Photophysiological variability of microphytobenthic diatoms after growth in different types of culture conditions

RODNEY M. FORSTER1,2 and VÉRONIQUE MARTIN-JÉZÉQUEL2

1Netherlands Institute of Ecology (NIOO-KNAW), Centre for Estuarine and Marine Ecology, Korringaweg 7, 4401 NT Yerseke, The Netherlands
2EA 2663, ISOMER, Université de Nantes, 2 rue de la Houssinière, BP 92208, Nantes 44322, France

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Microphytobenthic diatoms have great ecological importance in estuarine and coastal marine ecosystems, yet many aspects of their physiology have not been investigated under controlled conditions. This work describes patterns in growth rates and photosynthesis in different types of culture for several species of benthic diatom. Cells were grown as epipellic biofilms both in batch culture vessels and in a continuous culture system, and differences in growth and photosynthetic physiology were measured using optical techniques. Consistent differences were observed in the maximum photochemical efficiency of PS II, $Fv/Fm$, between species of diatoms, with highest values recorded early in the development of a culture. The maximum biomass produced also varied greatly between species, with the densest biofilms being formed by Amphora coffeaeformis. Batch cultures of benthic diatoms showed only small changes in $Fv/Fm$ but large decreases in the maximum rate of photosynthetic electron transport during the first week of culture development because conditions within the biofilm became unfavourable. A flow-through culture chamber was developed in order to increase the supply of nutrients to the biofilm and prevent the build-up of oxygen. In this system, high growth rates and a high maximum rate of photosynthesis was observed for periods of up to 1 wk. The flow chamber system could therefore be used for producing suitable material for comparative photophysiological experiments.

INTRODUCTION

Microphytobenthic algae contribute substantially to the total primary productivity in estuarine (Underwood & Kromkamp 1999) and coastal regions (Sagan & Thouzeau 1998; Cahoon 1999), with the input of carbon from benthic algae representing a large fraction of the total system production for intertidal and shallow areas (Buzzelli et al. 1999). Pennate diatoms are the main components of benthic microbial biofilms on muddy sediments in many European estuaries (Admiraal 1984). These epipellic cells can be found loosely attached to the sediment surface, and are also capable of moving through the upper layers of the substratum. Exudation of polysaccharides by diatoms can create a gel-like matrix which is more resistant to erosive forces than uncolonised sediment surfaces (Stal & de Brouwer 2003). For short periods of the year, benthic microalgal concentration may exceed 400 mg chlorophyll (chl) m$^{-2}$, or 10$^7$ cells m$^{-2}$, whereas areal concentrations of phytoplankton during blooms rarely exceed 150 mg chl m$^{-2}$ (Underwood & Kromkamp 1999). A much larger difference between the two habitats is apparent when algal concentration is expressed in volumetric units. The chl concentration in the upper centimetre of a diatom-colonised sediment can be over 10,000 mg m$^{-3}$ wet sediment volume, whereas maximum volumetric chl concentrations in the marine plankton are approximately 100 mg m$^{-3}$ of water. Hence, competition for space and nutrients is potentially greater by orders of magnitude for the microphytobenthic habitat.

Due to the intense biogeochemical activity within the illuminated zone of sediments, environmental conditions within the surface layers of intertidal sediments are in many respects adverse for photosynthesis (Admiraal et al. 1982). Photosynthetic activity in the densely populated microbial films described above will cause a rapid drawdown of dissolved CO$_2$ or HCO$_3^-$, or both, with increases in pH to over 9.9 in pore-water (Rasmussen et al. 1983; de Jong & Admiraal 1984). In addition, the uppermost layer of intertidal sediment is exposed to air at low tide, and rapid fluctuations in salinity can occur due to evaporation or rainfall. Sediment surface temperatures can also change by 15°C or more in the course of a sunny day (Guarini et al. 1997), potentially causing thermoinhibition (Morris & Kromkamp 2003). Frequent mechanical disturbance of the sediment by infauna, together with solar and tidally-driven fluctuations in surface irradiance will rapidly redistribute cells between supersaturating surface irradiances and darkness at depths of > 1 mm. At low tide, the sediment surface is also exposed to potentially damaging ultraviolet A and B radiation (Wulff et al. 2000).

These factors will disrupt the ability of microphytobenthic cells to balance the fluctuating supply of light energy with the demands of cellular metabolism and growth, thus exposing the photosynthetic machinery to the risks of over-excitation and oxidative damage. It is therefore likely that benthic microalgal species possess several mechanical and physiological adaptations that enable survival in a highly variable environment. Indeed, several authors have speculated on an inherently greater resistance to irradiance stress of microphytobenthic algae compared to phytoplankton (Blanchard & Montagna 1992; Kromkamp et al. 1998). In support of this idea, Peletier et al. (1996) found that seven species of benthic diatoms dis-

* Corresponding author (r.forster@nioo.knaw.nl).
played much greater resistance to ultraviolet-B radiation than certain phytoplanktonic species. However, physiological performance of microalgae is strongly dependent upon the external conditions during growth (Cullen 1992), which can potentially confound comparisons between species or functional groups. Physiological changes during growth in culture can be expected to be largest for benthic microalgae because of the aforementioned extreme variations in microclimatic conditions which occur as a biofilm develops.

It was the aim of this work to firstly compare growth rates and photosynthetic physiological responses of selected diatom species with time in the type of batch culture conditions which have most frequently been used for microphytobenthic studies to date. A second aim was to design and utilise a system in which peniculate benthic diatoms could be grown as a biofilm under controlled conditions of nutrient supply, temperature and irradiance, with continuous monitoring of algal biomass using non-invasive optical methods. A system is described in which the photosynthetic physiology of the cultured algae remained constant for a period of several days, which would facilitate physiological comparisons with phytoplankton species grown in balanced-growth continuous cultures. Finally, changes in physiological parameters during growth in the different types of culture are compared.

**MATERIAL AND METHODS**

**Organisms, culture media and growth conditions**

Unialgal cultures were obtained by serial isolations of benthic diatoms from locally collected sediments, and maintained in a culture collection at NIOO-CEME (Table 1). Stock cultures were maintained in an enriched artificial seawater medium (YMM; Wolfstein & Stal 2002), before transfer to f/2 medium (Guillard & Ryther 1962) for use in experiments.

Growth rates and physiological parameters were measured in situ in different growth vessels. In an initial series of experiments, batch cultures were set up by inoculation of 1–5 ml algal suspension into fresh medium. Flat-sided polystyrene cell culture bottles were used, allowing observation of growing biofilms with an inverted microscope. Later, conical, glass 50 or 125 ml Erlenmeyer flasks half-filled with medium were used for batch cultures. Fluorescence measurements were made to quantify the algal biomass by placing the tip of a fibre-optic cable either against the wall of the cell-culture vessel or against the flat bottom of a glass flask. Cultures were grown at 20–22°C with quantum irradiances of 50–150 μmol photon m⁻² s⁻¹, and a photoperiod of 16 h. Gentle shaking once per day ensured that diatoms were distributed evenly on the bottom of the containers, and cultures were observed for periods of up to 60 d after inoculation. All of the tested species grew as epipelic biofilms on the bottom of the culture vessels; with the exception of *Phaeodactylum tricornutum*, relatively few cells were found suspended in the medium. Initial tests showed for all species that the addition of a thin layer of quartz sand to the vessel allowed the production of a higher biomass (one tail t test, \(P < 0.04\)), therefore sand was always added. The epipsammic species *Achnanthes longipes* was the only species which was found attached to sand grains. The number of replicate cultures was \(n = 13\) for *Amphora coffeaeformis*, \(n = 11\) for *A. hybrida*, \(n = 15\) for *Achnanthes longipes*, \(n = 7\) for *Navicula mutica*, \(n = 4\) for *P. panduriformis* and \(n = 2\) for the other species.

Floating plastic rafts with a permeable membrane base (‘LifeRaft’, Osmotek, Rehovot, Israel), originally designed for the culture of plant callous tissues, were used in order to grow loosely-attached epipelic biofilms of benthic diatoms. Biofilms were grown at first by floating the rafts on a volume of medium, with the diatom cells exposed to air from above. For comparison, biofilms on permeable membranes were also grown fully submerged in a flowing supply of medium to increase growth rates (Admiraal et al. 1982). A clear polycarbonate chamber was used to hold the raft in a position facilitating optical measurements via fluorescence or reflectance (see below). Oxygen concentration and pH in the chamber were measured online with electrodes, and the medium was stirred with a Teflon-coated magnetic bar. The volume of the chamber was 275 ml, and flow rates through the chamber were controlled with a peristaltic pump. Replicate biofilm chambers were set up in a constant temperature room at 22°C and were illuminated from above by three fluorescent tubes giving an irradiance of 80–110 μmol photon m⁻² s⁻¹.

**Measurements of growth rates and photosynthesis**

**OPTICAL DETERMINATION OF GROWTH RATES:** Optical methods were used to quantify changes of microalgal biomass in time without disturbance of the growing biofilm. Room temperature fluorescence emission provided a quantitative measure of chlorophyll a concentration, which in turn is a useful proxy measure of microphytobenthic biomass (Karsten et al. 1996; Serödio et al. 1997; Honeywill et al. 2002). A PAM-2000 portable fluorometer (Walz, Germany) with a red light-emitting diode (LED) measuring light was used to measure dark-
acclimated chlorophyll fluorescence, $F_o$, which is the fluorescence parameter with least variability (Serôdio et al. 2001). The distance between the sample and the tip of the fluorometer fibre-optic cable was constant, and the instrument settings of the PAM were set to the highest sensitivity. As in previous microphytobenthic work, dark-acclimated minimum fluorescence, $F_o$, was found to be a good predictor of acetone-extractable pigment concentrations ($r^2 = 0.90$ was obtained for a linear regression of $F_o$ vs chlorophyll a from a range of diatom and green algal batch cultures in flasks); henceforth $F_o$ values are referred to as ‘biomass’.

Wavelength-resolved measurements of the light reflected from a microbial biofilm can also be used to quantify the pigment concentration (Hakvoort et al. 1997; Paterson et al. 1998). A Macam SR-9910 spectroradiometer with a 9° field-of-view radiance detector was used to measure the reflected irradiance from diatom biofilms. Reflectance was calculated as $L_o/L_u$ where $L_o$ is the upwelling radiance from the uncolonised surface of a white membrane and $L_u$ is the radiance emerging from an algal biofilm. Several regions of the reflectance spectrum can be used to quantify microphytobenthic algal pigments and hence biomass; here we use the normalised difference vegetation index based on mean reflectances in the red (R, 670–680 nm) and near infrared (NIR, 740–750 nm) wavebands (Thomson et al. 1998):

$$\text{NDVI} = \frac{(\text{NIR} - R)}{(\text{NIR} + R)} \quad (1)$$

Growth rates for the exponential phase of batch cultures or biofilms were calculated as

$$\mu = \frac{\ln(B^t) - \ln(B^0)}{t^1 - t^0} \quad (2)$$

where $B^0$ and $B^t$ represent the microphytobenthic biomass, measured with the fluorescence or reflectance methods, at time $t = 0$ and 1, respectively. For fluorescence estimates of biomass ($F_o$), a dark acclimation period of 15 min was necessary to reduce the effects of previous light exposure. Measurements were made at the same time of day, and values from three positions for each cell culture vessel or flask were averaged. A small, constant fluorescence signal was recorded in the absence of algae from culture vessels, and this value was subtracted from all other measurements.

**PHOTOSYNTHETIC PARAMETERS:** The variable fluorescence technique was used to make in situ measurements of photosynthetic parameters. The maximum photochemical efficiency of photosystem II ($F_v/F_m$) was measured with the saturating pulse technique (Schreiber 1986):

$$\frac{F_v}{F_m} = \frac{(F_m - F_o)}{F_m} \quad (3)$$

where $F_v$ is the dark-acclimated minimum fluorescence yield and $F_m$ is the maximum fluorescence obtained with all reaction centres closed (using a multiple-turnover, saturating pulse of light of 4000–6000 µmol photon m$^{-2}$ s$^{-1}$ for 0.6 s). For detailed fluorescence induction curves, a red LED of irradiance 1000 µmol photon m$^{-2}$ s$^{-1}$ was activated to induce maximum fluorescence. Measurements were made at an initial sampling rate of 150 µs then smoothed with a five-point moving average. For all $F_v/F_m$ measurements a dark acclimation of 15 min was used.

Relative electron transport (ETR) rates were estimated from the actual efficiency of PS II measured in continuous irradiance (Genty et al. 1989):

$$\frac{\Delta F}{F_m} = \frac{(F_m' - F)}{F_m'} \quad (4)$$

where $F$ is the light-acclimated steady-state fluorescence and $F_m'$ is the fluorescence obtained with all reaction centres temporarily closed. Relative ETR is given by

$$rETR = \frac{\Delta F}{F_m'} \times E \quad (5)$$

where $E$ is the measured quantum irradiance (see below). Conversion from relative to actual rates of ETR requires a measurement of the photon flux absorbed by photosystem II. Whereas this parameter was not measured, other optical parameters such as the total chl-specific absorption, and pigment ratios as determined by high-performance liquid chromatography were done for the flow chamber cultures. These values showed low variability ($a^* = 0.019 \pm 0.006$ m$^2$ (mg chl a)$^{-1}$ for biofilms of *Amphora coffeaeformis*, hence relative and absolute ETR rates would show similar trends.

For measurements of photosynthesis and fluorescence quenching, the inbuilt light sources of the PAM-2000 were used to generate a ‘Rapid Light Curve’ (Schreiber et al. 1994; White & Critchley 1999). Following a 10 min dark acclimation, each programmed irradiance curve had 14 irradiance steps with a red LED used for the first 11 irradiance steps, and a ‘white’ halogen lamp for the last three steps. The length of each step was 2 min, and a short dark recovery period was followed for 6 min at the end of the irradiance series. A steep gradient in irradiance exists within the short distance from the end of the fibre-optic and the photosynthetic target, and the potential for error using Equation 5 is serious unless irradiance measurements are done with high precision (Rascher et al. 2000). Irradiance readings were made at exactly the same distance, usually 4 mm, and in the same plane as the algal biofilm. Measurements were made through a layer of glass or plastic to account for attenuation by the vessel where appropriate. A calibrated micro quantum sensor with a sensor area of 3 mm$^2$ (2060-M, Walz, Germany) was used to measure irradiance because larger sensors (e.g. Licor Li 190 with a sensor area of 50 mm$^2$), underestimated the irradiance by a factor of two when working at such a close distance from the end of the fibre-optic.

In addition, irradiance-response curves were measured on suspensions of algal material. Cells were removed carefully from the loosely-attached epipelic biofilms with a pipette, and suspended in fresh medium. Measurements of cell suspensions were made in a purpose-built temperature-controlled aluminium housing containing a spectrophotometer cuvette with pathlength of 1 cm. A magnetic stirrer maintained an even distribution of cells in the cuvette. Fluorescence measurements were made via the front window of the cuvette, and irradiance was measured with the micro-sensor at the front and back of the suspension to account for self-shading. Mean irradiance within the suspension was calculated as:
Fig. 1. Growth curves of four species of benthic diatom in batch culture measured via fluorescence. The natural logarithm of dark-acclimated fluorescence ($F_o$), measured through the bottom of the culture vessel, is plotted vs the number of days after inoculation.

$$E_m = \frac{E_o - E_b}{\ln \left( \frac{F_o}{E_b} \right)}$$  \hspace{1cm} (Van Liere & Walsby 1982).  \hspace{1cm} (6)

The use of a dilute suspension, and short optical pathlength enabled accurate calculation of the irradiance received by the cells, and thus comparisons were possible for photosynthesis-irradiance curves made in situ in the growth vessels, on intact biofilms, or in suspensions of cells.

Photosynthesis-irradiance curves (ETR-E) were summarised using a two parameter fit:

$$r_{ETR} = \frac{r_{ETR_{max}}}{[1 - \exp(\alpha/E_{rETR_{max}})]}$$  \hspace{1cm} (7)

where $r_{ETR_{max}}$ is the light-saturated rate of ETR and $\alpha$ is the initial slope of $r_{ETR}$ at low irradiance (Webb et al. 1974). The $r_{ETR}$-E relationship was summarised using simultaneous least-squares curve fitting; the initial slope parameter thus determined is denoted $\alpha_e$. The initial slope was also determined separately by linear regression through the first four points at low irradiance and is presented for comparison as $\alpha_i$. The light saturation point of an $r_{ETR}$-E curve, $E_{r}$, was determined as $r_{ETR_{max}}/\alpha_e$.

**RESULTS**

**Growth rates, photosynthesis and chlorophyll fluorescence in batch culture**

**MAXIMUM BIOMASS AND GROWTH RATES:** Traits in growth rate and maximum biomass specific to each species were apparent during growth in batch culture. All species exhibited exponential increases in biomass during the first 3–7 d after inoculation, as shown by a log-linear plot of ($F_o$) vs time (Fig. 1). Thereafter, the algal biomass increased slowly during the second week of culture, and reached a maximum by day 15. The highest (and most consistent) levels of biomass were shown by *A. coffeaeformis*. For this species, the maximum biomass was found in 2–3 wk old cultures. A reduction of biomass occurred in older cultures. The lowest biomass was found in cultures of *N. mutica*, *N. phyllepta* and *Cylindrotheca closterium* (Fig. 2a). *Achnanthes longipes* cultures were the most variable and sometimes failed to develop at all, even though the inoculated cells still appeared healthy under the microscope. The instantaneous growth rate in early exponential phase was similar for all five species for which sufficient replication was available, with mean values ranging from 0.31 to 0.56 d$^{-1}$ (Fig. 2b). The type of culture vessel did not influence maximum growth rates, but differences in the maximum biomass were noticed between cell culture bottles and glass Erlenmeyer flasks, with the final biomass being higher in glass vessels than in plastic cell culture bottles. Algal density in the cell culture vessels often decreased rapidly after a period of
The corresponding $F_v/F_m$ values were 0.45 (Amphora coffeaeformis), 0.61 (Navicula mutica), 0.65 (Achnanthes longipes) and 0.66 (Amphora hybrida).

Consistent differences were observed between diatom species with regard to the highest values recorded for the maximum photochemical efficiency, $F_v/F_m$ (Table 1). Highest values were measured for Achnanthes longipes and Amphora hybrida, with $F_v/F_m$ values of 0.83 and 0.71, respectively. Navicula mutica had the highest variability in photochemical efficiency, with lowest and highest values ranging from 0.39 to 0.69. Consistently similar values of 0.58±0.60 were observed in cultures of A. coffeaeformis and Psammodyction panduriforme. Other benthic diatoms, and the tychoplanktonic Phaeodactylum tricornutum, had mean $F_v/F_m$ values between 0.54 and 0.66.

$F_v/F_m$ was low on the first day of inoculation of a new culture, but increased within 24 h so that the highest values were usually recorded on the second day after inoculation (Fig. 4a, b). Thereafter, the maximum photochemical efficiencies decreased as the culture developed. The rate of decrease was species-specific (Fig. 4a, b), with the greatest decrease in $F_v/F_m$ recorded for A. hybrida (Figs 4b, 5) and N. mutica (Fig. 5). The $F_v/F_m$ values had decreased to 0.31 and 0.35 by week 5 for these two species, respectively. In contrast, the decrease in $F_v/F_m$ during the first 5 wk in batch culture was less for
Table 2. Photophysiological characteristics of two species of *Amphora*. B indicates the batch cultures; R/A, membrane rafts floating on water; R/Sus, for R/A cultures resuspended in fresh medium; R/F, immersed membrane rafts in flowing medium; and R/NF, submerged rafts grown without replacement of the medium. Definitions of the photosynthetic parameters are given in Material and Methods.

| Species          | Culture | Age | $E_a$ | $a_{sp}$ | $a_{si}$ | $ETR_{max}$ |
|------------------|---------|-----|-------|----------|----------|-------------|
| *Amphora coffeaeformis* | B       | 2   | 345   | 0.43     | 0.51     | 177         |
|                   | B       | 3   | 236   | 0.58     | 0.49     | 116         |
|                   | B       | 5   | 114   | 0.71     | 0.49     | 55          |
|                   | B       | 7   | 111   | 0.76     | 0.50     | 55          |
|                   | R/A     | 3–18| 191   | 0.53     | 0.33     | 46          |
|                   | R/Sus   | 8–18| 215   | 0.40     | 0.34     | 71          |
|                   | R/F     | 1–8 | 400   | 0.57     | 0.51     | 223         |
|                   | R/NF    | 3–8 | 465   | 0.33     | 0.36     | 155         |
| *Amphora hybrida*  | B       | 2   | 559   | 0.29     | 0.64     | 360         |
|                   | B       | 3   | 191   | 0.78     | 0.59     | 113         |
|                   | B       | 7   | 178   | 0.57     | 0.46     | 81          |
|                   | R/A     | 9–18| 43    | 0.56     | 0.24     | 24          |
|                   | R/Sus   | 18  | 269   | 0.51     | 0.47     | 136         |

*Achnanthes longipes*, changing from 0.65 to 0.52. Older cultures of *A. longipes* even showed a small increase in efficiency values during late stationary phase (Fig. 5). For other species, there was little further change in $F_v/F_m$ values after week 5.

Biofilm development in batch culture was accompanied by large changes in photophysiological parameters derived from rETR-irradiance curves fitted with Equation 8 (values denoted ‘B’ in Table 2). Highest rates of light-saturated photosynthetic ETR ($rETR_{max}$) were measured early in the development of batch cultures, and rates decreased markedly in following days as the culture developed (Table 2). $rETR_{max}$ showed a proportionally larger decrease than the photosynthetic efficiency, $a$. By day 7, $rETR_{max}$ was reduced to 32% (*Amorpha coffeaeformis*) and 23% (*A. hybrida*) of the starting value. Photosynthetic efficiency calculated using the four lowest irradiance values, $a_{sp}$, was constant with time for *A. coffeaeformis*, or decreased to 71% of the initial value for *A. hybrida*. As a result, the derived light saturation parameter, $E_v$, was lowered by approximately 70% for both species after 7 d in batch culture.

**Growth rates, photosynthesis and quenching of biofilms in continuous flow culture**

Membrane rafts floating on f/2 medium, with the inoculated side of the membrane exposed to air, only allowed limited development of biofilms, typically less than 1 cell layer thick, with low chlorophyll concentrations (mean chl a concentration 26.4 mg m$^{-2}$ and s.d. 9.4 mg m$^{-2}$ for $n = 7$ biofilms grown for 4–21 d). Electron transport rates of these air-grown epipelagic biofilms was also low (mean $ETR_{max}$ 46 ± 9.5 for *A. coffeaeformis*; 24 ± 10 for *A. hybrida*; values denoted ‘R/A’ in Table 2; Fig. 8), and there was strong quenching of fluorescence during the irradiance-response curves. Resuspension of these biofilms in f/2 medium and measurement of the stirred suspension resulted in some restoration of $ETR_{max}$ (to 71 ± s.d. 42 for *A. coffeaeformis*; ‘R/Sus’ in Table 2), and a reduction in the amount of quenching during irradiance-response curves, suggesting that the limitation was not chronic but temporary in nature due to adverse conditions within the biofilm.

Growth rates were higher, and biofilms denser, when membrane rafts were first inoculated then gently submerged in medium (Fig. 6). Biofilms colonised the membrane rapidly under...
Fig. 7. (a) Growth of an *Amphora coffeaeformis* biofilm at a continuous irradiance of 100 μmol photon m$^{-2}$ s$^{-1}$ in submerged conditions (with stirring of the chamber but no exchange of medium) using the reflectance method. Upwelling radiance values are shown at 675 nm (chlorophyll red absorption maximum) and 750 nm (reference wavelength), together with the calculated vegetation index (Equation 1). (b) Growth of an *A. coffeaeformis* biofilm at 100 μmol photon m$^{-2}$ s$^{-1}$ with continuous supply of fresh medium, and registration of steady-state fluorescence signal. Irradiance was supplied with a 3 h : 1 h light : dark cycle. Darkening of the chamber caused visible decreases in steady-state fluorescence from the $F_o$ level to the $F_v$ level. Growth rates ($\mu$) are shown for selected 12 h periods of the trace. The trace is interrupted at 2 d by a pump failure, which allowed air into the chamber, and restarted at a new position after 3.5 d.

these conditions (Fig. 7a), and an areal chlorophyll concentration of 62 mg m$^{-2}$ was obtained after 3 d. Photosynthetic rates were higher than those for emersed rafts (‘R/NF’ in Table 2). However, in unstirred medium, photosynthetic oxygen evolution caused oxygen bubbles to form in the biofilm, which subsequently lifted clear of the membrane. Placement of the inoculated rafts in a flow-through chamber connected to a continuous supply of medium prevented bubble formation, and was convenient for allowing continuous monitoring of the pigment concentration via optical techniques (Fig. 7b). The submerged rafts produced a considerably higher biomass (range 60–151 mg chl m$^{-2}$ and cell concentration $2 \cdot 10^{10}$ cells m$^{-2}$) than floating rafts. Expansion of the biofilm, as monitored either by fluorescence or reflectance, occurred exponentially for periods of up to 8 d, as shown by the logarithmic plot of fluorescence vs time in Fig. 7b. The calculated growth rates
cies for the maximum photochemical efficiency of PS II, Fv/Fm is insensitive to small changes in the chlorophyll content, growth rates and photophysiological status of microphytobenthic cultures could be monitored rapidly and without the risk of contamination apparent during direct sampling. The growth of diatom biofilms in batch culture, although exponential at the beginning, could also be fitted with a logistic function, as reported previously for natural biofilms growing on sediments (Blanchard et al. 2001). Following inoculation, there was an initial, exponential phase of colonisation with similar rates of increase for all species. Maximum growth rates in the batch cultures reported here (of 0.1–0.8 d⁻¹) were similar to previous estimates made on batch cultures of natural microphytobenthic algae at different temperature and irradiances (μ = 0.11–0.76 d⁻¹; Admiraal et al. 1982), but were at the lower end of the range observed previously for microphytobenthic diatoms in culture. Underwood & Provot (2000) also used optical methods and showed growth rates varying from 1.0–2.8 d⁻¹ for cultures of N. phyllepta, 0.6–2.2 d⁻¹ for N. salinarum, and 0.2–0.8 d⁻¹ for N. perminuta, with growth rates varying as salinity and concentrations of nutrients were changed.

The exponential phase of growth was short in batch cultures, but could be prolonged by growing biofilms in a continuous flow of medium. For batch cultures under standard conditions of temperature, irradiance, culture vessel and starting nutrient concentration, the final amount of biomass produced, or carrying capacity, varied by a factor of 10 between species. This indicated large inherent differences in the ability of different diatom species to exploit environmental resources. In a previous study, maximum biomass (= absorption at 660 nm in stationary phase) also varied by a factor of four between the most productive species Cylindrotheca closterium and the least productive, N. salinarum (Underwood & Provot 2000). Although the experiments presented here did not address the issue of which resource was ultimately limiting for biomass, previous work with computer simulation (Ludden et al. 1985) or with cultured diatom biofilms grown under different flow rates and bicarbonate concentrations (Admiraal et al. 1982) has strongly suggested that the supply of dissolved inorganic carbon (DIC) becomes limiting as algal concentrations in the biofilm increase. Biological activity within a dense biofilm also generates high concentrations of dioxygen and the potentially more dangerous radical forms, O₂⁻, OH⁻ and H₂O₂ (Abele-Oeschger et al. 1997) as well as other toxic species such as NH₃ which form at high pH (Underwood et al. 1998).

The highest biomass was formed by the species A. coffeaeformis, which is a common biofouling diatom (Rasmussen & Ostgaard 2001). It is likely that this species possesses physiological acclimations such as a more efficient DIC concentrating mechanism that enable it to continue growth in a high pH–low DIC biofilm environment. In common with many other benthic diatoms, A. coffeaeformis was motile, and motility was retained at least in early exponential phase cultures. Motility is likely to be important in enabling dispersion away from crowded patches and minimising the deleterious effects of localised competition (Law et al. 2003).

There were significant differences between the diatom species for the maximum photochemical efficiency of PS II, Fv/Fm; the highest values of which were expressed in the early stages of cultures. Low values on the first day of batch culture could have been due to a time lag in physiological response of the inoculated cells to the resupply of nutrients. Alternatively, the low fluorescence signal due to low biomass at this stage of development may cause a systematic underestimation of variable fluorescence (Büchel & Wilhelm 1993; R.M. Forster & J.C. Kromkamp, unpublished observations). Consistent differences were observed regardless of the growth medium (YMM or f/2), or type of culture vessel (glass or plastic), with highest Fv/Fm in Acanthesthes longipes and lowest values in N. phyllepta. The differences in efficiencies were unlikely to be caused by photoinhibition because the irradiance levels used in the growth cabinets were moderate and sampling was done during the morning period of the daily light cycle. Furthermore, it is known that Fv/Fm is insensitive to small changes in external factors such as temperature (Morris & Kromkamp 2003), or salinity of the medium. Thus, the differences reported here were more likely to reflect inherent interspecific differences in the structure and function of PS II, rather than suboptimal growth conditions. Differences in maximum photochemical efficiency between algal groups have been known for some time (Büchel & Wilhelm 1993), but, to date, little information is available concerning interspecific differences within the diatoms. Dark-acclimated Fv/Fm values of natural microphytobenthic samples from intertidal areas in the Neth-
erlands vary in a range from 0.55 to 0.78 and investigations are currently in process to ascertain the extent to which these differences are due to environmental factors, or connected to changes in species and community composition.

There was no significant correlation between the maximum photochemical efficiency and the instantaneous growth rate ($P = 0.41$), and no correlation between $F_{v}/F_{m}$ and the ability of a species to produce a high biomass in culture ($P = 0.90$). This is troublesome because $F_{v}/F_{m}$ is widely accepted as a general ‘algal health’ indicator (Cordi et al. 1997). Phytoplankton physiological status can be predicted from $F_{v}/F_{m}$ under many conditions of nitrogen or iron limitation (reviewed in Beardall et al. 2001, but see also Parkhill et al. 2001). However, the use of this parameter as a physiological indicator may not be applicable for the case of benthic algae growing in biofilms. In situations where DIC is the limiting nutrient, $F_{v}/F_{m}$ will not be directly affected, and the occurrence of relatively high values of $F_{v}/F_{m}$ in 8 wk-old cultures of Acanthophora may not necessarily indicate that cells are still actively growing. $F_{v}/F_{m}$ was significantly less affected by culture conditions than the maximum rate of photosynthetic ETR. This indicated either that the nutrients that directly impact PS II (e.g. N, Fe) were not limiting in the medium, or that PS II activity was selectively preserved in adverse conditions. Long-term retention of basic photosynthetic competency could be an acquired survival strategy in soft sediment environments, where cells encounter periods without light due to burial in deeper sediment layers (Admiraal 1984).

Although batch cultures provide a useful system for examining biofilm formation and the production of EPS (de Brouwer et al. 2002) or responses to external factors (Underwood & Provot 2000), the large changes in photosynthetic parameters, such as $ETR_{max}$, which were observed within short time periods in batch culture would greatly limit the use of batch-grown material in comparative physiological experiments e.g. to investigate the effects of irradiance or inorganic carbon supply. For a biofilm growing in unstirred medium, the space in which cells grow and sense their environment is essentially two-dimensional and there is only a short time period before nutrient supply within the diffusive boundary layer is exhausted. Tremblin & Robert (2001) also reported a rapid decrease in photosynthetic activity for unstirred batch cultures of the benthic diatom Haslea ostrea, caused by a decline in the activity of the main C-fixation enzyme, RUBISCO. Decrease in Calvin cycle activity, or decreased DIC availability, could have caused the changes in $ETR_{max}$ reported here. The finding that resuspension of benthic cells in fresh medium could only partially restore photosynthetic activity suggests that a decrease in enzyme activity was indeed responsible for the low rates of $rETR$. Hence, batch cultures should not be used for physiological investigations of benthic species.

Cells grown on wetted rafts floating on medium did not develop high biomasses, possibly due to dehydration or diffusive limitation of nutrient supply from below. Alternation of emersion and immersion cycles may have improved growth rates and carrying capacity in this type of culture system. Electron transport rates in the air-exposed biofilms were low indicating a chronic reduction in photosynthetic competence. In contrast, cells grown in the flow-through chamber maintained high rates of photosynthesis for longer periods. Submerged biofilms colonised their substrate rapidly and could be continually harvested over a period of several days for use in comparative photophysiological experiments, thus satisfying one of the aims of this research. Similar systems have already been developed for determining the responses of freshwater periphyton to nutrients (Pringle 1987), and should prove useful in studying the photosynthetic physiology of microphytobenthos.

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