Quantifying primary production of microphytobenthos
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2005

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 3.

Growth and Photosynthesis during 
Microphytobenthic Biofilm Development: An 
Integrated Optical Approach in a Tidal 
Mesocosm

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Submitted to Limnology & Oceanography

Abstract

Reflected radiance and chlorophyll fluorescence were used to measure the growth rate and maximum biomass of an undisturbed microphytobenthic (MPB) biofilm in a tidal mesocosm. Growth of the biofilm followed a sigmoid, logistic curve with a maximum rate of population growth (R) being $0.47 \pm 0.1 \text{ d}^{-1}$ and a maximum biomass of $240 \pm 35 \text{ mg chl a m}^{-2}$ after three weeks. The photosynthetic parameters of the developing biofilm were estimated in-situ using fluorescence-based measurements of electron transport rate (ETR), as well as by ETR and $^{14}\text{C}$ assimilation measurements in optically-thin suspensions of algal cells. Absorption cross-sections were quantified by spectral reconstruction from HPLC pigment analysis. All three sets of photosynthetic parameters were used to estimate daily net primary carbon production ($P_{\text{n}}$). The maximum photosynthetic capacity ($P_{\text{B max}}$) estimated using suspensions decreased significantly with time, whereas $P_{\text{B max}}$ estimated on undisturbed sediments showed no significant trend. $P_{\text{B max}}$ always decreased towards the end of the daily emersion period. The maximum quantum yield of photosynthesis ($\Phi_m$) estimated using $^{14}\text{C}$ assimilation decreased with time, whereas $\Phi_m$ estimated using ETR did not change. Deviations between the methods were largest at very low biomass levels and during stationary phase, and are discussed with respect to optical artefacts e.g. interference from background fluorescence and the contribution of “deep layer fluorescence” or to incorrect physiological assumptions used in the calculation of ETR. $P_{\text{c n}}$ estimated from $^{14}\text{C}$ assimilation was
closely coupled to observed changes in biomass, which suggested that both C:chl \( \alpha \) ratios and respiration rates of the biofilm showed low variability over time.

**Introduction**

Benthic microalgae or microphytobenthos (MPB) form highly productive natural ecosystems in intertidal areas (Cahoon 1999). They are an important food source for both benthic and pelagic communities (Macintyre et al. 1996; Underwood and Kromkamp 1999) and have also been linked to sediment stabilisation (Underwood and Paterson 2003). Quantification of primary carbon production (\( P_P \)) and growth rates of MPB is important for understanding intertidal community dynamics and estuarine food-webs (Middelburg et al. 2000). The biomass of MPB on intertidal flats is controlled by a combination of bottom up and top down processes. When removal processes such as grazing and resuspension are minimal, the microalgal biomass will increase and eventually reach a steady state value that is determined primarily by light, temperature and nutrient availability. However, Blanchard et al. (2001) showed that the equilibrium biomass realised on a tidal flat tended to oscillate around a value of approximately half the maximum biomass (or carrying capacity) attainable for the environmental conditions prevalent. It was proposed that the dynamic nature of tidal flats, with continuous removal of algal biomass, ensured that MPB communities were always in a state of high growth rate.

Biomass measurements of MPB are typically quantified as concentration (g m\(^{-2}\)) or content (g g\(^{-1}\)) of chlorophyll \( \alpha \) (chl \( \alpha \)) within surface sediments (Flemming and Delafontaine 2000). The recognition that primary production of intertidal sediments was strongly controlled by the interaction of light and algal biomass at the sediment surface (Pinckney & Zingmark 1993), has led to a recent concept of ‘photosynthetically active biomass’ (PAB) (Guarini et al. 2000). Here, the concentration of chl \( \alpha \) within the narrow photic zone of the sediment must be modelled or determined directly. For this purpose, fine-scale slicing of frozen sediment cores can be used effectively (Wiltshire et al. 1997; Kelly et al. 2001). However, destructive sampling methods are time consuming, which limit their scope in spatio-temporal investigations of MPB dynamics. Optical methods may prove to be useful for rapid, non-intrusive quantification of MPB [chl \( \alpha \)]. Furthermore, as the optical depth of the sediment and the photic depth are coupled, optical methods provide a fast estimation of PAB (Serôdio 2003), which is a key parameter of current primary production models.

Two methods in particular have been used for this purpose: the measurement of the reflected light from the sediment surface, and the measurement of in-vivo chlorophyll fluorescence. Hyperspectral
reflectance spectra show the influence of light absorption by photosynthetic pigments in surface sediments (Paterson et al. 1998, Hakvoort et al 1998). This allows the accurate quantification of [chl \(a\)] (Meleder et al. 2003a) and identification of different MPB assemblages (Stephens et al. 2003). Time series analysis has shown that surface reflectance can change dramatically when motile diatom cells migrate upwards from deeper layers to the sediment surface (Kromkamp et al. 1998). Reflectance spectra at ground level can also be linked to aerial or satellite based remote sensing analysis of the intertidal environment (Hakvoort et al. 1998), thus enabling MPB biomass to be estimated over large spatial scales (Smith et al. 2004).

As with reflectance, measurement of chlorophyll fluorescence is rapid and non-destructive. Data processing can be automated allowing data capture with a high time resolution. For example, Serôdio et al. (1997) used fluorescence to study in detail the changes in surface chl \(a\) caused by vertical migration of diatoms. However, the quantum yield of fluorescence from algal chl \(a\) is not constant. Both photochemical, and especially at high irradiance, protective non-photochemical quenching (NPQ) mechanisms, induce changes in the fluorescence yield (Consalvey et al. 2004). These processes introduce variability into the relationship between chl \(a\) and steady-state fluorescence levels. Application of a short dark acclimation period before measurement is usually sufficient to relax quenching mechanisms. In the absence of photosynthesis, all reaction centres are open, and fluorescence yield stabilises at a minimum level (\(F_0\)). Variability in the fluorescence-chl \(a\) relationship is therefore reduced. Often a 15 min dark adaptation is used, and this \(F_0^{15}\) is taken as a proxy of the true \(F_0\). A study in the Tagus estuary showed that the \(F_0^{15}\) of diatom-dominated sediments did not vary significantly even after pre-treatment with a wide range of irradiances (Perkins et al 2001). Fluorescence measurements have shown in many studies close, linear correlations with the concentration of chl \(a\) in sediments or in biofilms. (Karsten et al. 1996; Serôdio et al. 1997; Honeywill et al. 2002). By using \(F_0\) to measure changes in PAB caused by diurnal vertical migration of MPB, the correlation between fluorescence and oxygen microelectrode-based estimates of benthic production was greatly improved (Serôdio 2003).

Various methods for quantifying the primary production of intertidal sediments have been developed (reviewed by Underwood and Kromkamp 1999). In-situ techniques such as the bell jar, measure the flux of oxygen or carbon dioxide from the sediment to the overlying atmosphere or water (Barranguet 1997; Schories and Mehlig 2000; Migne et al 2002), but may underestimate the total rate of photosynthesis because only the upwelling flux is measured (Berg et al. 2003). In-situ tracer incubations measure the fixation of \(^{13}\)C or \(^{14}\)C from bicarbonate solution applied to the...
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sediment surface (Middelburg et al. 2000), but calculations of fixation rates are difficult due to the unknown specific activity of the tracer in the sediment pore water (Vadeboncoeur and Lodge 1998). This problem may be serious in cohesive sediments (Jönsson, 1991), and or in dense biofilms where drawdown of the porewater DIC pool is extremely fast and atmospheric CO$_2$ is the main inorganic carbon source. In contrast, photosynthetic measurements using suspensions of MPB cells in a medium, typically filtered seawater, can give useful information about photosynthetic characteristics under controlled temperature and irradiance conditions (Blanchard 1994; Macintyre and Cullen 1998; Wolfstein et al. 2000). In this case, the photosynthetic rates obtained represent an upper bound, because the physico-chemical gradients existing in the sediments are destroyed.

Alternatively, analysis of variable chlorophyll fluorescence kinetics can be used to measure the effective quantum efficiency of PS II ($\Delta F/F_m$') and the rate of non-cyclic electron transport (ETR). The advantage of this technique is that it can be used in-situ, is non-destructive and is rather quick. ETR of algal suspensions has been linearly correlated to O$_2$-production or C-fixation at limiting irradiances, but, can at high irradiances sometimes under- or overestimates the maximum rate of photosynthesis (Flameling and Kromkamp 1998; Hartig et al. 1998; Kromkamp et al. 1998; Masojídek et al. 2001). Quantification and conversion of ETR requires knowledge about the irradiance absorbed by PSII and the electron use efficiency ($\Phi_e$, mol C fixed per mol electrons produced by PSII; Gilbert et al. 2000). Comparisons of ETR and standard measures for the quantification of undisturbed MPB biofilm photosynthetic rates are rare and the published results are contradictory (Barranguet and Kromkamp 2000; Perkins et al. 2001; Perkins et al. 2002; Brotas et al. 2003; Serôdio 2003). In comparison to measurements on phytoplankton, a number of additional complications are introduced when trying to assess ETR of undisturbed MPB biofilms.

Determination of the absorption properties of the biofilm is difficult. The severe attenuation of irradiance within sediments (Kuhl and Jorgensen 1992) combined with the highly structured distribution of chl $a$ within the photic zone is thought to be responsible for ‘deep-layer fluorescence’, where the fluorescence signal from deep chl $a$ layers contributes to fluorescence measurements made at the sediment surface (Serôdio 2004; Forster and Kromkamp 2004). Not correcting for ‘background fluorescence’ has also been shown to severely influence measurements of variable fluorescence yields in the water column (Cullen and Davis 2003), and the resultant artefacts apply also to sediment measurements. It has been argued that the combination of complex sediment optics and vertical migration of motile cells, either over tidal cycles or in response to
changing irradiance conditions (photo-taxis) can seriously complicate the measurement of ETR on undisturbed sediments (Oxborough et al. 2000; Perkins et al. 2001; Perkins et al. 2002).

Our aims were therefore to assess the effectiveness of using in-situ optical techniques to measure the growth rate, biomass and primary production of a MPB biofilm in semi-natural conditions at a high temporal resolution. Additional photosynthetic parameters were estimated using ETR and $^{14}$C assimilation measurements carried out on optically-thin suspensions of MPB in filtered seawater to simplify the comparison of methods. Fluorescence-based measurements were converted to rates of carbon fixation using previously published coefficients. All three sets of photosynthetic parameters were used to calculate the net daily primary carbon production ($P_{PB}^{c}$), thus allowing the biomass of the MPB biofilm to be predicted throughout the cultivation period.

**Methods**

**Tidal tank mesocosms**

Sediment was collected in February 2002 from an intertidal flat on the northern shores of the turbid, eutrophic Westerschelde estuary, SW Netherlands (Biezelingsche Ham, 51° 26'N, 3° 55'E). The sediment consisted of poorly sorted, fine sands (mean grain size 0.14 mm) with an organic matter content of 7.4 ± 2.7 % (Wolfstein, K., pers. comm.). The sediment was sieved (1 mm), to remove macrobenthos, and autoclaved, to kill the remaining meioebenthos and microbiota. Rectangular plastic trays (30 x 50 cm wide, 12 cm high) with drainage holes and a layer of fine mesh on the lower surface were filled with 7 cm of ‘silver’ sand. The sterilised intertidal sediment was placed on top of the sand, to form a 5 cm deep layer, so that the sediment surface was flush with the rim of the container. The containers were submerged in filtered seawater (0.2 µm) (obtained from the adjacent Oosterschelde estuary) and left for 7 d in the dark with alternate periods of tidal emersion and immersion. Four identical trays were placed in the tidal mesocosm. A computer controlled system was used to regulate the inflow and outflow of water to the 1000 litre seawater reservoir. The sediment containers were positioned so that the water level at ‘low’ tide was about 8 cm below the surface of the sediment, i.e. the base of the sediment container was always waterlogged. The emersion time of the sediment surface was 20 h per day (split into two 10 h low tides from 0900 to 1900 h and 2100 to 0700 h). The emersion times were comparable with the high shore collection site at Biezelingsche Ham, and were set at the same time of day throughout the experiment (i.e. no tidal cycle lag). The photo-period and irradiance were constant throughout the experiment at 12 h and 200 µmol quanta m$^{-2}$ s$^{-1}$, starting at 0800 h and ending at 2000 h, to give a daily photon dose of
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8.6 mol quanta m\(^{-2}\) equivalent to the February average incident irradiance (as measured at the logging station of the NIOO-CEME, 4 year time series using a LiCor LI192SA PAR sensor). A combination of cool white fluorescent tubes and halogen spot lamps were used to reach the required irradiance. Heterogeneity in the light field was approximately 10%. Less well illuminated areas at the corners of the tank were excluded from measurements. The whole system was constructed in a temperature controlled room set to 15°C. Salinity of the overlying seawater was 29. Initial nutrient concentrations of the sea water were: 54.9 µmol NO\(_3\) l\(^{-1}\), 6.9 µmol NH\(_4\) l\(^{-1}\), 1.2 µmol PO\(_4\) l\(^{-1}\), 25.0 µmol SiO\(_2\) l\(^{-1}\), 31.0 mmol C l\(^{-1}\) (TIC) and 119.6 µmol C l\(^{-1}\) (DOC).

MPB enriched sediment was collected by scraping the surface layer from the same intertidal site, from this a suspension of epipelic MPB was collected using the lens tissue method (Eaton and Moss 1966). The dilute suspension of MPB cells, which qualitatively appeared to be dominated by an epipelic *Navicula* sp., was then sprayed over the trays using a simple household plant mister. Viability (motility and damage) of cells was checked under the microscope after spraying to ensure the spraying had no effect. The trays were inoculated on 8\(^{th}\) February 2002, and growth of the MPB biofilm was monitored for 21 d. The tidal inflow/outflow was regulated by valves so that there was very little hydrodynamic disturbance to the sediment surface, thus preventing resuspension of the biofilm.

**Continuous optical measurement of surface sediment chlorophyll a concentration**

The fibre-optic inputs of two spectroradiometers (S2000, Ocean Optics, Dunedin, USA, full acceptance angle 20\(^{\circ}\) and SR-9910 Macam, Livingston, Scotland, full acceptance angle 8\(^{\circ}\)) were positioned so that the viewing apertures were facing the sediment surface whilst ensuring that the viewing area contained no shadow. The viewing area was 100 cm\(^2\) and fixed throughout the experimental period (i.e. the location of sediment viewed was constant). The integration time of radiance measurements was set at 100 ms, the optimum for the irradiance used. Regular dark scans were performed to check for instrument drift, which was minimal in both cases. Both instruments were set to automatically record the reflected upwelling radiance from the sediment (Lu\(_s\)) every 30 min throughout the experimental period. The reflected upwelling radiance (Lu\(_d\)) of a white polystyrene panel was also measured at least two times per day. White polystyrene plates were chosen as a reflectance standard because they are cheap and can easily be replaced. Cross calibration between the polystyrene standards and a commercially-available 99% white reflectivity standard plate from Spectralon (Photo Research model SRS-3) indicated that the spectral difference between a polystyrene plate and the calibrated standard was less than 5%. The bidirectional
reflectance factor (Milton 1987), or spectral reflectance \((R_\lambda)\) was calculated as \(\frac{L_u}{L_d}\) for wavelengths between 300 to 900 nm at 1 nm intervals. From the reflectance spectra the normalised difference vegetation index (NDVI) was calculated:

\[
\text{NDVI} = \frac{R_{750} - R_{675}}{R_{750} + R_{675}}
\]

where \(R_{750}\) and \(R_{675}\) are the average reflectance between 745-755 nm and 670-680 nm respectively. This index can be used as a proxy which is directly proportional to surface chl \(a\) concentration (Meleder et al 2003a; Carrère et al 2004).

Fluorescence parameters of the biofilm were logged every 10 min at two fixed locations using pulse amplitude modulated (PAM) chlorophyll fluorometers, DIVINGPAM and PAM2000 (H. Walz, Effeltrich, Germany). The ends of each of the fluorometer fibre optic probes were clamped 4 mm above the sediment surface at a 45 ° angle (to minimise self shading). The viewing area under each probe was 3.5 cm\(^2\). Chlorophyll fluorescence from the developing biofilm was excited by a weak red measuring light (1 \(\mu\)mol \(\text{m}^{-2}\) \(\text{s}^{-1}\), maximum emission at 650 nm) and fluorescence was detected at wavelengths above 695 nm. As fluorescence parameters were logged continuously throughout the dark periods, the mean minimum fluorescence \(F_{\text{b}}\) value during the night time emersion, from 0200 to 0600 h, was used as a proxy for the true \(F_{\text{o}}\).

In addition to the continuous daily measurements of fluorescence and reflectance, further measurements of \(F_{\text{o}15}\) (\(F_{\text{o}}\) after 15 min. dark adaptation) and NDVI were made at randomly selected locations across the mesocosm. During the midday emersion of selected days, a PAM fluorometer (MINIPAM, H. Walz, Effeltrich, Germany) and a diode array spectrometer (MMS-1, Carl Zeiss, Jena, Germany, full acceptance angle 22°) were used for this purpose. The viewing area of the spectrometer was 24 cm\(^2\). The height of the PAM-fluorometer fibre optic was fixed at 4 mm above the sediment surface using a special chamber designed for investigation of fluorescence parameters on the surface of sediments (Fig. 1). The chamber ensured that the sediment was darkened and, having a rotating lid allowed 5 measurements of \(F_{\text{o}15}\) to be taken on the sediment surface at each location allowing a total area of approximately 17.5 cm\(^2\) to be covered. Measurements were taken at 10 positions across the biofilm selected using random number tables. Measurements were processed using the same procedure as the static fluorometers and spectrometers.
In summary, the developing biofilm was monitored with a high time resolution at four fixed locations throughout the experiment, with additional optical measurements being taken every 2-3 d at randomly chosen positions to ensure maximum spatial coverage. Mean $F_o$ (after 6 h of darkness), $F_o^{15}$ (mean of 10 positions), NDVI (1000 to 1800 h) and NDVI (mean of 10 positions) from each day were transformed to chlorophyll $a$ units using a two-point regression based on the optical measurements of the initial autoclaved bare sediment (assumed to contain a $[\text{chl} \ a]$ of zero) and destructive measurements of the surface (2 mm) sediment pigment concentration at the end of the experiment (see below for details). Because of the machine specific nature of $F_o-[\text{chl} \ a]$ relationships each fluorometer was calibrated individually using sediment sampled from the field of view. NDVI values from spectrometers were converted into chlorophyll $a$ equivalent units (mg m$^2$) using the regression:

$$[\text{chl} \ a] = 699.29 \times \text{NDVI} - 47.36 \quad (2)$$
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Logistic growth curve

Mean daily [chl a] was fitted to a logistic growth model using least squares criterion (Blanchard et al. 2001):

\[
[chl a] = \frac{B_0 \times K}{B_0 + ((K - B_0) \times \exp(-R \times d))}
\] (3)

where, \(B_0\) is the initial [chl a], \(K\) is the final [chl a], \(R\) is the maximum rate of logistic growth and \(d\) is the day of growth. A 5 day lag period before logistic growth began was observed at the beginning of the experiment.

Sediment sampling and pigment determinations

Constant volume replicate samples of the sediment surface (2 mm depth, 24 cm\(^2\), \(n = 19\)) were collected at the end of the cultivation period using the contact core method (Ford and Honeywill 2002). Samples were transported in liquid nitrogen and stored at -80 °C until analysis (within one week). After freeze-drying, photosynthetic pigments were extracted from the homogenised sediment in 5 ml of DMF in the dark at –4 °C. Extracted pigment concentrations were quantified using HPLC (see below for details). Sub-samples of the MPB suspensions used in photosynthesis-irradiance (P-E) experiments were collected on Whatman GF/F filters for pigment extraction in DMF.

Reversed-phase HPLC analysis was performed with a C-18 column, and Waters 2690 separation module running a ternary gradient system consisting of 85% methanol: water, 90% acetonitrile: water, and ethyl acetate (Kraay et al. 1992). Pigments were detected using a Waters 996 photodiode array and a Waters 474 fluorescence detector and classified with Millennium software, according to a spectral library of known pigments. Quantifiable chlorophyll pigments included the chlorophylls \(a\), \(b\), \(c_1/c_2\), chlorophyllide \(a\), pheophorbide \(a\) and pheophytin \(a\). Photosynthetic accessory carotenoids (PSC) and photoprotective carotenoids (PPC) were assigned according to Macintyre et al. (2002). PSC included fucoxanthin and diadinoxanthin, and PPC comprised of \(\beta\) carotene, diatoxanthin, and lutein / zeaxanthin (not separable).

Photo-physiological fluorescence parameters of the microphytobenthic community

The maximum energy conversion efficiency or quantum efficiency of PSII charge separation (\(F_v/F_m\)) was calculated as (Genty 1989):
where $F_o$ is the minimum fluorescence and $F_m$ is the maximum fluorescence (during a saturating light pulse, 0.6 s pulse duration, 6000 µmol m$^{-2}$ s$^{-1}$) of a dark-adapted sample. As fluorescence parameters were logged continuously throughout the dark periods the mean $F_o/F_m$ from 0200 to 0600 h was used as a proxy for true $F_o/F_m$. The effective quantum efficiency of non-cyclic electron transport (i.e. the effective quantum efficiency of PSII) during the illuminated emersion period was calculated as:

$$\Delta F / F_m' = (F_m' - F) / F_m'$$

where $F$ is the steady-state fluorescence and $F_m'$ the maximum fluorescence after a saturating pulse when measured in the light. The mean $\Delta F/F_m'$ from 1130 to 1230 was used as a proxy for daily mean $\Delta F/F_m'$.

**Photosynthesis of the microphytobenthic community**

From day 6 to 15 after inoculation, measurements of the photosynthesis-irradiance (P-E) relationship were carried out around the midpoint of the daytime low tide. On day 7, 12 and 15, additional measurements of the P-E relationship were carried out throughout the whole of the daytime low tide in order to examine ‘within-day’ variations in photosynthetic parameters.

Photosynthetic electron transport rates were measured both in-situ on undisturbed sediment and on suspensions of MPB cells. The rate of carbon fixation was also measured on suspensions of MPB cells using a radiocarbon assay (see below). Small areas (10 cm$^2$) of lens tissue sample were taken throughout the day (the collection method was a mix of allowing migration into the lens tissue over approximately an hour, and by brushing the tissue lightly across the surface of the biofilm). It should be noted that the lens tissue method selects mainly motile species and therefore may influence the MPB assemblage of the suspensions. These samples were shaken in filtered seawater ([TIC] 3.1 ± 0.1 mmol C l$^{-1}$, $n = 48$, mean ± SD), mixed and refiltered through 1 mm gauze, to produce a dilute algal suspension for radiocarbon and PAM fluorescence measurements.

**PAM fluorescence methodology**

In-situ photosynthesis was measured at random positions using a portable PAM fluorometer (MINIPAM, H. Walz, Effeltrich, Germany) and the dark adaptation chamber (Fig 1). After 1 min of dark adaptation, a P-E curve was obtained with 8 sequential irradiance steps (96 to 600 µmol quanta m$^{-2}$ s$^{-1}$) each of 1 min duration, using the internal light source of the fluorometer.
Measurements of algal suspension photosynthesis using fluorescence were carried out using a WATERPAM (H. Walz, Effeltrich, Germany). A 2 ml sample of the algal suspension was placed in the quartz cuvette of the WATERPAM, the stirring lid was replaced, ensuring the sample was stirred and a P-E curve was initiated using 8 sequential irradiance steps (52 to 835 µmol quanta m\(^{-2}\) s\(^{-1}\), 15 ± 1 °C) each of 1 min duration, using the red LED light source of the fluorometer.

Using \(\Delta F/F_m\)', the linear electron transport rate (ETR, \(\mu\)mol e\(^-\) (mg chl \(a\))\(^{-1}\) s\(^{-1}\)) can be calculated per mg chl \(a\) (Hofstraat et al. 1994; Kromkamp and Forster 2003):

\[
ETR = n_{PSII} \times a^*_{PSII} \times E \times \Delta F / F_m
\]  

where \(n_{PSII}\) is the number of functional PSII centres per mg chl, \(a^*_{PSII}\) is the optical cross section of PSII (nm\(^2\)) and \(E\) is the incident irradiance (µmol quanta m\(^{-2}\) s\(^{-1}\)). As \(a^*_{PSII}\) and \(n_{PSII}\) are difficult to measure, ETR was calculated from the spectrally averaged (400-700 nm) chlorophyll specific absorption cross section in the absence of package effects (\(a^*_{ph}\)) assuming that the ratio of PSI:PSII cross-sections (\(\rho\)) was 0.5 (Gilbert et al. 2000):

\[
ETR = E \times a^*_{ph} \times \rho \times \Delta F / F_m
\]

Because of the difficulty of measuring MPB absorption spectra without sediment contamination \(a^*_{ph}\) was constructed from HPLC derived pigment concentrations. Hypsochromic shifts and weight-specific spectral absorption coefficients (in HPLC solvent) were made according to Bidigare et al. (1990). The spectrally-resolved absorption coefficient (\(a^*_{ph}(\lambda)\)) was calculated from:

\[
a^*_{ph}(\lambda) = \sum_{i=1}^{n} c_i a^*_i(\lambda)
\]

where \(c_i\) is the concentration of pigment \(i\) (mg m\(^{-3}\)) and \(a^*_i(\lambda)\) is the specific absorption coefficient of pigment \(i\) (m\(^2\) mg\(^{-1}\)) at wavelength \(\lambda\). \(a^*_{ph}\) was then calculated by normalising \(a^*_{ph}\) by the concentration of chlorophyll in the suspension. For the sake of simplification it was assumed that packaging was minimal, based on the observation by Bidigare et al. (1990) for phytoplankton, and the observation that reconstructed \(a^*_{ph}\) values were very close to those observed for the benthic diatom *Cylindrotheca closterium* (Morris and Kromkamp 2003). Also, the initial inoculation of diatoms consisted mainly of small *Navicula* sp., and the package effect is less important in small cells (Stramski and Morel 1990). \(a^*_{ph}\) did not significantly change during the cultivation period (one-way ANOVA, \(F_{1,7} = 0.15, p = 0.99\)), therefore a mean value of \(0.016 \pm 3.63 \times 10^{-4}\) m\(^2\) (mg chl \(a\))\(^{-1}\) (mean ± SD) was used in subsequent calculations.
Radiocarbon assay

The algal suspension was also used to measure 3 replicate P-E curves per sampling event, in a photosynthetron (Lewis and Smith 1983). 2 ml of algal suspension was dispensed into 20 ml glass vials into which 400 µl of Na$^{14}$HCO$_3$ (final activity of 3.7 x 10$^4$ Bq sample$^{-1}$) were added. Vials were kept in the dark for 5 min, prior to exposure for 30 min to 9 irradiances ranging from 0 to 1640 µmol photon m$^{-2}$ s$^{-1}$ at the same experimental temperature as the fluorescence measurements (15 ± 1 ºC). The incubation was terminated by adding glutaraldehyde (3 % final concentration) and non-incorporated C was removed by adding 100 µl of concentrated HCl. Packard scintillation cocktail was added to each sample, and $^{14}$C incorporation was measured with a Packard Tri-Carb 2300 TR scintillation counter, including quench correction. The dark incorporation rates were subtracted from the incorporation rates in the light. Dissolved inorganic C in the medium was determined by potentiometric titration of carbonate alkalinity (Parsons et al. 1984). It was assumed that due to the short incubation time, measured C-fixation rates represented gross photosynthesis.

Photo-physiological characteristics of the MPB community

To correct for the different spectral properties of each of the light sources used to derive photosynthetic parameters, the total spectral irradiance absorbed by the MPB community (AQ$_{ph}$, µmol quanta (mg chl a)$^{-1}$ s$^{-1}$) was calculated as the product of the reconstructed absorption spectrum $a^*_ph(\lambda)$ and spectrum of the light source Q(λ) for each light treatment (Kroon et al. 1993), where

$$AQ_{ph} = \int_{400}^{700} a^*_ph(\lambda) \cdot Q(\lambda) d\lambda \quad (9)$$

Rates of carbon incorporation ($P^B_g$, µmol C (mg chl a)$^{-1}$ h$^{-1}$) were calculated from the product of ETR (µmol e$^{-}$ (mg chl a)$^{-1}$ s$^{-1}$) and the electron yield ($\Phi_e$, mol C (mol e$^{-}$)$^{-1}$) where,

$$P^B_g = ETR \cdot \Phi_e \quad (10)$$

A constant $\Phi_e$ of 0.199 mol C (mol e$^{-}$)$^{-1}$ (i.e. 5 electrons required and produced by PSII per C molecule), calculated by Morris and Kromkamp (2003) from data derived by Barranguet and Kromkamp (2000) for natural populations of MPB from the Westerschelde estuary using $^{14}$C incorporation, was used throughout the experimental period (i.e. $\Phi_e$ was decided a priori).

The relationship between $P^B_g$ and AQ$_{ph}$ was fitted to the model proposed by Webb et al. (1974);
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\[
P^B_g = P^B_{\text{max}} \left(1 - e^{-\left(\frac{A Q_{\text{ph}}}{P^B_{\text{max}}}\right)}\right)
\]

(11)

From the fit, the maximum photosynthetic capacity \((P^B_{\text{max}}, \mu\text{mol C (mg chl } a^{-1}\text{ h}^{-1})\) and maximum quantum yield of photosynthesis \((\Phi_m, \text{mol C (mol quanta)}^{-1})\) were derived (Sakshaug et al. 1997). Curve-fitting was done using simultaneous least-squares regression. A decrease in \(P^B_g\) at high irradiance was not observed in the P-E curves.

**Primary production of the biofilm**

The daily integral of primary production for the intact biofilm was calculated by incorporating the chlorophyll \(a\) (chl \(a\)) specific photosynthetic parameters of the undisturbed MPB community and the optically thin MPB suspensions into a vertically-resolved optical model of the sediment. The distribution of algal biomass with sediment depth was calculated using an exponential decay \((0.0016 \mu\text{m}^{-1})\), as shown by recent experimental data (Perkins et al. 2003). Attenuation coefficients were calculated at each depth interval for biological and non-biological material, and the sum of both attenuation coefficients was used to determine the transmission of irradiance through each discrete sediment layer according to:

\[
AQ_{i+1} = AQ_{0} e^{\left(K_{d_{\text{algae}}}[\text{chl } a_{i}] - K_{d_{\text{sed}}}[\text{Sed}_{i}]\right)}
\]

(13)

where \(AQ_{0}\) is the absorbed quanta at the sediment surface; \([\text{chl } a_{i}]\) is the chlorophyll concentration in depth interval \(i\), \(K_{d_{\text{algae}}}\) is the attenuation coefficient of the algae (assumed to equal \(a^*_{\text{ph}}, \text{m}^2 (\mu\text{g chl } a^{-1})\)), \(K_{d_{\text{sed}}}\) is the attenuation coefficient of the sediment \((0.003 \text{m}^2 (\text{g sed})^{-1})\) (Forster and Kromkamp 2004) and \([\text{Sed}_{i}]\) is the concentration of sediment in depth interval \(i\). Mean absorbed quanta within each depth interval was calculated according to Van Lier and Walsby (1982):

\[
AQ_{i+1} = \frac{(AQ_{i} - AQ_{i+1})}{\ln\left(\frac{AQ_{i}}{AQ_{i+1}}\right)}
\]

(14)

where \(AQ_{i}\) is the absorbed quanta in depth interval \(i\) and \(i+1\) respectively. Finally, gross primary carbon production \((P^c_g)\) was calculated in each depth layer from knowledge of irradiance, biomass and photosynthetic parameters using the following equation:

\[
P^c_g (i-i+1) = \left[chl a_{i-i+1}\right] P^B_{\text{max}} \left(1 - e^{-\left(\frac{AQ_{i+1}}{P^B_{\text{max}}}\right)}\right)
\]

(15)
where $P_{B\text{max}}$ is the maximum photosynthetic capacity, $\Phi_m$ is the maximum quantum yield of C-fixation, $AQ_{i+i+1}$ is the mean absorbed quanta in the depth interval $i$ to $i+1$, and $[chl \alpha_{i+i+1}]$ is the chlorophyll concentration in the depth interval $i$ to $i+1$. The depth resolution of the model was 10 µm, and primary production was integrated by summing over all depths from 0 to 2000 µm. Hourly rates of areal production (mg C m$^{-2}$ h$^{-1}$) were converted into daily rates by multiplying by the photoperiod of the experiment. An assumed respiration rate (10% of $P_{B\text{max}}$, Collos 1997) for each depth interval ($R_{i+i+1}$) was calculated according to:

$$R_{i+i+1} = P_{B\text{max}} R_C [chl \alpha_{i+i+1}]$$

where $P_{B\text{max}}$ is the maximum photosynthetic capacity, $R_C$ is the respiration coefficient (0.1) and $[chl \alpha_{i+i+1}]$ is the chlorophyll concentration in the depth interval $i$ to $i+1$. As for the primary production calculations, respiration was integrated by summing over all depths from 0 to 2000 µm. Hourly rates of areal respiration (mg C m$^{-2}$ h$^{-1}$) were then converted into daily rates by multiplying by 24 h. Finally, daily areal net primary carbon production ($P_{\text{cn}}$) was calculated as the difference after subtracting respiration from $P_{\text{cg}}$. $P_{\text{cn}}$ estimated by each of the methods on each day of cultivation was used to predict the realised biomass of the MPB biofilm on the subsequent day.

**Statistical analyses**

Bartlett’s test was used to check for homogeneity of variances and the data were examined graphically for deviations from the normal distribution. Where statistical assumptions (homogeneity of variance and normal distribution) were valid, parametric statistical tests were used. One-way (model I) ANOVA was used to test for significant variation of the data. The post-hoc Tukey Unequal N HSD test was used to determine significant differences between group means within the one-way ANOVA setting. Pearson’s product moment correlation was used to derive significant correlations between variables. Model II regression was used to derive functional relationships between variables. Where statistical assumptions were not valid, non-parametric statistical tests were used. Kruskal-Wallis one way ANOVA by ranks was used to test for significant variation of the data. Kruskal-Wallis multiple comparisons test of mean ranks was used to determine significant differences between group means within the one-way ANOVA setting. Spearman’s rank correlation was used to derive significant correlations between variables. Curve fitting was carried out using minimum least squares criteria in SigmaPlot 8.0 (SPSS Inc., Chicago, USA, 2001). Confidence intervals were chosen as the expression of error for reported means within figures. All confidence
intervals (CI) are given at the 95% level. Statistical analyses were performed in Statistica 6 (StatSoft Inc., Tulsa, USA, 2001).

Results

Changes in surface sediment [chl a] during the cultivation period were successfully recorded using both fluorescence and reflectance methods irrespective of whether the instruments were deployed in continuous logging mode (static), or used for random sampling on selected days (Fig. 2). The biofilm formation clearly followed a sigmoid logistic-type curve in all cases reaching a maximum biomass of 240 ± 35 mg chl a m\(^{-2}\) (mean ± CI) after three weeks. Estimates of chl a from both reflectance and \(F_{o}^{15}\) taken at 10 random positions at the same time and position on selected days were not statistically different (Fig 2, T-test, \(t_{(1,118)} = -0.57, p = 0.57\)) and fell within the range of the estimates taken at the static locations.

Figure 2. Chlorophyll a concentrations (mg chl a m\(^{-2}\)) estimated using optical methods plotted as a function of cultivation time (days). PAM2000, DIVEPAM and MINIPAM (H. Walz, Effeltrich, Germany) are commercially available fluorometers, MACAM (SR-9910 Macam, Livingston, Scotland), OCOPTIC (S2000, Ocean Optics, Dunedin, USA) and MMS (Carl Zeiss, Jena, Germany) are commercially available reflectometers. [mean ± CI, n = 10 - 22]. A logistic curve (see methods for details) was fitted through all data points (dashed line).

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Daily biomass-specific production was estimated from the measured change in mean daily [chl a]. The highest rate of 0.48 d$^{-1}$ was found at the beginning of the logarithmic phase (Days 6-8), followed by a decline throughout the growth period (Fig. 3). Growth rates could also be predicted by using the logistic model ($r^2 = 0.74$, $n = 15$, $F = 40.3$, $p < 0.0001$). The maximum rate of logistic growth (R) estimated using all the different optical measures ranged from 0.32 to 0.62 d$^{-1}$ [mean ± SD, 0.47 ± 0.1 d$^{-1}$, $n = 6$] and there was no significant difference between the estimates based on fluorescence (mean ± SD, 0.52 ± 0.12 d$^{-1}$, $n = 3$) or reflectance (mean ± SD, 0.42 ± 0.09 d$^{-1}$, $n = 3$) (T-test, $t = -1.2$, $p = 0.3$).

![Figure 3. Predicted (from logistic equation) and measured biomass specific daily production (day$^{-1}$) plotted as a function of cultivation time (days).](image)

The maximum photosynthetic capacity per unit chlorophyll ($P^{\text{B max}}$), measured at midday on each day, calculated from $^{14}$C assimilation on a MPB suspension, significantly declined from a value of 1190 to 305 µmol C (mg chl a)$^{-1}$ h$^{-1}$ from day 6 to day 15 respectively (Fig. 4, Spearman’s Rank $r = -0.97$, $p < 0.0001$). $P^{\text{B max}}$ (ETR suspension) was also significantly negatively correlated to increasing days from inoculation (Spearman’s Rank, $r = -0.47$, $p < 0.001$). However, no significant correlation between $P^{\text{B max}}$ (ETR in-situ) and days from inoculation was observed during the growth period (Spearman’s Rank, $r = -0.02$, $p = 0.90$). A significant positive correlation was observed between daily mean $P^{\text{B max}}$ ($^{14}$C suspension) and $P^{\text{B max}}$ (ETR suspension) (Spearman’s Rank, $r =$
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0.83, \( p < 0.05 \). No significant correlation was observed between daily mean \( P^B_{\max} \) (\(^{14}\)C suspension) and \( P^B_{\max} \) (ETR in-situ) (Spearman’s Rank, \( r = 0.32, p = 0.48 \)). When daily \( P^B_{\max} \) estimates were examined for differences between methods on each day, daily mean \( P^B_{\max} \) (\(^{14}\)C suspension) values were significantly higher than \( P^B_{\max} \) estimated using both ETR methods (which were not sig. different) on days 6 and 7, whilst on day 15, \( P^B_{\max} \) derived by both suspension methods was not significantly different but significantly lower than \( P^B_{\max} \) estimated in-situ (Kruskal-Wallis multiple comparisons test, \( p < 0.05 \)).

Figure 4. Biomass specific maximum rates of carbon assimilation (\( P^B_{\max} \), \( \mu \text{mol C (mg chl } a^-1 \text{)} \ h^-1 \)) plotted as a function of cultivation time (days). Rates were measured as the rate of \(^{14}\)C-bicarbonate uptake in an algal suspension (\(^{14}\)C suspension), ETR in-situ converted to carbon assimilation (ETR in-situ) and ETR in an algal suspension converted to carbon assimilation (ETR suspension). For details of ETR conversions see methods. [mean ± CI, \( n = 3 - 6 \)]. Marked days (*) indicate significant differences between photosynthetic parameters estimated by each of the methods (Kruskal-Wallis multiple comparisons test, \( p < 0.05 \)).

\( P^B_{\max} \) estimated by the ETR methods were also examined at high temporal scale on three days selected to represent different growth stages of the biofilm (Fig. 5). On all of the days examined, the change in \( P^B_{\max} \) (ETR in-situ and suspension) during the emersion period were negatively correlated to increasing emersion time (Pearson’s product moment, \( p < 0.05 \)), except for ETR in-situ on day
15 which showed a strong pattern of induction, peaking at approximately 1500 h followed by a depression during the rest of the afternoon. No significant difference in mean $P_{\text{max}}$ ($^{14}$C suspension) measured at 1100, 1300 and 1500 h was observed during the emersion period on day 7 (Fig. 5) ($F_{(2, 6)} = 2.58, p = 0.16$). $P_{\text{max}}$ ($^{14}$C suspension) was constant between 1100 and 1300 h on day 12, but showed a significant reduction in mean $P_{\text{max}}$ ($^{14}$C) at 1500 h (Post-hoc Unequal N HSD test, $p < 0.05$). $P_{\text{max}}$ ($^{14}$C suspension) significantly declined during the emersion period on day 15 (Pearson’s product moment, $r = 0.79, p < 0.01$). When all measurements were considered over the whole emersion period on day 7, mean $P_{\text{max}}$ estimated by each of the 3 methods was significantly different to each other (Kruskal-Wallis ANOVA, $H_{(2, 73)} = 32.9, p < 0.0001$). On day 12, mean $P_{\text{max}}$ ($^{14}$C suspension) and $P_{\text{max}}$ (ETR in-situ) were not significantly different whereas $P_{\text{max}}$ (ETR suspension) was significantly lower than both other methods (Kruskal-Wallis multiple comparisons test, $p < 0.05$). On the final intensive day of sampling (day 15) $P_{\text{max}}$ (ETR in-situ) had a significantly higher mean $P_{\text{max}}$ value than estimated using ETR suspension or $^{14}$C assimilation, both of which were not statistically different to each other (Kruskal-Wallis multiple comparisons test, $p < 0.05$).

The maximum quantum yield ($\Phi_m$, mol C (mol quanta)$^{-1}$) derived from both the ETR methods was constant and did not significantly change from a mean value of 0.068 mol C (mol quanta)$^{-1}$ throughout the cultivation period (Fig. 6) (Kruskal-Wallis ANOVA, $H_{(7, 60)} = 3.68, p = 0.82$). The change in $\Phi_m$ derived from $^{14}$C assimilation over the cultivation period was significantly negatively correlated with increasing days from inoculation (Spearman’s rank, $r = -0.84, p < 0.0001$). No significant correlation between mean daily $\Phi_m$ estimated using both ETR methods and $^{14}$C was observed over the cultivation period. Daily mean $\Phi_m$ ($^{14}$C) values were significantly higher than $\Phi_m$ estimated using both ETR methods on days 6 and 7, and significantly lower on day 15 (Kruskal-Wallis multiple comparisons test, $p < 0.05$).

Examination of the within-day variation in $\Phi_m$ on days 7, 12 and 15 revealed no significant daily rhythms in $\Phi_m$ estimated using both ETR methods (Fig. 7). No significant difference was observed between $\Phi_m$ ($^{14}$C suspension) values measured at 1100, 1300 and 1500 h on day 7 and 12. $\Phi_m$ ($^{14}$C suspension) was significantly negatively correlated with time of day on day 15 (Spearman’s rank, $r = -0.58, p < 0.05$). On day 7 when all measurements made that day were pooled, no significant difference was observed between $\Phi_m$ estimated by each of the methods (Kruskal-Wallis multiple comparisons test, $p < 0.05$). On day 12 and 15, mean $\Phi_m$ estimated using both ETR methods was significantly higher than mean $\Phi_m$ ($^{14}$C) (Kruskal-Wallis multiple comparisons test, $p < 0.05$).
Figure 5. Biomass specific maximum rates of carbon assimilation ($P_B^{\text{max}}$, µmol C (mg chl a)$^{-1}$ h$^{-1}$) plotted as a function of time of day for cultivation day 7, 12 and 15. Each point represents a single P-E curve. Other details as in Fig. 4.
Figure 6. Maximum quantum yield ($\Phi_m$, mol C (mol quanta)$^{-1}$) plotted as a function of cultivation time [mean ± CI, $n = 3 - 6$]. Other details as in Fig. 4.

The fluorescence signal from the developing biofilm was high enough from the sixth day of cultivation to allow measurement of the maximum quantum efficiency of PSII charge separation ($F_v/F_m$) by taking the mean of dark period values between 0200 and 0600 h ($n = 16 - 35$) measured by both fluorometers viewing static locations (Fig. 8). $F_v/F_m$ (static) increased from a minimum value of 0.56 to a maximum value of 0.67 on day 6 and 7 respectively. $F_v/F_m$ was constant at a value of 0.67 until day 12, after day 12 values began to steadily decrease, eventually reaching a minimum value of 0.58 on day 20 (Kruskal-Wallis multiple comparisons test, $p < 0.05$).

The effective quantum efficiency of charge separation ($\Delta F/F_m'$) was estimated from the mean of all values logged by both fluorometers viewing static locations from 1130 to 1230 h each day ($n = 12$) (Fig. 8). $\Delta F/F_m'$ (static) steadily increased from a value of 0.44 on day 6 to a maximum value of 0.55 on day 11. From day 11, $\Delta F/F_m'$ steadily declined reaching a minimum value of 0.4 on day 20 (Kruskal-Wallis multiple comparisons test, $p < 0.05$).
Figure 7. Maximum quantum yield ($\Phi_m$, mol C (mol quanta)$^{-1}$) plotted as a function of time of day for cultivation day 7, 12 and 15. Each point represents a single P-E curve. Other details as in Fig. 4.
Total pigments ($p < 0.05$), photosynthetic carotenoids ($p < 0.05$), chl-c ($p < 0.001$) and breakdown products of chlorophyll ($p < 0.001$) expressed per mg chl $a$, all decreased marginally but significantly with increasing days from inoculation (Spearman’s rank, $n = 54$). The ratio of PPC : chl $a$ (Spearman’s rank, $r = 0.86, n = 54, p < 0.001$) and PPC:PSC (Spearman’s rank, $r = 0.83, n = 54, p < 0.001$) were significantly positively correlated with increasing days from inoculation, indicating that photosynthetic protective functions increased during the experiment (Fig. 9).

Net primary production of the mesocosm was calculated on an areal basis ($P^c_n, \text{g C m}^{-2} \text{d}^{-1}$) using, the measured biomass, a hypothetical depth distribution (see methods), incident irradiance, an assumed respiration rate and the measured photosynthetic parameters. The average of photosynthetic parameters from adjacent days was used when photosynthetic parameters were missing for a particular day. The cumulative estimates of net produced carbon (i.e. predicted biomass, g C m$^{-2}$) from the three different photosynthetic methods were compared to the kinetics of daily mean biomass (g C m$^{-2}$), estimated from the daily [chl $a$] predicted by the logistic growth curve using a C/chl $a$ -ratio of 50 (Fig. 10). Biomass estimated from daily mean [chl $a$] followed a sigmoid logistic-type curve, eventually reaching a value of 9.73 g C m$^{-2}$ on day 16. Predicted biomass ($^{14}$C suspension) showed a curvilinear pattern, with biomass estimates generally matching those predicted from the growth curve from days 7 to 10. From day 11, biomass ($^{14}$C suspension) began to reach a steady value and diverged from the biomass estimated from the growth curve, eventually reaching a value of 7.86 g C m$^{-2}$ on day 16. Biomass predicted from the ETR suspension method linearly increased throughout the cultivation period, meaning that for much of the growth period, biomass (ETR suspension) estimates were very similar to those derived from the growth curve. However, biomass (ETR suspension) showed no signs of saturation resulting in substantially higher biomass estimates than those predicted by all the other measures on day 16 (13.92 g C m$^{-2}$). ETR in-situ biomass estimates also increased linearly, but with a shallower slope than the ETR suspension estimates, meaning that from day 9, ETR in-situ biomass estimates were slightly lower than those estimated from the growth curve. Biomass (ETR in-situ) was higher than the biomass estimated from C-assimilation on days 14 and 15, leading to a substantially higher predicted biomass of 9.62 g C m$^{-2}$ on day 16. All of the biomass estimates by each of the primary production methods were significantly correlated to the biomass estimated from the growth curve (Pearson’s product moment, $r = 0.98, p < 0.001$).
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Figure 8. Daily mean maximum PSII quantum efficiency ($F_v/F_m$ static) measured on undisturbed sediment at a fixed location (0200 to 0600 hrs) and mean effective quantum efficiency ($\Delta F/F_m$ static) measured on undisturbed sediment at a fixed location (1130 to 1230 hrs, 200 µmol quanta m$^{-2}$ s$^{-1}$) [mean ± CI, $n = 3 - 65$].

Discussion

Measuring chlorophyll concentration and growth of a biofilm

The aim of this work was to investigate the suitability of optical methods for following the development of an intertidal phototrophic biofilm, to compare different methods to estimate photosynthesis, and to test the use of in-situ PAM fluorometry as a method for measuring microphytobenthic primary production. Relative changes in algal biomass could be satisfactorily followed using spectral reflectance or fluorescence, with both methods obtaining similar, not significantly different values for the maximum growth rate of the biofilm. Furthermore, chl $a$ concentration estimated from the two different methods did not differ significantly when measures of reflectance and $F_o^{15}$ fluorescence were made at the same time and position. Following careful calibration, optical methods can also be used for estimating absolute levels of MPB biomass, as shown here and in many previous studies (e.g. Honeywill et al. 2002; Meleder et al. 2003a; Carrère et al. 2004). However, there was considerable variation in the range of values recorded by the
different sampling methods at different locations across the biofilm. This may have been a result of patchiness between the different areas of the mesocosm, or incorrect calibration of the individual instruments.

Small changes in the distance of the PAM fibre optic from the sediment surface have a disproportionately large effect on the measured $F_o^{15}$. Despite efforts to keep the distance to the surface as constant as possible, deviations in surface topography was probably one of the major sources of the high variability in $F_o^{15}$. The influence of non-photochemical quenching or phototactic migration within the dark adaptation period may have also contributed to the variability of $F_o^{15}$ measures. Both surface topography and photobiological effects are likely to be of great importance for fluorescence measurements in the field, especially during periods of high irradiance. In contrast, the reflectance method is based on the in-situ measurement of an inherent optical property (pigment absorption) that is independent of the distance between sensor and target. Algorithms for the conversion of spectral reflectance to pigment concentration can be used for any spectroradiometer, including airborne and satellite based-instruments, whereas universal calibration of fluorometers is not possible due to machine-specific differences in response. The coefficient of variation (CV, SD/mean x 100) for the destructive sampling at the end of the experiment was higher than for the reflectance measurements [25 and 8 % respectively], perhaps due to the sampling process being more complicated in terms of analytical procedures. Errors may have been introduced during the sampling (variation in depths of cores), or during freeze-drying and weighing of samples, or during extraction and quantification of the pigments.

The growth of the biofilm followed a logistic pattern which converged towards a maximum value, which is the biotic capacity of the local environment. Similar shapes of growth curves for ‘natural’ populations of MPB were previously found by Blanchard et al. (2001) and Orvain et al. (2003) in tidal mesocosms. The highest growth rate of 0.47 d$^{-1}$ during early development of the biofilm was lower than a rate of 0.76 d$^{-1}$ recorded by Middelburg et al. (2000) for a low biomass MPB biofilm growing at an intertidal site in summer. The lowest growth rates of <0.1 from day 12 onwards are consistent with growth rates of 0.09 to 0.10 measured by Middelburg et al.(2000) at sites with dense MPB biofilms in the Westerschelde estuary. The maximum value of biomass found in the present study was 240 ± 35 mg chl $a$ m$^{-2}$ (mean ± CI) on day 19, which is at the upper end of the range reported for temperate estuarine MPB communities (Underwood and Kromkamp 1999). It was not the aim of this study to determine the limiting factor for the biotic capacity, however previous theoretical work has shown that inorganic carbon supply is most likely to become limiting (Ludden
et al. 1985). Our own modelling exercises have shown that the penetration of irradiance within the sediment, and the value assigned to respiratory losses are critical in determining whether net primary production and therefore biomass accumulation can occur (see later). The fact that the concentration of MPB on intertidal flats seldom reaches the biotic capacity (e.g. an annual mean [chl \(a\)] in the Westerschelde estuary of 81 mg m\(^{-2}\); Forster, R. M. pers. comm.) indicates that control factors such as grazing and resuspension are of great importance during most of the year in natural populations.

![Figure 9. Biofilm photoprotective carotenoids (PPC): chl \(a\) and PPC: photosynthetic accessory carotenoids (PSC) plotted as a function of cultivation time. See methods for explanation of pigment functional grouping [mean ± CI, \(n = 3\)].](image)

**Photosynthetic parameters**

It is important to note that rates of carbon fixation were calculated from PAM fluorescence measurements using measured absorption coefficients, and conversion factors determined a priori, and not via a post hoc empirical comparison. The maximum photosynthetic rates and maximum quantum yields thus determined were remarkably similar in absolute units to those determined with the radiocarbon method for the majority of the experiment. They are also in agreement with theoretical predictions of the minimum quantum requirement of carbon fixation (QR, \(1/\Phi_m\)).
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Mauzerall (1986) determined a QR of 10 quanta per O$_2$ using single turnover flashes, assuming a photosynthetic quotient (PQ) of 1.4, typical for growth on nitrate (Williams & Robertson 1991), the QR converted to carbon units would be 14. The mean QR calculated from ETR in this study was 14.7 ± 0.8 quanta (C)$^{-1}$ (mean ± SD), very close to the theoretical minimum. Therefore, the use of variable fluorescence seems to offer considerable promise as an alternative method for rapid in-situ estimates of MPB photosynthesis, a conclusion previously reached by other authors (Barranguet & Kromkamp 2000; Kuhl et al 2001; Serôdio 2003). The fluorescence method was refined in this paper by using spectral reconstruction of in vivo absorption spectra in order to calculate the quantity of absorbed photons for use in the electron transport rate calculation. In principle this technique could also be used in combination with PAM measurements in the field.

Figure 10. Biofilm daily mean biomass (g C m$^{-2}$) estimated from the logistic growth curve (Fig. 2) using a C:chl $a$ ratio of 50 and daily mean biomass (g C m$^{-2}$) predicted from net primary production estimates plotted as a function of cultivation time. Other details as in Fig. 4.

Measurements on optically-thin suspensions showed a decrease in photosynthetic capacity with increasing age of the biofilm, although the decrease in $p_{\text{B}}^{\text{R,max}}$ obtained from the C-fixation measurements was more pronounced than the decrease obtained from the ETR measurements. The marked decline in photosynthetic capacity and lesser declines in photosynthetic efficiency (Φ$_m$, Fv/Fm) could be indicators of photosynthetic down-regulation as the concentration of cells in the
surface layer of the sediment increased, and growth rates slowed. In batch cultures of benthic microalgae, both $P_{max}^B$ and RUBISCO activity decline markedly with increasing age (Tremblin & Robert 2001). Measurements on suspensions at high time resolution indicated a decrease in the photosynthetic capacity towards the end of the photoperiod. Several reasons might be responsible for the observed changes: an intrinsic rhythm of photosynthesis, which might be related to the cell cycle as shown for synchronized cultures of the diatom *Thalassiosira pseudonana* (Claquin et al. 2002) or a diurnal pattern of RUBISCO expression (Paul et al. 2000). A change in the dominant species at the sediment surface may have occurred, although no migration during the emersion period was observed using reflectance. Alternatively, unfavourable conditions at the sediment surface may have developed during the latter part of the photoperiod, such as depletion of pore water DIC, build-up of supersaturating oxygen concentrations, or dehydration of the sediment.

Our estimates of $P_{max}^B$ and $\Phi_m$ with three different techniques revealed some significant disagreement in trends and in absolute values at some time points. For example, the in-situ ETR measurements of $P_{max}^B$ remained constant over the whole course of the experiment in contrast to the decreases observed in suspensions. Closer examination of individual days with increased replication of photosynthesis measurements also showed some conflicting results, particularly on day 15, where the in-situ ETR measurements again deviated markedly from those made on suspensions.

There are a number of possible explanations for the divergence between $P_{max}^B$ estimates by each of the methods on days 6 and 7. The sediment chl $a$ on these days was still at a low concentration, and it is likely that the large errors bars associated with $P_{max}^B (^{14}\text{C})$ measurements on day 6 and 7, when compared to other days, were a result of inaccuracies in the determination of the [chl $a$] within the dilute suspensions used. PAM fluorescence measurements in very dilute suspensions may also have been subject to error. The low sediment surface and suspension [chl $a$] may have amplified errors associated with the background correction of fluorescence measurements, a problem previously discussed by Cullen and Davis (2003) and eluded to by Perkins et al. (2002). In the presence of “background fluorescence”, i.e. fluorescence not originating from PSII, correct calculation of the quantum efficiency is done by subtracting the background fluorescence ($F_b$) from the relevant PSII fluorescence parameters ($F, F_0, F_m, F_m'$). Thus, incorrect determination of $F_b$ (or no correction as was the case in this study) will lead to an underestimation of the true $F_v/F_m$ or, in actinic light, to an underestimation of the true $\Delta F/F_m'$ and thus ETR. Especially at low biomass, the error introduced by an incorrect estimation of the background fluorescence can be large, although its importance will decrease as the proportion of photosystem II-derived signal increases. This artefact may also
become important at high irradiance as quenching of fluorescence will increase the magnitude of $F_b$ relative to $F$. Although manufacturer’s instructions were followed, and low fluorescence values were discarded, the increase in both $F_v/F_m$ and $\Delta F/F_m$ observed in-situ and in suspensions at the beginning of the experiment does suggest an influence of $F_b$ on the calculated yields. Accurate determination of $F_b$ is a challenge to be tackled before PAM instruments can be used with confidence on sediments with different optical properties.

Higher values for $P_{\text{max}}^B$ ETR in-situ compared to suspensions at high sediment chl $a$ concentrations could be due to another optical artefact, the presence of ‘deep-layer fluorescence’, i.e. fluorescence originating from algae below the surface (Oxborough et al. 2000). This effect occurs when measurements are made on optically-complex structures such as plant leaves, macroalgal thalli or benthic biofilms. In the presence of light, subsurface algae are exposed to lower irradiances than algae at the sediment surface, and will have a correspondingly higher $\Delta F/F_m^*$. A fraction of the fluorescence from this layer will be detectable at the surface, causing an overestimation of $\Delta F/F_m^*$. The effect is more pronounced at irradiances exceeding $E_k$, and with higher biomass (Serôdio 2004; Forster and Kromkamp 2004). If present, this effect may have masked a decrease in $P_{\text{max}}^B$ with time in the ETR in-situ measurements.

The maximum quantum yield of photosynthesis ($\Phi_m$, mol C (mol quanta)$^{-1}$) estimated using carbon assimilation, significantly decreased with time, as did $P_{\text{max}}^B$. In contrast, $\Phi_m$ derived from both types of ETR gave equal values which did not differ between or within days. Contamination by background fluorescence may have caused in-situ $\Delta F/F_m^*$ values to be slightly underestimated on days 6 and 7, masking a decrease in $\Phi_m$ at the start of the experiment. As deep layer fluorescence is unlikely to be an important factor at the low irradiances used for calculation of $\Phi_m$ (Serôdio 2004; Forster and Kromkamp 2004), reasons for the deviations between methods at high biomass must be sought elsewhere.

The QR calculated from $^{14}$C assimilation ranged from a maximum of 29 quanta (C)$^{-1}$ on the final day of photosynthesis measurements to a minimum value of 8.3 quanta (C)$^{-1}$ on day 6. QRs calculated for days 6 and 7, were below the theoretical minimum of 14 (assuming PQ of 1.4, Williams & Robertson 1991, and minimum QR of 10 quanta (O$_2$)$^{-1}$, Mauzerall 1986), suggesting that the measured chlorophyll-normalised C-fixation rates on day 6 and 7 slightly overestimated the real rate of $P^B$. Alternatively, AQ could have been underestimated. This is unlikely, as the pigment packaging, if present, would have acted to decrease $a^*$ and was therefore unlikely to be the cause of the high QRs observed on day 6 and 7.
The differences observed between ETR- and $^{14}$C-derived $\Phi_m$ values, which were particularly noticeable at the end of the experiment, may have been caused by a combination of changes in the maximum quantum efficiency of PSII ($F_v/F_m$) (measured), the ratio of PSI:PSII functional cross sections ($\rho$, set a priori at 0.5) and the electron yield ($\Phi_e$, set a priori at 0.199 mol C (mol e$^-$)$^{-1}$).

The maximum quantum efficiency of PSII ($F_v/F_m$) reflects the functional state of the PSII reaction centre, and can be decreased by high irradiance or nutrient limitation, and by changes in the composition of pigments that are not associated with photosynthesis (Macintyre et al. 2002). A decrease in $F_v/F_m$ occurred after day 12, when biomass production of the biofilm began to decline, and the ratio of photoprotective carotenoids (PPC) to photosynthetic carotenoids (PSC) increased. This suggests that the observed trend of decreasing maximum quantum efficiency of photosynthesis ($\Phi_m$) measured using $^{14}$C assimilation was due to an accumulation of pigments that did not contribute to excitation energy transfer to reaction centres (Babin et al. 1996). Substrate limitation, which can lead to a reduction in the transfer efficiency from the antenna to RCII (Kolber et al, 1988; Greene et al. 1994) and/or an accumulation of damaged PSII reaction centres (Macintyre et al. 2002), may also be important. With the limited number of photosynthesis measurements carried out in the stationary growth phase (day 15 only) and no data on substrate availability, any conclusions about the cause of the changes in photo-physiological are currently speculative.

Changes in the efficiency of photosynthesis are likely to affect the electron yield ($\Phi_e$) and ratio of the PSI:PSII cross section ($\rho$). In this study, the combination of $\Phi_e$ and $\rho$ is equal to the ratio of the operational quantum yields of carbon incorporation and charge separation ($\Phi_c/\Delta F/F_m$'), which was previously termed EE by Barranguet and Kromkamp (2000). Days 6 and 7 had a mean EE of 0.17 mol C (mol e$^-$)$^{-1}$, higher than the theoretical minimum of 0.125 mol C (mol e$^-$)$^{-1}$, which is probably a result of the inaccuracies in the data (both $^{14}$C assimilation and PAM methodology) discussed earlier. For the majority of days (8 - 14), calculated EE values ranged from 0.087 to 0.099 mol C (mol e$^-$)$^{-1}$ [$0.091 \pm 0.006$ mol C (mol e$^-$)$^{-1}$, mean $\pm$ SD, $n = 12$], which was close to the mean EE calculated by Morris and Kromkamp (2003) for a benthic diatom culture [0.114 mol C (mol e$^-$)$^{-1}$] and the EE recalculated using the data of Barranguet and Kromkamp (2000) for natural MPB in field situations over a seasonal cycle [0.0995 mol C (mol e$^-$)$^{-1}$]. However, on day 15, mean EE appeared to be lower [0.053 $\pm$ 0.002 mol C (mol e$^-$)$^{-1}$]. In essence our results seem to indicate that the EE may be dependent on the growth stage of the MPB, however this result is based on uncertain values from days 6 and 7 and on day 15 only: further photosynthesis measurements during the stationary phase of growth would have been necessary to confirm this hypothesis, which we do not
have. Ratio’s of PSI:PSII cross section’s ($\rho$) can vary significantly between species, with growth irradiance and nutrient status (Babin et al. 1996; Kromkamp and Forster 2003) and it is likely that the $\Phi_e$ is affected by growth rate (Flameling and Kromkamp 1998) making it difficult to separate between which of these two factors were responsible for the observed change in EE on day 15.

Both $F_v/F_m$ (Spearman’s Rank, $r = -0.82$, $p < 0.01$) and PPC:PSC ratios (Spearman’s Rank, $r = 0.79$, $p < 0.01$) were significantly correlated to the change in photosynthetic efficiency and may be able to provide information on when EE changes, although further investigation is required with a range of MPB assemblages and environmental conditions in order to assess the this relationship. On the other hand, if, as proposed by Blanchard (2001) MPB community biomass dynamics are tightly coupled with removal processes, so that in natural situations, MPB communities are constantly in logarithmic growth, then a single mean EE value should be satisfactory.

**Areal net primary carbon production**

Cumulative net primary carbon production (i.e. the predicted biomass of the biofilm, g C m$^{-2}$) predicted by each of the methods basically followed the pattern of biofilm growth estimated from daily mean [chl $a$], although because of the overestimation of $\Phi_m$ (ETR) discussed above, the ETR estimates substantially over estimated $P_{\text{c}}$ (14C) and consequently over estimated the predicted biomass towards the end of the cultivation period (Fig. 10). $P_{\text{c}}$ (14C) values began to diverge away from those estimated from the growth curve (estimated using a constant C:chl $a$ ratio of 50) on day 11, suggesting that the algal C:chl $a$ ratio decreased. De Jonge and Colijn (1994) found C:chl $a$ ratio’s of natural MPB communities varied between 40 and 61 in the Ems Dollard estuary. C:chl $a$ ratios calculated in our study from the 14C assimilation biomass predictions ranged from 62 to 38 on day 8 and 16 respectively, suggesting that ratios found in this study were very comparable to those found in natural communities of MPB. The correlation between biomass estimated from 14C assimilation and the biomass estimated from [chl $a$] suggests that using net primary production as a proxy for biomass changes in undisturbed MPB biofilms is a promising approach. Although, improvements to the model used for the prediction of $P_{\text{c}}$ are still required. In particular a constant proportion (10%) of $P_{\text{B}}^{\text{max}}$ was chosen for the rate of respiration, which probably represents ‘maintenance’ respiration (Collos 1997) and does not represent variable respiration related to growth. $P_{\text{c}}$ became negative at an earlier stage of biofilm development when higher proportions of respiration (20%) were used in the model and considering the observed increase in biomass until day 15, this scenario is unlikely. However, it is likely that the proportion of respiration decreased as
the growth rate decreased (Collos 1997), indicating that $P_n$ could be higher. Further work on MPB respiration rates are required to help improve modelled $P_n$ estimations.

**Conclusions**

In this mesocosm experiment we demonstrated that optical methods, especially hyper-spectral reflectance, are useful tools to study MPB biofilm growth and development. The comparison of different methods to measure photosynthesis (in-situ versus in vitro and ETR vs. C-fixation) showed that the methods do not always give the same answers: in general in-situ ETR measurements provide slightly higher estimates of the rate of C-fixation than suspension measurements, which we attribute partly to optical artefacts, mainly the occurrence of deep layer fluorescence which causes an overestimate of the true $\Delta F/F_m'$, especially at high [chl $a$] and under high irradiance. At low biomass densities, background fluorescence can also cause the underestimation of both $F_v/F_m$ and $\Delta F/F_m'$. Spectral reconstruction of $a_{ph}$ using pigments determined with HPLC proved to be a very useful tool in estimating absolute rates of ETR, although uncertainties with respect to the magnitude of the package effect needs more study. As the biofilm reached a stationary phase, the production estimates obtained with suspensions using the different methods deviated, possibly indicating that either or both the electron yield ($\Phi_e$) and the ratio of PSI:PSII functional cross section’s ($\rho$) may have changed. Despite these uncertainties, the rates of photosynthesis obtained using the methods employed were rather similar during the main growth phase of the biofilm. Net primary production estimated from $^{14}$C assimilation measurements appeared to be a satisfactory proxy for daily biomass production and suggested that C:chl $a$ ratios of the MPB community declined during the cultivation period. Further work on MPB respiration rates will help to refine net primary production estimates.