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Relation between light absorption measured by the quantitative filter technique and attenuation of *Chlorella fusca* cultures of different cell densities: application to estimate the absolute electron transport rate (ETR)

Celia G. Jerez[1] · Carolina B. García[2,4] · Agustín Rearte[3,4] · Félix L. Figueroa[1]

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Abstract In order to estimate microalgal carbon assimilation or production of *Chlorella fusca* cultures based on electron transport rate (ETR) as in vivo chlorophyll *a* fluorescence, it is necessary to determine the photosynthetic yield and the absorbed quanta by measuring the incident irradiance and the fraction of absorbed light, i.e., absorptance or absorption coefficient in the photosynthetic active radiation (PAR) region of the spectra. Due to difficulties associated with the determination of light absorption, ETR is commonly expressed as relative units (rETR) although this is not a good estimator of the photosynthetic production since photobiological responses depend on the absorbed light. The quantitative filter technique (QFT) is commonly used to measure the absorbed quanta of cells retained on a filter (AbQ_f) as estimator of the absorbed quanta of cell suspensions (AbQ_s) determined by using integrating spheres. In this study, light attenuation of thin-layer cell suspensions is determined by using a measuring system designed to reduce the scattering. The light attenuation is related to the absorptance as the fraction of absorbed light by both indoor and outdoor *C. fusca* cultures of different cell densities. A linear relation between AbQ_f and AbQ_s (R^2= 0.9902, $p<0.01$) was observed, $AbQ_f = 1.98 \times AbQ_s$, being 1.98 an amplification factor to convert AbQ_s values into AbQ_f ones. On the other hand, depending on the culture system, the convenience of the use of the absorptance, light absorption or specific light absorption coefficient expressed per area (thin-layer cascade or flat panel cultivators), volume (cylindrical and tubular photobioreactors), or chlorophyll units (any type of cultivation system) is discussed. The procedure for the measurement of light absorption presented in this study for *C. fusca* could be applied in other phytoplankton groups. The absorbed quanta as determined in this study can be used to express absolute ETR instead of relative ETR, since the first one provides much more relevant photobiological information of microalgae culture systems.

Keywords Absorptance · *Chlorella fusca* · Chlorophyceae · Electron transport rate · In vivo absorption coefficient · Light attenuation coefficient

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✉ Félix L. Figueroa
felix_lopez@uma.es

[1] Departamento de Ecología, Universidad de Málaga, Facultad de Ciencias, Campus Universitario de Teatinos s/n, 29071 Málaga, Spain

[2] Laboratory a-Oil, Institutes MultiDisciplinary, University National of Cuyo, 5501 Mendoza, Argentina

[3] Laboratory of Analytical Chemistry, Department of Natural Resources and Environment, Faculty of Agronomy, University of Buenos Aires, C1417DSE Buenos Aires, Argentina

[4] CONICET, National Scientific and Technical Research Council, CABA, Buenos Aires, Argentina

Introduction

Microalgal biomass production has become an issue of great interest due to its big potential for many different uses as nutraceutical food, cosmetics and biofuels (Tredici 2010; Mayer et al. 2011). In order to enhance biomass and bioactive substance yields, it is necessary to increase photosynthetic efficiency and optimization of light absorption via adjustment of the light harvesting apparatus to achieve optimal balance of photosynthesis/photoprotection, rather than just maximizing light absorption (Grobbelaar 2009; Tredici 2010; Wilhelm and Jakob 2011).

Photosynthetic yield can be determined by using in vivo chlorophyll *a* fluorescence in microalgal cultures, i.e., effective or maximum quantum yields (Schreiber et al. 1995; Torzillo et al. 1996). Development of non-intrusive methodologies such as variable fluorescence techniques (pulse amplitude modulated fluorescence (PAM)) has led to rapid and sensitive measurements of changes in the physiological status of plants subjected to different environmental or culture systems (Schreiber et al. 1986) providing physiological information of microalgae under in situ growth conditions (Kromkamp et al. 2008; Masojidek et al. 2011). In order to estimate carbon assimilation or microalgal production, in addition to the photosynthetic yield, it is necessary to determine the absorbed quanta by measuring the incident irradiance and the fraction of absorbed light, i.e., absorptance or absorption coefficient (Ritchie and Runcie 2014). Thus, the electron transport rate (ETR), as estimator of photosynthetic capacity, can be determined as the product of photosynthetic yield, irradiance, absorptance, and the fraction of chlorophyll associated to photosystem II (Schreiber et al. 1986; Genty et al. 1989). The usefulness of the ETR as estimator of photosynthesis has been proved by its relation to gross photosynthesis (as oxygen evolution and carbon assimilation) (Flameling and Kromkamp 1998; Gilbert et al. 2000; Kromkamp et al. 2008; Suggett et al. 2009, 2011). However, a great number of authors did not determine light absorption, and therefore, ETR is only expressed as relative values (rETR). The use of rETR as estimator of the absolute photosynthetic capacity is not possible since the photobiological responses are not dependent of the incident irradiance but of the absorbed light (Suggett et al. 2004).

In biological oceanography, the estimation of light absorption of natural plankton and detrital matter is mainly by using the quantitative filter technique (Kishino et al. 1986; Arbones et al. 1996; Suggett et al. 2004), but it is not so frequent in cultured microalgae (Torzillo et al. 1998; Lippemeier et al. 2001; Obata et al. 2009; Masojídek et al. 2011). In mass algal cultivation, both closed and open systems, it is difficult to determine light absorption due to the high cell densities and heterogeneities in the cultures (Masojídek et al. 2011). Incident light is attenuated within the sample mainly due to absorption by the photosynthetic pigments enhanced by scattering that increases the path length of light within sample (Kirk 1994). The theory of radiation transfer in scattering suspensions of phytoplankton presents complex equations for wavelength-dependent extinction, and it is necessary to know the extinction and scattering coefficients and cell size distribution (Cleveland and Weidemann 1993; Fujiki and Taguchi 2002; Berberoglu et al. 2009).

The use of an integrating sphere for the determination inherent optical properties as absorption (a) and scattering (b) coefficient is the ideal approach, but it is not widely used among researchers due to the high cost of the equipment and because it is not always applicable in high-cell-density samples (Kirk 1994; Arbones et al. 1996). As an alternative to the integrating sphere method, the quantitative filter technique (QFT), which uses a glass fiber filter, both as an optical diffuser and to concentrate the particulate matter, is the most widely technique employed to measure light absorption in phytoplankton natural populations (Cleveland and Weidemann 1993; Arbones et al. 1996). In this method, the loss of scattered light is decreased by placing the filter close to the detector and by adding a correction factor (the so-called β factor) for the increase in the effective path length caused by scattering within the glass fiber. The absorption spectrum in natural samples concentrated on a filter needs correction for two aspects: (1) It is necessary to estimate absorption due to non-photosynthetic material, either by measuring the absorption of the matter collected on a filter once the pigments have been extracted with an organic solvent (Kishino et al. 1985), or by statistical estimation based on the relationship of optical densities of the particle absorption spectrum at wavelengths where the absorption is mainly due to non-algal material, i.e., blue wavelengths (Bricaud and Stramski 1990), and (2) the path length amplification effect or β factor evidenced by Butler (1962), which is defined as the ratio of the optical to geometrical path length which can be estimated as:

$$\beta = a_{filter}(\lambda) \Big/ a_{sus}(\lambda)$$

where $a_{filter}(\lambda)$ and $a_{sus}(\lambda)$ are the spectral absorption coefficient measured on filter and in suspension, respectively.

Another approach to determine the absorption cross section has been developed by using pulse amplitude modulated fluorescence. i.e., MulticolorPAM (Schreiber et al. 2012). Analysis of the fast fluorescence rise kinetics in saturating light allows determination of wavelength and sample specific functional absorption cross section of photosystem II, $\sigma_{II}(\lambda)$, with which the PSII turnover at given irradiance at a given incident photosynthetic active radiation (PAR) can be calculated (Schreiber et al. 2012). The study was conducted in low-cell-density cultures of *Chlorella vulgaris* (Chlorophyta) and *Synechocystis* PC683 (Cyanobacteria) at 200–300 mg Chl L^{-1}, where the light intensity gradient was small with good signal/noise ratios. Klughammer and Schreiber (2015) developed a new method to determine the absorption cross section also in optically dense samples. With increasing cell density, the apparent cross section $<\sigma>(\lambda)$ decreases when compared to σ_{II} (Schreiber et al. 2012), and when measuring light (ML) and actinic light (AL) are applied in the same direction, the decline of $<\sigma>(\lambda)/\sigma_{II}(\lambda)$ density is less steep than that of the theoretically $<PAR>(\lambda)/PAR(\lambda)$ (Schreiber et al. 2012). Thus, mean PAR in optically dense samples can be estimated via determination of $<\sigma>(\lambda)/\sigma_{II}(\lambda)$ (Klughammer and Schreiber 2015). Multicolor PAM in parallel with light microsensors has been also used to determine wavelength-specific effective absorption cross section of PSII photochemistry in corals (Szabó et al. 2014).

In this study, we present a method to estimate light absorption based on the measure of the absorptance and light

attenuation in algal suspensions under decreased scattering conditions. The absorptance of both indoor and outdoor *Chlorella fusca* cultures of different cell densities was measured. The validity of the approach is demonstrated by comparing these results with measurements of filtered samples (QFT) of the same cultures. Mercado et al. (1996) compared absorption of thin laminar macroalgae by using the integrating sphere and opal glass techniques (as analogous to the filter technique in phytoplankton), and they found very good relation between these two different techniques by determining the absorptance (A) of the thallus as defined by Kirk (1994). A similar approach was followed in this study in thin cell cultures by using a measuring system with quasi-collimated beam and two diffuser plates in order to have homogeneous light field with reduced scattering. According to different algal culture systems, applications of the expression of ETR in terms of area, volume, or chlorophyll by using the absorptance, light absorption, or specific light absorption coefficients, respectively, is discussed.

Material and methods

Culture conditions

Chlorella fusca BEA1005B (Shihira & Krauss, deposited in the Culture Collection of Marine Microalgae, ICMAN-CSIC, Cádiz, Spain and in the Spanish Bank of Algae, Gran Canaria, Spain) was grown in the laboratory in a culture chamber using Bold's basal medium modified with 3-fold nitrate content plus the addition of vitamins (3N-BBM-V) (Bischoff and Bold 1963; Andersen et al. 2005) at 25 °C and a photon fluence rate (PFR) of 150 μmol photons m^{-2} s^{-1} (12:12 h light/dark) until the culture reached the stationary phase (15×10^6 cells mL^{-1}). Photon fluence rate was determined by using a spherical quantum sensor of PAR (λ=400–700 nm) US-SQS (Walz GmbH, Germany) connected to a Licor radiometer model Li-250A (Licor Ltd., USA). At this point, part of the culture was kept in the laboratory and the rest was transferred to an outdoor thin-layer cascade (TLC) system. Details of the TLC system and culture conditions are given in Jerez et al. (2014). Experiments were carried out for 15 days in two periods: June and October 2011. We conducted the experiments in different seasons in order to get acclimation states to different light conditions: summer (June) and autumn (October). In addition, we used different cell densities in the thin-layer cascades to cover a wide range of absorption values associated to different levels of pigmentation and cell sizes, as we reported previously (Figueroa et al. 2013; Jerez et al. 2014). Triplicate samples were taken when the culture was 6–6.5 and 13–13.5 × 10^6 cells mL^{-1} three times a day (9:00, 15:00, and 19:00 h). The laboratory culture was diluted several times to obtain final concentrations of 10, 25, 50, and 100×10^4 cells mL^{-1} and 5, 10, 15×10^6 cells mL^{-1}, in order to have wide range of absorption or attenuation coefficients.

Pigment analysis and cell number

Pigment extraction was conducted by filtering triplicate samples (5 mL) of culture onto GF/F filters (Whatman) under low pressure (vacuum filtration). After the addition of 1 mL of dimethylformamide (DMF), filters were kept overnight (12 h) in dark conditions at 4 °C. The concentration of Chl*a* was determined by spectrophotometric measurements of the extracts at 647 and 664 nm using an UV-VIS spectrophotometer (UVmini-1240, Shimadzu, Japan) and calculated according to Wellburn (1994). Cell numbers were determined using a hemocytometer.

Absorption measurements

The absorption coefficient of phytoplankton on filter ($a_f(\lambda)$, m^{-1}) was determined by using the QFT according to the method and equation described by Arbones et al. (1996). This technique requires a single cell layer on the filter because the aggregation of cells provoked alterations in the apparent cross section related to the true absorption cross section as described by Klughammer and Schreiber (2015). To achieve this requirement, we previously checked the minimum volume necessary to get homogenous filtration, which was 5 mL. Secondly, we needed to know which cell density was necessary to achieve a single cell layer on the filter, considering that the filtration volume was 5 mL. We filtered 5 mL of samples of different cell densities, and afterwards, we observed by using a light microscope that filters had only one cell layer. The lowest cell density in which the filtration was homogeneous was 10^6 cells mL^{-1}. Therefore, samples were diluted or concentrated to this cell density (10^6 cells mL^{-1}), and 5 mL of the resulting dilution was filtered. In the case of lower cell densities, as 10^4–10^5 cells mL^{-1}, samples were concentrated to obtain 10^6 cells mL^{-1}, and at higher cell densities, up to 10^8 cells mL^{-1}, samples were diluted to get 10^6 cells mL^{-1}.

Optical densities (ODs) or absorbances were determined by using UV-VIS spectrophotometer (UVmini-1240, Shimadzu, Japan) with the baseline adjusted such that the value of OD_{750} was zero. The optical density of the phytoplankton in the filter ($OD_f(\lambda)$) was determined as the difference of $OD_p(\lambda)$ and $OD_d(\lambda)$, where $OD_p(\lambda)$ is the optical density of the particles, i.e., absorbance or attenuation (scattering + non-extractable-abs + extractable-abs), and $OD_d(\lambda)$ is the optical density after pigment extraction as the optical density of the non-pigmented matter of phytoplankton and detrital material, i.e., absorbance or attenuation of scattering + non-extractable-abs. All absorption spectra, both particulate and non-pigmented matter, were corrected for scattering

by subtracting the absorption at 750 nm from the entire spectrum. During all the process, the filter was kept moist.

$$OD_f(\lambda) = [(OD_p(\lambda) - OD_p(750)] - [(OD_d(\lambda) - OD_d(750)] \quad (1)$$

The absorption coefficient of the filter, $a_f(\lambda)$, expressed as m^{-1} was calculated as follows:

$$a_f(\lambda) = 2.303 \left[OD_f(\lambda) \Big/ (X^* \beta(\lambda)) \right] \quad (2)$$

with 2.303 being the conversion factor from $\log_{10}$ to ln, X the path length in meters, and $\beta(\lambda)$ the path length amplification factor (OD_f/OD_{sus}) where OD_f is the optical density of phytoplankton concentrated and fixed in filters and OD_{sus} is the optical density of cell suspension determined spectrophotometrically by using an integrating sphere.

Considering that $OD_p(\lambda) - OD_d(\lambda)$ is the optical density of cells in the filter, OD_f, and that β is OD_f/OD_{sus} (amplification factor) as defined above, the equation could be rearranged as

$$a_f(\lambda) = 2.303\, OD_f \Big/ \left[X \cdot \left(OD_f \Big/ OD_{sus} \right) \right] \quad (3)$$

where $OD_{sus} = 0.38\, OD_f + 0.42\, OD_f^2$ as reported by Arbones et al. (1996).

The Chl*a*-specific absorption coefficient of phytoplankton on filter ($a^*_f(\lambda)$, $m^2\ mg^{-1}$ Chl*a*) was calculated by dividing of $a_f(\lambda)$ by the [Chl*a*] expressed in mg m^{-3}.

The absorbed quanta of phytoplankton on filter (AbQ_f, μmol photons $m^{-3}\ s^{-1}$) were calculated as

$$AbQ_f = \int_{400}^{700} a_f(\lambda) E_0(\lambda) d\lambda \quad (4)$$

where $a_f(\lambda)$ is the absorption coefficient of phytoplankton on filter in m^{-1} and E_0 is the scalar irradiance in μmol photons $m^{-2}\ s^{-1}$.

The light attenuation coefficient in the algal suspension (k_d) was determined in a thin layer (3 mm) of cell suspension (2 mL volume) of the same cell densities as those used in the filter method. The measuring system (Fig. 1) consisted in a light source (150 W Halogen lamp) set perpendicularly to a cosine corrected PAR sensor (Licor 192SA) connected to a radiometer (Li-250A) both from Licor (USA). The irradiance used was 500 μmol photons $m^{-2}\ s^{-1}$. The light passed through a black cylindrical metal tube (20 mm diameter and 50 mm high) located 2 cm from the light source. A polyvinyl methacrylate diffuser plate (Plexiglass Weiss 0.17 of 3 mm thick) was placed between the black tube and the top of a plastic cuvette (polyethylene) of the same diameter containing 2 mL of microalgal suspension. The role of the black tube is to impose a restriction of the angular distribution of the light that hits the top of the diffuser, thus being restricted to a range of angles defined by the geometry. Therefore, the light that hit the top diffuser is expected to be quasi-collimated. The walls of the cuvette were covered with black foil excepting the area in contact with the PAR cosine corrected sensor. In order to decrease the reflected light and scattering (especially back scattering), in addition to the black color of the cuvette and the narrow area on the sensor, another diffuser plate was located at the base of the cuvette (Fig. 1). The light attenuation was determined taking into account only the light transmission and assuming a substantial reduction of scattering with the measuring system explained above. As control, previous measurements without the described black tube and diffuser plates produced a 5–8 % decrease of absorptance, probably due to increase of reflectance and scattering. This system mimics beam attenuation determination by using an orthogonal position of the light source whereas the diffuser plates, as in the opal glass technique, reduce the scattering (Mercado et al. 1996). The setup with two diffuser plates to achieve a more homogeneous light field and mimics an integrating sphere.

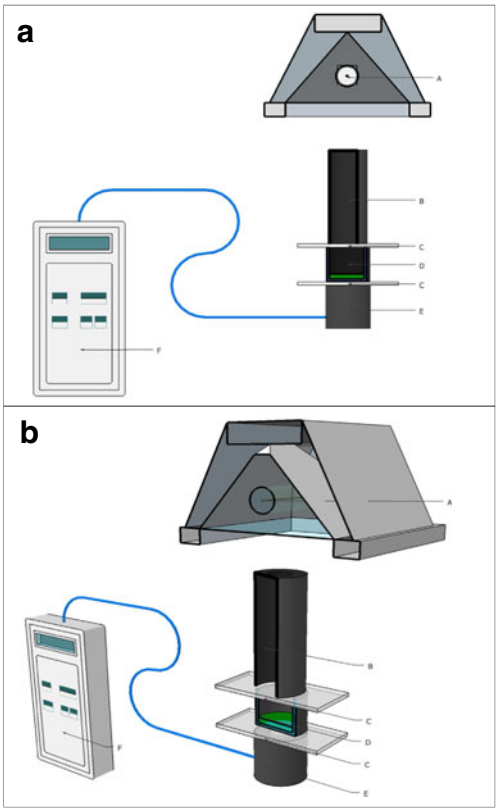

Fig. 1 Frontal (**a**) and isometric (**b**) view of the system used to measured light attenuation in cell suspension of *Chlorella fusca*. The different elements are marked by a capital letter: *A* halogen lamp, *B* black tube, *C* diffuser plate, *D* cuvette containing cell suspension, *E* cosine corrected PAR sensor, and *F* radiometer

The absorptance in the PAR range, (A_s(PAR), relative units (RUs)) was measured in the suspension according to the

approach reported by Mercado et al. (1996) and Figueroa et al. (2003, 2009) to determine the absorptance in laminar macroalgae but adapted to phytoplankton cultures. A_s(PAR) was determined as

$$A_S(PAR) = 1 - E_p \Big/ E_m \quad (5)$$

in which E_p/E_m is the transmittance (T), E_p is the transmitted irradiance by the phytoplankton suspension, and E_m is the transmitted irradiance by the culture medium. As explained previously, the designed measuring system considerably reduced (although not completely avoided) the scattering. Thus, the reflectance was considered negligible, and the error was assumed. A_s(PAR) could be related to the attenuation or extinction coefficient of phytoplankton in suspension (k_d, m^{-1}) taking into account the path length (m):

$$k_d = -\left[\ln(E_p) - \ln(E_m)\right] \Big/ D \quad (6)$$

By dividing k_d by the chlorophyll *a* concentration, the Chl*a*-specific absorption coefficient of phytoplankton in suspension (k_d*, m^2 mg^{-1} Chl*a*) was calculated.

The attenuated quanta of phytoplankton in suspension (AbQ_s, μmol photons m^{-3} s^{-1}) were calculated using k_d as follows:

$$AbQ_s = k_d \times E_{(PAR)} \quad (7)$$

where $E_{(PAR)}$ is the irradiance over the whole PAR range (μmol photons m^{-2} s^{-1}) and k_d is the attenuation coefficient of phytoplankton (m^{-1})

The terminology used in this study in relation to the absorption parameters is presented in Table 1.

Chlorophyll fluorescence measurements

In vivo chlorophyll *a* fluorescence parameters were determined by using Junior-PAM fluorometer (Walz GmbH, Germany), which employs blue light-emitting diodes as measuring and excitation light. As control, measurements at different cell densities were also conducted by using a Water-PAM fluorometer equipped with the red light version of the emitter-detector unit WATER-ED (Walz GmbH, Germany), and no differences were found (data not shown).

Samples were kept in darkness for 15 min to measure F_o (basal fluorescence in dark-adapted samples), and after that, a saturating flash (600 ms, ~9000 μmol photons m^{-2} s^{-1}) was applied in order to obtain the maximal fluorescence level (F_m). The maximal quantum yield of fluorescence (F_v/F_m) was determined according to Schreiber et al. (1986). The effective quantum yield ($\Delta F/F'_m$) was calculated as $\Delta F/F'_m = (F'_m - F_t)/F'_m$ (Schreiber et al. 1995), where F'_m is the maximal fluorescence and F_t the current steady-state fluorescence in light-adapted algae. Algae were exposed to 12 increasing E_{PAR} levels of actinic blue light for 20 s (25, 45, 66, 90, 125, 190, 285, 420, 625, 820, 1150, and 1500 μmol photons m^{-2} s^{-1}).

Prior to this study, different tests were carried out in order to choose the best protocol for rapid light curve measurements. Previously to this study, we compared different dark incubation periods (5, 10, 15, and 20 min) to decide the minimum time necessary to achieve relaxation and full oxidation of the photosynthetic apparatus. In addition, different incubation times under each actinic light intensity were also evaluated (10, 20, 30, and 120 s) to use the minimum time required to achieve the steady state. We did not observe any differences in the F_v/F_m of *C. fusca* after 15 and 20 min of dark incubation. On the other hand, steps of 10 s were not enough for the photosystem to reach the steady state, but no differences were found when the step duration was 20, 30, or 120 s. Consequently, the protocol for rapid light curve measurements included 15 min of dark incubation prior to the measurement and 20-s light steps.

In order to evaluate the suitability of the different methods explained above to estimate the absorbed light by *C. fusca* in the ETR determination, three of them (AbQ_f, AbQ_s, and A_s(PAR)) were used according to the equations presented in Table 2. It should be noted that there is one value of AbQ_f and AbQ_s for each light intensity provided by the Junior-PAM (n= 12) and that the ETR values calculated following the three equations presented in Table 2 can be, in turn, expressed per chlorophyll unit, i.e., specific ETR (ETR*).

ETR values were plotted against the incident irradiance and fitted using the model of Platt and Gallegos (1980) in order to calculate maximum ETR values (ETR_{max}).

Statistics

Pearson correlation coefficient (r) was determined to define the extent of a linear relation between the different forms of measuring and expressing light absorption by phytoplankton. A linear regression analysis was performed between those pairs of variables among which we wanted to find a linear predictive model. Statistical differences related to different cell densities and culture conditions (laboratory or outdoor) were tested by a two-way ANOVA. In the case of significant effects, the Student–Newman–Keuls post hoc test was applied ($p<0.05$) (Underwood 1997). The software Statistica for Windows (version 7.0, Statsoft, Inc., 1984–2004) was used for the analyses. ANOVA results are presented in the Supplementary material.

Table 1 Main symbols and units

Symbol	Definition	Formula	Units
E	Irradiance		μmol photons m^{-2} s^{-1}
E_0	Scalar irradiance		μmol photons m^{-2} s^{-1}
PAR	Photosynthetically available radiation (λ=400–700 nm)		μmol photons m^{-2} s^{-1}
OD(λ)	Optical density, wavelength dependent	$OD_{PAR} = -\log_{10}T = -\log_{10}(1-A_s(PAR))$	relative units (RU)
$a_f(\lambda)$	Spectral absorption coefficient of the pigmented fraction of phytoplankton on filter, wavelength dependent	$a_f(\lambda)=2.303\cdot[OD_p(\lambda)-OD_d(\lambda)]/X\cdot\beta(\lambda)$	m^{-1}
$\bar{a}_f(\lambda)$	Average wavelength absorption coefficient of the pigmented fraction of phytoplankton on filter	$\frac{\sum_{400-700} a_f(\lambda)}{n}$	m^{-1}
AbQ_f	Absorbed quanta by phytoplankton on filter, wavelength dependent	$AbQ_f = \int_{400}^{700} a_f(\lambda)E_0(\lambda)\,d\lambda$	μmol photons m^{-3} s^{-1}
$a^*_f(\lambda)$	Chl*a*-specific absorption coefficient of phytoplankton on filter, wavelength dependent	$a^*_f(\lambda)=a_f(\lambda)/[Chla]$	m^2 mg^{-1} Chl*a*
A_s(PAR)	Absorptance of phytoplankton in suspension (fraction of absorbed light)	$A_s(PAR)=1-E_p/E_m$	relative units (RU)
k_d	Attenuation coefficient of phytoplankton in suspension	$k_d=-[\ln(E_p)-\ln(E_m)]/D$	m^{-1}
k_d*	Chl*a*-specific attenuation coefficient of phytoplankton in suspension	$k^*_d=k_d/[Chla]$	m^2 mg^{-1} Chl*a*
AbQ_s	Absorbed quanta by phytoplankton in cell suspension	$AbQ_s=k_d\times E_0(PAR)$	μmol photons m^{-3} s^{-1}

Results

Absorption and light attenuation measurements

Both filter $\bar{a}_f(\lambda)$ and k_d of the suspension showed a linear relation with cell density with R^2=0.9881 and 0.9911, respectively (Fig. 2a). The AbQ_f and AbQ_s showed a linear relation with cell density at all irradiances supplied by the Junior-PAM. Only values of AbQ_f and AbQ_s corresponding to the highest irradiance used in this study (1500 μmol photons m^{-2} s^{-1}) and their linear relation to cell number (R^2=0.9863 and 0.9914, respectively) are shown in Fig. 2b.

All filter measurements that do not consider the irradiance showed a very good linear relation with AbQ_f (r=1.00, p<0.05) (Table 3). Suspension measurements presented a good positive correlation with filter ones regardless of the units in which they are expressed, i.e., RU, m^{-1} or μmol photons m^{-3} s^{-1} for A_s (PAR), k_d, and AbQ_s, respectively. Concerning values expressed per chlorophyll unit (m^2 mg^{-1} Chl*a*), both filter [$\bar{a}^*_f(\lambda)$] and suspension (k_d*) values were positively correlated although suspension ones were negatively correlated with filter measurements expressed both as μmol photons m^{-3} s^{-1} (AbQ_f) and m^{-1} [$\bar{a}_f(\lambda)$].

Optical density, absorption, and attenuation coefficient at different cell densities

Two samples were selected for each culture condition and cell density (n=12), i.e., laboratory and outdoor experiments in June and October (2011). One of the samples varied from 5 to 6.5×10^6 cells mL^{-1} (low density (LD), n=6) and the other ranged from 10 to 13.5×10^6 cells mL^{-1} (high density (HD), n=6). Optical densities of phytoplankton, pigmented, and non-pigmented fractions of LD and HD culture are shown in Fig. 3. The absorption of non-pigmented material was higher in shorter wavelengths (blue region of the spectra) than that in

Table 2 Equations used to calculate the electron transport rate (ETR) by measuring the absorbed light of cells immobilized on a filter or in suspension

ETR calculation	Units	Light absorption method
$ETR(1)=\Delta F/F'_m\times AbQ_f\times fAQ_{PSII}$	μmol electrons m^{-3} s^{-1}	Filter
$ETR(2)=\Delta F/F'_m\times AbQ_s\times fAQ_{PSII}$	μmol electrons m^{-3} s^{-1}	Suspension
$ETR(3)=\Delta F/F'_m\times E_{PAR}\times A_s(PAR)\times fAQ_{PSII}$	μmol electrons m^{-2} s^{-1}	Suspension

$\Delta F/F'_m$ effective quantum yield, AbQ_f absorbed quanta by the pigmented fraction of phytoplankton on filter (μmol photons m^{-3} s^{-1}), fAQ_{PSII} fraction of absorbed quanta to PSII (0.51, taken from Johnsen and Sakshaug 2007), E_{PAR} irradiance of PAR (μmol photons m^{-2} s^{-1}), $A_s(PAR)$ absorptance of phytoplankton in suspension (RU)

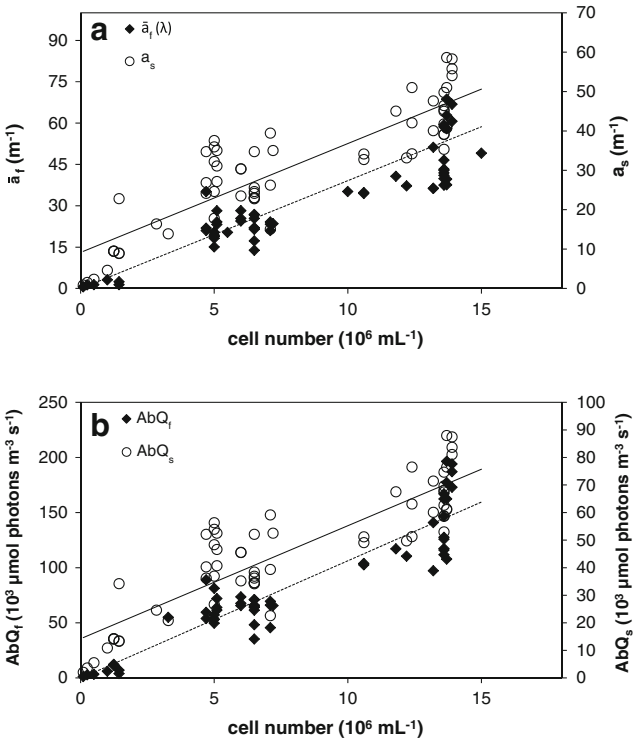

Fig. 2 Relationship between filter and suspension measurements of the absorbed light by *C. fusca* versus cell number (10^6 mL^{-1}). **a** Wavelength averaged absorption coefficient on filter [$\bar{a}_f(\lambda)$] and diffuse attenuation coefficient in suspension (k_d) both expressed in m^{-1}. **b** Absorbed quanta of *C. fusca* on filter (AbQ_f) and in suspension (AbQ_s) both as µmol photons m^{-3} s^{-1} taking into account only the highest irradiance (1500 µmol photons m^{-2} s^{-1}) provided by Junior-PAM as an example. The *line* shows the linear regression (n=60, p<0.01)

the red light region. It is noted that certain amount of chlorophyll (maximal in vivo optical density about 680 nm) remained in non-pigmented material after DMF extraction. This could be related to the thick cell wall of *C. fusca*, which made difficult the penetration of the solvent even when DMF is considered a very efficient extractant (Wellburn 1994).

Table 3 Pearson correlation for the different methods used to estimate the absorbed light by *Chlorella fusca* (p<0.05)

	Filter			Suspension			
	$\bar{a}_f(\lambda)$	AbQ_f	$\bar{a}^*_f(\lambda)$	A_s(PAR)	k_d	AbQ_s	k_d*
$\bar{a}_f(\lambda)$	1.00						
AbQ_f	1.00*	1.00					
$\bar{a}^*_f(\lambda)$	−0.68	−0.68	1.00				
A_s(PAR)	0.99*	0.98*	−0.70	1.00			
k_d	0.99*	0.99*	−0.71	1.00	1.00		
AbQ_s	0.99*	0.99*	−0.71	1.00	1.00	1.00	
k_d*	−0.98*	−0.88*	0.87*	−0.88	−0.88	−0.88	1.00

$\bar{a}_f(\lambda)$ wavelength averaged absorption coefficient on filter (m^{-1}), *AbQ_f* absorbed quanta by *C. fusca* on filter (µmol photons m^{-3} s^{-1}), $\bar{a}^*_f(\lambda)$ wavelength averaged Chl*a*-specific absorption coefficient on filter (m^2 mg^{-1} Chl*a*), *A_s(PAR)* absorptance (RU), k_d attenuation coefficient of phytoplankton in suspension (m^{-1}), *AbQ_s* absorbed quanta in suspension (µmol photons m^{-3} s^{-1}), k_d* Chl*a*-specific attenuation coefficient of phytoplankton in suspension (m^2 mg^{-1} Chl*a*)

*Indicate significant Pearson correlation values (p<0.05)

Chl*a* concentration (mg L^{-1}) was 2.30–2.93 or 1.76–2.5-fold higher in laboratory than that in outdoor systems, June and October, respectively (Table 4). Chl*a* content per cell was also higher in laboratory cultures (about 3-fold higher). However, Chl*a* content per cell was similar in HD and LD in both June and October outdoor cultures (Table 4). a^*_f values decreased with cell density in cultures in outdoor conditions. Concerning values measured in suspension, k_d*, the same pattern described for filter values was observed.

Relation between light absorption in filter and in suspension

A good linear relation between AbQ_f and AbQ_s (R^2=0.99, p<0.01) was obtained (Fig. 4). The function AbQ_f=1.98× AbQ_s can be used to convert AbQ_s values into AbQ_f ones. The values of 1.98 can be considered an amplification factor of cells concentrated in the filter compared to the cells in suspension. Sometimes, it is not possible to have the spectra of the excitation light emitted by the device (necessary to calculate AbQ_f), but it is possible to have the integrated irradiance. In this case, absorption coefficients given in m^{-1} can be used instead.

Chlorophyll fluorescence measurements

ETR values were calculated according to equations presented in Table 2 and then plotted versus the irradiance (Fig. 5). All curves showed a similar trend regardless of the method used to measure the absorbed light. The laboratory culture always had lower ETR values than the outdoor cultures. In the same culture conditions, HD cultures achieved higher ETR values than LD ones, both in laboratory and outdoor experiments. The highest ETR values were reached in October in HD cultures. ETR values were higher in October than that in June both in HD and LD cultures, although there is an exception at 1500 µmol photons m^{-2} s^{-1} in LD, i.e., similar ETR values in June and October (Fig. 5a, b, c). In June, in spite of the decrease of ETR values at irradiances higher than 800 µmol photons m^{-2} s^{-1} in HD cultures, at 1500 µmol photons m^{-2} s^{-1}, which gave values similar in HD and LD cultures (Fig. 5a, b). Concerning ETR calculations, values expressed per volume unit, ETR(1) and ETR(2), were higher than those expressed per area unit, ETR(3). Using AbQ_f, [ETR(1)] instead of AbQ_s [ETR(2)] resulted in higher values: Maximum values for outdoor cultures in October were around 12–14×10^3 µmol e^- m^{-3} s^{-1} for ETR(1) whereas they ranged from 4 to 5×10^3 µmol e^- m^{-3} s^{-1} for ETR(2). If we want to express ETR values per area unit, A_s(PAR) should be used

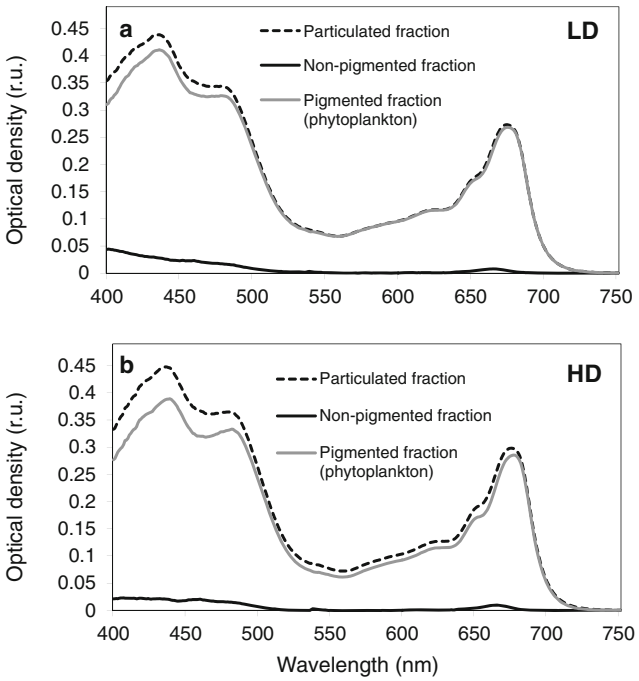

Fig. 3 Optical densities (ODs) or absorbances determined using UV-VIS spectrophotometer with the baseline adjusted such that the value of OD_{750} was zero. Measurements of the optical density of phytoplankton on filter, $OD_f(\lambda)$ (*continuous gray lines*), $OD_p(\lambda)$, optical density of the particles, i.e., absorbance or attenuation (scattering + non-extractable-abs + extractable-abs) (*discontinuous black lines*), $OD_d(\lambda)$, optical density after pigment extraction of the non-pigmented matter of phytoplankton and detrital material, i.e., absorbance or attenuation of scattering + non-extractable-abs (*continuous black line*) in low density (LD, 6–6.5 × 10^6 cells mL^{-1}) (**a**) and high density (HD, 13–13.5 million cell mL^{-1}) (**b**) cultures

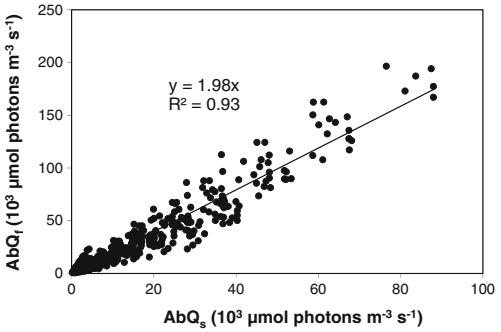

Fig. 4 Relationship between the absorbed quanta of *C. fusca* on filter (AbQ_f) and in suspension (AbQ_s) both expressed in µmol photons m^{-3} s^{-1}. The 11 irradiances supplied by the Junior-PAM were considered as well as samples from laboratory cultures under white light or from outdoor cultures grown under sunlight in a thin-layer cascade unit. The *line* shows the linear regression (n=735, p<0.01)

[ETR(3)], which gave values varying from 20 to 25 µmol e^- m^{-2} s^{-1}.

Regarding the three types of expression of ETR per chlorophyll unit, ETR^*_{max} (µmol e^- mg^{-1} Chl*a* s^{-1}), they did not present significant differences between laboratory cultures of different cell densities (Table 4). On the other hand, a significant effect of cell density was observed in outdoor cultures. Lower values of $ETR^*(1)_{max}$, $ETR^*(2)_{max}$, and $ETR^*(3)_{max}$ values were found at higher cell densities in all cases excepting October and June periods for $ETR^*(1)_{max}$ and $ETR^*(3)_{max}$, in which no significant differences were observed (Table 4). When ETR was expressed as relative values, which means that light absorption was not considered, no significant differences (p<0.05) were found, although a decrease was detected with cell density in laboratory and outdoor cultures in June (data not shown). No matter which of the three expressions is used, the highest ETR^*_{max} was always achieved in outdoor conditions: in LD cultures in June for $ETR^*(1)_{max}$ and $ETR^*(2)_{max}$ (4.24 and 1.55 µmol e^- mg^{-1} Chl*a* s^{-1}, respectively) and in HD cultures in October for $ETR(3)_{max}$ (31.35 µmol e^- m^{-2} s^{-1}).

Table 4 Cell density (× 10^6 cells mL^{-1}), Chl*a* concentration (mg L^{-1}), pg Chl*a* per cell, Chl*a*-specific absorption coefficients of *C. fusca* on filter (a^*_f, m^2 mg^{-1} Chl*a*) wavelength integrated [$\Sigma a^*_f(\lambda)$], averaged [$\bar{a}^*_f(\lambda)$] and maximum [$a^*_f(\lambda_{max})$]; Chl*a*-specific absorption coefficient in suspension (a^*_s, m^2 mg $Chla^{-1}$); $ETR^*(1)_{max}$ and $ETR^*(2)_{max}$ both expressed per chlorophyll unit (µmol electrons mg^{-1} Chl*a* s^{-1}); $ETR(3)_{max}$ expressed per area unit (µmol electrons m^{-2} s^{-1})

Sample	Million cells mL^{-1}	[Chl*a*] (mg L^{-1})	pg Chl*a*/cell	$\bar{a}^*_f(\lambda)$ (m^2 mg^{-1} Chl*a*)	k_d* (m^2 mg^{-1} Chl*a*)	$ETR^*(1)_{max}$ (µmol e^- mg^{-1} Chl*a* s^{-1})	$ETR^*(2)_{max}$ (µmol e^- mg^{-1} Chl*a* s^{-1})	$ETR(3)_{max}$ (µmol e^- m^{-2} s^{-1})
LAB	5	6.78[a]	1.36[a]	0.0022[a]	0.0011[a]	0.60[a]	0.13[a]	8.33[a]
	10	17.16[b]	1.72[a]	0.0020[a]	0.0009[a]	0.37[a]	0.08[a]	12.82[a]
June-LD	6.5	2.94[c]	0.41[b]	0.0096[b]	0.0093[b]	4.24[d]	1.55[b]	21.91[a]
June-HD	13	5.85[a]	0.45[b]	0.0068[c]	0.0067[c]	1.90[b]	0.70[c]	15.67[a]
Oct-LD	6	3.85[c]	0.55[b]	0.0074[c]	0.0094[b]	2.99[c]	1.24[b]	17.70[a]
Oct-HD	13.5	6.83[a]	0.51[b]	0.0086[b]	0.0057[b]	2.20[c]	0.83[c]	31.35[b]

Data from *Chlorella fusca* cultures grown in laboratory and outdoor conditions in two periods: June and October. In all cases, samples were taken when cell density was 5–6×10^6 cells mL^{-1} (low cell density (LD)) and 10–13.5×10^6 cells mL^{-1} (high cell density (HD))

*Different letters denote significant differences among different cell density cultures for each variable (p<0.05)

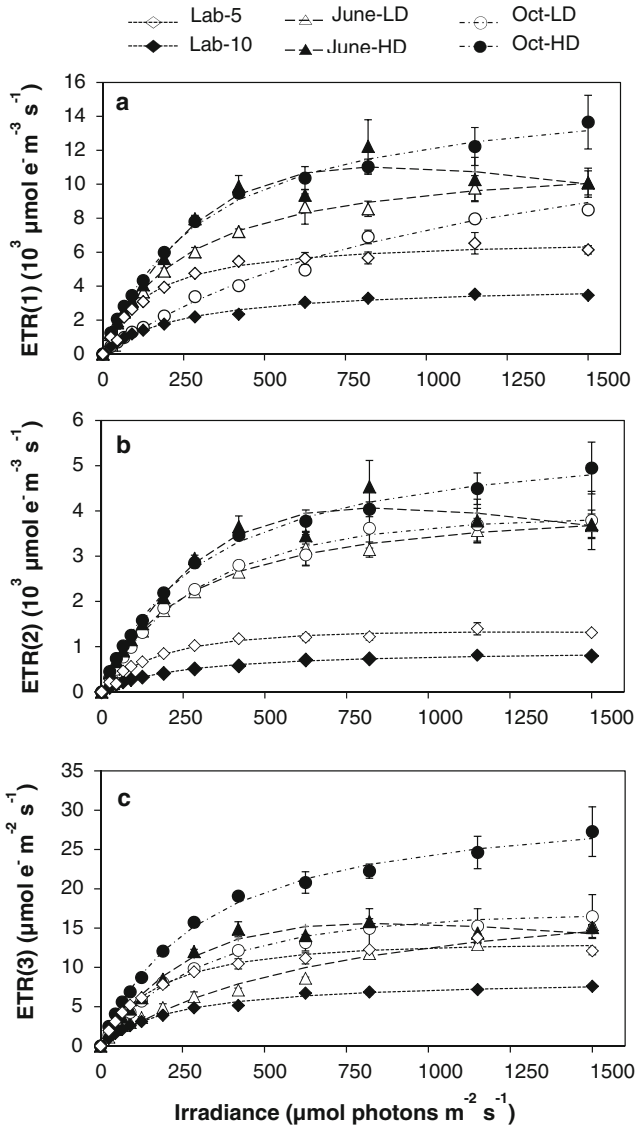

Fig. 5 Electron transport rate (ETR) as function of irradiance (µmol photons m^{-2} s^{-1}) for *C. fusca* grown in laboratory (diamonds) and outdoor conditions in a thin-layer cascade system in two different periods: June (*triangles*) and October (*circles*). In all cases, samples were taken when the cell density was 5–6×10^6 cells mL^{-1} (low cell density (LD), *open symbols*) and when it was 10–13.5×10^6 cells mL^{-1} (high cell density (HD), *closed symbols*). ETR was determined according to equations presented in Table 2: **a** ETR(1), in µmol e^- m^{-3} s^{-1} calculated using AbQ_f; **b** ETR(2), in µmol e^- m^{-3} s^{-1} calculated using AbQ_s; and **c** ETR(3), in µmol e^- m^{-2} s^{-1} calculated using A_s(PAR)

ETR_{max} values showed linear relation with cell number (Fig. 6). In the three cases, ETR_{max} was significantly and linearly related ($p<0.01$) to cell density, although $ETR(1)_{max}$ and $ETR(3)_{max}$ showed lower R^2 values (0.9611 and 0.9676, respectively) than $ETR(2)_{max}$ (R^2=0.9861). In addition, relations between ETR values expressed per volume or area unit, determined by measuring the absorbed light in filter or in suspension (see Table 2), resulted to be linear in all cases compared. The R^2 values of these relationships were 0.98 for ETR(1) vs. ETR(2), 0.92 for ETR(1) vs. ETR(2), and 0.94 for ETR(2) vs. ETR(3) (data not shown). $ETR(1)_{max}$, determined from filter measurements, varied from 415.08 to 9322.35 µmol photons m^{-3} s^{-1} whereas suspension values ranged from 96.45 to 2112.15 µmol photons m^{-3} s^{-1} if expressed per volume unit, $ETR(2)_{max}$, and from 0.93 to 13.74 µmol photons m^{-2} s^{-1} when expressed per area unit, $ETR(3)_{max}$.

Discussion

Measurement of absorbed irradiance is a requisite to calculate absolute ETR. Absorption coefficient determination is mainly conducted in oceanographic studies whereas in laboratory cultures, only a few number of studies have been carried out in outdoor high-density microalgal cultures (Gilbert et al. 2000; Wilhelm et al. 2004; Blache et al. 2011; Klughammer and Schreiber 2015). 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 in high-density cultures (Geider and Osborne 1992; Agustí et al. 1994). There are a variety of methods to measure the absorbed irradiance in laboratory such as the QFT (Mitchell and Kiefer 1984; Kishino et al. 1985; Arbones et al. 1996), the filter-transfer-freeze method which needs a double-beam spectrophotometer equipped with an integrating sphere attachment (Tassan and Allali 2002), or by using a reflective tube (Zaneveld et al. 1990). Despite its widespread use, the accuracy of the method remains controversial (Lohrenz 2000). Furthermore, for its use, it is necessary to take into account that variation in filter wetness and different filter batches can interfere with the result (Roesler 1998) and that a correction for the increased path length (β factor), still open to discussion and extensively used lately (Cleveland and Weidemann 1993; Hoepffner and Sathyendranath 1993; Arbones et al. 1996), must be applied. As Lohrenz (2000) reported before, we can confirm that cell morphology can be altered due to filtration and that changes in filter moisture can modify the spectrophotometric measurements. Thus, low pressures during filtration and rapid manipulation of samples are strongly recommended. Among these methods, the QFT is generally accepted and it shows negligible losses due to scattering (Perkins et al. 2011), but the methodology has still not been broadly applied in microalgal cultures. Recently, the methodology proposed by Klughammer and Schreiber (2015) regarding the analysis of the O-I_1 rise kinetics by using a Multicolor PAM opened the way for estimating mean PAR also in optically dense samples via measurement of $<\sigma>(\lambda)/\sigma_{II}(\lambda)$. Both the quantitative filter technique and the method proposed by Klughammer and Schreiber (2015) require certain expertise in bio-optical knowledge and also the need for

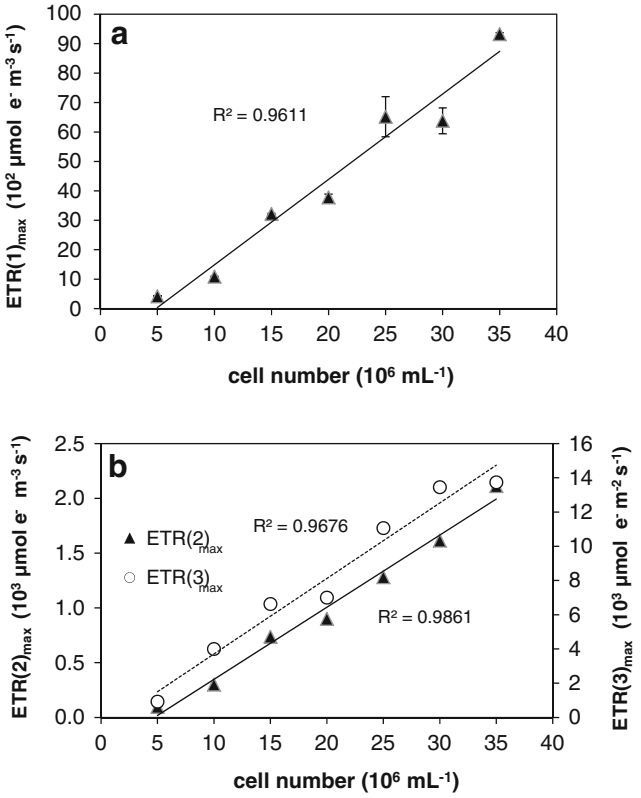

Fig. 6 Maximal electron transport rate values (ETR_{max}) versus cell number (10^6 mL^{-1}). ETR_{max} was calculated according to the equations presented in Table 2. **a** ETR(1) in µmol e^- m^{-3} s^{-1} calculated using AbQ_f, **b** ETR(2) in µmol e^- m^{-3} s^{-1} calculated using AbQ_s, and **c** ETR(3) in µmol e^- m^{-2} s^{-1} calculated using A_s(PAR). *Lines* show the linear regression in each case (n=7, p<0.01)

equipment not always available in laboratories or aquaculture companies.

In this study, we suggest a method based on the determination of the attenuation or extinction coefficient in a measuring system that mimics beam attenuation by using an orthogonal position of the light source and reduces scattering by using diffuser plates as in opal glass technique (Mercado et al. 1996) to estimate the absorptance of a thin layer of microalgal suspension analogous to an algal thallus (Mercado et al. 1996; Figueroa et al. 2003). For this measurement, the users only need to have an illumination system provided with lamps and elements as described in this study and a broadband PAR sensor connected to a radiometer. The illumination system is easy to set up, and the PAR sensor and radiometer are usually available in laboratory and algal aquaculture facilities. To validate this approach, the first requisite is to have a good relation between the absorption coefficient determined by using the QFT and the absorption measurement in the cell suspensions. A relation of 1.98 between the absorbed quanta of phytoplankton in filter (AbQ_f) and the absorbed quanta in cell suspensions (AbQ_s) was found. The value of 1.98 can be considered an amplification factor of the absorption of the cells in the filter compared to the cell suspension. Despite that our system pretends to reduce the scattering, it was lower when cells were concentrated in the glass filter following the quantitative filter technique. The ratio AbQ_f/AbQ_s can thus be considered analogous to the β factor, $a_{filter}(\lambda)/a_{sus}(\lambda)$, being $a_{filter}(\lambda)$ and $a_{sus}(\lambda)$ the spectral absorption coefficient measured on filter and in suspension, respectively as defined by Butler (1962). Several authors assumed the β factor to be constant (Lewis et al. 1985) whereas others reported that this factor varied depending on the species and light conditions. Kishino et al. 1985 reported that the β factor varied between 2.43 and 4.71 depending on the species. On the other hand, Mitchell and Kiefer (1988) demonstrated that the β factor is not constant but varies with the optical density of the particles on the filter and with different filter type. Arbones et al. (1996) gave evidence of a unique equation to correct the path length amplification on glass fiber filter, which we used in this study.

By using the amplification factor, the absorption of cell suspensions of *C. fusca* at different densities could be determined, and thus, absolute ETR could be calculated. This study could be extended to other microalgae groups and species with different cell sizes, so the bio-optical effect on the relation between AbQ_s and AbQ_f can be evaluated. Although the glass filter is located close to the detector to reduce the loss of scattered light and a correction for the increase in effective path length caused by scatter within glass fiber is applied (Kiefer and SooHoo 1982), the lower values of a^* in this study compared to other reports (Bunt 1995; Blache et al. 2011) can be explained by a residual scattering that reduces the light absorption.

The estimated absorption coefficient determined by measuring the attenuation or absorptance in cell suspension was already used by Figueroa et al. (2013). They estimated the biomass productivity of *C. fusca* grown in a thin-layer cascade system from absolute ETR values (µmol e^- m^{-2} s^{-1}), which were converted to fixed carbon and then to biomass productivity. Calculations were made based on several assumptions: mol of photons per mol of produced oxygen, mol of fixed CO_2 per mol of produced oxygen, and mg of carbon per g of algal biomass (Kromkamp et al. 2008; Figueroa et al. 2013). Figueroa et al. (2013) found a relationship between measured and estimated biomass productivity in *C. fusca* of 0.74–1.08. Obata et al. (2009) reported a relationship between fixed carbon measured by $NaH^{13}CO_2$ assimilation and rETR in *C. vulgaris* of ~6.6, which is far from 1 since ETR was expressed as relative units (µmol e^- m^{-2} s^{-1}), and thus, light absorption was not considered. In addition, ETR was not converted to fixed carbon although carbon assimilation was measured (mg C mg^{-1} Chl*a* h^{-1}). Therefore, conversion of ETR values to the same units, as carbon or biomass production, is essential if the study is aimed to make a comparison.

In the present study, we discussed the usefulness of different expressions of ETR on a volume, area, or chlorophyll basis. ETR can be expressed per volume unit (µmol

$e^- m^{-3} s^{-1}$) by using spectral absorption coefficient values determined either by the QFT [ETR(1)] or the absorptance of the suspension [ETR(2)]. Both ETR expressions are good estimators of photosynthetic production or carbon assimilation per unit of culture volume. However, few data of ETR per volume unit are found in the bibliography since most of ETR values are expressed as chlorophyll basis (ETR*) (mol $e^- mg^{-1}$ Chl*a* h^{-1}) or converted into oxygen production (mmol $O_2 mg^{-1}$ Chl*a* h^{-1}), both of which represent a specific productivity. They cannot be considered adequate for the estimation of the production of the whole culture, but they can be used as estimators of cell productivity since the chlorophyll concentration is used to estimate cell density. Chlorophyll is not always a good indicator of algal biomass since chlorophyll content per cell can be increased under low irradiance conditions or in high-cell-density cultures due to the photoacclimation to the self shading (Dubinsky et al. 1986; Kirk 1994; Blache et al. 2011). In addition, ETR* considers acclimation processes, i.e., decrease of ETR, as an acclimation to shade conditions by increasing chlorophyll density, and it also reflects the presence of the package effect (Dubinsky et al. 1986). In the present study, the ETR expressed per chlorophyll unit increased with cell numbers or Chl*a* per cell (Table 4). This result was not expected since cells should have the same physiological state. The lower ETR at $5–10 \times 10^6$ cells mL^{-1} compared to other cell densities can be explained by a lower photosynthetic efficiency since the penetration of light is higher than that at higher cell densities as reported by Masojídek et al. (2011). As expected, ETR_{max} as μmol $e^- m^{-3} s^{-1}$ was higher at 20, 25, and 30×10^6 cells mL^{-1} compared to lower cell densities. $ETR(1)_{max}$ determined by using the QFT ranged from 8000 to 11,000 μmol $e^- m^{-3} s^{-1}$ whereas by using the absorption coefficient, $ETR(3)_{max}$, ranged from 1600 to 2000 μmol $e^- m^{-3} s^{-1}$. The expression of ETR expressed per volume unit is of great utility to describe the photosynthetic performance of cultures in systems that have considerable volume such as raceways, open ponds, or tubular photobioreactors. In outdoor conditions, ETR was higher in HD than in LD cultures (see Fig. 4), which would indicate that in LD cultures, photoinhibition processes are likely to occur due to high penetration of light. In addition, the higher ETR values found in October could also be related to photoinhibition processes, since the daily integrated irradiance in this period was about 2-fold lower than that in June. In June, the daily integrated irradiance was ~12,000, 1500, and 72 kJ m^{-2} for PAR, UVA, and UVB, respectively, whereas in October, it was ~6300, 788, and 34 kJ m^{-2} for PAR, UVA, and UVB, respectively (measurements determined by UV-PAR Multi-filter radiometer NILU-6, Geminali AS, Oslo, Norway, located in the same place as the thin-layer cascade systems used in this study).

On the other hand, ETR(2), expressed per area unit (μmol $e^- m^{-2} s^{-1}$) by using the absorptance, would be very useful if applied to culture systems with high exposed surface (high surface to volume ratio) such as thin-layer cascades or flat panel photobioreactors (Masojidek et al. 2011). ETR (2) can be considered as ETR(3)*D according to Eq. 6. Thus, relative differences between ETR(2) and ETR(3) in Fig. 5 suggest that the optically thin assumption does not hold for some cultures. It is expected that the decrease in the thickness of cell cultures would improve the results.

The expression of ETR per area unit can be more extensively found in the literature, but most of the data are expressed without considering light absorption, i.e., relative units (rETR). Kromkamp et al. (2009) reported rETR values in *Nannochloropsis* sp. growing in outdoor raceways of 350 μmol $e^- m^{-2} s^{-1}$ and of 150 μmol $e^- m^{-2} s^{-1}$ when grown in a flat panel cultivator, which would represent an overestimation of the production since light absorption was not considered. In other studies of the same author, ETR is expressed as absolute values after the determination of absorption coefficient but in terms of chlorophyll (Flameling and Kromkamp 1998; Kromkamp et al. 2008). Masojídek et al. (2011) did not report ETR values but effective quantum yield and irradiance values in high-density thin-layer cultures of *Chlorella* sp.; considering the data corresponding to cultures of 10 g L^{-1}, rETR can be calculated being 66.25 μmol $e^- m^{-2} s^{-1}$, which is in the range of the rETR values achieved by *C. fusca* in the present study. Blache et al. (2011) reported rETR values in *C. vulgaris* of 30–90 μmol $e^- m^{-2} s^{-1}$ and a chlorophyll content of 0.25–0.9 pg Chl*a* $cell^{-1}$, whereas in this study, the rETR in *C. fusca* containing 1840 pg Chl*a* $cell^{-1}$ was 40.64 μmol $e^- m^{-2} s^{-1}$. Besides, ETR_{max} in *C. vulgaris* ranged from 200 to 800 μmol O_2 mg^{-1} Chl*a* h^{-1} (Blache et al. 2011), whereas values of 52.5 μmol O_2 mg^{-1} Chl*a* h^{-1} calculated from ETR values according to Figueroa et al. (2013) were found in *C. fusca* (35×10^6 cells mL^{-1}) in the present study. These discrepancies could be explained by the different cell diameter of *C. vulgaris* (3 μm) and *C. fusca* (6–8 μm) since consequently, big differences in cellular absorptivity are expected. Blache et al. (2011) indicated that the variability of bio-optical properties resulted in a great deviation of relative electron transport rate and oxygen basis-based photosynthesis. They concluded that P_{max} derived from rETR is strongly dependent on the specific cellular absorptivity, and it cannot be used to compare the photosynthetic performance of cells with different optical properties. Thus, it is necessary to compare the photosynthetic activity among species with different bio-optical properties, either by the determination of the absorption by the cell or light attenuation of the culture. Figueroa et al. (1997) compared cells with different bio-optical properties. i.e., cell volume ranged from 0.16 to 0.31 μm^3, chlorophyll content was 3.0–7.3 mg L^{-1}, and cell densities ranged from 1.1 to 672×10^6 cells mL^{-1}. They reported that the specific attenuation coefficient (K_c), which ranged from 0.01 to 0.03 m^2 mg^{-1} Chl*a*, explained the acclimation to increased

irradiance since the photoinhibition increased with K_c. They concluded that K_c could be a good indicator of the bio-optical properties of the cultures since it takes into account both the effect of cell size and pigment content on the light absorption. K_c is an apparent optical property of the culture whereas specific cell absorption (a^*) is an inherent optical property used to calculate the ETR per Chl*a* unit (μmol mg^{-1} Chl*a* s^{-1}). However, both variables represent the bio-optical properties of the cells. Johnsen and Sakshaug (2007) presented a method based on scaling fluorescence excitation spectra to the corresponding absorption spectra by matching them in the 540–650 nm range, estimates for the fraction of total Chl*a* that resided in PSII; the absorption of light by PSII, PSI, and photoprotective carotenoids was conducted. In addition, they showed that 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.

In summary, this study showed the usefulness of a procedure to determine light absorption of *C. fusca*, simply and rapidly, by measuring the light attenuation of a thin layer of cell suspension in a measuring system with reduced scattering conditions. Results were linearly related to the absorption coefficient determined by the QFT. The ratio AbQ_f/AbQ_s, which in this study was 1.98, can be used to convert absorption values from cell suspension to absorption values of cells retained on filter according to the quantitative filter technique, which would be interesting since this last technique is broadly accepted as it presented good relation with the absorption of cell suspensions determined by using an integrating sphere. In addition, the value of the ratio AbQ_f/AbQ_s showed in the present study (1.98) obtained from the absorption of cells concentrated in filters (determined by the use of a spectrophotometer) and the absorption of cell suspensions determined by using a broadband PAR sensor connected to a radiometer and an a illumination system designed to reduce the scattering is in the lowest range (2.43–4.71) reported by Kishino et al. (1985) for the amplification factors between measurements in filter and in suspension (the latter determined by using an integrating sphere).

The technique presented in this study can be easily applied to determine light absorption in order to express ETR as absolute values instead of the relative expression of ETR. Absolute ETR can be used to estimate photosynthetic activity as oxygen production, carbon assimilation, or biomass yield. We presented the different expressions of ETR, i.e., surface, volume, or chlorophyll units, which can be used according to the characteristics of the culture system. In thin-layer cascade systems or flat panel photobioreactors, it would be appropriate to express ETR per area unit (μmol e^- m^{-2} s^{-1}), whereas in raceways or tubular photobioreactors, the expression per volume unit would have more photobiological sense (μmol e^- m^{-3} s^{-1}) as reported by Bosma et al. (2007). In both culture systems, ETR can be expressed per chlorophyll unit, which would represent the specific productivity (ETR*, μmol e^- mg^{-1} Chl*a* h^{-1}) as it is an expression of the production per unit of pigment or cell. ETR* is interesting in terms of evaluating photoacclimation processes and package effect. More investigations on the use of the method here proposed to measure light absorption in microalgal cultures would be necessary in species with different bio-optical properties according to its pigment composition, morphology, and cell size in order to extend this approach to other studies. It is expected that the absolute ETR versus irradiance function would give information on the production and photoacclimation of phytoplankton species of different bio-optical characteristics.

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References

Agustí S, Enríquez S, Frost-Christensen H, Sand-Jensen K, Duarte CM (1994) Light harvesting among photosynthetic organisms. Ecology 8:273–379

Andersen RA, Berges JA, Harrison PJ, Watanabe MM (2005) Recipes for freshwater and seawater media. In: Andersen RA (ed) Algal culturing techniques. Elsevier Academic Press, London, pp 429–538

Arbones B, Figueiras FG, Zapata M (1996) Determination of phytoplankton absorption coefficient in natural seawater samples: evidence of a unique equation to correct the pathlength amplification on glass-fiber filters. Mar Ecol Prog Ser 137:293–304

Berberoglu H, Gómez PS, Pilon L (2009) Radiation characteristics of *Botryococcus braunii*, *Chlorococcum littorale* and *Chlorella* sp used CO_2 fixation and biofuel production. J Quant Spectrosc Radiat Transf 110:1879–1893

Bischoff HW, Bold HC (1963) Some soil algae from Enchanted Rock and related algal species. Phycol Stud Univ Tex IV:1–95

Blache U, Jakob T, Su W, Wilhelm C (2011) The impact of cell-specific absorption properties on the correlation of electron transport rates measured by chlorophyll fluorescence and photosynthetic oxygen production in planktonic algae. Plant Physiol Biochem 49:801–808

Bosma R, van Zessen E, Reith JH, Tramper J, Wijffels RH (2007) Prediction of volumetric productivity of an outdoor photobioreactor. Biotechnol Bioeng 97:1108–1120

Bricaud A, Stramski D (1990) Spectral absorption coefficients of living phytoplankton and non algal biogenous matter: a comparison between the Oeru upwelling area and Sargasso Sea. Limnol Oceanogr 35:562–568

Bunt J (1995) Light and photosynthesis in aquatic ecosystems. Aquat Bot 50:111–112

Butler WL (1962) Absorption of light in turbid materials. J Opt Soc Am 52:292–299

Cleveland JS, Weidemann AD (1993) Quantifying absorption by aquatic particles: a multiple scattering correction for glass-fiber filters. Limnol Oceanogr 38:1321–1327

Dubinsky Z, Falkowski PG, Wyman K (1986) Light harvesting and utilization by phytoplankton. Plant Cell Physiol 27:1335–1349

Figueroa FL, Mercado J, Jiménez C, Salles S, Aguilera J, Sánchez-Saavedra MP, Lebert M, Häder D-P, Montero O, Lubián L (1997) Relationship between bio-optical characteristics and photoinhibition of phytoplankton. Aquat Bot 59:237–251

Figueroa FL, Conde-Álvarez RM, Gómez I (2003) Relations between electron transport rates determined by pulse amplitude modulated chlorophyll fluorescence and oxygen evolution in macroalgae under different light conditions. Photosynth Res 75:259–275

Figueroa FL, Martínez B, Israel A, Neori A, Malta E, Put A Jr, Inken S, Marquardt R, Rachamim T, Arazi U, Frenk S, Korbee N (2009) Acclimation of Red Sea macroalgae to solar radiation: photosynthesis and thallus absorptance. Aquat Biol 7:159–172

Figueroa FL, Jerez CG, Korbee N (2013) Use of in vivo chlorophyll fluorescence to estimate photosynthetic activity and biomass productivity in microalgae grown in different culture systems. Lat Am J Aquat Res 41:801–819

Flameling IA, Kromkamp J (1998) Light dependence of quantum yields for PSII charge separation and oxygen evolution in eucaryotic algae. Limnol Oceanogr 43:284–297

Fujiki T, Taguchi S (2002) Variability in chlorophyll *a* specific absorption coefficient in marine phytoplankton as a function of cell size and irradiance. J Plankton Res 24:859–874

Geider RJ, Osborne BA (1992) Algal photosynthesis: the measurement of algal gas exchange. Chapman and Hall, Norwell, p 256

Genty B, Briantais JM, Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim Biophys Acta Gen Subj 990:87–92

Gilbert M, Domin A, Becker A, Wilhelm C (2000) Estimation of primary productivity by chlorophyll *a* in vivo fluorescence in freshwater phytoplankton. Photosynthetica 38:111–126

Grobbelaar JU (2009) Upper limits of photosynthetic productivity and problems of scaling. J Appl Phycol 21:519–522

Hoepffner N, Sathyendranath S (1993) Determination of the major groups of phytoplankton pigments from the absorption spectra of total particulate matter. J Geophys Res 98:22789–22803

Jerez CG, Navarro E, Malpartida I, Rico RM, Masojídek J, Abdala R, Figueroa FL (2014) Hydrodynamics and photosynthesis performance of *Chlorella fusca* grown in a thin-layer cascade (TLC) system. Aquat Biol 22:111–122

Johnsen G, Sakshaug E (2007) Biooptical characteristics of PSII and PSI in 33 species (13 pigment groups) of marine phytoplankton, and the relevance for pulse amplitude-modulated and fast-repetition-rate fluorometry. J Phycol 43:1236–1251

Kiefer D, SooHoo J (1982) Spectral absorbtion by marine particles of coastal waters of Baja California. Limnol Oceanogr 27:492–499

Kirk JTO (1994) Light and photosynthesis in aquatic ecosystems, 2nd edn. Cambrige University Press, Cambridge, **509 pp**

Kishino M, Takahashi M, Okami N, Ichimura S (1985) Estimation of the spectral absorption-coefficients of phytoplankton in the sea. Bull Mar Sci 37:634–642

Kishino M, Okami N, Takahashi M, Ichimura S (1986) Light utilization efficiency and quantum yield of phytoplankton in the sea. Bull Mar Sci 37:634–642

Klughammer C, Schreiber U (2015) Apparent PSII absorption cross-section and estimation of mean PAR in optically thin and dense suspensions of *Chlorella*. Photosynth Res 123:77–92

Kromkamp JC, Dijkman NA, Peene J, Simis SGH, Gons HJL (2008) Estimating phytoplankton primary production in Lake IJsselmeer (The Netherlands) using variable fluorescence (PAM-FRRF) and C-uptake techniques. Eur J Phycol 43:327–344

Kromkamp JC, Beardall J, Sukenik A, Kopecky J, Masojidek J, Van Bergeijk S, Gabai S, Shaham E, Yamshon A (2009) Short-term variations in photosynthetic parameters of *Nannochloropsis* cultures grown in two types of outdoor mass cultivation systems. Aquat Microb Ecol 56:309–322

Lewis MR, Warnock RE, Platt T (1985) Absorption and photosynthetic action spectra for natural phytoplankton populations implications for production in the open sea. Limnol Oceanogr 30:794–806

Lippemeier S, Hintze R, Vanselow KH, Hartig P, Colijn F (2001) In-line recording of PAM fluorescence of phytoplankton cultures as a new tool for studying effects of fluctuating nutrient supply on photosynthesis. Eur J Phycol 36:89–100

Lohrenz SE (2000) A novel theoretical approach to correct for pathlength amplification and variable sampling loading in measurements of particulate spectral absorption by the quantitative filter technique. J Plankton Res 22:639–657

Masojídek J, Kopecký J, Giannelli L, Torzillo G (2011) Productivity correlated to photobiochemical performance of *Chlorella* mass cultures grown outdoors in thin-layer cascades. J Ind Microbiol Biotechnol 38:307–317

Masojidek J, Vonshak A, Torzillo G (2011) Chlorophyll fluorescence applications in microalgal mass cultures. In: Suggett DJ, Prásil O, Borowitzka MA (eds) Chlorophyll *a* fluorescence in aquatic science: methods and applications. Springer, Dordrecht, pp 277–292

Mayer AMS, Rodríguez AD, Berlinck RGS, Fusetani N (2011) Marine compounds with antibacterial, antituberculosis, and antiviral activities; affecting the immune and nervous system, and other miscellaneous mechanisms of action. Comp Biochem Physiol C 153:191–222

Mercado JM, Jiménez C, Niell FX, Figueroa FL (1996) Comparison of methods for measuring light absorption by algae and their application to the estimation of the package effect. Sci Mar 60:39–45

Mitchell BG, Kiefer DA (1984) Determination of absorption and fluorescence excitation spectra for phytoplankton. In: Holm-Hansen O, Bolis L, Giles R (eds) Marine phytoplankton and productivity. AGU, Washington, DC, pp 157–169

Mitchell BG, Kiefer DA (1988) Chlorophyll *a* specific absorption and fluorescence excitation spectra for light-limited phytoplankton. Deep Sea Res 35:639–663

Obata M, Toda T, Taguchi S (2009) Using chlorophyll fluorescence to monitor yields of microalgal production. J Appl Phycol 21:315–319

Perkins RG, Kromkamp JC, Serôdio J, Lavaud J, Jesus B, Mouget JL, Lefebvre S, Forster RM (2011) The application of variable chlorophyll fluorescence to microphytobenthic biofilms. In: Suggett DJ, Prášil O, Borowitzka MA (eds) Chlorophyll *a* fluorescence in aquatic sciences: methods and applications. Springer, Dordrecht, pp 237–275

Platt T, Gallegos CL (1980) Modelling primary production. In: Falkowski PG (ed) Primary production in the sea. Plenum Press, NY, pp 339–351

Ritchie RJ, Runcie JW (2014) A portable reflectance-absorptance-transmittance meter for photosynthetic work on vascular plant leaves. Photosynthetica 52:614–626

Roesler CS (1998) Theoretical and experimental approaches to improve the accuracy of particulate absorption coefficients derived from the quantitative filter technique. Limnol Oceanogr 43:1649–1660

Schreiber U, Schliwa U, Bilger W (1986) Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. Photosynth Res 10:51–62

Schreiber U, Hormann H, Neubauer C, Klughammer C (1995) Assessment of photosystem II photochemical quantum yield by chlorophyll fluorescence quenching analysis. Aust J Plant Physiol 22:209–222

Schreiber U, Klughammer C, Kolbowski J (2012) Assesment of wavelength-dependent parameters of photosynthetic electron transport with a new type of multi-color PAM chlorophyll fluorometer. Photosynth Res 113:127–144

Suggett DJ, MacIntyre HL, Geider RJ (2004) Evaluation of biophysical and optical determinations of light absorption by photosystem II in phytoplankton. Limnol Oceanogr Methods 2:316–332

Suggett DJ, Moore CM, Hickman AE, Geider RJ (2009) Interpretation of fast repetition rate (FRR) fluorescence: signatures of phytoplankton community structure versus physiological state. Mar Ecol Prog Ser 376:1–19

Suggett DJ, Moore CM, Geider RJ (2011) Estimating aquatic productivity from active fluorescence measurements. In: Suggett DJ, Prášil O, Borowitzka MA (eds) Chlorophyll *a* fluorescence in aquatic sciences: methods and applications. Springer, Dordrecht, pp 103–127

Szabó M, Wangpraseurt D, Tamburic B, Larkum AWD, Schreiber U, Sugget DJ, Kühl M, Ralph PJ (2014) Effective light absorption and absolute electron transport rate in the coral *Pocillopora damicornis*. Plant Physiol Biochem 83:159–167

Tassan S, Allali K (2002) Proposal for the simultaneous measurement of light absorption and backscattering by aquatic particulates. J Plankton Res 24:471–479

Torzillo G, Accolla P, Pinzani E, Masojidek J (1996) In situ monitoring of chlorophyll fluorescence to assess the synergistic effect of low temperature and high irradiance stresses in *Spirulina* cultures grown outdoors in photobioreactors. J Appl Phycol 8:283–291

Torzillo G, Paola B, Masojídek J, Bernardini P (1998) On-line monitoring of chlorophyll fluorescence to assess the extent of photoinhibition of photosynthesis induced by high oxygen concentration and low temperature ans its effect on the productivity of outdoor cultures of *Spirulina platensis* (cyanobacteria). J Phycol 34:504–510

Tredici MR (2010) Photobiology of microalgae mass cultures: understanding the tools for the next green revolution. Biofuels 1:143–162

Underwood AJ (1997) Experiments in ecology. Their logical design and interpretation using analysis of variance. Cambridge University Press, Cambridge

Wellburn AR (1994) The spectral determination of chlorophyll *a* and chlorophyll *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. J Plant Physiol 144: 307–313

Wilhelm C, Jakob T (2011) From photons to biomass and biofuels: evaluation of different strategies for the improvement of algal biotechnology based on comparative energy balances. Appl Microbiol Biotechnol 92:909–919

Wilhelm C, Becker A, Toepel J, Vieler A, Rautenberger R (2004) Photophysiology and primary production of phytoplankton in freshwater. Physiol Plant 120:347–357

Zaneveld JV, Bartz R, Kitchen JC (1990) Reflective-tube absorption meter. Ocean Optics X, Proc SPIE 1302:124–136