

Effect of light and temperature on biomass, photosynthesis and capsular polysaccharides in cultured phototrophic biofilms

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Abstract Phototrophic biofilms seem to be suitable candidates for tertiary wastewater treatment due to their high uptake capacity for nutrients and other pollutants, also taking into account the time and cost savings derived from easy procedures for biomass harvesting. Biomass accrual, structure, and physiology of biofilms affect the efficiency of nutrient removal by its microbial community. Here, we construct a biofilm consisting of a cyanobacterium *Synechocystis* sp. and the green alga *Chlorococcum* sp. and determine the effect of combined variations of irradiance and temperature on the biofilm structure and function. The two species were isolated from phototrophic biofilms naturally developing in an Italian wastewater treatment plant and grown in a microcosm designed for biofilm investigations. Phototrophic biomass accumulation, percent species composition, photosynthetic response and the amount and composition of capsular polysaccharides (CPS), including anionic residues, are reported. The results showed that biofilm development required relatively moderate irradiances ($60 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) below which development was arrested. Both light and temperature had a

strong effect on the composition of each species to the biofilm. The CPS compositions also changed with temperature, light and species composition. The CPS of the green-algal-dominated biofilm had the higher uronic acid content indicating a potential to exploit green algae in the treatment of waste contaminated with heavy metals. Given the knowledge of the response of certain species to light and temperature combinations, it may be possible to construct biofilms of known species and CPS composition to use them for specific applications.

Keywords Phototrophic biofilm · Water treatment · Matrix · Capsular polysaccharides · Uronic acids · Photosynthesis

Introduction

Phototrophic biofilms are matrix-enclosed attached microbial communities of phototrophs (cyanobacteria and microalgae) and chemotrophs (Archaea, bacteria, fungi and protozoa), that can develop highly differentiated architectures and occur on most surfaces exposed to light given enough water including all submerged surface of outdoor wastewater treatment plants (WWTPs; Sládecková 1994; Congestri et al. 2006; Guzzon and Albertano 2009). From the 1990s, structure and function of biofilms has been increasingly investigated. Most of this research has focussed on freshwater biofilms because of their ecological importance and of the high potential biotechnological applications of these complex microbial associations, e.g. biomass production as energy source, wastewater treatment (Schumacher et al. 2003) and bioremediation (Cohen 2002; Chaillan et al. 2006). Phototrophic biofilms seem to be suitable candidates for tertiary wastewater treatment due to

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their high efficiency in removing inorganic nutrients, pollutants and xenobiotics (Hoffmann 1998; Guzzon et al. 2008). Moreover, field and culture experiments (Sládečková et al. 1983; Sládečková 1994; Guzzon et al. 2008) conducted to date have highlighted how biomass production and nutrient removal capacity of these periphytic communities are comparable to those of suspended algal cultures which are usually employed in wastewater remediation (Hoffmann 1998). The time and cost savings involved in biomass harvesting and cultivation of phototrophic biofilms make them viable alternatives to suspended algae for wastewater treatment (Hoffmann 1998; Gonzalez and Bashan 2000).

Phototrophic biofilms are efficient in removing nutrients from wastewater firstly because they use nitrogen and phosphorus for their growth and may store these elements inside cells when they are present in excess (Graham and Wilcox 2000). Secondly, the highly hydrated matrix being mainly made up of exopolysaccharides, is not only responsible for the structural and functional biofilm integrity (Mayer et al. 1999; Decho 2000; Flemming et al. 2007), but is also an important nutrient storage zone. The presence of anionic residues in the matrix exopolysaccharides allows cation binding and accumulation (De Philippis and Vincenzini 1998; Pereira et al. 2009). All these features have led to the exploitation of phototrophic biofilms in wastewater treatment (Cohen 2002; Bender and Phillips 2004; Roeselers et al. 2008). However, the potential biomass production of phototrophic biofilms cultured under different light and temperature conditions it still to be elucidated. Community development, in terms of biomass accrual and structure, and its physiology affect the efficiency of nutrient removal by microorganisms inside biofilm and by the biofilm as a whole (Sabater et al. 2002).

To date, studies have unravelled a wide array of physical and biological factors (irradiance, temperature, species composition) as being the key factors in phototrophic biofilm development, structural differentiation and physiology. Light has been shown to affect biofilm growth and physiology as well as community composition and biomass (Hill 1996; Stevenson 1996; Sabater et al. 2002). This is because light is the principal energy source fuelling biofilm primary production. Biofilm thickness causes a vertical gradient of light quantity and quality causing zonation (layering) of different species as a consequence, to have different photosynthetic responses (Hill 1996). Light is also known to exert an indirect effect on exopolysaccharide production by cultured diatoms and cyanobacteria, as production is closely linked to photosynthesis (Staats et al. 2000; Otero and Vincenzini 2003; Stal and Defarge 2005). The effect of the temperature on photosynthesis is understood but there is a scarcity of information on the effect of temperature on exopolysaccharide production by phototrophic biofilms with only a few studies on biofilms

containing diatoms (Wolfstein and Stal 2002) and cyanobacteria (Moreno et al. 1998). Among the biological factors that affect biofilm functioning, community composition is fundamental, shifting in space and time according to physical conditions such as light and temperature (Hill 1996; Stevenson 1996). Another driving factor of biofilm function is the quantity and quality of exopolysaccharides produced by the biofilm. Studies of exopolysaccharides produced by cultured and natural communities have shown that the proportions of monosaccharides, forming the complex anionic heteropolymers of the EPS matrix, depend on the specific organisms of the biofilm (Neu 1994; Bahulikar and Kroth 2008). Moreover, competition for light and temperature may affect the relative abundances of phototrophic species and this will affect the composition and production of exopolysaccharides at biofilm level.

It was decided to perform culture experiments using a mixed culture of two phototrophic species isolated from an Italian WWTP (Albertano et al. 1999) to create a synthetic phototrophic biofilm under different light and temperature conditions in a semi-continuous flow-lane incubator, specifically designed for studying biofilm development under closely controlled ambient conditions (Zippel et al. 2007). The aim of this research was to assess the effect of combined variations of irradiance and temperature on structure and functioning of the two-species cultured biofilms. Phototrophic biomass accumulation and composition, photosynthetic response and exopolysaccharide composition and production were estimated and results evaluated in view of the application of these artificial biofilms in wastewater remediation.

Materials and methods

The two benthic freshwater phototrophs, the coccoid cyanobacterium *Synechocystis* sp. and the green alga *Chlorococcum* sp., used in this study, were isolated from biofilms grown in the sedimentation tank of the WWTP located in Fiumicino (Rome, Italy) for the Airport ‘Leonardo da Vinci’. Each non-axenic stock culture was cultured in a 1,000-mL flask on 400 mL BG11 modified medium (Stanier et al. 1971; Guzzon et al. 2005) and grown in a controlled chamber at 20°C, at 60% RH and illuminated at 30 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (PPFD was measured with LI-Cor model LI-185B radiometer equipped with Quantum LI-190SB sensor) following a light: dark regime of 16:8 h. Four milliliters of each sample was taken every 48 h and growth was estimated by measuring optical density (Kontron Uvikon 860 spectrophotometer) at 730 nm for the cyanobacterium and at 678 nm for the green alga. When the stationary phase was reached, a mixed two-species biofilm

has been made after measurement of total cell biovolumes to ensure uniform inoculation at the start of the experiment. Four aliquots of ca. 100 mL of inoculum were added to 3.9 L of medium and the resulting mixture was continuously pumped (for 72 h at 100 L h⁻¹) through a flow-lane incubator prototype. After this period, the medium was refreshed twice a week and the flow rate was set at 25 L h⁻¹.

The flow-lane incubator prototype (PBI, Zippel and Neu 2005; Zippel et al. 2007) was especially designed and constructed by the Department of Inland Water Research, UFZ Centre for Environmental Research (Magdeburg, Germany) within the framework of the EU-project PHOBIA (PHototrophic BIofilms and their potential Application) for the development and investigation of aquatic phototrophic biofilms. It consisted of four separate chambers (LCs) each measuring 120×10 cm with four photosynthetic photon flux densities (PPFD): 15 (LC15), 30 (LC30), 60 (LC60) and 120 (LC120) μmol photons m⁻² s⁻¹. The BG11-modified medium was pumped through the inlet device within each lane with a flow rate of 25 L h⁻¹, resulting in a water velocity of 0.5 ms⁻¹. Two temperature settings, 20 and 30°C, were tested for each PPFD. Polycarbonate slides (76×25×1 mm) were used as artificial substrata for the biofilm adhesion. A turbulence reducer and a water temperature sensor were located at the inlet device of each chamber and the flow rate was valve regulated to give a uniform laminar flow over the surface of the slides. Light was provided by fluorescent lamps (True-light 36 W Auralight, Sweden) connected to a timer for a 16:8 h light–dark cycle. Four light sensors (one incident and three transmitted each composed of three diodes) were integrated per lane for the measure of incident light and for monitoring biomass accumulation as a function of percent light absorbed. Biofilm growth was monitored and recorded with three light sensors that were positioned directly under selected slides for monitoring the variations of transmittance (the percent of incident light attenuation through the biofilm biomass) during the experiments (Zippel et al. 2007). The development of the biofilm communities were monitored up to 45 d and samples were taken at the initial adhesion (90% transmittance), active growth phase (50%) and mature stage (<10%). For details of these standard values and for more details of the incubator design, see Zippel et al. (2007).

Biomass estimation

Biofilms at the three stages of development were scraped off the polycarbonate slides to evaluate the biomass. Phototrophic biomass was assessed by determining Chl *a* concentration and by calculating the biovolume of individual taxa. Chl *a* was extracted overnight in 90% acetone in the dark and then quantified spectrophotometrically accord-

ing to Jeffrey and Humphrey (1975). Biofilm samples for biovolume calculation were fixed in 2% formaldehyde in 0.1 M phosphate buffer (pH 7.2) and stored at 4°C. Preparation of samples for biovolume evaluation was carried out according to Congestri et al. (2006). The scrapings were sonicated twice for 3 min in a sonic water bath to disaggregate the samples. Aliquots of suspensions were diluted in phosphate buffer and left to settle for 24 h in 25-mL sedimentation chambers. Observations were made with an inverted Zeiss Axiovert 100 microscope. A Nikon CoolSnap digital photo-camera was used to acquire optical fields and digital images. Measurements of selected morphometric parameters were performed manually on digital images using Adobe Photoshop version CS3. To estimate the biovolume of single cells standard equations proposed for the different cyanobacterial and algal shapes were used (Hillebrand et al. 1999). All biomass analyses were performed in triplicate and per unit area calculated dividing biomass by the surface of sample substrate.

Determination of photosynthetic characteristics

Photosynthetic parameters of cultured biofilms at the three stages of development were assessed using a miniaturised pulse amplitude modulated (Mini-PAM) fluorometer, using WinControl Software (Walz GmbH, Germany) for operation and data analysis. Mini-PAM fluorometry was used as a non-invasive method to monitor the photosynthetic characteristics of the biofilm according to Barranguet et al. (2004). Further detailed information describing the measurements made using the PAM technique is available in Genty et al. (1989). Three slides were sampled at the initial stage of biofilm development and positioned on a specifically designed grid with the PAM fiber-optic positioned 10 mm above biofilm surface. After the measurements were made, the slides were put back in the same position in the lane and then re-used for fluorescence assessment in the following samplings, i.e. at the active and mature stage of biofilm development. In order to take into account heterogeneity of microalgal distribution, the light utilization efficiency $\Delta F/F'_m$ was measured at 27 points per slide (randomly without bias) for three slides (81 replicates). Thereafter, rapid light curves were made at three spots taken at random on each slide giving nine replicates for each sampling.

The relative electron transport rate (rETR) was calculated as follows: $\text{rel rETR} = \Delta F/F'_m \cdot E \cdot 0.5$ because it is assumed that light energy is equally distributed between both photosystems (Perkins et al. 2006; Herlory et al. 2007). Photosynthetic electron transport rate curves were fitted by means of an automatic spreadsheet based on linear regression for estimating ETR per light intensity and a Chi-square minimisation for the exponential function $\text{ETR} = \text{ETR}_{\text{max}} (1 - e^{-\alpha \text{E}/\text{ETR}_{\text{max}}})$ proposed by Webb et al. (1974,

in Henley 1993). From the fit of the maximum rate of relative ETR ($\text{relETR}_{\text{max}}$), the initial slope, i.e. photosynthetic efficiency (α) and the light saturation parameter ($I_k = \text{relETR}_{\text{max}}/\alpha$) were calculated.

Capsular polysaccharide extraction, quantification and composition

The bound/capsular polysaccharide extraction was performed following the procedure described in Barranguet et al. (2004, 2005). Cultured biofilms sampled at the active and mature stages of development were suspended in bi-distilled water, shaken for 5 min and centrifuged for 5 min at 3,500 rpm. The pellet was re-suspended in 0.1 M H_2SO_4 and incubated at 95°C in a water bath for 30 min. After the incubation, the samples were centrifuged for 5 min (3,500 rpm) and the resulting supernatant (considered as representative for the amount of ‘bound/capsular’ extracellular carbohydrates in the biofilm) was precipitated in cold 96% ethanol. The carbohydrate fractions were measured spectrophotometrically using the phenol–sulphuric acid method (Dubois 1956). The carbohydrate concentration in the samples was determined by comparing the measured absorption values with those of a calibration range of fresh glucose standards of known concentrations. After centrifugation, the pellet was stained with Alcian Blue at pH 2.5 and 0.5 to check the extraction efficiency of each eluent. Microscopic observation of the stained samples allowed us to determine the intactness of the cells and whether any capsular carbohydrates had remained after the extraction.

CPS extracts were analysed for their monosaccharide composition using ion exchange chromatography (IEC). Freeze-dried (2N trifluoroacetic acid, 120°C for 45 min) capsular polysaccharide samples were eluted in H_2O MilliQ, NaOH 0.185 M, CH_3COONa ($40 \mu\text{g mL}^{-1}$) with a flux of 1 mL min^{-1} and injected into a Carbon Pac A1 ion exchange chromatography column connected to a DIONEX.

Results

Biomass accumulation

Figure 1 shows the growth curves of the two-species biofilms at four different light intensities (15, 30, 60 and $120 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) and at 20 and 30°C . Biomass development is indicated as the increasing light absorbance of the biofilm. In the experiment performed at 20°C (Fig. 1 upper graph), exponential phase of phototrophic biofilm growth was detected at high ($120 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) and intermediate ($60 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) light intensities. The initial colonization phase (lag phase) was 10 days

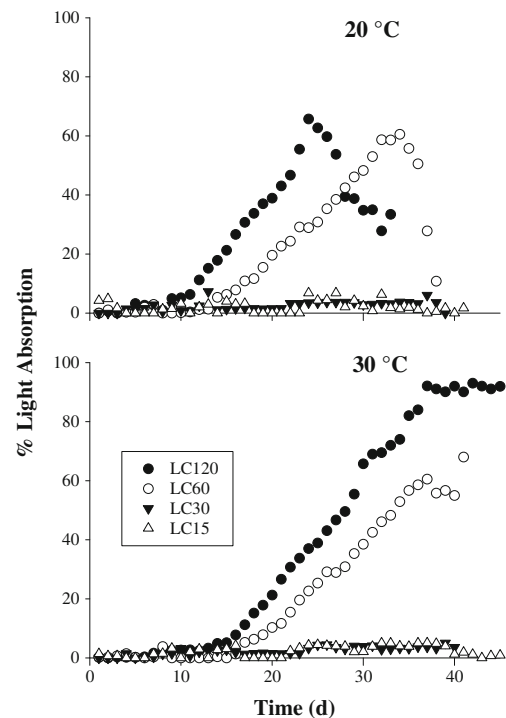


Fig. 1 Growth curves of cultured biofilms under 120, 60, 30, 15 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ at 20°C and 30°C indicating biofilm development as % of light absorption

for LC120 biofilms and 13 days for LC60 biofilms. Active phase occurred at day 20 in LC120 and day 25 in LC60 biofilms; no mature phase was attained in either case. Progressive detachment of biofilms from substrates was observed in both cultures grown at the highest irradiances. Biofilms grown under lowest irradiances (30 and $15 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) did not show any significant growth throughout the 40 day incubation period.

In biofilms grown at 30°C (Fig. 1 lower graph), only the LC120 and LC60 biofilms reached an exponential phase. In LC120 cultures, the lag phase lasted 15 days, and after 35 days cultures developed into mature communities. LC60 cultures showed a lag phase of 18 days and did not reach the mature phase within the experimental period. In both cases, there was no observed detachment of biofilms. Reduced absorbances recorded on days 37 to 40 were not due to general detachment of the biofilm, but due to localised minor detachment and rapid replacement growth occurred (as can be seen on day 41). Again, cultures grown under 30 and $15 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ did not show any significant growth throughout the 40 day incubation period. As these conditions thus yielded insufficient biomass for processing, all analyses were limited to samples obtained from LC60 and LC120 cultures.

Chl *a* and total biovolume values, used as estimation of phototrophic biomass, increased over time correlated with

the increasing light absorbance in each lane. Chl *a* values ranged between 1.51±0.07 and 68.81±21.89 mg m⁻² (Table 1). LC60 biofilms showed higher Chl *a* values than the LC120 biofilms in both runs. Higher phototrophic biomass and total biovolume was found at 30°C than at 20°C in both the final LC60 and LC120 biofilms. Phototrophic biomass in samples at the end of the lag phase was more abundant at 20°C than at 30°C. Total biovolume of phototrophic microorganisms ranged between 11.7±1.73 and 356.95±37.22 10⁶ μm³ cm⁻² (Tables 2 and 3). In the LC60 biofilm, biovolume was two times higher at 20°C than at 30°C at the active phase of development. Total biovolume values of active biofilms increased with increasing irradiances at 20°C while the opposite was observed at 30°C. In Tables 2 and 3 the percent contribution of the two phototrophs as biovolume is shown. At 20°C all biofilms were clearly dominated by the *Chlorococcum* sp. whilst at 30°C biofilm was almost entirely made up of the *Synechocystis* sp. (up to 99%).

Photosynthetic characteristics

PAM measurements were used to evaluate the photosynthetic performance of light-acclimated cultures and results are shown in Tables 4 and 5 and Fig. 2. The effective quantum yield of photosynthesis (Δ*F*/*F*_m'), measured on the surface of intact biofilms ranged between 0.431±0.015 and 0.660±0.003. Looking at effect of irradiance, cultures at the active phase showed yield values higher at 120 μmol photons m⁻² s⁻¹ than at 60 μmol photons m⁻² s⁻¹ at both temperatures tested. As for the effect of temperature, yield was higher in most cases at 20°C than at 30°C. reIETR_{max} ranged between 43.19±2.30 and 99.5±9.2 for LC120 and LC60 biofilms, respectively.

At 20°C the reIETR_{max} of LC120 and LC60 biofilms at the active phase of development were very similar (65 and 60, respectively). At 30°C, values of biofilms at the active phase of development were higher at lower intensity: 71.94 for LC60 cultures against 47.52 for LC120.

The LC120 cultures that were able to develop into mature biofilms at 30°C showed a reIETR_{max} of 74.94 and

it might depend on deep layer fluorescence, as reported in previous studies on microphytobenthos (Forster and Kromkamp 2004; Serodio 2004). From the analysis of α and *I*_K values, it emerges how photosynthesis was saturated slowly and inefficiently (corresponding to higher *I*_K values and lower α) in biofilms maintained at 30°C than in ones at 20°C. No evident photoinhibition occurred at all the experimental irradiances and temperatures.

Capsular polysaccharides

Light microscopy observations on stained pellets after the CPS extraction, showed the intactness of the cells and confirmed if any capsular carbohydrates had remained after the extraction.

Figure 3 shows the CPS values of active biofilms grown at 20 and 30°C under 120 and 60 μmol photons m⁻² s⁻¹. The highest CPS production occurred at 30°C and LC60. The biofilms grown under 20 and 30°C produced more capsular polysaccharide at lower irradiance (60 μmol photons m⁻² s⁻¹). Capsular polysaccharide samples analysed by means of IEC contained various proportions of four (LC120, T: 20°C) to 11 (LC60, T: 30°C) monosaccharides (Table 6). Across the two experiments, glucose was the main monosaccharide, with values ranging between 27.3 (LC60, T: 20°C) and 41.3 mol% (LC120, T: 30°C). Rhamnose was higher at 20°C compared to 30°C, while galactose was higher at 30°C. Between the two detected uronic acids, the proportion of glucuronic acid was higher than galacturonic acid, only found in LC120 biofilms grown at 30°C.

Discussion

These culture experiments using simple biofilms composed of a cyanobacterium (*Synechocystis* sp.) and a green alga (*Chlorococcum* sp.) showed promise in characterising the relevant conditions for growing biofilms for specific purposes. The responses of the biofilm to the combined effect of four different irradiance levels and two temper-

Table 1 Concentrations of Chl *a* (mg m⁻²) given as mean values (n=3)±standard deviation

Temperature (°C)	Biofilm stage	Irradiance (μmol photons m ⁻² s ⁻¹)	
		120	60
20	Initial	10.98±1.81	20.30±1.49
	Active	29.97±0.50	45.63±0.50
	Mature	–	–
30	Initial	1.84±0.71	1.51±0.07
	Active	54.43±6.13	68.81±21.89
	Mature	66.05±15.00	–

Table 2 Mean total biovolume values±standard deviation of the whole biofilm and the contribution (%) of the two single species to the total biovolume at 20°C

Irradiance ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$)	Stage	Total biovolume ($10^6 \mu\text{m}^3 \text{ cm}^{-2}$)	<i>Synechocystis</i> sp. contribution (%)	<i>Chlorococcum</i> sp. contribution (%)
120	Initial	12.3±2.30	3	97
	Active	113±1.99	8	92
	Mature	–	–	–
60	Initial	11.7±1.73	8	92
	Active	80.7±9.07	4	96
	Mature	–	–	–

atures were realised and used to evaluate the possibility to use mixed biofilms of known species composition in specialist wastewater treatment.

At both temperatures, *Synechocystis* sp. and *Chlorococcum* sp. biofilms formed at only the highest irradiances (120 and 60 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). Irradiance had therefore a strong effect on biofilm development. A similar result had been reported in previous studies for natural biofilms sampled from the same WWTP and grown in the same incubator prototype (Roeselers et al. 2006; Zippel et al. 2007; Di Pippo et al. 2009). Similar growth curves were also found between these studies and the present two-species biofilms under 120 and 60 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. However, biomass accumulation was limited to under 15 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ instead of 30 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ found here, indicating a lack of low light adaptive response of the two species used.

The two-species biofilms here did show some similarities to the multi-species biofilms at least in the initial stages of growth. However, some differences were observed in the two-species biofilms as the mature phase (corresponding to biofilm's light absorbance higher than 90%) was not reached at 20°C: biomass progressively detached from the slides after reaching the active phase both at 120 and 60 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ at 20°C. Only at 30°C and 120 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ did the cultures developed into mature biofilms. Higher temperatures and light intensities

therefore favoured higher biomass accumulation and were most likely linked to the increased EPS production (see below) which is involved in biofilm stability (Mayer et al. 1999).

Using constructed biofilms for the treatment of wastewater requires significant biomass quantities. We have shown here that irradiance and temperature are of vital importance when growing species specific biofilms. Using Chl *a* as an estimation of biomass, the two-species biofilms were within the range obtained for biofilms consisting of the natural species assemblages grown under the same conditions (0.16–125 mg m^{-2} from Guzzon et al. 2008). However, maximum values obtained here were much lower, indicating that species richness could also play an important role in biomass production. However, this is confounded when considering growth experiments conducted *in situ* on artificial substrates that gave the highest biomass range (4.3–252.6 mg m^{-2} , from Guzzon and Albertano 2009). In these *in situ* experiments, the light intensities were much higher (690–1,550 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ at water surface). It may be safe to assume that an increase in the light intensity of the incubator would lead to further increases in biomass especially if combined with a temperature increase, but the costs involved in doing so would reduce the economic feasibility of the application.

PAM measurements were carried out on light-acclimated cultures to assess the photosynthetic performance of the

Table 3 Mean total biovolume values±standard deviation of the two-species biofilms and the contribution (%) of the two single species to the total biovolume at 30°C

Irradiance ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$)	Biofilm stage	Total biovolume ($10^6 \mu\text{m}^3 \text{ cm}^{-2}$)	<i>Synechocystis</i> sp. contribution (%)	<i>Chlorococcum</i> sp. contribution (%)
120	Initial	38.91±3.47	93	7
	Active	127.23±5.94	97	3
	Mature	356.95±37.22	97	3
60	Initial	14.95±4.59	99	1
	Active	190.25±23.03	99	1
	Mature	–	–	–

Table 4 Mean values±standard deviation of reETR_{max}, α and I_k of mixed biofilms grown at 20°C in the incubator prototype

Irradiances (μmol photons m ⁻² s ⁻¹)	Stage	ΔF/F _m '	reETR _{max}	α	I _k
120	Initial	0.640±0.016	99.5±9.2	0.320±0.023	285.0±66.5
	Active	0.440±0.007	65.6±2.6	0.440±0.011	150.0±4.7
	Mature	–	–	–	–
60	Initial	0.660±0.003	71.3±1.4	0.550±0.011	131.0±4.4
	Active	0.509±0.006	60.4±1.3	0.420±0.07	144.6±3.8
	Mature	–	–	–	–

biofilms. Both the effective quantum yield of PSII (ΔF/F_m') and the reETR_{max} showed increasing values with increasing irradiance in the two experimental runs, indicating biofilm photoacclimation, which has also been observed in natural biofilm assemblages from different environments (Kromkamp et al. 1998; Underwood 2002; Barranguet et al. 2003; Rascher et al. 2003; Seródio 2003; Lugomela et al. 2005). When comparing between the two runs at a specific light intensity the values of ΔF/F_m' and reETR_{max} were higher at 20°C than at 30°C. This is because high light and/or high temperature, can lead to an imbalance between the energy supply and energy consumption by the redox reactions in the electron transport chain, resulting in a progressive photoinactivation of PSII reaction centers. This being so, in the LC60 samples the opposite occurred, as values of ΔF/F_m' and reETR_{max} were highest at 30°C. Values of photosynthetic parameters measured in the two runs reflected the response of the species dominating the biofilm. At 30°C *Synechocystis* sp. was the dominant species, while at 20°C it was *Chlorococcum* sp., which indicates strong competitive selection driven by temperature. In biofilms, competition for resources becomes increasingly important and some microorganisms are selected-out, leading to dominance by a few 'more-competitive' species (Battin et al. 2007). Here, the cyanobacterium is more competitive at the higher temperature which corresponds with the findings of other multi-species synthetic biofilms (Van der Grinten et al. 2004). The higher values of the photosynthetic parameters measured for *Synechocystis* sp. in the LC60 samples at 30°C suggested a higher capacity for light capture and if

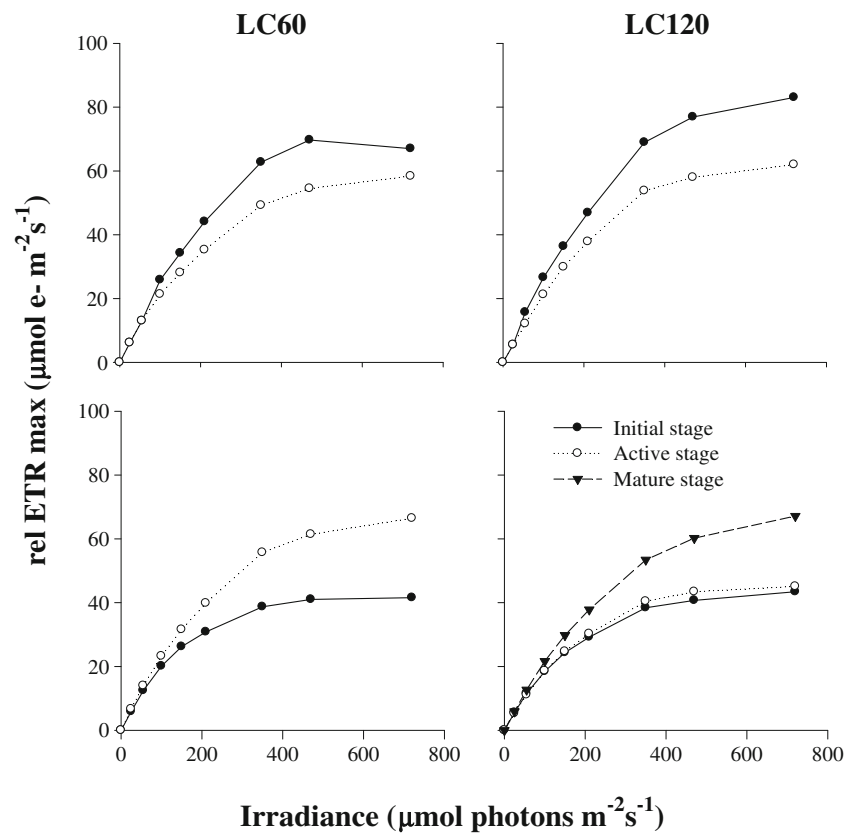
combined with a lower cellular maintenance constant associated with cyanobacteria over other algae (Mur et al. 1978; Van Liere et al. 1979) would give it the competitive edge over *Chlorococcum* sp.

The amount of CPS extracted from biofilms grown under 30°C was higher than those extracted from biofilms developed under 20°C and under both temperatures there was higher CPS under the lower light intensity. It has been shown that the production of EPS in biofilms is strongly influenced by species composition (Neu 1994; De Philippis and Vincenzini 1998; Di Pippo et al. 2009). However, here, we showed community composition to be temperature dependent, so it becomes difficult to determine if temperature or the species making up the biofilm, or a combination of both factors, drove CPS production. This would require detailed experiments on the growth and CPS production of single species biofilms under the tested conditions to elucidate. The amount of CPS produced by the two-species biofilm grown at 30°C at 60 μmol photons m⁻² s⁻¹, dominated by the cyanobacterium was nearly double those of CPS extracted from biofilms developed at 20°C, dominated by the green algae, as previously observed for natural phototrophic biofilms cultured in the same incubator (Di Pippo et al. 2009). There was detachment of the biofilm at 20°C which is most likely a result of the lower CPS production as exopolysaccharides are involved in the initial adhesion, formation and stability of biofilms (Barranguet et al. 2005). Moreover, the more complex monosaccharide composition, detected with IEC, of the CPS produced by biofilms at 30°C, was similar to that of exopolysaccharides synthesized by a variety of cyanobac-

Table 5 Mean values of reETR_{max}, α and I_k±standard deviation of mixed biofilms grown at 30°C in the incubator prototype

Irradiances (μmol photons m ⁻² s ⁻¹)	Stage	ΔF/F _m '	reETR _{max}	α	I _k
120	Initial	0.564±0.014	46.34±3.60	0.241±0.023	213.5±32.1
	Active	0.431±0.015	47.52±5.50	0.239±0.022	195.0±9.7
	Mature	0.473±0.004	74.12±3.00	0.259±0.004	285.5±10.4
60	Initial	0.477±0.004	43.19±2.30	0.272±0.007	161.0±12.8
	Active	0.533±0.006	71.94±3.19	0.287±0.006	250.93±10.63
	Mature	–	–	–	–

Fig. 2 Electron transport rates versus irradiance (ETR/I) curves of biofilms growing under 60 (left hand figures) and 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (right hand figures) biofilms grown at 20°C (upper two figures) and 30°C (lower two figures)



teria (De Philippis and Vincenzini 1998; Bellezza et al. 2006). The IEC studies showed the presence of two uronic acids, galacturonic and glucuronic acid, in the heteropolymeric matrix of the two-species biofilms, with their relative proportion higher in the biofilms grown at 20°C. This was an unexpected result as uronic acid production is normally associated with cyanobacteria (De Philippis and Vincenzini 1998; De Philippis et al. 2001; 2003; Pereira et al. 2009) or

diatoms (de Brouwer and Stal 2002; Bahulikar and Kroth 2008) yet this biofilm was dominated by *Chlorococcum* sp. However, there are a few studies on the production of EPS (including uronics) by green algae (Paulsen and Vieira 1994;

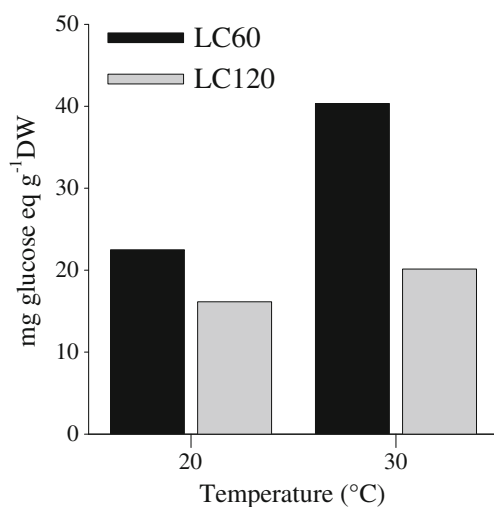


Fig. 3 Capsular polysaccharides quantity of active-phase biofilms grown in the incubator prototype at 20°C and 30°C under 60 and 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (LC60 and LC120)

Table 6 Monosaccharide composition (*Fuc* fucose, *Rhamn* rhamnose, *Ara* arabinose, *Gal* galactose, *Glc* glucose, *Man* mannose, *Xyl* xylose, *Rib* ribose, *GlcA* glucuronic acids, *GalA* galacturonic acids, *Galam* galactosamine, *Glcam* glucosamine) of CPS fractions extracted from the active biofilms

	20°C		30°C	
	LC120	LC60	LC120	LC60
Fuc	0		11.8	8.8
Rhamn	22.5	20.4	7.8	6.2
Ara	0	0	5.8	9.9
Gal	4	3.2	14.2	17.3
Glc	36.5	27.3	41.3	37.6
Man	0	0	7.3	9.1
Xil	0	2	4	5.6
Rib	0	13.9	1.8	1.7
GlcA	12	16	2.3	0
GalA	0	0	0.5	0
Galam	0	0	3.2	1.4
Glcam	25	17.2	0	2.4

Monosaccharide proportions are expressed as Mol%

Lombardi et al. 2002; 2005) and so it seems that this result was not an anomaly. If not, then this result suggests a wider potential for the use of green algae in water treatment such as for the removal of heavy metals in waste water treatment.

Although it is difficult to define the precise effect of light or temperature on biofilm structure and function due to interrelationships between metabolism and light saturation, it was possible to determine somewhat their synergistic effect. It was shown that changes in ambient conditions caused shifts in the species composition and that this had a large effect on the exopolysaccharide amount and composition. A potential application of this type of study would be in construction of a biofilm with a known composition and to have some control over the exopolysaccharide production in response to a particular need (e.g. heavy metal removal). It was also revealed that the potentially useful properties of green algal CPS should be given more consideration as there is very little information available in the literature to our knowledge.

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