



Changes in photosynthesis, growth and biomass composition in outdoor *Chlorella* g120 culture during the metabolic shift from heterotrophic to phototrophic cultivation regime

Jiří Masojídek^{a,b,*}, Karolína Ranglová^{a,c}, Tomáš Agustín Rearte^{d,e}, Paula S.M. Celis Plá^{f,g}, Giuseppe Torzillo^{h,i}, Ana Margarita Silva Benavidesⁱ, Amir Neori^{j,k}, Cintia Gómez^l, Félix Álvarez-Gómez^m, Martin Lukeš^a, Martín P. Caporgno^a, Roberto Abdala^m, Krystian Miazekⁿ, Thaís Fávero Massocato^o, Jaqueline Carmo da Silva^p, Richard Atzmüller^q, Hafidh Al Mahrouqi^r, Francisca Suárez Estrella^s, Felix L. Figueroa^m

^a Laboratory of Algal Biotechnology, Centre ALGATECH, Institute of Microbiology, Czech Academy of Sciences, Treboň, Czech Republic

^b Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic

^c Faculty of Agriculture, University of South Bohemia, České Budějovice, Czech Republic

^d Departamento de Recursos Naturales y Ambiente, Facultad de Agronomía, Universidad de Buenos Aires, CABA, Av. San Martín 4453, 1417 Buenos Aires, Argentina

^e Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Argentina

^f Laboratory of Aquatic Environmental Research (LACER), Center of Advanced Studies, University of Playa Ancha, Viña del Mar, Chile

^g HUB-AMBIENTAL UPLA, Vicerrectoría de Investigación Postgrado e Innovación, Universidad de Playa Ancha, Valparaíso 2340000, Chile

^h CNR, Institute of Bioeconomy, Sesto Fiorentino, Firenze, Italy

ⁱ Escuela de Biología, CIMAR – Centro de Investigación en Ciencias del Mar y Limnología, Universidad de Costa Rica, San Pedro, Costa Rica

^j Morris Kahn Marine Research Station, Marine Biology Department, The Leon H. Charney School of Marine Sciences, University of Haifa, Israel

^k The Interuniversity Institute for Marine Sciences, Eilat, Israel

^l Department of Chemical Engineering, University of Almería, Almería, Spain

^m University of Málaga, Institute of Blue Biotechnology and Development (IBYDA), Department of Ecology, Málaga, Spain

ⁿ University of Liege, Gembloux Agro-Bio Tech, Belgium

^o Department of Botany, Center of Biological Sciences, Federal University of Santa Catarina, Santa Catarina, Brazil

^p Department of Botany, Center of Biological Studies, Federal University of Sao Carlos, Sao Carlos, Brazil

^q University of Applied Sciences Upper Austria, Wels, Austria

^r Sultan Qaboos University, Muscat, Oman

^s Department of Biology and Geology, University of Almería, Almería, Spain

ARTICLE INFO

Keywords:

Chlorophyll fluorescence
Microalgae
Photosynthesis
Physiology
Thin-layer cascade
Trophic conversion

ABSTRACT

Up to now, scarce information has been available regarding major photobiochemical changes that accompany the metabolic shift from heterotrophy to phototrophy in microalgae cells. In the present trials the trophic conversion from heterotrophic to phototrophic growth regime was studied in the microalga *Chlorella vulgaris* g120 grown in outdoor thin-layer cascades. The crucial question was whether *Chlorella* g120 can undergo the photoacclimation of its photosynthetic apparatus under natural irradiance. As for comparison the phototrophic strain *Chlorella vulgaris* R-117 was cultured in parallel.

Various *in-situ* and *ex-situ* monitoring techniques namely oxygen production and chlorophyll fluorescence were tested to characterize physiological response of the *Chlorella* g120 culture during photoacclimation and correlate it with growth. We show that *Chlorella* g120 can undergo the metabolic shift from heterotrophic to phototrophic growth regime, but its conversion is. A rather high respiration rate was interpreted as a strategy to dissipate the unused light energy. *Chlorella* g120 was found as the strain characterized by a small-antenna size strain (low

Abbreviations: Chl, chlorophyll; Car, carotenoid; DO, dissolved oxygen; DW, dry weight; F₀, F_v, F_m, minimal, variable and maximal fluorescence in dark-adapted state; F_v/F_m, maximal photochemical yield of PSII; POE/RESP, photosynthetic oxygen evolution and respiration; OJIP curve, fast Chl fluorescence induction kinetics; PAM, pulse-amplitude modulation; PAR, photosynthetically active radiation; PQ, plastoquinone; PSII, photosystem II; ETR, relative electron transport rate through PSII; RLC, rapid light-response curve; TLC, thin-layer cascade; TL-RWP, thin-layer raceway pond.

* Corresponding author at: Centre ALGATECH, Institute of Microbiology, Novohradská 237, CZ-37901 Treboň, Czech Republic.

E-mail address: masojidekj@seznam.cz (J. Masojídek).

<https://doi.org/10.1016/j.algal.2021.102303>

Received 18 December 2020; Received in revised form 5 March 2021; Accepted 29 March 2021

2211-9264/© 2021 Elsevier B.V. All rights reserved.

chlorophyll/cell ratio < 1%) which should be favorable for light utilization in dense cultures. The high carotenoid/chlorophyll ratio (= 0.63) may be of interest from biotechnological point of view for carotenoid (namely lutein) production.

The build-up of dissolved oxygen (DO) concentration and high electron transport rate measured *in-situ* indicated high photosynthetic activity of outdoor microalgae cultures. These variables are usable *on-line* as reliable markers as they reflect the physiological status of microalgae culture. Highly productive cultures, like *Chlorella* R-117 have high photosynthesis which is inevitably accompanied by the build-up on high DO gradients. To maintain high biomass production in such strains we have to find an interplay among photosynthetic activity, gradients of DO concentration, biomass density and culture layer thickness that can secure high growth rate (and productivity).

1. Introduction

Since the 1950s microalgae have been used as a source of biomass proposed for production of food and feed supplements, pharmaceuticals, cosmetics and lately biofuels, as well as for wastewater treatment and a quest for mitigation of atmospheric CO₂ build-up [1]. These microorganisms produce a wide range of bioproducts — proteins, polysaccharides, lipids, pigments, antioxidants, vitamins, bioactive compounds and many others. Industrial cultivation of microalgae has developed considerably since the 1990s [2–4].

Most of microalgae strains grow phototrophically (in mineral media with addition of carbon dioxide) obtaining their energy *via* photosynthesis [5]. Certain species can grow also heterotrophically [6–9]. Heterotrophic cultivation of microalgae, *i.e.*, in the presence of organic substrates like glucose or acetate and other organic substrates — has to be carried out axenically in fermenters, similarly to the cultivation of bacteria. For example, some strains of *Chlorella* exhibit high growth rate under heterotrophic conditions using glucose in fed-batch cultivation which was employed for mass production [8,10]. Nevertheless, the content of pigments in heterotrophically-grown biomass is significantly lower (2–4 times) as compared with those from phototrophic growth [11–13]. The chlorophyll (Chl) content between 2.5 and 3.5% of dry weight (DW) was reported in phototrophically grown *Chlorella* while in heterotrophically grown cells can reach only 1–1.8% of DW [14,15].

Some microalgae strains are able to undergo a metabolic pathway shift (trophic conversion) from heterotrophic to phototrophic growth regime. In the 1990–2000s, a cultivation strategy called ‘sequential heterotrophy-dilution-photoinduction’ was developed [16,17]. Later, this procedure was scaled-up in *Chlorella* cultures to proof the feasibility in indoor and outdoor trials for biomass production [18,19]. Firstly, the culture was cultivated heterotrophically to achieve high cell densities. Upon glucose depletion, it was diluted and exposed to light for phototrophic growth. Using this strategy, the quality of the *Chlorella* biomass may approach that of cells cultivated phototrophically. A practical demonstration of the two-stage pilot and industrial production was managed as heterotrophically *Chlorella vulgaris* cells grown in fermenters (1st stage) were used to inoculate directly an outdoor flat-panel and tubular photobioreactors (2nd stage) [20]. This approach applied heterotrophic growth to obtain high-biomass inoculum for outdoor photobioreactors.

However, little information is available regarding the nature of photobiochemical changes that underlie the metabolic shift from heterotrophy to phototrophy in microalgae cultures. Recently we have examined physiological and photobiochemical changes during this two-stage process in laboratory *Chlorella vulgaris* g120 cultures [9]. Understanding the nature of photobiochemical changes that accompany the metabolic shift from heterotrophy to phototrophy in microalgae cultures is a pre-requisite to optimize the process.

Microalgae mass cultivation requires monitoring of culture’s physicochemical variables, namely its pH, temperature, dissolved oxygen (DO) concentration, and nutrient status as well as photosynthetic activity which is crucial for growth. One, direct approach is to measure photosynthesis as oxygen production or Chl fluorescence *in-situ* during the diel cycle to follow the actual situation in the culture. The other

possibility is to measure *ex-situ* using microalgae samples withdrawn from a cultivation unit. Some variables, like the maximum and actual quantum yield of PSII and electron transport rate measured by fluorescence quenching technique have been used to correlate photosynthesis and growth [21–28].

In this work the physiological and photobiochemical changes and growth were studied in the microalga *Chlorella vulgaris* g120 (Chlorophyta) that occurred during the transition from heterotrophic to phototrophic growth regime. For comparison, a fast-growing obligate phototroph *Chlorella* R-117 was investigated in parallel. We aimed to select techniques and marker variables that can be used to monitor photosynthetic activity and correlate it to culture performance, growth and biomass composition.

2. Material and methods

2.1. Strains, cultivation units and conditions

Two green microalgae were cultured side by side in thin-layer cascades (TLC) outdoors (Fig. 1): the strain *Chlorella* g120 (registered as *Chlorella vulgaris* BELJ., 1996/H 14, CCALA 30001, Culture Collection of Autotrophic Organisms, Institute of Botany, Třeboň, Czech Republic) further as *Chlorella* g120) which is used for mass heterotrophic production [8,9] and a fast-growing phototrophic strain *Chlorella* R-117 (registered as *Chlorella vulgaris* BELJ., CCALA 1107, Culture Collection of Autotrophic Organisms, Institute of Botany, Třeboň, Czech Republic; further as *Chlorella* R-117) [14,29] was used for comparison. The seed culture of *Chlorella* R-117 was grown outdoors and then diluted 10-times for the trial.

An axenic, seed culture of *Chlorella* g120 was firstly grown heterotrophically in a medium containing glucose (0.43 M), urea, macroelements (KH₂PO₄, MgSO₄, FeSO₄, CaCl₂) and other salts of micro- and trace elements (H₃BO₃, CuSO₄, ZnSO₄, CoSO₄, MgCl₂, (NH₄)₆Mo₇O₂₄) in 5-litre Erlenmeyer flasks placed on a shaker under low irradiance at 25 °C for 7–10 d as already described [8]. Then the culture was transferred to a 150-litre fermenter (Bioreaktor 150, SK Group) where was further grown heterotrophically in fed-batch regime in the same medium for 3 days at 35–37 °C.

For phototrophic cultivation outdoors both strains were grown in parallel TLCs (Fig. 1) using an inorganic medium as described previously [29]. Two outdoor TLCs were operated: one with a total surface of 24 m² (working volume of 200 L; Fig. 1a) and the other of 90 m² (working volume of 600 L; Fig. 1b) at culture layer thickness of 5–6 mm giving the surface-to-total-volume ratio of about 120–150 m⁻¹. The culture of *Chlorella* g120 was grown in the outdoor 24-m² TLC while the culture of the phototrophic *Chlorella* R-117 strain was grown in the TLC of 90 m² (Fig. 2) to carry out a 5-day trial.

The principle of TLC is that the microalgae culture grows in a thin-layer on an inclined flat surface with the declination of 0.5–1.7% [30]. The microalgae culture is pumped from the retention tank and gets evenly distributed to the top of the upper platform of TLC, flows down along the surface to troughs, from where it drains to the upper part of the lower platform and from there returns to the retention tank located under the unit, from where the cycle begins again. As the thin-layer

culture falls on the inclined wall of the retention tank, it is filtered through a screen where an efficient degassing is achieved. In this way, the DO concentration at the start of cultivation area is equilibrated with the air to about 100% saturation. The dark volume in the distribution tubing and retention tank is minimized. CO₂ is supplied based on pH stat system in the tubing (riser) after a pump. Centrifugal pumps (DWO 300, Ebara) are used for culture circulation in both units providing a flow speed of about 0.5 m s⁻¹.

2.2. Location of trial, period and weather data measured *in-situ*

Most of the trials were carried out during the 10th workshop of the International Group for Aquatic Productivity (GAP) organized at Centre ALGATECH located in Treboň, Czech Republic (GPS coordinates — 48°59'15" N; 14°46'40.630" E) in August starting on day 0 between 15:00 and 16:00 h to avoid photo-stress of freshly inoculated cultures. The time in figures corresponds to CEST (GMT + 1). Weather conditions (solar irradiance in PAR and ambient temperature) were monitored by a local meteorological station (IP Wariowether, model ME 13). For all days of the trial the weather conditions were rather fair with no rain; on some days there were cold mornings (day 0–day 3) or variable sunshine (Fig. 3). During days 0–3 morning temperatures were between 6 and 8 °C and midday maxima were between 20 and 23 °C. When morning temperatures (at about 07:00 h) were low, the cultures were kept in the retention tank until the ambient temperature increased to 15 °C (about 08:30–9:00 h) to avoid photoinhibition due to the synergism of high irradiance and low culture temperature. On days 4–5 morning temperatures significantly increased to about 14–15 °C and at midday between 30 and 34 °C, in this case the culture was exposed to sun at about 08:00 h (Fig. 3a). For all days the cultivation was usually stopped at 19:00 h when ambient temperature decreased to about 15 °C.

The irradiance conditions were favorable on days 2, 3 and 5 with the midday maxima between 1200 and 1500 μmol photons m⁻² s⁻¹ while on day 0, day 1 and 4 the sunshine intensity was lower, or variable, but still rather fair (Fig. 3b).

2.3. Photosynthesis measurements

Photosynthetic activity of the cultures was measured using two techniques — saturation pulse analysis of fluorescence quenching and oxygen production/respiration. Then, all records were analyzed to determine photosynthetic variables.

2.3.1. *In-situ* measurements

Some variables (DO concentration, ΔF'/F_m', ETR) were measured directly *in-situ* in cultivation units using a portable oximeter. Fluorescence *in-situ* data were recorded using PAM fluorimeters (Junior-PAM, PAM-2500, H. Walz GmbH, Effeltrich) controlled *via* USB interface by PC [31]. The WinControl-3.2 software was used for data acquisition and recording (H. Walz GmbH, Effeltrich, Germany). The fluorimeter was fitted with blue light-emitting diodes (LED, 460 nm) to apply measuring and actinic lights as well as saturating pulses. The measuring optical fiber (length of 100 cm and 1.5 mm in diameter) together with a spherical PAR sensor (US-SQS, H. Walz GmbH, Effeltrich) were submerged at a depth of about 3–5 mm to measure in the photic zone of the culture.

The incident photosynthetically active radiation E_{PAR} (400–700 nm) and the actual quantum yield [ΔF'/F_m' = (F'_m - F') / F'_m] were measured every 10 min during the day period where F'_m is the maximal fluorescence induced by a saturating light pulse and F' is the steady-state fluorescence level measured in the culture adapted to ambient irradiance before the application of saturation pulse [32]. The relative electron transport rate ETR (= ΔF'/F_m' × E_{PAR}) through PSII (μmol electrons m⁻² s⁻¹) was determined to estimate photosynthetic activity where E_{PAR} is the incident photosynthetically active irradiance (μmol photons m⁻² s⁻¹). This variable is easy to record and adequate for the purpose of this trial — to follow diurnal changes in microalgae cultures. In any case it represents a relative figure and should be cautiously considered as a comparative variable.

Dissolved oxygen (DO) concentration was measured *in-situ* directly in the culture at the start and end of the TLC cultivation area using a portable oximeter (model Oxi 330, WTW, Germany) during the day (10:00 to 18:00 h). The difference of DO (ΔDO) concentrations between the end and start was expressed as percentage of saturation (or mg O₂ L⁻¹).

Photosynthetically active radiation (PAR) was also measured *in-situ* as 10-s averaged values using the LI-250A light meter (Li-Cor, USA) with a flat quantum sensor (LI-190SA, cosine-corrected up to 80° angle of incidence).

2.3.2. *Ex-situ* measurements

For *ex-situ* measurements microalgae samples were taken from outdoor cultures at specified time intervals following already described procedures [9,28,33,34]. The rapid light response curve (RLC), maximum photochemical yield F_v/F_m, photosynthetic oxygen evolution/respiration (POE/R) were and specific absorption cross section measurements were carried out in the laboratory. For Chl fluorescence



Fig. 1. (a) Outdoor thin-layer cascade of 24 m² was inoculated with the heterotrophically grown *Chlorella* g120 and (b) thin-layer cascade of 90 m² was used to grow the phototrophic strain of *Chlorella* R-117.

and oxygen production measurements the cultures were diluted to 0.2 to 0.3 g DW L⁻¹ (corresponding to 3 to 7 mg Chl L⁻¹) with the growth medium and dark adapted for 10 min in the water bath keeping the same temperature as in the outdoor units. By dilution the re-absorption problems and light intensity gradient in dense cultures can be avoided. Data were recorded in duplicates or triplicates depending on the length of measurements.

The determination of the specific absorption cross-section a is based on light scattering effect from the cell surface [35]. To minimize the impact of the light scattering, the 1-cm cuvette with sample was placed close to a photomultiplier with a light diffuser (standard printer paper) in between. Culture samples were withdrawn from the outdoor cultures at three daytimes (8:30, 14:00 and 17:00 h) and diluted with fresh medium to obtain an optical density between 0.1 and 0.8. The optical density (OD) was measured *ex-situ* as an averaged value in the whole range of the visible spectrum (400–700 nm; OD at 750 nm was subtracted) by a double beam spectrophotometer (Shimadzu UV-3000). A specific absorption cross-section of the culture $a = 2.303 \times OD \times d \times 100$ (m⁻¹) is calculated where 2.303 is the conversion factor of decimal to natural logarithm (log₁₀/log_e), d is the dilution factor and 100 is the conversion to meter [36,37].

Photosynthetic oxygen evolution (POE) was measured polarographically using an oxygen monitoring system (Oxylab+, Hansatech, UK) consisting of a temperature controlled chamber DW2/2 with adjustable illumination and mixing which was connected to temperature-controlled water bath (Polystat, Cole & Parmer). Light-response curves were recorded using a stepwise increasing light intensities of 0, 200, 400, 600, 1200 and 1800 μmol photons m⁻² s⁻¹ of white light, each step lasting 2 min. The first step (2 min in the dark) provided the value of dark respiration. Oxygen production and respiration were calculated in μmol O₂ mg⁻¹ (Chl) h⁻¹ or in pmol O₂ cell⁻¹ h⁻¹.

For Chl fluorescence measurements, diluted samples were transferred to the measuring chamber of a fluorimeter to keep similar ‘light’

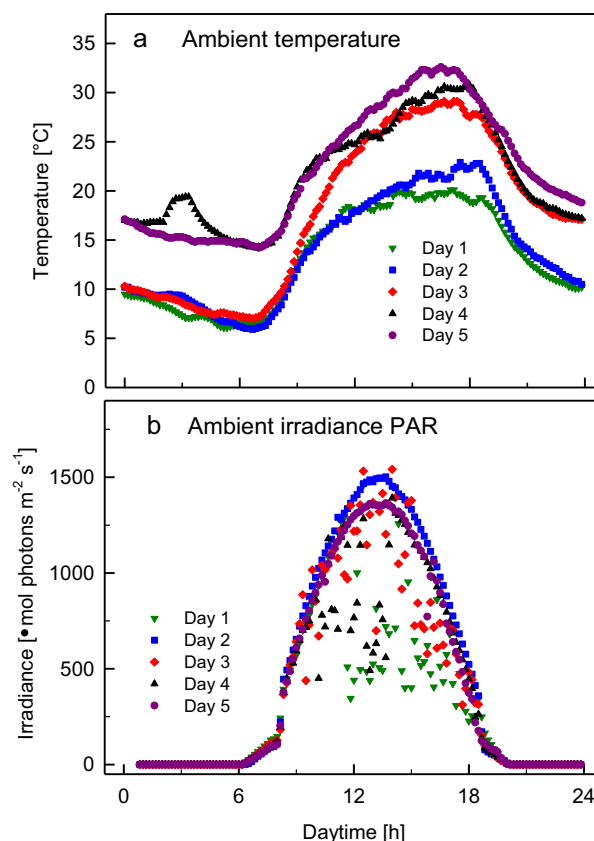


Fig. 3. The course of ambient temperature and irradiance during the 5-day outdoor trial. The ambient temperature and irradiance were recorded by a meteorological station placed next to the units.

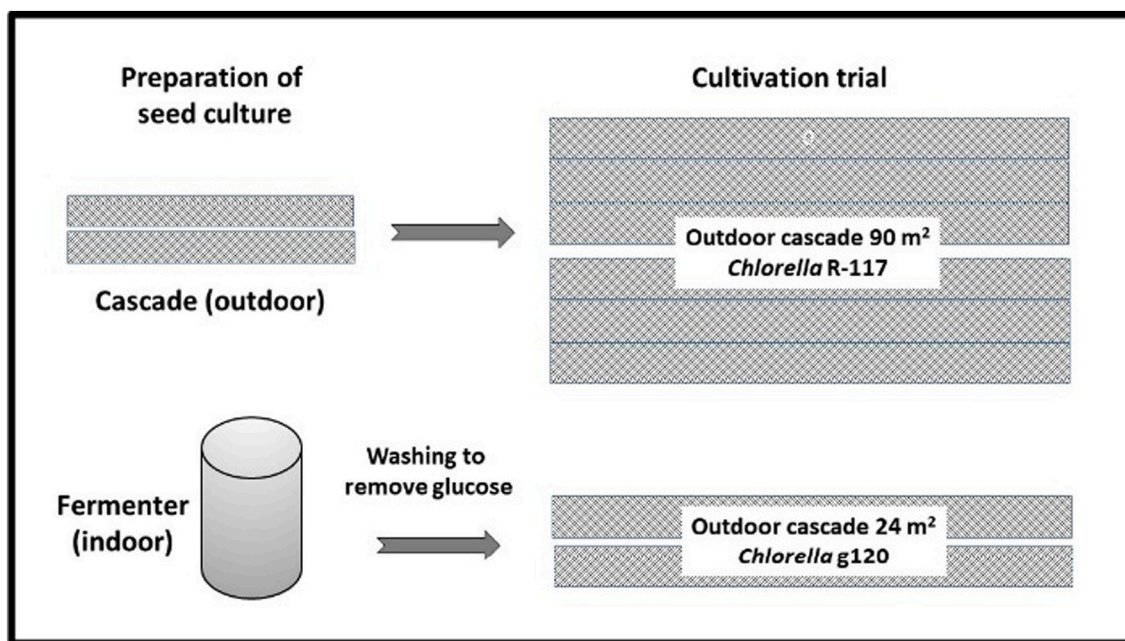


Fig. 2. The flow diagram of the experimental design. The seed cultures were prepared in an indoors fermenter (*Chlorella g120*) or in outdoor small cascade (*Chlorella R-117*). Then, the cultures were inoculated either to a 24 or 90 m² cascade as shown in the diagram. The culture of *Chlorella g120* was washed after heterotrophic growth to remove the rest of glucose and re-suspended in fresh BG-11 medium.

exposure history (the photoacclimation status of the cells). All other laboratory conditions were also well-defined. The fluorescence nomenclature in this paper follows [23,38]. By dilution the re-absorption problems in dense cultures were avoided and sufficient illumination was provided to reduce plastoquinone pool (closed PSII reaction centers). Values of the maximum photochemical yield of PSII, F_v/F_m of microalgae samples were measured using a pulse-amplitude-modulation fluorimeter (Junior-PAM or PAM-2500, H. Walz, Germany) in a light-protected measuring chamber. This variable was calculated as the ratio of variable and maximal fluorescence, $F_v/F_m = (F_m - F_0) / F_m$; it indicates physiological status of microalgae, i.e., the capacity of the system to absorb light through the reaction centers and the light harvesting complex and expresses the maximum quantum efficiency of primary photochemistry [32,39]. The F_0 (basal fluorescence from fully oxidized reaction centers of PSII) and F_m (maximal fluorescence from fully reduced PSII reaction center), were determined using a weak modulated light in the dark-acclimated samples.

Measurements of rapid light response curves of Chl fluorescence, RLCs [39] were carried out with dark-adapted samples (10 min) which were exposed to a series of stepwise increasing irradiance intensities using two PAM-fluorimeters: PAM-2500 and Junior-PAM to compare both measurements. RLCs by PAM-2500 were measured in a 2 mL culture sample (3–7 $\mu\text{g Chl mL}^{-1}$) placed in a liquid-phase chamber (DW2/2, Hansatech, thermostated at 30 °C and mixed by magnetic stirrer. A series of stepwise increasing light irradiances (red LEDs, in the range of 0–2000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) were automatically applied at 20 s intervals to obtain the light-adapted fluorescence level F' (fluorescence yield in the light), and at the end of each step a saturating pulse ($>10,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, 0.6 s duration) was triggered to reach the maximum fluorescence level F_m' (maximum fluorescence in the light). The RLCs by Junior-PAM were measured in a light-protected measuring chamber (15 mL) using a series of stepwise increasing light intensities in the range of 0–1500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (blue LEDs) applied at 20 s intervals. The variables – quantum efficiency of PS II electron transport α (the initial slope of the rapid light curve; which is related to the maximal quantum yield of PS II electron transport under light limited conditions) and ETR_{max} [$\mu\text{mol electrons m}^{-2} \text{s}^{-1}$] were calculated from RLCs by the WinControl-3 software (H. Walz, Germany).

2.4. Analytical measurements

2.4.1. Determination of dry weight, pigment concentration and number of cells

The measurement of biomass density was performed as previously described [33,34]. It was determined in triplicate as dry mass (DW) by filtering 5 mL of culture samples on pre-weighed glass microfiber filters (GC-50). The filters with the cells were washed twice with deionized water (approximately 50 mL total), oven-dried at 105 °C for 3 h; then these were transferred to a desiccator to equilibrate to laboratory temperature and weighed (precision of $\pm 0.01 \text{ mg}$).

Total chlorophyll (Chl) and carotenoids (Car) content was assayed in triplicates using 100% methanol according the procedure already described [9]. Calculation of concentration of pigments followed the Eqs. (1), (2) and (3) described by Wellburn [40].

$$\text{Chla} = 16.72_{A665.2} - 9.14_{A652.4} \quad (1)$$

$$\text{Chlb} = 34.09_{A652.4} - 15.28_{A665.2} \quad (2)$$

$$\text{Car} = (1000_{A470} - 1.63\text{Ca} - 104.96\text{Cb})/221 \quad (3)$$

The concentrations of individual carotenoids were estimated using a reverse-phase C-18 column in an HPLC system (Beckman System Gold module 125) equipped with a diode array detector [41].

For cell count the culture samples were fixed by 2.5% glutaraldehyde, diluted and cell number was determined using cell counter (Multisize4, Beckman Coulter).

2.4.2. Carbon allocation analysis by FTIR spectroscopy

Carbon allocation was determined by Fourier Transform Infrared Spectroscopy (FTIR) [42]. After centrifugation the cells were lyophilized (Lyovac GT 2) and dry samples were suspended in 50–100 μL of distilled water and deposited in a Si 384-well plate (10 μL per spot). The FTIR spectra were measured by a spectrometer (Nicolet IS10, Thermo) equipped with a microarray reader with a DTGS (Deuterated Tri-Glycine Sulphate) detector. Absorbance spectra were measured in the spectral range from 400 to 4000 cm^{-1} , at a resolution of 4 cm^{-1} . An Omnic software (Nicolet) was used for measurement and data processing (32 scans were averaged). Absorption bands were assigned as follows: the 1740 cm^{-1} line to C=O stretching of lipids, 1640 cm^{-1} line to vibration of C=O of Amide I, 1545 cm^{-1} to N-H of Amide II and 1150 cm^{-1} to C–O of carbohydrates [42]. Measured data were normalized to Amide I band and the results were expressed as ratios of absorption maxima of the bands corresponding to the main organic compounds (i.e., proteins, lipids, and carbohydrates).

2.5. Bioassays of antifungal and biostimulating activity

The freeze-dried biomass samples of both *Chlorella* g120 and R-117 cultures collected at the end of the trial were resuspended in distilled water (10 mg L^{-1} of DW) and sonicated (Branson sonicator 150, amplitude 40% for 3 min). The bioassay to evaluate potential activity of microalgae biomass was performed. The antimicrobial (biopesticide) activities of the samples were detected using the antagonism bioassay. The plant biostimulating activities of the samples were detected using seed germination and radicle elongation bioassays.

2.6. Antagonism bioassays by dual culture

The extracts were tested against the growth of phytopathogenic fungi and bacteria *in vitro* using the dual culture technique according to the protocol described previously [43,44]. The activity of the extracts was tested against three phytopathogenic fungi – *Fusarium oxysporum* f.sp. *melonis* (further as *Fusarium oxysporum*), *Rhizoctonia solani* and *Phytophthora capsici*. All strains were provided by the Spanish Type Culture Collection (STCC).

2.7. Germination index bioassays

The cultures of both *Chlorella* R-117 and g120 were harvested at the end of the trial. The biostimulant activity was determined as germination index (GI) using 100 cress seeds (*Lepidium sativum*) which were treated by the water extracts of 0.5 and 2 mg DW mL^{-1} of spray- or freeze-dried microalgae biomass after ultrasonication of microalgae biomass according to the procedure previously described [45]. Results are expressed as a percentage of means \pm standard deviation ($n = 3$) considering distilled water as blank = control (100%). The percentage of seed germination as well as the radicle elongation were considered for the calculation of the GI, based on the following formula: $\text{GI} (\%) = (G_S \times L_S) / (G_W \times L_W)$. Where G_S is the percentage of germinated seeds in the presence of microalgae extract, G_W is a percentage of germinated seeds in the presence of distilled water, L_S is a mean of radicle elongation (mm) in the presence of microalgae extract and L_W is a mean of radicle elongation (mm) in the presence of distilled water (blank).

2.8. Statistical analysis

Most measurements were performed in triplicate ($n = 3$); the means and calculated standard errors (SE) are reported. Sigma Plot 11.0 was used to determine significant differences between treatments. One-way analysis of variance (ANOVA) and the Holm-Sidak test were conducted for comparison of variables in the trials. P values lower than 0.05 ($P < 0.05$) were considered to be significantly different. In graphs, the mean values designated by the same letter did not differ significantly from

each other.

3. Results

One typical and complete trial was selected for this publication; most measurements were carried out in duplicate or triplicate. The cultures of *Chlorella* g120 and *Chlorella* R-117 were grown in two outdoor TLCs (Fig. 1). At the start of the 5-day trial the cultures were diluted with the growth medium to a similar optical density (about 15) measured at 685 nm (i.e., absorbance maximum of Chl measured *in vivo*) as to have comparable light absorbance; however, the starting biomass density was 7 and 3 g DW L⁻¹ for *Chlorella* g120 and *Chlorella* R-117, respectively (Fig. 4). The biomass density at the start was adjusted to 7 g L⁻¹ in *Chlorella* g120 culture (Fig. 4b) to have similar Chl concentration per area (about 210 mg Chl m⁻², i.e., 5 mg Chl mg⁻¹ DW) as that in *Chlorella* R-117 (214 mg Chl m⁻², i.e., 15 mg Chl mg⁻¹ DW). The growth curves showed that the *Chlorella* g120 culture had a lag phase and slowly photoacclimated to ambient irradiance. It started to increase the biomass density only on day 4 while *Chlorella* R-117 started to grow at once and much faster (Fig. 4b). In the trial the growth rate for *Chlorella* R-117 was 0.32 d⁻¹ while for *Chlorella* g120 it was only 0.08 d⁻¹. On day 5 the biomass density of *Chlorella* R-117 was double as compared with that of *Chlorella* g120 although at the start it was just opposite (Fig. 4b). When the areal productivity was calculated between days 3 and 5, it was 22 g DW m⁻² d⁻¹ for *Chlorella* R-117 which was about 5 times higher compared to 4.3 g DW m⁻² d⁻¹ found in *Chlorella* g120. Here, it is clear that the changes of biomass and cell number showed clear discrepancy

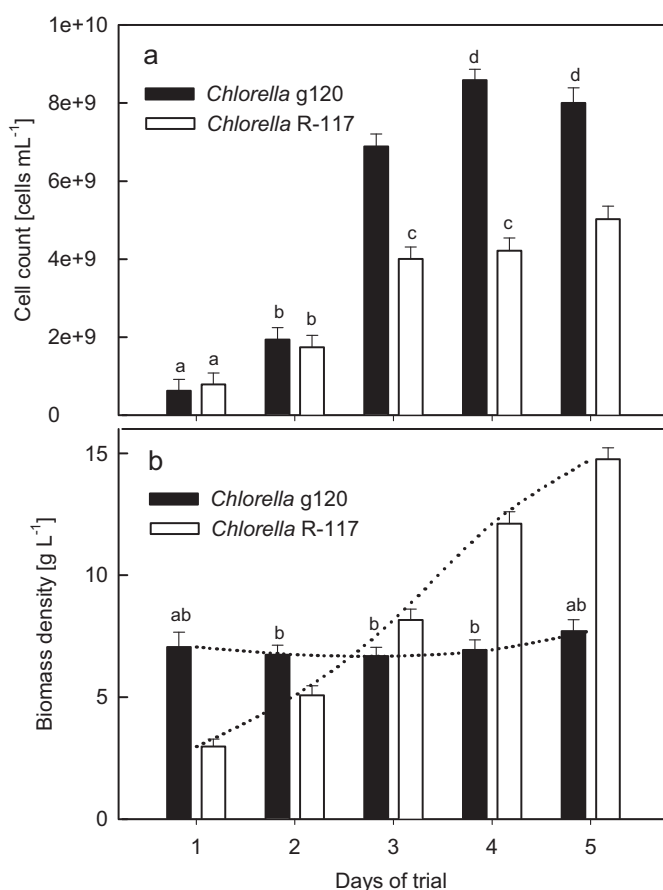


Fig. 4. The growth of *Chlorella* g120 and *Chlorella* R-117 during a 5-day outdoor trial estimated by (a) cell count/number and (b) biomass density. Samples were taken daily at 08:30 h. Values are presented as a mean ($n = 3$) with SE indicated by error bars. The values designated by the same letter did not differ significantly from each other.

between both cultures (Fig. 4). On day 5 the biomass density of *Chlorella* R-117 was double (15 g DW L⁻¹) as compared with that of *Chlorella* g120 (7.5 g DW L⁻¹) although at the start of the trial the latter was twice denser (3 vs. 7 g DW L⁻¹).

The changes of cell number showed that the values in both cultures were similar on days 1 and 2, about $6-8 \times 10^8$ cells mL⁻¹. At the start of the trial both cultures contained $6-8 \times 10^8$ cells mL⁻¹ and these remained similar on day 2. Then, on day 3, the cell number started to increase significantly in both cultures, but much faster in *Chlorella* g120 (about 3.7 times) whereas it only doubled in *Chlorella* R-117 (Fig. 4a). At the end of the trial the cell number was 1.4 times higher in *Chlorella* g120 compared to that in *Chlorella* R-117 (Fig. 4a). Judging from the course of cell number and biomass density, it looks like certain population of cells in the *Chlorella* g120 culture was divided after outdoor exposure and started to grow slowly only on day 4 when the cells were photo-acclimated and photosynthetic activity increased. As indicated by the various courses of biomass density and cell number changes, the reason was evidently better photoacclimation of *Chlorella* R-117 as compared to that of *Chlorella* g120.

Microscopic examination showed that the cells of *Chlorella* g120 cells were significantly smaller (about 3–4 μ m) than those of *Chlorella* R-117 (5–8 μ m) (Fig. 5).

Both cultures differed visibly in color as *Chlorella* g120 was milky yellow-green while *Chlorella* R-117 had bright dark green (Fig. 1). The yellowish color of *Chlorella* g120 reflected a much higher Car/Chl ratio of 0.63 as compared to the ratio of 0.26 in *Chlorella* R-117. The Chl content in biomass was 0.5–0.9% and 1.5–2% in *Chlorella* g120 and *Chlorella* R-117, respectively (Fig. 6a). The Car content in biomass was up to 0.48% and 0.68% in *Chlorella* g120 and *Chlorella* R-117, respectively (Fig. 6b). The higher Car/Chl in *Chlorella* g120 was due to the lower Chl content in the biomass compared to that in *Chlorella* R-117 (Fig. 6a). It means that the ratio of Car/Chl was nearly three times higher in *Chlorella* g120 than that in *Chlorella* R-117 (Fig. 6). The chromatographic analysis showed the quantitative difference in pigment composition of the *Chlorella* g120 and *Chlorella* R-117 cells (Fig. S1). The major carotenoid in both strains was the xanthophyll lutein, about 60% of total carotenoids. As both strains have similar amount of Car (Fig. 6) the pigment profiles were 'normalized' to the same height of the lutein peak (with the retention time of 13 min), they indicated that *Chlorella* g120 has similar spectrum of various carotenoids, but only about one half of Chl *b* compared with that of *Chlorella* R-117.

The different physiology of both *Chlorella* strains, g120 and R-117 affected their photosynthetic activity which was then reflected in their growth and photochemistry. It is important to note that the starting biomass densities of both cultures were different due to Chl content as these were adjusted to have similar absorption OD₆₈₅ per Chl (Fig. 4b vs. 6a). A good indicator of the photosynthetic activity measured *in-situ* is the oxygen production by the culture. This activity can be quickly estimated as the build-up of DO concentration, i.e., the difference in DO concentration (Δ DO) between the start and end of the cultivation area, the distance which the fast-flowing culture passes in tens of seconds. The photosynthetic activity depends on irradiance intensity as it is high at midday and much lower in the morning or close to sunset in late afternoon (18:00 h), even if the culture temperature was still about 25 °C (Fig. 7a). Thus, on sunny days when the microalgae cultures were in good condition, the Δ DO between the start and end of the cultivation track can be about 15 mg O₂ L⁻¹ (about 200%), or even higher. This was the case in *Chlorella* R-117 culture where photosynthetic activity was very high, producing the Δ DO of 12–15 mg L⁻¹ at midday from day 2 when the culture got acclimated (Fig. 7b). On the contrary, the DO concentration in the *Chlorella* g120 culture was only about 25–35% of that in *Chlorella* R-117 (Δ DO of 3–5 mg L⁻¹) on days 2–3. Only on day 5, when *Chlorella* g120 became better acclimated, the Δ DO reached about 55% compared to that of *Chlorella* R-117 (Fig. 7b) corresponding to the onset of the *Chlorella* g120 growth (Fig. 4b). The culture temperature should not play any significant role since it was similar in both cultures

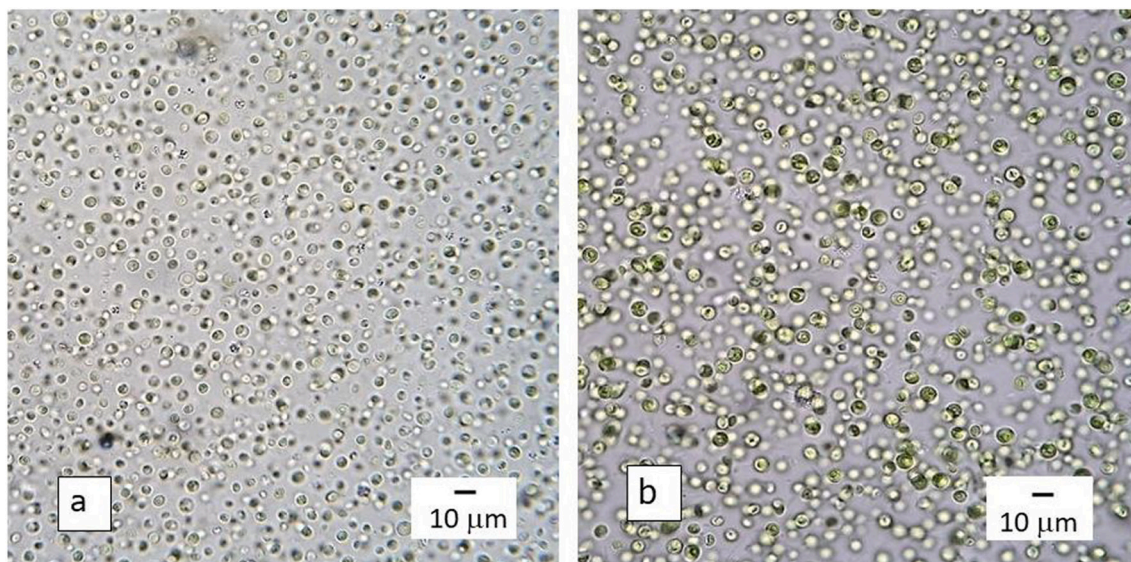


Fig. 5. Microscopic images of the cultures on day 2 of outdoor trial (magnification 600×). (a) Heterotrophic *Chlorella* g120; (b) phototrophic *Chlorella* R-117. The scale bars of 10 µm are shown.

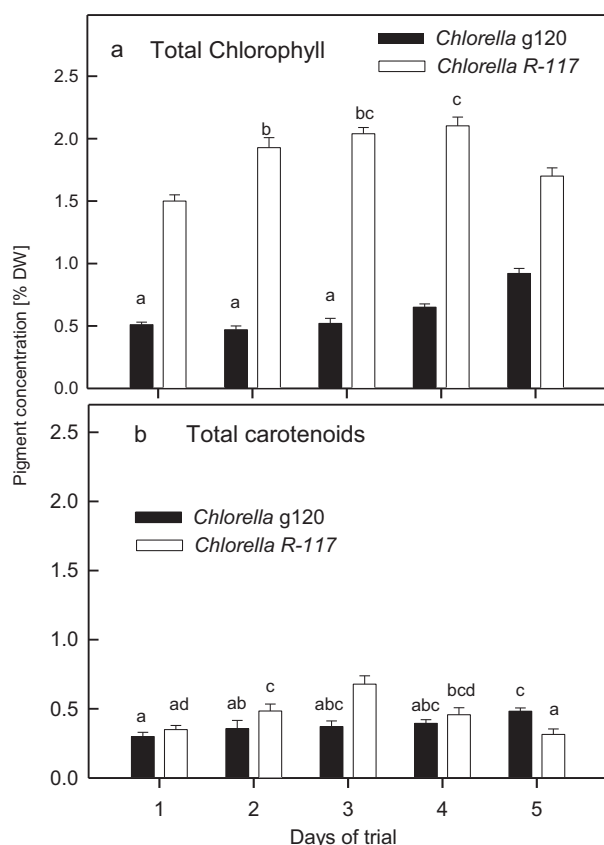


Fig. 6. Changes of total chlorophyll (a) and carotenoid (b) content measured as dry weight percentage in *Chlorella* g120 and *Chlorella* R-117 cultures during a 5-day outdoor trial. Samples were taken daily at 08:30 h. Values are presented as means ($n = 3$) with SE indicated by error bars. The values designated by the same letter did not differ significantly from each other.

with the morning minima of 17–22 °C and midday maxima between 30 and 35 °C on days 2–5 which represent the suitable physiological range (Fig. 7a).

When the photosynthetic activity was estimated as the relative electron transport rate ETR measured *in-situ* by Chl fluorescence techniques, the data corresponded to changes of Δ DO. On day 1 the ETR values were relatively low at midday, 240 and 360 $\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$ in *Chlorella* g120 and *Chlorella* R-117, respectively as the diluted cultures were acclimating to high irradiance outdoors (Fig. 8). Starting from day 2 to day 5 the ETR maxima at midday were very high — between 650 and 910 $\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$ in *Chlorella* R-117 (Fig. 8b) while in *Chlorella* g120 these values were only between 330 and 370 $\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$ (Fig. 8a). The averaged ETR for *Chlorella* g120 and *Chlorella* R-117 during days 2 to 5 were found 272 and 672 $\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$, respectively, which clearly illustrated the variance in the photosynthetic activity of both cultures. In average over the entire trial, the ETR values in *Chlorella* g120 were one third of those in *Chlorella* R-117.

The maximum photochemical yield of PSII, F_v/F_m ratios measured *ex-situ* in dark-adapted samples were significantly different between the two *Chlorella* strains. Generally, F_v/F_m was much lower in *Chlorella* g120 than in *Chlorella* R-117, especially during first three days (Fig. 9). On days 1 and 2, the morning F_v/F_m values were 0.11 to 0.13 in *Chlorella* g120 and then decreased at midday (Fig. 9a). On day 5 when *Chlorella* g120 started to grow, the F_v/F_m values were similar to those in *Chlorella* R-117 on day 1. In *Chlorella* R-117, the morning value on day 1 was 0.38 and it increased to 0.58 on day 2 (Fig. 9b). The *Chlorella* R-117 culture showed high F_v/F_m values around 0.6 starting from the day 2 which corresponded with its good growth (Fig. 4b). In this culture, the midday reduction of F_v/F_m was 47% and 35% on day 2 and 3, respectively, showing a certain degree of photo-stress due low biomass density as referred previously [29], whereas in denser cultures on day 4 and day 5 the reduction of F_v/F_m was only 23% and 14%, respectively. Nevertheless, in *Chlorella* g120 the midday depression of F_v/F_m was still about 50%. From these results it is evident that *Chlorella* g120 was much less acclimated to high irradiance in outdoor culture, even if this culture was inoculated at twice higher biomass density, to obtain similar areal Chl concentration as that in *Chlorella* R-117.

The F_v/F_m correlated well with Δ DO measured *in-situ* (Fig. 7). On days 2–3, Δ DO along the cultivation surface reached only 25–30% in *Chlorella* g120 compared to *Chlorella* R-117 (Fig. 7b) and F_v/F_m was very

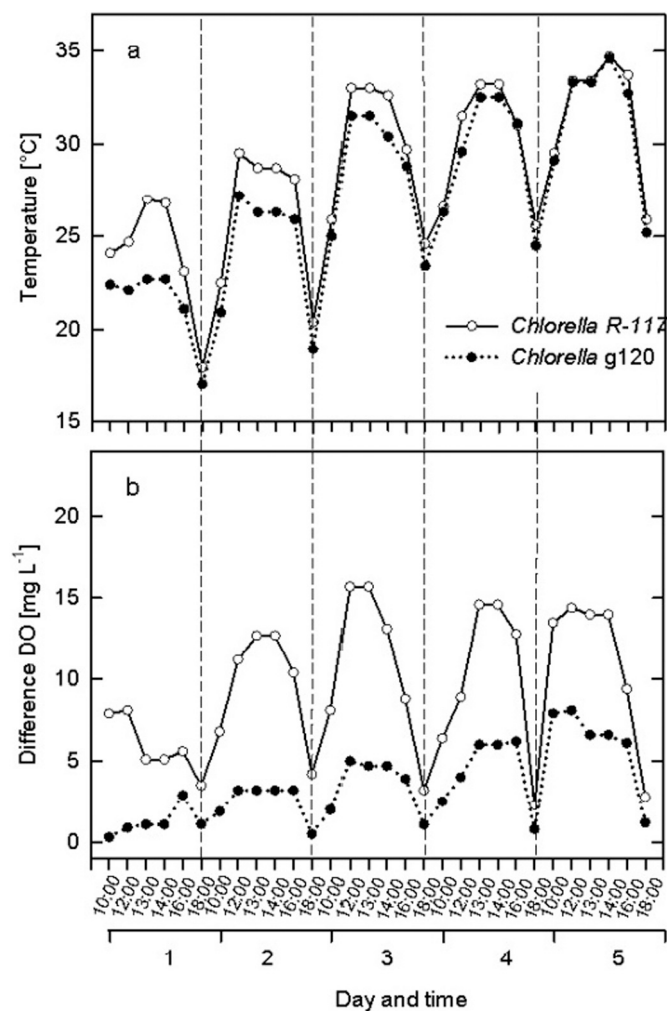


Fig. 7. Daily changes in the difference of DO concentration (b) measured *in-situ* between the start and end of cultivation area in *Chlorella g120* (dashed line with closed symbols) and *Chlorella R-117* (solid line with open symbols) cultures. Daily course of temperature (a) was measured in parallel with changes of DO concentration.

low compared with *Chlorella R-117* (Fig. 9a). During the cultivation trial, on days 4–5, ΔDO and F_v/F_m in *Chlorella g120* increased, but the values reached only about one half of these in *Chlorella R-117*. The courses of both variables clearly reflected the different growth rates of the *Chlorella g120* and *Chlorella R-117* cultures (Fig. 4b).

As the cultures differed greatly in their Chl content per cell, the values of oxygen production/consumption were measured *ex-situ* measurements using the measuring chamber and calculated per cell per hour for comparison (Fig. 10). On day 1 the morning value of respiration in the *Chlorella g120* culture reached $13 \text{ pmol cell}^{-1} \text{ h}^{-1}$ which was a higher value than the oxygen production ($10 \text{ pmol cell}^{-1} \text{ h}^{-1}$), while at midday the respiration slightly decreased. Then, the respiration values in *Chlorella g120* were usually below $5 \text{ pmol cell}^{-1} \text{ h}^{-1}$ while the oxygen production at midday increased from 11 on day 1 to about $20 \text{ pmol cell}^{-1} \text{ h}^{-1}$ on day 5. At midday the respiration rates were lower in *Chlorella g120* cultures, but the oxygen production was only 30–40% of that in *Chlorella R-117*. In the *Chlorella R-117* culture, the respiration values were between 0.4 and $2.2 \text{ pmol cell}^{-1} \text{ h}^{-1}$ during the trial, but the oxygen production significantly increased from 11 on day 1 to $34 \text{ pmol cell}^{-1} \text{ h}^{-1}$ on day 5. The higher value of oxygen production on day 1 in the morning later decreased probably due to photo-stress. At the end of the trial (days 4–5), the morning respiration rate in *Chlorella g120* was relatively low, but still about twice higher and oxygen evolution was

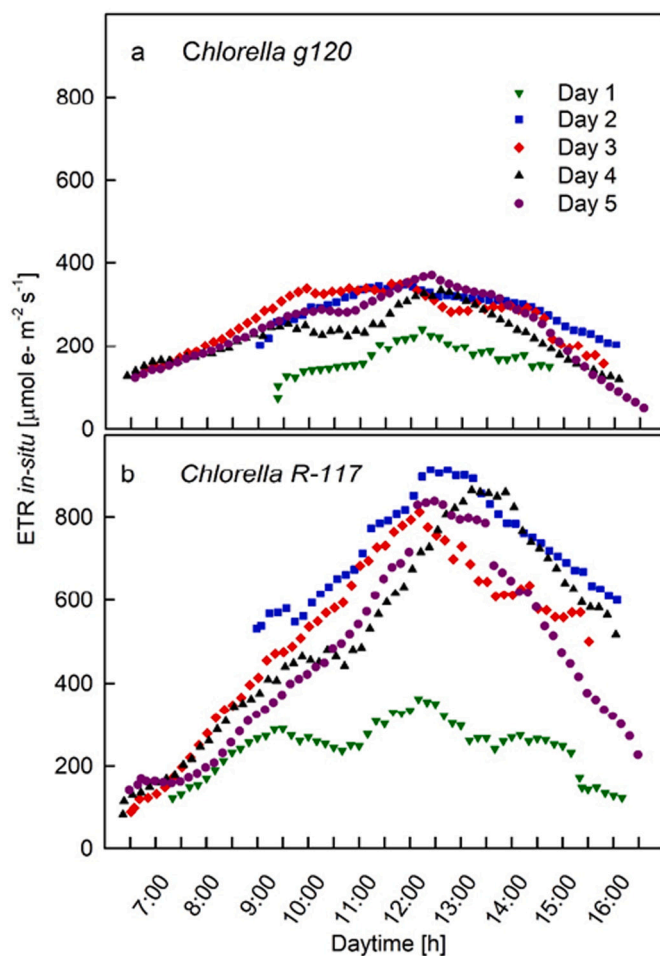


Fig. 8. Daily changes in relative electron transport rate, ETR measured *in-situ* in *Chlorella g120* (a) and *Chlorella R-117* (b) cultures during the 5-day trial.

about 5–10-times lower as compared to the values in *Chlorella R-117*.

Two additional variables were measured to clarify photochemical differences between the two *Chlorella* strains — specific absorption cross-section a (m^{-1}) and the quantum yield of PS II electron transport α (electron/photon). The a values in both strains were usually increased from the morning (08:30 h) till afternoon (17:00 h) on all days (Table 1). The values were in the range between 248 and 3619 m^{-1} and 662 and 6828 m^{-1} in *Chlorella g120* and *Chlorella R-117*, respectively. Thus, the specific absorption cross-section for light was in average about twice higher in *Chlorella R-117* that correlated with the increase in pigment content (Fig. 6). The data of the quantum yield of PSII electron transport α (photochemical efficiency) followed the trends observed with the specific absorption cross-section. From day 1 to day 5, the α values increased from 0.05 to 0.21 and from 0.18 to 0.28 in *Chlorella g120* and *Chlorella R-117*, respectively (Fig. 11). Thus, the values in *Chlorella g120* reached only between 26 and 75% of the values in *Chlorella R-117*. Only on days 4 and 5 the α values were similar in both cultures showing that *Chlorella g120* was partially acclimated to outdoor irradiance.

Biochemical analyses of microalgae using FTIR spectroscopy were carried for days 3–5 of the trial when the *Chlorella g120* culture started to grow (Fig. 4 vs. Fig. S2). The data showed significant differences between the two strains in the ratios of major components — proteins, lipids and carbohydrates. The carbohydrate:protein (C:P) ratio was between 0.23 and 0.38 and between 0.28 and 0.41 in *Chlorella g120* and *Chlorella R-117*, respectively (Fig. S2a). Between days 4 and 5 the difference in the C:P ratio rose in both strains. The carbohydrate:lipid (C:L) ratio was significantly smaller in *Chlorella g120* by about 30–40%

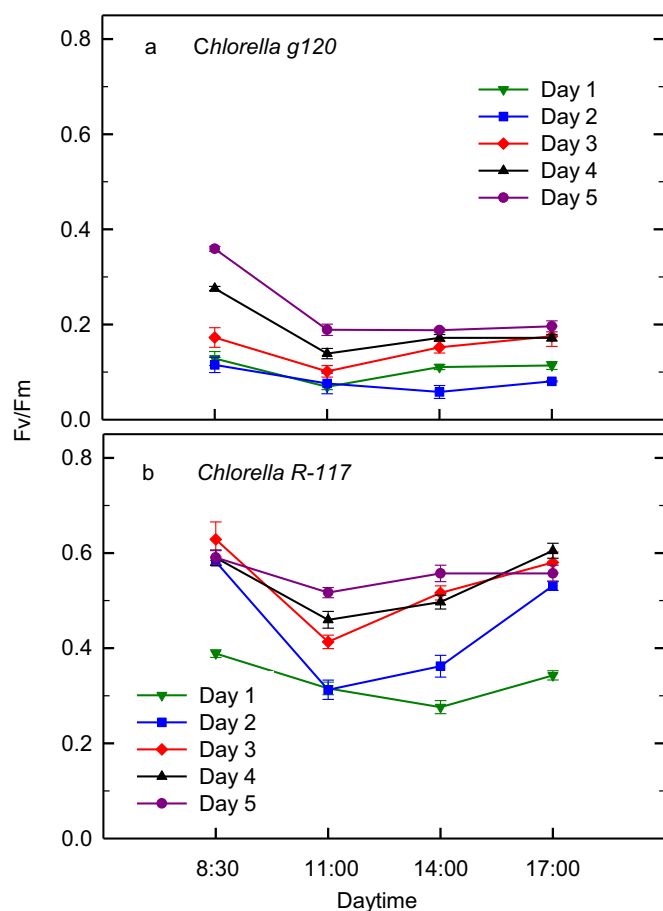


Fig. 9. Diel changes of the maximum PSII photochemical yield F_v/F_m measured *ex-situ* in the samples taken from outdoor cultures of *Chlorella* g120 (a) and *Chlorella* R-117 (b) during the 5-day trial.

(1.41–1.92) as compared to *Chlorella* R-117 (2.0–2.55) (Fig. S2b). The difference was the higher on day 3; on days 4–5 the C:L ratio was decreasing in both cultures, but more steeply in *Chlorella* g120. As concerns the lipid:protein (L:P) ratio, it was between 0.17 and 0.20 and 0.14 and 0.18 in *Chlorella* g120 and *Chlorella* R-117, respectively. The values L:P ratio were about 15% higher in *Chlorella* g120 as compared to those in *Chlorella* R-117. Important to note that the trends did not show significant changes as the difference was similar on days 3–5 (Fig. S2c). The comparison of the data revealed that the content of carbohydrates was changing during the period of days 3–5 when the cultures were photosynthetically active and growing.

Our experience from previous experiments (J. Masojćdek, unpublished) has shown that the healthy *Chlorella* cultures were rather resistant to contamination by other microorganisms, even if grown outdoors. Thus, we also examined antifungal and biostimulating activities in both outdoor cultures (Table S1). The biomass extracts from both strains inhibited the three tested fungi between 25 and 40%. Germination index (GI) was determined using lettuce or water cress seeds and it was calculated as percentage increase caused by microalgae extracts compared to the sterilized distilled water as blank. The water extracts prepared from the biomass of both *Chlorella* strains increase the GI by 8–10% (Table S1).

4. Discussion

The particular objective of this study was to elucidate the photo-biochemical changes which take place during the metabolic shift from heterotrophic to phototrophic growth regime in an outdoor culture of

the heterotrophic strain *Chlorella* g120. The crucial question was whether *Chlorella* g120 could undergo the photoacclimation of its photosynthetic apparatus outdoor under solar irradiance. For this purpose, we employed various methods (*in-situ* and *ex-situ*) to monitor changes of physiology and photosynthesis that can be correlated with growth. Monitoring techniques (and variables) – illustrative and relevant – were selected as markers to control and optimize growth.

Such data, as to follow photobiochemical changes in outdoor mass cultures during the trophic conversion from heterotrophy to phototrophy have been scarce in literature [46] although several authors used this two-step process to grow various microalgae [16,18,20,47]. Recently, photobiochemical changes vs. growth have been studied during the trophic conversion in laboratory cultures of *Chlorella* g120 [9]. The data suggested this strain to be a good model for outdoor mass cultivation. All evidence has shown that in practice the issue is not as straightforward. The culture of *Chlorella* g120 can grow photo-trophically but much slower than in the heterotrophic regime and slower than an obligate phototrophic strain, *Chlorella* R-117 [9]. *Chlorella* g120 can shift the metabolism from the heterotrophic to phototrophic growth regime [9] although the cells contain much less Chl and consequently a considerably higher ratio of carotenoid/chlorophyll (Car/Chl), compared to the phototrophic *Chlorella* R-117 [14].

The present study has identified differences in physiological and photosynthetic variables between the two *Chlorella* strains. The nearly three times higher ratio of Car/Chl was found in *Chlorella* g120 compared to that in *Chlorella* R-117 and the major carotenoid was the xanthophyll lutein, a valuable antioxidant. The decreased Chl content (<1% in DW) suggests that *Chlorella* g120 does not possess the fully competent photosynthetic apparatus, namely the light-harvesting antennae. The analyzes of pigments and chlorophyll-protein complexes indicated that *Chlorella* g120 has a modified, or missing part of the Chl *a/b* antennae which are responsible for the light-harvesting [9,48]. This hypothesis is supported by much lower values (about one half) of the specific absorption cross-section a and the quantum yield of PS II electron transport α measured in *Chlorella* g120 compared to those in *Chlorella* R-117 (Fig. 11, Table 1). The slowly increasing Chl content in the biomass of *Chlorella* g120 during outdoor growth still indicated some degree of photoacclimation (Fig. 6) which did not markedly express itself in biomass growth during the five days of this trial.

The microalgae strains with a regular Chl content may reach light saturation at lower irradiance intensities, but it practically means that the light energy not used for photochemistry is dissipated in the antennae. On the contrary, in the microalgae cultures showing a decreased Chl content (*i.e.*, reduced antennae and a smaller absorption cross-section) the light is allowed to penetrate deeper layers of micro-algae cells and irradiate a larger volume of the culture where can be used for photosynthesis. The strain *Chlorella* g120 presents some important features of the so-called “reduced antenna-size” strain which might be the characteristics desirable for mass culture growth [48–50]. Our data are in agreement with observation concerning the reduced antenna-size mutant of *Chlorella vulgaris* which contained lower Chl content due to a decreased expression of peripheral light-harvesting PSII antenna proteins [51]. The small antenna-size strains have been speculated to perform better as they may light-saturate at higher irradiance intensity [52]. Some authors considered such microalgae strains as potential candidates for enhanced bioproductivity in stirred highly-lighted photo-bioreactors, thanks to their high photon use efficiency and low susceptibility to high-light stress, mutual shading and the wasteful dissipation of absorbed sunlight [49,52].

High concentrations of dissolved oxygen can inhibit the growth of photosynthetic microorganisms in microalgal culture systems [53]. This study documented the correlation between photosynthetic activity and culture growth with an array of chemical and fluorescence methods. Several techniques were used that, directly or indirectly, show how the culture activity reflects in growth. The build-up of DO concentration (measured *in-situ* by an oxygen electrode) clearly provides an instant and

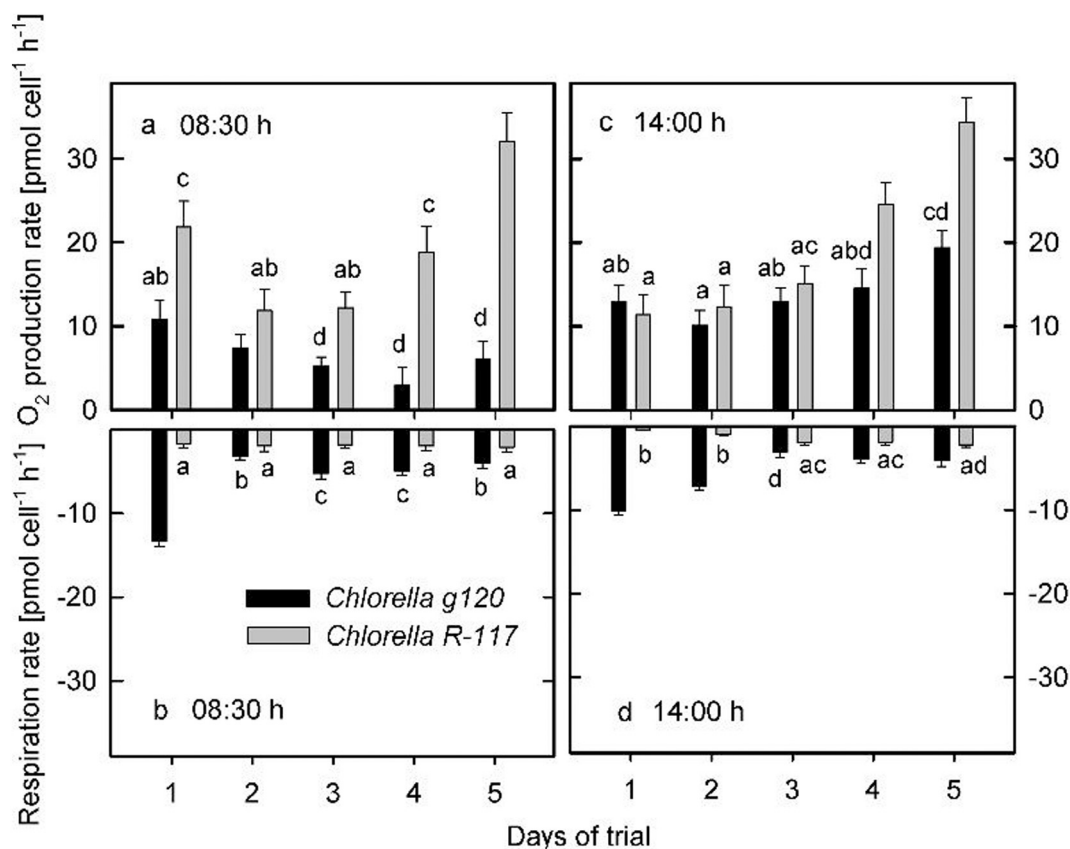


Fig. 10. Rates of the maximum photosynthetic oxygen evolution (panels a, c) and respiration (panels b, d) measured *ex-situ* in samples taken from the outdoor cultures of *Chlorella g120* and *Chlorella R-117* during the 5-day trial. Samples were taken daily at 8:30 (panels a, b) and 14:00 h (panels c, d). Values are presented as means ($n = 3$) with SE indicated by error bars. The values designated by the same letter did not differ significantly from each other.

Table 1

Diel changes of specific absorption cross-section a (m^{-1}) of the cultures *Chlorella g120* and *Chlorella R-117* measured daily during the 5-day outdoor trial at 8:00, 14:00 and 17:00 h. Values were calculated for the diluted culture as an average in the range between 400 and 700 nm.

Strain	Time	<i>Chlorella R-117</i>		<i>Chlorella g120</i>		Ratio
		a (m^{-1})		a (m^{-1})		
Day 1	8	662 ± 28	248 ± 16	2.67		
	14	541 ± 25	560 ± 26	0.97		
	17	719 ± 36	607 ± 28	1.18		
Day 2	8	739 ± 35	548 ± 25	1.35		
	14	1202 ± 52	602 ± 27	2.00		
	17	1785 ± 65	628 ± 19	2.84		
Day 3	8	1537 ± 61	622 ± 21	2.47		
	14	1851 ± 69	690 ± 35	2.68		
	17	1963 ± 79	767 ± 38	2.56		
Day 4	8	1967 ± 86	752 ± 35	2.62		
	14	3591 ± 135	1064 ± 53	3.38		
	17	3587 ± 123	1845 ± 85	1.94		
Day 5	8	2314 ± 111	1339 ± 51	1.73		
	14	6828 ± 181	3619 ± 121	1.89		

fast measure of photosynthetic activity (Fig. 7) that is directly linked to culture growth [27,54]. The much faster growth of *Chlorella R-117* (compared to *Chlorella g120*) correlated well with the ample build-up in DO concentration between the beginning and end of the cultivation area (Fig. 4 vs. Fig. 7). It is also demonstrated that a significant part of light energy is dissipated/consumed for respiration and not for photosynthetic production in *Chlorella g120* (Fig. 10).

Other variables used as reliable indicators of culture activity were the maximum PSII photochemical yield, F_v/F_m and the electron

transport rate, ETR measured by Chl fluorescence techniques. The latter variable measured *in-situ* in the upper exposed layer (photostage) corresponds to the build-up of DO concentration and culture growth. The reliable, easy to record variable F_v/F_m (although often underrated), or the actual photochemical yield $\Delta F'/F'_m$, both monitor the status and behavior of microalgae culture. The F_v/F_m measured *ex-situ* (Fig. 9) and the course of DO concentration increase (Fig. 7) and the course of ETR (Fig. 8) recorded *in-situ* showed similar trends. The small drop in F_v/F_m inversely corresponds to the highest ΔDO concentration and ETR values in both strains. This correlation is valid for days 3–5 when the cultures were denser and photoacclimated. *Per contra*, *Chlorella g120* presents a much higher respiration rate (Fig. 10) and lower photochemical efficiency (Fig. 11) compared to *Chlorella R-117*. The high respiration rate (oxygen consumption calculated per cell) is probably an inherited feature of heterotrophic strains. Respiration serves as an energy sink protecting the photosynthetic apparatus against surplus irradiance but in this case limiting biomass growth and production. In photosynthetic studies, the variable F_v/F_m is often used as a stress indicator [55]. The experience gathered in a number of experiments have demonstrated that a decrease in F_v/F_m or $\Delta F'/F'_m$ between 20 and 30% during the day – as also reported here – can be still considered physiological and represents a useful indicator of well-growing highly productive cultures [4,29,56,57]. The variation outside this range may indicate either photo-stress or photolimitation. This can be avoided by the adjustment of biomass density in cultures that are exposed to high irradiance as confirmed by the present experiments. If the higher or lower decrease are found, this may indicate either photo-stress or photolimitation.

The important issue coming from these trials is the correlation between high photosynthetic oxygen production rate causing an ample increase in DO concentration and PSII photochemical yield [58]. In fast

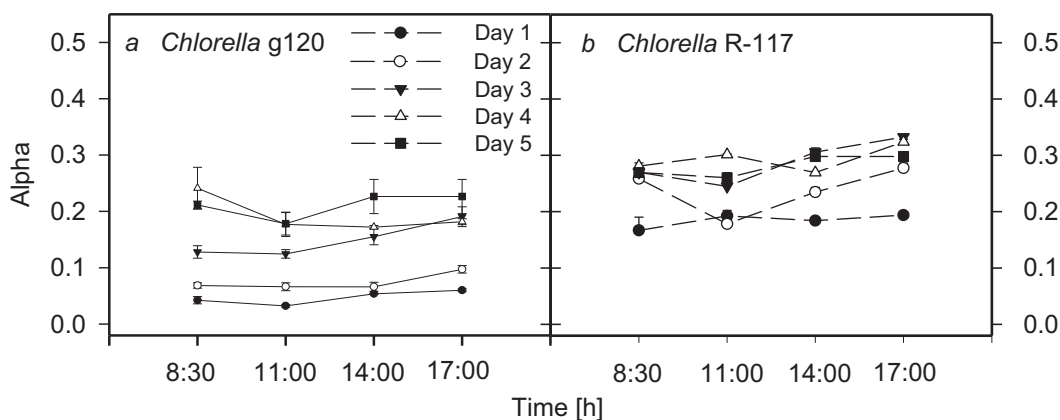


Fig. 11. Diurnal changes in the photochemical efficiency α (quantum yield of PSII electron transport; electrons/photon) measured *ex-situ* in the samples taken from the outdoor cultures of *Chlorella* g120 (a) and *Chlorella* R-117 (b) during the 5-day trial.

growing, highly productive cultures, the DO concentration at midday sometimes reached 300%, or higher (above 20 mg O₂ L⁻¹). From changes in the growth rate, the build-up of DO concentration, ETR and the dynamics of the F_v/F_m , it could be concluded that the high difference in DO concentration is not as inhibiting for photosynthesis in open units as in closed photobioreactors [21,22]. In TLC, the flow of culture is fast (about 0.5 m s⁻¹) in the thin layer of culture which facilitate the oxygen degassing to the ambient at the surface and in the retention tank. The microalgae cells are exposed to high DO concentration only for short periods (~20 s) during the circulation cycle, toward the end of the cultivation area, and then DO levels are quickly returned to equilibrium with the atmosphere. High DO concentration are found only at high ambient irradiance between 12:00 and 15:00 h. Due to the high photosynthetic activity at high culture densities, the rate of oxygen production exceeded the rate of oxygen diffusion out through the liquid/air interface. Despite the high surface to volume ratio in this TLC and an efficient degassing the high DO concentration was observed at the end of the cultivation area, and impacted the photosynthetic rate [58]. At high DO concentrations, photorespiration might also be induced due to oxygenase activity of Rubisco, reducing carbohydrate synthesis and enhancing the synthesis of phosphoglycolate [59]. A reduction of the photosynthetic rate above 200% DO saturation was observed in cultures of *Scenedemus almeriensis* [60,61].

Then, considering again the biomass production, the changes in DO concentration and F_v/F_m , we have to find the compromise between the rate of photosynthesis and the increase in DO concentration in large-scale microalgae mass cultures to secure high growth rate. In low activity cultures the build-up of DO concentration and the decrease in F_v/F_m was less dramatic, but the overall productivity was lower [29]. However, even in fast-growing cultures, maximal productivity depends on an optimal adjustment between biomass density and culture layer thickness (<10 mm), to prevent excessive mutual shading. This can be obtained in real time, with the use of the on-line measured photosynthetic fluorescence variables described here. In addition, in highly dense cultures (>10 g DW L⁻¹), turbulent flow is critical to ease degassing and maintain the intermittent light regime (light/dark cycles of about 0.5 s) match the turnover rate of the photosynthetic apparatus [29,56,62].

Biochemical analyses (the ratios of carbohydrate:protein and carbohydrate:lipid) showed that *Chlorella* R-117 accumulated more starch during the days 3 and 4 as compared to *Chlorella* g120. The lack of a significant change in the lipid:protein ratio on day 4 in this strain suggests that lipids were not markedly accumulated although the lipid:protein ratio was about 15% higher in *Chlorella* g120 as compared to those in *Chlorella* R-117. These results are in partial agreement with the report published by Li et al. [63]. They found that during a trophic conversion in *Chlorella protothecoides*, synthesis of starch transiently increased, followed by a phase of lipid accumulation [63]. In our

experiments, the data showed higher carbohydrate:lipid ratio in *Chlorella* R-117 as compared to *Chlorella* g120, but the difference was diminishing on days 4–5. Furthermore, the lipid:protein ratio was increasing in *Chlorella* g120 on days 4–5, possibly thanks to the use of the carbohydrates as precursors for lipid synthesis.

5. Conclusions

Physiological and photosynthetic changes were monitored in outdoor culture of *Chlorella* g120 during the metabolic pathway shift (trophic conversion) from heterotrophy to phototrophy. Various *in-situ* and *ex-situ* measuring techniques were tested as to follow the photoacclimation of *Chlorella* g120 during the trophic conversion as we aimed to select the most relevant methods and variables. The phototrophic growth of *Chlorella* g120 was slow due to low photosynthetic activity since the photosynthetic apparatus is not fully competent. The important resume is that the heterotrophic *Chlorella* g120 strain can grow phototrophically outdoors under ambient irradiance, but the shift was incomplete due to lengthy photoacclimation. Surprisingly, we recorded relatively high electron transport activity in *Chlorella* g120 which was not fully reflected by the growth rate. The results suggest that high photosynthetic activity is dissipated in futile processes like respiration and probably some kind of non-photochemical quenching [9] as the strategy to sink unused light energy and protect the photosynthetic apparatus against over-reduction. *Chlorella* g120 is also characterized by low Chl/cell ratio (below 1%) which suggests that *Chlorella* g120 does not possess the fully competent photosynthetic apparatus. It can be characterized as the typical strain with small-antenna size where the oligomers of light-harvesting antennae are missing [9]. As for comparison the phototrophic, fast-growing and highly productive strain *Chlorella vulgaris* R-117 was examined in parallel.

It was verified that some photosynthesis variables measured *in-situ* and *ex-situ*, in particular the maximum photochemical yield F_v/F_m , relative electron transport rate ETR and oxygen production (POE) can be used to estimate and optimize growth in large-scale units. The photosynthetic activity can be quickly estimated as the build-up of DO concentration, *i.e.*, the difference between the start and end of the cultivation area. As the culture flow is fast, the cells are exposed to high DO gradients only on the small part of cultivation area for which the culture passes in tens of seconds and then it is degassed.

The last but not least, the combination of culture grown in a fermenter followed by an outdoor cascade allows to avoid the dilution of the culture as the latter system can be operated at concentrations close to that used in fermenter. It may reduce the cost of harvesting of the phototrophic culture once the desired amount of pigment is reached. Another advantage of *Chlorella* g120 is the lack of an intense color of the biomass, a characteristic desirable when the microalgal biomass has to

be used in large percentage for the formulation of new foods or their ingredients. High carotenoid/chlorophyll ratio in biomass may be advantageous for biotechnological application.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.algal.2021.102303>.

Funding

This research was funded by the EU Program Horizon 2020 (project SABANA, grant no. 727874) and in part by National Sustainability Program of the Ministry of Education, Youth and Sports (project Alga-tech Plus LO1416) and cross-border InterReg Program between Czech Republic and Austria (Algenetics No. ATCZ15). Amir Neori was supported by BARD Research Grant Award No. US-4599-13R (The United States–Israel Binational Agricultural Research and Development Fund) and by the Israeli Ministry for Science and Technology and the Dead Sea–Arava Science Center (DSASC).

CRediT authorship contribution statement

Jirí Masojídek: Conceptualization, Methodology, Data curation, Writing – original draft, Supervision, Funding acquisition. **Karolína Ranglová:** Conceptualization, Investigation, Data curation, Visualization, Writing – review & editing. **Tomás Agustín Rearte:** Conceptualization, Investigation, Data curation, Visualization, Writing – review & editing. **Paula S.M. Celis Plá:** Conceptualization, Investigation, Data curation, Visualization, Writing – review & editing. **Giuseppe Torzillo:** Conceptualization, Investigation, Data curation, Visualization, Writing – review & editing. **Ana Margarita Silva Benavides:** Methodology, Investigation, Formal analysis, Data curation, Writing – review & editing. **Amir Neori:** Conceptualization, Investigation, Data curation, Visualization, Writing – review & editing. **Cintia Gómez:** Methodology, Investigation, Formal analysis, Data curation, Writing – review & editing. **Félix Álvarez-Gómez:** Methodology, Investigation, Formal analysis, Data curation, Writing – review & editing. **Martín Lukeš:** Methodology, Investigation, Formal analysis, Data curation, Writing – review & editing. **Martín P. Caporgno:** Methodology, Investigation, Formal analysis, Data curation, Writing – review & editing. **Roberto Abdala:** Methodology, Investigation, Formal analysis, Data curation, Writing – review & editing. **Krystian Miazek:** Methodology, Investigation, Formal analysis, Data curation, Writing – review & editing. **Thais Fávero Massocato:** Methodology, Investigation, Formal analysis, Data curation, Writing – review & editing. **Jaqueline Carmo da Silva:** Methodology, Investigation, Formal analysis, Data curation, Writing – review & editing. **Richard Atzmüller:** Methodology, Investigation, Formal analysis, Data curation, Writing – review & editing. **Hafidh Al Mahrouqi:** Methodology, Investigation, Formal analysis, Data curation, Writing – review & editing. **Francisca Suárez Estrella:** Methodology, Investigation, Formal analysis, Data curation, Writing – review & editing. **Felix L. Figueroa:** Conceptualization, Methodology, Data curation, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

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

Acknowledgement

The authors thank Ms. Soňa Pekařová, Mr. Jan Pilný, Mr. Michal Bureš and Mr. Petr Novotný for technical assistance and Richard Lhotský for experiment management.

References

- [1] J. Masojídek, E.S. Torzillo, Mass Cultivation of Freshwater Microalgae, Elsevier, 2014, <https://doi.org/10.1016/B978-0-12-409548-9.09373-8>.
- [2] M.A. Borowitzka, Commercial production of microalgae: ponds, tanks, tubes and fermenters, J. Biotechnol. 70 (1999) 313–321, [https://doi.org/10.1016/S0168-1656\(99\)00083-8](https://doi.org/10.1016/S0168-1656(99)00083-8).
- [3] M.A. Borowitzka, Algal biotechnology products and processes — matching science and economics, J. Appl. Phycol. 4 (1992) 267–279, <https://doi.org/10.1007/BF02161212>.
- [4] A. Richmond, Microalgal biotechnology at the turn of the millennium: a personal view, J. Appl. Phycol. 12 (2000) 441–451, <https://doi.org/10.1023/A:1008123131307>.
- [5] J. Masojídek, G. Torzillo, M. Koblížek, Photosynthesis in microalgae, in: A. Richmond, Q. Hu (Eds.), Handb. Microalgal Cult, John Wiley & Sons, Ltd., 2013, pp. 21–36, <https://doi.org/10.1002/9781118567166.ch2>.
- [6] H. Endo, H. Sansawa, K. Nakajima, Studies on *Chlorella regularis*, heterotrophic fast-growing strain II. Mixotrophic growth in relation to light intensity and acetate concentration, Plant Cell Physiol. 18 (1977) 199–205, <https://doi.org/10.1093/oxfordjournals.pcp.a075413>.
- [7] H. Endo, M. Shiota, Studies on the heterotrophic growth of *Chlorella* in mass culture, in: G. Terui (Ed.), Ferment. Technol. Today, 1972, pp. 533–541.
- [8] J. Doucha, K. Lívanský, Production of high-density *Chlorella* culture grown in fermenters, J. Appl. Phycol. 24 (2012) 35–43, <https://doi.org/10.1007/s10811-010-9643-2>.
- [9] A. Babaei, K. Ranglová, J.R. Malapascua, G. Torzillo, J. Shayegan, A.M. Silva Benavides, J. Masojídek, Photobiochemical changes in *Chlorella* g120 culture during trophic conversion (metabolic pathway shift) from heterotrophic to phototrophic growth regime, J. Appl. Phycol. (2020), <https://doi.org/10.1007/s10811-020-02137-w>.
- [10] H. Endo, H. Sansawa, Recent advances in the application of *Chlorella*, in: Second Asia-Pacific Conf. Algal Biotechnol, 1994, p. 22.
- [11] M. Sergejevoá, J. Masojídek, *Chlorella* biomass as feed supplement for freshwater fish: sterlet, *Acipenser ruthenus*, Aquac. Res. 44 (2012) 157–159, <https://doi.org/10.1111/j.1365-2109.2011.03011.x>.
- [12] J.R. Benavente-Valdés, C. Aguilar, J.C. Contreras-Esquivel, A. Méndez-Zavala, J. Montañez, Strategies to enhance the production of photosynthetic pigments and lipids in chlorophyceae species, Biotechnol. Rep. 10 (2016) 117–125, <https://doi.org/10.1016/j.btre.2016.04.001>.
- [13] M.A. Mohammad Mirzaie, M. Kalbasi, S.M. Mousavi, B. Ghobadian, Investigation of mixotrophic, heterotrophic, and autotrophic growth of *Chlorella vulgaris* under agricultural waste medium, Prep. Biochem. Biotechnol. 46 (2016) 150–156, <https://doi.org/10.1080/10826068.2014.995812>.
- [14] J.R. Malapascua, K. Ranglová, J. Masojídek, Photosynthesis and growth kinetics of *Chlorella vulgaris* R-117 cultured in an internally LED-illuminated photobioreactor, Photosynthetica (2018), <https://doi.org/10.32615/ps.2019.031>.
- [15] H. Endo, K. Nakajima, R. Chino, M. Shiota, Growth characteristics and cellular components of *Chlorella regularis*, heterotrophic fast growing strain, Agric. Biol. Chem. 38 (1974) 9–18, <https://doi.org/10.1080/00021369.1974.10861121>.
- [16] J. Ogbonna, H. Masui, H. Tanaka, Sequential heterotrophic/autotrophic cultivation — an efficient method of producing *Chlorella* biomass for health food and animal feed, J. Appl. Phycol. 9 (1997) 359–366.
- [17] N. Hata, J. Ogbonna, Y. Hasegawa, H. Taroda, H. Tanaka, Production of astaxanthin by *Haematococcus pluvialis* in a sequential heterotrophic-photoautotrophic culture, J. Appl. Phycol. 13 (2001) 395–402.
- [18] J. Fan, J. Huang, Y. Li, F. Han, J. Wang, X. Li, W. Wang, S. Li, Sequential heterotrophy–dilution–photoinduction cultivation for efficient microalgal biomass and lipid production, Bioresour. Technol. 112 (2012) 206–211, <https://doi.org/10.1016/j.biortech.2012.02.046>.
- [19] J. Fan, Y. Cui, J. Huang, W. Wang, W. Yin, Z. Hu, Y. Li, Suppression subtractive hybridization reveals transcript profiling of *Chlorella* under heterotrophy to photoautotrophy transition, PLoS One 7 (2012) 1–15, <https://doi.org/10.1371/journal.pone.0050414>.
- [20] A. Barros, H. Pereira, J. Campos, A. Marques, J. Varela, J. Silva, Heterotrophy as a tool to overcome the long and costly autotrophic scale-up process for large scale production of microalgae, Sci. Rep. 9 (2019) 13935, <https://doi.org/10.1038/s41598-019-50206-z>.
- [21] G. Torzillo, P. Accolla, E. Pinzani, J. Masojídek, In situ monitoring of chlorophyll fluorescence to assess the synergistic effect of low temperature and high irradiance stresses in *Spirulina* cultures grown outdoors in photobioreactors, J. Appl. Phycol. 8 (1996) 283–291, <https://doi.org/10.1007/BF02178571>.
- [22] G. Torzillo, P. Bernardini, J. Masojídek, On-line monitoring of fluorescence to access the extent of photoinhibition of photosynthesis induced by high oxygen concentration and low temperature and its effect on the productivity of outdoor cultures of *Spirulina platensis* (Cyanobacteria), J. Phycol. 34 (1998) 504–510, <https://doi.org/10.1046/j.1529-8817.1998.340504.x>.
- [23] J.C. Kromkamp, R.M. Forster, The use of variable fluorescence measurements in aquatic ecosystems: differences between multiple and single turnover measuring protocols and suggested terminology, Eur. J. Phycol. 38 (2003) 103–112, <https://doi.org/10.1080/0967026031000094094>.
- [24] P.J. Ralph, R. Gademann, Rapid light curves: a powerful tool to assess photosynthetic activity, Aquat. Bot. 82 (2005) 222–237, <https://doi.org/10.1016/j.aquabot.2005.02.006>.
- [25] S. Enríquez, M.A. Borowitzka, The use of the fluorescence signal in studies of seagrasses and macroalgae, in: Chlorophyll a Fluoresc. Aquat. Sci. Methods Appl.

- Springer Netherlands, Dordrecht, 2010, pp. 187–208, https://doi.org/10.1007/978-90-481-9268-7_9.
- [26] J. Masojídek, A. Vonshak, G. Torzillo, Chlorophyll fluorescence applications in microalgal mass cultures, in: D.J. Suggett, O. Prášil, M.A. Borowitzka (Eds.), *Chlorophyll a Fluoresc. Aquat. Sci. Methods Appl*, Springer Netherlands, Dordrecht, 2010, pp. 277–292, https://doi.org/10.1007/978-90-481-9268-7_13.
- [27] J.R.F. Malapascua, C.G. Jerez, M. Sergejevová, F.L. Figueroa, J. Masojídek, Photosynthesis monitoring to optimize growth of microalgal mass cultures: application of chlorophyll fluorescence techniques, *Aquat. Biol.* 22 (2014) 123–140, <https://doi.org/10.3354/ab00597>.
- [28] A.M. Silva Benavides, K. Ranglová, J.R. Malapascua, J. Masojídek, G. Torzillo, Diurnal changes of photosynthesis and growth of *Arthrospira platensis* cultured in a thin-layer cascade and an open pond, *Algal Res.* 28 (2017) 48–56, <https://doi.org/10.1016/j.algal.2017.10.007>.
- [29] J. Masojídek, J. Kopecký, L. Giannelli, G. Torzillo, Productivity correlated to photochemical performance of *Chlorella* mass cultures grown outdoors in thin-layer cascades, *J. Ind. Microbiol. Biotechnol.* 38 (2011) 307–317, <https://doi.org/10.1007/s10295-010-0774-x>.
- [30] J. Masojídek, M. Sergejevová, J.R. Malapascua, J. Kopecký, Thin-layer systems for mass cultivation of microalgae: flat panels and sloping cascades, in: *Algal Biorefineries*, Springer International Publishing, Cham, 2015, pp. 237–261, https://doi.org/10.1007/978-3-319-20200-6_7.
- [31] C.G. Jerez, J.R. Malapascua, M. Sergejevová, J. Masojídek, F.L. Figueroa, *Chlorella fusca* (Chlorophyta) grown in thin-layer cascades: estimation of biomass productivity by in-vivo chlorophyll a fluorescence monitoring, *Algal Res.* 17 (2016) 21–30, <https://doi.org/10.1016/j.algal.2016.04.010>.
- [32] U. Schreiber, T. Endo, H. Mi, K. Asada, Quenching analysis of chlorophyll fluorescence by the saturation pulse method: particular aspects relating to the study of eukaryotic algae and Cyanobacteria, *Plant Cell Physiol.* 36 (1995) 873–882, <https://doi.org/10.1093/oxfordjournals.pcp.a078833>.
- [33] A. Babaei, K. Ranglová, J.R.R. Malapascua, J. Masojídek, K. Ranglova, J.R. Malapascua, J. Masojídek, The synergistic effect of selenium (selenite, $-\text{SeO}_3^-$) dose and irradiance intensity in *Chlorella* cultures, *AMB Express* 7 (2017) 56, <https://doi.org/10.1186/s13568-017-0348-7>.
- [34] K. Ranglová, G.E. Lakatos, J.A.C. Manoel, T. Grivalský, J. Masojídek, Rapid screening test to estimate temperature optima for microalgae growth using photosynthesis activity measurements, *Folia Microbiol. (Praha)* (2019), <https://doi.org/10.1007/s12223-019-00738-8>.
- [35] K. Shibata, A.A. Benson, M. Calvin, The absorption spectra of suspensions of living micro-organisms, *Biochim. Biophys. Acta* 15 (1954) 461–470, [https://doi.org/10.1016/0006-3002\(54\)90002-5](https://doi.org/10.1016/0006-3002(54)90002-5).
- [36] F.L. Figueroa, R. Conde-Álvarez, I. Gómez, Relations between electron transport rates determined by pulse amplitude modulated chlorophyll fluorescence and oxygen evolution in macroalgae under different light conditions, *Photosynth. Res.* 75 (2003) 259–275, <https://doi.org/10.1023/A:1023936313544>.
- [37] F.L. Figueroa, C.G. Jerez, N. Korbee, Use of in vivo chlorophyll fluorescence to estimate photosynthetic activity and biomass productivity in microalgae grown in different culture systems, *Lat. Am. J. Aquat. Res.* 41 (2013) 801–819, <https://doi.org/10.3856/vol41-issue5-fulltext-1>.
- [38] U. Schreiber, U. Schliwa, W. Bilger, Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer, *Photosynth. Res.* 10 (1986) 51–62, <https://doi.org/10.1007/BF00024185>.
- [39] U. Schreiber, H. Hormann, C. Neubauer, C. Klughammer, Assessment of photosystem II photochemical quantum yield by chlorophyll fluorescence quenching analysis, *Funct. Plant Biol.* 22 (1995) 209, <https://doi.org/10.1071/PP950209>.
- [40] H.K. Lichtenthaler, A.R. Wellburn, Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents, *Biochem. Soc. Trans.* 11 (1983) 591–592, <https://doi.org/10.1042/bst0110591>.
- [41] A.M. Gilmore, H.Y. Yamamoto, Zeaxanthin formation and energy-dependent fluorescence quenching in pea chloroplasts under artificially mediated linear and cyclic electron transport, *Plant Physiol.* 96 (1991) 635–643, <https://doi.org/10.1104/pp.96.2.635>.
- [42] M. Giordano, M. Kansiz, P. Heraud, J. Beardall, B. Wood, D. McNaughton, Fourier transform infrared spectroscopy as a novel tool to investigate changes in intracellular macromolecular pool in the marine microalga *Chaetocerus muellerii* (Bacillariophyceae), *J. Phycol.* 37 (2001) 271–279, <https://doi.org/10.1046/j.1529-8817.2001.037002271.x>.
- [43] N. Sánchez San Fulgencio, F. Suárez-Estrella, M.J. López, M.M. Jurado, J.A. López-González, J. Moreno, Biotic aspects involved in the control of damping-off producing agents: the role of the thermotolerant microbiota isolated from composting of plant waste, *Biol. Control.* 124 (2018) 82–91, <https://doi.org/10.1016/j.biocontrol.2018.04.015>.
- [44] F. Suárez-Estrella, M. Ros, M. Vargas García, M. López, J. Moreno, Control of *Xanthomonas campestris* pv. *vesicatoria* using agroindustrial waste-based compost, *J. Plant Pathol.* 96 (2014) 243–248, <https://doi.org/10.4454/JPP.V96i2.028>.
- [45] F. Zucchini, A. Pera, M. Forte, M. de Bertoldi, Evaluating toxicity of immature compost, *Biocycle.* 22 (1981) 54–57.
- [46] S. Jaffri, Characterization of the Photosynthetic Apparatus of *Chlorella* BI sp., an Antartica Mat Alga Under Varying Trophic Growth States, 2011.
- [47] P. Bohutskyi, T. Kula, B.A. Kessler, Y. Hong, E.J. Bouwer, M.J. Betenbaugh, F.C. T. Allnutt, Mixed trophic state production process for microalgal biomass with high lipid content for generating biodiesel and biogas, *BioEnergy Res.* 7 (2014) 1174–1185, <https://doi.org/10.1007/s12155-014-9453-5>.
- [48] J. Masojídek, K. Ranglová, A. Silva Benavides, F. Charvát, M. Bečková, J. Pilyň, J. Komenda, G. Torzillo, Physiological shift from heterotrophic to phototrophic growth in a small-antenna-size strain of *Chlorella*, in: 6th Congr. Int. Soc. Appl. Phycol., 2017.
- [49] A. Melis, Solar energy conversion efficiencies in photosynthesis: minimizing the chlorophyll antennae to maximize efficiency, *Plant Sci.* 177 (2009) 272–280, <https://doi.org/10.1016/j.plantsci.2009.06.005>.
- [50] A. Melis, J. Neidhardt, J. Benemann, *Dunaliella salina* (Chlorophyta) with small chlorophyll antenna sizes exhibit higher photosynthetic productivities and photon use efficiencies than normally pigmented cells, *J. Appl. Phycol.* 10 (1999) 515–525, <https://doi.org/10.1023/A:1008076231267>.
- [51] W.-S. Shin, B. Lee, B. Jeong, Y.K. Chang, J.-H. Kwon, Truncated light-harvesting chlorophyll antenna size in *Chlorella vulgaris* improves biomass productivity, *J. Appl. Phycol.* 28 (2016) 3193–3202, <https://doi.org/10.1007/s10811-016-0874-8>.
- [52] J.M. Gordon, J.E.W. Polle, Ultrahigh bioproductivity from algae, *Appl. Microbiol. Biotechnol.* 76 (2007) 969–975, <https://doi.org/10.1007/s00253-007-1102-x>.
- [53] A. Kazbar, G. Cogne, B. Urbain, H. Marec, B. Le-Gouic, J. Tallec, H. Takache, A. Ismail, J. Pruvost, Effect of dissolved oxygen concentration on microalgal culture in photobioreactors, *Algal Res.* 39 (2019), 101432, <https://doi.org/10.1016/j.algal.2019.101432>.
- [54] J. Masojídek, A. Vonshak, G. Torzillo, Chlorophyll fluorescence applications in microalgal mass cultures, in: *Chlorophyll a Fluoresc. Aquat. Sci. Methods Appl*, Springer Netherlands, Dordrecht, 2010, pp. 277–292, https://doi.org/10.1007/978-90-481-9268-7_13.
- [55] H.M. Kalaji, G. Schansker, R.J. Ladle, V. Goltsev, K. Bosa, S.I. Allakhverdiev, M. Brestic, F. Bussotti, A. Calatayud, P. Dąbrowski, N.I. Elsheery, L. Ferroni, L. Guidi, S.W. Hogewoning, A. Jajoo, A.N. Misra, S.G. Nebauer, S. Pancaldi, C. Penella, D. Poli, M. Pollastrini, Z.B. Romanowska-Duda, B. Rutkowska, J. Seródio, K. Suresh, W. Szulc, E. Tambussi, M. Yannicari, M. Zivcak, Frequently asked questions about *in vivo* chlorophyll fluorescence: practical issues, *Photosynth. Res.* 122 (2014) 121–158, <https://doi.org/10.1007/s11120-014-0024-6>.
- [56] A. Richmond, Biological principles of mass cultivation, in: *Handb. Microalgal Cult.*, Blackwell Publishing Ltd, Oxford, UK, n.d.: pp. 125–177. doi:<https://doi.org/10.1002/9780470995280.ch8>.
- [57] J. Masojídek, Š. Papáček, M. Sergejevová, V. Jirka, J. Červený, J. Kunc, J. Korečková, O. Verbívková, J. Kopecký, D. Stys, G. Torzillo, A closed solar photobioreactor for cultivation of microalgae under supra-high irradiance: basic design and performance, *J. Appl. Phycol.* 15 (2003) 239–248, <https://doi.org/10.1023/A:1023849117102>.
- [58] T.A. Rearte, P.S.M. Celis-Plá, A. Neori, J. Masojídek, G. Torzillo, C. Gómez, A.M. Silva Benavides, F. Álvarez Gómez, R. Abdala, K. Ranglová, M. Caporgno, T. Fávoro Mascalzo, J. Carmo da Silva, H. Al Mahrouqui, R. Atzmüller, F.L. Figueroa, Diurnal changes of dissolved oxygen gradients in the highly productive strain *Chlorella vulgaris* R117 cultured in thin-layer cascades: effect on photosynthetic activity, This Issue. (n.d.).
- [59] M.R. Badger, T.J. Andrews, S.M. Whitney, M. Ludwig, D.C. Yellowlees, W. Leggat, G.D. Price, The diversity and coevolution of Rubisco, plastids, pyrenoids, and chloroplast-based CO₂-concentrating mechanisms in algae, *Can. J. Bot.* 76 (1998) 1052–1071, <https://doi.org/10.1139/b98-074>.
- [60] T.A. Costache, F.G. Acien Fernández, M.M. Morales, J.M. Fernández-Sevilla, I. Stamatin, E. Molina, Comprehensive model of microalgae photosynthesis rate as a function of culture conditions in photobioreactors, *Appl. Microbiol. Biotechnol.* 97 (2013) 7627–7637, <https://doi.org/10.1007/s00253-013-5035-2>.
- [61] M. Barceló-Villalobos, C.G. Serrano, A.S. Zurano, L.A. García, S.E. Maldonado, J. Peña, F.G.A. Fernández, Variations of culture parameters in a pilot-scale thin-layer reactor and their influence on the performance of *Scenedesmus almeriensis* culture, *Bioresour. Technol. Rep.* 6 (2019) 190–197, <https://doi.org/10.1016/j.biteb.2019.03.007>.
- [62] L. Nedbal, V. Tichý, F. Xiong, J.U. Grobbelaar, Microscopic green algae and cyanobacteria in high-frequency intermittent light, *J. Appl. Phycol.* 8 (1996) 325–333, <https://doi.org/10.1007/BF02178575>.
- [63] Y. Li, H. Xu, F. Han, J. Mu, D. Chen, B. Feng, H. Zeng, Regulation of lipid metabolism in the green microalga *Chlorella protothecoides* by heterotrophy-photoinduction cultivation regime, *Bioresour. Technol.* 192 (2015) 781–791, <https://doi.org/10.1016/j.biortech.2014.07.028>.