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

--Manuscript Draft--

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

Ferrara, 14th of December 2017

Dear Editor,

We are very grateful that our manuscript was very positively evaluated by reviewers, who we thank very much for their expert comments.
Please find our revised manuscript entitled "Effect of different CO2 concentrations on biomass, pigment content and lipid production of the marine diatom Thalassiosira pseudonana" by Alessandra Sabia, Esther Clavero, Simonetta Pancaldi and Joan 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

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Effect of different CO_2 concentrations on biomass, pigment content and lipid production of the marine diatom *Thalassiosira pseudonana*

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Keywords

Microalgae;
CO_2 concentrations;
Biomass productivity;
Fatty acid composition;
Biodiesel production.

Introduction

The global energy supply is drastically rising due to the continuous increase of modern industrialization. At the present, 90% of global energy demand is generated from fossil fuels and only 10% is fulfilled by renewable energy sources (Maity et al. 2014; Shen 2014). Based on the current projections, the total energy consumption is foreseen to increase year by year and by the year 2050, most of the fossil fuel reserves will be completely exhausted (Maity et al. 2014; Shen 2014). Besides this, the anthropogenic carbon dioxide (CO_2) emissions mainly come from the intensive burning of fossil fuels has contributed to a 40% increase in the atmospheric CO_2 levels, from 280 ppm in pre-industrial age to 400 ppm currently (Shen 2014; Cheah et al. 2015). Carbon dioxide, the principal greenhouse gas, is now widely considered as the major responsible of the global warming (Shen 2014; Cheah et al. 2015, Mondal et al. 2017). As a consequence of this global phenomenon, extensive studies have been focused on the development of renewable, carbon-neutral feedstock to displace petroleum and to mitigate anthropogenic emissions of CO_2 (Maity et al. 2014; Shen 2014, Singh et al. 2016, Mondal et al. 2017). In this perspective, microalgae have received much attention as renewable, energy-saving, sustainable approach to reduce the CO_2 emissions and simultaneously to produce biodiesel (Chisti 2007; Lam et al. 2012; Maity et al. 2014; Trobajo et al. 2014, Singh et al. 2016, Mondal et al. 2017). Microalgae have faster growth rates, higher photosynthetic efficiencies, higher rates of carbon dioxide fixation, and higher biomass and lipid productivities as compared to conventional oil crop plants (Chisti 2007; Sabia et al. 2015; Baldisserotto et al. 2016). Currently, extensive researches have been concerned the choice of the most adequate microalgal strain that could tolerate elevated CO_2 concentrations and, simultaneously, produce lipids suitable for biodiesel production (Lam et al. 2012; Trobajo et al. 2014; Nascimento et al. 2015). However, most of these microalgal strains studied for this purpose are freshwater species (Tang et al. 2011; Lam et al. 2012). With the aim of making the microalgal large-scale production increasingly sustainable, it is necessary to identify a species that can grow in saltwater to avoid the competition with freshwater resources (Doan et al. 2011; Baldisserotto et al. 2012). Marine diatoms may be considered as an attractive feedstock for combined CO_2 fixation and lipid production (Hildebrand et al. 2012; Wang et al. 2014; Vinayak et al. 2015, Manju et al. 2017). Diatoms, due to their high ~~er~~ trophic flexibility, are considered the most productive microalgae on the planet (Field et al. 1998; Hildebrand et al. 2012). These microorganisms, which are the main component of phytoplankton, significantly contribute to the global primary productivity and marine geochemical cycles (Field et al. 1998). Moreover, they are excellent lipid accumulators, especially triacylglycerides (TAGs), which are synthetized and accumulated in the stationary phase of growth (Hildebrand et al. 2012; d'Ippolito et al. 2015; Vinayak et al. 2015, Manju et al. 2017). It is well known that TAGs represent the best substrate for the biodiesel production (Chisti et al. 2007; Baldisserotto et al. 2016, Zienkiewicz et al. 2016). Nevertheless, only few studies have valorised the diatom lipid profile for biodiesel (Francisco et al. 2010; Zendejas et al. 2012~~1~~; Popovich et al. 2012, Manju et al. 2017). *Thalassiosira pseudonana*, a model diatom species, which genome has been recently sequenced (Armbrust et al. 2004), is one of the most studied species for the production of biofuels up to now (Yu et al. 2009; Zendejas et al. 2012~~1~~; d'Ippolito et al. 2015). In addition, recent studies have been concerned the effect of elevated CO_2 concentrations on growth and photophysiology of this diatom

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species (Crawfurd et al. 2011; Yang and Gao 2012; McCarthy et al. 2012; Li and Campbell 2013).

The present work is focused on the potential of the marine diatom *Thalassiosira pseudonana* as a promising ~~candidate~~ strain for the cultivation in the presence of elevated CO_2 concentrations. In particular, algal growth, cell morphology, photosynthetic pigment content, total nitrogen (TN), and dissolved inorganic (TIC) and organic carbon (TOC) content were monitored under increasing CO_2 concentrations (0.04 (ambient air), 1 and 5%). The lipid content and profile was also estimated in order to evaluate the effects of elevated CO_2 concentration on the composition of fatty acids useful for biodiesel production.

Materials and Methods

Algal strain and culture condition

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

T. pseudonana was inoculated in 6-L Erlenmeyer flasks with a 4-L working volume of filtered (0.45 µm) autoclaved seawater enriched with f/2 nutrients (Guillard and Ryther 1962) at 25±2°C and under an exposure to an irradiance of 250 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ provided by cool white fluorescent lamps (OSRAM L30W/865 Lumilux) under a cycle period of 16:8 h light-darkness. The *T. pseudonana* cultures were aerated at a rate of 250 mL min^{-1} (i.e., 0.0625 vvm, volume gas per volume media per min) of air enriched with CO_2 to obtain final concentration of 0.04, 1 and 5% CO_2. The air-CO_2 mixtures were obtained by combining rotameter flows of air and pure CO_2. The percentage of CO_2 in the air-CO_2 mixture was verified with an infrared analyzer (Servomex 4900C1). Experiments were performed in triplicate. Aliquots of cultures were collected at different times of cultivation, depending on the analysis.

Analyses

Growth, cell size and biomass evaluation

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

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

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

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

~~$P\ (mg_{AFDW} L^{-1} d^{-1}) = (X_t - X_0) / (t_t - t_0)$,~~

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

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

$P\ (mg_{AFDW} L^{-1} d^{-1}) = (X_1 - X_0) / (t_1 - t_0)$,

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

Photosynthetic pigment extraction and quantification

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

Biochemical analyses

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

Characterization of neutral lipids with Nile red

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

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

Lipid extraction

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

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

Methyl ester derivation and fatty acid analysis

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

Data treatment

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

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

Results

Effect of different CO_2 concentrations on T. pseudonana *growth*

In this study, as shown in Fig. 1a, the diatom *T. pseudonana* cultivated with 0.04 (ambient air), 1 and 5% CO_2 promptly entered the exponential phase and reached the stationary phase of growth during 4 days of cultivation. However, it was apparent from Table 1 that the highest CO_2 concentration increased the division rates of the diatom as compared to cells cultivated with 0.04 and 1% CO_2. Interestingly, at the end of the experiment, the biomass concentrations of cells cultivated with 1 and 5% CO_2 were 190 and 210 mg L^{-1} respectively, which were 1.58 and 1.75 times higher than that of air grown cultures (Table 1). Further, observations on diatom cell size during the experiment showed remarkable changes in response to the CO_2 treatments. In detail, a gradual but evident increasing trend was observed in cells cultivated with 1 and 5% CO_2 until the end of the experiment (Fig. 1b). Conversely, the cell volume of air grown cultures remained almost stable during the cultivation period. At 4th day of cultivation, the cell volume achieved by both cultures enriched with CO_2 was found 2 times higher than that of cell cultivated with 0.04% CO_2 (Fig. 1b). Therefore, these results reflected the higher biomass productivities achieved by cultures grown at 1 and 5% CO_2 (69 and 84%, ~~higher than 0.04% CO_2 treated cells,~~ respectively) ~~(Table 1)~~than 0.04% CO_2-treated cells (Table 1). As shown in Table 2, with the increase of CO_2 concentrations from 0.04% to 5%, the TIC concentration in the culture medium increased from 16.83 mg L^{-1} to 43.30 mg L^{-1} (+117 and 157% higher than air grown cultures in cells cultivated with 1% and 5% CO_2, respectively). In fact, despite, no great differences were reported between 0.04% and 1% of CO_2 treatments regarding TOC concentrations, the maximum TOC values in the culture medium were found under 5% CO_2 (14.63 mg L^{-1}, 19 and 17% higher than 0.04% and 1% CO_2-treated cells, respectively). On the other hand, higher CO_2 concentrations always resulted in lower pH. As shown in Table 2, the pH value of the culture medium decreased from pH 9.4 to pH 6.5 with the increase of CO_2 concentrations from 0.04% to 5%. Further, the TN concentration in the culture medium decreased by 12% and 30% in cells cultivated with 1% and 5% CO_2, respectively (Tab. 2).

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

In order to evaluate if the cultivation in presence of elevated CO_2 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 increase of CO_2 concentrations resulted in significant increases in Chl*a* and Car content as compared to those of air grown cultures (Table 3). In detail, Chl*a* concentrations in cells cultivated with 1% and 5% CO_2 were higher (28 and 77%, respectively) ~~higher~~ than 0.04% air grown

cultures. Despite, no great differences were reported between 0.04% and 1% of CO_2 treatments about Chl*c* content, the highest Chl*c* values were found in cells cultivated with 5% CO_2 (57 and 37% higher than air and 1% CO_2 grown cultures. About Car content, cells cultivated with 0.04% CO_2 contained ~~21 and 64%~~ lower quantities (21 and 64%, respectively) as compared to cultures enriched with elevated CO_2. The increases in total Chl content were greater than differences in the Car content, resulting in higher total Chl to Car molar ratios in cultures enriched with elevated CO_2 as compared to air grown cultures, but the ratios did not differ significantly at the 95% level. No significant effects on Chl*a* to *c* molar ratios were observed between the samples (Table 3).

Effect of different CO_2 concentrations on T. pseudonana *lipid quantification and characterisation*

In this study, to find a possible relation between CO_2 treatments and lipid synthesis, measurements of neutral lipid accumulation were performed by spectrofluorimetric quantification of the relative fluorescence intensity emitted by Nile Red-stained cells. After staining, cells were also observed by fluorescence microscopy. In detail, while cells cultivated with 0.04% CO_2 showed slightly positive reaction, the cultures enriched with elevated CO_2 accumulated abundant lipid droplets, giving an intense positive reaction with the fluorochrome (data not shown). Accordingly, in this study, as revealed by Nile Red staining, when 1 and 5% CO_2-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) and coincidently, decreased significantly the TN concentrations in culture medium (Table 2).

Nile red staining results were confirmed by Bligh and Dyer lipid extraction. Fluorescence values, normalized to the same cell concentration (A_{750} = 0.06), were proportional to the lipid content as % AFDW obtained with Bligh and Dyer extraction. The percentage of lipids in AFDW doubled when air was enriched with 1-5 % CO_2 from 7.3% (air) to 14.1% (1%) and 16.9% (5%) of CO_2 (Table 4).

The main fatty acid components of *T. pseudonana* under different CO_2 concentrations were determined by GC–MS analysis and are shown in Table 4. The predominant fatty acids were mirystic (C14:0), palmitic (C16:0), palmitoleic (C16:1), oleic (C18:1) acids (Table 4). Other fatty acids (C15:0, C16:2, C16:3, C18:0, C18:4, C20:5, C22:1 and C22:2) were minor components and presented concentration individually of less than 5%. However, the lipid classes showed differences in their proportions in response to the CO_2 treatments (Table 4). Despite, no great differences were reported between 1% (54.4%) and 5% (52.8%) of CO_2 treatments about the percentages of saturated fatty acids (SFAs), the highest SFAs values were found in cells cultivated with air (60.4%). The monounsaturated fatty acids (MUFAs) were the major class of fatty acids in cells cultivated ~~under~~ with 1% and 5% CO_2 ~~treatments~~. In detail, ~~, and~~ MUFAs in these cells under these CO_2 treatments account for more than 91% of the total fatty acids. They were significantly higher ($p<0.05$) than 0.04% air grown cultures ~~controls~~, reaching the average value (*ca.* 41%) under higher CO_2 treatments due to a high content of palmitoleic (*ca.* 29%) and oleic acids (*ca.* 9%) (Table 4). Regarding the polyunsaturated fatty acids (PUFAs), there was a significant decrease in 1 and 5% CO_2-treated cells compared to air grown cultures. In particular, PUFA levels

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decreased significantly due to the decline in the proportion of eicosapentaenoic acid, from 5.3% (air) to 4.5% (1%) and 2.2% (5%) of CO_2 (Table 4).

Discussion

This study clearly indicates that the marine diatom *T. pseudonana* could tolerate elevated CO_2 concentrations and simultaneously, produce lipids suitable for biodiesel production. In spite of extensive studies performed on this species, there is still no information on the possible effects of increasing CO_2 concentrations on lipid accumulation, which, if demonstrated, could allow a significant advancement for the biotechnological use of this alga. A recent review summarized how elevated or high CO_2 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 CO_2 concentrations (Wu et al. 2010; Yang and Gao 2012). In this study, the addition of 5% CO_2 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 CO_2 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 CO_2 treatments. At the end of the experiment, the cell volume achieved by both cultures enriched with CO_2 was found 2 times higher than that of cell cultivated with 0.04% CO_2. These results are coherent with previous studies, which found an evident increase in cell size in centric diatom cultivated under high CO_2 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 CO_2 concentrations resulted in high~~er~~ TIC values (Tang et al. 2011). Moreover, in this study, the cultivation with the highest CO_2 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 CO_2 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~~er~~ 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 CO_2 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 CO_2 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 CO_2 availability (Crawfurd et al. 2011; Baldisserotto 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 CO_2 concentrations (Crawfurd et al. 2011; McCarthy et al. 2012; Li and Campbell 2013).

The effects of elevated CO_2 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; Baldisserotto et al. 2016). Accordingly, in this study, as revealed by Nile Red staining, when 1 and 5% CO_2-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). The kinetics of lipid production in each CO_2 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 CO_2 enriched cultures and air grown cultures was remarkable. On the other hand, CO_2 enriched cultures, ~~and coincidently,~~ also presented ~~decreased significantly the~~lower TN concentrations in culture medium (Table 2).

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

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

Interestingly, the increase in CO_2 concentration resulted in an increase of monounsaturated acids (palmitoleic and oleic) and a decrease of the saturated myristic acid and polyunsaturated fatty acids (Table 4), suggesting that from the point of view of biodiesel production air enriched with CO_2 produces better distribution of fatty acid chains. The increase in unsaturated fatty acids and decrease in saturated fatty acids has previously been observed in Chlorophyceae species at increasing CO_2 concentrations up to 5% (Tang 2011; Nascimento et al. 2015). However, this trend did not appear in cultures of the same or other species in other works (Tsuzuki 1990; Muradyan et al. 2004). In this study, it is not observed a big difference in the fatty acid chain profile attending the use of 1% or 5% CO_2 enriched air (Table 4). Despite of this, the evident characteristic of the fatty acid composition of *T. pseudon*ana grown under 1-5% CO_2 is that the major fatty acids were C14–C18, and these account for more than 91% of the total fatty acids. The predominance of shorter-chain fatty acids (defined as alkyl chains with 12–18 carbon

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atoms) is very significant for the potential of *T. pseudon*ana for the production of biodiesel (Hu et al. 2008).

It has been reported that an increase in CO_2 concentration in culture medium tends to increase the accumulation of polyunsaturated fatty acids in the microalgal cells (Lam et al. 2012). However, compared to previous results (Tang et al. 2011; Wang et al. 2014), *T. pseudon*ana showed a decrease of the production of eicosapentaenoic (C20:5) acids (C20:5) from 5.3% (air) to 4.5% (1%) and 2.2% (5%) of CO_2. Despite, the cultivation with high levels of CO_2 showed a notable reduction of polyunsaturated fatty acids, this is still not enough to accomplish the biodiesel standards described in the EN14214 norm.

The increase of lipid content, the raise of monounsaturated and the reduction of polyunsaturated fatty acids when using 5% CO_2 may have great potential for the production of an ignition-quality biofuel with cold-flow properties and oxidative stability.

In conclusion, results presented here demonstrate that the maximum growth rate, biomass concentration and productivity, cell volume and photosynthetic pigment content per cell were obtained when the marine diatom *T. pseudon*ana was cultivated under 5% CO_2 . Moreover, treatments with 5% CO_2 gives an increase of total and neutral lipid content, the raise of monounsaturated and the reduction of polyunsaturated fatty acids. These results suggest that *T. pseudon*ana under 5% CO_2 showed ~~the mosta~~ suitable fatty acid composition for the production of biodiesel.

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Conflict of interest

The authors declare they have no conflict of interest.

Compliance with Ethical Standards

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

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References

Andersen RA (2005) Traditional microalgae isolation techniques In: Andersen RA (Ed), Algal culturing techniques, Academic press, pp. 269-285

Armbrust EV, Berges JA, Bowler C, Green BR, Martinez D, Putnam NH, Zhou S, Allen AE, Apt KE, Bechner M, Brzezinski MA, Chaal BK, Chiovitti A, Davis AK, Demarest MS, Detter JK, Glavina T, Goodstein D, Hadi MZ, Hellsten U, Hildebrand M, Jenkins BD, Jurka J, Kapitonov VV, Kröger N, Lau WWY, Lane TW, Larimer FW, Lippmeier JC, Lucas S, Medina M, Montsant A, Obornik M, Parker MS, Palenik B, Pazour GJ, Richardson MP, Rynearson TA, Saito MA, Schwartz DC, Thamatrakoln K, Valentin K, Vardi A, Wilkerson FP, Rokhsa DS (2004) The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. Science 306:79-86

Baldisserotto C, Ferroni L, Giovanardi M, Boccaletti L, Pantaleoni L, Pancaldi S (2012) Salinity promotes growth of freshwater *Neochloris oleoabundans* UTEX 1185 (*Sphaeropleales, Chlorophyta*): morphophysiological aspects. Phycol 51:700-710

Baldisserotto C, Popovich C, Giovanardi M, Sabia A, Ferroni L, Constenla D, Leonardi P, Pancaldi S (2016) Photosynthetic aspects and lipid profiles in the mixotrophic alga *Neochloris oleoabundans* as useful parameters for biodiesel production. Algal Res 16:255-265

Bligh EG, Dyer WM (1959) A rapid method of lipid extraction and purification. Can J Biochem Physiol 37:911-917

Cheah WY, Show PL, Chang JS, Ling TC, Juan JC (2015) Biosequestration of atmospheric CO_2 and flue gas-containing CO_2 by microalgae. Bioresource Technol 184:190-201

Chen W, Zhang C, Song L, Sommerfeld M, Hu Q (2009) A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. J Microbiol Methods 77:41-47

Chisti Y (2007) Biodiesel from microalgae. Biotech Adv 25:294-306

Crawfurd KJ, Raven JA, Wheeler GL, Baxter EJ, Joint I (2011) The response of *Thalassiosira pseudonana* to long-term exposure to increased CO_2 and decreased pH. PloS one 6:e26695-e26695

d'Ippolito G, Sardo A, Paris D, Vella FM, Adelfi MG, Botte P, Gallo C, Fontana A (2015) Potential of lipid metabolism in marine diatoms for biofuel production. Biotecnol Biofuels 8:1-28

Doan TTY, Sivaloganathan B, Obbard JP (2011) Screening of marine microalgae for biodiesel feedstock. Biomass Bioenergy 35:2534-44

~~Durrett TP, Benning C, Ohlrogge J (2008) Plant triacylglycerols as feedstocks for the production of biofuels. Plant J 54:593–607~~

Engel A (2002) Direct relationship between CO_2 uptake and transparent exopolymer particles production in natural phytoplankton. J Plankton Res 24:49-53

Field CB, Behrenfeld MJ, Randerson JT, Falkowski P (1998) Primary production of the biosphere: integrating terrestrial and oceanic components. Science 281:237-240

Francisco EC, Neves DB, Jacob-Lopes E, Franco TT (2010) Microalgae as feedstock for biodiesel production: carbon dioxide sequestration, lipid production and biofuel quality. J Chem Technol Biot 85:395-403

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Gao K, Campbell DA (2014) Photophysiological responses of marine diatoms to elevated CO_2 and decreased pH: a review. Funct Plant Biol 41:449-459

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 K, Liu N, Riebesell U (2012) Rising CO_2 and increased light exposure synergistically reduce marine primary productivity. Nat Clim Change 2:519-523

Giovanardi M, Baldisserotto C, Ferroni L, Longoni P, Cella R, Pancaldi S (2014) Growth and lipid synthesis promotion in mixotrophic *Neochloris oleoabundans* (*Chlorophyta*) cultivated with glucose. Protoplasma 251:115-125

Guillard RRL, Ryther JH (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve). Gran Can J Microbiol 8:229-239

Hempel N, Petrick I, Behrendt F (2012) Biomass productivity and productivity of fatty acids and amino acids of microalgae strains as key characteristics of suitability for biodiesel production. J Appl Phycol 24:1407-1418

Hildebrand D, Smith SR, Traller JC, Abbriano R (2012) The place of diatoms in the biofuels industry. Biofuels 3:221-240

Hoogstraten A, Timmermans KR, de Baar HJW (2012) Morphological and physiological effects in *Proboscia alata* (*Bacillariophyceae*) grown under different light and CO_2 conditions of the modern southern ocean. J Phycol 48:559–568

Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008) Microalgal triacylglycerols asa feedstocks for biofuel production: perspective and advances. Plant J 54:621-639

Hulatt CJ, Thomas DN (2010) Dissolved organic matter (DOM) in microalgal photobioreactors: a potential loss in solar energy conversion?. Bioresource Technol 101:8690-8697

Lam MK, Lee KT, Mohamed AR (2012) Current status and challenges on microalgae-based carbon capture. Int J Greenhouse Gas Control 10:456-469

Lee JY, Yoo C, Jun SY, Ahn CY, Oh HM (2010) Comparison of several methods for effective lipid extraction from microalgae. Bioresour Technol 101:S75-S77

Li G, Campbell DA (2013) Rising CO_2 interacts with growth light and growth rate to alter photosystem II photoinactivation of the coastal diatom *Thalassiosira pseudonana*. PLoS One 8:e55562

Maity JP, Bundschuh J, Chen CY, Bhattacharya P (2014) Microalgae for third generation biofuel production, mitigation of greenhouse gas emissions and wastewater treatment: Present and future perspectives - A mini review. Energy 78:104-113

McCarthy A, Rogers SP, Duffy SJ, Campbell DA (2012) Elevated carbon dioxide differentially alters the photophysiology of *Thalassiosira pseudonana* (*Bacillariophyceae*) and *Emiliania huxleyi* (haptophyta)[1]. J Phycol 48:635-646

~~Mehta SK, Gaur JP (2005) Use of algae for removing heavy metal ions from wastewater: progress and prospects. Crit Rev Biotechnol 25:113–152~~

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

Mejia LM, Isensee K, Méndez-Vicente A, Pisonero J, Shimizu N, González C, Monteleone B, Stoll H

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(2013) B content and Si/C ratios from cultured diatoms (*Thalassiosira pseudonana* and *Thalassiosira weissflogii*): relationship to seawater pH and diatom carbon acquisition. Geochim Cosmochim Ac 123:322-337

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

Muradyan EA, Klyachko-Gurvich GL, Tsoglin LN, Sergeyenko TV, Pronina NA (2004) Changes in lipid metabolism during adaptation of the *Dunaliella salina* photosynthetic apparatus to high CO_2 concentration. Russ J Plant Physl 51:53-62

Nascimento IA, Dominguez Cabanelas IT, Nunes dos Santos J, Nascimento MA, Sousa L, Sansone G (2015) Biodiesel yields and fuel quality as criteria for algal-feedstock selection: Effects of CO_2-supplementation and nutrient levels in cultures. Algal Res 8:53-60

Popovich CA, Damiani C, Constenla D, Leonardi PI (2012) Lipid quality of the diatoms *Skeletonema costatum* and *Navicula gregaria* from the South Atlantic Coast (Argentina): evaluation of its suitability as biodiesel feedstock. J App Phycol 24:1-10

Ritchie RJ (2006) Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. Photosynth Res 89:27-41

Sabia A, Baldisserotto C, Biondi S, Marchesini R, Tedeschi P, Maietti A, Giovanardi M, Ferroni L, 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 99:10597-10609

Shen Y (2014) Carbon dioxide bio-fixation and wastewater treatment via algae photochemical synthesis for biofuels production. RSC Adv 4:49672-49722

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

Sirin S, Clavero E, Salvadó J (2015) Efficient harvesting of *Chaetoceros calcitrans* for biodiesel production. Environ Technol 36:1902-1912

Tang D, Han W, Li P, Miao X, Zhong J (2011) CO_2 biofixation and fatty acid composition of *Scenedesmus obliquus* and *Chlorella pyrenoidosa* in response to different CO_2 levels. Bioresour Technol 102:3071-3076

Torstensson A, Chierici M, Wulff A (2012) The influence of increased temperature and carbon dioxide levels on the benthic/sea ice diatom *Navicula directa*. Polar Biol 35:205–214

Trobajo R, Ibañez C, Clavero E, Salvadó J, Jørgensen SE (2014) Modelling the response of microalgae to CO_2 addition. Ecol Model 294:42-50

Tsuzuki M, Ohnuma E, Sato N, Takaku T, Kawaguchi A (1990) Effects of CO_2 concentration during growth on fatty-acid composition in microalgae. Plant Physiol 93:851-856

Vinayak V, Manoylov KM, Gateau H, Blanckaert V, Hérault J, Pencréac'h G, Marchand J, Gordon R, Schoefs B (2015) Diatom Milking: A Review and New Approaches. Mar Drugs 13:2629-2665

Wang XW, Liang JR, Luo CS, Chen CP, Gao YH (2014) Biomass, total lipid production, and fatty acid

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Formatted: English (United Kingdom)

composition of the marine diatom *Chaetoceros muelleri* in response to different CO_2 levels. Bioresource Technol 161:124-130

Wellburn AR (1994) The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometer of different resolution. J Plant Physiol 144:307-313

Wu Y, Gao K, Riebesell U (2010) CO_2-induced seawater acidification affects physiological performance of the marine diatom *Phaeodactylum tricornutum*. Biogeosc 7:2915-2923

Yang G, Gao K (2012) Physiological responses of the marine diatom *Thalassiosira pseudonana* to increased pCO2 and seawater acidity. Mar Environ Res 79:142-151

Yu ET, Zendejas FJ, Lane PD, Gaucher S, Simmons BA, Lane TW (2009) Triacylglycerol accumulation and profiling in the model diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* (*Bacilariophyceae*) during starvation. J Appl Phycol 21:669-681

Zendejas FJ, Benke PI, Lane PD, Simmons BA, Lane TW (2012) Characterization of the acylglycerols and resulting biodiesel derived from vegetable oil and microalgae (*Thalassiosira pseudonana* and *Phaeodactylum tricornutum*) Biotechnol Bioeng 109:1146-1154

Zhu CJ, Lee YK (1997) Determination of biomass dry weight of marine microalgae. J Appl Phycol 9:189-194

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

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Figure captions

Fig 1: (a) Growth curves and **(b)** cell volumes of *T. pseudonana* cultivated under 0.04% (white circles and bars), 1% (grey diamonds and bars) and 5% CO_2 (dark squares and bars). Values are means ± s.d. (n=3), ANOVA $p<0.05$. Different superscripts denote significant ($p<0.05$) differences between samples within each day of cultivation

Fig 2: Nile red fluorescence of 4 day cultures of *T. pseudonana* grown under 0.04% (white), 1% (grey) and 5% CO_2 (dark). Fluorescence is given in Relative Fluorescence Units (RFU). Values are represented as means ± s.d. (n=3), ANOVA $p<0.05$. Different superscripts denote significant ($p<0.05$) differences between the samples

Tables

Table 1. The division rates (k), biomass concentrations (X) and biomass productivities (BP) of *T. pseudonana* cultivated under different CO_2 concentrations at 4th of cultivation. Values are means ± s.d. (n=3), ANOVA $p<0.05$, means in columns for each parameter analysed followed by the same superscripts are not significantly different at the 5% level according to Tukey's multiple comparison test.

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

Table 2. The pH, dissolved inorganic carbon (TIC), dissolved organic carbon (TOC) and total nitrogen (TN) concentration in the culture medium of *T. pseudonana* cultivated under different CO_2 concentrations at 4th of cultivation. Values are means ± s.d. (n=3), ANOVA $p<0.05$, means in columns for each parameter analysed followed by the same superscripts are not significantly different at the 5% level according to Tukey's multiple comparison test.

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

570 **Table 3.** Photosynthetic pigments content of *T. pseudonana* cultivated under different CO_2 concentrations
571 after 4 days of growth. Values are means ± s.d. (n=3), ANOVA $p<0.05$, means in columns for each
572 photosynthetic pigment analysed followed by the same superscripts are not significantly different at the
573 5% level according to Tukey's multiple comparison test.
574

CO_2 concentration (%)	Pigment content (µg 10^{-6} cells)				Chl*a* /Chl*c*	Chl tot/Car
	Chl*a*	Chl*c*	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]

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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% CO_2) and air enriched with 1 or
579 5 % CO_2.

	0.04% CO_2	1% CO_2	5% CO_2
Fatty acid			
C14:0	21.4 ± 0.09	15.1 ± 0.06	14.3 ± 0.11
C15:0	1.8 ± 0.12	1.4 ± 0.04	1.2 ± 0.07
C16:0	37.1 ± 0.29	34.8 ± 0.22	36.4 ± 0.25
C16:1	23.1 ± 0.13	29.2 ± 0.28	28.4 ± 0.08
C16:2	1.9 ± 0.04	0.8 ± 0.06	0.7 ± 0.04
C16:3	2.6 ± 0.06	0.7 ± 0.02	0.6 ±0.02
C18:0	ND*	1.0 ± 0.04	0.9 ± 0.03
C18:1	3.2 ± 0.15	8.0 ± 0.12	10.2 ± 0.17
C18:4	1.4 ± 0.10	ND	ND
C20:5	5.3 ± 0.26	4.5 ± 0.05	2.2 ± 0.09
C22:1	2.2 ± 0.06	2.9 ± 0.13	3.5 ± 0.33
C22:2	ND	1.5 ± 0.08	1.6 ± 0.20
Saturated	60.4	52.4	52.8
Mono-unsaturated	28.5	40.2	42.1
Poly-unsaturated	11.2	7.5	5.1
Lipid content (% AFDW)	7.3	14.1	16.9
Nile Red fluorescence (RFU) at Abs_{750} =0.06	422	911	1051

580 *ND, not detected

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Effect of different CO_2 concentrations on biomass, pigment content and lipid production of the marine diatom *Thalassiosira pseudonana*

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Abstract

The marine diatom *Thalassiosira pseudonana* 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 $mg_{AFDW}L^{-1}d^{-1}$) than air grown cultures (with 1.13 div d^{-1} and 26 $mg_{AFDW}L^{-1}d^{-1}$). The increase of CO_2 resulted in higher cell volume and pigment content per cell of *T. pseudonana*. Interestingly, lipid content doubled when air was enriched with 1-5% CO_2. Moreover, the analysis of the fatty acid composition of *T. pseudonana* 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 *T. pseudonana* seems to be an ideal candidate for biodiesel production using flue gases.

Keywords

Microalgae;

CO_2 concentrations;

Biomass productivity;

Fatty acid composition;

Biodiesel production.

Introduction

The global energy supply is drastically rising due to the continuous increase of modern industrialization. At the present, 90% of global energy demand is generated from fossil fuels and only 10% is fulfilled by renewable energy sources (Maity et al. 2014; Shen 2014). Based on the current projections, the total energy consumption is foreseen to increase year by year and by the year 2050, most of the fossil fuel reserves will be completely exhausted (Maity et al. 2014; Shen 2014). Besides this, the anthropogenic carbon dioxide (CO_2) emissions mainly come from the intensive burning of fossil fuels has contributed to a 40% increase in the atmospheric CO_2 levels, from 280 ppm in pre-industrial age to 400 ppm currently (Shen 2014; Cheah et al. 2015). Carbon dioxide, the principal greenhouse gas, is now widely considered as the major responsible of the global warming (Shen 2014; Cheah et al. 2015, Mondal et al. 2017). As a consequence of this global phenomenon, extensive studies have been focused on the development of renewable, carbon-neutral feedstock to displace petroleum and to mitigate anthropogenic emissions of CO_2 (Maity et al. 2014; Shen 2014, Singh et al. 2016, Mondal et al. 2017). In this perspective, microalgae have received much attention as renewable, energy-saving, sustainable approach to reduce the CO_2 emissions and simultaneously to produce biodiesel (Chisti 2007; Lam et al. 2012; Maity et al. 2014; Trobajo et al. 2014, Singh et al. 2016, Mondal et al. 2017). Microalgae have faster growth rates, higher photosynthetic efficiencies, higher rates of carbon dioxide fixation, and higher biomass and lipid productivities as compared to conventional oil crop plants (Chisti 2007; Sabia et al. 2015; Baldisserotto et al. 2016). Currently, extensive researches have been concerned the choice of the most adequate microalgal strain that could tolerate elevated CO_2 concentrations and, simultaneously, produce lipids suitable for biodiesel production (Lam et al. 2012; Trobajo et al. 2014; Nascimento et al. 2015). However, most of these microalgal strains studied for this purpose are freshwater species (Tang et al. 2011; Lam et al. 2012). With the aim of making the microalgal large-scale production increasingly sustainable, it is necessary to identify a species that can grow in saltwater to avoid the competition with freshwater resources (Doan et al. 2011; Baldisserotto et al. 2012). Marine diatoms may be considered as an attractive feedstock for combined CO_2 fixation and lipid production (Hildebrand et al. 2012; Wang et al. 2014; Vinayak et al. 2015, Manju et al. 2017). Diatoms, due to their high trophic flexibility, are considered the most productive microalgae on the planet (Field et al. 1998; Hildebrand et al. 2012). These microorganisms, which are the main component of phytoplankton, significantly contribute to the global primary productivity and marine geochemical cycles (Field et al. 1998). Moreover, they are excellent lipid accumulators, especially triacylglycerides (TAGs), which are synthetized and accumulated in the stationary phase of growth (Hildebrand et al. 2012; d'Ippolito et al. 2015; Vinayak et al. 2015, Manju et al. 2017). It is well known that TAGs represent the best substrate for the biodiesel production (Chisti et al. 2007; Baldisserotto et al. 2016, Zienkiewicz et al. 2016). Nevertheless, only few studies have valorised the diatom lipid profile for biodiesel (Francisco et al. 2010; Zendejas et al. 2012; Popovich et al. 2012, Manju et al. 2017). *Thalassiosira pseudonana*, a model diatom species, which genome has been recently sequenced (Armbrust et al. 2004), is one of the most studied species for the production of biofuels up to now (Yu et al. 2009; Zendejas et al. 2012; d'Ippolito et al. 2015). In addition, recent studies have been concerned the effect of elevated CO_2 concentrations on growth and photophysiology of this diatom

species (Crawfurd et al. 2011; Yang and Gao 2012; McCarthy et al. 2012; Li and Campbell 2013).

The present work is focused on the potential of the marine diatom *Thalassiosira pseudonana* as a promising strain for the cultivation in the presence of elevated CO_2 concentrations. In particular, algal growth, cell morphology, photosynthetic pigment content, total nitrogen (TN), and dissolved inorganic (TIC) and organic carbon (TOC) content were monitored under increasing CO_2 concentrations (0.04 (ambient air), 1 and 5%). The lipid content and profile was also estimated in order to evaluate the effects of elevated CO_2 concentration on the composition of fatty acids useful for biodiesel production.

Materials and Methods

Algal strain and culture condition

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

T. pseudonana was inoculated in 6-L Erlenmeyer flasks with a 4-L working volume of filtered (0.45 µm) autoclaved seawater enriched with f/2 nutrients (Guillard and Ryther 1962) at 25±2°C and under an exposure to an irradiance of 250 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ provided by cool white fluorescent lamps (OSRAM L30W/865 Lumilux) under a cycle period of 16:8 h light-darkness. The *T. pseudonana* cultures were aerated at a rate of 250 mL min^{-1} (i.e., 0.0625 vvm, volume gas per volume media per min) of air enriched with CO_2 to obtain final concentration of 0.04, 1 and 5% CO_2. The air-CO_2 mixtures were obtained by combining rotameter flows of air and pure CO_2. The percentage of CO_2 in the air-CO_2 mixture was verified with an infrared analyzer (Servomex 4900C1). Experiments were performed in triplicate. Aliquots of cultures were collected at different times of cultivation, depending on the analysis.

Analyses

Growth, cell size and biomass evaluation

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

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

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

where k is the division rate, N_t the cell number at time t_t, N_0 the cell number at time 0 and $t_t - t_0$ the time

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

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

$P\ (mg_{AFDW}\,L^{-1}\,d^{-1}) = (X_1 - X_0) / (t_1 - t_0)$,

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

Photosynthetic pigment extraction and quantification

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

Biochemical analyses

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

Characterization of neutral lipids with Nile red

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

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

Lipid extraction

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

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

Methyl ester derivation and fatty acid analysis

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

Data treatment

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

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

Results

Effect of different CO_2 concentrations on T. pseudonana *growth*

In this study, as shown in Fig. 1a, the diatom *T. pseudonana* cultivated with 0.04 (ambient air), 1 and 5% CO_2 promptly entered the exponential phase and reached the stationary phase of growth during 4 days of cultivation. However, it was apparent from Table 1 that the highest CO_2 concentration increased the division rates of the diatom as compared to cells cultivated with 0.04 and 1% CO_2. Interestingly, at the end of the experiment, the biomass concentrations of cells cultivated with 1 and 5% CO_2 were 190 and 210 mg L^{-1} respectively, which were 1.58 and 1.75 times higher than that of air grown cultures (Table 1). Further, observations on diatom cell size during the experiment showed remarkable changes in response to the CO_2 treatments. In detail, a gradual but evident increasing trend was observed in cells cultivated with 1 and 5% CO_2 until the end of the experiment (Fig. 1b). Conversely, the cell volume of air grown cultures remained almost stable during the cultivation period. At 4^{th} day of cultivation, the cell volume achieved by both cultures enriched with CO_2 was found 2 times higher than that of cell cultivated with 0.04% CO_2 (Fig. 1b). Therefore, these results reflected the higher biomass productivities achieved by cultures grown at 1 and 5% CO_2 (69 and 84%, respectively) than 0.04% CO_2-treated cells (Table 1). As shown in Table 2, with the increase of CO_2 concentrations from 0.04% to 5%, the TIC concentration in the culture medium increased from 16.83 mg L^{-1} to 43.30 mg L^{-1} (+117 and 157% higher than air grown cultures in cells cultivated with 1% and 5% CO_2, respectively). In fact, despite, no great differences were reported between 0.04% and 1% of CO_2 treatments regarding TOC concentrations, the maximum TOC values in the culture medium were found under 5% CO_2 (14.63 mg L^{-1}, 19 and 17% higher than 0.04% and 1% CO_2-treated cells, respectively). On the other hand, higher CO_2 concentrations always resulted in lower pH. As shown in Table 2, the pH value of the culture medium decreased from pH 9.4 to pH 6.5 with the increase of CO_2 concentrations from 0.04% to 5%.

Further, the TN concentration in the culture medium decreased by 12% and 30% in cells cultivated with 1% and 5% CO_2, respectively (Tab. 2).

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

In order to evaluate if the cultivation in presence of elevated CO_2 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 increase of CO_2 concentrations resulted in significant increases in Chl*a* and Car content as compared to those of air grown cultures (Table 3). In detail, Chl*a* concentrations in cells cultivated with 1% and 5% CO_2 were higher (28 and 77%, respectively) than 0.04% air grown cultures. Despite, no great differences were reported between 0.04% and 1% of CO_2 treatments about Chl*c* content, the highest Chl*c* values were found in cells cultivated with 5% CO_2 (57 and 37% higher than air and 1% CO_2 grown cultures. About Car content, cells cultivated with 0.04% CO_2 contained lower quantities (21 and 64%, respectively) as compared to cultures enriched with elevated CO_2. The increases in total Chl content were greater than differences in the Car content, resulting in higher total Chl to Car molar ratios in cultures enriched with elevated CO_2 as compared to air grown cultures, but the ratios did not differ significantly at the 95% level. No significant effects on Chl*a* to *c* molar ratios were observed between the

samples (Table 3).

Effect of different CO_2 concentrations on T. pseudonana *lipid quantification and characterisation*

In this study, to find a possible relation between CO_2 treatments and lipid synthesis, measurements of neutral lipid accumulation were performed by spectrofluorimetric quantification of the relative fluorescence intensity emitted by Nile Red-stained cells. After staining, cells were also observed by fluorescence microscopy. In detail, while cells cultivated with 0.04% CO_2 showed slightly positive reaction, the cultures enriched with elevated CO_2 accumulated abundant lipid droplets, giving an intense positive reaction with the fluorochrome (data not shown). Accordingly, in this study, as revealed by Nile Red staining, when 1 and 5% CO_2-treated cells were in stationary phase (4^{th} day of cultivation) showed a 3-fold increase in lipid content with respect to air grown cultures (Fig. 2) and coincidently, decreased significantly the TN concentrations in culture medium (Table 2).

Nile red staining results were confirmed by Bligh and Dyer lipid extraction. Fluorescence values, normalized to the same cell concentration (A_{750} = 0.06), were proportional to the lipid content as % AFDW obtained with Bligh and Dyer extraction. The percentage of lipids in AFDW doubled when air was enriched with 1-5 % CO_2 from 7.3% (air) to 14.1% (1%) and 16.9% (5%) of CO_2 (Table 4).

The main fatty acid components of *T. pseudonana* under different CO_2 concentrations were determined by GC–MS analysis and are shown in Table 4. The predominant fatty acids were mirystic (C14:0), palmitic (C16:0), palmitoleic (C16:1), oleic (C18:1) acids (Table 4). Other fatty acids (C15:0, C16:2, C16:3, C18:0, C18:4, C20:5, C22:1 and C22:2) were minor components and presented concentration individually of less than 5%. However, the lipid classes showed differences in their proportions in response to the CO_2 treatments (Table 4). Despite, no great differences were reported between 1% (54.4%) and 5% (52.8%) of CO_2 treatments about the percentages of saturated fatty acids (SFAs), the highest SFAs values were found in cells cultivated with air (60.4%). The monounsaturated fatty acids (MUFAs) were the major class of fatty acids in cells cultivated with 1% and 5% CO_2. In detail, MUFAs in these cells under these CO_2 treatments account for more than 91% of the total fatty acids. They were significantly higher ($p<0.05$) than 0.04% air grown cultures , reaching the average value (*ca.* 41%) under higher CO_2 treatments due to a high content of palmitoleic (*ca.* 29%) and oleic acids (*ca.* 9%) (Table 4). Regarding the polyunsaturated fatty acids (PUFAs), there was a significant decrease in 1 and 5% CO_2-treated cells compared to air grown cultures. In particular, PUFA levels decreased significantly due to the decline in the proportion of eicosapentaenoic acid, from 5.3% (air) to 4.5% (1%) and 2.2% (5%) of CO_2 (Table 4).

Discussion

This study clearly indicates that the marine diatom *T. pseudonana* could tolerate elevated CO_2 concentrations and simultaneously, produce lipids suitable for biodiesel production. In spite of extensive studies performed on this species, there is still no information on the possible effects of increasing CO_2 concentrations on lipid accumulation, which, if demonstrated, could allow a significant advancement for the biotechnological use of this alga. A recent review summarized how elevated or high CO_2

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 CO_2 concentrations (Wu et al. 2010; Yang and Gao 2012). In this study, the addition of 5% CO_2 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 CO_2 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 CO_2 treatments. At the end of the experiment, the cell volume achieved by both cultures enriched with CO_2 was found 2 times higher than that of cell cultivated with 0.04% CO_2. These results are coherent with previous studies, which found an evident increase in cell size in centric diatom cultivated under high CO_2 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 CO_2 concentrations resulted in high TIC values (Tang et al. 2011). Moreover, in this study, the cultivation with the highest CO_2 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 CO_2 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 CO_2 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 CO_2 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 CO_2 availability (Crawfurd et al. 2011; Baldisserotto 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 CO_2 concentrations (Crawfurd et al. 2011; McCarthy et al. 2012; Li and Campbell 2013).

The effects of elevated CO_2 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; Baldisserotto et al. 2016). Accordingly, in this study, as revealed by Nile Red staining, when 1 and 5% CO_2-treated cells were in stationary phase (4^{th} day of cultivation) showed a 3-fold increase in lipid content with respect to air grown cultures (Fig. 2).

The kinetics of lipid production in each CO_2 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 CO_2 enriched cultures and air grown cultures was remarkable. On the other hand, CO_2 enriched cultures also presented lower TN concentrations in culture medium (Table 2).

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

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

Interestingly, the increase in CO_2 concentration resulted in an increase of monounsaturated acids (palmitoleic and oleic) and a decrease of the saturated myristic acid and polyunsaturated fatty acids (Table 4), suggesting that from the point of view of biodiesel production air enriched with CO_2 produces better distribution of fatty acid chains. The increase in unsaturated fatty acids and decrease in saturated fatty acids has previously been observed in Chlorophyceae species at increasing CO_2 concentrations up to 5% (Tang 2011; Nascimento et al. 2015). However, this trend did not appear in cultures of the same or other species in other works (Tsuzuki 1990; Muradyan et al. 2004). In this study, it is not observed a big difference in the fatty acid chain profile attending the use of 1% or 5% CO_2 enriched air (Table 4). Despite of this, the evident characteristic of the fatty acid composition of *T. pseudon*ana grown under 1-5% CO_2 is that the major fatty acids were C14–C18, and these account for more than 91% of the total fatty acids. The predominance of shorter-chain fatty acids (defined as alkyl chains with 12–18 carbon atoms) is very significant for the potential of *T. pseudon*ana for the production of biodiesel (Hu et al. 2008).

It has been reported that an increase in CO_2 concentration in culture medium tends to increase the accumulation of polyunsaturated fatty acids in the microalgal cells (Lam et al. 2012). However, compared to previous results (Tang et al. 2011; Wang et al. 2014), *T. pseudon*ana showed a decrease of the production of eicosapentaenoic (C20:5) acids (C20:5) from 5.3% (air) to 4.5% (1%) and 2.2% (5%) of CO_2. Despite, the cultivation with high levels of CO_2 showed a notable reduction of polyunsaturated fatty acids, this is still not enough to accomplish the biodiesel standards described in the EN14214 norm.

The increase of lipid content, the raise of monounsaturated and the reduction of polyunsaturated fatty acids when using 5% CO_2 may have great potential for the production of an ignition-quality biofuel with cold-flow properties and oxidative stability.

In conclusion, results presented here demonstrate that the maximum growth rate, biomass concentration and productivity, cell volume and photosynthetic pigment content per cell were obtained when the marine diatom *T. pseudon*ana was cultivated under 5% CO_2 . Moreover, treatments with 5% CO_2 gives an increase of total and neutral lipid content, the raise of monounsaturated and the reduction of polyunsaturated fatty acids. These results suggest that *T. pseudon*ana under 5% CO_2 showed a suitable fatty acid composition for the production of biodiesel.

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Conflict of interest

The authors declare they have no conflict of interest.

Compliance with Ethical Standards

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

References

Andersen RA (2005) Traditional microalgae isolation techniques In: Andersen RA (Ed), Algal culturing techniques, Academic press, pp. 269-285

Armbrust EV, Berges JA, Bowler C, Green BR, Martinez D, Putnam NH, Zhou S, Allen AE, Apt KE, Bechner M, Brzezinski MA, Chaal BK, Chiovitti A, Davis AK, Demarest MS, Detter JK, Glavina T, Goodstein D, Hadi MZ, Hellsten U, Hildebrand M, Jenkins BD, Jurka J, Kapitonov VV, Kröger N, Lau WWY, Lane TW, Larimer FW, Lippmeier JC, Lucas S, Medina M, Montsant A, Obornik M, Parker MS, Palenik B, Pazour GJ, Richardson MP, Rynearson TA, Saito MA, Schwartz DC, Thamatrakoln K, Valentin K, Vardi A, Wilkerson FP, Rokhsa DS (2004) The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. Science 306:79-86

Baldisserotto C, Ferroni L, Giovanardi M, Boccaletti L, Pantaleoni L, Pancaldi S (2012) Salinity promotes growth of freshwater *Neochloris oleoabundans* UTEX 1185 (*Sphaeropleales, Chlorophyta*): morphophysiological aspects. Phycol 51:700-710

Baldisserotto C, Popovich C, Giovanardi M, Sabia A, Ferroni L, Constenla D, Leonardi P, Pancaldi S (2016) Photosynthetic aspects and lipid profiles in the mixotrophic alga *Neochloris oleoabundans* as useful parameters for biodiesel production. Algal Res 16:255-265

Bligh EG, Dyer WM (1959) A rapid method of lipid extraction and purification. Can J Biochem Physiol 37:911-917

Cheah WY, Show PL, Chang JS, Ling TC, Juan JC (2015) Biosequestration of atmospheric CO_2 and flue gas-containing CO_2 by microalgae. Bioresource Technol 184:190-201

Chen W, Zhang C, Song L, Sommerfeld M, Hu Q (2009) A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. J Microbiol Methods 77:41-47

Chisti Y (2007) Biodiesel from microalgae. Biotech Adv 25:294-306

Crawfurd KJ, Raven JA, Wheeler GL, Baxter EJ, Joint I (2011) The response of *Thalassiosira pseudonana* to long-term exposure to increased CO_2 and decreased pH. PloS one 6:e26695-e26695

d'Ippolito G, Sardo A, Paris D, Vella FM, Adelfi MG, Botte P, Gallo C, Fontana A (2015) Potential of lipid metabolism in marine diatoms for biofuel production. Biotecnol Biofuels 8:1-28

Doan TTY, Sivaloganathan B, Obbard JP (2011) Screening of marine microalgae for biodiesel feedstock. Biomass Bioenergy 35:2534-44

Engel A (2002) Direct relationship between CO_2 uptake and transparent exopolymer particles production in natural phytoplankton. J Plankton Res 24:49-53

Field CB, Behrenfeld MJ, Randerson JT, Falkowski P (1998) Primary production of the biosphere: integrating terrestrial and oceanic components. Science 281:237-240

Francisco EC, Neves DB, Jacob-Lopes E, Franco TT (2010) Microalgae as feedstock for biodiesel production: carbon dioxide sequestration, lipid production and biofuel quality. J Chem Technol Biot 85:395-403

Gao K, Campbell DA (2014) Photophysiological responses of marine diatoms to elevated CO_2 and decreased pH: a review. Funct Plant Biol 41:449-459

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 K, Liu N, Riebesell U (2012) Rising CO_2 and increased light exposure synergistically reduce marine primary productivity. Nat Clim Change 2:519-523

Giovanardi M, Baldisserotto C, Ferroni L, Longoni P, Cella R, Pancaldi S (2014) Growth and lipid synthesis promotion in mixotrophic *Neochloris oleoabundans* (*Chlorophyta*) cultivated with glucose. Protoplasma 251:115-125

Guillard RRL, Ryther JH (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve). Gran Can J Microbiol 8:229-239

Hempel N, Petrick I, Behrendt F (2012) Biomass productivity and productivity of fatty acids and amino acids of microalgae strains as key characteristics of suitability for biodiesel production. J Appl Phycol 24:1407-1418

Hildebrand D, Smith SR, Traller JC, Abbriano R (2012) The place of diatoms in the biofuels industry. Biofuels 3:221-240

Hoogstraten A, Timmermans KR, de Baar HJW (2012) Morphological and physiological effects in *Proboscia alata* (*Bacillariophyceae*) grown under different light and CO_2 conditions of the modern southern ocean. J Phycol 48:559–568

Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008) Microalgal triacylglycerols asa feedstocks for biofuel production: perspective and advances. Plant J 54:621-639

Hulatt CJ, Thomas DN (2010) Dissolved organic matter (DOM) in microalgal photobioreactors: a potential loss in solar energy conversion?. Bioresource Technol 101:8690-8697

Lam MK, Lee KT, Mohamed AR (2012) Current status and challenges on microalgae-based carbon capture. Int J Greenhouse Gas Control 10:456-469

Lee JY, Yoo C, Jun SY, Ahn CY, Oh HM (2010) Comparison of several methods for effective lipid extraction from microalgae. Bioresour Technol 101:S75-S77

Li G, Campbell DA (2013) Rising CO_2 interacts with growth light and growth rate to alter photosystem II photoinactivation of the coastal diatom *Thalassiosira pseudonana*. PLoS One 8:e55562

Maity JP, Bundschuh J, Chen CY, Bhattacharya P (2014) Microalgae for third generation biofuel production, mitigation of greenhouse gas emissions and wastewater treatment: Present and future perspectives - A mini review. Energy 78:104-113

McCarthy A, Rogers SP, Duffy SJ, Campbell DA (2012) Elevated carbon dioxide differentially alters the photophysiology of *Thalassiosira pseudonana* (*Bacillariophyceae*) and *Emiliania huxleyi* (haptophyta)[1]. J Phycol 48:635-646

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

Mejia LM, Isensee K, Méndez-Vicente A, Pisonero J, Shimizu N, González C, Monteleone B, Stoll H (2013) B content and Si/C ratios from cultured diatoms (*Thalassiosira pseudonana* and *Thalassiosira weissflogii*): relationship to seawater pH and diatom carbon acquisition. Geochim Cosmochim Ac 123:322-337

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

Muradyan EA, Klyachko-Gurvich GL, Tsoglin LN, Sergeyenko TV, Pronina NA (2004) Changes in lipid metabolism during adaptation of the *Dunaliella salina* photosynthetic apparatus to high CO_2 concentration. Russ J Plant Physl 51:53-62

Nascimento IA, Dominguez Cabanelas IT, Nunes dos Santos J, Nascimento MA, Sousa L, Sansone G (2015) Biodiesel yields and fuel quality as criteria for algal-feedstock selection: Effects of CO_2-supplementation and nutrient levels in cultures. Algal Res 8:53-60

Popovich CA, Damiani C, Constenla D, Leonardi PI (2012) Lipid quality of the diatoms *Skeletonema costatum* and *Navicula gregaria* from the South Atlantic Coast (Argentina): evaluation of its suitability as biodiesel feedstock. J App Phycol 24:1-10

Ritchie RJ (2006) Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. Photosynth Res 89:27-41

Sabia A, Baldisserotto C, Biondi S, Marchesini R, Tedeschi P, Maietti A, Giovanardi M, Ferroni L, 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 99:10597-10609

Shen Y (2014) Carbon dioxide bio-fixation and wastewater treatment via algae photochemical synthesis for biofuels production. RSC Adv 4:49672-49722

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

Şirin S, Clavero E, Salvadó J (2015) Efficient harvesting of *Chaetoceros calcitrans* for biodiesel production. Environ Technol 36:1902-1912

Tang D, Han W, Li P, Miao X, Zhong J (2011) CO_2 biofixation and fatty acid composition of *Scenedesmus obliquus* and *Chlorella pyrenoidosa* in response to different CO_2 levels. Bioresour Technol 102:3071-3076

Torstensson A, Chierici M, Wulff A (2012) The influence of increased temperature and carbon dioxide levels on the benthic/sea ice diatom *Navicula directa*. Polar Biol 35:205–214

Trobajo R, Ibañez C, Clavero E, Salvadó J, Jørgensen SE (2014) Modelling the response of microalgae to CO_2 addition. Ecol Model 294:42-50

Tsuzuki M, Ohnuma E, Sato N, Takaku T, Kawaguchi A (1990) Effects of CO_2 concentration during growth on fatty-acid composition in microalgae. Plant Physiol 93:851-856

Vinayak V, Manoylov KM, Gateau H, Blanckaert V, Hérault J, Pencréac'h G, Marchand J, Gordon R, Schoefs B (2015) Diatom Milking: A Review and New Approaches. Mar Drugs 13:2629-2665

Wang XW, Liang JR, Luo CS, Chen CP, Gao YH (2014) Biomass, total lipid production, and fatty acid composition of the marine diatom *Chaetoceros muelleri* in response to different CO_2 levels. Bioresource Technol 161:124-130

Wellburn AR (1994) The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometer of different resolution. J Plant Physiol 144:307-313

Wu Y, Gao K, Riebesell U (2010) CO_2-induced seawater acidification affects physiological performance of the marine diatom *Phaeodactylum tricornutum*. Biogeosc 7:2915-2923

Yang G, Gao K (2012) Physiological responses of the marine diatom *Thalassiosira pseudonana* to increased pCO2 and seawater acidity. Mar Environ Res 79:142-151

Yu ET, Zendejas FJ, Lane PD, Gaucher S, Simmons BA, Lane TW (2009) Triacylglycerol accumulation and profiling in the model diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* (*Bacilariophyceae*) during starvation. J Appl Phycol 21:669-681

Zendejas FJ, Benke PI, Lane PD, Simmons BA, Lane TW (2012) Characterization of the acylglycerols and resulting biodiesel derived from vegetable oil and microalgae (*Thalassiosira pseudonana* and *Phaeodactylum tricornutum*) Biotechnol Bioeng 109:1146-1154

Zhu CJ, Lee YK (1997) Determination of biomass dry weight of marine microalgae. J Appl Phycol 9:189-194

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

Figure captions

Fig 1: (a) Growth curves and **(b)** cell volumes of *T. pseudonana* cultivated under 0.04% (white circles and bars), 1% (grey diamonds and bars) and 5% CO_2 (dark squares and bars). Values are means ± s.d. (n=3), ANOVA $p<0.05$. Different superscripts denote significant ($p<0.05$) differences between samples within each day of cultivation

Fig 2: Nile red fluorescence of 4 day cultures of *T. pseudonana* grown under 0.04% (white), 1% (grey) and 5% CO_2 (dark). Fluorescence is given in Relative Fluorescence Units (RFU). Values are represented as means ± s.d. (n=3), ANOVA $p<0.05$. Different superscripts denote significant ($p<0.05$) differences between the samples

Tables

Table 1. The division rates (k), biomass concentrations (X) and biomass productivities (BP) of *T. pseudonana* cultivated under different CO_2 concentrations at 4th of cultivation. Values are means ± s.d. (n=3), ANOVA $p<0.05$, means in columns for each parameter analysed followed by the same superscripts are not significantly different at the 5% level according to Tukey's multiple comparison test.

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

Table 2. The pH, dissolved inorganic carbon (TIC), dissolved organic carbon (TOC) and total nitrogen (TN) concentration in the culture medium of *T. pseudonana* cultivated under different CO_2 concentrations at 4th of cultivation. Values are means ± s.d. (n=3), ANOVA $p<0.05$, means in columns for each parameter analysed followed by the same superscripts are not significantly different at the 5% level according to Tukey's multiple comparison test.

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

Table 3. Photosynthetic pigments content of *T. pseudonana* cultivated under different CO_2 concentrations after 4 days of growth. Values are means ± s.d. (n=3), ANOVA $p<0.05$, means in columns for each photosynthetic pigment analysed followed by the same superscripts are not significantly different at the 5% level according to Tukey's multiple comparison test.

CO_2 concentration (%)	Pigment content (μg 10^{-6} cells)				Chl*a* /Chl*c*	Chl tot/Car
	Chl*a*	Chl*c*	Car	Chl tot		
0.04	0.86 ±0.09[a]	0.14 ±0.02[a]	0.42 ±0.05[a]	1.00 ±0.11[a]	4.76 ±0.69[a]	1.58 ± 0.02[a]
1	1.10 ± 0.04[b]	0.16 ± 0.01[a]	0.51 ±0.02[b]	1.26 ± 0.05[b]	4.74 ±0.21[a]	1.64 ± 0.05[a]
5	1.52 ± 0.05[c]	0.22 ±0.02[b]	0.69 ± 0.02[c]	1.74 ± 0.07[c]	4.62 ±0.37[a]	1.68 ± 0.02[a]

Table 4. Lipid content, Nile red fluorescence and fatty acid composition (each value represents the mean ± SD of two replicates) of *T. pseudonana* cultured at ambient air (0.04% CO_2) and air enriched with 1 or 5 % CO_2.

	0.04% CO_2	1% CO_2	5% CO_2
Fatty acid			
C14:0	21.4 ± 0.09	15.1 ± 0.06	14.3 ± 0.11
C15:0	1.8 ± 0.12	1.4 ± 0.04	1.2 ± 0.07
C16:0	37.1 ± 0.29	34.8 ± 0.22	36.4 ± 0.25
C16:1	23.1 ± 0.13	29.2 ± 0.28	28.4 ± 0.08
C16:2	1.9 ± 0.04	0.8 ± 0.06	0.7 ± 0.04
C16:3	2.6 ± 0.06	0.7 ± 0.02	0.6 ±0.02
C18:0	ND*	1.0 ± 0.04	0.9 ± 0.03
C18:1	3.2 ± 0.15	8.0 ± 0.12	10.2 ± 0.17
C18:4	1.4 ± 0.10	ND	ND
C20:5	5.3 ± 0.26	4.5 ± 0.05	2.2 ± 0.09
C22:1	2.2 ± 0.06	2.9 ± 0.13	3.5 ± 0.33
C22:2	ND	1.5 ± 0.08	1.6 ± 0.20
Saturated	60.4	52.4	52.8
Mono-unsaturated	28.5	40.2	42.1
Poly-unsaturated	11.2	7.5	5.1
Lipid content (% AFDW)	7.3	14.1	16.9
Nile Red fluorescence (RFU) at Abs_{750} =0.06	422	911	1051

*ND, not detected

Figure 1

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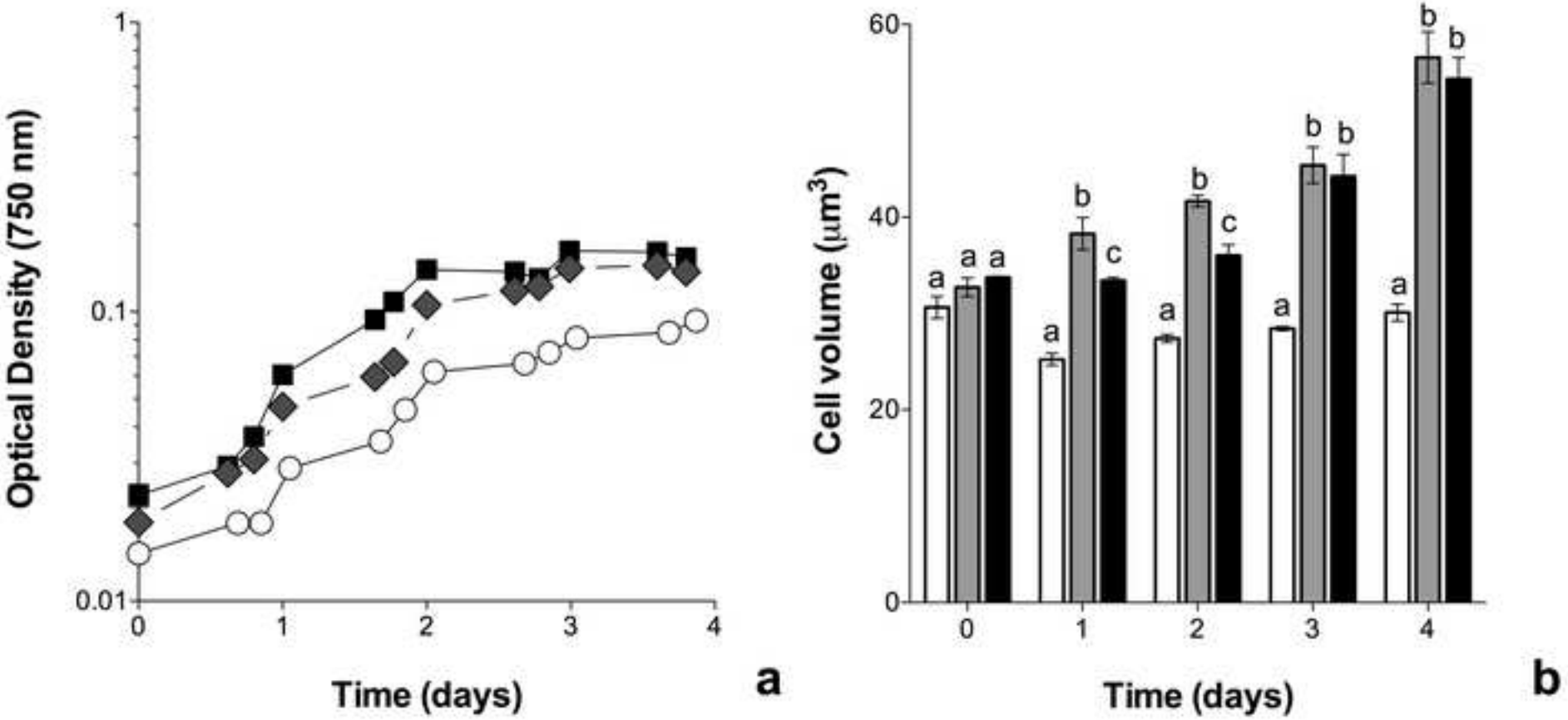

Figure 2

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