

Applied Microbiology and Biotechnology

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

--Manuscript Draft--

Manuscript Number:	AMAB-D-17-02373R1													
Full Title:	Effect of different CO ₂ concentrations on biomass, pigment content and lipid production of the marine diatom <i>Thalassiosira pseudonana</i>													
Article Type:	Original Article													
Section/Category:	Applied microbial and cell physiology													
Corresponding Author:	Simonetta Pancaldi Università degli Studi di Ferrara ITALY													
Corresponding Author Secondary Information:														
Corresponding Author's Institution:	Università degli Studi di Ferrara													
Corresponding Author's Secondary Institution:														
First Author:	Alessandra Sabia													
First Author Secondary Information:														
Order of Authors:	Alessandra Sabia Esther Clavero Simonetta Pancaldi Joan Salvadò Rovira													
Order of Authors Secondary Information:														
Funding Information:	<table border="1"><tr><td>University of Ferrara (Italy) (Bando Giovani Ricercatori 2015)</td><td>Dr Alessandra Sabia</td></tr><tr><td>University Institute of High Studies (IUSS)-1931 of Ferrara</td><td>Dr Alessandra Sabia</td></tr><tr><td>Spanish Ministry of Economy and Competitiveness (CTQ2014-56285-R)</td><td>Prof Joan Salvadò Rovira</td></tr><tr><td>Excma. Diputació de Tarragona (Fuels from Biomass)</td><td>Prof Joan Salvadò Rovira</td></tr><tr><td>European Regional Development Funds (Programa Competitividad de Catalunya 2007-2013)</td><td>Prof Joan Salvadò Rovira</td></tr><tr><td>Università degli Studi di Ferrara (Fondo per l'incentivazione alla Ricerca 2016)</td><td>Prof Simonetta Pancaldi</td></tr></table>		University of Ferrara (Italy) (Bando Giovani Ricercatori 2015)	Dr Alessandra Sabia	University Institute of High Studies (IUSS)-1931 of Ferrara	Dr Alessandra Sabia	Spanish Ministry of Economy and Competitiveness (CTQ2014-56285-R)	Prof Joan Salvadò Rovira	Excma. Diputació de Tarragona (Fuels from Biomass)	Prof Joan Salvadò Rovira	European Regional Development Funds (Programa Competitividad de Catalunya 2007-2013)	Prof Joan Salvadò Rovira	Università degli Studi di Ferrara (Fondo per l'incentivazione alla Ricerca 2016)	Prof Simonetta Pancaldi
University of Ferrara (Italy) (Bando Giovani Ricercatori 2015)	Dr Alessandra Sabia													
University Institute of High Studies (IUSS)-1931 of Ferrara	Dr Alessandra Sabia													
Spanish Ministry of Economy and Competitiveness (CTQ2014-56285-R)	Prof Joan Salvadò Rovira													
Excma. Diputació de Tarragona (Fuels from Biomass)	Prof Joan Salvadò Rovira													
European Regional Development Funds (Programa Competitividad de Catalunya 2007-2013)	Prof Joan Salvadò Rovira													
Università degli Studi di Ferrara (Fondo per l'incentivazione alla Ricerca 2016)	Prof Simonetta Pancaldi													
Abstract:	The marine diatom <i>Thalassiosira pseudonana</i> grown under air (0.04% CO ₂), 1 and 5% CO ₂ concentrations was evaluated to determine its potential for CO ₂ mitigation coupled with biodiesel production. Results indicated that the diatom cultures grown at 1 and 5% CO ₂ 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 CO ₂ 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% CO ₂ . 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 CO ₂ levels. These results suggested that <i>T. pseudonana</i> seems to be an ideal candidate for biodiesel production using flue gases.													

Response to Reviewers:**Answers to Editor's Comments:**

1. In Addition to the reviewers, I have the following request: All higher taxa names (e.g. Heterokontophyta, Thalassiosirales, Thalassiosiraceae) should also be italicized (in the text and the list of references).

Ok. Corrections has been added in the MS, all higher taxa name were italicized.

Reviewer 1:

We thank the Reviewer for its critical reading of the MS and for the precious contribution with his/her suggestion. The list of changes and specific comments are reported below:

1. First of all, I suggest an update of literature. There are some more recent papers on the topic that are not considered in this study (i.e. Manju et al. 2017 Biodiesel 8: 81-89; Mondal et al. 2017, 3 Biotech 7:99; Menezes, et al. 2016 J. Appl. Phycol. 28, 2679-2686; De Angelis, et al. 2016. PLOS ONE 11(11): e0165571; Singh, et al. 2016. Renew. Sust. Energ. Rev. 55, 1-16; Zienkiewicz, et al 2016. Biochim. Biophys. Acta 1861, 1269-1281; Silva, et al. 2015. Algal Res. 9, 312-321).

We introduced in the MS the following references:

Manju MJ, Renjith KR, John G, Nair SM, Chandramohanakumar N (2017) Biodiesel prospective of five diatom strains using growth parameters and fatty acid profiles. Biofuels, 8:81-89

Mandal M, Goswami S, Ghosh A, Oinam G, Tiwari ON, Das P, Gayen K, Mandal MK, Halder GN (2017) Production of biodiesel from microalgae through biological carbon capture: a review. 3 Biotech, 7:99

Singh P, Kumari S, Guldhe A, Misra R, Rawat I, Bux F (2016) Trends and novel strategies for enhancing lipid accumulation and quality in microalgae. Renew Sust Energ Rev 55:1-16

Zienkiewicz K, Du ZY, Ma W, Vollheyde K, Benning C (2016) Stress-induced neutral lipid biosynthesis in microalgae—Molecular, cellular and physiological insights. Biochim Biophys Acta (BBA)-Molecular and Cell Biology of Lipids, 1861:1269-1281

2. Check also the use of the comparative; sometimes is not used in the proper way (see line 44 pag. 2; or in different point at pag. 8).

Ok. Correction has been added (Page 2, line 45; page 8 line 279, line 280 in the present version).

3. Pag. 2 Line 54: Zendejas et al 2011; in the reference list is 2012.

Ok. Correction has been added: Zendejas et al. 2012 (Page 2, line 53 and 56 in the present version).

4. Pag. 3 line 58: I suggest to use 'promising candidate for..' or 'promising strain for..'

Ok. Correction has been added: "promising strain for" (Page 3 line 60 in the present version).

5. Pag. 4 lines 96-100: I think that it is better to move this paragraph after the following (101-107).

Ok. Correction has been added, the paragraph is moved (Page 4 line 110-114 in the present version).

Pag. 6 lines 184-185: I suggest 'These results reflected higher biomass productivities achieved by cultures grown at 1 and 5% CO₂ (69 and 84%, respectively) than 0.04% CO₂-treated cells'.

Ok. Correction has been added (Page 6 line 192-193 in the present version).

6.Line 205: add the bracket to close the parenthesis.

Ok. Correction has been added (Page 6 line 209, pag 7 lines 213 in the present version).

7.Pag. 8 line 277: correct as '..CO₂ concentrations' or 'CO₂ amount'.

Ok. Correction has been added: CO₂ concentrations (Page 9 lines 288 in the present version).

8.Pag. 10 line 325: I suggest to modify as 'These results suggest that *T. pseudonana* under 5% CO₂ showed a suitable fatty acid'

Ok. Correction has been added (Page 10 lines 340 in the present version).

9.References: Durret et al. 2008 and Mehta et al. 2005 are not cited in the test.

Ok. Correction has been added, these references were removed.

Reviewer 2:

We thank the Reviewer for its critical reading of the MS and for the precious contribution with his/her suggestion. The list of changes and specific comments are reported below:

1.There is one aspect of the work that I found questionable to some extent. The authors made the decision to analyze a single point of the growth curve in all cases to analyze the lipid content in the cells. They chose the 4th day of culture for the collection of cells and their analysis based on the results of a work by another author (see page 4-lines 124-126). But, the cultivation conditions used in both works were the same? According to Fig.1a, it can be estimated that the cultures under the three concentrations of CO₂ used entered the stationary phase after the 2nd day. How do the authors know if the intensity and dynamics of lipid production were the same when one of the experimental conditions in cultures varied, such as the concentration of CO₂? It would have been more appropriate, in my opinion, to analyze the kinetic of lipid production at the three concentrations of CO₂ used or at least analyze more than one point in the growth curve. I agree with the authors that on the 4th day of cultivation the production of lipids is higher in cells grown with higher concentrations of CO₂, however I think that at least one sentence should be included indicating that the maximum lipid content in the cells could vary over time depending on the CO₂ content used for cultivation.

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."

On the other hand, since the citation of Chen et al. (2009) refers to the methodology of Nile red fluorescence analysis, we also changed the position of this citation in the text (Page 4 line 133)

2.On page 6- line 205 is missing a parenthesis.

Ok. Correction has been added (Page 6 line 209, pag 7 lines 213 in the present version)

3.On page 7- lines 231-232: The paragraph "the monounsaturated FA were the major class under 1% and 5% CO₂ treatments, and these account for more than 91% of the total FA" is confusing. Please, clarify it.

Ok. Correction has been added: The monounsaturated fatty acids (MUFAs) were the major class of fatty acids in cells cultivated with 1% and 5% CO₂. In detail, MUFAs in these cells under these CO₂ treatments account for more than 91% of the total fatty

acids. (Page 7 lines 241-242 in the present version).

4.On page 7- lines 233: "They were significantly higher () than controls,..." Which are controls? Please, indicate.

Ok. Correction has been added: 0.04% air grown cultures (Page 7 lines 243 in the present version).



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 Salvadò 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

University of Ferrara
Laboratory of Plant Cytophysiology
Department of Life Sciences and Biotechnology
Corso Ercole I D'Este, 32
44121 Ferrara – Italy
e-mail: simonetta.pancaldi@unife.it; Phone: 0039 0532 293786; Fax: 0039 0532 208561



Answers to Editor's Comments:

1. In Addition to the reviewers, I have the following request: All higher taxa names (e.g. Heterokontophyta, Thalassiosirales, Thalassiosiraceae) should also be italicized (in the text and the list of references).

Ok. Corrections has been added in the MS, all higher taxa name were italicized.

Reviewer 1:

We thank the Reviewer for its critical reading of the MS and for the precious contribution with his/her suggestion. The list of changes and specific comments are reported below:

1. First of all, I suggest an update of literature. There are some more recent papers on the topic that are not considered in this study (i.e. Manju et al. 2017 Biodiesel 8: 81-89; Mondal et al. 2017, 3 Biotech 7:99; Menezes, et al. 2016 J. Appl. Phycol. 28, 2679-2686; De Angelis, et al. 2016. PLOS ONE 11(11): e0165571; Singh, et al. 2016. Renew. Sust. Energ. Rev. 55, 1-16; Zienkiewicz, et al 2016. Biochim. Biophys. Acta 1861, 1269-1281; Silva, et al. 2015. Algal Res. 9, 312-321).

We introduced in the MS the following references:

Manju MJ, Renjith KR, John G, Nair SM, Chandramohanakumar N (2017) Biodiesel prospective of five diatom strains using growth parameters and fatty acid profiles. Biofuels, 8:81-89

Mondal M, Goswami S, Ghosh A, Oinam G, Tiwari ON, Das P, Gayen K, Mandal MK, Halder GN (2017) Production of biodiesel from microalgae through biological carbon capture: a review. 3 Biotech, 7:99

Singh P, Kumari S, Guldhe A, Misra R, Rawat I, Bux F (2016) Trends and novel strategies for enhancing lipid accumulation and quality in microalgae. Renew Sust Energ Rev 55:1-16

Zienkiewicz K, Du ZY, Ma W, Vollheyde K, Benning C (2016) Stress-induced neutral lipid biosynthesis in microalgae—Molecular, cellular and physiological insights. Biochim Biophys Acta (BBA)-Molecular and Cell Biology of Lipids, 1861:1269-1281

2. Check also the use of the comparative; sometimes is not used in the proper way (see line 44 pag. 2; or in different point at pag. 8).

Ok. Correction has been added (Page 2, line 45; page 8 line 279, line 280 in the present version).

3. Pag. 2 Line 54: Zendejas et al 2011; in the reference list is 2012.

Ok. Correction has been added: Zendejas et al. 2012 (Page 2, line 53 and 56 in the present version).

4. Pag. 3 line 58: I suggest to use 'promising candidate for..' or 'promising strain for..'

Ok. Correction has been added: "promising strain for" (Page 3 line 60 in the present version).

5. Pag. 4 lines 96-100: I think that it is better to move this paragraph after the following (101-107).

Ok. Correction has been added, the paragraph is moved (Page 4 line 110-114 in the present version).

Pag. 6 lines 184-185: I suggest 'These results reflected higher biomass productivities achieved by cultures grown at 1 and 5% CO₂ (69 and 84%, respectively) than 0.04% CO₂-treated cells'.

Ok. Correction has been added (Page 6 line 192-193 in the present version).

6. Line 205: add the bracket to close the parenthesis.

Ok. Correction has been added (Page 6 line 209, pag 7 lines 213 in the present version).

7. Pag. 8 line 277: correct as '..CO₂ concentrations' or 'CO₂ amount'.

Ok. Correction has been added: CO₂ concentrations (Page 9 lines 288 in the present version).

8. Pag. 10 line 325: I suggest to modify as 'These results suggest that *T. pseudonana* under 5% CO₂ showed a suitable fatty acid'

Ok. Correction has been added (Page 10 lines 340 in the present version).

9. References: Durret et al. 2008 and Mehta et al. 2005 are not cited in the test.

Ok. Correction has been added, these references were removed.

Reviewer 2:

We thank the Reviewer for its critical reading of the MS and for the precious contribution with his/her suggestion. The list of changes and specific comments are reported below:

1. There is one aspect of the work that I found questionable to some extent. The authors made the decision to analyze a single point of the growth curve in all cases to analyze the lipid content in the cells. They chose the 4th day of culture for the collection of cells and their analysis based on the results of a work by another author (see page 4-lines 124-126). But, the cultivation conditions used in both works were the same? According to Fig.1a, it can be estimated that the cultures under the three concentrations of CO₂ used entered the stationary phase after the 2nd day. How do the authors know if the intensity and dynamics of lipid production were the same when one of the experimental conditions in cultures varied, such as the concentration of CO₂? It would have been more appropriate, in my opinion, to analyze the kinetic of lipid production at the three concentrations of CO₂ used or at least analyze more than one point in the growth

curve. I agree with the authors that on the 4th day of cultivation the production of lipids is higher in cells grown with higher concentrations of CO₂, however I think that at least one sentence should be included indicating that the maximum lipid content in the cells could vary over time depending on the CO₂ content used for cultivation.

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."

On the other hand, since the citation of Chen et al. (2009) refers to the methodology of Nile red fluorescence analysis, we also changed the position of this citation in the text (Page 4 line 133)

2. On page 6- line 205 is missing a parenthesis.

Ok. Correction has been added (Page 6 line 209, pag 7 lines 213 in the present version)

3. On page 7- lines 231-232: The paragraph "the monounsaturated FA were the major class under 1% and 5% CO₂ treatments, and these account for more than 91% of the total FA" is confusing. Please, clarify it.

Ok. Correction has been added: The monounsaturated fatty acids (MUFAs) were the major class of fatty acids in cells cultivated with 1% and 5% CO₂. In detail, MUFAs in these cells under these CO₂ treatments account for more than 91% of the total fatty acids. (Page 7 lines 241-242 in the present version).

4. On page 7- lines 233: "They were significantly higher () than controls,..." Which are controls? Please, indicate.

Ok. Correction has been added: 0.04% air grown cultures (Page 7 lines 243 in the present version).



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

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

a) University of Ferrara, Department of Life Sciences and Biotechnology, C.so Ercole I d'Este 32, 44121 Ferrara, Italy

b) Catalonia Institute for Energy Research, IREC, Marcel·lí Domingo, 2 43007, Tarragona, Catalonia, Spain

c) Departament d'Enginyeria Química, Universitat Rovira i Virgili, Av. Països Catalans, 26 43007, Tarragona, Spain

Formatted: English (United Kingdom)

Formatted: English (United States)

Alessandra Sabia email address: sabia.alessandra@gmail.com

Formatted: English (United States)

Esther Clavero email address: ester.clavero@gmail.com

Formatted: English (United States)

Simonetta Pancaldi email address: simonetta.pancaldi@unife.it

Formatted: English (United States)

Joan Salvadó Rovira email address: joan.salvado@urv.cat

Formatted: English (United States)

§ equally contributed

* Corresponding author. Tel.: +39 0532 293786; Fax: +39 0532 208561

University of Ferrara, Department of Life Sciences and Biotechnology, C.so Ercole I d'Este 32, 44121 Ferrara, Italy

E-mail address: simonetta.pancaldi@unife.it (S. Pancaldi).

Formatted: English (United States)

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
6 cultures (with 1.13 div d⁻¹ and 26 mg_{AFDW}L⁻¹d⁻¹). The increase of CO₂ resulted in higher cell volume and
7 pigment content per cell of *T. pseudonana*. Interestingly, lipid content doubled when air was enriched
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.*
11 *pseudonana* seems to be an ideal candidate for biodiesel production using flue gases.
12

13

14 **Keywords**

15 Microalgae;
16 CO₂ concentrations;
17 Biomass productivity;
18 Fatty acid composition;
19 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 (CO_2) emissions mainly come from the intensive burning of fossil fuels has contributed to
26 a 40% increase in the atmospheric CO_2 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 CO_2 (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 CO_2
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 CO_2 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 CO_2 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 er-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 synthetized 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. 2012⁴; Popovich et al. 2012⁴,
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. 2012⁴; d'Ippolito et al. 2015). In addition, recent studies have been
57 concerned the effect of elevated CO_2 concentrations on growth and photophysiology of this diatom

Formatted: Font: (Default) Times New Roman, Font color: Auto, Pattern: Clear

58 species (Crawfurd 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 Ryther 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:

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

Formatted: Font: Italic

96 where k is the division rate, N_t the cell number at time t, N₀ the cell number at time 0 and t_t-t₀ 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, X₀ the biomass
102 concentration at time 0 and t_t-t₀ 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, X₀ the biomass
114 concentration at time 0 and t_t-t₀ the time interval (days) (Hempel et al. 2012).

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 (Chla), 632 (Chlc) 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 (A₇₅₀).

134 After the algal suspension were stained with Nile Red (9-diethylamina-5Hbenzo[α]phenoxazine-5-one,
135 0.5 μ g 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 Sirin 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 μ m. 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 CO_2 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 CO_2 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 CO_2 concentration increased the
183 division rates of the diatom as compared to cells cultivated with 0.04 and 1% CO_2 . Interestingly, at the
184 end of the experiment, the biomass concentrations of cells cultivated with 1 and 5% CO_2 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 CO_2 treatments. In detail, a gradual but evident increasing trend was observed in cells cultivated
188 with 1 and 5% CO_2 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 CO_2 was found 2 times higher than that of cell cultivated with
191 0.04% CO_2 (Fig. 1b). Therefore, these results reflected the higher biomass productivities achieved by
192 cultures grown at 1 and 5% CO_2 (69 and 84%, higher than 0.04% CO_2 -treated cells, respectively) (Table
193 than 0.04% CO_2 -treated cells (Table 1)). As shown in Table 2, with the increase of CO_2 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% CO_2 , respectively).
196 In fact, despite, no great differences were reported between 0.04% and 1% of CO_2 treatments regarding
197 TOC concentrations, the maximum TOC values in the culture medium were found under 5% CO_2 (14.63
198 mg L⁻¹, 19 and 17% higher than 0.04% and 1% CO_2 -treated cells, respectively). On the other hand, higher
199 CO_2 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 CO_2 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% CO_2 , respectively (Tab. 2).

203

204 Effect of different CO_2 concentrations on *T. pseudonana* photosynthetic pigment content

205 In order to evaluate if the cultivation in presence of elevated CO_2 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 CO_2 concentrations resulted in significant increases in Chla and
208 Car content as compared to those of air grown cultures (Table 3). In detail, Chla concentrations in cells
209 cultivated with 1% and 5% CO_2 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 (A₇₅₀ = 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 (MUFA) were the major class of fatty acids in cells cultivated under with 1% and 5% CO₂ treatments. In
242 detail, and MUFA 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

Formatted: English (United States)

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 affect 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 (Crawfurd 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

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

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

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

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

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

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

213 Interestingly, the increase in CO₂ concentration resulted in an increase of monounsaturated acids
214 (palmitoleic and oleic) and a decrease of the saturated myristic acid and polyunsaturated fatty acids
215 (Table 4), suggesting that from the point of view of biodiesel production air enriched with CO₂ produces
216 better distribution of fatty acid chains. The increase in unsaturated fatty acids and decrease in saturated
217 fatty acids has previously been observed in Chlorophyceae species at increasing CO₂ concentrations up to
218 5% (Tang 2011; Nascimento et al. 2015). However, this trend did not appear in cultures of the same or
219 other species in other works (Tsuzuki 1990; Muradyan et al. 2004). In this study, it is not observed a big
220 difference in the fatty acid chain profile attending the use of 1% or 5% CO₂ enriched air (Table 4).
221 Despite of this, the evident characteristic of the fatty acid composition of *T. pseudonana* grown under 1-
222 5% CO₂ is that the major fatty acids were C14-C18, and these account for more than 91% of the total
223 fatty acids. The predominance of shorter-chain fatty acids (defined as alkyl chains with 12-18 carbon

Formatted: English (United States)

Formatted: Subscript

Formatted: Subscript

Formatted: English (United States)

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 Formatted: English (United States)

343

344 Acknowledgements

345 This work was supported by a research fellowship “Bando Giovani Ricercatori 2015” granted by the
346 University of Ferrara (Italy) to A.S. and by a fellowship granted by the University Institute of High
347 Studies (IUSS) [193+1391](#) of Ferrara for PhD student’s mobility to A.S.. [This work was also supported](#)
348 [by the University of Ferrara through the Fondo per l’Incentivazione alla Ricerca \(FIR 2016\) granted to](#)
349 [S.P..](#)

350 This work was supported by the projects CTQ2014-56285-R “Cultivo, concentración, fraccionamiento y
351 obtención de producto en refinería de microalgas” funded by the Spanish Ministry of Economy and
352 Competitiveness and “Fuels from Biomass” funded by Excmo. Diputació de Tarragona.

353 The research was also supported by the European Regional Development Funds (ERDF, FEDER
354 Programa Competitividad de Catalunya 2007-2013).

355

356

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

363
364
365
366
367

368 **References**

- 369
- 370 Andersen RA (2005) Traditional microalgae isolation techniques In: Andersen RA (Ed), Algal culturing
371 techniques, Academic press, pp. 269-285
- 372 Armbrust EV, Berges JA, Bowler C, Green BR, Martinez D, Putnam NH, Zhou S, Allen AE, Apt
373 KE, Bechner M, Brzezinski MA, Chaal BK, Chiovitti A, Davis AK, Demarest MS, Detter JK,
374 Glavina T, Goodstein D, Hadi MZ, Hellsten U, Hildebrand M, Jenkins BD, Jurka J, Kapitonov VV,
375 Kröger N, Lau WWY, Lane TW, Larimer FW, Lippmeier JC, Lucas S, Medina M, Montsant A,
376 Obornik M, Parker MS, Palenik B, Pazour GJ, Richardson MP, Ryneanson TA, Saito MA, Schwartz
377 DC, Thamatrakoln K, Valentim K, Vardi A, Wilkerson FP, Rokhsa DS (2004) The genome of the
378 diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. *Science* 306:79-86
- 379 **Baldisserotto C, Ferroni L, Giovanardi M, Boccaletti L, Pantaleoni L, Pancaldi S (2012)** Salinity promotes
380 growth of freshwater *Neochloris oleoabundans* UTEX 1185 (*Sphaeropleales, Chlorophyta*):
381 morphophysiological aspects. *Phycol* 51:700-710
- 382 Baldisserotto C, Popovich C, Giovanardi M, Sabia A, Ferroni L, Constenla D, Leonardi P, Pancaldi S
383 (2016) Photosynthetic aspects and lipid profiles in the mixotrophic alga *Neochloris oleoabundans* as
384 useful parameters for biodiesel production. *Algal Res* 16:255-265
- 385 Bligh EG, Dyer WM (1959) A rapid method of lipid extraction and purification. *Can J Biochem Physiol*
386 37:911-917
- 387 Cheah WY, Show PL, Chang JS, Ling TC, Juan JC (2015) Biosequestration of atmospheric CO₂ and flue
388 gas-containing CO₂ by microalgae. *Bioresource Technol* 184:190-201
- 389 Chen W, Zhang C, Song L, Sommerfeld M, Hu Q (2009) A high throughput Nile red method for
390 quantitative measurement of neutral lipids in microalgae. *J Microbiol Methods* 77:41-47
- 391 Chisti Y (2007) Biodiesel from microalgae. *Biotech Adv* 25:294-306
- 392 Crawfurd KJ, Raven JA, Wheeler GL, Baxter EJ, Joint I (2011) The response of *Thalassiosira pseudonana*
393 to long-term exposure to increased CO₂ and decreased pH. *PloS one* 6:e26695-e26695
- 394 d'Ippolito G, Sardo A, Paris D, Vella FM, Adelfi MG, Botte P, Gallo C, Fontana A (2015) Potential of
395 lipid metabolism in marine diatoms for biofuel production. *Biotechnol Biofuels* 8:1-28
- 396 Doan TTY, Sivaloganathan B, Obbard JP (2011) Screening of marine microalgae for biodiesel feedstock.
397 *Biomass Bioenergy* 35:2534-44
- 398 **Durrett TP, Benning C, Ohlrogge J (2008)** Plant triacylglycerols as feedstocks for the production of
399 biofuels. *Plant J* 54:593-607
- 400 Engel A (2002) Direct relationship between CO₂ uptake and transparent exopolymer particles production in
401 natural phytoplankton. *J Plankton Res* 24:49-53
- 402 Field CB, Behrenfeld MJ, Randerson JT, Falkowski P (1998) Primary production of the biosphere:
403 integrating terrestrial and oceanic components. *Science* 281:237-240
- 404 Francisco EC, Neves DB, Jacob-Lopes E, Franco TT (2010) Microalgae as feedstock for biodiesel
405 production: carbon dioxide sequestration, lipid production and biofuel quality. *J Chem Technol Biot*
406 85:395-403

Formatted: English (United Kingdom)

Formatted: Font: Italic

Formatted: English (United States)

Formatted: English (United Kingdom)

Formatted: English (United States)

- 407 Gao K, Campbell DA (2014) Photophysiological responses of marine diatoms to elevated CO₂ and
 408 decreased pH: a review. *Funct Plant Biol* 41:449-459
- 409 Gao K, Xu J, Gao G, Li Y, Hutchins DA, Huang B, Wang L, Zheng Y, Jin P, Cai X, Häder DP, Li W, Xu
 410 K, Liu N, Riebesell U (2012) Rising CO₂ and increased light exposure synergistically reduce marine
 411 primary productivity. *Nat Clim Change* 2:519-523
- 412 Giovannardi M, Baldisserotto C, Ferroni L, Longoni P, Celli R, Pancaldi S (2014) Growth and lipid
 413 synthesis promotion in mixotrophic *Neochloris oleoabundans* (*Chlorophyta*) cultivated with
 414 glucose. *Protoplasma* 251:115-125
- 415 Guillard RRL, Ryther JH (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and
 416 *Detonula confervacea* (Cleve). *Gran Can J Microbiol* 8:229-239
- 417 Hempel N, Petrick I, Behrendt F (2012) Biomass productivity and productivity of fatty acids and amino
 418 acids of microalgae strains as key characteristics of suitability for biodiesel production. *J Appl*
 419 *Phycol* 24:1407-1418
- 420 Hildebrand D, Smith SR, Traller JC, Abbriano R (2012) The place of diatoms in the biofuels industry.
 421 *Biofuels* 3:221-240
- 422 Hoogstraten A, Timmermans KR, de Baar HJW (2012) Morphological and physiological effects in
 423 *Proboscia alata* (*Bacillariophyceae*) grown under different light and CO₂ conditions of the modern
 424 southern ocean. *J Phycol* 48:559-568
- 425 Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008) Microalgal
 426 triacylglycerols asa feedstocks for biofuel production: perspective and advances. *Plant J* 54:621-639
- 427 Hulatt CJ, Thomas DN (2010) Dissolved organic matter (DOM) in microalgal photobioreactors: a potential
 428 loss in solar energy conversion?. *Bioresource Technol* 101:8690-8697
- 429 Lam MK, Lee KT, Mohamed AR (2012) Current status and challenges on microalgae-based carbon
 430 capture. *Int J Greenhouse Gas Control* 10:456-469
- 431 Lee JY, Yoo C, Jun SY, Ahn CY, Oh HM (2010) Comparison of several methods for effective lipid
 432 extraction from microalgae. *Bioresour Technol* 101:S75-S77
- 433 Li G, Campbell DA (2013) Rising CO₂ interacts with growth light and growth rate to alter photosystem II
 434 photoinactivation of the coastal diatom *Thalassiosira pseudonana*. *PLoS One* 8:e55562
- 435 Maity JP, Bundschuh J, Chen CY, Bhattacharya P (2014) Microalgae for third generation biofuel
 436 production, mitigation of greenhouse gas emissions and wastewater treatment: Present and future
 437 perspectives - A mini review. *Energy* 78:104-113
- 438 McCarthy A, Rogers SP, Duffy SJ, Campbell DA (2012) Elevated carbon dioxide differentially alters the
 439 photophysiology of *Thalassiosira pseudonana* (*Bacillariophyceae*) and *Emiliania huxleyi*
 440 (haptophyta)¹. *J Phycol* 48:635-646
- 441 Mehta SK, Gaur JP (2005) Use of algae for removing heavy metal ions from wastewater: progress and
 442 prospects. *Crit Rev Biotechnol* 25:113-152
- 443 Manju MJ, Renjith KR, John G, Nair SM, Chandramohanakumar N (2017) Biodiesel prospective of five
 444 diatom strains using growth parameters and fatty acid profiles. *Biofuels*, 8:81-89
- 445 Mejia LM, Isensee K, Méndez-Vicente A, Pisonero J, Shimizu N, González C, Monteleone B, Stoll H

Formatted: English (United Kingdom)

Formatted: Font: Italic

Formatted: Font: Italic

Formatted: English (United States)

Formatted: Font: Italic

Formatted: Font: (Default) Times New Roman, Font color: Auto, Pattern: Clear

- 446 (2013) B content and Si/C ratios from cultured diatoms (*Thalassiosira pseudonana* and
 447 *Thalassiosira weissflogii*): relationship to seawater pH and diatom carbon acquisition. *Geochim
 448 Cosmochim Ac* 123:322-337
- 449 Mondal M, Goswami S, Ghosh A, Oinam G, Tiwari ON, Das P, Gayen K, Mandal MK, Halder GN
 450 (2017) Production of biodiesel from microalgae through biological carbon capture: a review. *3
 451 Biotech.* 7:99
- 452 Muradyan EA, Klyachko-Gurvich GL, Tsoglin LN, Sergeyenko TV, Pronina NA (2004) Changes in lipid
 453 metabolism during adaptation of the *Dunaliella salina* photosynthetic apparatus to high CO₂
 454 concentration. *Russ J Plant Physl* 51:53-62
- 455 Nascimento IA, Dominguez Cabanelas IT, Nunes dos Santos J, Nascimento MA, Sousa L, Sansone G
 456 (2015) Biodiesel yields and fuel quality as criteria for algal-feedstock selection: Effects of CO₂-
 457 supplementation and nutrient levels in cultures. *Algal Res* 8:53-60
- 458 Popovich CA, Damiani C, Constenla D, Leonardi PI (2012) Lipid quality of the diatoms *Skeletonema
 459 costatum* and *Navicula gregaria* from the South Atlantic Coast (Argentina): evaluation of its
 460 suitability as biodiesel feedstock. *J App Phycol* 24:1-10
- 461 Ritchie RJ (2006) Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and
 462 ethanol solvents. *Photosynth Res* 89:27-41
- 463 Sabia A, Baldisserotto C, Biondi S, Marchesini R, Tedeschi P, Maietti A, Giovanardi M, Ferroni L,
 464 Pancaldi S (2015) Re-cultivation of *Neochloris oleoabundans* in exhausted autotrophic and
 465 mixotrophic media: the potential role of polyamines and free fatty acids. *Appl Microbiol Biotechnol*
 466 99:10597-10609
- 467 Shen Y (2014) Carbon dioxide bio-fixation and wastewater treatment via algae photochemical synthesis for
 468 biofuels production. *RSC Adv* 4:49672-49722
- 469 Singh P, Kumari S, Guldhe A, Misra R, Rawat I, Bux F (2016) Trends and novel strategies for enhancing
 470 lipid accumulation and quality in microalgae. *Renew Sust Energ Rev* 55:1-16
- 471 Sirin S, Clavero E, Salvadó J (2015) Efficient harvesting of *Chaetoceros calcitrans* for biodiesel
 472 production. *Environ Technol* 36:1902-1912
- 473 Tang D, Han W, Li P, Miao X, Zhong J (2011) CO₂ biofixation and fatty acid composition of *Scenedesmus
 474 obliquus* and *Chlorella pyrenoidosa* in response to different CO₂ levels. *Bioresour Technol*
 475 102:3071-3076
- 476 Torstensson A, Chierici M, Wulff A (2012) The influence of increased temperature and carbon dioxide
 477 levels on the benthic/sea ice diatom *Navicula directa*. *Polar Biol* 35:205–214
- 478 Trobajo R, Ibañez C, Clavero E, Salvadó J, Jørgensen SE (2014) Modelling the response of microalgae to
 479 CO₂ addition. *Ecol Model* 294:42-50
- 480 Tsuzuki M, Ohnuma E, Sato N, Takaku T, Kawaguchi A (1990) Effects of CO₂ concentration during
 481 growth on fatty-acid composition in microalgae. *Plant Physiol* 93:851-856
- 482 Vinayak V, Manoylov KM, Gateau H, Blanckaert V, Héault J, Pencréac'h G, Marchand J, Gordon R,
 483 Schoefs B (2015) Diatom Milking: A Review and New Approaches. *Mar Drugs* 13:2629-2665
- 484 Wang XW, Liang JR, Luo CS, Chen CP, Gao YH (2014) Biomass, total lipid production, and fatty acid

Formatted: English (United Kingdom)
Formatted: Font: (Default) Times New Roman, Font color: Auto, Pattern: Clear
Formatted: English (United Kingdom)
Formatted: Font: (Default) Times New Roman, Font color: Auto, Pattern: Clear
Formatted: Font: (Default) Times New Roman, Font color: Auto, Pattern: Clear
Formatted: Font: (Default) Times New Roman, Font color: Auto, Pattern: Clear
Formatted: Font: (Default) Times New Roman, Font color: Auto, Pattern: Clear
Formatted: English (United States)
Formatted: English (United Kingdom)

485 composition of the marine diatom *Chaetoceros muelleri* in response to different CO₂ levels.
486 Bioresource Technol 161:124-130
487 Wellburn AR (1994) The spectral determination of chlorophylls a and b, as well as total carotenoids, using
488 various solvents with spectrophotometer of different resolution. J Plant Physiol 144:307-313
489 Wu Y, Gao K, Riebesell U (2010) CO₂-induced seawater acidification affects physiological performance of
490 the marine diatom *Phaeodactylum tricornutum*. Biogeosc 7:2915-2923
491 Yang G, Gao K (2012) Physiological responses of the marine diatom *Thalassiosira pseudonana* to
492 increased pCO₂ and seawater acidity. Mar Environ Res 79:142-151
493 Yu ET, Zendejas FJ, Lane PD, Gaucher S, Simmons BA, Lane TW (2009) Triacylglycerol accumulation
494 and profiling in the model diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*
495 (*Bacillariophyceae*) during starvation. J Appl Phyc 21:669-681
496 Zendejas FJ, Benke PI, Lane PD, Simmons BA, Lane TW (2012) Characterization of the acylglycerols and
497 resulting biodiesel derived from vegetable oil and microalgae (*Thalassiosira pseudonana* and
498 *Phaeodactylum tricornutum*). Biotechnol Bioeng 109:1146-1154
499 Zhu CJ, Lee YK (1997) Determination of biomass dry weight of marine microalgae. J Appl Phyc 9:189-
500 194
501 Zienkiewicz K, Du ZY, Ma W, Vollheyde K, Benning C (2016) Stress-induced neutral lipid
502 biosynthesis in microalgae—Molecular, cellular and physiological insights. Biochim Biophys
503 Acta (BBA)-Molecular and Cell Biology of Lipids. 1861:1269-1281
504

Formatted: Font: Italic

Formatted: Font: Not Italic

Formatted: Indent: Left: -0.1", Hanging: 0.39", No
widow/orphan control, Don't adjust space between
Latin and Asian text, Don't adjust space between Asian
text and numbers

505 **Figure captions**

506

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

516

517

518

519

520

521

522

523

524

525

526

527

528

529

530

531

532

533

534

535

536

537

538

539

540

541

542

543

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

552

553

554

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.

561

562

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

563

564

565

566

567

568

569

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)				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

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

581
582
583
584
585
586
587
588
589
590

[Click here to view linked References](#)

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

3 Alessandra Sabia^{a§}, Esther Clavero^{b§}, Simonetta Pancaldi^{a*}, Joan Salvadó Rovira^c

4 a) University of Ferrara, Department of Life Sciences and Biotechnology, C.so Ercole I d'Este 32,
5 44121 Ferrara, Italy

6 b) Catalonia Institute for Energy Research, IREC, Marcel·lí Domingo, 2 43007, Tarragona,
7 Catalonia, Spain

8 c) Departament d'Enginyeria Química, Universitat Rovira i Virgili, Av. Països Catalans, 26 43007,
9 Tarragona, Spain

10 Alessandra Sabia email address: sabia.alessandra@gmail.com

11 Esther Clavero email address: ester.clavero@gmail.com

12 Simonetta Pancaldi email address: simonetta.pancaldi@unife.it

13 Joan Salvadó Rovira email address: joan.salvado@urv.cat

14 § equally contributed

15 * Corresponding author. Tel.: +39 0532 293786; Fax: +39 0532 208561

16 University of Ferrara, Department of Life Sciences and Biotechnology, C.so Ercole I d'Este 32,
17 44121 Ferrara, Italy

18 E-mail address: [\(S. Pancaldi\).](mailto:simonetta.pancaldi@unife.it)

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
6 cultures (with 1.13 div d⁻¹ and 26 mg_{AFDW}L⁻¹d⁻¹). The increase of CO₂ resulted in higher cell volume and
7 pigment content per cell of *T. pseudonana*. Interestingly, lipid content doubled when air was enriched
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.*
11 *pseudonana* seems to be an ideal candidate for biodiesel production using flue gases.

12 **Keywords**

13 Microalgae;

14 CO₂ concentrations;

15 Biomass productivity;

16 Fatty acid composition;

17 Biodiesel production.

1 **19 Introduction**

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

1 58 species (Crawford et al. 2011; Yang and Gao 2012; McCarthy et al. 2012; Li and Campbell 2013).
2 59 The present work is focused on the potential of the marine diatom *Thalassiosira pseudonana* as a
3 60 promising strain for the cultivation in the presence of elevated CO₂ concentrations. In particular, algal
4 61 growth, cell morphology, photosynthetic pigment content, total nitrogen (TN), and dissolved inorganic
5 62 (TIC) and organic carbon (TOC) content were monitored under increasing CO₂ concentrations (0.04
6 63 (ambient air), 1 and 5%). The lipid content and profile was also estimated in order to evaluate the effects
7 64 of elevated CO₂ concentration on the composition of fatty acids useful for biodiesel production.
8
9
10
11
12
13
14
15

67 Materials and Methods

68 Algal strain and culture condition

18 69 The strain used in this study was the marine centric diatom *Thalassiosira pseudonana* (Hustedt) Hasle
19 70 and Heimdal CCAP 1085/12 (*Heterokontophyta*, *Thalassiosirales*, *Thalassiosiraceae*), obtained from the
21 71 Culture Collection of Algae and Protozoa (CCAP; Scotland, United Kingdom; www.ccap.ac.uk).
22

23 72 *T. pseudonana* was inoculated in 6-L Erlenmeyer flasks with a 4-L working volume of filtered (0.45 µm)
24 73 autoclaved seawater enriched with f/2 nutrients (Guillard and Ryther 1962) at 25±2°C and under an
25 74 exposure to an irradiance of 250 µmol_{photons} m⁻² s⁻¹ provided by cool white fluorescent lamps (OSRAM
26 75 L30W/865 Lumilux) under a cycle period of 16:8 h light-darkness. The *T. pseudonana* cultures were
27 76 aerated at a rate of 250 mL min⁻¹ (i.e., 0.0625 vvm, volume gas per volume media per min) of air
28 77 enriched with CO₂ to obtain final concentration of 0.04, 1 and 5% CO₂. The air-CO₂ mixtures were
29 78 obtained by combining rotameter flows of air and pure CO₂. The percentage of CO₂ in the air-CO₂
30 79 mixture was verified with an infrared analyzer (Servomex 4900C1). Experiments were performed in
31 80 triplicate. Aliquots of cultures were collected at different times of cultivation, depending on the analysis.
32
33
34
35
36
37

81 Analyses

82 *Growth, cell size and biomass evaluation*

41 84 Algal cells were counted daily using an Improved Neubauer hemocytometer under a light microscope
42 85 (Zeiss, model Axioscope A1) equipped with DIC (Differential Interference Contrast) optics in order to
43 86 estimate the division rates (k, number of divisions per day). Cell density (cell mL⁻¹) was also measured by
44 87 the absorbance at 750 nm (A₇₅₀) in 96 well plates with the microplate reader (Infinite Tecan M200Pro).
45 88 Absorbance measures were converted to optical density by dividing absorbance values by the pathlength
46 89 following Beer-Lambert's law. Cell size measurements were done with the microscope described above
47 90 and measured with the aid of the software (ProgRes Capture Pro v. 2.8) and a digital camera (Jenoptik
48 91 ProgRes Speed XT Core5).

54 92 The division rates (k, number of divisions per day) during the exponential phase was calculated with the
55 93 following equation:

$$57 \quad 94 k (\text{div d}^{-1}) = (\log_2 N_t - \log_2 N_0) / (t_t - t_0),$$

58 95 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).

13 109 *Photosynthetic pigment extraction and quantification*

14 110 For photosynthetic pigment analysis, cell samples were collected by centrifugation and the extraction was
15 111 performed according to Giovanardi et al. (2014). The extracts were measured with the same microplate
16 112 reader, already described, at 665 (Chla), 632 (Chlc) and 480 nm (Car). Quantification of Chls was
17 113 performed according to equations reported in Ritchie (2006), whereas Car quantification according to
18 114 equations reported in Wellburn (1994).

19 116 *Biochemical analyses*

20 117 During the growth period, the pH in culture replicates was monitored daily using a pH meter (Crison
21 118 GLP21). The total nitrogen (TN), the dissolved inorganic (TIC) and dissolved organic carbon (TOC)
22 119 concentrations in the culture medium were measured by a Multi N/C 3100 Analytic Jena analyser. 20 mL
23 120 of the algal cultures were centrifuged at 4000 rpm for 15 min and the supernatant was used for TIC, TOC
24 121 and TN measurements.

25 123 *Characterization of neutral lipids with Nile red*

26 124 Cell lipid content was estimated by neutral lipid staining with the fluorochrome Nile Red at the 4th day of
27 125 cultivation, when all the microalgal cultures had reached the stationary phase of growth, according to
28 126 Chen et al. (2009). First, algal samples were diluted to attain a final cell concentration of 0.06 (A_{750}).
29 127 After the algal suspension were stained with Nile Red (9-diethylamina-5Hbenzo[α]phenoxyazine-5-one,
30 128 0.5 µg dissolved in 100 mL acetone) and 25% of absolute dimethyl sulphoxide (DMSO). Fluorescence
31 129 was measured with the microplate reader, already described, at excitation and emission wavelengths of
32 130 530 nm and 580 nm, respectively. The relative fluorescence was obtained by subtraction of the
33 131 autofluorescence of non-stained cells and self-fluorescence of the fluorochrome. After staining, cells were
34 132 also observed with a Zeiss AxioObserver Z1 equipped with epifluorescence. A metal halide lamp was
35 133 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
3
4

5
6 136 *Lipid extraction*
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

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

150
151 *Methyl ester derivation and fatty acid analysis*
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171 **Results**

150
151 A two-step protocol was used for the methylation process of all extracted lipids, according to Lee et al
152 (2010). The samples were saponified with 5 mL of 2% KOH in methanol at 75 °C for 10 min, and then
153 subjected to methanolysis with 5 mL of 5% HCl in methanol at 75 °C for another 10 min. Thereafter, the
154 phase containing the fatty acids was separated by adding 10 mL of distilled water and then recovered with
155 5 mL of heptane. The obtained fatty acid methyl esters (FAMEs) were analyzed according to EN 14103,
156 with an Agilent 6850 gas chromatograph equipped with a split/splitless inlet and a FID detector. The
157 column used was a HP-INNOWax (polyethylene glycol phase), a capillary column with a high polarity
158 and dimensions 30 m x 0.32 mm x 0.25 µm. Quantification of total FAMEs was accomplished by using
159 FAME C17:0 as internal standard. Identification of individual FAME components was based on
160 comparison of their retention times and fragmentation patterns with those for standards. The FAME
161 composition was calculated as the percentage of the total identified esters present in the sample.

162
163
164 **Data treatment**
165 Data were processed with Graphpad Prism 6 (Graph Pad Software, San Diego CA, USA). In each case,
166 means ± standard deviations for *n* number of samples are given.
167 The statistical significance of differences was determined by one-way ANOVA followed by a multiple
168 comparison test (Tukey's test). A significance level of 95% (*p* < 0.05) was accepted.

1 172 *Effect of different CO₂ concentrations on T. pseudonana growth*

2
3 173 In this study, as shown in Fig. 1a, the diatom *T. pseudonana* cultivated with 0.04 (ambient air), 1 and 5%
4 174 CO₂ promptly entered the exponential phase and reached the stationary phase of growth during 4 days of
5 175 cultivation. However, it was apparent from Table 1 that the highest CO₂ concentration increased the
6 176 division rates of the diatom as compared to cells cultivated with 0.04 and 1% CO₂. Interestingly, at the
7 177 end of the experiment, the biomass concentrations of cells cultivated with 1 and 5% CO₂ were 190 and
8 178 210 mg L⁻¹ respectively, which were 1.58 and 1.75 times higher than that of air grown cultures (Table 1).
9
10 179 Further, observations on diatom cell size during the experiment showed remarkable changes in response
11 180 to the CO₂ treatments. In detail, a gradual but evident increasing trend was observed in cells cultivated
12 181 with 1 and 5% CO₂ until the end of the experiment (Fig. 1b). Conversely, the cell volume of air grown
13 182 cultures remained almost stable during the cultivation period. At 4th day of cultivation, the cell volume
14 183 achieved by both cultures enriched with CO₂ was found 2 times higher than that of cell cultivated with
15 184 0.04% CO₂ (Fig. 1b). Therefore, these results reflected the higher biomass productivities achieved by
16 185 cultures grown at 1 and 5% CO₂ (69 and 84%, respectively) than 0.04% CO₂-treated cells (Table 1). As
17 186 shown in Table 2, with the increase of CO₂ concentrations from 0.04% to 5%, the TIC concentration in
18 187 the culture medium increased from 16.83 mg L⁻¹ to 43.30 mg L⁻¹ (+117 and 157% higher than air grown
19 188 cultures in cells cultivated with 1% and 5% CO₂, respectively). In fact, despite, no great differences were
20 189 reported between 0.04% and 1% of CO₂ treatments regarding TOC concentrations, the maximum TOC
21 190 values in the culture medium were found under 5% CO₂ (14.63 mg L⁻¹, 19 and 17% higher than 0.04%
22 191 and 1% CO₂-treated cells, respectively). On the other hand, higher CO₂ concentrations always resulted in
23 192 lower pH. As shown in Table 2, the pH value of the culture medium decreased from pH 9.4 to pH 6.5
24 193 with the increase of CO₂ concentrations from 0.04% to 5%.
25
26 194 Further, the TN concentration in the culture medium decreased by 12% and 30% in cells cultivated with
27 195 1% and 5% CO₂, respectively (Tab. 2).

28
29 196
30 197 *Effect of different CO₂ concentrations on T. pseudonana photosynthetic pigment content*

31
32 198 In order to evaluate if the cultivation in presence of elevated CO₂ concentrations could also affect the
33 199 photosynthetic activity of the diatom, the photosynthetic pigment content were also analysed at the end of
34 200 the cultivation period. The increase of CO₂ concentrations resulted in significant increases in Chla and
35 201 Car content as compared to those of air grown cultures (Table 3). In detail, Chla concentrations in cells
36 202 cultivated with 1% and 5% CO₂ were higher (28 and 77%, respectively) than 0.04% air grown cultures.
37 203 Despite, no great differences were reported between 0.04% and 1% of CO₂ treatments about Chlc content,
38 204 the highest Chlc values were found in cells cultivated with 5% CO₂ (57 and 37% higher than air and 1%
39 205 CO₂ grown cultures. About Car content, cells cultivated with 0.04% CO₂ contained lower quantities (21
40 206 and 64%, respectively) as compared to cultures enriched with elevated CO₂. The increases in total Chl
41 207 content were greater than differences in the Car content, resulting in higher total Chl to Car molar ratios
42 208 in cultures enriched with elevated CO₂ as compared to air grown cultures, but the ratios did not differ
43 209 significantly at the 95% level. No significant effects on Chla 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
16 222 Nile red staining results were confirmed by Bligh and Dyer lipid extraction. Fluorescence values,
17 223 normalized to the same cell concentration ($A_{750} = 0.06$), were proportional to the lipid content as %
18 224 AFDW obtained with Bligh and Dyer extraction. The percentage of lipids in AFDW doubled when air
19 225 was enriched with 1-5 % CO₂ from 7.3% (air) to 14.1% (1%) and 16.9% (5%) of CO₂ (Table 4).
20
21 226 The main fatty acid components of *T. pseudonana* under different CO₂ concentrations were determined
22 227 by GC-MS analysis and are shown in Table 4. The predominant fatty acids were mirystic (C14:0),
23 228 palmitic (C16:0), palmitoleic (C16:1), oleic (C18:1) acids (Table 4). Other fatty acids (C15:0, C16:2,
24 229 C16:3, C18:0, C18:4, C20:5, C22:1 and C22:2) were minor components and presented concentration
25 230 individually of less than 5%. However, the lipid classes showed differences in their proportions in
26 231 response to the CO₂ treatments (Table 4). Despite, no great differences were reported between 1%
27 232 (54.4%) and 5% (52.8%) of CO₂ treatments about the percentages of saturated fatty acids (SFAs), the
28 233 highest SFAs values were found in cells cultivated with air (60.4%). The monounsaturated fatty acids
29 234 (MUFAs) were the major class of fatty acids in cells cultivated with 1% and 5% CO₂. In detail, MUFAs
30 235 in these cells under these CO₂ treatments account for more than 91% of the total fatty acids. They were
31 236 significantly higher ($p < 0.05$) than 0.04% air grown cultures , reaching the average value (*ca.* 41%) under
32 237 higher CO₂ treatments due to a high content of palmitoleic (*ca.* 29%) and oleic acids (*ca.* 9%) (Table 4).
33
34 238 Regarding the polyunsaturated fatty acids (PUFAs), there was a significant decrease in 1 and 5% CO₂-
35 239 treated cells compared to air grown cultures. In particular, PUFA levels decreased significantly due to the
36 240 decline in the proportion of eicosapentaenoic acid, from 5.3% (air) to 4.5% (1%) and 2.2% (5%) of CO₂
37 241 (Table 4).
38
39 242 **Discussion**
40
41 243 This study clearly indicates that the marine diatom *T. pseudonana* could tolerate elevated CO₂
42 244 concentrations and simultaneously, produce lipids suitable for biodiesel production. In spite of extensive
43 245 studies performed on this species, there is still no information on the possible effects of increasing CO₂
44 246 concentrations on lipid accumulation, which, if demonstrated, could allow a significant advancement for
45 247 the biotechnological use of this alga. A recent review summarized how elevated or high CO₂

concentrations could affected the diatoms growth and physiology (Gao and Campbell 2014). Some Authors reported that they could have positive effects on diatoms growth (Wu et al. 2010; McCarthy et al. 2012; Wang et al. 2014), while others observed neutral (Crawfurd et al. 2011; Gao et al. 2012) or negative effects (Torstensson et al. 2012; Mejia et al. 2013). However, these conflicting results could be attributed to the varied experimental conditions such as the exposure to different light intensities (Li and Campbell 2013), pH (Wu et al. 2010), temperature (Torstensson et al. 2012), but also to the physiological complexity of algal responses to elevated CO₂ concentrations (Wu et al. 2010; Yang and Gao 2012). In this study, the addition of 5% CO₂ increased the diatom growth rates by 15% and biomass concentration by 75%. This was in agreement with such literature data, which reported a positive effect of elevated CO₂ concentrations on *T. pseudonana* growth (Yang and Gao 2012; McCarthy et al. 2012; Li and Campbell 2013). Interestingly, observations on diatom cell size during the experiment showed remarkable changes in response to the CO₂ treatments. At the end of the experiment, the cell volume achieved by both cultures enriched with CO₂ was found 2 times higher than that of cell cultivated with 0.04% CO₂. These results are coherent with previous studies, which found an evident increase in cell size in centric diatom cultivated under high CO₂ levels (Hoogstraten et al. 2012; Li and Campbell 2013).

The dissolved inorganic carbon (TIC) in culture medium represents the carbon sources for the microalgal growth. As expected, the cultivation of *T. pseudonana* under increasing CO₂ concentrations resulted in high TIC values (Tang et al. 2011). Moreover, in this study, the cultivation with the highest CO₂ resulted also in higher TOC concentrations in culture medium, which could be attributed to the frequently extracellular release of algal exudates during microalgal growth (Engel 2002; Hulatt and Thomas 2010). It has long been known that the inorganic carbon system is closely related to the pH, as it is the main buffering system in the culture medium. In this study, the higher CO₂ concentrations tested have led to higher diatom biomass productivities as compared to 0.04% air grown cultures, but also caused a decrease in the pH. Low pH values could be attributed to the buffering properties of the carbonate system (Wu et al. 2010; Yang and Gao 2012; Gao and Campbell 2014). In order to evaluate if the cultivation in presence of elevated CO₂ concentrations could also affect the photosynthetic activity of the diatom, the photosynthetic pigment content were also analysed at the end of the cultivation period. The overall higher pigment content of cells of *T. pseudonana* grown under increasing CO₂ concentrations as compared to 0.04% air grown cultures was more related to photoacclimation of cultures or as a consequence of the increase of cell volume rather than increase in CO₂ availability (Crawfurd et al. 2011; Baldisserto et al. 2012; Li and Campbell 2013). In fact, no significant effects on pigment content of this diatom species were observed after a long period of adaptation to elevated CO₂ concentrations (Crawfurd et al. 2011; McCarthy et al. 2012; Li and Campbell 2013).

The effects of elevated CO₂ concentrations on fatty acids composition and content of microalgae have been already reported (Muradyan et al. 2004; Tang et al. 2011; Trobajo et al. 2014; Wang et al. 2014). Nile Red fluorescence has been widely accepted as a valid method for analyzing TAGs in algal cultures (Yu et al. 2009; Giovanardi et al. 2014; Sabia et al. 2015; Baldisserto et al. 2016). Accordingly, in this study, as revealed by Nile Red staining, when 1 and 5% CO₂-treated cells were in stationary phase (4th 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
7 292 Several studies showed that diatoms accumulated lipids as a result of N limitation and increased the
8 293 proportion of TAGs in the stationary phase of growth (Yu et al. 2009; Hildebrand et al. 2012; d’Ippolito
9 294 et al. 2015).
10 295 For biodiesel purposes, fatty acid profiles rich in monounsaturated acids (MUFAs), which can be
11 296 transesterified to produce biodiesel (Hu et al. 2008), are the most interesting profiles. Biodiesel, which is
12 297 produced by the trans-esterification of triglycerides with methanol to yield the corresponding mono-alkyl
13 298 fatty acid esters, is an alternative to petroleum-based diesel fuel (Hu et al. 2008). The properties of biodiesel
14 299 are strongly determined by the characteristics of fatty acid chains, such as carbon chain length and
15 300 unsaturation extent, present in the fuel (Hu et al. 2008; Nascimento et al. 2015, Manju et al. 2017). For
16 301 example, saturated chains produce a biodiesel with superior oxidative stability and a higher cetane index
17 302 but rather poor low-temperature properties. In contrast, polyunsaturated fatty acids have good cold-flow
18 303 properties but are particularly susceptible to oxidation (Hu et al. 2008; Nascimento et al. 2015).
19
20 304 Interestingly, the increase in CO₂ concentration resulted in an increase of monounsaturated acids
21 305 (palmitoleic and oleic) and a decrease of the saturated myristic acid and polyunsaturated fatty acids
22 306 (Table 4), suggesting that from the point of view of biodiesel production air enriched with CO₂ produces
23 307 better distribution of fatty acid chains. The increase in unsaturated fatty acids and decrease in saturated
24 308 fatty acids has previously been observed in Chlorophyceae species at increasing CO₂ concentrations up to
25 309 5% (Tang 2011; Nascimento et al. 2015). However, this trend did not appear in cultures of the same or
26 310 other species in other works (Tsuzuki 1990; Muradyan et al. 2004). In this study, it is not observed a big
27 311 difference in the fatty acid chain profile attending the use of 1% or 5% CO₂ enriched air (Table 4).
28 312 Despite of this, the evident characteristic of the fatty acid composition of *T. pseudonana* grown under 1-
29 313 5% CO₂ is that the major fatty acids were C14–C18, and these account for more than 91% of the total
30 314 fatty acids. The predominance of shorter-chain fatty acids (defined as alkyl chains with 12–18 carbon
31 315 atoms) is very significant for the potential of *T. pseudonana* for the production of biodiesel (Hu et al.
32 316 2008).
33
34 317 It has been reported that an increase in CO₂ concentration in culture medium tends to increase the
35 318 accumulation of polyunsaturated fatty acids in the microalgal cells (Lam et al. 2012). However, compared
36 319 to previous results (Tang et al. 2011; Wang et al. 2014), *T. pseudonana* showed a decrease of the
37 320 production of eicosapentaenoic (C20:5) acids (C20:5) from 5.3% (air) to 4.5% (1%) and 2.2% (5%) of
38 321 CO₂. Despite, the cultivation with high levels of CO₂ showed a notable reduction of polyunsaturated fatty
39 322 acids, this is still not enough to accomplish the biodiesel standards described in the EN14214 norm.
40
41 323 The increase of lipid content, the raise of monounsaturated and the reduction of polyunsaturated fatty
42 324 acids when using 5% CO₂ may have great potential for the production of an ignition-quality biofuel with
43 325 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.
8
9
10
11
12
13
14
15

Acknowledgements

16 335 This work was supported by a research fellowship “Bando Giovani Ricercatori 2015” granted by the
17 336 University of Ferrara (Italy) to A.S. and by a fellowship granted by the University Institute of High
18 337 Studies (IUSS) 1391 of Ferrara for PhD student’s mobility to A.S.. This work was also supported by the
19 338 University of Ferrara through the Fondo per l’Incentivazione alla Ricerca (FIR 2016) granted to S.P..
20 339
21 340 This work was supported by the projects CTQ2014-56285-R “Cultivo, concentración, fraccionamiento y
22 341 obtención de producto en refinería de microalgas” funded by the Spanish Ministry of Economy and
23 342 Competitiveness and “Fuels from Biomass” funded by Excma. Diputació de Tarragona.
24 343 The research was also supported by the European Regional Development Funds (ERDF, FEDER
25 344 Programa Competitividad de Catalunya 2007-2013).
26
27
28
29
30
31
32
33
34
35
36
37
38

Conflict of interest

348 The authors declare they have no conflict of interest.

Compliance with Ethical Standards

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

42 353
43 354
44 355
45 356
46 357
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64

1 358 **References**

2 359

- 3 360 Andersen RA (2005) Traditional microalgae isolation techniques In: Andersen RA (Ed), Algal culturing
4 361 techniques, Academic press, pp. 269-285
- 5 362 Armbrust EV, Berges JA, Bowler C, Green BR, Martinez D, Putnam NH, Zhou S, Allen AE, Apt
6 363 KE, Bechner M, Brzezinski MA, Chaal BK, Chiovitti A, Davis AK, Demarest MS, Detter JK,
7 364 Glavina T, Goodstein D, Hadi MZ, Hellsten U, Hildebrand M, Jenkins BD, Jurka J, Kapitonov VV,
8 365 Kröger N, Lau WWY, Lane TW, Larimer FW, Lippmeier JC, Lucas S, Medina M, Montsant A,
9 366 Obornik M, Parker MS, Palenik B, Pazour GJ, Richardson MP, Rynearson TA, Saito MA, Schwartz
10 367 DC, Thamatrakoln K, Valentin K, Vardi A, Wilkerson FP, Rokhsa DS (2004) The genome of the
11 368 diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. *Science* 306:79-86
- 12 369 Baldissarotto C, Ferroni L, Giovanardi M, Boccaletti L, Pantaleoni L, Pancaldi S (2012) Salinity promotes
13 370 growth of freshwater *Neochloris oleoabundans* UTEX 1185 (*Sphaeropleales, Chlorophyta*):
14 371 morphophysiological aspects. *Phycol* 51:700-710
- 15 372 Baldissarotto C, Popovich C, Giovanardi M, Sabia A, Ferroni L, Constenla D, Leonardi P, Pancaldi S
16 373 (2016) Photosynthetic aspects and lipid profiles in the mixotrophic alga *Neochloris oleoabundans* as
17 374 useful parameters for biodiesel production. *Algal Res* 16:255-265
- 18 375 Bligh EG, Dyer WM (1959) A rapid method of lipid extraction and purification. *Can J Biochem Physiol*
19 376 37:911-917
- 20 377 Cheah WY, Show PL, Chang JS, Ling TC, Juan JC (2015) Biosequestration of atmospheric CO₂ and flue
21 378 gas-containing CO₂ by microalgae. *Bioresource Technol* 184:190-201
- 22 379 Chen W, Zhang C, Song L, Sommerfeld M, Hu Q (2009) A high throughput Nile red method for
23 380 quantitative measurement of neutral lipids in microalgae. *J Microbiol Methods* 77:41-47
- 24 381 Chisti Y (2007) Biodiesel from microalgae. *Biotech Adv* 25:294-306
- 25 382 Crawfurd KJ, Raven JA, Wheeler GL, Baxter EJ, Joint I (2011) The response of *Thalassiosira pseudonana*
26 383 to long-term exposure to increased CO₂ and decreased pH. *PloS one* 6:e26695-e26695
- 27 384 d'Ippolito G, Sardo A, Paris D, Vella FM, Adelfi MG, Botte P, Gallo C, Fontana A (2015) Potential of
28 385 lipid metabolism in marine diatoms for biofuel production. *Biotechnol Biofuels* 8:1-28
- 29 386 Doan TTY, Sivaloganathan B, Obbard JP (2011) Screening of marine microalgae for biodiesel feedstock.
30 387 *Biomass Bioenergy* 35:2534-44
- 31 388 Engel A (2002) Direct relationship between CO₂ uptake and transparent exopolymer particles production in
32 389 natural phytoplankton. *J Plankton Res* 24:49-53
- 33 390 Field CB, Behrenfeld MJ, Randerson JT, Falkowski P (1998) Primary production of the biosphere:
34 391 integrating terrestrial and oceanic components. *Science* 281:237-240
- 35 392 Francisco EC, Neves DB, Jacob-Lopes E, Franco TT (2010) Microalgae as feedstock for biodiesel
36 393 production: carbon dioxide sequestration, lipid production and biofuel quality. *J Chem Technol Biot*
37 394 85:395-403
- 38 395 Gao K, Campbell DA (2014) Photophysiological responses of marine diatoms to elevated CO₂ and
39 396 decreased pH: a review. *Funct Plant Biol* 41:449-459

- 1 397 Gao K, Xu J, Gao G, Li Y, Hutchins DA, Huang B, Wang L, Zheng Y, Jin P, Cai X, Häder DP, Li W, Xu
2 398 K, Liu N, Riebesell U (2012) Rising CO₂ and increased light exposure synergistically reduce marine
3 399 primary productivity. *Nat Clim Change* 2:519-523
4 400 Giovanardi M, Baldissarroto C, Ferroni L, Longoni P, Cella R, Pancaldi S (2014) Growth and lipid
5 401 synthesis promotion in mixotrophic *Neochloris oleoabundans* (*Chlorophyta*) cultivated with
6 402 glucose. *Protoplasma* 251:115-125
7 403 Guillard RRL, Ryther JH (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and
8 404 *Detonula confervacea* (Cleve). *Gran Can J Microbiol* 8:229-239
9 405 Hempel N, Petrick I, Behrendt F (2012) Biomass productivity and productivity of fatty acids and amino
10 406 acids of microalgae strains as key characteristics of suitability for biodiesel production. *J Appl*
11 407 *Phycol* 24:1407-1418
12 408 Hildebrand D, Smith SR, Traller JC, Abbriano R (2012) The place of diatoms in the biofuels industry.
13 409 *Biofuels* 3:221-240
14 410 Hoogstraten A, Timmermans KR, de Baar HJW (2012) Morphological and physiological effects in
15 411 *Proboscia alata* (*Bacillariophyceae*) grown under different light and CO₂ conditions of the modern
16 412 southern ocean. *J Phycol* 48:559–568
17 413 Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008) Microalgal
18 414 triacylglycerols asa feedstocks for biofuel production: perspective and advances. *Plant J* 54:621-639
19 415 Hulatt CJ, Thomas DN (2010) Dissolved organic matter (DOM) in microalgal photobioreactors: a potential
20 416 loss in solar energy conversion?. *Bioresource Technol* 101:8690-8697
21 417 Lam MK, Lee KT, Mohamed AR (2012) Current status and challenges on microalgae-based carbon
22 418 capture. *Int J Greenhouse Gas Control* 10:456-469
23 419 Lee JY, Yoo C, Jun SY, Ahn CY, Oh HM (2010) Comparison of several methods for effective lipid
24 420 extraction from microalgae. *Bioresour Technol* 101:S75-S77
25 421 Li G, Campbell DA (2013) Rising CO₂ interacts with growth light and growth rate to alter photosystem II
26 422 photoinactivation of the coastal diatom *Thalassiosira pseudonana*. *PLoS One* 8:e55562
27 423 Maity JP, Bundschuh J, Chen CY, Bhattacharya P (2014) Microalgae for third generation biofuel
28 424 production, mitigation of greenhouse gas emissions and wastewater treatment: Present and future
29 425 perspectives - A mini review. *Energy* 78:104-113
30 426 McCarthy A, Rogers SP, Duffy SJ, Campbell DA (2012) Elevated carbon dioxide differentially alters the
31 427 photophysiology of *Thalassiosira pseudonana* (*Bacillariophyceae*) and *Emiliania huxleyi*
32 428 (haptophyta)¹. *J Phycol* 48:635-646
33 429 Manju MJ, Renjith KR, John G, Nair SM, Chandramohanakumar N (2017) Biodiesel prospective of five
34 430 diatom strains using growth parameters and fatty acid profiles. *Biofuels*, 8:81-89
35 431 Mejia LM, Isensee K, Méndez-Vicente A, Pisonero J, Shimizu N, González C, Monteleone B, Stoll H
36 432 (2013) B content and Si/C ratios from cultured diatoms (*Thalassiosira pseudonana* and
37 433 *Thalassiosira weissflogii*): relationship to seawater pH and diatom carbon acquisition. *Geochim*
38 434 *Cosmochim Ac* 123:322-337
39 435 Mondal M, Goswami S, Ghosh A, Oinam G, Tiwari ON, Das P, Gayen K, Mandal MK, Halder GN
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1 436 (2017) Production of biodiesel from microalgae through biological carbon capture: a review. 3
2 437 Biotech, 7:99
3
4 438 Muradyan EA, Klyachko-Gurvich GL, Tsoglin LN, Sergeyenko TV, Pronina NA (2004) Changes in lipid
5 metabolism during adaptation of the *Dunaliella salina* photosynthetic apparatus to high CO₂
6 concentration. Russ J Plant Physl 51:53-62
7
8 441 Nascimento IA, Dominguez Cabanelas IT, Nunes dos Santos J, Nascimento MA, Sousa L, Sansone G
9
10 442 (2015) Biodiesel yields and fuel quality as criteria for algal-feedstock selection: Effects of CO₂-
11 supplementation and nutrient levels in cultures. Algal Res 8:53-60
12
13 444 Popovich CA, Damiani C, Constenla D, Leonardi PI (2012) Lipid quality of the diatoms *Skeletonema*
14
15 445 *costatum* and *Navicula gregaria* from the South Atlantic Coast (Argentina): evaluation of its
16 suitability as biodiesel feedstock. J App Phycol 24:1-10
17
18 447 Ritchie RJ (2006) Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and
19 ethanol solvents. Photosynth Res 89:27-41
20
21 449 Sabia A, Baldisserotto C, Biondi S, Marchesini R, Tedeschi P, Maietti A, Giovanardi M, Ferroni L,
22
23 450 Pancaldi S (2015) Re-cultivation of *Neochloris oleoabundans* in exhausted autotrophic and
mixotrophic media: the potential role of polyamines and free fatty acids. Appl Microbiol Biotechnol
24 451 99:10597-10609
25
26
27 453 Shen Y (2014) Carbon dioxide bio-fixation and wastewater treatment via algae photochemical synthesis for
28 biofuels production. RSC Adv 4:49672-49722
29
30 455 Singh P, Kumari S, Guldhe A, Misra R, Rawat I, Bux F (2016) Trends and novel strategies for enhancing
31 lipid accumulation and quality in microalgae. Renew Sust Energ Rev 55:1-16
32
33 457 Sirin S, Clavero E, Salvadó J (2015) Efficient harvesting of *Chaetoceros calcitrans* for biodiesel
34 production. Environ Technol 36:1902-1912
35
36 459 Tang D, Han W, Li P, Miao X, Zhong J (2011) CO₂ biofixation and fatty acid composition of *Scenedesmus*
37
38 460 *obliquus* and *Chlorella pyrenoidosa* in response to different CO₂ levels. Bioresour Technol
39 461 102:3071-3076
40
41 462 Torstensson A, Chierici M, Wulff A (2012) The influence of increased temperature and carbon dioxide
42 levels on the benthic/sea ice diatom *Navicula directa*. Polar Biol 35:205–214
43
44 464 Trobajo R, Ibañez C, Clavero E, Salvadó J, Jørgensen SE (2014) Modelling the response of microalgae to
45 CO₂ addition. Ecol Model 294:42-50
46
47 466 Tsuzuki M, Ohnuma E, Sato N, Takaku T, Kawaguchi A (1990) Effects of CO₂ concentration during
48 growth on fatty-acid composition in microalgae. Plant Physiol 93:851-856
49
50 468 Vinayak V, Manoylov KM, Gateau H, Blanckaert V, Héault J, Pencréac'h G, Marchand J, Gordon R,
51
52 469 Schoefs B (2015) Diatom Milking: A Review and New Approaches. Mar Drugs 13:2629-2665
53
54 470 Wang XW, Liang JR, Luo CS, Chen CP, Gao YH (2014) Biomass, total lipid production, and fatty acid
55 composition of the marine diatom *Chaetoceros muelleri* in response to different CO₂ levels.
56
57 473 Wellburn AR (1994) The spectral determination of chlorophylls a and b, as well as total carotenoids, using
58 various solvents with spectrophotometer of different resolution. J Plant Physiol 144:307-313
59
60
61
62
63
64
65

- 1 475 Wu Y, Gao K, Riebesell U (2010) CO₂-induced seawater acidification affects physiological performance of
2 476 the marine diatom *Phaeodactylum tricornutum*. Biogeosc 7:2915-2923
3
4 477 Yang G, Gao K (2012) Physiological responses of the marine diatom *Thalassiosira pseudonana* to
5 478 increased pCO₂ and seawater acidity. Mar Environ Res 79:142-151
6
7 479 Yu ET, Zendejas FJ, Lane PD, Gaucher S, Simmons BA, Lane TW (2009) Triacylglycerol accumulation
8 480 and profiling in the model diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*
9 481 (*Bacillariophyceae*) during starvation. J Appl Phycol 21:669-681
10
11 482 Zendejas FJ, Benke PI, Lane PD, Simmons BA, Lane TW (2012) Characterization of the acylglycerols and
12 483 resulting biodiesel derived from vegetable oil and microalgae (*Thalassiosira pseudonana* and
13 484 *Phaeodactylum tricornutum*) Biotechnol Bioeng 109:1146-1154
14
15 485 Zhu CJ, Lee YK (1997) Determination of biomass dry weight of marine microalgae. J Appl Phycol 9:189-
16
17 486 194
18
19 487 Zienkiewicz K, Du ZY, Ma W, Vollheyde K, Benning C (2016) Stress-induced neutral lipid
20 488 biosynthesis in microalgae—Molecular, cellular and physiological insights. Biochim Biophys
21 489 Acta (BBA)-Molecular and Cell Biology of Lipids, 1861:1269-1281
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 490 **Figure captions**
2 491
3
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.
6 (n=3), ANOVA p<0.05. Different superscripts denote significant (p<0.05) differences between
7 samples within each day of cultivation
8 495
9 496

10 496
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 represented as means ± s.d. (n=3), ANOVA p<0.05. Different superscripts denote significant (p<0.05)
14 differences between the samples
15 500
16 501
17 502
18 503
19 504
20 505
21 506
22 507
23 508
24 509
25 510
26 511
27 512
28 513
29 514
30 515
31 516
32 517
33 518
34 519
35 520
36 521
37 522
38 523
39 524
40 525
41 526
42 527
43 528
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 529 **Tables**

2 530

3
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 ±
6 s.d. (n=3), ANOVA p<0.05, means in columns for each parameter analysed followed by the same
7 533 superscripts are not significantly different at the 5% level according to Tukey's multiple comparison
8 534 test.
9 535

10 536

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

21 537

22 538

23 539

24 540

25 541

26 542 **Table 2.** The pH, dissolved inorganic carbon (TIC), dissolved organic carbon (TOC) and total nitrogen
27 543 (TN) concentration in the culture medium of *T. pseudonana* cultivated under different CO₂ concentrations
28 544 at 4th of cultivation. Values are means ± s.d. (n=3), ANOVA p<0.05, means in columns for each
29 545 parameter analysed followed by the same superscripts are not significantly different at the 5% level
30 546 according to Tukey's multiple comparison test.
31 547

32 548

33 549

34 550

35 551

36 552

37 553

38 554

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

39 555

40 556

41 557

42 558

43 559

44 560

45 561

46 562

47 563

48 564

49 565

50 566

51 567

52 568

53 569

54 570

55 571

56 572

57 573

58 574

59 575

60 576

61 577

62 578

63 579

64 580

65 581

1 555
 2 556
 3 557
 4 558
 5 559
 6
 7
 8
 9
 10
 11
 12
 13
 14
 15
 16
 17
 18
 19
 20
 21
 22
 23
 24
 25
 26
 27
 28
 29
 30
 31
 32
 33
 34
 35
 36
 37
 38
 39
 40
 41
 42
 43
 44
 45
 46
 47
 48
 49
 50
 51
 52
 53
 54
 55
 56
 57
 58
 59
 60
 61
 62
 63
 64
 65

Table 3. Photosynthetic pigments content of *T. pseudonana* cultivated under different CO₂ concentrations after 4 days of growth. Values are means \pm 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 ($\mu\text{g } 10^{-6} \text{ cells}$)				Chla /Chlc	Chl tot/Car
	Chla	Chlc	Car	Chl tot		
0.04	0.86 \pm 0.09 ^a	0.14 \pm 0.02 ^a	0.42 \pm 0.05 ^a	1.00 \pm 0.11 ^a	4.76 \pm 0.69 ^a	1.58 \pm 0.02 ^a
1	1.10 \pm 0.04 ^b	0.16 \pm 0.01 ^a	0.51 \pm 0.02 ^b	1.26 \pm 0.05 ^b	4.74 \pm 0.21 ^a	1.64 \pm 0.05 ^a
5	1.52 \pm 0.05 ^c	0.22 \pm 0.02 ^b	0.69 \pm 0.02 ^c	1.74 \pm 0.07 ^c	4.62 \pm 0.37 ^a	1.68 \pm 0.02 ^a

Table 4. Lipid content, Nile red fluorescence and fatty acid composition (each value represents the mean \pm 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 \pm 0.09	15.1 \pm 0.06	14.3 \pm 0.11
C15:0	1.8 \pm 0.12	1.4 \pm 0.04	1.2 \pm 0.07
C16:0	37.1 \pm 0.29	34.8 \pm 0.22	36.4 \pm 0.25
C16:1	23.1 \pm 0.13	29.2 \pm 0.28	28.4 \pm 0.08
C16:2	1.9 \pm 0.04	0.8 \pm 0.06	0.7 \pm 0.04
C16:3	2.6 \pm 0.06	0.7 \pm 0.02	0.6 \pm 0.02
C18:0	ND*	1.0 \pm 0.04	0.9 \pm 0.03
C18:1	3.2 \pm 0.15	8.0 \pm 0.12	10.2 \pm 0.17
C18:4	1.4 \pm 0.10	ND	ND
C20:5	5.3 \pm 0.26	4.5 \pm 0.05	2.2 \pm 0.09
C22:1	2.2 \pm 0.06	2.9 \pm 0.13	3.5 \pm 0.33
C22:2	ND	1.5 \pm 0.08	1.6 \pm 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

565 *ND, not detected

1 566
2 567
3 568
4 569
5 570
6 571
7 572
8 573
9 574
10 575
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Figure 1

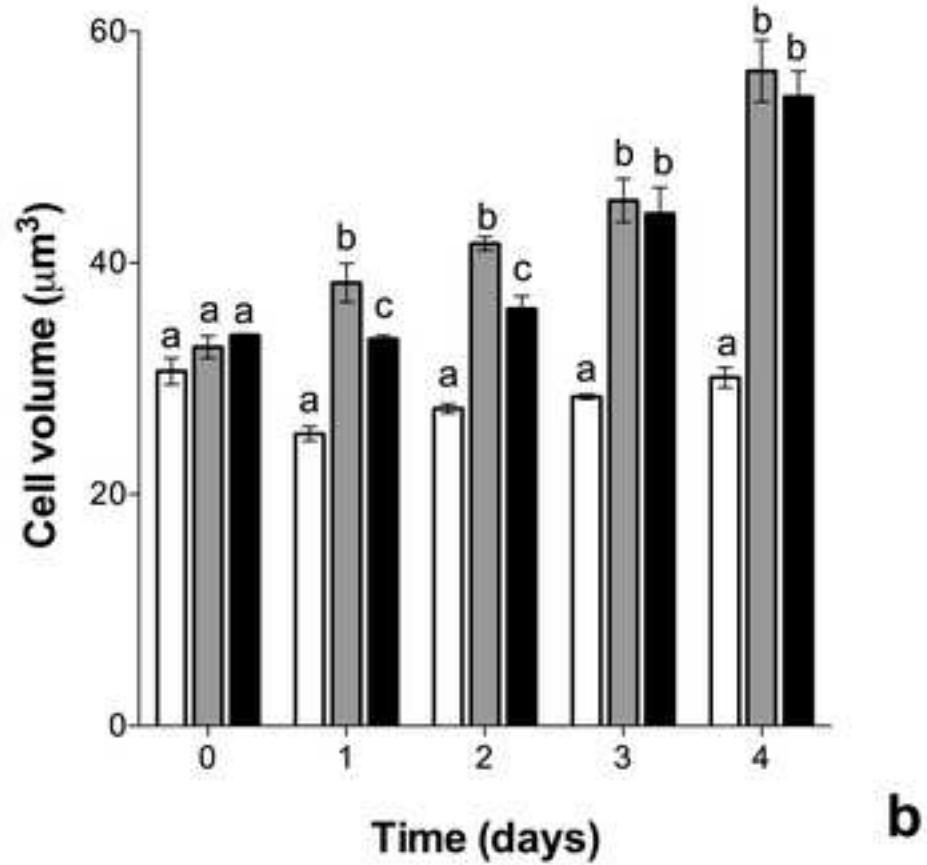
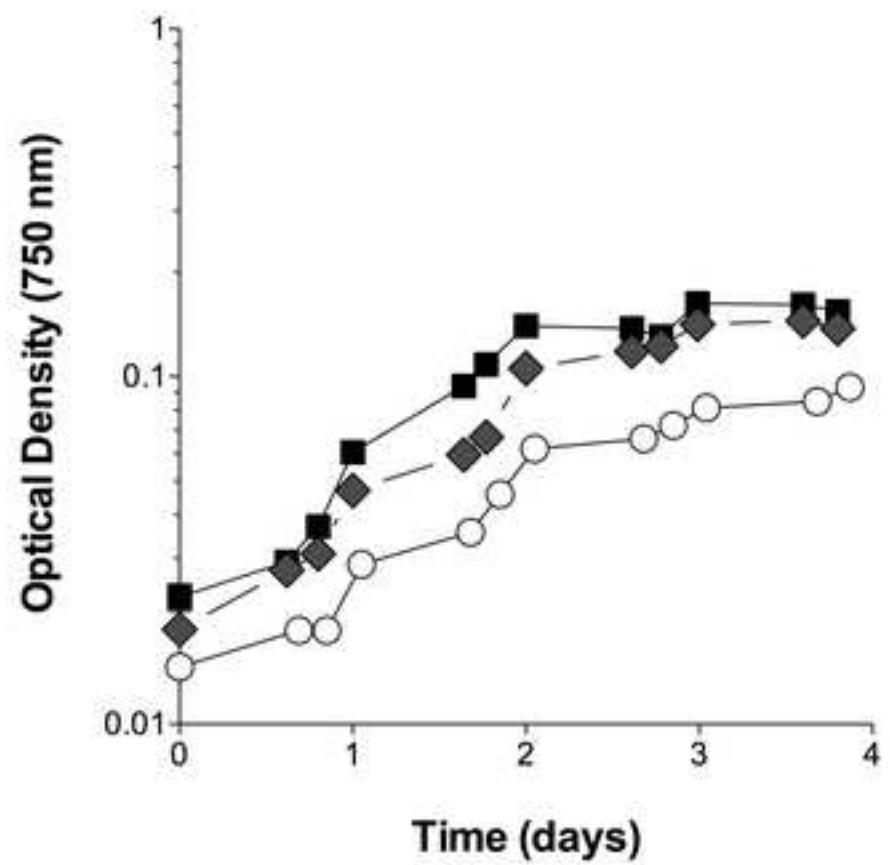
[Click here to download Figure Fig1.tif](#)

Figure 2

[Click here to download Figure Fig2.tif](#)