

# Photosynthesis and growth kinetics of *Chlorella vulgaris* R-117 cultured in an internally LED-illuminated photobioreactor

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## Abstract

The aim of this work was to correlate changes of photosynthesis activity vs. growth in *Chlorella vulgaris* R-117 (CCALA 1107), fast-growing and robust microalga cultured in an internally illuminated 10-L photobioreactor (PBR). The cultures were grown at high output irradiance provided by four LED light sources submerged in the culture when the light path was short, between 25–30 mm. The culture of *Chlorella* R-117 grown under 2,500  $\mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$  attained a doubling time of 3.5 d and biomass density of 3.5  $\text{g}(\text{DM}) \text{L}^{-1}$  after about 10-d period. When grown under 3,500  $\mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$ , the culture reached a doubling time of 1.7 d, and biomass density of  $\sim 5.5 \text{ g L}^{-1}$  before entering the stationary phase. Electron transport rate changes correlated well with the culture growth demonstrating the usefulness of chlorophyll fluorescence for photosynthesis monitoring. This can be crucial for potential scale-up to large indoor PBRs to optimise culture growth.

*Additional key words:* chlorophyll fluorescence; light-response curves; microalgae; OJIP induction kinetics; pigments.

## Introduction

Dense, well-mixed culture of microalgae [ $> 0.5 \text{ g}(\text{biomass}) \text{L}^{-1}$ ] with sufficient nutrition and gas exchange is completely different from optically thin natural phytoplankton populations. In principle, two basic approaches are used to grow microalgal mass cultures: the first applies to growth in ‘open’ reservoirs, while the second represents ‘closed’ systems – photobioreactors (PBRs, closed or semiclosed vessels with no direct contact between the culture and atmosphere). A variety of PBRs – using either natural or artificial illumination – have been designed to grow microalgae mass cultures phototrophically. These can consist of glass or transparent plastic tubes, columns or panels, positioned horizontally or vertically, arranged as serpentine loops, fences, flexible coils, or as a series of panels or columns in which the microalgae suspension is continuously mixed or circulated (for a review see e.g. Tredici 2004, Carvalho *et al.* 2006, Zittelli *et al.* 2013, Masojidek *et al.* 2015, Sergejevová *et al.* 2015, Acien *et al.* 2017).

PBRs can be illuminated naturally or using artificial

light sources. The latter – filament bulbs, fluorescent tubes or high-intensity discharge lamps (pressure sodium lamps) – have been used in indoor PBRs since the early days of microalgae biotechnology (for an overview see Pulz *et al.* 2013). In order to reduce the loss of light and to increase efficiency of artificial lighting, PBRs with internal illumination have been designed for microalgae culturing (e.g. Radmer 1989, Hirata *et al.* 1996, Ogbonna *et al.* 1996, 1999; An and Kim 2000, Csögör *et al.* 2001, Suh and Lee 2001, Gordon 2002, Chen and Chang 2006, Chen *et al.* 2006a,b, 2008; Chiang *et al.* 2011, Choi *et al.* 2011, Wang *et al.* 2014, Sergejevová *et al.* 2015). Moreover, the use of continuous illumination is advantageous since biomass losses due to respiration in diurnal dark periods can be avoided. One important point is to optimize illumination intensity, light path, and effective mixing to supply cells with light and nutrients evenly and facilitate efficient gas exchange.

Starting in the 1990s, novel light sources – light-emitting diodes (LED) – have been employed in laboratory panel or column PBRs for microalgae growth (e.g. Lee and Palsson 1994, 1995, Nedbal *et al.* 1996,

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*Abbreviations:* Car – carotenoid; Chl – chlorophyll; DM – dry mass; E – irradiance;  $F_0$ ,  $F_v$ ,  $F_m$  – minimal, variable, and maximal fluorescence in dark-adapted state;  $F'$ ,  $F_m'$  – steady-state and maximal fluorescence in light-adapted state;  $F_v/F_m$ ,  $\Delta F/F_m'$  – maximal, resp. actual photochemical yield of PSII; L/D cycle – light-dark cycle; LED – light-emitting diode; LED-PBR-10 – 10-L photobioreactor illuminated by light-emitting diodes; OD – optical density; OJIP curve – fast Chl fluorescence induction kinetics; P – biomass productivity; PAM – pulse-amplitude modulation; PBR – photobioreactor; rETR – relative electron transport rate through PSII; RLC – rapid light-response curve.

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2012, Cuaresma *et al.* 2009, Jacobi *et al.* 2012). Since, LED technology has been continuously improved over the last decade. In comparison to other lighting systems, LEDs have relatively low construction and operational costs, can operate with low electrical voltage and current, and due to its miniature size, can be easily fitted into any PBR design (Yam and Hassan 2005). The application of LEDs in PBRs represents a great advantage over existing indoor lighting as it allows the grower to control the light intensity (and frequency) in optimizing illumination.

In microalgae cultivation, the use of some monitoring technique (oxygen production, fluorescence) is advantageous to provide rapid and real-time control of culture physiological status (Havlik *et al.* 2013). When growing, microalgae cells change their photosynthetic activity according and hence this is a good indicator of growth (Malapascua *et al.* 2014). Since the mid-1980s chlorophyll (Chl) fluorescence has been used in assessing photosynthesis and physiological changes of microalgae mass cultures in various PBRs (Walker 1985, Schreiber *et al.* 1986, Vonshak *et al.* 1994, Torzillo *et al.* 1996, 1998; Figueroa *et al.* 1997, 2013; Maxwell and Johnson 2000, Kromkamp and Foster 2003, Ralph and Gademann 2005, Bischof *et al.* 2006, Obata *et al.* 2009, Masojidek *et al.* 2011a, White *et al.* 2011, Malapascua *et al.* 2014). One of the commonly used fluorescence techniques, pulse-amplitude-modulation (PAM), gives information on the balance between photosynthetic electron transport chain, dissipation of absorbed energy and the Calvin-Benson cycle. The so-called rapid light-response curves (RLC) of relative electron transport (rETR) show the dependency of photosynthesis on the irradiance intensity (*e.g.* Kromkamp *et al.* 1998, Ralph and Gademann 2005, Enriquez and Borowitzka 2010, Malapascua *et al.* 2014). It can be used to estimate photosynthetic performance and growth of microalgae cultures (Malapascua *et al.* 2014). The other approach is to examine fast fluorescence induction kinetics characterized by the so-called OJIP curve that provides information on the activity of the acceptor side of PSII and other partial electron transport events and the effective antenna size (Strasser *et al.* 2004, Ritchie 2008, Stirbet and Govindjee 2011).

In the present study, we correlated growth kinetics and photosynthesis changes (using Chl fluorescence monitoring techniques) of the microalga *Chlorella vulgaris*, strain R-117, in a column photobioreactor with high-intensity internal LED light sources submerged directly into culture. This is beneficial for potential scale-up to large indoor PBRs where monitoring of photosynthetic performance of microalgae cultures can be crucial to optimise growth.

## Materials and methods

**Strains and culture conditions:** The fast-growing and robust microalga *Chlorella vulgaris*, strain R-117 (CCALA 1107; Culture Collection of Autotrophic Organisms, Institute of Botany, Třeboň, Czech Republic), further as *Chlorella* R-117, was cultivated in a modified (double concentrated) inorganic medium, pH 7.4, containing the following compounds (in g L<sup>-1</sup>): KNO<sub>3</sub>, 4.04; KH<sub>2</sub>PO<sub>4</sub>,

0.68; MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.98, and some other micro- and trace compounds – ferric-sodium chelatonate, CaCl<sub>2</sub>, H<sub>3</sub>BO<sub>3</sub>, MnCl<sub>2</sub>·4 H<sub>2</sub>O, ZnSO<sub>4</sub>·7 H<sub>2</sub>O, CuSO<sub>4</sub>·5 H<sub>2</sub>O, CoSO<sub>4</sub>·7 H<sub>2</sub>O, (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, and NH<sub>4</sub>VO<sub>3</sub> (Šetlík *et al.* 1972, Zachleder and Šetlík 1982, Babaei *et al.* 2017). This nutrient-rich medium was used in order to maintain sufficient growth up to about 8 g of biomass per litre (contains about 7–8% nitrogen and 1–2% phosphorus). *Chlorella* R-117 was used in this trial due to its high growth rates and tolerance to high irradiance tested in outdoor cultures (Masojidek *et al.* 2011b). The light-acclimated stock cultures were grown in 2-L flat Roux bottles which were submerged in temperature-controlled water bath (30°C), mixed by bubbling air +1.5% CO<sub>2</sub> (v/v) and exposed to continuous illumination using day-light fluorescent lamps at an irradiance of about 150–250 μmol(photon) m<sup>-2</sup> s<sup>-1</sup>. For experiments the cells were harvested at mid-logarithmic phase of growth by centrifugation at 2,000 × g for 4 min and resuspended in the fresh medium.

### Photobioreactor with internal LED illumination:

A double-jacketed glass column (*B. Braun Biotech*, Germany) was used as a vertical vessel to set up a model photobioreactor, designated as LED-PBR-10 (Fig. 1A). The working volume was about 10 L (an internal diameter of 190 mm and a total height of 400 mm). The four specially constructed LED sources (*see* insert in Fig. 1A) were mounted vertically through the lid of the PBR and submerged in microalgae culture. To provide uniform illumination, the light sources were arranged 25 mm apart inside the PBR and ~30 mm from the inner wall (Fig. 1B). Each of the internal light sources (with a total power input of either 2 × 35 W + 2 × 80 W, or 4 × 80 W) was made of a rectangular aluminium rod (500 mm long, 10 × 10 mm profile) with a 300-mm long LED strips fastened along all four sides. The LED holder was then inserted into a glass tube (inner diameter of 37 mm) with a closed bottom end. Two types of LED strips – neutral-white and warm-white were combined (*see* an insert in Fig. 1A). In this arrangement all light was evenly dispersed in the microalgae suspension. Irradiance intensity could be adjusted using a voltage regulator. In cultivation trials continuous illumination was provided either of about 2,500 μmol(photon) m<sup>-2</sup> s<sup>-1</sup> (Trial A) or of about 3,500 μmol(photon) m<sup>-2</sup> s<sup>-1</sup> (Trial B). Important to note that these high light intensities (PAR) were measured directly at the outer surface submerged in water.

The production of heat by LEDs was rather low; necessary cooling of the light sources was maintained by heat exchange with microalgae culture which was controlled through the double jacket of the cultivation vessel connected to a thermo-regulated water circulation system. A stream of filtered air (+1.5% CO<sub>2</sub>) was bubbled from a stainless steel loop with evenly-spaced perforations (0.5 mm, 10 mm apart) placed at the vessel bottom maintaining mixing, sufficient supply of nutrients and the removal of oxygen produced *via* photosynthesis. Additionally, magnetic bar mixing can guarantee sufficient turbulence to maintain averaged cell irradiance. In this way, short light-dark cycles are produced which are crucial for

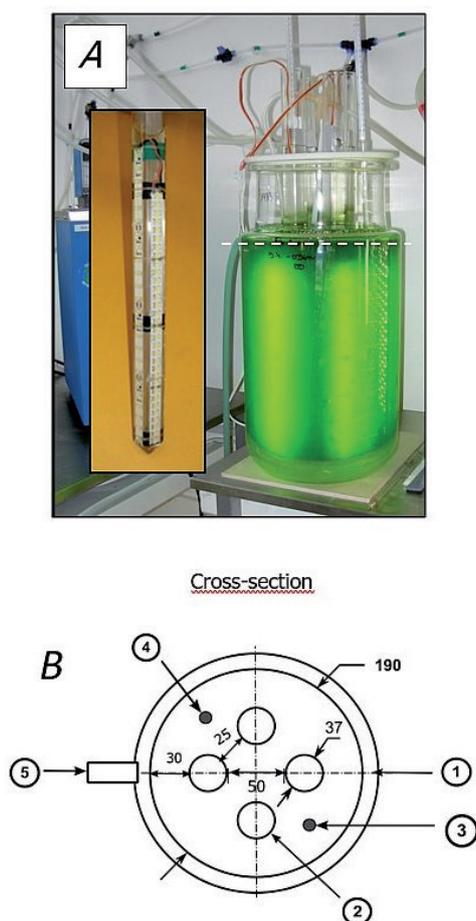


Fig. 1. (A) Column photobioreactor, LED-PBR-10 with internal illumination used in trials. The PBR consists of a double-jacketed, glass column (with a working volume of 8 L) and a total height of 400 mm. (Insert: Image of one of four tubular light LED sources that were submerged in the microalgae culture. Neutral-white and warm-white, high intensity LED strips (300 mm long) were evenly combined and glued along all four sides of a rectangular aluminium rod; the holder was then inserted into a glass tube of inner diameter of 37 mm with a closed bottom end.) (B) Cross-sectional view of a lid of the PBR with an internal diameter of 190 mm (1), in which four tubular light sources are mounted and submerged to microalgae culture (2). The stream of filtered air (containing 1.5% CO<sub>2</sub>) is supplied by a loop (3) to mix and supply CO<sub>2</sub> to cells and remove oxygen produced in photosynthesis (4). The temperature is controlled via the water circuit of a temperature controller connected to a double-jacket (5) of the cultivation vessel.

high photosynthetic activity (Zarmi *et al.* 2013). A slight overpressure inside the cultivation vessel was maintained (~10 kPa) that prevented microalgae culture from external contamination. All inputs for medium, air supply and temperature and pH sensors were piped through the lid of the PBR. The thickness of photic microalgae layer (light path) varied between 25 and 30 mm.

**Growth and pigment analyses:** Biomass density was measured as dry mass (DM) by filtering 5 ml of culture samples to preweighed fibre-glass filters (GC-50), which

were dried in an oven at 105°C for 3 h and then transferred to a desiccator to equilibrate at laboratory temperature and weighed. The growth rate [ $\mu = (\ln X_2 - \ln X_1)/\Delta t$ ; d<sup>-1</sup>] and doubling time ( $t_D = \ln 2/\mu$ ; d) of the culture were calculated.

The content of total Chls and carotenoids (Car) was determined in 80% acetone by breaking microalgae cells by intensive abrasion with glass beads using a vortex mixer for 2 min. The supernatant containing pigments was collected after centrifugation. If necessary, the extraction was repeated several times until the pellet was clear of pigments. The absorbance of the combined supernatants of all extraction steps was measured using a high resolution spectrophotometer (UV 2600 UV-VIS, Shimadzu, Japan; slit width of 0.5 nm) and the concentrations of pigments were calculated according to Wellburn (1994).

**Nutrient analysis:** Content of nutrients (nitrate, sulphate, and phosphate) in the culture medium were analysed during trials using an ion chromatography system (ICS-90, Dionex fitted with an AS22-Fast 4 × 150 mm, Dionex IonPac™ column). The samples were diluted twice with deionized water and 10- $\mu$ L aliquots were used for the analysis. The solutions of 1.4 mM NaHCO<sub>3</sub> and 4.5 mM Na<sub>2</sub>CO<sub>3</sub> were used as mobile phase for isocratic selection of compounds according to charge and molecular dimension. A solution (CZ 9102-IC MIX 22, Analytika, Ltd., Czech Republic) of known anion concentrations was used as standard to construct the calibration curve. Nutrient concentrations were calculated in mg L<sup>-1</sup> and expressed as percentage of the initial concentration (% of initial).

**Chl fluorescence measurements:** Samples were taken from the cultures and measured in triplicates (data presented as mean  $\pm$  SE) at specific time points. Before measurement, the cultures were diluted to 0.2 to 0.3 g(DM) L<sup>-1</sup> [corresponding to 5 to 7 mg(Chl) L<sup>-1</sup>] with growth medium, dark-adapted for 5–10 min, and then transferred to measuring chamber. Measurements were carried out under well-defined laboratory conditions with similar ‘light’ exposure history to avoid modifying the photoacclimation status of the cells. In this way, we prevented re-absorption problems with a dense culture by providing sufficient illumination in the dark-acclimated samples (with an oxidized plastoquinone pool). The fluorescence nomenclature in this paper follows Schreiber *et al.* (1986) as later elaborated by van Kooten and Snel (1990) and Kromkamp and Foster (2003).

**Rapid light-response curves (RLC)** of microalgae samples were measured using a pulse-amplitude-modulation fluorimeter (PAM-2500, H. Walz, Germany) in a light-protected measuring chamber with mixing (3-mL glass cuvette, light path of 10 mm). A series of stepwise increasing irradiance intensities [red LEDs; 0–2,700  $\mu$ mol(photon) m<sup>-2</sup> s<sup>-1</sup>] were applied in 20-s intervals to obtain the steady-state fluorescence level  $F'$  in the light-adapted state at respective irradiance level and then a saturating pulse [ $>10,000 \mu$ mol(photon) m<sup>-2</sup> s<sup>-1</sup>, 0.6 s duration] was triggered to reach the  $F'_m$ , the maximal fluorescence level. At each step the actual PSII photochemical quantum

yield in the light,  $Y_{II}$  was determined as  $(F_m' - F)/F_m'$ . The minimum and maximal fluorescence level ( $F_o$ ,  $F_m$ ) were determined using a weak modulated light [ $<0.15 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$ , frequency of 0.5–1 kHz] in the dark-adapted samples (actinic irradiance = 0; first step of RLC). The maximal PSII quantum yield was calculated as the ratio of variable and maximal fluorescence,  $F_v/F_m = (F_m - F_o)/F_m$ ; it indicates the capacity of the system to absorb light through the reaction centres and the light-harvesting complex and expresses the maximum quantum efficiency of primary photochemistry (Strasser *et al.* 2004). Analysis of RLC was used to calculate changes in important variables of  $rETR_{\text{max}}$  and nonphotochemical quenching NPQ calculated as  $(F_m - F_m')/F_m'$ . The variable, called the relative electron transport rate through PSII,  $rETR$ , was calculated as multiplication of the actual photochemical efficiency  $Y_{II}$  by the photosynthetically active radiation  $E_{\text{PAR}}$ ,  $rETR = Y_{II} \times E_{\text{PAR}}$  [dimensionless as it is called relative] (*e.g.* Hofstraat *et al.* 1994, Ralph and Gademann 2005, White *et al.* 2011). In order to estimate  $rETR_{\text{max}}$ , and the irradiance saturating photosynthesis the

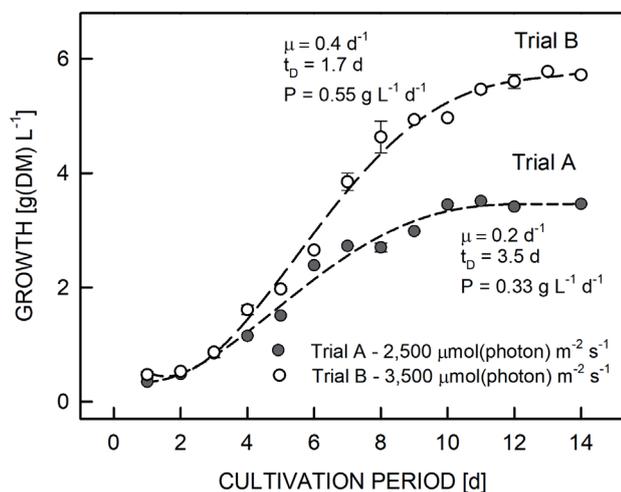


Fig. 2. Growth curves of *Chlorella* R-117 in the PBR exposed to continuous irradiance of  $2,500 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$  (close symbols), or  $3,500 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$  (open symbols). Values are presented as a mean ( $n = 3$ ) with SE indicated by error bars. Broken lines are the fitted curves according to logistic growth model.

$rETR$  vs. irradiance curves were fitted to the non-linear least-squares regression model by Eilers and Peeters (1998) using the Solver function of *Excel* (Microsoft, Redmond, USA) (Fig. 2).

**Fast fluorescence induction kinetics (OJIP-test):** A portable modulated fluorimeter (*AquaPen AP-100*, *P.S.I. Ltd.*, Brno, Czech Republic) adapted for liquid samples was used to follow rapid fluorescence induction kinetics ( $\sim 1$  s) in microalgae cultures. Dark-adapted samples were pipetted to a measuring cuvette (light path of 10 mm) which was mounted in a light-protected holder in front of the detector (adjustable measuring light pulses,  $\sim 2.5 \mu\text{s}$ ) while illuminating red LEDs served as high-intensity

continuous light from both sides of the cuvette [up to  $3,000 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$ ], perpendicular to the detector. The fast fluorescence induction kinetics was measured in the time range between 50  $\mu\text{s}$  to 1 s; it started upon illumination (saturating continuous light) of dark-adapted microalgae culture as the signal increased rapidly from the origin (O) to a peak (P) via two inflections – J and I (Strasser *et al.* 1995). The O point of the fluorescence induction curve represents a minimum value (designated as constant fluorescence yield  $F_o$ ) when plastoquinone electron acceptors ( $Q_A$  and  $Q_B$ ) of the PSII complex are fully oxidized. It is the signal emitted from excited Chl *a* molecules in the light-harvesting complex II before excitons have migrated to the PSII reaction centre. The inflection J occurs after  $\sim 2$ – $3$  ms of illumination and reflects a reduction of  $Q_A^-$  (photochemical phase). The second inflection I occurs some 30–50 ms after illumination and it is thought to reflect temporary maximum of  $Q_A^-Q_B^{2-}$ . The rise of fluorescence from J to the peak P represents the thermal phase influenced by the two-step reduction of  $Q_B$  ( $Q_B \rightarrow Q_B^- \rightarrow Q_B^{2-}$ ), and heterogeneity in the reduction status of plastoquinone pool. Finally, fluorescence yield reaches the peak P when the PQ pool becomes fully reduced (equivalent to maximal fluorescence level  $F_m$ ).

**Statistical analysis:** All measurements were performed three times ( $n = 3$ ) and the means and calculated standard errors (SE) are reported. *SigmaPlot 11.0* was used to determine significant differences between treatments. One-way analysis of variance (ANOVA) and Post-Hoc test was conducted for comparison of variables in trials. *P* values lower than 0.05 ( $P < 0.05$ ) were considered to be significantly different.

## Results

In order to test the photosynthetic performance of *Chlorella* R-117 in the LED-PBR-10, the light-adapted cultures were transferred to the fresh medium at a biomass concentration of about  $0.4$ – $0.5 \text{ g L}^{-1}$  and exposed to high irradiance intensities similar to those occurring outdoors. For the first 24 h (Day 0), the cultures were exposed to an irradiance intensity of  $1,200$ – $1,500 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$  to photo-acclimate (measured on the surface of light sources). Close to the wall of the PBR, it was about  $150$ – $200 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$  and about  $200$ – $400 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$  (measured with spherical light sensor) between light sources, when  $0.4$ – $0.5 \text{ g}$  culture was grown. Then, two series of experiments were carried out. There was a certain lag-phase period as a response of diluted culture to increased irradiance on Day 0 (Fig. 2), but the cultures were not severely photoinhibited and could quickly overcome – after 1–2 d – the lag phase, even if exposed to high irradiance.

On Day 1, the cultures in Trial A and Trial B received different irradiance treatment; therefore, their behaviour was different. In Trial A, the microalgae cultures were grown at an irradiance of  $2,500 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$  (Fig. 2; curve with close circles). At the end of the culti-

vation period, the culture attained a doubling time of 3.5 d [ $\mu = 0.2 \text{ day}^{-1}$ ,  $P = 0.33 \text{ g(DM) L}^{-1} \text{ d}^{-1}$ ] and reached the biomass concentration of  $3.4 \text{ g(DM) L}^{-1}$ . The exponential phase of growth was found between Day 3 and Day 9 (see comment above) when the biomass density was between  $0.5\text{--}3.0 \text{ g(DM) L}^{-1}$ ; then the culture showed the decelerating phase and reached the stationary phase after 10 d of the trial.

In the second series of cultivation experiments – Trial B, the irradiance was set to  $3,500 \mu\text{mol}(\text{photon}) \text{ m}^{-2} \text{ s}^{-1}$ . The culture showed a doubling time of 1.7 d, the growth rate  $\mu$  of 0.4 per day and biomass productivity  $P = 0.55 \text{ g(DM) L}^{-1} \text{ d}^{-1}$ . It reached the maximal biomass density of  $5.8 \text{ g(DM) L}^{-1}$  (Fig. 2; curve with open circles) which was by 66% higher than that in Trial A. Also in this case, the exponential phase of growth was found between Day 3 and 9 when the biomass density ranged between  $0.75$  and  $5.0 \text{ g(DM) L}^{-1}$ . Then the culture, similarly as in Trial A, started to enter the decelerating phase although there were still enough nutrients: 27% N, 40% P, and 48% S of the initial amount, respectively (Trial B in Fig. 1S, supplement). According to experimental setup, the medium contained

about 0.55 g of nitrogen and 0.16 g phosphorus per litre which should be sufficient for growth of about  $8 \text{ g(DM) L}^{-1}$  (biomass contains about 7–8% nitrogen and 1–2% phosphorus). On Day 9 (beginning of the stationary growth phase), the nutrients were still sufficient in both trials as there was still about 43–64% and 27–47% of the initial nutrient concentrations in Trial A and Trial B, respectively (Fig. 1S). Then, it implies that the lack of nutrients was not the main reason of photosynthetic activity and the slow-down of growth, but it was due to the decreased light availability and shade adaptation as the cultures were getting denser (Figs. 2, 4A vs. Fig. 1S). After all, in Trial B, in higher-irradiance culture, the growth rate was doubled as compared to the lower-irradiance culture in Trial A.

The growth kinetics in the cultures was reflected by changes of photosynthetic activities that significantly varied between Trial A [ $\sim 2,500 \mu\text{mol}(\text{photon}) \text{ m}^{-2} \text{ s}^{-1}$ ] and Trial B [ $\sim 3,500 \mu\text{mol}(\text{photon}) \text{ m}^{-2} \text{ s}^{-1}$ ]. Generally, in Trial A, rETR was much lower as compared to culture in Trial B. Rapid light-response curves RLC (measured by Chl fluorescence quenching technique) showed that maximal rETR activities at the beginning of treatment (Day 1–Day 3) were much higher, between 350 and 470 in Trial B [ $3,500 \mu\text{mol}(\text{photon}) \text{ m}^{-2} \text{ s}^{-1}$ ] as compared to the values between 240 and 280 found in Trial A [ $2,500 \mu\text{mol}(\text{photon}) \text{ m}^{-2} \text{ s}^{-1}$ ] (Fig. 3). Important to note that in Trial B, the  $\text{rETR}_{\text{max}}$  values decreased significantly during the experiment and finally at Day 9 they were lower than these in Trial A. This trend can be explained if we correlate the rETR curves with the growth kinetics; the cultures in Trial B were growing dense and evidently became low-light-adapted as compared to those in Trial A (Fig. 2 vs. Fig. 3).

The photosynthetic variables calculated from the RLC of the cultures in both trials changed over time. The maximal PSII quantum yield ( $F_v/F_m$ ) of the cultures in both trials showed a continuous decline regardless of the growth conditions. However, relatively high  $F_v/F_m$  ratio was observed at the start (between 0.75 in Trial A and 0.71 in Trial B) showing that the cultures were in good physiological conditions. During the two-week experiments,  $F_v/F_m$  showed a decreasing trend in Trial A while in Trial B it declined significantly by about 24% (Fig. 4A). In Trial B, in the cultures grown at high light intensity, the reduction in the maximum suggested that the culture in Trial B was slightly more tensed as compared that in Trial A.

It is worth noting that maximal relative electron transport rate  $\text{rETR}_{\text{max}}$  (Fig. 4B) and the onset of light saturation  $E_k$  (Fig. 5A) of the cultures in Trial B (higher irradiance culture) were significantly higher (by 30–40%) during Day 1–3 as compared to cultures grown in Trial A (low irradiance culture). Then, later on Day 6, the situation was reversed when the cultures reached the decelerating phase as the culture in Trial A was thinner than that in Trial B (Fig. 2), thus the cells received more light as also confirmed by higher  $E_k$  (Fig. 5A). Consequently, the  $\text{rETR}_{\text{max}}$  values on Day 9 were slightly lower in Trial B compared to that in Trial A (Fig. 4B). Statistically, the decrease of  $F_v/F_m$ ,  $\text{rETR}_{\text{max}}$ , and  $E_k$  was more significant in

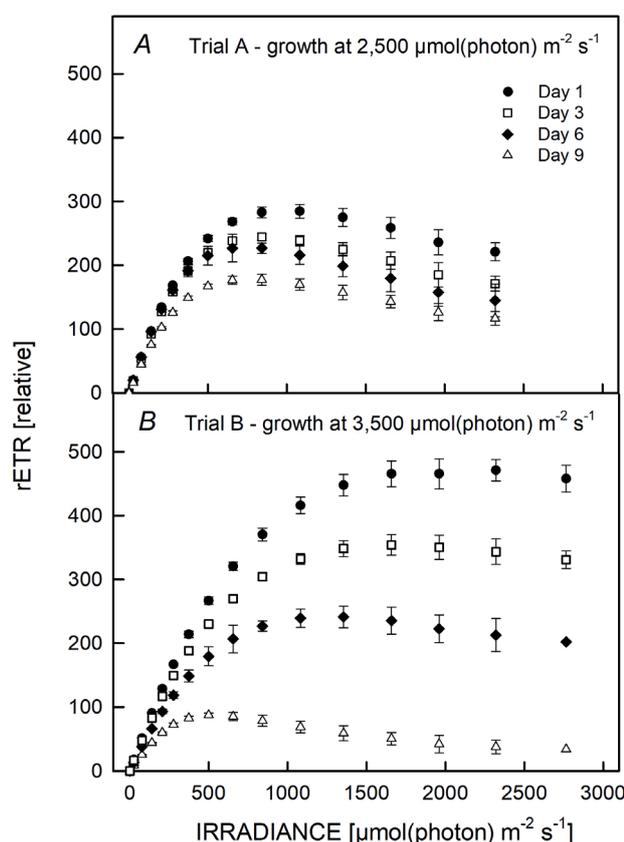


Fig. 3. Rapid light-response curves (RLCs) of the relative electron transport rate rETR vs. irradiance measured in *Chlorella R-117* cultures grown under high irradiance [A –  $2,500 \mu\text{mol}(\text{photon}) \text{ m}^{-2} \text{ s}^{-1}$ ] and [B –  $3,500 \mu\text{mol}(\text{photon}) \text{ m}^{-2} \text{ s}^{-1}$ ] recorded on Days 1, 3, 6, and 9.  $\text{rETR} = Y_{\text{II}} \times E_{\text{PAR}}$ , where  $Y_{\text{II}}$  is the actual photochemical yield of PSII and  $E_{\text{PAR}}$  is photosynthetically active radiation. Values are presented as a mean ( $n = 3$ ) with SE indicated by error bars.

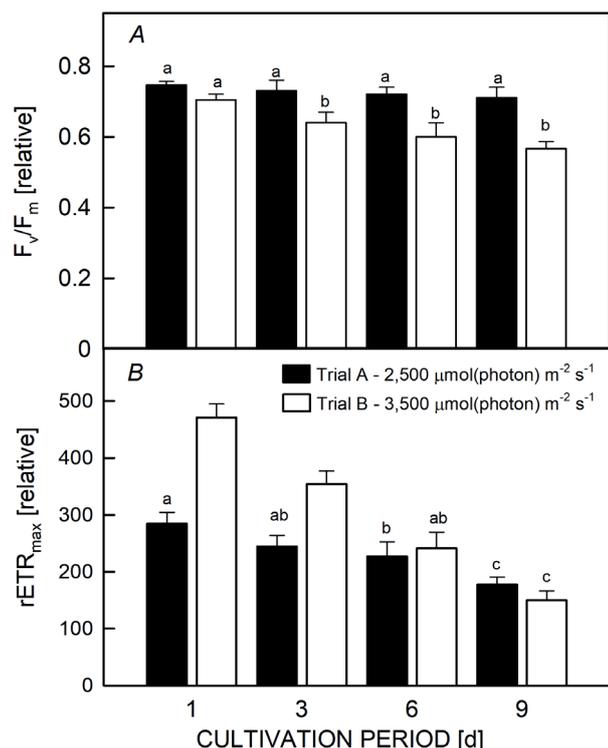


Fig. 4. Changes in the maximum effective quantum yield of PSII ( $F_v/F_m$ ) (A) and maximum relative electron transport rate ( $rETR_{max}$ ) (B), of *Chlorella* R-117 exposed to continuous illumination of  $\sim 2,500 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$  (black column) and  $\sim 3,500 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$  (grey column) during the 14-day cultivation period. Values are presented as a mean ( $n = 3$ ) with SE indicated by error bars. The mean values designated by the same letter did not differ significantly from each other.

Trial B as compared to Trial A. NPQ indicating dissipation of absorbed light energy showed somehow similar trend to that of  $rETR$ . NPQ response of both cultures was partially different as the values in Trial A showed slightly decreasing trend during the experiment, while the NPQ values in Trial B were not much varying. No dramatic increase of NPQ was found which may suggest that the cultures were not as much constrained as only a part of absorbed energy was dissipated as heat (Figs. 4B, 5B).

The cultures were further examined in detail to explain significant changes in photochemical performance which started in the stationary phase of growth during the second week of experiments. The contents of photosynthetic pigments of the cultures in both trials were estimated to determine whether the increased irradiance imposes some photo-stress (Fig. 2S, *supplement*). The total Chl (reaching the maximum of  $\sim 2.5\%$  DM, *i.e.* about  $25 \text{ mg L}^{-1}$ ) and total carotenoid (max.  $\sim 0.5\%$  DM, *i.e.*  $5 \text{ mg L}^{-1}$ ) contents increased from Day 1 and peaked at Day 9, which coincided with the end of the exponential growth phase (Fig. 3) and a significant decrease of  $rETR_{max}$  (Fig. 4B). The trends were very similar in Trials A and B. The changes in the Chl content of *Chlorella* R-117 examined in this experiment did not indicate stress imposed by high irradiance as there was no considerable

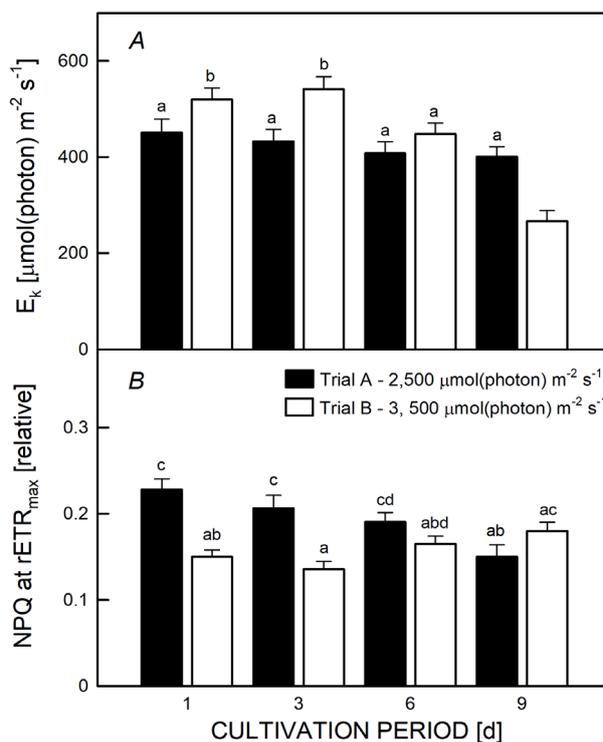


Fig. 5. Changes in the onset of light saturation  $E_k$  (A) and nonphotochemical quenching NPQ (B) of *Chlorella* R-117 exposed to continuous illumination of  $\sim 2,500 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$  (black column) and  $\sim 3,500 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$  (grey column) during the 14-day cultivation period. Values are presented as a mean ( $n = 3$ ) with SE indicated by error bars. The mean values designated by the same letter did not differ significantly from each other.

increase in the content of photoprotective Car during the first week of cultivation. The increase in the total Chl just reflected the (exponential and stationary) phases of the culture and might be slightly higher in Trial A as compared to Trial B (Fig. 2S).

Fast Chl fluorescence induction kinetics (OJIP test) was used to examine changes of photosynthetic performance, *i.e.* the substantial alterations of electron transport processes (Fig. 6). The shapes of the OJIP curves were significantly changed in the course of Trial B as these depend on the status of the photosynthetic apparatus in the cultivation period (Fig. 6B). The exposure to high irradiance, especially in the second week of the trial, showed that during Trial B, the variables  $V_j$  and  $V_i$  increased by about 50 and 38%, respectively. It indicated partial over-reduction of the electron transport carriers at the acceptor side of the PSII showing that the cultures were not fully photosynthetically competent as the cells have become low-light-adapted (Strasser *et al.* 1995, 2004). On the contrary no significant changes of the  $V_j$  and  $V_i$  variables were found in Trial A (Fig. 7A).

The changes of  $V_j$  and  $V_i$  variables may be an indicative of culture health, as they show a degree of electron transport capacity on the donor side, from  $Q_A$  to PQ pool in parallel with changes in the electron transport capacity

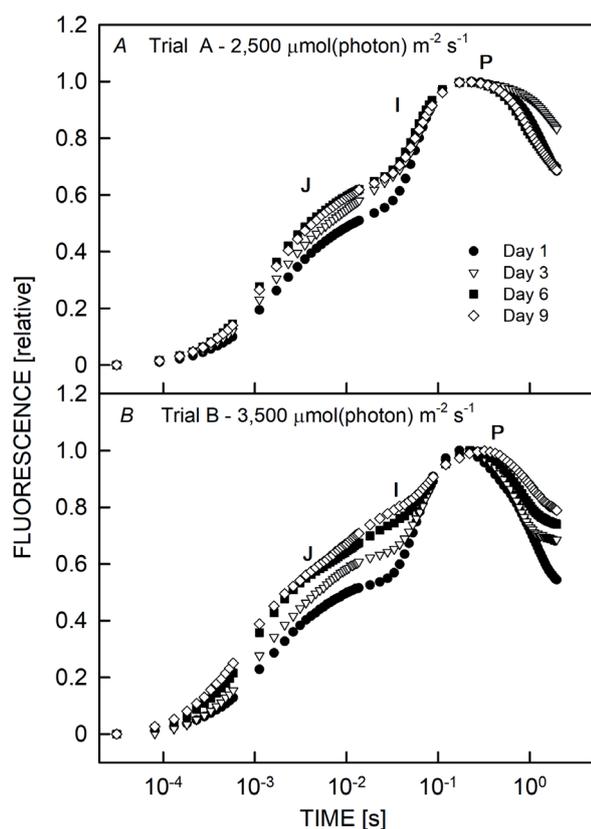


Fig. 6. Changes of rapid Chl fluorescence induction kinetics—OJIP test of *Chlorella* sp. R-117 grown under continuous illumination of 2,500  $\mu\text{mol}(\text{photon})\text{ m}^{-2}\text{ s}^{-1}$  (A) and 3,500  $\mu\text{mol}(\text{photon})\text{ m}^{-2}\text{ s}^{-1}$  (B) during the 14-day cultivation trial. The OJIP curves were double-normalized to on both O ( $F_0$ ) and P ( $F_m$ ) to distinguish the differences in the inflections J and I representing various reduction states of the PSII electron carriers. The curves were recorded in triplicates for each sample and averaged; they consist of different symbols without error bars as these can obscure kinetics if similar.

rETR. However, the increase of in the antenna size can also contribute to these changes since the concentration of Chl per dry mass was found in both trials (Fig. 2S).

### Discussion

In indoor PBRs, one of the constraints of microalgae cultured at high biomass density, is the efficient delivery of light as growth limitations are mostly caused by a significant reduction of cell irradiance. The use of artificial light sources is advantageous since they can provide continuous illumination preventing biomass losses due to respiration in diurnal dark periods. Compared to conventional, externally illuminated PBRs, those with internal illumination are more energy-efficient since all light is absorbed by the culture. If the conditions are set up properly, the light conversion efficiency and biomass productivity of microalgae cultures can be even higher than that in sunlight. The disadvantages of most

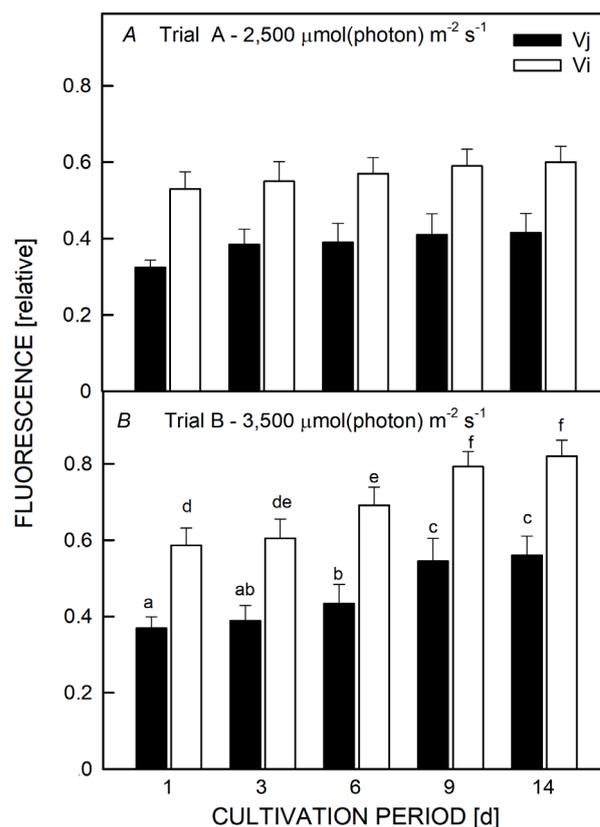


Fig. 7. Changes of the  $V_j$  and  $V_i$  variables calculated from fast Chl fluorescence induction kinetics OJIP test of *Chlorella* sp. R-117 grown under continuous illumination of 2,500  $\mu\text{mol}(\text{photon})\text{ m}^{-2}\text{ s}^{-1}$  (A) and 3,500  $\mu\text{mol}(\text{photon})\text{ m}^{-2}\text{ s}^{-1}$  (B) during the 14-day cultivation trial. The  $V_j$  and  $V_i$  variables corresponding to the J and I inflections were calculated as relative variable fluorescence yield.  $V_j$  represents the accumulation of the reduced "primary" acceptor  $Q_A^-$ , and it is calculated according to the formula  $V_j = (F_{2ms} - F_0)/(F_m - F_0)$ . The  $V_i$  parameter reflects different redox states (e.g.  $Q_A Q_B^{2-}$  or  $Q_A^- Q_B^{2-}$ ) and is calculated as  $V_i = (F_{30ms} - F_0)/(F_m - F_0)$ . Values are presented as a mean ( $n = 3$ ) with SE indicated by error bars. The mean values designated by the same small and capital letter did not differ significantly from each other for  $V_j$  and  $V_i$ , respectively.

conventional light sources (filament bulbs, fluorescent tubes or discharge lamps) have been overcome by the use of LED light sources as these have high energy to light conversion efficiency, low heat generation, high light intensity and recently also low cost for purchase and electricity consumption.

Generally, higher cell irradiance (up to photosynthesis saturating level) achieved inside the PBR, the higher biomass density (and productivity) can be reached. The ambient light maxima [about 2,000  $\mu\text{mol}(\text{photon})\text{ m}^{-2}\text{ s}^{-1}$ ] available for photosynthetic antennae represent an intensity that is roughly 5–10 times higher than that required to saturate growth. In other words, as much as 90% of the photons captured by the photosynthetic antennae may be dissipated as heat (Masojidek and Torzillo 2008).

The important point in microalgae mass culturing is the balance between biomass density and irradiance intensity,

light path and effective mixing to achieve sufficient average cell irradiance in order to reach high photosynthetic efficiency and biomass productivity (Masojídek *et al.* 2004, Richmond 2013, Zarmi *et al.* 2013, Sergejevoá *et al.* 2015). This is a relevant aspect for scale-up of PBRs with high cell densities. Due to the cell movement between illuminated and dark parts of the PBR volume, the microalgae cells experience the flashing (intermittent) light regime producing short light/dark cycles if the light path is short. Light intermittency of tens to hundreds of milliseconds corresponding to the time scale of the rate-limiting dark reactions of photosynthesis enhances light-use efficiency and subsequently growth rate as light energy is utilized with maximal efficiency (Phillips and Myers 1954, Grobbelaar *et al.* 1996, Janssen *et al.* 2000, Richmond 2013, Zarmi *et al.* 2013).

In our trials, we tested the novel PBR – a cylindrical vessel with internal LED illumination – for microalgae growth in which high-intensity light sources were placed evenly inside the culture to set up relatively short light path. Previously, various internally irradiated photobioreactors were designed, for example with an integrated solar (*e.g.* based on light collection by Fresnel lenses and optical fibres) and artificial light sources (metal halide lamps) in which light intensity was around  $100 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$  and the culture biomass density reached about  $1.2 \text{g}(\text{DM}) \text{L}^{-1}$  (Ogbonna *et al.* 1999). As compared to that, the presented LED-PBR-10 described here is technically rather simple as high-intensity LEDs were used for illumination. Thus, light intensities used in our trials were more than one order of magnitude higher and biomass density of microalgae cultures increased several times and the microalgae cells were exposed to high irradiance similar to summer light intensities (or more). In this setup, taking into consideration efficient mixing which can generate short light/dark cycles. Even high output irradiance intensity produced by light sources did not inhibit culture growth if biomass density was sufficient. The growth saturating light intensities for *Chlorella* R-117 cultures in our experiments were found between 400 and  $500 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$  at relatively high biomass densities (Fig. 5A) similarly as those measured in outdoor *Chlorella* R-117 cultures (Masojídek *et al.* 2011b). When the starting biomass density was about  $0.5 \text{g} \text{L}^{-1}$ , the culture did show short lag phase at the start of the trial as the seed culture was already high-light acclimated (Fig. 2). Then, the exponential growth was recorded during the first week that was comparable to that of *C. sorokiniana* grown in short light-path panel PBRs (Cuaresma *et al.* 2009). It is important to note that in fast-growing microalgae mass cultures, the increase in biomass density inevitably results in self-shading, and subsequent growth limitation (Masojídek *et al.* 2011b).

If we consider  $r\text{ETR}_{\text{max}}$  and  $E_{\text{K}}$  (Figs. 4B, 5A) vs. nutrient content in the media (Fig. 1S), one can illustrate that the primary limitation of the growth in our trials was caused by the decreased light availability due to increased cell density and not by nutrient limitation. Here, it is important to emphasize that on Day 6, the values of  $r\text{ETR}_{\text{max}}$  (Fig. 4B) were still higher in higher-irradiance culture

(Trial B) than those in the lower-irradiance culture (Trial A). However, on Day 9, the trend was inevitably reversed as the  $r\text{ETR}_{\text{max}}$  values were by about 33% lower in Trial B than those in Trial A (Fig. 4B); this coincided with the decelerating phase of the growth curve (Fig. 2) when biomass density reached about  $5 \text{g} \text{L}^{-1}$  in Trial B although there were still enough nutrients (28–48% of the initial amount, respectively; Fig. S1B). On Day 9, the decrease of  $E_{\text{K}}$  also indicated that the denser culture in Trial B was acclimated to more than 30% lower irradiance [ $E_{\text{K}} = 266 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$ ] as compared to the thinner culture [ $E_{\text{K}} = 400 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$ ] (Fig. 5A). These results suggested that the photosynthetic activity was limited by low cell irradiance when culture became dense (Masojídek *et al.* 2011a, Richmond 2013).

In our opinion the variables  $F_v/F_m$  and  $r\text{ETR}$  may express different degree of constrains – ‘distress’ when  $F_v/F_m$  changes dramatically (photoinhibition) and functional downregulation, ‘eustress’ when the  $r\text{ETR}$  values decrease. To say it in other words, the cultures in the presented experiments were quickly acclimated and benefited from higher irradiance as seen from high growth rate, but in the later phase of the trials they suffered low-light acclimation.

It is shown here that the primary reason of the down-regulation of photosynthetic activity in these trials was the significant increase in biomass density that prevents the light penetration to cells causing low-light adaptation and the depletion of the nutrients in the medium might be partially limiting in the final phase of the trial (Fig. 3B and 4B vs. Trial B in Fig. 1S).

**Conclusion:** In the tested PBR, we used high-intensity/short light-path internal illumination by the LED light sources which were submerged directly into culture. This PBR makes possible to adjust a wide range of culture conditions for growth of microalgae monocultures, *e.g.* irradiance intensity and temperature as well as efficient  $\text{CO}_2/\text{O}_2$  exchange, high cell turbulence, and good protection against contamination. The correlation between photosynthetic activity and growth kinetics can be used when evaluating optimal cultivation regime for microalgae strains.

Chlorophyll fluorescence variables resulting from saturating pulse analysis of fluorescence quenching and fast induction kinetics were used as a tool to measure photosynthetic activity. The knowledge of physiological responses of specific microalgae strain(s) and the estimation of relevant marker variables and set-up of measuring protocols in such small-scale PBRs can be used for scale-up, *i.e.* the design and optimisation of large-scale commercial plants. Here, we studied the situation when the seed culture is transferred from laboratory and exposed to ambient irradiance outdoors.

The photobioreactor LED-PBR-10 described here was used to test the construction of a pilot-scale 100-L photobioreactor, LED-PBR-100 (Sergejevoá *et al.* 2015) with one central LED light source which is now being used for production of sensitive strains or seed cultures for larger systems. Another step of scale-up has been a

1,000-L photobioreactor with flat LED-panels submerged in the culture.

If we consider practical use of such photobioreactors with internal illumination in larger-scale systems for biomass or bioactive compound production, then these should be operated in semibatch or continuous mode when a part of biomass is regularly harvested at the upper part of the exponential phase. At present, we are using this scheme to produce microalgae biomass containing particular bioactive compounds in various stages of culture development.

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