ABSTRACT

The Mass Spectrometry Analysis of *Chlamydomonas reinhardtii* During Nitrogen Starvation

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Chlamydomonas reinhardtii is a soil microalgae that has been found to produce useful biopharmaceutical and biofuel material. It is known that mutants of *Chlamydomonas reinhardtii* produce an abundance of lipid compounds during nitrogen starvation. This project aims to identify possible sources of energy for cellular processes and use mass spectrometry and carbon thirteen isotope labeling to characterize the process of lipid production during nitrogen starvation. To conduct this research, the sta6 mutant of *Chlamydomonas reinhardtii* was first grown in unlabeled acetate for 120 hours followed by nitrogen starvation in [1-^{13}C] labeled acetate for 72 hours. Mass spectrometry analysis of algal headspace at 24, 48, and 72 hours post-starvation shows that *Chlamydomonas reinhardtii* utilizes the labeled acetate to obtain carbons from outside the cell and incorporate them into fatty acid tails. Moreover, mass spectrometry characterization of post-starved algal contents revealed unique isotopic distributions for observed diacylglycerols and triacylglycerols. Comparisons of results from mass spectrometry analysis with published transcriptome and pathway analyses allows the identification of key enzymes involved in driving the lipid production during nitrogen starvation.

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THE MASS SPECTROMETRY ANALYSIS OF *CHLAMYDOMONAS REINHARDTII* DURING NITROGEN STARVATION

A Thesis Submitted to the Faculty of

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By

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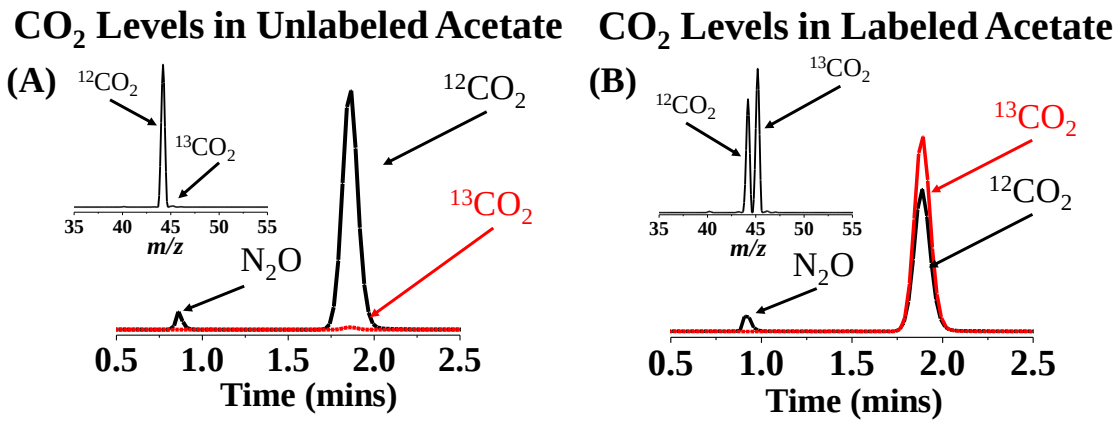

Figure 1: Chromatogram A displays the $^{12}CO_2$ and the $^{13}CO_2$ produced by the algae sample in the control group with its corresponding mass spectrum. Chromatogram A represents the $^{12}CO_2$ and the $^{13}CO_2$ produced by the algae in the experimental group with its corresponding mass spectrum.

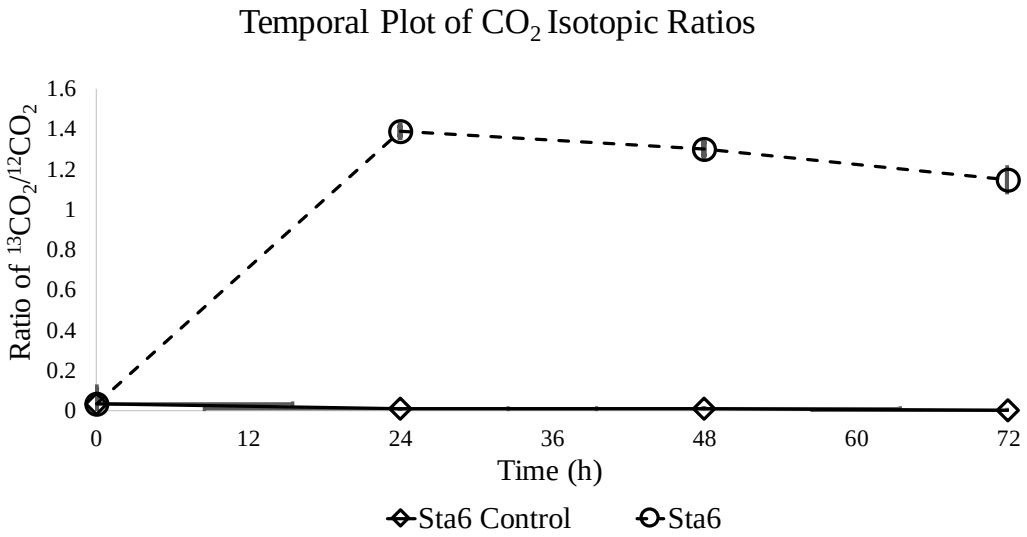

Figure 2: The ratio of $^{13}CO_2$ to $^{12}CO_2$ at time points 0h, 24h, 48h, and 72h for the control algae cultures and the experimental algae cultures done in triplicate. Error bars are reported at 95% confidence intervals for three independent systems each measured in triplicate.

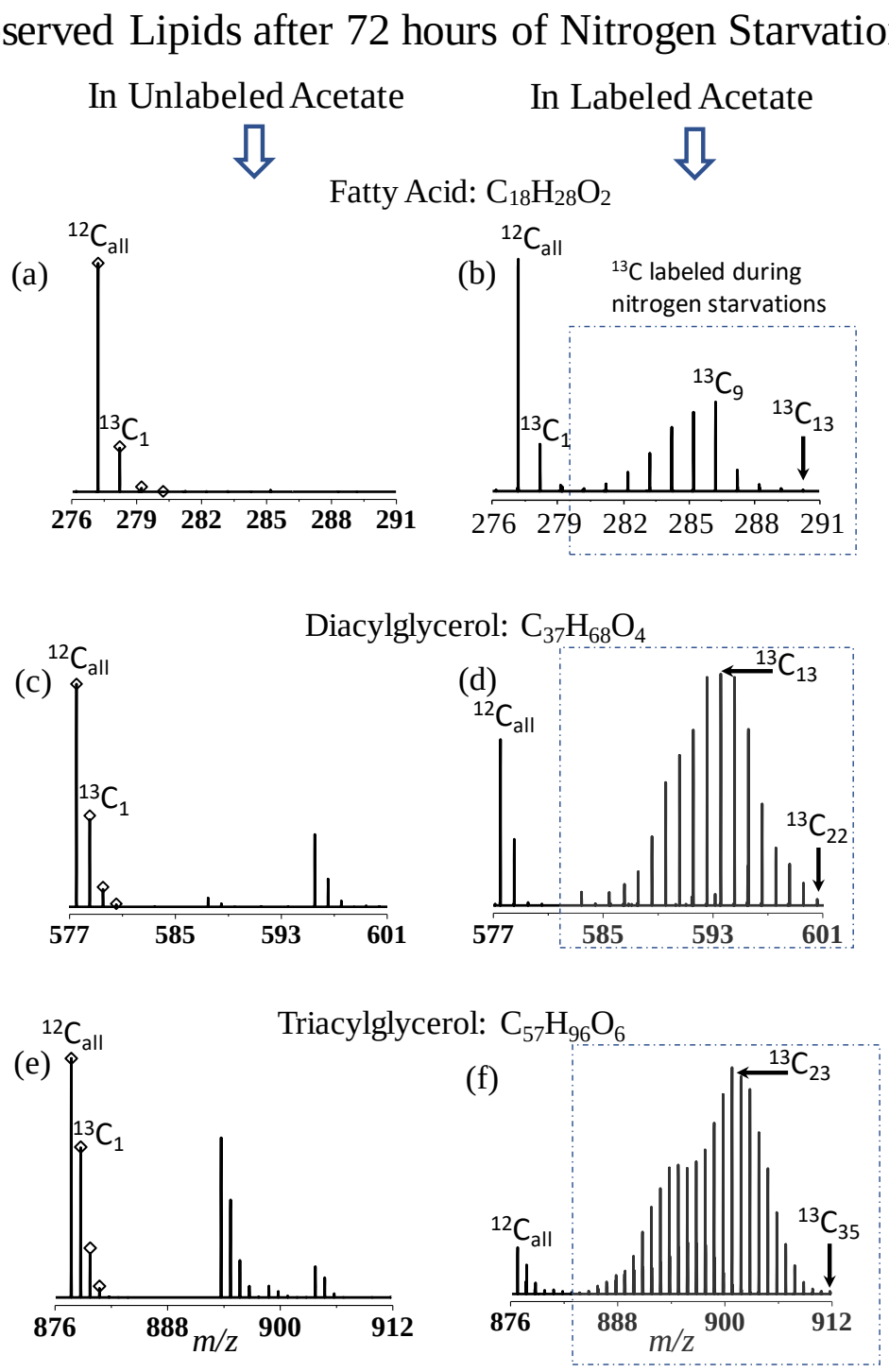

Figure 3: Lipids corresponding to a) fatty acid tail 18:4 (*m/z* 277.2163), diacylglycerol 37:3 (*m/z* 577.5193), and triacylglycerol 57:7 (*m/z* 877.7260) synthesized in algae grown in unlabeled acetate before nitrogen starvation then grown in unlabeled acetate (left) and [1-^{13}C] labeled acetate (right) during nitrogen starvation. Simulated isotopic distributions for unlabeled species are shown as diamonds (◊) in the left column.

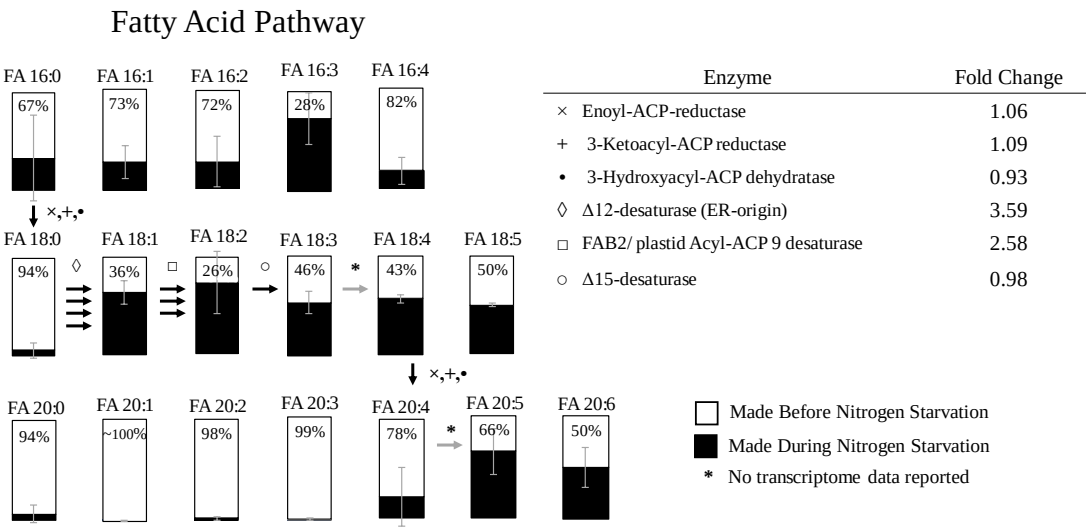

Figure 4: Relative intensities of fatty acids made before (white) and after (black) nitrogen starvation. Error bars are reported at the 90% confidence interval (n=3). Arrows represent previously reported fatty acid synthesis steps. Steps with published transcriptome results are represented as black arrows (steps not reported in transcriptome results shown as gray). A greater number of black arrows represent increasing up-regulation of RNA transcripts coding for requisite enzymes at each step.

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Table 1 Fatty Acids Found

Formula	Elem. Comp.	Observed *m/z*	Calculated *m/z*	Error (ppm)	Lipids Made BNS	Lipids Made DNS	90% Conf. Int.
FA 16:0	$C_{16}H_{33}O_2$	257.2477	257.2475	0.62	66.83%	33.17%	44.27%
FA 16:1	$C_{16}H_{31}O_2$	255.2318	255.2319	0.16	72.94%	27.06%	16.46%
FA 16:2	$C_{16}H_{29}O_2$	253.2162	253.2162	0.07	71.96%	28.04%	25.57%
FA 16:3	$C_{16}H_{27}O_2$	251.2006	251.2006	0.03	27.66%	72.34%	25.59%
FA 16:4	$C_{16}H_{25}O_2$	249.1849	249.1849	0.13	82.41%	17.59%	13.78%
FA 18:0	$C_{18}H_{37}O_2$	285.2787	285.2788	0.39	94.42%	5.58%	7.91%
FA 18:1	$C_{18}H_{35}O_2$	283.2633	283.2632	0.34	35.93%	64.07%	11.81%
FA 18:2	$C_{18}H_{33}O_2$	281.2476	281.2475	0.21	35.76%	64.24%	14.54%
FA 18:3	$C_{18}H_{31}O_2$	279.2319	279.2319	0.14	46.19%	53.81%	11.11%
FA 18:4	$C_{18}H_{29}O_2$	277.2163	277.2162	0.25	43.45%	56.55%	4.30%
FA 18:5	$C_{18}H_{27}O_2$	275.2007	275.2006	0.35	50.33%	49.67%	1.88%
FA 20:0	$C_{20}H_{41}O_2$	313.3101	313.3101	0.05	94.19%	5.81%	8.67%
FA 20:1	$C_{20}H_{39}O_2$	311.2944	311.2945	0.06	99.98%	0.02%	0.03%
FA 20:2	$C_{20}H_{37}O_2$	309.2788	309.2788	0.19	98.30%	1.70%	1.96%
FA 20:3	$C_{20}H_{35}O_2$	307.2631	307.2632	0.24	99.60%	0.40%	0.86%
FA 20:4	$C_{20}H_{33}O_2$	305.2471	305.2475	1.41	78.08%	21.92%	29.64%
FA 20:5	$C_{20}H_{31}O_2$	303.2317	303.2319	0.54	34.44%	65.56%	22.52%
FA 20:6	$C_{20}H_{29}O_2$	301.2162	301.2162	0.12	49.87%	50.13%	19.11%

Table 2 Diacylglycerols Found

Formula	Elem. Comp.	Observed *m/z*	Calculated *m/z*	Error (ppm)	Lipids Made BNS	Lipids Made DNS	90% Conf. Int.
DG 34:3	$C_{37}H_{69}O_4$	577.5194	577.5190	0.59	12.81%	87.19%	6.43%
DG 34:4	$C_{37}H_{67}O_4$	575.5035	575.5034	0.14	7.60%	92.40%	16.02%

Table 3 Triacylglycerols Found

Formula	Elem. Comp.	Observed *m/z*	Calculated *m/z*	Error (ppm)	Lipids Made BNS	Lipids Made DNS	90% Conf. Int.
TG 52:4	$C_{55}H_{99}O_6$	855.7412	855.7436	2.83	4.03%	95.97%	5.82%
TG 52:5	$C_{55}H_{97}O_6$	853.7255	853.7280	2.90	4.14%	95.86%	5.29%
TG 52:6	$C_{55}H_{95}O_6$	851.7100	851.7123	2.71	2.68%	97.32%	4.50%
TG 52:7	$C_{55}H_{93}O_6$	849.6941	849.6967	3.08	1.70%	98.30%	3.15%
TG 52:8	$C_{55}H_{91}O_6$	847.6786	847.6810	2.88	1.23%	98.77%	2.52%
TG 52:9	$C_{55}H_{89}O_6$	845.6629	845.6654	2.94	0.83%	99.17%	1.52%
TG 52:10	$C_{55}H_{87}O_6$	843.6474	843.6497	2.75	5.00%	95.00%	6.27%
TG 54:5	$C_{57}H_{101}O_6$	881.7568	881.7593	2.82	3.26%	96.74%	3.06%
TG 54:6	$C_{57}H_{99}O_6$	879.7411	879.7436	2.88	3.07%	96.93%	3.56%
TG 54:7	$C_{57}H_{97}O_6$	877.7256	877.7280	2.71	2.03%	97.97%	2.75%
TG 54:8	$C_{57}H_{95}O_6$	875.7096	875.7123	3.11	1.52%	98.48%	2.36%
TG 54:9	$C_{57}H_{93}O_6$	873.6941	873.6967	2.91	1.79%	98.21%	3.14%
TG 54:10	$C_{57}H_{91}O_6$	871.6785	871.6810	2.94	1.29%	98.71%	1.67%
TG 56:7	$C_{59}H_{101}O_6$	905.7567	905.7593	2.82	5.27%	94.73%	7.97%
TG 56:8	$C_{59}H_{99}O_6$	903.7413	903.7436	2.57	0.85%	99.15%	1.37%
TG 56:9	$C_{59}H_{97}O_6$	901.7255	901.7280	2.78	5.04%	94.96%	9.16%

CHAPTER ONE

Introduction

1. Overview

This thesis aims to learn more about the microalgae *Chlamydomonas reinhardtii* (*C. reinhardtii*) during nitrogen starvation. *C. reinhardtii* is a microalgae that is frequently used as a model organism.[1] It is known that *C. reinhardtii* produces a large quantity of lipids when starved of nitrogen containing compounds that are suitable for biofuel.[2] The data analysis provided in this work aims to provide insight into the lipid response of this microalgae during nitrogen starvation. Chapter one provides information relating to this project such as key biological concepts, relevant instrumentation, and previous data collected on *C. reinhardtii*. Chapter two includes information on the experimental procedure used to collect the data. Chapter three contains data and analysis on a possible source of energy utilized during nitrogen starvation. Chapter four includes the lipid compounds made by *C. reinhardtii* during nitrogen starvation and provides an explanation for this response.

2. Relevant Biological Science Topics

Biology is a field of study that encapsulates a variety of topics such as population genetics,[3] organism development,[4] and animal behavior.[5] To learn about the science of life, biologists study a variety of organisms from the smallest prokaryotic cells[6] to the largest mammals.[7] Research scientists often look at the chemistry of the cell to understand larger biological concepts such as protein function,[8] phenotype expression,[9] or even

behavioral interactions between organisms.[10] Study of the chemistry of biology or biochemistry has provided a deeper understanding of the ways biological materials can be used for medical, commercial, and industrial applications.

Biochemistry is a field of study that covers vast areas and topics such as the structural analysis and quantification of proteins,[8] the categorization of lipid compounds,[11] and the sequencing of the human genome.[12] In all of these areas of biochemistry, it is crucial to understand the complex chemical processes involved in cellular functions and hence characterization of different molecules in biological material is very important aspect of all research studies. Biological materials composed in the cell, such as proteins, lipids, and nucleic acids can be analyzed with modern instruments (*e.g.*, using nucleic acid sequencers,[13] mass spectrometers,[14] and nuclear magnetic resonance instruments[15]). Analytical data collected from modern instruments can be interpreted to learn about hidden molecular interactions involved in biochemical processes (*e.g.*, data from mass spectrometers lead to fragmentation pathway predictions of phospholipids[16] or x-ray crystallography provides structural information about proteins, such as Heme oxygenase,[8] *etc.*). Modern instruments, such as high resolution mass spectrometry[17] have also been utilized to identify of key compounds synthesized by organisms including bacteria,[18] yeast,[19] and algae.[20]

3. Background on Commercial Uses for Algae

Microalgae are a group of diverse organisms that exist in many different biomes on earth.[21] Many algae, such as *C. reinhardtii*, have had their genome fully sequenced.[22] Moreover, many of their genes that encode specific proteins associated with the function of organelles (*e.g.*, chloroplasts and mitochondria) have been identified.[23] In addition,

genomic and biochemical information have been used to identify the pathways of primary metabolism in algae such as *C. reinhardtii*.[24] Because these microalgae are ubiquitous and have been extensively studied, they have become excellent model organisms for research and experimentation.[1]

Because algae products (such as proteins, lipids, vitamins, *etc.*) can be used in a variety of commercial applications,[25] there is a significant interest in understanding the cellular compositions of algae at the molecular level. For instance, algae produce proteins, lipids, and vitamins[25] that can be used for human consumption as well as animal feedstock.[26] Furthermore, lipids produced by algae can serve as a renewable, commercial source of biofuel.[2, 27] Algae have been shown to grow in infertile and/or "undesirable" environments (such as waste water),[28] grow year-round, and produce non-toxic biofuel reserves.[29] Although algae can synthesize lipids suitable for use as biofuels, commercialization of algae as a fuel source has experienced limited success.[2]

4. Carbon Metabolism in Algae (CO_2 and Lipids)

Carbon metabolism provides energy for many organisms such as algae[30] and humans.[31] The carbon used for energy enters the Citric Acid Cycle (*i.e.* Krebs Cycle) in the form of pyruvic acid and is released as CO_2.[32] Organisms use the energy provided by the release of CO_2 to transfer electrons to molecules such as NADH.[33] The electrons are transferred from NADH to the electron transport chain in the mitochondria to produce ATP.[33] The ATP produced from the Krebs Cycle and the electron transport chain is used to carry out cellular processes such as enzyme function[34] and lipid efflux through membranes.[35] Hence, measurement of CO_2 output could provide important clues about its source. For instance, the CO_2 produced from organisms, such as mice, has been traced

through biochemical pathways by using carbon labeling techniques.[36] As we will discuss in chapter 3, carbon labeling can be utilized to trace carbon through various potential biochemical pathways. In addition to incorporation of labeled carbons (*e.g.*, carbon thirteen or ^{13}C) into the Krebs Cycle, as discussed in chapter 4, they can be incorporated into fatty acids, diacylglycerols, and triacylglycerols.

Lipids encompass a wide variety of compounds that have been described as hydrophobic molecules that are made from carbon sources in a living cell.[37] Some common lipids include sterol lipids, glycerolipids, sphingolipids, and fatty acyls.[37] Many of these lipids make up the bilayer of cell membranes,[38] and can form into structures such as lipid rafts, which can be used for cell membrane transport.[39] Lipids such as diacylglycerols can serve as signaling molecules for hepatic insulin resistance in people with diabetes.[40] In many organisms such as birds, lipids can also function as energy storage molecules.[41]

Lipid pathways have been identified in many organisms to understand how lipids are metabolized.[42] Fatty acid pathways have been identified to study the production of biofuel in algae,[2] phospholipid pathways in membrane damaged bacteria have been utilized to understand membrane generation,[43] and sphingolipid pathways have been studied to learn about obesity, cardiovascular disease, and diabetes in humans.[44] Understanding these lipid pathways can provide insight to address medical problems such as how diacylglycerols signal insulin resistance in humans.[45] Many lipid pathways that are found have been inserted into a KEGG database.[46]

Lipid production pathways in algae have been found to provide information on how algae make lipids such as fatty acids[42] and triacylglycerols.[2] These pathways give insight into how algae make polyunsaturated lipids that can be used for supplements[26a] and

triacylglycerols that can be used for biofuel production.[47] Manipulation of these algae pathways can increase lipid production so that these lipids can be used for commercial applications.[48] With the help of modern instrumentation and recent findings in the literature, it may be possible to maximize the number of lipids synthesized in a microalgae system and use them for human nutrition and/or other commercial applications.

5. High Performance Instruments Utilized in This Study

Modern instrumentation can help with the analysis of small molecules such CO_2 in complex sample mixtures. Gas Chromatography (GC) is a useful technique for separating multiple species in a gaseous sample.[49] The molecules separated by the GC technique can be transported through a stationary phase in a column by a gaseous mobile phase.[50] GC is useful for separating and identifying small molecules such as carbon dioxide, methane, and nitrous oxide.[49] The analytes separated by GC can be analyzed by a variety of detectors such an Atomic Fluorescence detector,[51] a flame ionization detector (FID),[52] a micro-discharge photoionization detector,[53] and a micro electron capture detector.[54] Time of Flight-Mass Spectrometry is another commonly used instrument for the detection of gas molecules that elute from a Gas Chromatograph.[55] In GC experiments, separated analytes present in a gaseous sample mixture can be accurately classified[55b] and quantified.[56]

High Performance Liquid Chromatography (HPLC) is a useful technique developed to separate multiple species in a liquid sample.[57] The molecules separated by the HPLC technique are transported through a stationary phase in a column by a liquid mobile phase.[58] Each compound elutes through the column at a different time depending on the polarity of the analyte and the polarity of the solvent.[58] The analytes in the sample

separated by HPLC can be analyzed by a variety of instruments including a fluorescence detector,[59] an ultraviolet detector,[60] a diode-array detector,[61] or an electrospray ionization mass spectrometer.[62] In HPLC experiments, separated analytes in a liquid sample mixture can be accurately classified[63] and quantified.[64]

Electron ionization is a technique that strips an electron from a neutral molecule upon impact with a filament.[65] It is a technique used to ionize gas molecules so that their mass to charge ratio can be detected.[66] Electron ionization has been used to ionize molecules such as pesticides[67] and small biological molecules.[68] EI can be coupled to a gas chromatograph to ionize the gaseous molecules that elute from the column.[67] Mass spectrometry can be used to detect analytes such as lipids that are ionized by an electron ionization source.[69]

Electrospray ionization is a sensitive ionization technique used for many mass spectrometers.[70] This technique uses a potential difference to produce an electric field of about 2-5kV.[71] Charges accumulate on the surface of the liquid, and the liquid is pulled in the direction of the electric field forming a Taylor Cone.[71] Once the droplets acquire an excess of charges on the surface of the liquid droplet, the molecules separate from the liquid droplet and enter the detector to be analyzed.[71] This technique can be used to ionize biological molecules for bottom-up analysis of proteins[72] and analysis of the lipid composition in a cell.[73] This technique is commonly coupled to HPLC to analyze molecules such as drugs in complex liquid samples.[74]

Quadrupole mass spectrometry uses radio frequency AC and DC voltages to transport ions from a source to a detector.[75] Alternating positive and negative voltages on each pole trap the ions radially and send them toward the detector.[75] DC voltages are

used to trap ions in an axial field.[75] Many quadrupole mass spectrometers have 3 separate quadrupoles.[76] The first quadrupole is for selecting ions for analysis, the second quadrupole is used for collision of ions with neutral molecules, and the third quadrupole is used for selecting ions and transporting them to the detector.[76] Quadrupole mass spectrometers have been used for the analysis of biological materials such as proteins[76] and lipids.[77]

Orbitrap Mass Spectrometry is a useful technique that is used to analyze a variety of compounds. Orbitrap Mass spectrometry operates by trapping ions radially through central and outer electrodes[78] and axially by the shape of the electrodes.[78] The receiver plates at opposite ends detects the current produced by the ions that oscillate in the cell.[78] A Fourier Transform is performed on the waveform generated by the oscillating ions.[78] The frequency domain is then converted into a mass spectrum.[78] The structure of the Orbitrap mass spectrometer gives it the ability to analyze ions such as polyphenols at high resolution.[79] Orbitrap Mass spectrometers can be used to identify biological compounds separated by liquid chromatography.[80] Orbitrap mass spectrometer can also be used to relatively quantify large biologica molecules such as proteins.[14]

6. Isotopic Labeling

Isotopic labeling can be used in biological systems to trace the progression of substances produced by that organism for kinetic information,[36] structural information,[81] and quantification.[81] Isotopic labeling has been used to study many different compounds such as the identification and quantification of proteins[81] and the creation of polyketides in *Amphidinium carterae*.[82] Isotopic labeling may also be useful when distinguishing a single compound synthesized at different timepoints.[83] Mass spectrometers are useful

analysis tools when identifying and quantifying molecules that have been labeled.[81] The metabolism of small molecules such as CO_2 have been traced with carbon labeling in order to quantify the catabolism of lipids in mice.[36]

7. Pathways Analyses (Transcriptomic and Bioinformatics Data)

Transcriptome data provides a way to analyze the genomic information expressed in the cell.[84] With the use of high-throughput sequencing technology, the genes that are expressed can be analyzed and quantified.[85] Transcriptome analysis been used to trace the development of plant gametophytes,[86] and study the expression of plant pathways during different environmental conditions.[87] Transcriptome analysis has also been used to look for genes that are heavily expressed in cancer cells[88] which can provide insight for cancer therapies.[89] Transcriptome work has even been done on algae to provide insight on the expression of genes during certain types of environmental stress.[13]

Bioinformatics and pathway analysis can be used for analyzing pathways common in organisms. KEGG is a database that holds the pathways of multiple metabolic pathways for a variety of organisms.[46] It also provides a comprehensive view of how metabolic pathways interact.[46] BLAST is a type of nucleotide and protein database that aligns nucleotide and protein sequences to identify sequence and structural similarities.[90] With this tool it is possible to detect similarities in a sequence of amino acids or nucleotides.[90] Information from these databases can be used to learn about the creation of small metabolites and other organic molecules such as lipids.

8. Background on Chlamydomonas reinhardtii

In recent years, it has been shown that *C. reinhardtii* produces a variety of responses to deal with environmental stress. Goold et al. showed that under saturated light conditions, algae tend to accumulate lipids.[48b] Moreover, it has been shown that *C. reinhardtii* produces higher concentrations of certain fatty acids under high salt conditions.[91] In addition, it has been reported that starvation from certain feedstock elements, such as sulfur or nitrogen, can induce unique responses in algae.[92] For example, under sulfur starvation conditions, it has been shown that an excess of hydrogen gas is released,[93] while under nitrogen starvation conditions the algae undergo an autophagy process.[92b]

If nitrogen starvation is induced, the algae undergo autophagy.[92b] In the literature, there is little known about the energy source for *C. reinhardtii* during nitrogen starvation. Because the algae undergo autophagy of large organelles and ribosomes during the nitrogen starvation process[92b] it was hypothesized that the algae obtain the energy needed during this time from the autophasized materials such as proteins.[94] Although, the data in the literature does not comment on whether outside carbon sources are used for energy during this process.

During nitrogen starvation and the autophagy process[92b] many types of genes expressed during normal growth are downregulated.[13] However, expression of genes coding for (a) trans-membrane proteins that transfer nitrogen-containing compounds into the cell from the environment and (b) proteins involved in lipid synthesis are shown to be upregulated.[95] Additionally, it has been reported that during nitrogen starvation, expression of some acyltransferases and nitrogen responsive regulators are upregulated.[48a,

[96] The most noticeable change during nitrogen starvation (as well as sulfur starvation) is that the algae accumulate a large density of lipid bodies.[92] In a study by Wang et al, a 15-fold increase in lipid production is observed in wild type *C. reinhardtii* while a 30-fold increase in lipid production is observed in starchless-mutants of *C. reinhardtii*.[92b]

The lipid composition of *C. reinhardtii* also changes under nitrogen starvation. Under ideal environment conditions (*i.e.*, where the algae can grow and carry on normal metabolism), starchless mutants of *C. reinhardtii* (*e.g.*, sta6-C2, sta6-C4, sta6-C6, and BAFJ5) synthesize relatively few triacylglycerols.[2, 94, 97] However, under nitrogen starvation conditions, starchless mutants of *C. reinhardtii* produce numerous triacylglycerols in high concentrations.[94, 98] Additionally, under standard conditions *C. reinhardtii* synthesize many types of short chain and long chain fatty acids.[99] Under nitrogen starvation conditions, however, a large volume of shorter chain fatty acids are observed.[99b, 100]

Previous lipidomics studies of *C. reinhardtii* have primarily utilized mass spectrometry [92b], staining with nile red for microscopy,[101] and relative lipid weight[102] for lipid analysis. For most experiments utilizing MS, relative fatty acid[99b] and triacylglycerols[94] abundances were compared before and after nitrogen starvation in effort to ascertain the types of lipids synthesized in result to nitrogen starvation. However, lipid constituents formed after nitrogen starvation are difficult to separate from those formed prior to nitrogen starvation. Isotopic labeling, in conjunction with mass spectrometry, has been utilized previously to identify compounds produced after a specific time point (*i.e.*, when the labeled compound is introduced).[103]

9. Overview of Project Details

In this report, we utilized mass spectrometry to pinpoint a possible energy source for the algae during nitrogen starvation. Unlabeled acetate was for algae growth in normal conditions but was replaced with heavy (*i.e.*, [1-^{13}C] labeled) acetate during nitrogen starvation conditions. The headspace of the algae was analyzed every 24 hours for 72 hours during the nitrogen starvation period. The ratio of $^{13}CO_2$ to $^{12}CO_2$ in the algal headspace may indicate whether external sources are being used for energy during nitrogen starvation. A change in the isotopic distribution of CO_2 may indicate where the algae obtain the energy necessary for lipid creation and other cellular processes.

Additionally, we have utilized isotopic labeling and mass spectrometry to identify lipids formed in response to nitrogen-starvation. Media exchange with nitrogen deplete media containing ^{13}C-labeled acetate was utilized to induce nitrogen starvation response in *C. reinhardtii*. Lipids synthesized after nitrogen-starvation were shown to readily incorporate ^{13}C present in the exchange media. Heavy (*i.e.*, ^{13}C-labeled) diacylglycerols (DAGs), triacylglycerols (TAGs), and fatty acids (FAs) were easily distinguished from their unlabeled counterparts in the mass spectra. Unlabeled DAGs, TAGs, and FAs were presumably synthesized prior to nitrogen-starvation. Meta-analysis of published lipid synthesis pathways for *C. reinhardtii*, published transcriptome data for *C. reinhardtii* under nitrogen starvation, and our observed trends in lipid production under nitrogen have illuminated key metabolic steps in lipid synthesis for *C. reinhardtii* under nitrogen starvation.

CHAPTER TWO

Experimental

This experiment was designed to gain information on possible energy sources and lipid production of *Chlamydomonas reinhardtii* during nitrogen starvation.

1. Sample Preparations

The *C. reinhardtii* mutant sta6 (CC-4348), a mutant that does not produce starch,[98a] was obtained from the University of Minnesota 140 Gorthner laboratory. The sta6 strain was placed on agar which was composed of Sueoka's high salt media with 15g of agar for every 1000ml of solution with and allowed to sit for a month. Next, 6 algae cultures were seeded from the agar and placed into 6 separate 150ml autoclaved flasks with 20ml of Sueoka's high salt media with unlabeled acetate (HSA). Immediately after the algae were placed in Sueoka's HSA, the 6 algae cultures were stirred by a Shaker Model 3500 from VWR (Radnor, Pa) at 110 rpm continuously at room temperature with a .8 amp, 120V, 60Hz light and grown for 120 hours.

After the algae had grown for 120 hours, nitrogen starvation (*i.e.* the absence of ammonium chloride) was induced. To induce nitrogen starvation, all 6 algae cultures were centrifuged by Beckman coulter allegra x-15 (Brea, CA) at 2400 rpm for 8 mins in 50 ml falcon tubes so that the algae accumulated at the bottom in a pellet. The supernatant containing ammonium chloride was removed from all the algae cultures. Three of the cultures were then suspended in Sueoka's high salt medium without ammonium chloride and unlabeled acetate. The other three cultures were suspended in 20ml of Sueoka's high

salt medium without ammonium chloride but contained [1-^{13}C] labeled acetate. Each of these cultures was covered with parafilm to provide a closed system. Immediately after the media exchange, the algae were stirred on a shaker continuously for 24 hours.

2. Gas Chromatography Mass Spectrometry (GC-MS) Instrumentation

Gas Chromatography Mass Spectrometry (GC/MS) Analysis: After 24 hours, 10µL of the headspace of the algae was collected from each of the 6 algae cultures. The 10µL of headspace from each of these samples was inserted into a SRI gas chromatograph instrument (model 8610C, SRI Instruments, Las Vegas, NV). The stationary phase was a Porapak-Q packed C_{18} column from Agilent technologies (Santa Clara, CA). The mobile phase was Helium (He) gas. The helium gas carried the headspace molecules to a 70eV filament which ionized the molecules. Then the ions were analyzed with a Varian 1200 mass spectrometer from Agilent technologies (Santa Clara, CA).

3. CO_2 Analysis

At 24 hours of nitrogen starvation, the $^{12}CO_2$ and $^{13}CO_2$ chromatographic peaks from each of the samples were obtained from the mass spectrometer software. The peaks were integrated and compared across the 3 different samples at the 95% confidence interval. The same analysis was done on the headspace of the 6 algae cultures after 48 hours of nitrogen starvation and 72 hours of nitrogen starvation. The chromatographic peaks of $^{12}CO_2$ and $^{13}CO_2$ at these intervals were also integrated and compared at the 95% confidence interval.

4. Extraction and High Performance Liquid Chromatography (HPLC):

It was hypothesized that acetate in the solution of the algae was being incorporated into lipids produced by the algae during nitrogen starvation. The Folch method of extraction was performed on the algae solution by inserting 0.5ml of lysed algae solution in 2ml of nanopure water and 4ml of 2:1 Chloroform:Methanol solution.[104] Two distinct liquid phases appeared in the mixture and a band formed at the liquid-liquid junction. The band at the liquid-liquid junction was removed with a pipet and placed into 2ml HPLC vials. Reverse-phase High Performance Liquid Chromatography (HPLC) was performed on the solution for 40mins. The column was a C-18 column (Agilent Technologies, Santa Carla, CA) and had an average particle size of 5µm. A gradient was performed in the first 20 minutes of the experiment. The gradient started at 10% Acetonitrile and 90% water with 0.1% Formic acid (HCOOH) and was ramped to 95% Acetonitrile and 5% water with 0.1% Formic acid. After the gradient, a solution of 95% Acetonitrile and 5% water with 0.1% formic acid continued to run through the sample for 15 mins. In the last 5 mins of the experiment, the column was flushed with a gradient that sloped from a solution of 95% Acetonitrile and 5% water with 0.1% formic acid to a solution of 10% Acetonitrile and 90% water with 0.1% Formic acid.

5. Electrospray Ionization Fourier transform Mass Spectrometry (ESI/FTMS)

An OrbitrapVelos Pro from Thermo Fisher (Waltham, MA), which is a Linear Trap Quadrapole FT Mass Spectrometer, was used to detect the compound that eluted from the solution for the entire 40-minute chromatogram.

6. Lipid Analysis

Structural information of lipids is often conveyed in short notation. Many lipids are represented with a few letters followed by a number and then a colon with another number (*i.e.* FA 18:0).[105] The letters stand for the type of lipids, the first number indicates the number of carbons in the lipid tails and the second number indicates the number of double bonds in the lipid.[105] For example, FA 18:4 lipid would be a fatty acid chain with 18 carbon and 4 double bond.[105] Other lipids such as diacylglycerols and triacylglycerols have similar notation but the acronym for these lipids are DAG[106] and TG[107] respectively. Using this notation, it is easy to display structural information of a lipid in a concise way. In this work, we used this notation when displaying lipid structural details.

The lipids separated by HPLC and analyzed by ESI/FTMS were pinpointed by looking for ^{13}C distributions in the labeled sample. Distributions resulting from the ^{13}C isotope were identified in the solution containing [1-^{13}C] acetate. Once the patterns had been identified the peak with no heavy carbons from each lipid was identified and the m/z value for that peak was inserted into a Fatty Acid Tool to gain some information on the elemental composition.[37, 108] Collision induced dissociation (CID) on the OrbitrapVelos Pro from Thermo Fisher (Waltham, MA) was performed on one lipid from each lipid category to confirm their composition. A fatty acid peak at 277.2163 *m/z* was fragmented with 23eV, a diacylglycerol peak at 577.5193 *m/z* was fragmented with 29eV, and a triacylglycerol peak at 877.7260 *m/z* was fragmented at 55eV. Once these peaks were fragmented, the fragmentation pattern was analyzed by the Sirius program to gain information on the elemental composition.[109]

Once the lipid compounds had been identified, the relative intensity of each lipid and each of its isotopes was recorded by averaging over the elution time of each lipid on the chromatogram. The lipid isotopes in the spectra that followed the normal carbon isotopic distribution were categorized as lipids made before nitrogen starvation. Any isotopes that did not follow the normal carbon isotopic distribution were categorized as lipids made after nitrogen starvation. The difference of those made before nitrogen starvation and after nitrogen starvation were found by comparing the relative abundance of the lipids. This mass spectrometry data was cross referenced with published transcriptome data[13] and known lipid pathways in eukaryotic algae[42, 110] to provide insight into this response.

CHAPTER THREE

Energy Reserves during Nitrogen Starvation

The data in this chapter indicates that *C. reinhardtii* obtains carbon sources for energy from outside of the cell when the algae are nitrogen starved. It is known that eukaryotic organisms, including algae, obtain energy by metabolizing carbon containing compounds through the Krebs cycle while releasing CO_2 in the process.[111] Therefore, it is possible to measure the metabolism of carbon used for energy by analyzing the CO_2 production from an organism.[112] At the beginning of the experiment, similar to previous studies, the algae were grown in unlabeled acetate; because all of the natural carbon sources inside the cell should have a normal carbon isotopic distribution, it was expected that CO_2 carbon isotope distribution would show natural carbon isotope distributions.[113] However, it was expected that during the nitrogen starvation, when unlabeled acetate in the algal solution was replaced with [1-^{13}C] labeled acetate, observed isotopic distribution would be altered. In other words, if the algae solely use internal carbon sources for energy during nitrogen starvation, the carbon isotope distribution of CO_2 should remain unchanged, but if the algae use the external acetate (provided in the solution) for carbon source, the isotopic distribution should shift.

Figure 1A is the gas chromatogram of the headspace above the "control" algae sample that was grown under normal conditions in the presence of unlabeled acetate and then grown under nitrogen starved conditions for 24 hours in the presence of unlabeled acetate. Figure 1B is the gas chromatogram of the headspace of the experimental algae

sample that was grown under normal conditions in the presence of unlabeled acetate and then grown under nitrogen starved for 24 hours in the presence of [1-^{13}C] labeled acetate. Black lines in both gas chromatograms represent selected ion chromatograms (SICs) for all molecules present in the headspace sample with *m/z* value of 44 (*i.e.*, $^{12}CO_2$) and profiles in red correspond to SICs of all molecules with *m/z* value of 45 (*i.e.*, $^{13}CO_2$) in the sample. Insets on the top left corner of Figures 1A and 1B correspond to mass spectra for GC elution time of CO_2. It was shown in the chromatogram that most of the gas molecules in the headspace sample including N_2O (shown in figure 1) eluted at 0.76 minutes while $^{12}CO_2$ and $^{13}CO_2$ eluted at 1.80 minutes indicating that the column successful separated out CO_2 from the rest of the sample. With a clear separation of $^{13}CO_2$ to $^{12}CO_2$ from the rest of the sample, it is possible to determine the ratio of $^{13}CO_2$ to $^{12}CO_2$ with their respective isotope intensities.

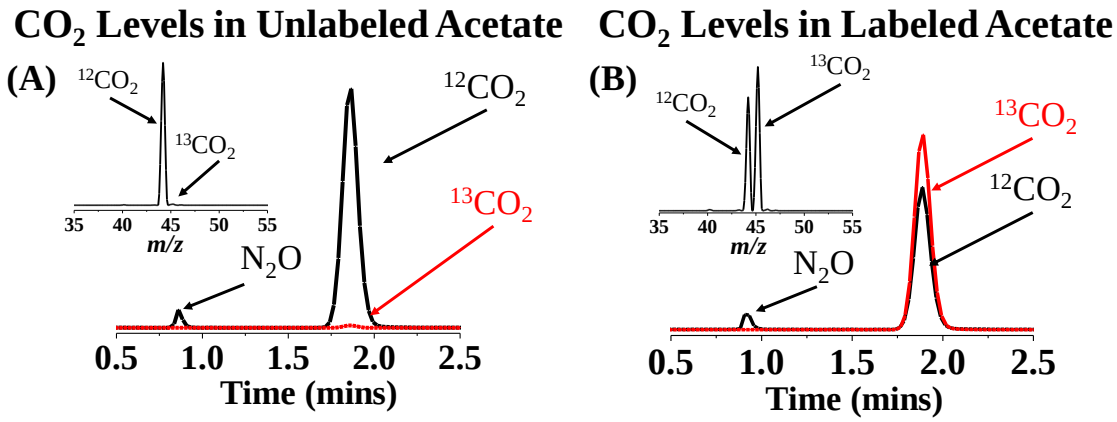

Figure 1: Chromatogram A displays the $^{12}CO_2$ and the $^{13}CO_2$ generated by the algae sample in the control group with its corresponding mass spectrum. Chromatogram B represents the $^{12}CO_2$ and the $^{13}CO_2$ produced by the algae (grown in isotopically labeled acetate) in the experimental group with its corresponding mass spectrum.

The isotopic distributions of CO_2 in the headspace of both algae solutions were determined after 24 hours of nitrogen starvation. The unlabeled acetate given to the algae

in the control group should not change the normal isotope distribution of CO_2 in the atmosphere which is approximately 0.01.[113] As expected, data shown in Figure 1A confirms that the ratio of the $^{13}CO_2$ to $^{12}CO_2$ is indeed 0.01. If the algae do not use the provided outside acetate (or the [1-^{13}C] acetate provided to the experimental group under nitrogen starvation), the observed isotopic distribution for the headspace CO_2 of algae should not remain unchanged and resemble the mass spectrometry data from the control group. However, if the algae metabolize outside acetate for energy, the carbon dioxide in the headspace should have a different isotopic distribution than the control group. Data in Figure 1B shows that the ratio of $^{13}CO_2$ to $^{12}CO_2$ is significantly different than the normal distribution shown in Figure 1A (viz., it changes from 0.01 to 1.38). The increase of the ratio of $^{13}CO_2$ to $^{12}CO_2$ indicates that the labeled acetate from the solution incorporates into the algae cells during nitrogen starvation, metabolizes through the Krebs cycle, and eventually is released as CO_2. These observations suggest that outside carbon sources are being utilized for energy in *C. reinhardtii* during the nitrogen starvation event.

Figure 1 also indicates that N_2O may be present in the headspace of the algae. N_2O (with an *m/z* value of 44) elutes from the column at 0.76 minutes. N_2O is known to be an intermediate in the pathway to produce NO.[114] NO is known to induce autophagy in organisms such as bacteria.[115] The presence of N_2O suggests that NO might be a key factor in determining the autophagy process of *C. reinhardtii* during nitrogen starvation. In the future, it might be possible to carry out additional experiments to confirm the importance of N_2O as a potential intermediate in the autophagy process of *C. reinhardtii* during nitrogen starvation.

The ratio of $^{13}CO_2$ to $^{12}CO_2$ in the headspace of the algae cultures was analyzed at 24, 48, and 72 hours of nitrogen starvation in a triplicate experiment. The ratio of $^{13}CO_2$ to $^{12}CO_2$ was calculated for each sample and displayed in Figure 2 with a 95% confidence interval. The solid line in Figure 2 represents the ratio of $^{13}CO_2$ to $^{12}CO_2$ of the algae grown in normal conditions with unlabeled acetate and nitrogen starved with unlabeled acetate. The dashed line in Figure 2 represents the ratio of $^{13}CO_2$ to $^{12}CO_2$ of the algae in normal conditions with unlabeled acetate and nitrogen starved with [1-^{13}C] labeled acetate.

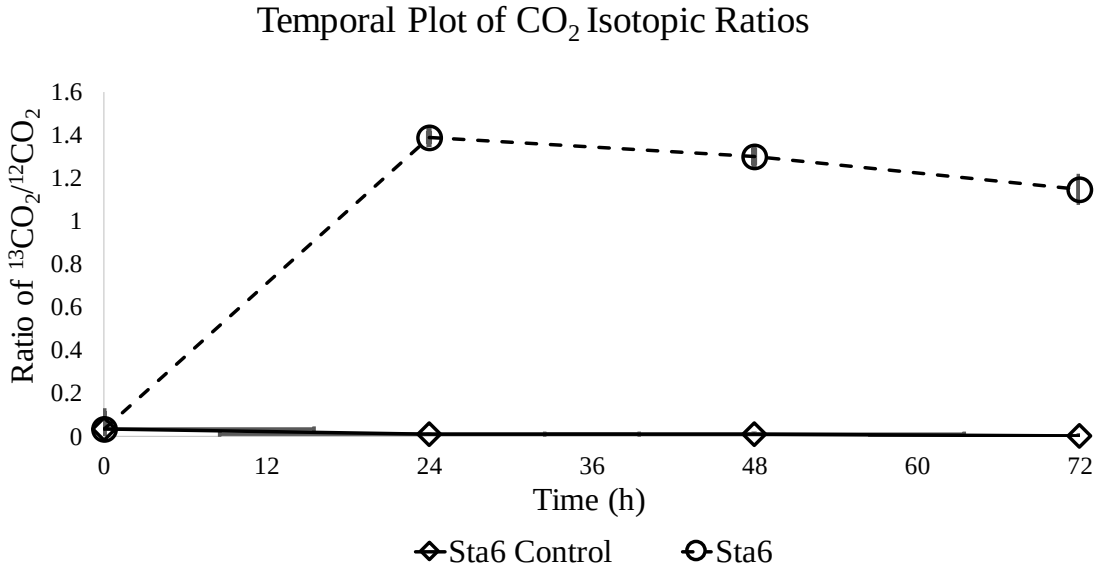

Figure 2: The ratio of $^{13}CO_2$ to $^{12}CO_2$ at time points 0h, 24h, 48h, and 72h for the control algae cultures and the experimental algae cultures done in triplicate. Error bars are reported at 95% confidence intervals for three independent systems each measured in triplicate.

In Figure 2, the ratio of $^{13}CO_2$ to $^{12}CO_2$ in the headspace above the algae nitrogen starved in unlabeled acetate hovers around 0.01 at 0 h, 24 h, 48 h, and 72 h; this is because the acetate that was given to the algae throughout the experiment had the same isotopic distribution as the environment. When the algae were nitrogen starved with [1-^{13}C] labeled acetate the ratio of $^{13}CO_2$ to $^{12}CO_2$ was 0.01 at 0 h because at this stage the algae in the solution had not had time to metabolize any [1-^{13}C] labeled acetate (time zero or starting

point for introduction of labeled acetate). However, after 24 hours of nitrogen starvation, the ratio of $^{13}CO_2$ to $^{12}CO_2$ increased to 1.39. As stated previously, this dramatic increase indicates that outside acetate is being used for energy during nitrogen starvation. After 24 hours, the ratio of $^{13}CO_2$ to $^{12}CO_2$ remains relatively constant. After 48 hours, the ratio of $^{13}CO_2$ to $^{12}CO_2$ was 1.30. After 72 hours the ratio of $^{13}CO_2$ to $^{12}CO_2$ was 1.15. The relatively constant ratio of $^{13}CO_2$ to $^{12}CO_2$ after 24 hours indicates that most of the outside acetate used for energy during nitrogen starvation was incorporated within the first 24 hours of nitrogen starvation. After 24 hours, metabolism of labeled acetate seemed to diminish. The slight decrease in the ratio of $^{13}CO_2$ to $^{12}CO_2$ could be attributed to the diffusing of CO_2 through the parafilm barrier, or the metabolism of CO_2 by the algae during nitrogen starvation (*i.e.*, concentration reduction of labeled acetate as a function of time due to consumption of labeled acetate by algae).

In the future, shorter time interval samplings (higher resolution temporal sampling) may provide additional details about the metabolism of outside acetate during nitrogen starvation. The data presented in Figure 2 suggest that the algae stop metabolizing labeled [1-^{13}C] acetate before 24 hours. This may be because It may also be interesting to record the isotopic distribution during an acetate boost.[116] Acetate boost may change the isotopic distribution because more labeled acetate could be provided in the grown solution.

CHAPTER FOUR

Lipid Creation During Nitrogen Starvation

1. Lipids Found

The lipid composition of *C. reinhardtii* was analyzed after 72 hours of nitrogen starvation. Labeled species were found by pinpointing unique isotopic distributions in the spectra of the chromatogram representing the incorporation of [1-^{13}C] labeled acetate. A few of the labeled species found in the sample are represented by the spectra in figure 3.

Figure 3 represents a fatty acid, a diacylglycerol, and a triacylglycerol found in the sample. Spectrum a) represents a fatty acid synthesized in algae grown with unlabeled acetate and then nitrogen starved with unlabeled acetate, while spectrum b) represents the same fatty acid species synthesized in algae grown with unlabeled acetate and then nitrogen starved with [1-^{13}C] labeled acetate. Spectrum c) represents a diacylglycerol species synthesized in algae grown with unlabeled acetate and then nitrogen starved with unlabeled acetate while spectrum d) is the same diacylglycerol synthesized in algae grown with unlabeled acetate and then nitrogen starved with [1-^{13}C] labeled acetate. Spectrum e) represents a triacylglycerol species synthesized in algae grown with unlabeled acetate and then nitrogen starved with unlabeled acetate and spectrum f) represents the same triacylglycerol species synthesized in algae grown with unlabeled acetate and then nitrogen starved with [1-^{13}C] labeled acetate. The isotopic distributions of these lipids were analyzed to gain information on the creation of these lipid species.

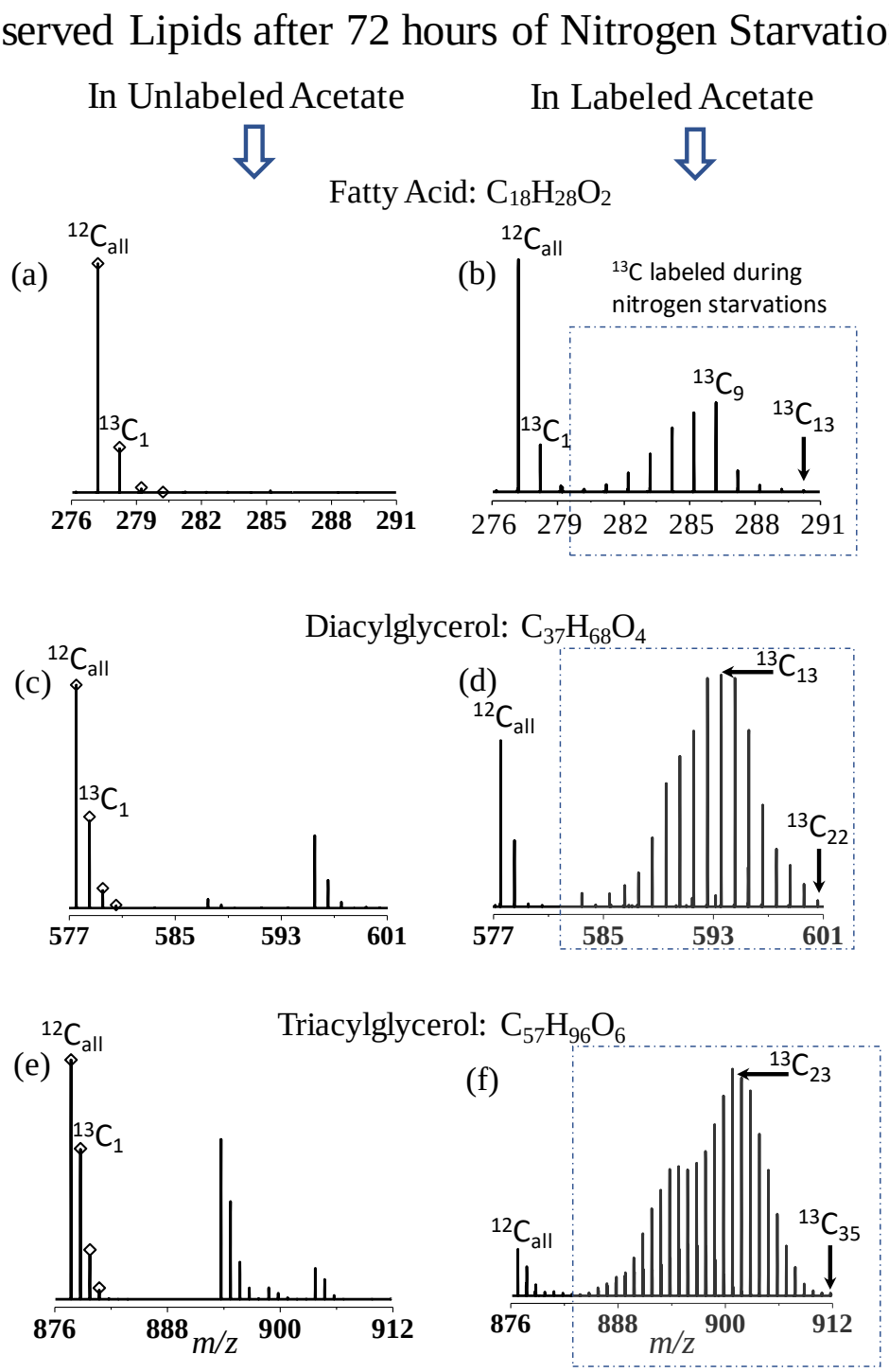

Figure 3: Lipids corresponding to a) fatty acid tail 18:4 (*m/z* 277.2163), diacylglycerol 37:3 (*m/z* 577.5193), and triacylglycerol 57:7 (*m/z* 877.7260) synthesized in algae grown in unlabeled acetate before nitrogen starvation then grown in unlabeled acetate (left) and [1-^{13}C] labeled acetate (right) during nitrogen starvation. Simulated isotopic distributions for unlabeled species are shown as diamonds (◊) in the left column.

The isotopic distributions in spectra in figure 3 provides a representation of when the lipids were made during the experiment. In spectrum b) of figure 3, the first 3 isotopes of the spectrum follow the isotopic distribution shown in spectrum a), which is the expected isotopic distribution. The isotopes in spectrum b) that follow the distribution in spectrum a) are presumed to be lipids made before nitrogen starvation. The isotopes in spectrum b) that did not follow the isotopic distribution in Spectrum a) incorporated labeled acetate and are presumed to have been made during the nitrogen starvation process. The same analysis was done for the diacylglycerols. The first 4 isotopes in Spectrum d) follow the isotopic distribution in spectrum c) which is the expected isotopic distribution and are presumed to represent the lipids that were made before nitrogen starvation. The isotopes in spectrum d) that do not follow the distribution in spectrum c) incorporated labeled acetate which indicates that those isotopic peaks represent lipid that were made during nitrogen starvation. The same analysis was done for the triacylglycerols. The first 4 isotopes of spectrum f) follow the isotopic distribution in spectrum e) and are presumed to represent the lipids made before nitrogen starvation. The isotopes that did not follow the isotopic distribution in spectrum e) incorporated labeled acetate which indicates that these lipids were made during nitrogen starvation.

In spectrum b) of figure 3, the isotopes that do not follow the normal isotopic distribution in spectrum a) account for 54% of the total isotope intensity of the 18:4 fatty acids. This indicates that approximately half of the total intensity of 18:4 fatty acids are made after 72 hours of nitrogen starvation. This analysis was done on all the fatty acids found in the sample. All the fatty acids found in the sample and the relative intensities of

the isotopes made before nitrogen starvation (BNS) and during nitrogen starvation (DNS)

Table 1 Fatty Acids Found

Formula	Elem. Comp.	Observed *m/z*	Calculated *m/z*	Error (ppm)	Lipids Made BNS	Lipids Made DNS	90% Conf. Int.
FA 16:0	$C_{16}H_{33}O_2$	257.2477	257.2475	0.62	66.83%	33.17%	44.27%
FA 16:1	$C_{16}H_{31}O_2$	255.2318	255.2319	0.16	72.94%	27.06%	16.46%
FA 16:2	$C_{16}H_{29}O_2$	253.2162	253.2162	0.07	71.96%	28.04%	25.57%
FA 16:3	$C_{16}H_{27}O_2$	251.2006	251.2006	0.03	27.66%	72.34%	25.59%
FA 16:4	$C_{16}H_{25}O_2$	249.1849	249.1849	0.13	82.41%	17.59%	13.78%
FA 18:0	$C_{18}H_{37}O_2$	285.2787	285.2788	0.39	94.42%	5.58%	7.91%
FA 18:1	$C_{18}H_{35}O_2$	283.2633	283.2632	0.34	35.93%	64.07%	11.81%
FA 18:2	$C_{18}H_{33}O_2$	281.2476	281.2475	0.21	35.76%	64.24%	14.54%
FA 18:3	$C_{18}H_{31}O_2$	279.2319	279.2319	0.14	46.19%	53.81%	11.11%
FA 18:4	$C_{18}H_{29}O_2$	277.2163	277.2162	0.25	43.45%	56.55%	4.30%
FA 18:5	$C_{18}H_{27}O_2$	275.2007	275.2006	0.35	50.33%	49.67%	1.88%
FA 20:0	$C_{20}H_{41}O_2$	313.3101	313.3101	0.05	94.19%	5.81%	8.67%
FA 20:1	$C_{20}H_{39}O_2$	311.2944	311.2945	0.06	99.98%	0.02%	0.03%
FA 20:2	$C_{20}H_{37}O_2$	309.2788	309.2788	0.19	98.30%	1.70%	1.96%
FA 20:3	$C_{20}H_{35}O_2$	307.2631	307.2632	0.24	99.60%	0.40%	0.86%
FA 20:4	$C_{20}H_{33}O_2$	305.2471	305.2475	1.41	78.08%	21.92%	29.64%
FA 20:5	$C_{20}H_{31}O_2$	303.2317	303.2319	0.54	34.44%	65.56%	22.52%
FA 20:6	$C_{20}H_{29}O_2$	301.2162	301.2162	0.12	49.87%	50.13%	19.11%

are recorded in Table 1. Some of these fatty acids that are made during nitrogen starvation are reported in the literature.[100]

Table 2 Diacylglycerols Found

Formula	Elem. Comp.	Observed *m/z*	Calculated *m/z*	Error (ppm)	Lipids Made BNS	Lipids Made DNS	90% Conf. Int.
DG 34:3	$C_{37}H_{69}O_4$	577.5194	577.5190	0.59	12.81%	87.19%	6.43%
DG 34:4	$C_{37}H_{67}O_4$	575.5035	575.5034	0.14	7.60%	92.40%	16.02%

In spectrum d) the isotopes that do not follow the expected isotopic distribution shown in spectrum c) accounted for 84% of the total 34:3 diacylglycerols. This indicates that some diacylglycerols are made before nitrogen starvation, but most are made during nitrogen starvation. The diacylglycerols that were found in the sample are shown in table 2. In spectrum f) the isotopes that do not follow the expected isotopic distribution in spectrum e) account for 99% of all the 54:7 lipids. This indicates that most triacylglycerol species are made during the nitrogen starvation process. The triacylglycerols that were found in the sample are shown in table 3. This confirms the

reports in the literature that triacylglycerols production increases rapidly in starchless mutants of *C. reinhardtii* during nitrogen starvation.[94, 98]

Table 3 Triacylglycerols Found

Formula	Elem. Comp.	Observed *m/z*	Calculated *m/z*	Error (ppm)	Lipids Made BNS	Lipids Made DNS	90% Conf. Int.
TG 52:4	$C_{55}H_{99}O_6$	855.7412	855.7436	2.83	4.03%	95.97%	5.82%
TG 52:5	$C_{55}H_{97}O_6$	853.7255	853.7280	2.90	4.14%	95.86%	5.29%
TG 52:6	$C_{55}H_{95}O_6$	851.7100	851.7123	2.71	2.68%	97.32%	4.50%
TG 52:7	$C_{55}H_{93}O_6$	849.6941	849.6967	3.08	1.70%	98.30%	3.15%
TG 52:8	$C_{55}H_{91}O_6$	847.6786	847.6810	2.88	1.23%	98.77%	2.52%
TG 52:9	$C_{55}H_{89}O_6$	845.6629	845.6654	2.94	0.83%	99.17%	1.52%
TG 52:10	$C_{55}H_{87}O_6$	843.6474	843.6497	2.75	5.00%	95.00%	6.27%
TG 54:5	$C_{57}H_{101}O_6$	881.7568	881.7593	2.82	3.26%	96.74%	3.06%
TG 54:6	$C_{57}H_{99}O_6$	879.7411	879.7436	2.88	3.07%	96.93%	3.56%
TG 54:7	$C_{57}H_{97}O_6$	877.7256	877.7280	2.71	2.03%	97.97%	2.75%
TG 54:8	$C_{57}H_{95}O_6$	875.7096	875.7123	3.11	1.52%	98.48%	2.36%
TG 54:9	$C_{57}H_{93}O_6$	873.6941	873.6967	2.91	1.79%	98.21%	3.14%
TG 54:10	$C_{57}H_{91}O_6$	871.6785	871.6810	2.94	1.29%	98.71%	1.67%
TG 56:7	$C_{59}H_{101}O_6$	905.7567	905.7593	2.82	5.27%	94.73%	7.97%
TG 56:8	$C_{59}H_{99}O_6$	903.7413	903.7436	2.57	0.85%	99.15%	1.37%
TG 56:9	$C_{59}H_{97}O_6$	901.7255	901.7280	2.78	5.04%	94.96%	9.16%

2. Explanation of Lipid Response

The lipids found in the sample indicate key changes in the lipid composition of *C. reinhardtii* during nitrogen starvation. Figure 4 represents all the fatty acids found in the sample and the relative intensity of the lipids made before nitrogen starvation and during nitrogen starvation. The error is reported at a 90% confidence interval. The arrows in figure 4 represent the lipid pathway in eukaryotic algae,[42] and the box at the top right displays the fold change of the RNA transcripts during nitrogen starvation.[13] The symbols ◊, □, and ○ are desaturase enzymes that are used to form double bonds in the fatty acid tails.[42] The symbols †, ‡, and • are fatty acid synthesis enzymes that are responsible for elongating the fatty acid tails.[110] This figure gives key clues into the fatty acids composition of *C. reinhardtii* during nitrogen starvation as well as what fatty acids may comprise the diacylglycerols and triacylglycerols.

Figure 4 indicates that before nitrogen starvation, all combinations of 16 carbon chain, 18 carbon chain, and 20 carbon chain fatty acids were synthesized. Lipid 16:0, a saturated lipid had the largest variation in lipid production because some samples indicated that this lipid was present after 72 hours of nitrogen starvation and other samples indicated that this lipid was not present after 72 hours of nitrogen starvation. More information is needed on this lipid before a claim can be made on its stability. During nitrogen starvation, unsaturated 16 chain fatty acids seem to synthesized considerably. There was quite a lot of variation among the relative amounts of lipid 16-carbon chain lipids as is seen by the large confidence interval. This suggests that 16-carbon chain lipids are not stable lipid species and may be readily converted to 18-carbon chain lipids or incorporated into other lipids such as diacylglycerols and triacylglycerols.

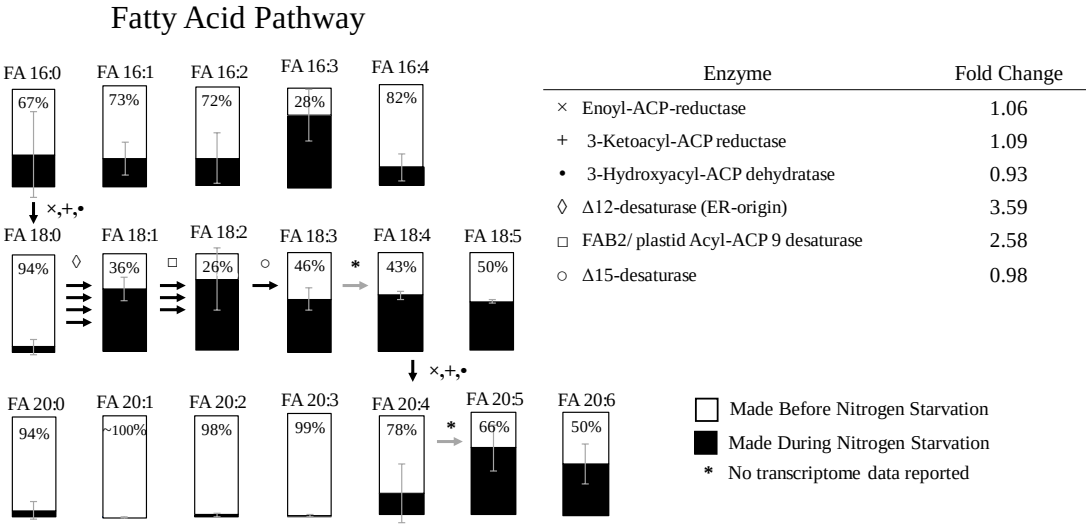

Figure 4: Relative intensities of fatty acids made before (white) and after (black) nitrogen starvation. Error bars are reported at the 90% confidence interval (n=3). Arrows represent previously reported fatty acid synthesis steps. Steps with published transcriptome results are represented as black arrows (steps not reported in transcriptome results shown as gray). A greater number of black arrows represent increasing up-regulation of RNA transcripts coding for requisite enzymes at each step.

Figure 4 also illuminates key aspects of the 18-carbon chain fatty acids. There were very few saturated 18 carbon fatty acids (FA 18:0) in the sample. Transcriptome data collected from Miller et al. reports a 3.59 fold increase in RNA transcripts of a Δ12 desaturase,[13] an enzyme that converts a saturated 18 carbon chain fatty acid to a monounsaturated fatty acid.[42] The increase in RNA transcripts indicates the creation of many Δ12 desaturase enzymes and the rapid conversion of saturated fatty acids to monosaturated fatty acids (FA 18:1). Because few labeled 18:0 fatty acids are shown in figure 4, the Δ12 desaturase may be responsible for creating double bonds on lipids made during nitrogen starvation. The transcriptome data also reports a 2.58 fold increase in RNA transcripts of the Δ9 desaturase[13] an enzyme that converts 18:1 fatty acids to 18:2 fatty acids.[42] This increase in RNA transcripts indicates the creation of many Δ9 desaturases and the rapid conversion of unlabeled 18:1 fatty acids to 18:2 fatty acids. Because few unlabeled 18:1 fatty acids are shown in figure 4, the Δ9 desaturase may be responsible for creating double bonds on lipids made before nitrogen starvation. The transcriptome data also reports a 0.02 fold decrease in the Δ15 desaturase which converts 18:2 fatty acids to 18:3 fatty acids as well as differentiates between the ω6 and the ω3 fatty acid pathways.[42] The small change in RNA transcripts of this enzyme indicates that this enzyme is unaffected and neither fatty acid pathway is significantly favored by nitrogen starvation. An increase in 18:1, 18:2, and 18:3 fatty acids is also reported by James et al.[100] The large 90% confidence interval of the 18:1, 18:2, and 18:3 fatty acids indicates that these fatty acids are not stable species but may be readily converted into fatty acids with a greater number of double bonds or incorporated into diacylglycerols and triacylglycerols.

The transcriptome data by Miller et al. did not report on the Δ6 desaturase shown in the fatty acid pathway,[42] but the mass spectra analysis does indicate that 18:4 fatty acids and 18:5 fatty acids are made during nitrogen starvation. These lipids have very little variation as is shown by the relatively small 90% confidence interval. This observation indicates that 18:4 and 18:5 fatty acids are terminal products that are not readily converted into other fatty acids or incorporated into diacylglycerols and triacylglycerols. The pathway shown indicates that 18:4 fatty acids are converted to 20:4 fatty acids, although, the intensity of 20 carbon chain lipids is small. This would account for the little variation that is seen among the 18:4 fatty acids.

In figure 4 some unsaturated 20 chain fatty acids are shown to be present during nitrogen starvation. A study by James et al. recorded that few 20:0, 20:1, 20:2, and 20:3 fatty acids are made.[100] This is consistent with the data in figure 4 which indicates that not many 20 chain fatty acids are produced during nitrogen starvation. Although the data collected reported that 20:4, 20:5, and 20:6 fatty acids are made during ntirgoen starvation. The large error on these lipids indicate that these lipids are readily incorporated into other lipids such as diacylglycerols and triacylglycerols. Algae do make fatty acid chains that have a tail lengths of 22 carbons or greater,[42] but these fatty acids were not found in high abundance in the mass spectra, though, and could not be confirmed. Because 22 carbon chain lipids were not found in high abundance, it is presumed that most of the 20 carbon chain fatty acids are not converted to 22 carbon lipids during nitrogen starvation but incorporated into other lipids.

It is interesting to note that enzymes used to elongate fatty acid do not seem to be affected by nitrogen starvation conditions. Three enzymes, namely an Enoyl-ACP-

reductase, a 3-Ketoacyl-ACP reductase, and a 3-Hydroacyl-ACP dehydratase, are crucial to fatty acid synthesis.[110] The transcriptome data reported that the fold change for the Enoyl-ACP-reductase was 1.06, the fold change for the 3-Ketoacyl-ACP reductase 1.09, and the fold change for the 3-Hydroacyl-ACP dehydratase was 0.93.[13] The small fold change from the transcriptome data indicates that the fatty acid synthesis pathway is unaffected by the nitrogen starvation process. More research will be needed to discern whether all fatty acid synthesis enzymes are unaffected by the nitrogen starvation process.

In table 2 it is shown that there are not many diacylglycerols in the sample. The absence of many diacylglycerols may indicate that their lifetime is short lived and they are converted to other materials. It is possible that the diacylglycerols that are seen in table 2 are not used to make triacylglycerols and are stored to make other lipids such as phospholipids.[2] The diacylglycerols that are seen in the sample have 3 or 4 double bonds. The lack of saturated diacylglycerols indicates that no saturated fatty acids are incorporated into diacylglycerols during nitrogen starvation. This indicates that the large upregulation of the Δ12 desaturase may convert the saturated fatty acids to monounsaturated fatty acids before these fatty acids can be incorporated into diacylglycerols or triacylglycerols. Table 2 also does not report any diacylglycerols that have an unsaturation number above 4. This indicates that fatty acids with large numbers of unsaturation points are not incorporated into diacylglycerols. This may explain the small amount of variation among the 18:4 and 18:5 fatty acids and further suggest that these fatty acids are terminal products.

In the literature, it has been shown that triacylglycerols are not made in high quantities during normal growth conditions, but made extensively during nitrogen starvation.[94, 98] Figure 3 spectrum f) confirms this finding because of the low abundance of the expected isotopic distribution. Transcriptome data indicates a 147.49 fold increase of a acylCoA Diacylglycerol Acyltransferase[13] which catalyzes the last step in triacylglycerol synthesis.[117] This enzyme may the enzyme that drives the creation of triacylglycerols during nitrogen starvation.

The triacylglycerols made during nitrogen starvation seem to incorporate unsaturated 16 carbon chain fatty acids, unsaturated 18 carbon chain fatty acids, and unsaturated 20 carbon chain fatty acids. Table 1 indicates that only 16, 18, and 20 chain fatty acids are present in the sample. Therefore, the triacylglycerols that are made are presumed to be combinations of these fatty acids. In table 3, triacylglycerols with 52 carbons in their tails are reported. The number of carbons in the tails of the lipids indicates that the triacylglycerols with 52 carbons in their tails are comprised of two 18 carbon chain fatty acids and one 16 carbon chain fatty acid. Table 3 also reports that some triacylglycerols have 54 carbons in their tails. This indicates that the triacylglycerols could either have three 18 carbon chain fatty acids or one 16 carbon chain fatty acid, one 18 carbon chain fatty acid, and one 20 carbon fatty acid chain. Table 3 also reports that three of the triacylglycerols found have 56 carbons in their tails. This indicates that there are two 18 carbon chain fatty acids and one 20 carbon chain fatty acid in the triacylglycerol. It is presumed that these triacylglycerols with these fatty acid tails make up most of the lipid bodies during nitrogen starvation.

The reported triacylglycerols reported in table 3 have between 4 saturations and 10 saturations. The lack of saturated triacylglycerols indicates that saturated fatty acids are not incorporated into triacylglycerols during nitrogen starvation. This corresponds with the fatty acid data in figure 4 because it is shown that saturated fatty acids may be rapidly converted to unsaturated fatty acids because of the upregulation of the Δ12 desaturase.[13] In addition, there are not any triacylglycerols that have greater than 10 double bonds even though the average maximum number of double bonds for these triacylglycerols is 15. This indicates that not many of the 18:4 fatty acids and 18:5 fatty acids are being used as triacylglycerol tails which further suggests that the 18:4 and 18:5 fatty acids are terminal products and not used for triacylglycerol synthesis. Table 3 also indicates that the three triacylglycerols that have 56 carbons in their tails contain no less than 7 double bonds. The presence of many double bonds in the 56 chain triacylglycerols correlates with figure 4 because only heavily unsaturated 20 chain lipids are made during nitrogen starvation. This information indicates that the unsaturated fatty acids that are unstable species are incorporated into triacylglycerols during nitrogen starvation.

In the future, it would be interesting to discuss the incorporation rate of outside carbon sources (i.e. [1-^{13}C] labeled acetate) into fatty acid tails during the nitrogen starvation process. The distributions in figure 3 spectrum b), d), and f) indicate that outside carbon sources are not the only source of carbon used for the synthesis lipid compounds. This analysis could provide insight into which carbon pathways are used for lipid creation during nitrogen starvation and *C. reinhardtii's* interaction with the environment during nitrogen starvation.

In the future, it would also be interesting to investigate the kinetics of triacylglycerol synthesis during nitrogen starvation. In figure 3 Spectrum f), the mass spectra peaks form what looks like a bimodal distribution with a local maximum at 18 labeled carbons and an absolute maximum at 24 labeled carbons. This bimodal distribution may indicate that the fatty acid tails made before nitrogen starvation (*i.e.* fatty acids tails that follow the normal isotopic distribution) are used at a later timepoint. The use of unlabeled tails at a different time point could point to the fact that fatty acids are stored during nitrogen starvation, and then released once they are needed to synthesize triacylglycerols.

These lipids made by microalgae have been reported to be good biofuel substances.[47] It has been stated that organisms that are viable for biofuel production must grow densely and synthesize a large amount of biofuel per cell.[92b] This project as well as many other projects have shown that algae can synthesize many biofuel lipids per cell under nitrogen starvation conditions,[92b, 100, 118] although it seems as if the algae populations themselves have density dependent factors.[119] Therefore, induce sustainable exponential growth of the algae so a large quantity of biofuel can be obtained from a small quantity of algae. Hopefully more research in the future can illuminate a solution to this problem so that algae can be used for commercial benefits.

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