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

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ABSTRACT

In this study the influence of day length on circadian rhythms of *N. oleoabundans* was investigated. *N. oleoabundans* was grown in a photobioreactor continuously operated (turbidostat), under various day/night (D/N) cycles; 20D4N, 16D8N and 12D12N. The following variables showed to be regulated by the circadian clock: maximum growth rate, start of starch synthesis and DNA replication to 4 and 8 copies. Timing of these processes was not influenced by day length. 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. This timing mechanism can be explored to produce biomass with a desired concentration of protein, lipids, carbohydrates or pigments.



## 1. Introduction

Circadian rhythms are generally believed to help organisms adapt to and anticipate on daily and seasonally fluctuating environments [1]. They help the organism to schedule certain processes within the temporal window that is most suitable [15,23]. For example, in microalgae, UV sensitive processes like DNA replication and cell division are often scheduled during the night [25]. Therefore, synchronous division can be triggered by exposing algae to a light/dark (L/D) cycle [19]. 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 less [25]. 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 [2]. In this way, synchrony can be maintained at higher growth rates (i.e., dividing more than once per day).

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) sunlight should be used as the sole source of light energy [3,27]. Day lengths (i.e. duration of light period) change over the course of a year and differ depending on the region. In the Netherlands, the longest days in summer last almost 17 h, while the shortest days in winter last only 8 h. In Norway (Hammerfest), the longest days last 24 h and the shortest days 0 h, while in Singapore days last more or less 12 h year round. Although it has long been recognized that biomass composition changes during the D/N cycle [7,20], not many studies have focused on the effect of day length on biomass composition. In order to optimize year round production, 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 [5,6]. Although under constant conditions circadian rhythms oscillate within a period of approximately 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, like the changing day lengths over the course of a year [8,18]. Light is one of the major cues for adjustment of the circadian clock [18] 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 [22]. Both dawn [1,16] and dusk [14,19] 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 [2], and thus these

*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, commitment point; $t_d$, doubling time; t, time; μ, growth rate; $N_t/N_0$, daughter cells per mother cell; V, volume overflow; $V_{pbr}$, volume reactor; D, dilution rate; a, absorption cross section.

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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 was to find out how different events, such as the start of DNA replication and cell division are scheduled during 24 h 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.

## 2. Materials and methods

### 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 [11] 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 (warm white lamp tubes). Four days before inoculation cultures were transferred to a second incubator at 120 RPM (Orbital Incubator, Sanyo, Japan) under continuous light at an intensity of 150 μmol $m^{-2}$ $s^{-1}$ (inoculum was used in exponential phase). Temperature was constant at 25 °C and the headspace of the incubator was enriched with 5% $CO_2$.

### 2.2. Photobioreactor set-up and experimental conditions

*N. oleoabundans* was cultivated in a flat panel photobioreactor (PBR) (Labfors 5 Lux, LED Flat Panel Option, Infors HT, Switzerland). The PBR had a light path of 20 mm and a working volume of 1800 mL. Light was supplied by 360 LEDs bulbs (warm white light, spectrum from 450 nm–620 nm). A black cover was placed on the back of the reactor to avoid environmental light. Additionally, a cover especially designed to fit the PBR was fitted in the space between the LED panel and the reactor, also preventing environmental light from entering the PBR. Ingoing and outgoing light intensity ($PFD_{in}$ and $PFD_{out}$) were measured at the illuminated surface using a Li-cor quantum sensor (LI250 light meter, LI-COR, USA).

Ingoing light intensity ($PFD_{in}$) was gradually increased after inoculation until the final set point, in order to allow the microalgae to adapt to the new light conditions. When $PFD_{out}$ reached the final set point, due to biomass growth, the light regime was changed from constant light to D/N cycles. Table 1 shows the different light settings of each experiment. $PFD_{in}$ and $PFD_{out}$ were chosen in order to keep an average photon flux density ($PFD_{av}$) at 200 μmol $m^{-2}$ $s^{-1}$ for experiments 1 till 3. For experiment 4 $PFD_{av}$ was kept at 70. 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. Experiments 2 and 4 were carried out under the same D/N cycle, but with the difference that 2 was done under high light (HL, 200 μmol $m^{-2}$ $s^{-1}$) while 4 was done under low light (LL, 70 μmol $m^{-2}$ $s^{-1}$). In that way we could evaluate the effect of the light intensity on the circadian cycle of *N. oleoabundans* grown under the same day length. 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 experienced 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 ($\mu_{24}$) is equal to the dilution rate (D), which was monitored by logging the amount of overflow produced ($V_{24}$) over 24 h (see Eq. (1)).

$$\mu_{24} = D = \frac{V_{24}}{V} \tag{1}$$

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)).

$$D = \mu + \frac{1}{a}\frac{da}{dt} \tag{2}$$

From the growth rate μ, also the doubling time $t_d$ ($day^{-1}$) and average number of daughter cells formed per mother cell $N_t/N_0$ during cell division were calculated according to Eqs. (2) and (3), in which t is time (days).

$$t_d = \frac{\ln(2)}{\mu} \tag{3}$$

$$\frac{N_t}{N_0} = 2^{\frac{t}{t_d}} \tag{4}$$

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.

### 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. [10]. During steady state, additional samples were taken over a 24 h period with 3 h 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

**Table 1**
Summary of the different light settings tested in the experiments.* Experiment 2 was done in duplicate. Experiments 2 and 4 were carried out under the same D/N cycle, but with the difference that 2 was done under incident high light (HL, 200 μmol $m^{-2}$ $s^{-1}$) while 4 was done under incident low light (LL, 70 μmol $m^{-2}$ $s^{-1}$).

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