

Methodology of light response curves: application of chlorophyll fluorescence to microphytobenthic biofilms

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Abstract The light response curve methodology for microphytobenthic biofilms was studied by comparing the two most usual approaches used in pulse amplitude modulated (PAM) fluorometry. The non-sequential light curve (N-SLC) method is characterized by independent measures of the photosynthetic activity across a light gradient whereas the rapid light curve (RLC) method consists of successive measures on the same sample exposed to a stepwise increase of light intensities. Experiments were carried out on experimental microphytobenthic biofilms prepared from natural assemblages and acclimated to dark conditions. In preliminary experiments, N-SLCs were constructed from fluorescence induction curves performed at 12 different photon flux densities (PFDs). A minimum of 50 s of illumination was necessary to reach a stable light response curve; shorter illumination times resulted in underestimating the physiological parameters (α the light utilization coefficient in light-limited conditions and $rETR_{max}$ the maximum rate of photosynthesis efficiency) of the light response curve. For the comparison between N-SLCs and RLCs, the same time of illumination (50 s) was used for each light step of RLCs so that N-SLCs differed from RLCs only by the way the amount of light was

delivered, i.e., a light dose accumulation for RLC. The experimental results showed the difference between the two photobiological response curves. In the lower range of PFDs, RLCs exhibited a larger value of α ; in this light-limited part of the response curve the incremental increase of PFDs limited the development of NPQ and resulted in a better optimization of electron transport rate for RLC. In the higher range of PFDs, the trend was reversed and the RLC showed a lower value of $rETR_{max}$ than the N-SLC did; this is attributed to the light dose accumulation which likely led to a more efficient dispersion of energy, as illustrated by a higher non-photochemical quenching (NPQ). In conclusion, these results confirm that parameters derived from both methods differ in their value and do not bear the same physiological information.

Introduction

Recent improvements of fluorescence measuring techniques have made the analysis of chlorophyll *a* (chl *a*) fluorescence quenching, by the pulse amplitude modulated (PAM) fluorometry, a powerful tool for assessing the physiological status of photosynthetic organisms (Schreiber and Bilger 1993; Schreiber 1998, 2004).

On intertidal mudflats devoid of macrophytes, where primary production is performed by sediment-inhabiting microalgal communities, commonly called the microphytobenthos (Round 1971; Colijn and De Jonge 1984; MacIntyre et al. 1996; Underwood and Kromkamp 1999), PAM fluorometry has been introduced by Serôdio et al. (1997) and widely applied to measure the photosynthetic activity of microphytobenthos (e.g., Hartig et al. 1998; Kromkamp et al. 1998; Perkins et al. 2002; Consalvey et al.

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2005; Serôdio et al. 2005b) or to evaluate the microalgal biomass (Serôdio et al. 1997; Honeywill et al. 2002; Consalvey et al. 2004a; Jesus et al. 2005, 2006a, b).

Microphytobenthos assemblages are frequently dominated by epipellic (i.e., free and mobile) diatoms (Round 1971) that migrate to the sediment surface during diurnal emersion periods forming transient biofilms (Gouleau 1976; Paterson 1986; Paterson et al. 1986; Paterson 1995). This biogenic structure, recognized as the primary production system in intertidal mudflats (Guarini et al. 2000) usually disappears at the end of emersion due to reburying of diatoms or to their resuspension with the incoming tide (Consalvey et al. 2004b; Easley et al. 2005; Herlory et al. 2005). Modulated fluorescence is a non-destructive technique which not only preserves the structure of the biofilm but also enables the assessment of the rapid response to light of microalgae induced by the high-frequency fluctuation of natural irradiance.

The methodology of “Rapid Light Curves” (RLCs) (Schreiber et al. 1997; White and Critchley 1999; Rascher et al. 2000; Ralph and Gademann 2005), specifically developed for the construction of light response curves relating the rate of photosynthetic electron transport (ETR) to photon flux density (PFD) within a few minutes, was widely applied to microphytobenthic assemblages (Serôdio et al. 2001; Defew et al. 2002; Perkins et al. 2002; Underwood 2002; Morris and Kromkamp 2003; Serôdio 2003, 2004; Serôdio et al. 2005a; Underwood et al. 2005; Perkins et al. 2006). However, despite its simplicity, particularly in field conditions, this method raises important questions as to the physiological interpretation of the response curve (Serôdio et al. 2005b, 2006; Perkins et al. 2006), particularly in comparison with other methods.

The rationale of RLCs is that the same microalgal sample is exposed to a stepwise increase or decrease of light intensity (tens of seconds light steps). The photochemical efficiency (Φ_{PSII}) of photosystem II (PSII) (Genty et al. 1989) is measured at the end of each irradiance level, and is dependent on the light environment experienced during the previous steps. The way the light is delivered and the duration of each step of PFD thus create an immediate light dose accumulation (cumulative effect of light) which influences the result of the measurement itself (Serôdio et al. 2005b; Perkins et al. 2006). Therefore, RLCs are fundamentally different from another category of light response curves based on independent measurements at each light level (Hawes et al. 2003; Ralph and Gademann 2005; Perkins et al. 2006); in such a case all measurements must be realized on different sub-samples, drawn from the same parent population, whose microalgae are in the same physiological state. In order to differentiate these light curves from RLCs, they have recently been called N-SLCs for Non-Sequential Light Response Curves

by Perkins et al. (2006); except the time scale of the measurement, they are a direct application of the classical Photosynthesis–Irradiance (P–E) curves. As a result, N-SLCs are a simple function of the light gradient whereas RLCs are a double function of light and time. Thus, N-SLCs aim at assessing experimentally in the laboratory a “steady state” response representative of stable light conditions while RLCs are more specifically designed to characterize in the field a dynamic response in a rapidly changing light environment.

Both RLCs and N-SLCs are necessary tools to elucidate the complex response of benthic microphytobenthos to light at different time scales, but users must be aware that they are fundamentally different in nature and that the physiological parameters derived from RLCs or N-SLCs (the light utilization efficiency in light-limited conditions, i.e., the initial slope α and the maximum rate of photosynthesis under light saturation or $r\text{ETR}_{\text{max}}$) cannot be compared directly, even though the shapes of the light curves are similar.

Therefore, to make it clear, the objective of our study was to compare experimentally both methods in order to point out their apparent similarities as well as their fundamental differences for a proper physiological interpretation of the light curves.

Recently, Perkins et al. (2006) initiated such a comparison on a monoculture of *Navicula phyllepta* (Kütz) but could not compare directly RLCs and N-SLCs because of practical reasons; they nevertheless clearly pointed out the sensitivity of the microalgal light response to the light dose accumulation generated by the RLC methodological protocols. Complementary to their work, we performed a direct comparison of both types of light response curves applied to experimental benthic biofilms re-created in vitro. Although our fluorescence measurements were made in controlled conditions to guarantee their replication, we attempted to create experimentally the microphytobenthic biofilm observed in situ (Herlory et al. 2004) from natural assemblage of epipellic cells, as realized by Consalvey et al. (2004a) or Jesus et al. (2006a, b). The dark-acclimated state was chosen as physiological reference because it represents the physiological state of microphytobenthos at the beginning of low tide when the biofilm forms after several hours in dark conditions.

Materials and methods

Isolation of epipellic diatoms from the sediment

Microphytobenthos was collected in the Aiguillon Bay (47°00'N, 1°05'W), an intertidal mudflat located along the French Atlantic coast and composed of very fine muds

(Lorin 1968). The sediment was collected on 25 January 2005 by scrapping the upper millimetres in areas of dense microalgal mats. On return to the laboratory, the epipellic fraction of the microphytobenthos was separated from the sediment using the procedure described in Riera et al. (1999). The collected sediment was spread on trays to reach a thickness of about 1 cm. Three nylon nets (100 μm mesh) were laid upon the sediment surface. Trays were held under continuous light while nets were kept wet by spraying filtered seawater (Sartorius membrane, 1.2 μm mesh, Göttingen, Germany). The day after, as a result of the vertical migration, epipellic cells had accumulated at the sediment surface, particularly within the upper nets. Epipellic microphytobenthos was then collected by rinsing the two upper nets with filtered seawater. The resulting suspension of the natural epipellic assemblage was kept overnight in the dark with a continuous stirring and at a temperature of 5°C to maintain the microphytobenthos in the same state of dark adaptation.

Preparation of experimental microalgal biofilms

All experimental biofilms were prepared from a single suspension of epipellic microphytobenthos in near-darkness to maintain cells in a dark-acclimated state, defined as the physiological reference for fluorescence measurements.

The principle of the method to prepare experimental biofilms consists of letting the suspension of microphytobenthos settle in a tube with a flat bottom for 15 min, time needed for the biofilm formation in situ (Herlory et al. 2004). In this way vertical movements through the mud are prevented and epipellic cells thus form a non-migratory biofilm on the bottom of the tube. Also, biofilms can be easily exposed to a controlled light environment and the photosynthetic response of microphytobenthos, as measured by chlorophyll fluorescence, can be assessed in vitro without the negative interferences in situ due to vertical migration of cells (Kromkamp et al. 1998; Perkins et al. 2002; Serôdio 2003) and depth integration of the fluorescence signal in the sediment (Forster and Kromkamp 2004; Serôdio 2004).

The biomass (or size) of experimental biofilms, expressed in $\text{mg chl } a \text{ m}^{-2}$, is assessed a priori from the quantity of chl *a* introduced in the tube and the surface area of the bottom of the tube. In the present experiment, the size of biofilms was set at about 35 $\text{mg chl } a \text{ m}^{-2}$ to reproduce in situ conditions corresponding to the date and site of sampling (Herlory et al. 2005). To reach this value, the concentration of the prepared microalgal suspension (see above) was adjusted to 930 $\mu\text{g chl } a \text{ L}^{-1}$, and 5 mL of this suspension was added to 13-mm diameter tubes, prior to settling of cells. The obtained non-migratory biofilms

were maintained in the dark at 20°C until fluorescence measurements.

Prior to the measurement of chlorophyll fluorescence of the experimental biofilms in each tube, 3 mL of seawater were removed to enable the introduction of the Diving PAM probe.

Finally, the exact biomass of each experimental biofilm was always checked a posteriori after the PAM fluorescence measurements: microphytobenthos was resuspended in the remaining 2 mL of seawater, it was filtered through glass fibre filter Whatman GF/F (pore size: 0.7 μm , Maidstone, England), the filter was placed in 9 mL of 90% acetone to extract pigments overnight at 5°C and then chl *a* and pheopigments were detected fluorimetrically and quantified using Lorenzen's equations (Lorenzen 1966).

Measurement of chlorophyll fluorescence parameters

Chlorophyll fluorescence measurements were made using a Diving PAM fluorometer (Walz, Effeltrich, Germany) and were performed the day following the extraction of cells from the sediment. A support device was specifically designed to maintain the Diving PAM fibre optic probe within the tube perpendicular to the biofilm surface and at a constant distance (5 mm).

Rapid light curves (RLCs, White and Critchley 1999) and non-sequential light curves (N-SLCs, Perkins et al. 2006) were constructed based on 12 actinic increasing light levels (20, 55, 100, 165, 230, 315, 445, 600, 885, 1,255, 1,805, and 2,880 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). These PFDs were delivered by the Diving PAM and assessed using the fibre quantum sensor connected to the PAM fluorometer.

The quantum efficiency of photosystem II (PSII) was measured by the saturation pulse technique, whereby a light flash (5,000 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ during 0.8 s with a halogen lamp: 8V/20W type Bellaphot, Osram, Munich, Germany) was emitted by the Diving PAM to measure the maximum fluorescence yield (F_m in the darkness and F'_m in the light).

In the dark-acclimated state, the maximum quantum efficiency of PSII was calculated as $F_v/F_m = (F_m - F_0)/F_m$ with F_0 the minimum fluorescence yield measured just prior to the saturation pulse. At each light level, the photochemical efficiency of PSII (Φ_{PSII}) was calculated as $(F'_m - F_t)/F'_m$ (Genty et al. 1989) (with F_t being the current fluorescence yield in the light measured just before the saturation pulse), which is equivalent to F'_q/F'_m (Oxborough et al. 2000; Perkins et al. 2006) and expresses the proportion of the light absorbed by chlorophyll associated with PSII that is used in photochemistry.

The relative electron transport rate (rETR) was then calculated as the product of light utilization efficiency

(Φ_{PSII}) and half of the photon flux density (PFD/2, it is assumed that light energy is equally distributed between both photosystems, Sakshaug et al. 1997). The non-photochemical quenching (NPQ), that reflects light energy dissipation by heat, was calculated as $(F_m - F'_m)/F'_m$ (Schreiber 2004).

Fluorescence induction curves following a dark-to-light transition and construction of Non-Sequential Light Curves (N-SLCs)

To construct the non-sequential light curves, the photochemical efficiency of PSII (Φ_{PSII}) has to be measured following a dark-light transition, independently at the 12 different PFDs required for the light response curve. As such transitions trigger characteristic transients in the fluorescence yield (the so-called Kautsky effect), it was first necessary to determine the minimum time required to get a stable photochemical efficiency of PSII (Φ_{PSII}).

Therefore, dark–light induction curves were realized for each of the 12 light intensities (from 20 to 2880 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). A single experimental biofilm was used for each induction curve. A repetitive application of saturating light pulses allowed to measure the photochemical efficiency of PSII (Φ_{PSII}) every 20 s from 10 to 130 s. Prior to each induction curve, a single saturation pulse was applied for assessment of the maximum quantum efficiency of PSII (F_v/F_m) of the biofilm in the dark-adapted state. Three replicates were performed for each actinic light level, that is 36 different experimental biofilms (3×12) with a biomass of 35 mg chl *a* m^{-2} .

N-SLCs were constructed using data from fluorescence induction curves. Relative electron transport rate (rETR) was calculated and plotted as a function of PFD. It was thus possible to construct seven N-SLCs, based on independent measurements at each actinic light level, for lighting durations of 10, 30, 50, 70, 90, 110, and 130 s.

Rapid Light Curves (RLCs) and comparison with N-SLCs

RLCs were performed in triplicate on different experimental biofilms prepared from the same parent epipelagic assemblage as for N-SLCs. During a rapid light curve, the same biofilm was exposed to a stepwise increase of light, using the same gradient as for N-SLCs (from 22 to 2880 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). RLCs were performed using the remote control function in the WinControl software (Walz, Effeltrich, Germany) from a computer to apply increasing 12 light steps.

The duration of each light step was set at 50 s, corresponding to the minimum time required to reach a stable photochemical efficiency of PSII (Φ_{PSII} , determined from the kinetics of light induction curves, see the ‘results’ section). The comparison between RLCs and N-SLCs was made on this basis, the only difference being the way the light dose was delivered: a cumulative effect of the light dose for RLCs and no accumulation of the light dose and independent measurements for N-SLCs.

Statistics for comparing RLCs and N-SLCs

RLCs and N-SLCs were modelled by fitting the model of Eilers and Peeters (1988), which was modified to introduce directly in the equation the physiological parameters α (the light utilization coefficient in light-limited conditions), rETR_{max} (the maximum rate of photosynthesis efficiency) and E_k (the light saturation coefficient):

$$\text{rETR} = \frac{E}{\frac{E^2}{\alpha \times E_k^2} + \frac{E}{\text{rETR}_{\text{max}}} - \frac{2 \times E}{\alpha \times E_k} + \frac{1}{\alpha}}$$

where E represents the Photon Flux Density (PFD).

This transformation enabled to estimate directly the values of α , rETR_{max} and E_k , and their standard error, using the Sigmaplot curve fitter (Systat Software Inc., San Jose, CA, USA). The software fits the curve to measurements using the Marquardt–Levenberg algorithm, which minimizes the residual sum of squares. All fittings were tested by analysis of variance ($P < 0.05$) and residues were tested for normality and homogeneity of variance ($P > 0.05$).

Although the shape of N-SLCs and RLCs is similar when rETR is plotted against PFD and parameters are estimated in the same way to allow a valuable comparison between N-SLCs and RLCs, the estimates do not bear the same physiological signification (cf. Introduction). Moreover the model of Eilers and Peeters (1988) is not appropriate for sequential light curves (RCLs) because the experimental data points are not independent (as required by the model), it is nevertheless commonly used for curve fitting and parameter comparison purposes (as no other model is currently available) (Macedo and Duarte 2006).

Light response curves were compared using the method of Ratkowski (1983) for nonlinear models. The principle is to calculate the “pooled” residual sum of squares from fittings of each individual data set and to compare it to the “common” residual sum of squares resulted from fitting of all data sets simultaneously (Zar 1999). It amounts to testing whether there is a difference in using separate or common parameter estimates. If two curves are different, each parameter estimate is tested individually, according to the method describes in Ratkowski (1983) and using

multiple nonlinear regression curve fitting of Sigmaplot (Systat Software Inc., San Jose, CA, USA).

Results

Fluorescence induction curves following a dark-to-light transition

Before the beginning of illumination periods, the PSII maximum photochemical efficiencies (F_v/F_m) of all biofilms were not significantly different (ANOVA, $P = 0.128$) and showed an average value of 0.721 (± 0.002 , 95% CI) (Fig. 1a).

For each actinic light intensity tested, the first 10 s of illumination were characterized by a decrease of the photochemical efficiency of PSII (Φ_{PSII}) tending towards 0 for the highest light levels (Fig. 1a). This was the result of a simultaneous increase of F_t (Fig. 1c) and a decrease of F'_m (Fig. 1d), the latter reflecting the increase of NPQ (Fig. 1b).

After 10 s of illumination, Φ_{PSII} tended to increase and then levelled off (Fig. 1a). A series of t -tests realized on the differences between the successive means of Φ_{PSII} showed that they were not statistically different from zero ($P > 0.05$) after 50 s of illumination onwards, and for each light level tested. This change in the trend of Φ_{PSII} was due to a decrease of F_t (Fig. 1c) while F'_m continued to decrease (Fig. 1d). The increase of NPQ slowed down after 30 s of illumination but continued to increase slightly and steadily until 130 sec for the highest light levels (Fig. 1b).

Stabilization of the shape of Non-Sequential Light Curves (N-SLCs)

N-SLCs constructed from results of the fluorescence induction curves (Fig. 1) are presented in Fig. 2. It is clear that the rETR vs. PDF curve converges towards a stable shape when the duration of illumination for each light level was at least 50 s. For shorter periods (10 and 30 s), rETR values were systematically lower (Fig. 2), due to the transient characteristics of Φ_{PSII} following a dark–light transition (Fig. 1a).

Thus, the initial slope (α) of the light curve (Fig. 3a) decreased from 0.347 relative units (± 0.049 , 95% CI) for 50 s down to 0.197 relative units (± 0.027 , 95% CI) for 10 s ($P = 0.003$). Similarly, $rETR_{max}$ (Fig. 3b) equalled 66 relative units (± 2 , 95% CI) for 50 s of illumination, whereas $rETR_{max}$ only reached 35 relative units (± 3 , 95% CI) for 10 s of illumination ($P < 0.0001$) (Fig. 3b).

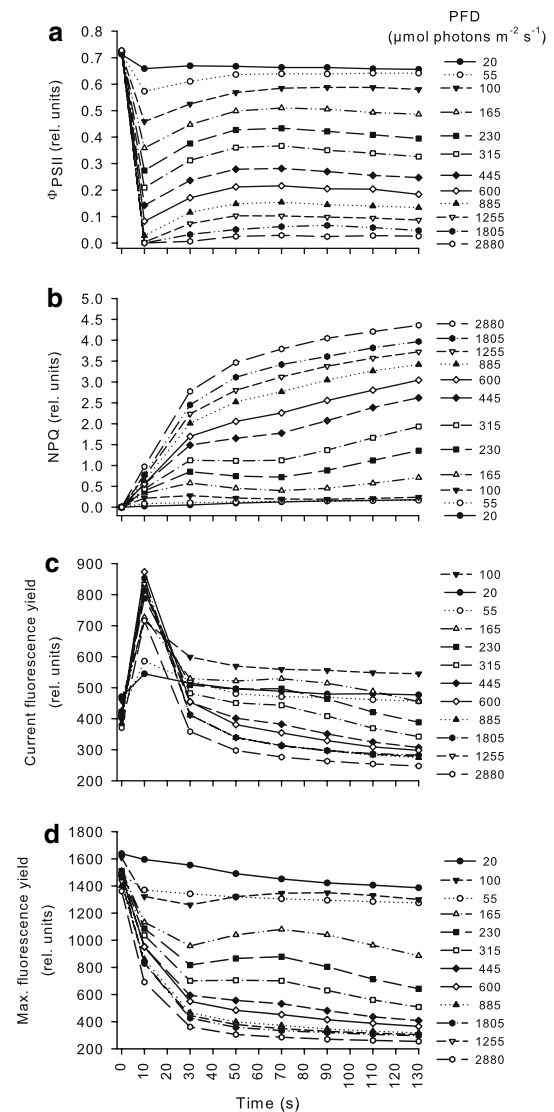


Fig. 1 Fluorescence induction curves following a dark–light shift at 12 different photon flux densities (PFDs). Different biofilms ($35 \text{ mg chl } a \text{ m}^{-2}$) were used in triplicate for the 12 different PFDs tested. Fluorescence parameters, expressed in relative unit (rel. unit), were measured every 20 s, from 10 to 130 s. **a** Kinetics of Φ_{PSII} , **b** kinetics of NPQ, **c** kinetics of current fluorescence yield (F_0 in the dark i.e., at 0 s then F_t in the light), **d** kinetics of maximum fluorescence yield (F_m in the dark i.e., at 0 s then F'_m in the light). Although each measure was realized in triplicate, vertical error bars are not represented for clarity

Comparing Rapid Light Curve (RLC) and Non-Sequential Light Curve (N-SLC)

Rapid Light Curve and Non-Sequential Light Curve could be compared directly because they were based on the same light levels (N-SLC) or light steps (RLC) with the same time of illumination for each PFD (50 s) and were prepared from the same parent community of microphytobenthos. Experimental biofilms used for this comparison were in the same physiological state as evidenced by the lack of

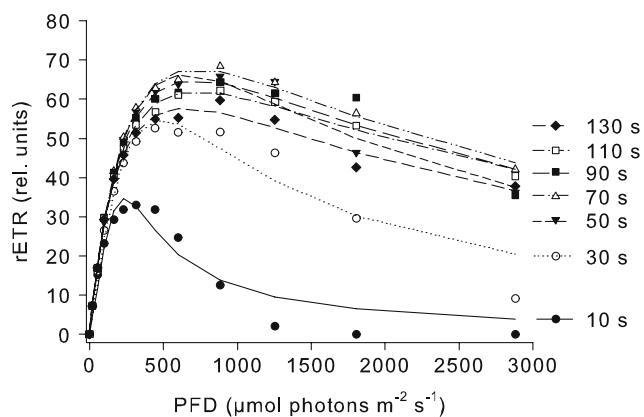


Fig. 2 Non-Sequential Light Curves of biofilms (35 mg chl *a* m⁻²) for 10, 30, 50, 70, 90, 110, and 130 s of illumination for each light level. Light curves were constructed using independent biofilms for each PFD. Mean values of rETR ($n = 3$) were calculated from data presented in Fig. 1a, vertical error bars are not displayed for clarity. Curves represent the model of Eilers and Peeters (1988) fitted to each series of data

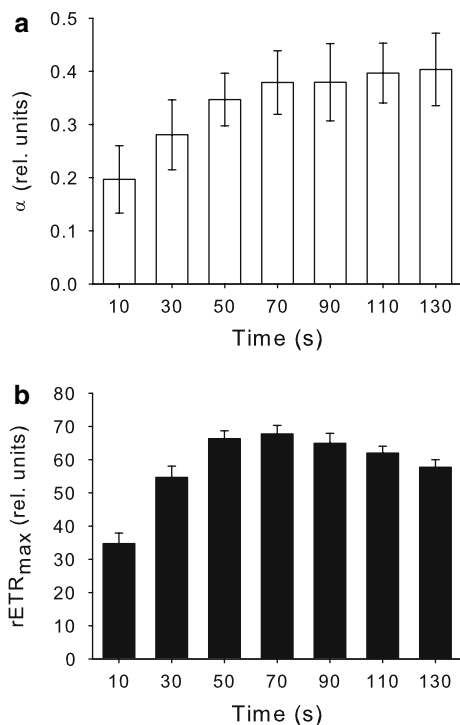


Fig. 3 Parameters (mean \pm 95%CI, $n = 3$) for Non-Sequential Light Curves shown in Fig. 2. **a** Maximum light utilization coefficient (initial slope α) and **b** maximum relative electron transport rate (rETR_{max}), are presented as a function of the duration of the illumination period at each light level (10, 30, 50, 70, 90, 110, and 130 s)

difference (unilateral *t*-test, $P = 0.359$, Table 1) between the values of the maximum quantum efficiency (i.e., in the dark-adapted state); F_v/F_m was $0.720 (\pm 0.001, 95\% \text{ CI})$ for RLC and $0.721 (\pm 0.011, 95\% \text{ CI})$ for N-SLC (Fig. 4a).

Table 1 Results of unilateral *t*-tests performed at each delivered photon flux density (PFD) to compare photochemical efficiency of photosystem II (Φ_{PSII}) and non-photochemical quenching (NPQ) of Rapid Light Curve (RLC) and Non-Sequential Light Curve (N-SLC)

PFD ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$)	Φ_{PSII}	NPQ
0.2	RLC = N-SLC $P = 0.359$	NPQ = 0
20	RLC = N-SLC $P = 0.092$	RLC < N-SLC $P = 0.011$
55	RLC = N-SLC $P = 0.265$	RLC < N-SLC $P = 0.022$
100	RLC > N-SLC $P = 0.005$	RLC < N-SLC $P = 0.005$
165	RLC > N-SLC $P = 0.033$	RLC < N-SLC $P = 0.025$
230	RLC = N-SLC $P = 0.427$	RLC > N-SLC $P = 0.003$
315	RLC = N-SLC $P = 0.057$	RLC > N-SLC $P < 0.001$
445	RLC < N-SLC $P = 0.040$	RLC > N-SLC $P < 0.001$
600	RLC < N-SLC $P = 0.026$	RLC > N-SLC $P < 0.001$
885	RLC < N-SLC $P = 0.008$	RLC > N-SLC $P = 0.003$
1,255	RLC < N-SLC $P = 0.02$	RLC > N-SLC $P = 0.002$
1,805	RLC < N-SLC $P = 0.014$	RLC > N-SLC $P = 0.004$
2,880	RLC = N-SLC $P = 0.151$	RLC > N-SLC $P = 0.004$

In the lower range of PFDs (below $165 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$), RLC exhibited significantly higher values of Φ_{PSII} at 100 and $165 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Fig. 4a; Table 1) and significantly lower values of NPQ between 20 and $165 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Fig. 4b; Table 1). During the RLC, the decrease of Φ_{PSII} between 20 and $100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ was related to the increase of F_t while F_m' remained relatively steady (Fig. 5).

Above $445 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ Φ_{PSII} became significantly higher for N-SLC than for RLC (Fig. 4a; Table 1), and NPQ values became significantly higher for RLC from $230 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ onwards (Fig. 4b; Table 1).

The rETR vs. PFD curves realized through RLC and N-SLC are presented in Fig. 4c. In terms of the physiological parameters derived from both types of response curves, the initial slope (α) was significantly higher for RLC (0.446 ± 0.069 relative units, 95%CI) than for N-SLC (0.347 ± 0.049 relative units, 95%CI) ($P < 0.017$). It was

Fig. 4 Comparison between Rapid Light Curve (*closed circles*) and Non-Sequential Light Curve (*open circles*) of experimental biofilms prepared from the same microalgal community. The step light duration of RLC was set at 50 s, and the duration of illumination at each light level of the N-SLC was also set at 50 s. **a** Comparison of Φ_{PSII} , **b** NPQ and **c** rETR variations. Mean values are reported ($n = 3$) with vertical error bars represent the 95% confidence interval. In **c** rETR vs. PFD curves represent the model of Eilers and Peeters (1988) fitted to RLC and N-SLC datasets. Insets display enlargement of the curves for the first five light intensities (between 0 and $250 \mu\text{mol photons m}^{-2} \text{s}^{-1}$)

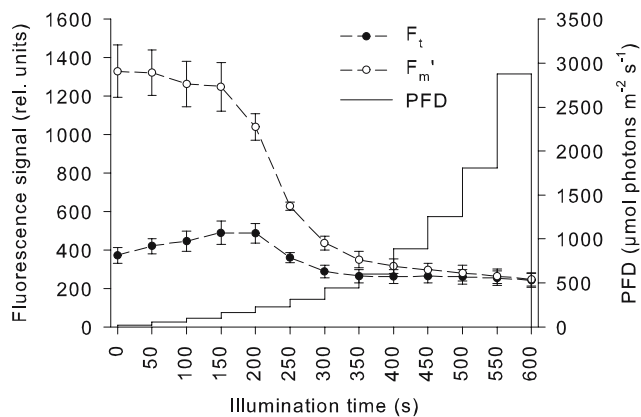
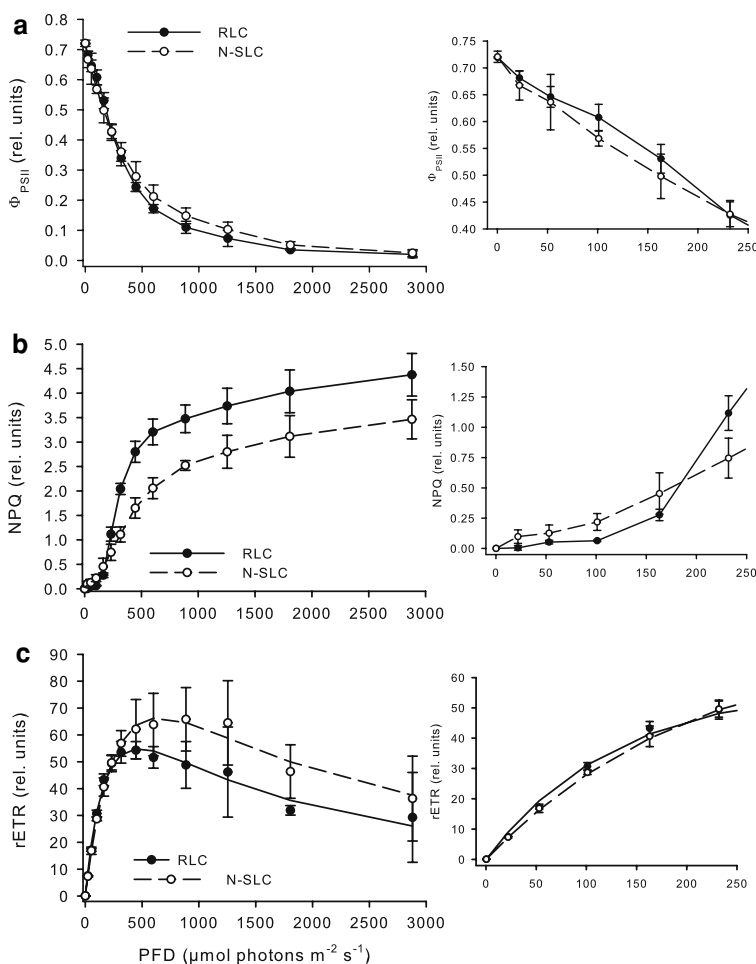


Fig. 5 Kinetics of fluorescence parameters during Rapid Light Curve. *Closed circles* represent kinetic of the current fluorescence yield (F_0 in the dark i.e., at 0 s then F_t in the light). *Open circles* represent kinetic of maximum fluorescence yield (F_m in the dark i.e., at 0 s then F'_m in the light). Measurements were realized in triplicate, each point represents and average value with the 95% confidence interval. Curve in the shape of stairs represents the increasing light steps of PFD during RLC, with an incremental time of 50 s

the reverse for $rETR_{max}$ with a mean value significantly higher ($P < 0.0001$) for N-SLC (66 ± 2 relative units, 95%CI) than for RLC, for which $rETR_{max}$ equalled 55 ± 2 relative units, 95%CI.

Discussion

Fluorescence induction curves following a dark-to-light transition

Before illumination periods, experimental biofilms kept in the dark had a maximum photochemical efficiency of PSII (F_v/F_m) greater than 0.7 (Fig. 1a), indicating that, at the most, 72% (± 0.2 , 95%CI) of photons absorbed by microalgae could be used in photochemistry. Although this maximum photochemical efficiency is lower than that recorded for higher plants in dark-adapted state (generally $F_v/F_m = 0.8$, Krause and Weis 1991; Schreiber 2004), it indicates a good physiological state of microalgae (Ting and Owens 1992; Ibelings et al. 1994; Kromkamp et al.

1998) and a lack of stress due to nutrient limitations (Parkhill et al. 2001).

Kinetics of PSII photochemical efficiency (Φ_{PSII}), non-photochemical quenching (NPQ) and fluorescence yields (F_t and F'_m) (Fig. 1) represent the basic information to analyze photoacclimation of the photosynthetic apparatus of microalgae during a dark-to-light transition. Such a sudden light exposure of dark-acclimated organisms reflects the field conditions when cells reach the sediment surface at the beginning of emersion periods.

The increase of the fluorescence yield (F_t) during the first 10 s of illumination (Fig. 1c) is characteristic of the closure of PSII reaction centres, when microalgae are transferred from darkness to light (Oxborough 2004): upon illumination, electron transport starts within milliseconds and, in the photosynthetic pathway downstream of PSII, the first quinone-type acceptors (Q_A) are reduced. However, the reduction of Q_A is faster than its oxidation, thus leading to an accumulation of reduced electron-acceptors between both photosystems. The result is a progressive closure (or reduction) of PSII reaction centres which leads to a dispersion of light energy, mainly by fluorescence (Oxborough 2004; Schreiber 2004).

After 10 s of illumination, the increase of Φ_{PSII} (Fig. 1a) indicates an optimization of the whole photochemical process, likely due to the induction of photoprotective mechanisms of excess energy dissipation, as suggested by the decrease of the fluorescence yields F_t and F'_m (Fig. 1c, d) and consequently the increase of non-photochemical quenching (NPQ) (Fig. 1d). Indeed, after a few seconds of illumination, the electron transport between the two photosystems induces the building up of a proton gradient through the thylakoïd membrane (Krause and Weis 1991). Then, the consecutive acidification of the lumen causes a conversion of diadinoxanthin (DD) into diatoxanthin (DT) in diatoms, thus leading to the dissipation of excessive light energy into heat (Casper-Lindley and Björkman 1998; Lavaud et al. 2002a, 2002b, 2004; Serôdio et al. 2005a). This process is called energy dependent quenching (q_E) and might explain the stabilization of Φ_{PSII} from 50 s of illumination onwards.

Stabilization of the shape of Non-Sequential Light Curves (N-SLCs)

An illumination period of 50 s at each light level appears to be the minimum time required to generate a stable response curve (N-SLC) for epipellic microphytobenthos organized in biofilm (Fig. 2). Shorter periods of illumination would induce a distortion of the light curve shape, hence an under-estimation of the basic physiological parameters α and $r\text{ETR}_{\text{max}}$ and a misinterpretation of the physiological

characteristics of microalgae, as also pointed out recently by Perkins et al. (2006). However, the illumination time required to get a stable N-SLC might depend of the composition and the light history of photosynthetic organisms. This period of time is therefore likely to change: 120 s are required to get a stable physiological response curve for cultures of light-acclimated benthic diatoms (*Navicula phyllepta*) (Perkins et al. 2006) and other previous studies have even reported higher values, between 5 and 45 min (Hartig et al. 1998; Perkins et al. 2001; Lavaud et al. 2002b).

Although we did not test the effect of the duration of each light step on RLCs' shape, Perkins et al. (2006) clearly demonstrated, using a similar experimental setup, that the duration of each light step and the way the light dose was delivered (stepwise increase or decrease) had a strong influence on the resulting light response curve. In particular, $r\text{ETR}_{\text{max}}$ was systematically higher for longer light steps and RLCs failed to saturate when applying incremental increases in irradiance (based on a light step of 60 s). In contrast, the RLCs that we measured in our study (Fig. 4c, based on a light step of 50 s) not only reached saturation but also exhibited a downturn at high irradiances, thus indicating down regulation of the microphytobenthic biofilm (Henley 1993). This discrepancy might be explained by the difference in the light history experienced by microalgae prior to the measurement of the light response curves. Indeed, our experimental biofilms were maintained in the dark overnight until the measurements while microalgal cultures were previously acclimated for 1 h to low light ($25 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) or high light ($400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) in Perkins et al. (2006) study. They further showed that the high light acclimated cultures were closer to saturation than those acclimated to low light.

Comparing Rapid Light Curve (RLC) and Non-Sequential Light Curve (N-SLC)

We have shown that an illumination period of 50 s was long enough to stabilize the PSII photochemical efficiency (Φ_{PSII}) and the shape of N-SLCs. In addition, referring to the recent studies of Perkins et al. (2006) and Serôdio et al. (2005b) who found close values (60 and 90 s, respectively), we further hypothesized that the same period of illumination would also be long enough to obtain 'steady state' RLCs. Therefore, we based our comparison between RLCs and N-SLCs on this time period. As expected from our theoretical analysis, our experimental test pointed out significant differences between both methods, which can be characterized by 2 phases.

In the lower range of PFDs (up to $165 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) or in the first step of RLC (up to 150 s of light

dose accumulation), the higher values of Φ_{PSII} in RLC reflect a better optimization of photon use in photochemistry. The incremental increase of PFDs is indeed expected to slow down the build up of the proton gradient through the thylakoïd membrane and, hence, minimize the energy-dependent quenching (qE) (White and Critchley 1999; Ralph and Gademann 2005). The lower NPQ levels observed in the lower RLC light levels support this assumption (Fig. 4b; Table 1). Therefore, during the first 150 s of illumination (or under a threshold of light dose accumulation), the excessive light energy is mainly dissipated by fluorescence (Fig. 5), which results on the light response curves in a higher value of α in RLC than in N-SLC.

However, in the upper range of PFDs (above $445 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) or in the second step of RLC (period of light dose accumulation longer than 150 s), the photochemical efficiency of PSII (Φ_{PSII}) became less efficient in RLC than in N-SLC. This reversal in the trend corresponds to a threshold in the light dose accumulation (350 s of light exposure for RLC) above which the excessive light energy is mainly dissipated by NPQ (Fig. 4b; Table 1). This results on the light response curves in higher values of $r\text{ETR}_{\text{max}}$ in N-SLC than in RLC (Fig. 4c).

Conclusion

The methodology of light response curves in conjunction with the technical advances of PAM fluorometry raises fundamental questions about the ecophysiological interpretation of the measurements. In the present work, we have demonstrated through an experimental approach that the photobiological response of epipellic microphytobenthos is directly influenced by their immediate light environment, i.e., the light dose accumulation during the measurement. In this regard, physiological parameters derived from RLCs are qualitatively and quantitatively different from those derived from N-SLCs. Therefore, the choice of one method or the other depends on the objective of the study. Although this study was performed on experimental microphytobenthic biofilm, which represents the primary production system of intertidal mudflats, it is also worth noting that our conclusions potentially concern the other domains of applications of PAM fluorometry.

Thus, N-SLCs can be considered as a “static” photobiological response, based on independent measurements, for which the photosynthetic activity varies as a function of the light gradient only (PFDs). N-SLCs would be a method of choice when the objective is to assess the potential photosynthetic activity (Schreiber 2004) and to compare it among communities from different habitats or periods.

However, this is a time-consuming method which is not appropriate for field studies. Users of this approach should take care to reach a steady state for each PFD tested even for short duration of illumination to avoid underestimation of the physiological parameters derived from the light curves.

RLCs are undoubtedly more appropriate to assess a “dynamic” photobiological response representative of field conditions with rapid light variations. RLCs allow to assess the effective photosynthetic activity (White and Critchley 1999; Ralph and Gademann 2005). This method is fundamentally different from N-SLCs because the light response is a double function of light and time. In the particular application to epipellic microphytobenthos, this approach is very useful in situ because it is rapid and easy to use, and allows detection of changes in the physiological status of microalgae under rapidly fluctuating light conditions (Serôdio et al. 2005b). However, users of this method should take care of the way the PFDs are delivered and the duration of the light steps because it implies a specific physiological response (Ralph and Gademann 2005; Serôdio et al. 2005b; Perkins et al. 2006).

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