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Flashing light does not improve photosynthetic performance and growth of green microalgae

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ABSTRACT

Light attenuation in photobioreactors is a major bottleneck in microalgal production. A possible strategy for artificial light-based microalgal production to deliver light deep inside the culture is through the periodical emission of high intensity light flashes (so-called flashing light). However, our results did not show improved photosynthetic rates compared to continuous light for dilute and concentrated *Tetraselmis chui* cultures exposed to flashing light with various repetition rates (frequencies 0.01 Hz–1 MHz), light-dark ratios (duty cycles: 0.001–0.7) or time-averaged light intensity (50–1000 μmol s^{-1} m^{-2}). Likewise, flashing light applied to *Chlorella stigmatophora* and *T. chui* batch cultures could not enhance growth. However, we observed flashing light effects at different duty cycles and frequencies, depending on cell acclimation, culture concentration, and light intensity. In conclusion, artificial flashing light does not improve microalgal biomass productivities in photobioreactors, but low frequencies ($f < 50$ Hz) may be still used to improve light harvesting-associated biomolecules production.

1. Introduction

Research and development on microalgal biotechnology dates back to the 19th century, and the first commercial cultivation was reported in the 1960s (Milledge, 2011). Since then, technologies have evolved and innovative production systems such as tubular or flat panel photobioreactors (PBRs) have been employed to improve the algal biomass throughput per area (Ruiz et al., 2016). Nevertheless, the limitation of any PBR is the inefficiency in delivering photons at optimum wavelengths and quantities to drive photosynthesis in all microalgal cells within a culture (Schulze et al., 2017; Schulze et al., 2014). The cells at the periphery prevent penetration of light into the PBR, limiting the photosynthetic efficiency and productivity of the whole culture (Abu-Ghosh et al., 2016).

To improve the delivery of photons to cells in a culture, light intensities as well as culture mixing velocities should be increased. While high-intense light penetrates deeper into a PBR, appropriate culture mixing rates allow the algal cells to move faster from the light-limited (or dark) to light-saturated (or inhibiting-) zones (Abu-Ghosh et al., 2016; Brindley et al., 2016). The fast transition from light- to dark zones helps to avoid photoinhibition of cells at the periphery but ensures the sufficient absorption of light energy by most cells to carry out photosynthesis and convert this energy into energy-bound molecules (e.g., sugars in the Calvin cycle, NADPH, ATP; Sivakaminathan et al., 2018).

In most production systems, as soon as a high cell concentration is reached, the proportion of light zones to dark zones becomes too low and the retention time of cells in the dark area becomes too long, which increases respiration, leading to biomass losses (Brindley et al., 2011). To avoid photo limitation and maximize the growth, the supplied light as well as the mixing velocity should be further increased (Brindley et al., 2011). However, this approach has its drawbacks; the high energy consumption of mixing pumps and light-emitting lamps increase the production costs, and high mixing velocities can damage the cells (Brindley et al., 2004). Alternatively, growth at high cell concentrations can be maintained by narrowing the light path between the light source and the culture (e.g., by light guides or decreasing thickness of the PBR) or tailoring wavelengths (Schulze et al., 2014; Sivakaminathan et al., 2018). Yet, another approach is the use of flashing (or pulsed-) light

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emitting diodes (LEDs) to generate high-light flashes artificially, which penetrate deep into the culture (Abu-Ghosh et al., 2015b; Schulze et al., 2017). Herein, flashing LEDs emit periodically flashing cycles, which are composed of a short light flash (or pulse; t_l) and an extended dark period (t_d). Choosing an ideal repetition rate (i.e., frequency) for the flashing cycle is important to accomplish the so-called "flashing light effect", where maximal photosynthetic rates are reached. The potential benefits of flashing light reported in previous studies differed considerably, and they usually focused on flashing conditions of low frequencies ($f < 100$ Hz) and relatively high duty cycles ($DC > 0.1$) that were used to mimic light regimes in mixed cultures (Brindley et al., 2011; Vejrazka et al., 2011). It was not certain if flashing light of high frequencies and short duty cycles (e.g., $f > 100$ Hz, $DC < 0.1$) can indeed improve the growth performance of microalgae. In Schulze et al. (2017), we reviewed the potential of flashing light to improve microalgal growth and suggested a minimum theoretical frequency threshold of 200–333 Hz which is necessary to obtain the biological flashing light effect in microalgae. To test these hypothesizes, we used the latest LED and solid-state technologies to examine the photosynthetic oxygen evolution rate of *Tetraselmis chui* under different frequencies (0.01 Hz–1 MHz), duty cycles (0.001–0.7), light intensities (50–1000 μmol s^{-1} m^{-2}) and culture concentrations. In addition, we exposed batch cultures of *Chlorella stigmatophora* and *T. chui* for 14 days to flashing light conditions at light intensities of 50 or 200 μmol s^{-1} m^{-2}, frequencies of 40 and 400 Hz at a shortest possible duty cycle of 0.05, which are practicable conditions for artificial light-based microalgal cultivation. Finally, we have compared previously reported results with our findings to understand if flashing light has a significant benefit on microalgal production.

2. Materials and methods

2.1. Stock cultures

Tetraselmis chui was cultivated continuously (dilution rate: 0.7 d^{-1}; target biomass concentration in dry weight per liter: 1.5 g DW L^{-1}) in a 2 L-bubble column PBR under a light intensity of 400 μmol s^{-1} m^{-2}, referred as *stock 1*. For the experiments with the dilute cultures, around 150 mL of *stock 1* was transferred into a second PBR containing 2 L fresh growth medium to obtain a final biomass concentration of ~0.13 g DW L^{-1}, referred as *stock 2*. This dilute culture was maintained at average light intensities of I_a = 50, 500 or 1000 μmol s^{-1} m^{-2}; referred as *stock 2.1*, *2.2* and *2.3*, respectively. After one day of acclimation to a given average light intensity, the culture was used for the flashing light experiments in the photosynthetic chamber as described further below.

For the experiments with the concentrated cultures, the overflow of *stock 1* was connected to a second PBR with an adjusted light intensity of 2000 μmol s^{-1} m^{-2} to obtain a higher biomass concentration of ~4.7 g DW L^{-1}, referred to as *stock 3*.

The growth medium for *stock 1* and *stock 2* was a modified F-medium consisting of 5.3 mM $NaNO_3$, 0.22 mM $NaH_2PO_4H_2O$, 35 μM FeCL3*6H2O, 35 μM $Na_2EDTA*2H_2O$, 0.12 μM $CuSO_4*5H_2O$, 0.078 μM $Na_2MoO_4*2H_2O$, 0.23 μM $ZnSO_4*7H_2O$, 0.126 μM $CoCl_2*6H_2O$, 2.73 μM $MnCl_2*4H_2O$, adjusted to a salinity of 35 ppt using artificial sea salt (PRODAC International S.r.l., Cittadella, Italy). Twice the aforementioned nutrient concentration was used for *stock 3*, in order to ensure nutrient-sufficient growth conditions. Non-flashing light (2700K, VALUE Flex Protect 1200S, Osram, GmbH, Munich, Germany) was supplied 24 h/day to all stock cultures. All cultures were aerated through an opening at the bottom of the cultivation vessel (0.5 vvm) and supplemented with CO_2 on demand at a pH of 7.5 (BL 931700 pH Mini Controller, Hanna Instruments, Bedfordshire, UK). The cultures were microscopically checked daily for contaminations and cell viability. All cultures were kept inside a climate chamber and maintained at 15 °C using a heat exchanger (F250, Julabo GmbH, Seelbach, Germany), which was connected to the water jacket of each PBR.

At different time points, culture samples were filtered through pre-dried glass fiber filters (pore size ø = 0.7 μm; VWR), washed twice with 0.5 M ammonium bicarbonate, dried (T = 70 °C) and weighed to determine the dry biomass weight per liter (DW L^{-1}). Daily, optical density at 750 nm (OD_{750}) was determined for all cultures (CM-3500D, Minolta Co. Ltd., Osaka, Japan). Upon plotting OD_{750} data vs. dry weight, a linear correlation was obtained ($p < 0.05$) and used to determine biomass concentrations of all cultures on a daily base.

2.2. Oxygen evolution trials in photosynthetic chamber

In the first trial, we tested the oxygen evolution response of *T. chui* (SAG 19.52) cultures to flashing light inside a photosynthetic chamber. Dilute *T. chui* cultures (*stock 2.1, 2.2., 2.3*) were exposed to three different average light intensities: I_a = 50, 500 and 1000 μmol s^{-1} m^{-2}. Concentrated *T. chui* cultures (*stock 3*) were exposed to two average light intensities: I_a = 500 and 1000 μmol s^{-1} m^{-2}. The lowest light intensity (I_a = 50 μmol s^{-1} m^{-2}) did not yield a positive oxygen evolution rate, and hence excluded from the concentrated culture trials. The range of tested frequencies and duty cycles ranged from 10 to 10,000 Hz and 0.01–0.7, respectively.

In addition to the above-mentioned broad-scale screening, supplementary trials were conducted to understand the effect of extreme flashing light conditions on dilute and concentrated cultures. Test conditions were f = 0.01 Hz–2 MHz, $DC < 0.01$ with maximal instantaneous flash intensities of up to I_l = 100,000 μmol s^{-1} m^{-2} whenever technically feasible. Also, the simultaneous supply flashing light (I_{a_fash} = 350 μmol s^{-1} m^{-2}) and continuous background light (I_{a_cont} = 150 μmol s^{-1} m^{-2}) was tested on concentrated *T. chui* cultures (f = 1–10,000 Hz, DC = 0.05 and 0.1, I_a = 500 μmol s^{-1} m^{-2}), as a promising approach for microalgal production (Abu-Ghosh et al., 2015a).

All oxygen evolution measurements were conducted according to Brindley et al. (2010), using a 200 mL flat panel PBR as the photosynthetic chamber. This chamber had side lengths of 10 × 10 cm, providing a front surface area of 100 cm^2 and a light path length of 2 cm. The chamber was lit from the front (100 cm^2), and the water jacket around the chamber was connected to a heat exchanger (F250, Julabo GmbH) to maintain the temperature of the culture inside the chamber at 15 °C. A rubber cover on the top of the chamber served as an insulation, minimizing gas exchange with the environment. The rubber cover had three openings to fit different accessories; (1) a mixer, (2) a funnel to fill the chamber with fresh culture and (3) an oxygen probe (6050 D.O. electrode, Crison Instruments S.A., Barcelona, Spain) to measure the oxygen evolution. The mixer kept the microalgal cells in suspension and facilitated the gas exchange between the culture and the oxygen probe. The oxygen probe was connected to a Mettler Toledo O24100 transmitter (Mettler-Toledo S.A.E., Barcelona, Spain), which transferred the obtained data to a logger (LabJack U12, LabJack ltd. Lakewood, US).

The following procedures were adopted for each trial: (1) filling of the photosynthetic chamber with the fresh stock cultures (*stock 2.1*, *2.2*, *2.3* or *3*); (2) monitoring the oxygen evolution rate under a given light condition for 10–20 min; (3) draining the culture through an opening at the bottom of the chamber into an Erlenmeyer flask; (4) washing and filling of the chamber with fresh stock culture for the next experiment; and (5) transferring the already tested culture back to the stock culture. Everyday, after the experiment, the chamber was cleaned and disinfected with ethanol (70% *v*/v).

A photosynthesis-irradiance (P-I) curve was generated to estimate the photo-acclimation stage of the stock cultures. Here, we measured the oxygen evolution rates of dilute cultures adapted to 50 (*stock 2.1*), 500 (*stock 2.2*) and 1000 (*stock 2.3*) μmol s^{-1} m^{-2} and concentrated cultures (*stock 3*) under incrementally increasing light intensities (0–10,000 μmol s^{-1} m^{-2}).

Every 3–4 h, we measured the oxygen evolution of the cultures under continuous light at the same average intensity as used for the flashing light trials (e.g., I_a = 50, 500 or 1000 μmol s^{-1} m^{-2}) or at I_a = 500 μmol s^{-1} m^{-2} on trials with concentrated cultures. These control measurements were necessary to detect possible shifts in oxygen evolution due to growth-stage changes, acclimation, or cell attachment to the corners or walls of the chamber.

2.3. Trials under batch conditions

To understand long-term effects of flashing lights on different microalgal species, we cultivated *C. stigmatophora* (RCC 661) and *T. chui* under batch conditions for 14 days with flashing light conditions that are technically feasible for industrial artificial light-based cultivation systems. Both strains were grown under flashing light at frequencies of 40 and 400 Hz and a duty cycle of 0.05 using an average light intensity of I_a = 200 μmol s^{-1} m^{-2}. To check the effects of flashing light at under-saturating average light intensities, *T. chui* was additionally grown at 40 and 400 Hz (*DC* = 0.05) with an average light intensity of I_a = 50 μmol s^{-1} m^{-2}. All batch cultures were carried out in 1-L borosilicate glass flasks (diameter; *d* = 9 cm) filled with 900 mL algal culture with an lit surface of 262 cm^2 (triplicates; lit-surface to volume ratio: ~0.3 m^{-1}) in a climate chamber maintained at 15 °C, using *stock 1* as inoculum. Air enriched with 1% CO_2 was used to mix the cultures at a flow rate of 0.5 vvm using glass Pasteur pipettes (VWR). The growth medium was the same as used for *stock 3*. These conditions previously resulted in a good growth for the tested strains (Meseck et al., 2005; Schulze et al., 2019).

2.4. Light supply

Sixteen warm-white high-power LEDs (MHD-G, 2700 K, 12.6 W, Opulent Americas, Raleigh, US)-mounted on an actively cooled aluminum heat sink (10 × 10 cm) lit the photosynthetic chamber. All LEDs were operated in parallel and were connected to the output of a pulse width modulator (PWM-OCX, RMCybernetics Ltd., Alsager, UK). The pulse signal was provided by a function generator (TG4001, TTi, Huntingdon, UK) and controlled via Waveform Manager Plus software (V. 4.01). Bench power supplies were used (EA-PS 2042-10B and EA-PS 2084-05B; EA Elektro-Automatik, Viersen, Germany) to power the pulse width modulator and LEDs. The voltages and currents supplied to the LEDs were regulated by the power sources to adjust the average light intensities and to compensate for switching and working losses by the LEDs and the pulse width modulator. The light flashes generated by this system were as short as 100 ns and the peak flash intensities were up to I_l = 100,000 μmol s^{-1} m^{-2} inside the photosynthetic chamber. We attained the highest flash intensities and maximal overloading potential only at a frequency threshold of $f \approx$ 400–1000 Hz. At frequencies beyond this threshold, maximum flash intensities decreased to 20,000 μmol s^{-1} m^{-2} (e.g., at f = 1 Hz; duty cycle depended). This reduced the possible range of test frequencies and duty cycles under a given average light intensity.

For the batch culture experiments, 36 LEDs (MHD-G, 2700 K) were mounted on an actively cooled aluminum heat sink (L = 300 cm, H = 75 cm, W = 40 cm), and the test flashing light conditions (f = 40, 400 Hz, *DC* = 0.05) and continuous light were adjusted as mentioned above.

The flashing light output of the LEDs was measured with a high-speed photodiode (OSI FCI-125G-006HRL, kindly provided by OSI Optoelectronics, Inc., Hawthorne, US) mounted waterproof inside a glass tube and connected to an electrical resistor. The voltage drop at the resistor, which corresponded to the light output of the LEDs, was measured by a Picoscope 3000 oscilloscope (Pico Technology Ltd., Cambridgeshire, UK). The applied average light intensities (I_a = 50–1000 μmol s^{-1} m^{-2}) were measured (QSL-100; Biospherical Instruments, San Diego, CA) inside the distilled water-filled photosynthetic chamber or cultivation flask. The average light intensities (I_a) under flashing light is the average of the light intensity during the light flash period (I_l) and the dark phase (I_d = 0 μmol s^{-1} m^{-2}) as detailed by Schulze et al. (2017).

2.5. Data treatment

The effects of flashing light on oxygen evolution of dilute and concentrated cultures inside the photosynthetic chamber were examined using analysis of variance (ANOVA). We conducted a stepwise analysis because not all flashing light conditions could be tested for all cultures due to technical and biological limitations. First (1), we evaluated effects of frequency (10–10,000 Hz; degrees of freedom, *DF* = 27) and duty cycle (0.001–0.3; *DF* = 9) on dilute cultures adapted to 50 μmol s^{-1} m^{-2} (*stock 2.1*) using F-statistic and Tukey's tests on the total number of samples (= 280). Next, from the results of the general ANOVA (2), effects of the factors culture concentrations (dilute and concentrated), average light intensities (I_a = 500 and 1000 μmol s^{-1} m^{-2}), frequencies (10–10,000 Hz; *DF* = 15) and duty cycles (*DF* = 6) on dilute (*stock 2.2, 2.3*) and concentrated (*stock 3*) cultures were inferred (total number of samples: 448). This analysis was done to identify the major factors (based on F-statistics) influencing the oxygen evolution rates. Thereafter, a third (3) and fourth (4) ANOVA was performed to test the effects of duty cycle (0.03–0.7; *DF* = 6), frequency (10–10,000 Hz; *DF* = 15) and light intensity (500 and 1000 μmol s^{-1} m^{-2}) on dilute (*stock 2.2, 2.3*) and concentrated (*stock 3*) cultures, respectively (total number of samples in each ANOVA: 224). Lastly, a fifth ANOVA (5) was conducted on all diluted cultures (*stock 2.1–2.3*) employing the factors light intensity (50, 500 and 1000 μmol s^{-1} m^{-2}), frequency (10–10,000 Hz; *DF* = 15) and duty cycle (0.03–0.3; *DF* = 5; total number of samples: 288).

The F- and *p*-values resulting from the Type III sum of squares analysis were used to describe the impact of the factors of interest on the response variable photosynthetic oxygen evolution. The adjusted means with standard error from Tukey's post-hoc tests after ANOVA (1), (3) and (4) were used to illustrate the impact of frequency and duty cycle on a given culture. Post-hoc results from ANOVA (5) are used to describe effects of different light intensities in the main text.

To conduct ANOVA on data from different days, culture concentrations or light intensities, data of all oxygen evolution rates under flashing light (P_f) were normalized (P_n) to the continuous light control measurements (P_c, Eq. (1)):

$$P_n = \frac{P_f}{P_c} \tag{1}$$

when P_n approaches a value of one, oxygen evolution rates under flashing- and continuous light become similar, while a $P_n < 1$ or > 1 indicates a lower or higher photosynthetic performance under flashing light compared to continuous light, respectively.

To quantify the photo-acclimation stage for each culture (*stock 2.1–2.3, 3*), oxygen evolution rates from the P—I curve were modelled, as described by Rubio et al. (2003). Their model provides the photosynthetic parameters (i) α, the saturation constant which is the light intensity (in μmol s^{-1} m^{-2}) where neither the metabolic rates nor light are limiting the photosynthetic rates, (ii) κ, the half saturation constant of the Calvin cycle (no unit) and (iii) P_m, the maximum rate of photosynthesis per gram of biomass (μmolO_2 g^{-1} s^{-1}; Eq. (2)):

$$P_{Gross} = P_m * \frac{I}{2\alpha} * \left[\left(1 + \kappa + \frac{\alpha}{I}\right) - \sqrt{\left(1 - \kappa - \frac{\alpha}{I}\right)^2 + 4\kappa}\right] \tag{2}$$

The parameters α, P_m and κ were estimated via curve fitting (Sigma Plot software 13.0.0.83, Systat Software Inc., San Jose, US) to the gross oxygen evolution rate (P_{Gross}) under a given continuous light intensity (I). Maintenance respiration (m) was subtracted from the P_n data prior to regression to obtain gross oxygen evolution rates (P_{Gross}).

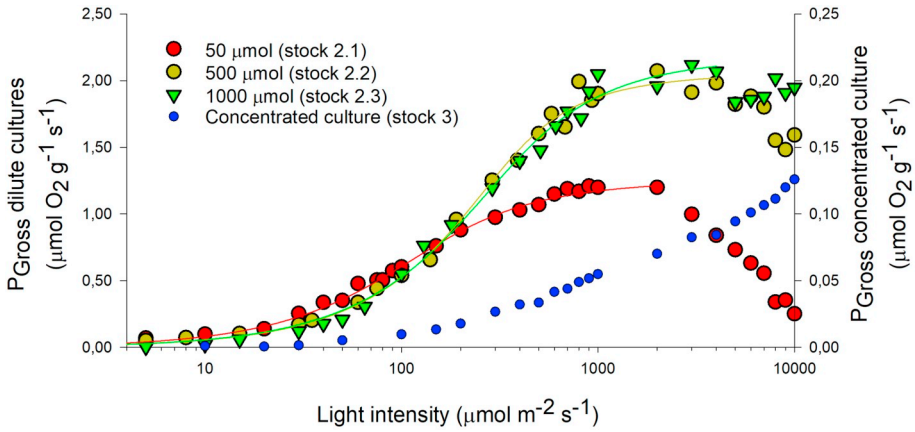

Fig. 1. Photosynthesis-irradiance (P–I) curves of *Tetraselmis chui* stock cultures used in the flashing light experiments. Dilute cultures were adapted to continuous light with a light intensity of 50 (*stock 2.1*), 500 (*stock 2.2*) and 1000 (*stock 2.3*) µmol s^{-1} m^{-2} while the concentrated culture (4.7 g L^{-1}; *stock 3*) was maintained under 2000 µmol s^{-1} m^{-2}. Oxygen evolution rates are plotted over incrementally increasing actinic light intensities. Solid lines are fitted to the gross photosynthetic rates according to Rubio et al. (2003) until maximal photosynthetic oxygen evolution was achieved ($r^2 > 0.99$). No model fit was obtained for concentrated cultures.

A Sigmoidal model (Eq. (3)) was applied to describe oxygen evolution response (P_n) to frequency or duty cycle (x).

$$P_n = y_0 + \frac{a}{1 + \exp\left(-\frac{x - x_0}{b}\right)} \tag{3}$$

where y_0, a, x_0 and b are parameters determined via iteration using Sigma Plot software.

In order to visualize and interpret data from the flashing light experiment, a mathematical model was designed based on Eq. (3) to predict the oxygen evolution rates obtained in the experiment (P_n) by the variables frequency (f) and duty cycle (DC) and four parameters a, b, c and d (Eq. (4)).

$$P_n = \frac{1 + e^{-a-b} * (DC - c + d)}{1 + \exp(-(a - b * \log 10(f))) * (DC - c + d * \log 10(f))} \tag{4}$$

Frequency (f) and P_n data from the low-light adapted cultures (*stock 2.1*) were applied to a model proposed by Fernández et al. (2018) (Eq. (5)) to identify the threshold frequency (f_β) where maximum photosynthetic performance (P_m) is reached:

$$\frac{P_n}{P_m} = \frac{f}{f_\beta} \bullet \left(1 - \exp\left(-\frac{f_\beta \bullet I_a}{f \bullet \alpha}\right)\right) \tag{5}$$

Data applied to this equation must come from flashing light conditions characterized by short duty cycles and non-saturating averaged light intensities ($I_a < \alpha$). The data sets of flashing light at moderate average light intensity (I_a = 500 µmol s^{-1} m^{-2}) and high average light intensity (I_a = 1000 µmol s^{-1} m^{-2}) were not suitable for fitting to Eq. (5) because in both cases $I_a > \alpha$, violating the non-saturating irradiance restriction.

Growth parameters for the batch cultivation experiments were estimated according to Ruiz et al. (2013) and the effects of flashing light on the maximal biomass productivity of the cultures was tested via ANOVA and Turkey's post-hoc test. A significance level of $p < 0.05$ was considered for all tests. The reader may refer to Supplementary Table S1 (Supplementary material) for test-statistics values, model parameters and original P_n data.

3. Results

3.1. P-I curve

A photosynthetic oxygen evolution rate vs irradiance curve (P-I curve) of *T. chui* cultures obtained under continuous irradiance shows the photo-acclimation stages of cultures exposed to the average light intensity used in the flashing light experiments (I_a = 50, 500 and 1000 µmol s^{-1} m^{-2}; *stock 2.1–2.3, 3*; Fig. 1). The maintenance respiration (oxygen evolution rate per gram of biomass measured in the darkness) of the 50, 500 and 1000 µmol s^{-1} m^{-2} adapted cultures

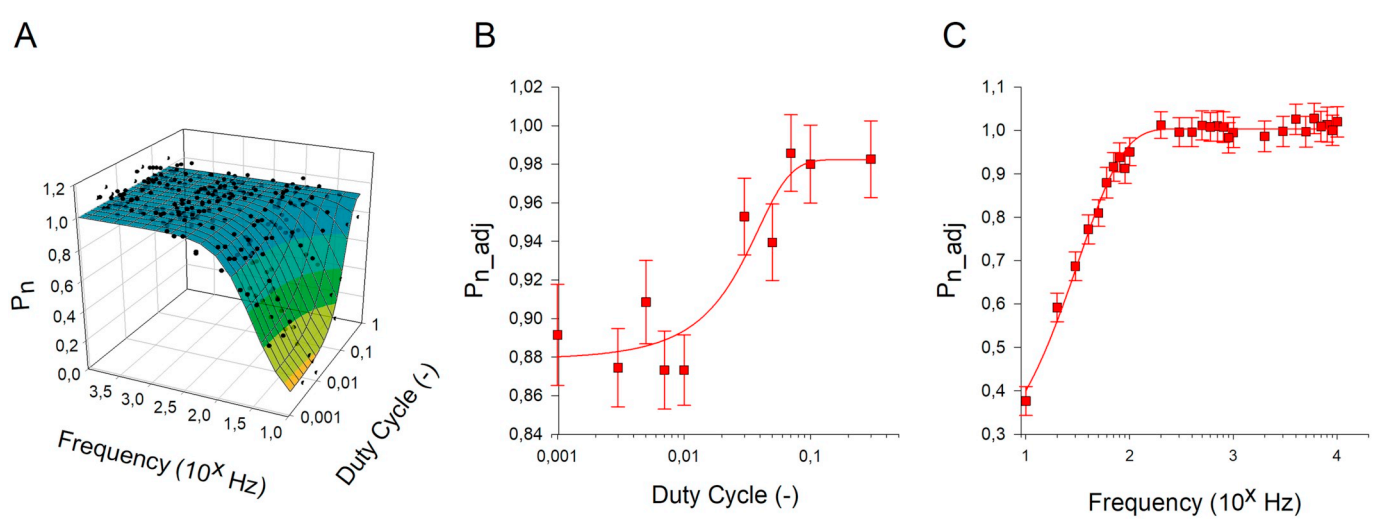

Fig. 2. Effects of flashing light with I_a = 50 µmol s^{-1} m^{-2} on dilute *Tetraselmis chui* cultures adapted to the same average light intensity (*stock 2.1*). Data (black points in A; 280 samples) of normalized oxygen evolution rates (P_n) are described by a mathematical model (surface area, r^2_{Model} = 0.88). An ANOVA (1, Supplementary Table S1) followed by Tukey's post-hoc tests was used to quantify the effects of duty cycle (B; DF = 9) and frequency (C; DF = 27) on P_n using the adjusted mean (P_{n_adj}). Error bars indicate the standard error from the adjusted means (r^2_{ANOVA} = 0.73).

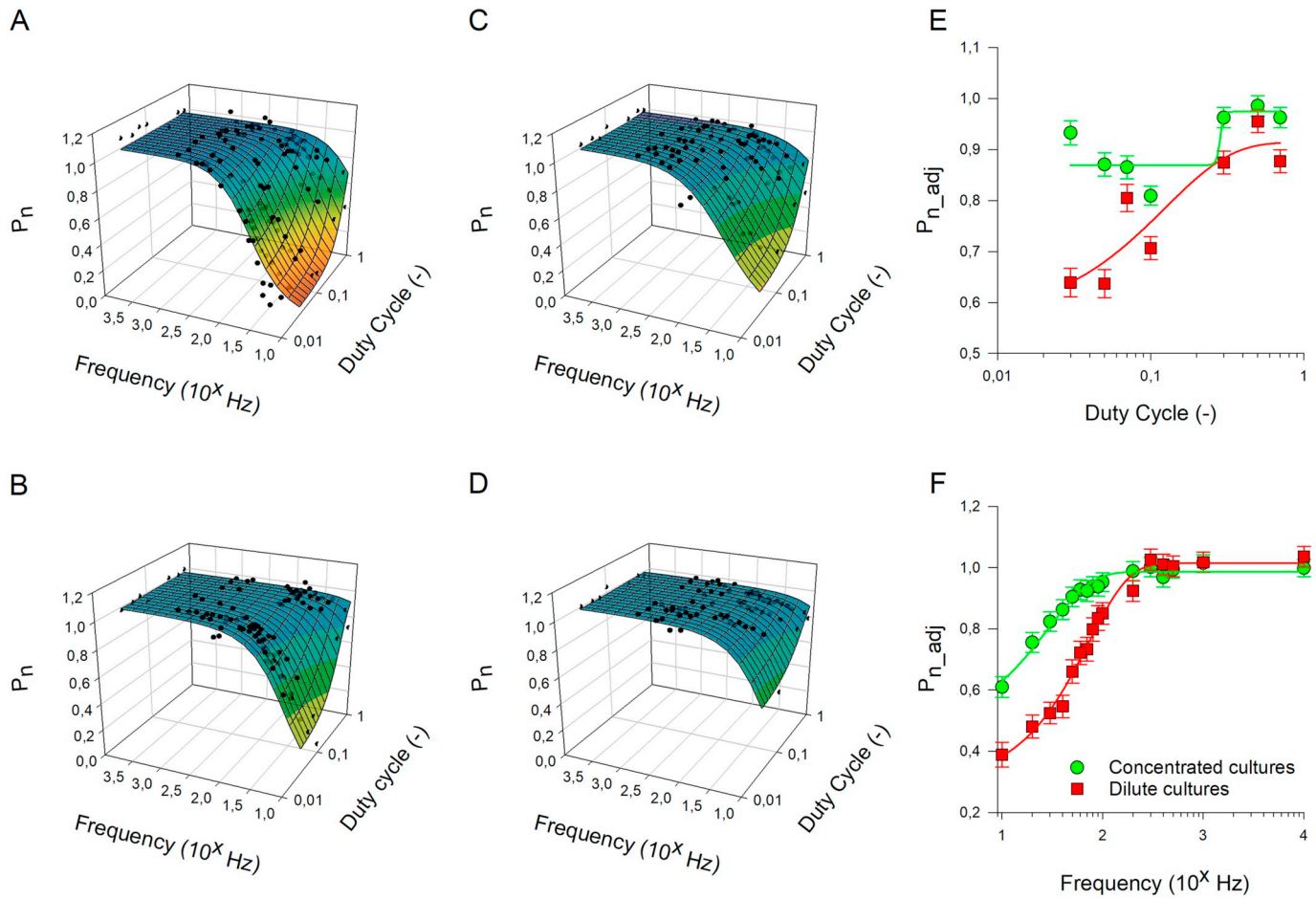

Fig. 3. Response of oxygen evolution for dilute and concentrated *Tetraselmis chui* cultures exposed to flashing light. Dilute cultures adapted to 500 μmol s^{-1} m^{-2} (*stock 2.2*, A) and concentrated cultures (*stock 3*; B) were exposed to I_a = 500 μmol s^{-1} m^{-2}. Dilute cultures adapted to 1000 μmol s^{-1} m^{-2} (*stock 2.3*; C) and concentrated cultures (*stock 3*; D) were exposed to I_a = 1000 μmol s^{-1} m^{-2}. Data (black points in A–D; samples:110, 102, 82, 82, respectively) of normalized oxygen evolution rates (P_n) are described by a mathematical model (surface area, r^2_{Model} > 0.68). ANOVA (3 and 4, Supplementary Table S1) followed by Tukey's post-hoc tests were used to quantify the effects of duty cycle (E) and frequency (F) on P_n for different culture concentrations using the adjusted mean (P_{n_adj}). Error bars indicate the standard error from the adjusted means (r^2_{ANOVA} = 0.60–0.80).

(*stock 2.1–2.3*) was m = −0.088, −0.16 and −0.26 μmol O_2 g^{-1} s^{-1}, respectively, and in concentrated cultures (*stock 3*) −0.2 μmol O_2 g^{-1} s^{-1}. The P-I curves of all dilute cultures followed a typical pattern, with the following characteristics: (1) an increase of oxygen evolution with increasing light intensities, (2) a maximum and (3) a subsequent decrease of photosynthetic performance with further rising light levels. The ratio between enzymatic and photochemical rate constants (α in μmol s^{-1} m^{-2}) increased with acclimation light intensity (α = 250, 456 and 559 μmol s^{-1} m^{-2} for the 50, 500 and 1000 μmol s^{-1} m^{-2} acclimatized cultures, respectively).

Lastly, we tested the oxygen evolution of concentrated cultures in response to increasing light (Fig. 1). Generally, photosynthetic rates per dry weight were ~10 times lower compared to dilute cultures and a minimum light intensity of 200 μmol s^{-1} m^{-2} was necessary to achieve photosynthetic oxygen evolution. The concentrated cultures did not show a plateau or any signs of decreasing oxygen evolution rates with increasing light intensities tested and thus no model fit was obtained.

3.2. Effect of flashing light

Dilute and concentrated cultures exposed to flashing light of different frequencies, duty cycles and light intensities did not exceed the photosynthetic rates obtained under continuous light (Figs. 2, 3). However, depending on culture concentration and average light intensity, inhibitory effects of flashing light on photosynthesis differed significantly.

3.2.1. Dilute low-light adapted cultures

Flashing light of a low average light intensity (I_a = 50 μmol s^{-1} m^{-2}) was tested on dilute microalgal cultures adapted to the same light intensity (*stock 2.1*). Various frequencies (10–10,000 Hz) and duty cycles (0.001–0.3) were tested (Fig. 2A). ANOVA (1) showed that duty cycle (F = 5.7) was affecting the oxygen evolution rate less than frequency (F = 20.9, p < 0.01). An analysis of the adjusted means from the Tukey's post-hoc test indicated that oxygen evolution rates rose sigmoidally with increasing duty cycles (Fig. 2B) and that duty cycles between 0.07 and 0.3 achieved the higher oxygen evolution rate, than compared to 0.003, 0.007 and 0.01 (p < 0.05; Fig. 2B, Supplementary Table S1). Likewise, oxygen evolution rates rose exponentially over frequencies (Fig. 2C, from 10 to 100 Hz), whereas frequencies between 10 and 50 Hz produced significantly lower oxygen evolution rates and higher frequencies (≥60 Hz) did not differ to continuous light treatment (P_n ≈ 1, Supplementary Table S1). Additional modelling according to Fernández et al. (2018) identified the threshold frequency under which the flashing light effect occurs in *stock 2.1* between ~130 and 180 Hz at duty cycles ≤0.01 (Supplementary Table S1).

3.2.2. Concentrated vs. dilute cultures

Concentrated and dilute *T. chui* cultures (*stock 2.2*, *2.3* and *3*) were exposed to average light intensities of I_a = 500 and 1000 μmol s^{-1} m^{-2} and frequencies of 10–10,000 Hz and duty cycles of 0.01–0.7 whenever technically feasible (Fig. 3A–D). An overall ANOVA (2) showed that all parameters significantly affected the oxygen evolution rates ($p < 0.01$), while the culture concentration had the strongest effect ($F = 62.2$), compared to duty cycle ($F = 23.8$), frequency ($F = 31.6$) and light intensity ($F = 11.1$; Supplementary Table S1). In a subsequent analysis, only oxygen evolution data from either concentrated (ANOVA 3) or dilute cultures (ANOVA 4) subjected to two average light intensities (I_a = 500 and 1000 μmol s^{-1} m^{-2}) were considered. In both models, oxygen evolution rates were mostly affected by duty cycle ($F_{dil_DC} = 24.7$, $F_{conc_DC} = 10.6$) and frequency ($F_{dil_f} = 34.6$, $F_{conc_f} = 10.6$) and to a lesser extent by average light intensity ($F_{dil_I} = 13.7$, $F_{conc_I} = 5.4$; Supplementary Table S1). A plot of the adjusted means of oxygen evolution in response to duty cycle and frequency (Tukey's test) revealed that concentrated cultures scored usually higher oxygen evolution rates at low frequencies (e.g., < 200 Hz) compared to dilute cultures (Fig. 3E, F). Concerning duty cycles, oxygen evolution rates were highest at DC = 0.3–0.7, while shorter duty cycles caused significantly lower P_n rates (Supplementary Table S1). Similar to experiments with *stock 2.1* exposed to 50 μmol s^{-1} m^{-2} (Fig. 2), adjusted means of oxygen evolution increased exponentially with frequency, approaching its maximum at around ~200 Hz (Fig. 3F).

A final ANOVA (5) was conducted on dilute cultures (*stock 2.1–2.3*) exposed to 50, 500 and 1000 μmol s^{-1} m^{-2} to quantify effects of different light intensities and acclimations (Supplementary Table S1). Generally, cultures exposed to average light intensity of 50 μmol s^{-1} m^{-2} showed higher oxygen evolution rates as compared to those exposed to 1000 μmol s^{-1} m^{-2} ($P_{adj} = 0.996$ vs. 0.850). Lowest oxygen evolution rates were found in 500 μmol s^{-1} m^{-2}-adapted cultures ($P_{adj} = 0.777$; $p < 0.01$).

3.2.3. Additional flashing light trials

We conducted additional experiments towards technical boundaries by extending the frequency range to 0.01 Hz-1 MHz, duty cycles < 0.01 (Fig. 4) and tested flashing light in combination with continuous light.

Results indicated that oxygen evolution rates of dilute cultures (*stock 2.2*, Fig. 3A) were reduced slightly (P_n = 0.7–0.9) at frequencies f = 200–500 Hz when the duty cycle was extremely short (0.005 and 0.007), while concentrated cultures (*stock 3*) remained unaffected at the same test conditions (I_a = 500 μmol s^{-1} m^{-2}). Low flashing light frequencies trials ($f \geq 1$ Hz, I_a = 500, 1000 μmol s^{-1} m^{-2} DC = 0.03, 0.1, Fig. 4B, C) revealed that oxygen evolution rates in dilute cultures approached zero at frequencies ≤ 10 Hz, indicating that rates of photosynthesis and respiration were similar, as reported earlier for other strains in dilute cultures (Brindley et al., 2010; Nedbal et al., 1996; Takache et al., 2015; Vejrazka et al., 2015). In concentrated cultures, frequencies ≤ 7 Hz caused a consumption of oxygen ($P_n < 0$; negative oxygen evolution rates), indicating that rates of respiration were higher than the photosynthetic rates (Fig. 4B, C). For example, oxygen evolution rates reached a $P_n = -0.32$ when exposed to f = 1 Hz, I_a = 1000 μmol s^{-1} m^{-2} and DC = 0.1 (Fig. 4B), while further frequency decrease to 0.1 and 0.01 Hz resulted always in higher oxygen evolution rates compared to dark (no light) conditions ($P_{n(Dark)} = -0.6$; Supplementary Table S1 in Supplementary material). Indeed, a negative growth for high average light intensities supplied in low frequencies was also reported previously (Brindley et al., 2011; Xue et al., 2011). As frequencies rise > 7–10 Hz, oxygen evolution rates increased sigmoidally in dilute and concentrated cultures until oxygen evolution rates became similar to those obtained under continuous light ($P_n \approx 1$) as reported earlier also for other strains (Brindley et al., 2010; Matthijs et al., 1996; Nedbal et al., 1996; Vejrazka et al., 2012; Vejrazka et al., 2011; Vejrazka et al., 2015). In concentrated cultures, the maximum photosynthetic rate was reached at lower frequencies ($f \approx$ 30–50 Hz) as compared to dilute cultures (f = 100–400 Hz; Fig. 4B, C). When frequencies were extremely high, the oxygen evolution rate of dilute cultures ($f \leq 1$ MHz, I_a = 50-μmol s^{-1} m^{-2}) and concentrated cultures ($f \leq 200$ KHz, I_a = 500 μmol s^{-1} m^{-2}, Supplementary Table S1) remained similar to continuous light. These findings are similar to our results (Figs. 2, 3) but are different from results of studies that reported better growth of microalgal cultures under flashing light of short duty cycles or high frequencies compared to continuous light (Liao et al., 2014; Park and Lee, 2001; Vejrazka et al., 2012; Yago et al., 2012). Likewise, the combination of continuously emitting LEDs (I_{a_cont} = 150 μmol s^{-1} m^{-2}) and flashing LEDs (I_{a_flash} = 350 μmol s^{-1} m^{-2}; DC = 0.05, 0.1) did not score higher oxygen evolution rates in concentrated cultures compared to continuous light alone (I_a = 500 μmol s^{-1} m^{-2}), in contrast to previous studies (Abu-Ghosh et al., 2015a).

3.3. Batch cultivation under flashing light

Previous studies about microalgae cultivation using flashing light indicated sometimes better growth as compared to continuous light (Katsuda et al., 2006; Liao et al., 2014; Lunka and Bayless, 2013; Park and Lee, 2000; Sastre, 2010; Vejrazka et al., 2012; Xue et al., 2011; Yago et al., 2014; Yoshioka et al., 2012). Even though photoproduction processes occur within milliseconds (Bernardi et al., 2017), the short exposure time of cultures to flashing light during the oxygen evolution trials (10–20 min; Fig. 1–4) could not detect possible beneficial effects that may come from cell acclimation to flashing light or from changing biomass concentration (i.e., optical properties). Our experiments with batch cultures showed that the results from the continuous light treatment did not differ significantly compared to 400 Hz-flashing light treatments (average: 0.21 ± 0.01 g DW L^{-1} d^{-1} for *C. stigmatophora*; Fig. 5A and 0.59 ± 0.02 g DW L^{-1} d^{-1} for *T. chui*; Fig. 5B, $p > 0.05$). Significantly lower biomass productivities were obtained for both strains when exposed to flashing light with I_a = 200 μmol s^{-1} m^{-2} and 40 Hz (*C. stigmatophora* 0.18 ± 0.01 g DW L^{-1} d^{-1} and *T. chui* 0.49 ± 0.01 g DW L^{-1} d^{-1}).

As suggested earlier, the effects of flashing light on algal growth depends also on supplied average light intensity (Figs. 2–4) and subsaturating intensities may be particular promising to improve photosynthesis of microalgae (Martín-Girela et al., 2017). However, an additional experiment with *T. chui* cultures revealed that flashing light (f = 40 and 400 Hz, DC = 0.05) with a lower average light intensity of I_a = 50 μmol s^{-1} m^{-2} did not cause significant effects on growth compared to the continuous light treatment (average: 0.19 ± 0.01 g DW L^{-1} d^{-1}; Fig. 5C; $p > 0.05$; Supplementary Table S1).

4. Discussion

4.1. Dilute cultures

All cells in dilute cultures received the flashing light with the adjusted average light intensity because light attenuation by self-shading was minimal (Brindley et al., 2010; Vejrazka et al., 2011), and the effect on the photosynthetic apparatus (i.e., biological flashing light effect; Schulze et al., 2017) could be tested for low, medium and high light-adapted cells (I_a = 50, 500 and 1000 μmol s^{-1} m^{-2}; *stock 2.1–2.3*). The α-value of cultures acclimatized to 50 μmol s^{-1} m^{-2} was higher than the average light intensity supplied (α = 250 μmol s^{-1} m^{-2} > I_a = 50 μmol s^{-1} m^{-2}), indicating that photosynthetic rates are limited by the supplied light but not by metabolic turnover rates (García-Camacho et al., 2012). The 500 μmol s^{-1} m^{-2}-adapted cultures had an α-value that was similar to the light intensity supplied (α = 456 μmol s^{-1} m^{-2}), indicating that neither the metabolic rates nor light intensity were limiting the photosynthetic performance of these cultures. On the other hand, the 1000 μmol s^{-1} m^{-2}-

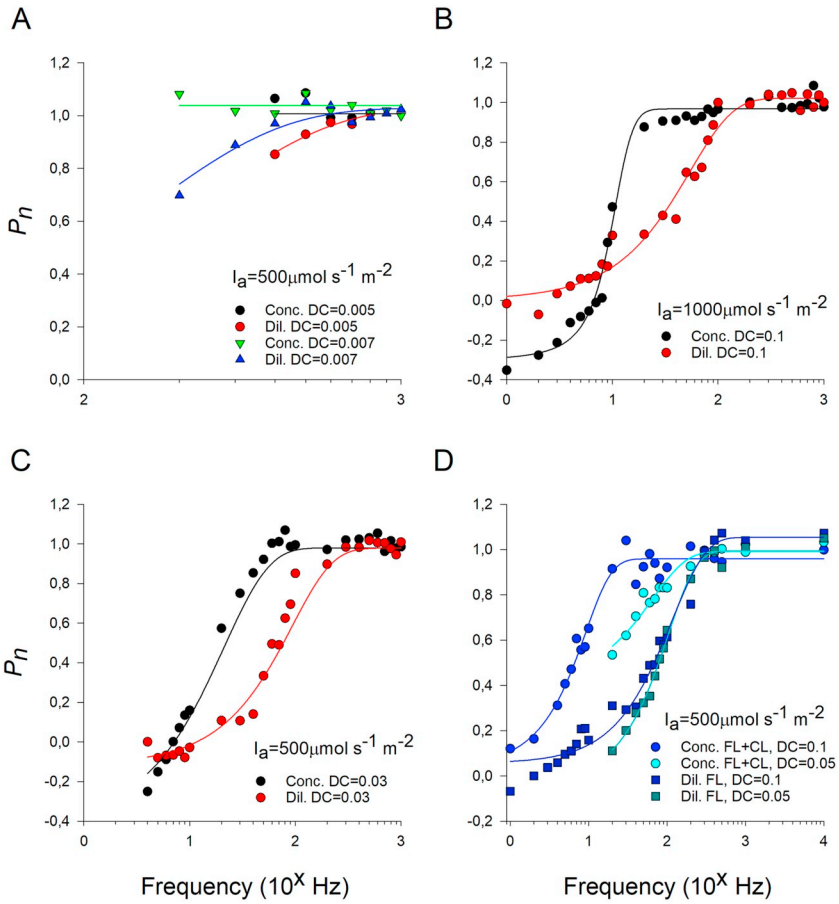

Fig. 4. Effects of extreme flashing light conditions with average light intensities of I_a = 500 and 1000 µmol s^{-1} m^{-2} on *Tetraselmis chui* cultures. Duty cycles < 0.01 were tested on cultures exposed to I_a = 500 µmol s^{-1} m^{-2} (A; f = 200–800 Hz; *stock 2.2, 3*). Frequencies < 10 Hz were tested on dilute and concentrated cultures with an average light intensity of I_a = 1000 µmol s^{-1} m^{-2} (B; *stock 2.3, 3*) and I_a = 500 µmol s^{-1} m^{-2} (C; *stock 2.2, 3*). Flashing light with I_{a_flash} = 350 µmol s^{-1} m^{-2} combined with continuous light with I_{a_cont} = 150 µmol s^{-1} m^{-2} (D; *stock 3*) was tested for a duty cycle of 0.1 and 0.05 and compared to dilute cultures exposed only to flashing light (*stock 2.2*) at the same average light intensity. Sigmoidal models (solid lines) were fitted to the normalized oxygen evolution rates (P_n).

adapted cultures showed a significantly lower α-value than the supplied light intensity (α = 559 < 1000 µmol s^{-1} m^{-2}), indicating that photosynthetic rates were maximal for *T. chui* cells and only metabolic turnover rates were limiting the photosynthetic performance. These low, medium and high α values indicate a low, moderate and high light adaption of stock 2.1, 2.2 and 2.3, respectively.

ANOVA (5) indicated that the normalized oxygen evolution rates in 50 µmol s^{-1} m^{-2}-adapted cells were higher compared to 500 or 1000 µmol s^{-1} m^{-2}-adapted cells ($P_{adj_50\mu mol}$ > $P_{adj_500\text{-}1000\mu mol}$) at low frequencies (e.g., f = 10–50 Hz; Supplementary Table S1). Such better light-use efficiency of cultures exposed to a limiting I_a (< $\alpha_{50\mu mol}$) at low-frequency flashing light compared to cultures exposed to I_a similar or higher than the saturation constant (e.g., $I_a \geq \alpha$ in 500 and 1000 µmol s^{-1} m^{-2}-adapted cultures) was previously suggested by Jishi et al. (2015) and Xue et al. (2011) for land plants and *Spirulina platensis*, respectively. Both studies reported a decrease of light-use efficiency under low-frequency flashing light as the supplied average light intensity increased. However, the present study showed that 1000 µmol s^{-1} m^{-2}-adapted cultures (*stock 2.3*) tolerated better low-frequency flashing light than 500 µmol s^{-1} m^{-2}-adapted cultures (P_{adj_500} < P_{adj_1000}; ANOVA 5, Supplementary Table S1). We suggest that the 1000 µmol s^{-1} m^{-2}-adapted cultures were high light-adapted (I_a > α; Fig. 1) and may have accumulated more photo-protective metabolites (e.g., β-carotene) that guard the cells from long-lasting high-light flashes (Katsuda et al., 2006; Mouget et al., 1995; Sastre, 2010; Schüler et al., 2017) compared to 500 µmol s^{-1} m^{-2}-acclimatized cells ($I_a \approx \alpha$). However, to our knowledge no such high average light intensities (I_a = 1000 µmol s^{-1} m^{-2}) were tested previously on high light-adapted cultures and further studies are needed to confirm the present findings.

4.2. Concentrated cultures

The P-I curve of concentrated cultures without saturation light intensity may be related to mixing and self-shading that caused a flashing light regime for the cells (Luzi et al., 2019; Terry, 1986) and a distribution of light to more cells compared to dilute cultures. In fact, the light regimes in concentrated cultures are complex and were studied extensively (Brindley et al., 2016; Loomba et al., 2018; Melis, 2009). We consider three light zones in concentrated cultures: (1) a high-light zone at the surface of the PBR where cells are exposed to most of the emitted light; (2) a low- to moderate-light zone i.e., a few millimeters below the high light zone, where cells are exposed to a low average light intensity; and (3), a dark zone where cells do not receive any light and respiration exceeds photosynthetic rates. Our concentrated cultures with biomass concentrations of ~4.7 g DW L^{-1} were characterized by a low proportion of light (1, 2) to dark (3) zones, leaving cells most of their time in the dark and only for short durations in the (low-) light zones (Loomba et al., 2018). This may have caused a low-light adaption of most cells in the culture (Brindley et al., 2010), which comes with a downregulation of photoprotective metabolisms (Schüler et al., 2017). Such low-light adapted microalgal cells passing through the high light zone (1) may have not been able to cope with intense, long-lasting light flashes (e.g., at low f < 7 Hz), leading to photodamage (Schulze et al., 2017) and consequently high respiration rates. In an additional experiment, we exposed low-light adapted *T. chui* cultures (*stock 2.1*) to flashing light with a higher average light intensity of I_a = 500 µmol s^{-1} m^{-2}: a negative oxygen evolution rate was obtained when the frequencies were low (P_n < 1 when f = 10–80 Hz, DC = 0.05, 0.07; Supplementary Table S1), confirming the incapacity of low-light adapted cells to utilize long-lasting intense light flashes.

When concentrated cultures were exposed to flashing light of higher

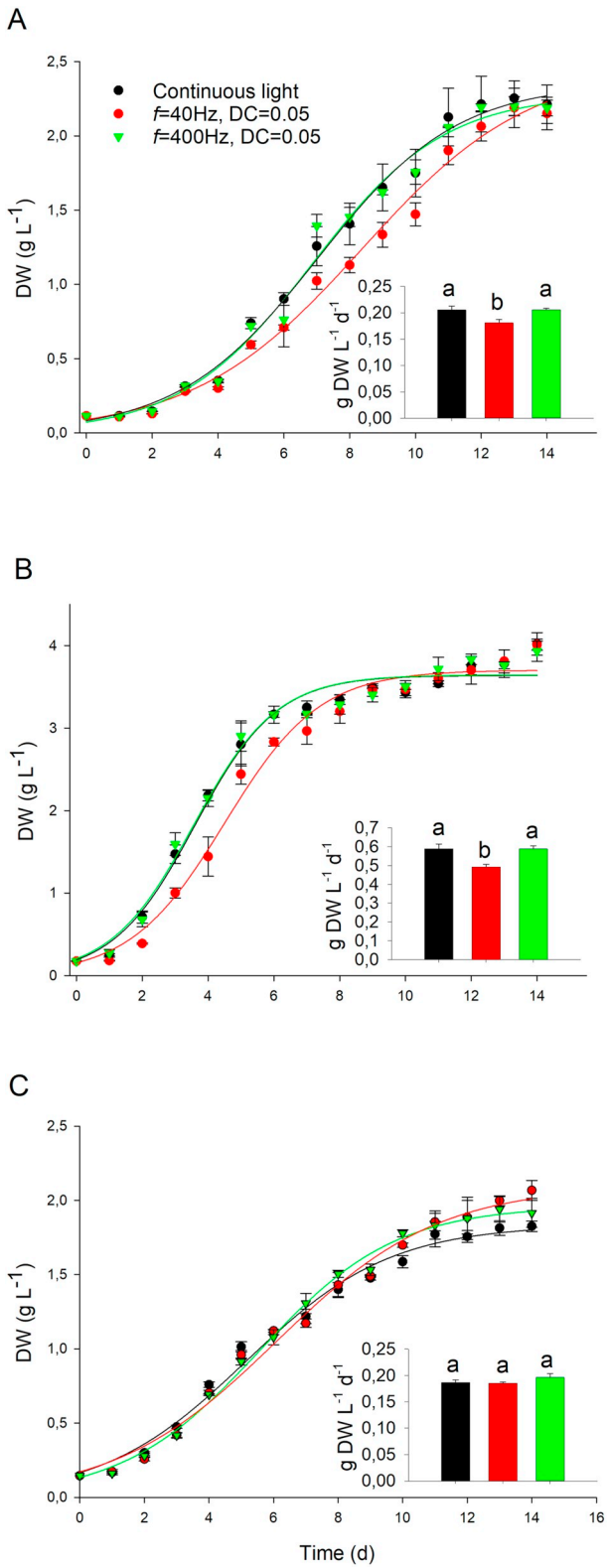

Fig. 5. Batch cultivation of *Chlorella stigmatophora* (A) and *Tetraselmis chui* (B) under flashing light with frequencies of 40 and 400 Hz at a duty cycle of 0.05. Both strains were cultivated under an average light intensity of I_a = 200 μmol s^{-1} m^{-2}. Additionally, *T. chui* was cultivated at I_a = 50 μmol s^{-1} m^{-2} (C). Solid lines are fitted to the growth data (symbols) using a sigmoidal growth model according to Ruiz et al. (2013). Data are shown as mean ± SD, n = 3.

frequencies from 10 to 100 Hz, the growth of cells in the periphery may be less inhibited, and cells in the low- to medium-lit zones displayed a good light use efficiency due to the reduced average light intensity (Xue et al., 2011). Such an effect would have helped concentrated cultures exposed to flashing light frequencies of f = 10–100 Hz achieve higher normalized oxygen evolution rates than dilute cultures in the same flashing light regime (e.g., Fig. 3E, F; ANOVA 2). Cells in dark zones received insufficient light for photosynthetic oxygen evolution and thus likely showed higher respiration than photosynthetic rates, adding up the total respiration of the culture. With the aid of a photodiode we could confirm that single light flashes indeed overcome mutual shading and penetrate until the dark layer of concentrated cultures (data not shown). However, the average light intensity of flashing light decreased to the same extent as continuous light with increasing light path. Since the (biological-) flashing light effect only causes similar photosynthetic rates as under continuous light (Jishi et al., 2015; Schulze et al., 2017) and cells respond to the average light intensity when frequencies are above the threshold of 200–500 Hz (Figs. 2–4), flashing light could not improve photosynthetic performance in the dark- or low-light layers.

Taken together, cells in each zone of concentrated cultures responded differently to flashing light, depending on the prevailing average light intensity. We suggest that the observations in concentrated cultures are a result of three factors: (1) inhibition of cells in high-light zones, (2) better light utilization efficiency of cells in low- or moderate-lit zones and (3) high respiration rates of cells in dark zones.

Lastly, batch experiments indicate that flashing light has no enhancing effects when culture concentrations change over time. During early growth stages, light attenuation in the culture was low and –similar to dilute cultures in the photosynthetic chamber– low-frequency flashing light of lower average light intensities were less growth inhibiting than high average light intensities (Fig. 5). The applied short duty cycle of 0.05 and high flash intensities (I_l = 1000 or 4000 μmol s^{-1} m^{-2}) were believed to deliver light deep into microalgal cultures with a high self-shading potential (Schulze et al., 2017). Such high self-shading potential can be assumed in the used cultivation flasks due to the low lit-surface to volume ratio (~0.3 m^{-1}), which causes significant light limitation for most cells in the cultures at increasing biomass concentrations (Geada et al., 2017; Pulz, 2001; Tredici et al., 1991). However, flashing light could not improve biomass productivities in these bubble column-type photobioreactor during 14 days of cultivation and cultures did not acclimatize to long-term exposure of high-frequency flashing light (Grobbelaar et al., 1996).

4.3. Comparison to the literature and sum-up

We derived P_n from several flashing light studies conducted on various microalgal species (Fig. 6, Supplementary Table S1). The studies employed different culture densities, light intensities and light adaption levels. For most studies that reported improved growth or photosynthetic performance, the P_n value was ~1.2, indicating a 20% better growth compared to continuous light (Fig. 6, Liao et al., 2014; Nedbal et al., 1996; Park and Lee, 2000; Vejrazka et al., 2012; Vejrazka et al., 2015; Yago et al., 2014; Yago et al., 2012). Only few studies have reported higher values (Lunka and Bayless, 2013; Luzi et al., 2019). It was often suggested that photosynthesis does not require continuous light and that short intense light flashes can penetrate deeper into the water column so that the cells in deeper layers can carry out photosynthesis (Park and Lee, 2000; Vejrazka et al., 2011; Vejrazka et al., 2015). At present, overcoming self-shading is one of the most discussed factors for explaining flashing light-induced growth enhancement in microalgal cultures (Abu-Ghosh et al., 2016; Brindley et al., 2011; Grobbelaar et al., 1996; Lunka and Bayless, 2013; Sastre, 2010). To our surprise, we did not find any improvements of flashing- over continuous light, even at the shortest, technically possible duty cycles (e.g., 0.005)

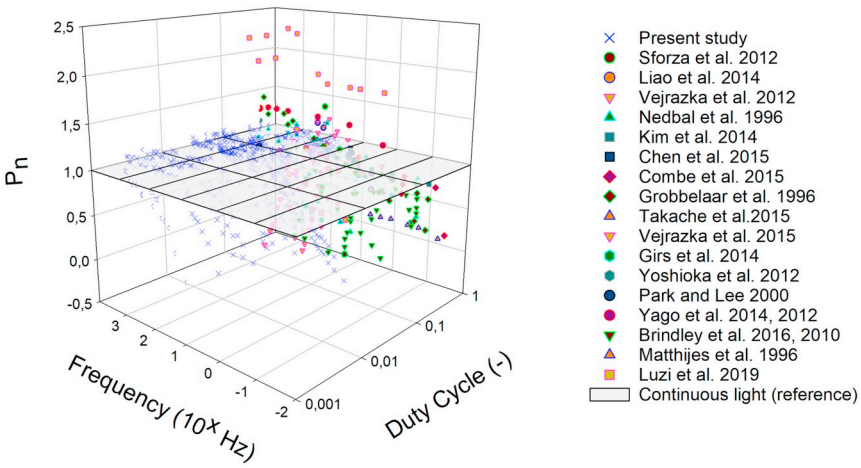

Fig. 6. Summary of studies with flashing light on microalgae and cyanobacteria. Data from different studies were normalized to a continuous light reference and is given as relative growth parameter (P_n). The horizontal plane that passes through $P_n = 1$ indicates the maximum value of the oxygen evolution rate obtained under continuous light. Details and references of each study are provided in the supplementary material (Supplementary Table S1).

with highest light flash intensities (e.g., I_l = 100,000 μmol s^{-1} m^{-2}) or frequencies (≤1 MHz). These conditions were thought to overcome self-shading and increase growth performance (Schulze et al., 2017). We could not identify the reasons for the different findings among studies, but may suggest (i) a specific flashing light condition (frequency and duty cycle) not detected in the present study that is coherent with reaction times of intracellular molecular processes and indeed enhances growth, (ii) measurement inaccuracies of the average light intensities under flashing light at different studies, leading to underestimation of the light intensity applied or (iii) photosynthetic measurements including cell attached to walls and corners of the photosynthetic chamber, which has been shown in the present study to have a non-linear positive effect over time on the photosynthetic performance of the culture (data not shown).

The threshold frequency where growth was inhibited (e.g., f = 200–500 Hz) for a culture depends on duty cycle, average light intensity, light acclimation stage of cells and the light attenuation potential of the culture. Towards higher frequencies (f > 200–500 Hz), oxygen evolution rates reached those obtained under continuous light, confirming findings about other strains (Brindley et al., 2010; Matthijs et al., 1996; Vejrazka et al., 2013; Vejrazka et al., 2012; Vejrazka et al., 2011; Vejrazka et al., 2015). Notably, frequencies < 200–500 Hz were most discriminative for changes of the oxygen evolution and growth performance declined sigmoidal with decreasing frequencies as previously described (Jishi et al., 2015; Sivakaminathan et al., 2018). The upper limit of this frequency threshold obtained in our experiments with extreme short duty cycles (Fig. 4) suggests a slightly faster turnover rate of the linear electron transfer chain than previously suggested (e.g., 200–330 Hz; Schulze et al., 2017). Nevertheless, the specific threshold flashing light condition at which photosynthetic rates become similar to continuous light differs among microalgal strains or species, depending on pool size of reducing equivalents (e.g., PQ-pool) or non-photochemical quenching mechanisms used by the phototroph (Jishi et al., 2015; Vejrazka et al., 2011). Notably, our study primary aimed to uncover possible beneficial effects of high frequencies and short duty cycle for artificial light-based microalgal production. Nevertheless, future studies should focus on applying frequencies < 500 Hz to different phototrophic species; to target species and acclimatization effects in relation to threshold frequency, duty cycle and average light intensity. Using low-light adapted cells exposed to flashing light of a higher average light intensities seem particularly promising to understand better threshold frequencies at which either the flashing light effect or damages on cell structures occur. This knowledge will allow the validation or further refinement of previously established photosynthetic response models of phototrophs exposed to flashing light and perhaps leads to a better understanding about reaction kinetics of the linear electron transfer chain (Fernández-Sevilla et al., 2018; Jishi et al., 2015; Schulze et al., 2017).

5. Conclusion

Our experiments with *T. chui* and *C. stigmatophora* cultures showed that flashing light does not improve light utilization of microalgal cultures in photobioreactors. Photosynthetic responses of *T. chui* cultures exposed to flashing light (frequencies = 0.01 Hz–1 MHz; duty cycles = 0.001–0.7; average light intensities: 50–1000 μmol s^{-1} m^{-2}) indicated a sigmoidal increase of growth performance over rising frequencies and duty cycles. The use of LEDs that emit low-frequency flashing light with high flash intensities that penetrate deep inside the culture (e.g., short duty cycles) may be still efficient to trigger a desired metabolic pathway and improve light-use efficiency in photobioreactors to produce biomolecules such as pigments.

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Declaration of authors' contributions

PS designed the experiments, performed the laboratory work, data analysis, and drafted the manuscript. CB, JF. and RR contributed to

discussion and critical revision of the article for important intellectual content. HP contributed to graphical design and revised the manuscript. RW and KV conceived the main project, contributed to the design, reviewed and edited the article and provided the administrative support for the research project. The authors agree with the authorship this work and give final approval of the version to be submitted and any revised version.

Declaration of competing interest

The authors confirm that there is no known conflict of interest related with this publication and there has been no significant financial support for this study that could have influenced its outcome.

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