BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Photosynthetic efficiency and oxygen evolution of *Chlamydomonas reinhardtii* under continuous and flashing light

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Abstract As a result of mixing and light attenuation in a photobioreactor (PBR), microalgae experience light/dark (L/D) cycles that can enhance PBR efficiency. One parameter which characterizes L/D cycles is the duty cycle; it determines the time fraction algae spend in the light. The objective of this study was to determine the influence of different duty cycles on oxygen yield on absorbed light energy and photosynthetic oxygen evolution. Net oxygen evolution of *Chlamydomonas reinhardtii* was measured for four duty cycles (0.05, 0.1, 0.2, and 0.5) in a biological oxygen monitor (BOM). Oversaturating light flashes were applied in a square-wave fashion with four flash frequencies (5, 10, 50, and 100 Hz). Algae were precultivated in a turbidostat and acclimated to a low photon flux density (PFD). A photosynthesis–irradiance (PI) curve was measured under continuous illumination and used to calculate the net oxygen yield, which was maximal between a PFD of 100 and 200 μmol m^{-2}s^{-1}. Net oxygen yield under flashing light was duty cycle-dependent: the highest yield was observed at a duty cycle of 0.1 (i.e., time-averaged PFD of 115 μmol m^{-2}s^{-1}). At lower duty cycles, maintenance respiration reduced net oxygen yield. At higher duty cycles, photon absorption rate exceeded the maximal photon utilization rate, and, as a result, surplus light energy was dissipated which led to a reduction in net oxygen yield. This behavior was identical with the observation under continuous light. Based on these data, the optimal balance between oxygen yield and production rate can be determined to maximize PBR productivity.

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Introduction

High photobioreactor (PBR) productivity is crucial to microalgae feed stock production and depends, among others, on a high biomass yield on light energy. If a PBR is exposed to (sun)light, then light is attenuated along the PBR depth as a function of algae concentration and pigmentation. A high biomass concentration results in an illuminated zone with net photosynthesis and a dark zone with possible respiration. As a result of mixing, algae experience flashing light or light/dark (L/D) cycles. These mixing-induced L/D cycles are suggested to enhance PBR efficiency (Degen et al. 2001; Hu and Richmond 1996; Richmond 1996)

Algae growing at (over-)saturating incident photon flux densities (PFDs) show a low photosynthetic efficiency. However, if algae are grown under light-limiting conditions, then photosynthetic efficiency is high (Tredici 2009). Photosynthetic efficiency can be expressed in different ways: biomass yield on light energy (i.e., gram of biomass produced per mole of PAR photons absorbed) or quantum yield of oxygen evolution (i.e., mole of oxygen produced per mole of PAR photons absorbed). With the knowledge of growth stoichiometry, oxygen yield can be converted to biomass yield and used to evaluate PBR performance.

The effect of a dynamic light regime on biomass yield can be assessed with the concept of light integration (Phillips and Myers 1954; Terry 1986). Under full light integration, algae do not respond to the actual PFD during the flash of an L/D cycle but to the lower, time-averaged PFD of the whole cycle (flash + dark time). In other words, biomass yield will be

comparable with cultivation under continuous and limiting PFD. Opposite to full light integration, algae can respond directly to the PFD during the flash and respire during the dark part of the L/D cycle. This behavior can be called growth integration and is not favorable because it leads to low biomass yields.

Photosynthetic rate measurements based on oxygen evolution have shown that subsecond L/D cycles are necessary to achieve (full) light integration (Kok and Burlew 1953; Matthijs et al. 1996; Nedbal et al. 1996; Terry 1986). These results are supported by the measurement of specific growth rate under flashing light (Phillips and Myers 1954; Rosello-Sastre 2010; Vejrazka et al. 2011; Xue et al. 2011). The major influence of L/D cycles on photosynthetic rate was found with oversaturation, incident PFDs during the flash (Vejrazka et al. 2011; Xue et al. 2011). However, most studies presented only results about photosynthetic rates and did not include photosynthetic efficiency, which is an important parameter because it incorporates the absorption cross section and the actual amount of light absorbed by the algae. As a consequence, the full impact of L/D cycles on PBR productivity is still not fully understood.

One parameter which characterizes and influences the amount of light integration is the duty cycle: it determines the time fraction algae spend in the light during one full L/D cycle. In a PBR, the duty cycle is dependent on the biomass concentration. An increase in biomass concentration will lead to an increase in a dark zone and thus a decrease in duty cycle, which might affect algae growth and productivity. The objective of our study was to determine the influence of different duty cycles on light integration.

The degree of light integration was assessed both in terms of photosynthetic rate and oxygen yield. Oxygen evolution rates in different (dynamic) light regimes were measured in a newly developed biological oxygen monitor (BOM).

To be able to properly discuss the results from flashing light, we measured net and gross oxygen production rates in continuous light and determined the corresponding oxygen yields (net and gross). The net oxygen production rate in continuous light could be described by a hyperbolic tangent model including maintenance respiration. This paper provides a systematic investigation on the effect of duty cycle in addition to flash frequency on oxygen production and, more importantly, photosynthetic efficiency.

Materials and methods

Organism and medium

Chlamydomonas reinhardtii CC-124 wild type mt− 137c was obtained from the *Chlamydomonas* culture collection at Duke University (now *Chlamydomonas* Resource Center). The microalgae were cultivated in a Sueka high salt (HS)-based liquid medium, composed of (amounts in gram per liter): NH_4Cl, 1.450; KH_2PO_4, 0.720; K_2HPO_4, 1.440; $MgSO_4·7H_2O$, 0.280; $CaCl_2·2H_2O$, 0.057; $Na_2EDTA·2H_2O$, 0.067 and 10 mLL^{-1} of a 100 times concentrated Hutner's trace elements solution (Hutner et al. 1950). Axenic cultures were maintained in shake flasks placed on an orbital shake incubator at pH 7, 25 °C, air enriched with 2.5 %$_{v/v}$ CO_2, and 60–80 μmol $m^{-2}s^{-1}$ continuous illumination for at least 1 week before use as PBR inoculum.

Photobioreactor setup

The PBR system (Photobioreactor FMT 150, Nedbal et al. 2008) was purchased from Photon System Instruments (PSI, Brno, Czech Republic) and equipped with a red LED panel peaking at 630 nm.

The PBR (375 mL working volume) was operated as a turbidostat (axenic) with an incident PFD of 100 μmol $m^{-2}s^{-1}$. The PFD leaving the PBR was set to 60 μm ol$m^{-2}s^{-1}$ to obtain low light acclimated algae because Vejrazka et al. (2011) showed that algae grown under flashing light conditions with a low time-averaged PFD (100 μmol $m^{-2}s^{-1}$) were acclimated to a low PFD.

Temperature was regulated at 25 °C and the culture was mixed by sparging air at a flow rate of 1 $L_{gas} L_{culture}^{-1}min^{-1}$. The pH was maintained at 7.0 via pulsewise CO_2 addition to the air stream. For this reason, the PBR medium was enriched with 0.420 gL^{-1} of $NaHCO_3$. After a steady state was reached, algae were used for oxygen evolution measurements. In this way, it was assured that algae were always acclimated to the same light regime. The general operation procedure for the PBR is described in Vejrazka et al. (2011).

Biological oxygen monitor setup

We designed a BOM for the oxygen evolution measurements. The BOM and the light modules (including control) were manufactured by the 'Ontwikkelwerkplaats' of Wageningen University. The BOM consisted of three chambers: two water jackets and one measurement chamber (see Fig. 1). The cylindrical measurement chamber had a diameter of 31 mm and a light path of 15 mm (liquid volume: 12.3 mL). It had three ports: one to inject the sample, one to release gas, and one to house the oxygen probe holder. Each side of the measurement chamber was coupled to a water jacket, which was connected to a water bath. The 4-mm thick windows of the BOM were made from the transparent polycarbonate resin Lexan.

The BOM setup was placed on a magnetic stirring plate to mix the algae suspension with a tumble stir disc (VP 719F-2, V&P Scientific, Inc., San Diego, CA, USA). The BOM was illuminated from each side with one round LED

Fig. 1 Biological oxygen monitor. *A* measurement chamber, *B* water jacket, *C* end piece, *D* LED module
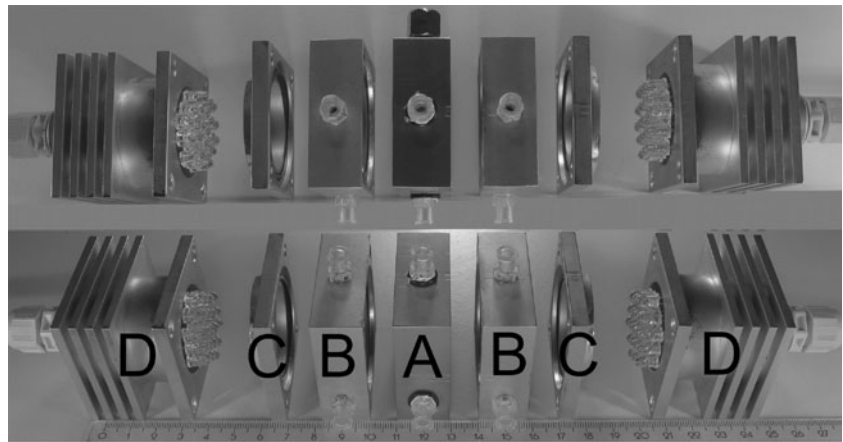
module emitting red light peaking at 620 nm. Each LED module was equipped with 19 Yoldal (YE-R5S15N) LEDs. The LED current was set to 20 mA and the brightness was controlled by pulse width modulation at a frequency of 10 kHz. The flash time and dark time of the LED modules could be set in 1-ms steps from 1 ms to 9,999 ms.

The dissolved oxygen concentration was measured with an optical oxygen microsensor (IMP-PSt1-L5-LIC0-BGF10-TF-OIW, PreSens, Regensburg, Germany). The measurement tip of the sensor was flat broken ($\varnothing \approx 140$ μm) and coated with an optical insulation to prevent influence of ambient light and chlorophyll fluorescence on the measurement. The signal was processed via a transmitter (Microx TX3, PreSens, Regensburg, Germany) and recorded on a PC with the program Microx TX3 for PSt1 (Ver. 6.02, PreSens, Regensburg, Germany).

Biological oxygen monitor operating procedure

All measurements and calibrations were done with an active water circulation through the water jacket to control the temperature at 25 °C. The oxygen probe was calibrated with a two-point calibration (0 %, 100 % air saturation) before the oxygen evolution measurement. The probe was calibrated inside the measurement chamber which was filled with medium (without $NaHCO_3$) and bubbled with air (100 % air saturation) or pure nitrogen (0 % air saturation).

Algae samples were taken during steady-state operation of the PBR for oxygen evolution measurements. In this way, it was assured that the algae were always acclimated to the same low light conditions. The PBR was mixed with pure nitrogen instead of air for 7 min to reduce the dissolved oxygen concentration in the algae suspension to a level between 20 % and 30 % before a sample (14 mL) was transferred to the BOM.

After sample injection into the BOM, algae were dark-acclimated for 5 min and then dark respiration (i.e., mainte-nance respiration) was measured for 10 min. Then, the light

was turned on and oxygen production was measured for 3 to 5 min. Next, lights were turned off and light respiration was measured for 5 min. Only the first 3 min of data were used to calculate the oxygen consumption rate. After the light respiration measurement, light was turned on again and the whole procedure was repeated at another PFD or duty cycle. In flashing light, no light respiration was measured.

Preliminary experiments showed that one sample could not be used for more than 1 h total measurement time or above a dissolved oxygen concentration of 130 %. Other-wise, oxygen production rates under the same conditions changed. Each setting was measured at least three times. Each measurement block (maximally 1 h of measurement) was designed as such that the PFD (time-averaged PFD in case of flashing light) was increased from first to last setting to prevent measuring oxygen evolution of photo-damaged algae at low PFDs.

The dissolved oxygen concentration was recorded in 1-s intervals and expressed as percent (%) air saturation. The conversion to micromole per liter was done based on Henry's law, thus correcting the maximum solubility of oxygen in water for the temperature influence: 100 % air saturation equals a dissolved oxygen concentration of 258 μmol L^{-1} at 25 °C. The biomass-specific (per gram dry weight) oxygen production rate $(P_{O_2}, \mu mol_{O_2} g^{-1} s^{-1})$ is the slope of dissolved oxygen concentration versus time divided by the biomass (dry weight) concentration $(c_x, g\ L^{-1})$.

Light regime biological oxygen monitor

The PFDs for continuous light can be found in Table 1. The light path in the BOM (15 mm) was shorter in comparison to the PBR (25 mm); therefore, only 20 % of the incident PFD was absorbed.

The highest PFD in continuous light was 1,079 μmol m^{-2}s^{-2}, which was also set as the flash PFD. This PFD was supplied by two LED modules: each LED module

Table 1 PFD settings for PI curve measurements

	PFD$_L$ (µmol m^{-2} s^{-1})	
	LED1	LED2
1	10	–
2	30	–
3	42	–
4	52	–
5	85	–
6	95	–
7	182	–
8	424	–
9	536	–
10	875	–
11	536	543

providing half the target PFD from each side of the measurement chamber. Net oxygen production was compared for different PFDs provided either from one LED module or half the target PFD from two modules (Fig. 2). Data shows that the oxygen production measured with either one or two LED modules are linearly correlated with a zero offset. Therefore, the PFD can be supplied either from one or from two sides and there is no influence on the net oxygen production rate.

The flashing light regime was defined by the length of the square-wave flash (t_1, microsecond), dark time (t_d, microsecond), cycle time ($t_c = t_1 + t_d$, microsecond), duty cycle ($\phi = t_l/t_c$, –), and flash frequency ($f = 1000/t_c$, hertz). The flash photon flux density (PFD$_L$, micromole per square meter per second) was provided by two sides and set to a total of 1,079 µmol m^{-2}s^{-1} (LED1: 536 µmol m^{-2}s^{-1}, LED2: 543 µmol m^{-2}s^{-1}). In flashing light, four different duty cycles (0.05, 0.1, 0.2, and 0.5)

and four different flash frequencies (5 Hz, 10 Hz, 50 Hz, and 100 Hz) were tested.

Photosynthesis–irradiance curve and respiration

The response to continuous light (photosynthesis–irradiance (PI) curve) is best described by the hyperbolic tangent model (Jassby and Platt 1976; Eq. 1): with the initial slope of the curve $\left(\alpha, \mu mol_{O_2} m^2 \mu mol_{ph}^{-1} g^{-1}\right)$ and the maximal net oxygen production rate $\left(P_{O_2,n}^{\max}, \mu mol_{O_2} s^{-1} g^{-1}\right)$. To account for oxygen consumption at PFDs below the compensation point, maintenance respiration $(R_{ms}, \mu mol_{O_2} g^{-1} s^{-1})$ was introduced in Eq. 1:

$$P_{O_2,n} = \left(\left(P_{O_2,n}^{\max} + R_{ms}\right) \cdot \tanh\left(\frac{\alpha \cdot PFD}{\left(P_{O_2,n}^{\max} + R_{ms}\right)}\right)\right) - R_{ms} \quad (1)$$

Algae respiration rate $(R, \mu mol_{O_2} g^{-1} s^{-1})$ can be determined with Eq. 2, with maintenance respiration $(R_{ms}, \mu mol_{O_2} g^{-1} s^{-1})$, net oxygen production rate $(P_{O_2,n}, \mu mol_{O_2} g^{-1} s^{-1})$, and a linear correlation factor ($c_R = 0.162$), which describes growth-associated respiratory activity. Maintenance respiration rate and linear correlation factor were determined by plotting the measured light respiration (i.e., postilluminated respiration, Kliphuis et al. (2011b)) versus the net oxygen production rate (Fig. 4a). Additionally, gross oxygen production rate $\left(P_{O_2,g}, \mu mol_{O_2} g^{-1} s^{-1}\right)$ can be defined as the sum of respiration and net oxygen production rate (Eq. 3):

$$R = R_{ms} + c_R \cdot P_{O_2,n} \quad (2)$$

$$P_{O_2,g} = P_{O_2,n} + R \quad (3)$$

Light measurement continuous light

The relative, spatial distribution of the incident PFD ($PFD_L^{sp}(i)$, micromole per square meter per second) was measured at nine different points (i) inside the measurement chamber of the BOM at the light-exposed surface and averaged into one relative PFD (PFD_L^{sp}, micromole per square meter per second). This measurement was done with an Avantes setup, which is a fiber optic CCD-based spectroradiometer (AvaSpec-2048 detector, Fiber FC-IR100-1-ME, 400–700 nm, Avantes, Eerbeek, The Netherlands). By dividing the PFD of position 9 ($PFD_L^{sp}(9)$, center position) with the average PFD (PFD_L^{sp}) a correlation factor cf was calculated.

The absolute PFD (PFD$_L$(9)) was measured at position 9 (center position) with a LI-COR 190SA 2π PAR (400–700 nm) quantum sensor (LI-COR, Lincoln, Nebraska). The absolute, average PFD (PFD$_L$) was calculated by
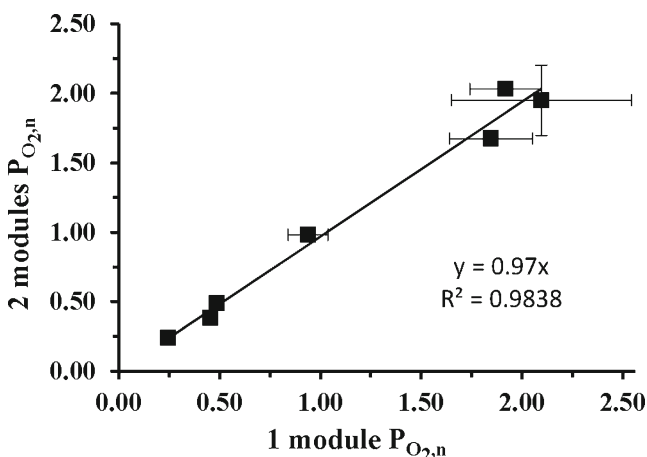

Fig. 2 Net oxygen production rate for two LED modules versus one LED module at different photon flux densities. *Error bars* represent the standard deviation from two measurements

y = 0.97x
R² = 0.9838

dividing the absolute PFD at position 9 ($PFD_L(9)$) with the correlation factor cf. In case of two LED modules, the total incident PFD was the sum of the PFD of each module.

The light transmission ($T(i)$, Eq. 4) was measured at three different positions behind the measurement chamber with the Avantes setup. First, a blank PFD ($PFD_{L,blank}^{sp}(i)$) was measured while the chamber was filled with medium, stirred, and illuminated. Second, an algae sample was injected and the PFD ($PFD_{L,algae}^{sp}(i)$) was measured again while the suspension was stirred and illuminated:

$$T(i) = \frac{PFD_{L,algae}^{(sp)}(i)}{PFD_{L,blank}^{(sp)}(i)} \tag{4}$$

In case of two modules, the light transmission was measured for each module.

Light measurement in flashing light

If LEDs are flashed with different frequencies, LED temperature and, as a result, LED light output can vary. For this reason, we measured the relative, time-averaged PFD ($PFD_{L/D}$) for each flashing light regime with the Avantes setup at position 9.

The integration time of the Avantes setup was set to 2 ms and a total of 2,500 measurements were averaged to obtain a stable signal in flashing light. First, a relative reference PFD ($PFD_L^{sp}(9)$) was measured for each LED module. For this measurement, the module was set to the flash intensity (continuous illumination). Second, a relative PFD ($PFD_{L/D}^{sp}(9)$) was measured for each flashing light setting. The absolute, time-averaged PFD ($PFD_{L/D}$, micromole per square meter per second) was then calculated according to Eq. 5:

$$PFD_{L/D} = PFD_L \cdot \frac{PFD_{L/D}^{sp}(9)}{PFD_L^{sp}(9)}. \tag{5}$$

This measurement was done for each module and the total PFD was the sum of both measurements.

Dry weight determination

For dry weight measurement, Whatman GF/F glass microfiber filters (Ø 55 mm, pore size=0.7 μm) were dried (24 h, 95 °C), cooled down to room temperature in a desiccator (> 2 h), and weighed. The sample (<5 mg dry weight) was filtrated with a constant vacuum (0.44 bar, absolute), washed three times with Milli-Q water, dried (24 h, 95 °C), cooled down in a desiccator (> 2 h), and weighed again. The weight difference between empty and algae-containing filter was the dry weight. Each sample was measured in triplicate.

Oxygen yield on absorbed light energy

The biomass-specific photon absorption rate ($r_{ph, abs}$, $\mu mol_{ph} g^{-1} s^{-1}$) was calculated based on Eq. 6:

$$r_{ph,abs} = PFD_L \cdot A_{BOM} \cdot \left(1 - \left(\sum_{i=1}^{3} T(i)\right) \cdot 3^{-1}\right) \cdot (c_x \cdot V_{BOM})^{-1} \tag{6}$$

with:

- Incident PFD (PFD_L, continuous light),
- The illuminated surface of the BOM (A_{BOM}, $7.55 \times 10^{-4} m^2$),
- The light transmission ($T(i)$),
- The biomass concentration (c_x, g m^{-3}), and
- The volume of the BOM (V_{BOM}, $1.2 \times 10^{-5} m^3$).

If two modules were used, then the photon absorption rate was calculated for each module separately and the total photon absorption rate was the sum of both calculations.

The photon absorption rate for continuous light showed a linear correlation with the incident PFD (Fig. 3). This linear correlation was used to determine the photon absorption rate for flashing light based on the measured, time-averaged PFDs.

The net (n) oxygen yield ($Y_{O_2/ph,n}$, $mol_{O_2} mol_{ph}^{-1}$) or gross (g) oxygen yield ($Y_{O_2/ph,g}$, $mol_{O_2} mol_{ph}^{-1}$) on absorbed photons is the quotient of net (n) oxygen production rate ($P_{O_2,n}$, $\mu mol_{O_2} g^{-1} s^{-1}$) or gross (g) oxygen production rate ($P_{O_2,g}$, $\mu mol_{O_2} g^{-1} s^{-1}$) and biomass-specific light absorption rate ($r_{ph,abs}$, Eq. 7):

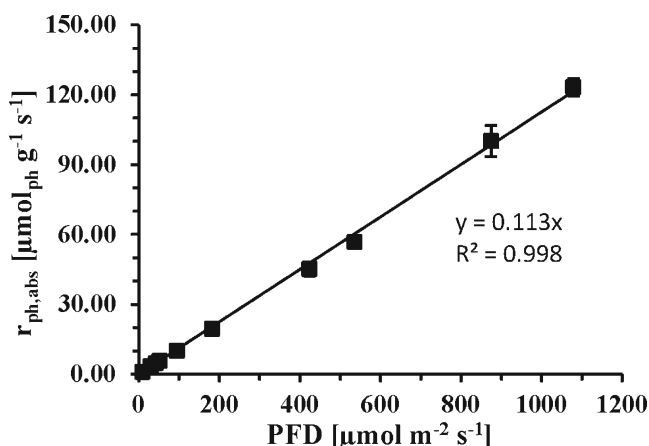$$Y_{O_2/ph,z} = \frac{P_{O_2,z}}{r_{ph,abs}}, z \in [n, g]. \tag{7}$$

Fig. 3 Biomass-specific photon absorption rate versus incident photon flux density in continuous light

Results

The first 200 h of PBR operation were necessary to reach steady-state, followed by a steady state operation of about 800 h. The average biomass-specific growth rate during the steady state was constant at 0.059 ± 0.002 h^{-1} and a biomass concentration of 0.095 ± 0.005 g L^{-1}. The average dry weight-specific absorption coefficient (see Vejrazka et al. 2011) was 0.21 ± 0.01 m^2 g^{-1} (determined after 250 h, 450 h, and 800 h of steady state). The resulting biomass yield on absorbed light energy was 0.99 ± 0.08 g$_{bm}$ mol$_{ph}^{-1}$. Based on growth stoichiometry and given elemental composition of the biomass (Vejrazka et al. 2011), the biomass yield could be recalculated into an net oxygen yield on absorbed light energy: 0.046 ± 0.004 $mol_{O_2} mol_{ph}^{-1}$.

Photosynthesis–irradiance curve in continuous light

To evaluate the effect of flashing light on oxygen production rate or oxygen yield on absorbed light energy, reference points under continuous illumination are necessary. Therefore, we measured a PI curve of *C. reinhardtii* under continuous illumination.

The hyberbolic tangent model fits the measured PI cruve well (Fig. 4b). The net oxygen production rate was zero at a PFD of 11 μmol m^{-2} s^{-1} which is similar to the compensation point (10 μmol m^{-2} s^{-1}) determined by Takache et al. (2010). After the compensation point, the net oxygen production rate increases linearly with increase in PFD until a PFD of about 100 μmol m^{-2} s^{-1}. With further increase in PFD, the net oxygen production rate becomes saturated and reaches its maximum (1.87 ± 0.20 $μmol_{O_2} s^{-1} g^{-1}$) at about 500 μmol m^{-2} s^{-1}. This maximal net oxygen production rate can be converted to a maximal-specific growth rate based on the growth stoichiometry and elemental composition shown

in Vejrazka et al. (2011). The maximal growth rate results in 0.14 ± 0.02 h^{-1} which is comparable to the measured value in Vejrazka et al. (2011).

The light respiration rate is linearly correlated with the net oxygen production rate (Fig. 4a) which is in agreement with Falkowski et al. (1985) and Geider and Osborne (1989). The intercept with the y-axis (Fig. 4a) results in the maintenance respiration rate (R_{ms}) which was 0.11 $μmol_{O_2} s^{-1} g^{-1}$. This maintenance respiration rate is similar to the rate (0.10 $μmol_{O_2} s^{-1} g^{-1}$) Kliphuis et al. (2011b) determined for *Chlorella sorokiniana* but differs from the rate (0.15 $μmol_{O_2} s^{-1} g^{-1}$) Kliphuis et al. (2011a) calculated for *C. reinhardtii* based on a metabolic growth model. Although, the maintenance respiration determined by Kliphuis et al. (2011a) is not exactly the same as in this study, it is in the same range. The difference might be due to the two completely different methods used to determine this parameter.

Based on the photon absorption rate and the oxygen production rate (gross rate and net rate), oxygen yield on absorbed photons (gross yield and net yield) was calculated (Fig. 5). Net oxygen yield increases with increase in PFD and reaches its maximum between 100 μmol m^{-2} s^{-1} and 200 μmol m^{-2} s^{-1}. Further increase in PFD results in a decrease in net oxygen yield. The low yield at low PFDs (<100 μmol m^{-2} s^{-1}) can be explained by the influence of maintenance respiration (i.e., dark respiration): the lower the oxygen production resulting from photosynthesis, the greater the relative part which has to be used for maintenance respiration is. At high PFDs (>500 μmol m^{-2} s^{-1}) photon absorption rate exceeds the maximal photon utilization rate, and, as a result, the surplus light energy must be dissipated.

The influence of respiration is best demonstrated by calculating the gross oxygen yield (Fig. 5, open squares) and comparing it to the net yield. The gross yield reflects the actual rate of linear electron transport by photosynthesis in the chloroplast. Data in Fig. 5 shows that this yield is slightly
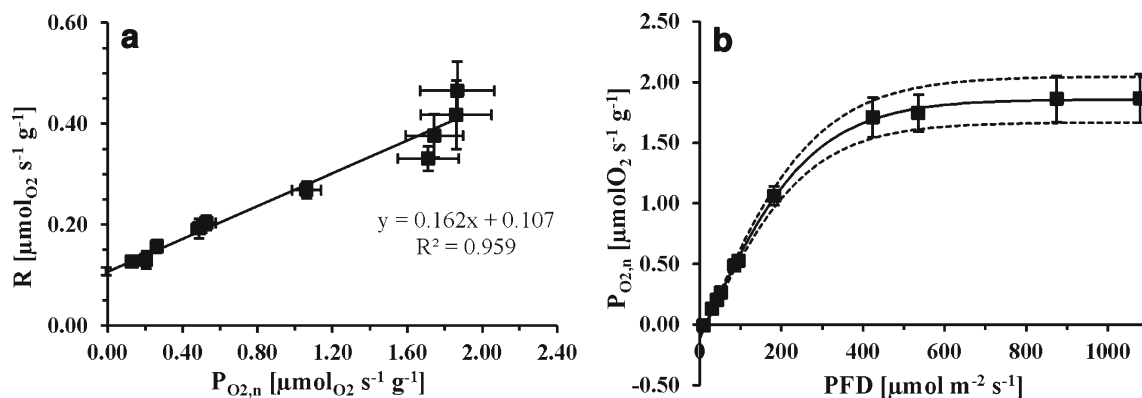

Fig. 4 **a** Light respiration versus net oxygen production rate. *Filled squares* measured values, *line* linear fit ($c_R = 0.162$, $R_{ms} = 0.107$ $μmol_{O_2} s^{-1} g^{-1}$). **b** Net oxygen production rate versus incident photon flux density. *Filled squares* measured values, *line* and

dashed line fit based on hyperbolic tangent model (Eq. 1), model parameters: $P_{O_2,n}^{max} = 1.86 \pm 0.19 μmol_{O_2} s^{-1} g^{-1}$, $\alpha = 0.0073 \pm 0.0004 μmol_{O_2} m^2 μmol_{ph}^{-1} g^{-1}$, $R_{ms} = 0.107$ $μmol_{O_2} s^{-1} g^{-1}$. *Error bars* represent the standard deviation of at least three measurements
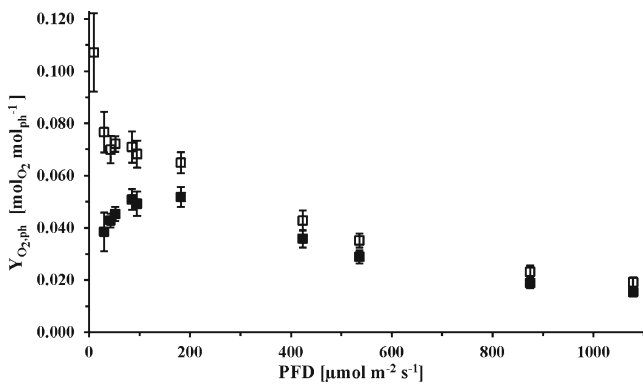
Fig. 5 Oxygen yield on absorbed photons versus incident photon flux density. *Filled squares* net yield, *open squares* gross yield (i.e., direct yield of photosystem II)

decreasing until a PFD of about 200 μmol m^{-2} s^{-1} and then it decreases more rapidly. This rapid decrease indicates that more photons are being absorbed than can be used and excess energy must be dissipated. The gross yield at 10 μmol m^{-2}s^{-1} shows a big standard deviation because measureable net oxygen production rate was around zero.

Flashing light

In total, four different duty cycles and four different flash frequencies were tested. The results of the measured net oxygen production rates ($P_{O_2,n,L/D}$) are summarized in Table 2. If only one duty cycle is considered, then the oxygen production rate

increases with increase in flash frequency, which is in agreement with other studies (Phillips and Myers 1954; Terry 1986; Vejrazka et al. 2011; Xue et al. 2011). Furthermore, if only a specific flash frequency is considered, then the net oxygen production rate increases with increase in duty cycle, which was expected because an increase in duty cycle coincides with an increase in time-averaged PFD.

To evaluate the amount of light integration in flashing light, the net oxygen production rate for continuous illumination with the time-averaged PFD has to be calculated with the hyperbolic tangent model shown in Eq. 1.

For this calculation, it is necessary to know exactly the measured, time-averaged PFD of one L/D cycle (Table 2, PFD$_{L/D}$). Measurements showed that especially at a frequency of 50 Hz and 100 Hz, the time-averaged PFD increased in comparison to 5 Hz and 10 Hz. However, also at the lower flash frequencies, the time-averaged PFD was higher than expected based on duty cycle and flash PFD. This increase in time-averaged PFD was caused by the LEDs heating up less while flashing compared to continuous light. At lower temperatures, LEDs convert more electricity into light.

As a result of the different, time-averaged PFD, the calculated net oxygen production rate for equivalent continuous illumination is not the same for one duty cycle, which was accounted for in the calculation of the reference oxygen production rates based on light integration (Table 2, $P_{O_2,n}$). A comparison of these rates with the measured rates from flashing light shows that the rate in flashing light does not exceed the rate in continuous light (based on light

Table 2 Results of oxygen evolution measurements in flashing light, and values in brackets are standard deviations for triplicate measurements

Φ	f (Hz)	t_l (ms)	t_d (ms)	PFD$_{L/D}$ (μmol m^{-2} s^{-1})	$P_{O_2,n,L/D}$ (μmol$_{O_2}$ s^{-1} g^{-1})	$P_{O_2,n}$ (μmol$_{O_2}$ s^{-1} g^{-1})
0.05	5	10	190	58	0.22 (0.02)	0.31 (0.03)
	10	5	95	58	0.27 (0.01)	0.31 (0.03)
	50	1	19	67	0.37 (0.01)	0.37 (0.03)
0.1	5	20	180	113	0.46 (0.02)	0.67 (0.05)
	10	10	90	114	0.55 (0.05)	0.68 (0.05)
	50	2	18	118	0.70 (0.04)	0.70 (0.05)
	100	1	9	132	0.80 (0.01)	0.78 (0.06)
0.2	5	40	160	227	0.82 (0.03)	1.24 (0.10)
	10	20	80	227	0.87 (0.07)	1.24 (0.10)
	50	4	16	232	1.02 (0.07)	1.26 (0.10)
	100	2	8	238	1.36 (0.02)	1.28 (0.10)
0.5	5	100	100	559	1.55 (0.04)	1.80 (0.17)
	10	50	50	559	1.45 (0.04)	1.80 (0.17)
	50	10	10	557	1.59 (0.15)	1.79 (0.17)
	100	5	5	561	1.66 (0.01)	1.80 (0.17)

integration). These results are in agreement with the hypothesis of Rabinowitch (1956) that the photosynthetic rate in flashing light does not exceed the one under continuous illumination with the same time-averaged PFD .

To properly asses the amount of light integration, it is possible to calculate an integration factor (IF, Eq. 8) similar to the one proposed by Terry (1986). This IF is based on the measured net oxygen production rate in flashing light ($P_{O_2,n,L/D}$, Table 2), net oxygen production rate based on growth integration ($P_{O_2,n,gi}$, Eq. 9) and net oxygen production rate based on light integration ($P_{O_2,n}$, Table 2). Oxygen production for growth integration (Eq. 9) is based on the assumption that algae photosynthesis during the flash is equivalent to the rate under continuous illumination with the flash PFD. Furthermore, it is assumed that maintenance respiration occurs during the dark part of the cycle:

$$IF = \frac{P_{O_2,n,L/D} - P_{O_2,n,gi}}{P_{O_2,n} - P_{O_2,n,gi}} \qquad (8)$$

$$P_{O_2,n,gi} = \phi \cdot P_{O_2,n} - (1-\phi) \cdot R_{ms}. \qquad (9)$$

The results of the IF are shown in Fig. 6: an IF of one equals full light integration and an IF of zero equals growth integration. During all tested L/D cycles, the rate of photosynthesis was above the rate expected for solely growth integration which is in agreement with observations that subsecond L/D cycles are necessary to achieve (partial) light integration.

In general, data shows that the amount of light integration depends on the duty cycle and also on flash frequency. A flash frequency of 100 Hz resulted in full light integration except for the duty cycle of 0.5. At this duty cycle, full light integration was not achieved at any frequency tested. A frequency of 50 Hz led to full light integration at a duty cycle of 0.05 and 0.1 but not at 0.2 as observed for 100 Hz. Frequencies below
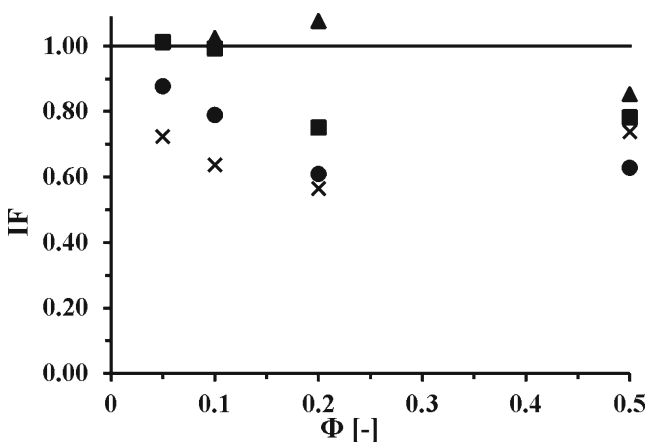

Fig. 6 Integration factor based on net oxygen evolution rates versus duty cycle for four selected frequencies, *crosses f*=5 Hz, *filled circles f*=10 Hz, *filled squares f*=50 Hz, *filled triangles f*=100 Hz
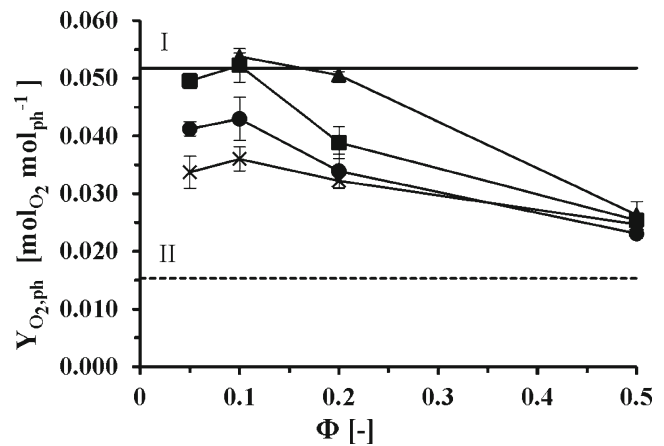

Fig. 7 Oxygen yield on absorbed light energy versus duty cycle for four selected frequencies, *crosses f*=5 Hz, *filled circles f*=10 Hz, *filled squares f*=50 Hz, *filled triangles f*=100 Hz, *I* maximal yield measured under continuous illumination, *II* yield measured at 1,079 μmol m^{-2}s^{-1}, which is the maximal yield expected for growth integration

50 Hz did not result in full light integration at any duty cycle but resulted in partial light integration, whereas the amount of light integration decreased with increase in duty cycle and decrease in flash frequency. An exception can be found for the duty cycle of 0.5, where light integration increased again in comparison to a duty cycles of 0.2 and 0.1.

In this study, oxygen yield on absorbed photons was determined (Fig. 7), which, so far, has not been reported for flashing light experiments in a BOM. The dependency of oxygen yield on duty cycle is shown in Fig. 7. The highest yield for each flash frequency was found at a duty cycle of 0.1, which relates to a time-averaged PFD of about 115 μmol m^{-2}s^{-1}. A lower duty cycle resulted in a decrease in yield which is similar to the observation in continuous light with PFDs below 100 μmol m^{-2}s^{-1}. In contrast to continuous light, where a high yield was also observed for a PFD of 200 μmol m^{-2}s^{-1}, a duty cycle of 0.2 (i.e., time-averaged PFD of 230 μmol m^{-2}s^{-1}) resulted in a decrease in yield except for the frequency of 100 Hz. The yield depended on the duty cycle and also on the flash frequency: the higher the flash frequency, the higher the yield. The lowest yields were determined for the highest duty cycle, where the flash frequency did not have an influence anymore.

Discussion

The maintenance respiration rate (R_{ms}) in continous light was $0.11 \, \mu mol_{O_2}s^{-1}g^{-1}$ as determined by the linear correlation between light respiration and net oxygen production rate. Each oxygen evolution measurement was started with a dark acclimaton period of 5 min followed by an intial maintenance respiration measurement of 10 min. The intial maintanance respiration measurement of each experiment

resulted on average in $0.10 \pm 0.02 \ \mu mol_{O_2} s^{-1} g^{-1}$, which is the same as derived from the data fit of Eq. 2. The intial maintanance respiration was measured at low dissolved oxygen concentrations: typically 15 % to 30 % air saturation. If the maintanance respiration of dark acclimated algae was measured at higher initial dissolved oxygen concentrations, then it resulted in $0.25 \pm 0.03 \ \mu mol_{O_2} s^{-1} g^{-1}$ at 100 % air saturation. This result was independent from the time of measurement: intial maintanance respiration or maintanance respiration after exposure to different PFDs inside the BOM, but only dependent on dissolved oxygen concentration. In other words, there is a dependency of maintanance respiration on dissolved oxygen concentration. However, at this moment, it is not clear what caused this dependency.

Maintanance respiration is a part of respiratiory activity during photosynthesis in the light. If this respiration is dependent on dissolved oxygen concentration, then it could add to the uncertainty of the oxygen evolution measurement. However, this uncertainy is not controllable because dissolved oxygen cannot be kept constant during the measurement, and therefore, each point of interest was measured at least as a triplicate.

The oxygen yield on absorbed light energy in continuous light is influenced by maintenance respiration. At a PFD below $100 \ \mu mol \ m^{-2} s^{-1}$, the yield decreased due to a relative increase in maintenance (absolute maintenance is constant) to support cell metabolism. The highest net oxygen yield in continuous light was observed between a PFD of $100 \ \mu mol \ m^{-2} s^{-1}$ and $200 \ \mu mol \ m^{-2} s^{-1}$. This range of PFDs is surprising because photosynthesis starts to become saturated already at $200 \ \mu mol \ m^{-2} s^{-1}$, which was confirmed by a decrease in gross yield at a PFD of $200 \ \mu mol \ m^{-2} s^{-1}$. As a result, the maximal yield would have been expected around $100 \ \mu mol \ m^{-2} s^{-1}$, where oxygen production rate still increases linear with PFD. Therefore, the influence of maintenance-associated respiration should not be underestimated at PFDs below $200 \ \mu mol \ m^{-2} s^{-1}$.

The flashing light experiments show that full light integration based on net oxygen production rate is only possible at high frequencies (>50 Hz) and there is strong dependency on the duty cycle that determines the time-averaged PFD. The duty cycles of 0.05 and 0.1 are favorable in terms of light integration and also with low flash frequencies, whereas at a duty cycle of 0.5, the flash frequency has almost no influence on the absolute amount of light integration. In summary, presented data show that for full light integration, the light flash should not be longer than 2 ms with a sufficient dark time not exceeding 20 ms.

Similar to the oxygen production rate, the oxygen yield was always higher than the yield expected for growth integration (i.e., yield at $1,079 \ \mu mol \ m^{-2} s^{-1}$ continuous light), and the yield under flashing light did not exceed the maximal yield measured under continuous light.

Data from this study show that light integration based on photosynthetic rate should not be the only criteria to evaluate the effect of flashing light. Based on the IF, it could be concluded that the duty cycles of 0.05 and 0.5 showed the highest level of light integration. However, from the yield data, it can be concluded that highest yields on light can be expected for a duty cycle of 0.1. In addition to the dependency of the yield on the duty cycle, our data also showed that for duty cycles below or equal to 0.2, the yield decreased with decrease in flash frequency. In summary, the duty cycle should be adjusted that the time-averaged PFD is photosynthetic rate limiting to achieve the highest yields.

Both oxygen production rate and yield data in flashing light show a similar trend than continuous illumination with the time-averaged PFD: if the incident PFD (continuous light) or time-averaged PFD (flashing light) is subsaturating, then the highest yields can be observed in continuous and in flashing light. However, higher oxygen production rates will be observed with higher duty cycles, whereas yields will decrease. If the PFD is too low (<100 $\mu mol \ m^{-2} s^{-1}$), then the yield will also decrease because a relatively large fraction of light is needed to fulfill maintenance requirements. Therefore, the application of proper L/D cycles (and duty cycles) can lead to an increase in light conversion efficiency even at flash frequencies of 5 Hz and 10 Hz, which can be found in current PBR designs (Luo et al. 2003; Perner-Nochta and Posten 2007; Pruvost et al. 2006).

The presented experiments represent a simplification of L/D cycles in PBRs. It was assumed that the incident PFD rather than local PFDs along the gradient or spatial average PFD of a gradient determines photosynthetic rate and oxygen yield (or biomass yield) on absorbed light energy. Under light attenuation conditions (i.e., high biomass density), which are relevant for commercial PBR operation, the light gradient, especially under oversaturating incident PFD, might lead to an increase in yield due to reduced exposure time to the incident-oversaturating PFD. Thus, less improvement in both rate and yield might be obtained by the application of L/D cycles. Nevertheless, this simplification is an important step to understand the fundamental influence of flashing light and to improve mathematical models which can describe this influence. These models should include a mechanistic approach to be applicable for a wide range of L/D cycles and different incident PFDs. Based on these models, it will be possible to find the optimal configuration of duty cycle and flash frequency to optimize yield and photosynthetic rate for a given PBR system and location.

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