

## Developments in microphytobenthos primary productivity studies

### Abstract

Primary production by microphytobenthos can be a significant fraction of the total estuarine primary production. The productivity can be measured using a variety of techniques, which are reviewed in this contribution. Photosynthesis of intertidal benthic microalgae is increasingly measured using the pulse amplitude modulated (PAM) fluorescence technique and we will discuss this technique in detail, putting emphasis on a number of inherent assumptions. PAM fluorescence is rapid, non-intrusive and can be used in situ. These measurements assume that the chlorophyll fluorescence is proportional to the inherent photosynthetic properties of the algae. We will demonstrate that algal fluorescence from below the surface ('deep layer' fluorescence) can contribute significantly to the fluorescence measured at the sediment surface. This will cause overestimates of the true apparent quantum efficiency and thus of the estimated rate of photosynthesis. This error, which is influenced by the pattern in depth distribution in algal biomass, is significant at higher irradiances and in most cases ranges between 20 to 40%. Most estuarine sediments show that microphytobenthos biomass decreases exponentially with depth during low tide. For these sediments calculated total production and modeled production showed a linear relationship, demonstrating that under these circumstances PAM fluorescence techniques can be used.

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### Introduction

Primary production by pelagic phytoplankton and by benthic algae make estuaries belong to the most productive ecosystems of the world. Estimates for microphytobenthos primary production range from 27 to 234 gC m<sup>-2</sup> year<sup>-1</sup> (Heip *et al.* 1995; Macintyre *et al.* 1996; Underwood and Kromkamp 1999) while those for phytoplankton range from 7 to 875 gC m<sup>-2</sup> year<sup>-1</sup> (Boynton *et al.* 1982; Underwood and Kromkamp 1999). This primary production drives a rich foodweb, making estuaries ecologically important areas and of crucial importance for shorebirds and fish(eries). However, microphytobenthos are not only important as primary foodsource for higher trophic levels; they also play a very important role in sediment dynamics as they are important stabilizers of estuarine sediments through the excretion of extracellular

polymeric substances (Blanchard *et al.* 2000; De Brouwer *et al.* 2000; Paterson 1989; Paterson and Black 1999).

Because estuaries are not steady state systems they change slowly over time. To follow these changes with respect to primary productivity good quantitative approaches are needed and this is not an easy task with respect to microphytobenthos biomass and production because of the complex interaction between physical, chemical and biological processes. As estuaries are often manipulated by man, discriminating the effects of 'normal' change and/or variability from year to year and anthropogenic induced changes is even more difficult, requiring good methods and avoiding the ever present danger of undersampling.

Microphytobenthos (MPB) primary productivity is a function of the biomass present, the photosynthetic characteristics, and the sediment characteristics (nutrient availability and sediment optics). However, total primary production is not only dependent on the productivity, but also in the case of intertidal MPB on the exposure time, water transparency (in case of submerged photosynthesis) and possible vertical migration (Paterson 1986; Pinckney and Zingmark 1993). These latter components will not be treated in this overview, but the reader is referred to the chapter from Forster & Kromkamp (this book).

### *Measurements of MPB biomass*

Although this overview will deal with MPB productivity measurements, some attention has to be paid to the different methods to determine MPB biomass. Routinely, samples are taken by using 'syringe like' corers of different type, and the sampling depth varies generally between 2 and 10 mm (see MacIntyre *et al.* 1997). In the more cohesive sediments most of the MPB biomass of intertidal mudflats is concentrated near the surface (De Brouwer and Stal 2001b; Kelly *et al.* 2001), and very little MPB is present below 2 mm depth (see Figure 1). In this case, the sampling depth is not so important as long as the chlorophyll concentration is expressed per unit surface. If it is expressed per weight, then adding more depth will 'dilute out' the chlorophyll content, making comparison difficult. In sandy sediments the chlorophyll is normally more evenly distributed with depth. In this case the chlorophyll content per unit weight is relatively independent of the sampling depth, but the chlorophyll concentration per surface area will vary with sampling depth. As significant compaction can occur during low tide as the water evaporates, this can add to the error in the estimate of the chlorophyll content of estuarine sediments (Perkins *et al.* 2003).

Estimating MPB biomass is also difficult because of the patchy nature of the algae. We investigated this briefly on several sites in the Eden estuary in Scotland, and found that the standard error ranged from 6-22% on samples (20 cm<sup>2</sup>) spaced 20 cm apart, from 6-53% on samples spaced 100 cm apart and from 6 to 145% on samples spaced 500 cm apart. More information on spatial distribution of MPB can be found in Seuront *et al.* (this book) and References therein.

Another factor complicating MPB biomass estimation is the occurrence of vertical migration (Kromkamp *et al.* 1998; Paterson 1986; Paterson *et al.* 1998; Serôdio *et al.* 1997). Pinckney and Zingmark (1991) developed a model, based on sun angle

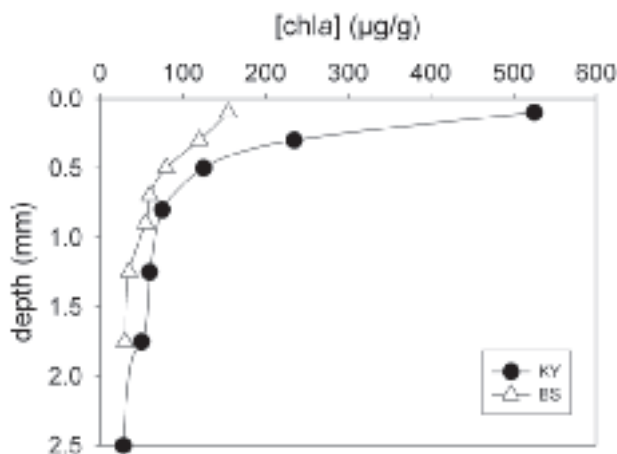


Figure 1. Two examples of vertical distribution of chlorophyll *a*. Profile KY is taken from (Kelly *et al.* 2001) and profile BS is taken from De Brouwer and Stal (2001).

and tidal stage, to describe vertical migration patterns. This model accurately predicted vertical migration of epipelagic diatoms on an intertidal mudflat in the Westerschelde estuary (Biezelingsche Ham, SW Netherlands, R.M. Forster pers. comm.). A preliminary conclusion from this model is that most of the migration takes place within 30-60 minutes after the tide leaves the mudflat, and that the algae start to migrate down 30-60 min before the tide returns. This leaves a sampling window open, centered around low tide, in which the vertical depth profiles are probably rather stable. Nevertheless caution is necessary, as the migration pattern, which seems to be driven by internal clock(s) as it persists for several days in darkness without tidal influence (Serôdio *et al.* 1997, own observation), can be modulated by environmental conditions. Perkins *et al.* (2002) observed that high light may drive MPB downwards, in contrast to the situation observed by Kromkamp *et al.* (1998) who found evidence that in high light the biomass stayed at the surface, but that a ‘micro-migration’ prevented MPB from being exposed to deleterious conditions too long.

## Measuring photosynthesis

Photosynthesis starts with the absorption of light by the light harvesting antenna complexes, which are phycobilosomes in cyanobacteria or red algae, and complexes intrinsic in the thylakoid membrane containing chl *a/b*- or chl *a/c*-binding proteins in eukaryotic algae. Absorbed light energy is passed on via several different light harvesting protein complexes to the reaction centre (RC) where it is used for charge separation. The charge separation occurring in the reaction centres of photosystem II (PSII), leading to the formation of oxygen by the splitting of water, can be measured using variable fluorescence techniques like the pulse amplitude modulated (PAM)

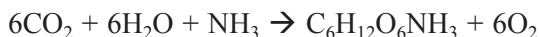
techniques (Schreiber *et al.* 1986), the pump and probe technique (Falkowski *et al.* 1986; Kolber and Falkowski 1993) and its successor the fast repetition rate fluorometer (FRRF, Kolber *et al.* 1998). These variable fluorescence techniques thus measure the initial steps in photosynthesis and can be used to estimate photosynthetic electron transport (ETR). Because PSII charge separation leads to the formation of O<sub>2</sub> it might be expected that rates of photosynthetic electron transport and rates of oxygen evolution are closely coupled (Kolber and Falkowski 1993). This is not necessarily the case though. Normal methods (Winkler titration, oxygen electrodes) measure the net oxygen exchange (NPP), i.e. the result of oxygen production (i.e. gross photosynthesis, GPP), minus the rate of oxygen consumption (R). In the dark, most of the oxygen consumption will be caused by respiratory electron transport (mitochondrial respiration), but in the light there may be other oxygen consuming processes like photorespiration (caused by the oxygenase activity of RUBISCO) and the Mehler reaction (i.e. O<sub>2</sub> uptake by PSI). In the latter case the oxygen produced from water by PSII is later consumed by PSI, producing water, (via O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub>) and this complex series of reactions is therefore also called the water-water cycle (see Asada 2000 for a recent review). Thus, although the Mehler reaction gives rise to ETR, there is no net O<sub>2</sub>-production. The Mehler reaction is likely to play a role in photoprotection: it keeps the redox state of the PQ-pool lower and increases the ΔpH, which might initiate the xanthophyll cycle (Neubauer and Yamamoto 1994; Schreiber and Neubauer 1990) which serves to protect PSII by over excitation because it dissipates absorbed irradiance as heat (for a recent review see Horton *et al.* 2000).

Photosynthesis can also be measured by the incorporation of CO<sub>2</sub>. In terrestrial situations CO<sub>2</sub> uptake is often measured directly by measuring changes in CO<sub>2</sub> concentration with an infrared gas analyzer (IRGA). In aquatic systems CO<sub>2</sub> fixation is measured indirectly by measuring the uptake of NaH<sup>14</sup>CO<sub>3</sub> into organic material, either in particulate form (i.e. the sample is filtered and the material on the filter is counted after acidification to remove any remaining NaH<sup>14</sup>CO<sub>3</sub>), or both the particulate and possible excreted DOC are measured after acidifying the solution.

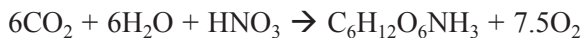
Photosynthesis can be described by the following, well known equation:



This suggests that both methods are interchangeable. This is not true, because it depends also on the nitrogen component used for growth (Williams 1993a): for growth on ammonia, and assuming a C: N-ratio of 6:



whereas for growth on nitrate the situation is different:



Thus, if the rate of C-fixation is to be calculated the photosynthetic quotient (PQ=CO<sub>2</sub>/O<sub>2</sub>) has to be known. For growth on ammonia a PQ of 1.1 is often observed, whereas

for growth on nitrate a PQ of 1.4 seems the best value. However, a larger range in PQ values has been observed in field observations, pointing to possible technical errors or to different regulation patterns in the N-metabolism leading to different C:N-ratios (Williams and Robertson 1991; Williams 1993b).

Thus, to measure photosynthesis and primary productivity in MPB, three principle techniques exist: Oxygen evolution measurements, C-fixation measurements, and measurements of variable fluorescence. For every principle method, a number of different approaches exist which we will very briefly review here. For all cases it can be said that comparisons are difficult, even if the same techniques are used, because different researchers use different incubation times

### *Oxygen-based techniques*

1. One of the older techniques is the bell-jar. With this technique a benthic chamber is pushed in the sediment, and the rate of change in oxygen in the overlying water over a specified period is measured (Lindeboom and De Bree 1982; Pomeroy 1959). A big advantage of this technique, assuming a larger size bell jar, is that it is less sensitive to small scale variation in patchiness. Another advantage of this technique is that it leaves the vertical chemical and physical gradients intact. This is important as these physical and chemical gradients might influence the rate of photosynthesis. Because the bell-jar technique measures net oxygen exchange, it measures net community production, which is not equal to net photosynthesis. Adding the respiration, measured in the dark, gives an estimate of gross primary production, but this assumes that the total rate of oxygen consumption is not influenced by light, an assumption which does not hold. Another disadvantage of this technique is that the measured rates are underestimates of the true rate of net oxygen production, because only the upward oxygen flux is measured, whereas part of the oxygen produced is consumed at greater depths.

2. Oxygen microelectrode techniques. These electrodes measure gross photosynthesis. The light-dark shift technique (Revsbech and Jørgensen 1983) measures the initial rate of oxygen consumption after darkening a sample, which equals the gross rate of photosynthesis. This can be done at several depths, so that a complete photosynthesis-depth profile can be measured. This technique is widely used (Glud *et al.* 1992; Kromkamp *et al.* 1995; Kühl *et al.* 1994; Yallop *et al.* 1994). The advantage of the light-dark shift technique is that it measures gross photosynthesis without destroying the chemical and physical gradients in the sediment. The technique can even be used *in situ*. When production profiles at different irradiances have to be measured it takes quite some time to finish the measurements because of the time needed for the oxygen gradients to reach steady state. As a result, the rates might be considerably influenced by vertical migration, if occurring during the measurements. Another disadvantage is that the method is very sensitive to patchiness, making it less suitable as a method to determine primary production at larger space scales, but very suitable to measure the occurrence of it.

Production rates can also be calculated from modeled oxygen depth profiles at fixed irradiances (Epping and Jørgensen 1996; Wiltshire *et al.* 1996). This method is

quicker than the light-dark shift technique and is therefore sometimes chosen when vertical migration might play a role, but it still takes several hours to complete a number of profiles at different irradiances. An additional disadvantages of the oxygen profile method are that several parameters have to be known or assumed (porosity, diffusivity and photic depth). An advantage of this method relative to the light-dark shift method is that a more robust electrode can be used because fast reaction times are not needed.

3. Optodes. Two different optodes techniques are described in the literature. Fiber-optic microprobes (Glud *et al.* 1999a; Holst *et al.* 1997) and planar optodes (Glud *et al.* 1999b; Glud *et al.* 1998; Glud *et al.*, this book). Optodes detect oxygen with a special chromophore, which then emits light, which is detected by a sensor. An advantage of the optodes is that they do not consume oxygen, which Clark-type oxygen electrodes do. Microfiber optodes are commercially available and can be operated analogous to oxygen microprofiles. This technique has the same advantages and disadvantages as estimating primary production by modelling of vertical oxygen profiles. Because the optode is based on a glass microfiber, they are flexible and will break less easily than the glass based Clark-type oxygen electrodes. In coarse sands they cannot be used, because the chromophore comes off too easily (personal experience).

The planar optode is a very recent development and is not commercially available yet. The big advantage of a planar optode is that it quantifies the oxygen field across an area of 10-100 cm<sup>2</sup>. For this reason the planar optode is ideal for scaling up productivity estimates because estimates are not sensitive to micro-small scale patchiness. For more information on optodes, especially planar optodes see Glud *et al.*, this book.

#### *<sup>14</sup>C based techniques*

1. Uptake of radioactive labeled bicarbonate can also be measured using the bell-jar technique, where the overlying water is labeled and the rate of uptake is measured (Colijn and De Jonge 1984; Rasmussen *et al.* 1983). A disadvantage of the technique is that is difficult to quantify the rates, because the interstitial DIC in the thin MPB layer is not known, leading to underestimates of the true rate (Heip *et al.* 1995; Smith and Underwood 1998). This technique seems hardly used nowadays.

When <sup>14</sup>C-labeled bicarbonate is added to the overlying waters in a bell-jar, the algae in the sediment are exposed to varying concentrations of the tracer. To overcome this problem Jönsson (1991) developed the percolation technique, allowing the <sup>14</sup>C-labelled bicarbonate to percolate through the sediment. He observed that the percolation tube gave 1-16x higher rates than obtained using a bell jar-technique, but the rates were still 3-8 times lower than observed when using slurries (see below). Percolation on porous, more sandy substrates is possible, but the technique does not work with very cohesive sediments (Sundback *et al.* 1996; Underwood and Kromkamp 1999). Because of difficulties with the specific activity, this method is not reliable for obtaining good estimates of photosynthetic activities.

2. Slurry technique. For this technique, the upper slices of sediment cores are used, often pooled, and diluted with filtered seawater. The pooled samples are then



incubated in a photosynthetron with a known concentration of  $^{14}\text{C}$ -labeled bicarbonate after which the samples are counted. This technique, employed by many authors (Barranguet and Kromkamp 2000b; Blanchard and Cariou-Le Gall 1994; Blanchard and Montagna 1992; Macintyre and Cullen 1998), is a good way to obtain reliable photosynthetic parameters. In principle the rate of oxygen evolution can also be measured with slurries (Wolfstein *et al.* 2000), but because the oxygen electrode is not so sensitive thick slurries are needed, causing a steep light gradient in the cuvet, thus giving rise to a poorly defined light climate. The drawback of the slurry technique is that it measures potential production, because the existing gradients in the sediments are destroyed. However, if no nutrients are limiting, especially  $\text{CO}_2/\text{HCO}_3^-$ , the measured potential photosynthetic rates might reflect real rates (Barranguet *et al.* 1998).

### *Variable fluorescence techniques*

Recently, photosynthetic activity of microphytobenthos is also measured with variable fluorescence techniques, either *in situ* (Barranguet and Kromkamp 2000a; Kromkamp *et al.* 1998; Perkins *et al.* 2002; Serodio 2003), or in slurries (Wolfstein *et al.* 2000). An exciting new development is the measurement of photosynthesis using variable fluorescence on single cells (Gorbunov *et al.* 1999), either using fluorescence microscopy (Snel and Dassen 2000) or in combination with imaging techniques (Baker *et al.* 2001; Oxborough *et al.* 2000; Perkins *et al.* 2002).

In the dark, when the primary electron acceptor of PSII,  $\text{Q}_\text{A}$ , is in its oxidized state, it is an efficient quencher of fluorescence. As a result, the measured minimal fluorescence ( $F_0$ ) is a good proxy for the chlorophyll concentration. In the light, part of the  $\text{Q}_\text{A}$  becomes reduced, causing a rise in fluorescence. When a single turnover flash (STF) is given (i.e. a flash of less than 2-5  $\mu\text{s}$  duration giving a reduction of  $\text{Q}_\text{A}$  only, thus without electron transport from  $\text{Q}_\text{A}$  to  $\text{Q}_\text{B}$ ) the fluorescence rises rapidly to a fluorescence level called  $I_1$  (Neubauer and Schreiber 1987; Schreiber *et al.* 1995) and which equals the level reached by the pump and probe and Fast Repetition Rate Fluorometer (Kolber and Falkowski 1993; Kolber *et al.* 1998; Kromkamp and Forster 2003; Samson *et al.* 1999; Schreiber *et al.* 1995). This rapid rise in fluorescence, called the photochemical phase, can be followed by a slower, thermal phase when a saturating multiple turnover flash (MTF) is used. Unlike the rapid photochemical phase, this slower phase in the fluorescence induction curve disappears partially at temperatures below 0 °C and is completely abolished at temperatures below -35 °C (see Neubauer and Schreiber 1987). Although the origin of the thermal phase is still uncertain (see reviews by Lazar 1999, and Samson *et al.* 1999) it suffices to say here that the  $F_\text{M}$  level reached by a STF ( $=F_{\text{M-ST}}$ ) is lower than the  $F_\text{M}$  level reached by a MTF ( $=F_{\text{M-MT}}$ ), and that the  $F_\text{M}$  reached with a MT-flash equals the fluorescence yield after application of the herbicide DCMU. The ratio of the variable fluorescence  $F_\text{V}$  ( $=F_\text{M}-F_0$ ) to  $F_\text{M}$  (i.e.  $F_\text{V}/F_\text{M}$ ) equals the maximum quantum efficiency of PSII charge separation and this is a valuable parameter as it reveals whether the algae are experiencing stressful conditions, as damage to PSII (Melis 1999; Ohad *et al.* 1994; Park *et al.* 1996; Parkhill *et al.* 2001; Young and Beardall 2003), which might be caused

by excess light or UV-radiation, as well as unbalanced growth conditions due to nutrient limitation (Green *et al.* 1994; Kolber *et al.* 1988; Kromkamp and Peene 1999; Lippemeier *et al.* 2001). All of these conditions will lead to a reduction in  $F_v/F_m$ .

The reaction centre of PSII is very susceptible to photoinhibitory damage, especially when  $Q_A$  is in the reduced (closed) state. It is therefore not surprising that several protection mechanisms exist. One of the prominent mechanisms is the activation of the xanthophyll cycle. At high rates of PSII electron transport, the lumen of the thylakoid membranes will acidify, causing a build up in the  $\Delta pH$ . This will in turn activate an enzyme called a de-epoxidase. In higher plants and green algae the de-epoxidation will convert in a two step way the carotenoid violaxanthin (V) via antheraxanthin (A) to zeaxanthin (Z). The conversion of  $V \rightarrow A \rightarrow Z$  occurs within a few minutes, causing a conformational state in the light harvesting protein complexes of PSII (LHC II) (Bassi and Caffarri 2000; Horton *et al.* 2000). Under low light, the reverse reaction takes place, but the rate of epoxidation is slower (10-20 min) compared to the de-epoxidation reaction. In diatoms and haptophytes a different xanthophyll cycle occurs: the xanthophyll diadinoxanthin (Dd) is de-epoxidized to diatoxanthin (Dt) in a one step process (Figure 2) (Young and Frank 1996). Like the conversion from  $V \rightarrow A \rightarrow Z$  the epoxidation from Dd to Dt is stimulated by an increase in the  $\Delta pH$  and occurs within a couple of minutes. Activation of the xanthophyll cycle induces loss of absorbed light as heat. This heat dissipation causes a decrease in the functional absorption cross section of PSII ( $\sigma_{PSII}$ ) and an increase in the quenching of fluorescence. This energy quenching ( $q_E$ ) is one of the components of non-photochemical quenching ( $q_N$ ), together with fluorescence quenching caused by a transition of the high fluorescent state 1 to the low fluorescent state 2 transition ( $q_T$ ) and quenching caused by photoinhibitory damage ( $q_I$ ). State transitions can be observed

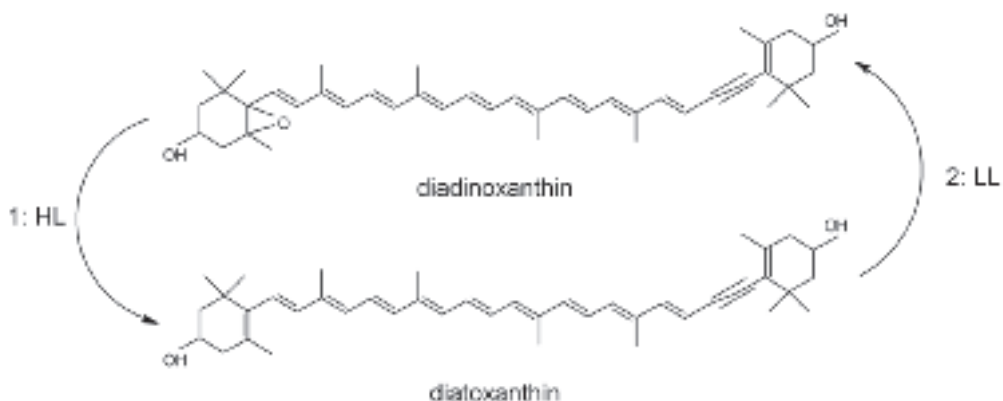


Figure 2. The xanthophylls cycle in diatoms and other chromophyte algae. In high light (HL) diadinoxanthin (DD) is de-epoxidized to diatoxanthin (DT), whereas in low light (LL) the reverse reaction takes place. In diatoms both  $\Delta pH$  and diatoxanthin are necessary to induce NPQ (Lavaud *et al.* 2002).



in green algae and are especially important in cyanobacteria, whereby the phycobilisomes can move from PSII (state 1) to PSI (state 2) as the redox state of the PQ becomes more reduced in high light (see Allan and Pfannschmidt 2000 for a recent review on state transitions and Campbell *et al.* 1998 for a review on chlorophyll fluorescence and photosynthesis in cyanobacteria). Non-photochemical fluorescence quenching can be calculated as follows:

$$q_N = 1 - \frac{F'_M - F}{F'_M - F'_0} \quad (1a)$$

or, in the absence of  $F'_0$  measurements as

$$NPQ = \frac{F_M - F'_M}{F'_M} \quad (1b)$$

Photochemical fluorescence quenching as can be calculated as:

$$q_P = \frac{F'_M - F}{F'_M - F'_0} \quad (2).$$

The photochemical quenching coefficient  $q_P$  is a proxy for the fraction of open reaction centres (Kolber and Falkowski 1993) although in the presence of excitation energy transfer between PSII units (connectivity) the relationship between  $q_P$  and the number of open RCII is not straightforward (Dau 1994; Lazar *et al.* 2001). Genty *et al.* (1989) demonstrated, using MT-flashes, that the effective quantum efficiency of linear PSII electron transport (ETR) equals the photochemical quenching coefficient and the efficiency of open PSII centres ( $F'_V/F'_M$ ):

$$\Delta F/F'_M = q_P \times F'_V/F'_M \quad (3)$$

Under optimal conditions  $\Delta F/F'_M$  often shows a linear relationship with the quantum efficiency of C-fixation (Genty *et al.* 1989). The rate of PSII electron transport can thus be calculated as:

$$ETR = E \times a^*_{PSII} \times \Delta F/F'_M \times 0.25 \quad (4)$$

$E$  is the irradiance (PAR) and  $a^*_{PSII}$  is the optical absorption cross section ( $\text{\AA}^2$ ) of a PSII. The product of  $E$  and  $a^*_{PSII}$  is the quantity of light absorbed by a PSII. These equations assumes that (minimally) 4 absorbed photons are needed to produce an electron. If the quantum requirement does not change, the rate of C-fixation can be calculated using a MTF-protocol (i.e. using a PAM-like instrument) as follows:

$$P^B \text{ (mg C (mg chl)}^{-1} \text{ h}^{-1}) = E \times a^* \times 0.5 \times \Delta F/F'_M \times 0.25 \times PQ^{-1} \quad (5a)$$

Here is  $a^*$  the optical absorption cross section per mg chl  $a$  ( $\text{m}^2 \text{ mg chl}^{-1}$ ). It is further assumed that 50% of the absorbed light is utilized by PSII. The photosynthetic quotient (PQ) is used so that the rate of oxygen production can be converted into a rate of C-fixation. Because the ratio of PSII to PSI can be variable, and the minimum

quantum requirement of both PSI and PSII can be greater than the 4 absorbed photons per photosystem, it is perhaps better to replace rewrite eq. 5a as follows:

$$P^B = E \times a^* \times \Delta F/F_M' \times \Phi_e \times PQ^{-1} \quad (5b)$$

Here  $\Phi_e$  is the electron requirement for oxygen production ( $O_2/e^-$ ). The minimal theoretical value according to the Z-scheme of photosynthesis is  $0.125 O_2/e^-$  (which is equal to  $0.5 \times 0.25$ , see eq. 5a), although it must be stressed that the electron produced by PSII is used again during charge separation in PSI: so  $\Phi_e$  is actually the reciprocal of the minimum quantum requirement of both PSI and PSII (i.e. the total number of charge separations needed by both PSI and PSII), and is closer to 0.1 than to 0.125 according to (Mauzerall 1986). The quantum requirement ( $\Phi_e^{-1}$ ) has been shown to vary often between 10 and 14 for a large variety of higher plants (Seaton and Walker 1990), and our own observations show that by using a value of 11-13 for charge separations of both PSI and PSII an accurate prediction of the rate of oxygen evolution can be predicted. An example is given for a benthic diatom in Figure 3.

For the pump and probe and FRRF fluorometers, which use a STF, a slightly different equation to predict the rate of C-fixation was developed (Kolber and Falkowski 1993):

$$P^B = E \times \sigma_{PSII} \times q_P \times n_{PSII} \times \Phi_e \times PQ^{-1} \quad (6).$$

Here,  $\sigma_{PSII}$  ( $\text{\AA}^2$ ) the functional absorption cross section (that fraction of the optical absorption cross section which is used to drive photochemistry) and  $n_{PSII}$  the number of the PSII units per mg chlorophyll *a* are required. For more information about the differences in the two methods see Kromkamp and Forster (2003).

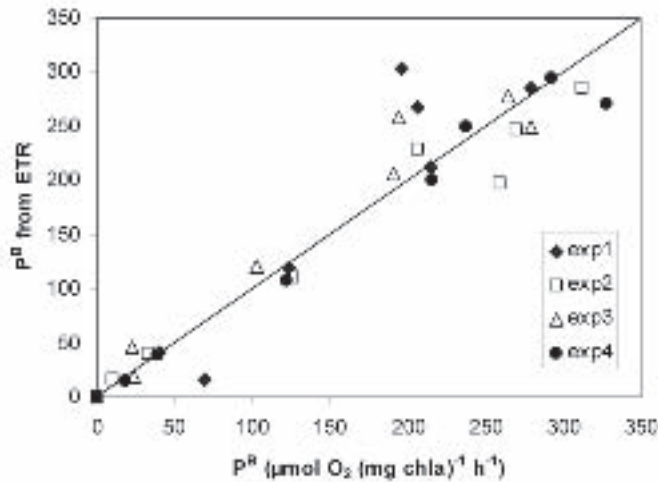


Figure 3. Rate of gross oxygen evolution measured using an oxygen electrode plotted against the gross rate of oxygen evolution calculated from ETR measurements for the diatom *Cyndrotheca closterium*.

## Estimating photosynthesis and primary production from PAM measurements

As described above the standard techniques using either oxygen evolution or C-fixation all have their pros and cons. Because of its non intrusive nature and the relative ease with which a portable PAM<sup>1</sup> fluorometer can be used to measure photosynthetic activity this fluorometric technique has found its way in *in situ* microphytobenthos studies. The minimal fluorescence signal has been used to derive chlorophyll content (Honeywill *et al.* 2002; Serôdio *et al.* 2001), to follow vertical migration (Kromkamp *et al.* 1998; Serôdio *et al.* 1997) and variable fluorescence has been used to estimate photosynthesis (Barranguet and Kromkamp 2000a; Kromkamp *et al.* 1998; Perkins *et al.* 2002). The results of these studies are mixed: Barranguet and Kromkamp (2000a) found in general a good relationship between areal production measured with PAM fluorescence and C-fixation (measured with the slurry technique, Figure 4), despite the fact that in a number of cases PAM measurements could overestimate C-fixation at high irradiances. Serodio (2003) also showed that total primary production ( $\Sigma PP$ ), estimated from oxygen microelectrode measurements could be adequately predicted by both biomass and photosynthetic activity:  $\Sigma PP = F_0/F_{0, \text{sed}} \times E \times \Delta F/F_M'$  ( $F_{0, \text{sed}}$  is the background fluorescence of sediments without chl). However, Perkins *et al.* (2002) found a poor relationship between ETR and C-fixation (measured with the percolation technique), which they attributed to downward migration of the epipellic

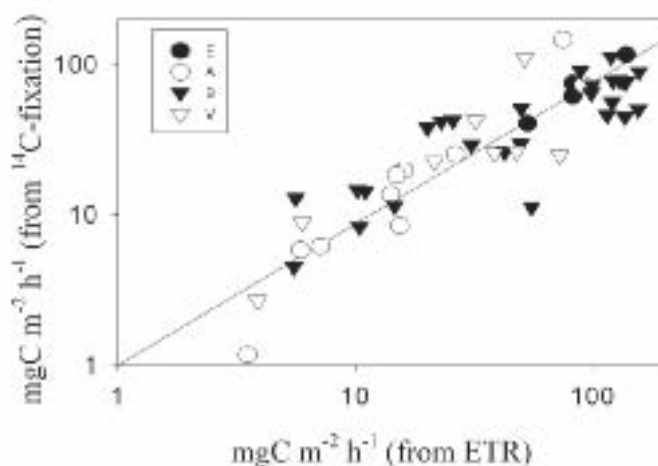


Figure 4. Relationship between C-fixation estimated using PAM surface measurements (ETR) and C-fixation measured using slurries in a photosynthetron. The symbols E,A,B are stations located on an intertidal flat in the Western Scheldt estuary (Molenplaat), whereas sampling location V was located on an intertidal flat in the central Eastern Scheldt. Both estuaries are located in the Netherlands. From Barranguet and Kromkamp (2000).

<sup>1</sup> There are several different manufacturers selling Pulse Amplitude Modulated (PAM) Fluorometers, and most of them use a MT-flash to measure  $F_M$ .

diatoms during the measurements of the PE-curve at irradiances exceeding 1200  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ .

It is important here to emphasize that there are a number of difficulties associated with working with a PAM fluorometer on benthic biofilms which might explain the varying results described in the literature.

- The signal measured by a PAM is depending on the sediment optics. The ‘measuring depth’ of the modulating light will depend on the (absorption by the) algal biomass, its vertical distribution and sediment characteristics such as grain size, water content, and organic matter content. For muddy sediments it is estimated that the measuring depth is approximately 150-200  $\mu\text{m}$ . Therefore, the total  $F_0$  signal measured depends not only on the algal biomass in the top layer of the sediment, but also by its vertical distribution. This is nicely demonstrated by Honeywill *et al.* (2002) who, using cryolandiers, showed that the regression coefficient of the relationship between  $F_0$  and [chl $a$ ] varied with the sampling depth.
- In high light, quenching of fluorescence can decrease the fluorescence yields, and this can falsely be mistaken for downward migration.
- For accurate measurements of ETR, the absorption in each layer of the sediment should be known. This requires accurate knowledge of the sediment light field, which is a function of the biomass density and depth distribution, algal absorption characteristics, sediment characteristics, and the incident irradiance and sun angle.
- It is assumed that the photosynthetic parameters describing the P/E-curve do not change with depth.
- If C-fixation of oxygen evolution is to be calculated from PAM measurements, the relationship *between*  $P^B$  and ETR should not be influenced by temperature.
- It is furthermore assumed that no significant depth integration of the fluorescence signal occurs, i.e. that  $\Delta F/F_M$  measured at the surface reflects the effective quantum efficiency from the cells at the surface and the signal is not influenced by variable fluorescence signals from below.

The latter two assumptions will be now investigated.

#### *Is the relationship between ETR and $P^B$ temperature dependent?*

This is an important question, as during sunny days the temperature on mudflats can easily rise by 10 °C during low tide. Harrison (1985) observed that temperatures can rise as quickly as 4 °C  $\text{h}^{-1}$ . At low light the rate of photosynthesis is limited by the rate of absorption of light, thus the light-limited region of the P/E-curve is hardly influenced by temperature (Davison 1991; Post *et al.* 1985), and light limited growth rates are therefore also hardly influenced by temperatures (Post *et al.* 1985). On the other hand, light saturated rates of photosynthesis are determined by enzymatic reactions and are therefore highly sensitive to changes in temperature. Generally  $P^B_{\text{max}}$ -values increase from low towards maximal values at an optimal temperature, after which it declines rapidly (Blanchard *et al.* 1996; Davison 1991). This temperature optimum can be quite wide, and be higher than the growth temperature (Morris and Kromkamp 2003).  $Q_{10}$  values for  $P^B_{\text{max}}$  can vary widely (Davison 1991) and be dependent on

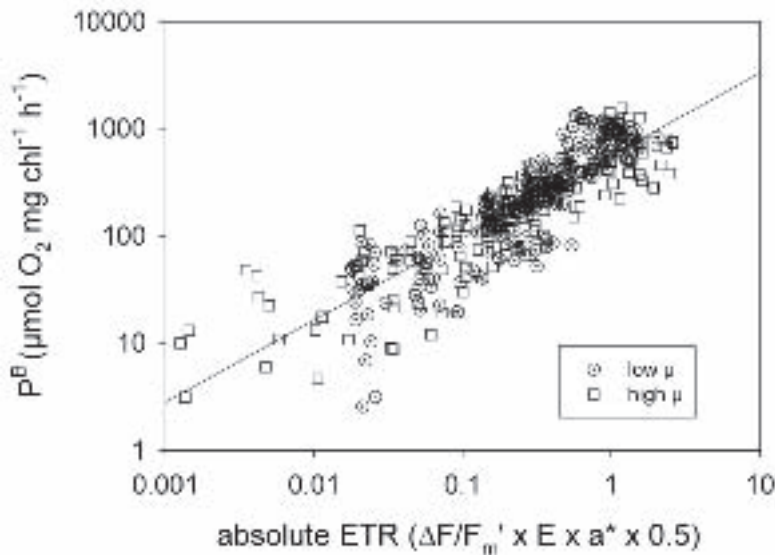


Figure 5. Relationship between ETR and oxygen evolution of light limited steady state grown *Cylindrotheca closterium* cells at a low and high irradiance. Photosynthesis was measured for each growth rate ( $\mu$ ) at temperature varying between 5 and 35 °C. From Morris and Kromkamp (2003)

growth conditions (Kromkamp *et al.* 1988). Morris and Kromkamp (2003) studied the short term acclimation in photosynthesis to different temperatures by simultaneously measuring the rate of oxygen evolution and ETR of the benthic diatom *Cylindrotheca closterium*, which was grown at two different light limited growth rates. They observed that the photosynthetic efficiency  $\alpha$  measured with both methods was hardly influenced by photosynthesis. The optimal quantum efficiency of PSII,  $F_v/F_m$ , showed a very small, but significant decrease at lower temperatures.  $P_{max}^B$  and  $ETR_{max}$  was very sensitive to changes in temperature, and the optimal temperature was 10 °C higher than the growth temperature of 20 °C. More interesting, the relationship between ETR and  $P^B$  was very robust (Figure 5) and only affected at the most extreme temperatures used and was not significantly affected by the growth rate. This indicates that temperature changes during a low tide will not influence microphytobenthos C-fixation estimates which are based on ETR measurements.

*Does fluorescence from subsurface influence the fluorescence measured at the surface of an intact microphytobenthos community?*

When performing fluorescence measurements on intact microphytobenthos communities it is implicitly assumed that upwelling fluorescence from algae below the surface does not distort the signal measured at the surface, and that the fluorescence values measured at the surface truly reflect the fluorescence yield under the ambient

environmental conditions. However, light is attenuated steeply within microphytobenthos communities, and as a result levels of  $F$  and  $F_M'$  may change rapidly with depth, and if part of this fluorescence will reach the sediment surface it will be part of the overall fluorescence measured. The effect of this was recently investigated by Forster and Kromkamp (2004) in a mathematical model with varying pigment depth profiles and a realistic simulation of the sediment light field. Four different situations were investigated: a) assuming an uniform distribution with depth; b), assuming an exponential decrease with depth, using 2 published profiles by Kelly *et al.* (2001) and De Brouwer and Stal (2001b) (resp. KY & BS in Figure 1); c), assuming a surface distribution only, with either a single surface monolayer (SSL) or a multilayer surface layer assemblage (MSL) and d), a multilayer subsurface assemblage (SUB). The first situation can be found in environments where the sediments are regularly stirred by the tides, normally the sandier sediments or in situation with high tide in the night. The profiles KY and BS are typical for epipellic diatoms in the more silty sediments during daytime low tide, where, according to low temperature scanning electron microscopy diatoms can also be present only at the surface in a single or multilayer organization (Paterson 1986). A subsurface situation is seldom encountered, but might arise when the epipellic pennate diatoms migrate away when the incoming tide approaches. Figure 6 shows what happens if fluorescence originating at increasing depth layers contributes via upwelling to the signal measured at the surface. In this case the vertical profile 'BS' was used as an example (see Figure 2). It can be seen that if the contributing depth increases, the measured values for  $F$ ,  $F_M'$  and  $\Delta F/F_M'$

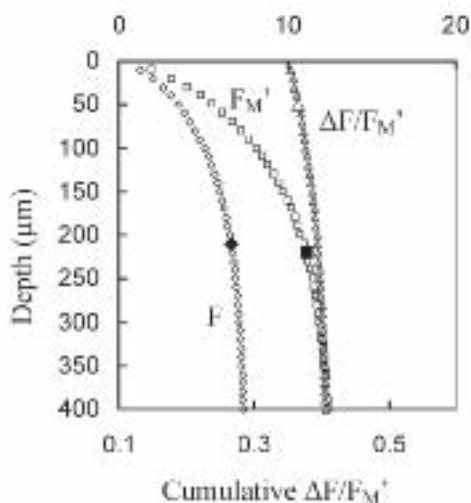


Figure 6. Cumulative  $\Delta F/F_M'$  measured at the surface with increasing depths contributing to the signals measured at the sediment surface. The filled symbols represent the depth where 90% of the final value at the surface is measured. Surface irradiance  $500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Data from Forster and Kromkamp (2004).



increase asymptotically: approximately 90 % of the maximum values is derived from the upper 210  $\mu\text{m}$ . Whereas the  $\Delta F/F_M'$  of the cells in the upper 10  $\mu\text{m}$  is 0.355, the cumulated  $\Delta F/F_M'$  measured at the surface reaches a value of 0.406: thus, in the light, especially in strong light, upwelling fluorescence from deeper layers cause an overestimation of the true  $\Delta F/F_M'$  of the cells at the surface. Similar results were obtained by Serôdio (2004). At an irradiance of 2000  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , this overestimation of the true  $\Delta F/F_M'$  can be as large as 46 %. In a dark adapted core contribution of fluorescence below also contributes to the  $F_0$  and  $F_M$  values measured at the surface, but the value of  $F_v/F_M$  does not change. Next these authors investigated the effect chlorophyll distribution pattern on changes in  $\Delta F/F_M'$  and rETR at different irradiances (Figure 7). As can be seen, the effect of the profile is quite limited: a uniform profile or a profile with monotonous increase in biomass towards the surface as shown by the BS, KY profiles hardly effect the shape of the  $\Delta F/F_M'$ -irradiance curves, and all overestimate the true ETR by approx. 40-50 % at the highest irradiance. The biggest overestimate in true  $\Delta F/F_M'$  occurs in a subsurface layer, which is caused by the fact that the true irradiance experienced by the algae below the surface is of course lower than the surface irradiance, which is used to calculate the electron transport rates. Interesting are the overestimates in the single and multilayered surface assemblages, both of which had a thickness of 50  $\mu\text{m}$ . It might be expected that a single cell surface layer might behave very similar to a leaf, but even here due to light attenuation within the upper 50  $\mu\text{m}$  caused a strong decrease in average irradiance, leading to overestimation of the true  $\Delta F/F_M'$ . The overestimation is stronger in the multilayered surface layer (MSL), where light absorption is stronger than in the SSL because the chlorophyll content is higher in the same depth stratum. It is thus clear that if the optical conditions in even a small layer are unknown, considerable errors (overestimates) might be made in the estimates of the inherent  $\Delta F/F_M'$ . This also suggest that effective PSII quantum efficiencies made on leaves and macroalgae might suffer from the same artefact, although the error is probably minimized by the focussing effect of the upper palisade parenchyma cells on the underlying mesophyll cells which contain most of the chlorophyll. A striking feature of the contribution of 'deep layer fluorescence' is that the ETR-irradiance (ETR-E) curves do show a varying degree of saturation at high irradiances: especially the conditions with a high surface biomass content like the MSL or the subsurface layer do not show a maximum rate of ETR at high irradiance.

The simulations shown in Figure 7 were carried out with a low degree of non-photochemical quenching. As diatoms can show a high degree of quenching this was also investigated, as quenching might decrease the signal of the upper layers, therefore making the contribution of deep layer fluorescence more pronounced, but the effects were only noticable at irradiance exceeding 1000  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  where the ETR-E curves showed an upward turn. Nevertheless, the effect was small and added only a 7% further increase in ETR at 2000  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  (Forster and Kromkamp 2004). The question now is: can we use a variable fluorescence to measure total primary production if the true ETR is overestimated by the contribution of deep layer fluorescence to the fluorescence measured at the surface. As most of the chlorophyll profiles in estuarine sediments show either a homogeneous or exponential

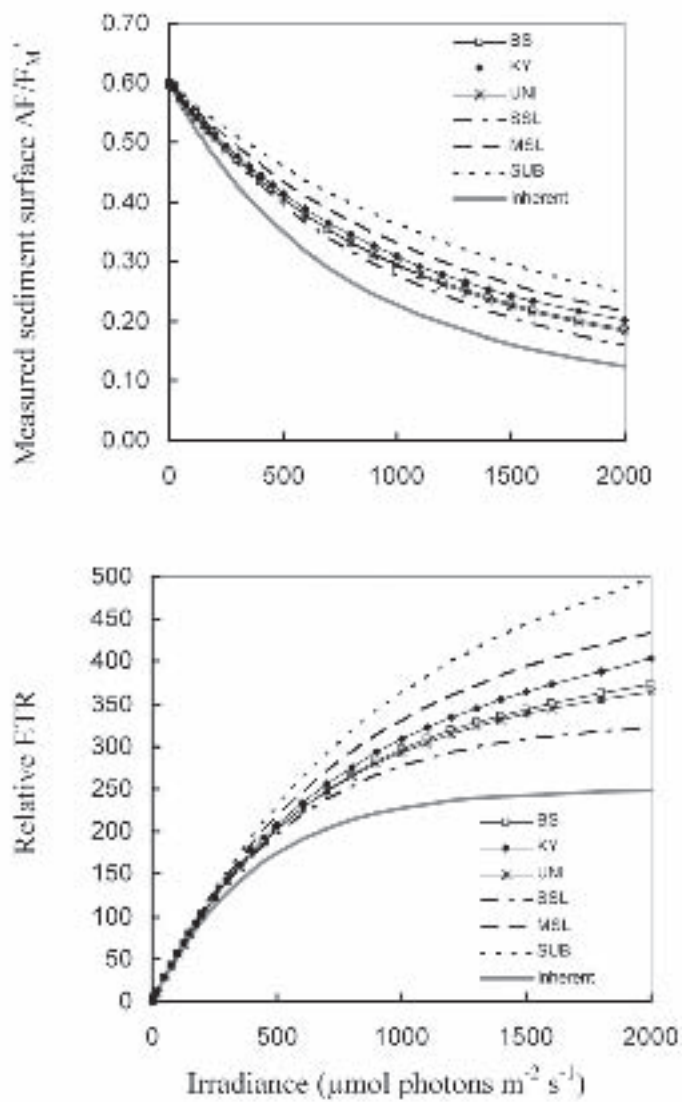


Figure 7. Change in  $\Delta F/F_M'$  (top panel) and rETR (bottom panel) with irradiance for different chlorophyll distributions with depth (see text). Data from Forster and Kromkamp (2004)

decrease with depth (De Brouwer and Stal 2001a; Kelly *et al.* 2001; Perkins *et al.* 2003, own observations), the model analysis by Forster and Kromkamp (2004) suggest that the effect can be quantified and is not very dependent on the shape of the exponential decrease in chlorophyll with depth. However, this is not the case with a surface assemblage, so knowledge of the local system is required to reduce potential errors caused by deep layer fluorescence. The results of the SSL and MSL also demonstrates that it is impossible to measure the light climate within an algal surface assemblage accurately because the sensor can actually be bigger than the layer involved. But suppose we know that we have homogenous distribution with depth or an exponential decrease with depth in biomass, is it then possible to derive an accurate prediction of total primary production? This was investigated Forster and Kromkamp (2004) by calculation of depth integrated primary production ( $\Sigma PP$ ) using the inherent photosynthetic properties and the sediment optical model against the depth integrated value of  $(\Delta F/F_M' \times E_z \times F_{0,z})$ . As can be seen in Figure 8, the nearly linear relationships between  $\Sigma PP$  and the composite term  $\Sigma(\Delta F/F_M' \times E_z \times F_{0,z})$  is very similar between the KY, BS and UNI profiles, corroborating earlier results by Serôdio (2003). However, the relationship between  $\Sigma PP$  and  $\Sigma(\Delta F/F_M' \times E_z \times F_{0,z})$  is not similar for all biomass depth distribution profiles, and as the results for the SUB profile indicate, are dependent on the attenuation coefficient of the sediment as well, again indicating that knowledge about the local biomass distributions is required for accurate depth integrated primary production estimates. However, with sufficient knowledge of the biomass distribution and sediment optics, surface PAM measurements can be used to estimate areal primary production.

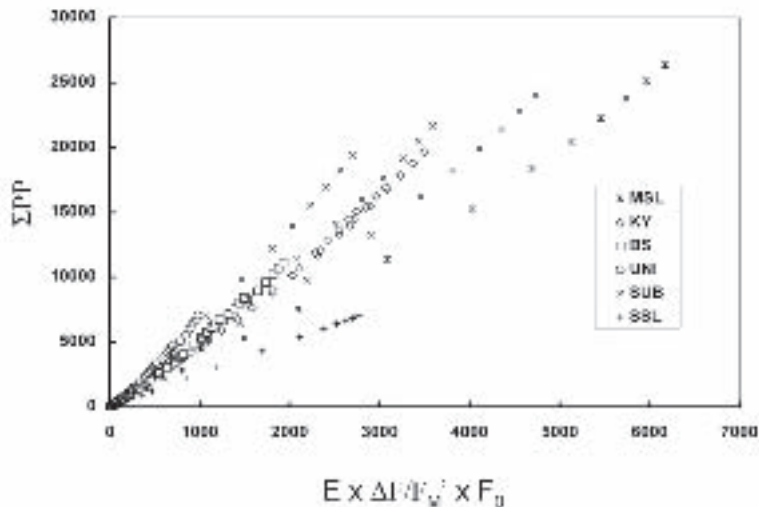


Figure 8. Comparison of calculated gross microphytobenthic primary production ( $\Sigma PP$ ) and estimated primary production using the composite parameter  $E \times \Delta F/F_M' \times F_0$  (Serôdio 2003). From Foster and Kromkamp (2004)

## Conclusions

Variable fluorescence measurements can be a useful way to estimate primary production of microphytobenthos, provided information about the depth distribution profiles and light attenuation coefficients is available. As in most cases MPB biomass in estuarine sediments will show an exponential decrease with depth during low tide, an a priori estimate of the overestimate of the inherent rates of electron transport can be assumed. It should be noted that for integration of radiocarbon measurements on suspensions the same knowledge is required.  $F_0$  measurements can be taken as a proxy for algal biomass, but the relationship between  $F_0$  and [chl] will depend very much on the biomass distribution in the sediment. Non-photochemical quenching will add to the uncertainty, as it will decrease the fluorescence yields, but not the [chl] concentration.

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