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

3
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14 **Running title:** architecture and physiology of microalgae biofilms

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51 **Abstract**

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

68

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

70

71

72 **Introduction**

73 Microalgae biofilms play a crucial ecological role in most aquatic ecosystems as they
74 contribute substantially to the primary production of shallow waters (Buhmann et al., 2012).
75 In most coastal and estuarine areas, they also stabilize sediments and promote the formation
76 of microbial communities essential for the functioning of the main biogeochemical cycles in
77 such environments. With respect to their planktonic counterparts, microalgae biofilms present
78 biomass densities that can be 10 to 100 times higher (Ozkan et al., 2012; Podola et al., 2017).
79 Such characteristic can provide strong resistance to grazing and pollutants but it can also
80 create very strong gradients of light and nutrients making them rapidly limiting in some
81 locations within the biofilm (Battin et al., 2016). For biotechnological applications, the high
82 productivity of microalgae biofilms has been recently recognized as a key advantage of
83 biofilm-based systems over classical photo-bioreactors where microalgae are in the planktonic
84 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

110 was tested using two biofilm-forming microalgae (a diatom and a green algae) that produce
111 biofilms with different architectural characteristics (Fanesi et al., 2019). Indeed, specific
112 architectures may translate in the formation of light and nutrient gradients (including gases
113 such as CO₂) as a function of biofilm complexity (Battin et al., 2016; Schnurr and Allen,
114 2015). We therefore tested the hypothesis that photosynthetic cells producing biofilms with
115 high structural complexities (e.g. high thickness and cell abundances) may need to acclimate
116 and change their physiological performances in order to optimize their fitness in the changing
117 local conditions (Stewart and Franklin, 2008). A consequence of this hypothesis is that a thick
118 biofilm would be expected to induce stronger gradients and therefore more changes in cell
119 physiology with respect to a thinner biofilm where light and nutrients gradients are weaker
120 and may impact less cell metabolic performances.

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

129

130 **Materials and Methods**

131 *Planktonic culture maintenance*

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

135 medium (Walne, 1970)) for *C. closterium*, 3N-Bristol for *C. vulgaris* (Bischoff and Bold,
136 1963) and were kept in the exponential phase by frequent re-inoculations (every 3 days). The
137 cultures were continuously shaken and grown in an incubator at 25°C under a continuous
138 photon flux density (PFD) of 20 $\mu\text{mol m}^{-2}\text{s}^{-1}$.

139

140 *Development of the cuvette flow-through system for biofilm cultivation*

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

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

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

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

165

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

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

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

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

190

191 *Light attenuation*

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

198

$$199 \text{ Light attenuation}_{(i)} (\%) = [1 - (\text{transmitted light}_{(i)} / \text{transmitted light}_{(0)})] \cdot 100 \quad (1)$$

200

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

202

203 *Chlorophyll a variable fluorometry (PAM)*

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

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

216

$$217 \quad F_v/F_m = (F_m - F_0)/F_m \quad (2)$$

$$218 \quad \Delta F/F'_m = (F'_m - F)/F'_m \quad (3)$$

219

220 where F_0 and F_m are the minimum and max fluorescence determined after 10 min dark-
221 adaptation, whereas F and F'_m are the minimum and max fluorescence during illumination.

222 The relative electron transport rate (rETR) was calculated using the Eq. (4):

223

$$224 \quad \text{rETR} = \Delta F/F'_m \times \text{PFD} \times 0.5 \quad (4)$$

225

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

232

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

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

260

261 *Confocal laser scanning microscopy and image analysis*

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

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

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

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

285

286 *Optical coherence tomography (OCT)*

287 The meso-structures of the biofilms were visualized using a spectral domain Optical
288 Coherence Tomograph (OCT; Ganymede 621, Thorlabs GmbH, Lübeck, Germany) with a
289 central light source wavelength of 900 nm. The images were acquired using a telecentric scan
290 lens (OCT-LK3-BB) at a speed of 25kHz per A-scan and an averaging of 6 A-scan. The field
291 of view was 4 x 4 mm (XY) and the axial depth 1 mm (Z). The lateral pixel resolution was 8
292 µm and the axial pixel resolution was 1.45 µm. For all the acquisitions a refractive index of
293 1.33 was used as the biofilms were imaged *in situ* in liquid medium (Wagner and Horn,
294 2017). Images were analysed using the software ThorImageOCT 5.4.4 (Thorlabs, Lübeck,
295 Germany). OCT measurements were performed only on one of the cuvettes to show the
296 versatility of the flow cell system.

297

298 *Statistics*

299 Statistics was performed using GraphPad prism 5.0 (San Diego, CA, USA) and the R software
300 (R Core Team, 2014). Before statistical analysis, the distribution of the data was investigated
301 using the Shapiro-Wilk's test. Data were transformed (log or square root) in the case they did
302 not followed a normal distribution ($P < 0.05$). To evaluate the significance of mean differences
303 over time, One-way and Two-way ANOVA followed by the Tukey's post-hoc test for
304 multiple comparisons were carried out (ns = $P \geq 0.05$; * = $P < 0.05$; ** = $P < 0.01$; *** =
305 $P < 0.001$). The level of significance was always set at 5%. The heatmap was performed in R
306 using the package "NMF" (Gaujoux and Seoighe, 2010). The analysis was conducted on the
307 mean values of all experiments performed in the study and the matrix of variables was further

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

310

311 **Results and Discussion**

312 *Biofilm architecture is species dependent*

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

316 The optical properties of the cuvettes allowed to acquire high quality images by means of
317 CLSM and OCT (Fig.2, Fig.S2, Fig.S3). The two species were chosen since they form
318 biofilms with different architectures (Fanesi et al., 2019; Fanesi et al., 2021; Le Norcy et al.,
319 2019) and were therefore good experimental organisms to test the hypothesis that complex
320 architectures may induce stronger physiological adjustments in the cells. Figure 2, Fig.S2 and
321 Fig.S3 show indeed that biofilm structure (both cells and EPS organizations) was species-
322 dependent, in line with previous results (Fanesi et al., 2019; Le Norcy et al., 2019) and it
323 appeared to be homogeneous along the length of the cuvettes (Fig.S4). *C. closterium*

324 produced biofilms that were twofold thicker and that produced 2.4 times more biomass than
325 those of *C. vulgaris* (Fig. 3a,b). Biofilms produced by *C. vulgaris* did not seem to grow much
326 in thickness (~20µm) but seemed mostly to become denser on the substrate surface (Fig.3a,b).

327 The increase in biomass in *C. closterium* on the contrary matched an increase in thickness of
328 the biofilms that reached up to 80 µm final thickness (Fig.2 and Fig.3a,b). These results are in
329 agreement with previous reports about microalgae biofilms (Fanesi et al., 2019; Fanesi et al.,
330 2021; Faÿ et al., 2010; Le Norcy et al., 2019) and are likely related to the preferential lifestyle
331 of the two species: *C. closterium* is commonly found forming microphytobenthos
332 communities in mudflats whereas *C. vulgaris* is generally a planktonic species. However,

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

337 The production over time of daughter cells led to a stronger light attenuation through the
338 biofilms in both species (Fig.3d). After four days of development, *C. closterium* attenuated
339 almost 60% of the incoming photons whereas *C. vulgaris* only 20%. Therefore, the lower
340 biomass produced by *C. vulgaris* was likely not related to a light limitation, indeed the
341 compensation point for *C. vulgaris* biofilms has been reported to be in the range 30-60 μmol
342 $\text{m}^{-2} \text{s}^{-1}$ (Rincon et al., 2019). Furthermore, recently Fanesi et al. (Fanesi et al., 2021) showed
343 that even when biofilm architecture changes, *C. vulgaris* maintains constant diffusion
344 characteristics in its biofilm. It results that a limitation by nutrients under dynamic conditions
345 should be excluded. On the other hand, the authors demonstrated that at low shear stress the
346 biofilms were very loose and that cells detached more easily from the biofilm. It is therefore
347 probable that the hydrodynamic conditions used did not allow the cells to build up strong and
348 biomass-rich biofilms.

349

350 *Biofilm architecture affects cell physiology*

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

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

358 and S3 show a strong dependency of the measurements on the species considered and on the
359 thickness of their biofilms. Regardless of the time point, the thin biofilms formed by *C.*
360 *vulgaris*, presented very similar photosynthetic responses both when measured *in situ* or in the
361 suspended mode (Fig.4). On the other hand, in *C. closterium* a strong deviation of the rETR
362 was observed between *in situ* and suspension measurements after two days of development
363 (Fig.4). This response has been extensively described in the literature for microphytobenthic
364 communities and it has been attributed to the attenuation of light as it penetrates through a
365 biofilm and to the integration of the fluorescence response over the whole biofilm depth
366 (Consalvey et al., 2005; Perkins et al., 2010). Thanks to the parallel measurements of
367 structures and photosynthesis, we identified a threshold thickness of 60 μm that would still
368 ensure linearity of the technique with our instrument (see Fig.S5). On the base of these
369 observations, only the results coming from the resuspension of *C. closterium* biofilms were
370 considered for interpretation, whereas the *in situ* measurements were retained for *C. vulgaris*.
371 As the biofilms produced by *C. closterium* developed, at day4 the photosynthetic capacity of
372 the cells was 40% lower ($P < 0.05$) with respect to that at day1 and the rETR saturated at lower
373 PFD values ($P < 0.05$; 46% lower E_k value) (Fig 4a,e). On the other hand, the photosynthetic
374 performance of the cells of *C. vulgaris* did not appear to be negatively affected as the biofilms
375 matured (Fig.4a). Since the thickness of *C. closterium* biofilms doubled at day4, the decrease
376 in photosynthetic capacity could have been induced by a rate limiting resource related to the
377 greater structural complexity of the biofilms. In accordance with Rincon et al.(Rincon et al.,
378 2019), the maximum quantum yield (F_v/F_m) in both species did not change over time ($P > 0.05$;
379 Fig.4g,h). The combination of a constant F_v/F_m and the decrease in $rETR_{\text{max}}$ in *C. closterium*
380 suggests that photosynthetic capacity could have been limited by DIC rather than nutrients
381 such as N and P. Forster and Martin Jézéquel (Forster and Martin-Jézéquel, 2005) came to
382 similar conclusions for diatoms biofilms grown under dynamic conditions. Furthermore, since

383 at day4 the attenuation of light by the biofilms reached 60% of the incident light, the decrease
384 in $rETR_{max}$ and the parallel increase in α could also be indicative of an acclimation of the
385 biofilms to lower photon flux densities (Dubinsky and Stambler, 2009; Li et al., 2021). This
386 interpretation would also fit with the progressive decrease of the Abs_{435} to Abs_{676} ratio over
387 time ($P < 0.05$; Fig.5a,b) that is a typical response of cells growing at lower light intensities
388 (Dubinsky et al., 1986; SooHoo et al., 1986). Therefore, we cannot rule out a combined effect
389 of light and nutrients as the biofilms of *C. closterium* became more complex from a structural
390 point of view.

391 Similarly to the photosynthetic parameters, substantial changes in the macromolecular
392 composition of the cells only occurred in *C. closterium* (Fig.5): a decrease over time in the
393 carbohydrates to proteins ratio ($P < 0.05$; 20%) and an increase in the lipids to proteins ratio
394 ($P < 0.05$; ~40%), whereas no compositional change was exhibited by *C. vulgaris* ($P > 0.05$;
395 Fig.5). This results further strengthen the hypothesis of a possible nutrient and light co-
396 limitation in *C. closterium*. Indeed, lower photosynthetic capacity and accumulation of
397 storage compounds such as lipids are typical strategies already described for the
398 phytoplankton growing under stressful/limiting conditions and of cultures entering the
399 stationary phase (Kamalanathan et al., 2016). Although for bacterial biofilms correlations
400 between macromolecules and structures have been described (Feng et al., 2015; Tan et al.,
401 2018), reports about photosynthetic biofilms are scarce (Fanesi et al., 2019; Wang et al., 2021;
402 Wijihastuti et al., 2017).

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

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

426 Further studies would be necessary to identify the precise rate-limiting resource inducing such
427 physiological reorganization as a function of changing biofilm architecture, for example by
428 integrating in the experimental set-up nutrients and pH sensors. Likely a combination of light
429 quantity and spectrum and nutrient limitation could be the principal reason (Fanesi et al.,
430 2019; Yuan et al., 2020; Zhang et al., 2019). It must be also kept in mind that the techniques
431 used in this study to characterize cell photosynthesis and macromolecular composition are
432 integrating the parameters over the whole cell population. Techniques with a higher spatial

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

436

437 **Conclusions**

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

453

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461

462 **Author Contributions**

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

467

468 **Data availability statement**

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

471

472 **Competing interests**

473 The authors declare no competing interests.

474

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619 **Figure legends**

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

623

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

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

632

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

638

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

645

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

652

653 **Figure 6.** Heatmap representing the changes in biofilm traits grouped in architecture,
654 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.**

659

660

661