

INVESTIGATION INTO THE METABOLIC CONTROL OF LIPID
ACCUMULATION IN THE MARINE DIATOM
PHAEODACTYLUM TRICORNUTUM

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree

of

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GLOSSARY

CO₂ --- Carbon Dioxide
O₂---Oxygen
GHG---Green House Gas
NADP⁺---Nicotinamide Adenine Dinucleotide Phosphate (Oxidized)
NADPH --- Nicotinamide Adenine Dinucleotide Phosphate (Reduced)
PSI---Photosystem I
PSII---Photosystem II
LHC---Light Harvesting Complex
P680---Pigment absorbing at 680nm
P700---Pigment absorbing at 700nm
PMF---Proton Motive Force
ATP---Adenosine triphosphate
RuBisCO---Ribulose 1,5 Bisphosphate Carboxylase/Oxygenase
TAG--- Triacylglyceride
FAME---Fatty Acid Methyl Ester
BGY---Billion Gallons per Year
C---Carbon
N---Nitrogen
P---Phosphorus
TE---Thermal Efficiency (the percentage of trapped energy stored photochemically)
PDH---Pyruvate Dehydrogenase
ACC---Acetyl-CoA Carboxylase
DOE---Department of Energy
ITS2---Internal transcribed spacer
Mb---Megabases
Kb---Kilobases
RNAi---interfering RNA
CCM---Carbon Concentrating Mechanism
RubisCO---Ribulose-1,5-bisphosphate carboxylase/oxygenase
CA---Carbonic Anhydrase
MDH---Malate Dehydrogenase
ME1---Malic Enzyme
PEPC---Phosphoenolpyruvate Carboxylase
PEPCK---Phosphoenolpyruvate Carboxykinase
PK---Pyruvate Kinase
PPDK---Pyruvate-phosphate Dikinase
PYC---Pyruvate carboxylase
RNA-Seq---RNA sequencing
TCA cycle---Tricarboxylic Acid
CPS---Carbamoyl phosphate synthase
CCMP---Culture Collection of Marine Phytoplankton

GLOSSARY- CONTINUED

NCMA---National Center for Marine Algae
NCGR---National Center for Genome Resources
NaCL---Sodium Chloride
ASP---Aquatic Species Program
nm---nanometer
TOC/TN---Total Organic Nitrogen/Total Nitrogen
NR---Nile Red
GC---Gas Chromatography
NIST---National Institute of Standards and Technology
PCR---Polymerase Chain Reaction
DAVID---Database for Annotation, Visualization, and Integrated Discovery
KEGG---Kyoto Encyclopedia of Genes and Genomes
JGI---Joint Genome Institute
NCBI---National Center for Biotechnology Information
CA---Carbonic Anhydrase
FPKM---Fragments Per Kilobase of exon per Million fragments mapped
RNAi---RNA interference
CPS---Carbamoyl Phosphate synthase
ACC---Acetyl CoA Carboxylase

ABSTRACT

The uncontrolled consumption of fossil fuels over the last two centuries has resulted in unprecedented rates of CO₂ (greenhouse gas) accumulation in the atmosphere, which caused an increased average global temperature and climate shifts. Replacing current fuel demands with a renewable and carbon neutral biofuel can alleviate some of the negative consequences of fossil fuel emissions. Algal biofuel can be that renewable energy source. Microalgae are unicellular photosynthetic organisms that can grow on CO₂ as the sole source of carbon by harvesting light energy. Under nutrient limited conditions microalgae will store carbon as oil or triacylglycerides (TAGs). Algal bio-oil can be harvested and converted to biodiesel, which can be put into any current diesel engine without modification. The objective of this dissertation is to characterize bio-oil accumulation in the marine diatom, *Phaeodactylum tricornutum* by transcriptomic profiling and to investigate metabolic control points during light and dark photoperiods. Extensive growth analysis in tandem with RNA sequencing provided insight into the metabolism of TAG accumulation in microalgae. Phosphate and nitrate depletion initiated lipid accumulation independently, but nitrate depletion resulted in greater lipid yields. Nutrient depletion caused cell cycle arrest, which is controlled by the differential expression diatom specific cyclins. During times of decreased inorganic carbon levels *P. tricornutum* will employ carbon-concentrating mechanisms to maintain carbon flow for photosynthesis during the light cycle. During the dark period up-expression of CCM genes is still maintained for replenishing carbon intermediates of the TCA and urea cycle. Acetyl-CoA carboxylase is the rate-limiting step in fatty acid biosynthesis, but during the light period it is not highly over-expressed. However, central carbon metabolism pathways are highly expressed throughout growth and during the light and dark cycle. It was postulated that elevated concentrations of precursor carbon pools are driving lipid accumulation without a highly expressed acetyl-CoA carboxylase. Thus, enhancement of lipid accumulation is controlled by upstream carbon flow from pathways like the urea and TCA cycle to fatty acid biosynthesis.

CHAPTER 1

INTRODUCTION

The Impacts of Unbalanced Combustion of Fossil FuelsEnvironmental Impacts.

Since the late 18th century at the start Industrial Revolution human kind as been releasing carbon dioxide (CO₂) into the atmosphere, primarily as a result of the combustion of fossil-fuels (Meinshausen et al., 2011). Carbon dioxide (CO₂) is a greenhouse gas (GHG) in the atmosphere that reflects thermal radiation back towards the earth that is necessary to maintain a living temperature(Guerrero-Lemus and Martínez-Duart, 2012). The excess CO₂ in the atmosphere from anthropogenic practices (Fig. 1.1); however, has caused an increase in the average global temperature by the decreased ability of the radiant heat to escape from the Earth's surface (IPCC, 2007). The increase in average temperature is better characterized as global climate change. Climate change is more specific to the changes that will and are occurring at more regional scales while the global temperature average increases. Elevated temperatures have lead to a decline in sea ice, particularly in the Arctic (Stroeve et al., 2007) and current environmental models have predicted that the Arctic will have no ice cover during the summer months by the end of the 21st century (Holland et al., 2008). Glacial melt is a consequence of elevated climate temperatures and accounting for a large portion of the increased sea levels (Meier et al., 2007; Cazenave et al., 2008; Guerrero-Lemus and Martínez-Duart, 2012). The consequences of increased sea levels occur at the long-term time scale, but

there are also immediate effects. For example, increased flooding of coastal land has been observed and is

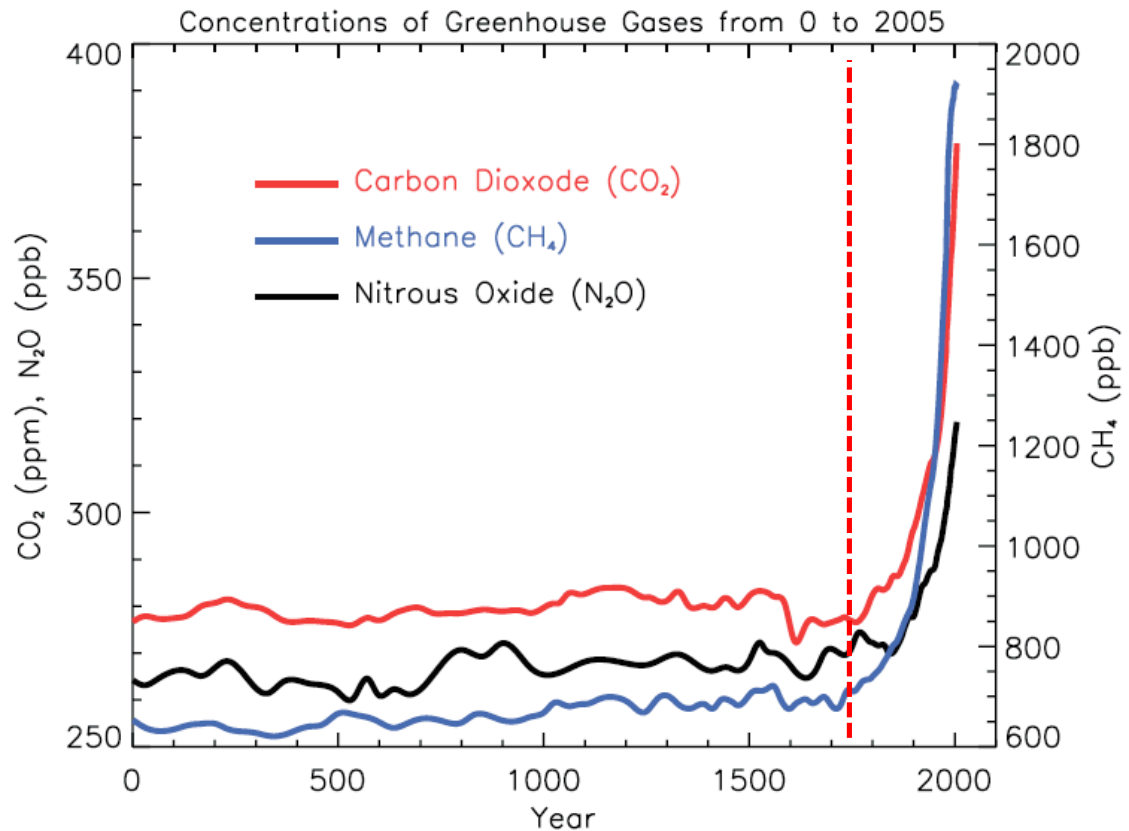


Figure 1.1. Atmospheric concentrations of important long-lived green house gases over the last 2,000 years. Increases since about 1750 (dashed red line) are attributed to human activities in the industrial era. Concentration units are parts per million (ppm) or parts per billion (ppb), indicating the number of molecules of the greenhouse gas per million or billion air molecules, respectively, in an atmospheric sample (reproduced from IPCC, 2007).

associated with the increased frequency of extreme weather events like hurricanes and tropical storms (IPCC, 2007; Church et al., 2008). Long-term effects of rising sea levels are expected to result in higher rates of coastal erosion, ecosystem changes, and salt water dissipation into water tables (Stroeve et al., 2007; Church et al., 2008). These long-term effects will effectively change land use, economic markets, and migration patterns.

The ocean is a sink for atmospheric CO₂ and with the increased levels of CO₂ from anthropogenic activities there has been a marked increase in ocean acidification (Sabine et al., 2004). As CO₂ dissolves into the ocean it associates with H₂O and produces carbonic acid. The carbonic acid dissociates with a proton producing bicarbonate and thus decreasing the ocean pH. The impact of acidification has been observed in the changing of marine ecosystems (Meinshausen et al., 2011; Doney et al., 2012). Acidification consequences range from species invasion to extinction to changing biogeochemical cycles, all of which can affect a broad range of interests from marine fisheries to tourism. The aforementioned consequences are a result of unsustainable human activities, particularly the combustion of fossil fuels.

Economic Impacts.

The U.S. is the largest energy producer and consumer as well as the largest net energy importer. The U.S. economy is dependent on petroleum not only for transportation needs but also for petroleum derivatives that are used for chemical derivatives and feedstocks. In addition to environmental impacts, dependence on imported oil continues to be financially problematic as unstable prices, declining reserves, and reliance on imports from unpredictable regions, like the Middle East, jeopardize the reliable supply of affordable energy to the U.S (Bang, 2010; Guerrero-Lemus and Martínez-Duart, 2012). To maintain a functioning modernized economy, a cheap and reliable supply of natural resource energy is required and has been termed, energy security. A review of the current energy policy, reduction of energy consumption, and replacement with more reliable energy sources (IPCC, 2007; Hughes, 2009) are

required to promote energy, economic, and environmental security. A comprehensive approach to address energy security and dependence will increase economic growth. Petroleum imports are the largest part of the U.S. trade deficit (Stroeve et al., 2007; Demain, 2009). Competition for energy from emerging economies like India and China increase the risk associated with foreign oil reliance. The direct costs of fossil fuel dependence can be measured in barrels per day and on import/export balance sheets; however, greater impacts on the economy may be the indirect costs attributed to climate change. For instance, in shifts in fisheries, food markets, droughts, water supply, or extreme weather events, all of which will have unpredictable impacts on the economy.

Health Impacts.

As the increase in CO₂ in the atmosphere causes shifts in climates and an overall increase in the average temperature, there are expected to be increasing heat related illnesses and deaths in urban areas. Particularly vulnerable are the elderly, sick, and young. Furthermore, urban areas tend to be 1-4°C warmer because of the heat island effect (Holland et al., 2008; O'Neill et al., 2009). In 2011, Sheffield and Landrigan summed up the climate sensitive health risks and challenges that will affect children (Meier et al., 2007; Cazenave et al., 2008; Landrigan and Sheffield, 2011; Guerrero-Lemus and Martínez-Duart, 2012). Changing microclimates means changing ecosystems that can become more conducive to increased infectious disease incidences as clean food and water availability may become limited in drought-associated climates. Increased occurrences of respiratory and cardiovascular diseases have also been noted as a result the higher concentrations of toxic GHGs like ozone or nitrogen dioxide that can

accumulate in the atmosphere (IPCC, 2007; Church et al., 2008; D'amato et al., 2010).

The health effects associated with climate change are not as predictable as economic or environmental impacts; however most experts agree that there will be direct and indirect effects and preparedness may reduce overall risks.

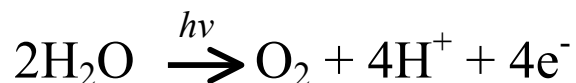
The unchecked human activities since the Industrial Revolution has lead to global climate change and increased average temperatures primarily due to the combustion of fossil fuels and the resulting releasing of CO₂ into the atmosphere. The fossil fuel burned in 1997 contained 44×10^{18} g of carbon, which is 400 times greater then what the globes current biota can fix in net primary productivity (Dukes, 2003; Stroeve et al., 2007; Church et al., 2008). Thus, the unbalance of carbon fixation and consumption has led problematic and challenging consequences to the environment, economy, and human health.

Microalgal Bio-Fuel to Mitigate Climate Change

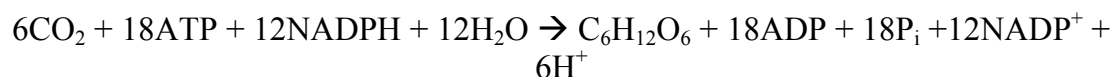
Potential of Microalgal Biodiesel Production.

Microalgae are a diverse group of unicellular eukaryotic O₂-evolving photosynthetic organisms. The machinery for photosynthesis evolved approximately 2.5 billion years ago and creatributed to oxygen rich environments for life to flourish away from anaerobic growth. There are two stages of photosynthesis: the light-dependent and light-independent stages. In light-dependent oxygenic photosynthesis organisms utilize light energy for the oxidation of water (H₂O), which generates electrons for reducing equivalents for redox reactions or driving electron flow through the electron transport

chain. Light reactions in algae occur in the thylakoid membranes of chloroplasts. The energy transfer of photosystems (PSI and PSII) occur at large protein centers that are surrounded by light-harvesting complexes (LHC) that funnel light energy to the reaction centers of PSI and PSII. The photons excite electrons in special chlorophyll molecules, P680 and P700, which, in conjunction with the reaction centers, oxidize H₂O. Electrons are transferred along electron transfer chains eventually reducing NADP⁺ to NADPH and in the process translocate protons across the membrane, forming a proton motive force (PMF) to drive ATP-synthesis by ATP-synthase as well as the production of oxygen (Sabine et al., 2004; Barsanti and Gualtieri, 2005). The photo-oxidation of water to oxygen can be summed in the following equation, requiring 4 photons to produce molecular oxygen (O₂):



The light-independent stage of photosynthesis is a series of complex reactions that fix inorganic carbon (CO₂) to glucose utilizing enzymes of the Calvin cycle and the energized compounds ATP and NADPH (Barsanti and Gualtieri, 2005; Farrelly et al., 2013). The overall formula utilizing RuBisCO (Ribulose 1,5 Bisphosphate Carboxylase/Oxygenase) as the initial fixing step is shown below:



RuBisCO is often consider the most abundant protein on the planet and photosynthetic CO₂ fixation and most carbohydrate production relies on this enzyme (Badger et al., 1998). Thus, the evolutionary combination of the light-dependent and light-independent

stages of photosynthesis allowed photosynthetic organisms to utilize inorganic carbon (CO_2) and electrons from the most abundant molecule on the planet (H_2O) in a light driven reaction to produce oxygen and organic carbon.

The process of producing organic carbon from inorganic carbon using abundant light energy has put algal biofuel in position as a potential mechanism for renewable energy (*e.g.*, lipids) and chemical feedstocks (*e.g.*, biomass) through CO_2 utilization. The fossil fuels of today are the direct result of photosynthesis and carbon fixation from millions of years ago (Farrelly et al., 2013). Microalgae produce different types of lipids, from phospholipids to triacylglycerides (TAGs) (Chisti, 2007; Hu et al., 2008). TAGs are long fatty acid chains esterified to a glycerol backbone and are a carbon storage molecule synthesized when stressed, particularly when CO_2 and light are still available but nutrients are limited (Chisti, 2007; Hu et al., 2008; Greenwell et al., 2010; Scott et al., 2010). Photosynthetic organisms can store energy as carbon chains as a way to maintain cellular processes during the dark. Microalgae have been recorded to produce a range of TAG levels per dry weight from 25% to >50% (Table 1.1.) (Chisti, 2007).

The transesterification of TAGs with methanol and an acid or base catalyst produce glycerol and three chains of fatty acid methyl esters (FAMEs) (Chisti, 2007; Scott et al., 2010). Therefore, biodiesel is essentially fatty acid methyl esters and can be put into any current diesel engine without modification making biodiesel a drop-in technology for the mass transportation infrastructure. The U.S. Military, NASA, and Yellowstone National Park are some of the users that have begun converting transportation fleets to biodiesel. Microalgae have been used as a feed source and energy

crop as long ago as the early 1900s (Huntley and Redalje, 2006; Greenwell et al., 2010) because of the high oil content. It was not until the oil crises of the 1970's that algae really become an option for fossil fuel replacement. In 1978, the U.S Department of Energy funded the Aquatic Species program with the purpose of developing renewable fuels from high lipid producing microalgae (Sheehan et al., 1998). Microalgae are again on the forefront of research as a renewable energy source that can produce biodiesel precursors.

Microalgae	Oil Content (% dry wt)
<i>Botryococcus braunii</i>	25-75
<i>Chlorella sp.</i>	28-32
<i>Cryptocodinium cohnii</i>	20
<i>Cylindrotheca sp.</i>	16-37
<i>Dunaliella primolecta</i>	23
<i>Isochrysis sp.</i>	25-33
<i>Monallanthus salina</i>	>20
<i>Nannochloris sp.</i>	20-35
<i>Nannochloropsis sp.</i>	31-68
<i>Neochloris oleoabundans</i>	35-54
<i>Nitzschia sp.</i>	45-47
<i>Phaeodactylum tricornutum</i>	20-30
<i>Schizochytrium sp.</i>	50-77
<i>Tetraselmis sueica</i>	15-23

Table 1.1. Oil content in % dry weight of some common microalgae. Oil content between 20-50% is common in many microalgae (reproduced from Chisti, 2007).

Specific Advantages of Growing Microalgae for Biodiesel.

Harnessing microalgae for biofuel production mitigates several concerns about sustainable renewable energy sources that can replace fossil fuels. The specific advantages include that algae utilize CO₂ (one of the main GHG causing global climate

change), require less land than terrestrial crops to produce the same amount of oil, will not directly affect the food supply, and can grow on water not deemed potable.

The ability of microalgae to fix CO₂ into biomass and lipids can directly take advantage of the excess CO₂ in the atmosphere but more importantly from the enormous emissions of power plants, cement plants, or fermentation industries that produce substantial amount of CO₂ (Pate et al., 2011). Microalgae are also some of the fastest growing photosynthetic organism with doubling rates that can be less than 24 hours during autotrophic growth (Kurano et al., 1995; Farrelly et al., 2013). Several studies have shown the possibility of using flue gas to supply algal cultures with CO₂ (Pokoo-Aikins et al., 2009). In 2005, using outdoor thin-layer photobioreactors Doucha *et. al.* showed that a 6-8% influx of flue gas into a algal culture would sequester 10-50% of injected CO₂ (Doucha et al., 2005). A flue gas removal ratio of 67% was achieved using a mutant *Scenedesmus* strain when aerating with 12% CO₂ (Li et al., 2011). Growth on CO₂ emission pipelines is considered a major requirement for capturing CO₂ and influencing the cost effective growth of algal biofuels.

The feasibility of microalgae replacing seed crops as the major source of renewable biofuel is evident in the land usage estimates required for replacing a substantial portion of transportation fuel needs. The land needed to produce corn or soybean for biofuel is considerably larger than that of microalgae. This is because of two prominent factors: first, each microalgal unit produces oil whereas soybean or corn produces a stalk and vegetation but only the seeds are harvested and secondly, the growth rate of microalgae is significantly faster than that of most terrestrial plants. Chisti in

2007 made a comparison of land area requirements between potential sources of biodiesel (e.g., corn, soybean, oil palm, etc.) versus microalgae. Conservative estimates based on demonstrated biomass productivity and oil yields showed that if 50% of the U.S. transportation fuel needs were to be replaced by biofuels, 846% or 326% of the U.S. arable crop land would be required when growing corn or soybean, respectively (Chisti, 2007). However, microalgae was estimated to only require 3% of the arable cropland to replace the same amount of transportation fuel (Chisti, 2007). Thus, microalgae are an achievable path to replace diesel fossil fuel at this time. In 2011, a study looking at the resource demand for microalgae to biodiesel scale up found that land use was the least restrictive requirement. Their predictions looked at land requirements for scenarios of algae biofuel feedstock targets ranging from 10 billion gallons per year (BGY) to 100 BGY. At 10 BYG 15% of the total U.S. diesel fuel demand would be met while 100 BYG would sufficiently meet the total diesel and aviation transport fuel demand (Pate et al., 2011). The results are shown in figure 1.2 and indicate the percentage of total land required per total land area in each region. The highest productivity is estimated to be in the western half of the country. These predictions offer a realistic estimate of the substantial promise of algal biofuels to replace transportation fuel.

Land usage estimates indicate microalgae are better suited as fuel crops rather than corn and soybean in terms of potential. In addition, microalgae do not directly affect food supplies, contrary to corn or soy crops. When fuel prices increase, farmers shift production away from soybean or wheat lands that are used for food production. This can in turn cause an increase in corn, soybeans, and wheat food prices by 40%, 20%, and

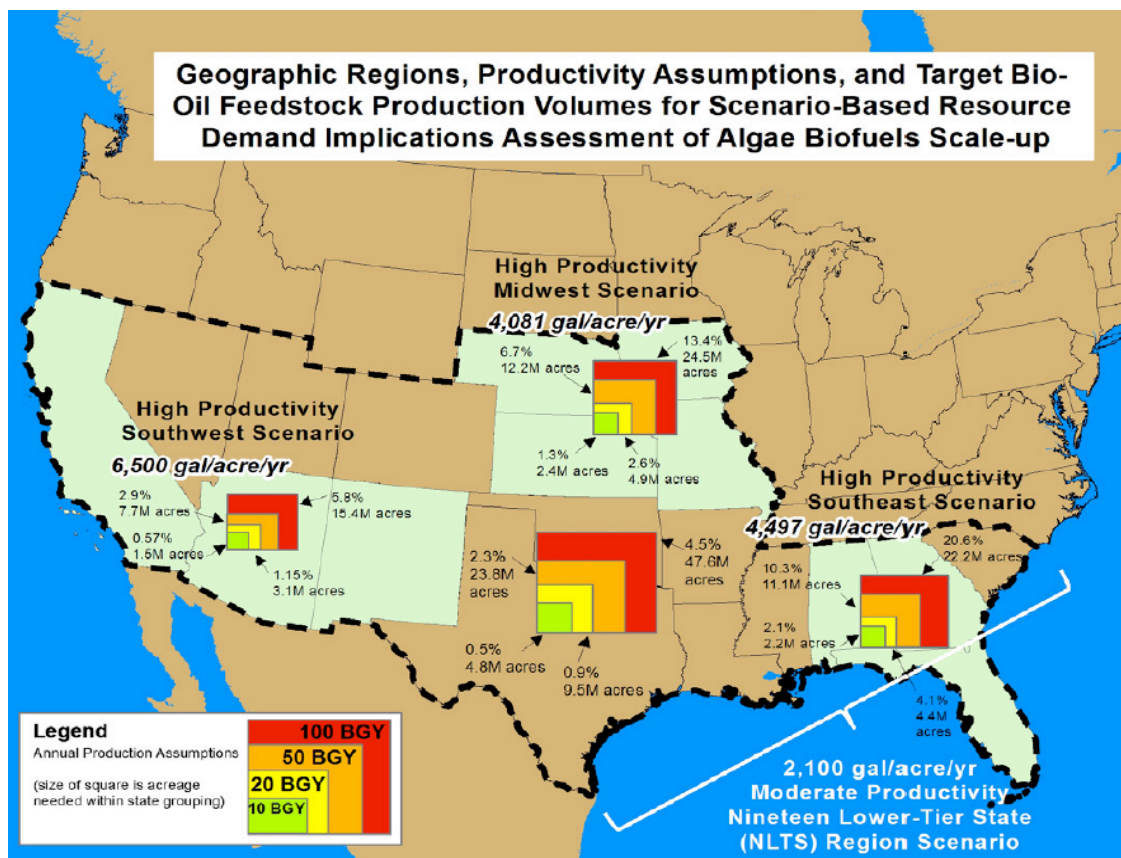


Figure 1.2. Projected land areas, shown in acres and in percentage of total land in each region, for the four algae oil production scenarios considered. Algal oil productivities are assumed to be optimistically high in the three distinct multi-state regions of the Southwest (SW), Midwest (MW), and Southeast (SE), with more moderate productivity assumed for the combined Nineteen Lower-Tier State (NLTS) region (reproduced from Pate et al., 2011).

17% respectively (Searchinger et al., 2008) because of decreases in supply. Production of biofuels from current food supply crops has been linked to rising food prices, thus making second generation biofuels such as algae much more resistant to food market influence (Carriquiry et al., 2011). In addition, improvements in policy and production efficiency can meet the global demand of biofuel and feed supply without having contrary effects on each other (Tilman et al., 2009).

A critical advantage of using microalgae as an energy crop is the fact that it can be grown on water sources other than freshwater, reducing the impact on the drinking water supply. Utilization of coastal marine water, brackish or high salt ground water, well water from oil, gas or coal bed methane processes, and wastewater from agricultural, municipal, and industrial practices will be critical for algal biofuel success (Pate et al., 2011). In a study comparing the water footprint of biofuels, it was estimated that the overall water demands including evaporative loss of bioethanol was 500-4000 gallons of water per gallon of fuel (Dominguez-Faus et al., 2009). However, for algal oil the estimates are between 240-600 gallons of water per gallon of bio-oil produced (Pate et al., 2011). This seems like a substantial amount of water to produce fuel, but it is substantially less than other feedstock biofuels. However, the algal biofuel water footprint can be drastically reduced if water recycling is employed in cultivation. Recycling water from the harvest can reduce water usage by 84% and nutrient usage by 55% (Yang et al., 2011). Also, employing sea or wastewater as growth medium can reduce the water requirement by 90% and can reduce the nutrient requirements to negligible except for phosphate, which would need to be supplemented (Yang et al., 2011). The integration of wastewater has been shown in multiple studies to produce viable algal cultures for biofuel production, from carpet industry wastewater to municipal or agricultural wastewater (Chinnasamy et al., 2010; Park et al., 2011; Dahiya et al., 2012).

Microalgal biofuel has the potential to be a realistic and achievable renewable and sustainable replacement for fossil fuel. Specifically, it takes advantage of light energy to

fix CO₂ into lipid that can be converted to biodiesel. Predictions and estimates indicate that it will require substantially less land to replace a large portion of U.S. transportation fuel needs compared to corn or soybean fuel. It would also not directly affect the food supply as farmers shift crops based on fuel prices. Algal biofuel requires less water to produce a gallon of oil compared other feed crop biofuels. Water from non-desirable sources like brackish water, wastewater (municipal, agricultural, and industrial), marine water, or industrial well water may be employed. Alternatively, recycling of nutrients and water can significantly decrease even further the requirements of water to produce fuel. Thus, algal biofuel is part of the solution to mitigate the unbalanced combustion of fossil fuels that is adversely effecting the environment, economy, and health in both the near- and long-term.

Increasing TAG Accumulation Efficiency

Optimization of Growth and Lipid Metabolism.

When considering algal biofuel as sustainable alternative to fossil fuel, every process of the production must be optimized as well as maximized. Optimization reduces costs and makes the overall process more efficient so that it maintains sustainability goals. A diagram of what an algal biofuel process entails is shown in Figure 1.3 (Scott et al., 2010). The focus of this dissertation focuses on the challenges associated with growth and nutrient optimization and its relationship to lipid metabolism. Investigation into these processes will add to the overall understanding of controlling growth and lipid yields for biofuel production.

In the field of algal lipid productivity, it is well researched that nutrient limitation or stress can increase lipid content in microalgae (Griffiths et al., 2011). Other factors such as temperature, light, and salinity have also been shown to affect lipid accumulation (Converti et al., 2009; Rodolfi et al., 2009; Pal et al., 2011). When using nutrient stress to induce lipid accumulation there is often a tradeoff in cellular growth (Griffiths and Harrison, 2009; Lardon et al., 2009). The depletion of nitrogen and/or phosphorus can induce lipid accumulation in microalgae, but silica can also be used to limit growth in diatoms.

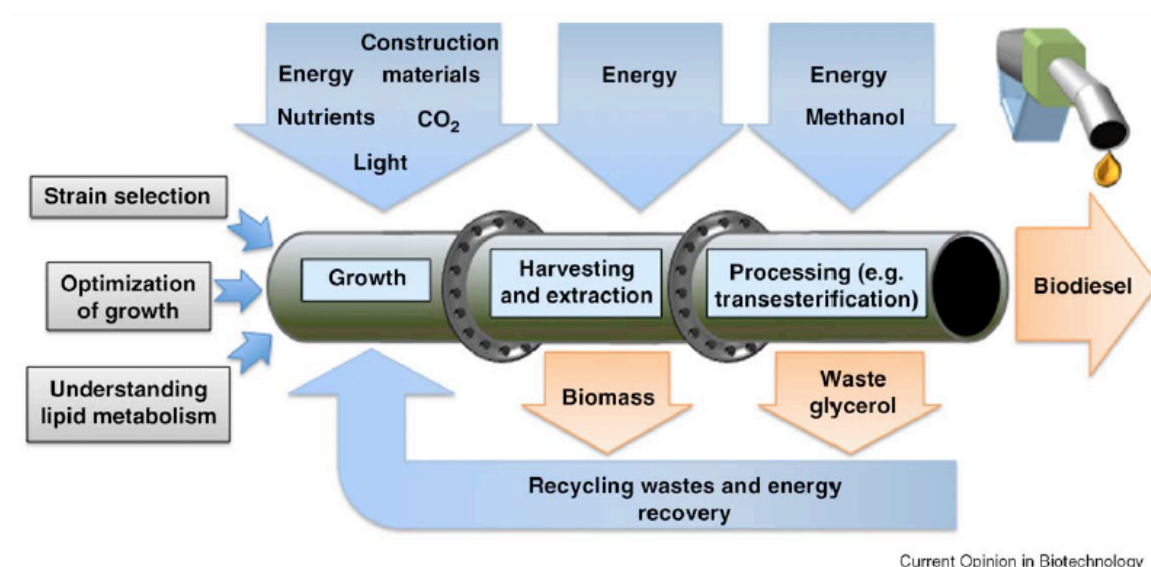


Figure 1.3. Algal biofuel pipeline, showing the major stages in the process, together with the inputs and outputs that must be taken into consideration by the life-cycle analysis (reproduced from Scott et al., 2010). My research focus is on optimization of growth and understanding lipid metabolism.

The Redfield ratio is a stoichiometric ratio that indicates nutrient limitation for phytoplankton based on average biomass composition (Redfield, 1958). Redfield predicted a ratio of C:N:P (Carbon:Nitrogen:Phosphorus) of 106:16:1 for phytoplankton, and the N:P ratio of 16:1 has been widely used as predictors for models of algal resource

allocation. Although ratios ultimately vary based on environmental conditions and species variation, the Redfield ratio has been a widely accepted indication of nutrient requirements and limitation. However, contemporary research suggests that the Redfield ratio is limited in its predictability based on nutrient requirements and the combined effects of N and P limitation together (Ptacnik et al., 2010). Based on cellular composition the major macromolecule in unicellular algae is protein, which requires a significant amount of reduced nitrogen for synthesis. On the contrary, phosphorus is a main contributor of nucleic acids, which make up far less of the cells composition (Falkowski, 2000). Both elements are critical for cellular growth and homeostasis but it is evident microalgae have a higher demand for nitrogen than phosphorus based strictly on elemental composition requirements.

Nitrogen Stress Lipid Accumulation in Microalgae.

Microalgae are well documented for the ability to accumulate triacylglycerol under nitrogen-stressed or -limited conditions. The nitrogen requirement for microalgae is estimated to be 55 to 111 times higher than terrestrial plants, meaning that nitrogen recycling is essential for algal biofuel feasibility (Halleux et al., 2008). The biofuel potential of algae under different nitrogen sources such as nitrate, ammonia, or urea has been investigated (Li et al., 2008; Eustance, 2011). In general, growth and lipid production seems to be more efficient when cells are grown on nitrate as the nitrogen source (Li et al., 2008). In a growth and lipid accumulation study with *Neochloris oleabundans*, a promising green microalgae for biofuel production, Li *et al.* (2008) observed nitrate was the optimum nitrogen source to produce lipid. The authors also

determined the tradeoff between lipid productivity and biomass productivity based on nitrate concentrations. The lower the nitrate concentration the higher the lipid productivity; however, the more nitrate available the more biomass that was produced, while excessive nitrate limited growth productivity presumably because of light limitation (Li et al., 2008). Thus, nitrate limitation has become a favorable choice for screening different algal strains of lipid productivity (Griffiths et al., 2011; Breuer et al., 2012).

The effect of nitrogen limitation on growth and lipid accumulation in algae is generally speculated to be attributed to reducing the ability to produce new amino acids for protein production and growth, so metabolism is shifted towards energy storage in the forms of TAG or carbohydrates (Jiang et al., 2012). Protein turnover decreases in conjunction with decreases in growth rate in microalgae (Quigg and Beardall, 2003), suggesting a shift in metabolism to energy accumulation from cellular growth. The effects of nitrogen starvation on three unicellular algae showed a decline in thermal efficiency (TE, the percentage of trapped energy stored photochemically). This is attributed to a decline in PSII, but not PSI (Berges et al., 1996). Such directed investigations begin to clarify the actual mechanisms that govern the decision-point between slower growth and lipid accumulation attributed to nitrogen starvation.

The advent of high-throughput RNA sequencing has allowed for deeper investigations into the metabolic consequences of nitrogen starvation in microalgae. The advantage of global profiling allows for the interconnecting of metabolic processes that play important roles in the cells homeostasis that may not directly be involved in lipid

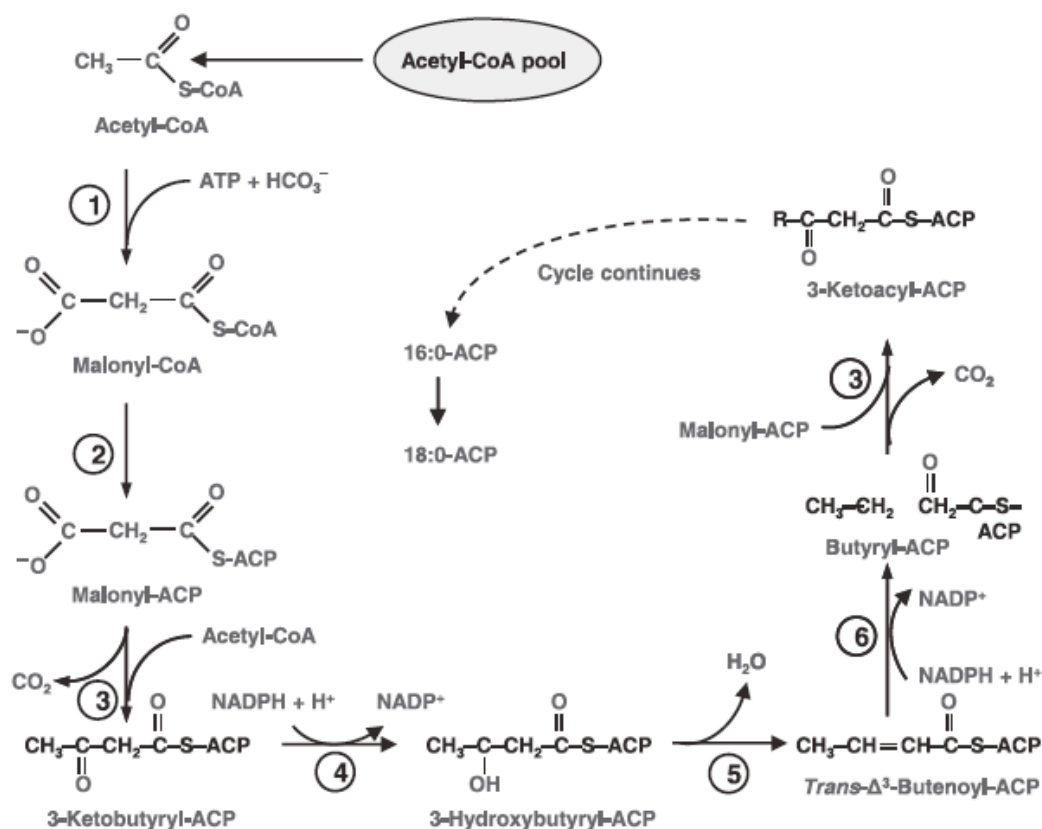


Figure 1.4. Fatty acid biosynthesis. Acetyl CoA enters the pathway as a substrate for acetyl CoA carboxylase (reaction 1) as well as a substrate for the initial condensation reaction (Reaction 3). Reaction 2, which is catalyzed by malonyl CoA:ACP transferase and transfers malonyl from CoA to form malonyl ACP. Malonyl ACP is the carbon donor for subsequent elongation reactions. After subsequent condensations, the 3-ketoacyl ACP product is reduced (Reaction 4), dehydrated (Reaction 5) and reduced again (Reaction 6), by 3-ketoacyl ACP reductase, 3-hydroxyacyl ACP dehydrase and enoyl ACP reductase, respectively (reproduced from Hu et al., 2008).

synthesis but indirectly play major roles. RNA-sequencing has been employed recently in three different studies with three different unicellular green algae, *Nannochloropsis sp.* (Liang et al., 2012a), *Neochloris oleoabundans* (Rismani-Yazdi et al., 2012), and *Chlamydomonas reinhardtii* (Lv et al., 2013). Some common conclusions in all three studies were that nitrogen depletion causes up-expression in nitrogen assimilation and

metabolism genes compared to replete conditions, presumably to maintain the cells readiness in case of encountering a nitrogen source that could easily incorporate into amino acid production. All three studies showed a net decrease in photosynthetic genes, which would be expected as cells decline in growth correlated with thermal efficiency. Another trend observed was the increase in pyruvate dehydrogenase (PDH) during lipid accumulation but not an increase in acetyl-CoA carboxylase (ACC). Acetyl-CoA carboxylase is the rate limiting step in fatty acid biosynthesis (Chisti, 2007; Hu et al., 2008; Greenwell et al., 2010; Rismani-Yazdi et al., 2012; Liang et al., 2012a; Lv et al., 2013) (Figure. 1.4, reaction 1), so it was hypothesized that during lipid accumulation it would be up-regulated, but many studies do not show that trend. During the DOE's Aquatic Species program, researchers successfully overexpressed acetyl-CoA carboxylase in mutant algae to test for increased lipid production; however, increased lipid accumulation did not occur (Sheehan et al., 1998). Thus, other routes need to be investigated to direct energy and carbon for increased lipid accumulation.

Phosphate Stress Lipid Accumulation in Microalgae.

Phosphate limitation can induce lipid accumulation similar to nitrogen stress, and the depletion of phosphate has also been used to screen for algal biofuel potential in different strains of microalgae (Rodolfi et al., 2009). The mechanisms of which nitrogen and/or phosphorous stress affect growth and lipid accumulation are different. In common with nitrogen stress, phosphate stress can cause the arrest of cell cycle division and chlorophyll synthesis (Khozin-Goldberg and Cohen, 2006). This is not unexpected, but the signal transduction by which phosphorus limitation causes cell cycle arrest is different

than that of nitrogen stress. The differences could be explained by the different responses of cell cyclins in algae that control cell cycle progression based on chemical signals. A genome wide analysis of diatom cyclins observed that there are distinctive cyclins that sense the availability of nitrogen or phosphorus and can control the cell cycle (Huysman et al., 2010). Thus, nitrogen and phosphorus limitation appear to arrest the cell cycle by different mechanisms.

In the microalga *Chlorella sp.*, phosphorus limitation induced lipid accumulation. However, phosphate starvation did not affect protein production in the stressed cells (Liang et al., 2012b), contrary to what is normally observed in nitrogen stressed cells. The results emphasize the different effects between the two macro- nutrients. The phosphorus stress response in the diatom *Thalassiosira pseudonana* was characterized globally via transcriptome and proteome profiling (Dyhrman et al., 2012). The authors observed that phosphorus allocation went through polyphosphate production. Classically, polyphosphate has been characterized as a result of high phosphate concentrations and stored into long chains of polyphosphate (Leitao et al., 1995; Diaz et al., 2008); however, based on transcripts and protein identifications it appears that even in phosphorus limited conditions, polyphosphate is still produced (Dyhrman et al., 2012). Transcriptome and proteome analysis showed that phosphate limitation causes a decrease in phospholipids and a corresponding increase of sulfolipids, presumably in an effort to conserve phosphate for cellular homeostasis. A down-regulation in ribosomal proteins was observed, indicating conservation of phosphate, as rRNA is by far the largest phosphate sink in microalgae (Dyhrman et al., 2012).

When focusing on the aspect of optimizing algal growth and lipid production the mechanisms involved in lipid production must be characterized to understand how accumulation is activated. The difference between 20% oil accumulation and 40% can make a significantly large impact when considering the up-scaling of the algal biofuel process. Thus, it is imperative to understand how lipid accumulation is a product of the global response to nutrient stress in the context of resource allocation (C, N, P) for both lipid-level optimization and conservation of N and P. Research in this area can provide insight into how to manipulate microalgae to produce greater lipid or how to trigger accumulation without nutrient stress. Regardless, a complete understanding of all aspects of growth is needed to maximize the algal biofuel potential of promising strains.

Research Directions

The climate and economic consequences of fossil fuel combustion has lead to a renewed interest in a clean, renewable, and a sustainable fossil fuel replacement. Algal biofuel is one of the second-generation biofuels that has substantial potential to replace a large portion of transportation fuel needs. However, at this point it is not commercially viable as the production costs are still high (Carriquiry et al., 2011). Thus, research and development must produce greater efficiency in the three main aspects of biofuel production: growth, harvesting, and processing. This dissertation focuses on understanding growth and lipid productivity by investigating the intrinsic aspects of lipid production as a consequence of nutrient limitation, specifically nitrogen and phosphorus. Analysis will focus on the marine diatom, *Phaeodactylum tricornutum*, in the phylum

Bacillariophyta, which contains one third of all known marine phytoplankton. *P. tricornutum* is a well studied and documented diatom, providing a wealth of knowledge to investigate and compare what is controlling the physiology and metabolism of stressed induced lipid accumulation in diatoms.

Phaeodactylum tricornutum, a Model
Organism for Studying C and N Metabolism.

Diatoms are the result of the secondary endosymbiosis of a red alga by a non-photosynthetic protist giving rise to the super group 'Chromalveolata' (Bhattacharya et al., 2007; Reyes-Prieto and Weber, 2007). This led to an evolutionary remnant unique to some diatoms. For example, *P. tricornutum* has a four layered membrane surrounding the plastid (Tanaka et al., 2005). Diatoms play an important role in the global inorganic carbon cycle. Estimates indicate that diatoms are responsible for up to one-fifth of the worlds primary production and approximately 40% of marine primary production (Falkowski and Raven, 1997; Field, 1998; Granum et al., 2005). Therefore, diatoms are of particular interest in algal biofuels because of there abilities to fix inorganic carbon and accumulate lipids.

Phaeodactylum tricornutum was first identified and characterized as a new species in 1897 by Bohlin from samples collected off the coast of the United Kingdom (Bohlin, 1897). The diatom was characterized as having a weakly silicified frustule, which required meager amounts of silica. Growth in a glass vessel showed no difference between media with and without silica (LEWIN et al., 1958). This is interesting as silica stress has been used before to increase lipid accumulation in diatoms, but this technique

does not work easily on *P. tricornutum* (Yu et al., 2009). *P. tricornutum* is a pleomorphic raphid pennate diatom. There are 10 different morphotypes that have been isolated from different areas around the world, from brackish waters to estuaries, tropical to Canadian coasts (Martino et al., 2007). The three major observable morphotypes are ovate, fusiform, and triradiated. In 2007, De Martiono *et al.* performed a thorough genetic and phenotypic characterization study of *P. tricornutum*'s 10 major morphotypes (Martino et al., 2007). The 10 strain accessions are listed from Pt1 to Pt10 and characterized based on morphological characteristics like size, shape, and growth rates. Genetic analysis was also performed to uncover the extent of the variability of the 10 different strains. Sequencing of the fast evolving ITS2 (Internal Transcribed Spacer) region at the start of the 28S rRNA gene sequences showed high similarity between 97-100%, but was able to distinguish 4 separate clades (Figure. 1.5) (Martino et al., 2007). Morphology and genetic analysis has revealed that there are distinctive genotypes but that all accessions belong to the same species, *P. tricornutum*.

To better understand diatom biology and evolution, *P. tricornutum* strain Pt1 was selected for complete genome sequencing by JGI (Joint Genome Institute, <http://genome.jgi-psf.org/Phatr2>). The completed genome was predicted to have 10,402 genes from a 27.4Mb sized genome (Bowler et al., 2008).

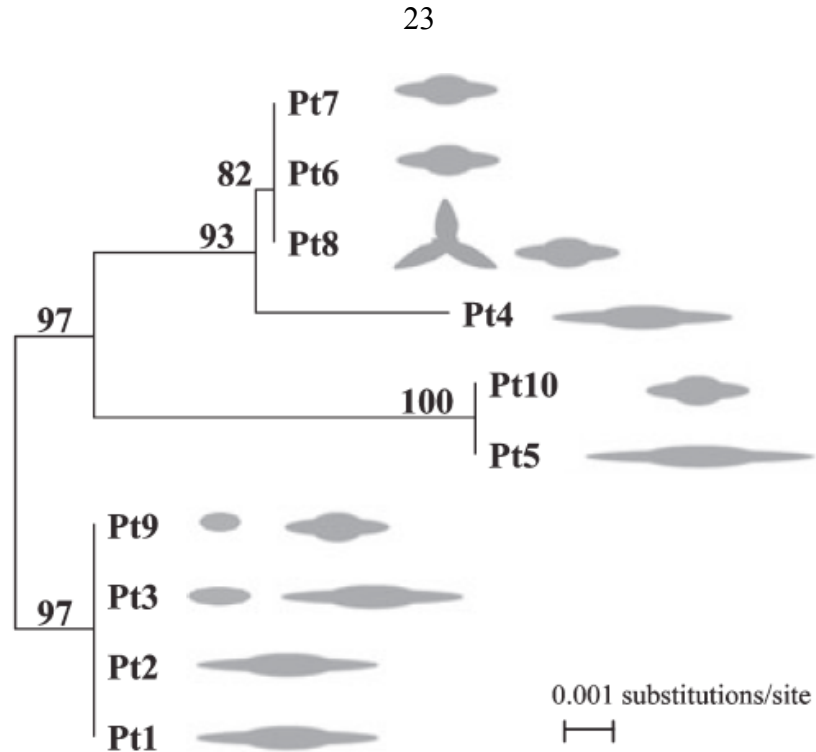


Figure 1.5. Intraspecies genetic and morphological variability among the 10 *Phaeodactylum tricornutum* accessions. Phylogenetic tree based on the neighbor-joining (NJ) method, obtained from analysis of the internal transcribed spacer 2 (ITS2) region (unrooted tree) (reproduced from Martino et al., 2007).

The chloroplast genome was also sequenced, and has a size of 117Kb with 130 protein coding genes (Oudot-Le Secq et al., 2007). Over 130,00 ESTs (expressed sequence tags) from different growth conditions have also been compiled into the Diatom EST Database (Scala, 2002; Maheswari, 2004). Thus, the genetic and expression data provides a wealth of background that can be used to better understand the metabolism of diatoms. Genetic analysis of *P. tricornutum* and *Thalassiosira pseudonana*, another diatom with a genome sequenced, (Armbrust, 2004) provided models for potential metabolism including a urea cycle, carbon concentrating mechanisms (CCM), and central carbon metabolism.

Comparison of the diatom genomes revealed genes and pathways of a urea cycle that is not observed in other photosynthetic organisms (Allen et al., 2006). The findings

indicated that the urea cycle could be playing a major role in driving the C and N fluxes. One of the main genes suspected of controlling such fluxes is the mitochondrial carbamoyl phosphate synthetase. Allen *et al.* in (2011) used metabolomics and RNAi to conclude that the urea cycle does indeed provide a pathway for C and N fluxes, particularly for redistribution during limiting conditions (Allen et al., 2011). The urea cycle in diatoms can act as an anaplerotic pathway that connects carbon fixation and nitrogen assimilation to maintain proper C and N homeostasis for diatom growth and productivity. Physiological control points of C and N flux are important for understanding how nitrogen stress can induce lipid accumulation in algae.

For photoautotrophic organisms such as diatoms, RuBisCO is the major route for fixation of inorganic carbon (CO_2). RubisCO is a carboxylase and oxygenase, meaning it can fix inorganic carbon during photosynthesis and the Calvin cycle but also has affinity for O_2 resulting in inefficient photosynthesis called photorespiration. In order to compensate, algae have either evolved RuBisCO with a higher affinity for CO_2 or developed carbon concentrating mechanisms (CCM) to increase the CO_2 concentration around RubisCO (Badger et al., 1998). The two major types of CCMs are a biophysical and a biochemical mechanism. In a biophysical CCM, the direct transport or pumping of HCO_3^- or CO_2 to the plastid for photosynthesis using transporters and carbonic anhydrases (CAs) that increase CO_2 in proximity to RuBisCO (Hopkinson et al., 2011). In this sense, diatoms can actively pump or translocate CO_2 across membranes to the plastid thereby creating and maintaining a high CO_2 concentration around RubisCO. A higher CO_2 concentration around RuBisCO results in greater carboxylation events and

more efficient photosynthesis (Reinfelder, 2011). In the marine diatom *P. tricornutum*, nine carbonic anhydrases were identified of which three are targeted to the plastid (Tanaka et al., 2005; Tachibana et al., 2011), providing a basis for a possible biophysical CCM. The second type of CCM is what is commonly observed in tropical plants, called a C4 (4-Carbon) biochemical CCM. In a C4 CCM, a three-carbon (C3) precursor is carboxylated with HCO_3^- by a carboxylase enzyme generating a C4 product. The C4 compound is then transported to the plastid and decarboxylated releasing and concentrating CO_2 around RubisCO for fixation and recycling the C3 precursor. Thus, a C4 CCM takes advantage of carboxylases high affinity (low K_m) for inorganic carbon to fix and consequentially release of CO_2 around RubisCO (Reinfelder, 2011).

In 2004, Reinfelder *et. al.* concluded that marine diatoms utilize an organic carbon concentrating mechanism using C4 intermediates, particularly oxaloacetate and malate (Reinfelder and Milligan, 2004). Using inhibitors and carbon isotope labeling Reinfelder deduced that unicellular algae employ a C4-type photosynthesis under low ambient CO_2 conditions. Comparative genome analysis of the marine diatoms *T. pseudonana* and *P. tricornutum* by Kroth *et al.* (2008) revealed a model for diatom carbohydrate metabolism. Their analysis indicated that the diatoms had the genes to run a C4 biochemical CCM as well as a inorganic biophysical CCM (Kroth et al., 2008). The resulting model for carbon concentrating mechanisms in diatoms is surmised in Figure 1.6. The relevant genes included transporters and carbonic anhydrases for an inorganic CCM. Carboxylases (pyruvate carboxylase, phosphoenolpyruvate carboxylase) and

decarboxylases (malic enzyme, phosphoenolpyruvate carboxykinase) were modeled to indicate a biochemical CCM.

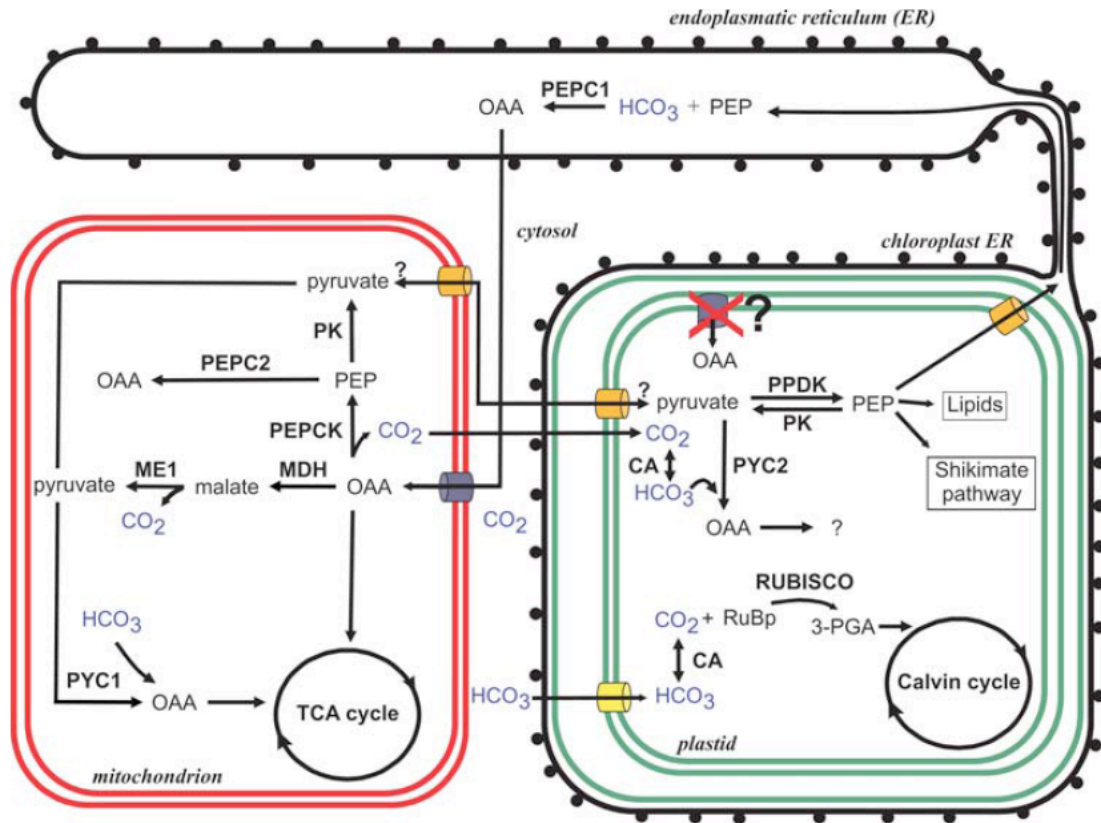


Figure 1.6. Model of carbon concentrating mechanisms (CCM) in diatoms based on annotations of the *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* genomes. For discussion of the pathways see text. Enzyme abbreviations: CA: carbonic anhydrase; MDH: malate dehydrogenase; ME1: NAD malic enzyme, mitochondrial; PEPC: phosphoenolpyruvate carboxylase; PEPCCK: phosphoenolpyruvate carboxykinase; PK: pyruvate kinase; PPDK: pyruvate-phosphate dikinase; PYC: pyruvate carboxylase; RUBISCO: ribulose-1,5-bisphosphate carboxylase (reproduced from Kroth et al., 2007).

This type of modeling is imperative to understand potential routes of C flux directly or indirectly into lipids especially utilizing photoautotrophic algae to fix carbon.

Optimization of growth and lipid accumulation will rely on understanding metabolic fluxes and control points.

Modeling allows for predictions of initial metabolic pathways and their possible control points. The optimization of biomass and lipid accumulation for large-scale biofuel production may fall on the regulation and manipulation of metabolic targets. Organization of carbon pathways, especially with the increase in transcriptomic, proteomic, and metabolomic data, is necessary for interpretation of the significance of the flux through pathways. Compartmentalization of carbon metabolism and partitioning was modeled using genomes from marine diatoms, with a focus on isoenzymes and their localization (Smith et al., 2012). A detailed diatom database for pathways and genes for *P. tricornutum* has been developed consisting of 286 metabolic pathways and 1613 assigned enzymes (<http://www.diatomcyc.org>) (Fabris et al., 2012a; Fabris et al., 2012b). These advances are imperative in integrating systems technology data.

The genomes and subsequent modeling of diatom metabolism in conjunction with the wealth of experimental data has made *P. tricornutum* an important model organism for algal lipid accumulation investigations. The advent of next generation sequencing, particularly RNA sequencing (RNA-seq) has shown to be a promising, providing an in-depth look into functional genomics. RNA-seq provides a more precise method of high-throughput transcript measurements over classical methods of ESTs or microarrays (Wang et al., 2009; Wilhelm et al., 2010). The advantage over microarrays is that hybridization tiles require existing genetic knowledge and there high background due to cross-hybridization (Wang et al., 2009).

This dissertation takes advantage of the extensive research data collected over the years on *P. tricornutum* as a model diatom and its subsequently sequenced genome.

Growth experiments are described in Chapter 2 and were essential to understand how cells respond to environmental parameters of interest (*e.g.*, dissolved inorganic carbon, nitrate, and phosphorus). Data collection was focused on the effects of nitrogen and phosphorus stress independently and together. Global transcriptomics was carried out using RNA-sequencing during growth and lipid accumulation of *P. tricornutum* from the light cycle (Chapter 3). RNA-seq along with growth data allowed for extensive interpretation of growth parameters and cell physiology and metabolism and the shifts in metabolism as a product of environmental changes based on nutrient availability. Data analysis allowed for the interpretation of C, N, and P fluxes through pathways, and the metabolic control points that may direct carbon to lipid accumulation as a result of nutrient stress.

Another aspect that is often overlooked in algal biofuel research and is addressed in this dissertation is the molecular responses of growth and lipid accumulation during the dark period. Lipid accumulation in unicellular algae is the net accumulation during the light period minus the lipid consumed during the dark cycle. For photoautotrophs during the dark cycle, stored carbon energy is oxidized to maintain cellular homeostasis. In 2013, Chaoton *et al.* utilized a microarray to investigate eight time points during a 26.5 hour light and dark period during growth in batch cultures of *P. tricornutum*. (Chaoton *et al.*, 2013). The authors observed clear temporal trends such as carbon sequestration during the light period and cell division during the dark period. The diel expression patterns uncovered in their analysis provide valuable insight into circadian rhythms. This dissertation looks at the broader diel cycle regulation throughout growth as cells

transition from biomass production to lipid accumulation (Chapter 4). Such insights in control points during the dark period could be targeted to reduce lipid consumption, thus increasing overall lipid accumulation. In conclusion, the efforts in this dissertation are to add to the knowledge base of algal biofuel production, focused on discovering metabolic control points of carbon flux towards lipid accumulation as a response to nutrient stress for the optimization and maximization of lipid yields.

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CHAPTER 2

NUTRIENT LEVEL CONTROL OF CELL GROWTH AND BIO-OIL ACCUMULATION IN *PHAEODACTYLUM TRICORNUTUM*

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Abstract

Phaeodactylum tricornutum is a marine diatom in the class *Bacillariophyceae* and is important ecologically and industrially with regards to ocean primary production and lipid accumulation for biofuel production, respectively. Triacylglyceride (TAG) accumulation has been reported in *P. tricornutum* under different nutrient stresses, and our results show that lipid accumulation can occur with nitrate or phosphate depletion. However, greater lipid accumulation was observed when both nutrients were depleted as observed using Nile Red assay and fatty acid methyl ester (FAME) profiles. Nitrate depletion had a greater effect on lipid accumulation than phosphate depletion. Lipid accumulation in *P. tricornutum* was arrested upon re-supplementation with the depleted nutrient. Cells depleted of nitrogen showed a distinct shift from a lipid accumulation mode to cellular growth post re-supplementation with nitrate, as observed through increased cell numbers and consumption of accumulated lipid. Phosphate depletion caused lipid accumulation that was arrested upon phosphate re-supplementation. This was followed by lipid consumption without increased cell numbers. Cells depleted in both nitrate and phosphate displayed cell growth upon the addition of both nitrate and phosphate and had the largest observed degree of lipid consumption upon re-supplementation. These results indicate that phosphate re-supplementation can shut down lipid accumulation but does not cause cells to shift into cellular growth, unlike nitrate re-supplementation. This data suggests that nutrient re-supplementation will arrest lipid accumulation and that switching between cellular growth and lipid accumulation can be regulated upon the availability of nitrogen and phosphorus.

Introduction

In modern societies, petroleum-based products and fuels have strongly influenced human society and infrastructure. In the case of fossil fuels, supply and demand over the past hundred years has been relatively equal; however, increased contemporary consumption has put strain on supply creating undesirable consequences (Brown, 2003). Petroleum markets have become increasingly unpredictable causing destabilized fuel prices. Additionally, the environmental impacts of the release of the greenhouse gas carbon dioxide (CO₂) from the combustion of fossil fuels without balanced CO₂ sequestration has likely contributed to increases in atmospheric CO₂ levels. For example, the amount of carbon released (44×10^8 g) in one year from the consumption of fossil fuels is 400-fold more than the amount of carbon that can be fixed via net global primary productivity (Dukes, 2003). In order to offset the massive influx of CO₂ into the atmosphere, the utilization of renewable biofuels (*e.g.*, ethanol, butanol, H₂, CH₄, biodiesel) is needed. Moreover, the use of autotrophic microorganisms for the production of biomass, biochemicals, and biofuels can further contribute via the direct utilization of atmospheric CO₂.

Diatoms are unicellular eukaryotic microalgae that account for approximately 40% of the total marine primary production each year (Falkowski and Raven, 1997; Field, 1998; Granum et al., 2005). Diatoms and green-microalgae are photoautotrophs that use CO₂ as a carbon source and sunlight as an energy source and many microalgae can store carbon and energy in the form of neutral lipids (*e.g.*, triacylglycerides, TAGs). Thus, the ability to fix and store carbon as long chained reduced hydrocarbons that can be

converted into biodiesel makes algal lipids a potential renewable and carbon neutral energy source. Chisti (2007) estimated conservatively (25-30% as lipid content, w/w) that it would only take 3% of the arable cropland in the U.S. growing microalgae for biodiesel to replace 50% of transportation fuel needs in the United States (Chisti, 2007). Although the interest in algal biofuels has been re-invigorated (Courchesne et al., 2009; Greenwell et al., 2010), significant fundamental and applied research is still needed to fully maximize algal biomass and biochemical production for biofuels. In addition to CO₂ utilization, algal biomass production can also be coupled to wastewater resources (e.g., water, N, and P), and wastewater from agricultural, industrial, and municipal activity may provide a cost effective source of nutrients needed for algal growth. Agricultural and municipal wastewater can be high in nitrogen (N) and phosphorus (P) (Hoffmann, 2002; Mallick, 2002; Aslan and Kapdan, 2006; Pittman et al., 2011), and thus, there is great potential for the integration of wastewater treatment and algal biofuel production. However, an improved understanding of the relationship between the effects of N and P availability on cellular resource allocation (cell growth versus lipid storage) in microalgae is needed.

Phaeodactylum tricornutum, is a marine diatom in the phylum *Bacillariophyta*, which makes up one third of all known phytoplankton (Hildebrand, 2008). *P. tricornutum* has been extensively studied since being isolated in 1956 and has been characterized into 10 different strains based upon genetic and phenotypic attributes (Martino et al., 2007). The genome sequence of *P. tricornutum* 8.6 (CCAP 1055/1; CCMP2561; morphological strain Pt1) was determined at 27.4 Mb with over 10,000 gene

predictions (Bowler et al., 2008). The chloroplast genome was also sequenced and 162 genes from 117,000 bp were predicted (Oudot-Le Secq et al., 2007). This extensive research background and availability of genomic data makes *P. tricornutum* an ideal model organism for understanding fundamentals of cellular processes and regulation (e.g., lipid accumulation) in diatoms.

TAG accumulation has been well documented as a result of nutrient stress or nutrient deficiency in microalgae (Sheehan et al., 1998; Gardner et al., 2010), and in particular, nitrate and/or phosphate stress can cause lipid accumulation in numerous algae (Hu et al., 2008). Recently, we observed that phosphorus limitation can initiate lipid accumulation and is magnified by nitrogen limitation in *P. tricornutum* Pt1 (Valenzuela et al., 2012). In this study we examined 1) the effects of nitrogen and phosphorus depletion on lipid accumulation and 2) the temporal effects of nutrient re-supplementation on biomass growth and lipid accumulation. Our results show that N and P levels contribute to cells switching from a cellular growth state to a lipid accumulation state and that lipid accumulation can be arrested and potentially reversed dependent upon nutrient availability. The work adds to the growing lipid accumulation studies by characterizing what conditions cause the cells to reverse lipid synthesis. This is an essential component of microalgae lipid cycling that has been largely overlooked.

Materials and Methods

Culture and Growth Conditions

The Bohlin Strain 8.6 of *Phaeodactylum tricornutum* (Pt1, CCMP2561 [Culture Collection of Marine Phytoplankton, now known as NCMA: National Center for Marine

Algae and Microbiota], genome sequenced) was used in all experiments. The diatom Pt1 was grown in ASPII medium (Tris-base buffered; pH of 8.0) as previously described (Provasoli et al., 1957; Cooksey and Cooksey, 1974; Valenzuela et al., 2012). Cells were grown in temperature controlled photobioreactors (1.25 L) at 20°C. Light was provided at $450 \mu\text{E}^{-1}\text{sec}^{-1} \text{ m}^2$ on a 14:10 light:dark cycle. Each reactor tube was aerated with sterile ambient air at a rate of $0.40 \text{ L} \times \text{min}^{-1}$ with the CO_2 from air as the sole source of carbon. Cells for inocula were grown photoautotrophically for two generations and then transferred to the 1.25L photobioreactors when cells were in exponential-phase to achieve a final concentration of approximately $1 \times 10^5 \text{ cells} \times \text{mL}^{-1}$. The only sources of nitrogen (N) and phosphorus (P) were nitrate (NaNO_3) and phosphate (H_2KPO_4), respectively. To test replete conditions of N, P, or both N + P, cells in photobioreactors were re-supplemented daily with approximately 1.5 mL of filter-sterilized 300 mM NaNO_3 or 28.7 mM H_2KPO_4 . Contamination was checked throughout the experiments by plating on R2A agar and ASPII medium supplemented with 0.5% glucose and yeast extract.

Experimental Design

The response of lipid accumulation in *P. tricornutum* (Pt1) to nutrient stress was tested in a temporal fashion under nutrient deplete and replete conditions. For replete conditions, N, P, or both N+P were kept in excess throughout batch growth. If N was replete then the limiting nutrient was P, and vice versa, in P replete conditions N became limited. For N+P replete conditions, both N and P were maintained in excess via sequential daily additions during growth. To test whether initial lipid accumulation was reversible, re-supplementation experiments were conducted. Cells were allowed to enter stationary

phase where lipid accumulation was observed under N+P depletion, then cells were re-supplemented (189hr) with N, P, or both N+P. Therefore, if re-supplemented with N, then cells remained P depleted and if re-supplemented with P the cells remained N depleted. In addition, both N+P was supplied to elucidate the effect of complete nutrient re-supplementation post-lipid accumulation. Control conditions for both nutrient replete and re-supplementation conditions only contained nutrients from the original medium and thus became depleted of N and P thus accumulating lipids. Each condition was performed with biological duplicates and compared to standard ASPII controls.

Physiological Parameter Analysis

During growth, samples were collected and analyzed daily for cell number, chlorophyll *a*, pH, dissolved inorganic carbon (DIC), nitrate, phosphate, and relative neutral lipid abundance via Nile Red (NR) fluorescence. Periodically cells were collected for fatty acid methyl ester (FAME) analysis using Gas Chromatography – Mass Spectrometry (GC-MS). Cell density was monitored via direct cell counts in duplicate with a hemocytometer. Cells were collected (1 ml in triplicate) and centrifuged (10,625 x g, 10 min, 4°C). The supernatant fraction was used for exogenous nitrate and phosphate determinations. To extract chlorophyll, 100% methanol (1 ml) was added to the cell pellet, vortexed (30 s) and incubated for 24 hours at 4°C in the dark. The cellular debris was then pelleted (10,625 x g, 5min) and extracts were measured on a spectrophotometer (UV-1700 PharmaSpec UV-vis, Shimadzu). Absorbance was read at 652nm and 665nm to measure chlorophyll *a* and calculated in µg/mL (Porra, 2002). The medium pH was monitored using a standard lab bench pH meter. Cell culture (7 ml) was filtered through

a 0.2 μm nylon membrane filter in preparation for DIC availability measurement on a Formacs^{HT} TOC/TN Analyzer (SKALAR, Buford, GA). Colorimetric assays were used according to manufacturer protocols to measure nitrate (Szechrome NAS, Polysciences, Inc.) and phosphate (SensoLyte MG Phosphate Assay Kit, AnaSpec). N and P assay absorbance was measured on a black walled clear bottom 96 well plate using a Synergy H1 Hybrid Reader (BioTek) spectrophotometer. For cellular lipid levels, the Nile Red assay protocol was used as previously described (Cooksey et al., 1987). Cell culture (1 ml) was diluted to an amount that fit within a linear relationship of Nile Red signal to cell abundance and stained with Nile Red as previously described (Valenzuela et al., 2012).

Microscopy

Fluorescent images were taken with a Nikon Eclipse E800 epifluorescence microscope. Nile red stained cells were imaged with a B2A (Ex 470/40, DM 500LP, EM515) filter with a 60x/1.20NA WI objective. Color images were captured using an Infinity 2 color camera and Infinity Capture software.

Fatty Acid Extraction and Identification via GC-MS

At different time points of growth, 10 mls of cell culture (in triplicate) was filtered over Whatman polycarbonate filters (22 mm, 0.2 μm), transferred to microcentrifuge tubes, and immediately flash frozen in liquid nitrogen. To extract lipids, cells were washed off filters with 10 mM Tris-HCl in glass culture tubes and lysed using sonication (1 min) in 2:1 dichloromethane:methanol (1 ml). Cell debris and phase separation was achieved via centrifugation (2 min at 2,750 x g). The aqueous layer was removed and the organic layer was transferred to a clean GC vial. Additional 2:1 dichloromethane:methanol (0.5

ml) was added to the cell debris, re-sonicated, and the organic layer added to the previous organic extract. An internal standard (pentadecanoic acid, $C_{15}H_{30}O_2$, SIGMA) was added to monitor the degree of transesterification. The organic layer was dried under a stream of nitrogen gas at 60°C. To the dried organic layer, Borontrifluoride-Methanol 10% w/w, 400 μ l (Sigma Aldrich) was added for fatty acid transesterification. Vials were incubated at 60°C for 30 minutes and then the reaction was quenched with nanopure water (200 μ l). Again the sample volatile material was dried under nitrogen gas and hexane (0.5 ml) was added to separate FAMES into the organic layer. Saturated NaCl was added to aid in separation of hydrophilic and hydrophobic phases. The hexane layer was then transferred into new a GC vial and dried down under nitrogen gas and re-suspended in hexane (100 μ l) concentrating FAMES. The contents were transferred into clean 150 μ L glass inserts within new GC vials. Identification and quantification was done on an Agilent Technologies 5975C inert EI/CI MSD with Triple Axis Detector with a Phenomex ZB-FFAP 250°C 60m x 250 μ m x 0.25 μ m column and Agilent 7693 autosampler. Standard curves were used to quantify FAMES based on integrated chromatogram peak area using Agilent MassHunter Qualitative Analysis software. FAMES were identified—by comparison to the spectral data in National Institute of Standards and Technology Mass Spectral Library (NIST 08) and only the “original” fatty acids were plotted. Only peaks identified with a minimal match score of 50 or greater were considered and were then cross-referenced with the NIST MS Search 2.0 program for confirmation of FAMES species.

Results

Control (N and P Depleted)

In *P. tricornutum* (Pt1), nutrient stress has been shown to cause lipid accumulation (Valenzuela et al., 2012; Mus et al., 2013). In control cultures inoculated with approximately 10^5 cells/mL in ASPII medium, exogenous phosphate was depleted at approximately 72 hours, while nitrate was depleted at approximately 100 hours (Fig. 2.1b). Cells did not reach stationary-phase until two doublings after phosphate was depleted and one doubling after nitrogen was depleted (119hrs) (Fig. 2.1a). This is most likely a result of intracellular phosphate storage, and Pt1 has been shown to store intracellular phosphate (Leitao et al., 1995). The doubling of cells after nitrogen depletion can be attributed to the repurposing of nitrogen rich molecules, possibly chlorophylls (Fig. 2.1c). Chlorophyll *a* reached a maximum level at approximately 100 hours and the levels declined throughout the rest of the growth curve. The decrease in chlorophyll content correlates to the decrease in nitrate availability, and these results suggest a shift to slower growth and eventual cell cycle cessation. Cells could also be recycling nitrogen from non-essential proteins, which could be a much greater nitrogen source than chlorophyll (Lee et al., 2012; Mus et al., 2013). This data suggest cells reallocated nitrogen resources to maintain functionality during a metabolism shift to lipid accumulation during N depletion.

Nile Red fluorescence intensity is an indicator of neutral lipid content and there was a slight increase in fluorescence after phosphate was depleted. However, a more drastic increase in NR signal was observed once nitrate was depleted and cells exit

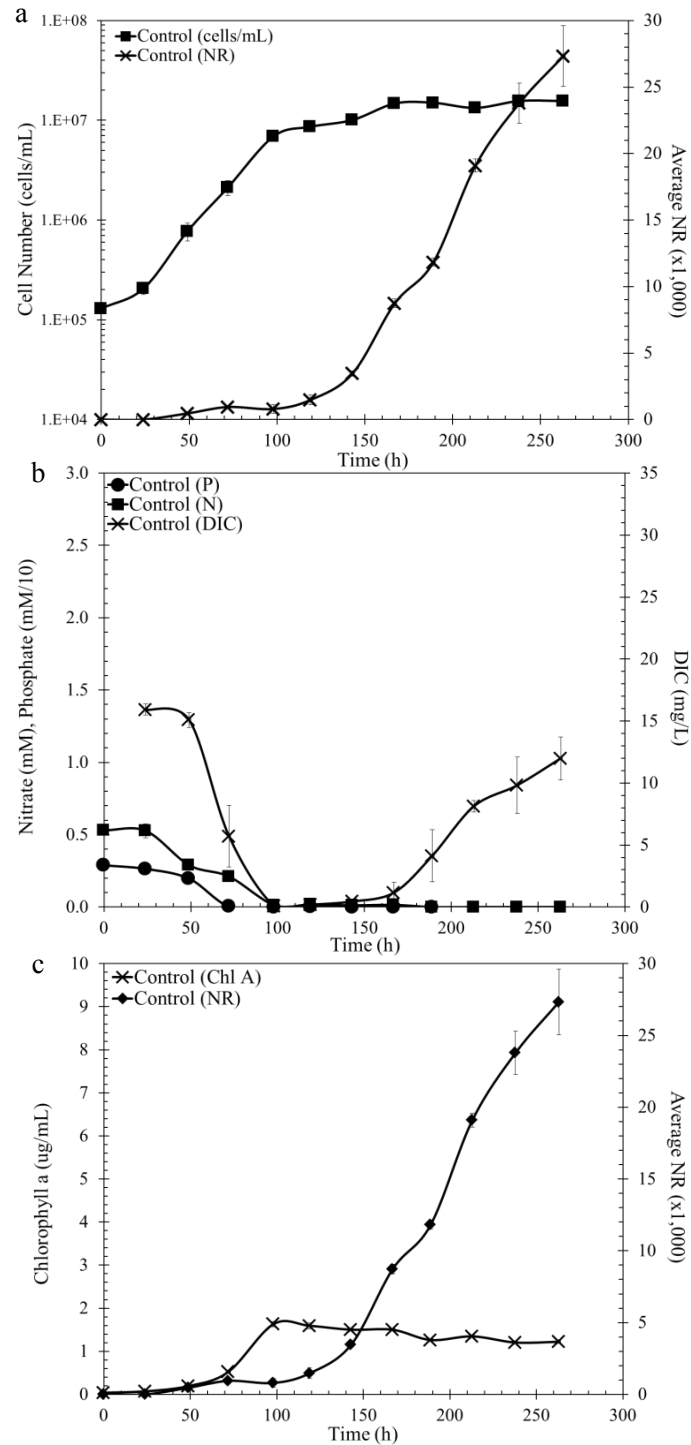


Figure 2.1. *P. tricornutum* growth parameters under control conditions, no nutrient re-supplementation. A) Growth curve cells/mL (■) and Nile Red fluorescence intensity (×). B) DIC (×), NO_3^- (■), PO_4^{3-} (●) throughout growth, (phosphate was multiplied by a scaling factor of 10). C) Chlorophyll a (×) concentrations and Nile Red fluorescence intensity (◆).

exponential-phase growth and entered stationary phase (Fig. 2.1a). The specific fluorescence is the NR signal divided by cell numbers. When cells exited exponential-phase (119hr), the specific fluorescence was 1.7 and increased 10.3-fold to 17.5 (263hr) as cells remained in stationary-phase (Fig. 2.7b). Cells did not have access to nitrogen or phosphorus but were still able fix carbon as it was continually being sparged into the system and reducing equivalents were presumably available from photosynthesis. In exponential-phase, the DIC became “limited,” but not depleted (Fig. 2.1b). However, the DIC recovered as cells entered stationary-phase during lipid accumulation. The fixed carbon is presumably going into lipid as cell number, protein, and carbohydrate content does not increase (Valenzuela et al., 2012). The DIC requirement for lipid accumulation may be lower than the DIC requirement during cellular exponential growth.

P depleted (N replete and re-supplementation effects)

To distinguish the effects of phosphate alone on lipid accumulation, we maintained nitrogen replete conditions as phosphate was depleted. There was no difference in the growth rates between control and nitrogen replete cells or the nitrogen re-supplemented cells. In nitrate replete conditions, phosphate was depleted at 92 hours and a slight increase in NR fluorescence was observed (approximately 2-fold, Fig. 2.2a). As nitrate remained in excess during N replete conditions, the DIC was limited as the carbon requirement for cellular growth was high and resulted in low lipid yields (Fig. 2.2b). To test nitrogen re-supplementation nitrate was re-supplied (189 h) after initial

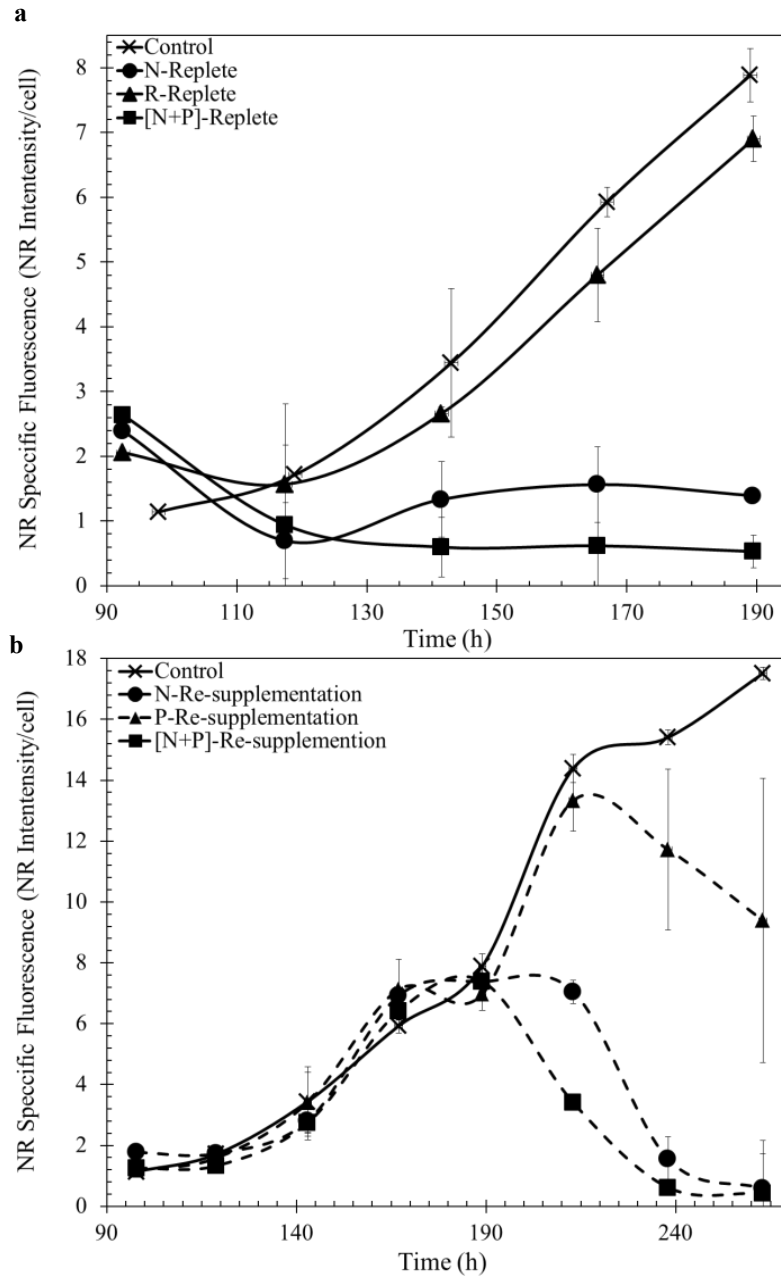


Figure 2.7. Nile Red specific fluorescence (Nile Red fluorescence intensity/cell) in *P. tricornutum*. Graphs start at 90 hours to show time-points of initial nitrate depletion (92.5 hours or 98 hours). A) Specific Nile Red fluorescence in control conditions and nutrient replete conditions: Control (×), N replete (●), P replete (▲), N+P replete (■). B) Specific Nile Red fluorescence in control conditions and nutrient re-supplemented conditions: Control (×), N re-supplemented (●), P supplemented (▲), N+P supplemented (■).

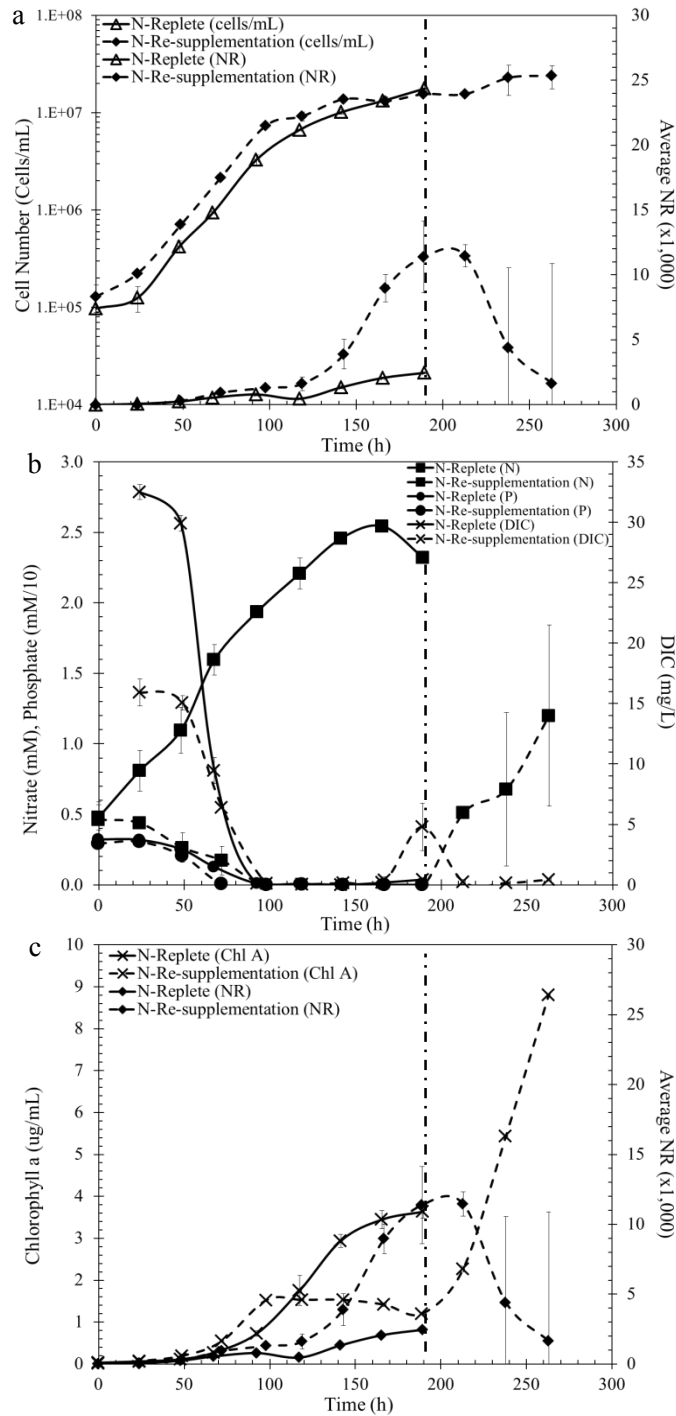


Figure 2.2. *P. tricornutum* growth parameters during continued N replete conditions (solid lines) and N re-supplemented (dashed lines), meaning phosphorus stress conditions. The dashed vertical line indicates where N was re-supplemented at 189 hours. A) Growth curve cells/mL (△, ◆) and Nile Red fluorescence intensity (△, ◆). B) DIC (×), NO_3^- (■), PO_4^{3-} (●) throughout growth, (phosphate was multiplied by a scaling factor of 10). C) Chlorophyll a (×) concentrations and Nile Red fluorescence intensity (◆).

nitrate depletion and an increase in cell number was observed (Fig. 2.2a). Congruently, a decrease in lipid accumulation was observed post-nitrate amendment (7-fold NR fluorescent intensity decreased between 189 and 263 hour time points) (Fig. 2.2a). As with nitrate replete conditions DIC concentrations rebounded as there was not a high carbon requirement for growth, but a smaller requirement for lipid accumulation prior to re-supplementation. However when re-supplemented with nitrate, the cells shifted metabolism to cellular growth (increased cell numbers) and the DIC usage increased (*i.e.*, DIC concentrations decreased) as lipid levels declined. Chlorophyll *a* increased along the growth curve with nitrogen replete conditions as expected and when nitrate was re-supplemented after stationary phase an increase in chlorophyll *a* (Fig. 2.2c) was observed, suggesting a switch from lipid accumulation to biomass production during nitrate assimilation.

N depleted (P replete and re-supplementation effects)

Growth rates during phosphate replete and re-supplemented conditions were similar to controls (Fig. 2.3a). Cell numbers did not increase when re-supplemented with phosphate as was observed when nitrogen was re-supplemented. During replete conditions phosphate remained elevated and nitrate was depleted at 92 hours (Fig 2.3b). After depletion of nitrogen, the lipid content increased 4.4-fold in specific NR fluorescence units (Fig. 2.3a, Fig. 2.7a). After phosphate re-supplementation (189hr), lipid content declined but not to the extent observed during nitrogen re-supplementation. Additionally, the DIC available during phosphate replete and re-supplementation

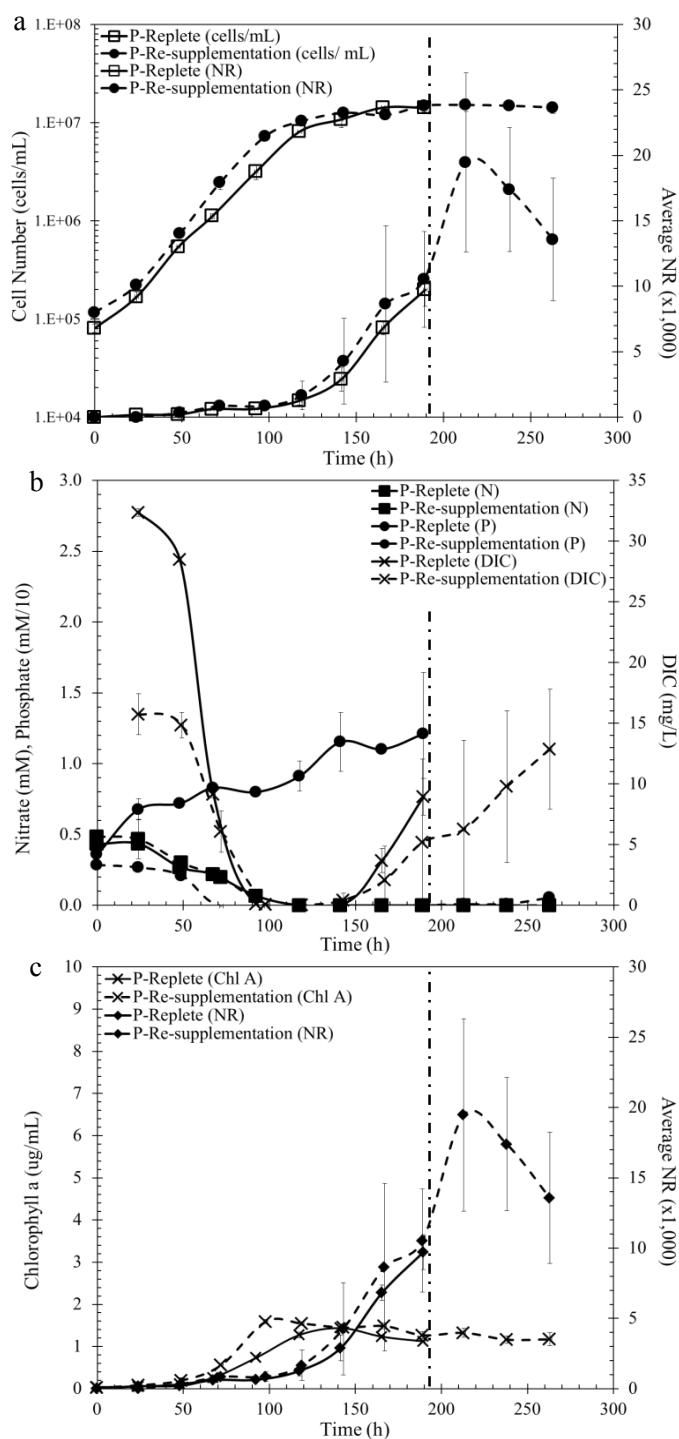


Figure 2.3. *P. tricornutum* growth parameters during continued P replete conditions (solid lines) and P re-supplemented (dashed lines), meaning nitrogen stress conditions. The dashed vertical line indicates where N was re-supplemented at 189 hours. A) Growth curve cells/mL ($\square$, $\bullet$) and Nile Red fluorescence intensity ($\square$, $\bullet$). B) DIC ($\times$), NO_3^- (n), PO_4^{3-} ($\bullet$) throughout growth, (phosphate was multiplied by a scaling factor of 10). C) Chlorophyll a ($\times$) concentrations and Nile Red fluorescence intensity ($\blacklozenge$).

conditions rebounded to DIC levels similar to control conditions (Fig. 2.3b). These results suggest phosphate re-supplementation cannot shift cells back to a cellular growth condition compared to the strong effects of nitrogen re-supplementation. Chlorophyll *a* was not affected by excess phosphate during replete conditions nor did levels change when the growth medium was re-supplemented with phosphate (Fig. 2.3c).

N + P Replete and Supplementation Effects

When cells were provided with excess nitrate and phosphate, the growth rate did not increase. However, when N and P were re-supplemented after reaching stationary-phase at 189 hours, cell numbers and chlorophyll increased (Fig. 2.4a and c). During replete conditions, N and P remained in excess and DIC remained limiting during growth and biomass production (Fig. 2.4b). This result is complementary to low specific NR fluorescence (0.53 NR specific fluorescence) (Fig. 2.4a, Fig. 2.7A). Cells remained in a cell growth state, as suggested by high chlorophyll content (Fig. 2.4c). When cells reached stationary-phase due to N and P depletion and initiated lipid accumulation, the re-supplementation of N and P caused a drastic decrease in lipid content. At 189 hours the NR fluorescence intensity was at 9,070 but declined to a fluorescence intensity of 1,067 within 74 hours (Fig. 2.4a). The 85% decrease in fluorescence indicated a shift to cellular growth associated with the arrest and consumption of lipids. The DIC levels decreased and remained low post re-supplementation with N and P, and these results indicate the carbon requirement during cellular growth is higher than for lipid accumulation, as would be predicted from a carbon mass balance (Fig. 2.4b). As with

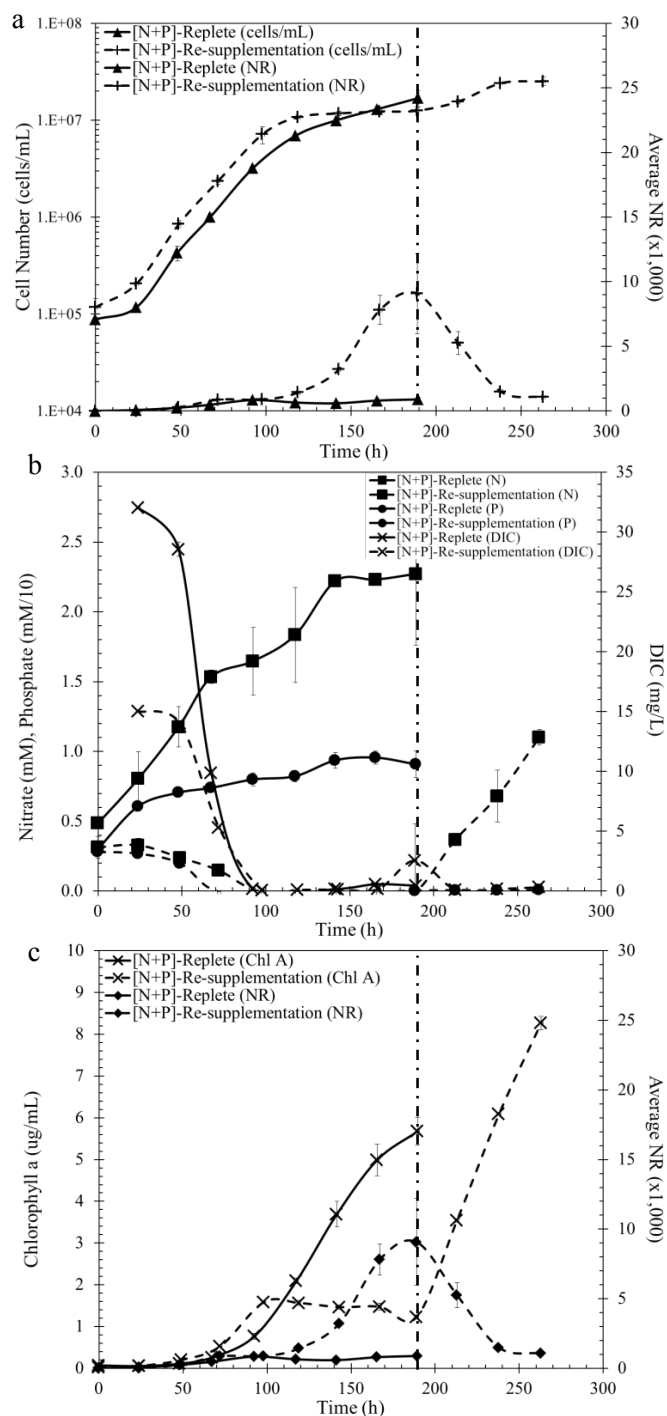


Figure 2.4. *P. tricornutum* growth parameters during continued N + P replete conditions (solid lines) and N + P re-supplemented (dashed lines), meaning no nutrient stress conditions. The dashed vertical line indicates where N was re-supplemented at 189 hours A) Growth curve cells/mL (▲,+) and Nile Red fluorescence intensity (▲,+). B) DIC (×), NO_3^- (■), PO_4^{3-} (●) throughout growth, phosphate was multiplied by a scaling factor of 10. C) Chlorophyll a (×) concentrations and Nile Red fluorescence intensity (◆).

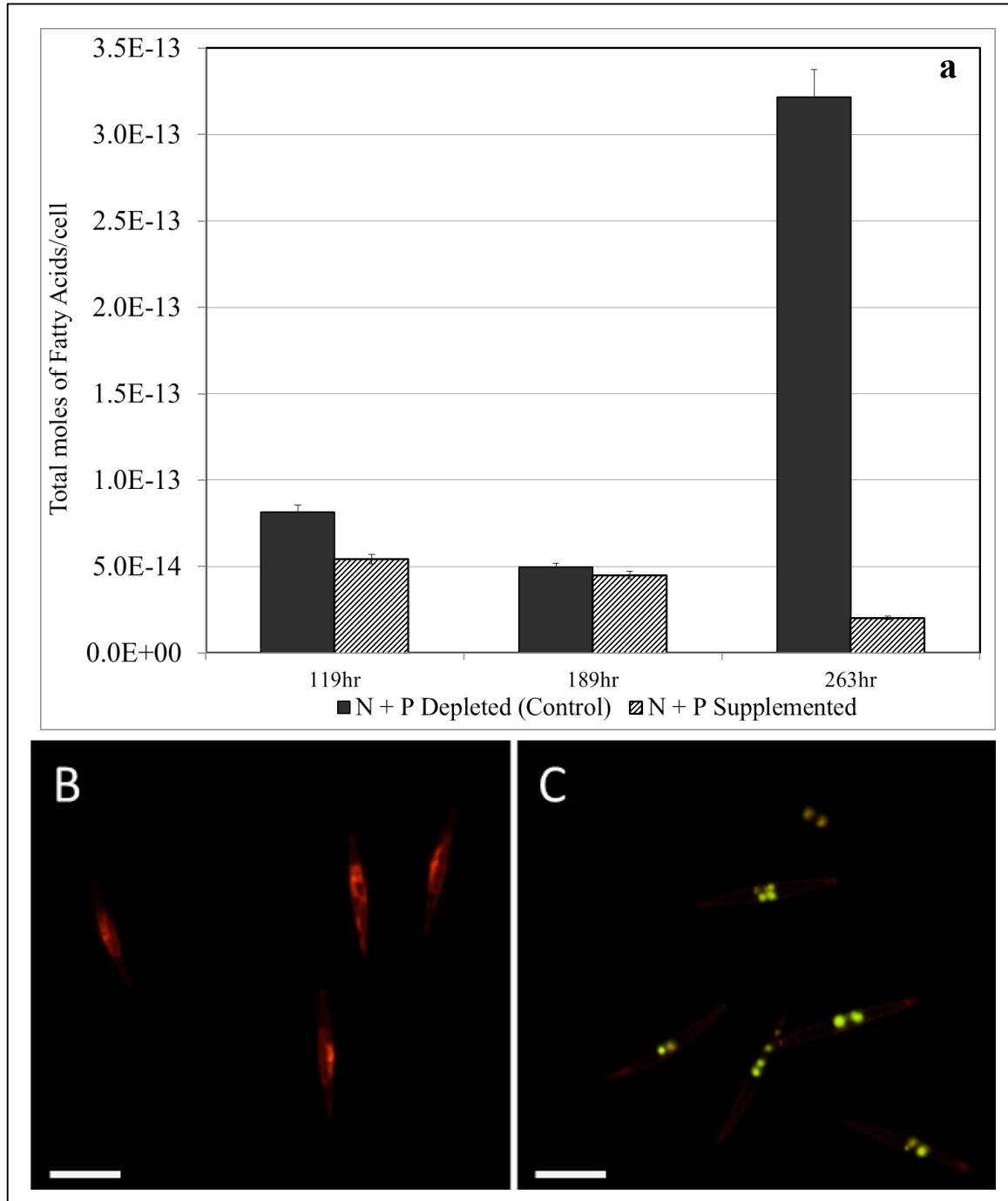


Figure 2.5. *P. tricornutum* lipid accumulation. A) Total moles of FAMES per cell in control (N + P depleted cells) compared to (N + P re-supplemented cells). B) Cells stained with Nile Red during N + P re-supplemented conditions. C) Cells stained with Nile Red under N + P depleted conditions, showing lipid bodies in yellow. Scale bars, 10 μ m.

nitrate re-supplementation alone, chlorophyll increased upon N and P re-supplementation (Fig. 2.4c). These results indicate a significant metabolic shift from stationary-phase lipid accumulation to cellular growth.

Fatty Acid Methyl Ester (FAME) analysis

The largest differences in NR fluorescence intensities were observed between control cells under N and P depletion and the N + P supplemented cells. The NR fluorescence of the control culture paralleled N + P re-supplemented cells at 119h and 189h, before differentiating at 263h making them good candidates for FAME analysis and quantification. Total FAMEs were quantified based on standard curves and calculated in moles of original fatty acids per cell. The fatty acid levels for control (N+P depleted) and N+P supplemented cells were not significantly different at the 119 and 189 h time points (Fig. 2.5a). However, at 263h the nutrient depleted cells had approximately 16-fold greater FAME levels compared to nutrient re-supplemented cells. This result was supported by the NR stained epifluorescence microscopy images (Fig. 2.5b and c). Cells under N + P deplete conditions have large visible lipid bodies that fluoresce yellow, while N + P re-supplemented conditions do not have visible lipid bodies (Fig. 2.5b and 2.5c). The FAME profile of the control culture for *P. tricornutum* is shown in Figure 2.6 on a logarithmic scale. The control profile indicates changes in fatty acid content during lipid accumulation. The most abundant three fatty acids were hexadec-9-enoic acid, hexadecanoic acid, and 5,8,11,14,17-eicosapentaenoic acid. Hexadec-9-enoic acid and hexadecanoic acid are both C16 hydrocarbons, while 5,8,11,14,17-eicosapentaenoic acid is a C20 unsaturated hydrocarbon.

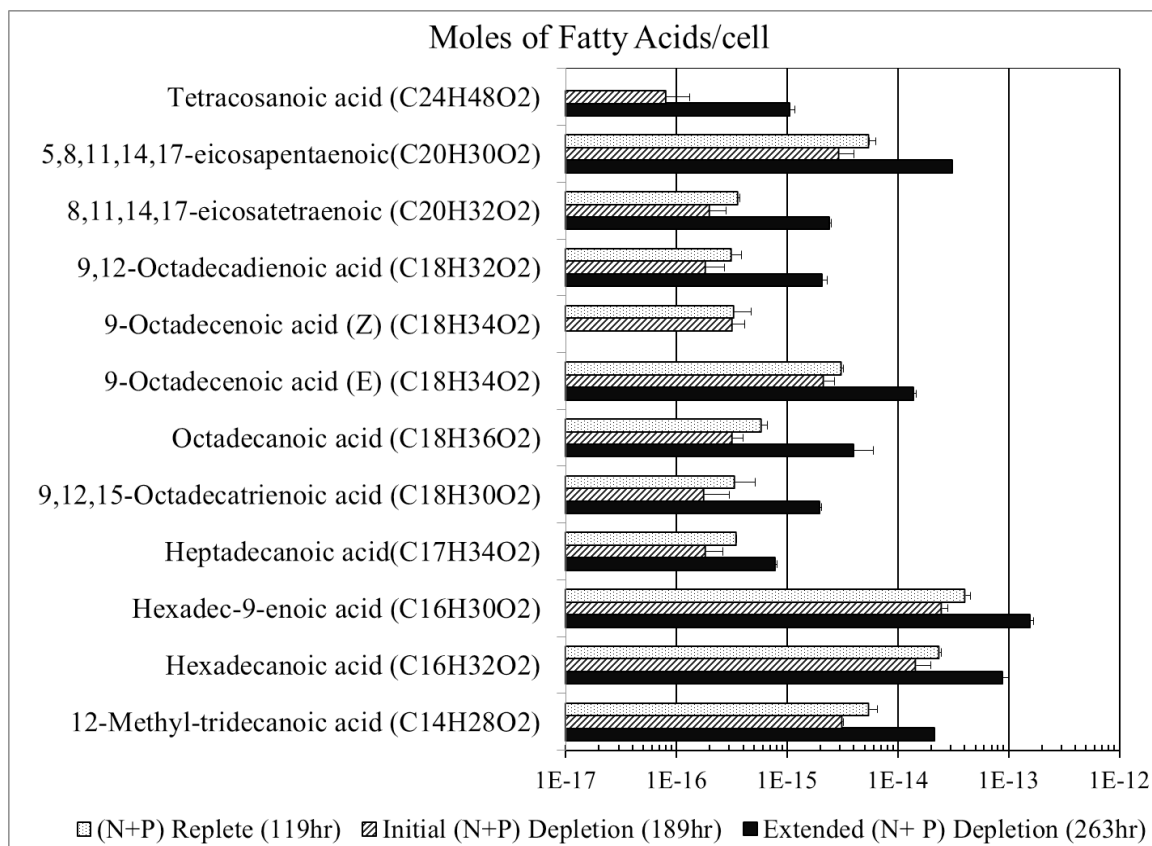


Figure 2.6. *P. tricornutum* fatty acid profiles and abundance (moles/cell) from control cells during hours 119, 189, and 263 of growth. Nutrient depletion occurred at hour 189. Results are shown on a logarithmic scale.

Discussion

P. tricornutum has been shown to increase TAG accumulation when nutrient stressed, particularly from nitrogen or phosphorus (Reitan et al., 1994; Larson and Rees, 1996; Yu et al., 2009; Burrows et al., 2012; Valenzuela et al., 2012). In the control condition, extracellular phosphate was depleted before nitrate but did not cause cell growth cessation. However, lipid accumulation was initiated to a small extent. This may be due to the ability of *P. tricornutum* to store phosphate internally as polyphosphate (Leitao et al., 1995). Thus, cells were most likely not completely limited because internal phosphate stores might have been available, but as exogenous nitrate depleted and the

degradation of chlorophyll and recycling of amino acids proceeded (Lee et al., 2012), lipid accumulation ensued. During exponential growth, DIC was limited due to high cell demand, but DIC concentration in the medium rebounded as growth stopped and lipids were accumulated. There is a clear distinction of lowered carbon fixation needs during lipid accumulation compared to cellular growth/replication. Protein production would be limited without available nitrogen: however carbon fixation still occurs and can be directed into energy storage molecules *i.e.* lipids. This represents a shift in metabolism from cellular growth to lipid accumulation in which the demand for DIC apparently decreases because cells require more carbon for biomass production than for lipid accumulation.

It has been shown that in nitrate deprived *P. tricornutum* cells, lipid biosynthesis was predominantly *de novo* (Burrows et al., 2012). *P. tricornutum* can shift carbon concentrating mechanisms (CCMs) based on DIC demand and use a C4-CCM when DIC concentration is low during growth at high cell densities (Valenzuela et al., 2012). Gene expression levels for TCA enzymes are largely maintained during nitrogen depletion to possibly provide precursors for nitrogen assimilation in the event more nitrogen is encountered (Hockin et al., 2012; Valenzuela et al., 2012). This explains the rapid reversal of lipid accumulation when nitrate is re-supplemented to the depleted cells. The diatom's metabolism is primed for assimilation of nitrogen and quick shifts back toward growth state conditions. As soon as nitrogen becomes available, carbon can be redirected from fatty acid storage to biomass growth and existing lipids are consumed.

In the presence of sufficient nitrogen, phosphate depletion (Fig. 2.2) can initiate lipid accumulation but the extent is limited compared to when nitrogen is depleted and phosphate is replete (Fig. 2.3). This may be explained by the Redfield ratio of 16:1 for N:P, or 15:1 that has been observed in *P. tricornutum* (Redfield, 1934; Zhang and Hu, 2011). The physiological response to phosphate limitation is different than nitrogen limitation. Both result in cell cycle cessation but the relative lipid accumulation response is different. This difference is observed as a 5-fold greater increase in specific fluorescence when cells were depleted of nitrate (6.9 NR specific fluorescence) compared to cells depleted of phosphate (1.4 NR specific fluorescence). However, when depleted together their effects combine for the greatest increase in lipid accumulation. (7.9 NR specific fluorescence) (Fig. 2.7A).

When cells had initiated lipid accumulation based on nutrient depletion, the re-supplementation with nitrate had more of an effect on shifting cells back to a growth state via cellular increases. This is evident in the approximately 13-fold decrease in NR specific signal (Fig. 2.7b), an increase in DIC demand and an increase in cell abundance when nitrate was re-supplemented (Fig. 2.2). However, when cells were re-supplemented with phosphate, the shift away from lipid accumulation was not as drastic. Lipid content did not increase but only slightly decreased post-phosphate re-supplementation. DIC consumption did not increase as observed in a high DIC demand state as seen during exponential growth, nor was there an increase in cell abundance. Similar results were observed by Liang *et. al.* in 2012 in a species of *Chlorella* (Liang et al., 2012) as well as by Feng *et. al.* in *Chlorella zofingiensis* (Feng et al., 2012). Thus, as it pertains to lipid

accumulation, nitrate depletion has a greater effect than phosphate depletion in regards to *P. tricornutum*, both in terms of lipid accumulation and subsequent lipid consumption after re-supplementation. One reason for this may be the difference in reducing equivalents required to utilize nitrate and phosphate. Unlike phosphate, nitrate needs to be reduced for utilization. Thus, the reduction of nitrate consumes electrons that would otherwise go towards carboxylation in the Calvin cycle or possibly fatty acid synthesis (Beardall et al., 2009; Oroszi et al., 2011). In addition, as mentioned above, nitrogen demand exerts a greater effect on resource allocation due to the ratio of C:N:P in biomass; and therefore, carbon and nitrogen metabolism is inherently linked via metabolites (*i.e.* α -ketoglutarate-glutamine-glutamate) and is enriched in biomass ($C_{106}:N_{16}:P_1$).

Independently, nitrate and phosphate depletion can cause lipid accumulation, but maintaining replete conditions or re-supplementation of media can drastically inhibit or reduce lipid accumulation, respectively (Fig. 2.4). In *P. tricornutum*, nitrogen and phosphorus depletion together have a greater impact on lipid accumulation than compared to the nutrient effects independently. The metabolic mechanisms between phosphate and nitrate stress are different but the metabolic consequences could be similar. Cyclins (*cycs*) and cyclin-dependent kinases (CDKs) work together to ensure correct passage through the cell cycle. Many different cyclins and CDKs have been shown to sense and respond to nutrient availability (Huysman et al., 2010), such as phosphate and nitrate. Our recent results showed that similar cyclins responded to nutrient stress as detected via transcriptome profiling in *P. tricornutum* (Valenzuela et al.,

2012). Thus, there is a molecular consequence to nutrient deprivation leading to cellular responses that favor lipid accumulation, such as the increase in Acetyl-CoA-Carboxylase during initial lipid accumulation (Valenzuela et al., 2012). However, the ability to accumulate lipids can be quickly reversed in response to nutrients and might suggest the presence of signal transduction systems to coordinate cellular growth versus lipid accumulation in a nutrient-dependent manner. Therefore, the manipulation of nutrient-dependent cyclins could be used to control resource allocation via cell cycle inhibition.

Fatty acid methyl ester analysis quantifies how specific nutrient depletion is critical for lipid accumulation in *P. tricornutum* (Fig. 2.5 and 2.6). The three major fatty acids that increased were hexadec-9-enoic acid, hexadecanoic acid, and 5,8,11,14,17-eicosapentaenoic acid. This coincides with results from a recent study by (Řezanka et al., 2012) where these three fatty acids were most prevalent in *P. tricornutum* as well. The authors reported an 85% increase in TAG assemblies with 5,8,11,14,17-eicosapentaenoic acid and two palmitic fatty acids (hexadec-9-enoate, and hexadecanoic acid) based on phosphate starvation. Our results are similar but not exactly the same and the difference may be attributed to the additional nitrogen depletion stress. In both studies, an increase in C16 and C16:1 fatty acids was observed in response to nutrient deprivation, and total lipids increased 16-fold with depletion of nitrate and phosphate compared to re-supplementation conditions (Fig. 2.5).

In conclusion, *P. tricornutum* accumulated lipid under phosphate depletion and under nitrate depletion independently, but the accumulation was magnified when both nutrients were depleted. Under N + P replete conditions the diatom does not accumulate

lipid because carbon may be directed into cellular biomass production and growth as evident by higher cell numbers, high DIC demand, and low to no lipid accumulation. Re-supplementation with phosphate after lipid accumulation can decrease accumulation and increase consumption of fatty acid stores as cells shift away from a lipid accumulating state. Nitrate re-supplementation shuts down lipid accumulation and presumably increases lipid consumption to a greater extent than phosphate re-supplementation as cells shift to a cellular growth state signified by increased cell numbers. The re-supplementation with both nitrate and phosphate arrested lipid accumulation and shifted cells to growth conditions with corresponding consumption of lipids. Cell numbers and DIC demand increases, signifying the metabolic shift back to biomass growth and away from lipid accumulation. FAME profiles remained similar throughout nutrient depletion growth with the largest abundance increases in the C16 fatty acids, hexadec-9-enoic acid, and hexadecanoic acid. Thus, when growing algae for the purpose of biofuel production, the control of exogenous nutrient levels is imperative to control cellular accumulation versus lipid accumulation regardless of whether algae are being cultivated in raceway ponds, photobioreactors, or wastewater effluents.

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CHAPTER 3

POTENTIAL ROLE OF MULTIPLE CARBON FIXATION PATHWAYS DURING LIPID ACCUMULATION IN *PHAEODACTYLUM TRICORNUTUM*

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

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Contributions: Collected and analyzed output data, and wrote the manuscript.

Co-Author: Type Co-author Aurelien Mazurie

Contributions: Constructed Cufflinks pipeline for expression value calculations

Co-Author: Ross P Carlson

Contributions: Localization of genes, obtained funding, discussed the results and implications, provided valuable insight, and edited the manuscript

Co-Author: Robin Gerlach

Contributions: Discussed the results and implications, provided valuable insight, and edited the manuscript

Co-Author: Keith E Cooksey

Contributions: Obtained funding, discussed the results and implications, provided valuable insight, and edited the manuscript

Co-Author: Brent M Peyton

Contributions: Obtained funding, discussed the results and implications, provided valuable insight, and edited the manuscript

Co-Author: Matthew W Fields

Contributions: Obtained funding, discussed the results and implications, provided valuable insight, and edited the manuscript

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Title: Potential Role of Multiple Carbon Fixation Pathways During Lipid Accumulation in *Phaeodactylum tricornutum*

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Abstract

Background. *Phaeodactylum tricornutum* is a unicellular diatom in the class *Bacillariophyceae*. The full genome has been sequenced (<30 Mb), and approximately 20 to 30% triacylglyceride (TAG) accumulation on a dry cell basis has been reported under different growth conditions. To elucidate *P. tricornutum* gene expression profiles during nutrient-deprivation and lipid-accumulation, cell cultures were grown with a nitrate to phosphate ratio of 20:1 (N:P) and whole-genome transcripts were monitored over time via RNA-sequence determination.

Results. The specific Nile Red (NR) fluorescence (NR fluorescence per cell) increased over time; however, the increase in NR fluorescence was initiated before external nitrate was completely exhausted. Exogenous phosphate was depleted before nitrate, and these results indicated that the depletion of exogenous phosphate might be an early trigger for lipid accumulation that is magnified upon nitrate depletion. As expected, many of the genes associated with nitrate and phosphate utilization were up-expressed. The diatom-specific cyclins *cyc7* and *cyc10* were down-expressed during the nutrient-deplete state, and cyclin B1 was up-expressed during lipid-accumulation after growth cessation. While many of the genes associated with the C3 pathway for photosynthetic carbon reduction were not significantly altered, genes involved in a putative C4 pathway for photosynthetic carbon assimilation were up-expressed as the cells depleted nitrate, phosphate, and exogenous dissolved inorganic carbon (DIC) levels. *P. tricornutum* has multiple, putative carbonic anhydrases, but only two were significantly up-expressed (2-fold and 4-fold) at the last time point when exogenous DIC levels had increased after the

cessation of growth. Alternative pathways that could utilize HCO_3^- were also suggested by the gene expression profiles (*e.g.*, putative propionyl-CoA and methylmalonyl-CoA decarboxylases).

Conclusions. The results indicate that *P. tricornutum* continued carbon dioxide reduction when population growth was arrested and different carbon-concentrating mechanisms were used dependent upon exogenous DIC levels. Based upon overall low gene expression levels for fatty acid synthesis, the results also suggest that the build-up of precursors to the acetyl-CoA carboxylases may play a more significant role in TAG synthesis rather than the actual enzyme levels of acetyl-CoA carboxylases *per se*. The presented insights into the types and timing of cellular responses to inorganic carbon will help maximize photoautotrophic carbon flow to lipid accumulation.

Key Words: algae, diatom, lipid-accumulation, transcriptomics, biofuel, carbon fixation, RNA-seq, bio-oil

Background

Since the industrial revolution, the infrastructure of our society has relied strongly on petroleum-based products for fuels, materials, and specialty chemicals. For the last hundred years, the use of petroleum has been possible due to a balance between supply and demand. However, the increased consumption of energy has created an environment where need could begin to exceed supplies (Brown, 2003). Perhaps even more important are the environmental impacts of petroleum consumption. In terms of carbon, the fossil fuels consumed in one year release 44×10^{18} g of carbon, and this is 400-fold the amount of annual carbon fixed during net primary productivity by the global biota (Dukes, 2003). This is a massive influx of carbon into the atmosphere mediated through the burning and

consumption of petroleum-based products, and it is becoming increasingly clear that renewable biofuels (*e.g.*, ethanol, butanol, H₂, CH₄, biodiesel) are needed to help replace petroleum-dependence in the United States and the world. However, while present technology can be used to help circumvent and reverse current environmental trends, both fundamental and applied research is needed to advance the feasibility and utility of renewable energy sources that use direct phototrophic CO₂-fixation into liquid biofuels.

Diatoms are a diverse group of eukaryotic unicellular microalgae that account for up to 40% of the total marine primary production each year (Falkowski and Raven, 1997; Field, 1998; Granum et al., 2005). In addition to photoautotrophic growth (*i.e.*, carbon fixation via sunlight), some green algae and diatoms can store carbon and energy in the form of lipids (*i.e.*, triacylglycerides, TAGs), and this fact has re-invigorated the possibilities of algal oil being used for the production of liquid fuels. Chisti (2007) estimates that biodiesel from microalgae would only take 3% of the arable crop land in the U.S. to replace 50% of the country's liquid transportation fuel needs (Chisti, 2007). Couchesne et. al. 2009 (Courchesne et al., 2009) reviewed many of the efforts focused on increasing microalgal lipid production, including biogeochemical and genetic approaches. Although we understand many aspects of carbon assimilation in diatoms, the direct responses and contributive flow of inorganic carbon to lipid accumulation are not known for many eukaryotic photoautotrophs under different growth conditions (Roberts et al., 2007a). Most approaches have had some success, but there is still much work to be done to fully understand the efficient and economical enhancement of lipid production in microalgae.

The marine diatom, *Phaeodactylum tricornutum*, is classified in the phylum *Bacillariophyta*, and this phylum comprises one-third of all known marine phytoplankton. *P. tricornutum* is a chlorophyll c-containing alga known as a heterokont (Allen et al., 2006), and has been studied as a ‘model’ diatom in the context of physiology, biochemistry, and genomics. *P. tricornutum* is a pleomorphic diatom that has been isolated and classified into 10 different strains over the last century based upon genetic and phenotypic differences (Martino et al., 2007). *P. tricornutum* 8.6 (CCAP 1055/1; CCMP2561; strain Pt1) has a major morphotype of fusiform and was selected for whole genome sequence determination. The Pt1 strain has a 27.4 Mb genome with over 10,000 predicted genes (Bowler et al., 2008), and the chloroplast genome sequence has also been determined (117,000 bp; 162 genes) (Oudot-Le Secq et al., 2007). This wealth of genomic knowledge has revealed the evolutionary lineage of diatoms and has also uncovered the physiological potential of lipid-accumulating diatoms and green algae. Accompanying the sequenced genomes are over 130,000 ESTs (Expressed Sequence Tags) from 16 different growth conditions of *Phaeodactylum tricornutum* (Maheswari et al., 2010), and the data is compiled in the Diatom EST Database (Maheswari, 2004).

This extensive research background is the foundation for using Pt1 as a model diatom system to better understand cellular responses during lipid-accumulation under different growth conditions. Nutrient deficiency or nutrient stress has been well documented to increase TAG accumulation in microalgae (Sheehan et al., 1998). Specifically, nitrogen- or phosphate-limitation can increase lipid accumulation in

numerous microalgae (Hu et al., 2008), and TAG accumulation in *Phaeodactylum* has been studied under nitrogen depletion (Hu et al., 2008). In the current study we used the

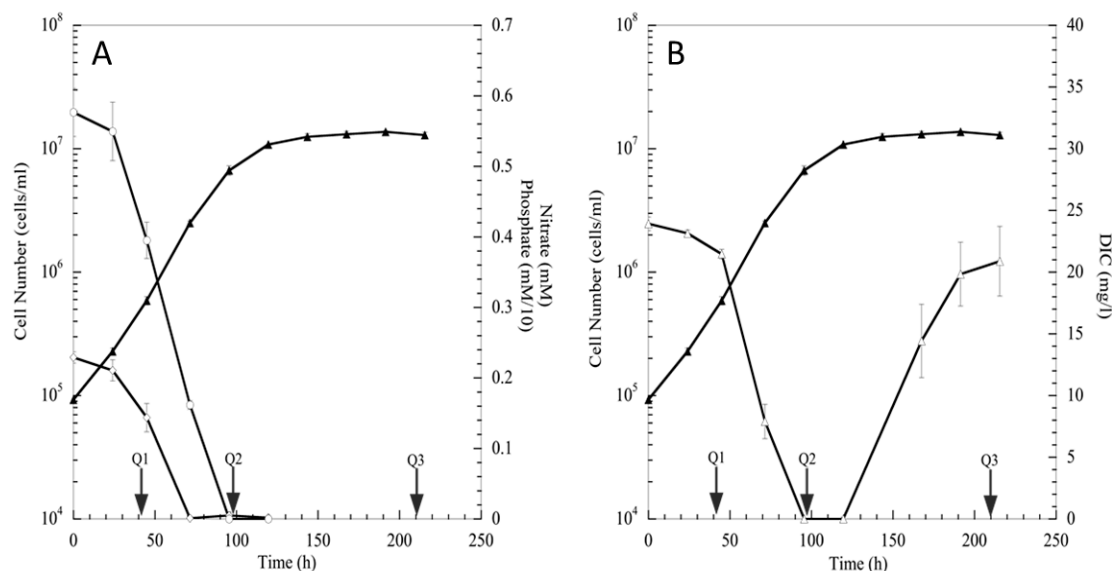


Figure 3.1. Growth characterization of *P. tricornutum*. Cell density growth curve of *P. tricornutum* cells (▲) showing depletion of exogenous nitrate (○) and phosphate (◇). Phosphate concentrations are multiplied by a factor of 10 for visualization (A). Cell density growth curve showing the depletion and rebound of dissolved inorganic carbon (△) throughout *P. tricornutum* growth (B). Arrows indicate time points at which cells were harvested for RNA sequencing analysis.

model diatom, *P. tricornutum* strain Pt1, to characterize global gene expression via RNA-seq during enhanced lipid production as a consequence of nitrogen- and phosphate-depletion.

Results and Discussion

Depletion of Nitrate and Phosphate. Sodium nitrate and potassium phosphate were the only sources of nitrogen or phosphorus available during growth of *P. tricornutum*, and ASPII medium was used as recently described (Gardner et al., 2012).

The exogenous nitrate and phosphate was monitored daily to determine nutrient availability. The classic Redfield ratio of nitrogen/phosphorus (N/P) is 16:1 (Redfield, 1934; Redfield, 1958) in phytoplankton, however it can be dependent on the source of

	%N	%P	%C	C:N	C:P	N:P	C:N:P
Q1	5.40	1.44	41.2	7.63	28.6	3.75	29:4:1
Q2	7.20	0.85	47.4	6.58	55.7	8.46	56:8:1
Q3	3.46	0.38	53.2	15.4	140.8	9.15	141:9:1

Table 3.1. Elemental analysis of *P. tricornutum* (carbon, nitrogen, and phosphorus) at the three time points (Q1, Q2, and Q3) during nutrient depletion and subsequent lipid accumulation. Values represent averages of duplicates.

nitrogen (Zhang and Hu, 2011). The growth medium in the described experiments had a N:P ratio of approximately 20.5:1 (Provasoli et al., 1957; Cooksey and Cooksey, 1974). Growth parameters and gene expression were measured at three time points, early-exponential (Q1), transition from exponential to stationary-phase (Q2), and stationary-phase (Q3). The growth data (Fig. 3.1a) suggested that nitrogen and not phosphate depletion coincided with the onset of stationary-phase. Exogenous phosphate was depleted after 72 h, but exponential growth continued for another 24 h. At 96 h, the exogenous nitrate was depleted and cells transitioned to stationary-phase within a doubling-period. At this time, a decrease in chlorophyll a content was observed (Fig. 3.10), and these results suggest a recycling of nitrogen rich compounds (e.g., chlorophyll). Similar results have been observed during nitrogen depletion in the green alga, *Neochloris oleoabundans* (Li et al., 2008). Elemental analysis (Table 3.1) revealed that cells in nutrient-replete Q1 had a N:P ratio of 4:1, but with the depletion of both

exogenous nitrate and phosphate, cellular N:P ratios shifted to 8:1 and 9:1 in Q2 and Q3, respectively. These results indicate that under the tested growth conditions of a starting

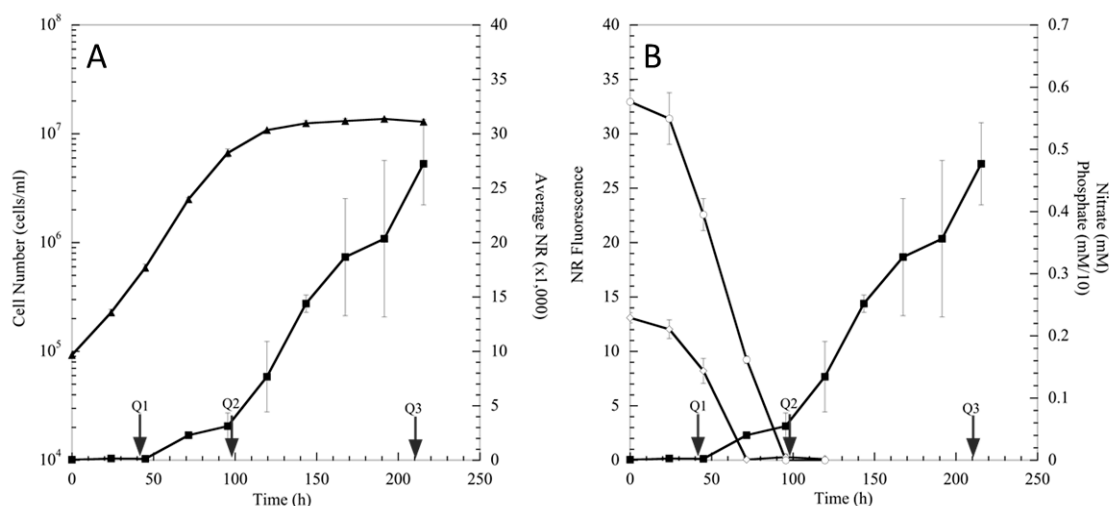


Figure 3.2. Characterization of lipid accumulation in *P. tricornutum* during increase in Nile Red fluorescence intensity (■) with respect to cell number (▲) (A). Nile Red fluorescence intensity indicating the increase in lipids is shown with the depletion of external nitrate (○) and phosphate (◇) (B). Phosphate concentrations are multiplied by a factor of 10 for scaling purposes. Arrows indicate time points at which cells were harvested for RNA sequencing.

exogenous N:P ratio of 20.5:1, cells transitioned to stationary-phase at an approximate cellular N:P ratio of 8 to 9.

The availability of dissolved inorganic carbon (DIC) during the light period was high in early-exponential growth, but decreased below detectable levels during the late-exponential phase (Q2) (Fig. 3.1b). Exogenous DIC declined during the exponential growth phase, and the decline continued past the depletion of exogenous nitrate and phosphate (Fig. 3.1b). The decline in DIC followed cell accumulation during exponential-growth. At the onset of nitrate depletion, the light-phase DIC remained low for approximately 25 h, and these results suggested the culture consumed the DIC at the

mass transfer rate from the air-spargers. The DIC began to increase when the cells entered stationary-phase. The DIC levels at Q3 (approximately 50 h after the depletion of nitrate) increased back to similar levels observed at the initiation of growth (Fig. 3.1). These results indicate lower carbon fixation under nutrient-deprivation and that biological activity was no longer limited by DIC mass transfer.

Lipid-Accumulation Under Nutrient Stress. Throughout *P. tricornutum* growth, lipid accumulation was monitored via the Nile Red (NR) assay as a way to measure relative abundance of triacylglycerols (Cooksey et al., 1987). The NR fluorescent intensities increased significantly as the cells transitioned to stationary-phase (approximately 95 h under the tested growth conditions; Fig. 3.2). The specific NR fluorescence (NR fluorescence intensity/cell) continued to increase after 96 h and these results indicated that the lipid accumulation was not merely a result of increasing cell numbers. After nutrient depletion, the NR specific fluorescence increased 4.5-fold (Fig. 3.2a), and the increased fluorescence coincided with a cessation of population growth (Fig. 3.2a). The data indicate that lipids started to increase as exogenous phosphate was depleted, but the rate (specific NR fluorescence/time) increased upon nitrate depletion (Fig. 2b and 3). A previous study reported that phosphate limitation could increase lipid content in *P. tricornutum*, but not green flagellates (Reitan et al., 1994). Our results suggest that depletion of exogenous phosphate might be an early trigger for lipid accumulation that is magnified upon nitrate depletion; and therefore, the N:P ratio could be an important parameter to monitor when examining mechanisms of lipid accumulation.

The lipid accumulation increased with cell numbers as exogenous DIC concentrations decreased, whereas specific lipid accumulation (NR/cell) increased with subsequent increases in exogenous DIC as biomass accumulation ceased (Fig. 3.3). This

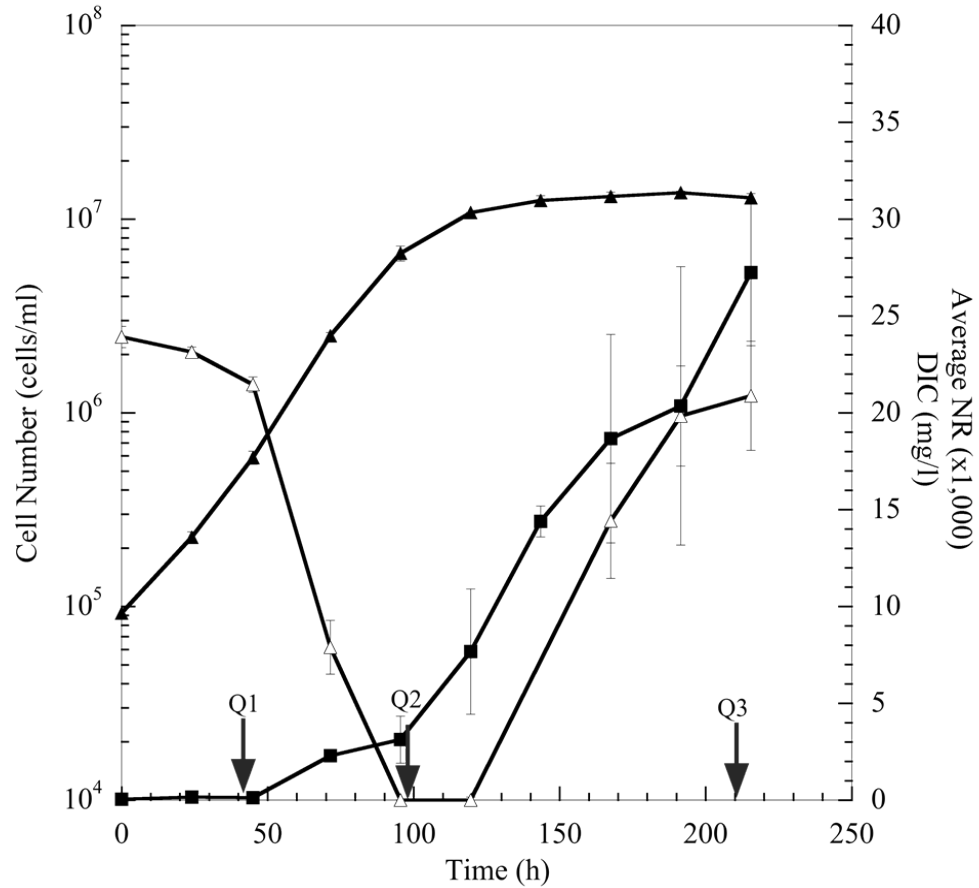


Figure 3.3. Nutrient depletion and cell count growth curve of *P. tricornutum*. Cell density (▲) during the depletion and rebounding of dissolved inorganic carbon (△) and increase in Nile Red fluorescence intensity (■). Arrows indicate time points at which cells were harvested for RNA sequencing.

increase in lipid accumulation could also be characterized in context of the increased cellular carbon to nitrogen ratio (C:N). At Q1 and Q2, the C:N ratio was 7.5:1 and 6.5:1, respectively, which is similar to the Redfield C:N ratio of 6.6. However, as lipids

accumulated, the C:N ratio increased to 15.4:1, and previous studies have documented a similar increase in the C:N ratio after exogenous nitrate depletion (Larson and Rees, 1996). Thus, elemental analysis indicated continued carbon influx after exogenous N and P depletion. In addition, specific cellular carbohydrate (μg carbohydrate/cell) did not

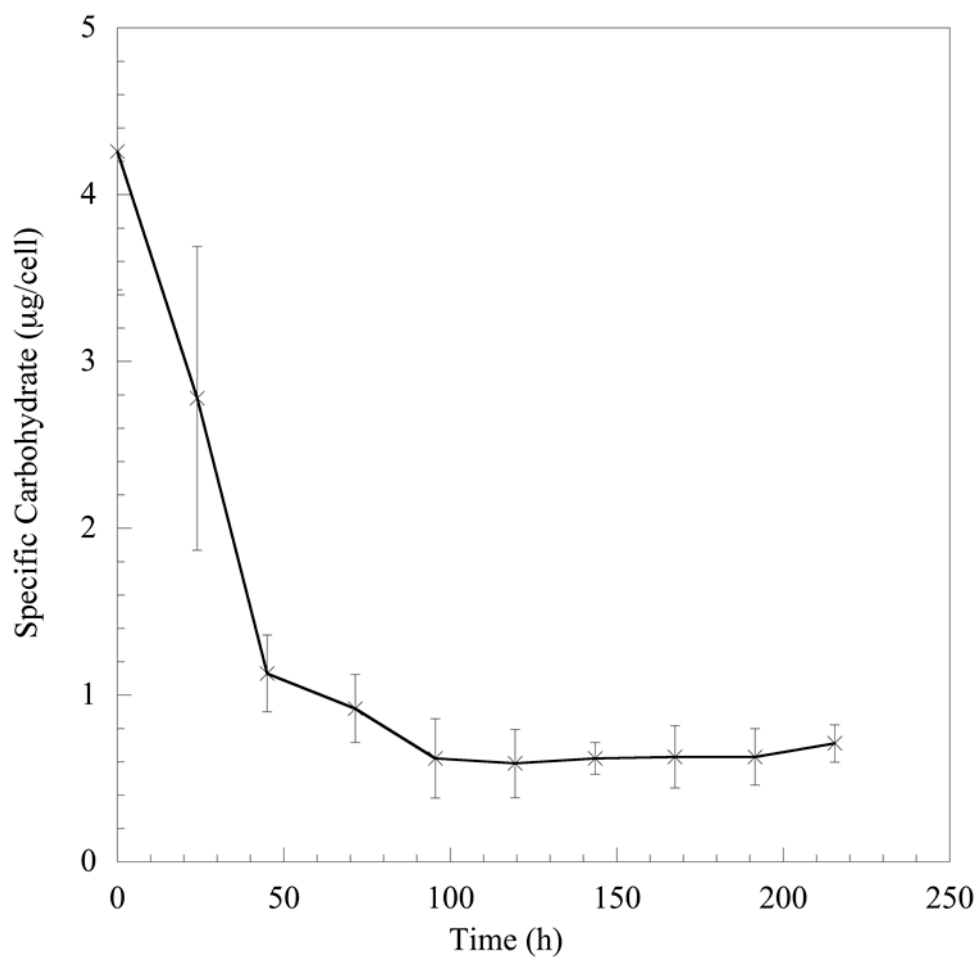


Figure 3.11. Specific carbohydrate (mg/cell) during growth of *P. tricornutum*

increase (Fig. 3.11), and intracellular lipid droplets were observed via epifluorescent microscopy (Fig. 3.12).

Analysis of RNA-sequence Data. A time course assessment strategy was employed to identify transcripts that were differentially expressed during growth and lipid accumulation from early-exponential (Q1), late-exponential (Q2), and stationary-phase cells (Q3) in conjunction with pH, nutrient availability, light, DIC, cell number, protein, carbohydrate, and lipid. Cultures were sampled in duplicate for each time point,

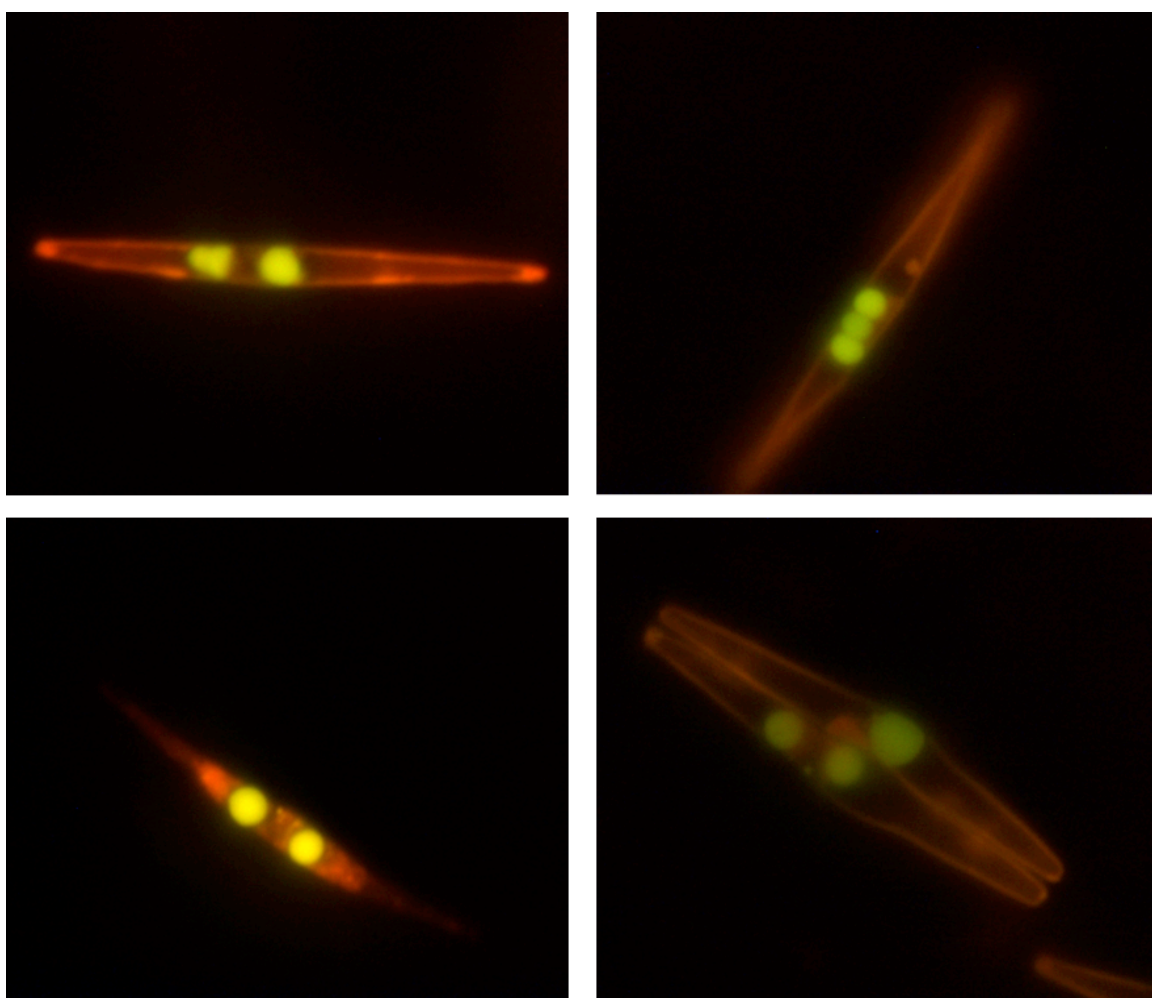


Figure 3.12. Color image of *P. tricornutum* stained with Nile Red. The image was taken with an Infinity 2 color camera (Lumera Corporation) at 600x-magnification using a Nikon Eclipse E800 epifluorescent microscope with a B-2A filter.

total RNA extracted, and each sample sequenced via an individual lane of Illumina sequence determination. RNA-sequence analysis was used to globally monitor gene expression during nutrient-depletion and lipid accumulation under the tested growth conditions. Using TopHat and Cufflinks (see Materials and Methods), the transcript relative abundance was calculated and reported as FPKMs (Fragments Per Kilobase of exon per Million fragments mapped), a normalized quantity that is directly proportional to transcript abundance as recently reported (Mortazavi et al., 2008; Trapnell et al., 2010; Twine et al., 2011). To compare transcript levels between the time points, Q2 and Q3 were reported as the relative ratio compared to Q1. The Q1 time point was considered the basal transcript level condition in which cells were growing exponentially under nutrient-replete conditions with low lipid levels.

Based upon work in bacteria, transcript and protein abundances are not necessarily correlated, although some genes can show similar transcript and protein trends (Clark et al., 2012). Therefore, different data sets (*i.e.*, transcript and protein) can reveal key aspects of the physiological state of the cells. Previous studies have shown differences between transcript and protein abundances for selected genes in diatoms and yeasts (Poulsen and Kröger, 2005; Xu et al., 2010; Foss et al., 2011) while a recent *P. tricornutum* study showed similar abundance trends (34). The presented data are based upon transcript abundances although other mechanisms of control most likely contribute to overall metabolism.

Global Transcript Differential Expression. Each sample (*i.e.*, biological replicate) was sequenced on a respective Illumina lane with an average of 31 million

reads per sample (1 x 54 nucleotides). Cufflinks assembled 30,373 transcripts to 10,125 mapped loci, and 1,259 genes were expressed at statistically significant levels (approximately 12.5% of the genome) at all three time points. Of all significant genes, approximately 180 genes were differentially expressed between all three time points, 546 genes between any two time points, and 177 genes between only two time points. Genes annotated as hypothetical proteins represented 37% of the significantly expressed genes (n=465). For example, the third most abundant transcript detected in Q2/Q1 (500-fold;

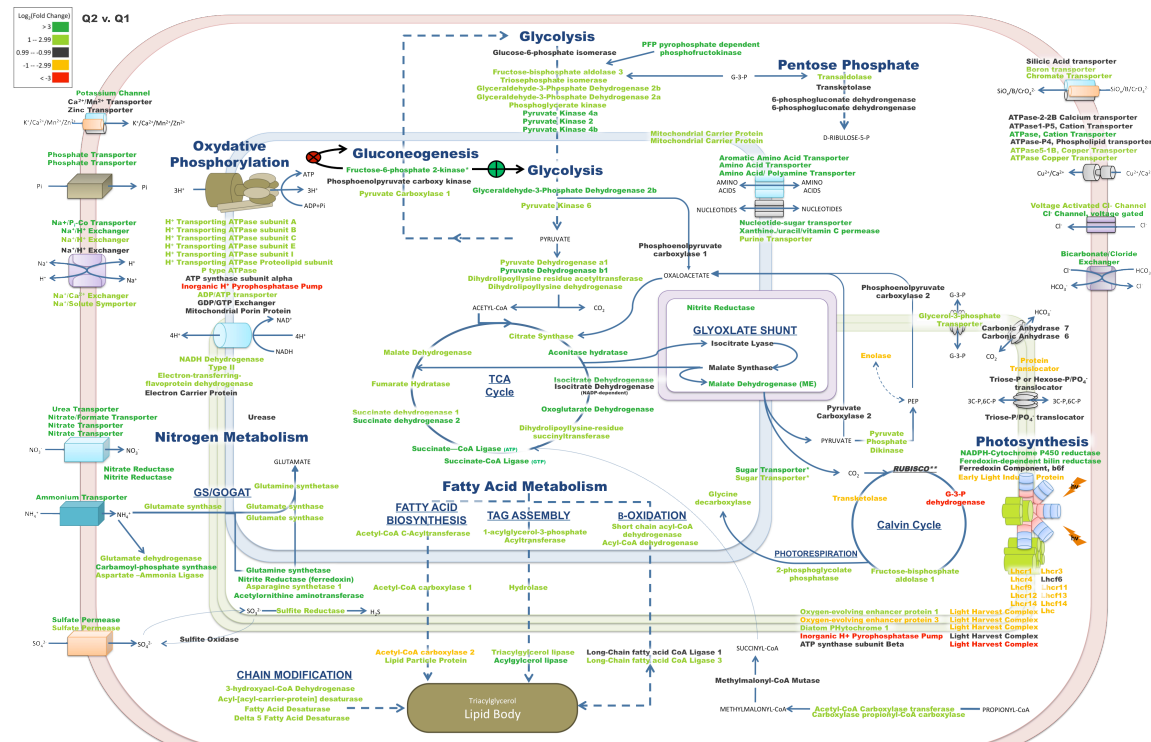


Figure 3.4. Proposed cellular metabolic map for *P. tricornutum* during nutrient depletion and initial lipid accumulation as compared to nutrient replete conditions (Q2 vs. Q1). Differences in fold change are based on log₂ scale. Color scale represents up-expressed (green) and down-expressed (red) genes. Genes are represented within organelles based on literature localizations including probable membrane bound genes.

$p < 0.05$) and Q3/Q1 (136-fold; $p < 0.05$) is a gene of unknown function (55010) that is annotated as a pyridoxal-dependent decarboxylase. The putative *P. tricornutum* protein has significant sequence homology (44%; 6×10^{-159}) with a coccolith-scale associated protein-1 (AB537972.1) from *Pleurochrysis carterae*. *P. carterae* has been shown to accumulate lipids (Moheimani et al., 2011). While the exact role of this putative protein in *P. tricornutum* is not known, it is interesting to speculate a possible role in inorganic carbon homeostasis.

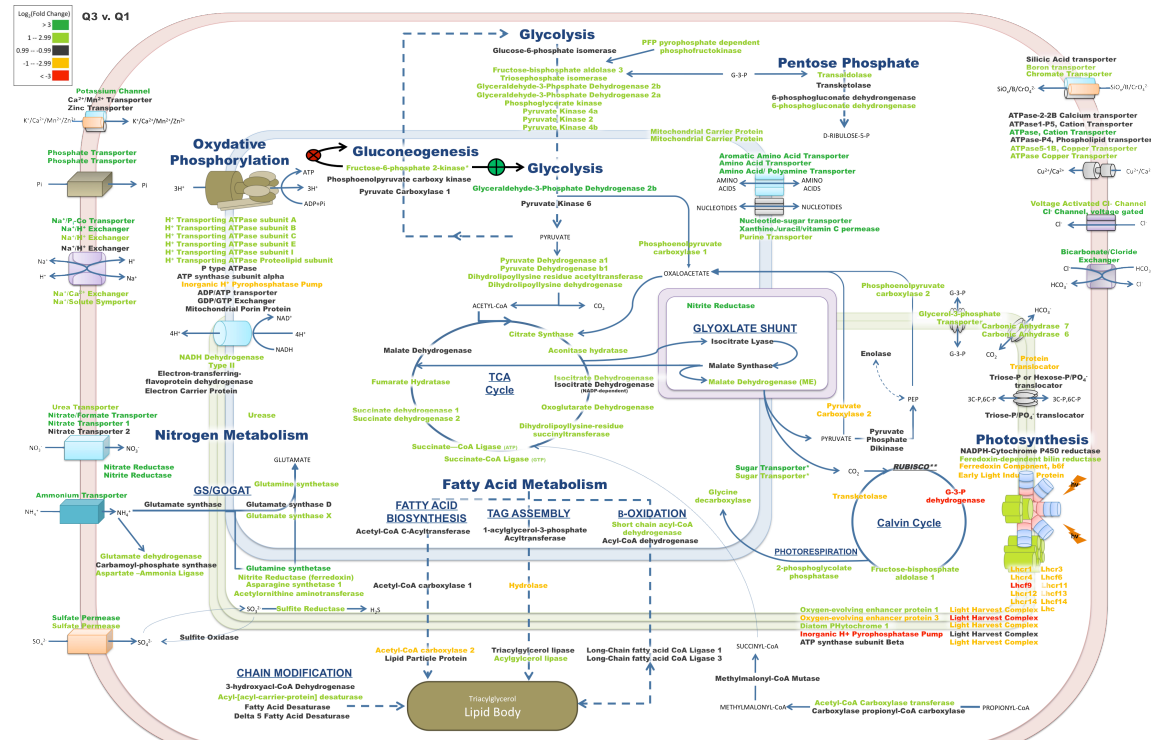


Figure 3.5. Proposed cellular metabolic map for *P. tricornutum* during extended nutrient depletion and lipid accumulation as compared to nutrient replete conditions (Q3 vs. Q1). Differences in fold change are based on log₂ scale. Color scale represents up-expressed (green) and down-expressed (red) genes. Genes are represented within organelles based on literature localizations including probable membrane bound genes.

Approximately 170 transcripts that showed significant changes in abundance were mapped with respect to major carbon pathways and cellular compartmentalization for Q2/Q1 and Q3/Q1 and included nitrogen metabolism, oxidative phosphorylation, photosynthesis, glycolysis, TCA cycle, and fatty acid metabolism (Fig. 3.4 and 3.5, respectively). A majority of the up-expressed genes during exponential-growth phase (Q2) were involved with nitrate and phosphate acquisition or utilization (*e.g.*, nitrate transporter, phosphate transporter, nitrite transporter, nitrate reductase, glutamine synthetase, and putative aspartate aminotransferase). Similar genes and/or functions remained up-expressed during transition to stationary-phase at Q3, but up-expressed genes also included a putative nucleotidase and alkaline phosphatases. The nucleotidase and alkaline phosphatases may be further responses to nutrient deprivation in order to sequester and recycle both nitrogen and phosphate. The largest down-expression at Q2 was the presumptive plastidic glyceraldehyde-3-phosphate dehydrogenase, which may coincide with a decline in photosynthetic carbon flow from 3-phosphoglycerate and phosphoglyceraldehyde phosphate to pentoses and hexoses. Light-harvesting complexes were also significantly down-expressed at Q2, and genes involved in light-harvesting complexes continued to be down-expressed into stationary-phase (Q3). These results suggest that overall energy-generation via light was down-expressed during the light cycle after exogenous N and P depletion.

Cell-cycle. Transcript analysis revealed significant differential expression of 31 transcription factors; however, the target genes are unknown. During Q2, one transcription factor had decreased expression while all others had increased expression

(2-fold to 48-fold; $p < 0.05$). During extended stationary-phase and lipid accumulation (Q3), 4 transcription factors had decreased expression and 20 transcription factors had increased expression (2-fold to 11-fold; $p < 0.05$). This also correlates to the cyclin expression and diatom specific cyclin (dsCYC) expression results. It has been proposed by Huysman *et al.* 2010 (Huysman et al., 2010) that dsCYCs may act as signal integrators for a fluctuating environment (*e.g.*, changes in light intensity, temperature, and nutrient availability). The Huysman *et al.* study reported that *dscyc7* and *dscyc10* were indicators of phosphate availability and that gene expression increased with increased phosphate availability. In our results, both *dscyc7* and *dscyc10* had increased expression (17-fold and 7-fold, respectively; $p < 0.05$) during Q2 when phosphate was internally available and most likely stored as polyphosphate. During Q3, *dscyc7* and *dscyc10* decreased in transcript abundance by 85% and 49% respectively, and coincided with the phosphate stress during Q3. Conserved cyclin (*cycB1*) is a late-phase cell cycle (G2/M) gene that shows a slight decrease in abundance in Q2 and a nearly 4-fold increase in transcript abundance during Q3 compared to Q1. The increased expression of *cycB1* suggests a role during cell-cycle arrest induced by nutrient deprivation of both nitrogen and phosphorus.

Nitrogen-limitation response. Genes involved in nitrogen metabolism were highly up-expressed at Q2 and Q3 (relative to Q1) as the cells experienced nitrate depletion and transitioned into stationary-phase (Fig. 3.6a and 3.6b). The up-expression of nitrogen metabolism genes including ammonium and nitrate transporters at Q2 is most likely a result of the fast growth and biomass accumulation during exponential growth.

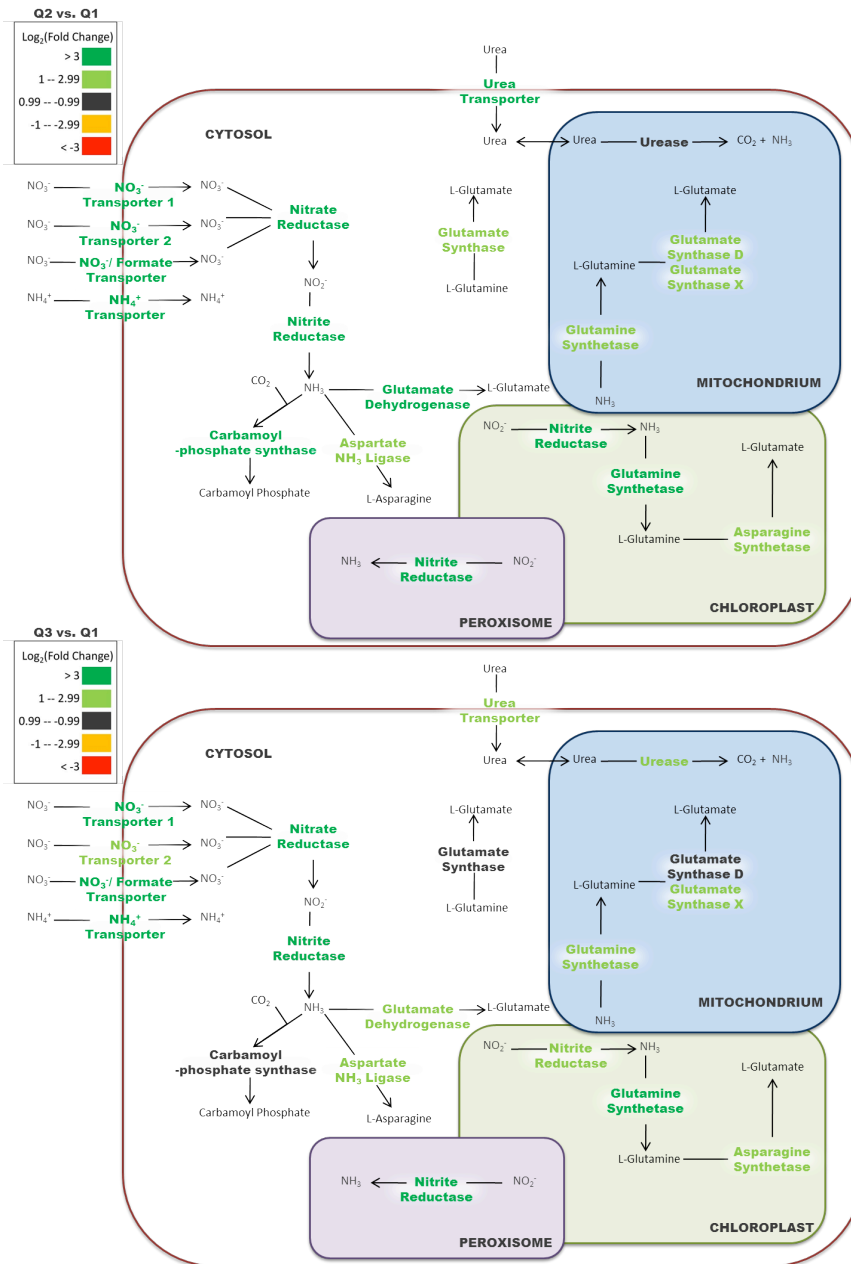


Figure 3.6. Nitrogen metabolism gene expression of *P. tricornutum* during nutrient depletion and lipid accumulation as compared to nutrient replete conditions (Q2 vs. Q1) (A). Nitrogen metabolism gene expression during extended nutrient depletion and lipid accumulation as compared to nutrient replete conditions (Q3 vs. Q1) (B). Genes are localized to organelles based upon reported literature. Differences in fold change are based on log₂ scale and the color scale represents up-expressed (green) and down-expressed (red) genes.

The up-expression of transporters coincided with enzymes that utilize ammonium ions for amino acid and nucleotide pools (Figure 3.6a and 3.6b). Most of the transporters remained up-expressed in Q3, most likely as a response to continued nitrogen deprivation. However, the putative carbamoyl-phosphate synthase, glutamate synthase (cytoplasmic), and glutamate synthase (mitochondria) were no longer up-expressed in stationary-phase and the transcript levels returned to initial Q1 abundances. The elevated transcript levels for nitrate, ammonium, and urea transporters in stationary-phase suggested a cellular strategy to scavenge externally available nitrogen while the internal ammonia-utilizing enzymes were at basal transcript levels.

A recent study demonstrated that phytoplankton altered the number of transporters at the cell surface compared to internal assimilatory enzymes (Smith et al., 2009), and this result is similar to observations of nitrogen deprivation in *Chlamydomonas reinhardtii*, in which some nitrogen acquisition genes remain strongly up-expressed after nitrogen deprivation (Miller et al., 2010). Interestingly, the expression pattern of carbamoyl-phosphate synthase that catalyzes carbon dioxide and ammonia condensation into carbamoyl phosphate is increased at Q2, but decreased in Q3. Carbamoyl-P synthase has been hypothesized to regulate carbon and nitrogen flow through the urea cycle in *P. tricornutum* (Allen et al., 2006). The urea cycle can fix carbon into nitrogenous compounds to effectively replenish compounds needed for cellular growth, and Allen et al. (2011) postulated that the urea cycle may serve as a distribution and repackaging hub for inorganic carbon and nitrogen (Allen et al., 2011).

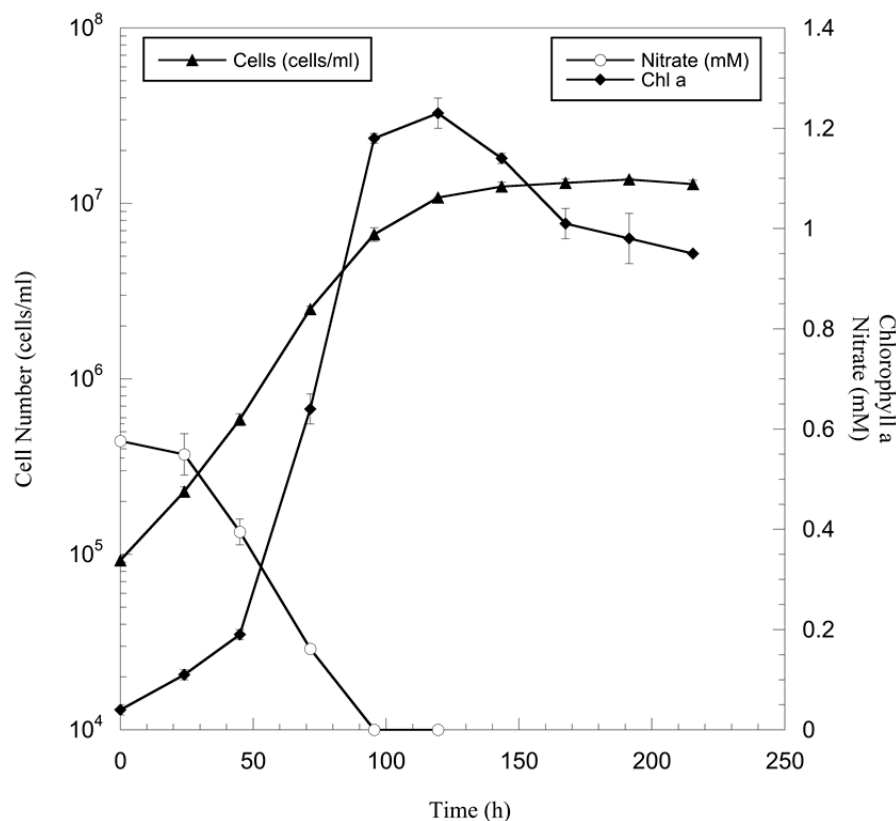


Figure 3.10. *P. tricornutum* growth curve (▲) showing nitrate (○) depletion and the chlorophyll a (◆) content.

Although the transcript expression results do not show up-expression of the complete urea cycle, it is possible that carbamoyl-P synthase could function as a control point for the sequestration of available ammonia for anaplerotic reactions (*e.g.*, pyrimidine biosynthesis) when exogenous nitrogen and DIC are low. Glutamate dehydrogenase and both glutamine synthetases remained at similar up-expressed levels during Q2 and Q3 and these results suggest that the cells expressed the GS/GOGAT (glutamine synthetase/glutamine oxoglutarate aminotransferase) pathway for ammonia sequestration possibly to recycle intracellular nitrogenous compounds (*e.g.*, proteins, chlorophyll).

When exogenous nitrogen was depleted and cells entered stationary-phase, chlorophyll *a* content decreased (Fig. 3.10) and this observation coincided with decreased gene expression for light-harvesting complex (LHC) genes (Fig. 3.4 and 3.5). These observations could be explained by nitrogen-deprivation-induced chlorophyll *a* degradation as the cells try to maintain nitrogen allocation into stationary-phase. In Q2 and Q3, we observed an overall decrease in presumptive photosynthetic genes including chlorophyll *a* and fucoxanthin. However, most of the photosynthetic reductase components did not decrease below exponential-phase levels, and this result may suggest a basal level of light-harvesting coupled to carbon fixation even during nutrient-deplete conditions. Photosynthetic transcripts were highly up-expressed in Q1 during nutrient replete conditions, but as depletion ensued and DIC consumption was high there was a metabolic shift to reduce overall photosynthesis via a decrease in Calvin cycle genes in the chloroplast. For example, glyceraldehyde phosphate dehydrogenase (22122) was down-expressed as well as a transketolase (50819) during Q2 and Q3.

Carbon Flow. The cells encountered low DIC availability during the exponential-phase, and most, if not all, phytoplankton have evolved carbon (CO_2)-concentration mechanisms (CCMs) to maintain photosynthetic carboxylation and reduce photorespiration via ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). Reinfelder (Reinfelder, 2011) recently published an extensive review on CCMs in eukaryotic marine phytoplankton. It is speculated that marine diatoms have two possible types of CCMs, a biophysical and a biochemical mechanism. A biophysical CCM is the direct transport of inorganic carbon across multiple membranes using transporters and

carbonic anhydrases. The other mechanism for concentrating inorganic carbon around the plastid is the use of a C4- (four carbon intermediate) CCM. In a biochemical C4-CCM, relatively high affinity carboxylases fix inorganic carbon to a C3 intermediate and form C4 compounds that can be transported to the chloroplast. The C4 intermediates are then decarboxylated to deliver the inorganic carbon to the relatively low-affinity RubisCO (Sage, 2004), and the C3 intermediate is recycled for another round of carbon fixation. In this sense, the CCM takes advantage of the C4 carboxylases low K_m for inorganic carbon and affords the cell an additional mechanism to concentrate carbon. In the debate of C3 versus C4 in diatoms, evidence for both a biophysical (Tanaka et al., 2005; Hopkinson et al., 2011; Moog et al., 2011; Tachibana et al., 2011) and biochemical CCM (Reinfelder and Milligan, 2004; Tanaka et al., 2005; McGinn and Morel, 2007; Roberts et al., 2007b; Kroth et al., 2008; Granum et al., 2009; Hopkinson et al., 2011; Moog et al., 2011; Tachibana et al., 2011) have been presented. The *P. tricornutum* genome encodes the presumptive genes to run both C3 and C4 mechanisms, (Bowler et al., 2008; Kroth et al., 2008) and *P. tricornutum* is most likely using both mechanisms in response to inorganic carbon levels (discussed further below).

The transcript analysis indicated a possible C4-CCM at Q2 when exogenous DIC was low, flowing through pyruvate carboxylase as the initial intermediate for carbon fixation followed by decarboxylation of malate by the malic enzyme (51970, B7FZD7) in the peroxisome (Fig. 3.7a). In the proposed model based upon gene expression, phosphoenolpyruvate (PEP) is converted to pyruvate by pyruvate kinase_6 (mitochondria), and pyruvate is then carboxylated to oxaloacetate via pyruvate

carboxylase (*pyc_1*, 30519) in the mitochondria. The oxaloacetate is then converted to malate from an up-expressed (6-fold) malate dehydrogenase (*mdh*, 51297) in the mitochondria. The malate is then decarboxylated by the malic enzyme (ME, 51970, B7FZD7) in the peroxisome (Fig. 3.7a). The malic enzyme is up-expressed 8-fold, and sequence data suggested that the malic enzyme could be located in the peroxisome. Peroxisomal proteins usually have a c-terminal peroxisomal targeting signal (PTS1) peptide consensus of (S/A/C)-(K/R/H)-(L/M) (Gonzalez et al., 2011). The c-terminus of the malic enzyme has a SKK motif, and thus may be targeted to the peroxisome. However, an *in silico* prediction of peroxisomal genes in the *P. tricornutum* genome did not locate the malic enzyme to the peroxisome (Gonzalez et al., 2011); therefore, it is possible that the malic enzyme is located in the mitochondria. The malic enzyme would decarboxylate the malate to pyruvate, thus concentrating inorganic carbon that can diffuse to the plastid for fixation by RubisCO. In addition to *pyc_1* and malic enzyme, the plastid pyruvate pyrophosphate di-kinase was up-expressed in Q2 relative to Q3 (Figure 3.7a and 3.7b). This enzyme could be involved in the moving of C3 intermediates back to the cytoplasm and/or the mitochondria for the C4-CCM. The data differ from most other proposed models in that the gene expression data suggest that the initial C3 + C1 carboxylation occurs mainly via pyruvate carboxylase_1 in the mitochondria and not through phosphoenolpyruvate carboxylase (*pepc_1*, 56026).

As the cells transitioned to stationary-phase (Q3) and the exogenous DIC increased due to decreased metabolic demand under nutrient deplete conditions, the *pyc_1* and malic enzyme remained up-expressed relative to Q1. The pyruvate kinase_6

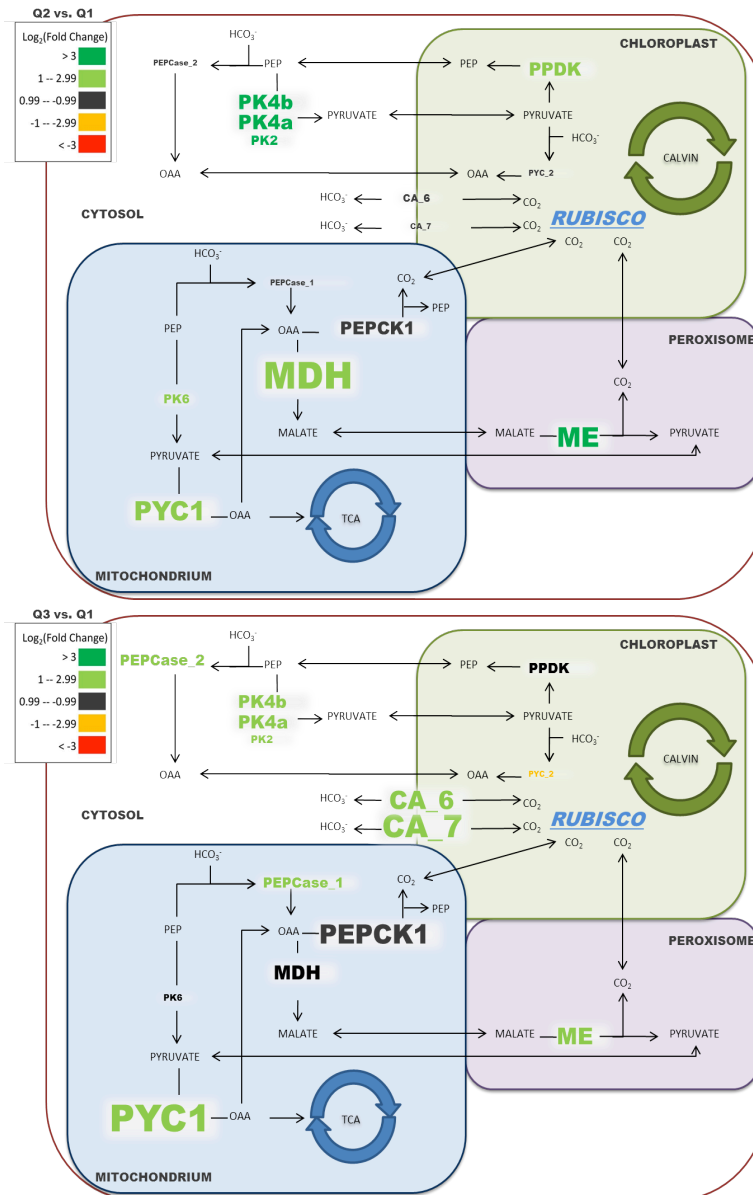


Figure 3.7. Proposed C4 metabolism of *P. tricornutum* based on gene expression and gene localizations. Carbon-assimilation gene expression during nutrient depletion and initial lipid accumulation as compared to nutrient replete conditions (Q2 vs. Q1) (A). Carbon-assimilation gene expression during extended nutrient depletion and lipid accumulation as compared to nutrient replete conditions (Q3 vs. Q1) (B). Differences in fold change are based on log₂ scale and the color scale represents up-expressed (green) and down-expressed (red) genes.

and the plastid *pyc_2* were down-expressed, and the mitochondria *pepc_1* and cytoplasmic *pepc_2* were up-expressed in Q3 (Figure 3.7b). However, the mitochondrial malate dehydrogenase was no longer up-expressed at Q3 (Fig. 3.7b). These results suggest that C4-CCM was more active during Q2 when exogenous DIC was low due to active population growth and lipid accumulation was initiated. At Q3, C4-CCM might become less important as the cell uses *pepc_2* to sequester bicarbonate in the cytoplasm.

Kroth *et al.* 2008 developed an annotated model for a biochemical CCM based on genome annotations of the marine diatoms, *P. tricornutum* and *Thalassiosira pseudonana*. The decarboxylating enzyme and subcellular localization were difficult to predict; however, the authors estimated the decarboxylation did not occur in the *P. tricornutum* plastid. The authors further suggested that carboxylation may occur in the mitochondria and the concentrated CO₂ could then diffuse to the chloroplast and be fixed by RubisCO. The presented expression data is in general agreement with the predicted model, but also provides a potential route for C4-CCM based upon gene expression. Malate could be transferred into the peroxisome for decarboxylation via the malic enzyme where the CO₂ could diffuse to the chloroplast and RubisCO.

It is not a new idea for C4 metabolism to go through PYC₁ and ME, but most models predict PEPC as the initial carboxylation step (McGinn and Morel, 2007; Kroth *et al.*, 2008; Granum *et al.*, 2009). However, under CO₂ limited-conditions in Q2 our expression data showed that *pepc_1* is expressed at a lower level compared to *pyc_1* (FPKM levels of 8.7 versus 102.9, respectively; $p < 0.05$). In addition, the presented data corroborate the study of McGinn and Morel (2007) that showed *P. tricornutum* *pepc*

transcripts did not significantly increase during low CO₂ conditions. In addition, bicarbonate addition was recently shown to increase the rate of lipid accumulation in *P. tricornutum* if added at the time of low N and low DIC (Gardner et al., 2012). The use of a C4-CCM would help explain this observation and also underscores the ability of *P. tricornutum* and similar photoautotrophs to respond to exogenous DIC for lipid accumulation.

As cells exited exponential-growth and entered stationary-phase, gene expression was maintained for most oxidative phosphorylation and glycolysis genes at Q2 and Q3 relative to Q1 (Fig. 3.4 and 3.5). However, a majority of TCA genes declined in expression from Q2 to Q3 (malate dehydrogenase, aconitase, isocitrate dehydrogenase, oxoglutarate dehydrogenase, succinyl-CoA ligase, and succinate dehydrogenase) (Fig. 3.4, 5 and 3.8). These results indicate an overall slower metabolism as the cells entered stationary-phase even though lipids continued to increase (Fig. 3.4, 3.5 and 3.8).

The α -carbonic anhydrase_6 and 7 (CA-6 and CA-7) were up-expressed at Q3 (6.8-fold and 3.3-fold, respectively; $p < 0.05$), and these results indicated a potential role for a biophysical CCM during lipid accumulation as exogenous DIC levels increased (Fig. 3.7b). The CA-6 and CA-7 are putatively localized in the periplastidic compartment (Moog et al., 2011; Tachibana et al., 2011). As growth slowed and DIC became more available during the nutrient deplete state, C4-CCM may not have been needed, but a biophysical CCM could still increase CO₂ flux to RubisCO by facilitating bicarbonate transport into the chloroplast. This mechanism would potentially concentrate CO₂ in the lumen surrounding the plastid for increased delivery to RubisCO (Kroth et al., 2008). In

addition, CA-6 and CA-7 in the periplastidic compartment could provide a pressure barrier for CO₂ efflux from the plastid (Tanaka et al., 2005). The increase in *pepc_1* and *pepc_2* transcript levels during Q3 may represent potential roles as anaplerotic enzymes to maintain TCA cycle intermediates and overall cellular maintenance. A presumptive Cl⁻/HCO₃⁻ exchanger gene (54405) was up-expressed (22-fold; p<0.05) at Q2 when exogenous DIC was low. At Q3, the gene expression for 54405 decreased back towards basal levels as exogenous DIC levels increased during stationary-phase, and these combined results could indicate a combined biophysical and biochemical strategy of CCM at Q2.

Fatty Acid Metabolism. Understanding how carbon is partitioned as nutrient conditions change is important to predict carbon flow for lipid accumulation. Previous work has shown that stress caused by low availability of nitrogen can increase lipid content in different microalgae (Li et al., 2008; Yu et al., 2009; Gardner et al., 2010; Yeesang and Cheirsilp, 2011), and we observed a similar response in *P. tricornutum* (Fig. 3.2 and 3.3). However, from a genome-wide expression standpoint, algal TAG accumulation is not well studied. Hu *et al.* (Hu et al., 2008) reviewed *de novo* fatty acid biosynthesis and TAG biosynthesis in microalgae, and proposed that a significant portion of TAGs are made from re-cycling of phosphoglycerides and glycolipids instead of *de novo* fatty acid biosynthesis *per se*. In fact, the activity and up-expression of acetyl-Co-carboxylase was a focus for previous studies, including the U.S. Department of Energy's Aquatic Species Program (Sheehan et al., 1998).

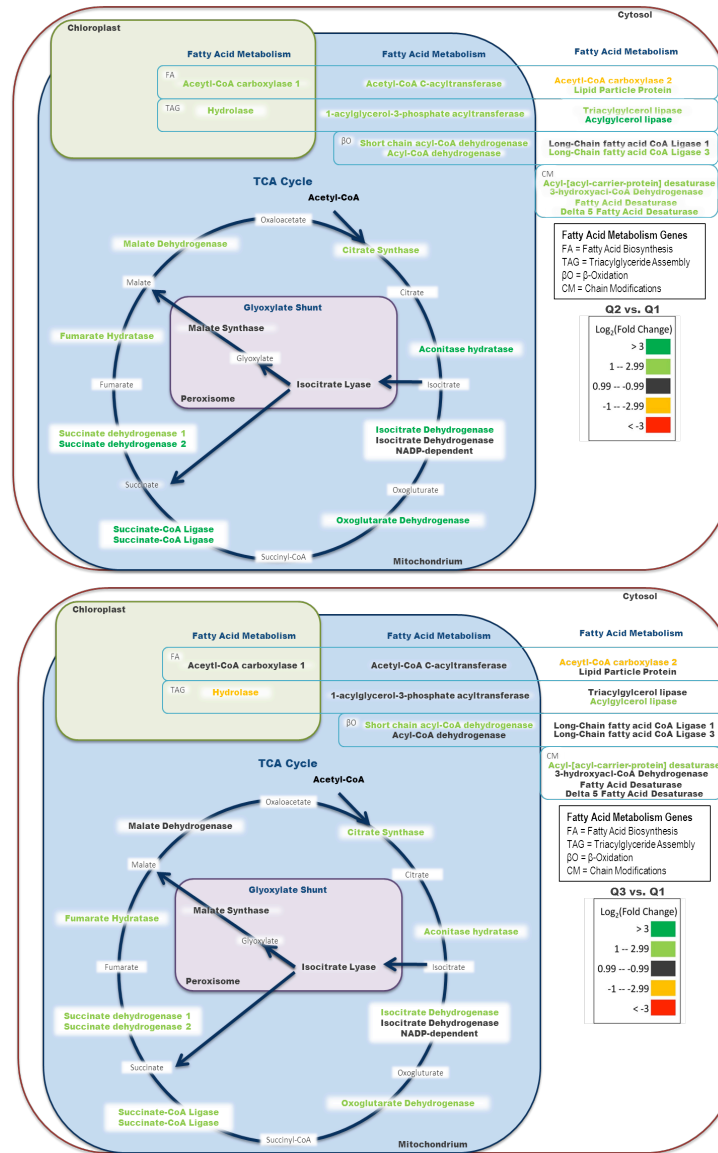


Figure 3.8. Fatty acid metabolism, tricarboxylic acid cycle, and glyoxylate shunt related gene expression in *P. tricornutum* during nutrient depletion and initial lipid accumulation as compared to nutrient replete conditions (Q2 vs. Q1) (A). Significant gene expression during extended nutrient depletion and lipid accumulation as compared to nutrient replete conditions (Q3 vs. Q1) (B). Fatty acid metabolism genes are denoted by presumptive roles in fatty acid biosynthesis, triacylglyceride assembly, β -oxidation, and chain modifications. Differences in fold change are based on log₂ scale and the color scale represents

At Q2, as exogenous nitrate and phosphate were depleted, the plastid acetyl-CoA carboxylase_1 and hydrolase were up-expressed (approximately 2.5-fold; $p < 0.05$) compared to Q1 (Fig. 3.8a). In the mitochondria, the acetyl-CoA acyltransferase (28068) and the acylglycerol acyltransferase (45551) genes were up-expressed (2.5-fold; $p < 0.05$) at Q2/Q1. Gene expression for the same genes declined in Q3 (*i.e.*, the levels were similar to Q1). The acetyl-CoA carboxylase_2 (55209) was down-expressed (approximately 2-fold; $p < 0.05$) and remained at similar levels at Q3 (Fig. 3.8b). A gene annotated as a lipid particle protein (45518) was up-expressed at Q2, but expression declined at Q3 (Fig. 3.8). These results suggest the plastid acetyl-CoA carboxylase showed greater expression changes (3-fold) than the cytoplasmic acetyl-CoA carboxylase, but neither was strongly up-expressed at the transcriptional level during prolonged lipid accumulation (*i.e.*, Q3). Similar results were recently reported when RNA-seq was used to evaluate gene expression in *Chlamydomonas reinhardtii*, in which only modest changes in gene expression of fatty acid metabolism genes were reported (Miller et al., 2010).

The results demonstrate modest increases in transcripts involved in fatty acid biosynthesis, TAG assembly, fatty acid chain modifications, and β -oxidation at Q2, but an overall return to early-exponential abundances at Q3 during an extended time period of lipid accumulation (Fig. 3.8). During the initial increase in lipid accumulation (Q2), a 3-fold increase in acetyl-CoA carboxylase_1 (*acc_1*, plastid) was observed. This is an expected result based on the fact that ACC is the enzyme involved in the committed step for fatty acid biosynthesis. In addition, 1-acylglycerol-3-phosphate acyltransferase (*agat*)

which is the second step in TAG formation was up-expressed 6-fold at Q2 (Fig. 3.4 and 3.8). Genes involved in β -oxidation and fatty acid chain modifications were also up-expressed at Q2, and these changes may be in response to changing membrane dynamics as well as recycling of nitrogen and phosphorus associated with different lipid classes (Fig. 3.8a). For example, an acyl-ACP desaturase was up-expressed 7.4-fold and 3.5-fold in Q2 and Q3, respectively and may be involved in incorporation and/or production of unsaturated fatty acids. However, even though lipid accumulation was at a faster rate (specific NR fluorescence/t) after Q2, most of the genes associated with fatty acid biosynthesis returned to basal levels (Q1), including *acc_1* and *agat* (Fig. 3.8).

During prolonged lipid accumulation (Q3), the direct fixation of C3 carbon might play a more significant role for overall carbon flow towards TAG accumulation (*i.e.*, higher exogenous DIC and lower expression of C4-related genes). Our results suggest that the build-up of precursors to the acetyl-CoA carboxylases (*i.e.*, acetyl-CoA) may be a more significant contribution toward TAG accumulation than the actual enzyme levels of acetyl-CoA carboxylases *per se*. In addition, there could be alternative routes of carbon intermediates towards overall TAG accumulation. For example, *P. tricornutum* has a putative methylmalonyl-CoA mutase gene that was slightly up-expressed in Q2 and Q3 (1.3 and 2.0-fold, respectively; $p < 0.05$). The putative methylmalonyl-CoA mutase (51830) might convert succinyl-CoA to methylmalonyl-CoA, and such a conversion could link TCA intermediates to fatty acid biosynthesis. In addition, a putative propionyl-CoA carboxylase gene (51245) that could carboxylate propionyl-CoA via bicarbonate to produce methylmalonyl-CoA was up-expressed 2-fold at Q2 and 1.8-fold

at Q3 ($p < 0.05$). These results suggested alternative routes for carbon fixation in addition to C3 and C4 intermediates.

Conclusions

P. tricornutum has evolved to survive the varying and often nutrient-limiting conditions encountered in marine habitats. In response to nitrogen and exogenous phosphate depletion, cells maintained up-expression of transcripts for nitrogen transport and assimilatory genes as well as presumptive phosphate transporters. Under the growth conditions tested, cell numbers continued to increase after the depletion of exogenous phosphate, and these results corroborated the notion that *P. tricornutum* can store internal phosphate (Leitao et al., 1995). The extracellular phosphate was depleted before nitrate, and Terry *et al.* (1985) (Terry et al., 1985) surmised that the ability of *P. tricornutum* to accumulate nitrogen was extremely restricted during phosphate limitation. Lipids started to accumulate when exogenous phosphate was approximately half the initial levels and the external N:P ratio was approximately 27. The rate of lipid accumulation (specific NR fluorescence/time) increased (3.3-fold) once external nitrate was depleted. These results indicate that phosphate depletion could be an initial trigger for lipid accumulation that was “magnified” upon nitrate depletion in *P. tricornutum*.

The lipid accumulation at Q2 did coincide with up-expression for acetyl-CoA carboxylase (*acc_1*) and 1-acylglycerol-3-phosphate acyltransferase. At Q2, *acc_1* was up-expressed 3.1-fold, the *acc* b-subunit was up-expressed 3.7-fold, and the acyltransferase was up-expressed 5.8-fold. However, the expression for these genes declined during Q3 when lipid was being accumulated at a faster rate (specific NR

fluorescence/time). In addition, *acc_2* was down-expressed 2-fold at Q2 and 5-fold at Q3. These results could suggest that carbon is being “pushed” into fatty acid synthesis via elevated acetyl-CoA and NADPH levels and thus not being “pulled” by a large abundance of fatty acid synthesis activity. While it is possible that protein levels are increased for fatty acid biosynthesis, the transcript data does not suggest this scenario.

A “push” condition is when flux processes occur prior to a pivot point (“hinge”), and a “pull” condition occurs following the “hinge” (Cortassa et al., 2009). With respect to lipid accumulation, the “hinge” is the committed step in fatty acid biosynthesis (*i.e.*, acetyl-CoA carboxylase), and we postulate that a “push” condition accounts for a significant portion of the metabolic flow based upon gene expression. If acetyl-CoA was being pulled, up-expression would be expected to be maintained throughout lipid accumulation (Q2 through Q3) for most fatty acid and TAG biosynthetic genes. This metabolic step has been tested by overexpression of *acc* (Sheehan et al., 1998; Courchesne et al., 2009), but overexpression did not result in an increase of overall lipid biosynthesis.

We conclude that during the transition from growth to lipid accumulation, *P. tricornutum* utilized pyruvate carboxylase in the mitochondria as the primary inorganic fixation step in a C4 pathway with subsequent decarboxylation of malate by the malic enzyme in the peroxisome to concentrate CO₂ for diffusion to the chloroplast. During the initial transition, exogenous DIC was low, and therefore, it appeared that a biochemical mechanism to concentrate carbon predominated. At the later time point (Q3), DIC levels had returned to initial levels, lipid accumulation continued, but two carbonic anhydrases

(CA_6 and CA_7) were up-expressed. These results indicate that a biophysical carbon-concentrating mechanism then predominated to maintain carbon flow for CO₂ reduction during nutrient deprivation.

The cells appeared to continue CO₂ reduction when population growth was arrested and different carbon-concentrating mechanisms were used dependent upon exogenous DIC levels. The fixed carbon as C3 and/or C4 intermediates can then be “pushed” into fatty acid biosynthesis. Alternative pathways that could utilize HCO₃⁻ were also suggested by the gene expression profiles (*e.g.*, propionyl-CoA and methylmalonyl-CoA). In addition, a putative decarboxylase with sequence homology to a coccolith-associated protein was significantly up-expressed. Coccoliths are external, CaCO₃ plates formed by some single-celled algae (coccolithophores) (Paasche, 2001), and the presumptive *P. tricornutum* protein could be involved in carbon homeostasis. Further work is needed (proteomic, metabolomic, modeling) to provide more insight into metabolic control points that are crucial for the routing of fixed carbon into lipid accumulation by photoautotrophs, but our results based upon genome expression analysis suggested multiple routes for inorganic carbon flow during lipid accumulation. Future work includes proteomic analyses for the identification of key proteins and enzymes for lipid accumulation and storage, as well as metabolomic analysis, particularly in response to dissolved carbon. It will be advantageous to consider the type and level of inorganic carbon for bio-oil production systems and how different microalgae respond during autotrophic, lipid accumulation. With respect to biofuel production, an improved understanding of how carbon is potentially “pushed” into fatty acid biosynthesis will

allow for the development of metabolic networks that can be engineered to maximize inorganic carbon flow into TAG production.

Methods

Culture and Growth Conditions. *Phaeodactylum tricornutum* (Pt1) Bohlin Strain 8.6 CCMP2561 (Culture Collection of Marine Phytoplankton, now known as NCMA: National Center for Marine Algae and Microbiota) was used in all experiments. Characterization of the *P. tricornutum* strain 8.6 (Pt1) and its relationship to other *P. tricornutum* strains was previously described (Martino et al., 2007). Pt1 was grown at 20°C in 1.25 L photobioreactors in ASPII media as previously described (Provasoli et al., 1957; Cooksey and Cooksey, 1974), and the medium was buffered at approximately pH 8.0 with Tris-base. Cells were grown on a 14:10 light ($450 \mu\text{E}^{-1}\cdot\text{sec}^{-1}$):dark cycle at 18°C. Tubes were aerated with ambient air at a regulated flow rate of $0.40 \text{ L} \cdot \text{min}^{-1}$ and CO_2 from air was the only available source of carbon for the diatoms. Initial inocula were grown in similar growth conditions and after two transfers were inoculated into photobioreactors during exponential-phase to a cell concentration of approximately $1 \times 10^5 \text{ cells} \cdot \text{mL}^{-1}$. Contamination checks were performed using ASPII medium supplemented with 0.5% yeast extract and 0.5% glucose as well as plating on R2A agar. Cultures remained axenic throughout experiments.

Physiological Parameter Analysis. Cells and media were collected and analyzed daily to measure chlorophyll a, pH, dissolved inorganic carbon (DIC), nitrate, phosphate, protein, carbohydrate, cell number, and Nile Red fluorescence. All measurements were done in triplicate. Cells (1 mL) were collected daily via

centrifugation (10,625 x g, 10 min) at 4°C. The supernatant was removed and stored for nitrate and phosphate analysis at 4°C. For chlorophyll a extraction, 100% methanol (1 mL) was added to the pellets followed by 30 seconds of mixing to extract chlorophyll a, and extracts were stored in the dark at 4°C for 24 hours. Cellular debris was pelleted (10,625 x g, 10 min) and the methanol extract was measured on a spectrophotometer. Absorbance was measured at 652 nm and 665.2 nm and concentrations ($\mu\text{g/mL}$) calculated using equations reviewed by Porra (2002) (Porra, 2002). Absorbance was measured on a spectrophotometer (UV-1700 PharmaSpec UV-Vis, Shimadzu). The medium pH was measured using a standard tabletop pH meter. Daily, cell culture (7 mL) was filtered (0.2 μm nylon membrane filters) in preparation for DIC measurement using a Formacs^{HT} TOC/TN Analyzer (SKALAR, Buford, GA). The analysis of nitrate and phosphate were done by following the manufacturer's colorimetric assay protocols Szechrome NAS (Polysciences, Inc.) and SensoLyte MG Phosphate Assay Kit (AnaSpec), respectively. The absorbance measurements were taken on a 96-well plate spectrophotometer (Synergy HT, Biotek). Protein was extracted by boiling 1 mL of pelleted (10,625 x g, 10 min) cells in 100 mM (Tris-HCl, 4% SDS, at pH 6.8) for 5 min (Rousch and Bingham, 2004). Proteins were precipitated overnight at 4°C in 10% TCA/acetone. Precipitated protein was pelleted at 4°C and supernatant was removed. Proteins were then washed with 100% acetone and re-pelleted. Supernatant was decanted and precipitated proteins were rehydrated in Tris-HCl. Protein concentration was measured by a Qubit Protein Assay and the Qubit Fluorometer (Invitrogen). The analysis of carbohydrates was performed by following a modified monosaccharide protocol

previously described by Chaplin et. al (1994) (Chaplin and Kennedy, 1994). Cells (1 mL) were pelleted and the supernatant decanted, and L-cysteine HCl (1 ml; 700 mg/L) in cold sulfuric acid was added to cells, vortexed, and boiled at 100°C for 3 minutes. Samples were rapidly cooled on ice and absorbance measured at 415 nm with a 96-well plate spectrophotometer (Synergy HT, Biotek).

For lipid accumulation, we followed the Nile Red assay protocol as previously described by Cooksey et al. (Cooksey et al., 1987). Cells were diluted with 1.8% NaCl (to minimize cell lysis) to an appropriate cell density that correlated to a linear relationship of cell density and Nile Red fluorescence that can be measured spectrofluorometrically. Nile Red (Sigma-Aldrich) solution (4 μ L of 250 μ g/mL in acetone) was added to a cell suspension (1 mL). Specific fluorescence was determined by dividing the Nile Red fluorescence intensity by the cell number. Cell density was monitored via cell counts in duplicate with a hemocytometer.

RNA Sampling, Extraction, and Sequencing. RNA extraction was done in duplicate from seven identical photobioreactors. At early exponential phase (Q1), 1.5 L of culture biomass (1 L from 1 reactor plus 0.5 L from a replicate reactor) were collected (5,856-rcf, 10 min at 4°C) for each duplicate, the supernatant was discarded, and the collected cells were immediately flash frozen in liquid nitrogen (Fig. 3.9). The use of three reactors for duplicates were necessary due to the lower cell density in the early exponential-phase. The same collection procedure was used for late exponential (Q2) and stationary phases (Q3) when cell numbers were higher except that 1 L was collected for each in duplicate. Flash frozen aliquots were stored at -80°C until RNA extraction.

Total RNA was extracted following the Spectrum Total RNA kit (Sigma Aldrich-STRN50) protocol with on-column DNAase digestion using DNase10 (Sigma-Aldrich) as suggested by the manufacturer. Initial lysis was done by a 30 second cell disruption via sonication. The protocol was performed in an RNase free-hood to prevent RNA degradation. Total RNA was not pooled but kept as respective duplicates, and samples were sent to the National Center for Genome Resources (NCGR, Santa Fe, NM). At NCGR, mRNA was isolated twice by polyadenylation purification, and first and second strand DNA was generated with random primers.

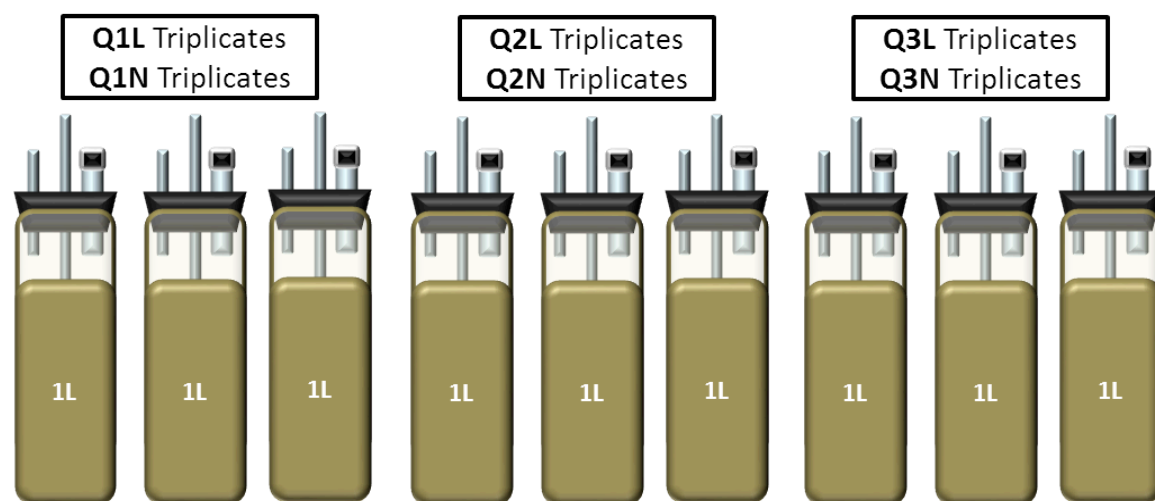


Figure 3.9. Schematic representation of temporal biomass sampling from replicate bioreactors

Gel products were purified and PCR enriched. Products were quantified on a Nanodrop fluorometer (Thermo Scientific) and checked for quality on a Bioanalyzer 2100 (Agilent). Using Illumina's standard protocols, flow cell construction and sequence determination was performed. Each duplicate was run on a single lane (6 total samples, 3 duplicates). Single reads were put into FASTQ format for analysis.

Transcript Assembly. Single-end Illumina reads were analyzed using the following pipeline. First, reads from each sample were aligned against the *P. tricornutum* genome (v. 8) from ENSEMBL (Kersey et al., 2009) with the TopHat algorithm (Trapnell et al., 2009). TopHat was configured to use known splice junctions for Pt1, as retrieved from genome annotation version 61 from ENSEMBL. Mapped reads were assembled into putative transcripts using Cufflinks (Trapnell et al., 2010). Abundance of each transcript was estimated in each sample then compared across samples using Cuffcompare and Cuffdiff, parts of the Cufflinks toolkit (Trapnell et al., 2010). Similar pipelines have been validated as well as the accuracy of RNA-seq with spike in experiments and qPCR comparisons (Marioni et al., 2008; Mortazavi et al., 2008; Nagalakshmi et al., 2008; Wang et al., 2009; Jiang et al., 2011; Twine et al., 2011).

Transcript Identification and Pathway Analysis. Cufflinks output files had transcripts identified by uniprot accessions. Using the DAVID (Database for Annotation, Visualization, and Integrated Discovery) (Huang et al., 2007) Gene ID conversion tool, uniprot accessions were converted to Locus Tag IDs and Protein IDs. Once all accessions were converted, the DAVID Functional Annotation tool was used to retrieve gene names as well as KEGG (Kyoto Encyclopedia of Genes and Genomes) Pathway information. For genes that were identified as hypothetical proteins, searches were performed on the JGI *Phaeodactylum tricornutum* v2.0 genome website (<http://genome.jgi-psf.org/Phatr2/Phatr2.home.html>) and based on best hits, %ID, score, and consistency of the top hits, genes were either identified or remained as hypothetical. Genes were also searched on NCBI and ENSEMBL genome browsers for cross-

referencing. To assign genes into pathways, we used KEGG maps for *P. tricornutum* as a backbone. Genes for major pathways were searched manually to find genes not directly annotated in the *P. tricornutum* KEGG maps. Gene lists were compiled for the major pathways and developed into network maps.

Organelle targeting of gene transcripts was done based on annotations from the databases of JGI, NCBI, and ENSEMBL. If no localization was found, eukaryotic organelle localizations were predicted with TargetP 1.1. Server (Emanuelsson et al., 2007) in both plant and non-plant mode. Amino acid sequences were also checked for a peroxisomal targeting sequence (SKL, serine-lysine-leucine). If nothing was established we assumed that the gene occurred in the cytoplasm. If the gene was an integral protein we again checked JGI, NCBI, and ENSEMBL. If nothing could be determined we located the gene in the most biologically relevant membrane, i.e. light harvesting complex in the plastid.

Elemental Analysis. To better understand the effects of nutrient limitation on lipid accumulation we wanted to determine the elemental carbon:nitrogen:phosphorus (C:N:P) make up of the cells at Q1, Q2, and Q3. Cells were grown in the same conditions and harvested at the same time points at which RNAseq occurred. Cells were collected via centrifugation (5,856 rcf, 10 min at 4°C). Supernatant was discarded and concentrated cells were transferred to 50 mL tubes and centrifuged (5,856-rcf, 15 minutes at 4°C). Supernatant was discarded and pelleted cells were placed in a 60°C incubator to dry. Dried cells were sent to the University of Missouri Soil Testing and Plant

Diagnostic Service Laboratories (Columbia, MO) for elemental analysis. Percentages of carbon, nitrogen, and phosphorus were used to calculate the C:N:P ratios in the cells.

List of Abbreviations

TAG, Triacylglyceride; NR, Nile Red; DIC, Dissolved Inorganic Carbon; CYC, cyclin; CCAP, Culture Collection of Algae and Protozoa, CCMP, formerly Culture Collection for Marine Phytoplankton, now NCMA, National Center for Marine Algae and Microbiota; Pt1, *Phaeodactylum tricornutum* strain 1; EST, Expressed Sequence Tag, RNA-seq, RNA-sequencing; FPKM, Fragments Per Kilobase of exon per Million reads; dsCYC, diatom specific cyclin; GS/GOGAT, glutamine synthetase/glutamine oxoglutarate aminotransferase; LHC, light harvesting complex; CCM, carbon concentrating mechanism; C3, 3-carbon metabolite; C4, 4-carbon metabolite; PEP, Phosphoenolpyruvate; PYC, pyruvate carboxylase; MDH, malate dehydrogenase; ME, Malic Enzyme; PTS1, Peroxisomal targeting signal; pepc, phosphoenolpyruvate carboxylase; RubisCO, Ribulose-1, 5-Bisphosphate carboxylase oxygenase; TCA, Tricarboxylic cycle; CA, carbonic anhydrase; ACC, acetyl-CoA carboxylase; ACP, acetyl carrier protein; NADPH, reduced form of Nicotinamide adenine dinucleotide phosphate; ASPII, Aquatic Species Program; TCA/Acetone, Trichloroacetic acid in acetone; NCGR, National Center for Genome Resources; DAVID, Database for Annotation, Visualization, and Integrated Discovery; KEGG, Kyoto Encyclopedia of Genes and Genomes; SKL, Serine-lysine-leucine

Competing Interests

The authors declare that there are no competing interests.

Author contributions.

JV carried out growth, biomass harvest, activity assays, RNA extraction, participated in RNA-Seq data analysis, and drafted the manuscript. AM assisted in RNA-Seq data analysis and statistics. RPC, RG, KEC, BMP, and MWF participated in study design, study coordination, data analysis/interpretation, and manuscript preparation. All authors read and approved the final manuscript.

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CHAPTER 4

GLOBAL LIGHT AND DARK TRANSCRIPTOMIC ANALYSIS OF LIPID
ACCUMULATION IN PHAEODACTYLUM TRICORNUTUMAbstract

The unicellular microalga *Phaeodactylum tricornutum* has an extensive research background and a fully sequenced genome, making it a model organism for diatom research. *P. tricornutum* is a marine diatom known to accumulate between 20 to 30% triacylglyceride (TAG) when grown under nutrient limitation. TAGs can be converted into biodiesel by a transesterification process that can be put into any diesel engine. Thus, algal biofuel is a realistic alternative renewable replacement for transportation fossil fuel. Lipid accumulation is the difference of lipids produced during photoautotrophic growth of the light cycle and the lipids consumed via β -oxidation during the dark period. This study investigates fatty acid production during phosphate and nitrate limitation while monitoring whole transcripts profiles via RNA-sequencing (RNAseq) during a time-course assessment of the light and dark periods.

Fatty acid production was initiated by phosphate depletion and intensified by nitrate depletion. Fatty acid profiles showed maximal lipid accumulation increased as cells were in stationary phase. RNAseq profiles indicated that central carbon metabolism like pyruvate, TCA cycle and pentose phosphate pathways maintained expression levels between light and dark periods, but changed expression over the time course of the experiment in response to growth conditions. A urea cycle was up-expressed in stationary

phase of the light period to maintain a central hub for metabolic precursors directed to nitrogen metabolism and carbon intermediates. A probable C₄ carbon concentration mechanism is employed during the light cycle during CO₂ limitation at the transition from exponential phase to stationary phase. Carbonic anhydrases showed unique expression levels during light and dark periods to maintain local inorganic carbon concentrations for fixation during light periods and for fatty acid biosynthesis reactions during the dark periods. Genes like carbamoyl phosphate synthase (CPS) and acetyl CoA carboxylase (ACC) play important roles in fatty acid biosynthesis and carbon homeostasis, which could potentially be harnessed to direct carbon into lipids. CPS is an important enzyme in nitrogen and carbon redistribution and shows little differential expression from the light to dark period, but changed overall expression through the time course of growth. ACC is the rate-limiting step in fatty acid biosynthesis and its expression correlated with the increase in accumulated fatty acids. The results presented provide greater detail into the cellular responses to inorganic carbon partitioning to fatty acids in the light and dark periods of photoautotrophic growth.

Background

Anthropogenic practices since the Industrial Revolution in the late 18th century have resulted in unprecedented release of CO₂ into the atmosphere (Meinshausen et al., 2011) resulting in problematic environmental impacts. CO₂ is a greenhouse gas (GHG) that traps thermal radiation from escaping the atmosphere and in turn reflects it back towards the Earth's surface (Guerrero-Lemus and Martínez-Duart, 2012). The increase

in GHG in the atmosphere has increased the planet's average temperature and dictated climate shifts. The carbon in fossil fuels that is consumed in one year is 400 times that of which the global biota can fix through primary production in a year, creating a substantial carbon deficit (Dukes, 2003). As environmental impacts mount there is also a substantial economical impact to fossil fuel consumption. The US economy is dependent on petroleum; America is the largest net energy importer in the world. The reliance on a stable supply of petroleum is cause for concern; as prices are unstable, reserves are declining, and instability in main importer regions causes unpredictable economic impacts (Bang, 2010). Thus, energy independence could alleviate the problematic environmental and economical impacts, providing energy security.

Diatoms are a diverse group of unicellular microalgae that can grow photoautotrophically (*i.e.* carbon fixation via sunlight). They account for up to 40% of carbon fixed by primary production in the oceans each year (Falkowski and Raven, 1997; Field, 1998; Granum et al., 2005). Microalgae can fix inorganic carbon and store it in lipids (TAGs) that can be harvested and processed into biodiesel, creating a sustainable carbon neutral biofuel (Greenwell et al., 2010; Fon Sing et al., 2011). Estimates indicate that 50% of the transportation fuel needs in the US could be replaced by algal biofuel only using 3% of the arable crop land (Chisti, 2007). Microalgae can also grow on water sources not considered part of the drinking supply, like brackish water, industrial wastewater, municipal wastewater, and marine water (Pate et al., 2011). Algal biofuel takes less water per gallon of biofuel than soy or corn biofuels and with water and nutrient recycling efficiency increases (Dominguez-Faus et al., 2009; Yang et al., 2011).

Thus, algal biofuel has provided a realistic alternative to fossil fuel and efforts to increase lipid production biochemically and genetically have been underway (Courchesne et al., 2009). Many aspects of carbon assimilation are understood in diatoms, the partitioning of inorganic carbon to lipid accumulation is not well understood from a whole cell perspective during different growth conditions (Roberts et al., 2007). Increasing lipid accumulation means greater biofuel productivity and lower costs. Global expression values during light and dark periods may give more insight into how carbon is assimilated into lipid during the light and what control points can be harnessed to decrease consumption at night.

Phaeodactylum tricornutum is marine diatom, with its whole genome sequenced (27.4Mb), with over 10,000 predicted genes (Bowler et al., 2008). The chloroplast genome is also sequenced in *P. tricornutum* with 162 predicted genes (Oudot-Le Secq et al., 2007). Over 130,000 ESTs (expressed sequence tags) during 16 different growth conditions was compiled into a diatom database (Maheswari, 2004), providing an enormous background of metabolic data. *P. tricornutum* has been genetically and morphologically characterized into 10 different strains (Martino et al., 2007), with strain Pt1 (*Phaeodactylum tricornutum* strain 1, CCMP 2561) being the ecotype chosen for genome sequencing. It has been observed in most algae and Pt1 that under under nitrogen or phosphate stress the algae will accumulate lipids (Khozin-Goldberg and Cohen, 2006; Yu et al., 2009; Gardner et al., 2010; Hockin et al., 2012; Valenzuela et al., 2012; Liang et al., 2012b). A complete understanding of the mechanisms and control points that direct carbon partitioning are still not well understood. Most studies focus on

light period dynamics and dismiss potential carbon and nitrogen fluxes that may occur during the dark period that may control overall lipid accumulation.

The extensive research background of *P. tricornutum* particularly at the genomic and transcript level makes it significant model organism to study the cellular responses of lipid accumulation through global transcriptomic profiling. RNA sequencing allows for deep profiling into the transcriptomes of organisms through high throughput next generation sequencing (Marioni et al., 2008; Mortazavi et al., 2008; Wang et al., 2009). In this study, the model diatom *Phaeodactylum tricornutum* is grown in a time course assessment of light and dark periods for transcription profiling under nitrate and phosphate stressed induced lipid accumulation.

Materials and Methods

To compare time course growth in both the light and dark periods 9 photobioreactors with the same culture and growth conditions were set up to give an n=3 for each time-point. Each time point was sampled during the middle of its cycle. For nutrient replete conditions, sampling occurred at hour 47 (L1, Light time-point 1) and hour 59 (D1, Dark time-point 1). During growth transition from exponential to stationary phase caused by nutrient depletion sampling occurred at hour 96 (L2, Light time-point 2) and hour 107 (D2, Dark time-point 1). The last time-points occurred at maximal lipid accumulation during extended stationary phase at hour 215 (L3, Light time-point 1) and hour 227 (D3, Dark time-point 1). This time course assessment resulted in 6 total time-points (3 Light, 3 Dark) analyzed into seven conditions.

Culture and Growth Conditions.

Phaeodactylum tricornutum (Pt1) Bohlin Strain 8.6 CCMP2561 (Culture Collection of Marine Phytoplankton, now known as NCMA: National Center for Marine Algae and Microbiota) was used in all experiments. Characterization of the *P. tricornutum* strain 8.6 (Pt1) and its relationship to other *P. tricornutum* strains was previously described (Martino et al., 2007). Pt1 was grown at 20°C in 1.25 L photobioreactors in ASPII media as previously described (Provasoli et al., 1957; Cooksey and Cooksey, 1974), and the medium was buffered at approximately pH 8.2 with Tris-base. Cells were grown on a 14:10 light ($450 \mu\text{E}^{-1}\cdot\text{sec}^{-1}$):dark cycle at 20°C. Tubes were aerated with ambient air at a regulated flow rate of $0.40 \text{ L} \cdot \text{min}^{-1}$ and CO_2 from air was the only available source of carbon for the diatoms. Initial inocula were grown in similar growth conditions and after two transfers were inoculated into photobioreactors during exponential-phase to a cell concentration of approximately $1 \times 10^5 \text{ cells} \cdot \text{mL}^{-1}$. Contamination checks were performed using ASPII medium supplemented with 0.5% yeast extract and 0.5% glucose as well as plating on R2A agar. Cultures remained axenic throughout experiments.

Physiological Parameter Analysis.

Cells and media were collected and analyzed daily and at dark period time-points to measure chlorophyll a, pH, dissolved inorganic carbon (DIC), nitrate, phosphate, cell number, and Nile Red fluorescence. All measurements were done in biological triplicate. Cells (1 mL) were collected daily via centrifugation ($10,625 \times g$, 10 min) at 4°C. The supernatant was removed and stored for nitrate and phosphate analysis at 4°C. For

chlorophyll a extraction, 100% methanol (1 mL) was added to the pellets followed by 30 seconds of mixing to extract chlorophyll a, and extracts were stored in the dark at 4°C for 24 hours. Cellular debris was pelleted (10,625 x g, 10 min) and the methanol extract was measured on a spectrophotometer. Absorbance was measured at 652 nm and 665.2 nm and concentrations ($\mu\text{g/mL}$) calculated using equations reviewed by Porra (2002) (Porra, 2002). Absorbance was measured on a spectrophotometer (UV-1700 PharmaSpec UV-Vis, Shimadzu). The medium pH was measured using a standard tabletop pH meter. Daily, cell culture (7 mL) was filtered (0.2 μm nylon membrane filters) in preparation for DIC measurement using a Formacs^{HT} TOC/TN Analyzer (SKALAR, Buford, GA). The analysis of nitrate and phosphate were done by following the manufacturer's colorimetric assay protocols Szechrome NAS (Polysciences, Inc.) and SensoLyte MG Phosphate Assay Kit (AnaSpec), respectively. The absorbance measurements were taken on a 96-well plate spectrophotometer (Synergy HT, Biotek).

For lipid accumulation, we followed the Nile Red assay protocol as previously described by Cooksey et al. (Cooksey et al., 1987). Cells were diluted with 1.8% NaCl (to minimize cell lysis) to an appropriate cell density that correlated to a linear relationship of cell density and Nile Red fluorescence that can be measured spectrofluorometrically. Nile Red (Sigma-Aldrich) solution (4 μL of 250 $\mu\text{g/mL}$ in acetone) was added to a cell suspension (1 mL). Specific fluorescence was determined by dividing the Nile Red fluorescence intensity by the cell number. Cell density was monitored via cell counts in duplicate with a hemocytometer.

Fatty Acid Extraction and Identification via GC-MS.

Cell density was lower at L1 and D1 so 100ml of culture in two 50ml tubes was centrifuged (15min at 4700rpm) using a bucket rotor. For time-points L2, D2, L3, and D3 only 50mL was centrifuged due to higher cell densities. Supernatant was decanted and cell pellet was re-suspended and transferred to 2ml microcentrifuge tubes. Cells were centrifuged ($10,625 \times g$, 5 min) at 4°C, supernatant was discarded and pellets were immediately flash frozen in liquid nitrogen. To extract lipids, lysed using sonication (1 min) in 2:1 dichloromethane:methanol (1 ml). Cell debris and phase separation was achieved via centrifugation (5 min at $18,000 \times g$). The aqueous layer was removed and the organic layer was transferred to a clean GC vial. Additional 2:1 dichloromethane:methanol (0.5 ml) was added to the cell debris, re-sonicated, and the organic layer was separated again via centrifugation (5 min at $18,000 \times g$) and added to the previous organic extract. The organic layer was dried under a stream of nitrogen gas at 60°C. To the dried organic layer, Borontrifluoride-Methanol 10% w/w, 500 μ l (Sigma Aldrich) was added for fatty acid transesterification. Vials were incubated at 60°C for 30 minutes and then the reaction was quenched with nanopure water (250 μ l). The volatile material was dried under nitrogen gas and hexane (0.5 ml) was added to separate FAMES into the organic layer. Saturated NaCl was added (0.250) to aid in separation of hydrophilic and hydrophobic phases. The hexane layer was then transferred into new a GC vial. Hexane (0.5 ml) was added again to remove any leftover FAMES into the aqueous layer, then transferred to organic hexane GC vial. The extracted FAMES in hexane were dried down under nitrogen gas and re-suspended in hexane (100 μ l) to

concentrate FAMES. The contents were transferred into clean 150 μ L glass inserts within new GC vials. Each time point was done in biological triplicates (n=3) for a total of 18 samples. Identification and quantification was done on an Agilent Technologies 5975C inert EI/CI MSD with Triple Axis Detector with a Phenomex ZB-FFAP 250°C 60m x 250 μ m x 0.25 μ m column and Agilent 7693 autosampler. Standard curves were used to quantify FAMES based on integrated chromatogram peak area using Agilent MassHunter Qualitative Analysis software. FAMES were identified-by comparison to the spectral data in National Institute of Standards and Technology Mass Spectral Library (NIST 08) and only the “original” fatty acids were plotted. Only peaks identified with a minimal match score of 50 or greater were considered and were then cross-referenced with the NIST MS Search 2.0 program for confirmation of FAMES species. Results were shown as fatty acids which were the original molecule transesterified.

RNA Sampling, Extraction, and Sequencing.

RNA extraction was done in duplicate from six identical photobioreactors. At early exponential phase (L1 and D1), 100ml of culture in two 50ml tubes was centrifuged (15min at 4700rpm) using a bucket rotor. For time-points L2, D2, L3, and D3 only 50mL was centrifuged due to higher cell densities. The supernatant was discarded, and cell pellets were re-suspended and transferred to 2ml microcentrifuge tubes. Cells were centrifuged (10,625 x g, 5 min) at 4°C, supernatant was discarded and pellets were immediately flash frozen in liquid nitrogen. Flash frozen aliquots were stored at -80°C until RNA extraction. Total RNA was extracted following the Spectrum Total RNA kit (Sigma Aldrich-STRN50) protocol with on-column DNAase digestion using DNase10

(Sigma-Aldrich) as suggested by the manufacturer. Initial lysis was done by a 60 second cell disruption via sonication on ice. The protocol was performed in an RNAse free-hood to prevent RNA degradation. Total RNA was not pooled but kept as respective duplicates, and samples were sent to the National Center for Genome Resources (NCGR, Santa Fe, NM). At NCGR, mRNA was isolated twice by polyadenylation purification, and first and second strand DNA was generated with random primers. Gel products were purified and PCR enriched. Nucleic acid barcodes were used to put multiple samples per lane. Products were quantified on a Nanodrop fluorometer (Thermo Scientific) and checked for quality on a Bioanalyzer 2100 (Agilent). Using Illumina's standard protocols, flow cell construction and sequence determination was performed. Four barcoded samples were run on a single lane (12 total samples, 6 time-points in duplicate). Single reads were put into FASTQ format for analysis.

Transcript Assembly.

Single-end Illumina reads were analyzed using the following pipeline. First, reads from each sample were aligned against the *P. tricornutum* genome (v. 17) from ENSEMBL (Kersey et al., 2009) with the TopHat algorithm (Trapnell et al., 2009). TopHat was configured to use known splice junctions for Pt1, as retrieved from genome annotation version 61 from ENSEMBL. Mapped reads were assembled into putative transcripts using Cufflinks (Trapnell et al., 2010). Abundance of each transcript was estimated in each sample then compared across samples using Cuffcompare and Cuffdiff, parts of the Cufflinks toolkit (Trapnell et al., 2010). Similar pipelines have been validated as well as the accuracy of RNA-seq with spike in experiments and qPCR

comparisons (Marioni et al., 2008; Mortazavi et al., 2008; Nagalakshmi et al., 2008; Wang et al., 2009; Jiang et al., 2011; Twine et al., 2011).

Transcript Identification and Pathway Analysis.

Cufflinks output files had transcripts identified by uniprot accessions. Using the DAVID (Database for Annotation, Visualization, and Integrated Discovery) (Huang et al., 2007). The DAVID Functional Annotation tool was used to retrieve gene names as well as KEGG (Kyoto Encyclopedia of Genes and Genomes) Pathway information. For genes that were identified as hypothetical proteins, searches were performed on the JGI *Phaeodactylum tricornutum* v2.0 genome website (<http://genome.jgi-psf.org/Phatr2/Phatr2.home.html>) and based on best hits, %ID, score, and consistency of the top hits, genes were either identified or remained as hypothetical. Genes were also searched on NCBI and ENSEMBL genome browsers for cross-referencing. To assign genes into pathways, we used KEGG maps for *P. tricornutum* as a backbone. Genes for major pathways (Urea Cycle, C4) were searched manually to find genes not directly annotated in the *P. tricornutum* KEGG maps. Gene lists were compiled for the major pathways and developed into network maps. Organelle targeting of gene transcripts was done based on annotations from the databases of JGI, NCBI, and ENSEMBL and literature. If no localization was found, eukaryotic organelle localizations were predicted with TargetP 1.1. Server (Emanuelsson et al., 2007) in both plant and non-plant mode. Amino acid sequences were also checked for a peroxisomal targeting sequence (SKL, serine-lysine-leucine). If nothing was established we assumed that the gene occurred in

the cytoplasm. If nothing could be determined we located the gene in the most biologically relevant membrane, i.e. light harvesting complex in the plastid.

Results and Discussion

Growth Analysis Under Nitrate and Phosphate Depletion.

Diatoms were grown on a 14:10 light:dark cycle and RNA and FAMES sampling was done in the middle of each light and dark period separated by approximately twelve hours between each sampling point. The sampling time-points are indicated by arrows in figures 4.1, 4.2, and 4.3 and 4.4a. L1 (Light time-point 1, hour 47) and D1 (Dark time-point 1, hour 59) are during exponential phase when cells are replete of C, N, and P. These time-points are considered the control time-point for which expression values are compared *i.e.* L2 (hour 96) and L3 (hour 107) compared to L1 expression and D2 (hour 215) and D3 (hour 227) are compared to D1 expression. Comparisons were also made between the diel cycle time-points, meaning L1 expression compared to D1 expression. This comparison tells us the differential expression of genes controlled by the diel cycle. Sodium nitrate and potassium phosphate were the only forms of nitrogen and phosphorus available for growth, respectively. Constant aeration with ambient air in photobioreactors provided the only source of carbon (CO₂) to *P.tricornutum*. The media was buffered at a pH of 8.2 to ensure most inorganic carbon is available as bicarbonate (HCO₃⁻). Nitrate and phosphate was monitored daily throughout growth. The first time-points (L1 and D1) were chosen during exponential growth when phosphate and nitrate were still available exogenously, meaning under nutrient replete conditions. Figure 4.1B

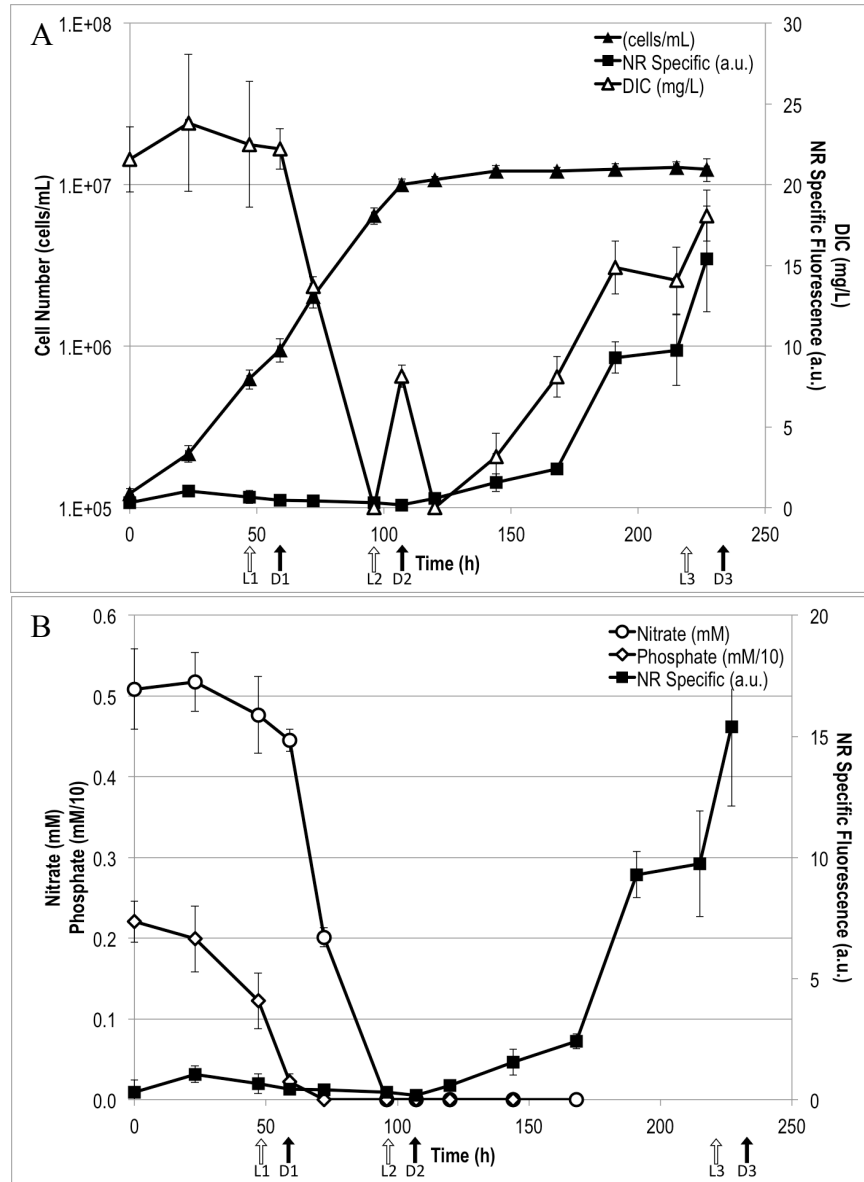


Figure 4.1 Growth characterization of *P. tricornutum*. A) Cell density ($\blacktriangle$) in cells/mL, DIC (Δ) mg/L and Nile Red specific (NR/cell) fluorescence ($\blacksquare$) in arbitrary units. DIC shows decreasing concentration at exponential phase and rebounding during stationary phase growth. B) Nitrate ($\circ$) and Phosphate ($\diamond$) depletion. Phosphate concentrations are multiplied by a factor of 10 to scale with nitrate. Nile Red specific (NR/cell) fluorescence ($\blacksquare$) in arbitrary units. Depletion of nutrients leads to increased lipid accumulation. Arrows indicate time-points for RNAseq sampling.

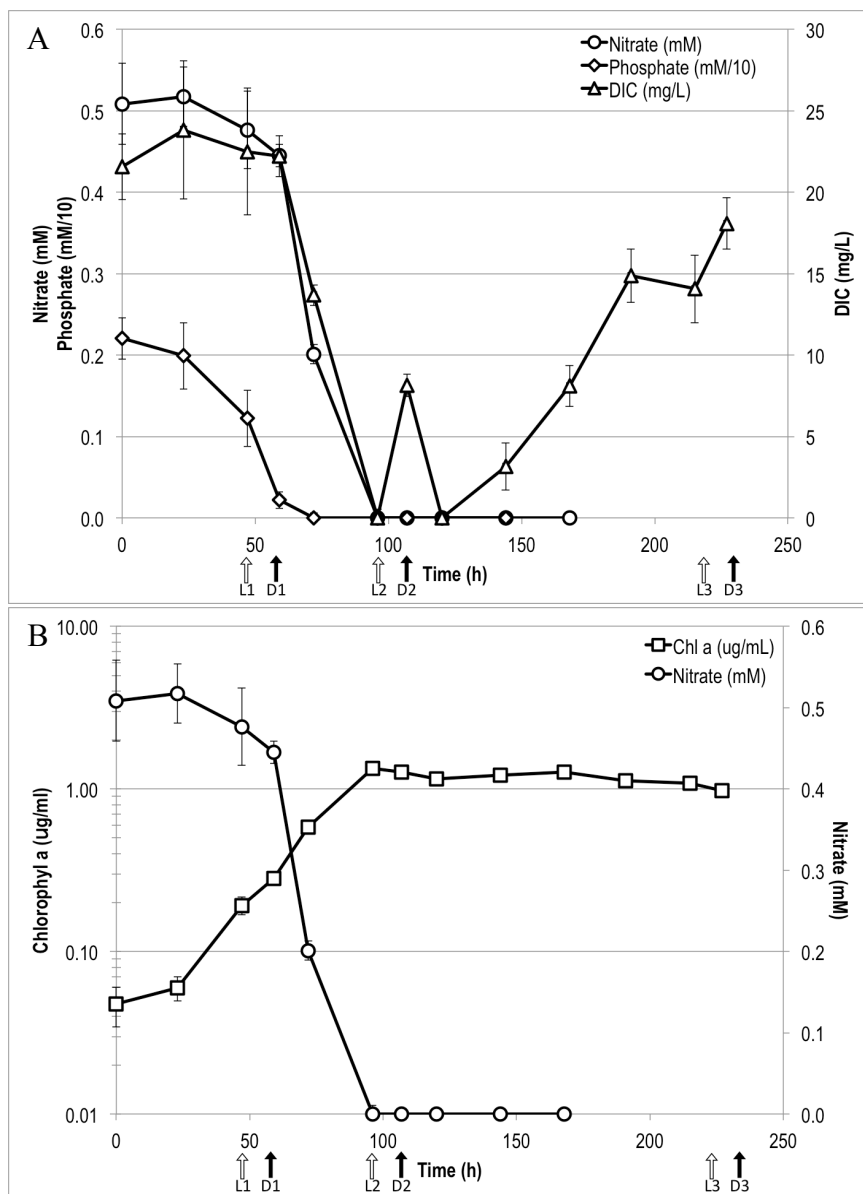


Figure 4.2 Growth characterization of *P. tricornutum*. A) Nitrate (●) and Phosphate (◇), and DIC (Δ) mg/L. During L1 and D1 media has available C, N, and P. At L2 depletion of N and P ensues with limitation of DIC. DIC rebounds at D2, L3 and D3, while N and P remain depleted. Phosphate concentrations are multiplied by a factor of 10 to scale with nitrate. B) Chlorophyll A (□) concentrations increased with available N, but level off as Nitrate (●) depletes. Arrows indicate time-points for RNAseq sampling.

shows nitrate and phosphate availability in the media. The nitrogen to phosphorus (N:P) ratio of ASPII is approximately 20.5:1 (Provasoli et al., 1957; Valenzuela et al., 2012). The Redfield ratio indicates that N:P ratio of phytoplankton to be approximately 16:1 (Redfield, 1958; Ptacnik et al., 2010), thus exogenous phosphate usually depletes before nitrate during growth on ASPII media. Figure 4.1b indicates depletion of phosphate occurs prior to nitrogen depletion. However, phosphate depletion does not cause cell cycle arrest. Internal polyphosphate storage has been shown in *Pt1* previously (Leitao et al., 1995; Diaz et al., 2008). Thus, although exogenous phosphate is not available internal stores may allow for cells to continue cellular processes unimpeded. From the previous chapters of this dissertation it has been shown that phosphate depletion can initiate lipid accumulation but nitrate depletion intensifies this process.

As nitrate depletes prior to hour 96, lipid accumulation is initiated (Figure 4.1A,B). Cells go through one more doubling and transition into stationary phase as indicated by cell density from figure 4.1A. At time points L2 and D2 cells are nutrient depleted and lipid accumulation is initiated as cells transition from growth to lipid storage. This transition is also represented by chlorophyll a content in figure 4.2B. Chlorophyll a can be considered an indicator of growth and when nitrate depletes there is a decrease of chlorophyll a abundance followed by a leveling off, as cells enter stationary phase. From time-points L2 and D2 until L3 and D3 lipid accumulation is occurring and is tracked by Nile Red fluorescence in figure 4.1. The increase in NR specificity is an indicator of lipid accumulation and it increases 16-fold from exponential phase transition (L2) to the late time-points L3 and D3 of extended stationary phase.

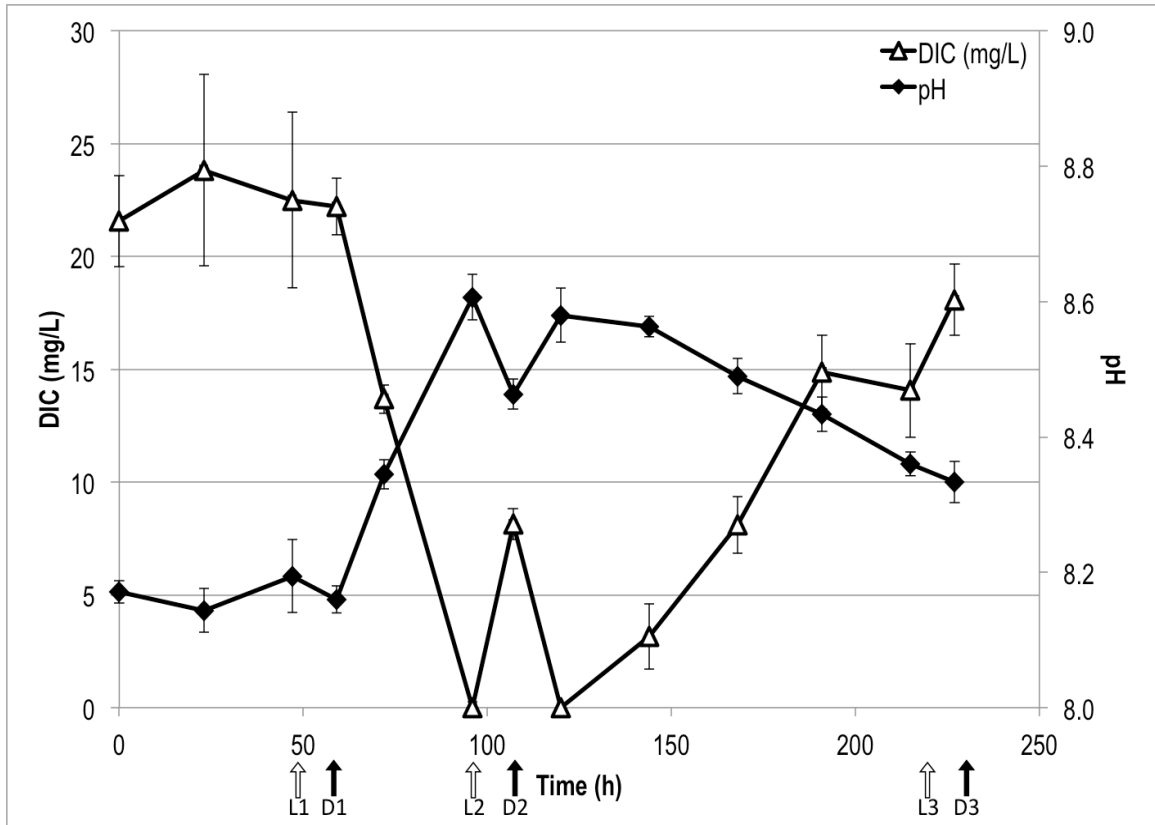


Figure 4.3 Relationship between pH and DIC availability in the media. DIC (Δ) mg/L and pH ($\blacklozenge$) throughout time course. The relationship is inverse and D2 (107 hours) shows the rebounding of DIC as photosynthesis is down-regulated during the dark period.

The availability of dissolved inorganic carbon is a critical component of understanding cellular growth compared to lipid accumulation. DIC is at equilibrium around 22mg/L when the pH is approximately 8.2 (Figure 4.3) in ASPII media. Most of the DIC is in the form of bicarbonate (HCO_3^-). During the early time-points DIC is available to cells at relatively high concentrations. Thus, at L1 and D1 cells are C, N, and P replete. But as exponential phase ensues there is a decrease in inorganic carbon availability as consumption has increased. As seen in figure 4.3 there is a minimal DIC available in the media, however it is limited not depleted as it is continually being aerated

into the photobioreactors. At D2 you see a spike in the DIC availability, this is an indication of a decrease in carbon fixation as light is not available to drive photosynthetic carbon assimilation. During lipid accumulation the DIC rebounds back towards equilibrium. Chapter 2 determined there is a distinctive difference in DIC requirement when cells are actively growing and dividing during replete conditions compared to nutrient depleted conditions and lipid accumulation. Cells require more carbon for biomass production and cellular growth, thus during exponential phase there is limitation in DIC. Availability of DIC rebounds as cellular growth is arrested and cells begin to accumulate lipids, indicating a smaller requirement for DIC. In summary, L1 and D1 cells are under replete conditions and do not accumulate lipids. At time-points L2 and D2 N and P is depleted resulting in cell cycle arrest and initiation of lipid accumulation, while DIC availability is limited. During extended stationary phase L3 and D3 cells have been shifted from a cellular growth phase to lipid storage condition as DIC rebounds toward equilibrium.

Fatty Acid Accumulation Under Nutrient Stress.

P. tricornutum growth on ASPII media results in lipid accumulation initiated by phosphate depletion and intensified by nitrate depletion. Figure 4.4A and B show lipid accumulation increasing as cells transition from exponential phase cellular growth to stationary phase indicated by Nile Red fluorescence and total fatty acids extracted at the six time-points. Many algae accumulate TAG during the light period and consume there energy stores via β -oxidation during the dark period to maintain ATP demands or cellular growth (Radakovits et al., 2010). Thus, it would be expected that fatty acid concentration

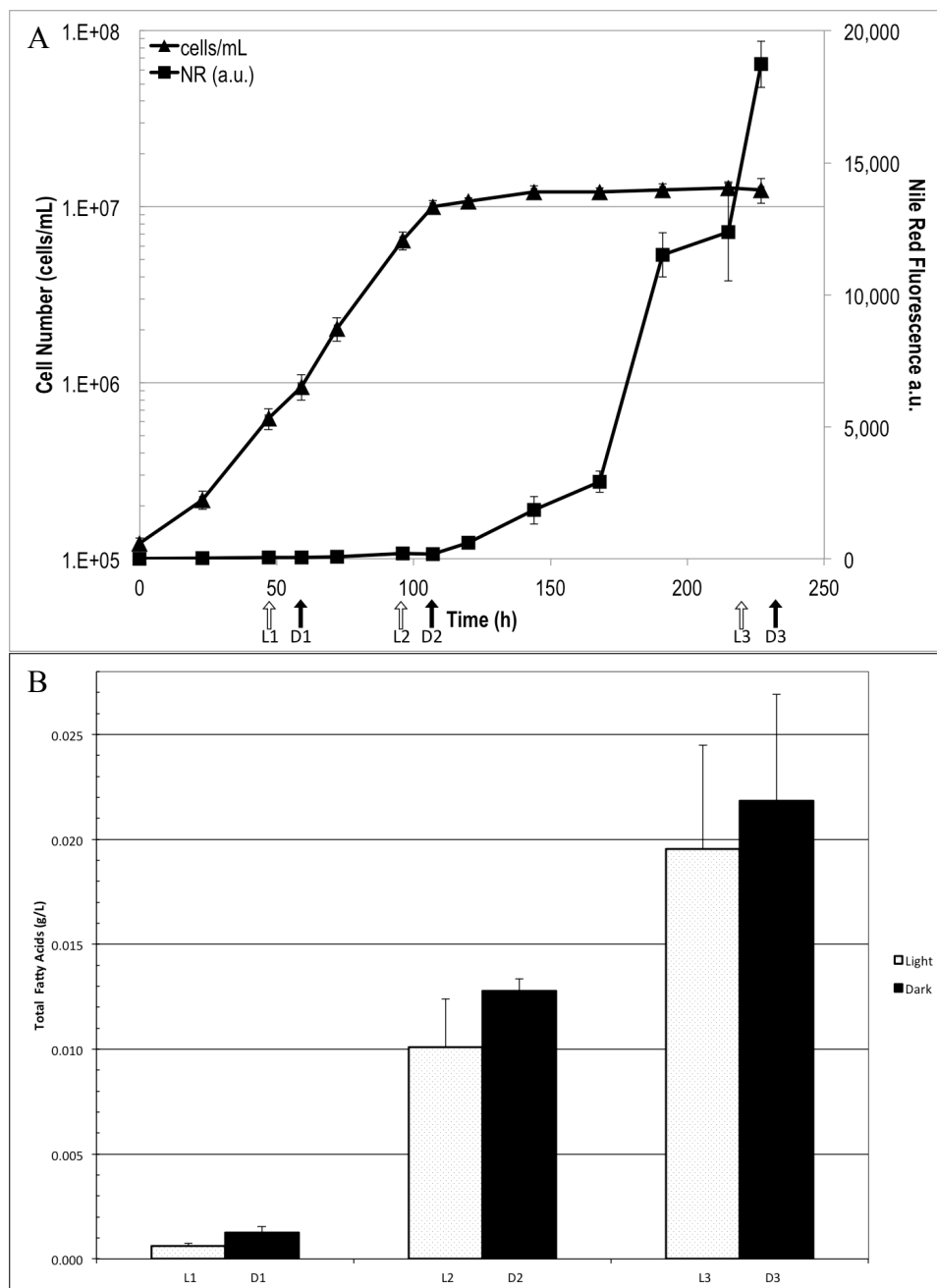


Figure 4.4 Lipid accumulation of *P. tricornutum*. A) Cell density (▲) in cells/mL and Nile Red fluorescence (■). An increase in NR fluorescence correlates to B) increased total fatty acids g/L.

would be less during the night time-points compared to the light time-points. Figure 4.4B does not indicate this, most likely based on when the samples were taken. For instance, maximal accumulation would be expected at the end of the light cycle when cells had 14 hours to accumulate lipid. In this study, cells were sampled in the middle of the light period, meaning only 7 hours of accumulation, so slightly reduced yields are observed. Conversely, maximum consumption would be observed at the end of the dark cycle, giving cells 10 hours to consume lipids. However, sampling during the middle of the dark cycle only allowed for 5 hours of consumption. Thus, strictly based on theoretical time differences the dark time points had a net of 2 hours of lipid accumulation (7 hours in light – 5 hours in dark). Regardless, the principal of total lipid accumulation is observed.

Fatty acid profiles (Figure 4.5A and B) indicate that the main fatty acid species accumulated is 9-hexadecanoic acid, hexadecanoic acid, and tetradecanoic acid, a 16:1, 16:0, and 14:0 respectively. These results for *P. tricornutum* are corroborated by chapter 2 findings and a nitrate limitation study done by Rezanka *et. al.* 2012. The authors concluded similar results with the same top fatty acids being hexadecanoic acid and 9-hexadecanoic acid (Řezanka et al., 2012). The accumulation of saturated fatty acids is an indication of energy storage as there is more energy stored in saturated fatty acids than polyunsaturated fatty acids. Interestingly the fatty acids 9,12-hexadecadienoic acid and 9,12-octadecadienoic acid had higher levels at L2 and D2 than L3 and D3. This could be an indication of selective consumption. Meaning at L3 cells shifted to more saturated fatty acid production and consumed the less energy stored molecules 9,12-hexadecadienoic acid and 9,12-octadecadienoic acid.

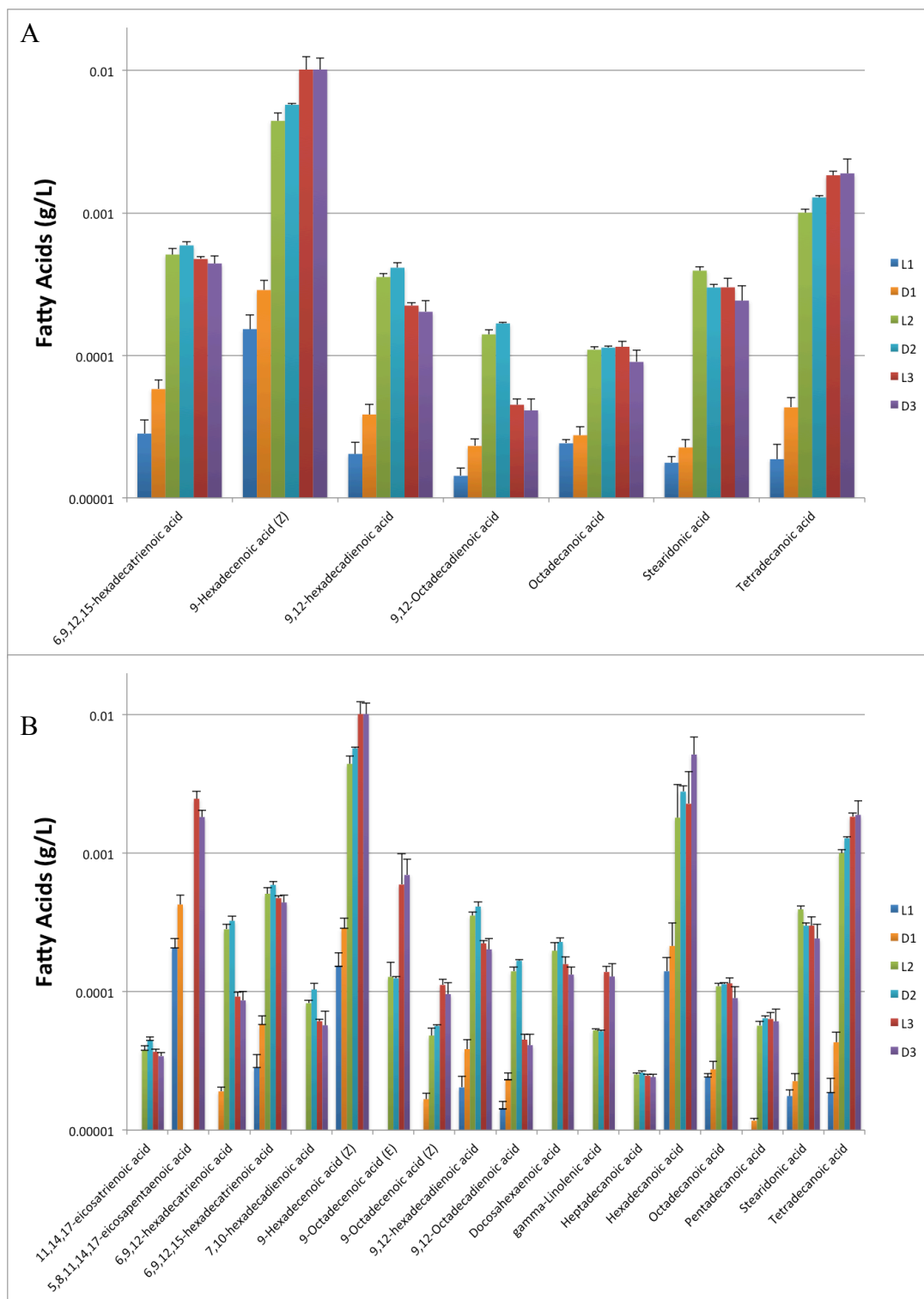


Figure 4.5 Fatty acid profiles of *P. tricornutum*. A) Fatty acid profiles of the common fatty acids in all time-points on a logarithmic scale. Fatty acids are listed in chronological order (L1, D1, L2, D2, L3, and D3). B) Expanded profiles showing all fatty acids that are detected in at least four of the six time-points on a logarithmic scale.

The polyunsaturated fatty acid 5,8,11,14,17-ecosapentaenoic acid (EPA) showed an interesting profile. EPA showed increased concentrations at early and late time-points with no detection during transition to lipid accumulation (figure 4.5B). This expression pattern follows the trend of DIC. DIC is available at the early time-points and is rebounding at late stages of accumulation. Similar results were shown in *Navicula saprophilia* and *Phaeodactylum tricornutum*, under increased CO₂ more EPA was produced compared to reduced CO₂ availability (Yongmanitchai and Ward, 1991; Kitano et al., 1998). The biological consequence of EPA regulation by DIC needs more investigation, but in terms of valuable products Omega-3 fatty acids are desirable. Thus, using environmental controls carbon could be directed into EPA as a way of producing high valued metabolites.

RNA Sequencing Analysis

A diel cycle time course assessment strategy was employed in this study to identify possible control points regulating the partitioning of carbon into lipids. The direct sequencing of RNA allows for in depth global transcript analysis. Global transcriptomic profiling is inherently a discovery-based technique but correlating it to extensive growth and genomic data allows for biologically relevant interpretation of physiology and metabolism. Using the Cufflinks open source software RNAseq produces transcript abundances reported as FPKM (Fragments Per Kilobase of exon per Million fragments mapped) (Mortazavi et al., 2008; Trapnell et al., 2010; Roberts et al., 2011). Essentially expression values of transcripts mapped to the genome. In this case, RNA sequencing produced 34,720 significantly (p-value <0.05) expressed genes from seven

conditions (L2vL1, L3vL1, D2vD1, D3vD1, L1vD1, L2vD2, and L3vD3). The average number of significant genes is 4,960 per condition. The amounts to almost 50% coverage of the *P. tricornutum* genome (approx.10,000 genes).

KEGG_Pathway Annotation Clusters	L2vL1	L3vL1	D2vD1	D3vD1
Tyrosine metabolism				5
Regulation of autophagy			4	
Pyruvate metabolism	7			10
Purine metabolism	15			
Phenylalanine, tyrosine and tryptophan biosynthesis		7		
Phenylalanine metabolism		5		
Pentose phosphate pathway		11		
Oxidative phosphorylation	12	23	11	14
Nitrogen metabolism	6	7		6
Glycolysis / Gluconeogenesis	9	15		11
Glycine, serine and threonine metabolism		10		
Fatty acid elongation in mitochondria				3
Endocytosis		14	11	16
Cysteine and methionine metabolism		10		
Citrate cycle (TCA cycle)	13	16	12	15
Arginine and proline metabolism	8	16		10
Alanine, aspartate and glutamate metabolism	7	12		8
Ribosome	77		77	
Pyrimidine metabolism		15		
Proteasome	18	13		
Porphyrin and chlorophyll metabolism	9		11	
Homologous recombination		8		6
Fatty acid biosynthesis	5		5	6
DNA replication		12		

Table 4.1 KEGG_Pathway Annotation Clusters of *P. tricornutum* time course growth. Pathways listed were significantly clustered based on DAVID annotation tool. Values indicate number of genes identified in each cluster. Only compared light to light and dark to dark periods throughout the time course of the experiment. Pathways in green were significantly up-regulated 2-fold or greater, Red clusters indicate down-regulation 2-fold or greater.

KEGG_Pathway Annotation Clusters	L1vD1	L2vD2	L3vD3
Porphyrin and chlorophyll metabolism	12	12	12
Valine, leucine and isoleucine degradation		4	6
Pyruvate metabolism			8
Arginine and proline metabolism			6
Sulfur metabolism			3
Cysteine and methionine metabolism	9		
Lysine biosynthesis	4		
Citrate cycle (TCA cycle)	10		
Aminoacyl-tRNA biosynthesis	13		
Pyruvate metabolism	10		
Ribosome		26	
Glyoxylate and dicarboxylate metabolism			4
Regulation of autophagy			4
Cysteine and methionine metabolism			5
Glycine, serine and threonine metabolism			5
Pentose phosphate pathway			5
Purine metabolism	24		
RNA polymerase	10		
Pyrimidine metabolism	15		7
Alanine, aspartate and glutamate metabolism	8		
Aminoacyl-tRNA biosynthesis	10		
Nitrogen metabolism	5		
RNA degradation	10		
Protein export	4	4	
Fatty acid biosynthesis		5	
Spliceosome			10
Glutathione metabolism			4
DNA replication			5
Purine metabolism			8

Table 4.2 KEGG_Pathway Annotation Clusters of *P. tricornutum* light verse dark periods. Pathways listed were significantly clustered based on DAVID annotation tool. Values indicate number of genes identified in each cluster. Green pathways indicated greater than 2-fold expression increases during light period of the time-points. Black indicates neutral expression changes (less than 2-fold) in either light or dark periods. Red pathways have a greater than 2-fold expression during the dark period compared to the light during the 24hr cycle.

To begin understanding the function of such large gene lists, DAVID (Database for Annotation, Visualization, and Integrated Discovery) bioinformatic database was used to annotate possible pathways. Gene lists partitioned into either up-regulation

(Green), down-regulation (Red), or neutral-regulation (Black) for each comparison and searched with the DAVID bioinformatics tool. By only focusing on genes annotated to KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways functionally annotated clusters were produced resulting in Tables 4.1 and 4.2. From the light and night time course cluster analysis (Table 4.1) it is clear that central carbon metabolism (*i.e.* pyruvate metabolism, glycolysis, TCA cycle) is up-regulated during the light phases along with nitrogen metabolism. Central carbon metabolism is need to maintaion cellular function throughout growth and could possible provide precursors to fatty acid biosynthesis (Valenzuela et al., 2012; Liang et al., 2012a). Nitrogen metabolism is up-regulated throughout light growth even under nitrogen depletion so that cells remain primed for nitrogen assimilation in the case of encountering more nitrogen. During the dark cycles TCA cycle and oxidative phosphorylation remain up-regulated presumable to continue producing ATP and carbon backbones for cellular homeostasis during the dark cycle.

Urea Cycle is Coupled to Central Carbon Metabolism.

The genome sequencing of *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* revealed a complete urea cycle in the marine diatoms (Parker et al., 2008). The urea cycle is an essential metabolic pathway for the homeostasis and anaplerotic reactions for carbon and nitrogen metabolism (Allen et al., 2011; Bender et al., 2012). The urea cycle links the TCA and the glutamine synthetase/glutamate synthase cycle via intermediates (Allen et al., 2011). It serves as a hub for redistribution of nitrogen and inorganic carbon to provide precursors for energy metabolism pathways. It also maintains

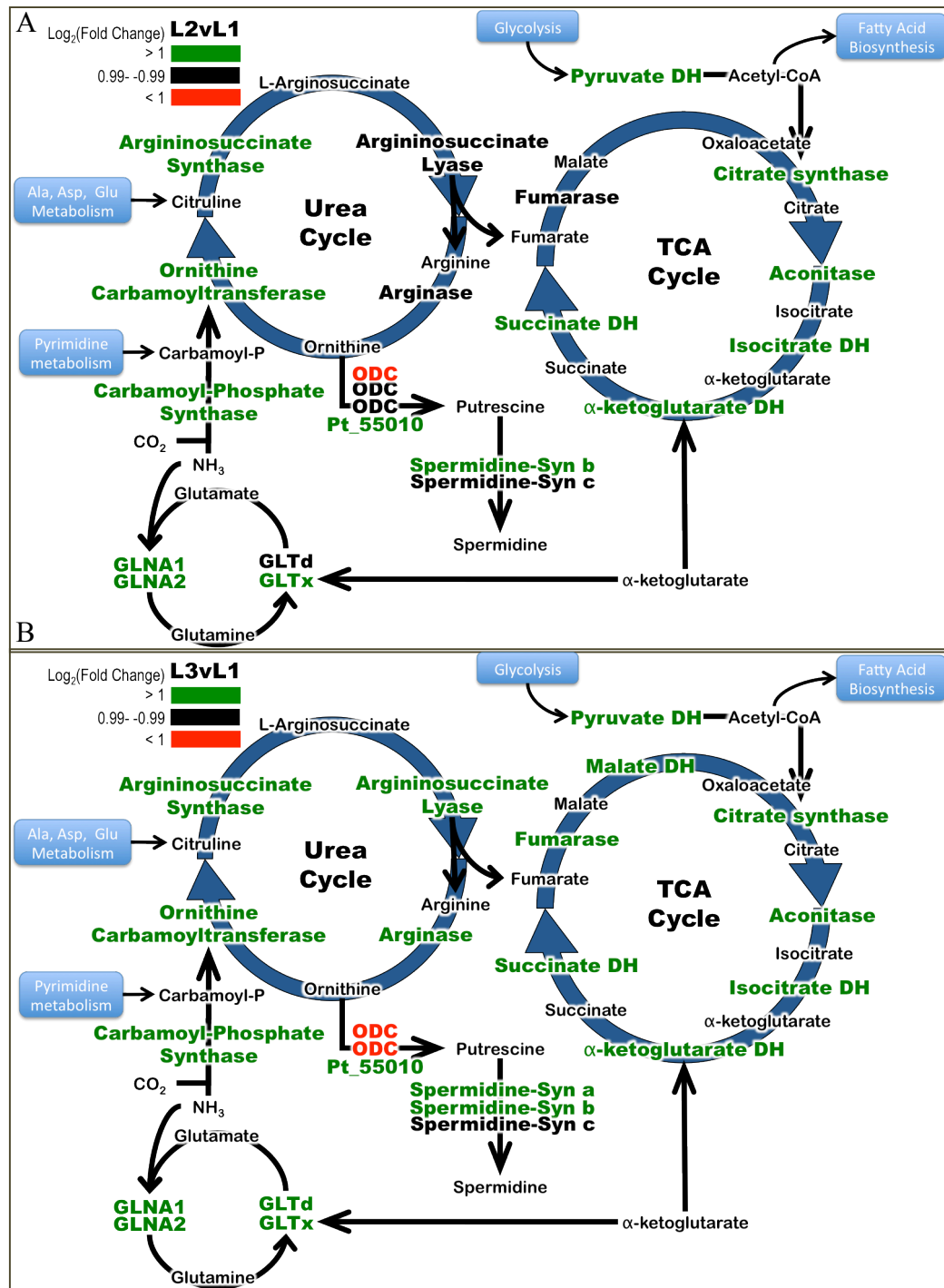


Figure 4.6 Urea and TCA cycle of *P. tricornutum* during light periods. A) L2 urea and TCA cycle expression during initial nutrient depletion compared to nutrient replete conditions (L1). B) Urea and TCA cycle during N+P depleted lipid accumulation of the light cycle (L3) compared to nutrient replete conditions of (L1).

its up-regulation in preparation for encountering nitrogen for the quick turnover of carbon

for growth (Allen et al., 2011). Figure 4.6 shows expression during the light phases of growth. At L2 during initial nitrogen depletion there is a semi up-regulated urea cycle, however, at L3 the entire cycle is up-regulated. This correlates to Allen *et. al.* 2011 findings that urea cycle is maintained as a response to episodic nitrogen availability. Cells are in extended stationary phase and are maintaining the urea cycle pathway for anaplerotic reactions in the case of encountering a nitrogen source. The urea cycle could play a key role in nitrogen depleted lipid accumulation by providing precursors that are up-stream of acetyl-CoA carboxylase that can “push” metabolites into fatty acid biosynthesis, as predicted in chapter 3. One of the main components of the urea cycle is carbamoyl phosphate synthase (CPS). CPS produces carbamoyl phosphate from CO_2 and NH_3 , which indicates its importance in cellular N and C homeostasis.

Carbon Concentrating Mechanisms

The role of carbon concentrating mechanisms in photoautotrophs is to concentrate CO_2 around RubisCO to increase photosynthetic carbon fixation and reduce photorespiration (Reinfelder, 2011). There are two types of CCMs, one based on a biophysical transport and another based on biochemical shuttling. In a biophysical CCM the direct transport of DIC via carbonic anhydrases and bicarbonate transporters to the plastid containing RubisCO. During a biochemical CCM the cell takes advantage of the low K_m of carboxylases to fix inorganic carbon to a C_3 (3-carbon molecule) making a C_4 (4-carbon) intermediate. The C_4 molecule is then transported into the plastid where it is decarboxylated releasing a CO_2 around RubisCO and recycling of the C_3 molecule for another round of fixation.

Kroth *et. al.* in 2008 postulated a CCM model in marine diatoms based on sequence analysis of the two marine diatom genomes. In chapter 4 RNAseq analysis proposed a C4 CCM during nitrate depleted and DIC limited conditions utilizing a pyruvate carboxylase (PYC1) as the primary carboxylase to fix inorganic carbon to oxaloacetate (C4). Eventually being decarboxylated by malic enzyme in the peroxisome. During extended stationary phase CAs showed increased expression indicating a possible biophysical CCM. In the current study at L2 a C4 CCM (Figure 4.7a) is not apparent as malate dehydrogenase and malic enzyme expression are not significant, thus there abundance cannot be expressed in the model. PYC1 is not up-expressed but phosphoenolpyruvate carboxylase 1 (PEPcase1) does have increased expression, so it may be functioning as the initial carboxylase. However, decarboxylation of oxaloacetate by phosphoenolpyruvate carboxykinase does not seem to be an up-regulated route for concentrating carbon. The transporters in L2vL1 do show increased expression around the plastid, thus possibly employing a partial biophysical CCM or to keep CO₂ in the plastid from diffusing out by converting it to the charged bicarbonate (HCO₃⁻) ion. However, at L3 during stationary phase, with DIC rebounding, there is indication of C4 CCM occurring through PEPcase1(C4 carboxylation), malate dehydrogenase, and the plastidal malic enzyme (decarboxylation). Plastid CAs are mostly down-regulated or at basal levels indicating a biophysical pathway is not participating, but could be reduce efflux of CO₂ out of the plastid.

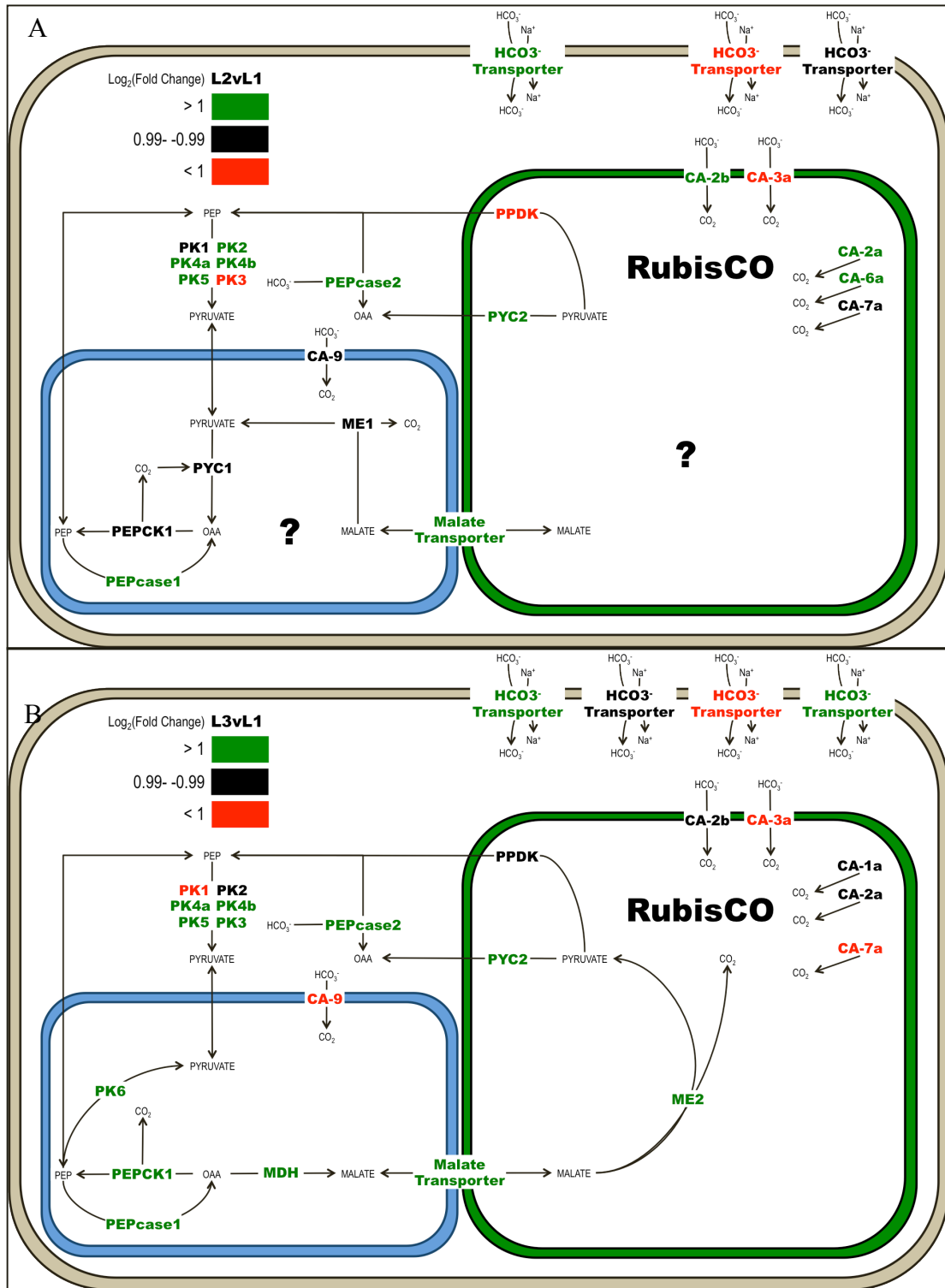
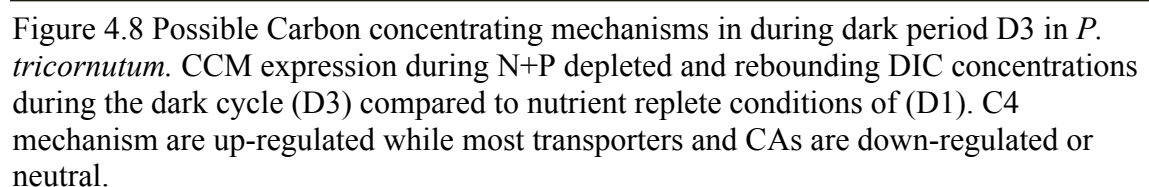


Figure 4.7 Possible carbon concentrating mechanisms (CCM) during light periods. A) L2 CCM expression during initial nutrient depletion compared to nutrient replete conditions (L1). B) CCM expression during N+P depletion and rebounding DIC concentrations during lipid accumulation of the light cycle (L3) compared to nutrient replete conditions of (L1).



Potential Metabolic Control Points for Lipid Accumulation.

This study was aimed at understanding control mechanisms that may direct carbon partitioning into lipid synthesis during accumulation. The balance between C and N homeostasis is critical for lipid accumulation as seen in chapter 2 of this dissertation. Three genes that have potential for controlling carbon flux during lipid accumulation in *P. tricornutum* are Acetyl-CoA-Carboxylase (ACC1), Carbamoyl Phosphate Synthase (CPS), and the Gene of Unknown function (55010). Their expression profiles are shown in figure 4.9.

ACC1 is the rate-limiting step in fatty acid biosynthesis and has been targeted in the past as a control point. In chapter 3, the case was made that it is upstream precursors that are driving fatty acid biosynthesis by “pushing” carbon flux towards ACC1. In this study ACC expression parallels that of lipid accumulation with greater increases in expression as lipid accumulation increases from L2 to L3 (figure 4.9A). However, expression at night is interesting with highest expression during nutrient replete conditions. This could be a consequence of cell division and the need for cellular membranes as mitosis usually occurs during the dark cycle (Chauton et al., 2013). As cell division subsides in D2 and D3 ACC expression during the dark cycle decreases. This correlates to the conclusion that the demand for carbon during biomass production is greater than during lipid accumulation and cell division may require more fatty acids than what is stored during TAG synthesis. Over expression of ACC did not produce greater lipid accumulation even though it did produce greater amounts of ACC (Sheehan et al., 1998).

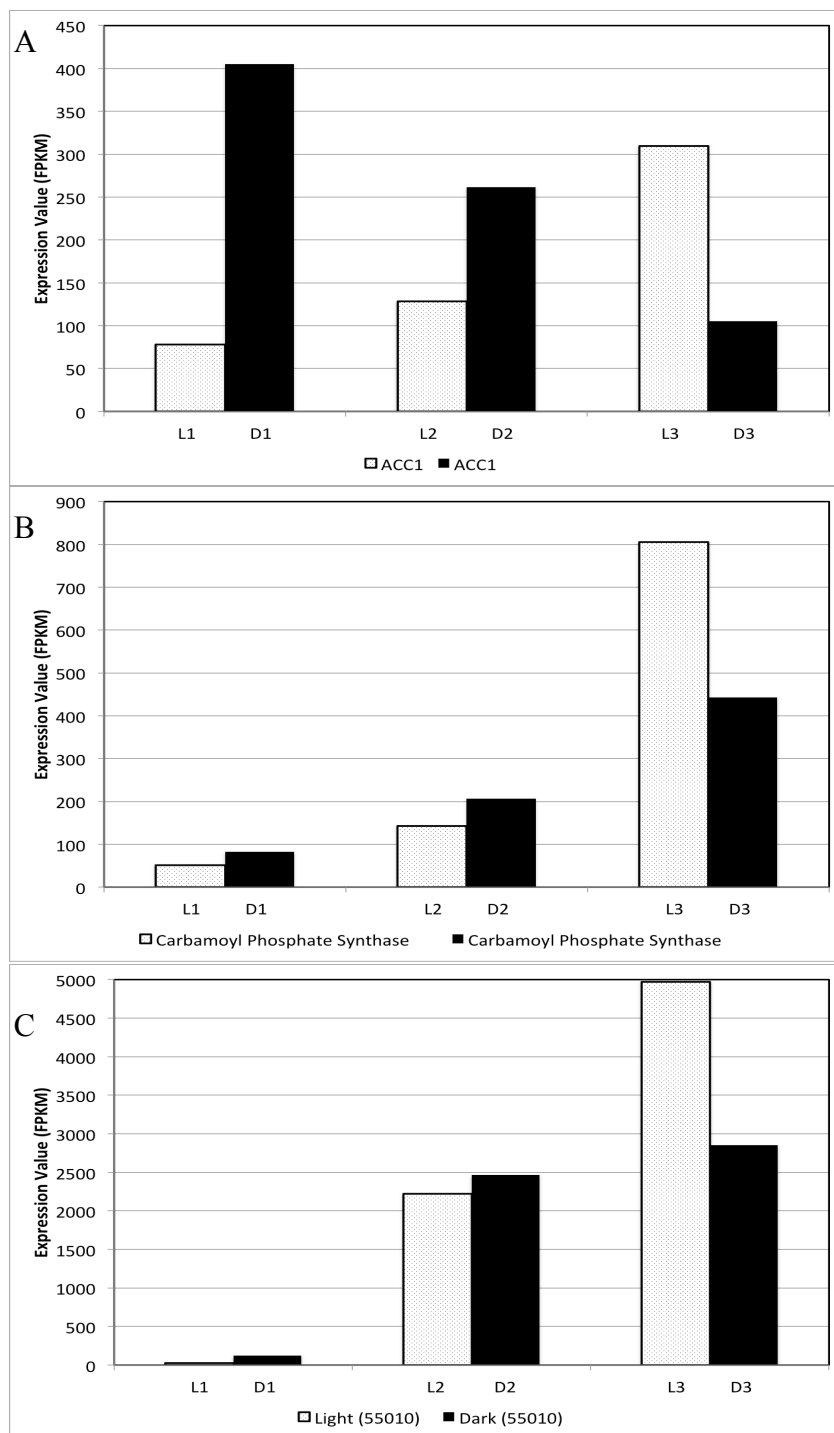


Figure 4.9 Possible metabolic control points for targeting in lipid production in *P. tricornutum* showing expression values throughout the time course experiment. A) ACC1 (plastid) is the rate limiting step in fatty acid biosynthesis, B) Carbamoyl Phosphate Synthase, is a integral enzyme in C and N homeostasis and the urea cycle, C) Protein of Unknown function (Phatr_55010) is one of the most highly expressed genes under N depletion and has significant sequence homology to coccolith scale associated protein-1 of *Pleurochrysis carterae*.

Tandem over expression of precursor genes like pyruvate dehydrogenase and ACC together could cause greater yields by having a large push from precursors and a over expression of ACC.

The expression of carbamoyl phosphate synthase (CPS) does not change between the daily light and dark cycles (Figure 4.9B). This suggest it is not light nor energy dependent but rather carbon and nitrogen dependent because its expression changes throughout the growth cycle, not between the diel cycle. CPS is playing a crucial part in C and N homeostasis by providing a go between CO₂ and NH₃ and the urea cycle (Allen et al., 2011). The more limited the cells are of nitrogen *i.e.* L3 the greater the expression of CPS. Light cycle expression follows that of ACC and since CPS provides anaplerotic reactions for TCA cycle and nitrogen metabolism it may indirectly effect carbon flow into lipid accumulation. Over-expression of CPS during nitrogen replete conditions could possibly manipulate metabolism into shifting carbon precursors to fatty acid accumulation.

One of the highest expressed genes during Q2 and Q3 of chapter 3 was a gene of unknown function (55010). It has sequence homology to a coccolith scale associated protein 1. In L2, D2, L3 and D3 it is a highly expressed gene. Expressed sequence tag data indicates it's a highly expressed gene during nitrate depletion as well (Maheswari, 2004). This could be a possible target gene for understanding carbon homeostasis in Pt1. Using RNAi to knock down translation could determine how it effects growth and possibly lipid accumulation.

Conclusions

Lipid accumulation is initiated by phosphate depletion and intensified by nitrate depletion in *P. tricornutum*. DIC availability decreases during exponential growth as higher carbon demand is required for cellular growth. Upon nitrate depletion cell cycle is arrested and the diatoms transition to lipid accumulation. DIC begins rebounding and lipid accumulation is observed. Fatty acid accumulation is led by 9-hexadecanoic acid, hexadecanoic acid, and tetradecanoic acid. Night cycle consumption seems to target 9, 12- hexadecadienoic acid and 9,12-Octadecanoic acid. The high value product 5,8,11,14,17-ecosapentaenoic acid (EPA) was produced during DIC replete conditions, thus providing a route for targeted production. The urea cycle is up-expressed during the light period during extend nitrate depletion (L3) as it provides important intermediates for redistribution of carbon and nitrogen molecules. CPS plays an import role in the urea cycle and may be involved in directing and accumulating precursor molecules for fatty acid biosynthesis. Incomplete C4 CCMs for concentrating CO₂ in the plastid for C1 fixation by RubisCO are observed during the light cycle. During the D3 many of the C4 genes are up-regulated to presumably decrease photorespiration and provide anaplerotic reactions to replenish central carbon cycles. ACC and the coccolith scale like protein (55010) play important roles in carbon homeostasis and lipid accumulation and are potential targets for controlling metabolic flux.

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CHAPTER 5

OVERALL CONCLUSIONS

In order for algal biofuel to become a feasible alternative to fossil fuel production must become more cost efficient. There are three areas of research focusing on increasing optimization and efficiency; growth, harvesting and processing (Scott et al., 2010). The focus of this dissertation has been on optimization of growth, specifically by understanding lipid metabolism. Increasing the lipid yield of a microalgae by as little as 10% translates to a significant amount of production when extrapolated to the scale of production. Thus, it is imperative that lipid production is pushed to the limit to maximize value.

To understand growth and lipid metabolism this study has focused on one organism, *Phaeodactylum tricornutum*. *P. tricornutum* is a unicellular marine diatom belonging the class *Bacillariophyceae* (Scala, 2002). There are 10 different strains based on genetic and phenotypic characteristics (Martino et al., 2007). The strain Pt1 was selected for whole genome sequencing. Over 10,000 genes were predicted from a 27.4Mb genome (Bowler et al., 2008). The combination of a sequenced genome and a large research background has made *P. tricornutum* a sufficient model organism for which to study growth and metabolism as it pertains to lipid accumulation.

The objectives of this dissertation were to characterize growth and lipid accumulation in *P. tricornutum*. After initial characterization, global transcriptomic

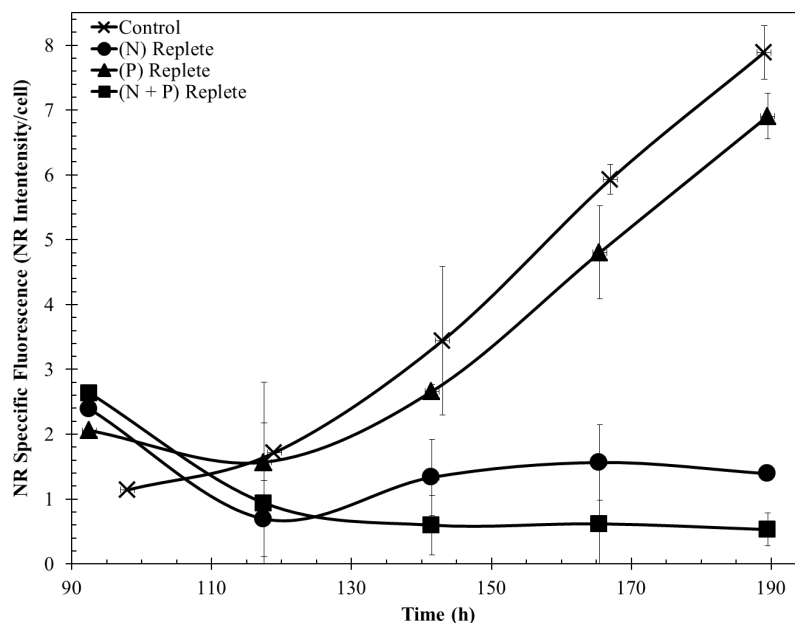


Figure 5.1 Effects of nitrate and phosphate depletion. N + P replete conditions (■), N replete or P depleted (●), P replete or N depleted (▲), N + P depleted (x).

profiling was employed to determine possible metabolic control points directing lipid accumulation. Identification of possible control points would become targets for modification to enhance total lipid yield. For many microalgae nutrient limitation causes increased TAG content. Nitrogen limitation will initiate lipid accumulation in *P. tricornutum* (Yu et al., 2009), but phosphate depletion can also be an initiator of TAG accumulation (Khozin-Goldberg and Cohen, 2006; Liang et al., 2012b).

Chapter 1 investigations focused on the difference in lipid accumulation during phosphate, nitrate, and combined nitrate and phosphate stress together. Results indicated that phosphate and nitrate depletion can initiate TAG accumulation independently, with nitrate depletion having a greater effect. The combined depletion of nitrate and phosphate had the greatest effect on TAG accumulation (Figure 5.1). The transition from

cellular growth to lipid accumulation during nutrient depletion is dependent on cell cycle arrest. Global transcriptomic profiling indicated that two diatom specific cyclins (*dscyc7* and *dscyc10*) that control cell cycle progression were down-expressed during extended stationary phase. In 2010, Huysman *et al.* suggested that *dscyc7* and *dscyc10* sensed phosphate, which correlated to the down-expression during stationary phase as cells were locked in the cell cycle and depleted of phosphate (Huysman et al., 2010). Also, *cycB1* had a 4-fold increase in abundance during lipid accumulation. *CycB1* is a late-phase cell cycle (G2/M) gene that must be degraded in order to progress through the cell cycle; its up-expression indicated it was preventing cell division. This suggests there is signal transduction between cell cycle inhibition and lipid accumulation. Based on re-supplementation experiments from chapter 2 addition of the depleted nutrient (N or P) to cell cycle arrested cells can reverse lipid accumulation. Only when N is re-supplied did cell numbers increase, indicating a greater importance of N to cell cycle progression than phosphate.

The interconnectivity of nitrogen availability controlling cell cycle and lipid accumulation was investigated by global transcriptomic analysis. Nitrogen assimilatory genes are constitutively up-expressed throughout nitrogen depletion as seen in chapter 3. The urea cycle is also maintained during the light and dark periods of extended stationary phase as cells are continually express C and N assimilatory genes for the episodic availability of nitrogen (Chapter 4). Thus, upon the re-supplementation with nitrate, cells primed for nitrogen assimilation and are shifted rapidly towards a growth (Figure 5.2) as cell cycle progression increases and lipid accumulation decreases. The urea cycle is

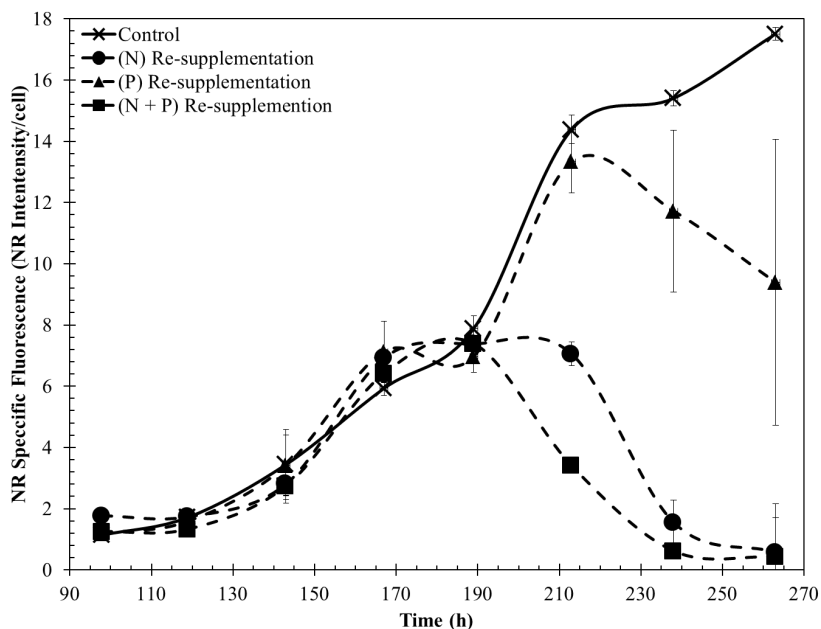


Figure 5.2 Effects of nitrate and phosphate depletion on lipid accumulation (NR). N + P re-supplementation conditions (■), N Re-supplementation or P depleted (●), P re-supplementation or N deplete (▲), N + P depleted (×).

playing a similar role as it is maintaining carbon and nitrogen intermediates during nitrogen depletion. From chapter 4, (Figure 4.6) there is a highly up-expressed urea cycle regulating N and C homeostasis so that when nitrogen is encountered it can readily have intermediates available to shift metabolisms from TAG biosynthesis to cellular growth

The balance between C and N homeostasis is highly linked. During N replete conditions cellular growth required more DIC than lipid accumulation during N depletion. The increased demand for DIC during exponential growth caused cells to utilize carbon concentration mechanisms. During the light period cells employed a biochemical C4 CCM utilizing carboxylases like PEPcase (Phosphoenolpyruvate carboxylase) or PYC1 (Pyruvate carboxylase) to fix inorganic carbon to a C3 precursor

producing a C4 intermediate. Decarboxylases like PEPCK or ME then concentrated CO₂ in the plastid. The C3 carbon can then be shuttled out of the plastid for another round of C4 fixation. Carbonic anhydrases are localized in different organelles. The α -CAs are localized in the periplastidal compartment of the plastid and function to limit CO₂ efflux out of the plastid (Tachibana et al., 2011). The expression patterns during light and dark indicate that during L2 more of the CAs were up-regulated, thus helped drive flux of CO₂ to RubisCO during DIC limitation. As DIC rebounds at L3 and D3 there is a general decrease in expression in CAs, indicating less of a requirement to concentrate CO₂ around RubisCO. However, at D3 the observed up-expression of the C4 CCM genes could be playing a role in reducing photorespiration by concentrating CO₂ around RuBisCO or providing anaplerotic reactions for replenishing TCA intermediates.

Central carbon metabolic pathways are generally constitutively up-expressed throughout growth and between the light and dark cycles. Cells maintain a constant supply of precursors so their metabolisms remain primed for quick responses to the addition of nutrients so that growth can be re-established. The carbon intermediates can be utilized to make new amino acids once nitrogen becomes available. Products between the urea cycle and TCA cycle provide a pool of essential intermediates for the rapid response to nitrogen assimilation. Glycolysis and TCA cycle intermediates provide precursors for fatty acid synthesis as carbon flux is driven to ACC by metabolite pools and not pulled by over-expression of ACC (Valenzuela et al., 2012; Liang et al., 2012a). During cellular growth under nutrient replete conditions cells divide at night. (Chauton et al., 2013). The expression profile of ACC1 has an inverse relationship between the dark

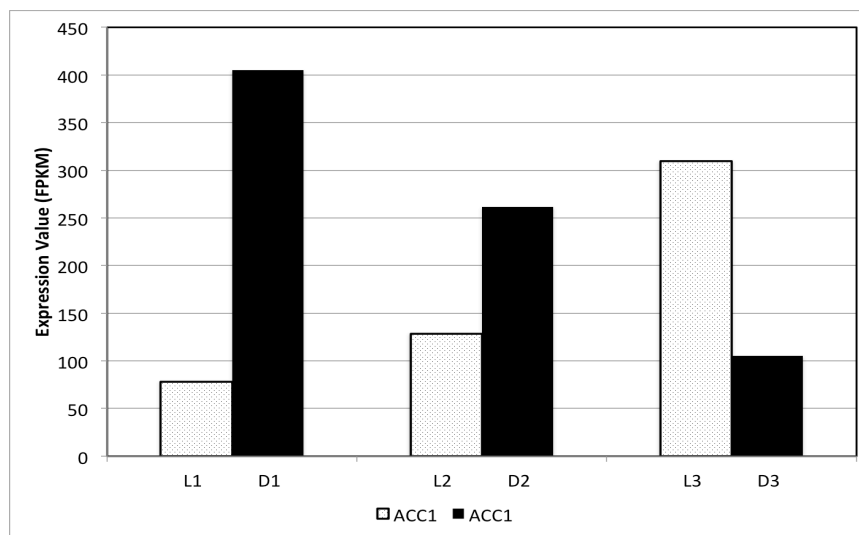


Figure 5.3 Acetyl CoA Carboxylase (ACC1) expression profile through out growth and diel cycle. Light bars indicate light period and black bars indicate dark period.

and light periods (Figure 5.3). The high expression of ACC1 at D1 compared to L1 may indicate a need for lipids during the night period for mitosis and based on the low availability of precursors an increased expression is required to “pull” acetyl-coA pools toward fatty acid biosynthesis. During the light period with photosynthesis and energy metabolism up-regulated the acetyl-CoA pools are being “pushed” towards fatty acid synthesis thus ACC has a lowered expression. At L2 and D2 there is an inflection point where cells are transitioning between cell growth and lipid accumulation. ACC expression increased during the light period and D2 showed decreased expression compared to D1, indicating a decreased requirement of dark period fatty acid biosynthesis. At L3, during extended stationary phase there is a significant increase in ACC expression compared to D3, indicating light period lipid accumulation. ACC functions as the rate-limiting step in fatty acid biosynthesis and its up-expression correlates with the increased lipid accumulation. However, increased expression doesn’t

necessarily mean increased lipid accumulation. As seen in chapter 3 the acetyl-CoA pools from carbon metabolism could play a more prominent role in driving fatty acid biosynthesis compared to ACC 1 expression.

In summary there is a distinctive metabolic shift from cellular growth to lipid accumulation that results in cell cycle arrest and initiation of lipid accumulation. Both cellular states have different DIC requirements with cellular proliferation requiring more DIC. Diatoms can utilize a biophysical or biochemical CCM to concentrate CO₂ for photosynthesis. Metabolic control points particularly involve carbon and nitrogen flux through urea cycle and central carbon pathways (*i.e.* glycolysis and TCA cycle). Carbon flux into lipids is driven more by upstream precursors than the pull of acetyl-coA carboxylase expression.

Future Directions

Specific Targeting of Predicted Metabolic Control Points.

Global transcriptomic profiling is in general a discovery-based technique. The possible control points that were postulated as playing a crucial role in lipid accumulation can be tested. For instance, RNAi has been used *in Chlamydomonas reinhardtii* to knock down *pepc2* expression resulting in increased oil content (Deng et al., 2011). The use of RNAi could be used to knock down CPS during lipid accumulation, where its expression is highest to observe what impact it would have on lipid yield. Conversely, to determine how over expression of CPS during early time-points of growth would have an impact on

lipid accumulation. Tools for over-expression in Pt1 have already been developed (Siaut et al., 2007). Phatr_55010, the coccolith scale-like protein homolog is one of the highly expressed genes during nitrate limitation. GUF_55010 is postulated to have a role in carbon and nitrogen homeostasis, decreased translation via RNAi experiments may confirm its importance to C and N partitioning.

Acetyl-CoA pools may have more influence on driving lipid accumulation from precursor metabolism into ACC when it comes to lipid yield. Testing of over expression and knock down of pyruvate dehydrogenase as it pertains to lipid accumulation could answer this question. Decreased pyruvate dehydrogenase abundance may result in reduced acetyl-coA pools and reduced TCA cycle intermediates, conversely the over expression may produce greater pools and TCA cycle intermediates that could drive fatty acid biosynthesis. Overproduction of PDH could signal excess energy and drive C into storage molecules.

Pushing the Limit of Fatty Acid Biosynthesis.

Microalgae accumulate lipids during nutrient stress but what is controlling the negative feedback that eventually inhibits the continued accumulation of lipids? Using a systems approach of transcriptomics, proteomics and metabolomics cells may be analyzed prior, during, and after maximal lipid accumulation. Data would provide insight into what is controlling maximal accumulation. Speculation could be turgor pressure. In this case, cytoskeletal rearrangements pathways would possible show increased expression. If the mechanism of feedback inhibition was deciphered, it could

be targeted for regulation. Theoretically, if you decrease the feedback inhibition of lipid accumulation you would increase TAG accumulation to a point of possible cell lysis or fatty acid leakage. Over production of TAG to a critical point of leakage or cellular disruption could reduce the harvesting and extraction costs associated with algal biofuel production, thus increasing efficiency of lipid accumulation and harvesting for biofuel production optimization.

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