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Growth and fatty acid content of *Rhodomonas* sp. under day:night cycles of light and temperature

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ABSTRACT

The biomass composition, namely the fatty composition, of microalgae used in aqua feed is of great importance for the nutritional value of the biomass. Day:night cycles of light and temperature could influence the growth and biomass composition of microalgae. To study the effect of these cycles on *Rhodomonas* sp. the algae were grown under 16:8 day:night cycles in lab-scale photobioreactors running as turbidostat. Different temperature and light intensities were applied during the light phase. Synchronized cell cycles were observed for *Rhodomonas* sp. under day:night conditions with oscillating cell size, cell number, biomass concentration and fatty acid content and composition. Cells increased in size during the light phase, storing energy, with cell division scheduled in the dark phase.

The introduction of a 16:8 day:night cycle did not affect the biomass yield on light, when operating at optimal conditions of light (150 μmol m^{-2} s^{-1}) and temperature (21 °C). However, under high light (600 μmol m^{-2} s^{-1}) or temperature (25 °C), an increased biomass yield on light of up to 22% was found under day:night cycles in comparison to continuous conditions under equal light and temperature levels. Implementation of a day:night cycle increased the maximum daytime temperature for *Rhodomonas* sp. from 25 °C to 30 °C. The fatty acid content and composition was influence by the implementation of day:night cycles. Daily fluctuations in total fatty acid content from 76 ± 2 mg gDW^{-1} at the end of the light phase to 94 ± 2 mg gDW^{-1} in the first hours of the light phase are found. The eicosapentaenoic acid and docosahexaenoic acid content fluctuated by 30% (12.1–16.1 mgEPA + DHA gDW^{-1}) on a daily basis. These daily fluctuations can be exploited in aqua feed applications by selecting a specific time of the day to harvest algae.

1. Introduction

Rhodomonas sp. is an important microalga for aquaculture as it is often used in live feed applications, specifically for copepods [1,2]. Applications of *Rhodomonas* sp. as copepod feed require a lower minimal feed concentration in ash free dry weight (1.13 μg AFDW ml^{-1}) than other algae species such as *Isochrysis* sp. (2.02 μg DW ml^{-1}) and *Pavlova salina* (1.86 μg AFDW ml^{-1}) [2]. A higher development index of the copepods is reached when feeding *Rhodomonas* sp. compared to any other algae species tested [2], whereas copepods grown on *Tetraselmis* sp. show fast growth but also development of growth anomalies [2,3]. The positive effects of *Rhodomonas* sp. on copepod development is mainly due to the requirements of fatty acid content in copepods during transformation and egg-production. *Rhodomonas* sp. is used as feed source in copepod production processes because they contain high levels of the essential fatty acids for copepod development; both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [4–7]. These fatty acids are specifically essential for fecundity, egg-production and copepod transformation [8]. Furthermore the biomass composition and therefore nutritional value of copepods can be manipulated by the microalgae diet [5]. Matching the nutritional value of *Rhodomonas* sp. with nutritional requirements of copepods is therefore of great importance for copepod production [9,10]. Optimization of *Rhodomonas* sp. cultivation towards favourable fatty acid composition could benefit copepod production. *Rhodomonas* sp. shows a high content for both EPA and DHA whereas other strains contain only EPA or

Abbreviations: C_x, Biomass concentration (g l^{-1}); D, Daily dilution rate (d^{-1}); DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; I_{ph}, Incident light intensity (μmol_{ph} m^{-2} s^{-1}); MUFA, Mono unsaturated fatty acid; PUFA, Poly unsaturated fatty acid; r_{ph}, Volumetric photon supply rate (mol_{ph} l^{-1} d^{-1}); SFA, Saturated fatty acids; TFA, Total fatty acid content of the biomass; $V_{dilution}$, Daily dilution volume (l); $V_{reactor}$, Total reactor volume (l); $Y_{x,ph}$, Biomass yield on light (g_x mol_{ph}^{-1})

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almost exclusively DHA [11]. A study comparing the fatty acid content of eight strains for aquaculture showed only three were able to produce DHA. *I. galbana* showed a higher DHA content (15–20 mgDHA gDW^{-1}) than *Rhodomonas salina* (5–15 mgDHA gDW^{-1}) but with very low EPA concentration in *I. galbana* (< 2 mgEPA gDW^{-1}) compared to *Rhodomonas salina* (15–20 mgEPA gDW^{-1}) [11]. This EPA and DHA content for Isochrysis is confirmed in multiple other studies [12–14]. Other microalgae strains used in aquaculture such as *Nannochloropsis* sp. *Tetraselmis* sp. and *Chaetoceros* sp. were shown to produce only EPA and no DHA [11–14].

For industrial production of microalgae a light limited growth strategy is typically applied. In these settings the productivity, biomass yield on light and fatty acid composition are mainly influenced by light intensity and cultivation temperature. The combined effect of these two parameters under continuous conditions, with both light and temperature at a constant level for 24 h day^{-1}, has recently been described in detail [15]. Maximal biomass yield on light was achieved at temperatures of 22–24 °C and light intensities of 110–220 μmol m^{-2} s^{-1} [15]. Lower temperatures (15 °C) result in higher EPA and DHA content of the cells, whereas highest biomass productivity was described at higher temperatures (> 21 °C) combined with high light intensities [15]. Daily fluctuation of the growth parameters could influence the biomass composition significantly compared to continues light conditions as cell components are used for energy storage in a day:night cycle [16–18]. When algae are produced at large scale under sunlight conditions a day:night cycle for light intensity occurs and potentially temperature oscillations will also occur, depending on the cultivation system and location.

A day:night cycle of light has a positive effect on the biomass yield on light for multiple microalgae strains. León-Saiki & Remmers et al. showed an increase of the biomass yield on light of approximately 20% under day:night cycles compared to continuous light cultivation for *Acutodesmus obliquus* [16]. De Winter et al. showed a 10–15% increase in biomass yield on light for *Neochloris oleobundans* grown under a day:night cycles compared to continuous light conditions [19,20]. The increase of the biomass yield on light is the result of a lower efficiency of light use during cell division than during other parts of the cell cycle. By synchronizing the cell division with the dark cycle, the overall biomass yield on light increases compared to continuous conditions [21]. Furthermore, it has been shown that during a day:night cycle the biomass composition of strains described in literature fluctuates significantly as the cells synchronize the cellular processes [16,22]. Cells under day:night cycles store energy rich components during the light period and utilize energy stored in these components during the dark phase when energy intensive and light sensitive processes like cell division take place [16,17,19,23,24]. The best described example of this process of diurnal energy storage is the use of starch in *Acutodesmus obliuquus* [16]. An identical daily fluctuation of starch is found in *Chlorella* sp., with contents varying between 13 and 45% of the biomass dry-weight depending on the time of the day [24]. Another study describes that content of other cell components, such as fatty acids, could be closely related to cell division processes. The fatty acid content has shown a daily fluctuation in *Nannochloropsis oceanica* [17]. A study of *Neochloris oleobundans* with synchronized cell cycles showed a daily fluctuation in starch, fatty acid and protein content even under continuous illumination [18]. The fatty acid content of *Isochrysis* sp. was dynamic under day:night cycles [23].

It is therefore hypothesized that the implementation of a day:night cycle for *Rhodomonas* sp. could affect the nutritional value of the biomass significantly. Understanding the effect of daily fluctuations of the biomass composition over a day:night cycle could result in improved nutritional value of the produced microalgae, namely for the essential feed ingredients, EPA and DHA content. Therefore, in this study we evaluate the effect of day:night cycles of both light and temperature on *Rhodomonas* sp., focussing on the biomass yield on light and fatty acid composition and content; specifically, the EPA and DHA content of the cells.

Table 1
Growth conditions of all eight experimental conditions and experiment names. Experimental names describe the conditions for Light and Temperature using: C = constant, F = fluctuating in a 16:8 day:night cycle, B = base level, H = high level and X = extreme, L = light and T = temperature.

Experiment name	Light intensity (μmol m^{-2} s^{-1})	Temperature day (°C)	Temperature night (°C)	Photon supply rate r_{ph} ($mol_{photons}$ l^{-1} d^{-1})
CBL-CBT	150	21	21	0.58
FBL-CBT	150	21	21	0.39
CBL-FBT	150	21	15	0.58
FBL-FBT	150	21	15	0.39
FHL-FBT	600	21	15	1.68
FHL-FHT	600	25	15	1.68
FBL-FXT	150	30	15	0.39
FHL-FXT	600	30	15	1.68

2. Materials and methods

Rhodomonas sp. was grown on a 16:8 day:night cycle for different light and temperature oscillations. A day:night cycle (for both light and temperature) was compared with growth experiments without any fluctuations, the baseline experiment, and to cells experiencing a day:night cycle for temperature or light only. The effect of the day:night cycle was investigated at more extreme conditions with higher light (600 μmol m^{-2} s^{-1}) and higher daytime temperature conditions (25 °C and 30 °C). A detailed list of all experimental conditions is given in Table 1.

Finally the full day:night cycle of one experiment was measured in detail to obtain insight in the daily fluctuations of the biomass composition.

2.1. Strains, cultivation medium and culture conditions

Rhodomonas sp. was obtained from commercial production in Dutch aquaculture and characterized by 18S sequencing as *Rhodomonas* sp. Cultures were maintained in Erlenmeyer flaks placed in an orbital shaker (100 rpm) maintained at 25 °C and 2.5% CO_2 enriched air with low light conditions (80 ± 10 μmol/m^2/s) under a 16:8 day:night cycle. Cultures were used for the inoculation of experimental reactors after 7–10 days of batch cultivation in flasks. The growth medium for reactor experiments was based on the L1-medium with an adjusted iron source and used in combination with artificial seawater [25]. Changes to the medium and concentration of the nutrients was done as described by Oostlander et al. [15].

2.2. Photobioreactor operation and experimental setup

Reactor experiments were performed in a heat-sterilized, flat-panel, photobioreactor (Labfors 5 Lux, Infors HT, Switzerland) with a total working volume of approximately 1.8 l. Aeration of the culture with 0.9 l min^{-1} filtered air (0.2 μm) was applied for mixing and CO_2 was injected on-demand to the airflow for pH control and carbon supply. The pH was maintained at 7.5. Cultivation temperature was controlled through a water jacket in direct contact with the cultivation chamber of the reactor with temperature profile controlled by the control computer. The incident light intensity was provided by the integrated LED-light system of the Labfors 5 Lux. A shroud between the reactor and light panel combined with a dark cover at the backside of the reactor protected the culture from any environment light entering the reactor. Light was applied 24 h per day or as 16:8 day:night cycles with a block pattern as described in Section 2.4. *Rhodomonas* sp. was inoculated

from flask cultures to a starting optical density of OD_{750} 0.15 ± 0.05.

2.2.1. Day:night cycles

Reactor operation started with a batch phase for biomass production followed by turbidostat operation under the experimental conditions to be tested. Not all experiment were started from a batch phase. Some reactors were started from a previous steady-state by changing the light and temperature set points. During turbidostat operation, on-demand dilution with fresh culture medium is applied to maintain the light leaving the reactor at 15 μmol m^{-2} s^{-1}, measured by the integrated light sensor (LI-250, Licor, USA). Turbidostat operation was only applied during the light hours of the experiments. Daily samples were taken from the onset of the experiment and were measured at sunset (t = 16 h after sunrise). For experiments with continuous light samples daily samples were taken at a fixed time of the day. Daily measurements for biomass concentration were performed by optical density and cell count measurements in duplicate. The daily dilution rate was calculated based on the added volume of medium in one day. Daily measurements were performed from the onset of the experiment until steady-state was reached. A reactor was defined to be in steady-state when a stable dilution rate was observed with a stable biomass concentration for the duration of at least 3 hydraulic retention times. Depending on the experimental settings, steady-state was achieved between 7 and 20 days after the start of the experiment. At steady-state the biomass concentration was determined by dry-weight (in triplicate), cell-count and optical density (in duplicate). The fatty acid composition and content of the biomass was determined at the end of the steady-state.

2.2.2. Detailed 24-hour cycle

A detailed analysis of the daily fluctuations of the fatty acid composition was performed using the growth parameters of the experiment with high light (I_{ph} = 600 μmol m^{-2} s^{-1}) and high temperature (25 °C); experiment FHL-FHT. These settings allowed for the largest oscillations in both growth parameters and were expected to show the largest fluctuations in biomass composition. Two independent reactors were operated in turbidostat mode until and during steady-state. The same settings for temperature and light for the day:night cycles were used in both reactors with the defined sunrise in the reactors shifted by 12 h to allow for measurements of the full 24 h cycle. Each reactor was sampled every two hours during a 14–15 h period. Sampling was performed for two separate days during steady-state in each reactor system. A two-sample overlap, sample t = 11 h and t = 23 h after sunrise, between the two reactors was used to show the reproducibility between the two systems. A visualization of the sample moments and temperature and light profile can be found in the supplementary files. All samples taken during these experiments were analysed for biomass concentration using optical density (OD_{750}) in duplicate, dry-weight in triplicate, cell-count in duplicate and cell-size in duplicate. The fatty acid content and composition was determined in duplicate for each sample.

2.3. Measurements and calculations

For daily measurements a 2.0 ml sample was taken directly from the reactor to measure optical density, in duplicate, at 750 nm using a UV-VIS spectrophotometer (Hach Lange DR-6000, light path 1 cm). The cell count and cell-size was determined, in duplicate, with a Multisizer II (Beckman Coulter) using a 50 μm aperture tube after diluting the sample using the original growth medium. The daily dilution rate (D_{24h}) was calculated from the difference in mass of the medium vessel used for dilution of the reactor, during one day ($V_{harvest24h}$), and the total reactor volume ($V_{reactor}$), given in Eq. (1).

$$D_{24h} = \frac{V_{harvest\ 24h}}{V_{reactor}} \quad (1)$$

The dry-weight and fatty acid content were determined at steady-state. Dry-weight was measured in triplicate as described by Oostlander et al. [15]. Fatty acid extraction and quantification was performed using GC-FID analysis based on the method described by Breuer et al. with samples collected according to method 1.1 [26]. The extraction method was adjusted for *Rhodomonas* sp. according to Oostlander et al. as no extended cell disruption is required [15].

The biomass yield on light ($Y_{x,ph}$) was calculated for each day using the dilution rate (D_{24h}), the dry-weight in the reactor system as measured at the time of sampling in steady-state (C_x) and the photon supply rate (r_{ph}), as described by Leon-Saiki & Remmers et al. and Eq. (2) [16]. Final results for the biomass yield on light are given as the average biomass yield on light over all measurements within the defined steady-state.

$$Y_{x,ph} = \frac{D_{24h} * C_x}{r_{ph}} \quad (2)$$

2.4. Experimental design

Eight different combinations of growth parameters were applied in the reactor experiments. Each experimental condition was performed in duplicate in two separate reactors. The first four experiments compare experimental conditions for a baseline experiment at continuous condition with equal levels for light and temperature under daily fluctuating conditions. The other four experiments combine fluctuating temperature and light conditions at higher levels for one or both growth conditions. Letters are used to describe the experimental conditions in the format: continuous (C) or fluctuating in a 16: 8 day:night cycle (F) followed by the level of the parameter indicated as baseline (B), high (H) or extreme (X) where extreme is only use in case of light and finally growth parameter: light (L) and temperature (T).The baseline experiment using continuous conditions is therefore referred to as CBL-CBT, see Table 1 for full list of experimental conditions and names.

The continuous conditions of the baseline experiment (CBL-CBT) are compared with a daily fluctuation for only temperature (CBL-FBT), only light (FBL-CBL) and both light and temperature (FBT-FBL). Fluctuations for light and temperature are tested at higher light levels as it is hypothesized that the impact of a day:night cycle on the biomass yield on light could be more pronounced under conditions that proved sub-optimal for continuous cultivation conditions. The impact of higher light levels is tested in the experiment FHL-FBT and the higher light level is coupled to the higher temperature in experiment FHL-FHT. The FXT-experiments test whether temperature conditions previously unviable for *Rhodomonas* sp. under continuous conditions would be viable if a night-phase (dark and cold) is implemented.

The growth conditions of the baseline experiments were set at an incident light intensity of I_{ph} = 150 μmol m^{-2} s^{-1} and temperature of T = 21.0 °C. These conditions were previously determined as the conditions that allow for maximum biomass yield on light for *Rhodomonas* sp. under continuous conditions [15]. A schematic representation of the light and temperature profile in the reactors for oscillation situations is given in Fig. 1. The solid line shows the daily oscillations of the light level. Light was applied in a block form (on/off) with a 16:8 day:night period and at baseline level (BL = 150 μmol m^{-2} s^{-1}) or at high light (HL = 600 μmol m^{-2} s^{-1}).

Temperature oscillations (dotted line Fig. 1) maintain a day and night temperature at fixed levels according to the experimental settings and the depicted pattern. Three levels for the day temperature are used in the experiments whereas the night temperature is maintained at a value of T = 15 °C for all experiments with a fluctuation temperature. The baseline temperature (BT) was set at 21 °C based on previous data that showed optimized biomass yield on light for baseline temperature. The upper temperature limit proved to show successful growth is set as the high temperature (HT = 25 °C) [15]. Extreme temperature conditions (XT = 30 °C) were proven as unviable growth conditions when

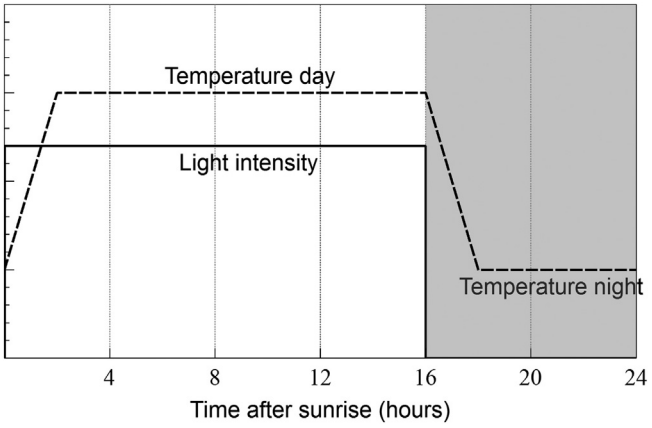

Fig. 1. Schematic representation of the light (continuous line) and temperature (dotted line) oscillations applied during reactor operation. Values for temperature day and Light intensity for each experiment can be found in Table 1. Temperature night is always maintained at 15 °C where applicable.

applied under continuous conditions for *Rhodomonas* sp. [15]. A gradual increase/decrease of temperature between the temperature during the day and night temperature was applied. The temperature transitioning period was kept constant (2 h) for all experimental conditions, regardless of the ΔT between the two temperature level, as depicted in Fig. 1.

2.5. Statistical analysis

Statistical analysis is performed by one-way ANOVA tests and two sample *t*-test with equal or unequal variance with the variance tested using an F-test by Microsoft Excel. The standard error of the mean or standard deviations are shown in all graphs, depending on the total sample size (see figure captions).

3. Results and discussion

3.1. Effect of day:night cycle on biomass yield on light

The results of the measured biomass yield on light are given in Fig. 2. Values represent the average of all days under steady state as measured in duplicate reactors with the standard deviation over all measurements. The biomass yield on light for the baseline experiment with continuous growth conditions for both light and temperature (CBT-CBL) showed a biomass yield on light of 0.87 ± 0.03 g mol^{-1}. This correlates exactly to the expected value with these growth conditions as described in previous work [15]. A day:night cycle for only light, but with a constant temperature (FBL-CBT), only temperature fluctuations but with continuous light (CBL-FBT) or a full day:night cycle for both temperature and light (FBL-FBT) did not show a significant difference for the biomass yield on light compared to the Baseline experiment, CBL-CBT ($P > 0.05$). The biomass yield on light of the full day:night cycle experiment (FBL-FBT) shows a slight but not significant increase from 0.87 ± 0.03 g mol^{-1} to 0.91 ± 0.07 g mol^{-1} ($P > 0.05$). This increase is not significant as a result of the relatively large standard deviation found on the experimental results. The results of these four experiments performed under base light (BL = 150 μmol m^{-2} s^{-1}) and temperature (BT = 21 °C) conditions show no effect of a day:night cycle when operating at the optimum conditions for biomass yield on light. It is suggested that under these light conditions there is no negative effect of the light intensity on the cell division processes or other light sensitive processes in the cell. Therefore, the addition of a dark cycle and the synchronization of the cellular processes does not result in an increase of the biomass yield on light. Literature describes an increased biomass yield on light

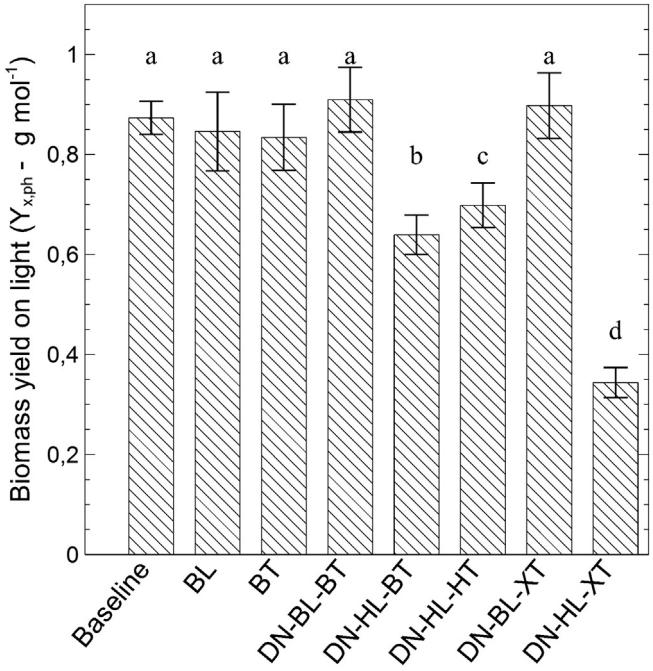

Fig. 2. Biomass yield on light results for *Rhodomonas* sp. grown under different day:night cycle conditions for light and temperature. Values show average value obtained over all days of steady-state from duplicate reactor experiments, Error bars depict standard deviation ($n = 8$ or $n = 10$). Means with the same letter are not significantly different ($P > 0.05$).
X-axis letters describe experimental conditions: C = constant, F = fluctuating in a 16:8 day:night cycle, B = base level, H = high level and X = extreme, L = light and T = temperature.

for both available studies on the effect of a day:night cycle [16,19]. It is unlikely that the baseline with continuous light conditions for the two strains tested (*Acutodesmus obliquus* and *Neochloris oleoabundance*) in literature were performed at the optimized growth conditions for maximum biomass yield on light of the applied strains, as done in this study. A light intensity of 500 μmol m^{-2} s^{-1} was used in both literature studies that showed increased biomass yield on light by the implementation of a day:night cycle [16,19].

Under high light conditions of 600 μmol m^{-2} s^{-1} in experiments FHL-FBT and FHL-FHT, the biomass yield on light is significantly reduced compared to the baseline scenarios for both temperatures studied ($P < 0.05$). Daytime temperature shows a significant effect on the biomass yield on light with the experiment at 25 °C (FHL-FHT) showing a higher biomass yield on light (0.70 ± 0.04 g mol^{-1}) than the experiment at 21 °C (FHL-FBT - 0.64 ± 0.04 g mol^{-1}) ($P < 0.05$). The significantly lower biomass yield on light for experiments with high light conditions (600 μmol m^{-2} s^{-1}) compared to the baseline experiment correlates to the negative effect of increased light intensity on biomass yield on light as described in literature [27]. Compared to previous experiments under continuous conditions for light and temperature performed at similar light and temperature levels, the results of the experiments including a day:night cycle, at high light levels (FHL-experiments) do show an increased biomass yield on light. A biomass yield on light of 0.53 ± 0.01 g mol^{-1} and 0.57 ± 0.01 g mol^{-1} for continuous conditions with equal high light conditions (600 μmol m^{-2} s^{-1}) was reported in a previous study at 20 °C and 25 °C, respectively [15]. In the current work we show a 20% increase of biomass yield on light for the FHL-FBT experiment compared to previous experiments at continuous conditions of 600 μmol m^{-2} s^{-1} and 20 °C. A 22% increase of the biomass yield on light for the FHL-FHT experiment in the present study is achieved compared to continuous conditions of 600 μmol m^{-2} s^{-1} and 25 °C in previous work [15]. These

results show that the biomass yield on light can be significantly improved with the implementation of a day:night cycle under suboptimal conditions such as high light intensities (600 μmol m^{-2} s^{-1}) or suboptimal temperature (25 °C). These results are in line with results in literature that showed 10–15% increase biomass yield on light for *Neochloris oleobundans* [19] and a 20% increase for *Acutodesmus obliquus* [16] when grown under day:night cycle conditions compared to continuous light using light intensities of 500 μmol m^{-2} s^{-1}.

Under extreme temperature conditions for *Rhodomonas* sp. (FXT = 30 °C in the FXT-experiments), the positive effect of day:night cycles on *Rhodomonas* sp. growth under suboptimal conditions is further confirmed. Good reproducible growth was observed at a temperature of 30 °C with oscillations to 15 °C during the dark phase at a light intensity of 150 μmol m^{-2} s^{-1} (FBL-FXT). The biomass yield on light under these conditions is not significantly different from the baseline experiment with 0.89 ± 0.06 g mol^{-1} ($P > 0.05$). Previous experiments showed that continuous light (light levels between 60 and 600 μmol m^{-2} s^{-1}) and a continuous temperature of 30 °C did not result in cell growth for *Rhodomonas* sp. [15]. The similar biomass yield on light of experiment FBL-FXT compared to the FBL-FBT emphasizes the benefits of synchronized cellular processes for microalgae growth under day:night conditions. The detrimental effects of temperature during the daytime are compensated during the dark phase with lower temperatures. The combination of high light (HL = 600 μmol m^{-2} s^{-1}) and the extreme temperature (XT = 30 °C in experiment FHL-FXT) shows that the regenerative capacity of the dark phase is limited. Unstable results were obtained and only one successful experiment with steady state was obtained with a low average biomass yield on light of 0.34 ± 0.03 g mol^{-1}.

The increased biomass yield on light by implementation of a day:night cycle has been described in literature before. Here we show that a positive effect is only present under sub-optimal growth conditions. This indicates that under temperature and light conditions where no detrimental effect of either of these growth parameters is present, no additional gains are obtained by including a day:night cycle. The optimal conditions for biomass yield on light, typically represent conditions with relatively low biomass production rates and these conditions would therefore be uninteresting for large scale microalgae production. Conditions with higher light intensities and therefore higher biomass production rates could benefit from the implementation of a day:night cycle.

3.2. Fatty acid composition

The fatty acid content and composition of the biomass was determined at the end of the steady state, and was measured at t = 16 h after sunrise, the transition moment between light and dark phase. The results are depicted in Fig. 3 where the fatty acid content is divided in four categories: Saturated fatty acids (SFA), Monounsaturated fatty acids (MUFA), Polyunsaturated fatty acids excluding EPA and DHA (PUFA) and EPA + DHA, the total fatty acid content (TFA) is the sum of all four categories.

The high TFA content for FHL-FHT does not fit with the other observed trends. This deviating TFA content is the most likely a stress response to the extreme conditions of light and temperature. As these growth conditions did not result in reproducible growth and only one reactor was sampled. Experiment FHL-FXT is not considered in further fatty acid results.

The TFA content of the experiment with fluctuating temperature and light at baseline conditions (FBL-FBT) is significantly lower ($P < 0.05$) at 76 ± 1 mg gDW^{-1} than the baseline experiment under continuous conditions (CBL-CBT) at 85 ± 1 mg gDW^{-1}. All other experiments with fluctuations in both light and temperature (FHL-FBT, FHL-FHT and FBL-FXT) appear to show a lower TFA content as well but these differences are not significant ($P > 0.05$) compared to the baseline (CBT-CBT) when one single experiment is compared to the

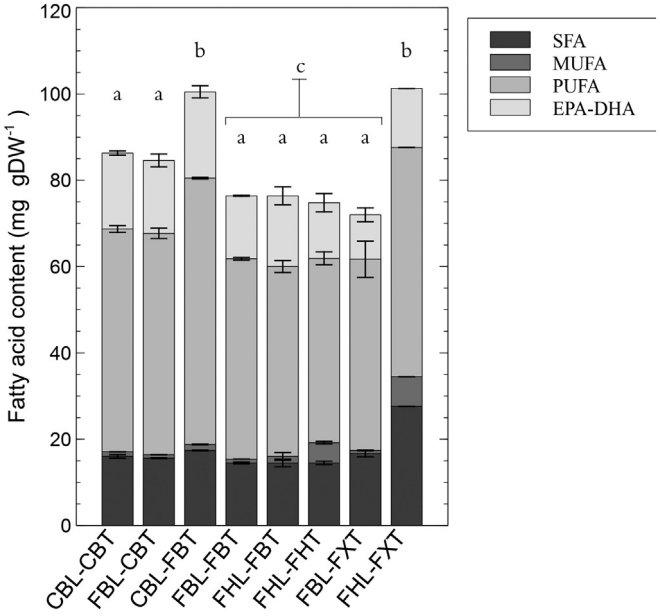

Fig. 3. Fatty acid content of *Rhodomonas* sp. grown under different day:night cycle conditions for temperature and light. Saturated fatty acids (SFA), Monounsaturated fatty acids (MUFA), Polyunsaturated fatty acids excluding EPA and DHA (PUFA) and EPA + DHA. Values are averages over two replicate reactor experiments, error bars depict standard error of the mean (n = 2). Means with the same letter are not significantly different ($P > 0.05$).
X-axis letters describe experimental conditions: C = constant, F = fluctuating in a 16:8 day:night cycle, B = base level, H = high level and X = extreme T = temperature and L = light.

baseline. The significance of these differences was not accurately determined as a result of the relatively large standard deviations and the low number of measurements for each individual experiment (n = 2). Comparing the average TFA content of all these experiments combined (FBL-FBT, FHL-FBT, FHL-FHT and FBL-FXT – n = 8) to the baseline, a significant difference was found (letter c in Fig. 3). A lower average TFA content of 75 ± 5 mg gDW^{-1} is found for the experiments with day:night cycle for both light and temperature compared to a TFA content of 85 ± 1 mg gDW^{-1} of the baseline with continuous conditions ($P < 0.05$ - letter c in Fig. 3). The results of EPA + DHA content show the same trend as found for the TFA. The average EPA + DHA content over all full day:night experiments (14 ± 3 mg gDW^{-1}) is significantly lower ($P > 0.05$) than the baseline (18 ± 1 mg gDW^{-1}). This lower TFA and EPA + DHA content in the full day:night cycle experiments indicates an effect of the fluctuating conditions and is further discussed in Section 3.3.2. A fluctuation in temperature only (CBL-FBT) results in a significant increase of the TFA and EPA + DHA content (P < 0.05). This increase is consistent with previous experiments with continuous conditions where a lowered temperature (15 °C) showed an increased TFA and EPA + DHA content [15]. The relatively large increase of TFA content as found here is higher than expected. The large increase found for the fluctuating temperature only experiment (CBL-FBT) could be the results of daily fluctuations in the cell size and the moment of sampling as described for the full 24 h cycle experiment in Section 3.3.2.

3.3. Daily oscillations

The 24 h-profile of the FHL-FHT experiment (Iph = 600 μmol m^{-2} s^{-1} and T = 25 °C) was determined in detail by sampling every two hours for the duration of 14 h in two reactor systems to represent the full 24 h cycle. A representation of sampling moments in the two reactor systems can be found in the supplementary files.

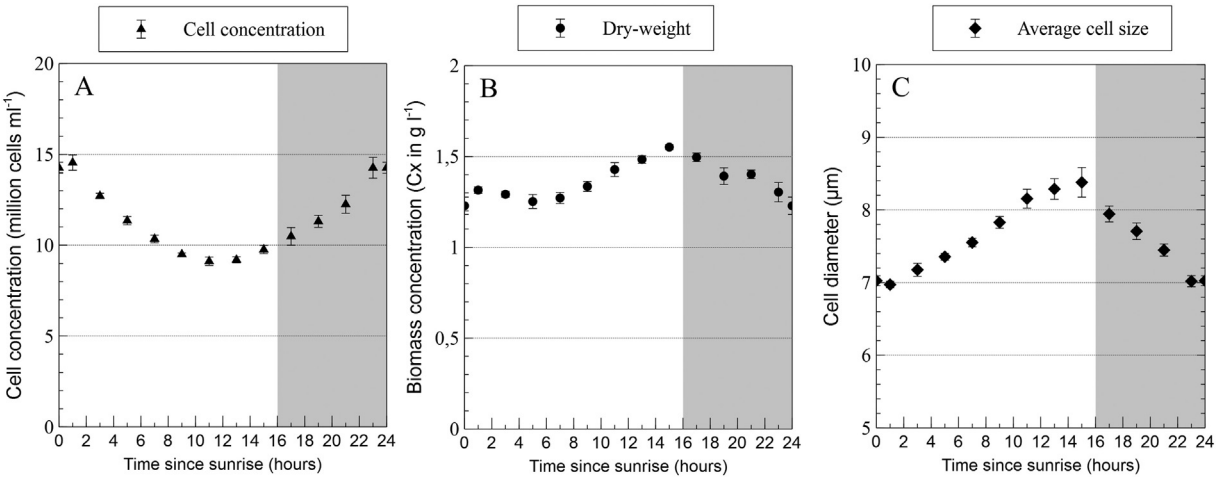

Fig. 4. Daily oscillation of average cell count (A -million cells ml^{-1}), Dry-weight (B) and the average cell size (C in µm). Grey area indicates dark hours. Error bars show standard deviation (4A and 4C $n = 4$, 4B $n = 6$with $n = 8$ in 4A and C and $n = 12$ in 4B for $t = 11$ h and $t = 23$ h).

3.3.1. Biomass concentration

Results of the cell-concentration (cells ml^{-1}) and biomass-concentration (Cx in g l^{-1}) are shown in Fig. 4A and B. Each datapoint represents the average of measurements from 2 separate days in steady-state. The datapoints of t = 11 h and t = 23 h were taken from two reactors and act as control for reproducibility between the reactors and represent data of duplicate measurement for two days of steady-state in two different reactors. The low standard deviation on these two measurements, relative to the other data points, shows that both reactors showed the exact same pattern. The biomass concentration (Fig. 4B) shows a slight fluctuation over the day:night cycle. The highest biomass concentration (1.55 ± 0.01 g l^{-1}) is found at $t = 15$ h followed by a 21% decrease in biomass concentration (to 1.23 ± 0.05 g l^{-1}) during the dark phase. The biomass loss during the night is the result of maintenance and energy used for cell-division. The fluctuating biomass concentration during the light period is the result of the turbidostat settings and changing absorption properties of the biomass (data not shown). During the daily fluctuations the average diameter of a cell increases from 7.03 ± 0.07 µm at $t = 0$ h to 8.38 ± 0.20 µm at t = 15 h after which the cell diameter decreases again during the dark phase (Fig. 4C). The increased average cell diameter during the first 15 lighthours results in a decrease of the cell concentration (cells ml^{-1} - Fig. 4A) and indicates the of cell preparation for cell-division. Similar behaviour of daily fluctuations in cell size has been described in literature for *Acutodesmus obliquus* [16,28]. Cell division occurs during the dark phase where cells decrease in size but increase in number. The oscilations from cell diameter and cell number are comparable to results described for *Acutodesmus obliquus* showing synchronized cell cycles under 16:8 day:night cycles [16].

The oscillation for dry-weight biomass concentration as found for *Rhodomonas* sp. have not been described for other species under similar growth conditions. This oscillation of biomass concentration over a 24 h period could have some implications for the results described in Section 3.1. All experiments were measured at $t = 16$ h, the moment with the highest biomass concentration for *Rhodomonas* sp. under day:night cycles, followed by a loss of biomass during the night. The moment of sampling could result in a slightly overestimated biomass yield on light for day:night cycle experiments as the biomass concentration was assumed to be constant during steady-state.

3.3.2. Fatty acid content and composition

During the 24 h cycle the fatty acid content of the biomass fluctuated (see Fig. 5). The fatty acid composition data shows a clear daily pattern with a maximum TFA content of 94 ± 2 mg gDW^{-1} at $t = 5$ h and minimum of 76 ± 2 mg gDW^{-1} at $t = 13$, 15 and 17 h after sunrise. All four defined fatty acid categories follow the same trend as the TFA content. The fluctuation in TFA content is not the result of fluctuations in one specific category. The TFA content seems to be mostly correlated to the average cell size (Fig. 4C). This indicates that *Rhodomonas* sp. does not utilize fatty acids as energy storage but TFA content is most likely a function of the fraction of membranes of the total biomass. With increased cell size the relative outside surface area to volume of the cells becomes smaller, resulting in a lower biomass content of the components that make up the cell membrane.

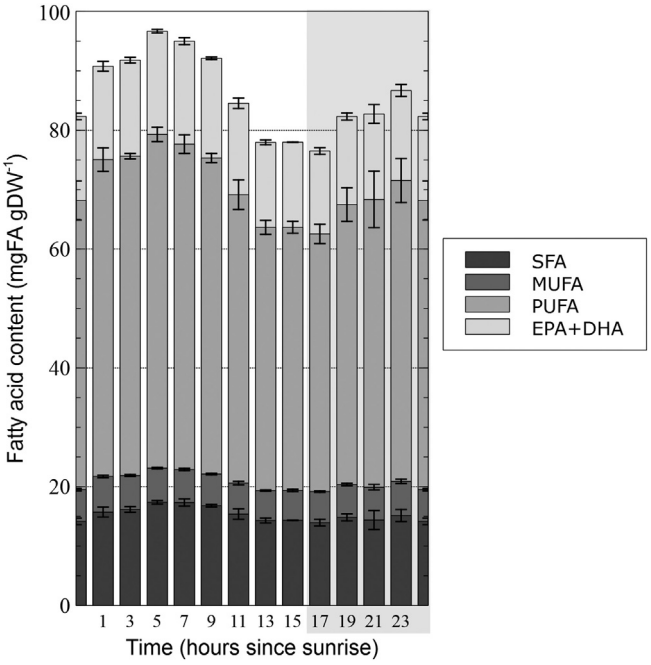

Fig. 5. Fatty acid content of *Rhodomonas* sp. over a full 16:8 day:night cycle. Grey background area indicates the 8 h dark phase. Saturated fatty acids (SFA), Mono unsaturated fatty acids (MUFA), Poly unsaturated fatty acids excluding EPA and DHA (PUFA) and EPA + DHA. Values are averages over duplicate samples taken on 2 separate days during steady-state, error bars depict standard deviation ($n = 4$ for all time points except $t = 11$ h and $t = 23$ h with $n = 8$).

This result suggests that fatty acids of *Rhodomonas* sp. are mainly present in the cell membrane resulting in the correlation between cell size and fatty acid content. This trend is opposite to the trend described by Lacour et al. for *Isochrysis* sp. which showed an increased fatty acid content for larger cells [23]. The EPA + DHA content shows small

deviations from the other fatty acids with the EPA + DHA content decreasing from the maximum content of 16 ± 1 mg gDW^{-1} at t = 24 h (instead of t = 5 h) to 12 ± 0 mg gDW^{-1} at t = 13, 15 and 17 h. Previous experiments under continuous conditions showed an EPA + DHA content of 12 mg gDW^{-1} at continuous conditions for 25 °C and 20 mg gDW^{-1} at 15 °C [15]. The current results correlate with those finding and show that EPA + DHA is mostly affected by temperature. During daytime temperatures (25 °C) the EPA + DHA content is equal to that of continuous growth conditions at this temperature. During the dark phase at 15 °C the EPA + DHA content reaches the highest level but not as high as the content of cell grown under continuous 15 °C conditions.

The daily oscillations show the lowest TFA content at t = 13, 15 and 17 h indicating that the decreased TFA content as found for the day:-night experiments of Section 3.2 are most likely the result of the moment of sampling, as all samples were taken at t = 16 h. The average TFA content of all day:night experiments of 75 ± 5 mgFA gDW^{-1} correlates with the TFA content found at t = 13, 15 and 17 h for the 24 h-cycle at 76 ± 2 mgFA gDW^{-1}. The daily oscillation of TFA content from 91 ± 4 mg gDW^{-1} at t = 5 h to 76 ± 1 mg gDW^{-1} at t = 13–17 h (Fig. 5) is larger than the total deviation found between the baseline experiment (CBL-CBT) and the day:night cycle experiments of Section 3.2 (Fig. 3). This shows that the moment of sampling during a day:night cycle is the most important parameter affecting the TFA content found in the biomass for all described scenarios in this study.

The average cell size of the baseline experiment was 7.68 ± 0.20 μm (data not shown), corresponding to the cells at t = 5 h and t = 7 h in the 24 h cycle experiment, the time with the highest TFA content (91 ± 4 mg gDW^{-1}) during the 24 h-cycle. The TFA content of the baseline experiment with continuous conditions (CBL-CBT) was 86 ± 1 mg gDW^{-1}. This further strengthens the hypothesis that TFA content of *Rhodomonas* cells is mostly linked to the cell size and explains the higher TFA content described for the continuous conditions as found for the CBL-CBT compared to the experiments with combined fluctuating temperature and light. In addition to cells size, the EPA + DHA content is also influenced by temperature. With higher levels of EPA + DHA found at lower temperatures, corresponding with previous experimental data under continuous temperatures [15]. The EPA + DHA concentration fluctuates significantly during a 24 h-cycle, these oscillations are not expected for continuous temperature conditions.

Under continuous production conditions, the TFA and EPA + DHA content can be adjusted with the growth conditions [15]. When *Rhodomonas* sp. is produced under day:night cycles an optimized harvesting strategy could be selected to utilize the daily fluctuation in biomass composition and maximize the nutritional value of the biomass in aqua feed applications. When maximized TFA content is required, the best harvesting moment is found between 5 and 9 h after sunrise. If a maximized EPA + DHA content is required the biomass should be harvested just before sunrise at t = 0 h. This harvesting optimization is not required or possible under continuous production conditions. The implementation of day:night cycle does allow for a higher TFA content or EPA + DHA content on the optimized harvesting moments compared to continuous operation using the same levels of light and temperature. In the case of the FHL-FHT experiment a 19% increase in the TFA content was achieved at the optimized harvesting hours (94.0 ± 1.8 mg gDW^{-1}) compared to continuous operation under the same growth conditions (79.1 mg gDW^{-1}) [15]. When optimizing the harvesting strategy for maximized EPA + DHA content a 13.5% increase (16.1 ± 1.0 mg gDW^{-1}) could be achieved compared to continuous conditions (14.1 mg gDW^{-1}) [15]. If such a strategy would be implemented at industrial scale it is worthwhile investigating if equal improvements are also found under growth conditions with smaller daily fluctuations in temperature and light.

With the implementation of a day:night cycle the total biomass productivity will decrease compared to the same growth conditions under continuous 24 h light per day, with the added benefit of potentially more optimized biomass composition for the selected aqua feed application. Nevertheless, our results show that the implementation of day:night cycle opens opportunities to further optimize the biomass composition in industrial microalgae production scenarios for aquaculture depending on the requirements for each specific application.

4. Conclusion

Rhodomonas sp. was grown under different day:night cycle conditions for light and temperature and compared to a baseline with all growth parameters at continuous levels. The cells showed clear synchronized cell cycles with cell growth during the light hours and cell division in the dark phase. It can be concluded that when growth conditions are maintained close to the optimized settings for biomass yield on light, no effect of a day:night cycle on the biomass yield on light is observed. An improved biomass yield on light was found for growth with a day:night cycle under suboptimal conditions for biomass yield on light with high light intensity (600 μmol m^{-1} s^{-1}) and high temperature (25 °C), compared to the same conditions applied for 24 h day^{-1}. High temperature (30 °C) that did not result in growth under continuous conditions did result in good growth when implementing a day:night cycle, but only at low light levels. The fatty acid content of *Rhodomonas* sp. seems to be correlated to cell size and growth temperature. Implementation of a day:night cycle for *Rhodomonas* sp. production at large scale for aqua feed applications could be optimized towards a specific fatty acid composition based on the time of harvesting. The implementation of day:night cycle can increase the TFA content of the biomass by 19% or the EPA + DHA content by almost 14%, compared to the same levels for light and temperature applied continuously. The moment at which EPA + DHA content is maximal in the biomass (just before sunrise) is different from the moment of the day with the highest TFA content in the biomass (between 5 and 7 h after sunrise) under the example conditions (Iph = 600 μmol m^{-1} s^{-1} and T = 25 °C).

Author contributions

PCO, JH, RHW and MJB conceptualization, Methodology, Supervision, Writing - reviewing and Editing. PCO Investigation, Formal analysis, Validation, Writing – original draft. All authors edited and approved the final manuscript.

Declaration of authors agreement

All authors agreed to the authorship and submission of the manuscript to Algal research for peer review.

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Declaration of competing interest

No conflicts, informed consent, or human or animal rights are applicable to this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2020.102034.

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