Effects of vertical migrations by benthic microalgae on fluorescence measurements of photophysiology

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ABSTRACT: During tidal emersion, microphytobenthic algae (principally pennate diatoms) migrate vertically within the top mm of sediment to form transient biofilms. This migration has been described as an evolutionary strategy, maximizing photosynthetic activity whilst minimizing photo-damage. This study investigated the effect of vertical migration on photophysiological parameters measured by pulse amplitude modulated (PAM) fluorescence, comparing migratory biofilms with artificially created non-migratory biofilms. Dark adaptation for 5 min, a light response curve and 18 min dark recovery period were sequentially applied to each biofilm treatment. Vertical migration and the variable chlorophyll fluorescence signal from cells migrating to the sub-surface significantly affected all variables measured (minimum fluorescence yield, F_0, and maximum yields, F_m or F_m' in the dark and light adapted states respectively). Maximum relative electron transport rates ($rETR_{max}$) calculated from light response curves were overestimated due to downward migration by cells in avoidance of high light. Changes in F_m' due to vertical migration caused underestimation of non-photochemical quenching (NPQ). F_0, often used as a proxy for algal biomass, was significantly lower on migrational biofilms after 5 min dark adaptation compared to non-migratory biofilms due to downward migration in the dark. This response to dark adaptation followed a temporal pattern, showing a stronger tendency for downward migration towards the end of the emersion period. Measurement of photophysiological variables of migrational microphytobenthic biofilms using variable chlorophyll fluorescence must take into account the effects of light induced vertical migration, in order to prevent errors in the calculation of derived parameters.

KEY WORDS: Endogenous rhythms · Microphytobenthos · NPQ · PAM fluorescence · Vertical migration

INTRODUCTION

Microphytobenthic (MPB) biofilms are important components of estuarine intertidal ecosystems that exhibit high rates of primary production (Brotas et al. 1995, Underwood & Kromkamp 1999), contribute substantially to carbon flux (Middelburg et al. 2000) and also influence sediment stability (Paterson & Hagerthey 2001). One of the major groups of microphytobenthos are biraphid pennate diatoms, which have the ability to migrate vertically within the sediment in response to environmental conditions (Admiraal 1984).

Patterns of vertical migration show diurnal and tidal rhythms (Round & Palmer 1966, Serôdio et al. 1997, Paterson et al. 1998, Consalvey 2002), and there is evidence that migration may also be a form of behavioural photoacclimation (Paterson et al. 1998, Underwood & Kromkamp 1999, Perkins et al. 2001, 2002, Consalvey 2002).

MPB biomass has traditionally been determined by taking samples of sediment for pigment extraction and subsequent analysis by spectrophotometry or high performance liquid chromatography, HPLC (Eaton & Moss 1966, Brotas & Plante-Cuny 1995). The recent intro-

duction of pulse-amplitude modulated (PAM) fluorometers has provided an alternative, non-intrusive method for examining microphytobenthos. PAM fluorometry allows migrational behaviour to be monitored over diel and tidal periods (Perkins et al. 2001, Consalvey 2002, Honeywill et al. 2002) and endogenous rhythms to be investigated (Serôdio et al. 1997, Consalvey 2002).

In a laboratory study, Serôdio et al. (1997) showed a positive linear relationship between minimum fluorescence yield (F_0) and the presence of MPB at the sediment surface. *In situ* studies have often used F_0 as a proxy for MPB biomass (Barranguet & Kromkamp 2000, Consalvey 2002, Honeywill et al. 2002, Serôdio 2003). However, F_0 is affected by photophysiological processes such as non-photochemical quenching (NPQ) and the oxidation/reduction status of photosystem reaction centres (Consalvey et al. 2004a). Studies on diatom suspensions have also shown that F_0 might be quenched in the dark due to the maintenance of a trans-thylakoid proton gradient in darkness (e.g. Caron et al. 1987, Ting & Owens 1993), and that the amount of NPQ may be proportional to the light intensity (Serôdio 2003, Fig.7). Thus, the time required to achieve a stable F_0 yield is dependent upon light history. As a practical compromise, the parameter F_0^{15} has been used as a substitute for the true F_0 (Honeywill et al. 2002). F_0^{15} is measured after a 15 min period of dark adaptation, which has shown a significant relationship with chlorophyll *a* (chl *a*) in the surface sediment (Barranguet & Kromkamp 2000, Honeywill et al. 2002).

The main application of variable chlorophyll fluorescence techniques has been in the study of photophysiology. Several parameters are usually measured: (1) photosystem II (PSII) quantum efficiency in the dark (F_v/F_m, where F_m is the maximum dark adapted fluorescence, and F_v is the variable fluorescence calculated as $F_m - F_0$), (2) PSII quantum efficiency in the light-adapted state (F_q'/F_m', where F_m' is the maximum light adapted fluorescence yield, F_q' is the fluorescence quenched by photochemistry calculated as $F_m' - F'$, and F' is the fluorescence yield just prior to application of the saturation pulse used to obtain F_m'; Oxborough et al. 2000), (3) relative electron transport rate (*r*ETR). All these parameters have the potential to offer ecologically relevant information, and are frequently used in primary productivity models (e.g. Barranguet & Kromkamp 2000, Perkins et al. 2001, Serôdio 2003, Forster & Kromkamp 2004, Serôdio 2004, Underwood et al. 2005). Under controlled situations F_v/F_m can also be used to infer microalgae nutrient stress: an example is the work by Geider et al. (1993), where diatoms showed a marked decrease in this parameter under nitrate and iron starvation. Thus, it is crucial that sources of variation in the measurement of all these

parameters are detected and minimized in order to improve our general knowledge of microphytobenthos ecology.

Of particular interest is the use of a high-resolution fluorescence imaging system (Oxborough & Baker 1997, Oxborough et al. 2000, Perkins et al. 2002), which allows fluorescence measurements and real time observations of cell-turnover at the sediment surface. Using this imaging system, Perkins et al. (2002) showed substantial errors in the calculations of *r*ETR for microphytobenthic biofilms caused by vertical migration of cells. If cell migration occurs during fluorescence measurements, calculations of *r*ETR will be affected by a variable error dependent upon the extent and direction of migration. Therefore, it is important to investigate how vertical migration affects fluorescence measurements of MPB photophysiology and the accuracy of F_0 as a biomass proxy for microphytobenthic biomass. Two other recent studies (Forster & Kromkamp 2004, Serôdio 2004) focused on modelling the effect of biomass and depth distribution in the integrated fluorescence measurements. Although reaching slightly different conclusions, both concluded that *r*ETR will be overestimated if no precaution is taken to account for biomass and depth distribution, with a maximum overestimation when sub-surface biomass maximum was observed. This was confirmed by our study (see below).

This study investigated the effect of vertical migration on the measurement of variable fluorescence parameters, including F_0 and others used in the calculation of NPQ and *r*ETR. In order to differentiate between the effects of cell migration and other photophysiological responses, an experimental system was devised in which cell migration was prevented.

MATERIALS AND METHODS

Study sites. Diatoms were collected by removing the upper 0.5 cm of intertidal sediments from 2 estuaries, the Tagus (Portugal, 38° 44′ N, 09° 08′ W) and the Eden (Scotland, 56° 22′ N, 02° 50′ W). The Tagus is a mesotidal estuary, with a mean depth of 5 m, and an intertidal area composed mainly of mudflats that comprise 20 to 40% of the total 320 km^2 area during neap and spring tidal exposure, respectively (Brotas et al. 1995). The Eden Estuary is located on the East Coast of Scotland and is a relatively small (4 km in length) and shallow (1 to 6 m) macro tidal system. The shores vary from sand towards the seaward extreme of the estuary with mudflats dominating the inner estuary. Samples were collected during November 2001 and April 2002 (Eden), and during January and February 2002 (Tagus). Additionally, 2 *in situ* studies were carried out in

the Tagus in March 2002. All experimental measurements were made on the 2 d following sampling.

Non-migratory biofilms. Non-migratory biofilms were prepared using phytotrays. These are small ($55 \times 55 \times 20$ mm) polypropylene membrane rafts with 0.3 µm pores in the basal membrane (SIGMA). Phytotrays are designed for the culture of plant callous material, and the rafts float on the selected media allowing nutrients to be absorbed through the membrane. In this study, filtered seawater collected from the sample site was used. Diatom cells, collected from the estuarine sediment by the lens tissue method (Eaton & Moss 1966), were re-suspended in seawater from the same sample site and, after removing fragments of lens tissue (by filtration through coarse muslin), the suspension was transferred into the phytotrays and allowed to settle to form a thin biofilm.

Phytotray (non-migratory) biofilms were established just prior to measurement, in order to minimise potential problems with desiccation, carbon and nutrient limitation. Also, establishment of the phytotray biofilms just prior to the measurements ensured that the diatom composition of the phytotray biofilm was as similar as possible to the cells at the sediment surface at that particular moment in time. F_v/F_m ratio values were taken as an indicator of cell stress; high F_v/F_m ratio values would indicate that cells were not substantially stressed by manipulation (Geider et al. 1993). Care was taken to ensure that the initial fluorescence yield was similar to its paired sediment biofilm (i.e. similar biomass). Excess seawater was removed by absorption with filter paper.

Vertical migration was prevented as cells were unable to pass through the phytotray membrane pores. The time required for cell settling together with the 31 min of each run were sufficient for a biofilm to form, which adhered strongly to the phytotray, such that active vigorous rubbing was required to remove the biofilm after each experiment. This indicated that during that period an EPS matrix was formed which would reduce the possibility of diatom lateral movements on a cm scale. This also indicated an active, healthy biofilm that bound to the phytotray, rather than a loose separate film free to move in the tray during experiments. Separate phytotrays were used as replicates, with replication varying among sites, but with a minimum of 4 (Tagus 1) and a maximum of 10 (Eden 2).

For fluorescence measurements, it was important to ensure that reflectance from the phytotrays did not significantly increase the light environments to which the biofilms were exposed to. This was achieved by comparing the down-welling light measured by a 2 pi light meter to measurements with a 4 pi meter, which measured the reflected light as well as the direct down-welling light. These measurements were made with a

biofilm in the phytotray of similar biomass to that during experimental measurements. It was found that the 4 pi meter gave integrated light readings between 10 and 80 µmol m^{-2} s^{-1} higher than the 2 pi meter for the light range used in the rapid light curves detailed below (0 to 1100 µmol photosynthetic photon flux density [PPFD] m^{-2} s^{-1}). This difference was not sufficient to induce the differences observed in the results, when non-migratory biofilms were compared to migratory biofilms.

Migratory biofilms. Sediments were brought to the laboratory, homogenised by re-suspension in water from the same sample site, and spread evenly in plastic trays to a depth of approximately 2 cm. The sediments were allowed to settle in the trays overnight, and excess overlying water was removed on the following morning during the period of virtual tide (i.e. coinciding with *in situ* low tide), during which time trays were exposed to 150 µmol PPFD m^{-2} s^{-1} from a sodium lamp, at room temperature (around 20°C). The sediment surface was kept moist during the experiment by regular spraying with sample site seawater. Measurements were replicated on separate sediment trays. The number of replicates varied from 8 (Tagus 1) to 14 (Eden 2).

The procedures described above were used to establish laboratory migratory (sediment trays) and non-migratory assemblages (phytotrays) for comparison with natural assemblages measured *in situ* (Table 1).

Variable chlorophyll fluorescence measurements. Fluorescence was measured using a Diving PAM (Walz). Fluorescence terminology followed Genty et al. (1989), van Kooten & Snell (1990), Oxborough et al. (2000) and Honeywill et al. (2002), except for $F_0{}^5$ and $F_m{}^5$, defined here as the minimum and maximum fluorescence yields, respectively, after 5 min of dark adaptation. The term fluorescence, used throughout the text, refers to variable chlorophyll fluorescence. We used 5 min of dark adaptation rather than the 15 min of Honeywill et al. (2002), to reduce the prob-

Table 1. Sampling sites, dates of sampling, type of biofilm studied and acronyms used throughout text

Site name	Date	Type	Acronym
Eden 1	30 Nov 2001	Phytotray	E1P
Eden 1	30 Nov 2001	Sediment tray	E1S
Eden 2	6 Apr 2002	Phytotray	E2P
Eden 2	6 Apr 2002	Sediment tray	E2S
Tagus 1	31 Jan 2002	Phytotray	T1P
Tagus 1	31 Jan 2002	Sediment tray	T1S
Tagus 2	6 Feb 2002	Phytotray	T2P
Tagus 2	6 Feb 2002	Sediment tray	T2S
Tagus 3	1 Mar 2002	Field sediment—*in situ*	T3F
Tagus 4	2 Mar 2002	Field sediment—*in situ*	T4F

lem of potential downward vertical migration during the darkness period. The saturating pulse was 9500 µmol m^{-2} s^{-1} for 800 ms. The measuring frequency was 0.6 KHz. The measuring fluorometer probe was fixed at a distance of 4 mm from the sample surface using a custom made holder that simultaneously covered the sample for dark adaptation (Honeywill et al. 2002). *In situ* measurements began immediately after tidal emersion and continued until tidal immersion. The number of temporal measurement between field sites varied due to different tidal exposure periods (n = 3 for Tagus 3, and n = 5 for Tagus 4). Laboratory measurements were made to coincide with the time of virtual low tide on the following 2 d after sediment collection.

Dark adaptation and light response curves were performed as follows. The fluorescence yield just prior to the application of the saturating pulse (F_0') and the maximum fluorescence yield during the saturating pulse (F_m') were measured 5 s after the transition of ambient light (150 µmol PPFD m^{-2} s^{-1} measured with the PAM light sensor) to darkness, followed by F_0^5 and F_m^5 after 5 min of dark adaptation. Light response curves with 8 light steps (0 to 1100 µmol PPFD m^{-2} s^{-1}) were then recorded with increases in PPFD at 1 min intervals. Photosynthesis-light response curves were followed by 18 min dark recovery, during which minimum and maximum fluorescence yields were measured after 10, 30, 60 s, 5 and 10 min.

NPQ was calculated from Eq. 1, modified from Bilger & Björkman (1994):

$$\text{NPQ} = \frac{F_m^5 - F_m'}{F_m'} \qquad (1)$$

rETR was calculated following Sakshaug et al. (1997) and Perkins et al. (2001, 2002):

$$r\text{ETR} = \frac{F_q'}{F_m'} \times \text{PPFD} \qquad (2)$$

rETR vs. light response curves were constructed using Eq. 2. The maximum rate of electron transport at light saturation ($r\text{ETR}_{max}$), the maximum light utilisation coefficient (α), and the photo-inhibition parameter (β) were calculated by iteration (Eq. 3, Platt et al. 1980):

$$r\text{ETR} = r\text{ETR}_{max} \left[1 - e^{\left(-\frac{\alpha \times \text{PPFD}}{r\text{ETR}_{max}} \right)} \right] \times e^{\left(-\frac{\beta \times \text{PPFD}}{r\text{ETR}_{max}} \right)} \qquad (3)$$

The fluorescence yields over the light response curves (F' and F_m') were recalculated using the recursive equations suggested by Serôdio (2004), which compensates the effect of sediment attenuation and

depth integration effects. Values reported by Serôdio (2004) were used as the source for the Tagus attenuation coefficients (k_p = 16.9 mm^{-1}; k_{meas} + k_f = 53.5 mm^{-1}), and values from Consalvey (2002) were used as the source for the Eden attenuation coefficients (k_p = 10.38 mm^{-1}; k_{meas} + k_f = 32.87 mm^{-1}). These values would emphasise differences resulting from hypothetical vertical migration and differences due only to sediment attenuation and depth integration.

Temporal patterns. Fluorescence measurements were taken at the beginning and end of the emersion period on each day for each site. Non-migrational biofilms were compared with migrational biofilms. The minimum period between initial and final measurements was 3 h for T3F and T4F, and the maximum period was 5 h for Eden 1. Patterns in F_0' and F_m' were investigated throughout the dark adaptation period (5 min), the light curve (8 min) and recovery phase (18 min), and the impact on the calculation of NPQ considered.

Statistical analysis. Data were analysed for statistical significance using Students *t*-test for unpaired samples and paired Students *t*-test for related samples. Due to

Table 2. Percentage change in dark-adapted maximum quantum efficiency (F_v/F_m) between values obtained after 5 min dark adaptation (prior to each light response curve) and those obtained after 18 min dark recovery period. *p < 0.01, ns: not-significant (paired *t*-test for means). Statistical tests performed on original F_v/F_m data (arcsine transformed); values in brackets are original means (5 min dark adaptation and 18 min after dark recovery, respectively)

Source	Phytotray	n	Sediment	n
Eden 1	−28%* (0.69, 0.50)	8	−8%* (0.73, 0.67)	10
Eden 2	−22%* (0.65, 0.53)	10	−13%* (0.67, 0.58)	14
Tagus 1	−3%ns (0.70, 0.68)	4	−3%* (0.74, 0.71)	8
Tagus 2	−11%* (0.66, 0.59)	6	−6%* (0.75, 0.70)	12

Table 3. Percentage change in fluorescence yield from F_0' to F_0^5 during initial 5 min dark adaptation for non-migratory biofilms (phytotrays) and migratory biofilms (sediment). *p < 0.01, ns: not-significant, (paired *t*-test for means). Statistical tests performed on absolute fluorescence yield values

Site	Phytotray	n	Sediment	n
Eden 1	−0.2%ns	8	−36%*	10
Eden 2	−3.5%*	10	−10%*	14
Tagus 1	2.1%ns	4	−10%*	8
Tagus 2	0%ns	6	−34%*	13
Tagus 3	–	–	−17%ns	6
Tagus 4	–	–	−22%*	6

the non-normal distribution of the proportional fluorescence data, values were arcsine transformed ($x' = $ arcsin$(\sqrt{x})$; Zar 1999). Analysis of variance (ANOVA) was used for multiple comparisons. Homogeneity of variances was confirmed using Bartlett's test, and post-hoc analysis of differences between groups was conducted using the Scheffé test.

RESULTS

Changes in photochemical efficiency (F_v/F_m)

PSII photochemical efficiency ratios (F_v/F_m) for sediment biofilms declined significantly between F_v^5/F_m^5 and the end of the dark recovery period (p < 0.01, n > 7) (Table 2). This was also true for 3 of the 4 non-migratory (phytotray) biofilms (p < 0.01, n > 3). Sediment biofilms under laboratory conditions showed a greater recovery of photochemical efficiency than phytotray biofilms, with one exception (Tagus 1).

Phytotray biofilms and laboratory sediment biofilms showed high values of F_v^5/F_m^5, which suggested that treatments did not stress the cells and that carbon or nutrient limitation was not a problem. Phytotray biofilms exhibited values between 0.62 to 0.73 and laboratory sediment biofilms showed values ranging from 0.69 to 0.74. *In situ* biofilms showed lower F_v^5/F_m^5 values ranging from 0.56 to 0.59.

Changes in the fluorescence yield from F_0' to F_0^5

Fluorescence yield significantly decreased over the 5 min dark adaptation period (from F_0' to F_0^5) for all biofilms maintained in the laboratory (Table 3), except for phytotray biofilms that showed no consistent pattern and a relatively small change in yield. For *in situ* biofilms, the decrease from F_0' to F_0^5 was significant for the T4F biofilms (p < 0.05, n = 6), but not for T3F (Table 3). Such a decrease in yield during dark adaptation strongly suggests downward migration as opposed to Q_A oxidation (see 'Discussion').

Changes in the maximum fluorescence yield (F_m' and F_m^5)

Maximum fluorescence yield increased after 5 min dark adaptation (from F_m' to F_m^5), for phytotray biofilms E1P ($F_{1,7} = 14.3$, p < 0.01) and T1P ($F_{1,4} = 10.04$, p < 0.05), whereas no changes were observed for phytotray biofilms E2P ($F_{1,9} = 2.1$, p = 0.18) and T2P ($F_{1,5} = 0.001$, p = 0.97) (Fig. 1A). F_m' then decreased as light
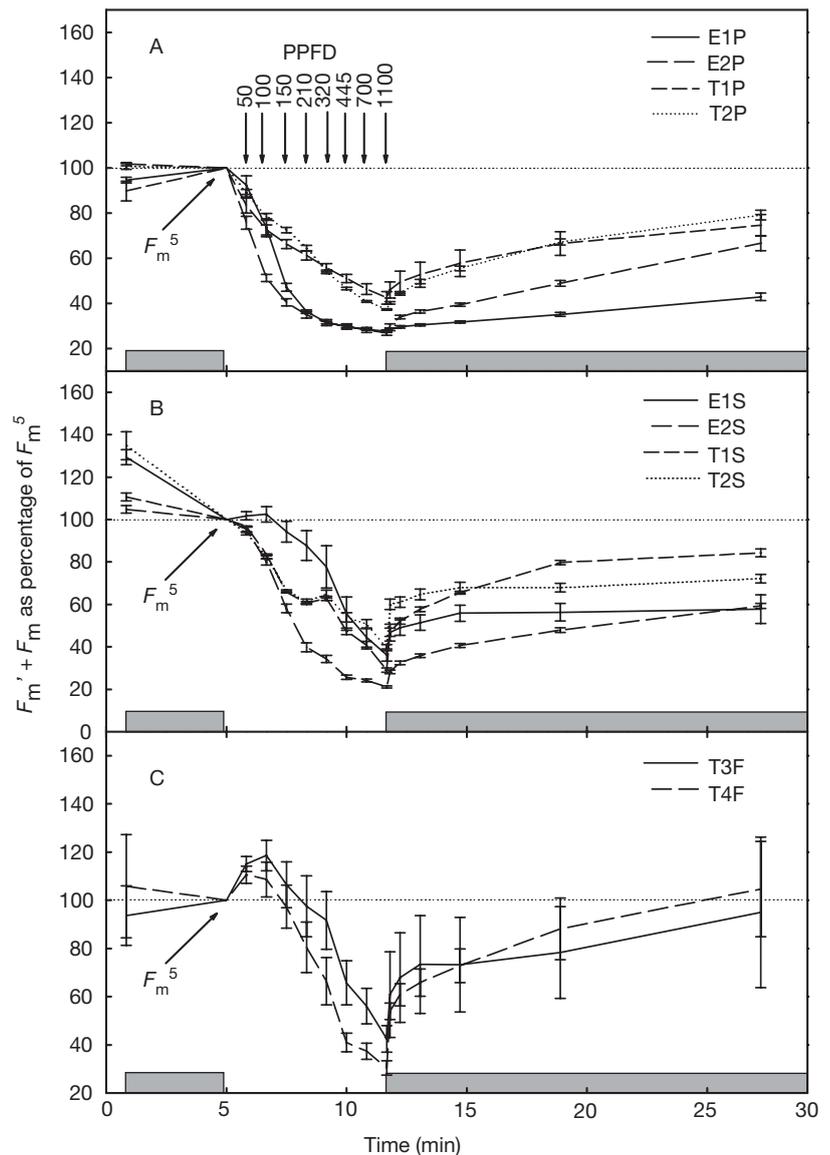

Fig. 1. Changes in F_m' and F_m expressed as a percentage of F_m^5 value; (A) phytotray, (B) sediment tray, and (C) field sediment. F_m^5: maximum fluorescence yield after 5 min dark adaptation, F_m': maximum fluorescence yield measured in light-adapted state, F_m: maximum fluorescence yield measured dark-adapted state during the recovery phase. Data, mean ± SE, plotted over time and as a function of irradiance (PPFD m^{-2} s^{-1}); grey bars indicate periods of dark adaptation
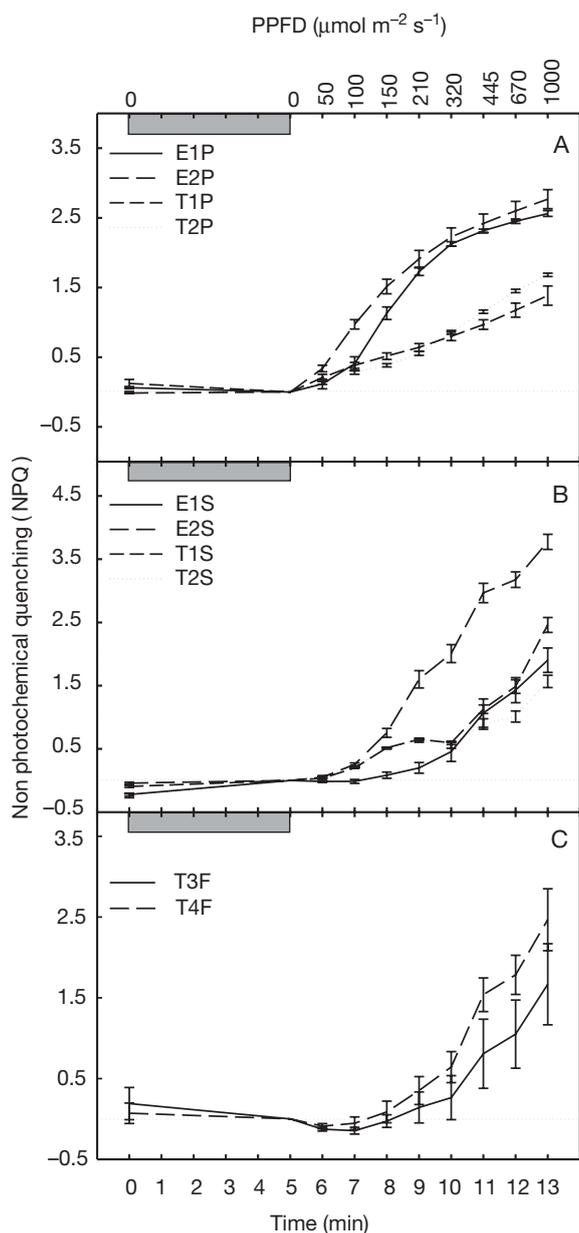
Fig. 2. Non-photochemical quenching (NPQ) after 5 min dark adaptation and during light response curves: (A) phytotray, (B) sediment tray, and (C) field sediment. Mean ± SE, grey bars indicate darkness periods

$F_{1,11} = 29.9$, p < 0.001, T2S) (Fig. 1B). In all treatments, F_m values over the recovery period failed to reach the original F_m^5 values (Fig. 1B). In some instances, e.g. T1S and T2S biofilms, the decrease in F_m' during the light curves showed an inflexion at around 150 to 210 μmol m^{-2} s^{-1}. Such an inflexion may have indicated downward migration, following the more rapid decrease in F_m' that resulted from NPQ induction (see 'Discussion').

For natural biofilms (Fig. 1C), a significant increase was observed from F_m' to F_m^5 during the dark adaptation period for T4F ($F_{1,4} = 14.8$, p < 0.05), whereas T3F decreased, but not significantly ($F_{1,5} = 0.23$, p = 0.65). During the light curves, F_m' increased for both biofilms until a light level of 150 μmol PPFD m^{-2} s^{-1} was
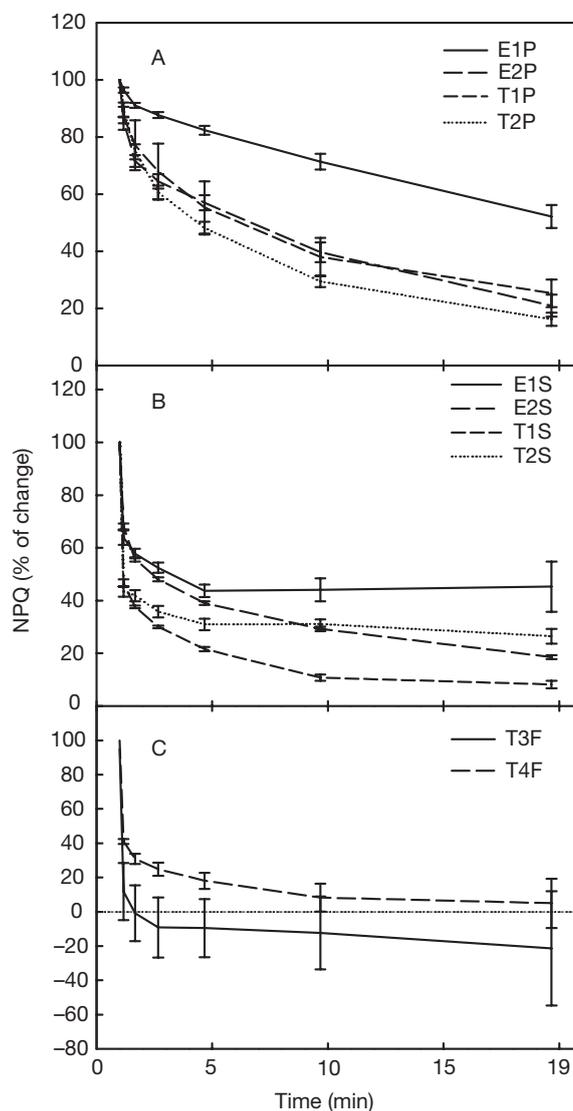

Fig. 3. NPQ during recovery phase, expressed as a percentage of the maximum value of NPQ achieved at highest irradiance during light response curves: (A) phytotray, (B) sediment tray, and (C) field sediment. Mean ± SE

levels increased during each of the rapid light curves. During the dark recovery phase, F_m increased rapidly over the first 2 min and then more slowly, with the exception of E1P that increased steadily over the whole recovery period. No biofilms showed a full recovery of F_m: all failed to reach the original F_m^5.

Migratory biofilms maintained in the laboratory all showed a significant decrease in maximum fluorescence yield from F_m' to F_m^5 ($F_{1,12} = 92.5$, p < 0.001, E1S; $F_{1,12} = 4.6$, p < 0.05, E2S; $F_{1,6} = 20.7$, p < 0.05, T1S;

reached, before decreasing as irradiance then increased further. A rapid increase in F_m was observed during the first 3 min of the recovery period for both biofilms, followed by a steady increase to an F_m yield similar to F_m^5.

Non-Photochemical Quenching (NPQ)

Light curve responses

During light response curves, both phytotray and sediment biofilms showed a steady increase in NPQ (Fig. 2A,B). Eden phytotray biofilms showed a steeper gradient of change and reached significantly higher final NPQ values than the Tagus phytotray biofilms (Scheffé test, p < 0.01; mean rate of increase: Eden 0.31 NPQ relative units min^{-1}; Tagus: 0.18 NPQ relative units min^{-1}). Phytotray biofilms from the Eden and the Tagus estuaries showed no within site variation in NPQ (Scheffé test, p = 0.39, p = 0.176, respectively; Fig. 2A). Three of the sediment biofilms (Fig. 2B) showed a slower initial increase in NPQ compared to phytotray biofilms. E1S showed no obvious increase in NPQ until an irradiance of 210 µmol PPFD m^{-2} s^{-1} was reached, while T1S and T2S showed an inflection point around 150 to 210 PPFD m^{-2} s^{-1}. E2S was similar in behaviour to phytotray biofilms, with a final value of NPQ significantly higher than that of the other non-migratory biofilms (Scheffé test, p < 0.01). Natural biofilms showed the same pattern in NPQ as the sediment biofilms, with no significant difference between T3F and T4F (Fig. 2C).

Recovery phase

NPQ decreased during the recovery period in phytotray biofilms (Fig. 3A). E1P exhibited a slow decrease in NPQ, whereas for the other 3 biofilms NPQ declined more rapidly during the first 2 min (Fig. 3A). Sediment biofilms also showed a rapid decrease in NPQ during the first 2 min (Fig. 3B). Natural biofilms showed the same general pattern (Fig. 3C), even though variation was introduced by changes in the NPQ response dependent on tidal stage (see below).

Light response curves (rETR)

The phytotray biofilms reached light saturation with a relatively stable rETR$_{max}$ (Fig. 4). E1S and E2S sediment

biofilms also showed evidence of a stable rETR$_{max}$ after ~500 µmol PPFD m^{-2} s^{-1}, whereas T1S and T2S exhibited rETR that increased above 1000 µmol PPFD m^{-2} s^{-1}. In the absence of saturation, values of rETR$_{max}$ were approximated using the last value of the light curve. rETR$_{max}$ of sediment biofilms were therefore consistently higher than for phytotray biofilms (Table 4). Eden biofilms showed significantly lower values of rETR$_{max}$ when compared to Tagus biofilms (Scheffé test, p < 0.01) and there were no difference within sites (Table 4).

Temporal patterns in NPQ

Laboratory biofilms

Patterns in NPQ during dark recovery were compared at the beginning and end of the virtual tidal emersion period. Both Tagus and Eden phytotray biofilms showed a biphasic pattern of NPQ with a rapid
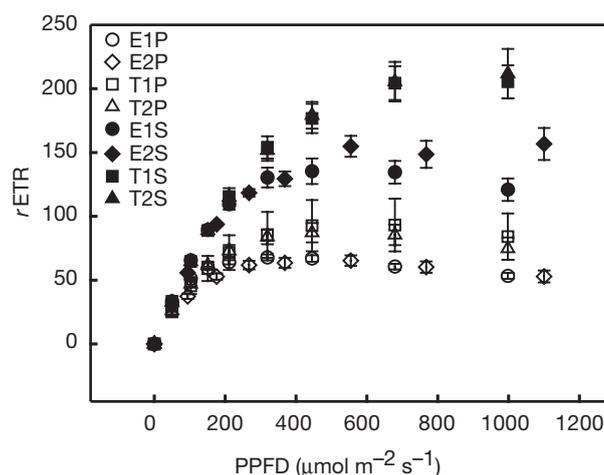

Fig. 4. Light response curves of relative electron transport rate (rETR) vs. irradiance (PPFD) obtained for laboratory sediment and phytotray biofilms. Open symbols represent phytotray biofilms, closed symbols sediment biofilms

Table 4. Maximum relative electron transport rates (rETR$_{max}$) and initial slope of light response curve (α, value of theoretical light use efficiency of PSII): comparison between non-migratory biofilms (phytotray) and migratory (sediment) biofilms. *p < 0.01, ns: not-significant (Student's t-test)

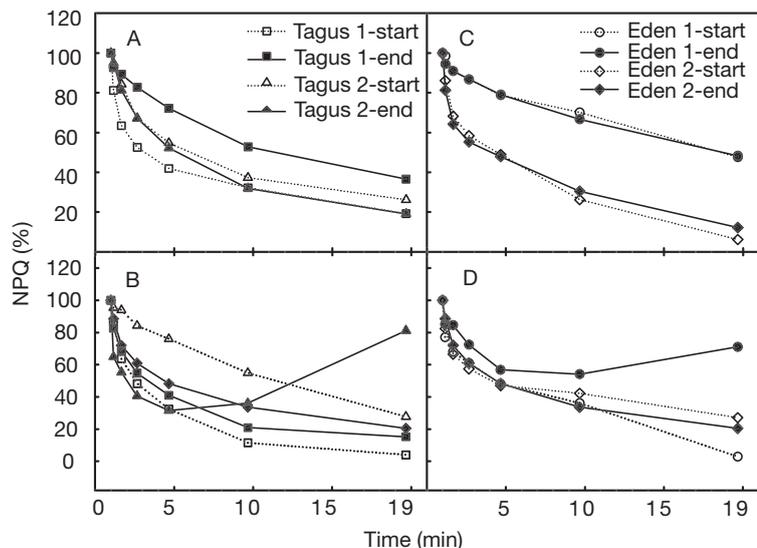| | rETR$_{max}$ | | | | α | | | |
	Phytotray	n	Sediment	n	Phytotray	n	Sediment	n
Eden 1	67	8	140	10*	0.81	8	0.87	10[ns]
Eden 2	66	10	160	14*	0.52	10	0.78	14*
Tagus 1	94	5	219	11*	0.58	5	0.76	11*
Tagus 2	88	6	215	13*	0.63	6	0.74	13*

Fig. 5. NPQ during recovery phase at start (open symbols, dashed lines) and end (closed symbols, continuous lines) of emersion period, expressed as a percentage of the maximum value of NPQ achieved at highest irradiance during light response curves; (A) Tagus phytotray, (B) Tagus sediment biofilms, (C) Eden phytotray, (D) Eden sediment biofilms

decrease during the first 2 min of dark recovery, followed by a gradual decline (Fig. 5A,C). For the sediment biofilms, this pattern was also observed at the beginning of the emersion period; however, at the end of the emersion period, T2S and E1S biofilms showed a large increase in NPQ after 5 min of darkness (Fig. 5B,D).

Natural biofilms

NPQ decreased over the dark recovery period for T3F and T4F biofilms measured at the beginning of the emersion period (Fig. 6). However, at the end of the emersion period, after initial rapid decreases in NPQ during the first 3 to 4 min, NPQ increased until the end of dark adaptation.

DISCUSSION

The regulation of photosynthesis by intertidal microphytobenthos in response to changes in ambient light can take the form of behavioural and/or physiological mechanisms. We define behavioural regulation as a relationship between light and cell position controlled by vertical migration, which optimises light availability whilst avoiding damaging high irradiances (Paterson 1994, Underwood & Kromkamp 1999, Perkins et al. 2001, 2002). Physiological regulation includes NPQ of energy by diversion of excess light energy away from

photosystem reaction centres using processes such as the xanthophyll cycle (Caron et al. 1987, Ting & Owens 1993, Müller et al. 2001). By comparing non-migrational systems (phytotrays) with migrational systems (sediment trays), we compared biofilms utilising NPQ without and with the ability to regulate their light environment through vertical migration.

Effect of vertical migration on the estimation of fluorescence yields

Light-induced vertical migration influenced fluorescence measurements at all stages of experimentation (dark adaptation, light curves and recovery period). Two non-migratory biofilms (E1P and T1P) exhibited a small increase in fluorescence yield from F_m' to F_m^5, which suggested a reversal of NPQ, whereas all migrational sediment tray biofilms (Fig. 1A,B) showed a decrease. Such a decrease in the dark can be indicative of downward migration (Perkins et al. 2001, 2002); in the dark, yield would be expected to increase through NPQ reversal. Supporting this was the observation that the decrease in F_m in sediment (migratory) biofilms was paralleled with a decrease in F_0' to F_0^5 (Table 3) — again, NPQ reversal should result in an increase in yield (in the case of F_0) partly balanced by a decrease in yield due to Q_A oxidation.

The decrease in the minimum fluorescence yield varied in magnitude between different biofilms, suggesting that it is not always possible to use F_0^5 or F_0^{15} to
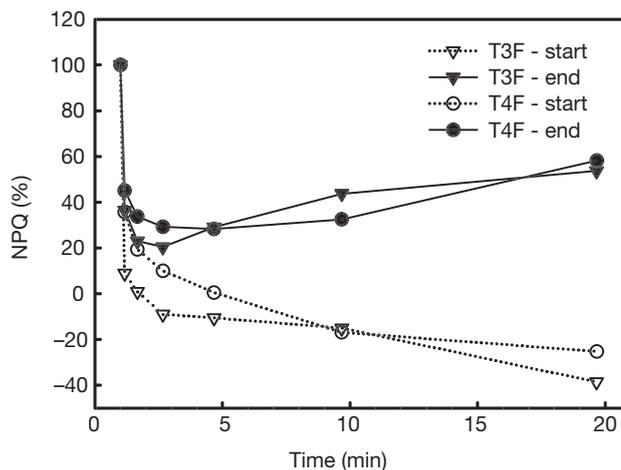

Fig. 6. NPQ for natural biofilms during recovery phase at start (open symbols, dashed lines) and end (closed symbols, continuous lines) of emersion period

accurately estimate biomass, even though this method could still be utilised to monitor changes in surface biomass due to migration (Consalvey et al. 2004, Jesus et al. 2005) and large scale difference in biomass. These variations may reflect differences in community composition as suggested by previous authors (Serôdio et al. 1997, Perkins et al. 2001, Honeywill et al. 2002). Our conclusion is that both F_0^5 and F_0^{15} may often underestimate biomass, dependent upon the migrational nature of the biofilm. The extent of biofilm migration will depend upon many variables including species composition, tidal stage and biofilm age, in addition to changes in the light environment (Pinckney & Zingmark 1991, Kingston 1999, Perkins et al. 2001, Consalvey 2002, Perkins et al. 2002). Therefore, it is suggested here that fluorescence proxy measurements of biomass (F_0^5 or F_0^{15}) should be limited to follow qualitative differences in surface biomass.

During light response curves, phytotray (non-migratory) biofilms showed a rapid decrease in F_m' with the onset of light and then a further slower decline typical of rapidly induced NPQ (Caron et al. 1987). Sediment (migratory) biofilms also initially showed a similar decline, followed by a possible inflexion in some instances (T1S and T2S) that may have indicated that vertical migration occurred to compensate for light exposure. In one case (E1S) an increase in F_m' over the first 2 steps of the light curve was observed. In the case of sediment biofilms, a decrease in yield could not be attributed with certainty to either NPQ induction or downward migration, it was likely to be a combination of both factors and dependent upon the taxa and environmental conditions. However, it seems likely that rapid induction of NPQ occurred once more, probably with slower induction of downward migration. Indeed, such a pattern in NPQ followed by downward migration has been observed for *Nitzschia dubia* using high resolution imaging (Paterson et al. 2003, Underwood et al. 2005).

Conversely, for sediment biofilms in which an increase in F_m' was observed, this could be the result of low light stimulating upward migration (Perkins et al. 2001) or reversal of NPQ (Kramer et al. 2004). NPQ reversal seemed unlikely in this instance, as an increase in F_m' was observed up to an irradiance of 150 µmol PPFD m^{-2} s^{-1} and no such increase was observed for phytotray biofilms. F_m' increased for natural biofilms (T3F and T4F, Fig. 1C) over the first irradiance steps used in the light response curves (up to 150 µmol PPFD m^{-2} s^{-1}), which also suggested that cells migrated towards the sediment surface. Nevertheless, the pattern of change in F_m' for natural biofilms over the light response curve was most similar to the sediment biofilm systems, whereas non-migratory phytotray biofilms showed a more rapid decline in F_m' over the light curve.

Effect of migration on the calculation of NPQ

NPQ during the light response curve

From Eq. 1, any change in F_m^5 or F_m' due to changes in biomass resulting from vertical migration to or away from the sediment surface will proportionally affect the calculation of NPQ. This was evident when comparing NPQ during the light curves (Fig. 2). Phytotray biofilms down-regulated physiologically on exposure to light, as indicated by rapid increases in NPQ (Fig. 2A), whereas NPQ for the sediment biofilms and natural biofilms increased only slowly or even declined (Fig. 2B,C). The patterns observed in NPQ for the migratory biofilms may be explained by several factors. Firstly, reversal of NPQ in response to low light (Caron et al. 1987, Mouget & Tremblin 2002). Secondly, attenuation of light by the sediment, resulting in cells being exposed to lower light than phytotray biofilms (Lassen et al. 1992, Consalvey 2002). However, this factor was strongly reduced or eliminated by using Serôdio's (2004) recursive equations to compensate for depth integration. Thirdly, a decrease in calculated NPQ due to an increase in F_m' resulting from upward vertical migration of cells in response to low light. The E2S biofilms were the least migrational biofilms, and both Eden biofilms showed higher levels of NPQ compared to the Tagus biofilms. This may reflect a photophysiological adaptation of Tagus biofilms, being better adapted to high light and less dependant upon physiological NPQ than Eden biofilms. In terms of the cellular energy budget, migration may be less costly than photophysiological adaptation due to the relatively 'cheap' mechanism of diatom locomotion (e.g. Underwood & Kromkamp 1999, Consalvey et al. 2004b). Some diatoms have been shown to use existing paths opened by larger diatom, thus decreasing the energy required to migrate downwards (Wenderoth et al. 2004).

NPQ during the recovery phase

The consistent decrease of NPQ in the phytotray biofilms (Fig. 3A) during the recovery phase was attributed to dissipation of the proton gradient in darkness and conversion of diatoxanthin to diadinoxanthin (Caron et al. 1987, Ting & Owens 1993). The relatively slow decrease of NPQ in E1P may have been the result of photo-inhibitory down regulation during the light curve, due to the prevention of vertical migration. Calculated NPQ for 2 of the sediment biofilms (E1S and T2S) and for *in situ* migratory biofilms (Fig. 3C) showed large variation by the end of the recovery period due to the variable response of NPQ at the end of tidal exposure (see discussion below).

Light response curves (rETR)

The maximum relative electron transport rate ($r\mathrm{ETR}_{max}$) was higher for sediment than for phytotray biofilms (Table 4). This was attributed to light-induced downward vertical migration of cells at the highest light levels of the light curves. Cells that moved to the sub-surface obviously received less irradiance than surface cells due to attenuation of light by the sediment. Thus, such cells will show a higher than expected photochemical efficiency for the light level they are, in theory, being exposed to: i.e. the light level they are experiencing is over-estimated. This results in an over-estimation of $r\mathrm{ETR}_{max}$, calculated using the surface light level (Perkins et al. 2001, 2002, Forster & Kromkamp 2004, Serôdio 2004). The lower $r\mathrm{ETR}_{max}$ of the phytotray biofilms provides further evidence that significant errors can be introduced in calculation of rETR due to vertical migration (Perkins et al. 2002).

An alternative hypothesis is that the measurement of $r\mathrm{ETR}_{max}$ is accurate, due to micro-migrations of cells within the biofilm (Kromkamp et al. 1998). Kromkamp et al. (1998) suggested that diatom cells from depth replaced those at the surface, resulting in higher quantum efficiencies at high light and hence higher values of $r\mathrm{ETR}_{max}$. However, this seems less likely than a simple downward migration of surface cells in response to the increasing light level. In addition, rapid decrease in PSII efficiency upon exposure to increasing irradiance suggests that microcycling cannot account for the high values of rETR observed. Such microcycling would have to be extremely rapid: once cells reached the surface, photochemical efficiency would decline as a result of continued exposure to the light and thus accumulation of light dose at the surface.

It is important to mention that our results were obtained after correction of the original values with Serôdio's (2004) model that compensates for fluorescence depth integration and sediment attenuation, which further strengthens the hypothesis that at higher light levels diatoms migrated down to photo-regulate exposure to light. Our results support reports by Serôdio (2004) and Forster & Kromkamp (2004) that depth integrated fluorescence measurements overestimates the intrinsic rETR, and also that a shift of cells from the surface to a sub-surface depth amplifies this overestimation. However, our observations calculate an overestimation in the rage of 108% (E1) and 144% (T2), in contrast with the maximum values estimated by other authors that ranged between 46% (Forster & Kromkamp 2004) and 62% (Serôdio 2004). Carbon supply was not monitored, so it could not be excluded as a source of variation; however, the phytotrays were developed for tissue growing and should have allowed nutrient re-supply through the basal membrane. The

difference might be attributable to the accumulated effect of the depth integration measurement effect with the simultaneous migration downwards, even though we attempted to compensate for the depth integration effect by using the recursive equations suggested by Serôdio (2004). Although the previous models (Forster & Kromkamp 2004, Serôdio 2004) did not account for a dynamic situation, where movement occurs during the measurement, they had already mentioned the potential effect that migrational movements might introduce in the final results.

Temporal patterns

For non-migratory biofilms, the decrease in NPQ over the dark recovery period did not vary between the beginning and end of the emersion period (Fig. 5A,C). However, 2 of the sediment biofilms (Fig. 5B,D) and 2 natural biofilms (Fig. 6) showed distinct increases in NPQ during dark recovery at the end of the emersion period. This accounted for the large variation in reversal of NPQ in biofilms in which migration could occur (Fig. 3B,C). This increase in NPQ was attributed to a decrease in F_m due to downward migration (data not shown). This showed that the migrational response to changes in light condition varied over the tidal cycle, and also that this variation may have been assemblage-specific, since not all the sediment biofilms exhibited identical migratory behaviour. The fact that phytotray biofilms (Fig. 5A,C) did not show any temporal pattern in NPQ excludes the hypothesis that the changes in the NPQ pattern were due to endogenous physiological mechanisms. Therefore, we conclude that the extent and direction of the vertical migration due to changes in light conditions were conditioned by the endogenous response of the cells to the tidal period. This supports the dual control mechanism of diatom migration proposed by Round (1981).

CONCLUSIONS

The use of PAM fluorescence to calculate photophysiological parameters (e.g. NPQ, $r\mathrm{ETR}_{max}$) with migrational microphytobenthic biofilms can be strongly biased by the type of biofilm and by the timing of measurements. Data in this study have shown that vertical migration can occur in the darkness (downward or upward migration, dependent on time of emersion), at low light (upward migration), and under high light (downward migration). These effects may be reduced if the measurements are carried out at the start of an emersion period, thus reducing possible endogenous

migrational effects. Even so, unless a comparison is made between similar migrational and non-migrational biofilms, it is not possible to quantify the effect of migrational behaviour on the calculation of photophysiological parameters. Further studies are needed to clarify the effect of light on vertical migration of microphytobenthic biofilm assemblages.

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