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1 **Biomass production and physiology of *Chlorella vulgaris* during the early**
2 **stages of immobilized state are affected by light intensity and inoculum**
3 **cell density**

4

5 **Su Fang Li, Andrea Fanesi, Thierry Martin, Filipa Lopes***

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29 **ABSTRACT**

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31 The interest for biofilm-based systems for microalgae and related compounds production
32 has been increasing lately. Although extensive literature has been reported on productivity,
33 the physiological characterization (photosynthetic activity and composition) of attached
34 cells at early stages of biofilm development has seldom been investigated. In this work, the
35 effect of light intensity and inoculum cell density on 3-days *Chlorella vulgaris* biofilms
36 developed on membranes was studied. Biomass production was clearly impacted by
37 mechanism of photo-limitation occurring in biofilms acclimated to low light intensity (50
38 $\mu\text{mol m}^{-2} \text{s}^{-1}$). A higher electron transport capacity and lower chlorophyll content in
39 biofilms at high light intensity (500 $\mu\text{mol m}^{-2} \text{s}^{-1}$) were also measured which are in line with
40 patterns observed for suspended microalgae cultures. In addition, optimal conditions in
41 terms of light (250 $\mu\text{mol m}^{-2} \text{s}^{-1}$) combined with low (4.8×10^6 cells cm^{-2}) or high inoculum
42 density (28.8×10^6 cells cm^{-2}) were identified to optimize biomass and lipids production,
43 respectively. On the whole, measuring physiological profiles of immobilized cells at the
44 initial stages of biofilm development provides information to efficiently operate and
45 optimize biofilm-based systems.

46

47 **Keywords:** *Chlorella vulgaris*, FTIR-spectroscopy, Macromolecular composition,
48 Microalgae biofilms, Photosynthetic activity

49

50 1.Introduction

51

52 Microalgae are a promising source of valuable compounds (e.g. proteins, pigments,
53 carbohydrates, lipids) produced by the conversion of photons into chemical energy via
54 photosynthesis [1–4]. Nowadays, biofilm-based systems for microalgae cultivation, where
55 cells are growing attached to a substrate, have gained attention due to their higher
56 productivity, lower water requirement and harvesting costs compared with conventional
57 suspended culture technology [5,6]. These systems have been widely used for
58 biomass/biofuel production, CO₂ fixation, and wastewater treatment [6–9]. Among those,
59 the twin-layer system [10,11], in which cells grow on porous supports such as membranes,
60 printing paper or synthetic nonwoven/textile combinations, is often used at the lab and
61 pilot scales [12,13]. In particular, a direct exposure to gas and light are supposed to enhance
62 gas mass transfer and light utilization due to the absence of the liquid phase [14,15]
63 improving therefore productivity. On the other hand, nutrients are transported by diffusion
64 through the porous material into the biofilm. Limitation in nutrients can therefore occur in
65 thick and/or compact biofilms, affecting in turn their behavior and productivity [11,16].

66 Recent studies investigated environmental and operational parameters that typically
67 affect the formation and development of microalgae biofilms [17,18]. Among those factors,
68 the impact of light, nutrients availability, temperature, pH and shear stress on growth rate
69 and productivity have been studied, but mostly over long-term period of development (i.e.
70 from weeks to months) [6,19–21]. On the other hand, other factors such as substrate
71 properties (e.g. surface energy, hydrophobicity, micro-pattern, etc.) have been studied on
72 the short-term formation of biofilms, especially during the initial adhesion phase (from

73 several hours to 2-3 days) [18,22–26]. However, little information is available regarding the
74 influence of process operational factors, such as light intensity and inoculum density, on
75 biofilm growth, activity and composition at the early stages of biofilm development which
76 in turn may affect biomass and compounds production. In our work, early stage of biofilm
77 development refers to a period during which the cells attach firmly to the support, grow and
78 fully acclimate to the new environmental conditions imposed by the sessile growth
79 associated to the support.

80 Ji et al. [27] have reported that both biomass production and growth rate could be
81 improved by increasing the inoculum density (from 0.05 to 3 g DW m⁻²). However, it must
82 be kept in mind that over a certain density threshold, the cells would not grow further due
83 to light attenuation and nutrients transfer limitation [14,16,18]. In this context, light
84 attenuation could be buffered by tuning the photon flux density (PFD) used for cultivation.
85 However care must be taken since like for planktonic cultures, the growth rate and biomass
86 productivity of microalgae biofilms increase with light intensity within a favorable range,
87 but decrease if the PFD exceeds the light saturation point because of photo-inhibition [28].
88 In order to avoid such operational problems, monitoring the physiological properties of
89 cells (activity and composition) in the early stages of immobilized growth might be of great
90 help to rapidly identify possible limiting operational factors (e.g. light, nutrients, CO₂,
91 humidity, etc.) and thus adjust them to optimize the bioprocess performance [11,14,15,29].
92 Nevertheless, although extensive literature has been reported on the productivity of biofilm-
93 based systems as summarized in [30] and reference therein, the characterization of cell
94 physiological parameters at early stages has seldom been mentioned.

95 The goal of our work was to assess the impact of light and initial cell density on
96 biofilm/compounds production and on cells physiology at early stages (3 days) of biofilm
97 formation in a twin layer system. In particular, we aimed at better understanding
98 photosynthetic mechanisms on biofilms that certainly allow a reasoned choice of process
99 operational parameters to optimize productivity. In order to do that, we immobilized *C.*
100 *vulgaris* on membranes with two initial cell densities and illuminated the biofilms with
101 three PFDs. A complete characterization of physiological parameters of the immobilized
102 cells, from photosynthetic performance to macromolecular composition was conducted
103 through which biofilm growth patterns and composition under different light and inoculum
104 density combinations were explained.

105

106 2. Materials and Methods

107

108 2.1. Planktonic culture maintenance

109

110 *Chlorella vulgaris* SAG 211–11b (Göttingen, Germany) was cultured at 25°C semi-
111 continuously in 1 L transparent bottles filled with 800 mL 3N-Bristol medium [31]. The
112 cultures were bubbled with filtered air under a continuous illumination of either 50, 250 or
113 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Viugreum 50W LED outdoor floodlights, the PFDs were measured by
114 QSL-2100 quantum scalar irradiance sensor, Biospherical Instruments, San Diego, CA,
115 USA). The cultures were kept in exponential phase with a max cell concentration of $6.9 \times$
116 10^6 cells mL^{-1} (Flow cytometer, Guava EasyCyte HT; Millipore, USA) by daily dilution in
117 order to maintain a chlorophyll (Chl) *a* concentration of 0.2-1.5 mg Chl *a* L^{-1} to ensure

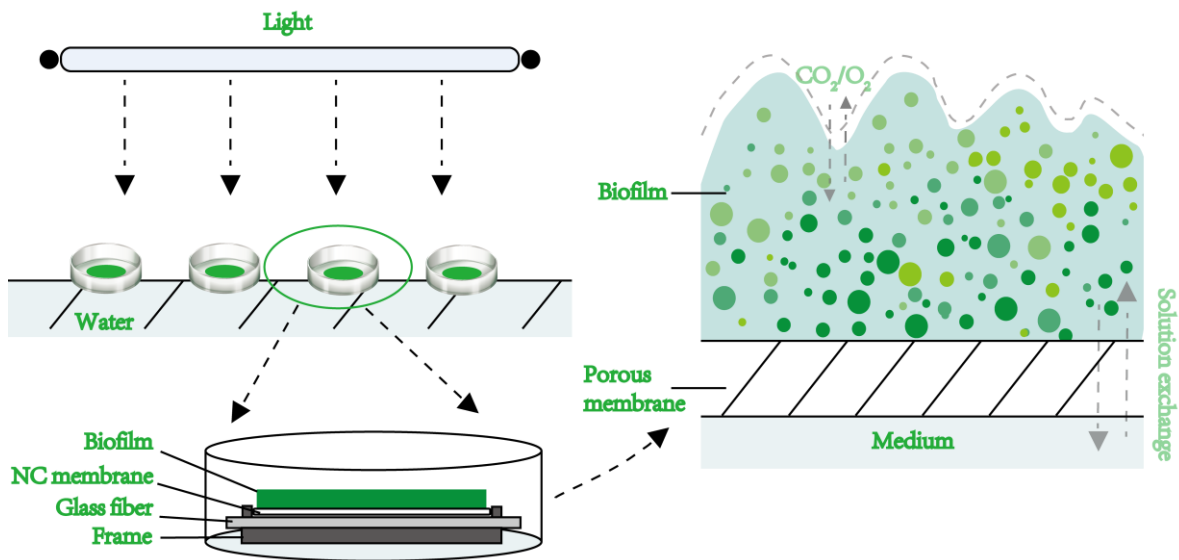
118 optimal light penetration. The planktonic cultures were pre-acclimated to each light
119 condition for at least 8 days before starting any experiment (for *C. vulgaris*, typically 5
120 days were enough to have a stabilization of Chl *a* content and growth rate).

121

122 2.2. Immobilization and growth of *C. vulgaris* on membranes

123

124 Sterile Petri dishes (55-mm diameter) filled with 3 mL of 3N Bristol medium were used
125 as bioreactors for *C. vulgaris* immobilized growth (Fig. 1). The support system consisted of
126 two glass fiber filters (working as an absorbing material for the medium; 47-mm diameter,
127 Whatman) and on top of that a nitrate cellulose filter (0.2- μm pore size; NC membrane, 25-
128 mm diameter, Whatman) on which the cells of *C. vulgaris* were immobilized.



129

130 **Figure 1.** Schematic representation of the cultivation system used for the immobilized
131 culture of *C. vulgaris* (NC membrane represents cellulose nitrate membrane filter).

132

133 In order to test the effect of PFD and the initial cell density on the immobilized growth
134 of *C. vulgaris*, the pre-acclimated planktonic cells (grown at 50, 250 and 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$)
135 were vacuum-filtered on the NC membranes with an effective colonization area of 2.01 cm^2 .
136 Two initial cell densities corresponding to $\sim 4.8 \times 10^6$ cells cm^{-2} (low initial cell density,
137 LC, $0.4 \pm 0.1 \text{ g m}^{-2}$) and $\sim 28.8 \times 10^6$ cells cm^{-2} (high initial cell density, HC, $2.6 \pm 0.8 \text{ g m}^{-2}$)
138 were obtained by filtrating specific volumes of planktonic cultures on the membranes.
139 Once the cells were immobilized, the membranes were placed on the glass fiber filters and
140 placed in Petri dishes illuminated at 50 (low light, LL), 250 (moderate light, ML) or 500
141 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (high light, HL; Hansatech Instruments Quantitherm light meter/thermometer,
142 Norfolk, England) depending on the experiments. The membranes were incubated for three
143 days and the medium was completely renewed every day. After three days of growth, the
144 cells were harvested from the membranes using Bristol medium and further measurements
145 were conducted to characterize a series of physiological parameters.

146

147 *2.3. Microscopic observation of initially immobilized cultures*

148

149 At day 0, the immobilized cultures at two cell density were scanned with a confocal
150 laser scanning microscope (CLSM) to acquire z-stacks over the whole culture depth (Fig.
151 S1). The confocal microscope set-up was as described in [19]. An inverted Zeiss LSM700
152 confocal microscope (Carl Zeiss microscopy GmbH, Jena, Germany) equipped with a LD

153 Plan-Neofluar 20×/0.4 Korr M27 was used to acquire images. Microalgae cells were
154 detected on the base of chlorophyll *a* auto-fluorescence which was excited at 639 nm.

155

156 *2.4. Relative biomass increase*

157

158 Cells were harvested with 10 mL fresh Bristol medium. For the dry weight
159 determination, the cell suspension was centrifuged and further washed in 10 mL of Bristol
160 medium. After a second centrifugation step, the pellet was dried at 100 °C until a constant
161 weight over time was reached. The relative biomass increase with respect to the initial level
162 (R_b) was determined according to Eq. (1):

163

$$164 R_b = (X_t - X_0) / X_0 \quad (1)$$

165

166 Where X_t is biomass areal density (g m^{-2}) after three days ($t = 3$ days), and X_0 is biomass
167 areal density (g m^{-2}) at the beginning of the immobilization.

168

169 *2.5. Variable chlorophyll a fluorescence measurements and relative electron transport rate* 170 *(rETR) estimation*

171

172 Photosynthetic parameters were determined using a portable pulse amplitude modulation
173 (PAM) fluorometer (AquaPen, AP 110-C, Photon Systems Instruments, Drasov, Czech
174 Republic). Measurements were performed in a 4 mL cuvette (light path of 10 mm).
175 Illumination was provided by a blue LED (455 nm), the measuring light was $0.02 \mu\text{mol m}^{-2}$

176 $^2 \text{ s}^{-1}$ and saturation pulses had an intensity of $3000 \mu\text{mol m}^{-2} \text{ s}^{-1}$. Prior to measurements, all
 177 samples were diluted to an appropriate concentration ($1 \times 10^6 \text{ cells mL}^{-1}$). After 10 min of
 178 dark-adaptation, the samples were exposed to a stepwise increase of seven actinic lights
 179 (from 0 to $1000 \mu\text{mol m}^{-2} \text{ s}^{-1}$) applied every 60s to construct the electron transport rate
 180 versus photon flux density (ETR/PFD) curves. The maximum quantum yield (F_v/F_m) and
 181 the effective quantum yield ($\Delta F/F'_m$) were calculated according to Eq. (2) and Eq. (3):

182

$$183 \quad F_v/F_m = (F_m - F_0) / F_m \quad (2)$$

$$184 \quad \Delta F/F'_m = (F'_m - F) / F'_m \quad (3)$$

185

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

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

189

$$190 \quad rETR = \Delta F/F'_m \times \text{PFD} \times 0.5 \quad (4)$$

191

192 Where PFD is the incident light and 0.5 is a factor assuming that two photons are
 193 required for linear electron transfer [32]. Light curves were quantitatively compared using
 194 the parameters of maximum rate of relative ETR ($rETR_{\text{max}}$), α and E_k ($E_k = rETR_{\text{max}} / \alpha$)
 195 obtained by fitting the rETR/PFD curves with the function $rETR = ETR_{\text{max}} (1 - e^{-\alpha I$
 196 $/rETR_{\text{max}})$ described by [33].

197

198 *2.6. Determination of cellular compounds*

199

200 Chl *a* was extracted from cells using dimethyl sulfoxide (DMSO) [34], and quantified
201 by measuring the absorption at 649 nm and 665 nm with an Evolution 60S UV–visible
202 spectrophotometer (Thermo Scientific, Madison, WI, USA). The Chl *a* concentration was
203 calculated by Eq. (5) [34]:

204

$$205 \text{ Chl } a \text{ (}\mu\text{g mL}^{-1}\text{)} = 12.19 \times \text{OD}_{665} - 3.45 \times \text{OD}_{649} \quad (5)$$

206

207 The Chl *a* content was normalized to the average cell bio-volume, which was estimated
208 with an AxioSkop 2 plus microscope (Carl Zeiss, Oberkochen, Germany).

209 The macromolecular composition of the cells was analyzed by means of an ATR-FTIR
210 PerkinElmer Spectrum-two spectrometer (PerkinElmer, Waltham, MA, USA). Biofilm cells
211 were re-suspended in 1 mL Milli-Q water and washed twice. 1-2 μL of the concentrated
212 sample was deposited on the crystal of the spectrometer, and dried at room temperature for
213 20 min. Infrared spectra were recorded in the range of 4000 to 400 cm^{-1} using an
214 accumulation of 32 scans at a spectral resolution of 4 cm^{-1} . Before loading algal samples,
215 the empty crystal was measured as background. The spectra were baselined and maximum
216 absorption values in the spectral ranges corresponding to specific macromolecular pool:
217 carbohydrates (C–O–C; 1200–950 cm^{-1}), lipids (C=O; 1750–1700 cm^{-1}), proteins (Amide
218 I; 1700–1630 cm^{-1}) were used to calculate the relative carbohydrates and lipids contents
219 with respect to the proteins signal (Fig. S2) [20,35].

220 Carbon and nitrogen contents of the biofilm samples were determined with an Elemental
221 Analyzer (Organic Elemental Analyzer FLASH 2000 CHNS/O, Thermo Scientific) using

222 1~2 mg of dried biomass collected from biofilms that have been previously washed twice
223 with miliQ water and dried at 100°C.

224

225 *2.7. Statistics analysis*

226

227 Statistical analysis was performed using IBM SPSS Statistics 25.0 (SPSS Inc., Chicago,
228 IL). Two-way ANOVA was used to test the influence of PFD and initial cell density on the
229 relative biomass increase, photosynthetic performance and cellular compounds of
230 immobilized microalgae. Bonferroni significant difference test for pairwise comparisons
231 testing was performed after the tests of normality and variance homogeneity. $P < 0.05$
232 indicates a statistically significant difference among tested values. Standard deviations were
233 calculated from at least four independent biological replicates.

234

235 **3. Results and discussion**

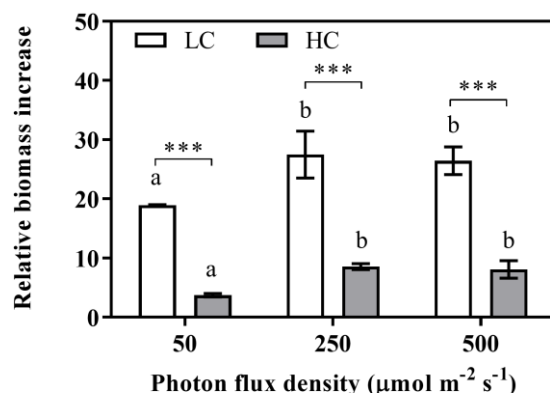
236

237 *3.1. Biomass production as a function of light intensity and initial inoculum density*

238

239 In biofilms, microalgae cell behavior is strongly impacted by the local conditions (light,
240 nutrients, biofilm properties such as density, thickness, gas exchange, ...) imposed by their
241 new life style (sessile mode) [14,20,21,36]. This may impact biomass and compounds
242 production and should be therefore studied in-depth.

243 Fig. 2 presents the relative biomass increase for the biofilms grown on membranes.
 244 Overall, our results show that when inoculated with lower cell density (LC), the
 245 immobilized cultures of *C. vulgaris* presented 3~5 times higher relative biomass increase
 246 than HC regardless of the light intensity ($P < 0.05$). On the other hand, light intensity
 247 stimulated biomass production, which is in agreement with other works [14,37].



248
 249 **Figure 2.** Effect of light intensity and initial cell density on the relative biomass increase of
 250 *C. vulgaris* biofilms after 3-days cultivation. All the results were shown as mean value \pm
 251 SD ($n \geq 4$). Bars with different letters represent the statistical differences among
 252 immobilized cultures under different light conditions at level of $P < 0.05$, while *** depicts
 253 the differences between biofilms with two inoculum cell densities at $P < 0.001$.

254
 255 Indeed, regardless of the initial cell density the immobilized cells under LL showed lower
 256 relative biomass increase compared with biofilms exposed to ML and HL ($P < 0.05$). This
 257 suggests that the immobilized growth of *C. vulgaris* under $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ may be photo-
 258 limited while that at higher light intensity were photo-saturated. This is not surprising as

259 similar results have been reported by Grenier et al. [28] for *Chlorella autotrophica*
260 biofilms.

261 It also appears that biomass increase was negatively affected by the initial cell density
262 (Fig. 2). These data can be explained by the fact that high cell density may affect light
263 penetration and/or limit the diffusion of nutrients [14,21,38]. Indeed, studies reported that
264 in immobilized cultures the biomass productivity may depend on the biofilm thickness due
265 to an exponential decrease of light [21,39]. In our study, the two initial cell conditions did
266 not produce biofilms with significantly different thickness (40 - 60 μm) but the HC culture
267 presented a denser initial population as detected by CLSM (Fig. S1) which might have
268 impacted biofilm growth. Finally, cell density dependent feedbacks (quorum-sensing) in
269 cyanobacteria are also known to influence biofilm development as a function of the
270 extracellular concentration of autoinducers (secreted by cells) which in turn affects the
271 expression of biofilm-related gene [40,41]. Therefore, we cannot rule out that similar
272 mechanisms may have occurred in our immobilized cultures. Other studies should be
273 further carried out to test such hypothesis and verify possible synergistic interactions of
274 light and nutrients availability and, quorum sensing mechanisms.

275 On the whole, these results show that with the proper combination of light intensity and
276 initial inoculum size the biofilm growth performance could be improved. For example, by
277 illuminating the biofilms with $250 \mu\text{mol m}^{-2} \text{s}^{-1}$ at low initial cell density we obtained a 28-
278 fold enhancement of biomass after 3 days of cultivation, whereas in [29], the authors only
279 obtained a 3-fold increase of *Chlorella kessleri* after 4-day cultivation.

280

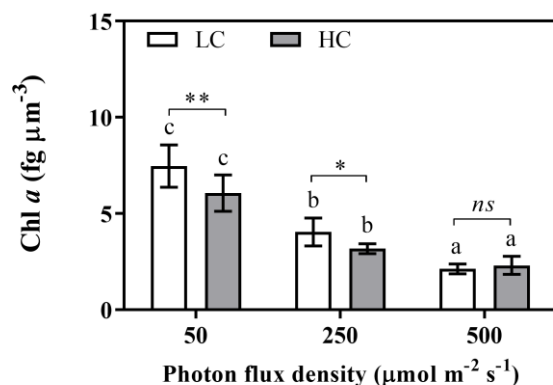
281 *3.2. Monitoring the physiological state of cells in order to improve biomass and energy-*
282 *rich compounds production*

283

284 Although photosynthetic performance and macromolecular composition of microalgae
285 have been measured in suspended cultures [42,43], only few studies estimated these
286 parameters for microalgae biofilm-based systems [44,45]. Indeed, estimating the health-
287 state of early-stage biofilms by monitoring their physiological state would be of paramount
288 importance to help the operator identifying stressful parameters in advance and to do
289 reasoned operation choices. For instance, if limiting factors are identified, cultivating
290 parameters or production strategies can be rapidly adjusted (light tuning, medium supply,
291 etc.) to prevent adverse effects in the long-term biofilm cultivation and process productivity.
292 Moreover, monitoring cell physiology also allows a better understanding of acclimation
293 strategies in photosynthetic biofilms that still remains unclear (such as the interplay
294 between photosynthetic activity and pools of macromolecules).

295 In biofilms, light availability and nutrient supply vary as a function of the inoculum size
296 and/or with the biofilm thickness [14,21]. From studies on the phytoplankton, we know that
297 variations of these two factors induce a physiological reorganization spanning from light
298 absorption to electron transport and to the final synthesis of macromolecules to acclimate
299 themselves to the new conditions and maximize growth [46–48]. Interestingly, similar
300 responses occurred in our immobilized cultures. Like their planktonic counterparts,
301 immobilized cells appear to acclimate to a range of light intensities and likely nutrients
302 availability through a series of physiological adjustments in a short term (3 days). This is

303 typically done to balance the incoming flux of energy and their energetic demand for
 304 growth in order to increase their fitness under each specific set of conditions [46,47,49].



305

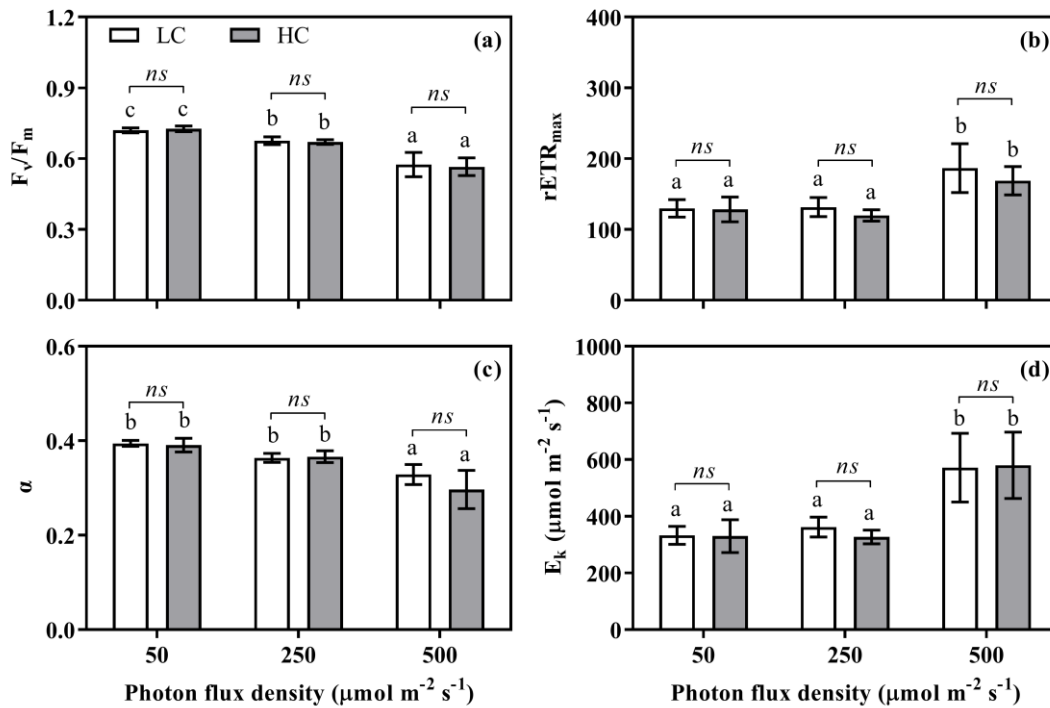
306 **Figure 3.** Effect of light intensity and initial cell density on Chl *a* content of *C. vulgaris*
 307 biofilms after 3-days cultivation. All the results were shown as mean value \pm SD ($n \geq 4$).
 308 Bars with different letters represent the statistical differences among immobilized cultures
 309 under different light conditions at level of $P < 0.05$ while ** and * respectively depict the
 310 differences between biofilms with two inoculum cell densities at $P < 0.01$ and $P < 0.05$, and
 311 *ns* represents no difference.

312

313 The first level at which microalgae can regulate excitation pressure is the absorption of
 314 light [50]. This is typically reached by changing the amount of pigment content in the cells.
 315 Fig. 3 shows that Chl *a* content declined with increasing light intensity (from 7 to 2 fg μm⁻³)
 316 ³) ($P < 0.05$), suggesting a lower amount of photons absorbed by the cells as a protective
 317 strategy to diminish photons absorption and to avoid photo-damage [51,52]. This is well
 318 described for planktonic microalgae cultures but no report of such a mechanism exists for

319 microalgae biofilms. However, changes in accessory pigments content in natural biofilms
 320 have been reported as a photo-protective mechanism [53].

321

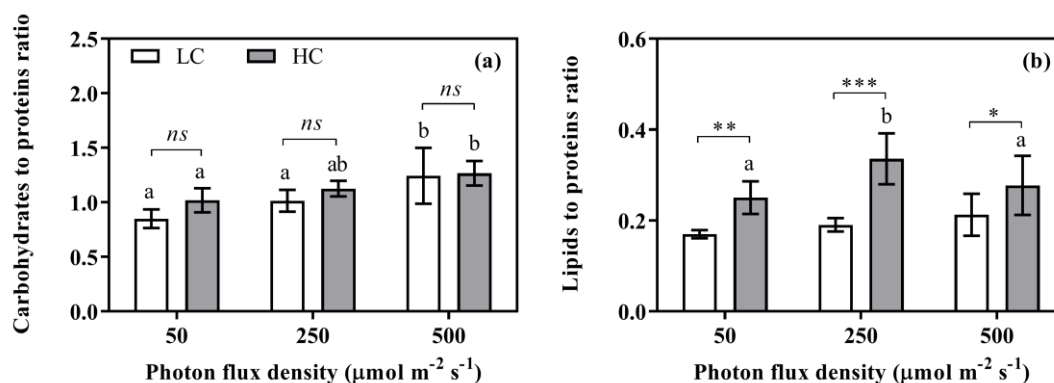


322

323 **Figure 4.** Effect of light intensity and initial cell density on photosynthetic parameters (a,
 324 F_v/F_m ; b, $rETR_{max}$; c, α ; d, E_k) of *C. vulgaris* biofilms after 3-days cultivation. All the
 325 results were shown as mean value \pm SD ($n \geq 4$). Bars with different letters represent
 326 statistical differences among immobilized cultures under different light conditions at $P <$
 327 0.05 while *ns* depicts no difference between biofilms with two inoculum cell densities.

328

329 Another level at which microalgae can regulate excitation pressure is by modulating
330 their photosynthetic capacity in order to meet the incoming flux of photons [48]. In this
331 context, photosynthetic parameters such as F_v/F_m , α , $rETR_{max}$ and E_k are often used to
332 describe the photo-acclimation state of microalgae when environmental conditions change,
333 especially with respect to irradiance and nutrient levels [42,49,54]. As depicted in Fig. 4,
334 the cells exposed to the LL and ML presented a higher α (*c.a* 0.4), lower $rETR_{max}$ and E_k
335 compared to those of HL acclimated cells, indicating that low-irradiance acclimated cells
336 modified their photo-physiology to maximize light harvesting efficiency [55]. According to
337 our expectations, algae photo-acclimated to LL and ML were more efficient in light
338 utilization than those at HL, as shown by the initial slope (α) of the $rETR/PFD$ curves.
339 Conversely, the higher $rETR_{max}$ (180 ± 10) and E_k ($575 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$) typically
340 associating with HL demonstrated that the cells exposed to $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ presented a
341 higher electron transport capacity to face the high flux of incoming photons. A similar
342 acclimation strategy with respect to light intensity has been described for fluvial biofilms
343 [56], suggesting that the cells at $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ were in a high-light acclimation state.
344 The maximum quantum yield (F_v/F_m) is typically used as a stress indicator to evaluate the
345 health state of suspended cultures and in natural photosynthetic biofilms [52,57,58]. After 3
346 days, the immobilized cells grown at $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ presented only a 0.14 lower F_v/F_m
347 value with respect to the cultures grown at LL ($P < 0.05$, Fig 4a), indicating that the
348 changes in Chl *a* content and in the photosynthetic parameters allowed the cells to optimize
349 their energy harvesting ability and using efficiency to protect from over-excitation and
350 photo-damage [48,55,59].



351

352 **Figure 5.** Effect of light intensity and initial cell density on carbohydrates (a) or lipids (b)
 353 to proteins ratio of *C. vulgaris* biofilms after 3-days cultivation. All the results were shown
 354 as mean value \pm SD ($n \geq 4$). Bars with different letters represent statistical differences
 355 among immobilized cultures under different light conditions at $P < 0.05$ while ***, ** and
 356 * respectively depict the differences between biofilms with two inoculum cell densities at P
 357 < 0.001 , $P < 0.01$ and $P < 0.05$, and *ns* represents no difference.

358

359 In planktonic cultures of microalgae, the photo-acclimation state of the cells is not only
 360 reflected in different pigment contents and photosynthetic efficiency, but often also in
 361 changes of their macromolecular and elemental composition [47]. Storage pools such as
 362 carbohydrates and lipids serve typically as carbon and energy sinks during unbalanced
 363 growth as a results of high excitation pressure and/or nutrient limitation [60,61]. In this
 364 study, a significant increase of the carbohydrates to proteins ratio with light intensity was
 365 observed (46.4% and 24.4% increases at LC and HC biofilms exposed to $500 \mu\text{mol m}^{-2} \text{s}^{-1}$,

366 respectively; Fig. 5a, $P < 0.05$). This is consistent with the high-light acclimation state of
367 the cells and in accordance with other works [62,63].

368

369 On the other hand, the relative lipids content (Fig. 5b) and the C/N ratio (Fig. S3), two
370 parameters that in microalgae are highly sensitive to N concentration in the environment
371 seemed to be affected by the initial cell density. The lipid to protein ratio increased by
372 47.3%, 76.3% and 30.4% when biofilms at HC were exposed to LL, ML and HL,
373 respectively, compared to those at LC (Fig. 5b). These results suggest an uncoupling of
374 carbon assimilation from nitrogen uptake which could be associated with nitrogen
375 limitation, in agreement with data reported in suspended cultures [64–66].

376 On the whole, the results show that the Chl *a* content, photosynthetic activity and the
377 carbohydrates to proteins ratio were highly related to light intensity, while the initial cell
378 density mostly affected the C/N and lipids to proteins ratio. In addition, optimal conditions
379 for biofilm-based cultivation of *C. vulgaris* were identified. Combined conditions of 250
380 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and low inoculum density ($4.8 \times 10^6 \text{ cells cm}^{-2}/0.4 \text{ g m}^{-2}$) promote biomass
381 production by avoiding effects caused by photo-limitation at $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 2). On
382 the other hand, if lipids are the target, combined conditions of $250 \mu\text{mol m}^{-2} \text{s}^{-1}$ and high
383 inoculum density ($28.8 \times 10^6 \text{ cells cm}^{-2}/2.6 \text{ g m}^{-2}$) should be applied. In conclusion,
384 monitoring photosynthetic activity and cellular composition help better understanding
385 photosynthetic mechanisms in biofilms but also allow to identify optimal strategies for
386 biomass and compounds production.

387

388 **4. Conclusion**

389

390 In this work, biofilm production and the physiological properties (photosynthetic
391 activity and composition) of sessile cells were assessed in response to two culture
392 operational factors, PFD and inoculum cell density. Results showed that light intensity
393 impacts biomass production. Mechanism of photo-limitation of biofilms exposed to LL was
394 highlighted. Acclimation of sessile cells to light and probably to nutrients were confirmed
395 by changes in the photosynthetic activity parameters and microalgae composition (Chl *a*
396 content and relative lipid/carbohydrates pools). Optimal conditions to produce biomass or
397 lipids were determined: 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ combined with LC or HC initial inoculum,
398 respectively. On the whole, monitoring physiological profiles at the early stage of biofilms
399 development provides information to better understand photosynthetic mechanisms in
400 biofilms and to operate efficiently biofilm-based systems in order to optimize biomass and
401 macromolecules productions.

402

403 **Author contributions**

404 S. F. Li, A. Fanesi, T. Martin and F. Lopes designed the experiments, S. F. Li conducted
405 the experiments, S. F. Li, A. Fanesi and F. Lopes analyzed the data, S. F. Li and A. Fanesi
406 wrote the manuscript, F. Lopes revised the article. All authors read and approved the final
407 manuscript.

408

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414 **Competing interests**

415 The authors declare no competing interests.

416

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