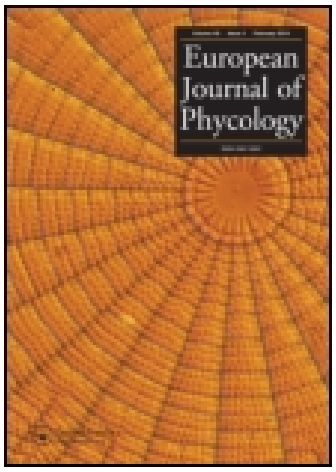

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Diversity and biomass accumulation in cultured phototrophic biofilms

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In the present study, biomass development and changes in community composition of phototrophic biofilms grown under different controlled ambient conditions (light, temperature and flow) were examined. Source communities were taken from a wastewater treatment plant and used to inoculate growth surfaces in a semi-continuous-flow microcosm. We recorded biofilm growth curves in cultures over a period of 30 days across 12 experiments. Biovolume of phototrophs and community composition for taxonomic shifts were also obtained using light and electron microscopy. Species richness in the cultured biofilms was greatly reduced with respect to the natural samples, and diversity decreased even further during biofilm development. *Diadesmis confervacea, Phormidium* spp., *Scenedesmus* spp. and *Synechocystis* spp. were identified as key taxa in the microcosm. While a significant positive effect of irradiance on biofilm growth could be identified, impacts of temperature and flow rate on biofilm development and diversity were less evident. We discuss the hypothesis that biofilm development could have been subject to multistability, i.e. the existence of several possible stable biofilm configurations for the same set of environmental parameters; small variations in the species composition might have been sufficient to switch between these different configurations and thus have contributed to overwriting the original effects of temperature and flow velocity.

Key words: diversity, flow, light, microcosm, multistability, phototrophic biofilms, temperature, variability

Introduction

Microorganisms growing in close association with each other and with a surface, commonly enclosed in a matrix composed of extracellular polymeric substances (EPS), are collectively known as biofilms. In these communities the production of EPS and phenotypic diversification allow biofilms to adapt to environmental variations. Biofilms are the prevailing mode of microbial life in most natural habitats and are ubiquitous in illuminated aquatic environments, where they contribute significantly to ecosystem function and even to large-scale carbon fluxes (Battin *et al*., 2003). The importance of their role in ecosystem function is based on high levels of primary production, nutrient transformation and sediment stabilization (Wetzel, 2001; Sabater *et al*., 2002; Stal, 2010). Phototrophic biofilms also have an interesting and not yet fully realized potential for biotechnological exploitation, e.g. in biomass production and wastewater treatment processes (Guzzon *et al*., 2008; Roeselers *et al*., 2008; Posadas *et al*., 2013). The capacity of biofilms to efficiently use and store excess quantities of nitrogen and phosphorus and, perhaps most importantly, their easy harvesting make them a viable alternative to conventional chemical and physical tertiary treatments of wastewater (Guzzon *et al*., 2008; Boelee *et al*., 2012; Christenson & Sims, 2012; Posadas *et al*., 2013). It has been shown that efficiency in the uptake and retention of nutrients can depend on biofilm development, species composition, biomass production and environmental conditions (Craggs *et al*., 1996; Sabater *et al*., 2002; Guzzon *et al*., 2008). Against this complex background of potential influences, defining the effect of environmental variation on biofilm development and potential biomass production is still challenging scientists in several fields of research (Singer *et al*., 2006; Besemer *et al*., 2007; Paule *et al*., 2011).

Attachment of cells to a surface, and hence biofilm initiation, is a complex process regulated by diverse hydrodynamic and chemical characteristics of the

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surrounding medium, preconditioning of the substratum, cell surface characteristics, EPS secretion and the composition of the source community (Donlan, 2002; Wolf *et al*., 2007; Besemer *et al*., 2012). As biofilms develop, competition for resources such as nutrients, light and space is believed to select those species which are better at competing for a limiting resource. However, with the increasing complexity of maturing biofilms, competition for resources is likely to affect species diversity as a result of the associated niche diversification within the biofilm (Besemer *et al*., 2007). Recently, studies on stream bacterial biofilms have highlighted how biofilm assembly reflects not only differences in the source community, but the importance of species sorting in the process (Besemer *et al*., 2012).

Identification of the key processes which control biofilm development in nature is hence fraught with difficulties. Undetected fluctuations and local gradients and their impact at the individual level make it difficult to determine significant factors driving changes in biofilm dynamics. Given this scenario, we performed biofilm growth experiments using a microcosm approach to unravel the synergistic effect of diverse factors on biofilm development. Tight control of ambient variables (light, temperature and flow) in the microcosm results in a 'simplified but stable' environment, which is essential for assessing biofilm development and species succession (Roeselers *et al*., 2006). This approach has the potential to detect significant links between biofilm development and environment conditions, ultimately supporting the design of optimized synthetic cultures for water treatment, based on adapted wastewater treatment plant biofilms (Di Pippo *et al*., 2012).

Materials and methods

Biofilm sampling and inoculum treatment

Phototrophic biofilm samples were scraped off the sedimentation tank (ST) of an Italian wastewater treatment plant (WWTP) located in Fiumicino (Rome, Italy) twice in spring (April, June) and summer (July and September) of 2004. The plant serves the 'Leonardo da Vinci' Airport (41° 47′ N 12° 15′ E) and receives municipal waste sewage water with an estimated capacity of 6000 m^3 d^{-1}. The physicochemical characteristics of the plant have been previously described in detail (Albertano *et al*., 1999; Guzzon *et al*., 2008). Samples were subject to rigorous pre-treatment to obtain safe-to-handle and homogeneous inocula, used as source communities, following Guzzon *et al*. (2005). Briefly, biofilms were washed in 2% sodium hypochlorite to eliminate macro- and microzoobenthos and then rinsed in modified BG11 medium (Stanier *et al*., 1971). The modification of the BG11 medium included the addition of silicates (57 mg l^{-1}) for diatom growth and the lowering of NO_3^- and PO_4^- concentrations to approximate wastewater levels ($NaNO_3$ 0.15 g l^{-1}, K_2HPO_4 4 mg l^{-1}). Thereafter, biofilms were homogenized with a blender and allowed to settle for 1 h before freezing for 2 d at −20°C, to kill any remaining fauna. These cell suspensions were then used as inocula for this study, where 100 ml of cell suspension was made up to 4 l with the modified BG11.

Microcosm

The semi-continuous flow-lane incubator system contained four separate light chambers. The inocula were circulated through each chamber over growth substrata of polycarbonate slides. The medium was changed at regular intervals (twice per week). In each chamber a different irradiance was maintained (30, 60 and 120 µmol photons m^{-2} s^{-1}) over a 16:8 h light:dark cycle. Two temperatures (20 and 30°C) and two flow rates of 25 and 100 l h^{-1} (equivalent to current speeds of 5 and 20 cm s^{-1}) were also tested. These four incubator experiments were referred to as 'Runs' (Table 1); biofilm communities grown under different irradiances are abbreviated as L30, L60 or L120. Transmitted light was measured in each chamber by nine light sensors positioned under the growth surface. Growth of the biofilm could be accurately tracked by the reduction in light transmittance; biomass accumulation has previously been shown to be linearly related to the decrease of subsurface light below the substratum (Zippel *et al*., 2007). The incubator design is described in more detail by Zippel *et al.* (2007). Biofilm samples were taken when initial settlement, active growth phase and mature stage were reached, identified by light absorbance values, here defined as $Absorbance = 100\% - percent\ transmitted\ light$ of 10, 50 and 90%, respectively. From absorbance data diverse growth phase parameters were calculated: the lag phase time (λ [Days]); maximum absolute growth rate (μ [%/Day]); maximum absorbance value (A_{max} [%]; see below for further explanation). The duration of each experimental run was around 30 d and on the last day, biofilms were collected irrespective of light absorbance and labelled as last-day sample.

Phototrophic composition and biovolume calculation

To assess the composition of phototrophs in the inocula and in cultured biofilms, samples were suspended in 0.1 M phosphate buffer (pH 7.2), fixed with formaldehyde at 2% and glutaraldehyde at 2.5% final concentrations and stored at 4°C until observed. Observations were made using a ZEISS Axioskop light microscope equipped with differential interference contrast (DIC) using 40× and 100× objectives (Zeiss). When ultrastructural details were necessary for identification, material was observed using Transmission Electron Microscopy (TEM, Zeiss CEM 902 electron microscope) at 80 kV. Preparation of samples for TEM and observations of phototrophs were carried out on thin sections of

Table 1. Experimental conditions of the performed incubator runs.

Incubator run	Temperature (°C)	Flow velocity (l h^{-1})
Run1	20	25
Run2	20	100
Run3	30	25
Run4	30	100

biofilms as previously reported in Albertano *et al*. (1999). Phototrophic biomass was assessed for biofilms scraped from a known area of the polycarbonate slides by calculating the biovolume of individual taxa in images of optical fields, according to the procedure of Congestri *et al*. (2006), using the equations reported in Hillebrand *et al*. (1999). Throughout this paper total biovolume values are denoted as V_{Total}. The low magnification meant that distinction of different species of the cyanobacteria *Phormidium* and *Synechocystis* and of the green algae *Desmodesmus* and *Scenedesmus* was not possible during biovolume analysis and they are referred to as the genus name followed by spp.

Data analysis

All data analyses were performed with scripts written in the R Language and Environment for Statistical Computing, version 3.0.0 for Mac OS X (R Core Team, 2013). To assess the significance of ambient variable effect and their interactions, the standard ANOVA provided by the R Language was used in combination with the Tukey HSD test or pairwise t-tests with Holm correction of P values for multiple post-hoc testing. The influence of environmental conditions on different phases of biofilm development was evaluated by repeated-measures ANOVA. To quantify similarities in observations across different runs, distance matrices, based on Euclidean distance in the case of continuous variables, and Jaccard distance, in the case of presence-absence data, were computed using the default function in R. Hierarchical clustering based on the complete-linkage clustering algorithm was then applied to the resulting distance matrices to obtain the dendrograms reported in the corresponding heatmaps.

Analysis of the biofilm growth curves was preceded by smoothing the raw data with local polynomial regression fitting, using the *loess* routine in R, applying second-degree polynomials and a robust fitting algorithm. To parameterize each smoothed growth curve, the lag phase time (λ), i.e. the time (in units of days) to reach 10% absorbance, was extracted together with the maximum absorbance A_{max}. To determine the maximum absolute growth rate μ (in units of per cent Absorbance per day), each time series was segmented, and the segment containing the interval of maximum growth was fitted by linear regression. The resulting slope was considered to represent μ.

To quantify the diversity of the phototrophic biomass we computed the Gini–Simpson Index of Diversity (D), according to the equation

$$D = 1 - \sum_i p_i^2,$$

where $p_i = V_i / V_{Total}$ is the proportion of the biovolume of species i (Peet, 1974). This value has a theoretical range from 0 (no diversity) to 1 (infinite, maximum diversity).

Results

Phototrophic composition of the inocula

Cluster analysis of presence/absence data of phototrophs in the inocula, which were primarily composed of diatoms (11–15 taxa), cyanobacteria (7–9 taxa) and green algae (4–6 taxa), showed similarity between Inocula (In) 2 and 3 and Inocula 1 and 4 (Fig. 1). The main characteristic of the first cluster is the presence of all cyanobacterial taxa, while some diatoms and green algae were not observed. In contrast, the second cluster contains all the observed green algae and diatoms, but a lower number of cyanobacteria.

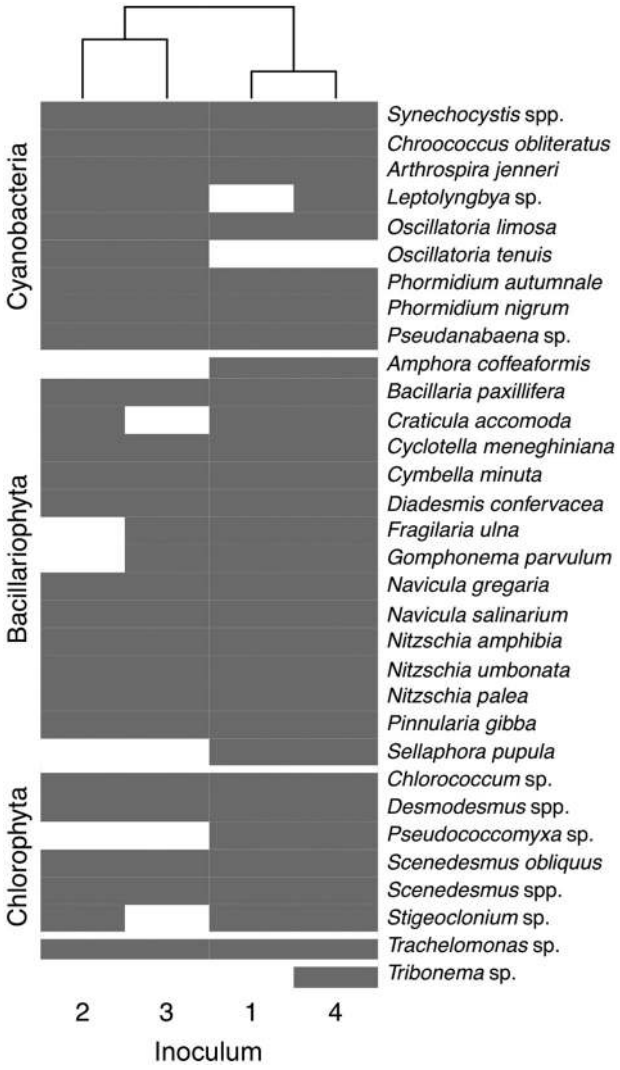


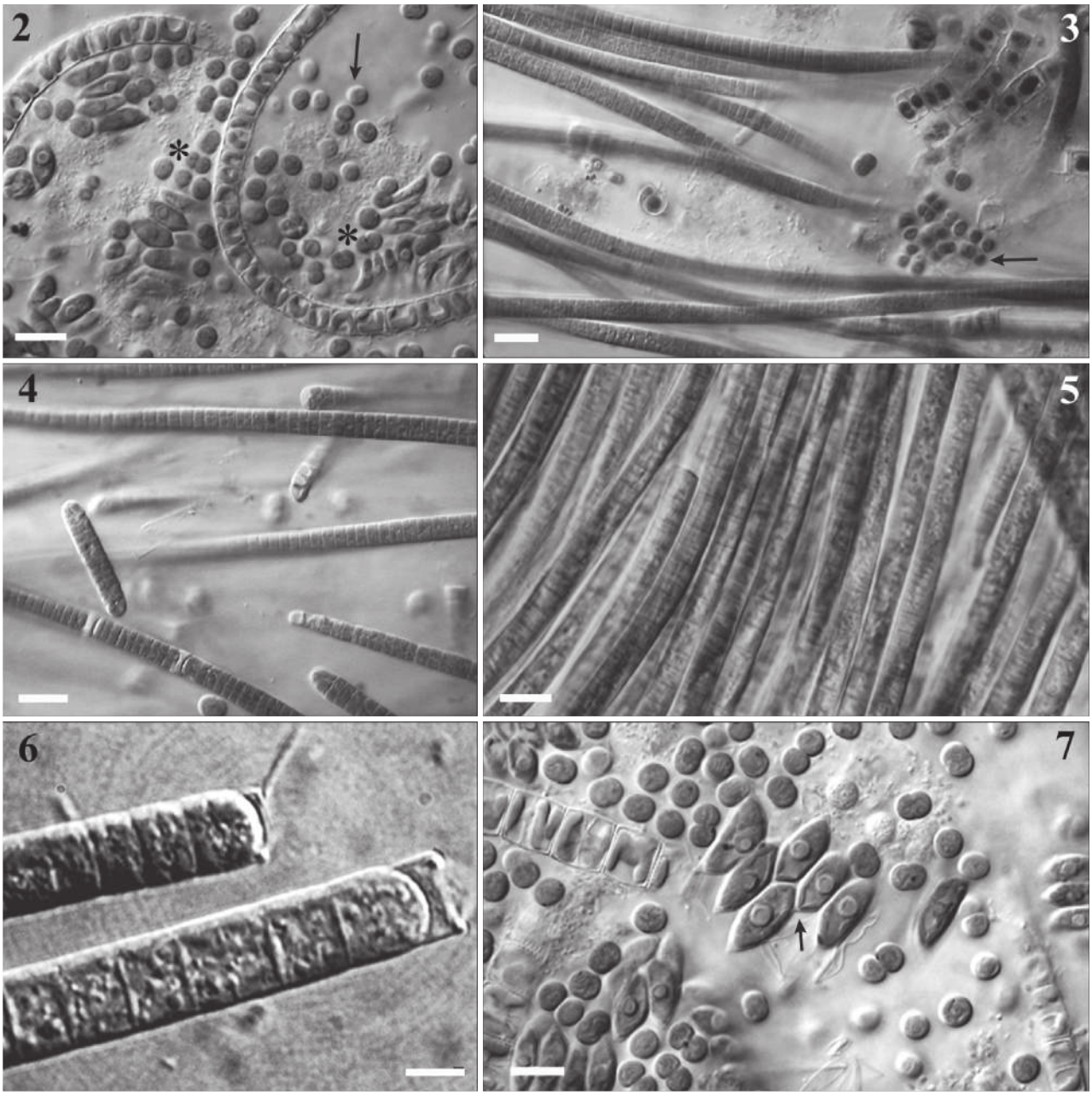
Fig. 1. Cluster analysis of the presence and absence of the major phototrophic taxa in the inocula. Grey boxes: presence; white boxes: absence. Inocula are ordered by their similarity, represented by the dendrogram on top.

Phototrophic composition of cultured biofilms

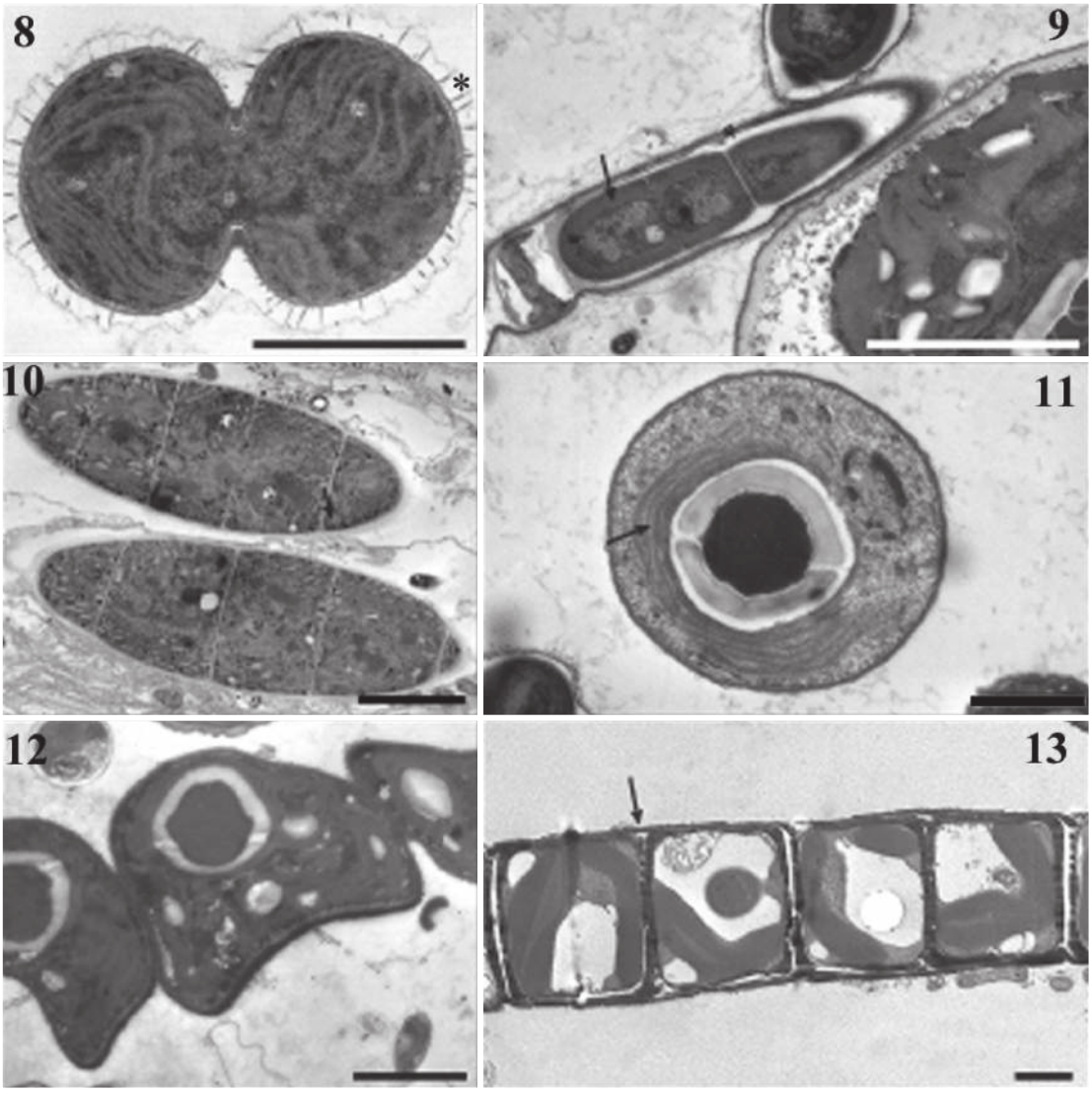
Taxon numbers in all biofilms were consistently lower than those of the inocula. In general, cultures were composed of up to six oscillatorialean morphotypes (Cyanobacteria), together with one to five Chroococcales (Cyanobacteria), raphid diatoms and unicellular or colonial forms of green algae. The euglenophyte *Trachelomonas* sp. was also observed.

Among the cyanobacteria there were two *Synechocystis* species. The most frequent was *S. aquatilis* Sauvageau with blue-green, rounded solitary cells (4.5–6 µm diameter), hemispherical after cell division (Figs 2 [arrow], 8). EPS cell envelopes were clearly seen during TEM analyses (Fig. 8). *Chroococcus obliteratus* Richter was also identified, with cells of 6–8 µm diameter, arranged in groups of two or four (Fig. 3, arrow). Filaments of *Leptolyngbya* sp. (2.5–2.7 µm width) and *Pseudanabaena* sp. (2.1–2.6 µm width) were also found, the latter with characteristic constrictions at cross walls and parietal arrangement of thylakoids, as visible in TEM longitudinal sections (Fig. 9). Three filamentous morphotypes belonged to the genus *Phormidium* (Figs 5, 6) based on trichome width, presence of isodiametric cells, absence of constricted cross walls and location of cell inclusions. The most frequent morphs were identified as *P. nigrum* (Vaucher ex Gomont) Anagnostidis et Komárek and *P. autumnale* (Agardh) Trevisan ex Gomont 1892 (Fig. 5). The latter had cells 5–7 µm wide and 3–5 µm long, with narrowed apex and thin EPS sheath (Fig. 10). Wider trichomes were attributed to *Oscillatoria* of two different morphotypes, *O. limosa* Agardh and *O. tenuis* Agardh (Fig. 4), found only rarely.

Unicellular and colonial chlorophytes represented the main green algae in the cultured biofilms. Their phenotypic plasticity made our taxonomic assessment



Figs 2–7. Light micrographs of the main phototrophs composing cultured biofilms. **Fig. 2.** Chains of *Diadesmis confervacea* are shown together with pointed *Scenedesmus* sp. cells, round (arrow) and dividing (asterisks) cells of *Synechocystis* sp.; **Fig. 3.** *Chroococcus* sp. (arrow); **Fig. 4.** *Oscillatoria tenuis*; **Fig. 5.** *Phormidium autumnale*; **Fig. 6.** *Phormidium* sp.; **Fig. 7.** *Scenedesmus* sp. colony (arrow). Scale bars: 10 µm.



Figs 8–13. TEM micrographs of the main phototrophs found in cultured phototrophic biofilms. **Fig. 8.** *Synechocystis* sp., showing a layered envelope with radial projections (asterisk); **Fig. 9.** *Pseudanabaena* sp., arrow indicates characteristic parietal arrangement of thylakoids in longitudinal section; **Fig. 10.** *Phormidium* sp.; **Fig. 11.** *Chlorococcum* sp., arrow indicates a single plastid and pyrenoid; **Fig. 12.** *Scenedesmus* sp.; **Fig. 13.** *Diadesmis confervacea*, overlap of girdle bands is visible in lateral view of a cell chain (arrow). Scale bars: 5 µm.

tentative: cells of about 15 µm in diameter, with one plastid occupying most of the cell lumen, and with a single pyrenoid were attributed to the genus *Chlorococcum* (Fig. 11). Members of the genera *Desmodesmus* and *Scenedesmus* (Figs 7, 12) were also observed; they occurred both as unicells (5–6 µm wide and 14–16 µm long) and as colonies, with cells in the colonies variably arranged.

The main representative diatom was the biraphid, filament-forming *Diadesmis confervacea* Kützing, which occurred in long chains maintained by a valve to valve connection between adjacent cells of 5–5.5 µm in width and 15–18 µm in length (Figs 2, 13). Members of the genus *Nitzschia*, namely *N. palea* (Kützing) W. Smith and *N. umbonata* (Ehrenberg) Lange-Bertalot, were extremely rare and in an unhealthy, senescent state.

Biomass accumulation

Biofilm attachment to the substratum in the initial stage of development was patchy when observed at a scale of ~1 cm. This was then followed by a stage in which growth occurred, filling in the spaces between the patches, resulting in a more homogeneous coverage of the substratum. On some occasions, biofilm detachment was noticeable as flocs within the circulating medium.

Biofilm growth was indicated by the increasing light absorbance derived from the decrease of subsurface light, as shown in the top panels of Fig. 14. Correlation analysis performed on the growth parameters λ, μ and A_{max} (lag phase time, maximum growth rate, maximum absorbance value), extracted from the smoothed growth curves, showed a significant negative correlation between λ and the other

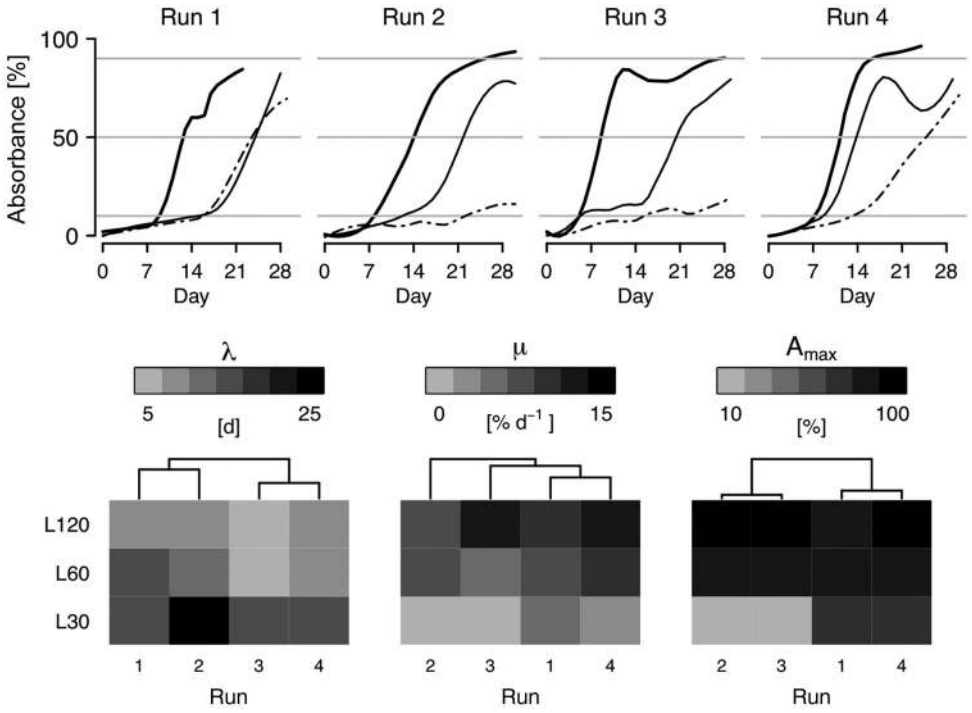


Fig. 14. Biomass accumulation. Top panels: time series of the biofilm absorbance along 30 d of observation in the four performed incubator runs. Bold: L120, Continuous: L60, Dashed: L30. Bottom panels: heatmaps of the growth curve parameters λ, μ, A_{max}.

parameters [r(λ:μ) = −0.77, P = 0.004; r(λ:A_{max}) = −0.79, P = 0.002)], while a significant positive correlation between μ and A_{max} was evident [r(μ:A_{max}) = 0.84, P = 0.001]. Hence, short lag times were positively correlated with high growth rates and maximum absorbance levels.

The heatmap of λ (Fig. 14, bottom left), obtained by clustering analysis, shows high similarities of Runs 1 and 2 and Runs 3 and 4. The first cluster (Runs 1 and 2) includes biofilms grown at 20°C and the second those obtained at 30°C. A two-way ANOVA of the λ values indicated that irradiance and temperature had the greatest significant effect (irradiance, $F_{2,6}$ = 14.7, P = 0.005; temperature $F_{1,6}$ = 8.9, P = 0.025), with no interaction between the two factors. Post-hoc testing indicated that at the lower temperatures (Runs 1 and 2), λ had an average value of 14.2 ± 5.5 d (mean ± SD) and was significantly longer than that of Runs 3 and 4 (9.8 ± 4.2 d, P = 0.025). Furthermore, λ of L30 (17.3 ± 3.9 d) was significantly higher than L60 (11.0 ± 4.4 d, P = 0.029) and L120 (7.8 ± 1.3 d, P = 0.004), but the difference between L60 and L120 λ values was not significant (P = 0.239).

The heatmap of μ (Fig. 14, bottom centre) indicates a high similarity between Runs 1 and 4, which form a separate cluster dissimilar to Runs 2 and 3. ANOVA indicated a significant impact of irradiance on μ ($F_{2,3}$ = 52.1, P = 0.01), expressed by a significantly lower mean value in L30 (3.1 ± 2.2 %/d) than in L60 (7.7 ± 1.5 %/d, P = 0.031) and L120 (10.2 ± 2.5 %/d, P = 0.009), with no significant difference between L60 and L120 (P = 0.149).

As with μ, the heatmap of A_{max} (Fig. 14, bottom right) suggests a high similarity between Runs 1 and 4 and Runs 2 and 3. The significant interaction in the two-way ANOVA between irradiance and clusters ($F_{2,6}$ = 71.1, P < 0.001), indicated that the dependence of A_{max} on irradiance was significantly different in the two clusters. Post-hoc testing showed that in the cluster Runs 1 and 4 there was a significant difference only between cultures grown at the highest (L120) and lowest (L30) irradiance tested (90.9 ± 7.6 vs. 70.5 ± 1.5%, P = 0.008), while in cluster Runs 2 and 3, there was a marginally significant difference between values obtained for L60 and L120 (78.9 ± 0.7 % vs. 91.9 ± 2.3 %; P = 0.07), and a highly significant difference between A_{max} in L30 (18.8 ± 2.7%) and in both L60 (P < 0.001) and L120 (P < 0.001).

Phototrophic biomass and diversity of cultured biofilms

In order to analyse taxon contributions to the total biofilm biomass, we considered only those species which had a proportion of more than 5% of the total phototrophic biovolume (V_{Total}) in at least one sample. This selection was composed of three green algal genera *Chlorococcum, Desmodesmus* and *Scenedesmus*, one diatom, *Diadesmis confervacea*, and three cyanobacterial taxa, *Leptolyngbya, Phormidium* and *Synechocystis*. V_{Total} of these taxa in the initial phase varied between 2.8 ± 0.3 10^6 μm^3 cm^{-2} (Run 4: L30) and 185.2 ± 17.1 10^6 μm^3 cm^{-2} (Run 2: L120, Fig. 15 bottom). In all cases, V_{Total} increased between the initial and

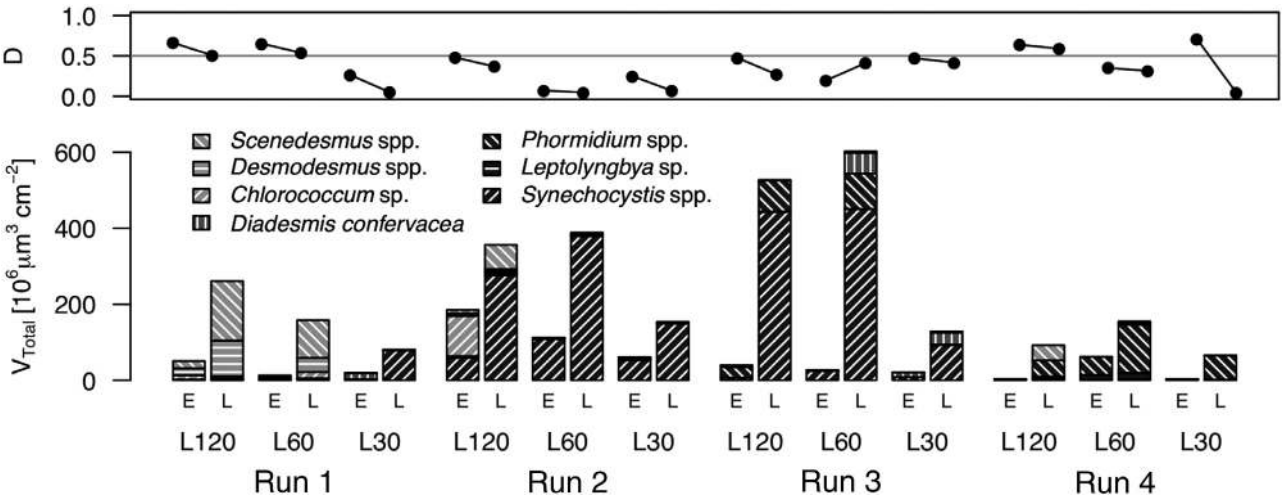


Fig. 15. Phototrophic diversity and biovolume values (V_{Total}). Top panel: Gini–Simpson Index of Diversity (D). Bottom panel: phototrophic biovolumes across all experiments. 'E' indicates the earliest observation, 'L' is the last, most mature sample.

mature/last day observations, at which V_{Total} ranged between a minimum of 65.2 ± 5.8 10^6 µm^3 cm^{-2} (Run 4: L30) and 602.6 ± 21.3 10^6 µm^3 cm^{-2} (Run 3: L60). As we missed two of the initial phase measurements (Run 1: L120 and Run 4: L60), we refrained from further analyses of V_{Total} values measured in the initial stage cultures. The phototrophic diversity of all biofilm cultures, assessed by the Gini–Simpson Index of Diversity (D), was calculated for both the earliest (initial) and the latest (mature/last day) phase in each experiment (Fig. 15, top). A reduction of diversity between the earliest and latest stage was observed in most experiments. Minimum and maximum values were retrieved in Run 4: L30, the maximum D (0.71 ± 0.02) in the initial phase and the minimum 0.04 ± 0.01, in mature stage cultures.

Figure 16 summarizes the analyses of potential effects of the environmental parameters on V_{Total}, D and species proportion. V_{Total} at the mature stage or on the last day showed a high similarity between Runs 1 and 4 and Runs 2 and 3 (Fig. 16, top right). The significant interaction between clusters and irradiance shown by two-way ANOVA ($F_{2,30}$ = 10.6, P < 0.001) revealed the different dependences of V_{Total} on irradiance. In Runs 1 and 4, values ranged between 72.8 ± 9.1 10^6 µm^3 cm^{-2} at L30 and 176.5 ± 94.3 10^6 µm^3 cm^{-2} at L120, but this difference was not significant (P = 0.182). In contrast, in Runs 2 and 3, V_{Total} of L30 (141.3 ± 14.5 10^6 µm^3 cm^{-2}) was significantly lower than values obtained for L60 (495.6 ± 118.9 10^6 µm^3 cm^{-2}, P < 0.001) and L120 (441.8 ± 98.9 10^6 µm^3 cm^{-2}).

Cluster analysis of diversity values suggested no association with temperature and flow, as evidenced by the clustering of Run 1 with Run 4 and of Run 2 with Run 3 (Fig. 16 middle row). While the association of Runs 2 and 3 was clear in both earliest and latest stages of development, the association of Runs 1 and 4 was weak in the latest stage biofilms. Repeated-measures ANOVA revealed a significant difference between the earliest and the latest stage diversity ($F_{1,8}$ = 6.9, P = 0.031) with post-hoc Tukey HSD test showing a lower diversity in the latest stage (P = 0.003). In spite of higher diversity values in Runs 1 and 4 (0.44 ± 0.23), no significant difference between Runs 1 and 4 and Runs 2 and 3 clusters were found. The effect of irradiance on diversity was marginally significant ($F_{1,4}$ = 4.6, P = 0.063) and post-hoc testing indicated that diversity in L120 was higher than in both L60 (P = 0.004) and L30 (P < 0.001), with no significant difference between L60 and L30 (P > 0.5).

Clustering the incubator runs based on the full set of species proportions again yielded the pattern of joining Runs 1 and 4 against Runs 2 and 3 both (Fig. 16 bottom row). Table 2 lists the species with the highest proportion p_i in each experiment. The high similarity of Run 2 and Run 3 is hence due to the overall dominance of *Synechocystis* spp. in all mature biofilms of both Runs (at least 72.3 ± 2.5%). By contrast, the green algae *Chlorococcum* sp. and *Scenedesmus* spp. prevailed in the initial stage of L60 and L120 of Run 1 and co-dominated, with cyanobacteria, the initial communities of Run 2: L60 and Run 2: L120. The only observed diatom, *Diadesmis confervacea*, had very low proportions in most experiments, but dominated under the lowest irradiance in the initial stages of both Run 1 and Run 3 (lower flow).

Discussion

Microcosm growth experiments using WWTP phototrophic biofilms as source communities revealed a complex interplay of biotic and abiotic factors acting on biomass accumulation and diversity, which was manifested in a few principal patterns of biofilm development observed across the 12 experimental conditions tested.

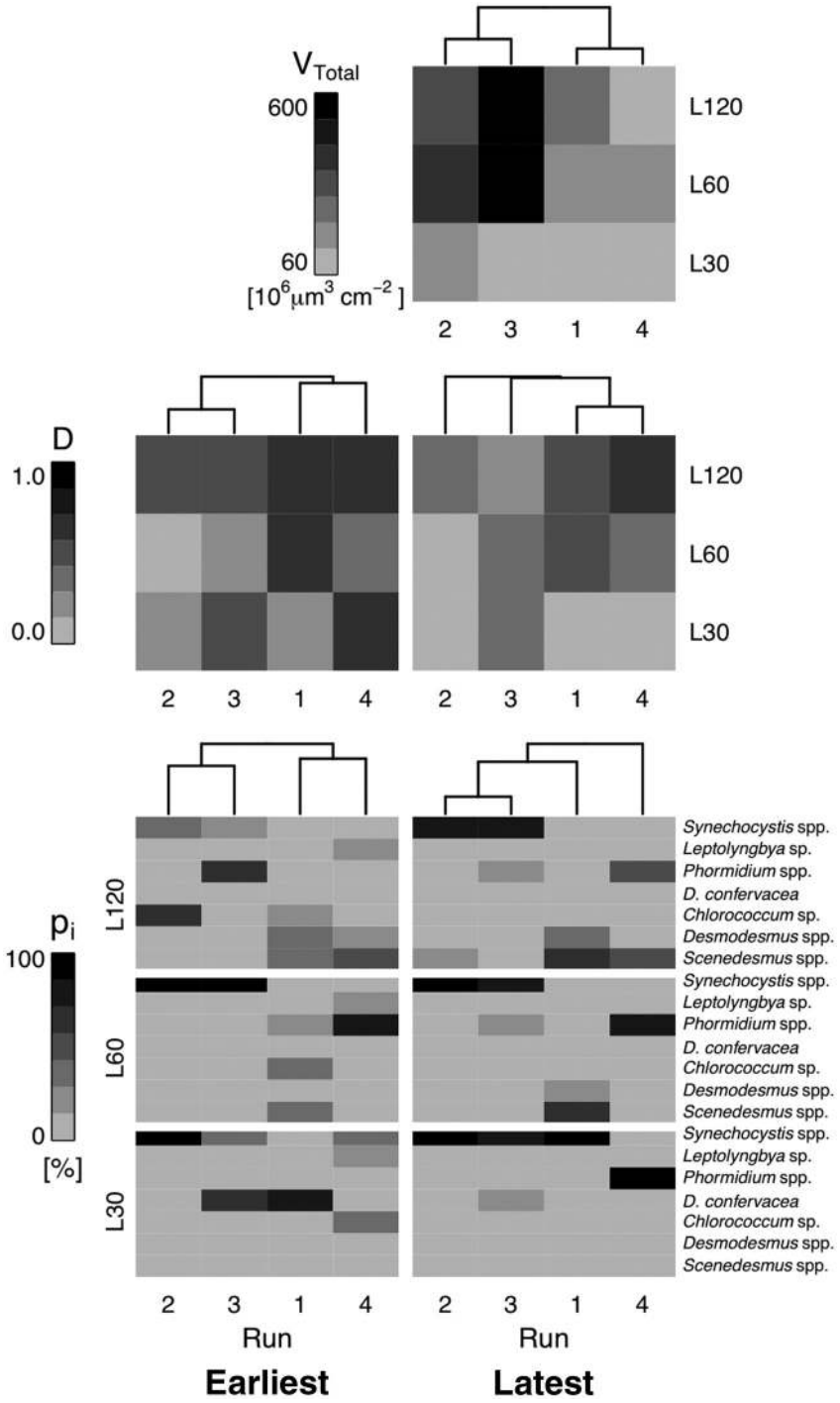


Fig. 16. Heatmaps of total biovolume (V_{Total}, top row), Gini–Simpson Index of Diversity (D, middle row) and species proportion (p_i, bottom row) data. Left column: earliest observation. Right column: latest observation.

One consistent phenomenon was the reduction in taxon richness of cultures, from the inocula through to initial- to mature-phase biofilms, which has also been reported in other experiments performed on phototrophic biofilms grown in reactors (Paule *et al.*, 2011). In a study on bacterial biofilms sampled from three streams (Besemer *et al.*, 2012) diversity was relatively consistent between sites and low in comparison to suspended communities, suggesting that: (i) biofilm assembly did not simply reflect differences in the source communities, but that certain microbial groups from the source community proliferate in the biofilm, (ii) the colonizers in each suspended community were similar and (iii) that the non-colonizers were mainly responsible for the differences in the three community diversities.

The initial inocula used in this study had the highest species richness with 26–31 phototrophic taxa, which was reduced to 7–10 taxa in the cultured biofilms. A contributing factor to this reduction may have been the inoculation period of 72 h, after which the medium was replaced, effectively removing the source community and preventing migration of more microorganisms into the biofilm

Table 2. Dominant taxa observed in each run. Values give mean and standard deviation of the proportion.

Incubator run	Irradiance (μmol photons m^{-2} s^{-1})	Earliest [%]	Latest [%]
1	120	*Scenedesmus* spp. 38.5±8.9	*Scenedesmus* spp. 60.1±2.4
1	60	*Chlorococcum* sp. 38.0±14.4	*Scenedesmus* spp. 62.7±1.3
1	30	*Diadesmis confervacea* 85.2±5.2	*Synechocystis* spp. 97.4±2.6
2	120	*Chlorococcum* sp. 59.3±23.7	*Synechocystis* spp. 77.4±2.4
2	60	*Synechocystis* spp. 96.4±0.9	*Synechocystis* spp. 97.6±0.7
2	30	*Synechocystis* spp. 86.3±2.5	*Synechocystis* spp. 96.5±0.6
3	120	*Phormidium* spp. 69.7±3.0	*Synechocystis* spp. 84.0±1.6
3	60	*Synechocystis* spp. 89.4±0.9	*Synechocystis* spp. 74.7±0.4
3	30	*Diadesmis confervacea* 62.1±1.6	*Synechocystis* spp. 72.3±2.5
4	120	*Scenedesmus* spp. 47.7±4.2	*Phormidium* spp. 46.3±1.8
4	60	*Phormidium* spp. 77.3±4.5	*Phormidium* spp. 82.0±1.3
4	30	*Synechocystis* spp. 31.6±3.5	*Phormidium* spp. 97.8±0.5

during the initial settlement phase. Moreover, the biofilm samples from the WWTP were mature and it is unlikely that the species within these communities had a high capacity to adhere to a bare substratum. So, whilst some species could colonize bare substrata, it is also credible that others could only adhere to existing biofilms due to the more heterogeneous surface texture and chemical characteristics. Thus, the composition of initial stage biofilms we cultured was possibly a function of the species' capacity to adhere to the surfaces; this initial settlement and growth might then have facilitated the adhesion of the other species.

Diversity of the community was also shown to decrease from the initial to mature phases of biofilm development, reflecting the constant microcosm environmental conditions (Sekar *et al*., 2002; Paule *et al*., 2011), which may have enhanced competitive exclusion. In most instances, species that colonized rapidly tended to be replaced by slower colonizing species (mainly cyanobacteria). Similar community changes, in combination with diversity reduction in ageing biofilms and cyanobacterial dominance, were also found in natural freshwater biofilms (Sekar *et al*., 2002). The decrease in diversity probably indicated a greater contribution to the community of the growth of species already present on the substrata compared with the settlement of new species, especially under nutrient replete conditions and reduced availability of bare substrata (Hillebrand & Sommer, 2000).

Of all environmental parameters tested, irradiance had the most significant effect on biofilm development across all incubator runs. At the lowest irradiance (30 μmol photons m^{-2} s^{-1}), the lag phase was longer and the augmentation of biofilm light absorbance was much reduced (in at least two runs) in comparison to high irradiance biofilms. At low- and mid-irradiance (30 and 60 μmol photons m^{-2} s^{-1}) there was a general dominance of cyanobacteria (except for the initial communities of the low flow runs), but at the highest irradiance (120 μmol photons m^{-2} s^{-1}) colonization by green algae was more evident. This probably reflects a combination of colonization ability and light resource competition but it is difficult to separate their effect on community composition. Bacteria and fast-growing microalgae are known pioneer species responsible for colonization of substrata (Sekar *et al*., 2002). The fast-growing green algae, *Chlorococcum, Desmodesmus* and *Scenedesmus,* are probably responsible for the shorter lag phase in biofilms grown at higher irradiances. This confirms findings from Roeselers *et al*. (2007) on the pioneering role of *Scenedesmus* attachment to polycarbonate substrata in parallel experiments on Fiumicino WWTP biofilms.

We also found that the lag phase was further shortened at higher temperature. Thus, an interaction between irradiance and temperature appeared to affect initial biofilm stages. High irradiance promotes the synthesis of EPS and a positive effect of high temperature on EPS production by benthic diatoms and cultured phototrophic biofilms has been documented (Staats *et al*., 2000; Di Pippo *et al*., 2009, 2012). It seems that increased EPS synthesis could have aided cell adhesion to the bare substrata in our system. Although there was no clear effect of flow rate on biofilm development, we observed some biofilm features that indicated a physical effect of low flow velocity. Cells in suspension under low flows generally have a thicker hydrodynamic boundary layer to overcome in order to attach to a surface; perhaps this is why initial colonization by the diatom *Diadesmis confervacea*, known to have a high settling rate and adhesion capacity (Congestri & Albertano, 2011), was predominant in the low flow (and low light) biofilms. Flow turbulence was kept to a minimum in order to be quasi-laminar, and so the hydrodynamic boundary layer probably had a large effect on cell–substratum interactions. Cells behave as particles in a liquid, and therefore the rate of settling will depend on flow velocity and cell density. Increased laminar-flow velocity will decrease the size of the boundary layer and therefore more rapid association with the surface would be expected, at least until velocities become high enough to exert substantial shear forces that prevent cell attachment. Flow of 100 l h^{-1} (20 cm s^{-1}) obviously did not

prevent colonization; in fact, one of the highest diversity values was detected at this flow rate.

Another pattern which emerged from statistical analyses of the growth descriptors μ and $A_{max}$was that increases in light intensity above 60 µmol photons m^{-2} s^{-1} did not produce concomitant increases in biofilm growth. In a similar experimental setup, saturation of photosynthesis was noted at 60 µmol photons m^{-2} s^{-1} along with an increase in the number of polyphosphate bodies at 120 µmol photons m^{-2} s^{-1} (Guzzon *et al*., 2008). These combined observations are indicative of the onset of cellular nitrogen and phosphorus storage, suggesting that light and nutrients must be in excess relative to what is needed for maximum growth.

Cluster analysis of the growth parameters, phototrophic biovolume and species diversity in culture, along with the composition of the inocula, revealed an unexpected pattern of high similarity between incubator runs performed under opposite temperature/flow combinations (Runs 2 and 3 cluster *vs* Runs 1 and 4). This clustering might be related to an unidentified random factor acting at some stage of the experimental procedure. Using the same incubators, source of natural biofilms, experimental procedures and culture conditions as the present study, Roeselers *et al*. (2006) assessed, by DGGE, the composition of mature biofilms grown in three incubators from different laboratories across Europe. The results showed a clear difference in communities between the different incubators and a low similarity within the corresponding irradiance. Roeselers *et al*. (2006) hypothesized that the large spatial heterogeneity of natural biofilms, providing many ecological microniches, in combination with inevitable turbulence and fluctuations occurring during biofilm development in culture, may lead to completely different microcosms even under otherwise constant conditions.

Recent experimental and modelling work by Bucci *et al*. (2012) suggested that, depending on the balance between fluctuations in the density of different bacterial groups and the strength of microbial interactions, complex communities may show multistability, i.e. even for a single set of experimental conditions, different microbial groups can potentially dominate the community. In the field of non-linear dynamics, such multistable systems are generally known to be highly sensitive to both noise and variations in initial conditions or system parameters (Feudel, 2008), as even small differences have the potential to switch the entire system from one configuration to another.

In both our study and that by Roeselers *et al*. (2006), data analysis showed unexpected but coherent patterns, which were less random than would be expected if biofilm development was completely noise-dominated. Therefore it seems more likely that high sensitivity to small differences in the initial community composition at least partially overwrote the specific effects of temperature and flow on biofilm development.

Experiments using natural phototrophic biofilms are important in understanding the function, evolution and development of these communities under realistic conditions. However, they come at a price of increased variability, which can hamper the investigation of the specific mechanisms that govern these communities of microbes. Microcosms can be a valuable and powerful approach to studying phototrophic biofilms, and provide much better control of the variability of the abiotic environment throughout the incubation period.

To complement studies with natural inocula, synthetic inocula containing well-defined proportions of some of the key species identified in our present work, such as *Synechocystis, Phormidium, Scenedesmus* or *Diadesmis confervacea* might help to further reduce complexity and randomness and gain important insights into biofilm development under certain environmental conditions as those of wastewaters.

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References

Albertano, P., Congestri, R. & Shubert, L.E. (1999). Cyanobacterial biofilms in sewage treatment plants along the Tyrrhenian coast (Mediterranean Sea), Italy. *Algological Studies/Archiv für Hydrobiologie*, **Suppl. 94**: 13–24.

Battin, T.J., Kaplan, L.A., Newbold, J.D. & Hansen, C.M.E. (2003). Contributions of microbial biofilms developing under various external factors. *European Journal of Phycology*, **40**: 1–8.

Besemer, K., Singer, G., Limberger, R., Chlup, A.-K., Hochedlinger, G., Hodl, I., Baranyi, C. & Battin, T.J. (2007). Biophysical controls on community succession in stream biofilms. *Applied Environmental Microbiology*, **73**: 4966–4974.

Besemer, K., Peter, H., Logue, J.B., Langenheder, S., Lindström, E. S., Tranvik, L.J. & Battin, T.J. (2012). Unraveling assembly of stream biofilm communities. *ISME Journal*, **6**: 1459–1468.

Boelee, N.C., Temmink, H., Janssen, M., Buisman, C.J. & Wijffels, R.H. (2012). Scenario analysis of nutrient removal from municipal wastewater by microalgal biofilms. *Water*, **4**: 460–473.

Bucci, V., Bradde, S., Biroli, G. & Xavier, J.B. (2012). Social interaction, noise and antibiotic-mediated switches in the intestinal microbiota. *PLOS Computational Biology*, **8**: e1002497.

Christenson, L.B. & Sims, R.C. (2012). Rotating algal biofilm reactor and spool harvester for wastewater treatment with biofuels by-products. *Biotechnology and Bioengineering*, **109**: 1674–1684.

Congestri, R. & Albertano, P. (2011). Benthic diatoms in biofilm culture. In *The Diatom World, Cellular Origin, Life in Extreme Habitats and Astrobiology*, Vol. 19, Part 2 (Seckbach, J. & Kociolek, P., editors), 227–243. Springer Science.

Congestri, R., Di Pippo, F., De Philippis, R., Paradossi, G. & Albertano, P. (2006). Seasonal succession of phototrophic biofilms in an Italian wastewater treatment plant: biovolume, spatial structure and exopolysaccharides. *Aquatic Microbial Ecology*, **45**: 301–312.

Craggs, R.J., Adey, W.H., Jenson, K.R., Green, F.B. & Oswald, W. J. (1996). Phosphorus removal from wastewater using an algal turf scrubber. *Water Science and Technology*, **33**: 191–198.

Di Pippo, F., Bohn, A., Congestri, R., De Philippis, R. & Albertano, P. (2009). Capsular polysaccharides of cultured phototrophic biofilms. *Biofouling*, **25**: 495–504.

Di Pippo, F., Ellwood, N.T.W., Guzzon, A., Siliato, L., Micheletti, E., De Philippis, R. & Albertano, P.B. (2012). Effect of light and temperature on biomass, photosynthesis and capsular polysaccharides in cultured phototrophic biofilms. *Journal of Applied Phycology*, **24**: 211–220.

Donlan, R.M. (2002). Biofilms: microbial life on surfaces. *Emerging Infectious Diseases*, **8**: 881–890.

Feudel, U. (2008). Complex dynamics in multistable systems. *International Journal of Bifurcation and Chaos*, **18**: 1607–1626.

Guzzon, A., Congestri, R. & Albertano, P. (2005). Light-induced changes in photosynthesis and structure of cyanobacterial cultured biofilms from an Italian wastewater treatment plant. *Algological Studies/Archiv für Hydrobiologie*, **Suppl. 117**: 223–238.

Guzzon, A., Bohn, A., Diociaiuti, M. & Albertano, P. (2008). Cultured phototrophic biofilms for phosphorus removal in wastewater treatment. *Water Research*, **42**: 4357–4367.

Hillebrand, H. & Sommer, U. (2000). Effects of continuous nutrient enrichment on microalgae colonizing hard substrates. *Hydrobiologia*, **426**: 185–192.

Hillebrand, H., Dürselen, C.D., Kirschtel, D., Pollingher, U. & Zohary, T. (1999). Biovolume calculation for pelagic and benthic microalgae. *Journal of Phycology*, **35**: 403–424.

Paule, A., Lauga, B., Ten-Hage, L., Morchain, J., Duran, R., Paul, E. & Rols, J.L. (2011). A photosynthetic rotating annular bioreactor (Taylor-Couette type flow) for phototrophic biofilm cultures. *Water Research*, **45**: 6107–6118.

Peet, R.K. (1974). The measurement of species diversity. *Annual Review of Ecology, Evolution, and Systematics*, **5**: 287–307.

Posadas, E., García-Encina, P.A., Soltau, A., Domínguez, A., Díaz, I. & Muñoz, R. (2013). Carbon and nutrient removal from centrates and domestic wastewater using algal-bacterial biofilm bioreactors. *Bioresource and Technology*, **139**: 50–58.

R Core Team (2013). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna. http://www.R-project.org/.

Roeselers, G., Zippel, B., Staal, M., van Loosdrecht, M.C.M. & Muyzer, G. (2006). On the reproducibility of microcosm experiments – different community composition in parallel phototrophic biofilm microcosms. *FEMS Microbiology Ecology*, **58**: 169–178.

Roeselers, G., van Loodsdrecht, M.C.M. & Muyzer, G. (2007). Heterotrophic pioneers facilitate phototrophic biofilm development. *Microbial Ecology*, **54**: 578–585.

Roeselers, G., van Loosdrecht, M.C.M. & Muyzer, G. (2008). Phototrophic biofilms and their potential applications. *Journal of Applied Phycology*, **20**: 227–235.

Sabater, S., Guasch, H., Romanì, A. & Munõz, I. (2002). The effect of biological factors on the efficiency of river biofilms in improving water quality. *Hydrobiologia*, **469**: 149–156.

Sekar, R., Nair, K.V.K., Rao, V.N.R. & Venugopalan, V.P. (2002). Nutrient dynamics and successional changes in a lentic freshwater biofilm. *Freshwater Biology*, **47**: 1893–1907.

Singer, G., Besemer, K., Hödl, I., Chlup, A., Hochedlinger, G., Stadler, P. & Battin, T.J. (2006). Microcosm design and evaluation to study stream microbial biofilms. *Limnology and Oceanography Methods*, **4**: 436–447.

Staats, N., Stal, L.J., de Winder, B. & Mur, L.R. (2000). Oxygenic photosynthesis as a driving process in exopolysaccharide production in benthic diatoms. *Marine Ecology Progress Series*, **193**: 261–269.

Stal, L.J. (2010). Microphytobenthos as a biogeomorphological force in intertidal sediment stabilization. *Ecological Engineering*, **36**: 236–245.

Stanier, R.Y., Kunisawa, R., Mandel, M. & Cohen–Bazire, G. (1971). Purification and properties of unicellular blue-green algae (Order Chroococcales). *Bacteriological Reviews*, **35**: 171–205.

Wetzel, R.G. (2001). Land-surfaces: attached microorganisms, littoral algae, and zooplankton. In *Limnology: Lake and River Ecosystems*, 577–623. Academic Press, San Diego.

Wolf, G., Picioreanu, C. & van Loosdrecht, M.C.M. (2007). Kinetic modeling of phototrophic biofilms: the PHOBIA model. *Biotechnology and Bioengineering*, **97**: 1064–1079.

Zippel, B., Rijstenbil, J. & Neu, T.R. (2007). A flow-lane incubator for studying freshwater and marine phototrophic biofilms. *Journal of Microbiological Methods*, **70**: 336–345.