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Photosynthesis monitoring to optimize growth of microalgal mass cultures: application of chlorophyll fluorescence techniques

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ABSTRACT: Since the mid-1990s, chlorophyll *a* (chl *a*) fluorescence measurement has become widespread to monitor photosynthetic performance of microalgal mass cultures. This paper aims to provide practical instructions for microalgal physiologists and biotechnologists on how fluorescence monitoring can be used to explain changes in photosynthetic activity of microalgal mass cultures. Emphasis is placed on the 2 most common fluorescence techniques—pulse-amplitude-modulation and fluorescence induction kinetics—and the interpretation of important variables that reflect changes of photosynthesis and physiological status of microalgal cultures. In particular, consideration is given to problems associated with the estimation of the photochemical yield of photosystem II and its relationship to linear electron transport rate and overall photosynthesis. Emphasis is also given to the applications and limitations of these techniques through several case studies. In model experiments with microalgal cultures, typical records and their interpretation under various culture conditions are illustrated. Changes of photosynthetic activity and selected variables monitored by chl *a* fluorescence techniques can thus be related to changes of cultivation conditions, physiological status and growth of microalgal cultures for a given microalgal strain and cultivation system. In this way, chl *a* fluorescence may be used as a rapid screening technique to monitor photosynthetic activity and subsequently to estimate growth rate in both indoor and outdoor studies. This text and results formed the basis of a key lecture at the 9th International GAP workshop held at the University of Málaga in September 2012.

KEY WORDS: Biomass · Chlorophyll fluorescence · Growth · Light-response curve · Microalga · OJIP curves · Productivity · Photosynthesis · Pulse-amplitude modulation (PAM)

INTRODUCTION

Microalgal culture

Microalgae—i.e. microscopic prokaryotic cyanobacteria and eukaryotic algae—represent important primary producers in nature and form the basis of the food chain in aquatic environments. Their substantial benefits over plants are based on their short life cycles and metabolic plasticity that offers the possibility of modifying their biochemical pathways and cellular composition by varying culture conditions. In microalgal biotechnology, suitable species can be grown as productive strains in aquacultures facilitating the efficient manipulation of the cultivation process. The 'mass culture of microalgae', dense (>0.5 g dry weight, DW, l^{-1}) and well-mixed, with sufficient nutrition and gas exchange, represents an artificial

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system that is completely different from natural, optically-thin phytoplankton populations, with biomass densities several orders of magnitude lower. Mass microalgal cultures should have their physiological status monitored operatively in order to optimize photosynthetic activity and growth.

Various cultivation systems and technologies have been developed to grow microalgal mass cultures. The choice of a suitable cultivation system and the adjustment of the cultivation regime must be determined for each individual microalgal strain and product. Two basic approaches to mass production are used: one applies to cultivation in open reservoirs (with direct contact between the microalgal culture and the environment), while the other involves closed or semi-closed vessels called photobioreactors (for a recent review, see Grobbelaar 2009, Zittelli et al. 2013). Open cultivation systems are natural or artificial ponds, raceways (shallow race tracks mixed by paddle wheels) and circulation cascades (i.e. inclined-surface systems). Similarly, for the closed/semi-closed systems a variety of photobioreactors (with either natural or artificial illumination) exists, consisting of glass or transparent plastic tubes, columns or panels, positioned horizontally or vertically, arranged as serpentine loops, flexible coils, a series of panels or column gardens, in which the microalgal suspension is circulated.

Principles of microalgal mass culturing

In every cultivation system, several basic cultivation requirements that affect growth must be considered: light, a suitable temperature and pH, and a sufficient supply of carbon and nutrients. 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 make nutrition available (e.g. Grobbelaar 2007, Richmond 2013).

Light is the most important factor for microalgal growth. The amount of photon energy received by each cell is a combination of several factors: photon fluence rate, cell density, length of optical path (thickness of culture layer), rate of mixing and acclimation state of microalgae. The ambient maxima (about 2 mmol 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 & Larkum 2012, Zarmi et al. 2013). Several areas can be conceived in a microalgal 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 3 areas, in which light is sufficient for net photosynthesis, make up the photic volume. In the most efficient systems—shallow ponds, thin-layer cascades or narrow flat panels—the microalgal cell concentration is so high that all 4 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 microalgal culture (oxygen production or uptake) is plotted as a function of irradiance (E), the so-called light-response curve of photosynthesis, or photosynthesis–irradiance (P/E) curve, is obtained (e.g. Torzillo & Vonshak 2013). The P/E curve can be divided into 4 distinct areas: respiratory, light-limited, light-saturated and light-inhibited (Fig. 1). This division fits the layout of the different areas in a dense microalgal 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 microalgal strain under the given conditions. At a certain area, the irradiance intensity becomes inhibiting and 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.

After irradiance, temperature is the most important variable to control microalgal culture growth. Some

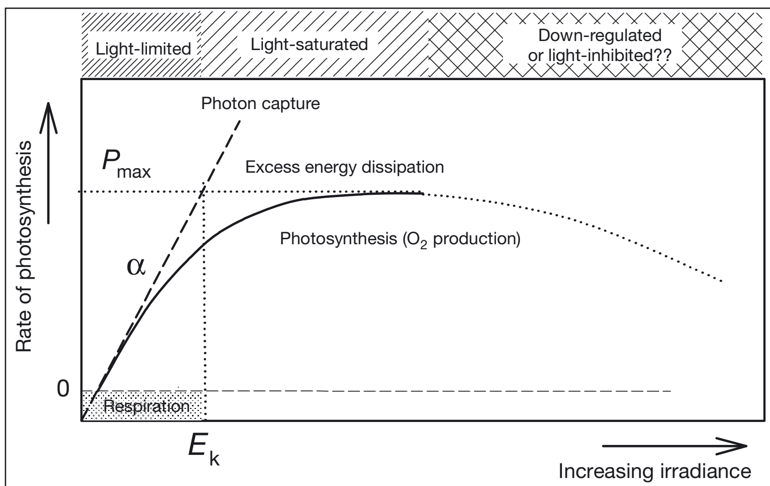

Fig. 1. Schematic representation of the photosynthetic light-response curve (solid line), i.e. the dependency of photosynthesis on irradiance. The initial slope of the curve (α, thick dashed line) shows maximum quantum efficiency of photosynthetic electron transport. The intersection between the maximum 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

strains tolerate a broad temperature range of between 15 and 40°C (e.g. *Chlorella vulgaris* or *C. sorokiniana*). By contrast, the freshwater Eustigmatophyceae strains (*Trachydiscus*) usually require a much narrower range (20 to 28°C). However, for the majority of freshwater microalgae the optimum temperature ranges between 25 and 30°C.

CO_2 serves as the main carbon source and is usually added on demand (for example, by using a pH-stat). Nutrient status can be followed by monitoring the concentration of nitrogen or phosphorus, using it as a measure for adding proportional amounts of the other nutrients.

Photosynthesis monitoring

Oxygen production, CO_2 uptake and chlorophyll *a* (chl *a*) fluorescence have been used as reliable and sensitive techniques to monitor the photosynthetic activity of various photosynthetic organisms (Bradbury & Baker 1984, Krause & Weis 1984, 1991, Walker 1987, Flameling & 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, 1995, Baker & Oxborough 2004, Suggett et al. 2011). While pulse-amplitude modulated (PAM) fluorescence can be used as a good proxy for photosynthetic performance under certain conditions, it must always be compared 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).

One direct approach is to measure photosynthesis on-line/*in situ* during the diel cycle to monitor the actual situation in a culture. Another possibility is to measure off-line, using dark-adapted microalgal samples taken from a cultivation unit at selected times (Masojídek et al. 2011b).

Presently, 2 basic chl *a* fluorescence techniques are used for monitoring photosynthetic efficiency in microalgal mass cultures: rapid fluorescence induction or relaxation kinetics, and the PAM method (for recent reviews, see Maxwell & Johnson 2000, Strasser et al. 2004, Schreiber 2004, Baker 2008, Masojídek et al. 2011b). While the rapid fluorescence induction provides us with 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. Soon after, fluorometry became one of the most common techniques to estimate photosynthetic performance both in terrestrial plants (e.g. Juneau et al. 2005) and microalgal mass cultures (e.g. Baker 2008, Masojídek et al. 2011b).

Care must be taken when measuring fluorescence and evaluating data in cyanobacteria. This is because the fluorescence emission of phycobilisomes, as well as state transition effects, contributes significantly to the total signal, and this affects the correct determination of certain variables (Ting & Owens 1992, Büchel & Wilhelm 1993, Schreiber et al. 1995).

The aim of this paper is to provide a simple, practical guide to the use of selected fluorescence techniques—PAM and fluorescence induction kinetics—

for biotechnologists who wish to monitor changes of photosynthetic activity and optimize the growth of microalgal mass cultures. In exemplary experiments with microalgal model strains *Chlorella* (Chlorophyceae) and *Trachydiscus* (Eustigmatophyceae) we have demonstrated typical records and their interpretation under various culture conditions as related to physiological status and growth.

MATERIALS AND METHODS

Organisms and culture conditions

For the present series of model experiments, cultures were grown in the laboratory or outdoors in inorganic media using various types of cultivation units (Fig. 2). For comparative purposes, green microalgae (commonly-used benchmark species for photosynthetic studies in our laboratories) have been included as experimental examples in this study. The experiments were carried out with the microalgae *Chlorella fusca* (Chlorophyta; UTEX 580 from Provasoli-Guillard Center CCMP), *C. vulgaris* (Chlorophyta) and *Trachydiscus minutus* (Eustigmatophyta) (both from the culture collection of the Laboratory of Algal Biotechnology at the Institute of Microbiology, Třeboň, Czech Republic). All microalgal cultures were grown phototrophically in a mineral medium using a semi-continuous regime, with part of the culture harvested at regular intervals. The growth optima are ~35°C for both strains of *Chlorella*, but they are able to grow up to 40°C, while *Trachydiscus* grows at 27°C up to 33°C. Laboratory experiments were carried out under continuous illumination.

At the start of the experiment, the cultures were diluted with fresh medium to the required biomass density. Microalgal mass cultures (>0.5 g dry biomass l^{-1}) were well-mixed and homogenous and therefore can be represented as an averaged cell population.

Cultivation equipment

Laboratory cultivation was carried out in glass cylinders (i.d. 35 mm, volume 0.4 l) placed in a temperature-controlled water bath with adjustable backside illumination from LED panels. Mixing of the microalgal suspension was maintained by bubbling through a mixture of air + 1% CO_2. One open unit (an outdoor thin-layer cascade) and 3 closed systems (photobioreactors; vertical flat-panel and annular column) were used for cultivation experiments (Fig. 2).

In the outdoor thin-layer cascade (24 m^2, volume 230 l, located at the Institute of Microbiology, Třeboň, Czech Republic) the illuminated surface to total volume (S/V) ratio was about 105. Most of the outdoor experiments in a thin-layer cascade were carried out between 07:00 and 19:00 h (GMT) on clear, sunny days with stable irradiance between May and July (for more information, see Doucha & Lívanský 1995, Masojídek et al. 2011a). In the outdoor cascade, the microalgal suspension was circulated during the day in a thin layer (about 6 mm) over a series of sloping planes exposed to solar irradiance using a centrifugal pump, and kept overnight in the retention tank, mixed by air bubbling. Nutrients were supplied once or twice a day according to culture demand (fed-batch regime). The pH was maintained at the optimal value of 7.6 ± 0.2 by automatic injection of pure CO_2,

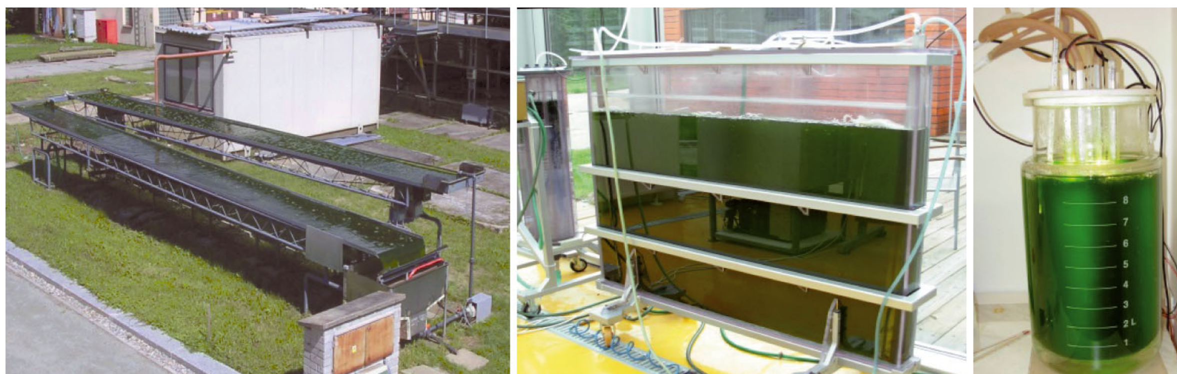

Fig. 2. Examples of experimental, open-outdoor systems or semi-closed photobioreactors for cultivation of microalgae, which can be scaled up to large production facilities. A thin-layer cascade of sloping planes (24 m^2, 220 l) at the Institute of Microbiology in Třeboň, Czech Republic (left); a vertical flat-plate photobioreactor (dimensions 1 × 1.4 m; 70 mm light path) at the Institute of Microbiology in Třeboň, Czech Republic (center); and a laboratory annular column photobioreactor (10 l) which is a double-jacket glass vessel with internal illumination by 4 internal LED sources (Institute of Microbiology in Třeboň, Czech Republic) (right)

or controlled manually in the range 7.4 to 7.9. In the morning (08:00 h), the irradiance intensity was about 400 µmol photons m^{-2} s^{-1}, reaching up to 2 mmol photons m^{-2} s^{-1} at midday (13:00 h). An outdoor thin-layer cascade (5 m^2, located at the University of Málaga, Spain) was also used in some experiments.

For comparison, 3 closed systems (photobioreactors), were also used (Fig. 2). The first, a laboratory annular column photobioreactor (10 l) was a double-jacket glass vessel with 4 internal light sources consisting of LED strips attached to an aluminum square profile protected by a glass cylinder. The maximum output irradiance was about 2 mmol photons m^{-2} s^{-1}. The light sources illuminated microalgal cultures equally, as these were mixed by bubbling with the mixture of air + 1% CO_2 with a light path of about 35 mm. The S/V ratio was about 1.5.

The second photobioreactor was a vertical thick flat-plate photobioreactor consisting of glass plates mounted in a stainless steel frame (dimensions 1 × 1.4 m; about 100 l), placed outdoors. Microalgal cultures were mixed by bubbling with the mixture of air + 1% CO_2 with a light path of about 70 mm. The S/V ratio was about 15.

The third photobioreactor was a laboratory annular column photobioreactor (10 l) which consisted of a doublejacket glass vessel with internal illumination by 4 internal LED sources (Institute of Microbiology in Třeboň, Czech Republic) (Fig. 2, right panel).

Chlorophyll *a* fluorescence and dissolved oxygen concentration measurements

For off-line fluorescence measurements, all samples were diluted (final biomass concentration 0.2 to 0.3 g DW l^{-1}, corresponding to 5 to 6 mg chl l^{-1}) and transferred to a measuring chamber (light path of 10 mm). In this way, we measured under standard conditions and avoided re-absorption problems with a dense culture by providing sufficient illumination in the dark-acclimated samples (with an oxidized plastoquinone, PQ, pool). Measurements were carried out under well-defined laboratory conditions within 5 to 10 min of a short dark adaptation, to avoid modifying the photoacclimation state of the cells. It should be noted that this procedure might generate slightly different results compared to those measured on-line/*in situ*, as the dark-adapted samples will have a part of their non-photochemical quenching coefficient (NPQ) relaxed, whereas this might be operational when measuring *in situ*. Hence, care should be taken that the handling/dark acclimation period should be tested with every microalgal culture investigated.

A pH/Oxi340i meter and electrodes (WTW) were used to control temperature, pH and dissolved oxygen concentration.

Rapid fluorescence induction kinetics (OJIP test)

The fast phase of fluorescence induction kinetics (~1 s) begins upon illumination (continuous light) of the dark-adapted microalgal culture. The signal rises rapidly from the origin (O) to a peak (P) called maximum fluorescence (F_m) via 2 inflections (J and I) (Fig. 3). This 'polyphasic fluorescence rise' is often designated as the 'Kautsky' curve (Kautsky & Hirsch 1931) or the OJIP test (Govindjee 1995, Strasser et al. 1995, 2004, Papageorgiou et al. 2007). It reflects the changes in the redox state of the reaction center of PSII (RCII) that reflect with the primary processes of photosynthesis (Govindjee & Papageorgiou 1971, Govindjee 1995, Stirbet & Govindjee 2011). The

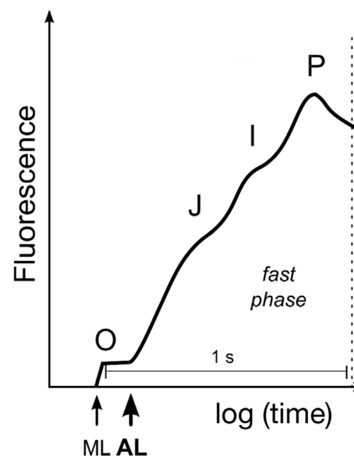

Fig. 3. Schematic illustration of the chl *a* fluorescence induction curve, known as the OJIP curve. The curve was recorded after short dark-adaptation (5 to 10 min) using weak modulated measuring light (ML). Upon application of strong actinic light (AL), fluorescence rises rapidly from the origin (O) to a peak (P), via 2 inflections (J and I), a process called the fast phase of fluorescence induction kinetics (<1 s). The rapid O–J rise (2 to 5 ms) in fluorescence represents the photochemical phase, and reflects a reduction of oxidized Q_A (gradual closing of open RCII). The rise of fluorescence from J to P shows the thermal phase, influenced by further reduction of plastoquinone (PQ) acceptors. The second inflection, I, occurs some 30 to 50 ms after illumination and it is thought to reflect a temporary maximum of Q_A^- Q_B^{2-}. Fluorescence yield then continues to rise, reaching the peak when the PQ pool becomes fully reduced (equivalent to maximum fluorescence yield. F_m; fully closed RCII). The *x*-axis (time) is in logarithmic scale

origin of the fluorescence induction curve is a base value, designated as the minimum fluorescence yield (F_0; measured after 10 to 50 µs). It is the signal emitted from excited chl *a* molecules in the light-harvesting complex (LHCII) before excitons have migrated to the RCII where PQ acceptors are fully oxidized. The F_0 or the F_m values are the most common empirical parameters. The difference between F_m and F_0, called variable fluorescence (F_V), and the ratio F_V/F_m are used extensively; the parameter F_V/F_m has been related to the maximum quantum yield of PSII photochemistry (Genty et al. 1989, Papageorgiou & Govindjee 2004, Masojídek et al. 2013).

After light exposure, the rapid O–J rise (2 to 5 ms) in fluorescence represents the photochemical phase, and reflects the accumulation of the reduced 'primary' acceptor of PSII, Q_A^- (gradual closing of the open RCII). The second inflection (I) occurs some 30 to 50 ms after illumination and it is thought to reflect different redox states, e.g. $Q_A\ Q_B^{2-}$ or $Q_A^-\ Q_B^{2-}$ (Govindjee 2004). The dip after the J inflection reflects the movement of electrons from one quencher to the next (e.g. $Q_A \rightarrow Q_B$). An important variable that is frequently used in graphical presentations of fluorescence induction data is the relative variable fluorescence at time *t*: $V_t = (F_t - F_0) / (F_m - F_0)$; it is a double normalization of the fluorescence induction curve that allows a comparison of transients measured under different conditions and/or on different samples (reviewed in Stirbet & Govindjee 2011). The rise of fluorescence from J to P (200 to 500 ms) shows the thermal phase influenced by the 2-step reduction of Q_B ($Q_B \rightarrow Q_B^- \rightarrow Q_B^{2-}$) and heterogeneity in the reduction of the PQ pool. Fluorescence yield then continues to rise, reaching P when the PQ pool becomes fully reduced (equivalent to F_m; fully closed RCII). Fast fluorescence induction curves can be normalized on both F_0 and F_m to better illustrate the reduction status of the J (V_j) and I (V_i) transients (Masojídek et al. 2011b, Fig. 4). Due to the complex nature of the numerous interactions influencing the chl *a* fluorescence induction curve, there has been some discussion over data interpretation; however, it is generally agreed which particular phase reflects the individual photosynthetic processes (Govindjee 1995, Strasser et al. 2004, Stirbet & Govindjee 2011).

As an example, we show a comparison of exemplary fluorescence induction kinetics of various microalgal strains which reflect their physiological features and photosynthetic activity (Fig. 4). The fluorescence induction curve from the cyanobacterium *Spirulina* (*Arthrospira*) *platensis* shows the distinct J inflection of the photochemical phase (curve with

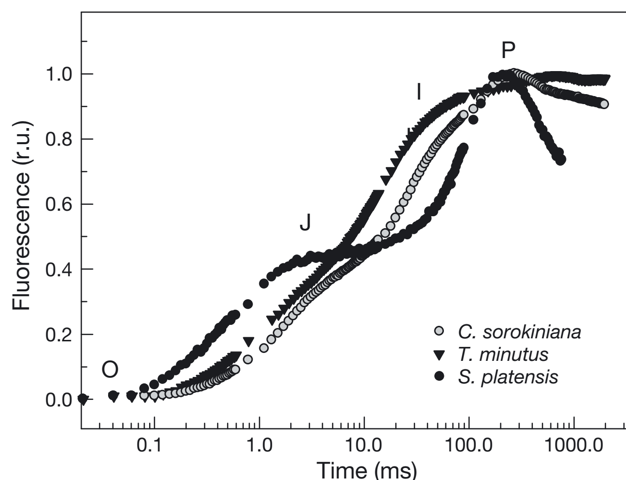

Fig. 4. Rapid chlorophyll fluorescence induction curves of various microalgae — *Chlorella sorokiniana* (Chlorophyceae), *Trachydiscus minutus* (Eustigmatophyceae) and *Spirulina* (*Arthrospira*) *platensis* (*Cyanobacteria*) — grown in an inorganic medium. The induction curves of outdoor cultures were measured at midday. Before measurement, the diluted cultures (DW = 0.2 to 03 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

filled circles in Fig. 4). 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 & Willims 1987). The OJIP kinetics among eukaryotic microalgae, e.g. the eustigmatophyt *T. minutus* (curve with closed triangles) compared to the chlorophyt *C. vulgaris* (curve with shaded circles) may also show entirely different kinetics. The slow growth of *T. minutus* 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 reoxidize the PQ pool (after 5 to 10 min of dark adaptation).

PAM-fluorometry — the saturation pulse method

The PAM technique was first described in the 1980s (Bradbury & Baker 1984, Schreiber et al. 1986) and further elaborated in the 1990s (Schreiber et al.

1995, Schreiber 2004). The most important contribution of PAM-fluorometry has been the development of quenching analysis (Schreiber et al. 1998), which provides the principal information on the distribution of the absorbed energy: its use in photochemistry (and growth) and/or its dissipation in non-photochemical processes.

PAM fluorometers usually employ 3 different light sources to examine the photosynthetic apparatus (Fig. 5). Firstly, there is a weak measuring light (ML; <0.5 µmol photons m^{-2} s^{-1}) that induces fluorescence emission without starting photosynthesis, and the detector is exclusively tuned to this emitter. The basic fluorescence signal (fluorescence yield) emitted as a response to ML in dark-adapted microalgae is F_0. The second light source used to assess maximum photosynthetic activity is a strong saturating pulse (SP; >10 000 µmol photons m^{-2} s^{-1}, 0.4 to 0.8 s in duration) which is sufficient to close all RCIIs (i.e. reduce all Q_A). The third is actinic light (AL), which can be provided by the sun or an artificial source, and which is used to induce photosynthesis.

Some variables (described below) are analogous to the fast induction kinetics introduced earlier. F_0 will occur when all RCIIs are open (Q_A in RCIIs is oxidized) and quantum energy reaching the reaction center has the maximum chance of being used for photochemistry (with only a small part being dissipated as heat and fluorescence). This state is generally considered to be achieved after dark adaptation and the release of the transthylakoid pH gradient. If a SP is applied to a dark-adapted sample, fluorescence yield will reach its true maximum F_m—hence photochemistry will be fully saturated and non-photochemical quenching negligible. The maximum quantum yield of PSII, F_V/F_m can be estimated by normalizing F_V to F_m (Table 1). The maximum values of the F_V/F_m ratio usually range between 0.7 and 0.8 in normal, non-stressed microalgae (Masojídek et al. 2013). This variable is often used as an indicator of photoinhibition: usually caused by a synergism between high irradiance and other forms of environmental stress (e.g. temperature extremes, high dissolved oxygen concentration, or nutrient limitation).

However, if the sample is exposed to any level of AL, non-photochemical quenching will inevitably lower the fluorescence yield (F' and F_m', where ′

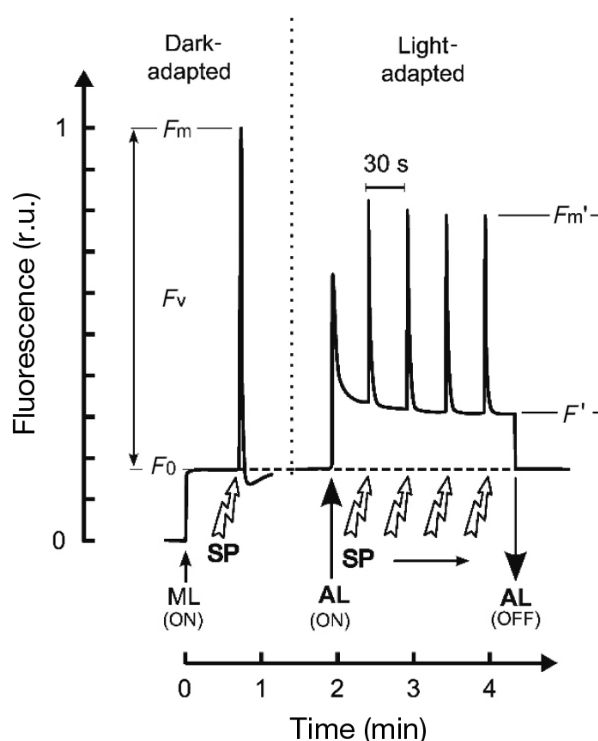

Fig. 5. Schematic representation of fluorescence quenching analysis using the saturation pulse method (pulse-amplitude modulation technique, PAM). The minimum and maximum fluorescence levels (F_0 and F_m) were measured after short dark-adaptation (5 to 10 min) using weak modulated measuring light (ML) and a saturating light pulse (SP) to calculate the maximum photochemical yield (F_V/F_m). Next, the sample was illuminated with actinic light (AL) and a series of saturating pulses in order to reach the steady state F' and F_m'

Table 1. Selected parameters calculated from chl *a* fluorescence measurements (see Fig. 5). F_0, F_V, F_m = minimum, variable and maximum fluorescence in the dark-adapted state; F', F_m' = steady-state and maximum fluorescence in the light-adapted state; a^* = optical cross-section of PSII; RCII = reaction center of PSII; E = irradiance; r.u. = relative units

Parameter	Symbol	Formula
Maximum photochemical yield of PSII (r.u.)	F_V/F_m	$F_V/F_m = (F_m - F_0) / F_m$
Actual or effective PSII photochemical yield (r.u.)	Y_{II} or $\Delta F/F_m'$	$Y_{II} = (F_m' - F') / F_m'$
Relative electron transport rate through PS II (rate of photochemistry)—correlated with primary productivity (r.u.)	rETR	$rETR = Y_{II} \times E_{PAR}$
Electron transport rate through PS II (µmol electrons m^{-2} s^{-1})	ETR	$ETR = Y_{II} \times E_{PAR} \times a^* \times 0.5$
Stern–Volmer coefficient of non-photochemical quenching (r.u.)	NPQ	$NPQ = (F_m - F_m') / F_m'$
Non-photochemical quenching (r.u.)	qN	$qN = (F_m - F_m') / (F_m' - F_0')$

denotes that the sample is measured under AL). Quenching analysis using the saturating pulse method compares the fluorescence yield during SP under AL, F_m' and F', with the dark-adapted values, F_m and F_0 (Fig. 5). The difference between F_m and F_m' can be used as a measure of non-photochemical quenching (NPQ). The actual photochemical yield of PSII, Y_{II}, which estimates the efficiency at which a certain light intensity is used for photochemistry, is calculated as Y_{II} or $\Phi_{PSII} = (F_m' - F')/F_m'$ (Genty et al. 1989). Proportionally, the non-photochemical quenching coefficient, $NPQ = (F_m - F_m')/F_m'$ can also be quantified (Bilger & Björkman 1990). The NPQ variable (Stern-Volmer coefficient of non-photochemical quenching) is considered more robust and frequently used in preference to qN (for calculation difference see Table 1), as the F_0' variable is not used in the calculation (Table 1; Ralph & Gademann 2005). NPQ is used to infer the activity of the xanthophyll cycle in microalgal cultures (Masojídek et al. 2004), although NPQ can also be caused by state transitions or photodamage (chronic photoinhibition). The primary site for the development of non-photochemical quenching is thought to be the light harvesting antennae (Ting & Owens 1992, Oxborough & Baker 1997). The down-regulation of the PSII photochemistry acts as a photoprotective mechanism. This prevents the development of triplet state of chl in RCII and the formation of reactive oxygen species. The evidence indicates that photoinhibition is moderated by the development of non-photochemical quenching (Krause & Weis 1991). A widely used variable in photosynthetic studies is the relative electron transport rate through PSII (rETR) (e.g. Hofstraat et al. 1994, Ralph & Gademann 2005, White et al. 2011). In this study, rETR (dimensionless) was calculated by multiplying the actual (or effective) photochemical efficiency by the photosynthetically active radiation: $rETR = Y_{II} \times E_{PAR}$.

The fluorescence nomenclature in this paper follows Schreiber et al. (1986) and as later elaborated by van Kooten & Snel (1990) and Kromkamp & Forster (2003). Although the terminology of chl *a* fluorescence variables is complex, reflecting the complexity of the fluorescence signal and the various ways of measuring it, the degree of standardization in nomenclature in recent years has made it easier to avoid misinterpretations. The comparison of results from the same instrument type, either induction or PAM fluorometers, should be made with some caution since the system arrangement and geometry (e.g. the position of a detector to the sample cuvette, dilution, stirring) can affect data (see e.g. Figueroa et al. 2013).

Rapid light-response curves (RLC) of photosynthetic electron transport

In comparison to the classic steady-state light-response curve (SLRC) (*P*/*E* curve; photosynthesis–oxygen production/CO_2 uptake vs. irradiance; Fig. 1), the so-called rapid light-response curve (RLC) looks similar, but shows the dependency of rETR on *E* (see Fig. 6B) (see also Kromkamp et al. 1998, White & Critchley 1999, Ralph & Gademann 2005). RLCs provide detailed information on the saturation characteristics of electron transport, as well as the actual performance of a microalgal culture. This technique can be used for analysis of changes in maximum photosynthetic rates (ETR_{max}), photosynthetic efficiency (α) and E_k (for a recent review see Enriquez & Borowitzka 2011). Using a RLC analysis we can calculate the following key variables similar to a classic SLRC:

- α (electrons/photon): the initial slope of RLC which reflects the quantum efficiency of photosynthetic electron transport.
- $rETR_{max} \sim P_{max}$ (relative units): maximum electron transport rate through the PSII complex.
- E_k (µmol photons m^{-2} s^{-1}): irradiance that is saturating photosynthesis.

The E_k value is determined from the interception point of the α slope with the maximum level of $rETR_{max}$; it defines the onset of light saturation (Fig. 1). RLCs also provide the threshold irradiance, showing the level at which down-regulation or photo-damage will occur and which manifests itself as a decrease in photosynthetic activity (Fig. 1).

RLCs measured by PAM fluorometers generate variables which describe the physiological state of variously-adapted microalgal cultures. Compared to the steady-state or 'slow' SLRC, the RLC is measured using increasing irradiances over very short time periods. Each AL intensity can last for 10 to 30 s to obtain the balanced fluorescence level F', and then a SP is triggered to reach the maximum F_m'. As an example, we show records of fluorescence quenching analysis to construct light-response curves of the low-light and high-light adapted *Chlorella* cultures grown outdoors (Fig. 6A). RLCs of the electron transport rate vs. irradiance curve (ETR/*E* curve, see below) can be completed within a few minutes compared to the several hours required for SLRC (White & Critchley 1999, Ralph & Gademann 2005, Ritchie & Larkum 2012). In this experiment, the high-light adapted cultures of *Chlorella* show values of $rETR_{max}$ that are twice as high as those of low-light adapted

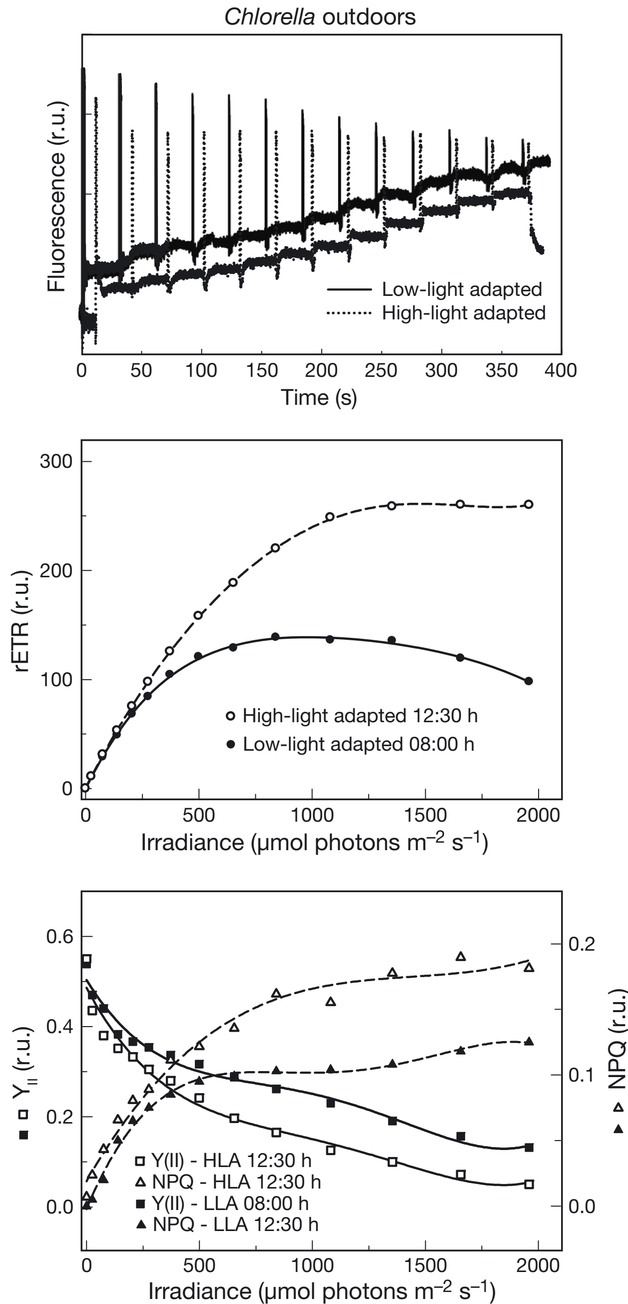

Fig. 6. (A) Fluorescence quenching records measured by the saturating pulse technique (see Fig. 5) using a modulated fluorometer in low-light and high-light adapted *Chlorella sorokiniana* cultures grown in a thin-layer cascade (6 mm light path) (see Fig. 2). A series of stepwise increasing irradiance intensities (0 to 2000 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 (F_m'). F' and F_m' were calculated at each step to construct rapid light-response ETR/E (electron transport rate vs. irradiance) curves. (B) Rapid light-response curves (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 & Peeters (1988)

ones (240 and 126, respectively; Fig. 6B). The information obtained concerns the energy distribution between photochemistry (Y_{II}) and non-photochemical dissipation (NPQ). In Fig. 6C, an antiparallel course of Y_{II} and NPQ is demonstrated. In the high-light acclimated culture of *Chlorella*, light-response kinetics showed a higher decrease of Y_{II} 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.

Case studies: photosynthesis under favorable and unfavorable conditions

In the case studies below, PAM fluorometers (PAM 101-103, PAM-2500 and Junior-PAM; Walz) were used for fluorescence quenching analysis, while the polyphasic kinetics of chl *a* fluorescence was monitored using dual-modulation fluorometers (Aquapen AP-100 and FL-2000; Photon Systems Instruments)

Here, we show several case studies to demonstrate some of the potentials of these techniques: how to estimate photosynthetic activity and growth from measurements of RLCs supported by fluorescence induction kinetics. In these studies, we usually examined the energy metabolism in various microalgal cultures to optimize the cultivation regime. On the other hand, some cultures were also exposed to unfavorable conditions (for example, nutrient starvation). The trials shown in these studies were carried out either for several days outdoors, or repeated several times in the case of laboratory measurements, and then one typical experiment (or a mean) was taken for presentation in the figures.

Trial 1 in Fig. 7 illustrates examples of the OJIP kinetics in model experiments, showing the physiological status of cultures of *C. vulgaris* at various biomass densities and light acclimation status grown in a series of flat-panel 'Hanging Garden' photobioreactors with a 30 mm light path (Fig. 1B). In the diluted shade-adapted cultures, freshly inoculated, the PQ pool was often over-reduced due to excess irradiance at midday; they thus showed a high and distinct J peak which might even be higher than the P peak

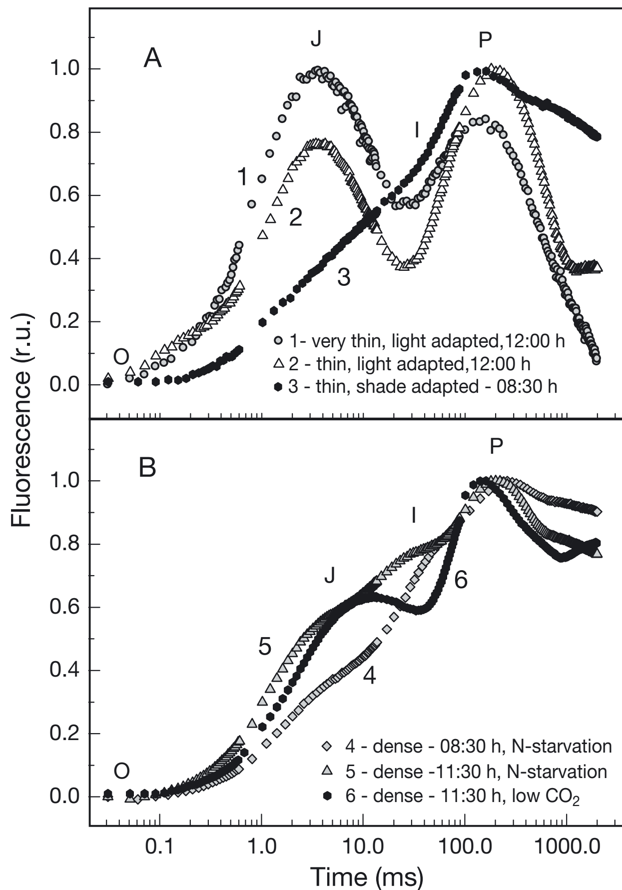

Fig. 7. Rapid fluorescence induction kinetics (OJIP-test) of *Chlorella sorokiniana* cultures measured in the morning (08:30 h) and at mid-day (11:30 to 12:00 h). (A) Diluted (0.1 to 0.33 g DW l^{-1}) and (B) dense (5 g DW l^{-1}) cultures were used to characterize typical fluorescence induction kinetics and demonstrate differences regarding the inflection points J and I (redox states of Q_A and Q_B). The measurement conditions and instrumentation are identical to that given in Fig. 4. The biomass density of cultures varied between 0.1–0.33 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 thin-layer cascade (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

(Fig. 7A, curves 1 and 2). The J inflection reflects the over-reduction of the PSII acceptors Q_A and Q_B and decrease of electron flow due to excessive irradiance as reducing power cannot be utilized in further reactions. As the cultures recovered overnight (Fig. 7A, curve 3), they seemed to be fully photosynthetically competent and able to grow well.

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. 7B, curve 4 with Fig. 7A, curve 3). If the nitrate-starved 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. 7B, 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. 7B, curve 6). These examples show some typical OJIP kinetics which can be used for diagnostics of the photosynthetic apparatus and physiological status of microalgal culture under various growth conditions.

Trial 2, as presented in Fig. 8, is an example of 2 microalgal strains, *C. fusca* (Chlorophyta) and *T. minutus* (Eustigmatophyta), grown in an outdoor experimental cascade (24 m^2, shown in Fig. 1). This near-horizontal system is based on microalgal 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, shown in Fig. 1). Nevertheless, *Chlorella* reached a biomass density that was 2-fold higher than that of *Trachydiscus*; after 10 d the biomass density of the former was 21.5 g l^{-1}, while the latter was 9.5 g l^{-1} (Fig. 8A). This higher growth rate corresponded with the diel courses of $rETR_{max}$, which were 2 to 3 times higher in *Chlorella* (about 795) than those of *Trachydiscus* (about 250) 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. 8B). 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, we also compared the diel courses of their OJIP kinetics simultaneously. 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. 8C). 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 ex-

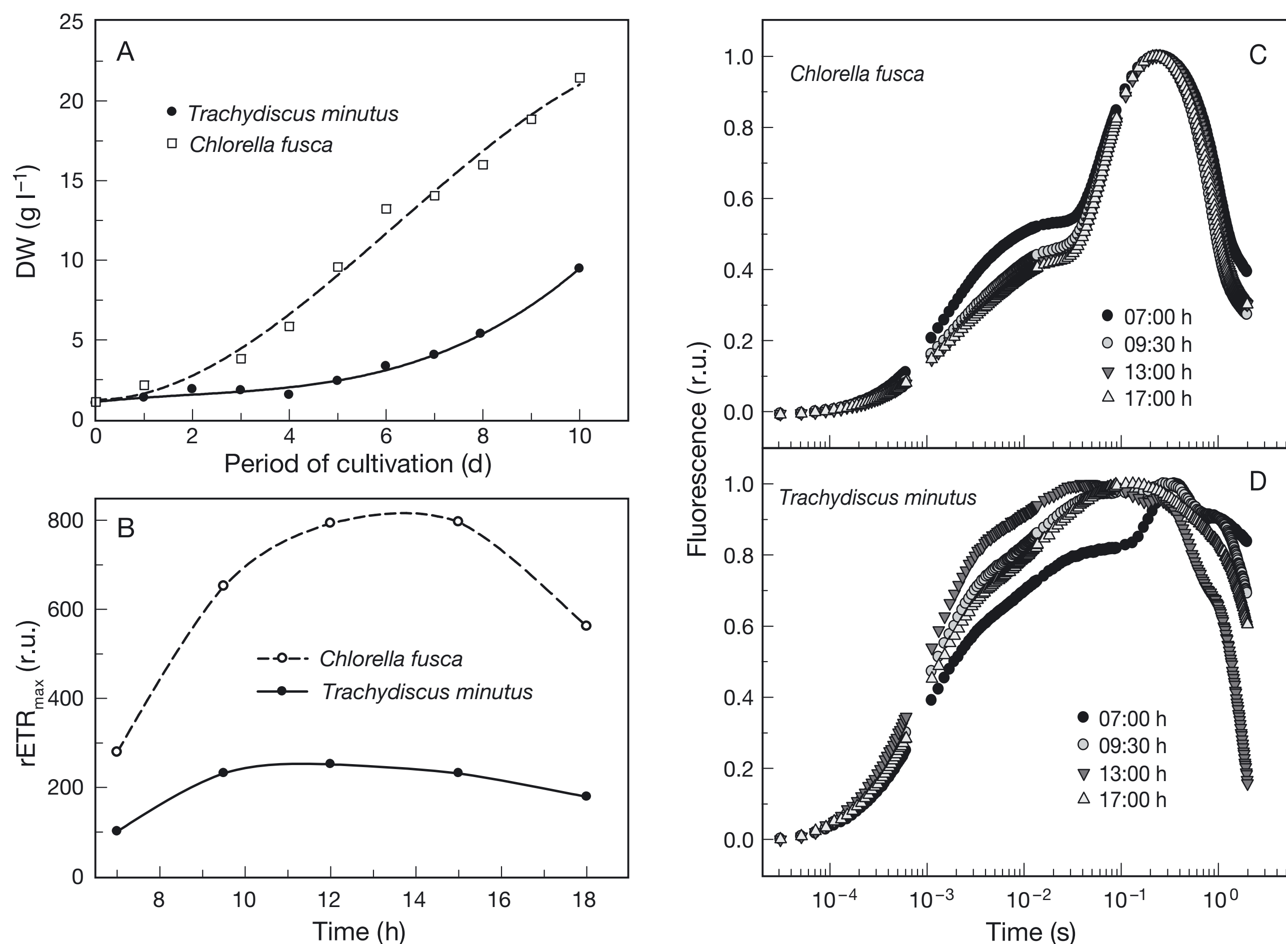

Fig. 8. (A) Growth curves of *Chlorella fusca* and *Trachydiscus minutus* cultivated in an outdoor thin-layer cascade during a 10 d experimental trial in June and July 2013. (B) Diel courses of maximum relative electron transport rate ($rETR_{max}$) of *C. fusca* and *T. minutus* on Days 3 and 7, respectively, 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 the stirred cuvette holder of a pulse-amplitude-modulation fluorometer (PAM-2500, Walz). (C) Fast fluorescence induction kinetics of *C.* and *T. minutus* grown outdoors in a thin-layer cascade during June and July 2013, 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)

plained 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 microalgal strains can be predicted even after a few experimental days.

Trial 3 represents a model laboratory experiment where we examined changes in RLCs in cultures of *T. minutus* and *C. fusca* grown under nutrient starvation and high irradiance (about 1.2 mmol photons m^{-2} s^{-1}) over a 24 h period. The photosynthetic activity of control cultures of *Trachydiscus* and *Chlorella* 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. 9A,C). In the nitrate-starved cultures, the photosynthetic activities of *Chlorella* and *Trachydiscus* were rather similar after 4 h of cultivation (Fig. 9B,D); but a dramatic difference was found after 24 h of the nitrate-starvation trial: 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 microalgal cultures is down-regulated when they experience nitrogen starvation and the products of the light-reactions cannot be used in the dark 'synthetic' processes.

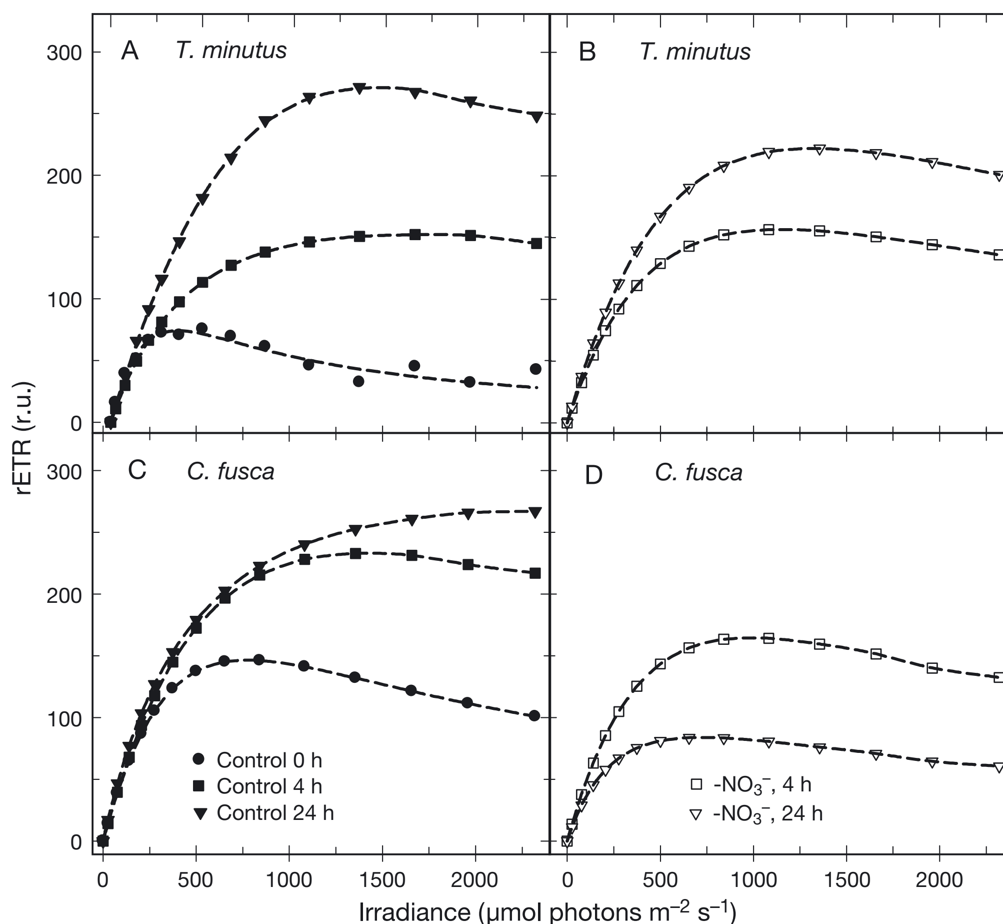

Fig. 9. Changes in the rapid light-response curves (RLCs) of the laboratory cultures of (A,B) *Trachydiscus minutus* and (C,D) *Chlorella fusca* induced by 4 and 24 h exposure to nitrogen starvation (25% of nitrogen level of full medium) under high irradiance (1200 µmol photons m^{-2} s^{-1}). (A,C) Closed symbols represent control cultures and (B,D) open symbols are the nitrate-limited treatments. Mixing of the microalgal suspension was maintained by bubbling through a mixture of air + 1% CO_2. Lines represent the fitted curves according to the model of Eilers & Peeters (1988). rETR: relative electron transport rate

The RLC of $rETR_{max}$ measured in the laboratory cultures of *Chlorella* and *Trachydiscus* during the 24 h experiment showed that we can obtain typical records which indicate behavior of the nutrient-replete and nitrate-starved 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.

In Trial 4, the photosynthetic activity of well-growing *C. fusca* cultures (3 to 4 g biomass l^{-1}) was examined when grown in various types of cultivation units (see examples in Fig. 1). These included an outdoor open thin-layer cascade (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 (T ebo , Czech Republic), in comparison to a 10 l laboratory annular column photobioreactor with internal LED-illumination (S/V about 1.5). 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. 10). The highest rETR activity (>300) was found in the thin-layer cascade 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 with the cultures grown in the flat-panel and cylindrical photobioreactor, but the $rETR_{max}$ values of the cascade culture were significantly higher (>300), in contrast to the latter ones (233 and 107, respectively; Fig. 6B). The culture in the column photobioreactor with internal illumination was evidently acclimated to low irradiance due

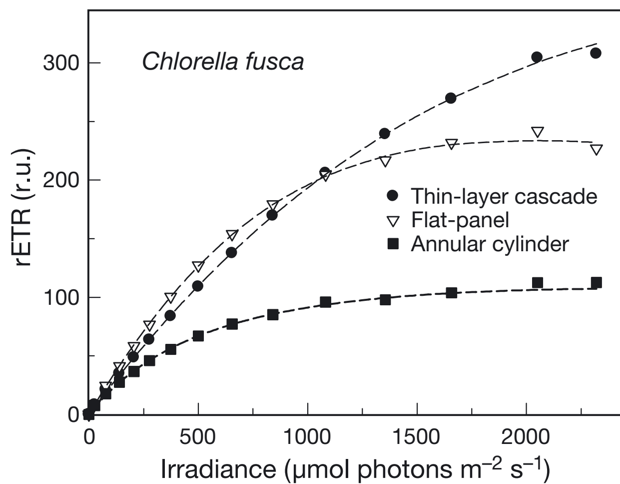

Fig. 10. Rapid light-response curves (RLCs) of *Chlorella fusca* cultures measured at midday to monitor photosynthetic performance of the cultures: relative electron transport rate (rETR) vs. irradiance. The cultures were grown in various types of cultivation units (see Fig. 2): outdoor thin-layer cascade (6 mm light path, cultivation surface of 24 m^2, 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 l laboratory annular column glass photobioreactor with continuous illumination by 4 cylindrical LED light sources (Institute of Microbiology, Třeboň, Czech Republic)

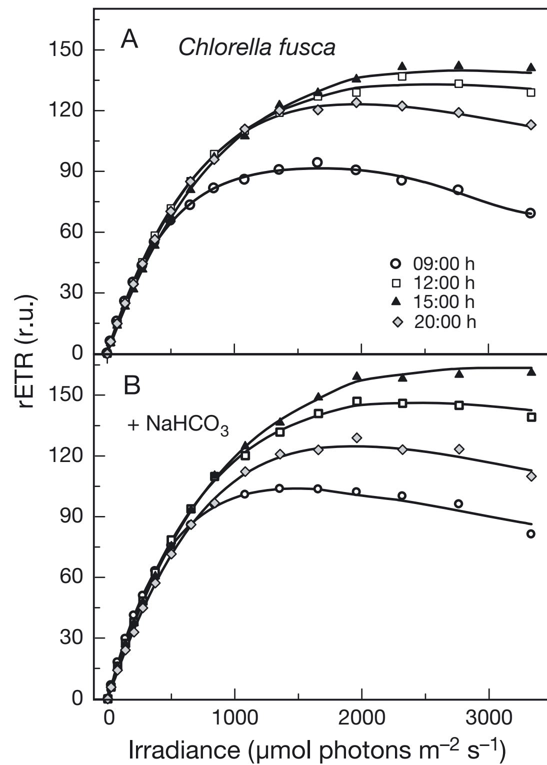

Fig. 11. Cultures of *Chlorella fusca* grown outdoors in thin-layer cascades (4 m^2; detail description by Jerez et al. 2014) in September at the University of Malaga, Spain. Comparisons were made between cultures grown in (A) an inorganic medium bubbled with CO_2, and (B) with an additional supply of 20 mM $NaHCO_3$. Rapid light-response curves (rETR vs. irradiance) of *C. fusca* were measured at 09:00 h (circle), 12:00 h (square) and 15:00 h (triangle) and at 20:00 h (diamond) to characterize the physiological state of the cultures. The measurements were carried out off-line in dark-adapted samples using the stirred cuvette holder of a pulse-amplitude-modulation fluorometer (PAM-2500, Walz)

to the low S/V ratio: photosynthesis saturation was noted at a much lower irradiance level than in the other 2 cultures, and $rETR_{max}$ was about 35% of that in the cascade. From the RLCs of rETR in this trial, we can estimate photosynthetic activity of various microalgal cultures in different cultivation units and adjust the optimum biomass density and cultivation regime. The RLCs show the varying photosynthetic performance of the cultures as a consequence of light availability in various cultivation units.

In Trial 5, we examined photosynthetic changes of *C. fusca* cultures grown in outdoor thin-layer cascades (4 m^2; for a more detailed description see Jerez et al. 2014, this Theme Section) with an additional supply of inorganic carbon. The aim was to find out whether the carbon supply (CO_2 bubbling into the retention tank) was sufficient for the culture. We followed the diel changes of the light-response curves in cultures grown in inorganic medium with and without the addition of sodium bicarbonate ($NaHCO_3$). In the morning at low irradiance, both cultures (no addition of $NaHCO_3$) showed a similar time course as well as similar $rETR_{max}$ values of about 90 to 100 (curves with open circles in Fig. 11). By the afternoon (15:00 h), the addition of 20 mM $NaHCO_3$ in the mid-morning to one of the cultures increased electron transport activity by about 15% (compare the curves with solid triangles in Fig. 11). By the evening (20:00 h), the $rETR_{max}$ of both cultures was similar again, at about 125 (compare curves with grey diamonds in Fig. 11). These results suggest that the photosynthetic activity of the culture where carbon was supplied by bubbling CO_2 to the retention tank was down-regulated, probably due to a lack of inorganic carbon.

The insufficient supply of carbon dioxide can represent a typical problem in outdoor open cultivation units exposed to full solar irradiance, as we often wish to supply only a necessary amount to keep production cost low. The lack of carbon for growth can be signaled by changes of the RLCs of rETR measured off-line in samples comparing the carbon-sufficient and carbon-limited cultures. In such a case, fluorescence measurement *in situ* as demonstrated below is more operative as we can monitor changes of photosynthesis on-line and correct the problem.

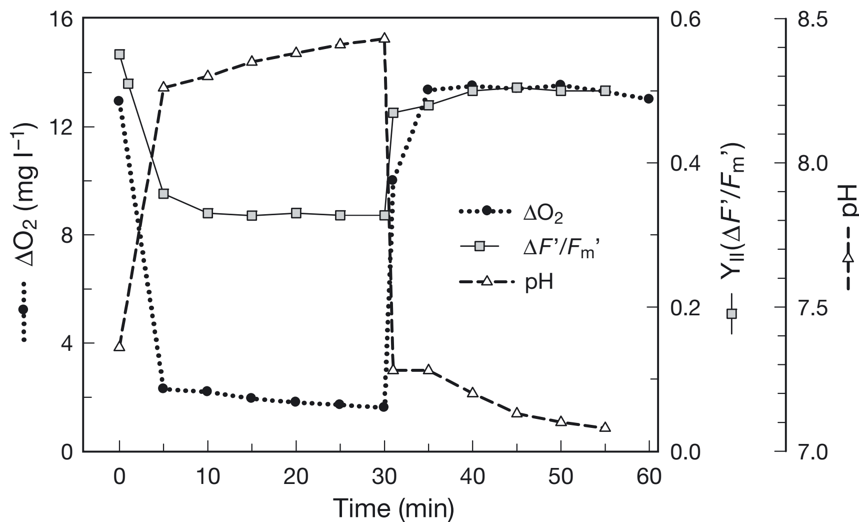

Fig. 12. Response of a *Chlorella sorokiniana* culture to insufficient CO_2 supply. The cultures were grown outdoors in an open thin-layer cascade (Institute of Microbiology, Třeboň; see Fig. 2) at midday on a sunny day in August. The well-growing culture had optimal biomass density for fast growth (about 6 g DW l^{-1}) and was exposed to about 1800 µmol photons m^{-2} s^{-1} at 25 to 30°C, which represents favorable conditions for this culture. The CO_2 supply was stopped for 30 min and the increasing deficiency/starvation was followed through changes in pH. Photosynthetic activity was monitored *in situ*/on-line, simultaneously, 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 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°)

In Trial 6, we show an experiment in which we studied the effect of CO_2 deficiency (CO_2 supply was stopped for 30 min) *in situ* on cultures of *C. vulgaris* grown in open thin-layer cascades outdoors (Fig. 12). The experiments were carried out in well-growing cultures under optimal growth conditions and biomass density at mid-day in summer (for more details see the legend to Fig. 12). Photosynthetic activity was monitored in the culture on-line, simultaneously, as photosynthetic oxygen production (ΔO_2; a difference of dissolved oxygen concentration between the start and end of the cultivation cascade) measured by the Clark-type electrode and Y_{II}, monitored by the saturation pulse technique of chl *a* fluorescence. The pH increase, which showed a development in CO_2 deficiency, was accompanied by a corresponding in-parallel decrease of Y_{II} that reflected an inhibited electron transport through the PSII complex. The course of Y_{II} was identical to the decrease in ΔO_2. At pH 8.4, the CO_2 partial pressure is reduced to about 0.2 kPa, which is insufficient for the fast-growing *Chlorella* culture (Lívanský 1993). Then, after 30 min when the CO_2 supply was restored, we observed the prompt recovery of photosynthesis (within 1 min), seen as an increase of Y_{II} and ΔO_2. This experiment clearly illustrates that chl *a* fluorescence measurements of photosynthetic activity (Y_{II}) can alternate/substitute for the monitoring of O_2 production, as the former technique is easier and more reliable.

DISCUSSION

Two basic factors must be fulfilled to obtain maximal photochemical efficiency and productivity: (1) cell density must be optimized, insuring that the mean photon irradiance per cell is close to the upper end of the linear phase of the light-response curve (E_k in Fig. 1); and (2) culture mixing must be sufficient to produce a 'flashing light effect' which is produced by high-frequency light-dark cycles of microalgal cells (10s to 100s of ms) and is correlated with high photosynthetic efficiency of energy conversion (Kok 1953, Laws et al. 1986, Hu et al. 1996, Richmond 2003, Janssen et al. 2001, Gordon & Polle 2007, Masojídek et al. 2011a, Zarmi et al. 2013).

Since the 1990s, chl *a* fluorometry has become one of the most common, non-invasive and rapid techniques to measure the variability of physiological status and photosynthetic performance in microalgal mass cultures (e.g. Vonshak et al. 1994, Torzillo et al. 1996, 1998, Masojídek et al. 1999, 2011b, Oxborough et al. 2000, Baker 2008, Kromkamp et al. 2009). Environmental conditions that impact the photosynthetic apparatus, especially PSII, directly or indirectly influence the measures of chl *a* fluorescence yield. The dominant factors include light availability, nutrient status and temperature. However, microalgal species might also exhibit endogenous diurnal patterns in fluorescence variables as a result of cellular metabolism changes. All these factors can confound possible interpretations of fluorescence variables (Kroon et al. 1993). Recent advances have allowed the careful interpretation of fluorescence data, which can provide detailed insights into photobiochemical changes of microalgal cultures. Converting the RLC and OJIP curves into a series of variables allows microalgal cultures to be optimized, or to stimulate physiological stress for secondary compound synthesis.

Photosynthetic efficiency measured by fluorescence was found to be related to quantum yields of other photosynthetic processes, such as O_2 evolution and CO_2 uptake (Genty et al. 1989, Flameling & Kromkamp 1998). The relationship between ETR and gross photosynthesis (as oxygen evolution) has been studied in microalgae (see reviews of Flameling & Kromkamp 1998, Gilbert et al. 2000, Kromkamp et al. 2009, Suggett et al. 2009).

Using fluorescence measurements, (relative) electron transport rate can be correlated with overall photosynthetic performance (Juneau et al. 2005, Baker 2008), and subsequently growth rate, giving rapid estimates of productivity (e.g. Torzillo et al. 1996). From a practical point in microalgal biotechnology, we can use used the simplified formula for the relative electron transport rate, rETR = $Y_{II} \times E_{PAR}$ (dimensionless), as we work with dense mass cultures (in contrast to relatively thin phytoplankton populations) where all light is absorbed in the microalgal layer. For a given irradiance intensity, rETR is an approximation of the rate of electron flow through the photosynthetic chain, and it has often been considered to be closely related to photosynthetic activity when measured by oxygen evolution or CO_2 uptake (Beer et al. 1998, Ralph & Gademann 2005). In this approximation (rETR = $Y_{II} \times E_{PAR}$), the absorptance (A) or ETR-factor is omitted, and equal electron transfer rates through PSII and PSI are expected (P_{PS2}/P_{PPS} or $\sigma_{PSII}/\sigma_{PSU}$ = 0.5). In this way, the incorrect assumptions of A, a^* (optical cross-section of PSII) and/or the absorption cross section $\sigma_{PSII}/\sigma_{PSU}$ can be avoided.

The calculation of absolute ETR rate is a complex point. Using Fast Repetition Rate fluorometry (FRRf), the so-called PSII electron flux per unit volume [RCII] can be estimated, which generally correlates well with photosynthetic O_2 evolution. A major limitation of using FRRf arises from the need to employ an independent method to determine the concentration of functional PSII reaction centers; a requirement that has prevented FRR fluorometers from being used as stand-alone instruments for the estimation of electron transport. Recently, a new algorithm for estimating the PSII electron flux per unit volume has been published (Oxborough et al. 2012), which does not require determination of [RCII]. This approach might also be possible with PAM, although it requires standardization of the ML and output gain settings, and involves an initial FRRf calibration as well. Once this is done, it is possible to calculate absolute ETR rates without knowledge of the optical cross section and the PSII/PSI ratio.

The correspondence of rETR and productivity of *Spirulina platensis* cultures was shown at low and high biomass densities grown at optimum and suboptimum temperatures (Torzillo et al. 1996). Recently, PAM fluorometry has also been applied to the monitoring of physiological stress caused by nutrient limitation; this induced the subsequent synthesis of cellular neutral lipids in laboratory cultures of a freshwater *Chlorella* strain (White et al. 2011). Physiological stress became evident when the photosynthesis RLC variables were decreased significantly in rETR, F_V/F_m and E_k to 75%, 36% and 60%, respectively, and increased in NPQ to 83%, marking the onset of neutral lipid synthesis. Complete nutrient stress induced the highest yield of cellular neutral lipids (~49%), compared to an absence of selected nutrients (~30%).

Photosynthetic carbon production (µmol C m^{-2} d^{-1}) of *Chlorella fusca* cultures in thin-layer cascade (TLC) cultivators has been converted into biomass productivity expressed as g DW m^{-2} d^{-1} (estimated productivity) using the total internal carbon content and the theoretical relationship between photons absorbed and oxygen production and the relationship between carbon assimilated per oxygen produced (Figueroa et al. 2013). In 2 cm layer cascades, the estimated biomass productivity was about 8% lower than the measured productivity, whereas in a 1 cm layer cascade no relationship was found (Figueroa et al. 2013).

Large-scale cultivation of microalgae will benefit from on-line/*in situ* monitoring of both physicochemical and biological variables to help achieve process control and improved productivity (Havlik et al. 2013). Such monitored variables could include irradiance intensity, temperature, pH, dissolved oxygen concentration, biomass density, chl *a* fluorescence, etc. Novel monitoring techniques available now should be reliable and non-invasive for most microalgal strains. All of the methods for on-line monitoring of biological variables require more sophisticated evaluation methods, implemented in software, in order to deliver meaningful results. Scientific experience and process understanding is an inevitable prerequisite.

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