JBC Papers in Press. Published on November 30, 2016 as Manuscript M116.760843
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M116.760843

Carbon and Acyl Chain Flux During Stress-Induced Triglyceride Accumulation by Stable Isotopic Labeling of the Polar Microalga *Coccomyxa subellipsoidea* C169

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Running Title: Quantifying Pathways of Stress-Induced TG Synthesis in Algae

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ABSTRACT

Deriving biofuels and other lipoid products from algae is a promising future technology directly addressing global issues of atmospheric CO_2 balance. To better understand the metabolism of triglyceride synthesis in algae, we examined their metabolic origins in the model species, *Coccomyxa subellipsoidea* C169, using stable isotopic labeling. Labeling patterns arising from ^{13}C-U-glucose, $^{13}CO_2$, or D_2O supplementation were analyzed by GC-MS and/or LC-MS over time courses during nitrogen (N) starvation to address the roles of catabolic carbon (C) recycling, acyl chain redistribution, and *de novo* fatty acid (FA) synthesis during the expansion of the lipid bodies. The metabolic origin of stress-induced triglyceride was found to be a continuous 8:2 ratio between *de novo* synthesized FAs and acyl chain transfer from pre-stressed membrane lipids with little input from lipid remodeling. Membrane lipids were continually synthesized with associated acyl chain editing during N stress in contrast to an overall decrease in total membrane lipid. The incorporation rates of *de novo* synthesized FAs into lipid classes were measured over a time course of N starvation. The synthesis of triglycerides, phospholipids and galactolipids followed a two-stage pattern where N starvation resulted in a 2.5-fold increase followed by a gradual decline. Acyl chain flux into membrane lipids was dominant in the first stage followed by triglycerides. These data indicate a level of metabolic control that determines acyl chain flux between membrane lipids and triglycerides during N stress primarily relies on the Kennedy Pathway and *de novo* FA synthesis with limited, defined input from acyl editing reactions.

Oil production from algal feedstocks is a promising answer to the dwindling supply of easily extractable petroleum, one that is readily transferrable to the current transportation infrastructure and recycling of CO_2 emissions. Algae have high growth rates and accumulate triglycerides (TG) in excess of crop seed oil when subject to abiotic stress, most notably nitrogen (N) deficiency (1). The caveat is a tradeoff between cellular TG quantities and reproductive growth. In nature, oil accumulation is linearly correlated with, and may be caused by, a cessation or slowing of the cell cycle (2-4). Engineering algae to produce oil on demand is needed for improving process yields and necessitates a thorough understanding of the biochemistry underlying oil accumulation for the development of strategies to attain this goal (5).

Most prokaryotic and all eukaryotic glycerolipids are commonly configured from two fatty acyl chains and one of a wide variety of polar functional groups esterified to a glycerol molecule. Differences in the acyl moiety chain length and number of double bonds, and in the size and charge of the polar head group, result in many thousands of theoretical variations. Chemical differences between membrane lipids result in unique intracellular environments required for the specialized functions of organelles and cellular

responsiveness to environmental changes (6). Specific enzymes mediate the replacement of membrane lipid acyl groups in plants, removing unsaturated FAs and replacing them with mono- or poly- unsaturated FAs and dynamically adjusting membrane chemistry through subtle alterations in acyl group architecture. An important result of this action is the efficient translocation of *de novo* synthesized fatty acids from the chloroplast to the extra-chloroplast membrane lipid pool (7). Acyl-editing reactions include the Lands Cycle (8), governing the removal of an unsaturated *sn*-2 position acyl moiety of phosphatidylcholine (PC) by hydrolysis via phospholipase A2 (PLA2) and replaced from the acyl-CoA pool by lyso-phosphatidylcholine acyltransferase (LPCAT). The *sn*-2 position of PC can also be directly transferred to the *sn*-3 position of diacylglycerol (DAG) to form triacylglycerol (TG) through the action of phosphatidylcholine: diacylglycerol transacylase (PDAT) (9). The LPCAT reaction is reversible in plants, so the Lands Cycle can be carried out by this enzyme alone (10). The reconversion of lyso-phosphatidylcholine (LPC) generated by PDAT into a PC requires LPCAT as well, making it a central enzyme in acyl editing reactions.

In contrast to acyl editing, lipid remodeling reactions alter membranes by removing and replacing the polar head group. In *Arabidopsis thaliana* the most extreme example of this is the majority replacement of PC during inorganic phosphate (Pi) stress with a galactolipid originating from the chloroplast (11). During Pi starvation, PC is hydrolyzed to phosphocholine and DAG by either phospholipase C or D, transported to the outer chloroplast membrane, and incorporated into the galactolipid synthetic pathway (12,13). Null mutations of LPCAT1/LPCAT2 in *A. thaliana* lead to the recycling of LPC by removal of the choline head group and reintegration into the Kennedy Pathway, resulting in significant alterations to the TG pool (14). These alterations could also be the result of increased activity of phosphatidylcholine diacylglycerol choline-phosphotransferase (PDCT), which catalyzes the removal of the choline head group from intact PC, allowing the conversion of the resulting DAG molecule to TAG through diacylglycerol acyltransferase (DGAT) (15). PDCT was found to be mainly responsible for the interconversion of PC and DAG, and shown to be required for about half of the incorporation of hydroxylated FAs into the TG pool in transgenic *A. thaliana* (16). Overexpression of PDCT in *A. thaliana* seeds also results in a 20% increase in polyunsaturated fatty acid (PUFA) content (17).

Lipid droplets are the repository of acyl chains thought to enter the TG pool through various pathways, but the relative inputs from membrane lipid recycling and *de novo* synthesis still remain unclear. In algae, protein and/or transcript abundance of the Kennedy Pathway enzymes glycerol phosphate acyltransferase (GPAT), lyso-phosphatidyl acyltransferase (LPAAT), phosphatidic acid phosphatase (PAP), and DGAT increase alongside acyl editing enzymes PDAT and LPCAT during N stress (9,18-21). The presence of PUFAs is also considered indicative of membrane acyl chain recycling into the TG pool since FA desaturation reactions in plants mostly occur on FAs incorporated into membrane lipids and there is no evidence to the contrary in algae (22-25). Although acyl-editing and lipid remodeling pathways are known to take part in TG accumulation, it is necessary to quantify these inputs to determine how important these pathways are to the overall yield. FA synthesis inputs from C recycled after the catabolism of biomolecules (*e.g.*, proteins) and C fixed through photosynthesis are similarly understudied, yet these aspects also remain important factors in determining how best to alter algal metabolism to enhance TG synthesis.

Coccomyxa subellipsoidea C-169 is a free-living asexual freshwater green algal species with a fully sequenced genome (26) and is closely related to the genus Chlorella, sharing 63% of genes with *Chlorella variabilis* NC64A (26,27). Chlorella species have emerged as strong candidates for algal biofuel production due to their high growth rates (28-31), and of note is that biodiesel derived from *C. subellipsoidea* has been favorably evaluated (32). Originally collected in Antarctica, *C. subellipsoidea* is being considered as a possible cold-tolerant production species (26). This is especially important for cooler geographical regions since controlling water temperature to maximize growth rate can be a major energy expense in large-scale production

(33). Aside from the optimal growth temperature, *C. subellipsoidea* exhibits lipid profiles and stress-induced metabolic trends consistent with other microalgal species (34,35). In a previous study, we demonstrated that acyl group chain length, positioning, and double bond content in TG presented as a fixed ratio during N stress (34). This suggests a significant role of acyl editing throughout N stress rather than synthesis through the sequential acylation of glycerol by newly synthesized acyl chains. N stress-induced TG was also highly enriched in oleic acid in *C. subellipsoidea*, suggesting that fatty acid synthesis still provides the majority of acyl chains for TG synthesis rather than redistribution from membrane lipids.

In the present work, pathways of acyl chain flux contributing to oil synthesis were analyzed during N stress using four different stable isotopic labeling schemes applied to *C. subellipsoidea* C169. Lipid classes were separated to distinguish between the biochemistry of membrane maintenance/catabolism and TG anabolism, enabling the indirect analysis of pathways contributing to oil accumulation. Neutral lipids containing >95% TG and a small amount of sterol ester were separated from crude lipid extracts by silica column chromatography. Membrane lipids were further separated into galactolipids intrinsic to plastids in algae and phospholipids that are mostly extrinsic of plastids (36). Significant structural changes in plastid architecture during N stress are associated with alterations in both total and relative amounts of the chloroplast-specific membrane lipids. This has been suggested to indicate that their metabolism different from other membrane lipids (36-38). Monogalactosyl-diacylglycerol (MGDG) is reduced dramatically during thylakoid degradation, likely to avoid destabilizing plastid membrane integrity and function due to its conical steric profile as the chloroplast decreases in size (34,39).

This study is the first showing that FA synthesis and subsequent incorporation into membrane lipids continues during N stress, and moreover demonstrates that the expansion of lipid droplets requires *de novo* FA synthesis and distinct patterns of acyl chain flux between membrane lipids and TGs. The contributions of acyl editing, membrane catabolism and lipid remodeling reactions were further quantified in relation to acyl chain flux into oil. A detailed analysis of FA synthesis and incorporation rates into lipid classes over the course of N starvation and recovery demonstrates two distinct stages of N stress where membrane lipids act as alternative sinks of newly synthesized FA, significantly outcompeting TG in the early stage.

RESULTS

Overview

Five labeling regimes were employed using ^{13}C and deuterium stable isotopes, which were designed to highlight specific aspects of acyl chain flux. Metabolites were generally labeled through mixotrophic growth using ^{13}C-U-glucose, and changes in the labeling patterns of lipids and lipid-associated glycerol analyzed during label-free N starvation. This approach facilitated study of acyl chain transfer from membrane lipids that could contribute to TG synthesis. Continuous labeling with $^{13}CO_2$ informed on the roles of photosynthetic C fixation and catalytic C recycling. D_2O labeling measured the general flux of acyl chains into TGs and through membrane lipids. Finally, much shorter time courses of D_2O labeling were used to quantify the incorporation rates of *de novo* synthesized FAs into complex lipids in the context of abiotic stress.

N-starvation after ^{13}C-U-glucose pre-labeling

The glycerol and FA moieties of triglyceride (TG), galactolipid (GL) and phospholipid (PL) pools were analyzed for the relative incorporation of pre-stress and newly synthesized glycerol into complex lipids during N starvation by measuring changes in percentage labeling and ^{13}C distribution. The goal of these experiments was to quantify the amount of TG derived from the interconversion of other complex lipids. *C. subellipsoidea* was ^{13}C labeled for this analysis under normal, unstressed conditions by mixotrophic growth with 25mM uniformly labeled ^{13}C-glucose for one week starting from a low initial cell density of 3 X 10^5 cells mL^{-1}. Labeling in the resulting stationary phase cultures was evident in the biomolecules analyzed but varied in

degree due to differences in the propensity of the associated biosynthetic pathways to incorporate C from the supplied ^{13}C-U-glucose or unlabeled, photosynthetically derived molecules (Figures 1 and 2). GC-MS analysis demonstrated the presence of ^{13}C in 85% of glycerol and 95% of FA molecules. The percentage labeling in glycerol was >63% and in FAs was >50%. The ^{13}C labeled biomass was subjected to N starvation without further label addition and changes in ^{13}C labeling in the TG, GL, and PL pools due to the addition of newly assimilated ^{12}C or the movement of labeled, previously synthesized moieties were measured after one week. Glucose was chosen for the simultaneous labeling of acyl chains and glycerol based on a recent plant-based study that demonstrated a lack of incorporation of label into fatty acids using glycerol itself (40). The incorporation of stable isotopically labeled glycerol into the TG pool as a proxy for the activity of complex lipid remodeling enzymes is an established technique (41).

One week of ^{13}C glucose labeling resulted in similar relative quantities of unlabeled, single, double, or fully ^{13}C labeled glycerol isotopologs hydrolyzed from neutral lipid, GL, and PL pools (Figure 1). Within the lipid classes analyzed, there was an approximate ratio of 3:3.5:2:1.5 between three ^{13}C labeled, two ^{13}C labeled, one ^{13}C labeled, and fully unlabeled glycerol. Following a week of N starvation in the absence of ^{13}C-glucose, there was an increase in the relative percentage of unlabeled glycerol synthesized during N stress, suggesting a dominant role of the Kennedy Pathway in complex lipid synthesis during this time. The total quantity of measured glycerol increased 2.1±0.2 fold with 99.8±9 % of this in the TG pool under N starvation conditions. More than half of the glycerol in the PL and GL pools was replaced by unlabeled glycerol during this period while there was essentially no change in quantity, indicating an equivalent efflux of existing and influx of newly synthesized glycerol. This data agrees with the FA labeling data and suggests an efflux of both preexisting acyl chains and glycerol, possibly esterified into complex lipids. However, virtually all of the TG produced during the week of N starvation was associated with either unlabeled or single ^{13}C labeled glycerol, suggesting a dominant role of the Kennedy Pathway rather than lipid remodeling reactions in complex lipid synthesis during this time. The double and triple ^{13}C-labeled glycerol quantities did not change appreciably in the TG pool during N stress (Figure 1B). In contrast, the quantity of singly labeled glycerol increased nearly two-fold, which is much more than can be accounted for by transfer from the phospholipids and is likely the result of new synthesis utilizing previously captured carbon. Loss of ^{13}C from the PL pool accounted for only 6.8% of the gains of singly ^{13}C-labeled glycerol in TG while the incorporation of unlabeled and singly ^{13}C-labeled glycerol together accounted for 92% of newly synthesized TG. The measured smaller changes in double and triple ^{13}C-labeled glycerol content support the conclusion there was little transfer of pre-existing glycerol from the membrane lipids into the TG pool under N starvation growth conditions.

We next determined the patterns of labeling and relative abundance of ^{13}C in 16C and 18C fatty acids in the TG, GL and PL pools after 7 days with ^{13}C-U-glucose in N replete media and after 7 days of N starvation in the absence of label to analyze the incorporation of *de novo* synthesized FA and the movement of FA moieties between lipid classes. The relative abundance of the different molecular ions for the 16C and 18C fatty acid was measured by GC-MS and molecular ion signal intensities normalized to the monoisotopic ion signal of an internal standard and the dry weights of the algal samples. Post-labeling molecular ion abundances of the 16C and 18C acyl chains in all three classes of lipid had parabolic patterns with apexes at M+13 for C16 FA and M+14 for C18 FA. After seven days of mixotrophic labeling with ^{13}C-U-glucose, approximately 94% of ion abundance counts in the 16C and 18C FAs were M+7 or greater (Figure 2). Of particular importance was label-free N starvation produced a second set of distinguishable ion abundances evident in all three classes of lipid between M+0 and M+7, which are indicative of *de novo* fatty acid synthesis and were easily distinguishable from pre-stress, highly labeled FAs. Quantitative analysis of these molecular ion groups showed a 78% reduction of pre-stress FAs in the PL pool, a 54% reduction in the GL pool, and a 12% increase in the TG pool. During N starvation the efflux of labeled acyl chains was

greatest from the PL pool; nearly all of the phospholipid acyl chains were replaced over the seven days of N starvation. The data indicates there is a significant flux of FA chains through membrane lipids during N starvation and that the majority of fatty acids in TG synthesis are from *de novo* synthesis from photosynthetically fixed carbon. An average of 11% of the carbon content of TG as 16C and 18C fatty acids synthesized during N starvation was labeled with ^{13}C suggesting the incorporation of previously reduced C also contributes to stress-induced lipogenesis (Table 1). These conclusions were further tested using $^{13}CO_2$ labeling.

$^{13}CO_2$ labeling during N starvation

Acyl chain remodeling was next addressed in relation to TG synthesis in *C. subellipsoidea* following $^{13}CO_2$ labeling during N starvation and analysis of intact, catalytically saturated molecules by LC-MS. There were three distinct molecular ion domains separable by approximately 15 m/z units, similar to the labeled acyl chains following growth in the presence of ^{13}C-U-glucose, which represent ^{13}C labeling from photosynthetically fixed carbon in one, two, or three of the acyl chains in TG (Figure 3A). The abundance of ^{13}C labeling in the acyl chains, noted with one, two, or three asterisks in Figure 3A, were analyzed at days 1, 3 and 7 over the 7 day time course of N-starvation and calculated as relative percentages (Figure 3B). The quantity of unlabeled acyl chains in each TG species analyzed were shown to be inversely correlated with the 16C FA content (Figure 3B). Approximately 2% of TG(18/18/18) and 24% of TG(16/16/16) acyl chains were unlabeled after 7 days. The total ^{13}C labeling of TG species varied with 16C FA; TG(18/18/18) had a maximum labeling with ^{13}C of 72% compared to 45% ^{13}C labeling in the TG(16/16/16) (Figure 3A). No statistically significant changes were measured by pairwise comparisons (ANOVA; $p < 0.05$) of time points within TG molecular species after day 1, and by day 3 further incorporation of labeled and unlabeled FAs were in proportional steady states (Figure 3B). These findings mirror our previous experimental results demonstrating the TG 16C/18C acyl chain ratio remained stable after 3 days and through 7 days of subsequent N deprivation (33), supporting the concept of a consistent and stable system-wide set of reactions leading to TG production during abiotic stress in this species.

The patterns of ^{13}C labeling related to the quantity of TG species specifically demonstrate that the unlabeled FA in TG is primarily 16C (Figure 3B). While the quantity of TG increases over the 7-day N starvation time course, the total ^{13}C-labeling of TG was 66%, 70%, and 68% on days 1, 3, and 7, respectively (Figure 4A). There was a continuous input of unlabeled C in the TG pool over the entire course of N starvation evident in both ^{13}C labeling studies. This was approximately 90% accounted for from the $^{13}CO_2$ labeling study as incorporation of entirely unlabeled FAs. Unlabeled C was also incorporated into labeled FAs, which can only be derived from catabolic recycling of pre-stress reduced C compounds, and ranged from 5-14% of the total TG pool. This range of unlabeled C corresponds well to the range of 9-12% determined by pre-labeling cultures with ^{13}C-U-glucose. This indicates both a continual insertion of acyl chains synthesized prior to N starvation and catabolically derived unlabeled C in fatty acid synthesis that contributes to the TG pool throughout the 7 day N starvation period (Figure 4B).

Analysis of membrane lipid flux using deuterium labeling

The experimental data presented above is fully consistent with the conclusion that *de novo* fatty acid synthesis continues during N starvation with the subsequent flux of these fatty acids into the TG pool. To address *de novo* fatty acid synthesis directly, continuously supplied D_2O (30%) was used to trace the flux of newly synthesized acyl chains between major lipid classes over an 8 day N starvation time course. Deuterium labeled lipid extracts were separated by column chromatography into TG, GL, and PL fractions as detailed above. These were either untreated to analyze specific FA species or catalytically hydrogenated to facilitate increased signal intensities by combining saturated and unsaturated 16C and 18C FAs into single peaks. Analysis of the resultant fatty acid methyl esters by GC-MS enabled the quantification of FAs with or without

deuterium incorporation due to the significant differences in time resolution between labeled and unlabeled peaks (Figure 5A). The relative quantification of deuterium labeled FAs was compared with absolute FA quantifications to analyze acyl chain flux through lipid pools (Figure 5). Cultures were analyzed every other day over the 8-day time course of N starvation. The TG pool increased by 5.3-fold between days 2 and 8, while the GL and PL pools decreased by 50% and 7%, respectively (Figure 5B). These quantitative changes were associated with increased deuterium labeling over the time course for all lipid pools examined (Figure 5C). In agreement with the ^{13}C-labeling data, *de novo* synthesis of 18C FA was much greater than 16C FA and 18C incorporation into the TG pool and accounted for ~85% of the total fatty acids synthesized by day 8. These results were comparable to the $^{13}CO_2$ labeling results that showed ~85% of the FAs were labeled at the end of the time course. While the absolute quantities decreased during N starvation, 51% and 34% of FA in the GL and PL pools, respectively, were synthesized during N stress. These results indicate the influx of *de novo* fatty acids was comparable to the efflux of acyl chains from the PL and GL pools, however there is discrepancy in the amount of PL synthesized between experiments. Data from the ^{13}C-U-glucose labeling experiment indicated a 38% increase in the absolute quantity of PL (Figure 1B). The GL pool was also not as reduced by N stress, likely because it was already somewhat reduced due to the availability of glucose in the media. The GL acyl chains became labeled despite the decrease in the amount of GL during N starvation, which supports the notion that both influx and efflux of acyl chains continues to occur with the coincident degradation of chloroplast membranes (34).

To further these studies, bioreactor based experiments were conducted over the 10-day starvation period to produce sufficient algal biomass for a more comprehensive analysis of the different unsaturated FA species. In these experiments, 30% D_2O was supplied at the beginning of N starvation and samples were then taken daily for 10 days (Figures 5D-E). In all three lipid pools analyzed, oleic acid (18:1$^{\Delta 9}$) was essentially completely labeled by day 4 and the patterns were strikingly similar between the membrane lipids and TGs. After day 1, pairwise comparisons (ANOVA; $p < 0.05$) showed no significant differences between labeling in the PL and TG pool oleic acid, however GL labeling was generally lower most days. Palmitic acid (C16:0) was not significantly different between the three lipid pools over the 10-day time course. This data suggests that the incorporation of *de novo* synthesized acyl chains is at equilibrium between lipid classes during N starvation. In the membrane lipids (PL and GL), linoleic acid (C18:2$^{\Delta 9,12}$) was nearly completely labeled by day 8; labeling of α-linolenic acid (C18:3$^{\Delta 9,12,15}$) was reduced and reached only ~50% labeling by day 10 (Figure 5E -F). By contrast, the labeling of α-linolenic acid was lower (32%) in the TG pool on day 10. The signal to noise ratio was too high to accurately measure labeling in unsaturated 16C FAs into the PL and TG pools. In the GL pool, palmitoleic acid (C16:1) was more rapidly labeled than palmitic acid (C16:0) (Figure 5F).

Analysis of FA synthesis and incorporation rates into the three lipid pools during N-stress

We next addressed the incorporation rates of *de novo* synthesized FAs into the TG, GL and PL pools over a total of ten short time courses where samples were taken at 3, 6, 9, and 12 hr after addition of 20% D_2O (Patton 1979). These time courses were conducted after 1, 2, 4, 6, 10, and 14 days of N starvation and 1, 2,and 4 days of N-recovery (Figure 6A). As expected from previous studies, the TG pool increased nearly 10-fold during N starvation and the total quantity of FA in the GL and PL pools were reduced by ~50% by day 14 of N stress (Figure 6B). Incorporation rates measured during N stress were compared to control rates measured during unstressed mid-exponential phase growth, referred to as time zero (Figure 6C-D).

The studies addressing FA synthesis revealed two distinguishing stages during N starvation. The first was characterized by an initial and rapid 2.4-fold increase in FA synthesis over the first two days and the second was a steady decline to the initial rate by day 14 (Figure 6C). This second phase decrease in the FA synthetic rate was linear from days 2 through 14 of N starvation (5% per day ($R^2 = 0.99$)) and coincided with previously

published reductions in both chlorophyll and MGDG content, diminishing photosynthetic capacity (22,34,42). Following the re-addition of N on day 14, *de novo* FA synthesis was not detectable by deuterium labeling on days 14-16 but returned to the pre-starvation rates within 4 days of N re-addition (Figure 6C). This initial cessation was seen in all lipid pools, indicating that the influx of 18C FA into GL's during recovery from N stress demonstrated previously in this species (34) originates from the TG pool and coincides with the total repression of FA synthesis.

The onset of N starvation led to an increase in the rate of *de novo* synthesized FA incorporation into both chloroplastic and extra-plastidic membrane lipids, with peak synthetic rates measured on day 2 for both GL and PL pools (Figure 6D), akin to total fatty acid synthesis rates (Figure 6C). The influx of *de novo* synthesized FAs into GL and PL as determined using continuous D_2O labeling (Figure 5, above) is apparently skewed toward the earlier phase rather than being uniform throughout N stress. Incorporation of fatty acids into the TG pool was delayed compared to PL and GL pools and increased in quantity peaking at day 6, through a linear increase of 0.5 % of DW algae day^{-1} (R^2 = 0.99). This suggests that incorporation into membrane lipids is essentially replaced by incorporation into TGs during the second phase of N starvation. Incorporation rates declined in the TG pool after day 6 in a linear fashion (R^2 = 0.99) at -0.083 µg lipid mg^{-1} DW algae hr^{-1}, indicating a decrease in carbon assimilation during extended N stress and thus, lower TG accumulation rates.

The high rates of *de novo* synthesized acyl chain incorporation into membrane lipids led us to further separate the TG, GL, and PL fractions into their major lipid class constituents, and define their relative contributions of *de novo* acyl chain incorporation. Nine lipid classes were isolated using TLC after 2 days of N starvation under D_2O labeling conditions coincident with peaks in membrane lipid incorporation and overall FA synthesis rates as noted above (Figure 6C-D). The fatty acids from the different lipid fractions were analyzed as their methyl esters by GC-MS at 3, 6, 9, 12, and 24 hr and the resultant data fitted to a least squares function; the resultant slopes were used to define incorporation rates of the newly synthesized fatty acids (Figure 7). Labeling was only detected for oleic, palmitic, and palmitoleic methyl esters due to the low concentrations of the individual lipid classes including the TG and sterol ester (St-E), galactolipids monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and trigalactosyldiacylglycerol (TGDG), and phospholipids phosphatidylcholine (PC), phosphatidylserine (PS), phsophatidylglycerol (PG), and phosphatidylethanolamine (PE). The total rates measured in these experiments were about 30% higher than those measured for the longer time course; however their relative ratios were very similar. The TG:GL:PL ratios of incorporation rates were 1.2:1.6:0.6 (µg lipid mg^{-1} DW algae hr^{-1}) in this experiment compared to 0.8:1.2:0.5 (µg lipid mg^{-1} DW algae hr^{-1}) on day 2 of the time course. A small amount (7%) of FA labeling measured in the neutral lipid pool was from sterol esters, but this pool otherwise was comprised of TG. The total incorporation rate into DGDG was more than 3-fold greater than MGDG, which was equivalent to TGDG. Incorporation into DGDG comprised 61% of the total GL pool rate, consistent with previous studies where MGDG declined faster than DGDG during N stress (33). In the PL pool, rates of incorporation into PC and PS were nearly equal, with PG about half of that. Half of the GL incorporation rate was from 16C FAs, demonstrating that palmitic acid is primarily incorporated into chloroplast derived complex lipids.

DISCUSSION

The economic viability of biofuel production from algal sources will largely depend on current scientific efforts to understand the mechanistic details underpinning TG accumulation under conditions where reproductive growth is limited, as occurs through N limitation, which will inform the development of strategies to replicate this phenotype free of growth restrictions (3,5). Stable isotopic labeling of *C. subellipsoidea* has yielded novel flux information especially related to TG synthesis that can be directly applied to developing strategies for maximizing oil production in algae. This is the first study that measures *de novo* FA synthesis during N stress,

defines the rate at which newly synthesized FAs are trafficked into complex lipids (triglycerides (TG), phospholipids (PL), galactolipids (GL)), and demonstrates both membrane lipid synthesis and turnover during N stress. Several results important to algal lipid biochemistry during N starvation have been revealed including the following: [1] There is very little to no membrane head group remodeling component to TG synthesis; [2] More than 90% of TG is the product of the Kennedy Pathway, however approximately one in five TG acyl moieties predate N stress, indicating a significant input from membrane lipid acyl editing or direct trans-acylation reactions; [3] Equivalent intrinsic rates of efflux of entire lipids including glycerol moieties are apparent for both plastid and non-plastid membrane lipid classes; [4] Phospholipid and not galactolipid acyl chains are subject to efflux via acyl editing at an approximate rate of one for every two removed by catabolism; [5] The rate of incorporation of *de novo* synthesized fatty acids more than doubles in the first two days of N stress with the majority entering membrane lipids, however TG incorporation is greater than any one specific lipid class; and [6] The rate of FA incorporation into TGs peaks after membrane incorporation rates decline, indicating competition between lipid classes as sinks for newly synthesized acyl chains.

TG is mainly produced by the sequential acylation of glycerol, not by membrane lipid remodeling reactions

To address the contribution of complex lipid remodeling between membrane lipids and the expanding TG pool, we tracked stable isotopically labeled glycerol after one week of labeling with ^{13}C-U-glucose followed by one week of label-free N stress. After 7 days in N stress, nearly half of the glycerol backbones in the membrane lipids were replaced while overall quantities remained constant, suggesting an efflux of esterified lipids and replacement by new synthesis. Only 8% of the stress-induced TG synthesized contained labeled glycerol, which is from the membrane lipid pool, demonstrating that 92% of the glycerol in the expanding TG pool was from new synthesis. Importantly, these data indicate the enzymes governing the conversion of membrane lipids to TG, such head group removal to generate diacylglycerol (DAG) that is subsequently converted to TG, do not play a major role in lipid droplet development during N stress. The $^{13}CO_2$ labeling experiments demonstrated that approximately 20% of the acyl chains of stress-induced TG synthesis were derived from membrane lipids, which coincides with the finding that 78% of acyl chains labeled with ^{13}C-U-glucose in the membrane lipids before N stress in were trafficked into the expanding TG pool. The trafficking of labeled acyl chains from the PLs is higher than can be accounted for by efflux of whole lipids, while in GLs, it is approximately equal.

Membrane lipid fatty acid moieties are incorporated into TGs

Membrane lipid acyl chain flux is currently poorly defined both spatially and temporally, yet relies on distinct enzymatic reactions that collectively provide flexibility in lipid metabolic homeostasis (43-46). The insertion and removal of fatty acids differing by chain length and number of double bonds defines membrane responses to temperature, salinity, and a variety of other abiotic conditions (47,48). In our previous work, we showed the rate of unsaturated FA accumulation in the TG pool was proportional with TG accumulation in *C. subellipsoidea* over 10 days of N stress (34). The flux of polyunsaturated fatty acids (PUFA) into TG during deuterium labeling was evident in the current study where there were gradual increases of di- and tri-unsaturated acyl chains in the expanding TG pool. While not strictly proven in algae, in other photosynthetic organisms polyunsaturated FAs come from membrane lipids (45,49,50). It is possible the incorporation of two or three double bonds occurs coincident with FA synthesis or in the fully formed TG but no such desaturase has been identified (51). The continual flux of PUFA into the TG pool therefore most likely reflects the rate of acyl chain transfer from membrane lipids. During deuterium labeling, the TG containing labeled PUFA was consistently 30-35% lower than in membrane lipids while GL and PL pools were essentially identical, which supports the conclusion that PUFAs in TG come from the membrane lipids in *C. subellipsoidea*. This is the

first study that demonstrates the transfer of intact acyl chains from membrane lipids into the TG pool in algae, specifically shown by tracking unlabeled FAs (prior to N starvation) and labeled FAs both before and during N stress.

Membrane lipids are an early sink for increasing FA synthesis induced by N stress

Changes in fatty acid synthesis during N stress followed a two-stage process with an initial 2.5-fold increase followed by a slow and linear decline. Our findings showing this early increase in FA synthesis is in disagreement with the generally accepted hypothesis that the time lag between the onset of N stress and TG accumulation is related to the partitioning of C between carbohydrate and lipid synthesis (52,53). Approximately two thirds of the lipidome-wide influx of newly synthesized FA moieties entered the membrane lipids in the first two days of N stress. The fine scale analysis of acyl chain incorporation into specific lipid classes revealed that this influx was spread across all membrane lipids, but only digalactosyl-diacylglycerol (DGDG) had a similar influx to TG after two days of N stress. This influx into DGDG could explain the disparity between monogalactosyl-diacylglycerol (MGDG) and DGDG degradation demonstrated previously (Allen 2015). The incorporation rates of the newly synthesized FAs into TG were shifted in time, peaking at 6 days of N stress, which was coincident with a sharp decline in the accumulation of newly synthesized acyl chains into membrane lipids. The time lag between the onset of N stress and the flux of acyl chains from membrane lipids into the TG pool likely reflects the upregulation of several phospholipases (54) and downregulation of membrane lipid synthetic genes predicted from transcriptomic studies comparing normal growth to N stress conditions (55,56).

Conclusions

Collectively, these data provide important mechanistic insights that show acyl chains are removed from PLs through two distinct mechanisms, the first includes the catabolism of glycerol and second, through specific acyl chain remodeling. Acyl chain remodeling in the PLs through an intermediate lyso-lipid, requires acyl-editing mechanisms that includes PDAT, the Land's Cycle, or LPCAT (8-10). Our findings measuring the quantity of intact pre-stressed acyl chains in the TG pool, the quantity of TG synthesized using glycerol by sequential acylation reactions, and showing the efficient removal of acyl chains from the PL pool demonstrate acyl-editing is a major source of acyl chains during stress induced TG synthesis. By comparison and in contrast, the predominant pathway involved in the trafficking of acyl chains from the GLs is accompanied by the degradation of the lipid in entirety. The recently characterized plant galactolipid galactosyltransferase Sensitive to Freezing 2 (SFR2) mediates the transfer of the galactose head group from one MGDG to another to form DAG and DGDG (57,58). GLs, especially MGDG, are prone to degradation in algae during N stress coincident with a reduction in chloroplast size and photosynthetic capacity (59,60). While *C. subellipsoidea* contains an SFR2 homolog and offers a plausible pathway for converting MGDG to TG, our labeling data shows this cannot be the case. As noted above nearly all of the newly synthesized TG contains newly synthesized glycerol while 20% of the acyl chains arise from the membrane lipid pool. There was acyl chain transfer from the GL pool, but no evidence of this proceeding through DAG. The Plastid Galactoglycerolipid Degradation 1 (PGD1) lipase, initially defined in *C. reinhardtii*, may contribute to TG synthesis through the hydrolysis of the acyl chain at the *sn*-1 position of glycerol in MGDG. However, the origins of the acyl chains in the expanding TG pools of *C. reinhardtii* likely differs significantly from *C. subellipsoidea* since the amount of 16C FA in the *sn*-2 position of the two species is approximately 80% and 10%, respectively (34,35). Our data demonstrates only a minor amount of acyl chain editing of the GLs in *C. subellipsoidea*. An uncharacterized mechanism is apparently active during N stress in *C. subellipsoidea* resulting in the transfer of acyl chains from membrane lipids coupled with the catabolism of the glycerol backbone, rather than via acyl-editing steps or lipid remodeling. Membrane lipids, including chloroplast-specific lipids, are instead continually synthesized and degraded during N starvation, leading to a general

flux of acyl chains through membranes to TGs. Since very little to no lipid head group remodeling was detected in this study, we believe that a viable strategy for enhancing lipid accumulation through genetic engineering may involve the introduction of reactions leading to DAG production from other complex lipids.

EXPERIMENTAL PROCEDURES

Growth conditions and stable isotopic labeling

General growth conditions

Coccomyxa subellipsoidea C169 was originally obtained from Dr. James VanEtten (University of Nebraska) and grown in Bold's Basal Medium either containing (BBM) or not containing an N source of 2mM sodium nitrate (N-BBM). Batch cultures were grown in photo-autotrophic conditions in 250mL Erlenmeyer flasks and 30mL of culture volume or otherwise varied volumes depending on the experimental parameters elaborated on below. Cultures were shaken at 120RPM and maintained at 25˚C and 60µmols photons $m^{-2}s^{-1}$ of light using a New Brunswick Innova 43 shaker. Transitioning algae from BBM to N-BBM involved centrifugation at 2500 X *g* for 5min, followed by removal of the BBM, a wash step in N-BBM to fully remove the N source, and finally suspending the washed cells in fresh, autoclave sterilized N-BBM. Bioreactor based experiments utilized a water jacketed 3L glass bioreactor (Applikon Biotechnology) in photo-autotrophic conditions (200µmols photons $m^{-2}s^{-1}$), bubbling with compressed air (330ppm CO_2 in 3L min^{-1}). The temperature (25˚C) and pH (6.6) were maintained using a circulating water bath and a set of peristaltic pumps automatically adjusting pH with 0.1M KOH and 0.1M HCl solutions based on continual input from a submerged pH probe. Initially 200mL of inoculum was grown for one week in 250mL shake flask cultures, then transferred to 1.8L of autoclave sterilized BBM and grown for one week before transferring the biomass to 2L of N-BBM.

Conditions of ^{13}C glucose labeling

Algal cultures were ^{13}C labeled with ^{13}C-U-glucose in BBM for one week and starved of N in the absence of label for one week to analyze transfer of pre-stress glycerol and acyl groups into the TG pool during lipid body development. Three cultures were initiated at 3 X 10^5 cells mL^{-1} in 30mL of BBM media and immediately provided 25mM ^{13}C-U-glucose by drop filtration. After one week, the cultures were split in half, one half collected by centrifugation and pellets stored immediately at -80˚C. Remaining cultures were washed of N and label by centrifuging, removing and replacing the supernatant with sterilized N-BBM, repeated twice. The cultures were re-suspended in 30mL N-BBM and incubated in a lighted shaking incubator as already described for one week, then collected by centrifugation and stored at -80˚C. Samples were lyophilized and dry weights measured prior to extraction as described below.

Conditions of D_2O labeling

Batch cultures were grown to early stationary phase and three cultures per time point transferred to N-BBM as described in 'General growth conditions' with the exception that the N-BBM experimental media was 30% by volume D_2O (Sigma Aldrich). Cultures were placed in a shaking lighted incubator and were collected every 2 days for a total of 8 days. A bioreactor culture was set up in 30% by volume D_2O N-BBM as described in the 'General growth conditions' section. 150ml samples were removed daily and split into three technical replicate samples of 50mL each. All samples were stored at -80˚C until they were lyophilized and weighed.

Conditions of $^{13}CO_2$ labeling

The method of $NaH^{13}CO_3$ labeling was adopted from Burrows *et al.* (61). Cultures were first grown in 100mL of BBM for 10 days in an Innova shaking incubator and transferred to N-BBM as described in 'General growth conditions'. These were added to clean, sterilized 250mL flasks and sparged with 0.22µm filtered N_2 gas for 10min to remove $^{12}CO_2$. A final concentration of 25mM $NaH^{13}CO_3$ was added to each flask and they were sealed with silicone stoppers to prevent gas exchange during the labeling period of one week. A parallel study was conducted using

unlabeled $NaHCO_3$ to assess the quantity of TG molecular species. Samples were taken immediately and 1, 3, and 7 days after the transfer to ^{13}C-labeled N-BBM, three cultures per time point. Samples were frozen and maintained at -80˚C until lyophilized and weighed prior to lipid extraction and lipid class separations as detailed below.

Analysis Methods

Lipid extraction and separation

Lipid extraction and separation methods were standardized for all experiments. Extractions were performed on lyophilized and weighed samples of algae using a modified version of the Bligh and Dyer method (62). *C. subellipsoidea* cell wall were disrupted prior to solvent extraction, performed using 0.7mm zirconia beads and a Qiagen Tissue-Lyser LT bead mill (50Hz for 5min). Extraction solvent (100µL; methanol/chloroform (2:1) containing 0.01% butylated hydroxytoluene (BHA)) was added with the necessary internal standards prior to milling. The disrupted samples were transferred to PTFE lined glass vials with 3mL of the extraction solvent and shaken using a vortex mixer for 1hr. 1mL of chloroform and 2mL of 0.08% aqueous potassium chloride was added and lipids partitioned by centrifugation (2,520 X *g*, 5 min). The organic layer was transferred to a fresh vial and sample dried at 40˚C under N_2 gas.

To separate neutral lipids, the crude lipid extracts were re-suspended in 1mL of methylene chloride and applied to a pre-equilibrated column containing 400mg of 200-450 mesh 60Å silica gel (Sigma-Aldrich); the neutral lipids were eluted with 2mL of methylene chloride. To separate the membrane lipids, the crude lipid extracts were re-suspended in 1mL of acetone/methanol (9:1) and applied to pre-equilibrated column containing 400mg of 200-450 mesh 60Å silica gel. The galactolipids were eluted using 2mL of the same solvent; PLs were then eluted with 3mL of methanol. The different lipid fractions were dried under an N_2 stream at 40˚C. The efficiency of lipid fractionation was routinely monitored using thin layer chromatography.

Fatty acid analysis using GC-MS

Fatty acid were analyzed as methyl esters (FAMEs) synthesized from the dried lipid extracts or directly from TLC scrapings and dried in fresh glass vials at 45˚C under N_2 gas as detailed previously (34). Hexanes (200µl) were added to the dried FAMEs, thoroughly mixed and 100µL transferred to GC vials containing 500µL inserts. The samples were analyzed using a SelectFAME column (Agilent Technologies; 200m x 271µm x 0.25µm) on an Agilent 7890A GC equipped with a 5975C VL MS triple axis detector. The initial oven temperature was 130˚C, held for 10min and successively ramped by 10˚C min-1 to 160˚C, 190˚C, 220˚C, and 250˚C incorporating holds of 7min, 7min, 22min, and 17min, respectively. The pressure and inlet temperatures were constant at 62.3psi and 250˚C. The FAME peaks corresponding to different fatty acids were identified by comparing fragmentation patterns with a standard NIST database and verified using a commercial standard mixture (37 FAME standard; Fisher Scientific). Quantification was accomplished using known amounts of internal standards.

Glycerol analysis using GC-MS

Esterified glycerol moieties were hydrolyzed from complex lipids during the initial saponification step of the FAME synthesis method detailed above. The aqueous phase of the solvent partitioning step after FAME synthesis was collected and evaporated to dryness under N_2 gas at 45˚C to isolate glycerol. The dried aqueous phase samples were lyophilized overnight prior to derivatization. Stable isotopic labeling in the glycerol moiety of TG, GL, and PL pools was then determined using a GC-MS method developed by Shen *et al*. (63). Glycerol samples were derivatized in sealed glass vials with 200µL of trimethylsilyl imidazole (TMSI; Sigma-Aldrich) and 50µL of acetonitrile for 45min at 60˚C. The samples were then dried under N_2 at room temperature, suspended in 100µL of hexane, transferred to fresh vials and separated in a 30m Agilent DB-5ms capillary column on an Agilent 7890A GC equipped with a 5975C VL MS triple axis detector. 5µL of each sample was injected with a 2:1 split flow of 6mL min^{-1} with a run time

of 16min with an inlet temperature of 280˚C and pressure set to 30psi. The initial oven temperature of 100°C was held for 3min followed by a 50˚C min-1 ramp to 250˚C, held for 10min. The column flow was held constant at 3mL min^{-1}. Percentage labeling was determined from MS scans averaged across the resulting total ion chromatographic peaks by comparison of the isotopic distributions of molecular ion signal intensities to unlabeled glycerol. The m/*z* 293 ion used for ^{13}C-labeling analysis is 15amu less than the intact molecular ion attributed to the loss of a methyl group from a TMSI moiety by comparative analysis of the theoretical and experimental isotopic distribution patterns of unlabeled glycerol.

Lipid quantification and analysis using LC-MS/MS

A SCIEX Q-Trap 4000 LC-MS/MS with a Shimadzu UFLC-XR system was used to quantify GL, PL, and TG pools after growth following isotopic labeling experiments as detailed above. An Agilent Poroshell 120 EC-18 4.6 x 50mm (2.7µm) column and an Agilent Eclipse Plus-C18 narrow bore 2.1 x 12.5mm (5µ) guard column (Agilent Technologies, Santa Clara, CA) were used to quantify mono-, di-, and tri-galactosyl-diacylglycerides and phosphatidylcholines as previously described (24). Neutral lipids were separated from total lipid extracts to reduce the possibility of spectral contamination by non-TG lipids. Catalytic saturation of the TG pool using platinum (IV) oxide and H_2 gas was also done as previously described (35), coalescing TG molecular species with equivalent FA chain lengths but varied unsaturation and removing confounding monoisotopic mass peaks. A binary solvent system consisting of methanol/1.6mM ammonium formate/0.7mM formic acid (solvent A) and chloroform (solvent B), and an Agilent Poroshell 120 EC-8 4.6 x 50mm (2.7µm) column equipped with a 4.6 x 5mm (2.7µm) guard column of the same type provided chromatographic separation. Solvent B was held for 1min at 7%, increased to 25% over 2min, 30% in 4min, then 7% in 1min and held 2min further. The column temperature was held constant at 40˚C. The MS settings and multiple reaction monitoring (MRM) used were the same as previously reported (35). MRM spectra were used to quantify TGs for the continuous batch culture deuterium labeling experiment and TG were run first to confirm spectral windows then used to inform Q1 MS analyses of isotopic distributions for the measurement of ^{13}C incorporation from $^{13}CO_2$.

FA synthesis and acyl chain incorporation into different lipid pools

Algae of various classes are long known to tolerate D_2O, even at concentrations reaching 100% (64), and its use in measuring FA synthesis rates in other organisms is established (65). The transfer of the label into FAs is tracked over time, the results graphed, and the synthesis rate measured as the slope of the resulting least-squares fitted line. Rate measurements were conducted by analyzing deuterium labeling in fatty acids converted to FAMEs using GC-MS after 0, 3, 6, 9, and 12hr of incubation with 20% D_2O. Rates were measured in TG, GL, and PL pools of algae undergoing a time course of N starvation and repletion to study changes in FA synthesis and allocation during lipid droplet production and catabolism. Cultures (n = 4) were N starved for a total of 14 days, at which point sodium nitrate (2mM) was added to initiate recovery from N stress. Rates were measured at 0, 1, 2, 4, 6, 10, and 14 days of growth in N-BBM, followed by 1, 2, and 4 days after N re-addition. Four 750mL batch cultures constituting biological repeats were initially grown for 2 weeks in BBM to produce cell biomass for the experiment. These were centrifuged and the pellets washed and re-suspended into four sets of ten 120mL N-BBM cultures housed in 250mL Erlenmeyer flasks kept sterile using plugs with inset 2µm pore size filters (Whatman Bugstopper venting closures). Due to the poor surface to volume ratio, the cultures were supplemented with 2.2mg (218µM) $NaHCO_3$ supplied once per day. The cultures were kept in an Innova shaking incubator (New Brunswick) at 120rpm, 25˚C, and 60µmols photons $m^{-2}s^{-1}$ and OD measurements taken daily at 550nm. Rate time courses were initiated by removing 4 biological replicate cultures from the N-starvation/repletion time course and transferring 20mL from each into 4 sterilized 250mL flasks containing 5mL of D_2O (99%; Sigma-Aldrich) in each and collecting

20mL for the zero time point. These flasks were placed back into the original shaking incubator through the labeling time course. Four biological replicates were removed at specified time points, collected by centrifugation, and the pellets frozen in liquid N_2. Samples were lyophilized overnight at the conclusion of each rate time course. Lipid extractions, separations of lipid classes, conversion to FAMEs, and GC-MS analysis were done using techniques already described.

Statistical analysis

Data presented are from experiments done in 3-4 times (biological replicates) as indicated; values are shown ± standard deviation. ANOVA was used for pairwise comparisons.

Acknkowedgements

This work was supported by grants from the National Science Foundation (EPS-1004094 to PNB and CCD and 1264409 to CCD) and the Nebraska Center for Energy Sciences Research (to PNB).We would also like to thank Drs. Paul Blum and Rahul Tevatia for bioreactor use and assistance.

Coflicts of interest

The authors declare no conflicts of interest.

Author Contributions

JWA and PNB developed the conceptual basis of the study; JWA completed the experiements detailed in the study; JWA, PNB and CCD analysed the data; JWA, CCD and PNB wrote the manuscript.

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Table 1. Percent of C from *de novo* synthesized FAs derived from the catabolism of pre-stress biomolecules

	16C FA	18C FA
Triglycerides	11.8 ± 0.44%	10.4 ± 0.23%
Galactolipids	9.0 ± 0.78%	9.4 ± 0.38%
Phospholipids	9.1 ± 0.35%	9.0 ± 0.27%

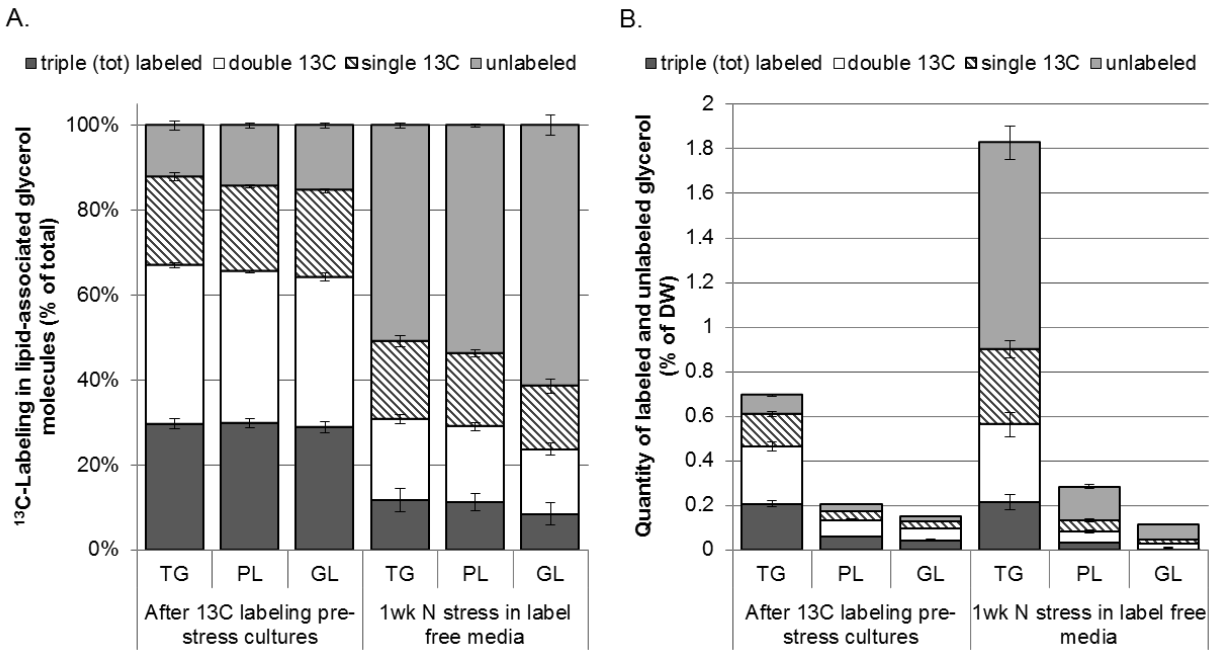

Figure 1. Relative and absolute quantities of ^{13}C-labeled glycerol from complex lipid hydrolysis after labeling with ^{13}C-U-glucose during stress-free growth and after one week of label-free N starvation. Data is shown for the glycerol moieties of three lipid classes separated by polarity, triglycerides (TG), galactolipids (GL), and phospholipids (PL) after pre-labeling with ^{13}C and a subsequent one week of growth in label-free N-free media. Isotopic distributions from GC-MS analysis of derivatized glycerol were used to generate the amount of unlabeled, single ^{13}C labeled, double ^{13}C labeled, and totally labeled glycerol as (A) a percentage of the total quantity and as (B) a percentage of the extracted dry weight of algae. Error bars represent the SD, n = 3.

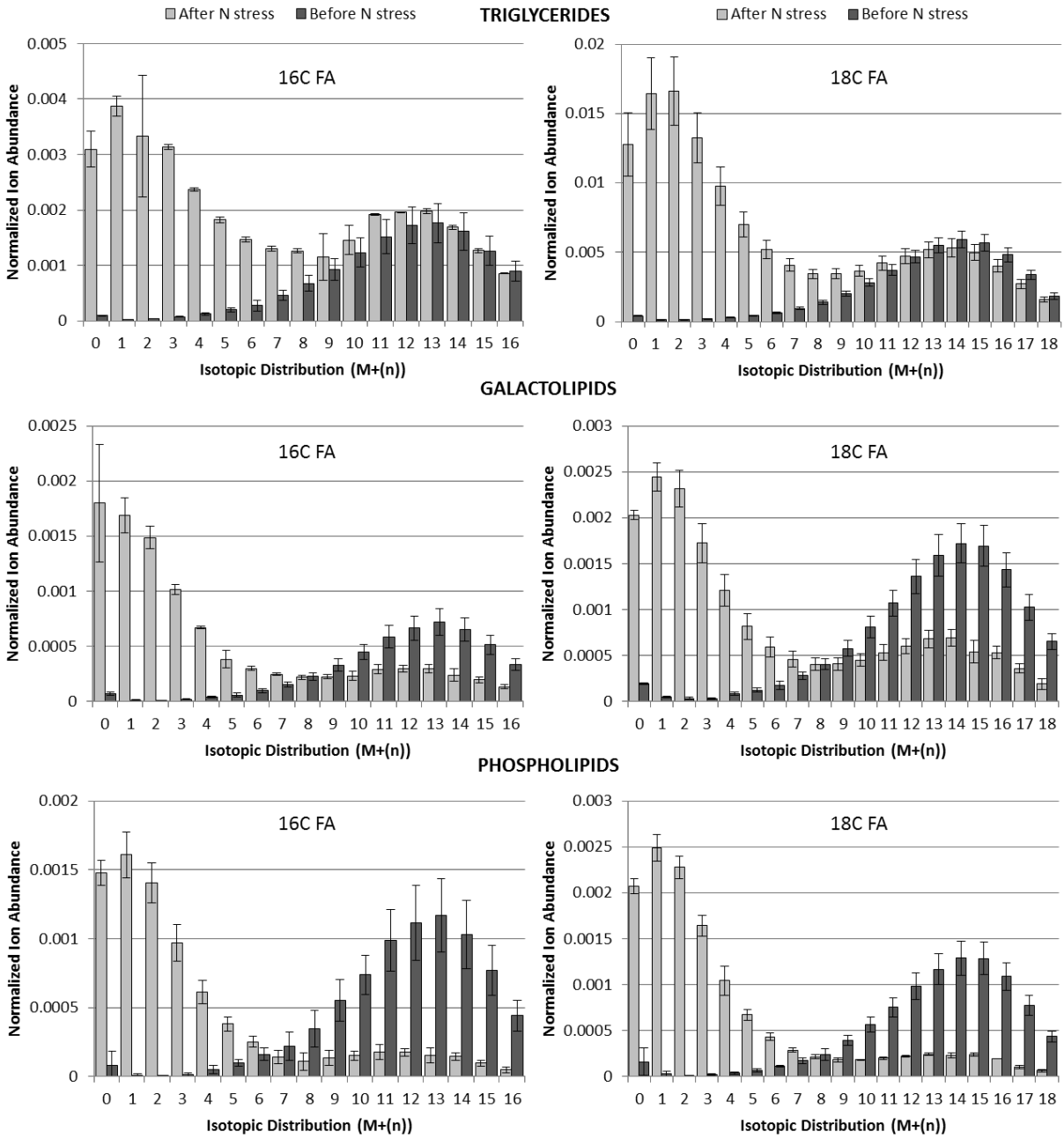

Figure 2. ^{13}C isotopomer distributions of catalytically saturated fatty acids from isolated lipid pools. FA labeling was analyzed by GC-MS from *C. subellipsoidea* labeled with ^{13}C-U-glucose for one week (before N stress) and after one week of N-starvation (after N stress). Lipid extracts were separated into triglyceride, galactolipid, and phospholipid pools by column chromatography, then saturated before conversion to methyl esters and analysis. Fatty acid isotopomers are normalized by monoisotopic mass (M) and their distribution as M+(n) where n is equal to the number of incorporated ^{13}C atoms. Error bars represent the SD of three replicate cultures.

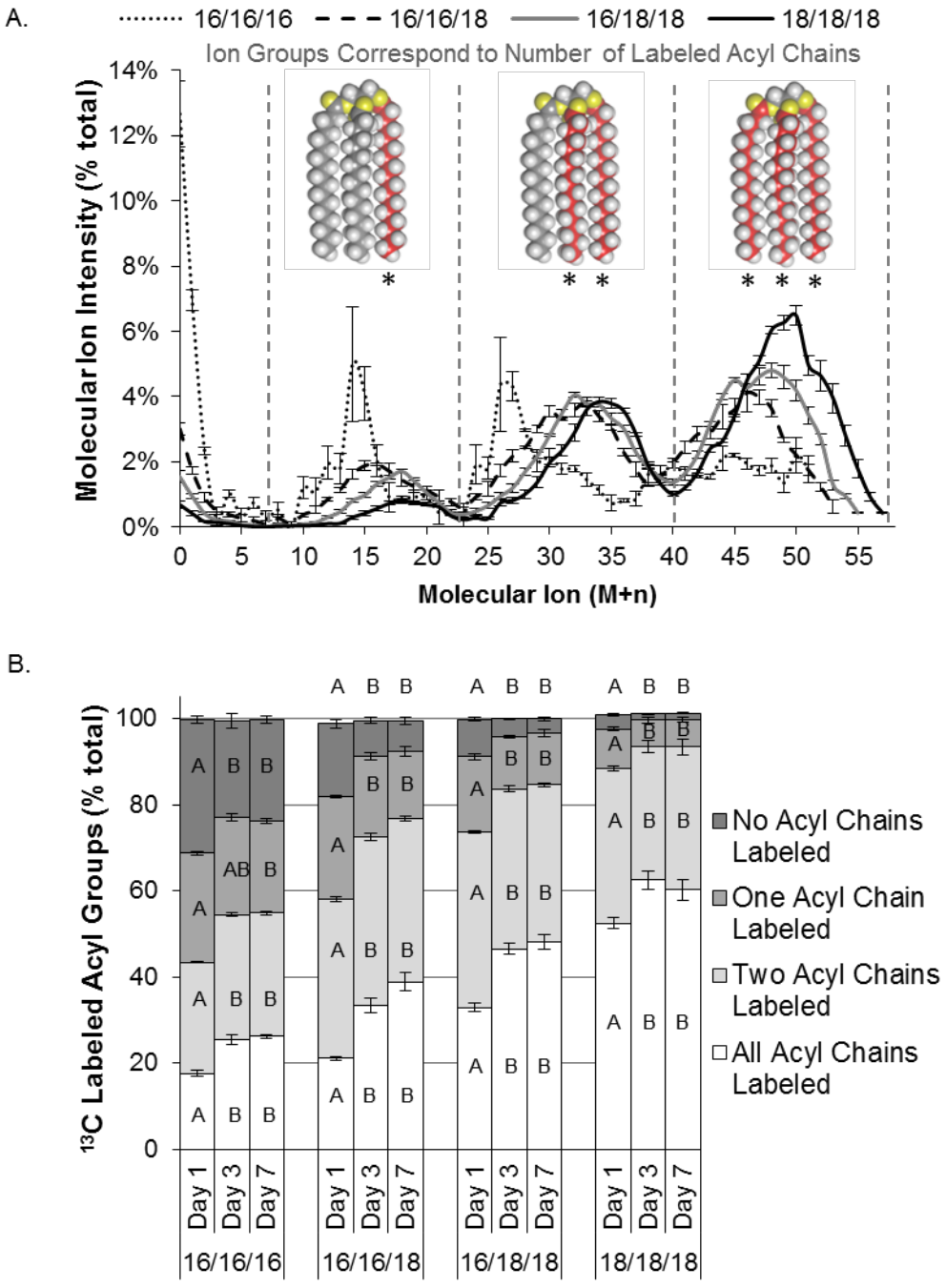

Figure 3. Incorporation of ^{13}C-labeled and unlabeled acyl chains in TAG molecular species from $^{13}CO_2$ labeling. Cultures of *C. subellipsoidea* were labeled with 100% ^{13}C-bicarbonate in N-free media with samples removed on days 1, 3, and 7. (A) Molecular ion intensities showing

peak distributions corresponding to TAG molecules with one, two, or three labeled fatty acyl groups (indicated by asterisks under space filling models of tri-stearin) after 7 days of ^{13}C labeling. (B) The sum of ion intensities within each peak representing molecules with three, two, one, or no labeled acyl chains were calculated relative to the total for each TG molecular species (% total). Error bars represent the SEM; statistical significance was determined by ANOVA one-way analysis and significant differences are shown here as A and B, whereas AB is not significantly different from A or B, n = 3.

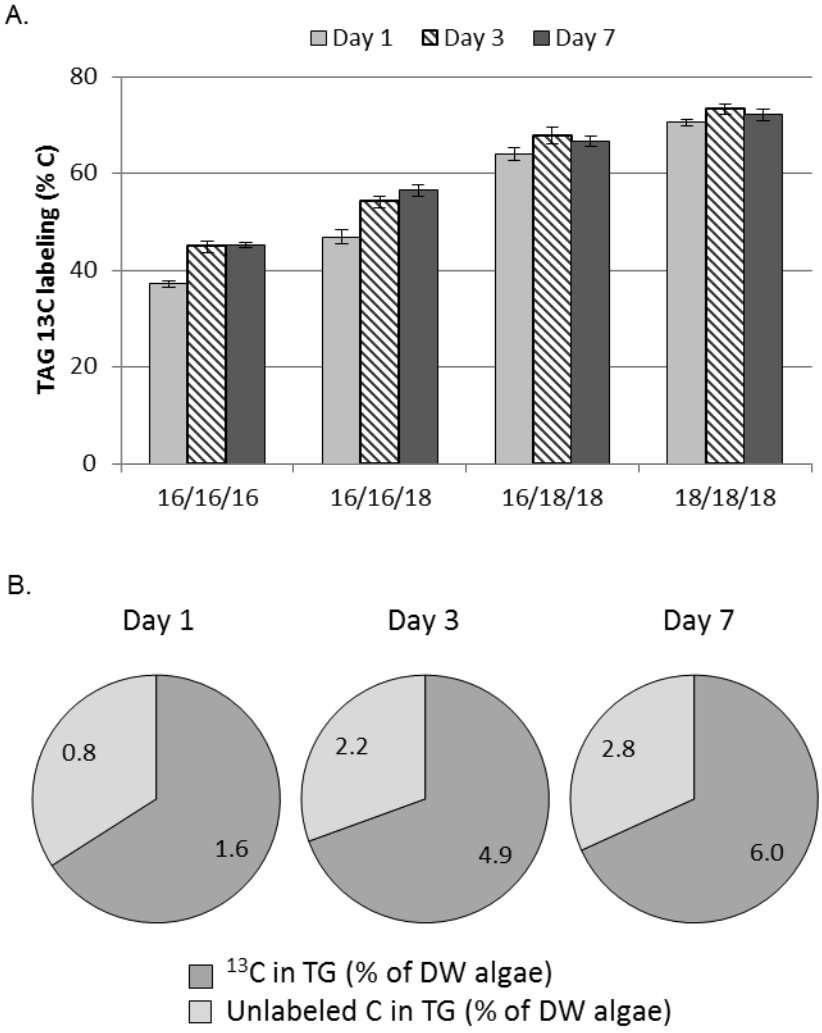

Figure 4. ^{13}C-labeling from $^{13}CO_2$ of TG molecular species during a time course of N starvation. Labeled TG molecular species were chromatographically separated, isolated and ^{13}C content measured by LC-MS. Data is shown as (A) ^{13}C labeling in TG molecular species and (B) the relative amount of ^{13}C and unlabeled ^{12}C in the total TG pool. Numbers indicate the quantity of ^{13}C (dark gray) and ^{12}C (light gray) in the TG pool as a percentage of the total dry weight of algae. Error bars represent the SD, n = 3.

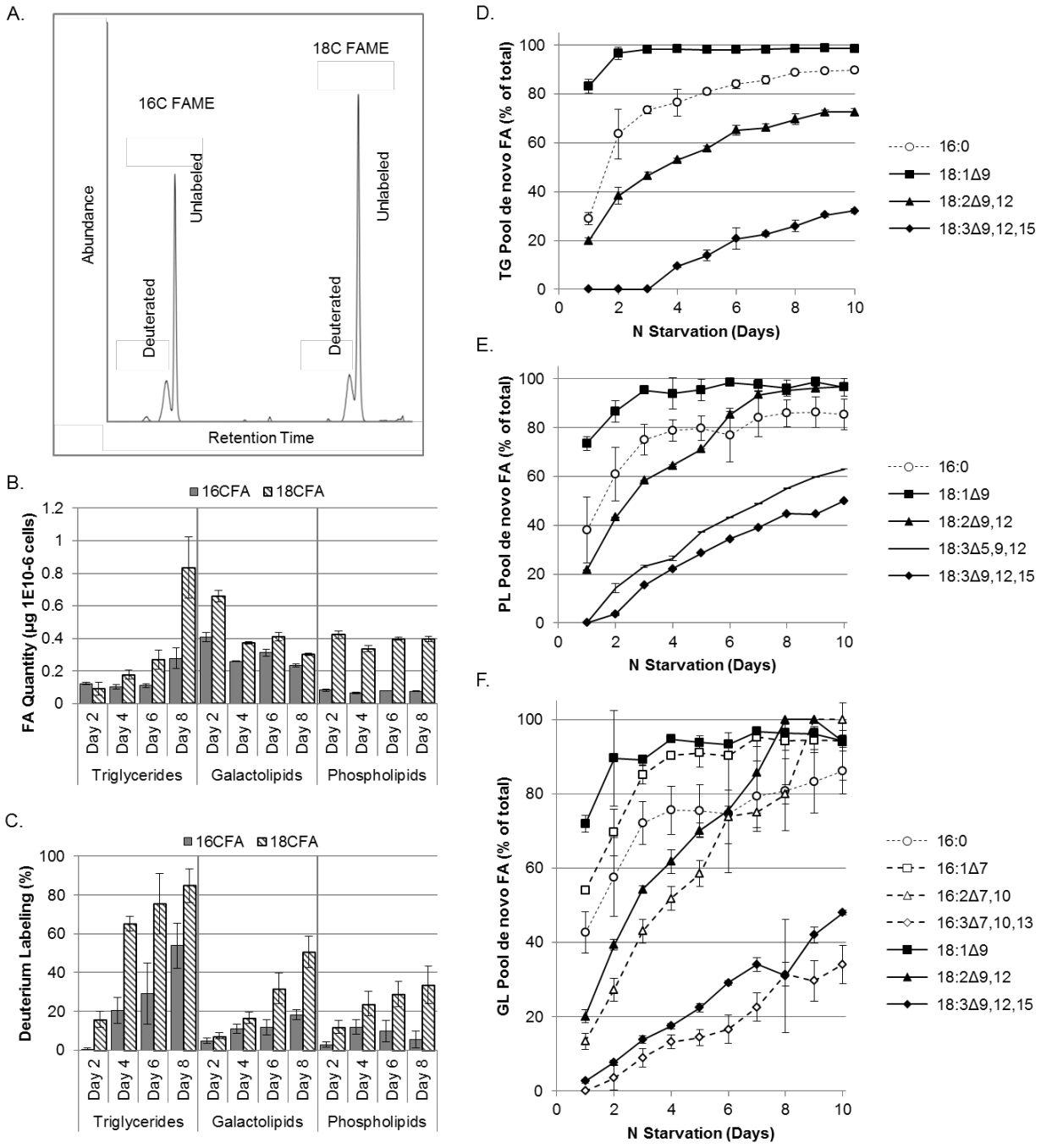

Figure 5. Deuterium labeling of membrane and storage lipid fatty acids during N starvation. (A) GC-MS chromatogram of hydrogenated, deuterium labeled FAMEs demonstrating the shift in retention time. (B-C) Batch cultures were labeled with 30% D_2O in N-free medium and lipids analyzed every 2 days for a total of 8 days. Lipids were extracted, class separated using silica columns, catalytically hydrogenated, then converted to fatty acid methyl esters. (B) Quantities of triglyceride, galactolipid, and phospholipid pools were measured by LC-MS/MS from parallel-grown unlabeled cultures. (C) The percent labeling was additionally measured by GC-MS analysis. Standard errors were calculated from five independent cultures

per condition. (D-F) *C. subellipsoidea* was labeled with 30% D_2O in N-free medium and grown in a bioreactor controlling light, temperature, gas availability, and pH. Samples were taken daily, lipids extracted and separated into (D) neutral, (E) galactolipid, and (F) phospholipid classes by silica column. FAMEs were generated and the percentage of labeled fatty acid measured by GC-MS from technical triplicate samples. Error bars represent the SD, n = 3.

Allen et al. Figure 6

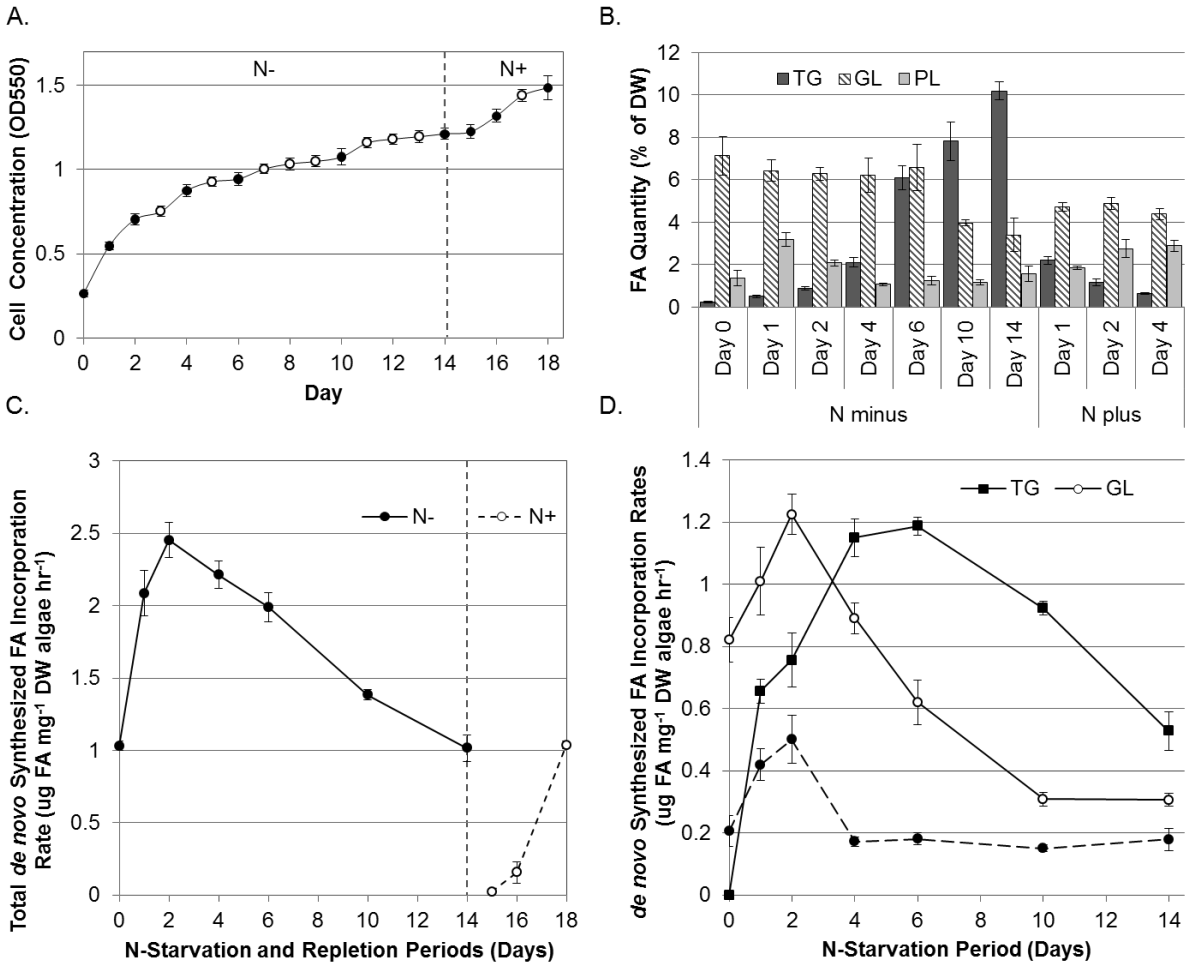

Figure 6. Incorporation rates of *de novo* synthesized acyl chains into major lipid classes over a time course of N-starvation and recovery. Cultures of *C. subellipsoidea* labeled with D_2O on days 1, 2, 4, 6, 10, and 14 of an N starvation time course and the rate of labeling in the FA's assessed after 3, 6, 9, and 12hr to determine the rate of incorporation. Samples were extracted and separated into triglycerides (TG), galactolipids (GL), and phospholipids (PL) by column chromatography, and the methyl esters analyzed by GC-MS. (A) OD was measured once daily over the 2 week N-starvation time course. Nitrate was added to the cultures on day 14 and the results analyzed over the subsequent 4 days. Closed circles indicate FA synthesis rate measurement time points. (B) The total FA quantity was analyzed for the major lipid pools assessed (triglycerides TG; galactolipids GL; phospholipids PL). (C) The total FA incorporation rate into all lipid classes analyzed was assessed as a measurement of FA synthetic rates over the time course. (D) Incorporation of *de novo* synthesized FA into the lipid pools analyzed. Error bars represent the SD, n = 4 independent cultures.

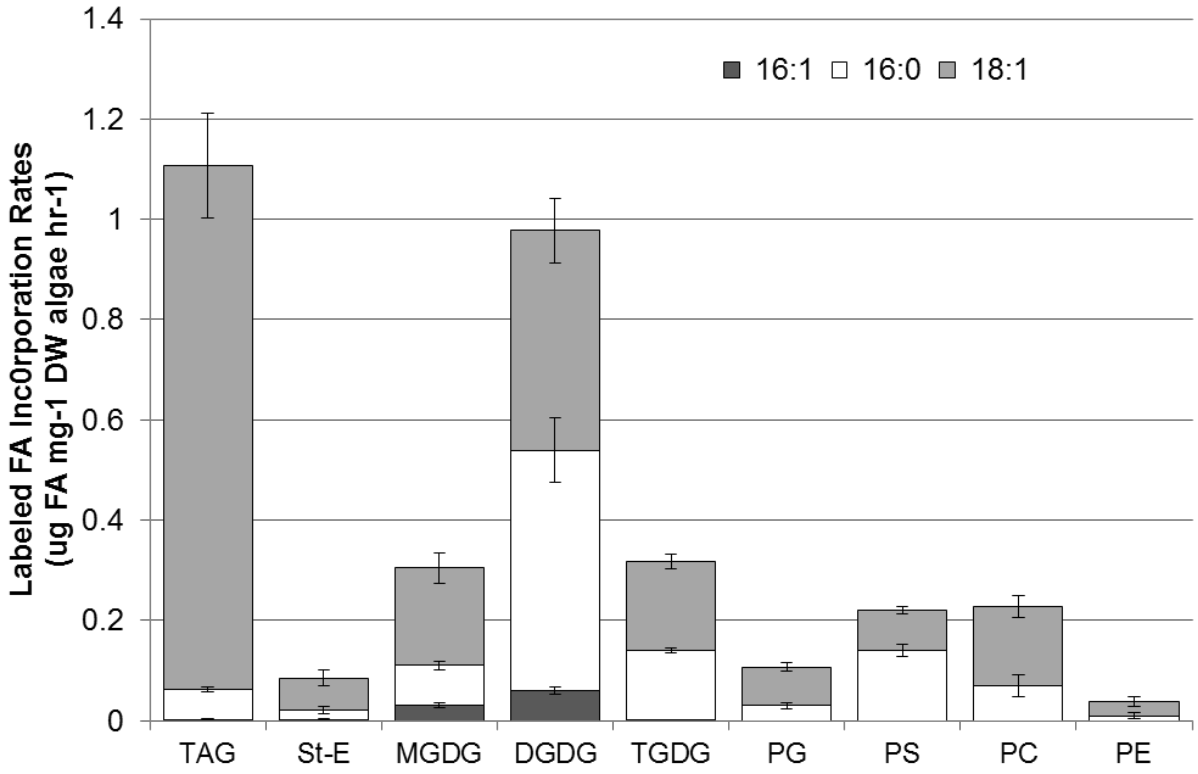

Figure 7. Incorporation rates of *de novo* synthesized FA in nine lipid classes after two days of N starvation. Cultures were labeled with D_2O after two days of N stress and acyl chain labeling analyzed in the lipidome at 3, 6, 9, 12, and 24hr to determine incorporation rates. Lipid extracts were separated by TLC before synthesis of methyl esters and GC-MS analysis of triglycerides (TAG), sterol esters (St-E), monogalactosyldiacylglycerides (MGDG), digalactosyldiacylglycerides (DGDG) trigalactosyldiacylglycerides (TGDG), phosphatidylglycerols (PG), phosphatidylserines (PS), phosphatidylcholines (PC), and phosphatidylethanolamines (PE). (A) Rates of incorporation of *de novo* synthesized oleoyl (18:1), palmitoyl (16:0), and palmitoleoyl (16:1) acyl chains into specified lipid classes. Error bars represent the SD, n = 4 independent cultures.

Carbon and Acyl chain flux during stress-induced triglyceride accumulation by stable isotopic labeling of the polar microalga *Coccomyxa subellipsoidea* C169

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J. Biol. Chem. published online November 30, 2016