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Effects of intertidal microphytobenthos migration on biomass determination via laser-induced fluorescence

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ABSTRACT: Laser-induced fluorescence (LIF) spectra of intertidal microphytobenthos (MPB) communities were obtained in the laboratory with a 532 nm pulsed Nd:YAG laser. The laser-induced chlorophyll (chl) fluorescence emission spectra of MPB in mud and sand sediments were characterized by a band in the red region with a maximum at ca. 685 nm. Biomass accumulation on the surface of the mud due to cell migration caused a shift to longer wavelengths (up to 5 nm) of the red emission maximum and the development and increase of an emission shoulder at the far-red region (maximum at ca. 732 nm), probably owing to increased re-absorption of chl fluorescence within the denser microalgae biofilm. Direct relations were observed between MPB biomass proxies (normalized difference vegetation index [NDVI] and phytobenthos index [PI]) and fluorescence intensity. LIF was used to track migratory rhythms of epipelic benthic microalgae in muddy sediments, which are absent in epipsammic communities in sand: progressive accumulation of biomass occurred at the sediment surface during diurnal low tide periods and was followed by a rapid downward migration before tides began to cover the sampling site. When exposed to high light, surface biomass decreased in migratory biofilms, indicating that diatom cells avoid photoinhibitory light levels. This phenomenon is known as behavioral photoprotection. For the first time, LIF was applied to study intertidal MPB communities to adequately describe surface biomass, which included changes due to migration.

KEY WORDS: Chlorophyll · Behavioural photoprotection · Benthic diatom · Microalgae biofilm

INTRODUCTION

Microphytobenthos (MPB) are a generic grouping of microalgae that colonize intertidal and shallow subtidal flats in estuarine and coastal ecosystems. MPB can form dense, micro-algal, diatom-dominated biofilms on the upper layers of intertidal sediments that range from fine silt and mud to sand and have a productivity that can exceed 300 g C m^{-2} yr^{-1} (MacIntyre et al. 1996, Underwood & Kromkamp 1999). MPB represent an important food source for benthic invertebrates (e.g. Montagna et al. 1995) and have been implicated in protecting sediments from erosion through the production of extracellular polymeric substances (e.g. Underwood & Paterson 2003). The large spatio-temporal variability of MPB and the difficulty of accessing tidal flats that cover many square kilometers of estuarine and coastal ecosystems make the use of remote sensing techniques particularly useful in assessing MPB distribution.

A unique feature of some of these benthic epipelic (motile, fine sediment-inhabiting) diatom communities

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is the exhibition of vertical migratory movements within the uppermost layers of the sediment (see review by Consalvey et al. 2004). Typically, motile diatoms move towards the surface of the sediment during diurnal low tide and then back to deeper layers before high tide or night, causing reversible several-fold changes in surface biomass (Round & Palmer 1966, Serôdio et al. 1997). Upward migration benefits microalgae by placing them on the surface during low tide when light is readily available for photosynthesis. However, rapid downward diatom movement has been observed upon exposure to high light levels (Kromkamp et al. 1998, Perkins et al. 2001, Cartaxana & Serôdio 2008), indicating that benthic diatoms may also use migration to avoid photoinhibitory light levels, a phenomenon known as behavioural photoprotection (Admiraal 1984, Serôdio et al. 2006). Recently, Perkins et al. (2010) have shown that vertical cell movement is a primary response of epipelic benthic biofilms to increasing light exposure. Epipsammic diatoms, i.e. those attached to particles of sandy sediments, depend exclusively on physiological mechanisms to photoregulate (Jesus et al. 2009).

Laser-induced fluorescence (LIF) has been successfully used in remote sensing of terrestrial plants (e.g. Subhash & Mohanan 1997, Richards et al. 2003, Anderson et al. 2004), phytoplankton (Barbini et al. 1998) and macroalgae (Kieleck et al. 2001). The chlorophyll (chl) fluorescence spectrum of plant leaves typically includes 2 maxima, one in the red (684 to 695 nm) and one in the far-red (730 to 740 nm) region, which are primarily dependent on the concentration of chl *a* (see review by Buschmann 2007). Changes in red to far-red fluorescence ratios (F_{685}/F_{735}), as well as shifts in the peak center of chl fluorescence bands, are correlated to changes in chl concentration in plants under stress (Lichtenthaler & Rinderle 1988, McMurtrey et al. 1994, Subhash & Mohanan 1997, Schuerger et al. 2003).

In the present study, LIF was used as a remote sensing technique to study MPB biomass of muddy and sandy sediments of the Tagus Estuary, Portugal. LIF was used successfully to estimate MPB biomass and, in the case of epipelic diatom communities, to track microalgal migration caused by diurnal and tidal cycles and changes in irradiance levels. To our knowledge this is the first application of LIF in the study of MPB communities.

MATERIALS AND METHODS

Sampling. Sediment samples were collect at Alcochete, Tagus Estuary, Portugal (38° 44′ N, 09° 08′ W), on several occasions between February and July 2010. This estuary has a large inner bay with extensive intertidal flats covering an area of approximately 100 km^2 (Brotas & Catarino 1995). The Tagus Estuary is mesotidal with a mean tidal range of 2.4 m, which ranges from about 1 m at neap tides to about 4 m at spring tides. Sampling was carried out during low tide periods at 2 stations with different sediment types: a mud site with 97% of particles <63 µm, and a sand site composed of a mixture of very fine to coarse sand ranging in diameter between 125 and 1000 µm, hereafter called mud and sand, respectively. Sediment samples were collected by means of plexiglass cores (8 cm internal diameter) and taken to the laboratory. All experimental measurements were carried out on the day after sampling. The sediment was left overnight in the laboratory in shallow water (±2 cm) collected from the site and carefully added to avoid re-suspending the sediment.

Effects of diurnal and tidal cycles on MPB surface biomass. Just before the start of the diurnal low tide emersion that was predicted to occur at the original sampled sites, the water was removed from the mud and sand cores in the laboratory. To promote cell migration to the sediment surface, the cores were exposed to low light (70 µmol photons m^{-2} s^{-1}) provided by a halogen lamp (Philips focusline, 250 W) through fiberoptics (model 460-F, Heinz Walz). Light intensity was measured with a quantum sensor (model QMSW-SS, Apogee). The *in vivo* LIF spectra of these 2 types of sediment were obtained every 30 min with a Nd:YAG laser (model NL303, EKSPLA) along a diurnal tidal cycle. Reflectance spectra were recorded in the same sediment area immediately before LIF measurements were taken. The experiment was repeated during a second diurnal tidal cycle for each sediment type. Additional measurements of reflectance spectra and LIF were recorded in areas covering a wide range of surface microalgal biomass.

Effects of irradiance levels on MPB surface biomass. Just before the start of the diurnal low tide emersion predicted for the original sampled site, the water was removed from the mud cores and the cores were exposed to low light as described in the above subsection. Sediment in the cores was sampled with plexiglass minicores (2 cm diameter) for the following treatments: addition of filtered water from the sampling site only (control) and addition of latrunculin A dissolved in filtered site water to inhibit diatom motility (Lat A). Three replicates were used for each treatment. Treatments were applied once the biofilm had become established at the sediment surface as assessed by the stabilization of the normalized difference vegetation index (NDVI) (see 'Spectral reflectance').

A concentrated Lat A solution (1 mM) was prepared as a fresh stock on the morning of the experiment by

dissolving purified Lat A (Sigma-Aldrich) in DMSO. A solution of 20 µM Lat A was prepared by diluting the stock solution in filtered water collected at the sampling site. Small volumes of this solution (total of 200 µl) were carefully pipetted directly onto the sediment surface of the minicores until a continuous thin layer completely covered the sample. The amount of Lat A needed to sufficiently inhibit diatom migration in benthic biofilms was previously determined (Cartaxana & Serôdio 2008, Perkins et al. 2010). Filtered site water (200 µl) without the addition of Lat A was added to all control cores to mimic chemical treatments. After 30 to 45 min, LIF spectra were obtained in control and Lat A-treated sediments for sequential light treatments in the following order: low light (LL1): 70 µmol photons m^{-2} s^{-1}; high light (HL): 1200 µmol photons m^{-2} s^{-1}; low light (LL2): 70 µmol photons m^{-2} s^{-1}; and dark (D). LIF spectra were recorded after a period of 30 min in each light level.

Laser-induced fluorescence. *In vivo* LIF spectra of sediment MPB communities were recorded with a Nd:YAG laser. The laser provided 30 mJ pulses of 4 ns at the wavelength of 532 nm (second harmonic), with a pulse repetition rate of 10 Hz. The distance of the laser to the sediment was ca. 1 m and the diameter of the laser spot hitting the sediment surface was ca. 1.5 cm. To obtain a good fluorescence signal, a relatively high laser excitation energy was necessary, which was sufficient to cause reaction center closure (Rosema et al. 1998).

Photosystem II (PSII) operating efficiencies, also known as the Genty factor (Genty et al. 1989), were measured with a pulse-amplitude modulated (PAM) fluorometer (model Diving-PAM, Walz) before and immediately after laser pulses. Mean (±SD) PSII operating efficiencies were 0.746 ± 0.056 and 0.705 ± 0.057 (n = 8), before and after the laser pulse, respectively (5.5 ± 3.9% lower after laser measurements). This showed that the laser pulse had an actinic effect on the samples. This effect was fully reverted before the next laser pulse, 30 min later, indicating that the decrease on the yield was not due to damages to the photosynthetic apparatus.

The fluorescence emission signal was collected by a telescope (model F810SMA, Thorlabs) situated ca. 40 cm from the sample. To protect the light-detecting electronics from very strong elastically scattered radiation of the second laser harmonic, the telescope was equipped with a long-wave pass filter of $\lambda \geq 550$ nm. The collected radiation was transmitted into a spectrometer (model USB4000, Ocean Optics) via an optical fiber. The spectrometer was synchronized with the laser pulse, which enabled the signal to be measured for about 10 µs (minimum exposure permissible by the spectrometer control software) after each laser pulse. To achieve a reliable signal-to-noise ratio, the fluorescence spectra were obtained by collecting and averaging signals from 100 to 1000 laser pulses.

Spectral reflectance. Reflectance spectra were measured over a 350 to 1000 nm bandwidth by means of a USB4000 spectrometer with a VIS-NIR optical configuration connected to a 400 µm diameter fiber optic (model QP400-2-VIS/NIR, Ocean Optics). The light spectrum reflected from the sample was normalized to the spectrum reflected from a clean polystyrene plate. The polystyrene plates differed by <3% from a calibrated 99% reflectance standard plate (Spectralon™, Forster & Jesus 2006). A reflectance spectrum measured in the dark was subtracted from both spectra to account for the dark current noise of the spectrometer. The fiber optic was positioned perpendicular to the sediment surface by means of a micromanipulator (model MM33, Diamond General) maintained at a fixed distance from the sample surface and set to match the area measured by LIF. Sample and reference spectra were measured under a constant irradiance of 70 µmol photons m^{-2} s^{-1}.

Microalgae biomass present in the surface layers of the sediment was estimated by calculating NDVI (Rouse et al. 1973) and a modified version of the phytobenthos index (PI) (Méléder et al. 2003, Jesus et al. 2006, Murphy et al. 2008) from reflectance spectra. Biomass indices NDVI and PI were calculated as follows:

$$\mathrm{NDVI} = (R_{750} - R_{675})/(R_{750} + R_{675})$$

and

$$\mathrm{PI} = (R_{750} - R_{636})/(R_{750} + R_{636}),$$

where R_{750}, R_{675} and R_{636} represent the diffusive reflectance (R) at 750, 675 and 636 nm, respectively.

Statistical analysis. Significant differences were determined with 2-way ANOVA for effects of irradiance levels (light treatment) and chemical treatment on MPB surface biomass (fluorescence peak area). Data complied with the assumptions of ANOVA. Multiple comparisons among pairs of means were performed with Tukey's Honestly Significant Difference (HSD) test.

RESULTS

LIF spectra of the 2 different intertidal sediments of the Tagus Estuary showed a chl fluorescence band in the red region, with a peak maximum between 684.7 and 689.9 nm. Typical fluorescence emission spectra for both sediments, with fluorescence maxima at 685.3 and 686.5 nm for mud and sand, respectively, are shown in Fig. 1. Emission spectra corresponding to mud sediments had consistently higher fluorescence intensities than those of sand sediments (Fig. 1).

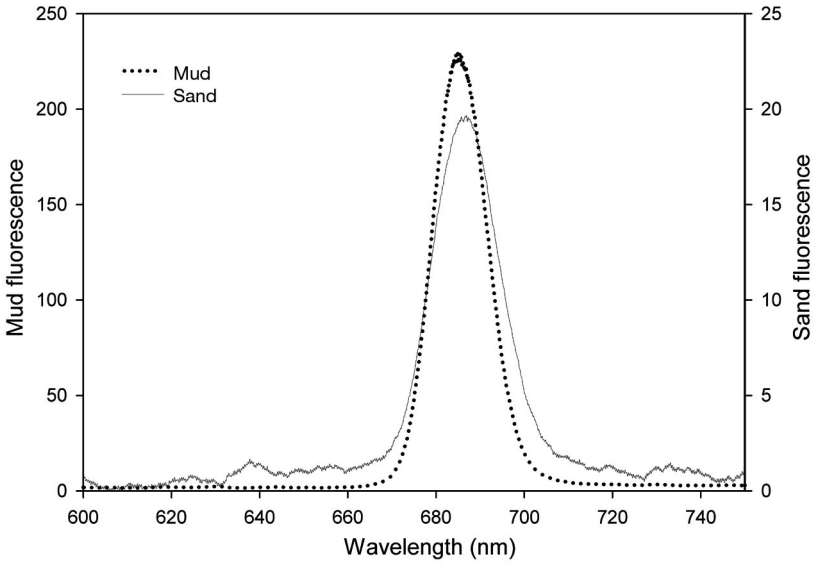

Fig. 1. Typical laser-induced fluorescence spectra of microphytobenthos in mud and sand intertidal sediments. Units on the *y*-axis are arbitrary

There was a positive correlation between peak area and the wavelength shift of peak maxima ($p < 0.001$, $r = 0.763$). In other words, for areas of sediment with higher surface microalgal biomass the red emission maxima occurred at longer wavelengths. This is depicted in Fig. 2, in which the sediment sample with less biomass (NDVI = 0.695) exhibits a maximum fluorescence at 686.3 nm, while samples with higher surface chl concentrations show maxima at 689.6 nm (NDVI = 0.827) and 689.9 nm (NDVI = 0.849). Furthermore, in the latter samples, there was a clear development and increase of an emission shoulder at the far-red region (maximum at ca. 732 nm, Fig. 2).

Fluorescence intensities, measured by calculating the ln-transformed data of peak area, were found to vary linearly with both biomass indices NDVI and PI (Fig. 3). Highly significant correlations were obtained between ln(peak area) and NDVI ($r = 0.968$, $p < 0.001$, Fig. 3A) and PI ($r = 0.943$, $p < 0.001$, Fig. 3B). Similar results were obtained using intensity calculated by ln-transforming data of peak height instead of peak area in these correlations (data not shown).

Fluorescence intensities measured in mud sediments were clearly related to migratory rhythms of epipelic benthic diatoms. A typical increase in relative fluorescence following exposure to low light that coincided with the emersion period is shown in Fig. 4. A rapid decrease of fluorescence was observed closer to—though clearly before—the start of the immersion (high tide) period in the natural environment (Fig. 4). These fluctuations of fluorescence intensity were not observed in epipsammic communities of sandy sediments (data not shown).

Effects of different irradiance levels on MPB surface biomass of mud sediments were investigated by applying the diatom motility inhibitor Lat A applied to the sediment surface after the biofilm was established during low tide. Fluorescence peak areas (intensity) were compared for a sequence of light treatments for control and Lat A-treated sediment samples (Fig. 5). There was a significant effect of light ($F_{3,16} = 4.604$, $p < 0.05$) and chemical ($F_{1,16} = 10.446$, $p < 0.01$) treatment on fluorescence intensity. Exposure to high light levels (1200 µmol photons m^{-2} s^{-1}) caused a significant (Tukey's test, $p < 0.05$) decrease of fluorescence peak area in control sediment (Fig. 5). Re-exposure of the control sediment to low light led to an increase in fluorescence peak area, which was followed by a decrease when samples were transferred to the dark near the time of arrival of high tide in the natural environment (Fig. 5). No significant differences were observed in fluorescence peak area for Lat A-treated sediment samples, as diatom migration was inhibited.

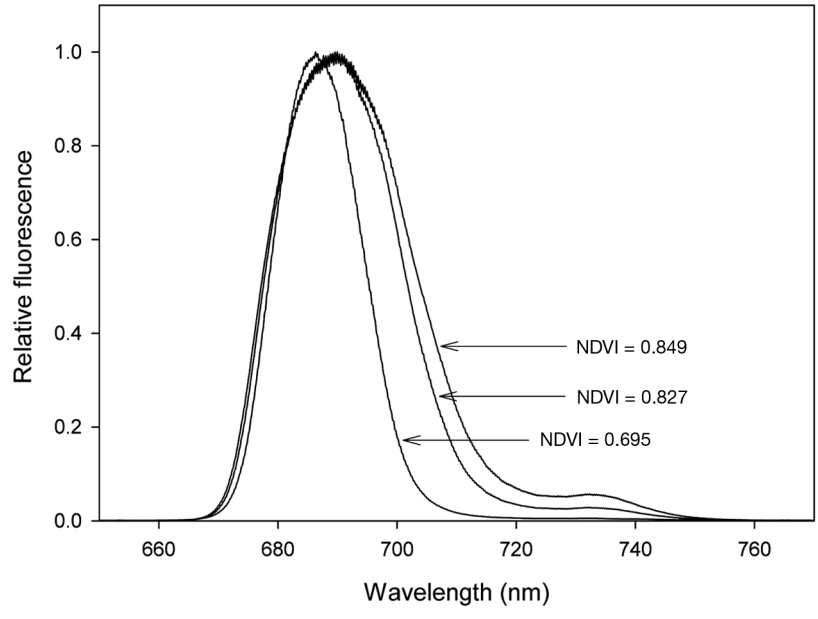

Fig. 2. Laser-induced relative fluorescence spectra of 3 samples (1, 2 and 3) of microphytobenthos with increasing biomass in mud intertidal sediments. Normalized difference vegetation index (NDVI) is shown for the 3 samples

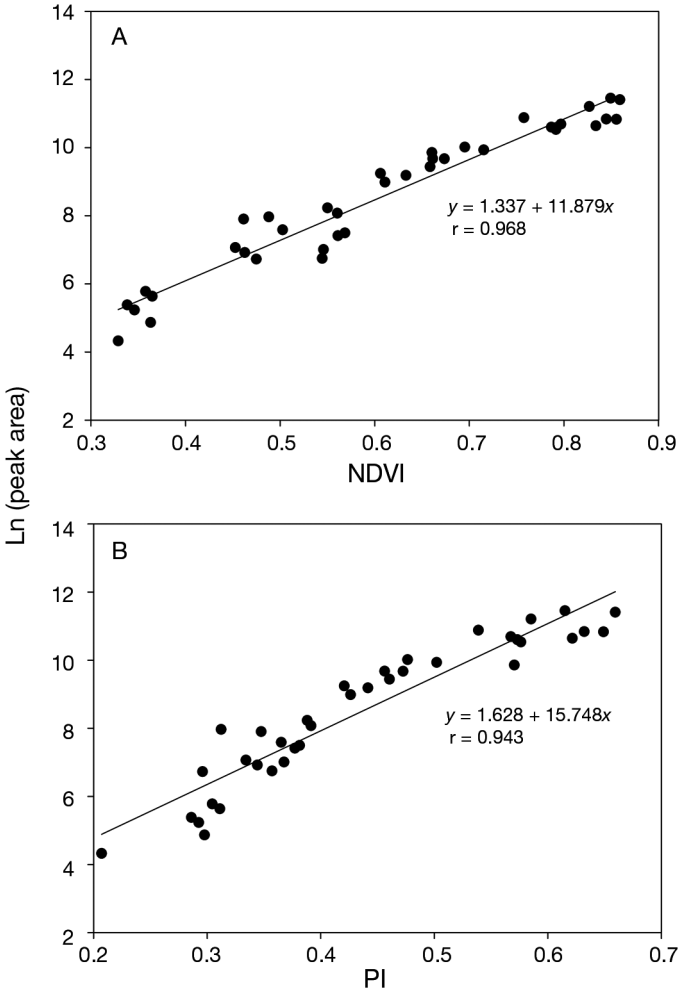

Fig. 3. Linear regressions between biomass indices, (A) normalized difference vegetation index (NDVI) and (B) phytobenthos index (PI) and laser-induced fluorescence measured as ln(peak area) of microphytobenthos of intertidal sediments

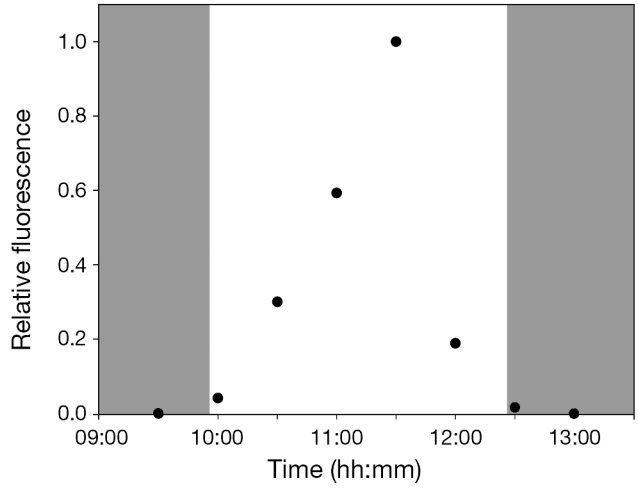

Fig. 4. Variation of laser-induced relative fluorescence of microphytobenthos in a mud intertidal sediment along a diurnal tidal cycle. The sample was kept emersed and under constant low light. Gray and white bars represent immersion and emersion periods, respectively, at the field site where the sample was collected

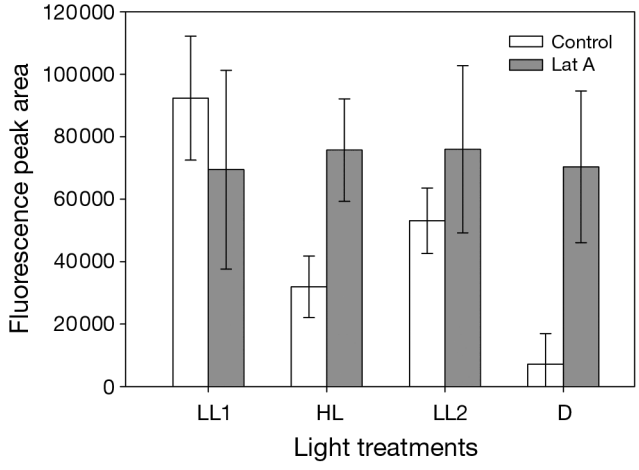

Fig. 5. Variation of laser-induced fluorescence measured as peak area (arbitrary units, mean ± SD) for control and Lat A-treated intertidal mud sediments during a sequence of 30 min light treatments: low light (LL1), 70 µmol photons m^{-2} s^{-1}; high light (HL), 1200 µmol photons m^{-2} s^{-1}; low light (LL2), 70 µmol photons m^{-2} s^{-1}; and dark (D)

DISCUSSION

Chl fluorescence emission spectra of MPB measured by means of LIF were characterized by a band in the red region (maximum at ca. 685 nm) and a shoulder at the far-red region (maximum at ca. 732 nm). Similar results were observed for leaves of higher plants, with 2 fluorescence emission bands, one in the red (684 to 695 nm) and one in the far-red (730 to 740 nm) region (see review by Buschmann 2007). It is generally assumed that most of this fluorescence arises from photosystem II (PSII) (Govindjee 1995), in which the 684 to 695 nm band arises from the main electronic transitions and the 730 to 740 nm band arises from vibrational sublevels whose relative intensities are increased *in vivo* through self-absorption (Franck et al. 2002).

Surface microalgal biomass accumulation on mud sediments caused a shift to longer wavelengths of the red emission maximum and the increase of the emission shoulder at the far-red region. Chl fluorescence can be partially reabsorbed within a cell or by other microphytobenthic cells. Naturally, at higher chl *a* concentrations in sediment, reabsorption of emitted chl fluorescence increases. The increase of reabsorption with increasing chl concentration leads to a shift in the position of the red chl fluorescence maximum of plant

leaves towards longer wavelengths as shown by Gitelson et al. (1998). Furthermore, since the red chl fluorescence maximum at around 685 nm is more strongly affected by the reabsorption than the long-wavelength maximum in the far-red region around 735 nm, the chl fluorescence ratio of F_{685}/F_{735} decreases with increasing leaf chl content (Buschmann 2007).

The MPB communities studied were composed exclusively of benthic diatoms as confirmed by microscopic examination of resuspended sediment samples and high performance liquid chromatography pigment analysis (data not shown). Differences in both the excitation and emission spectra related to differences in pigment composition have been used to characterize the taxonomic structure of microalgae *in vivo* (see review by MacIntyre et al. 2010). If cyanobacteria were present in the MPB, phycobilins would be able to absorb the excitation energy of the laser at 532 nm. In this case, emission peaks around 570 nm and/or 655 nm would be expected depending on the abundance of phycoerythrins and phycocyanins, respectively (MacIntyre et al. 2010).

In the present study, MPB biomass was estimated non-destructively with LIF by establishing a direct relationship between ln-transformed data on fluorescence intensity of MPB communities and the biomass proxies NDVI and PI. NDVI is based on the *in vivo* chl *a* absorption maximum around 675 nm. It constitutes a long-established index developed in the context of remote sensing of terrestrial vegetation and is currently the most commonly used index to quantify MPB biomass (Jesus et al. 2006, Kromkamp et al. 2006, Serôdio et al. 2006, Cartaxana & Serôdio 2008). Indices based on the absorption at 675 nm have the advantage of being specific for chl *a*, thus allowing detection and quantification of photosynthetic biomass without the interference of secondary pigments. However, saturation of NDVI for high MPB biomass has been pointed out and attributed to the saturation of light absorption (Méléder et al. 2003). The fact that a linear relationship of NDVI with fluorescence intensity was established with ln-transformed data suggests that the relationship between NDVI and chl *a* would approximate an exponential relationship. The PI is also commonly used to estimate MPB biomass (Méléder et al. 2003, Cartaxana & Serôdio 2008) and is based on the absorption of the diatom pigment chl *c* and the close relationship of the concentration of this pigment with reflectance at 636 nm (Murphy et al. 2008). Ln-transformed fluorescence intensity data was not fully linear with PI, which is less sensitive than NDVI to saturation at high chl *a* concentrations (Méléder et al. 2003, Barillé et al. 2007).

Complex diatom migratory patterns in epipelic motile biofilms can also be followed in a non-destructive way through chl fluorescence measurements with LIF. Motile diatoms moved towards the surface of the sediment during diurnal low tide and back to deeper layers before high tide causing several-fold changes in surface biomass as previously shown by other authors (e.g. Round & Palmer 1966, Serôdio et al. 1997). Upward migration during diurnal low tide periods allows cells to reach the photic zone and to absorb light to drive photosynthesis. The reasons for downward migration before high tide or darkness are less clear, but might include the prevention of cells being washed away during immersion or grazing by predators, and facilitating nutrient and carbon uptake or cell division (Admiraal 1984, Decho 1990, Saburova & Polikarpov 2003). Serôdio et al. (1997) have shown that these rhythms are partially endogenous as they were maintained in the absence of external stimuli.

Benthic epipelic diatoms were also found to migrate as a response to irradiance levels. Rapid downward diatom movement was observed upon exposure to 30 min of 1200 µmol photons m^{-2} s^{-1}, followed by upward migration when light levels were reduced to 70 µmol photons m^{-2} s^{-1}. Thus, diatoms exhibit behavioural photoprotection by avoiding photoinhibitory light levels (Admiraal 1984, Kromkamp et al. 1998, Perkins et al. 2001, 2010, Serôdio et al. 2006). This is in agreement with the 'microcycling' proposed by Kromkamp et al. (1998) in which a turnover of taxa at the sediment surface results in a reduction in photodose over time and emphasizes the role of vertical movement demonstrated in other experiments that used Lat A as a motility inhibitor (Cartaxana & Serôdio 2008). Cell migration may well be more energetically favorable than physiological photoprotection processes such as non-photochemical quenching induction (Perkins et al. 2010).

In the last 2 decades, research has increasingly focused on spectral reflectance and PAM fluorescence techniques that employ the optical properties of chl to remotely sense MPB biomass in intertidal flats of estuarine and coastal ecosystems (Serôdio et al. 2001, 2009, Méléder et al. 2003, Jesus et al. 2006, Kromkamp et al. 2006). LIF presents some advantages over these remote sensing techniques for the study of intertidal MPB. Spectral reflectance is a passive method of remote sensing that depends on stable and uniform illumination, making it difficult to take measurements under overcast and partly cloudy conditions. In contrast, LIF instruments use their own illumination source to actively excite fluorescence. Results obtained with hand-held LIF instruments studying beans and wheat have shown that this technique can be used for remote sensing under a diversity of light conditions, including full darkness, at dawn and dusk and under rapidly changing light environments similar to those encountered on partly cloudy days (Richards et al. 2003). The

lighting conditions described are generally unsuitable for spectral reflectance remote sensing systems.

PAM fluorometry (Schreiber et al. 1986) was first applied to MPB by Serôdio et al. (1997) and Kromkamp et al. (1998) and led to major advances in the comprehension of the ecophysiology and productivity of MPB communities. However, PAM techniques rely on short saturating pulses delivered at close range, making them impractical for most remote sensing applications. LIF techniques overcome this limitation and have been successfully used in the remote sensing of terrestrial plants (e.g. Subhash & Mohanan 1997, Richards et al. 2003, Anderson et al. 2004), phytoplankton (Barbini et al. 1998) and macroalgae (Kieleck et al. 2001). More recently, a laser-induced fluorescence transient (LIFT) fluorometer, which uses a fast repetition rate technique (Kolber & Falkowski 1993), has been developed to operate with relatively low excitation power with subsaturating flashes for measurement of fluorescence parameters from a distance of up to 50 m (Kolber et al. 2005, Pieruschka et al. 2010). The results shown in our study, together with the discussed advantages over spectral reflectance and PAM fluorometry, make LIF a promising technique for the remote sensing of intertidal MPB communities.

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