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The architecture and metabolic traits of monospecific photosynthetic biofilms studied in a custom flow-through system

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Running title: architecture and physiology of microalgae biofilms

Abstract

Microalgae biofilms have a great ecological importance and a high biotechnological potential. Nevertheless, an in-depth and combined structural (i.e. the architecture of the biofilm) and physiological characterization of microalgae biofilms is still missing. An approach able to provide at the same time physiological and structural information during biofilm growth would be of paramount importance to understand these complex biological systems and to optimize their productivity. In this study, monospecific biofilms of a diatom and a green alga were grown under dynamic conditions in custom flow cells represented by UV/Vis spectroscopic cuvettes. Such flow cells were conceived to characterize the biofilms by several techniques *mostly in situ* and in a non-destructive way. Physiological traits were obtained measuring variable chlorophyll *a* fluorescence by pulse amplitude modulated fluorometry (PAM) and by scanning the biofilms in a spectrometer to obtain *in vivo* pigments spectral signatures. The architectural features were obtained by imaging the biofilms with a confocal scanning laser microscope (CLSM) and an optical coherence tomograph (OCT). Overall, this experimental setup allowed us to follow the growth of two biofilm-forming microalgae showing that cell physiology is more affected in complex biofilms likely as a consequence of alterations in local environmental conditions.

Keywords: CLSM, flow cell, FTIR-spectroscopy, Microalgae biofilms, OCT, PAM

Introduction

Microalgae biofilms play a crucial ecological role in most aquatic ecosystems as they contribute substantially to the primary production of shallow waters (Buhmann et al., 2012). In most coastal and estuarine areas, they also stabilize sediments and promote the formation of microbial communities essential for the functioning of the main biogeochemical cycles in such environments. With respect to their planktonic counterparts, microalgae biofilms present biomass densities that can be 10 to 100 times higher (Ozkan et al., 2012; Podola et al., 2017). Such characteristic can provide strong resistance to grazing and pollutants but it can also create very strong gradients of light and nutrients making them rapidly limiting in some locations within the biofilm (Battin et al., 2016). For biotechnological applications, the high productivity of microalgae biofilms has been recently recognized as a key advantage of biofilm-based systems over classical photo-bioreactors where microalgae are in the planktonic state (Berner et al., 2015; Gross et al., 2015).

85 Despite the importance of characterizing the physiology of photosynthetic biofilms, the
86 majority of studies have been performed on mixed natural communities (e.g.
87 microphytobenthic biofilms with several microalgae taxa and bacteria), and only few studies
88 have investigated in parallel their physiology and architecture (i.e. specific 3D organization of
89 cells and matrix components) (Mallick et al., 2020; Stiefelmaier et al., 2020; Wijihastuti et al.,
90 2017). However, understanding how the metabolic activity of cells changes as biofilm 3D
91 structure matures might help identifying key biofilm traits to better comprehend such a
92 complex life style in nature and to optimize biotechnological processes. Nutrient limitation or
93 light acclimation might be identified on the basis of chlorophyll *a* variable fluorescence
94 (Beardall et al., 2001) or on the base of changes of specific macromolecular pools such as
95 lipids and carbohydrates (Dean et al., 2010; Jebsen et al., 2012). Furthermore, the parallel
96 study of both physiological and architectural biofilm traits might reveal structural-dependent
97 changes or species-specific acclimation strategies in photosynthetic biofilms. In this context,
98 the development of a system that allows measurements of 3D structures and physiological
99 properties of microalgae biofilms is of great importance.

100 In order to simulate environmental conditions, biofilms are experimentally grown under
101 dynamic conditions, typically reached by continuously pumping fresh medium into a flow cell
102 where the biofilm is developing (Fanesi et al., 2021; Le Norcy et al., 2019). Several systems
103 exist for the study of bacterial and photosynthetic biofilms (Buhmann et al., 2012; Forster and
104 Martin-Jézéquel, 2005; Heydorn et al., 2000; Lemke et al., 2021; Wijihastuti et al., 2017;
105 Zippel et al., 2007) but only few of them allow for multiple *in-situ* measurements. In this
106 study, we developed a user friendly flow-through system that can be used to grow
107 photosynthetic biofilms continuously under controlled laboratory conditions. The flow cell is
108 conceived using a four-face clear spectrophotometer cuvette that allows to easily reproduce
109 the system in different labs and to switch between several measurements modes. The system

was tested using two biofilm-forming microalgae (a diatom and a green algae) that produce biofilms with different architectural characteristics (Fanesi et al., 2019). Indeed, specific architectures may translate in the formation of light and nutrient gradients (including gases such as CO₂) as a function of biofilm complexity (Battin et al., 2016; Schnurr and Allen, 2015). We therefore tested the hypothesis that photosynthetic cells producing biofilms with high structural complexities (e.g. high thickness and cell abundances) may need to acclimate and change their physiological performances in order to optimize their fitness in the changing local conditions (Stewart and Franklin, 2008). A consequence of this hypothesis is that a thick biofilm would be expected to induce stronger gradients and therefore more changes in cell physiology with respect to a thinner biofilm where light and nutrients gradients are weaker and may impact less cell metabolic performances.

In order to test our hypothesis, the structural characteristics of photosynthetic biofilms were monitored by means of complementary techniques. Confocal Laser Scanning Microscopy (CLSM) at the microscopic scale and Optical Coherence Tomography (OCT) at the mesoscale allowing a higher penetration in the sample (Wagner and Horn, 2017). Photosynthetic efficiency was measured using a pulse amplitude modulated (PAM) fluorometer and pigments and macromolecules signatures were obtained using a UV-Visible and an ATR-FTIR spectrometer. All the techniques were chosen because they are non-destructive and **have the potential to** allow for *in situ* measurements.

Materials and Methods

Planktonic culture maintenance

The planktonic cultures of *Cylindrotheca closterium* AC170 (Caen, France) and *Chlorella vulgaris* SAG 211-12 (Göttingen, Germany) were grown in 250 mL Erlenmeyer flasks filled with 30 mL of growth medium (artificial sea water (Lyman and Fleming, 1940) + Walne's

medium (Walne, 1970)) for *C. closterium*, 3N-Bristol for *C. vulgaris* (Bischoff and Bold, 1963) and were kept in the exponential phase by frequent re-inoculations (every 3 days). The cultures were continuously shaken and grown in an incubator at 25°C under a continuous photon flux density (PFD) of 20 $\mu\text{mol m}^{-2}\text{s}^{-1}$.

Development of the cuvette flow-through system for biofilm cultivation

A bottle of sterilized medium is connected to the flow cell, which is connected to a waste bottle using silicon tubes (I.D. 1mm). The medium is pumped inside the system by means of a multichannel peristaltic pump 205S/CA (Watson and Marlow; La Queue Lez Yvelines, France).

The innovative part of the flow-through incubation system conceived in this study is the use of a spectrophotometer cuvette (Fig. 1a,b). Such a flow cell was conceived using a four face clear cuvette made of poly(methyl methacrylate) (PMMA) (01961-00, Kartell S.p.A., Italy; total volume of 4mL; 1 cm optical path length), a plastic cap (XK25.1, Roth) and two connectors: one for the inflow medium was made from a glass capillary (micro-hematocrit capillary tubes, 1.2 mm I.D.) whereas the second was made from a 10 μL pipet tip for the outflow. Two holes were made on the plastic cap (about 6-7 mm of distance). In one hole, the plastic tip was fitted in and in the second the glass capillary. The plastic tip has a length of about 10mm, 5 mm are inside the cuvette and 5mm are used to connect the silicone tube. The capillary was cut to a final length of 40mm: 35mm are inside the cuvette and 5mm are used to connect the silicon tube (Fig. 1a). The difference in length between the glass capillary and the plastic tip is necessary in order to avoid short circuit between the inflow and the outflow of medium. Once fitted in, the two connectors were sealed on the plastic cap using transparent silicon in order to avoid leakages. Once completed, the external edges of the cap were filled

with silicon and the whole cap inserted in the cuvette and the flow-through system was let dry overnight before using it for the experiments.

The optical characteristics and the standard design of the cuvette allow to perform *in situ* and in a non-destructive way measurements with multiple instruments. In our case, the choice of the measurements was done in order to get a complete characterization of biofilm structural, metabolic and compositional properties.

Inoculum and mode of operation of the cuvette flow-through cell for photosynthetic biofilms growth

Before each set of experiments, the complete system including the cuvette flow cells were sterilized by pumping a solution (0.5% v/v) of commercial bleach for two hours. The system was then washed with two liters of autoclaved MilliQ water and filled with the specific sterile growth media used for the two algae (see *planktonic culture* section).

For the inoculum of the system, an aliquot of planktonic culture containing 5×10^5 cell/mL for *C. closterium* and 10^6 cell/mL for *C. vulgaris* were injected using an in-line luer injection port (Ibidi GmbH, Germany) placed just before the cuvette. The cuvette inlet and outlet were then closed with clips and were gently shaken by hand to obtain a homogeneous suspension of cells. The cuvettes were then laid down horizontally for 24 hours (under $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) to induce the formation of the biofilm only on one face of the cuvette. After this time interval, the medium was flown at a flow rate of 0.2 mL min^{-1} (a good compromise to avoid large volumes of growth medium and leakages) and the light was continuously provided at a photon flux density of $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ from the top using an Alpheus illumination system that uses a mix of blue, white and red LEDs (Alpheus LED, Montgeron, France; see figure S1 for the emission spectra of the LEDs).

In this study, the biofilms were grown for four days, a time necessary (based on our inoculum size) to reach a *plateau* and to avoid colonization of the biofilms on all faces of the cuvette. For each experiment, a set of 6 flow-through cells was used. Three flow cells were used over the total length of the experiment (4 days) and measured every day at the spectrophotometer, CLSM, OCT, PAM and for light attenuation while of the remaining three, one flow cell for each day was opened and the biofilm scraped for FTIR measurements. Each experiment was repeated three times over three different weeks in order to obtain independent assays.

Light attenuation

Photon flux density was measured using a light meter (LI-190/R; LI-COR Biosciences GmbH, Bad Homburg, Germany). Light attenuation by the biofilms was monitored by placing the flow-cells on the sensor of the light meter. Light attenuation was measured in three positions along the length of each cuvette. The attenuation of light was expressed with respect to the transmitted light measured before the inoculation of the flow cells (transmitted light₍₀₎; measured at day 0) as follow:

$$\text{Light attenuation}_{(i)} (\%) = [1 - (\text{transmitted light}_{(i)} / \text{transmitted light}_{(0)})] \cdot 100 \quad (1)$$

Where *i* represents the day during the growth of the biofilms.

Chlorophyll a variable fluorometry (PAM)

The photosynthetic efficiency of the biofilms was determined using a portable pulse amplitude modulation (PAM) fluorometer (AquaPen, AP 110-C, Photon Systems Instruments, Drasov, Czech Republic) as described in Li et al. (Li et al., 2021). Briefly, the measurements were performed by placing the face of the cuvette colonized by the biofilms in contact with

the blue LED (455 nm) of the instrument, the measuring light was $0.02 \mu\text{mol m}^{-2} \text{s}^{-1}$ and saturation pulses had an intensity of $3000 \mu\text{mol m}^{-2} \text{s}^{-1}$. Furthermore, in order to understand if the biofilm lifestyle might interfere with the measurements, the biofilms of both microalgae were also measured after resuspension in the respective growth media (OD_{676} adjusted to 0.1 in order to have optically thin samples). Before each measurement, all samples were dark-adapted for 10 minutes. Afterward, the samples were exposed to seven actinic lights (from 0 to $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$) applied every 60s. The maximum quantum yield (F_v/F_m) and the effective quantum yield ($\Delta F/F'_m$) were calculated according to Eq. (2) and Eq. (3):

$$F_v/F_m = (F_m - F_0)/F_m \quad (2)$$

$$\Delta F/F'_m = (F'_m - F)/F'_m \quad (3)$$

where F_0 and F_m are the minimum and max fluorescence determined after 10 min dark-adaptation, whereas F and F'_m are the minimum and max fluorescence during illumination. The relative electron transport rate (rETR) was calculated using the Eq. (4):

$$\text{rETR} = \Delta F/F'_m \times \text{PFD} \times 0.5 \quad (4)$$

Where PFD is the incident photon flux density and 0.5 is a factor assuming that two photons are required for linear electron transfer. Light curves were quantitatively compared using the parameters of maximum rate of relative ETR (rETR_{max}), α (the initial slope of the curves) and E_k ($E_k = \text{rETR}_{\text{max}}/\alpha$; the photon flux density at saturation) obtained by fitting the rETR vs. PFD curves with the rectangular hyperbola $\text{rETR} = \text{rETR}_{\text{max}} (1 - e^{-\alpha \text{PFD}/\text{rETR}_{\text{max}}})$ (Ralph and Gademann, 2005).

233 *ATR-FTIR spectroscopy*

234 Each day, a biofilm was scrapped from one of the cuvettes. The samples were centrifuged at
235 8000 · g for 5 minutes and the supernatant removed. After that, 1 mL of distilled water in the
236 case of *C. vulgaris* and 1 mL of a solution of NaCl (35 g · L⁻¹) for *C. closterium* were used to
237 wash the biofilm suspensions. Afterward, the pellet was re-suspended in 5-10 µL of distilled
238 water or NaCl and 1.5 µL were transferred on a 45° ZnSe flat crystal of an ATR-FTIR
239 PerkinElmer Spectrum-two spectrometer (PerkinElmer, Waltham, USA) and the sample was
240 dried at room temperature for 20 minutes. Spectra were acquired in the range 4000 and 400
241 cm⁻¹ using 32 accumulations at a spectral resolution of 4 cm⁻¹. Before each measurement, the
242 empty crystal was measured using the same instrumental setting and used as a blank.
243 Spectra were baselined and the ratios carbohydrates to proteins, lipids to proteins and
244 carbohydrates to lipids were calculated as the ratios between the maximum absorption values
245 for the spectral ranges corresponding to proteins (Amide I, νC=O; 1700–1630 cm⁻¹), lipids
246 (νC=O; 1750–1700 cm⁻¹), and carbohydrates (νC–O–C, and νSi–O in diatoms; 1200–950
247 cm⁻¹). The band assignments were based on Sacket et al. (Sackett et al., 2013). The ratios
248 were calculated to follow over time the changes in the macromolecular composition of the
249 cells.

250

251 *In-vivo absorption visible spectroscopy*

252 Absorption spectra were measured using a UV-Vis spectrometer (Evolution 60S;
253 Thermoscientific). Absorption spectra were measured in the range 400-750 nm using a
254 spectral resolution of 1 nm. In the first days of growth each biofilm was scanned at least 8
255 times and the spectra averaged to increase the signal to noise ratio. The absorption at 750 nm
256 was further subtracted to all the wavelengths to remove the background. The ratio between the
257 absorption at 435 nm and that at 676 nm (Abs₄₃₅ to Abs₆₇₆ ratio) was further calculated as an

index of accessory pigments to chlorophyll *a* ratio in *in vivo* microalgae spectra (Dubinsky et al., 1986; SooHoo et al., 1986).

Confocal laser scanning microscopy and image analysis

The biofilms were scanned using a confocal laser scanning microscope (CLSM) to obtain z-stacks of cells signal. The images were acquired using an inverted Zeiss LSM700 confocal microscope (Carl Zeiss microscopy GmbH, Jena, Germany) equipped with a LD Plan-Neofluar 20x/0.4 Korr M27 objective with a 0.4 N.A. (numerical aperture). Each image (1568 x 1568 pixels; 12 bit) was 638 · 638 µm in size with a z-step of 3.9 µm and a lateral resolution of 0.41 µm. Each flow-cell was scanned in at least 6 random positions, from the inlet to the outlet, along the length of the cuvette.

Cells were detected by chlorophyll *a* auto-fluorescence. Chlorophyll *a* was excited at 639 nm and was detected using a long pass filter 615 nm. On the last day of growth, a biofilm cultivated in one of the cuvettes was also stained using a mix (5µL each diluted in 1 mL of respective growth media) of FITC-lectins (CON A, RCA I, SBA, PNA, DBA, WGA, UEA I; Vector Laboratories, Peterborough, UK) to visualize the matrix (i.e. the glycoconjugates) and to qualitatively compare the biofilms. This solution was later injected in a cuvette containing a biofilm that was incubated in the dark for 30 minutes before the acquisition of the images. FITC was excited using the 488 nm laser line.

A custom script running in Fiji (Schindelin et al., 2012) was used to extract from the z-stacks the quantitative parameters (based on the Chl*a* fluorescence) used to characterize biofilm architecture: biovolume, maximal and mean thickness and roughness (See supporting information for calculations; Tab.S1). The structural parameters were calculated according to the computations described in Heydorn et al. (Heydorn et al., 2000; see also Tab.S1) and the thresholding was performed using the Otsu algorithm (Otsu, 1979) considering the histogram

of the whole stack. The z-stacks were obtained over the whole length of the cuvette and the results averaged.

Optical coherence tomography (OCT)

The meso-structures of the biofilms were visualized using a spectral domain Optical Coherence Tomograph (OCT; Ganymede 621, Thorlabs GmbH, Lübeck, Germany) with a central light source wavelength of 900 nm. The images were acquired using a telecentric scan lens (OCT-LK3-BB) at a speed of 25kHz per A-scan and an averaging of 6 A-scan. The field of view was 4 x 4 mm (XY) and the axial depth 1 mm (Z). The lateral pixel resolution was 8 µm and the axial pixel resolution was 1.45 µm. For all the acquisitions a refractive index of 1.33 was used as the biofilms were imaged *in situ* in liquid medium (Wagner and Horn, 2017). Images were analysed using the software ThorImageOCT 5.4.4 (Thorlabs, Lübeck, Germany). OCT measurements were performed only on one of the cuvettes to show the versatility of the flow cell system.

Statistics

Statistics was performed using GraphPad prism 5.0 (San Diego, CA, USA) and the R software (R Core Team, 2014). Before statistical analysis, the distribution of the data was investigated using the Shapiro-Wilk's test. Data were transformed (log or square root) in the case they did not followed a normal distribution ($P < 0.05$). To evaluate the significance of mean differences over time, One-way and Two-way ANOVA followed by the Tukey's post-hoc test for multiple comparisons were carried out ($ns = P \geq 0.05$; $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$). The level of significance was always set at 5%. The heatmap was performed in R using the package "NMF" (Gaujoux and Seoighe, 2010). The analysis was conducted on the mean values of all experiments performed in the study and the matrix of variables was further

scaled using z-score scaling. Clustering of the variables was obtained using the Euclidean distance.

Results and Discussion

Biofilm architecture is species dependent

The flow cell developed in this study was used to easily cultivate microalgae biofilms and to use several optical approaches to characterize their structures and physiology (i.e. metabolic activity and biochemical composition).

The optical properties of the cuvettes allowed to acquire high quality images by means of CLSM and OCT (Fig.2, Fig.S2, Fig.S3). The two species were chosen since they form biofilms with different architectures (Fanesi et al., 2019; Fanesi et al., 2021; Le Norcy et al., 2019) and were therefore good experimental organisms to test the hypothesis that complex architectures may induce stronger physiological adjustments in the cells. Figure 2, Fig.S2 and Fig.S3 show indeed that biofilm structure (both cells and EPS organizations) was species-dependent, in line with previous results (Fanesi et al., 2019; Le Norcy et al., 2019) and it appeared to be homogeneous along the length of the cuvettes (Fig.S4). *C. closterium* produced biofilms that were twofold thicker and that produced 2.4 times more biomass than those of *C. vulgaris* (Fig. 3a,b). Biofilms produced by *C. vulgaris* did not seem to grow much in thickness (~20µm) but seemed mostly to become denser on the substrate surface (Fig.3a,b). The increase in biomass in *C. closterium* on the contrary matched an increase in thickness of the biofilms that reached up to 80 µm final thickness (Fig.2 and Fig.3a,b). These results are in agreement with previous reports about microalgae biofilms (Fanesi et al., 2019; Fanesi et al., 2021; Faÿ et al., 2010; Le Norcy et al., 2019) and are likely related to the preferential lifestyle of the two species: *C. closterium* is commonly found forming microphytobenthos communities in mudflats whereas *C. vulgaris* is generally a planktonic species. However,

under specific conditions, both have been reported to switch to the respective opposite lifestyle for example due to sediment resuspension for the first or contaminations and shift in light spectra for the latter (Aigner et al., 2020; Hultberg et al., 2014; Irving and Allen, 2011; Kingston, 2009).

The production over time of daughter cells led to a stronger light attenuation through the biofilms in both species (Fig.3d). After four days of development, *C. closterium* attenuated almost 60% of the incoming photons whereas *C. vulgaris* only 20%. Therefore, the lower biomass produced by *C. vulgaris* was likely not related to a light limitation, indeed the compensation point for *C. vulgaris* biofilms has been reported to be in the range 30-60 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ (Rincon et al., 2019). Furthermore, recently Fanesi et al. (Fanesi et al., 2021) showed that even when biofilm architecture changes, *C. vulgaris* maintains constant diffusion characteristics in its biofilm. It results that a limitation by nutrients under dynamic conditions should be excluded. On the other hand, the authors demonstrated that at low shear stress the biofilms were very loose and that cells detached more easily from the biofilm. It is therefore probable that the hydrodynamic conditions used did not allow the cells to build up strong and biomass-rich biofilms.

Biofilm architecture affects cell physiology

Interestingly, the different architectural features exhibited by the biofilms of the two algal species were reflected in different physiological responses of the cells.

The photosynthetic performance of the cells was assessed by PAM fluorometry and the cultivation of the biofilms on one of the faces of the flow cell-cuvette allowed to perform the measurements directly *in situ*. The samples were also resuspended to test possible interferences of the biofilm lifestyle with the measurement, in this case structural integrity of the biofilms was however lost. Interestingly, the results presented in Fig.4, Fig.S5 and Tab.S2

and S3 show a strong dependency of the measurements on the species considered and on the thickness of their biofilms. Regardless of the time point, the thin biofilms formed by *C. vulgaris*, presented very similar photosynthetic responses both when measured *in situ* or in the suspended mode (Fig.4). On the other hand, in *C. closterium* a strong deviation of the rETR was observed between *in situ* and suspension measurements after two days of development (Fig.4). This response has been extensively described in the literature for microphytobenthic communities and it has been attributed to the attenuation of light as it penetrates through a biofilm and to the integration of the fluorescence response over the whole biofilm depth (Consalvey et al., 2005; Perkins et al., 2010). Thanks to the parallel measurements of structures and photosynthesis, we identified a threshold thickness of 60 μm that would still ensure linearity of the technique with our instrument (see Fig.S5). On the base of these observations, only the results coming from the resuspension of *C. closterium* biofilms were considered for interpretation, whereas the *in situ* measurements were retained for *C. vulgaris*. As the biofilms produced by *C. closterium* developed, at day4 the photosynthetic capacity of the cells was 40% lower ($P < 0.05$) with respect to that at day1 and the rETR saturated at lower PFD values ($P < 0.05$; 46% lower E_k value) (Fig 4a,e). On the other hand, the photosynthetic performance of the cells of *C. vulgaris* did not appear to be negatively affected as the biofilms matured (Fig.4a). Since the thickness of *C. closterium* biofilms doubled at day4, the decrease in photosynthetic capacity could have been induced by a rate limiting resource related to the greater structural complexity of the biofilms. In accordance with Rincon et al. (Rincon et al., 2019), the maximum quantum yield (F_v/F_m) in both species did not change over time ($P > 0.05$; Fig.4g,h). The combination of a constant F_v/F_m and the decrease in rETR_{max} in *C. closterium* suggests that photosynthetic capacity could have been limited by DIC rather than nutrients such as N and P. Forster and Martin Jézéquel (Forster and Martin-Jézéquel, 2005) came to similar conclusions for diatoms biofilms grown under dynamic conditions. Furthermore, since

at day4 the attenuation of light by the biofilms reached 60% of the incident light, the decrease in $rETR_{max}$ and the parallel increase in α could also be indicative of an acclimation of the biofilms to lower photon flux densities (Dubinsky and Stambler, 2009; Li et al., 2021). This interpretation would also fit with the progressive decrease of the Abs₄₃₅ to Abs₆₇₆ ratio over time ($P<0.05$; Fig.5a,b) that is a typical response of cells growing at lower light intensities (Dubinsky et al., 1986; SooHoo et al., 1986). Therefore, we cannot rule out a combined effect of light and nutrients as the biofilms of *C. closterium* became more complex from a structural point of view.

Similarly to the photosynthetic parameters, substantial changes in the macromolecular composition of the cells only occurred in *C. closterium* (Fig.5): a decrease over time in the carbohydrates to proteins ratio ($P<0.05$; 20%) and an increase in the lipids to proteins ratio ($P<0.05$; ~40%), whereas no compositional change was exhibited by *C. vulgaris* ($P>0.05$; Fig.5). This results further strengthen the hypothesis of a possible nutrient and light co-limitation in *C. closterium*. Indeed, lower photosynthetic capacity and accumulation of storage compounds such as lipids are typical strategies already described for the phytoplankton growing under stressful/limiting conditions and of cultures entering the stationary phase (Kamalanathan et al., 2016). Although for bacterial biofilms correlations between macromolecules and structures have been described (Feng et al., 2015; Tan et al., 2018), reports about photosynthetic biofilms are scarce (Fanesi et al., 2019; Wang et al., 2021; Wijihastuti et al., 2017).

The heatmap summarizes the dynamics of the biofilm traits for the two species. *C. closterium*, the algae that built the greater biofilms, underwent the greater changes in terms of metabolic activity and cellular composition, as it can be seen by the more frequent changes in colors in the heatmap for most of the investigated traits (see Fig.6). On the other hand, the thinner biofilms developed by *C. vulgaris* did not induce any drastic change in cell physiology

(constant colors in Fig.6). Also, the clustering of structural parameters such as thickness (max and mean) and biovolume with light attenuation, photosynthesis and compositional parameters confirms that the decrease in PFD over time was probably responsible for the acclimation strategy undertaken by *C. closterium*. Changes in cell activity as a function of biofilm structural complexity have been previously reported for bacteria strains (Piculell et al., 2016; Stewart and Franklin, 2008; Sun et al., 2016; Torresi et al., 2016), but only few reports are available for photosynthetic biofilms (Stiefelmaier et al., 2020; Wang et al., 2021; Wijihastuti et al., 2017). The high physiological plasticity exhibited by benthic diatoms has long been recognized as a functional trait that allows the cells to rapidly acclimate in an extremely variable and harsh environment such as intertidal sediments (Forster and Martin-Jézéquel, 2005). Here, we suggest that such a trait might be advantageous in those species capable of building complex (i.e. thicker and with more biomass) biofilms, since it would allow the cells to promptly acclimate to the changing local conditions along biofilm maturation. In support to this, the ability of monospecific microalgae biofilms to promptly acclimate (both physiology and architecture) to changing environmental conditions (i.e. light intensity) has been recently demonstrated also by Wang et al. (Wang et al., 2021), suggesting that such a phenotypic plasticity must be taken into account also when designing biofilm-based systems for biotechnological applications.

Further studies would be necessary to identify the precise rate-limiting resource inducing such physiological reorganization as a function of changing biofilm architecture, for example by integrating in the experimental set-up nutrients and pH sensors. Likely a combination of light quantity and spectrum and nutrient limitation could be the principal reason (Fanesi et al., 2019; Yuan et al., 2020; Zhang et al., 2019). It must be also kept in mind that the techniques used in this study to characterize cell photosynthesis and macromolecular composition are integrating the parameters over the whole cell population. Techniques with a higher spatial

resolution (i.e. Imaging PAM and FTIR/Raman micro-spectroscopy) may help to discriminate the spatial distribution of cell phenotypes in photosynthetic biofilms and to properly explain acclimation strategies at a finer scale (Stewart and Franklin, 2008).

Conclusions

The use of UV/Vis-spectroscopic cuvettes as flow cell allowed a screening, by means of optical methods, of several biofilm traits (from metabolic activity to microscopic structures) mostly *in situ*, allowing to comprehend the interplay between structure development during the maturation of photosynthetic biofilms and their respective changes in photosynthetic activity and macromolecular composition. We were able to show that the development of more complex biofilms, from an architectural point of view, leads to greater changes in cell activity and composition likely because of changes in local environmental conditions as the biofilms grow in thickness and biomass. The diatom developed thicker and biomass-rich biofilms that attenuated more light, leading to a decrease in photosynthetic performance and a rearrangement of cell macromolecules. On the other hand, the thinner biofilms developed by *C. vulgaris* did not undergo any remarkable adjustment in their physiology. This system could be used to screen under controlled laboratory conditions several microalgae species at the same time, the interaction of mixed communities (microalgae and bacteria) and could provide a better comprehension of such complex communities for ecological and biotechnological purposes.

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Author Contributions

AF developed the flow cell, performed the experiments, analysed the data and wrote the article, TM and CB contributed in the realization of the experimental set-up. AF, OB, RB and FL designed the experiments. OB, RB and FL provided funding, analysed the data, supervised the project and revised the manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no competing interests.

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Figure legends

Figure 1. Schematic representation of the flow cell and example of biofilm growth. Panel (a) depicts the design of the cuvette flow cell, whereas in panel (b) an example of biofilm growth over time of *C. closterium* biofilms in the cuvettes is reported.

Figure 2. CLSM-topographic representation of the biofilms developed by *C. closterium* and *C. vulgaris* (a) and representative OCT B-scans of *C. closterium* biofilms over time (b). The colors in the topographic representations of the biofilms are calibrated with respect to biofilm height (in μm , from 0 to 100 μm): blue colors represent the bottom of the biofilm whereas purple colors represent the top of the biofilm (see the calibration bar). The height of the biofilms is based on the Chla signal, therefore it represents only the thickness of the cell

biomass. The z-stacks were obtained with a CLSM and the topography of the biofilm was computed in Fiji using the TopoJ plugin.

Figure 3. Evolution of structural parameters of the biofilms over time. Biovolume (a), max thickness (b) and roughness **coefficient** (c), obtained from CLSM z-stacks and light attenuation (d) as a function of time in biofilms of *C. closterium* and *C. vulgaris* grown in cuvettes flow cells. The results are reported as the mean and standard deviation of at least three independent assays.

Figure 4. Photosynthetic parameters obtained from *in situ* biofilms (in white) and from re-suspended biofilms (in black). The panels (a), (c), (e) and (g) refer to the photosynthetic parameters measured for the biofilms developed by *C. closterium* whereas the panels (b), (d), (f) and (h) refer to the biofilms developed by *C. vulgaris*. The results are reported as the mean and standard deviation of at least three independent assays. The statistical significance is reported with respect to day1. **See Tab.S2 and S3 for the detailed statistical results.**

Figure 5. Pigment signatures (a and b) and macromolecular composition (c,d,e and f) of *C. closterium* and *C. vulgaris* biofilms over time. The absorption ratio Abs435/Abs676 is an index of the accessory pigments to chl *a* ratio and was calculated from UV-Vis spectra whereas the macromolecular ratios were obtained from FTIR spectra. The results are reported as the mean and standard deviation of at least three independent assays. The statistical significance is reported with respect to day1.

Figure 6. Heatmap representing the changes in biofilm traits grouped in architecture, photosynthesis, macromolecular composition and optical properties of *C. closterium* and *C.*

655 *vulgaris* biofilms over time. Red colors in the heatmap depict higher values for a variable,
656 whereas blue colors refer to lower values for that variable. The dendrograms cluster variables
657 presenting similar trends over time using the euclidean distance. The heatmap was computed
658 using the mean values of the variables obtained from the experimental replicates.

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