Circadian rhythms in microalgae production

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Circadian rhythms in microalgae production

Lenneke de Winter

Thesis

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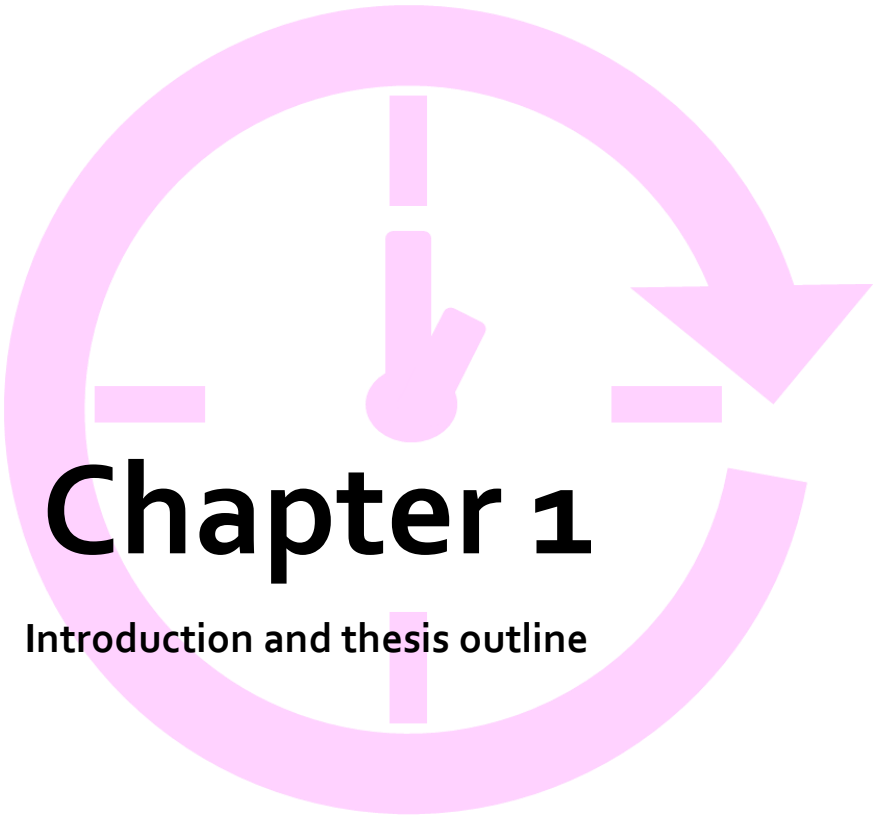


Chapter 1

Introduction and thesis outline

1.1 Circadian rhythms

The sun imposes a daily cycle of light and dark on nearly all organisms. The circadian clock evolved to help organisms program their activities at an appropriate time during this daily cycle. For example, UV sensitive processes, like DNA replication, can be scheduled to occur during the night (Nikaido and Johnson, 2000). In this way, the circadian clock causes rhythms in metabolic, physiological and/or behavioural events (Mittag et al., 2005). These circadian rhythms continue for some period of time following exposure to continuous light (Harding Jr et al., 1981) and have a duration of approximately 24 hours.

In microalgae, circadian rhythms were observed in many processes, like nitrogen fixation, chemotaxis, photosynthesis and the cell division cycle (Mittag, 2001), which might affect the production of microalgae. Microalgae biomass can be used as source for potential biofuels, chemicals, materials, foods, feeds and high-value bioactives (Chisti, 2007; Hu et al., 2008; Wijffels and Barbosa, 2010). However, the current production process of microalgae needs to be optimized in order to become economically feasible (Norsker et al., 2011). Researchers focussed on optimizing PBR design (Molina et al., 2001; Morweiser et al., 2010; Sierra et al., 2008), operating strategies (Cuaresma et al., 2011; Morweiser et al., 2010; Olivieri et al., 2014) and microalgae metabolism (Guschina and Harwood, 2006; Klok et al., 2013a), but as of yet did not consider the possible influence of circadian rhythms on microalgae production.

1.2 Circadian control of the cell cycle

The green microalgae *Neochloris oleoabundans* was grown under continuous red LED light in a photobioreactor (PBR) operated as a turbidostat, which means that the biomass concentration in the PBR was kept constant by automatic dilution with fresh medium once light intensity at the back of the reactor dropped below a certain set-point. In these constant conditions, cycles with a 24 hour duration were observed in the dilution rate (see figure 1.1) (de Winter et al., 2013). These 24h cycles in dilution rate suggested a circadian rhythm in the cell division cycle. Circadian control of the cell cycle has been observed in different species of microalgae and cyanobacteria. In the cyanobacterium *Synechococcus elongatus*, rhythmic cell division cycles occur due to 'gating' of cell division to a specific time frame in the circadian cycle (Mori and Johnson, 2001; Yang et al., 2010). In eukaryotic microalgae, cell division is often synchronized during the night (Bolige et al., 2005; Carre and Edmunds, 1993; Matsuo et al., 2010). Therefore, synchro-

nous cultures can be maintained under day/night cycles in which all cells go through the different stages of the cell cycle at the same time. In *N. oleoabundans*, it was possible to keep such a synchronous culture also under continuous red LED light, where presumably synchronization was triggered by some blue environmental light in the laboratory (see chapter 2).

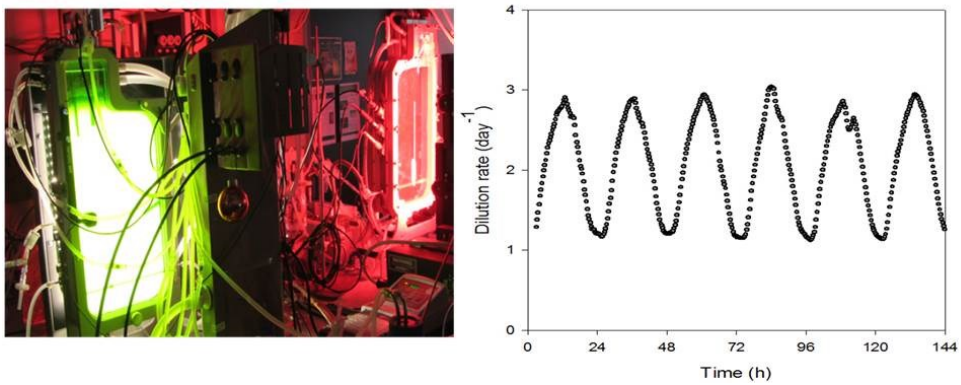


Figure 1.1 *Dilution rate (right) in a PBR operated as a turbidostat with Neochloris oleoabundans under continuous red LED light (left picture, right reactor). Under continuous white LED light (left picture, left reactor) the cycle disappeared*

When eukaryotic cells dividing by binary fission are in conditions that allow them to double more than once per day, the daily periodicity often disappears and synchrony is lost (Suzuki and Johnson, 2001). However, some green algae evolved a mechanism for cell division called multiple fission, in which the number of daughter cells per mother cell usually ranges from 4 to 16 (Bišová and Zachleder, 2014). This mechanism allows the cells to grow during the day, when light is available, and undergo multiple rounds of DNA replication and cell division in the dark. The number of daughter cells is dependent on external conditions, and can be described by 2^n, in which n is the number of doublings (see figure 1.2) (Bisova et al., 2005). In this way, synchronized cultures can be maintained under day/night cycles at high growth rates.

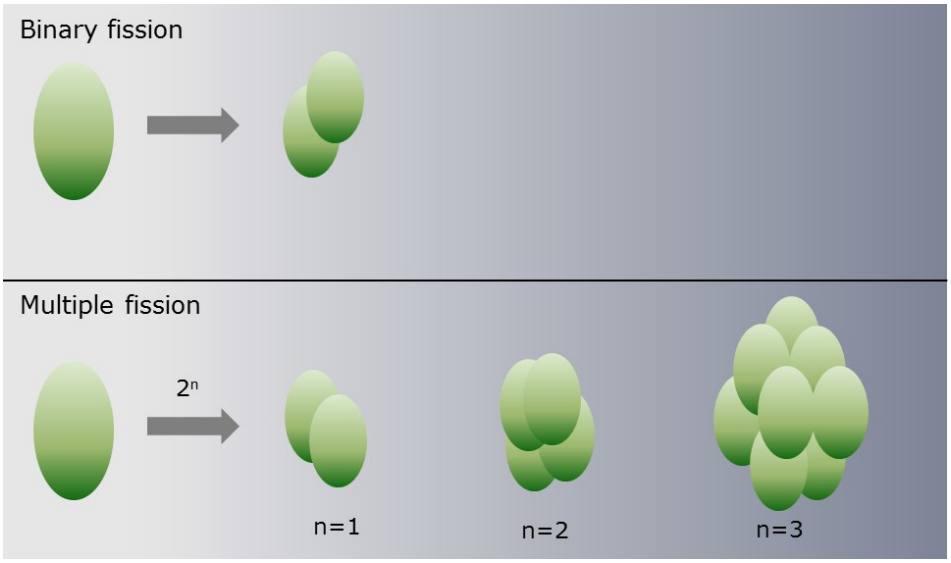


Figure 1.2 *Binary fission versus multiple fission, in which an algae mother cell divides into two or 2^n daughter cells, respectively*

1.3 Synchronous cultures in photobioreactors

In a synchronous culture, cells invariably reveal an increase in the carbon storage reserves like polysaccharides or lipids in the light period and a decrease of these reserves in the dark period (Falkowski and Raven, 2007). Therefore, the composition of cells in a synchronous culture varies throughout the day. In addition, there will be variation in chlorophyll content and photosynthetic rates during a 24h cycle (Falkowski and Raven, 2007). Nevertheless, research done on biomass composition and photosynthetic efficiency of microalgae often uses continuous light (Breuer et al., 2012; Kliphuis et al., 2010), which might not be representative for outdoor cultivation. Therefore, there is a need for research on biomass composition and yield on light done in PBRs under day/night cycles.

In the laboratory, day/night cycles can be supplied to PBRs in different ways. Light can be either switched on and off abruptly, or gradually increased and decreased during the light period, in which the latter choice would be more similar to the natural light cycles. In addition, the duration of the light period can be varied, like the varying day lengths over the course of a year. Different lighting regimes can have an influence on circadian behaviour of the microalgae cultures. For example, circadian rhythms can adapt to vary-

ing day lengths (Beneragama and Goto, 2010), and as such the timing of processes like photosynthesis and cell division might be influenced.

1.4 Aim and thesis outline

Biomass growth rate, biomass yield on light, and the biochemical composition of algal biomass are important factors in the production of microalgae. These factors are likely to be influenced by the day/night cycle and the circadian clock. Therefore, the aim of the work presented in this thesis is to obtain more insight in circadian rhythms in microalgae grown in photobioreactors.

In **chapter 2** it is described how the green microalgae *Neochloris oleoabundans* was grown in a photobioreactor operated as a turbidostat under continuous red LED light. Cell division in *N. oleoabundans* was shown to be under control of the circadian clock, and took place by multiple fission during the natural night. Due to the synchronized cell division, oscillations in biomass yield and composition were observed, despite the continuous red LED light. Synchronization disappeared under continuous white LED light, and therefore it was concluded that a blue light receptor might be involved in triggering synchronous cell division of *N. oleoabundans.*

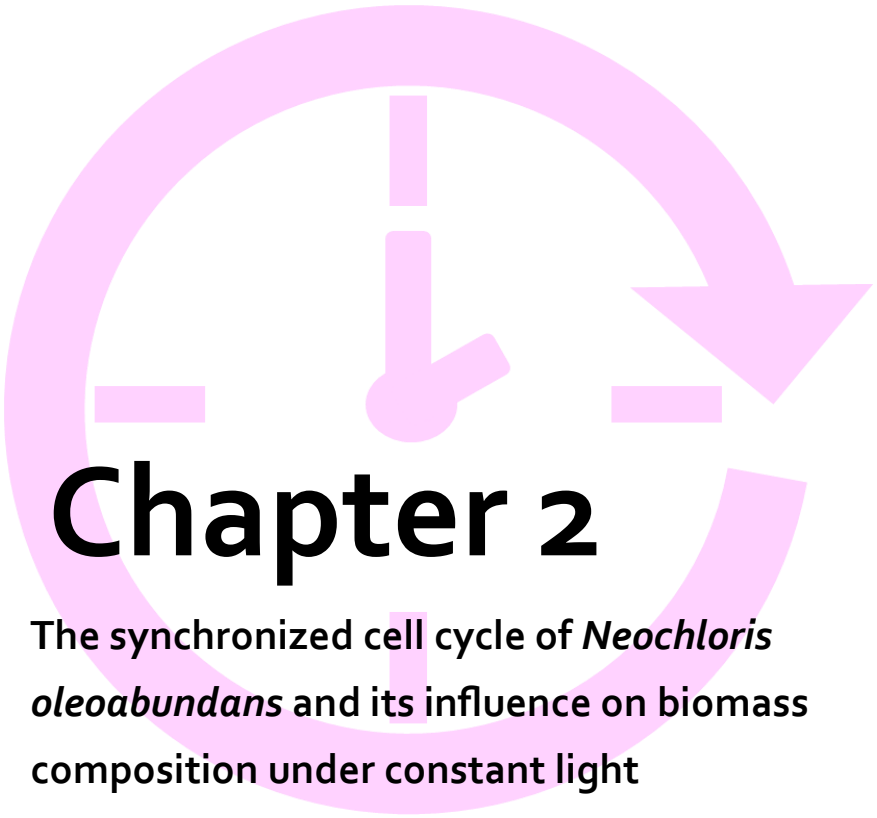
As biomass composition is also dependent on other culture conditions, the same set-up was used in **chapter 3**, only this time the culture was grown nitrogen-limited, as this is the most commonly used method for the production of storage components. In this way, it was shown that under nitrogen limitation the circadian clock was still timing cell division to the natural night. However, because of the lower growth rate, two subpopulations were observed which divided alternately every other day. Again, oscillations in biomass composition were observed. Neutral lipids were built up during the day, especially in cells that were arrested in their cell cycle.

After having studied the circadian clock under continuous light conditions, a step was made to day/night cycles. **Chapter 4** describes a comparison of biomass yield and composition between a synchronized culture under day/night cycles and a randomly dividing culture under continuous white LED light. In this way it was shown that circadian rhythms had a small influence on biomass yield, with biomass yield on light being 10-15% higher in synchronized cultures. Also biomass composition was influenced, as in continuous light starch never had to be spend for respiration during a dark period and therefore starch content remained higher. For the experiments with a day/night cycle, no difference was found between light supplied at constant intensity (block) or light sup-

plied in a more natural way (sine). Therefore, providing light in a block showed to be a good and easy to operate alternative to using sinuses when working with day/night cycles in the laboratory.

Chapter 5 takes a closer look at the multiple fission cell cycle of *N. oleaobundans*. Day/night cycles of different lengths and intensities were studied, as algae are exposed to different day lengths over the course of a year. Maximum growth rate and start of starch synthesis seemed to be regulated by the circadian clock and were scheduled after approximately 6-7 hours from sunrise. Therefore, they were not influenced by day length. However, day length did have an influence on biomass composition. In longer days, more starch was accumulated. The changes in biomass composition could also be correlated to the cell cycle of *N. oleoabundans*, and therefore knowledge about the timing of cell division showed to be important for the production of biomass with a desired concentration of protein, lipids, carbohydrates or pigments.

In **chapter 6** the implications of the overall results of this thesis for current research protocols and microalgae processes are discussed. First the occurrence of circadian rhythms in different species of microalgae is discussed, in order to establish the general nature of these rhythms. Based on the findings for *N. oleoabundans*, it is concluded that more research should be done using day/night cycles, as experiments under constant light are not representative for outdoor microalgae production. Still more knowledge is required on circadian rhythms in microalgae production and therefore some opportunities for future research are presented. Finally, it is discussed how manipulation of circadian rhythms might help to improve future microalgae production.



Chapter 2

The synchronized cell cycle of *Neochloris oleoabundans* and its influence on biomass composition under constant light

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The Synchronized cell cycle of neochloris oleoabundans and its influence on biomass composition under constant light

Algal research 2, pp.313-320

Abstract
The effect of cell cycle stage on biomass composition of the green microalgae *Neochloris oleoabundans* was investigated. *N. oleoabundans* was grown under constant light conditions in a flat panel photobioreactor operated as a turbidostat. Even though light conditions were constant, a synchronized cell division was observed with the cells dividing by multiple fission during the natural night. Presumably, the circadian clock was responsible for 'gating' cell division to this specific time frame. Oscillations in starch, protein and pigment content were observed during the cell cycle. These oscillations could be solely contributed to the cell cycle stage of the synchronized culture, since all experimental conditions were kept constant. A maximum in starch, protein and fatty acid content was obtained just before cell division. Biomass yield was also greatly influenced by the cell cycle and declined to a minimum during cell division. These findings highlight that knowledge of the cell cycle is of importance in microalgae process optimization.

2.1 Introduction

Microalgae can efficiently use sunlight energy to convert carbon dioxide into biomass. This biomass can be used as source for potential biofuels, chemicals, materials, foods, feeds and high-value bioactives (Chisti, 2007) (Wijffels and Barbosa, 2010). In any of these applications, the biochemical composition of the algal biomass is of importance, because the desired product will determine which part of the biomass will be used. In biofuel production, for example, biomass with a high lipid content is desired.

It has long been recognized that biomass composition during a day/night cycle is not constant. In general, carbon storage reserves like carbohydrates or lipids build up during the day and decrease during the night (Fábregas et al., 2002; Post et al., 1985b; Sukenik and Carmeli, 1990). Also chlorophyll content reaches a maximum during the day and decreases before the dark period (Falkowski and Raven, 2007). Important metabolic processes can also occur in the night. Frequently, carbon reserves and energy that built up during the day are used for net synthesis of proteins during the night (Cuhel et al., 1984). Consequently, biomass composition oscillates during the day/night cycle.

Diel oscillations, like the oscillation in chlorophyll content, are often not only driven by the external cycle of day and night, i.e. the photocycle, but actually are regulated by an endogenous biochemical oscillator called the circadian clock (Mittag, 2001). The circadian clock helps organisms schedule certain processes within a suitable temporal window. It might for example be beneficial to schedule UV sensitive processes like DNA synthesis or cell division during the night (Nikaido and Johnson, 2000). This could explain the fact that many microalgae species grown under day/night cycles synchronize their cell division cycle and divide during the night (Carre and Edmunds, 1993; Matsuo et al., 2010).

When cell division in a culture is synchronized, all cells in the culture are in the same stage of the cell cycle at a given time. This can cause significant fluctuations in biomass composition. For example, starch content has been shown to vary between 45% of DW before cell division to 4-13% of DW after cell division (Brányiková et al., 2011). However, it is difficult to distinguish between the influence of the cell cycle and the influence of the photocycle, since the photocycle itself partly controls the cell cycle (Lacour et al., 2012). To investigate the sole influence of cell cycle on biomass composition, experiments must be done under constant light conditions.

In this research *Neochloris oleoabundans* was grown under constant light conditions in a

flat panel photobioreactor operated as a turbidostat. We were able to maintain a synchronous population and observed oscillations in biomass composition and biomass yield during the cell division cycle, even though all experimental conditions were kept constant. Therefore, these oscillations could be contributed solely to the influence of the cell cycle, which highlights the importance of cell cycle research for microalgae process design.

2.2 Theoretical background

2.2.1 The cell cycle

In the classical cell division cycle a growing cell duplicates all of its components and divides them into two nearly identical daughter cells (Donnan et al., 1985) (Figure 2.1 A). A cell cycle can be separated into two phases: a pre- and a post-commitment phase. The pre-commitment phase (G1) consists out of growth processes leading to an increase in cell size, the building of cellular structures and the accumulation of energy reserves. This phase is dependent on energy and thus for microalgae dependent on photosynthesis. At a certain point during growth in the pre-commitment phase, algae reach a threshold size that triggers a sequence of events leading to cell division. This point is called the commitment point (CP). The post-commitment period (S, G2, M in Figure 2.1) is independent of light intensity and can be performed in the dark, using energy reserves built up during the pre-commitment phase.

Some Chlorococcal and Volvocean algae are able to divide into more than two daughter cells by a process called multiple fission (see figure 2.1 B). When growth is allowed to continue during the post-commitment phase, cells can attain additional commitment points, which makes them divide into multiple daughter cells at the end of the cell cycle (Bisova et al., 2005; Matsumura et al., 2003; Vítová et al., 2011a; Vítová et al., 2011b; Zachleder et al., 1997). In principle, the number of daughter cells is 2^n, in which n is the number of doublings, determined by the number of commitment points attained.

2.2.2 Circadian control of the cell cycle

Circadian rhythms are rhythms occurring with a frequency of about 24h that regulate metabolic, physiological and/or behavioural events to occur at optimal phases of the daily cycle (Mittag et al., 2005). Circadian rhythms persist in constant conditions and can be entrained to environmental cycles. Many processes in microalgae have been shown to follow a circadian rhythm. For example, nitrogen fixation, chemotaxis, photosynthe-

sis and cell division can be under control of the circadian clock (Mittag, 2001).

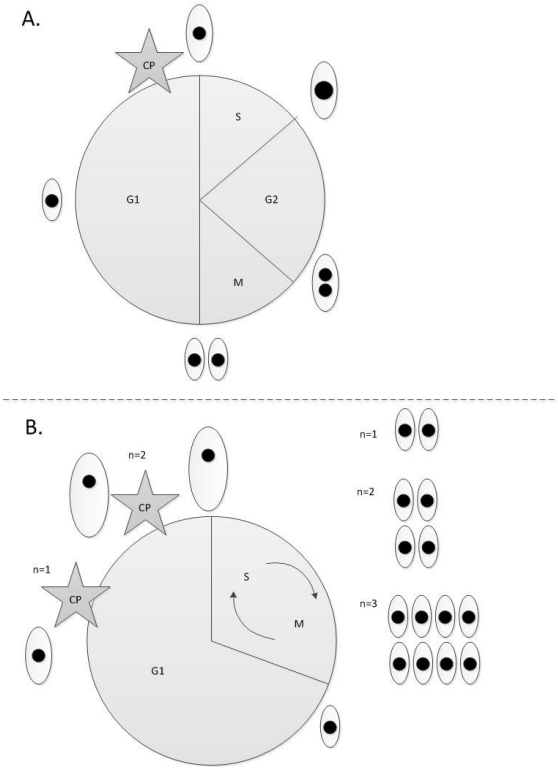

Figure 2.1 *Classical cell cycle (A) and multiple fission cell cycle (B) of Chlamydomonas after Bisova et al. (2005) (Bisova et al., 2005). G1=phase during which threshold size of the cell is attained, CP=commitment point, S=phase during which DNA synthesis takes place,G2=phase between DNA synthesis and nuclear division, M=phase during which mitosis takes place. Schematic pictures of the cells represent the size and the size and number of nuclei of the cells during the cell cycle. In the multiple fission cell cycle (B), number of commitment points determines n, generating* 2^n *daughter cells after rapid series of subsequent S and M cycles.*

Circadian control of the cell cycle has been observed in different species of microalgae and cyanobacteria. In *Synechococcus elongatus*, a cyanobacterium, rhythmic cell division cycles occur due to 'gating' of cell division to a specific time frame in the circadian cycle (Mori and Johnson, 2001; Yang et al., 2010). In eukaryotic microalgae, cell division is often synchronized during the night (Carre and Edmunds, 1993; Matsuo et al., 2010). A

synchronous cell division was for example observed in *Euglena,* and was shown to be under circadian control (Bolige et al., 2005; Carre and Edmunds, 1993). However, when eukaryotic cells are in conditions that allow them to double more than once per day, the daily periodicity often disappears (Suzuki and Johnson, 2001). This is to be expected for cells dividing by binary fission; a growth rate greater than 1 day^{-1} can never be maintained by dividing only once per daily cycle. However, in eukaryotic cells dividing by multiple fission, synchronized cell division can theoretically still be possible by increasing the number of daughter cells.

A model organism dividing by multiple fission is *Chlamydomonas.* The issue of circadian control over the cell cycle has been extensively studied in this green algae. Goto and Johnsson (Goto and Johnson, 1995) concluded that in *Chlamydomonas* cell division was gated by the circadian clock. The mechanism of circadian control was studied in more detail by Oldenhof et al. (2007) (Oldenhof et al., 2007). They concluded that the timing of cell division was regulated by a timer mechanism measuring a constant period from the onset of the light period. However, Vitova and coworkers (2011) found no evidence to support the presence of this hypothetical timer (Vítová et al., 2011a; Vítová et al., 2011b).

2.3 Materials and methods

2.3.1 Pre-culture and medium composition

Neochloris oleoabundans UTEX1185 was obtained from The Culture Collection of Algae, University of Texas, Austin. Cultures were maintained in a culture chamber on a shake incubator in 250 mL Erlenmeyer flasks containing 100 mL of defined medium at a pH of 7.5, as described by Klok et al. (2012) (Klok et al., 2013a). Temperature in the culture chamber was 25°C and light was provided in a 16/8h day/night cycle at an intensity of 20 -40 µmol m^{-2} s^{-1}. Three days before inoculation, cultures were placed in a shake-incubator with continuous light intensity of 280 µmol m^{-2} s^{-1} and a headspace enriched with 5% CO_2 to reach a sufficient cell density.

2.3.2 Reactor set-up and experimental conditions

N. oleoabundans was continuously cultivated in a sterile 1.7L flat panel photobioreactor operated as a turbidostat, as described by Klok et al. (2012) (Klok et al., 2013a). The light intensity at the front of the photobioreactor (I_{in}) was provided by a LED panel. Turbidostat control ensured dilution of the culture with fresh medium when light intensity at the back of the reactor (I_{out}) dropped below a set light intensity. In this way, the average

light intensity experienced by the culture (I_{av}) was be kept constant. Three different light settings were studied (see Table 2.1). To minimize environmental light, all windows in the laboratory were blinded.

Table 2.1 *Light settings in the photobioreactor in µmol m^{-2} s^{-1}*

	Light source	**I_{in}**	**I_{out}**	**I_{av}**
Experiment 1	Red LED	500	50	200
Experiment 2	Red LED	200	10	70
Experiment 3	White LED	500	50	200

Temperature of the culture was maintained at 30°C using an external cryostat connected to the photobioreactor's waterjacket. pH was maintained at 7.5±0.1 by an automatic supply of 1M HCl. The culture was also continuously sparged with 1200 mL min^{-1} N_2 enriched with 2%$_{(v/v)}$ CO_2 provided by a set of mass flow controllers (Brooks, Smart TMF SLA5850). Dissolved oxygen was measured online and foam formation was prevented by an automatic supply of a 1%$_{(v/v)}$ antifoam solution (Antifoam B® silicone emulsion, Mallinckrodt Baker B.V., Deventer, The Netherlands).
The reactor was inoculated to an optical density at 750 nm (OD_{750}) of 0.05. The system was operated in batch mode until a biomass concentration of 0.5 g L^{-1} was reached. At this biomass concentration, turbidostat control was started and the system was allowed to reach steady state. Steady state was defined as a constant biomass concentration and dilution rate for a period of at least 3 residence times. In steady state, the growth rate (µ) is equal to the dilution rate (D), which was monitored by logging the amount of overflow produced (V) over time (t) (see equation 1).

$$\mu = D = \frac{\Delta V}{\Delta t \cdot V_{pbr}} \qquad \text{(Eq. 1)}$$

2.3.3 Biomass analysis

Samples were taken at the same time every day to monitor biomass growth by measuring the optical density at 750 and 680 nm (OD_{750} and OD_{680}), cell number, cell size, total cell volume and dry weight (DW) as described by Kliphuis et al. (2010) (Kliphuis et al., 2010). When steady state was reached, sampling was done over a complete 24 hour cycle at 3 hour intervals for experiment 1, where the strongest cycle was observed. When

taking these samples, additional biomass was harvested to analyse DNA content and major biomass constituents (proteins, starch and fatty acids). The culture conditions were not influenced by sampling, as the growth rate of the algae was large enough to compensate for the volume taken from the reactor.

2.3.3.1 DNA content

DNA content was analysed by measuring fluorescence due to propidium iodide (PI) staining as described by Schippers et al. (2011) (Schippers et al., 2011). Samples taken from the reactor were diluted to a concentration of $10*10^6$ cells mL^{-1} using fresh culture medium. A volume of 900 µL of ice-cold methanol was added to 300 µL of the diluted sample. After vortexing, samples were fixated at -20°C for at least 1 hour. Subsequently, samples were centrifuged at 15.871 RCF for 1 minute and the pellets were washed twice in phosphate-buffered saline (PBS) buffer containing 0.1% bovine serine albumin (BSA). The pellets were then dissolved in 1mL of a 40 ug mL^{-1} PI solution (Sigma-Aldrich, Zwijndrecht, Netherlands) containing 3.8 mM sodium citrate, and 50 µL of a 1 mg mL^{-1} RNAse solution (Roche Diagnostics, Almere, Netherlands) was added. Samples were incubated for 30 minutes at 37°C. Fluorescence due to PI staining was measured using a flow cytometer (FACScalibur, Becton Dickinson, Franklin Lakes, NJ, USA). The instrument voltage was set to E+00, 500 and 740 for forward scatter (FSC), sideward scatter (SSC) and fluorescence (FL2) respectively. FSC signal was amplified 8.50 times and all measurements were done on a linear scale.

2.3.3.2 Major biomass constituents

Liquid samples were taken from the bioreactor in triplicate and centrifuged for 5 minutes at 2500 rpm. Pellets were stored at -20°C. After storage at -20°C, pellets were transferred to beat beater tubes and freeze dried overnight.

Proteins. Protein content was analysed with the DC protein assay (BioRad). Freeze dried pellets containing 1.5 mg of DW were dissolved in 1 mL of lysis buffer, containing 60 mM Tris, 2% SDS, 10% glycerol and 10 mM DTT. Subsequently, the cells were disrupted using a beat beater (Precellys 24, Bertin Technologies, France) at 6000 rpm for 3 cycles of 60 seconds with 120 seconds breaks on ice between each cycle. Another mL of lysis buffer was added and samples were incubated for 30 minutes at 100°C in a heating block. After incubation samples were centrifuged for 20 minutes at 10000 RPM. Supernatant was desalted using PD10 desalting columns (Sephadex G-25 M, GE-heatlhcare). Protein content of the eluent was analysed by measuring absorbance at 750 nm using a plate

reader (EL800, BioTek Instruments, USA).

Starch. Total starch content was determined using the Total Starch Kit of Megazyme (Ireland, 2011). Freeze dried pellets containing 9mg of DW were dissolved in 1mL 80%$_{(v/v)}$ ethanol. Beat beating was done at 6000 rpm for 3 cycles of 60 seconds with breaks on ice. Another 4 mL of 80%$_{(v/v)}$ ethanol were added and from this point the standard procedure of the Total Starch kit was followed. Absorbance was measured at 510 nm on a spectrophotometer (DU 640, Beckman, U.S.A.).

Fatty acids. Extraction of total fatty acids (TFA) with a 5:4 methanol:chloroform mixture and subsequent transesterification was done as described by Bligh and Dyer (1959) (Bligh and Dyer, 1959) with modifications as suggested by Lamers et al. (2010) (Lamers et al., 2010) and Santos et al. (2012) (Santos et al., 2012). Freeze dried pellets contained a minimum of 9 mg of DW. TFA content and composition were determined by gas chromatography.

2.4 Results

2.4.1 Synchronized cell division of Neochloris oleoabundans

2.4.1.1 Growth rate

For all three experiments, a stable 24h averaged growth rate (μ) was found using eq. 1. Using the red LED light with an I_{av} of approximately 70 μmol m^{-2} s^{-1} and 200 μmol m^{-2} s^{-1}, daily averaged growth rates were 1.15 d^{-1} and 1.92 d^{-1} respectively. Using white LED light with an I_{av} of approximately 200 μmol m^{-2} s^{-1} the obtained growth rate was 2.1 d^{-1}. However, calculation of the average μ over shorter time intervals (1h) revealed a cyclic pattern in the experiments using red LED light, as is illustrated in Figure 2.2. In this figure, a moving average of the growth rate of the cultures (d^{-1}) is plotted against time (t) in hours, where t=0 corresponds to midnight on the first day of steady state. Growth rate clearly peaked every day at midday with almost 3 d^{-1} for the high light experiment and 1.5 d^{-1} for the low light experiment, and was lowest at midnight with 1 d^{-1} and 0.8 d^{-1}. The phase of the oscillations was not influenced by light intensity and maintained to be approximately 24h. For the experiment using white LED light, no cycle was observed and a stable growth rate of 2.1 $d^{-1}$was obtained.

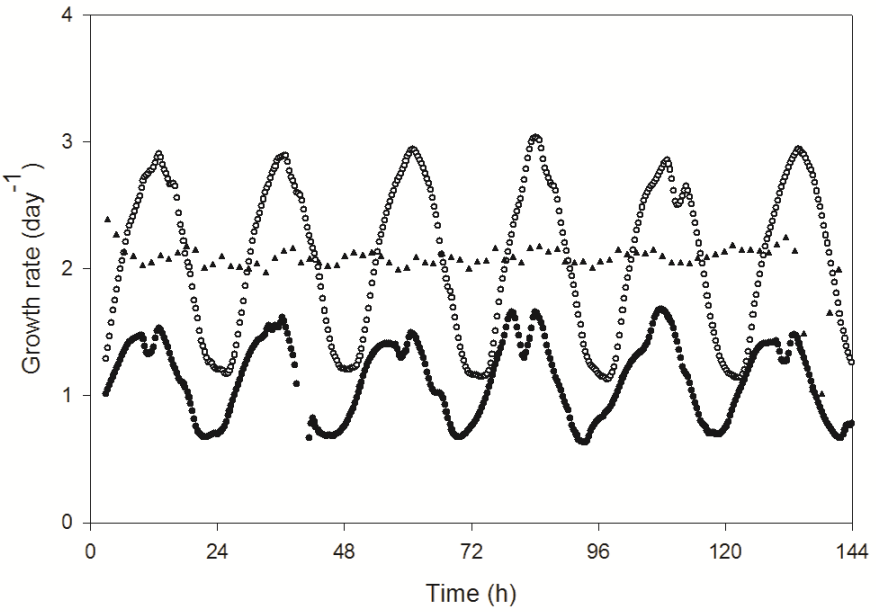

Figure 2.2 *Growth rate of Neochloris oleoabundans during steady state in three different experiments using LEDs: red LED light with an averaged light intensity over the reactor of 70 µmol m^{-2} s^{-1} (closed circles); red LED light with an averaged light intensity over the reactor of 200 µmol m^{-2} s^{-1} (open circles); white LED light with an averaged light intensity over the reactor of 200 µmol m^{-2} s^{-1} (triangles).*

2.4.1.2 Cell number and diameter

The cell division cycle of *N. oleoabundans* was clearly synchronized when using red LED light, which resulted in a cyclic behaviour in cell number and diameter throughout the experiment. In Figure 2.3, changes in cell size and cell number in the experiment done at an I_{av} of 200 µmol m^{-2} s^{-1} are shown during both the natural day (left) and the natural night (right). The cells in the first sample (7.00h) had an average diameter of 2.5 µm. In the next sample (10.00h) average cell diameter increased to 2.8 µm, while cell number slightly decreased. This is a result of cell growth and turbidostat control, in which the absorbed light in the culture is kept constant. The increase in cell size continued until 19.00h, when a new peak appeared with small daughter cells, indicating the onset of division. In the subsequent samples at 22.00h, 1.00h and 4.00h this population of small daughter cells increased in both size and number, while the number of large cells de-

creased (as indicated by the arrows). Clearly, cells of *N. oleoabundans* were growing during the day (left figure) and divided synchronously in the night (right figure).

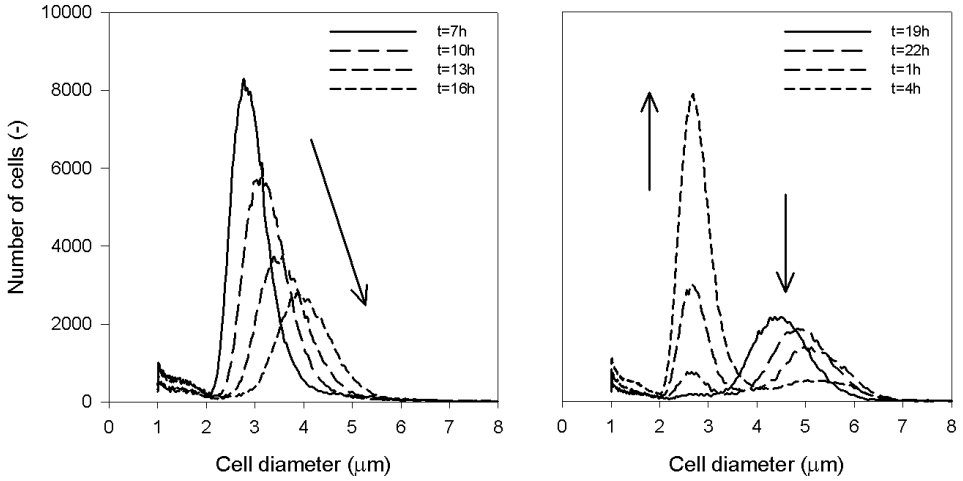

Figure 2.3 *Number of cells versus their cell diameter (in µm) in samples taken during the day when cells are growing (left) and during the night when division takes place (right). Arrows indicate increase in cell size and the appearance and disappearance of daughter and mother cells.*

2.4.1.3 DNA content

Because *N. oleoabundans* showed synchronized cell division during the night and the average growth rate was higher than 1 d^{-1}, multiple daughter cells had to be formed per mother cell. The average number of daughter cells released per mother cell (N_t/N_o) can be calculated according to equation 2, where t_d is the doubling time (d) and t is the time interval (d). Doubling time t_d is defined in equation 3, where µ is the specific growth rate (d^{-1}) which in steady state is equal to the dilution rate D. In the experiment using red LED light with an I_{av} of approximately 200 µmol m^{-2} s^{-1}, the average number of daughter cells released per mother cell was 6,82.

$$\frac{N_t}{N_0} = 2^{\frac{t}{t_d}} \qquad \text{(Eq. 2)}$$

$$t_d = \frac{\ln(2)}{\mu} \qquad \text{(Eq. 3)}$$

To confirm that *N. oleoabundans* divides by multiple fission, DNA content of the samples was analyzed by measuring fluorescence due to PI staining on the flow cytometer. Scatter plots of cell size (FSC-height) versus fluorescence (in the FL2 channel) were obtained (data not shown). Four populations of cells containing 1, 2, 4 or 8 copies of DNA were observed and the size of these populations was determined using the gate-tool of the WinMDI software (version 2.8). Figure 2.4 shows the summarized results for the samples taken during the 24 h cycle.

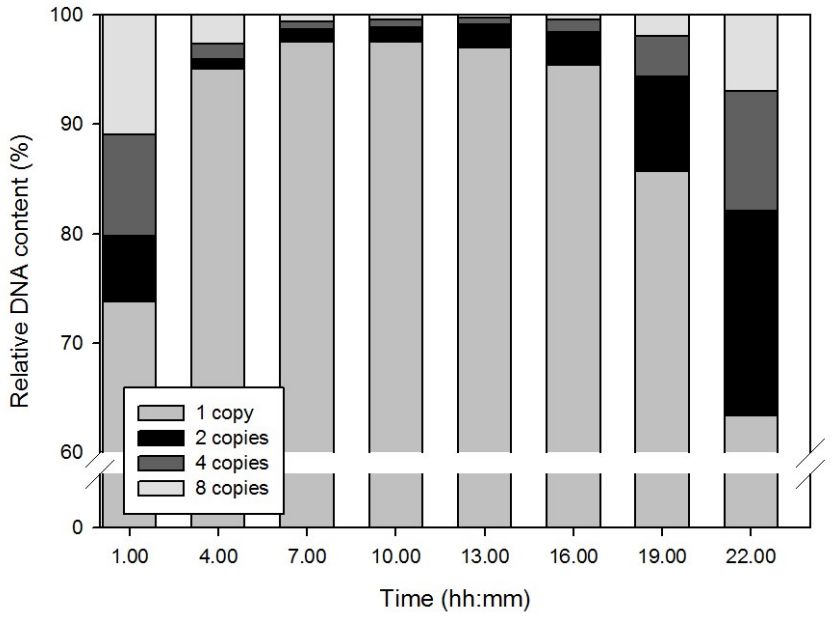

Figure 2.4 *Relative DNA content of N. oleoabundans cells in samples taken over a 24h cycle*

During the day, from 7.00h to 16.00h, the majority of cells only contained one copy of DNA. However, at 19.00h, two new populations of cells containing 2 and 4 copies of DNA appeared, indicating that these cells started to synthesize DNA in preparation for cell division. At 22.00h an additional population of cells containing 8 copies of DNA appeared. At this time, 40% of the counted cells contained more than 1 copy of DNA. At

1.00h this percentage decreased to about 25%, while the number of cells containing 8 copies of DNA kept on increasing to 10%. At 4.00h some cells remained in the 8 copy population, but the majority of cells contained only 1 copy of DNA. This shows that cells of *N. oleoabundans* were indeed dividing by multiple fission, releasing 2 to 8 daughter cells per mother cell.

2.4.2 Biomass composition

To study the effect of the synchronized cell cycle on biomass composition, the major biomass constituents were analysed during the 24h cycle in the experiment with an I_{av} of approximately 200 µmol m^{-2} s^{-1}. In Figure 2.5, protein, starch and total fatty acid content are plotted in gram per gram DW against time. Average biomass contained 33.5% protein, 20.6% starch and 8.2% total fatty acids. At 7.00h, when cells were at the beginning of their cell cycle, protein content was lowest at 30.7%. From this time on, protein content increased until 22.00h. An increase in starch content followed the increase in proteins, starting with 16% at 10.00h to its maximum value of 25% at 22.00h. Total fatty acid content showed the same cyclic pattern as starch, although the absolute changes in total fatty acid content were smaller.

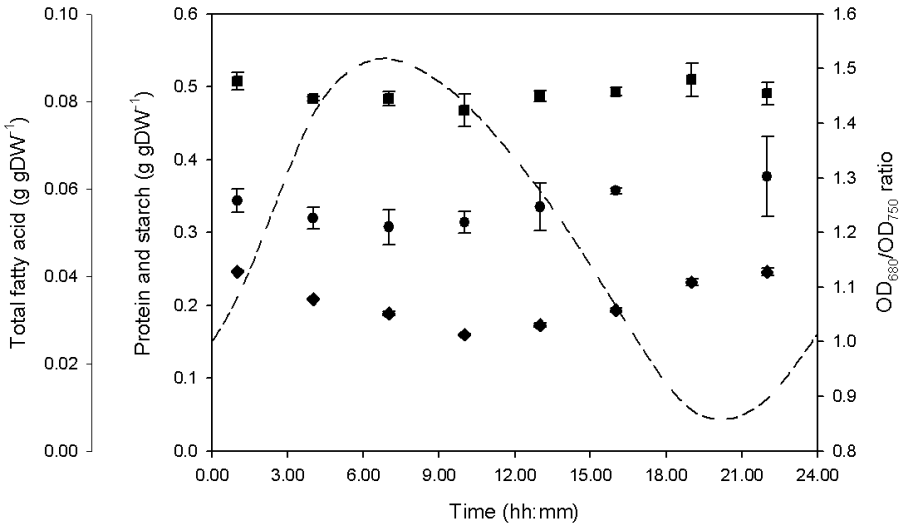

Figure 2.5 *Major biomass components - protein (circles), starch (diamonds) and total fatty acid (squares)- in g per g DW of N. oleoabundans during a 24 h cycle. Error bars represent standard deviation between triplicate measurements. Dashed line represents pigment content expressed as the OD_{680}/OD_{750} ratio.*

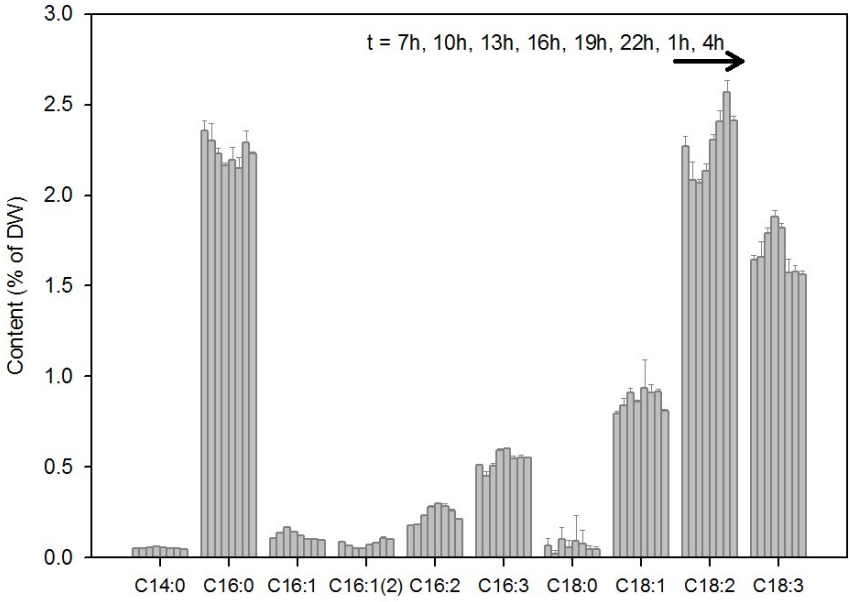

Figure 2.6 *Fatty acid profile in % of dry weight for N. oleoabundans during a 24 h cycle. The arrow indicates the time that samples were taken starting from 7.00h with 3 h intervals. Error bars represent standard deviation between triplicate measurements.*

In addition to total fatty acids, fatty acid composition was determined. In Figure 2.6 the fatty acid composition in % of DW is plotted for the samples taken during the 24 h cycle. While the total fatty acid content varied only slightly, the fatty acid composition changed during the 24 h cycle. During the day, 16:0, 16:1(2) and 18:2 decreased, while 16:1(1) 16:2 and 18:3 increased. During the night, the opposite pattern was observed.
As a relative measure for pigment content, the ratio OD_{680}/OD_{750} is plotted on the secondary y-axis (Figure 2.5), a high ratio indicating a higher pigment content. It can be seen that pigment content peaked at 7.00h, when the other major biomass components (proteins, starch, lipids) were at their minimum.

2.5 Discussion

2.5.1 The role of the circadian clock in cell division of Neochloris oleoabundans

The average light intensity of 200 µmol m^{-2} s^{-1}, provided by red LED light, allowed the cells of *Neochloris oleoabundans* to grow at a rate of 1.92 day^{-1}. Even though all experi-

mental conditions, including light intensity, were kept constant, growth rate was not constant over the day. A cyclic pattern was observed with growth rate peaking during the day and showing a dip in the night (Figure 2.2, open circles). Measurements of cell number and diameter showed a synchronous division during the night (Figure 2.3), as was also observed for other algae species (Carre and Edmunds, 1993; Matsuo et al., 2010). At a lower light intensity of 70 µmol m^{-2} s^{-1}, also provided by red LED light, the same cyclic behaviour in growth rate was observed (Figure 2.2, closed circles). The phase of the cycle was not altered by this change in light intensity, which suggests the involvement of the circadian clock.

Light is known to be the most important cue for resetting the circadian clock (Matsuo et al., 2010). To exclude the influence of natural environmental light on the cell cycle of the algae culture, windows in the laboratory were blinded and ingoing light intensity on the surface of the reactor was continuously monitored using a Licor sensor. No significant differences were detected in this ingoing light intensity during the natural day/night cycle (data not shown). All other abiotic conditions, such as temperature and pH, were strictly controlled in the photobioreactor. The fact that N. oleoabundans still synchronized its cell division during the natural night, suggests the involvement of a highly sensitive circadian clock. Possibly, spectral changes in the natural light in the laboratory, that were undetectable by our Licor sensor, were the trigger for this synchronisation (Oldenhof et al., 2006). This hypothesis is strengthened by the experiment using white LED light. In this experiment, no cyclic behaviour in growth rate was observed (Figure 2.2, triangles). Apparently, algae were not able to synchronize their cycles when they received constant white light, in which also blue light is present. A small difference in natural light could therefore not be detected. However, with constant red LED light, a small spectral change in natural light, including blue light, was easier to detect for the algae in the culture and enough to synchronize their division cycle. From these results, it is suggested that a blue light receptor is involved in this synchronization (Oldenhof et al., 2006).

The growth rate of 1.92 day^{-1}, combined with a synchronized daily division, implies that cells were dividing by multiple fission forming an average of 6.82 daughter cells per mother cell (section 2.4.1.3). This was confirmed by the flow cytometer data, showing DNA content of *Neochloris oleoabundans* increased to a maximum of 8-fold (Figure 2.4). This is comparable to the multiple fission cell cycle of *Chlamydomonas* (Donnan et al., 1985). However, there is no commonly accepted mechanism for circadian control of the

cell cycle in *Chlamydomonas.* Oldenhof et al. (2007) (Oldenhof et al., 2007) concluded the presence of a timing mechanism ensuring that cell division occurs at a constant time after the onset of the light period. That is, if all size requirements at the commitment point are met. If cells are able to reach multiple commitment points before the time of cell division occurs, multiple daughter cells will be formed (Zachleder et al., 1997). On the contrary, Vitova and coworkers (2011) (Vítová et al., 2011a; Vítová et al., 2011b) argued that the cell cycle is solely controlled by light intensity and temperature, thus excluding the presence of a timer mechanism.

In our photobioreactor, an average of 6.82 daughter cells were formed per mother cell to keep up with the average growth rate of 1.92 day^{-1}. DNA content confirmed that cells were dividing by multiple fission releasing 2 to 8 daughter cells per division. Possibly, also part of the cells did not divide at all. Assuming the cell size of all daughters is identical (Donnan et al., 1985) and cells are spheres, the smallest cells with a diameter of 2 µm have a volume of 4.2 μm^3. Therefore, the volumes required to reach the first, second and third commitment point can theoretically be calculated to be 8.4 μm^3, 16.8 μm^3 and 33.6 μm^3 respectively. This corresponds to cell diameters of 2.9 µm, 4 µm and 5.7 µm. It can be seen in Figure 2.3, that there was a proportion of cells smaller than 2.9 µm at 16.00h and 19.00h. Supposing there is a timer involved in circadian control of the cell division cycle, these cells would not be able to divide on this day, since they were not big enough to reach the first commitment point. However, on the next day, these cells are the largest cells in the culture and might be able to attain the 2^{nd} and 3^{rd} commitment point (the largest cells in Figure 2.3 at 19.00h with diameters over 5.7 µm). In this way, the average specific growth rate at the given light intensity could be maintained. In this reasoning, the experiment done at lower light intensity would result in a lower amount of daughter cells per mother cell, as less cells would be able to reach multiple commitment points, which would result in a lower overall growth rate. Indeed, the growth rate in this experiment was lower (1.15 d^{-1}) and the number of daughter cells per mother cell resulting from this growth rate was 3.15 (Eq. 2 and 3).

If the cell cycle would indeed be solely controlled by light intensity and temperature, as was proposed by Vitova and coworkers (2011) (Vítová et al., 2011a; Vítová et al., 2011b), the experiments done at high and low light intensity would not have the same phase (Figure 2.2). Therefore, our research supports the presence of a timer mechanism involved in circadian control of the cell division cycle in *N. oleoabundans*.

2.5.2 The influence of the cell cycle on biomass composition of N. oleoabundans

Usually, it is difficult to distinguish between the seperate effects of the photocycle and the cell cycle on biomass composition, since the photocycle is necessary to synchronize the cell cycle. However, in this experiment we were able to keep a synchronized population of *N oleoabundans* under constant light conditions. In this way, it was possible to investigate the sole influence of the cell cycle on biomass composition. Given the fact that not all cells were dividing every day (section 2.5.1), it must be kept in mind that the observed oscillations in biomass composition are an average for the entire population and might be larger in single cells. Non-dividing cells might have a different composition than dividing cells. However, since the non-dividing cells are the minority, observed trends are significant and solely due to the effect of the cell cycle. The variations in cellular biomass composition during 24 hours tell us how processes in the cell are regulated with respect to phasing of the cell cycle.

Protein content was varying between 31% and 38% of DW (Figure 2.5) and the minimum level was observed at 7.00h. At this time, all cells completed their cell division and entered the growth phase (G1). Protein levels increased while the cells were increasing in size (Figures 3 and 5) and attained a maximum around 22.00h. At the start of cell division, protein content decreased. A similar pattern was observed in *Nannochloropsis* cultures synchronized under day/night cycles (Sukenik and Carmeli, 1990). Sometimes, an increase in protein content is observed during the night (Cuhel et al., 1984). It is possible that the cells in our photobioreactor that did not attain their commitment point also increased their protein pool during the night. However, from the decrease in average protein content during cell division it can be concluded that there was probably no net production of proteins during cell division.

Net protein production started only after an increase in pigment content was observed, and preceeded a rise in starch and fatty acid content. This is in line with findings of Falkowski and Roche (1991) (Falkowski and LaRoche, 1991) and Sukenik et al. (1990) (Sukenik et al., 1990) and can be explained by the cell division cycle. During cell division, the chloroplast divides as well. It has to be increased in size to reach its full potential during the day, since cells need to be able to harvest energy for growth. Therefore, pigments are synthesized first. Next, proteins are synthesized in order to form the light harvesting chlorophyll protein complexes. Only then, when the cell is able to harvest energy, carbon can be increasingly allocated away from proteins toward carbohydrates and lipids.

Lipid and carbohydrate accumulation started at 10.00h. It is known that during photosynthesis, some fraction of the fixed carbon can be accumulated as storage compounds rather than being used immediately in growth and maintenance. The form of stored carbon is variable between algal classes and dependent on environmental conditions. Cells grown under day/night cycles, accumulate carbohydrates and neutral lipids during the photoperiod and use them in the dark period (Falkowski and Raven, 2007). The building up of storage compounds is in this case an anticipation on the arrival of a dark period., in which light energy will not be available for growth. In *N. oleoabundans*, carbon can be stored in the form of starch and neutral lipids (Tornabene et al., 1983). Our results show that starch is built up during the day until a maximum of 25% at 22.00h. This level clearly started decreasing from 1.00h, when cells were dividing. The minimum starch level of 16% was found at 10.00h after cell division. This is comparable with findings of Brányiková et al. (2011) (Brányiková et al., 2011) who found that starch levels in *Chlorella* decreased to 14% after cell division. These results indicate that starch synthesis in *N. oleoabundans* is under circadian clock control, as Ral et al. (2006) (Ral et al., 2006) also concluded for starch synthesis in *Chlamydomonas.* However, it remains difficult to distinguish between direct circadian control and circadian control exerted through the cell cycle.

Total fatty acid content did not strongly deviate during the day/night cycle. However, a small increase was observed during the day and a small decrease was measured during the night (Figure 2.5). This was also observed by Sukenik and Carmeli (1990) (Sukenik and Carmeli, 1990) and Fábregas et al. (2002) (Fábregas et al., 2002) for *Nannochloropsis* cells grown under day/night cycles. Interestingly, the fatty acid profile did change significantly during the day/night cycle (Figure 2.6). Fatty acids in microalgae are predominantly present in the form of membrane lipids or accumulated in lipid bodies as triacylglycerides (TAG) (Klok et al., 2013a). Not much is known about the fatty acid composition of the different membrane and storage lipids present in the cell. A fatty acid known to be present in membranes is 18:3, which is found particularly in chloroplast membranes (Guschina and Harwood, 2006). 18:3 content in our results built up during the day and clearly decreased once cells were dividing. Dividing cells divide their chloroplast equally over their daughter cells (Goodenough, 1970). At the same time, other membranes like cell membranes have to be synthesized. While 18:3 is decreasing, 16:0 is increasing. 16:0 is known to be present in membrane lipids (Guckert and Cooksey, 1990), which can possibly explain the observed decrease in 18:3 and increase in 16:0.

Together with 18:1, 16:0 is also the main constituent of TAG. TAG usually only accumulates under stress conditions (Li et al., 2011), (Rismani-Yazdi et al., 2012), (Klok et al., 2013a). As we are working in a non-stressed culture, it is expected that the contribution of TAG to the total fatty acid pool is minimal. Therefore, the observed variations in 16:0 and 18:1 were probably not arising from changes in the TAG content of the cells. However, high TAG contents are desired for biodiesel production (Hu et al., 2008). Potentially, the TAG content exhibits variations during the day under stress conditions, just as the other main components of biomass are produced in different phases of the cell cycle. Also, it is likely that the cell cycle itself is influenced by the adverse growth conditions necessary for TAG accumulation. Therefore, it would be interesting to see what happens to the cell cycle and biochemical composition of the algae once the culture is put under TAG accumulating stress conditions.

Proteins, starch and total fatty acids were the major biomass components measured in this study. All of these components followed more or less the same pattern; increasing during the day and decreasing during the night. Other biomass components, like pigments, must therefore behave antagonistically. This is confirmed by the OD_{680}/OD_{750} ratio, which was taken as a relative measure for chlorophyll content (Figure 2.5). The maximum pigment content was obtained when other biomass components were at their minimum level. Similar results were obtained by Post et al. (1984) (Post et al., 1984), who found that chlorophyll per cell reached a maximum in the middle of the photoperiod and began to decline before the dark period. Chlorophyll content was at a minimum when cells started dividing. Pigments are divided among daughter cells and therefore pigment content is diluted by cell division (Falkowski and LaRoche, 1991). Chlorophyll levels started to increase again before the next photoperiod. Apparently, cells exposed to a light/dark cycle do not acclimate to shade at night, but oscillate around the average irradiance during the photoperiod(Falkowski and LaRoche, 1991; Falkowski and Raven, 2007).. In this way, the cell is able to anticipate on changes in the environment, like sunset and sunrise (Suzuki and Johnson, 2001). This anticipation was even observed in our experiment, where light intensity was kept constant. This highlights once again the extent to which processes in *N. oleoabundans* are regulated by the sensitive circadian clock.

2.5.3 Application of cell cycle research in process design

As a result of the synchronized cell cycle, growth rate, biomass concentration and pigment content were not constant during the day, whereas daily averages were constant

during steady state (Figure 2.2). Biomass concentration in the experiment using red LED light with an I_{av} of 200 umol m^{-2} s^{-1} was between 0.67-0.8 g L^{-1} with a daily average of 0.74 g L^{-1}. However, due to turbidostat control, light absorbed by the culture in this experiment was constant at 1.69 mol L^{-1} d^{-1}. Since light absorbed was constant, yield of biomass on light was also varying throughout the circadian cycle. In Figure 2.7 the yield in grams of DW per mole of photons during the 24 h cycle is depicted. As can be seen, the biomass yield on light is severely influenced by the cell cycle. During cell division, yield declines to 0.5 g mol^{-1}, while at noon yield is almost 2.5 times higher (1.2 g mol^{-1}). Thus, the changes in yield observed throughout the day/night cycle in outdoor cultivation might not only be resulting from adaptational responses or photoinhibitory effects (Claquin et al., 2004; Kaftan et al., 1999; Strasser et al., 1999). Instead, the observed changes in yield might partly result from the internal cell cycle, which should be a consideration in process design. For example, shading of cultures when high light intensities are expected, would not necessarily prevent a dip in biomass yield in the afternoon. In addition, it might be possible to adapt photobioreactor control strategies to circadian rhythms in microalgae, by for example changing the light out settings in luminostat control as suggested by Cuaresma et al. (2011) (Cuaresma et al., 2011).

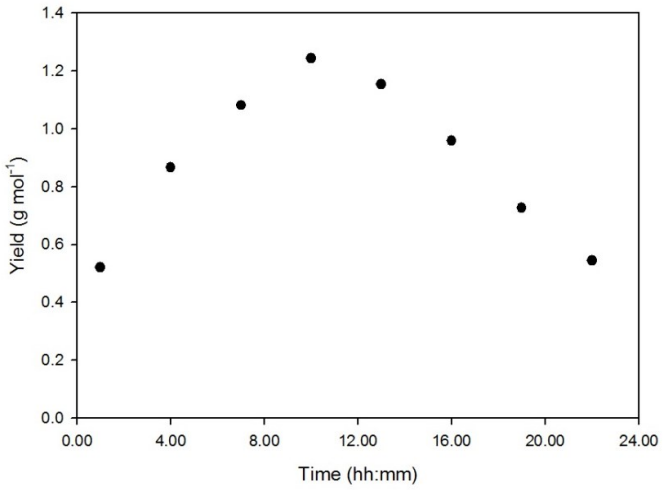

Figure 2.7 *Variation in yield (in g mol^{-1}) during the circadian cycle*

More possibilities for process optimization can be found in adjustment of harvesting times. According to the results of this study, biomass with a high lipid, starch and protein content should be harvested at the end of the day. As these components peak be-

fore cell division, it would be interesting to consider ways of postponing this event. Oldenhof et al. (2006) (Oldenhof et al., 2006) showed that blue light is capable of postponing the cell cycle. Providing blue light at the end of the day might therefore be a strategy to lengthen the growth phase and obtain biomass with an even higher lipid, starch and protein content.

2.6 Conclusions

Cell division in *N. oleoabundans* was proven to be under control of the circadian clock and took place by multiple fission during the night. Presumably, a timing mechanism 'gating' cell division is involved which makes use of a blue light receptor to keep track of time.

Oscillations in starch, protein and pigment content were observed during the synchronized cell division cycle. Maximal protein, starch and lipid content was obtained just before cell division. When growing outdoors under a natural day/night cycle, it must be kept in mind that oscillations observed in biomass composition might not simply be due to changes in environmental conditions, since the cell cycle stage of the algae has a severe influence as well. This research therefore highlights the importance of cell cycle research for microalgae process design.

Acknowledgements

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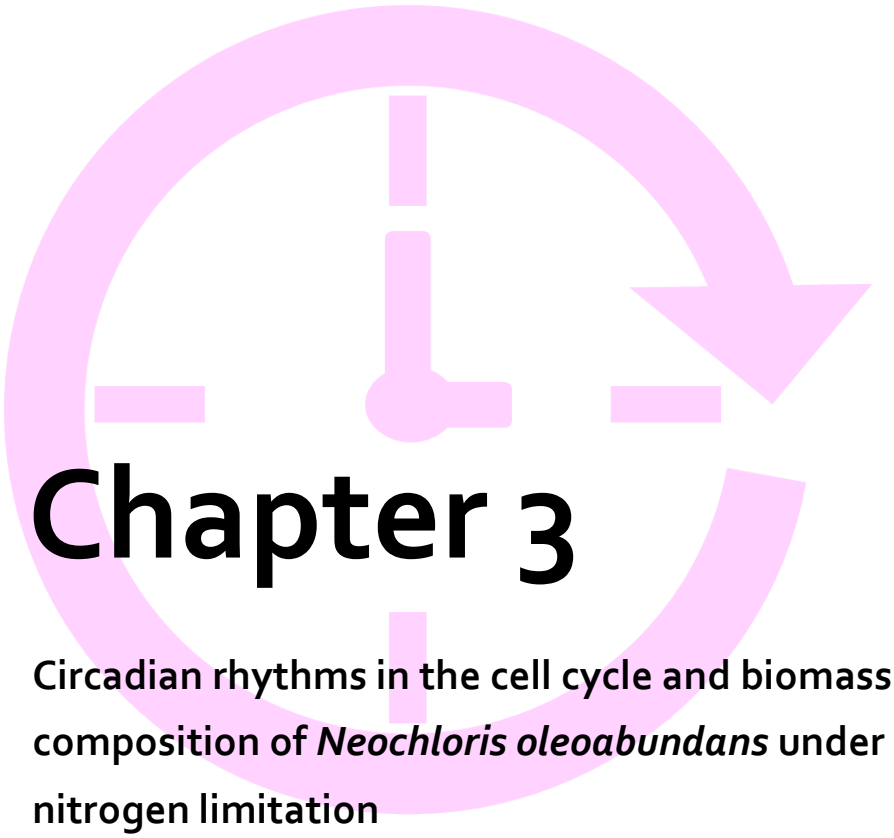
Abbreviations

BSA	Bovine Serine Albumine
CP	commitment point
D	Dilution rate (h-1)
DTT	Dithiothreitol
DW	Dry Weight (g L-1)
I_{in}	Light intensity on the front of the reactor (µmol m^{-2} s^{-1})
I_{out}	Light intensity at the back of the reactor (µmol m^{-2} s^{-1})
I_{av}	Average light intensity experienced by the culture (µmol m^{-2} s^{-1})
N_o	Number of cells per mL of culture at time = o
N_t	Number of cells per mL of culture at time = t
OD_{680}	Optical Density at 680 nm
OD_{750}	Optical Density at 750 nm
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
SDS	Sodium Dodecyl Sulphate
t	Time (h)
t_d	Doubling time (d)
TAG	Triacylglycerol
TFA	Total Fatty Acids
V	Volume of overflow produced (g)
V_{PBR}	Photobioreactor volume (g)
µ	Specific growth rate (h-1)



Chapter 3

Circadian rhythms in the cell cycle and biomass composition of *Neochloris oleoabundans* under nitrogen limitation

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Abstract

The circadian clock schedules processes in microalgae cells at suitable times in the day/night cycle. To gain knowledge about these biological time schedules, *Neochloris oleoabundans* was grown under constant light conditions and nitrogen limitation. Under these constant conditions, the only variable was the circadian clock. The results were compared to previous work done under nitrogen-replete conditions, in order to determine the effect of N-limitation on circadian rhythms in the cell cycle and biomass composition of *Neochloris oleoabundans*.

The circadian clock was not affected by nitrogen-limitation, and cell division was timed in the natural night, despite of constant light conditions. However, because of nitrogen-limitation, not the entire population was able to divide every day. Two subpopulations were observed, which divided alternately every other day. This caused oscillations in biomass yield and composition. Starch and total fatty acids (TFA) were accumulated during the day. Also, fatty acid composition changed during the cell cycle. Neutral lipids were built up during the day, especially in cells that were arrested in their cell cycle (G_2 and G_3). These findings give insight in the influence of circadian rhythms on the cell cycle and biomass composition.

3.1 Introduction

In the search for renewable energy sources, many researchers have focussed on microalgae. Algal biomass can consist out of 20-50% of triacylglyceride (TAG), which is suitable for biodiesel production (Hu et al., 2008; Mata et al., 2010). Also other biomass components, such as starch and proteins, can be used as source for chemicals, foods, feeds and high-value bioactives (Chisti, 2007; Wijffels and Barbosa, 2010). Biomass composition is not only dependent on culture conditions, like temperature, light and nutrient availability, but is also influenced by the circadian clock (de Winter et al., 2013).

In nature, microalgae are exposed to daily cycles of light and dark, which resulted in the evolution of a circadian clock. The circadian clock is able to schedule processes in suitable timeframes of the daily cycle (Nikaido and Johnson, 2000). Examples of processes under control of the circadian clock are chemotaxis, phototaxis, photosynthesis and cell division (Mittag and Kwang, 2001). Cell division, for example, is often scheduled in the night (Carre and Edmunds, 1993; Matsuo et al., 2010). As a result, most algae present in a culture are in the same stage of the cell cycle, in other words, the culture is synchronized. Recently, it was shown that this synchronization was even maintained under constant red light conditions, and that this had an influence on biomass composition (de Winter et al., 2013). Because the light intensity was constant, the sole influence of the circadian clock on cell cycle and biomass composition could be studied. However, that research was done under nutrient replete conditions, whereas production of storage components like starch and TAG is usually done using nutrient limitation or deprivation. Knowledge about the timing of processes in the cell as a function of the circadian clock under nutrient limited conditions might provide ways to improve the microalgae production process, for example by determining the right moment for harvesting of biomass with the desired composition.

To create nutrient limitation or deprivation, often nitrogen (N) is used as limiting nutrient. As nitrogen is an important constituent of biomass building blocks such as protein and pigment, nitrogen limitation will affect growth and the cell cycle. In case of N deprivation in batch cultures, growth is completely ceased (Ballin et al., 1988), and the increase in biomass observed is contributed to accumulation of carbohydrates and lipids (Breuer et al., 2012). However, accumulation of storage components can also be accomplished by continuous N-limitation in continuous cultures (Klok et al., 2013a). In this

case, the algae in the culture keep on dividing and growing at a lower rate, while simultaneously accumulating storage components under stable cultivation conditions.

In this work the influence of the circadian clock on the cell cycle and biomass composition of *Neochloris oleoabundans* was investigated. For this purpose, a continuous photobioreactor under continuous light and N-limitation was used, where the only variable factor was the circadian clock and its influence on the cell cycle and biomass composition. The results were compared to previous work done under N-replete conditions (de Winter et al., 2013). In this way, it was shown that under N-limitation the cell cycle was still under control of the circadian clock, which had its influence on biomass composition.

3.2 Materials and methods

3.2.1 Photobioreactor set-up and experimental conditions

Neochloris oleoabundans UTEX1185 (Culture Collection of Algae, University of Texas, Austin) was pre-cultivated in 250 mL erlenmeyer flasks as described in our previous work (de Winter et al., 2013). Then, the algae were transferred to a sterile 1.7 L flat panel photobioreactor, operated as a turbidostat, as described by (Klok et al., 2013a). The light intensity at the front of the photobioreactor was provided by a red LED panel and set to an intensity of 500 µmol m^{-2} s^{-1}. The reactor was inoculated to an optical density at 750 nm (OD_{750}) of about 0.05. The system was operated in batch mode until a biomass concentration of approximately 0.5 g L^{-1} (OD_{750} 1.5) was reached. At this biomass concentration, turbidostat control was started. Turbidostat control ensured dilution of the culture with fresh medium when the light intensity at the back of the reactor dropped below 50 µmol m^{-2} s^{-1}. In this way, the average light intensity in the reactor was kept constant at approximately 200 µmol m^{-2} s^{-1}, which for *N. oleoabundans* is a saturating light intensity (Sousa et al., 2012). Dilution medium was prepared without nitrogen and nitrogen was added separately by a continuous feed of 0.045 g L^{-1} day^{-1}, which resulted in nitrogen limited growth conditions (Klok et al., 2013a). All nitrogen in the system was consumed by the algae, so the nitrogen concentration inside the system was zero throughout the experiment.

The system was allowed to reach steady state. Steady state was defined as a constant biomass concentration at regular sample time (10.00h) and a constant dilution rate over

24h for a period of at least 3 residence times. In steady state, the growth rate (μ) is equal to the dilution rate (D), which was monitored by logging the amount of overflow produced (V) over time (t) (see equation 1).

$$\mu = D = \frac{\Delta V}{\Delta t \cdot V_{PBR}} \qquad \text{(Eq.1)}$$

Volume taken from the reactor by sampling was weighed and added to the overflow produced (V). The culture conditions were not influenced by sampling, as the dilution rate was high enough to compensate for the volume taken from the reactor. Temperature was maintained at 30°C and the culture was continuously sparged with 1200 mL min^{-1} N_2 enriched with 2%$_{(v/v)}$ CO_2. Dissolved oxygen was measured online and foam formation was prevented by an automatic supply of a 1%$_{(v/v)}$ antifoam solution (Antifoam B® silicone emulsion, Mallinckrodt Baker B.V., Deventer, The Netherlands). All as described by (de Winter et al., 2013).

3.2.2 Biomass analysis

Samples were taken at the same time every day to monitor biomass growth by measuring the optical density at 750 and 680 nm (OD_{750} and OD_{680}), cell number, cell size, total cell volume and dry weight (DW) as described by (Kliphuis et al., 2010). When steady state was reached, sampling was done over a complete 24 hour cycle at 3 hour intervals. For these samples taken during the 24 hour cycle additional biomass was harvested to analyse DNA content and major biomass constituents (proteins, starch and fatty acids).

DNA content. Relative DNA content was analysed by measuring red fluorescence due to propidium iodide (PI) staining on a flow cytometer. Samples were also stained with BODIPY 493/503. BODIPY fluorescence was used as a proxy to analyse the lipid content of the different cell cycle populations. Staining was done by diluting samples from the reactor to a concentration of $10*10^6$ cells mL^{-1} using fresh culture medium. A volume of 900 µL of ice-cold methanol was added to 300 µL of the diluted sample. After vortexing, samples were fixated at -20°C for at least 1 hour. Subsequently, samples were centrifuged at 15.871 RCF for 1 minute and the pellets were washed twice in phosphate-buffered saline (PBS) buffer containing 0.1% bovine serine albumin (BSA). After centrifuging again, the pellets were dissolved in 450 µL of fresh culture medium. Then 500 µL

of a 40 µg mL^{-1} PI solution (Sigma-Aldrich, Zwijndrecht, Netherlands) containing 3.8 mM sodium citrate and 66 µL of a 1 µg mL^{-1} BODIPY 493/503 solution in 0.02%$_{(v/v)}$ DMSO were added, together with 50 µL of a 1 mg mL^{-1} RNAse solution (Roche Diagnostics, Almere, Netherlands). Samples were incubated for 30 minutes at 37°C. Fluorescence due to PI and BODIPY staining was measured using a flow cytometer (FACScalibur, Becton Dickinson, Franklin Lakes, NJ, USA) and was recorded in the FL2 and FL1 channel respectively. The instrument settings can be found in table 3.1. FL2 was compensated by 33% of FL1 for overlap of the emission spectra. Data analysis was done with FlowJo sotfware (version 10.0.5, Tree Star Inc, USA).

Major biomass constituents. For analysis of major biomass constituents, liquid samples were taken from the bioreactor in triplicate and centrifuged for 5 minutes at 1204 RCF. The resulting pellets were stored at -20°C. After storage at -20°C, pellets were transferred to beat beater tubes (MP Biomedicals, Germany) and freeze dried overnight. Then, protein content was determined with the DC protein assay (BioRad). For the analysis of starch the Total Starch Kit of Megazyme (Ireland, 2011) was used. Total fatty acid content (TFA) were determined by extraction with a methanol:chloroform mixture and subsequent esterification as described by Bligh and Dyer (1959), with modifications of Lamers et al. (2010) and Santos et al. (2012). Further details are provided by (de Winter et al., 2013).

Table 3.1 *Instrument settings for the flow cytometer*

	Voltage	**Amplification**	**Scale**
FSC	E+00	8.50	Linear
SSC	500	1.00	Linear
FL1	650	1.00	Logarithmic
FL2	650	5.24	Linear

3.3 Results

3.3.1 Growth rate and biomass production rates

The growth rate of *N. oleoabundans* in the nitrogen limited turbidostat culture was calculated using equation 1. In steady state, the daily averaged growth rate was 0.6 day^{-1}. However, calculating the growth rate over 1h time intervals revealed a cyclic pattern during the 24h cycle, even though light intensity was kept constant. In figure 3.1 the

growth rate during 6 days of steady state is depicted (closed circles). For comparison, growth rate of the N-replete culture was included (open circles) (de Winter et al., 2013). Both in N-replete and in N-limited culture growth rate showed a cyclic increase and decrease during a 24h cycle, with high growth rates during the natural day and low growth rates during the natural night. The natural day started at 7.15 AM and 8.30 AM and ended at 20.15PM and 16.30PM for the N-replete and N-limited experiment, respectively. The average growth rate for these six oscillations in steady state is plotted in the top right corner. The observed pattern is similar but, as expected, growth rate was clearly lower in the N-limited culture.

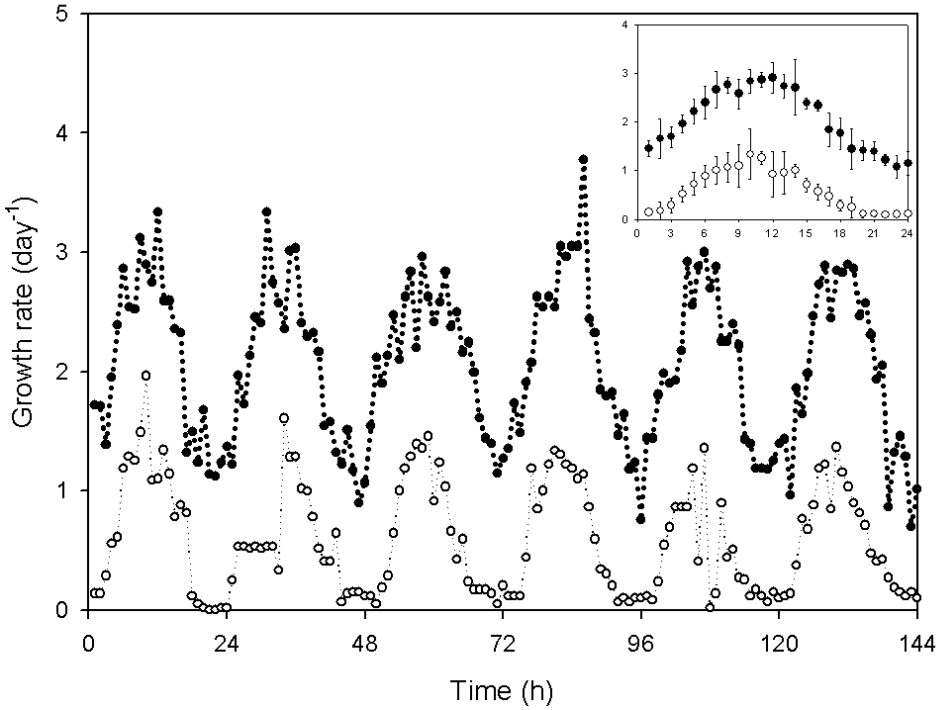


Figure 3.1 *Growth rate in steady state of N. oleoabundans under constant red light in a N-replete (closed circles) and N-limited (open circles) turbidostat culture showing a 24h oscillation. Insert on the top right: the average growth rate over 24h of the six shown oscillations in steady state.*

3.3.2 Cell cycle

Because the growth rate was oscillating, synchronized cell division was expected. There-

fore, measurements were done to determine cell size and number during a 24h cycle. In figure 3.2 the results are shown for the N-limited culture (left) and the N-replete culture from previous work (de Winter et al., 2013) (right). In the N-limited culture, two populations were observed in the sample at 7h in the morning, whereas the sample in the N-replete culture showed only 1 peak. When following the two populations of the N-limited culture during the 24h cycle, it was observed that both the population with small cells and the population with large cells increased in size during the day. At 1h a new peak with small daughter cells appeared, while the peak with the larger mother cells disappeared. The population of smaller cells in between the dashed lines did not divide this day. However, these cells increased further in size the next day and therefore became the dividing population at the end of that day. In the N-replete culture, all cells divided in one day, as can be seen from the single mother cell population disappearing completely, while a new and single population of daughter cells appeared (22h-4h). In other words, in N-replete conditions cell division was synchronized, while in N-limited conditions cell division was phased.

As it was found in previous work (de Winter et al., 2013) that *N. oleoabundans* divides by multiple fission under nutrient replete conditions, the multiple fission cell cycle of *N. oleoabundans* under N-limitation was evaluated as well. DNA content was determined on the flow cytometer. Populations were observed with 1, 2 and 4 copies of DNA. In addition, a population with less than 1 copy of DNA appeared. The populations were gated and the relative cell cycle distribution during a 24h cycle was calculated and is shown in figure 3.3 A. As a comparison, again the data for the N-replete culture are shown in figure 3.3 B. Clear differences were observed between the N-limited and the N-replete 24h cell cycle. No population with 8 copies of DNA (G_4) was found in the N-limited culture, which means that cells were not able to divide in 8 daughter cells like in the N-replete culture. Instead, cells divided in 4 daughter cells (G_3) at most. The average number of daughter cells released per mother cell (N_t/N_0) was 1.8, which was calculated according to Eq. 2, where t_d is doubling time (d) and t is the time interval (d).

$$\frac{N_t}{N_0} = 2^{\frac{t}{t_d}} \qquad \text{(Eq. 2)}$$

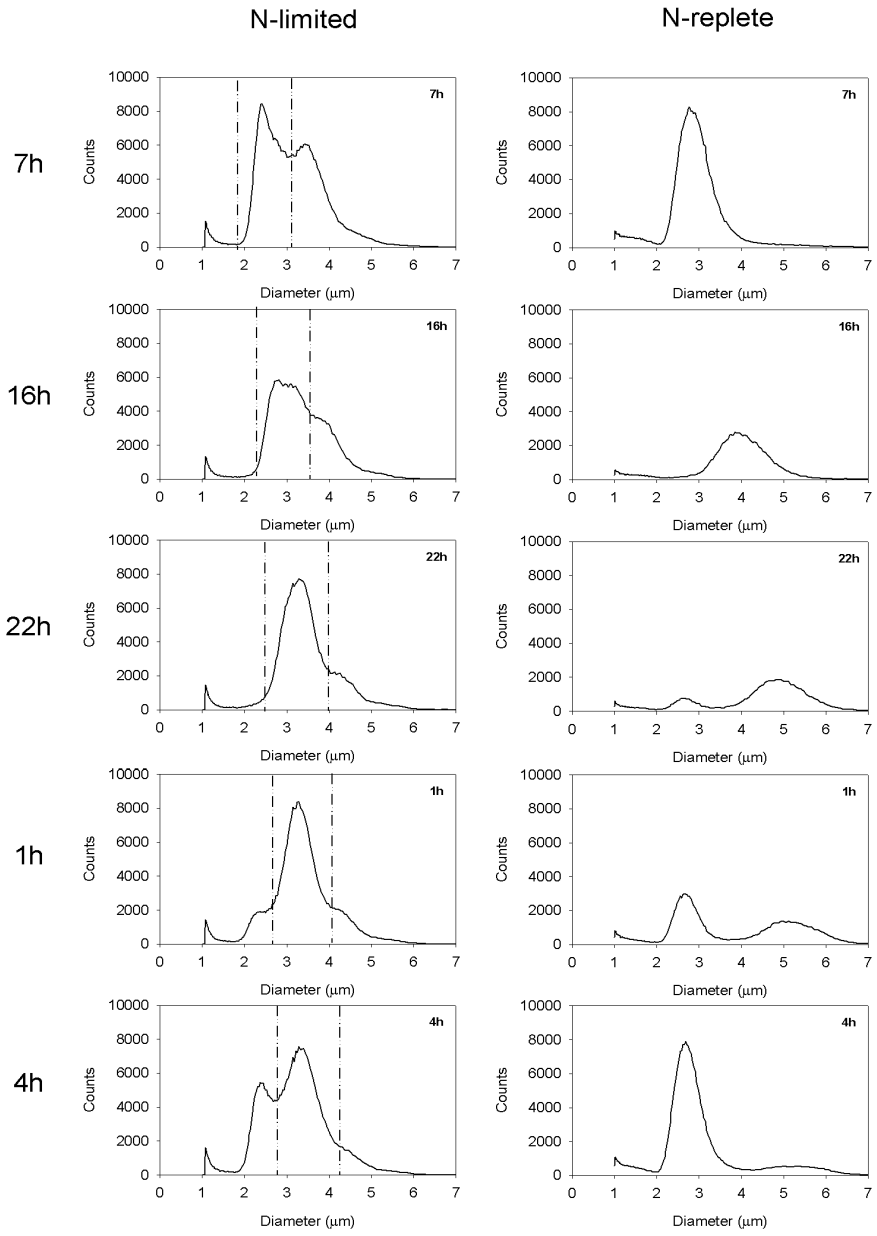


Figure 3.2 *Cell numbers as a function of cell size of samples taken at 7h, 16h, 22h, 1h and 4h in a N-limited (left) and N-replete (right) turbidostat culture of N. oleoabundans. The population between dashed lines in N-limited culture did not divide in this 24h cycle.*

Doubling time t_d is defined in Eq. 3, where μ is the specific growth rate (d^{-1}).

$$t_d = \frac{\ln(2)}{\mu} \qquad \text{(Eq. 3)}$$

DNA synthesis started around the same time (19h) in both cultures. However, in the N-limited culture it seemed not all cells in G2 or G3 divided during the night and thus part of the cells stayed in G2 and G3 during the day. These populations of G2 and G3 decreased during the day. Also, a new population was observed under N-limited conditions with cells containing less than 1 copy of DNA, referred to in figure 3.3 A as 'dead cells'.

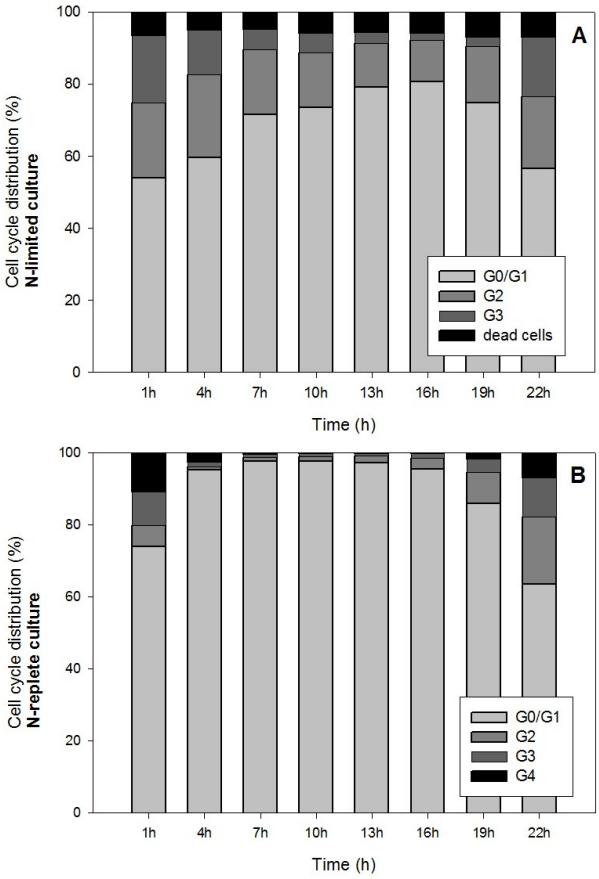


Figure 3.3 *Cell cycle distribution of N. oleoabundans during a 24h cycle in a N-limited (A) and N-replete (B) culture.*

3.3.3 Biomass composition of the culture

During the 24h cycle biomass composition was analyzed. The results are shown in figure 3.4 (open symbols). As a reference, the N-replete culture data are included in the pictures (closed symbols, (de Winter et al., 2013)). Data is presented in grams per gram dry weight, however, since dry weight per culture volume slightly differs over the 24h cycle, also a figure reflecting the data in grams per liter of culture volume is included in appendix figure 3.A.2. Starch content decreased during the night, and increased during the day. The same was true for the TFA content, although the oscillations in TFA content were less pronounced. Results were similar to the results obtained for the N-replete culture, but in the N-limited culture the starch and TFA contents were higher. For protein content, a different result was obtained in the N-limited culture. In the N-replete culture the protein content oscillated, with a slight increase in the day and a decrease during the night. In the N-limited culture the protein content remained stable during the 24h cycle and it was lower compared to the protein content in the N-replete culture. Please note that the fluctuations were only observed during the 24h cycle, whereas the daily samples gave constant values, showing that steady state was maintained throughout the experiments (see appendix figure 3.A.3).

In addition to the TFA content, fatty acid (FA) composition was determined for all samples during the 24h cycle. The FA composition is shown in figure 3.5 A with the N-replete culture data in figure 3.5 B. In general, FA content increased compared to the N-replete culture. Especially C16:0, C18:1 and C18:2 content increased. Furthermore, although the cyclic behaviour was not that apparent in TFA content, FA composition showed oscillations during the 24h cycle. In the N-limited situation, especially C18:1 showed a cycle, increasing during the day and decreasing during the night.

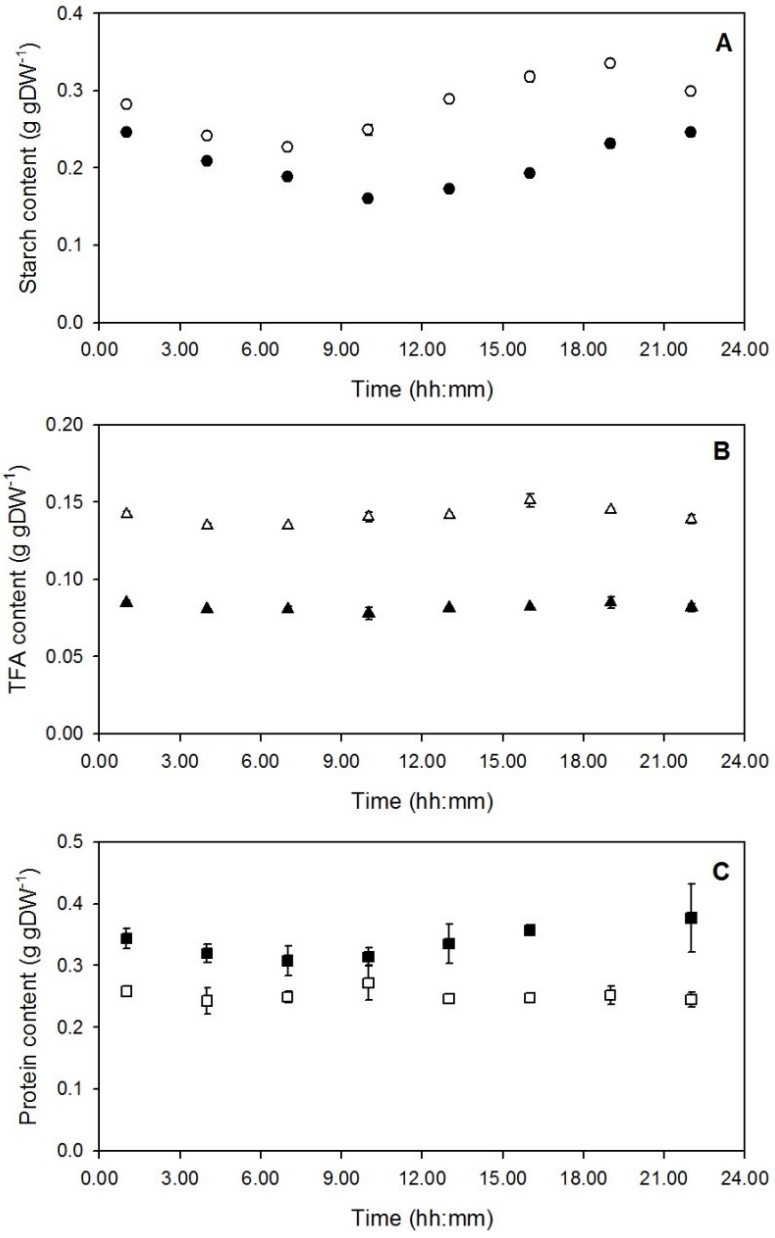


Figure 3.4 *Starch (A), TFA (B) and protein (C) content per DW in g gDW^{-1} of N. oleoabundans biomass during a 24h cycle in a N-limited (open symbols) and N-replete culture (closed symbols). Error bars represent standard deviation between triplicate measurements.*

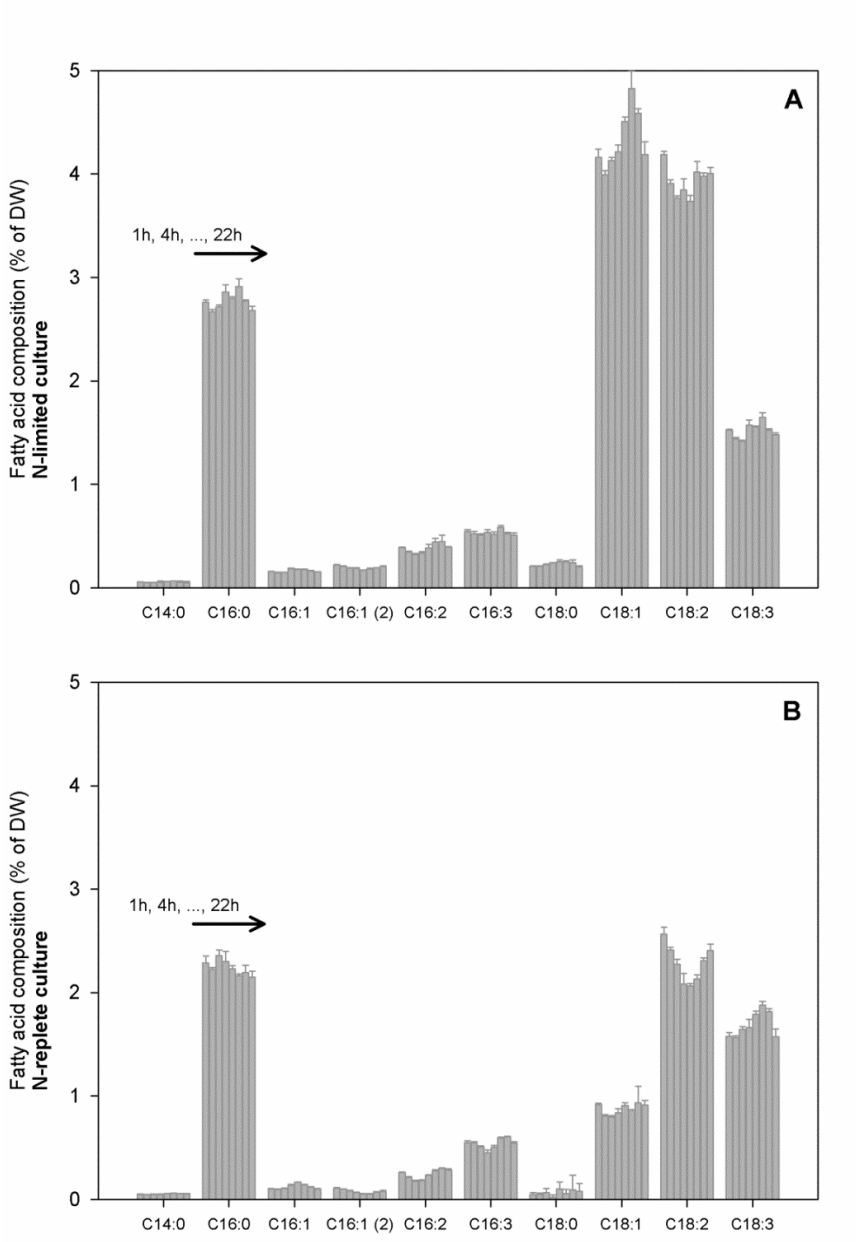


Figure 3.5 *Fatty acid composition of N-oleoabundans during a 24h cycle in a N-limited (A) and a N-replete culture (B). Error bars represent standard deviation between triplicate measurements.*

3.3.4 Biomass composition of single cells

Because the amount of cells was not constant in the turbidostat culture during the 24h cycle (see appendix figure 3.A.1), the content of biomass constituents in a single cell fluctuated differently from the content of these constituents per dry weight. Therefore, biomass content was also plotted per cell, by dividing the total starch, TFA and protein contents in the reactor by the amount of cells present at that moment in time. In figure 3.6 A, 3.6 B and 3.6 C, respectively the starch, TFA and protein contents per cell are shown for the N-replete culture (closed symbols) and the N-limited culture (open symbols). Also, the total dry weight (DW) per cell was plotted (figure 3.6D).

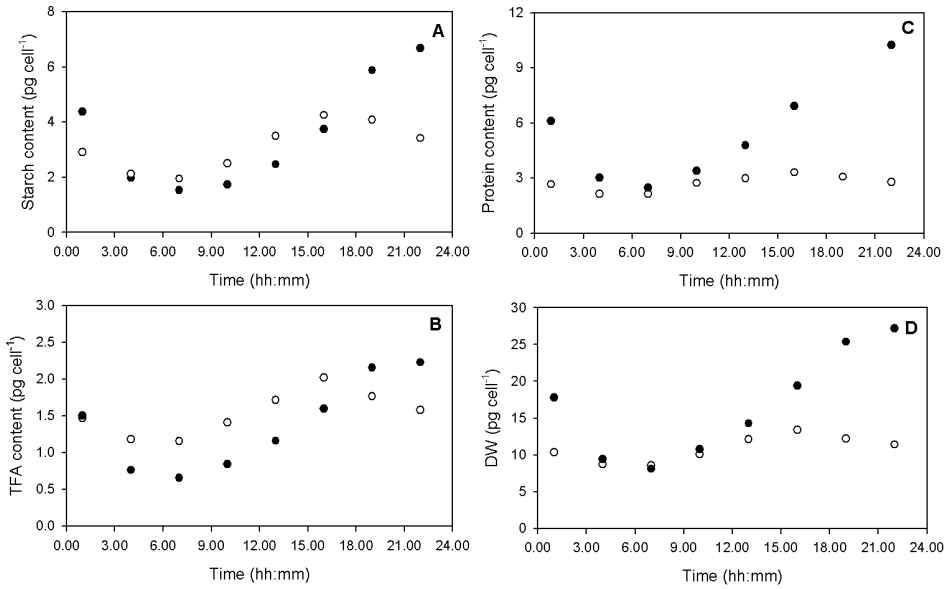


Figure 3.6 *Starch (A), TFA (B), protein (C) and total DW (D) content per cell in pg cell^{-1} of N. oleoabundans biomass during a 24h cycle in a N-limited (open symbols) and N-replete culture (closed symbols).*

Clearly, starch, TFA and protein contents increased during the day and decreased during the night, when cell division occurred. In the N-limited culture the oscillations were less pronounced than in the N-replete culture. Note that the number of cells was not fluctuating as much in the N-limited culture as it was in the N-replete culture (see appendix figure 3.A.1), since not all cells were dividing in one day. Because cell division was not synchronized, but phased, the oscillations in single cells that divide on that day were

possibly larger. However, it is clear that protein content and dry weight per cell did not increase as much during the day in the N-limited cells as they did in the N-replete culture (C and D). TFA content oscillated less in the N-limited culture, but was higher on average (B), while average starch content decreased compared to N-replete cells. Minimum amount of DW, protein and starch after cell division stayed the same for both cultures.

Since cells were present in different phases of the cell cycle during the 24h cycle (see figure 3.3), and thus were not completely synchronized, it was not possible to see in which cell cycle phase lipid accumulation took place. Therefore, cells of the N-limited culture were stained both with PI and BODIPY. The PI stain made it possible to identify the different cell cycle populations. These populations were then gated and the green fluorescence due to BODIPY was measured. This fluorescence is a measure for the amount of neutral lipids present in droplets in the cell (Cooper et al., 2010). As can be seen in figure 3.7, lipids in droplets in G0/G1, G2 and G3 increased during the natural day and decreased during the natural night. In the presumed dead cells the lipid content remained constant. It was also clear that lipid content was higher in the cells containing more DNA (G3 and G2) and that lipid content was lowest in dead cells.

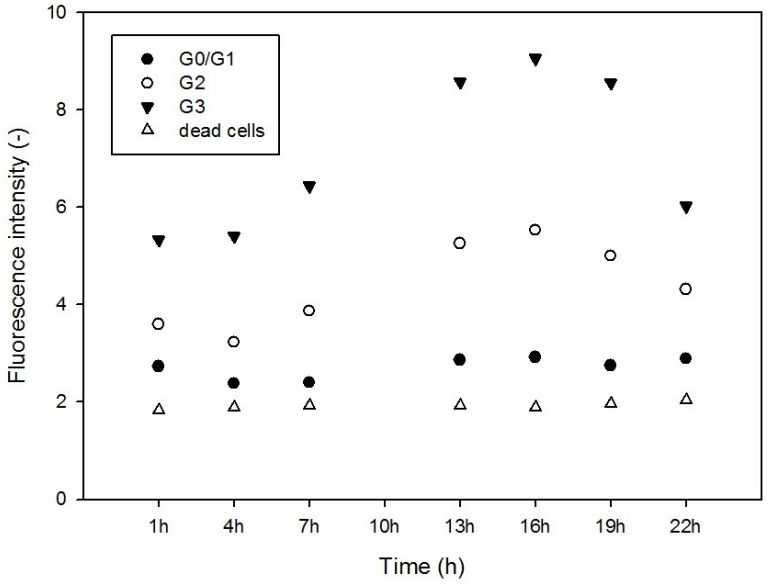


Figure 3.7 *Fluorescence due to BODIPY staining in the different cell cycle populations of N. oleoabundans during a 24h cycle*

3.4 Discussion

3.4.1 Circadian rhythm of the cell cycle under nitrogen limitation

The growth rate of *N. oleoabundans* under N-limitation and constant light was clearly oscillating during the natural day/night cycle (see figure 3.1). Because all conditions in the turbidostat, including light intensity, were constant, the circadian clock must be responsible for these oscillations. Previous work suggested that spectral changes in light intensity in the lab were responsible for synchronization of the cell cycle and regulation of cell division in the night (de Winter et al., 2013). Apparently, this cell cycle regulation by the circadian clock is not affected by nitrogen limitation. Cell division is still timed in the night (see figure 3.2), as is the case for many green microalgae (Lacour et al., 2012; Matsuo et al., 2010; Sukenik and Carmeli, 1990), and as was also the case in light/dark day/night experiments with *Neochloris oleoabundans* (data not shown). However, in contrast to the synchronized population observed in the N-replete situation, two populations arose in the reactor, who divided alternately every other night. This can be related to the growth rate of the culture, which was 0.6 day^{-1}, which corresponds to a doubling time of 1.15 day^{-1} according to Eq. 3 and an average of 1.8 daughter cells per mother cell (see Eq. 2). Consequently, not all cells will be able to divide in the one timeframe per day where the circadian clock allows cells to divide. *N. oleoabundans* divides by multiple fission (de Winter et al., 2013), like *Chlamydomonas* and *Scenedesmus*. In *Chlamydomonas*, cells can grow in G1 up to many times their original size. After this, rapid (n) series of S and M phases occur, yielding 2^n daughter cells. During early G1, cells must pass a commitment point in order to double their DNA and divide at least once during S and M phase (Bisova et al., 2005; Umen and Goodenough, 2001). When cells do not meet the size requirements for growth, for example due to nitrogen limitation, they will not be able to divide. This could explain the two populations observed in figure 3.2. One population reaches commitment the first day, but not the next day, and vice versa. However, in figure 3.3 we also observed cells with multiple copies of DNA present that did not divide during the night, which suggests that there is another control mechanism present after DNA replication that is affected by N-limitation. Arrest in the G2+M phase under nutrient limitation has been described before (Vaulot et al., 1987). Also, it was shown that in *Scenedesmus*, commitment to DNA replication is achieved by doubling of RNA content, while for commitment to nuclear division a doubling of protein content is required, which means that cells can double the amount of DNA without subsequently dividing, because the RNA commitment point is reached but the protein commitment

point is not (Zachleder and Šetlík, 1988). Possibly, the cell cycle in *N. oleoabundans* is regulated similarly, explaining the cells with double and four times DNA content (G2 and G3) present during daytime in the N-limited 24h cycle (figure 3.3). The amount of G2 and G3 cells decreased slightly during the day, which suggests that a limited number of cells divided during the day. In the N-replete culture, G2, G3 and G4 populations disappeared completely during the night, because of cell division.

Another difference with the N-replete culture was that a population with less than 1 copy of DNA became apparent (see figure 3.3). The lower DNA content suggests that cells were breaking down their DNA, which is an indicator of apoptosis, which has been shown to occur in microalgae cells (Moharikar et al., 2006; Segovia et al., 2003). Another possibility is that the DNA became more condensed, making it more difficult for PI to access the DNA, thus lowering the fluorescence signal. This irreversible condensation of DNA is part of necrosis and apoptosis, and was also observed for nitrogen starved *Dunaliella* cells (Carlos et al., 2009). The population of dead cells was constant, which means that a constant percentage of the cells was dying under N-limited conditions.

3.4.2 Biomass composition during the N-limited cell cycle

As discussed in section 3.4.1, a circadian rhythm in cell division was observed under N-limitation. Because all conditions were constant, the changes observed in growth rate (figure 3.1) were solely due to phasing of the cell cycle by the circadian clock. By comparing the biomass composition of the N-limited culture to the biomass composition in a N-replete culture (figure 3.4, 3.5, 3.6), the effect of N-limitation on the biomass composition could be determined during the cell cycle. Under N-replete conditions, storage components are accumulated during the day and decrease upon cell division (de Winter et al., 2013). Under nitrogen limitation, microalgae alter their metabolism and accumulate storage components like TAG and starch in higher amounts, because of an imbalance in energy absorbed in the photosystems and energy that can be used in anabolism (Ball et al., 1990; Hu et al., 2008). In this experiment indeed an increase in average TFA content and average starch content was observed, together with a decrease in protein content (see figure 3.4). However, from these data it was also possible to look at the changes in energy storage metabolism during the cell cycle. which to our knowledge has not been studied extensively in the past, except for a study of (Ballin et al., 1988).

In the N-limited culture, starch content increased during the natural day and decreased during the natural night, when cell division occurred. This is in line with previous findings in the N-replete culture. The oscillations in starch content are slightly less pronounced than in the N-replete culture, which might be related to the lower growth rate. Starch synthesis seems to be linked to growth (Klok et al.), and therefore with a lower growth rate, less starch will be produced and consumed. This was more apparent from the starch content per cell (figure 3.6 A), which was lower at the end of a cycle under N-limited conditions. Similar observations were done by (Ballin et al., 1988). The fact that average starch content per gram of DW was higher can be related to the fact that cells in the N-replete culture contained more dry weight than cells in the N-limited culture (see also figure 3.6 D).

The oscillations in TFA content were not that pronounced as the oscillations in starch content (see figure 3.4). However, also an increase was observed during the day, and a decrease during the night. This oscillation was more easily recognized when looking at the TFA content per cell (figure 3.6 C), and is common for cells grown under light/dark cycles (Lacour et al., 2012; Sukenik and Carmeli, 1990). Oscillations were similar to the oscillations in the N-replete culture, but the average TFA content was higher. From the FA composition (figure 3.5), it was shown that this was mostly due to a higher contribution of C16:0, C18:1 and C18:2, which are fatty acids commonly found in TAG (Hu et al., 2008; Klok et al., 2013a). Especially in C18:1 a cycle is visible, with an increase until 16.00h and a decrease during the night. So, oscillations in TFA content were small, but FA composition changed during the cell cycle. This suggests that different lipid classes are synthesized during different time frames. This is confirmed by the data in figure 3.7, where changes in lipid composition during the cell cycle were also observed. Lipid bodies, which contain TAG (Hu et al., 2008), were stained with BODIPY. The observed increase in BODIPY fluorescence until 16.00h exactly coincides with the increase of C18:1. So, neutral lipids seem to be accumulated during the day. Similar observations were done for neutral lipids in synchronized cultures of *Isochrysis* under light/dark cycles in presence of nitrogen (Lacour et al., 2012). The highest lipid content was found in bigger cells, that were in G2 and G3. Also Davis et al. (2012) found a correlation between neutral lipids and an increase in cell size (Davis et al., 2012). These big cells in G2 and G3 are likely to be arrested in their cell cycle (see section 3.3.2 and 3.4.1), and neutral lipids built up in these cells during the day. This shows again that neutral lipids can be used as an

energy sink when energy from photosynthesis exceeds the energy that can be used in growth (Hu et al., 2008).

The only major biomass component that did not show a cycle under nitrogen limitation was protein (figure 3.4). However, an increase in the content per cell was observed during the day and a decrease in the night, as also observed in the N-replete culture (figure 3.6). Because nitrogen supply rate was limiting, protein synthesis during the day was lower than in the N-replete culture, as also observed by (Ballin et al., 1988) and (Klok et al., 2013a). Also, there were two populations present in the culture, and therefore the oscillation in protein content was possibly levelled out in figure 3.5.

3.4.3 The cell cycle and the algal production process

Because the amount of light absorbed was constant due to turbidostat operation and the growth rate showed clear oscillations (figure 3.1), yield of biomass on light also showed major oscillations during the 24h cycle. Maximum yields were 1.2 g mol^{-1} and 0.9 g mol^{-1} and minimum yields were 0.5 g mol^{-1} and 0.07 in the N-replete and N-limited culture respectively. These changes in yield are solely caused by the circadian clock, and persist as long as cell division can occur. This should be kept in mind when, for example, lighting of photobioreactors during the night is considered (Chen et al., 2011). Providing extra light does not necessarily improve biomass production yields. The yield of storage components also oscillates during the 24h cycle, and for example maximum FA productivity is higher in the N-limited culture than in the N-replete culture. However, due to the lower growth rate in the N-limited situation, the minimum FA productivity during the 24h cycle in the N-limited culture is lower than the minimum FA productivity in the N-replete culture. Therefore, for a fair comparison between N-limited and N-replete cultivations, the productivity should be studied over 24h periods, which was done and discussed in detail by a.o. (Klok et al., 2013a).

The circadian clock caused changes in biomass composition of the culture (figure 3.5), also under N-limitation. Further research would be necessary to elucidate metabolic pathways and regulation of macromolecular metabolism by the circadian clock. However, these data show the result of this regulation and therefore the right moment to harvest cells with high lipid or starch content is before cell division, as was also concluded for cells growing in N-replete conditions (de Winter et al., 2013). When a certain FA com-

position is required, other harvesting times could be considered. Harvesting of proteins can be done at any point, since it was shown that protein content in the N-limited culture remained constant.

Timing could also be of importance in determining the moment for starting N-limitation in outdoor cultures. Figure 8 showed that neutral lipid content was highest in arrested cells at the end of the day, which implies that nutrients have to be taken away before cell division, or better said before the commitment to divide. In this way large cells unable to divide may be created, that will built up neutral lipids during the next photocycle. However, the exact timing cannot be derived from this research and further research is necessary to determine if there indeed is an effect of timing of N-stress on lipid content.

3.5 Conclusions

The circadian clock was not affected by N-limitation, and cell division was timed during the natural night under constant light conditions. Not the entire population was able to divide every day, because of the shortage of nutrients. Two subpopulations were observed in the reactor, which divided alternately every other day. Therefore, cell division was not synchronized but phased, but this phased cell division still caused changes in biomass yield and composition. Protein content was stable during the 24h cycle, but starch and TFA accumulated during the day and decreased during the night. The oscillation in TFA was not as pronounced as the oscillation in starch content, but FA composition changed during the cell cycle. Neutral lipids were built up during the day, especially in cells that were arrested in their cell cycle (G2 and G3). These findings should be kept in mind when optimizing the microalgae production process, for example by determining the right time for harvesting of biomass with the desired composition.

Acknowledgements

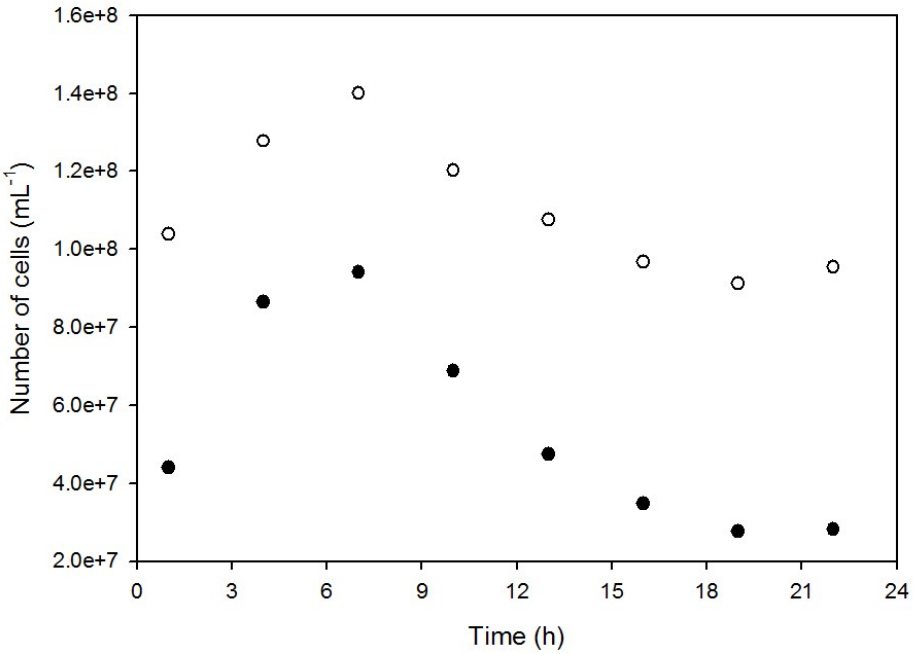
This work was performed within the TTIW-cooperation framework of Wetsus, Centre of Excellence for Sustainable Water Technology (www.wetsus.nl). We would like to thank the participants of the Wetsus research theme 'Algae' for the discussions and their financial support. Wetsus is funded by the Dutch ministry of Economic Affairs.

Abbreviations

BSA	Bovine Serine Albumine
D	Dilution rate (day^{-1})
DW	Dry Weight ($g\ L^{-1}$)
N_o	Number of cells per mL of culture at time = o
N_t	Number of cells per mL of culture at time = t
OD_{680}	Optical Density at 680 nm
OD_{750}	Optical Density at 750 nm
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
t	Time (h)
t_d	Doubling time (d)
TAG	Triacylglycerol
TFA	Total Fatty Acids
V	Volume of overflow produced (g)
V_{PBR}	Photobioreactor volume (g)
μ	Specific growth rate (day^{-1})

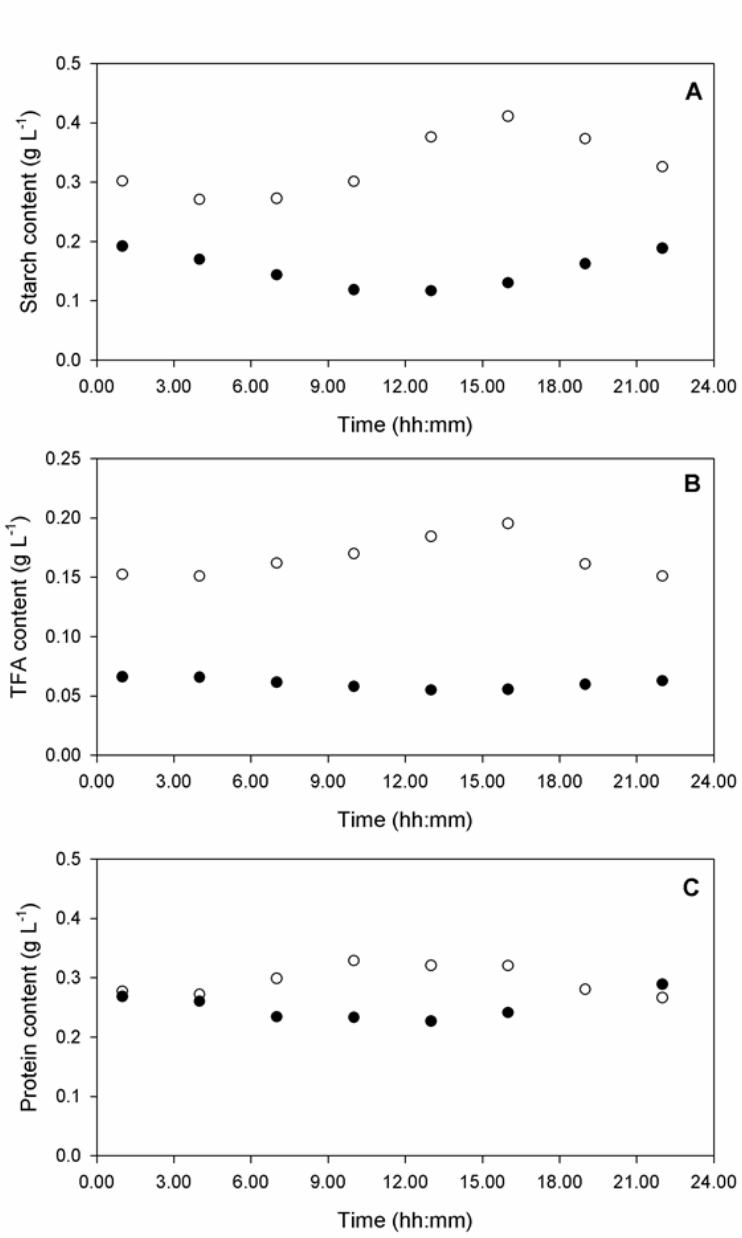
Appendix

Cell number in the turbidostat



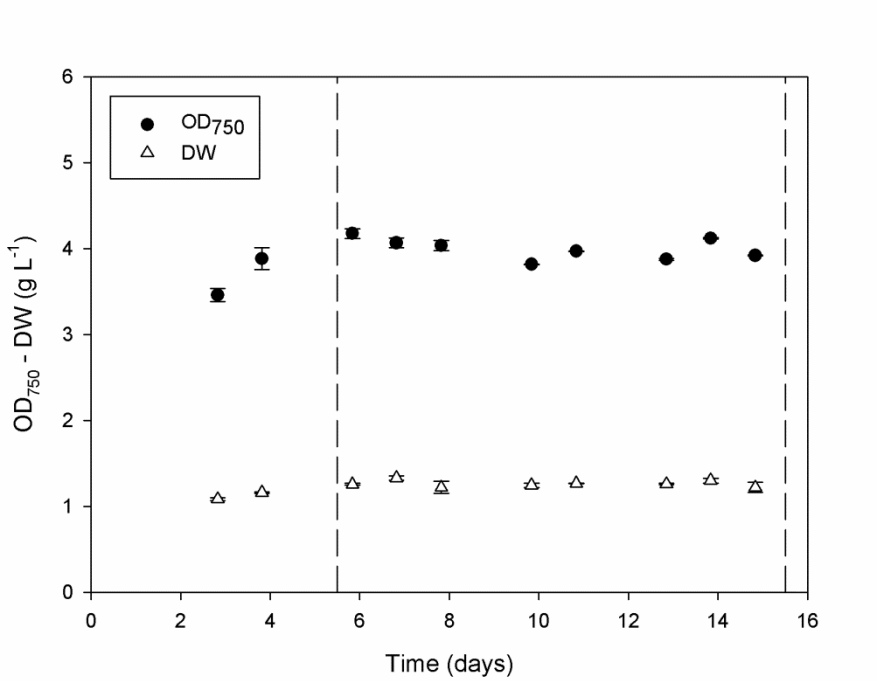
3.A.1 *Number of cells per mL in the turbidostat culture during the 24h cycle under N-replete (closed circles) and N-limited (open circles)*

Biomass composition of the culture in grams per litre of culture volume



3.A.2 *Starch (A), TFA (B) and protein (C) content of N. oleoabundans per liter of culture in g L^{-1} during a 24h cycle in a N-limited (open symbols) and N-replete culture (closed symbols).*

Optical density and DW concentration during steady state in the N-limited culture



3.A.3 *OD_{750} and DW concentration (g L^{-1}) during steady state in a N-limited culture of N. oleoabundans, operated by turbidostat control. Steady state is indicated by the dashed vertical lines.*

Chapter 4

The influence of circadian rhythms on biomass yield and composition of *Neochloris oleaobundans*: Day/night cycles vs. continuous light

This chapter has been submitted as:
Lenneke de Winter, Maria J. Barbosa, Dirk E. Martens, René H. Wijffels
The influence of circadian rhythms on biomass yield and composition of Neochloris oleoabundans : Day/night cycles vs. continuous light

Abstract

The influence of microalgae circadian rhythms on biomass yield and composition was studied by comparing continuous turbidostat cultures of *N. oleoabundans* grown under continuous light with cultures grown under simulated day/night cycles, using white LED light. Under day/night cycles, cultures were synchronized as cell division was scheduled in the night, whereas cell division occurred randomly in the continuous light culture. The synchronized cultures were able to use the light provided 10-15% more efficiently than the culture grown under continuous light. In other words, the efficiency of light usage varies over the cell cycle and the ability to schedule cell division during the night provides a fitness benefit to microalgae. Biomass composition under day/night cycles and continuous light was similar, with the exception of starch content, as under continuous light starch never had to be spend for respiration. No differences in biomass yield and composition were found when the light during the day/night cycle was provided in a 'block' or in a 'sine'. Therefore, providing light intensity in a block provides a good and easy to operate alternative for using sinuses when working with D/N cycles in the laboratory.

In conclusion, the biomass yield and composition of *N. oleoabundans* were influenced by the circadian clock when grown under day/night cycles. These results are important when translating research done under continuous light conditions to day/night cycle conditions and imply that more research should be done under day/night cycles.

4.1 Introduction

The circadian clock provides organisms with an internal estimate of the external time. In this way, it allows organisms to program activities at an appropriate time in the daily cycle. For example, UV sensitive processes like DNA replication can be scheduled to occur during the night. Such an 'escape from light' can provide a fitness benefit to organisms, and therefore is thought to be one of the major reasons for the evolution of the circadian clock (Nikaido and Johnson, 2000; Pittendrigh, 1993). Indeed, in plants it was shown that a substantial photosynthetic advantage was conferred by correct matching of the circadian clock period with that of the external day/night cycle (Dodd et al., 2005). Also in cyanobacteria a competitive advantage was shown for strains with a functioning circadian clock compared to strains with a disrupted clock grown in rhythmic environments (Woelfle et al., 2004).

In microalgae, the circadian clock can 'gate' cell division to take place during the night (Malinowski et al., 1985). Therefore, many species of microalgae have a synchronized cell division when grown under a day/night cycle. However, synchronized cultures of *Neochloris oleoabundans* were also maintained under continuous red LED light, where presumably some blue environmental light triggered synchronous division during the natural night (de Winter et al., 2013; de Winter et al., 2014). It was shown that during cell division the biomass yield on light energy was lower than during the rest of the 24h period (de Winter et al., 2013). This suggests that light provided during cell division is wasted, leading to a lower photosynthetic efficiency when cell division occurs in the light. Indeed, it was shown that microalgae make use of their internal starch reserves for cell division, even when division occurs in the light(Vítová et al., 2011b). Therefore, synchronization under day/night cycles would allow algae to grow during the day, when light is available, and undergo DNA replication and cell division in the dark, making optimal use of the available light energy (Bišová and Zachleder, 2014). This implies that the circadian clock, that schedules cell division in the night, may also provide a fitness benefit to microalgae by increasing the photosynthetic efficiency. Consequently, a randomly dividing culture under continuous white LED light might have a lower biomass yield than a synchronized culture under a simulated day/night cycle.

To verify that biomass yield is indeed influenced by the circadian clock, also fluctuations in biomass composition need to be considered. Biomass composition is influenced by

synchronized cell division and clearly oscillates during a 24h period (de Winter et al., 2013; de Winter et al., 2014; Fábregas et al., 2002). Consequently, biomass yield can be influenced, as for example more energy is needed for assimilation of 1 gram of total fatty acids (TFA) than for assimilation of 1 gram of starch.

A possible influence of the circadian clock on biomass yield and composition would have implications for microalgae research, as in this case it might not be possible to directly translate research under continuous conditions in the lab to day/night cycles in outside conditions. A lot of microalgae research, for example on biomass composition (Breuer et al., 2012; Gouveia et al., 2009) or photosynthetic efficiency (Kliphuis et al., 2010; Sforza et al., 2012), is done under continuous light conditions, where the circadian clock is not able to synchronize cell division and therefore algae divide randomly. Research also focussed on simulating outdoor conditions by applying light in light/dark cycles in 'block' form, i.e. lights on/off (Popovich et al., 2012) or 'sine' wave form, i.e. lights slowly on/off (Cuaresma et al., 2011). However, a solid comparison between cultures grown under continuous light and cultures grown under day/night cycles is lacking, as well as a comparison between cultures grown under 'sine' and block' lighting regimes.

The aim of this research was to investigate the influence of microalgae circadian rhythms on biomass yield and composition. Therefore, *N. oleoabundans* was grown in a continuous turbidostat photobioreactor under continuous white LED light, resulting in a randomly dividing culture. Biomass growth, oxygen production and biomass composition were monitored and compared with results from synchronized cultures obtained in similar photobioreactor runs under 16:8 day/night cycles (16D8N), with light either supplied in 'block' or in 'sine'. In this way, the influence of microalgae circadian rhythms on biomass yield was revealed.

4.2 Materials and methods

4.2.1 Preculture

Neochloris oleoabundans UTEX 1185 (The culture collection of Algae, University of Texas, Austin) was cultivated in 250 mL shake flasks containing 100 mL adjusted BBM medium with pH 7.5 (Klok et al., 2013a)on a shaking incubator (Max Q 3000, Barnstead) at 120 RPM at a temperature of 25°C. Light was provided at an intensity of 20-40 µmol m^{-2} s^{-1} through 16D:8N cycles. Four days prior to inoculation the cultures were transferred to a

second shaking incubator at 120 RPM (Orbital Incubator, Sanyo, Japan) with light provided continuously at an intensity of 150 µmol m^{-2} s^{-1}. Temperature was again kept at 25°C and the headspace of the incubator was enriched with 5% CO_2.

4.2.2 Photobioreactor set-up and experimental conditions

N. oleoabundans was continuously cultivated in a flat panel photobioreactor (PBR) (Labfors 5 Lux, LED Flat Panel Option, Infors HT, Switserland). A schematic overview of the PBR set-up is shown in figure 4.1. The light path of the PBR was 20 mm and the working volume was approximately 1800 L. The light was supplied by 360 LEDs (warm white light, spectrum from 450 nm – 620 nm). A black cover was placed on the back of the reactor to ensure no environmental light was able to enter the reactor. In addition, a cover especially designed to fit the PBR was placed in between the LED panel and the reactor, functioning as a light tunnel and again preventing environmental light from entering the PBR. Ingoing and outgoing light intensity (PDF_{in} and PDF_{out}) were measured at 44 points equally distributed over the lighted PBR surface using a Li-cor quantum sensor (LI250 light meter, LI-COR, USA).

After inoculation, PDF_{in} was gradually increased until it reached the final set point, in order to allow the microalgae to adapt to the new light conditions. When PDF_{out} reached its final setting due to biomass growth, the light regime was changed to the final light settings. Table 4.1 shows a summary of the different light settings that were tested. The maximum light intensity in experiment D was chosen such, that the total amount of photons provided to the algae was the same as in experiment B. In experiment C, the maximum light intensity of the sine was the same as the maximum light intensity in experiment A and B.

Table 4.1 *Light conditions used in the experiments*

Experiment	Photoperiod (h)	Light supply	Max intensity (µmol m^{-2} s^{-1})	PF (mol L^{-1} day^{-1})
A	24	Continuous	500	1.96
B	16	Block	500	1.30
C	16	Sine	500	0.86
D	16	Sine	770	1.30

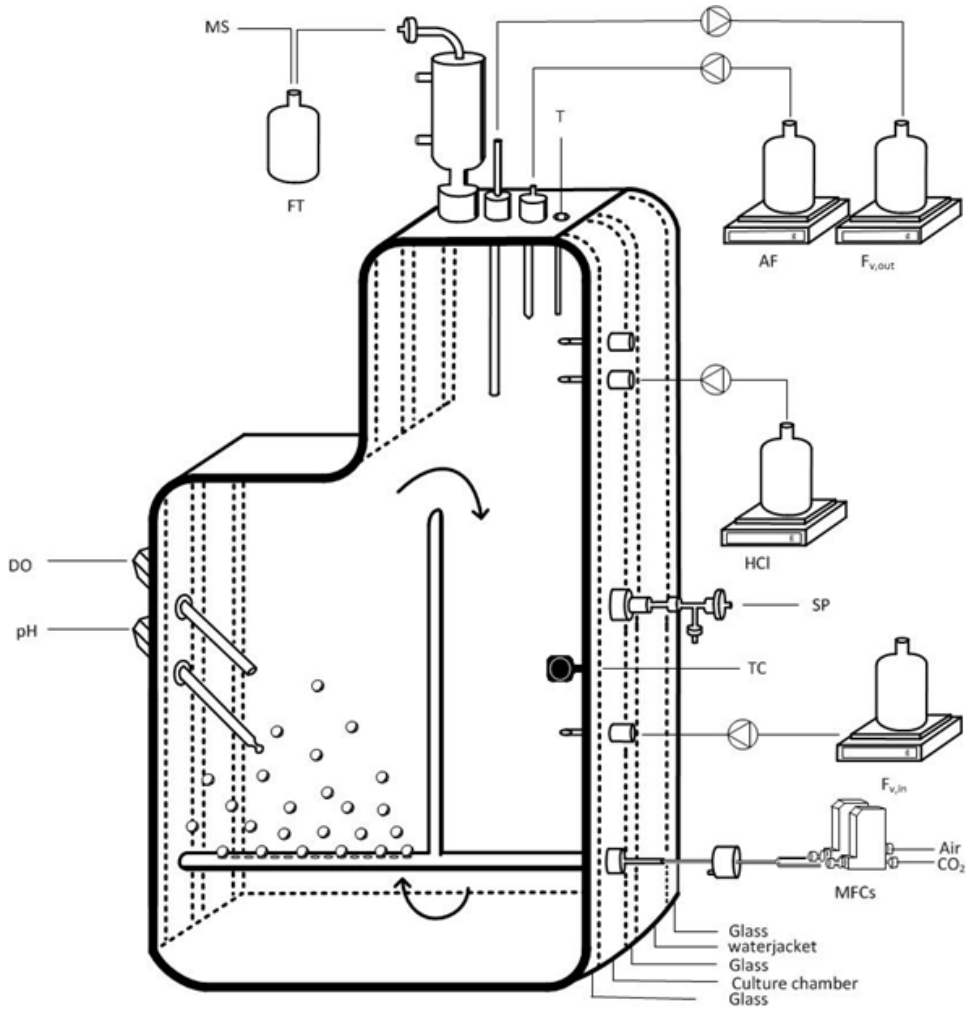


Figure 4.1 *Schematic overview of the PBR set-up. MS – mass spectrometer, AF – antifoam, Fv,out – overflow vessel on balance, HCl – pH control, T – temperature sensor, SP – sample port, TC – light sensor for turbidostat control, Fv,in – medium inflow, MFCs – mass flow controllers, DO – dissolved oxygen sensor, pH – pH sensor*

The temperature inside the PBR was maintained at 30°C using the internal temperature control system of the Infors HT system that was connected to the water jacket of the PBR. Water was provided to the Infors HT system at a constant temperature of 20°C through the use of an external cryostat (RE306/E300, Lauda, Germany). The pH was

maintained at 7.5±0.2 by an automatic supply of 1 M HCl. Dissolved oxygen was measured online and foam formation was prevented by manually supplying a 2%$_{(v/v)}$ antifoam solution (Antifoam B® silicone emulsion, Mallinckrodt Baker B.V., Deventer, The Netherlands) every morning and afternoon. The culture was continuously sparged with 1000 mL min^{-1} air enriched with 2%$_{(v/v)}$ of CO_2, provided by the set of mass flow controllers embedded in the Infors HT system. The air was leaving the reactor through a condenser, which was connected to a cryostat at 2°C.

When light was on, also the turbidostat control was turned on. Turbidostat control ensured dilution of the culture with fresh culture medium when PDF_{out} dropped below the set value. PDF_{out} was set as 10% of PDF_{in}. In this way, the light absorbed by the culture was always 90% of PDF_{in}. The system was allowed to reach steady state, which was defined as a constant biomass concentration measured at the same time every day, and a constant average daily dilution rate for a period of at least 3 residence times. In steady state, the daily growth rate (μ_{24}) is equal to the dilution rate (D), which was monitored by logging the amount of overflow produced (V_{24}) over 24 hours (see Eq. 1).

$$\mu_{24} = D = \frac{V_{24}}{V} \qquad \text{(Eq. 1)}$$

The total amount of light absorbed is kept constant by the turbidostat control, which means that changes in biomass concentration in the reactor are possible when light absorbing properties of the biomass change. Therefore, growth rate does not equal the dilution rate over smaller time intervals during the day, but becomes a function of biomass growth rate (μ) and the change in absorption cross section (a) of the biomass (Eq. 2). Derivation of Eq. 2 can be found in the appendix.

$$D = \mu + \frac{1}{a}\frac{da}{dt} \qquad \text{(Eq. 2)}$$

4.2.3 Sampling, biomass analysis and calculation of yield from overflow

Samples were taken daily at the same time to monitor biomass growth and steady state by measuring the optical density at 750 and 680 nm (OD_{750} and OD_{680}), cell number, cell size, total cell volume and dry weight (DW), as described by Kliphuis et al. 2010 (Kliphuis et al., 2010). During steady state, the total overflow of a 24h cycle was collected and kept on ice. This was done for 3 consecutive cycles. From the harvested biomass, DW samples were taken in order to determine biomass concentration (C_x) and calculate bio-

mass productivity (P_x) and yield ($Y_{E/x}$) over 24 hours according to eq. 3 and 4.

$$P_x = \mu_{24} \cdot C_x \quad \text{(Eq. 3)}$$

$$Y_{x/e} = \frac{\mu_{24} \cdot C_x}{PF} \quad \text{(Eq. 4)}$$

In which PF is the photon flux. The remainder of the biomass was centrifuged at 4500 RPM for 30 minutes. The pellets were washed with demi water and again centrifuged at 4500 RPM for 20 minutes. Pellets were then stored at -20°C until freeze drying. After freeze drying, pellets were grinded with a mortar and pestle and subsequently aliquots of the biomass were used to determine major biomass constituents (proteins, starch and fatty acids) as described by de Winter et al., 2013 (de Winter et al., 2013).

4.2.4 Gas analysis and calculation of yield from O_2 production

Outgoing air flow was analysed with a Prima dB mass spectrometer (Thermo Fisher Scientific). Oxygen production rate ($\Phi_{O2,prod}$) in mL min^{-1} was calculated according to eq. 5:

$$\phi_{O2,prod} = \phi_{N2,b} \cdot (\frac{x_{O2}}{x_{N2}})_{out} - \phi_{O2,b} \quad \text{(Eq. 5)}$$

In which $\Phi_{N2,b}$ and $\Phi_{O2,b}$ are the nitrogen and oxygen flow in mL min^{-1} measured in a baseline prior to inoculation of the reactor. x_{O2} and x_{N2} are the fractions of oxygen and nitrogen in the outgoing gas and the fraction of nitrogen is assumed constant. From this, the oxygen production rate (OPR) and CO_2 consumption rate (CUR) in mmol h^{-1} were calculated:

$$OPR = \frac{\phi_{O2,prod} \cdot p_0}{R \cdot T_0} \cdot 0.06 \quad \text{(Eq. 6)}$$

$$CUR = \frac{OPR}{PQ} \quad \text{(Eq. 7)}$$

In which PQ is the photosynthetic quotient followed from the stochiometric growth equation for growth of *N. oleoabundans* on nitrate (eq.8), in which molecular composi-

tion of *N. oleoabundans* biomass was taken from Pruvost et al. (Pruvost et al., 2009).

CO_{2} + 0.93 H_2O + 0.15 NO_3 --> $CH_{1.7}O_{0.43}N_{0.15}$ + 1.4 O_2 + 0.15 OH^- (Eq. 8)

Finally, with the molecular weight of *N. oleoabundans* biomass, $m_{biomass}$ (Pruvost et al., 2009) the biomass yield on light ($Y_{X/e}$) in g mol^{-1} was calculated (eq. 8) to be compared with the yield calculated in eq. 3.

$$Y_{x/e} = \frac{CUR \cdot m_{biomass}}{PF} \quad \text{(Eq. 9)}$$

4.3 Results and discussion

3.3.1 Growth rate

Daily dilution rate and biomass concentration of the cultures at a set time were followed untill steady state was reached. During steady state, the daily dilution rate is equal to average specific growth rate over a day (eq.1). However, as in turbidostat cultures the light absorbed is kept constant, slight changes in biomass concentration are possible due to changes in biomass composition (eq.2). As a result, dilution rate does not directly translate into growth rate in the cultures under 16D8N cycles, where oscillations in biomass composition occur that are not observed under continuous white LED light. Therefore, the term dilution rate will be used instead of specific growth rate.

The dilution rate was calculated from the amount of overflow produced using a 1 hour time intervals and plotted in figure 4.2. As a reference, the light intensity applied to the culture was added in the figures (dashed line). The average 24h dilution rate was 2.2, 1.7, 1.4 and 1.7 for experiments A, B, C and D respectively. In the experiments done under a 16D8N cycle (see figure 4.2B, 4.2C, 4.2D) the dilution rate varied from 0 day^{-1} in the night till approximately 4 day^{-1} during the day. The maximum dilution rate was slightly higher in experiment D, where maximum light intensity applied to the culture was the highest, and therefore maximum dilution rate was dependent on light intensity. This is an expected result, as growth rate is a function of light intensity (Sorokin and Krauss, 1958).

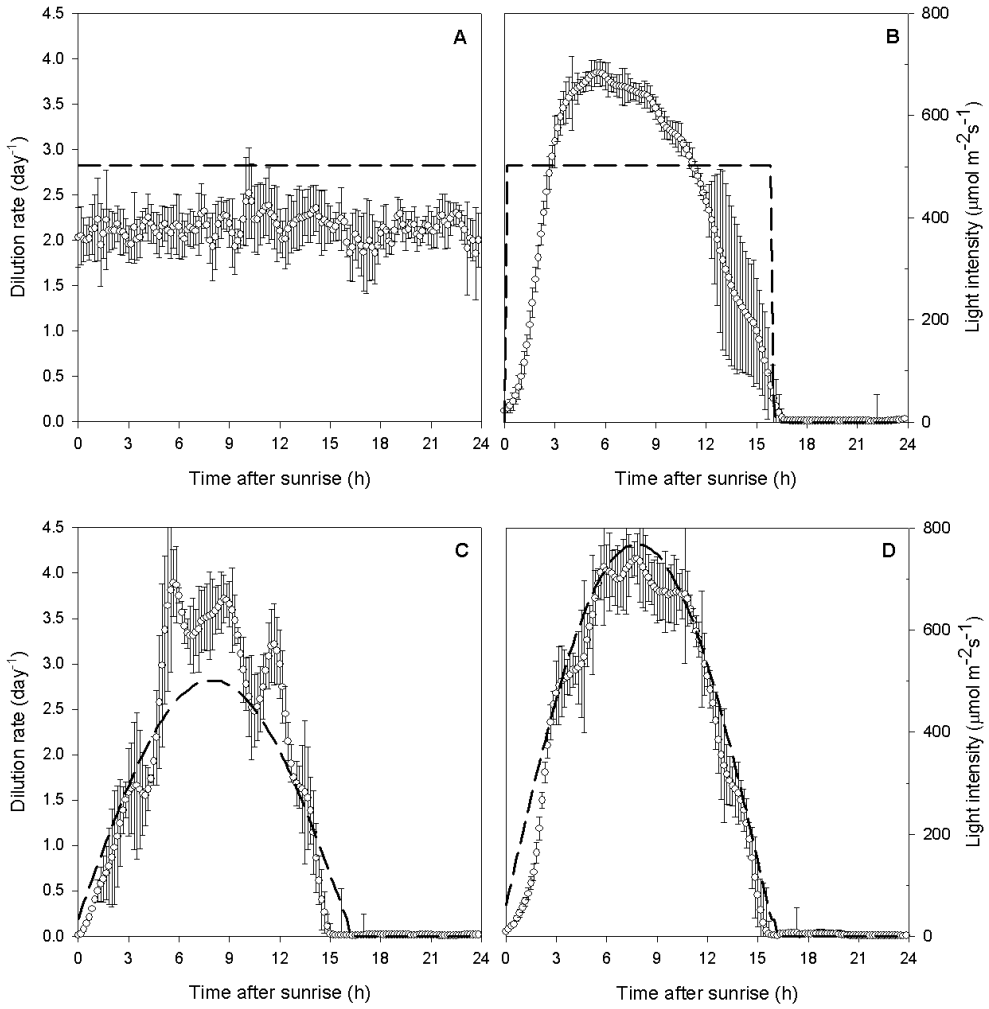


Figure 4.2 *Daily variation in dilution rate in steady state in cultures grown under continuous light (A), 16D8N cycles applied in block form (B), sine with low light intensity (LL) (C) and sine with high light intensity (HL) (D). Dilution rate was averaged over at least 5 days in steady state and error bars represent standard deviation between those days. Ingoing light intensity is plotted in dashed lines.*

However, a more interesting observation was that in all the D/N cycle experiments dilution rate increased untill approximately 6 hours after 'sunrise' and started to decrease after 9 hours of light. Strikingly, also in experiment B, where light was applied with con-

stant intensity during the day period, dilution rate more or less followed a sine curve. The maximum in dilution rate between 6 and 9 hours of light suggests that a timing mechanism is involved in growth of *N. oleoabundans*. Before reaching the maximum dilution rate, the total amount of light received by the cultures in experiment B, C and D was different. Therefore, the attainment of the maximum dilution rate was not a function of the amount of light received, and more likely resulted from a process timed by the circadian clock. Photosynthesis is well known to be under control of the circadian clock (Matsuo et al., 2010) and daily variations in photosynthetic rate were already described in earlier research (Gerath and Chisholm, 1989; Post et al., 1985a). To see if the maximum dilution rate coincided with a maximum in photosynthesis rate, gas flow coming from the reactor was analysed on a mass spectrometer. In figure 4.3, the oxygen production rates in mL min^{-1} during a 24h cycle in steady state, as calculated from eq. 5 are shown. As can be seen, oxygen production rates in experiments C and D closely follow a sine curve, indicating that the culture as a whole was growing light limited with higher light intensities leading to higher oxygen production rates (MacIntyre et al., 2002). As such a maximum oxygen production rate was expected when ingoing light intensity was at its top. However, in experiment B, where light intensity was kept constant for a 16h period, a maximum oxygen production rate was also obtained. In combination with the strong sine that was observed in dilution rate (figure 4.2B), this again suggests the influence of the circadian clock, which synchronizes cell division during the night in the 16D8N cycle (Tamiya, 1966). The synchronized cell division influences biomass composition (de Winter et al., 2013), which can in turn influence dilution rate in the turbidostat (eq. 2). An interesting observation is that the increase in oxygen production rate in experiment B was a lot faster than the increase in dilution rate (figures 2B and 3). This means that growth rate increased immediately when light was turned on, but apparently the production of light absorbing material (a), and therefore the dilution rate, lagged behind. Also the decrease in dilution rate after the maximum was steeper than the decrease in oxygen production rate. This indicates that after the maximum in dilution rate the absorption cross section of the biomass decreases, again meaning that other biomass constituents, like starch, were synthesized at higher rates than the light absorbing material.

Under the continuous white LED light conditions in experiment A all timing processes were clearly lost or randomized (figure 4.2A), resulting in an average dilution rate of 2.2

day^{-1}. This was expected when applying a constant intensity of white LED light, as a synchronized cell cycle can not be maintained under these conditions (de Winter et al., 2013). Also, oxygen production rate in experiment A was constant, at 2.3 mL min^{-1}. In experiment B, where light was supplied with the same intensity as in experiment A, oxygen production rate during the day was higher with a maximum oxygen production rate of 2.8 mL min^{-1}. When biomass composition in experiment A and B is similar, the higher oxygen production rate already indicates a higher photosynthetic efficiency during the day in experiment B, as more oxygen was produced using the same amount of photons. In section 3.3, the biomass yield and productivity of the synchronized cultures (experiment B,C,D) and the randomly dividing culture (experiment A) will be further addressed.

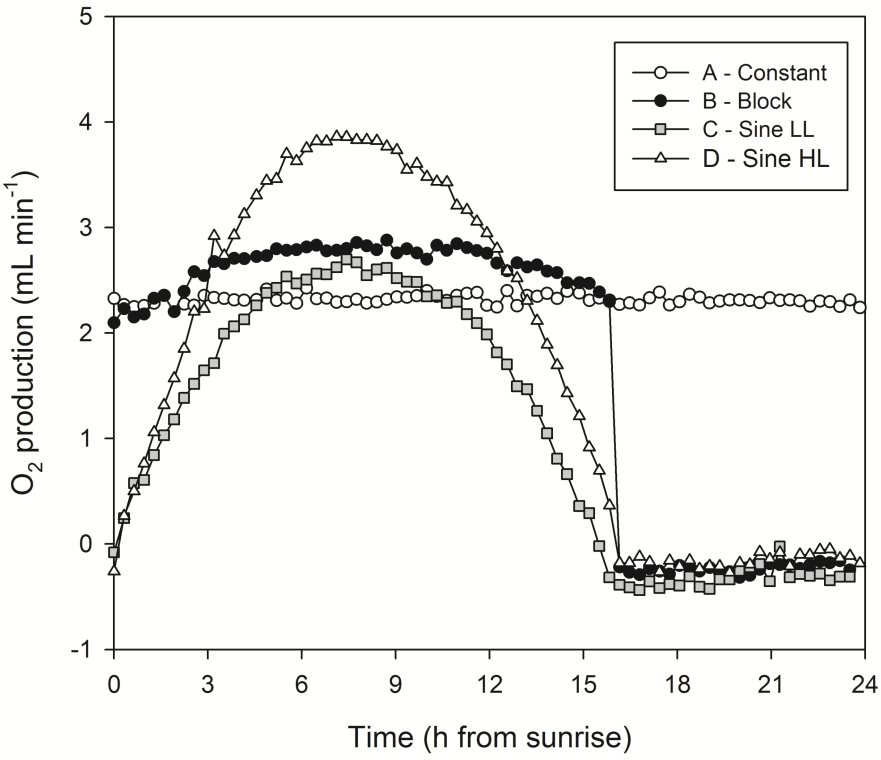


Figure 4.3 *Oxygen production rate ($\Phi_{O2,prod}$) during a day in steady state for experiments A (white circles), B (black circles), C (grey squares) and D (white triangles)*

4.3.2 Biomass composition

Biomass yield on light can also be influenced by biomass composition, as will be further discussed in section 4.3.3. Therefore, major biomass constituents of the harvested biomass were determined. Samples were taken from the overflow of 3 subsequent days in steady state and analysed for protein content, TFA content and starch content . In figure 4 the results are presented in % of DW and error bars represent the standard deviation between the 3 days.

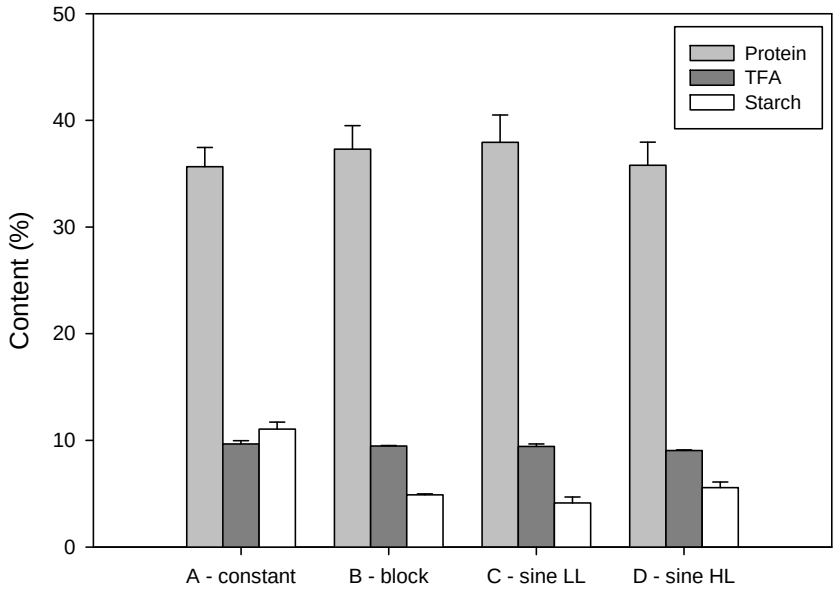


Figure 4.4 *Biomass composition of N. oleoabundans with different lighting regimes as a percentage of dry weight. Biomass was pooled over the 24h cycle and error bars represent standard deviation between triplicate 24h cycles.*

Protein content and TFA content were the same in all experiments. However, starch content was twice as high in the continuous light culture. During a 24h period under day/night cycles, biomass composition clearly oscillates (Fábregas et al., 2002). Storage molecules like starch are built up during the day, and respired during the night (Bránykiová et al., 2011). Possibly, starch content in the continuous light culture remains high because there is no dark period during which storage molecules have to be spend for maintenance. This has to be kept in mind when translating research on the biomass

composition of microalgae done under continuous light conditions in indoor experiments (Breuer et al., 2012; Gouveia et al., 2009) to outdoors. For *N. oleoabundans* grown in nutrient replete conditions the only difference was observed in starch content. However, it should be noted that the sum of the measured biomass constituents only accounts for approximately 50% of total dry weight, which means that other biomass constituents can also show differences. In addition, under other process conditions or in other microalgae also differences in TFA might be expected between continuous light and day/night cycle cultures, as also fatty acids might be used in respiration (Lacour et al., 2012). There was no difference in protein, starch and TFA content between experiment B and experiment C and D, which underlines that providing light energy in block is a good alternative for using sine forms when working with D/N cycles in the laboratory.

4.3.3 Biomass yield and productivity

Biomass yield in grams of DW per mole of photons was calculated from eq. 4 and plotted in grey bars in figure 4.5 (left), with error bars representing the standard deviation between three subsequent days in steady state.

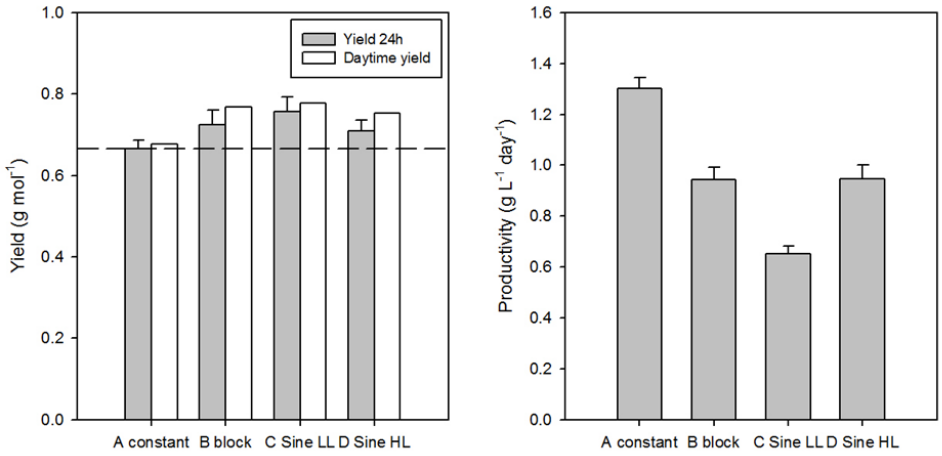


Figure 4.5 *Yield and productivity of the cultures grown under continuous light and 16D8N cycles applied in block, sine LL and sine HL. Grey bars represent productivity and yield calculated over 24h, using the biomass harvest of three days and error bars represent standard deviation between triplicate measurements. White bars represent yield calculated over just the daytime period, using the O_2 production rates.*

The biomass yield in continuous light was slightly lower (approximately 10%) than the biomass yield in the D/N cycle experiments (T-test, $P<0.05$). Furthermore, in the D/N cycle experiments, a negative oxygen production rate was measured during the night, indicating that oxygen was consumed through respiration of biomass (figure 4.3). Therefore, in the D/N cycle cultures (experiments B, C, D) it is likely that some biomass is lost during the night (J.Geider and Osborne, 1989), and as such the biomass yield on light during the daytime period could be even higher than the value measured here over a whole day. To confirm this, the biomass yield on light during the light period (16h) was calculated (eq. 6-9), by integration of the oxygen production rates in figure 4.3 and converting this to biomass production with the stochiometric growth equation for growth of *N. oleoabundans* on nitrate. The results are plotted as white bars in figure 4.5 and referred to as daytime yield. As expected, the daytime yield in the D/N cycle cultures calculated from the oxygen production rates was higher than the 24h yield, whereas no difference was found in the continuous light experiment. The difference in biomass yield between continuous light and D/N cycle cultures became approximately 15%. The increased biomass yield in the D/N cycle cultures is not due to the lower starch content in these cultures. Less energy is required for the assimilation of starch than for the assimilation of most other biomass components, like protein, lipids, DNA and RNA. As starch content under day/night cycles is lower, the amount of energy needed for assimilation of biomass in the day/night cycle cultures is higher than in the continuous light culture. This means that yield of biomass on photons would be even higher in the day/night cycle cultures when correcting for the energy density of the biomass.

Interestingly, no clear difference in yield was observed between the experiment where light was provided in a block of constant light intensity (B) and the experiments where light was provided in a sine form (C and D). It was expected that when light was provided in lower intensities in the beginning of the day, algae had more time to adapt to the increasing light intensity and therefore would be able to use the light more efficiently (MacIntyre et al., 2002) . Indeed, in experiment C, where light intensities were the lowest, yield was slightly higher. In experiment D, where light at the maximum was the highest, yield was slightly lower. However, the obtained differences were within the measurement error, which means that providing light in a block to controlled indoor experiments can provide a good and easy to operate alternative to providing light in sine form.

In summary, light energy was used 10-15% more efficiently during the day in the synchronized cultures, where cell division occured during the dark period, than in the continuous light culture, where cell division occured randomly. A possible explanation for the lower yield in the continuous light culture is that during cell division light is used less efficiently for a certain period of time. Possibly, cells use starch as an energy source for cell division in the light, like they also do during the night(Vítová et al., 2011b). Therefore, some of the light energy provided during cell division might be wasted. In nature, UV light might even cause damage to the cells during cell division. As a consequence, scheduling cell division during a dark period provides a fitness benefit to microalgae. However, as the difference in biomass yield was only 10-15%, biomass productivity was still mainly dependent on the amount of light provided to the culture. Daily biomass productivity (24h) was calculated according to eq.3. In figure 4.5 (right figure), it can be seen that productivity was exactly the same in the experiments with light provided in a block (B) and in a sine (HL) (D). Those two experiments received the same amount of photons, whereas the continuous light culture (A) received more, and the sine LL (C) culture received less. So, productivity was mainly a function of the amount of photons received, and therefore providing more light will result in a higher productivity.

4.4 Conclusions

Microalgae cultures of *N. oleoabundans* that were synchronized by day/night cycles were able to use the light provided 10-15% more efficiently than cultures grown under continuous light. In other words, the efficiency of light usage varies over the cell cycle and the ability to schedule cell division during the night provides a fitness benefit to microalgae. Protein, TFA and starch contents of the 16D8N cycle cultures were the same. However, a higher starch content was found when continuous light was provided. The microalgae under these conditions never had to spend starch for respiration during a dark period, and therefore starch content remained higher. No difference in biomass yield and composition was found when the light during the 16D8N cycle was provided in a block or in a sine. Therefore, providing light intensity in a block provides a good and easy to operate alternative for using sinuses when working with D/N cycles in the laboratory.

In conclusion, the biomass yield and composition of *N oleoabundans* were influenced by the circadian clock when grown under D/N cycles. These results have to be kept in mind when translating research done under continuous light conditions to outdoor D/N cycle conditions.

Acknowledgements

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Abbreviations

TFA	Total fatty acids	-
D/N	Day/night	-
PFD_{in}	Ingoing light intensity	µmol m^{-2} s^{-1}
PFD_{out}	Outgoing light intensity	µmol m^{-2} s^{-1}
μ_{24}	Daily average specific growth rate	Day^{-1}
µ	Specific growth rate	Day^{-1}
D	Dilution rate	Day^{-1}
a	Absorption cross section	m^2 gDW^{-1}
V_{24}	Volume overflow collected over 24h	L h^{-1}
V	Volume of bioreactor	L
C_x	Biomass concentration	g L^{-1}
P_x	Productivity	g L day^{-1}
$Y_{x/e}$	Yield of biomass on light	g mol^{-1}
PF	Photon flux	mol L^{-1} day^{-1}
$\Phi_{O2,prod}$	Oxygen production rate	mL min^{-1}
$\Phi_{N2,b}$	Nitrogen flow in baseline	mL min^{-1}
$\Phi_{O2,b}$	Oxygen flow in baseline	mL min^{-1}
x_{O2}	Fraction of oxygen in outgoing gas	-
x_{N2}	Fraction of nitrogen in outgoing gas	-
P_o	Ambient pressure	Pa
R	Gas constant	(Pa L^{-1})/(mmol K^{-1})
T_0	Absolute temperature	K
OPR	Oxygen production rate	mmol h^{-1}
CUR	Carbon dioxide consumption rate	mmol h^{-1}
PQ	Photosynthetic quotient	-
$m_{bio-mass}$	Molecular weight of the biomass	g

Appendix

Derivation of equation 2

Balance over the amount of absorbing material a (m^2.gDW^{-1}):

$$V\frac{d(C_x \cdot a)}{dt} = V \cdot (a \cdot \frac{dC_x}{dt} + C_x \cdot \frac{da}{dt}) = V \cdot r_a \cdot C_x - F \cdot C_x \cdot a = 0$$

$$D = \frac{r_a}{a}$$

$$\frac{dC_x}{dt} = -\frac{C_x}{a} \cdot \frac{da}{dt}$$

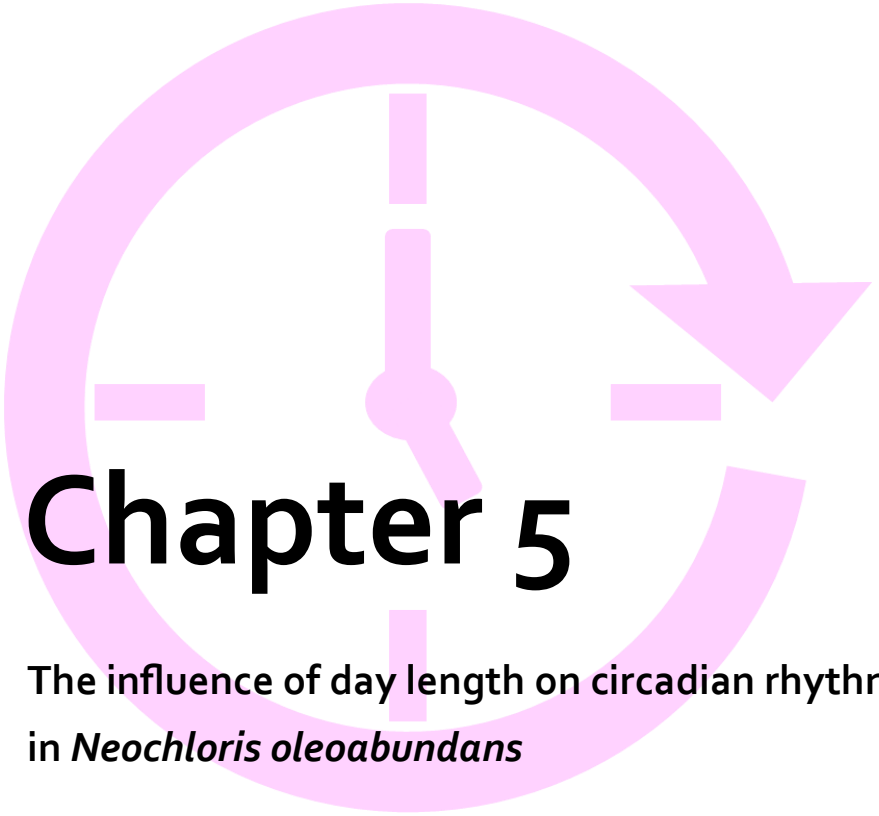
Dry weight balance:

$$V\frac{dC_x}{dt} = V \cdot \mu \cdot C_x - F \cdot C_x = 0$$

$$D = \mu - \frac{1}{C_x}\frac{dC_x}{dt}$$

Combining both balances gives:

$$D = \mu + \frac{1}{a}\frac{da}{dt}$$



Chapter 5

The influence of day length on circadian rhythms in *Neochloris oleoabundans*

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The influence of day length on circadian rhythms in Neochloris oleoabundans

Abstract

In this study the influence of day length on circadian rhythms in *N. oleoabundans* was investigated. *N. oleoabundans* was grown in a continuous photobioreactor operated as a turbidostat under various D/N cycles; 20D4N, 16D8N and 12D12N. Maximum growth rate and start of starch synthesis were scheduled after approximately 6-7 hours from sunrise and therefore timing of these processes was not influenced by day length. Also, a timing mechanism seemed to be involved in DNA replication to 4 and 8 copies, which was always initiated approximately 16 hours after 'sunrise'. Cell division seemed to be 'gated' to the night, except during very long photoperiods where synchrony was partly lost. The length of the photoperiod caused changes in biomass composition, especially due to variations in starch content. In longer days, more starch was accumulated. Starch was used for cell division probably also when cell division occurred in the light. Therefore, also the timing of cell division should be considered in the production of microalgae biomass with a desired concentration of protein, lipids, carbohydrates or pigments.

5.1 Introduction

Circadian rhythms are generally believed to help organisms adapt to and anticipate on daily and seasonally fluctuating environments (Beneragama and Goto, 2010). They help the organism to schedule certain processes within the temporal window that is most suitable for the organism (Schulze et al., 2010). For example, in microalgae, UV sensitive processes like DNA replication and cell division are often scheduled during the night (Suzuki and Johnson, 2001). Therefore, by exposing algae to a daily light/dark (L/D) cycle, synchronous division can be triggered (Post et al., 1985a). If cells divide by binary fission, this circadian rhythm in cell division can usually only be maintained when algae are dividing once per day or more slowly (Suzuki and Johnson, 2001). However, some species of green microalgae can divide by multiple fission. This means that cells can increase in biomass during the day, when light is available, and undergo multiple cycles of DNA replication and cellular division in the dark (Bišová and Zachleder, 2014). In this way, synchrony can be maintained at higher growth rates.

This daily cycle of light and dark plays a big role when microalgae are grown outdoors. For the sustainable production of microalgae biomass as potential source for commodities such as biofuels and chemicals (Wijffels and Barbosa, 2010) sunlight should be used as the sole source of light energy (Blanken et al., 2013). Day lengths change over the course of a year and differ depending on the region. In the Netherlands, the longest days in summer are almost 17 hours, while the shortest days in winter are only 8 hours. In Norway (Hammerfest), the longest days are 24 hours and the shortest days 0 hours, while in Singapore days last more or less 12 hours year round. Although it has long been recognized that biomass composition changes during the D/N cycle(Fábregas et al., 2002; Post et al., 1985b), not many studies have focussed on the effect of day length on biomass composition. In order to find ways to optimize production year round, the effect of day length on biomass composition and yield has to be understood.

The biomass composition and yield on light of the green alga *Neochloris oleoabundans* is influenced by a circadian rhythm in cell division (de Winter et al., 2013; de Winter et al., 2014). Although in constant conditions circadian rhythms oscillate with a period of approximately (≈circa) 1 day, the period length needs to be adjusted to the environmental day/night cycle. This daily adjustment of the circadian clock, also called entrainment, is an essential mechanism to adapt to environmental changes (Hirschie Johnson et al.,

2003; Niwa et al., 2013), like the changing day lengths over the course of a year. Light is one of the major cues for adjustment of the circadian clock (Niwa et al., 2013) and several aspects of the day/night (D/N) cycle could be used for the entrainment, like dawn, dusk, increasing light intensity, decreasing light intensity, continuous presence of light during daytime, or spectral changes (Roenneberg and Foster, 1997). Both dawn (Beneragama and Goto, 2010; Moulager et al., 2007) and dusk (Matsumura et al., 2003; Post et al., 1985a) have been suggested to be most important for the timing of cell division of microalgae. However, the specific organization of the multiple fission cell cycle differs in distinct species (Bišová and Zachleder, 2014), and thus these mechanisms of entrainment are likely to be species specific as well. It is not clear how cell division in *Neochloris oleoabundans* is scheduled during the day/night cycle and if the circadian rhythm in cell division entrains to different day lengths.

Knowing the timing of cell division can provide valuable information for the design of a microalgae production process, as for example the right moment for harvesting of the culture can be determined. Therefore, the aim of this study is to find out how different events like start of DNA replication and cell division are scheduled during 24 hours at different day lengths and how this affects the biomass composition. *N. oleoabundans* was grown in a continuous photobioreactor operated as a turbidostat under various D/N lengths; 20D4N, 16D8N and 12D12N. Light was provided at a constant intensity and growth, biomass composition and the cell cycle were monitored, in order to gain insight in the behaviour of synchronized cultures under various day/night cycles.

5.2 Materials and methods

5.2.1 Pre-culture

Neochloris oleoabundans UTEX 1185 (The culture collection of Algae, University of Texas, Austin) was cultivated in 250 mL shake flasks containing 100 mL adjusted BBM medium with pH 7.5 (Klok et al., 2013a) on a shaking incubator (Max Q 3000, Barnstead) at 120 RPM at a temperature of 25°C. Light was provided at an intensity of 20-40 µmol m^{-2} s^{-1} through 16D:8N cycles. Four days prior to inoculation the cultures were transferred to a second shaking incubator at 120 RPM (Orbital Incubator, Sanyo, Japan) with light provided continuously at an intensity of 150 µmol m^{-2} s^{-1}. Temperature was again kept at 25°C and the headspace of the incubator was enriched with 5% CO_2.

5.2.2 Photobioreactor set-up and experimental conditions

N. oleoabundans was continuously cultivated in a flat panel photobioreactor (PBR) (Labfors 5 Lux, LED Flat Panel Option, Infors HT, Switserland). The light path of the PBR was 20 mm and the working volume was approximately 1800 mL. The light was supplied by 360 LEDs (warm white light, spectrum from 450 nm – 620 nm). A black cover was placed on the back of the reactor to ensure no environmental light could enter the reactor. In addition, a cover especially designed to fit the PBR was placed in between the LED panel and the reactor, functioning as a light tunnel and again preventing environmental light from entering the PBR. Ingoing and outgoing light intensity (PFD_{in} and PFD_{out}) were measured at 44 points equally distributed over the lighted PBR surface using a Li-cor quantum sensor (LI250 light meter, LI-COR, USA).

After inoculation, PFD_{in} was gradually increased until it reached the final set point, in order to allow the microalgae to adapt to the new light conditions. When PFD_{out} reached its final setting due to biomass growth, the light regime was changed from constant light to D/N cycles. Table 5.1 shows a summary of the different light settings that were tested. PFD_{in} and PFD_{out} were chosen such, that the average photon flux density (PFD_{av}) was 200 µmol m^{-2} s^{-1} for experiments 1 till 3 and 70 for experiment 4. The experiment with the 16D8N cycle under high light (HL) conditions (experiment 2) was done in duplicate, in order to check reproducibility of the steady states.

Table 5.1 *Summary of the different light settings tested in the experiments*

Experiment	**D/N cycle**	**PDF_{in}**	**PDF_{out}**	**PDF_{av}**
1	20D4N	500	50	200
2	16D8N* *in duplicate	500	50	200
3	12D12N	500	50	200
4	16D8N	200	10	70

When switching to D/N cycles, also the turbidostat control was turned on during day time. Turbidostat control ensured dilution of the culture with fresh culture medium when PDF_{out} dropped below the set value. In this way, the average light intensity experi-

enced by the culture (PDF_{av}) during day time was kept constant. The system was allowed to reach steady state, which was defined as a constant biomass concentration measured at the same time every day, and a constant average daily dilution rate for a period of at least 3 residence times. In steady state, the daily growth rate (μ_{24}) is equal to the dilution rate (D), which was monitored by logging the amount of overflow produced (V_{24}) over 24 hours (see Eq. 1).

$$\mu_{24} = D = \frac{V_{24}}{V} \qquad \text{(Eq. 1)}$$

The total amount of light absorbed is kept constant by the turbidostat control, which means that changes in biomass concentration in the reactor are possible when light absorbing properties of the biomass change. Therefore, growth rate does not equal the dilution rate over smaller time intervals during the day, but becomes a function of biomass growth rate (μ) and the change in absorption cross section (a) of the biomass (Eq. 2). Derivation of Eq. 2 can be found in the appendix of chapter 4.

$$D = \mu + \frac{1}{a}\frac{da}{dt} \qquad \text{(Eq. 2)}$$

From the growth rate μ, also the doubling time t_d (day^{-1}) and average number of daughter cells formed per mother cell N_t/N_o during cell division were calculated according to equation 2 and 3, in which t is time (days).

$$t_d = \frac{\ln(2)}{\mu} \qquad \text{(Eq. 3)}$$

$$\frac{N_t}{N_0} = 2^{\frac{t}{t_d}} \qquad \text{(Eq. 4)}$$

The temperature inside the PBR was maintained at 30°C using the internal temperature control system of the Infors HT system that was connected to the water jacket of the PBR. Water was provided to the Infors HT system at a constant temperature of 20°C through the use of an external cryostat (RE306/E300, Lauda, Germany). The pH was maintained at 7.5±0.2 by an automatic supply of 1 M HCl. The culture was continuously sparged with 1000 mL min^{-1} air enriched with $2\%_{(v/v)}$ of CO_2, provided by the set of mass flow controllers embedded in the Infors HT system. The air was leaving the reactor

through a condensor, which was connected to a cryostat at 2°C. Dissolved oxygen was measured online and foam formation was prevented by manually supplying a 2%$_{(v/v)}$ antifoam solution (Antifoam B® silicone emulsion, Mallinckrodt Baker B.V., Deventer, The Netherlands) every morning and afternoon.

5.2.3 Sampling and biomass analysis

Samples were taken daily at the same time to monitor biomass growth and steady state by measuring the optical density at 750 and 680 nm (OD_{750} and OD_{680}), cell number, cell size, total cell volume and dry weight (DW), as described by Kliphuis et al. 2010 (Kliphuis et al., 2010). During steady state, additional samples were taken over a 24h period with 3h intervals. For these samples, more biomass was harvested to analyse DNA content and major biomass constituents (proteins, starch and fatty acids). Because the dilution rate in the 12D12N experiment and the 16D8N experiment under low light (LL) was lower than in the other experiments, less samples could be taken during the 24h cycle for analysis of the major biomass constituents (6 and 4 samples, respectively). In the other experiments, the dilution rate was large enough to compensate for the volume taken from the reactor.

DNA content was analysed by measuring fluorescence due to propidium iodide (PI) staining using a flow cytometer (FACScalibur, Becton Dickinson, Franklin Lakes, NJ, USA). The staining procedure and instrument settings were used exactly as described by de Winter et al., 2013 (de Winter et al., 2013). For the analysis of major biomass constituents liquid samples were taken from the PBR in triplicate and centrifuged for 5 minutes at 2500 RPM. Pellets were stored at -20°C. After storage, pellets were transferred to beat beater tubes and freeze dried overnight. Protein, starch and total fatty acid (TFA) contents of the dried biomass were then determined as described by de Winter et al., 2013 (de Winter et al., 2013). Also, chlorophyll content was analysed using an Accela UHPLC system (Thermo Scientific, USA) as described by Mulders et al. (2014)(Mulders et al., 2014).

For the duplicate experiment of 16D8N HL, cell division was studied in more detail by taking measurements of cell number, cell size and DNA content at a higher frequency. During the night, the reactor was sampled every hour and every half hour around the time of cell division.

5.3 Results

5.3.1 Growth rate

N. oleoabundans was grown under 20D4N, 16D8N and 12D12N cycles and the biomass density and daily dilution rate were monitored until steady state was reached. In steady state, the daily average dilution rate is equal to average specific growth rate over a day (eq.1). The daily average dilution rate, and therefore the average specific growth rate over a day (24h) was constant at 1.9, 1.6 and 1.2 day^{-1} for the 20D4N, 16D8N and 12D12N cycles, respectively.

However, when monitoring the dilution rate over shorter time intervals during a 24h cycle, oscillations were observed. In figure 4.1A and 1B the dilution rate over a 24h cycle is shown. As in turbidostat cultures the light absorbed is kept constant, slight changes in biomass concentration are possible due to changes in biomass composition (eq.2). As a result, there is a large difference between the way the dilution rates change (figure 5.1A and 5.1B) as compared to the way the oxygen production rates change(figure 5.1C and 5.1D) in response to turning the lights on and off. The increase in oxygen production rates was a lot faster than the increase in dilution rate. This means that growth rate increased immediately when light was turned on, but apparently the production of light absorbing material (a), and therefore the dilution rate, lagged behind. Consequently, the dilution rate over short time periods is not equal to the specific growth rate in day/night cycle cultures.

A maximum in oxygen production rate and dilution rate was reached in between 6 and 9 hours after sunrise in all experiments. Therefore, timing of this maximum was not dependent on light intensity nor on day length. The maximum values, on the other hand, were dependent on light intensity, as was shown by the experiment done at a lower light intensity where maximum dilution rate and maximum oxygen production rate were lower (figure 5.1 B and D). After the peak, both oxygen production rate and dilution rate decreased. Again, the decrease in dilution rate was steeper than the decrease in oxygen production rate. This indicates that the absorption cross section of the biomass decreased, meaning that other biomass constituents, like starch, were synthesized at higher rates than the light absorbing material.

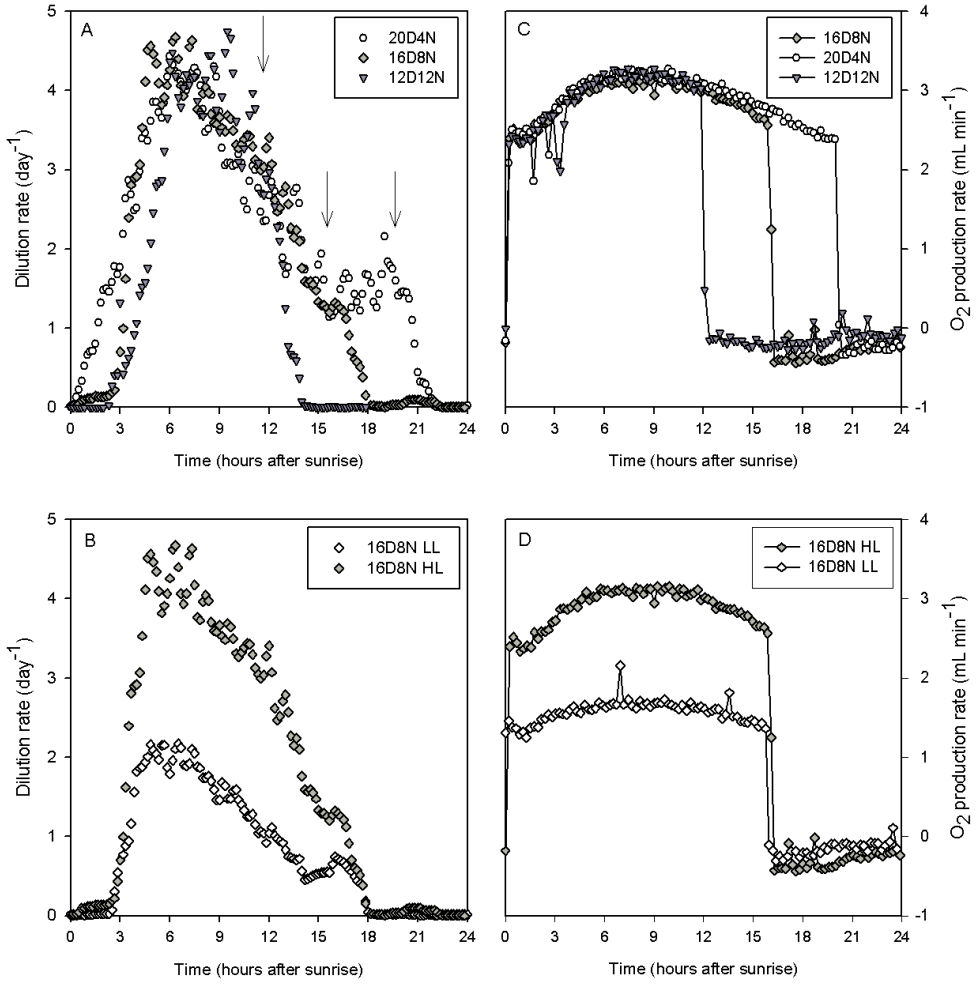

Figure 5.1 *Dilution rate (day^{-1}) (A and B) and oxygen production rates (mL min-1) (C and D) in steady state during 24 hours in the 20D4N (white circles), 16D8N HL (grey diamonds), 16D8N LL (white diamonds) and 12D12N (dark grey triangles) experiments with N. oleo-abundans. Zero on the x-axis represents the point where light was turned on and arrows in figure A indicate where light was turned off for the 20D4N, 16D8N and 12D12N cycles (from left to right respectively)*

5.3.2 Biomass composition and cell division

Samples were taken from the reactor with 3 h intervals for determination of TFA, starch, protein and chlorophyll content. In Figure 5.2 the biomass composition during the 20D4N, 16D8N and 12D12N experiment are plotted in % of DW. In appendix figure 5.A.1 the contents are also plotted in pg per cell. Due to the lower dilution rate, less data points are available for the 12D12N and 16D8N LL experiments, as more frequent sampling would have influenced the steady states. Biomass composition data for experiment 16D8N LL can be found in appendix figure 5.A.2 and 5.A.3. The moment of cell division was determined by analysing cell size and cell number. Arced areas in figure 5.2 represent the dark period, and cell division took place between the times indicated by vertical dashed lines. Cell division was timed in the dark, except in the 20D4N experiment.

From the moment light was turned on, i.e. sunrise, biomass components started to increase in all experiments, illustrating that cells were increasing in size (see appendix figure 5.A.1). The biomass composition in % of DW remained relatively constant in the first hours. After approximately 6-7 hours from sunrise, starch levels in the cells started to increase, indicating that the production rate of starch was increasing faster than that of the other measured biomass components.

Although the trends in the start of the day were similar in all the experiments, differences were observed later in the day. In the 12D12N experiment no clear oscillation in starch content was observed, possibly due to the short day length. In the 16D8N experiment (figure 5.2, middle graph), a decrease was observed in starch content when light was turned off and before cell division started, which coincided with an increase in protein content. In the 20D4N experiment, the starch content still increased after cell division started (15h), although the amount of starch accumulated in the end of the day was similar to the amount measured in the 16D8N experiment, being 20% of DW. During cell division, all cell components in pg per cell decreased, indicating the decreasing cell size (appendix figure 5.A.1).

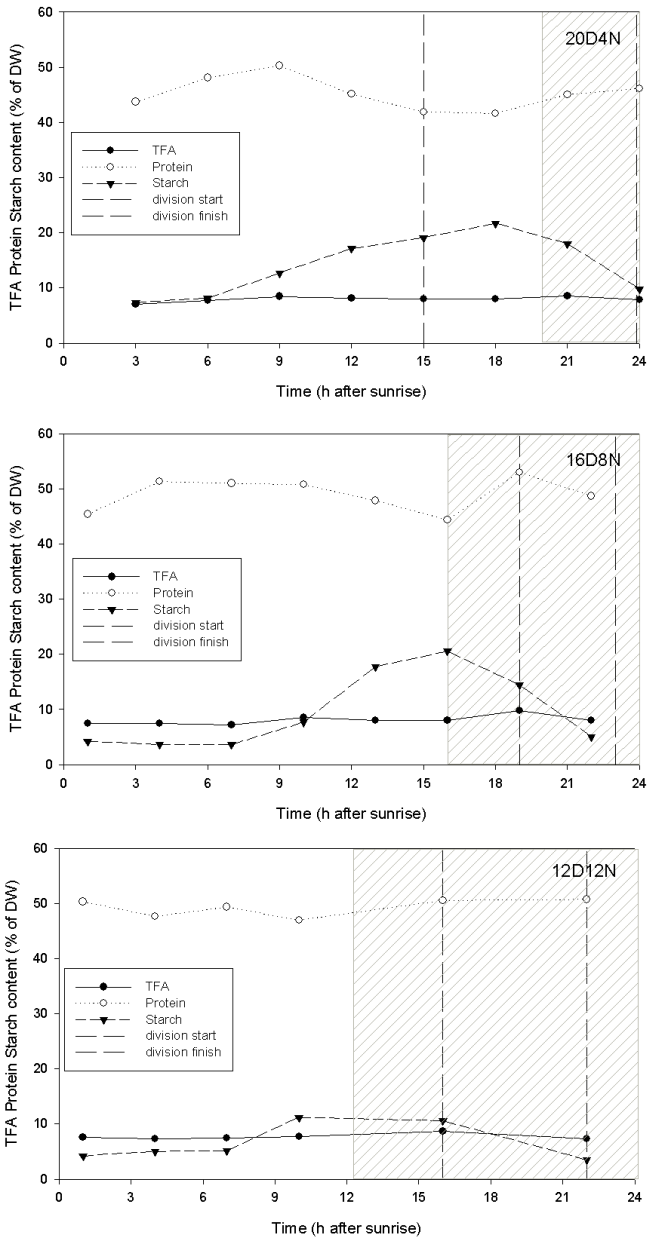

Figure 5.2 *TFA, protein and starch content expressed in % of DW during a 24h cycle in the 20D4N, 16D8N and 12D12N experiments. Arced areas represent the dark period. Start and end of cell division are marked by the dashed vertical lines.*

5.3.3 DNA content

For a better insight in the regulation and timing of cell division, DNA content was analysed. In figure 5.3A the DNA content of the 16D8N HL experiment is shown for samples taken during part of the day and the entire night. Light was turned off at t=16. DNA replication to 2 copies was initiated from approximately 10 hours after sunrise. Further replication to 4 and 8 copies started after 16 hours of light, in this case the moment when light was turned off. Cell division started at 19 hours after sunrise, i.e. 3 hours after dusk, which was determined by coulter counter measurements and confirmed by the increasing population of cells with 1 copy of DNA in figure 5.3A.

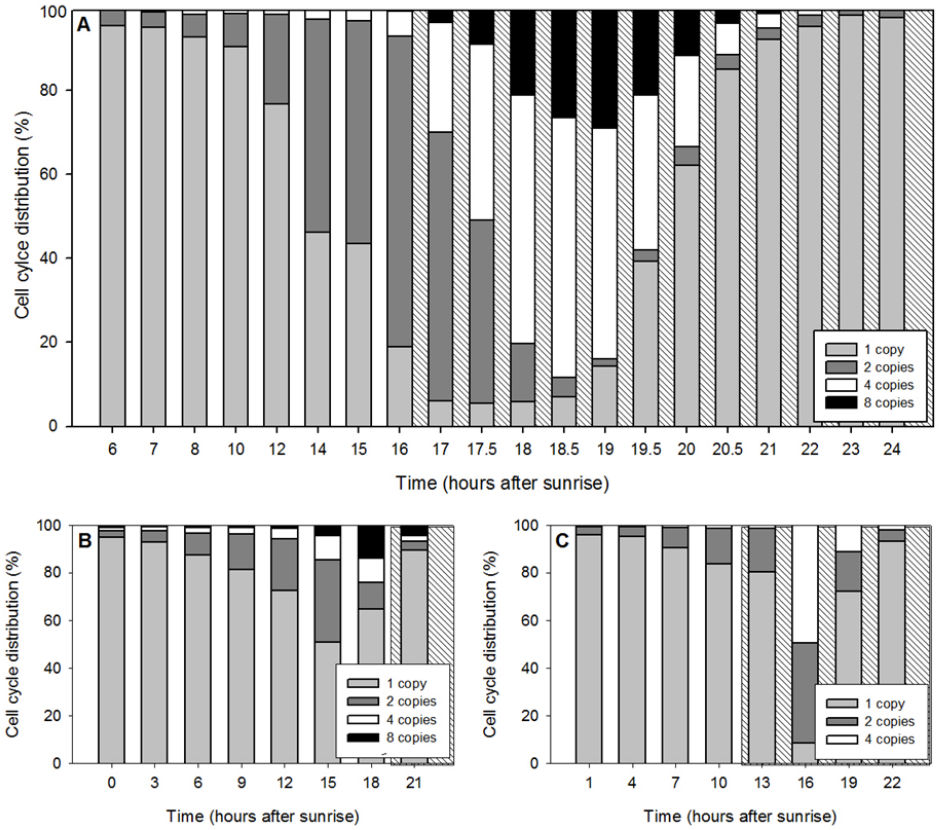

Figure 5.3 *Cell cycle distribution of N. oleoabundans during the day night cycle in the 16D8N HL experiment (A), the 20D4N experiment (B) and the 12D12N experiment (C) showing the amount of copies of DNA per cell in the sample. Arced areas represent the dark period. Note that the time points on the x-axis in 3A are not distributed evenly. Measurement error between duplicate measurements was approximately 1%.*

Data of the 20D4N and 12D12N experiments can be found in figure 5.3B and 5.3C respectively. Also in the 20D4N and the 12D12N experiments it can be seen that DNA replication to two copies started during the day, and further replication to 4 and 8 copies occurred after approximately 16 hours from sunrise. In the 16D8N LL experiment (see appendix figure 5.A.4), replication to two copies was not initiated during the day but started after approximately 16 hours from sunrise. Replication to 8 copies did not occur in the 12D12N and the 16D8N LL experiments, which is consistent with the average amount of daughter cells formed per mother cell as calculated from equation 3 and 4. On average 6.7, 5.0, 3.3 and 2.1 daughter cells were formed per mother cell in the 20D4N, 16D8N HL, 12D12N and 16D8N LL experiments respectively.

5.4 Discussion

5.4.1 Growth rate

Both dilution rate and oxygen production rate reached a maximum in between 6 and 9 hours after sunrise. However, the dilution rate was clearly influenced by the rates at which the different biomass components were synthesized, as was explained in 3.1 and equation 2. Therefore, the oxygen production rate is a better indication of specific growth rate. Since in turbidostat the absorbed light is constant, the oxygen production rate is a direct measure for the efficiency with which light is used to fix electrons into biomass. As the biomass composition is more or less constant in the first hours, this directly translates into growth rate. Thus, the growth rate is at a maximum between 6 and 9 hours after 'sunrise'.

The timing of this maximum in oxygen production rate was not dependent on light intensity nor on day length. Therefore, it seems that the circadian clock was involved in timing of this process. The maximum in oxygen production rate did not adjust to the photoperiod, as is the case for some other circadian rhythms where the period of the graph shortens or lengthens with changing photoperiod (Beneragama and Goto, 2010). Rather, the signal given by turning the lights on, i.e. dawn, seems to be the only cue for entrainment of this rhythm. Sometimes, maxima of oxygen production are attributed to the attainment of the commitment point (CP), an important point in the cell cycle after which cells are able to divide without any external energy supply when cells are put in the dark (Bišová and Zachleder, 2014; Šetlík et al., 1972). However, in this case it is not likely that the maximum oxygen production rate is caused by the attainment of the CP, as attainment of a CP is dependent on light energy. The maximum in oxygen production

was observed at the same time in both high and low light 16D8N experiments (figure 5.1D) and therefore was not depending on the amount of light received and thus must be caused by another cue. Daily variations in photosynthetic rate were already described by Post et al. (1985)(Post et al., 1985a), and photosynthesis is well known to be under control of the circadian clock. So, it is likely that the rhythm in oxygen production rate was caused by a circadian rhythm in photosynthesis.

Although timing was the same, the values for maximum oxygen production rate and maximum dilution rate were lower in the experiment done at lower light intensity (figure 5.1B and 5.1D). Daily averaged dilution rate was also lower at shorter photoperiods. Therefore, the daily averaged growth rate was mainly determined by light intensity and length of the photoperiod (see also (Jacob-Lopes et al., 2009)).

5.4.2 Biomass composition

All biomass components expressed in pg per cell started to increase after light was turned on (appendix figure 5.A.1), indicating an increase in cell size during the day as was also observed for other species (Fábregas et al., 2002; Lacour et al., 2012; Šetlík et al., 1972). Interestingly, starch content of the DW started to increase after approximately 6-7 hours after sunrise (figure 5.2), which was the same moment that the maximum in dilution rate was observed (figure 5.1A and B). This illustrates that the increase in starch coincided with a decrease in the absorption cross section of the biomass, and as such a decrease in dilution rate. Starch is a storage molecule that is assimilated during photosynthesis and can be respired during dark periods for maintenance processes and cell division (Bišová and Zachleder, 2014). Indeed, starch content of the DW started to decrease when light was turned off, indicating that starch was respired in the dark. Lipids can also be respired during the night (Fábregas et al., 2002). However, TFA content of the DW remained stable throughout the day/night cycles. Possibly, this is because mainly neutral lipids are respired (Lacour et al., 2012), and in *N. oleoabundans* neutral lipids are not accumulated under nitrogen replete conditions (Klok et al., 2013a).

During cell division, the contents of all biomass components expressed in pg per cell were decreasing (appendix figure 5.A.2), indicating that these components were divided over the daughter cells. In the 20D4N experiment cell division already started in the light (15h). Starch is supposed to be used for cell division even if cell division occurs in light

(Bišová and Zachleder, 2014; Brányiková et al., 2011). However, starch content per cell remained constant and starch content as % of DW kept increasing (figure 5.2). This can be explained by a loss of synchrony in this culture (as will be described further in section 5.4.3), and as such the increasing starch content in the DW can be the result of a decrease in starch in the cells that started cell division combined with an increase in starch per cell in cells that were not dividing yet.

The rhythm in starch synthesis might be regulated by the circadian clock, like in *Chlamydomonas reinhardtii* (Ral et al., 2006). This rhythm, like the rhythm in oxygen production, did not adjust to photoperiod, and the increase in starch always started after approximately 6-7 hours of light, independent of day length. The increase in starch coincided with the decrease in oxygen production rate, which implies that this is an important switch regulated by the circadian clock. However, it is not possible to exclude the effect of light intensity on the start of starch synthesis, as it was not possible to determine the moment of starch increase in the 16D8N LL experiment due to the limited amount of samples that could be analysed. Because there was no effect of day length on the start of starch synthesis, differences in biomass composition arose between experiments only later in the day. For example, less starch was accumulated in the 12D12N cycle than in the longer days.

5.4.3 Cell division and DNA replication

Cell division started approximately 3-4 hours after dusk in the 16D8N experiments under HL and LL and the 12D12N experiment. However, in the 20D4N experiment cell division started when light was still on, 15 hours after 'sunrise'. This means that cell division is not regulated solely by the amount of light received as was proposed by Vitová (Vítová et al., 2011b), since in the 16L8D HL experiment cells received the same light as the 20D4N experiment after 15 hours but did not start to divide. Therefore, it is still plausible that a timing mechanism is involved in regulation of the cell cycle in *Neochloris*. In this case, it is possible that the clocks of some algae in the 20D4N experiment were not synchronized with the light/dark cycle supplied, which caused them to divide earlier. To entrain to a daily light/dark cycle, circadian clocks respond differently to light at different phases of their cycle. Light pulses given to the algae during the first 12h after sunrise will have little or no effect on the circadian clock, as algae are experiencing their day period, i.e. their subjective day. During the second half of the daily cycle, light pulses can

either advance or delay the rhythm (Hirschie Johnson et al., 2003). Especially in the hours before sunrise, the rhythm is sensitive to light pulses (Malinowski et al., 1985). As algae in the 20D4N experiment were experiencing long photoperiods, with only 4h of dark before sunrise, it might be that the clocks of some algae were reset by the light supplied during the last part of the day. These algae will have a different subjective day and therefore synchrony of the culture will be partially lost. This hypothesis was strengthened when looking at the cell size distributions (data not shown), as there was a larger spread on cell diameter in the 20D4N experiment than in the other experiments, which suggests that cell division was less synchronized. Although this theory can explain why cell division already happened 15 hours after sunrise in the 20D4N whereas nothing happens in the 16D8N HL experiment after 15 hours, it remains unclear what actually makes the cells 'decide' to start cell division. Transcription of the main cell cycle regulators in *Ostreococcus* are regulated by the circadian clock (Moulager et al., 2007) and therefore can influence the timing of cell division. Research on cell cycle regulators in *Neochloris* would be necessary to know more about the timing mechanism.

Also in the start of DNA synthesis to two copies no clear timing mechanism seemed to be involved. According to theory, DNA replication is started when cells meet the requirements for cell division, i.e. when cells reach the commitment point (CP)(Vítová et al., 2011b). Light energy is required to attain a CP, as cell size needs to be roughly doubled. It has been suggested that also the amount of starch plays an important role in attainment of the CP (Bišová and Zachleder, 2014). In the experiments done at higher light (20D4N, 16D8N HL and 12D12N) DNA replication to two copies started during the day, whereas in the 16D8N experiment under lower light DNA replication started only after 16 hours, i.e. at the moment that light was turned off. This indicates that indeed the start of DNA synthesis to two copies is depending on the amount of light energy received. However, further replication to 4 and 8 copies seemed to be starting after approximately 16 hours from sunrise, independent on day length and light intensity. Therefore, it is possible that initiation of DNA synthesis is regulated by the combination of a timer mechanism and reaching a commitment point.

5.4.4 Timing during the D/N cycles

In sections 5.4.1 and 5.4.2 it was discussed that photosynthesis and starch synthesis seemed to be regulated by the circadian clock and how timing of these processes was

dependent on the moment light was switched on. Also timing of DNA synthesis to 4 and 8 copies seemed to depend on 'sunrise'. To be able to relate the timing of processes to 'sunrise' or 'sunset', the data can be plotted relative to the photo- and scotoperiods received in the different D/N cycle regimes. In figure 5.4 this was done for the maximum in oxygen production rate, the start of the increase in starch, the start of cell division, the end of cell division and timing of DNA replication to 4 and 8 copies. The highest points in the graph represent data of the 12D12N cycle experiment, the middle points the data of the two 16D8N cycle experiments and the lowest points are data of the 20D4N cycle experiment. It should be noted that sampling was done at 3h intervals, and therefore the position of the data points along the x-axis has at most a 3 hours uncertainty to the left, except for the data points for maximum dilution rate and data points from the duplicate 16D8N HL experiment. Shaded areas in the plot represent the dark period, and therefore the angled lines represent 'sunrise' and 'sunset'. In this way, the relation between the timing of the maximum in oxygen production, the start of the starch increase and the start of DNA synthesis to 4 and 8 copies can be clearly related to 'sunrise', as they can be seen as a parallel to the 'sunrise line, as indicated by the dashed grey lines. This parallel to 'sunrise' suggests the presence of a timing mechanism, that was reset by 'sunrise'.

Both dawn and dusk both were suggested to be important for the timing of cell division in microalgae (Beneragama and Goto, 2010; Matsumura et al., 2003; Post et al., 1985a). However, for the timing of cell division no clear parallels were observed with 'sunrise' or 'sunset', although it was clear that cell division was scheduled in the night, with the exception of the 20D4N experiment where synchrony was partly lost. Data collected for more different day lengths, light intensities, and with a higher sampling frequency would be necessary to draw more conclusions on the exact mechanism involved in timing of the cell cycle of *N. oleoabundans*.

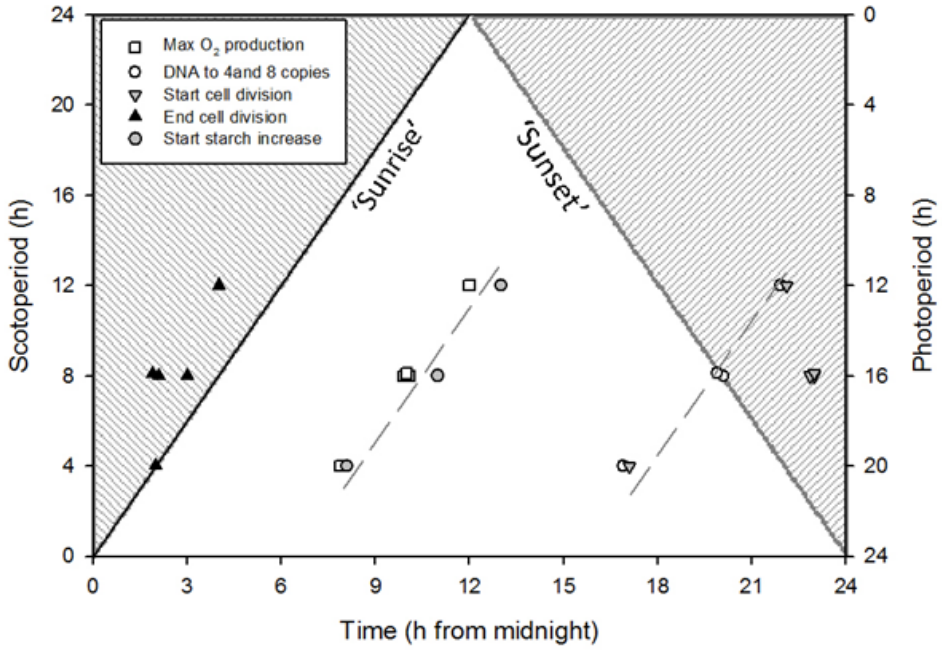

Figure 5.4 *Maximum oxygen production rate, start of DNA replication to 4 and 8 copies, start of cell division and end of cell division in N. oleoabundans relative to the photo- and scotoperiod received during different 24h D/N cycle regimes. Shaded areas represent dark periods and therefore the lines of the triangle represent 'sunrise' and 'sunset'. Dashed grey lines show parallels to 'sunrise'.*

5.5 Conclusions

In *N. oleoabundans*, maximum growth rate and start of starch synthesis occurred after approximately 6-7 hours from sunrise and therefore seemed to be regulated by the circadian clock. Also, a timing mechanism seemed to be involved in DNA replication to 4 and 8 copies, which was always initiated approximately 16 hours after 'sunrise'. Timing of these processes was not affected by day length. Cell division seemed to be 'gated' to the night, except during very long photoperiods where synchrony was partly lost. However, the exact mechanism involved in timing of the start of DNA synthesis to 2 copies and timing of cell division remains unknown.

The length of the photoperiod caused changes in biomass composition, especially due

to oscillations in starch content. In longer days, more starch was accumulated. Starch was used for cell division probably also when cell division occurred in the light. Therefore, also knowledge on the timing of cell division is important for the production of biomass with a desired concentration of protein, lipids, carbohydrates or pigments.

Acknowledgements

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Abbreviations

D/N	Day/Night
PBR	Photobioreactor
PDF_{in}	Ingoing light intensity
PDF_{out}	outgoing light intensity
PDF_{av}	average light intensity
OD_{750}	Optical density at 750 nm
OD_{680}	Optical density at 680 nm
DW	dry weight
HL	high light
LL	low light
MS	mass spectrometer
CP	commitmentpoint
t_d	doubling time
t	time
μ	specific growth rate
N_t/N_o	daughter cells per mother cell
V	volume overflow
V_{pbr}	volume reactor
D	dilution rate
a	absorption cross section

Appendix

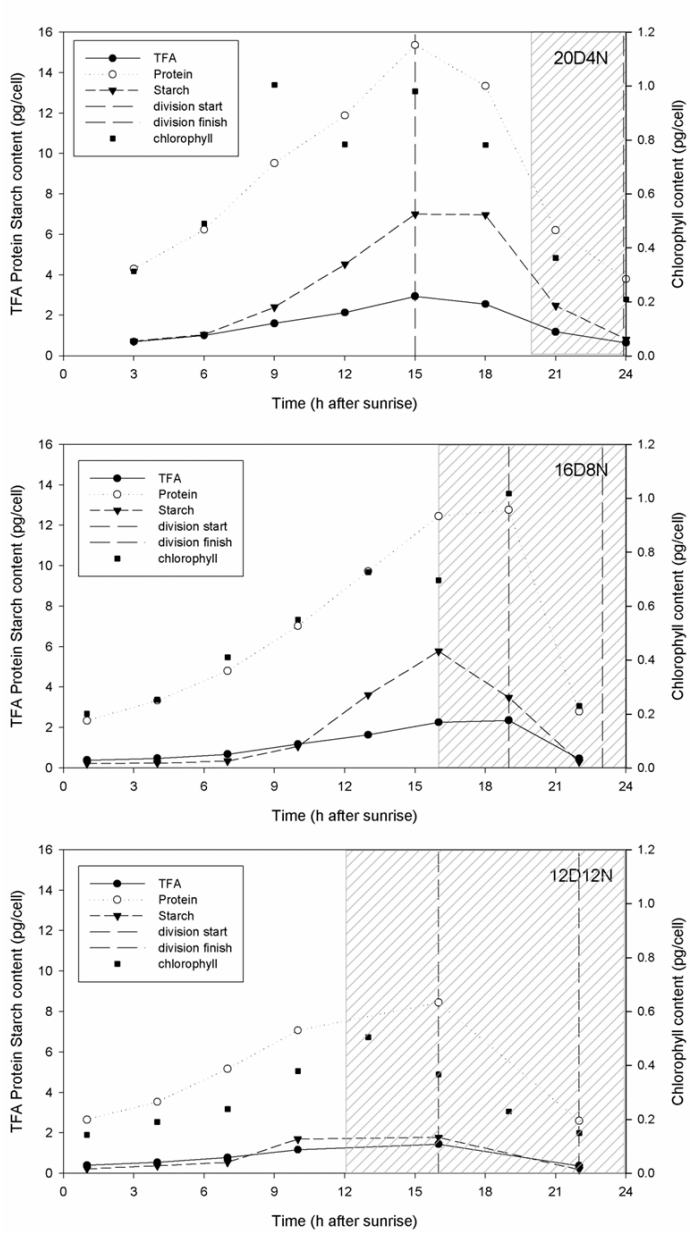


Figure 5.A.1 *TFA, protein, starch and chlorophyll content expressed in pg/cell during a 24h cycle in the 20D4N, 16D8N and 12D12N experiments. Arced areas represent the dark period. Start and end of cell division are marked by the dashed vertical lines.*

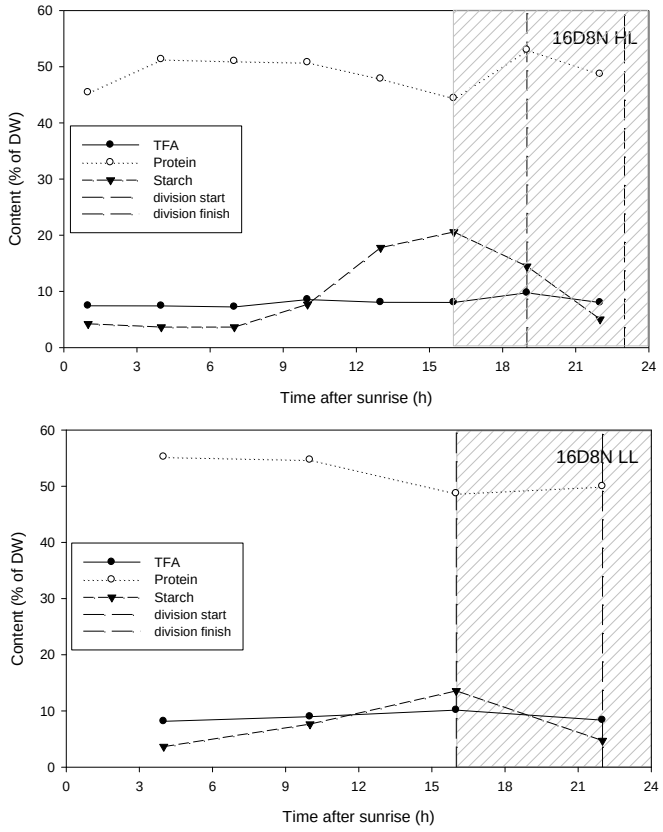


Figure 5.A.2 *TFA, protein, starch and chlorophyll content expressed in % of DW during a 24h cycle in the 16D8N HL and 16D8N LL experiments. Arced areas represent the dark period. Start and end of cell division are marked by the dashed vertical lines.*

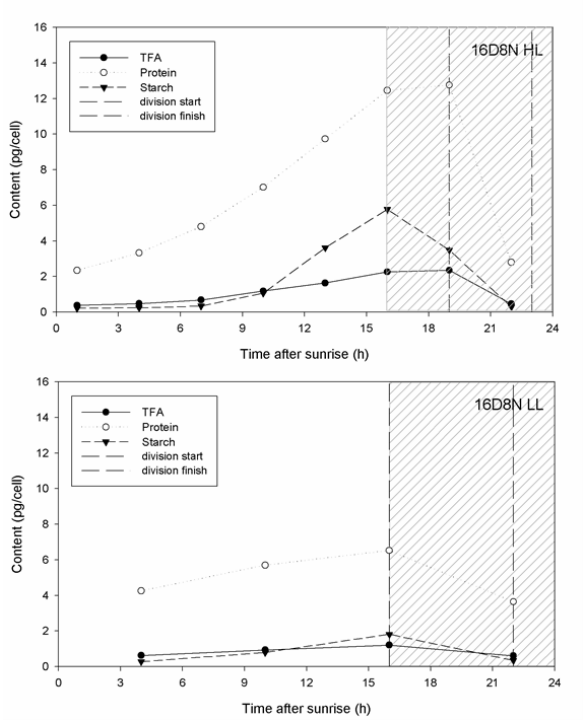


Figure 5.A.3 *TFA, protein, starch and chlorophyll content expressed in pg/cell during a 24h cycle in the 16D8N HL and 16D8N LL experiments. Arced areas represent the dark period. Start and end of cell division are marked by the dashed vertical lines.*

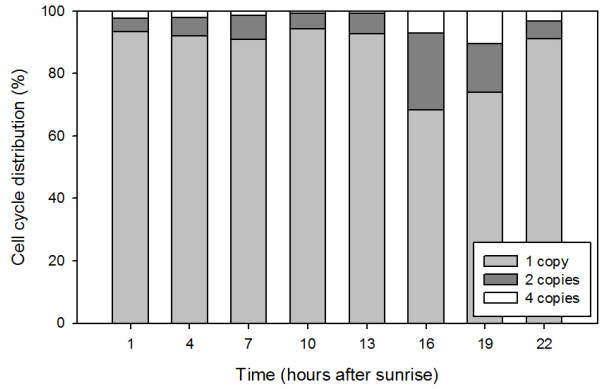
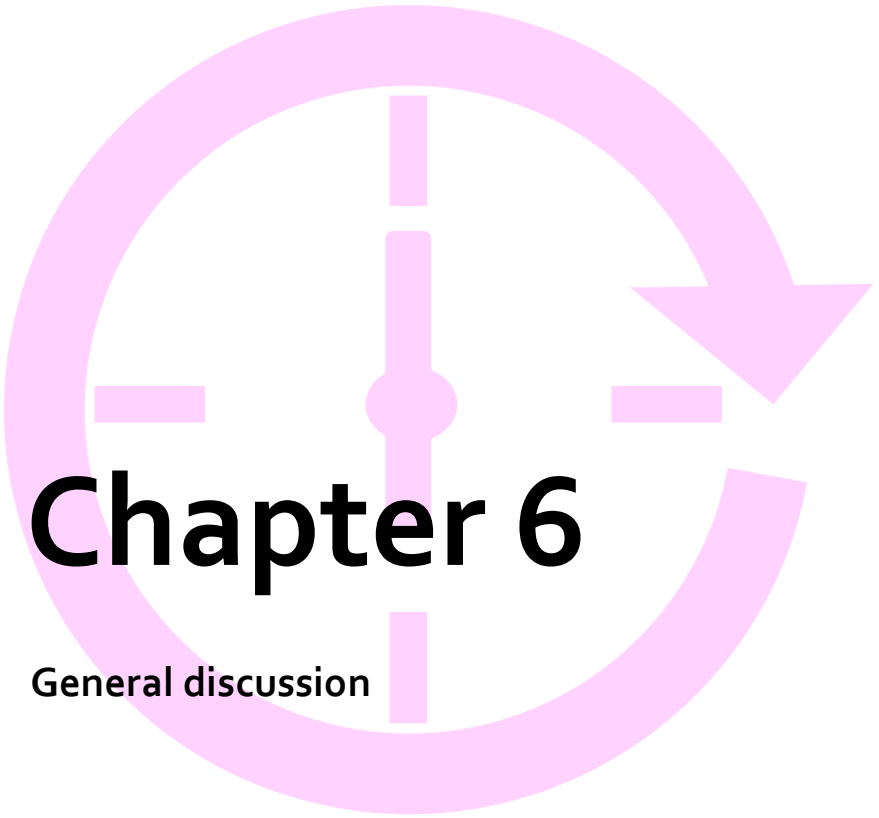


Figure 5.A.4 *Cell cycle distribution of N. oleoabundans during 24h in the 16D8N LL experiment, showing the amount of copies of DNA per cell in samples taken at 3h intervals. Measurement error between duplicate measurements was approximately 1%.*



Chapter 6

General discussion

6.1 Introduction

More or less all organisms are exposed to the daily cycle of light and dark. The circadian clock evolved to help organisms program their activities at an appropriate time during this daily cycle. The rhythms in metabolic, physiological and/or behavioural events caused by the circadian clock are called circadian rhythms. Circadian rhythms persist even under constant conditions and occur with a frequency of approximately 1 day (Mittag et al., 2005). In microalgae, circadian rhythms were observed in many processes, like nitrogen fixation, chemotaxis, photosynthesis and the cell division cycle (Mittag, 2001).

Microalgae are a potential source for biofuels, chemicals, materials, foods, feeds and high-value bioactives (Chisti, 2007; Hu et al., 2008; Vanthoor-Koopmans et al., 2013; Wijffels and Barbosa, 2010) and as such a lot of research is conducted in order to optimize the microalgae production process. Biomass growth rate, biomass yield on light and biochemical composition of the microalgae are investigated by growing microalgae in different types of photobioreactors (PBRs) (Molina et al., 2001; Morweiser et al., 2010; Sierra et al., 2008). However, the possible influence of the circadian clock on these factors received scant attention and therefore the exact influence of the circadian clock on production processes remains unknown.

The aim of the work presented in this thesis was to obtain more insight in circadian rhythms of microalgae grown in PBRs. In this final chapter, first the occurrence of circadian rhythms in different species of microalgae is discussed in order to establish the general nature of these rhythms, after which a brief summary of the observed circadian rhythms in *Neochloris oleoabundans* is presented. Based on the findings for *N. oleoabundans* some ideas for research protocols and production processes are proposed. Finally, the knowledge gaps and opportunities for future research and possible manipulation of the circadian clock will be presented.

6.1.1 Circadian rhythms in many different microalgae

Circadian rhythms are ubiquitous in nature and have been described in a variety of microalgae across a range of phyla. The first reported circadian rhythm in a unicellular organism was the phototaxis rhythm in the alga *Euglena* in 1948 (Tollin and Robinson, 1969). From then on, rhythmic phenomena in chemotaxis, photosynthesis, bioluminescence, gene expression, sensitivity to UV light, chloroplast movement and cell division

were described in different microalgae ((Mittag, 2001; Suzuki and Johnson, 2001) and refereces therein). With respect to circadian rhythms, amongst the most well-studied microalgae are *Gonyaulax polyedra* representing the Dinophyta (Hastings, 2007; Homma and Hastings, 1989), *Euglena gracilis* representing the Euglenophyta (Hagiwara et al., 2002; Malinowski et al., 1985) and *Chlamydomonas reinhardtii* representing the Chlorophyta (Goto and Johnson, 1995; Matsuo and Ishiura, 2010; Schulze et al., 2010). In cyanobacteria, circadian rhythms were studied most thoroughly in *Synechococcus* (Woelfle et al., 2004; Yang et al., 2010).

Circadian rhythms can be observed in cell division, which results in synchronization of the cell division cycle. Several researchers have reported on synchronized cultures and circadian control of the cell cycle. In *Euglena gracilis* cell division is 'gated' to a specific timeframe of the circadian cycle (Hagiwara et al., 2002; Malinowski et al., 1985). Also the cell cycles of *Scenedesmus* and *Chlorella* can be synchronized by light/dark cycles (Vítová and Zachleder, 2005; Zachleder et al., 2002), ref chlorella). *Nannochloropsis gaditana* was shown to divide during the night (Fábregas et al., 2002) and recently it was shown that there was circadian influence on growth and chlorophyll accumulation in *N. gaditana* (Braun et al., 2014). Circadian control of the cell cycle was studied most extensively in *Chlamydomonas reinhardtii*. Goto and Johnson (Goto and Johnson, 1995) concluded that cell division in *Chlamydomonas* was gated by the circadian clock and the mechanism of circadian control was studied in more detail by Oldenhof et al. (Oldenhof et al., 2007). In this thesis, most work was done on *Neochloris oleoabundans*, but some additional studies were done on *Nannochloropsis sp* (unpublished results). In figure 6.1 the average size and number of cells in a turbidostat PBR under 16D8N cycles with *Nannochloropsis sp*. and *N. oleoabundans* is shown. Both species showed an increasing cell size during the day and an increase in cell number during the night, although the increase in cell number started only in the last hours of the night for *Nannochloropsis.* The oscillations in cell size and cell number were less pronounced in *Nannochloropsis,* which can be due to the lower growth rate in this turbidostat culture. In addition, *Nannochloropsis* divides by binary fission which results in smaller differences in cell size. Nevertheless, figure 6.1 shows that both *N. oleoabundans* and *Nannochloropsis* were synchronized by the light/dark cycle.

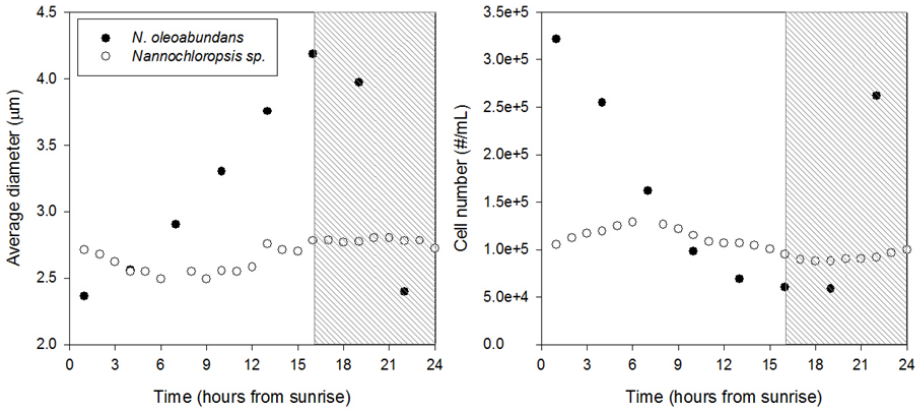


Figure 6.1 *Synchronized cell division in Neochloris oleoabundans (closed circles) and Nannochloropsis sp. (open circles) as shown by average cell diameter (left) and cell number (right) in a PBR operated as a turbidostat with 16D8N cycles. Arced area represents the dark period.*

So, it is clear that microalgae can become synchronized when grown under day/night cycles. As this is a general observation that holds for many different species of microalgae, the study of circadian rhythms in cell division is very relevant for outdoor microalgae production.

6.1.2 Circadian rhythms in Neochloris oleoabundans

For *N. oleoabundans* it was shown that cell division was taking place at night. As discussed in chapter 2, the circadian clock was 'gating' cell division to the natural night in experiments done under constant red LED light. Probably, a blue light receptor was involved in this regulation, as synchrony disappeared under constant white LED light. This indicates that a small amount of blue light in the laboratory environment could have been responsible for synchronization under the constant red LED light conditions. Cell division occurred via multiple fission with cells dividing in 2 to 8 daughter cells, depending on growth conditions. Under nitrogen limitation (chapter 3), growth rate declined, but cell division was still phased, meaning two populations arose in the reactor which divided alternately every other day.

Both phased and synchronized cell division were shown to have an influence on biomass composition under constant red LED light. Fatty acid composition, starch content, protein content and pigment content were oscillating with a cycle period of 24 hours. The

observed oscillations were solely attributed to the effect of the circadian clock and synchronized cell division, as it was clear that the changes in biomass composition were not due to changes in light intensity.

Also biomass yield on light was influenced by the circadian clock, which was investigated further in chapter 4. It was shown that the biomass yield on light was 10-15% higher in synchronized cultures under day/night cycles, compared to randomly dividing cultures under constant white LED light. In other words, scheduling cell division during the night provided the day/night cycle cultures with an advantage.

Algae are exposed to different day lengths over the course of a year. Therefore, the influence of day length on timing of cell division and biomass composition was investigated (chapter 5). Maximum growth rate and start of starch synthesis were scheduled after approximately 6-7 hours from sunrise and this timing was not influenced by day length. Also DNA replication to 4 and 8 copies was always initiated approximately 16 hours after sunrise and was therefore not influenced by day length. However, changes in biomass composition were observed because of differences in photoperiod and timing of cell division. Cell division seemed to be 'gated' to the night, with the exception of very long photoperiods where synchrony was partly lost.

A graphical summary of the influence of the circadian clock on cultures of *N. oleoabundans* can be found in figure 6.2. When grown under day/night cycles, processes like cell growth and starch accumulation occur simultaneously in all cells and therefore the culture is synchronized (left). When grown under continuous light the circadian clock is not able to synchronize these processes, which results in a randomly dividing culture with cells in different stages of the cell cycle (right).

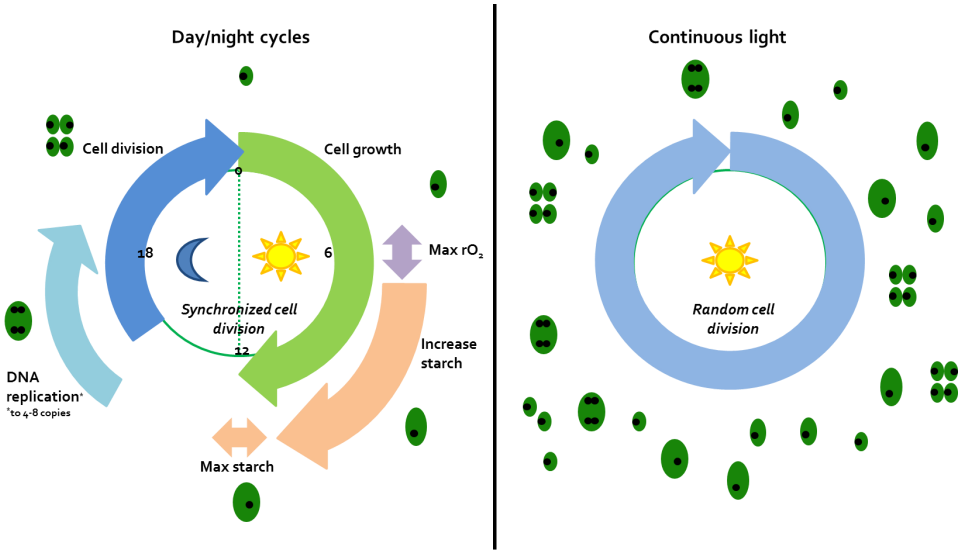


Figure 6.2 *The difference between a synchronized culture under day/night cycles where processes are influenced by the circadian clock in N. oleoabundans (left) and a randomly dividing culture under continuous light (right). rO_2 - oxygen production rate*

6.2 Implications for the growth of microalgae in photobioreactors

6.2.1 Constant light versus day/night cycles

Microalgae can either be grown under constant light or light dark cycles. However, the production of microalgae will mainly be done outside under natural day/night cycles. Nevertheless, a lot of research on the effect of cultivation conditions on biomass composition (Breuer et al., 2012; Cerón García et al., 2005; Pruvost et al., 2009) or photosynthetic efficiency (Kliphuis et al., 2010; Sforza et al., 2012) is done under continuous light. In photobioreactors under continuous light a steady state can be reached, which is a very good research strategy as the experiments are highly controllable and cell physiology becomes constant. As such the influence of individual variables on the microalgae culture can be investigated. However, as early as in 1959 a paper was published comparing a steady state culture of *Chlorella* under constant light with a synchronized culture under day/night cycles *(Sorokin and Krauss, 1959)*. It was concluded that a higher growth rate could be reached in the synchronized culture, which is in agreement with the higher biomass yield on light that was found for synchronized cultures in chapter 4 of this thesis. Therefore, the results of this thesis underline that one should be careful with the

extrapolation of research done under constant light to outdoor cultures under day/night cycles.

Values for photosynthetic efficiency and growth rate, often obtained under constant light in laboratory conditions, are usually incorporated into models predicting outdoor growth of microalgae, e.g. (Breuer et al., 2015; Quinn et al., 2011; Sforza et al., 2014; Slegers et al., 2011). In chapter 4 it was shown that a 10-15% higher biomass yield on light was obtained in synchronized cultures compared to constant light cultures. This means that average photosynthetic efficiency during the day in a day/night cycle is not the same as the average photosynthetic efficiency under constant light. Therefore, average values for model parameters obtained under constant light are not representative for the changes that occur in these parameters during the day. A sensitivity analysis by Quinn et al. (Quinn et al., 2011) showed that maximum photosynthetic rate and maximum growth rate, as well as the photon efficiency, had a large impact on the biomass productivity that was predicted by the growth model. Also biomass productivity calculated by the model of Breuer et al. (Breuer et al., 2015) showed to be sensitive to variations in the maximum photosynthetic rate. As such, it will have significant impact on predicted productivities if these parameters are estimated wrongly. Therefore, it would be better to use input parameters obtained under light/dark cycles in growth models simulating the outdoor cultivation of microalgae.

Also the average biomass composition was different in the constant light culture compared to the day/night cycle cultures (chapter 4). During the day/night cycle biomass composition and growth rate showed to be highly dynamic (chapter 5). Therefore, also models describing biomass composition should include day/night cycles. This holds for black box models but also for metabolic flux models. Metabolic flux models can give an understanding of the regulation of metabolic fluxes in response to environmental conditions (Kliphuis et al., 2012). These models usually describe steady states under continuous light. Under continuous light conditions the dynamics in biomass composition are probably maintained to a certain extend in individual cells, but as all cells undergo the different stages at different times a stable average biomass composition is observed. This is not representative for a day/night situation where metabolism is affected by the circadian clock and the day/night cycle. For example, in *N. oleoabundans* starch content ranged from 5-21% of DW under a 16D8N cycle, and averaged over 24 hours the content

was twice as high under continuous light conditions as under day/night cycles. Therefore, microalgae metabolism should be studied under day/night cycles with multiple samples taken over the 24 hour cycle. Recently, a new framework for metabolic modelling under non-balanced growth was published, accurately describing biomass growth and composition of *Isochrysis sp.* grown under day/night cycles (Baroukh et al., 2014). These kind of approaches are necessary to get a realistic understanding of the dynamics of microalgae metabolism during day/night cycles.

As indicated above, research should be done under simulated day/night cycles in the laboratory in order to get reliable data that translate to outdoor day/night cycles. In this thesis it was shown that 'block' experiments, in which lights are switched on and off abruptly, are sufficiently representative for outdoor conditions, where light intensity follows a 'sinewave'. Providing light in a ' block' is technically easier in continuous experiments than providing light in a 'sinewave', i.e. slowly building light intensity up and down. Different light intensities and different durations can be used for the blocks. This will increase understanding of the behaviour of synchronized cultures under different operational conditions. During these experiments, multiple samples should be analysed over the 24h cycle. The frequency of sampling depends on the dynamics of the parameter of study. For some parameters, like total starch accumulated, a sample before and after the day period might be sufficient, whereas for others, like DNA synthesis, intervals of 1 hour during the night are already too long (see chapter 5). Intensive sampling during the night can be circumvented by switching the subjective day for the algae to the night period. This approach was proven to be successful during data acquisition of chapter 5, by blinding the reactor and using white LED light during the natural night.
In conclusion, microalgae growth and metabolism need to be studied using day/night cycles, in order to increase the understanding of the dynamics of microalgae growth under outdoor conditions and to serve as reliable inputs to models. These experiments can be done by using 'block' light regimes and sampling at several time points during 24h cycles.

6.2.2 Possibilities for optimization of the production process

One of the most obvious possibilities for improvement of microalgae production processes, based on knowledge gathered in this thesis for *N. oleoabundans*, lies in ***choosing the optimal harvest time***. Biomass composition is influenced by circadian rhythms, as

was shown in chapters 2, 3 and 5. Under day/night cycles, starch content starts to increase after approximately 6-7 hours from sunrise and decreases after light is shut down, as it is needed for respiration. Therefore, harvesting for biomass with a high starch content should be done before sunset. In case of the 16D8N cycle this would yield biomass with a starch content of 21% of DW instead of 5% of DW at sunrise. On the other hand, if proteins are the product of interest, cells should be harvested when starch content starts to increase, as a higher starch content coincided with a lower protein content in g mL^{-1} or g gDW^{-1}.

Choosing the optimal harvest time of synchronized cultures can also benefit ***downstream processing (DSP)***. Larger cells are easier to harvest (Salim et al., 2013) and therefore harvesting cells from a synchronized population towards the end of the day, when the cells are significantly larger in size, might reduce the energy needed for harvesting (Salim et al., 2011). Also further into the DSP there can be an influence of cell cycle phase on the process, as for example the characteristics of the cell wall change (Yamamoto et al., 2004; Yamamoto et al., 2007), which might affect the extraction of valuable components.

Nitrogen (N) limitation is the most commonly used method to stimulate accumulation of neutral lipids. ***The timing of N-starvation*** might have an influence on lipid accumulation. Neutral lipids were accumulated during the subjective day period in larger cells that were unable to divide (chapter 3). Therefore, starting N-starvation in the morning might yield a faster accumulation of neutral lipids than starting N-starvation in the afternoon or evening. Developing N-feeding and N-limitation regimes will be an interesting subject for future research (section 6.3.1).

When producing microalgae year round, the varying day lengths have an influence on biomass growth and composition. For *N. oleoabundans* it was shown that starch started to accumulate approximately 6-7 hours after sunrise. Therefore, less starch accumulated in shorter days, even though light intensity was kept the same. In a day of 12h, only 10% of DW consisted of starch, opposed to 20% of DW in the 16h and 20h days. In the longest day of 20h starch content did not increase after 16 hours of light. A possibly strategy might be to increase day length up to 16 hours by giving some ***extra light to photobioreactors*** at the end of short days. In this way, the production of starch can be increased.

The same may apply to other storage components, like neutral lipids. Spectral colour of the light might be of importance when trying to keep a synchronized culture and 'fooling' the algae in a longer day. Both blue and red light have an influence on the cell cycle in Chlamydomonas (Oldenhof et al., 2006). Blue light can delay cell division (Carroll et al., 1970; Oldenhof et al., 2004) and it is likely that a blue light receptor is involved in this inhibition, although the exact receptor in *Chlamydomonas* has not been identified yet (Nishihama and Kohchi, 2013). Also in *N. oleoabundans* a blue light receptor seemed to be involved in synchronization of the cell cycle (chapter 2) and therefore blue, or white LEDs that contain a big part of the blue spectrum, would be recommended when providing extra light to photobioreactors. Obviously, process economics should be taken into account to find out if the profit from these extra storage components outweighs the energy required for the extra lighting of the photobioreactors, in a study comparable to the study of Blanken et al. (Blanken et al., 2013).

The changes in biomass composition due to different behaviour of the synchronized cultures at different day lengths might also have implications for the ***choice of microalgae species***. It is sometimes suggested that certain algae species have a preference with respect to duration of the light period, resulting from the environmental conditions in which the particular species was isolated in nature (Jacob-Lopes et al., 2009; Sicko-Goad and Andresen, 1991). Nevertheless, for *N. oleoabundans,* that was isolated from a desert in Saudi Arabia where day lengths vary from 10-14 hours, there was not a large difference in biomass yield between different day lengths. Biomass yield on light was slightly lower in the long day experiments. However, a lot more light is provided in long days, and therefore productivity will still be highest in longer days (chapter 5).

Summarizing, adaptations to harvesting strategies and process design can be made based on the observed changes in cell composition and cell size during 24h cycles. This highlights the importance of research to the cell cycle and circadian clock with respect to the production of microalgae.

6.3 Future research and opportunities

In the next section opportunities for future research with regard to process design and regulation of the multiple fission cell cycle are given. Also, a search on the circadian clock in cyanobacteria and plants shows us opportunities for future manipulation of cir-

cadian rhythms in microalgae that might help improving the microalgae production process.

6.3.1 The circadian clock in process design

The discussed possibilities for future optimization of the production process were all based on findings for *N. oleoabundans*. In other species with a potential for production circadian rhythms are not well studied yet. The effect of synchronized cell division is likely to be higher in species dividing by multiple fission, like *N. oleoabundans, Scenedesmus* and *Chlorella*, than in species dividing by binary fission, like *Nannochloropsis* (see figure 6.1). Possibly, higher productivities can be obtained in species dividing by multiple fission, as they can reach high growth rates while still being synchronized to the day/night cycle. Likewise, effects on biomass composition may be less pronounced in binary fission algae as compared to multiple fission algae. Clearly, there is a need for research into circadian rhythms in other species that are considered promising for outdoor cultivation. Variables to include in the study are temperature, light intensity and duration of the light period.

In addition to studying other microalgae species, more work remains in determining the effect of circadian rhythms on the cell cycle and metabolism during nutrient limitation, like nitrogen, phosphorus or sulphur limitation. Uptake of nitrate and following reduction of nitrate to nitrite and ammonium are energy dependent processes and the enzyme nitrate reductase was shown to be most active in the middle of the day (Falkowski and Raven, 2007). Therefore, it would be interesting to study nitrogen assimilation under light/dark cycles, as well as circadian rhythms in nitrogen starved batch cultivations. This would help the development of N-feeding and N-limitation regimes as discussed in section 6.2.2. Brányiková et al. (Brányiková et al., 2011) showed that stopping the cell cycle in *Chlorella* by sulfur limitation or specific cell cycle inhibitors was a successful strategy to increase starch contents up to 60% of DW. This shows that either delaying or stopping the cell cycle, for example with extra light or nutrient limitation, is an interesting approach.

Finally, the effect of circadian rhythms on harvesting and extraction of valuable compounds can be considered in future studies. Also, models can be used to study the economic feasibility of the proposed methods.

6.3.2 Unravel the mechanism of cell division and clock control

Cell division in *N. oleoabundans* was shown to occur once every 24 hours under a range of different light conditions and was unaffected by nitrogen limitation in turbidostat cultures. The oscillation in cell division exhibited several characteristics of circadian rhythms (Mittag, 2001; Suzuki and Johnson, 2001); the oscillations were visible under constant conditions, had a period of 24h and it was possible to entrain them to the light/dark cycle. It should be noted that temperature compensation, the process that takes care of keeping a stable rhythm at a range of temperatures (Mittag, 2001), was not investigated in this study although it is an important characteristic defining a circadian rhythm. In addition, it is not clear to what extend the oscillation in cell division was self-sustained under constant light conditions, as the oscillation was lost in constant white light. However, a 24h oscillation was observed under a range of different conditions and also in other species of microalgae it was shown that there was a circadian rhythm in cell division (Goto and Johnson, 1995; Hagiwara et al., 2002; Malinowski et al., 1985). Therefore, we can assume that the circadian clock influenced cell division of *N. oleoabundans*. In addition, starch content and oxygen evolution showed 24h oscillations. The observed circadian rhythms in these processes suggest that the underlying gene expression is also regulated by a circadian clock (Matsuo and Ishiura, 2011). It would be interesting to look further into the mechanism of circadian regulation of the cell cycle, starch content and oxygen evolution. Systematic genetic studies were used to identify the circadian clock components in *Chlamydomonas.* However, it is not clear yet how output pathways, like cell division, are influenced by these clock components. Recent work showed that in the red alga *Cyanidioschyzon merolae* the G1/S transition was linked to a circadian rhythm and cell cycle progression was inhibited during the daytime (Miyagishima et al., 2014). However, regulation of the cell cycle differs in distinct species (Bišová and Zachleder, 2014) and much work remains to elucidate the genetic and biochemical interactions of circadian genes and proteins (Matsuo and Ishiura, 2011) with cell cycle genes and proteins.

Besides genetic studies, more knowledge on the mechanism of multiple fission can be gathered in an approach similar to the approach taken in this thesis. For example, commitment points and cell characteristics like cell size and composition at those points can be determined for experiments done at different light intensities, temperatures and photoperiods. This will provide information regarding the timing of cell division and re-

quirements for cells to reach the commitment point. However, as was experienced in this study, data coming from these kind of experiments can be hard to interpret, as there is still a spread on the timing of different events in different cells in the culture. In addition, not all parameters involved in regulation of the cell cycle are known and therefore it is possible that important parameters are left unmeasured. One way to deal with these difficulties is mathematic modelling, as was stressed by Bišová and Zachleder in their review (Bišová and Zachleder, 2014). Mathematic modelling of the multiple fission cell cycle can provide knowledge on the underlying mechanisms regulating cell division. Models exist for mammalian and yeast cells (Tyson and Novak, 2008) and for microalgae dividing by binary fission (Mocquet et al., 2010). However, up till now no models were published for the multiple fission cell cycle, except for a model by Rading et al.(Rading et al., 2011) describing stationary size distributions of cells growing by multiple fission.

During data acquisition of chapter 5 of this thesis, it was tried to create a simple model describing the multiple fission cell cycle of *N. oleoabundans* (unpublished results). This model was based on the hypothesis that the amount of daughter cells is ultimately determined by mother cell size (Matsumura et al., 2010). It was assumed that all cells increased in size with the same growth rate during the day and that cell division into 2, 4 or 8 cells took place once during the night. Although it was not possible to accurately describe our steady states with this model, it already provided us with a deeper understanding of the mechanism of multiple fission by rejecting the hypothesis that the amount of daughter cells is dependent solely on mother cell size (Matsumura et al., 2010). This was confirmed experimentally by taking samples from the reactor and comparing size distributions in these samples directly after sampling with size distributions after 1 day of incubation in the dark, during which cell division took place.

As is illustrated in figure 6.3, not all cells with a diameter above 3.3 µm divided after 7 hours of light in the reactor, whereas after 10 hours of light all cells above 3.3 µm did divide. This means that on top of cell size additional requirements, possibly a certain starch content (Bišová and Zachleder, 2014; Vítová et al., 2011b), have to be met before cell division can occur. This example illustrates how models can be used in further exploration of the mechanism behind multiple fission.

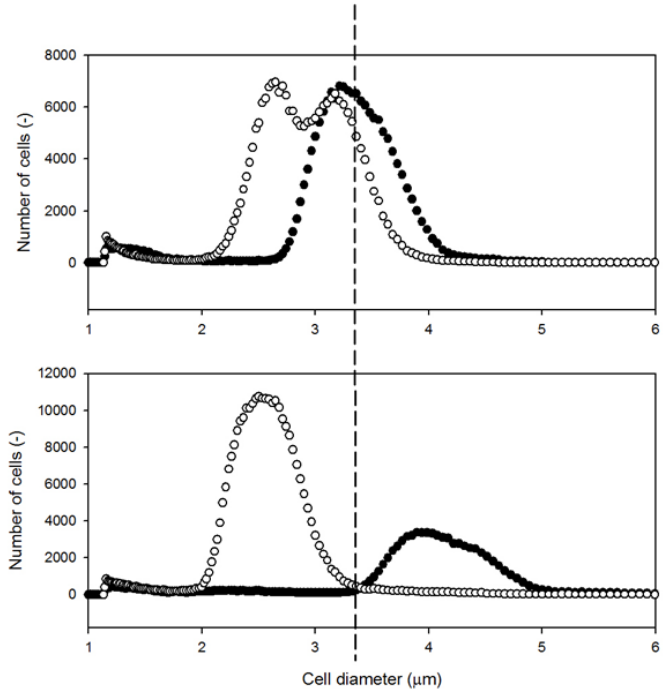


Figure 6.3 *Size distributions of N. oleoabundans in samples taken from a 16D8N turbidostat after 7 (upper) and 10 (lower) hours of light, directly after sampling (closed circles) and after incubating the same sample for 1 day in the dark (open circles). Vertical line illustrates that cell size is not the only requirement for a cell to divide.*

6.3.3 Manipulation of circadian rhythms

Circadian clocks are ubiquitous and it was long thought that they evolved independently across phylogenetic kingdoms, as there are apparently no common clock components. However, researchers discovered that oxidation-reduction cycles of peroxiredoxin proteins show conserved circadian oscillations in cells from all phylogenetic kingdoms (Edgar et al., 2012). In addition, for all organisms for which the molecular timing mechanism was studied a common model was found, namely a transcription-translation feedback loop (TTFL). Despite a difference in TTFL components, there are similarities between systems (Haydon et al., 2013) and therefore a sneak peak at cyanobacteria and plants can yield more insight in the future opportunities for possible manipulation of the circadian clock of microalgae to improve microalgae production processes.

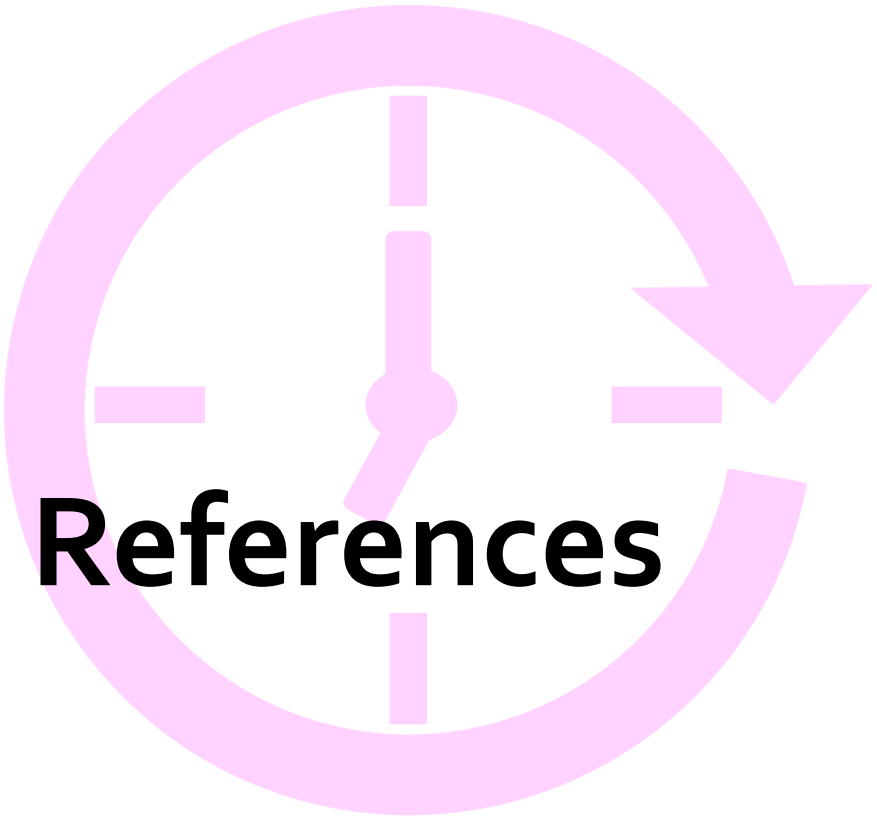
In cyanobacteria, the clock is modelled around three proteins, KaiA, KaiB and KaiC. KaiA activates transcription of KaiB and KaiC, with KaiC repressing KaiA. Furthermore, phosphorylation and dephosphorilation of KaiC play a key role in the clock mechanism (Haydon et al., 2013; Kageyama et al., 2006). KaiC and KaiA have been shown to complementarily contribute to the regulation of circadian gene expression. Therefore, circadian rhythms can be reprogrammed by overexpression of either *kaiA* or *kaiC* to enhance gene expression of either 'dusk' or 'dawn' genes, which can improve production of heterologous proteins (Xu et al., 2013). This theory was demonstrated by overexpression of KaiA in *Synechococcus elongatus* that was used to produce human pro-insulin. A fivefold increase relative to cells not overexpressing KaiA was found, which shows that 'pausing' the circadian clock in the most productive time window is a viable strategy for maximizing production of exogenous proteins in cyanobacteria (Hoyle and O'Neill, 2013).

In horticulture, knowledge on the circadian clock is already used to influence plant growth. The plant circadian clock not only synchronizes physiological and developmental events with daily changing environments, it also synchronizes with seasonally changing environments (Song et al., 2010). For the latter, the duration of the day and/or night in the environment is measured, which is called photoperiodic time measurement or photoperiodism. Photoperiodism can be used by horticulturalists as a way to induce flowering (Imaizumi and Kay, 2006). In addition, LEDs have made their introduction into horticulture due to their contributions in growth in case of red LEDs, and morphogenesis in case of blue LEDs (Massa et al., 2008; Morrow, 2008). Plant circadian clocks might also be manipulated in other ways to improve crop yield. The plant circadian clock was shown to influence photosynthesis and crop yield (Dodd et al., 2005) and a recent study confirmed the importance of the plant circadian clock in yield (Kusakina et al., 2014). A higher yield was reached at high temperatures in accessions of *Arabidopsis* that had a circadian clock that was not perfectly temperature compensated compared to accessions that had a clock that was perfectly temperature compensated.

When more knowledge becomes available on the circadian clock of microalgae, approaches similar to the ones in cyanobacteria and plants can be adopted, in order to improve the microalgae production process.

6.4 Conclusions

Circadian rhythms in cell division in the green microalgae *N. oleoabundans* were shown to influence biomass composition and yield. Not only *N. oleoabundans* but also many other microalgae synchronize their cell cycles under day/night cycles. Therefore, the study of circadian rhythms in microalgae in photobioreactors is very relevant for microalgae production. Yet, this subject received scant attention up till now. Experiments done with microalgae often use continuous light. However, in this thesis it was demonstrated that average values obtained under continuous light are different from average values obtained under day/night cycles. This means that laboratory experiments should be done under day/night cycles in order to accurately describe outdoor microalgae production. Based on the current knowledge of circadian rhythms clear directions for the improvement of microalgae production were given. More research on circadian rhythms and the underlying mechanisms is necessary to realise some of the proposed ideas and may result in other approaches to further improve microalgae production.



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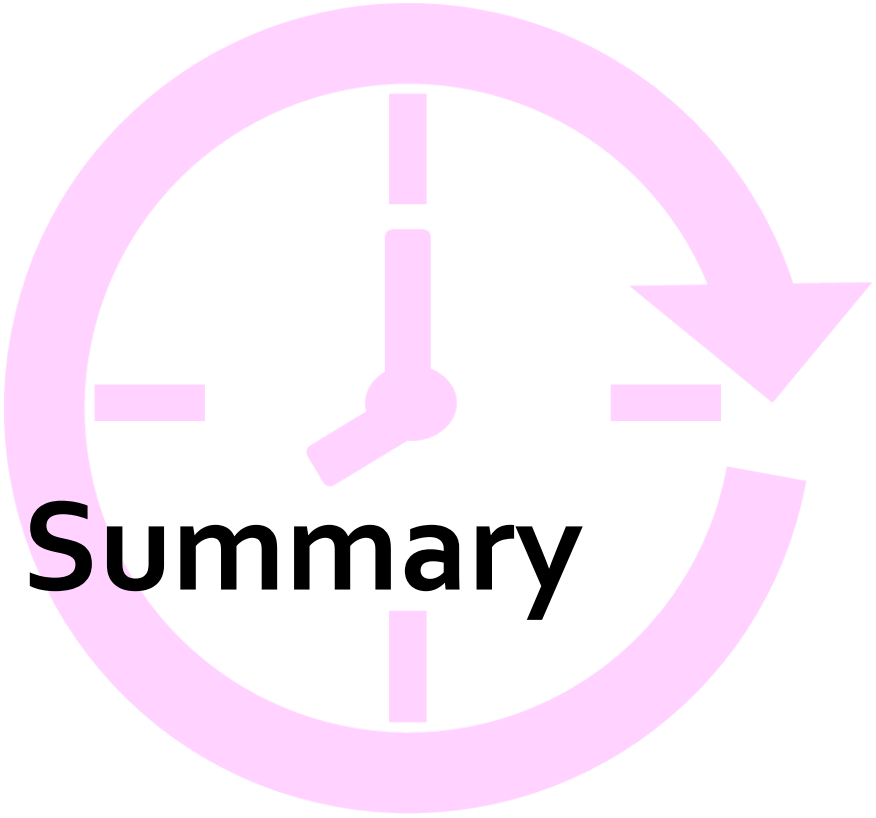


Summary

The sun imposes a daily cycle of light and dark on nearly all organisms. The circadian clock evolved to help organisms program their activities at an appropriate time during this daily cycle. For example, UV sensitive processes, like DNA replication, can be scheduled to occur during the night. In this way, the circadian clock causes rhythms in metabolic, physiological and/or behavioural events. These 24 hour rhythms are called **circadian rhythms** and continue for some period of time following exposure to continuous light.
In microalgae, circadian rhythms were observed in many processes, like nitrogen fixation, chemotaxis, photosynthesis and the cell division cycle, which might affect the **production of microalgae**. Microalgae biomass can be used as source for potential biofuels, chemicals, materials, foods, feeds and high-value bioactives and as such a lot of research is conducted in order to optimize the microalgae production process. Biomass growth rate, biomass yield on light and biochemical composition of the microalgae are investigated by growing microalgae in different types of photobioreactors. However, the possible influence of the circadian clock on microalgae production received scant attention. Therefore, the focus of this thesis was to obtain more insight in circadian rhythms in microalgae grown in photobioreactors and their relevance for microalgae production.

In **chapter 2** *N. oleoabundans* was grown under continuous red LED light in a flat panel photobioreactor operated as a turbidostat. A synchronized cell division was observed during the natural night. Under continuous white LED light the synchrony disappeared. Presumably, the circadian clock was triggered by some blue environmental light in the laboratory, and in this way was able to schedule cell division during the natural night even though the red light conditions were constant. Cell division occurred by multiple fission into 2 to 8 daughter cells, allowing the culture to remain synchronized at high growth rates. The effect of the synchronized cell division on biomass composition was investigated. Oscillations in starch, protein and pigment content were observed during the cell cycle. These oscillations could be solely contributed to the circadian clock, since all experimental conditions were kept constant. A maximum in starch, protein and fatty acid content was obtained just before cell division. Biomass yield was also greatly influenced by the cell cycle and declined to a minimum during cell division. These findings highlighted that knowledge of the cell cycle is of importance in microalgae process optimization.

The most commonly used method for the accumulation of storage compounds in microalgae is nitrogen limitation. Therefore, in **chapter 3** the effect of N-limitation on circadian rhythms in the cell cycle and biomass composition of *Neochloris oleoabundans* was investigated. N. oleoabundans was grown under continuous red LED light and nitrogen limitation. Under these constant conditions, the only variable was the circadian clock. The results were compared to the results of chapter 2. It was shown that the circadian clock was not affected by nitrogen-limitation, and cell division was still timed in the natural night, despite of continuous red LED light conditions. However, because of nitro--limitation a lower growth rate was obtained and therefore not the entire population was able to divide every day. Two subpopulations were observed, which divided alternately every other day. This phased cell division was still causing oscillations in biomass yield and composition. Starch and total fatty acids (TFA) were accumulated during the day. Also, fatty acid composition changed during the cell cycle. Neutral lipids were built up during the day, especially in cells that were arrested in their cell cycle (G_2 and G_3). These findings can potentially be used in future research to develop nitrogen limitation and nitrogen feeding strategies under day/night cycles.

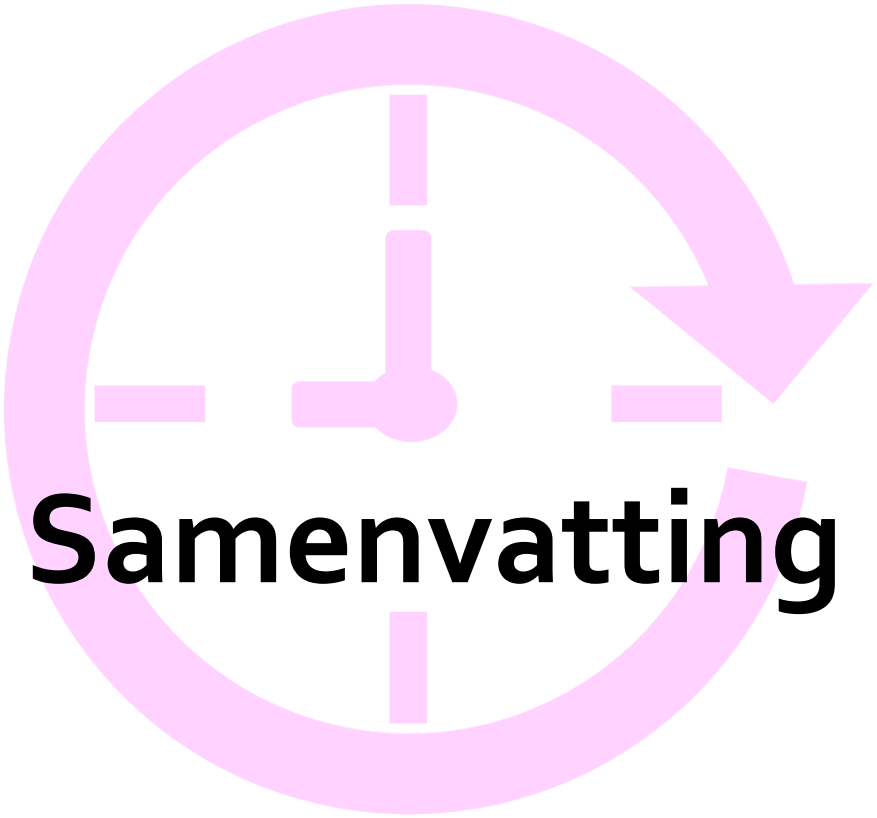
After having studied the circadian clock under continuous light conditions, a step was made to day/night cycles. In the laboratory, day/night cycles can be supplied to PBRs in different ways. Light can be either switched on and off abruptly, or gradually increased and decreased during the light period, in which the latter choice would be more similar to the natural light cycles. In **chapter 4** *N. oleoabundans* was grown under different simulated day/night cycles using white LED light. This resulted in synchronized cultures with cell division occurring during the dark period. Biomass yield and composition were compared with biomass yield and composition of a continuous light culture, in which cell division occurred randomly. In this way the influence of microalgae circadian rhythms on biomass yield and composition was revealed. The cultures that were synchronized by day/night cycles were able to use the light provided 10-15% more efficiently than the culture grown under continuous light. In other words, the efficiency of light usage varied over the cell cycle and the ability to schedule cell division during the night provided a fitness benefit to microalgae. Biomass composition under day/night cycles and continuous light was similar, with the exception of starch content. Microalgae under continuous light never had to spend starch for respiration during a dark period, and therefore starch content remained higher in the continuous light culture. No differences in biomass yield

and composition were found when the light during the day/night cycle was provided in a 'block' of constant intensity or in a 'sine' with gradually increasing and decreasing intensity. Therefore, it was concluded that providing light intensity in a block provides a good and easy to operate alternative for using sinuses when working with day/night cycles in the laboratory. In addition, the differences in biomass yield and composition between continuous light and D/N cycles implied that research under continuous light is not representative for outdoor day/night cycles. Therefore, more microalgae research should focus on day/night cycles.

In outdoor day/night cycles, day lengths vary over the course of a year. Varying day lengths can have an influence on circadian behaviour of the microalgae cultures. For example, circadian rhythms can adapt to varying day lengths, and as such the timing of processes like photosynthesis and cell division might be influenced. In **chapter 5** the influence of day length on circadian rhythms in *N. oleoabundans* was investigated. *N. oleoabundans* was grown in a continuous photobioreactor operated as a turbidostat under various D/N cycles; 20D4N, 16D8N and 12D12N. It was shown that maximum growth rate and start of starch synthesis were scheduled after approximately 6-7 hours from sunrise and therefore were not influenced by day length. Also, a timing mechanism seemed to be involved in DNA replication to 4 and 8 copies, which was always initiated approximately 16 hours after 'sunrise'. The exact mechanism of the timing of cell division remained unknown, although cell division seemed to be 'gated' to the night, except during very long photoperiods where synchrony was partly lost. The length of the photoperiod caused changes in biomass composition, especially due to oscillations in starch content. In longer days, more starch was accumulated. Starch was used for cell division probably also when cell division occurred in the light. Therefore, changes in biomass composition could also be correlated to the cell cycle of *N. oleoabundans*. These findings can be used to adjust harvesting times over the course of a year to harvest biomass with a desired concentration of protein, lipids, carbohydrates or pigments.

Finally, in **chapter 6** the implications of the overall results of the thesis for future research and microalgae production were discussed. First, the occurrence of circadian rhythms in different species of microalgae was discussed, in order to establish the general nature of these rhythms. Not only *N. oleoabundans* but also many other microalgae synchronize their cell cycles under day/night cycles. Therefore, the study of circadian

rhythms in microalgae in photobioreactors is very relevant for microalgae process engineering. Circadian rhythms in cell division in *N. oleoabundans* were shown to influence biomass composition and yield. Based on these findings for *N. oleoabundans*, ideas were proposed for the optimization of current research protocols and production processes. A case was made for performing research under day/night cycles instead of under continuous light, as in chapter 4 it was demonstrated that values obtained under continuous light are different from the values obtained under day/night cycles. In addition, some opportunities were discussed for improvement of the microalgae production process using knowledge on circadian rhythms. Possibly, future research will reveal ways of manipulating circadian rhythms for further improvement of microalgae production.



Samenvatting

De zon dwingt alle organismen op aarde in een cyclus van donker en licht. De biologische klok is ontstaan om organismen te helpen hun activiteiten op een geschikte tijd in te plannen tijdens deze dagelijkse cyclus. Zo kunnen bijvoorbeeld UV gevoelige processen, zoals het vermenigvuldigen van DNA, ingepland worden gedurende de nacht. Op deze manier regelt de biologische klok allerlei ritmes gedurende een 24 uur durende cyclus. Deze ritmes worden **circadiaanse ritmes** genoemd en ze kunnen voor een bepaalde tijd voortduren wanneer organismen worden bloot gesteld aan continue licht.
In algen zijn circadiaanse ritmes geobserveerd in vele processen, zoals stikstof fixatie, chemotaxis, fotosynthese en de celdeling. Dit zou de **productie van microalgen** kunnen beïnvloeden. Microalgen kunnen gebruikt worden als mogelijke bron van biobrandstoffen, chemicaliën, materialen, voedsel en hoogwaardige bioactieve stoffen. Daarom wordt er veel onderzoek gedaan naar het optimaliseren van het productie proces van algen. Zo worden bijvoorbeeld de groeisnelheid, de biomassa opbrengst op licht en de biochemische compositie van de algen onderzocht door algen in verschillende typen bioreactoren te groeien. Toch is er in onderzoek nog weinig aandacht geweest voor de mogelijke invloed van de biologische klok op de productie van algen. Daarom was het doel van deze thesis om meer inzicht te krijgen in circadiaanse ritmes in microalgen en te kijken in hoeverre dit belangrijk is voor het productie proces van microalgen.

In **hoofdstuk 2** werd de groene alg *N. oleoabundans* gegroeid onder continue rood LED licht in een vlakke plaat fotobioreactor die werd gedraaid als een turbidostat. Een gesynchroniseerde cel deling vond plaats gedurende de nacht. Wanneer de cellen onder continue wit LED licht werden gegroeid verdween deze synchronie. Waarschijnlijk werd de biologische klok van de algen getriggerd door een kleine hoeveelheid blauw omgevingslicht in het laboratorium. Op deze manier konden algen onder continue rood licht nog wel het verschil tussen dag en nacht waarnemen, en daardoor hun cel cyclus synchroniseren in de nacht, terwijl de algen onder continue wit licht de kleine extra hoeveelheid blauw licht niet op konden merken. Moeder cellen deelden in 2 tot 8 dochter cellen, waardoor de cultuur ook gesynchroniseerd kon blijven bij hogere groeisnelheden. Het effect van de gesynchroniseerde cel deling op de biomassa compositie werd onderzocht. Oscillaties in de hoeveelheden zetmeel, eiwit en pigment werden geobserveerd en deze oscillaties konden toegeschreven worden aan de circadiaanse klok, omdat alle experimentele condities constant werden gehouden. Een maximum in de hoeveelheid zetmeel, eiwit en vetzuur werd bereikt vlak voor cel deling. De biomassa op-

brengst op licht werd ook beïnvloed door de cel cyclus en deze opbrengst werd het laagste gedurende de cel deling. Deze bevindingen laten zien dat het belangrijk is om de cel cyclus te bestuderen voor de optimalisatie van het productie proces van microalgen.

De meest gebruikte methode om microalgen vetten en zetmeel te laten ophopen is stikstof limitatie. Daarom werd in **hoofdstuk 3** het effect van stikstof limitatie op circadiaanse ritmes in de cel cyclus en biomassa compositie van *Neochloris oleoabundans* onderzocht. In dit experiment werd continue licht gebruikt onder continue limitatie van stikstof. In deze omstandigheden was de enige variabele weer de biologische klok. De resultaten werden vergeleken met de resultaten van hoofdstuk 2. Er werd aangetoond dat de biologische klok niet beïnvloed werd door de stikstof limitatie, en dat de cel deling nog steeds in de nacht plaats vond, ondanks het gebruik van continue rood licht. Maar als gevolg van de stikstof limitatie werd er een lagere groeisnelheid bereikt en daarom kon niet de gehele populatie elke dag delen. Twee subpopulaties werden geobserveerd, welke om de dag deelden. Deze gefaseerde cel deling veroorzaakte nog steeds oscillaties in de biomassa opbrengst en samenstelling. Zetmeel en vetzuur gehaltes stegen gedurende de dag. Ook de vetzuur samenstelling veranderde. Neutrale vetten stegen gedurende de dag, vooral in cellen die gestopt waren in hun cel cyclus (G2 en G3 cellen). Deze bevindingen kunnen gebuikt worden in toekomstig onderzoek om stikstof limitatie en stikstof voeding strategieën te ontwikkelen onder dag/nacht cycli.

Nadat de biologische klok onder continue licht was bestudeerd, werd de stap gemaakt naar dag/nacht cycli. In het laboratorium kunnen dag/nacht cycli op verschillende manieren worden toegepast op de fotobioreactoren. Licht kan ofwel plotseling aan en uit geschakeld worden, ofwel langzaam opbouwend en afbouwend zoals in de natuurlijke dag/nacht cyclus. In **hoofdstuk 4** werd *N. oleoabundans* blootgesteld aan gesimuleerde dag/nacht cycli van wit LED licht. Dit resulteerde in gesynchroniseerde culturen waarbij cel deling plaats vond in het donker. De biomassa opbrengst en samenstelling van deze dag/nacht cycli culturen werd vergeleken met de biomassa opbrengst en samenstelling van een cultuur onder continue licht, waar de cel deling ook continue en gerandomiseerd plaats vond. Op deze manier werd de invloed van de biologische klok op biomassa opbrengst en samenstelling onthuld. De culturen onder dag/nacht cycli gebruikten het licht 10-15% efficiënter dan de cultuur onder continue licht. Met andere woorden, de efficiëntie waarmee licht gebruikt werd varieerde tijdens de cel cyclus en daarom was

het een voordeel voor de algen om hun cel deling in de nacht de plannen. De biomassa samenstelling onder dag/nacht cycli en continue licht was vergelijkbaar, behalve de hoeveelheid zetmeel. De microalgen onder continue licht hadden hun zetmeel voorraden niet nodig voor respiratie tijdens een donkere periode, en daarom was de hoeveelheid zetmeel hoger onder continue licht. Er was geen verschil in biomassa opbrengst en samenstelling tussen de proeven waarin het licht langzaam aan en uit werd gezet (sinus) en de proeven waarin het licht abrupt aan en uit werd gezet (blok). Daarom werd geconcludeerd dat het abrupt aan en uit zetten van het licht in experimenten een goed en makkelijk alternatief is voor het gebruik van sinussen als er met dag/nacht cycli gewerkt moet worden in het laboratorium. Bovendien lieten deze resultaten zien dat experimenten met continue licht niet representatief zijn voor de situatie onder natuurlijke dag/nacht cycli. Daarom zou het onderzoek naar microalgen zich moeten focussen op experimenten onder dag/nacht cycli.

In een natuurlijke dag/nacht cyclus varieert de dag lengte in de loop van het jaar. Deze variërende dag lengtes kunnen een invloed hebben op het circadiaanse gedrag van culturen van microalgen. Zo kunnen circadiaanse ritmes zich bijvoorbeeld aanpassen aan de variërende dag lengtes, en op die manier kan de timing van processen zoals fotosynthese en cel deling worden beïnvloed. In **hoofdstuk 5** werd de invloed van dag lengte op circadiaanse ritmes in *N. oleoabundans* onderzocht. De experimenten werden gedaan in een continue fotobioreactor die werd gedraaid als een turbidostat onder variërende dag/nacht cycli: 20D4N, 16D 8N en 12D12N. De maximum groeisnelheid en de start van de zetmeel synthese vonden ongeveer 6-7 uur na zonsopkomst plaats en werden dus niet beïnvloed door dag lengte. Er leek ook een timer betrokken te zijn in de start van DNA synthese tot 4 en 8 kopieën, die altijd ongeveer 16 uur na zonsopkomst plaats vond. Het exacte mechanisme waarmee de cel deling getimed werd blijft onbekend, maar cel deling vond altijd plaats in de nacht, behalve bij hele lange dagen waarin de synchronie in de cultuur gedeeltelijk verloren ging. De dag lengte veroorzaakte ook verschillen in biomassa samenstelling, vooral door oscillaties in de hoeveelheid zetmeel. In langere dagen werd meer zetmeel geaccumuleerd. Zetmeel werd gebruikt voor de cel deling, zelfs als de cel deling in het licht plaats vond. Daarom konden de verschillen in biomassa samenstelling ook gelinkt worden aan de cel cyclus van *N. oleoabundans*. Deze bevindingen kunnen gebruikt worden om oogst momenten gedurende het jaar te bepalen om biomassa te oogsten met een gewenste concentratie aan eiwitten, vetten, zet-

meel of pigmenten.

Uiteindelijk werden in **hoofdstuk 6** de implicaties van de resultaten van deze thesis voor toekomstig onderzoek en voor de productie van microalgen bediscussieerd. Allereerst werd de aanwezigheid van circadiaanse ritmes in andere soorten algen bediscussieerd. Niet alleen *N. oleoabundans* maar vele soorten microalgen synchroniseren hun cel cyclus als ze worden blootgesteld aan dag/nacht cycli. Daarom is de studie van circadiaanse ritmes erg belangrijk voor het ontwerpen van processen om algen te produceren. In deze thesis werd bewezen dat circadiaanse ritmes in de cel deling van *N. oleoabundans* de biomassa samenstelling en opbrengst beïnvloedde. Gebaseerd op deze bevindingen werden wat voorstellen gedaan om huidige onderzoeksprotocollen en productie processen te verbeteren. Er werd gepleit voor het gebruiken van dag/nacht cycli in experimenten in plaats van het gebruik van continue licht, omdat het in hoofdstuk 4 was bewezen dat de waarden verkregen onder continue licht anders zijn dan de experimentele waarden verkregen onder dag/nacht cycli. Verder werden er mogelijkheden gegeven voor het verbeteren van het productieproces van algen met gebruik van de kennis over circadiaanse ritmes. Toekomstig onderzoek zou kunnen uitwijzen of het mogelijk is om circadiaanse ritmes te manipuleren om het productie proces van algen nog verder te verbeteren .

Dankwoord

Mislukte experimenten, lekkende reactoren, besmette culturen, neergeslagen medium, biofilms, verstopte spargers, kapotte pompen, falende pH of DO probes, vastlopende computers, overstromende overflowvaten, opgeblazen leidingen, stroomstoringen, gestolen lichtmeters, geplunderde voorraden, verstopte condensors, op hol geslagen antifoam regelaars, oververhitte lampen, lekkende gasleidingen, gebroken glasplaten, kromme plastic platen, kapotte autoclaven, gesprongen klemmen, geblokkeerde coultercounter buisjes, of gewoon een net iets TE dunne reactor... Tijdens een PhD heb je soms wat mensen nodig om je eraan te herinneren hoe LEUK een PhD is!! Deze mensen wil ik nu graag bedanken. Staat je naam er niet tussen? Dan kun je me opzoeken aan de bar voor de gepaste tranen met tuiten, beloftes van eeuwige vriendschappen en dronken liefdesverklaringen.

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Supervising students was always one of my favourite things, except of course when they

asked questions before coffee time. **Ward,** ik ben trots dat mijn eerste studentje nu ook bijna klaar is met zijn PhD! Je hebt me leren relativeren ('oh, ik laat dat altijd gewoon ontploffen'). Dank je voor je vriendschap en natuurlijk de D-factor! **Richard,** ik ben blij dat we collega-PhDs zijn geworden! **Lutz,** thanks for your enthusiasm and your very big smile. **André,** I often think about our nights at the Biotechnion, the elevator races and how well you took care of our 'baby'. Thanks for all your hard work! **Imma,** thanks for your work on *Nannochloropsis*! **Evelien,** ik was blij dat jij er was tijdens de laatste fase van mijn PhD. Dankzij jou is hoofdstuk 5 veel beter geworden!
Alle problemen in het lab kon ik natuurlijk niet alleen oplossen. **Fred,** jij was mijn held! Zelfs in het weekend stond je me vrolijk te woord en hielp je mij van een afstand met welke stekkertjes ik eruit moest trekken en op welke knopjes ik moest drukken. **Wendy,** jij bent onmisbaar in het lab! Maar daarnaast was het heel gezellig met jou in koffiepauzes en borrels. Je bent een schat! **Sebas,** een korte of lange vraag, het maakte niet uit; ik kreeg altijd uitgebreid antwoord. Bedankt voor alle hulp, BPE kan niet zonder jullie!
Toen ik aan mijn onderzoek naar dag/nacht cycli begon, besefte ik mij niet volledig welke consequenties dit zou hebben voor mijn eigen dag/nacht ritme. Spook nachten in het Biotechnion met loeiende alarmen, films in 628, irritante wekkers, een tent in de koffiehoek… bedankt voor onze slapeloze nachten **Sina, Anne, Ward, André, Jacqueline!**

Ze zeggen weleens work hard, play hard! En dat hebben we gedaan! Fijn ook van die lange vakanties die aan aio reizen vastgeplakt konden worden. Met **Saskia** in Californië op zoek naar beren, met **Mariette** op een handdoekje aan het strand in Kroatië, met **Jacqueline** zigzaggend op de fiets naar Barcelona, met **papa** op de fiets richting Rome (jammer dat hij de alpen te zwaar vond) en Denemarken en met **Anne** op zoek naar koala's en kangoeroes in Australië. Bedankt voor deze ontspannende reizen!
Er was ook altijd nog ruim voldoende tijd voor het organiseren van borrels, BBQs, labuitjes en kerstdiners. Het begon allemaal toen **Klaske** en **Sina** mij inwijdden in de borrelcommissie. Na enige verbazing (eten mensen van BPE écht zoveel chips tijdens de borrels??) was ik er niet meer weg te slaan en ik heb met veel plezier ontelbare borrels georganiseerd. Bedankt voor jullie inzet voor de borrelcommissie **Jeroen, Ward, Ilse, Agi, Laura, Lenny, Guido, Jacqueline,** en natuurlijk de nieuwe garde die tegenwoordig zo enthousiast de borrels regelen. Mijn absolute favoriete bezigheid werd het organiseren van kerstdiners. Winter wonderland, Tapas, Sprookjesgala, Christmas with the Stars, Gourmetten met de helden van vroeger tot nu, BPE Christmas Cook Off, BPEs got

Talent… in totaal 7 prachtige diners waarvoor ik de **kerstcommissies** hartelijk wil bedanken. Op het organisatie lijstje kan natuurlijk ook de Young Algaeneers niet ontbreken. Ik ben ontzettend trots op wat we hebben neergezet met YAS 1.0 **Tweety (**ehm **Tim), Kim, Lenny, Anne, Guido en Marjon**!

Soms heb je van die dagen… dan ben je echt blij met je flexibele werktijden! Eerst een rondje hardlopen met **Gerard** en **Joao** (trouwens ook de rondjes op zaterdag werden gewaardeerd **Mark**), dan uitgebreid douchen en ontbijten en precies op tijd naar werk voor de koffiepauze. Koffiepauzes konden soms een tikkeltje uitlopen… Voor deze gezelligheid wil ik graag iedereen bij **BPE** bedanken en uiteraard ook de **foodies** met in het bijzonder **Pieter, Jacqueline, Marta, Nicholas en Laura.** Dan was het al weer snel tijd voor lunch. Bedankt **Pieter** voor de gezellige lunch afspraken die we hadden toen onze groepen uit elkaar gingen, en bedankt **Ellen** voor de vele lunches waarin jij altijd een luisterend oor bood. Tussen de pauzes door was er gelukkig nog meer tijd om te kletsen, bijvoorbeeld met **Miranda, Marina** en **Marjon** over de nieuwste roddels of met **Tim** over de tips en tricks van fietsvakanties. Of in kantoor met mijn vele roomies **Anne, Packo, Petra, Nicholas, Ana, Abdulaziz, Lenny, Guido, Rupali, Catalina** en **Rouke**.

Ook buiten werk was er wat welkome afleiding. Bedankt lieve, fantastische, succesvolle, knappe, slimme, mooie, en vooral ook grappige **MEISJES; Belle, Ariël, Sneeuwwitje en Assepoester**! Ik zou jullie relativerende humor en steun niet meer kunnen missen! Lieve **Marge**, al sinds onze 15e delen wij lief en leed en ik vind het super fijn en bijzonder dat je me ook op het podium bij zult staan. **Nora**, lieve schat wat fijn dat jij altijd voor me klaar staat, zelfs al moet je mijn voicemail wel drie keer per dag vol zingen. Sommige vriendinnen blijven altijd!

سینا جان خیلی ممنون . توعزیز زیبا با مزه با هوش و خوش تیپ هستی . من عاشقتم .

Lieve **zussen**, jullie zijn de meest fantastische en grappige zussen die ik mij kan wensen! **Heike**, wat fijn dat je altijd in de buurt was als ik je nodig had en **Willemijn** bedankt dat je mij kon laten zien dat er nog zoveel andere dingen in het leven zijn dan promoveren (zoals de liefste neefjes en het liefste nichtje van de wereld, **Menno, Ruben, Olivier** en **Sam**!). Lieve **papa** en **mama**, jullie hebben ook heel wat moeten doorstaan voor dit proefschrift. Toch hebben jullie me altijd gesteund en interesse getoond in die rare algen. Bedankt voor jullie onvoorwaardelijke liefde. Ik hou van jullie.

- Lenneke -

Curriculum Vitae

Lenneke de Winter was born in Zaltbommel, The Netherlands, on March 13, 1985. She attended Scholengroep Cambium in Zaltbommel, where she obtained her diploma in 2003.

In the same year, she started her study Biotechnology at Wageningen University. In her minor thesis project at the Food Microbiology group of Wageningen University, she worked on the thermal inactivation of Bacillus spores in chocolate milk. During her major thesis at the Bioprocess Engineering group of Wageningen University, she investigated the effect of mixing on algal productivity in a novel photobioreactor. Lenneke completed her studies by doing an internship at Solix Biofuels in Colorado, USA, where she designed a mathematical model for the growth of microalgae in outdoor photobioreactors. She graduated in 2008 (BSc) and 2009 (MSc).

After completing her studies, she continued working as a PhD at the Bioprocess Engineering group of Wageningen University, on the project Maximization of photosynthetic efficiency. After 2 years she started investigating circadian rhythms in microalgae and their effect on microalgae production.

List of Publications

Kliphuis, A.M.J., **de Winter, L.,** Vejrazka, C., Martens, D.E., Janssen, M.G.J., Wijffels, R.H. 2010. Photosynthetic efficiency of *Chlorella sorokiniana* in a turbulently mixed shirt light-path photobioreactor. Biotechnology Progress 26, 687-696

Quinn, J., **de Winter, L.,** Bradley, T. 2011. Microalgae bulk growth model with application to industrial scale systems. Bioresource Technology 102, 5083-5092

Sousa, C., **de Winter, L.,** Janssen, M., Vermuë, M.H., Wijffels, R.H. 2012. Growth of the microalgae *Neochloris oleoabundans* at high partial oxygen pressures and sub-saturating light intensity. Bioresource Technology 104, 565-570

de Winter, L., Klok A.J., Cuaresma Franco, M., Barbosa, M.J., Wijffels, R.H. 2013. The synchronized cell cycle of *Neochloris oleoabundans* and its influence on biomass composition under constant light conditions. Algal Research 2 (4), 313-320

de Winter, L., Schepers, L.W., Cuaresma, M., Barbosa M.J., Martens, D.E., Wijffels, R.H. 2014. Circadian rhythms in the cell cycle and biomass composition of *Neochloris oleoabundans* under nitrogen limitation. Journal of Biotechnology 187, 25-33

de Winter, L., Barbosa, M.J., Martens, D.E., Wijffels, R.H. 2015. The influence of circadian rhythms on biomass yield and composition of *Neochloris oleoabundans*: Day/night cycles vs. constant light. Submitted for publication.

de Winter, L., Órfão, A.N., Vaessen, E., Barbosa, M.J., Martens, D.E., Wijffels, R.H. 2015. The influence of day length on circadian rhythms in *Neochloris oleoabundans*. Submitted for publication.

Overview of completed training activities

Discipline specific activities

Courses

SolarH2 Photobioreactor School (Nantes, France, 2009)
Bioreactor design and Operation (Wageningen, 2010)
Advanced Course on Microbial Physiology and Fermentation Technology (Delft, 2010)

Symposia

Netherlands Biotechnology Congress (Ede, 2009, 2014[2])
Wetsus congres and Wetsus internal congres (Leeuwarden, 2010)
1st International Young Algaeneers Symposium (Wageningen, 2012) [1,3]
Mini-symposium Microalgae Biotechnology: Towards industrial strains and processes (Wageningen, 2013)[2]
2nd International Young Algaeneers Symposium (Narbonne, 2014) [2]
International Symposium Applied Phycology 2014 (Sydney, Australia, 2014) [2]

General courses

PhD Competence Assessment (Wageningen, 2009)
VLAG PhD Week (Baarlo, 2010)
Teaching and Supervising Thesis Students (Wageningen, 2010)
Basic Statistics (Wageningen, 2011)
PhD Scientific Writing (Wageningen, 2013)
Career perspectives (Wageningen, 2014)

Optionals

Wetsus Team Meetings (Leeuwarden, 2009-2015) [2]
Bioprocess Engineering Brainstorm Days (2009-2014) [2]
Bioprocess Engineering PhD excursion USA (2010) [1,2]
Bioprocess Engineering PhD excursion Spain (2012) [1,2]

[1] Poster
[2] Presentation
[3] Organisation

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