# Taxonomy and growth conditions concur to determine the energetic suitability of algal fatty acid complements

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**Abstract** We hypothesize that, in algae, carbon allocation to lipids depends on the combined effects of the metabolic constraints imposed by the genotype (i.e., species-specific differences in composition) and on the acclimation responses (phenotype) to changes in the stoichiometry of available inorganic carbon (for photosynthesis) and nitrogen (primarily for amino acids, protein, and nucleic acid synthesis). We thus cultured three taxonomically distinct algae, the green alga *Dunaliella salina*, the diatom *Thalassiosira pseudonana*, and the dinoflagellate *Protoceratium reticulatum*, at four nitrate concentrations and constant inorganic carbon. Since energy availability also directly impacts carbon partitioning, we studied the effect of irradiance on the oil quality of *P. reticulatum*. We used Fourier transform infrared (FTIR) spectroscopy to study carbon allocation and biomass reduction level and gas chromatography for fatty acid analysis. The fatty acid complements of the three species were different; within each species, growth conditions substantially altered oil quality. We ranked the oils in terms of their suitability as biodiesels, using international standards as reference. We believe that this approach may help to identify the appropriate combination of taxa and culture conditions for algal biodiesel production and in general offers insight on carbon allocation to fatty acids.

**Keywords** Algae · Biodiesel · C allocation · Cell composition · Elemental stoichiometry · Fatty acids · Phytoplankton

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## Introduction

Microalgae constitute a very promising feedstock for the production of oils to be used as fuel (Pandey et al. 2014). In addition to their substantial (although highly variable) oleogenic capacity, their potential as source of biofuel is associated to the fact that they can be cultivated in marginal lands or on wastewater, with minimal (if any) competition with key crops. Microalgae, therefore, fit the definition of sources of "second-generation biofuels" (Schenk et al. 2008). It should be mentioned, however, that the cost of algal biofuel production is presently not competitive with that of fossil fuels (Sun et al. 2011), although technological improvements and the unavoidable increase of fossil fuel price may change this in the future. In the short term, economical viability of algal biofuel productions appears to depend on multiple use of the biomass (Sun et al. 2011).

Because of the general interest in this topic, extensive (although still very limited in relation to the number of known algal species) screening has been conduced to identify the most oleogenic algal species and strains (e.g., Griffiths and Harrison 2009; Palmucci and Giordano 2012; Picardo et al. 2013 and references therein). The results of these studies suggest that special attention must be devoted to the study of the impact of culture conditions on oleogenesis, since microalgae carbon partitioning varies greatly in response to environmental perturbations. It should be noted, however, that the extent by which algae acclimate to changes in the external milieu are very species-specific and, while some algae promptly respond to even moderate changes of their growth environment, others are capable of maintaining compositional homeostasis in spite of substantial external changes (Giordano 2013).

It is generally accepted that oil production in microalgae is enhanced by nutrient, especially nitrogen, limitation. This is due to the fact that nitrogen shortage stimulates the allocation

of excess carbon (with respect to nitrogen) into nitrogen-poor macromolecules such as lipids and carbohydrates (Palmucci et al. 2011). This occurs to different extents in different species (Giordano 2013; Palmucci et al. 2011). The availability of nitrogen not only does affect the relative size of the lipid pool but also impacts on the quality of lipids, especially fatty acid composition (Flynn et al. 1992; Ota et al. 2009; Solovchenko et al. 2008). In some studies, it was observed that the production of octadecenoic acid was upregulated when nitrogen was absent or little available, at the expenses of octadecadienoic and octadecatrienoic acids (Flynn et al. 1992; Ota et al. 2009). This is especially important because oleic acid is the first monounsaturated acid that is produced during fatty acid biosynthesis and is at the origin of various other fatty acids (Choi et al. 2011) and because it appears to confer properties to the oil that increase its suitability as fuel (Knothe 2005).

The preferential allocation of carbon to lipids rather than to carbohydrates under nitrogen limitation (and in general terms) is connected to the availability of energy (i.e., irradiance; Norici et al. 2011; Montechiaro et al. 2006). Irradiance also affects the quality of fatty acids; for instance, the degree of unsaturation of C16 and C18 fatty acids increases with increasing irradiance (Hu et al. 2008). The accumulation of polyunsaturated fatty acids (PUFA) under excess light may be a way to prevent photodamage of sensitive cell components (Solovchenko et al. 2008).

The quality and relative abundance of microalgae fatty acids are different from those of vascular plants, with a higher amount of PUFA with long carbon chains, such as eicosatetraenoic acid (C20:4), eicosapentaenoic acid (C20:5), and docosahexaenoic acid (C22:6), in algae (Guschina and Harwood 2006; Hu et al. 2008). Within algae, the presence of long-chain PUFAs appears to be connected to the taxonomic position of the species: in diatoms, dinoflagellates, rhodophytes, and chrysophytes, PUFAs with C >20 may constitute a relatively large fraction of cell fatty acids, whereas in chlorophytes and prasinophytes, the main PUFAs have 16 to 18 atoms of carbon (Guschina and Harwood 2006; Hu et al. 2008).

Fatty acid composition has a strong impact on the physical properties of the oil obtained from the algae and on the biodiesel that derives from the oil (Knothe 2005). The cetane number (CN) whose value influences the delay between the fuel injection and ignition in an engine is positively correlated to the length of carbon chains (Klopfenstein 1985) and decreases when the unsaturation level of the hydrocarbon in the oil increases (Harrington 1986). Thus, a fuel with higher CN should combust more easily in a diesel engine than one with lower CN. The viscosity of the oil also has a direct impact on the quality of the biodiesel (the lower the better); it decreases when the number of carbon atoms in the hydrocarbon chain becomes smaller and when the level of unsaturation increases (Srivastava and Prasad 2000). Another physical property

that has a direct influence on the quality of biodiesel is the melting temperature of the oil; this parameter is lower when the number of double bonds between carbon atoms is higher (Knothe 2005). A high level of unsaturation of hydrocarbon chains also confers a greater sensitivity to oxidation to the biodiesel and therefore constitutes a problem for its storage (Schenk et al. 2008).

The assessment of the energetic suitability of algal oil obtained from a large number of species cultured in a wide range of conditions requires simple, rapid, and inexpensive methodologies that minimize the introduction of the artifacts intrinsically associated with complex extractive and analytical procedures. Fourier transform infrared (FTIR) spectroscopy may be able to meet the abovementioned objective and was therefore proposed as an appropriate methodology for the screening of oleogenic algae (Palmucci et al. 2011). However, proper validation of FTIR spectroscopy as a screening methodology for oleogenic algae is still missing in the literature. In this work, we attempted to achieve a dual aim: (1) provide evidence of the interplay of species-specific (genotypic) metabolic properties and culture conditions (and the correspondent phenotype) in determining oil quality, this was done through the analysis of algal oil from different species grown under different conditions, and (2) validate the use of FTIR on whole algae cells (with no need for cumbersome extraction procedures) for the screening of the oleogenic potential of algae and of the energetic suitability of their oils; to achieve the latter objective, FTIR data were directly compared with those obtained by traditional gas chromatographic techniques.

## Material and methods

Three species representing three major groups in algal phylogeny were selected for this study: the green alga *Dunaliella salina* CCAP 19/25, the centric diatom *Thalassiosira pseudonana* CCMP 1335, and the dinoflagellate *Protoceratium reticulatum* PRA 0206. In a previous paper, we reported that the lipid content of an alga is also inversely related to the cell size (Palmucci et al. 2011); we therefore also chose our experimental organisms to cover a wide size range, approximately between 50 and 15,000 $\mu m^3$, with the size of *T. pseudonana* cells in the order of 54–56 $\mu m^3$, that of *D. salina* cells between 152 and 164 $\mu m^3$, and that of *P. reticulatum* cells in the range of 12,204 to 23,332 $\mu m^3$ (Palmucci et al. 2011). The algae were grown semicontinuously and diluted daily at a rate that equaled the maximum growth rate achieved in batch cultures, under all the experimental conditions. This afforded rather constant cell concentrations throughout the experiments. All cells were cultured in AMCONA growth medium (Fanesi et al. 2014), which was modified for the $NO_3^-$ content, to obtain final

concentrations of 80, 800, 1600, and 3200 μmol L$^{-1}$. The cultures were shaken several times per day to help the maintenance of equilibrium between dissolved inorganic C and atmosphere. The persistence of such equilibrium was verified through frequent measurements of the medium pH and total inorganic carbon content. The measurements of the inorganic carbon content were conducted with an InfraRed Gas Analyzer (IRGA, LI-840A, Licor, USA); the calculations of inorganic carbon speciation (not shown because they were constant throughout the experiments) were conducted according to Ratti et al. (2007). Cultures of *D. salina* and *T. pseudonana* were maintained in 25×200 mm glass tubes containing 50 mL of growth medium; *P. reticulatum*, which appeared to be negatively affected by the low surface to volume ratio of the tubes, was cultivated in 250-mL flasks containing 200 mL of growth medium. All cultures were maintained at a temperature of 20 °C and under a constant photon flux density (PFD) of 100 μmol photons m$^{-2}$ s$^{-1}$ (PAR). *P. reticulatum* was also cultivated at PFDs of 25, 50, and 300 μmol photons m$^{-2}$ s$^{-1}$ (PAR). After a steady growth rate was reached in the semicontinuous culture regime, cells were acclimated to the different NO$_3^-$ condition and, for *P. reticulatum*, PFDs for at least four generations prior to be used for the experiments.

The cell numbers in *D. salina* and *T. pseudonana* cultures were determined by counting the cells with a Burker hemocytometer; the larger cells of *P. reticulatum* were counted using a Sedgwick-Rafter chamber. *D. salina* and *T. pseudonana* cells were harvested by centrifugation at 12,000×*g* for 10 min. *P. reticulatum* cells tend to break when centrifuged (Ratti et al. 2007) and were therefore harvested by filtration through 11-μm pore size nylon filters (Millipore, USA). For each condition, the cell number and the growth rate were determined for three distinct cultures. Only for *P. reticulatum* were the growth rates previously published by the authors and collaborators used (Jebsen et al. 2012).

FTIR spectroscopy

Cells were harvested as described in FTIR spectroscopy. They were then washed twice with a solution of ammonium formate isosmotic to the culture medium, in order to get rid of the medium salts and of any organic matter that may have been released by the cells during growth. The cell pellet was finally resuspended in a small volume of the same ammonium formate solution. An aliquot of 50 μL of this cell suspension was deposited on infrared transparent silica windows. The spectra were acquired, processed, and deconvoluted as described by Palmucci et al. (2011), using a Tensor 27 FTIR spectrometer and the Opus 6.5 software (Bruker Optics, Germany).

Semiquantification of cell lipid content and estimate of oleogenesis from FTIR spectroscopy data

Lipids were semiquantified according to Palmucci et al. (2011). The oleogenic potential per cell was calculated as the product of the lipid content estimated with the semiquantification procedure and the specific growth rate; the oleogenic potential per volume of culture was estimated as the cell oleogenic potential multiplied by the maximum cell concentration in the culture. In all cases, results were normalized to the lowest value and are therefore adimensional. We wish to clarify that the oleogenic potential per volume unit of culture is not necessarily the highest attainable, since, in this study, the cell carrying capacity of the growth media was not maximized.

Lipid extraction and fatty acid analysis

Cells were homogenized on ice with four 20-s cycles of sonications at 12 J, with an MSE Soniprep 150 sonicator (Sanyo, UK). Lipid extraction was performed according to Bligh and Dyer (1959), in a mixture of chloroform/methanol/water (2:2:1 *v/v*). The lipids partitioned in the chloroform phase. Chloroform was then evaporated under a stream of N$_2$, and the dry lipid extract was stored at −20 °C until ready for further processing.

Transesterification was performed on 5–10 mg of lipid extract that were resuspended in 1 mL of 3 mM CH$_3$COCl (acetyl chloride) in methanol, at 60 °C, with reflux of solvent for 45 min. The amount of reagent used and the duration of the reaction were in excess with respect to those required from the reaction stoichiometry. This was verified by the NMR analysis of transesterified samples (data not shown). The methanol in excess was then eliminated with a rotary evaporator, and the transesterified fatty acids were resuspended in 50 μL of chloroform. Samples containing fatty acid methyl esters (FAMEs) were analyzed immediately or kept in the dark and at 4 °C until analyzed.

FAMEs were separated, identified, and quantified using a FOCUS gas chromatograph (GC) equipped with a flame ionization detector (FID) (Thermo Fischer Scientific, USA). The separation was effected through a TR-FAME column (Thermo Fischer Scientific). The identification of the FAMEs in the algal oil was effected by comparison with a commercial standard mix (Sigma-Aldrich cat. #47885-U). The quantification of the individual FAMEs was done by interpolating their peak area into a standard curve constructed with the areas of known amounts of heptacosanoic acid methyl ester (Sigma-Aldrich cat. #H6639). Heptacosanoic acid methyl ester was also used as an internal standard to minimize the risk of artifacts associated with the extraction and transesterification procedures.

Validation of FTIR determination of the level of reduction of fatty acids

In order to ascertain if the FTIR determination of biomass reduction provides a good representation of the fatty acid unsaturation, we compared the data obtained by FTIR spectroscopy with the level of unsaturation deduced from the GC analysis of FAME. The level of reduction of organic constituent was calculated from FTIR spectra according to Palmucci et al. (2011) as the ratio between the sum of the integrals of the peaks at 2950±5 and 2920±5 cm$^{-1}$ (due to $CH_2$ and $CH_3$, respectively) and those at 3015±10 cm$^{-1}$ (CH of C=C). The overall level of reduction of fatty acids from GC data was obtained by multiplying the moles of each fatty acid (obtained as described in the previous paragraph) by the number of $-CH_n$ ($-CH_2$, $-CH_3$, and $-CH$) it contained.

Assessment of biodiesel properties

The cetane number (CN) of the fatty acids methyl esters obtained from the algae in the various culture conditions was calculated using the following equation (Ramos et al. 2009):

$$CN \ = \ X_{ME} \bullet CN_{ME}$$

where $X_{ME}$ is the weight fraction of each methyl ester and $CN_{ME}$ is its cetane number. Most FAME CN values were obtained from the literature (Bamgboye and Hansen 2008 and references therein). Some of the fatty acids produced by microalgae, however, are substantially longer and with higher degrees of unsaturation than those in the vegetable oils commonly used to produce biodiesel (Baba and Shiraiwa 2013), and we were unable to find CN values for polyunsaturated fatty acids longer than C20, in the literature. Therefore, the cetane numbers of these FAMEs were calculated using the formula proposed by Stansell et al. (2012):

$$CN \ = \ -21.157 + \left(7.965 – 1.785 \bullet db + 0.235 \bullet db^2\right) \bullet N – 0.099 \bullet N^2$$

where $N$ is the number of carbon atoms in the fatty acid chain and $db$ is the number of double bonds.

The viscosity ($\eta$) of the transesterified oil was calculated as follows (Allen et al. 1999):

$$\ln(\eta) \ = \ X_{ME} \bullet \ln(\eta_{ME})$$

where $\eta$ is the viscosity of the oil at 40 °C, $X_{ME}$ is the weight fraction of each FAME, and $\eta_{ME}$ is its viscosity of each FAME

at 40 °C; $\ln(\eta_{ME})$ was calculated according to Ramírez-Verduzco et al. (2012):

$$\ln(\eta_{ME}) \ = \ -12.503 \ + \ 2.496 \bullet \ln(mw_{ME}) – 0.178 \bullet N$$

where $mw_{ME}$ is the molecular weight of individual FAMEs and $N$ is the number of carbon atoms in the FAME.

The "higher heating value" ($\delta$) of the fatty acids mixture, i.e., the amount of heat produced by the complete combustion of a unit quantity of fuel, was calculated as follows:

$$\delta \ = \ X_{ME} \bullet \delta_{ME}$$

The $\delta$ of each FAME ($\delta_{ME}$) was calculated using the following equation (Ramírez-Verduzco et al. 2012):

$$\delta_{ME} = 46.19 – 1794 \bullet mw_{ME} – 0.21 \bullet N$$

The average length of saturated carbon chains in fatty acid mixtures was estimated as the long-chain saturated factor (LCSF). LCSF was calculated applying the empirical formula proposed by Ramos et al. (2009):

$$LCSF \ = 0.1 \bullet X_{C16} \ + \ 0.5 \bullet X_{C18} \ + \ 1 \bullet X_{C20} \ + \ 1.5 \bullet X_{C22} \ + \ 2 \bullet X_{C24}$$

where $X_{C16}$, $X_{C18}$, $X_{C20}$, $X_{C22}$, and $X_{C24}$ are the weight fraction of saturated fatty acids with respectively 16, 18, 20, 22, and 24 carbon atoms. The LCSF was used for the calculation of the cold filter plugging point (CFPP; Ramos et al. 2009), defined as the lowest temperature at which a given volume of diesel passes through a standardized filter. CFPP is used as a way to estimate the performance of biodiesel in a diesel engine at low temperatures. In this paper, CFPP was not measured but calculated as follows:

$$CFPP = 3.1417 \bullet LCSF – 16.477$$

*Evaluation of biodiesel produced by different species* A score calculated with the equation that follows was assigned to the putative biodiesels produced by each species cultured in each condition:

$$B_s \ = \ CN_s \bullet V_s \bullet CFPP_s \bullet HV_s$$

where $B_s$ is the biodiesel score, $CN_s$ is the cetane number score, $V_s$ is the viscosity score at 40 °C, $CFPP_s$ is the CFPP score, and $HV_s$ is the heating value score.

The scores were assigned using as reference the standard values for biodiesel suggested by the international reference standard UNI EN 14214:2008. The $B_s$ values ranged from 0.500 to 1296, with the highest score attributed to the oil that, according to the International Standards, should give the best overall performance. Table 1 shows the criteria used for score assignments.

Statistical methods

Results are expressed as the means±standard deviations of measurements conducted on at least three biological replicates (i.e., distinct cultures). The statistical significance of the correlation between unsaturation level of fatty acids estimated by GC and unsaturation level of fatty acids estimated by FTIR was analyzed by means of a two-tailed Pearson correlation ($r$), with a significance threshold of 0.01, using GraphPad Prism 4.03 (GraphPad Software, USA).

## Results

### Impact of nitrate availability on growth rate and lipid content

In our experimental conditions, the growth rates of *P. reticulatum* and *D. salina* (Table 2) were strongly dependent on $NO_3^-$ availability ($r=0.82$ and $r=0.93$, respectively). Such relationship was not observed for *T. pseudonana* ($r=-0.23$). This, to some extent, may reflect the metabolic response of these species to limited nitrogen supply (Hockin et al. 2012; Lomas and Gilbert 2000), but it is also possible that it is a consequence of the size difference, the largest cells being saturated at higher $NO_3^-$ concentration (Edwards et al. 2012).

*D. salina* and *T. pseudonana* had comparable average lipid contents (Fig. 1a). *P. reticulatum* lipid content was, in most conditions, one order of magnitude higher than in the other two species, and the difference was even larger (64- to 83-fold) at the lowest $NO_3^-$ concentration (Fig. 1a). This difference is certainly associated to the fact that *P. reticulatum* cells are about two orders of magnitude bigger than *D. salina* and *T. pseudonana* cells (Palmucci et al. 2011). Interestingly,

**Table 1** Criteria used to calculate the parameter scores

| Parameter | Reference value (UNI EN 14214:2008) | Criteria used to identify score classes | Score class | Score |
|---|---|---|---|---|
| Cetane number | >51 | Percentage of increase with respect to minimum reference standard | <10 % | 1 |
| | | | 11–20 % | 2 |
| | | | 21–30 % | 3 |
| | | | 31–40 % | 4 |
| | | | 41–50 % | 5 |
| | | | >50 % | 6 |
| Viscosity at 40 °C | 3.50–5.00 mm$^2$ s$^{-1}$ | Actual viscosity within the standard range (mm$^2$ s$^{-1}$) | 3.51–3.75 | 1 |
| | | | 3.76–4.00 | 2 |
| | | | 4.01–4.25 | 3 |
| | | | 4.26–4.50 | 4 |
| | | | 4.51–4.75 | 5 |
| | | | 4.76–5.00 | 6 |
| Cold filter plugging point | <0 °C | Actual cold filter plugging point (°C) | >0 | 0.5 |
| | | | −1 | 1 |
| | | | 2 | 2 |
| | | | −3 | 3 |
| | | | −4 | 4 |
| | | | −5 | 5 |
| | | | <−5 | 6 |
| Heating value | 42 MJ kg$^{-1}$ | Percentage of the maximum heating value of petroleum diesel | <75 % | 1 |
| | | | 75÷79 % | 2 |
| | | | 80÷84 % | 3 |
| | | | 85÷89 % | 4 |
| | | | 90÷94 % | 5 |
| | | | >95 % | 6 |

As a reference, the International Standards UNI EN 14214:2008 was used. A scale between 1 and 6 was used for all parameters; only for the "cold filter plugging point," a 0.5 score was assigned to those values that were below the lower end of the standard range

**Table 2** Specific growth rates, protein contents (measured according to Peterson 1977) and maximum cell concentrations in the culture media of *D. salina*, *T. pseudonana*, and *P. reticulatum* cultured at 80, 800, 1600, and 3200 μM $NO_3^-$

| Species | $[NO_3^-]$ (μM) | Specific growth rate (day$^{-1}$) | Protein content (pg cell$^{-1}$) | Maximum cell concentration ($10^3$ cells mL$^{-1}$) |
|---|---|---|---|---|
| *D. salina* | 80 | 0.34 (0.04) | 17.9 (0.70) | 2432 (147) |
| | 800 | 0.72 (0.03) | 30.4 (0.38) | 4855 (448) |
| | 1600 | 1.04 (0.04) | 27.8 (0.73) | 4227 (106) |
| | 3200 | 1.00 (0.04) | 52.6 (18.0) | 4436 (625) |
| *T. pseudonana* | 80 | 1.19 (0.04) | 4.35 (0.09) | 3348 (313) |
| | 800 | 1.51 (0.08) | 9.11 (0.54) | 3293 (97) |
| | 1600 | 1.57 (0.07) | 9.36 (1.14) | 3292 (59) |
| | 3200 | 1.16 (0.12) | 11.8 (0.65) | 3464 (11) |
| *P. reticulatum* | 80 | 0.11 (0.02) | 580 (83.4) | 5.5 (0.9) |
| | 800 | 0.13 (0.02) | 1742 (40.41) | 12.2 (2.2) |
| | 1600 | 0.16 (0.01) | 1021 (103.3) | 6.3 (1.3) |
| | 3200 | 0.17 (0.02) | 1461 (61.70) | 10.6 (3.2) |

Standard deviations are shown in brackets (*n*=3)

when the lipid content was normalized to the cell volume, however, it was appreciably higher in *D. salina* and *T. pseudonana* than in *P. reticulatum*. This observation agrees with what we reported in a previous paper (Palmucci et al. 2011), i.e., that smaller cells are more prone to use lipids as the storage pool for excess carbon because lipids have a higher volume-based energy content than carbohydrates.

Impact of nitrate availability on oleogenesis

The cell oleogenic potential was estimated by multiplying the cell lipid content by the specific growth rate (Fig. 1b). At the two intermediate $NO_3^-$ concentrations, oleogenesis is fairly similar for all species (Fig. 1b). If the maximum cell concentration attainable for the three species in our growth media is considered (again, we did not optimize our media for maximum carrying capacity, though; Table 2), *D. salina* and *T. pseudonana* were much more productive than *P. reticulatum* (Fig. 1c). In terms of productivity per unit volume of growth medium, *D. salina* was the most oleogenic species at high $NO_3^-$ concentration (3600 μmol L$^{-1}$), with an ~220-fold and an ~2-fold higher productivity per unit of culture volume by this species than by *P. reticulatum* and *T. pseudonana*, respectively.

Impact of nitrate availability on fatty acid composition

*D. salina* In the green alga *D. salina*, a higher percentage of saturated fatty acids (SFAs) was observed when it was cultivated at 80 μmol L$^{-1}$, whereas at higher $[NO_3^-]$, the relative percentage of monounsaturated fatty acids (MUFAs) increased (Fig. 2, Table 3). Cells cultured at 80 μmol L$^{-1}$ $NO_3^-$ had a 3.5- to 5-fold higher percentage of SFAs than cells grown at higher $[NO_3^-]$ (Fig. 2, Table 3). The proportion of PUFAs and MUFAs, instead, was higher when the $[NO_3^-]$ in the medium was higher (Fig. 2, Table 3). At 80 μmol L$^{-1}$ $NO_3^-$, the more abundant SFAs were hexadecanoic (C16:0, 19 %) and dodecanoic (C12:0, 16.3 %) acids; octadecanoic acid (C18:0) was not detected. Interestingly, C12:0 was absent in cells cultured at $[NO_3^-]$ greater than 80 μmol L$^{-1}$ $NO_3$, which instead contained low relative amounts of C18:0. The most abundant MUFAs were hexadecenoic acid (C16:1) and *cis* and *trans* octadecenoic acid (C18:1) (Table 3). Their amounts relative to the total fatty acids were little affected by the growth conditions, except for the lowest $[NO_3^-]$, where C16:1 and C18:1 *cis* were present in lower proportions. Octadecatrienoic acid (C18:3 n-3) was the most abundant PUFA (Table 3) and was responsible for most of the differences in the percentage of total PUFAs (Fig. 2). Our data for *D. salina* comprise a rather large quota of unidentified fatty acids; with the methods at our disposal, we were unfortunately unable to overcome this problem.

*T. pseudonana* In the cells of this alga, the percentages of SFAs, MUFAs, and PUFAs were not significantly affected by the growth $[NO_3^-]$. In all conditions, SFA percentage was roughly half the sum of MUFAs and PUFAs (Fig. 2, Table 3). The SFA with the highest relative abundance was C16:0 (17.4–20.3 %) (Table 3). MUFAs constituted the largest fraction of fatty acids (up to 44 % of the total fatty acids; Fig. 2, Table 3). Hexadecenoic acid (C16:1) represented about 50 % of all MUFAs; however, also docosahexaenoic (C22:1) and tetracosenoic (C24:1) acids were present in substantial amounts (up to 3 and 9 %, respectively). The PUFA pool was dominated by hexadecatrienoic (C16:3, 8.9–10.2 %) and octadecadienoic (C18:2, 5.5–6.8 %) acids; percentages always below 1.5 % were found for PUFAs with chain length between 20 and 24C (Table 3).
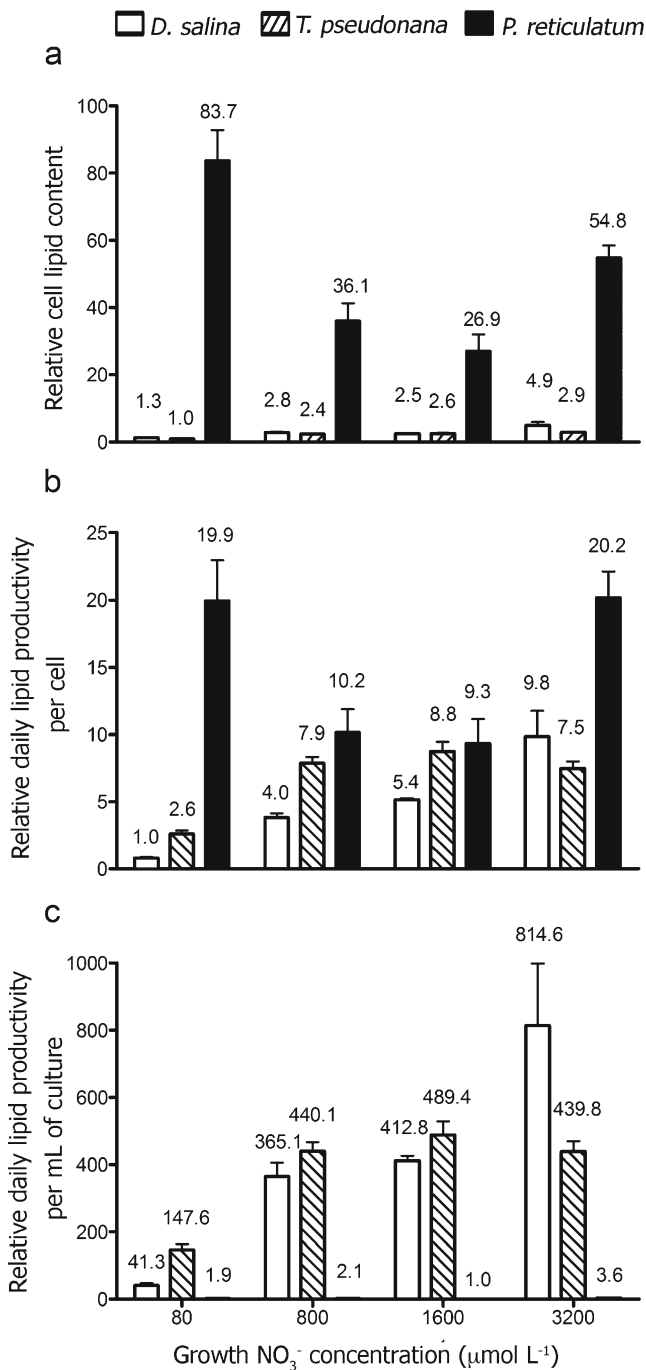
**Fig. 1** Lipid cell content, lipid productivity per cell, and lipid productivity per milliliters of culture of *Dunaliella salina*, *Thalassiosira pseudonana*, and *Protoceratium reticulatum* cultured in the presence of different nitrate concentrations (80, 800, 1600, and 3200 μmol L$^{-1}$). The *number on top of each bar* indicates the mean value; the *error bars* show the standard deviations ($n=3$). In each panel, the lowest value is set to 1 and it is used as the base for the normalization of all other values

*P. reticulatum* Also in this organism, the variations in relative abundance were such that the overall ratio between SFAs and MUFAs+PUFAs changed little (at 800 μmol L$^{-1}$) or not at all as a function of [NO$_3^-$] (Fig. 2, Table 3). The availability of

NO$_3^-$ in the culture medium affected the abundance of SFAs with 14 and 16 atoms of C (Table 3). At low NO$_3^-$ (80 μmol L$^{-1}$), C14:0 was 10 % of the total fatty acids and C16:0 was 35 % of total fatty acids; at higher [NO$_3$], the relative abundance of C14:0 increased and that of C16:0 decreased by a similar percentage (Table 3). The relative abundance of C18:0 was similar in all growth conditions, while C23:0 was higher when NO$_3^-$ availability was higher. The most abundant MUFA was C18:1 n-9 *cis*, whose relative amount represented up to 22 % of total extracted fatty acids (Table 3).

**Impact of light on unsaturation level and fatty acid composition**

In *P. reticulatum*, the different PFDs did not elicit a change in the overall level of reduction of fatty acids (Table 4). Irradiance also had little impact on *P. reticulatum* fatty acid composition (Table 4). Among the saturated fatty acids, only the hexadecanoic acid (C16:0) was slightly higher when cells were cultured at lower (25 μmol photons m$^{-2}$ s$^{-1}$) than at higher irradiances, whereas the relative abundance of octadecanoic acid (C18:0) was greater at high irradiance (300 μmol photons m$^{-2}$ s$^{-1}$). Also, the pool of unsaturated fatty acids was quite homeostatic, with the exception of octadecenoic (C18:1) and docosahexaenoic (C22:6 n-3) fatty acids, whose content was slightly higher at low PFD than at high PFD. As a consequence of these changes, MUFAs decreased slightly at the two highest irradiances and PUFAs did the opposite. The percentage of SFA was not appreciably altered by the light regime.

**Validation of FTIR determination of the level of reduction of fatty acids**

In order to assess if information on unsaturation could be obtained by FTIR spectroscopy without the need for FA extraction and GC analyses, we compared the level of unsaturation derived by the FA profile and the biomass reduction level deduced from the FTIR spectra (Palmucci et al. 2011). The results are shown in Fig. 3; a strong correlation exists between the two measurements (Pearson $r=0.8$), and a very low $P$ value ($P=0.0071$) confirms the robustness of the correlation.

**Impact of fatty acid composition on biodiesel properties**

The biodiesels obtainable from the three experimental species are overall rather different, and the influence of the growth treatment on the oil quality is also species-specific (Table 5).

**Fig. 2** This figure depicts the percent content of saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids in *Dunaliella salina*, *Thalassiosira pseudonana*, and *Protoceratium reticulatum* cultured in the presence of different nitrate concentrations (80, 800, 1600, and 3200 μmol L$^{-1}$). The *numbers in parentheses* represent the standard deviations of three independent replicates; *n.i.* indicates the fraction of fatty acids that were not identified



*Dunaliella salina*

*Thalassiosira pseudonana*

*Protoceratium reticulatum*
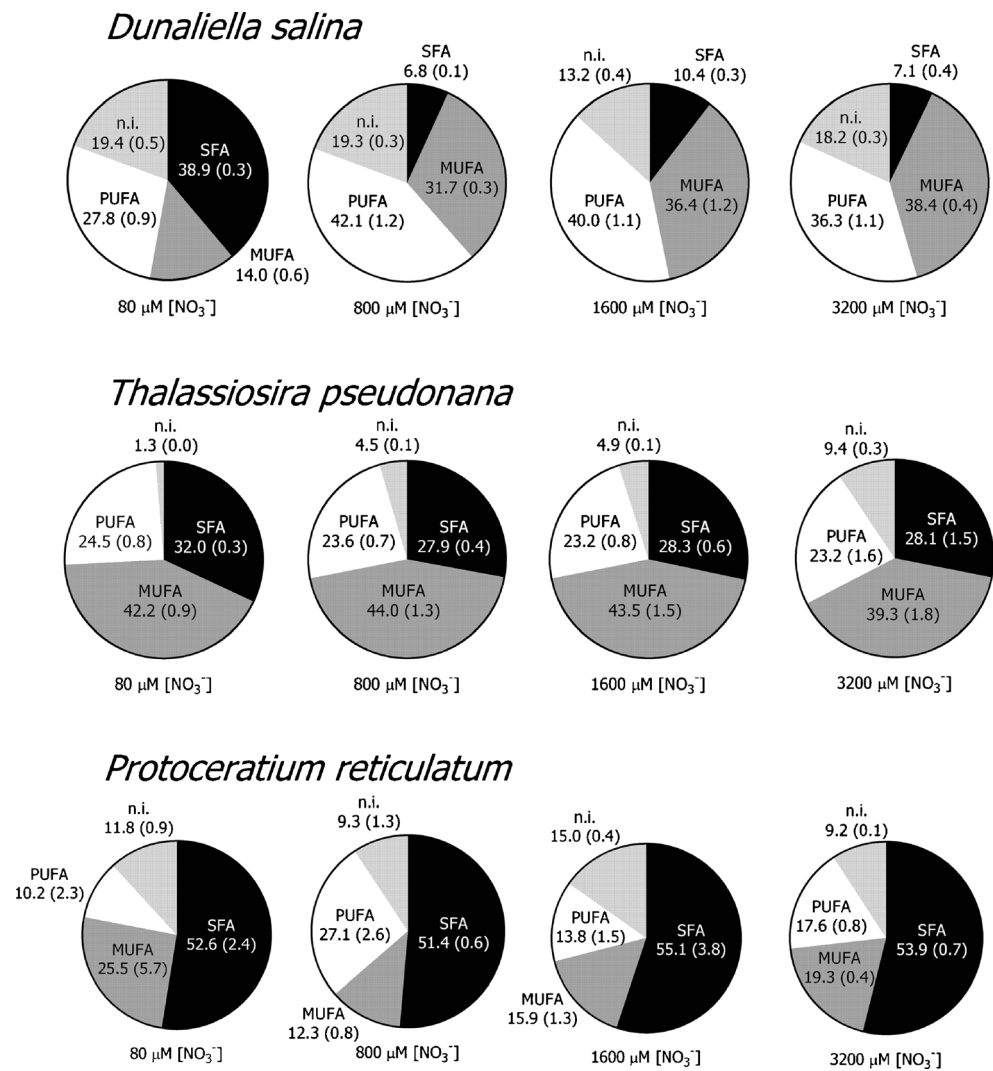
## Discussion

### Impact of nitrate availability on growth rate and lipid content

Algae cultured in media containing relatively low amount of nitrogen often allocate the carbon in excess with respect to the requirement for subsistence and growth to storage pools (Giordano 2013). It is often assumed that such condition leads to lipid accumulation. This is in fact not always true: Very often, C assimilation is downregulated to match nitrogen assimilation (Kaffes et al. 2010), and/or the carbon in excess can (also) be allocated to carbohydrates (Palmucci et al. 2011). Recent studies made it clear that the strategies of carbon allocation under nitrogen limitation are species-specific (Palmucci et al. 2011; Palmucci and Giordano 2012). Also in this study, the relationship of the lipid content with nitrogen availability and growth rate is very different in the three species (Fig 1a). In *D. salina*, the lipid content was closely related to both NO$_3^-$ availability and growth rate ($r=0.95$ for [NO$_3^-$]; $r=0.72$ for growth rate;

Fig. 1a). In *T. pseudonana*, the amount of lipids per cell appeared to be rather closely related to the concentration of NO$_3^-$ in the growth media ($r=0.85$), but not with growth rate ($r=0.28$) (Fig. 1b). In *P. reticulatum*, the lipid content was weakly and inversely related to the growth rate ($r=-0.58$), and the correlation coefficient with NO$_3^-$ availability was only $-0.32$ (Fig. 1c). This may reflect the mode of nutrient utilization by these algae: *T. pseudonana* requires low concentrations of nitrogen for growth (Table 2), and it may consequently not take up nitrogen beyond growth requirement, therefore maintaining a rather constant carbon to nitrogen ratio and carbon allocation pattern (Palmucci et al. 2011). The same cannot be said for *D. salina*. Much less is known about the physiological responses of dinoflagellates to changes in the environmental rate of supply of carbon and nitrogen; the fact that, in *P. reticulatum*, the lipid content increases substantially only at the lowest nitrogen concentration suggests that this alga is able to maintain a balanced intracellular carbon and nitrogen ratio, in most conditions.

**Table 3** Fatty acid percent composition of *D. salina*, *T. pseudonana*, and *P. reticulatum* cultured at 80, 800, 1600, and 3200 µM NO$_3^-$

| Fatty acid | [NO$_3^-$] used for *D. salina* growth (µM) | | | | [NO$_3^-$] used for *T. pseudonana* growth (µM) | | | | [NO$_3^-$] used for *P. reticulatum* growth (µM) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 80 | 800 | 1600 | 3200 | 80 | 800 | 1600 | 3200 | 80 | 800 | 1600 | 3200 |
| C8:0 | 1.2±0.6 | n.d. | n.d. | n.d. | 0.3±0.0 | n.d. | 0.2±0.1 | n.d. | n.d. | n.d. | n.d. | n.d. |
| C12:0 | 16.3±0.8 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| C13:0 | n.d. | n.d. | n.d. | n.d. | 0.3±0.0 | 0.4±0.0 | 0.4±0.0 | n.d. | n.d. | n.d. | n.d. | n.d. |
| C14:0 | 1.0±0.1 | 1.9±0.1 | 2.9±0.3 | 0.6±0.0 | 6.4±0.0 | 6.2±0.3 | 6.4±0.3 | 6.5±0.6 | 10.2±1.4 | 12.2±0.8 | 15.8±1.8 | 15.7±0.1 |
| C14:1 | 1.0±0.0 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d | n.d. | n.d. |
| C15:0 | n.d. | n.d. | n.d. | n.d. | 0.3±0.0 | n.d. | 0.3±0.0 | n.d. | n.d. | n.d | n.d. | n.d. |
| C15:1 | n.d. | n.d. | n.d. | n.d. | 1.3±0.1 | 1.3±0.1 | 1.0±0.1 | 1.2±0.1 | n.d. | n.d | 0.38±0.0 | n.d. |
| C16:0 | 19.0±0.8 | 1.4±0.0 | 1.8±0.1 | 1.3±0.4 | 20.3±0.3 | 18.1±0.3 | 17.9±0.5 | 17.4±1.3 | 35.4±0.2 | 25.7±0.6 | 28.5±3.2 | 25.9±0.7 |
| C16:1 | 0.9±0.2 | 15.0±0.3 | 18.2±1.0 | 13.4±0.4 | 25.7±0.2 | 25.4±0.4 | 24.8±0.6 | 22.1±1.5 | 3.5±4.9 | n.d. | 0.4±0.0 | 0.3±0.0 |
| C16:2 | n.d. | n.d. | n.d. | n.d. | 2.1±0.0 | 2.2±0.0 | 1.9±0.1 | 2.2±0.1 | n.d. | n.d. | n.d. | n.d. |
| C16:3 | 3.9±0.6 | 7.6±0.5 | 2.3±0.3 | 2.6±0.1 | 8.9±0.1 | 10.2±0.2 | 9.9±0.3 | 10.2±1.6 | n.d. | n.d. | n.d. | n.d. |
| C17:0 | n.d. | n.d. | n.d. | n.d. | 0.3±0.0 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| C18:0 | n.d. | 1.6±0.0 | 2.3±0.2 | 2.5±0.0 | 3.7±0.1 | 3.2±0.0 | 2.8±0.1 | 3.7±0.1 | 5.7±0.8 | 4.7±0.4 | 5.9±0.6 | 5.5±0.1 |
| C18:1 n-9 *cis* | 2.9±0.2 | 8.1±0.1 | 11.1±0.6 | 10.9±0.2 | n.d. | n.d. | n.d. | n.d. | 21.9±2.8 | 12.2±0.8 | 14.7±1.3 | 18.6±0.4 |
| C18:1 n-9 *trans* | 8.4±0.5 | 8.6±0.1 | 7.2±0.2 | 14.1±0.0 | 2.8±0.1 | 3.1±0.2 | 2.9±0.2 | 3.2±0.5 | n.d. | n.d | n.d. | n.d. |
| C18:2 n-6 *cis* | 8.4±0.4 | 9.3±0.3 | 4.1±0.2 | 3.1±0.1 | 3.3±0.2 | 3.4±0.0 | 3.4±0.0 | 3.1±0.2 | n.d. | 1.6±0.1 | 1.3±0.1 | 1.2±0.0 |
| C18:2 n-6 *trans* | n.d. | n.d. | n.d. | n.d. | 6.8±0.2 | 5.6±0.1 | 6.1±0.1 | 5.8±0.5 | n.d. | n.d | n.d. | n.d. |
| C18:3 n-6 | 3.3±0.2 | 3.8±0.1 | 4.1±0.2 | 3.8±0.1 | n.d. | n.d. | n.d. | n.d. | n.d. | 0.9±0.1 | 0.5±0.0 | 0.4±0.0 |
| C18:3 n-3 | 7.6±0.4 | 15.2±0.3 | 24.2±0.8 | 22.4±0.5 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d | n.d. | n.d. |
| C20:0 | 1.4±0.7 | 2.0±0.0 | 3.3±0.1 | 2.8±0.0 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d | n.d. | n.d. |
| C20:1 n-9 | 0.8±0.1 | n.d. | n.d. | n.d. | 1.8±0.0 | 2.8±0.7 | 2.9±0.9 | 2.2±0.3 | n.d | n.d | n.d | n.d |
| C20:2 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | n.d | n.d. | 1.8±0.7 | 0.5±0.1 | 0.4±0.1 |
| C20:3 n-3 | n.d. | n.d. | n.d. | n.d. | 0.7±0.0 | 0.9±0.0 | 0.8±0.0 | 0.6±0.0 | n.d. | n.d | n.d. | n.d. |
| C20:4 n-6 | n.d. | n.d. | n.d. | n.d. | 1.5±0.0 | 1.1±0.0 | 0.8±0.0 | 1.3±0.1 | n.d. | n.d | n.d. | n.d. |
| C20:5 n-3 | 3.7±0.2 | 2.2±0.1 | 2.5±0.1 | 1.7±0.1 | n.d. | n.d. | n.d. | n.d. | 2.5±0.2 | 4.8±1.9 | 0.4±0.1 | n.d. |
| C21:0 | n.d. | n.d. | n.d. | n.d. | 0.4±0.0 | n.d. | 0.4±0.1 | 0.7±0.3 | n.d. | n.d | n.d. | n.d. |
| C22:1 | n.d. | n.d. | n.d. | n.d. | 7.9±0.2 | 8.4±0.2 | 8.8±0.0 | 8.1±0.5 | n.d. | n.d | n.d. | n.d. |
| C22:6 n-3 | 0.8±0.1 | 4.0±1.0 | 2.8±0.7 | 2.7±1.0 | 1.0±0.0 | 0.4±0.0 | 0.2±0.0 | n.d. | 7.7±2.3 | 17.9±0.7 | 11.7±1.5 | 16.1±0.9 |
| C23:0 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 1.3±1.8 | 8.7±0.3 | 4.9±0.6 | 6.7±0.1 |
| C24:1 | n.d. | n.d. | n.d. | n.d. | 2.8±0.1 | 3.0±0.1 | 2.9±0.1 | 2.6±0.1 | n.d. | n.d. | n.d. | n.d. |
| Not Identified | 19.4±0.5 | 19.3±0.3 | 13.2±0.4 | 18.2±0.3 | 1.3±0.0 | 4.5±0.1 | 4.9±0.1 | 9.4±0.3 | 11.8±0.9 | 9.3±1.3 | 15.0±0.4 | 9.2±0.1 |

The data are reported as the mean±standard deviations of three biological replicates (i.e., three independent cultures); *n.d.* (not detected) indicates that the concentration was below the limit of detection

## Impact of nitrate availability on oleogenesis

The patterns of oleogenesis as a function of NO$_3^-$ concentration, estimated as the product of the lipid content and the specific growth rate, reflect the lipid content of the cells, although the difference among the species with respect to oleogenesis is much smaller than for their lipid content. It should be considered, however, that species-specific resource-use strategies and elemental stoichiometries (Giordano 2013) can lead to rather large differences in the amount of cells/biomass that a given growth medium can support. It goes without saying that a nitrogen-limited culture medium cannot support the same biomass as a nitrogen-replete medium. In a production plant, the actual productivity will be strongly dependent on the cell concentration that the culture system can sustain. For this reason, a two-phase cultural system, in which the highest biomass is produced in (nearly) optimal conditions and oleogenesis is subsequently induced, is often considered as a good option. A two-phase cultivation, based on the data reported here, seems of little use (and economically nonsensical) for *D. salina* and *Thalassiosira weissflogii*, since their lipid content increases with nitrogen concentration (a nitrogen starvation—rather than nitrogen limitation—treatment may give different results). In the case of *P. reticulatum*,

**Table 4** Fatty acid percent composition of *P. reticulatum* cultured at 25, 50, 100, and 300 $\mu mol$ photons $m^{-2}$ $s^{-1}$

| Fatty acid | Irradiance used for growth ($\mu mol$ photons $m^{-2}$ $s^{-1}$) | | | |
|---|---|---|---|---|
| | 25 | 50 | 100 | 300 |
| C12:0 | 0.5±0.1 | n.d. | n.d. | n.d. |
| C14:0 | 12.7±0.2 | 13.5±0.1 | 12.2±0.8 | 12.9±0.3 |
| C15:0 | 0.5±0.0 | n.d. | n.d. | n.d. |
| C16:0 | 27.3±0.3 | 26.1±0.0 | 25.7±0.6 | 25.2±0.5 |
| C16:1 | n.d. | 0.1±0.0 | n.d. | n.d. |
| C18:0 | 3.5±0.0 | 3.9±0.0 | 4.7±0.4 | 4.2±0.1 |
| C18:1 n-9 *cis* | 14.7±0.1 | 14.8±0.1 | 12.2±0.8 | 12.8±0.2 |
| C18:2 n-6 *cis* | 1.5±0.0 | 1.5±0.0 | 1.6±0.1 | 1.6±0.0 |
| C18:3 n-6 | 0.3±0.0 | 0.4±0.0 | 0.9±0.1 | 0.7±0.0 |
| C18:3 n-3 | n.d. | n.d. | n.d. | 0.4±0.0 |
| C20:1 n-9 | 0.3±0.0 | 0.3±0.0 | n.d. | n.d. |
| C20:2 | 0.9±0.0 | 0.8±0.0 | 1.8±0.7 | 1.0±0.0 |
| C22:0 | n.d. | n.d. | n.d. | 1.2±0.1 |
| C23:0 | 8.8±0.1 | 8.8±0.1 | 8.7±0.3 | 8.5±0.1 |
| C20:5 n-3 | 2.3±0.0 | 2.1±0.0 | 4.8±1.9 | 2.5±0.0 |
| C22:6 n-3 | 19.2±0.3 | 20.3±0.2 | 17.9±0.7 | 18.0±0.4 |
| C24:0 | 0.3±0.0 | 0.4±0.1 | n.d. | 0.4±0.0 |
| Not identified | 7.3±0.7 | 7.1±0.1 | 9.3±1.3 | 10.7±0.9 |
| SFA | 53.5±0.6 | 52.7±0.2 | 51.4±0.6 | 52.4±0.7 |
| MUFA | 15.0±0.1 | 15.1±0.1 | 12.3±0.8 | 12.8±0.2 |
| PUFA | 24.2±0.2 | 25.0±0.1 | 27.1±2.6 | 24.1±0.4 |
| MUFA+PUFA | 39.2±0.2 | 40.1±0.1 | 39.3±1.9 | 36.9±0.5 |
| SFA/(MUFA+PUFA) | 1.37±0.0 | 1.31±0.0 | 1.31±0.1 | 1.42±0.0 |

The data are reported as the mean±standard deviations of three biological replicates (i.e., three independent cultures); *n.d.* (not detected) indicates that the concentration was below the limit of detection

*SFA* saturated fatty acid, *MUFA* monounsaturated fatty acid, *PUFA* polyunsaturated fatty acid
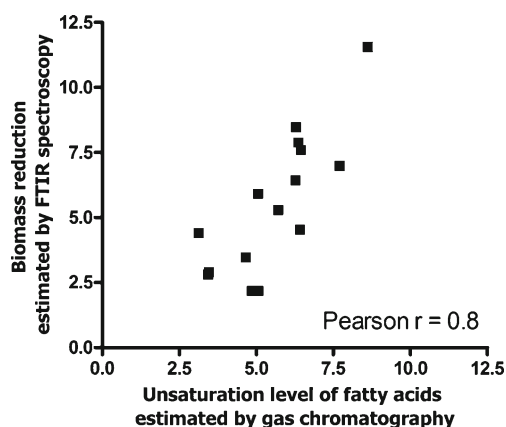


**Fig. 3** Correlation between the values of biomass reduction obtained by Fourier transform infrared (FTIR) spectroscopy and those obtained by gas chromatography (GC). The means of all experimental data were used for this analysis

the cell lipid content was about 35 % higher at the lowest than at the highest nitrogen concentration used in this work. A thorough assessment of the economic cost of a dual-phase cultivation may be necessary to verify if such increment in the lipid content would make a two-phase cultivation worthwhile.

Impact of nitrate availability on fatty acid composition

In *D. salina*, SFAs were more abundant at low $[NO_3^-]$, whereas unsaturated fatty acid and especially PUFAs were more abundant at high $[NO_3^-]$ (Fig. 2). PUFAs are mainly esterified in polar lipids (i.e., monoacyldiglyceride, diacyldiglyceride) and are membrane components (Guschina and Harwood 2006). A change in the fatty acid composition of membranes has been reported in the case algae are exposed to low temperatures; in this case, membrane lipids tend to get desaturated to confer higher fluidity to the membrane (the melting point of fatty acids is significantly lowered by the addition of double bonds; Klyachko-Gurvich et al. 1999 and references therein). Very little information, however, exists on the effect of the availability of nutrients on the unsaturation level of fatty acids. An increase of the relative percentage of unsaturated fatty acids may also be due to an increase in the extension of cell membranes, such as in the case of an increase in cell size (Goss and Wilhelm 2010). *D. salina* cells had a higher cell volume and surface when they were cultivated at 800, 1600, and 3200 $\mu mol$ $L^{-1}$ of $NO_3^-$ than at 80 $\mu mol$ $L^{-1}$ of $NO_3^-$. Therefore, the greater abundance of unsaturated fatty acids in this alga may be related to the fact that they contain more membranes. Using a similar argument, the relative invariability of *T. pseudonana* and *P. reticulatum* SFAs, MUFAs, and PUFAs as a function of growth $[NO_3^-]$ (Fig. 2) may be associated to the fact that in this species, cell size is not significantly affected by the growth conditions.

Use of FTIR spectroscopy to evaluate the quality of fatty acids

In previous papers (Palmucci et al. 2011; Palmucci and Giordano 2012), we used the ratio between the integrals of the FTIR peaks attributed to $-CH_3$, $-CH_2$, and $-CH$ as a proxy of the reduction level of the whole biomass. In principles, these molecular groups are present in all main organic molecules, even if they are especially abundant in the aliphatic chains of fatty acids. For this reason, we did not recommend the use of the ratio of these spectroscopic features to determine the unsaturation level of fatty acid, specifically (Palmucci et al. 2011). In this work, we directly compared the biomass reduction level obtained by FTIR spectroscopy with the degree of unsaturation in fatty acids determined by GC and found the two to be highly correlated (Fig. 3). FTIR estimation of the unsaturation level thus appears to provide a reliable measure of the number of C double bonds in the fatty acid mixture. Our previous reluctance to use this spectroscopic

**Table 5** Cetane number (CN), viscosity at 40 °C ($V$, in $mm^2 s^{-1}$), cold filter plugging point (CFFP), and heating value (HV, in $MJ kg^{-1}$) of the oils obtained from *D. salina*, *T. pseudonana*, and *P. reticulatum* cultured at 80, 800, 1600, and 3200 $\mu mol L^{-1} NO_3^-$

| Species | $[NO_3^-]$ ($\mu mol L^{-1}$) | CN | CN score | $V$ | $V$ score | CFPP (°C) | CFPP score | HV | HV score | Overall biodiesel score |
|---|---|---|---|---|---|---|---|---|---|---|
| *D. salina* | 80 | 55.5 | 1 | 3.4 | 1 | −6.1 | 6 | 35.9 | 3 | 18 |
| | 800 | 52.8 | 1 | 3.8 | 2 | −2.9 | 3 | 36.3 | 3 | 18 |
| | 1600 | 57.1 | 2 | 4.1 | 3 | 3.2 | 0.5 | 38.2 | 4 | 12 |
| | 3200 | 56.7 | 2 | 4.2 | 3 | 0.5 | 0.5 | 37.6 | 4 | 12 |
| *T. pseudonana* | 80 | 58.3 | 2 | 3.9 | 2 | −4.3 | 3 | 36.2 | 5 | 60 |
| | 800 | 58.5 | 2 | 3.9 | 2 | −5.8 | 3 | 36.2 | 6 | 72 |
| | 1600 | 58.6 | 2 | 3.9 | 2 | −6.5 | 3 | 36.0 | 6 | 72 |
| | 3200 | 55.7 | 1 | 3.7 | 1 | −5.3 | 2 | 34.4 | 6 | 12 |
| *P. reticulatum* | 80 | 67.7 | 4 | 4.1 | 3 | 3.6 | 3 | 36.2 | 0.5 | 18 |
| | 800 | 67.5 | 4 | 4.1 | 3 | −1.0 | 1 | 36.3 | 3 | 36 |
| | 1600 | 73.8 | 4 | 4.1 | 3 | 1.8 | 3 | 36.2 | 0.5 | 18 |
| | 3200 | 69.1 | 5 | 4.6 | 5 | 0.3 | 5 | 39.8 | 0.5 | 62.5 |

The score columns show the relative quality of the biodiesel for the various parameters and the overall score resulting from the sum of the scores for the individual parameters. See "Material and methods" for details

information as a proxy of the unsaturation level of fatty acids may thus have been excessive. FTIR spectroscopy may therefore be applied as a screening method to evaluate the level of reduction of fatty acid mixtures and consequently their sensitivity to oxidation (Schenk et al. 2008). FTIR spectroscopy is probably not a good method to determine CN, viscosity, and melting point of the biodiesel derived from algal oils, because these properties not only are exclusively affected by the number of double bonds between atoms of carbon but also depend on the length of the carbon chains of the fatty acids (Knothe 2005 and references therein).

Impact of light on unsaturation level and fatty acid composition

Since the level of unsaturation may reflect the energy content of biomass (and specifically of lipids), we also wanted to test if this could be affected by the light regime. In many studies, an increase of the unsaturated fraction of fatty acids was reported in response to an increase of irradiance. Microalgae may increase the level of reduction of their fatty acids, at high irradiances, as a way to cope with excess energy (Palmucci et al. 2011). On the other hand, an increase of the unsaturation level of fatty acids can also be expected as a consequence of the proliferation of thylakoid membranes at low light (Goss and Wilhelm 2010). In *P. reticulatum*, both the ratio between the percentage of saturated fatty acids (SFAs) and that of unsaturated fatty acids (MUFAs+PUFAs) and fatty acid composition were little affected by an even 12-fold difference in PFD, and the small differences observed did not show any clear trend with respect to irradiance. These results confirm the tendency of *P. reticulatum* to strictly control compositional homeostasis (Montechiaro and Giordano 2010).

Impact of fatty acid composition on biodiesel properties

The biodiesel that can potentially be obtained from microalgae is quite different from that obtained from traditional crops, and some algal biodiesels do not fit the specifications required internationally for this kind of fuels (UNE-EN 14214, 2003: Automotive fuels; fatty acid methyl esters (FAME) for diesel engines) (Requirements and test methods are available at http://www.novaol.it/novaol/export/sites/default/allegati/EN14214.pdf; also see Ramos et al. 2009 and references therein). Many groups of microalgae have high percentages of polyunsaturated fatty acids (e.g., Guschina and Harwood 2006); a high content of PUFA makes biodiesel highly susceptible to oxidation (Monyem and van Gerpen 2001). Unfortunately, it is not possible to give a value of oxidative stability only on the basis of the fatty acid composition as we did for the other biodiesel properties (Stansell et al. 2012), but it is reasonable to assume that the higher is the level of unsaturation of fatty acids, the lower is the oxidative stability. Our estimated level of reduction may therefore be an approximate indicator of the susceptibility to oxidation of a defined mixture of fatty acids (Knothe 2007). If this is true, the biodiesel that can be produced from *D. salina* would be especially prone to oxidation, given the high content of PUFA in this species (Table 3). Also, the biodiesel produced from the two other species may suffer of poor oxidative stability, even if they have lower percentage of PUFA than *D. salina*: In the oil from both *P. reticulatum* and *T. pseudonana*, at least one very abundant long-chain PUFA is present (i.e., C22:6 in *P. reticulatum* and C16:3 in *T. pseudonana*). Our results thus support the warning by Stansell et al. (2012) about the low oxidative stability of alga oils; although the problem can be eliminated by post-

extraction hydrogenation (Moser et al. 2007), the added cost of this treatment has to be considered.

On the other hand, the presence of many double bonds decreases the viscosity and the cetane number (CN) of the oil. However, the CN of the algae used in this work, as it is common for algal oils, was substantially higher than the standards required from the international organization (Table 1), which is usually considered a desirable feature in a diesel fuel. The predicted CN of *P. reticulatum* was surprisingly high if compared to those measured for biodiesel from vascular plants (e.g., Blin et al. 2013 and references therein): This was mainly due to the high content of C16:0, which has a very high CN (~75). The CN values of *T. pseudonana* (55.7–58.6) and *D. salina* (52.8–57.1) oils were much lower than those of the oil from the dinoflagellate (Table 5).

The high abundance of C16:0 also made the viscosity of the mixture of fatty acid methyl esters from *P. reticulatum* (4.1–6.1 mm$^2$ s$^{-1}$, at 40 °C) much higher than that from *D. salina* (3.4–4.2 mm$^2$ s$^{-1}$, at 40 °C) and *T. pseudonana* (3.7–4.0 mm$^2$ s$^{-1}$, at 40 °C; Table 5).

The maximum heating value calculated for the hypothetical biodiesel produced from the three algae was rather similar and in the range between 34.3 and 38.3 MJ kg$^{-1}$ (Table 5). Only the oil from *P. reticulatum* cultured at a PFD of 50 μmol photons m$^{-2}$ s$^{-1}$ had an appreciably higher heating value (46.4 MJ kg$^{-1}$).

The cold filter plugging point (CFPP) was remarkably lower for the hypothetical biodiesel from *T. pseudonana* (−6.5 to −4.3 °C) than for that from *D. salina* (−6.1 to + 3.22 °C) and *P. reticulatum* (−0.99 to +6.4 °C; Table 5).

Ranking of algal biodiesel

In most cases, the biodiesel derived from the three species used for this work has CN, viscosity, and heating value above the standard minimum requirements (Tables 1 and 4). The biodiesel derived from *D. salina* cultured at 1600 and 3200 μmol L$^{-1}$ NO$_3^-$ and from *P. reticulatum* cultured at all [NO$_3^-$] did not match the requirements for CFPP. The FAME complement of *T. pseudonana* obtained high total scores ($B_s$) (except when the cells of this species were cultured at 3200 μmol L$^{-1}$ NO$_3^-$) and appears to be the most suited for utilization in today's diesel engines. The FAMEs extracted by *T. pseudonana* cultured at any [NO$_3^-$] obtained especially high scores for CFPP and satisfied all other standards.

**Conclusion**

N deprivation is commonly used to enhance oleogenesis in algal cultures. However, the outcome of such treatment is strongly species-specific and depends on the metabolic strategies cells adopt to allocate carbon, which may change appreciably under different culture conditions.

Among the three species used, *D. salina* is the most oleogenic. However, the oil that is most suited for utilization in extant diesel engines is that obtained from *T. pseudonana*. FTIR spectroscopy appears to be a good method for the rapid and inexpensive screening and monitoring of oleogenic algae.

## References

Allen CAW, Watts KC, Ackman RG, Pegg MJ (1999) Predicting the viscosity of biodiesel fuels from their fatty acid ester composition. Fuel 78:1319–1326

Baba M, Shiraiwa Y (2013) Biosynthesis of lipids and hydrocarbons in algae. In: Dubinsky Z (ed.) Photosynthesis, InTech, doi: 10.5772/56413

Bamgboye AI, Hansen AC (2008) Prediction of cetane number of biodiesel fuel from the fatty acid methyl ester (FAME) composition. Int Agrophys 22:21–29

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

Blin J, Brunschwig C, Chapuis A, Changotade O, Sidibe SS, Noumi ES, Girard P (2013) Characteristics of vegetable oils for use as fuel in stationary diesel engines—towards specifications for a standard in West Africa. Renew Sust Energ Rev 22:580–597

Choi G-G, Kim B-Y, Ahn C-Y, Oh H-M (2011) Effect of nitrogen limitation on oleic acid biosynthesis in *Botryococcus braunii*. J Appl Phycol 23:1031–1037

Edwards KF, Thomas MK, Klausmeier CA, Litchman E (2012) Allometric scaling and taxonomic variation in nutrient utilization traits and maximum growth rate of phytoplankton. Limnol Oceanogr 57:554–566

Fanesi A, Raven JA, Giordano M (2014) Growth rate affects the responses of the green alga *Tetraselmis suecica* to external perturbations. Plant Cell Environ 37:512–519

Flynn KJ, Garrido JL, Zapata M, Opikl H, Hipkin CR (1992) Changes in fatty acids, amino acids and carbon/nitrogen biomass during nitrogen starvation of ammonium- and nitrate-grown *Isochrysis galbana*. J Appl Phycol 4:95–104

Giordano M (2013) Homeostasis: an underestimated focal point of ecology and evolution. Plant Sci 211:92–101

Goss R, Wilhelm C (2010) Lipids in Algae, Lichens and Mosses. Advances in Photosynthesis and Respiration, 30:117–137

Griffiths MJ, Harrison STL (2009) Lipid productivity as a key characteristic for choosing algal species for biodiesel production. J Appl Phycol 276:23–25

Guschina IA, Harwood JL (2006) Lipids and lipid metabolism in eukaryotic algae. Prog Lipid Res 45:160–186

Harrington KJ (1986) Chemical and physical properties of vegetable oil esters and their effect on diesel fuel performance. Biomass 9 (1):1–17

Hockin NL, Mock T, Mulholland F, Kopriva S, Malin G (2012) The response of diatom central carbon metabolism to nitrogen starvation is different from that of green algae and higher plants. Plant Physiol 158:299–312

Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008) Microalgal triacylglycerols as feedstock for bio-fuel production: perspectives and advances. Plant J 54:621–639

Jebsen C, Norici A, Wagner H, Palmucci M, Giordano M, Wilhelm C (2012) The FTIR spectra of algal species can be used as physiological fingerprints to assess their actual growth potential. Physiologia Plantarum, 146:427–438, doi: 10.1111/j.1399-3054.2012.01636.x;

Kaffes N, Thoms S, Trimborn S, Rost B, Richter K-U, Köhler A, Norici A, Giordano M (2010) Carbon and nitrogen fluxes in the marine coccolithophore Emiliania huxleyi grown under different nitrate concentrations. Journal of Experimental Marine Biology and Ecology 393:1–8. doi: 10.1016/j.jembe.2010.06.004.

Klyachko-Gurvich GL, Tsoglin LN, Doucha J, Kopetskii J, Shebalina (Ryabykh) IB, Semenenko VE (1999) Desaturation of fatty acids as an adaptive response to shifts in light intensity. Physiol Plant 107:240–249

Klopfenstein WE (1985) Effect of molecular weights of fatty acid esters on cetane numbers as diesel fuels. Journal of the American Oil Chemists Society 62 (6):1029–1031

Knothe G (2007) Some aspects of biodiesel oxidative stability. Fuel Proc Technol 88(7):669–677

Lomas MW, Gilbert PM (2000) Comparison of nitrate uptake, storage, and reduction in marine diatoms and flagellates. J Phycol 36:903–913

Montechiaro F, Giordano M (2010) Compositional homeostasis of the dinoflagellate Protoceratium reticulatum grown at three different $pCO_2$. J Plant Physiol 167:110–113

Montechiaro F, Hirschmugl C, Raven JA, Giordano M (2006) Homeostasis of cell composition during prolonged darkness. Plant Cell Environ 29:2198–2204

Monyem A, van Gerpen JH (2001) The effect of biodiesel oxidation on engine performance and emissions. Biomass Bioenergy 20:317–325

Moser BR, Haas MJ, Winkler JK, Jackson MA, Erhan SZ, List GR (2007) Evaluation of partially hydrogenated methyl esters of soybean oil as biodiesel. Eur J Lipid Sci Technol 109:17–24

Norici A, Bazzoni AM, Pugnetti A, Raven JA, Giordano M (2011) Impact of irradiance on the C al location in the coastal marine diatom Skeletonema marinoi Sarno and Zingone. Plant Cell Environ 34:1666–1677

Ota M, Kato Y, Watanabe H, Watanabe M, Sato Y, Smith RL Jr, Inomata H (2009) Fatty acid production from a highly $CO_2$ tolerant alga, Chlorocuccum littorale, in the presence of inorganic carbon and nitrate. Bioresour Technol 100:5237–5242

Palmucci M, Giordano M (2012) Is cell composition related to the phylogenesis of microalgae? An investigation using hierarchical cluster analysis of Fourier transform infrared spectra of whole cells. Environ Exp Bot 75:220–224

Palmucci M, Ratti S, Giordano M (2011) Ecological and evolutionary implications of carbon allocation of marine phytoplankton as a function of nitrogen availability: a Fourier transform infrared spectroscopy approach. J Phycol 47:313–323

Pandey A, Lee D-J, Chisti Y, Soccol CR (eds) (2014) Biofuels from algae. Elsevier, Burlington

Peterson GL (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83:346–356

Picardo MC, de Medeiros JL, Garcia J, Monteiro M, Moreira Chaloub R, Giordano M, de Queiroz Fernandes Araujo O (2013) A methodology for screening of microalgae as a decision making tool for energy and green chemical process applications. Clean Technol Environ Pol 15:275–291

Ramírez-Verduzco LF, Rodríguez-Rodríguez JE, del Rayo Jaramillo-Jacob A (2012) Predicting cetane number, kinematic viscosity, density and higher heating value of biodiesel from its fatty acid methyl ester composition. Fuel 1:102–111

Ramos MJ, Fernández CM, Casas A, Rodríguez L, Pérez A (2009) Influence of fatty acid composition of raw materials on biodiesel properties. Bioresour Technol 100:261–268

Ratti S, Giordano M, Morse D (2007) $CO_2$-concentrating mechanisms of the potentially toxic dinoflagellate Protoceratium reticulatum (Dinophyceae, Gonyaulacales). J Phycol 43:693–701

Schenk PM, Thomas-Hall SR, Stephens E, Marx UC, Mussgnug JH, Posten C, Kruse O, Hankamer B (2008) Second generation biofuels: high-efficiency microalgae for biodiesel production. Bioenergy Res 1:20–43

Solovchenko AE, Khozin-Goldberg I, Didi-Cohen S, Cohen Z, Merzlyak MN (2008) Effects of light intensity and nitrogen starvation on growth, total fatty acids and arachidonic acid in the green microalga Parietochloris incisa. J Appl Phycol 20:245–251

Srivastava A, Prasad R (2000) Triglycerides-based diesel fuels. Renew Sust Energ Rev 4:111–133

Stansell GR, Gray VM, Sym SD (2012) Microalgal fatty acid composition: implications for biodiesel quality. J Appl Phycol 24:791–801

Sun A, Davis R, Starbuck M, Ben-Amotz A, Pate R, Pienkos PT (2011) Comparative cost analysis of algal oil production for biofuels. Energy 36:5169–5179