Review

Use of *in vivo* chlorophyll fluorescence to estimate photosynthetic activity and biomass productivity in microalgae grown in different culture systems

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ABSTRACT. *In vivo* chlorophyll fluorescence associated to Photosystem II is being used to evaluate photosynthetic activity of microalgae grown in different types of photobioreactors; however, controversy on methodology is usual. Several recommendations on the use of chlorophyll fluorescence to estimate electron transport rate and productivity of microalgae grown in thin-layer cascade cultivators and methacrylate cylindrical vessels are included. Different methodologies related to the measure of photosynthetic activity in microalgae are discussed: (1) measurement of light absorption, (2) determination of electron transport rates versus irradiance and (3) use of simplified devices based on pulse amplitude modulated (PAM) fluorescence as Junior PAM or Pocket PAM with optical fiber and optical head as measuring units, respectively. Data comparisons of *in vivo* chlorophyll fluorescence by using these devices and other PAM fluorometers as Water-PAM in the microalga *Chlorella* sp. (Chlorophyta) are presented. Estimations of carbon production and productivity by transforming electron transport rate to gross photosynthetic rate (as oxygen evolution) using reported oxygen produced per photons absorbed values and carbon photosynthetic yield based on reported oxygen/carbon ratio are also shown. The limitation of ETR as estimator of photosynthetic and biomass productivity is discussed. Low cost:quality PAMs can promote monitoring of chlorophyll fluorescence in algal biotechnology to estimate the photosynthetic activity and biomass productivity.

Keywords: absorptance, biomass productivity, electron transport rate, PAM fluorescence, photosynthetic yield.

Uso de la fluorescencia de la clorofila *in vivo* para estimar la actividad fotosintética y productividad de la biomasa en microalgas crecidas en diferentes sistemas de cultivo

RESUMEN. La fluorescencia de la clorofila *in vivo* asociada al Fotosistema II se emplea para la determinación de la actividad fotosintética en microalgas crecidas en distintos tipos de fotobiorreactores pero existe controversia sobre la metodología más apropiada. En este trabajo se hacen varias recomendaciones sobre su uso para estimar la tasa de transporte electrónico (ETR) y la productividad de la biomasa en algas crecidas en cultivadores de cascada de capa fina y en recipientes cilíndricos de metacrilato. Se discuten diferentes medidas asociadas a la metodología de la actividad fotosintética: (1) medidas de absorción de luz, (2) determinación de ETR *versus* irradiancia, y (3) uso de equipos simplificados basados en fluorescencia por pulso de amplitud modulada (PAM) como el Junior PAM o Pocket PAM, con fibra óptica o cabezal óptico como unidades de medida, respectivamente. Se comparan los datos de fluorescencia *in vivo* en la microalga *Chlorella* sp. (Chlorophyta) con los de otros fluorímetros como el Water PAM. Se estima la producción de carbono y productividad de la biomasa mediante la transformación del ETR en producción bruta de oxígeno usando valores teóricos de oxígeno producido por fotones absorbidos y la tasa de asimilación de carbono considerando la relación oxígeno producido/carbono asimilado. Se discuten las limitaciones del uso del ETR como estimador la productividad de la biomasa. Los fluorímetros con alta relación calidad:precio pueden promover la monitorización de la fluorescencia *in vivo* de la clorofila en biotecnología de algas para estimar la actividad fotosintética y productividad de la biomasa.

Palabras clave: absorptancia, productividad de la biomasa, tasa de transporte electrónico, fluorescencia de clorofila *in vivo*, rendimiento fotosintético.

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INTRODUCTION

Accurate determination of algal photosynthesis presents difficulties in both short and long-term scale due to diverse photoacclimation mechanisms and complex regulation systems (MacIntyre *et al.,* 2002). In mass algal cultivation both closed photobioreactors and open cultivation systems, difficulties to determine the photosynthetic yield increase due to high light absorption and high cell density and heterogeneity (spatial-temporal variations) in the cultivation systems (Masojídek *et al.,* 2011). Development of non-intrusive methodologies has led to rapid and sensitive measurements of changes in the physiological status of aquatic plants subjected to light stress (Suggett *et al.*, 2011). Pulse Amplitude Modulation (PAM) chlorophyll fluorescence of photosystem II (PSII) was primarily developed to assess photosynthetic primary reactions and quenching mechanisms in plant physiology studies of higher plants (Schreiber *et al.,* 1986; Baker & Oxborough, 2004) and later it has been used in aquatic macrophytes and phytoplankton under different natural and artificial light conditions (Hanelt, 1992; Schreiber *et al.,* 1995a; Flameling & Kromkamp, 1998; Figueroa *et al.,* 2003; Wilhelm *et al.*, 2004; Suggett *et al.*, 2011). An optimization of the PAM instrumentation was needed to meet accurately the low chlorophyll fluorescence emission of microalgae in oligotrophic waters, *i.e.,* Water-PAM (Korbee *et al.,* 2012). The high diversity of phytoplankton according to the pigment composition (Johnsen & Sakshaug, 2007; Babin, 2008) requires care with the interpretation since the standard of higher plant models are not sufficient. For example, the presence of phycobilisomes in the light-harvesting system of red algae and cyanobacteria results in generally lower maximal quantum yields (F_v/F_m) than that measured in green and brown algae (Büchel & Wilhem, 1993). On the other hand, the high diversity of carotenoids and other accessory pigments in the phytoplanktonic photosynthetic antenna together with the high variety of photoprotectors produces high diversity fraction of absorbed quanta to PSII, package effect and consequently of irradiance acclimation capacity (Dubinsky *et al.,* 1986; Suggett *et al.,* 2004; Johnsen & Sakshaug, 2007).

The introduction of a simple parameter to use fluorescence for measuring photosynthetic efficiency in light by Genty *et al*. (1989), and subsequent findings that this parameter is related to quantum yields of other photosynthetic processes, such as O_2 evolution and CO_2 fixation (for algal examples, see Flameling & Kromkamp, 1998; Figueroa *et al.,* 2003), have led to the widespread use of protocols for

converting the quantum yield of PSII into actual rates of electron flow. Electron transport rate (ETR), as originally described by Schreiber *et al.* (1986), can be determined in field samples by using values of $\Delta F/Fm'$ and simultaneously measuring the incident irradiance (E) between 400 and 700 nm (photosynthetic active radiation, PAR) and the absorptance (A).

The usefulness of chlorophyll fluorescence as an indicator of photosynthesis has been reported as the relationship to the quantum yield of gas evolution (O_2 or CO_2). The relation between ETR and gross photosynthesis (as oxygen evolution) has been studied in terrestrial plants (Asada, 1999; Genty *et al.,* 1989; Krall & Edwards, 1990), microalgae (see review of Flameling & Kromkamp, 1998; Gilbert *et al.*, 2000b; Kromkamp *et al.,* 2008; Suggett *et al.,* 2009) and macroalgae by using oxygen electrodes (Hanelt & Nultsch, 1995; Beer *et al.,* 2000; Carr & Björn, 2003; Figueroa *et al.,* 2003) and mass spectrometry (Franklin & Badger, 2001). Therefore, extrapolations of quantum yield of PSII (Φ_{PSII}) to the absolute photosynthetic electron transport rate (ETR) depend on the specific correlation among quantum yields of oxygen evolution (Φ_{O2}), carbon fixation (Φ_{CO2}) and any other competing sinks for electrons.

The excess energy can be dissipated as heat, *i.e.,* non photochemical quenching (NPQ) caused by xanthophyll cycle via heat dissipation during transport form antenna to reaction centers of PSII (RCII) and it is caused by conformational changes in PSII complex (Horton & Ruban, 2005; Lavaud & Kroth, 2006). The excess of energy can be dissipated as heat form inactivated PSII centers indicating that the first line of defense against excess of light failed.

A linear relation between ETR and CO_2 exchange can be demonstrated, as in the case of C_4 and C_3 plants at various irradiances under non-photorespiratory conditions (Weis & Berry, 1987; Krall & Edwards, 1990). The relationship can be curvilinear at low irradiance at normal levels of O_2 and CO_2 when electrons flow to O_2 via the photosynthetic carbon oxidation cycle and/or Mehler-ascorbate-peroxidase reaction. This reaction competes in the water cycle with carbon fixation and confounds measurements of O_2 evolution (Genty *et al.,* 1989, 1992; Asada, 1989). Linear relation between ETR and gross photosynthesis (GP) has been found until certain growth irradiances, *i.e.,* lower than E_k with a relation of about 4-5 electrons (associated to PSII activity) per oxygen of molecule. A loss of linearity between GP-ETR is observed at high irradiances because the decrease in Φ_{O2} with increasing growth irradiance is much higher than that observed for Φ_{PSII} both in microalgae (Flameling & Kromkamp, 1998) and macroalgae

(Figueroa *et al.*, 2003). This decrease in Φ_{O2} can be explained, in part, as a consequence of an increase in cyclic phosphorylation with respect to non-cyclic phosphorilation under high light (Dubinsky *et al.*, 1986). In addition, under high irradiance pseudocyclic electron transport (Mehler reaction) with the transfer of electrons from the reduced ferredoxin at the donor site of PSI to oxygen instead of $NADP^+$ occurs (Robinson, 1988). Other explanations for the non-linearity between Φ_{PSII} and Φ_{O2} can be attributed to the PSII heterogeneity (Melis, 1991), the increase of photorespiration (Raven & Johnston, 1991) and the underexplored phenomena of PSII cycling (Feikema *et al.*, 2006). Longstaff *et al.* (2002) found that *in situ* measurements of photosynthesis in *U. lactuca* revealed a good correlation between ETR and O_2 evolution at moderate light but at higher irradiances they found higher ETR than the expected ones. Figueroa *et al.* (2003) suggested applying a tangential function instead of a linear function to fit the relation between ETR and GP as for a wide range of irradiances it presents a tangential function form. In any case, the number of algal species in which both variables have been simultaneously analyzed is still scarce so far (Hartig *et al.,* 1998; Sugget *et al*., 2011).

Phytoplankton studies in the laboratory have been conducted mainly by using PAM fluorescence whereas in biological oceanographic studies both PAM fluorescence and Fast Repetition Rate Fluorescence had been used (Flameling & Kromkamp, 1998; Johnsen & Sakshaug, 2007; Blache *et al.,* 2011). These devices present different characteristics according to the quality of actinic lamps (halogen, red or blue light emitted diodes), sensitivity and measuring units (cuvettes *versus* fiber). On the other hand, measurements of *in vivo* chlorophyll fluorescence in algal mass cultures in open or closed photobioreactors are still scarce (Vonshack *et al.,* 1994; Torzillo *et al.,* 1996; Masojídek *et al.,* 1999, 2011; Kromkamp *et al.,* 2009).

In this review, several technical aspects on the use of chlorophyll fluorescence are discussed and several recommendations are presented on: (1) Measurement of light absorption. (2) optimal procedure to determine electron transport rate. (3) use of relatively new devices based on PAM fluorescence as Junior PAM (Walz GmbH, Effeltrich Germany) or Pocket PAM (Gademann Instruments, Würzburg, Germany) with optical fiber and optical head as measuring units, respectively. Data of several experiments in culture vessels or thin-layer cascades by using these devices and the comparisons with Water-PAM are presented. (4) estimation on carbon production and biomass yield of phytoplankton by transforming electron transport rates using theoretical and empirical data on number of photons necessary to produce an oxygen molecule and the carbon assimilated/oxygen production ratio.

In order to release general advances on the use of *in vivo* chlorophyll fluorescence in plants, several reports and reviews on phytoplankton have been considered (Kromkamp & Forster, 2003; Suggett *et al.*, 2011).

Light absorption

Irradiance is expressed as incident energy per unit of time and area, as energy units (W m^{-2}), but it can be also expressed as photon units (μmol photons m^{-2} s^{-1}). Irradiance is the term used when a sensor with a cosine response (2π) is used as measuring unit, in contrast to 4π sensor in which the correct term is photon fluence rate (Björn *et al.*, 1996). Incident radiance in the 400-700 nm waveband is generally considered the photosynthetic active component of total spectral irradiance [$E(\lambda)$] and it is refer to as Photosynthetic Active Radiation (PAR or E_{PAR}). Photosynthetic irradiance is usually denoted with the symbol *I*, however, as *I* is used for radiant intensity (W sr^{-1}), the symbol *E* for irradiance is recommended by the International Union of Pure and Applied Chemistry (IUPAC) (Braslavsky, 2007). Algae can use irradiance at wavelengths as low as 350 nm for photosynthesis, however, difficulties of measuring the 350-400 nm waveband and its very small contribution (~5-7%) to total irradiance (either solar or from commonly used emission sources) means this is usually ignored (Sakshaug *et al.*, 1997). Photosynthetic active radiation is generally given in units of μmol quanta m^{-2} s^{-1} or μmol photons m^{-2} s^{-1}. Units of μEinsteins m^{-2} s^{-1} are also used but it is incorrect since it is not included in the IUPAC for E_{PAR} (Kirk, 1994) whereas the irradiance of UV radiation (UVR) is usually presented as energy units (W m^{-2}). For phytoplankton cells that collect radiant energy equally for all sides, the photosynthetically active scalar irradiance, E^0_{PAR}, measured with a spherical (4π) sensor should be used (Kirk, 1994).

In order to obtain accurate estimations of the quantum yields of electron transport through PSII (Φ_{PSII}), oxygen evolution (Φ_{O2}) or carbon fixation (Φ_{CO2}), one must know the amount of E_{PAR} that is absorbed by the studied organism. Difficulties associated with accounting for the scattering component of attenuation in optical measurements have limited the determination of absorbed irradiance as distinct from incident irradiance (Agustí *et al.*, 1994). Conventional spectrophotometers measure transmittance (T) and/or optical density (OD):

$OD\ (\lambda = Log_{10}\ (E^o_\lambda\ /E_\lambda) = -log_{10}\ T\ (\lambda)$ (absorbance units)   (1)

where E^o_λ is the incident (prior to absorption) spectral irradiance and E_λ is the transmitted spectral irradiance.

By using techniques with negligible losses due to scattering, such as the opal glass (Shibata et al., 1954), diffusing plate and minimal sample-detector distance (Bricaud et al., 1983), or an integrating sphere method (Bricaud et al., 1983; Maske & Haardt, 1987; Fujiki & Taguchi, 2002), absorptance (A) can be calculated as follows:

$A = 1-10^{-OD}$ (relative units respect to one)   (2)

Absorptance can be converted into absorption coefficient (a, m^{-1}) taking into account the path-length. The absorption coefficient is expressed as an exponential function of the optical density (OD) and the path length (l, m^{-1}) through the suspension. Rearranging the above equations, the absorption coefficient of phytoplankton, $a(\lambda)$, can be calculated directly from OD as:

$a\ (\lambda) = 2.303\ OD\ l^{-1}\ (m^{-1})$ (3)

being 2.303 the transformation factor of decimal to natural logarithm (log_{10}/log_e).

when expressed per mass unit of Chl-a, the Chl-a specific absorption coefficient by phytoplankton, a (λ) is:

$a\ (\lambda) = a\ [Chl\text{-}a]^{-1}$ m^{-1} mg Chl-a^{-1} (4)

where Chl-a is the Chl-a concentration (mg m^{-3}). The Chl-a specific absorption coefficient varies from 0.004-0.043 m^2 mg Chl-a^{-1} (Arbones et al., 1996).

Absorption spectra measured for aquatic particles concentrated onto glass-fiber filters require a correction for the increase in path length caused by multiple scattering in the filter. Controversy of the multiple scattering corrections has been reported (Kishino et al., 1985). Kieffer & SooHoo (1982) refined the filter methods, minimizing loss of scattered light by placing the filter close to the detector and adding a correction for the increase in effective path length caused by scatter within glass-fiber filter. This path length amplification factor (β) is defined as the ratio between optical to geometrical path length (Kieffer & SooHoo, 1982). Cleveland & Weidemann (1993) in absence of wavelength dependent effect suggested a general quadratic relationship ($r^2 = 0.988$):

$OD_{susp}\ (\lambda) = 0.378\ *\ OD_{filter}\ (\lambda) + 0.523\ [OD_{filter}\ (\lambda)]^2$ (absorbance units)   (5)

Other similar equation for the amplification factor has been reported by using a great number of phytoplankton species and replicates (n = 15,600; $r^2 =$ 0.95) from natural waters but taking into account the hysteresis effect, i.e., for the same $OD_{filter}\ (\lambda)$, the β value varied with the wavelength (Arbones et al., 1996) as follows:

$OD_{susp}\ (\lambda) = 0.38\ *\ OD_{filter}\ (\lambda) + 0.42\ [OD_{filter}\ (\lambda)]^2$ (absorbance units)   (6)

$OD_{filter}\ (\lambda)$ only differs from $OD_{susp}\ (\lambda)$ in the path length amplification factor, β. $OD_{susp}\ (\lambda)$ is determined in a integrating sphere or 1 cm cuvette modified by opal glass technique (Kieffer & SooHoo, 1982) and OD_{filter} by filtration phytoplankton culture onto the filter.

We suggest to conduct the filter technique by using the filter placed directly against the photomultiplier tube of the spectrophotometer. Ideally, an integrating sphere should be used but this optical accessory is expensive and not always available in laboratories dedicated to microalgal production. The filter technique is extensively used in biological oceanography but not in microalgal studies with production purposes. Arbones et al. (1996) reported the determination of absorption coefficient in phytoplankton collected from the field as follows (Equation 6): cell suspension is filtered by using a vacuum water pump under low pressure on GF/F filter taking a variable volume according to the phytoplankton concentration. The filter is placed close to the photomultiplier of the spectrophotometer, attached to the hole of the photomultiplier tube. The same volume of prefiltered culture medium or seawater is filtered also through a GF/F filter and this filter is used as a blank.

Optical density of the filter is calculated as follows:

$OD_{ph}(\lambda) = [(OD_p(\lambda)\ -\ OD_p(750)] - [(OD_d(\lambda)\ OD_d(750)]$ (absorbance units)   (7)

being OD_{ph} the optical density due to phytoplankton cells, OD_p the optical density of the pigmented and no pigmented fraction of particulate matter and OD_d the optical density of detritus. The conversion filter to suspension used is indicated in equation 6.

Then, the conversion of OD to absorption coefficient is calculated as:

$a(\lambda) = 2.3\ *\ OD_{susp}\ (\lambda)\ /\ (V\ *\ S)$ (m^{-1}) (8)

being $a(\lambda)$ the spectral absorption coefficient of phytoplankton (m^{-1}), V the volume of sample filtered and S the area of the filter.

An alternative to the use of the filter technique in microalgal production due to its difficulty and costs could be the approach reported by Figueroa et al. (2009) to determine the absorptance in laminar

macroalgae. This method is based on the use of a light source (high halide pressure lamp) set perpendicularly to a cosine corrected PAR sensor. Absorptance (A) is determined as A = 1 - E_t/E_o, being E_t/E_o the transmittance (T). The transmitted irradiance by the phytoplankton suspension placed on a Petri dish situated on top of the PAR sensor is E_t and E_o is the incident irradiance without any sample. Transmittance of algal suspension was conducted using the necessary volume of culture in order to have a 2 mm layer of algal suspension placed in a Petri dish all covered in black adhesive band except the area of the teflon window of the PAR sensor. Reflectance (R) is not considered since it is generally less than 5-8%, except in calcareous algae (Salles *et al.,* 1996). This procedure has been used also to determine the absorptance of phytoplankton cultures *i.e.,* good linear relationship between absorption coefficient determined by glass-fiber filter technique and this simplified method in *Chlorella fusca* cultures (Jerez *et al.,* unpublished). A summary of the absorption and *in vivo* chlorophyll fluorescence terminology is indicated in Table 1.

Electron transport rate

The electron transport rate (ETR) is determined according to the following formula (see Kromkamp *et al.,* 2008):

ETR = $\Delta F/F'_m * AbQ * F_{II}$ (μmol electrons m^{-3} s^{-1}) (9)

The effective quantum yield, ($\Delta F/F'_m$, also noted as Φ_{PSII} or Y(II)) was calculated according to Schreiber *et al.* (1995b). $\Delta F/F'_m = (F'_m - F_t)/F'_m$, being F'_m the maximal fluorescence which is induced with a saturating white light pulse (400-800 ms, approx. 9,000 μmol m^{-2} s^{-1}); F_t is the current steady-state fluorescence in light adapted algae. AbQ is the absorbed quanta calculated as the product of the integration of the spectral absorption coefficient aph$_\lambda$ between 400 and 700 nm and spectral irradiance of the light source (E_λ). Instead of AbQ, absorbed irradiance can be expressed as $E_{PAR}*A_{PAR}$, where A is the absorptance in the PAR region of the spectra calculated as: A_{PAR} = 1 - T_{PAR} - R_{PAR} (Figueroa *et al.,* 2003) being T_{PAR} the transmittance and R_{PAR} the reflectance both in the PAR range. In this case the absorptance is expressed as relative units (dimensionless) and thus ETR is presented as μmol electrons m^{-2} s^{-1}. A_{PAR} can be also calculated in microalgae using the filter technique or following the simplified method explained above (Jerez *et al.,* unpublished).

F_{II} is the fraction of cellular Chl-*a* in PSII and it is associated to LHCII (dimensionless). Suggett *et al.* (2003) estimated chlorophyll-*a* specific light absorption

and the proportion of total light absorbed by PSII (*P*, dimensionless) using both biophysical (Fast Repetition Rate Fluorescence) and optical (chlorophyll-*a* specific light absorption coefficient, a*) techniques on cultures of phytoplankton from diverse taxa. On the other hand, Johnsen & Sakshaug (2007) determined *in vivo* absorption coefficients and PSII-scaled fluorescence excitation (fl-ex) spectra of high light (HL) and low light (LL) acclimated cultures of 33 phytoplankton species that belonged to 13 different pigment groups (PGs) and thus a simple approach is presented to quantify intracellular self-shading and evaluating the impact of photoacclimation on bio-optical characteristic of the different PGs examined. In addition, Johnsen & Sakshaug (2007) discussed on the parameters used in the calculation of oxygenic photosynthesis based on Pulse Amplitude Modulated (PAM) and Fast Repetition Rate (FRRF) fluorometers in contrast to Suggett *et al.* (2003) that discussed only on the application of FRRF. In both reports, the ratio between light available to PSII and total absorption, essential for the calculation of the oxygen release rate (using the PSII-scaled fluorescence spectrum as a proxy) was dependent on species and photoacclimation state. The study of Johnsen & Sakshaug (2007) presented a greater number of phytoplankton groups and light conditions in the cultures than that of Sakshaug & Johnsen (2005) *i.e.,* three subgroups of chromophytes exhibited 70-80%, 60-80%, and 50-60% chl-*a* in PSII-LHCII; two subgroups of chlorophytes, 70 or 80%; and cyanobacteria, only 12%. In contrast, the mean fraction for chromo- and chlorophytes of quanta absorbed by PSII was 73% in LL and 55% in HL acclimated cells; thus, the corresponding ratios 0.55 and 0.73 might be used as correction factors adjusting for quanta absorbed by PSII for PAM and FRRF measurements. In several phytoplankton species *P* values were unexpectedly lower in low compared to high grown light algae in contrast to the values of AbQ$_{PSII}$ reported by Johnsen & Sakshaug (2007) in low light grown algae. We have preference for the F_{II} values reported by Johnsen & Sakshaug (2007) due to their possible application both for PAM and FRR fluorometers and because of the inclusion of a greater number of phytoplankton species and photoacclimation and bio-optical state.

ETR *versus* irradiance curves can be fitted using different functions as hyperbolic tangent without photoinhibition as Jassby & Platt (1976) and Eilers & Peters (1988) or with photoinhibition (Platt & Gallegos, 1980) and fitting-in-line model (Ritchie, 2008) to obtain ETR parameters as maximal ETR (ETR$_{max}$), irradiance for the initial saturation of ETR

Table 1. Symbols, definitions, units and general abbreviations

Symbol	Definition	Unit
General terms		
E	Irradiance	$\mu mol\ m^{-2}\ s^{-1}$
LHC	Light harvesting complex bonded to PSII or PSI (denoted as LHCII and LHCI, respectively)	
Bio-optical coefficients		
A	Absorptance, fraction of light absorbed by the algae	dimensionless
$a\lambda$	Spectral *in vivo* absorption coefficient by phytoplankton	m^{-1}
$a(\lambda)$	*In vivo* chl-*a*-specific absorption coefficient	$m^2\ mg\ chl\text{-}a^{-1}$
AbQ	Absorbed quanta calculated as the product of the integration of the spectral absorptance , $a(\lambda)$, between 400 and 700 nm and spectral irradiance of the light source (E_λ)	$\mu mol\ photons\ m^{-3}\ s^{-1}$
$OD\lambda$	Spectral optical density	relative units
V	Volume of filtered cell suspension	m^3
S	Area of the filter	m^2
F_{II}	Fraction of cellular chl-*a* in PSII associated to LHCII	dimensionless
K_d	Attenuation coefficient of downward radiation	m^{-1}
K_c	Specific absorption coefficient ($K_c=K_d/$ chl-*a*)	$m^2\ mg\ chl\text{-}a^{-1}$
In vivo chlorophyll fluorescence parameters		
F_m	Maximal fluorescence from fully reduced PSII reaction center	mv
F_m'	Maximal quantum yield of algae adapted to determined irradiance	mv
F_o	Intrinsic fluorescence from the antenna of fully oxidized PSII	mv
F_v	F_m-F_o	mv
F_t	Current steady-state fluorescence in light adapted algae	
F_v/F_m	Maximal quantum yield	dimensionless
$\Delta F/Fm`$	Effective quantum yield, $\Delta F = F_m$' - F_t	dimensionless
rETR	Relative Electron transport rate	$\mu mol\ e^-\ m^{-2}\ s^{-1}$
$aETR_v$	Absolute electron transport rate (as volumetric basis)	$\mu mol\ e^-\ m^{-3}\ s^{-1}$
$aETR_{chla}$	Absolute Electron transport rate (as chl-*a* basis)	$\mu mol\ e^-\ mg\ chl\text{-}a^{-1}\ s^{-1}$
$aETR_{max}$	Absolute Maximal ETR (as surface basis)	$\mu mol\ e^-\ m^{-2}\ s^{-1}$
α_{ETR}	Photosynthetic efficiency (slope of the ETR *versus* E curve)	units of ETR/units of E

(E_k) and the initial slope of the function ETR *versus* irradiance (α_{ETR}).

Use of different chlorophyll fluorometers

Available Pulse Amplitude Modulated (PAM) instruments present different spectral characteristics of measuring and actinic light, sensitivity and other technical characteristics (Table 2). The first fluorometers were developed with red light (LEDs, Light-Emitting Diodes) as measuring or actinic light, *i.e.*, PAM 2000 substituted by PAM 2500 and later fluorometers with broad band blue light (LEDs) were introduced *i.e.*, Mini PAM, Water PAM, Junior PAM and Monitoring PAM (Walz GmbH, Effeltrich, Germany) and Pocket PAM (Gademann Instruments Würzburg, Germany) or with narrow wavelengths as measuring and actinic light as Phyto PAM and

Multicolor PAM (Walz GmbH, Effeltrich, Germany) (Table 2). The AquapenC AP-C100 fluorometer (Photon System Instruments, Brno, Czech Republic) uses red and blue LEDs as actinic light and blue (455 nm) and orange (620 nm) as excitation light being suitable for cyanobacteria. The broad band blue light fluorometers are not recommended for red algae due to their low photosynthetic yield in blue light compared to that measured under green or red light spectra (Lüning & Dring, 1985; Büchel & Wilhelm, 1993; Schreiber *et al.*, 1995a). Several reports have presented data of photosynthetic yield in cyano-bacteria by using different excitation light wavelengths as orange (590 nm) for chlorophyll-*a* fluorescence induction (FI) kinetics using a FL-100 Fluorometer (Photon Systems Instruments, Czech Republic) (Kaňa *et al.*, 2009) or blue light avoiding

Table 2. Technical characteristics of PAM fluorometers used to determine *in vivo* chlorophyll fluorescence in algal suspensions including the type of measuring system, approximate limit of detection (mg Chl-*a* L^{-1}), measuring and actinic light color and configuration. All fluorometers are produced by Walz GmbH (Effletrich, Germany) except the Pocket PAM (Gademann Instruments, Würzburg, Germany). AquapenC AP-C100 is a fluorometer produced by Photon Systems Instruments (Brno, Czech Republic). LEDs: light emitted diodes.

PAM Fluorometer	Measurement of algal suspensions	Approximate limit of detection, μg Chl-*a* L^{-1}	Measuring light color	Actinic light color	Configuration
PAM-2500	Immersion of fiber optics in suspension or aliquot in cuvette	500	Red (LEDs)	Red and blue (LEDs)	Fiber optics, cuvette accessory for suspensions
DIVING-PAM	Immersion of fiber optics in suspension or aliquot in cuvette	3000	Blue or red (Halogen lamps with filters)	Blue or red (Halogen lamps with filters)	Fiber optics, cuvette accessory for suspensions
MINI-PAM	Immersion of fiber optics in suspension or aliquot in cuvette	3000	Blue or red (LEds)	Blue or red (LEDs)	Fiber optics, cuvette accessory for suspensions
WATER-PAM	Immersion of fiber optics in suspension or aliquot in cuvette	0.025	Blue or red (LEDs)	Blue or red (LEDs)	Cuvette or fiber optics version
JUNIOR-PAM	Immersion of fiber optics on suspensions	500	Blue or white (LEDs)	Blue or white, far red (LEDs)	Fiber optics
POCKET-PAM	Emitter-Detector Unit (ED) optimized to look through a glass window into an algae suspension cultivators	500	Blue (LEDs)	Blue (LEDs)	Optical head
MONITORING-PAM	Immersion of fiber optics in suspension or emitter detector unit on vessel surface	Data not available	Blue (LEds)	Blue (LEDs)	Leaf clip, fiber optics, or emitter detector units
PHYTO-PAM	Immersion of fiber optics in suspension or aliquot in cuvette	0.1	470, 520, 645 and 665 nm (LEDs)	665 nm (LEDs)	Cuvette or fiber optics version
MULTI-COLOR-PAM	Aliquot in standard 1 x 1 cm cuvette	100-300	400, 440, 480, 540, 590 and 625 nm. 365 nm replacing 400 nm on request (LEDs)	440, 480, 540, 590 and 625 nm. Also, far red and white (LEDs)	Cuvette, Perspex optics for accessory macroalgae
AQUAPEN-CAP-C100	Aliquot in standard 1 x 1 cm cuvette	0.5	Blue (455 nm) and orange (620 nm) LED	Blue and red (LEDs)	Cuvette

the excitation of phycobiliproteins (since the cellular phycobiliprotein content influences the *Fo* level fluorescence, particularly when phycobiliprotein levels are high). This leads in some cases to downward distortion of Fv/Fm, which is widely used as an index of PSII activity (Campbell *et al.*, 1998).

Recently, it has been developed a very versatile instrument, *i.e.*, the Multicolor PAM (Walz GmbH, Effeltrich, Germany) that offers a wide color range of measuring and actinic lights so measurements can be adjusted to very different phytoplankton groups, including cyanobacteria, according to bio-optical

characteristics including cyanobacteria. This fluoro-meter provides six colors of pulse-modulated measuring light (400, 440, 480, 540, 590 and 625 nm) and five colors of actinic light (440, 480, 540, 590, 625). In addition, it also has white (420-640 nm) and far-red (730 nm) light sources. On the other hand, the Phyto PAM allows discriminating between differently pigmented groups of algae (green algae, diatoms and cyanobacteria) by exciting the fluorescence alterna-tingly at high repetition rates by μsec pulses of 470, 520, 645 and 665 nm light originating from light emitting diodes (LED). Finally, the photosynthetic yield of the different phytoplankton groups mixed in the sample can be distinguished according to models of excitation and emission fluorescence spectra.

In this study, photosynthetic yield determined in different experimental sets by using two low cost:quality fluorometers as Junior PAM and Pocket PAM were compared to the data measured by the Water PAM in the same samples.

Pocket PAM to analyze the photosynthetic activity in *Chlorella* sp. growing in lecheate of urban sewage and the comparison with Water PAM (fiber configuration)

In vivo chlorophyll fluorescence is a good approach to evaluate the effect of toxic substances or effluents on photosynthetic activity or physiological status of phytoplankton (Shelly *et al.,* 2011). The measurement can be conducted directly in the experimental chamber by introducing the standard optical fiber of several PAMs, as Diving PAM or Mini PAM, but also by taking samples and measuring chlorophyll fluores-cence of cell suspension in cuvettes, as Water PAM (Büchel & Wilhelm, 1993; Figueroa *et al.*, 1997; Kromkamp *et al.*, 2008).

In order to avoid contact of the fiber with potential toxic substances or the transfer of samples to cuvettes with certain risks of contaminating the culture, an alternative is to measure chlorophyll fluorescence from outside the culture by placing the optical fiber directly in the wall of the experimental vessel. Recently, a new PAM instrument was released, Pocket-PAM (Gademann Instruments, Würzburg, Germany), provided with an Emitter-Detector Unit (ED) optimized to look through a glass window into an algae suspension as Erlenmeyer flasks or methacrylate cylinders (Fig. 1). The PAM sensor has two components: (1) the emitter, a blue light emitting diode (LED) and (2) the detector, a photodiode to sense the fluorescence signal and a preamplifier emitter and detector which cross 10 mm apart of the surface of the sensor.
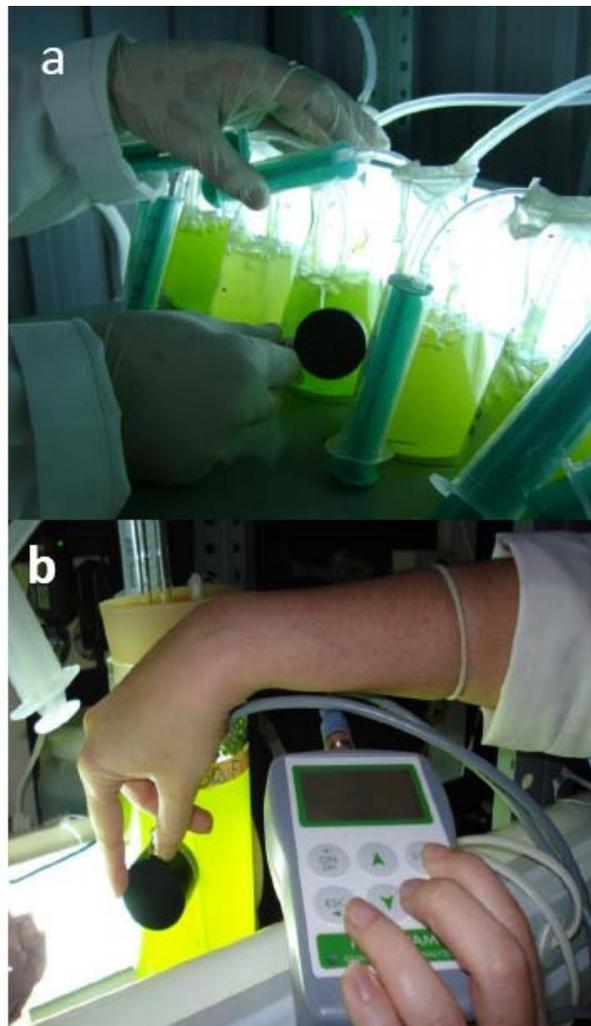

Figure 1. Details of Pocket-PAM (pulse amplitude modulated fluorometer). a) Measuring head, b) device unit. The measuring head is placed on the external part of cylindrical methacrylate vessels of *Chlorella fusca* cultures.

In order to test the usefulness of this new approach, *Chlorella fusca* was grown for nine days in 250 mL Erlenmeyer vessels in Basal Bold's Medium (BBM) and under 100, 50 and 10% lecheate from urban sewage diluted in distilled water and light transmission in the culture, cell number and ETR_{max} were determined. Results are shown in Figure 2 whereas the chemical composition of BBM medium and the lecheate are indicated in Table 3. Culture was bubble with air and irradiances of 200 μmol m^{-2} s^{-1} provided by three daylight white fluorescent lamps (Philips DL-36W) were used. E_{PAR} was measured inside the culture (in the central part of the Erlenmeyer vessel) by immersing a spherical quantum sensor (US-SQS/L, Walz GmbH).

Culture samples were taken once each day (3rd, 7th and 9th) in order to determine absorption by using the absorptance technique as explained previously, *i.e.*, absorptance determination of a cell suspension (2 mm layer) in the PAR range λ = 400-700 nm) using a cosine corrected PAR sensor.

Photon fluence rate (E_{PAR}) in the culture decreased (Fig. 2a) as a consequence of the increase in cell densities throughout the time (Fig. 2b). The E_{PAR} in the first two days of experiment was lower in 100% and 50% lecheate compared to 10% lecheate and BBM culture medium (Fig. 2a). This can be explained due to the absorption of light by dissolved substances present in the lecheate, what affects the turbidity of the medium. After three days, cell number was higher in lecheate treatments than in BBM. E_{PAR} diminished after 7 days culture in lecheate because of the increase in cell density (Fig. 2b), however, in BBM, E_{PAR} remained high after 7 days. This can be related to the low cell density and turbidity associated to BBM treatment. The lowest E_{PAR} is reached after 9 days in 100% lecheate culture that is also the treatment with the highest cell number (about 45 million cells mL^{-1}). The increase of the cell number throughout the time was higher under lecheate treatment than that in BBM (Fig. 2b). ETR presented similar pattern as cell number in the 7th and 9th days of culture (Figs. 2b, 2c) but not in the 3th day. ETR increased from the 3rd to the 7th day as well as the number of cells. However, ETR decreased in spite of the increase in cell number in day 9. A possible uncoupling between growth and photosynthesis, *i.e.*, increase in the division rate without any raise of photosynthetic activity per cell could explain this result. In order to evaluate the uncoupling between growth and photosynthetic rate, the expression of growth as biomass, *i.e.*, g DW per volume is suggested to be better than number of cells (Tredici, 2010). However, in this case it was not possible to determine g DW per volume as the sampling volume used in the experiment was too small. The positive effect of the lecheate of urban sewage on the growth of *Chlorella fusca* could be explained by the efficient use of the ammonium, at high concentration in lecheate medium (Table 2).

In order to assess the quality of the data obtained with the Pocket PAM fluorometer, *in vivo* chlorophyll fluorescence was also measured by another and more commonly used fluorometer, Water PAM (emitter-detector unit of fiber version, Water EDF). The fiber of the Water PAM (red light version) was located in the wall of the vessels, the same as for the Pocket PAM. The correlation index of the effective quantum yield by using Pocket PAM *versus* Water PAM was significant (r = 0.88, $P < 0.01$) being the values about 7.5% higher by using Water PAM compared to Pocket PAM. No significant differences ($P > 0.01$) were found in the effective quantum yield determined either by using the fiber version of Water PAM (unit EDF) located in the wall of the vessels or by measuring the cell suspension using a cuvette (Water ED measuring unit). Thus, possible artifacts by scattering or reabsorption of fluorescence seem not to be significant.

Junior PAM to monitor chlorophyll fluorescence in a thin-layer cascade (TLC)

Junior PAM is a small PAM fluorometer (Walz GmbH, Effeltrich, Germany) with a good price-performance ratio. Modulated fluorescence excitation is produced by blue LED (maximum wavelength emission: 465 nm) under two modulation frequencies (5 and 100 Hz). Actinic light is provided by the same power LED as for modulated light. Saturating pulses are produced by the same power LED as for modulated light being the maximum photon fluence rate 10,000 µmol m^{-2} s^{-1} at 1 mm distance from the tip of the guide. The light guide is made of plastic fiber and it has 40 cm x 1.5 mm (length x diameter).

In this study, Junior PAM is used online to estimate photosynthetic activity of algae growing outdoor in a thin-layer cascade (TLC). Open systems, such as TLC, present several advantages compared to closed systems such as their higher efficiency in light absorption and operating higher biomass densities (Masojídek *et al.*, 2011).

C. fusca was grown TLC cultivators with an exposure surface of 4 m^2 and S/V of 27 m^{-1} (Table 4). The TLC located in Málaga, Spain (36º43'N, 4º25'W) has three different parts: (1) a compartment with a surface of 0.43 m^2 and capacity for 20 L, where suspended cells are mixed after being injected from PVC tubes with several holes (Figs. 3a, 3b, 3f); (2) a thin-layer cascade on a plate of fiber glass of 4 m^2 where cell culture circulates through (Figs. 3c, 3g) and (3) a fiber glass tank with a surface of 0.72 m^2 and 145 L capacity into where cell suspension falls in cascade (Figs. 3c, 3e). The height of the layer in the thin compartment (2) can be changed by locating a fiber-glass rectangular piece at the end of the plate (before the cascade). Cells were pumped from the tank (compartment 3) through PVC tubes back again to compartment 1 (Fig. 3).

Algae were cultivated in 850 mg L^{-1} NaNO$_3$, 120 mg L^{-1} MgSO$_4$ and 540 mg L^{-1} KH$_2$PO$_4$. Micro-nutrients were added (60 µL L^{-1}) using a commercial mixture (Welgro Hydroponic, Comercial Química Massó, Spain).
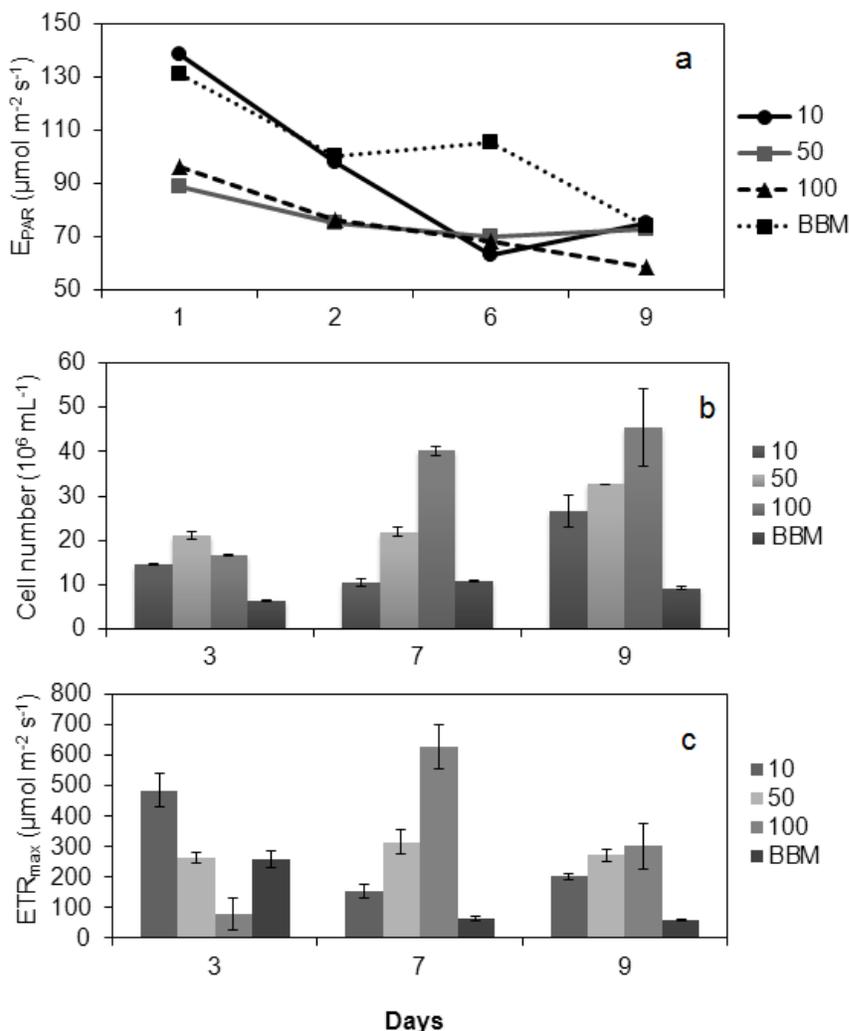
Figure 2. Photon fluence rate (E_{PAR}) expressed as a) μmol photons m^{-2} s^{-1}, b) cell number as 10^6 cells mL^{-1}, c) and maximal electron transport rate (ETR_{max}) expressed as μmol electrons m^{-2} s^{-1} grown for 9 days in 250 mL Erlenmeyer vessels under Basal Bold's Medium (BBM) and under 100%, 50% and 10% of lecheate from urban sewage.

Algal biomass productivity expressed as g DW m^{-2} day^{-1} and photosynthetic activity as *in vivo* chlorophyll-*a* fluorescence of PSII electron transport rate (ETR) *i.e.*, estimator of production were determined in *C. fusca* growing outdoor in the above described TLC. The production is expressed per unit area (m^2) instead of using volume units (m^3) as the TLC is provided with a fine culture film flowing on a plate that provides a high exposure surface. Effective quantum yield was measured every two minutes by introducing the light guide of Junior PAM into the compartment 1 (Figs. 3a, 3b). In addition, photon fluence rate of PAR was determined by using a spherical quantum sensor (US-SQS/L, Walz GmbH) also introduced in compartment 1. Relative ETR (rETR) was calculated as:

$$rETR = \Delta F/F_m' * E \quad (\text{μmol electron m}^{-2}\,\text{s}^{-1}) \quad (10)$$

In this case, absorptance was not estimated and thus it is not possible to determine the photosynthetic rate. rETR shows only a relative capacity of production in the culture as the actual production cannot be estimated since photosynthetic activity is not related to incident irradiance but to absorbed irradiance.

The principle of *in situ* modulated fluorescence measurement in turbulent microalgal cultures is based on the assumption that signal modulation is two-orders of magnitude faster than that of the suspension movement in the cultivation unit (Masojídek *et al.*, 2011), *i.e.*, 600-800 ms of saturating pulses as applied in this study are faster than that of suspension
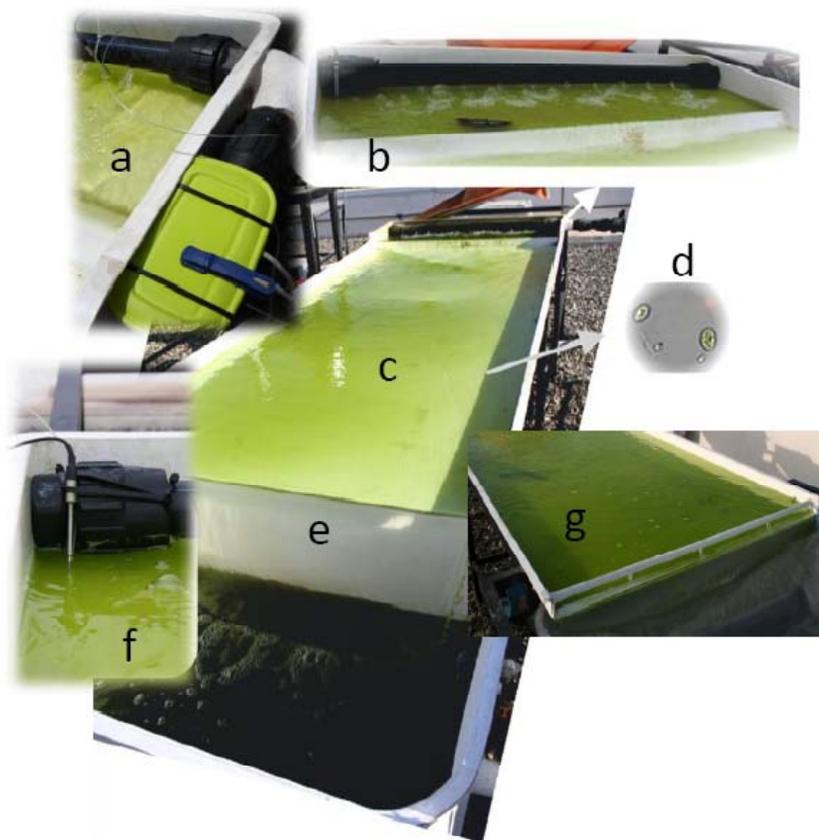
Figure 3. Thin layer cascade (TLC) cultivator located in Málaga, Spain (36º43'N, 4º25'W). The TLC cultivator has three different parts: Compartment-1 with a surface of 0.43 m^2 and capacity for 20 L, in which suspended cells are mixed after being injected from PVC tubes with several holes a, b) Compartment-2, a plate of fiber glass of 4 m^2 surface where the cell culture, c) of *Chlorella fusca*, d) circulates and the compartment-3, a fiber glass tank with a surface of 0.72 m^2 and 145 L of volume where the cell suspension falls in cascade, e) online measurements of photon fluence rate by using spherical quantum sensor (US-SQS/L, Walz GmbH), f) and effective quantum yield by using the fluorometer Junior PAM (Walz GmbH) with the fiber placed in the compartment-1 (A) were conducted, g) the height of the layer in the compartment-2 can be changed by the use of a fiber-glass rectangular piece at the end of the plate (before the cascade).

movement. To integrate the bio-optical properties of the algae in the culture media, the specific attenuation coefficient (K_c, m^2 mg^{-1} Chl-*a*) has been considered since it takes into account all variables affecting light absorption as cell size, chlorophyll concentration and algal density (Figueroa *et al.*, 1997).

Water PAM measurements (emitter-detector unit of fiber version, Water EDF) were also carried out in order to assess the quality of the data obtained with the Junior PAM fluorometer. The fiber of the Water PAM (red light version) was located in the compartment 1 of the TLC the same as the fiber of Junior PAM and simultaneously measurements of effective quantum yield were conducted at six different periods in the day from the morning to the afternoon (10 min measurements in each period). The

correlation index of the effective quantum yield by using Pocket PAM *versus* Water PAM was significant (r = 0.85, *P* < 0.01) being values about 12.5% higher by using Water PAM compared to Junior PAM.

Data of effective quantum yields, photon fluence rates (E_{PAR}) and rETR in two late afternoon periods, 16th and 18th February 2011, are presented in Figure 4. rETR ranged from 20 to 40 µmol m^{-2} s^{-1} on 16th February 2011 (Figs. 4a, 4c). On 18 February 2011, photon fluence rates decreased, showing higher variations than that observed on 16 February as a consequence of intermittent clouds (Fig. 4d). $\Delta F/F_m$' presented a reverse pattern compared to E, *i.e.*, increase of $\Delta F/F_m$' under decreasing irradiances (Fig. 4e). rETR ranged from 40-80 µmol m^{-2} s^{-1} in the first period of 18 February 2011 (Fig. 4f) in which there
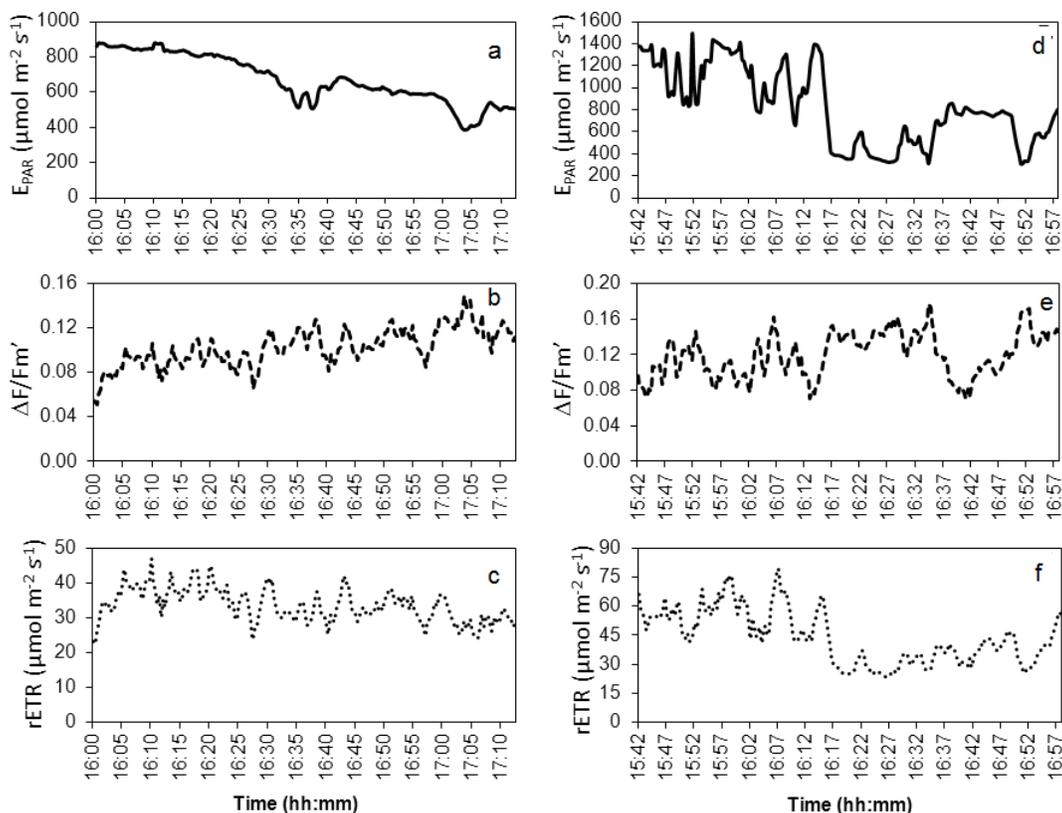
Figure 4. (a, d) Photon fluence rates (E_{PAR}) in µmol photons m^{-2} s^{-1}, effective quantum yield ($\Delta F/Fm`$) and relative electron transport rate (rETR) (b, d) in µmol electrons m^{-2} s^{-1} of *Chlorella fusca* cultures in thin-layer cascade cultivators as described in Fig. 3 in two evening periods, 16th (a, b, c) and 18th (d, e, f) February 2012.

are high variations in E (Fig. 4d). On the other hand, in the second period of time, E presented lower variations and rETR oscillated from 20-50 µmol m^{-2} s^{-1}.

In order to obtain absolute ETR (aETR) values, *i.e.*, including absorptance in the calculation (equation 9), an experiment with *C. fusca* grown in TLC with different layer heights of 2 or 1 cm was conducted (Fig. 5). In the first period, cells were grown in 2 cm layer culture on the exposure area until it reached the stationary phase. Measurements were carried out in this phase during two days. After this time, the culture was diluted to the density necessary to be in exponential phase again. After that, a layer height of 1 cm was applied to the culture for another two days (Fig. 5). The whole experiment was conducted from 1 to 15 July 2011. Irradiance and effective quantum yield were measured online. Results are shown in Figure 4 and they were described above. In addition, culture samples were taken throughout the day in order to determine absorption by using the absorptance technique as it was explained previously.

aETR was higher using a culture layer of 2 cm than that using 1 cm (Fig. 5b). These differences cannot be explained by different cell densities among the cultures since the average cell density in 2 cm culture was similar to that in 1 cm layer culture, *i.e.*, 0.528 and 0.522 g L^{-1} respectively. However, in 1 cm layer culture, cell weight per number of cells or per culture volume was higher than that in 2 cm culture layer (Table 5). This can be related to the higher level of pigments and proteins per cell found in 1 cm layer culture than that found in 2 cm one (data not shown). The decrease of daily integrated irradiance in the culture during the 8 and 9 days of experiment is related to the bio-optical characteristics in 1 cm culture, *i.e.*, increase of cell weight per cell number or culture volume and increase of specific attenuation coefficient (K_c) compared to 2 cm layer culture (Table 5). In this study, K_c and cell weight per cell and volume were negatively related to photosynthetic production (ETR). This result suggests photaccli-mation of the cell culture. Increase in photoinhibition (decrease of ETR_{max}) with increasing K_c was
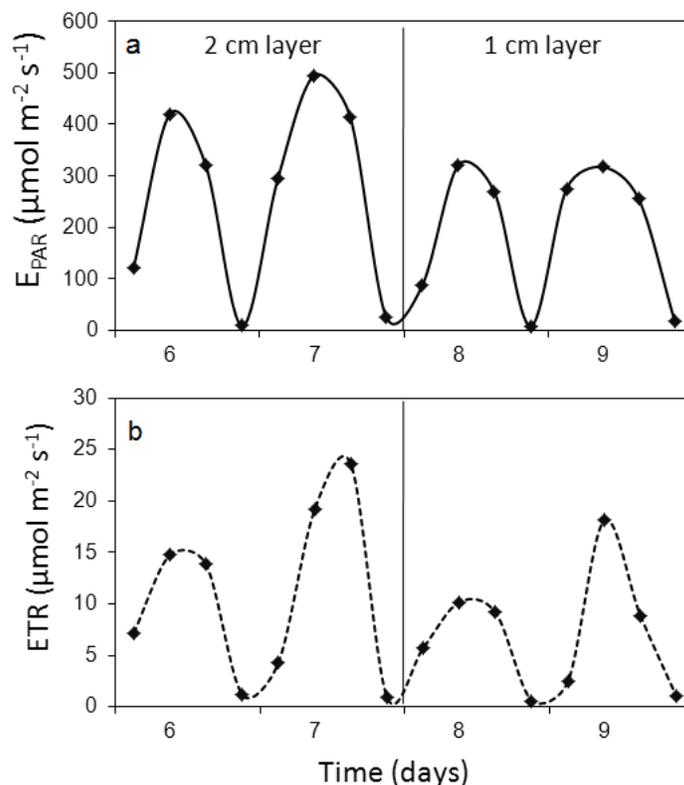
Figure 5. a) Photon fluence rates (E_{PAR}) expressed in μmol photons m^{-2} s^{-1} and b) electron transport rate (ETR) in μmol electrons m^{-2} s^{-1} of *Chlorella fusca* cultures in thin-layer cultivators as described in Fig. 3 during periods of two days: 6-7th July, 2011 in 2 cm, on 8-9th July, 2011 in 1 cm culture layers.

previously reported by Figueroa *et al.* (1997) in different phytoplankton species, *i.e.*, different morphology, size and pigment density. Figueroa *et al.* (1997) suggested that K_c is a good bio-optical indicator since it integrates the effect of cell size and pigmentation, as it has been observed in this study.

Photosynthetic efficiency (%) calculated from measured biomass productivity (mBP) as mol Carbon per mol of photons absorbed was higher using a layer height of 2 cm than that using only 1 cm (Table 5). Thus, the increase in light absorption in 1 cm culture and increase of K_c are related to the acclimation and reduction of algal productivity, *i.e.*, decrease in the photosynthetic capacity. The maximum value of 12.5% is 1/8, hence assumes the maximum efficiency predicted by the Z-scheme. The NADP:ATP ratio is then sufficient to run the Calvin cycle, but more ATP is needed for anabolic reaction, requiring some extra PSI activity, reducing the photosynthetic activity (Wilhelm & Jakob, 2011). Although theoretically 12.5% of photosynthetic efficiency is possible, the maximal photosynthetic efficiency under optimal conditions in the field is 8-9% (Grobbelaar, 2009).

According to Tredici (2010), maximal efficiency of photosynthesis in microalgae is 12.4%, but taking into account photorespiration, respiration, photosaturation and photoinhibition, the efficiency decrease to 5.4%. This maximal value can be reached in short-term experiment under low irradiance but it can be maintained during long-term period under other stress condition as increased temperature (Tredici, 2010). In our study the efficiency was lower than 5.4 *i.e.*, it ranged from 1.6 to 2.1%.

The measured biomass productivity in the 2 cm layer culture (*C. fusca*) presented in this study was lower than that of *Chlorella* sp. growing in thin-layer cascade systems in other studies (Table 4). This result can be explained because the exposed area of the thin-layer cascade used in the present study is much smaller (only 4 m^2) than that used by Masojídek *et al.* (2011). Biomass productivity increased with higher S/V, *i.e.*, an increase of 23% in the S/V ratio produced an increase of 30% in the biomass productivity according to Table 4 (Masojídek *et al.*, 2011). However, in spite of the smaller S/V ratio of the cultivator used in this study (a reduction of 483.6%

compared to the 133 m^{-1} S/V ratio cultivator used by Masojídek *et al.* (2011), only 36.9% of reduction in the biomass productivity was observed (Table 4). The huge differences among the physical variables measured in both sites could explain this result. The daily integrated irradiance in Málaga in the algal culture (at 0.3 cm depth) in July was 9704-11685 kJ m^{-2} and 6094-7306 kJ m^{-2} in the algal culture (0.3 cm depth) of Třeboň (Southern Czech Republic) in summer time (Masojídek *et al.*, 2011) (Table 4). In addition, the range of temperature inside the water was 20-34°C (Table 4), higher that that reported by Masojídek *et al.* (2011). A more productive strain, in the case of this study, under high irradiance and temperature (Table 3) compared to the *Chlorella* sp. used by Masojídek *et al.* (2011) is not excluded.

Estimation of carbon production by using chlorophyll fluorescence

ETR can be converted to oxygen evolution rates assuming that four (the theoretical minimum), or five electrons (the measured minimum), equivalent to 10 absorbed photons by both PSII and PSI (Ley & Mauzerall, 1982) are needed to produce one oxygen molecule. ΦO_2 is the electron yield for oxygen (the number of charge separations in both PSII and PSI needed to produce an oxygen molecule). Although the theoretical value of ΦO_2 is 0.2-0.25, most of the authors report 0.1 as a more reasonable value, *i.e.*, there are 10 absorbed photons needed to produce one oxygen molecule (Williams & Robertson, 1991; Kromkamp *et al.*, 2008). On the other hand, the oxygen production can be converted into carbon

Table 3. Chemical composition of Basal Bold's Medium (BBM) and lecheate of urban sewages.

Chemical characteristics	BBM	Lecheate
Ammonium (mg L^{-1})	-	98
Nitrate (mg L^{-1})	547.12	12.4
Nitrite (mg L^{-1})	-	-
Phosphate (mg l^{-1})	153.23	303.4
Sulfates (mg L^{-1})	29.23	133.8
Chloride (mg L^{-1})	15.17	402.7
Fluorides (mg L^{-1})	-	-
Sodium (mg L^{-1})	212.62	292
Potasium (mg L^{-1})	75.96	91.8
Calcium (mg L^{-1})	353.60	1.3
Magnesium (mg L^{-1})	7.39	0.02
Manganese (mg L^{-1})	-	-
Conductivity (µS cm^{-1})	-	1.5
pH	7.2	6.3
DBO$_5$ (mg L^{-1})	-	>500

assimilation taking into account the values of the photosynthetic quotient (PQ) as reported by Kromkamp & Forster (2003). Thus, C assimilation can be estimated from ETR as follows:

Carbon assimilation rate = $F_{PSII}*AbQ*F_{II}*FO_2*PQ^{-1}$
(µmol C m^{-2} s^{-1})   (11)

The unit of carbon assimilation rate is expressed in terms of surface (m^2) instead of volume (m^3) since the productivity in thin layer cascade is better expressed in terms of area than that in volume. In this study, the considered value of ΦO_2 was 0.1 (*i.e.*, there are 10 absorbed photons needed to produce one oxygen molecule). The PQ value used was 1.4 since nitrate was the main nitrogen source in the culture (Williams & Robertson, 1991). PQ should be 1.1 mol O_2 / mol CO_2 in medium with ammonium as main inorganic nitrogen source since it is necessary a lower number of electrons to assimilate it than that under nitrate supply (Williams & Robertson, 1991).

The photosynthetic carbon production (P$_c$, µmol C m^{-2} day^{-1}) of *C. fusca* cultures in TLC cultivators (Fig. 3) was converted into biomass productivity expressed as g DW m^{-2} day^{-1} using the total internal carbon content after its determination (n = 3) by combustion in an elemental analyzer (CNHS LECO-932, Michigan, USA). The average internal content of C in *C. fusca* was 40.5 ± 3.8 mg per g DW of biomass. The estimated biomass productivity (eBP) was higher in 2 cm layer culture than that under 1 cm (Table 5) as occurred with the measured biomass productivity (mBP). eBP was about 8% lower in 2 cm layer culture than the mBP whereas in 1 cm layer culture, eBP was 25.8% higher than the mBP. Thus, in 1 cm layer culture, the difference between measured and estimated values was much higher than that in 2 cm layer culture .The reason of this discrepancy cannot be explained but it could be related to the bio-optical variation in 1 cm layer culture, *i.e.*, the increase of cell weight per number of cells and volume. Interestingly, K$_c$ is higher in 1 cm layer culture and it has been previously shown that an increase of K$_c$ in phytoplankton (different species, chlorophyll content and cells sizes) was related to an increase in photoinhibition (Figueroa *et al.*, 1997). The discrepancy between measured and estimated algal productivity can be explained by assuming that the electron requirement for O_2 production/C fixation is overestimated and they differ between 1 and 2 cm culture layer. Using the values of ΦO_2 and PQ reported by Williams & Robertson (1991) and Kromkamp *et al.* (2008), an overestimation of the measured rate of C-fixation was found. However, Kromkamp *et al.* (2008) reported that the degree of

Table 4. Comparison of the thin-layer cascade (TLC) cultivator situated in University of Málaga (UMA) (Málaga, Spain, 36º43'13"N, 04º25'13"W) and in the Institute of Microbiology (IM) (Treboň, Czech Republic, 49º0'13"N, 14º46'14"E) (Masojídek *et al.,* 2010), respectively. Exposure surface (S, m^{-2}), volume of the medium (V, m^{-3}), S/V ratio (m^{-1}), maximal measured biomass productivity (mBP, g DW m^{-2} d^{-1}) of *Chlorella fusca* (UMA) and *Chlorella* spp. (IM), daily integrated irradiance of PAR (DII, kJ m^{-2}) and temperature interval during the day (light period) (T, ºC) during experiments conducted in July 2011 in the case of UMA and in June-July in IM (year not specified) are indicated.

TLC cultivator	S	V	S/V	mBP	DII	T	Reference
UMA	4	145	27.5	11.8 ± 2.9	9704-11685	20-34	This study
IM-1	24	180	133.0	18.7 ± 5.1	6094-7306	16-26	Masojídek *et al*. (2010)
IM-2	224	2196	102.0	14.3 ± 4.1	6094-7306	16-26	Masojídek *et al*. (2010)

Table 5. Bio-optical and biological variables of cell cultures of *Chlorella fusca* in a thin-layer cultivator of 4 m^2 of exposure surface and surface:volume of 27.5 m^{-1}. The initial cell density was 1.24 million cells mL^{-1}. Effective quantum yield and photon fluence rate of PAR were measured for 2 days in 2 cm water layer when the stationary phase was reached, the culture was followed for another 2 days but the layer height of the culture in the planar plate of the cultivator was 1 cm. Cell weight/number cells (mg DW10^6 cells^{-1}), cell weight/culture volume (mg DW L^{-1}),electron transport rate (ETR, μmol e$^-$ m^{-2} s^{-1}), average measured (mBP) and average estimated (eBP) biomass productivity expressed as g DW m^{-2} day^{-1}, photosynthetic efficiency as mBP per mol photons absorbed (%) and specific absorption coefficient (Kc, m^2 mg Chl-a^{-1}) are presented. The experiments were conducted in July, 2011 (n = 6).

Variables	2 cm layer	1 cm layer
Average electron transport rate (ETR)	36.95 ± 6.35	26.66 ± 4.55
Average measured biomass productivity (mBP)	5.62 ± 0.4	3.73 ± 0.35
Average estimated biomass productivity (eBP)	5.17 ± 0.53	4.98 ± 0.30
Average cell weight/number of cells	0.033	0.044
Average cell weight/culture volume	0.049	0.067
Specific attenuation coefficient (K_c)	0.0044 ± 5.4 10^{-4}	0.0027 ± 3.2 10^{-4}
Photosynthetic efficiency (%)	2.11 ± 0.35	1.63 ± 0.18

overestimation is rather constant, despite considerable changes in phytoplankton composition. Spectral correction of fluorescence data caused a reduction of 30% or an increase of 44% in the estimated C-fixation, depending how C-fixation was estimated (Kromkamp *et al*., 2001). In this study with *C. fusca,* less deviation among estimated and measured biomass yield than that between carbon fixation and ETR reported by Kromkamp *et al*. (2001) in other species was found.

Linear relation between estimated and measured biomass productivity has been determined in *Chlorella* sp. grown in TLC cultivators in different seasonal periods (Jerez & Figueroa, unpublished data). No good correlation was found in the culture in which 1 cm layer was applied (cells with higher cell weight per number of cells compared to 2 cm layer culture). This indicate that the use of ETR as estimator of biomass yield cannot be generalized since it does not take into account the variability of alternative electron cycling and the variability of the reduction degree of the biomass. The higher cell weight per number of cells or culture volume in 1 cm compared to 2 cm

layer culture (Table 5) could be related with the accumulation of lipids or proteins and it is not adequate to assume a constant photosynthetic quotient.

As final conclusion, chlorophyll fluorescence can be considered as a good tool to control microalgal cultivation by using online monitoring of photo-chemical activity to optimize the cultivation regime, *i.e.*, biomass density, turbulence, nutrient availability or CO$_2$ supply. PAM devices with low cost/quality ratio can contribute to a broader use of *in vivo* chlorophyll fluorescence in mass microalgal culture to monitor and control the physiological status of cultured organisms. More investigations are necessary for the estimation of biomass yield by using ETR data taking into account the contribution of cyclic and non cyclic electron transport and the bio-optical and biochemical characteristics of different species under different culture and environmental conditions.

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