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In situ photosynthetic activity and xanthophylls cycle development of undisturbed microphytobenthos in an intertidal mudflat

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ABSTRACT

The photosynthetic performances of microphytobenthos as well as the xanthophylls cycle development were monitored directly *in situ* on undisturbed intertidal mudflats in the Authie Bay (Northern France) during the two daylight emersion periods of the same day. The effective quantum yield of PSII was measured regularly using the PAM fluorescence technique at the same three locations on the sediment, allowing the estimation of the relative electron transport rate (rETR) and the establishment of two light curves. The photosynthetic responses of microalgae at a given light level differed for one emersion period to the other. The maximum rETR ($rETR_m$) calculated from the afternoon light curve were significantly higher than in the morning. Pigments analyses show evidence of the progressive conversion of diadinoxanthin (DD) to diatoxanthin (DT) with increasing light. We demonstrate for the first time, with *in situ* measurements, the involvement of the xanthophylls cycle in the photoacclimation process in microphytobenthos at high irradiances.

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1. Introduction

Despite being submitted to extreme environmental variations (light, pH, temperature, salinity, nutrients, oxygen, etc.) due to diurnal and tidal cycles, estuaries are highly productive zones (Pinckney and Zingmark, 1991; Blanchard and Guarini, 1996; Serôdio and Catarino, 1999; Barranguet and Kromkamp, 2000). In intertidal flats, benthic microalgal photosynthesis can account for up to a third of total estuarine carbon fixation (Sündback et al., 1997; Sullivan and Currin, 2000) and occurs essentially during the diurnal emersion period (Pinckney and Zingmark, 1991). Microalgae in this severe habitat have developed different behavioural and physiological adaptations that optimize their primary production. One adaptation to these harsh conditions is vertical migration through the sediment (for a review, see Consalvey et al., 2004). The movement of motile microalgae, essentially diatoms, is linked to the excretion of extracellular polymeric substances (EPS), mainly carbohydrates, which also contribute to biofilm development at the sediment surface (Smith and Underwood, 1998; Underwood and Paterson, 2003). Within this biofilm, microalgae are protected against external stresses (Underwood and Paterson, 1993). The downward migration can occur before immersion to reduce wash-away of cells with the tide but also during emersion to reduce grazing by predators and to avoid high light exposures (Underwood, 2002; Forster and Kromkamp, 2004; Serôdio et al., 2005b). Another adaptation to high light stress is the development of photoprotective mechanisms such as the development of xanthophylls cycle which allows the thermal dissipation of excess energy in the antennae of photosystem II and has the effect of decreasing the photosynthetic efficiency of microalgae. It consists, in diatoms, of the de-epoxidation of diadinoxanthin (DD) to diatoxanthin (DT) (Young et al., 1997). This cycle has been demonstrated in the laboratory, most often in monocultures (Lavaud et al., 2002, 2003, 2004) but only a few studies have investigated its development *in situ*, on the whole microalgal community (van Leeuwe et al., 2008). Despite all the studies done on microphytobenthic communities, the main problem remains on the development of reliable techniques that allow the estimation of *in situ* photosynthetic activity and the contribution of the community to primary production in estuaries. Among the various methods tested (Glud et al., 1992; Hartig et al., 1998; Migné et al., 2004), pulse amplitude modulated (PAM) fluorometry (Schreiber et al., 1986) has been the most widely used technique (Serôdio et al., 1997; Kromkamp et al., 1998; Barranguet and Kromkamp, 2000; Perkins et al., 2002; Brotas et al., 2003; Barranguet et al., 2004; Serôdio et al., 2008). This non-invasive technique is generally used to estimate the effective quantum yield of photosystem II (Φ_{PSII}) (Genty et al., 1989)

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of microalgae. This parameter reflects the short-term variability of photosynthetic activity and can be used to assess the linear electron transport rate (ETR) through photosystem II. This technique has been used successfully to follow photosynthetic activity of the microphytobenthic communities on intact sediment cores or cultivated, isolated microalgae (Barranguet and Kromkamp, 2000; Perkins et al., 2001; Serôdio et al., 2005b). However, only a few studies have been performed directly in the field (Brotas et al., 2003; Migné et al., 2007). Measuring photosynthetic activity directly *in situ* is essential as variations in environmental factors strongly modify the behaviour of microalgae and, as a consequence, may influence the production of the communities.

In the present work, we propose (1) to study the photochemical behavioural changes of natural biofilms during emersion periods by *in situ* measurements of chlorophyll fluorescence associated with assessments of pigment contents (2) to estimate the light history influence by comparing the response of microphytobenthic communities during two daylight emersion periods within the same day and finally (3) to evaluate the importance of the xanthophylls cycle in the photosynthetic efficiency and photoacclimation development of microalgae.

2. Material and methods

Experiments were carried out directly *in situ* during emersion in the estuary of Authie Bay located in Northwestern France (50°N22′ 255, 1°E37′525; Fig. 1). The study site was selected in mudflats dominated by muddy sands (80% silt) (J-P Debenay, unpublished data) on which microphytobenthos, mainly diatoms, occurred in some parts as a biofilm. The experiment described here has been repeated on various days and the obtained results showed similar patterns. We present the results of a unique day of experiment, representative of all the other ones performed in similar conditions in this study site. The tidal cycle is semi diurnal and during the day of the presented experiment (11th August 2007), the gap between tidal and nychtemeral cycles lead to two daylight emersions. Low tides occurred at 6:23 h (UT + 2) and 18:49 h and high tide at 11:44 h. Photosynthetic photon flux density (PPFD) was measured continuously *in situ* using an SA-190 quantum sensor connected to a Li-1400 datalogger (Li-cor) placed on the muddy sands. Sediment surface temperature (SST) and air temperature (AT) were also measured during the daytime of the experiment.

2.1. Fluorescence parameters and light curves

Chlorophyll fluorescence was measured *in situ* using a Diving-PAM fluorometer (Walz) and a system of three custom made supports that allowed measurements in triplicate at the three locations on the sediment during the whole experiment. The tip of the fibre optic of the fluorometer was fixed at a constant distance (2 mm) from the biofilm at a 60° angle. The effective PSII quantum efficiency (Genty et al., 1989) was measured under ambient light and was calculated as:

$$\Phi_{PSII} = \frac{F'_m - F_t}{F'_m}$$

where F_t is the fluorescence steady-state level under ambient light and F'_m is the maximal level determined with a single saturating light pulse (0.8 s, 2500 µmol photons $m^{-2} s^{-1}$) for light-acclimated samples. Φ_{PSII} can be used to calculate the relative electron transport rate (rETR), according to the estimation of Genty et al. (1989):

$$rETR = \Phi_{PSII} \times PPFD \times 0.5$$

where PPFD is the value of the ambient light and 0.5 is the factor that accounts for the partitioning of energy between the two photosystems. The term relative ETR is used here as an approximation of the ETR, for which a measure of the chlorophyll *a*-specific absorption is required but not possible for intact biofilms (Underwood, 2002; Forster and Kromkamp, 2004).

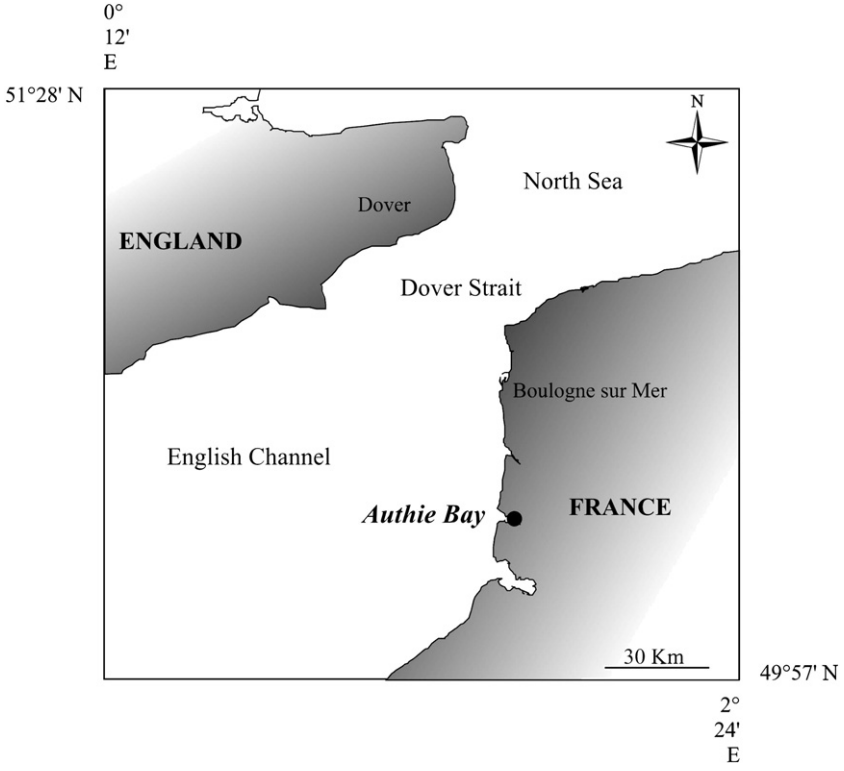

Fig. 1. Map of the study area on the French coasts of the eastern English Channel.

The "relative Non-Photochemical Quenching" (rNPQ) was calculated for each replicate according to Serôdio et al. (2005a) as:

$$rNPQ = \frac{F'_{m_m} - F'_m}{F'_m}$$

where $F'_{m\,m}$ is the maximum value of F'_m measured during the emersion period, under low actinic irradiance, for each replicate. We calculated this "relative Non-Photochemical Quenching" in order to evaluate the thermal dissipation of excess energy during the emersion periods but it can not be compared to the "real" NPQ as it is defined in Bilger and Björkman (1990) for instance. The difficulty in calculating the "real" NPQ in our case was due to the fact that F_m values, measured in darkness, were often lower than F'_m values because not all microalgae had migrated to the surface.

rETR values as a function of the ambient light (rETR-irradiance curves also called light curves) were fitted using the model of Eilers and Peeters (1988). The least square estimation method (fitting procedure of the "SYSTAT 10" software) was used to assess the characteristic photosynthetic parameters of the microphytobenthic communities, i.e. the initial slope of the non-saturated photosynthetic rate (α), the light saturation parameter (E_k) and the maximum rETR ($rETR_m$) (Coutinho and Zingmark, 1987; Henley, 1993).

2.2. Pigments content analyses

To access pigments content of the microphytobenthic communities, sediment cores were collected in triplicate at four different times during each emersion period, corresponding to different ambient light. Cores were taken from the same areas that photosynthetic activities were measured. Copper tubing (diameter 1 cm) fitted with a cotton piece was sunk into the mud, as described in Wiltshire et al. (1997). Liquid nitrogen was poured onto the tubing to freeze the sediment over 1 cm depth. The samples were kept frozen and transported to the laboratory in darkness in liquid nitrogen and stored at −80 °C until further analysis.

Pigments from the first 1 mm sediment were extracted by sonicating for 30 s in 1.5 mL cold 100% acetone and incubated for 3 h in darkness at 4 °C under constant agitation. Acetone was chosen as the extraction solvent according to the recommendations of Buffan-Dubau and Carman (2000). Extracts were separated from sediment by centrifugation (5000×*g*), filtered on PTFE membrane (0.2 µm; Pall, Acrodisc) and evaporated to dryness under a stream of nitrogen. Pigments were then redissolved with a mixture of methylene chloride/distilled water (50/50, v/v) and after decantation, the aqueous phase was discarded with the aim of removing salt and water before injection. The pigment extract was completely evaporated under a stream of nitrogen and then recovered in methanol before injection. Pigment contents were analyzed by a reverse-phase High Performance Liquid Chromatography (HPLC) according to Arsalane et al. (1994), using a Beckman 32 Karat system equipped with a diode array detector and an Allure C18 Restek column. The molar extinction coefficients used to calibrate the system are those cited by Berkaloff et al. (1990). The de-epoxidation ratio (DR) was calculated as:

$$DR = \frac{Dt}{Dt + Dd + cisDd}$$

where Dt = diatoxanthin, Dd = diadinoxanthin and cisDd = cis-diadinoxanthin amounts.

The relationship between rNPQ and DR was established with the mean values of these parameters at the four different times during each emersion period.

3. Results

3.1. Physical variables

The results presented here come from an experiment carried out on 11th August 2007 during a sunny day without clouds with measurements performed from sunrise to sunset, except during the immersion period. Fig. 2 shows the emersion/immersion periods and the time courses of PPFD, SST and air temperature during the experiment. The maximal irradiances measured during the emersion periods, in the morning and in the afternoon, were 1315 µmol quanta $m^{-2}\,s^{-1}$ and 1630 µmol quanta $m^{-2}\,s^{-1}$, respectively. The SST during the morning rose from 13.4 °C at 6:21 h to 23.0 °C at 10:51 h. During the afternoon, the SST reached 27.7 °C at 15:30 h and then decreased to 18.5 °C at 21:28 h.

3.2. Fluorescence parameters during the two diurnal periods of emersion

Fig. 3 illustrated the time courses of rETR and PPFD during the morning and afternoon periods of emersion. During the morning emersion, the values of rETR increased when PPFD increased and the highest mean rETR value was 111 ± 19 for a PPFD of 1100 µmol quanta $m^{-2}\,s^{-1}$. In the afternoon, the highest rETR values were not measured just after immersion, at the maximum irradiances, but later. Just after immersion, the mean value of rETR was 184 ± 28 for a PPFD of 1630 µmol quanta $m^{-2}\,s^{-1}$. Then, rETR increased (even if the PPFD decreased) and reached a value of 297 ± 22 (at 1315 µmol quanta $m^{-2}\,s^{-1}$) before decreasing until sunset. The light curves obtained by fitting the data from the two emersion periods to the model of Eilers and Peeters (1988) and the corresponding light curve parameters are presented in Fig. 4 and Table 1, respectively. $rETR_m$ obtained for the afternoon light curve (258 ± 33) was significantly higher than that in the morning (136 ± 68) (Test *U* Mann–Whitney, $p<0.05$). α and E_k showed no significant difference between the morning and afternoon periods.

3.3. Pigment contents

Chlorophyll *a* contents measured in the first sediment millimeter during this experiment were relatively constant during the day with a mean value of 16.80 ± 3.15 mg m^{-2} ($n = 23$). Fig. 5 shows the DR and the PPFD changes during the emersion periods. For the fourth time sample (10:51 h), one of the three replicates was lost, reducing the number of analyzed samples to two. During the morning emersion, DR increased from 0.123 ± 0.016 at 6:20 h to 0.207 ± 0.025 at 10:50 h. After immersion, DR was 0.217 ± 0.018 at 15:00 h and then decreased to 0.102 ± 0.008 at 21:30 h. A significant linear regression was

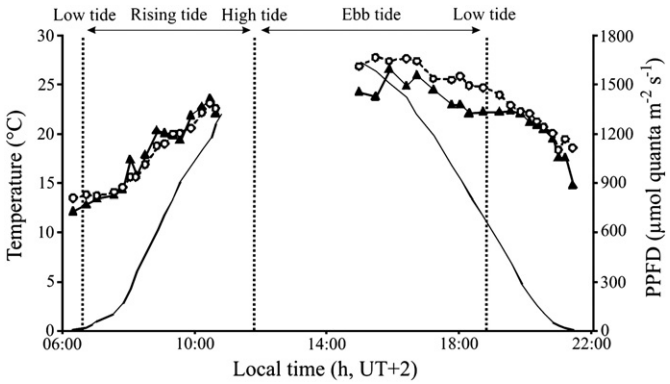

Fig. 2. Diurnal course of incident irradiance (PPFD, solid line), sediment surface temperature (○) and air temperature (▲) during the tidal cycle.

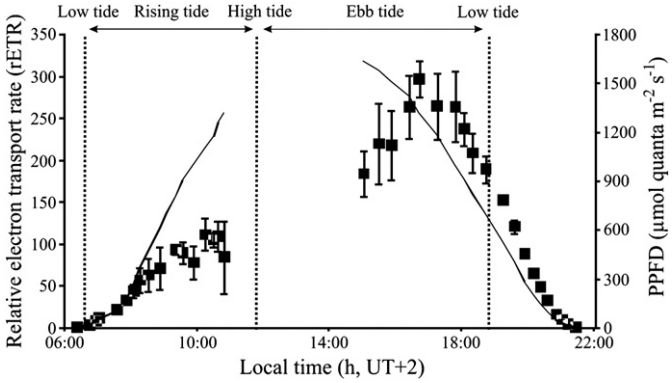

Fig. 3. Diurnal course of mean relative electron transport rate (rETR, ■ and vertical bars: SD) measured on three spots on the sediment and incident irradiance (PPFD, solid line) during the tidal cycle.

observed between DR and PPFD ($r=0.936$, $n=23$, $p<0.001$) (Fig. 6), with no significant difference occurring in the development of DR with light between the morning and afternoon periods of emersion (analysis of covariance). A significant linear regression was also observed between DR and rNPQ ($r=0.934$, $n=8$, $p<0.001$) (Fig. 7). The differences between DR values obtained at different times of the experiment were evaluated using non parametric statistics (Test *U* Mann–Whitney). Significant differences were observed between the two first measurements of DR (at 6:20 h and 8:15 h) in the morning and the third DR measurement (at 9:55 h) ($p<0.05$), but also between the first two DR values in the afternoon (at 15:00 h and 16:45 h) and the last two DR values (at 19:55 h and 21:30 h) ($p<0.05$). No significant difference was observed between the last values of DR in the morning (just before immersion) and the first values of DR in the afternoon (just after emersion).

4. Discussion

The experiment described in this paper was performed during one day in which the two periods of emersion presented different specific environmental conditions for instance in terms of light and temperature. During the morning rising tide, after a long period (around 8.5 h) of darkness (corresponding to the night and to immersion), the biofilm was exposed to progressively increasing light, with a maximal PPFD of 1300 µmol quanta $m^{-2}\,s^{-1}$, before the sediment was submerged around high tide. In contrast, during the afternoon ebb tide, after an immersion of 3.5 h, the biofilm was more rapidly submitted to high light (1630 µmol quanta $m^{-2}\,s^{-1}$) and then to decreasing light until sunset. These environmental and tidal conditions caused some differences in the photosynthetic activity of the

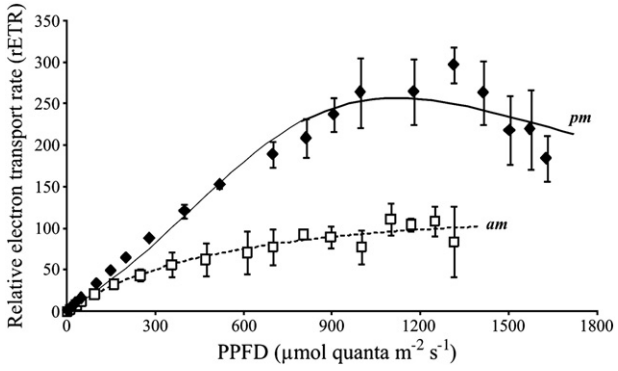

Fig. 4. Light curves obtained by fitting the data from the two emersion periods by the model of Eilers and Peeters (1988): am (morning emersion period — rising tide, □, and dotted line) and pm (afternoon emersion period — ebb tide, ◆, and solid line).

Table 1
Photosynthetic parameters assessed with the model of Eilers and Peeters (1988) from the two (morning and afternoon) rETR-irradiance curves. α = the initial slope of the non-saturated photosynthetic rate, E_k = the light saturation parameter and $rETR_m$ = the maximum rETR. SD are in brackets ($n=3$).

Photosynthetic parameters	Morning emersion period	Afternoon emersion period
α	0.16 (0.10)	0.21 (0.04)
$rETR_m$	136 (68)	258 (33)
E_k	932 (145)	1115 (60)

microphytobenthos which was globally much higher in the afternoon compared to the morning (i.e. a systematic higher rETR at a given value of PPFD). Even if the range of light is not the same between the rising tide and ebb tide (lower PPFD levels during the morning), the difference between the two light curves may also be explained by the specific environmental factors of each emersion period. The lower photosynthetic activity observed in the morning period of emersion could be caused by a depletion of nutrients or carbon (Glud et al., 1992; Kromkamp et al., 1998), but also by stressful conditions, i.e. prolonged exposure to high light and temperature, supersaturation in oxygen, dessication of sediment or high salinity (Rijstenbil, 2003). The tidal and environmental conditions could also influence the composition of microalgal communities at the sediment surface. This change in species composition at the surface is allowed by the ability of motile microalgae to migrate into the sediment (Kromkamp et al., 1998; Perkins et al., 2001). However, for both periods, the rETR values could have been overestimated due to the difference between inherent physiological parameters and depth-integrated parameters (Forster and Kromkamp, 2004; Serôdio, 2004). Indeed, at high light, some cells migrated downward to a depth where light is strongly attenuated and then certainly had a higher effective quantum yield of PSII. rETR were then calculated with the Φ_{PSII} values resulting from the combination of the surface sediment and the upper sediment layer signals, and with incident PPFD measured at the sediment surface but not with attenuated light values within the sediment. This overestimation can occur for both periods, even if this phenomenon is probably higher during the afternoon emersion, when the light levels were higher than in the morning. However, this overestimation can only partly explain the difference between the two light curves.

In the beginning of the afternoon ebb tide, rETR values at 15:30 h (when PPFD are the highest) were lower than those obtained at 17:00 h (when the irradiances were lower) (Fig. 3). These lower values were due to lower PSII yields and could be the result of the worst physiological state of episammic microalgae that remained at the sediment surface. Indeed, at 15:30 h, motile microalgae could migrate downward to protect themselves from photodamage in a layer where they are not detected by the PAM, i.e. deeper than the first 200 µm. On the contrary, without the ability to migrate inner the sediment, the episammic microalgae have to stand at the surface layer and cope with high light, developing photoprotection mechanisms like the xanthophylls cycle. With decreasing irradiances, between 15:30 h and 17:00 h, photophilic microalgae migrated up to a shallow subsurface layer or to the surface and contributed then to the increase of the fluorescence yield. The increasing rETR between 15:30 h and 17:00 h is then explained by a modification of community composition which was enriched with photophilic microalgae.

Some studies have estimated Non-Photochemical Quenching by calculating a parameter called NPQ using the maximal fluorescence for dark-adapted algae and for light-adapted algae, in parallel with measurements of photosynthetic activity of benthic microalgae (Serôdio et al., 2005a). But to our knowledge, no studies have evaluated the development of the xanthophylls cycle and photosynthetic activity at the same time, directly *in situ* on undisturbed sediment. NPQ processes include rapidly reversible mechanisms (energy-dependent quenching, q_E) and slowly reversible changes

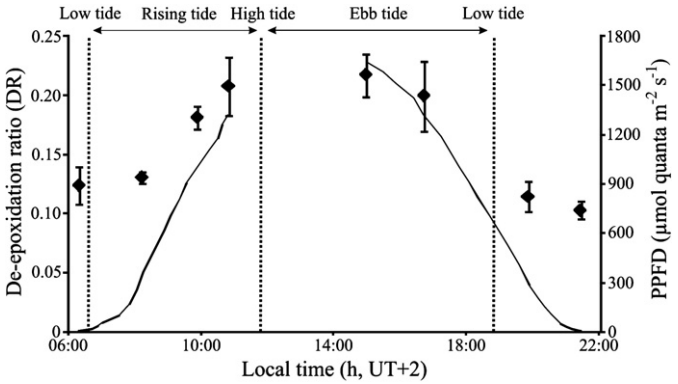

Fig. 5. Diurnal course of de-epoxidation ratio (DR, ♦, and vertical bars: SD) and incident irradiance (PPFD, solid line) during the tidal cycle.

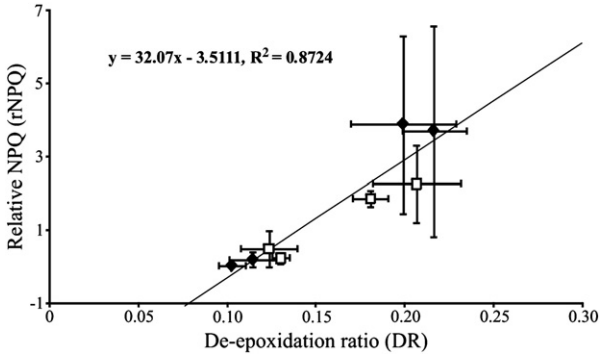

Fig. 7. Mean relative NPQ (rNPQ) as a function of mean de-epoxidation ratio (DR) for the morning emersion period (□) and the afternoon emersion period (♦) (horizontal and vertical bars: SD). Solid line: linear regression.

(photoinhibitory quenching, q_I) (Müller et al., 2001). q_E development involves the development of the xanthophylls cycle which is considered to be the most dominant and rapid of the NPQ processes (Serôdio, 2004). The xanthophylls cycle is a photoprotection process that involves the de-epoxidation of diadinoxanthin to diatoxanthin and induces an increase in the thermal dissipation of excess photons absorbed by the light-harvesting complexes (Olaizola et al., 1994; Bertrand et al., 2001; Lavaud et al., 2002; Ruban et al., 2004). We showed in our experiments that the xanthophylls cycle occurs *in situ* in microphytobenthos during the tidal cycle. We demonstrated that the cycle was reversible *in situ* and that DR was directly correlated to the daily course of light. According to Olaizola et al. (1994), an increase in light results, within 15 min, in an increase in DT and in a change in DR. We cannot completely exclude the possibility of DD and DT *de novo* synthesis as it has been demonstrated in cultures on microalgae submitted to high lights (Olaizola et al., 1994; Lavaud et al., 2004). However, this phenomenon is difficult to evaluate *in situ*, due to the possible upward migration of microalgae from deeper sediment layers. The linear correlation between DT accumulation and NPQ has already been observed *in vitro* (Lavaud et al., 2002, 2004; Dimier et al., 2007). In the case of *in situ* experiments, it is very difficult to measure a real NPQ, as it is currently defined, because a dark measurement of F_m is necessary. If F_m measurements are done at dawn, the microalgae are still in deep sediment layers and are not detected by the fluorometer, giving very low F_m values. If measurements are done during the emersion period, it is possible that the necessary dark period induces migratory processes in the microalgae which could modify the fluorescence signals. Consequently, we decided, as proposed in Serôdio et al. (2005a) to calculate "relative" NPQ values with the highest F'_m value of the replicate obtained at low light. In this study, we demonstrate for the first time that the rNPQ is positively correlated to the DR during the whole day.

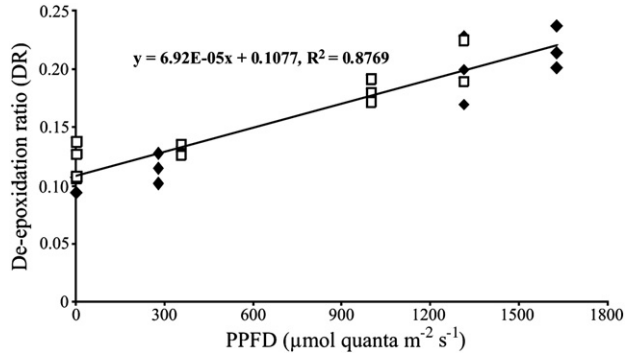

Fig. 6. De-epoxidation ratio (DR) as a function of incident irradiance (PPFD) for the morning emersion period (am, □) and the afternoon emersion period (pm, ♦). Solid line: linear regression.

The averaged DR observed at PPFD around 3 and 300 µmol quanta m^{-2} s^{-1} during both emersion periods is 0.118 ± 0.014 and is in agreement with the results obtained *in situ* by van Leeuwe et al. (2008) at the sediment surface of the Barrow estuary (DR of 0.1 at low irradiance). The xanthophylls cycle was settled during the two emersion periods, with DR equivalent at a given irradiance, even if, as previously demonstrated, the two rising and ebb tides present different ranges of light. One hypothesis is that this photoprotection process is mainly developed by non-motile microalgae, which stay in the same layer of the sediment during the whole tidal cycle and which respond to the same light exposure with an equivalent DR. The motile microalgae have the ability to modulate their light exposure by moving through the sediment by 'micro-migrations' (Kromkamp et al., 1998; Perkins et al., 2001; Underwood, 2002). Nevertheless, they probably contribute to a part of the xanthophylls cycle development.

The combination of fluorescence measurements and pigments content analysis, even if they were not obtained at the same scale (200 µm and 1 mm, respectively), confirmed that microalgae are able to respond to the environmental changes occurring during emersion by short-term photoacclimation to optimise their photosynthetic activity during the day. This photoacclimation takes place either by migration into the sediment layers, inducing changes in the micro-algal community at the surface, or by the development of NPQ processes like the xanthophylls cycle, or by both processes. The range of information obtained with our experiments indicate the importance of *in situ* studies on undisturbed microfilms that take into account the natural physical and biological parameters which characterized each emersion period. Indeed, each emersion period differs from another in relation to specific environmental conditions such as: time of high tide and low tide, increasing or decreasing light. The differences observed in the two light curves for the two different periods of emersion within the same day show the necessity for regular monitoring of photosynthetic activity during the whole emersion period to make the most accurate assessment of production, and which is representative of the true daily production.

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