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

O. Herlory · P. Richard · G. F. Blanchard

Received: 17 April 2007 / Accepted: 6 August 2007 / Published online: 25 August 2007 © Springer-Verlag 2007

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 ($\alpha$ the light utilization coefficient in light-limited conditions and $\text{rETR}_{\text{max}}$ 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 $\alpha$; 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 $\text{rETR}_{\text{max}}$ 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 Seroüdo 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.)

Communicated by S.A. Poulet.

O. Herlory · P. Richard · G. F. Blanchard
Centre de Recherche sur les Ecosystèmes Littoraux Anthropisés (CRELA), UMR 6217, Université de La Rochelle-CNRS-IFREMER, Avenue Michel Crépeau, La Rochelle 17042, France

O. Herlory (✉)
Netherlands Institute of Ecology (NIOO-KNAW), P.O. Box 140, Yerseke 4400 AC, The Netherlands
e-mail: o.herlory@nioo.knaw.nl
Microphytobenthos assemblages are frequently dominated by epipelic (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. 2004). 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 photosynthetic efficiency (ΦPSII) 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 rETRmax) 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 phylepta (Kutz) 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 epipelic 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 epipelic 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 scraping the upper millimetres in areas of dense microalgal mats. On return to the laboratory, the epipelagic 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 hold 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, epipelic cells had accumulated at the sediment surface, particularly within the upper nets. Epipelic microphytobenthos was then collected by rinsing the two upper nets with filtered seawater. The resulting suspension of the natural epipelagic 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 epipelagic 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 epipelagic 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. Biofilms can be prevented and epipelic cells thus form a non-migratory biofilm on the bottom of the tube. In the present experiment, the quantum efficiency of photosystem II (PSII) was measured by the saturation pulse technique, whereby a light flash (5,000 μmol photons m–2 s–1) was 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 using the Diving PAM fluorometer (Walz, Effeltrich, Germany) at a constant light and temperature of 5°C to maintain the microphytobenthos in the same state of dark adaptation. 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).

Measurement of chlorophyll fluorescence parameters

The photosynthetic efficiency of PSII (PSII) was measured by the saturation pulse technique, whereby a light flash (5,000 μmol photons m–2 s–1) was emitted by the Diving PAM to measure the maximum fluorescence yield (Fm in the darkness and Fm′ in the light).

The dark-acclimated state, the maximum quantum efficiency of PSII was calculated as

\[ \frac{F_m - F_s}{F_m} \]

with F0 the minimum fluorescence yield measured prior to the saturation pulse. At each light level, the photochemical efficiency of PSII (ΦPSII) was calculated as

\[ \frac{F_m - F_s}{F_m} \]

with F0 being the current fluorescence yield in the light measured just before the saturation pulse, which is equivalent to Fm′ in the light (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 with PSII that is used in photochemistry.
(ΦPSII) 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-photocatalytic 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 (ΦPSII) 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 (ΦPSII).

Therefore, dark–light induction curves were realized for each of the 12 light intensities (from 20 to 2880 μmol photons m\(^{-2}\) 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 (ΦPSII) 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 \((F_m/F'_m)\) of the biofilm in the dark-adapted state. Three replicates were performed for each actinic light level, that is 36 different experimental biofilms (3 x 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 epipelic 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 μmol photons m\(^{-2}\) 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 (ΦPSII, 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 \(z\) (the light utilization coefficient in light-limited conditions), \(rETR_{max}\) (the maximum rate of photosynthesis efficiency) and \(E_k\) (the light saturation coefficient):

\[
rETR = \frac{E}{2 \times rETR_{max} + \frac{2 \times E_k}{E_k - 2 \times E_k} \frac{1}{2}}
\]

where \(E\) represents the Photon Flux Density (PFD).

This transformation enabled to estimate directly the values of \(z\), \(rETR_{max}\) 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 (RLCs) 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 ($\pm 0.002$, 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 ($\Phi_{PSII}$) 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 $F_0m$ (Fig. 1d), the latter reflecting the increase of NPQ (Fig. 1b).

After 10 s of illumination, $\Phi_{PSII}$ tended to increase and then levelled off (Fig. 1a). A series of $t$-tests realized on the differences between the successive means of $\Phi_{PSII}$ 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 $\Phi_{PSII}$ was due to a decrease of $F_t$ (Fig. 1c) while $F_0m$ 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 $\Phi_{PSII}$ following a dark–light transition (Fig. 1a).

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

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...
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$0.001, 95% CI) for RLC and 0.721 ($\pm$0.011, 95% CI) for N-SLC (Fig. 4a).

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

Above 445 $\mu$mol photons m$^{-2}$ s$^{-1}$ $\Phi_{PSII}$ became significantly higher for N-SLC than for RLC (Fig. 4a; Table 1), and NPQ values became significantly higher for RLC from 230 $\mu$mol photons m$^{-2}$ 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 ($\alpha$) was significantly higher for RLC (0.446 $\pm$ 0.069 relative units, 95% CI) than for N-SLC (0.347 $\pm$ 0.049 relative units, 95% CI) ($P < 0.017$). It was
the reverse for $r\text{ETR}_{\text{max}}$ with a mean value significantly higher ($P < 0.0001$) for N-SLC (66 ± 2 relative units, 95%CI) than for RLC, for which $r\text{ETR}_{\text{max}}$ 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. 2003).
Kinetics of PSII photochemical efficiency ($\Phi_{PSII}$), 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 $\Phi_{PSII}$ (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 thylakoid 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 excess 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 $\Phi_{PSII}$ 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 epipelic 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 $z$ and rETR$_{max}$ 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, rETR$_{max}$ 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 μmol photons m$^{-2}$ s$^{-1}$) or high light (400 μmol photons m$^{-2}$ 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 ($\Phi_{PSII}$) 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 μmol photons m$^{-2}$ s$^{-1}$) or in the first step of RLC (up to 150 s of light...
dose accumulation), the higher values of $\Phi_{PSII}$ 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 thylakoid membrane and, hence, minimize the energy-dependent quenching ($q_E$) (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 $z$ in RLC than in N-SLC.

However, in the upper range of PFDs (above 445 pmol photons m$^{-2}$ s$^{-1}$) or in the second step of RLC (period of light dose accumulation longer than 150 s), the photochemical efficiency of PSII ($\Phi_{PSII}$) 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 $rETR_{\text{max}}$ 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 epipelic 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 epipelic 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).

Acknowledgments This study forms part of the PhD thesis of O. Herlory. This work was financially supported by “Ministère de la Jeunesse, de l’Éducation Nationale et de la Recherche”, by the program “ACI Ecologie Quantitative”, and by the Région Poitou-Charentes. We thank S. Lefebvre for his helpful advices in curve fitting.

References

Casper-Lindley C, Björkman O (1998) Fluorescence quenching in four unicellular algae with different light-harvesting and xanthophyll-cycle pigments. Photosynth Res 56:277–289
Colijn F, De Jonge VN (1984) Primary production of microphytobenthos in the Ems-Dollard estuary. Mar Ecol Prog Ser 14:185–196
Consalvey M, Jesus B, Perkins RG, Brotas V, Underwood GJC, Paterson DM (2004a) Monitoring migration and measuring biomass in benthic biofilms: the effects of dark/far-red adaptation and vertical migration on fluorescence measurements. Photosynth Res 81:91–101
Consalvey M, Paterson DM, Underwood GJC (2004b) The ups and downs of life in a benthic biofilm: migration of benthic diatoms. Diatom Res 19:181–202
Consalvey M, Perkins RG, Paterson DM, Underwood GJC (2005) PAM fluorescence: a beginners guide for benthic diatomists. Diatom Res 20:1–22
Defew EC, Paterson DM, Hagerthey SE (2002) The use of natural microphytobenthic assemblages as laboratory model systems. Mar Ecol Prog Ser 237:15–25
Easley JT, Hymel SN, Plante CJ (2005) Temporal patterns of benthic microalgal migration on a semi-protected beach. Estuar Coast Shelf Sci 64:486–496
Eilers PHC, Peeters JCH (1988) A model for the relationship between light-intensity and the rate of photosynthesis in phytoplankton. Ecol Model 42:199–215
Forster RM, Kromkamp JC (2004) Modelling the effects of chlorophyll fluorescence from subsurface layers on photosynthetic
efficiency measurements in microphytobenthic algae. Mar Ecol Prog Ser 284:9–22

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

Gouleau D (1976) The role of benthic diatoms in rapid fixation of muddy Atlantic tidal flats. C R Hebd Seances Acad Sci Paris Ser D 283:21–23

Guarini J-M, Blanchard GF, Gros P, Gouleau D, Bacher C (2000) Dynamic model of the short-term variability of microphytobenthic biomass on temperate intertidal mudflats. Mar Ecol Prog Ser 195:291–303

Hartig P, Wolfstein K, Lippemeier S, Colijn F (1998) Photosynthetic activity of natural microphyto-benthos populations measured by fluorescence (PAM) and 14C-tracer methods: a comparison. Mar Ecol Prog Ser 166:53–62

Hawes I, Sutherland D, Hanelt D (2003) The use of pulse amplitude modulated fluorometry to determine fine-scale temporal and spatial variation of in situ photosynthetic activity within an Isoetes-dominated canopy. Aquat Bot 77:1–15

Henley WJ (1993) Measurement and interpretation of photosynthetic light-response curves in algae in the context of photoinhibition and diel changes. J Phycol 29:729–739

Herlory O, Guarini J-M, Richard P, Blanchard GF (2004) Microstructure of microphytobenthic biofilm and its spatio-temporal dynamics in an intertidal mudflat (Aiguillon Bay, France). Mar Ecol Prog Ser 282:33–44

Herlory O, Blanchard GF, Planche S, Huet V, Richard P (2005) Does the size of the temporary microphytobenthic biofilm on intertidal mudflats depend on the available photosynthetic biomass? Mar Ecol Prog Ser 298:95–100

Honeywill C, Paterson DN, Hagerthey SE (2002) Determination of microphytobenthic biomass using pulse-amplitude modulated minimum fluorescence. Eur J Phycol 37:485–492

Ibelings BW, Kroon BMA, Mur LR (1994) Acclimation of photosystem-II in a cyanobacterium and a eukaryotic green-alga to high and fluctuating photosynthetic photon flux densities, simulating light regimes Induced by mixing in lakes. New Phyol 128:407–424

Jesus B, Brotas V, Marani M, Paterson DM (2005) Spatial dynamics of microphytobenthos determined by PAM fluorescence. Estuar, Coast Shelf Sci 65:30–42

Jesus B, Perkins RG, Consalvey M, Brotas V, Paterson DM (2006a) Effects of vertical migrations by benthic microalgae on fluorescence measurements of photosphyiology. Mar Ecol Prog Ser 315:55–66

Jesus B, Perkins RG, Mendes CR, Brotas V, Paterson DM (2006b) Chlorophyll fluorescence as a proxy for microphytobenthic biomass: alternatives to the current methodology. Mar Biol 150:17–28

Krause GH, Weis E (1991) Chlorophyll fluorescence and photosynthesis: the basics. Annu Rev Plant Physiol Plant Mol Biol 42:313–349

Kromkamp J, Barranguet C, Peene J (1998) Determination of microphytobenthos PSII quantum efficiency and photosynthetic activity by means of variable chlorophyll fluorescence. Mar Ecol Prog Ser 162:45–55

Lavaud J, Rousseau B, Etienne A-L (2002a) In diatoms, a trans-thylakoid proton gradient alone is not sufficient to induce a non-photochemical fluorescence quenching. FEBS Lett 523:163–166

Lavaud J, Rousseau B, van Gorkom HJ, Etienne A-L (2002b) Influence of the diadinoxanthin pool size on photoprotection in the marine planktonic diatom Phaeodactylum tricornutum. Plant Physiol 129:1398–1406

Lavaud J, Rousseau B, Etienne A-L (2004) General features of photoprotection by energy dissipation in planktonic diatoms (Bacillariophyceae). J Phycol 40:130–137

Lorenz CJ (1966) A method for the continuous measurement of in vivo chlorophyll concentration. Deep Sea Res Oceanogr Abstracts 13:223–227

Lorin J (1968) Contribution à l’étude des transit sédimentaires dans la partie orientale du Pertuis Breton et la baie de l’Aiguillon. Bulletin de l’Institut Géologique du Bassin d’Aquitaine 5:111–139

Macedo MF, Duarte P (2006) Phytoplankton production modelling in three marine ecosystems - static versus dynamic approach. Ecol Modell 190:299–316

MacIntyre HL, Geider RJ, Miller DC (1996) Microphytobenthos: the ecological role of the “secret garden” of unvegetated, shallow-water marine habitats. J. Distribution, abundance and primary production. Estuaries 19:186–201

Morris EP, Kromkamp JC (2003) The influence of temperature on the relationship between oxygen- and fluorescence-based estimates of photosynthetic parameters in a marine benthic diatom (Cylindrotheca closterium). Eur J Phycol 38:133–142

Oxborough K, Hanlon ARM, Underwood GIC, Baker NR (2000) In vivo estimation of the photosystem II photochemical efficiency of individual microphytobenthic cells using high-resolution imaging of chlorophyll a fluorescence. Limnol Oceanogr 45:1420–1425

Oxborough K (2004) Imaging of chlorophyll a fluorescence: theoretical and practical aspects of an emerging technique for the monitoring of photosynthetic performance. J Exp Bot 55:1195–1205

Parkhill J-P, Maillet G, Cullen JJ (2001) Fluorescence-based maximal quantum yield for PSII as a diagnostic of nutrient stress. J Phycol 37:517–529

Paterson DM (1986) The migratory behaviour of diatom assemblages in a laboratory tidal micro-ecosystem examined by low temperature scanning electron microscopy. Diatom Res 1:227–239

Paterson DM, Crawford RM, Little C (1986) The structure of benthic diatom assemblages: a preliminary account of the use and evaluation of low-temperature scanning electron microscopy. J Exp Mar Biol Ecol 95:279–289

Paterson DM (1995) Biogenic structure of early sediment fabric visualized by low-temperature scanning electron microscopy. J Geol Soc London 152:131–140

Perkins RG, Underwood GIC, Brotas V, Snow GC, Jesus B, Ribeiro L (2001) Responses of microphytobenthos to light: primary productivity and carbohydrate allocation over an emersion period. Mar Ecol Prog Ser 223:101–112

Perkins RG, Oxborough K, Hanlon ARM, Underwood GIC, Baker NR (2002) Can chlorophyll fluorescence be used to estimate the rate of photosynthetic electron transport within microphytobenthic biofilms? Mar Ecol Prog Ser 228:47–56

Perkins RG, Mouget J-L, Lefebvre S, Lavaud J (2006) Light response curve methodology and possible implications in the application of chlorophyll fluorescence to benthic diatoms. Mar Biol 149:703–712

Ralph PJ, Gademann R (2005) Rapid light curves: a powerful tool to assess photosynthetic activity. Aquat Bot 82:222–237

Rascher U, Liebig M, Luttgé U (2000) Evaluation of instant light-response curves of chlorophyll fluorescence parameters obtained with a portable chlorophyll fluorometer on site in the field. Plant Cell Environ 23:1397–1405

Ratkowski DA (1983) Nonlinear regression modeling. A unified practical approach. Marcel Dekker Inc., New-York

Riera P, Stal LJ, Nieuwenhuize J, Richard P, Blanchard G, Gentil F (1999) Determination of food sources for benthic invertebrates in a salt marsh (Aiguillon Bay, France) by carbon and nitrogen...
stable isotopes: importance of locally produced sources. Mar Ecol Prog Ser 187:301–307
Round FE (1971) Benthic marine diatoms. Oceanogra Mar Biol. An Annu Rev 9:83–139
Sakshaug E, Bricaud A, Dandonneau Y, Falkowski PG, Kiefer DA, Legendre L, Morel A, Parslow J, Takahashi M (1997) Parameters of photosynthesis: definitions, theory and interpretation of results. J Plankton Res 19:1637–1670
Schreiber U, Bilger W (1993) Progress in chlorophyll fluorescence research: major developments during the past years in retrospect. Prog Bot 54:151–173
Schreiber U, Gademann R, Ralph PJ, Larkum AWD (1997) Assessment of photosynthetic performance of Prochloron in Lissoclinum patella in hospite by chlorophyll fluorescence measurements. Plant Cell Physiol 38:945–951
Schreiber U (1998) Chlorophyll fluorescence: new instruments for special applications. In: Garab G (ed) Photosynthesis: mechanisms and effects. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 4253–4258
Schreiber U (2004) Pulse-Amplitude-Modulation (PAM) fluorometry and saturation pulse method: an overview. In: Papageorgiou GC, Godvindjee (eds) Chlorophyll a fluorescence: a signature of photosynthesis. Kluwer Academic Publishers, Dordrecht The Netherlands, pp 279–319
Seródio J, Silva JM, Catarino F (1997) Nondestructive tracing of migratory rhythms of intertidal benthic microalga using in vivo chlorophyll a fluorescence. J Phycol 33:542–553
Seródio J, Da Silva JM, Catarino F (2001) Use of in vivo chlorophyll a fluorescence to quantify short-term variations in the productive biomass of intertidal microphytobenthos. Mar Ecol Prog Ser 218:45–61
Seródio J (2003) A chlorophyll fluorescence index to estimate short-term rates of photosynthesis by intertidal microphytobenthos. J Phycol 39:33–46
Seródio J (2004) Analysis of variable chlorophyll fluorescence in microphytobenthos assemblages: implications of the use of depth-integrated measurements. Aquat Microb Ecol 36:137–152
Seródio J, Cruz S, Vieira S, Brotas V (2005a) Non-photochemical quenching of chlorophyll fluorescence and operation of the xanthophyll cycle in estuarine microphytobenthos. J Exp Mar Biol Ecol 326:157–169
Seródio J, Vieira S, Cruz F, Barroso F (2005b) Short-term variability in the photosynthetic activity of microphytobenthos as detected by measuring rapid light curves using variable fluorescence. Mar Biol 146:903–914
Seródio J, Vieira S, Cruz F, Coelho H (2006) Rapid light-response curves of chlorophyll fluorescence in microalgae: relationship to steady-state light curves and non-photochemical quenching in benthic diatom-dominated assemblages. Photosynth Res 90:29–43
Ting CS, Owens TG (1992) Limitations of the pulse-modulated technique for measuring the fluorescence characteristics of algae. Plant Physiol 100:367–373
Underwood GJC, Kromkamp J (1999) Primary production by phytoplankton and microphytobenthos in estuaries. In: Nedwell DB, Raffaelli DG (eds) Estuaries. Advances in ecological research. Academic Press, London, pp 93–153
Underwood GJC (2002) Adaptations of tropical marine microphytobenthic assemblages along a gradient of light and nutrient availability in Suva Lagoon, Fiji. Eur J Phycol 37:449–462
Underwood GJC, Perkins RG, Consalvey MC, Hanlon ARM, Oxborough K, Baker NR, Paterson DM (2005) Patterns in microphytobenthic primary productivity: species-specific variation in migratory rhythms and photosynthetic efficiency in mixed-species biofilms. Limnol Oceanogr 50:755–767
White AJ, Critchley C (1999) Rapid light curves: a new fluorescence method to assess the state of the photosynthetic apparatus. Photosynth Res 59:63–72
Zar JH (1999) Biostatistical analysis, 4th edn. Prentice-Hall, Inc., Upper Saddle River, New Jersey