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## **Control of the pH for marine microalgae polycultures: a key point for CO<sub>2</sub> fixation improvement in intensive cultures**

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1 Control of the pH for marine microalgae polycultures: a key point for CO<sub>2</sub> fixation  
2 improvement in intensive cultures

3

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12

13 Keywords: carbon yield, microalgal diversity, pH, predators

14 Declarations of interest: none

15

## 16 **Abstract**

17 Recently, CO<sub>2</sub> recycling for the production of valuable microalgae has acquired  
18 substantial interest. Most studies investigating CO<sub>2</sub> conversion efficiency in algal cultures  
19 were based on single species, although a stabilising effect of algal diversity on biomass  
20 production was recently highlighted. However, addition of CO<sub>2</sub> into polyalgal cultures  
21 requires a careful control of pH; performance of CO<sub>2</sub> conversion, growth and carbon biomass  
22 production are affected by pH differently, depending on the species of microalgae. This study  
23 investigates the efficiency of CO<sub>2</sub> conversion by natural marine algal assemblage cultivated in  
24 open, land-based raceways (4.5 m<sup>3</sup>, 10 m<sup>2</sup>), working as high rate algal ponds (HRAP). Ponds  
25 were enriched with nitrogen and phosphate, pure CO<sub>2</sub> was added and algal cultures were

26 grown under three different fixed pH levels: pH 6, 7 and 8. The highest conversion of  
27 photosynthetically fixed CO<sub>2</sub> into carbon biomass (40%) was reached at pH 7, an intermediate  
28 level, due to the partial CO<sub>2</sub> asphyxiation of algal predators (copepods, ciliates), while being  
29 under the suboptimal conditions for the development of marine amoebae. Under this pH, the  
30 theoretical maximal biological conversion of available CO<sub>2</sub> into carbon biomass was  
31 estimated to be 60% in naturally inoculated open ponds.

32

### 33 1. Introduction

34 Algae has become a focal point in the circular economy to valorise industrial waste in order to  
35 continually use resources and reduce industrial CO<sub>2</sub> emissions to the atmosphere (Lai et al.  
36 2019; Stiles et al. 2018). Through photosynthesis, microalgae can convert CO<sub>2</sub> from industrial  
37 flue gas into biomass that can potentially be used for the production of biofuel, biochemical  
38 products or green chemistry (Bhola et al. 2014; Brune et al. 2009; Wang et al. 2008).  
39 Industrial flue gas contains potentially toxic contaminants which can lead to reductions in the  
40 production of biomass (Hess et al. 2017). Additionally, injection of CO<sub>2</sub> acidifies algal culture  
41 medium by modifying the balance of carbonates in the water (Galloway and Krauss 1961).  
42 This acidification could counterbalance CO<sub>2</sub> fixation by microalgae which alkalises their  
43 growth environment (Galloway and Krauss 1961). A controlled injection of CO<sub>2</sub> would  
44 maintain an optimal pH level that could be adjusted to optimise for metabolic activity of algal  
45 species (Galloway and Krauss 1961). For example, the marine microalgae *Nannochloris* sp.  
46 achieves maximal growth rate at pH 6, maximum carbon content at pH 7 and maximal carbon  
47 conversion efficiency at pH 8 (Vasseur et al. 2012). Other example, the optimal growth of the  
48 cyanobacteria *Spirulina platensis* and the green algae *Chlorococcum littorale* are 9 and 4,  
49 respectively (Zeng et al. 2011). In microalgal predators, lower pH (associated with CO<sub>2</sub>  
50 injection) negatively affects the densities and grazing activity of rotifers (Montemezzani et al.

51 2017a, b, c). If we ignore the potential effects of associated contaminants, using CO<sub>2</sub> from  
52 industrial flue gas can combine pH control during high photosynthetic CO<sub>2</sub> fixation and  
53 reduction of algal grazing pressure.

54 Large-scale industrial cultivation of marine microalgae appears to be one of the most  
55 promising approaches of sustainable sources of food (lipids and proteins) and energy (Greene  
56 et al. 2016). Narwani et al. (2016) demonstrated stabilising effects of biodiversity on biocrude  
57 production from polycultures of several assembled algal species using raceway ponds under  
58 variable environmental conditions. However, higher performance can be reached using  
59 natural algal polyculture than assembled polyculture (Thomas et al. 2019).

60 We suggest that an optimal pH can be reached for maximising carbon conversion  
61 efficiency of a natural marine polyculture of microalgae while reducing grazers development.  
62 Natural marine polycultures were cultivated in High Rate Algal Ponds (HRAP) in a  
63 Mediterranean climate with different levels of pure CO<sub>2</sub>. We measured algal diversity,  
64 photosynthetic CO<sub>2</sub> uptake, carbon biomass accumulation, and associated development of  
65 algal predators under three different pH values. We developed performance curves of  
66 biological CO<sub>2</sub> conversion efficiency a function of pH. Results from this study provide a  
67 comprehensive assessment of the biological CO<sub>2</sub> conversion efficiency from marine  
68 microalgae cultivated in open ponds. **The results from this experiment can inform engineering  
69 projects for industrial CO<sub>2</sub> sequestration or mass production of marine microalgae.**

70

## 71 **2. Material & Methods**

### 72 2.1. Experimental design

73 An intensive culture of natural microalgae from Mediterranean seawater was  
74 performed outdoors in high rate algal ponds (HRAP) as described in Metaxa et al. (2006).  
75 HRAPs are oval-shaped ponds (6 m long, 2 m wide and 0.6 m deep) with a working volume

76 of 4.46 m<sup>3</sup> and surface area of 10 m<sup>2</sup>. Water mixing in the HRAPs was maintained at 0.2 m s<sup>-1</sup>  
77 using a vacuum airlift column developed and patented by COLDEP® (Barrut et al. 2013).  
78 Briefly, the column was connected to the HRAP and was composed of a central tube, the top  
79 of which was hermetically closed and connected to a vacuum pump. Water was raised to top  
80 of the central tube with a vacuum and was allowed to flow over the central tube so that it  
81 could be returned to the HRAP. We maintained pH using an automatic JBL pH-controller  
82 system (JBL ProFlora m1003) connected to a pure CO<sub>2</sub> bottle using a solenoid valve. We  
83 diffused CO<sub>2</sub> through a ceramic gas diffuser in the HRAP. The JBL ProFlora system used a  
84 combined Ag/AgCl KCl-electrolyte pH electrode at operational IUPAC scale calibrated  
85 monthly using commercial NIST buffers with precision of 0.05 pH units with an additional  
86 systematic error of about 0.15 pH units relative to seawater scale (Riebesell et al. 2011).

87 Experiments were conducted at the Ifremer Station of Palavas-les-Flots, Hérault,  
88 France during spring and early summer 2016 to coincide with the climatic period of  
89 favourable algal growth. The first run of experiment (run 1) occurred from 17 May to 22 June  
90 2016 (36 days); run 2 occurred from 27 June to 26 July 2016 (29 days). For each experimental  
91 run, three HRAPs were monitored simultaneously on batch feeding mode and maintained at  
92 pH of 6, 7 and 8, respectively. We assessed cumulated photosynthetic CO<sub>2</sub> fixation,  
93 production of carbon biomass, microalgal diversity and predator identification.

94 For all experiments, seawater was pumped from the Mediterranean Sea, inoculated  
95 using non-specific microalgae inoculant and filtered through a 100 µm sand filter before  
96 entering HRAPs. All HRAPs were supplemented with phosphorus to have final  
97 concentrations of 5 mg L<sup>-1</sup> of phosphate and nitrogen to achieve 80 mg L<sup>-1</sup> of nitrate and  
98 ammonium.

99

100 2.2 Physical and chemical variables

## 101 2.2.1 Meteorological variables

102 Global, direct and diffuse solar radiation were recorded every hour at the Météo-  
103 France station at Fréjorgues airport, 7 km from the experimental site.

104

## 105 2.2.2 Water pH, salinity, light and temperature measurements

106 Water temperature (accuracy of 0.5°C) in HRAPs were recorded every 10 minutes and  
107 salinity (accuracy of 0.1 PSU) in HRAPs was recorded once a day using a PONSEL ODEON  
108 X meter. In addition to JBL ProFlora system, water pH was measured every 10 minutes in one  
109 point of the well-mixed HRAPs (water circulation of 0.2 m.s<sup>-1</sup>) using a combined Ag/AgCl  
110 KCl electrolyte ODEON electrode with a resolution of 0.01 pH units and an accuracy of 0.1  
111 pH units. The underwater light intensity in HRAPs was recorded every 15 min at three depths  
112 (sub-surface, 20 and 40 cm) using waterproof light data loggers (UA-002-64 HOBO, Onset).

113

## 114 2.3 Biological variables

### 115 2.3.1 Microalgae biomass and diversity

116 The algal biomass was assessed daily when possible for Chlorophyll *a* (Chl*a*) and  
117 weekly for particulate organic carbon (POC) measurements. For Chl*a* measurements, between  
118 3 and 800 ml samples (depending on sample loading) were filtered onto 13 mm GF/F  
119 fibreglass fibre filters to determine the total Chl*a* concentrations. For size-fractionated Chl*a*  
120 measurements, between 50 and 800 ml samples were filtered using 20 µm, 5 µm and 2 µm  
121 membrane filters and GF/F fibreglass (0.7 µm nominal pore size). Filters were stored frozen  
122 at -20°C until extraction with 5 ml of 100% ethanol. Extractions were performed after  
123 ultrasonication in an ice bath followed by overnight extraction at 4°C. Samples were then  
124 filtered and extract absorbance was measured at 632, 649 and 665 nm (with absorbance at 750  
125 nm used for a baseline correction) using a Shimadzu UV1800 zeroed with 100% ethanol.

126 Chlorophyll concentration was calculated according to Ritchie (2008). For the POC  
127 measurements, between 5 and 100 mL samples were filtered onto precombusted Whatman  
128 GF/F filters and stored at -80°C for analysis. The filters were then dried at 60°C for 24 h,  
129 pelleted and analysed using an ANCA mass spectrometer (Europa Scientific). Because of the  
130 small volume of samples (<100 ml), we assumed that microalgae were mainly collected and  
131 retained onto the filters (nominal pore size of 0.7 µm) and the POC measurement was  
132 therefore representative of microalgae carbon biomass. This is supported by positive linear  
133 correlation between Chl $a$  and POC measurements ( $r^2= 0.85$ ,  $p < 0.001$ ,  $n=33$ ) leading to a  
134 Chl $a$ :POC ratio of 55, similar to the value measured for marine green algae and  
135 Prymnesiophyceae in the Mediterranean Sea (Latasa 2005).

136 Microalgal community structure was determined in all HRAPs at the beginning (Day 0) and at  
137 the end of each experimental run (Day 36 and Day 29) using 18S rRNA gene analysis. For  
138 each experimental run, 10 mL samples were filtered onto 0.2 µm membranes (PALL ALL  
139 Supor® 200 PES) and stored at -20°C for subsequent DNA extractions. The DNA was  
140 extracted using DNeasy PowerWater Kit (Qiagen) according to the manufacturer's  
141 instructions. The V4 region of the 18S rRNA gene was amplified over 30 amplification cycles  
142 at an annealing temperature of 65°C, with forward and reverse primers (5'-  
143 CTTTCCCTAACGACGCTCTTCCGATCTGCGGTAATTCCAGCTCCAA-3' and 5'-  
144 GGAGTTCAGACGTGTGCTCTTCCGATCTTTGGCAAATGCTTTCGC-3', respectively) with  
145 their associated linkers. The resulting products were purified and loaded onto an Illumina  
146 MiSeq cartridge for sequencing, paired with 300 bp reads following manufacturer's  
147 instructions (v3 chemistry). Sequencing and library preparation steps were carried out at the  
148 Genotoul Lifescience Network Genome and Transcriptome Core Facility in Toulouse, France  
149 ([get.genotoul.fr](http://get.genotoul.fr)). A modified version of the standard operation procedure for MiSeq data  
150 (Kozich 2013) in Mothur version 1.35.0 (Schloss 2009) was used for alignment and

151 taxonomic outline. Mothur was also used to identify representative sequences of operational  
152 taxonomic units (OTUs).

153

### 154 2.3.2 Algal predator diversity

155 To characterise the presence and abundance of protozoan and metazoan organisms, 2 L water  
156 samples were filtered sequentially through a 100  $\mu\text{m}$  and 35  $\mu\text{m}$  filters to separate 35 to 100  
157  $\mu\text{m}$  and  $> 100 \mu\text{m}$  size fractions, which were then stored in neutralised formalin (4% final  
158 concentration). Protozoan and metazoan taxa from a subsample of the 35 to 100  $\mu\text{m}$  fraction  
159 were identified, sized and enumerated using an Olympus AX70 dissecting microscope with a  
160 Nageotte counting chamber. Protozoan and metazoan taxa from a subsample of the  $> 100 \mu\text{m}$   
161 fraction were identified and enumerated using an Olympus Stereomicroscope SZX7 with a  
162 Bogorov counting chamber.

163

### 164 2.3.3. HRAP photosynthetic $\text{CO}_2$ uptake

165 The photosynthetic  $\text{CO}_2$  uptake by microalgae in HRAPs was computed from the relationship  
166 between biomass-specific gross photosynthetic oxygen production and the light intensity  
167 (photosynthesis-irradiance P-I model). The weekly photosynthetic oxygen production was  
168 measured at the laboratory as detailed below. **Algal biomass (Chl $a$ ) was measured every day**  
169 **when possible in the HRAPs.** Average light intensity received by microalgae was measured  
170 every 15 min at mid-depth (0.2 m) in each HRAP.

171 Samples were collected weekly from HRAPs, distributed to air-tight 30 mL  
172 polycarbonate flasks and placed in a custom-made, controlled-temperature photosynthetic  
173 with a light density gradient up to 400  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  using an array of 4 W white  
174 LEDs. Photosynthetically active radiation (PAR) was measured using a US-SQS/L spherical  
175 quantum microprobe (Walz, Effeltrich, Germany). The temperature was kept constant within



176 the HRAPs by heating or cooling. Dissolved oxygen (to determine net photosynthesis) was  
177 measured using a FireSting optode system (Pyro-Science, Aachen, Germany) every 20 min  
178 for 6 h of incubation in all illuminated flasks. Net photosynthetic rate was calculated at all  
179 light intensities. Samples were also incubated in the dark at the same temperature to  
180 determine respiration rate (oxygen consumption). Gross photosynthetic rates for all light  
181 intensities were calculated as the sum of net photosynthetic rates and respiration rates. Gross  
182 photosynthetic rates were expressed in carbon units using a photosynthetic quotient of 1  
183 (Falkowski and Raven 1997) and in algal biomass units using Chlorophyll *a* measurements.  
184 These were plotted against irradiance using the P-I model described by Eilers and Peters  
185 (1988) using the phytotools R package (<https://cran.r-project.org/package=phytotools>). The  
186 instantaneous gross photosynthetic CO<sub>2</sub> uptake rates were derived from the P-I model  
187 obtained every week for each HRAP, the Chl*a* biomass measurement measured every day and  
188 the light measurements taken every 15 min at mid-depth during P-I model assessment. For the  
189 calculation of instantaneous photosynthetic rates, we assumed there was no significant change  
190 of P-I parameter values during a week and the Chl*a* biomass during a day.

191 The total gross photosynthetic CO<sub>2</sub> uptake for HRAPs was calculated as the sum of all  
192 instantaneous gross CO<sub>2</sub> uptake rates obtained during the whole experiment in each HRAP.

193

### 194 2.3.3. Prediction of minimal CO<sub>2</sub> losses

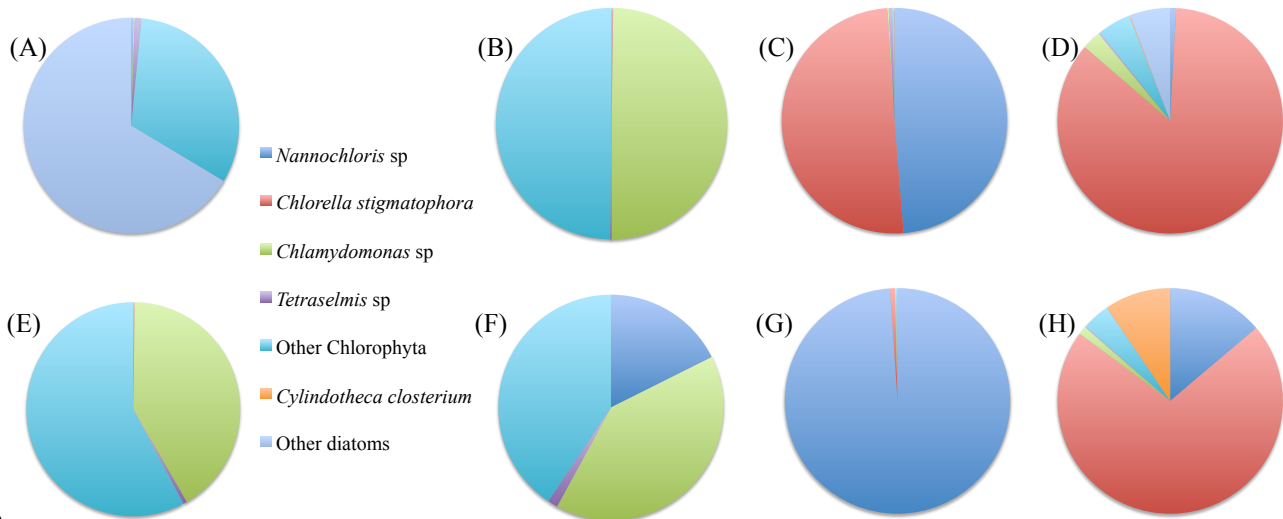
195 We attempted to predict the minimal carbon losses through biological activity (respiration,  
196 exudation, predation) using a generalised additive model (GAM) in R (R Core Team, 2019)  
197 with the mgcv package (Wood, 2017). Carbon lost through biological activity was calculated  
198 as the fraction of gross photosynthetically fixed CO<sub>2</sub> not accumulated into the C biomass over  
199 time series from HRAPs at pH 7.

200

### 201 3. Results & Discussion

#### 202 3.1. Influence of pH on algal diversity and predation

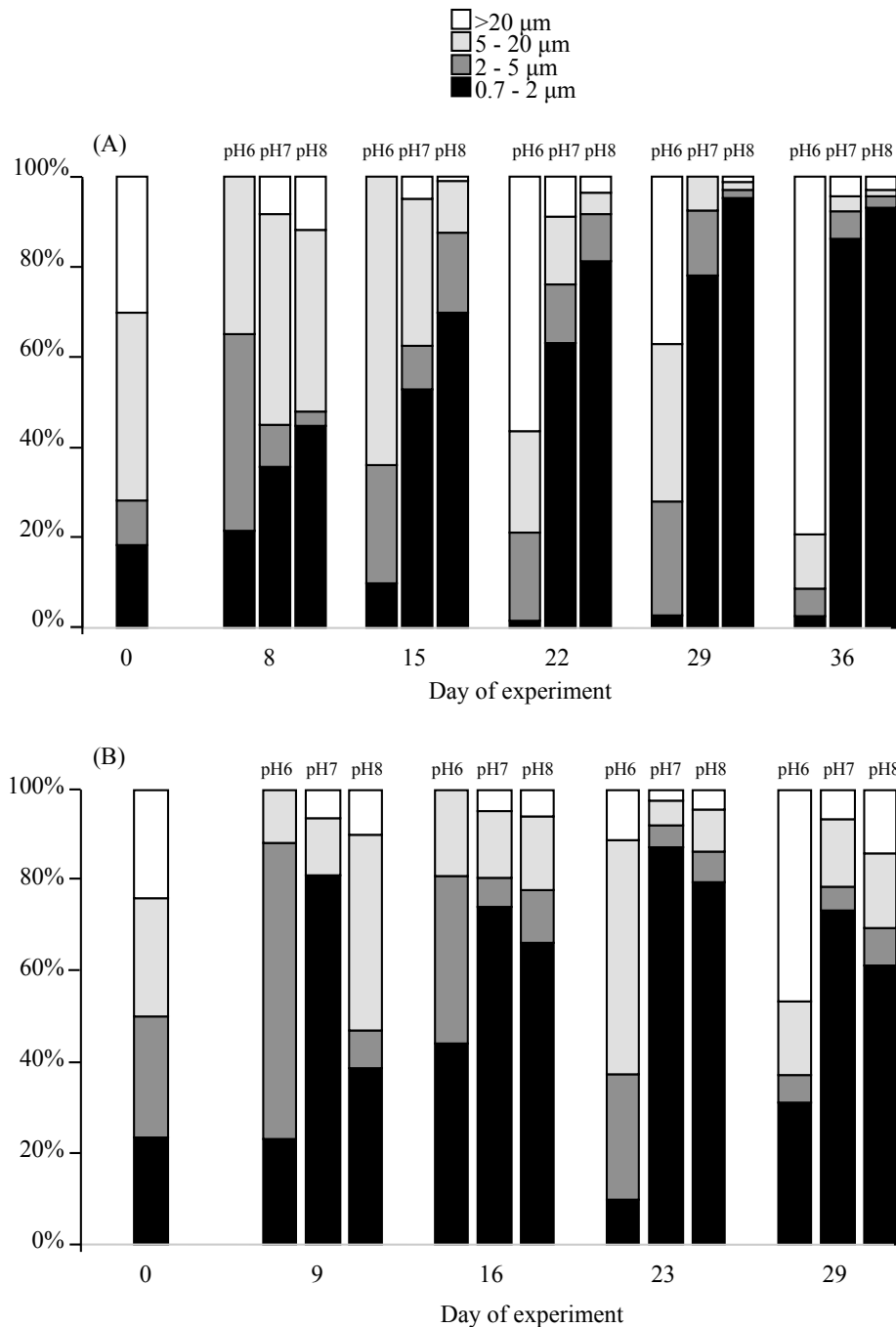
203 In the pH-6 HRAPs, pH averaged  $6.2\pm 0.2$  (Figure S1). Lower pH variability was  
204 observed in the pH-7 HRAPs (Figure S1), where pH averaged  $6.7\pm 0.1$  and  $7.0\pm 0.1$  during run  
205 1 and run 2, respectively. In the pH-8 HRAPs, pH averaged  $8.2\pm 0.1$  and  $7.9\pm 0.1$  during run 1  
206 and run 2, respectively. When pooling pH measured during both experimental runs, pH  
207 averaged  $6.2\pm 0.2$ ,  $6.9\pm 0.2$ , and  $8.1\pm 0.2$  in HRAPs with pH targeted at 6, 7 and 8 respectively.  
208 The addition of CO<sub>2</sub> acidifies seawaters and impacts the growth of marine planktonic  
209 organisms found in naturally buffered seawater at pH 8.07 in the Mediterranean Sea (Hassoun  
210 et al 2015). Larger microalgae ( $> 5 \mu\text{m}$ , Fig. 1), such as *Chlamydomonas* sp., favoured the  
211 growth at pH 6, while smaller microalgae ( $< 2 \mu\text{m}$ , Fig. 1), such as *Nannochloris* sp. and  
212 *Chlorella stigmatophora*, attained maximal growth rates at pH 7 and 8, respectively (Fig. 2).  
213 Those two species have been reported to be highly resilient to environmental variability,  
214 rapidly growing, and living in marine polluted waters (Butcher 1952, Fabregas 1986, Cho et  
215 al. 2007). Of economic interests for industries, *Nannochloris*-like species (Henley et al. 2004)  
216 show a high potential for removing nitrogen and phosphorus from nutrient-polluted waters  
217 whilst maintaining a stable biochemical profile under environmental changes (von  
218 Alvensleben et al. 2013). Our results suggest that adding CO<sub>2</sub> to control pH is a viable tool for  
219 favouring the development of one specific microalgal species among a natural assemblage.



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Figure 1. The diversity of microalgae was determined using 18S rRNA gene analysis. The first experiment ran from 17 May to 22 June 2016 (A-D). Organisms identified in the beginning of the experiment (A) and those identified at the end of the experiment in HRAPs at pH 6 (B), pH 7 (C) and pH 8 (D). The second experiment ran from 27 June 27 to 26 July 2016 (E-H). Organisms identified at the beginning of the experiment (E) and at the end of the experiment in HRAPs at pH 6 (F), pH 7 (G) and pH 8 (H).

231 Although differences in microalgal diversity were observed in HRAPs, no significant  
 232 differences were found among pH treatments for the photosynthetic parameters,  $P_{max}$  and  $I_k$   
 233 (Table S1). However, photoinhibitory effect was significantly higher in HRAPs at pH 7 and 8  
 234 than in HRAPs at pH 6 (ANOVA  $p < 0.05$ , Tukey post hoc; Table S1). As expected,  
 235 microalgae in all HRAPs were acclimated to higher  $I_k$  values and lower  $w$  values in July than  
 236 in June during the experiments (ANOVA  $p < 0.05$ , Tukey post hoc; Suppl. Table S1).



237

238 Figure 2. Size fractions of algal biomass measured in HRAPs at pH 6, 7  
 239 and 8 during the first experimental run 1 (A) and the second experimental  
 240 run 2 (B).  
 241

242 Interestingly, rhizopod development in amoebae was observed in HRAPs at pH 6 (Table 1).

243 Rhizopod amoebae are known to feed on organic matter, bacteria, and small algal cells

244 (Carney and Lane 2014; Day et al. 2017); they also have higher growth rates at pH lower than

245 seawater (Rodriguez-Zaragoza 1994). The rapid development of amoebae would prevent the

246 abundance of small algal cells observed under the other pH conditions. In contrast, ciliates  
 247 (both attached and free) grew rapidly at pH 8 and dominated the community of predators in  
 248 HRAPs. Copepods (adults and nauplii) were also observed in HRAPs at pH 7 and 8 but were  
 249 not observed at pH 6. The pH, therefore, seems to have a direct impact on the presence of  
 250 predators of algae. Low pH has a negative effect on ciliates and copepods but a positive effect  
 251 on the presence of amoebae. As a consequence, low levels of microalgal accumulation were  
 252 observed at pH 6. The development of the typical marine trophic food web (ciliates,  
 253 copepods) was observed in the HRAP at pH 8, leading to the dominance of very small algal  
 254 cells. At intermediate pH of 7, the abundance of predators was the lowest (Table 1) and the  
 255 accumulation of microalgal biomass was affected by the initial community structure of  
 256 predators and temperature. Indeed, a lower level of algal biomass accumulation was observed  
 257 in the HRAP at pH 7 during run 2 when the initial presence of rhizopod amoebae (Table 1)  
 258 and water temperature (average level was  $> 25^{\circ}\text{C}$ ) was significantly higher ( $p < 0.05$ , which  
 259 was determined using a t-test).

260

261 Table 1: Abundance of all the main groups of protozoans and metazoans identified by  
 262 microscopy in the HRAP at pH 6, 7 and 8 at the beginning and at the end of two experimental  
 263 runs of the study.

Size fraction	Group (ind. L <sup>-1</sup> )	20th of May 2016 (T0)	22rd of June 2016 (Tf)			28th of June 2016 (T0)	26th of July 2016 (Tf)		
			pH 6	pH 7	pH 8		pH 6	pH 7	pH 8
35-100 $\mu\text{m}$	Free ciliates		208	312	3952	103	3328	1664	34320
	Attached ciliates	52	3432	832	120016			1040	43888
	Rhizopods		18408	624	1040	11	57928	8008	1352
	Actinopods				416		1976	416	208
	Nematods	10	520	936	416		104	2184	
	Rotifers								520
	Copepods			1664	104			312	2600
> 100 $\mu\text{m}$	Nematods larvae	9	104		351	5	50	513	169
	<i>Tisbe</i> sp.			299	156				871
	<i>Tisbe</i> nauplius	0							104
	Copepod nauplii			59					

264

265

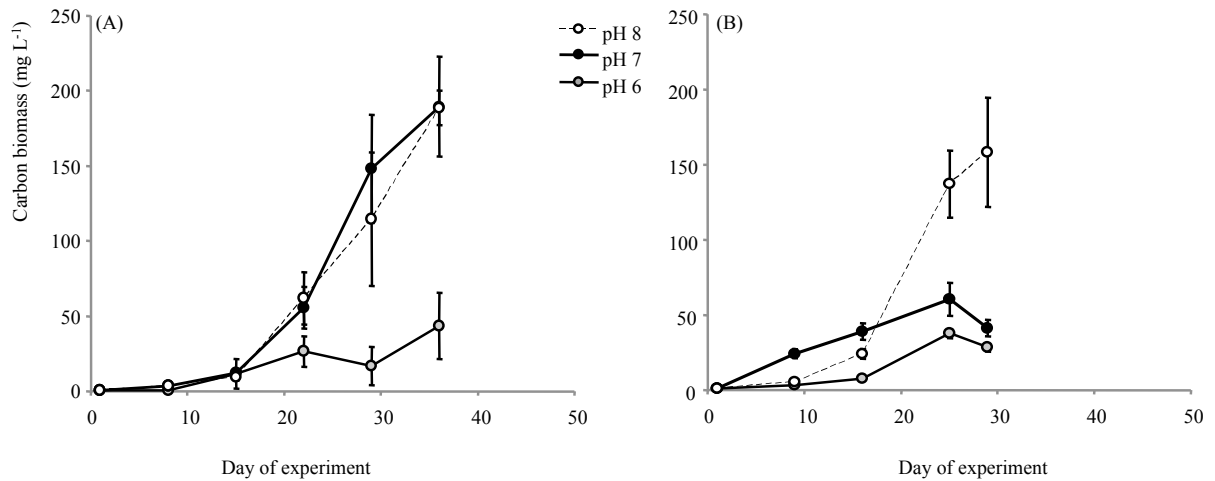
266 Injection of CO<sub>2</sub> into HRAPs negatively affects zooplankton (Cladocceans, copepods, rotifers)  
 267 through asphyxiation, allowing an increase in algal productivity and biomass (Montemezzani

268 et al. 2017a, b). Similarly, in this study, no copepods grew in HRAPs where a continuous  
269 injection of CO<sub>2</sub> was done in order to maintain a pH of 6. In addition, the final abundance  
270 protozoans and metazoans >100 µm decreased as injected CO<sub>2</sub> accumulated (lower pH) in the  
271 HRAPs (Table 1). This highlights the difficulty that large-sized aquatic organisms had  
272 maintaining a costly mode of respiration in media where CO<sub>2</sub> replaced O<sub>2</sub> availability. In  
273 contrast, the reductions in pH through the CO<sub>2</sub> injection favoured the development of  
274 rhizopods (mainly amoebae) that strongly limited algal biomass accumulation in the HRAPs.  
275 This is the first report of this observation in the literature, and it may have dramatic  
276 consequences on marine microalgae cultures in large-scale facilities.

277

### 278 3.2. Influence of pH on algal biomass accumulation

279 In this study, the highest accumulation of carbon as algal biomass (> 150 mg L<sup>-1</sup>) was  
280 observed in HRAPs at pH 8 and the lowest values (< 50 mg L<sup>-1</sup>) were observed in HRAPs at  
281 pH 6 (Fig. 3). Interestingly, algal biomass in HRAPs at the intermediate pH 7 had more  
282 variability and was influenced by seasonality, greater for run 1 than run 2 (Fig. 3). In all  
283 HRAPs, lower algal biomass was observed during run 2. Initial algal diversity was different  
284 between runs with abundance of *Nannochloris* sp. observed in June relatively to July. Water  
285 temperature was higher in July (average of 25.3°C) than in June (average of 21.8°C) and  
286 influenced predator development (Table 1), leading to higher predatory pressure on  
287 microalgae.



289

290 Figure 3. Concentration of carbon biomass (POC measurements) measured in  
 291 HRAPs (average and standard deviation of sample triplicates) during run 1 from 17  
 292 May to 22 June 2016 (A) and during run 2 from 27 June to 26 July 2016 (B).  
 293

294

295 The loss of carbon that was photosynthetically fixed but not ultimately accumulated  
 296 into algal carbon biomass was estimated from the difference between the total photosynthetic  
 297 CO<sub>2</sub> uptake and the algal carbon biomass accumulated over time (Table 2). When differences  
 298 were larger, algal biomass lost greater amounts of carbon through algal CO<sub>2</sub> respiration, algal  
 299 carbon exudation and algal mortality by predation or viral lysis.

300

301 Table 2. Gross photosynthetic CO<sub>2</sub> uptake, produced carbon biomass and carbon losses (the  
 302 fraction of the gross photosynthetic CO<sub>2</sub> uptake that was not accumulated into carbon biomass)  
 303 measured in HRAPs at pH 6, 7 and 8 during the two experimental runs.

Date	Day of experiment	pH 6			pH 7			pH 8		
		Gross photosynthetic CO <sub>2</sub> uptake (mgC L <sup>-1</sup> )	Produced C biomass (mgC L <sup>-1</sup> )	C loss (%)	Gross photosynthetic CO <sub>2</sub> uptake (mgC L <sup>-1</sup> )	Produced C biomass (mgC L <sup>-1</sup> )	C loss (%)	Gross photosynthetic CO <sub>2</sub> uptake (mgC L <sup>-1</sup> )	Produced C biomass (mgC L <sup>-1</sup> )	C loss (%)
20/05/16	3	0	0.0		0	0		0	0	
25/05/16	8	10	0.1	99	10	3	68	19	3	85
01/06/16	15	600	11	98	159	12	93	147	9	94
08/06/16	22	2270	26	99	767	55	93	1079	61	94
15/06/16	29	2328	16	99	1431	148	90	1985	114	94
22/06/16	36	2541	43	98	2602	189	93	3760	188	95
28/06/16	1	0	0.0		0	0		0	0	
06/07/16	9	22	2.1	91	57	23	60	15	4	73
13/07/16	16	371	6.2	98	386	38	90	203	23	89
22/07/16	25	877	36	96	875	59	93	1246	136	89
26/07/16	29	1074	27	97	1180	40	97	1620	157	90

304

305

306 In all HRAPs, carbon loss increased rapidly over time, reaching more than 90% of the gross  
307 photosynthetic CO<sub>2</sub> uptake measured throughout the experimental period (Table 2). Most CO<sub>2</sub>  
308 loss is related to high microalgal respiration rate which has been reported to be 60-90% of the  
309 photosynthetic fixed CO<sub>2</sub> (Herzig and Falkowski 1989). Carbon loss through respiration is  
310 combined with the carbon loss through algal exudation and predation increasing with  
311 substrate limitation and time, respectively. Interestingly, the lowest carbon loss (60 and 68%)  
312 was observed within the first week of the experiments at pH 7 (Table 2). This supports our  
313 observations that intermediate pH reduces grazing on marine microalgal growth as a result of  
314 partial asphyxiation of ciliates and copepods, but maintains optimal conditions for  
315 development of amoebae that prefer more acidic waters.

316

### 317 3.3 Predictions of maximal biological CO<sub>2</sub> conversion efficiency in HRAP

318 We predicted the theoretical level of minimal carbon loss through biological activity  
319 (respiration, exudation, predation) using a GAM model with a smooth function of time and  
320 four degrees of freedom. Biological activity explained 77% of deviance ( $R^2 = 0.64$ ) for  
321 biological carbon loss. Using this model, a new time series and confidence interval were  
322 predicted for time points that varied from 0 to 44 days using a step of 1 day (Fig. 3). The  
323 minimal fraction of gross photosynthetically fixed CO<sub>2</sub> that was not accumulated into biomass  
324 was predicted using the value reached at day 0 (Figure 4). This fraction ( $44 \pm 25\%$ ) is  
325 considered the minimal loss of carbon due to CO<sub>2</sub> respiration required for algal metabolism  
326 (typically 20–30% of the maximal algal growth rate; Geider and Osborne 1989) and algal  
327 carbon exudation, or natural phytoplankton (7–50% of total photosynthetically fixed CO<sub>2</sub>;  
328 Fogg et al. 1965).



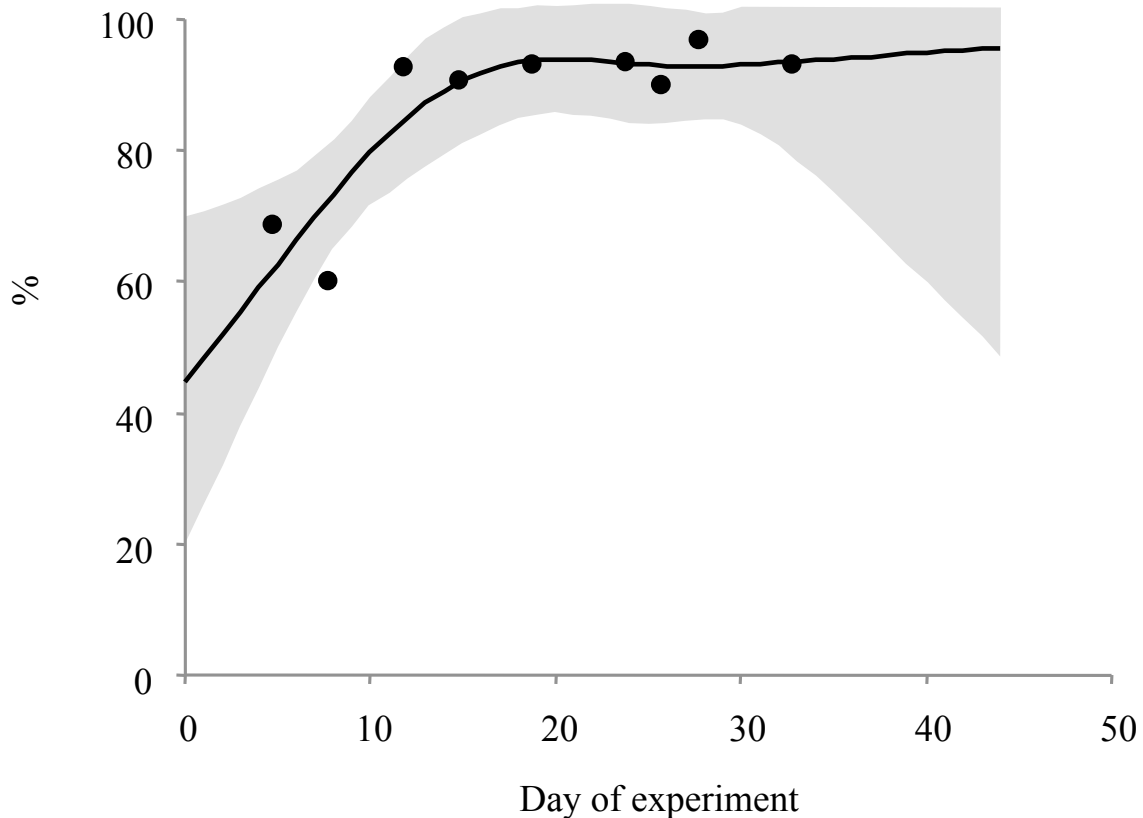


Figure 4. The fraction (%) of gross photosynthetically fixed CO<sub>2</sub> that was not accumulated into the C biomass (biological losses) measured in HRAPs at pH 7 during run 1 and run 2. The time series was modelled using a generalised additive model (GAM) and was plotted (full lines) along with their confidence intervals (grey areas) at time points 0 to 44 days using 1-day intervals.

Therefore, the maximal theoretical CO<sub>2</sub> utilisation efficiency would be 56%, corresponding to lowest biological CO<sub>2</sub> loss (Fig.4) in an open raceway using natural marine inoculate. The maximal CO<sub>2</sub> utilisation efficiency reported during this study (40%) was measured one week after the beginning of the experiment. This value corresponds to values (31–49%) determined for CO<sub>2</sub> utilisation efficiency reported for *Nannochloris* sp. cultivated in batch mode indoors in a 10 m<sup>2</sup> raceway at pH 7.5 for 14 days (Asadollahzadeh et al. 2014). Similarly, the highest CO<sub>2</sub> utilisation efficiency observed by Asadollahzadeh et al. (2014) occurred one week after the beginning of the experiment when algal productivity was at its highest level. From our results, we show a positive correlation between water resident time and loss of fixed CO<sub>2</sub> through biological activity, especially through predation. We suggest

that microalgal cultivation time should be sufficiently short to avoid the substantial development of predators while maintaining high algal productivity. The present experiment was run under batch culture mode where microorganisms are inoculated to a fixed volume of medium and they gradually consumed nutrients until exhaustion. A continuous culture mode seems more adapted than batch culture mode, because fresh medium is continuously added to the culture while used medium and cells are harvested at the same time. For continuous culture mode, the dilution rate (inverse of the resident time of growing cells) can be adjusted according to climatic variations for maintaining algal cells at high productivity rates and for removing slow-growth predators. Our results also show that CO<sub>2</sub> injection can be used for fixing pH at requested values to control the development of algal predators and to favour rapid-growth stress-tolerant microalgae species such as *Nannochloris* sp. (Henley et al. 2002, Vasseur et al. 2012). This supports previous observations reported in studies with CO<sub>2</sub> addition in HRAPs and performed in various geographical areas (New Zealand, Southern France, Southern Spain) using natural biological inoculates (de Godos et al. 2016; Mehrabadi et al. 2017; Uggetti et al. 2018). This suggests a common response of different natural-inoculate biological communities to CO<sub>2</sub> injection in HRAPs. The use of artificial algal assemblages, well-adapted to CO<sub>2</sub> capture under low pH (Piiparen et al. 2018), may facilitate experimental procedures and industrialisation. However, a very recent study shows that a natural algal polyculture was more productive and stress resilient than an optimised artificially assembled polyculture when cultivated with anaerobic digester effluent and with or not grazer additions (Thomas et al. 2019). Therefore, assembled polycultures may not be as resilient as natural assemblages to cope with grazing pressure, variable growth conditions and potential contaminants when using industrial flue-gas and/or wastewater

#### 4. Conclusion

Injection of CO<sub>2</sub> for microalgae production in HRAPs requires pH control. Our results show that pH 7, controlled by CO<sub>2</sub> addition with a variability of 0.2 pH unit, favoured the growth of *Nannochloris* sp., reduced populations of marine algal predators and photosynthetically fixed accumulated CO<sub>2</sub> at 40 % efficiency into carbon biomass. More acidic pH values led to the development of amoebae and restrict algal biomass accumulation. By extrapolating our experimental results, we predict that the maximal CO<sub>2</sub> conversion efficiency in these systems would be 60% of the available CO<sub>2</sub>. Such value may be achievable if running HRAPs in a continuous mode, with a water resident time required for maintaining a rapid algal growth whilst reducing the development of algal predators. When using an industrial CO<sub>2</sub> source, pH regulation using injection of industrial fumes could be an effective tool for driving an algal polyculture toward more desired species. Further investigations should assess the impact of flue gas-associated contaminants on microalgal diversity, biomass production and resilience, as well as the effects of seasonal variability throughout the year on annual capability of fixing CO<sub>2</sub> and algal species selection using pH control in HRAPs.

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#### References

- [1] Y.-C. Lai, C.-H. Chang, C.-Y. Chen, J.-S. Chang, I-S. Ng, Towards protein production and application by using *Chlorella* species as circular economy, *Biores. Technol.* 289 (2019) 121625.
- [2] W.A.V. Stiles, D. Styles, S.P. Chapman, S. Esteves, A. bywater, L. Melville, A. Silkina, I. Lupatsch, C.F. Grünewald, R. Lovitt, T. Chaloner, A. Bull, C. Morris, C.A. Llewellyn, Using microalgae in the circular economy to valorise anaerobic digestate: challenges and opportunities, *Biores. Technol.* 267 (2019) 732-742.
- [3] V. Bhola, F. Swalaha, R. Ranjith Kumar, M. Singh, F. Bux, Overview of the potential microalgae for CO<sub>2</sub> sequestration, *Int. J. Environ. Sci. Technol.* 11 (2014) 2103-2118.
- [4] D.E. Brune, T.J. Lundquist, J.R. Benemann, Microalgal biomass for greenhouse gas reductions: potential for replacement of fossil fuels and animal feed. *J. Environ. Eng.* 135 (2009) 1136-1144.
- [5] B. Wang, Y. Li, N. Wu, C.Q. Lan, CO<sub>2</sub> bio-mitigation using microalgae. *Appl. Microbiol. Biotechnol.* 79 (2008) 707-718.
- [6] D. Hess, K. Napan, B.T. McNeil, E.L. Torres, T. Guy; J.E. McLean, J.C. Quinn, Quantification of effects of flue gas derived inorganic contaminants on microalgae growth system and end fate of contaminants, *Algal Res.*, 25 (2017) 68-75.
- [7] R.A. Galloway, R.W. Krauss, The effect of CO<sub>2</sub> on pH in culture media for algae, *Plant Cell Physiol.*, 2 (1961) 331-337.
- [8] C. Vasseur C, G. Bougaran, M. Garnier, J. Hamelin, C. Leboulanger, M. Le Chevanton, B. Mostajir, B. Sialve, J.-P. Steyer, E. Fouilland, Carbon conversion efficiency and population dynamics of a marine algae-bacteria consortium growing on simplified synthetic digestate:

First step in a bioprocess coupling algal production and anaerobic digestion, *Biores. Technol.* 119 (2012) 79-87.

[9] X. Zeng, M.K. Danquah, X.D. Chen, Y. Lu, Microalgae bioengineering: from CO<sub>2</sub> fixation to biofuel production. *Ren. Sustain. Energy rev.* 15 (2011) 3252-3260.

[10] V. Montemezzani, I.C. Duggan, I.D. Hogg, R.J. Craggs, Screening of potential zooplankton control technologies for wastewater treatment High Rates Algal Ponds, *Algal Res.* 22 (2017a) 1-13.

[11] V. Montemezzani, I.C. Duggan, I.D. Hogg, R.J. Craggs, Control of zooplankton populations in a wastewater treatment High Rate Algal Pond using overnight CO<sub>2</sub> asphyxiation, *Algal Res.* 26 (2017b) 250-264.

[12] V. Montemezzani, I.C. Duggan, I.D. Hogg, R.J. Craggs, Assessment of potential zooplankton control treatments for wastewater treatment High Rate Algal Ponds, *Algal Res.* 24 (2017c) 40-63.

[13] C.H. Greene, M.E. Huntley, L.N. Gerber, D.L. Sills; J. Granados, J.W. Tester, C.M. Colin, M.J. Walsh, R.R. Bidigare, S.L. Brown, W.P. Cochlan, Z.I. Johnson, X.G. Lei, S.C. Machesky, D.G. Redalje, R.E. Richarson, V. Kiron, V. Corless, Marine microalgae: Climate, energy, and food security from the sea, *Oceanography* 29 (2016) 10–15.

[14] A. Narwani, A.R. Lashaway, D.C. Hietala, P.E. Savage, B.J. Cardinale, Power of plankton: effects of algal biodiversity on biocrude production and stability, *Environ. Sci. Technol.* 50 (2016) 13142-13150.

- [15] P.K. Thomas, G.P. Dunn, A.R. Good, M.P. Callahan, E.R. Coats, D.T. Newby, K.P. Feris, A natural algal polyculture outperforms an assembled polyculture in wastewater-based open pond biofuel production, *Algal Res.*, 40 (2019) 101488.
- [16] E. Metaxa, G. Deviller, P. Pagand, C. Alliaume, C. Casellas, J.-P. Blancheton, High rate algal pond treatment for water reuse in a marine fish recirculation system: Water purification and fish health, *Aquaculture*, 252 (2006) 92-101.
- [17] B. Barrut, J.-P. Blancheton, A. Muller-Feuga, F. René, C. Narvaez, J.-Y. Champagne, A. Grasmick, Separation efficiency of a vacuum gas lift for microalgae harvesting, *Biores. Technol.* 128 (2013) 235-240.
- [18] U. Riebesell, V. J. Fabry, L. Hansson, J. P. Gattuso, Guide to best practices for ocean acidification research and data reporting. Office for Official Publications of the European Communities, (2011).
- [19] R.J. Ritchie, Universal chlorophyll equations for estimating chlorophylls a, b, c, and d and total chlorophylls in natural assemblages of photosynthetic organisms using acetone, methanol, or ethanol solvents. *Photosynthetica*, 46 (2008) 115-126.
- [20] M. Latasa, X. A. G. Moran, R. Scharek, M. Estrada. Estimating the carbon flux through main phytoplankton groups in the northwestern Mediterranean. *Limnol. Oceanogr.* 50 (2005) 1447-1458.
- [21] J.J Kozich, S.L. Westcott, N.T. Baxter, S.K. Highlander, P.D. Schloss, Development of a dual -index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina Sequencing Platform, *Appl. Environ. Microbiol.* 79 (2013) 5112-5120.

- [22] P.D. Schloss, S.L. Westcott, T. Ryabin, J.R. Hall, M. Hartmann, E.B. Hollister, R.A. Lesniewski, B.B. Oakley, D.H. Parks, C.J. Robinson, J.W. Sahl, B. Stres, G.G. Thallinger, D.J. Van Horn, C.F. Weber Schloss, Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities, *Appl. Environ. Microbiol.* 75 (2009) 7537-7541.
- [23] P.G. Falkowski, J.A. Raven, *Aquatic photosynthesis*, Blackwell Science, 1997.
- [24] P.H.C. Eilers, J.C.H. Peters, A model for the relationship between light intensity and the rate of photosynthesis in phytoplankton, *Ecol. Model.* 42 (1988) 199-215.
- [25] R Core Team, *R: A language and environment for statistical computing*, R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>, 2019.
- [26] S.N. Wood, *Generalized Additive Models: An Introduction with R* (2nd edition), Chapman and Hall/CRC, 2017.
- [27] A. E. R. Hassoun, E. Gemayel, E. Krasakopoulou, C. Goyet, M. A. A. Saab, V. Guglielmi, F. Touratier, C. Falco, Acidification of the Mediterranean Sea from anthropogenic carbon penetration. *Deep Sea Res. Part I: Oceanographic Research Papers* 10 (2015) 1-15.
- [28] R.W. Butcher, Contributions to our knowledge of the smaller marine algae. *J. Mar. Biol. Ass. United Kingdom* 31 (1952) 175-191.
- [29] J. Fabregas, C. Herrero, J. Abalde, B. Cabezas, The marine microalga *Chlorella stigmatophora* as a potential source of single cell protein: enhancement of the protein content in response to nutrient enrichment, *J. Indus. Microbiol.* 1 (1986) 251-257.

- [30] S. H. Cho, S.-C. Ji, S. N. Hur, J. Bae, I-S. Park, Y.\_C. Song, Optimum temperature and salinity conditions for growth of green algae *Chlorella ellipsoidea* and *Nannochloris oculata*, 73 (2007) 1050-1056.
- [31] W.J. Henley, J.L. Hironaka, L. Guillou, M.A. Buchheim, J.A. Buchheim, M.F. Fawley, K. Fawley, Phylogenetic analysis of the ‘*Nannochloris*-like’ algae and diagnoses of *Picochlorum oklahomensis* gen. et sp. nov. (Trebouxiophyceae, Chlorophyta), *Phycologia*, 43 (2004) 641-652.
- [32] N. von Alvensleben, K. Stookey, M. Magnusson, K. Heimann, Salinity Tolerance of *Picochlorum atomus* and the use of Salinity for contamination control by the freshwater cyanobacterium *Pseudanabaena limnetica*, *Plos One* 8 (2013) e63569.
- [33] L.T. Carney, T.W. Lane, Parasites in algae mass culture, *Frontiers Microbiol.* 5 (2014) 278.
- [34] J. Day, Y. Gong, Q. Hu, Microzooplanktonic grazers – A potential devastating threat to the commercial success of microalgal mass culture, *Algal. Res.* 27 (2017) 356-365.
- [35] S. Rodriguez-Zaragoza, Ecology of free-living amoebae, *Crit. Rev. Microbiol.* 20 (1994) 225-241.
- [36] R. Herziz, P. G. Falkowski, Nitrogen limitation in *Isochrysis galbana*. I. Photosynthetic energy conversion and growth efficiencies, 25 (1989) 462-471.
- [37] R.J. Geider, B.A. Osborne, Respiration and microalgal growth: a review of the quantitative relationship between dark respiration and growth, *New Phytol.* 112 (1989) 327-341.



- [38] G.E. Fogg, C. Nalewajko, W.D. Watt, Extracellular products of phytoplankton photosynthesis. *Proc. Royal Soc. B* 162 (1965) 517-534.
- [39] M.J. Asadollahzadeh, M. Ardjmand, A.A. Seafkordi, S.M. Heydarian, Efficient storage and utilization of CO<sub>2</sub> in open raceway ponds for cultivation of microalgae, *Korean J. Chem. Eng.* 31 (2014) 1425-1432.
- [40] W. J. Henley, K. M. Major, J. L. Hironaka, Response to salinity and heat stress in two halotolerant chlorophyte algae, *J. Phycol.* 38 (2002) 757-766.
- [41] I. de Godos, Z. Arbib, E. Lara, F. Rogalla, Evaluation of High Rate Algal Pond for treatment of anaerobically digested wastewater: Effect of CO<sub>2</sub> addition and modification of dilution rate, *Water Res.* 220 (2016) 253-261.
- [42] A. Mehrabadi, M. M. Farid, R. C. Craggs, Effect of CO<sub>2</sub> addition on biomass energy yield in wastewater treatment high rate algal mesocosms, *Algal Res.* 22 (2017) 93-103.
- [43] E. Uggetti, B. Sialve, J. Hamelin, A. Bonnafous, J.-P. Steyer, CO<sub>2</sub> addition to increase biomass production and control microalgae species in high rate algal ponds treating wastewater, *J. CO<sub>2</sub> Util.* 28 (2018) 292-298.
- [44] J. Piiparen, D. Barth, N. T. Eriksen, S. Teir, K. Spilling, M. G. Wiebe, Microalgal CO<sub>2</sub> capture at extreme pH values, *Algal Res.* 32 (2018) 321-328.
- [45] P. K. Thomas, G. P. Dunn, A. R. Good, M. P. Callahan, E. R. Coats, D. T. Newby, K. P. Feris, A natural algal polyculture outperforms an assembled polyculture in wastewater-based open pond biofuel production, *Algal Res.* 40 (2019) 101488.

## Supplementary material

Figure S1. Values of pH measured in the HRAPs with pH targeted at 6, 7 and 8 during run 1 (from 17 May to 22 June 2016) and run 2 (from 27 June to 26 July 2016).

