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Towards industrial production of microalgae without temperature control: The effect of diel temperature fluctuations on microalgal physiology

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ABSTRACT

Regions that offer high levels of sunlight are ideal to produce microalgae. However, as a result of high light intensities, the temperature in photobioreactors can reach temperatures up to 50 °C. Control of temperature is essential to avoid losses on biomass productivity but should be limited to a minimum to avoid high energy requirements for cooling. Our objective is to develop a production process in which cooling is not required. We studied the behaviour of thermotolerant microalgae *Picochlorum* sp. *(BPE23)* under four diel temperature regimes, with peak temperatures from 30 °C up to a maximum of 47.5 °C. The highest growth rate of 0.17 h^{-1} was obtained when applying a daytime peak temperature of 40 °C. Operating photobioreactors in tropical regions, with a maximal peak temperature of 40 °C, up from 30 °C, reduces microalgae production costs by 26.2 %, based on simulations with a pre-existing techno-economic model. Cell pigmentation was downregulated under increasingly stressful temperatures. The fatty acid composition of cell membranes was altered under increasing temperatures to contain shorter fatty acids with a higher level of saturation. Our findings show that the level of temperature control impacts the biomass yield and composition of the microalgae.

1. Introduction

Microalgae are photosynthetic microorganisms that can be grown using sunlight as an energy source. These microalgae contain high levels of protein, lipids, pigments and carbohydrates and are therefore interesting for the production of a wide range of products such as food, feed, biofuels, cosmetics and bioplastics (Mulders et al., 2014; Wijffels et al., 2010). Stable climate and high light intensities are favourable conditions to achieve yearly high productivities (Ruiz et al., 2016). Cultivation of microalgae is best done in photobioreactors, where high process control, high cell densities and high productivities can be obtained (Ruiz et al., 2016; Vree et al., 2015; Wijffels and Barbosa, 2010). However, the disadvantages of such systems compared to traditional open ponds are higher capital and operational costs, and the absence of natural cooling through water evaporation. As a result of exposure to high light intensities, the culture temperature in a photobioreactor can increase up to 50 °C (Bechet et al., 2010; Bleeke et al., 2014). This exceeds the optimum temperature of most industrial microalgae, which is between 20 °C and 30 °C (Mata et al., 2010; Ras et al., 2013). As a consequence, this will result in losses of productivity or even culture collapse. While mechanical cooling, shading or spraying of water can offer a solution, applying these cooling methods should be avoided as much as possible due to their negative economic and environmental impact (Ruiz et al., 2016). These high temperatures only occur for a few hours each day, at midday. However, this still affects most microalgae severely. Microalgal species that tolerate and remain productive under such high temperatures can significantly reduce process costs. If species are able to grow optimally at a diel peak temperature of 40 °C instead of 30 °C then production costs decrease by 26.2 %, while a peak temperature of 45 °C would reduce production costs by 28.4 %, as calculated by the techno-economic model of Ruiz et al., using climatological conditions as found in Curacao (Ruiz et al., 2016).

At peak temperatures exceeding the microalgae's lethal growth temperature, microalgal cells will die quickly due to denaturation, dissociation, and destabilisation of nucleic acid structures, proteins, and cell membranes (Kobayashi et al., 2014; Ras et al., 2013). Also temperatures below the lethal, but above the optimal growth temperature cause cell stress. To survive, cells have to rebalance their composition. Membrane fluidity is elevated at higher temperatures, which is rebalanced by alterations in sterol composition and by fatty acid remodelling. Higher saturation levels are favourable at increased temperature (Los et al., 2013; Renaud et al., 2002). Photosynthesis is impaired

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severely as temperatures past the optimum growth temperature unbalance the energy equilibrium within the cell. Excessive energy which cannot be transferred into the photoelectron transfer chain is dissipated by which reactive oxygen species (ROS) are formed (Markou and Nerantzis, 2013). ROS are highly reactive and cause damage to DNA, proteins and membrane lipids. As a response, photoprotective pigments are produced to filter light, to support in non-photochemical quenching and to scavenge ROS (Demmig-Adams and Adams, 2000). Examples of these photoprotective pigments that can be found in green algae are lutein, carotene and the xanthophyll cycle pigments: zeaxanthin, antheraxanthin and violaxanthin (Mulders et al., 2014). The mentioned adaptation mechanisms protect against changes in environmental conditions both throughout seasons and during shorter periods (Dimier et al., 2007; Leya et al., 2009; Minhas et al., 2016).

The temperature in a photobioreactor fluctuates if no cooling is applied (Gaidarenko et al., 2019; Ras et al., 2013; Srirangan et al., 2015; Tamburic et al., 2014; Willette et al., 2018). However, most studies done in the field of microalgae cultivation are done under stable temperatures (Converti et al., 2009; Ras et al., 2013; Renaud et al., 2002). A stable temperature does not reflect the situation in photobioreactors at industrial scale. To understand the physiology of microalgae in representative industrial settings, a fluctuating diel temperature regime should be used. Research on the effect of diel, above optimal temperature fluctuations on microalgae growth and composition are therefore very relevant to both academia and industry. The objective of this study is to develop a microalgal production process in which photobioreactor cooling can be greatly reduced or avoided, in order to improve the sustainability and economics of the process. In this research paper, we study the response of the green temperature-tolerant microalgae strain *Picochlorum* sp. *(BPE23)* to diel fluctuating temperatures (Henley et al., 2004). The interest and number of studies on *Picochlorum* sp. species have recently increased due to their robustness and tolerance for high light, temperature and salinity levels, also in combination with fluctuating conditions (Krasovec et al., 2018; Krishnan et al., 2021; Mucko et al., 2020; Weissman et al., 2018). *Picochlorum* sp. *(BPE23)* was selected for our study because of its high growth rate in combination with its thermotolerance (Barten et al., 2020). To the authors' knowledge, this is the first study in which Picochlorum is subjected to simulated industrial growth conditions with a diel temperature profile. To compare different temperature scenarios, four diurnal temperature cycles with maximal peak temperatures of 30 °C, 40 °C, 45 °C and 47.5 °C during daytime and a constant 30 °C during night-time were studied in highly controlled flat panel photobioreactors. These temperature profiles were based on temperatures that can be found in photobioreactors on Bonaire, as a reference for regions that harbour similar climatological conditions, since these are favourable for microalgae growth (Ruiz et al., 2016). This study provides insight on the physiological response of the green microalgae *Picochlorum* sp. *(BPE23)* to diel temperature cycles in a photobioreactor production process, up to lethal temperatures.

2. Materials and methods

2.1. Microalgae cultivation

2.1.1. Microalgae and growth medium

The green microalga *Picochlorum* sp. *(BPE23)*, belonging to the phylum *Chlorophyceae*, was used for this research (Barten et al., 2020). For photobioreactor cultivation, a filter-sterilized growth medium was created using artificial seawater, enriched with 20 mL of nutrient solution and 1 mL of trace element solution per kilogram of growth medium. The artificial seawater was created using 24.5 $g.l^{-1}$ NaCl, 3.2 $g.l^{-1}$ Na_2SO_4, 0.8 $g.l^{-1}$ $CaCl_2{\cdot}2H_2O$, 0.85 $g.l^{-1}$ K_2SO_4 and 9.8 $g.l^{-1}$ $MgCl_2{\cdot}6H_2O$. The nutrient solution contained 106 $g.l^{-1}$ $NaNO_3$, 11.5 $g.l^{-1}$ KH_2PO_4 and 3 $g.l^{-1}$ $Na_2EDTA{\cdot}2H_2O$. The trace element solution contained 45 $g.l^{-1}$ $Na_2EDTA{\cdot}2H_2O$, 30 $g.l^{-1}$ $FeSO_4{\cdot}7H_2O$, 1.71 $g.l^{-1}$ $MnCl_2{\cdot}2H_2O$, 0.66 $g.l^{-1}$ $ZnSO_4{\cdot}7H_2O$, 0.07 $g.l^{-1}$ $Co(NO_3)_2{\cdot}6H_2O$, 0.02 $g.l^{-1}$ $CuSO_4{\cdot}5H_2O$ and 0.24 $g.l^{-1}$ $Na_2MoO_4{\cdot}2H_2O$. During inoculum preparation, HEPES buffer was added at a final concentration of 4.77 $g.l^{-1}$. The pH of the growth medium was set to 7.4 both for inoculum and photobioreactor cultivation.

2.1.2. Inoculum preparation and photobioreactor inoculation

The inoculum for all experiments was prepared in 250 mL erlenmeyers with a liquid volume of 100 mL. Light was set at ~100 $\mu mol.m^{-2}.s^{-1}$. The CO_2 level in the atmosphere was regulated at 2% and the relative humidity at 60 %. A day/night cycle of 12/12 h was applied with 40 °C during daytime and 30 °C during night-time.

The flat panel photobioreactor was inoculated with microalgae biomass at a concentration of 0.1 $g.l^{-1}$. Bicarbonate was added at a concentration of 0.168 $g.l^{-1}$ at the moment of inoculation to ensure sufficient carbon. To avoid light stress during the initial batch growth phase, the light level was set at 200 $\mu mol.m^{-2}.s^{-1}$ and increased periodically with steps of 200 $\mu mol.m^{-2}.s^{-1}$ until a level of 813 $\mu mol.m^{-2}.s^{-1}$ was reached after two days. The bioreactor was operated in batch mode until an outgoing light level of 35 $\mu mol.m^{-2}.s^{-1}$ (PAR) was reached, after which photobioreactor dilution was started.

2.1.3. Photobioreactor setup and experimental conditions

Cell cultivation experiments were done in heat sterilized flat-panel photobioreactors with a 1.8 L working volume, a 20.7 mm light path and a 0.08 m^2 surface area for irradiation (Labfors 5 lx, Infors HT, Switzerland). A gas mixture was sparged at the bottom of the flat panel photobioreactor, consisting of 480 mL min^{-1} fresh air and 500 mL min^{-1} recycled gas. Extra CO_2 was added to the gas stream on-demand with a maximum flow rate of 25 mL min^{-1} to compensate for pH changes as a result of CO_2 and NO^{-3} consumption. Illumination was done from one side by 260 warm white LED lights with a spectrum of 450−620 nm. The incoming light level was set constant at 813 $\mu mol.m^{-2}.s^{-1}$ under a 12/12 h/h day/night cycle. The outgoing light level was maintained at 35 $\mu mol.m^{-2}.s^{-1}$ (PAR) by light-controlled dilution of the cell culture (turbidostat mode). The culture dilution rate was determined by logging the feed addition.

Temperature was controlled in a sinusoid pattern during day time to mimic outdoor temperature fluctuations as can be found near the equator. Four peak temperatures were applied: 30 °C, 40 °C, 45 °C, and 47.5 °C. These are displayed in Fig. 1 as measured in the photobioreactor. The temperature during night time was kept constant at 30 °C.

2.1.4. Sampling regime

Microalgae were grown until a cycling steady state was reached. Cultures were determined to be in a cycling steady state when the deviation between days, for all measured on-line and off-line parameters was less than 15 % for a period of at least 3 days. In the situation where peak temperatures of 45 °C and 47.5 °C were applied, a period of adaptation and stabilization of several weeks was required before a cycling steady state situation was reached. Once cultures reached this state, samples were taken for at least two days (n = 2). Two flat panel photobioreactors were operated with a shifted time-schedule which differed 12 h. Sampling was done every two hours for a period of 14 h. The data of the two different algae growth schedules were connected and overlaid to allow for an analysis of 24 h of cultivation, which represents a full day of growth.

2.2. Offline measurements

2.2.1. Biomass concentration

The biomass concentration (in $g.l^{-1}$) was measured in duplicate by dry weight determination. Empty Whatman glass microfiber filters (θ 55 mm, pore size 0.7 μm) were dried overnight at 95 °C and placed in a desiccator for 2 h. Filters were then weighed and placed in the mild vacuum filtration setup. Cell culture containing 1–10 mg of microalgae

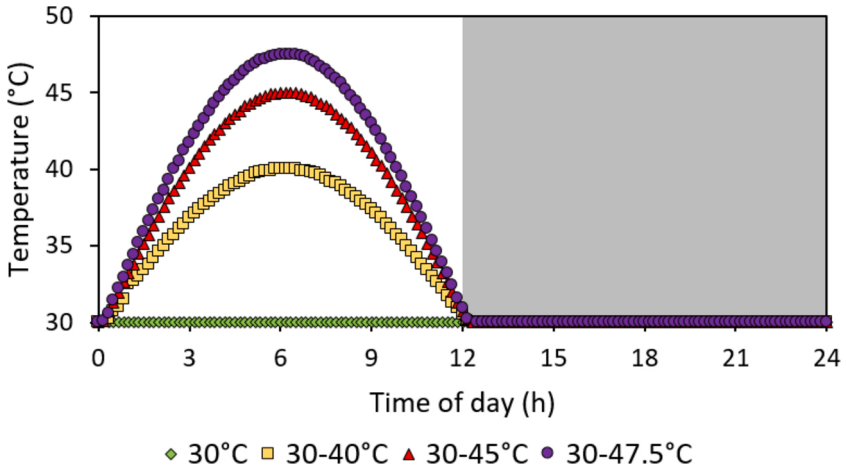

Fig. 1. The temperature and light profile as measured in the flat panel photobioreactor during microalgae cultivation. The night and the peak culture temperature, found 6 h after 'sunrise' are displayed in the legend. The x axis displays hours after 'sunrise', with day and night time indicated in white and grey background, respectively.

biomass was diluted in 25 mL 0.5 M ammonium formate and filtered. The filter was washed twice with 25 mL 0.5 M ammonium formate to remove residual salts. The wet filter was dried overnight at 95 °C, placed in a desiccator for 2 h, and weighed. Biomass concentration was calculated from the difference in filter weight before and after filtration and drying.

2.2.2. Cell size and cell number

Cell size and cell number were determined in duplicate with the Multisizer III (Beckman Coulter Inc., USA, 50 μm aperture). Samples were diluted in two steps before analysis, initially by dilution of 5x in fresh medium, followed by dilution of 100x in Coulter Isoton II.

2.2.3. Quantum yield

The cell culture's quantum yield (F_v/F_m), representing the maximum photosynthetic capacity of photosystem II was determined. Cells were measured after dark adaption at room temperature for 15 min (Aqua-Pen-C 100, PSI; excitation light 455 nm (blue), saturating light pulse: 3000 $\mu mol.m^{-2}.s^{-1}$).

2.3. Compositional analysis

2.3.1. Harvest and lyophilising

Biomass samples for compositional analysis were taken every 2 h, at the same moment as when offline measurements were performed. Microalgae cells were pelleted by centrifugation at 4000 *g* for 5 min and washed with 0.5 M ammonium formate. The centrifugation/washing cycle was repeated twice more after which the cell pellet was frozen at −20 °C. Samples were then lyophilised for 24 h and stored at −20 °C until further processing.

2.3.2. Pigment analysis

Pigment extraction was done using 10 mg of lyophilized biomass. Cells were disrupted by bead beating (Precellys 24, Bertin Technologies, France) at 5000 rpm for three cycles of 90 s with 60 s breaks on ice between each cycle. The extraction was done through five washing steps with methanol containing 0.1 % butylhydroxytoluene. Separation, identification and quantification of pigments were performed using a Shimadzu (U)HPLC system (Nexera X2, Shimadzu, Japan), equipped with a pump, degasser, oven (25 °C), autosampler, and photodiode array (PDA) detector. Separation of pigments was achieved using a YMC Carotenoid C30 column (250 × 4.6 mm 5 μm ID) coupled to a YMC C30 guard column (20 × 4 mm, 5 μm ID)(YMC, Japan) at 25 °C with a flow rate of 1 $mL.min^{-1}$. A sample injection volume of 20 μL was used. The mobile phases consisted of Methanol (A), water/methanol (20/80 (v/v %)) containing 0.2 % ammonium acetate (B), tert-methyl butyl ether (C) (all solvents were purchased at Sigma Aldrich). The elution protocol started with 0−12 min isocratic A:95 % B:5% C:0%, with at 12 min a step to A:80 %, B:5%, C:15 %, followed by a linear gradient 12−30 min to A:30 %, B:5%, C:65 %, finally followed by a conditioning phase 30−40 min at the initial concentration. Analytical HPLC standards for Antheraxanthin, Chlorophyll- a, Chlorophyll-b, Lutein, Violaxanthin, Zeaxanthin had a purity of >99 % (Carotenature, Switzerland).

2.3.3. Fatty acids analysis

Fatty acids within the triacylglycerol (TAG) and polar lipids (PL) fraction were quantified through GC-FID analysis (Breuer et al., 2013). 10 mg of lyophilised biomass was disrupted by bead beating. The fatty acids were extracted from the disrupted biomass in a mixture of chloroform/methanol (1:1.25, v:v) containing Glyceryl tripentadecanoate (C19:0 TAG) (T4257, Sigma-Aldrich) and 1,2-didecanoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt) C10:0 PG (840434, Avanti Polar Lipids Inc) as internal standards for the TAG and the PL fraction, respectively. Separation of TAG and PL was done by the use of Sep-Pak Vac silica cartridge (6 cc, 1000 mg; Waters). TAGs were eluted from the column with a solution of hexane:diethylether (7:1, v:v) and the PL were eluted with a solution of methanol:acetone:hexane (2:2:1, v:v:v). The extracts were methylated for 3 h at 70 °C in methanol containing 5% H_2SO_4.

2.4. Data and statistical analysis

The error of the cell diameter, cell density, dry weight, quantum yield data, displayed per hour, was calculated using the average deviation of both technical and biological duplicate measurements. The error on the reactor dilution rate data per hour was calculated using the standard deviation, while the dilution rate data per day was calculated by propagation of errors as calculated per hour through the standard deviation. The error for the fatty acid groups and pigment species, as displayed as an average per growth condition, was calculated by the standard deviation of eight (fatty acids) and fourteen (pigments) samples which were taken throughout the day from biological duplicates. The error on the total average fatty acid content over the entire day was calculated by propagation of errors through the error as calculated by the standard deviation for each of the fatty acid groups.

3. Results and discussion

3.1. Growth and cell size

Diurnal growth conditions were applied to the microalgae culture with 4 different peak temperatures as shown in Fig. 1. These temperature profiles were selected to mimic temperature conditions as found in outdoor photobioreactors placed under Caribbean climatological conditions. Cell density was maintained at a stable level through adaptive dilution based on light transmission through the cell culture (turbidostat mode).

The measured dilution rates throughout the day are displayed as average per day in Table 1 and per hour in Fig. 2. The culture grown at a stable temperature of 30 °C shows a daily dilution rate of 1.05 ± 0.02 d^{-1} with a peak growth rate of 0.13 h^{-1} at 6 h after sunrise. The maximum growth rate was found when a diel oscillation between 30 °C and 40 °C was applied. In this scenario, the peak in growth (0.17 h^{-1}) was found 6 h after sunrise, in similarity to the experiment under a stable temperature of 30 °C. The daily growth rate was 1.22 ± 0.06 d^{-1}. The growth rate of these two cultures follow a sinusoid pattern while the cultures grown with diel peak temperatures of 45 °C and 47.5 °C clearly show different profiles. These cultures show a decrease in growth rate during midday, which becomes more severe as temperature increases. The daily dilution rate was found to be respectively, 0.61 ± 0.03 d^{-1} and 0.28 ± 0.02 d^{-1} at 45 °C and 47.5 °C. With 47.5 °C being the maximum peak day-temperature at which *Picochlorum* sp. *(BPE23)* showed active growth, as cells died when a diel peak temperature of 48 °C was applied (data not shown). While *Picochlorum* sp. *(BPE23)* was able to grow when a diel peak temperature of 47.5 °C was applied, the productivity was found with a diel peak temperature of 40 °C. Allowing an increased maximum temperature during the operation of a photobioreactor located in tropical regions, can lead to a cost reduction of 26.2 %, based on techno-economic model simulations using the model as described by (Ruiz et al., 2016). For these model simulations Bonaire was chosen as model location for light and temperature, seawater at a temperature of 27 °C was chosen as cooling medium, and a photosynthetic efficiency of 2.6 % was assumed. The ability to grow optimally at 40 °C is a major improvement compared to other industrially applied species which commonly exhibit optimal growth temperatures of 20−30 °C (Ras et al., 2013).

The sinusoid trend observed for the dilution rate throughout the day found for two of the cultures (Fig. 2) is not solely due to changing temperatures as this trend is also observed with the stable temperature of 30 °C (Fig. 2). All cultures show a slight delay before dilution starts directly after sunrise, due to the need to recover from nightly biomass losses associated to cell maintenance (Fig. 4a) (Edmundson and Huesemann, 2015). Interestingly, the dilution rate declines from midday to sunset despite light being supplied at a continuous level throughout the 12 h daytime. It is thought that the cell cycle plays a role in the observed growth pattern, as is shown by cell size (Fig. 3) (de Winter et al., 2017).

An increase in cell size is observed from sunrise to sunset (Fig. 3a). Cell division was initiated around sunset as indicated by the rapid decrease in cell size. Cell division of the culture was concluded within 8 h after the first cell division event was observed, based on the increase in cell number (Fig. 3b). For the culture grown at the diel cycle of 30−40 °C, the onset of cell division was observed sooner than for the other cultures. The cell division took place at a cell diameter 3.2 µm which is the same size at which cell division occurred in the culture grown at the diel cycle of 30 °C. Cultures grown under non-optimal conditions such as 30−45 °C and 30−47.5 °C, show reduced increase in cell size during the day. Furthermore, these cultures showed less cell division events during the night. Interestingly, the cell size continued to increase at a steady rate throughout daytime, even at a stressful peak temperature of 47.5 °C. This contradicts the observations for the reactor dilution rate in which a reduction in growth was observed from 3 to 9 h after sunrise. This continuing cell size increase is partly explained by the accumulation of storage compounds such as fatty acids (Fig. 7). In addition, It was found that due to depigmentation of microalgae biomass the concentration of microalgae (in g.L^{-1}) within the photobioreactor kept growing despite the halted reactor dilution (Figs. 4a and 5).

A decrease in biomass concentration was observed in the night for every culture which is accounted for by respiration and maintenance requirements of the culture (Fig. 4a). Based on literature, the rate of biomass decrease during night was expected to increase with increasing growth rate during daytime, and thus when the cells have a more active metabolism (Edmundson and Huesemann, 2015). However, this was not observed in our data, in which the decline in biomass is the same for each condition. During daytime the biomass concentration remains stable between 0.8–1.0 g.l^{-1} for all cultures, except for the culture grown with a peak temperature of 47.5 °C in which the biomass concentration was 1.34 g.l^{-1}. This was caused by depigmentation of the cell due to temperature stress. This influenced the turbidostat control of the flat panel photobioreactor. Species of *Picochlorum* can rapidly adapt their photosystems to modulate the photon uptake rate (Dimier et al., 2007). Under stressful temperatures as seen in this experiment, *Picochlorum* sp. *(BPE23)* downregulated its photon uptake capacity accordingly, meaning that higher biomass concentrations were required to maintain the absorbed light level constant at 35 µmol.m^{-2}.s^{-1}.

Quantum yield, as a measure for the efficiency with which light energy is converted into assimilated carbon, was measured throughout the day (Table 1, Fig. 4b). This analysis can serve as a qualitative indicator for cell health. We generally observe that quantum yield values for non-stressed cultures of *Picochlorum* range between 0.7 to 0.75. The algae grown at 30 °C exhibited an average quantum yield of 0.73 ± 0.01. The algae cultures grown at diel cycles of 30−40 °C and 30−45° showed an increasing trend that continued until the peak temperature at midday was reached. While the culture grown at the diel cycle of 30−47.5 °C showed a decrease, 5 h after sunrise, when a temperature of 46 °C was reached.

Table 1
Average dilution rate (d^{-1}) and quantum yield (Fv/Fm) of the cell cultures at different diel temperatures. Data corresponds to the average ± SD of 24 h of data acquisition (N = 2).

Diel temperature (°C)	Dilution rate (d^{-1})	Quantum yield (Fv/Fm)
30	1.05 ± 0.02	0.73 ± 0.01
30−40	1.22 ± 0.06	0.70 ± 0.02
30−45	0.61 ± 0.03	0.68 ± 0.02
30−47.5	0.29 ± 0.02	0.67 ± 0.02

3.2. Pigment composition

The role of pigments in cells is diverse. Their role in photosynthesis involves light harvesting, photoprotection, ROS scavenging, excess energy dissipation, and structure stabilization (Frank and Cogdell, 1996; Mulders et al., 2014). In addition, each pigment can fulfil multiple functions within the cell. Primary and secondary carotenoids synthesis is shown to be affected by exposure to temperature stress conditions, and significantly enhanced by the presence of ROS (Markou and Nerantzis, 2013). The concentration of six pigments present in the microalgae was measured throughout the day for all four experimental conditions (Fig. 5).

Overall, pigment concentrations were highest for microalgae grown at 30−40 °C. The cells showed decreasing levels of chlorophyll when diel peak temperatures increased up to 45 °C and 47.5 °C, but also when a stable temperature of 30 °C was applied. Modification of the pigment composition is often seen during adaptation to new environmental conditions (Dimier et al., 2007; Leya et al., 2009; Minhas et al., 2016). When the growth rate decreases as a result of non-optimal growth temperatures, the cell is unable to utilize all the energy that is absorbed by the photosystems. Primary light-harvesting pigments are

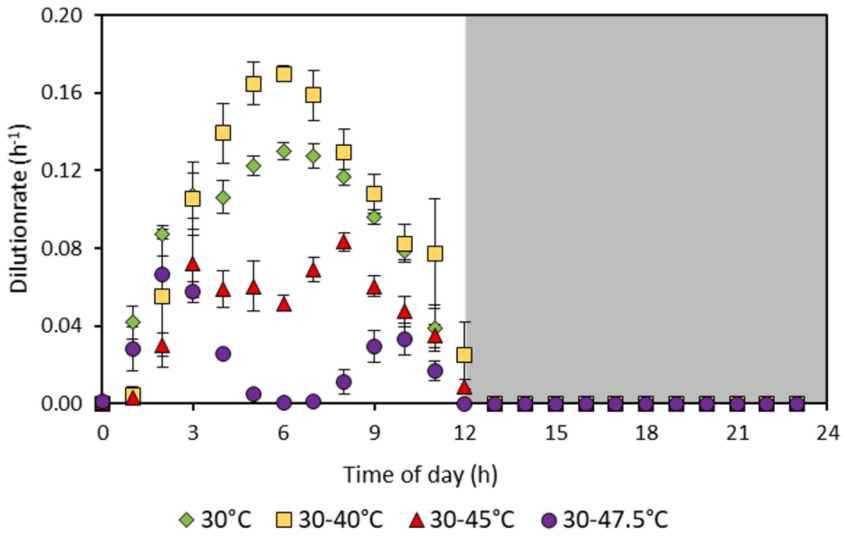

Fig. 2. Measured dilution rates (h^{-1}) of the microalgal cell culture. The night and the peak culture temperature, 6 h after 'sunrise' are displayed in the legend. The x axis displays hours after 'sunrise', with day and night periods indicated in white and grey background, respectively.

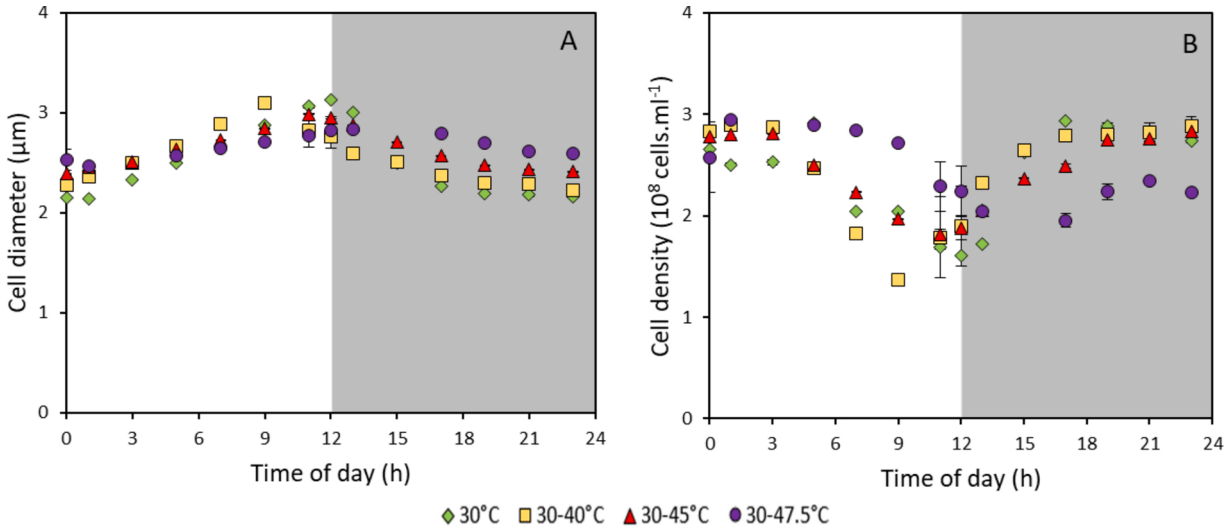

Fig. 3. cell diameter (a) and cell density values (b) over time in the microalgal cell culture during 24 h of cultivation. The night and the peak culture temperature, found 6 h after 'sunrise' are displayed in the legend. The x axis displays hours after 'sunrise', with day and night periods indicated in white and grey background, respectively. Data is composed of the average ± average deviation of both biological and technical duplicate measurements.

downregulated to prevent cell damage as a result of an energy overflow within the metabolism. Excess energy within the metabolism results in the formation of harmful ROS which is neutralised by scavenging compounds with an antioxidant function (Jahns and Holzwarth, 2012). Three of these compounds present in *Picochlorum* sp. *(BPE23)* are the xanthophyll cycle pigments violaxanthin, antheraxanthin and zeaxanthin. With zeaxanthin being the most efficient ROS scavenger, followed by antheraxanthin and violaxanthin, respectively. The xanthophyll cycle serves as an important short term stress acclimation mechanism in higher plants, while its importance varies significantly amongst algae species (Masojidek et al., 2004). As stressful temperatures have a disturbing effect on the electron transport chain, oversaturation of the photosystem likely caused excess energy in the microalgae cells grown at 30−47.5 °C (Mulders et al., 2014). Increased concentrations of all xanthophyll cycle pigments, but especially zeaxanthin were expected to protect against cell damage (Leya et al., 2009; Minhas et al., 2016). However, the opposite was observed (Fig. 5), all measured pigments, including the pigments which are known as strong ROS scavengers, showed lower levels when stress conditions increased. The xanthophyll cycle appears to have an insignificant role in protecting against stressful diurnal temperature cycles in *Picochlorum* sp. *(BPE23)*, in similarity with other green microalgae species (Chlorophyceae) such as *Chlorella*, *Scenedesmus* and *Haematococcus* (Masojidek et al., 2004). These pigments must, therefore, have a different function. The most obvious being involvement in light-harvesting complexes, which are hypothesised to be downregulated to decrease the energy inflow into the disrupted metabolism based on Dimier et al. and Polle et al. (Dimier et al., 2007; Polle et al., 2002). This hypothesis is strengthened by the fact that the ratio of chlorophyll per zeaxanthin is stable at 1.5 ± 0.05 under all temperature conditions (Demmig-Adams and Adams, 2000). Two exceptions on this decrease in pigment concentration are lutein and violaxanthin which are found at constant levels throughout all experimental conditions. These pigments must therefore fulfil a function other than light-harvesting, such as light-filtering or as an antioxidant (Mulders et al., 2014).

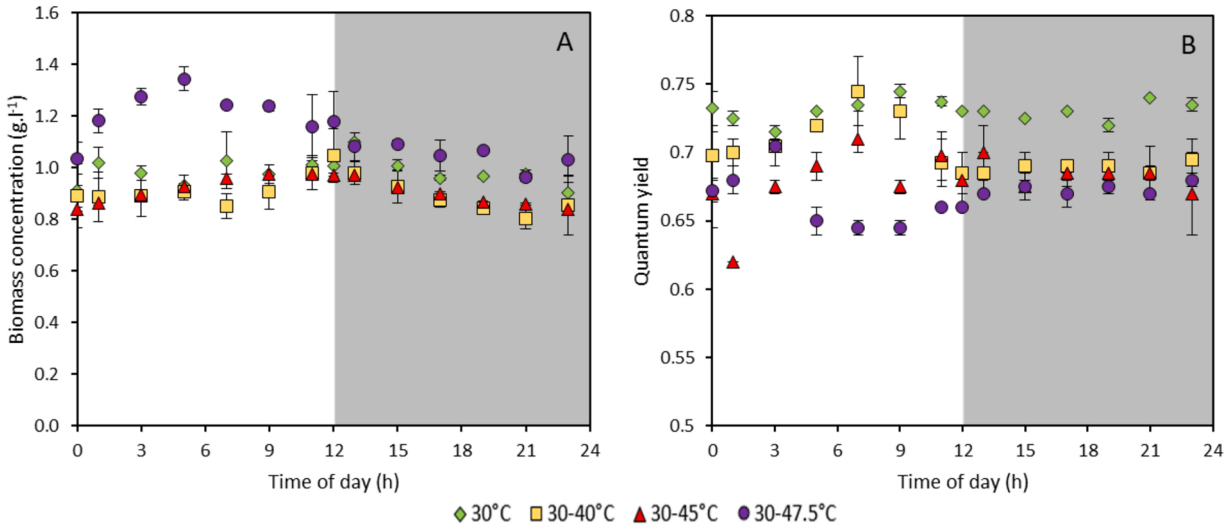

Fig. 4. Dry weight (a) and Quantum yield (b) values of the microalgal cell culture during 24 h of cultivation. The night and the peak culture temperature, found 6 h after 'sunrise' are displayed in the legend. The x axis displays hours after 'sunrise', with day and night periods indicated in white and grey background, respectively. Data is composed of the average ± average deviation of both biological and technical duplicate measurements.

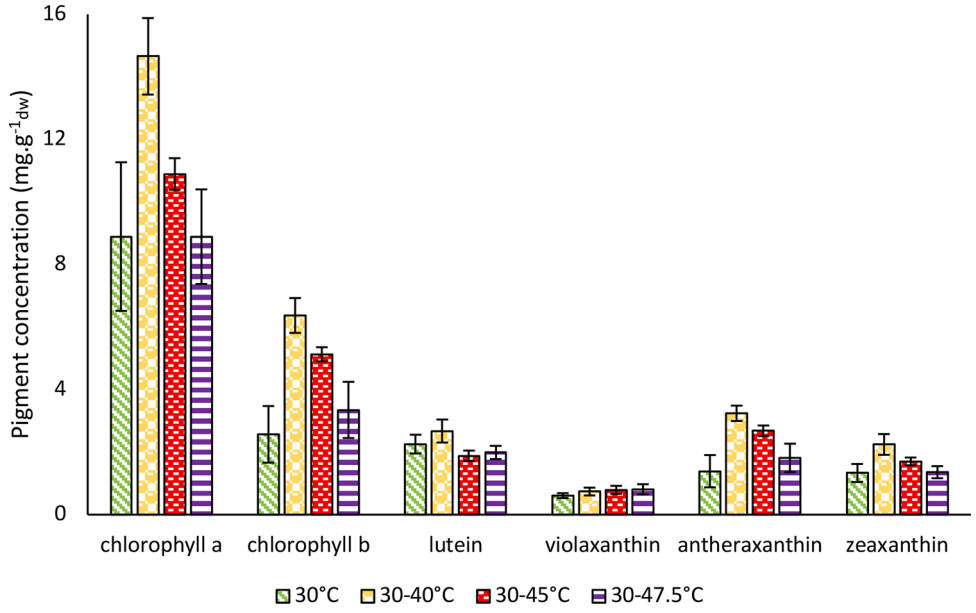

Fig. 5. Average pigment concentration of microalgae cells throughout the day under different temperature conditions. Every point represents the average of 14 samples measured throughout the day which were obtained as biological duplicate. The night and the peak culture temperature, found 6 h after 'sunrise' are given in the legend.

3.3. Fatty acid composition

Fatty acid levels of the polar lipid fraction (PL) and triacylglycerol fraction (TAG) were measured throughout the day and presented as averaged content due to the absence of apparent trends within a cultivation day (Fig. 6). The total lipid content was found highest for 30−45 °C, at 186 mg.g^{-1}. The lipid content at 30−47.5 °C was 162 mg.g^{-1} and the content at 30 °C and 30−40 °C was 149 and 152 mg.g^{-1}, respectively. Lipids in the polar lipid fraction show a decrease with increasing temperature. TAG is found at the highest concentration at 30−45 °C.

The content of C16:0, C16:3 and C18:3 in the polar lipid fraction decreases largely with increasing temperatures (Fig. 7a). While C16:2 and C18:2 fractions show an increase. This increase in double unsaturated fatty acids is at the cost of triple unsaturated fatty acids. Comparable trends are found in literature, in which most species, including species of *Nannochloropsis*, *Rhodomonas* and *Isocrysis*, show increasing fatty acid saturation with increasing temperature (Aussant et al., 2018; Renaud et al., 2002). Changes in temperature may have a disruptive effect on the stability of the cell membrane due to changes in membrane fluidity. Remodelling of cell membrane components such as fatty acids and sterols is done to counteract this effect. With increasing fluidity, the level of saturated fatty acids in the cell membrane was upregulated at the cost of unsaturated fatty acids to maximize hydrophobic interactions, restoring membrane stability (Falcone et al., 2004).

In the TAG fraction, the total amount of fatty acids increases with increasing temperature (Fig. 7b). Indicating a larger reserve of lipids under more stressful conditions. This is mainly due to an increase of the fatty acids C16:0, C16:2 and C18:2. The other fatty acid groups show a comparable pattern, at a lower concentration. The effect of temperature on the level of TAG differs from species to species, some species show an

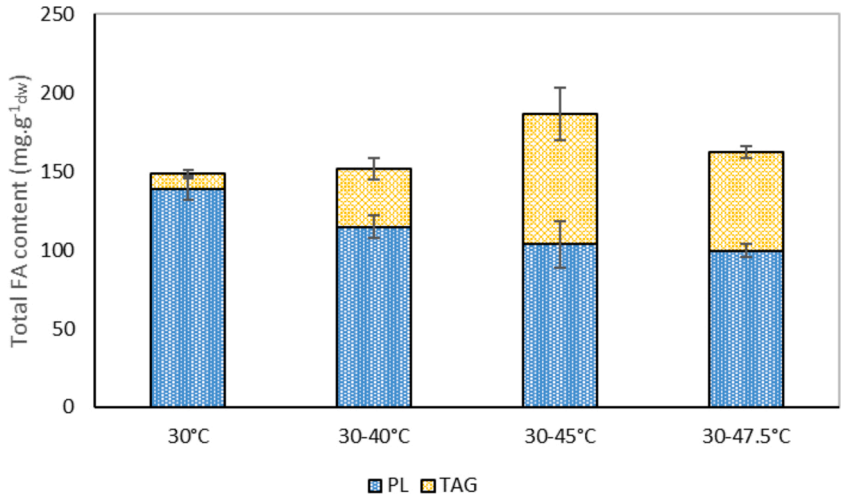

Fig. 6. Polar lipid fraction (PL) and Triacylglycerol fraction (TAG) content in the biomass of *Picochlorum* sp. *(BPE23)*.

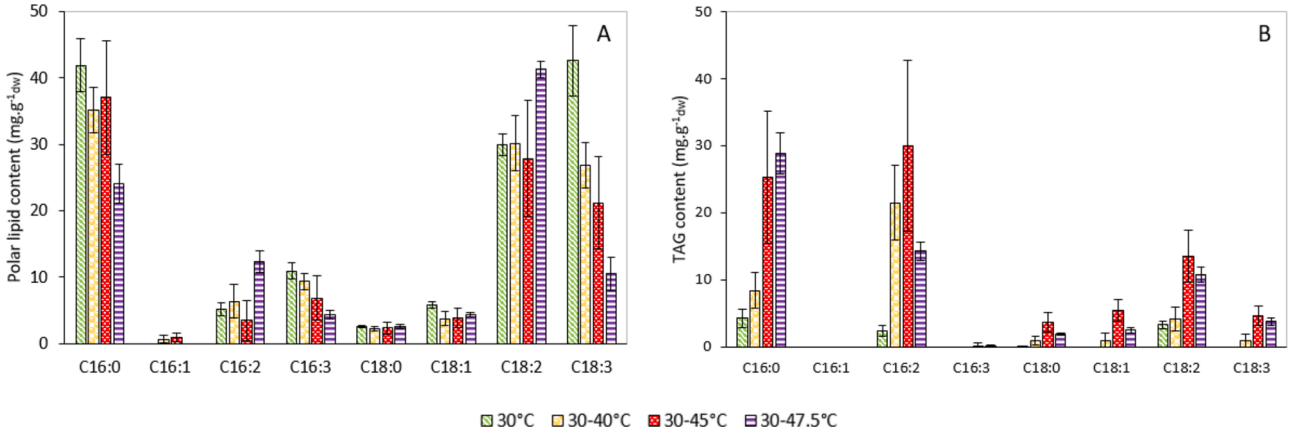

Fig. 7. Effect of temperature on the Polar lipid (a) and Triacylglycerol (TAG)(b) content of *Picochlorum* sp. *(BPE23)*. The night and the peak culture temperature, found 6 h after 'sunrise' are displayed in the legend. Data is presented as the average ± SD fatty acid content over a day as calculated through 8 timepoints within the day (biological replication: N = 2).

increase in TAG with increasing temperature, while other species exhibit a decreased amount (Converti et al., 2009; Renaud et al., 2002). In agreement with our data, studies on *Arabidopsis* and *Dunaliella* show increased TAG content with increased temperature (Falcone et al., 2004; Srirangan et al., 2015). In both studies this was mainly caused by increased levels of saturated fatty acids. Correspondingly, increased temperature caused a decrease of the fatty acid 18:3 in *Arabidopsis* (Falcone et al., 2004).

4. Conclusions

Studies on the physiological response of microalgae to fluctuating temperatures as found in industrial production systems are scarce. Our objective is to develop a microalgae production process in which cooling could be avoided. Therefore *Picochlorum* sp. *(BPE23)* was subjected to four diel temperature cycles and its physiological response was studied. Temperature was found to affect the growth and biomass composition of microalgae severely. When exposed to temperatures other than the optimum growth temperature which was found at 40 °C during daytime, a decrease in total pigment content was observed. Additionally, the fatty acid composition showed a tendency towards higher saturation levels with increasing temperature. Our study shows that the highest productivity of *Picochlorum* sp. *(BPE23)* is reached when the daytime temperature increased to a maximum of 40 °C. Growth rate and productivity decreased with an increased diel peak temperature and under a continuous diel temperature of 30 °C. Despite the reduced productivity, *Picochlorum* sp. *(BPE23)* was able to grow when exposed to a maximum diel temperature of 47.5 °C. The ability to grow optimally at maximum diel temperature of 40 °C instead of 30 °C, can reduce costs for industrial microalgae production in tropical regions by 26.2 %.

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Author contributions

Robin J.P. Barten: Conceptualization, Investigation, Data acquisition, Data analysis, Methodology, Writing – original draft. **Yovita A. Djohan**: Investigation, Data acquisition, Data analysis. **Wendy A.C. Evers:** Methodology, Data acquisition. **Rene H. Wijffels:** Writing - review & editing, Funding acquisition. **Maria J. Barbosa:** Writing – review & editing, methodology, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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