Growth and structure of phototrophic biofilms under controlled light conditions

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Abstract Phototrophic biofilms may be defined as interfacial microbial communities mainly driven by light as energy source. Structure, productivity and taxonomic composition of freshwater phototrophic biofilms under different growth conditions were investigated within the EU-project PHOBIA with the following aims: 1) optimisation of wastewater treatment in wetlands, 2) control and prevention of biofouling on submersed objects, and 3) modelling of phototrophic biofilm development. Experiments were carried out in a flow-lane incubator with precise control of external light, temperature, velocity conditions and nutrient-adapted artificial medium. Structure and architecture of phototrophic biofilms at different developmental stages were examined by using multi-channel confocal laser scanning microscopy (CLSM). The development of phototrophic biofilms was clearly light dependent. Fast growing phototrophic biofilms were mostly dominated by single species algae and formed less stable structures of up to 900 μm thickness. Biofilms with these dimensions had to be cryo-sectioned and post-stained for CLSM. Laser microscopy analysis also revealed a stratification of phototrophic organisms which was more pronounced in slow growing biofilms. In contrast, at very low light intensity the development of phototrophic biofilms was strongly delayed. In conclusion, structural features and subsequent functional relationships may be key parameters for exploitation, control and modelling of phototrophic biofilms.

Keywords CLSM; growth; light intensity; nutrients; phototrophic biofilm

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Introduction

In many aquatic ecosystems, most microbes live in matrix-enclosed biofilms and contribute substantially to energy flow and nutrient cycling (Battin *et al.*, 2003). Due to their regulating function of organic carbon content in shallow environments, phototrophic biofilms form a crucial link at the base of the food web (Steinman, 1996), purification of water (Percival *et al.*, 2000) or conversely cause a nuisance in the form of biofouling (Gawne *et al.*, 1998). Contrary to bacteria-dominated biofilms, light is one of the main energy sources for development of phototrophic biofilms. Phototrophic biofilms can develop into thick (mm) compact layers of cells which are embedded into a matrix of extracellular polymeric substances (EPS) (Barranguet *et al.*, 2004). Photosynthetically produced organic compounds are released into the biofilm matrix and form an additional pool of substrates for degradation by associated bacterial communities. The aim of this study was to use different light intensities during cultivation of artificial phototrophic biofilms in order to estimate the influence on growth characteristics as well as cellular and polymeric biofilm constituents.

Materials and methods

Biofilm cultivation

Experimental studies on the development of phototrophic biofilms were carried out in a special flow-lane incubator at four different light intensities (10, 25, 85, 125 μmol m^{-2} s^{-1}), flow conditions of 100 L h^{-1} and a temperature of 20 °C. Detailed description of the experimental setup is described elsewhere (Zippel *et al*. in preparation). Phototrophic biofilm material from wastewater treatment of Fiumicino Airport, Rome, Italy was used

as inoculum. Biofilms were incubated with re-circulating artificial medium BG11 for cyanobacteria (Rippka and Herdman, 1992) with a 16/8 h light/dark cycle. In addition, nutrients were added to the artificial medium in order to simulate conditions in waste-water treatment plants. The final nutrient concentrations in freshly prepared medium were: nitrate 24.5 mg N L^{-1}; soluble reactive phosphorus 3.5 mg P L^{-1}; silicate 6.5 mg Si L^{-1}. The volume of 4 l re-circulating medium per lane was changed twice a week. The main nutrients (NO_3-N, SRP, DOC) and pH were analysed in freshly prepared and re-circulated medium on days 29, 33, 36, 40, 43 and 47 according to DIN EN 26777, DIN EN 6878 and DIN EN 1484 of the German Institute of Standardisation. Biofilm development was monitored with specially adapted light sensors which were directly mounted under selected polycarbonate slides used as substratum (Figure 1a, b). Decrease of subsurface light was used as an indicator for different biofilm growth stages (mean of three light sensors along 1.2 m length of each flow lane). Light sensor readings allowed the differentiation between initial adhesion (>90%), exponential growth phase (90–10%) and matured stage (<10%) in phototrophic biofilms.

Preparation of samples and microscopy

Biofilm samples of different developmental stages were collected on days 8, 12, 26, 33 and 41 of the experiment. Fully hydrated samples were stained with 1 μg ml^{-1} nucleic acid specific stain (SYTO 9) or 100 μg ml^{-1} lectin solution (Alexa488 conjugated *Aleuria aurantia* lectin; Molecular Probes, Eugene, Oregon) for 5 or 20 min at room temperature, respectively (Neu *et al.*, 2001). Lectin samples were carefully washed three times with tap water to remove unbound lectins. Biofilms were analysed by confocal scanning laser microscopy (CLSM) using visible lasers (488, 568, 633 nm) for excitation and multi-channel recording of nucleic acid signal, glycoconjugates, chlorophyll a and

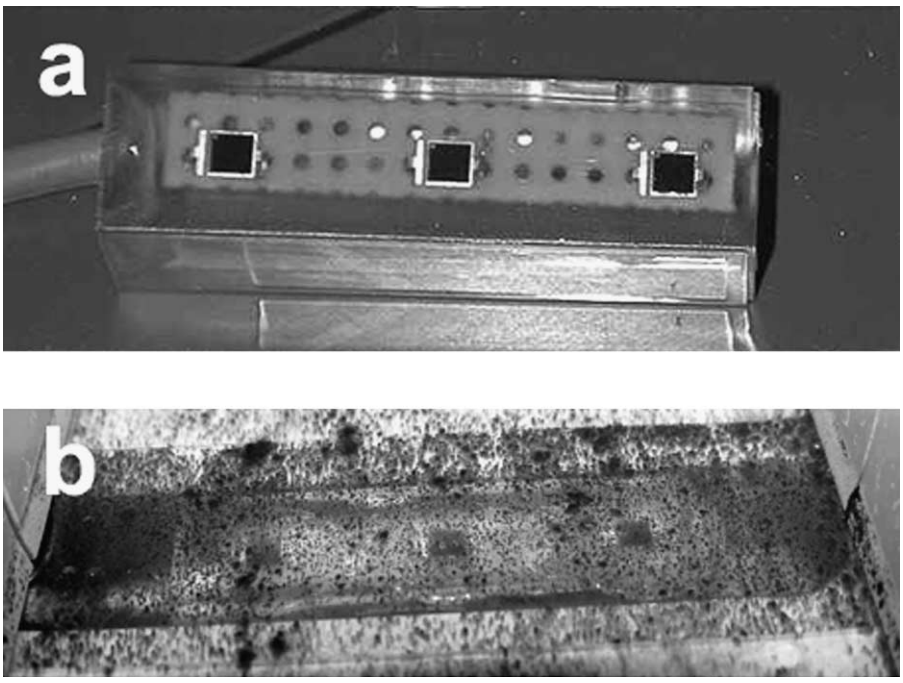

Figure 1 a) Light sensor for measuring subsurface light below polycarbonate slide (substratum), three photodiodes were embedded in a polystyrene cuvette with epoxy resin. b) Light sensor mounted in a flow lane with attached phototrophic biofilm at 25 μmol m^{-2} s^{-1} (60% of subsurface light)

phycobilin autofluorescence (Neu *et al.*, 2004). Samples were examined with 20 × 0.5 NA, 40 × 0.8 NA and 63 × 0.9 NA water immersible lenses. Quantification of data sets was done with the PHLIP software tool developed in Matlab (Mueller *et al.*, submitted). Vertical distribution of phototrophic organisms and lectin-specific glycoconjugates at mature stage was investigated on cryo-sectioned, post-stained biofilm samples. For this purpose the biofilm samples were embedded in Tissue Tek, frozen at −25 °C and sectioned together with the polycarbonate substratum into 100 μm slices. The air-dried slices were finally stained as described above, covered with Citifluor and used for CLSM. Samples were examined by CLSM across the whole biofilm depth. The series of connected image stacks were finally mounted in Photoshop (Adobe).

Results and discussion

Exponential growth of phototrophic biofilms at high (125 μmol m^{-2} s^{-1}) and intermediate (85 μmol m^{-2} s^{-1}) light intensities, indicated by decrease of subsurface light, started approximately 8 days after inoculation (Figure 2a, thick black and dashed lines). For biofilms grown at low light intensity (25 μmol m^{-2} s^{-1}) we observed a lag time of 17 days (Figure 2a, dotted line). At very low light intensity (10 μmol m^{-2} s^{-1}) biofilm development was even more delayed and started after 43 days (Figure 2a, thin black

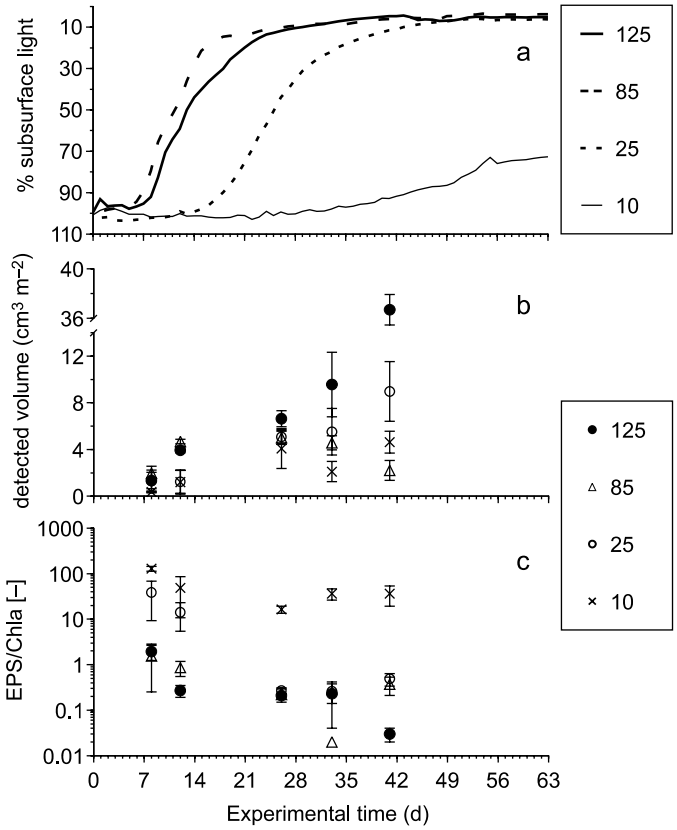

Figure 2 a) Growth curves at different incident light intensities indicating biofilm development as decrease of subsurface light. b) Detected biovolume (cm^3 m^{-2}) as the sum of biofilm constituents (algae, cyanobacteria, bacteria and lectin-specific glycoconjugates), error bars show standard deviation of three quantified CLSM data sets (take notice of break in y-axis). c) EPS/Chla ratio calculated from quantified CLSM data sets. Legend for light intensities 10, 25, 85 and 125 μmol m^{-2} s^{-1} is given on the right-hand side of the figures

line). Maturation of phototrophic biofilms, indicated by subsurface light values lower than 10%, was reached after 28 days at high and intermediate light intensity, whereas biofilms at low light intensity reached this stage 2 weeks later due to their slower development.

After quantification of CLSM images, the volumetric development of biofilm was compared graphically (Figure 2b). During the initial stage (day 8) biofilm volumes of 1.2–2.0 cm^3 m^{-2} were detectable at low, intermediate and high light intensities, whereas at very low light intensity a biovolume of only 0.4 cm^3 m^{-2} was recorded. In the middle of the exponential growth phase (50% of subsurface light) biofilm volumes reached 3.9–5.1 cm^3 m^{-2}. Results recorded for phototrophic biofilms at matured stage (day 26 for 85 and 125 μmol m^{-2} s^{-1} as well as day 33 and 41 for 25–125 μmol m^{-2} s^{-1}) were not representative (Figure 2b). Due to limited diffusion of the staining solutions and laser penetration only the surface areas of phototrophic biofilms could be detected by means of CLSM (Barranguet *et al.*, 2004). Nevertheless, a comparison of the proportional content of biofilm constituents showed obvious differences depending on light intensities during biofilm development (Figure 3a–d).

Exponentially growing phototrophic biofilms at low, intermediate as well as high light intensity consisted of up to 50–60% phototrophic constituents (algae and cyanobacteria). Lectin-specific EPS glycoconjugates were detectable in the range of 30–50%. Heterotrophic bacteria reached values up to 17% at intermediate and high light intensity but only 2–7% at low light intensity. At the beginning of maturation (10% of subsurface light), proportional contents of all detected biofilm constituents showed nearly the same values (Figure 3a,b: day 26 for high and intermediate light, and Figure 3c: day 41 at low light). However, due to higher biofilm volume at low light intensity (Figure 2b), the lectin-specific glycoconjugate volume was approximately 2 times higher (3.7 cm^3 m^{-2} in comparison with 1.5–2.2 cm^3 m^{-2} at intermediate and high light intensity). Glycoconjugate volume decreased down to 0.2 or 1.2 cm^3 m^{-2} with further biofilm maturation (days 33, 41), whereas the proportional content of phototrophic organisms increased up to more than 95%.

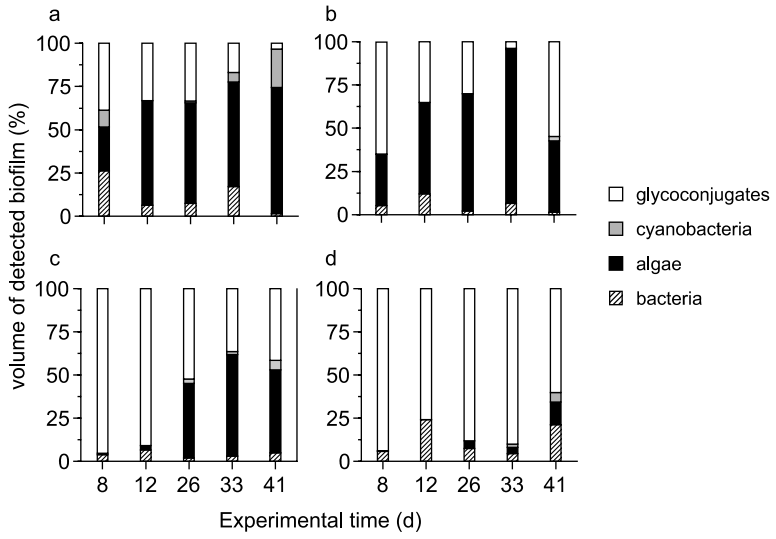

Figure 3 Proportional comparison of different biofilm constituents cultivated at: a) high (125 μmol m^{-2} s^{-1}), b) intermediate (85 μmol m^{-2} s^{-1}), c) low (25 μmol m^{-2} s^{-1}) and d) very low (10 μmol m^{-2} s^{-1}) incident light intensity

In contrast to higher light intensities, phototrophic biofilms developed at very low light intensity were clearly dominated by lectin-specific EPS glycoconjugates and heterotrophic bacteria (Figure 3d). After 41 days of experimental time, algae and cyanobacteria reached only 15% of detected volume. CLSM showed that the volume of lectin-specific glycoconjugates was usually higher than the volume of bacteria. The EPS/bacteria ratio ranged from 15:1 to 3:1. Similar values were also found during cultivation of heterotrophic and chemoautotrophic bacteria-dominated biofilms grown with glucose or ammonium as substrate (Staudt *et al.*, 2004). The very slow growth and low volume of phototrophic organisms during the experimental time may indicate light-limited conditions for the development of phototrophic biofilms. Calculated EPS/Chla ratios of the phototrophic biofilms varied clearly depending on light intensity during cultivation (Figure 2c). At very low light intensity, EPS/Chla ratio ranged from 10:1 to 100:1. This was caused by very low occurrence of phototrophic organisms. Nearly the same ratios were calculated for the initial stage at low light intensity (Figure 2c, days 8 and 12 for 25 μmol m^{-2} s^{-1}). However, during light-driven development of phototrophic biofilms these values decreased down to 0.3:1 and at mature stage the lowest values (0.02:1) were recorded at intermediate and high light intensity.

The fast increase of biovolume as a result of high light conditions indicated a high photosynthetic activity and consequently a high release of dissolved organic carbon into the bulk medium. This was also confirmed by the increase in pH due to photosynthesis (Figure 4). However, the concentrations of N and P decreased down to the detection limit. This was very obvious at the high and intermediate light intensities (125 and 85 μmol m^{-2} s^{-1}). However, at 25 μmol m^{-2} s^{-1} the increase in pH was lower, the DOC increase in the bulk medium was close to zero and less nutrients were consumed (Figure 4). A further observation was that biofilms developed under intermediate and high light conditions showed a more pronounced sloughing behaviour if compared with biofilms grown under low light conditions. Again this may be explained by the fast growth resulting in less stable biofilm structures (Loosdrecht *et al.*, 2002). Furthermore, these biofilms consisted of nearly pure cultures of scenedesmus-like algae (Figure 5a).

In Figure 5 two examples of the vertical structure of matured phototrophic biofilms developed at high and low light intensities are presented. Both biofilms were cryo-sectioned and several image series had to be collected in order to show the large dimension of the biofilm structure. At high light intensities the biofilm grew up to

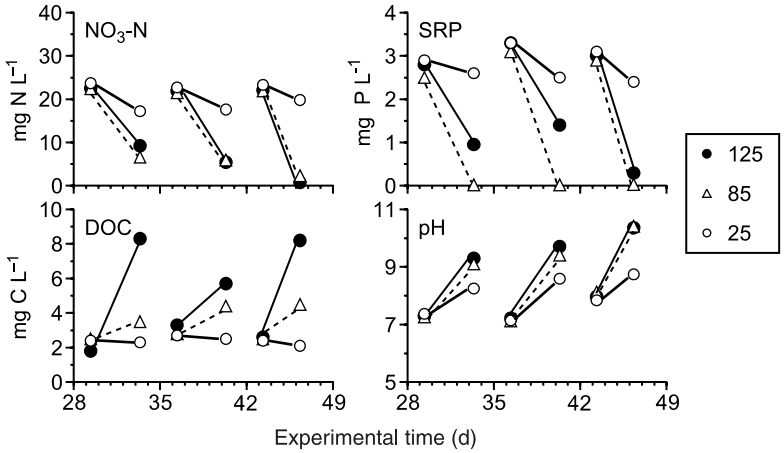

Figure 4 Changes in nutrient concentrations and pH during three different medium re-circulating intervals (96 h); values are shown for 25, 85 and 125 μmol m^{-2} s^{-1}

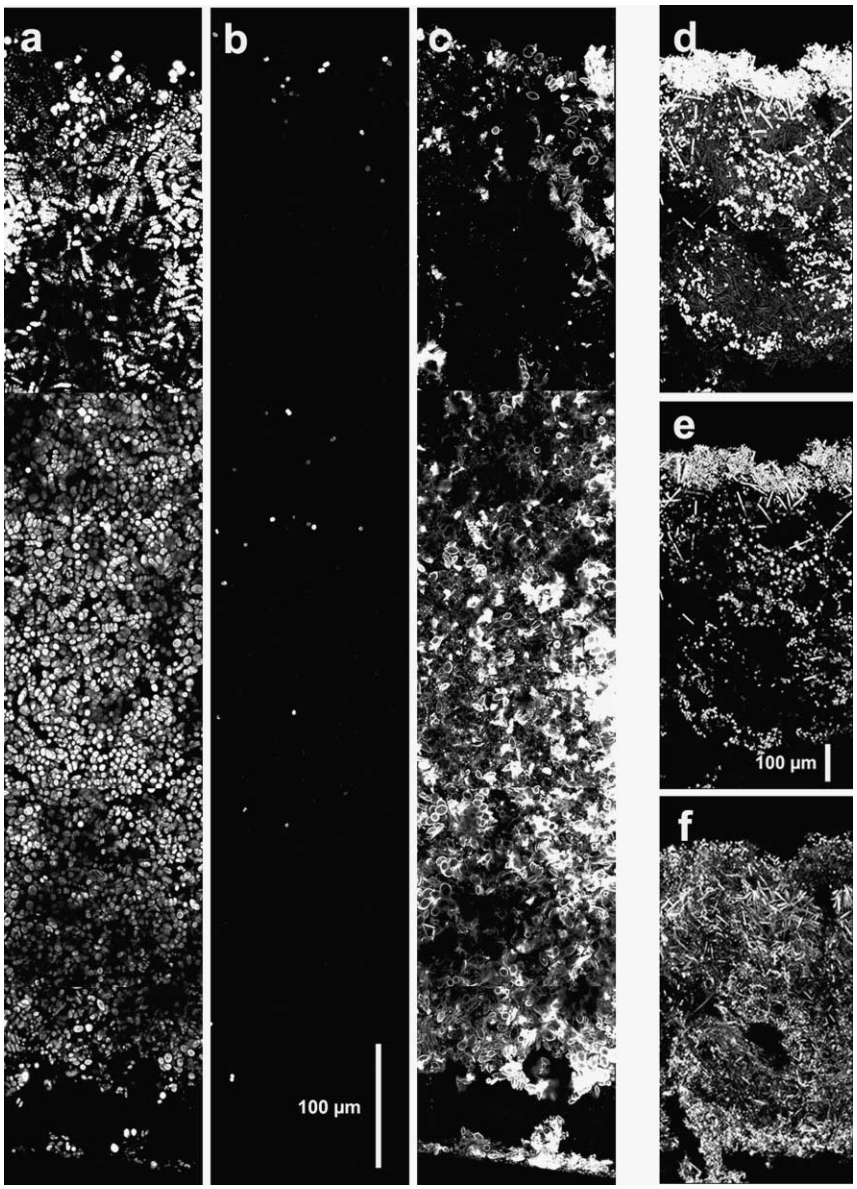

Figure 5 Comparison of phototrophic biofilms at matured stage (75 days old) after cryo-sectioning. Maximum intensity projection of thin sections showing single channel signals. Several connected image stacks were mounted to demonstrate the dimension of the biofilms. a–c) high light intensity (a – algae, b – cyanobacteria, c – glycoconjugates) d–f) low light intensity (d – algae, e – cyanobacteria, f – glycoconjugates)

900 µm thickness and was built up to a major degree by scenedesmus-like algae from the substratum to the biofilm surface (Figure 5a). Only very few cyanobacteria were present (Figure 5b). The glycoconjugates were concentrated in the lower half of the biofilm (Figure 5c). The biofilm developed at low light intensities showed a thickness of about 500 µm and had a layered structure. The phototrophic cell constituents were dominated by *Desmodesmus* sp. and coccal cyanobacteria (Figure 5d, e). Compared to the biofilms grown at higher light conditions these biofilms contained more cyanobacteria. They were present throughout the biofilm and in addition they formed a dense layer on top of the biofilm (Figure 5e). Lectin-specific glycoconjugates were present across the whole

biofilm also indicating a very stable biofilm structure (Figure 5f). The light-driven succession, growth characteristic and physiological activity of different phototrophic organisms resulted in a clear differentiation between vertical layers within the biofilm matrix from the substratum to the biofilm surface. The data from these experiments showed the differences between fast and slow growing phototrophic biofilms, which have been demonstrated in theory with an individual-based model by Kreft (2004). In the case of fast growing phototrophic biofilms and an almost complete depletion of the available resources, the development of single species biofilms is supported. In case of slow growing biofilms, coexistence of different species is favoured probably due to a more economic consumption of the available resources.

Conclusions

In conclusion, the growth characteristics and structure of phototrophic biofilms are mainly influenced by light conditions. Growth rate increased, whereas species diversity and glycoconjugate content decreased with light intensity. The persistence of phototrophic biofilms was more pronounced under low light conditions. It is suggested that lower productivity of the phototrophic biofilms favoured stability of the whole biofilm matrix due to lower nutrient and pH fluctuations in the bulk liquid. These results may have implications for the application of phototrophic biofilms in wastewater treatment and the control of biofouling dominated by phototrophic biofilms.

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