University of South Bohemia in České Budějovice
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Photosynthesis Monitoring in Microalgae Mass Cultures

Ph. D. Thesis

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Annotation: This Ph.D. thesis deals with principles of microalgae cultivation in laboratory as well as outdoor aquacultures (Chapter 1) using various cultivation systems and photobioreactors (Chapter 2). Case studies illustrate the main research topic as to correlate changes in growth rate with variation of photosynthetic activity, physiological features and biomass composition (Chapter 3). Special attention was paid to elaboration of protocols of chlorophyll a fluorescence techniques for monitoring the physiology and photosynthetic performance of microalgae mass cultures maintained under various growth conditions (Chapter 4).

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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In České Budějovice, on 17th August 2018

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Partnership

This Ph.D. thesis originated from a partnership of the Faculty of Science, University of South Bohemia in České Budějovice supporting doctoral studies in the P1701 Physics/P1513 Biophysics study program in collaboration with the Institute of Microbiology of the Czech Academy of Science, Algatech Centre in Třeboň, the University of Málaga in Spain, the Institute of Ecosystem Study, National Research Council in Sesto Fiorentino, Italy and Ecoduna AG in Bruck an der Leitha, Austria.

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Motto:

In life there is neither rewind nor fast forward, so live life to the fullest and make each day count.

To Lauren and Jose Gabriel,

My Father (+), Rolando and Mother, Gregoria; brothers, Jose Rene and Jose Roy

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

1. **Malapascua JR**, Ranglová K, Masojídek J (2018) Photosynthesis and growth kinetics of *Chlorella vulgaris* R-117 cultured in an internally LED-illuminated photobioreactor. Photosynthetica (accepted) (50%; IF 1.507)
   JRM was responsible for a set-up of photobioreactor, design of experiments, performed measurements of growth rate, biochemical analyses and photosynthetic activities, carried out interpretation of collected data, and assisted in preparation of figures, and writing and revision of the manuscript.

2. Benavides AM, Ranglová K, **Malapascua JR**, 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)
   JRM was responsible for the measurements of photosynthetic activities of the cultures and interpretation of the collected data, preparation of figures and revision of the MS.

3. 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)
   JRM contributed to the design of the experiment, setting-up of indoor cultivation units, measurements of photosynthetic activities of the cultures, interpretation of the collected data, and preparation, writing and revision of the manuscript.

4. Jerez CG, **Malapascua JR**, Sergejevová M, Masojídek J, Figueroa FL (2016) *Chlorella fusca* (Chlorophyta) grown in thin-layer cascades: Estimation of biomass productivity by *in-vivo* chlorophyll a fluorescence monitoring. Algal Research - Biomass Biofuels and Bioproducts 17:21-30 (30%; IF 3.994)
   JRM participated in the setting-up and maintenance of outdoor cultures, measurements of photosynthetic activities, interpretation of the data, preparation of figures, and writing and revision of the manuscript.

5. Jerez, CG, **Malapascua JR**, Sergejevová M, Figueroa FL, Masojídek, J (2016) Effect of nutrient starvation under high irradiance on lipid and starch accumulation in *Chlorella fusca (Chlorophyta).* Marine Biotechnology 18:24-36 (30%; IF 2.748)
   JRM contributed equally to the design of the experiment, setting-up of indoor cultivation units, measurements of photosynthetic activities of cultures, interpretation of the data, and preparation, writing and revision of the manuscript.

6. Masojídek J, Sergejevová M, **Malapascua JR**, Kopecký J (2015) Thin-layer systems for mass cultivation of microalgae: flat panels and sloping cascades. In: Prokop et al. (eds) Algal Biorefineries Volume 2: Products and Refinery Design. Springer International, Switzerland, pp 237-261 (20%; book chapter)
   JRM assisted in the measurement of photosynthetic activities, evaluation of data, preparation of figures and writing/revision of the manuscript.

7. Sergejevová M, **Malapascua JR**, Kopecký J, Masojídek J (2015) Photobioreactors with internal illumination. In: Prokop et al. (eds) Algal Biorefineries Volume 2: Products and Refinery Design. Springer International, Switzerland, pp 213-236 (20%; book chapter)
   JRM prepared a set-up of photobioreactors, performed measurements of growth rate and photosynthetic activities of cultures, made interpretation of collected data, and assisted in preparation of figures, and writing and revision of the manuscript.

8. Ihnken S, Beardall J, Kromkamp JC, Gómez SC, Torres, MA Masojídek J, Malpartida I, Abdala R, Jerez CG, **Malapascua JR**, Navarro E, Rico RM, Peralta E, Ezequil JPF, Figueroa FL (2014) Light acclimation and pH perturbations affect photosynthetic performance in Chlorella mass culture. Aquatic Biology 22:95-110 (10%; IF 1.258)
   JRM performed measurements of photosynthetic activities of the cultures and assisted in the interpretation of the collected data and preparation of the manuscript text.

9. Malpartida I, Jerez CG, Morales MM, Nascimento P, Freire I, Ezequiel J, Rico RM, Peralta E, **Malapascua JR**, Florez Y, Masojídek J, Abdala R, Figueroa FL, Navarro E (2014) Synergistic effect of UV radiation and nutrient limitation on *Chlorella fusca* (Chlorophyta) cultures grown in outdoor cylindrical photobioreactors. Aquatic Biology 22:141-158 (10%; IF 1.258)
   JRM performed measurements of photosynthetic activities of the cultures and assisted in the interpretation of the collected data and preparation of the manuscript text.

10. **Malapascua JRF**, Jerez CG, Sergejevová M, Figueroa FL, Masojídek J (2014) Photosynthesis monitoring to optimize growth of microalgal mass cultures: application of chlorophyll fluorescence techniques. Aquatic Biology 22:123-140 (50%; IF 1.258)
    JRM made major contributions to this article; was responsible for the measurements of photosynthetic activities of the cultures, interpretation of collected data, preparation of figures, and writing and revision of the manuscript.

Contents

Foreword

This work mainly deals with the use of chlorophyll a fluorescence in assessing the physiology and photosynthetic performance of microalgae cultures maintained under various conditions in indoor and outdoor cultivation systems. This approach was determined by research interests of the Laboratory of Algal Biotechnology starting from the time when Centrum Algatech was established in Třeboň, Czech Republic.

This work could have not become a reality if not for the assistance of my colleagues and friends from Třeboň, who were kind enough to accept me in their group. I am eternally grateful to my exceptional supervisor Jiří Masojídek who tirelessly and patiently expounded my understanding of the use of chlorophyll a fluorescence in microalgae biotechnology and whose open- and broad-mindedness has kept me grounded. I am also forever thankful to Magda Sergejevová who is always ready to extend a hand in times of need and who always assisted me in all the aspects of my stay in Třeboň. I will forever be indebted to the both of them for making me feel part of a family and at home in the Czech Republic.

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Abbreviations

AL	actinic light
Chl	chlorophyll
DW	dry weight
E	irradiance
E_k	saturation irradiance
E_{PAR}	intensity of photosynthetically active radiation
ETR	electron transport rate through PSII
ETR/E	electron transport rate vs. irradiance curve
ETRa	absolute electron transport rate
F’	steady-state fluorescence
F_0	basic Chl fluorescence yield recorded under sub saturating light intensities for photosynthesis
F_m	maximal Chl fluorescence yield when photosystem II reaction centers are closed by a saturation pulse
F_m’	maximal Chl fluorescence yield when photosystem II reaction centers are closed by a strong light pulse
F_v/F_m	variable fluorescence/ maximum fluorescence, maximum photochemical yield of PSII
F_v’/F_m’	(F_m’ − F_o’) / Fm’, effective quantum yields
HL	high light
HLA	high light acclimated
LED	light emitting diode
LL	low light
LLA	low light acclimated
mBP	measured biomass productivity
NPQ	non-photochemical quenching
NPQ_{max}	maximum non-photochemical quenching
OCP	open circular pond
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
PQ	photosynthetic quotient
PQ	plastoquinone
PSII	photosystem II
PUFA	polyunsaturated fatty acids

Q_A	primary quinone acceptor
Q_B	secondary quinone acceptor
QR	quantum requirement
rETR	relative electron transport rate
$rETR_{max}$	maximum relative electron transport rate
RLC	rapid light-response curve
S/V	surface-to-volume ratio
LRC	steady-state or “slow” light-response curve
SP	saturating pulse
TLC	thin-layer cascade
UVR	ultraviolet radiation
Y(II)	(Fm’-F)/Fm’; effective photochemical quantum yield of photosystem II
Y(NPQ)	quantum yield of non-photochemical energy quenching in PS II due to down-regulation in the light-harvesting antennae as pH- and xanthophyll re dissipation processes
Y(NO)	quantum yield of non-photochemical energy quenching in PS II other than that caused by consecutive (energy trapped in closed reaction center of PS II) thermal dissipation processes
α or αETR	maximum photosynthetic efficiency of light conversion
$\Delta F/F_m'$	effective quantum yield of PSII
ΔO_2	photosynthetic oxygen production

Objectives of this thesis

In microalgae biotechnology, the major goal is to achieve higher yields and productivity of biomass, or valuable compounds. The culture conditions have to be optimized for selected microalgae strain and cultivation unit. Thus the physiological status of microalgae mass cultures should also be monitored operatively in order to optimize productivity. Monitoring techniques must reflect the general status of the cell physiology to adjust the appropriate cultivation conditions. In this thesis measuring protocols were elaborated to characterize photosynthetic performance of various strains as to optimize growth of selected microalgae strains in various cultivation units.

The general objective of this thesis was to correlate changes of physiology and photobiochemical activity and growth rate (and productivity) of microalgae strains in order to find suitable cultivation conditions (Chapter 1).

This approach was also applied to examine the requirements of microalgae cultures in various indoor and outdoor cultivation systems in order to optimize growth regimes which is illustrated in several case studies (Chapter 2).

In this thesis the practical use of Chl fluorescence techniques was developed to monitor the physiological status of microalgae mass cultures to estimate growth and biomass productivity, or even finding marker processes of certain compound synthesis (Case Studies 3.1.1-3.1.5). The purpose was to elaborate measuring protocols for microalgae biotechnologists for monitoring microalgae 'health' (Chapter 3).

The last series of experiments/case studies - included in this thesis was aimed to study changes of photobiochemical activities under unfavorable conditions that induce/reflect the production of certain valuable compounds (Chapter 4).

Chapter 1

Chapter 1. The State-of-the-art: Microalgae Mass Cultures

Microalgae[1] represent microscopic, unicellular, filamentous or colonial (coenobial) prokaryotic cyanobacteria and eukaryotic algae that are capable of converting inorganic nutrients, water, carbon dioxide and light energy (sunlight) into biomass *via* photosynthesis. The photosynthetic mechanisms of microalgae are similar to that of terrestrial plants but a much simpler cellular organization together with the absence of non-supporting structures such as stems or roots make them very efficient solar energy converters. The microalgae represent important primary producers in nature and form the basis of the food chain in aquatic environments (Masojídek and Torzillo, 2008).

In their biomass, microalgae produce various bioactive and valuable substances such as pigments, antioxidants, lipids, polyunsaturated fatty acids (PUFA), polysaccharides, or immunologically effective compounds (e.g. Koller et al. 2014a, Tredici 2010, Cakmak et al. 2012, Sharma et al. 2012) which are used for many different biotechnological applications, hence microalgae have a large biotechnological potential for producing valuable substances for biotechnological processes.

Microalgae's substantial advantages over plants are based on their short life cycles, simple anatomy and metabolic plasticity that offers the possibility of modifying their biochemical pathways and cellular composition by varying culture conditions. Thus, microalgae cultures are an ideal platform for the large-scale production of biomass because they are fast-growing, solar-powered 'biofactories' with low nutrient requirements. In the last decade, microalgae biotechnology has received great attention as it can potentially reach high biomass yield (Gordon and Polle 2007) and extract valuable products (Spolaore et al. 2006, Tredici 2010, Stengel et al. 2011, Wilhelm and Jakob, 2011, Sharma et al. 2012). Although many microalgae strains are cultivated worldwide for different purposes, the bulk of annual biomass production is represented by only a few species.

Over the last 60 years, microalgae biotechnology has shown a range of applications: from the traditional extensive biomass production in human and animal nutrition, health food products, soil conditioning in agriculture, aquaculture colorants, technologies for waste-water treatment, products for pharmacy and health food industry (Pulz and Gross 2004), an important commodity for novel food (e.g. Plaza et al. 2008), as a base of biodegradable plastics (e.g.

[1] According to applied phycology

Koller et al. 2014b) and most recently the possible production of a 'third' generation biofuels (for a recent review e.g. Wijffels and Barbosa 2010).

Increase in the yield of microalgae biomolecules: can be done through the following: (1) by increasing microalgae cell density, (2) by increasing the intracellular accumulation of such products or (3) a combination of both. These methods may depend, largely, on the type of photobioreactor and/or may rely more on the growing conditions and stress to which algae are exposed. Therefore, knowledge about the effects of changes in culture conditions that may, in turn, change the synthesis and quantity of certain molecules would be of great interest for both basic and applied research.

The metabolic plasticity of microalgae to varying cultivation ("unfavorable") conditions (e.g. irradiance intensity, nutrient limitation, temperature extremes, salinity) provides opportunities to alter, control and thereby maximize the formation of targeted compounds by modifying key biochemical pathways towards the production of various bioactive and high-value compounds through the manipulation of culture conditions that directly affect biomass composition.

In many microalgae, the accumulation of storage compounds can be enhanced through macronutrient (N, P, and S) limitation (insufficient supply) or starvation (no supply) but at the expense of biomass productivity. Whereas there is a prevalence of studies on the effects of N-starvation on the accumulation of targeted compounds and changes in photosynthetic activity, several studies on S- and P- deprivation also showed that these elements can also be used as a means of increasing the yield of valuable compounds in microalgae. On the one hand, sulfur deprivation may result in the cessation of microalgae cell division (Hase et al. 1959) and in the last few years some authors have observed higher starch and lipid content in S-deficient compared to N-limited cultures (Brányiková et al. 2011, Cakmak et al. 2012, Sharma et al. 2012, Li et al. 2013). On the other hand, increased accumulation of cellular triacylglycerols (TAGs) was observed in phosphate limited cultures of *Monodus subterraneus* (Khozin-Goldberg and Cohen, 2006), *Dunaliella tertiolecta* (Chen et al. 2011) and *Chlorella sp.* (Praveenkumar et al. 2012). Depletion of phosphate was also observed to increase the susceptibility to photoinhibition and reduce the capacity for photoprotection against UV radiation (Carrillo et al. 2008). Depletion of nutrients in mass cultivation of microalgae rarely occurs with increased light stress. While light intensities >200 μmol photons m^{-2} s^{-1} are usually saturating for microalgae growth (depending on cultivation systems, parameters and conditions), higher irradiance is essential for the induction of secondary metabolites (Carvalho

and Malcata 2005, Friedman et al. 1991, Solovchenko et al. 2008); therefore, cultivation process should include a sunlight stage (i.e. 700–2000 μmol photons m^{-2} s^{-1}) in which high biomass densities are achieved and the effect of high irradiance on biomass productivity and composition can be evaluated.

1.1 Principles of Microalgae Mass Culturing

Mass cultivation of microalgae using man-made technology has been as early as 1948 at Stanford (USA), Essen (Germany) and Tokyo (Burlew 1953). Microalgae mass culture are generally dense (>0.5 g biomass per liter), well-mixed, with sufficient nutrition and gas exchange represents an artificial system which is completely different from optically-thin natural phytoplankton populations, with biomass densities which is usually several orders of magnitude lower. The first commercial large-scale culture of microalgae started in Japan and Czechoslovakia in the early 1960's with the culture of *Chlorella* and *Scenedesmus* followed by several establishment of culturing facility in the USA (Sosa Texcoco S.A. in 1977), in Thailand (Dai Nippon Ink and Chemicals Inc.) and Australia (Western Biotechnology Ltd and Betatene Ltd now Cognis Nutrition & Health) in the 1980s and were soon followed by other commercial plants in Israel and the USA. In microalgae biotechnology, suitable and desirable species can be grown as productive strains in aquacultures through the efficient manipulation of the cultivation process.

In every cultivation system, several basic cultivation requirements that affect growth must be considered: light, suitable temperature, pH, sufficient supply of carbon and nutrients, and dissolved oxygen concentration. Most of currently used systems for large-scale cultivation of microalgae are outdoor units and are exposed to a variation of environmental conditions that directly influence biomass productivity. Thus, it is desirable to monitor immediate response to unfavorable conditions that may decrease culture biomass productivity. However, since mass cultures grow in dense suspensions, efficient mixing is necessary to expose cells to light evenly and to allow for an efficient gas exchange (CO_2 supply/O_2 removal) and mak**e** nutrition available (e.g. Grobbelaar 2007, Richmond 2003).

1.1.1 Light

The most important factor affecting photoautotrophs is light. On the other hand, in order to survive the constantly changing light fields, they utilize different strategies including chromatic

adaptations, vertical distribution (in the case of macroalgae) and vertical migration through the water column with respect to microalgae (Kirk 1994). The amount of photon energy received by each microalgae cell is a combination of several factors: irradiance intensity, cell density, length of optical path (thickness of culture layer), rate of mixing and acclimation state. The ambient maxima (about 2,000 μmol photons m^{-2} s^{-1}) of photosynthetically active radiation (PAR) available for photosynthetic antennae are roughly 5 to 10 times higher than those required to saturate growth. At high irradiance, the rate of photosynthetic electron transport significantly exceeds the rate of the dark enzymatic reactions (the Calvin-Benson cycle) (Masojídek et al. 2013, Richmond 2013). In other words, the culture density must be adjusted for an optimal light regime and growth; otherwise as much as 90% of the photons captured by the photosynthetic antennae may be dissipated as heat.

In mass cultures, due to the self-shading of cells, light through the culture is attenuated exponentially with depth according to the Beer-Lambert law — from full sunlight at the surface to darkness at the bottom (Ritchie and Larkum 2012, Zarmi et al. 2013). Several areas can be conceived in a microalgae culture exposed to solar irradiance (Tredici 2010): (1) the surface layers in which irradiance is supra-saturating and photoinhibition is taking place; (2) the deeper area, in which irradiance is saturating and a high photosynthetic rate is achieved; (3) a light-limited area, in which irradiance is used with maximum efficiency; and (4) an area below a certain irradiance intensity (the compensation point), at which photosynthetic rates are too low to compensate for respiration. The first three areas, in which light is sufficient for net photosynthesis, make up the photic zone. In the most efficient systems — shallow ponds, thin-layer cascades or narrow flat panels — the microalgae cell concentration is so high that all four layers can occur over a short distance, and some form of efficient stirring is crucial to maintain optimal production.

If the rate of photosynthesis of a microalgae culture (oxygen production or uptake) is plotted as a function of increasing irradiance (*E)*, the so-called light-response curve of photosynthesis, or photosynthesis−irradiance (*P*/*E)* curve, is obtained (e.g. Torzillo and Vonshak 2013). The *P*/*E* curve can be divided into four distinct areas: respiratory, light-limited, light-saturated and light-inhibited (Fig. 1). This division fits the layout of the different areas in a dense microalgae culture exposed to sunlight mentioned above (Tredici 2010). The so-called initial slope (α) of the *P*/*E* curve is a measure of the maximum photosynthetic efficiency of light conversion. The intercept of α and the maximum photosynthetic activity (P_{max}) represents the saturation irradiance (E_k). Beyond this point, photosynthetic rate is slowed down and

counteracted by energy dissipation. The system reaches P_{max} for that microalgae strain under the given conditions. At a certain area, the irradiance intensity becomes inhibiting and the so-called down-regulation occurs which might be considered as a complex mechanism to defend against over-saturation and potential damage to the photosynthetic apparatus.

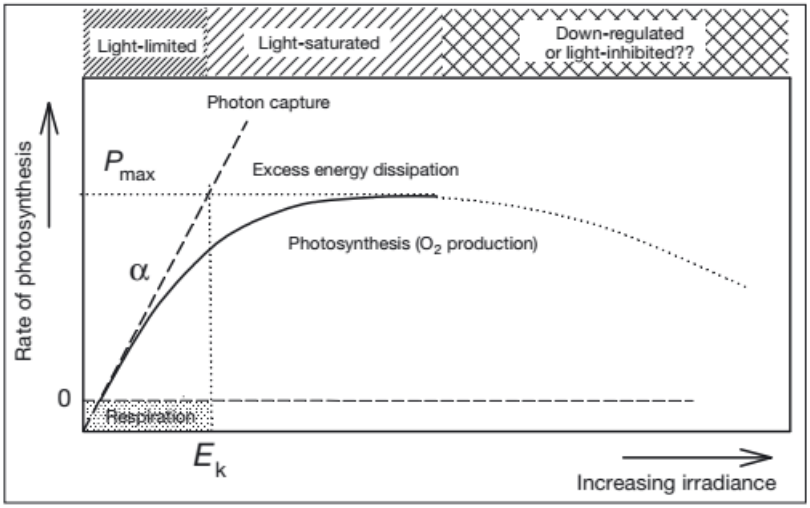


Fig. 1. Schematic diagram of the photosynthetic light-response curve (solid line), i.e. the dependency of photosynthesis on irradiance. The initial slope of the curve (α, thick dashed line) shows the maximum quantum efficiency of photosynthetic electron transport. The intersection between the maximum rate of photosynthesis (P_{max}) and α is the light saturation (optimum) irradiance, E_k. When photosynthesis reaches maximum, surplus energy above this value is dissipated. At supra-optimum irradiance, photosynthesis declines (dotted part of the light-response curve) which is commonly called down-regulation or photoinhibition (Masojídek et al. 2013).

1.1.2 Temperature

After irradiance, temperature is the most important variable to control microalgae culture growth. Temperature affects all biological processes, and thus growth. Ambient temperature is a fundamental physical parameter which can fluctuate substantially in nature; thus, photosynthetic organisms are expected to exhibit complex adaptive and acclimation processes to changes in temperature. Some strains tolerate a broad temperature range of between 15°C and 40°C (e.g. *Chlorella vulgaris* or *Chlorella sorokiniana*). By contrast, the freshwater *Eustigmatophyceae* strains (*Trachydiscus*) usually require a much narrower range (20°C to 28°C). However, for the majority of freshwater microalgae the optimum temperature ranges between 25 and 30°C.

The role of both light and temperature on photosynthesis is of great importance. While light provides the primary source of energy for photosynthesis, temperature often dictates the

rate of how the absorbed light energy will be utilized. Photosynthetic organisms must perceive the fluctuations in the environment and thus, increase their survival by employing various adaptive and acclimation mechanisms to cope with the changes in the environmental factors that would otherwise be pernicious to the organism. In nature, changes in environmental variables rarely occur exclusively of other factors such as irradiance. Therefore, acclimation to long-term temperature stress frequently leads to an accompanying tolerance to photoinhibition. In order to optimize the productivity in mass cultures, layer thickness should be optimal while attaining the maximum possible photosynthetic efficiency attained in the culture. It is generally accepted that high concentrations of microalgae can be attained at shallow culture depth (or thickness). However, shallow cultures are more prone to major temperature fluctuations and changes in medium composition due to evaporation, therefore, the actual culture depth (or thickness) must be a compromise.

1.1.3 Carbon and Nutrient Supply

Carbon dioxide (CO_2) serves as the main carbon source and is usually added on demand (for example, by using a pH-stat). Since microalgae are generally found in aquatic habitat, inorganic carbon exists at CO_2, HCO_3^- and CO_3^{2-}. Microalgae can take up CO_2 by diffusion and several microalgae species have active carbon uptake systems which can take up HCO_3^-. However, no microalgae can take up CO_3^{2-} ions. It is worth noting that the total solubility of inorganic carbon also depends on the ionic strength (or salinity) of the medium, i.e. the solubility of inorganic carbon is reduced in medium with extremely high ionic strengths. It is a common practice that CO_2 is added to large-scale cultures to improve growth and yields. However, care must be observed since CO_2 addition can result to acidification of the medium and must therefore be implemented in a controlled manner.

For mass cultivation phototrophic, heterotrophic and mixotrophic microalgae production is feasible, however, microalgae are typically grown in large-scale using inorganic medium (i.e. taking advantage of the photoautotrophic nature of microalgae which greatly reduces the cost of operation) (Brennan and Owende 2010). The nutritional requirement of microalgae is reflected in their great taxonomic diversity and for most microalgae species, the exact nutrient requirements are still yet undetermined. The composition of the culture media that is used in large-scale culture of microalgae are usually the same media used in the laboratory, albeit with a few small modifications. The growth requirements of the microalgae,

how the constituents of the medium may affect final product quality, and cost are some the several factors considered in the choice of the medium to be used for outdoor cultures of microalgae (Borowitzka 2005). Nutrients are generally classified into two major groups depending on the amount at which the nutrients are present in the medium. Those required at g L^{-1} concentrations are considered macronutrients while those required at mg L^{-1} or μg L^{-1} concentrations are considered micronutrients (for a detailed review of the nutritional requirements of microalgae, see Richmond 1986, Richmond 2004, Anderson 2005). It is a common practice that nutrient status is followed by monitoring the concentration of nitrogen or phosphorus, using it as a measure for adding proportional amounts of the other nutrients.

1.1.4 Dissolved Oxygen

One product of photosynthesis is oxygen. Though the production of oxygen is sometimes overlooked in large-scale cultures, high concentrations of dissolved oxygen in the medium which can occur especially in dense cultures under high irradiance intensity at noon in outdoor cultures can result in photoinhibition and photorespiration and a reduction in photosynthesis and growth (e.g. Pope, 1975, Marquez et al., 1995, Moheimani and Borowitzka, 2007). Dissolved oxygen concentrations equivalent to 3-4 times saturation with respect to air are inhibiting, or even toxic to many photoautotrophs. The maintenance of O_2 levels below this concentration requires degassing or culture mixing produced by high flow rates. In contrast, anoxia which may occur in dense cultures, especially outdoors, at night can be tolerated by most microalgae for short periods but may have a growth-reducing effect. Oxygen supersaturation or anoxia can be alleviated, in part, by good mixing or aeration of the culture (Malapascua et al. 2014).

1.1.5 Culture Monitoring

Successful cultivation requires continuous monitoring of physicochemical parameters. One of the basic biological methods used is microscopic examination to detect morphological changes and contamination by other microalgae and protozoa. In mass cultivation of microalgae, monocultures are usually required for biomass exploitation. The appearance of ‘contaminants’ (other microalgae as well as protozoa, bacteria, or fungi) might indicate that the cultivated culture has come under stress (Masojídek and Torzillo 2008).

Biophysical and biochemical monitoring methods generally reflect the status of the cells’

photosynthetic apparatus and are used to adjust the appropriate cultivation conditions for the production of biomass or certain compounds. Oxygen production, CO_2 uptake and chlorophyll *a* (Chl) fluorescence have been used as reliable and sensitive techniques to monitor the photosynthetic activity of various photosynthetic organisms (Bradbury and Baker 1984, Krause and Weis 1984, 1991, Walker 1987, Flameling and Kromkamp 1998, Gilbert et al. 2000, Figueroa et al. 2003, Wilhelm et al. 2004). All these methods reflect the performance of processes in the photosynthetic apparatus, and consequently physiological status and growth of a culture. They provide analogous information, but compared with measurements of O_2 production and/or CO_2 uptake, fluorescence methods are considerably faster, more sensitive and can give information on energy use between the photochemical and non-photochemical (heat dissipation) processes (Schreiber et al. 1986, Schreiber et al. 1995, Baker and Oxborough 2004, Suggett et al. 2011).

Since the mid-1990s, Chl fluorescence measurement has become widespread to monitor photosynthetic performance of microalgae mass cultures due to its non-invasiveness, sensitivity and a wide availability of commercial fluorimeters that are easily utilized *in situ* (Maxwell and Johnson 2000, Baker 2008, Hout and Babin 2010). In the 2000s, fluorometry became one of the most common techniques to estimate photosynthetic performance both in terrestrial plants (e.g. Juneau et al. 2005) and microalgae mass cultures (e.g. Baker 2008, Masojídek et al. 2011a). One approach is to measure photosynthesis *on-line/in situ* during the diel cycle to monitor the actual situation directly in microalgae culture. Another possibility is to measure *off-line*, using dark-adapted samples taken from a cultivation unit at selected times (Masojídek et al. 2011b).

Presently, two basic Chl fluorescence techniques are used for monitoring photosynthetic efficiency in microalgae mass cultures: rapid fluorescence induction or relaxation kinetics, and the PAM method (for recent reviews, see Maxwell and Johnson 2000, Strasser et al. 2004, Schreiber 2004, Baker 2008, Masojídek et al. 2011a). While the rapid fluorescence induction provides information on the reduction of the photosynthetic electron transport chain, the PAM technique gives information on the balance between photosynthetic electron transport and the Calvin-Benson cycle. The latter method was a considerable leap forward as it made photosynthetic activity very easy to estimate *in situ* under ambient irradiance. Still it should be compared with some other standard method: for example, oxygen production measurement which is an appropriate and well-established technique as it can measure both photosynthetic and respiratory rates (Walker 1987). *In vivo* Chl fluorescence reflects the utilization of light

energy, i.e. its distribution between photochemical and non-photochemical processes. PAM fluorometry has been successfully used on-line (*in situ*) to monitor the photosynthetic performance of mass cultures and give rapid evidence of stress affecting growth (Torzillo et al. 1998, Lippemeier 2001, Masojídek et al. 2011a, White et al. 2011, Figueroa et al. 2013).While pulse-amplitude modulated (PAM) fluorescence can be used as a good proxy for photosynthetic performance under certain conditions, it should always be matched with some other standard method: for example, an oxygen electrode measurement which is an appropriate and well-established technique as it can measure both photosynthetic and respiratory rates (Walker 1987).

Several variables can be estimated using a pulse amplitude modulated (PAM) technique: the effective quantum yield (ΔF/Fm′), used as an indicator of acclimation of photosystem II (PSII) as it depends on the redox state of the reaction centers; the maximal quantum yield (F_v/F_m') which is an estimate of the maximal photochemical PSII efficiency and is used as an indicator of the physiological state of the culture, and the electron transport rate (ETR) through PSII, used as a proxy of the photosynthetic capacity and productivity (Figueroa et al. 2013, Kromkamp et al. 2008). Care must be taken when measuring fluorescence and evaluating data in cyanobacteria because the fluorescence emission of phycobilisomes as well as higher proportion of PSI complexes contribute significantly to the total signal, and this affects the correct determination of certain variables (Ting and Owens 1992, Büchel and Wilhelm 1993, Schreiber et al. 1995).

Chapter 2

Chapter 2. Microalgae Cultivation Systems

2.1 Photobioreactor design (with respect to maximizing light utilization)

Various cultivation systems and technologies have been developed to grow microalgae mass cultures. The key problem to solve in a cultivation unit design is how to maximize the utilization of light, i.e. how to allow each single microalgae cell to get access to an optimum number of photons every time.

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 (for a recent review, see Zittelli et al. 2013, Masojídek et al. 2015, Acién et al. 2017). Large-scale outdoor PBRs for commercial production are usually designed as modules. 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, 2012).

Several parameters or variables involved in large-scale cultivation of microalgae are oftentimes overlapping and efficient microalgae mass production cannot be properly maintained without adequate knowledge of the physiology in mass culture of the organisms to be cultivated. Generally, the main design criteria for culture systems include: light availability in the culture, orientation and inclination, surface-to-volume (S/V) ratio and gas exchange. One primary objective in any large-scale cultivation system is to expose cells equally to intermittent illumination (i.e. to move the cells in and out of the photic volume or zone) that would allow using light most efficiently. Cells in the illuminated part of any cultivation system should be optimally exposed to light for the duration required for the turnover the light reaction, and then be replaced by cells from the dark zone. Optimally the cells in the dark zone should be returned to the light zone when the dark reaction is complete (which usually takes ~1–10 ms) to match the turnover of the photosynthetic units. This predicament should be considered when designing PBRs to improve light regime and achieve higher biomass yields (Richmond 2004).

One way of maximizing the utilization of light in mass cultures is by orienting and inclining PBRs at various angles and positions (with respect to the sun) to vary irradiance at the cultivation surface. Generally, sun-oriented systems (south-facing and tilted so as to intercept maximum solar radiation) achieve higher cell concentrations.

Another way of maximizing light use (therefore higher yields) is by increasing the ratio between the illuminated surface area of a culture system and its volume (S/V). The surface to volume ratio (S/V) determines the amount of light that enters the system per unit volume; and in thin-layer systems it is easy to reach short light/dark cycles of cells in culture layer (Richmond 2004, Zarmi et al. 2013, Masojídek et al. 2015). Generally, the higher the S/V, the higher the cell concentration. Higher biomass densities reduce the cost of harvesting. For this, culture systems with high S/V are advantageous. High S/V ratio is attained in open thin-layer systems, where, microalgae suspension is circulated over a flat surface exposed to solar irradiance (Šetlík et al. 1970, Masojídek et al. 2015). More efficient light utilization and therefore high densities of biomass (~25–35 g L^{-1}) can be achieved in open thin-layer systems due to the short optical path (<10 mm). These units have high ratio of exposed surface area to total culture volume (S/V of approximately 100 m^{-1}) and highly turbulent flow that enables high volumetric and areal productivity. Its short light path in combination with the high cell density and intensive turbulence enables cells to be exposed to intermittent light with short light/dark cycles, thus avoiding over-reduction of photosynthetic electron carriers (Masojídek et al. 2011b). Furthermore, high turbulent flow resulting to sufficient mixing of the microalgae suspension is ensures nutrient diffusion, as well as to prevent the accumulation of oxygen in the culture. On the other hand, care must be taken to avoid excessive mixing which can cause hydrodynamic or sheer stress to the cells, and consequently a similar reduction of productivity.

There is no universal all-purpose cultivation unit. The choice of a suitable cultivation system and the adjustment of the cultivation regime must be worked out for each individual microalgae strain and production purpose. Crucial variables have to be considered in growing microalgae in various systems include: irradiance intensity, temperature, optical depth, turbulence, light acclimated state of the organism, nutrient availability and gas exchange (supply of CO_2 and O_2 degassing). In choosing the cultivation system for commercial production of biomass, the price of the final product is often an important consideration. Currently, open reservoirs are more preferred culture systems for the production of tons of biomass that is cheaper than a culture from closed PBRs. However, the use of open systems is restricted to a relatively small number of microalgae species due to the limited control of cultivation conditions and contamination. Hence, open systems are suitable for “robust” microalgae strains (e.g., *Chlorella* or *Scenedesmus*) that grow rapidly or to these cultured under very selective conditions of high alkalinity or salinity media (e.g., *Spirulina* or *Dunaliella*).

2.1.1 Open Outdoor Systems

Open cultivation systems represent artificial ponds, tanks, raceways (shallow race tracks mixed by paddle wheels) and sloping cascades (i.e. inclined-surface platforms). An overview of open culture systems used for the mass cultivation of microalgae outdoors has been presented recently (Zittelli et al. 2013, Acién et al. 2017). Cultivation of microalgae in open ponds has been extensively studied in the past decades (Boussiba et al. 1988, Tredici and Materassi 1992, Hase et al. 2000). The simplest example is an unmixed pond, which consists of a shallow ditch dug into the ground pond. Raceway pond is the next improvement as it is draped with plastic sheets up to the sloping earth ridges and microalgae culture is circulated around the racetrack by paddlewheels. This construction is relatively cheap and is favored in commercial plants. The culture depth may vary from 10 to 30 cm. In large raceway ponds, water level is kept not much lower than 15 cm, otherwise a severe reduction of flow and turbulence would occur. Increasing the culture depth from 20 to 30 cm to improve turbulence (mixing) and minimize evaporation, the microalgae are exposed to inadequate irradiance due to the limited depth at which sunlight can penetrate the pond. Hence, cultures are considered photo-limited (low-light adapted), since these systems have to be operated at an optimum concentration rather than at a maximum growth rate. Generally, in raceway ponds, the biomass concentrations 0.5-1 g DW L^{-1} are achieved and long-term yields in large commercial raceways rarely exceeds 12 – 13 g m^{-2} day^{-1} (Tredici 2004).

Compared to another type of open-system – sloping thin-layer cascades (TLCs) – several factors and improvements have been considered. This system is known worldwide as the Třeboň's or Šetlík's type (Šetlík et al. 1967, 1970). In this cultivation unit microalgae suspensions flow in thin-layer from the top to the bottom over open, inclined-surface platforms in such a way that the layer thickness of the suspension remain below 1 cm and efficient mixing is created by fast circulation; high turbulence prevents self-shading and ensures optimal light/dark cycles, i.e. exposure of the cells to photic and dark zones. This cultures system has high S/V ratio of up to 130 m^{-1} compared to raceway ponds (<10 m^{-1}), thus in the former high areal or volumetric productivities could be achieved. At times, such high productivities as over 40 g dry matter m^{-2} day^{-1} can be obtained in cascade cultivation units, even in temperate climate zones. Due to the very short optical path, high biomass densities between 15 and 35 g DW L^{-1} can be reached, thus much lower volume of dense microalgae suspension can be handled during biomass processing (Masojídek et al. 2015). TLCs combine the benefits of open systems (direct sun irradiance, easy heat dissipation, simple cleaning and maintenance, lower biomass costs

and efficient degassing) with advantages of closed systems (operation at high biomass densities achieving high volumetric productivity). Recently, outdoor TLCs were used in pilot trials to study the growth of the cyanobacterium *Arthrospira platensis* (Benavides et al. 2017) and the freshwater microalga *Trachydiscus (Eustigmatophyceae)* (Malapascua et al. 2014). These systems, similar to raceway ponds are limited by several problems such as sedimentation of the cells at points of lower turbulence, strong evaporative losses, high rates of CO_2 desorption and considerable requirement of energy for continuously pumping the culture to the head of the declined surface.

Most microalgae cannot be maintained long enough in outdoor open systems because of the risk of contamination by fungi, bacteria and protozoa, and competition by other microalgae that tend to dominate regardless of the original species used as inoculum (Richmond 1999). Excessive evaporative losses, particularly in hot dry climates, and lack of temperature control are other major drawbacks of open systems. However, raceway ponds are mostly used as commercial microalgae production systems (Benemann, 2008).

2.1.2 Closed and Semi-closed Photobioreactors

PBRs can be defined as culture systems for microalgae growth in which light does not directly impinge on the culture surface but has to pass through the transparent wall to reach the cultivated cells. Consequently, PBRs do not allow or significantly limit, direct exchange of gases and contaminants (e.g. microorganisms, dirt, etc.) between the culture and the atmosphere. Compared to open systems PBRs are more flexible and can be better optimized according to the biological and physiological features of a selected microalgae strain. A variety of PBRs (using either natural or artificial illumination) has been designed consisting of glass or transparent plastic tubes, columns or panels, positioned horizontally or vertically, arranged as serpentine loops, fences, flexible coils, a series of panels or column ‘gardens’. These arrangements act as a photostage in which the microalgae suspension is continuously mixed. The PBRs have been constructed as flat-plate (Hu et al. 1996), horizontal/serpentine tubular loop (Camacho Rubio et al. 1999), inclined tubular PBRs (Ugwu et al. 2002), bubble column (Degen et al. 2001, Ogbonna et al. 2002, Chini Zittelli et al. 2003), airlift column (Harker et al. 1996, Kaewpintong et al. 2007), stirred-tank (Ogbonna et al. 1999), helical tubular (Hall et al. 2003), conical (Watanabe and Saiki 1997), torus (Pruvost et al. 2006), or seaweed-type (Chetsumon et al. 1998). Due to limited evaporative losses of PBRs, these systems are prone

to overheating. As closed systems, the PBRs can be temperature-controlled (i.e., maintaining at required temperature). Nevertheless, large-scale outdoor systems cannot be easily tempered without high technical efforts. Necessary cooling can also be provided by submerging the tubes in a pool of water, by heat exchangers, or by water spray onto the surface. In PBRs, much higher biomass density can be maintained than in open systems (except thin-layer systems). Slowly-growing strains sensitive to contamination are grown in PBRs (e.g. *Nannochloropsis, Haematococcus, Tetraselmis, Phaeodactylum, Skeletonema, Pavlova, Thalassiosira, Nostoc*, *Navicula, Isochrysis, Chaetoceros,* etc.). At present, panel or tubular PBRs are often mounted in greenhouses to maintain culture conditions for all-year cultivation.

Despite the higher biomass yields attainable with PBRs (as compared to open systems), their high construction and maintenance costs still make them uncompetitive for the industrial production of microalgae biomass. Their use can be foreseen for the production of high-value bioactive substances which require the adoption of controlled conditions. Due to better control of cultivation conditions, productivities in closed systems are generally higher than open systems. Productivities from 20 to 25 g DW m^{-2} d^{-1} have been attained with red microalga *Porphyridium cruentum* (Tredici 2004), while in *Arthrospira platensis*, volumetric yields of up to 1.3 g DW L^{-1} d^{-1} and areal yields of more than 28 g DW m^{-2} d^{-1} were obtained (Tredici and Zittelli 1998) and daily yields of 1.3 g DW L^{-1} and 28 g DW m^{-2} were reported with *Chlorella* (Pulz and Scheibenbogen 1998).

2.2. Case Studies

2.2.1. Growth and Photosynthesis of *Arthrospira* in Thin-layer Cascades and Open Ponds

Diel changes in photosynthetic performance (measured by Chl fluorescence) and biomass productivity were examined in outdoor pilot trials with subtropical microalga *Arthrospira platensis* cultures grown in an open circular pond (OCP) and a thin-layer cascade (TLC). The experiments were carried out in Sesto Fiorentino, Italy in August. The two cultures were grown at the same areal biomass density, but temperature maxima were adjusted either to optimal (33°C) or suboptimal (25°C) temperatures.

The cultures grown in both OCP and TLC showed good physiological condition with a maximum photochemical yield of PSII, (F_v/F_m) ranging from 0.60 to 0.65 at 0800 h (Fig. 2.1). In OCP, the culture at optimal temperature did show certain decrease at 1400 h, while at

suboptimal temperature a significant decrease by about 45% was observed (Fig. 2.1b). Compared to TLC, there was much less difference in F_v/F_m between the cultures grown at 33 °C and 25 °C; showing only a decreased in F_v/F_m of ~13% in suboptimal temperature at midday (Fig. 2.1b). Data analysis indicated that the synergism between light and temperature on F_v/F_m was significant in both cultivation 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 $m^{-2}s^{-1}$).

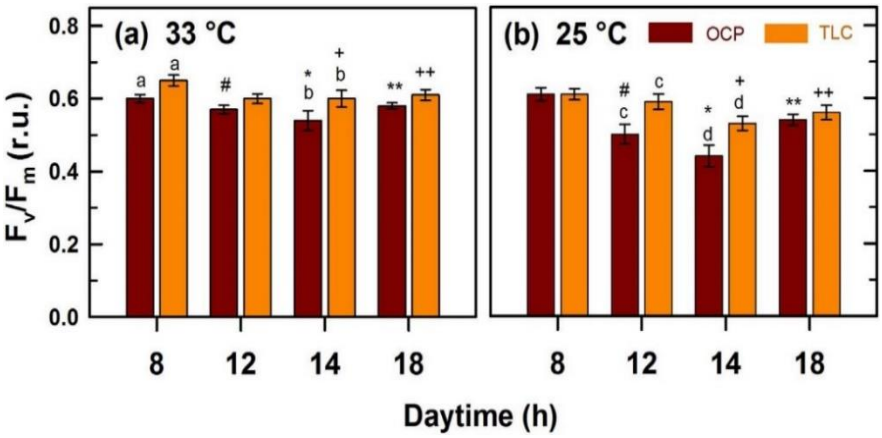


Fig. 2.1. Diel changes of the maximum photochemical quantum yield of PSII (F_v/F_m) measured in *Arthrospira* cultures grown in TLC and OCP at 33 °C (a) and 25 °C (b). (Data are the average of 3–4 measurements; columns labelled by the same letter differ significantly from each other; the same symbols mean significant differences between the two temperatures) (Benavides et al. 2017).

Rapid light response curves (RLCs) provide valuable information on the photosynthetic activity of the cells grown in various cultivation systems and conditions. RLCs of the cultures grown in OCP and TLC indicated significant differences in photosynthetic activity (Fig. 2.2). Grown at optimal temperatures the *Arthrospira* cultures showed stable photosynthetic activity during the day although daily rETR values in TLC were about 50% higher compared to those measured in OCP (~ 100) (Fig. 2.2a vs Fig. 2.2b). When the *Arthrospira* cultures were grown at suboptimal temperature, the maximum rETR values decreased in both units. However, in the TLC culture, the sub-optimal 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. 2.2b and 2.2d).

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. 2.2a and 2.2c). The average rETR activity in OCP was about 14% lower compared to the cultures grown at optimal temperature. At suboptimal temperature, the rETR activity decreased proportionally in both

cultivation units, but the average photosynthetic activity was still significantly lower in the OCP culture compared to that in TLC. Highest values of $rETR_{max}$ were found in the TLC cultures at midday at optimal temperatures and were only 16% lower at suboptimal temperature whereas the rETR values in the OCP were about 30% lower at both optimal and suboptimal temperature (Fig. 2.3a, b).

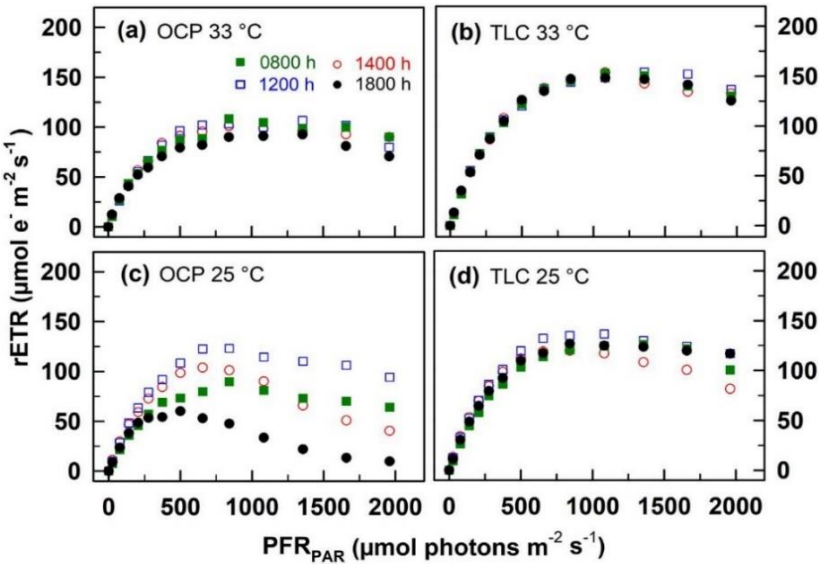


Fig. 2.2. Diel changes in rapid light-response curves (RLC) of rETR in *Arthrospira* cultures grown at optimal (33 °C) (a, b) and suboptimal temperature (25 °C) (c, d) in OCP (a, c) and TLC (b, d). The data were calculated by PAM software using Eilers and Peeters model (1988) (Benavides et al. 2017).

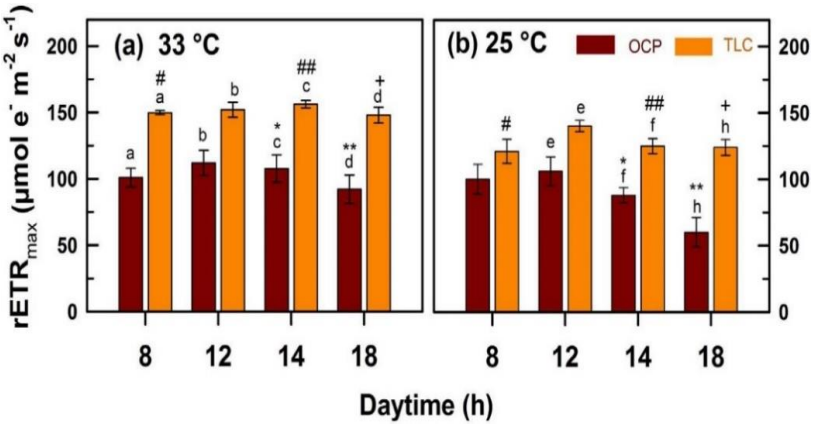


Fig. 2.3. Diel changes of maximum relative electron transport rate $rETR_{max}$ (calculated from RLC) of *Arthrospira* cultures grown at optimal (a) and suboptimal (b) temperature in OCP (brown columns) and TLC (orange columns). (Data are the average of 3–4 measurements; columns labelled by the same letter differ significantly from each other; the same symbols mean significant differences between the two temperatures) (Benavides et al. 2017).

In the two culture systems, biomass productivity is influenced by their very different light path, regimes and interaction of light and temperature. Biomass productivity in TLC at optimal temperature is higher compared to OCP at either optimal or sub-optimal temperatures (Fig. 2.4). The highest biomass productivity over 20 g m^{-2} d^{-1} was found in the TLC at optimal temperature while at the sub-optimal one it decreased by 20%. In the OCP, biomass productivity was about one third lower than that at the same temperature regime in the TLC.

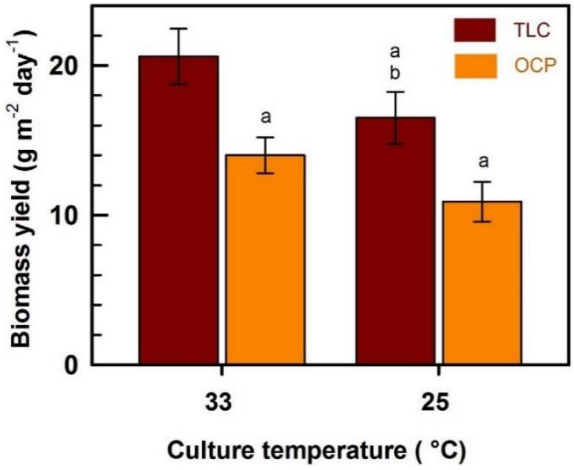


Fig. 2.4. Biomass yields of *Arthrospira* cultures grown in TLC and OCP at 33 °C and 25 °C. Data are the mean values at each temperature during August; columns labelled by the same letter differ significantly from each other. Daily biomass yield was calculated on a 24-h basis, from dawn to dawn (Benavides et al. 2017).

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 (Fig. 2.4). When compared with rETR curves it indicates that more energy is absorbed by TLC and used for electron transport (Figs. 2.2 and 2.5), 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 depressed as the NPQ values are much lower. This was especially observed in OCP at sub-optimal temperatures where the cultures showed much lower NPQ values starting from mid-morning.

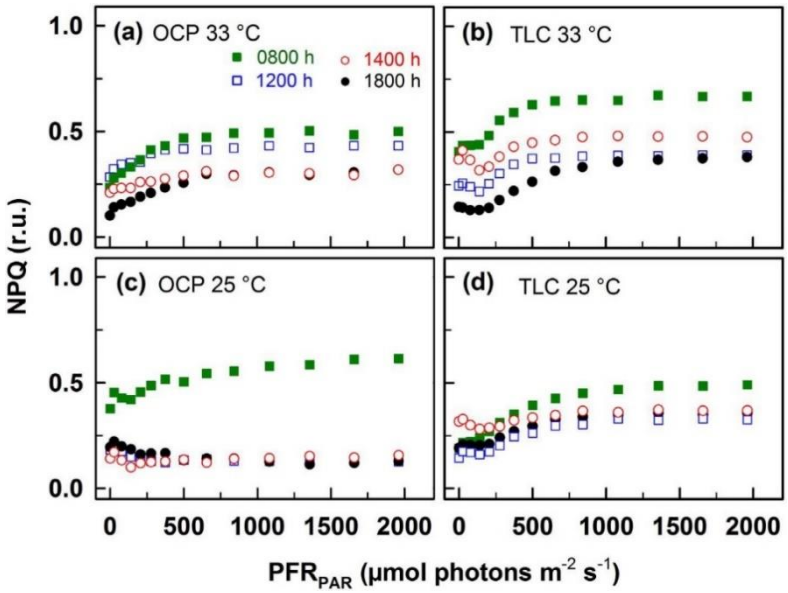


Fig. 2.5. Diel changes in rapid light-response curves (RLC) of non-photochemical quenching, NPQ in *Arthrospira* cultures grown at optimal (a, b) and sub-optimal (c, d) temperature in OCP (a, c) and TLC (b, d). The data calculated by PAM software (Benavides et al. 2017).

2.2.2 Estimation of Biomass Productivity in Thin-layer Cascades

Estimation of biomass productivity of *Chlorella fusca* using *in vivo* Chl fluorescence measurements (*in situ* and *off-line*) was studied in thin-layer cascades conducted in two trials in Málaga (Southern Spain) in July 2012 (E1) and October 2012 (E2), using TLCs with a S/V ratio of 27 m^{-1} and one trial in Czech Republic in July 2013 using another TLC with an S/V ratio of 120 m^{-1} (Jerez et al. 2016b).

The irradiance (Fig. 2.6a) measured inside the culture showed a typical diurnal pattern in which values increased from morning to midday and then decreased during the afternoon. Diel courses of the actual photochemical yield ΔF/Fm′ (Fig.2.6b) showed a midday depression in all cultures that was more evident in the first days of cultivation when biomass density was relatively low which was less pronounced as the biomass density increased. Relative electron transport rate (rETR) in the three trials was similar in the three trial during the first week and was significantly increased in E3 at the end of the cultivation period (Fig. 2.6c).

All cultures showed a typical course of P–E curve in maximum electron transport rate ($rETR_{max}$) is achieved as irradiance increased (Fig. 2.7). All photosynthetic variables ($rETR_{max}$, E_k and αETR) generally increased during the trial as the culture grew and cells acclimated to outdoor conditions, achieving the highest value at day 14 in all three cultures. In E3 culture, photosynthetic variables showed characteristic behavior of high-light adapted cells (higher

$rETR_{max}$ and E_k and lower α_{ETR}) while in the E1 and E2 trials, the cultures exhibited a shade-adapted pattern (decreased $rETR_{max}$ and E_k and increased α_{ETR}). These results clearly show different light acclimation patterns according to the physiological state of the culture.

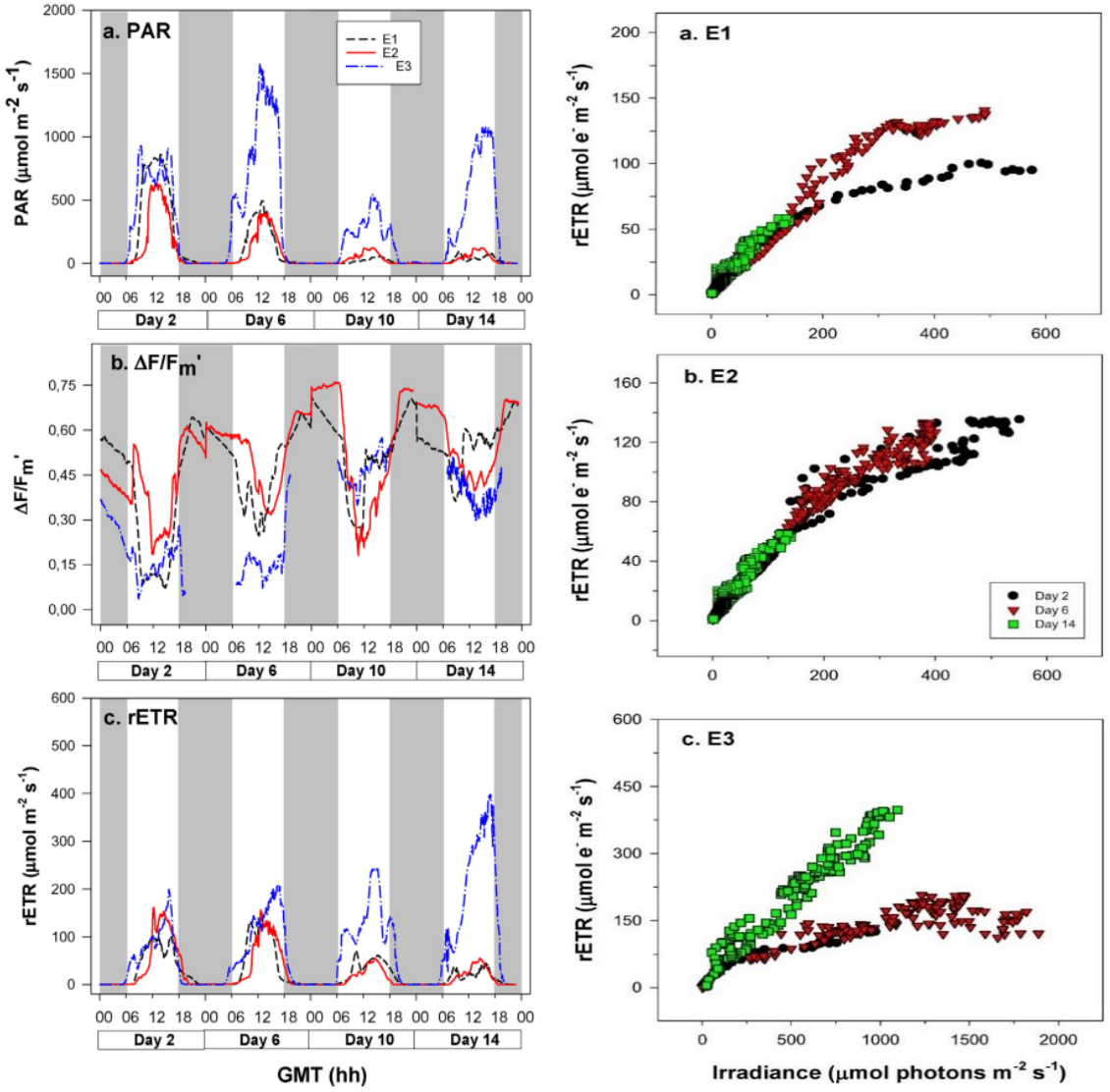


Fig. 2.6. Diurnal changes of irradiance, ΔF/Fm and rETR recorded in situ at 5-min intervals during a 14-day cultivation period of *Chlorella fusca* grown in outdoor thin-layer cascades (TLCs). (a) Irradiance (E_{PAR}, µmol photons m^{-2} s^{-1}); (b) effective quantum yield (ΔF/Fm′, r.u.) and (c) relative electron transport rate (rETR; r.u.) calculated as ΔF/Fm′ × E_{PAR}. Experiment 1 (E1, dashed curve) and Experiment 2 (E2, solid line) were conducted in Málaga (Southern Spain) during July and October 2012, respectively in a 4-m^2 TLC with a S/V ratio of 27 m^{-1}. Experiment 3 (E3, dash-

Fig. 2.7. Relative electron transport rate (rETR, r.u.) *versus* PAR irradiance (E_{PAR}, µmol photons m^{-2} s^{-1}) measured inside the culture every 5 min using Junior-PAM fluorometer during a 14-day cultivation period of *C. fusca* grown in outdoor thin-layer cascades (TLCs). Experiments 1 and 2 (a and b, E1 and E2, respectively) were conducted in Southern Spain during July and October 2012, respectively in a TLC with 27 m^{-1} surface/volume (S/V) ratio. Experiment 3 (c, E3) was conducted in Czech Republic

dot line) was conducted in Třeboň (Czech Republic) during July 2013 in a TLC of 24 m^2 with a S/V ratio of 120 m^{-1} (Jerez et al. 2016a).

during July 2013 in a TLC with S/V ratio of 120 m^{-1} (Jerez et al. 2016a).

2.2.3 Growth and Photosynthetic Activity of Microalgae in Internally Illuminated PBRs

The growth and photosynthetic activity of the freshwater microalga *Choricystis* sp. strain 1983 (Trebouxiophyceae) were studied in a mineral medium at 30 °C in model experiments in two PBRs - LED-PBR-10 (10 L volume; with four internal light sources which produced two light regimes differing in the maximum irradiance intensity of 2,500 and 3,500 μmol photons m^{-2} s^{-1}, respectively) and LED- PBR-100 (100 L volume; maximum irradiance intensity produced by one central light source was 2,200 μmol photons m^{-2} s^{-1} which was comparable to the low-light regime of the smaller LED-PBR-10) (Sergejevová et al. 2015). In the LED-PBR-10 the growth of the *Choricystis* cultures was directly dependent on light intensity (mean cell irradiance). At higher irradiance the biomass density increased from a diluted culture (0.1 g L^{-1}) to about 3.4 g L^{-1} in 2 weeks which was twice higher than that in the culture grown at a lower irradiance (1.9 g L^{-1}) (Fig. 2.8). The sigmoidal shape of the growth curve in the culture exposed to higher irradiance showed the lag phase which was probably caused by photo-stress in the diluted culture. In the larger LED-PBR-100, the culture growth was slower as the biomass density reached only 1.4 g L^{-1} in 2 weeks, less than that in the low light culture and the culture was still growing. The slower growth was apparently caused by a long light path.

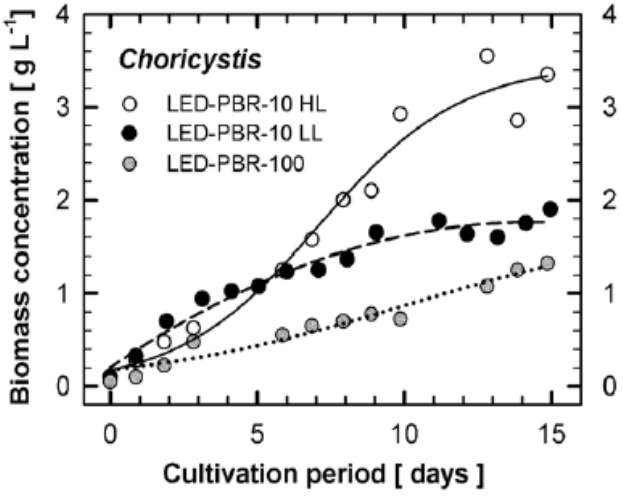


Fig. 2.8. Growth curves of *Choricystis* cultures cultivated in two internally illuminated column PBRs, LED-PBR-10 under low light (LL, close circles) and high light (HL, open circles) regimes and LED-PBR-100 (gray circles) during a 14-day experimental trial (Sergejevová et al. 2015).

In the cultivation trials, all cultures were in relatively good physiological state as the maximum photochemical efficiency of PSII, F_v/F_m was between 0.65 and 0.75 (Fig. 2.9a). In the culture grown in the LED-PBR-10 at higher irradiance, the subsequent decrease of the F_v/F_m value found during the trial was probably caused by shade adaptation of the dense culture and its decreased maximum photosynthetic performance compared to the start of the trial. The highest F_v/F_m values were found in the culture grown in the LED-PBR-100 as this was in a good physiological state, growing at lower irradiance with maximum photosynthetic efficiency even after two weeks of growth. The courses of the relative electron transport rate rETR coincided with previous results. The culture grown in the low-irradiated LED-PBR-10 was photosynthetically very active due to lower biomass density allowing more light penetration to the photic zone, but rETR was continuously decreasing during the trial as the culture became dense (Fig. 2.9b). The culture grown in the LED-PBR-100 showed an increase of rETR after one week of experiment, but due to a long light path the supply of light was not sufficient. The trends of rETR measurements correlated with lower F_v/F_m values.

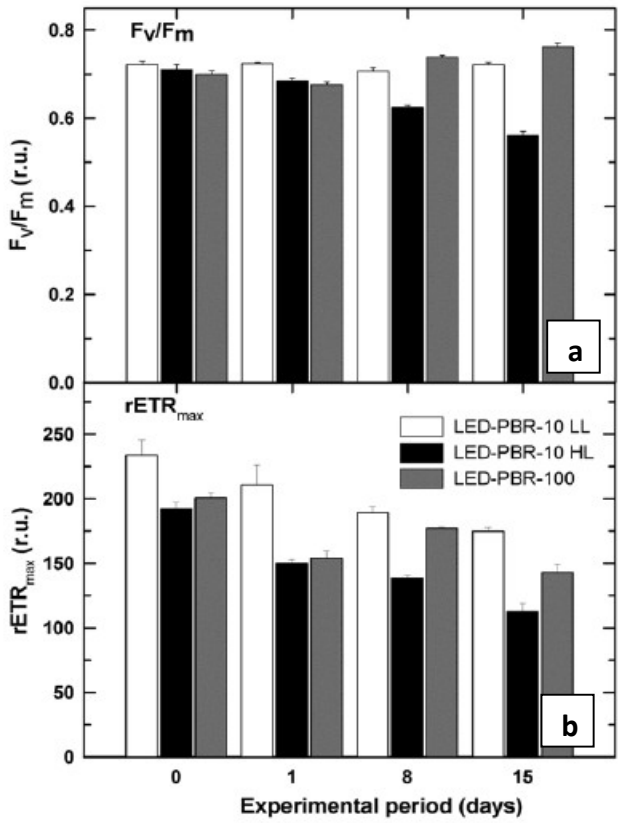


Fig. 2.9. Changes in the maximum quantum yield of PS II, F_v/F_m and relative electron transport rate, rETR of *Choricystis* cultures grown in two internally illuminated column PBRs, LED-PBR-10 under low light (LL, open column) and high light (HL, close column) regimes and LED-PBR-100 (gray column) during a 14-day experimental trial. Values are presented as mean (n=3). SE indicated by error bars (Sergejevová et al. 2015).

The measurement of RLCs clearly indicated the photosynthetic performance of cultures (Fig. 2.10). On Day 2, when the cultures were already acclimated to growth conditions, the highest photo- synthetic activity was found in low-light culture in the LED-PBR-10 as it showed higher initial slope (photochemical efficiency) of RLC and higher rETR while the high-light exposed culture had lower photochemical efficiency and ~ 30% lower rETR. In comparison to the latter, the culture grown in the LED-PBR-100 revealed a similar $rETR_{max}$ value, but higher photochemical efficiency (slope) and the curve was 'bending' earlier (~800 µmol photons m^{-2} s^{-1}) due to downregulation as it was acclimated to lower irradiance.

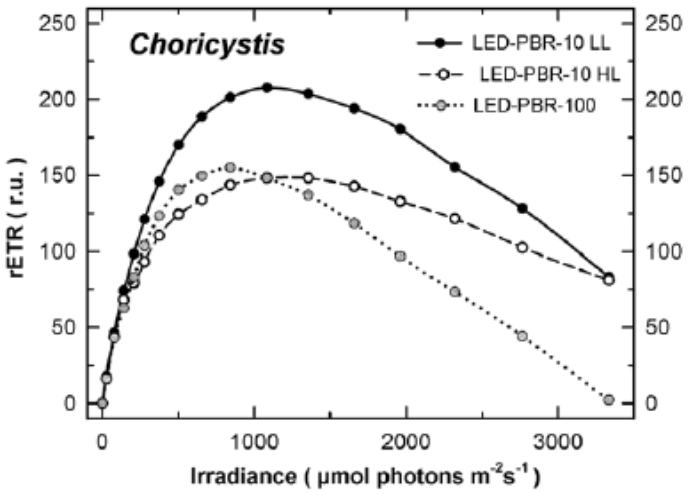


Fig. 2.10. Rapid light-response curve of rETR (= Y_{PSII} x E) of *Choricystis* cultures grown in two internally illuminated column PBRs, LED-PBR-10 under low light (LL, close circles) and high light (HL, open circles) regimes and LED-PBR-100 (gray circles) during a 14-day experimental trial (Sergejevová et al. 2015).

Chapter 3

Chapter 3. Photosynthesis Monitoring in Microalgae Cultures: Chl Fluorescence Techniques

3.1 Case Studies

In order to show the usefulness of Chl fluorescence in monitoring photosynthetic activity in microalgae, typical records and their interpretation under various culture conditions as related to physiological status and growth are demonstrated in exemplary experiments of microalgae model strains *Chlorella* (*Chlorophyceae*) and *Trachydiscus* (*Eustigmatophyceae)* (Malapascua et al. 2014).

3.1.1. Fast Chl Fluorescence Induction Curves of Various Microalgae (OJIP kinetics)

As an example, a comparison of typical fluorescence induction kinetics of various microalgae species which reflect their physiological features and photosynthetic activity are shown in Fig. 3.1. The fluorescence induction curve from the cyanobacterium *Arthrospira* revealed the distinct J inflection of the photochemical phase (curve with filled circles in Fig. 3.1). This inflection probably reflects the reduction of the PQ acceptor Q_A since cyanobacteria generally reduce PQ acceptors in the dark due to respiratory electron transport (Dominy and Willims 1987). The OJIP kinetics among eukaryotic microalgae, e.g. the eustigmatophyte *Trachydiscus minutus* compared to the chlorophyte *Chlorella vulgaris* may also show entirely different kinetics. The slow growth of *Trachydiscus* is reflected by a high I inflection, suggesting a high level of Q_A and Q_B reduction and electron transport being slowed down beyond the P maximum. Green microalgae like *Chlorella* grow quickly, since photosynthetic electron transport is not delayed compared to that of *Trachydiscus*. The J variable of *Chlorella* was low as it can quickly re-oxidize the PQ pool (after 5 to 10 min of dark adaptation).

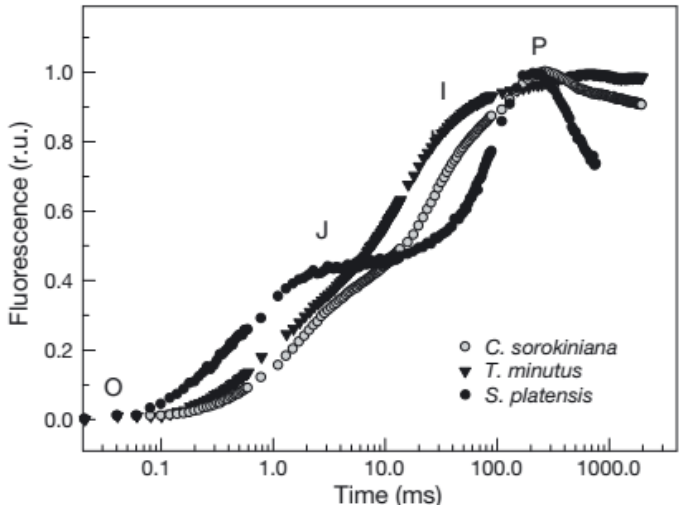


Fig. 3.1. Rapid Chl fluorescence induction curves of various microalgae – *Chlorella sorokiniana* (Chlorophyceae), *Trachydiscus minutus* (Eustigmatophyceae) and *Arthrospira platensis* (Cyanobacteria) – grown in an inorganic medium. The induction curves of outdoor cultures were measured at midday. Before measurement, the diluted cultures (DW = 0.2 to 0.3 g L^{-1}) were dark adapted (5 to 10 min). Fast fluorescence induction kinetics was recorded in a 3 ml cuvette (light path of 10 mm) within a time range of 50 μs to 2 s from the onset of the saturation light using a dual-modulation induction fluorometer (Aquapen AP-100; Photon Systems Instruments). The recorded curves (n = 3 to 5) were averaged and double-normalized to F_0 and F_m in order to distinguish changes in the intermediate steps (J and I) that represent various reduction states of the PSII electron carriers. r.u. = relative units (Malapascua et al. 2014).

3.1.2 Photosynthesis in Low Light and High Light Adapted Cultures

RLCs measured by PAM fluorometers generate variables which describe the physiological state of variously-adapted microalgae cultures. Compared to the steady-state or 'slow' light response curves of oxygen production, LRC, the RLC is measured using increasing irradiances over very short time periods. Each actinic light (AL) intensity can last for 10 to 30 s as fluorescence signal at the level *F'*, is quickly stabilized. Then, a saturating pulse (SP) is triggered to reach the maximum fluorescence yield *F*m*'*. As an example, records of fluorescence quenching analysis are shown in Fig. 3.2A to construct light-response curves of the low-light and high-light adapted *Chlorella* cultures grown outdoors (Malapascua et al. 2014). RLCs of the electron transport rate vs. irradiance curve (ETR/*E*) can be completed within few minutes compared to tens of minutes required for LRC (White and Critchley 1999, Ralph and Gademann 2005, Ritchie and Larkum 2012). In this experiment, the high-light adapted cultures of *Chlorella* showed values of $rETR_{max}$ that were twice as high compared to those of low-light adapted ones (Fig. 3.2B). In Fig. 3.2C, an antiparallel course of Y_{II} and NPQ is found. In the high-light acclimated culture of *Chlorella*, light-response kinetics showed a

higher decrease of about 20% compared to the low-light acclimated culture. Inversely, the increase of NPQ was less abundant in the low-irradiance acclimated culture compared with the other one.

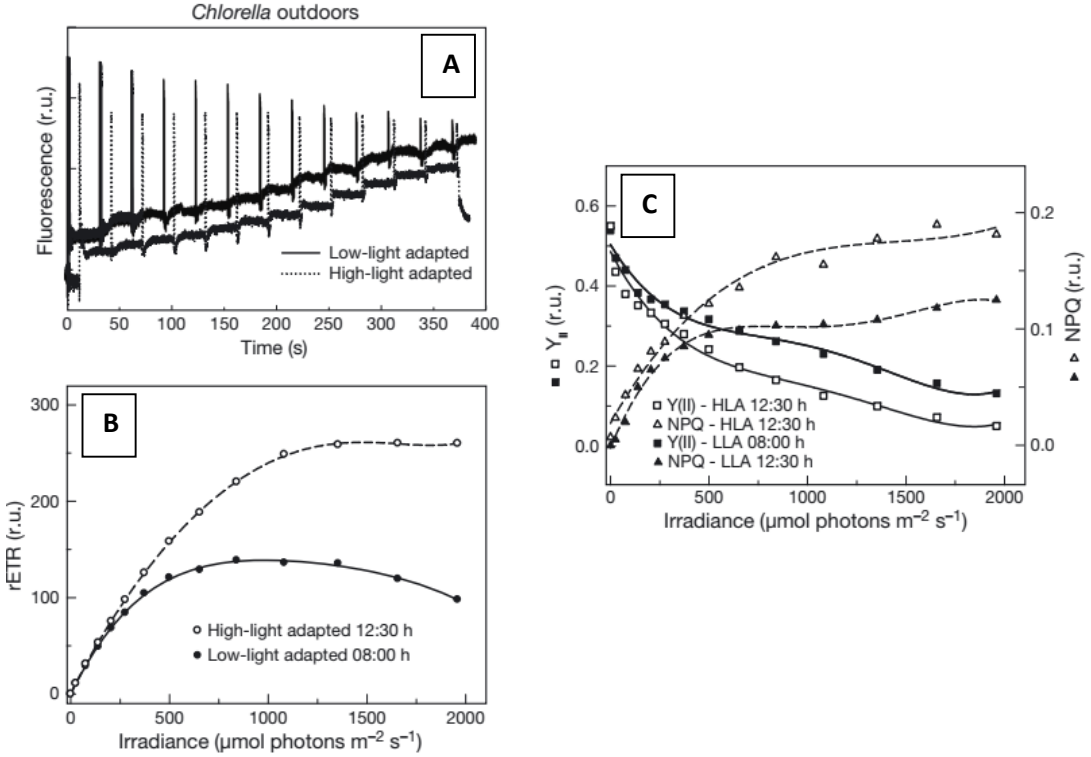


Fig.3.2. (A) Fluorescence quenching records measured by the saturating pulse technique using a modulated fluorometer in low-light and high-light adapted *Chlorella sorokiniana* cultures grown in a TLC (6 mm light path). A series of stepwise increasing irradiance intensities (0 to 2,000 µmol photons m^{-2} s^{-1}) were applied in 30 s intervals to obtain the steady-state fluorescence level (F') and then a saturating pulse was triggered to reach the maximum (Fm'). F' and Fm' were calculated at each step to construct rapid light-response ETR/E (electron transport rate vs. irradiance) curves. (B) RLCs of relative electron transport rate (rETR = $Y_{II} \times E_{PAR}$) of low-light and high-light adapted outdoor cultures of *C. sorokiniana*, measured at 08:00 and 12:30 h. Y_{II} = actual photochemical yield of PSII; E_{PAR} = photosynthetically active radiation. (C) RLCs of Y_{II} and the corresponding non-photochemical quenching coefficient (NPQ) of low-light (LLA, solid symbols) and high-light (HLA, open symbols) adapted outdoor cultures of *C. sorokiniana* measured at 08:00 and 13:00 h. RLCs were calculated by PAM WIN 3 software according to Eilers and Peeters (1988) (Malapascua et al. 2014).

3.1.3 Rapid Chl Fluorescence Induction Curves of Cultures under Various Cultivation Conditions

The present exemplary experiments show OJIP kinetics illustrating the physiological status of cultures of *Chlorella* at various biomass densities and light acclimation status which grown in flat-panel 'Hanging Garden' photobioreactors with a 30-mm light path (Fig. 3.3). In the diluted

shade-adapted cultures, exposed to light, the PQ pool is often over-reduced due to excess irradiance at midday which showed a high and distinct J peak which might even be higher than the P peak (Fig. 3.3A, curves 1 and 2). The J inflection reflects the over-reduction of the PSII acceptors Q_A and Q_B and the decrease of electron flow due to excessive irradiance as reducing power cannot be utilized in further reactions. After recovery overnight, the cultures seemed to be fully photosynthetically competent and able to grow well (Fig. 3.3A, curve 3). A different situation was observed when *Chlorella* cultures which were even denser, were exposed to nitrate starvation. In the morning when light intensity was low, only a slight increase in the J and I inflections was found compared to the nutrient-replete culture (compare Fig. 3.3B, curve 4 and Fig. 3.3A, curve 3). When the nitrate-limited culture was exposed to high irradiance at midday, the J and I inflections were clearly visible, suggesting over-reduction of the Q_A and Q_B electron acceptors of PSII due to the metabolic insufficiency of the culture (Fig. 3.3B, curve 5). However, in the culture limited by CO_2 supply, only the J peak was distinct and the shape of the OJIP kinetics was different. This suggests that the inhibition of Q_A to Q_B electron transport due to the Calvin-Benson cycle was slowed down and unable to utilize reduction equivalents (Fig. 3.3B, curve 6). These examples demonstrate some typical OJIP kinetics which can be used for diagnostics of the photosynthetic apparatus and physiological status of microalgae culture under various growth conditions.

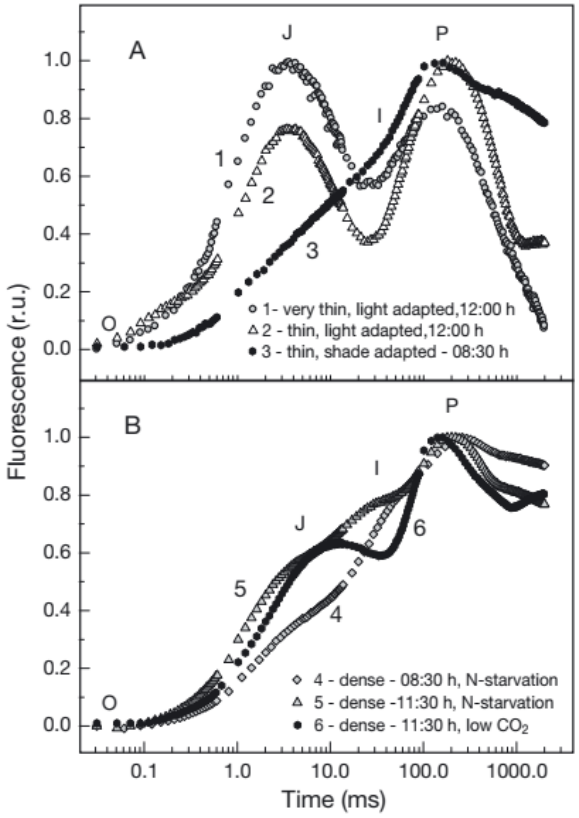


Fig. 3.3. Rapid fluorescence induction kinetics (OJIP-test) of *Chlorella sorokiniana* cultures measured in the morning (08:30 h) and at midday (11:30 to 12:00 h). (A) Diluted (0.1 to 0.33 g DW L^{-1}) and (B) dense (5 g DW L^{-1}) cultures were used to characterize typical fluorescence induction kinetics and demonstrate differences regarding the inflection points J and I (redox states of Q_A and Q_B). The biomass density of cultures varied between 0.1−0.3 and 5 g DW L^{-1}. The cultures were grown in (A) a flat-panel series 'Hanging Gardens'© photobioreactor with a light path of 30 mm (Ecoduna), and (B) in a TLC (curve 6) at the Institute of Microbiology in Třeboň, Czech Republic. Off-line fluorescence measurements were carried out under well-defined laboratory conditions within 5 to 10 min after short dark adaptation. Between 3 and 5 records were averaged to construct each OJIP curve (Malapascua et al. 2014).

3.1.4 Photosynthesis and Growth of *Chlorella* and *Trachydiscus* in Thin Layer Cascade

Example of two microalgae species, *Chlorella fusca* (*Chlorophyta*) and *Trachydiscus minutus* (*Eustigmatophyta*), were grown in an outdoor experimental cascade (24 m^2). This near-horizontal system is based on microalgae growth in a thin-layer (6 mm) that supports very high areal and volumetric productivity (Masojídek et al. 2011a). Both cultures had high photosynthetic activity and grew well in comparison to other cultivation systems (vertical flat-panel or annular cylinder). Nevertheless, after 10 days, *Chlorella* reached a biomass density (21.5 g L^{-1}) that was 2-fold higher than that of *Trachydiscus* (9.5 g L^{-1}) (Fig. 3.4A). This higher

growth rate corresponded with the diel courses of $rETR_{max}$ which were 2 to 3 times higher in *Chlorella* than those of *Trachydiscus* when both cultures began growing exponentially at a biomass density of about 4 g L^{-1} (representing the optimum starting biomass density for this cascade system) (Fig. 3.4B). It matched the specific growth rate of 0.43 and 0.28 d^{-1} and daily productivity 2 g L^{-1} d^{-1} and 1.3 g L^{-1} d^{-1} for *Chlorella* and *Trachydiscus*, respectively. To analyze the photobiochemical changes in more detail, the diel courses of their OJIP kinetics were also compared in parallel. The kinetics of both strains showed rather typical differences, as the J and I inflections were much higher in *Trachydiscus* than in *Chlorella* – especially at midday (Fig. 3.4C). This suggests that the PQ electron acceptors of PSII were reduced in the *Trachydiscus* culture, showing an inability to utilize the high energy input for growth in this outdoor culture in contrast to the *Chlorella* culture. The difference in energy utilization to biomass production can be explained by the fact that *Trachydiscus* produces about 30% of oils in the biomass (Řezanka et al. 2010), the synthesis of which is rather energy demanding. The values of $rETR_{max}$ and OJIP kinetics of *Chlorella* and *Trachydiscus* cultures measured during the cultivation period showed that using these characteristic records of growth rate and productivity of microalgae strains can be predicted even after a few experimental days.

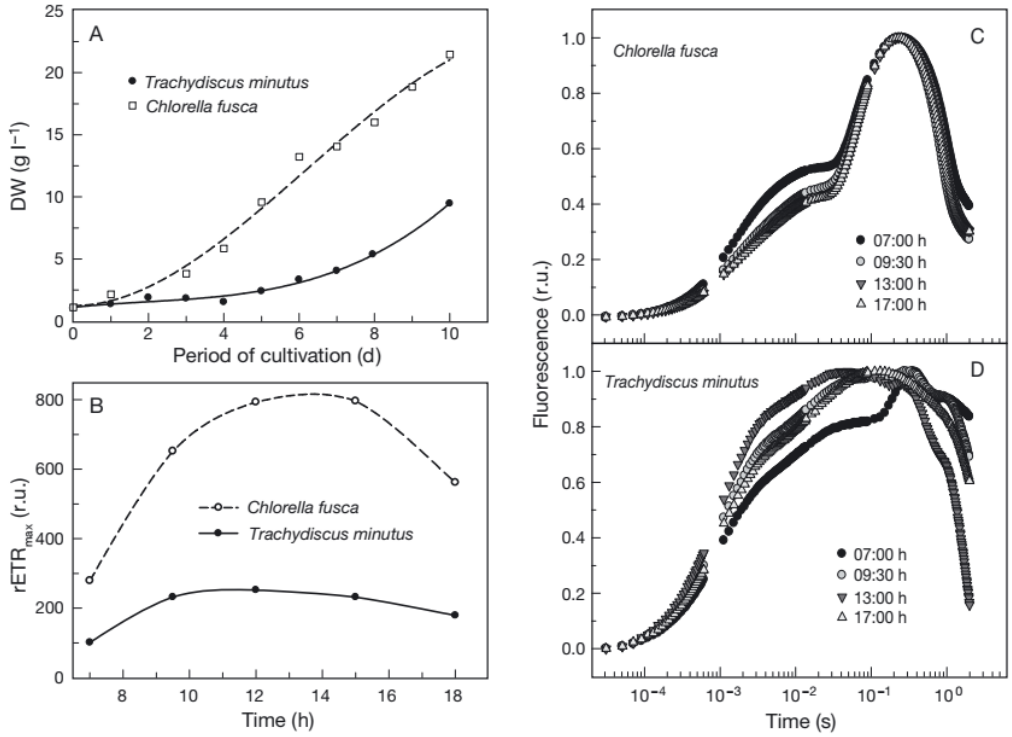


Fig. 3.4. (A) Growth curves of *Chlorella fusca* and *Trachydiscus minutus* cultivated in an outdoor TLC during a 10 d experimental trial in June and July 2013. (B) Diel courses of maximum relative electron transport rate ($rETR_{max}$) on Days 3 and 7, respectively, when both cultures started to grow exponentially at a biomass density of about 4 g L^{-1} (i.e. the optimum starting biomass density for the cascade system). Measurements were taken in culture samples *off-line* using a pulse-amplitude-modulation fluorometer (PAM-2500, Walz) connected by fiberoptics to the stirred cuvette holder. (C) Fast fluorescence induction kinetics measured at selected times of the day (07:00, 09:30, 13:00 and 17:00 h), using an induction fluorometer (Aquapen AP-100; Photon Systems Instruments) (Malapascua et al. 2014).

3.1.5 Photosynthesis Measurements of *Chlorella* Cultures in Various Cultivation Systems

The photosynthetic activity of well-growing *C. fusca* cultures (3 to 4 g biomass L^{-1}) was examined when cultivated in various types of cultivation units. These included an outdoor open (S/V about 105) and an outdoor vertical flat-plate photobioreactor (S/V about 15), both under a diurnal regime in July under a moderate climate (Třeboň, Czech Republic), in comparison to a 10-L laboratory annular column photobioreactor with internal LED-illumination (S/V about 1.5) (Malapascua et al. 2014). In the outdoor units, photosynthetic activity was measured at the maximum daily irradiance at midday (13:30 h), whereas the laboratory column photobioreactor was sampled at random times since the culture was grown under continuous illumination. The RLCs showed the varying photosynthetic performance of the cultures grown in various

cultivation units (Fig. 3.5). The highest rETR activity was found in the TLC culture since that was acclimated to high irradiance as the S/V ratio was the highest (about 105) which pre-determines the culture for fast growth. The saturation of photosynthesis was noted in the cultures grown in the flat-panel and cylindrical photobioreactor compared to the $rETR_{max}$ values of the cascade culture which are significantly higher. The culture in the column photobioreactor with internal illumination was evidently acclimated to low irradiance due to the low S/V ratio: photosynthesis saturation was noted at a much lower irradiance level than in the other two cultures and $rETR_{max}$ was about 35% of that in the cascade. From the RLCs of rETR in this trial, photosynthetic activity of various microalgae cultures in different cultivation units can be estimated and adjust the optimum biomass density and cultivation regime. The RLCs show the varying photosynthetic pperformance of the cultures as a consequence of light availability in various cultivation units.

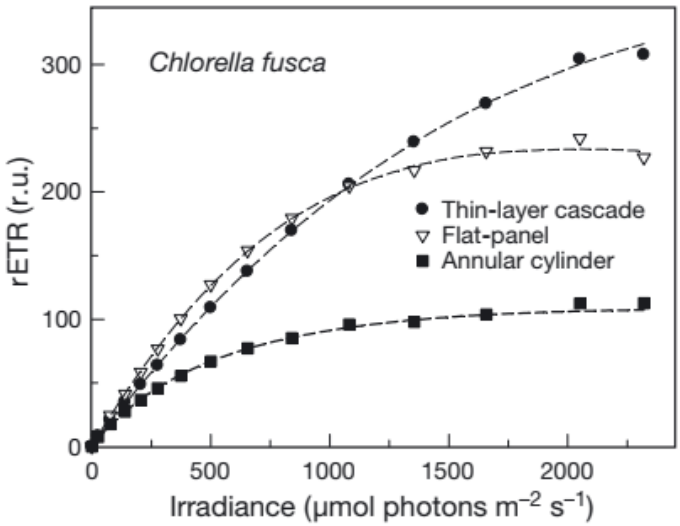


Fig. 3.5. Rapid light-response curves (RLCs) of *Chlorella fusca* cultures measured at midday to monitor photosynthetic performance of the cultures: relative electron transport rate (rETR) vs. irradiance. The cultures were grown in various types of cultivation units (see Fig. 2): outdoor TLC (6 mm light path, cultivation surface of 24 m2, total volume 220 l) and vertical flat-plate photobioreactor (dimensions 1 × 1.4 m; 70 mm light path, total volume 100 l) as well as in a 10-liter laboratory annular column glass photobioreactor with continuous illumination by four cylindrical LED light sources (Institute of Microbiology, Třeboň, Czech Republic) (Malapascua et al. 2014).

Chapter 4

Chapter 4. Growth of Microalgae under Unfavorable Conditions

Microalgae mass cultures are primarily aimed for high biomass yields, but often, through by manipulation of growth conditions. Large-scale outdoor production of microalgae is carried out in cultivation systems which allow for some degree of control of environmental variables affecting microalgae growth. However, in reality, culture conditions are frequently difficult to control and to reach optima (i.e. light, temperature and nutrition) which result in microalgae stress and acclimation, requiring biochemical and metabolic adjustments resulting in the synthesis and accumulation of some secondary metabolites of interest (Humby et al. 2013).

Nutrient deprivation (−S, −P, −N, etc.) results in a decrease of growth rate and photosynthetic rates by both direct (reduction of the synthesis of certain biomolecules), but on the other side in indirect effects (reduction of protection or repair mechanisms) which can be desirables from biotechnological point of view. For example, nitrogen limitation results in a decrease of photosynthetic efficiency and growth rate (Berges et al. 1996, Berges and Falkowski 1998, Young and Beardall 2003, Jiang et al. 2012), changes of pigment composition, reallocation of protein resources to maintain cellular processes (Geider et al. 1998, Young and Beardall 2003) in parallel with the increase in carbohydrate and/or lipid production (Rodolfi et al. 2009, Dean et al. 2010, Praveenkumar et al. 2012, Yeh and Chang 2012, Jacobsen et al. 2010, Liu et al. 2013).

4.1 Case Studies

4.1.1. Growth of Laboratory Cultures under Nutrient Starvation

When laboratory cultures of *Chlorella fusca* were grown under high light and nutrient limited conditions, they (even nutrient-limited) were able to maintain almost linear growth for the first two days under relatively high irradiance (1200 μmol photons $m^{-2}s^{-1}$; about a half of the ambient maxima), even when the substantial part (50–70%) of nitrogen or sulfur was consumed (Figs. 4.1 and 4.2). As the nutrient level is substantially decreased to ~5–10% of the initial value, growth was slowed down and all cultures entered the stationary phase by the end of the experiment. The full nutrient culture had by about 70 and 40% higher biomass density compared to N- and S-limited cultures (Fig. 4.1a), which showed minimal cell division compared to the full nutrient culture (Fig.4.1b). Regardless of the treatment, all the cultures were gradually increasing in biovolume which then decreased at Day 8 (Fig. 4.1c). In the stationary phase, the full nutrient culture exhibited higher cell number and biovolume than N-

and S-limited culture, respectively. The decrease in cell density and biovolume at the end of the experiment in nutrient-limited cultures was more significant under S-starvation.

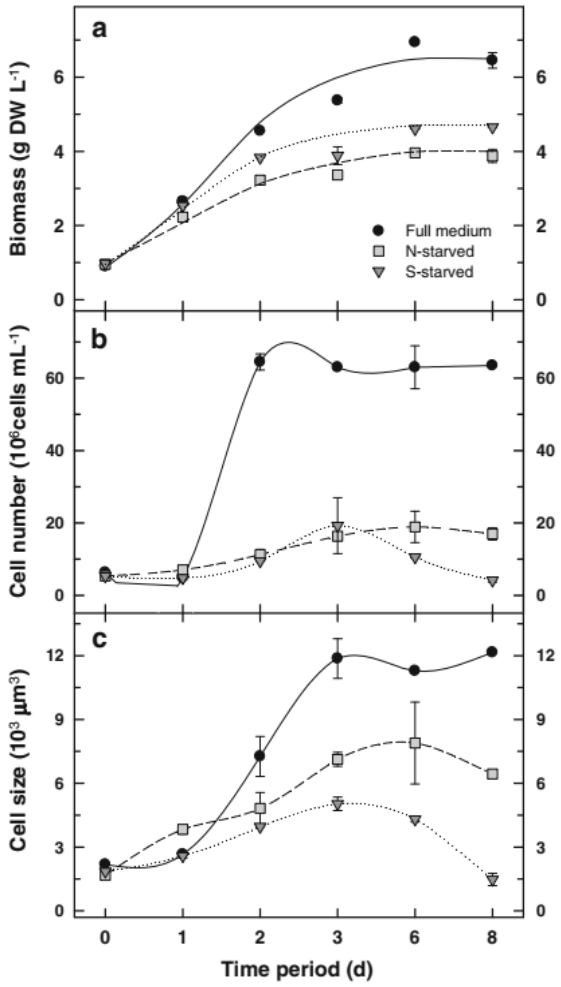


Fig. 4.1. Changes in (a) biomass density (as dry weight, DW), (b) cell number and (c) biovolume of *C. fusca* cultures grown in full nutrient (circle), N-limited (square) and S-limited (triangle) medium during the 8-day cultivation period. Values presented as mean ± SE (n=2) (Jerez et al. 2016b).

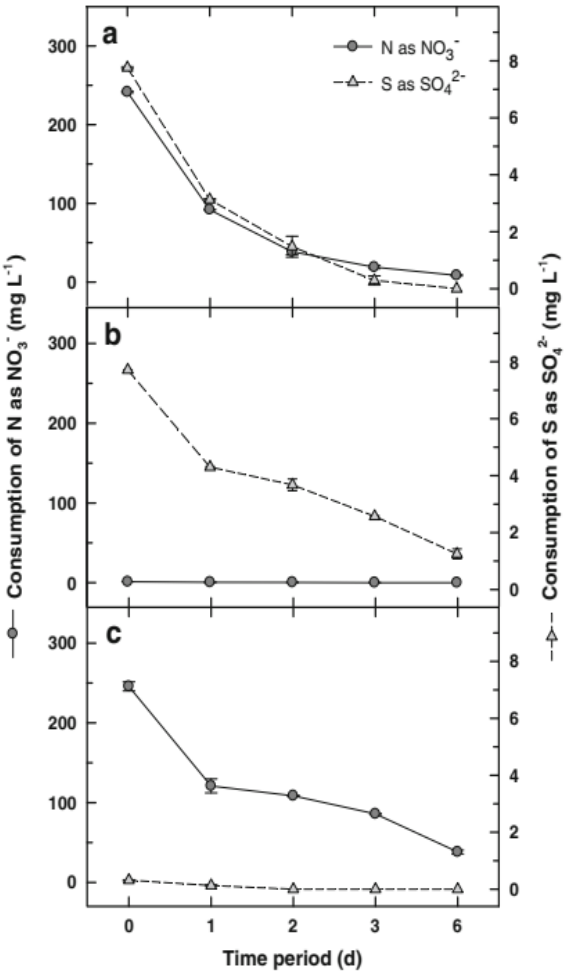


Fig. 4.2. Changes of nitrogen (circles) and sulfur (triangles) concentrations in the medium measured as variation of NO_3^- and SO_4^{2-}, respectively, in *C. fusca* cultures grown under (a) full medium, (b) N-limited and (c) S-limited conditions during the first 6 days of the cultivation period. Values presented as mean ± SE (n=2) (Jerez et al. 2016b).

Photosynthetic activities showed significant differences between the full nutrient limited cultures of *C. fusca.* The full nutrient culture showed a significant increase the F_v/F_m during the first three days which then decreased by the end of trial as the culture became denser and nutrient-limited (Fig. 4.3a). Changes in rETR corresponded to F_v/F_m; however, the increase from Day 0 to Day 3 was much pronounced (Fig. 4.3b). The full nutrient culture became

stressed at the end of the experiment due to nutrient limitation as NPQ_{max} significantly increased ~10-times between Day 0 and Day 6 (Fig. 4.3c). In the N-limited culture, F_v/F_m (Fig. 4.3a) and $rETR_{max}$ (Fig. 4.3b) steadily decreased during the experiment. Compared to S-limited culture, the changes were not as pronounced (Fig. 4.3b). The NPQ_{max} values in the S-limited culture showed similar trend as the full nutrient culture, whereas under N-starvation, the quenching was significantly decreased by ~45% from Day 6 to Day 8 (Fig. 4.3c).

The partitioning of absorbed excitation energy in PSII could be expressed in terms of the unity of the complementary quantum yields of photochemical energy conversion, Y(II), and non-photochemical losses, Y(NPQ) and Y(NO). As shown in Fig. 4.4, Y(II) decreased in all treatments starting from Day 3 towards the end of the experiment, achieving the minimum values (<0.1) on Day 8 and 6 in N- and S-limited cultures, respectively. Y(NPQ) increased significantly in the full nutrient and S-limited cultures, showing maximum values at Day 6, respectively, whereas only a slight increase was observed in the N-limited culture. Contrary to Y(NPQ), Y(NO) was higher at the beginning of the experiment, presenting similar values in all treatments up to Day 1. However, under N-starvation, Y(NO) was as significant as Y(NPQ) on Day 3–6 while Y(NO) was mostly lower in the full nutrient and S-limited cultures.

Lipid content (% of DW) decreased in all treatments after the first day (Fig. 4.5a). Nevertheless, it significantly increased in all cultures at the end of the trial, from ~21% at day 0 (indicated by the dashed line) to ~27–31% at day 8. The highest increase (about a third of the initial content) was found in the full nutrient culture. Starch accumulation was present in all cultures regardless of the treatments showing significant differences over time. In the full nutrient culture, starch content doubled from the initial content (~24% indicated by the dashed line) to 50% during the first day which then continuously decreased until the end of the trial (Day 8) to ~30% (Fig. 4.5b). In nutrient-limited treatments, starch content doubled, reaching ~50 and 45% in N- and S-limited cultures, respectively. On the other hand, protein content (% of DW) markedly decreased in all treatments after the first day of experiment, from the initial value, 28% (indicated by the dashed line), to ~20% in full nutrient, ~18% in S- and ~9% in N-limited cultures. Maximum biomass, lipid and starch productivities (g L^{-1} day^{-1}) were achieved on Day 3 in all cultures (Fig. 4.6a). Biomass productivity was the highest in the full nutrient culture and it significantly lowers in N- and S-limited cultures, respectively. Lipid productivity was almost twofold higher in the full nutrient culture than under nutrient starvation. Starch productivity was significantly higher in limited cultures compared to the full nutrient culture. Mean productivity (8-day average) of biomass, lipids and starch (g DW L^{-1} day^{-1}) was 3.3–4.8

times higher in the full nutrient culture compared to the nutrient-limited cultures (Fig. 4.6b). Lipid and starch productivities were rather similar in all treatments although mean starch productivity was significantly higher than lipid productivity.

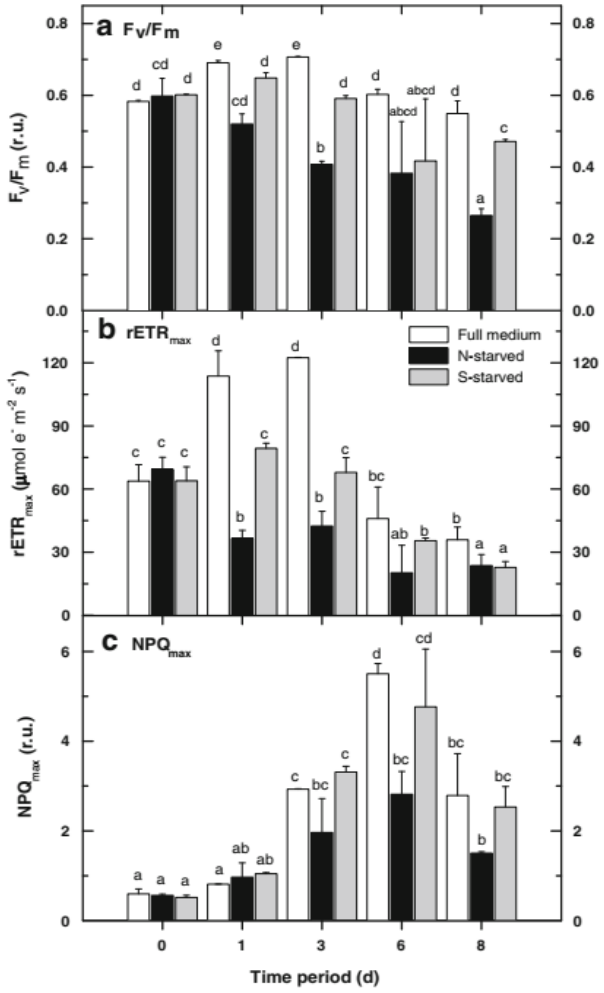


Fig. 4.3. Changes in the (a) maximum quantum yield of PSII, F_v/F_m, (b) maximum relative electron transport rate, $rETR_{max}$ (µmol $e^{-}m^{-2}$ s^{-1}) and (c) maximum non-photochemical quenching, NPQ_{max}, of *C. fusca* grown in full (clear column), N-limited (black column) and S-limited (grey column) medium during the 8-day cultivation period. Values presented as mean ± SE (n=2). Different letters denote significant differences ($p<0.05$) (Jerez et al. 2016b).

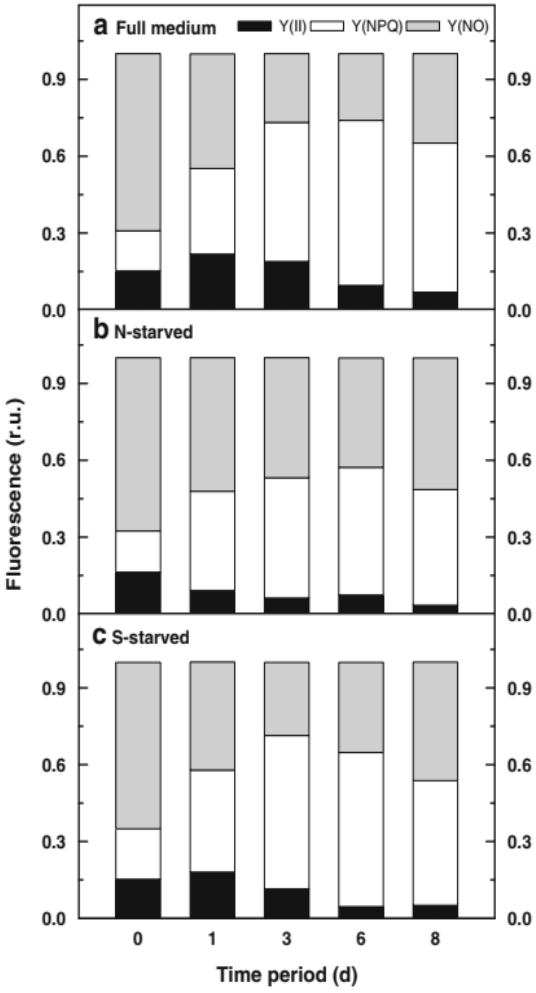


Fig. 4.4. Interplay of photochemical and non-photochemical complementary quantum yields: Y(II)+Y(NPQ)+Y(NO)=1. Changes in the actual quantum yield of PSII, Y(II) (black column), regulated non-photochemical quenching, Y(NPQ) (white column) and consecutive non-photochemical quenching, Y(NO) (grey column) of *C. fusca* grown in (a) full nutrient, (b) N-limited and (c) S-limited medium during the 8-day cultivation period. Values were taken at the point (~840 µmol photons m^{-2} s^{-1}) of the rapid light curve, at which maximal relative electron transport rate ($rETR_{max}$) was achieved. Values presented as mean (n=2) (Jerez et al. 2016b).

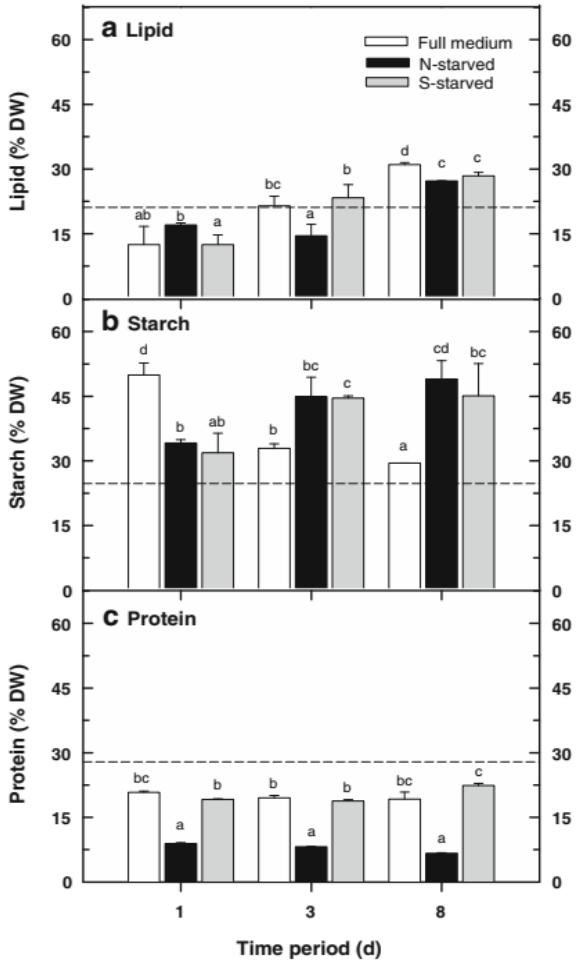


Fig. 4.5. Changes in the (a) lipid, (b) starch and (c) protein content of *C. fusca* cultures grown in full (clear column), N-limited (black column) and S-limited (grey column) medium during the 8-day cultivation period. Values presented as mean ± SE (n=2). Different letters denote significant differences ($p<0.05$). Dashed lines correspond to the starting concentrations of individual compounds at Day 0 (Jerez et al. 2016b).

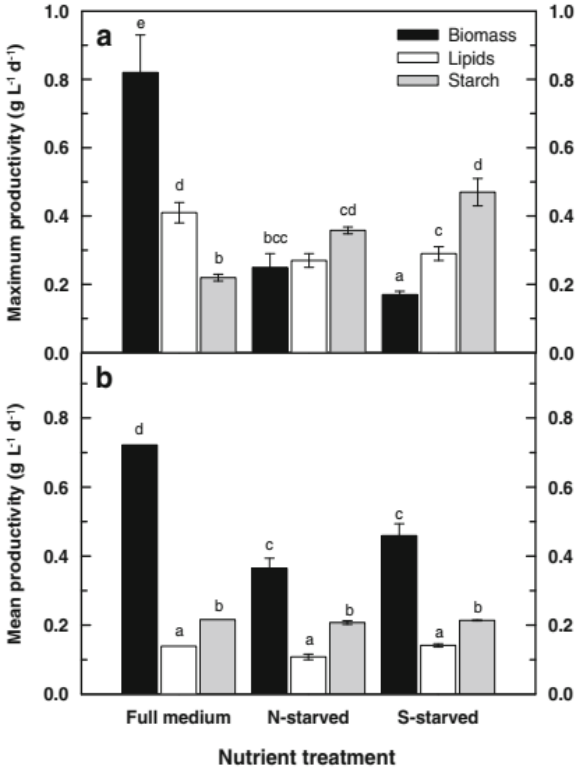
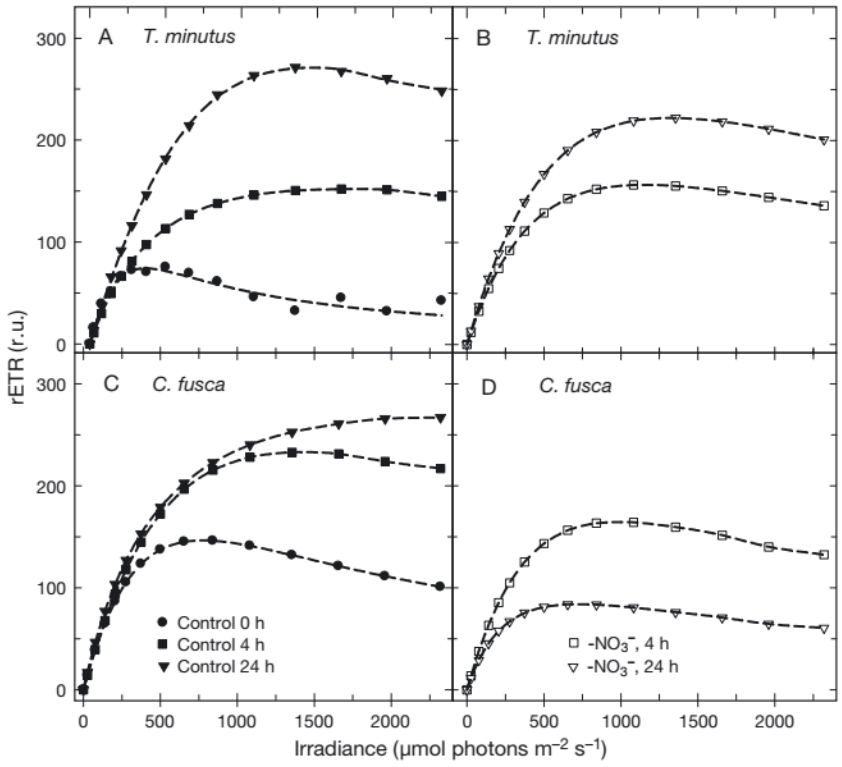


Fig. 4.6. Changes of (a) maximum and (b) mean biomass (black column), lipid (clear column) and starch (grey column) productivities of *C. fusca* cultures grown in full medium, N-limited and S-limited treatments. Maximum productivities were achieved on Day 3 in all treatments. Mean productivities were calculated as the 8-day overall value (mean± SE; n=2) (Jerez et al. 2016b).

In another series of laboratory experiments, changes in RLCs in cultures of *Trachydiscus minutus* and *Chlorella fusca* grown under nutrient starvation and high irradiance (~1,200 μmol photons m^{-2} s^{-1}) were studied over a 24-h period. The photosynthetic activity of the control cultures of both strains developed differently: in the *Chlorella* culture it was relatively high right from the start, reaching high values after just 4 h, while the activity of *Trachydiscus* was about 40% lower. However, after 24-h the $rETR_{max}$ values were similar in

both cultures, but *Chlorella* was tolerating the high irradiance level better, while the *Trachydiscus* culture became down-regulated at cultivation light intensities (Fig. 4.7 A, C). The photosynthetic activities of *Chlorella* and *Trachydiscus* were rather similar after 4 h of cultivation in the nitrate-limited cultures (Fig. 4.7B, D); but a dramatic difference was found after 24-h: the activity of *Trachydiscus* was over 60% higher than that of *Chlorella* as the latter's faster growth at the beginning had exhausted the medium's nutrient content. The photosynthetic activity of microalgae cultures is down-regulated when they experience nitrogen starvation and the products of the light-reactions cannot be used in the dark 'synthetic' processes. The RLC of $rETR_{max}$ measured in the laboratory cultures of *Chlorella* and *Trachydiscus* during the 24-h experiment showed typical records indicative of behavior of nutrient-replete and nitrate-limited cultures. This can be used to manipulate growth of these cultures under nutrient-starvation which induces production of some valuable metabolites, e.g.



carotenoids, polysaccharides or fatty acids.

Fig. 4.7. Changes in the rapid light-response curves (RLCs) of the laboratory cultures of (A,B) *Trachydiscus minutus* and (C, D) *Chlorella fusca* induced by 4 and 24-h exposure to nitrogen starvation (25% of nitrogen level of full medium) under high irradiance (1,200 µmol photons m^{-2} s^{-1}). (A, C) Closed symbols represent control cultures and (B, D) open symbols are the nitrate-limited treatments. Mixing of the microalgae suspension was maintained by bubbling through a mixture of air + 1% CO_2. Lines represent the fitted curves according to the model of Eilers and Peeters (1988). rETR: relative electron transport rate (Malapascua et al. 2014).

4.1.2 The Effect of Na_2CO_3 Addition or CO_2 Limitation in Outdoor *Chlorella* Cultures

The effect of CO_2 deficiency (CO_2 supply was stopped for 30 min) was examined *in situ* in outdoor cultures of *Chlorella vulgaris* grown in open thin-layer cascades. The experiments were carried out in well-growing cultures under optimal growth conditions and biomass density at midday in summer. Photosynthetic activity was monitored in the culture *on-line*, simultaneously, as photosynthetic oxygen production ΔO_2 (means a difference of dissolved oxygen concentration between the start and end of the cultivation platform) and the actual fluorescence quantum yield Y_{II} monitored by the saturation pulse technique. CO_2 deficiency recorded as the pH increase, was reflected in-parallel, by a decrease Y_{II} was identical to the decrease in ΔO_2 (Fig. 4.8). At pH 8.4, the partial pressure of CO_2 is reduced to about 0.2 kPa which is limiting for the fast-growing Chlorella culture (Lívanský 1993). When the CO_2 supply was restored, a prompt recovery of photosynthesis (within 1 min) was seen as an increase of Y_{II} and ΔO_2. This experiment clearly illustrates that Chl fluorescence measurements of photosynthetic activity (Y_{II}) can alternate/substitute for the monitoring of O_2 production, as the former technique is easy to measure and reliable.

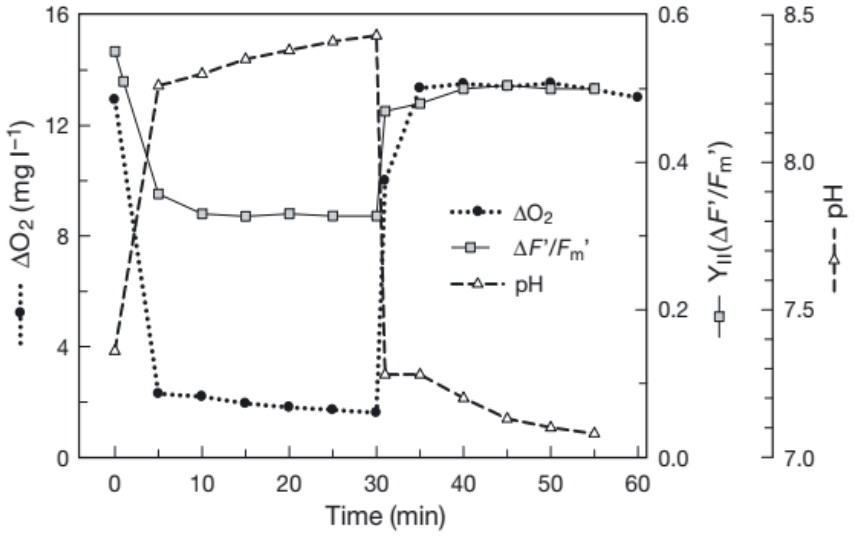


Fig. 4.8. Response of a *Chlorella* culture to insufficient CO_2 supply. The cultures were grown outdoors in TLC (Institute of Microbiology, Třeboň) at midday on a sunny day in August. The well-growing culture had optimal biomass density for fast growth (about 6 g DW L^{-1}) and was exposed to about 1,800 μmol photons m^{-2} s^{-1} between 25-30 °C which represents favorable conditions for this culture. The CO_2 supply was stopped for 30 min and the increasing deficiency was followed through changes in pH. Simultaneously, photosynthetic activity was monitored *in situ/on-line*, as ΔO_2 (the difference of dissolved oxygen concentration between the upper and lower ends of the cultivation surface) measured by the Clark-type electrode and the actual PSII photochemical yield (Y_{II}), by a PAM-101-103 fluorometer. The variables *F*' and *F*m' designate the steady state and maximum fluorescence in the light-adapted state. The fiber optics of the fluorometer was protected by a glass tube and placed directly,

3 mm deep into the suspension using solar irradiance as actinic light (at an angle of about 60°) (Malapascua et al. 2014).

4.1.3. Growth of *Chlorella* in the Presence of Selenium

Microalgae can play a crucial role in selenium(Se)-metabolism due to the uptake and biotransformation, and the subsequent transfer upwards into the food chain through, mostly by biosorption and incorporation of Se to biomass. In a series of laboratory as well as outdoor trials with *Chlorella vulgaris* cultures, the synergistic effects of Se doses and irradiance intensity were evaluated (Babaei et al. 2017). It was found that the rate of Se incorporation/tolerance by microalgae depends on photosynthetic activity. Various concentrations of Se (2.5, 8.5, 25 and 85 mg g^{-1} DW) were introduced to *Chlorella* cultures under low irradiance (250 μmol photons m^{-2} s^{-1}) to determine effective Se dose that inhibits photosynthesis and subsequently growth and correlated these variables with Se uptake (Trial 1). 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 growth curve was found with an initial slower phase, followed by acceleration of growth then it started to decelerate after 48 h (Fig. 4.9a). The results showed that in the control culture (no Se added), the biomass concentration increased by about 2.5 times 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.

In the other series of laboratory experiments, 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 (Trial 2). The biomass density at the start of both experiments was 1.2–1.6 g DW L^{-1}. 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. 4.9b). 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. Similar to trial 1, a reduction of growth and photosynthetic activity of the cultures was always accompanied by a release of volatile Se compounds; indicating that Se was not metabolized.

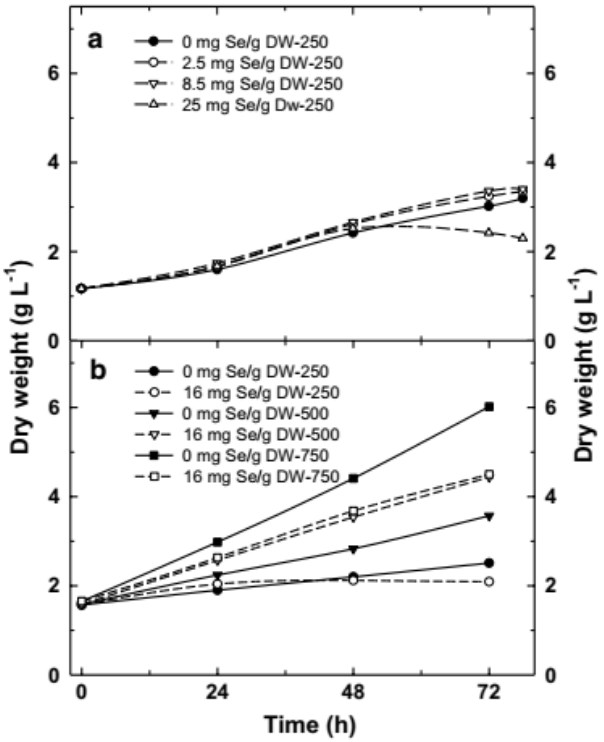


Fig. 4.9. Changes in the biomass density (DW) of the *Chlorella* cultures treated with various Se doses (2.5, 8.5 and 25 mg Se per g of DW): **a** with addition of various Se concentrations at 250 μmol photon m^{-2} s^{-1}; **b** with addition of 16 mg Se g^{-1} exposed to various light intensities (250, 500 and 750 μmol photons m^{-2} s^{-1}) during cultivation period of 72 h (control - no Se added) (Babaei et al. 2017).

The growth analysis was accompanied by fluorescence measurements of RLCs and OJIP kinetics. 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 stimulation after 24-h (Fig. 4.10). 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. 11a). 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. 11 a, b, c). These variables were affected differently as a function of Se concentration. 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 stimulation after 24-h (Fig. 4.10). 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%. 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, F_v/F_m, maximum of relative electron transport rate $rETR_{max}$ and photosynthesis-saturating irradiance E_k were calculated from RLCs (Fig. 4.11a, b, c).

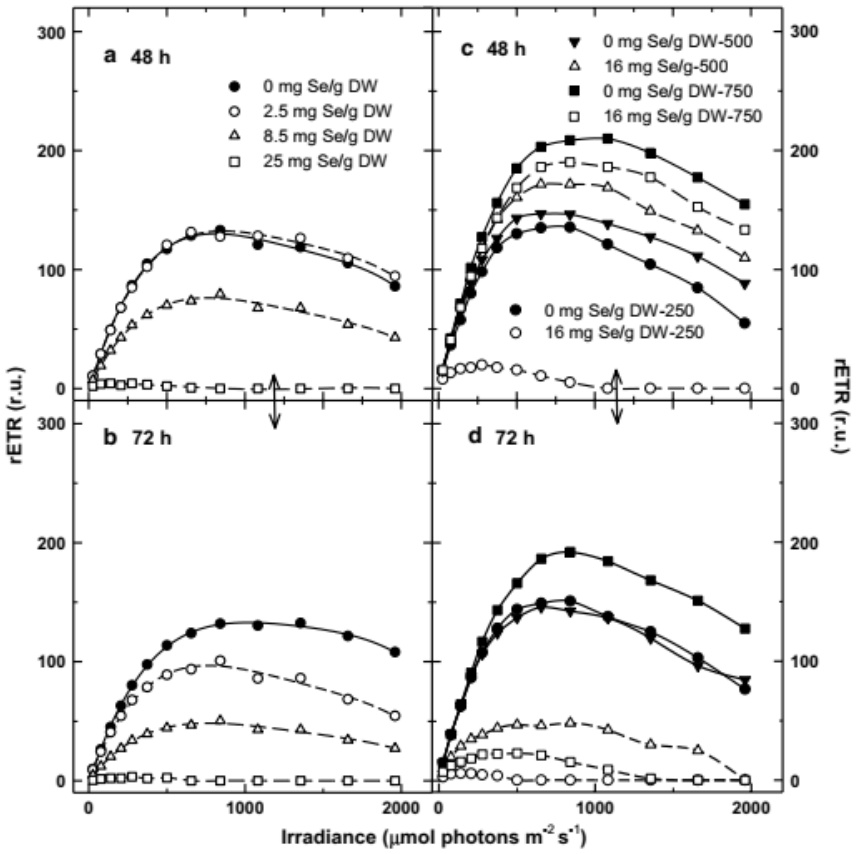


Fig. 10. Rapid light response curves (RLCs) of relative electron transport rate measured in the *C. vulgaris* cultures treated with Selenium in mg per g of cell biomass (a, b) 0 (black circle), 2.5 (white circle), 8 mg/g (white triangle) and 32 mg/g (white square) selenium for 48 and 72 h; (c, d) and treated with Selenium in mg per g of cell biomass – 0 (black symbol) and 10 mg g^{-1} (white symbol) exposed to 250 (circle), 500 (triangle) and 750 (square) µmol photons m^{-2} s^{-1} during the cultivation period after 72 h of experiment (Babaei et al. 2017).

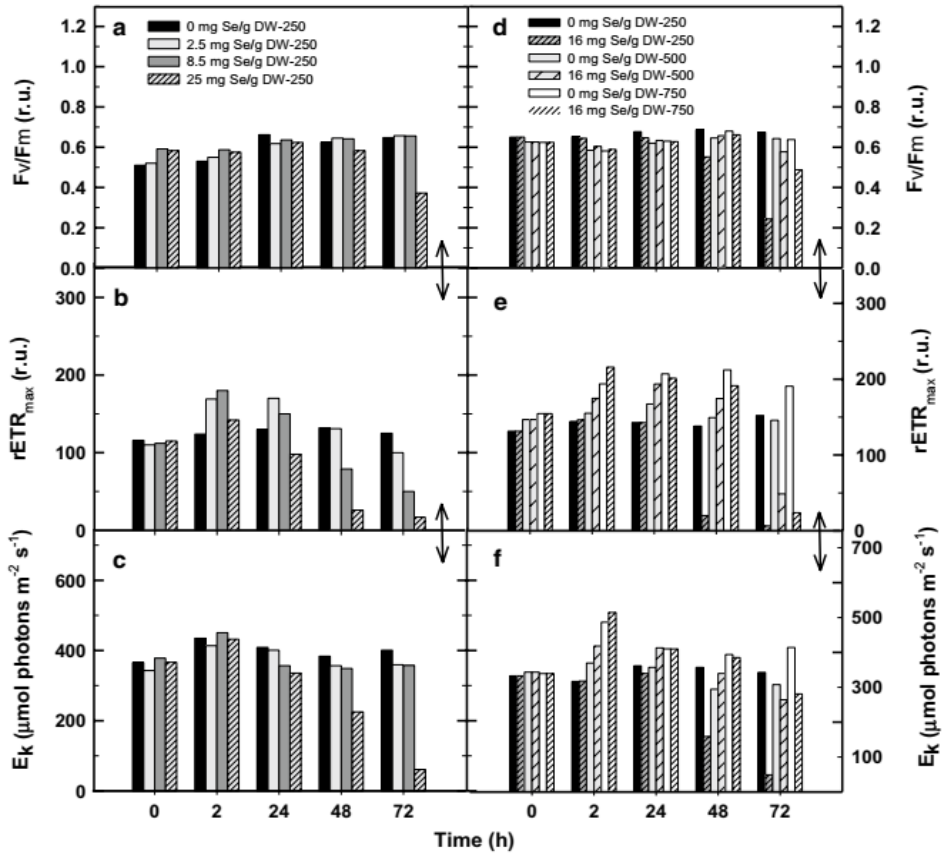


Fig. 4.11. Changes of variables F_v/F_m (**a**) $rETR_{max}$ (**b**) and E_k (**c**) calculated from rapid light-response curves (RLC) from cultures supplemented with various Se concentrations (2.5, 8.5 and 25 mg Se per g of DW) after 72 h of experiments. The effect of various light intensities of 250, 500 and 750 µmol photons m^{-2} s^{-1} in the presence of exposure to 16 mg g^{-1} selenium on F_v/F_m (**d**) $rETR_{max}$ (**e**) and E_k (**f**) variables is shown after 72 h. Control means no Se addition (Babaei et al. 2017).

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. 4.12c, 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. 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 and 72 h, respectively. It was manifested by the increased Vj and Vi values in the cultures starting from Day 2 (Figs. 4.12a, 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.

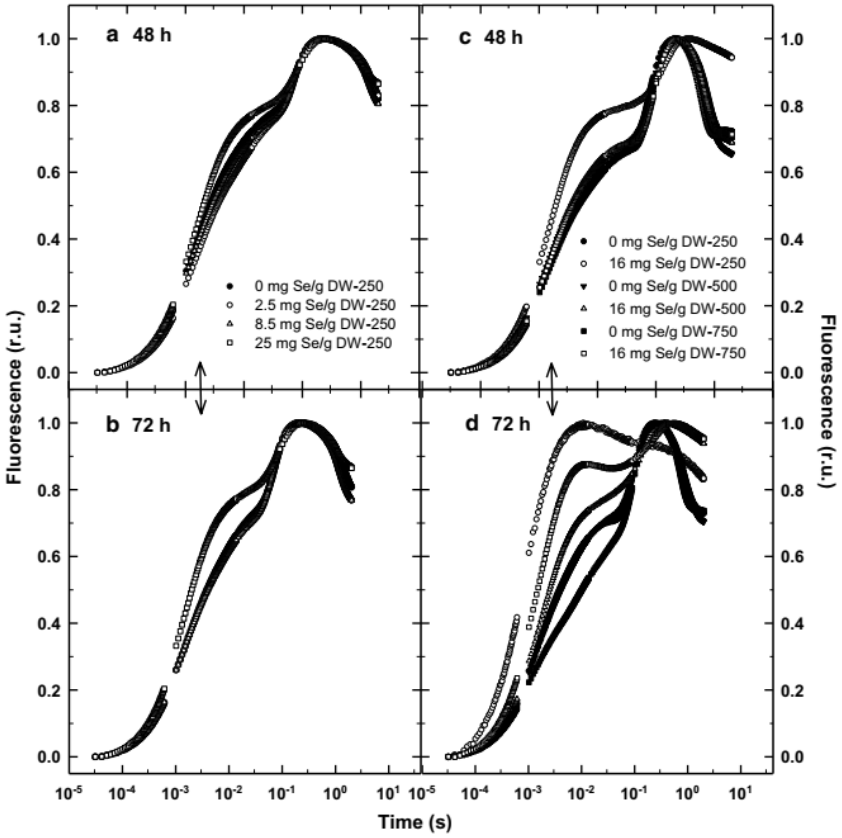
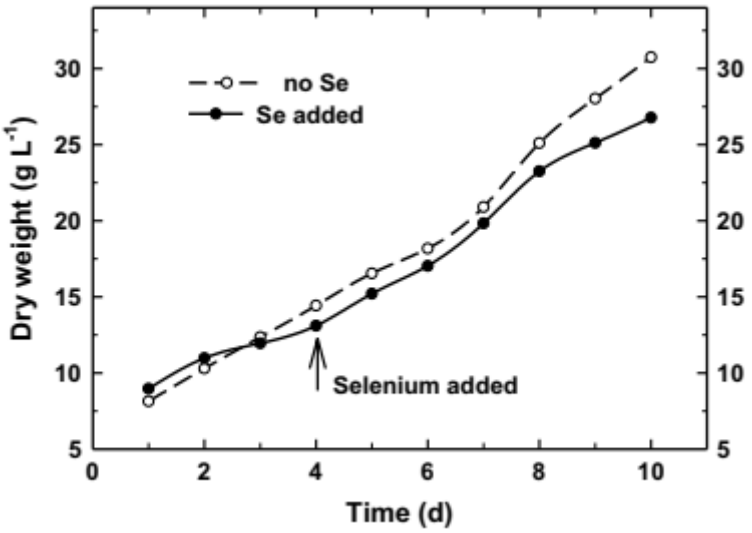


Fig. 4.12. Rapid Chl fluorescence kinetics of *Chlorella* cultures treated with various Se concentrations (2.5, 8.5 and 25 mg Se per g of DW) at 250 μmol photon m^{-2} s^{-1} after 48 h (**a**) and 72 h (**b**) of experiment. The C*hlorella* cultures were also exposed to various light intensities (250, 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) (Babaei et al. 2017).

Finally, laboratory experimental data were used in outdoor cultivation of *Chlorella* (Trial 3) aiming at producing *Chlorella* biomass enriched in Se in longer trial while minimizing growth inhibition and element losses. The starting biomass density was adjusted to 13 g L^{-1} taking into account the light path in outdoor thin-layer cascades (6 mm) and the diel period (higher daily irradiance and longer diel period). 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 it was 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. 4.13). 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. One can conclude that it is possible 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 Chl fluorescence was used as fast and suitable technique to monitor doses of Se salts.



F**ig. 4.13.** 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-d trial (Babaei et al. 2017).

5. Summary and Conclusions

One important feature of microalgae, which is of great importance to biotechnological use, is their metabolic flexibility (plasticity) as it allows the production of a wide variety of metabolites under various environmental (cultivation) conditions. The production of certain metabolites by microalgae arises from the fact that culture physiology can be modified – to a certain degree – by varying one or more environmental variables such as irradiance, temperature or nutrition. For 'adjustment' of required conditions the control of culture activity is mandatory.

The increased interest in microalgae biotechnology, aimed to the production of biomass, high-value products, has prompted the application of control measurements. Microalgae biotechnology still presents several obstacles related to biological and engineering limitations that need to be overcome, the case studies presented in this work provide some attempts in identifying these constraints to help achieve process control and improve productivity.

As photosynthetic activity is the crucial point for biomass productivity, its monitoring has always been the easiest way of growth control. Using *on-line/in-situ* measurements, we can obtain immediate evidence of culture status and potentially detect unfavorable conditions. For this purpose, the use of Chl fluorescence has been developed since the 1990s as fast, sensitive and non-invasive technique to control the growth of microalgae mass cultures.

This thesis represents an effort to show the usefulness and importance of Chl fluorescence techniques to monitor both physiological and photosynthetic variables in various indoor and outdoor large-scale cultivation system. The use of Chl fluorescence provides a simple, rapid, versatile and non-invasive tool for the monitoring of the photosynthetic activity, performance and physiological status of microalgae under various cultivation conditions. As concerns practical maintenance of cultivation units the principles of mass cultivation of microalgae are presented followed by the analysis of important variables affecting the growth and productivity of microalgae in large-scale cultivation.

Various culturing systems have been developed since the early period of microalgae biotechnology in the 1950s; none of them has been of universal use and their application has to be customized according the strain, purpose and environment. In any case it is crucial to monitor large-scale cultures *in-situ* in order to prevent growth problems, or even culture loss. Such monitored variables could include not only Chl fluorescence data, but also irradiance intensity, temperature, pH, dissolved oxygen concentration, biomass density, etc. Chl fluorescence

monitoring techniques are reliable for most microalgae strains, but for *on-line* screening of biological variables require more sophisticated evaluation methods, implemented in software, in order to deliver meaningful results. Scientific experience and process understanding also described in this thesis is an inevitable prerequisite.

One of the objectives of this thesis has been to test Chl fluorescence diagnostics for optimization of large-scale cultivation of microalgae which can benefit from *on-line/in-situ* measurement of selected variables to achieve process control and improved productivity. The information in this thesis and enclosed articles aims to provide practical instructions for microalgae physiologists and biotechnologists how fluorescence monitoring can be used to interpret changes of photosynthetic activity of microalgae mass cultures. One, direct approach is to measure photosynthesis *on-line/in-situ* during the diel cycle to monitor the actual situation in a culture. The other possibility is to measure Chl fluorescence (and other variables) *off-line* using microalgae samples taken from a cultivation unit at selected times. For this purpose, simple protocols were elaborated which showed typical records for certain strains and can help in monitor culture situation and identify possible constrains before culture is damaged or even lost. The applicability of fluorescence measurements was tested in various microalgae strains cultured in various indoor and outdoor cultivation system.

The emphasis is placed on the two most common fluorescence techniques – pulse-amplitude-modulation and fast fluorescence induction kinetics – and the interpretation of important variables that reflect changes of photosynthesis and physiological status of microalgae cultures. In particular, consideration is given to problems associated with estimation of the photochemical yield of photosystem II, electron transport rate and non-photochemical energy dissipation and its relationship to overall photosynthetic processes. In model experiments with microalgae cultures, typical records and their interpretation under various culture conditions are illustrated. Changes of photosynthetic activity and selected variables monitored by Chl fluorescence techniques can thus be related to changes of cultivation conditions, physiological status and growth of microalgae cultures for a given microalgae strain and cultivation system. In this way Chl fluorescence may be used as rapid screening technique to monitor photosynthetic activity and subsequently to estimate growth rate in both indoor and outdoor trials.

Some recommendations can be considered. (i) Though Chl fluorescence represents a rapid technique for physiological status detection in plants and microalgae cultures, it must always be accompanied by other physiological measurements. (ii) Indeed, although it is

relatively easy to generate fluorescence data, care must always be taken to select and calculate sensible variables. This is particularly true when dealing with microalgae cultures outdoors, where growth limitations, such as light, temperature and other unfavorable factors can occur side by side. As long as this is kept in mind, Chl fluorescence is a powerful technique which allows rapid monitoring of physiological status, the use of which has been steadily increasing in both laboratory and field studies of microalgae cultures.

Several cultivation systems were used in exemplary trials. TLCs represent 'open' cultivation systems which can guarantee high areal or volumetric productivity due to high exposed surface to volume ratio (S/V ratio) as shown in trials with *Arthrospira* cultures compared to cultures grown in open pond. The better culture performance in TLCs is ascribed to higher photosynthetic activity and significantly shorter light path which promoted much faster light/dark cycles favorable for photosynthesis as well as faster warming of the cultures in the morning as compared to the culture grown in the pond. Due to its biomass productivity in favorable latitudes, the thin-layer cascades are a promising system for sunlight energy conversion into microalgae biomass with low operation costs.

On the one hand, photobioreactors (PBRs; closed or semi-closed vessels with no direct contact of the microalgae culture with the environment) compared to open systems, have certain advantages: reproducible cultivation conditions with respect to environmental influences, reduced risk of contamination, low CO_2 losses, lower cost of biomass down-stream processing and smaller area requirements. On the other hand, closed systems are: more difficult to clean, the construction material might partially decrease sunlight penetration and the system must be cooled and degassed effectively since excessive oxygen produced by the growing cultures can reduce growth. Furthermore, the cost of construction is about one order of magnitude higher than that of open ponds.

Internally-illuminated PBRs is the recent improvement of closed systems as light sources are embedded in microalgae culture in order to minimize light losses and maximize light-use efficiency for photosynthesis. The features respected in the design should involve: easy maintenance and cleaning, sterilisability, light intensity regulation, adjustment of culture regime in a wide range of conditions, thorough mixing, effective gas exchange, efficient temperature regulation and the control of contamination. The tested PBRs with internal illumination can be used for the growth of delicate microalgae strains (containing bioactive compounds) that require preciously controlled culture conditions. Another application of these PBRs can be the preparation of seed monocultures to inoculate large cultivation systems.

Moreover, these internally illuminated PBRs can be scaled up to large commercial systems of thousands of liters for mass microalgae production under well-controlled conditions. Here, the test costs and technical feasibility of large PBR construction have to be carefully considered.

In outdoor cultures, microalgae are subjected to environmental changes and unexpected unfavorable conditions which may reflect in microalgae response occurring sometimes within only several seconds or minutes. It is therefore imperative that culture performance has to be monitored in order to detect warning signals that may entail decrease of productivity or culture loss. Monitoring photosynthetic performance via Chl fluorescence diagnostics can provide early signs of stress during cultivation. Microalgae have developed numerous protective and regulatory mechanisms to maximize the light energy interception, utilization in the process of CO_2 fixation and several mechanisms that minimize the light dependent injury caused to the electron transport chain by over-excitation of the photosynthetic apparatus. Monitoring of Chl fluorescence allows the evaluation of the effects of various cultivation conditions such as CO_2 supply, temperature, high light, pH, mixing, nutrients, etc. on the physiology of the culture. Using monitoring techniques such as Chl fluorescence, it is possible to optimize the photosynthetic performance of the culture by relating changes in the culture conditions to the acquired photosynthetic data. *On-line*/*in situ* and *off-line* monitoring of both physiological and photosynthesis variables (e.g. irradiance intensity, temperature, pH, dissolved oxygen concentration, biomass density, etc.) in large-scale mass cultures can help to control the growth and improved productivity. These monitoring techniques should be reliable, and non-invasive/-destructive for most microalgae species. All of the methods for monitoring of biological variables require more sophisticated evaluation methods, implemented in software, in order to deliver meaningful results, but these are already well established. Scientific experience and process understanding is an inevitable prerequisite.

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Jerez CG, Malapascua, JR, Sergejevová, M, Masojídek, J Figueroa, FL (2016b) *Chlorella fusca* (Chlorophyta) grown in thin-layer cascades: Estimation of biomass productivity by in-

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Annexes

Annex I
(related to Chapter 1 Microalgae Mass Culture, Chapter 3 Photosynthesis Monitoring in Microalgae Culture and Chapter 4 Growth of Microalgae under Unfavorable Conditions)

Article

Malapascua JRF, Jerez CG, Sergejevová M, Figueroa FL, Masojídek J (2014) Photosynthesis monitoring to optimize growth of microalgae mass cultures: application of chlorophyll fluorescence techniques. Aquatic Biology 22:123-140

Educational and application relevance

This work is a product of several projects of Algatech Centre in Třeboň (AlgaTech CZ.1.05/2.1.00/03.0110, AlgaIn CZ.1.07/2.3.00/30.0059 and ALGAMAN Czech Republic (1.07/2.3.00/20.0203) and partnership with the University of Málaga, Andalusia and Ecoduna AG in Bruck an der Leitha, Austria. This paper is also one of the outcomes arising from the author's participation in the 9th International Group for Aquatic Primary Productivity (GAP) Workshop in Málaga. Furthermore, the case studies gave the author an opportunity to work with several colleagues from academic as well as the business sector of microalgae biotechnology. Furthermore, the results of the case studies presented in this paper provided the necessary knowledge, which the author is benefiting from in his current endeavor.

Summary

This paper presets several case studies from several academic institutions and company from the business sector using different microalgae species (e.g. *Chlorella* and *Trachydiscus*) in several cultivations systems (e.g. thin-layer cascades, panel photobioreactors and annular cylinder) under various growth conditions (e.g. high- and low- light adapted, dense and dilute cultures). The photosynthetic activities and performance of the cultures were measured as a means of evaluating the design of the cultivation unit and growth condition using the two basic Chl fluorescence techniques (i.e. rapid fluorescence induction or relaxation kinetics, and the pulse amplitude modulation (PAM) method), which can either be used on-line/in situ or off-line/ex situ.

From the case studies presented in this paper, large-scale cultivation of microalgae could benefit from on-line/*in situ* monitoring of both physicochemical and biological variables to help achieve process control and improved productivity.

Annex II
(related to Chapter 2 Microalgae Cultivation Systems)

Article

Masojídek J, Sergejevová M, **Malapascua JR**, Kopecký J (2015) Thin-layer systems for mass cultivation of microalgae: flat panels and sloping cascades. In: Prokop et al. (eds) Algal Biorefineries Volume 2: Products and Refinery Design. Springer International, Switzerland, pp 237-261

Educational and application relevance

This paper resulted from several of the projects of Algatech Centre in Třeboň, Czech Republic (AlgaTech CZ.1.05/2.1.00/03.0110 and AlgaIn CZ.1.07/2.3.00/30.0059 and ALGAMAN Czech Republic (1.07/2.3.00/20.0203), which partly supported the author's study. The author was given a chance to work on the new design of thin-layer cascade made of stainless steel and gained valuable information not just on the evaluating the photosynthetic performance of cultures grown in thin-layer systems but also acquired knowledge on the vital designs of thin-layer cascade.

Summary

In large-scale cultivation of microalgae for biomass production, two basic approaches are used. One applies to cultivation in closed or semi-closed vessels – photobioreactors, while the other involves open reservoirs with direct contact of the microalgae culture with the environment. It has been shown that highest growth rate and productivity have been achieved in cultivation systems with microalgae layer lower than 50 mm, which can be achieved in open sloping cascades (≈ thin-layer cascade). In such cultivation units, high productivity is obtained by reducing the layer thickness of the microalgae culture and improved mixing (turbulence).

Annex III
(related to Chapter 2 Microalgae Cultivation Systems)

Article

Benavides AM, Ranglová K, **Malapascua JR**, 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

Educational and application relevance

This paper originates from the collaboration and partnership between Algatech Centre in Třeboň, Czech Republic and the Institute of Ecosystem Study, National Research Council in Sesto Fiorentino, Italy. The experiments resulting from this paper gave the author the opportunity to gain experience working on small- to medium-scale open circular ponds and expound his knowledge on the application of Chl fluorescence measurements and comparison of two open cultivation system (thin-layer cascade vs open-circular pond).

Summary

In this work, the productivity of the commercially important microalgae species, *A. platensis* was examined in two culture units – thin-layer cascade (TLC) and open circular pond (OCP) with different light paths.

The large difference in the S/V ratio between TLC and OCP strongly influenced photosynthetic activity. When grown at optimal and suboptimal temperatures, the photosynthesis and biomass productivity in the TLC was by about 30% higher than that obtained in the OCP. Based on the results, dense cultures in TLC showed better acclimation to high irradiance outdoors compared to the cultures in OCP. 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 utilize absorbed light energy compared to those in the OCP. When the diel course of NPQ was compared, much higher values were found in the cultures grown in TLC than in OCP, indicating that the cultures in TLC more energy is absorbed and used for electron transport and at the same time more energy that not used for photochemistry is dissipated as heat to avoid damage to the photosynthetic apparatus.

This study also demonstrated that the 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 (e.g. OCP) and therefore cells may benefit from an intermittent light pattern, which may explain the higher productivity of microalgae cultures in TLC compared to OCP. In this study, *A. platensis* cultures grown in the TLC showed higher productivity compared to cultures in OCP, which is related to a higher photosynthetic activity.

Annex IV
(related to Chapter 2 Microalgae Cultivation Systems)

Article

Jerez CG, **Malapascua JR**, Sergejevová M, Masojídek J, Figueroa FL (2016) Chlorella fusca (Chlorophyta) grown in thin-layer cascades: Estimation of biomass productivity by *in-vivo* chlorophyll a fluorescence monitoring. Algal Research - Biomass Biofuels and Bioproducts 17:21-30

Educational and application relevance

This paper originates from the collaboration and partnership between Algatech Centre in Třeboň, Czech Republic and the University of Málaga in Andalusia, Spain. Through these series of experiments, the author learned valuable information on the estimation of biomass productivity through data obtained from physiochemical measurements and photosynthetic activities through Chl fluorescence measurements.

Summary

In microalgae biotechnology it is a common practice to measure biomass productivity by taking the dry weight of microalgae cultures per square meter and day. In this study, the photosynthetic performance of outdoor cultures of the microalga *Chlorella fusca* in different thin-layer cascades (TLC) from Spain and Czech Republic was monitored using *in vivo* Chl fluorescence. This manuscript presents a first attempt to predict biomass yield and estimate biomass productivity from the daily integrated electron transport rate by using *in situ* measurements of *in vivo* Chl fluorescence in mass cultures.

The microalga *Chlorella fusca* showed high biomass productivity in TLC systems with large light-exposed surface to culture volume ratio (high S/V ratio). I*n-situ* monitoring of Chl fluorescence provided data that revealed essential information about the photosynthetic performance of the culture. Moreover, simultaneous measurement of irradiance and effective quantum yield allowed the determination of daily electron transport rate ETR, which led to the estimation of biomass productivity. Here, we present a first attempt at estimating biomass productivity based on ETR measurements in microalgae mass cultures that correlates with the measured values. However, studies on different species and culture systems may improve the accuracy of areal estimates of biomass productivity and the factors that are used to calculate the rate of carbon fixation necessary to estimate the biomass productivity. Due to its biomass productivity in favorable latitudes, the TLCs are a promising system for sunlight energy conversion into microalgae biomass with low operation costs.

Annex V
(related to Chapter 2 Microalgae Cultivation Systems)

Article

Sergejevová M, **Malapascua JR**, Kopecký J, Masojídek J (2015) Photobioreactors with internal illumination. In: Prokop et al. (eds) Algal Biorefineries Volume 2: Products and Refinery Design. Springer International, Switzerland, pp 213-236

Educational and application relevance

This paper resulted from several of the projects of Algatech Centre in Třeboň, Czech Republic (AlgaTech CZ.1.05/2.1.00/03.0110 and AlgaIn CZ.1.07/2.3.00/30.0059 and ALGAMAN Czech Republic (1.07/2.3.00/20.0203), which partly supported the author's study. In this paper, the author actively contributed in improvising the design of the PBR. Aside from the knowledge acquired in the evaluation of the PBR as concerns the growth performance of microalgae cultures, the author also actively contributed in improvising the design of the PBR.

Summary

Two photobioreactor (PBR) models with internal LED illumination (10- and 100-liter) were designed and constructed at the Laboratory of Algal Biotechnology, Algatech Center, for the cultivation of microalgae. Both PBRs were designed as vertical columns made of glass with LED light sources placed in cultivation chamber (i.e. submerged in the microalgae culture). Internally-illuminated PBRs are mostly designed as closed reservoirs with light sources embedded in microalgae culture in order to minimize light losses and maximize light-use efficiency for photosynthesis. One possible solution is to construct a PBR with light sources placed inside the cultivation vessels. Another design option of internally-illuminated PBRs represents an annular arrangement of two concentric cylinders where the suspension of microalgae is kept in the interspace and the lighting system is placed in the inner cylinder. One of the important presumptions was a possibility to scale-up to thousands of liters for mass microalgae production under well-controlled conditions. A transparent vessel made of glass allowed the use of a combination of natural light and artificial illumination for microalgae cultivation to compensate for respiration losses at night, or low-irradiance periods. Thus, we have constructed and successfully tested two models of glass-column PBRs for microalgae cultivation in which LED light sources are placed submerged in the microalgae culture in the cultivation chamber.

One of the outcomes of this study has been to test the PBRs with LED interior illumination in a pilot scale which can be scaled up to large-scale commercial systems of thousands of liters for mass microalgae production under well-controlled conditions.

Annex VI
(related to Chapter 4 Growth of Microalgae under Unfavorable Conditions)

Article

Jerez, CG, **Malapascua JR**, Sergejevová M, Figueroa FL, Masojídek, J (2016) Effect of nutrient starvation under high irradiance on lipid and starch accumulation in *Chlorella fusca (Chlorophyta).* Marine Biotechnology 18:24-36

Educational and application relevance

The results presented in this study could provide a better understanding of physiological processes that can be applied in microalgae biotechnology to enhance lipid and starch accumulation. The knowledge of the relationships between nitrogen (N) and sulphur (S) cell content, growth and biomass composition assists in the prediction of the nutrient regime required for optimal productivity. The combination of *in vivo* Chl fluorescence measurements and biochemical analysis allow us to integrate a more detailed vision of cell physiology when microalgae are exposed to N- or S-starvation.

Summary

Microalgae have been shown to be a potential source of high-value compounds such as pigments, vitamins, lipids, proteins, polysaccharides and antioxidant substances. Moreover, the production and accumulation of these compounds is enhanced by modifying the cultivation condition of the culture. In this study, the effect of combined nitrogen and sulphur limitation under high irradiance (PAR) was studied in the green microalga *Chlorella fusca* (Chlorophyta) in order to follow lipid and/or starch accumulation. Growth, biomass composition and the changes in photosynthetic activity (*in vivo* Chl fluorescence) were analyzed in the trials. The full nutrient culture showed high biomass production and starch accumulation at Day 1, when photosynthetic activity was high. Significant suppression of photosynthesis became evident as the growth medium was gradually depleted of nutrients. Suppression of photosynthesis was reflected in the decrease of maximum relative electron transport rate ($rETR_{max}$) and increase of non-photochemical quenching (NPQ), accompanied by the onset of lipid accumulation and decline in starch content. By the end of the trial, all treatments showed high lipid content (~30% of DW). Our results showed that we could enrich biomass of *C. fusca* (% DW) in lipids using a two-stage strategy (a nutrient replete stage followed by gradual nutrient limitation) while under either N- or S-starvation, both high lipid and starch contents could be achieved.

Annex VII
(related to Chapter 4 Growth of Microalgae under Unfavorable Conditions)

Article

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

Educational and application relevance

This paper is an outcome of joint research with a PhD student from the University of Tehran, Iran supported by UNESCO stipend. From this work, the author gained a better understanding of the overall effect of Se enrichment of microalgae biomass in a dose-dependent manner. Biotechnological processes were developed for the production of 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 procedures and key variables in order to optimize doses of Se salts preventing growth inhibition, or even culture loss in large-scale mass cultivation.

Summary

Microalgae are able to metabolize inorganic selenium (Se) to organic forms (e.g. Se-proteins); nevertheless, at certain Se concentration culture growth is inhibited. In this paper, the limit of Se tolerance in *Chlorella* cultures exposed to different light intensities was determined. 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}) in laboratory cultures, the daily dose of Se below 8.5 mg per g of biomass 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 outdoor cultures, no substantial change in the photosynthetic activity of the Se-treated cultures compared to the control. At the end of the one-week trial outdoors, the total yield of biomass harvest was about 50 kg containing 650 mg Se kg^{-1} DW (~80% of the added selenite was incorporated in the 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.