The background of the cover is a light blue gradient with a fine, textured pattern. A large, semi-transparent blue circle is centered in the upper half, containing the title text. In the lower half, there are faint, stylized illustrations of microalgal cells and structures, including oval-shaped cells with internal details and elongated, filamentous structures.

PHOTOSYNTHETIC EFFICIENCY IN MICROALGAL LIPID PRODUCTION

Ilse M. Remmers

Propositions

1. Diurnal light cycles do not affect the lipid yield.
(this thesis)
2. Wasting energy on by-products improves the overall solar-to-biomass conversion efficiency.
(this thesis)
3. The term 'nutrient limitation' is mal defined.
4. Impact in science is only obtained when disciplines are integrated.
5. Public acceptance of controversial innovations is only possible if the outcomes are fulfilling a need.
6. Leisure on a daily basis is essential.

Propositions belonging to the thesis entitled:

'Photosynthetic efficiency in microalgal lipid production'

Ilse M. Remmers

Wageningen, 15 September 2017

PHOTOSYNTHETIC EFFICIENCY IN MICROALGAL LIPID PRODUCTION

Ilse M. Remmers

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PHOTOSYNTHETIC EFFICIENCY IN MICROALGAL LIPID PRODUCTION

Ilse M. Remmers

Thesis

submitted in fulfilment of the requirement for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus,

Prof. Dr A.P.J. Mol,

in the presence of the

Thesis Committee appointed by the Academic Board

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Chapter 1

Introduction and thesis outline

1.1 Microalgae as renewable feedstock in a biobased economy

As a result to the growing world population and emerging welfare, the demand for food, feed and fossil-fuel derived products is increasing (Bozell et al., 2010). Consequently initiatives are taken to reduce our environmental footprint by shifting from a fossil-fuel based economy to a biobased economy, where biomass is used as renewable feedstock (EU, 2010; Metz et al., 2007). Resulting from these initiatives, large scale biofuel production using first generation feedstocks such as corn, grain and palm oil have already been introduced. Biofuel production, however, is under critical debate due to the competition for arable land and freshwater with food- and feed-production (Mitchel, 2008; Tenenbaum, 2008).

In the search for renewable feedstocks, which are not in competition with our current food- and water supply, microalgae are reported as one of the most promising ones (Wijffels et al., 2010). Microalgae are eukaryotic unicellular organisms capable to convert sunlight and CO₂ into chemical energy using photosynthesis. As microalgae can grow on non-arable land using waste- or salt water, they are not in competition with food and feed crops, while they have a superior areal productivity compared to terrestrial crops (Chisti, 2007; Draaisma et al., 2013; Wijffels et al., 2010). Depending on the used species and applied cultivation conditions, microalgae can produce a wide range of products that can be used for different market applications (Figure 1.1).

Currently, microalgae are already commercially produced for specialty markets such as feedstock for aquaculture and for human health and cosmetics (i.e. PUFAs and pigments) (Ruiz et al., 2016; Spolaore et al., 2006; Williams and Laurens, 2010). Besides serving high-value applications, they have a great potential as feedstock for bulk-products as they predominantly consist out of proteins, carbohydrates and lipids (Wijffels et al., 2010). However, current production costs are still too high to make production of these bulk commodities commercially feasible. For example, production costs should decrease at least by a factor 10 for bulk applications such as lipids for biodiesel (Ruiz et al., 2016). To move microalgae from current niche-

applications to large scale bulk-applications, research should initially focus on increasing the solar-to-product conversion efficiency.

Figure 1.1 – Different potential market applications for microalgal derived products.

1.2 Lipid production in microalgae

Microalgae have evolved to survive or proliferate over a wide range of environmental conditions. As a result, they have a tremendous diversity in metabolism and biomass composition (Cardozo et al., 2007). Among the different fractions, lipids are considered as a very interesting one. Besides their potential application in the food-feed- and chemical market, they can also fulfil our growing demand for energy and fuels (Figure 1.1).

Under optimal growth conditions, the lipid fraction of microalgae mainly consists of structural membrane lipids (5-20% of their dry cell weight). Depending on the species, these structural lipids can contain high amounts of high-valuable polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA, C20:5) or docosahexaenoic acid (DHA, C22:6) (Adarme-Vega et al., 2012; Doughman et al., 2007). When microalgae are exposed to unbalanced growth conditions, they can accumulate up to 60% of their cell-dry weight in triacylglycerol (TAG). TAG predominantly consists of fatty acids with medium chain length (C16 and C18), which

makes microalgal TAG suitable for biodiesel production but also as replacement for edible vegetable oils (Draaisma et al., 2013). TAG-accumulation goes, however, at the expense of growth and biomass production (Hu et al., 2008; Klok et al., 2014).

Nitrogen is the most critical nutrient affecting lipid metabolism in microalgae (Hu et al., 2008; Sharma et al., 2012). Under nitrogen deficient growth conditions, microalgal cells are not able to convert light energy into functional, nitrogen containing, biomass anymore. As a consequence, the cells adapt their metabolism by: decreasing the energy intake through photosynthesis (reduced pigmentation, activity of photosystems and number of photosynthetic antennae), increasing photon dissipation (e.g., in the form of heat or fluorescence) and through production of energy rich storage compounds (e.g., carbohydrates and TAG) (Figure 1.2).

Figure 1.2 – Schematic illustration of microalgal cells grown under balanced (A) and nitrogen deprived (B) growth conditions. Under balanced growth conditions (A), cells receive sufficient light and nutrients to produce functional biomass. When exposed to nitrogen deprivation (B), cells are not able to produce functional biomass and thus adapt their metabolism by: reducing pigmentation and photosynthetic machinery (1), increasing photon dissipation in the form of heat or fluorescence (2) and increasing the flux towards energy dense storage molecules such as TAG or carbohydrates (3).

1.3 Cultivation strategies for microalgal lipid production

Nitrogen deprivation is most commonly induced using batch wise nitrogen starvation. In a batch strategy, biomass is first produced under balanced growth conditions ('growth phase') followed by a complete exhaustion of nitrogen to initiate TAG accumulation ('stress phase'). Batch operation allows for relative high TAG contents (30-60% of dry weight) and is simple in operation (Benvenuti et al., 2016b). To reach high TAG productivities, cultures should have high photosynthetic efficiencies and channel the majority of the energy towards TAG during the nitrogen starvation phase (Figure 1.2). Unfortunately, batch wise TAG production is still accompanied with impaired growth rates and severely reduced photosynthetic efficiencies under nitrogen starvation. As a consequence, highest TAG productivities are only reached in the first days of the starvation when the cellular TAG content is still low (Breuer et al., 2014).

To overcome the reduced photosynthetic efficiencies during nitrogen starvation, a continuous nitrogen limitation strategy was proposed (Klok et al., 2013). Here, nitrogen limitation refers to growth under a constant, but limiting nitrogen supply rate leading to simultaneous growth and TAG accumulation. Continuous processes have the advantage (compared to batchwise cultivation) of high operation flexibility under outdoor conditions (e.g., fluctuating insulation or day-time length), as the biomass concentration and nutrient feed rates can be controlled via the dilution rate. However, a quantitative comparison of batch versus continuous TAG production is still lacking.

1.4 Current bottlenecks in microalgal bulk lipid production

Although commercial production of bulk lipids from microalgae is not yet profitable, Ruiz et al. (2016) estimated that cultivation costs for algal biomass can reduce to 0.5 € per kg and hereby allowing economically feasible production of bulk microalgal lipids. A sensitivity analysis showed that the photosynthetic efficiency is the most crucial parameter that influences the biomass production costs. Detailed

understanding of the physiology behind photosynthetic efficiency under nitrogen deprivation is limited and makes it therefore difficult to target further improvements in microalgal TAG production. A better understanding of photosynthetic efficiency and metabolism under nitrogen deprivation is therefore crucial to further improve microalgal TAG production.

1.5 Aim and thesis outline

This thesis aims to identify targets for improving microalgal lipid production, focussing specifically on improving the solar-to-lipid conversion efficiency in microalgae. To identify targets for optimization, a thorough understanding of diurnal light-dark (LD) cycles, cultivation strategy (batch versus continuous nitrogen limitation) and regulation of metabolism is needed.

Lipid production in microalgae is highly dependent on the applied light intensity (Breuer et al., 2013b). Therefore, in **chapter 2**, we first focussed on the effect of light on biomass and lipid production during batch-wise nitrogen starvation of *Phaeodactylum tricornutum* (Figure 1.3A and B). This salt-water diatom is already commercially produced at large scale for the high EPA content, however, it is not yet known how nitrogen starvation affects the EPA-accumulation dynamics. Therefore, we also studied the partitioning of EPA into membrane lipids and TAG under nitrogen deprivation.

To obtain more insight into the mechanism of microalgal lipid accumulation and to define targets for future strain improvements, we continued in **chapter 3** with studying the regulatory processes (e.g., transcriptome, proteome and metabolome) for lipid accumulation in *P. tricornutum*. Here, we used a continuous, turbidostat operated, photo bioreactor setup that allows for highly reproducible and defined conditions. Cultures grown in LD cycles, under either nitrogen replete or limited conditions, were compared on biochemical composition, transcription and translation of proteins. Such an integrated omics approach presents novel knowledge on the molecular interaction between transcription, translation of proteins and expression of metabolites for TAG enhanced culture conditions.

Figure 1.3 – Pictures of the salt-water diatom *Phaeodactylum tricornutum* (A and B) and the freshwater microalgae *Acutodesmus obliquus* (C and D) cultivated in a flat panel photobioreactor (B and D).

Despite the commercial potential for EPA production using *P. tricornutum*, **chapter 2 and 3** show low volumetric TAG productivities resulting from the low photosynthetic efficiency and suboptimal carbon distribution during nitrogen starvation. Therefore, in **chapter 4**, we selected *Acutodesmus obliquus* (Figure 1.3C and D) as alternative microalga to investigate the impact of two different cultivation strategies on TAG production. The green freshwater microalgae *A. obliquus* was selected because it is able to retain a high photosynthetic efficiency and shows improved carbon distribution under nitrogen starvation. In addition, de Jaeger et al. (2014) created a starchless mutant with further improved carbon partitioning to TAG under nitrogen starvation. By using both both strains to evaluate the potential of continuous lipid accumulation, we could also study the role of carbon partitioning (i.e. presence of starch) for both processes.

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, fixated carbon is used for growth and is partially stored in storage compounds (e.g., carbohydrates or lipids). It is commonly assumed that such temporary storage is essential for fuelling cell division at night. To further optimize

overall cultivation strategies, it is essential to understand the impact of temporary storage systems on growth. **Chapter 5** therefore investigates the impact of temporary storage systems on growth of *A. obliquus* wildtype and the starchless mutant. These findings assist targeted approaches (using starchless mutants) towards improved lipid producing strains.

This thesis investigates different TAG-accumulation strategies from a process engineering and metabolic point of view. The combination of all findings are used in the general discussion (**chapter 6**) to thoroughly evaluate the current lipid-accumulation strategies. Based on this evaluation, **chapter 6** discusses the main bottlenecks and proposes new strategies to move beyond the current existing limitations in phototrophic lipid production with microalgae.

Chapter 2

Triacylglycerol and EPA production in
Phaeodactylum tricornutum under different light
intensities

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ABSTRACT

Lipid production in microalgae is highly dependent on the applied light intensity. However, for the EPA producing model-diatom *Phaeodactylum tricornutum*, clear consensus on the impact of incident light intensity on lipid productivity is still lacking. This study quantifies the impact of different incident light intensities on the biomass, TAG and EPA yield on light in nitrogen starved batch cultures of *P. tricornutum*. The maximum biomass concentration and maximum TAG and EPA contents were found to be independent of the applied light intensity. The lipid yield on light was reduced at elevated light intensities ($>100 \mu\text{mol m}^{-2} \text{s}^{-1}$). The highest TAG yield on light ($112 \text{ mg TAG mol}_{\text{ph}}^{-1}$) was found at the lowest light intensity tested ($60 \mu\text{mol m}^{-2} \text{s}^{-1}$), which is still relatively low to values reported in literature for other algae. Furthermore, mass balance analysis showed that the EPA fraction in TAG may originate from photosynthetic membrane lipids.

2.1 Introduction

Lipid production by phototrophic microalgae is only economically feasible today for high-value products, such as pigments and ω -3-fatty acids (Ruiz et al., 2016). One of the major hurdles for commercial bulk microalgal lipid production is the low lipid yield on light (Chauton et al., 2015; Norsker et al., 2011; Ruiz et al., 2016).

Typically, microalgal lipid production is induced by nitrogen deficiency. When exposed to nitrogen deficient conditions, some microalgal species can accumulate triacylglycerol (TAG) up to 30-60 % of dry weight (Breuer et al., 2012; Mulders et al., 2014; Santos et al., 2014). However, nitrogen deficiency is often accompanied with impaired or even fully inactivated photosynthesis (Benvenuti et al., 2016b; Breuer et al., 2012) and therefore results in a reduced TAG yield on light. For commercial production of TAG, the TAG yield on light should be increased.

One of the most advertised microalgae for commercial lipid production is *Phaeodactylum tricornutum*. *P. tricornutum* is a salt-water diatom that is already cultivated at large scale for aquaculture. This microalgae produces large amounts of eicosapentaenoic acid (EPA; a long chain polyunsaturated fatty acid) and can produce TAG up to 30% of dry weight (Breuer et al., 2012). It is generally acknowledged, however, that high light exposure can severely affect the biomass and lipid productivity of this diatom (Bitaubé Pérez et al., 2008; Breuer et al., 2013b; Molina-Grima et al., 1994; Reis et al., 1996; Yongmanitchal and Ward, 1992). However, clear consensus regarding the impact of light intensity on TAG and EPA productivity in *P. tricornutum* is still lacking. The aim of this work is to obtain insight into the relation between incident light intensity and the accumulation of TAG and EPA under nitrogen starvation.

2.2 Material and methods

2.2.1 Strain, medium and precultivation

Phaeodactylum tricornutum SAG1090-1b was obtained from the Culture Collection of Algae Goettingen University, Germany. Cultures were maintained in 250 mL shake flasks containing 100 mL filter sterilized (pore size 0.2 μm) medium. The medium was designed to reach a biomass concentration of 2.5 g L⁻¹ and consisted out of 252 mM NaCl, 16.8 mM KNO₃, 3.5 mM Na₂SO₄, 5 mM MgSO₄ 7H₂O, 2.4 mM CaCl₂ 2H₂O, 2.5 mM K₂HPO₄, 10 mM NaHCO₃, 28 μM NaFeEDTA, 80 μM Na₂EDTA 2H₂O, 19 μM MnCl₂ 4H₂O, 4 μM ZnSO₄ 7H₂O, 1.2 μM CoCl₂ 6H₂O, 1.3 μM CuSO₄ 5H₂O, 0.1 μM Na₂MoO₄ 2H₂O, 0.1 μM Biotin, 3.7 μM vitamin B1 and 0.1 μM vitamin B12. The medium contained 100 mM, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) as a pH buffer. Prior to cultivation, the pH was adjusted with KOH to pH 7.2.

Cultures were maintained in a cultivation chamber at 25 °C, orbitally shaken at 120 rpm, illuminated at an incident light intensity of 40-60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and a 16:8h light-dark (LD) cycle under enriched air (2% CO₂). Three days prior to the start of the experiment, cultures were transferred to an orbital shaker incubator with an incident light intensity of 160 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 16:8h LD cycles and 2.5% CO₂ enriched air.

2.2.2 Photo bioreactor setup and operation

Experiments were performed in aseptic, flat-panel, airlift-loop reactors (Algaemist, Technical Development Studio, Wageningen University, the Netherlands). The reactor has a working volume of 400 mL, a light path of 14 mm and an illuminated area of 0.028 m² (Schematic illustration of the reactor design is given in Breuer et al. (2013b)). Illumination was provided using 6 LED lamps with a warm white light spectrum, (BXRA W1200, Bridgelux, USA, emission spectrum given in the Supplementary information: Figure S2.1) under a 16:8h LD cycle. The incident photon flux density (PFD_{in}) was measured with a LI-COR 190-SA 2 π PAR (400-700 nm) quantum sensor (PAR, USA) and determined as the average over 28 points evenly distributed over the illuminated surface of the inside of the front glass panel of the reactor. The culture was continuously supplied with sterile moisturized air at 0.4 L

min^{-1} . pH was maintained at 7.2 by on-demand sparging of CO_2 to the airflow and temperature was kept constant at 20 °C. Reactor medium was similar to the preculture medium, with modifications to the amount of KNO_3 (5 mM instead of 16.8 mM) and exclusion of HEPES. 5 mM of KNO_3 is sufficient to reach a biomass concentration of 1.2 g L^{-1} (assuming a cellular nitrogen fraction of 10% per dry weight, measurements not shown). Per day, a maximum of 2 drops of 1% (v/v) antifoam (Antifoam B, J.T. Baker) were added manually to the reactor when foaming was visible.

Reactors were heat sterilized (60 min at 121 °C) and inoculated at a biomass concentration of approximately 0.5 g L^{-1} of dry weight and grown at an incident light intensity of $100 \mu\text{mol m}^{-2} \text{ s}^{-1}$. Once the biomass concentration reached 1 g L^{-1} , which was typically three days after inoculation, the set points for incident light intensity were changed to the desired values (60, 100, 250, 500, or $750 \mu\text{mol m}^{-2} \text{ s}^{-1}$ incident light intensity). The day at which the light intensity was changed to the experimental set point was regarded as start of the experiment, and therefore referred to as $t=0$. Periodically, samples (approximately 20 mL culture volume) were taken aseptically and analysed for dry weight concentration, triacylglycerol (TAG) and total fatty acid (TFA) concentration and composition, dissolved (residual) nitrate, maximum efficiency of PSII chemistry (F_v/F_m), and absorption cross section. After taking a sample, the culture volume was restored by adding $0.2 \mu\text{m}$ -filtered reactor medium to ensure that the pH probe remained submerged. Depending on whether the culture was in N-replete phase or N-depleted phase, this medium contained either 5 mM KNO_3 or no nitrogen, respectively.

Five different incident light intensities were selected that cover both low light and light saturation (light saturation point for *P. tricornutum* was found at $150\text{--}200 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (J. Geider et al., 1985; Ritchie, 2008; Wiegman et al., 2003). Three photo bioreactor experiments were conducted in biological duplicate to test reproducibility (Postma et al., 2015).

Measurements for dry weight, F_v/F_m and dissolved nitrogen were performed in technical triplicates. Measurements for the light absorption spectrum, lipid content and composition were performed in technical duplicate.

2.2.3 Biomass Analysis

Samples were taken at regular time points to monitor biomass dry weight concentration gravimetrically as described by Lamers et al. (2010). Light absorption spectrum was measured in a double-beam spectrophotometer (UV-2600, Shimadzu, Japan) equipped with an integrated sphere (ISR-2600). The average dry weight-specific optical cross section (α_c in $\text{m}^2 \text{g}^{-1}$) was calculated from the obtained absorption spectrum as described by (de Mooij et al. (2014). The F_v/F_m ratio was measured using an AquaPen-C AP-C 100 FluorPen fluorometer (Photon Systems Instruments, Czech Republic) equipped with an orange LED emitter (excitation light 620 nm) as described by Benvenuti et al. (2014).

The triacylglycerol (TAG), membrane lipid (ML) and total fatty acid (TFA) content and fatty acid composition were determined by lipid extraction (chloroform:methanol mixture), lipid class separation using a silica column, transesterification and subsequently quantified using gas chromatography (GC-FID) as described by Breuer et al. (2013b). The dissolved nitrate concentration was measured with an AQ2 nutrient analyser (Seal Analytical, USA) as described by Benvenuti et al. (Benvenuti et al., 2016a).

2.2.4 Calculating yield and productivity

The time averaged volumetric productivity ($r_i(t_0 \rightarrow t)$) was calculated by dividing the amount of formed product (i: biomass, triacylglycerol, EPA) over cultivation time according to Equation 2.1;

$$r_i(t_{N=0} \rightarrow t) = \frac{C_i(t) - C_i(t_{N=0})}{t - t_{N=0}} \quad (\text{Eq. 2.1})$$

The time averaged yield of TAG on light ($Y_{TAG,ph}(t_0 \rightarrow t)$) on each time point was calculated by dividing the amount of TAG produced in that period by the amount of light supplied in that period (Breuer et al., 2015a). In addition, the photon costs for initial biomass production until nitrogen depletion were incorporated by assuming that this biomass was made with a theoretical net photosynthetic yield, $Y_{x,ph}$, of 1 g mol_{ph};

$$Y_{TAG,ph}(t) = \frac{TAG(t) - TAG(t_{N=0})}{\frac{PFD_{in} \cdot (t - t_{N=0})}{z} + \frac{C_{x,N=0}}{Y_{x,ph}}} \quad (\text{Eq. 2.2})$$

In Equation 2.2, TAG(t) and TAG($t_{N=0}$) represent the TAG concentration at time point t and the moment of nitrogen starvation, respectively. The exact time of nitrogen starvation ($t_{N=0}$) was estimated by interpolation of the biomass concentration to 1.2 g L⁻¹. PFD_{in} is the incident light intensity, z is the reactor depth and C_{x,N=0} is the biomass concentration at the onset of nitrogen starvation.

2.3 Results and discussion

2.3.1 Effect of light on biomass growth

The impact of five different light intensities on biomass, TAG and EPA productivity during nitrogen starvation was assessed in batch-wise operated flat-panel photo bioreactors that were subjected to 16:8h light-dark (LD) cycles. Results for dry weight, maximum efficiency of PSII chemistry and TAG content are shown in Figure 2.1.

Sufficient nitrogen was added to reach a biomass concentration of 1.2 g L⁻¹. After that biomass concentration was reached, all nitrogen was used and the culture was exposed to nitrogen starvation. The biomass concentration further increased up to 2.2 g L⁻¹ of dry weight, which was due to the accumulation of TAG (Figure 2.1) and carbohydrates (Supplementary material: Table S2.1). The maximum achieved biomass concentration was reached 5 days after nitrogen starvation started, except for the culture exposed to the lowest light conditions, which reached the maximum biomass 14 days after nitrogen starvation started. The maximum reached biomass concentration was 2.15±0.1 g L⁻¹, independent of the supplied light intensity (Figure

2.1A). Other studies showed similar findings for the freshwater microalgae *Acutodesmus obliquus* under nitrogen starvation (Breuer et al., 2012).

Cultures that are light limited are expected to show an increase in biomass productivity upon increasing light intensities (Breuer et al., 2013b). However, our results show no differences in productivities for the cultures exposed to $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ and higher (Figure 2.1). This similarity in productivity might be explained by photo inhibition that occurred from $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ onwards. As soon as the photosynthetic machinery becomes inhibited, further increasing light intensity would not enhance the productivity anymore (Dillschneider et al., 2013). According to Figure 2.1B, a strong decline in F_v/F_m was observed (from 0.6 to 0) for cultures exposed to light intensities above $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ immediately after nitrogen

starvation. A decline in F_v/F_m is generally acknowledged with reduced photosynthetic efficiency or photo damage (Benvenuti et al., 2014; Parkhill et al., 2001; Zhao et al., 2015). Geider et al. (1985) presented a light curve for *P. tricornutum*. Similar to our findings, they reported that from $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ onwards light saturation occurs in *P. tricornutum*. In addition, Acién Fernández et al. (2003) performed outdoor growth experiments with *P. tricornutum*, where the maximum biomass productivity was reached at average irradiances of $50\text{-}150 \mu\text{mol m}^{-2} \text{s}^{-1}$ and decreased at light intensities higher than $150 \mu\text{mol m}^{-2} \text{s}^{-1}$. Altogether, it is likely that in this work *P. tricornutum* becomes photo inhibited at light intensities above $100 \mu\text{mol m}^{-2} \text{s}^{-1}$.

2.3.2 Changes in TAG accumulation, productivity and yields

Independent of incident light intensity, the TAG concentration increased from 0.05 g L^{-1} (3 % of dry weight) under nitrogen replete conditions to $\sim 0.41 \text{ g L}^{-1}$ (~ 25 % of dry weight) after prolonged nitrogen starvation (Figure 2.1 and Table S2.2). These maximum TAG contents are in agreement with previous observations in literature (Table 2.1).

Table 2.1 – Comparison of the maximum TAG and total fatty acid content obtained in this study to literature values. *TAG contents were estimated from the total fatty acid content, assuming 8% of membrane lipids per dry weight.

Strain	TAG content (% of dry weight)	Total fatty acid content (% of dry weight)	Reference
<i>P. tricornutum</i>	25	31	This study
<i>P. tricornutum</i>	22*	30	Breuer et al. (2012)
<i>P. tricornutum</i>	20*	28	Griffiths et al. (2011)
<i>P. tricornutum</i>	26*	34	Santos-Bllardo et al. (2016)

Except for the culture exposed to the lowest light intensity ($60 \mu\text{mol m}^{-2} \text{s}^{-1}$), TAG accumulation did not differ significantly for the tested conditions (Figure 2.1). The culture exposed to high incident light intensity ($750 \mu\text{mol m}^{-2} \text{s}^{-1}$) showed a decline in TAG content from day 11 onwards, which might indicate photo damage or even cell death.

The TAG yield on light is directly proportional to areal productivity and is therefore also strongly related to investment and variable production costs. The maximum time-averaged TAG yield on light is often used as indicator to determine optimal harvest moments (Benvenuti et al., 2016b). Our study shows that large differences were observed in the maximum time-averaged TAG yield on light among the different tested incident light intensities (Figure 2.2). Of all tested conditions, the cultures exposed to low light ($60 \mu\text{mol m}^{-2} \text{s}^{-1}$) reached the highest maximum TAG yield on light ($112 \text{ mg TAG mol}_{\text{ph}}^{-1}$). Increasing the incident light intensity only resulted in lower TAG yields on light: $96 \text{ mg TAG mol}_{\text{ph}}^{-1}$ for the culture exposed to $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $36\text{--}48 \text{ mg TAG mol}_{\text{ph}}^{-1}$ for cultures exposed to even higher light intensities. In comparison with other studies (Table 2.2), *P. tricornutum* shows relative low TAG yields on light compared to other strains at similar or higher incident light intensities.

Figure 2.2 – Time averaged TAG yield on light for five different incident light intensities over time. Incident light intensities of $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ (blue circles), $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ (red squares), $250 \mu\text{mol m}^{-2} \text{s}^{-1}$ (green triangles), $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ (black circles) and $750 \mu\text{mol m}^{-2} \text{s}^{-1}$ (open diamonds). Sufficient nitrogen was added to reach biomass concentrations of 1.2 g L^{-1} , thereafter cells were exposed to nitrogen starvation. Error bars indicate the standard deviation of both cultures from the average value.

Table 2.2 — Comparison of the maximum time-averaged TAG yield on light obtained in this study to literature values. CL: continuous light, LD16:8: light dark cycles with 16h of light followed by 8h of darkness. ¹ was calculated by assuming that the total lipid fraction existed for 90% out of TAG. ² Assumed that 42% (J/J) of the total irradiance has a wavelength in the photosynthetic active radiation spectrum with an energy content of $0.217 \text{ MJ mol}_{\text{ph}}^{-1}$. ³ Data given only for the starvation period. ⁴ Light was supplied with a sinus function with a solar noon at $1500 \mu\text{mol m}^{-2} \text{ s}^{-1}$ and a daily averaged light intensity of $636 \mu\text{mol m}^{-2} \text{ s}^{-1}$. ⁵ the calculated range is depends on initial biomass concentration at the moment of nitrogen deprivation and includes measurement errors.

Species	TAG yield on light ($\text{g mol}_{\text{ph}}^{-1}$)	Incident light intensity ($\mu\text{mol m}^{-2} \text{ s}^{-1}$)	Operational strategy	Reference
<i>Phaeodactylum tricornutum</i>	0.11	60	Batch, LD 16:8	This study
<i>Acutodesmus obliquus</i>	0.24, 0.13, 0.09, 0.04	200, 500, 800, 1500	Batch, CL	Breuer et al. (2012)
<i>Acutodesmus obliquus</i>	0.15	500	Batch, CL	Breuer et al. (2014)
<i>Acutodesmus obliquus slm1</i>	0.22	500	Batch, CL	Breuer et al. (2014)
<i>Acutodesmus obliquus</i>	0.16	500	Batch, LD 16:8	Remmers et al. (2017a)
<i>Acutodesmus obliquus slm1</i>	0.20	500	Batch, LD 16:8	Remmers et al. (2017a)
<i>Chlorella zofingensis</i>	0.32	245	Batch, CL	Mulders et al. (2014)
<i>Chlorella vulgaris</i>	0.16 ¹	615 ²	Batch, outdoor	Münkel et al. (2013)
<i>Chlorococcum litrorale</i>	0.16 ³	636 ⁴	Batch, sinusoidal LD 16:8	Cabanelas et al. (2016a)
<i>Chlorococcum litrorale</i> , S5 sorted population	0.32 ³	636 ⁴	Batch, sinusoidal LD 16:8	Cabanelas et al. (2016a)
<i>Neochloris oleoabundans</i>	0.16	218	Batch, CL	Santos et al. (2014)
<i>Nannochloropsis oculata</i>	0.17-0.25 ⁵	250	Batch, CL	Van Vooren et al. (2012)
<i>Nannochloropsis</i> sp.	0.10	636 ⁴	Batch, sinusoidal LD 16:8	Benvenuti et al. (2016a)

Sustained biomass productivity upon nitrogen starvation is a key selection parameter for efficient TAG production. As shown in Figure 2.1A, *P. tricornutum* only showed a 2-2.2-fold biomass increase upon nitrogen starvation. Other oleaginous green microalgae (e.g., *Acutodesmus obliquus*, *Chlorella zofingiensis*) showed a 6-8 fold increase in biomass upon nitrogen starvation (Benvenuti et al., 2014; Breuer et al., 2012; Mulders et al., 2014). High photon to TAG conversion yields are only possible when light energy is efficiently used to fix carbon (e.g., measured as biomass productivity) and when the majority of fixed carbon is channelled towards storage lipids (e.g., TAG content). Literature reports that diatoms are specifically known to mobilize alternative electron transport (AET) pathways, that can eradicate up to 50% of the electrons released by PS II (Jallet et al., 2016; Wagner et al., 2015; Waring et al., 2010). Also, increased Non-Photochemical Quenching (NPQ) rates were found for *P. tricornutum* when exposed to increased light intensities (Bailleul et al., 2010; Matilde Skogen Chauton et al., 2013; Lavaud et al., 2002). Both processes are known to protect the cells from photo inhibition but to also result in inherent productivity losses. For *P. tricornutum*, these mechanisms might explain the reduced photosynthetic efficiency compared to other microalgae under low light nitrogen starvation.

Considering the abovementioned light sensitivity of *P. tricornutum* during nitrogen starvation, precautions should be taken to prevent the culture from extensive photo inhibition when working in high irradiance conditions (Richmond, 2008). Examples are to increase the culture cell density to create a dark zone in which the photo inhibitory damage can be repaired (Olivieri et al., 2014; Tredici and Zittelli, 1998) or to execute strain improvement for reduced antenna size (de Mooij et al., 2016; Perin et al., 2015).

2.3.3 FA composition

The fatty acid composition of TAG and membrane lipids (ML) did not substantially change over time nor with incident light intensity (the complete fatty acid composition of both fractions during the time-course of the experiments can be found in Table 2.3). The most abundant fatty acids in TAG were found to be C16:1 (44% of total TAG fatty acids) and C16:0 (30% of total TAG fatty acids). Only a small

fraction of C20:5 (EPA) was found in the TAG pool (6-10% of total TAG fatty acids). The membrane lipid fraction was primarily comprised of C16:0 (20% of total ML fatty acids), C16:1 (25-30% of total ML fatty acids) and C20:5 (25-30% of total ML fatty acids) (Table 2.3). Literature reports similar fatty acid profiles in *P. tricornutum* (Abida et al., 2015; Alonso et al., 2000; Breuer et al., 2012; Gatenby et al., 2003). As a consequence of TAG accumulation (Figure 2.1) and the lower EPA content of TAG compared to membrane lipids, the EPA fraction of the total fatty acid pool in the biomass dropped from 25% to 9% of the total fatty acids (Supplementary material: Table S2.3). Because the biomass productivity declined at the onset of nitrogen depletion, EPA productivity was highest (6-12 mg L⁻¹ day⁻¹) under nitrogen replete growth conditions.

Table 2.3 – Average fatty acid composition of *P. tricornutum* in the TAG and ML lipid fraction for five different light intensities: 60, 100, 250, 500 and 750 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Values are averaged over the entire time course of the experiment (n=7). Standard deviations are shown between brackets.

	% of fatty acids in TAG fraction					% of fatty acids in ML fraction				
	60	100	250	500	750	60	100	250	500	750
C14:0	5.1 (0.7)	5.0 (0.5)	5.5 (0.9)	5.2 (0.5)	5.8 (0.4)	6.5 (0.3)	5.7 (0.7)	5.5 (0.8)	6.4 (0.4)	6.0 (1.2)
C16:0	24.8 (3.6)	26.3 (4.5)	31.8 (1.4)	29.8 (3.7)	25 (0.4)	19.1 (3.3)	21.2 (3.7)	25.7 (5.6)	26.3 (7.9)	31.1 (8.5)
C16:1	40.4 (5.1)	44.8 (3.2)	43.1 (3.1)	42 (5.5)	49.1 (3.4)	26.4 (2.3)	26.5 (2.1)	26.6 (2.2)	25.8 (1.5)	21.8 (4.3)
C16:2	0.7 (0.5)	1.3 (1.3)	1.9 (0.4)	0.7 (0.3)	2.1 (3.1)	3.0 (1.1)	2.5 (1.9)	1.2 (0.6)	1.7 (1)	1.2 (0.5)
C16:3	1.1 (0.5)	1.3 (0.8)	1.2 (0.6)	0.9 (0.3)	1.4 (0.7)	5.2 (2.3)	4.3 (1.7)	3.7 (1.9)	4.1 (2.5)	3.4 (2.3)
C18:0	1.8 (0.2)	1.7 (0.3)	1.6 (0.5)	1.4 (0.2)	1.5 (0.5)	2.4 (1)	3.6 (1.5)	5.2 (2.8)	3.4 (1.3)	7.6 (5.3)
C18:1	5.8 (1.2)	5.7 (1.4)	5.1 (1.1)	4.2 (0.6)	4.6 (1.1)	2.7 (1.1)	3.8 (2.4)	4.1 (1.7)	4 (1.7)	4.5 (1.6)
C18:2w6	1.4 (0.3)	1.3 (0.3)	1.3 (0.2)	1.1 (0.3)	1.3 (0.2)	2.0 (0.6)	1.4 (0.4)	2.2 (0.8)	2.0 (0.8)	2.0 (1.1)

Table 2.3 (continued) – Average fatty acid composition of *P. tricornutum* in the TAG and ML lipid fraction for five different light intensities: 60, 100, 250, 500 and 750 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Values are averaged over the entire time course of the experiment (n=7). Standard deviations are shown between brackets.

	% of fatty acids in TAG fraction					% of fatty acids in ML fraction				
	60	100	250	500	750	60	100	250	500	750
C18:3w3	6.7 (4.3)	3.3 (2.4)	2.8 (0.6)	5.6 (5.4)	2.7 (0.6)	1.5 (0.4)	1.5 (0.6)	1.7 (0.5)	1.3 (0.3)	1.5 (0.8)
C20:3-n3	0.9 (0.8)	0.4 (0.3)	0.1 (0.1)	1.7 (4)	0.4 (0.8)	5.8 (1.7)	5.7 (2.7)	3.8 (1.3)	3.7 (0.7)	3.0 (2.1)
C20:5-n3	7.8 (1.4)	7.1 (1.4)	6.7 (0.8)	7.4 (1)	7.2 (1.1)	25.5 (5.7)	23.8 (7)	20.3 (5.5)	21.3 (7.1)	18.0 (8.9)

2.3.4 EPA incorporation in membrane lipids and triacylglycerol

Figure 2.3 shows the EPA concentration per litre culture with respect to the TAG and membrane lipid fraction. Apart from a small increase until day 5, neither a substantial net synthesis nor breakdown of EPA upon N starvation is observed. However, the distribution of EPA over TAG and membrane lipids changes over time. Upon nitrogen depletion, a substantial increase in EPA distribution towards TAG can be observed. Simultaneously, we observed a decrease in EPA that was bound to membrane lipids. Therefore, we hypothesize that the EPA found in TAG under nitrogen starvation could partially be derived from EPA-containing membrane lipids that were restructured upon nitrogen depletion. Similar observations have been made for *Nannochloropsis oceanica* (Jia et al., 2015), *Nannochloropsis oculata* and *Pavlova lutheri* (Guihéneuf and Stengel, 2013).

For many species, nitrogen depletion results in an inherent degradation of photosynthetic membrane lipids with simultaneous accumulation of TAG. Part of the fatty acids originating from membrane lipids could thus be used as fatty acyl donors for TAG synthesis under nutrient deprivation (Figure 2.3). However, detailed knowledge on the mechanisms behind carbon partitioning in microalgae under nitrogen deprivation is still lacking (Guihéneuf and Stengel, 2013; Juergens et al., 2016; Klein, 1987; Mühlroth et al., 2013; Tonon et al., 2002) and further research using ^{13}C labelling should confirm these observations. To further improve EPA productivity

by means of metabolic engineering or process development (Adarme-Vega et al., 2012), it is crucial to understand the regulation of EPA towards different lipids classes (e.g., TAG or structural membrane lipids) under different environmental conditions.

Figure 2.3 – EPA concentration in both TAG and ML lipids over time at an incident light intensity of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$. The moment of nitrogen starvation is indicated with a dashed line.

2.4 Conclusion

Elevated light intensities ($>100 \mu\text{mol m}^{-2} \text{s}^{-1}$) reduce the photosynthetic efficiency and the TAG yield on light during nitrogen starvation, likely caused by photo inhibition. Compared to other microalgae strains, relative low TAG yields on light were observed for *P. tricornutum* as the maximum yield was only $112 \text{ mg TAG mol}_{\text{ph}}^{-1}$ (at $60 \mu\text{mol m}^{-2} \text{s}^{-1}$). TAG contents were similar for all light intensities tested (i.e. 25 % of dry weight). Further analysis on the fatty acid composition revealed that the TAG fraction of *P. tricornutum* contains 6-10% of EPA. Mass balance analysis revealed this EPA fraction may originate partly from photosynthetic membrane lipids that were restructured after nitrogen depletion.

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Supplementary file 2.1– Figures and tables

Figure S2.1 – Emission spectrum of the Algaemist Bridgelux LED panel, data obtained from de Mooij et al. (2014).

Table S2.1 – Carbohydrate content over time for five different incident light intensities. For all experiments $n=2$, except for incident light intensity of 250 and 750 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with $n=1$. Values in brackets represent the standard deviation from the two biological duplicates.

Days	Total carbohydrate content (% of dry weight)				
	60 $\mu\text{mol m}^{-2} \text{s}^{-1}$	100 $\mu\text{mol m}^{-2} \text{s}^{-1}$	250 $\mu\text{mol m}^{-2} \text{s}^{-1}$	500 $\mu\text{mol m}^{-2} \text{s}^{-1}$	750 $\mu\text{mol m}^{-2} \text{s}^{-1}$
0	15.23 (1.18)	12.10 (1.09)	12.9	12.53 (0.70)	15.00
2	18.18 (0.59)	20.64 (2.45)	21.83	25.64 (6.64)	23.2
5	28.61 (1.99)	24.17 (6.19)	20.08	25.78 (10.41)	18.53
8	32.21 (0.62)	28.22 (4)	23.97	26.92 (6.26)	26.42
11	33.01 (1.46)	26.96 (4.22)	23.31	25.29 (5.11)	25.10
15	27.48 (0.80)	22.98 (2.44)	22.76	23.85 (1.94)	27.56
17	28.82 (0.89)	19.37 (2.66)	19.37	21.69 (5.56)	26.11

Table S2.2 – TAG concentration over time for five different incident light intensities. For all experiments $n=2$, except for incident light intensity of 250 and 750 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with $n=1$. Values in brackets represent the standard deviation from the two biological duplicates.

Days	TAG concentration (g L^{-1})				
	60 $\mu\text{mol m}^{-2} \text{s}^{-1}$	100 $\mu\text{mol m}^{-2} \text{s}^{-1}$	250 $\mu\text{mol m}^{-2} \text{s}^{-1}$	500 $\mu\text{mol m}^{-2} \text{s}^{-1}$	750 $\mu\text{mol m}^{-2} \text{s}^{-1}$
0	0.04 (0.01)	0.03 (0)	0.05	0.05 (0.01)	0.04
2	0.04 (0)	0.13 (0.09)	0.16	0.26 (0.07)	0.21
5	0.14 (0.03)	0.33 (0.05)	0.23	0.39 (0.11)	0.30
8	0.26 (0.03)	0.36 (0.04)	0.31	0.43 (0.06)	0.39
11	0.4 (0.06)	0.48 (0.05)	0.36	0.44 (0.02)	0.43
14	0.45 (0.07)	0.49 (0.11)	0.31	0.46 (0.06)	0.27
17	0.54 (0.07)	0.53 (0.16)	0.30	0.45 (0.1)	0.26

Table S2.3 – EPA content in the total lipid fraction over time for five different incident light intensities. For all experiments n=2, except for incident light intensity of 250 and 750 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with n=1. Values in brackets represent the standard deviation from the two biological duplicates.

Days	EPA content in total fatty acids (% of dry weight)				
	60 $\mu\text{mol m}^{-2} \text{s}^{-1}$	100 $\mu\text{mol m}^{-2} \text{s}^{-1}$	250 $\mu\text{mol m}^{-2} \text{s}^{-1}$	500 $\mu\text{mol m}^{-2} \text{s}^{-1}$	750 $\mu\text{mol m}^{-2} \text{s}^{-1}$
0	24.97 (0)	18.86 (8.64)	12.75	21.81 (4.47)	12.75
2	23.08 (4)	22.13 (10.53)	15.71	13.36 (0)	22.07
5	23.43 (3.48)	17.47 (4.67)	13.92	12.93 (0.86)	10.79
8	16.42 (0.4)	10.44 (3.64)	11.50	12.15 (1.02)	12.21
11	14.96 (0.76)	11.04 (1.42)	11.25	11.88 (0.4)	12.16
15	11.21 (0.35)	9.09 (2.06)	9.90	9.66 (0.89)	8.06
17	9.11 (0.26)	8.23 (2.17)	8.72	8.77 (0.72)	7.89

Chapter 3

Orchestration of transcriptome, proteome
and metabolome in a nitrogen limited
diatom *Phaeodactylum tricornutum*

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ABSTRACT

Nitrogen deprivation increases the triacylglycerol (TAG) content in microalgae but also severely decreases their growth rate. Most approaches that attempted to increase TAG productivity by overexpression or knockdown of specific genes related to the regulation of the lipid synthesis pathway have failed. More insight into the molecular mechanisms related to lipid accumulation and impaired growth rate is needed to find targets for improving TAG productivity. We comprehensively profiled the physiology, transcriptome, proteome and metabolome of the diatom *Phaeodactylum tricornutum* during steady state growth at both nitrogen limited and replete levels during light:dark cycles. Nitrogen limitation resulted in an increased TAG content, from 3% to 16% of dry weight, and a reduced growth rate and photosynthetic efficiency. Under nitrogen limited conditions, 22% (2699) of the total identified transcripts, 17% (543) of the proteins and 44% (345) of the metabolites were significantly differentially regulated compared to nitrogen replete growth conditions. Although nitrogen limitation was responsible for the majority of significant differential transcript, protein and metabolite expression, we also observed substantial differential expression over a diurnal cycle. Nitrogen limitation mainly induced an upregulation of nitrogen fixation and central carbon metabolism, while photosynthetic, ribosomal and protein synthesis were mainly downregulated. Regulation of lipid metabolism suggests that overexpression of DGAT and the entire acyl-CoA – fatty acid pathway might be an important target for improving TAG productivity. However, TAG biosynthesis is also clearly limited by the reduced carbon flux through central metabolism. Future strain improvement should therefore not only focus on lipid metabolism, but also on improving the carbon flux through central carbon metabolism.

3.1 Introduction

Microalgae are regarded as a promising production platform for specialty fatty acids and the bulk production of triacylglycerol (TAG) for the biofuel industry (Adarme-Vega et al., 2012; Matilde S. Chauton et al., 2013; Draaisma et al., 2013; Wijffels et al., 2010). Currently, microalgae are already commercially applied for heterotrophic production of fatty acids for the food- and feed industry (DSM, 2015; Sprague et al., 2015). However, algae are also able to grow phototrophic, converting sunlight directly into chemical energy by the fixation of CO₂ into biomass and useful products like TAG. TAG accumulation occurs when microalgae are exposed to suboptimal growth conditions (e.g., nitrogen deprivation). Microalgae can accumulate lipids up to 60% of dry weight, although at the expense of growth (Breuer et al., 2012; Rodolfi et al., 2009). The impaired growth rate at suboptimal culture conditions is one of the key limitations in commercializing photosynthetic algal bulk lipid production (Ruiz et al., 2016).

A lot of research has been published on the effect of nitrogen deprivation on lipid accumulation in different microalgal strains (Benvenuti et al., 2014; Breuer et al., 2012; Griffiths et al., 2011). However, the exact mechanism for the induction of TAG synthesis, nitrogen deprivation and loss of photosynthetic efficiency is not yet known. Moreover the influence of light:dark (LD) cycles, which are present at (large scale) outdoor production, are not known. The increasing availability of sequenced genomes and molecular techniques allowed more studies to focus on the molecular mechanisms behind the regulation of TAG accumulation in microalgae. Rational (metabolic) engineering strategies to improve TAG production are, however, still ambiguous due to lacking understanding of microalgal lipid metabolism (Dunahay et al., 1995; Li et al., 2010a; Niu et al., 2013; Work et al., 2010). With the rapid development of 'omics' technologies (e.g., transcriptomics, proteomics, metabolomics), new possibilities arise allowing for a more comprehensive description of microalgal metabolism and its regulation.

While the effect of nitrogen deficiency on microalgal metabolism has been predominantly studied at the transcriptomic level, the effects on protein and metabolite level are often neglected (Bromke et al., 2015; Garnier et al., 2014; Ho et al., 2015; Longworth et al., 2016; Muhseen et al., 2015; Willamme et al., 2015). However, some regulated changes on the expression level may not lead to comparable changes on the protein or metabolite level (Nie et al., 2007; Zhang et al., 2010). An 'integrated' omics approach may provide a complete picture and novel insight on the orchestration of the stress response in a cell. To our knowledge, only few microalgal studies exploited the combination of transcriptome, proteome and/or metabolome data (Dyhrman et al., 2012; Guarnieri et al., 2011; Schmollinger et al., 2014). For example, Schmollinger et al. (2014) identified a target to increase the nitrogen-use efficiency in the nitrogen-starved *Chlamydomonas reinhardtii*.

Knowledge on metabolism is important to improve lipid production using metabolic engineering approaches. Transcriptome studies have been done to obtain more understanding (Corteggiani Carpinelli et al., 2014; Guarnieri et al., 2011; Nie et al., 2007; Rogers et al., 2008; Schmollinger et al., 2014; Smith et al., 2016). However, these studies often do not include the role of LD cycles. Moreover, they often assume that gene expression directly translates to post-translation regulation and do not consider regulation on the protein and metabolite level.

The marine diatom *Phaeodactylum tricornutum* is currently considered as a diatom model organism in the field for physiology, biochemistry and genomics studies (Valenzuela et al., 2012). The availability of the whole genome sequence and molecular toolboxes have recently provided opportunities to use this strain for metabolic engineering (Bowler et al., 2008; Daboussi et al., 2014; De Riso et al., 2009; Eilers et al., 2016; Hamilton et al., 2014; Niu et al., 2013; Nymark et al., 2016; Seo et al., 2015). Therefore, *P. tricornutum* serves as a solid model system to better understand the cellular responses related to nitrogen deprivation and the trade-off between growth and lipid accumulation.

Most of the microalgal 'omics' studies, focussing on improving lipid productivity, have focussed on nitrogen starved batch cultures (Dyhrman et al., 2012; Guarnieri et

al., 2011; Longworth et al., 2016; Smith et al., 2016). Such processes allow to study molecular mechanism over time and thus give insight into the order of regulatory events related to lipid induction. However, the main disadvantage of batch is the continuous changing culture conditions (e.g., light per cell, progressive cell death under nitrogen starvation) that can influence the measurements. Alternatively, synchronized continuous cultures can be used to study single parameter variations, such as the impact of diurnal light:dark (LD) cycles, nitrogen deficiency or iron deficiency (de Winter et al., 2014; Klok et al., 2013; Smith et al., 2016).

The aim of this study is to obtain a comprehensive insight into the metabolism of TAG production induced by nitrogen limitation at the transcriptome, proteome and metabolome level under LD cycles. Nitrogen limitation was induced in continuous, turbidostat controlled, photo bioreactors that allow for highly reproducible culture conditions with simultaneous growth and TAG production. Cultures were acclimated to a 16:8h LD cycle and samples were taken during both the light and right after the dark period for analysis of biomass composition, transcriptome expression, proteome expression, and metabolome composition.

3.2 Material and methods

3.2.1 Strain, medium and precultivation

Phaeodactylum tricornutum SAG1090-1b was obtained from the Culture Collection of Algae Göttingen University (Sammlung von Algenkulturen, Göttingen, Germany). Liquid cultures were maintained in a controlled incubator (25 °C, 30-40 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 16:8h light:dark (LD) cycle, 100 rpm, enriched air with 2% CO_2) in 250 mL Erlenmeyer flasks containing 100 mL of filter sterilized (pore size 0.2 μm) defined medium. The medium was designed to reach a biomass concentration of 2.5 g L^{-1} and consisted of 252 mM NaCl, 16.8 mM KNO_3 , 3.5 mM Na_2SO_4 , 100 mM, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), 5 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.4 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.5 mM K_2HPO_4 , 10 mM NaHCO_3 , 28 μM NaFeEDTA, 80 μM $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, 19 μM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 4 μM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.2 μM $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 1.3 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.1 μM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.1 μM Biotin, 3.7 μM vitamin B1 and 0.1 μM vitamin B12. The

pH was adjusted with KOH to pH 7.2. Prior to the start of the experiments, cultures were transferred to an incubator with a light intensity of $180 \mu\text{mol m}^{-2} \text{s}^{-1}$, a 16:8 h LD cycle and a headspace enriched with 2.5% CO_2 to reach the desired inoculation cell density.

3.2.2 Photobioreactor setup and experimental conditions

P. tricornutum was cultivated aseptically in a 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 similar reactor setup was described previously by Remmers et al. (2017a). Cultures were continuously purged with 1.7 L min^{-1} air enriched with 1% CO_2 . The temperature was controlled at 20°C and the pH was maintained at 7.2 using 5% H_2SO_4 . 1-2 drops of 1%w/w antifoam (Antifoam B, Baker, the Netherlands) were added once per day. Light was provided by 260 LED lamps with a warm white spectrum (450-620 nm) at the culture side of the reactor. As the culture was exposed to 16:8h light dark (LD) cycles, a black cover was placed on the rear of the reactor to prevent external light falling on the culture. After inoculation, the light intensity falling through the back side of the culture was continuously measured by a light meter (LI-250, Licor, USA). The reactor was inoculated at $0.5 \text{ g dry weight L}^{-1}$ at an incident light intensity of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ and along with cellular growth the incident light intensity was stepwise increased until it reached the final set point of $260 \mu\text{mol m}^{-2} \text{s}^{-1}$. Hereafter, continuous turbidostat control was initiated. The culture was diluted with fresh medium (containing no nitrogen) when the outgoing light intensity dropped below $10 \mu\text{mol m}^{-2} \text{s}^{-1}$. Control was only active during the light period. The culture was exposed to two feeding regimes using a separate nitrogen (N) feed (Figure 3.1A): nitrogen replete growth (control experiment, nitrogen supply rate of 0.11 gN day^{-1}) and nitrogen limited growth (0.02 gN day^{-1}). Under nitrogen replete conditions more nitrogen was added than consumed by the algae, resulting in an excess of nitrogen in the culture supernatant. The medium composition was kept equal to that of the preculture except for HEPES (0 mM) and KNO_3 . The dilution medium contained no KNO_3 while the separate nitrogen feed medium contained either 37.5 mM (control experiment) or 21.3 mM (nitrogen limitation) KNO_3 .

Figure 3.1 – Experimental setup (A) and sampling scheme (B). 8 independent cultures were exposed to either nitrogen limited (N=4) or nitrogen replete (control; N=4) continuous growth conditions. When the culture reached steady state, samples were taken at 5-6h intervals for biomass composition (B), proteome (P), metabolome (M) and transcriptome (T) analysis. Light was always supplied in a 16h:8h LD cycle with the lamps either switched on at a fixed intensity of $260 \mu\text{mol m}^{-2} \text{s}^{-1}$ or switched off (dark period; shaded areas).

3.2.3 Reproducibility and sampling

Reproducibility of each experimental condition (e.g., control and nitrogen limited experiment) was verified by running 4 independent replicates. Each experiment was sampled daily at a fixed time point to monitor growth. Steady state was defined as a constant dilution rate and stable biomass concentration, averaged over 24h periods, for at least 6 consecutive days. During steady state, 24h overflow was collected on ice and used to determine the dilution rate and average growth rate per day.

Samples of the overflow were taken to determine biomass concentration ($C_{x,OF}$), absorption coefficient, and the macromolecular biomass composition (TAG, carbohydrates, protein, ash), as described by Remmers et al. (2017a). Productivity (r_i) and yield ($Y_{i,ph}$) on light of biomass and TAG were calculated from the 24h averaged dilution rate (D_{24}), concentration of biomass or TAG in the 24h collected overflow ($C_{i,OF}$) and the photon supply rate (r_{ph}):

$$r_i = D_{24} \cdot C_{i,OF} \quad (\text{Eq. 3.1})$$

$$Y_{i,ph} = \frac{r_i}{r_{ph}} \quad (\text{Eq. 3.2})$$

The photosynthetic conversion efficiency (in % of the absorbed photons) was calculated using Equation 3.3. Biomass was divided in functional biomass, TAG and

storage carbohydrates. The theoretical yields of TAG (1.33 g TAG mol⁻¹ photon), storage carbohydrates (3.24 g storage carbohydrates mol⁻¹ photon) and functional biomass (1.63 g biomass mol⁻¹ photon) on photons were derived using a simplified metabolic flux model (Breuer et al., 2015a; Kliphuis et al., 2012).

Photosynthetic conversion efficiency (%)

$$= \frac{\frac{r_{\text{TAG}}}{1.33} + \frac{r_{\text{carbohydrates}}}{3.24} + \frac{r_{\text{functional biomass}}}{1.63}}{r_{\text{ph}}} \cdot 100 \quad (\text{Eq. 3.3})$$

To study diurnal fluctuations, each culture was also sampled directly from the culture broth during a 24h cycle with 5-6 hour intervals (Figure 3.1B). These samples were analysed for biomass composition (e.g., TAG, membrane lipids, protein, total carbohydrates, ash) and for transcriptome, proteome and metabolome analysis. Biomass for transcriptome, proteome and metabolome analysis was immediately centrifuged (4200rpm, 1 min, 0°C, supernatant discarded), flash frozen in liquid nitrogen and kept at -80°C until analysis. Pellets for metabolomics analysis were freeze-dried prior to metabolite extraction.

3.2.4 Transcriptomics

RNA isolation and sequencing

Pelleted algae cultures were homogenized in liquid nitrogen by grinding. The ruptured cells were incubated in 400 µl TriPure (Roche) for 30 minutes on a rotor at RT. Subsequently, total RNA was isolated using Direct-Zol RNA Miniprep kit (Zymo Research) according to the manufacturer's instructions. Finally the RNA was eluted from the column in 2 steps with 50 µl DNase/RNase-free water. To check the RNA quality, approximately 10 µl was loaded on 1.5% agarose gel. Yield and purity were subsequently analysed by determining OD260/230 ratio using Nanodrop (Thermo Scientific). The polyA-RNA was recovered using an oligo-dT beads capture (Tru-Seq, Illumina). The RNA was converted into cDNA from which paired end sequence libraries were constructed that were subsequently sequenced with Illumina HiSeq 2500 sequence technology according to protocols provided by Illumina.

RNA transcriptome, differential expression and gene annotation

The re-annotated reference genome for *P. tricornutum* JGI genebuild (Ensembl build 29, 2015) was retrieved from EnsemblProtist (http://protists.ensembl.org/Phaeodactylum_tricornutum/Info/Index). To determine RNA expression levels of nitrogen replete (N+) and nitrogen limited samples (N-), RNA-seq data were mapped against the annotated reference genome with CLC Genomics Server 7.5.1 software (<http://CLCbio.com>) using the following parameter settings; Mapping type = Map to gene regions only (fast mode), Mismatch cost = 2, Insertion cost = 3, Deletion cost = 3, Length fraction = 0.9, Similarity fraction = 0.8, Global alignment = No, Auto-detect paired distances = Yes, Strand specific = Both, Maximum number of hits for a read = 10, Count paired reads as two = No, Expression value = RPKM, Calculate RPKM for genes without transcripts = No, Create report = Yes, and Create fusion gene table = No, Create list of unmapped reads = Yes.

Subsequently, a multigroup experiment for sample sets was designed and compared for N replete (N+; control) versus nitrogen limitation (N-) at t=0, and N+ versus N- at t=10 using CLC Genomics Workbench 9.5.3 software. Original expression data was normalized using Quantile normalization. Subsequently, the normalized expression values were corrected for multiple testing using false discovery rate (FDR) correction. The following additional parameter settings were used for differential gene expression analysis in CLC; Total count filter cutoff = 5.0, Estimate tagwise dispersions = Yes.

Gene ontology annotation were retrieved with the ensembl REST api <http://rest.ensemblgenomes.org>. Additional annotation was based on protein sequences by extracting coding sequences from the annotated reference genome from *P. tricornutum* in CLC. Briefly, 11522 CDS sequences with an ATG start codon from a total of 12178 extracted elements were translated into proteins using a standard translation table. Next, predictive information on proteins was retrieved using InterProScan 5.16-55.0 screening against ProDom, HAMAP, Panther, SMART, SuperFamily, PRINTS, PIRSF, Pfam, Gene3d, Coils, TIGRFAM, and PrositePatterns databases. Subsequently, KEGG identifiers and additional gene ontology annotations were retrieved from Interproscan XML output.

3.2.5 Proteomics

Pelleted diatom cultures were lysed by addition of 1 ml of chloroform:MeOH (1:2 v/v) and homogenised by vigorous shaking in FastPrep (2x 20 sec, position 5). After centrifugation (16,000 g, 10min at RT) the supernatant was removed. This homogenisation and extraction was repeated 2 more times. Pellet material was dried in a vacuum centrifuge (SpeedVac) and was subsequently resuspended in 0.5 ml of 8 M urea, 5 mM DTT in 0.1 M ammonium bicarbonate pH7 (ABC) with vigorous shaking in FastPrep (2 times 20 sec, position 5) and 9hr rotating incubation at 37 °C. Iodoacetamide was added (15 µl of a 0.5 M stock) for alkylation of free sulfhydryl groups (cysteine and quenching of DTT, overnight incubation). After centrifugation (16,000 g, 10min at RT) the supernatant was transferred to a fresh Eppendorf tube. The pellet was re-extracted twice with 0.5 ml 0.1 M ABC. The supernatants were pooled with the first (urea) extract. Protein content was determined by Qubit Fluorometer protein assay (ThermoFisher scientific, product Q33211). An aliquot equivalent to 50 µg of protein was taken and diluted again with equal volume of 0.1M ABC before 0.5 µg trypsin (Promega Proteomics grade) was added. Digestion was incubated overnight at 37 °C and stopped by adding 3 µl 10% trifluoro-acetic acid. Peptides were purified by solid-phase extraction (SPE) in Waters OASIS HLB micro-elution plates. Eluted peptides were dried by SpeedVac centrifugation and dissolved in 2% acetonitrile, 20 mM ammonium formate pH10. Approximately 1 µg aliquot was injected on a 2D-nanoAcquity LC system with 2 step peptide capture and elution in four subsequent fractions. In short, peptides were first loaded onto an XBridge Peptide BEH C18 NanoEase column and subsequently eluted with steps of 15%, 19%, 25% and 65% acetonitrile in 20mM ammonium formate pH10, on-line diluted 10 fold with 2% acetonitrile in 0.1% formic acid (pH2), and peptides were trapped on a second trap column nanoACQUITY HSS T3 (1.8 µm particles; 2cm x 100 µm). Subsequently, per fraction an elution gradient was performed over analytical nanoACQUITY HSS T3 column (1.8 µm particles; 15 cm x 75µm) using a 120 min gradient with separation between 5% to 40% acetonitrile in 100 min followed by a steep gradient to 80% for washing and return to 2% acetonitrile, all at a flow rate of 300 µl/min. Eluting peptides were on-line injected to a Q-Exactive Plus FTMS

instrument (ThermoFisher Scientific). The Q-Exactive was operated in Data Dependent Analysis (DDA) positive ESI mode for automated MS/MS acquisition. Settings were: MS1: resolution=70k, AGC target=3e6, MaxIT=50mS and scan range 400-1500 m/z.; MS2: resolution=35k, AGC target=1e5, MaxIT=110mS, loop count =8, ion isolation width 1.6 m/z no offset, NCE=28, centroid mode and automatic scan range from 140 m/z upwards. Charge exclusion was set to include only 2, 3 and 4+ charged ions, preferred peptide match, excluded isotopes and dynamic exclusion during 30sec.

LC-MS/MS raw data files were processed using MaxQuant (version 1.5.3.) using carbamidomethylation of cysteine as fixed and oxidized methionine as variable modifications. Searches were performed with the proteome equivalent of *P. tricornutum* JGI genebuild (Phatr3 Ensembl build 29, 2015) retrieved from EnsemblProtist

(http://protists.ensembl.org/Phaeodactylum_tricornutum/Info/Index). During processing, the data were re-calibrated, aligned, matched between runs and normalised using the LFQ quantitation algorithm (Cox et al., 2014; Cox and Mann, 2008). The resulting ProteinTable (Supplemental digital file 3.2) was loaded, filtered and visualised using Perseus software (Tyanova et al., 2015).

3.2.6 Metabolomics

Metabolite extraction

Lipid-soluble, apolar compounds were extracted by mixing 25 mg freeze-dried algae with 4.6 ml of methanol/chloroform (1:1 v/v) containing 0.1% (w/v) butylhydroxytoluene as antioxidant and 10 µM of 1,2-didecanoyl-sn-glycero-3-phosphocholine as internal standard in a 10 ml glass tube. The mixture was sonicated for 15 min, crushed with a glass rod and again sonicated for 15 min. After centrifugation, 400 µl of the supernatant was transferred to an Eppendorf tube, dried in a speed-vac and redissolved in 1 ml of ethanol, after which the sample was sonicated for 5 min and centrifuged once more before transferring to an HPLC glass vial for LCMS analysis of extracted apolar compounds.

Polar compounds were extracted using a protocol similarly as described by Carreno-Quintero et al. (2012). In brief, 15 mg of freeze-dried algae were mixed 210 μ l of 80% methanol, containing ribitol (32 ng μ l⁻¹) as an internal standard, in an Eppendorf tube. Samples were vortexed, sonicated for 15 min and centrifuged, after which 125 μ l was transferred into a new Eppendorf tube containing 188 μ l ice-cold H₂O and 94 μ l ice-cold chloroform. After vortexing, sonication and centrifugation, 50 μ l of the upper polar phase was taken and dried in a speed-vac before derivatization and analysis by GCMS.

Untargeted metabolite profiling

Apolar compounds present in the crude methanol/chloroform extracts were detected by high resolution LCMS using an Acquity UPLC (Waters) connected to an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo). A Waters Acquity HSS T3 column (1.8 μ m particles; 2.1 x 150 mm) at 55°C was used to separate compounds. A solvent gradient of 20% acetonitrile in water containing 10 mM ammoniumacetate and 0.1% formic acid (eluent A) and 10% acetonitrile in isopropanol containing 10 mM ammoniumacetate and 0.1% formic acid (eluent B) was used, starting with an increase from 35-70% B in 3 min, then from 70-85% B in 6 min and from 85-90% B in 13 min, followed by a washing step at 90% B for another 5 min; the flow rate was 200 μ l/min. Masses were recorded at a resolution of 60,000 in positive electrospray ionization mode and in the m/z range of 112-1400. Mass calibration and other settings of the LTQ-Orbitrap were as described by (Hooft et al., 2012).

Polar compounds were analysed by GCMS after automated TMS-derivatization of the dried polar extract, as described by Carreno-Quintero et al. (2012). A series of alkanes (C10-C32) was automatically added to each sample in order to calculate retention indices of all eluting compounds using a third order polynomial function.

LCMS and GCMS data processing

Raw mass spectrometry data were processed in an essentially untargeted manner using an in-house developed dedicated metabolomics workflow, which consists of applying MetAlign software (Lommen and Kools, 2012) for noise estimation, peak picking and alignment, selecting for reproducible signals, i.e. being present in at least

3 samples with an intensity of at least 15 times the local noise, and MSClust software (Tikunov et al., 2012) to cluster all mass signals originating from the same metabolite, including fragments, adducts and isotopes. The resulting metabolite tables, containing the relative signal intensity of each compound detected in each sample analysed, was corrected for variation in the internal standards and used for statistical analyses after log2 transformation of metabolite intensities (Supplemental digital file 3.3). The identification of selected polar metabolites was based on comparison of their mass spectrum and retention index with those of authentic reference standards and of commercial and in-house mass spectral libraries. Annotation of selected apolar compounds from the LCMS analysis was performed by matching the observed accurate mass of the molecular ions with available metabolite databases, including those provided by LipidMaps (<http://www.lipidmaps.org>) and the Human Metabolite Database (<http://www.hmdb.ca>), within a mass deviation of 3 ppm.

3.2.7 Statistical analysis of omics

Principal Component Analyses (PCA) of omics datasets were performed using SIMCA-P version 14.0 (MKS solutions, Umea, Sweden). Protein and transcriptome data were merged based on the gene identifiers from Phatr3 Ensembl and analysed in Perseus software (Tyanova et al., 2015). Genes, proteins and metabolites that met the criterion of an FDR corrected p-value < 0.05 were considered to be significantly differentially regulated in nitrogen limited conditions in either the light or dark period. Differential expressed genes and proteins were mapped to KEGG pathways using a tool to map multiple KEGG identifiers to the pathway (MapOmics, access to the test version can be obtained from linda.bakker@wur.nl). Pathway p-values were calculated according to Equation 3.4 where K equals the unique features known in this pathway, k for the searched features uniquely mapped on this pathway, N as the unique features known in all pathways and n for the searched features uniquely mapped on all pathways.

$$P(X = k) = f(k; N, K, n) = \frac{\binom{K}{k} \binom{N-K}{n-k}}{\binom{N}{n}} \quad (\text{Eq. 3.4})$$

3.3 Results and discussion

3.3.1 Effect of nitrogen limitation on physiological responses in *Phaeodactylum tricornutum*

Nitrogen limitation was induced in continuous, turbidostat controlled reactors with a separate continuous nitrogen supply as described by Remmers et al. (2017a). Turbidostat control ensures a fixed light absorption over the culture throughout the entire experiment, because the in- and out-coming light intensity are kept constant during the light period through automated dilution of the culture. The control culture was exposed to an excess of nitrogen, meaning that growth was limited by the supplied light. The nitrogen limited cultures were exposed to a constant, but limiting nitrogen supply. This turbidostat-nutristat approach allowed for simultaneous growth and lipid production (Klok et al., 2013; Remmers et al., 2017a). Diurnal cycles of 16 hours of light and 8 hours of dark were applied to the cultures, and, as a consequence, 24h repeating diurnal oscillations in dilution rate and biomass concentration were observed. Average values for the relevant physiological parameters (Table 3.1) were determined on the culture overflow, which was collected on ice over 24 hours.

Under nitrogen replete conditions, i.e. when nitrate is not limiting, an average growth rate (i.e. dilution rate) of $0.42 \pm 0.02 \text{ day}^{-1}$ and biomass concentration of $1.60 \pm 0.13 \text{ g L}^{-1}$ was found, while approximately 57% of the absorbed photons was used to produce biomass (Table 3.1 and Figure 3.2B). The dry matter of nitrogen replete cells consists primarily of proteins (57% w/w), carbohydrates (13% w/w), membrane lipids (ML; 7% w/w), triacylglycerol (TAG; 3% w/w) and ash (10% w/w) (Figure 3.2A). Other studies found similar biomass compositions for nitrogen replete cultures of *P. tricornutum* (Breuer et al., 2012; Matilde Skogen Chauton et al., 2013; Mirón et al., 2003), highlighting the reliability of our applied cultivation method.

Table 3.1 – Physiological parameters of continuous cultures of *P. tricornutum* under nitrogen replete and nitrogen limited growth conditions and exposed to 16:8h LD cycles. The values represent the mean and standard deviation of 24h-collected culture overflow of four biological replicate experiments (N=4) for each tested condition.

	N replete	N limitation
Dilution rate (day ⁻¹)	0.42±0.02	0.11±0.02
Biomass concentration (g L ⁻¹)	1.60±0.13	2.28±0.10
Biomass productivity (g L ⁻¹ day ⁻¹)	0.68±0.07	0.25±0.04
Biomass yield on light (g mol _{ph} ⁻¹)	0.95±0.09	0.35±0.05
Absorption cross section (kg m ⁻²)	0.16±0.02	0.09±0.01
Photosynthetic conversion efficiency (% of absorbed photons used for biomass production)	58±3	20±1
TAG content (% of dry weight)	3±1	16±1
TAG productivity (g L ⁻¹ day ⁻¹)	0.02±0.00	0.04±0.01
TAG yield on light (g mol _{ph} ⁻¹)	0.03±0.01	0.06±0.01

When the culture was subjected to nitrogen limitation, the pigmentation (i.e. absorption cross section) decreased with a consequent turbidostat-mediated increase in biomass concentration to 2.28±0.10 g L⁻¹. Simultaneously, the specific growth rate decreased to 0.11 ± 0.02 day⁻¹, leading to a 3 fold decrease in both biomass productivity and yield on light for the nitrogen limited cultures as compared to the nitrogen replete cultures. Under nitrogen limitation, only 20% of the absorbed photons were used to form biomass (Figure 3.2B). However, the TAG productivity and TAG yield on light increased more than two-fold upon nitrogen limitation as reflected in the changed biomass composition. The relative TAG (16% w/w) and storage carbohydrate (25% w/w) content increased at the expense of protein (38% w/w) and membrane lipids (5% w/w) (Figure 3.2A). With our turbidostat-nutristat approach, we were able to induce TAG accumulation in continuous growing cultures under a daily repeating light:dark (LD) cycle. Both conditions showed steady state oscillating patterns synchronized to the LD cycle. The oscillations were very reproducible, both between the six days of each single steady state and between the four biological replicates of each condition (e.g., nitrogen replete and limited). This allows for an

accurate determination of the transcriptome, proteome and metabolome in each condition on different time points of the LD cycle.

Figure 3.2 – Mass and photon distribution for nitrogen replete and nitrogen limited continuous cultures of *P. tricornutum*. A: Macromolecular biomass composition in % of dry weight (w/w) for nitrogen replete (left; control) and nitrogen limited (right) cultures. B: Photon conversion efficiency into biomass. The values represent the mean of biological replicates (N=4). Functional biomass consists of membrane lipids (ML), carbohydrates, proteins, ash and others. The increase in carbohydrate productivity upon nitrogen limitation is considered as additional storage carbohydrate accumulation.

3.3.2 Physiological response during diurnal light:dark cycle

The cultures were synchronized to diurnal LD cycles (16h of light and 8h of dark), which resulted in differences in dilution rate (i.e. growth) throughout a daily 24h cycle (Figure 3.3). A clear peak in culture dilution rate, equivalent to specific growth rate due to the observed constant biomass concentration, was found around mid-day (i.e. 6-10h after switching on the light) for both nitrogen replete and nitrogen limited conditions. Similar patterns were reported for continuous turbidostat cultures of

Neochloris and *Acutodesmus* (de Winter et al., 2014; León-Saiki et al., 2017). The biomass composition remained constant over 24h for both treatments, except for the total carbohydrate fraction in nitrogen replete cultivations (Figure 3.3). The total carbohydrate fraction increased significantly and specifically during the last 6 hours of the light period: from 12% at t=10 to 18% at t=16 h of light Figure 3.3B). Similar findings for carbohydrate profiles were found in continuous nitrogen replete cultures of *Neochloris oleoabundans* (de Winter et al., 2014). Both TAG and carbohydrates were significantly higher in the nitrogen limited cultures as compared to the nitrogen replete cultures, throughout the LD cycle. This result indicates that the largest differences in biomass composition were found between the treatments (nitrogen limitation versus nitrogen replete), whereas only small or no diurnal effects were observed on biomass production.

Figure 3.3 – Dilution rate and biomass composition (TAG and total carbohydrate [CHO]) of nitrogen replete (closed symbols) and nitrogen limited (open symbols) cultures of *P. tricornutum*. The values represent the mean of four biological replicates (N=4) after 6 consecutive days of ‘stable’ steady state conditions. TAG is shown in squares and total carbohydrates (CHO) in diamonds. The shaded areas indicate the dark period.

3.3.3 Differential expression of transcripts, proteins and metabolites due to nitrogen limitation

In this study we analysed in total 12179 transcripts, 3171 proteins and 779 metabolites. Similar to the macromolecular biomass composition (Table 3.1 and

Figure 3.2), the strongest effects in the transcriptome, the proteome and the metabolome were related to the two nitrogen treatments (Supplemental file 3.6). Principal component analysis showed distinct separate clusters of the nitrogen limited samples and the nitrogen replete samples on the first principle component (PC) explaining 28.7%, 27.8% and 54.4% of the total variance in the transcriptome, proteome and metabolome, respectively (Supplemental file 3.6). Interestingly, only the transcriptome data showed a clear LD effect (second PC, explaining 18.2% of the total variance): here all samples taken in the light period (5, 10, or 16h of illumination) are separated from samples taken after the 8h of darkness (0h of illumination). Therefore, we focussed our further data analyses on the overall differences caused by nitrogen supply as well as the differences between dark and light (i.e. 0 versus 10h of illumination).

Statistical analysis showed that 22% (2699) of the transcripts, 17% (543) of the proteins and 44% (345) of the metabolites in our dataset were differentially expressed under nitrogen limitation compared to the nitrogen replete culture (N=4 for both nitrogen replete and limited conditions, FDR corrected). These differentially expressed genes, proteins and metabolites were subsequently compared for their expression profiles in light and dark. Interestingly, the Venn diagrams (Figure 3.4) indicate that 22% of these genes, 37% of these proteins and 70% of these metabolites were differentially regulated during both time points of the day (e.g., differentially up- or down regulated in both the dark and light period). Thus, a large part of the nitrogen-regulated genes, proteins and metabolites were differentially expressed over the light-dark cycle, thereby highlighting that this LD cycle has a significant influence on the nitrogen-regulated biomolecules (transcripts, proteins and metabolites) in *P. tricornutum*. Figure 3.4 further shows that, despite the strong diurnal regulation in both transcriptome and proteome, the majority of the significant differential expressed metabolites under nitrogen limitation only show limited diurnal regulation. Diurnal transcript and translational regulation, thus, does not automatically lead to diurnal physiological changes.

Figure 3.4 – Effect of nitrogen limitation on the number of differential expressed genes, proteins and metabolites. Differential expression was calculated by comparing the average expression values for nitrogen limited growth to nitrogen replete growth. The sample set was further separated for both light and dark periods to illustrate the light-independent nitrogen-regulated differential expression (grey zone) and light-dependent nitrogen-regulated differential expression in either the dark or light period of the day. Upward arrows indicate upregulation of genes, proteins or metabolites under nitrogen limitation, whereas downwards arrows indicate downregulation under nitrogen limitation, as compared to nitrogen replete conditions.

3.3.4 Pathway enrichment

1147 out of 12179 identified transcripts (9.4%) and 731 out of identified 3171 proteins (23%) could be mapped onto KEGG pathways. Due the limited possibility to annotate *P. tricornutum* transcripts with KEGG, 340 out of the 2699 (13%) differential nitrogen-responsive transcripts and 158 out of the 543 (29%) differential nitrogen-responsive proteins could be mapped onto KEGG pathways (Figure 3.5, Supplementary file 3.7). Most distinct differences occurred in pathways linked to photosynthesis, nitrogen fixation, carbon metabolism, amino acid metabolism and lipid metabolism (Figure 3.5). Since the metabolomics data were primarily produced in an untargeted manner, annotation was performed manually for a selected set of the most significantly affected compounds only. Unfortunately, for only a few differential compounds a KEGG identifier could be retrieved and mapped onto KEGG pathways, so metabolomics data could not be included in the pathway analysis of Figure 3.5 (data available in supplemental file 3.3).

Figure 3.5 – KEGG pathway analysis for the transcript (A) and proteome (B) dataset. Colour bars indicate the percentage of genes or proteins within each pathway that are significant differentially expressed under nitrogen limited growth conditions in both light and dark period. Differential up-regulation is shown in green, no differential expression is shown in grey and differential down-regulation is shown in red. KEGG pathways are listed in random order with the total number of annotated genes or proteins in the KEGG database in brackets.

Figure 3.5 and Supplemental file 3.8 show some minor discord in the regulation of transcript and proteome expression. The discrepancy between transcript, protein and/or metabolite levels has also previously been reported (Longworth et al., 2016; Rogers et al., 2008) and can be explained by the fact that there is always a time delay between changes in gene expression and changes in protein content (Waldbauer et al., 2012). We could, however, not detect stronger correlations between transcript and proteins levels when taking the time interval of our samples (i.e. 0h and 10h of illumination) into account. Alternatively, the discord could have also been caused by regulation on the translation of messenger-RNA into proteins. In either case, to fully understand microalgal metabolism, a comprehensive multi-omics study including proteomics is essential.

3.3.5 Nitrogen limitation affects a range of biochemical pathways

Photosynthesis

Under nitrogen limited, turbidostat controlled, conditions, the cells are exposed to an energy imbalance between supply of light and the demand by cellular metabolism and growth. Transcriptome and proteome analysis showed that the majority of genes and proteins associated to photosynthesis are significantly downregulated under nitrogen limitation for both the dark and light period (Table 3.2). In line with this observation, chlorophyll levels were lowered (data not shown). The observed down-regulation of genes and proteins associated to photosynthesis is in accordance with previous studies on both the transcriptome and proteome of nitrogen-deficient cultures of *P. tricornutum* and other microalgae (Alipanah et al., 2015; Levitan et al., 2015; Longworth et al., 2016; Shang et al., 2017).

An energy imbalance due to overexposure to light, also under nitrogen limited conditions, can result in photo-damage (Domingues et al., 2012; Erickson et al., 2015; Remmers et al., 2017b). It is thus likely that nitrogen limited cells prevent light-induced damage by downregulating their photosynthetic machinery. Additionally, the down-regulation of biosynthesis of photosynthetic proteins may preserve

nitrogen for other vital metabolic processes in times of nitrogen scarcity (Longworth et al., 2016; Nie et al., 2007). Similar observations were done for nitrogen-limited shake flask cultures of both *P. tricornutum* and *Dunaliella parva* (Bai et al., 2016; Longworth et al., 2016; Shang et al., 2017).

Table 3.2 – Differential expression of photosynthesis-related genes and proteins for nitrogen limited cultures of *P. tricornutum* compared to nitrogen replete growth . The values refer to the fold change in nitrogen limited cultures in the dark period (after 8h darkness) or the light period (after 10h of illumination) compared to nitrogen replete cultures that were sampled at identical time points. NDE represents annotated transcripts or proteins that were not significantly differentially expressed. A hyphen indicates that the transcript or protein was not found in the dataset. Full descriptions of the annotations, fold-changes and FDR-corrected p-values can be found in the Supplemental digital file 3.1 and 3.2.

Cluster	Phatr3 Identifier	Fold change			
		Transcriptome		Proteome	
		Dark	Light	Dark	Light
Photosystem II	Phatr3_J48359	-1.66	-3.65	-1.23	-1.41
Photosystem II	Phatr3_J20331	NDE	NDE	NDE	-0.77
Photosystem II	Phatr3_J26293	-3.60	NDE	NDE	NDE
Photosystem II	Phatr3_J55057	-3.67	-1.90	-	-
Photosystem II	Phatr3_J9078	-4.06	-3.00	NDE	NDE
Photosystem II	Phatr3_J54499	-3.23	NDE	NDE	NDE
Cytochrome b6f complex	Phatr3_J13358	NDE	NDE	NDE	NDE
Cytochrome b6f complex	Phatr3_J46657	NDE	NDE	-	-
Photosynthetic electron transport	Phatr3_J44056	-9.20	-2.08	NDE	NDE
Ferredoxin-NADP ⁺ reductase	Phatr3_EG02321	-1.39	NDE	-	-
Ferredoxin-NADP ⁺ reductase	Phatr3_J15777	1.54	NDE	NDE	NDE
Ferredoxin-NADP ⁺ reductase	Phatr3_J23717	-3.22	-2.77	NDE	-0.52
Ferredoxin-NADP ⁺ reductase	Phatr3_J42018	NDE	-2.68	NDE	NDE
ATP-ase	Phatr3_J20657	NDE	-2.49	-0.92	-0.65

Nitrogen metabolism

In response to nitrogen limitation, most nitrogen metabolism-related transcripts and proteins were upregulated (Figure 3.6). Acquisition of nitrogen is essential for growth and maintenance in microalgae and is, especially for nitrate, energetically expensive

requiring both ATP and NAD(P)H (Lambers et al., 2013). Therefore, it is thus likely that the nitrogen assimilation pathway is upregulated under nitrogen scarcity. Interestingly, we noticed a strong repression (-39.4 fold) in the transcript and protein encoding ferredoxin-nitrite reductase (EC 1.7.7.1, Phatr3_J12902) in the nitrogen limited culture after 8 hours of darkness when compared to nitrogen replete conditions. This observation disagrees with a transcriptome study on the nitrogen-starved microalga *Neochloris oleoabundans* (Rismani-Yazdi et al., 2012), where it was found that all genes related to nitrogen assimilation were upregulated. Interestingly, Smith et al. (2016) observed strong diurnal fluctuations in genes related to nitrogen assimilation in both iron replete and starved cultures of *P. tricornutum*. They found hardly any effect of iron deficiency on nitrogen assimilation genes, and it was thus proposed that nitrogen assimilation is strongly affected and regulated by the photoperiod. Further research should focus on understanding diurnal nitrogen assimilation in both nitrogen replete and limited cultures.

Figure 3.6 – Differential expression of nitrogen metabolism related genes (T) and proteins (P) for nitrogen limited cultures of *P. tricornutum* compared to nitrogen replete growth. The values in boxes illustrate the fold change in nitrogen limited cultures in either the dark period (after 8h darkness) or the light period (after 10h of illumination) compared to nitrogen replete cultures that were sampled at identical time points. NDE represents annotated transcripts or proteins that were not significantly differentially expressed. An empty box indicates that the transcript or protein was not found in the dataset. Full descriptions of the annotations, fold-changes and FDR-corrected p-values can be found in the Supplemental digital file 3.1 and 3.2.

Simultaneously, nitrogen limitation set a global constraint on the translation, DNA replication and amino acid biosynthesis (Figure 3.5). Corresponding to the altered

transcript and protein expression related to amino acid biosynthesis, the levels of several free amino acids are significantly decreased under nitrogen limitation (such as lysine, glycine, glutamic acid, proline, asparagine, threonine, serine and proline; Supplemental digital file 3.3). Downregulation of ribosome assembly and translation are often the first and most drastic responses of cells when they experience stress such as nutrient limitation (Longworth et al., 2016; Schmollinger et al., 2014; Smith et al., 2016). All changes in nitrogen metabolism and photosynthesis can be directly related to the reduced growth rate under nitrogen limitation (Table 3.1).

Primary carbon metabolism

Glycolysis is a key primary pathway as it provides substrate for energy production in the cell. In the glycolysis, glyceraldehyde 3-phosphate (G3P) is converted into pyruvate, which can be further converted into acetyl-CoA, the precursor of fatty acids. Most differentially expressed transcripts and proteins were upregulated under nitrogen limitation. Possibly, this is a response to the reduction in photon conversion efficiency, and thus the flux through the central carbon pathways, under nitrogen limitation. By upregulation of pathways related to primary carbon metabolism, the cells might attempt to compensate for the shortage of carbon for downstream metabolism.

Some redundancy in transcript expression was found, where multiple transcripts encoding for an enzyme with the same function (i.e. isozymes) show opposite regulation (Figure 3.7). Similar to our work, Smith et al. (2016) also showed opposite regulation of isozymes associated to glycolysis/gluconeogenesis. Our results suggest that proteins that catalyse the same reaction can either have different kinetics or require expression in a different compartment.

Figure 3.7 – Differential expression of central carbon metabolism related genes (T) and proteins (P) for nitrogen limited cultures of *P. tricornutum* compared to nitrogen replete growth. The values refer to the fold change in nitrogen limited cultures in the dark period (after 8h darkness) or in the light period (after 10h of illumination) compared to nitrogen replete cultures that were sampled at identical time points. NDE represents annotated transcripts or proteins that were not significantly differentially expressed. An empty box indicates that the transcript or protein was not found in the dataset. Red arrows illustrate that the corresponding gene was not annotated in the KEGG database. Full descriptions of the annotations, fold-changes and FDR-corrected p-values can be found in the Supplemental digital file 3.1 and 3.2.

Most annotated transcripts and proteins associated to the TCA cycle were upregulated upon nitrogen limitation (Figure 3.7), conform earlier studies (Alipanah et al., 2015; Levitan et al., 2015; Shang et al., 2017). Despite the upregulation of transcript and protein expression under nitrogen limitation, most of the TCA intermediate metabolites were not differentially regulated (Supplemental digital file 3.3). As mentioned earlier, the biomass productivity (or photosynthetic conversion efficiency) is reduced under nitrogen limitation (Table 3.1 and Figure 3.2), which points towards a reduced net carbon flux through central carbon metabolism. As more net carbon is directed towards TAG under nitrogen deprivation (Figure 3.2B), the flux through(at least part of) the TCA cycle is increased for the production of the precursor of fatty acids and lipids: acetyl-CoA (Ho et al., 2015; Levitan et al., 2015).

Lipid metabolism

When exposed to nitrogen deprived growth conditions, microalgae start to accumulate TAG (Figure 3.8, Supplemental digital file 3.3). In accordance, the gene encoding for diacylglycerol O-acyltransferase (DGAT; Phatr3_9794) was 1.6-fold upregulated under nitrogen limited conditions (Figure 3.8). DGAT is responsible for the last step from diacylglycerols (DAG) to TAG and is regarded as a promising target for improving TAG content in microalgae. For example, Niu et al (2013) showed that overexpression of DGAT in *P. tricornutum* resulted in 35% higher TAG levels. Interestingly, in our study the DGAT gene (Phatr3_9794) was only upregulated by nitrogen limitation after the dark period and not during the light period. The TAG content was not significantly changed at the end of the dark period (Figure 3.3 and Supplemental digital file 3.3), however, suggesting no direct relation to DGAT activity and TAG levels.

Contradicting with other studies (Ge et al., 2014; Longworth et al., 2016; Park et al., 2015), we did not observe a significant change in either transcript or protein expression for Acyl-CoA synthase (ACC1; Phatr3_J55209), the enzyme that catalyses the reaction from Acetyl-CoA and Malonyl-CoA to form long-chain acyl-CoA molecules. Moreover, under nitrogen limitation most lipid pathway genes were downregulated, while protein levels associated to fatty acid biosynthesis did not

differ from the nitrogen replete condition (Figure 3.8). In correspondence to our results, a transcriptome study on nitrogen deprived *Nannochloropsis gaditana* also showed generally unchanged fatty acid biosynthesis gene expression levels (Corteggiani Carpinelli et al., 2014).

It is often hypothesized that the assembly of membrane lipids is downregulated under nitrogen starvation (Abida et al., 2015; Goncalves et al., 2013; Levitan et al., 2015). Coherently, we observed a clear downregulation of metabolites related to membrane lipid metabolism, e.g., a 5 fold decrease for two species of phosphatidylinositol (38:6; total acyl carbons:total double bonds), a 2 fold decrease of diacylglycerol (32:3) and a 2-fold decrease of a phosphatidic acid-type of glycerophospholipid (40:4) (Supplemental digital file 3.3). In accordance, pathways related to membrane lipids (glycerolipid and glycerophospholipid metabolism) are mostly downregulated at both gene and protein expression level (Figure 3.8). Similar findings are reported in a proteome study of nitrogen-starved *P. tricornutum* (Longworth et al., 2016).

Even though a substantial increase in the content and productivity of TAG was found (Table 3.1 and Supplemental digital file 3.3), the overall fatty acid productivity was decreased under nitrogen limitation (66 ± 1 mg TFA L⁻¹ day⁻¹ for nitrogen replete conditions versus 51 ± 2 mg TFA L⁻¹ day⁻¹ for nitrogen limited conditions). In accordance, the genes and proteins relation to conversion of acyl-CoA into fatty acids was downregulated. We have also observed a strong downregulation in pathways related to the synthesis of membrane lipids (e.g., MGDG) and upregulation of the DGAT, the last concomitant step in TAG biosynthesis (Figure 3.8). Thus, this regulation supports the reduced membrane synthesis rate and strong channelling of fatty acids to TAG instead of membrane lipids under nitrogen limitation (Figure 3.2). DGAT is therefore an important target for improving TAG productivity, as also shown in a study by Niu et al. (2013). Overexpression of the entire acyl-CoA – fatty acid pathway might also assist in increasing TAG productivity, as the whole pathway is currently downregulated. In addition, TAG biosynthesis is clearly limited by the reduced carbon flux through central metabolism. Future strain improvement should

therefore not only focus on lipid metabolism, but also on improving the carbon flux through central carbon metabolism.

Figure 3.8 – Differential expression of genes (T) and proteins (P) involved in glycerolipid metabolism for nitrogen limited cultures of *P. tricornutum*. The values in boxes illustrate the fold change in nitrogen limited cultures in either the dark period (after 8h darkness) or the light period (after 10h of illumination) compared to nitrogen replete cultures that were sampled at identical time points. NDE represents annotated transcripts or proteins that were not significantly differentially expressed. An empty box indicates that the transcript or protein was not found in the dataset. Full descriptions of the annotations, fold-changes and FDR-corrected p-values can be found in the Supplemental digital file 3.1 and 3.2.

3.4 Conclusion

This study reveals, for the first time, the combined transcriptome, proteome, and metabolome of the diatom *P. tricornutum* under nitrogen limited (TAG accumulating) cultivation conditions. Nitrogen limitation resulted in a 5-6 fold increase in TAG content, but with a concomitant loss in growth rate and photosynthetic efficiency. Though nitrogen limitation was responsible for the majority of differentially expressed transcripts, protein and metabolites, we also observed substantial differential gene expression over a diurnal cycle. Global expression data showed that nitrogen limitation in *P. tricornutum* suppresses photosynthesis, ribosomal and protein synthesis. On the contrary, pathways involved in nitrogen fixation and central carbon metabolism (glycolysis and TCA cycle) were mainly upregulated. Regulation of lipid metabolism suggests that overexpression of DGAT and the entire acyl-CoA – fatty acid pathway might be an important target for improving TAG productivity. However, TAG biosynthesis is also clearly limited by the reduced carbon flux through central metabolism. Future strain improvement should therefore not only focus on lipid metabolism, but also on improving the carbon flux through central carbon metabolism.

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Digital material – Untill publication, all supplemental digital files can be downloaded from: <http://bit.ly/2qs0obO>

Supplemental digital file 3.1: Transcriptomics.xls

Supplemental digital file 3.2: Proteomics.xls

Supplemental digital file 3.3: Metabolomics.xls

Supplemental digital file 3.4: Pathway counts.xls

Supplemental file 3.5 – Relative fatty acid composition of TAG and membrane lipids

Table S3.1 – Average fatty acid composition of *P. tricornutum* in the TAG and ML lipid fraction for nitrogen replete and nitrogen limited continuous growth conditions. Values are averaged over multiple days of 4 independent reactor runs. Standard deviations are shown between brackets.

	% of fatty acids in TAG fraction		% of fatty acids in ML fraction	
	Nitrogen replete	Nitrogen limited	Nitrogen replete	Nitrogen limited
C14:0	6.0 (0.5)	5.4 (0.3)	7.0 (0.3)	7.5 (0.4)
C16:0	34.8 (5.2)	28.3 (1.3)	18.3 (0.3)	16.0 (0.8)
C16:1	22.9 (7.2)	49.8 (1.4)	30.8 (1.3)	24.7 (0.8)
C16:2	1.4 (0.7)	1.0 (0.2)	2.2 (0.5)	4.5 (0.3)
C16:3	3.3 (2.6)	1.2 (0.3)	8.4 (0.6)	11.3 (0.9)
C18:0	1.7 (0.3)	1.2 (0.1)	1.2 (0.3)	0.6 (0.1)
C18:1	16.8 (8.0)	4.7 (0.4)	2.1 (0.3)	1.2 (0.1)
C18:2w6	0.7 (0.3)	1.2 (0.1)	0.9 (0.2)	0.7 (0.2)
C18:3w3	2.1 (0.5)	1.0 (0.2)	0.7 (0.2)	1.0 (0.3)
C20:5-n3	10.4 (3.0)	5.9 (0.1)	28.4 (1.1)	32.4 (1.6)

Supplemental file 3.6 – Transcript, proteome and metabolome sample arrangement

Figure S3.1 – Principle component analysis of the transcriptome of nitrogen replete cultures (control, N+) and nitrogen limited (N-) cultures exposed to a diurnal 16:8h LD cycle. Samples were taken after 0h, 5h, 10h and 16h of illumination. Clustering was found for the effect of treatment (clear separation visible on PC1) and for the effect of light period (PC2) between time point 0(dark adapted cultures) and the other time points. PCA plot was generated using Simca version 14.0.

Figure S3.2 Principle component analysis of the proteome of nitrogen replete cultures (control, N+) and nitrogen limited (N-) cultures exposed to a diurnal 16:8h LD cycle. Samples were taken after 0h, and 10h of illumination. Clustering was only found for the effect of treatment (PC1). PCA plot was generated using Simca version 14.0.

Figure S3.3 – Principle component analysis of the polar and apolar metabolomics of nitrogen replete cultures (control, N+) and nitrogen limited (N-) cultures exposed to a diurnal 16:8h LD cycle. Samples were taken after 0h, 5h, 10h and 16h of illumination. Clustering was only found for the effect of treatment (PC1). PCA plot was generated using Simca version 14.0.

Supplemental file 3.7 – Mapping to the KEGG metabolic pathways

Figure S3.4 – Metabolic pathway diagram from KEGG for transcriptome up and down regulation. The map shows proteins with differential expression (black highlighted) under nitrogen limited growth conditions compared to nitrogen replete growth. PPP: pentose phosphate pathway, TCA: tricarboxylic acid cycle.

Figure S3.5 – Proteome Metabolic pathway diagram from KEGG for proteome up and down regulation. The map shows proteins with differential expression (black highlighted) under nitrogen limited growth conditions compared to nitrogen replete growth. PPP: pentose phosphate pathway, TCA: tricarboxylic acid cycle.

Supplemental file 3.8 – Transcript vs proteome expression

Figure S3.6 – Log₂ fold change of transcript and protein expression under nitrogen limited growth conditions. Each dot in black represents a Phatr3 identifier that was found in both the transcriptome and proteome dataset. Significant differential expression is shown in red. Fold change was calculated for both light and dark timepoints for nitrogen limited versus nitrogen replete cultures. The blue dotted line fits a theoretical scenario where transcript and protein show identical fold changes.

Chapter 4

Continuous versus batch production of lipids

This chapter is published as:

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ABSTRACT

This work provides a novel quantitative comparison of batch versus continuous microalgal lipid production in the wildtype 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 nitrogen limitation in *A. obliquus*, whereas TAG is only accumulated after starch reaches a cellular maximum of 40% of dry weight.

4.1 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., 2016b; Breuer et al., 2015b; 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., 2016b; 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 *Scenedesmus 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 ($0.2 \text{ g TAG mol}^{-1}_{\text{ph}}$) 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 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.

4.2 Methods

4.2.1 Strains, pre-cultivation conditions and cultivation medium

Wildtype *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. (2013b).

4.2.2 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 Supplemental file 4.2. The culture was continuously sparged with 1 L min⁻¹ air enriched with 1% CO₂. The temperature was controlled at 27.5 °C and the pH was maintained at 7 using 5% H₂SO₄. 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 the Supplementary material: Figure S4.2). 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 g L⁻¹ with an incident light intensity (PFD_{in}) of 200 μmol m² s⁻¹. After three days, PFD_{in} was increased to the final set point of 500 μmol m² s⁻¹. Light was supplied in block form with 16h of constant illumination followed by 8h 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. (2013b), with the exception that the KNO₃ concentration was 3.24 g L⁻¹. After reaching a biomass concentration of approximately 2 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 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 $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ by automatically diluting the culture with fresh medium. The composition of this medium was identical to Breuer et al. (2013b), 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 24h period. Therefore, steady state conditions were defined when the average dilution rate, dilution pattern and biomass concentration in the reactor were stable over a 24h 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 (Equation 4.1).

$$\begin{aligned} \text{Nitrogen to photon ratio} &= \frac{r_N}{r_{\text{ph,absorbed}}} \\ &= \frac{(F_{N,\text{in}} C_{N,\text{in}}) - (F_{\text{out}} C_{N,\text{out}})}{(\text{PFD}_{\text{in}} - \text{PFD}_{\text{out}}) 3600 16 10^{-9} \frac{1}{d_r}} \end{aligned} \quad (\text{Eq. 4.1})$$

Multiple nitrogen feed regimes were tested for both strains: 7 for the wildtype 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 24h periods and used to determine the biomass concentration, biomass composition (TAG, starch, carbohydrates, proteins, ash) and elemental composition. We confirmed that samples stored for 24h 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.

4.2.3 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. (2017b). Lipid extraction was done with a chloroform:methanol (1:1.25) solution containing two internal standards: $180 \mu\text{g ml}^{-1}$ glyceryl trinonadecanoate (T4632; Sigma-Aldrich) and $300 \mu\text{g ml}^{-1}$ 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, 1,000 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 TAG, 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 Dubois et al. (1956) and 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. Maximum efficiency of PSII chemistry (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 minutes prior to the measurements. The biomass-specific absorption cross section (α_c) was measured according to de Mooij et al. (2014).

4.2.4 Calculations

Biomass (r_x) and TAG (r_{TAG}) productivity ($\text{g L}^{-1} \text{ day}^{-1}$) were calculated by dividing the amount of biomass or TAG per litre reactor volume (g L^{-1}) over the cultivation time. The biomass ($Y_{x,\text{ph}}$) and TAG ($Y_{\text{TAG},\text{ph}}$) 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., 2013b). 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: $0.85 \text{ mol}_{\text{ph}} \text{ g biomass}^{-1}$ for *A. obliquus* wildtype and $1.12 \text{ mol}_{\text{ph}} \text{ g biomass}^{-1}$ 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.

4.3 Results and Discussion

4.3.1 Batch cultivation under LD cycles

Duplicate batch experiments were performed under day night cycles for both *A. obliquus* wildtype 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* wildtype and up to 8.6 g L^{-1} for the slm1 (Figure 4.1A). This biomass increase was due to the production of starch, TAG and other carbohydrates. The wildtype simultaneously produced starch and TAG after nitrogen depletion (Figure 4.1C). Starch was initially accumulated at a higher rate compared to TAG, reaching a maximum content of 34% of dry weight after 40h. Thereafter starch was degraded while TAG synthesis continued, reaching a maximum TAG content of 41% of dry weight at the end of the experiment (30 days). The non-starch carbohydrate fraction increased in the first 24h 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 24h 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 48h to 20% of dry weight (Figure 4.1D). 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).

Figure 4.1 – Batch nitrogen starvation of *A. obliquus* wildtype (squares) and *slm1* (circles) under 16:8h LD cycle. (A) biomass concentration in g L⁻¹, (B) time-averaged yield of TAG on photons in g mol_{ph}⁻¹ and the biomass composition (TAG, triacylglycerol; TFA, total fatty acid; TC, total carbohydrate; starch) for the wildtype (C) and *slm1* (D) in % of dry weight. The cells were exposed to nitrogen starvation at t=0. Error bars show the minimum and maximum values of duplicate reactors

The observed biomass productivity of the *slm1* was lower than the wt, while equal amounts of light were supplied (Figure 4.1A). 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 gram TAG requires 0.75 mol_{ph} while 1 gram starch only requires 0.31 mol_{ph} (Breuer et al., 2014; Kliphuis et al., 2012). When calculating the metabolic requirements for biomass for both strains (detailed calculations available in Supplementary file 4.3), 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 wildtype 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 wildtype and 0.20 g TAG mol_{ph}⁻¹ within 7.7 days for the *slm1* (Figure 4.1B). These yields correspond to volumetric TAG productivities of 0.23 g L⁻¹ day⁻¹ for the wildtype 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 wildtype and 0.23 g TAG mol_{ph}⁻¹ for *slm1* for continuous light experiments, calculations available in Supplementary file 4.4). Additional biomass analysis revealed that dark respiration was marginal and only occurred during the first three nights of starvation (Supplementary file 4.5). The wildtype 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.

4.3.2 Continuous nitrogen limitation under DN cycles

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 24h periods, the culture overflow was collected on ice for 24 hour 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 wildtype. This difference can be explained by a reduced specific growth rate and lower protein content in *slm1* ($54\pm2\%$ versus $66\pm4\%$ of dry weight for the wildtype 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* wildtype and *slm1*, respectively. Similar steady state biomass concentrations were found for both strains (1.41 ± 0.01 g L^{-1} for the wildtype and 1.48 ± 0.17 g L^{-1} for the *slm1*). As a consequence, the observed biomass yield on light (Figure 4.2A) of the nitrogen-replete wildtype experiments was substantially higher than that of *slm1* (1.12 $\text{g mol}_{\text{ph}}^{-1}$ versus 0.85 $\text{g mol}_{\text{ph}}^{-1}$). 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 (Figure 4.2B). Here, we also found a lower electron fixation efficiency for *slm1* compared to the wildtype under nitrogen replete conditions (Figure 4.2B, 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., 2010b).

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 $\text{mgN mol}_{\text{ph}}^{-1}$) resulted in equal electron fixation efficiencies for both strains (Figure 4.2B). Contrary to observations under

nitrogen replete growth (red data points in Figure 4.2), 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 wildtype to the starchless mutant strain.

Figure 4.2 – Continuous nitrogen limitation of *A. obliquus* wildtype and slm1 under 16:8h LD cycle. Variation in (A) biomass yield on light, (B) theoretical electron fixation efficiency and biomass composition for (C) *A. obliquus* wildtype (squares) and (D) *A. obliquus* slm1 (circles) as a function of the nitrogen to photon uptake ratio. The nitrogen to photon ratio was calculated according to section 0, where 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.

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 (wildtype) or solely TAG (slm1), as shown in Figure 4.2. 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 $7 \pm 1 \text{ mgN mol}_{\text{ph}}^{-1}$. Conversely, the wildtype accumulated solely starch at moderate nitrogen limitation (range 100-50 $\text{mgN mol}_{\text{ph}}^{-1}$), and accumulated both starch and TAG at severe nitrogen limitation (range 50-10 $\text{mgN mol}_{\text{ph}}^{-1}$). The starch content reached its maximum of 40% of dry weight at 50 $\text{mgN mol}_{\text{ph}}^{-1}$ 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 $\text{mgN mol}_{\text{ph}}^{-1}$).

Figure 4.2C shows that TAG synthesis in the wildtype 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., 2015b; 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., 2015b). 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.

4.3.3 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 (Figure 4.3). Experimental maxima of $0.08 \text{ g TAG mol}_{ph}^{-1}$ for the wildtype and $0.12 \text{ g TAG mol}_{ph}^{-1}$ for the *slm1* were achieved at $32 \pm 1 \text{ mgN mol}_{ph}^{-1}$ and $22 \pm 0 \text{ mgN mol}_{ph}^{-1}$, respectively. An evidence-based interpolation (Supplementary file 4.6) of the TAG yield on light showed optima of $0.14 \text{ gTAG mol}_{ph}^{-1}$ for the wildtype and $0.16 \text{ gTAG mol}_{ph}^{-1}$ for *slm1*. Further reduction of the nitrogen supply rate resulted in increased cellular TAG contents (Figure 4.2C and D) but not in a higher TAG yield on light (Figure 4.3). 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.

Figure 4.3 – TAG yield on light ($Y_{TAG,ph}$) for continuous cultivations of *A. obliquus* wildtype (closed squares) and *slm1* (open circles). The nitrogen to photon ratio was calculated according to section 0, where the red data points illustrate nitrogen replete growth. Both lines (dotted line for the wildtype and straight 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 4.6). Error bars show the standard deviation over three different cultivation days.

In addition, the variable 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* wildtype and *slm1* was found to be approximately 0.7 (Figure 4.4), 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 (Figure 4.4B), even though the photosynthetic rate did decrease substantially under nitrogen limitation (Figure 4.2 and 4.3). Parkhill 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.

Figure 4.4 – Impact of batch nitrogen starvation (A) and continuous nitrogen limitation (B) on the F_v/F_m ratio of *A. obliquus* wildtype (closed squares) and *slm1* (open circles). Error bars show the standard deviation over three different cultivation days.

4.3.4 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 wildtype 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 4.1). 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 wildtype and 0.16 g TAG mol_{ph}⁻¹ for slm1, traditional batch nitrogen starvation still outcompetes continuous nitrogen limitation.

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 wildtype 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 Dominquez 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.

Table 4.1 – Comparison of batch and continuous lipid production in *A. obliquus* wildtype and slm1. The maximum yield TAG yield on light with corresponding TAG content and productivity are shown for batch and continuous cultivation with *A. obliquus* wildtype and slm1. The TAG productivity and yield on light were calculated over the entire cultivation period, including inoculum production. *Data interpolation showed maximum TAG yields on light of 0.14 g TAG mol_{ph}⁻¹ for the wildtype and 0.16 g TAG mol_{ph}⁻¹ for the slm1 under continuous nitrogen limitation (Supplementary file 4.6). Standard deviation was found to always be lower than 5%.

	Batch		Continuous	
	wildtype	slm1	wildtype	alm1
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

4.4 Conclusions

This study provides a quantitative comparison of batch versus continuous microalgal lipid production. Batch nitrogen starvation of *A. obliquus* results in an almost two-fold 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.

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Digital material - Supplementary digital File 4.1 can be found at <http://dx.doi.org/10.1016/j.biortech.2017.04.093>.

Supplementary File 4.2 – Reactor design

Figure S4.1 – Schematic overview of the experimental setup for batch and continuous cultivations. Continuous cultures were turbidostat controlled by the addition of nitrogen free medium. Nitrogen was continuously (but only in the light period) supplied via a separate medium port (N-feed). The nitrogen feed rate never exceeded 20% of the total dilution rate.

Figure S4.2 – Emission spectrum of the warm-white LEDs (specs). The parameter $E_{n,\lambda}$ represents the relative fraction of PAR photons present in 1 nm wavelength interval.

Supplementary File 4.3 – Photosynthetic requirement for batch cultivations

The theoretical electron fixation rate for biomass formation was calculated using Equation S4.1. Biomass was divided in functional biomass, TAG, starch and non-starch carbohydrates. Functional biomass consists out of a fixed amount of proteins, carbohydrates and membrane lipids. Upon nitrogen starvation, the increase in dry weight was solely caused by the accumulation of the storage compounds.

$$r_{e-} = \frac{r_{TAG}}{Y_{TAG,e-}} + \frac{r_{starch}}{Y_{starch,e-}} + \frac{r_{carbohydrates}}{Y_{carbs,e-}} + \frac{r_{functional\ biomass}}{Y_{funct.biomass,e-}} \quad (\text{Eq. S4.1})$$

With the productivity (r_i in $\text{g L}^{-1} \text{ day}^{-1}$) of TAG, starch, carbohydrates and functional biomass calculated from the reactor product concentrations over time. The theoretical yields ($Y_{i,e-}$ $\text{g mol}^{-1} e^-$) of TAG, starch, carbohydrates and functional biomass on electrons derived from photosynthesis were calculated with a simplified metabolic flux model (Breuer et al., 2015a; Kliphuis et al., 2012). The photosynthetic yields were estimated to be $2.66 \text{ g TAG mol}^{-1} e^-$, $6.48 \text{ g starch mol}^{-1} e^-$, $6.48 \text{ g carbohydrates mol}^{-1} e^-$ and $3.24 \text{ g biomass mol}^{-1} e^-$. We used the quantum mechanistic minimum of 2 mol_{ph} per mole of electron released from H_2O , based on the Z scheme of photosynthesis.

Figure S4.3 shows that no differences between the wildtype and *slm1* could be observed with regard to the theoretical amount of electrons fixed in biomass after N-depletion. Although DN cycles were applied, both strains have identical photosynthetic efficiency and only differ in terms of carbon partitioning (starch versus TAG).

Figure S4.3 – Theoretical amount of electrons fixed in *A.obliquus* wildtype (closed squares) and *slm1* (open circles) after a wash-down ($t=0$). The production rates for the calculations are based on the measured product concentrations.

Supplementary File 4.4 – Correction of TAG yield on light for inoculum production

Previously reported data (Breuer et al., 2014) was modified and corrected for inoculum production according to Equation S4.2. Corrections were done with a fixed theoretical biomass yield on light according to numbers found in the present study ($Y_{x,ph}$ of 1.12 g mol_{ph}⁻¹ for slm1 and $Y_{x,ph}$ of 0.85 g mol_{ph}⁻¹ for the wildtype). $M_x(t_{start\ starvation})$ represents the amount of biomass present in the reactor at the moment of nitrogen starvation. Table S4.7 shows the differences between previously published data and corrected data.

$$Y_{TAG,ph} = \frac{C_{TAG}(t) - C_{TAG}(t_{start\ starvation})}{\text{mol photons supplied } (t - t_{start\ starvation}) + \frac{M_x(t_{start\ starvation})}{Y_{x,ph}}} \quad (\text{Eq. S4.2})$$

Table S4.7 – Calculation example of TAG yield on light with corrections for inoculum production. Breuer et al. (2014) calculated the TAG yield on light using the actual supplied light for pre-cultivation. Our modifications corrected the inoculum production with yields observed in this study ($Y_{x,ph} = 0.85$ g mol_{ph}⁻¹ for slm1 and $Y_{x,ph} = 1.12$ g mol_{ph}⁻¹ for the wildtype).

	$Y_{TAG,ph}$ (g mol _{ph} ⁻¹) (Breuer et al., 2014)*	$Y_{TAG,ph}$ (g mol _{ph} ⁻¹) Only starvation	$Y_{TAG,ph}$ (g mol _{ph} ⁻¹) corrected
Wildtype	0.144	0.178	0.158
Starchless mutant	0.217	0.286	0.230

Supplementary File 4.5 – Overnight change in metabolite content

Figure S4.4 – Overnight change in metabolite content in batch nitrogen starvation experiments for *S. obliquus* wildtype (A) and *slm1* (B). Error bars show the standard deviation of duplicate runs. Both strains show significant losses in dry weight in the first days after N starvation. For the wt, the loss in dry weight is partially explained by the decrease in carbohydrates (e.g., starch). For *slm1* it remains unclear which biomass constituent is degraded overnight.

Supplementary File 4.6– Interpolation of TAG yield on light

Measured data was interpolated to estimate the maximal possible TAG yield on light over the entire range of nitrogen limitation (e.g., nitrogen to photon ratio). Interpolation of the dilution rate, TAG concentration and content was done by linear regression between the time points (Figure S4.5 and S4.6). The TAG yield on light was calculated based on the interpolated results for dilution rate and TAG concentration.

Figure S4.5 – Interpolation of the dilution rate (A), TAG concentration (B), TAG content (C) and TAG yield on light (D) for *A.obliquus* wildtype (D). The nitrogen to photon ratio was calculated according to section 4.2.2. The interpolation (dotted line) was based on the slope between the measured data points (closed squares).

Figure S4.6 – Interpolation of the dilution rate(A), TAG concentration (B), TAG yield on light (C) and TAG content for *A.obliquus* slm1 (D). The nitrogen to photon ratio was calculated according to section 4.2.2. The interpolation (dotted line) was based on the slope between the measured data points (open circles).

Chapter 5

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

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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 wildtype and starchless mutant strain of *Acutodesmus obliquus* under light-dark (LD) cycles and nitrogen replete conditions.

A. obliquus (formerly known as *Scenedesmus obliquus*) wildtype utilized light 20% more efficiently under LD regimes compared with continuous light. When exposed to LD regimes, the wildtype 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 triacylglycerol 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 wildtype. 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*.

5.1 Introduction

Diurnal cycles of biological activities are ubiquitous and allow many organisms to anticipate and adapt to changing environmental conditions (de Winter et al., 2013; Farré, 2012; Goldbeter et al., 2012; McClung, 2006). 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 (Bishop and Senger, 1971; de Winter et al., 2013; Graf et al., 2010). 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 (Pulz and Gross, 2004; Ruiz et al., 2016; Spolaore et al., 2006; Wijffels and Barbosa, 2010). One key bottleneck in reducing these costs is to improve biomass growth and yield (Wijffels and Barbosa, 2010). 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 (Caspar et al., 1985; Stitt and Zeeman, 2012). Therefore, a thorough understanding of the role of storage metabolites under diurnal, outdoor conditions might contribute to commercialization of large-scale microalgal production systems (de Winter, 2015).

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 (de Winter et al., 2013; Edmundson and Huesemann, 2015; Fábregas et al., 2002; Michels et al., 2014; Nikaido and Johnson, 2000). Specifically for microalgae, some strains use starch as the primary storage metabolite (Ballin et al., 1988; Brányiková et al., 2011; de Winter et al., 2014; Klein, 1987; Vitova et al., 2015), while others use non-starch carbohydrates (e.g., chrysolaminarin) or lipids (Matilde Skogen Chauton et al., 2013; Lacour et al., 2012; Poliner et al., 2015). The presence of such transitory energy

storage (TES) compounds allows efficient use of sunlight under diurnal light-dark (LD) conditions, as was shown for *Neochloris oleoabundans* (de Winter et al., 2013). The role of TES compounds and the regulation of the cell cycle have been studied extensively (Bišová and Zachleder, 2014; Garz et al., 2012; Schulze et al., 1991; Sulpice et al., 2009; Vitova et al., 2015; Wanka et al., 1970). 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 (Bišová and Zachleder, 2014; Jia et al., 2015; Klein, 1987; Li et al., 2011; Ma et al., 2016; Merchant et al., 2012).

In recent years, many microalgal strains have been suggested as promising candidates for the production of food, fuel, or chemicals (Mata et al., 2010; Rodolfi et al., 2009; Rosenberg et al., 2008; Wijffels and Barbosa, 2010). Among them, *Acutodesmus obliquus* (formerly known as *Scenedesmus obliquus* (Krienitz and Bock, 2012) is considered as an industrially relevant strain for food and fuel production (Breuer et al., 2012). To further improve triacylglycerol (TAG) productivity in *A. obliquus*, a starchless mutant was created using random mutagenesis (de Jaeger et al., 2014). This mutant showed a 51% higher TAG yield on light compared to the wildtype (0.144 ± 0.004 in the wildtype to 0.217 ± 0.011 g TAG·mol_{ph}⁻¹ in the starchless mutant) under batch wise nitrogen starvation, while maintaining its photosynthetic efficiency (Breuer et al., 2014), 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 (Li et al., 2010a; Ramazanov and Ramazanov, 2006; Siaut et al., 2007; Sirikhachornkit et al., 2016; Vonlanthen et al., 2015; Wang et al., 2009; Work et al., 2010). 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* wildtype 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.

5.2 Material and methods

5.2.1 Strains, pre-culture conditions and cultivation medium

Wildtype *Acutodesmus obliquus* UTEX 393 was obtained from the Culture Collection of Algae, University of Texas. *A. obliquus* was recently reclassified from *Scenedesmus obliquus* (Krienitz and Bock, 2012). The starchless mutant of *A. obliquus* (slm1) was generated as described by de Jaeger et al. (2014). Liquid cultures were maintained in a culture chamber with shaker (25 °C, 100 rpm, air in headspace, continuous illumination at 30-40 $\mu\text{mol m}^{-2} \text{s}^{-1}$) in 250 mL Erlenmeyer flasks containing 100 mL of filter sterilized (pore size 0.2 μm) defined medium, as described in (Breuer et al., 2013b). Prior to the start of the experiments, cultures were placed in a shake incubator operating at 23 °C with a light intensity of 180 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 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.

5.2.2 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$. 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 ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$). 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 to determine the 24 h average dilution rate ($D_{24\text{h}}$ in day^{-1}) and biomass composition according to de Winter et al. (2017).

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 ($< 15\text{min}$), as shown with Equation 5.1:

$$\frac{dC_{\text{cells}}}{dt} = -D_t \cdot C_{\text{cells}} + \mu_t \cdot C_{\text{cells}} \quad (\text{Eq. 5.1})$$

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 $1200 \times g$ 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.

5.2.3 Wet biomass analysis

Biomass concentration

Optical density was measured at 750 nm (OD₇₅₀). 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. (2012).

5.2.4 Biochemical analysis

Total carbohydrate and starch content

Total carbohydrates were extracted and quantified according to Dubois et al. (1956) and Herbert et al. (1971). 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. (2014) 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. 5.2). With the change over 1 or 3 h time intervals in starch content ($\frac{dC_{starch}}{dt}$), dilution rate (D_t), starch content ($C_{starch,t}$) and the starch productivity ($r_{starch,t}$):

$$\frac{dC_{starch}}{dt} = -D_t \cdot C_{starch,t} + r_{starch,t} \quad (\text{Eq. 5.2})$$

Lipid analysis

Triacylglycerol (TAG) content, total fatty acid (TFA) content, and fatty acid composition were determined as described by Breuer et al. (2013a) 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 $\mu\text{g mL}^{-1}$ glyceryl trinonadecanoate (T4632; Sigma-Aldrich) and 300 $\mu\text{g mL}^{-1}$ 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 cc, 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 TAG, 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. (Breuer et al., 2013a). The TFA profile and content were calculated as the sum of each individual fatty acid in the neutral and polar lipid fraction.

Protein

The total protein concentration was determined using the colorimetric assay (Bio-Rad DC protein assay) as described by Postma et al. (2015) with the difference that 10-15 mg of freeze dried biomass was used for analysis.

5.2.5 Cell number, diameter and division rate

A. obliquus is characterized by the formation of coenobia (Bišová and Zachleder, 2014). 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).

5.2.6 Biomass yield on light

Biomass yield on light ($Y_{x,ph}$ in $g\ mol_{ph}^{-1}$) 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 $mol_{ph}\ L^{-1}\ day^{-1}$) using Equation 5.3:

$$Y_{x,ph} = \frac{D_{24h} \cdot C_X}{r_{ph}} \quad (\text{Eq. 5.3})$$

5.2.7 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.

5.3 Results and Discussion

5.3.1 *A. obliquus* wildtype 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* wildtype under 16h:8h light-dark (LD) cycles. Duplicate reactors were subjected to LD cycles at an incident light intensity of $500\ \mu mol\ m^{-2}\ s^{-1}$. 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 ($10\ \mu mol\ m^{-2}\ s^{-1}$). During the light period, constant turbidity was maintained by automatically adding fresh media.

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

The time specific dilution rate fluctuates clearly in a cyclic pattern (Figure 5.1A), 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) (de Winter et al., 2014). The biomass concentration remained constant at $1.25 \pm 0.25 \text{ g L}^{-1}$ (Figure 5.1B) 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} (Figure 5.1C) during the last 2 h of light (14 h after 'sunrise') and the first 2 h of dark (16-17 hours 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 (Figure 5.1C).

By calculating the cell division rate (Figure 5.1D) 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 hours after 'sunrise') and thereafter decreased (from 4.2 to 3.3 μm) due to cell division (Supplementary Figure S5.1). These results show that LD cycles result in synchronization of cell division in *A. obliquus* wildtype. Similar results have been described previously (Bongers, 1958; Senger, 1970). Bongers (1958) 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.

Figure 5.1 – Daily variation in (A) dilution rate, (B) biomass concentration, (C) cell number and (D) cell division rate for *A. obliquus* wildtype under LD 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.

5.3.2 Biomass composition of *A. obliquus* wildtype 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 (Figure 5.2A). Starch was accumulated during the second half of the light period and consumed throughout the entire dark period. Starch started accumulating 7 hours after 'sunrise', simultaneous with a decrease in biomass productivity, and reached its maximum content of 20% of dry weight 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 S5.2). This supports the hypothesis that starch is respired overnight, as also observed in other diurnal studies on photosynthetic organisms (Blanken et al., 2017). 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* wildtype. Previous work suggested that other storage metabolites such as triacylglycerol (TAG) or non-starch carbohydrates (NSC) could also act as TES (de Winter et al., 2014; Jia et al., 2015). However, these metabolite concentrations remained stable throughout the LD cycle (Figure 5.2). The NSC fraction, which is defined as the total carbohydrate content minus the starch content, was stable at approximately 20% of dry weight. In addition, the TAG content was lower than 1% of dry weight (Figure 5.2B) 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 (Figure 5.2C). 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* wildtype.

Figure 5.2 – Daily variation in (A) starch content, (B) starch productivity, (C) triacylglycerol (TAG, squares) and non-starch carbohydrates (NSC, triangles) content, and (D) TAG (circles) and NSC (diamonds) productivity for *A. obliquus* wildtype under LD 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.

5.3.3 Continuous illumination abolishes synchronized growth in *A. obliquus* wildtype

To further study the impact of LD cycles on cell physiology, we also cultivated *A. obliquus* wildtype under continuous light conditions. Here, as with the LD cycles, the biomass concentration remained stable due to turbidostat control (Supplementary Material: Figure S5.2). 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 33% of dry weight, proteins 47% of dry weight, TAG 0.2% of dry weight and a dilution rate of $1.54 \pm 0.03 \text{ day}^{-1}$ (Supplementary material: Figure S5.2C). In addition, no apparent fluctuations in the biomass productivity and composition (protein, total carbohydrates, and TAG content) were observed (Supplementary material: Figure S5.2E 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 (Lu, 2005; Smith, 2004). In addition, also no growth rhythms were found in maize (*Zea mays*), rice (*Oryza sativa*) (Poiré et al., 2010), and the microalga *N. oleoabundans* (de Winter et al., 2013) when grown under constant light conditions.

5.3.4 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 (Caspar et al., 1985; de Winter et al., 2013; Webb and Satake, 2015; Weise et al., 2011). In *A. obliquus* wildtype, we showed that starch acts as the sole transitory energy storage (TES) molecule under nitrogen replete conditions (Figure 5.2A and 5.2C). The starch and TAG biosynthesis pathways are competing for carbon precursors (Fan et al., 2012). 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 (Poliner et al., 2015), 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* (de Jaeger et al., 2014) under continuous light and diurnal LD cycles. *Slm1* is not capable of producing starch due to a mutation in the small subunit of ADP-glucose pyrophosphorylase (de Jaeger, 2015), 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 $0.90 \pm 0.01 \text{ day}^{-1}$ was found. Dilution rate patterns of *slm1* were similar to those observed for the wildtype (Figure A) but absolute values were approximately 20% lower (Figure 5.3A). As for the wildtype, biomass concentration remained constant over a 24 h cycle (Figure 5.3B); therefore, biomass productivity is proportional to dilution rate. Also here, the biomass productivity of the *slm1* were approximately 20% lower compared to the wildtype under LD cycles.

As for the wildtype, the *slm1* was also grown under continuous light. However, under continuous light the dilution rate was even lower for *slm1* when compared to the wildtype ($1.03 \pm 0.06 \text{ day}^{-1}$ for *slm1* compared with $1.50 \pm 0.10 \text{ day}^{-1}$ for the wildtype, Supplementary material: Figure S5.2D). Similar reduced growth rates were also observed for starchless mutants of *Chlamydomonas reinhardtii* (Li et al., 2010b). 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 Figure 5.3C and 5.3D, *A. obliquus* *slm1* shows cyclic behaviour in cell division rate and cell diameter (data available in Supplementary material: Figure S5.1) throughout the LD cycle. During each cycle, cell number doubled from $22.0 \pm 0.7 \text{ million cells mL}^{-1}$ to $41.4 \pm 0.5 \text{ million cells mL}^{-1}$ (Figure 5.3C). Cell division started 2 h before the dark period (14 h after 'sunrise') as for the wildtype. However, while for the wildtype cell division was confined to a 4-hour 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 material: Figure S5.1) and decreased due to cell division afterwards (from 14 h after 'sunrise'). Interestingly, the mutant *slm1* and the wildtype show different subpopulation patterns in cell diameter. The wildtype 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 wildtype all three subpopulations increase in size during the day and decrease in size due to division in the night (Supplementary material: Figure S5.1). 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 (Figure 5.3E). Although the non-starch carbohydrate content was slightly higher in the starchless mutant (22% versus 27% for the wildtype and starchless mutant, respectively), there was no diurnal pattern observed under either LD cycles or continuous light (Figure 5.3E and Supplementary material: Figure 5.2F). Therefore, in contrast to our expectations, these findings indicate that neither TAG nor any alternative carbohydrates (Figure 5.3E) are used in *slm1* to replace starch as TES agent.

Figure 5.3 – Daily variation in (A) dilution rate, (B) biomass concentration, (C) cell number, (D) cell division rate, (E) carbohydrates (CHO, diamonds) and triacylglycerol (TAG, circle) content (E), and (F) CHO (diamond) and TAG (circle) productivity for *A. obliquus* slm1 under LD 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).

5.3.5 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 (de Winter et al., 2013; Klein, 1987; Lacour et al., 2012; Vitova et al., 2015). To elaborate on this, we calculated the biomass yield on light (Equation 5.3). *A. obliquus* wildtype uses light more efficiently under LD cycles compared to continuous light (Figure 5.4), as it produces approximately 20% more biomass per mol_{ph} absorbed when grown under LD cycles ($0.98 \pm 0.00 \text{ g mol}_{\text{ph}}^{-1}$ for continuous light compared to $1.18 \pm 0.03 \text{ g mol}_{\text{ph}}^{-1}$ for LD cycles). In contrast, the *slm1* showed similar biomass yields on light for both continuous and LD conditions ($0.72 \pm 0.04 \text{ g mol}_{\text{ph}}^{-1}$ for continuous light compared to $0.80 \pm 0.04 \text{ g mol}_{\text{ph}}^{-1}$ 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 5.2.6). Similar trends as for biomass yield on light were found for the energy conversion efficiencies under the different light regimes (Supplementary File 5.2). We can therefore conclude that *A. obliquus* wildtype 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 wildtype 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 wildtype immediately after switching on the light (Figure 5.1A).

Figure 5.4 – 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.

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 wildtype (Supplementary File 5.2) under nitrogen replete conditions. Interestingly, under nitrogen starvation and continuous light, Breuer et al. (2014) observed that the mutant *slm1* has a similar energy conversion efficiency compared with the wildtype. 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.

5.4 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* wildtype. 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 wildtype 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 wildtype, 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 wildtype. Therefore, by removing the 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.

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Supplementary File 5.1 – Figures and Tables

Figure S5.1 – Cell diameter distribution for *A. obliquus* wildtype (top) and *slm1* (bottom). The length of the diagram represent the size of the population, the width represents the density of the population on each size value, and the white dot represents the average. The x axis shows hours after ‘sunrise’. Dark period starts at 16 h.

The role of starch in synchronized microalgal growth

Figure S5.2 – Trends in biomass concentration (A and B), dilution rate (C and D), carbohydrate (CHO in diamonds) and TAG (circles) (E and F, and proteins (G and H) for *A. obliquus* wildtype (A, C, E, G) and *slm1* (B, D, F, H) under continuous light. Open and closed symbols represent duplicate cultures.

Table S5.1 – Experimental values for the wildtype and *slm1* under continuous and LD regimes. Average steady state values (at least 3 consecutive days) are given for duplicate reactor runs. Standard deviation is given in brackets.

Strain	Wildtype				Slm1			
	Continuous light		LD cycles		Continuous light		LD cycles	
Dry weight concentration (g L ⁻¹)	1.38 (0.06)	1.34 (0.05)	1.42 (0.05)	1.40 (0.03)	1.64 (0.10)	1.53 (0.03)	1.17 (0.07)	1.15 (0.08)
Dilution rate (day ⁻¹)	1.52 (0.08)	1.49 (0.14)	1.14 (0.01)	1.14 (0.02)	0.97 (0.02)	1.07 (0.04)	0.84 (0.02)	0.84 (0.01)
TAG (% of dry weight)	0 (0)	0 (0)	0 (0)	0 (0)	2 (0)	3 (0)	0 (0)	1 (0)
CHO (% of dry weight)	25 (3)	22 (4)	19 (1)	19 (1)	26 (2)	29 (1)	21 (2)	25 (4)
Energy fixation rate (mol _{ph} L ⁻¹ day ⁻¹)	1.30 (0.00)	1.16 (0.00)	1.00 (0.00)	0.98 (0.00)	1.02 (0.00)	1.01 (0.00)	0.58 (0.00)	0.60 (0.00)
Photon absorption rate (mol _{ph} L ⁻¹ day ⁻¹)	2.05		1.36		2.05		1.36	
Energy fixation efficiency (% photons used)	63.35 (0.01)	56.80 (0.01)	73.72 (90.00)	72.34 (0.01)	49.79 (0.04)	49.30 (0.03)	42.63 (0.00)	43.99 (0.02)

Figure S5.4 – Diurnal biomass productivity under LD cycles for *A. obliquus* wildtype (A) and starchless mutant (B). Open and closed symbols represent duplicate cultures. The x axis shows hours after sunrise. Shaded areas indicate the dark period.

The role of starch in synchronized microalgal growth

Figure S5.5 – Absorption cross section for continuous light (A and B) and LD cycles (C and D) for *A. obliquus* wildtype (A and C) and starchless mutant *slm1* (B and D). Open and closed symbols represent replicate cultures. The absorption spectrum was measured in diluted cell suspensions (OD_{750} of approximately 0.5) according to de Mooij et al.(2016). Shaded areas indicate the night period for the DN cultures.

Table S5.2 – Overnight difference in dry weight and starch concentration for duplicate reactor runs. Standard deviation is shown in brackets.

Strain	wildtype		slm1
	Dry weight concentration (g L^{-1})	Starch (g L^{-1})	Dry weight concentration (g L^{-1})
Sunset	1.43 (0.02)	0.26 (0.03)	1.20 (0.00)
Sunrise	1.24 (0.00)	0.08 (0.01)	1.24 (0.02)
Overnight difference	0.19 (0.02)	0.19 (0.04)	-0.04 (0.03)

Supplementary File 5.2 – Energy conversion efficiency

The theoretical energy conversion efficiency was calculated from the theoretical photon requirements for biomass production and the photon consumption rate, as illustrated by Equation S5.1. Biomass was considered to consist of TAG, starch and functional biomass. The functional biomass was determined by subtracting the measured fractions of TAG and starch from the measured total biomass. The photon requirements for functional biomass, TAG and starch were calculated based on the 24 h average productivity (r_{TAG} for TAG productivity, r_{starch} for starch productivity and $r_{\text{functional biomass}}$ for functional biomass productivity in $\text{g L}^{-1} \text{ day}^{-1}$) and theoretical yields on photons of these biomass fractions. These theoretical yields were estimated using flux balance analysis according to (Breuer et al., 2015a; Kliphuis et al., 2012) and were $1.62 \text{ g functional biomass mol}_{\text{ph}}^{-1}$ ($Y_{\text{functional biomass,ph}}^{\text{max}}$), $3.24 \text{ g starch mol}_{\text{ph}}^{-1}$ ($Y_{\text{carbs,ph}}^{\text{max}}$) and $1.33 \text{ g TAG mol}_{\text{ph}}^{-1}$ ($Y_{\text{TAG,ph}}^{\text{max}}$). The photon consumption rate ($r_{\text{ph,absorbed}}$ in $\text{mol}_{\text{ph}} \text{ L}^{-1} \text{ day}^{-1}$) was calculated by subtracting the incoming from the outgoing light intensity.

$$\text{Theoretical energy conversion efficiency} = \frac{\frac{r_{\text{TAG}}}{Y_{\text{TAG,ph}}^{\text{max}}} + \frac{r_{\text{starch}}}{Y_{\text{starch,ph}}^{\text{max}}} + \frac{r_{\text{functional biomass}}}{Y_{\text{functional biomass,ph}}^{\text{max}}}}{r_{\text{ph,absorbed}}} \quad (\text{Eq. S5.1})$$

Wildtype cultures exposed to LD cycles used $73 \pm 1\%$ of the photons supplied to create biomass, compared with $60 \pm 5\%$ when grown under continuous light conditions. In contrast to the wildtype, the efficiency was lower for the starchless mutant *slm1* under LD cycles compared with continuous light ($43 \pm 1\%$ under LD cycles compared with $50 \pm 0.4\%$ under continuous light regime, T-test, $p < 0.05$).

Chapter 6

General discussion

ABSTRACT

The productivity of lipids in microalgae is high. Nevertheless, the yield obtained in practice is 10 times lower than the theoretical yield. There is an enormous potential to improve the lipid productivity in microalgae further. In this paper, we analyzed the major factors causing this gap. The technology for production under controlled conditions is well developed, however, there is still a major loss in yield when transferred to outdoor conditions. The lower practical yield is mainly caused by the fluctuating insolation in outdoor reactors. Therefore, there is a need for process improvement and control under outdoor conditions at pilot scale. Another bottleneck is the lipid production capacity of the strains used. Knowledge on microalgal strain improvement, however, is limited. The coverage of genes that can be annotated is too low to fully understand the metabolic routes and strain-specific molecular toolboxes are still lacking. As a consequence it is hard to identify the target genes which need to be improved. Combining gene discovery with genome based models will allow identification of (multiple) genetic targets for improved microalgal lipid production.

Altogether, only by concerted efforts on strain and process improvement the potential of microalgae for lipid production can be fully exploited.

6.1 Introduction

Microalgae can contain large amounts of lipids which make them a promising feedstock for sustainable production of food, feed, fuels and chemicals (Klok et al., 2014; Ruiz et al., 2016; Vanthoor-Koopmans et al., 2013). Microalgae have an aerial productivity superior to that of terrestrial crops and can be grown on waste- and sea water (Wijffels et al., 2010). Although some microalgal strains can accumulate up to 60% of triacylglycerol (TAG) per dry cell weight (Breuer et al., 2012; Spolaore et al., 2006), current production costs for cultivation are still too high for bulk lipid production from microalgae (Ruiz et al., 2016).

Microalgal TAG accumulation is most commonly induced using nitrogen starvation (Benvenuti et al., 2016a; Griffiths et al., 2011; Klok et al., 2014). Typically, biomass is first produced under nitrogen replete conditions followed by nitrogen depletion where TAG is accumulated and cell growth is hampered. As a consequence to nitrogen deprivation, microalgae severely decrease their photosynthetic capacity by reducing pigmentation, photosynthetic proteins and active photosystem reaction centres to minimize damage through over-excitation (Rhiel et al., 1985; Simionato et al., 2013). One of the major causes for the high costs of bulk lipid production is the reduced solar-to-lipids conversion efficiency (Box 6.1). Attempts to increase the solar-to-lipid conversion efficiency include rational strain selection, strain improvement, and optimizing process conditions (Benvenuti et al., 2016b; Breuer et al., 2013b; Cabanelas, 2016b; de Jaeger et al., 2014; Gan et al., 2016; Ghosh et al., 2016; Griffiths et al., 2011; Remmers et al., 2017a; Trentacoste et al., 2013). In this chapter we evaluate the recent findings on microalgal lipid production. Additionally, we also provide an outlook on future research needed to further improve the solar-to-lipids conversion efficiency in microalgae and to improve TAG productivity in microalgae.

Box 6.1 – Evaluation of theoretical maximum solar-to-TAG efficiency**Theoretical maxima**

The theoretical maximum TAG yield on light, calculated from the photosynthetic maximum ($0.125 \text{ Cmol CO}_2 \text{ mol}_{\text{ph}}^{-1}$) and the molecular weight of TAG ($15.71 \text{ g TAG Cmol}^{-1}$), is $1.96 \text{ g TAG mol}_{\text{ph}}^{-1}$. When incorporating the reduction of CO_2 to $\text{CH}_{1.82}\text{O}_{0.11}$ (triolein, a TAG molecule with three oleic acid tails) and concomitant energy requirements for the metabolic steps, the theoretical maximum TAG yield on light decreases to $1.33 \text{ g TAG mol}_{\text{ph}}^{-1}$ (Breuer et al., 2015a). This yield was based on flux balance analysis (FBA) where all photons are used for TAG production with maximum photosynthetic efficiency (i.e. 8 photons are converted into 2 electrons to fix 1 mol of CO_2 , Z-scheme for photosynthesis).

Theoretical maxima including losses in photosynthesis and metabolism

The maximum theoretical yield based on FBA, however, does not include losses through photo-inhibition, respiration, or other metabolic processes. As a consequence to these concomitant losses, the maximum theoretical yield of $1.33 \text{ g TAG mol}_{\text{ph}}^{-1}$ can never be reached in practice. The empirical maximum TAG production could be estimated from the maximum observed sunlight-to-biomass conversion efficiency. For this calculation, we used our maximum observed lab-scale sunlight-to-biomass yield for continuous cultures of *A. obliquus* ($1.17 \text{ g biomass mol}_{\text{ph}}^{-1}$ (Remmers et al., 2017a)). Note that higher conversion yields could be obtained when working at lower incident light intensities, but low light conditions do not represent outdoor environments. We converted the maximum observed lab-scale sunlight-to-biomass yield to an empirical maximum TAG yield using theoretical conversion yields from FBA ($1.62 \text{ g biomass mol}_{\text{ph}}^{-1}$ and $1.33 \text{ g TAG mol}_{\text{ph}}^{-1}$). When assuming that all carbon is directed into TAG (i.e. the cell is 100% TAG), the empirical maximum is $0.96 \text{ g TAG mol}_{\text{ph}}^{-1}$. Though, in practice a microalgal cell consists also of functional biomass (proteins, pigments, membrane lipids, and more) and TAG only contributes to a maximum of 50% of dry weight at the time of harvest (Remmers et al., 2017a). An empirical maximum of $0.53 \text{ g TAG mol}_{\text{ph}}^{-1}$ ($5.5 \text{ L TAG m}^{-2} \text{ year}^{-1}$, calculated for Southern Europe according to Breuer et al., 2013b) seems therefore more realistic.

Empirical limits

The maximum experimental TAG yields on light in our research (Remmers et al., 2017a)

Box 6.1 (continued) – Evaluation of theoretical maximum solar-to-TAG efficiency

were found at 0.16 g TAG mol_{ph}⁻¹ for the wildtype and 0.20 g TAG mol_{ph}⁻¹ for the starchless mutant. Outdoor experimental data even showed a maximum TAG yield on light of 0.09 g TAG mol_{ph}⁻¹ (Benvenuti et al., 2016a) and 0.93 L TAG m⁻² year⁻¹ (calculated from the TAG yield on light for production in Southern Europe according to Breuer et al., 2013b). Similar average year-round outdoor TAG productivities (1.07 L TAG m⁻² year⁻¹) were found by Quinn et al. (2012). Maximum TAG yield on light differs depending on species and supplied light intensity (Remmers et al., 2017b, 2017a). Mulders et al. (2014) reported the highest TAG yields on light so far, up to 0.3 g TAG mol_{ph}⁻¹ for the wildtype strain of *Chlorella zofingiensis* when exposed to lower light conditions than in our study (Remmers et al., 2017a). Still, the large discrepancy between the current maximum practical yield and the theoretical maximum illustrates the necessity to improve the solar-to-TAG conversion efficiency during nitrogen starvation.

Figure 6.1 — The different solar-to-TAG yield based on theoretical maxima and empirical limits.

6.2 Operation mode

The most sensitive parameter in microalgal lipid production is the photosynthetic efficiency (PE) under nitrogen deprivation (Ruiz et al., 2016). In order to improve the PE, recent studies have addressed the importance of mode of operation for lipid production (i.e. batch, repeated batch, or continuous lipid accumulation) (Benvenuti et al., 2016b; Fernandes et al., 2015; Griffiths et al., 2014; Klok et al., 2013; Pruvost et al., 2009; Remmers et al., 2017a; Rodolfi et al., 2009)

In the most applied strategy (batch operation), first biomass is produced in the presence of nitrogen, followed by a nitrogen starvation phase to induce TAG accumulation. Batch processes allow high TAG contents and are simple in operation. Unfortunately, batch wise nitrogen starvation is accompanied with impaired growth rates and reduced photosynthetic activities (Breuer et al., 2014; Griffiths et al., 2014). To overcome this loss, (semi-) continuous lipid production was proposed (Benvenuti et al., 2016b; Klok et al., 2013). Batch nitrogen starvation showed, however, in all cases to be more efficient compared to (semi-) continuous limitation (Table 6.1). Cells exposed to (semi-) continuous nitrogen limitation always suffered from reduced photosynthetic capacity that led to inefficient light use in either the regrowth of biomass (repeated batch, Benvenuti et al., 2016b) or the continuous exposure to nitrogen limited conditions (Remmers et al., 2017a).

The operation mode has a significant effect on the solar-to-TAG conversion efficiency, whereas batch cultivations always outcompete (semi-) continuous cultivations, both in terms of TAG content and in TAG productivity. However, even with lab-scale process improvements, there is still a gap between the current empirical and theoretical maximum TAG yield on light (0.20 vs 0.53 gTAG mol_{ph}⁻¹; Box 6.1). To bridge the gap between the empirical and theoretical maximum TAG yield, there should be a focus on strain improvement (Box 6.1).

Table 6.1 — Different reported approaches to improve microalgal lipid productivity. slm: starchless mutant, ALE: adaptive laboratory evolution, FACS: fluorescence-activated cell sorting. *calculated only for the starvation period, ‡ lipid measurements were fluorescence based, therefore no contents or productivities are given.

Method	Condition	Algal strain	Outcome	Reference
Operational mode				
Batch (vs repeated batch)	(repeated) Nitrogen starvation	<i>Nannochloropsis sp.</i>	Two-fold higher TAG yield on light under batch conditions: 54 %w/w TAG, 0.13 g TAG mol _{ph} ⁻¹	Benvenuti et al. (2016b)
Batch (vs continuous)	Nitrogen starvation and limitation	<i>A. obliquus</i>	Two-fold higher TAG yield on light under batch conditions: 59 %w/w TAG, 0.29 g TAG L ⁻¹ day ⁻¹ , 0.20 g TAG mol _{ph} ⁻¹	Chapter 4
Stain selection and improvement				
Random mutagenesis: defective in the small subunit of ADP-glucose pyrophosphorylase	Nitrogen starvation	<i>C. reinhardtii</i> , BAFJ5	8 fold higher neutral lipid content: 46%w/w lipids, 0.10 g lipids L ⁻¹ day ⁻¹	Li et al. (2010b)
Random mutagenesis: SNP in the small subunit of ADP-glucose pyrophosphorylase	Nitrogen starvation	<i>A. obliquus</i> slm1	51% improved TAG productivity: 57 %w/w TAG, 0.36 g TAG L ⁻¹ day ⁻¹ , 0.22 g TAG mol _{ph} ⁻¹	Breuer et al. (2014); de Jaeger et al. (2014)
FACS	Nitrogen starvation	<i>C. littorale</i>	2 fold increase in TAG productivity: 34 %w/w TAG, 0.34 g TAG L ⁻¹ day ⁻¹ , 0.32 g TAG mol _{ph} ⁻¹ *	Cabanelas et al. (2016a)
Random mutagenesis: mutation not known	Nitrogen starvation	<i>N. oceanica</i>	30% higher lipid productivity: 62 %w/w lipids, 0.27 g lipid L ⁻¹ day ⁻¹	Ma et al. (2013)
Targeted strain improvement through metabolic or genetic engineering				
Knockdown of a multifunctional lipase/phospholipase/acyltransferase	Silica starvation	<i>T. pseudonana</i>	3.2-4.1 fold higher total lipid content ‡	Trentacoste et al. (2013)
Antisense knockdown of Pyruvate dehydrogenase kinase		<i>P. tricornutum</i>	33–82% more neutral lipids ‡	Ma et al. (2014)
Overexpression of malic enzyme (<i>PtME</i>)	Nitrogen deprivation	<i>P. tricornutum</i>	2.4- to 3.2-fold more neutral lipids ‡	Xue et al. (2015)
Overexpression of AAE		<i>C. reinhardtii</i>	Increase in lipid content of 24-34%: 12 %w/w TAG	Deng et al. (2012)
Overexpression of DGAT		<i>P. tricornutum</i>	35% more neutral lipids, 76.2% more EPA ‡	Niu et al. (2013)
Overexpression of thioesterase (PtTE)		<i>P. tricornutum</i>	72% more total lipids: 13 %w/w total fatty acids	Gong et al. (2011)
Overexpression of NAD(H) kinase from <i>Arabidopsis</i>		<i>C. pyrenoidosa</i>	Up to 2-fold increase in total lipid content: 38 %w/w lipids	Fan et al. (2015)

6.3 Translation of lab-scale to outdoor production

Cultivation parameters, such as temperature, pH, light intensity, have significant impact on the TAG productivity and yield on light (Remmers et al., 2017b). Breuer et al. (2013b) showed, for small scale indoor experiments, that an optimal pH and temperature can boost TAG productivity and yield 5-fold in *Acutodesmus obliquus*. Further optimization of the incident light intensity improved the TAG yield even further (2-fold increase) to 0.27 g TAG mol_{ph}⁻¹ (Breuer et al., 2013b). Similar results were found in *Neochloris oleaobundans* (Santos et al., 2014).

These optimizations were reported for indoor lab scale systems, but, despite the advanced predictive growth models for outdoor production (Benvenuti, 2016; Cabanelas, 2016a; Slegers, 2014), the gap between indoor and outdoor observed TAG productivities and yield in light is still large (Box 6.1). Outdoors, the incident solar intensity and temperature can fluctuate severely throughout a day, with consequently (negative) effects on the lipid productivity.

Benvenuti et al. (2016a) showed, for example, a reduction of 26% in TAG yield on light for batch nitrogen starvation and 71% for repeated batch nitrogen starvation in pilot scale outdoor reactors compared to indoor experiments. As temperature can be controlled at the cost of energy, the decrease in yield for outdoor cultivations is mainly caused by the fluctuating insolation. Therefore, locations with highly fluctuating weather conditions are less suitable for algal cultivation. To minimize the impact of fluctuating insolation, it is important to develop new reactor designs (e.g., tilting reactors; GWP-III, (Tredici et al., 2011)) combined with dynamic process control techniques (Marsullo et al., 2015). Dynamic control models can anticipate on the variations in both irradiance and temperature that occur during the day and in the different seasons. To develop models for dynamic process control, in depth knowledge is required on algal growth, photo acclimation, and daily- and yearly cycles of irradiance. It is very important to translate expertise developed under controlled laboratory conditions to outdoor conditions and to control the process under these outdoor conditions to bridge the gap between laboratory experiments and industrial applications (Bosma et al., 2014).

6.4 Strain selection and improvement

Recently, many screening studies have focussed on selecting wildtype strains with both high photosynthetic efficiency and high TAG content under nitrogen deprivation (Benvenuti et al., 2014; Breuer et al., 2012; Griffiths and Harrison, 2009). Such screening studies are often elaborate, but also crucial to identify industrial promising strains based on predetermined selection criteria. By identifying a few model strains as chassis (Table 6.2), joint research efforts will accelerate the transition towards commercial large scale production. This is well illustrated for the production of industrial enzymes, where only a few (fungal and bacterial) strains are used (Nielsen and Keasling, 2011). Also, for the production of second generation biofuels most research efforts were targeting the use of the yeast *Saccharomyces cerevisiae* (Alper and Stephanopoulos, 2009). Specifically for the field of synthetic biology, such focused efforts will accelerate the identification of targets to improve microalgal metabolism.

The TAG productivity of microalgal strains can be further improved using a variety of techniques for strain selection and improvement. Examples are selective cell sorting, random mutagenesis, and targeted engineering strategies.

6.4.1 Selective cell sorting

Cabanelas et al. (2016a) and Klok et al. (2013) showed that nitrogen starved cultures consist of subpopulations with variable lipid contents. This natural variability allows for the selection of subpopulations with (potential) higher lipid productivities. Selection and sorting of subpopulations requires careful selection of the sorting criteria. Besides the apparent selection for lipid-dependent fluorescence, Cabanelas et al. (2016) used flow cytometry to also select for cell size (using forward scatter) and chlorophyll-dependent fluorescence (Auto fluorescence, AF). Inclusion of chlorophyll AF allowed sorting of cells with high chlorophyll content that, potentially, show sustained photosynthetic efficiency after a period of nitrogen starvation compared to populations with low AF.

Cabanelas et al. (2016a) and Doan et al. (2011) showed significantly enhanced lipid productivities in fluorescence sorted subpopulations. For example, *Chlorococcum littorale* showed a 2 fold higher TAG yield on light for the sorted subpopulation compared to the original population. Interestingly, the sorted subpopulation of *C. littorale* showed no alterations in carbon partitioning between TAG and starch. Further transcriptome and proteome studies on the sorted subpopulation should be performed to identify genes that play a role in the enhanced TAG content, productivity and yield on light. In addition, similar cell sorting studies should also be applied on industrially interesting strains for lipid production, such as *Nannochloropsis* (Table 6.2).

Table 6.2 – Comparison of different microalgal strains for large scale, outdoor lipid production. Different screening criteria were scored from 0 (poor) to 5 (excellent). Information in table is based on: Remmers et al. (2017a, 2017b), León-Saiki et al. (2017), Teles et al. (2016), Benvenuti et al. (2016), Breuer et al. (2014, 2013b, 2012), Zheng et al. (2013), Vigeolas et al. (2012), Blanken et al. (2016), Perrineau et al. (2014), and Ruiz et al. (2016). ¹ Comprises of: *Nannochloropsis* sp., *Nannochloropsis oceanica* and *Nannochloropsis gaditana*.

Screening criteria	Weight factor (1-3)	Strains					
		<i>Chlorella sorokiniana</i>	<i>Chlamydomonas reinhardtii</i>	<i>Acutodesmus obliquus</i>	<i>Nannochloropsis spp.</i> ¹	<i>Phaeodactylum tricornutum</i>	<i>Chlorococcum littorale</i>
Cultivation characteristics							
Tolerance to salt water	2	2	1	0	5	5	5
Tolerance to high temperature (>30 °C)	3	5	1	1	3	0	3
High maximum specific growth rate	3	5	4	4	3	2	3
Ability to accumulate TAG	3	2	0	5	5	3	5
Molecular Biology							
Sequenced genome	1	0	5	3	3	5	0
Tailored molecular toolbox	1	2	5	0	4	5	0
Knowledge on metabolism	2	1	5	2	3	3	2
Down stream processing							
Large cell size	2	1	3	3	1	3	3
Cell disruptability	2	2	2	2	2	4	2
Total score		50	47	47	62	55	57

6.4.2 Classical strain improvement

Besides the natural diversity of the current explored microalgal strains, strain improvement can further enhance the volumetric TAG productivity. A well-established method for strain improvement is random mutagenesis using UV light, X-ray, or chemicals (Fu et al., 2016; Lee et al., 2014). Random mutagenesis is a time consuming process where first random mutants are generated, then selected and thereafter further characterized (Cagnon et al., 2013; de Jaeger et al., 2014; Kwaroo et al., 2015; Liu et al., 2015; Sandesh Kamath et al., 2008). The most successful strategy is reported by de Jaeger et al. (2014), where random mutagenesis resulted in a starchless mutant of *Acutodesmus obliquus* with one of the highest TAG yields on light reported so far at moderate light intensities ($0.22 \text{ g TAG mol}_{\text{ph}}^{-1}$ with an incident light intensity of $500 \mu\text{mol m}^{-2} \text{ s}^{-1}$). Despite the success with *A. obliquus*, other starchless mutants did not show increased TAG productivities (Li et al., 2010a; Ramazanov and Ramazanov, 2006; Work et al., 2010).

Alternatively, the availability of more than 20 whole algal genomes and recent developments in molecular toolboxes allows for (targeted) genetic engineering strategies to improve (lipid) metabolism (Hamed, 2016; Radakovits et al., 2010; Randhawa et al., 2017). Although many attempts were made, only a few studies resulted in strains with improved TAG productivities (Table 6.1). This clearly illustrates that a systematic approach is lacking so far.

6.4.3 Targeted engineering strategies: localizing the rate-limiting step

To improve the efficiency of (genetic) strain improvement, it is crucial to know which pathways to target. Many studies suggest that the reduced sunlight-to-TAG conversion efficiency is mainly caused by an impaired activity of the photosynthetic machinery, however, clear scientific evidence is lacking so far. The reduced PE under nitrogen starvation might be a result of many factors, such as limitations in light absorption (antenna systems or pigment composition), CO_2 fixation, or even further downstream in the metabolism (anabolism and/or catabolism). To identify targets for optimization it is thus crucial to determine the rate limiting pathway(s) or step(s). We

should therefore uncouple energy harvesting and subsequent conversion of sugar precursors from further downstream metabolism (anabolism and catabolism).

One approach to decouple the photosystem from downstream metabolism in microalgae is by heterotrophic lipid production (Figure 6.2). When microalgae are grown heterotrophically, the conversion of organic carbon into carbon intermediates can replace photosynthetically produced molecules (Morales-Sánchez et al., 2015). Many microalgae can be grown heterotrophically on glucose or acetate as substrate (Lowrey et al., 2016; Morales-Sánchez et al., 2015). Heterotrophic, nitrogen limited, cells can produce TAG (Liang et al., 2009; Xu et al., 2006), and thus after optimizing carbon and nitrogen feed strategies, heterotrophic growth experiments can be used to measure the maximum rate and yield of TAG on either glucose or acetate (Marudhupandi et al., 2016). A comparison of both heterotrophic and phototrophic lipid accumulation in microalgae will provide a quantitative overview on the efficiency of both up- and downstream metabolism. Hereby it will become possible to determine whether strain improvement should target either up- or downstream metabolism.

Figure 6.2 – Phototrophic and heterotrophic lipid production in microalgae.

6.4.4 Targeted engineering strategies: improving annotation and multi-gene approaches

When the critical parts of either up- or downstream metabolism have been identified, research should continue on improving the flux through the specific pathway(s) with bottlenecks. To do so, transcriptomic and proteomic platforms have proven to be very successful to identify targets for strain improvement (Ishii et al., 2007; Kalita et al., 2014; Pomraning et al., 2016). The use of transcriptomics and proteomics platforms for microalgae is increasing, but, as mentioned before, its effectiveness is strongly restricted by the limited information on gene annotation and function. For example, a multi-omics study on nitrogen limited cultures of *Phaeodactylum tricornutum* (Chapter 3) shows that, from all differentially expressed transcripts, only 26% could be matched to a corresponding protein. Furthermore, functional description and/or location of (isomeric) genes are also often limited (Chapter 3, this thesis). Identification and subsequent targeted genetic manipulation of key enzymes to improve algal lipid production is therefore difficult.

Chapter 3 (this thesis), for example, showed mainly differential regulation of the ribosomal, central carbon, photosynthetic and proteins synthesis pathways in *P. tricornutum* during lipid accumulation. Up to now, some targeted omics studies revealed biological functions of specific genes or proteins, but clear insight into metabolism is lacking (Bai et al., 2016; Ge et al., 2014; Nobusawa et al., 2017). Also, targeting individual genes, which were earlier reported as rate limiting in fatty acid synthesis of microalgae, have resulted into limited success (Table 6.1). The absence of increased yields might be due to the lack of insight into regulatory pathways. However, it could also be that single-gene overexpression/deletion is not sufficient enough to improve lipid yields, and that a multi-gene approach is actually required. Recent work (Blazeck et al., 2014) in the yeast strain *Yarrowia lipolytica* showed that simultaneous expression of ACL, ME, and DGAT with simultaneous deletions of genes in the β -oxidation pathway resulted in cells with lipid contents up to 90%w/w and titres exceeding 25 g L⁻¹ lipids (60-fold improvement compared to the wildtype). This clearly illustrates the benefits of a multi-gene approach rather than single gene overexpression.

It is evident that many breakthrough developments are required to facilitate targeted genetic and metabolic engineering strategies in microalgae. To do so, future research should focus on gene discovery and identification of the biological function of relevant genes in lipid production. Discovery of gene candidates to improve lipid production in microalgae can take place by combining traditional mutagenesis (such as UV-mutagenesis), high-throughput (HT) screening studies and whole genome resequencing. Traditional mutagenesis allows for a large variety of multiple genetic altered mutants that can subsequently be screened for interesting phenotypes (e.g. sustained or increased PE and/or increased lipid content) as also described earlier (Cabanelas et al., 2015; Chen et al., 2009; Duong et al., 2012; Terashima et al., 2015). The recent technological advances in microfluidic HT selection (Kim et al., 2016) will allow for faster and more comprehensive screening of oleaginous strains. Subsequently, the corresponding genotype can be determined via whole genome resequencing (Schierenbeck et al., 2015). This approach generates potentially mutants with both knock-outs and overexpressed genes in multiple places, which makes identification of the responsible mutation still laborious and expensive.

Alternatively, traceable random insertional mutagenesis combined with HT screening can also be used as tool for identifying gene candidates. Here, knockout mutants are generated by random-site integration of DNA with a traceable sequence (Goodman et al., 2009; Li et al., 2016; Zhang et al., 2014) and antibiotic resistance site for fast selection of mutants. After selection of a potentially interesting phenotype (again using HT methods combined with FACS), the responsible knock-out gene can quickly be identified by sequencing of short genomic regions that are cut by a type II restriction endonuclease (Li et al., 2016). Important to note here is that random insertional mutagenesis only generates knock-out strains. Phenotypes with strongly decreased PE or TAG content should also be selected for further analysis. The knock-out genes can be crucial to either efficient photosynthesis or TAG biosynthesis, and could play as well an important role in boosting lipid productivity.

Additionally, novel genetic tools, such as CRISPR/Cas9, reveal great potential for targeted metabolic engineering of microalgal metabolism. However, only a small number of strains have been successfully engineered with CRISPR/Cas9 (Nymark et

al., 2016; Shin et al., 2016; Wang et al., 2016). More resources should be invested in characterizing, standardizing and sharing successful toolboxes that can perform targeted changes in the metabolism. In the past decade, *Chlamydomonas* and *Phaeodactylum* were often used as model organisms strains to demonstrate the success of new genetic tools (Schierenbeck et al., 2015). However, these strains are not representative for industrially relevant lipid producing strains (Table 6.2). Therefore, also here, focus should lie on developing molecular toolboxes specifically for model platform strains (e.g., *Nannochloropsis*).

All abovementioned methods will lead to a tremendous amount of information on geno- and phenotypical correlations. The field of systems biology will accelerate the identification of target genes when detailed genome based models for microalgal metabolism are developed (Kliphuis et al., 2012). These models can be used to predict the success of engineered mutants (with either single or multiple genetic alterations) and the effect of, for example, inducible expression under specific growth conditions (Heyde et al. 2015, Iwai et al. 2015, Doron et al. 2016).

6.5 Conclusion

Although large improvements have been made in the selection and cultivation of microalgae for industrial applications, commercial large scale bulk lipid production is not yet feasible. Outdoor production still shows reduced TAG productivities and yields compared to small scale, indoor cultivations. This decrease in yield is mainly caused by the fluctuating daily and seasonal insolation. To cope with this, innovative (i.e. tilting) reactor designs combined with dynamic control models should be used to anticipate on the fluctuations. To build proper dynamic control models, pilot-scale outdoor experiments should be performed to gain more knowledge on the physiological response to daily- and yearly cycles of irradiance.

Alternatively, advances in microalgal synthetic biology can offer tremendous opportunities to improve TAG productivity in tailor made microalgal cell factories. Microalgal strain improvement, however, is still strongly inhibited by the lack of knowledge on gene annotation and transcriptional regulation, and by the absence

of strain specific molecular toolboxes. Therefore, future research should first focus using a few industrial relevant strains. For these selected strains, initial research efforts should target gene discovery relevant to lipid production using, for example, traceable mutagenesis combined with HT phenotype screening and sequencing. Combining gene discovery with genome based models will allow identification of (multiple) genetic targets for improved microalgal lipid production. Subsequently, to include these targets into the microalgal genome, molecular toolboxes (i.e. CRISPR/Cas9) need to be developed specifically for the industrially relevant oleaginous strains. Only concerted efforts of the community on a selection of industrial relevant oleaginous strains will lead to commercial feasible bulk lipid production.

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Summary

Microalgae have received a lot of attention as promising sustainable feedstock for the food-, feed-, fuel-, and chemical industry. They are able to photosynthetically store fixed carbon in the form of proteins, lipids and carbohydrates. Microalgae have an aerial biomass productivity superior to that of traditional terrestrial crops, while they lack the competition for arable land with agriculture and they can be grown on waste- and sea water.

Microalgae mainly produce functional biomass when grown under optimal growth conditions. However, under so called 'stress conditions' (e.g., nutrient limitation or high light intensity), some microalgae can accumulate neutral lipids up to 30-60% of their dry cell weight. These neutral lipids are comprised of triacylglycerol (TAG) that can easily be converted into biodiesel. Although this is a promising and sustainable route for lipid-production, the costs are still too high. At the moment, commercial production of microalgae is only economically feasible for high value added products, such as β -carotene and ω -3-fatty acids. There is still a need for improvements in the whole microalgal production chain (i.e. cultivation and downstream processing) to move microalgal derived products from current niche-applications to large scale bulk-applications (**chapter 1**).

This thesis focussed on TAG production in microalgae. The most applied cultivation strategy to induce TAG accumulation in microalgae is batch-wise nitrogen starvation. Here, biomass is first produced in a growth phase followed by complete nitrogen exhaustion to initiate TAG accumulation. Batch-wise nitrogen starvation allows for high TAG contents (up to 60% TAG per dry weight). It is, however, also accompanied with impaired growth rates and severely reduced photosynthetic efficiencies. Previous studies showed that the photosynthetic efficiency is the most crucial parameter that influences the biomass production costs.

In depth understanding of the physiology under nitrogen deprivation is, however, still limited and it is thus currently difficult to further improve microalgal TAG production. This thesis therefore aimed to gain insight into the physiology of microalgae under nitrogen deprived growth conditions with the ultimate goal to identify targets for improving microalgal TAG production (**chapter 1**). Specific focus

was put on examining the regulation of lipid metabolism, different cultivation strategies and impact of diurnal light-dark (LD) cycles.

Among all known microalgal species, *Phaeodactylum tricornutum* is one of the few species that is already commercially produced in large scale outdoor reactors. *P. tricornutum* has the ability to produce large amounts of ω -3-fatty acids, and when it is exposed to nitrogen deprivation it produces TAG up to 30% of its dry weight. However, it is also known that high light exposure can negatively affect the biomass and lipid productivity of this marine diatom. **Chapter 2** therefore quantified the impact of different incident light intensities on the biomass, TAG and EPA yield on light in nitrogen starved batch cultures of *P. tricornutum*. The maximum biomass concentration and maximum TAG and EPA contents were found to be independent of the applied light intensity. The lipid yield on light was reduced at elevated light intensities ($>100 \mu\text{mol m}^{-2} \text{s}^{-1}$). The highest TAG yield on light ($112 \text{ mg TAG mol}_{\text{ph}}^{-1}$) was found at the lowest light intensity tested ($60 \mu\text{mol m}^{-2} \text{s}^{-1}$), but is still relatively low compared to values reported in literature for other algae. Further analysis on the fatty acid composition revealed that the TAG fraction of *P. tricornutum* contains 6-10% of EPA. Mass balance analysis revealed this EPA fraction may originate partly from photosynthetic membrane lipids that were restructured after nitrogen depletion.

The cellular response to nitrogen deprivation in *P. tricornutum* was studied in detail in **chapter 3**. The physiology, transcriptome, proteome and metabolome profiles of the diatom *P. tricornutum* were profiled during diurnal steady state growth at nitrogen replete and limited levels. Nitrogen limitation resulted in an increase in TAG content from 3 to 16% of dry weight, but at the expense of growth rate and photosynthetic efficiency. Transcript and proteome profiling revealed that in total 2699 transcripts, 543 proteins and 345 metabolites were differentially expressed under nitrogen limitation. Functional analysis revealed that nitrogen limitation mainly induced upregulation of nitrogen fixation and central carbon metabolism, while photosynthetic, ribosomal and protein synthesis pathways were mainly downregulated. This integrated omics study provides a detailed overview of the

metabolic response of *P. tricornutum* to nitrogen limitation and can be used as useful guide to target future strain improvements for increased TAG accumulation.

One of the main aims of this thesis was to study the impact of different lipid accumulation strategies with each other (i.e. batch and continuous lipid production). To do so, an appropriate microalgal strain should be used. **Chapter 2** and **3** showed that *P. tricornutum* has relatively low volumetric TAG productivities compared to other microalgal species. These low productivities were predominantly caused by low photosynthetic efficiency and suboptimal carbon distribution during nitrogen starvation. This made *P. tricornutum* inappropriate for further detailed characterization studies. For that reason, in **chapter 4**, *Acutodesmus obliquus* wildtype and its starchless mutant were selected. The green freshwater microalgae *A. obliquus* was selected because it is able to retain a high photosynthetic efficiency and shows strong carbon distribution towards TAG under nitrogen starvation. Furthermore, the starchless mutant showed an even further improved carbon partitioning to TAG under nitrogen starvation. **Chapter 4** provides a quantitative comparison of continuous versus batch wise lipid production under diurnal light conditions. By using both strains to evaluate the potential of continuous lipid accumulation, it was also possible to study the role of carbon partitioning (i.e. presence of starch) for both processes.

Both the wildtype and starchless mutant strain of *A. obliquus* showed higher TAG yields on light under batch ($0.20 \text{ g TAG mol}_{\text{ph}}^{-1}$ for the starchless mutant and $0.16 \text{ g TAG mol}_{\text{ph}}^{-1}$ for the wildtype) operation compared to continuous nitrogen limitation ($0.12 \text{ g TAG mol}_{\text{ph}}^{-1}$ for the starchless mutant and $0.08 \text{ g TAG mol}_{\text{ph}}^{-1}$ for the wildtype). Also, 30-50% higher TAG contents were found for batch starvation compared to continuous cultivations. **Chapter 4** therefore concludes that batch wise nitrogen starvation outcompetes continuous nitrogen limitation in terms of TAG yield on light in this alga. 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.

Literature often suggests that lipids and/or carbohydrates act as transitory energy storage (TES) to support metabolism in diurnal light:dark cycles. Phototrophic microalgae can use parts of their biomass to fuel cell division or respiration at night. **Chapter 4** describes, however, that the lipid productivity under batch wise nitrogen starvation was not negatively influenced by LD cycles for both the wildtype and starchless mutant of *A. obliquus*. To unravel the role of transitory energy storage compounds, such as starch, in growth, **chapter 5** investigates the effect of TES compound on synchronized cell division and in *A. obliquus*. Experiments with the wildtype under LD cycles and nitrogen replete conditions showed that starch acted as major transitory energy storage (TES) compound. Starch was accumulated during the last part of the light period and was consumed throughout the entire dark period. Also, *A. obliquus* wildtype utilized light 20% more efficient under LD regimes compared with continuous light. In parallel, the starchless mutant of *A. obliquus* was also studied to test if the presence of starch exclusively fuels cell division, as often hypothesized in literature. In the absence of starch, the cell division of the starchless mutant was still scheduled at night while no alternative TES compound was found (i.e. a compound that is accumulated and subsequently degraded during a 24h diurnal light cycle). This suggests that neither starch nor any other major alternative TES are required for synchronized growth and cell division in *A. obliquus*. The starchless mutant did show, however, a reduced growth and cell division rate compared to the wildtype. 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.

The general discussion (**chapter 6**) evaluates the current achievements in microalgal lipid production and provides an outlook to future research needed to further improve the solar-to-lipid conversion efficiency in microalgae. The evaluation showed that recent developments in strain selection, strain improvement, culture conditions and operational strategy did result in improved TAG productivities. At the same time, however, commercial large scale bulk lipid production is not yet feasible. Process engineering resulted in an advanced understanding of physiology, and this has been incorporated in advanced growth models. These models allow us to

simulate and predict optimal microalgae TAG production strategies under outdoor conditions. To further enhance microalgae TAG-production, cellular optimization should be established through targeted genetic engineering. The field of molecular microalgal biology is, however, still strongly inhibited by the lack in knowledge on gene annotation, function of genes in regulation and the presence of strain specific genetic tools.

Chapter 6, therefore continues by providing concrete suggestions to further improve the physiological limitations of microalgal metabolism under nutrient deprivation. For example, by decoupling the upstream (photon capture and conversion into sugars) from downstream metabolism (use of sugars for respiration and anabolism), targets can be identified for future strain improvement. However, to fully exploit the potential of microalgae, improved characterization of the metabolic pathways is necessary (i.e. targeted gene and pathway discovery). With improved annotation information, omics technologies can be used to targetedly overexpress or delete multiple genes in both central carbon and lipid metabolism.

Samenvatting

Microalgen zijn microscopisch kleine, eencellige organismen die als een interessante alternatieve grondstof voor de voedsel-, brandstof- en chemie-industrieën dienen. Microalgen zijn in staat om, door middel van fotosynthese, CO₂ om te zetten in voor de mens interessante producten zoals eiwitten, vetten, koolhydraten, en pigmenten. Deze producten kunnen vervolgens weer gebruikt worden voor onder andere de productie van biobrandstoffen, voedingssupplementen, visvoer, of additieven voor de cosmetische- en verfindustrie.

Microalgen gebruiken zonlicht als energiebron om te groeien. De voedingsstoffen die nodig zijn voor groei (zoals stikstof en fosfaat) halen ze direct uit zoet- of zeewater, of zelfs uit afvalwater. Daarnaast kan de opbrengst van microalgen per vierkante meter landoppervlak veel hoger liggen dan die van traditionele landbouwgewassen. Door deze voordelen kunnen algen dus op veel verschillende manieren bijdragen aan een duurzame economie.

Het hier beschreven onderzoek richt zich op de productie van biobrandstoffen met behulp van algen. Wanneer microalgen voldoende voedingsstoffen krijgen zullen ze normaal groeien. De gevormde microalgen biomassa zal overwegend bestaan uit eiwitten, koolhydraten, en membraanvetten. Echter, wanneer één of meerdere voedingsstoffen onvoldoende aanwezig zijn in het groeimedum en de algen nog steeds energie in de vorm van zonlicht ontvangen, kunnen sommige microalgen vetten ophopen tot wel 30-60% van hun drooggewicht. Deze neutrale vetten bestaan uit *triacylglycerolen* (TAG). Deze TAGs zijn de grondstof voor de productie van biodiesel. Maar hoewel de biodieselproductie met algen veelbelovend is, zijn de huidige productiekosten nog steeds te hoog om commerciële productie haalbaar te maken.

Op dit moment worden microalgen alleen commercieel gebruikt voor de productie van hoogwaardige componenten zoals beta-caroteen en omega-vetzuren. Om de productiekosten van microalgen te reduceren zodat we in de toekomst ook op een economisch rendabele manier laagwaardige bulkchemicaliën (zoals biobrandstof) met algen kunnen maken is meer onderzoek nodig (**hoofdstuk 1**).

TAG wordt met name gemaakt tijdens zogenoemde *stresscondities* waarbij één of meerdere voedingsstoffen onvoldoende aanwezig zijn in het groeimedium. TAG wordt dan door microalgen gebruikt als energieopslag, waarbij het overschot van de energie uit zonlicht opgeslagen wordt voor later gebruik. De meest toegepaste methode voor TAG-productie met microalgen is stikstofuithongering onder zogenaamde *batch-condities*. In een batchproces voor TAG-productie wordt eerst biomassa gevormd in een groeifase waarbij de voedingsstoffen in overmaat aanwezig zijn. Na de groeifase volgt een productiefase waarbij de hoeveelheid voedingsstoffen, en met name stikstof, afneemt. Omdat een microalgencel stikstof nodig heeft om te blijven delen, kan de cel de energie uit zonlicht niet meer gebruiken voor groei. De energie wordt opgeslagen als TAG. Daarom maken algen in de productiefase grote hoeveelheden TAG (tot wel 60% TAG per droog gewicht). Helaas is een bijeffect van dit batchproductieproces dat de fotosynthetische efficiëntie (maat waarin zonlicht omgezet wordt in biomassa met behulp van fotosynthese) sterk gereduceerd wordt (immers, de cel kan geen stikstof meer opnemen en dus niet meer groeien en/of onderhoud plegen).

Een goed begrip van het *algenmetabolisme* (stofwisseling), het effect van groeistrategieën, en het effect van buitencondities (denk hierbij aan grote variaties in de lichtintensiteit vanwege bewolking en zonsterkte, daglengte, etc.), is nodig om de kosten van TAG-productie met behulp van microalgen te kunnen reduceren. In dit proefschrift is onderzoek gedaan naar het metabolisme van algen onder stikstofarme groeicondities. De opgedane kennis is daarna gebruikt om nieuwe doelen te identificeren om TAG-productie op grote schaal met microalgen te verbeteren (Hoofdstuk 1).

In dit proefschrift zijn twee verschillende microalgen gebruikt. De eerste, *Phaeodactylum tricornutum*, is een zoutwater diatoom die momenteel al gebruikt wordt voor commerciële productie van biomassa. Deze alg heeft een bruine kleur dankzij het pigment fucoxantine, kan grote hoeveelheden omega 3-vetzuren maken, en kan TAG tijdens stikstofarme groeicondities maken. Vanuit literatuur is bekend dat hoge lichtintensiteiten (zoals die ook in buiten op grote schaal productie behaald kunnen worden) een negatief effect op zowel de biomassa als TAG productie kunnen

hebben. In **hoofdstuk 2** hebben we daarom het effect van licht op TAG-productie gekwantificeerd. Hoewel de maximaal behaalde biomassa en TAG-concentratie niet afhingen van de toegevoegde hoeveelheid licht, vonden we wel verlaagde omzettingsefficiënties van energie van zonlicht naar TAG bij hoge lichtintensiteiten. Daarnaast was de omzetting van zonlicht naar TAG minder efficiënt in vergelijking tot andere algensoorten.

In **hoofdstuk 3** is het metabolisme van *P. tricornutum* in detail bestudeerd. Om het metabolisme op de juiste manier te bestuderen is er gebruik gemaakt van zogenaamde continue systemen, waarbij de toevoer van medium wordt geregeld op basis van de groeisnelheid van de alg. Hierdoor waren we in staat om hele stabiele en reproduceerbare experimenten uit te voeren onder zowel stikstofrijke als stikstofarme condities. Van beide condities zijn vervolgens biomassacompositie, en RNA- (boodschappers van genetische informatie), eiwit-, en metaboliet concentraties bestudeerd. Op deze manier is getracht om nieuwe informatie te verkrijgen over cruciale stappen in het metabolisme die kunnen leiden tot verbeterde TAG-productie. Onder stikstofrijke groeicondities bestond de alg voor 3% uit TAG, terwijl dit onder stikstofarme condities toenam tot 17% van het drooggewicht. We hebben 2699 RNA-transcripten, 543 eiwitten en 345 metabolieten kunnen identificeren die significant en differentieel verschillend waren onder stikstofarme groeicondities. Dit betekent dat er grote veranderingen plaatsvinden in het metabolisme wanneer een alg blootgesteld staat aan stikstofarmoede. De grootste veranderingen vonden plaats in het vastleggen van stikstof, in de centrale routes van het metabolisme, en bij fotosynthese en eiwitproductie. De inzichten uit deze studie fungeren als leidraad voor nieuw onderzoek voor stamverbetering van microalgen met verbeterde TAG-productie.

Er zijn meerdere manieren om TAG met behulp van microalgen te maken. De eerste is door middel van een batch cultivatieproces (**hoofdstuk 2**) of een continu cultivatieproces (**hoofdstuk 3**). In een batchproces zijn er twee fases: eerst biomassagroei, en daarna TAG-productie onder complete stikstoflimitatie. In een continu proces wordt er een beperkte hoeveelheid stikstof toegediend aan de alg, waardoor er gelijktijdig groei en TAG-productie kan plaatsvinden. Een bijkomend

voordeel van een continu proces is dat de kweekcondities uiterst reproduceerbaar en stabiel zijn, zoals nodig was voor het onderzoek beschreven in **hoofdstuk 3**.

Het is echter nog niet duidelijk welke van deze twee kweekstrategieën tot het beste resultaat (namelijk: wat is de meest efficiënte TAG-productie) leidt. In **hoofdstuk 4** hebben we daarom beide kweekstrategieën geëvalueerd met een efficiëntere TAG-producerende alg: *Acutodesmus obliquus*. *A. obliquus* is een groene zoetwateralg die tot wel 60% van zijn drooggewicht aan TAG kan maken. Alhoewel er hoge verwachtingen geschapen waren voor het continue kweekproces, bleek dat de traditionele batch twee-staps-strategie voor de meest efficiënte TAG-productie zorgde. Verder onderzoek, waarin ook het oogsten, zuiveren van TAG, en grote schaal productie worden meegenomen, moet uitgevoerd worden om het kostenplaatje van beide kweekstrategieën te schetsen.

Wanneer algen op grote schaal onder buitencondities gekweekt worden, zijn er een hoop variabele parameters (temperatuur, variabele lichtintensiteit, daglengte). Bij laboratoriumproeven op kleine schaal kunnen experimenten onder ieder gewenste conditie worden uitgevoerd. Het is daarom mogelijk om buitenexperimenten te simuleren met variabele dag-nacht cycli, lichtintensiteiten en temperatuur. Om hoge efficiëntie in buitenreactoren te halen, is het cruciaal om het metabolisme van algen ook onder dag-nachtcycli te begrijpen. **Hoofdstuk 5** beschrijft de impact van dag-nachtcycli op de energiehuishouding van *A. obliquus*. In het *wildtype* (de natuurlijk voorkomende variant van een organisme) wordt gedurende de dag zetmeel geproduceerd. Tijdens de nacht wordt deze opgebouwde reserve van zetmeel geconsumeerd en vindt celdeling plaats. Echter, bij de zetmeelloze *mutant* (dat is een variant van het organisme met een mutatie zodat deze, in dit geval, geen zetmeel meer kan produceren) van *A. obliquus* vindt de celdeling nog steeds exclusief in de nacht plaats. Deze bevindingen bewijzen dat, ondanks eerder geformuleerde hypothesen, zetmeel niet uitsluitend nodig is voor gesynchroniseerde celgroei onder dag-nachtcycli.

Alhoewel er het laatste decennium grote stappen zijn gemaakt richting optimalisatie van TAG-productie met microalgen is commerciële bulkproductie nog niet haalbaar

(hoofdstuk 6). Een grote tekortkoming is het ontbreken van microalgen die geschikt zijn voor industriële productie op grote schaal. Er is daarom behoefte aan verdergaande studies waarin moleculaire biologie wordt ingezet om de huidige microalgen verder te optimaliseren richting voor de industrie geschikte soorten. Belangrijke criteria hierin zijn met name de vetproductiviteit, cultivatie, en verwerkbaarheid van de microalgen. Ook hebben we in de afgelopen jaren veel kennis van grote schaal openlucht-algencultivatie opgedaan. Wanneer we nog beter kunnen inspelen op de verschillen in openluchtcondities (bijvoorbeeld fluctuerend zonlicht en wisselende temperatuur), kan de productiviteit nog verder stijgen. Dit vereist verdere studies richting geavanceerde cultivatiestrategieën. Alles tezamen zullen deze onderzoeksinspanningen uiteindelijk resulteren in een competitief en duurzaam olieproductieproces met microalgen als productieplatform.

Nomenclature

C_{cells}	Concentration of cell number
CHO	Carbohydrates
C_i	Concentration of component i
CL	Continuous light
$C_{x,N=0}$	Biomass concentration at nitrogen starvation
D_{24}	Average dilution rate over 24 hours
DO	Dissolved oxygen
D_t	Dilution rate over small time intervals
DW	Dry weight
$E_{n,\lambda}$	Relative fraction of PAR photons present in 1 nm wavelength interval
EPA	Eicosapentaenoic acid
FACS	Fluorescent active cell sorting
F_i	Flow rate of component i
F_v/F_m	Maximum efficiency of PSII chemistry
HT	High throughput
LD	Light-dark
ML	Membrane lipid
mol_{ph}	Mol photon
M_x	Amount of biomass
N	Nitrogen
NSC	Non starch carbohydrate
OD	Optical density
PAR	Photosynthetic active region
PCA	Principle component analysis
PFD_{in}	Incident light intensity
PFD_{out}	Outgoing light intensity
PS II	Photosystem 2
PUFA	Polyunsaturated fatty acid
r_i	Volumetric productivity of component i
slm1	Starchless mutant 1
t	Time
TAG	Triacylglycerol
TES	Temporary energy storage
TFA	total fatty acids
$t_{N=0}$	Time at nitrogen starvation
wt	Wildtype
$Y_{\text{TAG,ph}}$	Triacylglycerol yield on light
$Y_{x,\text{ph}}$	Biomass yield on light
z	Reactor dept
α_c	Dry weight-specific optical cross section
μ_t	Time specific cell division rate
ω -3-fatty acids	Omega 3 fatty acids

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About the author

Curriculum Vitae

Ilse Marije Remmers was born on July 7th, 1989 in Groningen, the Netherlands. She attended 'het Drachtster Lyceum' in Drachten where she obtained her HAVO diploma in 2006. That same year she started her Bachelor of Applied Sciences in Biotechnology at Van Hall-Larenstein and Noordelijke Hogeschool Leeuwarden. She worked during her internships at DSM, MSD and Wageningen UR on topics related to 2nd generation feedstock fermentations, pharmaceutical antibody production and microalgae cultivation. She obtained her BSc in 2010 and she continued studying for a MSc in Biotechnology at Wageningen UR.

In Wageningen she focussed on bioprocess engineering with special interest in cell factory design. In her MSc thesis at the Bioprocess Engineering group of Wageningen UR, she cultivated microalgae for lipid production. For her internship she visited the Nielsen laboratory at Chalmers University in Göteborg, Sweden, where she worked on substrate preference in fungi for organic acid production.

After obtaining her MSc degree in 2012, she started her doctorate (PhD) research at the Bioprocess Engineering group of Wageningen UR. Her PhD project was carried out within the EU FP7 funded 'Fuel4ME' program, where she studied continuous lipid production in microalgae. The results of her PhD research are described in this thesis.

List of publications

IM Remmers, RH Wijffels, MJ Barbosa, PP Lamers. Can we approach theoretical lipid yields in microalgae?, submitted for publication

IM Remmers, DE Martens, RH Wijffels, RCH de Vos, R Mumm, AHP America, JHG Cordewener, LV Bakker, SA Peters, PP Lamers. Orchestration of transcriptome, proteome and metabolome in the nitrogen limited diatom *Phaeodactylum tricornutum*, submitted for publication

GM León-Saiki*, **IM Remmers***, DE Martens, PP Lamers, RH Wijffels, D van der Veen (2017). The role of starch as transient energy buffer in synchronized microalgal growth in *Acutodesmus obliquus*. *Algal Research* 25, 160–167

*Authors contributed equally

Remmers IM, Hidalgo-Ulloa A, Brandt BP, Evers WAC, Wijffels RH, Lamers PP (2017). Continuous versus batch production of lipids in the microalgae *Acutodesmus obliquus*, *Bioresource Technology*, in press

Remmers IM, Martens DE, Wijffels RH, Lamers PP (2017). Dynamics of triacylglycerol and EPA production in *Phaeodactylum tricornutum* under nitrogen starvation at different light intensities. *PLOS ONE* 12(4): e0175630

Knuf C, Nookaew I, **Remmers I**, Koomrung S, Brown S, Berry A, Nielsen J (2013).

Physiological characterization of the high malic acid-producing *Aspergillus oryzae* strain 2103a-68. *Appl Microbiol Biotechnol* 98: 3517

Overview of completed training activities

Discipline specific activities

Conferences

Minisymposium: Microalgae biotechnology:	2013
towards industrial strains and processes	
Symposium 'Pandora's box: what came out' ¹	2014
Netherlands Biotechnologie symposium, (Ede, the Netherlands) ²	2014
MiniSymposium Oil production in algae	2014
2 nd edition AlgnChem, (Montpellier, France) ²	2014
2 nd edition Young Algaeneers Symposium, (Montpellier/Narbonne, France) ²	2014
EU Conference Algae Roadmap, (Olhão, Portugal) ²	2016
European Symposium on Biochemical Engineering Sciences (Dublin, Ireland) ²	2016

Courses and meetings

EPS PhD course 'Systems Biology: statistical analysis of `omics data'	2012
VLAg Microalgae process design: from cell metabolism to photo bioreactors	2013
BSDL Advanced Course Microbial Physiology and Fermentation Technology	2016
Fuel4Me EU Consortium meetings ²	2013-2016

General courses

VLAg PhD week	2013
WGS PhD Competence Assessment	2013
WGS Teaching and Supervising Thesis Students	2014
WGS Voice Matters ²	2014
WGS Carrousel	2014
WGS The Essentials of Scientific Writing and Presenting	2015
WGS Stress identification and management	2015
WGS Project and Time Management	2015
WGS Career Perspectives	2016

Optionals

Preparation of research proposal	2013
International PhD study trip (Portugal) ^{1,2,3}	2014
BPE Symposia and brainstorm days ^{1,2}	2013-2016

1 – Poster; 2 – Oral presentation; 3 – Organisation

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