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Ph.D. THESIS

Cultivation, Monitoring and Application of Microalgae Cultures

(Kultivace, sledování a využití mikrořas)

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Declaration

I, hereby, declare that this Ph.D. thesis is my work alone and that I have used only those sources and literature detailed in the list of references.

Further, I declare that, in accordance with Article 47b of Act No. 111/1998 Coll. in the valid wording, I agree to the publication of my Ph.D. thesis - unabbreviated - in electronic form in a publically accessible part of the STAG database operated by the University of South Bohemia in České Budějovice on its webpage, with the preservation of my rights of authorship to the submitted text of this thesis.

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Annotation: This thesis deals with microalgae mass cultures grown in laboratory as well as outdoor cultivation systems and a potential use of their biomass. Case studies illustrate the correlation of changes in growth rate with photosynthetic activity, physiological features and biomass composition under various conditions (high irradiance intensity, optimal/suboptimal temperature and the presence of some metalloids). The special attention was also paid to evaluation of the bioavailability of Se-enriched *Chlorella* biomass which is commonly used as a food supplement. As concerns methodology, photosynthesis monitoring techniques, namely chlorophyll fluorescence were employed as an important tool to estimate microalgae activity.

Anotace: Předmětem této práce jsou mikrořasy pěstované jak v laboratorních, tak i ve venkovních kultivačních systémech a jejich potencionální využití. Případové studie ilustrují korelaci změn rychlosti růstu s fotosyntetickou aktivitou, fyziologickým stavem a složením biomasy za různých podmínek (vysoká světelná ozářenost, optimálníúsuboptimální teplota a přítomnost některých polokovů). Zvláštní pozornost byla věnována také vyhodnocení biologické dostupnosti biomasy mikrořasy *Chlorella* obohacené selenem, které se běžně užívá jako doplněk stravy. V rámci metodiky, jako důležitý nástroj pro odhadnutí aktivity mikrořas, byly použity techniky monitorování fotosyntézy, zejména měření fluorescence chlorofylu.

Partnership

This thesis originated from the collaboration of Algatech Centre in Třeboň as a part of the Institute of Microbiology of the Czech Academy of Sciences, with the Institute of Ecosystem Study, Italian National Research Council in Sesto Fiorentino with the University of Technology, Tehran, Iran and with the RECETOX Research Centre of the Faculty of Science, Masaryk University's in Brno.

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List of author's publications (with % of his contribution to individual papers and journal impact factors) upon which this thesis is based

1. **Ranglová K**, Lakatos GE, Manoel JAC, Grivalský T, Masojídek J (2019) Rapid screening test to estimate temperature optima for microalgae growth using photosynthesis activity measurements. Folia Microbiol 64:615-625 (50%; IF 1.730)
2. Babaei A, **Ranglová K**, Malapascua JR, Masojídek J (2017) The synergistic effect of Selenium (selenite, -SeO_3^{2-}) dose and irradiance intensity in *Chlorella* cultures. AMB Express 7 (56):1-14 (20%; IF 1.825)
3. Malapascua JR, **Ranglová K**, Masojídek J (2019) Photosynthesis and growth kinetics of *Chlorella vulgaris* R-117 cultured in an internally LED-illuminated photobioreactor. Photosynthetica (20%; IF 1.507)
4. Grivalský T, **Ranglová K**, Manoel JAC, Lakatoe GE, Lhotský R, Masojídek J (2019) Development of thin-layer cascades for microalgae cultivation: milestones (review). Folia Microbiol 64:603-614 (10%; IF 1.730)
5. Benavides AM, **Ranglová K**, Malapascua JM, Masojídek J, Torzillo G (2017) Diurnal changes of photosynthesis and growth of *Arthrospira platensis* cultured in thin-layer cascade and an open pond. Algal Research 28:48-56 (20%; IF 3.994)
6. Vu DL, Saurav K, Mylenko M, **Ranglová K**, Kuta J, Ewe D, Masojídek J, Hrouzek P (2019) *In vitro* bioaccessibility of selenoamino acids from selenium (Se)-enriched *Chlorella vulgaris* biomass in comparison to selenized yeast; a Se-enriched food supplement; and Se-rich foods. Food Chemistry 279:12-19 (15%; IF 6.306)
7. **Ranglová K**, Lakatos GE, Manoel JAC, Grivalský T, Suárez Estrella F, Acién Fernández G, Molnár Z, Ördög V, Masojídek J (2020) Growth, biostimulant and biopesticide activity of the MACC-1 *Chlorella* strain cultivated outdoors in inorganic medium and wastewater. (submitted to Algal Research)

All co-authors agreed on the the author's contribution to the mentioned publications.

On behalf of author's team: …….
prof. RNDr. Jiří Masojídek, CSc.

Supervisor: …….
prof. Ing. Vladislav Čurn, Ph.D.

Abbreviations

AL	actinic light
Chl *a*	chlorophyll *a*
CFU	colony-forming unit
CGF	*Chlorella* growth factor
DW	dry weight
E	irradiance
E_{inhib}	inhibiting irradiance
E_k	saturation irradiance
E_{PAR}	intensity of photosynthetically active radiation
ETR	electron transport rate through PSII
ETR_{max}	maximum electron transport rate
F_0	minimum chlorophyll fluorescence yield recorded under subsaturating light intensity for photosynthesis
F′	steady state fluorescence in the light-adapted state at respective irradiance level
F_m	maximal chlorophyll fluorescence yield when PSII reaction centres are closed by a saturation pulse
F_m'	maximal chlorophyll fluorescence yield when photosystem II reaction centres are closed by a strong light pulse
F_v/F_m	variable fluorescence/maximum fluorescence, maximum photochemical yield of PSII
GC-APCI-HRMS	gas chromatography atmospheric pressure chemical ionization mass
GI	germination index
HPLC-ICP-MS	high performance liquid chromatography with inductively coupled plasma mass spectrometry
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
KIN	kinetin
L/D cycle	light/dark cycle
LED	light emitting diode

LRC	steady-state or “slow” light-response curve
MeSeCyst	methylselenocysteine
MIC	minimum inhibitory concentration
MBC	minimum bactericidal concentration
MFC	minimum fungicidal concentration
NPQ	non-photochemical quenching
OCP	open circular pond
OD	optical density
OJIP curve	rapid fluorescence induction kinetics
P/E	photosynthesis−irradiance
PAM	pulse-amplitude modulation
PAR	photosynthetically active radiation
PBR	photobioreactor
P_{max}	maximum photosynthetic activity
POE	photosynthetic oxygen evolution
PQ	plastoquinone
PSII	photosystem II complex
PUFA	polyunsaturated fatty acids
Q_A	primary quinone acceptor
Q_B	secondary quinone acceptor
R	respiration
rETR	relative electron transport rate
$rETR_{max}$	maximum relative electron transport rate
RLC	rapid light-response curve
S/V	surface-to-volume ratio
Se-AAs	selenoaminoacids
SeCys	selenocysteine
SeMet	selenomethionine
TLC	thin-layer cascade
α	maximum photosynthetic efficiency of light conversion
$\Delta F'/F_m'$	$(F_m' - F')/F_m'$ effective quantum yield of PSII

Contents

Foreword

This work deals with the application of chlorophyll fluorescence assessment and other techniques for photosynthetic activity monitoring in microalgae mass cultures in the field of biotechnology. It is based on 6 articles which focus on the growth and photosynthetic performance of microalgae cultures maintained under various conditions (optimal and suboptimal temperature, high light irradiance, the presence of selenium to obtain Se-enriched biomass) in indoor and outdoor cultivation systems. This work also summarizes the development of the most microalgae productive systems, thin-layer cascades, since 1960s when this type of units was designed. This thesis also deals with the cultivation of microalga *Chlorella* in the presence of selenium which biotechnologically important procedure to analyse bioaccessibility of Se-enriched biomass as food or feed supplement.

The main objective of this thesis was to get introduced to the field of microalgae biotechnology, especially monitoring of the growth of microalgae cultures. The Chapter 1 and 2 are devoted to the study of variables influencing selected microalgae growth and further the Chapter 2 deals in more detail with the chlorophyll fluorescence techniques for monitoring of the physiological status of microalgae cultures in order to estimate suitable growth regimes. The use and comparison of various laboratory and outdoor cultivation systems for growth of selected microalgae in order to optimize growth regimes for production of required biomass and/or bioactive compounds is described in the Chapter 3. As the microalgae biomass has a wide range of application, the examples of the use of microalgae in human and animal nutrition and in agriculture as biostimulants and biopesticides are described in the last chapter of this thesis in the Chapter 4.

Chapter 1

Chapter 1. Microalgae

Microalgae (prokaryotic cyanobacteria and eukaryotic algae) are fast growing microorganisms since their doubling time can be as little as a few hours. These, vastly mainly photosynthetic microorganisms are one of the most efficient converters of solar energy to biomass (e.g., Masojídek and Torzillo 2014). Microalgae as well as plants use nutrients (most important are carbon, nitrogen and phosphorus), water and light energy to produce biomass while releasing oxygen to atmosphere according the scheme below (e.g., Hall and Rao 1999):

$$\mathbf{CO_2 + H_2O + nutrients + light\ energy \rightarrow biomass + O_2}$$

Representatives of microalgae can be found in both in eukaryotes and prokaryotes (Corrêa et al. 2017). Cyanobacteria (blue-green algae) are the oldest group of microalgae as they have started to operate oxygenic photosynthesis about 2.5 billion years ago (e.g., Masojídek and Torzillo 2014). They have prokaryotic cell structure closely related to bacteria. Eukaryotic microalgae cells have organelles: a nucleus, one or more chloroplasts, mitochondria, Golgi apparatus, endoplasmic reticulum and other organelles (Andersen 2013). In eukaryotes, the photosynthetic apparatus is organised in a special organelle, chloroplast which contain alternating layers of lipoprotein membranes (thylakoids) and aqueous phase, stroma (Masojídek et al. 2013). The differences between these two types of cells are shown in Fig. 1.

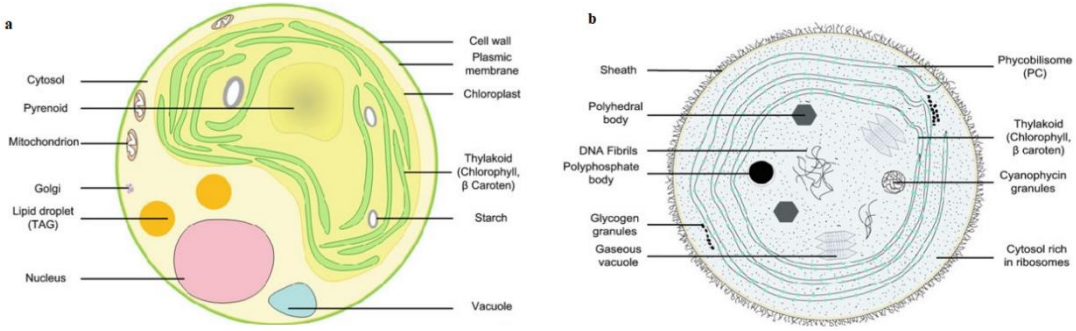

Fig. 1 The differences between eukaryotic and prokaryotic cells (Pignolet et al. 2013)

Microalgae can be divided to four groups according their light-harvesting photosynthetic pigments: Rhodophyta (red algae), Chromophyta (brown algae), Chlorophyta (green algae) and cyanobacteria (Masojídek at al. 2013). Although more than 30 000 of microalgae species are known, only about 10-20 strains are commercially exploited (Barka and Blecker 2016, Gouveia et al. 2008). They represent a variety of sizes and forms ranging from one to tens of micrometres (Correa et al. 2017).

1.1 Microalgae biotechnology

Microalgae grown in mass cultivations produce various storage or protective compounds such as polyunsaturated fatty acids (PUFAs), lipids, antioxidants or immunologically effective, virostatic and cytostatic compounds. Therefore, they are cultivated commercially in aquaculture for biomass used as food and feed additives, as a source of bioactive and novel products for pharmacology, cosmetics and chemical industry, as well as research or diagnostic compounds (Masojídek and Torzillo 2014). The extent of production of these compounds depends on the environmental factors and conditions.

Light intensity and temperature are the most important environmental variables influencing the microalgae growth (Ranglová et al. 2019) which is also affected by nutrient availability in the media. Appropriate pH is usually maintained by CO_2 addition which serves as a source of carbon. Since microalgae mass cultures grow in dense suspensions, some kind of mixing is essential to expose cells to light and to allow for an efficient mass transfer (Richmond 2004). The cultivation units for culturing highly productive and high density cultures are characterised by turbulent cell mixing – high frequency of the light/dark cycles (L/D cycle) which can match the turnover of photosynthetic apparatus and short optical path important for improved light utilisation (Masojídek et al. 2010). To maximize the use of the light, the ratio between the illuminated cultivation surface and total culture volume (S/V) needs to be high (in some highly productive systems between 50 and 100). This is the case of thin-layer systems where the short L/D cycle can be easily reached (Richmond 2004; Zarmi et al. 2013; Masojídek et al. 2015).

Several microalgae strains producing biopesticides and biostimulant can be used for the crop treatment to minimise the use of chemicals and to enhance agricultural sustainability (Kulik 1995; Romero-Villegas 2018; Ronga et al. 2019). Some microalgae (e.g., *Chlorella* and *Arthrospira* strains) are also able to accumulate heavy metals and metalloids (Cd, Zn, Cu, Cr, Fe, Pb, As, Se, I and others) from aqueous solutions (e.g., Sandau et al. 1996).

1.2 Cultivation variables influencing the microalgae growth

1.2.1 Light intensity

Light intensity is the most important environmental variable influencing the microalgae growth (Masojídek and Torzillo 2014; Ranglová et al. 2019). The wavelengths of visible light between 400 and 700 nm corresponds to photosynthetically active radiation (PAR)

which is used by microalgae in the process of photosynthesis. The ambient maxima during sunny day can reach up to 450 W m^{-2} which is equal to about 2000 µmol photons m^{-2} s^{-1} and represents about 45% of direct solar irradiance. This is still 5 to 10 times higher than needed for the saturation of the photosynthetic apparatus (Masojídek et al. 2013; Richmond 2013). The rest of the absorbed energy is dissipated as heat (Barsanti and Gualtieri 2006; Masojídek and Torzillo 2014). Photosynthetic pigments (chlorophylls, carotenoids and phycobilins) absorb energy of photons and transfer in to the reaction centre where it is utilised for photochemistry (Masojídek et al. 2013). All chlorophylls have two major absorption bands: blue or blue-green (440-450 nm) and red (630-675 nm) which results in characteristic green colour. Carotenoids are biological chromophores with the absorption range between 400-550 nm resulting in yellow-orange colour. Phycobilins in cyanobacteria absorbs blue-green, green, yellow or orange light (500-650 nm) (Masojídek et al. 2013).

During the cultivation the amount of photon energy received by cell is a combination of several factors such as photon flux density, cell density, thickness of the culture (light path) and mixing rate (Masojídek and Torzillo 2014). Biochemical composition of microalgae cells is affected by the intensity of light. High intensity tends to enhance the content of storage compounds – polysaccharide and lipids. However, polyunsaturated fatty acids (PUFAs) are inversely related to the light intensity (Hu 2013).

1.2.2 Temperature

After light, temperature is the second most important variable influencing the microalgae growth. Some microalgae strains tolerate a broad range of temperatures between 15 and 40 °C (e.g., *Chlorella* and *Arthrospira*) (Masojídek and Torzillo 2014) but for the majority of freshwater microalgae strains the optimum temperature ranges between 25°C and 30°C (Masojídek and Torzillo 2014; Malapascua 2018; Ranglová et al. 2019).

A decrease in growth temperature below an optimum level decreases the total amount of lipids but increases the degree of unsaturation of lipids in membrane systems. By an increasing level of unsaturated fatty acids in membranes the cells protect the photosynthetic apparatus from photoinhibition at low temperature. Also the amount of photosynthetic pigments (e.g., chlorophyll *a*, astaxanthin, carotenoids) is related to the cultivation temperature. The content of pigments increases with increasing temperature until the optimum is reached. An optimal temperature for growth may result in cells with minimal cell size, low carbon and nitrogen content during the cultivation, and on the other hand the temperature values above or below the optimal level may lead to an increase in

cell volume and the presence of some compounds produced at unfavourable conditions (Hu 2013).

1.2.3 Nutrients

Nitrogen (N) is an essential element of all proteins in microalgae cells and represents about 7-10% of cell dry weight (DW) (Hu 2013). It is also crucial for the synthesis of amino acids, DNA and pigments. Nitrogen can be added to the culture in several forms as nitrate, ammonia or urea (Grobbelaar 2013; Hu 2013).

Nitrogen starvation has negative impact on protein synthesis and could cause serious stress in microalgae leading to the growth inhibition (Lakatos et al. 2019) and as a result, photosynthetically fixed carbon is diverted from protein synthesis into storage compounds such as carbohydrates and lipids (Hu 2013; Li et al. 2011; Lakatos et al. 2019). Nitrogen limitation also stimulates the synthesis and accumulation of pigments (e.g., ß-carotene, astaxanthin) (Ben-Amotz et al. 1982; Borowitzka et al. 1991; Hu 2013).

Phosphorus (P) is another essential macronutrient necessary for the synthesis of DNA, RNA, ATP, proteins and phospholipids (Lakatos et al. 2019). Microalgae contain about 1% of P in their biomass. Inorganic phosphate occurs in the cells in the form of polyphosphates under cell-sufficient growth but disappear when phosphate becomes scarce. Some of the symptoms of P depletion are similar to those observed in nitrogen-deficient cultures resulting in the accumulation of storage carbohydrates, pigments (e.g., *ß*-carotene, astaxanthin), but not in such extent as under nitrogen deficiency (Hu 2013; Brányiková et al. 2011; Lakatos et al. 2019).

Sulphur (S) is another macronutrient essential for normal cell development and growth. This element is required for the synthesis of S-containing amino acids (cysteine, methionine). The starvation results in the inhibition of cell division (Lakatos et al. 2019).

Chapter 2

Chapter 2. Culture Monitoring and Maintenance

Successful cultivation requires monitoring of physicochemical variables of cultures such as irradiance, temperature, dissolved oxygen concentration, nutrient concentration, and pH. The basic controlling method is to use biological examination under the microscope to detect morphological changes, mechanical cell damage (caused by mixing) and contamination by other microorganisms. The nutrient status can be followed by monitoring the concentration of essential compound in cultivation media. The appropriate amount of nutrients can be added accordingly. In the mass cultivation of microalgae, 'monocultures' are usually required. The appearance of substantial populations of contaminants might indicate that the culture is stressed. Such contaminants often represent one of the major limitations of large-scale production, particularly in outdoor cultured strains that cannot be grown in selective conditions. Another important condition is sufficient mixing of microalgae cultures in order to ensure a homogeneous light supply to the cells, nutrient diffusion and sufficient gas exchange to prevent the accumulation of oxygen in the culture, particularly when grown in a closed system.

Culture growth might be estimated as changes in the optical density (OD) at 750 nm, the biomass dry weight, or the number of cells. The specific growth rate is usually calculated as μ (h^{-1}, day^{-1}) = (ln X_2− ln X_1)/ (t_2− t_1), where X is cell number, or dry weight, at the final and starting time points. Biomass productivity can be expressed as the areal or volumetric yield per unit time, that is, in g m^{-2} day^{-1} or in g L^{-1} day^{-1}, respectively.

Biophysical and biochemical monitoring techniques reflect the general status of the photosynthetic apparatus of the cells and are thus often used to adjust the appropriate cultivation conditions (Masojídek et al. 2014).

One, direct approach is to measure photosynthesis *in-situ* during the diel cycle to monitor the actual situation in the culture (Maxwell and Johnson 2000; Baker 2008). The other possibility is to measure *ex-situ* using microalgae samples taken from a cultivation unit at selected times (Masojídek et al. 2011b).

2.1 Chlorophyll Fluorescence monitoring

In the 1990s, chlorophyll (Chl) fluorescence has become one of the most common method used for monitoring the physiological status of microalgae cultures as it is fast, sensitive and easy to measure and a choice of commercial fluorimeters is available (Torzillo et al. 1998; Masojídek et al. 2001; Masojídek et al. 2011b; Malapascua et al. 2014).

Chl fluorescence can be estimated by measurement of photosynthesis *in-situ* to monitor the actual situation in microalgae cultures and by using *ex-situ* measurements using dark-adapted samples taken from a cultivation unit (Masojídek et al. 2011b).

Two basic Chl fluorescence techniques are used for photosynthetic performance monitoring in microalgae cultures – rapid fluorescence induction kinetics and saturating pulse analysis of fluorescence quenching (for recent reviews see Maxwell & Johnson 2000; Strasser et al. 2004; Masojídek et al. 2011b; Malapascua et al. 2014). While the rapid fluorescence induction kinetics provide the information on the redox status of the photosynthetic electron transport chain, the saturating pulse analysis technique gives information on the balance between photosynthetic electron transport and the Calvin-Benson cycle. Chl fluorescence variables can be well correlated with changes of cultivation conditions, physiological status and growth and the suitability of a selected cultivation system (Torzillo et al. 1998; Malapascua et al. 2014; Ranglová et al. 2019).

The most frequently used and measured fluorescence variable characterizing the culture state is the maximum photochemical yield F_v/F_m which is related to the performance of the photochemical processes in photosystem II (PSII). A decline in the F_v/F_m ratio by about 20% of the morning value can be considered as a reliable warning signal of a certain culture stress that may affect the productivity. The effective (actual) quantum yield ($\Delta F'/F_m'$) is an indicator of actual acclimation of PSII as it depends on the redox state of the reaction centres and the electron transport rate through PSII (Figueroa et al. 2013; Kromkamp et al. 2008). The relative electron transport rate rETR (the outcome of $\Delta F'/F_m'$ multiplied by the photosynthetically active radiation E_{PAR} in the culture) proved to be a simple and reliable variable to estimate the photosynthetic performance of outdoor microalgae cultures. Chl fluorescence measurements *in-situ* indicate that changes of the ETR integrated values can be well correlated with analogous changes in the daily productivities of the cultures grown under different conditions (Torzillo et al. 1998).

Chl fluorescence and oxygen production monitoring have often been used complementary to optimise growth regimes as well as to examine effects of adverse environmental conditions – high irradiance, temperature extremes, high dissolved oxygen concentration and their synergism on microalgae growth (Torzillo et al. 1998; Lippemeier 2001; Masojídek et al. 2011a; 2011b; White et al. 2011; Figueroa et al. 2013; Malapascua et al. 2014; Ranglová at al. 2019). Both techniques provide analogous information, but Chl fluorescence techniques are considerably faster, more sensitive and can also provide data on energy distribution between the photochemical and non-photochemical (heat

dissipation) processes (for a review see Baker 2008; Masojídek et al. 2011b; Malapascua et al. 2014). The special attention must be paid to the fluorescence measurement in cyanobacteria as the photosynthetic and respiratory chain are shared (Vermaas 2001) which can contribute to the total signal and affect the correct determination of fluorescence variables (Schreiber 1995).

2.1.1 Light-response curves measured by Chlorophyll Fluorescence

The response of the photosynthetic apparatus of microalgae to light intensity can be measured as a light-response curve (*P/E* curve) by saturating pulse analysis technique using pulse-amplitude modulation (PAM) fluorimeters (Masojídek et al. 2011b; 2013). This curve can be divided into three regions: a light-limited where photosynthesis increases with light intensity; a light-saturated and the down-regulated region where photosynthesis decreases due to photo-stress (Torzillo and Vonshak 2013). The initial slope α represents the maximum photosynthetic efficiency. The rate of photosynthesis is increasing in parallel with the light intensity until the photosynthetic apparatus is saturated. The intersection between the variable α and the maximum rate of photosynthesis P_{max} give the information about light saturation intensity E_k (optimum irradiance). Under high irradiance beyond the inhibiting value (E_{inhib}) the photosynthetic apparatus of microalgae can be damaged (Fig. 2) (Masojídek et al. 2013; Malapascua et al. 2014). By saturation pulse analysis of fluorescence quenching the information about the energy distribution between photochemistry and heat dissipation (measured as non-photochemical quenching NPQ); the latter represents one of the most important photoprotective process (Serôdio and Lavaud 2011).

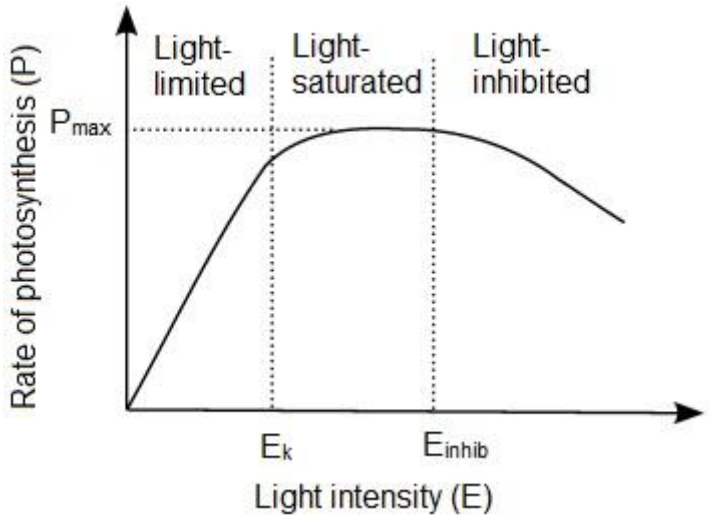

Fig. 2 Schematic diagram of the photosynthetic light-response curve divided into three parts (light-limited, light-saturated and light-inhibited) according the corresponding light intensity (modified from Malapascua et al. 2014).

2.1.2 Fast fluorescence induction kinetics (Kautsky curve)

The Kautsky curve (also known as fast fluorescence induction kinetics, or the OJIP curve) characterises rapid changes in Chl *a* fluorescence yield after illumination of a dark adapted sample (Krause and Weis 1984; Govindjee 1995; Cosgove and Borowitzka

2011). The fast fluorescence induction kinetics is usually measured in the time range between 50 μs to 1 s; it starts right after the illumination (saturating continuous light) of a dark-adapted microalgae culture as the signal increases rapidly from the origin (O) to a peak (P) via two inflections – J and I (Strasser et al. 1995). The O point (50 μs) of the fluorescence induction curve represents a minimum value (equivalent to minimum fluorescence yield F_0) when the plastoquinone electron acceptors (Q_A and Q_B) of the PSII complex are fully oxidized. The inflection J occurs after ~2-3 ms of the start of illumination and reflects a reduction of Q_A^- (photochemical phase). The further inflection I occurs in between 30–50 ms after illumination and it reflects a 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) (Malapascua et al. 2014; 2019). The J and I peaks are characterized by V_j and V_i transients. Their increasing height indicates the unfavourable growing conditions as the PQ pool remains reduced (Babaei et al. 2017; Benavides et al. 2017; Ranglová et al. 2019).

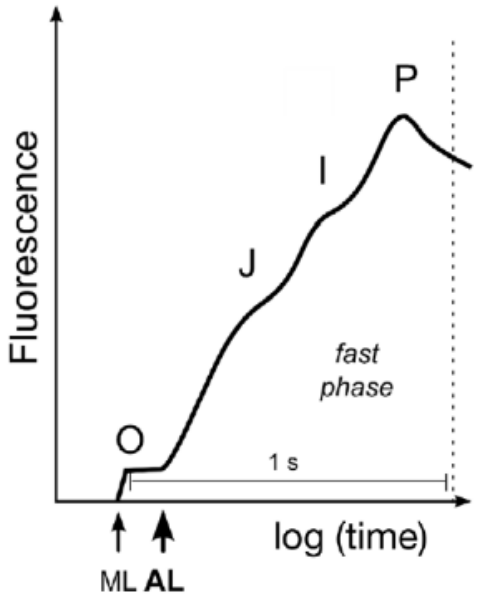

Fig. 3 Schematic diagram of the fast Chl *a* fluorescence induction curve measured by a double-modulation fluorescence technique. The smaller arrow represents the start of the weak measuring light (ML) and the other arrow represents the application of strong continuous actinic light (AL) when the fluorescence emission rises from the origin (O) to the peak (P) (Malapascua et al. 2014).

2.1.3 Measurement of photosynthetic oxygen evolution

Measurement of photosynthetic oxygen production together with Chl fluorescence techniques mentioned above are used as a reliable and sensitive technique to monitor photosynthetic activity of various photosynthetic organisms. Contrary to fluorescence techniques it provides information about photosynthetic oxygen evolution (POE) and also respiration (Gilbert et al. 2000; Figueroa et al. 2003; Wilhelm et al. 2004; Malapascua 2018). Routine measurements of POE in microalgae cultures are usually carried out with a Clark-type oxygen electrode (Walker 1993). The measured current is proportional to the concentration of oxygen in the sample (van Gorkom and Gast 1996). Gross photosynthesis is considered as the sum of net photosynthesis (O_2 evolution) and respiration (O_2 uptake). Oxygen production is usually expressed in µmol or mg O_2 per mg (Chl) $^{-1}$ h^{-1} or per cell h^{-1} (Masojídek et al. 2013).

2.2 Dissolved oxygen concentration

The concentration of dissolved oxygen measured *in-situ* is considered as a reliable, fast and sensitive indicator of photosynthetic activity and physiological status of microalgae cultures (Richmond 2013). Namely in high density cultures exposed to high irradiance the concentration of dissolved oxygen could be very high, exceeding the culture saturation level several times (Malapascua 2018). Photosynthetic activity becomes inhibited at oxygen concentrations exceeding 3-4 times saturation with respect to air (Moheimani and Borowitzka 2006) and this situation may cause the reduction of daily productivity (Torzillo et al. 1998). In order to maintain efficient degassing, the culture turbulence is rather crucial which can be maintained by various mechanical mixers, pumps or air bubbling (Sydney et al. 2019) as to decrease the concentration of dissolved oxygen below critical level (Malapascua et al. 2014; Malapascua 2018).

2.3 Case studies

2.3.1 Main objectives

Each microalgae strain is characterized by suitable growth conditions, namely irradiance and temperature which function together. The range of the suitable growth temperature correlates to the maxima of photosynthetic activity which indicate high biomass productivity. The main objective of the first case study (Annex I) was to find out whether a one-day long procedure based on photosynthesis measurements can be used for preliminary tests of suitable cultivation temperature of selected microalgae strains. In this way we can substitute timely growth tests which may take a week or more. Photosynthetic activity was measured using three techniques: oxygen production/respiration, saturation pulse analysis of fluorescence quenching and fast fluorescence induction kinetics. Not only the non-optimal growing temperature, but also other unfavourable conditions like irradiance, high dose of certain compounds and other factors are causing the changes of photosynthetic activity. For example, we can find out how the photosynthetic activity of a microalgae culture is affected by the synergism of selenium dosage and irradiance intensity when various biomass density is used. The objective of the second case study (Annex II) was to set-up the protocol for large-scale trials to produce Se-enriched biomass.

2.3.2 Photosynthesis measurements for determination of temperature optima in microalgae cultures (Annex I)

The growth of microalgae is affected by various environmental factors such as light, temperature, nutrition, pH, the presence of contaminants and others. Light intensity and temperature are the most important variables influencing the microalgae growth. Each microalgae strain is characterized by an optimal growth temperature which influences its photosynthetic activity and subsequently growth. Thus, it is obvious that unfavourable temperature causes a decrease of biomass productivity.

Four green microalgae strains (*Scenedesmus* MACC-677, *Chlorella* MACC-1, *Scenedesmus almeriensis* and *Chlorella vulgaris* R-117) and four cyanobacteria strains (*Cylindrospermum alatosporum*, *Synechocystis* sp., *Nostoc* MACC-612 and *Nostoc* MACC-683) were selected for cultivated tests in 100-mL glass columns which were illuminated by continuous irradiance of 100 µmol photons m^{-2} s^{-1}. The cultures were acclimated for 2 hours to the range of temperatures from 15 to 35°C using 5°C steps. At each temperature step, photosynthetic activity of all strains was monitored. Then, the suitable cultivation temperature based on photosynthetic performance was confirmed by a 5-day growth test.

Temperature optima for individual microalgae strains were estimated from measurements of the maximum photochemical yield of PSII (F_v/F_m), relative electron transport rate ($rETR_{max}$), fluorescence induction transients V_j/V_i and photosynthetic oxygen evolution and respiration (POE/R). The average values for all variables indicating the temperature optima were compared with the temperature optima obtained from the growth tests (Tab. 1).

Table 1 Temperature optima for selected microalgae strains based on photosynthesis measurements were compared with the temperature optima obtained from a 5-day growth test. Values are presented as a mean (n=3) with SD. The same letters mean that the values do not differ significantly from each other.

Variable	F_v/F_m	$rETR_{max}$	V_j, V_i	POE/R	Mean	Growth optimum
Temperature optima	[°C]	[°C]	[°C]	[°C]	[°C]	[°C]
Nostoc 612	28.3±2.9	30.0±0	28.3±2.9	28.3±2.9	28.7±2.2[a,b]	30
Nostoc 683	28.3±2.9	33.3±2.9	33.3±2.9	35.0±0	32.5±2.2[a,b]	35
Cylindrospermum 988	28.3±2.9	28.3±2.9	33.3±2.9	33.3±2.9	30.8±2.9[a,b]	30
Synechocystis 6803	26.7±2.9	28.3±2.9	26.7±2.9	31.7±2.9	28.4±2.9[a,b]	30
Scenedesmus 677	33.3±2.9	33.3±2.9	33.3±2.9	33.3±2.9	33.3±2.9[a,b]	30
Chlorella 1	33.3±2.9	35.0±0	33.3±2.9	31.7±2.9	33.3±2.2[a,b]	30
S. almeriensis	25.0±0	26.7±2.9	26.7±2.9	31.7±2.9	27.5±2.2[a,b]	25
Chlorella R-117	31.7±2.9	35.0±0	33.3±2.9	33.3±2.9	33.3±2.2[a,b]	25

The average temperature optima obtained from photosynthetic activity measurements correlated with the regular growth test for most of the strains. Only one exception was *Chlorella vulgaris* R-117. This microalgae strain is used as a fast-growing production strain in outdoor large-scale cultivation cascades where the temperatures can range from 15˚C in the morning up to almost 40˚C at midday. Such temperature adaptability can influence the infallibility of tests carried out in laboratory cultivations. To conclude our tests, the described procedure can be used as a rapid one-day pre-screening test to estimate suitable growth regime of microalgae strains.

2.3.3 The effect of selenium dose and irradiance intensity in Chlorella cultures (Annex II)

Selenium (Se) is a natural trace element that may alternate from an essential micro-nutrient to toxic compound within a narrow concentration level. Microalgae are able to metabolize inorganic Se (selenite, selenate) to organic forms (e.g., Se-proteins) as a part of detoxification process. Organic forms of Se in microalgae are more beneficial and less toxic to humans and animals and can be used as food or feed supplement. Low Se concentration stimulate the microalgae growth, but it can be inhibited at certain levels. The aim of these trials was to confirm that the limit of Se tolerance in *Chlorella* cultures is related to the photosynthetic performance, i.e. if it depends on the light intensity.

Various Se concentration (as selenite, SeO_3^{2-}) was added to the culture illuminated with the same light intensity of 250 µmol photons m^{-2} s^{-1}. The Se concentration of 2.5-8.5 mg g^{-1} DW slightly stimulated the growth while the concentration of 25 mg L^{-1} was highly inhibiting compared to the control (no Se added) (Fig. 4a). According to these results, the effective concentration of 16 mg Se g^{-1} DW was used to study changes of photosynthetic activity under various light intensities (250, 500 and 750 µmol photons m^{-2} s^{-1}). In this trial, the addition of Se to the low and high irradiance (250 and 750 µmol photons m^{-2} s^{-1}) caused a 20-25% inhibition of growth as compared to the control cultures. The growth in the presence of Se exposed to the moderate irradiance was stimulated being higher by about 24% after 72 h of experiment as compared to the control culture (Fig. 4b).

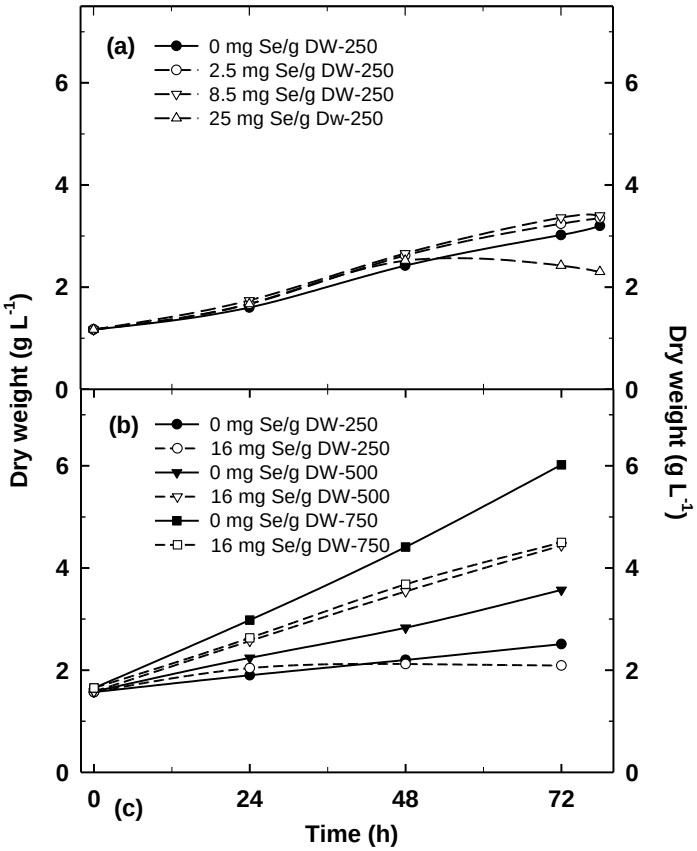

Fig. 4 Changes in the biomass density (DW) in the *Chlorella* cultures treated (a) with various Se doses at light intensity of 250 µmol photons m^{-2} s^{-1} and (b) after the addition of 16 mg Se g^{-1} DW when exposed to various light intensities (250, 500 and 750 µmol photons m^{-2} s^{-1})

The growth analysis was accompanied by fluorescence measurements of RLCs and OJIP kinetics. Three variables – maximum PSII quantum yield, F_v/F_m, maximum of relative electron transport rate $rETR_{max}$ and photosynthesis-saturating irradiance E_k were calculated from RLCs (Fig. 5a-f). In the trial using various Se concentration added to the culture, in some cases the F_v/F_m value decreased after 3 days of cultivation by about 40% reflecting worsened physiological state (Fig. 5a). The value of $rETR_{max}$ also decreased by 20 and 80% after 24 and 48 h, respectively (Fig. 5b). The variable E_k corresponding to light saturation is used to characterized the photoacclimation status of microalgae cultures; a significant drop was found at highest concentration of Se meaning that this culture was strongly inhibited (Fig. 5c).

In the second trial using the same Se concentration in the cultures exposed to various light intensities the F_v/F_m values correlated with the growth (Fig. 5d and Fig. 4b) as in the previous experiment. At the medium irradiance intensity (500 µmol photons m^{-2} s^{-1})

$rETR_{max}$ was higher in the Se-treated culture after 48 h as compared to the control (Fig. 5e).

A slow-down of growth and photosynthetic activity in the *Chlorella* cultures in both trials was always accompanied by a release of volatile Se compounds indicating that Se was not metabolized.

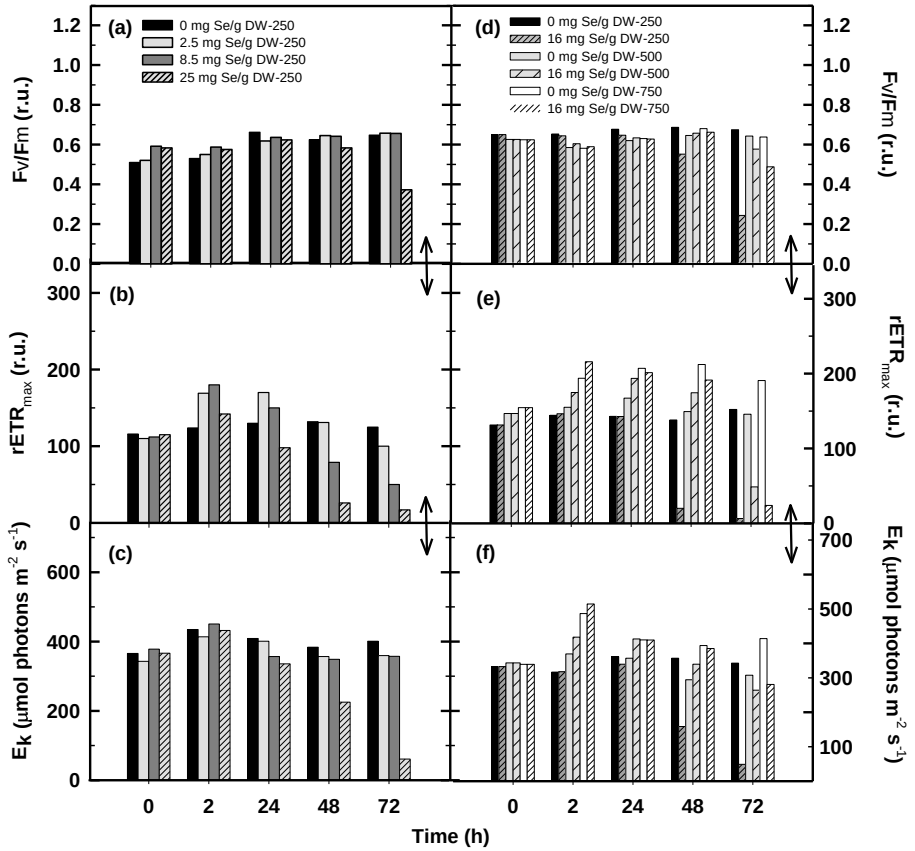

Fig. 5 Changes of photochemical activity caused by various Se concentration (a-c) and exposed to various light intensity in the presence of 16 mg g^{-1} Se (d-f) were calculated from rapid light-response curves: F_v/F_m (a, d), $rETR_{max}$ (b, e) and E_k (c, f).

On the basis of these experiments, outdoor large-scale trials were performed. We estimated the dose of Se for longer-term trials to produce the Se-enriched *Chlorella* biomass. The starting biomass density was about 13 g L^{-1} and the cultivation was performed in thin-layer cascade units with the culture layer of only 6 mm that guaranties sufficient irradiance (light penetration) even in such dense cultures (Babaei et al. 2017; Grivalský et al. 2019). The outdoor cultures were exposed to the Se concentration of about 20 µmol L^{-1} (i.e. about 0.8 mg g^{-1} biomass) in a 10-day trial. Only the slight decrease (5-10%) of the culture growth was observed in the presence of Se as compared to the control (Fig. 6).

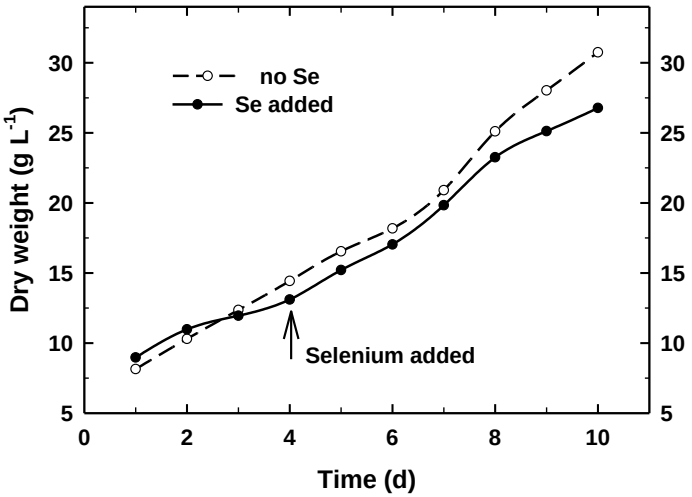

Fig. 6 Growth curves of *Chlorella* cultures grown in outdoor thin-layer cascades in the presence (solid line) and absence (dashed line) of Se during the 10-days trial.

It can be concluded (Table 2) that we were able to select the suitable Se dose that was only slightly inhibiting photosynthetic activity and growth, but still the incorporation of Se to biomass was relatively high (650 mg Se kg^{-1} biomass, i.e. about 80% of the total amount added).

Table 2 Changes of fluorescence variables reflecting the photosynthetic activity of both control and Se treated culture.

Culture treatment	**Fluorescence variables *(r.u.)***	**Day 1**	**Day 4**	**Day 7**
	F_v/F_m	0.77	0.75	0.80
	$rETR_{max}$	461	404	359
Control (no Se)	E_k	758	714	531
	V_j	0.21	0.22	0.21
	V_i	0.43	0.43	0.43
	F_v/F_m	0.74	0.74	0.78
	$rETR_{max}$	436	380	332
Se treated culture	E_k	665	660	625
	V_j	0.24	0.26	0.23
	V_i	0.46	0.46	0.46

2.4 Conclusions

Monitoring of photosynthetic performance using Chl fluorescence techniques can provide early stress warning during microalgae cultivation. The F_v/F_m variable (maximum photochemical yield of PSII) decreases under stress reflecting the reduction of PSII activity; it correlates well with growth rate. The same trend was observed when the microalgae strains were characterized by fast fluorescence induction kinetics. The temperature at which the lowest values of V_j and V_i were achieved coincided with the temperature at which the growth rate of microalgae was the fastest. The changes of photosynthetic performance correlate not only with the growth, but also with Se incorporation into the biomass. A suitable dose of Se can be estimated by photosynthesis monitoring using Chl fluorescence techniques in mass cultivation to produce a bulk of Se-enriched biomass for nutrition purposes.

Thus, the use of Chl fluorescence provides a simple, rapid, versatile and non-invasive tool to estimate photosynthetic performance and physiological status of microalgae under various cultivation conditions. In this case study, we conclude that Chl fluorescence measurements can be used as a fast and suitable technique to monitor doses of Se salts.

Chapter 3

Chapter 3. Cultivation Systems

Numerous cultivation systems and technologies have been developed to grow microalgae cultures, using both natural and artificial light (for a recent review, see Zittelli et al. 2013; Masojídek and Torzillo 2014; Masojídek et al. 2015; Sergejevová et al. 2015; Acién-Fernández et al. 2017). Microalgae can be grown in open reservoirs (mixed ponds, raceways or cascades) with direct contact of the microalgae culture with the environment or in closed photobioreactors (PBRs). The major disadvantage of open systems with deep culture layer (10-30 cm) is inefficient mixing causing poor light utilization. More advantageous have been shallow thin-layer systems like sloping cascades or cascade raceways which are highly efficient for biomass production (Becker 1994; Masojídek et al. 2011a; Masojídek et al. 2015). These systems are characterized by simple cleaning, maintenance and efficient degassing where the short light-dark cycles of cells due to short light path maximize the productivity (Richmond 2003; Masojídek et al. 2011a; Masojídek et al. 2015). On the other hand, there exists a risk of contamination by other microorganisms (Ugwu et al. 2008). These systems can also be placed in greenhouses for better control of ambient conditions and contamination (Morales-Amaral et al. 2015; Acién-Fernández et al. 2017).

3.1 Laboratory cultivation

The simplest cultivation vessel is an illuminated flask with the microalgae culture placed on the shaker. In this way stock cultures are often prepared and maintained. For laboratory experiments glass columns kept in a temperature-controlled water bath are often used (Fig. 7). The microalgae culture is mixed by bubbling CO_2 enriched air to facilitate faster growth. Stepwise, the culture is then diluted (ratio approximately 1:5) to larger volumes in open or closed cultivation units. The starting biomass density of 10 g m^{-2} is usually recommended to avoid photoinhibition in outdoor unit exposed to ambient irradiance (Masojídek and Torzillo 2014).

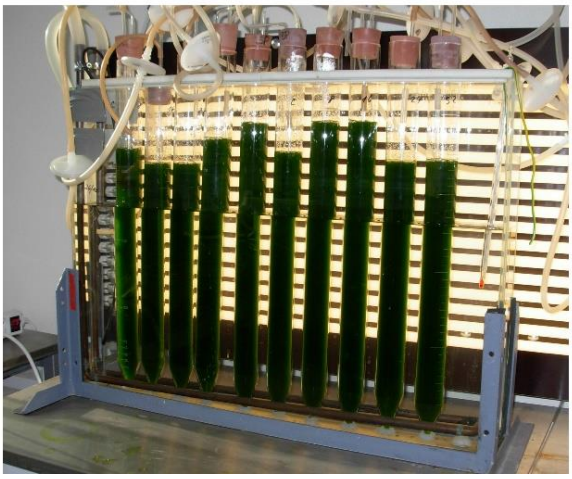

Fig. 7 Laboratory cultivation using glass columns placed in a temperature-controlled water bath with adjustable LED illumination panel (Institute of Microbiology, Academy of Sciences, Třeboň, Czech Republic) (© Karolína Ranglová)

3.2 Outdoor culturing in open systems

Open cultivation systems include natural ponds, raceway ponds and inclined cascades (Zittelli et al. 2013) where the culture in the direct contact with the environment. Cultivation of microalgae for commercial purposes is mainly carried out in these types of units as they are easier to maintain and less expensive to build than large closed PBRs. Due to the limited control of cultivation conditions, open systems are suitable only for fast growing microalgae or strains growing in selective environments, e.g., alkaline pH (Masojídek and Torzillo 2014). The slowly-growing microalgae may be overgrown by other competing strains and also easily contaminated by fungi, bacteria and protozoa.

The simplest example of raceway ponds is a shallow ditch dug to the ground, covered with plastic sheets mixed by paddle wheels or air bubbling (Tredici 2004). The depth of raceway ponds may vary between 10 and 30 cm where the culture is photo-limited at higher biomass density resulting in the productivity of 10 to 25 g m^{-2} d^{-1} (Pulz 2001). Open ponds are mainly used for *Arthrospira* and *Chlorella* production in Japan, Thailand, California, Hawaii, Taiwan, India and China (see example in Fig. 8a) (Zitelli et al. 2013; Masojídek and Torzillo 2014).

For a better light utilization, the system of thin-layered units is advantageous (Fig. 8). The system of declined thin-layer cascades has been developed by the group led by Ivan Šetlík at the Institute of Microbiology of the Czech Academy of Sciences in Třeboň in the 1960s (Šetlík et al. 1967; 1970; Masojídek et al. 2015; Malapascua 2018; Grivalský et al. 2019). In thin-layer, the microalgae suspension flows from the top to the bottom of sloping surface to the retention tank, from where it is pumped back to the upper part of the

cultivation surface. The suspension thickness remains below 1 cm and efficient mixing is achieved by fast circulation, high turbulence prevents self-shading and ensure optimal L/D cycle of microalgae cells. The very thin layer allows better utilization of light by cells and biomass densities up to 60 g L^{-1} can be achieved. The cultivation in thin-layer systems allows to achieve productivity up to 40 g m^{-2} d^{-1} (Masojídek et al. 2011a).

Although open cultivation systems have the advantage of low construction and operation costs, they have few limitations that have been discussed above.

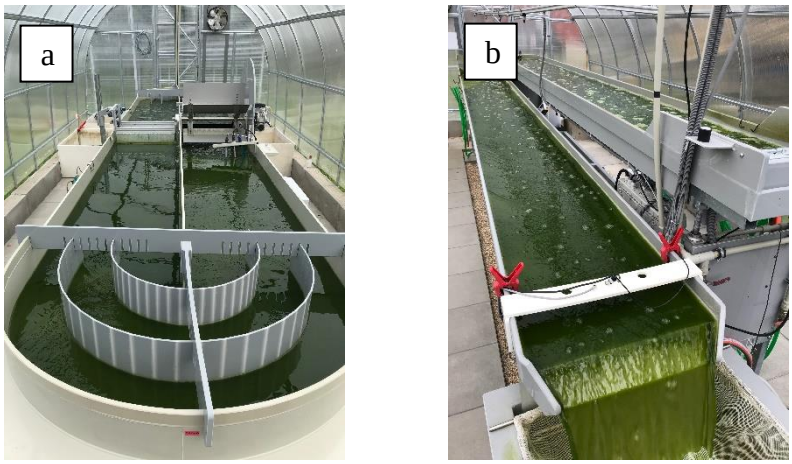

Fig. 8 (a) Microalgae cultivation in a raceway pond with paddle wheel mixing (volume of 100-150 L; 5 m^2, culture layer 15-25 mm) and (b) a thin-layer cascade consisting of two sloping platforms (volume of 60-100 L; 5 m^2, culture layer 7-10 mm) at Centre Algatech, Institute of Microbiology, Třeboň, Czech Republic (b) (© Karolína Ranglová)

3.3 Closed Systems – Photobioreactors

Photobioreactors (PBRs) can be defined as closed and semi-closed culture systems in which microalgae culture is not in direct contact with the environment and the light needs to pass through the transparent wall (Zitelli et al. 2013; Malapascua 2018; Acién-Fernández et al. 2017). In PBRs, a direct exchange between the culture and atmosphere is significantly limited as for oxygen stripping, but on the other hand contamination is prevented (dust, microorganisms, etc.). Artificial and/or natural illumination can be used depending if PBRs are placed indoors or outdoors. Compared to the open systems, the culturing conditions in PBRs can be better controlled according to biological and physiological characteristics of a selected microalgae strain (Masojídek and Torzillo 2014; Malapascua 2018). It is important to mention that closed systems are more difficult to clean and the material of PBR might reduce the light penetration. A variety of closed PBRs has been designed for microalgae cultivation: (i) vertical column

tubular systems (glass, plastic, Plexiglas) (Fig. 9a); (ii) flat panels (Fig. 9b) or tubular loops (Pulz 2001).

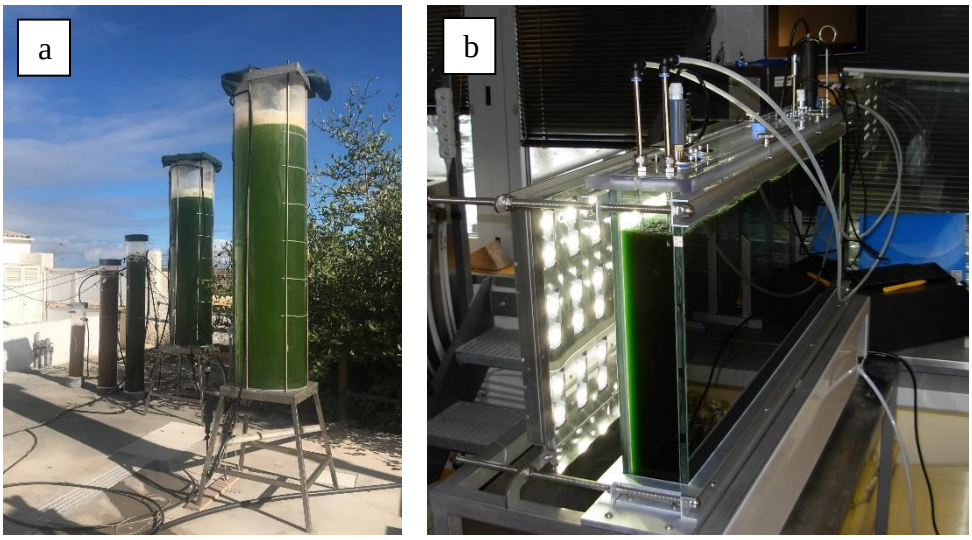

Fig. 9 Examples of closed photobioreactors for cultivation of microalgae (a) horizontal column photobioreactor (University of Las Palmas, Gran Canaria), (b) flat-panel photobioreactor (Centre Algatech, Institute of Microbiology, Třeboň) (© Karolína Ranglová)

The culture suspension is mixed by a pump or by air-lifting. Due to high possibility of overheating the system must be effectively cooled (Masojídek and Torzillo 2004). Nevertheless, large-scale outdoor PBRs cannot be easily tempered without high technical efforts. The cooling can be provided by submerging the system in a pool of water, by heat exchangers, or by spraying of water on the surface (Malapascua 2018).

In PBRs, despite some disadvantages, higher biomass density can be achieved than in open systems (with the exception of thin-layer systems). The productivity of 25 g m^{-2} d^{-1} has been achieved during cultivation of *Porphyridium cruentum* (Tredici 2004) and even more than 28 g m^{-2} d^{-1} has been achieved in *Arthrospira* cultures (Tredici and Zitelli 1998). Despite the higher biomass yields which can be achieved in PBRs, their high construction and maintenance costs still make them uncompetitive for the industrial production of microalgae biomass.

3.4 Case studies

3.4.1 Main objectives

Several cultivation systems and technologies have been developed to grow microalgae cultures. In the first trial the main objective was to study the growth and photosynthetic performance of microalgae *Chlorella vulgaris* cultivated in a 10 L PBR with internal illumination (Annex III). In this type of PBR with the short light path of 25 mm, the irradiance intensity can be simulated as the seed culture is transferred from laboratory to outdoors as the light intensity up to 3500 µmol photons m^{-2} s^{-1} can be operated. In these experiments physiological responses of microalgae strain and the estimation of relevant fluorescence variables were studied which can be used for outdoor scale-up.

Open cultivation systems are represented basically by two types: open ponds or raceways (shallow race tracks mixed by a paddle-wheel) and thin-layer sloping cascades (declined surface systems). The main objective of the second trials was to review the development of thin-layer cascades (since 1960s when the thin-layer cascade technology was introduced for microalgae culturing) which are frequently used for mass cultivation due high biomass productivity (Annex IV).

Open systems are the most common types of cultivation units used for mass microalgae cultivation as their construction cost are rather low. They differ in light path (deeper culture layer as compared to the cascades) which can affect the photosynthetic activity of cultivated microalgae. In the third trial (Annex V) the behaviour of *Arthrospira* was compared when grown outdoors in open circular pond (OCP) and thin-layer cascade (TLC) under optimal and suboptimal temperature regimes as to mimic weather changes.

3.4.2 Growth and photosynthetic performance of Chlorella in photobioreactor with internal illumination (Annex III)

The microalgae *Chlorella vulgaris* R-117 (CCALA 1107) was grown in 10 L laboratory photobioreactor (PBR) with internal illumination. The light path about 25 mm was illuminated by high irradiance (2500 and 3500 µmol photons m^{-2} s^{-1}) provided by LED sources to mimic the situation when the seed culture is transferred from laboratory and exposed to ambient irradiance outdoors. The changes of photosynthesis activity and growth of *Chlorella* was correlated.

At the beginning of the experiment, the cultures in Trial A (2500 µmol photons m^{-2} s^{-1}) and Trial B (3500 µmol photons m^{-2} s^{-1}) were exposed to different irradiance. The doubling time of microalgae during the Trial A was 3.5 d [µ= 0.2 day^{-1}, P = 0.33 g DW L^{-1} d^{-1}] and reached the biomass concentration of 3.4 g DW L^{-1}. The exponential phase of the growth was observed between Day 3 and Day 9, then the culture showed the decelerating phase and reached the stationary phase after Day 10 of the trial. More than twice higher doubling time of 1.7 d was found for the culture in Trial B [µ= 0.4 day^{-1}, P = 0.55 g DW L^{-1} d^{-1}]. It reached the maximal biomass density of 5.8 g DW L^{-1}. The exponential phase of the growth was observed during the same days like in Trail A between Day 3 and 9 (Fig. 10).

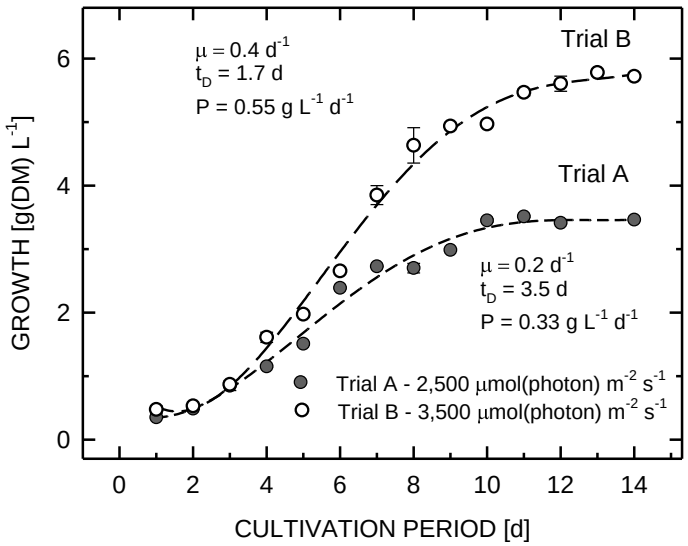

Fig. 10 Growth curves of *Chlorella* in the PBR exposed to the continuous irradiance of 2500 µmol photons m^{-2} s^{-1} (white symbols) and 3500 µmol photons m^{-2} s^{-1} (grey symbols). Values are presented as a mean (n=3) with SE indicated by error bars. The lines are the fitted curves according to logistic growth model.

A slow-down of the growth was not caused by a lack of nutrients (according the nutrient analysis in the media), but evidently by the decreased light availability and shade adaptation as the culture was getting denser.

In Trial A, rETR was much lower as compared to the culture in Trial B. How the cultures were getting thicker in both trials, the rETR was decreasing. At Day 9 (Trial B) the rETR dropped significantly. The correlation with growth kinetics (Fig. 7), the culture in Trial B were growing faster and evidently became low-light adapted as compared to the culture in Trial A (Fig. 10 vs. Fig. 11).

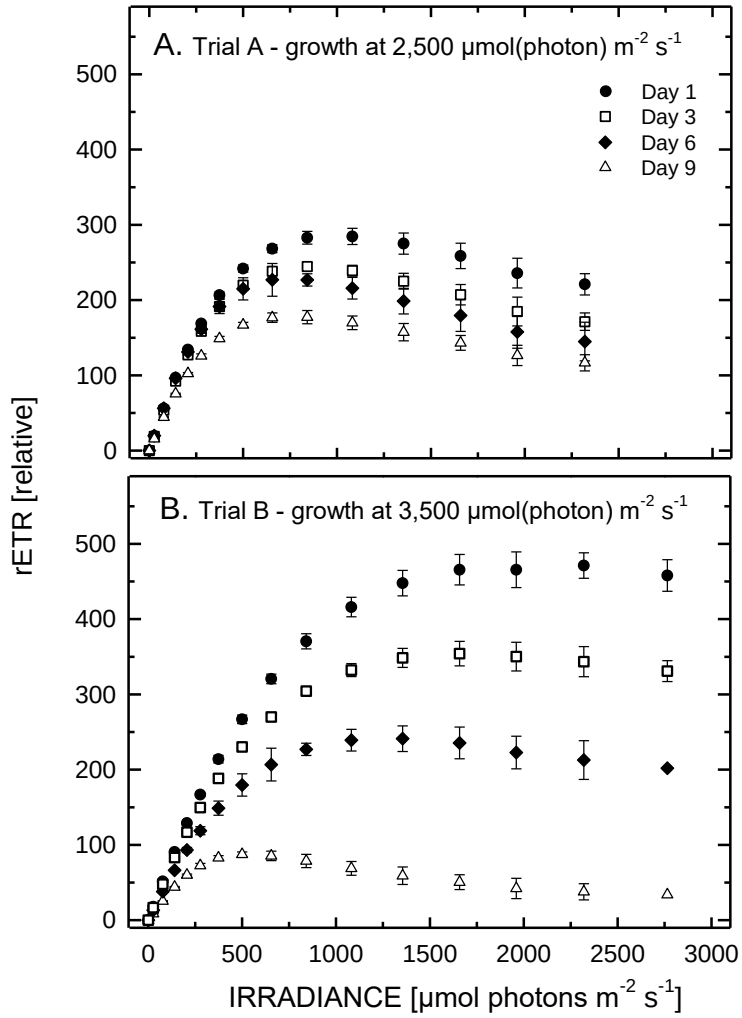

Fig. 11 Rapid light-response curves of relative electron transport rate (rETR) vs. irradiance in *Chlorella* cultures grown under high irradiance (A - 2500 µmol photons m^{-2} s^{-1}, B - 3500 µmol photons m^{-2} s^{-1}) recorded on Days 1, 3, 6 and 9. Values are presented as a mean (n=3) with SE indicated by error bars.

It is shown here that the primary reason of the down-regulation of photosynthetic activity in these trial was the significant increase in biomass density that prevents the light penetration to the cells and causes low-light adaptation. We simulated outdoor cultivation

conditions where the ambient irradiance maxima may be above 2000 µmol photons m^{-2} s^{-1}. Generally, the higher cell irradiance, the higher biomass density can be reached. One has to keep in mind that it is valid when the starting biomass density is about 0.5 g L^{-1} (as in this trial). For more diluted cultures high irradiance could cause severe photo-stress and eventually culture death.

3.4.3 Genesis of thin-layer cascades for microalgae cultivation (Annex IV)

Open cultivation units are the most widely used cultivation systems for commercial large-scale production due to the low costs of their construction and maintenance. As there is a limited control of cultivation conditions and biological contamination, the open systems are suitable for fast growing strains or those requiring specific growth conditions (e.g., *Arthrospira, Dunaliella*). Open cultivation raceway ponds are characterized by a low light utilization as the culture depth varies from 10 to 30 cm. It results in a low productivity. Thin-layer cascades (TLCs) were designed to overcome disadvantages of deep ponds and raceways which were characterised by low turbulence of low-density cultures resulting in low biomass productivity. TLCs bring together the advantages of open systems (e.g., direct sun irradiance, evaporative self-cooling, simple cleaning, low maintenance costs) with the positive features of closed systems in which the high productivity can be achieved as they are better mixed and the light path is short. The typical feature of thin-layer systems is low depth (< 10 mm) and fast flow (0.4 – 0.5 m s^{-1}) of the culture. It guarantees a high exposed surface to total culture volume ratio, typically between 50-150 m^{-1} and high turbulence of cells compared to open reservoirs and raceways.

In 1960 the first generation of cascades was built as a system of shallow troughs s in Třeboň (Fig. 12). The disadvantage of this system was that the technical design was not suitable to prevent the sedimentation of cells.

Fig. 12 First generation of outdoor cascades built in Třeboň in 1960.

In 1962 and 1963, the second generation of cultivation units was constructed in which the plain surface was fitted by transversal baffles (Fig. 13).

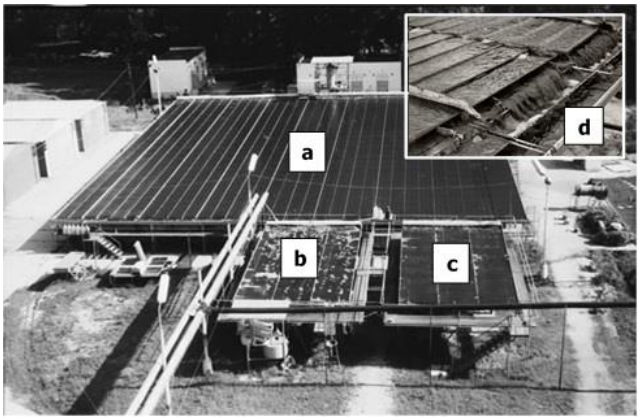

Fig. 13 The second generation of outdoor cascades consisted of a pilot unit of 900 m^2 (a) and of two experimental units (b and c), each of 50 m^2. Inserted (d): Microalgae culture flowing over baffles to the collecting trough.

At the early 1990s, the third generation of TLCs was developed. (Fig. 14). The surface declination was reduced from 3 to 1.7%, cultivation platforms were divided into parallel lanes to support lateral turbulence and mixing baffles were later removed (laborious installation and difficult cleaning of cultivation surface were required).

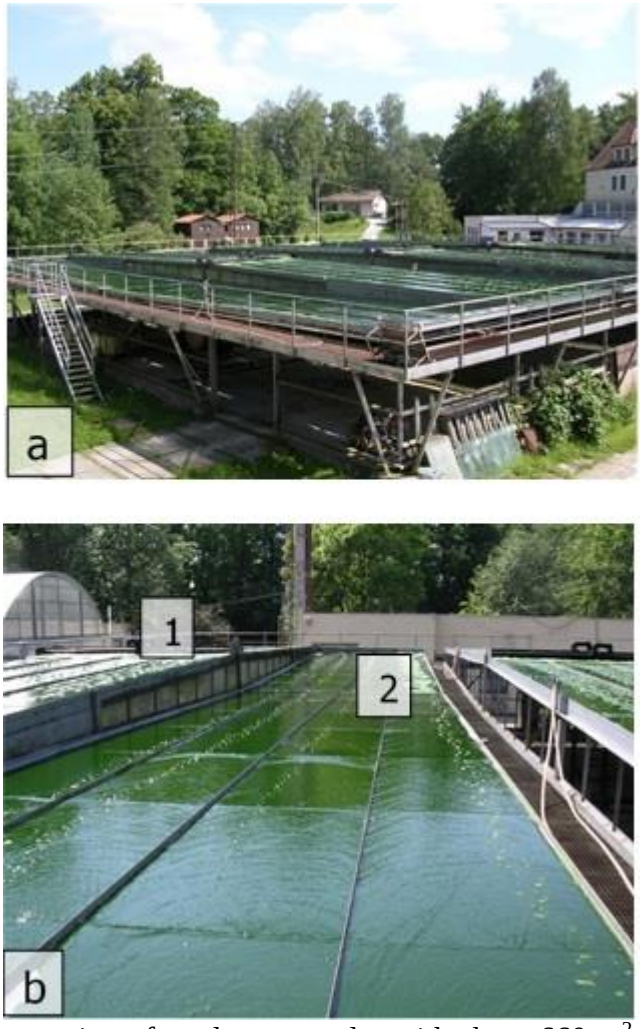

Fig. 14 The third generation of outdoor cascades with about 660 m^2. It consists of three units (a) with each 220 m^2 arranged as a cascade of four planes arranged symmetrically in a roof-shape design (b).

The latest generation of TLC has been built in the 2010s. Compared to the previous generation made of glass plates, the cultivation surface is made of stainless steel plates (Fig. 15).

Fig. 15 The latest generation of cultivation unit with the area of 90 m^2 can be operated with a volume between 500-1500 L. The total surface to volume ratio can be operated in the range between 60 and 180 m^{-1}.

3.4.4 Growth and photosynthetic performance of Arthrospira in thin-layer cascade and an open circular pond (Annex V)

The growth and productivity of microalgae cultures is mostly influenced by light availability (the surface to volume ratio S/V). In the trials presented in this paragraph diel changes in photosynthetic performance and biomass productivity were examined in *Arthrospira platensis* cultures grown in an open circular pond (OCP) and thin-layer cascade (TLC), when the temperature was adjusted to optimal (33˚C) and suboptimal value (25˚C). The experiments were carried out in Sesto Fiorentino, Italy. The starting biomass density was about 5 g L^{-1} in TLC and 0.5 g L^{-1} in OCP to achieve the same areal biomass density of about 50 g m^{-2}.

Under both optimal and suboptimal temperature regime the photosynthesis and biomass productivity was by about 30% higher in TLC as compared to that obtained in OCP (Fig. 16).

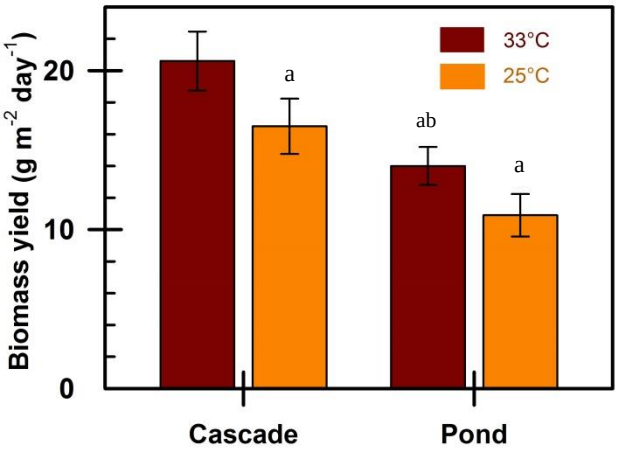

Fig. 16 Biomass yield of *Arthrospira* cultures grown in TLC and OCP at 33˚C and 25˚C. Columns labelled by the same letter differ significantly from each other.

Dense, well mixed *Arthrospira* cultures in the TLC acclimated better to the high irradiance as compared witho the OCP cultures. The trends of rETR, NPQ and OJIP curves correlated well to changes in biomass productivity. Higher values of rETR and NPQ in TLCs indicated that more energy was absorbed and used for electron transport, more energy was used for photochemistry, but more was also dissipated as heat to avoid damage to the photosynthetic apparatus. The maximum photochemical yield of PSII, F_v/F_m ranged at optimal temperature from 0.60 to 0.65 in the morning (0800 h) indicating good physiological condition. A considerable interaction between irradiance (reached up to 1740 µmol photons m^{-2} s^{-1} at 1400 h) and temperature was observed in the culture

cultivated in the OCP at suboptimal temperature where F_v/F_m dropped to 0.33 (a decrease by 33%) while in the culture F_v/F_m it only dropped from 0.61 to 0.53 (by about 13%) (Fig. 17). The cells in the deeper layer of the OCP comparing to thin layer in the TLC were low-light acclimated which resulted in an increase of phycocyanin content in the OCP-grown cultures.

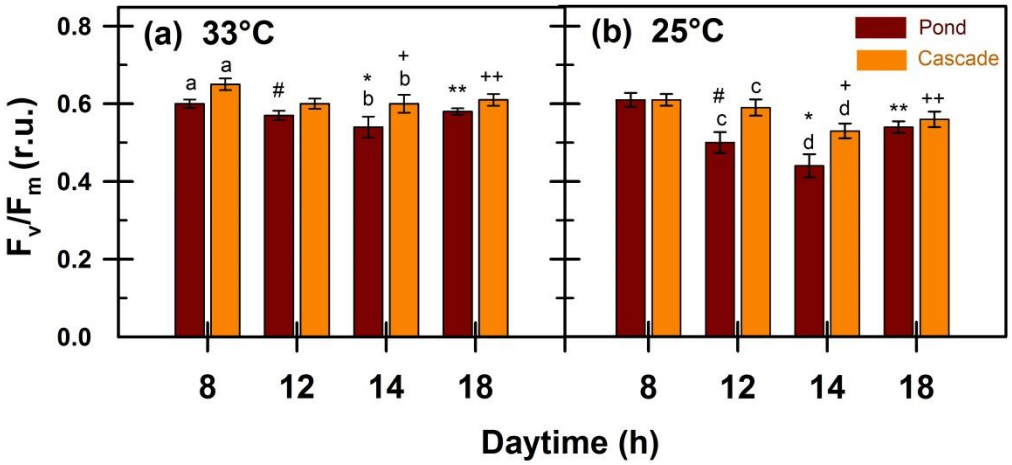

Fig. 17 Diel changes of the maximum photochemical quantum yield of PSII (F_v/F_m) measured in the *Arthrospira* cultures grown in the TLC and OCP at 33˚C (a) and 25˚C (b). Columns marked with the same letter differ significantly from each other. The same symbols mean significant differences between the two temperatures.

These experiments reveal that the cultures grown in the TLC showed higher productivity than those in the OCP which can be related to higher photosynthetic activity. The shorter light path promoted faster L/D cycle favourable for better photosynthetic performance and thus higher productivity.

3.5 Conclusions

The two-week experiment was carried out to study the growth and photosynthetic performance of *Chlorella* R-117 in 10-L PBR with internal LED illumination when the cultures were to two irradiance intensities (Trial A - 2500 µmol photons m^{-2} s^{-1} and Trial B - 3500 µmol photons m^{-2} s^{-1}) simulating outdoor conditions during summer. Both cultures had the same starting biomass density but in Trial B the culture reached 5.8 g DW L^{-1} which was by 66% more than that in Trial A. On Day 9 there were still enough nutrients in both cultures and thus the growth and also the photosynthetic activity decreased significantly in Trial B due to low light availability in the denser culture. By simulating outdoor conditions we could see that the culture exposed to higher irradiance could reach higher biomass density, but after several days became light limited.

Therefore, it is necessary to grow microalgae in thin-layer system where the availability of the light is sufficient, or use semi-continuous growth regime with regular biomass harvesting.

Thin-layer cascades (TLCs) are characterized by low depth (< 10 mm). They bring together the advantages of open systems (direct sun irradiance, evaporative self-cooling, simple cleaning, low maintenance costs) with the positive feature of closed systems (operation at high biomass densities achieving high biomass productivity). The advanced design is based on plain surface made of stainless-steel in which the productivity of 40 g m^{-2} d^{-1} can be achieved.

When we compared the growth and photosynthetic performance of the *Arthrospira* cultures in two outdoor cultivation units – open circular pond (OCP) and thin-layer cascade (TLC) cultured at two different temperatures – optimal and suboptimal (as the outdoor cultivation conditions are not fully controllable), the TLC was found far more productive regardless of the temperature as compared to the growth in the OCP. The former culture showed higher productivity than that in the latter that was related to the higher photosynthetic activity. The better culture performance in TLC was ascribed to significantly shorter light path that promoted much faster L/D cycles favourable for the light phase of photosynthesis as well as better temperature regime (faster warming of cultures in the morning) compared to the OCP cultures.

Chapter 4

Chapter 4. Use of Microalgae

Microalgae have been utilized by humans for hundreds of years as food, feed, remedies and fertilizers (Barsanti and Gualtieri 2006). The use is dated back 2000 years to Chinese who consumed *Nostoc* to survive the periods of famine (Spolaore et al. 2006). The microalga *Arthrospira* was used in Mexico where Aztecs harvested the biomass from Lake Texcoco to prepare dry cake called *tecuitlatl* (Barsanti and Gualtieri 2006). Due to the potential of microalgae to enhance nutritional value of conventional food and feed supplement, there were many attempts to commercialize microalgae production (Masojídek and Torzillo 2014). Large-scale commercial cultivation is limited to *Dunaliella, Haematococcus, Arthrospira* and *Chlorella*. These microalgae are source of various pigments (chlorophylls and carotenoids), proteins, polysaccharides, lipids, vitamins and other compounds that can be used further for the production of nutraceuticals, pharmaceuticals, feed additives, cosmetics and recently also biofuels (Barsanti and Gualtieri 2006).

Microalgae (e.g., *Chlorella* and *Arthrospira*) can also strip heavy metals (Cd, Zn, Cu and Pb) from aqueous solutions (Sandau et al. 1996). In case of metalloid selenium (Se), microalgae are able to incorporate it to organic compounds (e.g., proteins) as a part of detoxification process. Selenium is bound in the form of Se-amino acids via sulphur substitution (Babaei at al. 2017). A low dosage of organic Se compounds brings numerous health benefits having anti-inflammatory, immunostimulating, antiaging and cytotoxic activities (Vu et al. 2019; Mylenko et al. 2020). Important is that Se can alternate from an essential micro-nutrient to a toxic compound (could cause selenosis or cancer) within a narrow concentration range (Babaei et al. 2017). The limit of Se tolerance by microalgae is related to photosynthetic activity that can be used for dose control (Babaei et al. 2017). When the Se concentration is in excess, a significant decrease of photosynthetic activity was monitored by oxygen evolution or Chl fluorescence.

Some microalgae also produce other biologically active compounds – biopesticides and biostimulants that can be applied as an alternative to enhance rooting and protection of agricultural crops as to minimize the application of chemical and enhance encironmental sustainability (Kulik 1995; Ördög et al. 2014; Garcia-Gonzalez 2015; Romero-Villegas 2018; Ronga et al. 2019).

4.1 Biochemical composition of microalgae

Microalgae are able to enrich nutritional content of conventional food and hence, positively affect the health of humans and animals. For ages the microalgae are considered as a unique source of protein (Tab. 3). Their cells are also able to synthesize all essential amino acid for human and animal nutrition. Carbohydrates are present in microalgae cells in the form of starch, glucose or other sugars. Microalgal lipids, as storage compounds and energy sources are composed of glycerol, sugars or bases esterified to saturated or unsaturated fatty acids. The average lipid content varies between 5 to 40% and under certain condition could be as high as 85% of DW (Becker 2004; Spolaore et al. 2006).

Table 3 General composition of various food sources [% DW] (Becker 2004)

	Protein	Carbohydrates	Lipids
Meat	43	1	34
Milk	26	38	28
Rice	8	77	2
Chlorella vulgaris	51-58	12-17	14-22
Dunaliella salina	57	32	6
Arthrospira maxima	60-71	13-16	6-7

Microalgae also represent a source of almost all important vitamins (e.g., A, B, B1, B2, B6, B12, C, E and biotin). The downstream processing of microalgae biomass may affect the content of vitamins in the biomass especially in case of heat sensitive vitamins B1, B2, C (for a comparison with common foods see Becker 2004). Microalgae are also rich in pigments like chlorophylls (1-2.5% of DW), carotenoids (usually 0.3-0.5%; up to 14% of DW for ß-carotene of *Dunaliella*) and phycobiliproteins (Spolaore et al. 2006). Thus, their composition gives microalgae interesting qualities, which can be applied in human and animal nutrition.

4.2 Microalgae in human and animal nutrition

4.2.1 Human nutrition

Microalgae biomass for human nutrition is supplied in various forms, e.g., tablets, capsules or drinks, but can be consumed in the form of dried powder (Liy and Hu 2013).

The commercial applications are dominated by *Arthrospira* and *Chlorella*. The former is used in human nutrition because of its high protein content (Tab. 3) and the consumption of these microalgae have also various health-promoting effect, e.g., suppression of hypertension (Spolaore et al. 2006). Apart from chlorophyll, *Arthrospira* contains phycocyanin which is a blue pigment known to have an interesting antioxidant power (Adiba et al. 2011). The advantage of *Arthrospira* is that the biomass is directly digestible (e.g., in our stomach) in contrast to *Chlorella* which needs disintegration of cellulose cell wall before consumption (Kuznetsova et al. 2019). The latter has also been widely used as food additive due to the high content of proteins, fatty acids, vitamins and carotenoids (Liu and Hu 2013). Various health-promoting effects have been reported for *Chlorella* – to boost immune function, prevent against tumour and cancer and lower blood pressure (Iwamoto 2004). The important bioactive product represents *Chlorella* Growth Factor (CGF) which is a water-soluble extract containing proteins, amino acids and vitamins with immunostimulating effect (Merchant and Andre 2001).

4.2.2 Animal nutrition

Chlorella is also used as feed supplement for various animals. Due to high content of carotenoids it is used as colouring agent for ornamental birds and fish. Fresh or dried biomass is also fed to crustaceans (e.g., rotifers) that serve as feed for fish or larvae (Becker 2004; Liu and Hu 2013). The rotifers (*Brachionus plicatilis*), live prey for pikeperch larvae, fed with *Chlorella*, increased survival rate by 50% compared to those fed with *Nannochloropsis* and by 10% than the larvae receiving the commercial feed (Yanes-Roca et al. 2020). The *Chlorella* biomass fed to fish also enhanced the skin pigmentation due to the accumulation of secondary carotenoids and it increase their market value (Gouveia et al. 2003; Sergejevová and Masojídek 2012). *Chlorella* also finds application in hens feeding resulting in enhanced colour of egg yolk (Gouveia et al. 1996).

4.3 Microalgae as biopesticides

Increasing global population and a greater demand for food have led to intensive agriculture resulting in a greater use of synthetic pesticides (Albuquerque et al. 2018; Gomiero 2018). There are more than 200 bacteria that infect plants. Almost one half belong to the genus *Pseudomonas* and cause rots, blights and wilts. The microalgae of the genus *Chlorella* have a high antimicrobial activity against both gram-positive and gram-negative bacteria strains (Ördög et al. 2004). The use of bioactive microalgae

minimizing the use of chemicals and enhancing the agricultural sustainability (Romero-Villegas 2018). A number of metabolites are considered to have biopesticide activity and hence the microalgae are used as means to control harmful microorganisms *in-situ*. These activities are attributed to phenolic compounds, polyphenols, tocopherols, carbohydrates, proteins, oils, saponins and nitrogen-rich peptides. These compounds act by disruption of the cytoplasmic membrane, enzyme inactivation and inhibition of synthesis in the targeted microorganisms and in addition the microalgae stimulate the immune system in relation to pathogens. Especially, the microalgae *Chlorella vulgaris* and *Arthrospira platensis* produce phenolic compounds which are responsible for antifungal (Costa et al. 2019).

A variety of laboratory methods can be used to evaluate the *in-vitro* antimicrobial and antifungal activity of extract or a pure isolated compounds. They can be divided into two groups – semi-quantitative diffusion methods and quantitative dilution methods. The most known basic methods are the disk-diffusion and broth or agar dilution methods (Balouiri et al. 2016).

Agar disk-diffusion bioassay

Agar disk-diffusion method is the approved method used for routine antimicrobial testing. The plate surface that is used for bioassay is inoculated by spreading a certain volume of microbial pathogen (Balouiri et al. 2016) or the fungal pathogen in placed in the centre of agar plate (Velusamy and Das 2014). Then, filter paper discs of the diameter of 6 mm (Balouiri et al. 2016), containing the test compound with a required concentration, are placed on agar. The Petri dishes are incubated at specific temperature for certain time period (37°C, 16 h for bacteria and 30°C for 48 h for fungi) (Saurav et al. 2019). Antimicrobial agent diffuses into the agar and inhibits the growth of the test pathogen. The diameters of clear inhibition zones are measured (Fig. 18a; Balouiri et al. 2016).

Agar well-diffusion method

After spreading the microbial pathogen over the agar plate, holes with a certain diameter are made aseptically with a sterile cork borer. Then, the defined volume of the potentially active extract is introduced into the well. After incubation, the size of clear zones is measured (Fig. 18b).

Disk-diffusion assays are simple and cheap to perform and the interpretation of results is easy, but on the other hand these techniques are not adequate to determine the minimum

inhibitory concentration (MIC) as it is problematic to quantify the amount of the antimicrobial sample diffused into the agar medium (Balouiri et al. 2016).

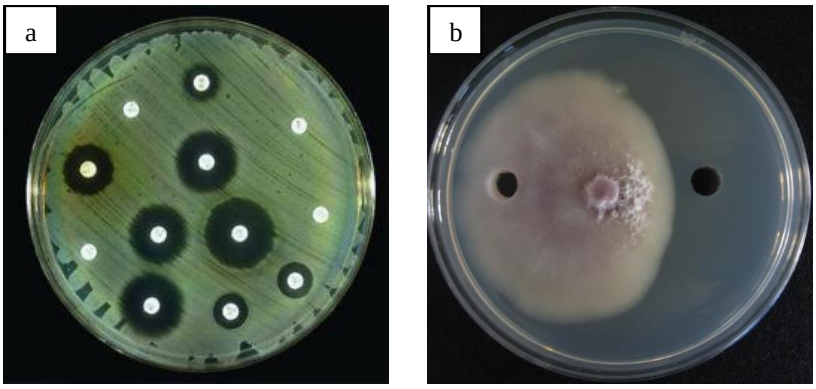

Fig. 18 Agar diffusion methods (a) disk-diffusion bioassay (Sandle 2016); (b) well-diffusion method (Velusamy and Das 2014).

Broth dilution bioassay

Dilution bioassays are the most appropriate methods for the determination of MIC value which is the lowest concentration of the potentially active sample that inhibits the visible growth of the pathogen (usually expressed in μg mL^{-1} or mg mL^{-1}). They give the possibility to estimate the concentration of the tested antimicrobial agent in the agar (agar dilution) or broth medium (macrodilution or microdilution). Both broth and agar dilution methods are used to measure antimicrobial and antifungal activity *in vitro* quantitatively. Broth micro- or macro-dilution is one of the basic anti- microbial susceptibility testing methods. The procedure is based on two-fold dilutions of the antimicrobial agent (e.g., 1, 2, 4, 8, 16 and 32 μg mL^{-1}) in a liquid growth medium in tube with minimum volume 2 mL (macrodilution, Fig. 19a) or with smaller volumes in 96-well microtiter plate (microdilution, Fig. 19b). Then each tube or well is inoculated with the pathogen adjusted to the concentration of 10^6 colony-forming units (CFU) mL^{-1} (Balouiri et al. 2016). The concentration of microbial cultures is measured by determining the optical density (OD_{600}) which can be correlated to CFU for each microbial pathogen. For example, $OD_{600} = 1$ is equal to 3.13×10^7 CFU mL^{-1} in case of *Mycobacterium tuberculosis* (Peñuelas-Urquide et al. 2013). After well mixing and incubation, spectrophotometric methods are used to determine the MIC. By broth dilution methods minimum bactericidal (MBC) or minimum fungicidal concentration (MFC) can be determined. MBC and MFC are defined as the lowest concentration of antimicrobial/antifungal agent needed to kill 99.9% of the pathogen.

The broth macrodilution method requires larger amount of antimicrobial agent to be tested and the method itself is time-consuming (Kadlec et al. 2015) Thus, the reproducibility, costs of reagents and laboratory space favour test miniaturization the major advantages of the microdilution method (Balouiri et al. 2016).

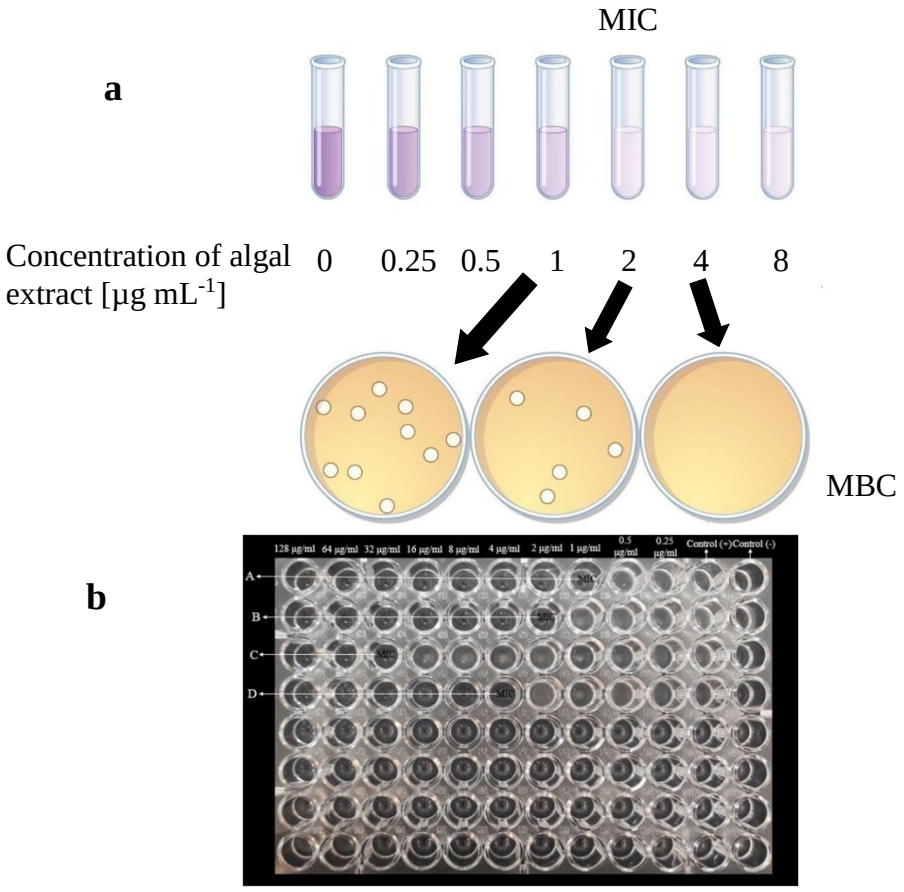

Fig. 19 Broth dilution bioassays (a) macrodilution method (Tankeshwar 2020); (b) microdilution method for 4 tested samples (Nezhadi et al. 2019)

Agar dilution method

The agar dilution method involves the incorporation of the antimicrobial agent (e.g., algal extract) into an agar medium on the plate, using serial two-fold dilutions as in the previous method. The plate is then inoculated with tested pathogen.

4.4 Microalgae as biostimulants

A number of microalgae produce biologically active compounds and thus could be used as alternative techniques to enhance rooting and protect agricultural crops in the field (Garcia-Gonzalez 2015; Kulik 1995; Ronga et al. 2019). The extracts responsible for plant development contain a variety of plant hormones such as cytokinins, auxins,

abscisic acid, gibberellic acid and salicylic acid (Plaza 2018; Schwarz and Krienitz 2005; Tarakhovskaya et al. 2007; Mógor et al. 2018; Calvo 2014). Phytohormones are a class of small molecules which are produced in extremely low concentration and coordinate cellular activities in higher plants. Phytohormones including auxin, cytokinin, abscisic acid, ethylene and gibberellins have been found in a broad spectrum of microalgae (Lu and Xu 2015). Auxins and cytokinins are essential for plant growth as they are involved in almost all aspects of plant growth and development (Stirk et al. 2013). Indole-3-acetic (IAA) acid as a natural auxin synthesized in chloroplasts of young leaves and induces the process of root formation and cytokinins control cell division (Tarakhovskaya et al. 2007). IAA has been identified in the microalgae *Scenedesmus armatus* and *Chlorella pyrenoidosa* (Stirk et al. 2013). Another natural auxin is indole-3-butyric acid (IBA) which is mainly used as a standard for comparison when the bioassays regarding auxin-like activity are performed.

Determination of auxin-like activity

The seedlings of mung bean (*Vigna radiate*) are used for the determination of auxin-like activity of microalgae. They are placed in the vial containing the algal suspension and after several days of incubation the number of roots are recorded and compared to standard auxin of IBA. The results are then given in IBA equivalent concentrations (Hess 2013). The auxin-like activity can be also determined by seed germination already described by Zucconi et al. 1981. The microalgae extract is applied on 100 cress seeds and they are incubated at 25°C for 36 h. The number of germinated seeds and the average length of roots is recorded in order to calculate a Germination Index (GI; in %) according to the following formula:

$$GI = ((G \times L) / (G_C \times L_C)) \times 100$$

where G is the average of germinated seeds in the sample, G_C is the average of germinated seeds in control plates (distilled water is used), L is the average length of the radicle in the sample (mm), and L_C is the average length of the radicle in the negative control (mm) (Ronga et al. 2019).

Determination of cytokinin-like activity

Cytokinins stimulate the chlorophyll production. Therefore, the cytokinin-like activity of microalgae is determined using the seeds of wheat cultivar (*Triticum aestivum* L.). Small segments of leaves are placed for several days in vials containing algal suspension and

kept in the dark. Chlorophyll content is then evaluated and compared to standard cytokinin of kinetin (KIN). The results are then given in KIN equivalent (Kuhnle et al. 1977).

4.5 Case studies

4.5.1 Main objectives

Some population groups are advised to take specific supplements as a source of vitamins (e.g., A, C, D, B_{12}), antioxidants and proteins. *Chlorella* has an impressive nutritional profile – over 50% of protein. Moreover, it can accumulate heavy metals (Cr, Zn, Fe, I, etc.) as well as metaloids (e.g., Se, As). For example selenium (Se) is metabolized mostly to protein bound selenomethionine (SeMet), selenocysteine (SeCys) and methylselenocysteine (MeSeCys) by replacing sulphur. In a dose dependent manner, Se can act either an essential microelement or a toxic compound. At low dosage Se brings numerous health benefits, such as a decrease of cancer incidence, protection against cardiovascular diseases and boosting immune function. The required dose of Se can be also obtained from food such as nuts, seafood, meat and vegetable.

The main objective of these trial was to quantify and compare the amount of SeMet, SeCys and MeSeCys in Se-enriched microalgae biomass and the bioaccessibility of Se-amino acids (Se-AAs) found in several Se rich food sources (salmon, brazil nut, mustard seed), Se-supplement and Se-enriched microalgae (Annex VI).

Some microalgae produce biologically active compounds such as biopesticides and biostimulants and thus the biomass in becomming usable in agriculture for crop treatment to minimize the application of chemicals and for enhancing of environmental sustainability. By using wastewater as a growing media (which is rich in nitrogen mainly in the form of ammonium) the cultivation cost can be reduced. The main objective of the second case study in this chapter was to study the growth and bioactivity of *Chlorella* when grown in inorganic medium and in centrate. Then, the bioactivity was correlated with the photosynthetic activity (Annex VII).

4.5.2 Bioaccessibility of selenoamino acids from Se-enriched Chlorella in comparison to other Se-enriched foods (Annex VI)

Selenium is a part of more than 30 distinct selenoproteins, including glutathione peroxidases, a group of antioxidant enzymes that help to protect cells from damage by free radicals. Traditionally, Se is made available in human diet via consumption of Se rich foods such as nuts and seafood or from Se containing food supplements including Se-enriched yeast and *Chlorella* biomass which accumulates Se in the form of Se-amino acids (Se-AAs). Despite its popular uses, data about bioaccessibility of Se-AAs from Se-*Chlorella* are not abundant. Gastrointestinal digestion times and bioaccessibility of Se-AAs in Se-*Chlorella* was studied and compared to Se-yeast, a commercially available Se-enriched food supplement and Se-enriched food (salmon, brazil nut, mustard seed). The optimized gastrointestinal transit time, 2h for gastric digestion and 4h for intestinal digestion, was used for further comparison of bioaccessibility.

Se-enriched biomass of *Chlorella* was obtained from the cultivation outdoors in thin-layer cascade (working volume 2200 L) when sodium selenite was added to the culture every day (the case study of Chapter 2; Babaei et al. 2017). After the analysis and quantification of Se-AAs in Se-*Chlorella*, Se-yeast and Se-supplement (GC-APCI-HRMS) and in Se-foods (HPLC-ICP-MS), the bioaccessibility (the fraction of a substance that can be theoretically released from the food into the gastrointestinal tract) of all samples was determined and compared (Tab. 4).

Table 4 Amount of SeMet, SeCys, MeSeCys and total organic Se (µg g^{-1} biomass) and their bioaccessibility (%) in Se-*Chlorella*, Se-yeast and Se-foods.

Se-AAa	Content (µg g^{-1})				Bioaccessibility (%)				Daily diet (g)
	SeMet	SeCys	MeSeCys	Organic Se	SeMet	SeCys	MeSeCys	Organic Se	
Se-*Chlorella**	281 ± 48.5	43 ± 5.8	115 ± 16.2	183 ± 24.3	12.2 ± 2.14	11.3 ± 4.14	13.3. ± 2.64	12 ± 3.2	~ 2.5
Se-*Chlorella*#	282 ± 24.5	49 ± 4.6	110 ± 9.3	185 ± 15.5	50.5 ± 3.73	50.5 ± 4.23	48.6 ± 6.11	49 ± 4.8	~ 0.61
Se-yeast	3186 ± 335.3	354 ± 44.5	39 ± 5.4	1467 ± 153.4	22.87 ± 2.01	20.9 ± 3.11	19.8 ± 4.87	21 ± 3.5	~ 0.18
Se-supplement	61 ± 6.1	15 ± 4.5	ND	34 ± 3.4	34.7 ± 6.84	30.1 ± 4.78	ND	32 ± 5.9	~ 5.1
Salmon	1.5 ± 0.17	0.4 ± 0.14	ND	0.8 ± 0.09	52.7 ± 4.71	51.3 ± 6.11	ND	52 ± 5.9	~ 132.2
Brazil nut	12 ± 1.7	0.8 ± 0.22	ND	5.2 ± 0.6	8.2 ± 3.34	8.1 ± 2.89	ND	8.2 ± 3.70	~ 129.0
Mustard seed	1.1 ± 0.31	0.4 ± 0.1	ND	0.6 ± 0.14	7.2 ± 2.37	ND	ND	7.2 ± 2.37	~ 1273

ND: not detected.
* Non-disintegrated, lyophilized Se-*Chlorella*
Disintegrated, spray-dried Se-*Chlorella*

The disintegration process to access intracellular products of *Chlorella* using disintegration and spray-drying (when the initial temperature of about 200°C might cause degradation of Se-AAs content) is a necessary process for the preservation of biomass. Nevertheless, the stability of Se-AAs was confirmed as the amount of Se-AAs was similar between lyophilized and spray-dried samples. The bioaccessibility of Se-AAs from

disintegrated, spray-dried Se-*Chlorella* (~ 50%) was similar to data obtained from salmon (~ 52%), but it was significantly higher than that from lyophilized, non-disintegrated Se-*Chlorella* (~ 13%). The pressure during the disintegration process disrupts the cell wall of *Chlorella* and therefore enhanced the hydrolysis activity of pepsin and pancreatin during gastrointestinal digestion. In any case, the drying process did not affect the bioaccessibility of Se-AAs from Se-*Chlorella*. Even if the bioaccessibility of disintegrated, spray-dried Se-*Chlorella* is similar to the bioaccessibility of Se from salmon, we have to consider two-orders of magnitude higher content of organic Se in the former material. To achieve the recommended daily intake of 55 µg of Se per person, one has to consume about 0.6 g of disintegrated, spray-dried Se-*Chlorella* biomass or more than 130 g of salmon (Table 4).

4.5.3 Biopesticide and biostimulant activity of Chlorella cultivated in inorganic medium and wastewater (Annex VII)

A number of microalgae strains produce biologically active compounds, like plant hormones (responsible for biostimulating activity) and antimicrobial compounds (responsible for biopesticide activity). Hence, the biomass can be applied as an alternative to enhance plant growth and for protection of agricultural crops. Cultivation of microalgae involves open reservoir but a thin-layer systems are more adequate for light utilization. The growth of *Chlorella* during the cultivation in thin-layer cascade and a novel, thin-layer raceway pond was followed and photosynthetic activity was correlated to find suitable optimal point of biomass harvesting with high bioactive properties.

Chlorella was firstly grown in a batch mode for 7 days to get well-growing dense culture and then semi-continuous growth regime was operated for 4 d at 25% dilution rate (0.25 d^{-1}) to follow a model of large-scale production where microalgae are grown in semi-continuous/continuous regime replacing daily a part of culture with fresh medium/WW. During the first week of cultivation in BG-11 medium in batch regime, both cultures grew well, linearly between Day 1 and 7. Nevertheless, the *Chlorella* culture grown in TLC showed about 35% higher (μ = 0.19 d^{-1}) as compared to the culture grown in TL-RWP (μ = 0.14 d^{-1}). The slower growth in TL-RWP could be caused by deeper culture layer causing lower average cell irradiance compared to that in TLC. The specific growth rate in TLC when cultivated in WW was higher by about 21% (μ = 0.23 d^{-1}) compared to that in BG-11 medium. It could be caused by better light conditions during cultivation in WW.

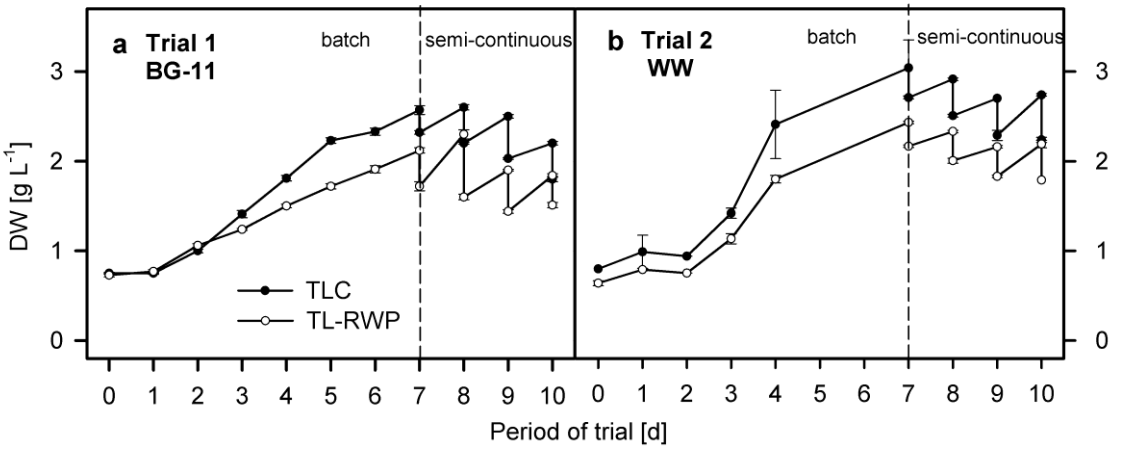

Fig. 20. Changes in the biomass density (DW) of *Chlorella* MACC-1 cultures grown in batch and semi-continuous regime in thin-layer cascade (TLC; black circle) and thin-layer raceway pond (TL-RWP; white circle) grown in: a) BG-11 medium (Trial 1) and b) centrate of municipal wastewater (Trial 2).

The changes in the photosynthetic activity were estimated as the maximum PSII photochemical rate, F_v/F_m by Chl fluorescence (Fig. 21). The lower F_v/F_m values were observed in Trial 1 (in BG-11 medium) in both units during the Day 0 and Day 1 which indicated that laboratory cultures were partially photo-stressed after the exposure to outdoor irradiance and needed some time for acclimation. It corresponded to the lag phase also seen in growth curves for the first two days. Compared to high photochemical performance of the cultures grown in Trial 1, the F_v/F_m values of cultures grown in Trial 2 (in WW) were lower at the end of the cultivation, probably indicating decreased photosynthetic activity due to some stress (e.g., shade adaptation in dense cultures).

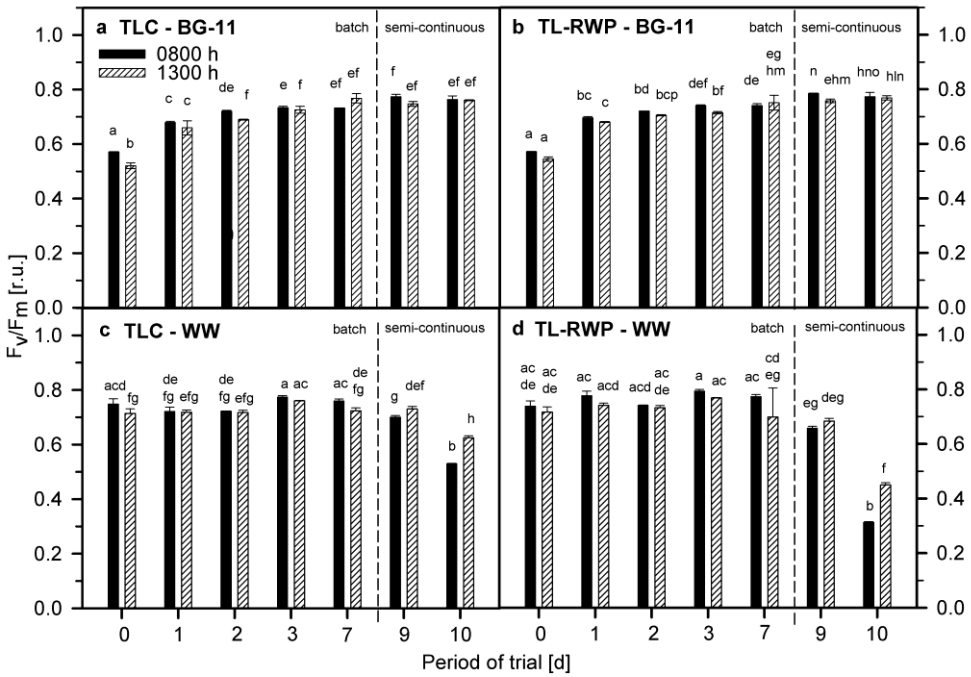

Fig. 21. Changes in maximum effective quantum yield of PSII (F_v/F_m) during the cultivation of *Chlorella* cultures in batch and semi-continuous regime: a) cultures grown in TLC in BG-11 media; b) cultures grown in TL-RWP in BG-11 media; c) cultures grown in TLC in WW; d) cultures grown in TL-RWP in WW. The black columns represent the measurement at 0800 h and dashed columns represent the measurement at 1300 h. Values with the same symbol did not differ significantly from each other (P>0.05).

The dual culture bioassay for determination of biopesticide activity was performed. Out of the total number of 8 pathogenic microorganisms (two fungi – *Fusarium oxysporum* f.sp. *melonis, Rhizoctonia solani* (further as *F. oxysporum, R. solani*), two oomycetes – *Phytophthora capsici Pythium ultimum* (further as *P. capsici* and *P. ultimum*) and four bacteria strains – *Clavibacter michiganensis* subsp. *michiganensis, Xanthomonas campestris* pv. *vesicatoria, Pseudomonas syringae* pv. *tomato, Pectobacterium*

carotovorum (*C. michiganensis, X. campestris, P. syringae* and *P. carotovorum*) the biomass extracts of *Chlorella* cultivated in BG-11 medium were active only against one bacterial strain of *C. michiganensis* and three fungal strains, *F. oxysporum*, *R. solani* and *P. capsici.* Antifungal activity up to 37% against *F. oxysporum* was determined for the biomass harvested from TLC. Generally, the antibacterial and antifungal activity was higher when *Chlorella* cultures was grown in WW as compared to those grown in BG-11 (Table 5). It is important to note that antifungal activity of the *Chlorella* cultures grown in WW was about twice higher compared to antibacterial activity. Antifungal activity against all fungal pathogens was almost always higher when *Chlorella* biomass was harvested at midday. The highest antifungal activity of *Chlorella* of 41.4% (0800 h) and 50.4% (1300 h) was observed against *P. capsici* when grown in WW.

Table 5 Antibacterial and antifungal activity of *Chlorella* extracts. The biomass was harvested at the end of semi-continuous regime in the morning (0800 h) and in the afternoon (1300 h). Statistical analysis was performed between the rows. Values with the same letter did not differ significantly from each other ($P>0.05$).

	Inhibition index [%]							
	Trial 1 (BG-11)				**Trial 2 (WW)**			
Pathogen	TLC		TL-RWP		TLC		TL-RWP	
	0800 h	1300 h	0800 h	1300 h	0800 h	1300 h	0800 h	1300 h
Bacteria								
C. michiganensis	25.6±0.6^{a}	21.7±0.3^{a}	22.8±0.3^{a}	22.8±0.3^{a}	0^{b}	20.8±3.3^{a}	0^{b}	23.1±7.3^{a}
X. campestris	0^{a}	0^{a}	0^{a}	P	0^{a}	18.3±5.3^{b}	16.9±5.3^{b}	21.9±5.3^{b}
P. syringae	0^{a}	0^{a}	0^{a}	0^{a}	12.2±5.3^{b}	16.7±0.1^{bc}	0^{a}	17.8±2.0^{c}
P. carotovorum	0^{a}	0^{a}	0^{a}	0^{a}	14.7±4.7^{b}	15.8±6.7^{b}	15.6±4.0^{b}	17.3±7.5^{b}
Fungi								
R. solani	11.3±3.0^{a}	23.8±4.8^{b}	0^{c}	10.1±1.8^{a}	27.2±0.2^{b}	42.3±4.0^{d}	28.8±0.2^{b}	44.2±0.2^{d}
F. oxysporum	37.5±8.9^{a}	33.3±7.1^{a}	1.2±1.2^{b}	32.1±8.3^{a}	28.7±1.3^{a}	26.8±2.0^{a}	31.6±2.0^{a}	32.0±2.0^{a}
Oomycetes								
P. capsici	19.6±2.6^{a}	34.6±2.5^{ab}	0^{c}	3.7±3.7^{c}	41.4±0.7^{b}	50.4±0.1^{b}	46.9±6.7^{b}	41.4±17.3^{b}
P. ultimum	0^{a}	0^{a}	0^{a}	0^{a}	25.7±2.7^{b}	34.3±1.3^{c}	33.3±4.0^{c}	35.6±1.3^{c}

Higher biostimulant activity was observed when a lower concentration of the biomass extract (0.5 mg DW mL^{-1}) was applied as it ranged from 108 to 115% as compared to the 2 mg DW L^{-1} extract. As concerns daytime, the higher biostimulating activity of all extracts was mostly observed when the *Chlorella* biomass was harvested in the morning (0800 h) using less concentrated biomass extract with the exception of the sample harvested at 0800 h from TLC (Table 6). Surprisingly, no biostimulating activity was found in the *Chlorella* cultures grown in WW.

Table 6 Biostimulating activity of *Chlorella* extracts. The biomass was harvested from TLC and TL-RWP at the end of semi-continuous regime in the morning (0800 h) and in the afternoon (1300 h) when cultivated in BG-11 medium, lyophilised and then studied. No activity of biomass harvested at the end of Trial 2 (WW) was found. Values with the same letter did not differ significantly from each other ($P>0.05$).

c [mg/mL]	Germination index [%] **Trial 1 (BG-11)**			
	TLC		TL-RWP	
	0800 h	1300 h	0800 h	1300 h
0.5	108.5±0.2[a]	108.5±0.1[a]	**115.7±0.5[b]**	108.6±1.2[a]
2	**100.8±0.2[c]**	85.5±0.2[d]	**105.4±0.5[e]**	86.0±2.1[d]

4.6 Conclusions

The optimal time for *in-vitro* gastric and intestinal digestion were found to be 2h and 4h, respectively. The content of SeMet, SeCys and MeSeCys in Se-*Chlorella* was quantified; SeMet was present at the highest concentration of 280 µg g^{-1} biomass. The other, SeCys and MeSeCys were present at concentrations between 40 and 50 µg g^{-1} biomass and between 110 and 115 µg g^{-1} biomass, respectively. In general, the content of Se-AAs was significantly higher in Se-*Chlorella* compared to Se-supplement and Se-foods but significantly lower as compared to Se-yeast. The bioaccessibility of SeMet, SeCys and MeSeCys from Se-*Chlorella* biomass was found to be higher as compared to Se-yeast, Se-foods and Se-supplement. The disintegration process influenced the bioaccessibility of Se-AAs in the Se-*Chlorella* biomass. It increased upon disintegration and stayed unchanged during drying process. Even if the bioaccessibility of the disintegrated, spray-dried Se-C*hlorella* was similar to the bioaccessibility of Se from salmon (~ 50%), we have to consider the different content of organic Se (which is more than 200 times less in the salmon). To achieve the recommended daily intake of 55 µg of Se, we have to consume about 0.6 g of disintegrated, spray dried Se-*Chlorella* and more than 130 g of salmon.

The microalga *Chlorella* shows the biostimulating and biopesticide activity, but cultivation conditions inducing the differences. The cultures grew well in both nutrients sources, inorganic nutrient medium and in undiluted centrate from municipal wastewater as well. The growth rate was 10-15% higher in TLC compared to that in TL-RWP which was probably caused by higher culture layer and lower light utilization. As concerns antifungal and antibacterial activity, these were generally higher when *Chlorella* cultures were grown in WW as compared to those grown in BG-11. The antifungal activity of the *Chlorella* cultures grown in WW was about twice higher compared to antibacterial activity. The highest biostimulating activity has been found for *Chlorella* cultures grown in the inorganic BG-11 medium. Surprisingly, no biostimulant activity was found in the *Chlorella* cultures grown in WW. There is no clear correlation between photosynthetic activity and bioactivity and further study is needed in this field.

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Annexes

Summary

Annex I - **Ranglová K**, Lakatos GE, Manoel JAC, Grivalský T, Masojídek J (2019) Rapid screening test to estimate temperature optima for microalgae growth using photosynthesis activity measurements. Folia Microbiol 64:615-625 (50%; IF 1.730)

Annex II - Babaei A, **Ranglová K**, Malapascua JR, Masojídek J (2017) The synergistic effect of Selenium (selenite, -SeO_3^{2-}) dose and irradiance intensity in *Chlorella* cultures. AMB Express 7 (56):1-14 (20%; IF 1.825)

Annex III - Malapascua JR, **Ranglová K**, Masojídek J (2019) Photosynthesis and growth kinetics of *Chlorella vulgaris* R-117 cultured in an internally LED-illuminated photobioreactor. Photosynthetica (20%; IF 1.507)

Annex IV - Grivalský T, **Ranglová K**, Manoel JAC, Lakatoe GE, Lhotský R, Masojídek J (2019) Development of thin-layer cascades for microalgae cultivation: milestones (review). Folia Microbiol 64:603-614 (10%; IF 1.730)

Annex V - Benavides AM, **Ranglová K**, Malapascua JM, Masojídek J, Torzillo G (2017) Diurnal changes of photosynthesis and growth of *Arthrospira platensis* cultured in thin-layer cascade and an open pond. Algal Research 28:48-56 (20%; IF 3.994)

Annex VI - Vu DL, Saurav K, Mylenko M, **Ranglová K**, Kuta J, Ewe D, Masojídek J, Hrouzek P (2019) *In vitro* bioaccessibility of selenoamino acids from selenium (Se)-enriched *Chlorella vulgaris* biomass in comparison to selenized yeast; a Se-enriched food supplement; and Se-rich foods. Food Chemistry 279:12-19 (15%; IF 6.306)

Annex VII – **Ranglová K**, Lakatos GE, Manoel JAC, Grivalský T, Suárez Estrella F, Acién Fernández G, Molnár Z, Ördög V, Masojídek J (2020) Growth, biostimulant and biopesticide activity of the MACC-1 *Chlorella* strain cultivated outdoors in inorganic medium and wastewater. Submitted to Algal Research on 3 August 2020 (15 %; IF 4.008 in 2019)

Annex I

(related to Chapter 2 Culture Monitoring and Maintenance)

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ORIGINAL ARTICLE

Rapid screening test to estimate temperature optima for microalgae growth using photosynthesis activity measurements

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Abstract

We have worked out a rapid 1-day test based on photosynthesis measurements to estimate suitable growth temperature of microalgae cultures. To verify the proposed procedure, several microalgae—*Chlorella*, *Nostoc*, *Synechocystis*, *Scenedesmus*, and *Cylindrospermum*—were cultured under controlled laboratory conditions (irradiance, temperature, mixing, CO_2, and nutrient supply) to find the optima of photosynthetic activity using the range between 15 and 35 °C. These activities were recorded at each temperature step after 2 h of acclimation which should be sufficient as oxygen production and the PQ cycle are regulated by fast processes. Photosynthetic activity was measured using three techniques—oxygen production/respiration, saturating pulse analysis of fluorescence quenching, and fast fluorescence induction kinetics—to estimate the temperature optima which should correspond to high growth rate. We measured all variables that might have been directly related to growth—photosynthetic oxygen evolution, maximum photochemical yield of PSII, F_v/F_m, relative electron transport rate $rETR_{max}$, and the transients V_j and V_i determined by fast fluorescence induction curves. When the temperature optima for photosynthetic activity were verified in growth tests, we found good correlation. For most of tested microalgae strains, temperature around 30 °C was found to be the most suitable at this setting. We concluded that the developed test can be used as a rapid 1-day pre-screening to estimate a suitable growth temperature of microalgae strains before they are cultured in a pilot scale.

Keywords Chlorophyll fluorescence · Electron transport rate · Microalgae · Photosynthesis measurements · Rapid test · Temperature optimisation

Abbreviations

Chl	Chlorophyll
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
DM	Dry mass
F_0, F_v, F_m	Minimal, variable, and maximal fluorescence in dark-adapted state
F_v/F_m	Maximal photochemical yield of PSII
POE/R	Photosynthetic oxygen evolution and respiration
OJIP curve	Fast Chl fluorescence induction kinetics
PAM	Pulse-amplitude modulation
PAR	Photosynthetically active radiation
PQ	Plastoquinone pool
PSII	Photosystem II
PTFE	Polytetrafluoroethylene
rETR	Relative electron transport rate through PSII
RLC	Rapid light-response curve

Dedicated to the memory of Prof. Ivan Šetlík

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Introduction

Microalgae are fast-growing photosynthetic organisms since their cell doubling time can be as little as few hours. Growth of microalgae is affected by various environmental variables such as light, temperature, nutrition, pH, presence of contaminants and others (Cho et al. 2007; Bernard and Rémond 2012). Light intensity and temperature are the most important

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environmental variables that influence microalgae growth. As concerns temperature regime, some microalgae strains tolerate a broad range of temperatures (15 to 40 °C; e.g. *Chlorella*), while marine diatoms such as *Phaeodactylum* usually require stricter regulation (20 to 25 °C), where at 30 °C growth is completely inhibited. For the majority of freshwater microalgae, the optimum temperature ranges from 25 to 35 °C (Masojídek and Torzillo 2014). Each microalgae strain is characterized by an optimal growth temperature which should correlate to its photosynthetic activity indicating its maximum photosynthetic efficiency (Béchet et al. 2013; Ras and Steyer 2013; He et al. 2018). Sub-optimal (or supra-optimal) temperatures usually cause a decrease in photosynthetic activity and subsequently also microalgae growth (Torzillo et al. 1991; Benavides et al. 2017).

Suitable cultivation regimes have to be worked out for each microalgae strain and particular cultivation unit because the optimum temperature may vary depending on the construction details (light path, mixing, etc.). Since the nineteenth century (e.g. Martinus Beijerinck in 1890) classical growth tests have been employed for optimisation of cultivation regimes determining changes of microalgae biomass density in illuminated cultivation vessels. Regular growth tests may take several days, or even weeks in order to combine various conditions. Later, in the 1980s, cross-gradient cultivation techniques were developed which significantly improved the estimation of the light/temperature regime. In these tests, microalgae cultures are usually grown in Petri dishes placed on a surface with a temperature and light intensity gradient. This approach can give a number of combinations of temperature and light regimes to be tested at the same time (Lukavský 1982). Still, it is rather a preliminary test which can take days and has the great disadvantage that the strains are grown on agar plates, not in mixed liquid media with a supply of CO_2.

During the growth of microalgae, monitoring of photosynthetic activity changes is advantageous to monitor the culture physiological status (Havlik et al. 2013; Malapascua et al. 2014). Recently, chlorophyll (Chl) fluorescence techniques (quenching and induction curve analyses) have been used in the rapid monitoring of photosynthetic activity to detect various unfavourable effects of environmental stressors on microalgae and to optimise culture growth (Babaei et al. 2017; Maxwell and Johnson 2000; Strasser et al. 2004; Baker 2008; Masojídek et al. 2011a, 2011b; Malapascua et al. 2014). Relative electron transport curves (rETR) measured by Chl fluorescence techniques were used to monitor the dependency of photosynthesis on culturing conditions, mostly irradiance and temperature (e.g. Torzillo et al. 1996, 1998; Kromkamp et al. 1998; Ralph and Gademann 2005; Enríquez and Borowitzka 2010; Malapascua et al. 2014; Benavides et al. 2017). Generally, the trends of rETR correlated well with the culture growth. Another monitoring approach is to examine fast fluorescence induction kinetics characterized by the fast chlorophyll fluorescence induction curve (so-called OJIP curve). While fluorescence quenching analysis gives information on the balance between photosynthetic electron transport and the Calvin-Benson cycle, fast fluorescence induction kinetics provides information on the reduction of the photosynthetic electron transport chain (Malapascua et al. 2014). Measurement of photosynthetic oxygen evolution (POE) provides similar data to that of Chl fluorescence techniques with one important advantage, the possibility to also monitor dark respiration (R) and correlate these to electron transport measurements by Chl fluorescence (Figueroa et al. 2003).

In this work, a fast preliminary test based on photosynthetic measurements (Chl fluorescence and POE) has been correlated with classical growth tests. The experiments showed which photosynthetic variables can be closely related to culture growth. Based on this comparison, the suitable growth regime (temperature) for microalgae strains could be estimated within 1 day.

Materials and methods

Strains

Several microalgae strains were used in laboratory trials. Four green microalgae (*Scenedesmus* MACC-677, *Chlorella* MACC-1 (Algal Culture Collection, Szechényi István University, Mosonmagyárovár, Hungary), *Scenedesmus almeriensis* CCAP 276/24 (University of Alméria, Spain), and *Chlorella vulgaris* R-117 (CCALA 1107, Culture Collection of Autotrophic Organisms, Institute of Botany, Třeboň, Czech Republic)) and four cyanobacteria strains *Cylindrospermum alatosporum* (CCALA 988 Culture Collection of Autotrophic Organisms, Institute of Botany, Třeboň), *Synechocystis* sp. PCC 6803 (Pasteur Collection, Paris), *Nostoc* MACC-612, and *Nostoc* MACC-683 (Mosonmagyaróvár Algal Culture Collection, Szechényi István University, Hungary) were used in laboratory experiments. The cultures were grown in BG-11 medium (Rippka et al. 1979). Further in the text, the strains are abbreviated as *Scenedesmus* 677, *Chlorella* 1, *S. almeriensis*, *Chlorella* R-117, *Cylindrospermum* 988, *Synechocystis* 6803, *Nostoc* 612 and *Nostoc* 683.

Cultivation equipment and conditions

Stock cultures were grown in glass tubes (400 mL) bubbled with air + 1% CO_2 in inorganic BG-11 medium under controlled laboratory conditions: biomass density was between 1 and 2 g/L, temperature was set to 25 °C and light intensity was 80–100 μmol/m^2 photons per s. Established cultures of each strain were diluted with fresh medium to the initial biomass

density of ~ 0.5 g/L DM and transferred into 100-mL glass test tubes (∅30 × 200 mm) which were submerged into a temperature-controlled water bath and exposed to the light intensity of 100 μmol/m^2 photons per s (Fig. 1).

Several cultivation units were used in parallel. The cultures were acclimated to temperatures in the range between 15 and 35 °C using 5° steps under continuous irradiance of 100 μmol/m^2 photons per s for 2 h. At each temperature step, a new batch from the stock culture was prepared. Photosynthetically active radiation (PAR) provided by a panel of high-frequency cool fluorescent tubes with adjustable light intensity (36 W/830 Lumilux, Osram, Germany) was measured at the front surface of the water bath. The photosynthetic performance of all strains at different temperatures was followed.

Then, the suitable cultivation regime was confirmed by a 5-day growth test carried out in triplicate in the temperature range (25–35 °C) close to the optimum under continuous moderate irradiance (100 μmol/m^2 photons per s).

Photosynthesis measurements

Photosynthetic activity of tested cultures was measured using three techniques—saturation pulse analysis of fluorescence quenching to record rapid light-response curves (RLC), fast fluorescence induction kinetics (OJIP), and photosynthetic oxygen evolution and respiration (POE/R) to construct steady-state light-response curves. All records were analysed to determine photosynthesis variables.

Microalgae samples taken from the glass tubes were diluted to 0.2 to 0.3 g/L DM (corresponding to 5 to 7 mg/L Chl) with growth medium, dark adapted for 10 min, and measured *off-situ*. In this way, light re-absorption problems in dense cultures were prevented by dilution and sufficient illumination was available to reduce the plastoquinone pool (closure of PSII reaction centres). Photosynthesis measurements were carried out using standardised procedures at temperatures corresponding to growth values. Data were recorded in triplicate by measuring of three separate samples ($n = 3$).

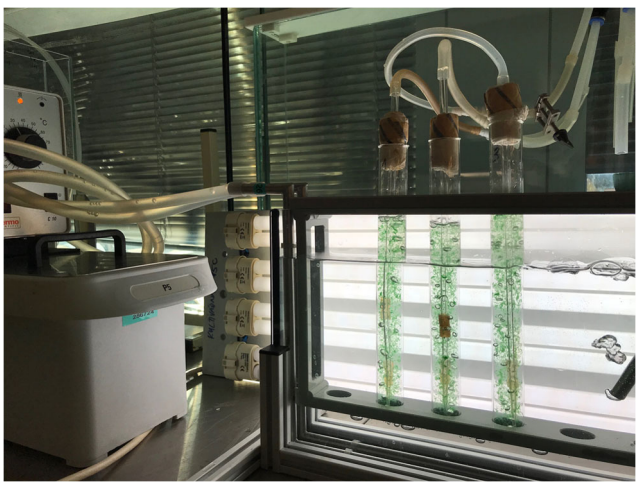

Fig. 1 Laboratory cultivation unit with side illumination panel fitted with high-frequency fluorescent tubes. Glass 100-mL columns with microalgae cultures are submerged in temperature-controlled water bath

Rapid light-response curves

Rapid light-response curves (RLCs) of microalgae samples taken from the cultures were measured in a light-protected measuring chamber with mixing (3-mL glass cuvette with light path of 10 mm) using a pulse-amplitude modulation fluorimeter (PAM-2500, H. Walz, Germany). A series of stepwise increasing irradiance intensities (red LEDs; 0–2700 μmol/m^2 photons per s) were applied in 20-s intervals to obtain the steady-state fluorescence level F' and then a saturating pulse (> 10,000 μmol/m^2 photons per s, 0.6-s duration) was triggered to reach the maximum F_m'. At each step, the actual PSII photochemical quantum yield in the light, Y_{II} was determined as $(F_m' - F')$ F_m' where F_m' is the maximum fluorescence level and F' is the steady-state fluorescence in the light-adapted state at respective irradiance level. Analysis of RLCs was used to estimate changes of the relative electron transport rate through PSII, rETR, which was calculated by multiplication of the actual photochemical efficiency Y_{II} and the photosynthetically active radiation E_{PAR}, $rETR = Y_{II} \times E_{PAR}$ (dimensionless) (e.g. Hofstraat et al. 1994; Ralph and Gademann 2005; White et al. 2011). In order to determine $rETR_{max}$ and the irradiance saturating photosynthesis, light response curves were fitted to the non-linear least-squared regression model by Eilers and Peeters (1998).

The minimum and maximum fluorescence levels (F_0, F_m) were determined using a weak modulated light (< 0.15 μmol/m^2 photons per s, 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). For cyanobacteria, the plastoquinone pool (PQ) is shared by photosynthetic and respiratory electron transport chains. Thus, the "true" F_m has to be determined under low actinic illumination (~ 150 μmol/m^2 photons per s) in the presence of 10^{-5} M herbicide DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) which blocks electron transport behind the PSII complex. In the absence of DCMU, the apparent F_m in dark-adapted culture samples are usually 15–20% lower than the "true" value.

Fast chlorophyll fluorescence induction kinetics

Fast chlorophyll fluorescence induction kinetics (OJIP curves) were measured *off-situ* using a portable fluorimeter (AquaPen

AP-100, P.S.I. Ltd. Brno, Czech Republic). The sample for Chl fluorescence induction kinetics (OJIP curve) was followed in diluted (0.2 to 0.3 g/L DM) sample, dark-adapted for 10 min before measuring. The samples were transferred to a 3-mL measuring cuvette (light path of 10 mm) which was mounted in a light-protected holder in front of the detector (adjustable measuring light pulses, ~2.5 μs) while red LEDs served as high-intensity actinic light from both sides of the cuvette (up to 3000 μmol/m^2 photons per s), perpendicular to the detector. The OJIP curve was measured in the time range between 50 μs and 1 s when the signal rises rapidly from the origin (*O*) to highest peak (*P*) via two inflections—*J* and *I* (Strasser et al. 1995). The *O* point (50 μs) of the fluorescence induction curve represents a minimum value (designated as constant fluorescence yield F_0) when PQ electron acceptors (Q_A and Q_B) of the PSII complex are oxidized. The inflection *J* occurs after ~2–3 ms of illumination and reflects the dynamic equilibrium (quasi-steady state) between Q_A and Q_A^-. The *J*–*I* phase (at 30–50 ms) corresponds to the closure of the remaining centres, and the *I*–*P* (ending at about 300–500 ms) corresponds to full reduction of the plastoquinone pool (equivalent to maximum fluorescence level F_m) (Strasser et al. 2004; Goltsev et al. 2016). From the fluorescence levels at the *J* and *I* points, the variables V_j and V_i were calculated as follows:

$$V_j = (F_{2ms} - F_0)/(F_m - F_0) \text{ and } V_i = (F_{30ms} - F_0)/(F_m - F_0)$$

Measurement of oxygen production and respiration

Photosynthetic oxygen evolution (POE) was measured polarographically using a temperature-controlled chamber with adjustable illumination and mixing connected to temperature-controlled bath and a control unit (Oxygen Monitoring System Oxylab+; Hansatech, UK). Samples taken from experimental cultures were adapted for 10 min in the dark at the desired temperature. Light-response curves were recorded using a stepwise increasing light intensity of 0, 200, 400, 600, 1200, and 1800 μmol/m^2 photons per s where each step was adjusted to 2 min. The first step (2 min in the dark) provided the value of the dark respiration. Oxygen production and respiration were calculated in micromole of O_2 per milligramme (Chl) per hour.

Analytical procedures

Biomass was measured as dry mass (DM) by filtering 5 mL of culture samples on pre-weighed glass microfiber filters (GC-50). The pre-weighted filters with the cells were washed twice with deionized water, dried in an oven at 105 °C for 8 h, then transferred to a desiccator to equilibrate to laboratory temperature and weighed (precision of ± 0.01 mg). The growth rate $\mu = (\ln X_2 - \ln X_1) \quad \Delta t$ (1/d) of microalgae cultures was calculated over the whole period of 5-day growth trial to confirm the optimal temperature obtained from measuring changes in the photosynthetic apparatus.

Chlorophyll concentration was determined spectrophotometrically in methanol extracts. Samples of 500 μL were collected in 2-mL Eppendorf tubes and centrifuged at 13,000 rpm for 3 min (centrifuge Minispin, Eppendorf). The pellet was resuspended in 500 μL of 100% methanol, 100 μL of sea sand was added, and the tubes were put into a laboratory ultrasound bath (Kraintek 6) for 2 min, then cooled down in an ice bath and centrifuged at 10,000 rpm for 1 min. If necessary, the extraction was repeated several times until the pellet was colourless. The absorbance of the combined supernatants from all extraction steps was measured at 665 and 750 nm using a high-resolution spectrophotometer (UV 2600 UV-VIS, Shimadzu, Japan, slit width of 0.5 nm) and the concentration of chlorophyll was calculated according to Wellburn (1994).

Irradiance measurements

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

Statistical analysis

Sigma Plot 11.0 was used to determine significant differences between treatments. One-way ANOVA and the Holm-Sidak test were conducted for every binary combination of data. *P* values lower than 0.05 were considered significantly different.

Results

The laboratory experiments were carried out in order to find suitable culturing conditions (temperature) of several microalgae strains using fluorescence measuring techniques. At each temperature step, a new batch from the stock culture was taken and acclimated for 2 h to the irradiance of 100 μmol/m^2 photons per s. Then, photosynthetic activity was measured as POE/R, RLC, and OJIP kinetics. In the following series of trials, growth tests were carried out where microalgae were cultured for 5 days at different temperatures (25, 30, and 35 °C).

The maximum photochemical yield of PSII, F_v/F_m (measured in the presence of DCMU) was recorded in the range from 15 to 35 °C in cultures of cyanobacteria strains *Nostoc* 612, *Nostoc* 683, *Cylindrospermum* 988, and *Synechocystis* 6803. The F_v/F_m values ranged from 0.17 at low temperatures

up to 0.63 at favourable temperature (Fig. 2a). The highest value (0.63) was observed in the *Nostoc* 612 culture while all the other strains showed lower maxima between 0.31 and 0.38. In all cyanobacteria strains, the maximum values of F_v/F_m were measured when the temperature was between 26.7 and 28.3 °C. It is important to note that the F_v/F_m values increased from low up to optimum temperature and then decreased as the condition became unfavourable.

In the case of eukaryotic microalgae *Scenedesmus* 677, *Chlorella* 1, *S. almeriensis*, and *Chlorella* R-117, the course of F_v/F_m was different. Only *Scenedesmus* 677 showed increasing fluorescence values from low temperature up to the maxima at temperature 25 °C and then started to decrease (Fig. 2b). In all other cultures, little variation (near-flat curves) was found in the temperature dependence curves of F_v/F_m suggesting a broad temperature range for activity. Similar results were found in *S. almeriensis* where the curves of temperature dependence for F_v/F_m of about 0.7 were found between 10 and 40 °C (Sánchez et al. 2008). Thus, measurements of other photosynthetic variables were necessary to clarify the question of temperature dependence.

Light-response curves of relative electron transport rate (rETR) were recorded using saturating pulse analysis of fluorescence quenching to evaluate photosynthetic activity of microalgae cultures. The rETR–temperature dependence curve maximum values were calculated for all microalgae strains. Among cyanobacteria, the highest $rETR_{max}$ value of about 140 was found in *Nostoc* 612 at 30 °C, the other cyanobacteria strains showed much lower activities (Fig. 3a). These results reflected the F_v/F_m values measured for cyanobacteria strains (Fig. 2a). For eukaryotic microalgae, the highest $rETR_{max}$, 1350, was found in *S. almeriensis* at 25 °C, which was still about one order of magnitude higher than that in *Nostoc* 612 (Fig. 3b). The $rETR_{max}$ value

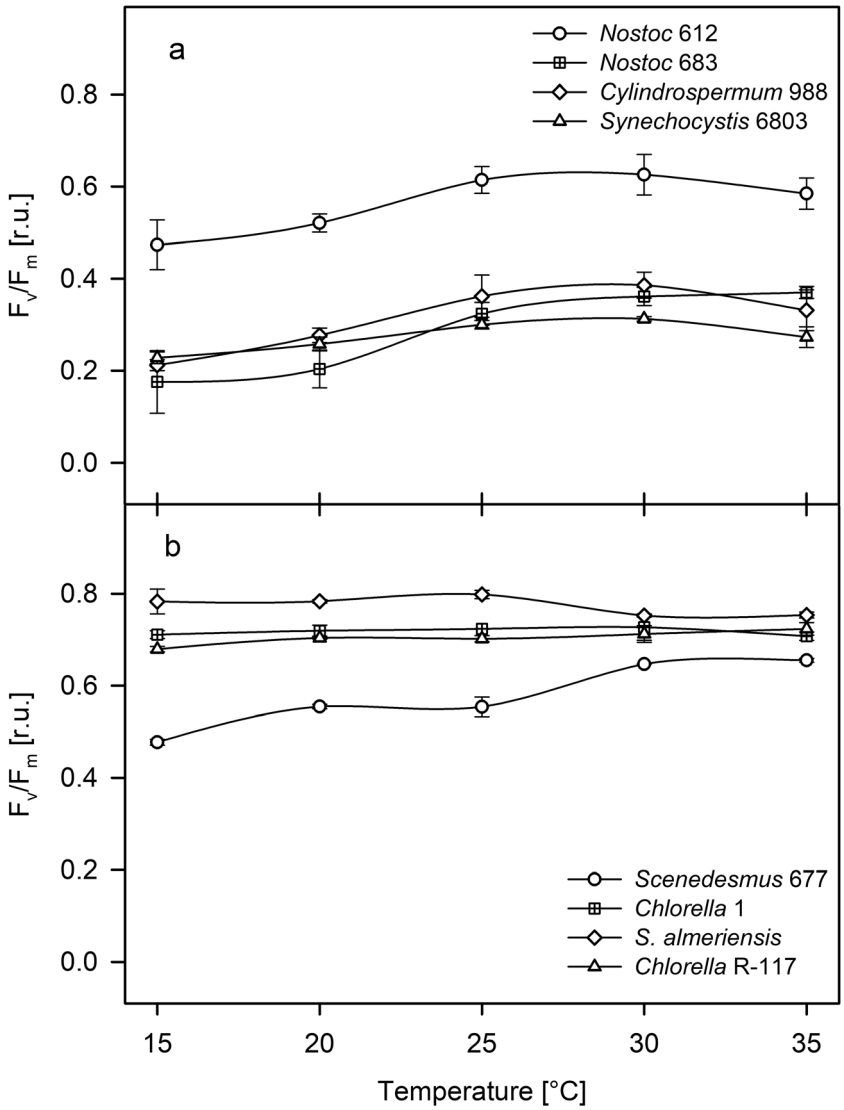

Fig. 2 Changes of the maximum photochemical quantum yield of PSII (F_v/F_m) as a function of temperature in the range of 15–35 °C for selected microalgae cultures. (a) *Nostoc* 612, *Nostoc* 683, *Cylindrospermum* 988, and *Synechocystis* 6803. (b) *Scenedesmus* 677, *Chlorella* 1, *S. almeriensis*, and *Chlorella* R-117. Values are presented as a mean ($n = 3$) with SD indicated by error bars

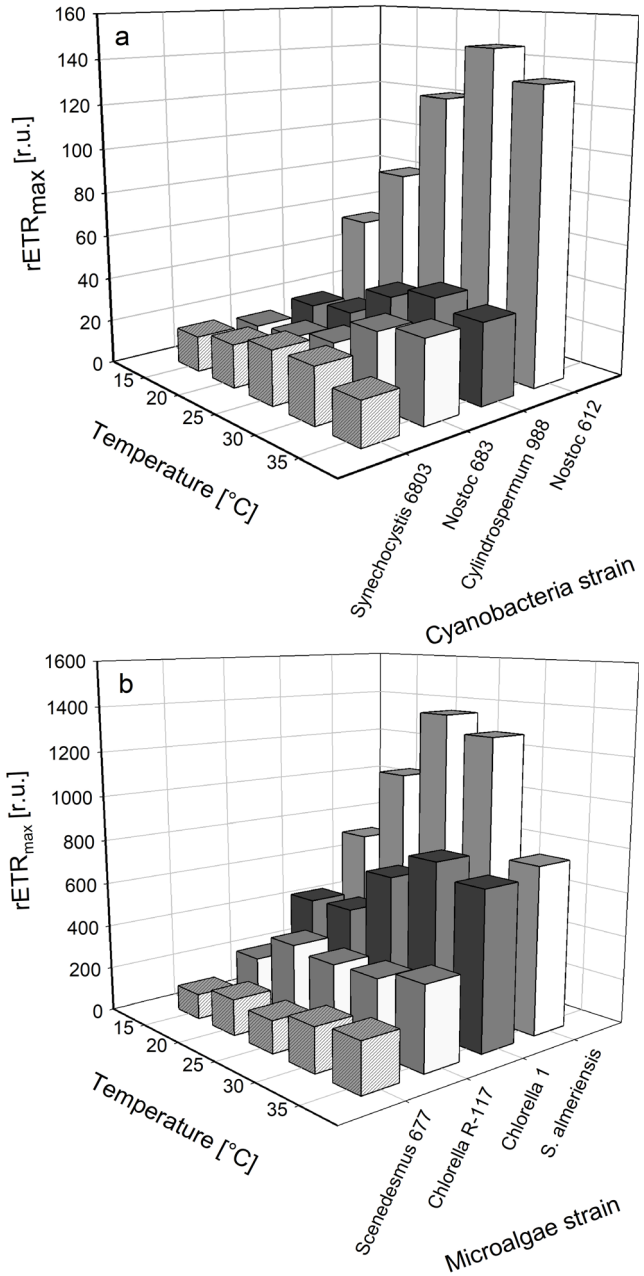

Fig. 3 Changes of the maximum electron transport rate $rETR_{max}$ (calculated from RLCs) as a function of temperature measured at 15, 20, 25, 30, and 35 °C for selected microalgae cultures. (a) *Nostoc* 612, *Nostoc* 683, *Cylindrospermum* 988, and *Synechocystis* 6803. (b) *Scenedesmus* 677, *Chlorella* 1, *S. almeriensis*, and *Chlorella* R-117

measured in the *Chlorella* 1 culture was much lower, about 770 (at 30 °C). In *Chlorella* R-117 and *Scenedesmus* 677 cultures, the $rETR_{max}$ values were lower, 375 and 224, respectively.

Fast fluorescence induction kinetics, the so-called OJIP curves indicating electron transport steps through the PSII complex, show the two inflection points *J* and *I*. These are represented by the V_j and V_i variables which indicate the redox status of the acceptor side of the PSII complex (downregulation of photosynthetic electron transport). If increased, V_j and V_i indicated a slowdown of electron transport due to overreduction of the plastoquinone pool. When the V_j and V_i variables were evaluated for all strains, the minimum values indicated the most favourable conditions (Fig. 4). The V_j and V_i variables were higher for cyanobacteria (0.6–0.9) as compared with V_j (0.3–0.4) and V_i (0.5–0.7) measured in eukaryotic microalgae. For *Nostoc* 612 and *Synechocystis* 6803, the lowest values indicating the favourable conditions were found in the range between 25 and 30 °C (Fig. 4a, d) while for *Cylindrospermum* 988 and *Nostoc* 683, the favourable conditions were achieved at 30 and 35 °C (Fig. 4b, c). The lowest value of both variables was measured when temperatures of 30 and 35 °C was set (Fig. 4e–h). The maxima of photosynthetic oxygen evolution (POE) and respiration were achieved under the conditions which can be considered suitable for photosynthetic oxygen production activity of individual microalgae strains. The highest activity was achieved in the range between 28.7 and 33.3 °C (Table 1).

In the second series of experiments, the microalgae cultures were grown in laboratory experiments at 25, 30, and 35 °C for 5 days to verify the temperature optima estimated from photosynthetic activity measurements (Fig. 5). The optimum temperature was estimated in the range between 30 and 35 °C for most of tested strains (*Nostoc* 612, *Nostoc* 683, *Cylindrospermum* 988, *Synechocystis* 6803, *Scenedesmus* 677, and *Chlorella* 1). Only for *S. almeriensis* and *Chlorella* R-117 the optimum growth temperature was 25 °C.

The growth rates μ of all microalgae strains were calculated over the whole period. The highest growth rate, 0.48 1/d, was found for *Nostoc* 612, followed by *Cylindrospermum* 988 at 0.33 1/d, *Chlorella* 1 at 0.28 1/d, *Scenedesmus* 677 at 0.27 1/d, and *Synechocystis* 6803 at 0.23 1/d when they were grown at 30 °C. Two strains—*S. almeriensis* and *Chlorella* R-117—showed the highest growth rates (0.25 and 0.29 1/d, respectively) at 25 °C. For *Nostoc* 683, the highest rate (0.28 1/d) was found at 35 °C.

Finally, the data from all measurements—maximum photochemical yield of PSII, F_v/F_m, $rETR_{max}$, fluorescence induction transients V_j/V_i, and POE/R—were summarised for each microalgae strain and averaged (Table 1). The photosynthesis optima for the examined strains were found in the range between 27.5 and 33.3 °C. We have not found any remarkable discrepancies between photosynthesis and growth optima, i.e. the averaged optima obtained from measurements of photosynthetic activity correlated with the regular growth tests for most of the strains. Only one exception was *Chlorella* R-117 where the mean temperature obtained from the measurement of photosynthetic activity was found to be 33.3 °C and the growth optimum obtained from regular cultivation test was found to be 25 °C. It is important to mention that *Chlorella* R-117 is used as a fast-growing

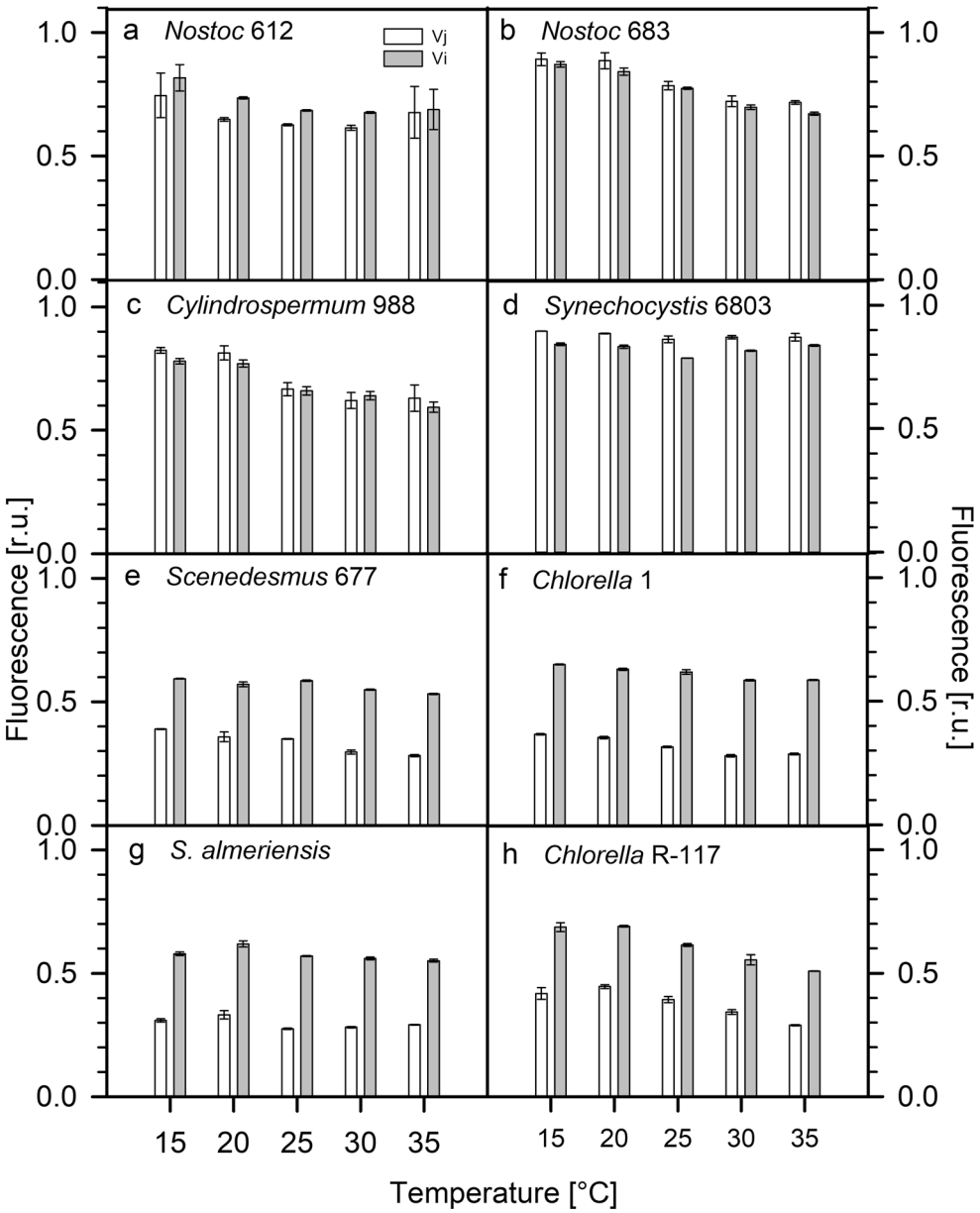

Fig. 4 Changes of the V_j and V_i variables (calculated from the curves of fast fluorescence induction kinetics) as a function of temperature at 15, 20, 25, 30, and 35 °C for selected microalgae cultures. (a) *Nostoc* 612, (b) *Nostoc* 683, (c) *Cylindrospermum* 988, (d) *Synechocystis* 6803, (e) *Scenedesmus* 677, (f) *Chlorella* 1, (g) *S. almeriensis*, and (h) *Chlorella* R-117. Values are presented as a mean ($n = 3$) with SD indicated by error bars

Table 1 Temperature optima for individual microalgae strains were estimated from measurements of the maximum photochemical yield of PSII, F_v/F_m, relative electron transport rate $rETR_{max}$, fluorescence induction transients V_j/V_i, and photosynthetic oxygen evolution and respiration POE/R. The temperature optima data for all variables—F_v/F_m, $rETR_{max}$, V_j/V_i, and POE/R—were then averaged (mean). The growth optima were verified in a 5-day cultivation trial. Values are presented as a mean ($n = 3$) with SD. The same letters mean that the values do not differ significantly from each other

Variable Temperature optima	F_v/F_m [°C]	$rETR_{max}$ [°C]	V_j, V_i [°C]	POE/R [°C]	Mean [°C]	Growth optimum [°C]
Nostoc 612	28.3 ± 2.9	30.0 ± 0	28.3 ± 2.9	28.3 ± 2.9	28.7 ± 2.2^{a,b}	30
Nostoc 683	28.3 ± 2.9	33.3 ± 2.9	33.3 ± 2.9	35.0 ± 0	32.5 ± 2.2^{a,b}	35
Cylindrospermum 988	28.3 ± 2.9	28.3 ± 2.9	33.3 ± 2.9	33.3 ± 2.9	30.8 ± 2.9^{a,b}	30
Synechocystis 6803	26.7 ± 2.9	28.3 ± 2.9	26.7 ± 2.9	31.7 ± 2.9	28.4 ± 2.9^{a,b}	30
Scenedesmus 677	33.3 ± 2.9	33.3 ± 2.9	33.3 ± 2.9	33.3 ± 2.9	33.3 ± 2.9^{a,b}	30
Chlorella 1	33.3 ± 2.9	35.0 ± 0	33.3 ± 2.9	31.7 ± 2.9	33.3 ± 2.2^{a,b}	30
S. almeriensis	25.0 ± 0	26.7 ± 2.9	26.7 ± 2.9	31.7 ± 2.9	27.5 ± 2.2^{a,b}	25
Chlorella R-117	31.7 ± 2.9	35.0 ± 0	33.3 ± 2.9	33.3 ± 2.9	33.3 ± 2.2^{a,b}	25

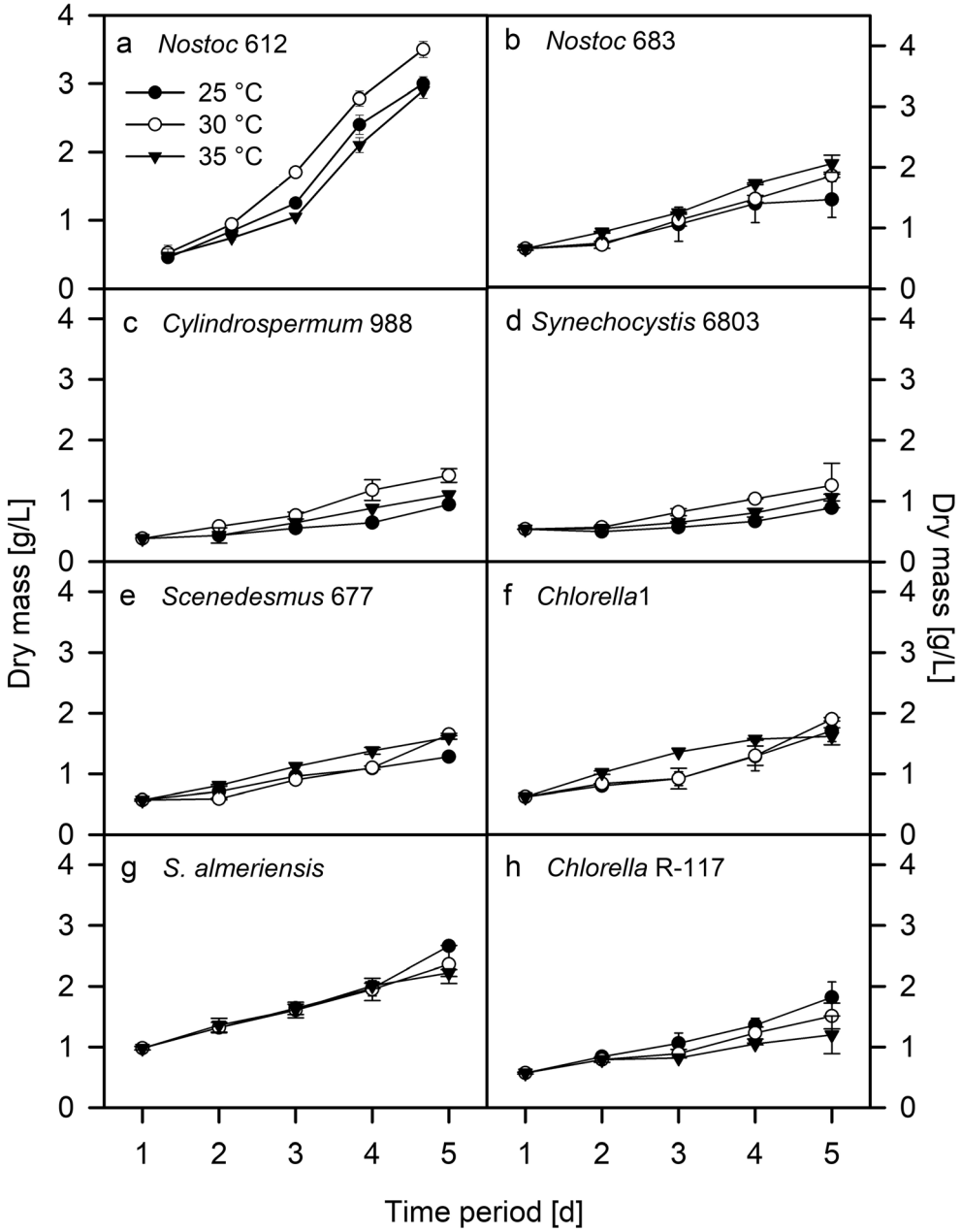

Fig. 5 Growth curves of selected microalgae strains cultivated for 5 days at 25, 30, and 35 °C at the light intensity of 100 μmol/m^2 photon per s. (a) *Nostoc* 612, (b) *Nostoc* 683, (c) *Cylindrospermum* 988, (d) *Synechocystis* 6803, (e) *Scenedesmus* 677, (f) *Chlorella* 1, (g) *S. almeriensis*, and (h) *Chlorella* R-117. Values are presented as a mean ($n = 3$) with SD indicated by error bars

production strain in outdoor large-scale cultivation cascades where the temperatures can be 15 °C in the morning while at midday these reach up to 35 °C. We can speculate that such temperature adaptability can influence the verity of correlation if carried out in laboratory experiments.

Discussion

Our results suggest that rapid measurement of photosynthetic activity can provide a first estimate of optimum growth temperature. In this way, we can quickly evaluate the growth regime for a specific strain using measurements of photosynthetic activity which have been confirmed by regular growth tests and correlate with the growth. For most of the examined strains considered both photosynthetic activity and growth rate tests, the most favourable temperature of 30 °C was found. The optimum temperature may vary depending on the cultivation unit used for trial, especially on the diameter and hence the light path (microalgae can adapt to higher light intensity and higher temperature over time). Due to the variety of cultivation vessel availability, the correlation between photosynthetic activity and growth rate has to be done for each cultivation unit separately.

In our case, we started with a biomass density of ~ 0.5 g/L DM and a light intensity of 100 μmol/m^2 photons per s (d = 3 cm). The optimum growing temperature for *S. almeriensis* was determined to be 25 °C when it was cultivated in 100-mL glass tubes (∅30 × 200 mm). When it was cultivated in a 2-L column with diameter of 60 cm, the light intensity could be increased to 650 umol/m^2 photons per s and the highest growth rate was observed at 35 °C (Sánchez et al. 2008). The microalgae *S. obliquus* was cultured in flasks (LI = 60 μmol/m^2 photons per s) and has a temperature optimum of 31–32 °C, temperatures up to 45 °C did not cause cell death (Hanagata et al. 1992). When we compared the conditions of our experiments and the data found for *S. almeriensis* culture with the data published (Sánchez et al. 2008; Costache et al. 2013), we found that their maxima for biomass productivity was achieved at the temperature of 35 °C at high irradiance (1625 μmol/m^2 photons per s) while at 650 μmol/m^2 photons per s, the highest biomass productivity was found at the temperature of 20 °C. This data suggests that high irradiance plays crucial role in determination of temperature optima. In our experiments, we used a much lower irradiance and the glass tube with the diameter of 30 mm. This might be the source for discrepancy between both data sets.

Concerning the cyanobacteria *Synechocystis* sp., a similar discrepancy was observed where the growth rate was dependent on the light intensity (and thus on the light path in the cultivation unit) as noted by Martínez et al. 2011. In the study of CO_2 biofixation, the experiment was performed in a 1-L column photobioreactor with the diameter of 8 cm. The highest CO_2 biofixation and highest growth were found at the temperature of 35 °C. According to Dauta et al. (1990), the highest growth of *Synechocystis minima* is achieved when the temperature is set to 32 °C.

If we compare a mean activity maximum for all four photosynthetic variables—F_v/F_m, $rETR_{max}$, inflections V_j/V_i, and POE/R for individual strains, these as well as the maxima of growth rate, we cannot see any significant differences ($P < 0.05$) (Table 1). It is important to note that *Chlorella* R-117 is used as a fast-growing production strain in outdoor large-scale cultivation cascades where the temperature can reach up to 35 °C at midday and the culture can adapt to the set conditions without large differences in the growth rates (Converti et al. 2009; Dauta et al. 1990).

The F_v/F_m value is not varying under favourable conditions, but decreases under stress as it reflects a reduction of PSII activity. Temperature regime has been shown to influence not only F_v/F_m but also the oxygen-evolving activity (Bayro-Kaiser and Nelson 2016; Sharkey and Zhang 2010; Valledor et al. 2013). In our experiments, the temperature at which the maximum of F_v/F_m was achieved well correlated with the high growth rate. The same trend was observed when the microalgae strains were characterised by fast fluorescence induction kinetics. The temperature at which the lowest value of V_j and V_i was achieved correlated with the temperature at which the growth rate of microalgae was fastest. For POE/R, these measurements are more sensitive and laborious and take longer time to perform when compared with the much faster fluorescence measurements. We have not found any significant differences ($P < 0.05$) between the optimum temperature obtained from these measurements and the optimum temperature obtained from regular growth tests.

Conclusions

The photosynthetic performance of eight microalgae strains (*Nostoc* 612, *Nostoc* 683, *Cylindrospermum 988*, *Synechocystis* 6803, *Scenedesmus* 677, *Chlorella* 1, *S. almeriensis*, and *Chlorella* R-117) was studied in laboratory experiments through a range of temperatures from 15 to 35 °C. We aimed to develop a fast preliminary test based on photosynthetic measurements (Chl fluorescence and POE/R) in order to estimate a suitable temperature range for growth. Photosynthetic variables—the maximum photochemical yield of PSII, F_v/F_m, $rETR_{max}$, the inflection points V_j/V_i—were chosen to be directly related to the growth. Then, the estimated temperature range was verified in 5-day growth tests, within the results showing rather good correlation without any significant differences ($P < 0.05$). For most of the examined microalgae, a temperature around 30 °C was considered most favourable. Generally, the described procedure can be used as a rapid 1-day pre-screening test to determine suitable growth regime of examined microalgae. Nevertheless, the growth regime in a particular cultivation unit has to be adjusted to the individual microalgae strain.

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Author's contribution Karolína Ranglová, Gergely Lakatos, João Artur Câmara Manoel and Tomáš Grivalský carried out joint experiments, evaluated data and took a part in the preparation of the manuscript. Karolína Ranglová prepared the manuscript text and figures. Jiří Masojídek revised and finalized the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Annex II

(related to Chapter 2 Microalgae Monitoring and Maintenance)

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ORIGINAL ARTICLE Open Access

The synergistic effect of Selenium (selenite, $-SeO_3^{2-}$) dose and irradiance intensity in *Chlorella* cultures

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Abstract

Microalgae are able to metabolize inorganic selenium (Se) to organic forms (e.g. Se-proteins); nevertheless at certain Se concentration culture growth is inhibited. The aim of this work was to confirm the hypothesis that the limit of Se tolerance in *Chlorella* cultures is related to photosynthetic performance, i.e. depends on light intensity. We studied the relation between the dose and irradiance to find the range of Se tolerance in laboratory and outdoor cultures. At low irradiance (250 µmol photons m^{-2} s^{-1}), the daily dose of Se below 8.5 mg per g of biomass (<20 µM) partially stimulated the photosynthetic activity (relative electron transport rate) and growth of *Chlorella* cultures (biomass density of ~1.5 g DW L^{-1}) compared to the control (no Se added). It was accompanied by substantial Se incorporation to microalgae biomass (~0.5 mg Se g^{-1} DW). When the Se daily dose and level of irradiance were doubled (16 mg Se g^{-1} DW; 500 µmol photons m^{-2} s^{-1}), the photosynthetic activity and growth were stimulated for several days and ample incorporation of Se to biomass (7.1 mg g^{-1} DW) was observed. Yet, the same Se daily dose under increased irradiance (750 µmol photons m^{-2} s^{-1}) caused the synergistic effect manifested by significant inhibition of photosynthesis, growth and lowered Se incorporation to biomass. In the present experiments Chl fluorescence techniques were used to monitor photosynthetic activity for determination of optimal Se doses in order to achieve efficient incorporation without substantial inhibition of microalgae growth when producing Se-enriched biomass.

Keywords: *Chlorella*, Chlorophyll fluorescence, Growth, Irradiance intensity, Photosynthesis, Selenium incorporation

Introduction

Selenium (Se) is a natural trace element that may alternate from an essential micro-nutrient to a toxic compound within a narrow concentration level. Microalgae are able to metabolize inorganic selenium (Se) salts to organic forms as a part of detoxification process, but at certain concentration culture growth becomes inhibited. At nanomolar concentrations Se salts can be beneficial for growth of some microalgae (Li et al. 2003), but further up, at micromolar concentrations may result in a decreased growth rate, photosynthesis inhibition and disturbance to the cell ultrastructure (Morlon et al. 2005; Geoffroy et al. 2007; Fournier et al. 2010; Vítová et al. 2011; Gojkovic et al. 2015). Typical Se content in freshwaters is found in the range of 0.1–2 nM (equivalent to 0.08–0.16 µg Se L^{-1}), but much higher concentrations reaching 5 µM (equivalent to 400 µg Se L^{-1}) have been observed in contaminated areas (Conde and Sanz Alaejos 1997; Sun et al. 2014). Both selenite (Se^{IV}, $-SeO_4^{2-}$) and selenate (Se^{VI}, $-SeO_3^{2-}$), two major inorganic forms of Se, are toxic to microalgae at higher concentrations (Geoffroy et al. 2007; Morlon et al. 2005) although selenite was found to be more lethal than selenite (Wheeler et al. 1982). These salts are readily incorporated by microalgae, though selenate is accumulated about ten times more efficiently than selenate due to different membrane transporters (Vriens et al. 2016). For nutritional purposes, Se is often supplied in the form of inorganic salts (selenite, selenate), but organically bound selenium in microalgae biomass (Se-proteins) is more beneficial and less toxic to humans and animals and can be used as food and feed

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supplement (Becker 2007; Doucha et al. 2009; Gojkovic et al. 2015; Kouba et al. 2014; Novoselov et al. 2002).

Microalgae play a crucial role in Se-metabolism due the uptake and biotransformation, and the subsequent transfer upwards into the food chain. Basically, three mechanisms are associated with Se removal by microalgae: (i) biosorption of Se ions on the cell surface, (ii) intracellular uptake of Se ions, and (iii) chemical transformation of Se ions (Neumann et al. 2003; Pardo et al. 2003; Pelah and Ephraim 2005; Oh et al. 2009; Umysová et al. 2009; Gojkovic et al. 2014, 2015). The effects of Se, and its metabolism and bioaccumulation have recently been studied in various groups of microalgae including *Spirulina* (Li et al. 2003; Mane et al. 2013; Pronina et al. 2002), *Scenedesmus* (Umysová et al. 2009), *Chlorella* (Sun et al. 2014; Gojkovic et al. 2014) or *Chlamydomonas* (Geoffroy et al. 2007; Morlon et al. 2005). At low concentration levels, up to 10 mg Se L^{-1}, i.e. ~12 μM, cell growth and functioning are not inhibited (Umysová et al. 2009) and microalgae are able to incorporate and transform Se salts to bioavailable organic compounds—proteins containing selenated amino acids, replacing sulphur in methionine and cysteine (for review see ref. Araie and Shiraiwa 2016). Some species also metabolize Se to volatile compounds—dimethylselenide, dimethyldiselenide and dimethylselenenylsulfide (Fan et al. 1997; Larsen et al. 2001; Neumann et al. 2003; Guadayol et al. 2016). Adverse effects of Se on microalgal cultures are usually observed as decrease of population densities and/or growth rates, in proportion to Se concentration in the culture medium. Toxicity to microalgae culture is usually reflected as the corresponding EC50 value. For example, the EC50 values for selenite were found 80 μM in *Chlamydomonas* cultures (Morlon et al. 2005), and 418 μM in *Scenedesmus* (Vítová et al. 2011). At higher doses (above tens of micromoles), Se accumulation may result in inhibited photosynthesis, decreased growth rate, and changes of the cell ultrastructure (Geoffroy et al. 2007; Morlon et al. 2005; Vítová et al. 2011). The uptake of Se in excess to requirements may cause metabolic reactions, possibly some photo-oxidative damage (Chen et al. 2005) that can eventually lead to cell death (Schiavon et al. 2012; Sun et al. 2014).

Fast and sensitive monitoring techniques are employed to detect early stress effects in microalgae cultures. These can be promptly assessed as changes of the photosystem II (PSII) photochemical performance that is reliably determined by chlorophyll (Chl) fluorescence, a sensitive and easy-to-use technique. Recently, we have used fast fluorescence induction kinetics and rapid light-response curves of electron transport activity for detection of various stressors in microalgae cultures (Masojídek et al. 2011a; Malapascua et al. 2014). These techniques reflect the absorption, distribution and utilization efficiency of energy by cells and can indicate Se effect on photosynthetic activity in microalgae cultures (e.g. Geoffroy et al. 2007; Gojkovic et al. 2015).

In this work, we aimed to study the synergistic effect of Se (selenite, SeO_3^{2-}) doses and irradiance intensity on *Chlorella vulgaris* cultures. Changes of photosynthetic performance (measured as Chl fluorescence variables) were investigated in detail in correlation with growth, pigment composition and Se incorporation to biomass. Here, photosynthesis measurements were used in order to control microalgae activity during Se treatment and incorporation. The experiments at various irradiance intensities showed that the rate and extent of Se uptake depends on photosynthetic performance of the culture. In this way suitable doses of Se can be estimated for mass cultivation to produce a bulk of Se-enriched biomass for nutritional purposes.

Materials and methods

Organism

The fast-growing microalga *Chlorella vulgaris*, strain *R117* (*CCALA 10258,* Culture Collection of Autotrophic Organisms, Institute of Botany, Třeboň, Czech Republic) was cultivated in a modified inorganic medium (Šetlík et al. 1972; Zachleder and Šetlík 1982), containing the following compounds (in mg L^{-1}): KNO_3, 2021; KH_2PO_4, 340; $MgSO_4{\cdot}7H_2O$, 989; ferric-sodium chelatonate, 18.4; $CaCl_2$, 16; H_3BO_3, 1.9; $MnCl_2{\cdot}4H_2O$, 7.3; $ZnSO_4{\cdot}7H_2O$, 7; $CuSO_4{\cdot}5H_2O$, 2; $CoSO_4{\cdot}7H_2O$, 1.4; $(NH_4)_6Mo_7O_{24}$, 0.08; and NH_4VO_3, 0.06, pH 7.4.

The cultures were grown in glass columns (working volume 330 mL; internal diameter 35 mm) that were submerged in temperature-controlled water bath (28–29 °C) and mixed by bubbling with air +1.5% CO_2 (v/v) (Fig. 1). The batch cultures were exposed to continuous illumination between for 4 days. Photosynthetically active radiation (PAR) provided by a horizontal panel of high frequency cool fluorescent tubes (36 W/830 Lumilux, Osram, Germany) was measured directly inside an empty cultivation column to adjust light intensity using a quantum sensor (LI-190SA, cosine-corrected up to 80° angle of incidence) coupled to a light meter (LI-250, Li-Cor, USA).

Outdoor pilot experiment was carried out under ambient irradiance light in thin-layer cascades (area of 220 m^2 with total cultivation volume of 2200 L) for 7 days in June 2015. More detailed description of the outdoor units and cultivation regime is shown elsewhere (Masojídek et al. 2011b).

Growth and selenium treatments

Optical density (OD_{750}) of the culture was measured at 750 nm using a spectrophotometer as it is approximately

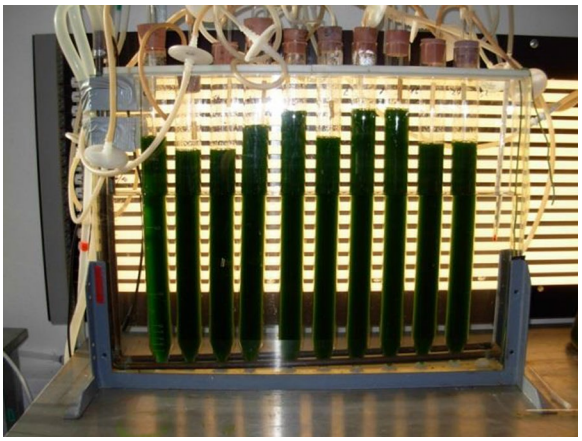

Fig. 1 Laboratory cultivation of *Chlorella* in 400 mL vertical columns (mixed by bubbling of air +CO_2) that were placed in temperature controlled bath with back illumination by high-frequency fluorescent tubes

linearly related to biomass density (DW). Samples of microalgae cultures were diluted accordingly prior to measurement to keep the absorbance in a linear range. Biomass density was assayed as dry weight (DW) by filtering 5 mL of culture samples on pre-weighed fiberglass filters (GC-50). Then, these were dried in an oven at 105 °C for 3 h, transferred to a desiccator to equilibrate to laboratory temperature and weighed. In all laboratory experiments, well-growing cultures of *Chlorella* were suspended in the fresh medium to the initial biomass density of about 1.5 g/L.

The robust strain R117 of *Chlorella* was selected as model microalga in this work as in preliminary trials it showed tolerance to high doses of selenite (not shown here). In order to select appropriate Se dose in our experiments we have considered the tested concentration range of Se salts in cultivation media that was usually between 0.1 and 80 mg L^{-1} (1–500 µM) (e.g. Bennett 1988; Morlon et al. 2005; Geoffroy et al. 2007; Umysová et al. 2009; Fournier et al. 2010; Gojkovic et al. 2014, 2015).

In one series of experiments, Se was added as a stock solution of sodium selenite (Na_2SeO_3) once a day to each cultivation column (250 µmol photons m^{-2} s^{-1}) at various concentration (2.5, 8.5 and 25 mg Se per g of starting biomass density; representing final concentrations of about 19, 65 and 190 µmol L^{-1}, respectively) to determine effective concentration. In the other series of experiments, various light intensities (250, 500 and 750 µmol photons m^{-2} s^{-1}, respectively) were used to study the irradiance-dependent effect of Se treatment (16 mg Se g^{-1} biomass added twice a day as Na_2SeO_3; final concentration of about 122 µmol L^{-1}). All Se treatments were performed in duplicates. Control cultures were grown in the absence of Se. Distilled water was added to columns before each sampling to compensate volume lost by evaporation.

In outdoor trial Se was added to *Chlorella* cultures in the cascade units in the form of Na_2SeO_3 twice a day (at 8 a.m. and 1 p.m.) to keep its concentration of about 20 µmol L^{-1}.

Analysis of selenium content

For Se analysis culture samples (50 mL) were centrifuged and sediments of cells were freeze-dried before analysis. Biomass was mineralized in the microwave decomposition equipment (Milestone 1200 Mega) using nitric acid and hydrogen peroxide. The total amount of Se in biomass was determined using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700×) by a commercial company (Healthcare Institute Ltd., Ústí nad Labem, Czech Republic).

Chlorophyll fluorescence measurements

For Chl fluorescence measurements, samples were taken from the cultures and measured in triplicates (data presented as mean ± SE) at specified daytimes. Before measurement, the cultures were diluted to 0.2–0.3 g DW L^{-1} (corresponding to 5–7 mg Chl L^{-1}) with distilled water, dark adapted for 5–10 min and then transferred to a measuring chamber. In this way, we prevented re-absorption problems in a dense culture and provided sufficient illumination in the dark-acclimated samples (with an oxidized plastoquinone pool). Measurements were carried out under well-defined laboratory conditions and 'light' exposure history to avoid modifying the photo acclimation state of the cells.

Two Chl fluorescence techniques were used for culture monitoring. While rapid fluorescence induction kinetics provides information on the reduction of the photosynthetic electron transport chain, fluorescence quenching analysis gives information on the balance between photosynthetic electron transport and Calvin-Benson cycle (Malapascua et al. 2014). The fluorescence nomenclature in this paper follows Schreiber et al. (1986) as later elaborated by Kooten and Snel (1990) and Kromkamp and Foster (2003).

Rapid light-response curves

Rapid light-response curves (RLCs) of microalgae samples were measured using a pulse-amplitude-modulation fluorometer (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; 0-2000 µmol photons m^{-2} s^{-1}) were applied in 20 s intervals to obtain the 'steady-state' fluorescence level (F′) and then

a saturating pulse (>10,000 µmol photons m^{-2} s^{-1}, 0.6 s duration) was triggered to reach the maximum fluorescence level (F_m'). At each irradiance intensity, the actual (or effective) photosystem II (PSII) photochemical quantum yield in the light-adapted state, designated as Y_{II} was calculated as $(F_m'–F')/F_m'$; it represents actual operational ability of photosynthesizing cells to convert light power into chemical energy. The so called relative electron transport rate through PSII, rETR (dimensionless) was calculated as multiplication of Y_{II} by the photosynthetically active radiation E_{PAR}, $rETR = Y_{II} \times E_{PAR}$ and plotted against E_{PAR} (e.g. Hofstraat et al. 1994; Ralph and Gademann 2005; White et al. 2011). RLCs for ETR were constructed and analyzed to determine important variables. In order to estimate maximum value, $rETR_{max}$, and the value of irradiance saturating photosynthesis, or onset of light saturation (E_k), the rETR vs. irradiance curves were fitted to the non-linear least-squares regression model by Eilers and Peeters (1988) (PAM Win–3 Software provided by H. Walz), The minimum and maximum fluorescence levels (F_o, F_m) were determined using a weak modulated light (<0.15 µmol photons m^{-2} s^{-1}, frequency of 0.5–1 kHz) in the dark adapted samples (1st step of RLC; actinic irradiance = 0). The maximum PSII quantum yield was calculated as the ratio of variable to maximum fluorescence, $Fv/Fm = (F_m–F_o)/F_m$.

Fast fluorescence induction kinetics (OJIP-test)

A hand-held fluorometer (AquaPen AP-100, produced by P.S.I. Ltd, Brno, Czech Republic) adapted for liquid samples was used to follow rapid fluorescence induction kinetics (~1 s) in microalgae cultures. Dark-adapted samples (5–10 min) were applied to a 3 mL measuring cuvette (light path of 10 mm) that was mounted in a light-protected holder in front of the detector (adjustable measuring light pulses, ~2.5 µs) while illuminating red LEDs served as high-intensity continuous light source from both sides of the cuvette (up to 3000 µmol photon m^{-2} s^{-1}), perpendicular to the fluorescence detector. The fast fluorescence induction kinetics was measured in the time range between 50 µs to 1 s; it started upon illumination (saturating continuous light) of dark-adapted microalgae culture as the signal rises rapidly from the origin (O) to a peak (P) via two infections—J (Vj) and I (Vi) (Strasser and Srivastava 1995; Goltsev et al. 2016). The O point of the fluorescence induction curve is a minimum value (designated as constant fluorescence yield F_o at 50 µs) when plastoquinone (PQ) electron acceptors (Q_A and Q_B) of the PSII complex are fully oxidized. It represents the signal emitted from excited Chl-a molecules in the light-harvesting complex II before excitons have migrated to the PSII reaction center. The inflection J occurs after ~2–3 ms of illumination and reflects the dynamic equilibrium (quasi-steady state) between the Q_A reduction and its oxidation. The J–I phase (at 30–50 ms) is due to the closure of the remaining centers (further reduction of Q_A and various redox states of temporary maximum of $Q_A^- Q_B^{2-}$) and the I–P phase (ends at about 300–500 ms) corresponds to the full reduction of the plastoquinone pool (equivalent to maximum fluorescence level F_m).

Pigment analysis

Chlorophyll and total carotenoid contents were determined in 80% acetone by breaking microalgae cells by intensive abrasion with sea sand for 2 min using a vortex mixer. The supernatant containing pigments was collected after centrifugation. 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 with a slit width of 0.5 nm (UV 2600 UV–VIS, Shimadzu, Japan) and the concentrations of pigments were calculated according to Wellburn (1994).

Statistical analysis

Sigma Plot 11.0 was used to determine significant differences between treatments. One-way ANOVA and Post-Hoc test was conducted for comparison of Se treatment to control cultures. *P* values lower than 0.05 were considered to be significantly different. Statistical analysis was only done for pigment contents and Se accumulation in microalgae biomass.

Results

In one series of laboratory experiments (Trial 1), various concentrations of Se (2.5, 8.5, 25 and 85 mg g^{-1} DW) were applied to *Chlorella* cultures under low irradiance of 250 µmol photons m^{-2} s^{-1} to determine effective Se dose that inhibits photosynthesis and subsequently growth and correlate these variables with Se uptake. In the other series of laboratory experiments (Trial 2) the concentration of 16 mg Se g^{-1} DW was used under various light intensities (250, 500 and 750 µmol photons m^{-2} s^{-1}) to study the irradiance-dependent rate of Se uptake. The biomass density at the start of both experiments was 1.2–1.6 g DW L^{-1}. Finally, laboratory experimental data were used in outdoor cultivation of *Chlorella* (Trial 3) to produce a bulk of Se-enriched biomass for nutritional purposes.

Trial 1: changes in *Chlorella* cultures caused by various Selenium concentrations

In this series of experiments the *Chlorella* growth and physiology was studied after the addition of various Se concentration (added as selenite) from low and

stimulating (2.5 mg g^{-1} DW) to highly inhibiting concentration (85 mg g^{-1} DW) and compared to the control cultures (no Se addition). The Se concentration of 2.5 and 8.5 mg Se g^{-1} DW caused a slight stimulation of growth during 3 day trial. A sigmoidal course was found with an initial slower phase, followed by acceleration of growth and it started to decelerate after 48 h (Fig. 2a). The results showed that in the control culture (no Se added), the biomass concentration increased by about 2.5 times and it reached stationary phase after 72 h. In the cultures treated with 2.5 and 8.5 mg Se g^{-1} DW, the growth rate was slightly faster. In case of the 25 mg Se g^{-1} DW, the culture grew up to 48 h and then the biomass density started to decrease. The higher concentration of Se (85 mg Se g^{-1} DW) caused a strong inhibition of growth and photosynthetic activity (not shown here).

The growth analysis was accompanied by fluorescence measurements of RLCs and OJIP kinetics. Although the growth rate was still similar during first 48 h in the Se treated cultures, we found clear changes in RLCs as compared to the control (Fig. 3). The courses of rETR calculated from RLCs were similar in the control and 2.5 mg Se treated culture, even in the latter there was found slight

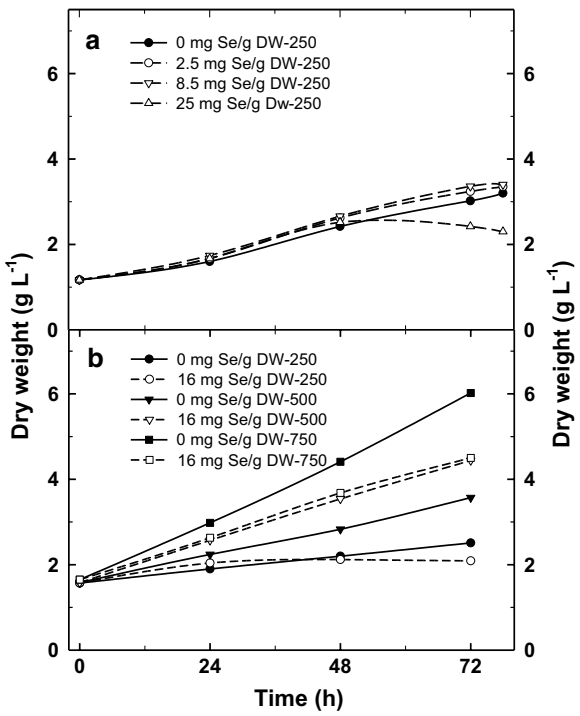

Fig. 2 Changes in the biomass density (DW) of the *Chlorella* cultures treated with various Se doses (2.5, 8.5 and 25 mg Se per g of DW): **a** with addition of various Se concentrations at 250 µmol photon m^{-2} s^{-1}; **b** with addition of 16 mg g^{-1} selenium exposed to various light intensities (250, 500 and 750 µmol photons m^{-2} s^{-1}) during cultivation period of 72 h (control—no Se added)

stimulation after 24 h (Fig. 4). After 48 h, the culture treated with 8.5 mg Se showed about 40% decrease of the rETR activity and the one treated with 25 mg Se g^{-1} DW dropped to 18% (Fig. 3a). Nevertheless, even in the cultures treated with 2.5 and 8.5 mg Se g^{-1} DW, the rETR decreased by 22 and 60%, respectively after 72 h, as compared to the control, most probably due to the onset of Se inhibition. Three variables—maximum PSII quantum yield, Fv/Fm, maximum of relative electron transport rate $rETR_{max}$ and photosynthesis-saturating irradiance E_k were calculated from RLCs (Fig. 4a–c). These variables were affected differently as a function of Se concentration. The Fv/Fm values varied a little between 0.61 and 0.66 on Day 1–3, showing that the cultures were in a good physiological state, slightly increasing from Day 1 to Day 3 by 11–27%; only the Fv/Fm values in the culture containing 25 mg Se g^{-1} DW decreased by about 40% on Day 3 (Fig. 4a). The values of rETR in the control culture were rather stable during the trial—between 115 and 130. In the cultures treated with 2.5 and 8.5 mg Se g^{-1} DW, the $rETR_{max}$ values were stimulated up to about 166–177 for the first 24 h, but not in the culture treated with 25 mg Se as it decreased by 20 and 80% on Day 1 and 2, respectively (Fig. 4b). The variable E_k corresponding to light saturation is used to characterize the photo acclimation status of microalgae cultures (Serodio et al. 2006). These values showed a decreasing trend after 24 h that probably reflects increasing biomass density and culture acclimation to lower irradiance. A significant drop of the E_k values was found only in the cultures treated with 25 mg Se g^{-1} DW; after 48 h of the trial meaning that this culture was strongly inhibited by Se addition (Fig. 4c).

Rapid fluorescence induction kinetics recorded as OJIP curves in the control and cultures treated with various concentrations of Se indicated inhibitory changes of PSII redox status only at Se concentration of 8.5 and 25 mg Se g^{-1} DW after 48–72 h, respectively. It was manifested by the increased Vj and Vi values in the cultures starting from Day 2 (Figs. 5a, b, 6a, b) which means that the electron transport through the PSII complex was inhibited due to higher reduction of the electron acceptors Q_A and Q_B and the cultures were not able to utilize the energy input for growth.

As concerns Chl content in biomass, it was about 2.2% at the start and the maximum values between 3.4 were reached in these laboratory cultures after 24 h; then these decreased to 2.5–2.8 being relatively stable on Days 2 and 3 (Fig. 7a). Higher Se concentration did not significantly affect the total Chl content in the cultures even if the $rETR_{max}$ values were significantly decreased (72 h). The content value of carotenoids was about 0.23% at the start and the maximum value of 0.5% were reached during exposure to 8.5 mg g^{-1} Se after 72 h (data are not shown).

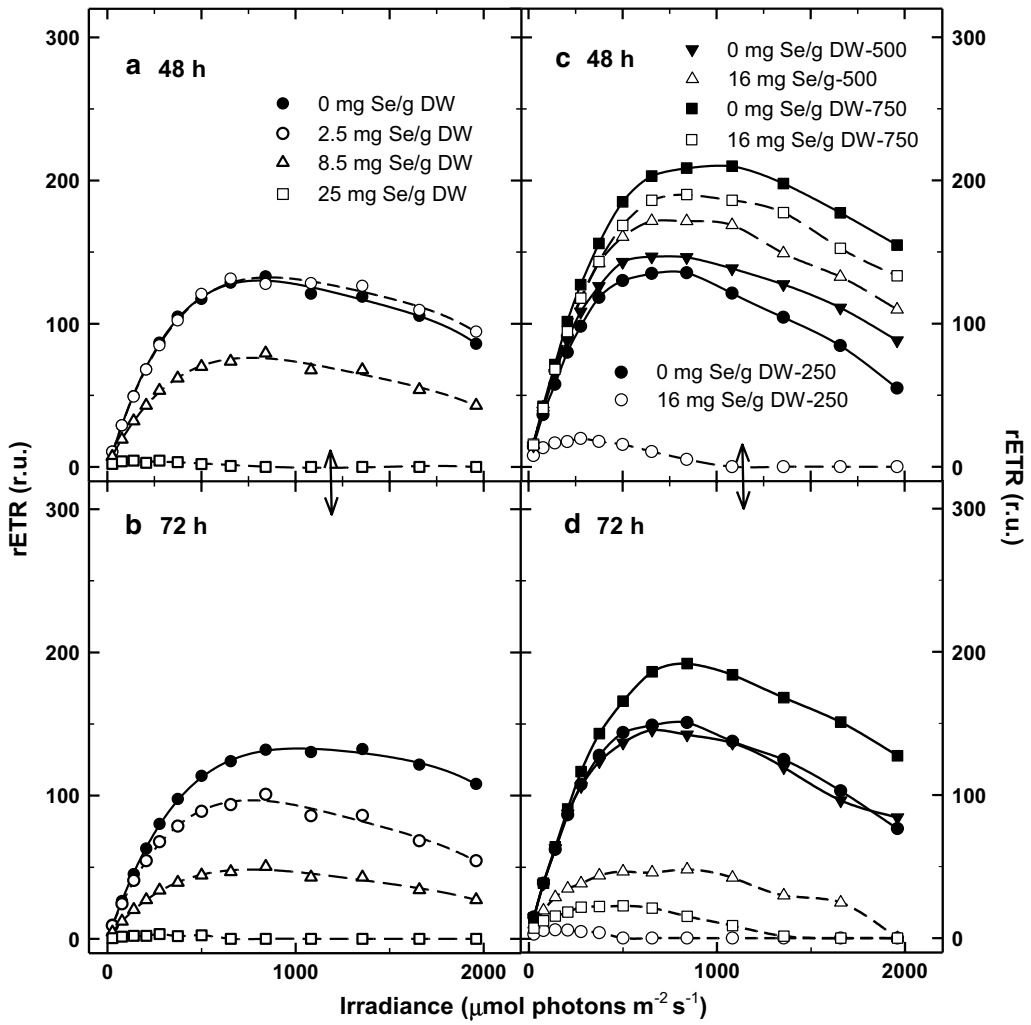

Fig. 3 Changes of rapid light-response curves of rETR monitored in the *Chlorella* cultures treated with various Se concentrations (2.5, 8.5 and 25 mg Se per g of DW) at 250 µmol photon m^{-2} s^{-1} after 48 h (**a**) and 72 h (**b**) of experiments. The *Chlorella* cultures were also exposed to various light intensities of 250, 500 and 750 µmol photons m^{-2} s^{-1}, respectively in the presence of 16 mg Se g^{-1} DW for 48 h (**c**) and 72 h (**d**) (control—no Se addition)

This increase in the carotenoids pigments was attributed to the protection provided by carotenoids to chloroplasts against oxidative damage (Schiavon et al. 2012).

As concerns Se accumulation in *Chlorella* biomass, at low dose (2.5 mg g^{-1} DW) added to the culture, the content was 101 µg Se g^{-1} DW after 24 h that was about 50% of that (208 µg Se g^{-1} DW) found in the culture treated with 8.5 mg g^{-1} DW (Fig. 8a). In the cultures treated with low and medium dose of Se, its accumulation reflected the partial stimulation of $rETR_{max}$ (Fig. 8a vs. Fig. 4b). After 72 h the content of Se in biomass was increased to 251 and 281 µg g^{-1} DW, respectively while about 10 and 55% decrease of rETR, respectively was observed showing the onset of Se inhibition. At high Se dose to the culture (25 mg g^{-1} DW) the course of experiments was different; after 24 and 48 h, the level of Se accumulation was considerably higher—508 and 1370 mg g^{-1} DW, respectively (5–10 times more than in the presence of 2.5 and 8.5 mg Se g^{-1}). Here, the inhibitory affect was clearly visible as decrease of $rETR_{max}$ after 24 h (Fig. b). After 72 h Se content in the biomass was too high (not shown here) that we consider it as an artefact, probably due to unspecific adsorption of Se since ETR activity was negligible (Fig. 4b).

The results revealed that at low irradiance intensity (250 µmol photons m^{-2} s^{-1}) the doses of Se above 8.5 mg g^{-1} DW were growth-inhibiting. The 'background'

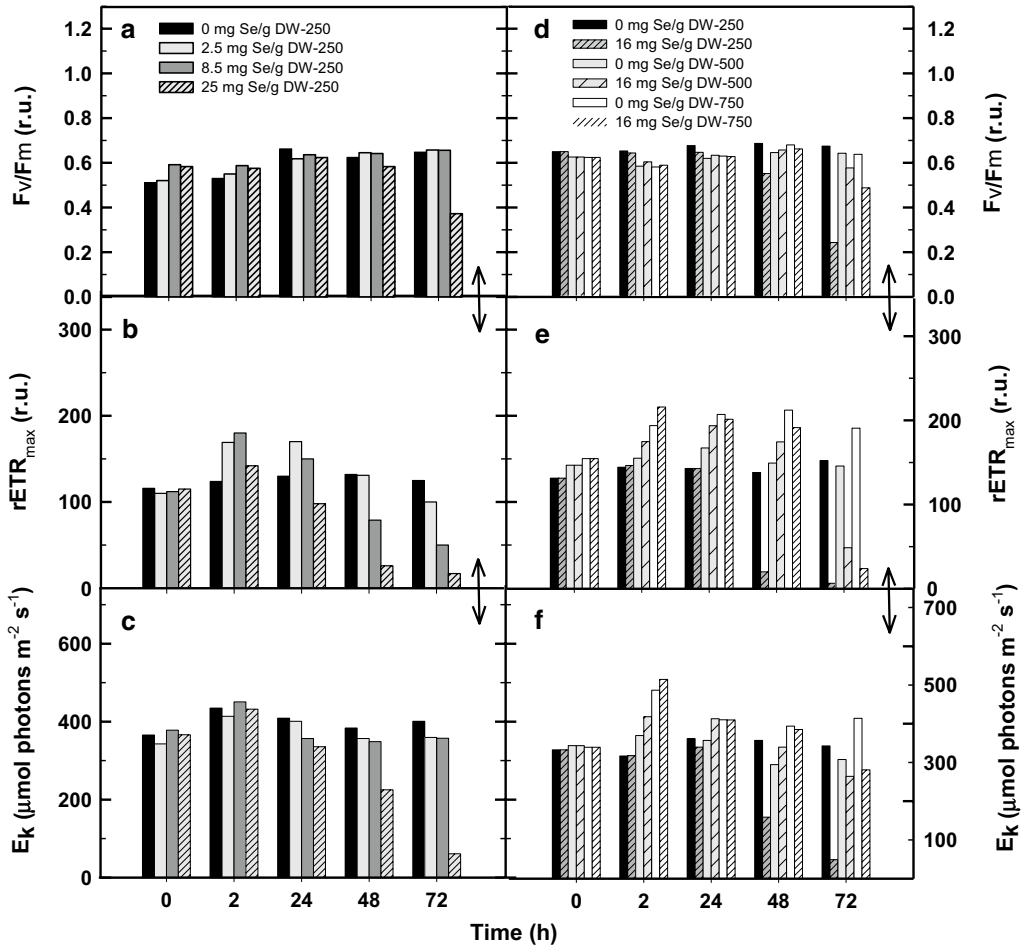

Fig. 4 Changes of variables Fv/Fm (**a**) $rETR_{max}$ (**b**) and E_k (**c**) were calculated from rapid light-response curves (see Fig. 3) caused by various Se concentrations (2.5, 8.5 and 25 mg Se per g of DW) after 72 h of experiments. The effect of various light intensities of 250, 500 and 750 µmol photons m^{-2} s^{-1} in the presence of exposure to 16 mg g^{-1} selenium on Fv/Fm (**d**) $rETR_{max}$(**e**) and E_k (**f**) variables is shown after 72 h. Control means no Se addition

content of Se in the control cultures was between 15 and 17 µg g^{-1} DW.

Trial 2: the effect of Selenium on microalgae cultures under various irradiance intensities

Preliminary experiments showed that the limit of Se tolerance is related to photosynthetic activity, i.e. depends on irradiance intensity. Thus, in Trial 2, the effective concentration of 16 mg Se g^{-1} DW was used (~235 µmol L^{-1}) to study changes of photosynthetic activity and the extent of Se incorporation into biomass under various light intensities (250, 500 and 750 µmol photons m^{-2} s^{-1}). This range of irradiance intensities was assessed from outdoor experiments where we found the average diel irradiance of ~400 µmol photon m^{-2} s^{-1} close to the surface (Masojídek et al. 2011b). The control cultures (no Se added) for each irradiance intensities were set-up in parallel. At low irradiance (250 µmol photons m^{-2} s^{-1}), the biomass density increased from 1.57 to 2.5 g L^{-1} during a 3-day trial (Fig. 2b). The rise of light intensity to 500 and 750 µmol photons m^{-2} s^{-1} increased the growth rate of *Chlorella* cultures more than 2 and 4 times, respectively to reach 3.5 and 6 g biomass L^{-1} at the end of the trial. It is important to note that similarly as in Trial 1, a slow-down of growth and photosynthetic activity of the cultures was always accompanied by a release of volatile Se compounds; it indicated that Se was not metabolized.

The courses of growth curves were reflected by RLCs and OJIP kinetics. After 24 h, RLCs measured in the control cultures revealed high rETR values of about 210 at 750 µmol photons m^{-2} s^{-1} while this activity was by 10–20% lower at 500 and 250 µmol photons m^{-2} s^{-1}

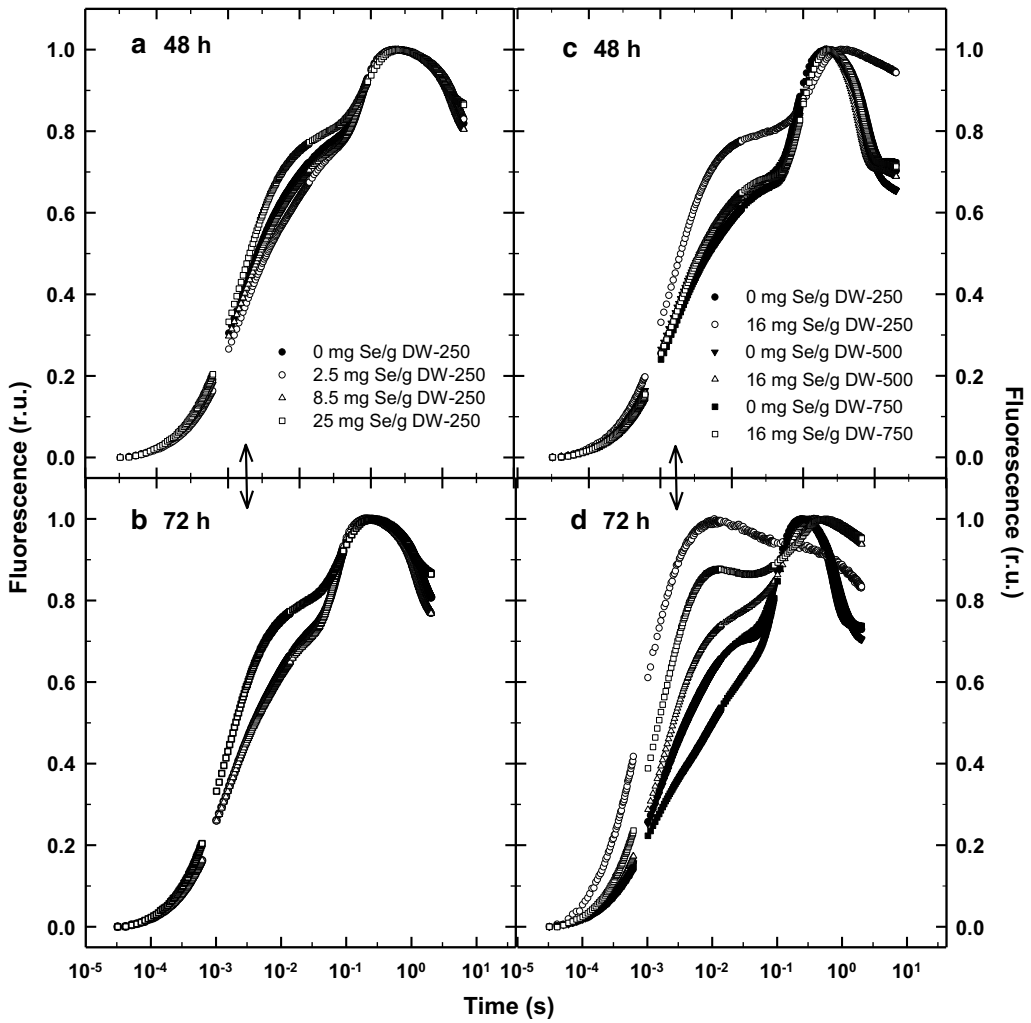

Fig. 5 Rapid chlorophyll fluorescence kinetics of *Chlorella* cultures treated with various Se concentrations (2.5, 8.5 and 25 mg Se per g of DW) at 250 µmol photon m^{-2} s^{-1} after 48 h (**a**) and 72 h (**b**) of experiment. The *Chlorella* cultures also were exposed to various light intensities (250, 500 and 750 µmol photons m^{-2} s^{-1}, respectively) in the presence of 16 mg Se g^{-1} DW for 48 h (**c**) and 72 h (**d**) (control—no Se addition)

(Figs. 3c, d, 4e). In the presence of Se, the growth at the medium irradiance (500 µmol photons m^{-2} s^{-1}) was stimulated being higher by about 24% after 72 h as compared to the control culture (Fig. 2b). (Interestingly, the course of growth at 500 µmol photons m^{-2} s^{-1} in the Se presence was almost identical as in the control at 750 µmol photons m^{-2} s^{-1}). At the medium irradiance rETR was higher in the Se-treated culture after 48 h as compared to the control; the significant inhibition was seen only after 72 h as it dropped to 33% of the initial value (Figs. 3d, 4e). The addition of Se to the low and high irradiance (250 and 750 µmol photons m^{-2} s^{-1}) caused a 20–25% inhibition of growth as compared to the control cultures, but we have to consider different final biomass densities (Fig. 2b). Similarly at in the case of RLC, the course of the OJIP curves revealed that the Se-treated cultures at 500 µmol photons m^{-2} s^{-1} were least inhibited as compared to those exposed to 750 and 250 µmol photons m^{-2} s^{-1}. At medium irradiance, the Vj and Vi variables were only slightly increased in the presence of Se after 72 h (Fig. 6c, d) while at low and high irradiance these increased significantly indicating a more reduced state of the PSII reaction center, i.e. a block of electron transport.

Chlorophyll content in biomass of the control cultures was about 2.2–2.3% at the start and the maximum values of were reached in low irradiance culture (3.2) while, logically these maxima were 2.1 and 1.8 in the cultures exposed to medium and high irradiance, respectively

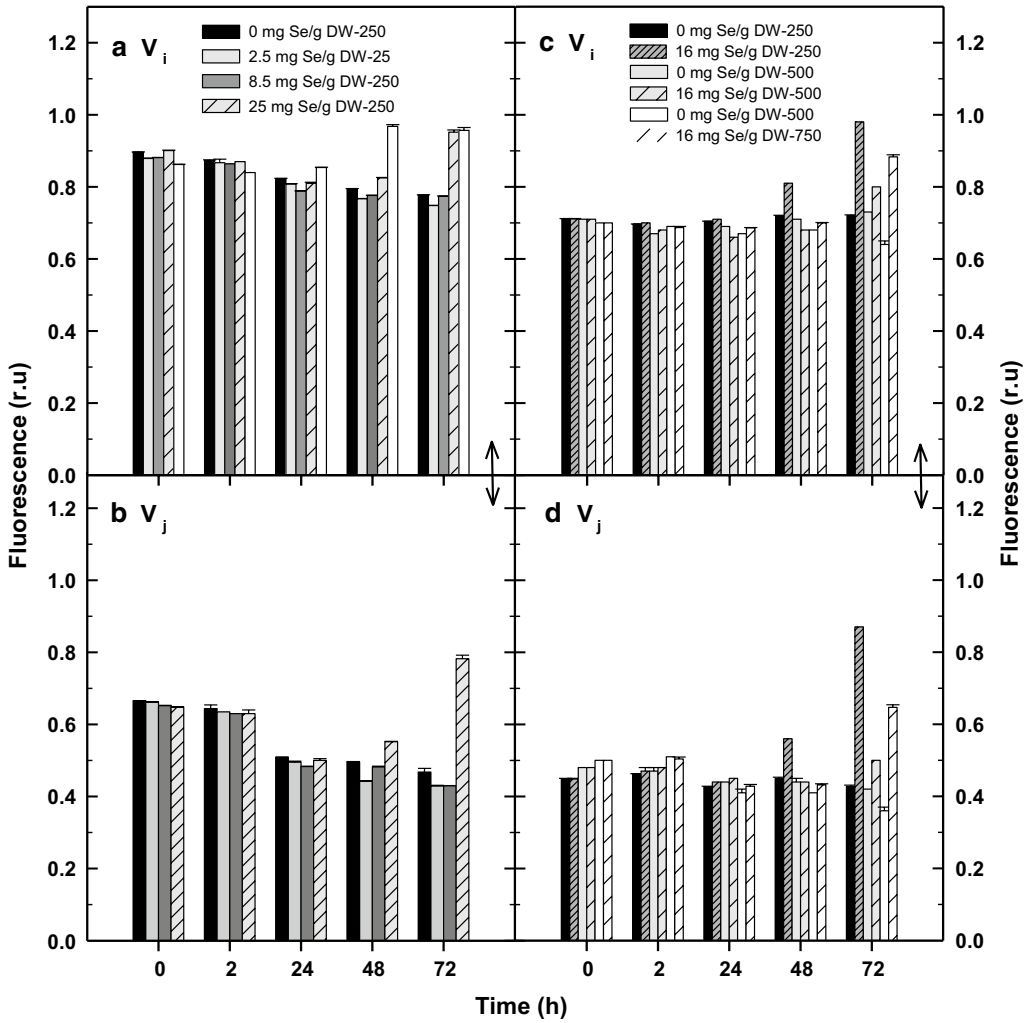

Fig. 6 Changes of the Vj (**a**, **c**) and Vi variables (**b**, **d**) calculated from rapid fluorescence induction curves (see Fig. 5) caused by various Se doses (2.5, 8.5 and 25 mg Se per g of DW) after 72 h of experiment (**a**, **b**). The effect of various light intensities of 250, 500 and 750 µmol photons m^{-2} s^{-1} in the presence of exposure to 16 mg g^{-1} selenium on the Vi (**c**) and Vj (**d**) variables shown after 72 h. Control means no Se addition

(Fig. 7b). In the Se−treated cultures, the total Chl content did not changed substantially.

In low-irradiance cultures, Se incorporation was high on Day 2; but after 72 h, similarly as in Trial 2 at high Se dose, the content of Se in biomass was extremely high (not shown here) that can be consider as an artefact, probably due to unspecific adsorption of Se to deprived cells as ETR activity was negligible (Fig. 4d). The highest Se accumulation in biomass (7100 µg g^{-1} DW) was found in *Chlorella* cultures under medium irradiance after 72 h (Fig. 8b) when rETR was still 27% of the maximum activity. As the Se-content of Se in the control cultures was between 15 and 17 µg g^{-1} DW, it means that well-growing *Chlorella* can accumulate Se up to thousand-times in their biomass. At high irradiance the Se incorporation into biomass was low that we consider as the consequence of activity inhibition by the Se+ high irradiance synergism.

Trial 3: cultivation of outdoor *Chlorella* culture with selenium

In Trials 1 and 2, laboratory *Chlorella* cultures grown under continuous illumination were able to well tolerate certain concentrations of Se (2.5 mg g^{-1} DW, corresponding to 37 µmol L^{-1}) without substantial inhibition of growth for 3 days (Figs. 2, 3, 4). On the basis of Trial 1 and 2 and some preliminary outdoor studies (not shown here) we estimated the dose of Se for outdoor longer-term trial test presented here. The aim of this experiment was to produce Se-enriched *Chlorella* biomass in longer run with high amount of Se incorporated into biomass while minimizing growth inhibition and Se losses.

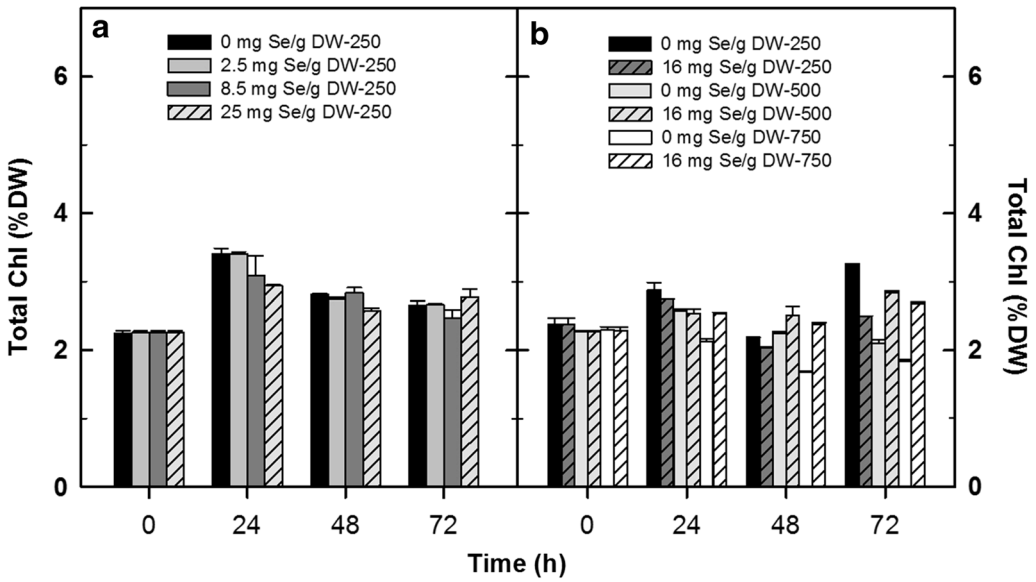

Fig. 7 Changes of total Chlorophyll concentrations in *Chlorella* cultures treated with various Se doses (2.5, 8.5 and 25 mg Se per g of DW) exposed to 250 µmol photons m^{-2} s^{-1} after 72 h of experiments (**a**). The effect of various light intensities (250, 500 and 750 µmol photons m^{-2} s^{-1}) in the presence of 16 mg Se g^{-1} DW on chlorophyll concentration is shown after 72 h (**b**). *$P < 0.05$ means significant difference compared to the control (no Se added)

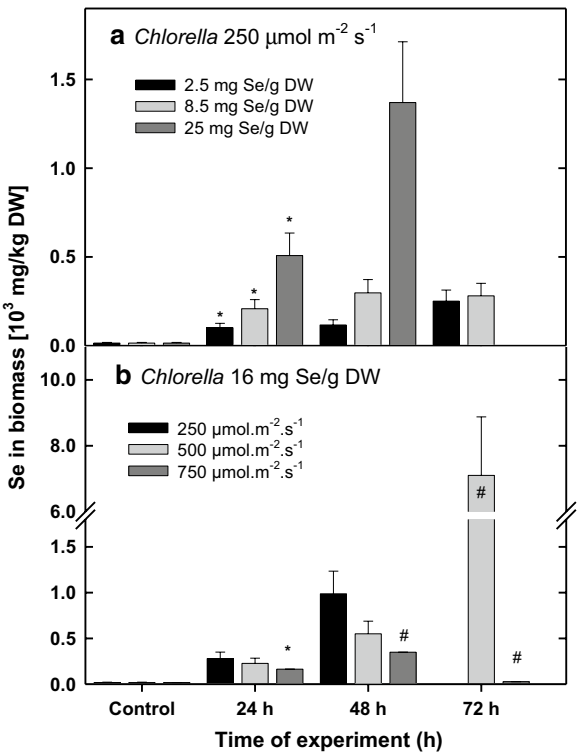

Fig. 8 Changes of total Se incorporation to biomass during a 3-day experiment in *Chlorella* cultures exposed to various Se doses (2.5, 8.5 and 25 mg Se per g of DW) under 250 µmol photons m^{-2} s^{-1} (**a**) and various light intensities (250, 500 and 750 µmol photons m^{-2} s^{-1}) in the presence of 16 mg Se g^{-1} DW (**b**). *$P < 0.05$ means significant difference compared to the control (no Se added)

The starting biomass density was about 13 g L^{-1} as we took in consideration that light path in outdoor thin-layer cascades was only 6 mm and the trial was carried out at the beginning of June when daily irradiance is relatively high and the diel period is long. In this trial the average concentration of Se was about 20 µmol L^{-1}, i.e. total dose of Se was about 0.8 mg g^{-1} biomass supplied during 7 days. This dose was adjusted significantly lower than the lowest dose (2.5 g Se g^{-1} DW) in laboratory Trial 1 that still caused inhibition of rETR after 3 days of experiment and volatile Se compounds were released. In Trial 3 we intended to avoid substantial inhibition of growth. Indeed, in the Se-treated culture was only slightly decreased (5–10%) as compared with the control culture (Fig. 9). No substantial changes of RLC were found in the middle and at the end of the trial (Day 4 and 7) as compared to Day 1 (Additional file 1: Figure S1). The variables $rETR_{max}$ and E_k showed the trends of slightly decreasing activity in the Se-treated culture at midday (Table 1) that probably reflected increased biomass density. Changes of the OJIP-curves (Additional file 1: Figure S2) and the derived Vj and Vi variables also indicated only a negligible decrease of photosynthetic activity of the Se-treated culture as compared to the control (Table 1). Nevertheless, at the end of 1-week trial the biomass density was about 26.7 g L^{-1} (about 15% lower than in the control). The total yield of biomass harvest was about 50 kg containing 650 mg Se kg^{-1} DW; about 80% of the added selenite amount was incorporated. We can conclude that we

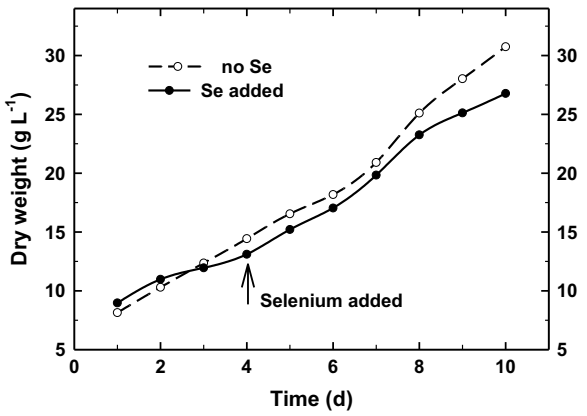

Fig. 9 Growth curves of *Chlorella* cultures grown in outdoor thin-layer cascades in the presence (*solid line*) and absence (*dashed line*) of Se during the 10-days trial

Table 1 Changes of variables of the maximum PSII quantum yield, F_v/F_m, maximum electron transport rate $rETR_{max}$ and the irradiance saturating photosynthesis E_k were estimated from rapid light-response curves (Additional file 1: Figure S1) monitored at midday on Day 1, 4 and 7 during a 7-day experiment in the presence and absence of Se

Culture treatment	Fluorescence variables *(r.u.)*	Day 1	Day 4	Day 7
Control (no Se)	F_v/F_m	0.77	0.75	0.80
	$rETR_{max}$	461	404	359
	E_k	758	714	531
	V_j	0.21	0.22	0.21
	V_i	0.43	0.43	0.43
Se treated culture	F_v/F_m	0.74	0.74	0.78
	$rETR_{max}$	436	380	332
	E_k	665	660	625
	V_j	0.24	0.26	0.23
	V_i	0.46	0.46	0.46

Changes of variables Vj and Vi were calculated from fast fluorescence induction kinetics (Fig. S2) monitored on Day 1, 4 and 7 during a 10-day experiment in the presence and absence of Se. Vj reflects a reduction of Q_A^- (photochemical phase) while Vi is thought to correspond reflect various redox state of temporary maximum of $Q_A^- Q_B^{2-}$

were able to select suitable Se dose that was only mildly inhibiting photosynthetic activity and growth but still the incorporation of Se to biomass was relatively high (compare with Fig. 8). Chl fluorescence was used as fast and suitable technique to monitor doses of Se salts.

Discussion

Toxic effect of Se salts is dependent on microalgae species as well as culturing conditions. Thus, prior to production of Se-enriched biomass, specific toxicity analysis for the selected species is required as seen in our trials. As an example published data for the microalga *Chlorella* can be taken for comparison. She can tolerate Se concentrations of up to tens of mg L^{-1} depending on culturing conditions (Pelah and Ephraim 2005; Gojkovic et al. 2014; Sun et al. 2014). It is important to consider that reported laboratory cultures were mostly grown in flasks in rather diluted suspensions exposed to 50–100 µmol photons m^{-2} s^{-1}. Not much attention has been paid to the dependence of Se effect at varying irradiance intensities that have been proven as crucial in our trials. Se doses have been usually expressed in mg per liter or molar concentration, but we have to consider biological effect which depends on the ratio between Se concentration and biomass density (g Se per g DW).

Scarce reports can be found describing the physiological effects of Se in detail (added in the form of selenite or selenate) in correlation to biomass productivity and element uptake in microalgae cultures. Here photosynthesis monitoring is essential technique for cultivation control and evaluation of Se tolerance/toxicity in microalgae cultures. When culturing in excessive Se concentrations the early response of microalgae culture is significant decrease of photosynthetic activity monitored as oxygen evolution or Chl fluorescence quenching (e.g. Geoffroy et al. 2007; Gojkovic et al. 2014; Morlon et al. 2005).

In the present experiments with *Chlorella* cultures, a range of Se concentrations (2.5–85 mg L^{-1}, i.e. 0.03–1 mM) was used as referred for various microalgae in the literature (for a review see Gojkovic et al. 2015) and we exposed *Chlorella* cultures to higher irradiance intensity, closer to values measured inside the culture outdoors (250–750 µmol photons m^{-2} s^{-1}; Masojídek et al. 2011b) at higher biomass densities (~1.5 g L^{-1}). The extent of Se effect was dependent on the culture treatment, i.e. Se dose, irradiance intensity and time of exposure. According to our results, *Chlorella* was able to accumulate Se concentration in her cells up to 1000-fold more than it is normally found during cultivation (Fig. 8). This process was, to a certain extent, stimulated by photosynthetic activity (irradiance intensity) until the synergism of increasing irradiance intensity and of Se dose caused photo inhibition (Figs. 3b, c, 4d–f, 5c, d). It is a typical situation described by Lichtenthaler (1988) as *eu-stress,* a mild stress activating cell metabolism and increasing physiological activity vs. *dis–stress,* having a negative, damaging effect. We have demonstrated that low Se concentration of about 2.5–8.5 mg g^{-1} DW (19–65 µM) partially stimulated photosynthetic activity (rETR) and growth of *Chlorella* for the first 24 h (Fig. 4b). Further on, Se above the tolerable level showed its toxic effect that was indicated by the significantly decreased growth (Fig. 2). Similar trend was also found by Sun et al.

(2014) as the growth of *Chlorella* was promoted by Se at lower concentrations. The inhibitory effect of Se salts on photosynthetic yield has been related to ultrastructural changes (Geoffroy et al. 2007; Morlon et al. 2005; Vítová et al. 2009; Schiavon et al. 2016). Chloroplasts are probably the first target of Se cytotoxicity affecting the stroma, thylakoids and pyrenoids as electron microscopy showed the over-accumulation of starch and the formation of dense granules containing Se (Morlon et al. 2005).

As the Se concentration was increased (25 mg g^{-1} DW, i.e. 190 µmol L^{-1}), it led to a significant reduction in photosynthetic activity and subsequently growth rate (Figs. 2a, 3a, b). The decrease of photosynthetic activity was accompanied, for some period, by increased Se incorporation to biomass (0.5–1.5 mg g^{-1} DW). This is in agreement with the results obtained with different microalgae species like *Spirulina, Chlamydomonas* or *Chlorella sorokiniana* (Geoffroy et al. 2007; Gojkovic et al. 2014; Li et al. 2003; Morlon et al. 2005). In our experiments growth inhibition at higher Se doses, which resulted in decreased incorporation of Se, was also accompanied by "sulphurous" smell of volatile compounds released during cultivation. At higher Se doses when the culture activity was significantly inhibited (and Se was not incorporated) was this physiological state always accompanied by "sulphurous" smell of volatile Se compounds released during cultivation as mentioned by Guadayol et al. (2016). Se uptake probably employs the enzymatic metabolism resulting in the substitution of some of the sulphur atoms by Se in both free amino acids and proteins as demonstrated by the presence of Se-analogues of the sulphur amino acids during Se accumulation (Li et al. 2003; Araie and Shiraiwa 2016). The higher Se doses can also result in an enhanced accumulation, probably, due to the sorption to cell walls (Pronina et al. 2002) as these consist of variety of extracellular organic compounds such as polysaccharides and proteins that offers a number of active sites capable of binding metal ions (Mane and Bhosle 2012).

When we exposed *Chlorella* cultures to medium light intensities of 500 µmol photons m^{-2} s^{-1}, about a 40% stimulation of growth was observed in a 3-day experiment even in the presence of 16 mg Se g^{-1} DW (Fig. 2). However, further increase of light intensity to 750 µmol photons m^{-2} s^{-1} was photo inhibitory, as it was revealed by lowered Fv/Fm and $rETR_{max}$ values (compared to the control), indicating that high irradiance amplified the effect of Se presence on the PSII complex (Fig. 4e).

It is important to note that rETR was more sensitive variable to the Se treatment as compared to the maximum photochemical yield Fv/Fm (Fig. 4). Actually, the effective PSII photochemical yield Y_{II} (used for rETR calculation) was more sensitive to Se treatment as compared to Fv/Fm (Fig. 4a, b; Geoffroy et al. 2007). In any case, we consider the variable rETR more suitable as compared to as Y_{II} as the former takes into account also irradiance intensity E_{PAR} that influence Se incorporation. Based on our Chl fluorescence data—RLCs and OJIP kinetics, changes of Chl fluorescence variables Fv/Fm, $rETR_{max}$, E_k, Vj and Vi (Figs. 3, 4, 5, 6) we believe that higher Se doses obstruct photosynthetic electron transport between PSII and PSI due to the over reduction of plastoquinone electron carriers. Thus the balance between excitation and electron transfer rate is changed and results in a more reduced state of the PSII reaction center as also reported by Chen et al. (2016). These conclusions in supported by our results as the increase of Vj and Vi (Fig. 6a and c) indicated accumulation of the reduced plastoquinones that cannot transfer electrons further (Strasser et al. 2004). Some authors hypothesize that the presence of Se probably also affects the Cyt b-$_6$f complex (Geoffroy et al. 2007; Gojkovic et al. 2015). Because of their chemical similarity, Se can substitute sulphur in an iron–sulphur protein that is a part of the complex. This may eventually lead to disruption of the photosynthetic electron transport chain as Cyt b_6f plays the role as an electron carrier from PS II to PS I. Eventually, this also indirectly affects the O_2 evolution rate as the water-splitting complex is also associated with PS II. These are the early photochemical events affecting photosynthesis, supporting the fact that the PSII complex is very sensitive to abiotic stresses (Jajoo et al. 2016), for example the presence of Se in excess.

A better understanding of the overall effect of Se on microalgae has been needed to develop biotechnological processes for the production of Se-enriched biomass containing valuable organic Se–compounds. The main motivation of this work was to study the interplay between the dose and irradiance in order to find the range of Se tolerance in laboratory and outdoor cultures. Therefore, we wished to find fast and suitable methods and key variables in order to optimize doses of Se salts preventing growth inhibition, or even culture loss in large-scale mass cultivation. Monitoring photosynthetic performance via Chl fluorescence diagnostics can give us the early warning to prevent toxic effect (and also release of unpleasant volatile Se compounds) during cultivation. It can also help to follow metabolic pathway of Se in the cells, but it is out of the scope of this work. From a biotechnological point of view, we are able to achieve efficient incorporation of Se by microalgae cells (Se-enriched of biomass) controlled by photosynthesis monitoring without substantial decrease of growth rate.

Additional file

Additional file 1: Fig. S1. Examples of rapid light-response curves of rETR recorded in *Chlorella* cultures on Day 1, 4 and 7 during the outdoor trials. **Fig. S2**. Examples of fast fluorescence induction kinetics (OJIP curves) recorded in *Chlorella* cultures on Day 1, 4 and 7 during the outdoor trials

Abbreviations

Chl: chlorophyll; DW: dry weight; ETR: electron transport rate; OJIP: fast fluorescence induction curve; PSII: photosystem II; RLC: rapid light curve; Se: selenium.

Authors' contributions

Design of the experiments: JM, AB, KR, JRM. Experiments performed by: AB, KR, JRM, JM. Data analyses: AB, JRM, KR, JM. The article written by: JM, AB, JRM, KR. Graphics: AB, KR, JM. All authors read and approved the final manuscript.

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Competing interests

The authors declare that the research was supported by grants listed below. No commercial or financial can be construed as a potential conflict of interest.

Availability of data and materials

The original data supporting the final conclusions are kept by the authors. *Chlorella vulgaris*, strain *R117* was designated as *CCALA 10258* and deposited in the Culture Collection of Autotrophic Organisms, Institute of Botany, Třeboň, Czech Republic.

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Annex III

(related to Chapter 3 Cultivation Systems)

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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 μmol(photon) m^{-2} s^{-1} attained a doubling time of 3.5 d and biomass density of 3.5 g(DM) L^{-1} after about 10-d period. When grown under 3,500 μmol(photon) m^{-2} s^{-1}, the culture reached a doubling time of 1.7 d, and biomass density of ~5.5 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 g(biomass) 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 microalgae 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, Masojídek *et al.* 2015, Sergejevová *et al.* 2015, Acién *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, Masojídek *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, Enríquez 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^{-1}): KNO_3, 4.04; KH_2PO_4, 0.68; $MgSO_4 \cdot 7H_2O$, 1.98, and some other micro- and trace compounds – ferric-sodium chelatonate, $CaCl_2$, H_3BO_3, $MnCl_2 \cdot 4\,H_2O$, $ZnSO_4 \cdot 7\,H_2O$, $CuSO_4 \cdot 5\,H_2O$, $CoSO_4 \cdot 7\,H_2O$, $(NH_4)_6Mo_7O_{24}$, and NH_4VO_3 (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 (Masojídek *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_2 (v/v) and exposed to continuous illumination using day-light fluorescent lamps at an irradiance of about 150–250 μmol(photon) m^{-2} s^{-1}. 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.1*A*). 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. 1*A*) 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. 1*B*). 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. 1*A*). 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^{-2} s^{-1} (Trial A) or of about 3,500 μmol(photon) m^{-2} s^{-1} (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_2) 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

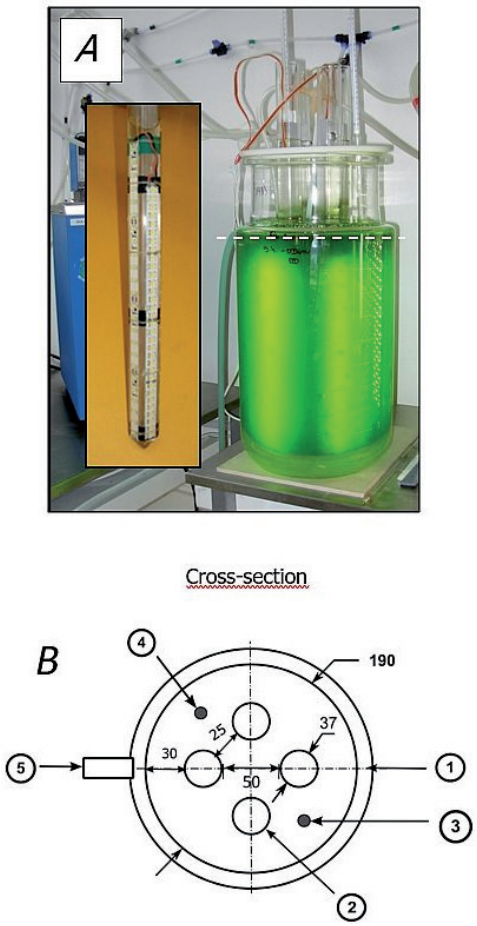

Fig. 1. (*A*) Column photobioreactor, LED-PBR-10 with internal illumination used in trials. The PBR consists of a double-jacketed, glass column (with a working volume of 8 L) and a total height of 400 mm. (Insert: Image of one of four tubular light LED sources that were submerged in the microalgae culture. 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_2) is supplied by a loop (3) to mix and supply CO_2 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^{-1}] 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*^{TM} column). The samples were diluted twice with deionized water and 10-µL aliquots were used for the analysis. The solutions of 1.4 mM $NaHCO_3$ and 4.5 mM Na_2CO_3 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^{-1} 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 ± SE) at specific time points. Before measurement, the cultures were diluted to 0.2 to 0.3 g(DM) L^{-1} [corresponding to 5 to 7 mg(Chl) L^{-1}] 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 µmol(photon) $m^{-2} s^{-1}$] 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 µmol(photon) $m^{-2} s^{-1}$, 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 μmol(photon) m^{-2} s^{-1}, frequency of 0.5–1 kH] 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 $rETR_{max}$ 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_{PAR}, $rETR = Y_{II} \times E_{PAR}$ [dimensionless as it is called relative] (*e.g.* Hofstraat *et al.* 1994, Ralph and Gademann 2005, White *et al.* 2011). In order to estimate $rETR_{max}$, and the irradiance saturating photosynthesis the

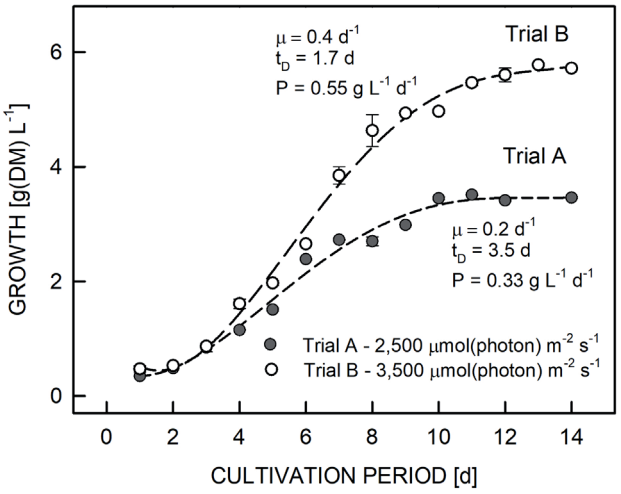

Fig. 2. Growth curves of *Chlorella* R-117 in the PBR exposed to continuous irradiance of 2,500 μmol(photon) m^{-2} s^{-1} (*close symbols*), or 3,500 μmol(photon) m^{-2} s^{-1} (*open symbols*). Values are presented as a mean (n = 3) with SE indicated by error bars. Broken lines are the fitted curves 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 (~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, ~2.5 μs) while illuminating red LEDs served as high-intensity continuous light from both sides of the cuvette [up to 3,000 μmol(photon) m^{-2} s^{-1}], perpendicular to the detector. The fast fluorescence induction kinetics was measured in the time range between 50 μs 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_0) 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 ~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 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 μmol(photon) m^{-2} s^{-1} to photo-acclimate (measured on the surface of light sources). Close to the wall of the PBR, it was about 150–200 μmol(photon) m^{-2} s^{-1} and about 200–400 μmol (photon) m^{-2} s^{-1} (measured with spherical light sensor) between light sources, when 0.4–0.5 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 μmol(photon) m^{-2} 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 [μ = 0.2 day^{-1}, P = 0.33 g(DM) L^{-1} d^{-1}] and reached the biomass concentration of 3.4 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 0.5–3.0 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 μmol(photon) m^{-2} s^{-1}. The culture showed a doubling time of 1.7 d, the growth rate μ of 0.4 per day and biomass productivity P = 0.55 g(DM) L^{-1} d^{-1}. It reached the maximal biomass density of 5.8 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 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 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, 4*A 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.

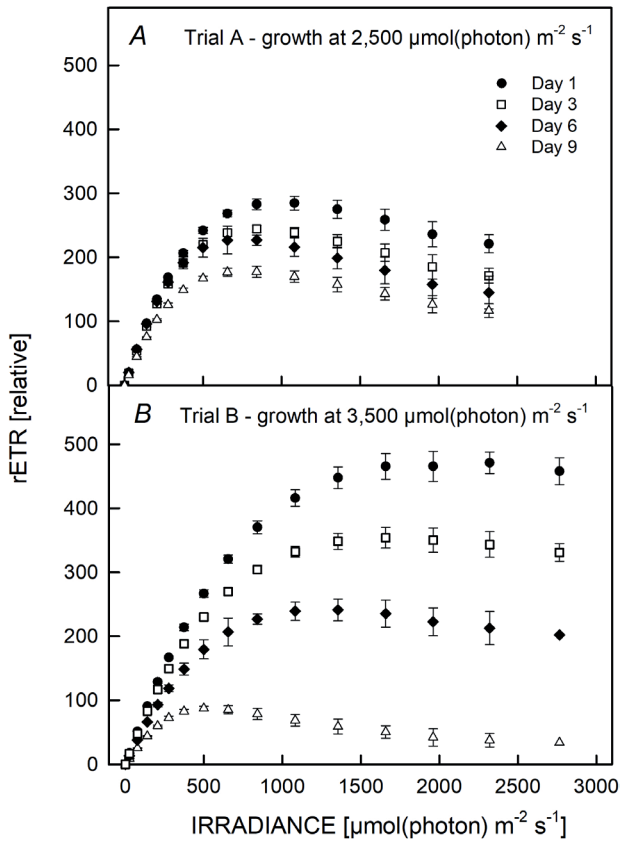

Fig. 3. Rapid light-response curves (RLCs) of the relative electron transport rate rETR *vs*. irradiance measured in *Chlorella* R-117 cultures grown under high irradiance [*A* – 2,500 μmol(photon) m^{-2} s^{-1}] and [*B* – 3,500 μmol(photon) m^{-2} s^{-1}] recorded on Days 1, 3, 6, and 9. rETR = Y_{II}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

The growth kinetics in the cultures was reflected by changes of photosynthetic activities that significantly varied between Trial A [~2,500 μmol(photon) m^{-2} s^{-1}] and Trial B [~3,500 μmol(photon) m^{-2} 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 μmol(photon) m^{-2} s^{-1}] as compared to the values between 240 and 280 found in Trial A [2,500 μmol(photon) m^{-2} s^{-}1] (Fig. 3). Important to note that in Trial B, the $rETR_{max}$ 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. 4*A*). 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 $rETR_{max}$ (Fig. 4*B*) and the onset of light saturation E_K (Fig. 5*A*) 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. 5*A*). Consequently, the $rETR_{max}$ values on Day 9 were slightly lower in Trial B compared to that in Trial A (Fig. 4*B*). Statistically, the decrease of F_v/F_m, $rETR_{max}$, and E_k was more significant in

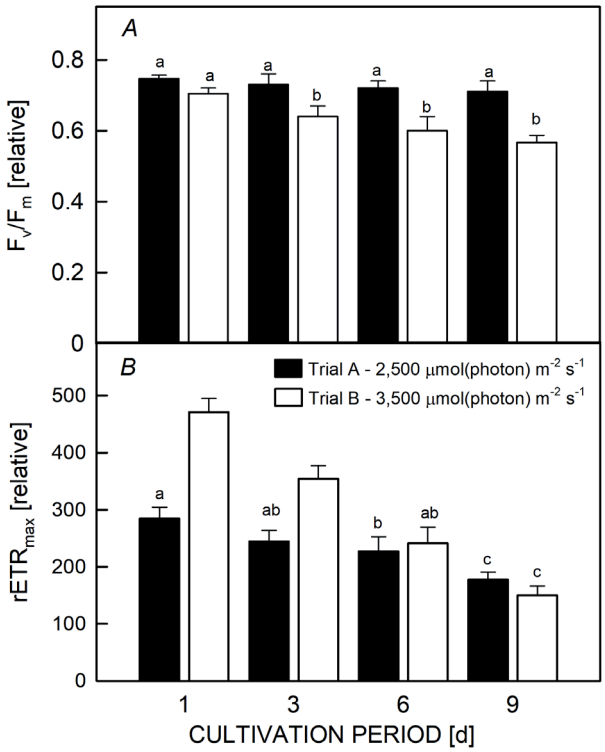

Fig. 4. Changes in the maximum effective quantum yield of PSII (F_v/F_m) (*A*) and maximum relative electron transport rate ($rETR_{max}$) (*B*), of *Chlorella* R-117 exposed to continuous illumination of ~2,500 μmol(photon) m^{-2} s^{-1} (*black column*) and ~3,500 μmol(photon) m^{-2} s^{-1} (*grey 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.

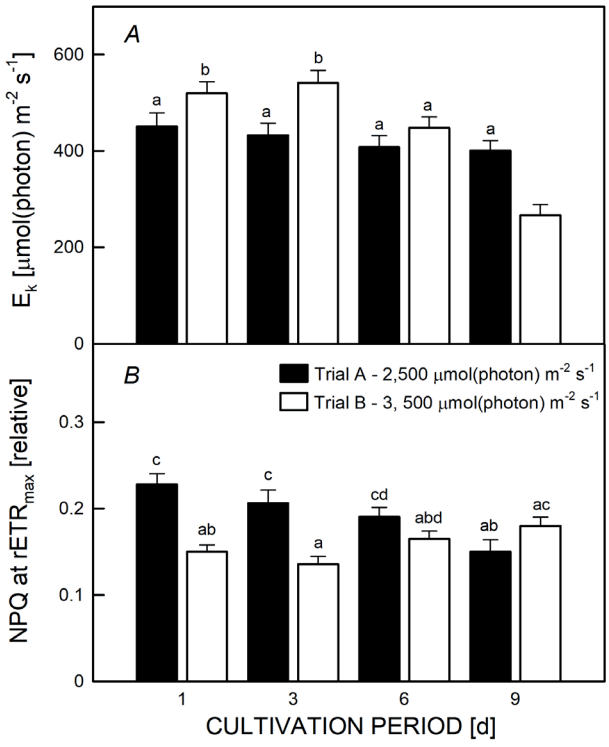

Fig. 5. Changes in the onset of light saturation E_K (*A*) and nonphotochemical quenching NPQ (*B*) of *Chlorella* R-117 exposed to continuous illumination of ~2,500 μmol(photon) m^{-2} s^{-1} (*black column*) and ~3,500 μmol(photon) m^{-2} s^{-1} (*grey column*) during the 14-day cultivation period. 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. 4*B*, 5*B*).

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 ~2.5% DM, *i.e.* about 25 mg L^{-1}) and total carotenoid (max. ~0.5% DM, *i.e.* 5 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. 4*B*). 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 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. 6*B*). 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. 7*A*).

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

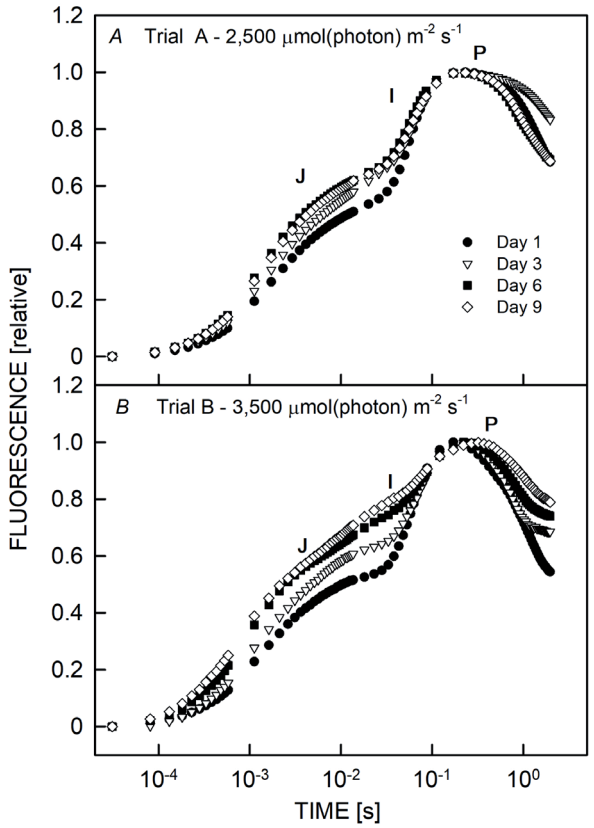

Fig. 6. Changes of rapid Chl fluorescence induction kinetics – OJIP test of *Chlorella* sp. R-117 grown under continuous illumination of 2,500 μmol(photon) m^{-2} s^{-1} (*A*) and 3,500 μmol(photon) m^{-2} s^{-1} (*B*) during the 14-day cultivation trial. The OJIP curves were double-normalized to on 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.

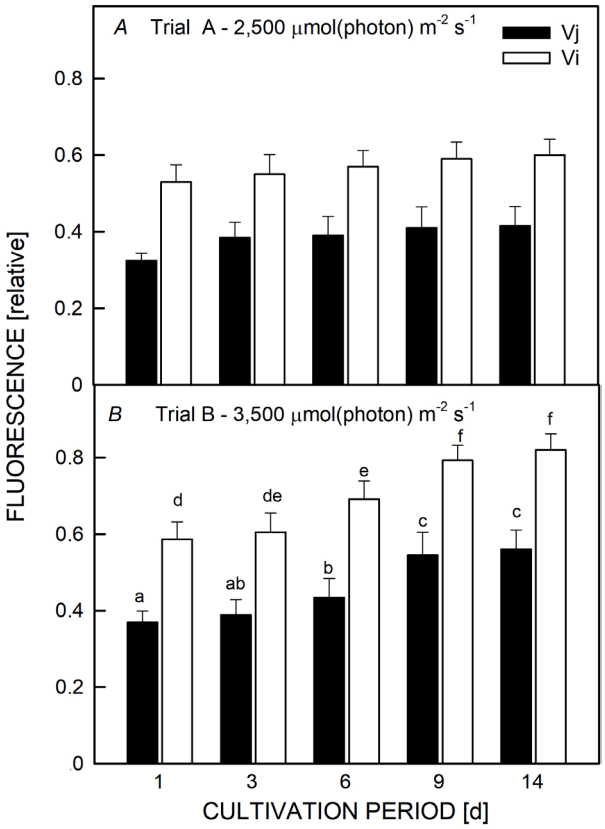

Fig. 7. Changes of the V_j and V_i variables calculated from fast Chl fluorescence induction kinetics OJIP test of *Chlorella* sp. R-117 grown under continuous illumination of 2,500 μmol(photon) m^{-2} s^{-1} (*A*) and 3,500 μmol(photon) m^{-2} s^{-1} (*B*) during the 14-day cultivation trial. The V_j and V_i variables corresponding to the J and I inflections were calculated as relative variable fluorescence yield. VJ 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.* $Q_AQ_B^{2-}$ 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.

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 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 μmol(photon) m^{-2} 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 (Masojídek 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, Sergejevová *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 μmol(photon) m^{-2} s^{-1} and the culture biomass density reached about 1.2 g(DM) 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 μmol(photon) m^{-2} s^{-1} at relatively high biomass densities (Fig. 5*A*) similarly as those measured in outdoor *Chlorella* R-117 cultures (Masojídek *et al.* 2011b). When the starting biomass density was about 0.5 g 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 $rETR_{max}$ and E_K (Figs. 4*B*, 5*A*) *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 $rETR_{max}$ (Fig. 4*B*) 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 $rETR_{max}$ values were by about 33% lower in Trial B than those in Trial A (Fig. 4*B*); this coincided with the decelerating phase of the growth curve (Fig. 2) when biomass density reached about 5 g L^{-1} in Trial B although there were still enough nutrients (28–48% of the initial amount, respectively; Fig. S1*B*). On Day 9, the decrease of E_K also indicated that the denser culture in Trial B was acclimated to more than 30% lower irradiance [E_K = 266 μmol(photon) m^{-2} s^{-1}] as compared to the thinner culture [E_K = 400 μmol(photon) m^{-2} s^{-1}] (Fig. 5*A*). 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 rETR may express different degree of constrains – 'distress' when F_v/F_m changes dramatically (photoinhibition) and functional downregulation, 'eustress' when the rETR 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. 3*B* and 4*B 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 CO_2/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 (Sergejevová *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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Annex IV

(related to Chapter 3 Cultivation Systems)

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REVIEW

Development of thin-layer cascades for microalgae cultivation: milestones (review)

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Abstract

In this work, the key moments of the development of the so-called thin-layer cascades (TLC) for microalgae production are described. Development started at the end of the 1950s when the first generation of TLCs was set-up in former Czechoslovakia. Since, similar units for microalgae culturing, which are relatively simple, low-cost and highly productive, have been installed in a number of other countries worldwide. The TLCs are characterized by microalgae growth at a low depth (< 50 mm) and fast flow (0.4–0.5 m/s) of culture compared to mixed ponds or raceways. It guarantees a high ratio of exposed surface to total culture volume (> 100 1/m) and rapid light/dark cycling frequencies of cells which result in high biomass productivity (> 30 g/m^2/day) and operating at high biomass density, > 10 g/L of dry mass (DW). In TLCs, microalgae culture is grown in the system of inclined platforms that combine the advantages of open systems—direct sun irradiance, easy heat derivation, simple cleaning and maintenance, and efficient degassing—with positive features of closed systems—operation at high biomass densities achieving high volumetric productivity. Among significant advantages of thin layer cascades compared to raceway ponds are the operation at much higher cell densities, very high daylight productivities, and the possibility to store the culture in retention tanks at night, or in unfavourable weather conditions. Concerning the limitations of TLCs, one has to consider contaminations by other microalgae that limit cultivation to robust, fast-growing strains, or those cultured in selective environments.

Introduction

Microalgae can be grown in controlled mass aquaculture (algaculture) using various cultivation units. These primarily photosynthetic microorganisms represent a diverse group of tremendous ecological importance since they inhabit all major Earth ecosystems—aquatic and terrestrial, oceanic and freshwater, from arctic regions, through alkaline or saline habitats, to hot springs. Thus, they can tolerate a wide range of light intensities, temperatures, pH values and salinities. Microalgae are efficient biomass producers responsible for a substantial part of global primary production, being at the base of the food chain. Annually, they assimilate 30–50% of the inorganic carbon and produce about 50% of oxygen from/to the atmosphere (Chapman 2013; Yan et al. 2013). In 2017, annual worldwide microalgae production reached about 30,000 tons of dry biomass based on companies' reports (lecture by Claudia Grewe at the 6th Congress of the International Society for Applied Phycology 2017, Nantes, France), but a qualified estimate is several times higher due to ample production in China. Microalgae production for human use has become scarce to cover current annual demand. Microalgae in general are a potent source of natural substances such as proteins, carotenoids, oils, antioxidants, prebiotics and other compounds that are widespread used as food and feed supplements, in agriculture as biofertilisers, biostimulants and biopesticides, in wastewater treatment, as well as cosmetics and pharmacology, and most recently as a feedstock for biofuels (Khan et al. 2018).

Initiated in the 1950s (Burlew 1953), numerous cultivation systems have been designed for microalgae growth (Chaumont 1993; Borowitzka 1999; Tredici 2004; Zittelli

Dedicated to the memory of Prof. Ivan Šetlík

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et al. 2013; Masojídek et al. 2015; Acién et al. 2017). The choice of a suitable cultivation system and the adjustment of the cultivation regime must be worked out for each individual microalgae strain, purpose, or product. Two basic approaches to mass production are used: one applies to cultivation in open reservoirs (with direct contact of the microalgae culture with the environment), while the other involves closed or semi-closed vessels—photobioreactors (PBRs) with no direct contact between the culture and the atmosphere. There are major operational differences between open reservoirs and PBRs and, consequently, the growth physiology of the microalgae is different between the two systems (Grobbelaar 2009). Important variables of a cultivation system are the light intensity, temperature, length of optical path mixing, light acclimated state of the organism, nutrient availability, and gas exchange (supply of CO_2 and O_2 degassing). At present, open reservoirs are the most widely used cultivation systems for commercial large-scale production of tons of biomass due to their low cost of construction, maintenance and operation (Zittelli et al. 2013; Costa and de Morais 2014; Masojídek et al. 2015; Acién et al. 2017). Unfortunately, the use of open ponds is restricted to a small number of microalgae species due to the limited control of cultivation conditions and biological contamination. Hence, open systems are suitable for "robust" microalgae strains (mostly belonging to the *Chlorophyta*) that grow rapidly (Doucha et al. 2005; Doucha and Lívanský 2009; Masojídek et al. 2011a), or under very selective conditions (e.g., the cyanobacteria *Spirulina—Arthrospira*, or the green microalga *Dunaliella*) (Belay 2013; Borowitzka 2013).

Open cultivation systems represented by artificial ponds, tanks, raceways (shallow race-tracks mixed by paddle wheels) and sloping cascades (i.e., inclined-surface systems). An overview of open culture systems used for the mass cultivation of microalgae outdoors has been published recently (for a recent review, see Zittelli et al. 2013; de Vree et al. 2015; Acién et al. 2017). Productivity in these open systems is usually low, ~ 1 g/m^2/day of dry mass (DW), due to limited light, the lack of mixing, and CO_2 supply and the cultures are usually grown at a biomass density ranging between 0.5 and 1 g/L depending on the culture depth. To improve mixing, open systems are mixed by impellers, rotating arms, paddle wheels, or by a stream of air enriched with CO_2 supplied into the culture. The culture depth may vary between 10 and 30 cm.

The aim of this review is to review the milestones of thin-layer cascade (TLC) technology starting from the early development in the 1960–1970s, through "hard" times in the 1980s, its resurrection in the 1990–2000 up to today when various modifications have been used worldwide. The history of microalgae cultivation systems in former Czechoslovakia is presented in more detail, including construction characteristics of three TLC generations, their productivity, and advantages and limitations of TLC system.

Initial period of microalgae mass culturing

In the 1940s, cultivation trials with microalgae were carried out by the Carnegie Institution for Science. The possibility of growing the unicellular green microalga *Chlorella* (*Chlorophyta*) was considered in large-scale units. The early cultivation studies of several research groups were summarized in the "bible" of microalgae biotechnology edited by John S. Burlew of the Carnegie Institution in Washington, DC (Burlew 1953). The first attempts for large-scale microalgae cultivation and design of early pilot plants focused on closed systems in order to isolate cultures from the natural environment as to prevent contamination of cultures by other microorganisms. One of the productive pilot plants for mass cultivation of *Chlorella* was devised and tested at Arthur D. Little, Inc. in Cambridge, Massachusetts, in collaboration with the Carnegie Institution in 1952 (Fig. 1). In Germany, Gummert and co-workers experimented with large-scale cultures of *Chlorella* grown in open cultivation units using concrete trenches (reservoirs) with a plastic lining (Gummert et al. 1953). In Israel, a small-scale pilot plant to produce biomass of *Chlorella* or *Scenedesmus* as green fodder for cattle was set-up as a closed, mixed reservoir mounted in a greenhouse taking advantage of climatic conditions with a year-round availability of sunlight (Evenari et al. 1953). In Japan, at the Tokugawa Institute of Biological Research, an early attempt was made to use a shallow trough covered with transparent polyethylene sheets (Mituya et al. 1953).

By the mid-1950s, it was proven that open outdoor cultures were feasible and that they would probably not suffer from contamination if fast-growing strains (e.g., *Chlorella* or *Scenedesmus*) were cultivated. Once the feasibility of open cultures for outdoor cultivation was confirmed, it substantiated this concept for outdoor microalgae culture. This is without

Fig. 1 One of the first pilot-scale units (about 4000 L) was devised and tested at Arthur D. Little, Inc. in Cambridge, Massachusetts in collaboration with the Carnegie Institution in 1951. The photobioreactor was formed by a loop of thin-walled polyethylene tube of a 7–8-cm depth with continuous circulation of the culture by a pump

doubt due to the much simpler design of the open type units, whose construction is therefore much cheaper.

The overall goal of these early researchers strictly was to produce protein for potential human and animal consumption.

Start of thin-layer culturing in the 1960–1970s

In former Czechoslovakia, the concept of "cascade" cultivation units which was quite different from "deep" open ponds emerged in the late 1950s. The first pilot units were built at the Botanical Garden of the Slovak Academy of Sciences in the city of Košice (Eastern Slovakia) in 1958 by the research team lead by Ivan Šetlík. Soon after in January 1960, the research team joined the Laboratory for Algae Research of the newly established Institute of Microbiology, Czechoslovak Academy of Sciences in Třeboň. At the laboratory in Třeboň, the research was aimed at defining the scientific basis for commercial exploitation of microalgae cultivated on a large scale. In the 1960s, the efforts of the laboratory were focused mostly on the technological development of microalgae mass culture.

The idea was to grow microalgae on a system of shallow sloped troughs of about 2–5 cm deep arranged one below another, to form a cascade of hydraulic jumps, over which microalgae cultures were continuously circulated to secure sufficient mixing for solar irradiation attenuation and gas exchange to cells. As compared to mixed ponds, a specific feature of cascade design was the difference between day and night operation modes which is used until now. The culture was kept circulating only during hours when there was sufficient photosynthetically active radiation. During the dark or unfavourable weather periods, the culture was kept in a retention tank in which it was aerated and mixed. In principle, the culture was continuously recirculated to the highest trough from the lowest by pumping so that the culture cascaded by gravity in a thin layer. In these culture units, it was expected that cultures could reach higher biomass density as well as productivity. A number of outdoor production units (Fig. 2) was developed and constructed in Třeboň as well as

Fig. 2 First generation of outdoor cascades built in Třeboň in early 1960s

cultivation procedures based on the principle of complex approach to study microalgae production, instrumentation development, biotechnology, physiology, cell biology, genetics and ecophysiology. The major drawback of this construction was that the culture motion in the troughs was not turbulent enough to prevent the sedimentation of cells completely. This concept was not appropriate for application on a larger scale due to slow eddy motion, sedimentation of microalgae cells, high energy consumption for pumping due to high mean slope, or high CO_2 desorption caused by the jumps.

As a next step, the design of cascade cultivation units was modified in order to establish a steady and uniform flow of relatively thin layer of culture. In 1962–1963, the second generation of original cultivation units was constructed, as a unique outdoor pilot plant with 2 U of 50 m^2 (volume of 2500 L) and one of 900 m^2 (culture volume of 40,000 L) were built on the new laboratory campus "Opatovický mlýn" (Fig. 3) (Šetlík et al. 1970) which was one of the first large-scale plants for microalgae cultivation in Europe. These highly productive units were based on sloping planes, which are known worldwide as the Třeboň type units—cascades (Fig. 3). The principle of microalgae cultivation was to maintain turbulent flow of a relatively thin culture layer using a plain surface fitted with transversal baffles. The pilot plant was constructed as dual-purpose units that were used during the winter as a greenhouse for hydroponic vegetable and flower cultivation and in summer for microalgae biomass production.

As compared to open reservoirs (ponds, raceways) with a depth of suspension in the range of 15 to 30 cm where diluted cultures of microalgae (0.5–1 g/L DW) were grown under limited mixing and gas exchange, the main advantage of the cascade system constructed at the Laboratory for the Algae Research in Třeboň was the growth of well-mixed dense (10–

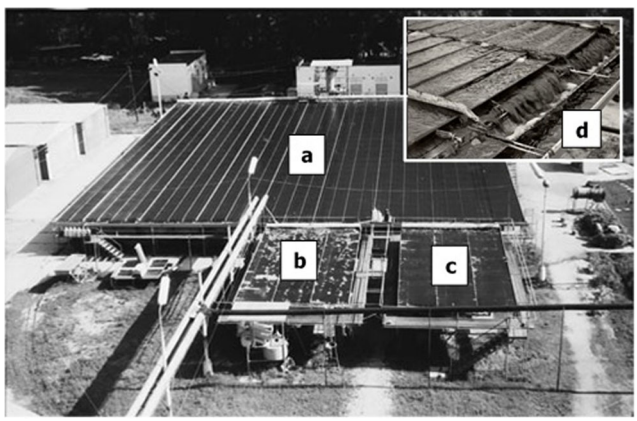

Fig. 3 The 2nd generation of outdoor cascades units for microalgae cultivation installed in Třeboň consisted of a pilot unit of 900 m^2 (a) and of two experimental units (b and c), each of 50 m^2. Insert (d): microalgae culture flowing over baffles to the collecting trough. The units were based on sloping glass planes with a slope of 3%, supported by a steel structure. The surface was fitted with transverse baffles, 3.5 cm high and 15 cm apart. The principle was to maintain turbulent flow of a relatively thin layer for high biomass productivity

15 g/L DW) microalgae culture in a relatively thin layer (< 5 cm) (Masojídek and Prášil 2010). In deep raceway ponds, the low turbulence of microalgae culture may result in lower efficiency of solar energy use due to photoinhibition of the microalgae cells in the upper layer exposed to high irradiance. Also, a much lower volume of dense microalgae suspension can be treated at harvest. Novel, thin-layer cultivation units were constructed of glass plates with a slope of 3%, supported by a steel structure. Additionally, the cultivation planes were fitted with transverse baffles 3.5 cm high and 15 cm apart to create intensive turbulence in the microalgae suspension which flows at a velocity of 7 cm/s. At the lower end of the culture area, the suspension was collected by a trough to a reservoir and conveyed by the return tube to the circulation pump. Then, it was lifted back to the upper part of the culture surface. The culture was circulated over the surface during the day while during the night was kept in the aerated retention tank to reduce heat losses, or during rainfall to avoid dilution by rainwater. As compared to deep ponds, the thin layer system was characterized by an intensive mixing which prevented culture sedimentation and increased the frequency of light/dark periods. Due to sufficient culture turbulence, high average irradiance per cell and good gas exchange can guarantee higher areal productivity for thin-layer systems.

In outdoor cultivations experiments as well as in most laboratory studies, the green microalgae *Scenedesmus* and *Chlorella* were used. Later, in the 1970s, thin-layer cultivation systems were constructed in Poland, Cuba and Italy to compare growth under various climatic conditions. A large-scale cultivation plant with a total area of 3000 m^2, using thin-layer sloped platforms placed on the ground, was constructed in Rupite in southwest Bulgaria (Becker 1994). The concrete platforms over which microalgae flowed had a slope of 3% and were fitted with transversal baffles to increase culture turbulence (Fig. 4). The advantage of the Rupite location was a high number of sunny days and a source of CO_2 from carbonic mineral springs.

The cultivation units in Třeboň were operated during the cultivation seasons from 1963 to 1970. After the Soviet invasion in 1968, large-scale microalgae cultivation was intentionally suppressed for almost 20 years. A partial resurrection of large-scale microalgae culturing in Czechoslovakia came at the end of the 1980s.

Thin-layer cascades in the 1990s

Only after political changes in 1989, support for Czech microalgae biotechnology was renewed and steady development has occurred since. In September 1993, the 6th International Conference on Applied Algology was held at Třeboň which helped to renew collaborations broken in the 1960s. Since the 1990s, the industrial cultivation of

Fig. 4 The large-scale cultivation plant with a total area of 3000 m^2 made of concrete was constructed in Rupite, southwest Bulgaria. It consisted of thin-layer sloped platforms placed on the ground over which microalgae would flow. The platforms had a slope of 3% and were fitted with transversal baffles to increase culture turbulence

microalgae to produce biofuels and bioproducts has increased dramatically worldwide (Khan et al. 2018).

Starting at the end of the 1980s and early 1990s, the third generation of thin-layer units was developed and various cultivation units were tested in Třeboň—from experimental to large scale (Fig. 5). Each unit of 225 m^2 was constructed as a cascade of four planes arranged symmetrically in a mirror-like design. The microalgae culture is pumped from the retention tank to a distributor tube at the high point of the upper roof-like platforms and moves down along the surface to troughs which transfer it to lower platforms. Then, it flows in the opposite direction and at the end is collected back to the

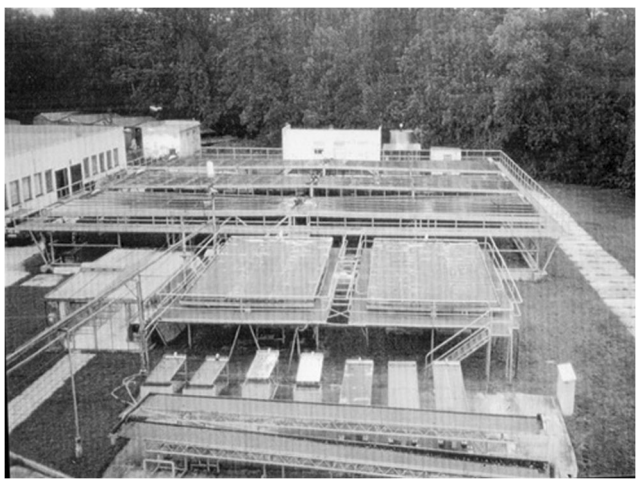

Fig. 5 In the 1990s, various thin-layer cultivation units with an area from 2 to 225 m^2 were tested. An important improvement was that in the largest units, the system of transversal baffles was replaced with plastic rods (diameter of 13 mm) mounted over a distance of 150 cm perpendicularly to the culture flow. This guaranteed high turbulence due to the flow of suspension under and over the rods with an average layer thickness of about 10–15 mm

retention tank located just under the unit. In this way, the dark volume in distribution tubing and in the retention tank can be minimized due to the short distances (Masojídek et al. 2011a).

Compared to the previous generation, some modifications were made: a reduction of surface inclination from 3 to 1.7%, mixing baffles were removed, but cultivation planes were divided into parallel lanes to support lateral mixing (Fig. 6). The main disadvantage of the previous, second-generation thin-layer units was the presence of baffles which required laborious installation and difficult cleaning of the cultivation surface. Thus, based on the experience from the TLCs used in the 1970s, the third generation of TLCs employs a much thinner layer of microalgae—less than 10 mm. Instead of densely spaces baffles, plastic rods with a diameter of 13 mm were placed 1.5 m apart and thus the flow velocity could be increased to 0.4–0.5 m/s (Doucha and Lívanský 1995; Grobbelaar et al. 1995). The rods were placed 3 mm the above cultivation surface perpendicularly to the culture flow. High turbulence was reached due to culture flow under and over the rods with an average layer thickness of about 10 mm.

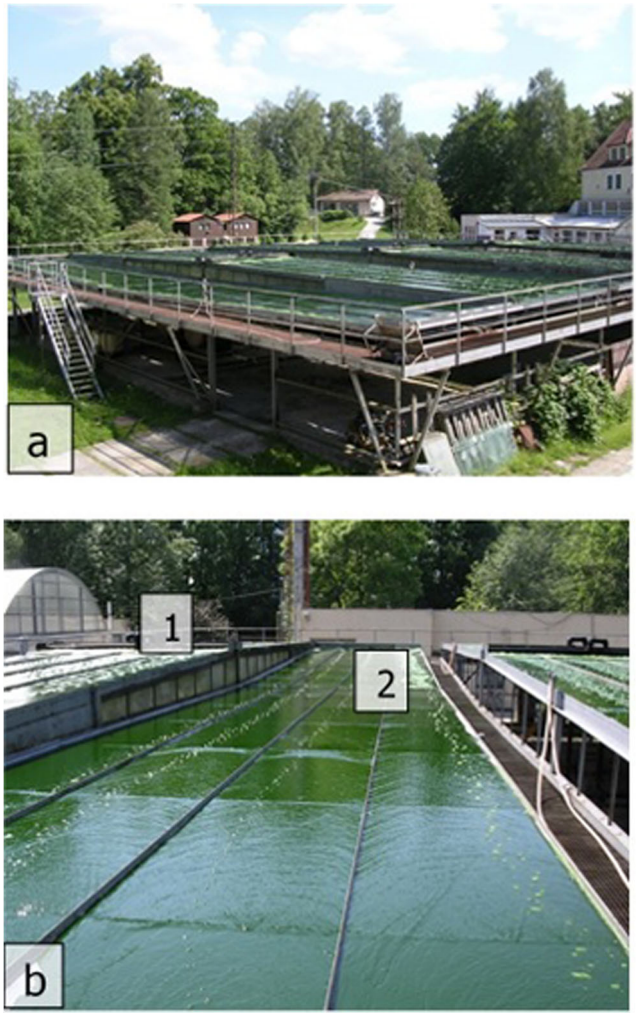

Fig. 6 At the beginning of the 1990s, a cultivation plant of about 660 m^2 was constructed which is still under operation. It consists of 3 units (**a**) with each (220 m^2) being made of glass plates glued in metal frames and arranged as a cascade of four planes arranged symmetrically in a roof-shape design (**b**). The culture is pumped to the distributor of upper cultivation planes (1), flows along to the end and turns back to the lower cultivation planes to be collected by a gutter (2) to a retention tank

Later, experiments in Rupite and Třeboň demonstrated that microalgae cultures can be grown on a smooth inclined surface (glass plates glued in metal frames) without any baffles or rods (Fig. 6). The thickness of culture layer could be decreased only to 6–8 mm with a flow speed of about 0.4–0.5 m/s reaching high biomass density, 15 g/L or more (Lívanský et al. 1993; Doucha and Lívanský 1995) as compared to 1–2 g/L in the culture grown in 40–50 mm with baffles (Figs. 3 and 4). The high flow velocity of the culture in thin layer resulted in a Reynolds number of about 4500, while the value characteristic for laminar flow in open channels is only about 500 (Masojídek et al. 2011a). The detailed principle of thin-layer cascade functioning is described in the legend of Fig. 7. Due to the high ratio of the exposed surface to total volume (> 100 1/m), the cell average irradiance is high even at high biomass densities under short light/dark cycles which support high productivity. The highest photosynthetic activities were achieved in cultures of 6.5–12.5 g/L DW (when operated in semi-batch regime), which was reflected in a maximum daylight productivity of up to 55 g/m^2/day of dry biomass under optimal conditions during few favourable days. The culture growth can result in maximal biomass densities up to 50 g/L DW (Masojídek et al. 2011a). First and foremost, the cleaning and maintenance of smooth surface units are much simpler, as compared with the baffled system. Another advantage of TLCs is temperature self-regulation as the culture is quickly heated by solar irradiance on cold mornings, and on the other hand cooled by increased evaporation when overheating. In this respect, TLC is more effective compared to deep culture systems due to the large surface and thin layer operation. Experiments have shown that the modification of this type of unit can make production more efficient, using semi-batch regimes in the range of biomass density between 15 and 35 g/L, simplifying cleaning and maintenance.

Starting in the mid-1990s, modern techniques like chlorophyll (Chl) fluorescence were introduced for microalgae culture monitoring to support semi-empirical methods (Knoppová et al. 1993; Torzillo et al. 1996). Photosynthesis measuring techniques were applied to monitor photosynthetic activity of microalgae mass cultures in situ. The pilot experiments were carried out in cascades and closed photobioreactor systems in the Czech Republic, Italy and Israel. Photosynthesis measuring techniques have been used to optimize culturing regimes and to estimate adverse environmental conditions—high irradiance, temperature extremes, high dissolved oxygen concentration and their synergism on

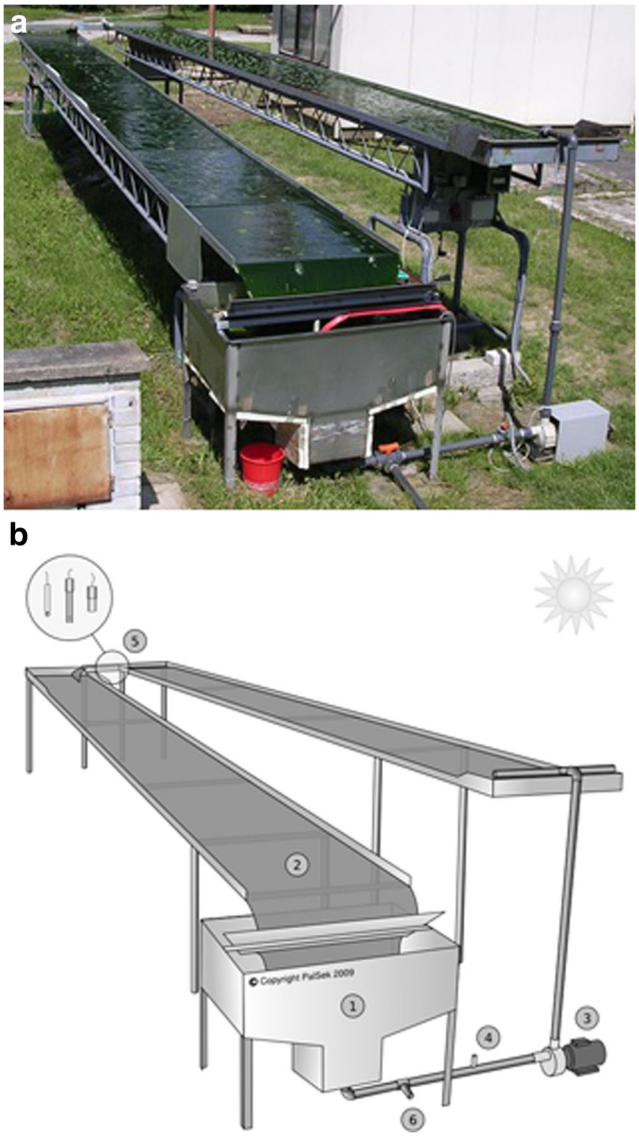

Fig. 7 (**a**) Experimental thin-layer cascade (total surface 24 m^2, working volume 220 L, flow speed 0.4–0.5 m/s, culture layer thickness 6 mm) was built in 1989 and served to verify construction and function of large thin-layer cascades. As soon as the first functional tests had been performed with this unit, the construction of the large culture unit started (Fig. 6). The experimental unit (surface of 24 m^2; volume 225 L) consists of two declined cultivation lanes made of glass plates (declination of 1.7%) supported by a scaffolding (Institute of Microbiology, Academy of Sciences, Třeboň, Czech Republic). (**b**) Schematic diagram of a cascade where a thin layer (6 mm) of suspension flows along declined surface. The microalgae culture is pumped (3) from the retention tank (1) and evenly distributed to the high point of the upper plane, flows down along the surface to the trough which transfers the culture to the upper part of lower lane (2), and at the end, the culture is collected back to the retention tank located under the unit. As the culture falls on a plate in a thin layer and then passes through a filter screen, efficient degassing takes place. As the start of one lane and the end of the other are close, the dark volume in distribution tubing and retention tank can be minimized. CO_2 is supplied in the tubing before the pump (4) based on pH stat system (5).

microalgae growth (for a review see Masojídek et al. 2011b; Malapascua et al. 2014).

Productivity of thin layer cascades

From the 1960s, large-scale microalgae cultivation in Třeboň has mostly been aimed at biomass production for use in nutrition—health food and supplements for human and animal nutrition in collaboration with commercial companies. A portion of the produced biomass has also been used for cosmetic purposes and isolation of bioactive compounds.

Starting in 1963, when the second generation of thin-layer cascades was set in operation, biomass productivity steadily increased from 7 g/m^2/day in 1963 to 12 g/m^2/day in 1967 (Fig. 8) (Šetlík et al. 1967; Nečas and Lhotský 1968). Important to note is that the data was calculated as an average figure during cultivation season between May and September, for about 150 days. Fast-growing robust microalgae *Scenedesmus obliquus* and two strains of *Chlorella* were cultured for production. Microalgae cultures were circulated on the cultivation surface only during the day (about 10–13 h) and during the night, the suspension was kept in an aerated tank. The decreased temperature during the night reduced the biomass consumption caused by respiration. The lower daily yield values during this year were probably caused by a reduced supply of CO_2 adopted on a practical economical basis (Nečas and Lhotský 1969). The growing season in 1970 is the last archived before large-scale production was virtually out of operation for several years. The microalgae increments were periodically harvested by centrifugation every 2 to 4 days, and the final product was mainly spray-dried. In 1964, 1.5 ton of dry biomass was produced which was sent to other institutes for further analysis. The overall objective was to determine the potential of microalgae as a replacement for human high-protein diet and as a feed for cattle and poultry, due to the

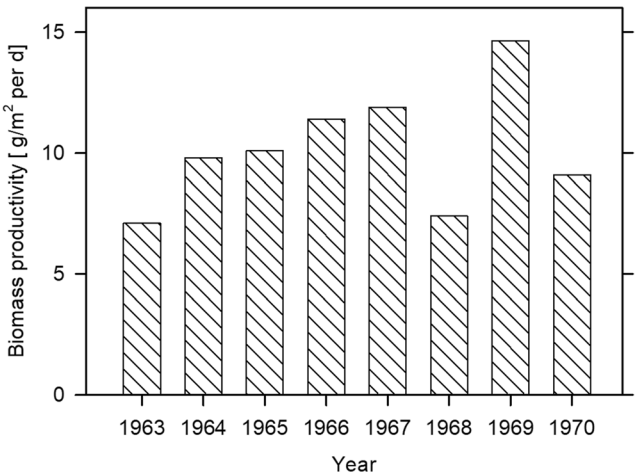

Fig. 8 Overview of averaged biomass productivity (g/m^2/day) of the second generation of thin-layer cascades (area 900 m^2) in Třeboň in the period of 1993–1970. The data were evaluated as an average during cultivation season between May and September regardless of the strain (Šetlík et al. 1967; Nečas and Lhotský 1968; Nečas and Lhotský 1969; Nečas and Lhotský 1973)

expected food crisis. After the political turmoil in 1968, research activities were substantially inhibited.

The productivity of the third generation of thin-layer cascades (area of 650 m^2) was monitored between 2000 and 2017 (Fig. 9a). We observe the trend of increasing averaged biomass productivity from about 14 g/m^2/day in 2000 to about 17–18 g/m^2/day in 2016–2017. It is important to note that biomass productivity in moderate climate zones varies according to weather conditions in the particular year, and it is responsible for decreased productivities to about 10 g/m^2/day in 2012 and to about 13 g/m^2/day in 2014 which also caused low biomass harvest (Fig. 9b).

After the turn of millennium

Compared with the units used in the 1990–2000, the latest generation of TLC has been innovated to improve the cultivation process and ease of maintenance (Fig. 10) (Masojídek et al. 2013; Masojídek et al. 2015). The latest unit has an area of 90 m^2 and is made up of two identical platforms where microalgae culture is exposed to sunlight in a north-south orientation. Compared to the previous generation made of fragile glass plates glued to metal frames, the cultivation surface was made of stainless steel plates which can be easily cleaned and maintained, avoiding corrosion and damage by hailstone or snow. The slope of each platform is (independently) adjustable between 0 and 3% which in combination with a variable flow (20–50 cm/s) maintained by a pump make it possible to set-up suspension layer thickness between 5 and 15 mm. Thus, we can adjust layer thickness in order to study the optimal conditions at varying biomass densities regulating optimum culture irradiance. The shape of the retention tank was designed to minimize the dark volume of the microalgae culture which can be lower than 10%. The exposed surface to total volume ratio (S/V) can be maintained in the range of 60–180 1/m in this unit. All

Fig. 10 Outdoor thin-layer cascade for cultivation of microalgae constructed from stainless steel. It has an area of 90 m^2 and can be operated with a volume between 500 and 1500 L. The surface-to-total-volume ratio can be operated in the range between 60 and 180 1/m corresponding to a layer thickness of suspension between 5 and 15 mm

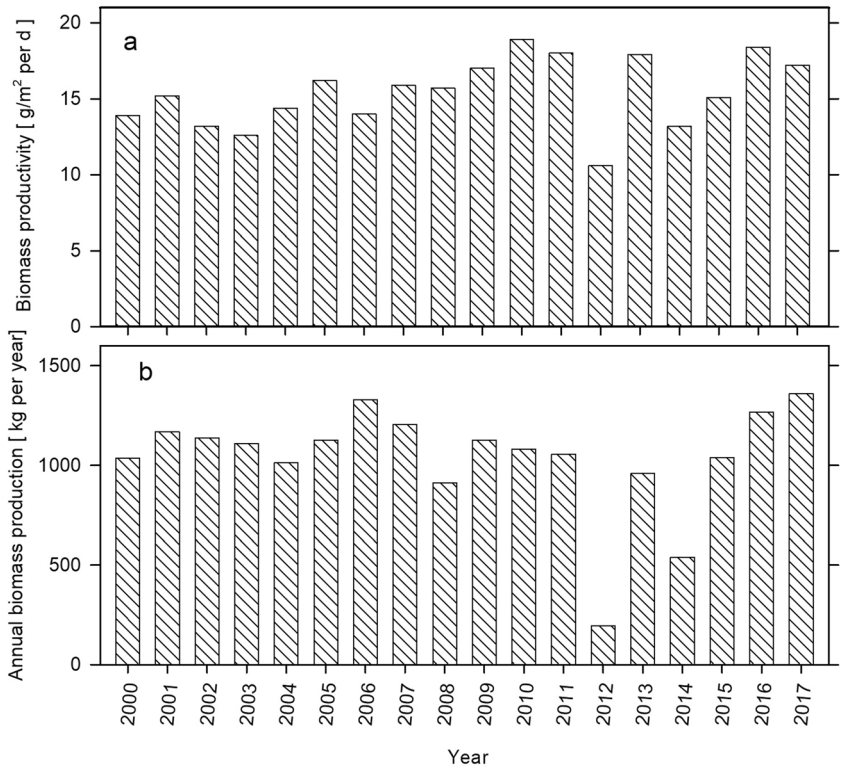

Fig. 9 Overview of biomass productivity (g/m^2/day; panel **a**) and total biomass harvest (kg/year; panel **b**) of the third generation of thin-layer cascades (area 660 m^2) in Třeboň in the period of 2000–2017. The data were calculated as an average during cultivation season between May and September

materials used for construction are biocompatible (stainless steel, PVC, PE, zinc-galvanized parts). The construction is durable to climate conditions and corrosive environmental factors for long time periods.

Compared to cultivation surfaces made of glass or polypropylene, the great advantage of stainless steel cultivation surface was solidness, easy cleaning and resistance of the material to harsh weather or culture conditions (UV radiation in summer, frost in winter). The use of this demonstration unit was intended for microalgae production as food and feed additives, especially enriched in certain bioactive compounds (e.g., carotenoids, polyunsaturated fatty acids, etc.) or chemical elements (Se, Cr, I, Fe, Zn) as well as for wastewater treatment or marine strain culturing.

In the 1990–2000s, massive development of microalgae biotechnology has occurred and returned to the stage again due to the potential shortage of fossil fuels for transportation and their ever-increasing costs. The aim should be at least partial replacement of fossil fuels, especially oil and natural gas by renewable biofuels. Unfortunately, biodiesel from oil crops, waste cooking oil and animal fat cannot realistically satisfy even a small fraction of the existing demand for transport fuels. Agro-fuels (i.e., bio-diesel and bio-ethanol) for transport vehicles have been produced almost exclusively from food and feed crops grown on agricultural land such as rape, sugar beet, corn, or wheat which has a number of very serious negative consequences: Huge monocultures, high water consumption and the use of high doses of fertilizers and pesticides also increase food price. Microalgae have been considered as one of the potential sources of biofuels (Chisti 2008). Although the economic feasibility of biofuel production has still been questionable, it has also supported massive research and development of microalgae biotechnology. The quest for cheap production of biomass led to use of wastewaters as nutrient sources—animal wastes, municipal wastewaters and industrial wastes (including CO_2 from flue gases) for biomass production. Thin-layer cascades as high production cultivation units were tested for this purposes.

A special, multiple cascade pilot plant was built in Třeboň to produce microalgae biomass as a potential source of the third-generation biofuels (Doucha et al. 2005). The cascade was similar to a semi-closed system as it was placed in a greenhouse to use solar radiation as the source of light and heat and CO_2 was obtained from flue gas in order to decrease production costs. In this system, the culture was circulated over the cascade of 8 polypropylene lanes connected by troughs (total area of 56 m^2 and total volume of 500–600 L) which were arranged in a meandering way with an inclination of 1.6% (Fig. 11). The flow velocity was about 0.5 m/s with culture layer thickness of 6–7 mm. It was estimated that about 50% of flue gas decarbonisation can be attained in the photobioreactor and 4.4 kg of CO_2 is needed for the production of 1 kg (dry mass) microalgae biomass. A scheme of a combined farm unit was proposed including anaerobic digestion of organic agricultural wastes, production and combustion of biogas, and utilization of flue gas for production of microalgae biomass, which could be used in animal feeds. The placement of the cultivation unit in a greenhouse can partially prevent cross-contamination by invasive microorganisms and also control culture conditions, especially during bad weather days.

Fig. 11 A multiple-lane cascade system constructed as a semi-closed system placed in a greenhouse (Institute of Microbiology, Třeboň, Czech Republic). The culture was circulated over the cascade of 8 smooth polypropylene platforms arranged in a meandering way with a declination of 1.6% (total area of 56 m^2 and total volume of 500–600 L) which were connected by troughs. The flow velocity was about 0.5 m/s with a culture layer thickness of about 6–7 mm

A similar labyrinth-like thin-layer system of 32 m^2 with total volume of 1200 L was constructed at the University of Almería, Spain (Fig. 12) (Morales-Amaral

Fig. 12 Labyrinth-like thin-layer system of 32 m^2 in area with total volume of 1200 L was constructed at the University of Almeria, Spain (Morales-Amaral del Mar et al. 2015). The layer thickness of culture is about 20 mm. The culture is pumped from the aerated tank to the first layer using a low-stress centrifugal pump; then it is circulated by gravity at 0.2 m/s until it returns back to the tank

Fig. 13 A large-scale plant consisting of cascade raceway modules of 1500 m^2 (volume of 100,000 L) was installed in Pataias, Portugal, for the BIOFAT project (designed and built by the company A4F EU). The unit consists of two sloped platforms (declining of 0.5%), 10 m wide and 75 m long which form a cascade-like system running in opposite directions. This facility is a hybrid between a raceway pond and a sloping cascade since the layer thickness is about 40 mm, resulting in the S/V ratio of about 15 1/m. In this case, the operating biomass density is about 4 g/L DW (courtesy by Luis Costa)

del Mar et al. 2015). The culture was pumped from the aerated tank to the first layer and back to the tank using a low-shear centrifugal pump at a flow velocity of about 0.2 m/s. The layer thickness was about 20 mm. Side-by-side in one greenhouse, this system was compared with a raceway pond of the same area having a layer thickness of 120 mm circulated by a paddle wheel. Both systems were used to study outdoor production of the freshwater microalga *Scenedesmus* sp. using centrate from anaerobic digestion as a sole nutrient source. The aim was to estimate feasibility of large-scale microalgae production systems reducing the biomass cost by the use of waste instead of expensive fertilizers and disposing abundant, nutrient-rich wastes (Morales-Amaral del Mar et al. 2015; Acién et al. 2017). Such a combination may help to increase the possibility of producing commodities, like biofuels, from microalgae biomass by coupling their production to treatment processes.

A large-scale production plant, consisting of two cascade raceway modules of 1500 m^2 (total volume of 100,000 L), was built within the FP7 EU project BIOFAT (www.biofat-project.eu) by the company A4F in Pataias, Portugal, in 2011–2016 (Fig. 13). Each unit consisted of two sloped platforms, 10 m wide and 75 m long (declining by 0.5%), which form a cascade-like system running in opposite directions. This facility is a hybrid technology between raceway pond and sloping cascade since the layer thickness is about 40 mm, resulting in the S/V ratio of about 15 1/m. In this case, the operating biomass density is about 4 g/L DW. The unit was used for production of biomass for nutritional use.

In 2016, an international team led by the University of Almería opened the EU H2020 project SABANA (http://www2.ual.es/sabana/project), the goal of which is to develop and demonstrate an integrated microalgae-based sustainable biorefinery to produce a range of value-added products

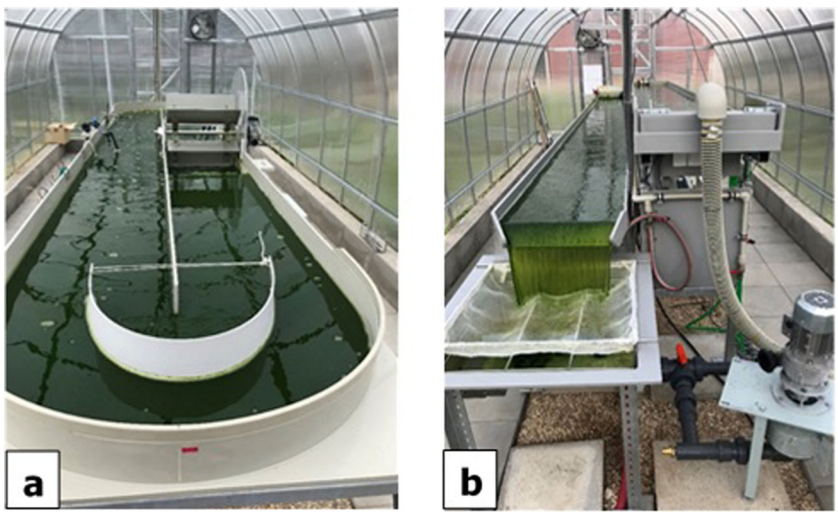

Fig. 14 (**a**) Thin-layer raceway pond mixed by a paddle wheel of 5 m^2 with a working volume of 100–600 L, culture layer thickness between 15 and 60 mm, and flow velocity about 0.2 m/s (manufactured by F&M s.r.l., Firenze, Italy). (**b**) Thin-layer cascade of 5 m^2 with working volume 60–70 L, culture depth 7–10 mm and flow velocity 0.5 m/s (manufactured by Agrico Ltd., Třeboň, Czech Republic) placed in a polycarbonate greenhouse with temperature control by electric fans and heaters for better control of suitable cultivation conditions (Centre Algatech Třeboň). The raceway set-up (panel **a**) was modified to enable cultivation in a thin layer (minimum of about 15 mm), as a paddle wheel was placed in a sump to secure gentle mixing (flow speed about 0.2 m/s) giving a total surface to volume ratio about 40 1/m. In the thin-layer cascade (panel **b**) with adjustable surface declination (0–3%) where the ratio of total surface to volume is about 80 1/m, various types of pumps have been tested for culture circulation. Both units are equipped with automatic addition of CO_2 on the basis of pH (8 ± 0.2)

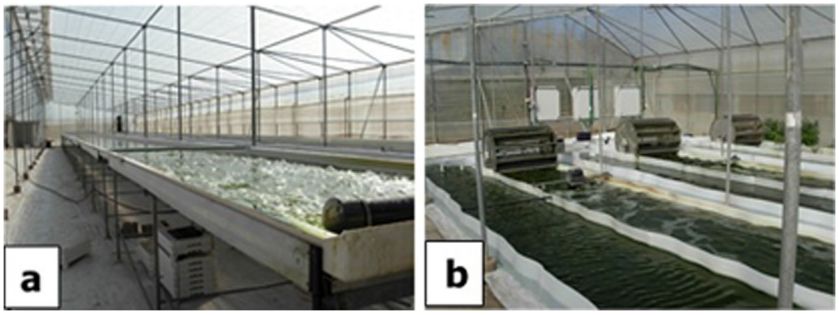

Fig. 15 R&D facilities consisting of two thin-layer cascades (**panel a** ;area of 60 and 140 m^2, layer thickness of 15–20 mm, 2400 and 3400 L, respectively) and three raceway ponds (**panel b**; 13.5-cm culture depth, each 80 m^2 and 12,000 L) mounted in a PE greenhouse were constructed by the University of Almería, Spain

(biostimulants, biopesticides and aquafeed additives) and low-value products (biofertilizers, aquafeed) for agriculture and aquaculture recovering nutrients from wastewaters (sewage, centrate, or pig manure). The final goal to achieve is to build and operate a demonstration facility for producing biofertilizers/biopesticides and aquafeed on a 5-ha scale.

Two experimental cultivation systems—raceway ponds and thin-layer cascades—have been tested at Algatech Centre Třeboň for their suitability to culture selected microalgae strains. In laboratory and pilot-scale trials, the most promising freshwater microalgae strains with biostimulating and biopesticide activities were characterized and examined for large-scale production in different cultivation systems—raceway ponds and thin-layer cascades which basically differ only in circulation devices—paddle wheel or centrifugal pumps (Fig. 14). It is an important point when either single-cell or filamentous microalgae are cultured as concerns their sensitivity to friction. Both experimental units—raceway ponds and thin-layer cascades—were placed in greenhouses to protect cultures from cross-contamination and maintain the same cultivation conditions. Monitoring techniques based on physiological behaviour and photosynthetic activity (Chl fluorescence techniques, dissolved oxygen measurements) to optimize culture growth in outdoor large-scale units were also tested in these trials.

Lately, experimental R&D systems of raceway ponds and thin-layer cascades have been constructed at the University of Almería to test microalgae cultivation based on seawater and wastewaters (Fig. 15). Large-scale DEMO plants of 1 ha including biomass processing technology will be finished in 2019.

Conclusions

Thin-layer, cascade-like systems have been used in a number of countries worldwide (Bulgaria, Italy, Germany, Portugal and Spain) as relatively simple, low-cost and highly productive units for microalgae culturing. TLCs bring together the advantages of open systems (direct sun irradiance, evaporative self-cooling, simple cleaning, low maintenance costs and increased interface for light dilution and oxygen stripping) with the positive features of closed systems (operation at high biomass densities achieving high productivity). Among the most significant advantages of TLCs compared to raceway ponds are the operation at much higher cell concentrations, very high daylight productivities, and the possibility to store the culture in a retention tank at night or in the case of unfavourable weather conditions. Among the limitations of these systems is the possibility of contamination by other microalgae resulting in growing preferentially fast-growing strains or those cultivated in selective environments to overcome this. The other is limited control of cultivation regime which depends on ambient conditions, but this problem can be overcome when cultivation units are located in countries with favourable climatic conditions.

TLCs are usually characterized by low depth (< 10 mm) and fast flow (0.4–0.5 m/s) of culture. It guarantees a high ratio (> 100 1/m) of exposed surface to total culture volume and high turbulence of cells compared to mixed ponds or raceways. Rapid light/dark cycles of cells (frequency of 10–100 Hz) are required to match turnover of the photosynthetic apparatus in order to utilize irradiance efficiently and facilitate higher biomass productivity (Hu et al. 1998; Richmond 2013; Zarmi et al. 2013). Applied intermittently to the individual cells in turbulent cultures, high irradiance is diluted by being available in smaller doses to more cells within a given time span and volume. Thus, the light is used more effectively, compared with poorly stirred cultures.

These mentioned features of thin-layer systems are important from a biotechnological point of view in order to optimize the growth of outdoor microalgae mass cultures under varying climatic conditions.

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Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Annex V

(related to Chapter 3 Cultivation Systems)

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Diurnal changes of photosynthesis and growth of *Arthrospira platensis* cultured in a thin-layer cascade and an open pond

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ABSTRACT

Diel changes in photosynthetic performance and biomass productivity were thoroughly examined in *Arthrospira platensis* cultures grown outdoors in an open circular pond (OCP) and a thin-layer cascade (TLC). The two cultures were grown at the same areal biomass density, but temperature maxima were adjusted to optimal (33 °C) and suboptimal (25 °C). At the optimal temperature, the cultures grown in TLC showed about 20% higher photosynthetic activity than those in OCP, while at the suboptimal one photosynthetic activity dropped by 20% and 35% in the TLC and OCP, respectively. Accordingly, the highest biomass productivity over 20 g m^{-2} d^{-1} was attained in the TLC at the optimal temperature, while at the suboptimal temperature the productivity decreased by 20%. In the OCP, the biomass productivity at both temperatures was about one third lower compared to those in the TLC.

The better culture performance in the TLC was mainly ascribed to the shorter light path that promoted much faster light/dark cycles favourable for photosynthesis, as well as the faster warming of the cultures in the morning as compared to the OCP cultures. Monitoring photosynthesis performance of a culture can indicate design improvements, which may capitalize this photochemical advantage, increasing biomass productivity further.

1. Introduction

The cyanobacterium *Arthrospira platensis* (*Spirulina*) is one of the most cultivated microalga at large-scale, mostly as food and feed supplement. The annual biomass production of *Arthrospira* in outdoor ponds has been estimated in about 13,000 metric tons, with a mean daily yield ranging between 8 and 10 g DW $m^{-2}d^{-1}$ [1–3]. Various environmental factors can affect growth, and among them, suboptimal temperature and light availability to the cells are the most important. Cultures in open ponds are characterised by a low surface to total volume ratio (S/V), usually in the range between 5 and 10 m^{-1}, due to the necessity to maintain a sufficient culture depth for hydraulic reasons. Volumetric productivity is therefore reduced due to the long light path and this increases the harvesting costs as cultures are usually maintained at low biomass density, ranging between 0.3 and 0.5 g L^{-1}. Another, important limitation encountered in open ponds is the daily temperature profile. Suboptimal values are usually common in the morning while excessive values may be reached for a large part of the day and at night. De-synchronization between temperature and light courses in the morning can create the conditions for low-temperature photoinhibition [4]. Temperatures around 35 °C are considered favourable, while 25 °C is suboptimal; on the other hand, high temperature at night increases the loss of biomass due to respiration [5].

Microalgae cultivated under certain irradiance intensities exhibit a corresponding growth rate and an array of pigments arranged in antenna complexes, as a result of acclimation to a given irradiance profile [6]. Culture mixing represents the most practical way to achieve a uniform exposure of the cells to light. However, mixing rates generating frequencies higher than tens of Hz are impossible to reach in open ponds due to their long optical light path. In this respect, thin-layer systems have a more favourable set-up for faster light/dark cycles.

The aim of this work was to examine how different light paths, and

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consequently different light exposure patterns of the cells, affect their photosynthetic activity and consequently the productivity of cultures grown in thin-layer cascade (TLC) and open circular pond (OCP). The behaviour of outdoor *Arthrospira* cultures was compared when grown under optimal and suboptimal temperature regimes. Chlorophyll fluorescence techniques (fast induction kinetics and pulse-amplitude modulation) were used to monitor diel changes in photosynthetic performance.

2. Materials and methods

2.1. Strain and culture conditions

The filamentous cyanobacterium *Arthrospira platensis*, strain M2 from the culture collection of the Institute of Ecosystem Study, CNR at Sesto Fiorentino (Italy), was grown in Zarrouk's medium [7]. The cultivation trials were carried out outdoors in an open circular pond (OCP; 100 mm deep, total exposed surface of 1 m^2; S/V ratio = 10 m^{-1}), and in an inclined thin-layer cascade (TLC; 10 mm deep, total exposed surface of 5 m^2; S/V = 83 m^{-1}). The two culture units were exposed to diurnal irradiance pattern and only upper temperature limit was controlled at either optimal (33 ± 1 °C) or suboptimal (25 ± 1 °C) values using a cooling system; otherwise temperature rise was spontaneous as promoted by sunshine. The suboptimal temperature of 25 °C was considered sufficiently distant from the optimal (8–10 °C below), to demonstrate its effects on photosynthesis and productivity.

The experiments were carried out in Sesto Fiorentino (GPS coordinates 43°49′6.81″ N; 11°12′7.08″ E). The starting biomass density was about 5 g L^{-1} in TLC, while it was about 0.5 g L^{-1} in OCP. Thus, in this set-up, both cultures were cultured at similar areal biomass density of about 50 g m^{-2}. The pH of cultures was maintained at 9.4 by automatic addition of CO_2.

2.2. Outdoor culture units

One type of culture unit (short light path) used in these experiments, an inclined thin-layer cascade (TLC), was based on open circulation units for large scale culturing of microalgae (Fig. 1, left) [8,9]. A unique feature of TLC is that the microalgal suspension circulates in a thin layer (about 10 mm thick) over a sloped glass plane (5 m long and 1 m wide) exposed to solar radiation in a southwards orientation. The unit consisted of 6 parts: cultivation surface (photostage), reservoir (degasser), pump for culture circulation, PVC tube riser with manifold distributor, cooling system (heat exchanger) and measurement and control sensors. At the lower end the culture was collected in a reservoir and continuously delivered by a pump to the upper edge (start) of the inclined surface through a perforated distributor. Cultures were circulated night and day using a special type of horizontal centrifugal pump that does not break *Arthrospira* filaments even when operated at the maximum speed (up to 1000 rpm). It was made of PVC equipped with a paddle wheel enclosed in the housing. The design of the pump was described elsewhere [10]. For the experiments, the microalgal suspension flowed at a speed of 8 cm s^{-1} down an inclined surface with a 3% slope. Heat exchange was achieved by circulating cold water in a coaxial stainless steel pipe (i.d. of 1.5 cm, 4 m long) running inside the PVC tube riser (5 cm diameter) and connected to the cooling system. The working volume of the cascade was 60 L, 50% of which was exposed on the cascade and 50% in the darkened part of the system (*i.e.*, 2 L in the pump, 15 L in the retention tank, and 13 L in the PVC pipes).

The cultures were cooled by evaporation and additionally the heat exchanger was used when limiting the temperature to 25 °C. To minimize CO_2 loss, the injection was performed in the rising tube, downstream of the pump. In case of bad weather, the cultures these were stored in the reservoir and mixed by air bubbling.

The other type of cultivation unit was an open circular pond (OCP). A central, perforated rotating PVC arm (15 rpm) was used for culture mixing, air bubbling and CO_2 supply. Temperature control was achieved *via* a stainless steel loop (connected to water cooling system) that was placed at the bottom of the pond (Fig. 1, right).

Both TLC and OCP units were equipped with probes to measure culture temperature and pH. The addition of CO_2 to the culture was controlled electronically to maintain the pH at 9.4 (software designed by Chemitec, Florence, Italy).

2.3. Analytical procedures

Determination of dry biomass weight (DW) was performed in duplicates using 10-mL samples, using pre-weighted glass microfiber filters (Whatman GF/F, Maidstone, England). The filters with the cells were washed twice with deionized water (approximately 50 mL total), oven-dried at 105 °C and weighted (precision of ± 0.1 mg). Volumetric biomass productivity (g $L^{-1}d^{-1}$) was calculated as the difference of biomass (dry weight) concentrations between two consecutive days. The areal biomass productivity (g m^{-2} d^{-1}) was calculated taking into consideration the total volume of the cultures and the illuminated area of the cascade (5 m^2) or pond (1 m^2). Outdoor cultures were operated in a semi-continuous regime as they were diluted in the early morning with fresh medium to adjust the desired biomass concentrations.

For chlorophyll concentration measurements, 5-mL samples were collected in glass tubes and centrifuged at 2600 × *g* for 8 min. The pellets were re-suspended in 5 mL of methanol and the tubes were heated in a water bath at 70 °C for 3 min, then these were centrifuged. Absorbance of the supernatant was measured at 665 and 750 nm.

The average specific chlorophyll optical absorption cross-section (a^*) of the cells (m^2 mg^{-1} chl*a*) was determined from *in vivo* absorption spectra (in the range from 400 to 750 nm) according to reference [11] using a spectrophotometer (Varian Cary50 UV–visible). To minimize the impact of the light scattering effect from the cell surface, the sample cuvette was placed close to the detector window.

For the measurement of phycocyanin (PC) content, 5 mL samples were collected in glass tubes and centrifuged at 2600 × *g* for 8 min. The pellet was resuspended in 0.2 mL of phosphate-buffer and glass beads (diameter 0.17–0.18 mm, B. Braun Biotech Int., Germany) were added to the sample. The mixture was vortexed for 10 min in order to break down the cells, and then phosphate buffer was added to reach the final volume of 5 mL. The mixture was centrifuged, the supernatant was

Fig. 1. Cultivation units used in trials – inclined thin-layer cascade (TLC; left) and open circular pond (OCP; right).

transferred to 15 mL Falcon tubes and centrifuged again at 12500 × *g* for 10 min. The absorbance of the extract was measured at 615 nm and the concentration of phycocyanin was calculated according to Bennett and Bogorad [12]. The assays of chlorophyll and phycocyanin were performed in duplicates.

2.4. Chlorophyll *a* fluorescence measurements

Changes of the physiological status and photosynthetic performance of cultures were monitored by two fluorescence techniques – fast fluorescence induction kinetics and saturation pulse analysis of fluorescence quenching.

For chlorophyll fluorescence measurements *off-situ* (rapid light-response curves RLC and fluorescence induction kinetics OJIP) samples were taken from the cultures, diluted to 0.2 to 0.3 g DW L^{-1} (corresponding to 5 to 7 mg Chl L^{-1}) with fresh medium and then dark adapted for 5–10 min. By dilution we prevented re-absorption problems in dense cultures and provided sufficient illumination to reduce plastoquinone pool (close PSII reaction centres). Measurements were carried out under well-defined laboratory conditions retaining the same 'light' exposure history of samples to avoid modifying the photoacclimation status of the cells. Data were recorded in duplicates (RLC) or triplicates (OJIP kinetics) at specified daytimes (CEST). The fluorescence nomenclature follows [13] as later elaborated by [14,15].

2.4.1. In-situ chlorophyll fluorescence measurements

Chlorophyll fluorescence measurements were carried out in parallel in TLC and OCP in order to compare the photosynthetic performance between the two culture systems. The fibre optics of the a pulse-amplitude-modulation fluorimeter (PAM-2100, H. Walz, Effeltrich, Germany) was placed about 3 mm deep in the culture with an inclination of about 45–60° to sun direction. To allow a reliable determination of the F_0 and F_m* (maximum fluorescence yield after a short dark adaptation), a part of the surface of TLC or OCP (about 1 m × 1 m surface) were covered with a dark polyethylene sheet and a train of light pulsed were applied to the culture until constant values were reached. The variables F′ and F_m′ were recorded in the light exposed cultures to calculate the actual photochemical yield Y_{II} [(F_m′ − F′) / F_m′]. The rETR values were calculated as (F_m′ − F′) / F_m′ × PFD. We consider this to be a relative measure useful to compare culture activities. Measurements were carried out at 1–2 h intervals during sunny days.

2.4.2. Maximum PSII photochemical yield F_v/F_m

The maximum PSII photochemical yield F_v/F_m[= (F_m − F_0) / F_m] was determined *off-situ* in the dark-adapted samples withdrawn at time intervals from the outdoor cultures. A low-intensity modulated measuring light (LEDs of a peak wavelength at 650 nm; < 0.3 μmol photons m^{-2} s^{-1}, frequency of 0.5–1 kHz) that is not inducing any photosynthetic activity, was used to measure F_0. In case of cyanobacteria, the 'true' F_m was determined under low actinic illumination (~150 μmol photons m^{-2} s^{-1}) in the presence of 10^{-5} M DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] that stops electron transport behind the PSII complex. In the dark, cyanobacteria are in State 2, with high non-photochemical quenching since the plastoquinone (PQ) pool is reduced by respiratory electron transport. When illuminated, cyanobacterial cells rapidly shift to State 1, with lower non-photochemical quenching and increased PSII fluorescence [16]. In the absence of DCMU, the apparent value in dark-adapted *Arthrospira* cells was usually 15%–20% lower than the 'true' F_m.

2.4.3. Rapid light-response curves

Rapid light-response curves (RLCs) of microalgae samples taken from the outdoor cultures were measured *off-situ* in a light-protected measuring chamber with mixing (3 mL glass cuvette, light path of 10 mm) using a pulse-amplitude-modulation fluorimeter (PAM-2500, H. Walz, Germany). A series of stepwise increasing irradiance intensities (red LEDs; 0–2500 μmol photons m^{-2} s^{-1}) were applied at 20-s intervals to obtain the light-adapted fluorescence level F′ and at the end of each step a saturating pulse (> 10,000 μmol photons m^{-2} s^{-1}, 0.6 s duration) was triggered to reach the maximum fluorescence level F_m′. The actual PSII photochemical quantum yield in the light, Y_{II} was determined as (F_m′ − F′) / F_m′ in the light-adapted state at respective irradiance level.

Analysis of RLC was used to calculate changes in important parameters, namely the maximum electron relative electron transport rate through PSII ($rETR_{max}$), the initial slope of rETR *vs.* PFD curve, α, the onset of light saturation (irradiance saturating photosynthesis) E_k, and non-photochemical quenching NPQ [17–19]. The curves were fitted to the non-linear least-squares regression model by Eilers and Peeters [20] using PamWin 3 *software*.

2.4.4. Non-photochemical quenching (NPQ)

In principle, non-photochemical quenching NPQ [= (F_m − F_m′) / F_m′ = Y(NPQ) / Y(NO)] is a measure of energy dissipation as heat. It is inversely related to photochemistry (Y_{II}) and is considered a safety valve protecting PSII reaction centres from damage by excess irradiance. Non-photochemical quenching can be resolved into two components: Y (NPQ) [= F′/F_m′ − F′/F_m′] which quantify regulated (in the light-harvesting antennae as pH- and xanthophyll regulated quenching) and non-regulated Y(NO) [= F′/F_m] (energy trapped in closed PSII reaction centre) thermal dissipation processes [21]. Semi-empirical evaluation has indicated that the sum Y(PS II) + Y(NPQ) + Y(NO) = 1.

2.4.5. Fast fluorescence induction kinetics (OJIP-test)

Fast chlorophyll fluorescence induction kinetics (OJIP test) was measured *off-situ* using a hand-held fluorimeter (AquaPen AP-100, P.S.I. Brno, Czech Republic). Diluted (0.2 to 0.3 g DW L^{-1}) samples taken from the cultures at regular intervals, dark-adapted for 5–10 min were transferred to a 3-mL measuring cuvette (light path of 10 mm) that was mounted in a light-protected holder in front of the detector (adjustable measuring light pulses, ~2.5 μs) while red LEDs served as high-intensity actinic light source from both sides of the cuvette (up to 3000 μmol photons m^{-2} s^{-1}), perpendicular to the detector. In case of rapid induction curves it was necessary to dilute cultures to prevent high Chl concentration from overloading the signal of the fluorimeter.

The fast fluorescence induction kinetics (OJIP-test) was recorded in the time range between 50 μs to 1 s; the signal rises rapidly from the origin (O) to a peak (P) *via* two inflections – J and I [22]. The O point of the fluorescence induction curve represents a minimum value (designated as constant fluorescence yield F_0) when PQ electron acceptors (Q_A and Q_B) of the PSII complex are oxidized, *i.e.* the signal emitted from excited Chl-a molecules in the light-harvesting antennae before excitons have migrated to the PSII reaction centre. The inflection J occurs after ~2–3 ms of illumination and reflects the dynamic equilibrium (quasi-steady state) between the Q_A/Q_A^-. Following the gradual reduction of consecutively positioned acceptors in the electron transport chain (the mobile plastoquinone PQ, the cytochrome b_6f complex and PSI acceptors), the intensity of Chl fluorescence increases from the point J to the point I; this process lasts from 30 to 50 ms. At the level I of the fluorescence induction kinetics, the rate of electron transport toward the PQ pool and from the PQ pool to PSI acceptors ($NADP^+$, *etc.*) become equal [23,24]. After complete reduction of available acceptors, fluorescence reaches its maximum (F_P) at the point P (ends at about 300–500 ms), which corresponds (at a given intensity of actinic light) to the maximum reduction of PSII acceptors and to the lowest yield of photochemical reactions.

The recorded OJIP curves were double-normalized to F_0 and F_m in order to better distinguish changes in the intermediate steps J and I that represent various reduction states of the PSII electron carriers. V_j and V_i represent the chlorophyll fluorescence levels at the corresponding points on the fluorescence induction curve. From the fluorescence levels

at J (F_j) and I (F_i) the variables V_j and V_i were calculated as follows: $V_j = (F_j - F_o) / (F_m - F_o)$ and $V_i = (F_i - F_o) / (F_m - F_o)$.

2.4.6. Light measurements

Values of photosynthetically active radiation (PAR) in the laboratory were measured as 15-s average values using the LI-250A light meter (Li-Cor, USA) equipped with spherical sensor model SQSA0191 (H·Walz, Germany) placed in the cuvette, or with a flat LI-190SA quantum sensor (cosine-corrected up to 80° angle of incidence) outdoors.

2.4.7. Statistical analysis

Sigma Plot 11.0 was used to determine significant differences between treatments. One-way ANOVA and Holm-Sidak test was conducted for every binary combination of systems. *P* values lower than 0.05 were considered different significantly.

3. Results

Outdoor cultivation trials were carried out in order to follow changes in cultures of *A. platensis* grown in the TLC and OCP units. The experiments were carried out on sunny days in August at optimal (33 °C) and suboptimal cultivation temperature (25 °C) regimes to examine how photosynthetic performance, and culture productivity were affected.

3.1. Diel changes of chlorophyll fluorescence quenching recorded off-situ

In "healthy" *A. platensis* cultures grown in TLC, the maximum photochemical yield of PSII, F_v/F_m ranged from 0.60 to 0.65 in the morning (0800 h) showing their good physiological condition (Fig. 2A). In OCP, the culture at optimal temperature did show certain decrease at 1400 h, while at suboptimal temperature a significant decrease to 0.33 (by about 45%) was observed (Fig. 2B). In the TLC there was much less difference in F_v/F_m between the cultures grown at 33 and 25 °C (as compared to those in OCP); only at suboptimal temperature F_v/F_m decreased from 0.61 to 0.53 (by about 13%) at midday. Statistical data analysis (two-way Anova) indicated that the interaction between light and temperature on F_v/F_m was significant in both units with very different light regime (compare F_v/F_m at 33 and 25 °C at 1400 h when irradiance reached 1740 µmol photons $m^{-2} s^{-1}$).

Rapid light response curves (RLCs) indicated significant differences in photosynthetic activity of cultures grown in OCP and TLC (Fig. 3). RLCs in the cultures grown at optimal growth temperatures showed stable photosynthetic activity during the day although daily rETR values in TLC were about 50% higher (~150) as compared to those measured in OCP (~100) (compare Fig. 3A, and B). When *A. platensis* cultures were grown at suboptimal temperature, the maximum rETR values decreased in both units. However, in the TLC culture, the suboptimal temperature did not heavily affect the electron transport activity (rETR) compared to that measured at the optimal temperature, as it was 17% lower (compare Fig. 3B and Fig. 3D).

In contrast, diel courses of rETR curves in the OCP at suboptimal temperature had significantly different trends compared to those grown at optimal one (Fig. 3 A, C). The rETR maximum of 106 was found at 1200 h at suboptimal temperature, but at 1800 h, the $rETR_{max}$ values were lowered to 60 (Fig. 3C), probably due to low light acclimation in the deep layer culture. The average rETR activity in OCP was about 14% lower compared to the cultures grown at optimal temperature. In any case, at suboptimal temperature the rETR activity proportionally decreased in both cultivation units, but the average photosynthetic activity was still significantly lower (rETR = 90) in the OCP culture compared to that in TLC (rETR = 127). To sum up, the highest values of $rETR_{max}$, around 154 were found in the TLC cultures at midday at optimal temperatures and the values were only 16% lower at suboptimal temperature. Compared to the TLC cultures, the rETR values in the OCP were about 30% lower at both optimal and suboptimal temperature (Fig. 4 a,b).

The values of E_k (the saturation irradiance) for both cultures showed very similar trends (not shown here) to those found for $rETR_{max}$.

Light response curves of non-photochemical quenching NPQ showed that the extent and course of NPQ was very different between the OCP and the TLC. Much higher values were found in TLC compared to OCP (Fig. 5a,b). Comparing these results with rETR curves indicates that more energy is absorbed by TLC and used for electron transport (Figs. 3 and 4), but also more energy is dissipated as heat if not used for photochemical processes to avoid damage to the photosynthetic apparatus. At suboptimal temperatures the protective role of NPQ is weakened as the NPQ values are much lower. This was especially observed in OCP at suboptimal temperatures where the cultures showed much lower NPQ values starting from mid-morning.

NPQ [= Y(NPQ) / Y(NO)] can be decomposed into two distinct components: Y(NO), non-regulated thermal dissipation and fluorescence, reflecting the fraction of energy that is passively dissipated in form of heat and fluorescence, mainly due to closed PS II reaction centres while Y(NPQ) corresponds to the fraction of energy dissipated in form of heat *via* the regulated photoprotective mechanisms.

In all experiments, Y(NO) was a dominant part of non-photochemical dissipation, usually 2–3 times bigger than Y(NPQ) (Fig. 6.). At the optimal temperature the values of Y(NO) were lower in the TLC culture (0.5–0.6) than in the OCP (0.6–0.7), but in the latter a significant increase was found at suboptimal temperature. The other component, Y(NPQ) was much lower in both cultures and the suboptimal temperature caused a further decrease of this parameter, especially in OCP. Taking this into account, we may conclude that non-regulated thermal processes (and fluorescence) are a main means of

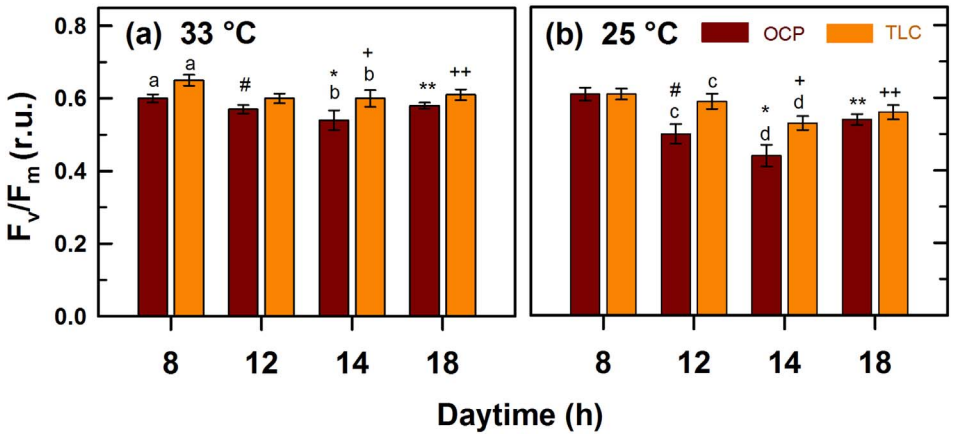

Fig. 2. Diel changes of the maximum photochemical quantum yield of PSII (F_v/F_m) measured in *A. platensis* cultures grown in TLC and OCP at 33 °C (a) and 25 °C (b). (Data are the average of 3–4 measurements; columns labelled by the same letter differ significantly from each other; the same symbols mean significant differences between the two temperatures). During the experiments, photon flux density (PFD) reached 1740 µmol $m^{-2} s^{-1}$ in the middle of day. Daytime corresponds to CEST.

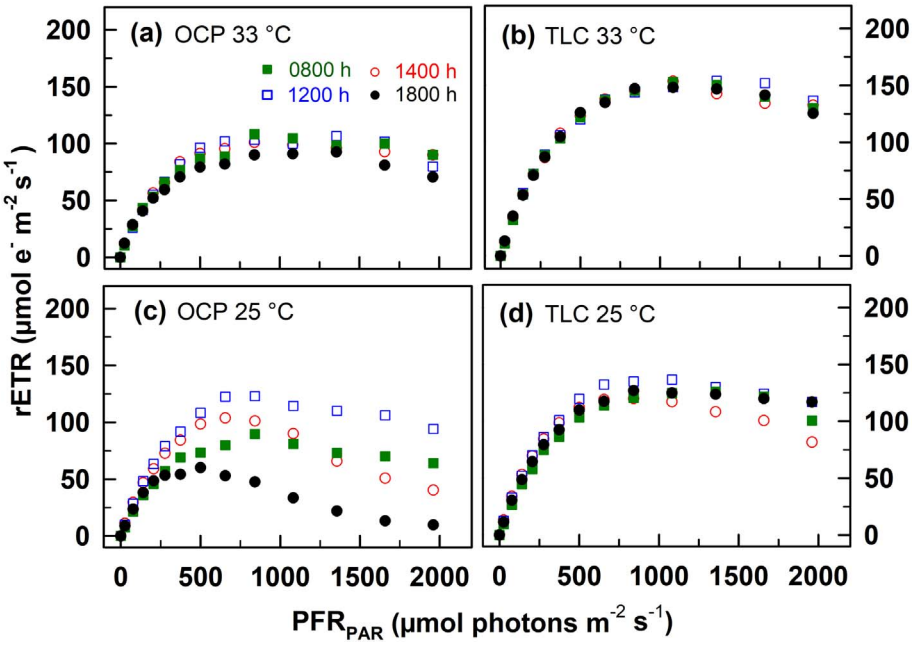

Fig. 3. Diel changes in rapid light-response curves (RLC) of rETR in *A. platensis* cultures grown at optimal (a, b - 33 °C) and suboptimal (c, d - 25 °C) temperature in OCP (a, c) and TLC (b, d). The data calculated by PAM software using Eilers and Peeters model. Light-response curves are shown as colour symbols without error bars as these obscure data point measured at the same irradiance intensities.

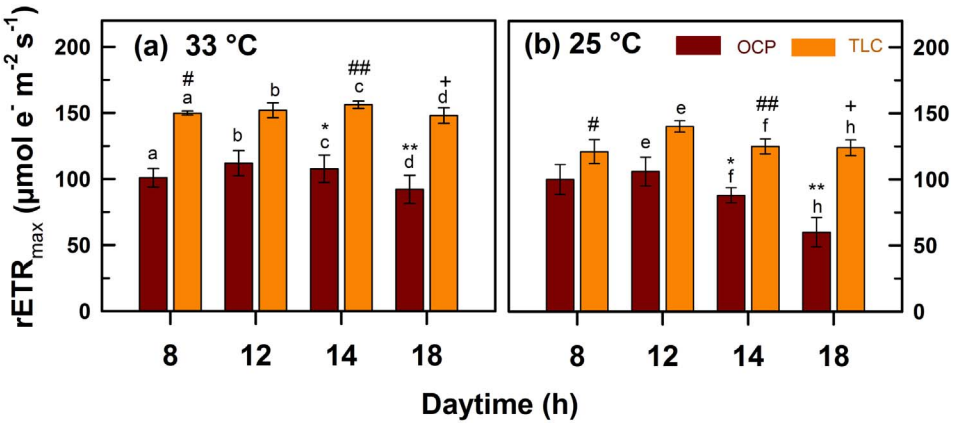

Fig. 4. Diel changes of maximum relative electron transport rate $rETR_{max}$ (calculated from RLC) of *A. platensis* cultures grown at optimal (a - 33 °C) and suboptimal (b – 25 °C) temperature in OCP and TLC. (Data are the average of 3–4 measurements; columns labelled by the same letter differ significantly from each other; the same symbols, *e.g.* $^{\#}$, *, $^{+}$ mean significant differences between two temperatures). Daytime corresponds to CEST.

energy dissipation while regulated photoprotective processes are minor; the latter are less significant in OCR and even more suppressed at suboptimal temperatures.

To analyse diel photochemical changes in further detail, the fast fluorescence induction kinetics (OJIP curves) were recorded at various daytimes in parallel to RLCs. The kinetics showed rather typical differences between TLC and OCP cultures grown at optimal temperature and those grown at suboptimal one (Fig. 7).

Typically, at midday in both OCP and TLC cultures grown at suboptimal temperature, fast fluorescence induction curves showed a significant increase in the J inflection which reflects the accumulation of the reduced "primary" acceptor of PSII, Q_A^-) (Fig. 7). Generally, the Vj and Vi values were higher in the OCP than in the TLC indicating that in the former the cultures were more stressed due to a slow-down in the electron transport within the PSII complex, especially at suboptimal temperature (Fig. 8).

3.2. Changes of chlorophyll fluorescence quenching recorded in-situ

The daily courses of rETR rates measured by Chl fluorescence quenching technique *in-situ* showed that the cultures grown in the OCP at 33 °C (integrated on the daily basis) exhibited about 20% lower values compared to the TLC cultures (Fig. 9a). The differences in the rETR between the two cultures were more pronounced during the first half of the day, than in the afternoon. Most likely this behaviour reflected the different morning temperature profiles as these were different between TLC and OCP cultures. Indeed, culture temperature control was active only in preventing overheating of the cultures (*i.e.*, above 35 °C) while the rise to the experimental temperature (33 or 25 °C) in the morning was spontaneous. However, the temperature of the two cultures at sunrise was similar, but it rose more quickly in the TLC culture than that in OCP (Supplementary data: Fig. S1). Indeed, optimal temperature for growth was reached sooner (by 1100 h) in the TLC than in the OCP (by 1400 h). The differences in the rETR values between the two cultures increased to one third when the cultures were grown at the suboptimal temperature of 25 °C (Fig. 9b). In this case, the differences in the rETR values between the cultures were more uniformly distributed throughout the day. The set level of temperature of 25 °C was reached by 1000 h in both the cultures, thus remaining constant for the rest of the day (results not shown).

3.3. Culture productivity

Biomass productivity was influenced by the two culture systems with their very different light path, and by temperature regimes. The highest biomass productivity over 20 g m^{-2} $d^{-1}$and rETR daily integral was found in the TLC at optimal temperature, while at the suboptimal one it decreased by 20% (Fig. 10). In the OCP, biomass

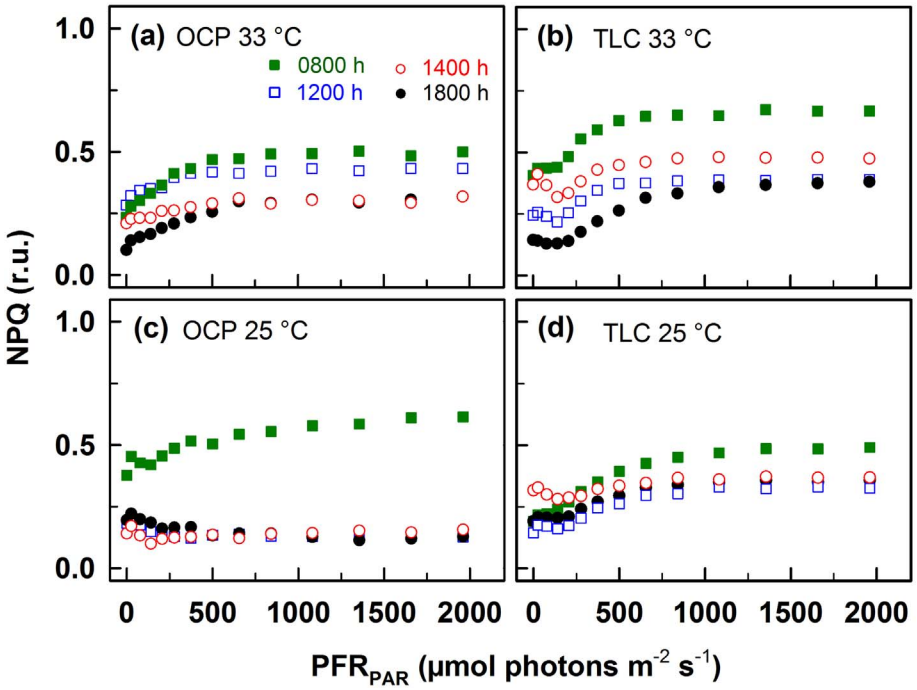

Fig. 5. Diel changes in rapid light-response curves (RLC) of non-photochemical quenching NPQ in *A. platensis* cultures grown at optimal (a, b – 33 °C) and suboptimal (c, d – 25 °C) temperature in OCP (a, c) and TLC (b, d).The data calculated by PAM software. Light-response curves are shown as colour symbols without error bars as these obscure data point measured at the same irradiance intensities.

productivity was about one third lower than that at the same temperature regime in the TLC (Fig. 10). The results indicated that the interaction between culture temperature and culture systems with two very different S/V ratio was not significant. In other words, increasing, or decreasing culture temperature did not result in larger differences in productivity between the two culture systems.

3.4. Pigment content

Pigment content measured in the morning and in the evening showed some differences between the cultures grown in the two systems (Supplementary data in Fig. S2). Phycocyanin concentration was 30% higher in the biomass grown in the OCP compared to that grown in the TLC but there was not big difference between morning and afternoon samples. Chlorophyll content was below 1 and 1.4% in the biomass grown in the OCP and the TLC, respectively.

4. Discussion

In this work we have examined whether different light exposure of *A. platensis* cultures grown in the two culture units – TLC and OCP – with very different light paths, was primarily responsible for difference in their productivity.

As pointed out by Grobbelaar [25,26], microalgal cells growing in a dense outdoor culture can be subjected to three ranges of intermittent illumination (L/D cycles): (i) low frequency cycles of hours to days and years; (ii) medium frequency fluctuations of seconds to minutes, and (iii) high frequency fluctuations of 100 ms (> 10 Hz) and lower. The productivity of a microalgal mass culture is mostly influenced by medium and high frequency fluctuations. Medium frequency fluctuations are the norm in open raceway ponds, and their benefit on microalgal productivity is controversial, while high frequency fluctuations are possible to achieve in closed short light path photobioreactors and in the thin-layer cultures at high mixing rates.

An important prerequisite for enhancing light conversion efficiency

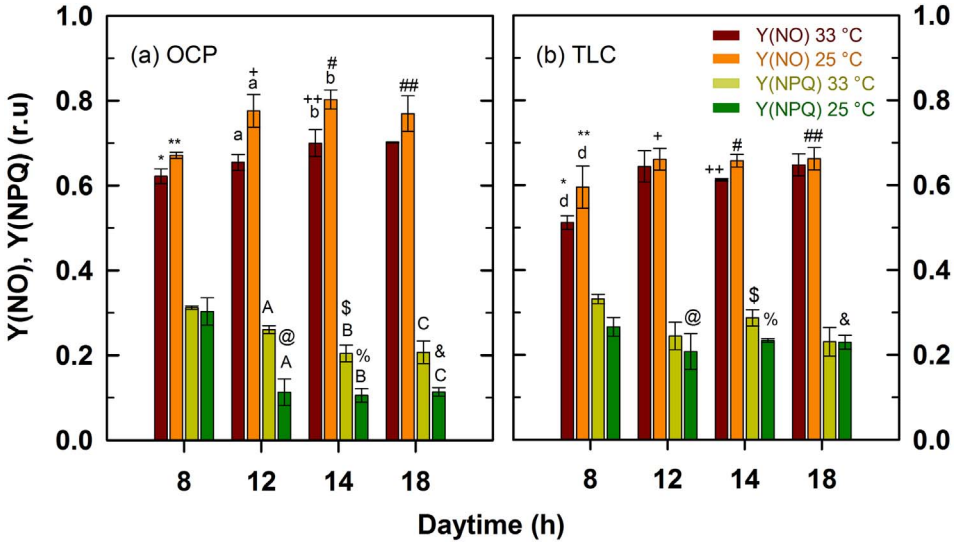

Fig. 6. Diel changes of two components of non-photochemical quenching, Y(NO) and Y(NPQ) (calculated from RLC) in *A. platensis* cultures grown in OCP and TLC at optimal (a – 33 °C) and suboptimal (b – 25 °C) temperature. The values of Y(NO) and Y(NPQ) correspond to rETR$_{max}$. Data are the mean of 3–4 measurements. Statistical analysis showed that columns labelled by the same small and capital letter differ significantly from each other for the Y(NO) and Y (NPQ) at 33 and 25 °C, respectively; the same graphical symbols mean significant differences between the two units, OCP and TLC. Daytime corresponds to CEST.

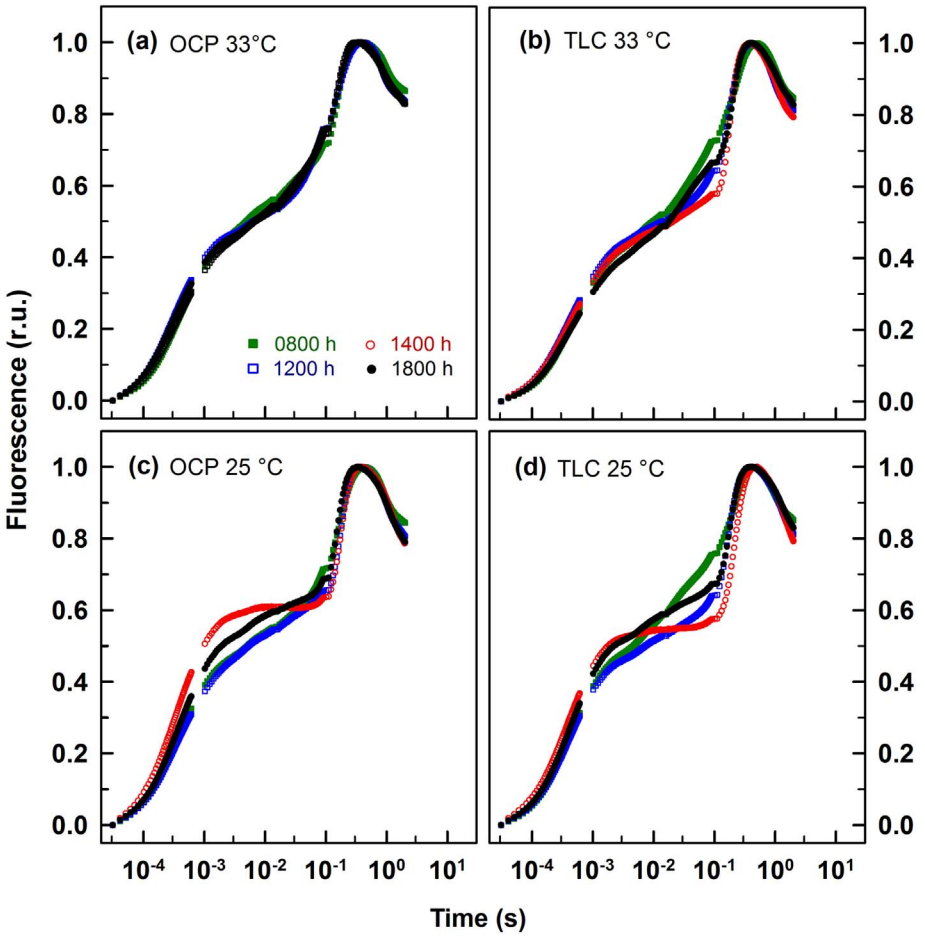

Fig. 7. Diel course of OJIP curves (normalized to F_0-Fm) of *A. platensis* cultures grown in OCP (a, c) and in TLC (c, d) at the optimal (a, b - 33 °C) and suboptimal (c, d - 25 °C) temperature.

would be shortening the L/D cycles to milliseconds, *i.e.* to the time scale of the Q_A (the primary electron acceptor of PSII) electron turnover. Should this not be the case, then the excess energy will be dissipated non-photochemically as heat, or *via* some futile processes. In TLCs, cell turbulence in the thin layer of culture flowing quickly on the tilted surface, promotes short light/dark (L/D) cycles compared to mixing in deep ponds, and therefore cells may benefit from an intermittent light pattern. The importance of the turbulence in outdoor open systems was clearly demonstrated by placing aerofoil pieces in flowing cultures that generated vortices of 0.5–1.0 Hz at flow rate of 30 cm s^{-1} resulting in higher photosynthetic conversion efficiencies from 3.7 to up to 10% [27].

The large difference in the S/V ratio between TLC and OCP strongly influenced photosynthetic activity. Indeed, at both optimal and sub-optimal temperatures the photosynthesis and biomass productivity in the TLC was by about 30% higher than that obtained in the OCP. To better understand the mechanisms behind this, both *off-situ* and *in-situ* measurements of photosynthetic activity were carried out using Chl

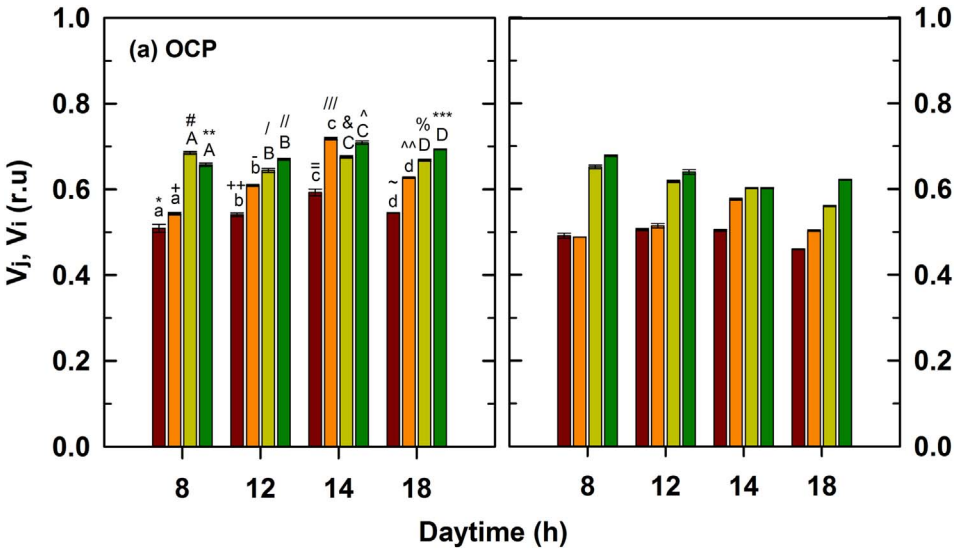

Fig. 8. Diel changes of fluorescence variables Vj (2 ms) and Vi (30 ms) were calculated from fast fluorescence induction kinetics measured in *A. platensis* cultures grown in OCPs (a) and TLCs (b) grown at 33 °C and 25 °C. Daytime corresponds to CEST.

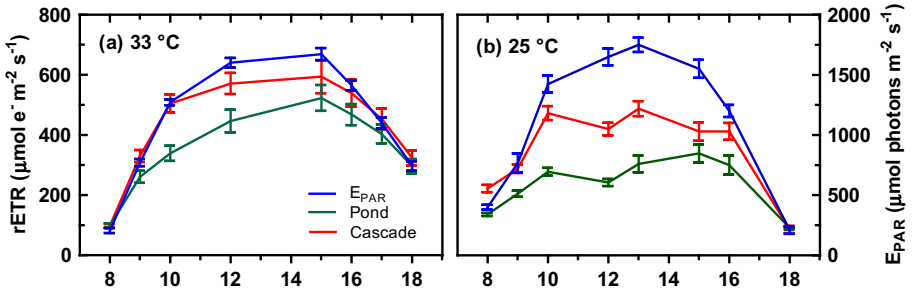

Fig. 9. Diel courses of the rETR measured *in situ* in *A. platensis* cultures grown in TLC and OCP at optimal (33 °C) and suboptimal temperature (25 °C). Daytime corresponds to CEST.

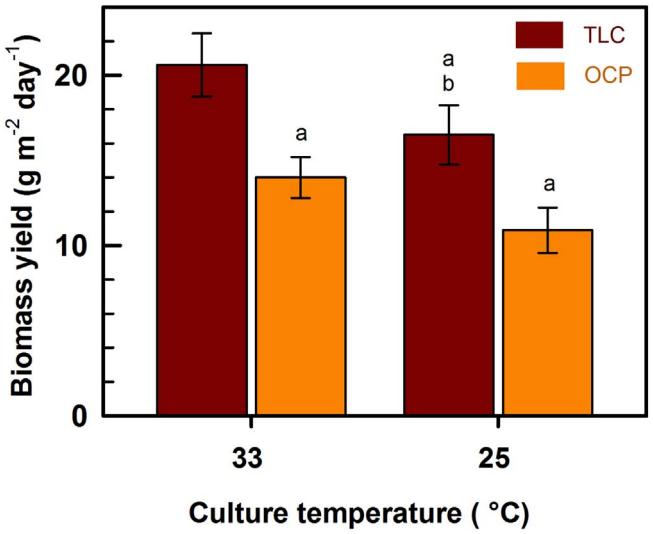

Fig. 10. Biomass yields of *A. platensis* cultures grown in TLC and OCP at 33 and 25 °C. Data are the mean values of 14 measurements performed at each temperature during the months of August; columns labelled by the same letter differ significantly from each other. Daily biomass yield was calculated on a 24-h basis, from dawn to dawn.

fluorescence techniques. The results showed a better acclimation of dense cultures in TLCs to high light irradiance outdoors as compared to the OCP cultures. Moreover, the trends of rETR, NPQ and OJIP curves correlated well to changes in biomass productivity, indicating that the cultures in the TLC could better utilise absorbed light energy compared to those in the OCP. Comparing the daily course of NPQ in the TLC and OCP cultures much higher values were found in the former. It indicated that in the TLC, more energy is absorbed and used for electron transport, but also more energy is not used for photochemistry and is dissipated as heat to avoid damage to the photosynthetic apparatus. Further analysis of NPQ showed that the Y(NO) component, *i.e.*, the non-regulated thermal dissipation reflecting passively dissipated energy fraction in form of heat, mainly due to closed PS II reaction centres, was much bigger than the other Y(NPQ) that corresponds to the fraction of energy dissipated in form of heat *via* the regulated photoprotective mechanisms. At lower irradiances, in cyanobacteria, a certain dip in NPQ light-response curves usually indicates the state transitions; the minimum corresponds to the irradiance intensity to which cyanobacteria culture are acclimated [28,29]. This supports the hypothesis that non-regulated thermal dissipation is the way how *Arthrospira* cells protect themselves against excess irradiance under unfavourable environmental conditions when grown outdoors. The signal for this mechanism is probably overreduction of plastoquinone electron acceptors of PSII as it can be observed from the kinetics of fast fluorescence induction (Fig. 7). The OCP cells suffer from too slow movement through the culture layer which does not generates sufficiently short light/dark cycles. Thus some cells can be either overexposed to light (with closed PSII complex) or remain too long in the dark.

In TLC, because of their short light path, cells can travel quickly between photic and dark zones generating suitable photon utilisation and they benefit of the higher light availability per unit of volume, rapid light/dark cycling due to short optical light path to match electron turnover in the photosynthetic apparatus, better mass transfer and more efficient oxygen stripping. High S/V ratio, coupled with the faster light/dark cycle in TLCs can prevent either PSII down-regulation (surface layer) or low-light acclimation of the cells in the bottom layer that usually causes an increase in the accessory pigments and consequently a reduction of light penetration. Indeed, we observed a higher concentration of phycocyanin in the cultures grown in OCP (Fig. S2). These changes were also accompanied by a decrease in the optical cross-section of the cultures in OCP (8.7×10^{-3} m^2 mg Chl^{-1}) compared to TLC (9.7×10^{-3} m^2 mg Chl^{-1} TLC), indicating that cells in OCP tended to acclimate to low light.

Interestingly, we did observe much synergism between culture system and temperature regime if F_v/F_m is considered as a reference, while photoinhibition was indicated only in OCP at suboptimal temperature (Fig. 2, at 14.00) which was reflected in lower biomass productivity (Fig. 10).

The rETR measured *in situ* was up to 4–5 times higher than that measured in the culture samples transferred in the lab. However, it must be pointed out that the measuring approach between *in-situ* and *off-situ* fluorescence measurements was quite different. *In-situ* rETR measurements were carried out by pointing the fibre optic of the fluorimeter directly onto the culture surface, while *off-situ* measurements were performed on culture samples taken from the outdoor cultures and placed under well-defined conditions (*i.e.*, same Chl *a* concentration, same light irradiance, well mixed suspension, and same light path of 1 cm), to allow for direct comparison of the parameters extracted from the rETR *vs*. PFR_{PAR} curves. Therefore, for a given light irradiance supplied to the sample, cells in the laboratory were exposed to a much higher average light irradiance compared to the cells in the dense cultures outdoors, resulting in a remarkably lower effective quantum yield. Moreover, transferring samples to the laboratory, although the measurements were performed after a very short time (10–15 min), may have caused a reduction in fluorescence due to state transition quenching (state 1 to state 2 transition) which occurs in cyanobacteria maintained in the dark [28]. However, *off-situ* and *in-situ* measurements provide complementary information; *off-situ* ones provide complex information on the physiological and acclimation status of the cultures, through analysis of initial slope (α), of the saturation irradiance (E_k), and the maximum $rETR_{max}$ of the RLC, while *in-situ* measurements of rETR provides timely information about their potential productivity [30,31].

It was also interesting to note that the interaction between culture temperature and culture system was not as significant, that is, increasing the culture temperature to the optimum did not result in a larger difference in productivity between the two culture systems, indicating that to attain maximum benefit from the intermittent light patter in TLC, temperature should be kept at the optimum since the early morning hours.

To conclude, the first trials of *A. platensis* cultures grown in the TLC presented here showed that the productivity was higher than that in the

OCP and it was related to a higher photosynthetic activity. The better culture performance in TLC was ascribed to significantly shorter light path that promoted much faster light/dark cycles favourable for photosynthesis as well as faster warming of the cultures in the morning as compared to the culture grown in the pond. Monitoring culture performance can uncover design improvements which may further exploit this photochemical advantage, increasing biomass productivity of TLCs further.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2017.10.007.

Conflict of interest

The authors declare no conflicts, informed consent, human or animal rights applicable.

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Annex VI

(related to Chapter 4 Use of Microalgae)

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Research Article

In vitro bioaccessibility of selenoamino acids from selenium (Se)-enriched *Chlorella vulgaris* biomass in comparison to selenized yeast; a Se-enriched food supplement; and Se-rich foods

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ABSTRACT

Selenium (Se) is an indispensable microelement in our diet and health issues resulting from deficiencies are well documented. Se-containing food supplements are available on the market including Se-enriched *Chlorella vulgaris (Se-Chlorella)* which accumulates Se in the form of Se-amino acids (Se-AAs). Despite its popular uses, data about the bioaccessibility of Se-AAs from Se-*Chlorella* are completely missing. In the present study, gastrointestinal digestion times were optimized and the *in vitro* bioaccessibility of Se-AAs in Se-*Chlorella*, Se-yeast, a commercially available Se-enriched food supplement (Se-supplement) and Se rich foods (Se-foods) were compared. Higher bioaccessibility was found in Se-*Chlorella* (~49%) as compared to Se-yeast (~21%), Se-supplement (~32%) and Se-foods. The methods used in production of Se-*Chlorella* biomass were also investigated. We found that disintegration increased bioaccessibility whereas the drying process had no effect. Similarly, temperature treatment by microwave oven also increased bioaccessibility whereas boiling water did not.

1. Introduction

Selenium (Se) is an essential microelement required by a variety of organisms including humans (Schiavon, Ertani, Parrasia, & Vecchia, 2017). It is an integral part of more than 30 distinct selenoproteins, including glutathione peroxidases, a group of antioxidant enzymes that help to protect cells from damage by free radicals (Hatfield, Tsuji, Carlson, & Gladyshev, 2014). Traditionally, Se is made available in human diet *via* consumption of Se rich foods such as nuts, seafood, meat and vegetables or from inorganic Se salts (Moreda-Pineiro et al., 2013; Moreda-Piñeiro, Moreda-Piñeiro, & Bermejo-Barrera, 2017). However, Se content in foodstuffs varies in different regions and may be insufficient for daily requirement (Moreda-Pineiro et al., 2013). A recent trend in the food industry aims to tackle this problem by culturing yeast or microalgae in Se-enriched medium to produce biomass with an increased content of organically bound Se, which is more suitable for human consumption than the potentially toxic inorganic Se salts (Gojkovic, Garbayo, Ariza, Márová, & Vílchez, 2015; Schiavon et al., 2017).

The applications of microalgal biomass range from food and feed

Abbreviations: Se, selenium; MA, methanesulfonic acid; ME, mercaptoethanol; SeMet, selenomethionine; SeCys, selenocysteine; MeSeCys, methylselenocysteine; Se-AAs, selenoamino acids; Se-supplement, Se-enriched food supplement; Se-yeast, selenized yeast; Se-*Chlorella*, selenium enriched *Chlorella vulgaris*; SD, standard deviation; GC-APCI-HRMS, gas chromatography atmospheric pressure chemical ionization high resolution mass spectrometry; HPLC, high performance liquid chromatography; GC, gas chromatography; MS, mass spectrometry; HFB-CF, 2,2,3,3,4,4,4-heptafluorobutyl chloroformate; HFB-OH, 2,2,3,3,4,4,4-heptafluoro-1-butanol; ICP-MS, inductively coupled plasma mass spectrometry

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production to high value food supplement products (Vaz, Moreira, Morais, & Costa, 2016). Among the various groups of microalgae, *Chlorella vulgaris* (*Chlorella*) is widely used for human consumption as it provides high protein content with balanced amino acid composition, vitamins and essential trace elements (Salati et al., 2017). Moreover, *Chlorella* cultures take up inorganic Se salts and metabolize them mostly to protein bound selenomethionine (SeMet), selenocysteine (SeCys) and methylselenocysteine (MeSeCys) (Sun, Zhong, Huang, & Yang, 2014). Se-enriched *Chlorella* (Se-*Chlorella*) biomass can be used as antioxidative food/feed supplement (Safi, Zebib, Merah, Pontalier, & Vaca-Garcia, 2014). A low dosage of organic Se compounds brings numerous health benefits, such as decreasing the incidence of cancer (Rahmanto & Davies, 2012), protecting against cardiovascular diseases (Flores-Mateo, Navas-Acien, Pastor-Barriuso, & Guallar, 2006), treating several muscle disorders (Rederstorff, Krol, & Lescure, 2006), delaying the onset of AIDS in HIV-positive patients (Watanabe, Barbosa Junior, Jordao, & Navarro, 2016) and boosting immune function (Hatfield et al., 2014). At high dosage however, it may cause adverse health effects, including selenosis and cancer (Cai et al., 2016). Since Se acts either as an essential microelement or as a toxic compound in a dose dependent manner, it is important to investigate the bioavailability of organic Se in Se-*Chlorella* biomass.

Under *in vivo* bioavailability tests, foodstuffs are directly administered/fed and the amount of certain serum Se-proteins such as glutathione peroxidase and Se-protein P are measured for the evaluation of bioavailability (Moreda-Pineiro et al., 2013). By comparison, under *in vitro* tests, the physiological variables such as: temperature, shaking, pH, enzyme/chemical composition of saliva, gastrointestinal juices and the sequence of events that happen in the human gastrointestinal tract are simulated (Moreda-Piñeiro et al., 2012). The simple protocol employs pepsin at pH 2 and a mixture of pancreatin and bile salts to mimic the gastric and intestinal digestion conditions, respectively (Moreda-Pineiro et al., 2013). The first approach, commonly referred as bioaccessibility, measures the maximum fraction of a substance that can be theoretically released from the food into the gastrointestinal tract (Lavu, Van De Wiele, Pratti, Tack, & Du Laing, 2016). Although very simple, the correlation between results from this approach and the *in vivo* test showed good agreement for both the stomach and the intestinal extraction phases (Intawongse & Dean, 2006). Another approach, formally called bioavailability, refers to the fraction of the substance that reaches the systemic circulation (blood) from the gastrointestinal tract (Moreda-Pineiro et al., 2013).

Although bioavailability and bioaccessibility studies can be performed either by *in vivo* or *in vitro* methods, the former approach limits itself with a few drawbacks concerning high operational costs, long experiment duration and difficult handling processes compared to *in vitro* methods (Lavu et al., 2016). *In vitro* approaches are therefore often more advantageous and have been used in many studies reported recently (Carbonell-Capella, Buniowska, Barba, Esteve, & Frígola, 2014). There have been some reports about bioavailability and bioaccessibility of Se from different sources such as seafood (Moreda-Piñeiro et al., 2012), grains (Mombo et al., 2016) and vegetables (Funes-Collado, Rubio, & Lopez-Sanchez, 2015; Khanam & Platel, 2016). However, such studies on Se-*Chlorella* are completely missing.

In this study, we aimed to evaluate the bioaccessibility of Se-AAs from Se-*Chlorella* in comparison with selenized yeast (Se-yeast), a commercially available Se-enriched food supplement (Se-supplement) and Se-rich foods (Se-foods) using an *in vitro* approach. The effect of disintegration and the drying process during the production of Se-*Chlorella*, temperature treatment and gastrointestinal transit time on the bioaccessibility of Se-AAs from Se-*Chlorella* were also investigated. We hypothesized that bioaccessibility of Se-AAs from disintegrated, spray-dried Se-*Chlorella* could be higher than those from Se-yeast, Se-supplement and Se-foods.

2. Materials and methods

2.1. Chemicals and reagents

Sodium selenite was purchased from Alfa Aesar (Karlsruhe, Germany). SeMet, SeCys, MeSeCys, L-methionine-13C_5-15 N, sodium hydroxide (NaOH), sodium bicarbonate ($NaHCO_3$), hydrochloric acid (HCl), isopropanol, mercaptoethanol (ME), methanesulfonic acid (MA), pyridine, isooctane, 2,2,3,3,4,4,4-heptafluoro-1-butanol (HFB-OH), 2,2,3,3,4,4,4-heptafluorobutyl chloroformate (HFB-CF), LC-MS grade water, formic acid, pepsin (lyophilized, salt-free), bile salt (microbiological grade) and pancreatin (from porcine pancreas) were obtained from Merck (Czech Republic). All the solvents and solutions used were of liquid chromatography mass spectrometry (LC-MS) grade. Se-AAs (0.1% formic acid) and MA stock solutions were prepared using LC-MS grade water. Lyophilized Se-yeast (certified reference material) was obtained from National Research Council Canada (Ottawa, Canada). A Se-supplement was purchased from Česká Řasa (Czech Republic). Se-foods (salmon, Brazil nut and mustard seed) were bought from Tesco supermarket (Czech Republic).

2.2. Cultivation of Se-Chlorella

The fresh water microalgae *Chlorella vulgaris* R-117 (CCALA 1107, Culture Collection of Autotrophic Organisms, Institute of Botany, Třeboň, Czech Republic) was cultivated in a fed-batch mode using pre-established inorganic medium (Doucha & Lívanský, 1995). Cultures were grown phototrophically outdoors in thin-layer cascades (working volume of 2200 L) as described previously (Babaei, Ranglova, Malapascua, & Masojidek, 2017; Masojidek, Kopecky, Giannelli, & Torzillo, 2011), during June 2018. The cultivation was performed mostly on sunny days during which the temperature of the microalgae suspension varied between 22 °C and 33 °C. Overnight or during rainy days, the culture was kept in a retention tank. Se in the form of sodium selenite was added to the culture once or twice per day, with the dosage from 2.25 to 4.50 mg/L, depending on the physiological state of the cultures (growth rate, photosynthetic activity and weather conditions). The starting biomass culture density was ~7 g DW/L and after 9 days, it reached ~21 g DW/L. Then, Se-*Chlorella* cultures were harvested by centrifugation using the DeLaval BRPX-309-34S industrial continuous centrifuge at 1300 rpm and biomass was washed using a 1:1 ratio of water to culture. The dense suspension of the *Chlorella* cells (~100 g DW/L) were disrupted using an Ariete NS3006 homogenizer (GEA Niro Soavi), at a pressure of 800 bar with a flowrate of 30 L/h. Then, the disintegrated cells were spray dried using a GEA Niro VSD-6.3-R, equipped with a centrifugal atomizer unit working at the speed of 30000 rpm (inlet and outlet temperatures of drying air were kept at 210 °C and 80 °C, respectively).

2.3. Sample preparation for Se-AA quantification

Homogenized samples (from 5 to 25 mg) were weighed in 10 mL glass tubes and 1.0 mL of MA at 3 M was added for the hydrolysis. To keep the reducing environment during the hydrolysis, 20 μL of ME was added to the mixture. The mixture was then vortexed and incubated at 95 °C for 24 h in a conventional oven followed by an addition of 20 μL of ME to prevent the potential oxidation of amino acids. After that, the samples were diluted to 5 mL with LC-MS grade water and centrifuged (4000 rpm for 10 min). The supernatant was then collected for derivatization prior to gas chromatography atmospheric pressure chemical ionization mass spectrometry (GC-APCI-HRMS) or for direct high performance liquid chromatography inductively coupled plasma mass spectrometry (HPLC-ICP-MS) measurements.

2.4. In vitro gastrointestinal digestion

In vitro gastrointestinal digestion was performed following the pre-established protocol (Moreda-Pineiro et al., 2013) with minor modifications. Homogenized samples (approximately 100 mg) and 5 mL of LC-MS grade water were mixed and vortexed thoroughly in a 15 mL centrifugation tube and then incubated for 15 min at room temperature. The mixture was then adjusted to pH 2 using 6 M HCl before adding 30 µL of gastric solution (6% pepsin, dissolved in 6 M HCl) followed by incubation for various time intervals (0.5, 1, 2, 4 and 6 h) at 37 °C.

After the respective incubation, the gastric digest was adjusted to pH 5 using 6 M NaOH. A further 40 µL of intestinal juice (bile salt and pancreatin, both at the concentration of 30 g/L in 0.1 M $NaHCO_3$) was added into the mixture. The intestinal digestion was carried out for various time periods (1, 2, 4 and 8 h) at 37 °C. The samples were then centrifuged (4000 rpm for 10 min) and the supernatants were collected for derivatization prior to GC-APCI-HRMS or for direct HPLC-ICP-MS measurements.

2.5. Derivatization of samples for GC-APCI-HRMS

Derivatization of samples for GC-APCI-HRMS measurements was done using HFB-CF derivatization (Husek et al., 2018) following the previously described protocol (Vu, Ranglova, Hajek, & Hrouzek, 2018). Briefly, 25 µL of MA hydrolysate or gastrointestinal hydrolysate plus 1 µL of internal standard (10 µg/mL L-methionine-$13C_5$-15 N) were mixed with 25 µL of 0.1 M $NaHCO_3$ in a glass tube (6 × 50 mm) and left to stand for 2 min. Then, 50 µL of the reactive organic medium (a mixture of isooctane: HFB-OH: HFB-CF, 15:1:4 v/v/v) and 25 µL of the catalytic medium (0.75 M NaOH: pyridine 24:1, v/v) were added and the contents were vortexed for 3 s. Next, 25 µL of the catalytic medium was added again and vortexed to generate a clear two-phase bilayer. Finally, 60 µL of isooctane and 25 µL of 1 M HCl were added to the sample and vortexed for a few seconds. The upper organic phase was collected for the GC-APCI-HRMS measurement with a sample injection volume of 1 µL.

2.6. GC-APCI-HRMS

Gas chromatography (Bruker GC-456) coupled with a high-resolution mass spectrometer equipped with an APCI source (Impact HD mass spectrometer, Bruker, Billerica, MA, USA) was used for the analysis of Se-AAs in derivatized samples (Se-*Chlorella*, Se-yeast and Se-supplement). A DB-XLB fused silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness from Agilent Technologies, USA) was operated at a helium flow rate of 1.1 mL/min with a temperature gradient from 45 °C to 250 °C (at 10 °C/min) and then to 330 °C (at 20 °C/min). The mass spectrometer was calibrated using heptacosafluorotributylamine. The operating parameters of the mass spectrometer were as follows: the spray needle voltage was set at 4200 V, nitrogen was used as nebulizing gas (0.3 bar) and drying gas (4 L/min), and the drying temperature was 210 °C. The scanning range was 400–1200 *m/z* and the scanning rate was 4 Hz, acquired in the positive ion mode.

Mass spectrometric data was processed by Skyline software version 4.1. Compound identification was based on the retention time compared to standards (retention time window was set to ± 0.2 min), mass accuracy (mass error was set down to the threshold of 20 ppm) and isotope distribution (checked manually). Limit of detection (LOD) and limit of quantification (LOQ) of the GC-APCI-HRMS method were calculated as:

$$LOD = 3sd_{blank}/m$$

$$LOQ = 10sd_{blank}/_{m}$$

where sd_{blank} stands for standard deviation of the blank response and m is the slope of the calibration curve.

Table 1
Analytical parameters of SeMet, SeCys and MeSeCys using GC-APCI-HRMS.

Compound	Retention time (min)	Theoretical *m/z* of HFB-CF derivate	LOD (µg/mL)	LOQ (µg/mL)
SeMet	9.05	604.9787	0.03	0.1
SeCys	9.10	802.9339	0.15	0.5
MeSeCys	9.15	816.9496	0.06	0.2

Analytical parameters such as: LOD, LOQ, retention time, theoretical *m/z* of Se-AAs post derivatization by HFB-CF can be found in Table 1.

2.7. HPLC-ICP-MS

Se-AAs in Se-foods (salmon, Brazil nut and mustard seed) were analyzed following a well-established HPLC-ICP-MS protocol in a laboratory routinely carrying out Se speciations. Separation was performed on an Agilent 1100 HPLC system using a Hamilton PRPX-100 column (strong anion exchange resin, 150 × 4.1 mm, 10 µm particles). Species were separated in 10 mM ammonium citrate buffer at pH 4.5 with 2% methanol (*v*/v), based on protocol reported previously (Bednarik et al., 2018). MS quantification was performed using an Agilent 7700x ICP-MS on the ^{78}Se isotope operated in the helium collision mode. The retention times for SeMet, SeCys and MeSeCys were 3.33, 2.7 and 1.83 min, respectively. LOD and LOQ for all the analytes were 0.008 and 0.026 µg/mL, respectively.

2.8. Data analysis

Bioaccessibility was calculated as the ratio of Se-AA obtained from the gastrointestinal digestion divided by the total amount of corresponding Se-AA, as quantified from the MA digestion. Statistical analysis was performed using R (3.5.1) and RStudio (1.1.456). Outliers were eliminated using Grubbs test. Statistical difference was confirmed by Student's *t* test or analysis of variance (ANOVA) while the test of equivalence was done following two-one-sided t-tests (TOST) procedure. The significance level was set at 0.05. Average and standard deviation (SD) were calculated from at least 5 replicates.

3. Results and discussion

3.1. Hydrolysis conditions using MA for the quantification of Se-AAs

In order to determine the bioaccessibility of Se-AAs, it is necessary to first quantify the amount of Se-AAs in the samples prior to simulated gastrointestinal digestion. Most of the recently reported methods employed either protease or MA for the hydrolysis of proteins and/or peptides to free amino acids before quantification by HPLC-MS, HPLC-ICP-MS or GC-MS. Our previous study reported a high recovery of Se-AAs following the digestion of the sample in a microwave oven using MA (Vu et al., 2018). We therefore aimed to optimize the following conditions: temperature; time; concentration of MA; and sample: MA ratio for the digestion of samples in a conventional oven. In the present study, the efficiency of the protein digestion in a conventional oven using MA was evaluated by the recovery of SeMet from the certified reference material of Se-yeast, with a defined SeMet content.

The four variables affecting the hydrolysis of proteins into their constituent amino acids using MA were investigated (Fig. 1). Firstly, we tested hydrolysis efficiency under a range of temperatures from 80 to 105 °C, while the other variables were fixed to: time of hydrolysis, 24 h; MA concentration, 4 M; amount of sample, 25 mg in 1.0 mL of 4 M MA. As shown in Fig. 1A, the temperature significantly affected hydrolysis efficiency as the recovery of SeMet was statistically different between various temperatures ($p < 0.05$, ANOVA). The highest recovery was

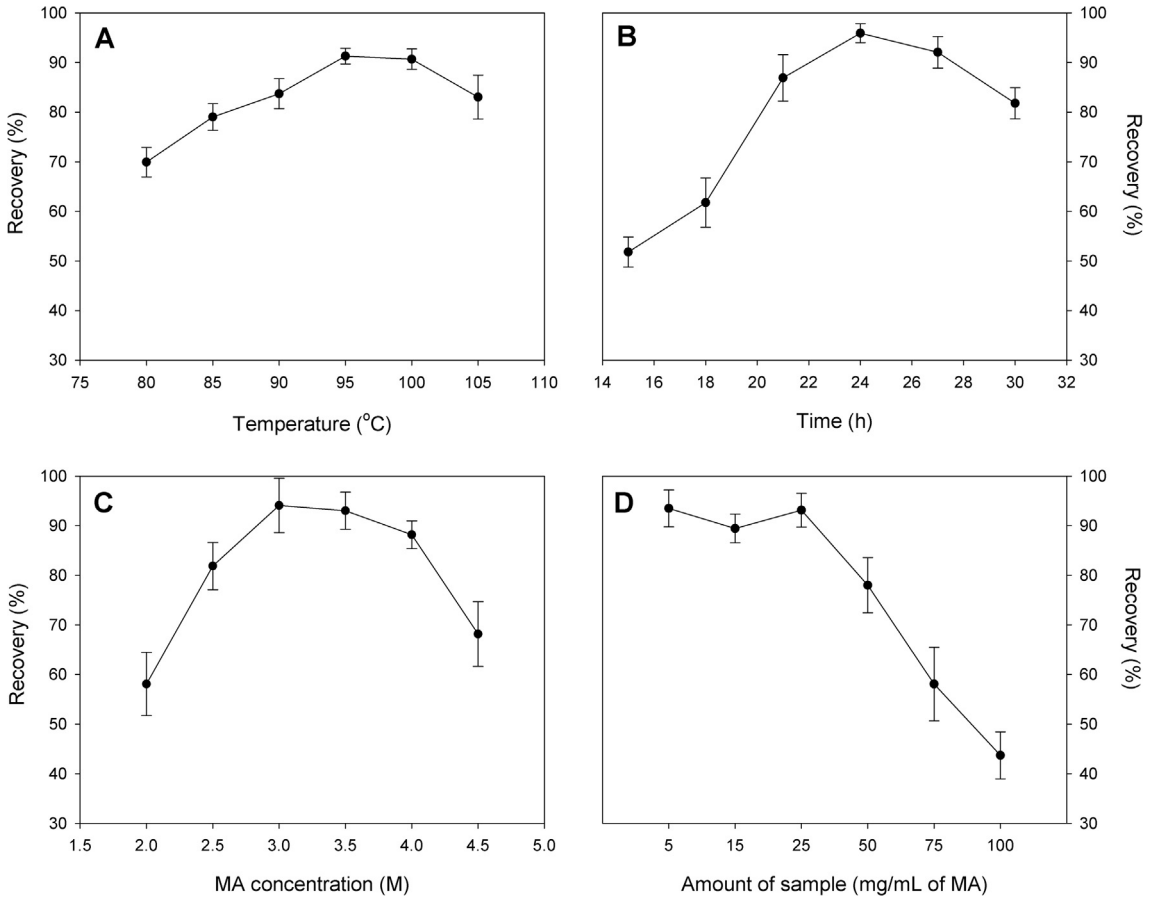

Fig. 1. Recovery of SeMet in Se-yeast at different digesting conditions: A) Varied temperatures from 80 to 105 °C; hydrolysis period, 24 h; MA concentration, 4 M; amount of sample, 25 mg (in 1.0 mL of 4 M MA). B) Digestion times from 15 to 30 h; temperature, 95 °C; MA concentration, 4 M; 25 mg of sample (in 1.0 mL of 4 M MA). C) Varied MA concentrations from 2 M to 4.5 M; temperature, 95 °C; a 24 h hydrolysis period; 25 mg of sample (in 1.0 mL of MA). D) Different sample amount from 5 mg to 100 mg (in 1.0 mL of 3 M MA); temperature, 95 °C; a 24 h hydrolysis period; MA concentration, 3 M.

obtained using 95 to 100 °C and the former was chosen as the optimal value. Subsequently, the effect of time on hydrolysis was investigated (Fig. 1B). The period given for hydrolysis varied from 15 to 30 h while the other variables were kept as: 95 °C; 4 M MA; and 25 mg of sample (in 1.0 mL of 4 M MA). After 24 h of hydrolysis, the recovery of SeMet increased by almost twice compared to after 15 h. Even longer hydrolysis treatment led to a slight decrease of the SeMet recovery ($p < 0.05$, TOST procedure), probably due to the degradation of amino acids (including Se-AAs) as noted also previously (Pedrero & Madrid, 2009). The MA concentration was tested in the range of 2 M to 4.5 M (Fig. 1C), while the other variables were maintained as: 95 °C; a 24 h hydrolysis period; 25 mg of sample (in 1.0 mL of MA). Lower or higher concentration of 3–3.5 M of MA gave a significantly lower recovery of SeMet ($p < 0.05$, ANOVA). Finally, we studied the effect of the amount of sample subjected to MA hydrolysis on SeMet yield. Quantities varied from 5 mg to 100 mg (in 1.0 mL of 3 M MA) while the other variables were kept at their optimized values. We found that the amount of sample processed was inversely proportional to the recovery yield (Fig. 1D). Statistical tests confirmed the different recovery of SeMet obtained from various amounts of Se-yeast subjected to hydrolysis ($p < 0.05$, ANOVA) and the sample amount from 5 mg to 25 mg provided highest recoveries. We did not use sample amounts lower than 5 mg to ensure that the amount of SeMet and other Se-AAs released during MA digestion was significantly higher than the LOQ of those analytes.

In summary, the optimized conditions for sample hydrolysis using MA in a conventional oven were: a hydrolysis temperature of 95 °C, 24 h of hydrolysis, a concentration of 3 M MA and 5–25 mg of sample per 1 mL of MA 3 M. Although the hydrolysis using MA in a conventional oven required a longer time than a microwave assisted method (Yang, Maxwell, & Mester, 2013), it provided very high reproducibility and is easily applied without the need of an expensive microwave-assisted extraction system. Typical relative standard deviations for replicated analysis of samples ranged between 6 and 12 %. In addition, the recovery of SeMet in Se-yeast using the optimized conditions in a conventional oven (95.6 ± 3.7%) was even higher than that obtained from the microwave assisted method (92.5 ± 4.1%), both having undergone the same derivatization and GC-APCI-HRMS measurement conditions. Furthermore, the hydrolysis used in this work was shown to give high analytical recovery compared to an enzymatic method using protease XIV (83 ± 16.1%), already reported (Vu et al., 2018). The same analytical method was applied to distilled water (blank) and no Se-AAs was found.

We also calculated the relative recovery of other amino acids. The conditions optimized for the hydrolysis of SeMet also provided the highest relative recovery of almost all amino acids including SeCys and MeSeCys. Cysteine was an exception which was found to degrade (relative recovery was ~20%) at temperatures higher than 90 °C, or when using hydrolysis periods longer than 18 h.

3.2. *Effect of disintegration and the drying process during biomass processing on content and bioaccessibility of Se-AAs from Se-Chlorella*

In microalgae biotechnology, the disintegration process is used to access intracellular products of *Chlorella* (Postma et al., 2017) while drying is a necessary process for the preservation of biomass. Drying of

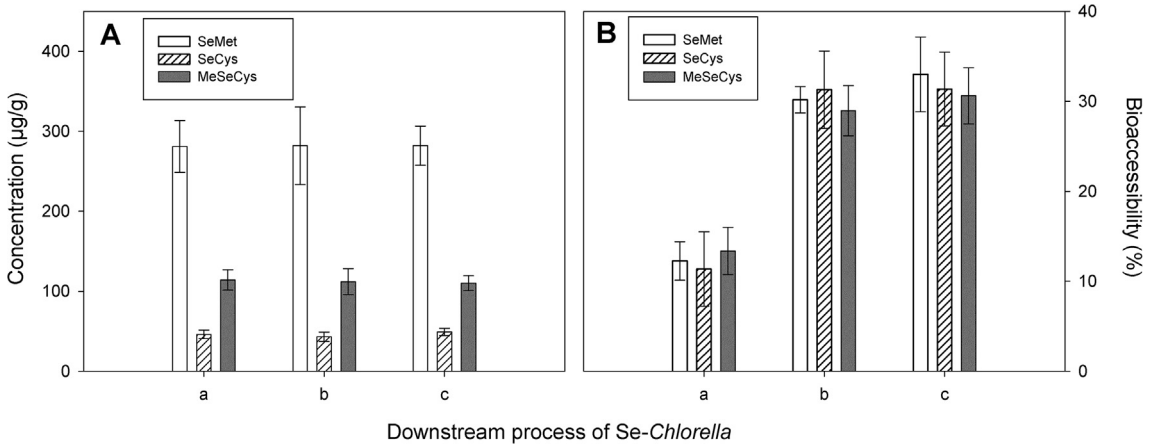

Fig. 2. Comparing Se-*Chlorella* biomass preparation methods. A) The concentration of Se-AAs in three differently prepared Se-*Chlorella* biomasses and B) the corresponding bioaccessibility. (a), (b) and (c) refer to Se-*Chlorella* biomass that was non-disintegrated and lyophilized; disintegrated and lyophilized; and disintegrated and spray-dried, respectively.

harvested microalgae from aqueous broth to biomass (< 5% moisture) using spray driers (inlet temperature of about 200 °C) represents a certain risk of nutrient degradation, including Se-AAs. Therefore, we quantified the amount of Se-AAs in Se-*Chlorella* biomass that was a) non-disintegrated and lyophilized, b) disintegrated, and lyophilized and c) disintegrated and spray-dried. The results were related to the corresponding Se-AA bioaccessibility data (Fig. 2). The amount of all the Se-AAs was similar between the differently prepared samples (Fig. 2A) confirming the stability of Se-AAs during the preparation of Se-*Chlorella* biomass. Bioaccessibility of Se-AAs (Fig. 2B) from the disintegrated, lyophilized sample (~30%) was significantly higher than that from non-disintegrated, lyophilized sample (~12%; $p < 0.05$, Student's *t* test). This result was not surprising as the high pressure inevitably disrupted the cell walls of Se-*Chlorella* and therefore enhanced the hydrolysis activity of pepsin and pancreatin during gastrointestinal digestion. Our results confirmed the importance of the disintegration process for improving nutrient bioaccessibility from Se-*Chlorella* biomass, which is in good agreement with data in the literature (Postma et al., 2015). The drying process did not affect the bioaccessibility of Se-AAs from Se-*Chlorella* ($p < 0.05$, TOST procedure), although the temperature used for drying in our trials was higher than that recommended previously (Hosseinizand, Sokhansanj, & Lim, 2018). Therefore, only disintegrated, spray-dried Se-*Chlorella* samples were used in further experiments, unless otherwise specified.

3.3. Effect of gastrointestinal transit time on Se-AA bioaccessibility from Se-Chlorella

The gastrointestinal transit time is a variable which strongly depends on gender, age, dietary habits and health status (Faaborg, Christensen, Rosenkilde, Laurberg, & Krogh, 2010). Therefore, *in vitro* methods for the comparison of bioaccessibility among the samples may suffer from bias especially when the actual transit time is longer than that in the simulated method. Although methods for the determination of bioaccessibility have been reported recently (Khanam & Platel, 2016; Lavu et al., 2016; Moreda-Piñeiro et al., 2012; Moreda-Pineiro et al., 2013) the effect of gastrointestinal time on bioaccessibility of Se-AAs is still unknown. In our study, we investigated the effect on bioaccessibility of different simulated gastric digestion times, from 0.5 h to 6 h, typical of the transit time for food in the human stomach (Faaborg et al., 2010). A simulated intestinal digestion time of 2 h was used throughout. The results showed that bioaccessibility of SeMet, SeCys and MeSeCys in Se-*Chlorella* samples were maximum when the gastric digestion period was 2 h (Fig. 3A). For longer periods, the bioaccessibility remained at similar levels of about 43% for all analyzed Se-AAs ($p < 0.05$, TOST procedure).

Changes in Se-AA bioaccessibility as a result of different simulated intestinal digestion periods varying from 1 to 8 h, typical of intestinal transit time in humans (Faaborg et al., 2010), were also studied. A 2 h gastric digestion period was maintained throughout. We found that different simulated intestinal digestion times strongly affected the bioaccessibility of all Se-AAs and the maximal bioaccessibility values (~49%) were reached after 4 h of intestinal digestion (Fig. 3B). The optimized gastrointestinal transit time, 2 h for gastric digestion and 4 h for intestinal digestion, was used for further comparison of bioaccessibility of SeMet, SeCys and MeSeCys in Se-*Chlorella*, Se-yeast, Se-foods and a Se-supplement. In several recent articles, gastric and intestinal digestion times of 1 and 2 h, respectively were reported, even for materials with a very strong cell wall such as cereals (Khanam & Platel, 2016; Mombo et al., 2016). Based on the results obtained in this study, we propose to prolong the simulated gastrointestinal transit time for *in vitro* bioaccessibility and bioavailability studies for such materials.

Although the amounts of individual Se-AAs in Se-*Chlorella* biomass were different, all of the Se-AAs had equivalent bioaccessibility indicating similar behavior of those analytes under gastrointestinal as well as MA digestion. This result was in good agreement with previous studies on bioaccessibility of Se-AAs obtained from various foodstuffs (Khanam & Platel, 2016; Moreda-Piñeiro et al., 2017; 2013).

3.4. Se-AA content and bioaccessibility from Se-Chlorella in comparison with Se-yeast, a Se-supplement and Se-foods

In this series of experiments, we first quantified the amount of SeMet, SeCys and MeSeCys (calculated in µg/g, expressed as average ± 1.96 × SD) in Se-*Chlorella*, Se-yeast, a Se-supplement and Se-foods (Table 2). In Se-*Chlorella*, the major Se-AAs were SeMet and MeSeCys, while in other samples were SeMet and SeCys. MeSeCys was in low amount in Se-yeast and was not detected at all in Se-supplement and Se-foods. The total organic Se (calculated from Se-AAs) in Se-yeast (1467 ± 153.4 µg/g) was about 8 times higher than that in disintegrated, lyophilized Se-*Chlorella* (185 ± 15.5 µg/g) and about 43 times higher compared to Se-supplement (34 ± 3.4 µg/g). Nevertheless, the amount of total organic Se in Se-*Chlorella* was still very high in comparison with those found in Se-foods (0.5–6 µg/g). Cultivated rice, wheat and chickpea had total organic Se contents of only 0.01–0.3 µg/g (Khanam & Platel, 2016), only a fraction of the content in Se-*Chlorella*. Moreover, Se content in nuts, vegetables, meat or seafood strongly depends on the amount of Se in the environment and therefore, it varies a lot in different regions while the content in Se-*Chlorella* can be controlled during cultivation.

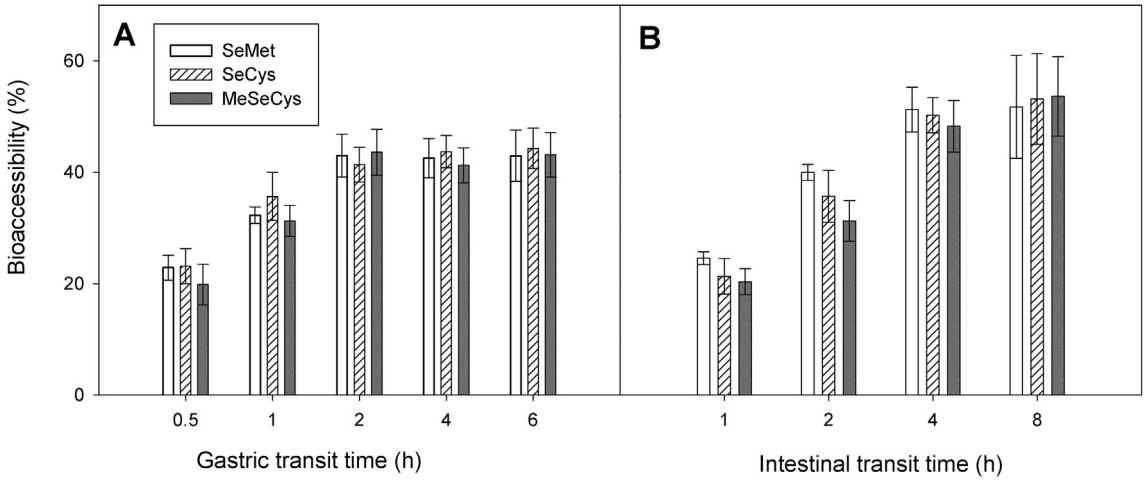

Fig. 3. Effect of gastric (A) and intestinal (B) digestion time on the bioaccessibility of SeMet, SeCys and MeSeCys in Se-*Chlorella*.

We also compared bioaccessibility of Se-AAs from: disintegrated, spray-dried Se-*Chlorella*; non-disintegrated, lyophilized Se-*Chlorella*; Se-yeast; Se-supplement; and Se-foods, using the optimized procedure described above (Table 2). Bioaccessibility of Se-AAs from disintegrated, spray-dried Se-*Chlorella* (~50%) was similar to those obtained from salmon (~52%) and was significantly higher than that from non-disintegrated, lyophilized Se-*Chlorella* (~13%), Se-yeast (~20%), Se-supplement (~30%), Brazil nut (8.1%) and mustard seed (7.2%) ($p < 0.05$, ANOVA). Disintegrated, spray-dried Se-*Chlorella* could be digested more easily in the human gastrointestinal system in comparison to the mentioned products.

Although the *Chlorella* cell wall is tough compared to that of yeast (Gerken, Donohoe, & Knoshaug, 2013), the cell disruption during the processing of Se-*Chlorella* biomass evidently enhanced the efficiency of its gastrointestinal digestion. This presumption was supported by the fact that bioaccessibility of Se-AAs from non-disintegrated Se-*Chlorella* was significantly lower than that from Se-yeast ($p < 0.05$, Student's *t* test). Disintegrated, spray-dried Se-*Chlorella* obviously contains very high amount of Se-AAs with high bioaccessibility, making it an outstanding food Se-supplement for preventing Se deficiency, especially in Eastern Europe and the Middle East where the dietary Se intake is relatively low compared to other regions (Stoffaneller & Morse, 2015).

The recommended daily intake of Se is 55 μg (US National Institute of Health for adult daily diet). This quantity, considering bioaccessibility of each product, corresponds to an intake of ~0.18 g of yeast, the equivalent of which can be found in ~0.61 g of disintegrated, spray-dried Se-*Chlorella*; ~2.5 g of non-disintegrated, lyophilized Se-*Chlorella*; ~5.1 g of Se-supplement; ~132 g of salmon or ~129 g of Brazil nut, as calculated from our data in Table 2. Although the bioaccessibility of Se-AAs from disintegrated, spray-dried Se-*Chlorella* was among the highest in all of the investigated materials, Se-yeast was the most efficient supplement of Se-AAs due to the high content of Se-AAs. This result indicated the need to develop the cultivation process of Se-*Chlorella* for a higher incorporation rate of Se to amino acids (AAs).

Microalgae vary in their ability to metabolize inorganic Se (mainly from selenate and selenite) to protein-bound Se-AAs or other Se containing biomolecules depending on their strains (Ponton, Fortin, & Hare, 2018; Schiavon et al., 2017; Umysova et al., 2009). Therefore, different microalgae strains could be isolated from nature and subjected to selection, genetic engineering or mutagenesis (Larkum, Ross, Kruse, & Hankamer, 2012) to obtain better strains for Se-AA production. Moreover, sulfate, silicate and phosphate were reported to compete with selenate and selenite for the membrane transporters (Gojkovic et al., 2015; Ponton et al., 2018; Schiavon et al., 2017). Therefore, cultivation of microalgae in the medium depleted in these competing ions, may enhance accumulation of Se-AAs. Nevertheless, as some of these competing constituents are essential for the cell growth, it is important to keep their concentrations at a certain level (Gojkovic et al., 2015; Schiavon et al., 2017; Umysova et al., 2009). Sufficient irradiance, optimal ambient temperature and pH supporting adequate photosynthetic activity also facilitate Se incorporation in amino acids (Babaei et al., 2017; Fernandes, Mota, Teixeira, & Vicente, 2015; Ponton et al., 2018; Schiavon et al., 2017). All the above mentioned variables combined with the optimization of cultivation system parameters, i.e., an open pond design or photobioreactor geometry (Fernandes et al., 2015) may significantly increase the incorporation rate of Se in Se-AAs during biomass cultivation.

Table 2
Amount of SeMet, SeCys, MeSeCys and total organic Se (μg/g) and their bioaccessibility (%) in Se-*Chlorella*, Se-yeast, Se-supplement and Se-foods.

Se-AAs	Content (μg/g)				Bioaccessibility (%)				Daily diet (g)
	SeMet	SeCys	MeSeCys	Organic Se	SeMet	SeCys	MeSeCys	Organic Se	
Se-*Chlorella*[*]	281 ± 48.5	43 ± 5.8	115 ± 16.2	183 ± 24.3	12.2 ± 2.14	11.3 ± 4.14	13.3 ± 2.64	12 ± 3.2	~2.5
Se-*Chlorella*[#]	282 ± 24.5	49 ± 4.6	110 ± 9.3	185 ± 15.5	50.5 ± 3.73	50.5 ± 4.23	48.6 ± 6.11	49 ± 4.8	~0.61
Se-yeast	3186 ± 335.3	354 ± 44.5	39 ± 5.4	1467 ± 153.4	22.87 ± 2.01	20.9 ± 3.11	19.8 ± 4.87	21 ± 3.5	~0.18
Se-supplement	61 ± 6.1	15 ± 4.5	ND	34 ± 3.4	34.7 ± 6.84	30.1 ± 4.78	ND	32 ± 5.9	~5.1
Salmon	1.5 ± 0.17	0.4 ± 0.14	ND	0.8 ± 0.09	52.7 ± 4.71	51.3 ± 6.11	ND	52 ± 5.9	~132.2
Brazil nut	12 ± 1.7	0.8 ± 0.22	ND	5.2 ± 0.6	8.2 ± 3.34	8.1 ± 2.89	ND	8.2 ± 3.70	~129.0
Mustard seed	1.1 ± 0.31	0.4 ± 0.1	ND	0.6 ± 0.14	7.2 ± 2.37	ND	ND	7.2 ± 2.37	~1273

ND: not detected.
[*] Non-disintegrated, lyophilized Se-*Chlorella*.
[#] Disintegrated, spray-dried Se-*Chlorella*.

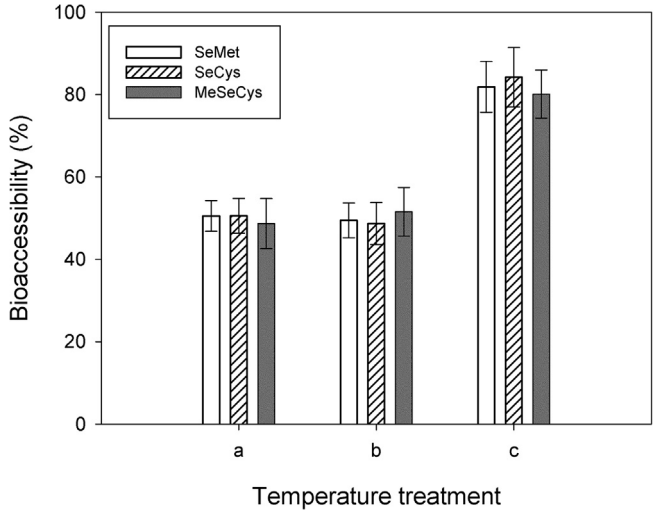

Fig. 4. Effect of temperature treatment (a: room temperature; b: boiling water; c: microwave oven) on bioaccessibility of Se-AAs from Se-*Chlorella*.

3.5. Effect of temperature treatment on bioaccessibility of Se-AAs in Se-Chlorella

Finally, the effect of temperature treatment on bioaccessibility of Se-AAs in Se-*Chlorella* was studied (Fig. 4). The use of a boiling water bath (100 °C for 5 min) did not affect the bioaccessibility of any Se-AAs ($p < 0.05$, TOST procedure) while treatment in a microwave oven (500 W for 5 min) significantly increased the efficiency of gastrointestinal digestion and therefore increased the bioaccessibility of those Se-AAs ($p < 0.05$, Student's *t* test). We assume that microwave treatment may have further disrupted the cell wall of Se-*Chlorella* so that gastrointestinal enzymes could penetrate better through the cell wall and therefore efficiently hydrolyzed proteins in Se-*Chlorella* into amino acids, including those Se-AAs investigated. According to previous studies, bioaccessibility of Se-AAs from vegetables and seafood has also changed significantly after various temperature treatments, either in a positive or negative manner (Funes-Collado et al., 2015; Khanam & Platel, 2016). Khanam and Platel (2016) found, like us, that boiling foodstuffs (100 °C, less than 30 min) did not significantly change the bioaccessibility of Se-AAs while temperature treatment using a microwave oven and high pressure cooker, in general, increased their bioaccessibility. However, the use of a microwave oven to support hydrolysis should be closely managed as longer treatment times and/or higher power can potentially affect other nutrients such as vitamins and essential fatty acids (Dejoye et al., 2011).

4. Conclusion

Gastrointestinal transit time strongly affected bioaccessibility of Se-AAs from Se-*Chlorella*. The optimal time for *in vitro* gastric digestion and intestinal digestion were found to be 2 h and 4 h, respectively. The bioaccessibility of SeMet, SeCys and MeSeCys from Se-*Chlorella* biomass compared with data obtained from Se-yeast, Se-foods and Se-supplement showed that the former had higher bioaccessibility. Se-*Chlorella* cultivated with our outdoor unit contained significant higher amount of Se-AAs compared to Se-supplement, Se-food, but less than Se-yeast. Bioaccessibility of Se-AAs from Se-*Chlorella* increased upon disintegration and stayed unchanged during drying process. The temperature treatment trial indicated that the use of a microwave oven could increase the efficiency of gastrointestinal digestion, while boiling water did not have any effect. Finally, the method for the determination of bioaccessibility as well as the method for quantification of Se-AAs elaborated in this study can, with minor modifications, be applied to other materials, especially to the variety of newly arising Se-supplements.

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Declaration of interests

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

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Annex VII

(related to Chapter 4 Use of Microalgae)

Manuscript Details

Manuscript number	ALGAL_2020_837
Title	Growth, biostimulant and biopesticide activity of the MACC-1 Chlorella strain cultivated outdoors in inorganic medium and wastewater
Article type	Full Length Article

Abstract

Growth, physiological performance and bioactivity of the microalga strain Chlorella vulgaris MACC 1 was studied outdoors in two cultivation units – thin-layer cascade and a novel, thin layer raceway pond. Two nutrient sources were compared – inorganic BG-11 medium and centrate from municipal wastewater (WW). The main objective of this work was to study bioactivity of Chlorella when grown in inorganic medium and in centrate. The correlation between the bioactivity and photosynthetic activity was also studied. The results revealed a clear interplay among ambient irradiance intensity, growth rate, PSII photochemical efficiency Fv/Fm and oxygen production/respiration. Samples were harvested at the end of trial at different daytimes (0800 h and 1300 h) in the semi-continuous cultivation regime from both units for determination of bioactivity using water extracts of freeze dried biomass. The highest biostimulating activities detected by different bioassays were found in Chlorella cultures grown in BG 11, but not in WW. On the other hand, the antibacterial and antifungal activities were significantly higher when grown in WW. We expect that antimicrobial activities can be induced by WW-microbes and biostimulating effects depend on physiological status of the algae cells. Here, we found certain correlation between photosynthetic activity and bioactivity as significant differences in both activities coincided. Thus, photosynthesis monitoring can be used as an indication of microalgae cultures as to indicate biomass harvesting in large-scale unit for agricultural use.

Keywords	Chlorella, outdoor cultivation, chlorophyll fluorescence, bioactivity, pathogens, wastewater
Taxonomy	Biological Sciences, Environmental Science, Agricultural Biotechnology, Applied Sciences
Manuscript category	Algal Biotechnology

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Cover letter to the article: Growth, biostimulant and biopesticide activity of the MACC-1 *Chlorella* strain cultivated outdoors in inorganic medium and wastewater

Dear Editorial office,

Please find the manuscript entitled "Growth, biostimulant and biopesticide activity of the MACC-1 *Chlorella* strain cultivated outdoors in inorganic medium and wastewater" for consideration in Algal Research.

A number of microalgae produce biologically active compounds, like plant hormones responsible for plant biostimulating activity and antimicrobial compounds, which can be used as biopesticides to control plant diseases. Their use in field treatments minimise the amount of required chemicals, thus indirectly improves agricultural sustainability. Unicellular microalgae *Chlorella vulgaris* MACC-1 was cultivated in inorganic medium and in wastewater in two outdoor cultivation units – thin-layer cascade and a novel, thin-layer raceway pond. The antimicrobial (biopesticide) activity of the *Chlorella* biomass samples were detected with the antagonism bioassay. The responsible plant hormones for plant biostimulating activity were detected by three different bioassays, which measure the auxin-like (seed germination, mung bean rooting) and cytokinin-like (chlorophyll retention) activities. The growth and photosynthetic activity were correlated to find suitable optimal point of biomass harvesting with high bioactivity.

We anticipate that our study is interesting for the broad readership of Algal Research.

On behalf of the authors

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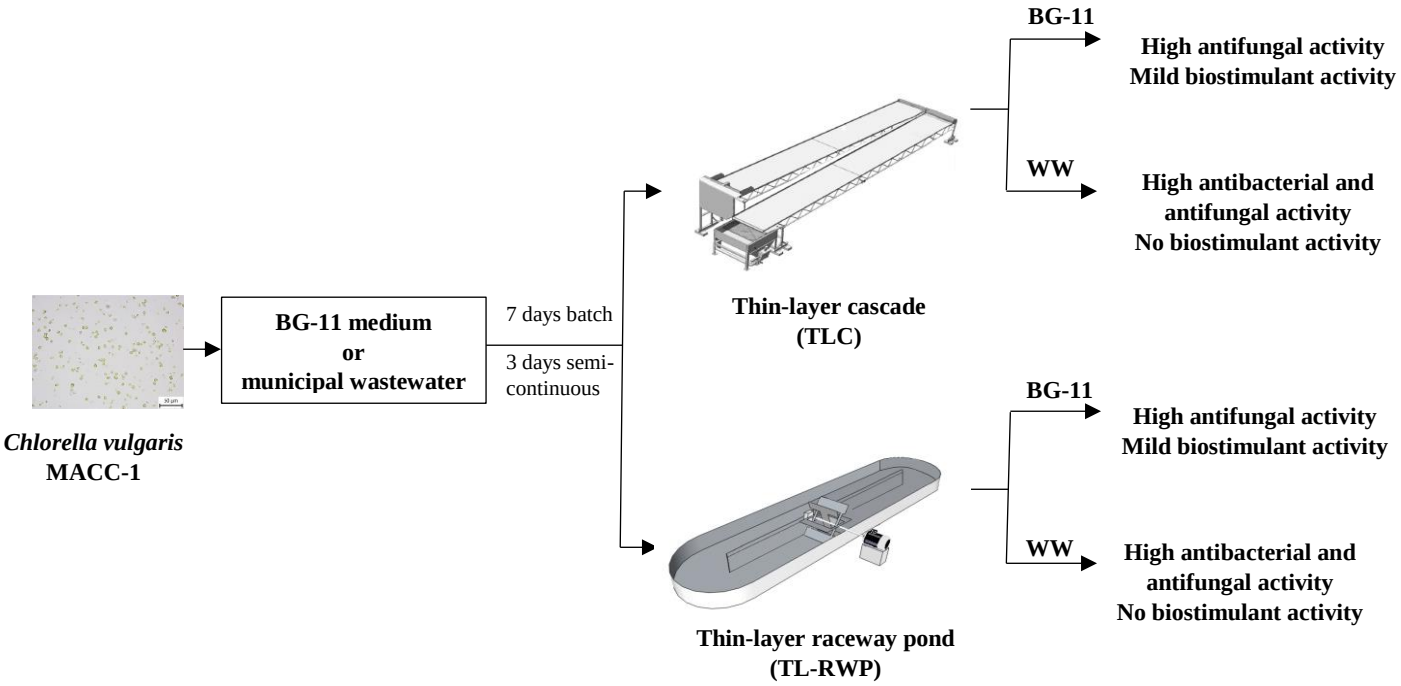
BG-11
High antifungal activity
Mild biostimulant activity
WW
High antibacterial and
antifungal activity
No biostimulant activity
Thin-layer cascade
(TLC)
BG-11 medium
or
municipal wastewater
7 days batch
3 days semi-
continuous
Chlorella vulgaris
MACC-1
BG-11
High antifungal activity
Mild biostimulant activity
WW
High antibacterial and
antifungal activity
No biostimulant activity
Thin-layer raceway pond
(TL-RWP)

Growth, biostimulant and biopesticide activity of the MACC-1 *Chlorella* strain cultivated outdoors in inorganic medium and wastewater

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Abstract

Growth, physiological performance and bioactivity of the microalga strain *Chlorella vulgaris* MACC-1 was studied outdoors in two cultivation units – thin-layer cascade and a novel, thin-layer raceway pond. Two nutrient sources were compared – inorganic BG-11 medium and centrate from municipal wastewater (WW).

The main objective of this work was to study bioactivity of *Chlorella* when grown in inorganic medium and in centrate. The correlation between the bioactivity and photosynthetic activity was also studied. The results revealed a clear interplay among ambient irradiance intensity, growth rate, PSII photochemical efficiency F_v/F_m and oxygen production/respiration. Samples were harvested at the end of trial at different daytimes (0800 h and 1300 h) in the semi-continuous cultivation regime from both units for determination of bioactivity using water extracts of freeze dried biomass. The highest biostimulating activities detected by different bioassays were found in *Chlorella* cultures grown in BG-11, but not in WW. On the other hand, the antibacterial and antifungal activities were significantly higher when grown in WW. We expect that antimicrobial activities can be induced by WW-microbes and biostimulating effects depend on physiological status of the algae cells. Here, we found certain correlation between photosynthetic activity and bioactivity as significant differences in both activities coincided. Thus, photosynthesis monitoring can be used as an indication of microalgae cultures as to indicate biomass harvesting in large-scale unit for agricultural use.

Keywords: Chlorella, outdoor cultivation, chlorophyll fluorescence, bioactivity, pathogens, wastewater

1. Introduction

A number of microalgae strains produce biologically active compounds, like plant hormones (responsible for biostimulating activity) and antimicrobial compounds (responsible for biopesticide activity). The biomass can be applied as an alternative to enhance plant growth and for protection of agricultural crops [1–5] as use of chemical pesticides or fertilizers cause pollution and their residues may be harmful to humans [6,7]. Alternative technologies to increase productivity in sustainable agricultural systems needs to be found [8] and microalgae represent one of them [4].

Cultivation in aquaculture involves open reservoirs (mixed ponds, raceways or cascades) with direct contact of the microalgae culture with the environment and closed or semi-closed vessels – photobioreactors (PBR) as well [9–12]. The biomass production in open systems is cheaper than closed PBRs as the former are easier to clean and requires lower operation costs [13]. In microalgae culturing the biomass composition may be affected by modulating of various environmental factors and conditions [14]. Microalgae cultivation requires major nutrients such as nitrogen, phosphorus and carbon. Urban wastewaters contain high amounts of nitrogen (mainly in the form of ammonium) and phosphorus which can replace these expensive mineral fertilizers used for microalgae cultivation and the cultivation costs are reduced [1,2,15–18]. *Chlorella* is often used in wastewater treatments as it possess a great tolerance to high ammonia concentration [1,19,20].

For the successful microalgae cultivation monitoring of physicochemical variables (pH, temperature, dissolved oxygen concentration, nutrient status, etc.) and photosynthesis monitoring in order to optimise growth regime are required. Chlorophyll fluorescence measurement has become one of the most frequently used techniques for monitoring microalgae mass cultures [21–30]. Most frequently used variable - maximum quantum yield of PSII (F_v/F_m) - has been used to correlate the photosynthesis and growth [22,23,31–35]. Measurements of photosynthetic oxygen production give similar information as measurement of F_v/F_m by saturation pulse analysis of fluorescence quenching, but one important advantage concerns the possibility to distinguish between photosynthetic oxygen production (POE) and dark respiration (R) [36].

In the present experiments we cultured the microalga *Chlorella* in inorganic medium and in municipal wastewater using two outdoor cultivation units – thin-layer cascade and a novel, thin-layer raceway pond as thin-layered systems are more adequate for light utilization. The growth and nutrient removal were followed and photosynthetic activity was correlated to find suitable optimal point of biomass harvesting with high bioactive properties.

2. Materials and methods

2.1. Strain and inoculum preparation

For these trials the unicellular green microalga *Chlorella vulgaris* MACC-1 (abbreviated as *Chlorella*) was selected due its high growth rate and potential biostimulating and biopesticide activity. It was obtained from the Algal Culture Collection of the Széchenyi István University in Mosonmagyaróvár, Hungary. Seed culture (inoculum) of *Chlorella* was grown in laboratory at 28-30 °C in 10 L Pyrex glass bottles using the inorganic BG-11 medium [37,38] which was bubbled with air + 1% CO_2 (v/v) until the stationary phase. Photosynthetically active radiation (PAR) of about 120 µmol photons m^{-2} s^{-1} was provided continuously by a panel of dimmable warm-white tubes (55 W, Dulux L, Osram, Germany) placed vertically behind the cultivation unit.

2.2. Wastewater preparation

In a local wastewater treatment plant (WWTP) of Třeboň's municipality, automatic addition of flocculant is used for sludge separation which causes microalgae cell clogging when centrate is used for cultivation. This problem may be overcome by centrifugation of the sludge before addition of flocculant. Therefore, the activated sludge after the secondary aerobic digestion (secondary-treated wastewater) was taken from the WWTP and centrifuged at 3000 g for 5 min (centrifuge Sigma 8KS) to separate the liquid centrate (WW) from the solid matter. Then, the cleared WW (see the composition in Table 1) of brownish colour was collected and was used undiluted for growth in outdoor cultivation units. The ratio of total nitrogen (N) to total phosphorus (P) was about 1.5. Compared to WW, the nitrogen content is similar in BG-11 (250 mg DW L^{-1}) while phosphorus is about 25 times lower, about 7 mg DW L^{-1}.

2.3. Outdoor trials

Once the stationary phase in the laboratory PBRs was reached, the cultures were mixed in a big plastic tank with 4 parts of either the BG-11 medium (Trial 1) or wastewater (Trail 2) and then these were transferred to outdoor cultivation units. The *Chlorella* culture was grown in a thin-layer cascade (TLC) and a thin-layer raceway pond (TL-RWP) placed in polycarbonate greenhouses with controlled environment (Fig. 1). The TLC (surface of $5m^2$; working volume of 70 L; culture depth of 8 – 10 mm and a flow speed of 0.5 m s^{-1}) was circulated by a pump only during the day (between 7.30 a.m. and 7.30 p.m.); at night the culture was stored in a retention tank and mixed by air using a blower [39]. The TL-RWP was operated continuously (surface of $5m^2$; work volume of 120 L at a culture depth of 15-20 mm) at a flow speed of 0.2 m s^{-1}. In both units an automatic addition of CO_2 kept the pH value at 8.0 ± 0.2 (pH-stat).

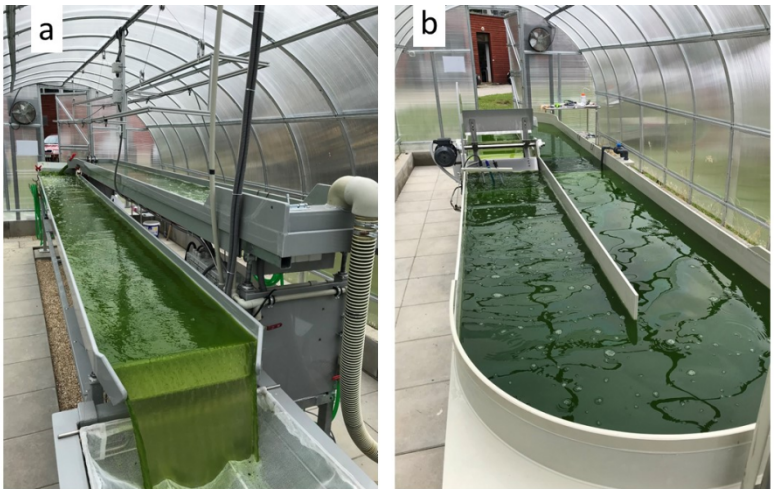

Fig. 1. Outdoor cultivation units used in trials were placed in polycarbonate double-layer greenhouses to protect cultures from cross-contamination and unfavourable outdoor conditions: a) thin-layer cascade (TLC) with the ratio of total surface to volume about 80 m^{-1} and b) thin-layer raceway pond (TL-RWP) with the ratio of total surface to volume about 40 m^{-1}.

The initial biomass density in outdoor trials was set to 0.7-0.8 g DW L^{-1}; the cultures in WW had brown-green colour due to the wastewater addition. The dark colour of cultivation media or dense culture could cause substantial light absorption when cultivated in a unit with thick culture layer but the thin-layer systems used in these trials are advantageous for these purposes.

Chlorella was firstly grown in a batch mode for 7 days to get well-growing dense culture and then semi-continuous growth regime was operated for 4 d at 25% dilution rate (0.25 d^{-1}). Every morning at 0800 h, one-quarter of the culture volume was harvested from each unit and replaced by fresh BG-11 medium or centrate. At the end of the semi-continuous cultivation regime, 20 L of microalgae culture was collected in the morning (0800 h) and the same amount in the afternoon (1300 h), centrifuged and freeze-dried for bioassays. In these trials we used a model of large-scale production where microalgae were grown in semi-continuous/continuous regime replacing daily a part of culture with fresh medium/WW. Thus, samples for bioassays were taken in semi-continuous culturing regime in the morning and in the afternoon when physiological status and photosynthetic activity of *Chlorella* cultures may differ because of the temperature and light stress during midday.

2.4. Location and weather conditions

The outdoor trials were carried out in the Centre Algatech (48°59'16.6"N, 14°39.9"E) in August and September. In both units the morning temperatures ranged between 12-20°C (TL-RWP) and 11-20 °C (TLC) while midday temperature varied between 23-37 °C (TL-RWP) and 25-37 °C (TLC) in Trial 1 and 2, respectively. This range of temperatures is not limiting for *Chlorella*.

Photosynthetically active radiation (PAR) inside greenhouses was recorded by a weather station (IP Warioweather, model ME 13). During Trial 1 with the BG-11 medium the weather conditions

were fair; daily irradiance maxima inside the greenhouses were between 600-800 μmol photons m^{-2} s^{-1} during the first week and around 400-500 μmol photons m^{-2} s^{-1} during the second week (Fig. 2a). The light intensity inside the greenhouses was much higher during Trial 2 when the cultivation of *Chlorella* in undiluted WW was performed. During the first week daily irradiance maxima were between 1000-1700 μmol photons m^{-2} s^{-1} and between 1100-1500 μmol photons m^{-2} s^{-1} during the second week (Fig. 2b). The evaporation was compensated every morning by the addition of tap water.

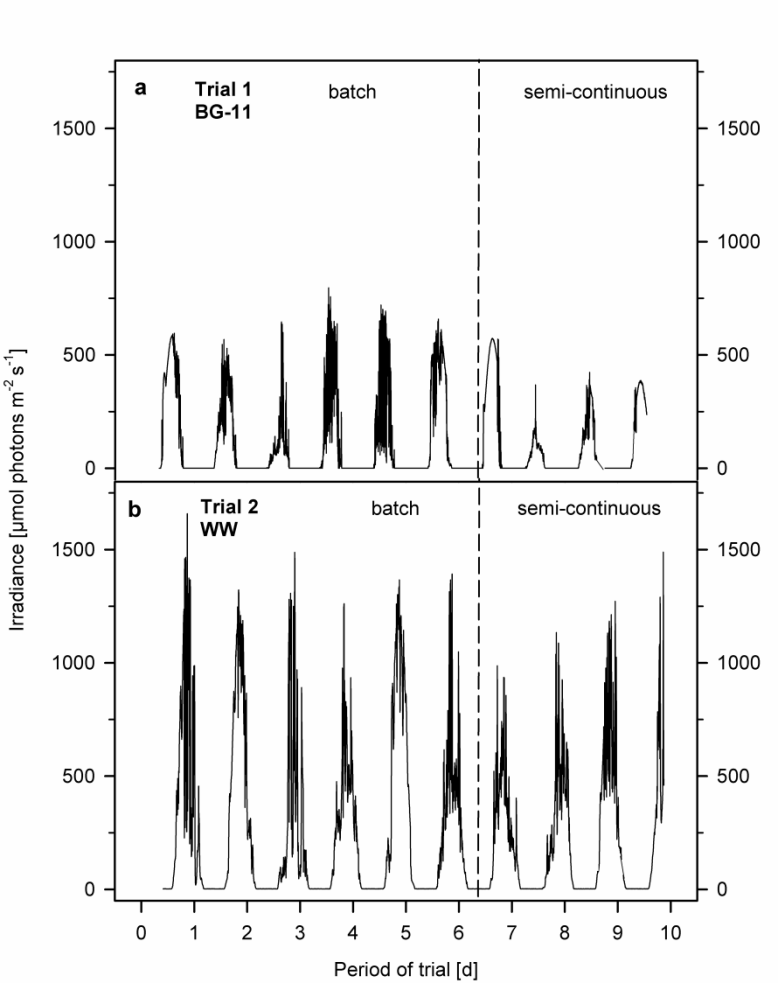

Fig. 2. Light intensity measured inside the greenhouses during the growth of *Chlorella:* a) Trial 1 with BG-11 medium; and b) Trial 2 with centrate (WW) in batch and semi-continuous regime.

2.5. Dry weight determination

The measurement of biomass density was performed as previously described [29,30]. The biomass was measured in triplicates once a day in the morning (0800 h) after compensation of evaporation when cultivation was performed in batch regime and twice a day (in the morning after compensation of evaporation and after dilution) during the semi-continuous regime. The specific growth rate $\mu = (\ln X_2 - \ln X_1)/\Delta t$ [d^{-1}] of microalgae cultures was calculated over the period of linear growth phase in batch regime.

2.6. Nutrient analysis

The centrate (25 mL) was filtered using a syringe filter and then kept at -20 ºC until analysis (laboratory of the company Povodí Vltavy Ltd., České Budějovice). The following components and variables were determined (Table 1): BOD (Biological Oxygen Demand) by suppression of nitrification, COD (Chemical Oxygen Demand) using an analytical commercial kit (Merck), TOC (Total Organic Carbon) by thermal decomposition with Pt catalyst, nitrite content (NO_2-N) by automatic discrete photometry, ammonium nitrogen (NH_4-N) acidimetrically after distillation, total nitrogen content (N_{tot}) by thermal oxidation with electrochemical detection and nitrate concentration (NO_3-N) by addition to the total nitrogen content. The content of orthophosphate-phosphorus (PO_4-P) was assayed by automatic discrete photometry and total phosphorus content (P_{tot}) by photometry after mineralization.

Table 1 Averaged composition (n=3) and variables of the centrate of municipal wastewater taken from the Wastewater Treatment Plant in Třeboň which was used for cultivation of *Chlorella* in outdoor units. Total nitrogen and phosphorus content in the BG-11 medium are shown for comparison.

	Centrate [mg L^{-1}]	**BG-11 [mg L^{-1}]**
BOD*	197±38	-
COD**	1367±208	-
TOC***	607±130	-
N-NO_3	0.11±0.14	250
N-NO_2	0.005±0.002	-
N-NH_4	207±6	-
Total N	**273±32**	**250**
P-PO_4	163±12	7
Total P	**183±21**	**7**

*Biological oxygen demand; **Chemical oxygen demand; ***Total organic carbon

2.7. Photosynthesis measurements

Photosynthetic activity of the cultures was estimated using two techniques: Chl fluorescence to follow changes in maximum photochemical yield F_v/F_m and by measurement of POE and R to construct steady-state light-response curves. These techniques have been were already described in detail before [29,30,32,40]

2.7.1. Rapid light-response curves

Rapid light-response curves (RLCs) of relative electron transport (rETR) of microalgae samples were measured in a light-protected measuring chamber with mixing (3 mL glass cuvette, light path of 10 mm) using a pulse-amplitude-modulation fluorimeter (PAM-2500, H. Walz, Germany). Before measurements the culture was diluted to 0.2-0.3 g DW L^{-1} to avoid re-absorption problems in a dense culture and the sample was 10 min dark adapted.

The minimum and maximal fluorescence level (F_0, F_m) were determined using a weak modulated light [<0.15 μmol photons m^{-2} s^{-1}, frequency of 0.5–1 kHz] in the dark-adapted samples (actinic irradiance = 0; first step of RLC). A stepwise increasing irradiance intensities (between 0-2500 μmol photons m^{-2} s^{-1}) were applied in 20-s intervals to obtain the steady state fluorescence level. The maximal PSII photochemical was calculated as the ratio of variable and maximal fluorescence, $F_v/F_m = (F_m - F_o)/F_m$. It is frequently used as a convenient estimate of the photochemical performance as it indicates the maximum quantum efficiency of primary photochemistry [26].

2.7.2. Measurement of photosynthetic oxygen evolution and respiration rates

POE and R measurements were carried out in a temperature-controlled chamber with adjustable illumination and mixing (DW2/2, Hansatech, UK) connected to temperature-controlled bath and a control unit (Oxygen Monitoring System Oxylab+, Hansatech, UK). The samples were diluted to 0.2-0.3 g DW L^{-1} and dark adapted and measured at temperature corresponding to outdoor unit. Steady-state light-response curves were recorded using a stepwise increasing light intensity of 0, 200, 400, 600, 1200 and 1800 μmol photons m^{-2} s^{-1}, when each step lasted 2 min. The first step (in the dark) provided a value of the dark RESP. Oxygen production and RESP were calculated in pmol O_2 $cell^{-1}$ h^{-1}.

2.8. Bioactivity determination

The antimicrobial (biopesticide) activity of the *Chlorella* biomass samples were detected with the antagonism bioassay. The responsible plant hormones for plant biostimulating activity were detected by three different bioassays, which measure the auxin-like (seed germination, mung bean rooting) and cytokinin-like (chlorophyll retention) activities.

2.8.1. Antagonism Bioassay by Dual Culture

The protocol followed in this work has been described [41,42]. The amount of 10 mg DW mL^{-1} lyophilized *Chlorella* biomass was sonicated (Branson sonicator 150, amplitude 40%, 3 min) and

subsequently 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 The activity of all extracts was tested against two fungi – *Fusarium oxysporum* f.sp. *melonis, Rhizoctonia solani* (further as *Fusarium, Rhizoctonia*) and two oomycetes – *Phytophthora capsici Pythium ultimum* (further as *Phytophthora* and *Pythium*) and four bacteria strains – *Clavibacter michiganensis* subsp. *michiganensis, Xanthomonas campestris* pv. *vesicatoria, Pseudomonas syringae* pv. *tomato, Pectobacterium carotovorum* (*Clavibacter, Xanthomonas, Pseudomonas* and *Pectobacterium*). All strains were provided by the Spanish Type Culture Collection (STCC). In the case of bacteria, the growth inhibition clear zones were measured in the presence of *Chlorella* extract. The inhibition of the growth of phytopathogenic fungi was calculated according to the growth diameter in the presence of *Chlorella* extract.

The inhibition index was calculated according the following formula:

$$I = [(C-T)/C] \times 100,$$

where I is the inhibition index in %, C is the diameter of grown pathogen in the absence of microalgae extract (mm) and T is the diameter of pathogen in the presence of algal extract (mm). In all cases, control bioassays were prepared using distilled water.

2.8.2. Seed Germination Bioassays

The biostimulant activity was tested on 100 cress (*Lepidium sativum*) seeds using extracts of 0.5 and 2 mg DW mL^{-1} of microalgae biomass according to the procedure previously described [43]. To carry out this bioassay, samples were taken from lyophilized *Chlorella* biomass and prepared as mentioned above. The percentage of seed germination as well as the radicle elongation were taken into account for the calculation of the Germination Indexes, based on the following formula:

$$GI = (G_S\% * L_S) / (G_w\% * L_w),$$

where GI is Germination Index in %, G_S% is a 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.

2.8.3. Determination of auxin-like activity

The bioassay was performed according to reference [44]. The mung bean (*Vigna radiata* (L.) Wilczek) seeds were soaked for 4 min in 0.33% sodium hypochlorite solution, then removed and rinsed under running tap water for 24 h. The seeds were planted at 1 cm depth in moistened perlite in plastic trays. The trays were placed in the growth chamber maintained at 27 °C and about 60 to 65% relative humidity, illuminated with fluorescent lamps (120 µmol photons m^{-2} s^{-1}) for 7 to 10 days. The seedlings are then cut with clean razor 12 cm below the two leaves. Five uniform cuttings were placed

in vials of 25 x 90 mm (three vials per treatment) containing 10 ml of distilled water or algal nutrient solution, as controls, and algal suspensions (0.5, 1.0, 2.0 and 3 g DW L^{-1}), as treatment. Freeze-dried *Chlorella* biomass was suspended in water and sonicated for 3 minutes and used in the bioassays. A standard curve to each bioassay was also prepared by using indol-3-butyric acid (IBA) at concentrations of 0, 0.3, 0.5, 0.7 and 1 mg DW L^{-1}. The vials were placed in the growth chamber in the original growth conditions for one day. Than all plants were removed from the vials, washed thoroughly with distilled water and putted back to the growth chamber for 8 days. The solution (lost by transpiration) was brought up to its original level with distilled water daily. After the incubation period, the number of roots (longer than 1 mm) were recorded on each hypocotyl. The mean number of roots were compared to IBA which was used as a standard auxin. The results are given in IBA equivalent concentrations.

2.8.4. Determination of cytokinin-like activity

Cytokinins accelerate chloroplast differentiation as well as regulate and stimulate the chlorophyll production. The use of wheat leaves as a specific bioassay for cytokinins was already reported [45]. Seeds of a wheat cultivar (*Triticum aestivum* L.) were soaked for 5 min in sodium hypochlorite solution (2%), then removed and rinsed under running tap water for 24 h. The seeds were planted at 1 cm depth in moistened perlite in plastic trays. The trays were placed in growth chamber maintained at 25 °C and about 60 to 65% relative humidity, illuminated with fluorescent lamp (120 μmol photons m^{-2} s^{-1}) for 7 days. Leaves from seedlings (about 10 cm height) were collected and then cut 35 mm below their apical tip into 10 mm segments. The fresh weight of ten cuttings are measured with an analytical grade balance and placed in glass vials of 25 x 90 mm size (four vials per treatment) containing 10 mL of distilled water or algal nutrient medium as controls, and algal suspensions (0.5, 1.0, 2.0 and 3.0 g DW L^{-1}), as treatment. A standard curve to each bioassay was prepared by using kinetin (KIN) at the concentration of 0, 0.3, 0.5 and mg DW L^{-1}. The vials were placed in the dark growth chamber for 4 days. After the incubation period the leaves were blotted dry and transferred into centrifuge tubes containing 8 mL of 80% ethanol. The tubes were kept in a water bath (warmed up to 80-90 °C) for 10 min than cooled down to room temperature and completed with 80% ethanol up to 10 mL. The chlorophyll extract was then carefully poured, without the segments, into spectrophotometer cuvettes. The optical density was measured at 645 nm and compared to the KIN. The results are given in KIN equivalent.

2.9. Statistical analysis

Sigma Plot 11.0 was used to determine significant differences between treatments. One-way ANOVA and Holm-Sidak test was conducted for every binary combination of data. *P* values lower than 0.05 were considered significantly different.

3. Results

3.1. Growth

The *Chlorella* cultures were grown in the outdoor cultivation units – TLC and TL-RWP either in inorganic BG-11 medium or undiluted WW. During the first week of cultivation in BG-11 medium in batch regime, both cultures grew well, linearly between Day 1 and 7 when cultivated, only short lag phase was observed between Day 0 and Day 1 as the laboratory cultures acclimated to higher irradiance outdoors (Fig. 3). Nevertheless, the *Chlorella* culture grown in TLC showed about 35% higher ($\mu = 0.19$ d^{-1}) as compared to the culture grown in TL-RWP ($\mu = 0.14$ d^{-1}). The slower growth in TL-RWP can be caused by deeper culture layer causing lower average cell irradiance compared to that in TLC. When *Chlorella* culture was grown in WW the lag phase was observed between Day 0 and Day 2. The specific growth rate in TLC was higher by about 21% ($\mu = 0.23$ d^{-1}; Fig. 3b) compared to that in BG-11 medium (Fig. 3a). Here, we consider that as a result of better light conditions during cultivation in WW (Fig. 2). At the end of the batch regime the culture grown in BG-11 reached biomass density 2.6 and 2.1 g DW L^{-1} in TLC and TL-RWP, respectively while in WW it was 3.1 and 2.4 g DW L^{-1} (Fig. 3). Nevertheless, it is important to note that the WW used as a source of nutrients did not have any negative effect on growth, but rather positive.

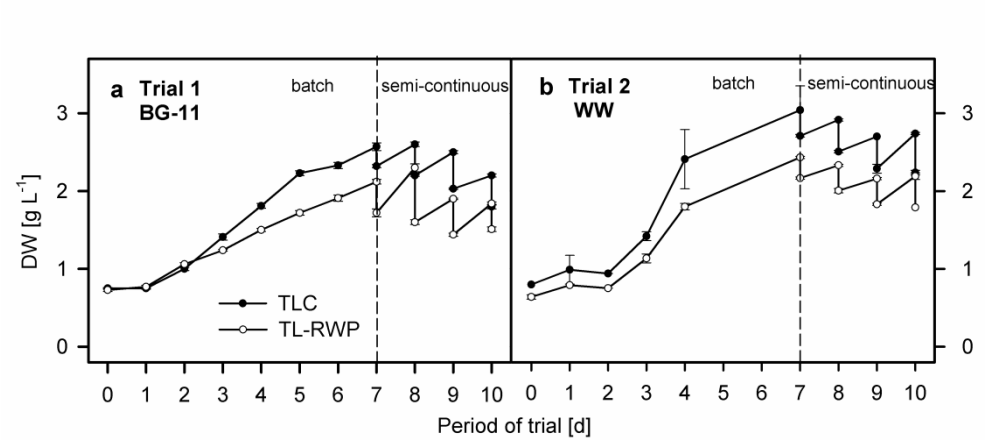

Fig. 3 Changes in the biomass density (DW) of *Chlorella* MACC-1 cultures grown in batch and semi-continuous regime in thin-layer cascade (TLC; black circle) and thin-layer raceway pond (TL-RWP; white circle) grown in: a) BG-11 medium (Trial 1) and b) centrate of municipal wastewater (Trial 2).

During the second week starting from Day 7 afternoon, the cultures were grown in semi-continuous regime, removing 25% (dilution rate DR = 0.25 d^{-1}) of culture volume every day and replacing by either BG-11 medium or undiluted WW. On the next day the cultures were almost able to reach the biomass density measured before dilution indicating that the DR can be lower to 0.2 d^{-1} in the future. We have to take into account that higher growth of *Chlorella* culture in both units using WW (Fig. 3b) was probably caused by higher irradiance intensity in this period (Fig. 2b). During the cultivation of *Chlorella* cultures in WW, the concentrations of total N and P were decreasing during the batch regime (Day 0 – Day 7) in both units (Fig. 4a).From Day 0 until Day 7 the concentration of total N

decreased from 290 mg DW L^{-1} to 170 mg DW L^{-1} in TLC and from 190 to 140 mg DW L^{-1} in TL-RWP (Fig. 4). The uptake of P during the batch regime was not so efficient (Fig. 4a-b). This could be caused by fast N-NH_4 exhaust [46]. The concentration of N-NH_4 in both cultivation units dropped during first two days almost to zero while the uptake of N-NO_3 was efficient only in TLC. The concentration of N-NO_3 in TL-RWP was still 3.4 mg DW L^{-1} at the end of batch regime.

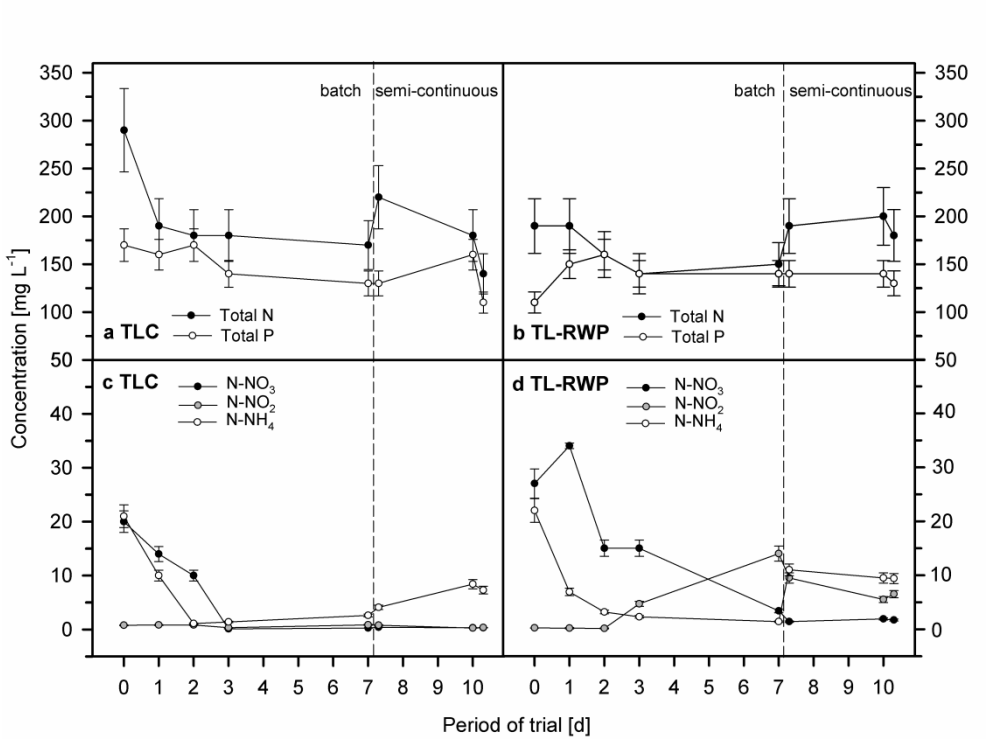

Fig. 4. Nutrient concentration during the growth of *Chlorella* in municipal wastewater: a) total nitrogen (black circle) and phosphorus (white circle) during the cultivation in thin-layer cascade (TLC); b) total nitrogen (black circle) and phosphorus (white circle) during the cultivation in thin-layer raceway pond (TL-RWP); c) nitrate-nitrogen (N-NO_3; black circle), nitrite-nitrogen (N-NO_2; grey circle) and ammonium-nitrogen (N-NH_4; white circle) amount during the cultivation in TLC; d) nitrate-nitrogen (N-NO_3 black circle), nitrite-nitrogen ((N-NO_2; grey circle) and ammonium-nitrogen (N-NH_4; white circle) amount during the cultivation in TL-RWP.

3.2. Photosynthesis performance

The changes in the photosynthetic activity were estimated as the maximum PSII photochemical rate, F_v/F_m by Chl fluorescence (Fig. 5). The data were measured twice a day in the morning (0800 h) and at midday (1300 h). The lower F_v/F_m values were observed in Trial 1 (in BG-11 medium) in both units during the Day 0 and Day 1 which indicated that laboratory cultures were partially photo-stressed after the exposure to outdoor irradiance and needed some time for acclimation. It corresponded to the lag phase also seen in growth curves for the first two days (Fig. 3).

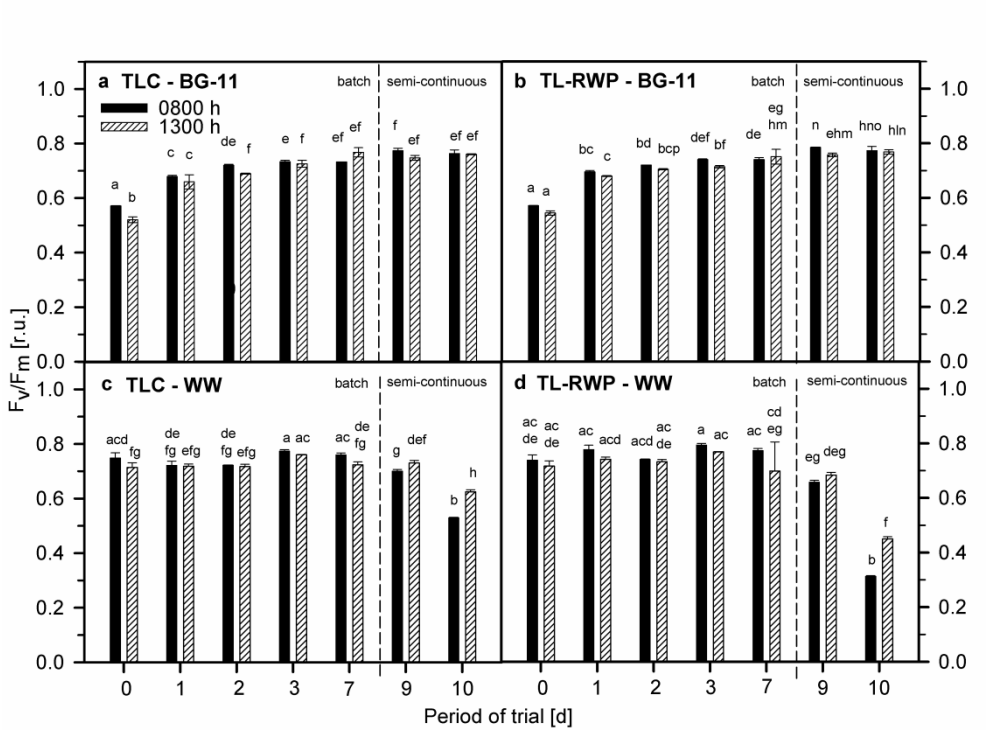

Fig. 5. Changes in maximum effective quantum yield of PSII (F_v/F_m) during the cultivation of *Chlorella* cultures in batch and semi-continuous regime: a) cultures grown in TLC in BG-11 media; b) cultures grown in TL-RWP in BG-11 media; c) cultures grown in TLC in WW; d) cultures grown in TL-RWP in WW. The black columns represent the measurement at 0800 h and dashed columns represent the measurement at 1300 h. Values with the same symbol did not differ significantly from each other (P>0.05).

The variable F_v/F_m did not change much during both trials, it mostly ranged between 0.70 and 0.79. In green microalgae such values show that the cultures are in good physiological conditions [47]. Starting from Day 7 after the transfer to the semi-continuous regime, the measurements were carried out before the dilution to avoid any problems of culture disturbance. Compared to high photochemical performance of the cultures grown in Trial 1, the F_v/F_m values of cultures grown in Trial 2 (in WW) were lower at the end of the cultivation, probably indicating decreased photosynthetic activity due to some stress (e.g. shade adaptation in dense cultures). In Trial 2 the F_v/F_m values ranged from 0.70 to 0.77 and dropped significantly to 0.3-0.5 on the last day of cultivation which indicated higher degree of stress. As this stress was pronounced in the morning samples, we suppose it is caused by the growth in WW, maybe because of the presence of some microorganisms.

3.3. Photosynthetic oxygen production and respiration

When the *Chlorella* cultures were grown in BG-11 the maxima of POE activity in both units after inoculation was between 60 and 100 pmol O_2 cell^{-1} h^{-1} (Day 0) as the cultures were acclimating to the high light comparing to the light intensity in the laboratory. The maxima POE reached up to 150 pmol O_2 cell^{-1} h^{-1} (Day 3) after the acclimation during the batch regime (Fig. 6a-b). In the semi-continuous regime, the POE activity was higher in TLC as compared to that in TL-RWP. In TLC, the R rates

were between 50 and 80 pmol O_2 $cell^{-1}$ h^{-1} in the batch regime; in semi-continuous regime these increased from 80 to 200 pmol O_2 $cell^{-1}$ h^{-1}. In semi-continuous regime the R rate even increased due to higher biomass density and lower ambient irradiance. The same trends were observed in TL-RWP culture, only the photosynthesis rate was lower and R rate higher which is probably due to the growth in deeper-layer culture (Fig. 6b). Important to note that R rate in both cultures was quite high, in semi-continuous regime usually higher than photosynthesis, most probably due to the growth in high biomass density and relatively low ambient irradiance.

When *Chlorella* culture was cultivated in WW in batch regime, higher activity of POE was observed in both units – up to 340 pmol O_2 $cell^{-1}$ h^{-1} on Day 3 compared to Trial 1 performed in BG-11 media. When the units were operated in semi-continuous regime, POE was still high in both units and then it was decreasing. The R rate was high, especially in TL-RWP which could be caused by deeper culture layer and less cell exposed to the light.

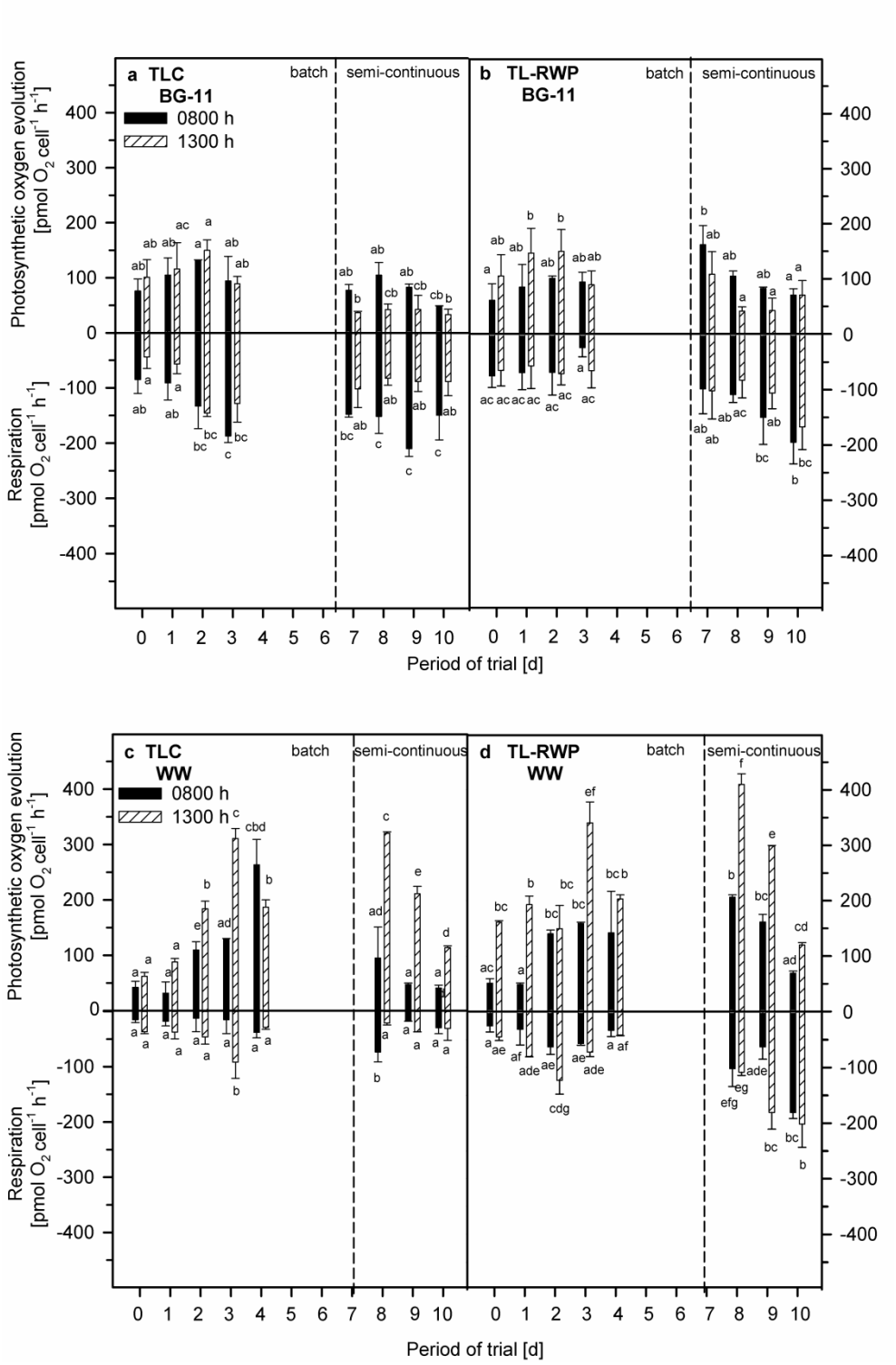

Fig. 6. Changes of photosynthetic oxygen evolution and respiration measured in *Chlorella* cultures in two outdoors cultivation units in batch (Day 0 – Day 6) and semi-continuous cultivation regime (Day 7 – Day 10): a) culture grown in TLC in BG-11; b) culture grown in TL-RWP in BG-11; c) culture grown in TLC in WW centrate; d) culture grown in TL-RWP in WW centrate. The black columns represent the measurement at 0800 h and dashed columns represent the measurement at 1300 h. Values with the same symbol did not differ significantly from each other (P>0.05).

If we compare the growth rate (Fig. 3), maximum photochemical PSII yield (Fig. 5), POE and R rate (Fig. 6), we found the correlation as the trends are similar. In batch regime we found relatively good

growth rate in both units although TLC was performing better. There was not much difference in F_v/F_m and POE between both units, only R was higher in TL-RWP. In semi-continuous regime when the *Chlorella* culture was grown in WW, we could find a clear interplay among ambient irradiance intensity, growth rate, PSII photochemical efficiency F_v/F_m and POE/R. In both units the growth rate was quite high due to sufficient ambient irradiance, increasing or decreasing stepwise according to dilution, but still the growth rate was 10-15% higher in TLC compared to that in TL-RWP. At the end of Trial 2, F_v/F_m significantly decreased in both units as well as POE; on the contrary R significantly increased compared to batch regime.

3.4. Bioactivity determination

Results of the dual culture assay for determination of biopesticide activity were varying as the extracts from biomass samples at different daytimes were active against individual bacteria and fungi (Table 2). Out of the total number of 8 pathogenic microorganisms, the biomass extracts of *Chlorella* cultivated in BG-11 medium were active only against one bacterial strain of *C. michiganensis* and three fungal strains, *F. oxysporum, R. solani* and *P. capsici.* Antifungal activity up to 37% against *F. oxysporum* was determined for the biomass harvested from TLC. Generally, the antibacterial and antifungal activity was higher when *Chlorella* cultures was grown in WW as compared to those grown in BG-11 (Table 2). It is important to note that antifungal activity of the *Chlorella* cultures grown in WW was about twice higher compared to antibacterial activity. Antifungal activity against all fungal pathogens was almost always higher when *Chlorella* biomass was harvested at midday. The highest antifungal activity of *Chlorella* of 41.4 (0800 h) and 50.4% (1300 h) was observed against *P. capsici* when grown in WW.

Table 2 Antibacterial and antifungal activity of *Chlorella* extracts. The biomass was harvested at the end of semi-continuous regime in the morning (0800 h) and in the afternoon (1300 h). Statistical analysis was performed between the rows. Values with the same letter did not differ significantly from each other (P>0.05).

	Inhibition index [%]							
	Trial 1 (BG-11)				**Trial 2 (WW)**			
Pathogen	TLC		TL-RWP		TLC		TL-RWP	
	0800 h	1300 h	0800 h	1300 h	0800 h	1300 h	0800 h	1300 h
Bacteria								
C. michiganensis	25.6±0.6^{a}	21.7±0.3^{a}	22.8±0.3^{a}	22.8±0.3^{a}	0^{b}	20.8±3.3^{a}	0^{b}	23.1±7.3^{a}
X. campestris	0^{a}	0^{a}	0^{a}	P	0^{a}	18.3±5.3^{b}	16.9±5.3^{b}	21.9±5.3^{b}
P. syringae	0^{a}	0^{a}	0^{a}	0^{a}	12.2±5.3^{b}	16.7±0.1^{bc}	0^{a}	17.8±2.0^{c}
P. carotovorum	0^{a}	0^{a}	0^{a}	0^{a}	14.7±4.7^{b}	15.8±6.7^{b}	15.6±4.0^{b}	17.3±7.5^{b}
Fungi								
R. solani	11.3±3.0^{a}	23.8±4.8^{b}	0^{c}	10.1±1.8^{a}	27.2±0.2^{b}	42.3±4.0^{d}	28.8±0.2^{b}	44.2±0.2^{d}
F. oxysporum	37.5±8.9^{a}	33.3±7.1^{a}	1.2±1.2^{b}	32.1±8.3^{a}	28.7±1.3^{a}	26.8±2.0^{a}	31.6±2.0^{a}	32.0±2.0^{a}
Oomycetes								
P. capsici	19.6±2.6^{a}	34.6±2.5^{ab}	0^{c}	3.7±3.7^{c}	41.4±0.7^{b}	50.4±0.1^{b}	46.9±6.7^{b}	41.4±17.3^{b}
P. ultimum	0^{a}	0^{a}	0^{a}	0^{a}	25.7±2.7^{b}	34.3±1.3^{c}	33.3±4.0^{c}	35.6±1.3^{c}

Bioactivities were determined by two methods – antimicrobial activity using dual culture assay as inhibition index and biostimulating activity as germination index. The biomass for seed germination bioassay was harvested on Day 10, the last day of the growth in semi-continuous regime at two daytimes (0800 h and 1300 h) from both units and both treatments. Higher biostimulant activity was observed when a lower concentration of the biomass extract (0.5 mg DW mL^{-1}) was applied as it ranged from 108 to 115% as compared to the 2 mg DW L^{-1} extract. As concerns daytime, the higher biostimulating activity of all extracts was mostly observed when the *Chlorella* biomass was harvested in the morning (0800 h) using less concentrated biomass extract with the exception of the sample harvested at 0800 h from TLC (Table 3). Surprisingly, no biostimulating activity was found in the *Chlorella* cultures grown in WW. This could be caused probably because of some inhibitors present in WW which remain in the freeze-dried biomass sample and inhibit the seed germination [48].

Table 3 Biostimulating activity of *Chlorella* extracts. The biomass was harvested from TLC and TL-RWP at the end of semi-continuous regime in the morning (0800 h) and in the afternoon (1300 h) when cultivated in BG-11 medium, lyophilised and then studied. No activity of biomass harvested at the end of Trial 2 (WW) was found. Values with the same letter did not differ significantly from each other (P>0.05).

c [mg/mL]	Germination index [%] **Trial 1 (BG-11)**			
	TLC		TL-RWP	
	0800 h	1300 h	0800 h	1300 h
0.5	108.5±0.2[a]	108.5±0.1[a]	**115.7±0.5[b]**	108.6±1.2[a]
2	**100.8±0.2[c]**	85.5±0.2[d]	**105.4±0.5[e]**	86.0±2.1[d]

Auxin-like activities equivalent to 0.3 and 0.5 mg DW L^{-1} of IBA were measured in 2.0 and 3.0 g DW L^{-1} *Chlorella* suspensions (biomass harvested from TLC at 0800 h), respectively. Both auxin- and cytokinin-like activities were detected in the *Chlorella* suspensions tested in the same concentrations, when the biomass was harvested from TL-RWP at 1300 h. In this case the auxin-like activity was equivalent to 0.5 mg DW L^{-1} of IBA and was independent from the concentrations of algal suspension (1.0, 2.0 and 3.0 g DW L^{-1}). This was the only sample demonstrating cytokinin-like activity equivalent to 0.3 mg DW L-1 of kinetin (Table 4).

The results obtained in these experiments show clearly that the cultivation in WW has a positive effect on the accumulation of bioactive compounds responsible for antibacterial and even more for antifungal activity (Table 2) but no biostimulating effects were detected in the seed germination bioassay (Table 3) and in the bioassays for detection of auxin- and cytokinin-like activities (Table 4).

Table 4 Auxin- and cytokinin-like activity (equivalent to IBA and KIN concentration in mg L^{-1}) of *Chlorella* samples detected by mung bean root development and wheat chlorophyll retention test. No activity of biomass harvested at the end of Trial 2 (WW) was found.

Sample	Time of harvesting	**Auxin- and cytokinin-like activity** Concentration of microalgae extract [g L^{-1}]							
		0.5		1.0		2.0		3.0	
		IBA	KIN	IBA	KIN	IBA	KIN	IBA	KIN
TLC	0800 h	0	0	0	0	0.3	0	0.5	0
	1300 h	0	0	0	0	0	0	0	0
TL-RWP	0800 h	0	0	0	0	0	0	0	0
	1300 h	0	0	0.5	0	0.5	0.3	0.5	0.3

4. Discussion

To survive at adverse conditions, microalgae are able to synthetize numerous compounds [49] and many of them possess antibacterial and antifungal activity [50,51]. The loss of yields from agricultural production due to the presence of pathogens has been prevented over the years by synthetic pesticides, but the use of these substances negatively affects the environment and presents health risk for consumers and animals [77]. Microalgae can be used as one of the main biological agents for the control of pathogenic fungi and soil-borne diseases in plants [78]. In microalgae several amino acids, vitamins and polyamines can act as the growth-promoting substances [8,54,73–75]. Also extracts and hydrolysates of high protein containing microalgae biomass (up to 50-60% in dry weight) contain the active substances, mainly amino acids and small peptides can act as plant biostimulants. Microalgae also contain polysaccharides (such as α-glucan) that may be also involved in the improvement of plant growth [71,76]. The antimicrobial substances include unsaturated lactones, glycosides, sulphur containing compounds, phenols and phenolic glycosides, saponins and phytoalexins as well as fatty acids, acrylic acid, halogenated aliphatic compounds, terpenes, sulphur containing heterocyclic compounds, carbohydrates and phenols [49]. A range of results of in vitro anti-fungal activities of extracts of green algae, diatoms, and dinoflagellates have been reported [52,53]. The first report of compounds with biopesticide activity from microalgae was about a bioactive compound from the green alga *Chlorella* the chlorellin which acts inhibiting the growth of Gram-negative and Gram-positive bacteria [83].

Microalgae can be grown in controlled processes in constructed cultivation units and they do not need arable land compared to higher plants. They can reach the higher production of biomass per unit area [54]. In order to increase sustainability, the microalgae can be grown using alternative sources of nutrients to reduce production costs [55]. In outdoor deep-culture systems for example open ponds, low turbulence results in unsuitable cell irradiance regime and subsequently low biomass productivity [17]. A more advantageous is the use of shallow, thin-layer systems like sloping cascades or cascade raceways which are more efficient for biomass production [10,56–58]. To avoid any contamination and for better control of ambient conditions the units can be placed in greenhouses [39,59,60].

In this work, we have tested two open, thin-layer cultivation systems – TLC and modified shallow TL-RWP placed in greenhouses whose design is suitable for good light utilisation and high biomass productivity can be reached, they are also quite suitable for *Chlorophyta* as these can tolerate high average cell irradiance [39]. Moreover, thin-layer systems are advantageous when working with dark-coloured centrate to avoid substantial light absorption. In our experiments, the TLC was performing slightly better than the TL-RWP due to lower layer thickness, but on the other hand, the latter is more universal as it can be operated at higher volume per area and paddle wheel is a gentle mixing device for some susceptible strains. [10,57,58]. Based on our findings, the freshwater *Chlorella* can be grown in undiluted centrate from municipal wastewater as the only nutrient source. It is evident that

Chlorella used in this study represents a robust, well-growing strain suitable for cultivation in WW as it can tolerate and assimilate high content of ammonium (about 180 mg DW L^{-1}) and organic carbon (TOC, about 490 mg DW L^{-1}). Moreover, in these particular trials the *Chlorella* culture was growing better in WW compared to that in BG-11, presumably due to higher ambient irradiance during the cultivation in WW. The culture irradiance is a crucial variable as the light availability has been definitely the limiting factor, not nutrition as shown in our previous reports [33,61]. Our results are confirmed by previous reports [59,62] although they used diluted centrate after anaerobic digestion for outdoor culturing of *Scenedesmus*. Data in the literature has confirmed that microalgae can be grown in wastewater, recovering nutrients and recycling water for further use [1,16,55,63,64]. In addition, microalgae can uptake CO_2 from flue gas, reducing greenhouse gas emissions.

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5. Conclusions

To conclude our trials, the microalga *Chlorella* shows biostimulating and biopesticide activity, but cultivation conditions inducing the differences. The cultures grew well in both nutrients sources, inorganic nutrient medium and in undiluted centrate from municipal wastewater as well.

In these trials we used a model of large-scale production where microalgae are to be grown in semi-continuous/continuous regime replacing daily a part of microalgae culture with fresh medium. Samples for bioactivity assay were taken in semi-continuous culturing regime in the morning and in the afternoon when the physiological status and photosynthetic activity of *Chlorella* cultures differ significantly.In semi-continuous regime when the *Chlorella* culture was grown in BG-11 medium or WW, we could find a clear interplay among ambient irradiance intensity and growth rate vs. photochemical efficiency of PSII, F_v/F_m and POE/R. In both units the growth rate was related to ambient irradiance as we used thin-culture-layer units, increasing or decreasing stepwise according to dilution. Still, the growth rate was 10-15% higher in TLC compared to that in TL-RWP.

As concerns antifungal and antibacterial activity, these were generally higher when *Chlorella* cultures were grown in WW as compared to those grown in BG-11. These activities in all *Chlorella* samples grown in both BG-11 and WW and taken at midday (1300 h) were mostly higher than those sampled in the morning (0800 h). Somehow this activity might be related/induced by the physiological status of the culture when exposed to higher irradiance. It is also important to note that antifungal activity of the *Chlorella* cultures grown in WW was about twice higher compared to antibacterial activity.

The highest biostimulating activity has been found for *Chlorella* cultures grown in the inorganic BG-11 medium. As concerns daytime, the highest activity of all extracts was observed when the *Chlorella* biomass was harvested in the morning using the lower concentration of the biomass extract. Surprisingly, no biostimulant activity was found in the *Chlorella* cultures grown in WW. This activity may be related to relaxed physiological status of *Chlorella* cultures.

The highest auxin-like activity equivalent to 0.3 mg DW L^{-1} of IBA has been detected for *Chlorella* (2.0 g DW L^{-1}) when cultivated in TLC and harvested in the morning (0800 h). Both auxin- and cytokinin-like activities (in the range between 0.3 and 0.5 mg DW L^{-1} of IBA or KIN) have been found for the biomass harvested from TL-RWP in the afternoon (1300 h).

At the proper set cultivation condition selected microalgae strains can be grown in order to obtain biomass either with biostimulating or antibacterial and antifungal properties.

Author Contribution

Karolína Ranglová, Jiří Masojídek, Vince Ördög and Francisco Gabriel Acién-Fernández conceived research. Karolína Ranglová, Gergely Ernö Lakatos, João Artur Câmara Manoel, Tomáš Grivalský, Francisca Suárez Estrella, Vince Ördög and Zoltán Molnár carried out joint experiments, analysed data. Karolína Ranglová prepared the text and figures of the manuscript. Jiří Masojídek, Francisco Gabriel Acién-Fernández and Vince Ördög revised and finalized the manuscript. All authors read and approved the manuscript.

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Ethical statements

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be understood as a potential conflict of interest.

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