Dynamics of Lipid Biosynthesis and Redistribution in the Marine Diatom Phaeodactylum tricornutum Under Nitrate Deprivation

ARTICLE *in* BIOENERGY RESEARCH · DECEMBER 2012

Impact Factor: 3.54 · DOI: 10.1007/s12155-012-9201-7

9 AUTHORS, INCLUDING:

Damian Carrieri
Matrix Genetics, Seattle Washington
18 PUBLICATIONS **619** CITATIONS
SEE PROFILE

Anita Brinker
Rutgers, The State University of New Jersey
23 PUBLICATIONS **1,064** CITATIONS
SEE PROFILE

Miguel Frada
Hebrew University of Jerusalem - Inter Univ…
14 PUBLICATIONS **296** CITATIONS
SEE PROFILE

Gerard Charles Dismukes
Rutgers, The State University of New Jersey
223 PUBLICATIONS **9,099** CITATIONS
SEE PROFILE

Bioenerg. Res. (2012) 5:876–885
DOI 10.1007/s12155-012-9201-7

Dynamics of Lipid Biosynthesis and Redistribution in the Marine Diatom *Phaeodactylum tricornutum* Under Nitrate Deprivation

Elizabeth H. Burrows · Nicholas B. Bennette · Damian Carrieri · Joseph L. Dixon · Anita Brinker · Miguel Frada · Steven N. Baldassano · Paul G. Falkowski · G. Charles Dismukes

Published online: 4 May 2012

Abstract One approach to achieve continuous overproduction of lipids in microalgal "cell factories" relies upon depletion or removal of nutrients that act as competing electron sinks (e.g., nitrate and sulfate). However, this strategy can only be effective for bioenergy applications if lipid is synthesized primarily de novo (from CO_2 fixation) rather than from the breakdown and interconversion of essential cellular components. In the marine diatom, *Phaeodactylum tricornutum*, it was determined, using ^{13}C-bicarbonate, that cell growth in nitrate (NO_3^-)-deprived cultures resulted predominantly in de novo lipid synthesis (60 % over 3 days), and this new lipid consisted primarily of triacylglycerides (TAGs). Nearly complete preservation of ^{12}C occurred in all previously existing TAGs in NO_3^--deprived cultures and thus, further TAG accumulation would not be expected from inhibition of TAG lipolysis. In contrast, both high turnover and depletion of membrane lipids, phosphatidylcholines (PCs), were observed in NO_3^--deprived cultures (both the headgroups and fatty acid chains), while less turnover was observed in NO_3^- replete cultures. Liquid chromatography-tandem mass spectrometry mass spectra and ^{13}C labeling patterns of PC headgroups provided insight into lipid synthesis in marine diatoms, including suggestion of an internal pool of glycine betaine that feeds choline synthesis. It was also observed that 16C fatty acid chains incorporated into TAGs and PCs contained an average of 14 ^{13}C carbons, indicating substantial incorporation of ^{13}C-bicarbonate into fatty acid chains under both nutrient states.

Keywords Algae · Biodiesel · Nitrate · Nutrients · Fatty acid metabolism · De novo lipid biosynthesis · *Phaeodactylum tricornutum*

Electronic supplementary material The online version of this article (doi:10.1007/s12155-012-9201-7) contains supplementary material, which is available to authorized users.

E. H. Burrows · N. B. Bennette · G. C. Dismukes (✉)
Department of Chemistry and Chemical Biology,
Rutgers University,
610 Taylor Rd.,
Piscataway, NJ 08854, USA
e-mail: dismukes@rci.rutgers.edu

D. Carrieri · S. N. Baldassano
Department of Chemistry, Princeton University,
Piscataway, NJ, USA

J. L. Dixon · A. Brinker
Department of Nutritional Sciences, Rutgers University,
Piscataway, NJ, USA

J. L. Dixon · A. Brinker · P. G. Falkowski
Rutgers Center for Lipid Research, Rutgers University,
Piscataway, NJ, USA

M. Frada · P. G. Falkowski
Environmental Biophysics and Molecular Ecology Program,
Rutgers University,
Piscataway, NJ, USA

Introduction

Photosynthesis stores solar energy as chemical bond energy in the form of carbohydrates, lipids, and other cellular material, and photosynthetically derived lipids can be extracted or secreted and readily saponified and transesterified to form biodiesel [1]. Although lipid-rich plants, such as soybean, jatropha, and oil palm, have received much attention, some natural strains of aquatic microalgae have 6- to 12-fold higher energy yield per unit area per year than terrestrial crops [2] and the theoretical maximum per unit area per year is almost 60 times reported yields for the most productive terrestrial crops [3].

Although there has been extensive research on lipid production in microalgae, including diatoms, for decades [4, 5], several hurdles remain before the technology becomes economically viable [6]. One is increasing the quantum efficiency for de novo production of lipids. Although nutrient deprivation, in particular the removal of nitrate (NO_3^-) from growth media, causes an increase in cellular lipid accumulation on the order of 1.5- to 2-fold in many species of algae [7], the mechanisms behind this phenomenon have not been elucidated. The magnitude of the increase in lipid production depends on multiple factors, including but not limited to period and degree of deprivation, cell age and density, and light conditions.

The depletion of nitrate without replacement with another utilizable nitrogen source is accompanied by retardation, if not complete cessation, of cell growth. Therefore, in order for the strategy of NO_3^- deprivation to be practical for industrial scale-up, a two-stage "cell factory" system would likely be required, in which cells would be first grown to final desired (maximum) biomass, and the subsequently growth arrested cells would be continuously "milked" for lipids. The lipids could either be extracted from live cultures using low-energy methods, for example via solvents [8, 9], or cells could be genetically engineered for secretion of lipids [10]. Several comprehensive reviews discuss this approach [11, 12]. The strategy of NO_3^- deprivation in conjunction with the milking approach would only be viable for sustainable cell culturing if the observed lipid increase were due to de novo synthesis from CO_2 rather than the breakdown of nitrogen-rich intracellular components.

A study to determine the extent of de novo lipid synthesis upon NO_3^- deprivation in the alga *Nannochloropsis* found that bioconversion of other cell components to lipid was insignificant even after 10 days of deprivation [13]. Another study, examining silicon deprivation in the marine diatom *Cyclotella cryptica*, concluded that there was a slow conversion of non-lipid components to lipid [14]. In the present study, we aimed to evaluate de novo lipid synthesis under NO_3^- deprivation in the marine diatom, *Phaeodactylum tricornutum*, for which a fully sequenced genome [15] and genetic tools are available [16], making it particularly promising for biotechnological applications. We employed ^{13}C labeling and liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques to determine the extent of de novo lipid synthesis and the molecular location of labeling under NO_3^- deprivation.

Methods

Culture Conditions

P. tricornutum strain CCAP1055/1 was grown on F/2 media [17] at approximately 18 °C on a shaker table at 200 rpm [18] illuminated from above with approximately 100 μmol quanta m^{-2} s^{-1}.

Experimental Setup

Samples of *P. tricornutum* were pre-inoculated in F/2 media, with initial concentration of about 8×10^5 cells mL^{-1} and grown for 4 days prior to experimentation. At this point, "Time zero", the first round of assays was performed, which included measurements of cell numbers, chlorophyll, lipid contents, ash (inorganic) percent, and percent of ^{13}C, ^{12}C, and N in both whole cells and dried lipid extracts, measured via elemental analysis (conducted at the Stable Isotope Laboratory, the Ecosystems Center, Marine Biological Laboratory in Woods Hole, MA, USA, measured on a Europa 20–20 CF-IRMS interfaced with the Europa ANCA-SL elemental analyzer). The measured elemental abundance of ^{13}C in the unlabeled cultures was 1.1 % which matches the natural abundance. The remaining cultures were then pelleted and resuspended in F/2 media with ^{13}C-labeled 16 mM $NaH^{13}CO_3$, totaling three 500 mL flasks (250 mL culture volume) of NO_3^- replete media and three of NO_3^--deprived media. NO_3^--deprived media was identical to NO_3^- replete media, except that there was no $NaNO_3$ added (NO_3^- replete media contains 0.88 mM $NaNO_3$ constituting a negligible amount of the sodium present in F/2 media, which contains 400 mM NaCl). Even though $^{12}CO_2$ constituted less than 0.01 % of the C in the flask, cultures were bubbled with nitrogen gas for 10–15 min to remove much of the $^{12}CO_2$. To maintain axenic cultures, sparging was conducted with 0.22 μm filtered N_2 in a biosafety cabinet using 8.5-in. long stainless steel syringe needles with rubber stoppers almost completely sealing the flasks. The syringe needles were then quickly removed and the rubber stoppers were used to seal the flasks to minimize gas exchange with the environment. Approximately 3-fold more $NaH^{13}CO_3$ than would be needed to maintain diatom growth over 3 days was added to account for the small amount lost as $^{13}CO_2$ during the sparging. Based on reported oxygen evolution rates of marine diatoms, the O_2 concentration in the closed system would not have increased from near zero after N_2 sparging to higher than ambient, 20.9 %, over 3 days. Prior trials were conducted to ensure that *P. tricornutum* cells multiplied at the same rate with labeled bicarbonate as the exclusive carbon source, as has been shown by Granum et al. [19]. After 3 days of growth in the test conditions, the assays performed at "Time zero" were repeated. The entire experimental procedure was completed twice to ensure reproducibility.

Chlorophyll *a* Assay

Four milliliters of sample was pelleted, resuspended in methanol, and incubated for 30 min in the dark. The samples were then centrifuged for 5 min, the supernatant was collected, and the optical density was measured at 665 nm. The

chlorophyll *a* content, plus some chlorophyll *c*, was determined using the extinction coefficient in Porra et al. [20].

Cell Counts

Cells were counted using a hemacytometer, in which 9.5 μL of cells were inserted, and allowed to settle for 10 min before counting. Between 20 and 200 cells were counted, in duplicate.

Lipid Analysis

For LC-MS/MS and elemental analysis, 100 mL of cells were filtered on glass fiber filters for use in the lipid assay, and frozen at −80 °C until analysis. Both the Slayback et al. [21] and the Bligh and Dyer [22] methods of lipid extraction were tested prior to the experiment and it was determined that the Bligh and Dyer method was preferable, since both methods gave similar results and the Bligh and Dyer method was shorter and used less toxic chemicals.

For quantification of total fatty acid methyl esters (FAMEs), a hexane/acetyl chloride/methanol extraction-transmethylation step followed by GC analysis was used. The method was adapted from Rodriguez-Ruiz et al. [23].

For examination of labeling patterns in various lipid classes, a normal phase LC-MS/MS method was used, which emphasized separation of triacylglycerides (TAGs) and phosphatidylcholine (PCs) using a previously developed technique [24]. Briefly, lipid samples extracted via the Bligh and Dyer [22] method and suspended in isooctane/tetrahydrofuran (9:1 *v*/*v*) were injected, without preseparation, into a Dionex UltiMate 3000 HPLC (Dionex, Sunnyvale, CA, USA) coupled to an ABSciex 4000Q-TRAP® mass spectrometer equipped with a Turbo V™ ESI source (ABSciex, Foster City, CA, USA). The UltiMate system used a Waters (Milford, MA, USA) Spherisorb® S5W 4.6×100 mm silica column, 5 μm particle size, with a Spherisorb® S5W 4.6×10 mm guard cartridge. The solvent gradient was modified from that used by Homan and Anderson [25]. The autosampler temperature was 20 °C and the column oven temperature was 45 °C. TAGs (eluted in a single peak from 8 to 9 min) were monitored with a Q1 scan covering 500–1,000 *m*/*z* and were observed as intact TAG-plus-sodium cations. PCs eluted in the same chromatographic run in a single peak from 23.5 to 27 min. The labeling of the phosphocholine (not to be confused with phosphatidylcholine, abbreviated PC) headgroups was quantifiable, as PCs were detected based on the production of the phosphocholine fragment ion, $C_5H_{15}NO_4P^+$, with a singly protonated mass of 184. The precursor ion scans for positive ions 184, 185, 186, 187, 188, and 189 were monitored, in order to account for the incorporation of ^{13}C into the five carbon atoms in the choline head group. Amounts of total TAGs and PCs were calculated using Analyst 1.4.2 software by integrating the entire TAG and PC peaks and utilizing standard curves. Peak areas of individual molecular species within each TAG and PC lipid class were identified and determined using Lipid Profiler/LipidView software (ABSciex).

Ash Percents

Thermogravimetric analysis was used to determine ash percent (noncombustible solids). In this method, a small sample of cells (approximately 5 mg) was dried overnight at 60 °C, weighed, and then heated at a rate of 40 °C per min over a range starting at 40 °C and increasing to 900 °C under an argon purge. After cooling, the sample was reweighed and the percent ash was calculated. The percent ash was used solely to calculate the ash-free quantities of dry weight, since original dried cells were centrifuged and washed, but not rinsed with ammonium formate to dissolve inorganic silica attached to cells or in the media. Results were compared to overnight combustion at 450 °C of 30 mL of dried cells.

Calculations

For Table 1, ^{13}C and total C in lipid were calculated by multiplying the total FAME measurements by the proportion of ^{13}C and total C in lipid (obtained via elemental analysis), respectively. ^{12}C was calculated by subtracting ^{13}C from total C (and propagating the error). Rates of increase were calculated from these values.

For Fig. 3, measured quantities are displayed next to projected quantities assuming no interconversion of the lipid present at "Time zero". This was done to make it apparent that a certain amount of unlabeled lipid is expected based on the cell biomass existing in the flasks at "Time zero". For example, if cell count doubled over 3 days of exposure to ^{13}C, the unlabeled lipid per cell should be half, assuming no interconversion. Both the measured and predicted bars involved some simple calculations (see Fig. 1). The labeled *m*/*z* range represents any molecule that elutes at the correct retention time with an *m*/*z* larger than the "previously existing" *m*/*z* range, and thus contains new molecules that are

Table 1 Total ^{13}C and ^{12}C lipid increase rate

	3 Days NO_3^- replete	3 Days NO_3^- deprived
^{12}C in lipid increase (day^{-1})	0.035±0.033	0.205±0.037
^{13}C in lipid increase (day^{-1})	1.34±0.016	1.56±0.022

Values were calculated as specific growth rate is calculated. See "Methods" section for details. Error represents one standard error of the mean (SEM, n=3)

either partially or fully ^{13}C enriched; therefore, the "new" notation refers to new molecules, meaning that they did not exist before in their current state because they contain some degree of ^{13}C. Also, as previously described in the "Methods" section, the ^{13}C label content in the PC headgroups was able to be quantified. Therefore, as was described in Fig. 1, the "Previously existing headgroup, new acyl chain" was calculated by simply integrating the labeled range (735–765 *m/z*) for solely the 184 precursor ion scan. The "New headgroup, previously existing acyl chains" bars were calculated by integrating the unlabeled range (725–735 *m/z*) and summing these peak areas for the 185–189 *m/z* precursor ion scans, representing 1–5 ^{13}Cs in the headgroup (see Fig. 4). Similarly, the "New headgroup and acyl chain(s)" bars were calculated by integrating the 735–765 *m/z* peaks and summing the values for the 185–189 *m/z* precursor ion scans, since each of these molecules could be considered "new" because there was some label in both the headgroup and the acyl chains.

Results and Discussion

De Novo Lipid Biosynthesis

Cultures completely deprived of NO_3^- for 3 days doubled at half the control rate (0.23 ± 0.02 day^{-1}) yet accumulated three times as much total lipid per cell on a weight basis, as compared to cells in NO_3^- replete media with a growth rate of 0.46 ± 0.02 day^{-1} (Tables 2 and 3). The total lipid content of NO_3^- replete cultures of *P. tricornutum* agreed with Tonon et al. [26] at the same cell density and growth phase, although these values are approximately 3-fold lower than other reports under similar, nutrient replete conditions [27–29].

Unlabeled C in lipid increased at a rate of only 0.035 ± 0.033 day^{-1} (essentially zero) over 3 days in NO_3^- replete media containing ^{13}C-bicarbonate as expected, while the ^{13}C in lipid increased at a rate of 1.34 ± 0.02 day^{-1} (Table 1). Likewise, although cell growth was twice as slow under NO_3^- deprivation (Table 3), ^{12}C in lipid increased at a rate of 0.205 ± 0.037 day^{-1} and ^{13}C in lipid increased at a rate of 1.56 ± 0.02 day^{-1}. In NO_3^- replete cultures, 85 % of new lipid resulted from de novo synthesis from ^{13}C-bicarbonate and the remainder from conversion of nonlipid precursors, while in NO_3^--deprived cultures 60 % of new lipid was synthesized de novo. NO_3^- deprivation over 3 days resulted in about 2.8-fold more total lipid biosynthesis than in NO_3^- replete media. The 16 % higher rate of ^{13}C increase in lipid in NO_3^--deprived cultures (Table 1) is particularly promising for the prospect of lipid cell factories.

However, the observed de novo synthesis and greater ^{13}C accumulation in NO_3^--deprived cultures could not continue indefinitely under total NO_3^- removal, due to requisite protein repair and cell maintenance. Ultimately, some optimal, low NO_3^- concentration may exist that would permit continual culture maintenance and repair in parallel with continuous lipid overproduction. Alternatively, NO_3^- availability could be varied temporally or an approach could be

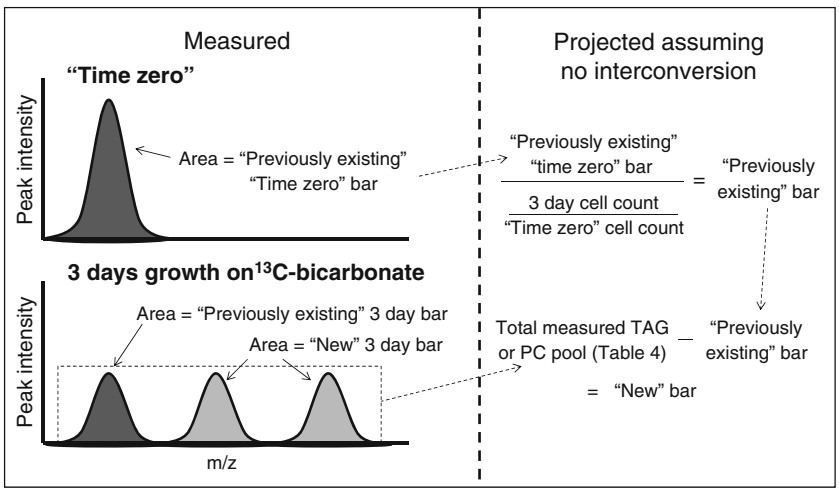

Fig. 1 Visual representation of the origin of the values used to create Fig. 3. The "Previously existing" (unlabeled, primarily ^{12}C) measured bars were calculated by integrating the area under the curve for "Time zero", and the area under the curve over the same *m/z* range as "Time zero" for NO_3^- replete and deprived cultures (the representative TAG group chosen was 820–830 *m/z*, and the representative PC group chosen was 725–735 *m/z*). The "Previously existing" projected bars were calculated by dividing the "Time zero" TAG or PC value by the % increase in cell number, since for example, if cell number doubled over 3 days, then the maximum "Previously existing" lipid per cell could only be half of the "Time zero" amount *if* there was no conversion of other cell pools to lipid. The "new" TAG and PC projected bars were calculated by simply subtracting the "Previously existing" (unlabeled) amount from the total TAG or PC (presented in Table 4). The "new" measured bars were calculated by integrating the area under the curve for the ^{13}C-labeled *m/z* range (for TAG the labeled range was 830–880 *m/z* and for PC it was 735–765 *m/z*)

Table 2 Ash-free dry weight nitrogen and carbon elemental analysis and total lipid (determined as total FAME) of *P. tricornutum* after growth for 3 days, in both NO_3^- replete and NO_3^- deprived culture media

	3 Days NO_3^- replete	3 Days NO_3^- deprived
AFDW N (%)	5.6±0.8	2.8±0.6
AFDW C (%)	40.7±3.7	36.8±2.0
Lipid (pg cell^{-1})	0.95±0.08	3.10±0.38

Error represents one SEM (n=3)

employed that mimics the lipid "trigger" caused by NO_3^- deprivation without actually depriving the cells of nitrogen, such as ammonium or tungstate addition (M. Frada, personal communication).

After 3 days of growth, the lipid fractions in NO_3^--replete and NO_3^--deprived cultures were enriched in ^{13}C to approximately 40 % of total C (Fig. 2). Additionally, both conditions produced a greater proportion of ^{13}C as lipid than as whole cell biomass, with a larger difference for the NO_3^--deprived cultures. Hence, NO_3^--deprived cells allotted a greater proportion of assimilated C to lipid biosynthesis than to other cell biomass. Although the mean percentage of ^{13}C in total carbon for NO_3^- replete was higher than for NO_3^--deprived cultures, the difference was not statistically significant (Fig. 2), indicating that cells assimilated a similar amount of ^{13}C per cell over 3 days.

The appreciable rate of increase of unlabeled lipid present in the NO_3^--deprived cultures (Table 1) may arise from redirection of intracellular metabolite pools away from protein and chlorophyll synthesis into lipid storage. Consistent with this observation, chlorophyll a per cell decreased in NO_3^--deprived cells over 3 days (Table 3). The higher chlorophyll a and lower TAG per cell in NO_3^--replete cells after 3 days indicates that cells at "Time zero" came from different conditions than those that the cells were exposed to over the 3-day experiment. The precise comparison, upon which the conclusions of this study depend, is between NO_3^--deprived and NO_3^--replete cells, which came from the same "Time zero" culture and were treated identically. "Time zero" data are important for calculations such as quantification of the total ^{12}C expected after 3 days.

Table 3 Growth parameters of *P. tricornutum* at "Time zero" and after growth for 3 days, in both NO_3^- replete and NO_3^- deprived culture media

	Time zero	3 Days NO_3^- replete	3 Days NO_3^- deprived
Cell count (× 10^6 cells mL^{-1})	2.52±0.04	10.00±0.38	4.99±0.16
Chl a (pg cell^{-1})	0.67±0.036	0.80±0.033	0.41±0.015

Error represents one SEM (n=3)

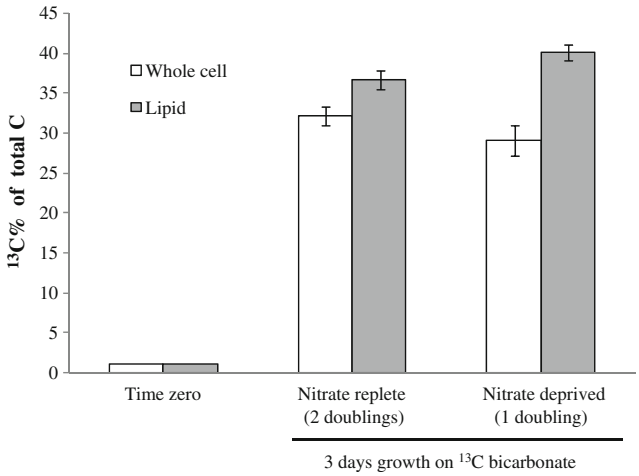

Fig. 2 Percent ^{13}C of total C pool in whole cells and lipid fraction showing that a greater proportion of C assimilated was allotted to lipid biosynthesis under nitrate deprivation. Values were measured directly by elemental analysis. "Time zero" represents prelabeling natural abundance. *Error* 1 SEM (n=3)

LC-MS/MS data suggest that the composition of the lipids changed and the relative yield of TAGs per cell increased 4-fold in NO_3^--deprived cultures, whereas PCs, a main component of cell outer membranes, decreased approximately 35 % (Table 4, Fig. 3). Also, it can be seen from the LC-MS/MS data that both newly synthesized PCs and TAGs contain a portion of previously existing carbon (^{12}C); the evidence for TAGs and PCs is different, and explained as follows. For TAGs, evidence for this claim is seen by the fact that although the previously existing fraction of TAGs (99 % ^{12}C plus 1 % natural abundance ^{13}C) is the same size as expected based on cell count, assuming no degradation, there is some ^{12}C contained in the "new" lipid molecules (recall: "new" is defined as a molecule with some degree of ^{13}C label as was discussed in the "Methods" section), as evidenced by the labeling pattern (see Fig. 5). Therefore, the amount of ^{12}C in TAGs at day 3 surpassed the amount present at "Time zero" by the amount of ^{12}C in the "new TAG" fraction (Fig. 3a).

The evidence for ^{12}C in newly synthesized PCs at day 3 is apparent in the unexpected labeling pattern in the choline headgroups. The abundance of phosphocholine molecules containing between zero and five ^{13}C atoms was measured for each set of samples (Fig. 4), and the fully unlabeled fraction greatly exceeded that which was predicted if all new phosphocholines were synthesized using ^{13}C bicarbonate. Specifically, only 16 % of the total phosphocholine pool contained one or more ^{13}C carbons in NO_3^--deprived cultures, which is significantly lower than the 50 % that would be projected assuming no interconversion (due to one cell doubling, as was explained in the "Methods" section). The value is similarly depressed in NO_3^- replete cultures, which show only 35 % ^{13}C in the total phosphocholine pool as

Table 4 Total TAG and PC amounts calculated using LC-MS/MS

	Total TAG (pg cell^{-1})	Total PC (pg cell^{-1})
Time zero	2.25±0.20	0.087±0.0048
3 d NO_3^- replete	0.89±0.06	0.090±0.0078
3 d NO_3^- deprived	4.02±0.59	0.058±0.0014

compared to a projected value of 75 % (due to two cell doublings), indicating that there was synthesis of phosphocholine from an intracellular pool present at "Time zero". One possible explanation for the unexpected values could be the existence of a glycine betaine (GBT) pool that can act as a reserve form of choline, and has been reported to accumulate to high amounts in marine phytoplankton [30–32] and *P. tricornutum* in particular [33]. As such, we presume a large fraction of phosphocholine in NO_3^--deprived and replete cultures is synthesized from GBT already present at "Time zero". Thus, this observation is critical to interpretation of Fig. 3b, providing an explanation for why, in the NO_3^- replete case, the amount of fully ^{12}C PC headgroups was significantly greater than the projected "previously existing" pool. The synthesis of PCs from previously existing, ^{12}C, could also partially be a sign of cell stress and breakdown of proteins, rather than of a large GBT pool that buffers the ^{13}C from immediate incorporation into PC head groups. Direct measurement of GBT is proposed to determine the extent to which this could be the case.

Total NO_3^- deprivation for 3 days resulted in cells with half as much nitrogen as the NO_3^- replete cells (Table 2). Cells were still able to divide once, which could have been possible due to this GBT pool or to other internal N storage, including NO_3^- previously observed to be stored inside vacuoles in diatoms [34–36]. Ash-free cellular carbon percent did not change (Table 2).

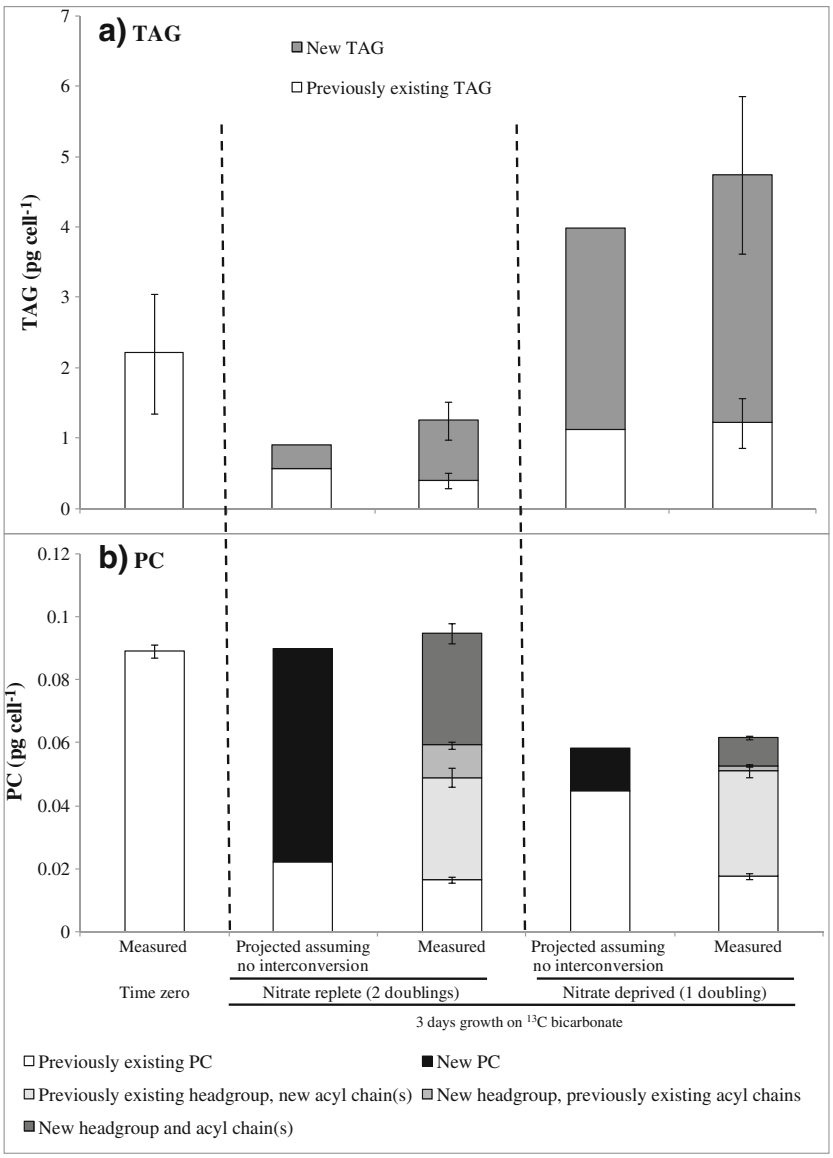

Fig. 3 LC-MS/MS results showing much more turnover of previously existing (^{12}C) in PCs than in TAGs in NO_3^--deprived cultures. This figure displays per cell previously existing and new molecules (defined as molecules with some degree of ^{13}C label) of the main TAG (**a**) and PC (**b**) groups in the lipid extract of *P. tricornutum* cultures. Three datasets are presented: "Time zero", nitrate replete, and nitrate deprived. In order to illustrate the concept that less unlabeled lipid per cell is expected since cell count increased and presumably the ^{12}C pool per flask did not change substantially, the measured values are compared to projected previously existing and new values assuming no interconversion of the lipid present at "Time zero". Projected values were calculated based on the increase in cell number and the total per-cell TAG or PC masses measured for nitrate replete and nitrate-deprived cultures (see Table 4). See "Methods" section for detailed calculations. *Error* 1 SEM (n=3)

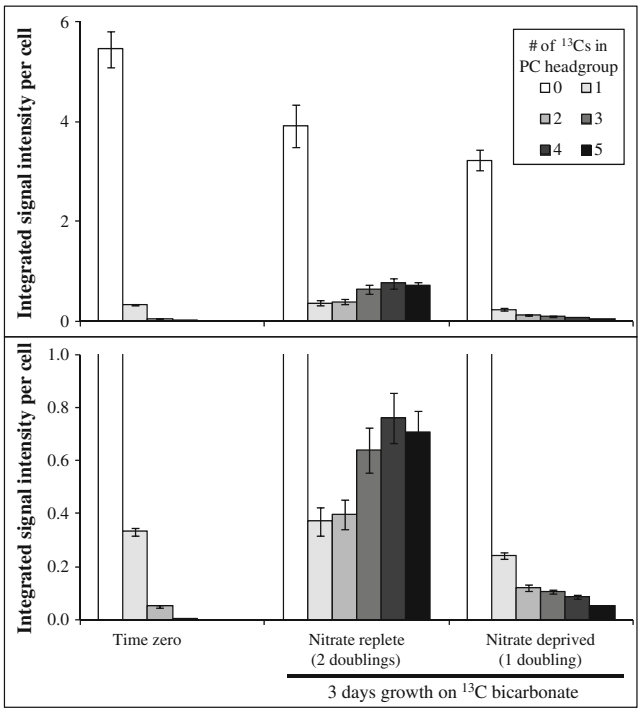

Fig. 4 The phosphocholine headgroup labeling pattern showing an unexpectedly low amount of label in both nitrate deprived and replete conditions, 16 and 35 % of total respectively, indicating an internal N pool used to synthesize choline (based on one and two doublings in NO_3^--deprived and replete cultures over the time exposed solely to ^{13}C, expected values would be 50 and 75 % of the total, for NO_3^--deprived and replete cultures respectively). This figure displays the LC/MS-MS peak areas for the phosphocholine subunits, $C_5H_{15}NO_4P^+$, with a singly-protonated mass of 184 vs. peak areas for phosphocholine subunits with 1–5 ^{13}C labeled PC cholines (the lower panel shows the *y*-axis over a narrower range to display the lower abundance headgroups). Total PC headgroup signal intensity per cell is 5.8, 6.8, and 3.8 for "Time zero", nitrate replete, and nitrate deprived respectively, which mirrors the total PC values in Table 4, as expected. *Error* 1 SEM (n=3)

Turnover of Lipid Fractions

The main group of TAG peaks observed likely corresponds to combinations of palmitic (P, C16:0) and palmitoleic (Po, C16:1) acyl chains, the two most common fatty acids in *P. tricornutum* (Electronic Supplementary Material (ESM) 1). This group of TAGs (measured as sodium ion adducts) was measured in the *m/z* range 820−830 for previously existing peaks, and in the 830−880 window for new peaks (see Fig. 5). There was essentially complete preservation of fully ^{12}C TAG present at "Time zero" in NO_3^--deprived cultures (indicated by the similar size white areas between projected and measured TAG in Fig. 3a), whereas about 28 % turnover appeared in NO_3^- replete cells. Active metabolism of TAG in the presence of NO_3^- is further evidenced by the larger than projected amount of new TAG, whereas new TAG measured in NO_3^--deprived cultures agreed with the value projected assuming no turnover of initial pools (Fig. 3a). If any interconversion occurred in TAG in NO_3^--deprived cultures, it was not measurable by the methods used in this study. Solely ^{12}C fatty acids may have been transferred to TAGs for new synthesis from PCs and other phospholipids under NO_3^--deprived conditions. These data clearly support the role of TAGs as a storage molecule in NO_3^--deprived cultures, and additionally illustrate a more active role in metabolism under nutrient replete growth. A practical outgrowth of this observation is that inhibition of TAG lipolysis may not be required to further increase TAG storage in NO_3^--deprived cultures.

There was much more turnover of PCs than TAGs in NO_3^--deprived cultures, and thus a bigger difference between measured and projected values assuming no conversion from other cellular pools of the unlabeled lipids existent at "Time zero" (Fig. 3b). As with TAGs, PC acyl chain composition of primarily PP and PPo was consistent with mass spectrometric observations, wherein unlabeled PC was measured in the 725–735 *m/z* region and labeled PC in the 737–765 range (see Fig. 5). In NO_3^--deprived cultures, there was significant turnover of the fatty acid moieties in these PCs, with the "previously existing" value 61 % lower than that projected for complete retention of PCs initially present at "Time zero" (Fig. 3b). This demonstrates replacement of newly synthesized fatty acid subgroups onto existing phosphocholine headgroups, expected under conditions of low nitrogen in which such moieties would be obligatorily recycled. In NO_3^- replete conditions, significantly less turnover was evidenced, with the previously existing PCs only 26 % below the projected value for complete fidelity of "Time zero" PC. NO_3^- replete cultures synthesized a much larger fraction, more than half, of their PCs with some degree of labeling in the headgroup, while in NO_3^--deprived cultures less than 25 % of the labeled PCs had labeling in the headgroup (Fig. 4). This result supports the observation that there was greater PC synthesis in the NO_3^- replete cultures, as expected. In addition, 23 % of PCs in the NO_3^- replete cultures with labeling in the headgroup possessed totally unlabeled acyl chains, whereas this value was 14 % for NO_3^--deprived cultures. This indicates generally high mixing of headgroups and acyl chains, even to some extent in cells starved of nitrogen, which have less demand for membrane lipids. Indeed, per cell volume of the NO_3^--deprived culture was approximately twice as large, as evidenced by microscopy and dry weight estimations (data not shown), indicating a smaller surface area to volume ratio and thus requiring less membrane lipid.

Acyl Chain Labeling Pattern

In examining the "Time zero" versus labeled mass spectra, it was observed that the label appeared in groups with maxima at multiples of 14 *m/z* higher than observed for the previously synthesized ^{12}C lipids (Fig. 5, the pattern was the

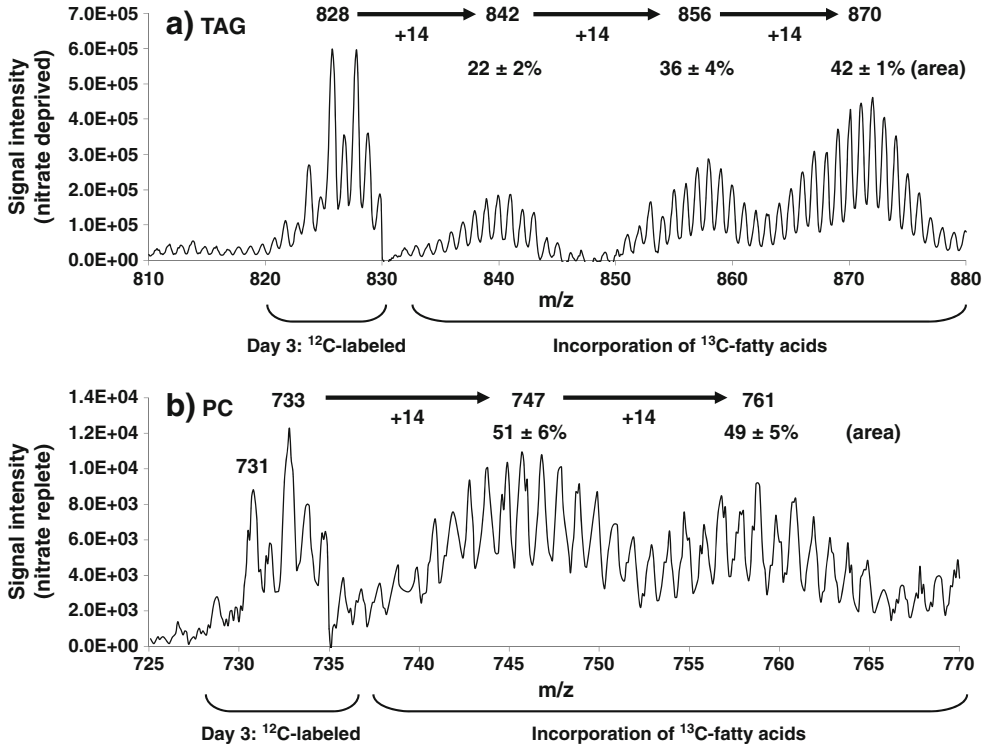

Fig. 5 Portions of the mass spectra for TAGs (**a**) and PCs (**b**) showing that newly synthesized lipids increase in multiples of 14 *m/z* units, indicating that the added newly synthesized fatty acids were, on average, 7/8 labeled with ^{13}C (14 ^{13}C/16 total carbons). This was observed under both nutrient states for both TAG and PC. **a**) A representative Q1 mass spectral scan of a TAG cluster from NO_3^--deprived cells after 3 days with previously synthesized ^{12}C TAGs (sodium ions of 48:3, 48:2, and 48:1; *m/z* 823.7, 825.7, and 827.7 (828 in figure)) and newly synthesized TAGS with incorporation of either 1, 2, or 3 partially ^{13}C labeled fatty acids. **b**) A representative PC cluster from NO_3^- replete cultures after 3 days showing previously synthesized ^{12}C labeled PCs (H^+ ions of 32:2 and 32:1, *m/z* 730.8, 732.8 (731 and 733 in figure)) and newly synthesized PCs with incorporation of either 1 or 2 ^{13}C labeled fatty acids. This figure also shows that there were mixtures of previously and newly synthesized fatty acids. From the mean areas, the distribution of the incorporation of newly synthesized fatty acids can be calculated. In the case of TAGs, 42 % of newly synthesized TAG molecules contained three newly synthesized fatty acids, representing 57 % of ^{13}C-labeled TAG fatty acids. For PCs, 49 % of newly synthesized PC molecules contained two newly synthesized fatty acids, representing 66 % of ^{13}C-labeled PC fatty acids

same for both NO_3^--deprived and replete cultures, but NO_3^--deprived cultures were shown for TAG, since there were more TAGs in NO_3^--deprived cultures, and likewise, NO_3^- replete cultures were shown for PC). As TAGs have three acyl chains and PCs have two, there were correspondingly three groups of plus 14 *m/z* peaks for TAGs and two for PCs. Since the fatty acid profile did not change appreciably over 3 days (ESM 1), and assuming most of the acyl chains are Po or P, both 16 Cs in length, this indicates synthesis of these fatty acids. This synthesis pathway could begin with ribulose-1,5-bisphosphate plus CO_2, yielding glyceraldehyde-3-phosphate (GAP) that would expectantly become fully ^{13}C-labeled following the switch to labeled bicarbonate. GAP is converted to pyruvate, which is decarboxylated to form ^{13}C acetyl CoA, which is carboxylated by acetyl CoA carboxylase in the plastid to form malonyl CoA, which serves as the scaffold for subsequent acetyl additions to a growing fatty acid. The data suggest that, on average, one acetyl CoA from degradation or a carbon pool otherwise existent at "Time zero" is incorporated into a palmitic acid, along with seven labeled acetyl CoAs resulting from de novo assimilation. Full labeling of acyl chains would never be achieved unless the algae were grown in ^{13}C bicarbonate from the very beginning. Therefore, 7/8 labeling of acyl chains is reasonable.

In NO_3^--deprived cultures, 42±0.6 % of the TAGs appeared to have all three acyl chains occupied with newly synthesized fatty acids, whereas, in NO_3^- replete cultures, 48.7±5 % of the PCs had both acyl chains replaced, calculated by summing the areas under the distinct groups of peaks in the 3-day mass spectra (see Fig. 5). This observation corroborates the PC headgroup labeling results that show high-mixing even in decreasing lipid pools.

Promise for Cell Factories

Since carbon uptake does not increase upon NO_3^- deprivation, an emphasis should be placed on elucidating the mechanism(s) that cause lipid versus carbohydrate to accumulate

in order to eventually redirect all storage carbon into TAGs or other lipid classes. This diatom carbon "decision tree" is poorly understood, as some studies have shown no increase in carbohydrate upon NO_3^- deprivation in *P. tricornutum* [37, 38], whereas Granum et al. [19] reported an increase in the marine diatom, *Skeletonema costatum*, under similar conditions. One approach to decrease starch accumulation is to knock out the starch accumulation pathways [39] through alternative mutagenesis of signaling mechanisms, or alternatively, control flux distributions using environmental cues. As we found little turnover in TAGs under NO_3^- deprivation, there may be no need to inhibit genes involved in lipolysis. The combination of a starchless, hydrocarbon excreting mutant grown under conditions of maximal biomass accumulation and subsequently exposed to low nitrogen or other conditions known to achieve high lipid [7, 11, 12, 40] is a reasonable near-term goal, given the recent genetic variants and the critical finding that the majority of lipid is synthesized de novo upon NO_3^- deprivation in *P. tricornutum*.

Conclusions

The combined results showing that about 60 % of lipid is synthesized de novo during 3 days of total NO_3^- deprivation, and that these lipids are primarily TAGs, highlights the promise of using NO_3^- deprivation with marine diatoms as a strategy for optimizing lipid production. LC-MS/MS analysis of isotopically labeled lipids revealed (1) low TAG and high PC turnover (of both headgroups and acyl chains) in NO_3^--deprived cultures, (2) a glycine betaine pool feeding PC headgroup synthesis, and (3) a general pattern of 14 out of 16 labeled carbons in both TAG and PC acyl chains.

Acknowledgments This work was funded by the Air Force Office of Scientific Research, grant # FA9550-05-1-0365. The LC/MS instrument was obtained through National Center for Research Resources (NIH) (grant # RR021120). EHB was additionally funded by the Busch-Waksman Postdoctoral Fellowship, and NBB was funded by the Sustainable Fuels NSF-IGERT program (award #0903675). MF was funded by a gift from James Gibson. We would also like to thank Char Fuller and Kevin Wyman. Elemental analysis was performed by Marshall Otter at the Marine Biological Laboratory Ecosystems Center, Woods Hole, MA, USA.

References

1. Chisti Y (2007) Biodiesel from microalgae. Biotechnol Adv 25 (3):294–306
2. Dismukes GC, Carrieri D, Bennette N, Ananyev GM, Posewitz MC (2008) Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. Curr Opin Biotechnol 19(3):235–240. doi:10.1016/j.copbio.2008.05.007
3. Weyer KM, Bush DR, Darzins A, Wilson BD (2010) Theoretical maximum algal oil production. Bioenergy Res 3:204–213
4. Sheehan J, Dunahay T, Benemann J, Roessler PG (1998) A Look Back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae. US DoEnergy National Renewable Energy Laboratory
5. Bozarth A, Maier U-G, Zauner S (2009) Diatoms in biotechnology: modern tools and applications. Appl Microbiol Biotechnol 82 (2):195–201. doi:10.1007/s00253-008-1804-8
6. Wijffels RH, Barbosa MJ (2010) Outlook on microalgal biofuels. Science 329:796–799
7. Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M et al (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J 54(4):621–639. doi:10.1111/j.1365-313X.2008.03492.x
8. Frenz J, Largeau C, Casadevall E (1989) Hydrocarbon recovery by extraction with a biocompatible solvent from free and immobilized cultures of *Botryococcus braunii*. Enzyme Microb Technol 11 (11):717–724
9. Sayre RT (2009) Optimization of biofuel production. United States Patent, International patent number 20090181438
10. Radakovits R, Jinkerson RE, Darzins A, Posewitz MC (2010) Genetic engineering of algae for enhanced biofuel production. Eukaryot Cell 9(4):486–501. doi:10.1128/ec.00364-09
11. Ramachandra TV, Mahapatra DM, Karthick B, Gordon R (2009) Milking diatoms for sustainable energy: biochemical engineering versus gasoline-secreting diatom solar panels. Ind Eng Chem Res 48(19):8769–8788. doi:10.1021/ie900044j
12. Hejazi MA, Wijffels RH (2004) Milking of microalgae. Trends Biotechnol 22(4):189–194
13. Suen Y, Hubbard JS, Holzer G, Tornabene TG (1987) Total lipid production of the green-alga *Nannochloropsis* Sp Qii under different nitrogen regimes. J Phycol 23(2):289–296
14. Roessler PG (1988) Effects of silicon deficiency on lipid-composition and metabolism in the diatom *Cyclotella-Cryptica*. J Phycol 24(3):394–400
15. Bowler C, Allen AE, Badger JH, Grimwood J, Jabbari K, Kuo A et al (2008) The *Phaeodactylum* genome reveals the evolutionary history of diatom genomes. Nature 456(7219):239–244. doi:10.1038/nature07410
16. Siaut M, Heijde M, Mangogna M, Montsant A, Coesel S, Allen A et al (2007) Molecular toolbox for studying diatom biology in *Phaeodactylum tricornutum*. Gene 406(1–2):23–35
17. Guillard RR, Ryther JH (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (cleve) Gran. Can J Microbiol 8:229–239
18. Camacho FG, Grima EM, Miron AS, Pascual VG, Chisti Y (2001) Carboxymethyl cellulose protects algal cells against hydrodynamic stress. Enzyme Microb Technol 29(10):602–610
19. Granum E, Kirkvold S, Myklestad SM (2002) Cellular and extracellular production of carbohydrates and amino acids by the marine diatom *Skeletonema costatum*: diel variations and effects of N depletion. Mar Ecol Prog Ser 242:83–94
20. Porra RJ (2002) The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b. Photosynth Res 73(1–3):149–156
21. Slayback JRB, Cheung LWY, Geyer RP (1977) Quantitative extraction of microgram amounts of lipid from cultured human cells. Anal Biochem 83(2):372–384
22. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Phys 37(8):911–917
23. Rodriguez-Ruiz J, Belarbi EH, Sanchez JLG, Alonso DL (1998) Rapid simultaneous lipid extraction and transesterification for fatty acid analyses. Biotechnol Tech 12(9):689–691
24. Johnson DR, Brinker A, Thackray J, Dixon J Normal phase LC/MS analysis of triglyceride concentration and composition in liver cells. In: 58th ASMS Conference on Mass Spectrometry, 2010. J. Am Society Mass Spectrometry, p S44

25. Homan R, Anderson MK (1998) Rapid separation and quantification of combined neutral and polar lipid classes by high-performance liquid chromatography and evaporative light-scattering mass detection. J Chrom B 708:21–26
26. Tonon T, Harvey D, Larson TR, Graham IA (2002) Long chain polyunsaturated fatty acid production and partitioning to triacylglycerols in four microalgae. Phytochem 61(1):15–24
27. Viso A-C, Marty J-C (1993) Fatty acids from 28 marine microalgae. Phytochem 34(6):1521–1533
28. Chrismadha T, Borowitzka M (1994) Effect of cell density and irradiance on growth, proximate composition and eicosapentaenoic acid production of *Phaeodactylum tricornutum* grown in a tubular photobioreactor. J Appl Phycol 6(1):67–74. doi:10.1007/bf02185906
29. Parrish CC, Wangersky PJ (1987) Particulate and dissolved lipid classes in cultures of *Phaeodactylum tricornutum* grown in cage culture turbidostats with a range of nitrogen supply rates. Mar Ecol Prog Ser 35:119–138
30. Park YI, Buszko ML, Gander JE (1999) Glycine betaine: Reserve form of choline in *Penicillium fellutanum* in low-sulfate medium. Appl Environ Microbiol 65(3):1340–1342
31. Keller MD, Kiene RP, Matrai PA, Bellows WK (1999) Production of glycine betaine and dimethylsulfoniopropionate in marine phytoplankton. I Batch cultures Mar Bio 135(2):237–248
32. Keller MD, Kiene RP, Matrai PA, Bellows WK (1999) Production of glycine betaine and dimethylsulfoniopropionate in marine phytoplankton. II. N-limited chemostat cultures. Mar Bio 135(2):249–257
33. Dickson DMJ, Kirst GO (1987) Osmotic adjustment in marine eukaryotic algae—the role of inorganic-ions, quaternary ammonium, tertiary sulfonium and carbohydrate solutes. 1. Diatoms and a Rhodophyte. New Phytol 106(4):645–655
34. Lomas MW, Glibert PM (2000) Comparisons of nitrate uptake, storage, and reduction in marine diatoms and flagellates. J Phycol 36(5):903–913
35. Dortch Q, Clayton JR, Thoresen SS, Ahmed SI (1984) Species differences in accumulation of nitrogen pools in phytoplankton. Mar Bio 81(3):237–250
36. Eppley RW, Coatsworth JL (1968) Nitrate and nitrite uptake by *Ditylum brightuellii*. Kinetics and mechanisms. J Phycol 4:151–156
37. Larson TR, Rees TAV (1996) Changes in cell composition and lipid metabolism mediated by sodium and nitrogen availability in the marine diatom *Phaeodactylum tricornutum* (bacillariophyceae). J Phycol 32(3):388–393
38. KaiXian Q, Borowitzka MA (1993) Light and nitrogen deficiency effects on the growth and composition of *Phaeodactylum tricornutum*. Appl Biochem Biotechnol 38(1–2):93–103
39. Work VH, Radakovits R, Jinkerson RE, Meuser JE, Elliott LG, Vinyard DJ et al (2010) Increased lipid accumulation in the *Chlamydomonas reinhardtii* sta7-10 starchless isoamylase mutant and increased carbohydrate synthesis in complemented strains. Eukaryot Cell 9:1251–1261
40. Yongmanitchai W, Ward OP (1991) Growth of and omega-3-fatty-acid production by *Phaeodactylum tricornutum* under different culture conditions. Appl Environ Microbiol 57(2):419–425