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Triacylglycerol accumulation and profiling in the model diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* (Baccilariophyceae) during starvation

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Abstract Although substantial economic barriers exist, marine diatoms such as *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* hold promise as feedstock for biodiesel because of their ability to manufacture and store triacylglycerols (TAGs). The recent sequencing of these two marine diatom genomes by the United States Department of Energy Joint Genome Institute and the development of improved systems for genetic manipulation should allow a more systematic approach to understanding and maximizing TAG production. However, in order to best utilize these genomes and genetic tools, we must first gain a deeper understanding of the nutrient-mediated regulation of TAG anabolism. By determining both the yield and molecular species distribution of TAGs we will, in the future, be able to fully characterize the effects of genetic manipulation. Here, we lay the groundwork for understanding TAG production in *T. pseudonana* and *P. tricornutum*, as a function of nitrate and silicate depletion. Diatoms were starved of either nitrate or silicate, and TAGs were extracted with hexane from lyophilized samples taken at various time intervals following starvation. The timing of TAG production and the relative abundance of TAGs were estimated by fluorescence spectroscopy using Nile red and the total yield per biomass determined by gravimetric assay. TAGs were analyzed using thin layer chromatography, gas chromatography–mass spectrometry, and electrospray ionization mass spectrometry to identify the major TAG species produced during the growth curve. Under our conditions, the TAG yield from *T. pseudonana* is about 14–18% of total dry weight. The TAG yield from *P. tricornutum* is about 14% of total dry weight. Silicate-starved *T. pseudonana* accumulated an average of 24% more TAGs than those starved for nitrate; however, the chemotypes of the TAGs produced were generally similar regardless of the starvation condition employed.

Keywords Biodiesel · Mass spectrometry · Nitrate · Silicate · Triacylglycerols

Introduction

In developed nations, concerns around global warming have led to programs focused on displacing fossil fuels with renewable low-carbon biofuels as part of the overall energy portfolio. Previous research carried out by the Aquatic Species Program (ASP) at the United States Department of Energy National Renewable Energy Laboratory was initially targeted at identifying the naturally occurring species that were the most promising for use as a feedstock for biodiesel production (Sheehan et al. 1998). During this program, certain strains of algae known as diatoms were shown, under appropriate conditions, to produce up to 60% of their cellular mass as triacylglycerols (TAG) under certain growth conditions. These TAGs can be easily converted

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into biodiesel through a transesterification reaction. These species were therefore identified as strong candidates for significant biodiesel production. The marine species of diatoms were judged to be particularly attractive because they grow in saline water and thus will not compete with freshwater resources nominally reserved for human consumption and agriculture. In the final years of the ASP, initial attempts were made to improve the TAG production in diatoms through genetic engineering, but these efforts were hampered by a lack of genetic information and appropriate molecular biology tools to carry out the manipulations.

Optimal production of TAGs occurs in diatoms under conditions of environmental stress, such as nutrient deficiency or low temperature (for review, see Hu et al. 2008). There is a broad range of variability in TAG yields between species, as well as a fair amount of strain-to-strain variation within the same species. Although TAGs are formed under a variety of environmental stress conditions, there have not been systematic comparisons of the yield and molecular makeup of TAGs produced under these different conditions in the same species. One would expect that different forms of environmental stress will have differing effects on the cellular machinery required for TAG synthesis (Mock et al. 2008). Nitrate starvation likely has a different impact on metabolic activity than silicate limitation. For example, previous work has shown that *de novo* protein synthesis is required for starvation-induced increase in acetyl CoA carboxlase (ACCase) activity in the diatom *Cyclotella cryptica* (Roessler 1988). If the protein synthesis required to create enzymes for TAG formation is impacted by nitrate limitation, then there is a possibility that induction by nitrate starvation could result in a different pattern of TAG formation than that found under silicate starvation.

In this study, we identified and compared the molecular species of triacylglycerols formed by the two strains of marine diatoms for which there are genomic data. *T. pseudonana* was studied under nitrate and silicate starvation conditions and *P. tricornutum*, which has little if any requirement for silica, under nitrate starvation. The goals were to profile TAG synthesis in terms of their molecular composition and quantities in these wild-type strains under optimal growth conditions and to investigate changes in TAG synthesis in response to manipulation of nitrate and silica levels. This information, together with the recent genome sequences of *Thalassiosira pseudonana* (Hustedt) Hasle and Heimdal clone 3H (Armbrust et al. 2004) and *Phaeodactylum tricornutum* Bohlin clone CCAP1055/1 and significant advances in the development of molecular genetic tools for the manipulation of these strains (Poulsen and Kroger 2005; Poulsen et al. 2007; Zaslavskaia et al. 2000, 2001), will facilitate the development of these strains both as model systems for the study of the biological mechanisms of TAG synthesis and as commercial strains for the production of algal oil-based products.

Materials and methods

Strains and culture conditions Axenic cultures of *Thalassiosira pseudonana* 3H (CCMP1335) and *Phaeodactylum tricornutum* CCAP1055/1 (CCMP632) were obtained from the Provasoli-Guillard Center for the Culture of Marine Phytoplankton at Bigelow Laboratory for Ocean Sciences (West Boothbay Harbor, MA, USA). Cultures were maintained in enriched seawater–artificial water (ESAW) medium (Harrison et al. 1980) under constant light 1,000 μmol photons m^{-2} s^{-1} and 21°C. Cell numbers were determined by direct counting using a Coulter Z2 particle counter (Beckman Coulter Inc, Fullerton, CA, USA). Experimental cultures were grown in variants of ESAW (reduced nitrate or silicate) in 8-L polycarbonate carboys with aeration under the conditions described above. All experiments consisted of three biological replicates of each strain and condition grown on separate days. Experimental cultures were inoculated (2×10^4 cells mL^{-1}) from midlogarithmic phase cultures that had been maintained for at least three passages under the same nutrient conditions as the experimental cultures. For silicate-limited cultures (*T. pseudonana* only), standard ESAW was used (105 μM $NaSiO_3$). For nitrate-limited cultures of *T. pseudonana* and *P. tricornutum*, the concentration of nitrate was lowered in ESAW from 549 to 50 μM and the silicate was doubled (210 μM $NaSiO_3$). One-liter aliquots were withdrawn from the cultures at the time points indicated, and biomass was harvested for further analysis by gentle filtration (5 mm Hg vacuum) onto three micron pore size nucleopore filters (Whatman, USA). Cells were washed off of the filters, pelleted, and lyophilized. Lyophilized material was stored in polypropylene centrifuge vials at −80°C until solvent extraction.

Nile red assay The relative abundance of intracellular triacylglycerols present in samples from diatom cultures was estimated by fluorometric assay using the dye Nile red (Cooksey et al. 1987). Nile red dissolved in acetone (250 μg mL^{-1}) was added to a final concentration of 1 μg mL^{-1} to 2 mL of cells (600,000 cells mL^{-1}). The samples were allowed to incubate in the presence of the dye at room temperature (RT), for 140 s and subsequently excited at 530 nm while the fluorescent emission was measured from 550 to 750 nm. Emission curves from three separate scans were averaged and corrected for the fluorescence contribution due to Nile red alone (in ESAW) and chlorophyll.

Lipid extraction All solvents used for extraction and analysis were GC grade (≥95%, Sigma Aldrich, St Louis, MO, USA). To minimize polymer contamination, solvent extractions were carried out in Teflon-lined screw-capped 20-mL glass scintillation vials, and glassware was used for all subsequent steps. High purity acylglycerol standards 1-oleoyl-rac-glycerol (~99%), 1,3-diolein (~99%), and glyceryl-trioleate (≥99%), (mono-, di-, and tri-acylglycerols, respectively), and chlorophyll *a* standards (≥96%) were obtained from Sigma-Aldrich.

The lyophilized cell pellet was first ground into a fine power and weighed. Each vial was filled with 3 mL of hexane, as the extraction solvent. The scintillation vials were sonicated in a water bath for 5 min at room temperature in order to disrupt the cell membranes. The main hexane extraction step was completed by placing the sample vial in a 60°C water bath for 1 h while continuously stirring the sample with a Teflon stir bar at 500 rpm.

The hexane extracts were cooled to room temperature then the supernatant was filtered through a 20-nm PTFE filter and transferred into 2 mL vials. To determine the lipid extraction yields, it was necessary to account for the chlorophyll *a* present in each sample. The chlorophyll *a* absorbance of each sample was measured using a UV–Vis spectrophotometer (UNICO 3802) at a wavelength of 660 nm (Beer et al. 1982), and the concentration in each sample was determined using a chlorophyll *a* standard curve. The hexane extracts also contained sterol lipids which we included in our extraction yields for two reasons. First, we found that during the stationary phase, the relative abundance of the sterols present in each of the cultures was constant and therefore would not affect our overall extraction yield comparisons. Secondly, although sterol lipids are not acylglycerols, we included them in our extraction yields as it has been reported that sterols can be converted into biodiesel through the same transesterification process used to convert the acylglycerols (Nota et al. 1999; Mansour et al. 1999). After determining the extraction yields for each sample, the solvent was then evaporated under nitrogen, and the lipid content determined gravimetrically. The samples were stored under nitrogen at −20°C until lipid analysis (Mansour et al. 2005). This step was important since lipids of marine organisms are highly sensitive to hydrolysis and oxidation processes during storage (Sasaki and Capuzzo 1984).

Thin layer chromatography All of the lipid extract samples were reconstituted in heptane to make stock solutions. The stock solutions along with standards (monoacylglycerols, etc) were spotted three times onto silica gel TLC plates (Fisher Scientific). The mobile phase consisted of a solvent mixture of hexane/diethyl ether/acetic acid (70:30:1 by volume) system (GC grade (≥95%) Sigma-Aldrich; Maloney 1996). In order to visualize the lipids, the plates were developed by heating iodine crystals and allowing the vapor to stain the plates.

Gas chromatography–mass spectrometry analysis After the initial thin layer chromatography (TLC) lipid screening, the standards and extracts were analyzed using an Agilent 5975C series high-temperature gas chromatography–mass spectrometer (GC–MS), fitted with an autoinjector. The main focus of using a GC–MS was exclusively for lipid identification rather than quantification. For GC–MS analysis, a high-temperature column (DB-5ht; 30 m×0.25 mm id×0.1 μm film thickness) was purchased from Agilent Technologies. By employing a high-temperature column, we eliminated the need for derivatization of each sample, which can result in artifacts due to overlapping peaks and cause inaccurate identification of the TAGs (Laakso 2002; Malavolta et al. 2004).

A modified European Standard protocol (EN 14105:2003), used for diesel analysis, was employed for the analysis operating conditions. The injector and detector temperatures were set at 400°C while the initial column temperature was set at 65°C. A 1-μL sample volume was injected into the column and ran using a 50:1 split ratio. After 2 min, the oven temperature was raised to 170°C at a ramp rate of 15°C min^{-1}. The oven temperature was then raised to 270°C at a ramp rate of 8°C min^{-1}, and finally the oven temperature was raised to 400°C at a ramp rate of 15°C min^{-1} and maintained at this temperature for 40 min. The helium carrier gas was programmed to maintain a constant flow rate of 3 mL min^{-1}, and the mass spectra were acquired and processed using both Agilent ChemStation (Agilent, USA) and AMDIS32 software.

Electrospray ionization mass spectrometry of lipid extracts Triheptadecenoin (TAG 51:3) was obtained from Nu Chek Prep, Inc (Elysian, MN, USA). All solvents used in mass spectrometric analyses were HPLC grade. Extracted nonpolar lipids were dissolved in 100 μL of chloroform. Analyte solutions were prepared in electrospray buffer (1:200 dilution with 1:4 (*v*/*v*) chloroform/methanol). Lithium acetate was added to achieve a final [Li^+] of 2 mM to aid in the detection of the triacylglycerols (Hsu and Turk 1999). TAG 51:3 (0.4 μg) was also added to each sample and used as an internal standard.

All mass spectrometric determinations were performed on a Waters Q-TOF Ultima MS equipped with a nanospray assembly. Analyte solutions were infused directly into the source via a syringe pump at a flow rate of 1 μL min^{-1}. The mass spectrometer was operated in positive mode and typical MS settings were as follows: 3.5 kV electrospray voltage, 200 V cone voltage, and a 220°C capillary temperature. Spectra were acquired over a 3-min period of

signal averaging for each sample extract. TAG molecular species were quantitated according to the method reported by Han and Gross (2001). Briefly, ion peak intensities of individual TAG species were compared with that of the internal standard (TAG 51:3) after correction for C^{13} isotope and sensitivity (differences in carbon length and level of unsaturation) effects. Percentage TAG composition was also calculated per time point as ion intensity of the individual TAG relative to the total ion intensity of identified TAGs.

Tandem mass spectrometry of the lithiated TAG species was performed to characterize the fatty acid composition. For ion isolation, the LM/HM was set to 15/15. Ions subjected to 30–35 eV collision energy were sufficient to achieve optimal fragmentation. MS/MS data analysis was performed with the aid of an on-line tool in the LipidMaps (Schmelzer et al. 2007) website (http://www.lipidmaps.org/tools/ms/triacylglycerols.php). All possible fatty acid combinations consistent with the molecular TAG (acyl carbon number:total number of double bonds, ACN:DB) structure are reported.

Results

Growth rate and assaying of non-polar lipid production Under our growth conditions, *T. pseudonana* achieved a specific growth rate (μ) of 2 per day. *P. tricornutum* achieved a specific growth rate of 1.5. Silica-limited cultures of *T. pseudonana* reached a final cell density of 1.5×10^6 cells mL^{-1} after approximately 50 h. Nitrate-limited cultures achieved a density of 1.2×10^6 cells mL^{-1} in approximately the same amount of time. It took 60 h before the accumulation of TAGs was detected within the cells by Nile red assay. *P. tricornutum* attained a final density of 5×10^6 cells mL^{-1} after approximately 90 h. Nutrient assays taken from the culture media at the beginning and end of the time course demonstrated the complete consumption of the limiting nutrient and the presence of residual non-limiting nutrients (data not shown). As expected, both silica and nitrate starvation resulted in an accumulation of TAGs as initially detected by Nile red assay. In all cases, an increase in TAG content was first detected by Nile red assay approximately 10 h after the cultures entered the stationary phase (Fig. 1).

Hexane extraction yields The hexane extraction yields, based on the dry weight of biomass, were determined gravimetrically. We verified, by serial re-extraction of the biomass with either hexane or chloroform, that a consistent

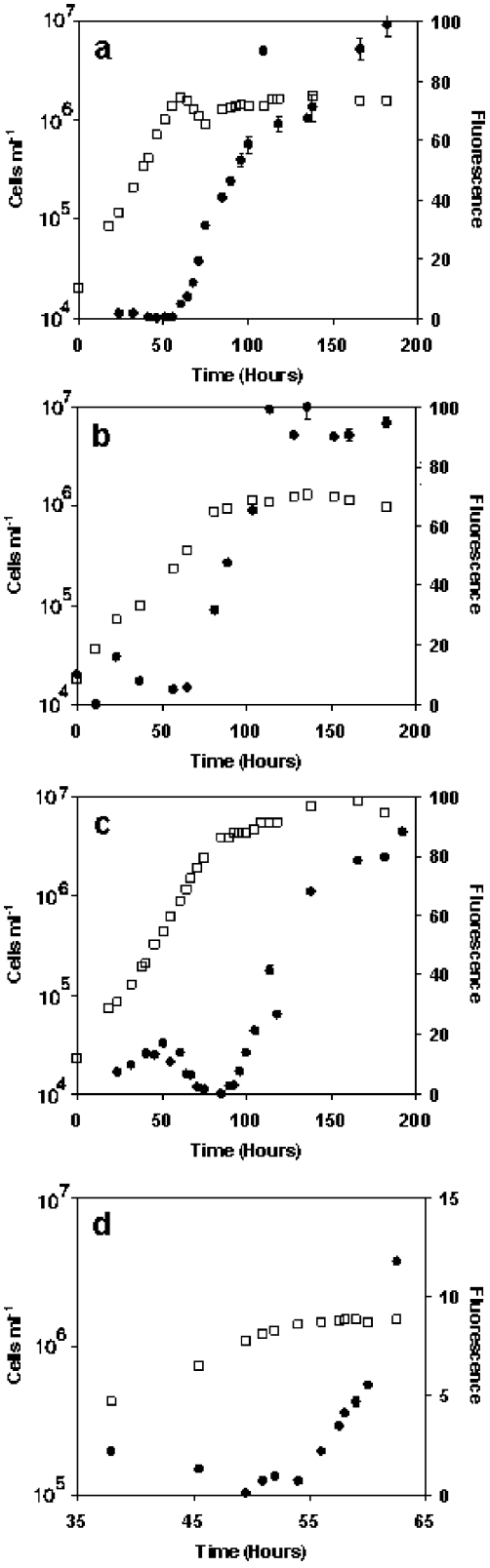


Fig. 1 Typical growth curves and Nile red assay data taken from nutrient starvation experiments. *Open symbols* are cell counts and *closed symbols* are Nile red fluorescence (arbitrary units). **a** *T. pseudonana* silicate starvation, **b** *T. pseudonana* nitrate starvation, **c** *P. tricornutum* nitrate starvation, and **d** high temporal resolution time course of *T. pseudonana* silicate starvation focusing on the transition from the exponential phase to the stationary phase and the onset of TAG accumulation (note the different fluorescence scale)

fraction of the non-polar lipids (80%) were extracted from our samples by a single hexane extraction (data not shown). Maximum yields of hexane-extractable material resulted when the cultures reached the stationary phase. Average yields of 17.85±1.55%, 14.36±0.93%, and 14.67±1.88% were determined for the silicate-limited *T. pseudonana*, nitrate-limited *T. pseudonana*, and nitrate-limited *P. tricornutum* cultures, respectively.

We identified the principal contaminants in the hexane-extracted material for each of the time points. In the early exponential phase, the predominant contaminant we identified in the hexane extracts was chlorophyll *a*, which was accounted for in our total extraction yield calculations (see "Materials and methods"). In addition, vitamin E was identified in all of the extracted samples; however, the abundance measured was consistently negligible compared to the measured acylglycerol abundances and therefore not taken into account in the yield values. Also identified in each of the samples was 24-methyl cholest-5,22-dien-3β-ol, which is a sterol lipid often found in unicellular algae. During the stationary phase, the relative abundance of the sterol lipid present in each of the cultures was found to be constant; hence, we included it in the reported extraction yield values. Despite the fact that the sterol lipid identified is not an acylglycerol, we included it in our extraction yield values as it has been reported that these sterols can be converted into biodiesel using a transesterification technique (see "Materials and methods").

Lipid analysis Hexane extract samples were analyzed for lipid content using TLC as a means of verifying and extending the Nile red assay results and identifying those samples that warranted further analysis. The acylglycerol standards (mono-, di-, and tri-acylglycerols), along with solutions of the extracted samples, were spotted onto silica gel TLC plates. Figure 2 shows a developed plate which includes the acylglycerol standards in the first column, along with the first and last days for each of the three culture conditions studied.

All of the trends observed in both the Nile red data (Fig. 1) and the TLC plates (Fig. 2) indicate that TAG accumulation is observed to start after nutrient starvation and increases with time regardless of the species. The TLC plate methodology used in the analysis of these extracts provides a quick and inexpensive technique to initially screen for the presence of targeted lipids against a very proscribed standard composition; however, in order to achieve the resolution necessary for absolute identification and speciation of the lipids present, gas chromatography–mass spectrometry (GC–MS) and electrospray ionization mass spectrometry (ESI-MS) were employed.

The results of the lipids identified by GC–MS from the hexane extracts of silica-limited *T. pseudonana*, nitrate-limited *T. pseudonana*, and nitrate-limited *P. tricornutum*

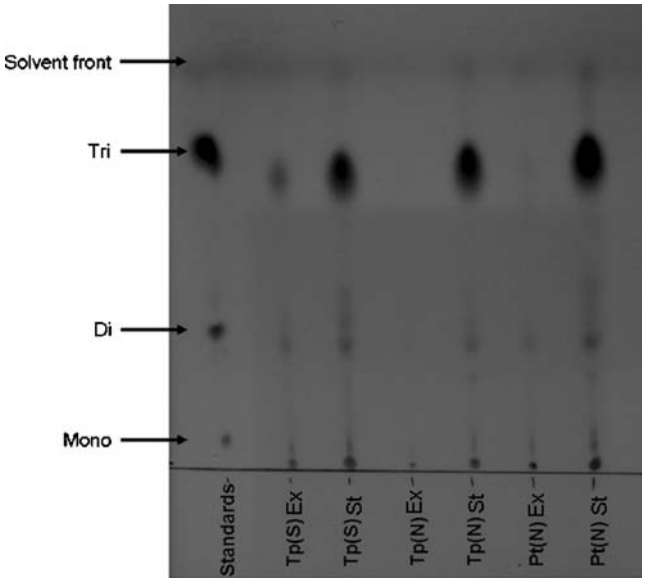


Fig. 2 TLC plate showing hexane extractions from cell cultures as a function of time after nutrient starvation. Mono-, di-, and tri-acylglycerol standards were run (denoted as standard), along with hexane extracts taken at exponential (*Ex*) and stationary (*St*) phases for silica-limited *T. pseudonana* (*Tp(S)*), nitrate-limited *T. pseudonana* (*Tp(N)*), and nitrate-limited *P. tricornutum* (*Pt(N)*). The hexane extraction yields were determined gravimetrically. Maximum extraction yields for silicate-limited *T. pseudonana*, nitrate-limited *T. pseudonana*, and nitrate-limited *P. tricornutum* cultures were 17.85±1.55%, 14.36±0.93%, and 14.67±1.88%, respectively

cultures during the stationary phase are shown in Table 1. There are a number of common chemical moieties present in all of the samples, but differences are also observed. For instance, in both the silicate- and nitrate-limited *T. pseudonana* cultures, triacylglycerols in the form of 1,2,3-propanetriyl tetradecanoic acid, 2-(tetradecanoyl)propane-1,3-diyl dihexadecanoate,3-(hexadecyloxy)propane-1,2-dihexadecanoate, and 1,2,3-propanetriol tris(hexadecanoate) were identified along with propanoic acid mono-acylglycerols. Moreover, the silicate-limited culture also contained additional monoacylglycerols esterified with tetradecanoic, hexadecanoic, and octadecanoic acids that are not observed in the nitrate-limited culture of the same species. The nitrate-limited cultures of both species contained triacylglycerols in the form of 1,2,3-propanetriyl tetradecanoic acid, 2-(tetradecanoyl)propane-1,3-diyl dihexadecanoate, and 1,2,3-propanetriol tris(hexadecanoate), along with propanoic acid monoacylglycerols. The *P. tricornutum* hexane extract contained more monoacylglycerols esterified with hexadecanoic, 4,7,10,13,16,19-hexaenoic acid, and 5,9-pentacosadienoic acid, than that observed in *T. pseudonana*. As mentioned earlier, also identified in each sample was 24-methyl cholest-5,22-dien-3β-ol. While these results using GC–MS add a higher level of fidelity in identifying the lipid species present as a function of nutrient starvation than either

Table 1 Lipids identified by GC–MS from the hexane extracts of silicate-limited *T. pseudonana*, nitrate-limited *T. pseudonana*, and nitrate-limited *P. tricornutum* cultures during the stationary phase

Lipid identification from hexane extracts		
T. pseudonana (silica-limited)	*T. pseudonana* (nitrate-limited)	*P. tricornutum* (nitrate-limited)
Tetradecanoic acid	Oxalic acid	Hexadecanoic acid
Hexadecanoic acid	Propanoic acid	4,7,10,13,16,19-Hexaenoic acid
4,7,10,13,16,19-Hexaenoic acid	1,2,3-Propanetriyl tetradecanoic acid	5,9-Pentacosadienoic acid
Octadecanoic acid	2-(Tetradecanoyl)propane-1,3-diyl dihexadecanoate	Propanoic acid
Propanoic acid	3-(Hexadecyloxy)propane-1,2-dihexadecanoate	1,2,3-Propanetriyl tetradecanoic acid
1,2,3-Propanetriyl Tetradecanoic acid	1,2,3-Propanetriol tris(hexadecanoate)	2-(Tetradecanoyl)propane-1,3-diyl dihexadecanoate
2-(Tetradecanoyl)propane-1,3-diyl dihexadecanoate		3-(Hexadecyloxy)propane-1,2-dihexadecanoate
3-(Hexadecyloxy)propane-1,2-dihexadecanoate		
1,2,3-Propanetriol tris(hexadecanoate)		

Also identified in all the samples but not included in the table was the sterol lipid 24-methyl cholest-5,22-dien-3β-ol, which is often found in unicellular algae (Ponomarenko et al. 2004)

the Nile red assay or TLC, resolving each TAG molecular species is more commonly analyzed using ESI-MS, which has higher mass resolution and is capable of TAG structural elucidation.

Profiling of TAG molecular species from algal oil extracts In order to fully characterize the molecular species of TAGs produced by these model diatoms under nutrient stress, they were analyzed using ESI-MS. The mass spectrum obtained from direct infusion ESI-MS of algal lipid extracts shows that a majority of the molecular ions are observed between 750 and 950 mass/charge (*m*/*z*), and is indicative of a mixture of lithiated triacylglycerols (Fig. 3). While we observed the same triacylglycerols identified by GC–MS, ESI-MS analysis provided substantially better resolution of the TAG molecular species in algal oils. Product ions generated from tandem MS (MS^2) of individual TAG molecular ions corresponded to neutral loss of each fatty acid constituent in the parent TAG ion (Fig. 3, inset). Therefore, the TAG acyl composition (ACN:DB) (total acyl carbon number (ACN) and total number of double bonds (DB)) can be deduced from mass/charge ratios of parent and product ions obtained from MS^2 analysis. TAG molecular species (and their fatty acid compositions) identified in stationary phase time points are summarized in Table 2, and a more comprehensive list for all the TAG species identified is in the Electronic supplementary material. Based on the MS^2 analysis, a number of the TAG species expressed may appear as isomers, having multiple possible fatty acid compositions. The most we have observed is TAG 52:8, which can have up to six possible FA compositions (Table 2).

The ion abundances of the product ions can provide some insight into the molar ratio of different isomers. For example, there are two possible fatty acid compositions for TAG 50:5 (14:0/16:0/20:5, 16:0/16:1/18:4) based on the tandem mass spectra (parent ion *m*/*z* 831.7, Supplementary Figure 1). The relative abundances of the product ions from MS^2 of TAG 50:5 (Supplementary Figure 1) show that the predominant product ions resulted from the loss of fatty acids 16:0, 16:1, and 18:4 (more than 50% difference in intensity units), and we can infer that the predominant FA composition for TAG 50:5 is 16:0/16:1/18:4. It appears that the fatty acid composition profiles for each TAG isomer remain constant through the growth curve, as the relative abundance of each product ion in the spectra obtained from various time points was similar. While quantitation of each isomer present in a specific TAG is possible, it is beyond the scope of this study and therefore was not pursued.

There was some variation in the TAG species found at the exponential phase in cultures designed for silicate-limited growth versus those designed for nitrate-limited growth of *T. pseudonana*. We believe that this difference arises from the fact that the cells were passaged through the same medium as used for the experiment and may have different levels of residual TAGs. Although there are some detectable differences, the pattern of TAGs produced by *T. pseudonana* under silicate or nitrate starvation is remarkably similar (Fig. 4a, b). Low levels of TAGs were found in *T. pseudonana* cells in the exponential growth phase by the more sensitive techniques of TLC and MS but not by Nile red. Despite such low concentrations, ESI-MS analysis revealed a diverse group of TAG species (Fig. 4a, b). It is likely that these are either residual TAGs from previous periods of starvation or represent a low constitutive level of TAG formation. Major triacylglycerols detected, common to all exponential growth phase samples, included TAGs 46:1, 46:2, 48:1–48:4, 50:5, 52:6, and 52:7 (comprising

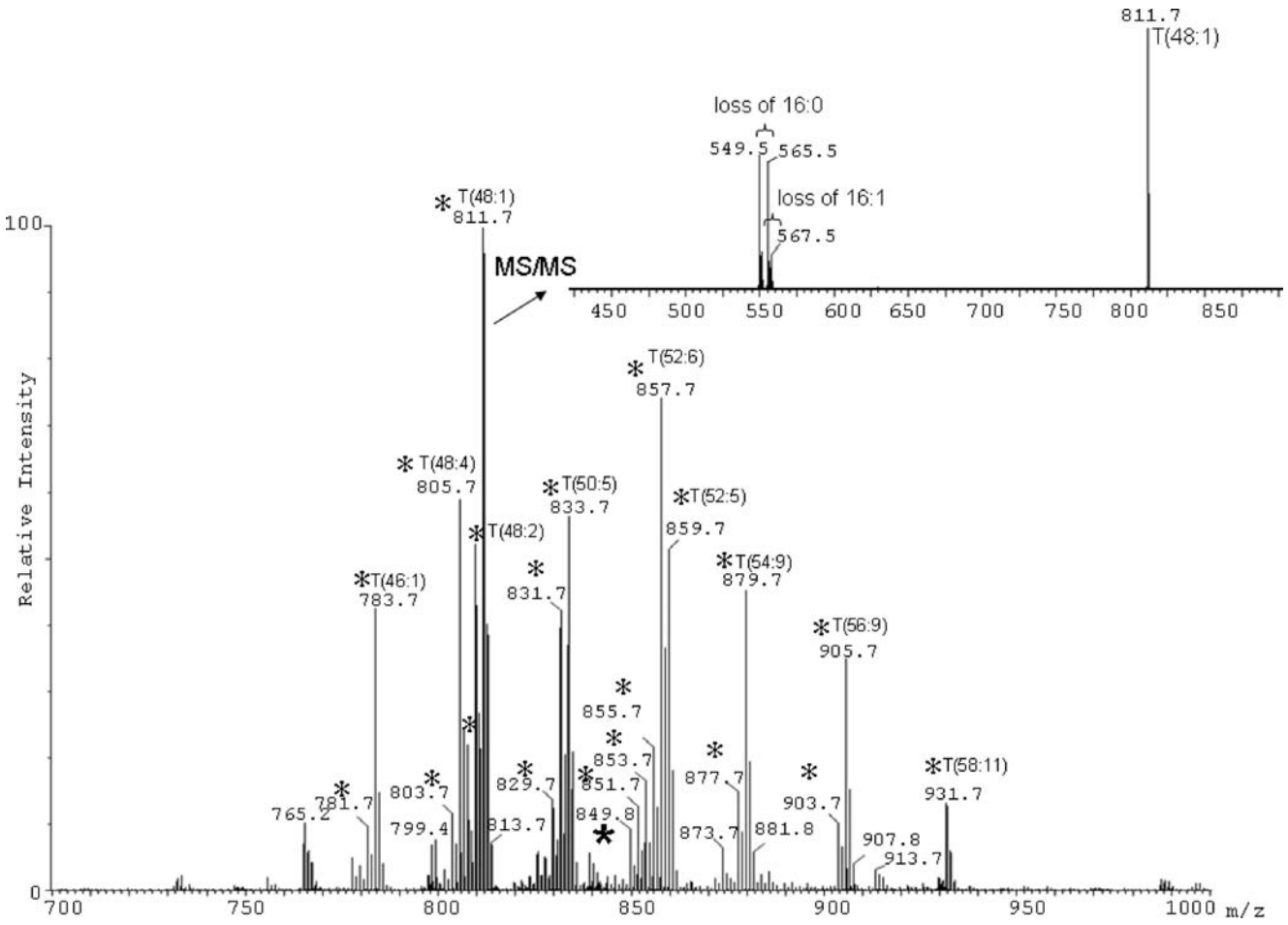


Fig. 3 Typical ESI-MS of non-polar lipids extracted from *T. pseudonana* cells. *Zoom inset* of the region spanning the 700–1,000 mass/charge (*m*/*z*) range shows TAG species verified by MS/MS (*asterisk*). Triheptadecenoin standard, TAG (51:3) is also shown at *m*/*z* 849.8 (*star*). Shorthand for TAG molecular species follows acyl carbon number:double bond (ACN:DB) notation. *Inset* shows MS^2 of lithiated TAG species (48:1) at *m*/*z* 811.7. The major product ions arise from loss of intact fatty acid chain, allowing the length of each chain to be determined (16:0/16:0/16:1)

almost 60% of identified TAGs), having palmitic (16:0), palmitoleic (16:1), and myristic (14:0) acid as major fatty acid substituents. In addition, we also detected higher molecular weight (MW) TAGs (54:9, 54:10, 56:10–56:12, and 58:11), which have polyunsaturated fatty acids (PUFA) incorporated into these TAG species. For example, ω-3 fatty acids, such as docosahexaenoic acid (22:6) and eicosapentanoic acid (20:5), are shown to be major components of these higher MW TAG species (Table 2). Although both cultures were sampled at mid logarithmic phase, TAGs extracted from nitrate limitation cultures are less diverse, as we failed to detect many of the low abundance species found in the silicate-limited cultures (Fig. 4b).

As the diatoms entered early stationary phase, overall TAG levels increased but distribution of TAG species remained constant. Only a few new TAG molecular species were detected at the onset of the stationary phase (46:3, 48:6, 52:9, 54:6, 54:7, and 58:12), albeit as minor constituents. We also observed temporal variation in the levels of some TAG species in the silicate-limited samples. For some TAGs (48:5, 50:4, 50:5, 50:6, 50:8, 52:5, and 52:6), levels appear to spike during onset of the stationary phase before following a decreasing trend during late stationary phase.

Among the TAGs detected at the onset of the stationary phase, the majority persisted through the late stationary phase and had similar percentage values between time points (44:1, 50:7, 52:9, 54:5 through 54:8, 56:13). However, there seems to be temporal variations in some TAG species, with 48:5, and 50:8, appearing to spike during early stationary phase then decreasing in the late phase. In contrast, some higher molecular weight TAGs (50:3, 56:10, 58:11, 58:12, and 60:15) increased in proportion during the late stationary phase.

TAG distribution and fatty acid composition in P. tricornutum oil extracts TAG speciation in algal oils extracted from *P. tricornutum* cells during exponential growth phase was significantly less complex than the *T. pseudonana* counter-

Table 2 Major TAG molecular species extracted from *T. pseudonana* and *P. tricornutum* during the stationary phase

TAG[a]	FA composition[b]	*T. pseudonana* Silica-limited	*T. pseudonana* Nitrate-limited	*P. tricornutum* Nitrate-limited
(46:1)	14:0/16:0/16:1	+	+	+
(46:2)	14:0/16:1/16:1	+	+	+
	14:0/16:0/16:2		+	
(48:0)	16:0/16:0/16:0	+	+	+
(48:1)	16:0/16:0/16:1	+	+	+
(48:2)	16:0/16:1/16:1	+	+	+
	16:0/16:0/16:2		+	
(48:3)	16:1/16:1/16:1	+	+	+
	16:0/16:0/16:3	+	+	
	16:0/16:1/16:2	+	+	
	14:0/16:0/18:3	+		
(48:4)	16:0/16:1/16:3	+	+	+
	14:0/16:0/18:4	+	+	+
	14:0/16:1/18:3	+		+
	16:1/16:1/16:2			+
	14:0/14:0/20:4	+		
(48:5)	16:1/16:1/16:3	+	+	+
	14:0/16:1/18:4	+	+	+
	14:0/14:0/20:5	+	+	+
(50:4)	16:0/16:0/18:4	+	+	+
	16:0/16:1/18:3	+		+
	16:1/16:1/18:2			+
(50:5)	16:0/16:1/18:4	+	+	+
	14:0/16:0/20:5	+	+	+
	16:1/16:1/18:3	+		+
(50:6)	14:0/16:1/20:5	+	+	+
	16:1/16:1/18:4	+		+
(52:5)	16:0/16:0/20:5	+	+	+
	16:0/16:1/20:4		+	+
	16:1/16:1/20:3		+	
(52:6)	16:1/16:0/20:5	+	+	+
(52:7)	16:1/16:1/20:5	+	+	+
	16:0/16:2/20:5		+	
	14:0/16:1/22:6		+	
(52:8)	16:0/16:3/20:5	+	+	+
	16:0/18:4/18:4	+	+	+
	16:1/16:2/20:5	+	+	+
	16:1/18:3/18:4	+		+
	14:0/18:3/20:5			+
	16:2/18:3/18:3			+
(54:9)	16:0/18:4/20:5	+	+	+
	16:1/18:3/20:5			+
(54:10)	16:1/18:4/20:5	+	+	
	14:0/20:5/20:5		+	+
	18:3/18:3/18:4	+		
	14:0/18:4/22:6		+	
(56:10)	16:0/20:5/20:5	+	+	+
(56:11)	16:1/20:5/20:5	+	+	+
(58:11)	16:0/20:5/22:6	+	+	+
(58:12)	16:1/20:5/22:6	+	+	+

TAG and fatty acid compositions observed in samples are indicated by (+). Regiospecificity of the fatty acids in the TAG is not determined

[a] TAG molecular species are reported as ACN:DB

[b] Possible fatty acid compositions consistent with the TAG molecular composition and MS^2 analysis are listed for each TAG

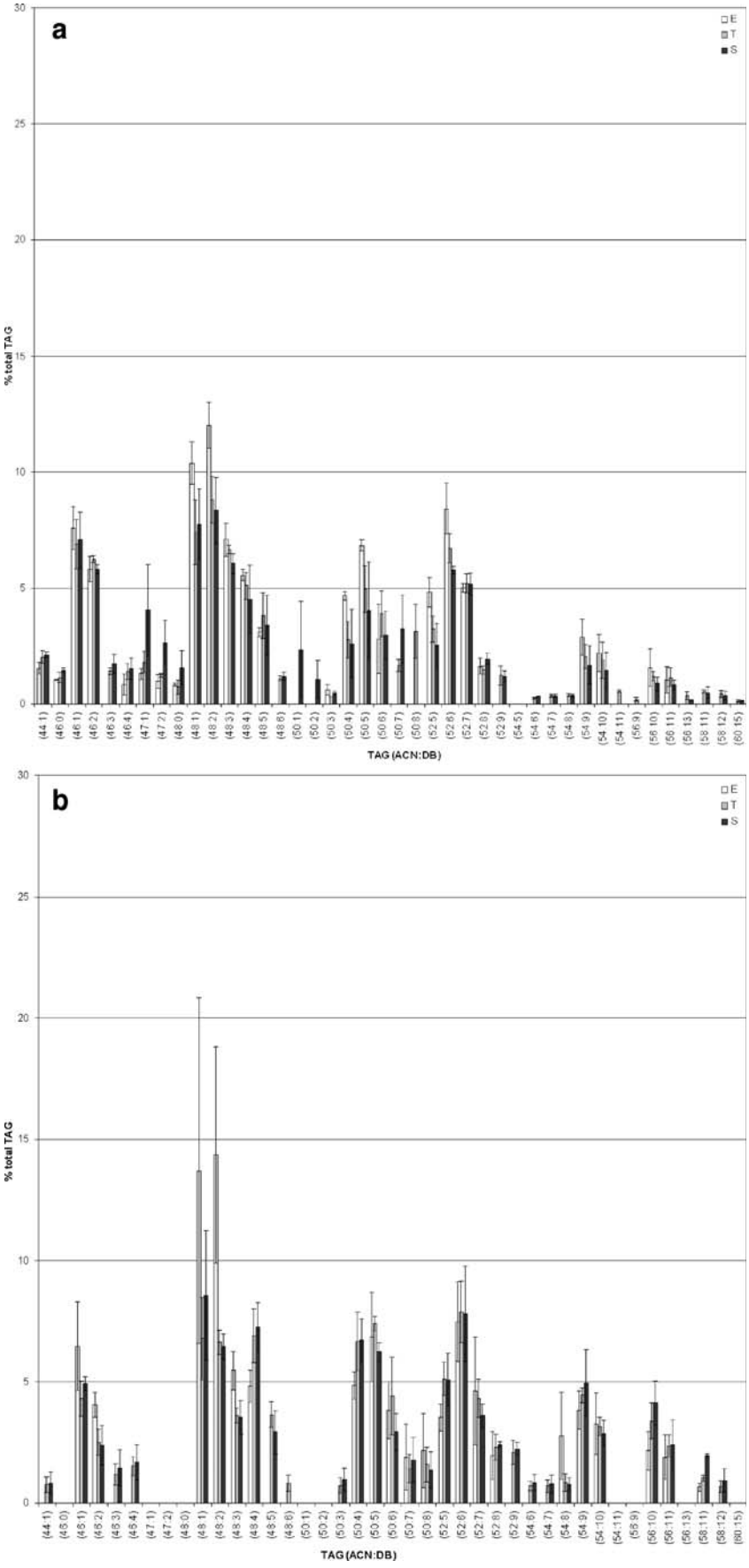
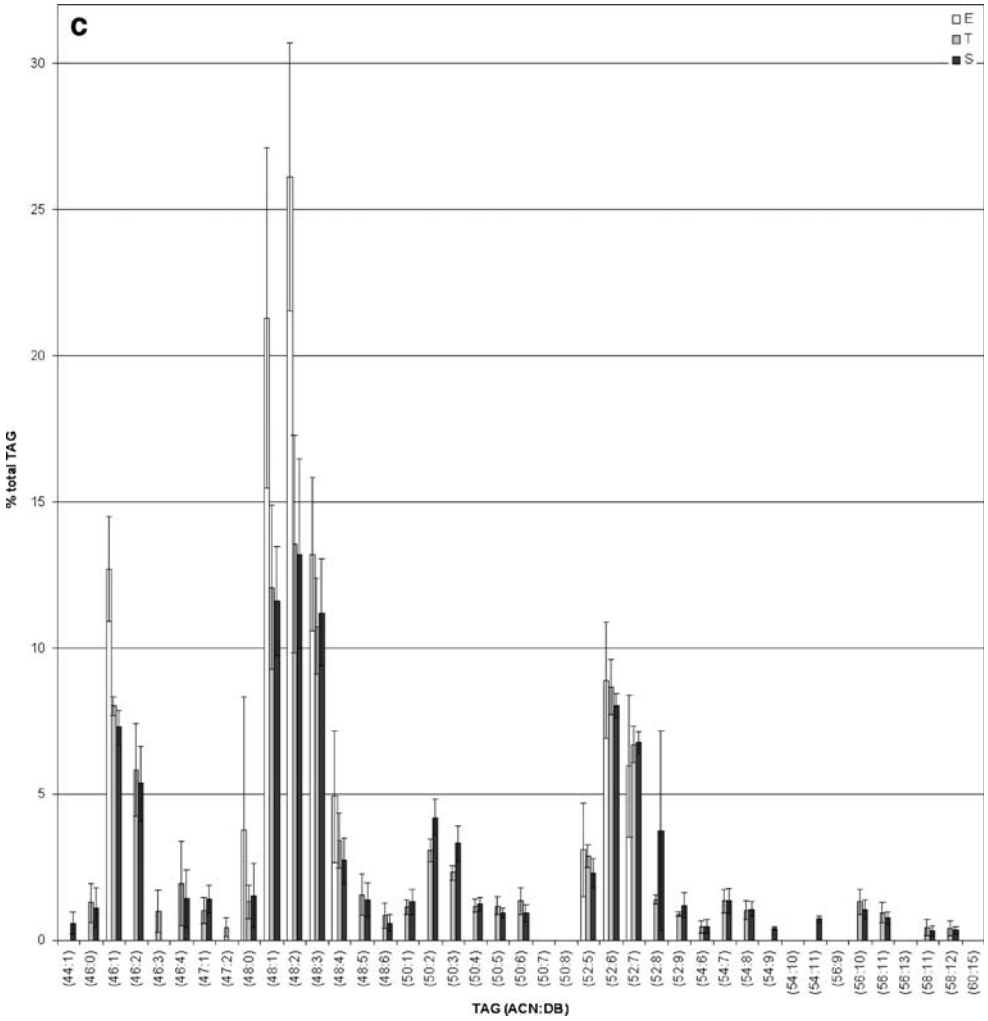


Fig. 4 The distribution of TAG species from (**a**) silicate-limited *T. pseudonana*, (**b**) nitrate-limited *T. pseudonana*, and (**c**) nitrate-limited *P. tricornutum* at different stages of the growth cycle. *E* (*white bars*), *T* (*gray bars*), and *S* (*black bars*) indicate exponential growth, transition to (or onset of) stationary, and stationary phase of growth, respectively. Each value represents the mean of three replicates. Error bars are standard deviation

Fig. 4 (continued)



parts. In fact, we were only able to consistently detect approximately ten individual TAG species. The oil extracts were predominantly composed of TAGs 46:1, 48:1, 48:2, and 48:3, having palmitic (16:0), palmitoleic (16:1), and myristic (14:0) acid substituents. TAG 48:1 (16:0/16:0/16:1) and 48:2 (16:0/16:1/16:1) constitute the main TAG molecular species that is expressed throughout the time course analysis of *P. tricornutum* cells grown in nitrate-limited media (Fig. 4c).

An increase in the diversity of TAG molecular species (with as much as 40 individual TAGs) was consistently detected from cells harvested during the onset of the stationary phase (Fig. 4c). Cells harvested at the onset of the stationary phase show accumulation of TAG species with a higher degree of unsaturation and higher ACN. Specifically, TAG species 50:1 through 50:6, 52:8, 52:9, 54:6 through 54:8, 56:10, 56:11, 58:11, and 58:12 were observed. The time course also revealed that a significant number of the expressed TAGs appear to have more PUFA incorporated into TAGs as the cells shift from the exponential growth phase to the stationary phase. Fatty acids 20:5 and 22:6 were incorporated in TAGs 52:6 (16:0/16:1/20:5), 56:10 (14:0/20:5/20:5), 58:11 (16:0/20:5/22:6), and 58:12 (16:1/20:5/22:6) (for a complete list, see Supplementary Table 1). In addition to these two long-chain PUFAs, fatty acids 16:3, 18:1 through 18:4, and 20:4 were also detected as TAG substituents. This increase in PUFA incorporation in TAGs from *P. tricornutum* is consistent with known trends in other microalgae grown under unfavorable environmental conditions. TAG molecular species observed during the onset of the stationary phase persisted throughout the resting state of cells, with the majority of the TAG species having more or less constant TAG proportions (Fig. 4c). In contrast, some TAGs (48:6, 50:1 through 50:3, 50:6) increase in the stationary phase.

Discussion

The overall goal of our research is to take advantage of genomic information and utilize genetic engineering to enhance TAG yields in diatoms. In order to fully charac-

terize the effects of particular genetic modifications, it will be important to understand not only the effect on TAG yield, but also on the distribution of molecular species within the TAG population. The utility of algal oils as biodiesel is largely dictated by the composition of fatty acids in triacylglycerols. Profiling the basal TAG species and fatty acid incorporation under nutrient starvation conditions for these two model diatoms provides a foundation for future genetic manipulations to make more suitable biodiesel feedstocks.

We have carried out time courses of TAG formation, comparing silicate starvation in *T. pseudonana* to nitrate starvation in *T. pseudonana* and *P. tricornutum*. This work provides the basis for further characterization of non-polar lipid synthesis in these strains and establishes these strains as model systems for the study of lipid metabolism. This characterization includes rapid screening techniques for the presence and identification of TAGs, such as Nile red dye assay and TLC of hexane extracts, which allow us to rapidly screen TAG formation trends, as well as robust GC–MS and ESI-MS techniques, which allow full identification of TAG chemotypes. By approaching our TAG analysis in a sequential fashion, of increasing analytical complexity, we were able to validate our results with multiple methods. The TLC analysis provided evidence of lipid content in the logarithmic phase which was not completely evident using only the Nile red fluorometric assay, due to the background noise present in the measurement. By using a combination of analytical techniques, we have in each case determined that TAG accumulation initiates approximately 10 h after the cells enter stationary phase and continues for several days after onset. We have utilized hexane extraction to purify non-polar lipids from lyophilized biomass harvested at daily time points during the exponential growth and stationary phases. Under silicate starvation conditions, the TAG yield from strain 3H of *T. pseudonana* is about 18% of total dry weight. Biomass from silica starved cultures contained 24% more non-polar lipids than that from nitrate-starved cultures. The TAG yield from *P. tricornutum* is about 14% of total dry weight.

Using a modified diesel analysis GC–MS standard (EN 14105:2003) we found that the lipids identified, and the trends observed in their formation and relative abundance, by GC–MS were consistent with those identified by ESI-MS. In most cases, before GC–MS analysis, the TAGs undergo a derivatization step to increase their volatility (Murphy et al. 2001). However, we did not use a derivatization process because a drawback of this technique is that it can modify the structure of the polyunsaturated TAGs and cause inaccurate identification, or artifacts that overlap with other peaks (Eder 1995; Malavolta et al. 2004). To eliminate the derivatization step, we employed a high-temperature (400°C), non-polar (5%-phenyl)-methylpolysiloxane column. Non-polar columns are traditionally used for lipid analysis because the higher temperatures allow for the volatilization of higher molecular weight TAGs and high-temperature stability.

Using ESI-MS, we have identified the TAG molecular species, determined individual acyl groups present in each TAG, as well as determined the relative abundances of the individual TAGs in the hexane extracts (Fig. 4 and Table 2). It takes nitrate-starved cultures of *T. pseudonana* about 24 h longer than it does for silicate-starved cultures to achieve maximal levels of TAGs. The TAG profile produced from each strain and culture condition was well defined and consistent over multiple cultures grown on separate days. While the TAG molecular species profile has not been determined for *T. pseudonana,* fatty acid profiling has been performed on the microalgae at various culture conditions (Fisher and Schwarzenbach 1978; Tonon et al. 2002; Zhukova 2004). We observed the same predominant fatty acids (i.e., 14:0, 16:0, 16:1, 16:3, and 20:5) incorporated in TAGs as described in these earlier studies. In general, the distribution of the major fatty acids we found for *T. pseudonana* clone 3H agrees with that observed by Fisher and Schwarzenbach (1978), but the yield of TAGs we obtained in the stationary phase was higher (12% versus 18%). Profiling of TAG species in *P. tricornutum*, albeit a different strain (UTEX 640), has been previously reported (Yongmanitchai and Ward 1993). After 7 days of cultivation, only 18 TAG molecular species were identified via reverse-phase HPLC analysis in their study. Due to the high resolution and sensitivity of ESI-MS, we were able to detect twofold more species in our algal oil extracts. In our study, we detected 14 of the 18 species they detected by HPLC, at comparable percentage compositions. However, we did not detect TAGs 48:7, 48:9, 48:12, and 54:10. It is possible that we did not observe these TAG species because of the difference in the *P. tricornutum* strains and culture conditions. For the molecular species that we did not observe, these TAGs were composed predominantly of 16:3 and 16:4 fatty acids, which were constituents in some of the other minor TAGs we analyzed by MS^2 (Table 2). Our results are consistent with previous studies reporting fatty acid profiles of *P. tricornutum* at various nutrient limitation conditions (Alonso et al. 2000; Ben-Amotz et al. 1985).

Our mass spectrometry results indicate that the lipids present depend on both the type of nutrient limitation employed, as well as the species of diatom analyzed. Overall, *T. pseudonana* produced almost the same number of TAGs whether it was grown in silicate- or nitrate-limited media during stationary phase. In *T. pseudonana*, the TAG chemotypes produced under nitrate and silicate starvation were generally quite similar, but we did observe some differences in the pattern of TAGs formed under nitrate starvation versus those observed under silicate starvation. Under nitrate starvation, we observed a greater production

of high MW TAGs, and in silicate-starved cultures, we observed the presence of several low MW species that were not detected under nitrate starvation. Nitrate limitation seems to result in either production and/or incorporation of longer fatty acyl chains in TAGs while silicate limitation seems to either cease to produce or begin to utilize these longer fatty acyl chains. The suitability of algal oils for biodiesel production depends largely on the composition of fatty acids and their distribution in triacylglycerols (Knothe 2005). Generally, saturated fatty acids of longer acyl chains are preferred for increased energy yield, superior oxidative stability, and higher cetane numbers. However, these oils are prone to solidify at ambient temperature, limiting their utility as biodiesel. While oils rich in PUFAs have very good cold-flow properties, they are, on the other hand, more susceptible to oxidation. Profiling the basal TAG species and fatty acid incorporation under nutrient starvation conditions for these two model diatoms provides a foundation for future genetic manipulations to make more suitable biodiesel feedstocks.

The attractiveness of utilizing diatoms as feedstock for the commercial production of biodiesel and other biofuels is limited by their requirement for silica. However, there are species such as *P. tricornutum*, that have little, if any, silica requirement thereby minimizing the cost associated with providing that nutrient. We have identified a possible benefit of this silica requirement. Starving *T. pseudonana* for silica resulted in the production of 24% more TAGs than found in nitrate-starved cultures. It seems reasonable to suggest that nitrate starvation has a far more significant impact on the metabolic capacity of cells than silicate starvation. If so, this could explain the difference in TAG yields. If this result represents a general characteristic of diatoms, it would suggest that it may be more effective and economical to produce TAGs under silica starvation rather than under nitrate starvation.

Previous researchers have suggested that TAG synthesis is constant through the logarithmic and stationary phases of growth, and when cell division slows as cultures enter stationary phase, the amount of TAGs increases on a per cell basis and that there is an increase in TAG accumulation per cell rather than an induction of TAG synthesis (Sheehan et al. 1998). In our experiments, there was a 10-h delay between the end of the logarithmic growth and the increase in the per cell concentration of TAGs as determined by Nile red assay. This delay in the onset of TAG accumulation argues that in the two strains examined, TAG biosynthesis is a regulated process. This is in agreement with previous studies carried out on *C. cryptica* (Roessler 1988). They demonstrated that the ACCase activity increased two- and fourfold after 4 and 16 h of silica limitation. This increase in ACCase activity was dependent on de novo protein synthesis. A time course of gene expression over the transition from logarithmic growth to early stationary phase would give a more complete picture of transcriptional regulation of lipid production and will be the subject of future research.

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References

14105, E., BSI BS EN 14105 (2003) Fat and oil derivatives fatty acid methyl esters (FAME). Determination of free and total glycerol and mono-, di-, triglyceride contents (Reference method)

Alonso DL, Belarbi EH, Fernandez-Sevilla JM, Rodriguez-Ruiz J, Molina Grima E (2000) Acyl lipid composition variation related to culture age and nitrogen concentration in continuous culture of the microalga *Phaeodactylum tricornutum*. Phytochemistry 54:461–471

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

Beer S, Stewart AJ, Wetzel RG (1982) Measuring chlorophyll a and C-labeled photosynthate in aquatic angiosperms by the use of a tissue solubilizer. Plant Physiol 69:54–57

Ben-Amotz A, Tornabene TG, Thomas WH (1985) Chemical profile of selected species of microalgae with emphasis on lipids. J Phycol 21:72–81

Cooksey KE, Guckert JB, Williams SA, Callis PR (1987) Fluorometric determination of the neutral lipid content of microalgal cells using Nile red. J Microbiol Methods 6:333–345

Eder K (1995) Gas chromatographic analysis of fatty acid methyl esters. J Chromatogr B 671(1–2):113–131

Fisher NS, Schwarzenbach RP (1978) Fatty acid dynamics in *Thalassiosira pseudonana* (Bacillariophyceae): implications for physiological ecology. J Phycol 14:143–150

Han X, Gross RW (2001) Quantitative analysis and molecular species fingerprinting of triacylglyceride molecular species directly from lipid extracts of biological samples by electrospray ionization tandem mass spectrometry. Anal Biochem 295:88–100

Harrison PJ, Waters R, Taylor FJR (1980) A broad spectrum artificial seawater medium for coastal and open ocean phytoplankton. J Phycol 16:28–35

Hsu F-F, Turk J (1999) Structural characterization of triacylglycerols as lithiated adducts by electrospray ionization mass spectrometry using low-energy collisionally activated dissociation on a triple stage quadrupole instrument. J Am Soc Mass Spectrom 10:587–599

Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J 54 (4):621–639

Knothe G (2005) Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. Fuel Process Technol 86:1059–1070

Laakso P (2002) Mass spectrometry of triacylglycerols. Eur J Lipid Sci Tech 104:43–49

Malavolta M, Bocci F, Boselli E, Frega NG (2004) Normal phase liquid chromatography-electrospray ionization tandem mass spectrometry analysis of phospholipid molecular species in blood mononuclear cells: applications to cystic fibrosis. J Chromatogr B 810(2):173–186

Maloney M (1996) Thin-layer chromatography in bacteriology. In: Fried B, Sherma J (eds) Practical thin-layer chromatography: a multidisciplinary approach. CRC, Boca Raton, p 336

Mansour MP, Volkman JK, Jackson AE, Blackburn SI (1999) The fatty acid and sterol composition of five marine dinoflagellates. J Phycol 35:710–720

Mansour MP, Frampton DMF, Nichols PD, Volkman JK, Blackburn SI (2005) Lipid and fatty acid yield of nine stationary-phase microalgae: applications and unusual C_{24}–C_{28} polyunsaturated fatty acids. J Appl Phycol 17:287–300

Mock T, Samanta MP, Iverson V, Berthiaume C, Robison M, Holtermann K, Durkin C, Bondurant SS, Richmond K, Rodesch M, Kallas T, Huttlin EL, Cerrina F, Sussman MR, Armbrust EV (2008) Whole genome expression profiling of the marine diatom *Thalassiosira pseudonana* identifies genes involved in silicon bioprocesses. Proc Natl Acad Sci USA 105:1579–1584

Murphy RC, Fiedler J, Hevko J (2001) Analysis of non-volatile lipids by mass spectrometry. Chem Rev 101(2):479–526

Nota G, Musso SS, Naviglio D, Romano R, Sabia V (1999) Determination of sterols and their esters in fats by way of transesterification in different solvents. Anal Lett 32:811–825

Ponomarenko LP, Stonik IV, Aizdaicher NA, Orlova TY, Popovskaya GI, Pomazkina GV, Stonik VA (2004) Sterols of marine microalgae *Pyramimonas* cf. *cordata* (Prasinophyta), *Attheya ussurensis* sp. nov. (Bacillariophyta) and a spring diatom bloom from Lake Baikal. Comp Biochem Physiol B 138(1):65–70

Poulsen N, Kroger N (2005) A new molecular tool for transgenic diatoms: control of mRNA and protein biosynthesis by an inducible promoter-terminator cassette. FEBS J 272:3413–3423

Poulsen N, Berne C, Spain J, Kroger N (2007) Silica immobilization of an enzyme through genetic engineering of the diatom *Thalassiosira pseudonana*. Angew Chem 46:1843–1846

Roessler PG (1988) Changes in the activities of various lipid and carbohydrate biosynthetic enzymes in the diatom *Cyclotella cryptica* in response to silicon deficiency. Arch Biochem Biophys 267:521–528

Sasaki GC, Capuzzo JM (1984) Degradation of *Artemia* lipids under storage. Comp Biochem Physiol B 78:525–531

Schmelzer K, Fahy E, Subramaniam S, Dennis EA, Brown HA (2007) The lipid maps initiative in lipidomics. Methods Enzymol 432:171–183

Sheehan J, Dunahay T, Benemann J, and Roessler P (1998) A look back at the U.S. Department of Energy's Aquatic Species program: biodiesel from algae. National Renewable Energy Laboratory, Report NREL/TP-580-24190

Tonon T, Harvey D, Larson TR, Graham IA (2002) Long chain polyunsaturated fatty acid production and partitioning to triacylglycerols in four microalgae. Phytochemistry 61:15–24

Yongmanitchai W, Ward OP (1993) Molecular species of triacylglycerols from the freshwater diatom, *Phaeodactylum tricornutum*. Phytochemistry 32:1137–1139

Zaslavskaia LA, Lippmeier JC, Kroth PG, Grossman AR, Apt KE (2000) Transformation of the diatom *Phaeodactylum tricornutum* (Bacillariophyceae) with a variety of selectable marker and reporter genes. J Phycol 36:379–386

Zaslavskaia LA, Lippmeier JC, Shih C, Ehrhardt D, Grossman AR, Apt KE (2001) Trophic conversion of an obligate photoautotrophic organism through metabolic engineering. Science 292:2073–2075

Zhukova NV (2004) Changes in the lipid composition of *Thalassiosira pseudonana* during its life cycle. Russ J Plant Physiol 51:702–707