

Applied Microbiology and Biotechnology

Effect of different CO₂ concentrations on biomass, pigment content and lipid production of the marine diatom *Thalassiosira pseudonana* --Manuscript Draft--

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Full Title:	Effect of different CO ₂ concentrations on biomass, pigment content and lipid production of the marine diatom <i>Thalassiosira pseudonana</i>	
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Section/Category:	Applied microbial and cell physiology	
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	Università degli Studi di Ferrara (Fondo per l'incentivazione alla Ricerca 2016)	Prof Simonetta Pancaldi
Abstract:	<p>The marine diatom <i>Thalassiosira pseudonana</i> grown under air (0.04% CO₂), 1 and 5% CO₂ concentrations was evaluated to determine its potential for CO₂ mitigation coupled with biodiesel production. Results indicated that the diatom cultures grown at 1 and 5% CO₂ showed higher growth rates (1.14 and 1.29 div d⁻¹, respectively) and biomass productivities (44 and 48 mgAFDWL-1d⁻¹) than air grown cultures (with 1.13 div d⁻¹ and 26 mgAFDWL-1d⁻¹). The increase of CO₂ resulted in higher cell volume and pigment content per cell of <i>T. pseudonana</i>. Interestingly, lipid content doubled when air was enriched with 1-5% CO₂. Moreover, the analysis of the fatty acid composition of <i>T. pseudonana</i> revealed the predominance of monounsaturated acids (palmitoleic-16:1 and oleic-18:1) and a decrease of the saturated myristic acid-14:0 and polyunsaturated fatty acids under high CO₂ levels. These results suggested that <i>T. pseudonana</i> seems to be an ideal candidate for biodiesel production using flue gases.</p>	

Response to Reviewers:

Answers to Editor's Comments:

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We agree with the reviewer that providing the kinetic of lipid production would have been best. According to the reviewer suggestion we introduced the sentence in line 296 page 9 in the present version:

"The kinetics of lipid production in each CO2 condition was not monitored and hence the 4th day of cultivation may not correspond to the maximum lipid content in any treatment. Nevertheless, the difference in lipid content between CO2 enriched cultures and air grown cultures was remarkable."

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UNIVERSITÀ DEGLI STUDI DI FERRARA
DIPARTIMENTO di SCIENZE della VITA e BIOTECNOLOGIE
Corso Ercole I d'Este, 32 - 44121 Ferrara (Italia)

Ferrara, 14th of December 2017

Dear Editor,

We are very grateful that our manuscript was very positively evaluated by reviewers, who we thank very much for their expert comments.

Please find our revised manuscript entitled “Effect of different CO₂ concentrations on biomass, pigment content and lipid production of the marine diatom *Thalassiosira pseudonana*” by Alessandra Sabia, Esther Clavero, Simonetta Pancaldi and Joan Salvadó Rovira.

Changes in response to reviewers’ comments are tracked in the revised text.

Hoping that our work is now eligible for publication in your Journal, I convey you our very best regards.

Simonetta Pancaldi

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Laboratory of Plant Cytophysiology
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Alessandra Sabia^{a§}, Esther Clavero^{b§}, Simonetta Pancaldi^{c*}, Joan Salvadó Rovira^c

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1 **Abstract**

2 The marine diatom *Thalassiosira pseudonana* grown under air (0.04% CO₂), 1 and 5% CO₂
3 concentrations was evaluated to determine its potential for CO₂ mitigation coupled with biodiesel
4 production. Results indicated that the diatom cultures grown at 1 and 5% CO₂ showed higher growth rates
5 (1.14 and 1.29 div d⁻¹, respectively) and biomass productivities (44 and 48 mg_{AFDW}L⁻¹d⁻¹) than air grown
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8 with 1-5% CO₂. Moreover, the analysis of the fatty acid composition of *T. pseudonana* revealed the
9 predominance of monounsaturated acids (palmitoleic-16:1 and oleic-18:1) and a decrease of the saturated
10 myristic acid-14:0 and polyunsaturated fatty acids under high CO₂ levels. These results suggested that *T.*
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12
13 **Keywords**

14 Microalgae;
15 CO₂ concentrations;
16 Biomass productivity;
17 Fatty acid composition;
18 Biodiesel production.

19 **Introduction**

20 The global energy supply is drastically rising due to the continuous increase of modern industrialization.
21 At the present, 90% of global energy demand is generated from fossil fuels and only 10% is fulfilled by
22 renewable energy sources (Maity et al. 2014; Shen 2014). Based on the current projections, the total
23 energy consumption is foreseen to increase year by year and by the year 2050, most of the fossil fuel
24 reserves will be completely exhausted (Maity et al. 2014; Shen 2014). Besides this, the anthropogenic
25 carbon dioxide (CO₂) emissions mainly come from the intensive burning of fossil fuels has contributed to
26 a 40% increase in the atmospheric CO₂ levels, from 280 ppm in pre-industrial age to 400 ppm currently
27 (Shen 2014; Cheah et al. 2015). Carbon dioxide, the principal greenhouse gas, is now widely considered
28 as the major responsible of the global warming (Shen 2014; Cheah et al. 2015, [Mondal et al. 2017](#)). As a
29 consequence of this global phenomenon, extensive studies have been focused on the development of
30 renewable, carbon-neutral feedstock to displace petroleum and to mitigate anthropogenic emissions of
31 CO₂ (Maity et al. 2014; Shen 2014, [Singh et al. 2016](#), [Mondal et al. 2017](#)). In this perspective, microalgae
32 have received much attention as renewable, energy-saving, sustainable approach to reduce the CO₂
33 emissions and simultaneously to produce biodiesel (Chisti 2007; Lam et al. 2012; Maity et al. 2014;
34 Trobajo et al. 2014, [Singh et al. 2016](#), [Mondal et al. 2017](#)). Microalgae have faster growth rates, higher
35 photosynthetic efficiencies, higher rates of carbon dioxide fixation, and higher biomass and lipid
36 productivities as compared to conventional oil crop plants (Chisti 2007; Sabia et al. 2015; Baldisserotto et
37 al. 2016). Currently, extensive researches have been concerned the choice of the most adequate
38 microalgal strain that could tolerate elevated CO₂ concentrations and, simultaneously, produce lipids
39 suitable for biodiesel production (Lam et al. 2012; Trobajo et al. 2014; Nascimento et al. 2015). However,
40 most of these microalgal strains studied for this purpose are freshwater species (Tang et al. 2011; Lam et
41 al. 2012). With the aim of making the microalgal large-scale production increasingly sustainable, it is
42 necessary to identify a species that can grow in saltwater to avoid the competition with freshwater
43 resources (Doan et al. 2011; Baldisserotto et al. 2012). Marine diatoms may be considered as an attractive
44 feedstock for combined CO₂ fixation and lipid production (Hildebrand et al. 2012; Wang et al. 2014;
45 Vinayak et al. 2015, [Manju et al. 2017](#)). Diatoms, due to their high e₊-trophic flexibility, are considered
46 the most productive microalgae on the planet (Field et al. 1998; Hildebrand et al. 2012). These
47 microorganisms, which are the main component of phytoplankton, significantly contribute to the global
48 primary productivity and marine geochemical cycles (Field et al. 1998). Moreover, they are excellent
49 lipid accumulators, especially triacylglycerides (TAGs), which are synthesized and accumulated in the
50 stationary phase of growth (Hildebrand et al. 2012; d'Ippolito et al. 2015; Vinayak et al. 2015, [Manju et
51 al. 2017](#)). It is well known that TAGs represent the best substrate for the biodiesel production (Chisti et al.
52 2007; Baldisserotto et al. 2016, [Zienkiewicz et al. 2016](#)). Nevertheless, only few studies have valorised
53 the diatom lipid profile for biodiesel (Francisco et al. 2010; Zendejas et al. 2012⁺; Popovich et al. 2012,
54 [Manju et al. 2017](#)). *Thalassiosira pseudonana*, a model diatom species, which genome has been recently
55 sequenced (Armbrust et al. 2004), is one of the most studied species for the production of biofuels up to
56 now (Yu et al. 2009; Zendejas et al. 2012⁺; d'Ippolito et al. 2015). In addition, recent studies have been
57 concerned the effect of elevated CO₂ concentrations on growth and photophysiology of this diatom

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58 species (Crawford et al. 2011; Yang and Gao 2012; McCarthy et al. 2012; Li and Campbell 2013).
59 The present work is focused on the potential of the marine diatom *Thalassiosira pseudonana* as a
60 promising ~~candidate~~ strain for the cultivation in the presence of elevated CO₂ concentrations. In
61 particular, algal growth, cell morphology, photosynthetic pigment content, total nitrogen (TN), and
62 dissolved inorganic (TIC) and organic carbon (TOC) content were monitored under increasing CO₂
63 concentrations (0.04 (ambient air), 1 and 5%). The lipid content and profile was also estimated in order to
64 evaluate the effects of elevated CO₂ concentration on the composition of fatty acids useful for biodiesel
65 production.

66
67

68 **Materials and Methods**

69 Algal strain and culture condition

70 The strain used in this study was the marine centric diatom *Thalassiosira pseudonana* (Hustedt) Hasle
71 and Heimdal CCAP 1085/12 (*Heterokontophyta, Thalassiosirales, Thalassiosiraceae*), obtained from the
72 Culture Collection of Algae and Protozoa (CCAP; Scotland, United Kingdom; www.ccap.ac.uk).

73 *T. pseudonana* was inoculated in 6-L Erlenmeyer flasks with a 4-L working volume of filtered (0.45 µm)
74 autoclaved seawater enriched with f/2 nutrients (Guillard and Rytner 1962) at 25±2°C and under an
75 exposure to an irradiance of 250 µmol_{photons} m⁻² s⁻¹ provided by cool white fluorescent lamps (OSRAM
76 L30W/865 Lumilux) under a cycle period of 16:8 h light-darkness. The *T. pseudonana* cultures were
77 aerated at a rate of 250 mL min⁻¹ (i.e., 0.0625 vvm, volume gas per volume media per min) of air
78 enriched with CO₂ to obtain final concentration of 0.04, 1 and 5% CO₂. The air-CO₂ mixtures were
79 obtained by combining rotameter flows of air and pure CO₂. The percentage of CO₂ in the air-CO₂
80 mixture was verified with an infrared analyzer (Servomex 4900C1). Experiments were performed in
81 triplicate. Aliquots of cultures were collected at different times of cultivation, depending on the analysis.

82

83 Analyses

84 *Growth, cell size and biomass evaluation*

85 Algal cells were counted daily using an Improved Neubauer hemocytometer under a light microscope
86 (Zeiss, model Axioscope A1) equipped with DIC (Differential Interference Contrast) optics in order to
87 estimate the division rates (k, number of divisions per day). Cell density (cell mL⁻¹) was also measured by
88 the absorbance at 750 nm (A₇₅₀) in 96 well plates with the microplate reader (Infinite Tecan M200Pro).
89 Absorbance measures were converted to optical density by dividing absorbance values by the pathlength
90 following Beer-Lambert's law. Cell size measurements were done with the microscope described above
91 and measured with the aid of the software (ProgRes Capture Pro v. 2.8) and a digital camera (Jenoptik
92 ProgRes Speed XT Core5).

93 The division rates (k, number of divisions per day) during the exponential phase was calculated with the
94 following equation:

95 $k \text{ (div d}^{-1}\text{)} = (\log_2 N_t - \log_2 N_0) / (t_t - t_0)$,

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96 where k is the division rate, N_t the cell number at time t_t , N_0 the cell number at time 0 and $t_t - t_0$ the time
97 interval (days) (Andersen 2005). ~~The biomass productivity ($\text{mg}_{\text{AFDW}} \text{L}^{-1} \text{d}^{-1}$) was calculated from the~~
98 ~~variation in the biomass concentration ($\text{mg}_{\text{AFDW}} \text{L}^{-1}$) within a specific cultivation time (d) according to the~~
99 ~~following equation:~~

100
$$P (\text{mg}_{\text{AFDW}} \text{L}^{-1} \text{d}^{-1}) = (X_t - X_0) / (t_t - t_0);$$

101 ~~where P is the biomass productivity, X_t the biomass concentration at time t_t , X_0 the biomass~~
102 ~~concentration at time 0 and $t_t - t_0$ the time interval (days) (Hempel et al. 2012).~~

103 Algal biomass concentration, expressed as ash free dry weight (AFDW) was measured according to the
104 methods as described by Zhu and Lee (1997), with some modifications. In detail, after the filtration of
105 culture replicates (30-50 mL) (onto tare GF/F Whatman filters which had been previously precombusted),
106 the filtered samples were washed with sodium chloride to remove adhering salts and dried in the oven for
107 72 h at 60°C. Subsequently, the filters were placed in a muffle furnace for 4 h at 550°C and re-weighed.
108 AFDW corresponds to the difference of the filter weight after the oven treatment minus the filter weight
109 after the muffle.

110 The biomass productivity ($\text{mg}_{\text{AFDW}} \text{L}^{-1} \text{d}^{-1}$) was calculated from the variation in the biomass concentration
111 ($\text{mg}_{\text{AFDW}} \text{L}^{-1}$) within a specific cultivation time (d) according to the following equation:

112
$$P (\text{mg}_{\text{AFDW}} \text{L}^{-1} \text{d}^{-1}) = (X_t - X_0) / (t_t - t_0);$$

113 where P is the biomass productivity, X_t the biomass concentration at time t_t , X_0 the biomass
114 concentration at time 0 and $t_t - t_0$ the time interval (days) (Hempel et al. 2012).

115

116 *Photosynthetic pigment extraction and quantification*

117 For photosynthetic pigment analysis, cell samples were collected by centrifugation and the extraction was
118 performed according to Giovanardi et al. (2014). The extracts were measured with the same microplate
119 reader, already described, at 665 (Chl a), 632 (Chl c) and 480 nm (Car). Quantification of Chls was
120 performed according to equations reported in Ritchie (2006), whereas Car quantification according to
121 equations reported in Wellburn (1994).

122

123 *Biochemical analyses*

124 During the growth period, the pH in culture replicates was monitored daily using a pH meter (Crison
125 GLP21). The total nitrogen (TN), the dissolved inorganic (TIC) and dissolved organic carbon (TOC)
126 concentrations in the culture medium were measured by a Multi N/C 3100 Analytic Jena analyser. 20 mL
127 of the algal cultures were centrifuged at 4000 rpm for 15 min and the supernatant was used for TIC, TOC
128 and TN measurements.

129

130 *Characterization of neutral lipids with Nile red*

131 Cell lipid content was estimated by neutral lipid staining with the fluorochrome Nile Red at the 4th day of
132 cultivation, when all the microalgal cultures had reached the stationary phase of growth, according to
133 Chen et al. (2009). First, algal samples were diluted to attain a final cell concentration of 0.06 (A_{750}).

134 After the algal suspension were stained with Nile Red (9-diethylamina-5Hbenzo[*a*]phenoxazine-5-one,
135 0.5 µg dissolved in 100 mL acetone) and 25% of absolute dimethyl sulphoxide (DMSO). Fluorescence
136 was measured with the microplate reader, already described, at excitation and emission wavelengths of
137 530 nm and 580 nm, respectively. The relative fluorescence was obtained by subtraction of the
138 autofluorescence of non-stained cells and self-fluorescence of the fluorochrome. After staining, cells were
139 also observed with a Zeiss AxioObserver Z1 equipped with epifluorescence. A metal halide lamp was
140 used as the fluorescence excitation source (Lumen 200Pro, Prior Scientific), and a filter cube containing a
141 450-490 nm excitation and a 510-515 nm collection filter was used for light filtration.

142

143 *Lipid extraction*

144 For fatty acids analysis, the total final algal cultures were harvested by flocculation with NaOH 2M at the
145 end of the cultivation period, according to Şirin et al. (2015). The settled down volume was further
146 centrifuged (3000 rpm, 5min) to obtain a more reduced volume. Subsequently, the microalgal pellets
147 were frozen (-80°C) and lyophilized drying in Telstar Lyoquest-85 freeze dryer for dry weight
148 measurement and fatty acids characterization.

149 Lipids were extracted from the microalgal samples with the Bligh and Dyer method (Bligh and Dyer
150 1959), which uses a ternary system of chloroform/methanol/water and is the most commonly used
151 method for the quantitative extraction of lipids from microalgae at analytical level. Briefly, 2g of sample
152 were dissolved in 20 mL of water and then were mixed with 75 mL of a mixture chloroform-methanol
153 (1:2 v/v) using a magnetic stirrer at 300 rpm for 10 min. Then 25 mL of chloroform and 25 mL of
154 distilled water were added to form a two-phase system. The phases were separated by 5 min
155 centrifugation at 3500 rpm. The chloroform phase was then separated and the amount of lipid obtained
156 was gravimetrically measured after drying overnight in an oven at 70 °C.

157

158 *Methyl ester derivation and fatty acid analysis*

159 A two-step protocol was used for the methylation process of all extracted lipids, according to Lee et al
160 (2010). The samples were saponified with 5 mL of 2% KOH in methanol at 75 °C for 10 min, and then
161 subjected to methanolysis with 5 mL of 5% HCl in methanol at 75 °C for another 10 min. Thereafter, the
162 phase containing the fatty acids was separated by adding 10 mL of distilled water and then recovered with
163 5 mL of heptane. The obtained fatty acid methyl esters (FAMES) were analyzed according to EN 14103,
164 with an Agilent 6850 gas chromatograph equipped with a split/splitless inlet and a FID detector. The
165 column used was a HP-INNOWax (polyethylene glycol phase), a capillary column with a high polarity
166 and dimensions 30 m x 0.32 mm x 0.25 µm. Quantification of total FAMES was accomplished by using
167 FAME C17:0 as internal standard. Identification of individual FAME components was based on
168 comparison of their retention times and fragmentation patterns with those for standards. The FAME
169 composition was calculated as the percentage of the total identified esters present in the sample.

170

171 **Data treatment**

172 Data were processed with Graphpad Prism 6 (Graph Pad Software, San Diego CA, USA). In each case,
173 means \pm standard deviations for n number of samples are given.
174 The statistical significance of differences was determined by one-way ANOVA followed by a multiple
175 comparison test (Tukey's test). A significance level of 95% ($p < 0.05$) was accepted.

176
177

178 **Results**

179 *Effect of different CO₂ concentrations on T. pseudonana growth*

180 In this study, as shown in Fig. 1a, the diatom *T. pseudonana* cultivated with 0.04 (ambient air), 1 and 5%
181 CO₂ promptly entered the exponential phase and reached the stationary phase of growth during 4 days of
182 cultivation. However, it was apparent from Table 1 that the highest CO₂ concentration increased the
183 division rates of the diatom as compared to cells cultivated with 0.04 and 1% CO₂. Interestingly, at the
184 end of the experiment, the biomass concentrations of cells cultivated with 1 and 5% CO₂ were 190 and
185 210 mg L⁻¹ respectively, which were 1.58 and 1.75 times higher than that of air grown cultures (Table 1).
186 Further, observations on diatom cell size during the experiment showed remarkable changes in response
187 to the CO₂ treatments. In detail, a gradual but evident increasing trend was observed in cells cultivated
188 with 1 and 5% CO₂ until the end of the experiment (Fig. 1b). Conversely, the cell volume of air grown
189 cultures remained almost stable during the cultivation period. At 4th day of cultivation, the cell volume
190 achieved by both cultures enriched with CO₂ was found 2 times higher than that of cell cultivated with
191 0.04% CO₂ (Fig. 1b). Therefore, these results reflected the higher biomass productivities achieved by
192 cultures grown at 1 and 5% CO₂ (69 and 84% ~~higher than 0.04% CO₂-treated cells,~~ respectively) (Table
193 ~~1) than 0.04% CO₂-treated cells (Table 1).~~ As shown in Table 2, with the increase of CO₂ concentrations
194 from 0.04% to 5%, the TIC concentration in the culture medium increased from 16.83 mg L⁻¹ to 43.30 mg
195 L⁻¹ (+117 and 157% higher than air grown cultures in cells cultivated with 1% and 5% CO₂, respectively).
196 In fact, despite, no great differences were reported between 0.04% and 1% of CO₂ treatments regarding
197 TOC concentrations, the maximum TOC values in the culture medium were found under 5% CO₂ (14.63
198 mg L⁻¹, 19 and 17% higher than 0.04% and 1% CO₂-treated cells, respectively). On the other hand, higher
199 CO₂ concentrations always resulted in lower pH. As shown in Table 2, the pH value of the culture
200 medium decreased from pH 9.4 to pH 6.5 with the increase of CO₂ concentrations from 0.04% to 5%.
201 Further, the TN concentration in the culture medium decreased by 12% and 30% in cells cultivated with
202 1% and 5% CO₂, respectively (Tab. 2).

203

204 *Effect of different CO₂ concentrations on T. pseudonana photosynthetic pigment content*

205 In order to evaluate if the cultivation in presence of elevated CO₂ concentrations could also affect the
206 photosynthetic activity of the diatom, the photosynthetic pigment content were also analysed at the end of
207 the cultivation period. The increase of CO₂ concentrations resulted in significant increases in Chl_a and
208 Car content as compared to those of air grown cultures (Table 3). In detail, Chl_a concentrations in cells
209 cultivated with 1% and 5% CO₂ were higher (28 and 77%, respectively) higher than 0.04% air grown

210 cultures. Despite, no great differences were reported between 0.04% and 1% of CO₂ treatments about
211 Chlc content, the highest Chlc values were found in cells cultivated with 5% CO₂ (57 and 37% higher
212 than air and 1% CO₂ grown cultures. About Car content, cells cultivated with 0.04% CO₂ contained ~~21~~
213 ~~and 64%~~ lower quantities (21 and 64%, respectively) as compared to cultures enriched with elevated CO₂.
214 The increases in total Chl content were greater than differences in the Car content, resulting in higher
215 total Chl to Car molar ratios in cultures enriched with elevated CO₂ as compared to air grown cultures,
216 but the ratios did not differ significantly at the 95% level. No significant effects on Chla to c molar ratios
217 were observed between the samples (Table 3).

218

219 *Effect of different CO₂ concentrations on T. pseudonana lipid quantification and characterisation*

220 In this study, to find a possible relation between CO₂ treatments and lipid synthesis, measurements of
221 neutral lipid accumulation were performed by spectrofluorimetric quantification of the relative
222 fluorescence intensity emitted by Nile Red-stained cells. After staining, cells were also observed by
223 fluorescence microscopy. In detail, while cells cultivated with 0.04% CO₂ showed slightly positive
224 reaction, the cultures enriched with elevated CO₂ accumulated abundant lipid droplets, giving an intense
225 positive reaction with the fluorochrome (data not shown). Accordingly, in this study, as revealed by Nile
226 Red staining, when 1 and 5% CO₂-treated cells were in stationary phase (4th day of cultivation) showed a
227 3-fold increase in lipid content with respect to air grown cultures (Fig. 2) and coincidentally, decreased
228 significantly the TN concentrations in culture medium (Table 2).

229 Nile red staining results were confirmed by Bligh and Dyer lipid extraction. Fluorescence values,
230 normalized to the same cell concentration ($A_{750} = 0.06$), were proportional to the lipid content as %
231 AFDW obtained with Bligh and Dyer extraction. The percentage of lipids in AFDW doubled when air
232 was enriched with 1-5 % CO₂ from 7.3% (air) to 14.1% (1%) and 16.9% (5%) of CO₂ (Table 4).

233 The main fatty acid components of *T. pseudonana* under different CO₂ concentrations were determined
234 by GC-MS analysis and are shown in Table 4. The predominant fatty acids were mirystic (C14:0),
235 palmitic (C16:0), palmitoleic (C16:1), oleic (C18:1) acids (Table 4). Other fatty acids (C15:0, C16:2,
236 C16:3, C18:0, C18:4, C20:5, C22:1 and C22:2) were minor components and presented concentration
237 individually of less than 5%. However, the lipid classes showed differences in their proportions in
238 response to the CO₂ treatments (Table 4). Despite, no great differences were reported between 1%
239 (54.4%) and 5% (52.8%) of CO₂ treatments about the percentages of saturated fatty acids (SFAs), the
240 highest SFAs values were found in cells cultivated with air (60.4%). The monounsaturated fatty acids
241 (MUFAs) were the major class of fatty acids in cells cultivated under with 1% and 5% CO₂ treatments. In
242 detail, and MUFAs in these cells under these CO₂ treatments account for more than 91% of the total
243 fatty acids. They were significantly higher ($p < 0.05$) than 0.04% air grown cultures controls, reaching the
244 average value (ca. 41%) under higher CO₂ treatments due to a high content of palmitoleic (ca. 29%) and
245 oleic acids (ca. 9%) (Table 4). Regarding the polyunsaturated fatty acids (PUFAs), there was a significant
246 decrease in 1 and 5% CO₂-treated cells compared to air grown cultures. In particular, PUFA levels

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247 decreased significantly due to the decline in the proportion of eicosapentaenoic acid, from 5.3% (air) to
248 4.5% (1%) and 2.2% (5%) of CO₂ (Table 4).

249
250

251 Discussion

252 This study clearly indicates that the marine diatom *T. pseudonana* could tolerate elevated CO₂
253 concentrations and simultaneously, produce lipids suitable for biodiesel production. In spite of extensive
254 studies performed on this species, there is still no information on the possible effects of increasing CO₂
255 concentrations on lipid accumulation, which, if demonstrated, could allow a significant advancement for
256 the biotechnological use of this alga. A recent review summarized how elevated or high CO₂
257 concentrations could affected the diatoms growth and physiology (Gao and Campbell 2014). Some
258 Authors reported that they could have positive effects on diatoms growth (Wu et al. 2010; McCarthy et al.
259 2012; Wang et al. 2014), while others observed neutral (Crawford et al. 2011; Gao et al. 2012) or negative
260 effects (Torstensson et al. 2012; Mejia et al. 2013). However, these conflicting results could be attributed
261 to the varied experimental conditions such as the exposure to different light intensities (Li and Campbell
262 2013), pH (Wu et al. 2010), temperature (Torstensson et al. 2012), but also to the physiological
263 complexity of algal responses to elevated CO₂ concentrations (Wu et al. 2010; Yang and Gao 2012). In
264 this study, the addition of 5% CO₂ increased the diatom growth rates by 15% and biomass concentration
265 by 75%. This was in agreement with such literature data, which reported a positive effect of elevated CO₂
266 concentrations on *T. pseudonana* growth (Yang and Gao 2012; McCarthy et al. 2012; Li and Campbell
267 2013). Interestingly, observations on diatom cell size during the experiment showed remarkable changes
268 in response to the CO₂ treatments. At the end of the experiment, the cell volume achieved by both
269 cultures enriched with CO₂ was found 2 times higher than that of cell cultivated with 0.04% CO₂. These
270 results are coherent with previous studies, which found an evident increase in cell size in centric diatom
271 cultivated under high CO₂ levels (Hoogstraten et al. 2012; Li and Campbell 2013).

272 The dissolved inorganic carbon (TIC) in culture medium represents the carbon sources for the microalgal
273 growth. As expected, the cultivation of *T. pseudonana* under increasing CO₂ concentrations resulted in
274 higher TIC values (Tang et al. 2011). Moreover, in this study, the cultivation with the highest CO₂
275 resulted also in higher TOC concentrations in culture medium, which could be attributed to the frequently
276 extracellular release of algal exudates during microalgal growth (Engel 2002; Hulatt and Thomas 2010).
277 It has long been known that the inorganic carbon system is closely related to the pH, as it is the main
278 buffering system in the culture medium. In this study, the higher CO₂ concentrations tested have led to
279 higher diatom biomass productivities as compared to 0.04% air grown cultures, but also caused a
280 decrease in the pH. Lower pH values could be attributed to the buffering properties of the carbonate
281 system (Wu et al. 2010; Yang and Gao 2012; Gao and Campbell 2014). In order to evaluate if the
282 cultivation in presence of elevated CO₂ concentrations could also affect the photosynthetic activity of the
283 diatom, the photosynthetic pigment content were also analysed at the end of the cultivation period. The
284 overall higher pigment content of cells of *T. pseudonana* grown under increasing CO₂ concentrations as

285 compared to 0.04% air grown cultures was more related to photoacclimation of cultures or as a
286 consequence of the increase of cell volume rather than increase in CO₂ availability (Crawford et al. 2011;
287 Baldisserotto et al. 2012; Li and Campbell 2013). In fact, no significant effects on pigment content of this
288 diatom species were observed after a long period of adaptation to elevated CO₂ concentrations (Crawford
289 et al. 2011; McCarthy et al. 2012; Li and Campbell 2013).

290 The effects of elevated CO₂ concentrations on fatty acids composition and content of microalgae have
291 been already reported (Muradyan et al. 2004; Tang et al. 2011; Trobajo et al. 2014; Wang et al. 2014).

292 Nile Red fluorescence has been widely accepted as a valid method for analyzing TAGs in algal cultures
293 (Yu et al. 2009; Giovanardi et al. 2014; Sabia et al. 2015; Baldisserotto et al. 2016). Accordingly, in this
294 study, as revealed by Nile Red staining, when 1 and 5% CO₂-treated cells were in stationary phase (4th
295 day of cultivation) showed a 3-fold increase in lipid content with respect to air grown cultures (Fig. 2).

296 The kinetics of lipid production in each CO₂ condition was not monitored and hence the 4th day of
297 cultivation may not correspond to the maximum lipid content in any treatment. Nevertheless, the
298 difference in lipid content between CO₂ enriched cultures and air grown cultures was remarkable.
299 On the other hand, CO₂ enriched cultures, and coincidentally, also presented decreased significantly
300 the lower TN concentrations in culture medium (Table 2).

301 Several studies showed that diatoms accumulated lipids as a result of N limitation and increased the
302 proportion of TAGs in the stationary phase of growth (Yu et al. 2009; Hildebrand et al. 2012; d'Ippolito
303 et al. 2015).

304 For biodiesel purposes, fatty acid profiles rich in monounsaturated acids (MUFAs), which can be
305 transesterified to produce biodiesel (Hu al. 2008), are the most interesting profiles. Biodiesel, which is
306 produced by the trans-esterification of triglycerides with methanol to yield the corresponding mono-alkyl
307 fatty acid esters, is an alternative to petroleum-based diesel fuel (Hu al. 2008). The properties of biodiesel
308 are strongly determined by the characteristics of fatty acid chains, such as carbon chain length and
309 unsaturation extent, present in the fuel (Hu et al. 2008; Nascimento et al. 2015, Manju et al. 2017). For
310 example, saturated chains produce a biodiesel with superior oxidative stability and a higher cetane index
311 but rather poor low-temperature properties. In contrast, polyunsaturated fatty acids have good cold-flow
312 properties but are particularly susceptible to oxidation (Hu et al. 2008; Nascimento et al. 2015).

313 Interestingly, the increase in CO₂ concentration resulted in an increase of monounsaturated acids
314 (palmitoleic and oleic) and a decrease of the saturated myristic acid and polyunsaturated fatty acids
315 (Table 4), suggesting that from the point of view of biodiesel production air enriched with CO₂ produces
316 better distribution of fatty acid chains. The increase in unsaturated fatty acids and decrease in saturated
317 fatty acids has previously been observed in Chlorophyceae species at increasing CO₂ concentrations up to
318 5% (Tang 2011; Nascimento et al. 2015). However, this trend did not appear in cultures of the same or
319 other species in other works (Tsuzuki 1990; Muradyan et al. 2004). In this study, it is not observed a big
320 difference in the fatty acid chain profile attending the use of 1% or 5% CO₂ enriched air (Table 4).
321 Despite of this, the evident characteristic of the fatty acid composition of *T. pseudonana* grown under 1-
322 5% CO₂ is that the major fatty acids were C14–C18, and these account for more than 91% of the total
323 fatty acids. The predominance of shorter-chain fatty acids (defined as alkyl chains with 12–18 carbon

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324 atoms) is very significant for the potential of *T. pseudonana* for the production of biodiesel (Hu et al.
325 2008).

326 It has been reported that an increase in CO₂ concentration in culture medium tends to increase the
327 accumulation of polyunsaturated fatty acids in the microalgal cells (Lam et al. 2012). However, compared
328 to previous results (Tang et al. 2011; Wang et al. 2014), *T. pseudonana* showed a decrease of the
329 production of eicosapentaenoic (C20:5) acids (C20:5) from 5.3% (air) to 4.5% (1%) and 2.2% (5%) of
330 CO₂. Despite, the cultivation with high levels of CO₂ showed a notable reduction of polyunsaturated fatty
331 acids, this is still not enough to accomplish the biodiesel standards described in the EN14214 norm.

332 The increase of lipid content, the raise of monounsaturated and the reduction of polyunsaturated fatty
333 acids when using 5% CO₂ may have great potential for the production of an ignition-quality biofuel with
334 cold-flow properties and oxidative stability.

335
336 In conclusion, results presented here demonstrate that the maximum growth rate, biomass concentration
337 and productivity, cell volume and photosynthetic pigment content per cell were obtained when the marine
338 diatom *T. pseudonana* was cultivated under 5% CO₂. Moreover, treatments with 5% CO₂ gives an increase
339 of total and neutral lipid content, the raise of monounsaturated and the reduction of polyunsaturated fatty
340 acids. These results suggest that *T. pseudonana* under 5% CO₂ showed ~~the most~~ suitable fatty acid
341 composition for the production of biodiesel.

342

343

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355

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357 **Conflict of interest**

358 The authors declare they have no conflict of interest.

359

360 **Compliance with Ethical Standards**

361 This article does not contain any studies with human participants or animals performed by any of the
362 authors

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505 **Figure captions**

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507 **Fig 1: (a)** Growth curves and **(b)** cell volumes of *T. pseudonana* cultivated under 0.04% (white circles
508 and bars), 1% (grey diamonds and bars) and 5% CO₂ (dark squares and bars). Values are means ± s.d.
509 (n=3), ANOVA p<0.05. Different superscripts denote significant (p<0.05) differences between
510 samples within each day of cultivation

511

512 **Fig 2:** Nile red fluorescence of 4 day cultures of *T. pseudonana* grown under 0.04% (white), 1%
513 (grey) and 5% CO₂ (dark). Fluorescence is given in Relative Fluorescence Units (RFU). Values are
514 represented as means ± s.d. (n=3), ANOVA p<0.05. Different superscripts denote significant (p<0.05)
515 differences between the samples

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544 **Tables**

545

546 **Table 1.** The division rates (k), biomass concentrations (X) and biomass productivities (BP) of *T.*
 547 *pseudonana* cultivated under different CO₂ concentrations at 4th of cultivation. Values are means ±
 548 s.d. (n=3), ANOVA p<0.05, means in columns for each parameter analysed followed by the same
 549 superscripts are not significantly different at the 5% level according to Tukey's multiple comparison
 550 test.

551

CO ₂ concentration (%)	k (div d ⁻¹)	X (mg _{AFDW} L ⁻¹)	BP (mg _{AFDW} L ⁻¹ d ⁻¹)
0.04	1.13 ± 0.08 ^a	120 ± 0.005 ^a	26 ± 0.001 ^a
1	1.14 ± 0.09 ^a	190 ± 0.010 ^b	44 ± 0.002 ^b
5	1.29 ± 0.08 ^a	210 ± 0.015 ^b	48 ± 0.003 ^b

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555

556 **Table 2.** The pH, dissolved inorganic carbon (TIC), dissolved organic carbon (TOC) and total nitrogen
 557 (TN) concentration in the culture medium of *T. pseudonana* cultivated under different CO₂ concentrations
 558 at 4th of cultivation. Values are means ± s.d. (n=3), ANOVA p<0.05, means in columns for each
 559 parameter analysed followed by the same superscripts are not significantly different at the 5% level
 560 according to Tukey's multiple comparison test.

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CO ₂ concentration (%)	pH	TIC (mg L ⁻¹)	TOC (mg L ⁻¹)	TN (mg L ⁻¹)
0.04	9.36 ± 0.03 ^a	16.83 ± 0.52 ^a	12.25 ± 0.12 ^a	18.84 ± 0.32 ^a
1	7.34 ± 0.03 ^b	36.64 ± 0.38 ^b	12.52 ± 0.39 ^a	16.59 ± 0.38 ^b
5	6.49 ± 0.03 ^c	43.30 ± 0.19 ^c	14.63 ± 0.64 ^b	13.16 ± 0.38 ^c

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570 **Table 3.** Photosynthetic pigments content of *T. pseudonana* cultivated under different CO₂ concentrations
 571 after 4 days of growth. Values are means ± s.d. (n=3), ANOVA p<0.05, means in columns for each
 572 photosynthetic pigment analysed followed by the same superscripts are not significantly different at the
 573 5% level according to Tukey's multiple comparison test.
 574

CO ₂ concentration (%)	Pigment content (µg 10 ⁻⁶ cells)				Chl <i>a</i> /Chl <i>c</i>	Chl tot/Car
	Chl <i>a</i>	Chl <i>c</i>	Car	Chl tot		
0.04	0.86 ± 0.09 ^a	0.14 ± 0.02 ^a	0.42 ± 0.05 ^a	1.00 ± 0.11 ^a	4.76 ± 0.69 ^a	1.58 ± 0.02 ^a
1	1.10 ± 0.04 ^b	0.16 ± 0.01 ^a	0.51 ± 0.02 ^b	1.26 ± 0.05 ^b	4.74 ± 0.21 ^a	1.64 ± 0.05 ^a
5	1.52 ± 0.05 ^c	0.22 ± 0.02 ^b	0.69 ± 0.02 ^c	1.74 ± 0.07 ^c	4.62 ± 0.37 ^a	1.68 ± 0.02 ^a

575
 576
 577 **Table 4.** Lipid content, Nile red fluorescence and fatty acid composition (each value represents the mean
 578 ± SD of two replicates) of *T. pseudonana* cultured at ambient air (0.04% CO₂) and air enriched with 1 or
 579 5 % CO₂.

	0.04% CO ₂	1% CO ₂	5% CO ₂
Fatty acid			
C14:0	21.4 ± 0.09	15.1 ± 0.06	14.3 ± 0.11
C15:0	1.8 ± 0.12	1.4 ± 0.04	1.2 ± 0.07
C16:0	37.1 ± 0.29	34.8 ± 0.22	36.4 ± 0.25
C16:1	23.1 ± 0.13	29.2 ± 0.28	28.4 ± 0.08
C16:2	1.9 ± 0.04	0.8 ± 0.06	0.7 ± 0.04
C16:3	2.6 ± 0.06	0.7 ± 0.02	0.6 ± 0.02
C18:0	ND*	1.0 ± 0.04	0.9 ± 0.03
C18:1	3.2 ± 0.15	8.0 ± 0.12	10.2 ± 0.17
C18:4	1.4 ± 0.10	ND	ND
C20:5	5.3 ± 0.26	4.5 ± 0.05	2.2 ± 0.09
C22:1	2.2 ± 0.06	2.9 ± 0.13	3.5 ± 0.33
C22:2	ND	1.5 ± 0.08	1.6 ± 0.20
Saturated	60.4	52.4	52.8
Mono-unsaturated	28.5	40.2	42.1
Poly-unsaturated	11.2	7.5	5.1
Lipid content (% AFDW)	7.3	14.1	16.9
Nile Red fluorescence (RFU) at Abs ₇₅₀ = 0.06	422	911	1051

580 *ND, not detected

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1 **Effect of different CO₂ concentrations on biomass, pigment content and lipid production of the**
2 **marine diatom *Thalassiosira pseudonana***

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19 **1 Abstract**

20 The marine diatom *Thalassiosira pseudonana* grown under air (0.04% CO₂), 1 and 5% CO₂
21 concentrations was evaluated to determine its potential for CO₂ mitigation coupled with biodiesel
22 production. Results indicated that the diatom cultures grown at 1 and 5% CO₂ showed higher growth rates
23 (1.14 and 1.29 div d⁻¹, respectively) and biomass productivities (44 and 48 mg_{AFDW}L⁻¹d⁻¹) than air grown
24 cultures (with 1.13 div d⁻¹ and 26 mg_{AFDW}L⁻¹d⁻¹). The increase of CO₂ resulted in higher cell volume and
25 pigment content per cell of *T. pseudonana*. Interestingly, lipid content doubled when air was enriched
26 with 1-5% CO₂. Moreover, the analysis of the fatty acid composition of *T. pseudonana* revealed the
27 predominance of monounsaturated acids (palmitoleic-16:1 and oleic-18:1) and a decrease of the saturated
28 myristic acid-14:0 and polyunsaturated fatty acids under high CO₂ levels. These results suggested that *T.*
29 *pseudonana* seems to be an ideal candidate for biodiesel production using flue gases.

30 **13 Keywords**

31 **14 Microalgae;**

32 **15 CO₂ concentrations;**

33 **16 Biomass productivity;**

34 **17 Fatty acid composition;**

35 **18 Biodiesel production.**

19 Introduction

20 The global energy supply is drastically rising due to the continuous increase of modern industrialization.
21 At the present, 90% of global energy demand is generated from fossil fuels and only 10% is fulfilled by
22 renewable energy sources (Maity et al. 2014; Shen 2014). Based on the current projections, the total
23 energy consumption is foreseen to increase year by year and by the year 2050, most of the fossil fuel
24 reserves will be completely exhausted (Maity et al. 2014; Shen 2014). Besides this, the anthropogenic
25 carbon dioxide (CO₂) emissions mainly come from the intensive burning of fossil fuels has contributed to
26 a 40% increase in the atmospheric CO₂ levels, from 280 ppm in pre-industrial age to 400 ppm currently
27 (Shen 2014; Cheah et al. 2015). Carbon dioxide, the principal greenhouse gas, is now widely considered
28 as the major responsible of the global warming (Shen 2014; Cheah et al. 2015, Mondal et al. 2017). As a
29 consequence of this global phenomenon, extensive studies have been focused on the development of
30 renewable, carbon-neutral feedstock to displace petroleum and to mitigate anthropogenic emissions of
31 CO₂ (Maity et al. 2014; Shen 2014, Singh et al. 2016, Mondal et al. 2017). In this perspective, microalgae
32 have received much attention as renewable, energy-saving, sustainable approach to reduce the CO₂
33 emissions and simultaneously to produce biodiesel (Chisti 2007; Lam et al. 2012; Maity et al. 2014;
34 Trobajo et al. 2014, Singh et al. 2016, Mondal et al. 2017). Microalgae have faster growth rates, higher
35 photosynthetic efficiencies, higher rates of carbon dioxide fixation, and higher biomass and lipid
36 productivities as compared to conventional oil crop plants (Chisti 2007; Sabia et al. 2015; Baldissarotto et
37 al. 2016). Currently, extensive researches have been concerned the choice of the most adequate
38 microalgal strain that could tolerate elevated CO₂ concentrations and, simultaneously, produce lipids
39 suitable for biodiesel production (Lam et al. 2012; Trobajo et al. 2014; Nascimento et al. 2015). However,
40 most of these microalgal strains studied for this purpose are freshwater species (Tang et al. 2011; Lam et
41 al. 2012). With the aim of making the microalgal large-scale production increasingly sustainable, it is
42 necessary to identify a species that can grow in saltwater to avoid the competition with freshwater
43 resources (Doan et al. 2011; Baldissarotto et al. 2012). Marine diatoms may be considered as an attractive
44 feedstock for combined CO₂ fixation and lipid production (Hildebrand et al. 2012; Wang et al. 2014;
45 Vinayak et al. 2015, Manju et al. 2017). Diatoms, due to their high trophic flexibility, are considered the
46 most productive microalgae on the planet (Field et al. 1998; Hildebrand et al. 2012). These
47 microorganisms, which are the main component of phytoplankton, significantly contribute to the global
48 primary productivity and marine geochemical cycles (Field et al. 1998). Moreover, they are excellent
49 lipid accumulators, especially triacylglycerides (TAGs), which are synthesized and accumulated in the
50 stationary phase of growth (Hildebrand et al. 2012; d'Ippolito et al. 2015; Vinayak et al. 2015, Manju et
51 al. 2017). It is well known that TAGs represent the best substrate for the biodiesel production (Chisti et al.
52 2007; Baldissarotto et al. 2016, Zienkiewicz et al. 2016). Nevertheless, only few studies have valorised
53 the diatom lipid profile for biodiesel (Francisco et al. 2010; Zendejas et al. 2012; Popovich et al. 2012,
54 Manju et al. 2017). *Thalassiosira pseudonana*, a model diatom species, which genome has been recently
55 sequenced (Armbrust et al. 2004), is one of the most studied species for the production of biofuels up to
56 now (Yu et al. 2009; Zendejas et al. 2012; d'Ippolito et al. 2015). In addition, recent studies have been
57 concerned the effect of elevated CO₂ concentrations on growth and photophysiology of this diatom

species (Crawford et al. 2011; Yang and Gao 2012; McCarthy et al. 2012; Li and Campbell 2013).
The present work is focused on the potential of the marine diatom *Thalassiosira pseudonana* as a promising strain for the cultivation in the presence of elevated CO₂ concentrations. In particular, algal growth, cell morphology, photosynthetic pigment content, total nitrogen (TN), and dissolved inorganic (TIC) and organic carbon (TOC) content were monitored under increasing CO₂ concentrations (0.04 (ambient air), 1 and 5%). The lipid content and profile was also estimated in order to evaluate the effects of elevated CO₂ concentration on the composition of fatty acids useful for biodiesel production.

Materials and Methods

Algal strain and culture condition

The strain used in this study was the marine centric diatom *Thalassiosira pseudonana* (Hustedt) Hasle and Heimdal CCAP 1085/12 (*Heterokontophyta*, *Thalassiosirales*, *Thalassiosiraceae*), obtained from the Culture Collection of Algae and Protozoa (CCAP; Scotland, United Kingdom; www.ccap.ac.uk).

T. pseudonana was inoculated in 6-L Erlenmeyer flasks with a 4-L working volume of filtered (0.45 µm) autoclaved seawater enriched with *f/2* nutrients (Guillard and Ryther 1962) at 25±2°C and under an exposure to an irradiance of 250 µmol_{photons} m⁻² s⁻¹ provided by cool white fluorescent lamps (OSRAM L30W/865 Lumilux) under a cycle period of 16:8 h light-darkness. The *T. pseudonana* cultures were aerated at a rate of 250 mL min⁻¹ (i.e., 0.0625 vvm, volume gas per volume media per min) of air enriched with CO₂ to obtain final concentration of 0.04, 1 and 5% CO₂. The air-CO₂ mixtures were obtained by combining rotameter flows of air and pure CO₂. The percentage of CO₂ in the air-CO₂ mixture was verified with an infrared analyzer (Servomex 4900C1). Experiments were performed in triplicate. Aliquots of cultures were collected at different times of cultivation, depending on the analysis.

Analyses

Growth, cell size and biomass evaluation

Algal cells were counted daily using an Improved Neubauer hemocytometer under a light microscope (Zeiss, model Axioscope A1) equipped with DIC (Differential Interference Contrast) optics in order to estimate the division rates (k, number of divisions per day). Cell density (cell mL⁻¹) was also measured by the absorbance at 750 nm (A₇₅₀) in 96 well plates with the microplate reader (Infinite Tecan M200Pro). Absorbance measures were converted to optical density by dividing absorbance values by the pathlength following Beer-Lambert's law. Cell size measurements were done with the microscope described above and measured with the aid of the software (ProgRes Capture Pro v. 2.8) and a digital camera (Jenoptik ProgRes Speed XT Core5).

The division rates (k, number of divisions per day) during the exponential phase was calculated with the following equation:

$$k \text{ (div d}^{-1}\text{)} = (\log_2 N_t - \log_2 N_0) / (t_t - t_0),$$

where k is the division rate, N_t the cell number at time t_t, N₀ the cell number at time 0 and t_t-t₀ the time

1 96 interval (days) (Andersen 2005). Algal biomass concentration, expressed as ash free dry weight (AFDW)
2 97 was measured according to the methods as described by Zhu and Lee (1997), with some modifications. In
3 98 detail, after the filtration of culture replicates (30-50 mL) (onto tare GF/F Whatman filters which had
4 99 been previously precombusted), the filtered samples were washed with sodium chloride to remove
5 100 adhering salts and dried in the oven for 72 h at 60°C. Subsequently, the filters were placed in a muffle
6 101 furnace for 4 h at 550°C and re-weighed. AFDW corresponds to the difference of the filter weight after
7 102 the oven treatment minus the filter weight after the muffle.

8 103 The biomass productivity ($\text{mg}_{\text{AFDW}} \text{L}^{-1} \text{d}^{-1}$) was calculated from the variation in the biomass concentration
9 104 ($\text{mg}_{\text{AFDW}} \text{L}^{-1}$) within a specific cultivation time (d) according to the following equation:

10 105
$$P (\text{mg}_{\text{AFDW}} \text{L}^{-1} \text{d}^{-1}) = (X_1 - X_0) / (t_1 - t_0),$$

11 106 where P is the biomass productivity, X_1 the biomass concentration at time t_1 , X_0 the biomass
12 107 concentration at time 0 and $t_1 - t_0$ the time interval (days) (Hempel et al. 2012).

13 108

14 109 *Photosynthetic pigment extraction and quantification*

15 110 For photosynthetic pigment analysis, cell samples were collected by centrifugation and the extraction was
16 111 performed according to Giovanardi et al. (2014). The extracts were measured with the same microplate
17 112 reader, already described, at 665 (Chl*a*), 632 (Chl*c*) and 480 nm (Car). Quantification of Chls was
18 113 performed according to equations reported in Ritchie (2006), whereas Car quantification according to
19 114 equations reported in Wellburn (1994).

20 115

21 116 *Biochemical analyses*

22 117 During the growth period, the pH in culture replicates was monitored daily using a pH meter (Crison
23 118 GLP21). The total nitrogen (TN), the dissolved inorganic (TIC) and dissolved organic carbon (TOC)
24 119 concentrations in the culture medium were measured by a Multi N/C 3100 Analytic Jena analyser. 20 mL
25 120 of the algal cultures were centrifuged at 4000 rpm for 15 min and the supernatant was used for TIC, TOC
26 121 and TN measurements.

27 122

28 123 *Characterization of neutral lipids with Nile red*

29 124 Cell lipid content was estimated by neutral lipid staining with the fluorochrome Nile Red at the 4th day of
30 125 cultivation, when all the microalgal cultures had reached the stationary phase of growth, according to
31 126 Chen et al. (2009). First, algal samples were diluted to attain a final cell concentration of 0.06 (A_{750}).
32 127 After the algal suspension were stained with Nile Red (9-diethylamina-5Hbenzo[α]phenoxazine-5-one,
33 128 0.5 μg dissolved in 100 mL acetone) and 25% of absolute dimethyl sulphoxide (DMSO). Fluorescence
34 129 was measured with the microplate reader, already described, at excitation and emission wavelengths of
35 130 530 nm and 580 nm, respectively. The relative fluorescence was obtained by subtraction of the
36 131 autofluorescence of non-stained cells and self-fluorescence of the fluorochrome. After staining, cells were
37 132 also observed with a Zeiss AxioObserver Z1 equipped with epifluorescence. A metal halide lamp was
38 133 used as the fluorescence excitation source (Lumen 200Pro, Prior Scientific), and a filter cube containing a

1 134 450-490 nm excitation and a 510-515 nm collection filter was used for light filtration.

2 135

3
4 136 *Lipid extraction*

5
6 137 For fatty acids analysis, the total final algal cultures were harvested by flocculation with NaOH 2M at the
7 138 end of the cultivation period, according to Şirin et al. (2015). The settled down volume was further
8
9 139 centrifuged (3000 rpm, 5min) to obtain a more reduced volume. Subsequently, the microalgal pellets
10 140 were frozen (-80°C) and lyophilized drying in Telstar Lyoquest-85 freeze dryer for dry weight
11 141 measurement and fatty acids characterization.

12
13 142 Lipids were extracted from the microalgal samples with the Bligh and Dyer method (Bligh and Dyer
14 143 1959), which uses a ternary system of chloroform/methanol/water and is the most commonly used
15 144 method for the quantitative extraction of lipids from microalgae at analytical level. Briefly, 2g of sample
16 145 were dissolved in 20 mL of water and then were mixed with 75 mL of a mixture chloroform-methanol
17 146 (1:2 v/v) using a magnetic stirrer at 300 rpm for 10 min. Then 25 mL of chloroform and 25 mL of
18 147 distilled water were added to form a two-phase system. The phases were separated by 5 min
19 148 centrifugation at 3500 rpm. The chloroform phase was then separated and the amount of lipid obtained
20 149 was gravimetrically measured after drying overnight in an oven at 70 °C.

21 150

22 151 *Methyl ester derivation and fatty acid analysis*

23
24 152 A two-step protocol was used for the methylation process of all extracted lipids, according to Lee et al
25 153 (2010). The samples were saponified with 5 mL of 2% KOH in methanol at 75 °C for 10 min, and then
26 154 subjected to methanolysis with 5 mL of 5% HCl in methanol at 75 °C for another 10 min. Thereafter, the
27 155 phase containing the fatty acids was separated by adding 10 mL of distilled water and then recovered with
28 156 5 mL of heptane. The obtained fatty acid methyl esters (FAMES) were analyzed according to EN 14103,
29 157 with an Agilent 6850 gas chromatograph equipped with a split/splitless inlet and a FID detector. The
30 158 column used was a HP-INNOWax (polyethylene glycol phase), a capillary column with a high polarity
31 159 and dimensions 30 m x 0.32 mm x 0.25 µm. Quantification of total FAMES was accomplished by using
32 160 FAME C17:0 as internal standard. Identification of individual FAME components was based on
33 161 comparison of their retention times and fragmentation patterns with those for standards. The FAME
34 162 composition was calculated as the percentage of the total identified esters present in the sample.

35 163

36 164 **Data treatment**

37 165 Data were processed with Graphpad Prism 6 (Graph Pad Software, San Diego CA, USA). In each case,
38 166 means ± standard deviations for *n* number of samples are given.

39 167 The statistical significance of differences was determined by one-way ANOVA followed by a multiple
40 168 comparison test (Tukey's test). A significance level of 95% ($p < 0.05$) was accepted.

41 169

42 170

43 171 **Results**

172 *Effect of different CO₂ concentrations on T. pseudonana growth*

173 In this study, as shown in Fig. 1a, the diatom *T. pseudonana* cultivated with 0.04 (ambient air), 1 and 5%
174 CO₂ promptly entered the exponential phase and reached the stationary phase of growth during 4 days of
175 cultivation. However, it was apparent from Table 1 that the highest CO₂ concentration increased the
176 division rates of the diatom as compared to cells cultivated with 0.04 and 1% CO₂. Interestingly, at the
177 end of the experiment, the biomass concentrations of cells cultivated with 1 and 5% CO₂ were 190 and
178 210 mg L⁻¹ respectively, which were 1.58 and 1.75 times higher than that of air grown cultures (Table 1).
179 Further, observations on diatom cell size during the experiment showed remarkable changes in response
180 to the CO₂ treatments. In detail, a gradual but evident increasing trend was observed in cells cultivated
181 with 1 and 5% CO₂ until the end of the experiment (Fig. 1b). Conversely, the cell volume of air grown
182 cultures remained almost stable during the cultivation period. At 4th day of cultivation, the cell volume
183 achieved by both cultures enriched with CO₂ was found 2 times higher than that of cell cultivated with
184 0.04% CO₂ (Fig. 1b). Therefore, these results reflected the higher biomass productivities achieved by
185 cultures grown at 1 and 5% CO₂ (69 and 84%, respectively) than 0.04% CO₂-treated cells (Table 1). As
186 shown in Table 2, with the increase of CO₂ concentrations from 0.04% to 5%, the TIC concentration in
187 the culture medium increased from 16.83 mg L⁻¹ to 43.30 mg L⁻¹ (+117 and 157% higher than air grown
188 cultures in cells cultivated with 1% and 5% CO₂, respectively). In fact, despite, no great differences were
189 reported between 0.04% and 1% of CO₂ treatments regarding TOC concentrations, the maximum TOC
190 values in the culture medium were found under 5% CO₂ (14.63 mg L⁻¹, 19 and 17% higher than 0.04%
191 and 1% CO₂-treated cells, respectively). On the other hand, higher CO₂ concentrations always resulted in
192 lower pH. As shown in Table 2, the pH value of the culture medium decreased from pH 9.4 to pH 6.5
193 with the increase of CO₂ concentrations from 0.04% to 5%.
194 Further, the TN concentration in the culture medium decreased by 12% and 30% in cells cultivated with
195 1% and 5% CO₂, respectively (Tab. 2).

197 *Effect of different CO₂ concentrations on T. pseudonana photosynthetic pigment content*

198 In order to evaluate if the cultivation in presence of elevated CO₂ concentrations could also affect the
199 photosynthetic activity of the diatom, the photosynthetic pigment content were also analysed at the end of
200 the cultivation period. The increase of CO₂ concentrations resulted in significant increases in Chl_a and
201 Car content as compared to those of air grown cultures (Table 3). In detail, Chl_a concentrations in cells
202 cultivated with 1% and 5% CO₂ were higher (28 and 77%, respectively) than 0.04% air grown cultures.
203 Despite, no great differences were reported between 0.04% and 1% of CO₂ treatments about Chl_c content,
204 the highest Chl_c values were found in cells cultivated with 5% CO₂ (57 and 37% higher than air and 1%
205 CO₂ grown cultures. About Car content, cells cultivated with 0.04% CO₂ contained lower quantities (21
206 and 64%, respectively) as compared to cultures enriched with elevated CO₂. The increases in total Chl
207 content were greater than differences in the Car content, resulting in higher total Chl to Car molar ratios
208 in cultures enriched with elevated CO₂ as compared to air grown cultures, but the ratios did not differ
209 significantly at the 95% level. No significant effects on Chl_a to *c* molar ratios were observed between the

1 210 samples (Table 3).
2 211
3
4 212 *Effect of different CO₂ concentrations on T. pseudonana lipid quantification and characterisation*
5
6 213 In this study, to find a possible relation between CO₂ treatments and lipid synthesis, measurements of
7 214 neutral lipid accumulation were performed by spectrofluorimetric quantification of the relative
8 215 fluorescence intensity emitted by Nile Red-stained cells. After staining, cells were also observed by
9 216 fluorescence microscopy. In detail, while cells cultivated with 0.04% CO₂ showed slightly positive
10 217 reaction, the cultures enriched with elevated CO₂ accumulated abundant lipid droplets, giving an intense
11 218 positive reaction with the fluorochrome (data not shown). Accordingly, in this study, as revealed by Nile
12 219 Red staining, when 1 and 5% CO₂-treated cells were in stationary phase (4th day of cultivation) showed a
13 220 3-fold increase in lipid content with respect to air grown cultures (Fig. 2) and coincidentally, decreased
14 221 significantly the TN concentrations in culture medium (Table 2).
15 222 Nile red staining results were confirmed by Bligh and Dyer lipid extraction. Fluorescence values,
16 223 normalized to the same cell concentration ($A_{750} = 0.06$), were proportional to the lipid content as %
17 224 AFDW obtained with Bligh and Dyer extraction. The percentage of lipids in AFDW doubled when air
18 225 was enriched with 1-5 % CO₂ from 7.3% (air) to 14.1% (1%) and 16.9% (5%) of CO₂ (Table 4).
19 226 The main fatty acid components of *T. pseudonana* under different CO₂ concentrations were determined
20 227 by GC-MS analysis and are shown in Table 4. The predominant fatty acids were mirystic (C14:0),
21 228 palmitic (C16:0), palmitoleic (C16:1), oleic (C18:1) acids (Table 4). Other fatty acids (C15:0, C16:2,
22 229 C16:3, C18:0, C18:4, C20:5, C22:1 and C22:2) were minor components and presented concentration
23 230 individually of less than 5%. However, the lipid classes showed differences in their proportions in
24 231 response to the CO₂ treatments (Table 4). Despite, no great differences were reported between 1%
25 232 (54.4%) and 5% (52.8%) of CO₂ treatments about the percentages of saturated fatty acids (SFAs), the
26 233 highest SFAs values were found in cells cultivated with air (60.4%). The monounsaturated fatty acids
27 234 (MUFAs) were the major class of fatty acids in cells cultivated with 1% and 5% CO₂. In detail, MUFAs
28 235 in these cells under these CO₂ treatments account for more than 91% of the total fatty acids. They were
29 236 significantly higher ($p < 0.05$) than 0.04% air grown cultures, reaching the average value (*ca.* 41%) under
30 237 higher CO₂ treatments due to a high content of palmitoleic (*ca.* 29%) and oleic acids (*ca.* 9%) (Table 4).
31 238 Regarding the polyunsaturated fatty acids (PUFAs), there was a significant decrease in 1 and 5% CO₂-
32 239 treated cells compared to air grown cultures. In particular, PUFA levels decreased significantly due to the
33 240 decline in the proportion of eicosapentaenoic acid, from 5.3% (air) to 4.5% (1%) and 2.2% (5%) of CO₂
34 241 (Table 4).
35 242 **Discussion**
36 243 This study clearly indicates that the marine diatom *T. pseudonana* could tolerate elevated CO₂
37 244 concentrations and simultaneously, produce lipids suitable for biodiesel production. In spite of extensive
38 245 studies performed on this species, there is still no information on the possible effects of increasing CO₂
39 246 concentrations on lipid accumulation, which, if demonstrated, could allow a significant advancement for
40 247 the biotechnological use of this alga. A recent review summarized how elevated or high CO₂

1 248 concentrations could affected the diatoms growth and physiology (Gao and Campbell 2014). Some
2 249 Authors reported that they could have positive effects on diatoms growth (Wu et al. 2010; McCarthy et al.
3 250 2012; Wang et al. 2014), while others observed neutral (Crawfurd et al. 2011; Gao et al. 2012) or negative
4 251 effects (Torstensson et al. 2012; Mejia et al. 2013). However, these conflicting results could be attributed
5 252 to the varied experimental conditions such as the exposure to different light intensities (Li and Campbell
6 253 2013), pH (Wu et al. 2010), temperature (Torstensson et al. 2012), but also to the physiological
7 254 complexity of algal responses to elevated CO₂ concentrations (Wu et al. 2010; Yang and Gao 2012). In
8 255 this study, the addition of 5% CO₂ increased the diatom growth rates by 15% and biomass concentration
9 256 by 75%. This was in agreement with such literature data, which reported a positive effect of elevated CO₂
10 257 concentrations on *T. pseudonana* growth (Yang and Gao 2012; McCarthy et al. 2012; Li and Campbell
11 258 2013). Interestingly, observations on diatom cell size during the experiment showed remarkable changes
12 259 in response to the CO₂ treatments. At the end of the experiment, the cell volume achieved by both
13 260 cultures enriched with CO₂ was found 2 times higher than that of cell cultivated with 0.04% CO₂. These
14 261 results are coherent with previous studies, which found an evident increase in cell size in centric diatom
15 262 cultivated under high CO₂ levels (Hoogstraten et al. 2012; Li and Campbell 2013).
16 263 The dissolved inorganic carbon (TIC) in culture medium represents the carbon sources for the microalgal
17 264 growth. As expected, the cultivation of *T. pseudonana* under increasing CO₂ concentrations resulted in
18 265 high TIC values (Tang et al. 2011). Moreover, in this study, the cultivation with the highest CO₂ resulted
19 266 also in higher TOC concentrations in culture medium, which could be attributed to the frequently
20 267 extracellular release of algal exudates during microalgal growth (Engel 2002; Hulatt and Thomas 2010).
21 268 It has long been known that the inorganic carbon system is closely related to the pH, as it is the main
22 269 buffering system in the culture medium. In this study, the higher CO₂ concentrations tested have led to
23 270 higher diatom biomass productivities as compared to 0.04% air grown cultures, but also caused a
24 271 decrease in the pH. Low pH values could be attributed to the buffering properties of the carbonate system
25 272 (Wu et al. 2010; Yang and Gao 2012; Gao and Campbell 2014). In order to evaluate if the cultivation in
26 273 presence of elevated CO₂ concentrations could also affect the photosynthetic activity of the diatom, the
27 274 photosynthetic pigment content were also analysed at the end of the cultivation period. The overall higher
28 275 pigment content of cells of *T. pseudonana* grown under increasing CO₂ concentrations as compared to
29 276 0.04% air grown cultures was more related to photoacclimation of cultures or as a consequence of the
30 277 increase of cell volume rather than increase in CO₂ availability (Crawfurd et al. 2011; Baldisserotto et al.
31 278 2012; Li and Campbell 2013). In fact, no significant effects on pigment content of this diatom species
32 279 were observed after a long period of adaptation to elevated CO₂ concentrations (Crawfurd et al. 2011;
33 280 McCarthy et al. 2012; Li and Campbell 2013).
34 281 The effects of elevated CO₂ concentrations on fatty acids composition and content of microalgae have
35 282 been already reported (Muradyan et al. 2004; Tang et al. 2011; Trobajo et al. 2014; Wang et al. 2014).
36 283 Nile Red fluorescence has been widely accepted as a valid method for analyzing TAGs in algal cultures
37 284 (Yu et al. 2009; Giovanardi et al. 2014; Sabia et al. 2015; Baldisserotto et al. 2016). Accordingly, in this
38 285 study, as revealed by Nile Red staining, when 1 and 5% CO₂-treated cells were in stationary phase (4th
39 286 day of cultivation) showed a 3-fold increase in lipid content with respect to air grown cultures (Fig. 2).

1 287 The kinetics of lipid production in each CO₂ condition was not monitored and hence the 4th day of
2 288 cultivation may not correspond to the maximum lipid content in any treatment. Nevertheless, the
3 289 difference in lipid content between CO₂ enriched cultures and air grown cultures was remarkable.
4 290 On the other hand, CO₂ enriched cultures also presented lower TN concentrations in culture medium
5 291 (Table 2).
6 292 Several studies showed that diatoms accumulated lipids as a result of N limitation and increased the
7 293 proportion of TAGs in the stationary phase of growth (Yu et al. 2009; Hildebrand et al. 2012; d'Ippolito
8 294 et al. 2015).
9 295 For biodiesel purposes, fatty acid profiles rich in monounsaturated acids (MUFAs), which can be
10 296 transesterified to produce biodiesel (Hu al. 2008), are the most interesting profiles. Biodiesel, which is
11 297 produced by the trans-esterification of triglycerides with methanol to yield the corresponding mono-alkyl
12 298 fatty acid esters, is an alternative to petroleum-based diesel fuel (Hu al. 2008). The properties of biodiesel
13 299 are strongly determined by the characteristics of fatty acid chains, such as carbon chain length and
14 300 unsaturation extent, present in the fuel (Hu et al. 2008; Nascimento et al. 2015, Manju et al. 2017). For
15 301 example, saturated chains produce a biodiesel with superior oxidative stability and a higher cetane index
16 302 but rather poor low-temperature properties. In contrast, polyunsaturated fatty acids have good cold-flow
17 303 properties but are particularly susceptible to oxidation (Hu et al. 2008; Nascimento et al. 2015).
18 304 Interestingly, the increase in CO₂ concentration resulted in an increase of monounsaturated acids
19 305 (palmitoleic and oleic) and a decrease of the saturated myristic acid and polyunsaturated fatty acids
20 306 (Table 4), suggesting that from the point of view of biodiesel production air enriched with CO₂ produces
21 307 better distribution of fatty acid chains. The increase in unsaturated fatty acids and decrease in saturated
22 308 fatty acids has previously been observed in Chlorophyceae species at increasing CO₂ concentrations up to
23 309 5% (Tang 2011; Nascimento et al. 2015). However, this trend did not appear in cultures of the same or
24 310 other species in other works (Tsuzuki 1990; Muradyan et al. 2004). In this study, it is not observed a big
25 311 difference in the fatty acid chain profile attending the use of 1% or 5% CO₂ enriched air (Table 4).
26 312 Despite of this, the evident characteristic of the fatty acid composition of *T. pseudonana* grown under 1-
27 313 5% CO₂ is that the major fatty acids were C14–C18, and these account for more than 91% of the total
28 314 fatty acids. The predominance of shorter-chain fatty acids (defined as alkyl chains with 12–18 carbon
29 315 atoms) is very significant for the potential of *T. pseudonana* for the production of biodiesel (Hu et al.
30 316 2008).
31 317 It has been reported that an increase in CO₂ concentration in culture medium tends to increase the
32 318 accumulation of polyunsaturated fatty acids in the microalgal cells (Lam et al. 2012). However, compared
33 319 to previous results (Tang et al. 2011; Wang et al. 2014), *T. pseudonana* showed a decrease of the
34 320 production of eicosapentaenoic (C20:5) acids (C20:5) from 5.3% (air) to 4.5% (1%) and 2.2% (5%) of
35 321 CO₂. Despite, the cultivation with high levels of CO₂ showed a notable reduction of polyunsaturated fatty
36 322 acids, this is still not enough to accomplish the biodiesel standards described in the EN14214 norm.
37 323 The increase of lipid content, the raise of monounsaturated and the reduction of polyunsaturated fatty
38 324 acids when using 5% CO₂ may have great potential for the production of an ignition-quality biofuel with
39 325 cold-flow properties and oxidative stability.

1 326
2 327 In conclusion, results presented here demonstrate that the maximum growth rate, biomass concentration
3 328 and productivity, cell volume and photosynthetic pigment content per cell were obtained when the marine
4 329 diatom *T. pseudonana* was cultivated under 5% CO₂. Moreover, treatments with 5% CO₂ gives an increase
5 330 of total and neutral lipid content, the raise of monounsaturated and the reduction of polyunsaturated fatty
6 331 acids. These results suggest that *T. pseudonana* under 5% CO₂ showed a suitable fatty acid composition
7 332 for the production of biodiesel.
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26 347 **Conflict of interest**

27 348 The authors declare they have no conflict of interest.
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30 349

31 350 **Compliance with Ethical Standards**

32 351 This article does not contain any studies with human participants or animals performed by any of the
33 352 authors
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1 490 **Figure captions**

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4 492 **Fig 1: (a)** Growth curves and **(b)** cell volumes of *T. pseudonana* cultivated under 0.04% (white circles
5 and bars), 1% (grey diamonds and bars) and 5% CO₂ (dark squares and bars). Values are means ± s.d.
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7 494 (n=3), ANOVA p<0.05. Different superscripts denote significant (p<0.05) differences between
8 495 samples within each day of cultivation

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11 497 **Fig 2:** Nile red fluorescence of 4 day cultures of *T. pseudonana* grown under 0.04% (white), 1%
12 (grey) and 5% CO₂ (dark). Fluorescence is given in Relative Fluorescence Units (RFU). Values are
13 498 represented as means ± s.d. (n=3), ANOVA p<0.05. Different superscripts denote significant (p<0.05)
14 499 differences between the samples

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1 529 **Tables**

2 530
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 4 531 **Table 1.** The division rates (k), biomass concentrations (X) and biomass productivities (BP) of *T.*
 5 532 *pseudonana* cultivated under different CO₂ concentrations at 4th of cultivation. Values are means ±
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 7 533 s.d. (n=3), ANOVA p<0.05, means in columns for each parameter analysed followed by the same
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 9 534 superscripts are not significantly different at the 5% level according to Tukey's multiple comparison
 10 535 test.

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CO ₂ concentration (%)	k (div d ⁻¹)	X (mg _{AFDW} L ⁻¹)	BP (mg _{AFDW} L ⁻¹ d ⁻¹)
0.04	1.13 ± 0.08 ^a	120 ± 0.005 ^a	26 ± 0.001 ^a
1	1.14 ± 0.09 ^a	190 ± 0.010 ^b	44 ± 0.002 ^b
5	1.29 ± 0.08 ^a	210 ± 0.015 ^b	48 ± 0.003 ^b

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 26 541 **Table 2.** The pH, dissolved inorganic carbon (TIC), dissolved organic carbon (TOC) and total nitrogen
 27 542 (TN) concentration in the culture medium of *T. pseudonana* cultivated under different CO₂ concentrations
 28 543 at 4th of cultivation. Values are means ± s.d. (n=3), ANOVA p<0.05, means in columns for each
 29 544 parameter analysed followed by the same superscripts are not significantly different at the 5% level
 30 545 according to Tukey's multiple comparison test.

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CO ₂ concentration (%)	pH	TIC (mg L ⁻¹)	TOC (mg L ⁻¹)	TN (mg L ⁻¹)
0.04	9.36 ± 0.03 ^a	16.83 ± 0.52 ^a	12.25 ± 0.12 ^a	18.84 ± 0.32 ^a
1	7.34 ± 0.03 ^b	36.64 ± 0.38 ^b	12.52 ± 0.39 ^a	16.59 ± 0.38 ^b
5	6.49 ± 0.03 ^c	43.30 ± 0.19 ^c	14.63 ± 0.64 ^b	13.16 ± 0.38 ^c

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Table 3. Photosynthetic pigments content of *T. pseudonana* cultivated under different CO₂ concentrations after 4 days of growth. Values are means ± s.d. (n=3), ANOVA p<0.05, means in columns for each photosynthetic pigment analysed followed by the same superscripts are not significantly different at the 5% level according to Tukey's multiple comparison test.

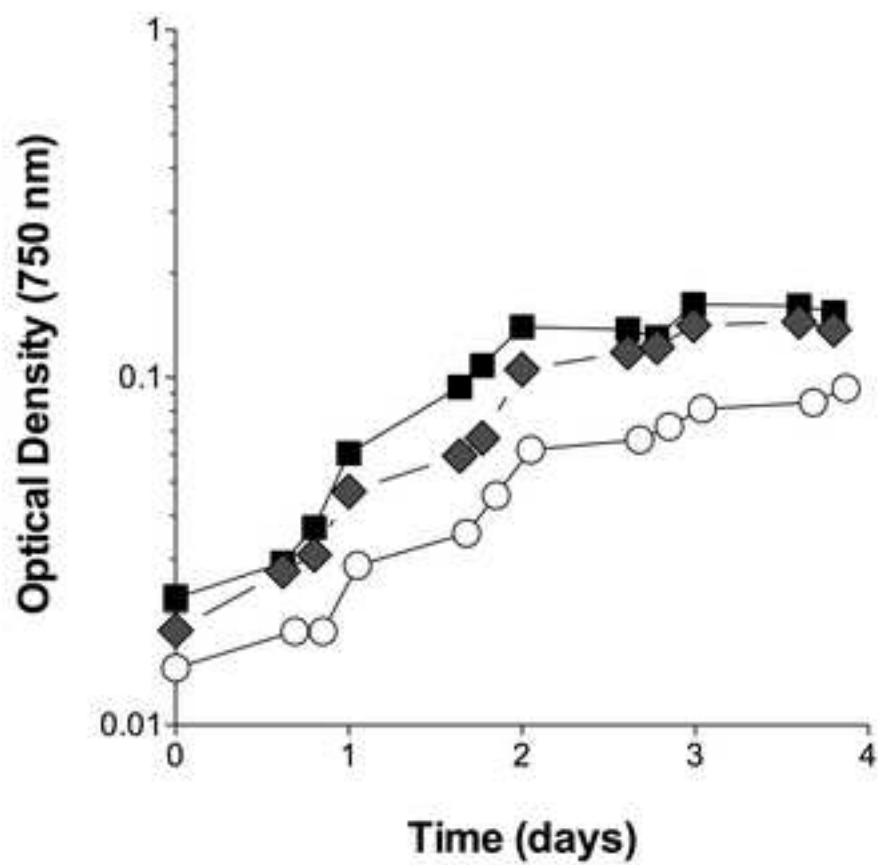
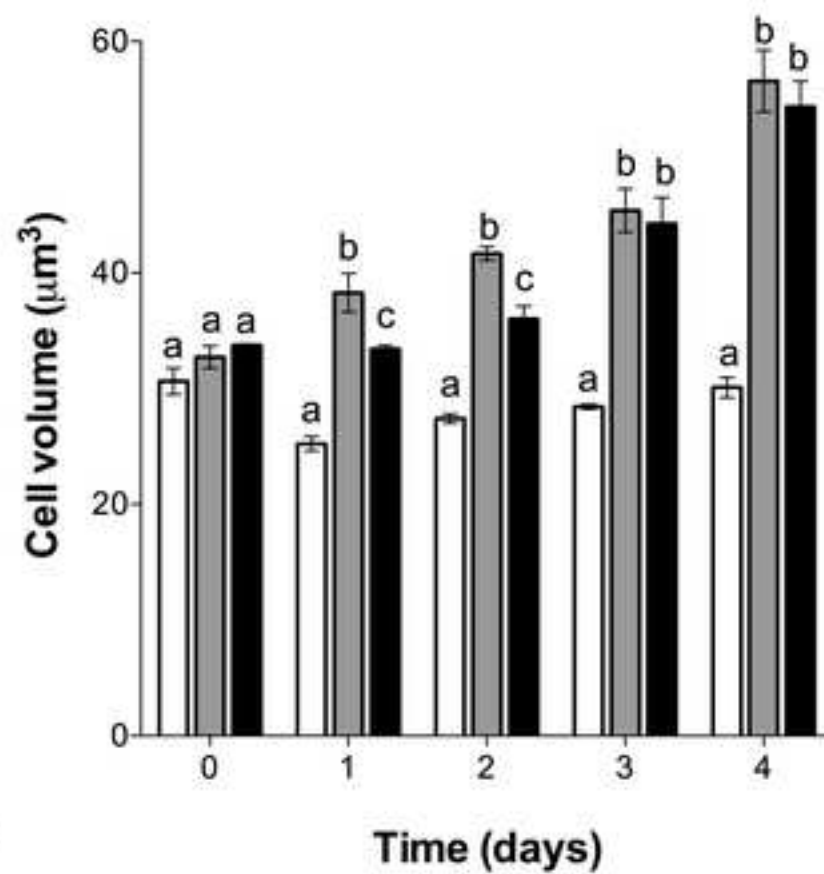
CO ₂ concentration (%)	Pigment content (µg 10 ⁻⁶ cells)				Chla /Chlc	Chl tot/Car
	Chla	Chlc	Car	Chl tot		
0.04	0.86 ± 0.09 ^a	0.14 ± 0.02 ^a	0.42 ± 0.05 ^a	1.00 ± 0.11 ^a	4.76 ± 0.69 ^a	1.58 ± 0.02 ^a
1	1.10 ± 0.04 ^b	0.16 ± 0.01 ^a	0.51 ± 0.02 ^b	1.26 ± 0.05 ^b	4.74 ± 0.21 ^a	1.64 ± 0.05 ^a
5	1.52 ± 0.05 ^c	0.22 ± 0.02 ^b	0.69 ± 0.02 ^c	1.74 ± 0.07 ^c	4.62 ± 0.37 ^a	1.68 ± 0.02 ^a

Table 4. Lipid content, Nile red fluorescence and fatty acid composition (each value represents the mean ± SD of two replicates) of *T. pseudonana* cultured at ambient air (0.04% CO₂) and air enriched with 1 or 5 % CO₂.

	0.04% CO ₂	1% CO ₂	5% CO ₂
Fatty acid			
C14:0	21.4 ± 0.09	15.1 ± 0.06	14.3 ± 0.11
C15:0	1.8 ± 0.12	1.4 ± 0.04	1.2 ± 0.07
C16:0	37.1 ± 0.29	34.8 ± 0.22	36.4 ± 0.25
C16:1	23.1 ± 0.13	29.2 ± 0.28	28.4 ± 0.08
C16:2	1.9 ± 0.04	0.8 ± 0.06	0.7 ± 0.04
C16:3	2.6 ± 0.06	0.7 ± 0.02	0.6 ± 0.02
C18:0	ND*	1.0 ± 0.04	0.9 ± 0.03
C18:1	3.2 ± 0.15	8.0 ± 0.12	10.2 ± 0.17
C18:4	1.4 ± 0.10	ND	ND
C20:5	5.3 ± 0.26	4.5 ± 0.05	2.2 ± 0.09
C22:1	2.2 ± 0.06	2.9 ± 0.13	3.5 ± 0.33
C22:2	ND	1.5 ± 0.08	1.6 ± 0.20
Saturated	60.4	52.4	52.8
Mono-unsaturated	28.5	40.2	42.1
Poly-unsaturated	11.2	7.5	5.1
Lipid content (% AFDW)	7.3	14.1	16.9
Nile Red fluorescence (RFU) at Abs ₇₅₀ = 0.06	422	911	1051

*ND, not detected

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