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Can chlorophyll fluorescence be used to estimate the rate of photosynthetic electron transport within microphytobenthic biofilms?

R. G. Perkins, K. Oxborough*, A. R. M. Hanlon, G. J. C. Underwood, N. R. Baker

Department of Biological Sciences, University of Essex, Wivenhoe Park, Colchester, Essex CO4 3SQ, United Kingdom

ABSTRACT: Chlorophyll fluorometry has frequently been used to estimate the photosynthetic electron transport rate (ETR) within oxygenic organisms. One of the requirements of this method is that the absorptivity of the photosynthetic system is known. In the specific case of microphytobenthos within biofilms, it is known that cells migrate vertically over relatively short time periods. This can radically alter the absorptivity of the photosynthetic biomass as a whole and, potentially, could result in highly inaccurate values of ETR being calculated. In this study, both modulated, integrating fluorometry and high resolution imaging of fluorescence have been used to investigate the error introduced into the calculation of ETR by the vertical migration of cells. Estimates of ETR derived from fluorescence data were compared with rates of primary production measured using a ^{14}C-radiotracer method. The effect of fluorescence from photosystem I (PS I) on the calculated value of ETR was also assessed. Overall, these data suggest that PS I fluorescence can introduce significant errors into the estimation of ETR from diatom cultures and in the estimation of ETR from individual cells at the biofilm surface, when using high resolution imaging of chlorophyll fluorescence. Measurements made on intact biofilms under incident light showed an extremely poor correlation between estimated ETR (derived from fluorescence data) and primary production (measured by ^{14}C incorporation). The simplest explanation for this result is that the downward migration of cells decreased the amount of light absorbed by the photosynthetic biomass, which led to substantial errors in the calculation of ETR. The clear implication is that conventional (integrating) fluorometers cannot be used to determine rates of ETR from intact, migratory biofilms and that any realistic estimation of ETR within intact biofilms is likely to involve high resolution imaging of fluorescence.

KEY WORDS: Fluorescence imaging · Diatoms · Photosystem II · Photosystem I fluorescence · ETR

INTRODUCTION

Microphytobenthic biofilms in estuarine sediments exhibit high rates of primary production (Underwood & Kromkamp 1999), contribute to sediment stability (Paterson 1989, Underwood & Paterson 1993, Yallop et al. 1994) and directly affect nutrient fluxes across the sediment-water interface (Thornton et al. 1999, Dong et al. 2000). One adaptation to the sediment habitat exhibited by benthic diatoms is to migrate vertically in response to diurnal and tidal rhythms (Serôdio et al. 1997, Underwood & Kromkamp 1999). Downward migration reduces wash-away of cells during immersion and grazing by predators and may increase nutrient availability (Decho 1990). Migration may also occur as a response to changes in ambient PPFD (for abbreviations used, see Table 1) and hence act as a behavioural method of photoacclimation (Paterson et al. 1998, Underwood & Kromkamp 1999).

The application of chlorophyll fluorometry as a non-invasive technique for the estimation of photosynthetic efficiency in algae, including microphytobenthos, has increased in recent years (Serôdio et al. 1997, Hartig et

*Corresponding author. E-mail: koxbor@essex.ac.uk

Table 1. Abbreviations

α	Initial slope of the relationship between PPFD and ETR
E_k	Light saturation parameter
ETR	Electron transport rate
ETR_{max}	Maximum electron transport rate
F'	Chlorophyll fluorescence signal in the light-adapted state
F_m'	Chlorophyll fluorescence signal when all PS II centres are closed in the light-adapted state
F_q'	Difference between F' and F_m'
F_q'/F_m'	Fluorescence parameter that provides an estimate of the operating efficiency of PS II photochemistry
ϕ_{CO_2}	Quantum yield of CO2 assimilation
PPFD	Photosynthetically active photon flux density

al. 1998, Kromkamp et al. 1998, Underwood et al. 1999). Estimates of the photosynthetic electron transport rate (ETR) from fluorescence data have been compared with estimates of primary production, using ^{14}C-radiotracers (Hartig et al. 1998, Wolfstein & Hartig 1998, Barranguet & Kromkamp 2000), and O_2 evolution (Flameling & Kromkamp 1998). In such studies, the so-called 'Genty factor' (Genty et al. 1989) has been used in estimation of the ETR. This factor provides an estimate of the proportion of absorbed photons that are used to drive photochemistry at PS II (the PS II operating efficiency) and is expressed (in the terminology used here) as the fluorescence parameter, F_q'/F_m'. This method of estimating ETR requires accurate measurement of both the incident PPFD and absorptivity of the sample. It also assumes that the photons absorbed are divided equally between PS II and PS I and that the value of F_q'/F_m' provides a good estimate of the photon yield of electron transfer into the photosynthetic electron transport chain.

In most situations, errors in the measurement of PPFD and/or absorptivity and/or an uneven distribution of absorbed photons between PS II and PS I will result in proportional errors in the calculation of ETR. That is to say, while the calculated values of the initial slope (α) of the relationship between PPFD and calculated ETR and the maximum rate of ETR (ETR_{max}) will both be inaccurate, the shape of the relationship between PPFD and ETR (and the value of E_k) will not be affected. One way in which a non-proportional error (which will affect the shape of the relationship between PPFD and ETR) could be introduced is if the absorptivity of the sample changed with PPFD. Among the objectives of this study was to determine the extent to which the vertical migration of diatoms within biofilms might affect the absorptivity of the photosynthetic biomass as a whole. This part of the study employed high-resolution imaging of chlorophyll fluorescence (Oxborough & Baker 1997, Oxborough et al. 2000), which makes it possible to assess the species composition and cell density at the surface of the biofilm at the same time that measurements of F_q'/F_m' are being made.

The relationship between F_q'/F_m' and the true value of the PS II operating efficiency can be affected by fluorescence from PS I (Genty et al. 1990, Pfündel 1998). The fluorescence yield of PS I is thought to be reasonably constant and independent of PPFD (Dau 1994, Pfündel 1998). In contrast, the fluorescence yield of PS II is known to be affected by 2 processes: PS II photochemistry, which results in photochemical quenching, and down-regulation, which results in non-photochemical quenching. Down-regulation, which tends to increase with PPFD, decreases the yield of fluorescence at F_m', while the combined effect of down-regulation and photochemistry result in a reasonably stable value of F' with changes in PPFD (Havaux et al. 1991, Genty et al. 1992, Laisk et al. 1997, Oxborough & Baker 2000). Consequently, the relative effect of PS I fluorescence on the value of F_q'/F_m' might be expected to increase with PPFD, introducing a non-proportional error into the relationship between PPFD and estimated values of ETR.

Data from a number of studies show a strong linear relationship between the value of F_q'/F_m' and the quantum yield of CO_2 assimilation (ϕ_{CO_2}) (Genty et al. 1989, 1990, di Marco et al. 1990, Krall & Edwards 1990, Edwards & Baker 1993). This would seem to indicate that, in leaves of higher plants at least, PS I fluorescence frequently does not contribute enough to the overall fluorescence signal to induce a significant non-proportional error of the type just described. Another objective of this study was to determine whether or not PS I fluorescence introduces a significant non-proportional error into the estimation of ETR from intact biofilms. This was achieved by comparing fluorescence data that were collected using 1 of 2 different filters in front of the fluorescence detector. The first of these (680 nm bandpass) filters out most of the PS I fluorescence, resulting in a very high PS II to PS I fluorescence ratio, while the second (a 695 nm longpass) allows a much higher proportion of PS I fluorescence to pass through while filtering out a proportion of PS II fluorescence, resulting in a substantially lower PS II to PS I fluorescence ratio. If the contribution of PS I fluorescence to the overall signal is high enough to introduce a non-proportional error, this would be expected

to show up as a difference between the estimated values of ETR, derived from the measurements, which increases with PPFD.

Finally, values of ETR, estimated from fluorescence data, were compared with rates of primary production, as measured by a ^{14}C-radiotracer method. Measurements were made on intact biofilms over a 14 h period to allow for an assessment of the comparative effects of diel and tidal rhythms on these parameters.

MATERIALS AND METHODS

Diatom cultures. Cultures of *Cylindrotheca closterium*, (Ehr.) Reimann et Lewin. were grown at a PPFD of 180 µmol m^{-2} s^{-1} on a 14/10 h light/dark cycle at 18°C in a growth cabinet using f/2 media (Guillard & Ryther 1962), salinity 20 and with an added antibiotic cocktail to inhibit bacterial growth (Smith & Underwood 1998). Stationary phase was reached on Day 5. Fluorescence measurements were made during the logarithmic phase, on Days 3 and 4.

Sampling of intact biofilms. Microphytobenthic biofilms were sampled at low tide using 7.5 cm diameter cores: from 2 sites in the Colne estuary, Alresford Creek (51° 50.2′ N, 0° 59.5′ E) and The Hythe (51° 52.5′ N, 0° 56.2′ E), Colchester, Essex, UK. Alresford Creek is situated midway along the estuarine salinity gradient and supports diatom-dominated biofilms with some euglenoids. The Hythe is at the head of the estuary, with a low salinity range and high nutrient concentrations, and is dominated by euglenoids with some cyanobacteria and diatoms (Underwood et al. 1998). Cores were carefully transported back to the laboratory and maintained in shallow trays containing site water to prevent drying of the sediment surface. Light response curves, from intact cores, were started within 2 h of sampling and within the low tide period (when the sediments would not have been immersed). Comparison of ^{14}C-radiotracer and fluorescence measurements were started the day after sampling. All samples were taken during July 1999, when the ambient temperature was between 22 and 25°C.

Measurements of chlorophyll fluorescence from cultures and intact biofilms. The fluorescence terminology employed here is the same as used by Oxborough et al. (2000). Chlorophyll fluorescence was measured using a Xenon PAM fluorometer (Walz GmbH, Effeltrich, Germany) or a high resolution fluorescence imaging system (Oxborough & Baker 1997, Oxborough et al. 2000). With the PAM system, the saturating pulses used for measurement of F_m' were at a PPFD of 8600 µmol m^{-2} s^{-1} for 300 ms. With the imaging system F_m' was imaged over the last 100 ms of a 350 ms saturating pulse at a PPFD of 8100 µmol m^{-2} s^{-1}. With both the PAM and imaging systems, chlorophyll fluorescence was defined by either a 680 nm bandpass filter (Coherent, Watford, UK) or an RG 695 longpass filter (Schott, Mainz, Germany). One complication that arises in comparing data from measurements using the 2 filters is that fluorescence of shorter wavelengths (between approximately 675 and 685 nm) is strongly reabsorbed, because these wavelengths are close to the peak of the chl *a* absorption spectrum. Consequently, use of the 680 nm filter could, in principle, decrease the measured value of F_q'/F_m' from relatively dense systems (such as leaves or dense biofilms) because fluorescence from the cells at depth will be reabsorbed by the cells at the surface (which will normally be operating at a lower efficiency, because they are absorbing more light than the underlying cells).

A detailed description of the high resolution imaging system used in this study is given in Oxborough & Baker (1997). The method used to isolate individual cells from images is described in Oxborough et al. (2000). Integrated values of F_q'/F_m' across whole images were calculated by summing all values within the F' and F_m' images used and then calculating a single mean value. Values of F_q'/F_m' from isolated cells were calculated as the sum of individual pixel values for this parameter (Oxborough & Baker 1997).

Estimation of ETR, ETR_{max}, α and E_k. ETR was estimated according to the method of Sakshaug et al. (1997), which is essentially the same as the method originally described within the Walz PAM 2000 fluorometer handbook. In Eq. (1), a^* is the specific absorption coefficient for chl *a*. Values of a^* were determined using an integrating sphere and cosine-corrected light meter. Biofilms were sampled using the lens tissue technique (Eaton & Moss 1966). Cells were re-suspended in f/2 media in repli-dishes (Bibby Sterlin). This was serially diluted to construct a standard curve of absorption versus chl *a* concentration (over a range of 50 to 800 g l^{-1}), from which a^* could be determined.

$$\mathrm{ETR} = \frac{F_q'}{F_m'} \cdot \frac{\mathrm{PPFD}}{2} \cdot a^* \quad (1)$$

Values for the maximum ETR (ETR_{max}), light utilisation coefficient (α) and the light saturation parameter (E_k = ETR_{max}/α) were determined through iteration (Long & Hällgren 1993), from light response curves (ETR vs PPFDs of 40 to 920 µmol m^{-2} s^{-1}).

Comparison of ^{14}C-radiotracer and fluorescence measurements. Sample cores were collected from Alresford Creek, as above, on July 15 and 22, 1999, and incubated the following day in a temperature-controlled glasshouse (22°C). Light curves were obtained hourly, using the PAM system, between 06:00 and 20:00 h. In addition, values of F_q'/F_m' were determined from measurements made with the PAM system under

ambient PPFD every hour and at a PPFD of 220 µmol m^{-2} s^{-1} with the high resolution imaging system). The 680 nm bandpass filter was used in both cases. Ambient PPFD was logged every 10 min and hourly averages used in calculations. Fluorescence data were compared to bi-hourly measurements of CO_2 incorporation using the ^{14}C-radiotracer method.

For the measurements of CO_2 incorporation, 1 ml of ^{14}C-labelled sodium bicarbonate (370 kBq) was added to minicores (internal area 3.14 cm^2) taken from each large core. This was allowed to diffuse into the biofilm during a 1 h dark period, which was followed by 1 h incubation under ambient PPFD, terminated by the addition of gluteraldehyde (5% v/v). The surface 2 mm of biofilm was carefully sliced off and freeze-dried. Hydrochloric acid was added to drive off inorganic label. After the addition of scintillant cocktail (Optiphase Safe, Fisons, Loughborough, UK), rates of CO_2 incorporation were calculated from counts obtained from a Packard Tricarb 460C scintillation counter with internal quench correction. Counts were corrected for self-quenching by the sediment using radiation standard curves, with and without sediment addition. Self-quenching reduced counts by less than 5%. Rates of CO_2 incorporation were corrected for dark uptake using dark controls (Smith & Underwood 1998). Paired measurements of chl *a* were made for each core. For spectrophotometric determination, chl *a* was extracted in cold methanol and samples were acidified to correct for phaeopigments (Lorenzen 1967).

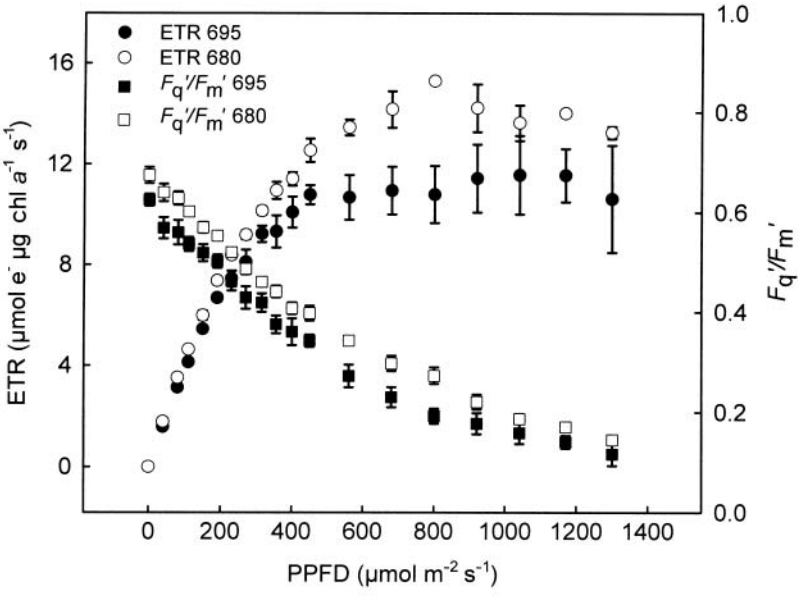

Fig. 1. Comparison of values (mean ± SE, n = 3) for F_q'/F_m' and estimated ETR, derived from measurements of chlorophyll fluorescence from cultures of *Cylindrothea closterium*. Measurements were made using a Xenon PAM fluorometer, with the 680 nm bandpass filter or the 695 nm longpass filter

RESULTS

Effect of PS I fluorescence on the estimated value of ETR

The light response curves in Fig. 1 illustrate the effect of removing most of the PS I fluorescence from the overall fluorescence signal (by using the 680 nm bandpass filter, instead of the 695 nm longpass filter) on F_q'/F_m' and the estimated value of ETR from cultures of *Cylindrotheca closterium*. Values of F_q'/F_m' were significantly higher throughout the light curve when the 680 nm bandpass filter was used ($F_{2(1,56)}$ = 97.4, $p < 0.001$). Because these cultures had a very short optical path, the re-absorption of fluorescence by overlying cells (discussed in 'Methods') was minimised and the effect of removing the PS I fluorescence was maximised. Between 0 and 900 µmol m^{-2} s^{-1}, the difference between the 2 sets of measurements increased with PPFD. At PPFDs >900 µmol m^{-2} s^{-1}, this difference in calculated rates of ETR levels off and even decreased slightly. ETR_{max} was 24% higher with the 680 nm bandpass filter, compared to the 695 nm longpass filter. There was no significant difference in α between the 2 filters. Consequently, E_k was higher with the 680 nm bandpass filter. Values of

Table 2. Comparison of electron transport parameters derived from measurements of chlorophyll fluorescence made using either a 680 nm bandpass filter or 695 nm longpass filter. Some of the original data that were used to calculate the values in this table are shown in Figs. 1 & 2

Filter/Site	Estimated ETR_{max} (µmol e^- µg chl a^{-1} s^{-1})	α (µmol e^- m^{-2} µg chl a^{-1} µmol photons^{-1})	E_k (µmol photons m^{-2} s^{-1})
Cylindrotheca closterium, PAM fluorometer			
680 nm/culture	14.3 ± 0.68	0.037 ± 0.0003	390
695 nm/culture	11.5 ± 0.35	0.037 ± 0.0040	310
Measurements made using the high resolution imaging system			
680 nm/Alresford	10.0 ± 0.46	0.024 ± 0.0022	420
695 nm/Alresford	9.8 ± 0.67	0.027 ± 0.0028	360
680 nm/Hythe	7.1 ± 0.00	0.021 ± 0.0030	340
695 nm/Hythe	4.5 ± 0.10	0.028 ± 0.0020	160

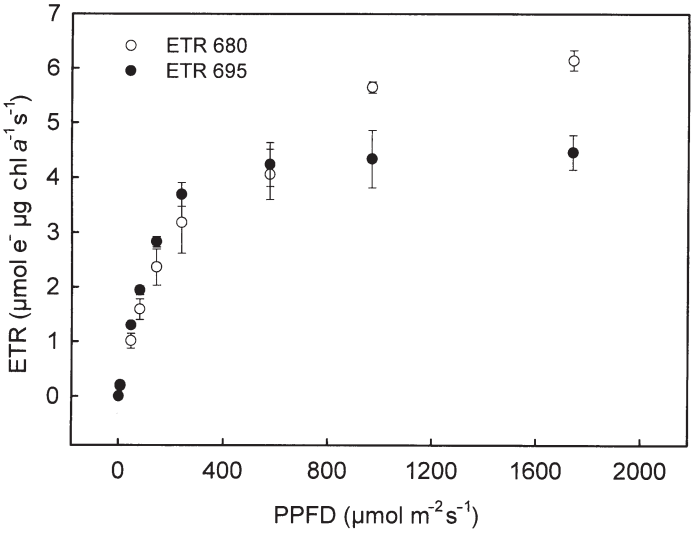

Fig. 2. Comparison of estimated ETR (mean ± SE, n = 3) derived from images of chlorophyll fluorescence from an intact biofilm sampled from The Hythe. Measurements were made using the 680 nm bandpass filter or the 695 nm longpass filter

ETR_{max}, α and E_k derived from these light response curves are shown in Table 2.

Similar calculated rates of ETR derived from light response curves from an intact biofilm, sampled from The Hythe, are shown in Fig. 2. These measurements were made using the high resolution imaging system. In contrast to the data from the cultures of *Cylindrotheca closterium* (Fig. 1), the estimated values of ETR using the 680 nm bandpass are actually lower than those using the 695 nm longpass filter between 0 and approximately 500 µmol m^{-2} s^{-1} and only become higher at PPFDs >500 µmol m^{-2} s^{-1}. This pattern of relative values of ETR with the 2 filters is reflected in the higher values of ETR_{max} and lower values of α for the 680 nm bandpass filters. Values of ETR_{max}, α and E_k derived from these light response curves are shown in Table 2, along with similar data collected from an intact biofilm sampled from the Alresford Creek site.

Selected fluorescence images (at F') used in the construction of the light response curves from Alresford Creek and The Hythe are shown in Fig. 3. At Alresford Creek, there was a very obvious decrease in the density of cells of *Staurophora amphioxus* at the surface of the biofilm with increasing PPFD, in combination with an overall decrease in cell density (Fig. 3a,c,e). At The Hythe, the surface of the biofilm was increasingly dominated by euglenoids with increasing PPFD (Fig. 3b,d,f), although the overall cell density did not change appreciably, due to the downward movement of diatoms (mainly *Gyrosigma* sp.). These changes in species composition occurred on the time scale of 10 min or less.

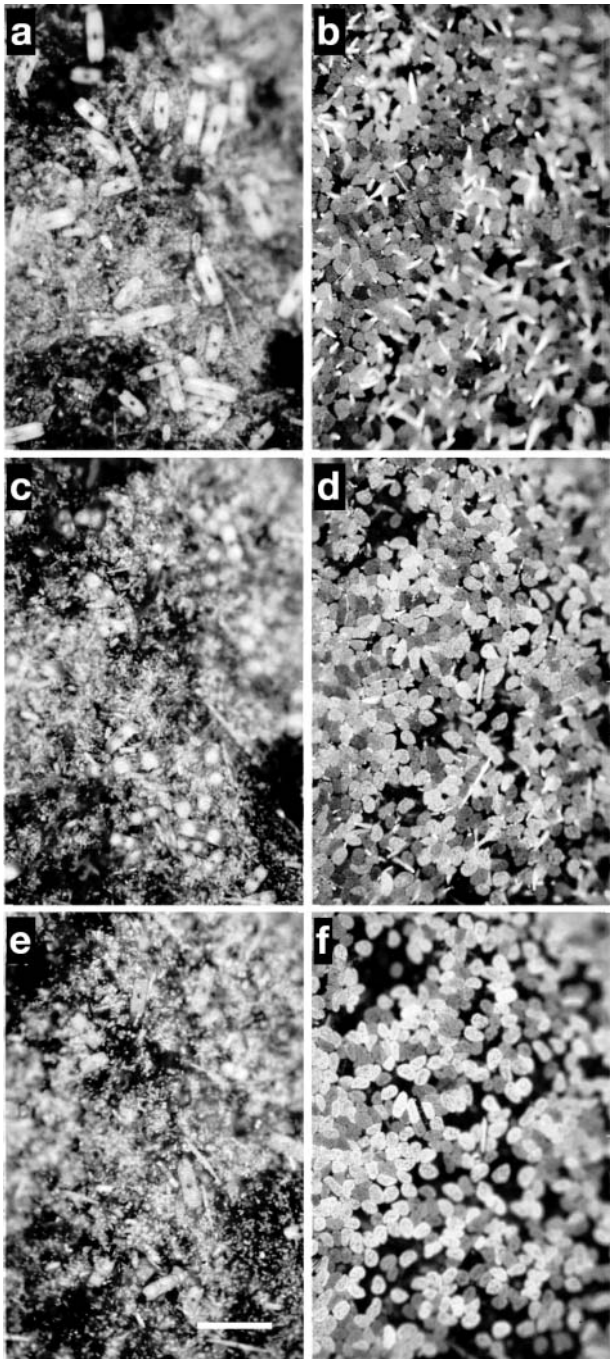

Fig. 3. Selected fluorescence images (at F') used in the construction of the light response curves from (a,c,e) Alresford Creek and (b,d,f) The Hythe. Images were taken at a PPFD of 180 µmol m^{-2} s^{-1} (a,b), 380 µmol m^{-2} s^{-1} (c,d) or 1150 µmol m^{-2} s^{-1} (e,f). All images were taken using the 680 nm bandpass filter. Scale bar in (e) is 100 µm long

The images in Fig. 4 illustrate the differences in F_q'/F_m' for cells at the surface of biofilms taken from Alresford Creek or The Hythe, at a PPFD of 180 or 1150 µmol m^{-2} s^{-1}, when fluorescence was defined with the 680 nm band-

pass or the 695 nm longpass filter. At the lower PPFD, the cells at the surface showed lower values of F_q'/F_m' when the 680 nm bandpass filter was used, rather than the 695 nm longpass filter. Conversely, the 680 nm bandpass filter gave higher values of F_q'/F_m', compared to the 695 nm longpass filter, at the higher PPFD.

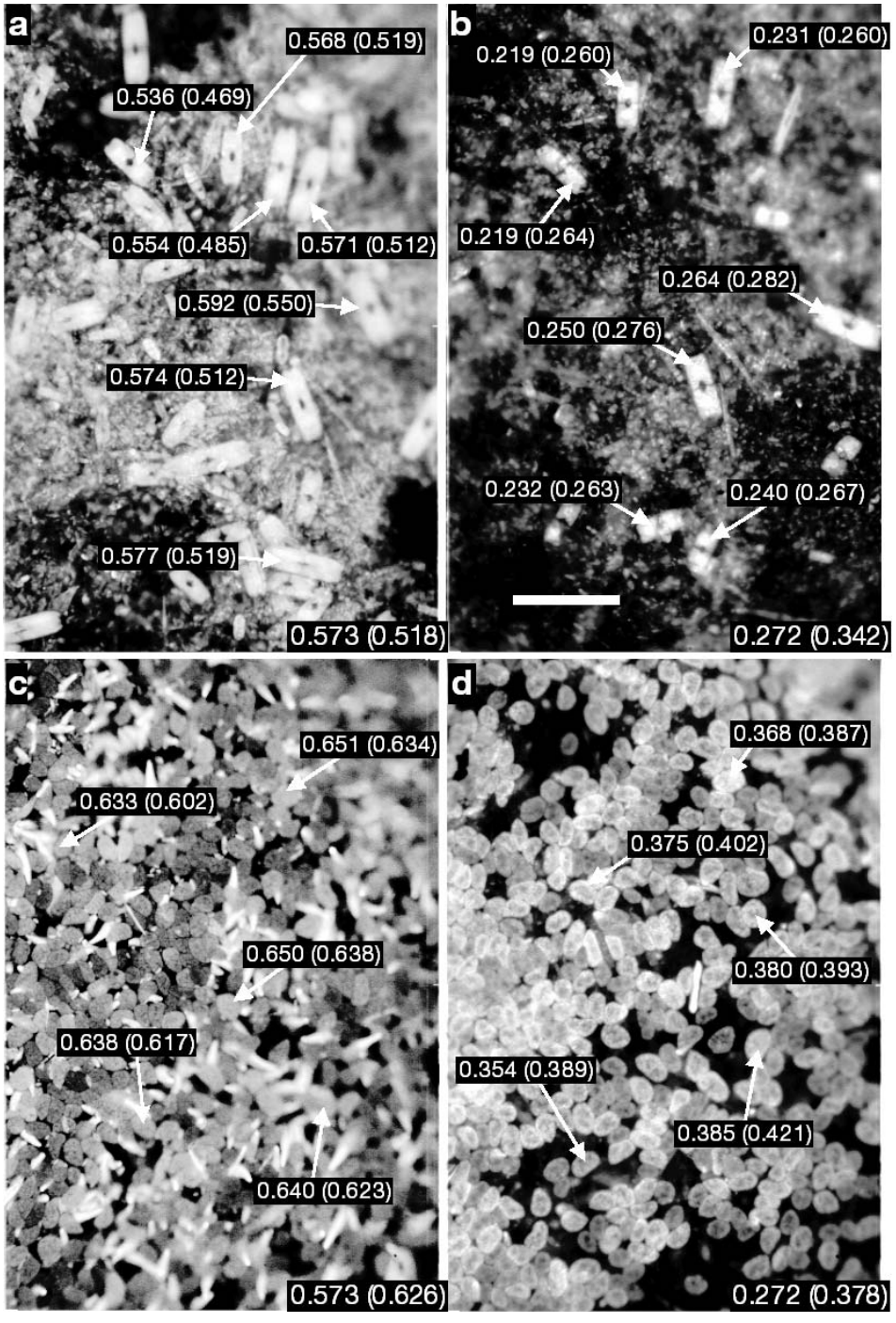

Fig. 4. Images of F' taken at a PPFD of 180 µmol m^{-2} s^{-1} (a,c) or 1150 µmol m^{-2} s^{-1} (b,d). Images are from (a,b) Alresford Creek and (c,d) The Hythe and were taken using the 695 nm longpass filter. Numbers that accompany the arrows within images indicate the value of F_q'/F_m' for the cell to which the arrow is pointing. Numbers in the bottom right hand corner of each image are mean values for the whole image. In all cases, the initial value is calculated from images of F' and F_m' taken using the 695 nm longpass filter while the numbers in brackets are from images of F' and F_m' taken using the 680 nm bandpass filter. Values of F_q'/F_m' from individual cells were calculated using the method described in Oxborough et al. (2000). Scale bar in (b) is 100 µm long

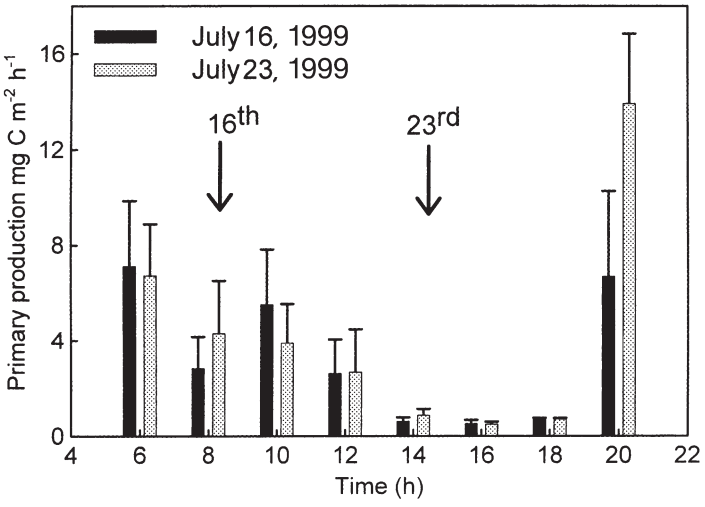

Fig. 5. Rates of primary production (mean ± SE, n = 3) followed using the ^{14}C-radiotracer method. Intact biofilms were sampled from Alresford Creek on July 15 and 22, 1999. In both cases, measurements were made under greenhouse conditions over 16 h periods on the following day. Arrows indicate the time that the biofilms would have experienced low tide on the days that measurements were made

Effect of vertical migration of cells on the estimated value of ETR

The rate of primary production by an intact biofilm from Alresford Creek was followed using the ^{14}C-radiotracer method (Fig. 5). Measurements were made on emersed cores under glasshouse conditions over 16 h periods, between 06:00 and 22:00 h, on July 16 and 23. Measurements of F_q'/F_m', estimated ETR and incident PPFD over the same 16 h period on July 16 are shown in Fig. 6. It is clear from the data in Fig. 5 that there was no obvious relationship between primary production and tide time, as the pattern of ^{14}C incorporation was very similar on both dates, even though the tide was roughly 6 h later on July 23. Nor did the rate of primary production correlate with PPFD, since the highest rates of ^{14}C incorporation (Fig. 5) were seen in the early morning and late evening, while the highest PPFDs were seen over the middle part of the day (Fig. 6).

The data in Figs. 5 & 6 also reveal that the rate of primary production did not correlate with the calculated values of ETR over the course of the day. The difference between ^{14}C incorporation and calculated rates of ETR was most evident at 2 peaks in PPFD, at approximately 12:00 and 15:00 h (Fig. 6). At these points, the rate of ^{14}C incorporation was relatively low (particularly at 15:00 h) while the calculated rates of ETR were very high (2 to 3 times higher than at any other time). It is also noticeable that the very large increases in PPFD at 12:00 and 15:00 h were accompanied by increases in F_q'/F_m'.

Although the integrated values of F_q'/F_m' under ambient PPFD did not correlate with rates of ^{14}C-primary production, rates of primary production under ambient PPFD did correlate with values of F_q'/F_m' measured from individual cells of *Staurophora amphioxus* and *Pleurosigma angulatum* at the surface of the biofilm, at a constant PPFD of 220 µmol m^{-2} s^{-1} ($r = 0.67$, $p < 0.01$, $n = 21$ for *S. amphioxus* and $r = 0.92$, $p < 0.01$, $n = 15$ for *P. angulatum*) (Fig. 7). Changes in the community structure of the biofilm were also observed. On July 16, the biofilm was dominated by *S. amphioxus* between 07:00 and 11:00 h. Thereafter *P. angulatum* and *Gyrosigma* spp. were more evident until 15:00 h, when the population became very mixed. By 18:00 h, *S. amphioxus* was again dominant.

DISCUSSION

The simplest system examined here was the culture of *Cylindrotheca closterium* (Fig. 1). With such a system, the re-absorption of fluorescence from underlying

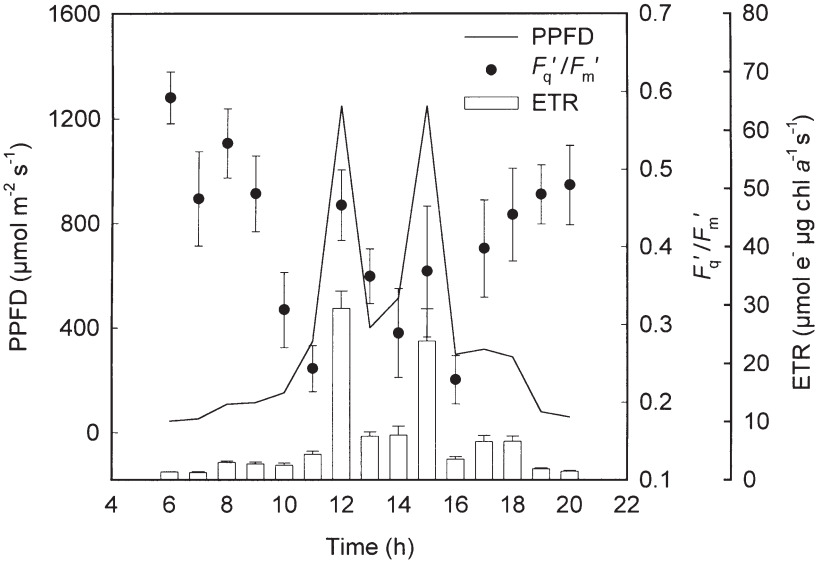

Fig. 6. Estimated values of ETR, values of F_q'/F_m' and changes in ambient PPFD measured on July 16, 1999, from biofilm samples taken from Alresford Creek on the previous day. Measurements were made under greenhouse conditions. Rates of primary production from the same samples are shown in Fig. 5

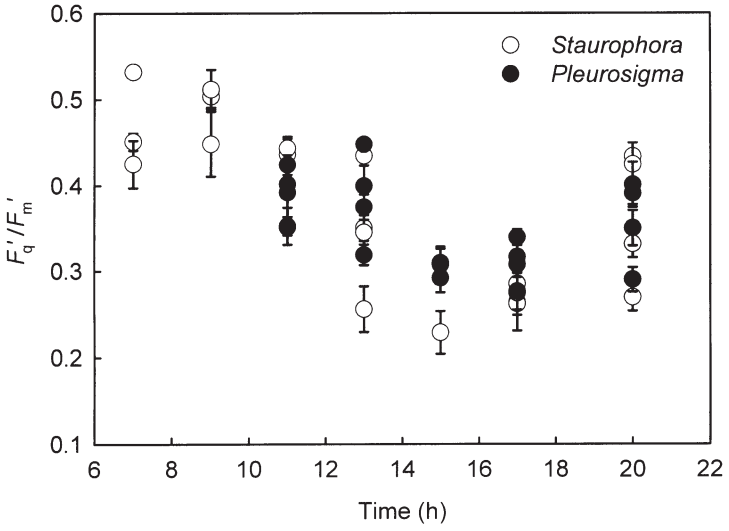

Fig. 7. Integrated values of F_q'/F_m' from cells of *Staurophora amphioxus* and *Pleurosigma angulatum* that were present at the surface of biofilms sampled from Alresford Creek on July 15, 1999. Measurements were made using the high resolution imaging system at a constant PPFD of 220 µmol m^{-2} s^{-1} on July 16, 1999. Rates of primary production, estimated values of ETR, values of F_q'/F_m' and changes in ambient PPFD made from the same biofilm samples under glasshouse conditions on the same day are shown in Figs. 5 & 6

cells is minimal and there is a clear advantage to removing most of the PS I fluorescence by using the 680 nm bandpass filter, rather than the 695 nm longpass filter. With intact biofilms the situation is more complex, since the 680 nm bandpass filter removes most of the PS I fluorescence but also decreases the fraction of the total fluorescence signal coming from the cells at depth within the biofilm. In many instances, the cells deeper within the biofilm have a higher PS II operating efficiency. Consequently, the re-absorption of a proportion of the fluorescence signal from these cells can actually decrease the overall, integrated value of this parameter.

The light curves in Fig. 2 and light curve data in Table 2 show that the overall effect of using the 680 nm bandpass filter, rather than the 695 nm longpass filter, is to increase the estimated values of ETR_{max} and E_k while decreasing the value of α. These data suggest that in these instances the increase in F_q'/F_m' resulting from the removal of most of the PS I fluorescence when the 680 nm bandpass filter is used is more than offset by the removal of part of the fluorescence signal from the cells at depth at low to moderate PPFDs, but not at high PPFDs. Clearly, these data illustrate that use of the 680 nm bandpass filter, rather than the 695 nm longpass filter, has a non-proportional effect on the relationship between PPFD and estimated ETR. However, it is equally clear that neither filter is providing an accurate picture of the true relationship between these parameters and that, in the context of integrated fluorescence measurements, there is no clear advantage to using either filter.

The images in Fig. 4 illustrate the dual effects of decreasing PS I fluorescence and background fluorescence through use of the 680 nm bandpass filter, rather than the 695 nm longpass filter. At the lower PPFD of 180 µmol m^{-2} s^{-1}, F_q'/F_m' was lower when the 680 nm bandpass filter was used while at the higher PPFD of 1150 µmol m^{-2} s^{-1} the situation was reversed. This was true for cells isolated from the surface of both biofilms and for the integrated values. The most obvious explanation for these results is that, at low PPFD, the increase in F_q'/F_m' due to the removal of PS I fluorescence by the 680 nm bandpass filter is more than offset by the decrease in F_q'/F_m' due to the removal of fluorescence from deep within the biofilm, while at high PPFD, the removal of PS I fluorescence has more effect on the overall value of F_q'/F_m' than does the removal of fluorescence from deep within the biofilm. It is these competing effects of the 680 nm bandpass filter on F_q'/F_m' that result in the complex relationship between PPFD and estimated ETR that is revealed by the data in Fig. 2 and Table 2.

Although PS I fluorescence clearly introduces an error into the calculation of ETR (through F_q'/F_m'), this error is minor compared to the size of the error that can result from the vertical migration of cells and (to a lesser extent) changes in species composition at the surface of biofilms. Of particular note are the large increases in the estimated values of ETR that were observed at 12:00 and 15:00 h on July 16 (Fig. 6). These data are almost certainly due to the migration of cells deeper into the sediment at high PPFD, which has greatly reduced the proportion of incident photons that are actually absorbed by the cells and which has resulted in values of F_q'/F_m' that are higher than those on either side of the peaks. Since the change in absorptivity of the cells is not taken into account (and cannot realistically be accounted for when using an integrating fluorometer system) the estimated value of ETR is very much higher than the true value.

Previous studies have assessed the relationship between ETR and rates either of ^{14}C assimilation or O_2 evolution using slurries (Hartig et al. 1998, Wolfstein & Hartig 1998, Barranguet & Kromkamp 2000). With slurry systems, the cells are suspended in a light environment that is radically different from that experienced within the intact biofilm (Underwood & Kromkamp 1999) and obviously take no account of the impact of vertical migration on the fraction of incident light that is actually absorbed. Clearly, imaging of chlorophyll fluorescence provides a powerful method for assessing vertical migration within intact biofilms.

The rate of primary production measured by ^{14}C radiotracer (Fig. 5) showed a diel pattern that was independent of PPFD or tidal rhythm. The observation that this pattern correlated significantly with F_q'/F_m' measured from individual cells at the biofilm surface, at constant PPFD (Fig. 7), suggests that some form of endogenous rhythm may be operating at the cell level. Although pH-mediated changes of the CO_2 equilibrium would effect the ^{14}C-specific activity within biofilms and could account for apparent decreases in the calculated rate of primary production, this does not seem to be the case here. pH-mediated shifts will be most apparent in dense biofilms under high irradiance (Underwood & Kromkamp 1999). However, we have consistently measured similar significant patterns of high morning rates of primary production in biofilms from a range of sites and sampled at different times of the year, independent of biofilm biomass or light intensity (Perkins et al. pers. obs.). This pattern remains to be further investigated.

In conclusion, the data presented in this study demonstrate that PS I fluorescence introduces an error into the calculated value of ETR. Although most of the PS I fluorescence can be removed from the overall signal by using a 680 nm bandpass filter, this introduces a second error (which operates in the opposite direction) by giving undue weighting to fluorescence from cells at the surface of the biofilm. Consequently, there is no clear advantage to using the 680 nm bandpass filter with an integrating fluorometer, such as the Xenon PAM system used in this study. In many situations, a more substantial source of error than PS I fluorescence is the vertical migration of cells within biofilms, which can have an enormous impact on the calculated value of ETR and, consequently, on estimates of primary production within biofilms. While these data do not rule out the application of chlorophyll fluorometry to the estimation of ETR and rates of primary production within biofilms, they do present a very clear demonstration of how important it is to know the vertical location of cells within the biofilm and to be able to measure values of F_q'/F_m' from individual cells. This can be realistically achieved only when using high resolution imaging of chlorophyll fluorescence.

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