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Extracellular hydrocarbon and intracellular lipid accumulation are related to nutrient-sufficient conditions in pH-controlled chemostat cultures of the microalga *Botryococcus braunii* SAG 30.81

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1 **Extracellular hydrocarbon and intracellular lipid accumulation are related to nutrient-**
2 **sufficient conditions in pH-controlled chemostat cultures of the microalga *Botryococcus***
3 ***braunii* SAG 30.81**

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8 **Highlights:**

- 9 1. Effect of pH in continuous cultures studied in successive steady-states.
10 2. Hydrocarbon productivities in *B. braunii* cultures were pH-dependent.
11 3. Increase of biomass and hydrocarbon productivities induced by 3N6P-AF-6 medium.
12 4. Extracellular hydrocarbon content appears related to total intracellular lipid content.
13 5. Hydrocarbon productivities were related to steady-state DIC speciation.

14
15
16 **Abstract**

17 Here we investigate the effect of environmental pH on biomass and hydrocarbon productivity
18 in photoautotrophic cultures of *Botryococcus braunii* SAG 30.81 (race A). Successive steady-
19 states of continuous cultures were used to study the effects of pH controlled by carbon dioxide
20 feeds in a closed photobioreactor. At a fixed dilution rate of 0.2 d^{-1} , hydrocarbon productivity
21 was found to be pH-dependent in the range 5.5 to 8.0, with an optimum at pH 6.5. pH
22 variations from pH 6.0 to pH 8.0 had little effect on biomass productivity, nutrient
23 requirement for biomass production, photosynthetic yield and pigment ratios, whereas acidic
24 pH 5.5 conditions induced a slight decrease in biomass and hydrocarbon productivities as well
25 as stressing the cells . The pH effect occurred even after a threefold increase in nitrate and

26 phosphate concentrations in the feed culture medium, resulting in biomass productivity up to
27 $8.9 \text{ g m}^{-2} \text{ d}^{-1}$ and hydrocarbon productivity up to $0.5 \text{ g m}^{-2} \text{ d}^{-1}$. Dissolved inorganic carbon data
28 suggest that a maximum hydrocarbon content of 5.7% dry weight was reached at and above 1
29 mmol L^{-1} dissolved CO_2 and that hydrocarbon production was linearly related to CO_2 up to 2.5
30 mmol L^{-1} , whatever the N and P enrichment of AF-6 culture medium.

31

32 **Keywords:** *Botryococcus*; pH effect; continuous culture; dissolved CO_2 ; hydrocarbon; lipids

33

34 1. Introduction

35 Microalgal biofuels are gaining increasing attention as an alternative energy source. Among
36 microalgae, *Botryococcus braunii* strains have emerged as the most promising potential
37 renewable resource due to the huge amount of extracellular hydrocarbon (HC) they produce,
38 which can reach 75% of dry cell weight [1]. Strains of *B. braunii* are classified into four races,
39 A, B, L and S, according to nature of the hydrocarbons they produce[2,3]. Hydrocarbon oils
40 of *B. braunii* make a suitable feedstock material for hydrocracking to transport fuels [1]. CO_2
41 supplementation improves growth and hydrocarbon oil production in *B. brauni* cultures [4–6],
42 which means that *B. braunii*, like other photosynthetic microalgae, can bio-mitigate CO_2 from
43 industrial exhaust gases to help reduce greenhouse gas emissions [7].

44 However, successful mitigation of CO_2 and improved production of hydrocarbon oils depend
45 on CO_2 tolerance and the associated decrease in culture pH. Environmental pH is one of the
46 key operational parameters driving algal growth, as it is known to affect photosynthetic
47 activity, availability of inorganic nutrients through carbon and metal speciation, potential
48 enzyme activities within cell walls, and oil production rates [8]. Low pH could influence
49 intracellular pH and thereby modify enzyme activities [9], and acidification of the chloroplast
50 is known to cause inhibition of photosynthesis [10]. This makes it vital to achieve appropriate

51 pH control to improve the productivity of microalgal cultures, as demonstrated for the
52 triacylglycerol-accumulating microalgae *Nannochloropsis salina* [8]. Nevertheless, few
53 studies have investigated the influence of pH on *B. braunii* cultivation [4,6,11–13]. It has
54 been reported that maximal production of both biomass and hydrocarbon could be achieved at
55 a pH around 6.3 [4] or at pH 7.5 [12], whereas another study showed higher biomass could be
56 obtained at pH 6.0 but that hydrocarbon content showed little response to pH in the range 6.0
57 to 8.5 [11]. Other studies have demonstrated pH effects on pigments [13] and growth rate [6]
58 but did not address pH effect on hydrocarbon production. It appears that *B. braunii* race A are
59 generally cultivated at around pH 7.5 [14] whereas *B. braunii* race B strains are cultivated at
60 around pH 6.5 [6]. However, in these studies, culture pH was only adjusted before cultivation
61 [11,12,15] or monitored [4,6], never held constant by either automatic pH regulation or
62 stoichiometrically-balanced growth media as was done for triacylglycerol-accumulating
63 microalgae [16]. Moreover, these experiments were often carried out in batch cultures for
64 relatively short durations. Batch cultures are non-ideal model systems as they carry a number
65 of inherent drawbacks for quantitative studies on the effects of environmental parameters on
66 microalgal culture performances. Chemostat cultivation makes a far better tool for studies on
67 quantitative aspects under controlled cultivation conditions.

68 We therefore investigated the effect of pH on biomass and hydrocarbon productivities
69 in continuous controlled cultures of a *B. braunii* race A strain. Chemostat cultures were used
70 at a fixed dilution rate to determine the relation between culture performances at steady-state,
71 and environmental pH was regulated by automatic CO₂ injection into the cell suspensions.
72 This is the first time this approach has been applied to investigate the behavior of *B. braunii*
73 cultures. A change in nitrate and phosphate nutrient composition of the feeding medium was
74 used to compare the effects of increasing residual nitrate and phosphate concentrations in the
75 cultures on biomass and hydrocarbon productivities according to pH setpoints.

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2. Materials and Methods

2.1. Strain and medium

Experiments were conducted with the strain *B. braunii* Kützing SAG 30.81 obtained from the Culture Collection of Algae at Gottingen University (Sammlung von Algenkulturen, SAG). This strain is classified as a race A strain as identified by their characteristic hydrocarbons using GC-MS [11,15,17] and further confirmed by comparison with a typical race A strain LB 572 using random amplified polymorphic DNA analysis [18]. The strain was kept on buffered AF-6 medium with 2-(N-morpholino) ethanesulfonic acid (MES, 2 mmol L⁻¹) agar plates and sub-cultured in a 300 mL column. The subcultures were regularly checked for absence of bacteria and fungi by controls on Plate Count Agar (Fluka).

The modified AF-6 medium was used for preliminary cultivations [19]. Final concentration contains NaNO₃ 140 mg L⁻¹, NH₄NO₃ 22 mg L⁻¹, MgSO₄·7H₂O 30 mg L⁻¹, KH₂PO₄ 10 mg L⁻¹, K₂HPO₄ 5 mg L⁻¹, CaCl₂·2H₂O 10 mg L⁻¹, FeCl₃·6H₂O 1 mg L⁻¹, Fe-citrate 2 mg L⁻¹, citric acid 2 mg L⁻¹, Na₂EDTA·2H₂O 8 mg L⁻¹, micronutrients (MnCl₂·4H₂O 200 µg L⁻¹, ZnSO₄·7H₂O 40 µg L⁻¹, CoCl₂·6H₂O 8 µg L⁻¹, Na₂MoO₄·2H₂O 20 µg L⁻¹, NH₄VO₃ 1 µg L⁻¹, H₂SeO₃ 5µg L⁻¹) and vitamins (biotin 2 µg L⁻¹, thiamine HCl 10 µg L⁻¹, vitamin B₆ 0.1 µg L⁻¹, vitamin B₁₂ 1 µg L⁻¹). Before autoclaving, pH was adjusted to 6.6 using KOH. For a first set of experiments, feed medium for continuous cultures was AF-6 medium without MES buffer but enriched with two-fold phosphate concentration (2P-AF-6) in order to avoid the phosphate limitation observed during preliminary batch cultures. A second set of experiments was done with a 3-fold sodium nitrate 6-fold phosphate concentration-enriched medium (3N6P-AF-6, without MES) to improve biomass production.

101 2.2. Culture system and experimental set-up

102 2.2.1 Batch culture

103 Experiments were conducted in 300-mL bubble columns. Aeration was provided by injecting
104 0.2 μm -filtered air at the bottom of the columns at a rate of 0.1 vvm (volume per volume per
105 minute). Continuous photon irradiance was provided by cool-white fluorescent lamps
106 (Philipps, Master TLD 18W) and its intensity-adjusted to 150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ as
107 photosynthetic photon flux density (PPFD) corresponding to photosynthetically active
108 radiation (PAR). Measurements were performed at the surface of the columns using a Li-Cor
109 light meter (LI-250A). Batch cultures were maintained at a temperature of $23 \pm 2^\circ\text{C}$.

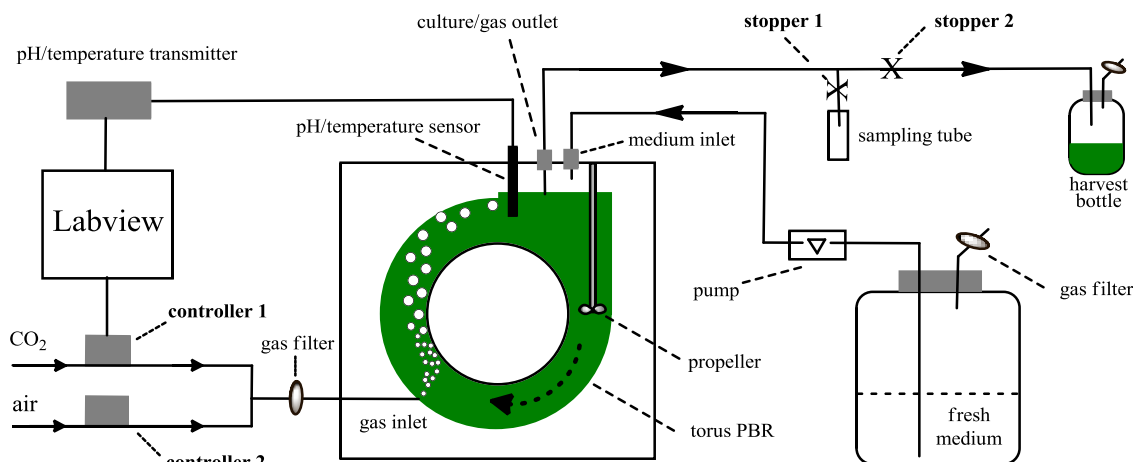
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111 2.2.2 Continuous culture

112 The torus photobioreactor (PBR) system (Fig. 1), as described by Pruvost et al. [20], was
113 sterilized by vapor stream. Working volume (V_w) was 1.4 L with a light incident area A_l of
114 0.035 m^2 and a A_l/V_w ratio of 25 m^{-1} . Speed of the axial flow impellers was 200 rpm. The
115 chemostat cultures were fed with a medium flow rate (F) of 0.28 L d^{-1} , corresponding to a
116 dilution rate $D = F/V_w$ of 0.2 d^{-1} .

117 Temperature was regulated at $23 \pm 1^\circ\text{C}$. The photobioreactor was continuously illuminated at
118 a photon flux density of 150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ in the 400–700 nm waveband (PAR). Air
119 flow rate was 450 mL min^{-1} corresponding to an aeration rate of 0.32 vvm. pH was controlled
120 by automatic CO_2 injection (Mettler Toledo M200 pH-controller) for values set sequentially
121 at 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0, after reaching a steady-state at the operating pH (mean
122 duration of 10 days).

123



124

125

126 **Fig. 1.** Sketch map of the torus PBR system. At sampling, stopper 1 is open and stopper 2 is
 127 closed. At other times, stopper 1 is closed and stopper 2 is open. Sampling tube and harvest
 128 bottle were autoclaved (121°C, 25 min) before being connected. Controller 1 (CO₂ flow
 129 controller) will be open when the pH value detected online is higher than the setpoint pH
 130 value, or closed when the pH value is lower than setpoint pH. Controller 2 (air flow controller)
 131 is open all the time at a constant air flow rate (450 mL min⁻¹), with culture pH controlled at
 132 the setpoint pH ± 0.1 pH unit.

133

134

135 2.3. Analytical methods

136 2.3.1 Biomass concentration and productivity

137 Biomass concentration (X , g L⁻¹ or g m⁻²) was determined by dry weight (dw) measurement as
 138 described in [21]. Amounts of samples (10 mL) were filtered over pre-weighed Whatman
 139 GF/F-grade glass fiber filters and washed with distilled water. The filters were dried at 105°C
 140 for 24 h in aluminum trays, cooled down in a desiccator, and dry weight was measured.
 141 Biomass productivity P_x (g L⁻¹ d⁻¹ or g m⁻² d⁻¹) was calculated using the formula:

142

$$P_x = D \cdot X, \text{ (g L}^{-1} \text{ d}^{-1} \text{ or g m}^{-2} \text{ d}^{-1}\text{)}$$

143

144 *2.3.2 Pigment contents*

145 The samples (2 mL) were first centrifuged (12,100 g, 10 min, room temperature) then the
 146 cells were suspended in 2 mL methanol solution (90 % v/v) by sonication and vortexing
 147 (18,000 rpm) followed by incubation in the dark for 2 h. The supernatant was clarified by
 148 centrifugation (12,100 g, 10 min, room temperature) and the optical densities at 470 nm,
 149 652.4 nm, 665.2 nm and 750 nm (, Perkin Elmer Lambda 2S spectrophotometer) were used to
 150 determine the concentrations of chlorophylls (Chl, including chlorophyll *a* (Chl *a*) and
 151 chlorophyll *b* (Chl *b*) and carotenoids (Car) using the spectrophotometric equations of
 152 Lichtenthaler & Buschmann [22] and those below:

$$153 \quad \text{Chl } a = (OD_{665.2nm} - OD_{750nm}) \cdot 16.82 - (OD_{652.4nm} - OD_{750nm}) \cdot 9.28, (\text{mg L}^{-1})$$

$$154 \quad \text{Chl } b = (OD_{652.4nm} - OD_{750nm}) \cdot 36.92 - (OD_{665.2nm} - OD_{750nm}) \cdot 16.54, (\text{mg L}^{-1})$$

$$155 \quad \text{Car} = \frac{1000 \cdot (OD_{470nm} - OD_{750nm}) - 1.91 \cdot \text{Chl } a - 95.15 \cdot \text{Chl } b}{225}, (\text{mg L}^{-1})$$

156

157 *2.3.3 Photosynthetic activities*

158 *In vivo* chlorophyll fluorescence of photosystem II (PSII) was measured with a Water-PAM
 159 (Walz GmbH, Germany). Samples from steady-state cultures were dark-adapted for 20 min to
 160 determine F_0 then exposed to a saturating pulse (800 ms; irradiance of $\sim 2400 \mu\text{mol photons}$
 161 $\text{m}^{-2} \text{s}^{-1}$) to determine F_m . Photochemical efficiency (F_v/F_m) was calculated with WinControl-
 162 3 Software (Walz GmbH). For determinations of photosynthesis-irradiance (P/I) curves,
 163 samples of microalgal cells were exposed to 8 incremental levels of irradiance (75, 109, 165,
 164 245, 346, 479, 773 and $1127 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), each with a 3-min light-adaptation
 165 duration [23]. Electron transport rate (ETR) vs photosynthetic active radiation (PAR) values
 166 were fitted as P/I curves according to Platt's equation [24], i.e.:

$$\text{ETR} = \text{ETR}_{\text{mPot}} \cdot \left(1 - e^{-\frac{\alpha \cdot \text{PAR}}{\text{ETR}_{\text{mPot}}}}\right) \cdot e^{-\frac{\beta \cdot \text{PAR}}{\text{ETR}_{\text{mPot}}}}$$

167
 168 where α is initial slope of P/I curve, which is related to quantum efficiency of photosynthesis
 169 (electrons/photons), and β is photoinhibition parameter. The values of α , β and ETR_{mPot} are
 170 estimated by the fitting procedure.

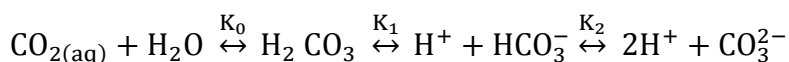
171 Maximum ETR (ETR_{max} , $\mu\text{mol electrons m}^{-2} \text{ s}^{-1}$) is determined by the equation:

$$ETR_{max} = ETR_{mPot} \cdot \left(\frac{\alpha}{\alpha + \beta}\right) \cdot \left(\frac{\beta}{\alpha + \beta}\right)^{\frac{\beta}{\alpha}}$$

172
 173 *2.3.4 Nutrient concentrations*
 174 *2.3.4.1 Dissolved inorganic carbon concentrations and calculation for $CO_{2(aq)}$*
 175 Dissolved inorganic carbon (DIC) was determined using a TOC Analyzer (Shimadzu, TOC-
 176 5000A). The supernatants of the samples were injected after centrifugation (12,100 g, 10 min,
 177 10°C). The relative concentrations of all DIC species, dissolved carbon dioxide ($CO_{2(aq)}$),
 178 carbonic acid (H_2CO_3), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) in the culture were
 179 calculated from the DIC and the pH values at each steady-state with equations derived from
 180 the following reactions:

$$181 \quad [DIC] = [CO_{2(aq)}] + [H_2CO_3] + [HCO_3^-] + [CO_3^{2-}];$$

182 At constant pressure and temperature, the following equilibria between $CO_{2(aq)}$, H_2CO_3 ,
 183 HCO_3^- and CO_3^{2-} species apply:



$$184 \quad \text{where } K_0 = \frac{[H_2CO_3]}{[CO_{2(aq)}]}, K_1 = \frac{[HCO_3^-][H^+]}{[H_2CO_3]}, K_2 = \frac{[CO_3^{2-}][H^+]}{[HCO_3^-]},$$

185 The calculations used the constants from Millero & Roy [25,26]. At equilibrium, $K_0 \approx 1.7 \times$
 186 10^{-3} ; then only a small fraction, less than 1% of the dissolved CO_2 , is converted to H_2CO_3 ,
 187 with the main fraction as aqueous molecular $CO_{2(aq)}$. K_1 and K_2 were calculated from the
 188 following equations:

$$pK_1 = \frac{6320.813}{T} + 19.568224 \cdot \ln T - 126.34048$$

$$pK_2 = \frac{5143.692}{T} + 14.613358 \cdot \ln T - 90.18333$$

189 Then, $[CO_{2(aq)}] = [DIC] \cdot \frac{[H^+]^2}{[H^+]^2 + K_1 \cdot [H^+] + K_1 \cdot K_2}$

190 *2.3.4.2 Nitrate and phosphate concentrations*

191 Nitrate as N-NO₃ and phosphate as P-PO₄ concentrations were determined in the culture
 192 medium using an ionic chromatography system including a cationic chromatograph (Dionex-
 193 1100-IonPac) equipped with a CG16 guard column and CS16 separation column, an anionic
 194 chromatograph (Dionex-ICS 900-IonPac) equipped with a AG9-HC guard column and
 195 AS9HC separation column, and an external AMMS (Anion-ICE MicroMembrane Suppressor
 196 300, Dionex) supplied with sulfuric acid (H₂SO₄, 25 mmol L⁻¹, 1.8 mL min⁻¹). Eluents were a
 197 solution of 6 mmol L⁻¹ Na₂CO₃ and 1 mmol L⁻¹ NaHCO₃ at a flow of 0.9 mL min⁻¹ for anionic
 198 chromatography and a solution of 20 mmol L⁻¹ H₂SO₄ at a flow of 0.9 mL min⁻¹ for cationic
 199 chromatography. Detection was by conductivity, and data acquisition and processing were
 200 accomplished on Chromeleon software. Nutrient requirement for biomass production (R_{SX},
 201 mg gX⁻¹) in terms of biomass production was calculated as:

202
$$R_{SX} = \frac{S_{in} - S_{out}}{X}, \text{ (mg gX}^{-1}\text{)}$$

203 where: S_{in} is ion concentration of the fresh medium flow into the photobioreactor and S_{out} is
 204 ion concentration of culture sampled from the PBR outlet.

205

206 *2.3.5 Hexane extract contents*

207 Hexane extracts, regarded as representative of crude hydrocarbon, were measured using a
 208 gravimetric method [4]. The lyophilized biomass was soaked in n-hexane at room temperature

209 then sonicated and vortexed (18,000 rpm) for 5 minutes. The upper hexane layer containing
210 hydrocarbon was collected by centrifugation (12,100 g, 10 min, 10°C), and the supernatants
211 were evaporated under a gentle flow of nitrogen gas. Dried hexane extracts were weighed to
212 determine percent of biomass dry weight.

213

214 *2.3.6 Total lipid and hydrocarbon contents*

215 Total lipids and hydrocarbons were determined according the method described by Ishimatsu
216 et al. [27]. Quantity of total lipids was determined gravimetrically and expressed as percent of
217 biomass dry weight. After drying and weighing for the calculation of total lipids, the
218 remaining residue (total lipids) was dissolved in a small amount of hexane and then purified
219 by chromatography on a silica-gel column. Four bed volumes of n-hexane were applied to the
220 column, and the purification of hydrocarbon was confirmed by thin-layer chromatography
221 (TLC) using squalene as standard. The eluate was evaporated and dried under a gentle flow of
222 nitrogen gas, and quantity of purified hydrocarbon content [HC] (% dw) was determined.
223 Hydrocarbon productivity was calculated as:

$$224 \quad P_{\text{HC}} = P_X \cdot [\text{HC}], \text{ (g L}^{-1} \text{ d}^{-1} \text{ or g m}^{-2} \text{ d}^{-1}\text{)}$$

225 and specific rate of hydrocarbon production was determined as:

$$226 \quad q_{\text{HC}} = \frac{P_{\text{HC}}}{X}, \text{ (gHC gX}^{-1} \text{ d}^{-1}\text{)}$$

227

228 *2.4. Statistical analysis*

229 Results were expressed as mean \pm standard deviation (SD). Data was processed by R software
230 to test homogeneity of variance using leveneTest (Car package). Differences between groups
231 were analyzed statistically using one-way analysis of variance (ANOVA) and Tukey's
232 'Honest Significant Difference' method (TukeyHSD) at 95% family-wise confidence level.

233

234 3. Results and Discussion

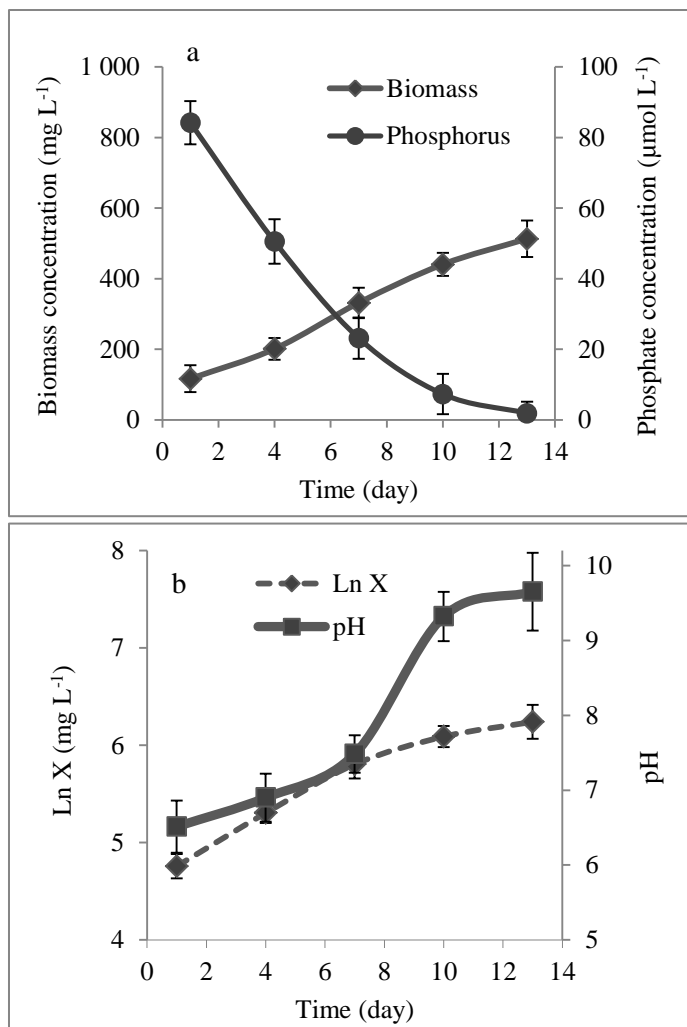
235 The effect of pH on efficiency of *B. braunii* cultures was essentially studied using continuous
236 cultures after preliminary assays in batch cultures to determine the value of the dilution rate
237 and the enrichment level of the basic AF-6 culture medium. The results thus correspond to N
238 and P-enrichment levels of the culture medium at a fixed dilution rate. All data obtained under
239 continuous cultures correspond to steady-state equilibrium, i.e. < 10% variation on at least 10-
240 day periods.

241

242 3.1. Preliminary characterization of *B. braunii* batch cultures in AF-6 medium

243 Preliminary cultures were maintained in 300 mL columns with AF-6 medium, as this medium
244 has been demonstrated as suitable for *B. braunii* [27]. During 12-day batch cultures, pH
245 increased from 6.6 to 9.5, although the AF-6 medium contained 2 mmol L⁻¹ MES, a pH buffer.
246 This corresponds to a specific pH variation from 1.8 UpH to 4.2 UpH per g biomass produced.
247 This alkalization of the culture medium, routinely observed in any microalgal cultures with
248 nitrate as N-source, has been reported in other *B. braunii* cultures [11,13].

249 Ionic analysis of the culture supernatants after 12-day incubation periods indicated that almost
250 all the initial phosphate concentration (103 μmol L⁻¹), was consumed (Fig. 2a). Another study
251 has already shown that phosphate was a potent limiting factor in batch cultures of *B. braunii*
252 765 with an initial concentration of 175 μmol L⁻¹ [4]. The residual concentrations of the other
253 macronutrients after the 12-day cultivations were still relatively high, such as nitrate at about
254 300 μmol L⁻¹, except for DIC which in some cultures was under 2 mg L⁻¹. These measured
255 DIC values indicated that inorganic carbon could be limited. Then, during the first set of
256 experiments, the continuous culture was fed with a 2-fold phosphate-enriched AF-6 medium,
257 named 2P-AF-6. Moreover, the end of the log phase seemed to coincide with pH values over
258 7.5 (see Fig. 2b). Therefore, the pH effects on *B. braunii* biomass and hydrocarbon production



259

260

261 **Fig. 2.** Variations of (a) biomass, X, and phosphate concentrations and (b) Ln X and pH as a
 262 function of time in a *B. braunii* batch culture. Culture medium was AF-6. The residual
 263 concentrations of the other nutrients were non-negligible after 13 days of incubation.

264

265 were studied with microalgae grown in a photobioreactor where pH was regulated using
 266 controlled injection of CO₂ gas flow. Through this approach, the inorganic carbon supply of
 267 the cultures was provided, as after injection a fraction of the CO₂ gas dissolves as CO₂ (aq)
 268 according to Henry's law and is then converted to HCO₃⁻ and CO₃²⁻. The dilution rate of the
 269 continuous cultures was fixed at 0.2 d⁻¹, as the specific growth rates of *B. braunii* cultures are
 270 usually in the range 0.1 d⁻¹ to 0.3 d⁻¹, with a maximal growth rate of 0.5 d⁻¹ [6].

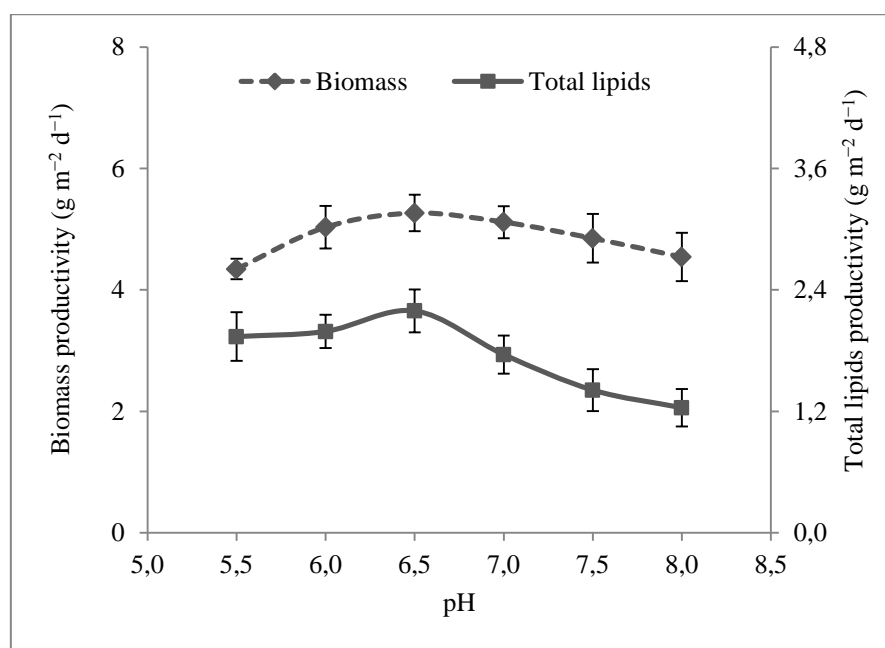
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272 3.2. Characterization of *B. braunii* continuous cultures in 2P-AF-6 medium

273 3.2.1 Biomass and hydrocarbon productivities in 2P-AF-6 medium as a function of pH

274 To highlight the effect of environmental pH on production rates, a continuous culture of *B.*
 275 *braunii* was started in a 1.4 L photobioreactor. All the parameters were fixed, i.e. irradiance
 276 ($150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), temperature ($23 \text{ }^\circ\text{C}$), stirring (200 rpm), aeration rate (0.32 vvm)
 277 and 2P-AF-6 feed medium at a dilution rate of 0.2 d^{-1} , except the pH value which was
 278 changed stepwise in 0.5 unit increments from pH 5.5 to pH 8.0. The biomass and lipid
 279 concentrations were determined after reaching a steady-state at each pH value. The effects on
 280 biomass and total lipid productivities were deduced from these steady-state values.

281



282

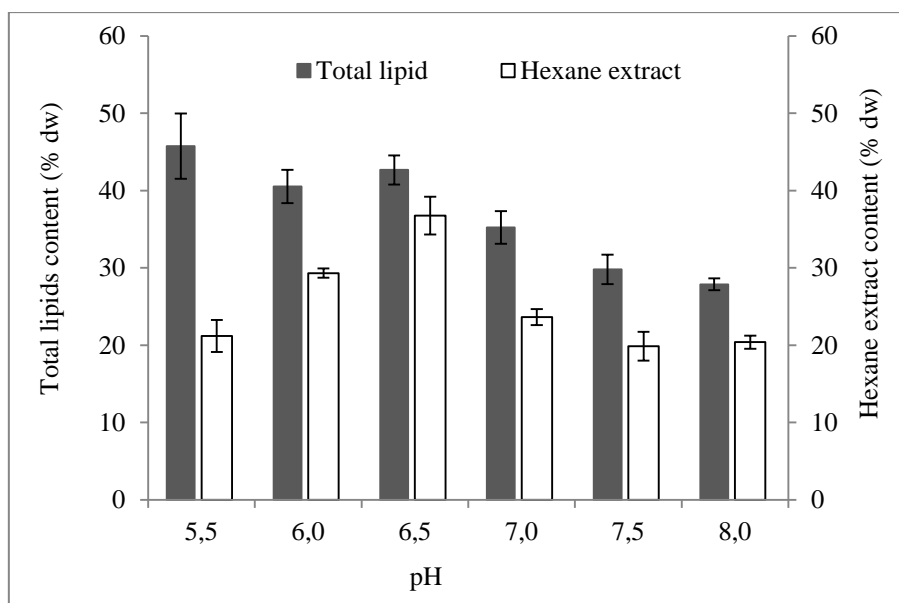
283 **Fig. 3.** Biomass and total lipid productivities as a function of pH in steady-state continuous
 284 cultures fed with 2P-AF-6.

285

286 Environmental pH had a significant effect on total lipid productivity ($P < 0.05$) while having
 287 little effect on biomass productivity (Fig. 3). According to the geometric features of the PBR,
 288 maximal biomass productivity reached $5.2 \text{ g m}^{-2} \text{d}^{-1}$ and total lipids areal productivity reached

289 2.2 g m⁻² d⁻¹ at pH 6.5. Environmental pH not only had an effect on total lipids productivity
 290 but also on the total lipids and hexane extract contents of dry biomass (Fig. 4). Total lipid
 291 contents were significantly different according to whether microalgal biomass was produced
 292 at a pH over or under the threshold of 7.0.

293

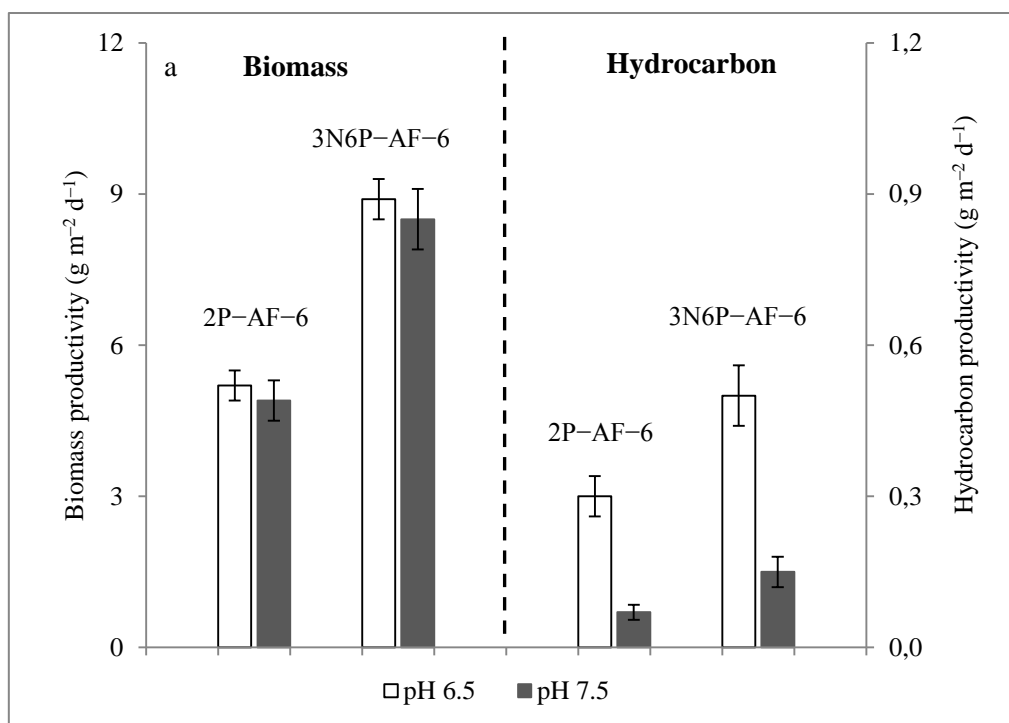


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295 **Fig. 4.** Total lipids and hexane extracts contents as a function of pH in steady-state continuous
 296 cultures fed with 2P-AF-6.

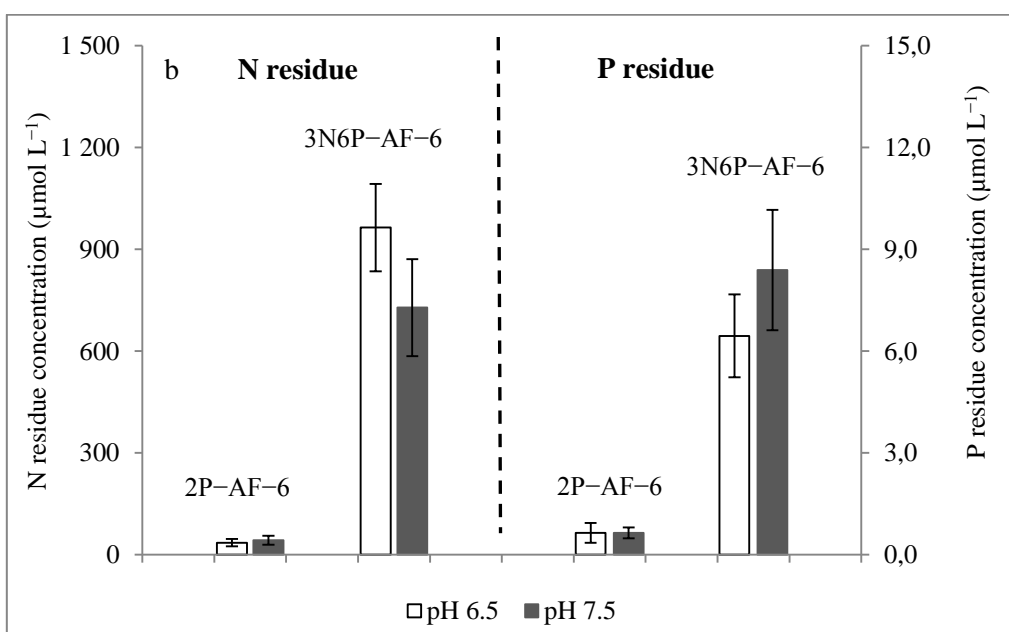
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298 The hexane extracts are generally regarded as crude hydrocarbon fractions. As shown in Fig.
 299 4, in contrast with total lipids, the highest values for hexane extract contents were obtained at
 300 pH 6.5. A change of just one pH unit, from pH 7.5 to 6.5, was enough to induce a significant
 301 ($P < 0.05$) increase in total lipid and hexane extract contents. Hexane extract content was
 302 between 19.9% dw and 36.8% dw, which is consistent with Dayananda et al. [15] who found
 303 that hexane extract as hydrocarbon ranges from 15% to 50.6% dw of the same SAG 30.81
 304 strain. Note that the hexane extracts could contain other lipids, such as triacylglycerol and
 305 aldehydes, and that hydrocarbon accounted for only 20.4% of hexane extracts from an Austin
 306



307

308



309

310 **Fig. 5.** Comparison of biomass productivity, purified hydrocarbon productivity, nutrient
 311 consumption at pH 6.5 and nutrient consumption at pH 7.5 in steady-state continuous cultures
 312 fed with 2P-AF-6 or 3N6P-AF-6. a) biomass and hydrocarbon productivities; b) N and P
 313 residue concentrations. N = nitrogen; P = phosphate.

314

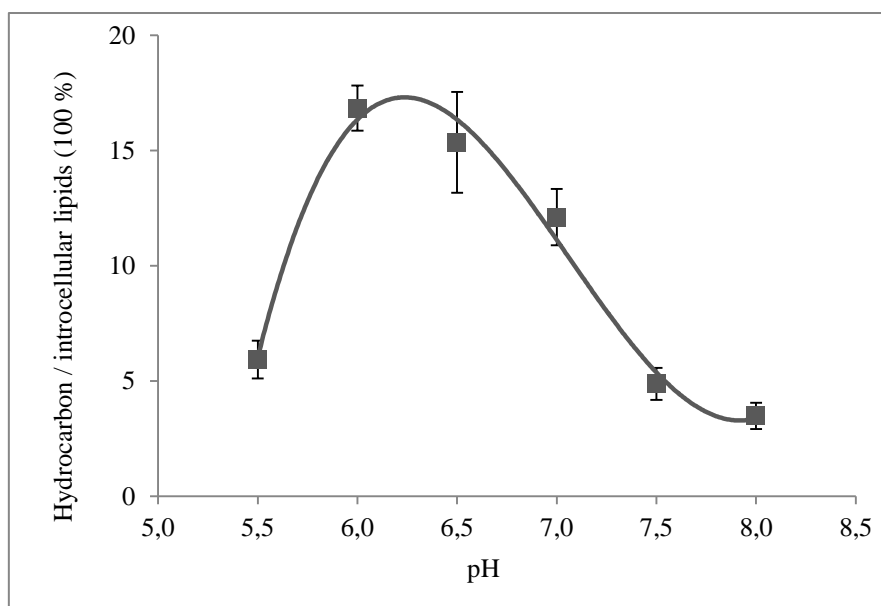
315 strain of *B. Braunii* [28]. In order to quantify the exact hydrocarbon content of this strain, we
316 purified the hydrocarbon using silica gel chromatography.

317 In addition, pH also had a significant effect on purified-hydrocarbon productivity, which
318 increased fourfold with the change from pH 7.5 to pH 6.5 (Fig. 5a).

319 At pH 6.5, hydrocarbon productivity reached up to $0.291 \text{ g m}^{-2} \text{ d}^{-1}$ with a hydrocarbon content
320 of 5.7% dwh whereas at pH 7.5, hydrocarbon productivity was only $0.065 \text{ g m}^{-2} \text{ d}^{-1}$ and with a
321 much lower hydrocarbon content of 1.3% dw. Moreover, hydrocarbon-specific production
322 rate was significantly higher at pH 6.5 ($11.1 \text{ mg HC g X}^{-1} \text{ d}^{-1}$) than at pH 7.5 ($2.7 \text{ mg HC g X}^{-1}$
323 d^{-1} ; $P < 0.05$). The hydrocarbon contents measured in this study were not so high, peaking at
324 5.7% dw, they are still within the range of values reported in the literature. Hydrocarbon
325 contents in *B. braunii* race A biomass are generally from 1% dw to 10% dw, although reports
326 claim some race-A strains accumulate up to 61% hydrocarbons while others accumulate less
327 than 0.1% dw [2]. As hydrocarbons are mainly accumulated in the extracellular matrix of *B.*
328 *braunii*, it was possible to estimate intracellular lipid contents by subtracting purified
329 hydrocarbons content from total lipids content [2]. The calculation of the ratio of extracellular
330 hydrocarbon to intracellular lipids as a function of pH then confirmed that optimal pH was
331 around pH 6.0–6.5 (Fig. 6).

332 According to the literature, one of the main precursors for hydrocarbon biosynthesis in *B.*
333 *braunii* race A is oleic acid, probably via an elongation mechanism involving conversion to
334 very-long-chain fatty acids, as revealed by comparative gene expression analysis in a race A
335 strain of *B. braunii* [29,30].

336



337

338 **Fig. 6.** Ratio of purified hydrocarbons to intracellular lipids as a function of pH in steady-state
 339 continuous cultures fed with 2P-AF-6. Intracellular lipids = total lipids – purified hydrocarbon.

340

341 There could therefore be a relation between intracellular lipid accumulation and extracellular
 342 hydrocarbon accumulation in *B. braunii* cultures. Indeed, when taking in account only the pH
 343 range from 6.0 to 8.0, there is a linear relation between extracellular hydrocarbon and the
 344 intracellular lipid contents:

345

$$[\text{HC}] = 0.534 \cdot [\text{Intracellular lipid}] - 13.398, R^2 = 0.939$$

346 The assumption of a linear relationship between hydrocarbon and lipid contents is only valid
 347 for pH values between 6.0 and 8.0. This relationship is not valid for the values corresponding
 348 to pH 5.5. The hypothesis for this is that the physiological state of *B. braunii* cells could be
 349 modified when the algae is grown in such acidic environmental conditions.

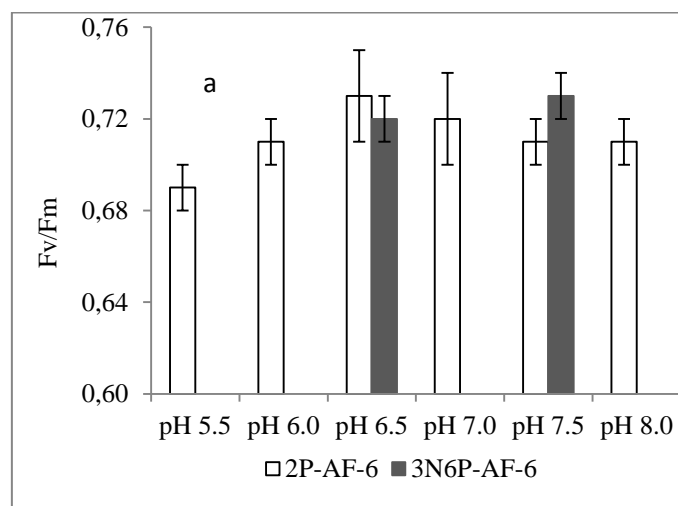
350

351 3.2.2. Steady-state photosynthetic activity at pH from 5.5 to 8.0

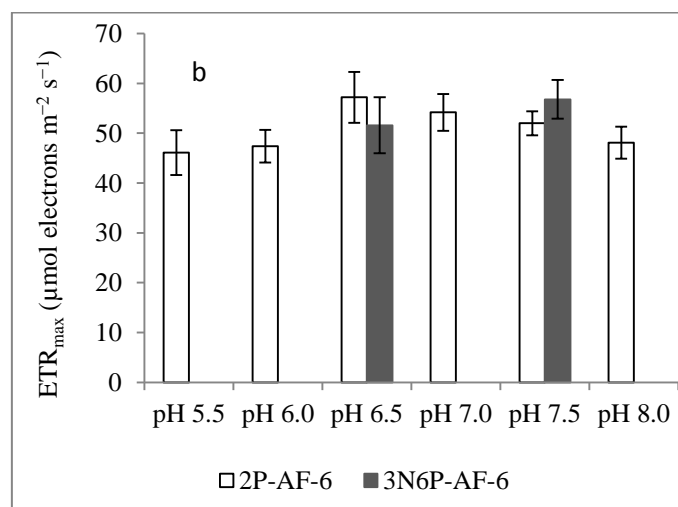
352 As *B. braunii* SAG 30.81 was cultivated under photoautotrophic conditions, photosynthetic
 353 activity is essential to provide energy and reductive carbon for biomass and hydrocarbon
 354 production [31]. The photosynthetic characteristics of the cell populations were determined at

355 each steady-state. The photochemical efficiency of photosystem II (PSII), expressed as Fv/Fm
 356 and ETR_{max} , was relatively unaffected by pH of the culture from pH 6.0 to 8.0, even if a slight
 357 increase was observed at pH 6.5 for the continuous culture fed with 2P-AF-6 (Fig. 7)

358



359



360

361 **Fig. 7.** Steady-state Fv/Fm and ETR_{max} of the P/I curve as a function of pH in continuous
 362 cultures of *B. braunii* at a fixed dilution rate of 0.2 day^{-1} . Means \pm SD. P/I curve =
 363 photosynthesis-irradiance curve.

364

365 The parameters Fv/Fm and ETR_{max} are commonly used to evaluate the impact of stresses
 366 such as nutrient or heat stress on microalgae [32,33]. In our experimental conditions, the
 367 absence of any sharp decline in Fv/Fm and ETR_{max} values suggests *B. braunii* were not

368 stressed at any pH in the range 6.0 to 8.0 (Fig. 7). However, the slight decrease of Fv/Fm at
369 pH 5.5 indicates an inhibition of microalgal photosynthesis by this more acidic
370 environment—an effect that could be related to the decrease in biomass concentration at pH
371 5.5. The decrease of extracellular pH induced by a high CO₂ supply could also affect
372 intracellular pH, although green microalgae are known to maintain their intracellular pH at
373 around 7.2 even when grown in relatively acidic media of pH down to 3.0 [34]. For example,
374 when cells of *Chlorococcum littorale* were transferred from low CO₂, i.e. 0.04% in air, to
375 high CO₂, i.e. up to 40%, intracellular pH value decreased from 7.0 to 6.4 with an
376 environmental pH value of 5.5 [9]. This acidification induced an inhibition of photosynthetic
377 carbon fixation in *C. littorale* cells exposed to excess concentrations of CO₂ [9].

378

379 3.2.3. Steady-state pigment composition at pH from 5.5 to 8.0

380 Microalgae like *B. braunii* have two major classes of pigments, chlorophylls (Chl) and
381 carotenoids (Car), that are generally associated to photosynthetic activities, except in some
382 race B strains that accumulate Car within the extracellular matrix [35]. The race A strain SAG
383 30.81, like other green algae, contains Chl *a*, Chl *b* and Car. Chl *a*, Chl *b* and Car contents are
384 known to depend on culture conditions, particularly nutrients and light. Eroglu et al. [36]
385 observed values from 0.38% to 0.86% (w/w) for chlorophylls and from 0.10% to 0.54% (w/w)
386 for carotenoids according to species of *B. braunii* grown under air. Sydney et al. [7] found
387 13.05% (w/w) pigments in *B. braunii* SAG 30.81 cultivated under CO₂-enriched (5%) air. As
388 shown in Table 1, the pigment contents obtained here at each steady-state were within the
389 range of values obtained by Ranga Rao et al. [5], at 1.16% w/w total Chl and 0.27% w/w Car.
390 Chl *a*/Chl *b* ratio and Car/Chl ratios are widely used indicators of photosynthetic cell response
391 to changes in irradiance, nutrients, temperature or salinity [37]. Plant cells present Chl *a*/Chl *b*

392 ratios of 3.2–4.0 when exposed to high irradiance and 2.5–2.9 when grown under low
393 irradiance [38].

394

395 **Table 1**

396 Steady-state pigment composition of biomass as a function of pH in continuous cultures of *B.*
397 *braunii* at a fixed dilution rate of 0.2 d⁻¹.

	Feeding medium	Total Chl (%dw)	Chl <i>a</i> / Chl <i>b</i> (mol / mol)	Total Car (% dw)	Car / Chl (w / w)
pH 5.5	2P-AF-6	1.3±0.1*	2.1±0.2	0.24±0.02*	0.19±0.02
pH 6.0	2P-AF-6	2.0±0.2	2.3±0.2	0.37±0.03	0.19±0.02
pH 6.5	2P-AF-6	2.1±0.1	2.4±0.3	0.37±0.03	0.18±0.01
pH 7.0	2P-AF-6	1.8±0.1	2.2±0.1	0.33±0.02	0.18±0.01
pH 7.5	2P-AF-6	1.8±0.2	2.3±0.3	0.33±0.02	0.18±0.01
pH 8.0	2P-AF-6	1.7±0.1	2.2±0.2	0.32±0.02	0.19±0.02
pH 6.5	3N6P-AF-6	2.2±0.2	2.1±0.3	0.38±0.03	0.18±0.01
pH 7.5	3N6P-AF-6	1.8±0.2	2.1±0.2	0.34±0.02	0.19±0.02

398 Means ± SD. Chl = chlorophylls; Car = carotenoids; * = significantly different from other
399 conditions (P < 0.05).

400

401 As reported for other Chl *b*-containing plant cells, the Chl *a*/Chl *b* values of microalgae are
402 generally between 2 and 3, with little if any effect of pH [39].

403 The Car/Chl ratio (0.24) of *B. braunii* obtained by Ranga Rao et al. [5] in cultures fed at a 2%
404 CO₂ concentration is relatively close to our data. Note that a lower Car/Chl ratio (0.15) was
405 also observed in other *B. braunii* species in absence of CO₂ supplementation [36]. The
406 evidence thus suggests there was apparently no stress from pH 5.5 to 8.0, as environmental

407 pH had no significant influence on Chl *a*/Chl *b* and Car/Chl ratios. However, under steady-
 408 state, total chlorophyll and carotenoid contents were significantly decreased at pH 5.5 ($P <$
 409 0.05), thus confirming that the cells were stressed by the more acidic conditions (Table 1).

410

411 3.2.4. Nutrient requirement for biomass production at steady-state from pH 5.5 to 8.0

412 As residual concentrations of macronutrients—mainly phosphate and nitrate—in the outflow
 413 from the continuous culture were relatively low (Fig. 5b), we cannot exclude that the pH
 414 effect could be related to some nutrient limitations. Nutrient limitations are known to induce
 415 physiological responses, particularly in certain photosynthetic characteristics related to PSII
 416 [40]. Indeed, nitrogen (N-NO₃⁻) and phosphorus (P-PO₄³⁻) count among the main components

417

418 **Table 2**

419 Nutrient requirement for biomass production (Rxs mg g X⁻¹) during growth in steady-state
 420 continuous cultures at a fixed dilution rate of 0.2 d⁻¹ but at different pH levels.

	Feed medium	N-NO ₃ ⁻	P-PO ₄ ³⁻
pH 5.5	2P-AF-6	57.5±3.2	13.5±1.2
pH 6.0	2P-AF-6	59.0±2.2	13.0±1.0
pH 6.5	2P-AF-6	56.5±4.0	12.0±0.9
pH 7.0	2P-AF-6	58.5±2.8	12.5±1.0
pH 7.5	2P-AF-6	61.5±2.6	12.5±1.1
pH 8.0	2P-AF-6	63.5±4.4	13.5±1.0
pH 6.5	3N6P-AF-6	42.2±3.8	15.9±1.2
pH 7.5	3N6P-AF-6	48.1±5.1	16.6±1.5

421 Means ± SD.

422 of many microalgal culture media as well as the most effective factors for regulating
423 secondary metabolism in microalgal cells [41].

424 N and P requirement for biomass production were calculated for all the successive steady-
425 states of the continuous culture fed with 2P-AF-6 medium (Table 2). pH changes had no
426 significant effect on nutrient consumption yields determined from data on each steady-state
427 continuous culture.

428

429 *3.3. Biomass and hydrocarbon productivities at pH 6.5 and 7.5 with the 3N6P-AF-6 medium*

430 We led a second set of experiments characterized by threefold-higher N and P concentrations
431 in the feed medium (3N6P-AF-6) and focusing on two successive steady-states at pH 6.5 then
432 7.5. In these conditions, N and P concentrations increased in the PBR outlet regardless of pH
433 setpoint (Fig. 5b). As shown in Fig. 5a, both biomass and hydrocarbon productivities were
434 increased by feeding the culture with the 3N6P-AF-6 nutrient-enriched medium compared to
435 2P-AF-6 medium. Biomass and hydrocarbon productivities increased up to $8.9 \text{ g m}^{-2} \text{ d}^{-1}$ and
436 $0.50 \text{ g m}^{-2} \text{ d}^{-1}$, respectively, at pH 6.5, and up to $8.5 \text{ g m}^{-2} \text{ d}^{-1}$ and $0.15 \text{ g m}^{-2} \text{ d}^{-1}$, respectively,
437 at pH 7.5. Note that the increase in purified hydrocarbon productivity was related to the
438 increase in biomass productivity by increasing the N and P supply. Indeed, purified
439 hydrocarbon content showed no significant change, at 5.6% dw at pH 6.5 and 1.7% dw at pH
440 7.5. These data confirm the results of Xu et al. [42] showing that higher biomass and
441 hydrocarbon productivities in *B. braunii* cultures were obtained by increasing the N and P
442 supplies. In contrast, according to these data, the effect of pH on biomass and hydrocarbon
443 contents was similar whatever the N and P concentrations.

444 However, nitrogen requirement for biomass production significantly decreased when the
445 culture medium contained threefold more N (Table 2). It seems that enriching N and P in the
446 feed medium could decrease the N needs for biomass. Sydney et al. [7] worked on the same *B.*

447 *braunii* SAG 30.81 strain as here and reported a relatively similar nitrogen requirement for
 448 biomass production of 40.72 mg g X⁻¹. However, phosphorus requirement for biomass
 449 increased slightly but significantly in the cultures grown on 3N6P-AF-6 medium (Table 2).
 450 This observation could be interpreted by a relatively classical luxury phosphate uptake
 451 process, with intracellular accumulation of polyphosphate as an intracellular nutrient pool in
 452 different microalgae [43], particularly in *B. braunii* [44]. Further studies are needed to improve
 453 the formulation of a culture medium for an optimal production of biomass for the different *B.*
 454 *braunii* strains.

455 There was no significant difference in Fv/Fm and ETR_{max} between feeding with 2P-AF-6 and
 456 3N6P-AF-6 media in steady-state continuous cultures at pH ≥ 6.0 (Fig. 7). Total lipid
 457 productivity was significantly higher at pH 6.5 (3.4 g m⁻² d⁻¹) than pH 7.5 (2.8 g m⁻² d⁻¹).
 458 Hydrocarbon productivities were also significantly higher at pH 6.5 (P < 0.05) regardless of
 459 media used (2P-AF-6 or 3N6P-AF-6)(Fig. 5a).

460 In addition, extracellular hydrocarbons-to-intracellular lipids ratio was also higher at pH 6.5
 461 than at pH 7.5, in line with the results on feeding with 2P-AF-6 medium. Extracellular
 462 hydrocarbons and intracellular lipids were fitted together with data previously obtained and
 463 appeared to follow a linear relationship:

$$464 \quad [\text{HC}] = 0.557 \cdot [\text{Intracellular lipids}] - 141,$$

465 although with a low of R² value (= 0.769). Either way, this trend needs to be confirmed.

466
 467 There are relatively few published studies on *B. braunii* cultures within photobioreactors, and
 468 only two in which pH was controlled by automated CO₂ injection, at setpoint values of pH 7.2
 469 and 7.5, respectively [7,14] ; see Table 3). Our data at pH 7.5 were similar to results from
 470 another study at pH 7.2 on the same strain but much higher on biomass productivity and lipids
 471

472 **Table 3**

473 Comparison of *B. braunii* biomass productivity, total lipid productivity and hydrocarbon
 474 productivity during studies within pH-controlled photobioreactors.

Strain	Race	pH control	Biomass productivity (mg L ⁻¹ d ⁻¹)	Hydrocarbon productivity (mg L ⁻¹ d ⁻¹)	Total lipid productivity (mg L ⁻¹ d ⁻¹)	Reference
GUBIOTJTBB1	--	7.5	13	6.8 ^a	7.4	[14]
SAG 30.81	A	7.2	207 ^b	--	68	[7]
SAG 30.81	A	6.5	223	12.5 ^c	84	This study
SAG 30.81	A	7.5	212	3.8 ^c	70	This study

475 ^a, hexane extract as crude hydrocarbon; ^b, average productivity calculated from figure; ^c,
 476 purified hydrocarbon; --, no data available.

477

478 productivity and different on hydrocarbon productivity compared to strain GUBIOTJTBB1 at
 479 pH 7.5. This difference could be due to other cultivation conditions or could be strain-
 480 dependent. Further, our study found that the highest hydrocarbon and total lipid productivities
 481 were reached at pH 6.5, but pH is also known to influence the speciation and bioavailability
 482 of different nutrients in the culture media, particularly DIC forms.

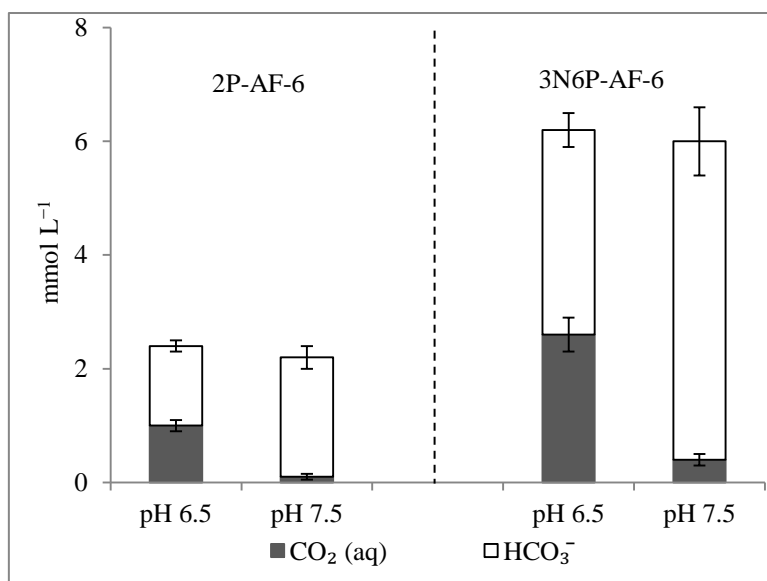
483

484 *3.4 Relation between dissolved carbon dioxide and hydrocarbon production*

485 Hydrocarbon is considered as a growth-associated product in *B. braunii* cultures [6]. However,
 486 our preliminary results suggest that environmental pH can stimulate hydrocarbon production
 487 with little effect on biomass productivity. The large increase (>70%) in biomass concentration
 488 found here when continuous cultures were fed with 3N6P-AF-6 medium compared to 2P-AF-
 489 6 medium indicates that certain N and P limitations control biomass accumulations without
 490 change of hydrocarbon content within the PBR. The controlling factor here was not light

491 availability, as PFD at the PBR was constant. Indeed higher productivities could be reached
492 by increasing PFD and decreasing the light path within the PBR, as already shown in a
493 trickle-film configuration [2].

494 *B. braunii* hydrocarbon content is mainly related to its exocellular hydrocarbons [1,45].
495 Indeed, the biomass of this colonial species is essentially composed of cells surrounded by a
496 complex extracellular matrix of polymerized hydrocarbons [46] that accumulates liquid
497 hydrocarbons, mainly alkadienes and alkatrienes in race A strain SAG 30.81. The
498 extracellular fraction has been evaluated to about 95% of total hydrocarbon [36]. We can use
499 these characteristics to assume that the liquid hydrocarbons as exocellular products could be
500 dissociated from the biomass and the matrix. Then, as biomass concentration at steady-state
501 seems relatively unaffected by pH compared to the change in hydrocarbon biosynthesis, the
502 stimulation of hydrocarbon productivity at pH 6.5 could be triggered by either increased
503 hydrocarbon biosynthesis activities, the excretion process, or the adsorption capacity of the
504 matrix. In the first case, pH 6.5 would be optimal for hydrocarbon biosynthesis activities that
505 resulting in overproduction. Another hypothesis is that this stimulation could be caused by an
506 effect of external pH on internal pH, as some intracellular enzymes, such as the decarboxylase,
507 associated with hydrocarbon biosynthesis, are highly pH-dependent [47]. In the second case,
508 pH 6.5 could impact the rate of globule content exocytosis through the plasma membrane and
509 cell wall of *B. braunii*. In the third case, the polymer's capacity to trap extracellular
510 hydrocarbon would presumably be pH-dependent, like the effect of NaCl on efficiency of
511 hydrocarbons extraction from *B. braunii* biomass observed by Furuhashi et al. [48]. Moreover,
512 we cannot rule out an effect of pH on chemical composition of the liquid hydrocarbon fraction.
513



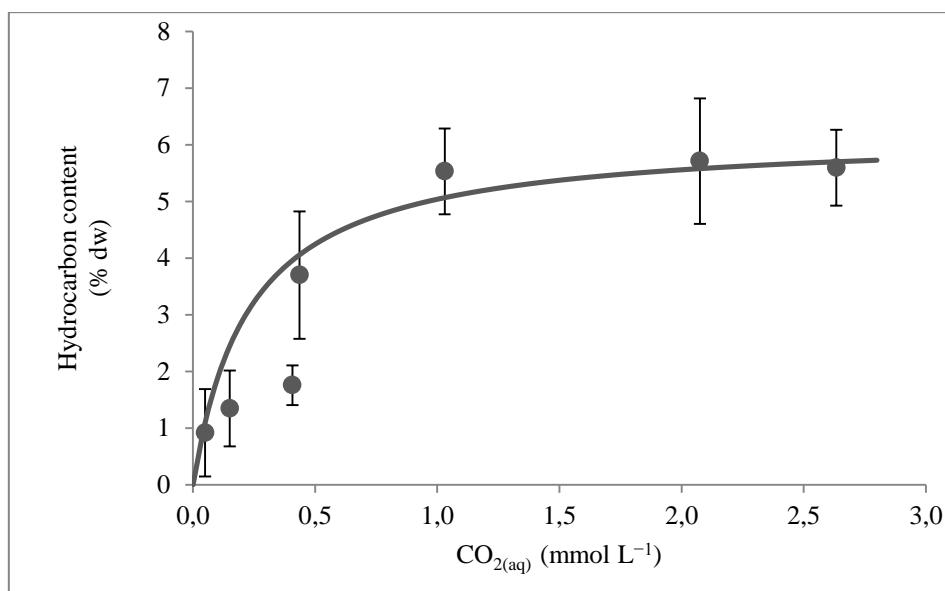
514

515 **Fig 8.** Inorganic carbon dioxide composition of the supernatant of *B. braunii* cultures in 2P-
 516 AF-6 and 3N6P-AF-6 media at pH 6.5 and 7.5 at the constant dilution rate of 0.2 d⁻¹; CO_{2(aq)}
 517 values were calculated from DIC data.

518 However, we observed that with pH controlled by CO₂ injection, DIC concentrations in the
 519 supernatants of steady-state cultures seemed to be relatively constant (i) at pH 6.5 and 7.5
 520 regardless of feed medium (2P-AF-6 or 3N6P-AF-6) (Fig. 8), and (ii) between pH 6.0 and 8.0,
 521 with a net RSD of 13% and very little fluctuations of DIC concentration during each steady
 522 state (RSD ≤ 6.8%). Thus, at first glance, there was visibly no DIC limitation of growth and
 523 hydrocarbon production occurred in our experimental conditions. On the basis of biomass
 524 elemental composition, stoichiometry equations on *B. braunii* have shown that hydrocarbon-
 525 enriched biomass production needs about 30% more CO₂ for strain BOT 22 than for *Chlorella*
 526 sp. or *Spirulina* sp. [2]. Not only the necessary amounts but also species of DIC have to be
 527 provided to drive increased growth of hydrocarbon-enriched biomass. The significant
 528 difference in hydrocarbon production at pH 6.5 vs pH 7.5 could thus be related to the effect of
 529 pH on inorganic carbon speciation, i.e. CO_{2(aq)} or H₂CO₃, HCO₃⁻, and CO₃²⁻ [49–52]. Fig. 9
 530 shows that hydrocarbon accumulation in *B. braunii* colony biomass of seems to be limited at
 531 dissolved CO₂ concentrations < 1 mmol L⁻¹. A maximal hydrocarbon content of 5.7% dw was

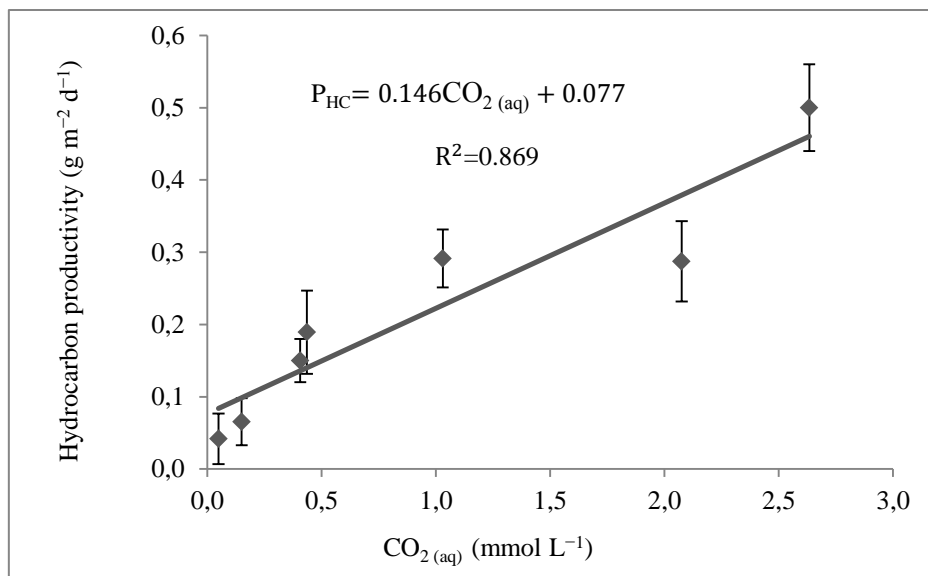
532 reached at and above this concentration. This DIC range is comparable with other DIC values
 533 related to biomass productivities in cultured microalgae such as *Isochrysis galbana* and
 534 *Phaeodactylum tricornutum*, where DIC limitations but not CO₂ limitations were observed for
 535 biomass productivity but not metabolite productivity at DIC values < 0.5 mmol L⁻¹ [53].
 536 After factoring in all the data obtained here but excluding data with physiological inhibition
 537 on cells at pH 5.5, hydrocarbon productivity appears linearly related to CO_{2(aq)} up to 2.5 mmol
 538 L⁻¹, whatever the N and P content of the culture medium (Fig. 10), according to the relation:

$$539 \quad P_{\text{HC}} = 0.146 \cdot \text{CO}_{2(\text{aq})} + 0.077, R^2=0.869$$



540
 541 **Fig. 9.** Hydrocarbon content of *B. braunii* biomass as a function of residual CO_{2(aq)} in the
 542 culture under steady-states conditions from pH 6.0 to 8.0 in continuous cultures fed with 2P-
 543 AF-6 or 3N6P-AF-6; CO_{2(aq)} values calculated from DIC data.

544



545

546 **Fig. 10.** Relation between hydrocarbon productivity and residual dissolved carbon dioxide in
 547 the culture medium at each steady state; CO_{2(aq)} values calculated from DIC data.

548 As a result, pH effects on hydrocarbon productivity are probably related to how pH influences
 549 distribution of DIC speciation, and CO_{2(aq)} could be the key driver of hydrocarbon production.

550

551 4. Conclusion

552 Successive steady-state continuous cultures of *B. braunii* SAG 30.81 at a constant dilution
 553 rate have shown that hydrocarbon productivity but not biomass productivity is pH-dependent
 554 in the range 5.5 to 8.0. At pH 5.5, the inhibition of PSII activities could be related to a
 555 reduction in hydrocarbon production. Maximal hydrocarbon productivities were obtained at
 556 pH 6.5 in two modified AF-6 media with nitrate and phosphate enrichments. The pH effect on
 557 hydrocarbon production was shown to be mainly related to dissolved CO_{2(aq)} concentration.
 558 This study also indicated that in unstressed, i.e. stable PSII activity, microalgae, extracellular
 559 hydrocarbon accumulation appeared to be linked to intracellular lipid accumulation. Further
 560 comparative studies between race-A and race-B *B. braunii* strains cultivated at different
 561 dilution rates and PFD could valuably help determine how dissolved CO₂ availability,
 562 hydrocarbon productivity and intracellular lipid productivity are co-related.

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564

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568

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