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1 Article

2 **The architecture of monospecific microalgae biofilms**3 **Andrea Fanesi**¹, **Armelle Paule**¹, **Olivier Bernard**², **Romain Briandet**³ and **Filipa Lopes**^{1,*}4 ¹ Laboratoire Génie des Procédés et Matériaux (LGPM), CentraleSupélec, Université Paris-Saclay, 91190 Gif-
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10 **Abstract:** Microalgae biofilms have been proposed as an alternative to suspended cultures in
11 commercial and biotechnological fields. However, little is known about their architecture which
12 may strongly impact biofilm behavior, bioprocess stability and productivity. In order to unravel the
13 architecture of microalgae biofilms, four species of commercial interest were cultivated in
14 microplates and characterized using a combination of confocal laser scanning microscopy and FTIR-
15 spectroscopy. In all the species, the biofilm biovolume and thickness increased over time and
16 reached a *plateau* after 7 days, the final biomass reached was very different though. The roughness
17 decreased during maturation, reflecting cell division and voids filling. The extracellular polymeric
18 substances content of the matrix remained constant in some species and increased over time in some
19 others. Vertical profiles showed that young biofilms presented a maximum cell density at 20 μm
20 above the substratum co-localized with matrix components. In mature biofilms, the maximum
21 density of cells moved at a greater distance from the substratum (30–40 μm) whereas the maximum
22 coverage of matrix components remained in deeper layer. Carbohydrates and lipids were the main
23 macromolecules changing during biofilm maturation. Our results revealed that the architecture of
24 microalgae biofilms is species-specific. However, time is similarly affecting the structural and
25 biochemical parameters.

26 **Keywords:** biofilm; microalgae; architecture, confocal laser scanning microscopy, FTIR-
27 spectroscopy

28 **1. Introduction**

29 In the last decades microalgae have been recognized as a valuable source of bio-products such
30 as pigments, anti-oxidants and food supplements and they have gained popularity in a wide range
31 of commercial activities. In conventional photobioreactors (PBRs), microalgae present low biomass
32 concentrations (1–3 $\text{g} \cdot \text{L}^{-1}$; [1,2]) and 12 to 2000 L of liquid medium are required for the production of
33 1 Kg of microalgae dry mass [3]. This high water fraction requires energetic expenses for culture
34 agitation (up to 385.71 $\text{MJ} \cdot \text{Kg}^{-1}$; [2]) and for biomass harvesting, dewatering and drying (up to 82 MJ
35 $\cdot \text{Kg}^{-1}$; [2]). Biomass production in typical PBRs is therefore constrained by high energy and operating
36 costs [4].

37 Biofilm-based cultivation systems are promising technologies overcoming the drawbacks of
38 conventional PBRs. Such systems can reach high productivities (up to 35 $\text{g DW} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$) and
39 biomass concentration (up to 96 $\text{g} \cdot \text{L}^{-1}$; [2]). Furthermore, harvesting is simply carried out by scraping
40 the attached biomass with minimal energy demand [3]. Finally, there is an increasing interest from
41 the industrial sector about the great variety of molecules excreted by microalgae when developing
42 biofilms [5]. Biofilm-based systems seem therefore to address most of the challenges of suspended
43 cultures.

44 In a biofilm, microbial cells are associated with a surface and enclosed in a matrix, which is
45 mainly composed of water, polysaccharides, proteins and nucleic acids [6]. The spatial arrangement
46 of microorganisms and matrix components define the size and quantity of voids and channels,

47 altering in turn the transport of nutrients and gases [7]. The biofilm architecture induces therefore
48 marked gradients of nutrients, gases and light along depth, inducing the cells to acclimate or displace
49 in order to maintain an optimal growth [8,9]. Therefore, structural data are of major importance to
50 better understand the complex behavior of biofilms (i.e. development and activity) and to improve
51 productivity of biofilm-based technologies.

52 Structural changes in bacteria biofilms have been well characterized experimentally under
53 several growth conditions, and it has been shown that the architecture is strongly species and strain
54 dependent [10–13]. From a compositional point of view, it has been shown that changes of structural
55 parameters in bacterial biofilms are strongly correlated to the biochemical composition of
56 exopolymers [14–17]. Phototrophic biofilms, especially microalgae biofilms, have been far less
57 studied [8,18–22]. In particular, little is known about the link between architecture and extracellular
58 polymeric substances production for different species. In addition, the role played by the matrix in
59 biofilm development has been only addressed in studies regarding mixed communities (i.e. bacteria,
60 microalgae etc.) [8,23,24], even though microalgae are known to excrete exopolymers with specific
61 carbohydrates:proteins:lipids:nucleic acids ratios [25,26].

62 In this work, we aimed therefore at better understanding how various microalgae monospecific
63 biofilms differ in their architecture and composition depending on the species. Four biofilm-forming
64 microalgae species of actual or potential biotechnological interest were selected, including two green
65 algae, a red algae and a diatom. Biofilm structural dynamics and macromolecular composition were
66 characterized by a combination of non-destructive techniques including confocal laser scanning
67 microscopy (CLSM, which allows to characterize several structural parameters such as biovolume,
68 thickness, roughness and diffusion distance) and vibrational spectroscopy (ATR-FTIR spectroscopy).

69 2. Materials and Methods

70 2.1. Microalgae strains and planktonic culture maintenance

71 *Chlorella vulgaris* SAG 211-11b (Göttingen, Germany) was grown in 3N-Bristol [27], the marine
72 strain *Chlorella autothrophica* CCMP 243 (Bigelow, Maine, USA) was grown in Artificial Sea Water
73 [28], whereas the diatom *Cylindrotheca closterium* AC170 (Caen, France) and *Porphyridium purpureum*
74 SAG 1380-1e (Göttingen, Germany) were cultivated in filtered natural seawater. The marine media
75 were supplemented with Walne's medium [29]; $1\text{ mL} \cdot \text{L}^{-1}$). All biofilms were inoculated from
76 suspended stock cultures grown in a PSI MC1000 multicultivator (Photon systems instruments,
77 Czech Republic) in borosilicate tubes filled with 70 mL of growth medium. The cultures were bubbled
78 and maintained semi-continuously at 25°C under a continuous photon flux density of $80 \mu\text{mol}$
79 $\text{photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Cells from the stock cultures were harvested during the exponential phase (cell
80 density of $2\text{-}3 \cdot 10^6 \text{ cell} \cdot \text{mL}^{-1}$).

81 2.2. Biofilms cultivation: inoculum, initial adhesion and growth

82 Biofilms were grown in polystyrene $\mu\text{Clear}^{\circledR}$ 96-well microplates (Grenier Bio-one, France). The
83 inoculum was prepared by diluting a volume of suspended cells (see above) to a final concentration
84 of $1 \cdot 10^6 \text{ cell} \cdot \text{mL}^{-1}$ ($1 \cdot 10^5 \text{ cell} \cdot \text{mL}^{-1}$ for *C. closterium*) and by transferring 250 μL of such suspension
85 in the wells. This cell concentration corresponded to similar starting biovolume for all the species (~ 5
86 $\mu\text{m}^3 \cdot \mu\text{m}^{-2}$). The cells were then left for 24 hours adhering to the surface of the wells and subsequently
87 200 μL of the medium were removed in order to eliminate any unattached cell. After the first 24
88 hours, 80 μL of medium were removed and replaced with new medium every two days to
89 compensate for evaporation and to buffer nutrient and CO_2 limitations. A continuous photon flux
90 density of $100 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ was used. Light (PAR 400-700 nm) was homogeneously
91 provided by two sets of light emitting diode (Alpheus LED, Montgeron, France). Biofilms growth
92 was monitored for 11 days and each day the biofilms were scanned using CLSM to detect cells signal.
93 At day 2, 7 and 11 the biofilms were also stained to characterize the matrix and samples were
94 harvested for ATR-FTIR spectroscopy.

95 2.3. Confocal laser scanning microscopy (CLSM): cells and matrix characterization

96 Images (512 · 512 pixels) were acquired using an inverted Zeiss LSM700 confocal microscope
97 (Carl Zeiss microscopy GmbH, Germany) controlled using the Zen 10.0 software black edition (Carl
98 Zeiss microscopy GmbH, Germany). All biofilms were scanned with a LD Plan-Neofluar 20x/0.4 Korr
99 M27 objective with a 0.4 N.A. (numerical aperture). Each image was 638 · 638 µm in size with a z-step
100 of 3.94 µm. The settings of the confocal microscope are reported in Table S1. After preliminary trials,
101 a low magnification lens was preferred to a higher one (e.g. 63x), because allowing to scan wider
102 biofilm areas (essential to properly capture microalgae cell patterns) and to acquire z-stacks over the
103 whole biofilm depth. However, we have to point out that this choice comes with side effects, such as
104 a greater point spread function and a lower resolution of the images.

105 Two laser lines were used to respectively detect microalgae and the matrix surrounding them.
106 Microalgae cells were observed by detecting chlorophyll *a* auto-fluorescence. Lectins and dextran
107 (3kDa), both labelled with fluorescein (FITC), were added at day 2, 7 and 11 to detect glycoconjugates
108 and to visualize non-specifically the matrix, respectively [30–33]. The pool of extracellular
109 glycoconjugates (i.e. exopolysaccharides, glycoproteins etc.) specifically detected by the lectins will
110 be identified here as EPS (extracellular polymeric substances). Dextran on the other hand is supposed
111 to diffuse into the channels, voids and to get finally non-specifically trapped into the matrix [30–33].
112 A fresh cocktail of 20 lectins (Kit I, II and III, Vector Laboratories, Peterborough, UK) or dextran were
113 supplied at a final concentration of 13 µg · mL⁻¹. The microplates were then incubated in the dark for
114 30 minutes. Afterwards, the excess of dyes present in the wells was removed by carefully removing
115 140 µL of the medium and by adding other 140 µL of fresh medium (specific for each species) in each
116 well. This process was repeated one time for the dextran and two times for the lectins. We have to
117 point out that since a mixture of lectins was used to detect the matrix, it is possible that the interaction
118 among the single lectins and the competition for similar targets may have occurred.

119 Chlorophyll *a* was excited with the 639 nm line of a 5 mW solid state diode laser and the emission
120 of chlorophyll *a* auto-fluorescence was observed using the long pass (LP) filter 615 nm. Lectins and
121 dextran were excited with the 488 nm laser line of a 10 mW solid state diode laser and their
122 fluorescence detected using the band pass (BP) filter 490–530 nm. Unlabeled organisms and wells
123 filled with growth media but not inoculated with microalgae were used as a staining control. Each
124 well was scanned on at least three random positions (three z-stacks) resulting in a total surface area
125 of at least 1.2 mm².

126 2.4. Image analysis

127 The plug-in COMSTAT 2.1 (Technical University of Denmark; [11]) running in ImageJ 1.48v [34]
128 was used to extract from the images the quantitative parameters typically used to characterize biofilm
129 structures. The complete list of parameters is reported in Table S2. Images binarization was
130 automatically computed in the plug-in by selecting a threshold value using the Otsu algorithm [35]
131 and the function “connected volume filtering” was unchecked.

132 Since the auto-fluorescence of the cells comes from the chlorophyll within the chloroplasts, we
133 have to point out that the structural parameter calculated from the images reflect such organelle
134 rather than the whole cell, even though the overlapping of fluorescence and transmission images
135 revealed good matching of the two acquisition mode (data not shown). However, to be consistent
136 with the terminology present in most of the literature, we considered that the auto-fluorescence of
137 the chlorophyll quantifies the cells.
138

139 2.5. ATR-FTIR spectroscopy

140 At day 2, 7 and 11, the biofilms were scrapped from four wells for each species. The samples
141 were centrifuged at 8000 · g for 5 minutes and the supernatant removed. After that, 1 mL of distilled
142 water in the case of *C. vulgaris* and 1 mL of a solution of NaCl (35 g · L⁻¹) for the marine species were
143 used to wash the biofilm suspensions from salts that would otherwise interfere with the cell and

144 matrix spectral signature. Afterward, the pellet was re-suspended in 5-10 μL of distilled water or
145 NaCl and 1.5 μL were transferred on a 45° ZeSe flat crystal of an ATR-FTIR PerkinElmer Spectrum-
146 two spectrometer (PerkinElmer, Waltham, USA) and the sample was dried at room temperature for
147 20 minutes. Spectra were acquired in the range 4000 and 400 cm^{-1} using 32 accumulations at a spectral
148 resolution of 4 cm^{-1} . Before each measurement, the empty crystal was measured using the same
149 instrumental setting and used as a blank.

150 Spectra were baselined using the rubberband algorithm and the ratios between the main
151 macromolecular pool (proteins, lipids and carbohydrates) were calculated as the ratios between the
152 maximum absorption values for the spectral ranges corresponding to each macromolecular pool:
153 proteins (Amide I; 1700-1630 cm^{-1}), lipids (C=O; 1750-1700 cm^{-1}) and carbohydrates (C-O-C, C-C and
154 Si-O-Si in diatoms; 1200-950 cm^{-1}). Since no separation between cells and matrix components was
155 performed, the spectra reflected both the physiological changes occurring in the cells and those
156 related to EPS.

157 2.6. Statistics

158 Statistics was performed using GraphPad prism 5.0 (San Diego, CA, USA) and R [36]. One-way
159 and two-way ANOVA were used to test the statistical significance of mean differences among
160 different species and over time. The level of significance was always set at 5%. The logistic function
161 [37,38] was fit to the biovolume vs. time curves and the maximal cell biovolume (i.e. the biovolume
162 at the *plateau*) and the specific growth rate (μ) were obtained in order to make quantitative
163 comparisons between the species.

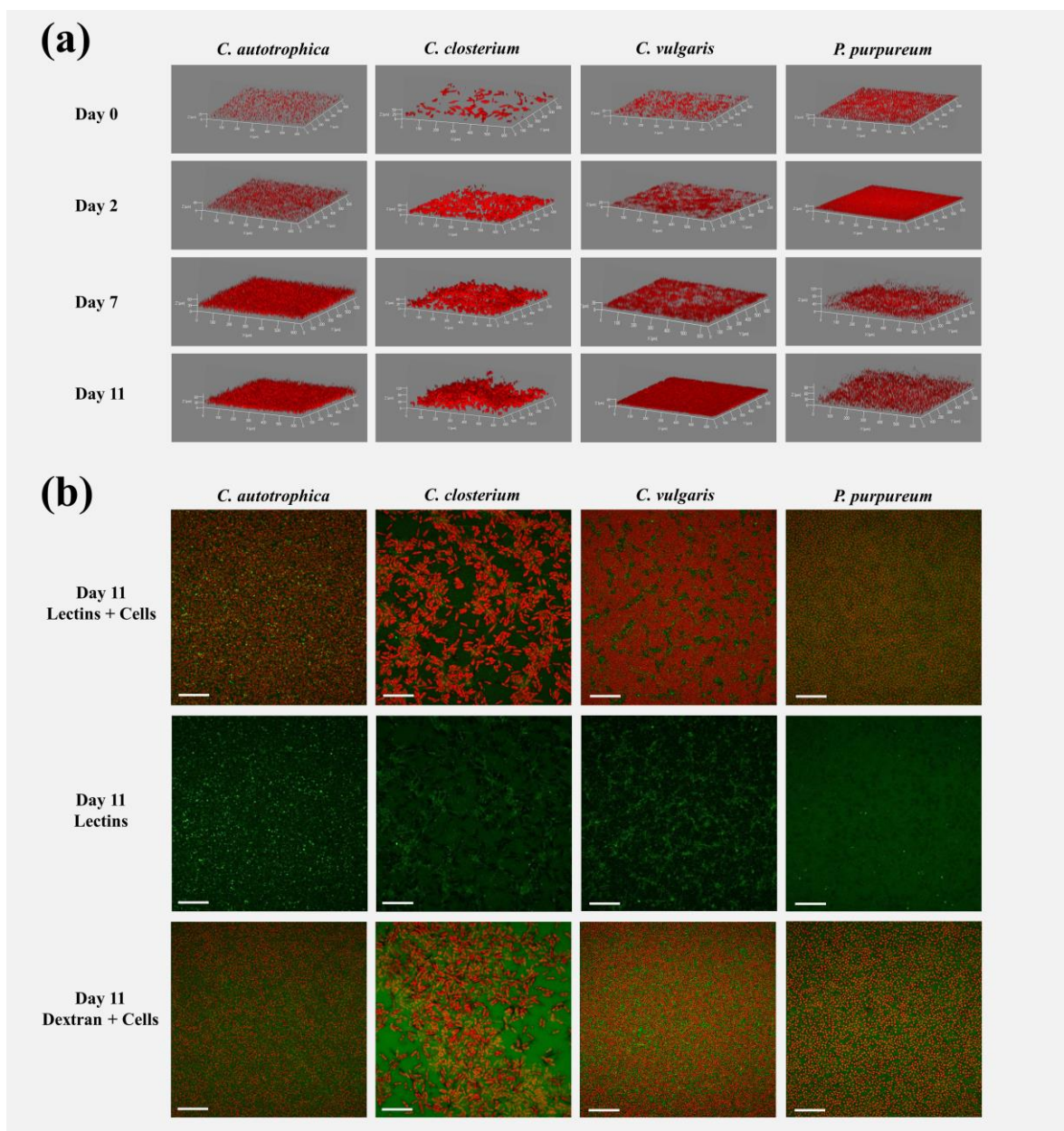
164 Correlation matrices, using the Pearson's coefficient, were computed using the package
165 "corrplot" [39] present in R to investigate the relationship among structural parameters (obtained
166 from CLSM) and biofilms macromolecular composition (FTIR ratios).

167 All results are reported as mean and standard deviations of several independent biological
168 replicates. Biofilm dynamics were repeated on at least four independent microplates. In each
169 microplate, for each species and for each time point, at least three separate wells were analyzed by
170 CLSM. In each well, at least three randomly chosen biofilm areas were scanned.

171 3. Results

172 3.1. Biofilm development over time: structural characteristics

173 Examples of 3D biofilm reconstructions are reported in Figure 1a. The biovolume of the cells
174 within the biofilm increased over time and after seven days the *plateau* was reached for all the species
175 (Figure 2a). *C. autotrophica* and *P. purpureum* exhibited the highest biovolume, whereas *C. vulgaris* and
176 *C. closterium* presented almost 50% lower biovolume. *C. autotrophica* also exhibited the highest growth
177 rate whereas no significant difference was found among the other microalgae (Table 1 and Figure 2a;
178 $p > 0.05$).



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Figure 1. Representative three-dimensional reconstructions of the microalgae biofilms (a) and maximum intensity projection (b) of cells (red signal), lectins (green signal) and dextran (green signal) signals of the biofilms at day 11. In panel (a), day 0 represents the inoculum, day 2, 7 and 11 are the days at which the biofilms were stained and analyzed by ATR-FTIR spectroscopy. The brightness of the images was adjusted for better visualization. Images size in (a) and (b) is 638 · 638 μm . The XY ticks interval in (a) is 100 μm and the scale bar in (b) is 100 μm .

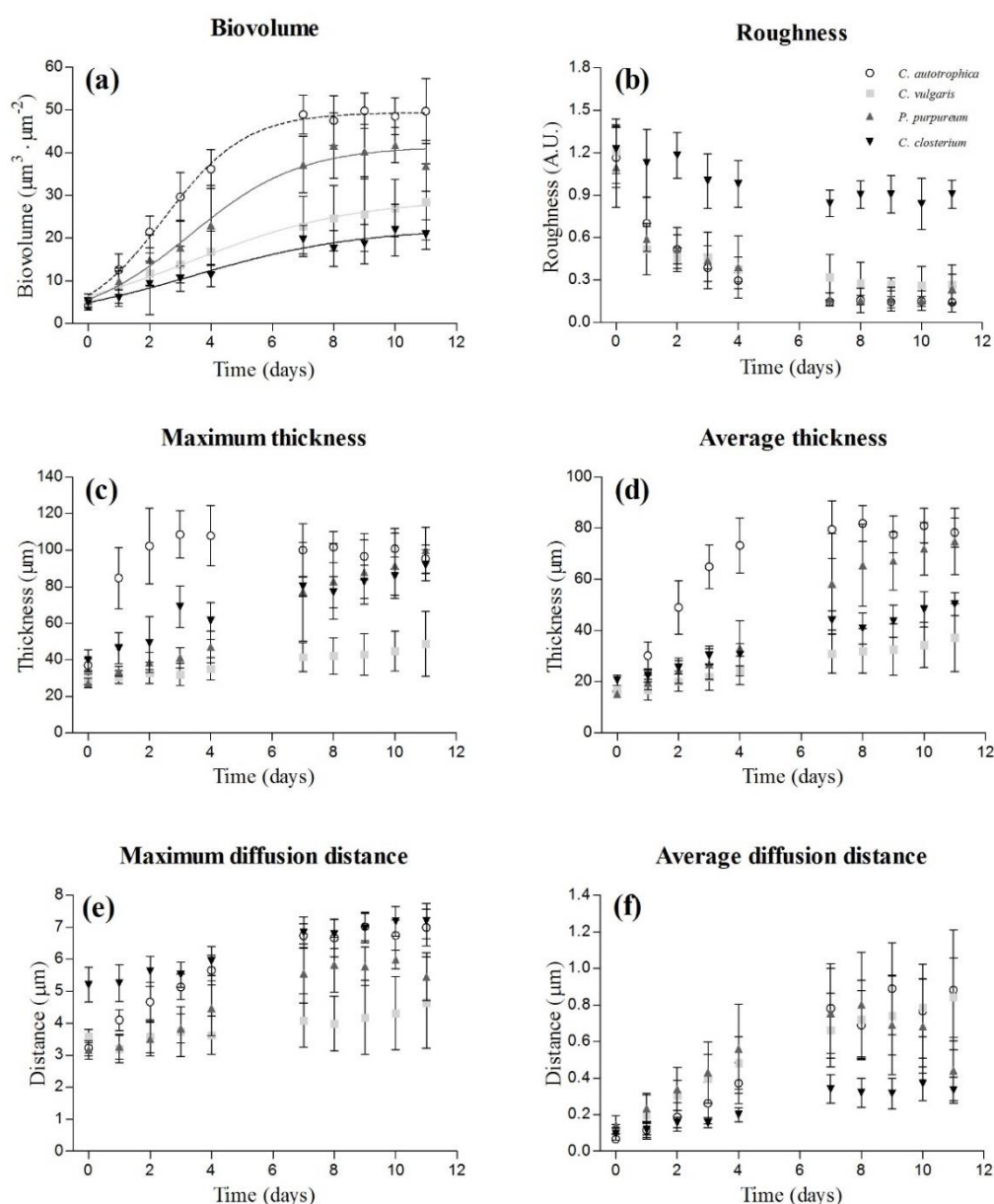
197 **Table 1.** Growth parameters (growth rate and maximal biovolume) obtained by fitting the logistic
 198 regression to the biovolume vs. time curves. Data are reported as the mean and standard deviation of at least
 199 nine independent biological replicates. Different letters represent statistically different means ($p < 0.05$) as
 200 determined by pair-wise comparisons after one-way ANOVA.

Microalgae species	μ (d ⁻¹)	Maximal biovolume ($\mu\text{m}^3 \cdot \mu\text{m}^{-2}$)
<i>C. autotrophica</i>	0.72 ^a (0.24)	50.47 ^a (4.09)
<i>C. vulgaris</i>	0.45 ^b (0.14)	31.22 ^c (8.49)
<i>P. purpureum</i>	0.65 ^{ab} (0.20)	43.02 ^b (4.92)
<i>C. closterium</i>	0.43 ^b (0.13)	22.42 ^d (2.59)

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 202 Roughness presented an opposite trend to biovolume and decreased (from 1.2 to 0.2 a.u.) over
 203 time in *C. autotrophica*, *C. vulgaris* and *P. purpureum*. The roughness coefficient of *C. closterium* instead,
 204 remained stable at values around one (Figure 2b).

205 Over time, the increase in biomass resulted in a thickening of the biofilms (Figure 2c,d). *C.*
 206 *autotrophica* developed the thickest biofilms (~100 μm) and its maximum thickness remained stable
 207 over time. *P. purpureum* and *C. closterium* presented a similar increase of the maximum thickness
 208 reaching values like those of *C. autotrophica*. *C. vulgaris* presented thinner biofilms with a maximum
 209 thickness around 30–40 μm . The average thickness of *C. autotrophica* increased rapidly during the first
 210 four days and then levelled off around 80 μm at day 7. *P. purpureum*, *C. closterium* and *C. vulgaris*
 211 exhibited a more linear increase of the average thickness. *P. purpureum* reached a similar thickness to
 212 that of *C. autotrophica* whereas *C. closterium* and *C. vulgaris* at the end presented 40–50% lower
 213 thicknesses with respect to *C. autotrophica*.

214 The maximum diffusion distance increased over time in all the species but *C. vulgaris* (Figure
 215 2e). At the end of the assay *C. autotrophica* and *C. closterium* presented similar values and reached the
 216 highest values among all species, followed by *P. purpureum* and *C. vulgaris*. The average diffusion
 217 distance followed a similar pattern to biovolume: it increased rapidly the first four days and then
 218 leveled off from day 7 (Figure 2f). *C. autotrophica*, *C. vulgaris* and *P. purpureum* presented comparable
 219 average diffusion distance (0.6–0.9 μm) whereas *C. closterium* exhibited the lowest (0.1–0.3 μm ; $p < 0.05$).



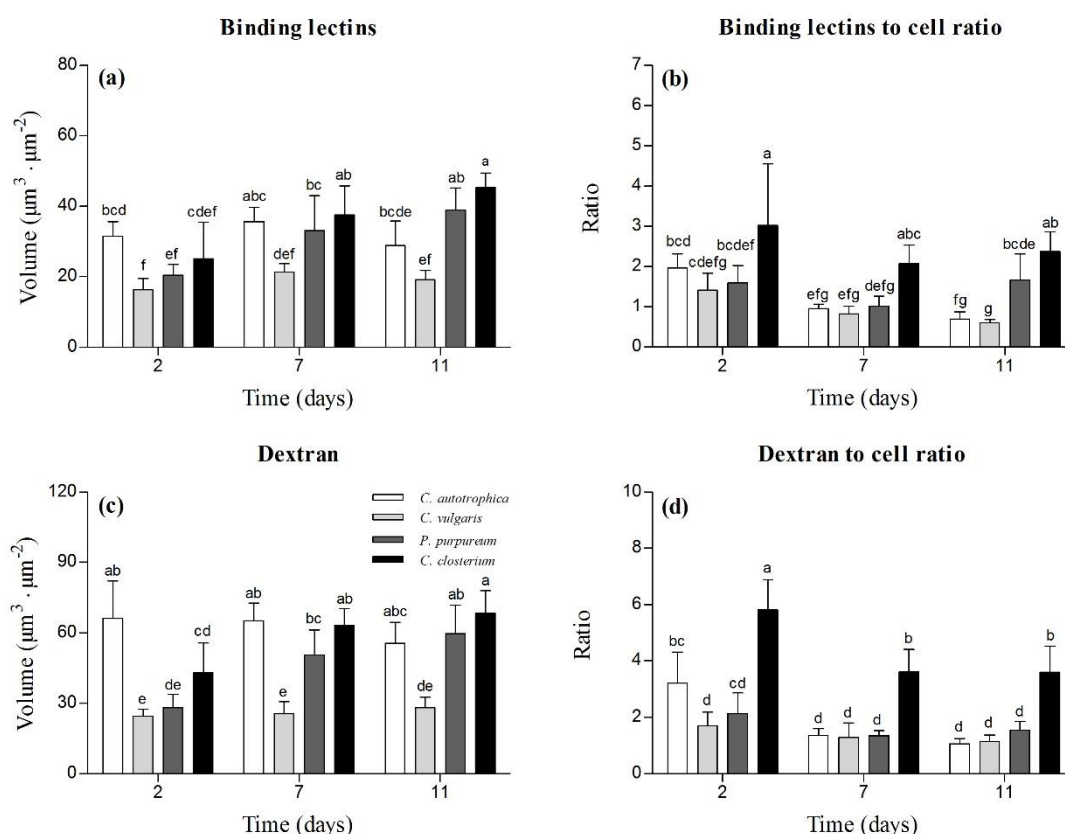
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221 **Figure 2.** Dynamics of the structural parameters obtained from the z-stacks acquired at the CLSM:
 222 biovolume (a), roughness (b), maximum thickness (c), average thickness (d), maximum diffusion
 223 distance (e) and average diffusion distance (f). The results are reported as the mean and standard
 224 deviation of 12 independent biological replicates. The fitting of the logistic model is also presented
 225 for the biovolume.

226 3.2. Matrix characterization: lectins and dextran volumes

227 In order to characterize the matrix of the biofilms, the volume of binding lectins and of dextran
 228 was quantified. The volume of lectins reflected the fraction of glycoconjugates (EPS) in the matrix
 229 and dextran was used to quantify voids, water channels and non-specifically the whole matrix
 230 (Figure 1b).

231 *C. vulgaris* presented the lowest amount of EPS (Figure 3a), whereas it increased over time in *P.*
 232 *purpureum* and *C. closterium* biofilms, which volume of EPS doubled from day 2 to day 7. No temporal
 233 change was observed for *C. autotrophica* and *C. vulgaris* ($p > 0.05$). *C. closterium* presented the highest
 234 lectins to cells ratio (Figure 3b). Over time, *C. autotrophica* exhibited a decrease over time in the lectins
 235 to cells ratio ($p < 0.05$) whereas in the other species the ratio remained stable.



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Figure 3. Structural parameters characterizing the biofilm matrix at day 2, 7 and 11. Volume of binding lectins ((a); specifically binding to glycoconjugates), lectins to cells ratio (b), volume of dextran ((c); used to stain non-specifically the matrix), and dextran to cell ratio (d). The results are reported as the mean and standard deviation of at least four independent biological replicates. Bars with different letters represent statistically different means ($p < 0.05$) as determined by pair-wise comparisons after two-way ANOVA.

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At day 2, the volume of dextran was the highest in *C. autotrophica* biofilms ($\sim 65 \mu\text{m}^3 \cdot \mu\text{m}^{-2}$). *P. purpureum* and *C. closterium* presented 50% and 30% lower volume of dextran in their matrix, but at day 7 and 11, they approached the values of *C. autotrophica* (Figure 3c). *C. vulgaris* always presented the lowest volume of dextran in the matrix ($\sim 30 \mu\text{m}^3 \cdot \mu\text{m}^{-2}$). In *C. autotrophica* and *C. vulgaris*, no significant change was observed over time (Figure 3c). Similarly to lectins, the dextran to cells ratio was always the highest in *C. closterium* and it decreased from day 2 to 7 and then levelled off. A similar ratio was found for *C. autotrophica*, *C. vulgaris* and *P. purpureum*. *C. autotrophica* presented a decreasing trend from day 2 to day 7 similar to *C. closterium*. *C. vulgaris* and *P. purpureum* biofilms did not show any significant change over time of the dextran to cells ratio (Figure 3d).

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3.3. Areal coverage over depth: cells vs. matrix vertical profiles

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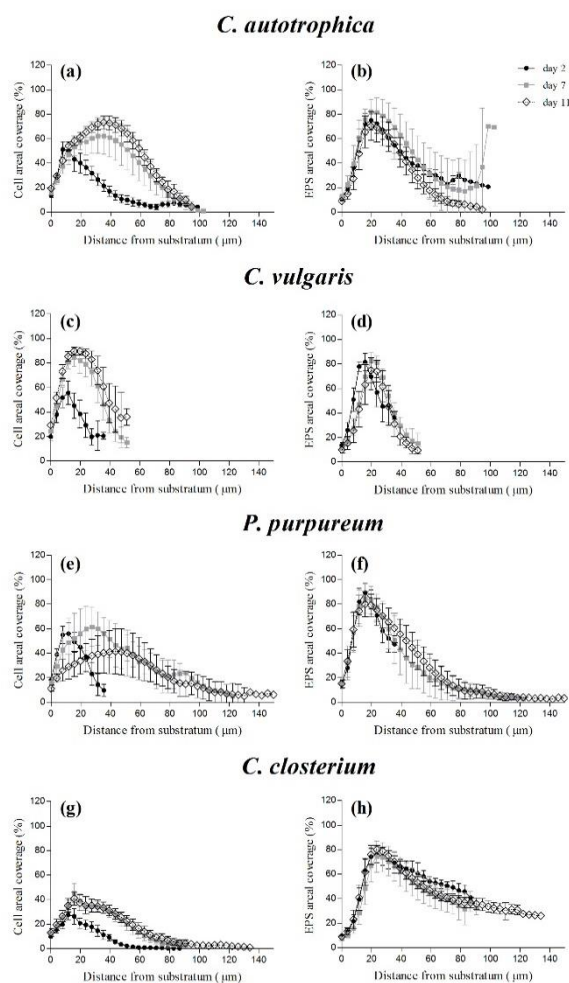
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Regardless of the species, the maximum of cell density was reached at day 2, at a distance from the substratum of 10 μm , where the cells were already covering almost 50% of the area, except for *C. closterium* that only covered 30% of the area (Figure 4g). On the other hand, during maturation, the maximal percentage of cell coverage moved at a greater distance (30–40 μm) above the substratum and the depth at which it was reached and the percentage of coverage were species-specific (ranging from 40 to 90% of covered area; Figure 4).



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Figure 4. Vertical profiles of cells (a, c, e, g) and EPS (b, d, f, h) coverage of four different monospecific microalgae biofilms after 2, 7 and 11 days of maturation. The vertical profiles are reported as the percentage of coverage of cells or of EPS obtained from the z-stacks acquired at the CLSM. The vertical profiles are reported as the mean and standard deviation of at least four independent biological replicates.

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Dextran and lectins presented comparable distributions over depth (Figure 4b,d,f,h and Figure S1). Interestingly, although the cells profile over depth changed as a function of time, the highest density of EPS and voids seemed to be positioned close to the substratum (20–30 µm) at all time points and for all the species.

In addition, the depth of maximum cells density presented a positive correlation with the average biofilm thickness ($R^2 = 0.78$, $p=0.0001$). On the other hand, for the EPS the regression slope was not significantly different from zero (Figure S2; $R^2 = 0.005$, $p=0.81$).

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3.4. Biofilm biochemical characterization by ATR-FTIR spectroscopy and correlation analysis with structural data

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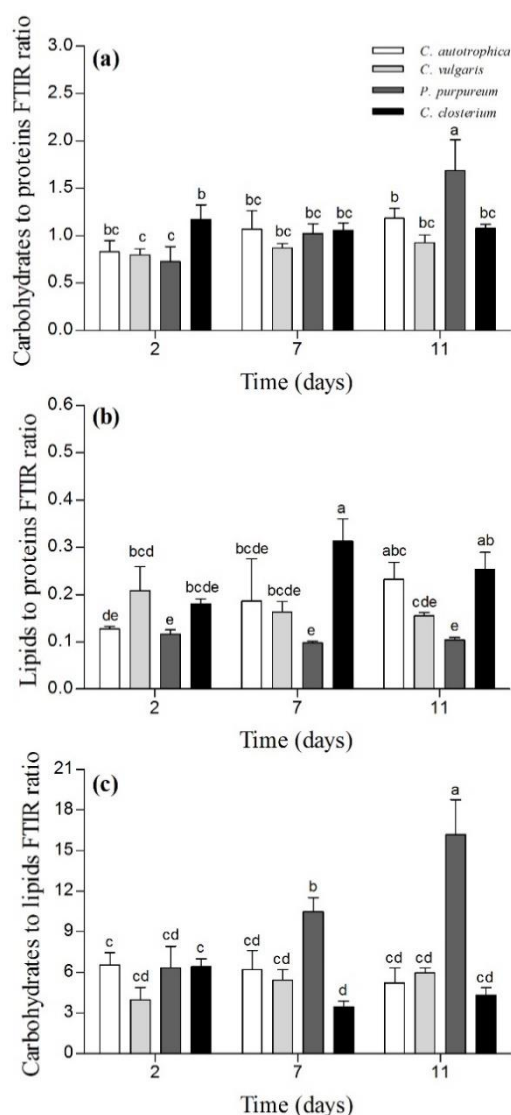
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The average FTIR spectra of the biofilms at the different sampling times (day 2, 7 and 11) are reported in Figure S3. *C. autotrophica* and *P. purpureum* were the species that presented the greatest macromolecular changes along time.

Similar carbohydrates to proteins ratios were observed for all the samples and the ratio did not change in *C. vulgaris*, *C. closterium* and *C. autotrophica* (Figure 5a; $p>0.05$). In contrast for *P. purpureum*, the carbohydrates to proteins ratio was 50% higher at day 11 than that at day 2 (Figure 5a; $p<0.05$). *P. purpureum* and *C. vulgaris* biofilms did not exhibit any change in the lipids to proteins ratio (Figure

281 5b; $p>0.05$). For *C. autotrophica* biofilms, the lipids to proteins ratio increased by 50% at day 11
 282 compared to day 2 (Figure 5b; $p<0.05$) and for *C. closterium* it increased by 30% at day 7 compared to
 283 day 2.



284

285 **Figure 5.** Macromolecular composition of the biofilms after 2, 7 and 11 days of maturation. The panel
 286 (a) depicts the carbohydrates to proteins ratio, the panel (b) depicts the lipids to proteins to ratio and
 287 the panel (c) represents the carbohydrates to lipids ratio. The results are reported as the mean and
 288 standard deviation of at least four independent biological replicates. Bars with different letters
 289 represent statistically different means ($p<0.05$) as determined by pair-wise comparisons after the two-
 290 way ANOVA.

291 In *C. autotrophica* and *C. vulgaris* biofilms, the carbohydrates to lipids ratio did not change over
 292 time ($p>0.05$). For *P. purpureum* it tripled between day 2 and day 11. In *C. closterium*, the ratio was
 293 lower at day 7 compared to day 2 (almost 50%), but no difference was found between day 11 and day
 294 2 (Figure 5c).

295 A correlation analysis was carried out to identify the possible correlations among the
 296 macromolecular ratios and the matrix of structural data (Figure S4). In *P. purpureum*, the EPS and
 297 dextran volumes were positively correlated to the carbohydrates to proteins and to the carbohydrates
 298 to lipids ratios whereas they were negatively correlated to the lipids to proteins ratio. In all the other

299 species, only minor correlations were found between matrix components and the macromolecular
300 pools (Figure S4). In contrast, for these species the macromolecular ratios were correlated to the
301 structural parameters obtained from cells autofluorescence such as thickness, biovolume, diffusion
302 distance and roughness (Figure S4).

303 4. Discussion

304 Our results show that the architecture of microalgae biofilms is species dependent. But the
305 structure evolution over time seemed to follow some general common rules that resemble those
306 described for bacteria and fungi [11,40–42].

307 During the first stages of substrate colonization the biofilms were irregular in their surfaces (i.e.
308 high roughness), thin and for some of the species the volume of EPS was higher than that of the cells
309 (Figure 2 and 3). As the biofilms matured, the biovolume increased reflecting active cell division,
310 similarly to what was reported for phototrophic biofilms by Mueller et al. [8] and Kernan et al. [20].
311 Mueller et al. [8] reported a linear increase whereas our growth curves and those from Kernan et al.
312 [20] reached a *plateau* (Figure 2a). Mueller et al. [8] focused on a natural mixed community including
313 bacteria and different species of microalgae. Therefore, the species succession over time with different
314 physiological requirements may have led to a continuous growth. In our case and for Kernan et al.
315 [20], the biofilms were composed by a single species that may have experienced over time energy or
316 nutrients limitation leading to a slowdown of the growth. Since nutrients were replenished every two
317 days, light was probably the limiting factor. Barranguet et al. [23] reported for instance that light was
318 attenuated up to 90% in mature phototrophic biofilms [23], whereas Schnurr et al. [43] reported that
319 the transmitted light for a biofilm with a thickness of 100 μm (similar to the ones reported in this
320 study) was only 12% of the incident light. This means that for a photon flux density of 100 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$
321 the cells in the deeper layers of the biofilm would receive only 12 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, an intensity far
322 below the light compensation point measured for example in *C. vulgaris* biofilm (30-60 $\mu\text{mol photons}$
323 $\cdot \text{m}^{-2} \cdot \text{s}^{-1}$; [44]).

324 The roughness of the biofilms decreased over time along with the progressive filling of the initial
325 voids by new daughter cells (Figure 2b). A similar behavior has been described for natural multi-
326 specific river biofilms [45] and for bacteria biofilms [10,41]. Mueller et al. [8] on the other hand,
327 reported a positive correlation between biovolume and roughness and a parallel increase of diatoms
328 over time. This is in agreement with the biofilm of the diatom *C. closterium* that exhibited only minor
329 changes of roughness over time (Figure 2b). The progression from rough to smoother surfaces as a
330 biofilm mature has been proposed to be dependent either on cells metabolic rate or on the maximum
331 internal transport rate of nutrients [46,47]. In the case of cells with low metabolic activity (and
332 therefore high nutrient availability) the valley between the biofilm peaks grow and merge with
333 adjacent peaks decreasing the roughness of a biofilm. Cells with high metabolic rate might become
334 nutrient limited and division will proceeds only at the biofilm peaks (i.e. along the vertical nutrient
335 gradients; [46,47]), inducing the formation of finger-like structures. Based on these observations, the
336 development of smoother biofilms along time, in *P. purpureum*, *C. vulgaris* and *C. autotrophica*, as
337 compared to the rougher structured biofilms in *C. closterium*, may reflect different cell metabolic rates.
338 Further metabolic investigations, such as intracellular measurements or O_2 evolution, will be
339 necessary to validate these conclusions.

340 As the majority of studies on photosynthetic biofilms focused on natural mixed communities
341 (i.e. formed by bacteria and microalgae), little is known about EPS dynamics in microalgae biofilms.
342 Here, we report the ability of microalgae to set up a supporting matrix and the dynamics of EPS
343 during biofilm development. Interestingly, whereas the dynamics of cells were similar among all the
344 species (Figure 2), those of EPS were more species-specific (Figure 3a): the two green algae did not
345 show any quantitative change of the EPS whereas *P. purpureum* and *C. closterium* presented a greater
346 volume of EPS over time. These different trends are in agreement with the great variety of dynamics
347 reported elsewhere as a function of the community composition. Trends of EPS production in
348 photosynthetic biofilms present in the literature are indeed very disparate [8,23,24]. Ratios of matrix
349 components (either considering lectins or dextran) to cells were not significantly different over time

350 for all species, which may indicate a stable physiological state of the cells within the biofilm.
351 Interestingly, they were positively correlated to the biofilm roughness coefficient, indicating that
352 irregular surfaces were associated with high EPS content and low cell biovolume. Similar ratios of
353 EPS to cells have been reported for stream mixed biofilms by Battin et al. [45], which proposed that
354 high EPS content may be advantageous for the attachment of the cells and first colonization of the
355 substratum.

356 During biofilm development, several processes such as cell growth, EPS excretion or
357 consumption [48], as well as the establishment of chemical and physical gradients may be responsible
358 for the vertical distribution of cells and matrix components [9]. The few literature data about z-
359 profiles in photosynthetic biofilms indicate that the distribution of cells and EPS is very much
360 dependent on the culture conditions and on the biofilm nature (bacteria, algae or mixed
361 communities), nevertheless some patterns can be drawn [8,18,20,23]. The maximal coverage for
362 photosynthetic organisms seems to occur within the first 40 μm from the substratum, whereas EPS
363 seem to match the cells position in young biofilms and to be mostly placed in layers above the cells
364 in older biofilms. In our work, at day 2 the cell z-profile was consistent with the patterns described
365 in the literature: maximal areal coverage of the cells positioned at 20 μm from the substratum (Figure
366 4) and greatest coverage of EPS typically co-localized with the cells in proximity of the substratum
367 (~20 μm). This means that the matrix components were mostly interspersed between the cells [49].
368 Along time, the maximal areal coverage of the cells moved at greater distances from the substratum
369 as described in Cole et al. [18] but the location of the maximum EPS density remained stable over
370 time (Figure 4). It is therefore likely that the production of EPS in deep layers may have been
371 responsible for the upward growth of the biofilm by global advection [48,50] This is also supported
372 by the fact that the maximum cell coverage moved upward as a function of the biofilm thickness
373 whereas the EPS maximal coverage remained stable (Figure S2).

374 In bacteria, changes in biofilms structures have been reported to be strongly related to qualitative
375 shift in the macromolecular composition of the EPS, which in turn may alter biofilms functions and
376 properties [14,15,17,51]. In order to evaluate the relationships among macromolecules and structures
377 in microalgae biofilms, a correlation analysis using the CLSM and FTIR results was performed. Lipids
378 and/or carbohydrates changed in concentration as the biofilms matured and their architecture
379 became more complex (Figure 5 and S3). The lack of correlation between the macromolecular changes
380 and the EPS and dextran volumes (except for *P. purpureum*) might indicate a reallocation of carbon in
381 the cells rather than changes in matrix components. *P. purpureum* behaved differently, and such
382 changes were positively correlated to the EPS and dextran volumes (Figure S4), suggesting an
383 increase in glycoconjugates in the matrix as the biofilm matured [52].

384 Concluding, the development, architecture and macromolecular composition of monospecific
385 microalgae biofilms seem to be strongly species-dependent. The selection of a microalgae strain for
386 further cultivation in biofilm-based systems is therefore a crucial step for the whole process as specific
387 structural features maybe more or less advantageous under a certain set of culture conditions. Future
388 studies will be required to address how hydrodynamics and the fluctuating light conditions,
389 characteristic of outdoor cultivation systems, influence the structures and composition of algal
390 biofilms. The data recorded in this study may be used to feed and calibrate photosynthetic growth
391 models to better understand the mechanisms behind biofilm development under different conditions
392 [53]. Extension of these models for large-scale production can be used for optimal process design or
393 to guide the process operations. Furthermore, microfluidic tools or larger flow-cells are promising
394 tools for completing the picture and eventually providing an overview of the advantages and pitfalls
395 of using microalgae biofilm-based system [22]

396 **Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1, Figure S1: Vertical
397 profiles of cells and dextran, Figure S2: Dependency of the depth of maximal coverage of cells and
398 glycoconjugates (EPS) as a function of the average biofilm thickness, Figure S3: ATR-FTIR spectra of microalgae
399 biofilms, Figure S4: Correlation plot, Table S1: Instrumental settings of the confocal microscope, Table S2: Biofilm
400 structural parameters calculated using COMSTAT 2.1.

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