Microalgal primary producers and their limiting resources

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CHAPTER 6

A two-dimensional analysis of photosynthetic activity and vertical migration of microphytobenthos using imaging pulse amplitude modulated (PAM) fluorescence

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Abstract

The photosynthetic activity and vertical migration of microphytobenthos (MPB) were investigated in two types of sediment: a muddy location (Vista Alegre: VA) and a sandy location (Costa Nova: CN) at the Ria de Aveiro (Portugal). The MPB biomass (using fluorescence as a proxy), photosynthetic activity (effective PSII photochemical efficiency: \( \Delta F/F_m' \)) and relative photosynthetic electron transport rate (rETR) were measured at different sediment depth layers (0-0.5, 0.5-1, 1-1.5 and 1.5-2 mm) on a flat vertical section of a sediment core with the non-invasive technique imaging pulse amplitude modulated fluorometer (iPAM). In addition, the hyperspectral reflectance was measured at the sediment surface which allowed the calculation of the normalized difference vegetation index (NDVI). The results showed that biomass and photosynthetic activity were the highest at the turning of the low tide (LW) in all measured depth layers at the muddy site. An upward movement was already observed in the few hours before the emersion period under the dark condition, an indication that the vertical movement of the cells originated from an endogenous driven vertical migration. In the sandy sediment, the MPB biomass was homogeneously distributed over the sediment depth. The upward movement observed of the MPB biomass in the homogenized sediment of CN site is likely to be attributed to a positive phototaxis response. Complementary to the fluorescence measurements, in situ measurement of the C-incorporation rates into particulate organic carbon (POC), carbohydrate and phospholipid fatty acid (PLFA) were also measured at the two locations. The incorporation of \(^{13}\text{C}\) stable isotope into different PLFAs was used to give an indication on MPB activity of specific groups. The POC and carbohydrate concentrations indicated a higher standing stock of biomass at the CN site compared to the VA site. This agreed with the iPAM measurements, but remarkably, the C-incorporation rate into PLFAs from the sandy site (CN) was nearly twice as high as in the muddy site (VA). Hence, the epipelic diatoms appeared to be more productive compared to the epipsammic microalgae. At both sites glucose was the main form of synthesized carbohydrate. The high activity of the epipelic diatoms at the muddy site VA might have been the result of the exudation of extracellular polymeric substances (EPS) during vertical migration of the epipelic diatoms.
Introduction

Estuarine intertidal microphytobenthos (MPB) communities contribute up to 50% of the total estuarine primary production (Underwood & Kromkamp 1999). In intertidal sediment, MPB needs to adapt rapidly to fluctuations in environmental parameters such as light, temperature, salinity, nutrient availability, grazing and sediment exchange with the water column (Barranguet et al. 1998, Blanchard et al. 2001). Two main types of MPB communities are distinguished: an epipsammic community, consisting of immotile cells attached to the sand grains, and an epipelic community, consisting of highly motile cells that can move between sediment grains and which are generally found in muddy sediment. A third type, consisting of tychoplanktonic species, i.e. species which combine a benthic and a pelagic life styles are less common and not much is known about this group (Marcus & Boero 1998). As a common behaviour, epipelic diatoms migrate toward the sediment surface during emersion period in daytime and migrate down to deeper layer before the onset of immersion or sunset. Evidence suggests that the vertical migration in MPB is driven by an endogenous clock as the migratory patterns continue to synchronize with diurnal and tidal cycles for a few days in the absence of tides or other environmental stimuli (light and tides) (Serôdio et al. 1997). Seasonal and short term temporal variations of migratory behaviour of MPB have been modelled as the combined results of the daily rhythm superimposed on the tidal cycle (Pinckney & Zingmark 1991).

During a tidal cycle a maximum photosynthetic activity is found at low tide during daytime when cells migrate to the surface in order to perform photosynthesis (Serôdio et al. 2008). Before immersion, the MPB cells migrate downward and the biofilm disintegrates at the sediment surface. Only when conditions are too extreme (high irradiance, extreme temperature, and desiccation), cells may migrate downward early to avoid physiological stress in deeper layers. Due to this complex migratory behaviour that is the result of biological clock driven migration and photo- and chemotaxis, MPB cells move up and down the sediment during the day and, as a consequence, alter the photosynthetic activity at the sediment surface (Consalvey et al. 2004). Other factors trigger and modify the endogenous vertical migration of MPB, in particular sediment re-working from abiotic factors such as wave action or from bioturbation, and influence the depth where MPB cells are present. Several methods have been used to study the MPB vertical migration. These include the lens tissue method by which migrating cells are trapped (Eaton & Moss 1966). Optical techniques like hyperspectral reflectance and PAM fluorescence have been used to study the variability of MPB biomass at the sediment surface (Kromkamp et al. 1998, Paterson et al. 1998, Serôdio 2003, Kromkamp et al. 2006). During the last few decades, the use of active fluorescence measurements to estimate primary production has become increasingly popular as it is a quick and non-invasive method (Kromkamp et al. 1998, Barranguet & Kromkamp 2000, Consalvey et al. 2004). However, optical modelling studies have shown that active fluorescence techniques can seriously overestimate photosynthesis activity in the MPB community (Forster & Kromkamp 2004, Serôdio 2003), which is due to the contribution of ‘deep layer’ fluorescence. Signals emanating from the subsurface and the deeper layers are still under investigated (Perkins et al. 2010). In deeper layers where dark and
anaerobic conditions can occur, some biofilms inactivate their photosynthetic process (Kromkamp et al. 2007).

The technique used in this study is the imaging pulse-amplitude modulated (iPAM) chlorophyll fluorescence. The use of the iPAM to study photosynthetic process has the advantage to cover a larger study area compared to oxygen measurements with microsensor techniques or to the more common PAM fluorometers used such as the Diving-PAM or the mini-PAM. With the iPAM the average spatial heterogeneity in photosynthetic parameters of MPB during a daily and tidal cycle can be investigated in two dimensions.

In this study, we developed a method to quantify the vertical migration and study photosynthetic activity at different depth intervals. The goal was to develop a technique which can obtain measurements of the MPB vertical migration biomass and photosynthetic activity at different depths simultaneously by using an imaging fluorescence technique that allows larger surface areas to be measured simultaneously. For this reason a special coring device was developed that produces a flat cross section suitable for the iPAM measurements. In addition to the photosynthetic activity measurements, we assessed MPB communities (migrating MPB species composition and phospholipid fatty acid (PLFA) composition) and compared the rate of C-fixation by quantifying $^{13}$C-incorporation rates in bulk of particulate organic carbon (POC) and carbohydrate for each location.

**Material and methods**

**Study site**

Ria de Aveiro is a mesotidal shallow lagoon located at the Atlantic north-west Portuguese coast that is characterized by symmetrical tides. Two distinct locations were sampled (Fig. 1): one muddy site, Vista Alegre, VA (40° 38’ 1.98 N, 8° 39’33.40 W) and a sandy site: Costa Nova, CN (40°38’ 1.70 N, 8°39’33.3W) in November 2010. The days of measurements were chosen to coincide with daytime low tide.

**Fluorescence measurements**

From each site (CN and VA), sediment samples were collected using custom made Perspex corers of 7.5 cm diameter. The sediment was kept overnight in the dark at ambient temperature. The measurements were done the next morning. For each station, two types of measurements were made, one on an intact sediment core with an undisturbed MPB community, while the other was made on material that was scraped from the surface (included most of the MPB), was homogenized, and subsequently the MPB community was allowed to reposition itself.

In order to create a flat vertical cross section, a specially designed device was pushed over the sediment core (Fig. 2). The upper half of this device was removed and fitted with a flat and sharp Perspex plate (“knife”) that sliced the upper part of the core into two halves. After removing the unsupported half, the other part provided an intact core with a smooth cross
section. The flat cross section was tightly fitted to an imaging pulse amplitude modulated (iPAM) fluorometer equipped with red LEDs and actinic light (IMAG-MIN PAM, Walz Effeltrich, Germany).

The minimal fluorescence as proxy of MPB biomass and photosynthetic parameters were measured in the four different successive layers (L1, L2, L3 and L4) up to 2 mm in the sediment depth (Fig. 3). For each selected layer, the fluorescence intensities were measured on a rectangular area of 0.5 mm width and 6.6 mm length. The iPAM monitored fluorescence parameters during 10 hours in the laboratory. During the period when emersion took place in the field, the light was switched on (100 µmol photons m\(^{-2}\) s\(^{-1}\)) in order to measure the spectral reflectance (see below) at the time of the measurement. The overlying water on the core was removed at the onset of the emersion period, but it was not added back when the emersion period ended. A saturating pulse was applied every 15 minutes in order to measure steady state fluorescence (F), the maximum fluorescence in the presence of actinic light (F\(_{m}'\)) and effective quantum efficiency of the photosystem II (ΔF/F\(_{m}'\)). ΔF was calculated as: F\(_{m}'\)-F at the subsurface layer (L1) (see paragraph on NDVI measurements). Before the measurements were started, a correction procedure as suggested by the manufacturer was followed in order to correct for none homogeneity in the light field. Measurements performed in darkness corresponded to the minimal fluorescence F\(_{o}\) and the maximum fluorescence F\(_{m}\). All measurements were done using identical settings, facilitating the comparison of microalgal biomass between the cores.

Although we were unable to measure the light penetration in the sediment during the emersion period, we assumed that the light intensity in the deeper layers was low and therefore the effective PSII efficiencies measured in L1 (muddy site), L2, L3 and L4 were probably close to F\(_{v}/F_{m}\).

Rapid light curve (RLC) measurements

Every hour, rapid light curves were recorded with 12 incremental irradiance steps (1, 36, 84, 141, 235, 350, 444, 527, 631, 756, 912 and 1100 µmol photon m\(^{-2}\) s\(^{-1}\)) of 10 seconds duration. RLCs were recorded for each depth layer (L1-L4). Before each RLC, the samples were placed in the dark condition for at least two minutes. Because we were unable to determine the absorption coefficient of the MPB cells, the relative photosynthetic electron transport rate (rETR) was calculated by multiplying the effective PSII photochemical efficiency ΔF/F\(_{m}'\) with the irradiance E. From the RLC the maximum photosynthesis electron transport rate (rETR\(_{max}\)), the light utilization coefficient in the light limited region of the RLC (α), and light saturating irradiance (E\(_{k}\) = rETR\(_{max}\)/α) were determined by fitting RLCs to a modified version of the equation of Eilers and Peeters (1988):

\[ rETR = E / (aE^2 + bE + c), \]

where \( a = (\alpha \times E_k)^{-1}; \) \( b = (rETR_{max})^{-1} - 2 \times (\alpha \times E_k)^{-1}; \) \( c = \alpha^{-1}. \)

Because \( rETR = ΔF/F_{m}' \times E, \) the photosynthetic parameters rETR\(_{max}\) and α can also be obtained after normalizing rETR to E, taking out the dependency of rETR on E:

\[ ΔF/F_{m}' = (aE^2 + bE + c)^{-1}. \]

By fitting ΔF/F\(_{m}'\) as a function of irradiance, the RLC were fitted according to an R script developed by Silsbe and Kromkamp (2012).
Spectral reflectance (NDVI index)

The changes in MPB biomass were recorded using normalized difference vegetation index (NDVI) as a biomass proxy targeting an area with a homogeneous microalgal biomass on the sediment surface. During the NDVI measurement, light was applied on the sediment surface using a Schott KL 2500 LCD fibre-optic light source (Schott, Marlborough, MA). The artificial light was switched on (100 µmol photons m$^{-2}$ s$^{-1}$) and off at the respective time corresponding to the start and end of the emersion period at the sampled sites. Spectral reflectance was measured every five minutes using an Ocean optics USB2000 spectrometer (Ocean Optics, Dunedin, USA). During the simulated emersion period, the targeted area was a circle of approximately 5 cm diameter. From the reflectance measurements, the NDVI was calculated in order to estimate microalgal biomass. The NDVI was calculated as follows:

$$\text{NDVI} = \frac{\text{Infrared} - \text{red}}{\text{Infrared} + \text{red}},$$

where infrared is the average reflectance in the range of 748–752 nm and red the average reflectance in the range of 673–677 nm. The reflectance of the sediment was measured against a diffuse white calibrated standard (WS-1-SL Spectralon Reference Standard, Ocean Optics).

$^{13}$C-labeling experiments (POC, PLFA and carbohydrate)

In order to measure incorporation rates of $^{13}$C stable isotope into different pools (POC, PLFA and carbohydrate), $^{13}$C-labeling experiments were performed in situ at each site. At the two locations an area of 0.25 m$^2$ of the sediment surface was sprayed with 250 ml of $^{13}$C-NaHCO$_3$ solution (99% $^{13}$C; Cambridge Isotope Laboratories, Inc) at ambient salinity, giving a final concentration of 1 g m$^{-2}$ DI$^{13}$C. Samples of unlabeled and labeled particulate organic carbon (POC), phospholipid fatty acid (PLFA) and bulk carbohydrate were taken before and after 4h of incubation. The $^{13}$C stable isotope composition of the POC samples was analyzed with a Carlo Erba elemental analyser coupled inline to a Finnigan Delta S isotope ratio mass spectrometer (EA-IRMS) according to Middelburg et al., (2000).

PLFA samples were extracted according to a modified Bligh and Dyer method (1959) (Middelburg et al. 2000). First, total lipids were extracted and separated into different polarity classes using a silicic acid column. The column eluted several solvents (chloroform, acetone and methanol) representing different lipid fractions. The methanol fraction was collected and contained most of the PLFA. Derivatives of PLFA were synthesized in order to measure the methyl ester derivatives of the fatty acids (FAMEs) by gas chromatography-combustion isotope ratio mass spectrometry (GC-c-IRMS) (Dijkman et al. 2010). The FAMEs were identified according to their retention times and were compared to internal standards (12:0 and 19:0).

Carbohydrate in the sediment was extracted according to Boschker et al. (2008). Freeze dried sediment was treated with 1.1 mol L$^{-1}$ H$_2$SO$_4$ for one hour at 120 °C in order to hydrolyze the carbohydrates. The solution was brought to pH 5.5-6 by adding SrCO$_3$, and the precipitated SrSO$_4$ was removed by centrifugation (15 min, 4500 g). Monosaccharide concentrations and isotope ratios were analyzed with high-performance liquid chromatography combined with
isotope ratio mass spectrometry (HPLC-IRMS) equipped with a Carbopac PA20 (Dionex Benelux, Amsterdam, The Netherlands).

**MPB species**

The migrating MPB was collected from the surface of the homogenized sediment at the two sites by the lens tissue method (Eaton & Moss 1966). On the surface of the sediment, the lens tissues were left during a part of the emersion period, removed after 3h, suspended in filtered sea water and then fixed with 10% (v/v) glutaraldehyde for identification of the species. Epipelic microalgae taxonomic groups were determined using a light microscopy.

**Results**

*General observation of the MPB biomass with fluorescence (F) images*

Fig. 4 shows selected images generated by iPAM. The two columns of selected images on the left were taken from cores (intact and homogenized) obtained at the muddy site VA. Emersion period started at ~11:00h and the turning of the low tide (LW) was ~13:30h. Immersion started at ~18:00h, whereas sunset was at ~17:00h. At 09:00h, no MPB biomass was visible near the surface, but at 12:00h an accumulation of the benthic algae was found at the surface. The single yellow/orange dots observed in deeper layers were the colonies of the cyanobacterium *Merismopedia* sp. which showed an upward movement. At 16:00h, the MPB biomass density at the sediment surface decreased again, and the biomass had migrated away from the surface at 20:00h. The homogenized sediments of a core of station VA showed a visible accumulation of the MPB biomass at 09:00h and also the bioturbation occurred at the sediment surface. At 12:00h, the MPB biomass in the upper layer had increased due to an upward vertical migration. Most of the MPB biomass was visible just below the surface at a depth of 200 µm. The bioturbation was also visible as the fluorescence in the burrow increased. Most of the MPB biofilm on the surface had disintegrated at 20:00h, showing a similar MPB biomass to those observed at 09:00h.

The right two columns of selected images in Fig. 4 were obtained from the sandy sediments of station CN. The turning of the low tide at this station was ~15:45h. No particular changes in MPB biomass was observed through time. As the measurement on both types of sediments were performed using the same settings of the iPAM, a higher MPB biomass was observed at CN which was distributed over a larger depth compared to the MPB biomass at VA. As the light penetrated deeper in sandy sediment the photosynthetically active biomass was thus substantially higher at CN than at VA. However, near LW, the MPB biomass in the upper depth layers was similar to the surface biomass. In contrast to the intact core of CN, F in the homogenized core showed an increase near LW, which continued after immersion (20:00h).
**MPB biomass (F) in different depth layers**

Vertical migration in the muddy sediment of station VA (Fig 5, A1) showed that the steady state fluorescence (F) at the four different layers was similar at 09:00h two hours before the onset of emersion. This suggested that up to 2 mm depth the biomass was equally distributed at VA. Between the start of the measurement at 09:00h and the start of emersion period at 11:00h F values increased, demonstrating that the upward movement was initiated before the onset of the emersion period. When the emersion period started, which coincided with the onset of actinic light, F values stopped increasing in the layers L1, L2 and L3. F in L4 fluctuated in the first half of the emersion time but reached a maximum around LW. After LW, the values of F decreased in all layers and were similar to those observed two hours before the onset of emersion. Fluorescence measurements in the homogenized sediment of station VA showed F values which were four times higher than in intact sediment of VA (Fig 5, A2). Like the situation for the intact core, F values in the homogenized sediment increased before the onset of emersion and showed a peak in all layers which coincided with LW around noon. In contrast to the situation in the intact core, the migration in L1 was not as high as in L2-L4 and this led to the formation of a subsurface maximum. Immediately after LW, MPB in all layers showed a downward migration. Measurements on the intact sediment of station CN (Fig 5, B1), showed that F was constant in all layers before and during the emersion period. F was lowest in L1 and fluctuated without showing a trend during the emersion period. F slightly decreased in all layers at 13:00h when the light was switched on (start of emersion period). In the homogenized sediment of CN station (Fig 5, B2) F showed a gradually increase during the measurement at all depth layers, without any apparent peak around LW. However, this upward migration only started after the actinic light was switched on and when the water was removed to stimulate the emersion period.

**Variation of the biomass at the sediment surface in cores of station VA: F of L1 compared to NDVI**

The changes in biomass at the subsurface layer (L1) as revealed by using the proxies F, obtained from measurements with the iPAM, and NDVI, obtained from the spectral reflectance measurements, showed a similar pattern. F did not increase between the onset of emersion and LW in the intact sediment (Fig. 6A). However, NDVI increased between the onset of emersion period and LW. As shown in Fig. 5A, the upward migration based on the fluorescence (F) measurements took place in the hours before the onset of emersion. After LW, F started to decrease whereas the decrease in NDVI due to the downward vertical migration only commenced two hours after LW. Thus, both measurements showed downward migration of the MPB after LW but the migration patterns differed in their timing. In the homogenized sediment (Fig. 6B), both types of measurement showed upward migration of the MPB at different speed. NDVI increased rapidly reaching a plateau at 11:00h before LW whereas F showed a maximum at 13:00h (LW). Then, a decrease was observed in F just after LW whereas the NDVI showed hardly a decrease.
Maximum quantum efficiency of photosystem II ($F_v/F_m$)

To simplify the nomenclature between the subsurface (L1) and the deeper layers (L2, L3 and L4), we used $F_v/F_m$ for all layers even if the PSII quantum efficiency was measured in the light. In the intact sediments of VA station (Fig. 7A1), $F_v/F_m$ showed a steep increase in all layers until the beginning of the emersion period. Before the emersion, $F_v/F_m$ in L1 was higher than in other layers. After the beginning of emersion $F_v/F_m$ did not change further. Two hours before LW, $F_v/F_m$ decreased in L1 but remained constant in the other layers. Near the end of the emersion period $F_v/F_m$ started to decrease in all layers. In the homogenized sediment of station VA (Fig. 7A2), $F_v/F_m$ increased in L2, L3 and L4 until LW and then decreased. $F_v/F_m$ in L1 was higher than in other layers during most of the emersion period, as was also found in the intact core. In contrast to other layers, $F_v/F_m$ in layer L1 decreased from the beginning to the end of the measurement.

Measurements made in an intact core from station CN showed a small increase in $F_v/F_m$ in all layers before the emersion period (Fig. 7B1). During the emersion period, $F_v/F_m$ in all layers remained constant. $F_v/F_m$ in L1 was slightly higher than in L2, L3 and L4. At the end of emersion period (when the light went off) $F_v/F_m$ remained constant until the end of the measurement.

In the homogenized sediment from station CN (Fig. 7B2) in all layers, $F_v/F_m$ fluctuated in the dark before the onset of emersion. During the emersion period, $F_v/F_m$ increased in all layers until LW, became constant and decreased when the emersion period ended. During the measurement, the values of $F_v/F_m$ in L2, L3 and L4 were lower than those observed for L1 during the measurement.

Rapid Light Curves (RLC)

Relative $\text{ETR}_{\text{max}}$ increased slowly before the emersion at all depth layers on an intact core from station VA (Fig. 8A1). After the onset of emersion period, $\text{ETR}_{\text{max}}$ in the L1 and L2 showed a rapid increase and reached high values. In L3 and L4, $\text{rETR}_{\text{max}}$ slowly increased and reached a high at LW. After LW, $\text{rETR}_{\text{max}}$ decreased in all layers. At the end of the emersion period and after the light went off (17:30) $\text{rETR}_{\text{max}}$ was equal to the values found at the start of the emersion period. A similar pattern was observed in the homogenized sediment of VA (Fig. 8A2) $\text{rETR}_{\text{max}}$ values were similar between different layers under the dark periods in both the intact and homogenized sediments. $\text{rETR}_{\text{max}}$ in the homogenized sediment from station VA increased before the emersion period and until LW. Measurements on an intact core from station CN revealed that $\text{rETR}_{\text{max}}$ in L1 was constant before the emersion period (Fig. 8B1). After LW, a slight increase of $\text{rETR}_{\text{max}}$ was observed. A depth gradient in $\text{rETR}_{\text{max}}$ was observed with the highest values at the surface (L1) and the lowest values in L4. The homogenized sediment of the CN site showed the lowest values of $\text{rETR}_{\text{max}}$ in all sediment types (Fig. 8B2). $\text{rETR}_{\text{max}}$ did not differ between the different depth layers, with the exception of the situation ~08:00h, when it was higher in L1 than in the other layers. Before emersion started, $\text{rETR}_{\text{max}}$ increased in all layers. The increase of $\text{rETR}_{\text{max}}$ in L2, L3 and L4 was faster than in L1.
During the emersion period rETR$_{\text{max}}$ fluctuated without a particular trend regarding the tidal cycle.

In the intact sediment of station VA, $\alpha$ (light utilization coefficient in the light limited region of the RLC) values slightly increased in all layers until LW although the trend in L4 was less clear (Fig. 9A1). After LW, $\alpha$ values were similar and slowly decreased in all layers. The decrease of $\alpha$ in all layers apparently continued after the emersion period had ended. The patterns of $\alpha$ were similar in the homogenized sediment as in the intact core of VA (Fig. 9A2). The values of $\alpha$ were lower in L1 when compared to the other layers. In the intact sediment of station CN, the $\alpha$ values were more or less constant in all layers until LW when $\alpha$ decreased (Fig. 9B1). The values of $\alpha$ were similar in the layers L1-L3 and $\alpha$ was lower in L4. In the homogenized sediment of station CN (Fig. 9B2) $\alpha$ showed a different trend. It decreased until 10:30h and then increased and reached a maximum one hour after LW. Thereafter, $\alpha$ decreased in all layers. The value of $\alpha$ was highest in L1. Because the magnitude of changes in $\alpha$ was smaller than that of rETR$_{\text{max}}$, changes in E$_k$ reflected similar patterns as described for rETR$_{\text{max}}$ (Fig. 10).

C- incorporation

The rate of $^{13}$C-incorporation in POC and in different carbohydrates in VA (4213 ± 75 mg $^{13}$C m$^{-2}$ h$^{-1}$) was twice that in CN (2439 ± 249 mg $^{13}$C m$^{-2}$ h$^{-1}$) (Table 1). Glucose was the main carbohydrate product labeled during the first 4h of the incubation and contributed 48% (CN) and 64% (VA) of the bulk of POC C-fixation. The $^{13}$C-incorporation rates in other carbohydrates were lower than 80 mg $^{13}$C m$^{-2}$ h$^{-1}$.

Because PLFA can be used as a chemotaxonomic marker for microalgae, we were able to identify the active groups of the MPB community at both stations (Fig. 11). Contrary to rates of $^{13}$C-labeling in POC and carbohydrate, the $^{13}$C-incorporation rate in most of the individual PLFA was higher at CN than at VA, reflecting higher biomass at the sandy site. At CN, the ubiquitous 16:0 and 16:1$\omega$7 revealed the highest $^{13}$C-incorporation rates. A noticeable exception was 20:5$\omega$3, a PLFA found in high abundance in diatoms. The $^{13}$C-incorporation rate into 20:5$\omega$3 was higher in VA than in CN. On the opposite, $^{13}$C-incorporation in C$_{18}$ polyunsaturated fatty acids (18:2$\omega$6, 18:3$\omega$3, 18:3$\omega$6 and 18:4$\omega$3) were higher at CN than at VA.

Dominant microalgae species composition

At the muddy VA location, large epipelic diatoms were found which belong to Pleurosigma sp. The cyanobacterium Merismopedia sp. was observed but did not pass through the lens tissue. At the other location, MPB was composed of epipsammic diatoms at CN that could not be harvested by lens tissue. However, some epipellic MPB species were observed such as the green algae Euglena sp., the filamentous cyanobacterium Oscillatoria sp., and small epipellic diatoms belonging to Gyrosigma sp.
Discussion

MPB migration

Sediment type is an important abiotic factor in structuring MPB communities (Underwood & Kromkamp 1999, Forehead et al. 2013). In muddy environments, 90% of the photosynthetic activity occurs within the upper 400 µm depth (Kühl et al. 1997). At the two studied sites vertical migration took mainly place in the muddy environment of station VA. The MPB migration was measured from the sediment surface up to 2 mm deep, thus deeper than the photic zone. A previous study by Coelho et al. (2011) at VA showed that the MPB cells accumulated at the sediment surface under incident PAR ranging between 50-250 µmol m$^{-2}$ s$^{-1}$. This is in contrast to our observations, which showed that in the dark upward migration starting two hours before emersion. This suggests that the upward migration was controlled by an endogenous rhythm of the epipelic diatoms and was most likely related to the tidal cycle at the VA location.

Epipelic diatoms need to migrate to the sediment surface in order to capture sufficient light, perform photosynthesis and replenish intracellular organic carbon stores (storage lipids, storage carbohydrate chrysolaminaran) (Stal & de Brouwer 2003). However, at the sediment surface, prolonged light exposure can also cause photo-damage and depletion of CO$_2$ and bicarbonate. These stress conditions might induce downward migration where cells at the surface are replaced by those from greater depth (Kromkamp et al. 1998). The unicellular, colony-forming cyanobacterium *Merismopedia* sp. was visible as the orange spots (on fluorescence false colour scale) in the cores of the muddy station VA (Fig. 4A). They were observed in deeper layers and migrated upward without reaching the subsurface layer. As far as we know vertical migration of *Merismopedia* sp. has not been reported previously. Vertical migration of MPB may also be affected by biotic activity such as bioturbation (Reise 2002, Orvain et al. 2004). This was visible in our results with the accumulation of MPB inside the burrow of a bioturbator in the homogenized muddy sediment. Bioturbation can re-oxygenate the sediment and can transport cells into deeper layers (Larson & Sundbäck 2008, Middelburg & Levin 2009).

Light penetrates deeper in sandy than in muddy sediments (Kühl & Jørgensen 1994). Therefore, in sandy sediments MPB cells might also find suitable conditions for photosynthetic activity in deeper layers. At CN, the presence of photosynthetic biomass at greater depths indicates that light penetrates deeper in sandy sediment. This suggests that the photosynthetic active biomass was substantially higher at CN than at the muddy site. The homogenous distribution of the MPB at CN can also be explained by frequent sediment reworking caused by the tides.

The lens tissue technique only captured the migrating species while the majority of the community at CN was categorized as epipsammic. The MPB biomass increased in all layers after homogenization of the sandy sediment of a CN core. However, the upward migration only started after the onset of the emersion period when the actinic light was switched on. Thus, in contrast to the situation in the muddy sediment, the MPB in the sandy sediment apparently
required an exogenous stimulus (i.e. light) in order to start the upward movement. For this reason we conclude that the migration in the homogenized sediment of the sandy site is probably under control of positive phototaxis.

MPB photo-acclimation and C-fixation

From previous studies, differences between the photo-acclimation strategies were observed between muddy and sandy sediments (Consalvey et al. 2004, Jesus et al. 2009). In the muddy environment (VA) where light is strongly attenuated, 1% of the surface light level occurred between 0.5 and 1 mm. Hence, the layers L3 and L4 of the muddy site were probably in darkness during the measurement. All layers showed tidally driven changes in rETR\textsubscript{max} and E\textsubscript{k} at the muddy location of VA. These changes were more pronounced in L1 and L2 than in L3 and L4. Both the maximum rate of photosynthetic electron transport rETR\textsubscript{max} and the light saturation coefficient E\textsubscript{k} were highest near LW. This indicates that more light was needed to saturate the rate of photosynthetic electron transport and was taken as evidence of photo-acclimation of the cells to high light. In sandy sediments, light penetrates deeper and the 1% of surface light may be found up to 4 mm (Kromkamp unpublished). No changes were observed in rETR\textsubscript{max} and E\textsubscript{k} that could be related to the tidal cycle in sandy site.

In sediment cores from both sites, rETR\textsubscript{max} and E\textsubscript{k} were highest at the surface (L1) and lowest in L4, except the homogenized sediment of CN. This suggests a different photo-acclimation status between the surface and deeper layers. It is unlikely that the light levels played a role at CN in explaining depth differences in RLC parameters (rETR\textsubscript{max}, E\textsubscript{k} and \(\alpha\)) with depth because the deeper layers (L2, L3 and L4) were always in darkness. The concentration of oxygen concentrations plays a role in determining the depth related RLC parameters. Oxygen is apparently required for efficient photosynthesis (Kromkamp et al. 2007, Cox et al. 2010). However, the top 2 mm of the CN cores were oxygenated (as judged from the absence of black sediment), and we nevertheless observed depth-dependent changes in rETR\textsubscript{max} and E\textsubscript{k}. Therefore, oxygen was not a major factor that could explain photo-acclimation at the different depths. The most likely explanation for photo-acclimation at different depths is that more cyanobacteria are found at greater depth and that this caused a lower quantum efficiency of electron transport rates compared to diatoms. The phycobilins in the cyanobacteria add to the “background” fluorescence and this will decrease \(F_\text{v}/F_\text{m}\) and, consequently, the value of rETR (Campbell et al. 1998).

According to the fluorescence measurements, MPB biomass was highest at the sandy site and was distributed over a greater depth. In accordance with the fluorescence measurements, the net growth rates of the population, as measured from the labeling patterns in the PLFA, were also higher at the sandy site than at the muddy site. On the opposite, C-incorporation rates into the POC- and carbohydrate pools were higher than in the muddy sediments. This indicates that MPB cells at sandy and muddy locations partitioned the C-fixed in different ways. At the sandy site, the fixed C was used for MPB growth, whereas at the muddy site it was channelled to carbohydrate. Epipelic diatoms produce copious amounts of extracellular polymeric substance
(EPS) which is mainly composed of glucose (De Brouwer & Stal 2001). This EPS is exuded through the raphe of the diatom frustules and is involved in motility (Lind et al. 1997).

Microscopic observations and the rate of $^{13}$C-incorporation in specific PLFA, distinguished two different MPB communities: one in the muddy environment and one in the sandy environment. The muddy environment hosted mainly large migrating diatoms such as *Pleurosigma* sp. This station has a high specific activity as indicated in $^{13}$C-incorporation in POC and in 20:5ω3, which is a specific PLFA of diatoms. In contrast, the sandy environment supported large numbers of different MPB taxa (the green alga *Euglena* sp., the cyanobacterium *Oscillatoria* sp. and diatoms, especially *Gyrosigma* sp). Green algae is the main active group in the MPB of the sandy site as suggested from C-incorporation in potential biomarker of green algae (18:2ω6, 18:3ω3, 18:3ω6 and 18:4ω3) (Dijkman et al., 2009). Cyanobacteria and green algae have the capacity to synthesize a variety of PUFA from C$_{18}$ (18:2ω6, 18:3ω3, 18:3ω6 and 18:4ω3), making it difficult to separate cyanobacteria from green algae as there are no PLFAs that are unique for one of these groups (Kelly & Scheibling, 2012, Dijkman et al., 2009). However, green algae normally have a higher content of these PLFAs than cyanobacteria, and we therefore assume that most of the PLFA found at CN originated from green algae.

**Surface biomass measurements**

Vertical migration of MPB biomass has been followed using changes in fluorescence (F) as a proxy of biomass (Honeywill et al. 2002) or by measuring changes in surface reflectance, using NDVI as a proxy of biomass (Kromkamp et al. 2006, Serôdio et al. 2006). Both methods may yield qualitatively similar results (Serôdio et al. 2006), as demonstrated in this study. However, differences between fluorescence and surface reflectance results may occur because the fluorescence signal is not only influenced by biomass, but also by the photosynthetic characteristics of the cell. In high light, photoprotective reactions such as the induction of the xanthophyll cycle may induce non-photochemical quenching (NPQ), which affects the linear relationship between F and Chla (Honeywill et al. 2002). The NDVI measurements do not suffer from this problem as this index is based on the absorption of light rather than its utilisation (Kromkamp et al. 2006). As the light intensity applied when measuring NDVI was below E$_k$, the intrusive effect was expected to be minimal. Although non-photochemical quenching (NPQ) can occur in diatoms even at low rates of ETR, light applied at the sediment surface did not induce NPQ and was expected to be limited (Jakob et al. 2001).

In addition, the changes in F were also observed in deeper layers in dark condition, where the light induced NPQ cannot take place. Therefore, it is unlikely that the differences between F and NDVI in the speed of vertical migration at the sediment surface were caused by light-induced NPQ.

In summary, we have shown that vertical migration and photosynthetic activity in muddy sediments seem to be controlled by an endogenous rhythm triggered by the tides. The upward migration started at all depth layers investigated (0-2 mm) already before the onset of emersion in the absence of any external trigger. This is in contrast to the situation found in the sandy sediment, where we observed little if any migration in the intact core. However, when the
sediment was homogenized, MPB migrated to the sediment surface. However, as this migration only started after switching on the light, it is probably positive phototaxis.

Acknowledgments

We would like to thank Greg Silsbe for his help in fitting RLC curves and Tanja Moerdijk for carbohydrate analysis. This work received financial support from Schure-Beijerinck-Popping (SBP) Fonds.
Figures and tables

Figure 1. Map of the Ria de Aveiro and sampling locations: muddy site, VA (Vista Alegre) and sandy site, CN (Costa Nova).
Figure 2. Schematic drawing of the core device.
Figure 3. Measuring areas chosen: Layer 1 (L1), Layer 2 (L2), Layer 3 (L3) and Layer 4 (L4).
<table>
<thead>
<tr>
<th>Time</th>
<th>VISTA ALEGRE</th>
<th>COSTA NOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact sediment</td>
<td>Homogenized sediment</td>
</tr>
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<td><img src="image2" alt="Image" /></td>
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<tr>
<td>20:00h</td>
<td><img src="image13" alt="Image" /></td>
<td><img src="image14" alt="Image" /></td>
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</tbody>
</table>
Figure 4. Two dimensional images showing steady state fluorescence (F) at different times of the day (9h, 12h, 16h and 20h) at station Vista Alegre (VA)-intact sediment (peak of LW 14:17h), VA-homogenized sediment (peak of LW 12:57h), Costa Nova (CN)-intact sediment (peak of LW 16:51h), CN-homogenized sediment (peak of LW 15:44h). Relative values ranging from 0 to 100 are displayed using an identical false color scale of relative biomass. Two units in the false color scale correspond to 500 µm.

Figure 5. Changes in steady state fluorescence (F) at two stations: Vista Alegre (VA) and Costa Nova (CN) measured of two types of sediment: intact (VA: A1 and CN: B1) and homogenized (VA: A2 and CN: B2). Note the difference of y-axis scale in graphic (A1). Arrows indicate the turning of the low tide (LW) and horizontal dashed grey lines indicate emersion period.
Figure 6. NDVI index at the sediment surface and steady state fluorescence (F) at the subsurface layer (L1) measured of two types of sediment: intact (A) and homogeneous (B) during the illuminated period. Arrows indicate the turning of the low tide (LW) and horizontal dashed grey lines indicate emersion period. Note the differences in Y-axis scale for F between both figures.
Figure 7. Changes in maximum quantum efficiency of photosystem II ($F_v/F_m$) at two stations: Vista Alegre (VA) and Costa Nova (CN) measured of two types of sediment: intact (VA: A1 and CN: B1) and homogenized (VA: A2 and CN: B2). Arrows indicate the turning of the low tide (LW) and horizontal dashed grey lines indicate emersion period.
Figure 8. Comparison of maximum electron transport rate ($r\text{ETR}_{\text{max}}$) from rapid light curves fitting (RLC) derived from Eilers and Peeters (1988) at two stations: Vista Alegre (VA) and Costa Nova (CN) measured of two types of sediment: intact (VA: A1 and CN: B1) and homogenized (VA: A2 and CN: B2). Arrows indicate the turning of the low tide (LW) and horizontal dashed grey lines indicate emersion period.
Figure 9. Comparison of alpha (α) from rapid light curves fitting (RLC) derived from Eilers and Peeters (1988) at two stations: Vista Alegre (VA) and Costa Nova (CN) measured on two types of sediment: intact (VA: A1 and CN: B1) and homogenized (VA: A2 and CN: B2). Arrows indicate the turning of the low tide (LW) and horizontal dashed grey lines indicate emersion period.
Figure 10. Comparison of light saturation coefficient ($E_k$) from rapid light curves fitting (RLC) derived from Eilers and Peeters (1988) at two stations: Vista Alegre (VA) and Costa Nova (CN) measured on two types of sediment: intact (VA: A1 and CN: B1) and homogenized (VA: A2 and CN: B2). Arrows indicate the turning of the low tide (LW) and horizontal dashed grey lines indicate emersion period.
Figure 11. $^{13}$C-incorporation of individual PLFAs (nmol C g DW$^{-1}$ h$^{-1}$) normalized with total C-incorporation in total PLFA at two sites Costa Nova (CN) and Vista Alegre (VA). Data are represented as average with standard deviation error bars ($n=2$).

<table>
<thead>
<tr>
<th>Carbon pool</th>
<th>Sites</th>
<th>CN</th>
<th>VA</th>
</tr>
</thead>
<tbody>
<tr>
<td>POC</td>
<td></td>
<td>2439 ± 249</td>
<td>4213 ± 75</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>Fuc</td>
<td>13</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Rha</td>
<td>5</td>
<td>6</td>
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<tr>
<td></td>
<td>Gal</td>
<td>55</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Glc</td>
<td>1181</td>
<td>2713</td>
</tr>
<tr>
<td></td>
<td>Xyl</td>
<td>23</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>Man</td>
<td>14</td>
<td>44</td>
</tr>
</tbody>
</table>

Table 1. Increase of $^{13}$C-incorporation rate (nmol $^{13}$C m$^{-2}$ h$^{-1}$) into bulk organic matter (average ± standard deviation, $n=2$) and different types of carbohydrate: fructose (Fuc), rhamnose (Rha), galactose (Gal), glucose (Glc), xylose (Xyl) and mannose (Man) in the two studied locations (CN: Costa Nova and VA: Vista Alegre) during four hours of $^{13}$C-labeling.