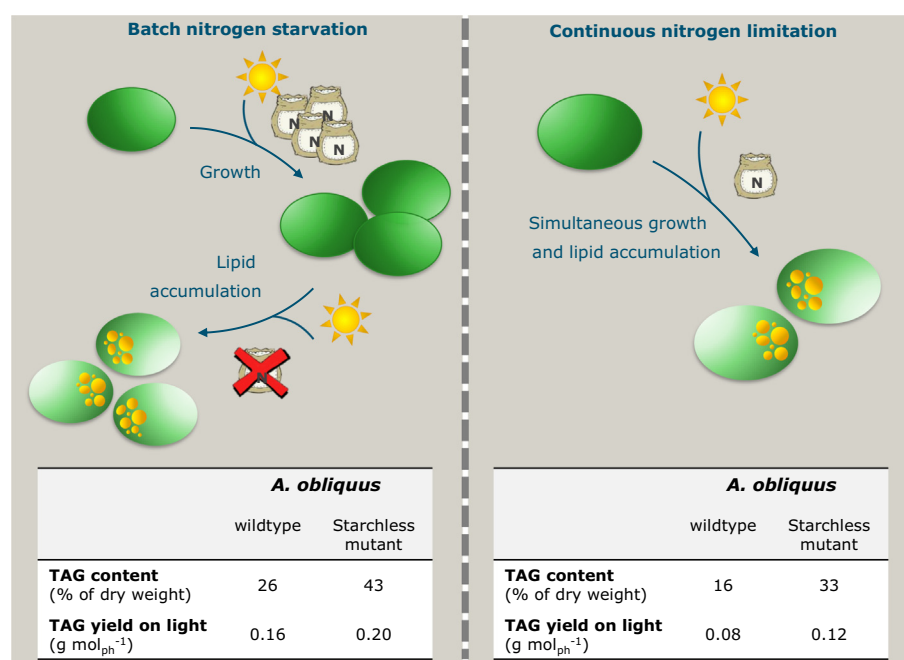# Continuous versus batch production of lipids in the microalgae *Acutodesmus obliquus*

I.M. Remmers [a,*], A. Hidalgo-Ulloa [a], B.P. Brandt [a], W.A.C. Evers [a], R.H. Wijffels [a,b], P.P. Lamers [a]

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HIGHLIGHTS

- Quantitative study on batch and continuous TAG production is presented.
- Batch outcompetes continuous TAG production by a ~2-fold higher TAG yield on light.
- Starch acts as primary storage metabolite in *Acutodesmus obliquus*.
- Diurnal light cycles do not influence the TAG yield under batch nitrogen starvation.

GRAPHICAL ABSTRACT

	A. obliquus	
	wildtype	Starchless mutant
TAG content (% of dry weight)	26	43
TAG yield on light (g mol$_{ph}^{-1}$)	0.16	0.20

	A. obliquus	
	wildtype	Starchless mutant
TAG content (% of dry weight)	16	33
TAG yield on light (g mol$_{ph}^{-1}$)	0.08	0.12

ARTICLE INFO

ABSTRACT

This work provides a novel quantitative comparison of batch versus continuous microalgal lipid production in the wild type and starchless mutant strain of *Acutodesmus obliquus*. Both strains showed higher TAG yields on light under batch operation compared to continuous nitrogen limitation. The starchless mutant showed 0.20 g TAG mol$_{ph}^{-1}$ for batch and 0.12 g TAG mol$_{ph}^{-1}$ for continuous operation, while the wildtype only showed 0.16 g TAG mol$_{ph}^{-1}$ for batch and 0.08 g TAG mol$_{ph}^{-1}$ for continuous operation. Also, higher TAG contents were found under batch starvation (26% of dry weight for the wildtype and 43% of dry weight for starchless mutant) compared to continuous cultivations (16% of dry weight for the wildtype and 33% of dry weight for starchless mutant). Starch acts as the favoured storage metabolite during

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Triacylglycerol (TAG)
Starch
Diurnal LD cycles

nitrogen limitation in *A. obliquus*, whereas TAG is only accumulated after starch reaches a cellular maximum of 40% of dry weight.

1. Introduction

Microalgae are a promising and sustainable feedstock of triacylglycerol (TAG) for food, feed, and biofuel applications (Chisti, 2013; Draaisma et al., 2013; Wijffels et al., 2010). However, the production costs are still too high for commercialization and the areal TAG productivity needs further improvement (Collet et al., 2014; van Boxtel et al., 2015).

Typically, under optimal growth conditions, microalgae produce low amounts of TAG. When microalgae are exposed to unfavourable growth conditions such as nitrogen limitation, TAG accumulation is initiated (Hu et al., 2008). TAG production commonly proceeds by using a batch process (Benvenuti et al., 2016; Breuer et al., 2015; Mulders et al., 2012; Rodolfi et al., 2009; Santos et al., 2014). In a batch strategy, first biomass is produced under nitrogen replete conditions which is followed by a nitrogen starvation phase to initiate TAG accumulation. Besides the advantage of simplistic operation, batch processes allow TAG contents up to 60% of dry weight (Breuer et al., 2012). These high contents are often reached after prolonged nitrogen starvation which is accompanied with decreased photosynthetic activity (Breuer et al., 2014; Griffiths et al., 2014). Consequently, maximum TAG productivity is only reached within the first days of cultivation while the cellular TAG content is still low. Maintaining photosynthetic activity throughout a prolonged period of nitrogen starvation could result in improved lipid productivities, which subsequent contributes to improved process economics.

In an effort to overcome this loss in photosynthetic activity and concomitant losses in TAG-productivities, recent studies on (semi) continuous cultivation processes for lipid production were done (Benvenuti et al., 2016; Fernandes et al., 2015; Griffiths et al., 2014; Klok et al., 2013; Pruvost et al., 2009; Rodolfi et al., 2009). In all studies, growth and photosynthesis were restricted by a limited nitrogen supply leading to either simultaneous growth and TAG production (continuous nitrogen limitation) or sequenced growth and TAG accumulation (semi continuous). Recently, Klok et al. (2013) provided a proof-of-concept for simultaneous growth and TAG production using *Neochloris oleoabundans* in a continuous setup. Although the potential of continuous lipid production was shown, this work is still lacking a systematic comparison under optimized and diurnal conditions to the benchmark batch strategy. Therefore, further in-depth research on continuous lipid production is necessary.

For continuous TAG production under optimized conditions, microalgal strains should not only be selected on their capacity to accumulate high amounts of TAG, but also on their ability to retain biomass productivity under nitrogen deficiency. Recent screening studies indicated the high potential of *Acutodesmus obliquus* (formerly known as *Scenedemus obliquus* (Krienitz and Bock, 2012)) as TAG producer, as it showed a 2.5-fold higher biomass productivity under nitrogen starvation compared to other species such as *N. oleoabundans* (Breuer et al., 2012). Literature showed that the TAG yield on light could be further improved by using biologically improved strains, as Breuer et al. (2014) showed one of the highest light to TAG conversion efficiencies reported so far ($0.2 \, \text{g TAG mol}_{\text{ph}}^{-1}$) in a starchless mutant of *A. obliquus*. Although the starchless mutant shows improved carbon partitioning towards TAG, more detailed studies are required to identify the impact of starch deficiency on cultivation strategies.

In this study, we present a detailed and quantitative comparison of lipid production with the wildtype (wt) and starchless mutant (slm1) of *A. obliquus*, cultivated under simulated outdoor conditions in both batch and continuous cultivations. Based on this comparison, the potential of a continuous lipid-accumulation strategy will be evaluated.

2. Methods

2.1. Strains, pre-cultivation conditions and cultivation medium

Wild type *A. obliquus* UTEX 393, formerly known as *Scenedesmus obliquus* (Krienitz and Bock, 2012), was obtained from the University of Texas Culture collection of algae. The starchless mutant (slm1) was developed by de Jaeger et al. (2014). Pre-cultivation was performed in shake flasks as described by Breuer et al. (2013).

2.2. Photo bioreactor setup and experimental conditions

Algae were cultivated in an aseptic flatpanel airlift-loop reactor with a working volume of 1.7 L and a light path of 0.02 m (Labfors 5 Lux, Infors HT, Switzerland). A schematic overview of the photobioreactor is provided in Supplementary Fig. A1. The culture was continuously sparged with $1 \, \text{L min}^{-1}$ air enriched with 1% CO_2. The temperature was controlled at 27.5 °C and the pH was maintained at 7 using 5% H_2SO_4. A few drops of a 1% solution of antifoam (Antifoam B, Baker, the Netherlands) were daily added when foaming was visible. Illumination was provided at the culture side of the reactor by a light panel with 260 LEDs with a warm white spectrum (Emission spectrum given in Supplemental Fig. A2). The outgoing light was corrected for the light absorbed by the water jacket and the rear glass panels and continuously measured using a LI-COR sensor (LI-COR 190-SA 2π PAR 400–700 nm, Licor, USA). In all experiments, reactors were inoculated at a biomass concentration of $0.5 \, \text{g L}^{-1}$ with an incident light intensity (PFD_{in}) of $200 \, \mu\text{mol m}^2 \, \text{s}^{-1}$. After three days, PFD_{in} was increased to the final set point of $500 \, \mu\text{mol m}^2 \, \text{s}^{-1}$. Light was supplied in block form with 16 h of constant illumination followed by 8 h of darkness. For simplicity in lab-scale experiments, light is often supplied in block form and previous research confirmed that such light:dark (LD) cycles are appropriately representing simulated outdoor conditions (de Winter et al., 2017). To allow a fair comparison between the different cultivation strategies, culture parameters related to light absorption (outgoing light intensity, biomass concentration, absorption cross section) were kept identical at start of nitrogen starvation (batch operation) and under steady-state light-limited growth conditions (continuous operation).

For batch cultivations, first biomass was grown in fresh water medium identical to medium used by Breuer et al. (2013), with the exception that the KNO_3 concentration was $3.24 \, \text{g L}^{-1}$. After reaching a biomass concentration of approximately $2 \, \text{g L}^{-1}$, the culture was harvested and centrifuged (for 15 min at 900g). Thereafter the cell pellet was resuspended in nitrogen free medium and then transferred back to the reactor at a biomass concentration of $1.5 \, \text{g L}^{-1}$. Hereby we were able to accurately determine the moment of nitrogen depletion for all batch experiments to reduce the effect of circadian rhythms (de Winter et al., 2014). In all experiments, the moment of medium-exchange was scheduled three

hours before sunrise and considered as t = 0. Batch experiments for both strains were performed in duplicate. Sampling was done at two fixed time points during the light period (at the start and end of the light period). Samples were taken directly from the reactor and analysed for biomass concentration, cell count, residual nitrate concentration, biomass-specific absorption cross section (α_c), maximum efficiency of PSII photochemistry (F_v/F_m ratio) and biomass composition (TAG, starch, carbohydrates).

Continuous cultures were turbidostat controlled, where the outgoing light intensity (PFD_{out}) was kept constant at 10 μmol m^2 - s^{-1} by automatically diluting the culture with fresh medium. The composition of this medium was identical to Breuer et al. (2013), with the exception that it did not contain any KNO_3. Nitrogen was added separately and the nitrogen dilution rate never exceeded 10% of the total dilution rate. Control was only active during the light period. As a consequence, the cultures showed variable growth rates and biomass composition over a 24 h period. Therefore, steady state conditions were defined when the average dilution rate, dilution pattern and biomass concentration in the reactor were stable over a 24 h LD cycle for at least 14 consecutive days. Nitrogen replete growth was achieved when the growth rate was only limited by light supply, as nitrogen was supplied in excess. Different levels of nitrogen limitation were imposed on the culture by reducing the supply rate of nitrogen. We calculated the nitrogen to photon ratio for each steady state to illustrate the degree of nutrient depletion to the system. The nitrogen to photon ratio was defined as gram assimilated nitrogen per mol absorbed photon (Eq. (1)).

$$\text{Nitrogen to photon ratio} = \frac{r_N}{r_{ph,absorbed}}$$
$$= \frac{(F_{N,in}C_{N,in}) - (F_{out}C_{N,out})}{(PFD_{in} - PFD_{out})3600\ 16\ 10^{-9}\frac{1}{d_r}} \quad (1)$$

Multiple nitrogen feed regimes were tested for both strains: 7 for the wild type and 8 for slm1. Reproducibility was ensured by running independent duplicate experiments at two nitrogen feed regimes for both strains: light limited growth with an excess in nitrogen supply and nitrogen limitation at 30% of the nitrogen consumption rate observed under light limitation. When stable steady state conditions were reached, culture overflow was collected on ice for 24 h periods and used to determine the biomass concentration, biomass composition (TAG, starch, carbohydrates, proteins, ash) and elemental composition. We confirmed that samples stored for 24 h on ice or taken directly from the reactor did not show significant differences. Additionally, absorption cross section and F_v/F_m were measured on samples originating directly from the culture broth.

2.3. Biomass analysis

Dry weight was measured as described by Lamers et al. (2010). Cell number and diameter were determined using a Beckman Coulter Multisizer 3 (Beckman Coulter Inc., USA) according to Kliphuis et al. (2012). Triacylglycerol (TAG) and total fatty acid (TFA) content were determined as described by Remmers et al. (2017). Lipid extraction was done with a chloroform:methanol (1:1.25) solution containing two internal standards: 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). Apolar and polar lipids were separated using a Sep-Pak Vac silica cartridge (6 cc, 1000 mg; Waters). The silica cartridges were prewashed with 10 mL of hexane and the lipid extract was dissolved in 1 mL hexane:diethylether (7:1 v/v) and loaded to the column. The neutral lipid fraction, containing TAGs, was eluted with 10 mL of hexane:diethylether (7:1 v/v).

The polar lipid fraction, mainly containing membrane lipids, was eluted with 10 mL of methanol:acetone:hexane (2:2:1%v/v). Both extracts were methylated and quantified using gas chromatography (GC-FID). Total fatty acid composition and content were calculated by taking the sum of all fatty acids in both fractions. Starch content was determined using a AA/AMG Total Starch kit (Megazyme, Ireland) with modifications as described by Mulders et al. (2015). Total carbohydrates were extracted and quantified using a phenol-sulphuric-acid solution according to Klok et al. (2013). Total protein concentration was determined using the DC protein assay (Bio-Rad Laboratories, USA) according to de Winter et al. (2014) with the exclusion of the desalting step. The dissolved nitrate concentration was measured using a Seal analytical AQ2 nutrient analyser (SEAL Analytical Inc., USA) according to the standard operating instructions. KNO_3 was used as standard and two samples with known concentrations of $NaNO_3$ were used as positive control. Photosystem II (PSII) maximum quantum yield (F_v/F_m) was measured using the pulse-amplitude modulated fluorimeter (AquaPen-C AP-C 100, Photon Instruments, Czech Republic) as described by (Benvenuti et al., 2014) with the exception that cultures were diluted to an OD750 of approximately 0.3 and adapted to dark conditions for at least 15 min prior to the measurements. The biomass-specific absorption cross section (α_c) was measured according to de Mooij et al. (2014).

2.4. Calculations

Biomass (r_x) and TAG (r_{TAG}) productivity (g L^{-1} day^{-1}) were calculated by dividing the amount of biomass or TAG per litre reactor volume (g L^{-1}) over the cultivation time. The biomass ($Y_{x,ph}$) and TAG ($Y_{TAG,ph}$) yield on light were calculated by dividing the biomass or TAG productivity by the average light supply rate during that period. The yield of biomass or TAG on light in batch conditions was always calculated between the measured time point and the start of the experiment (t = 0), also known as time-averaged yield (Breuer et al., 2013). All batch data presented in this paper, including values obtained from Breuer et al. (2014), were corrected for inoculum production. Corrections were done by accounting for the photons used to produce the initial amount of biomass present at each batch experiment (1.5 g L^{-1} biomass at t = 0). We used a fixed photon cost for biomass: 0.85 mol$_{ph}$ - g biomass^{-1} for A. obliquus wt and 1.12 mol$_{ph}$ g biomass^{-1} for the starchless mutant (as found in this study). More details are explained in the Supplementary material. A T-test with a significance level of $p < 0.05$ was used for statistical analysis.

3. Results and discussion

3.1. Batch cultivation under LD cycles

Duplicate batch experiments were performed under day night cycles for both A. obliquus wild type (wt) and the starchless mutant (slm1). Nitrogen depletion was induced by exchanging the cultivation medium with nitrogen-free medium. Starvation experiments were started at a biomass concentration of 1.5 g L^{-1}. The moment of nitrogen depletion was regarded as t = 0.

No cell division was observed after the start of nitrogen starvation. Nonetheless, the biomass concentration kept increasing from 1.5 g L^{-1} up to 10.8 g L^{-1} for A. obliquus wild type (wt) and up to 8.6 g L^{-1} for the slm1 (Fig. 1A). This biomass increase was due to the production of starch, TAG and other carbohydrates. The wild type simultaneously produced starch and TAG after nitrogen depletion (Fig. 1C). Starch was initially accumulated at a higher rate compared to TAG, reaching a maximum content of 34% of dry weight after 40 h. Thereafter starch was degraded while TAG synthesis continued, reaching a maximum TAG content of 41% of
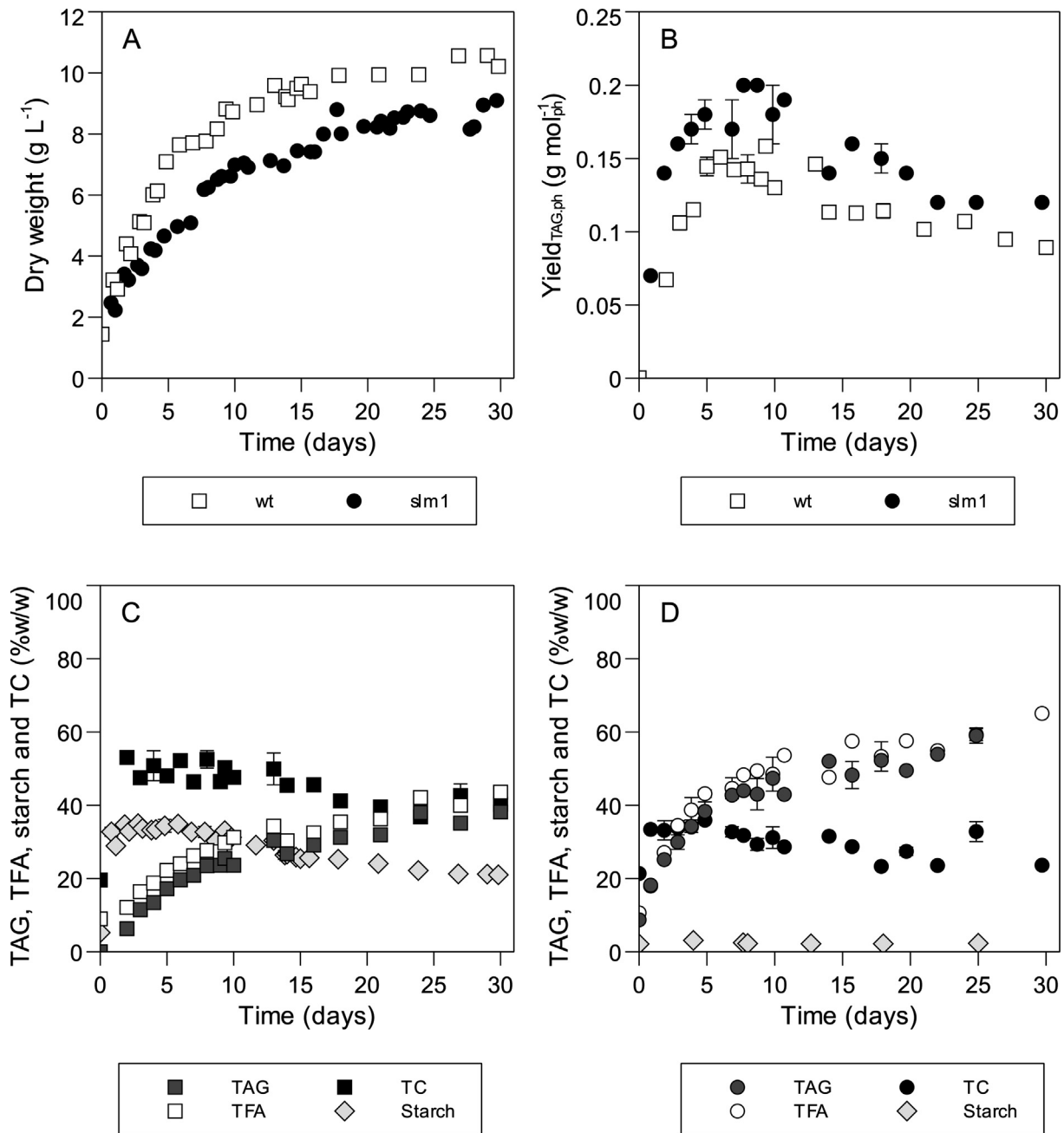
Fig. 1. Batch nitrogen starvation of *A. obliquus* wt (squares) and slm1 (circles) under 16:8 h LD cycles. Variation in biomass concentration (A), time-averaged TAG yield on light (B) and the biomass composition for the wt (C) and slm1 (D). The cells were exposed to nitrogen starvation at t = 0. Error bars show the minimum and maximum values of duplicate reactors. TAG: triacylglycerol; TFA: total fatty acid; TC: total carbohydrates.

dry weight at the end of the experiment (30 days). The non-starch carbohydrate fraction increased in the first 24 h from 14% to 23% of dry weight and thereafter remained constant over the entire cultivation period. Similarly, the starchless mutant's total carbohydrate content increased in the first 24 h from 19% to 26% of dry weight and then remained equal. With no starch being produced by the starchless mutant, the TAG content increased rapidly in the first 48 h to 20% of dry weight (Fig. 1D). The absolute maximum TAG content of 59% of dry weight was reached at the end of the experiment. Similar trends in TAG and starch content were observed for cultivations done under continuous light cultivations in *A. obliquus* (Breuer et al., 2014) and *Desmodesmus* sp. (Ho et al., 2014).

The observed biomass productivity of the slm1 was lower than the wt, while equal amounts of light were supplied (Fig. 1A). As also described by Breuer et al. (2014), differences in biomass productivity can partially be explained by differences in requirements of metabolites (e.g. 1 g TAG requires 0.75 mol photons while 1 g starch only requires 0.31 mol photons (Breuer et al., 2014; Kliphuis et al., 2012). When calculating the metabolic requirements for biomass for both strains (detailed calculations available in Supplemental file C), we found that the theoretical electron fixation rate is equal for both strains. Therefore, the difference in biomass productivities can be completely explained by alterations in carbon partitioning to starch and TAG and subsequently the photon requirements

to produce these metabolites. Thus, upon nitrogen starvation, the photosynthetic capacity to convert photons into biomass is equal for the wt and slm1.

The time averaged TAG yield on light was calculated for each time point using the measured TAG concentration and the amount of light supplied to the reactor up to that time point. The TAG yield on light increased to a maximum of 0.16 g TAG mol_{ph}^{-1} within 9 days for the wt and 0.20 g TAG mol_{ph}^{-1} within 7.7 days for the slm1 (Fig. 1B). These yields correspond to volumetric TAG productivities of 0.23 g L^{-1} day^{-1} for the wild type and 0.29 g L^{-1} day^{-1} for slm1.

Many microalgae lose culture performance due to nocturnal respiration when exposed to LD cycles (Acién Fernández et al., 2003; de Winter et al., 2017; He et al., 2015; Lacour et al., 2012; Mairet et al., 2011; Michels et al., 2014). Interestingly, no clear differences in maximum TAG yield on light were found between DN and continuous supplied light (Breuer et al. (2014) found TAG yields on light of 0.16 g TAG mol_{ph}^{-1} for the wt and 0.23 g TAG mol_{ph}^{-1} for slm1 for continuous light experiments, calculations available in Supplementary file D). Additional biomass analysis revealed that dark respiration was marginal and only occurred during the first three nights of starvation (Supplementary file E). The wt predominantly respired starch during the night, whereas the mutant probably respired functional biomass such as proteins. Both strains did not show any TAG respiration overnight.

3.2. Continuous nitrogen limitation under DN cycles

3.2.1. Growth

To allow a fair comparison between continuous and batch-cultivation, initial set-points were kept identical (pH, temperature, incident light intensity, outgoing light intensity). Thereafter, the optimal nitrogen supply rate for the continuous experiments needs to be determined to find the maximum TAG yield on light. To do so, medium was continuously supplied in turbidostat mode to keep the amount of absorbed light constant throughout the experiments. Each experiment was then fed with a different amount of nitrogen to reach multiple levels of nitrogen limitation. As soon as steady state oscillations were reached over 24 h periods, the culture overflow was collected on ice for 24 h periods. The culture overflow was used for analysis of biomass content, composition (TAG, TFA, starch, carbohydrates) and maximum photosystem II efficiency.

Under nitrogen replete conditions, lower nitrogen uptake rates were observed for slm1 compared to the wt. This difference can be explained by a reduced specific growth rate and lower protein content in slm1 ($54 \pm 2\%$ versus $66 \pm 4\%$ of dry weight for the wt and slm1, respectively). Light limited specific growth rates of 1.14 ± 0.02 and 0.80 ± 0.06 day^{-1} were found for *A. obliquus* wt and slm1, respectively. Similar steady state biomass concentrations were found for both strains (1.41 ± 0.01 g L^{-1} for the wt and 1.48 ± 0.17 g L^{-1} for the slm1). As a consequence, the observed biomass yield on light (Fig. 2A) of the nitrogen-replete wild type experiments was substantially higher than that of slm1 (1.12 g mol_{ph}^{-1} versus 0.85 g mol_{ph}^{-1}). Similar to batch nitrogen starvation, the discrepancy in observed biomass yield on light between the two strains might be caused by differences in requirements for metabolites. To take the differences in biomass composition into consideration, we determined the photosynthetic electron fixation efficiency (Fig. 2B). Here, we also found a lower electron fixation efficiency for slm1 compared to the wt under nitrogen replete conditions (Fig. 2B, red points). Therefore, the difference in biomass yield on light between both strains cannot be explained by a difference in energy content of the biomass. The difference is therefore most likely caused by an increased rate of dissipation for the

slm1 under nitrogen replete conditions. This observation is in accordance with earlier presented studies on starchless mutants of *Chlamydomonas* and the terrestrial crop *Nicotiana* (Huber and Hanson, 1992; Li et al., 2010).

When decreasing the nitrogen supply rate (e.g. lower nitrogen to photon ratio), both the biomass yield on light and electron fixation efficiency decreased. Interestingly, strong nitrogen limitation (range 0–30 mgN mol_{ph}^{-1}) resulted in equal electron fixation efficiencies for both strains (Fig. 2B). Contrary to observations under nitrogen replete growth (red data points in Fig. 2), these findings suggests that LD cycles have a little to no impact on photosynthetic efficiency under nitrogen starvation or severe nitrogen limitation when comparing the wt to the starchless mutant strain.

3.2.2. TAG and starch accumulation

As a result to lower nitrogen to photon ratios (e.g. lower nitrogen feed rates) both microalgae started to accumulate either starch and TAG (wt) or solely TAG (slm1), as shown in Fig. 2. The starchless mutant initiated TAG synthesis under nitrogen limitation, reaching TAG contents up to 46% of dry weight at a nitrogen to photon ratio of 7 ± 1 mgN mol_{ph}^{-1}. Conversely, the wt accumulated solely starch at moderate nitrogen limitation (range 100–50 mgN mol_{ph}^{-1}), and accumulated both starch and TAG at severe nitrogen limitation (range 50–10 mgN mol_{ph}^{-1}). The starch content reached its maximum of 40% of dry weight at 50 mgN mol_{ph}^{-1} and remained constant at lower nitrogen to photon ratios. A maximum TAG content of 21% of dry weight was observed at the lowest nitrogen supply rate tested (10 mgN mol_{ph}^{-1}).

Fig. 2C shows that TAG synthesis in the wt is only initiated when the maximum storage capacity of starch is reached, which suggest product inhibition. It is well known that starch and TAG compete for a common carbon precursor. Both biosynthetic pathways have been well studied and characterized, although the interaction and regulation of carbon partitioning into starch and lipid synthesis is not yet well understood (Busi et al., 2014; Fan et al., 2012; Johnson and Alric, 2013; Rawsthorne, 2002; Wang et al., 2009). In batch experiments starch and TAG are produced simultaneously (Breuer et al., 2015; Chu et al., 2014; Sundberg and Nilshammar-Holmvall, 1975), where starch was always considered as the dominant carbon sink due to its abundance under nitrogen starvation. The observation of subsequent production of starch and TAG under nitrogen limitation could also well explain carbon partitioning during batch nitrogen starvation. Here, initially most carbon is used for synthesizing starch, with an increasing gradual shift towards complete use of carbon for TAG production (Breuer et al., 2015). We therefore conclude that starch is the primary storage product in *A. obliquus* wt, and that TAG is only produced when the imbalance between supplied energy versus nutrient availability exceeds the capacity of the starch synthesis pathway.

3.3. Overall yields and productivities

Whereas the biomass yield on light decreased with decreasing nitrogen supply rate, the TAG yield on light ($Y_{TAG,ph}$) had an optimum within the tested range of conditions (Fig. 3). Experimental maxima of 0.08 g TAG mol_{ph}^{-1} for the wt and 0.12 g TAG mol_{ph}^{-1} for the slm1 were achieved at 32 ± 1 mgN mol_{ph}^{-1} and 22 ± 0 mgN mol_{ph}^{-1}, respectively. An evidence-based interpolation (Supplementary file F) of the TAG yield on light showed optima of 0.14 g TAG mol_{ph}^{-1} for the wt and 0.16 g TAG mol_{ph}^{-1} for slm1. Further reduction of the nitrogen supply rate resulted in increased cellular TAG contents (Fig. 2C and D) but not in a higher TAG yield on light (Fig. 3).
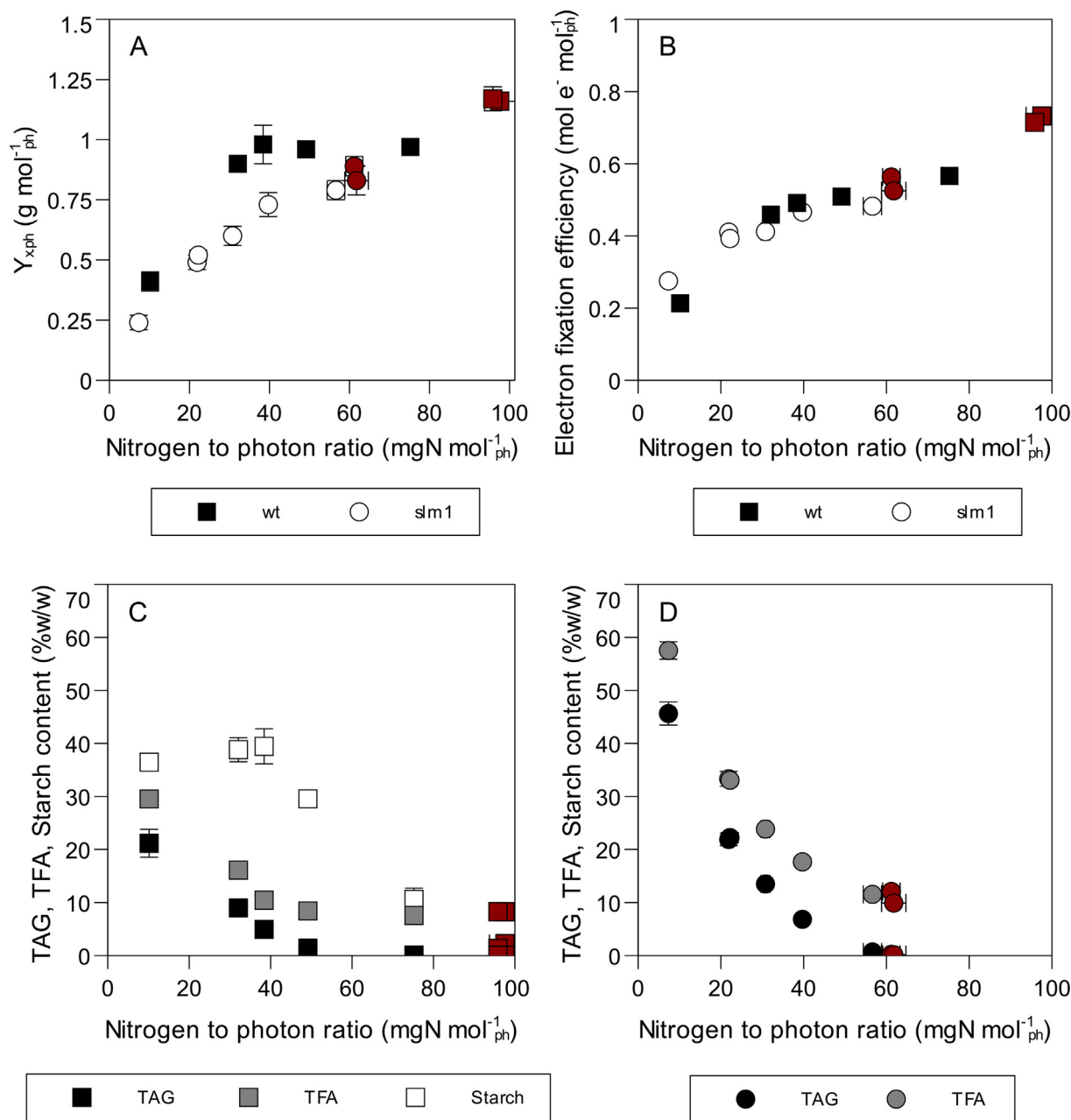
Fig. 2. Continuous nitrogen limitation of *A. obliquus* wt (squares) and slm1 (circles) under 16:8 h LD cycles. Variation in biomass yield on light (A), theoretical election fixation efficiency (B) and biomass composition for *A. obliquus* wt (C) and *A. obliquus* slm1 (D) as function of the nitrogen to photon uptake ratio. The nitrogen to photon ratio was calculated according to Section 2.2. The red data points illustrate nitrogen replete (light limited) growth. Error bars show the standard deviation over three different cultivation days. TAG: triacylglycerol; TFA: total fatty acids. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A similar observation was done for *Neochloris oleoabundans* at lower light intensity (Klok et al., 2013). We conclude that severe N-limitation does lead to improved partitioning of the fixed carbon towards TAG, but that this positive effect is outweighed by a large reduction in the overall carbon fixation rate, leading to a nett reduction in the TAG yield on light.

In addition, the variable fluorescence/maximum fluorescence ratio (F_v/F_m) was measured and used as an estimate for the maximum efficiency of PSII (Baker, 2008; de Mooij et al., 2014). Under nitrogen replete growth conditions, independently of cultivation mode (e.g. batch, or continuous), the F_v/F_m ratio of *A. obliquus* wt and slm1 was found to be approximately 0.7

(Fig. 4), similar to findings of (Benvenuti et al., 2014; Breuer et al., 2014; Parkhill et al., 2001). Commonly, the proteins related to PSII are damaged under batch wise nitrogen starvation. This damage leads to a decrease in F_v/F_m (Berges et al., 1996) and it will eventually result in a decrease in biomass yield on light. Therefore, F_v/F_m ratios are often used as a diagnostic of nutrient stress (Benvenuti et al., 2014; Parkhill et al., 2001). Interestingly, in our continuous experiments, F_v/F_m ratios were not influenced by the different degrees of nitrogen limitation and always remained higher than 0.6 for both strains (Fig. 4B), even though the photosynthetic rate did decrease substantially under nitrogen limitation (Figs. 2 and 3). Parkhill
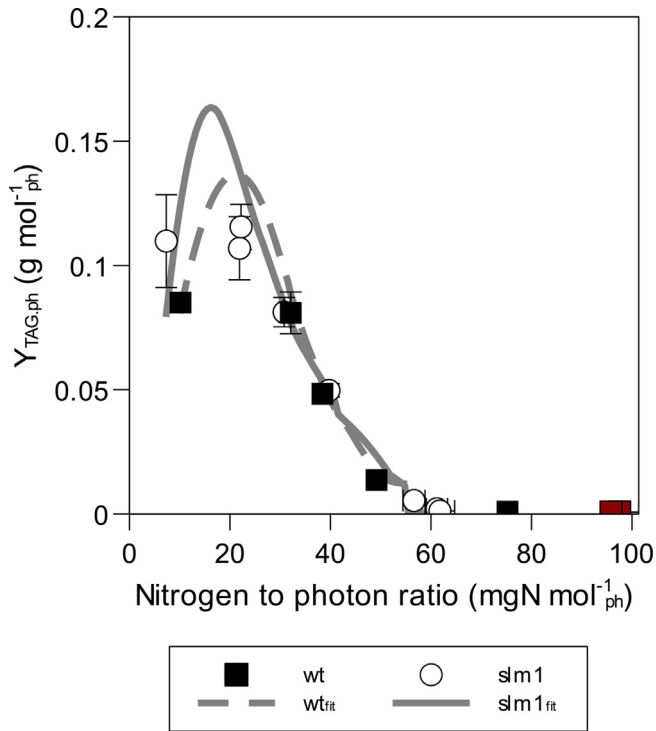
Fig. 3. TAG yield on light ($Y_{TAG,ph}$) for continuous cultivations of *A. obliquus* wt (closed squares) and slm1 (open circles). The nitrogen to photon ratio was calculated according to Section 2.2. The red data points illustrate nitrogen replete (light limited) growth. Both lines (dotted line for the wt and straight line for slm1) show an interpolation of the $Y_{TAG,ph}$, based on the estimated dilution rate and TAG concentration at each point (Further details available in Supplementary file F). Error bars show the standard deviation over three different cultivation days. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al. (2001) and Cullen et al. (1992) support these findings. This shows that the F_v/F_m ratio is not always a suitable indicator of nutrient stress. Indeed, many varying factors in the photosystem can attribute to a reduced photosynthetic efficiency (Babin et al., 1996) which do not necessarily result in altered F_v/F_m values.

3.4. Evaluation of batch versus continuous lipid production

A key characteristic for commercial TAG production with microalgae is the ability of a strain to accumulate high amounts of product (content) at highest possible light-to-product conversion (i.e. yield on light). This study showed a detailed and quantitative comparison of lipid production with the wt and starchless mutant of *A. obliquus*, cultivated under simulated outdoor conditions in both batch and continuous cultivations. The maximum time-averaged TAG yield on light of the batch experiments was almost 2 times higher than that of the continuous experiments (Table 1). Batch cultivations also allow for higher TAG contents.

Although data interpolation of the continuous experiments revealed a potential increase in TAG yield on light to 0.14 g TAG mol_{ph}^{-1} for the wt and 0.16 g TAG mol_{ph}^{-1} for slm1, traditional batch nitrogen starvation still outcompetes continuous nitrogen limitation. Outdoor pilot experiments should be performed to validate these lab-scale outcomes, as shown by Benvenuti (2016) and Dominguez Teles (2016). In addition, further techno-economic studies and life cycle analysis should determine the degree of competitiveness of a continuous strategy over a batch strategy for outdoor microalgal lipid production. Such studies can be performed using a modelling approach similar to Ruiz et al. (2016), but should also be based on year round numbers collected from a pilot scale demonstration plant.
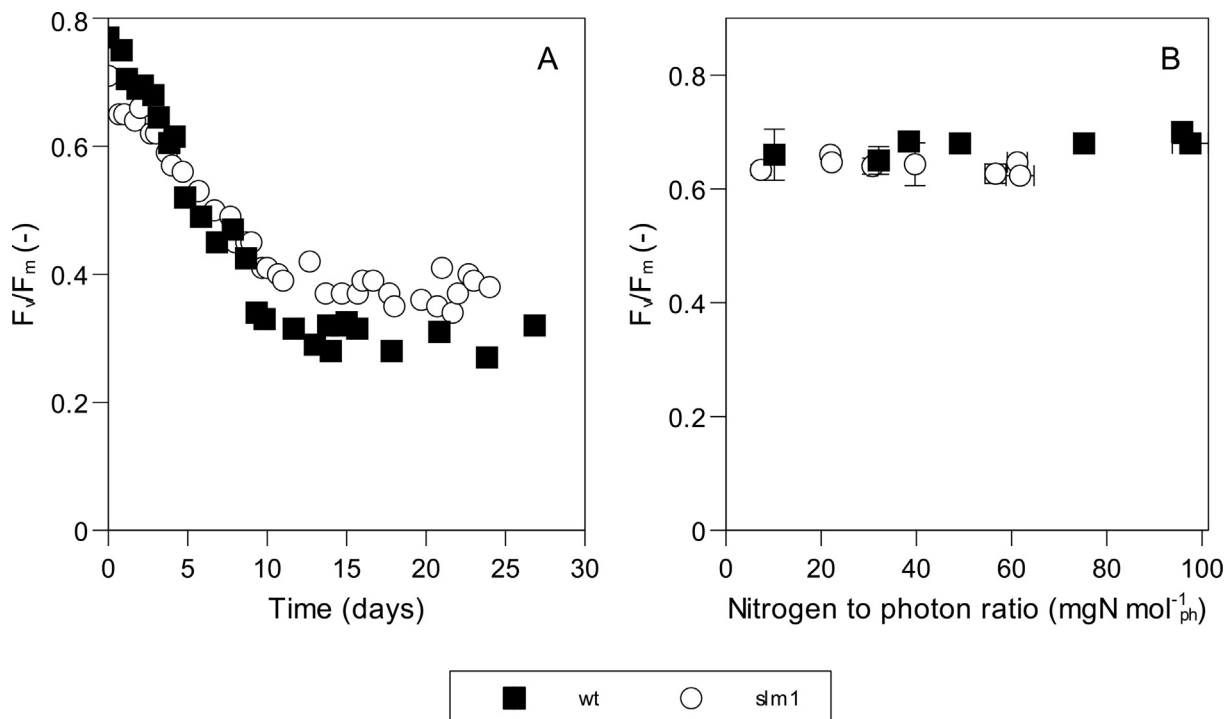
Fig. 4. The F_v/F_m ratio for batch nitrogen starvation (A) and continuous nitrogen limitation (B) of *A. obliquus* wt (closed squares) and slm1 (open circles). Error bars show the standard deviation over three different cultivation days.

Table 1

Comparison of batch and continuous lipid production in *A. obliquus* wt and slm1. The TAG productivity and yield on light were calculated over the entire cultivation period, including inoculum production. The standard deviation was found to always be lower than 5%. [*]Data interpolation showed maximum TAG yields on light of 0.14 g TAG mol_{ph}^{-1} for the wt and 0.16 g TAG mol_{ph}^{-1} for the slm1 under continuous nitrogen limitation (Supplementary file F).

	Batch		Continuous	
	Wild type	Slm1	Wild type	Slm1
Maximum TAG yield on light (g mol_{ph}^{-1})	0.16	0.20	0.081[*]	0.115[*]
Maximum TAG productivity (g L^{-1} day^{-1})	0.23	0.29	0.110	0.157
TAG content at maximum productivity (% of dry weight)	26	43	16	33

4. Conclusions

This study provides a quantitative comparison of batch versus continuous microalgal lipid production. Batch nitrogen starvation of *A. obliquus* results in an almost twofold higher TAG yield on light compared to continuous cultivations. Also, batch starvation allows higher TAG contents. Batch is therefore the preferred cultivation strategy for TAG production using *A. obliquus*.

Also, by gradually increasing nitrogen limitation in turbidostat controlled reactors, we found that starch act as the primary storage metabolite in *A. obliquus*, with TAG only accumulating when the starch synthesis rate is limited.

Funding

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.04.093.

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