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Studies on nitrogen and silicon deficiency in microalgal lipid production

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STUDIES ON NITROGEN AND SILICON DEFICIENCY IN MICROALGAL LIPID PRODUCTION

by

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ABSTRACT

Studies on Nitrogen and Silicon Deficiency in Microalgal Lipid Production

by

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Major Professor: Dr. Bruce Bugbee
Department: Plants, Soils, and Climate

Microalgae are a rich, largely untapped source of lipids. Algae are underutilized, in part, because lipid formation generally is stimulated by stress, such as nutrient deficiency. Nutrient deficiencies reduce growth, resulting in a tradeoff between elevated cellular lipids and abundant cell division. This tradeoff is not well understood. We also have a poor understanding of the physiological drivers for this lipid formation. Here we report on three sets of research: 1) Assessment of species differences in growth and lipid content tradeoffs with high and low level nitrogen deficiency; 2) Investigation of physiological drivers of lipid formation, by mass balance accounting of cellular nitrogen with progressing deficiency; 3) Examination of the effects of sodium chloride and silicon on lipid production in a marine diatom.

1) Nitrogen deficiency typically had disproportionate effects on growth and lipid content, with profound differences among species. Optimally balancing the tradeoff required a wide range in the rate of nitrogen supply to species. Some species grew first

and then accumulated lipids, while other species grew and accumulated lipids concurrently—a characteristic that increased lipid productivity. High lipid content generally resulted from a response to minimal stress.

2) Commonalities among species in cellular nitrogen at the initiation of lipid accumulation provided insight into the physiological drivers for lipid accumulation in nitrogen deficient algae. Total nitrogen uptake and retention differed widely among species, but the ratio of minimum retained nitrogen to nitrogen at the initiation of lipid accumulation was consistent among species at 0.5 ± 0.04. This suggests that lipid accumulation was signaled by a common magnitude of nitrogen deficiency. Among the cellular pools of nitrogen at the initiation of lipid accumulation, the concentration of RNA and the protein to RNA ratio were most similar among species with averages of 3.2 ± 0.26 g L^{-1} (8.2% variation) and 16 ± 1.5 (9.2% variation), respectively. This implicates critical levels of these parameters as potential signals initiating the accumulation of lipids.

3) In a marine diatom, low levels of either sodium chloride or silicon resulted in at least 50% increases in lipid content. The synergy of simultaneous, moderate sodium chloride and silicon stress resulted in lipid content up to 73%. There was a strong sodium chloride/silicon interaction in total and ash-free dry mass densities that arose because low sodium chloride was inhibitory to growth, but the inhibition was overcome with excessive silicon supply. This suggests that low sodium chloride may have affected metabolism of silicon.

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PUBLIC ABSTRACT

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Microalgae are single celled plants that inhabit aquatic and terrestrial environments across the planet. Many species are oleaginous, which means they are capable of producing oils, similar to many higher plants we are familiar with like canola, safflower and coconut. Different from higher plants, however, algae have simple structures that allow them to grow at very high rates. Due to these characteristics—oil production and rapid growth rates—algae are considered a promising future source of oil. Algal oils could be useful for production of food for people, feed for animals, biodiesel, detergents, and many other applications.

Algae have not been heavily used to this point as a source of lipids for a variety of reasons. One primary reason is that algal lipid formation generally is prompted by stress, such as nutrient deficiency. Nutrient deficiencies reduce growth, resulting in a difficult tradeoff between elevated cellular lipids and abundant cell division. This tradeoff is not well understood. We also have a poor understanding of what happens in the cell

physiologically in response to nutrient deficiency that drives this lipid formation. To make algae useful as lipid producers on commercial scales, research is needed.

This dissertation is a report on three sets of research: 1) An assessment of species differences in growth and lipid content tradeoffs with high and low level nitrogen deficiency; 2) Investigation of physiological drivers of lipid formation, by mass balance accounting of cellular nitrogen pools with progressing deficiency; 3) Examination of the effects of sodium chloride and silicon on lipid production in a marine diatom.

1) Nitrogen deficiency typically had disproportionate effects on growth and lipid content, with profound differences among species. Optimally balancing the tradeoff required a wide range in nitrogen supply among species. Some species grew first and then accumulated lipids, while other species grew and accumulated lipids concurrently—a characteristic that increased lipid productivity. High lipid content generally resulted from a response to minimal stress.

2) Commonalities among species in cellular nitrogen at the initiation of lipid accumulation provided insight into the physiological drivers for lipid accumulation in nitrogen deficient algae. Total nitrogen uptake and retention differed widely among species, but the ratio of minimum retained nitrogen to nitrogen at the initiation of lipid accumulation was consistent among species at 0.5 ± 0.04. This suggests that lipid accumulation was signaled by a common magnitude of nitrogen deficiency. Among the cellular pools of nitrogen at the initiation of lipid accumulation, the concentration of RNA and the protein to RNA ratio were most similar among species with averages of 3.2 ± 0.26 g L^{-1} (8.2% variation) and 16 ± 1.5 (9.2% variation), respectively. This implicates

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These studies give insight into how nutrient deficiency can be used effectively to enhance lipid production in oleaginous algae.

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ABBREVIATIONS

Non-standard abbreviations used in this dissertation are defined at their first instance of use, with the exception of nutrient elements. These abbreviations are used extensively and include: nitrogen (N), silicon (Si), carbon (C), phosphorus (P), potassium (K), sodium (Na), sulfur (S), magnesium (Mg), calcium (Ca), boron (B), iron (Fe), chloride (Cl), manganese (Mn), zinc (Zn), copper, (Cu), molybdenum (Mo). The nutrient elements are fully spelled out in all abstracts and titles.

CHAPTER 1

INTRODUCTION & LITERAURE REVIEW

1.1. Introduction

Microalgae are a rich, largely untapped source of lipids. Algae are capable of lipid productivity far greater than higher plants (Mata et al. 2010), but are not heavily used commercially (Roessler 1990, Borowitzka 1999, Spolaore et al. 2006). Several challenges have hampered the development and implementation of reliable, large-scale production of lipids by algae. Foremost among these challenges is that algal lipid accumulation is generally promoted by stress conditions—primarily nutrient deficiency—and we have insufficient understanding of stress optimization (Sheehan et al. 1998). The potential of algae is well appreciated, however, as evidenced by the recent surge in research into their use as a biodiesel feedstock (Chisti 2007, Hu et al. 2008, Griffiths and Harrison 2009, Brennan and Owende 2010, Mata et al. 2010, Verma et al. 2010, Wijffels and Barbosa 2010), which was a primary motivation of the research summarized in this dissertation. In a recent published opinion, Michel (2012) suggested that development of biofuels is "nonsense" and that more efficient solar/battery technologies are the more sensible route. But this opinion overlooks the indispensable contemporary need for transportable and energy-dense liquid fuels. In addition to fuel markets, tremendously diverse algal lipids could have greater place in the higher-value markets for food, detergent lipids, and specialty lipids (Roessler 1990, Guschina and Harwood 2006, Mata et al. 2010). The following literature review covers some history of algal cultivation, the physiology of nutrient stress-induced lipid accumulation, and aspects and challenges of cultivation.

1.2. Literature Review

1.2.1. History of Artificial Algal Culture

For academic and commercial purposes, algae have been cultured for more than a century. Bold (1942) suggested that A. Famintzin was likely the first to report the culture of algae in liquid media (Famintzin 1871). Early researchers such as Beyerinck (1890), who is credited with being the first to culture bacteria-free green algae, employed the common sense method of culturing algae in water directly from natural habitats. Growth was poor in natural waters in these early studies, however. In response to the apparent lack of essential growth factors in early culture media, the use of soil extracts was introduced by Pringsheim (1912), which greatly extended the number of algae that could be cultivated. From his work developing artificial seawater media, Allen (1914) concluded that soil extracts contained unknown organic substances that are required for algal growth. "We now know that soil extract performs numerous functions in culture media, and [those functions] have largely been replaced by specific compounds. Soil extract provides various elements and vitamins needed for plant growth, metal complexing by organic compounds that sequester potentially toxic metals, and organic compounds that keep iron in solution" (Harrison and Berges 2005).

Modern algal media are greatly improved, most are completely artificial and defined, and many allow for the culture of a large diversity of algal organisms. Some media appear to be designed for production, while others are designed to approximate conditions in nature. In addition to the macronutrient elements (e.g. N, P), many micronutrient elements (e.g. Zn, Mn, Cu), some vitamins (vitamin B_{12}, thiamine, and

biotin are most common), and chelating agents are often added to modern media. As an excellent example of the development of a modern marine culture media, Provasoli et al. (1957) reviewed historical developments in media and offered physiologically and research-based justifications for the composition of their popular ASP-2 media. The Bold Basal Medium (Bischoff and Bold 1963), which is a variation of the original Bristol's solution (Bold 1949), is an example of a widely used freshwater media capable of culturing a wide variety of freshwater algae.

Today, however, there are numerous published recipes for algal media. Many of the recipes are simple manipulations of other recipes that accommodate specific research needs (e.g. varied N concentration). Some recipes vary slightly from other published recipes to suit particular physiological needs of specific organisms (e.g. dilution of micronutrient metals). Somewhat recently, Andersen et al. (2005) compiled dozens of the more common marine and freshwater media recipes in a format that lends itself to comparison among media (i.e. molar component concentrations); they also comment briefly on the derivation of each media and mention some organisms that have been grown in each.

1.2.2. Algal Lipid Formation is Stimulated by Stress

All algae have membrane phospholipids and glycolipids that are a relatively constant fraction of their biomass. Additionally, algae produce neutral, storage lipids called triacylglycerides (TAG). Some algae will accumulate relatively small amounts of neutral lipid in non-stress conditions, but large increases have been observed in many algal species under stress conditions—primarily nutrient deficiencies (Shifrin and

Chisholm 1981, Griffiths and Harrison 2009). Nutrient deficiencies necessarily reduce growth and therefore, from a lipid production standpoint, there is a tradeoff between nutrient deficiency that results in high cellular lipid content and nutrient sufficiency that results in an abundance of cells. The literature is full of reports on the lipid content achievable with various species in high stress conditions; there is less information on the optimization of the growth and lipid content tradeoff. The scientific community is recognizing the need to select species and to study cultural conditions that lead, not just to high lipid content, but high lipid productivity (Zhukova and Aizdaicher 1995, Griffiths and Harrison 2009, Rodolfi et al. 2009, Huerlimann et al. 2010, Araujo et al. 2011, Mutanda et al. 2011).

Overwhelmingly more than other nutrient deficiencies, N deficiency has been shown to induce lipid accumulation in algae (Shifrin and Chisholm 1981, Tornabene et al. 1983, Utting 1985, Larson and Rees 1996, Kilham et al. 1997, Alonso et al. 2000, Illman et al. 2000, Zhila et al. 2005, Li et al. 2008, Converti et al. 2009, Gouveia et al. 2009, Hsieh and Wu 2009, Wang et al. 2009). After N, effects of P have been reported at least several times (Lombardi and Wangersky 1991, Reitan et al. 1994, Kilham et al. 1997). Specifically in diatoms, Si deficiency has been shown to induce lipid accumulation, with an effect more rapid and severe than N or P deficiencies in those organisms (Shifrin and Chisholm 1981, Taguchi et al. 1987, Mortensen et al. 1988, Lombardi and Wangersky 1991).

1.2.3. Physiology of Lipid Accumulation under Nitrogen Deficiency

Nitrogen is central to the proper function of algal cells. It is a key component of amino acids, proteins, enzymes, nucleic acids, and photosynthetic pigments and as such is taken up among the highest levels of all nutrient elements. McGlathery et al. (1996) and Dortch et al. (1984) investigated algal responses to N deficiency and found that individual N pools (e.g. protein, chlorophyll, inorganic N) are depleted at different rates. This observation indicates that some N pools serve more of a storage function, while others are more critical. Dortch et al. (1984) showed that endogenous inorganic N was accumulated in periods of excessive N supply, then rapidly and drawn down below detectable levels with limiting N supply. The concentration of free amino acid was shown to be considerable in N-sufficient algae, but was often still present in reduced concentrations in N deficient conditions (Dortch et al. 1984). The concentration of RNA typically decreased dramatically with N deficiency, while the concentration of DNA remained relatively steady (Dortch et al. 1984). Considerable species differences were identified in protein losses with N deficiency, with some species maintaining most of their N-sufficient protein levels and others experiencing dramatic decreases (Dortch et al. 1984, McGlathery et al. 1996). Photosynthetic pigments (e.g. chlorophylls) and enzymes (e.g. Rubisco) generally decreased in concentration per cell, which ultimately leads to reductions in photosynthetic capacity (Dortch et al. 1984, Turpin 1991).

An N-sufficient algal cell typically has a molar C/N ratio of ~10/1 and approximately 20% of photosynthetic electron flow is devoted just to support N assimilation (Turpin et al. 1991). Given the importance of N, algal cells have adapted

strategies to help them to moderate the effects of temporal variation in supply. Two categories of strategies have been identified: those that regulate N supply and those that regulate energy supply for N assimilation.

Luxury consumption of N and autophagy are examples of strategies that regulate N supply. Luxury consumption is when an organism takes up something essential in excess of immediate need. This process allows algae to store up N in times of plenty for use in times of scarcity; it has been well documented and described in algae (Droop 1975). Nitrogen consumed in luxury may be stored in inorganic forms ($NO3^-$ and $NH4^+$) or may also be rapidly assimilated to form simple N-containing compounds that act as a reservoir for the nutrient. Autophagy is the process by which organisms degrade cellular components, such as organelles and proteins, to recycle the resources for more critical purposes. The use of genetic markers has shown that autophagy is active at a low levels in plants in non-stress conditions for routine nutrient recycling and that up-regulation of the process occurs in stress conditions, such as N limitation (Thompson et al. 2005). Thompson et al. (2005) described plants in which autophagy was disabled as "hypersensitive" to N or C starvation. The process is non-selective (general degradation), but it allows essential cell processes to proceed despite exhaustion of exogenous resource supply.

The production of organic storage compounds is an example of a strategy that regulates the supply of energy for N assimilation. In physiological N sufficiency, the molar rate of photosynthetic C fixation is seven to 10 times the rate of N assimilation, which is a suitable ratio of the elements for synthesis of essential N-containing cellular

components. In the initial phases of N deficiency—before photosynthetic capacity is significantly diminished—C fixation may exceed demands for assimilation of N-containing compounds and excess C may be diverted into storage compounds, such as lipids and carbohydrates. When N is resupplied, the energy and C from storage compounds is used, in part, for N assimilation, until photosynthetic capacity is restored (Turpin 1991).

A growing body of evidence is showing a connection between the cellular lipid accumulation observed in N deficiency and autophagy. In the green alga *Chlamydomonas reinhardtii,* Perez-Perez et al. (2010) identified that autophagy was active in this alga in stress conditions, including N deficiency, oxidative stress, or the presence of misfolded proteins in the endoplasmic reticulum. Wang et al. (2009) extended this finding, identifying a pathway linking autophagy activity and the production of storage lipids and carbohydrates in this alga. The proteins and signaling pathways known to mediate the autophagy process have been described in yeast, mammals, plants, algae, and others (Wullschleger et al. 2006, Diaz-Troya et al. 2008, Perez-Perez et al. 2010). In mammals, autophagy-related proteins have been found to sense the energy and nutrient status of cells, signaling the *metabolism* of storage lipids at the onset of stress (Wullschleger et al. 2006, Singh et al. 2009).

Flynn (1990) speculated that the biochemical responses to N deficiency, such as autophagy and lipid accumulation, would likely be triggered by reaching critical levels or proportions of key metabolites of C and N. As discussed earlier, changes in the cellular concentrations of these metabolites have been measured in N deficiency studies, but none

have sought to identify critical levels or interactions of these related to storage lipid accumulation.

1.2.4. Lipid Profiles

The types of lipid produced by different algae appears to be an intrinsic characteristic which may be influenced somewhat by environmental and cultural conditions. For example, many diatoms have been shown to produce a high lipid fraction of C16 fatty acids relative to green algae, which tend to produce a higher fraction of C18 fatty acids (Volkman et al. 1989). Factors such as temperature, pH, nutrient limitations, and growth phase have been found to exert an influence on these unique lipid profiles (Benamotz et al. 1985, Volkman et al. 1989, Dunstan et al. 1993, Zhu et al. 1997, Zhila et al. 2011). Thus, species selection and cultural management can be used to encourage desired lipid products.

1.2.5. Lipid Quantification

Most literature on algal lipids use a gravimetric method of lipid determination (Folch et al. 1957) to report mass lipid content of their algae. In this method, a mixture of polar and non-polar solvents is used to extract lipids from biomass, which are dried and weighed. The dry mass of the lipid extract over the total biomass gives the mass lipid content. The lipid extract contains all lipid-soluble cellular contents (e.g. chlorophyll), however, which typically leads to an overestimation of lipids. A more accurate and direct method of lipid quantification is by gas chromatograph (Wahlen et al. 2011). Gas chromatography can also be used to determine lipid profiles.

1.2.6. Lipid Conversion to Biodiesel

Algal storage lipids (triacylglycerol or TAG) can be extracted from algal cells with organic solvents and a type of biodiesel can be produced by a transesterification reaction in which the lipids are converted to fatty acid alkyl esters. Lipids other than TAG (e.g. free fatty acids, phospholipids, and glycolipids) have also been shown to be convertible into this form of biodiesel, depending on the method of transesterification. Generally the transesterification reaction takes place in the presence of a catalyst and an alcohol. When methanol is the alcohol used, the fatty acid alkyl ester products are called fatty acid methyl esters or FAME.

Four general classes of transesterification methods have been reported in literature: base-catalyzed, lipase-catalyzed, acid-catalyzed, and supercritical methanol biodiesel production. Only a superficial discussion of these method classes will be presented here, referring to the more extensive literature review of Wahlen (2011). Base-catalyzed transesterification methods are the most commonly used methods and allow for fast and complete conversion of TAG to FAME. However, samples containing even low concentrations of free fatty acids foul FAME production with base catalysis. This characteristic precludes the use of lower-quality lipid sources using these methods. The lipase-catalyzed method, while being a robust and efficient method of transesterification that will convert free fatty acids to FAME, is too costly for commercial biodiesel production due to high lipase production costs. The supercritical methanol method does not require a catalyst and involves heating methanol above its critical point. The method is not ideal for commercial scales because of costly requirements for infrastructure and

equipment capable of generating and sustaining high temperature and pressure. Finally, acid-catalyzed methods have the benefit of being able to convert free fatty acids to FAME, but have traditionally had the drawbacks of long conversion time and high alcohol requirements. Canakci and Van Gerpen (2001) developed a two-step method combining acid- and base-catalyzed techniques to allow faster conversion of samples containing free fatty acids. This method has the drawbacks of high alcohol and catalyst requirements and complication inherent in being multi-step.

A novel, acid-catalyzed method in which lipid extraction and transesterification occur in a single step was recently developed (Wahlen et al. 2011), building on the work of Lepage and Roy (1986). This method efficiently combines lipid extraction and transesterification, reducing the need for organic solvent; the reaction occurs significantly faster than traditional acid-catalyzed methods. As in other acid-catalyzed methods, there is a high alcohol requirement, but the author states that the alcohol may be recycled for use in multiple transesterification reactions. Free fatty acids are transesterified in the reaction, making biodiesel with low quality lipid sources possible. Reaction conditions were optimized to obtain 100% conversion of TAG to FAME. Using samples of cyanobacteria known to produce no TAG, the author demonstrated that this method is also capable of transesterifing phospholipids and glycolipids to FAME.

1.2.7. Algal Culture Management

An algal culture consists of two general components: the medium and the biomass. These components may be managed in a variety of ways to obtain desired culture growth.

Commercially, most algae are grown in batch cultures (Borowitzka 1999).[1] In a batch culture, all nutrients and other growth factors are added at once in fixed quantities to make a medium, the medium is inoculated with algae, and growth proceeds to an application-specific point at which the entire culture is harvested. The batch culture process is the simplest form of culture management. Simplicity in culture management, however, is traded off for some drawbacks (Borowitzka 1997). Since all nutrients must be added up front, initial nutrient concentrations may be at or near harmful levels in order to meet demands for all expected growth. "Fed-batch" culture was designed to overcome this problem. A fed-batch is a culture in which nutrients are added in stages, more closely matching nutrient supply with demand, but harvest occurs only at the end of the growth phase like a regular batch culture.

As a batch culture grows it first experiences a lag phase, a period in which culture density is little and growth is slow. Light capture efficiency (light absorbed by algae / incident light) is low due to the diluteness of the culture. After proceeding through an exponential growth phase, batch cultures become limited by some factor (light, if the medium is replete with nutrients) and enter a stationary phase in which growth slows to an eventual halt. In this phase, the dense culture may begin to self-shade and just enough light may be received to provide energy for maintenance respiration demands and growth cannot occur. Available light is not efficiently used also in this phase. Batch culture is

[1] Although published 14 years ago, this article provides the most recent comprehensive discussion of commercial algal production, including details on production practices, that we were able to find. Short-term batch cultures are still the most effective method for growing a quality product, as they are simply managed and provide the grower with lower likelihood of culture contamination, culture death, etc.

largely popular due to its ease of use, rather than to it being an optimized procedure. Nevertheless, batch culture is useful from a lipid-production standpoint in that dense cultures may be grown up and starved for a lipid-inducing nutrient during the stationary phase.

Other common methods of cultural management are forms of continuous culture. Continuous culture techniques approach some level of ongoing biomass density and media component regulation, which allows maintenance of growth in the exponential phase. True continuous culture is the maintenance of absolutely steady cultural conditions, but requires a complex system and is generally not practical on large scales. Semi-continuous culture techniques regulate biomass density and media component concentrations at some frequent interval (e.g. daily biomass harvest and nutrient replacement in response to growth). Semi-continuous culture has the benefits of being relatively simple (no complex system required) and, compared to batch culture, more efficient use of light and a more steady-state, low-level nutrient supply. But only nutrient limitation, not starvation, is possible in continuous cultures, which may preclude the possibility of obtaining the high lipid content for most species of algae.

1.2.8. Production Systems

In algal production systems, the terms open and closed are often applied to describe a system's exposure to the surrounding environment. Open systems are any of several variations of pond that are exposed to the natural environment and typically rely on natural sunlight for growth (Fig. 1–1). Algal competitors (such as wild algal strains, cyanobacteria and bacteria) and predators (such as rotifers) are ubiquitous in the

environment and always pose a risk of contamination of open culture systems. Closed systems provide an enclosed (or largely enclosed), artificial environment in which environmental conditions are more easily optimized and the risks of culture contamination by foreign species are reduced. Closed systems are often referred to as bioreactors. It is important to note, however, that large-scale closed systems are not sterile systems in which axenic algal culture is absolutely ensured (such as small-scale systems in which fundamental research is conducted), although system design and treatment can help to reduce risks of contamination. Closed system designs include those that rely on natural sunlight and others in which light is supplied by electric lights. Fig. 1–2 is an image of plate bioreactors, designed to improve light penetration and gas exchange. Mata et al. (2010) provided a comprehensive discussion of pros and cons on the use of open and closed systems.

Borowitzka (1999) provided a review of systems for commercial algae production, both large and small, and discussed considerations in the use of each system. At the time of his review (1999), Borowitzka stated that all large commercial culture systems were open systems, due simply to economics. Closed systems can increase productivity, but intensive energy and infrastructure demands make these options cost ineffective for most lower-value applications.

With the recent biofuel-based interest in algae production, there has been a surge in research on cultivation systems (Brennan and Owende 2010), including open and closed system designs. If algae are to be a viable biofuel feedstock that provide an appreciable portion of human energy demands, algae cultivation must happen on

unprecedentedly large scales and must be cost competitive on world fuel markets (Chisti 2007). This is also true if algae are to be grown for lipid supply to the food, detergent, and specialty lipid industries. This reality seems to favor lower input open systems, but adequate production quantity or quality may not be possible in this type of system. Obtaining lipid-productive algae capable of thriving in extreme conditions that exclude competitors and predators, such as high or low pH, may provide an answer. Innovation in bioreactor technologies may also provide an answer.

1.2.9. Species Selection for Cultural Stability

There are thousands of characterized algal species, each with characteristics that allow them to exploit various freshwater, marine, and soil environments around the world. The environments where algae have been found include extreme habitats, such as terrestrial Antarctica, (Davey 1989), on dry desert soils (Chantanachat and Bold 1962), the most saline waters on Earth (Brock 1975), in high pH solutions (Vonshak and Richmond 1988), and in the absence of nutrients (such as Ca in *Chlorella pyrenoidosa*) essential to most biological organisms (Hopkins and Wann 1926). From an algae production standpoint, the ability to survive in extreme environments is an advantage—adaptation to an extreme cultural environment allows a species of algae to grow with decreased predation and less microbiological competition for space or resources (e.g. nutrients, light). Such a characteristic may be critical in the success of large-scale algae production systems, both open and closed. The species must also be lipid-productive in their extreme environment.

1.2.10. Conclusions

Algal oils could be useful for production of food for people, feed for animals, biofuels, detergents, and many other applications. This literature review discussed many of the elements and challenges involved in large-scale production of algal lipids, including the role of nutrient deficiency. Nutrient deficiency is a primary stress that stimulates the accumulation of storage lipids in algae, but nutrient deficiencies also reduce growth resulting in a tradeoff between elevated cellular lipids and abundant cell division. This tradeoff is not well understood. We also have a poor understanding of what happens in the cell physiologically in response to nutrient deficiency that drives this lipid formation. To make algae useful as lipid producers on commercial scales, research is needed to address these gaps in our understanding.

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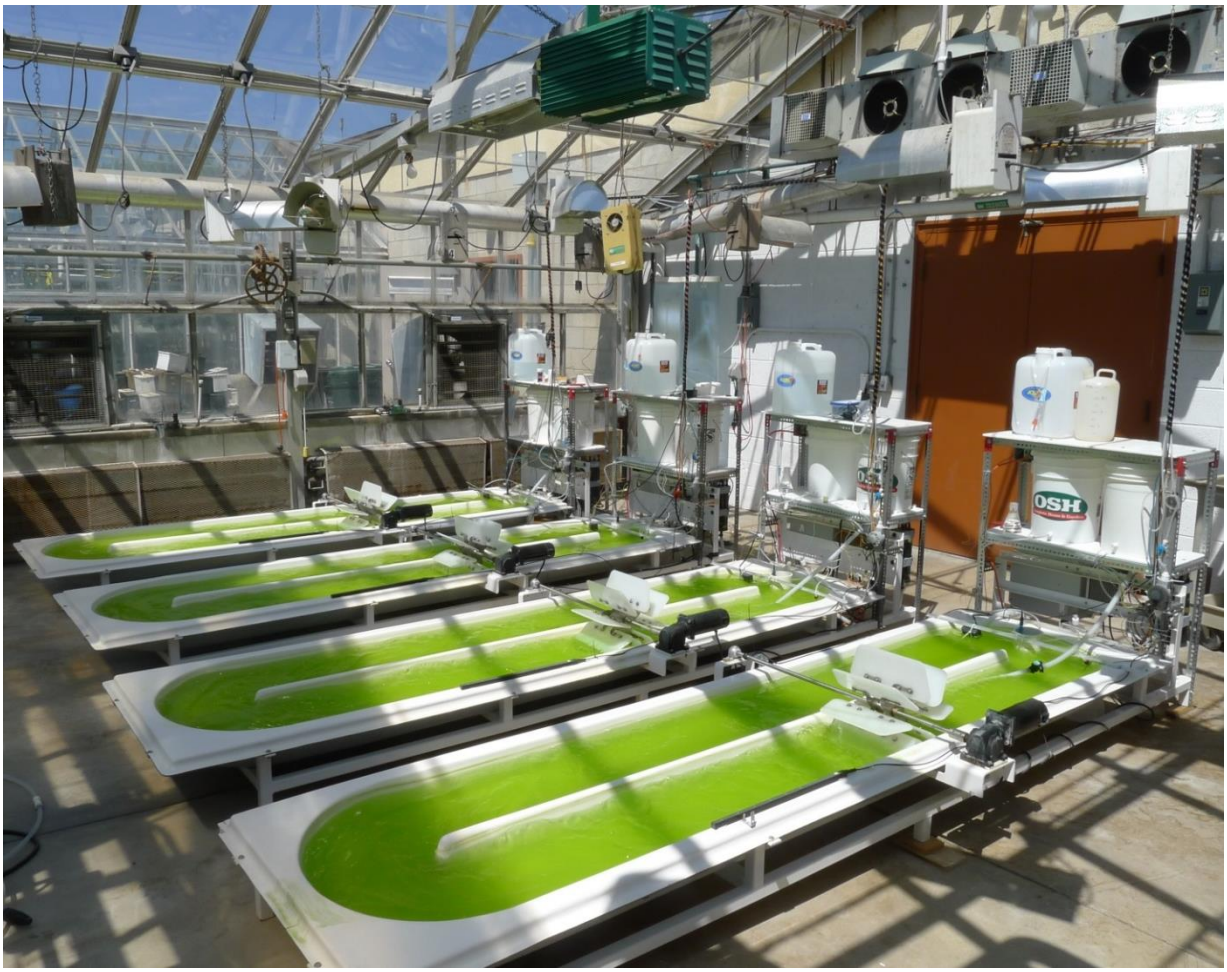

Fig. 1–1: An example of an open raceway algae growth system, located at the Utah State University Research Greenhouse Complex.

Fig. 1–2: A plate bioreactor system at the Utah State University Research Greenhouse Complex, designed to improve light penetration and gas exchange.

CHAPTER 2

UNDERSTANDING PRECISION NITROGEN STRESS TO OPTIMIZE THE GROWTH AND LIPID CONTENT TRADEOFF IN OLEAGINOUS GREEN MICROALGAE[2]

2.1. Abstract

Nitrogen deficiency promotes lipid formation in many microalgae, but also limits growth and lipid productivity. In spite of numerous studies, there is poor understanding of the interactions of growth and lipid content, the time course of lipid accumulation and the magnitude of nitrogen deficiency required to stimulate lipid formation. These relationships were investigated in six species of oleaginous green algae, comparing high and low levels of deficiency. Nitrogen stress typically had disproportionate effects on growth and lipid content, with profound differences among species. Optimally balancing the tradeoffs required a wide range in nitrogen supply rate among species. Some species grew first and then accumulated lipids, while other species grew and accumulated lipids concurrently which resulted in increased lipid productivity. Accumulation of high lipid content generally resulted from a response to minimal stress. The data highlight the tremendous biodiversity that may be exploited to optimally produce lipids with precision nitrogen stress.

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2.2. Introduction

Microalgae have been recognized as a potential source of lipids for multiple outlets. This is because some algae can accumulate energy-dense neutral lipid (triacylglycerides or TAG). TAG can be chemically extracted and simply converted to fatty acid methyl esters (FAME), or biodiesel. For this reason—in the worldwide quest to increase use of renewable biofuels—there is currently focus on algae as a biodiesel feedstock (Chisti 2007, Hu et al. 2008, Griffiths and Harrison 2009, Brennan and Owende 2010, Verma et al. 2010, Wijffels and Barbosa 2010). Environmental stress has been shown to elevate the production of lipid bodies containing TAG (Thompson 1996). Synthesizing dozens of reports, Hu et al. (2008) calculated an average algal lipid content of ~46% in stress conditions, up from an average of ~26% in non-stress conditions; several high lipid content values, from 70 to 90%, were reported. The primary stress applied to the algae was N deficiency.

In N sufficiency, the molar rate of photosynthetic C fixation is seven to 10 times the rate of N assimilation, which is a suitable ratio of the elements for synthesis of essential N-containing cellular components. In the initial phases of N deficiency—before photosynthetic capacity is significantly diminished—C fixation may exceed C demands for N assimilation and excess C may be diverted into storage compounds, such as lipids and carbohydrates. As storage compounds accumulate due to N deficiency, the growth rate is diminished. When N is resupplied, the energy and C from storage compounds is used, in part, for N assimilation, until photosynthetic capacity is restored (Turpin 1991).

If N deficiency is to be an effective tool for the production of algal lipids, we must have a thorough understanding of the resulting tradeoff between growth and lipid accumulation. The executive summary of the Aquatic Species Program (Sheehan et al. 1998)—a nearly two decade effort supported by the U.S. Department of Energy to develop biofuel from algae—stated that "The common thread among the studies showing increased oil production under stress seems to be the observed cessation of cell division…The increased oil content of the algae does not lead to increased overall productivity of oil. In fact, overall rates of oil production are lower during periods of nutrient deficiency." The validity of this claim is important for the future prospects of algal biodiesel, because culturing either many cells with low lipid content (nutrient replete conditions) or few cells with high lipid content (severe nutrient stress), will not result in an economically viable biodiesel feedstock. High biomass density (growth) is needed to increase yield per unit culture area and high lipid content is needed to reduce processing costs per unit of biomass product (Griffiths and Harrison 2009). Achieving the best economic scenario will require the proper balance of growth and lipid content.

Two trends in the biofuels-related algae literature, which are evident in the articles reviewed by Griffiths and Harrison (2009) and Hu et al. (2008), illustrate that the need to balance growth and lipid content has not been appreciated. The first is the common comparison of lipid productivity in N-deficient conditions to productivity in N-replete conditions. This comparison implicitly makes the assumption that the relatively stable production of structural phospholipids and glycolipids is an appropriate benchmark for the productivity of storage lipids. The second is that reports of high lipid content are

often observed in stressful cultural conditions that severely limit growth. Even large increases in lipid content will not pay off energetically or economically if growth is severely reduced.

Little is known quantitatively about the interactions of growth and lipid content, the time course of lipid accumulation and the magnitude of N deficiency required to stimulate lipid formation. The objective of this study was to investigate these relationships in six species of oleaginous green algae, comparing high and low levels of N deficiency. We sought to identify physiological characteristics among the algae that lead to the highest productivity of high-lipid content biomass. We also sought to compare lipid production dynamics with batch and semi-continuous cultural techniques.

2.3. Materials and Methods

2.3.1. Batch Culture Studies

Experimental design and setup are described here; apparatus, species and measurement details are described in the sections that follow. These studies were designed to track the progress of physiological changes to N deprivation in six species of green algae, with two N treatments—low N stress and high N stress (higher N supply and lower N supply, respectively). Daily measurements were facilitated by running 12 replicate cultures per N treatment in air-lift, glass bioreactors and harvesting one daily, a form of trend analysis. For each species, culture growth was started by filling the 12 reactors with media and 100 mL of axenic inoculation culture to a 1.2 L volume. Daily measurements of cellular N and lipid content were taken on harvested biomass. Dry mass density was measured daily during the dark period on every reactor. Solution N

concentration was measured daily during the dark period only on the reactors to be harvested on a given day.

2.3.2. Semi-Continuous Culture Study

Experimental design and setup are described here; apparatus and measurement details are described in the sections that follow. A 7-d semi-continuous culture study was done with *N. oleoabundans* to compare lipid productivity by this method with batch culture. This species was chosen because it required the least N stress to prompt lipid accumulation in batch culture, as evident in preliminary tests. There were four N treatments that were defined by the initial concentration of N in the media and the concentration of N in the refill media: 11, 5.5, 2.8, and 1.4 mM N. The N treatments were designed to supply N in a range from abundant to severely limiting for growth. There were two replicate air-lift, glass bioreactors per N treatment. Partial culture removal and media renewal were done once daily during the dark period in response to growth. For each reactor, culture was removed at a volume calculated to bring transmission at 750 nm to 1% (0.43 g L^{-1}) after renewal of media, thus minimizing effects of differential light absorption among treatments with different growth rates. Daily measurements of cellular N and lipid content were taken on harvested biomass. Solution N concentration was measured daily during the dark period on all reactors.

2.3.3. Algal Strains

Six species were studied: *Chlorella sorokiniana* (UTEX #1602); *Chlorella vulgaris* (UTEX #265); *Chlorococcum oleofaciens* (UTEX #105); *Neochloris*

oleoabundans (UTEX #1185); *Scenedesmus dimorphus* (UTEX #417); *Scenedesmus naegelii* (UTEX #74).

2.3.4. Culture Apparatus

Algal cultures were grown in glass, air-lift bioreactor tubes that were autoclaved before use. The bioreactors had an outer diameter of 50 mm, inner diameter of 45 mm and were filled to an approximate height of 75 cm, giving a culture volume of 1.2 L. The bioreactor tubes were placed in a plexiglass water tank maintained at 25˚C. CO_2-enriched (1%), filtered air (Whatman PolyVENT 0.2 µm PTFE filters, L#639) was bubbled into the bottom of each bioreactor through a 1 mm glass capillary tube at a rate of 0.5 L min^{-1}. For the semi-continuous study, the bioreactor tubes were altered with valves fixed to the reactor bottoms, for simple and clean removal of culture. Light was supplied by banks of fluorescent tubes that ran perpendicular to the bioreactor tubes, completely covering one side. The photosynthetic photon flux (PPF) was 300 µmol m^{-2} s^{-1} (300 µE m^{-2} s^{-1}) for the batch cultures and 350 µmol m^{-2} s^{-1} for the semi-continuous cultures, both with a 16-h photoperiod. (The Einstein, commonly used in algae literature, is defined as a mole of photons, but is not an SI unit and does not facilitate comparison with the photochemical, photobiology and plant biology literature.) A 16-h photoperiod was chosen to approximate the natural photoperiod of summers days in the mid-latitudes and to provide a regenerative dark period. Growth of photosynthetic organisms is best determined by the daily integrated PPF (Bugbee and Monje 1992), which was 17.3 mol m^{-2} d^{-1} in the batch cultures and 20.2 mol m^{-2} d^{-1} in the semi-continuous cultures. This is less than 50% of the average daily PPF of 45 to 55 mol m^{-2} d^{-1} in the summer months in North America. For

conversion of measurements made by volume (e.g. g L^{-1}) to a unit of area (e.g. g m^{-2}), the illuminated area of the bioreactor tubes was used as a conversion factor as follows: 1.2 L / πrh = 1.2 L / (3.14 x 2.25 cm x 75.5 cm) x (1 m^2 / 100^2 cm^2) = 22.5 L m^{-2} and 22.5 L m^{-2} x g L^{-1} = g m^{-2}.

2.3.5. Media Composition and Preparation

To make media, all ingredients other than P were added to deionized water in 20 L carboys. The carboys were autoclaved, maintaining a liquid temperature of 130˚C for 1 h. A P stock solution was autoclaved separately and added to the media after cooling. The freshwater culture media was a custom recipe with the following composition: 0.61 mM $CaCl_2 \cdot 2H_2O$; 0.63 mM $MgSO_4 \cdot 7H_2O$; 0.34 mM KH_2PO_4; 21.4 µM DTPA-Fe; 11.4 µM H_3BO_3; 7.0 µM $MnCl_2 \cdot 4H_2O$; 0.79 µM $CuSO_4 \cdot 5H_2O$; 3.1 µM $ZnSO_4 \cdot 7H_2O$; 0.16 µM $Na_2MoO_4 \cdot 2H_2O$; 0.34 µM $CoCl_2 \cdot 6H_2O$. For all N treatments, 1 mM N was added as $NaNO_3$ and the remaining N was added as KNO_3.[3] For the high N stress treatment (4 mM N): 1 mM $NaNO_3$ and 3 mM KNO_3. For the low N stress treatment (11 mM N): 1 mM $NaNO_3$ and 10 mM KNO_3 (except in the case of *C. sorokiniana*, where 15 mM KNO_3 was added for a final concentration of 16 mM N). The media had the following elemental concentrations: 4 mM N (high N stress) or 11 mM N (low N stress); 0.34 mM P; 1.34 mM K (high N stress; low as a counter ion to N) or 10.34 mM K (low N stress; high as a counter ion to N); 0.61 mM Ca; 0.63 mM Mg; 0.64 mM S; 1.2 mM Cl; 1.0 mM Na; 21.4 µM Fe; 11.4 µM B; 7.0 µM Mn; 3.1 µM Zn; 0.79 µM Cu; 0.16 µM Mo; 0.34 µM Co. In

[3] We were concerned about putting up to 10 mM Na in our algal media for the potential of toxicity. We opted for higher levels of K instead.

the semi-continuous culture of *N. oleoabundans* the same media was used, but in each treatment (11, 5.5, 2.8, and 1.4 mM N) N was split evenly by moles between $NaNO_3$ and KNO_3 because of the high tolerance of this organism of Na (Arredondo-Vega et al. 1995). Based on common levels of K in published media, K was assumed to be at adequate levels in both low and high N stress treatments (Andersen et al. 2005) and as such would not be expected to affect growth or lipid accumulation.

2.3.6. Growth/Algal Density Measurement

Measurements of culture density were made spectrophotometrically at 750 nm with a Shimadzu UV-2401 PC, UV-VIS recording spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Dilution of algal suspensions was made if absorbance measurements exceeded a value of 1.0. Spectral measurements were converted to dry mass densities by relationships developed individually for each species. Dry mass was determined by filtering 10 mL suspensions of algae with Whatman GF/C filters that were dried for one to two days at 105˚C. Virtually all increases in dry mass are necessarily due to photosynthetic production, whether in the form of lipids or cellular components. This work focused on photosynthetic production. Cell counts were not made, limiting the ability to make conclusions on rates of cell division.

2.3.7. Biomass Harvesting and Drying

Cells suspended in media were concentrated for harvest with a Sorvall RC6 Plus centrifuge (ThermoFisher Scientific, Waltham, MA). Spinning speed and duration varied somewhat among species, depending on pelleting characteristics. Generally, a speed of

7500 rpm for 5 mintes was sufficient for full recovery of the algae. Following centrifugation the biomass was loaded into 15 mL plastic sample vials and frozen at -80˚C. Frozen biomass was freeze-dried with a Labconco Freezone 4.5 freeze-drier (Labconco, Kansas City, MO).

2.3.8. Solution and Cellular Nitrogen Measurements

Samples of algal suspension (3 mL) were collected daily from the bioreactor tubes to be harvested and filtered with Whatman GF/C filters to monitor N consumption rates. Solution N concentration was measured on a Lachat QuikChem 8500 Automated Ion Analyzer using the total N, in-line persulfate digestion, imidazole buffer method that is available from the manufacturer (Lachat Instruments, Loveland, CO). Total C and N in algae were measured by a Perkin-Elmer Model 2400 CHN analyzer (Waltham, MA).

2.3.9. Lipid Extraction, Conversion to FAME and Quantification

Simultaneous conversion and extraction of algal lipids to fatty acid methyl esters (FAME) was done by the method of Wahlen et al. (2011). This method effectively converts to FAME the fatty acids contained in membrane phospholipids and glycolipids, as well as free fatty acids and storage lipid triglyceride. The lipid or FAME content of 100 mg freeze-dried algal samples was determined with a gas chromatograph (Model 2010, Shimadzu Scientific, Columbia, MD) equipped with a programmable temperature vaporizer (PTV), split/splitless injector, flame ionization detector (FID) (GCMS-QP2010S, Shimadzu Scientific, Columbia, MD), and autosampler. The FID detector was used in this analysis. Analytes were separated on an RTX-Biodiesel column (15 m, 0.32

mm ID, 0.10 μm film thickness, Restek, Bellefonte, PA) using a temperature program of 60˚C for 1 min followed by a temperature ramp of 10˚C per min to 360˚C for 6 min. Constant velocity of helium as a carrier gas was set at 50 cm s^{-1} in velocity mode. Sample sizes of 1 μL were injected into the PTV injector in direct mode that followed an identical temperature program to that of the column. The FID detector was set at 380˚C. Each sample contained octacosane (10 μg mL^{-1}) as an internal standard. FID detector response to FAME was calibrated using methyl tetradecanoate (C14:0), methyl palmitoleate (C16:1), and methyl oleate (C18:1) at concentrations ranging from 0.1 mg mL^{-1} to 1 mg mL^{-1} and tripalmitin at concentrations ranging from 0.05 mg mL^{-1} to 0.5 mg mL^{-1}. Standards were obtained as pure compounds (Nu-Chek Prep, Inc., Elysian MN) and were diluted with chloroform to obtain the needed concentrations. A standard (GLC-68A, Nu-Chek Prep, Inc.) containing methyl esters ranging from methyl tetradecanoate (C14:0) to methyl nervonate (C24:1) was used to identify the retention time window for FAME peak integration. Peaks within this region were integrated using GC solution postrun v. 2.3 (Shimadzu) and concentrations were determined by linear regression analysis.

2.4. Results & Discussion

2.4.1. Introduction

This is an analysis of progressive N deprivation in batch cultures of six species of oleaginous green microalgae at two N supply rates, keeping other nutrients constant between treatments. The treatment with a lower supply of N was designed to apply significant N stress while allowing reasonable growth and is hereafter referred to as “high N stress.” The treatment with a higher supply of N was designed to apply only minimal N

stress and is hereafter referred to as “low N stress.” We also investigated the effectiveness of semi-continuous culture for yielding high productivity of high-lipid content biomass. In studies of the effect of N on algal lipids it is common to quantify lipids by a gravimetric method, a portion of which is lipid soluble material. To ensure an accurate representation of the lipid production potentials of the algae and cultural methods studied here, the *in situ* transesterification method of Wahlen et al. (2011) was used to extract and convert lipids to FAME for quantification by gas chromatography. This method effectively converts to FAME the fatty acids contained in membrane phospholipids and glycolipids, as well as free fatty acids and storage lipid triglyceride. Because algae rapidly consume and store N (luxury consumption (Dortch et al. 1984)), measurements of cellular N were necessary to gauge the magnitude of N stress in the cells. Cellular N was thus used in analysis of lipid and growth responses.

2.4.2. Growth and Lipid Content Tradeoffs in Batch Culture

Nitrogen stress generally had disproportionate effects on growth and lipid content, with profound differences among species (Fig. 2–1). Conceptually, the organisms can be separated into three categories of response (comparing from low to high N stress): 1) the increase in lipid content exceeded the decrease in growth, resulting in higher lipid content and productivity with high N stress; 2) the decrease in growth exceeded the increase in lipid content, resulting in higher lipid productivity with low N stress and higher lipid content with high N stress; and 3) the increase in lipid content was offset by the decrease in growth, resulting in approximately equal lipid productivity with high or low N stress but higher lipid content with high stress.

N. oleoabundans and *S. dimorphus* fell into the first category. In particular, *N. oleoabundans* exhibited only a small decrease in growth (-21%) and drastically higher lipid content (100%) with high N stress relative to low. *C. vulgaris* and *C. oleofaciens* fell into the second category, with drastic decreases in growth and relatively modest increases in lipid content. This may seem like the case in which N stress was not effective in increasing lipid productivity, but there is another, more appropriate perspective (at least for *C. vulgaris* and *C. oleofaciens*). Rather than a high degree of N stress, these species required only a low level of stress to achieve high lipid productivity with relatively high lipid content. With low N stress—where growth was substantially higher—these species were able to accumulate 35 and 40% lipids (only 24 and 17% less than with high stress, respectively). In approximately one-for-one tradeoffs in growth and lipid content, *C. sorokiniana* and *S. naegelii* fell into the third category. If grown as lipid feedstock crops, higher N stress would always be the preferred cultural condition for third category species because of the higher lipid content and thus lower processing costs per unit lipid.

These categories are somewhat arbitrary, as species may be categorized differently depending on the exact N supply rates tested. However, the differences among species identified here may signify differences in N metabolism and/or strategies for handling N deficiency. First category species may have a panic response, making significant physiological sacrifices to generate adequate lipids to supply energy and C needs for times of hardship. *N. oleoabundans*, a first category species, was apparently first isolated in an arid, sand dune environment in Saudi Arabia (Chantanachat and Bold 1962) and would be expected to have adaptations for managing prolonged periods of

resource scarcity. In second category species, cellular function and thus growth may be particularly sensitive to severe N deficiency; when N deficiency is less severe, the photosynthetic mechanism may be functioning and capable of high rates of lipid production. In the event of N deficiency, third category species may directly divert energy and C intended for growth into lipids.

Table 2–1 displays percent changes in peak values of growth, lipid content, and lipid productivity between the stress treatments for all species. The lipid content values listed for "No N stress" were taken from our initial measurements of lipids in N-replete conditions. Because the measurements of lipid includes fatty acids contained in membrane phospholipids and glycolipid, free fatty acids and storage lipid triglyceride, the "No N stress" values may be reasonable estimates of relatively stable phospholipid and glycolipid background levels for each species.

These data allow a clearer picture of differences among oleaginous green algal species in their growth and lipid content responses to N stress. The data indicate that optimally balancing lipid content and growth tradeoffs required a wide range in N supply rate among species—lower levels of stress for some, higher for others.

2.4.3. Stress Quantification and the Timing of Lipid Accumulation

There were tremendous differences among the species in the magnitude of N stress required to stimulate the onset of lipid accumulation in the population. Fig. 2–2 shows how the lipid content of the cells varied as a function of total N in the algae (cellular N concentrations are used here as a proxy for levels of N stress). The level of N stress at which lipid accumulation was triggered was unique for the populations of each

species, ranging from minimal to severe stress. *N. oleoabundans* began accumulating lipids with the application of minimal stress (~7.5% cellular N), just following exhaustion of exogenous N; *C. vulgaris*, *C. sorokiniana*, *C. oleofaciens*, and *S. dimorphus* were intermediate (~4 to 5% cellular N); and *S. naegelii* required the most severe N deficiency (~3% cellular N). The coincidence of these lipid development trends as a function of cellular N in independent N treatments provides strong evidence for the roll of N in the response, rather than counter ions of N or other effects. In general, the species that accumulated the highest lipid content were the species that responded to the least stress.

A response to minimal N stress was less well correlated to high rates of lipid productivity, however, due to species differences in growth cessation with stress. There was a three-fold variation among species in the cellular N content at which growth stopped, from ~1% to 3% N. The algae that stopped growing with higher cellular N presumably employ a more conservative growth strategy, to preserve their physiological state at a higher level of function (e.g. maintenance of higher cellular protein concentrations). But early cessation of growth resulted in stalled rates of lipid productivity, and not all species continued to accumulate lipids in the stationary phase. With a finite supply of N as in batch cultures, where cellular N decreases with growth, the range in cellular N over which lipid accumulation occurred is proportional to the amount of growth and lipid accumulation that occurred concurrently. Among the six species in this study, those that exhibited the highest values of lipid productivity were the species in which there was more concurrent growth and lipid accumulation. This trend is evident by comparing lipid productivities in Table 2–1 with the ranges in cellular N

where lipids accumulated in Fig. 2–2. At the extremes, some algae grow first and then accumulate lipids, while other species grow and accumulate lipids at the same time.

Comparing *C. sorokiniana*—which grew and then accumulated lipids—and *N. oleoabundans*—which grew and accumulated lipid concurrently—illustrates the large impact these timing characteristics have on lipid productivity (Fig. 2–3). Despite having similar peak values of lipid content and growth (with high N stress), lipid productivity reached only up to 85 mg L^{-1} d^{-1} in *C. sorokiniana*, but up to 131 mg L^{-1} d^{-1} in *N. oleoabundans*. Time-series progress of cultural parameters—including solution N concentration, cellular N content, growth, lipid content and lipid productivity—for all species are shown in Fig. 2–4. This data shows wide variation in the extent to which each species combined or separated their growth and lipid accumulation phases. *C. oleofaciens*, *C. vulgaris*, *N. oleoabundans* and *S. dimorphus* were the species with the highest lipid productivity and the greatest amount of concurrent growth and lipid accumulation. The data clearly shows the importance of harvest timing, as an optimal harvest window may be brief, just prior to growth and lipid-accumulation cessation.

Selection of species as lipid feedstock crops should be broadened to include concurrent lipid accumulation and growth, a characteristic that resulted in higher lipid productivity in this study. The most promising biodiesel feedstock species will combine this characteristic with the ability to accumulate high lipid content. The data indicate that the species capable of accumulating higher lipid content generally form lipids in response to more minimal N stress.

2.4.4. Extrapolating to the field

Radiation is the ultimate limiting factor in all photosynthetic systems. Algal productivities are often expressed per unit of culture volume, but this does not allow extrapolation to the field without knowing the productivity per unit area. The results of these studies are expressed in both units of volume and area to facilitate extrapolation. However, as indicated previously, the daily integrated PPF was less than half of that in the field in the summer months. An even better way to express productivity is per unit of photosynthetic light. This is done by taking the ratio of productivity per unit area and time (g m^{-2} d^{-1}) and the daily PPF integral (mol m^{-2} d^{-1}), yielding the productivity in units of g mol^{-1} of photons. The best biomass productivity achieved in higher plants is approximately 1 g mol^{-1} in CO_2-enriched controlled environments (Bugbee and Monje 1992). The highest biomass productivities in the batch cultures in this study were about 10 g m^{-2} d^{-1} with a PPF of 17.3 mol m^{-2} d^{-1}, which is 0.58 g mol^{-1}. Higher algal biomass productivities have been reported (Williams and Laurens 2010). Algal systems are uniquely valuable for their lipid productivity, however. The highest lipid productivity in this study was 3.3 g m^{-2} d^{-1}, which is 0.19 g mol^{-1} of photons. The highest lipid productivity from higher plants is typically reported from oil palm (*Elaeis guineensis*) at about 5950 L ha^{-1} (Chisti 2007). This equates to about 535 g of oil m^{-2}, with a specific density of 0.9 g ml^{-1}. Assuming continuous production over a year, this is 1.5 g m^{-2} d^{-1}. Assuming an average daily integrated PPF near the equator of 40 mol m^{-2} d^{-1} gives 0.036 g mol^{-1} of photons. This makes oil production by oil palm about 20% of what we achieved in this study with algae, per unit light. Our values are similar to the peak values

reported by others (Amaro et al. 2011). The challenge is to achieve high lipid productivity at scale.

2.4.5. Semi-Continuous Culture

Because *N. oleoabundans* required the least N stress to prompt lipid accumulation in batch cultures, this species was chosen to compare its lipid productivity in batch and semi-continuous cultures. Semi-continuous culture yielded relatively low lipid content at all levels of N limitation tested and, in fact, lipid content and lipid productivity were inversely related (Fig. 2–5). To attain lipid productivity approximately equivalent to the peak productivity in batch culture, N replete conditions were required, a condition in which there was likely no storage lipid accumulation. Rather, lipid productivity directly mirrored biomass productivity and the production of structural lipids.

This comparative analysis indicated that batch culture was a far more effective cultural method for generating high productivity of high lipid content biomass than semi-continuous culture. The lack of storage lipid accumulation in semi-continuous culture may suggest that lipid production was inhibited by frequent supply of N. This may also suggest that the kinetics of lipid accumulation were not fast enough to overcome the effects of frequent, partial-culture removal or some other species-specific interaction. Cellular N content did not descend below ~3% in semi-continuous culture as compared to ~2% in batch culture, which indicates that semi-continuous culture did not apply N stress with the same severity as batches. Some species, however, as suggested by the research

of Hsieh and Wu (2009), may be capable of high productivity of high-lipid content biomass in semi-continuous culture.[4]

2.5. Conclusions

Nitrogen stress generally had disproportionate effects on growth and lipid content, with tremendous differences among species. A wide range among species in N supply rate was required for optimal production of high-lipid content biomass. Concurrent growth and lipid accumulation resulted in increased lipid productivity. The most promising biodiesel feedstock organisms will combine this characteristic with the ability to accumulate high lipid content, which typically occurred in species that accumulated lipids in response to minimal N stress. Batch culture is more effective than semi-continuous culture for optimizing growth and lipid content tradeoffs, even for species requiring the least N stress.

2.6. References

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[4] When it became clear in our studies that semi-continuous culture was not promoting high lipid content, we decided to stop pursuing this method. We took the data we had generated from *N. oleoabundans* in semi-continuous and batch culture to highlight a large contrast in the paper. The reference, Hsieh and Wu (2009), is included to make it clear that our results do not preclude the potential for achieving high lipid content and productivity by this method with other species.

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	Algal Density (g L^{-1})		Density Decrease (%)	Lipid Content (%)			Lipid Content Increase (%)		Lipid Productivity (mg L^{-1} d^{-1})	
Species	Low Stress	High Stress	Low Stress to High	No N Stress	Low Stress	High Stress	No Stress to Low	No Stress to High	Low Stress	High Stress
C. sorokiniana	4.1	1.8	-55	15	21	47	46	222	68	85
C. vulgaris	4.3	2.1	-51	10	40	48	302	377	146	94
C. oleofaciens	4.3	2.0	-55	12	35	46	195	286	127	86
N. oleoabundans	2.4	1.9	-20	13	29	58	132	358	91	131
S. dimorphus	5.3	4.0	-25	9	20	34	117	277	86	111
S. naegleii	4.8	2.0	-57	10	21	39	118	306	83	83

Table 2–1: Peak values and calculations on algal density, lipid content and lipid productivity in 12-d batch cultures. Nearly three-fold differences among species were observed in growth between the high and low N stress treatments. Lipid content increased over baseline levels up to 377% and as little as 222% with high N stress. Even with low N stress, lipid increased from 46 to 302%. Lipid productivities reflect the independent effects of N supply on growth and lipid content on each species. "No stress" lipid content values were obtained from the high and low stress treatment data, prior to the onset of lipid accumulation shown in Fig. 2–4. To convert "per L" to "per m^2," multiply by 22.5 L m^{-2}.

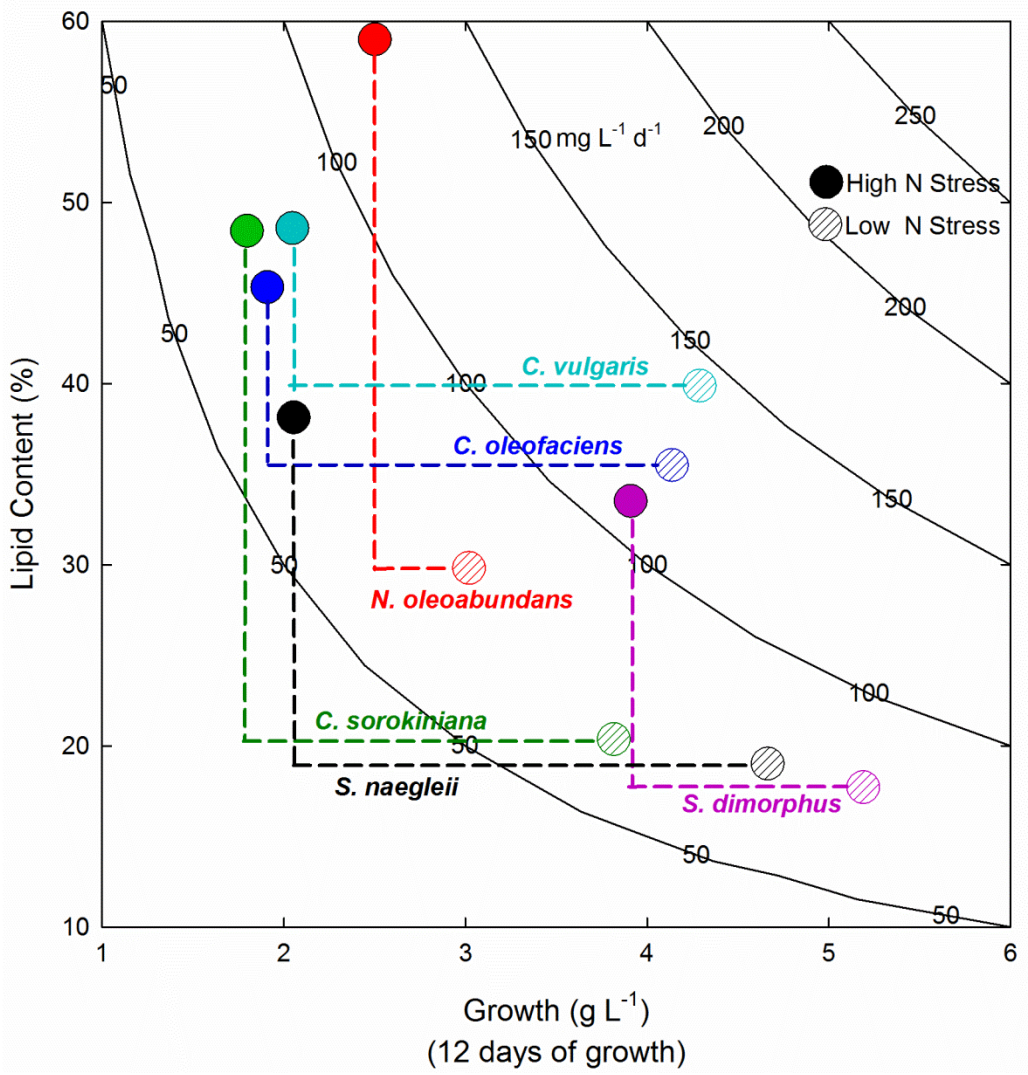

Fig. 2–1: Tradeoffs in growth and lipid content observed between high and low N stress treatments in six species of oleaginous green algae. For each species, the cross-hatched bubble on the right is peak lipid productivity with low N stress and the solid bubble on the left is peak lipid productivity with high N stress. Lipid productivity is calculated for 12 days and therefore the position of the bubbles is only approximate for species whose peak productivity occurred prior to 12 days. The data highlights tremendous biodiversity and the need for species-specific N optimization in production of lipid feedstock algae—higher N stress for some, lower for others. To convert "per L" to "per m^2," multiply by 22.5 L m^{-2}.

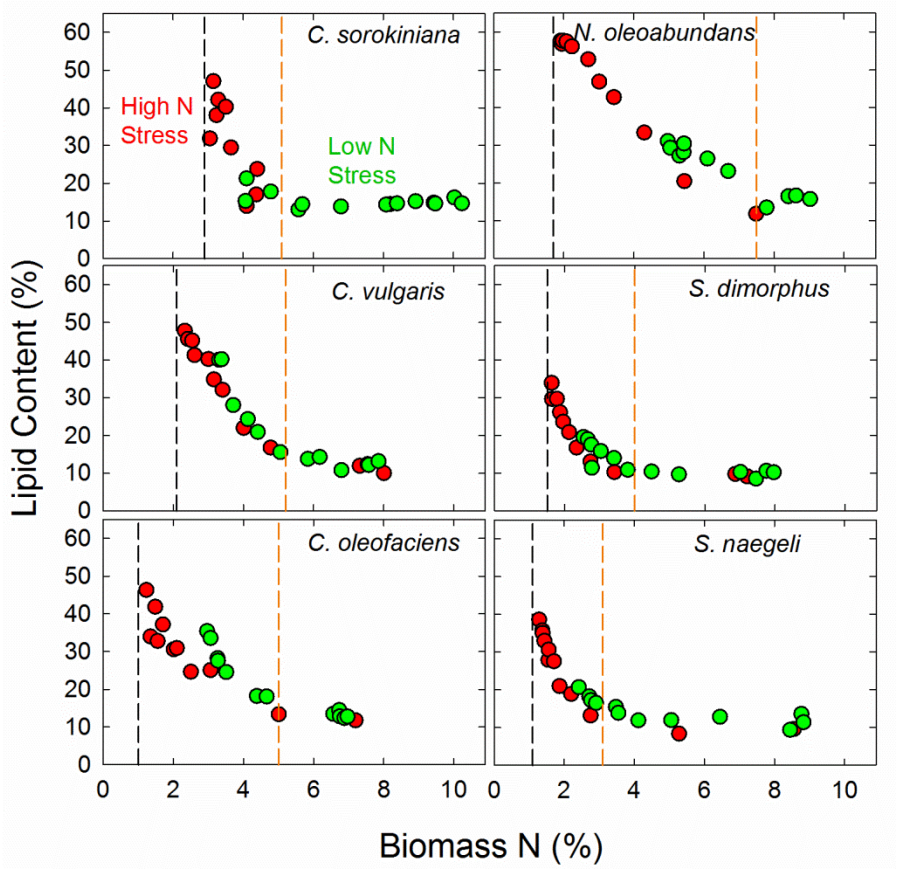

Fig. 2–2: The lipid content of the cells as a function of total N in algae. Cellular N is used here as a proxy for N stress. The vertical, dotted lines signify the levels of N stress at which lipid accumulation began and ceased in each species. The level of N stress that triggered the onset of lipid accumulation was unique for each species and ranged from minimal to severe stress. In general, the species that accumulated the highest lipid content were the species that responded to the least stress. The range in cellular N over which lipid accumulation occurred is proportional to the amount of growth and lipid accumulation that occurred concurrently. Species that grew and accumulated lipids concurrently had higher lipid productivity.

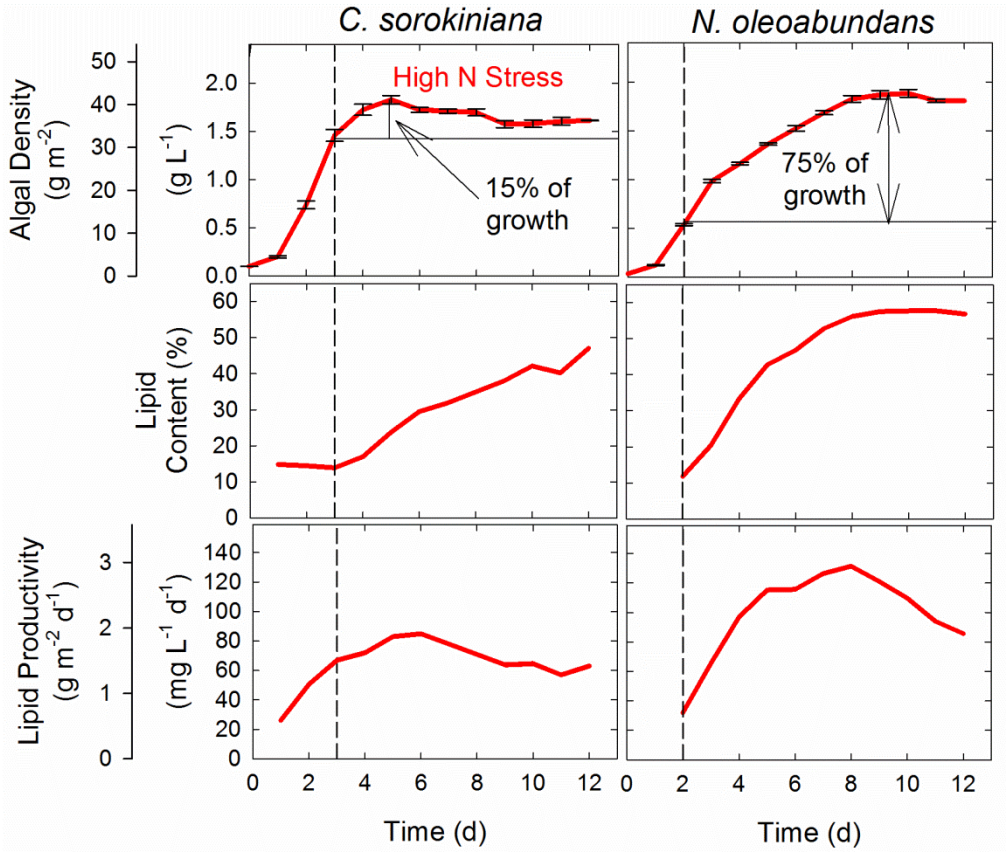

Fig. 2–3: The impact on lipid productivity of the timing of lipid accumulation relative to the timing of growth is evident in comparing *C. sorokiniana* and *N. oleoabundans*. The vertical, dotted line in each graph signifies the point at which lipid accumulation began. *C. sorokiniana* grew and then accumulated lipids—only 15% of growth occurred as lipids were accumulating. *N. oleoabundans* grew and accumulated lipids at the same time—75% of growth occurred as lipids accumulated. Concurrent growth and lipid accumulation resulted in higher lipid productivity.

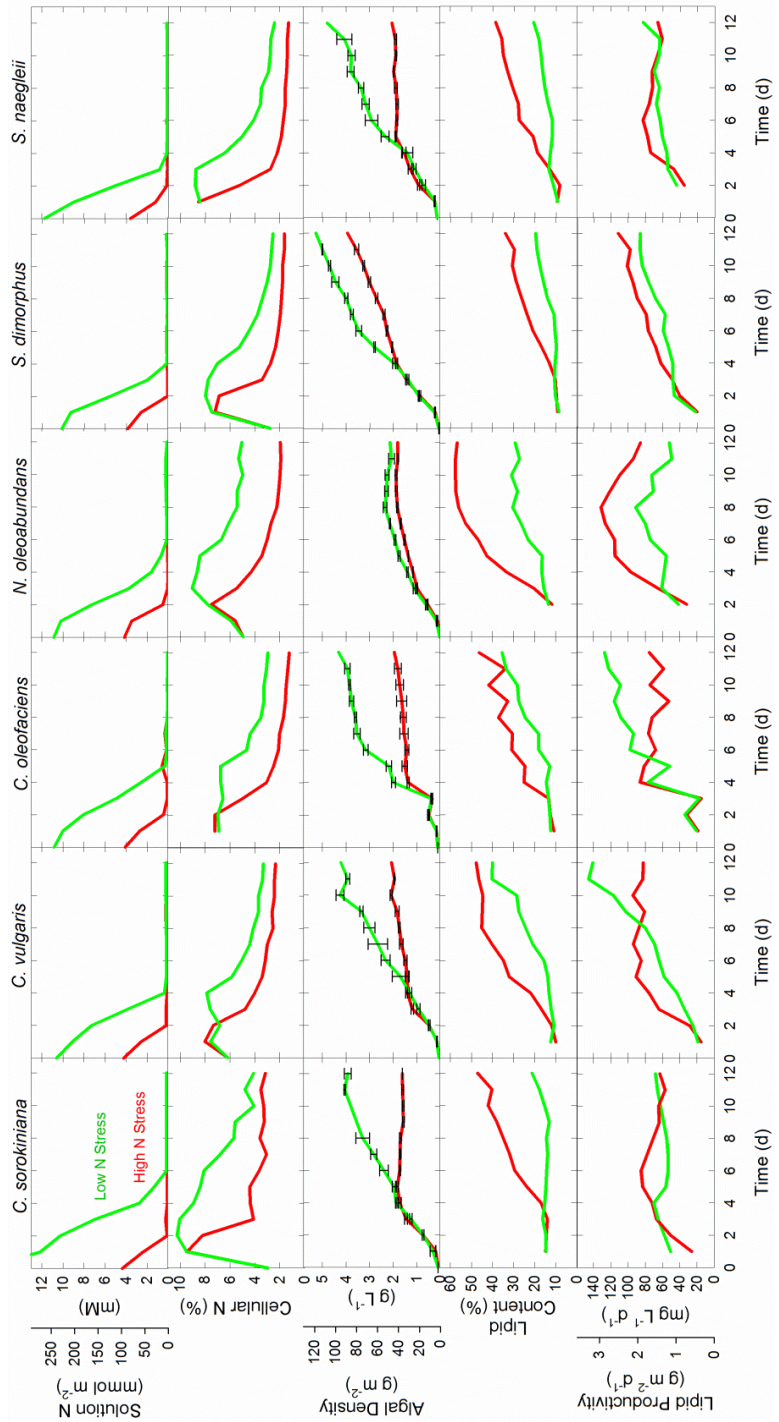

Fig. 2–4: Time-series data on growth, cellular N, lipid content, and lipid productivity for six species of oleaginous green algae. The data shows wide variation in the extent to which each species combined or separated their growth and lipid accumulation phases. The importance of harvest timing is clear, as an optimal harvest window may be brief, just prior to growth and lipid-accumulation cessation.

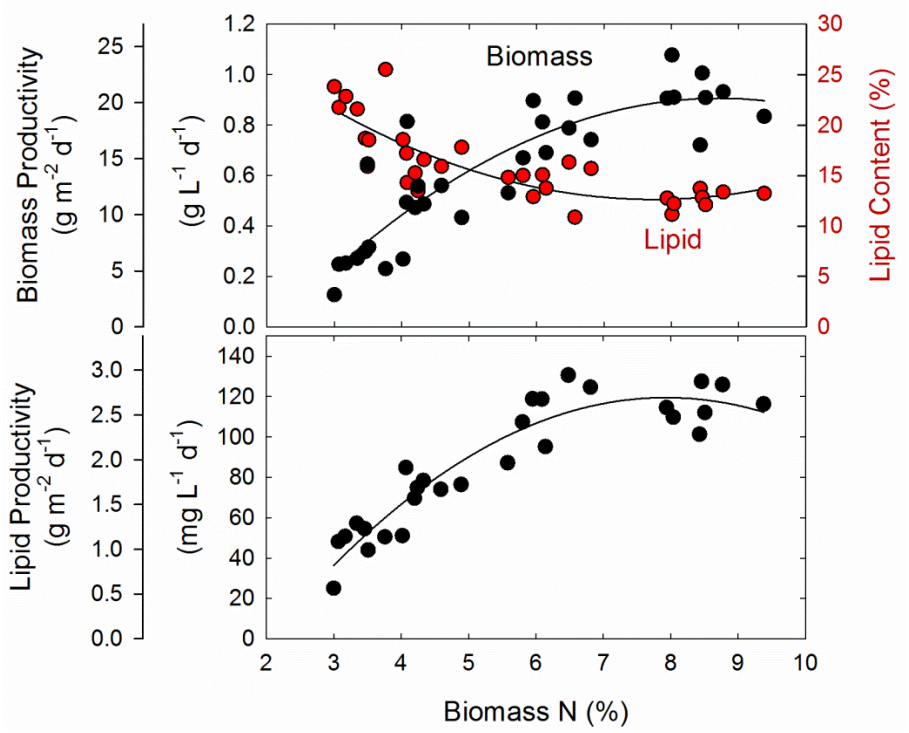

Fig. 2–5: Because it required the least N stress to prompt lipid accumulation in batch cultures, *N. oleoabundans* was chosen to evaluate the effectiveness of semi-continuous culture for production of high-lipid content biomass. A limiting supply of N resulted in drastically diminished growth and only small increases in the lipid content of the biomass. In fact, lipid content and lipid productivity were inversely related across the scope of N supply rates studied. The data suggests that this cultural method is unsuitable for production of high-lipid content biomass, at least for this species.

CHAPTER 3

POTENTIAL SIGNALS FOR INITIATION OF MICROALGAL LIPID ACCUMULATION ARE IMPLICATED BY COMMONALITIES AMONG SPECIES IN CELLULAR NITROGEN

3.1. Abstract

Changes in concentration of the primary nitrogen-containing cellular components in microalgae have been analyzed in nitrogen deficiency studies, but studies identifying critical levels or interactions of the components associated with initiation of lipid accumulation are lacking. Here we report on total cell nitrogen and changes in five cellular pools of nitrogen (protein, free amino acids, DNA, RNA, chlorophyll) and lipids during progressive nitrogen deficiency in three species. Total nitrogen uptake and retention differed widely among species, but the ratio of minimum retained nitrogen to nitrogen at the initiation of lipid accumulation was consistent among species at 0.5 ± 0.04. This suggests that lipid accumulation was signaled by a common magnitude of nitrogen deficiency. Among the cellular pools of nitrogen at the initiation of lipid accumulation, the concentration of RNA and the protein to RNA ratio were most similar among species with averages of 3.2 ± 0.26 g L^{-1} (8.2% variation) and 16 ± 1.5 (9.2% variation), respectively. This implicates critical levels of these parameters as potential signals initiating the accumulation of lipids. These results provide insight into the physiological drivers for lipid accumulation in nitrogen deficient algae.

3.2. Introduction

3.2.1. Background

Algae are increasingly being recognized as an important source of lipids. Owing to the wide diversity among species in native habitat and physiology, the types of lipids algae produce are many (Guschina and Harwood 2006). This diversity and their potential for high rates of lipid productivity (Mata et al. 2010) make them candidates for production of lipids for many applications. Accumulation of lipids generally occurs in stress conditions, including N deficiency, but the physiological drivers of the effect are not well understood.

3.2.2. Algal Strategies for Managing Nitrogen Deficiency

Nitrogen is central to the proper function of algal cells. It is a key component of amino acids, proteins, enzymes, nucleic acids, and photosynthetic pigments and as such is taken up among the highest levels of all nutrient elements. Depending on species and the form of N supplied, a N-sufficient algal cell typically has a molar C/N ratio of ~10/1 and approximately 20% of photosynthetic electron flow is devoted just to support N assimilation (Turpin et al. 1991). Given the importance of N, algal cells have adapted strategies to help them to moderate the effects of temporal variation in supply. Among previous studies it is possible to identify two types of strategies: those that regulate N supply and those that regulate energy supply for N assimilation.

Luxury consumption of N and a process called autophagy are examples of strategies that regulate N supply. Luxury consumption is when an organism takes up something essential in excess of immediate need. This process allows algae to store up N

in times of plenty for use in times of scarcity; it has been well documented and described in algae (Droop 1975). Nitrogen consumed in luxury may be stored in inorganic forms (NO_3^- and NH_4^+) or may also be rapidly assimilated to form simple N-containing compounds that act as a reservoir for the nutrient. Autophagy is the process by which organisms degrade cellular components, such as organelles and proteins, to recycle the resources for more critical purposes. The use of genetic markers has shown that autophagy is active at a low levels in higher plants in non-stress conditions for routine nutrient recycling and that up-regulation of the process occurs in stress conditions, such as in N deficiency (Thompson et al. 2005). Thompson et al. (2005) starved plants for N and C in which autophagy had been purposely disabled and described their response as "hypersensitive." Autophagy appears to be non-selective (general degradation), but it allows the most critical cell processes to proceed despite depletion of exogenous resource supply.

The production of organic storage compounds is an example of a strategy that regulates the supply of energy for N assimilation. In physiological N sufficiency, the molar rate of photosynthetic C fixation is seven to 10 times the rate of N assimilation, which is a suitable ratio of the elements for synthesis of essential N-containing cellular components. In the initial phases of N deficiency—before photosynthetic capacity is significantly diminished—C fixation may exceed demands for assimilation of N-containing compounds and excess C may be diverted into storage compounds, such as lipids and carbohydrates. When N is resupplied, the energy and C from storage compounds is used, in part, for N assimilation, until photosynthetic capacity is restored

(Turpin 1991). Many literature reports have demonstrated that N deficiency is a key stress that promotes accumulation of lipids in many algae (Shifrin and Chisholm 1981, Zhila et al. 2005, Li et al. 2008, Converti et al. 2009, Gouveia et al. 2009, Hsieh and Wu 2009, Wang et al. 2009, Adams et al. 2013).

3.2.3. Cellular Nitrogen Distribution in Physiological Deficiency

McGlathery et al. (1996) and Dortch et al. (1984) investigated algal responses to N deficiency and found that individual N pools (e.g. protein, chlorophyll, inorganic N) are depleted at different rates. This observation suggests that some pools of N may serve a storage function, while the maintenance of other pools is more critical. Dortch et al. (1984) showed that inorganic N (NO_3^- and NH_4^+) was taken up and stored during periods of excessive N supply, then rapidly drawn below detectable levels with limiting N supply. The concentration of free amino acids was often considerable in N-sufficient algae, and, surprisingly, a free amino acid pool remained in N deficient conditions in some algae (Dortch et al. 1984). RNA usually decreased dramatically with N deficiency, while DNA content remained relatively steady (Dortch et al. 1984). There were considerable species differences in protein losses with N deficiency, with some species maintaining most of their N-sufficient protein levels and others experiencing dramatic decreases (Dortch et al. 1984, McGlathery et al. 1996). Photosynthetic pigments (e.g. chlorophylls) and enzymes (e.g. RUBISCO) generally decreased, which would ultimately lead to reductions in photosynthetic capacity (Dortch et al. 1984, Turpin 1991).

3.2.4. How does Nitrogen Deficiency Signal Lipid Accumulation?

A growing body of evidence indicates a connection between the cellular lipid accumulation observed in N deficiency and autophagy. In the green alga *Chlamydomonas reinhardtii,* Perez-Perez et al. (2010) identified that autophagy was active in stress conditions, including N deficiency, oxidative stress, or the presence of misfolded proteins in the endoplasmic reticulum. Wang et al. (2009) extended this finding in this species, identifying a pathway linking autophagy activity and the production of storage lipids and carbohydrates. The proteins and signaling pathways known to mediate the autophagy process have been described in yeast, mammals, plants, algae, and other forms of life (Wullschleger et al. 2006, Diaz-Troya et al. 2008, Perez-Perez et al. 2010). In mammals, autophagy-related proteins have been found to sense cellular energy and nutrient status, signaling the metabolism of storage lipids at the onset of stress (Wullschleger et al. 2006, Singh et al. 2009).

Flynn (1990) speculated that the physiological responses to N deficiency, such as autophagy and lipid accumulation, would likely be triggered by reaching critical levels or proportions of key metabolites of C and N. As discussed earlier, changes in concentration of the primary N-containing cellular components have been analyzed in N deficiency studies, but studies identifying critical levels or interactions of these components associated with the initiation of lipid accumulation are lacking.

3.2.5. Objectives and Hypotheses

Our objective was to investigate associations between critical levels or interactions of specific pools of N and the initiation of lipid accumulation. We

hypothesized that there are commonalities among species in N concentration and/or partitioning associated with initiation of lipid accumulation.

3.3. Materials and Methods

3.3.1. Experimental Design

Three species of algae were studied in batch cultures, each given two N treatments: 4 and 10 mM N (hereafter referred to as low N and high N, respectively). Measurements were taken on all significant pools of N in the cell (protein, free amino acids, DNA, RNA, chlorophyll) and lipid content as the algae became progressively more N deficient. For 10 days of growth, once-daily measurements were facilitated by running 10 replicate, 1.2 L cultures per N treatment and running analysis on one culture per day. This is a form of trend analysis and did not include daily treatment replicates; the validity of the data can be assessed and verified by observation of the time-series trends. Standard deviations are included for measurements of cell count and volume, which are discussed in a later section. The experimental setup was designed to deliver algal biomass with a wide range of levels of N stress—from N-replete to severely N deficient. For each species, culture growth was started by filling the 10 reactors with media and 100 mL of axenic inoculation culture to a 1.2 L volume. Details on the growth apparatus and experimental methods are in the sections that follow.

3.3.2. Algal Strains

Three species were studied: *Chlorella sorokiniana* (Shihira & R.W. Krauss), UTEX #1602; *Ettlia oleoabundans* (S. Chantanachat & H.C. Bold) J. Komarek (formerly

Neochloris oleoabundans), UTEX #1185; *Nannochloropsis salina* (D.J. Hubbard), CCMP #1776.

3.3.3. Culture Apparatus

Algal cultures were grown in glass, air-lift bioreactor tubes that were autoclaved before use. The bioreactors had an outer diameter of 50 mm, inner diameter of 45 mm and were filled to an approximate height of 75 cm, giving a culture volume of 1.2 L. The bioreactor tubes were placed in a plexiglass water tank maintained at 25˚C. CO_2-enriched (1%), filtered air (Whatman PolyVENT 0.2 µm PTFE filters, L#639) was bubbled into the bottom of each bioreactor through a 1 mm glass capillary tube at a rate of 0.5 L min^{-1}. Light was supplied at a photosynthetic photon flux (PPF) of 250 µmol m^{-2} s^{-1} for 16 h per day by banks of fluorescent tubes that ran perpendicular to the bioreactor tubes, completely covering one side. A 16-h photoperiod was chosen to approximate the natural photoperiod of summers days in the mid-latitudes. The daily integrated PPF, a better indicator of growth potential than the instantaneous PPF (Bugbee and Monje 1992), was 14.4 mol m^{-2} d^{-1} in these studies. For conversion of measurements made by volume (e.g. g L^{-1}) to a unit of area (e.g. g m^{-2}), the illuminated area of the bioreactor tubes containing algae was used as a conversion factor as follows: 1.2 L / πrh = 1.2 L / (3.14 x 2.25 cm x 75.5 cm) x (1 m^2 / 100^2 cm^2) = 22.5 L m^{-2} and 22.5 L m^{-2} x g L^{-1} = g m^{-2}.

3.3.4. Media Composition and Preparation[5]

The study included two freshwater organisms (*C. sorokiniana* and *N. oleoabundans*) and a salt water organism (*N. salina*) that were treated with two levels of N: 4 and 10 mM N (low N and high N). Differences in ionic strength due to the N treatments were compensated for by adjusting levels of Cl, resulting in equal levels of all ions between treatments except N and Cl. The freshwater media components and concentrations were: 4.0 mM NaNO3 (low N) or 6.0 mM KNO3 and 4.0 mM NaNO3 (high N); 6 mM KCl (low N) or 0 mM KCl (high N); 0.60 mM $CaCl_2 \cdot 2H_2O$; 0.60 mM $MgSO_4 \cdot 7H_2O$; 0.40 mM KH_2PO_4; 15 μM DTPA-Fe; 11 μM H_3BO_3; 7.0 μM $MnCl_2 \cdot 4H_2O$; 0.70 μM $CuSO_4 \cdot 5H_2O$; 3.0 μM $ZnSO_4 \cdot 7H_2O$; 0.15 μM $Na_2MoO_4 \cdot 2H_2O$; 0.20 μM $CoCl_2 \cdot 6H_2O$. The seawater media components and concentrations were: 4.0 mM NaNO3 (low N) or 10 mM KNO3 (high N); 6.0 mM KCl (low N) or 0 mM KCl (high N); 1.0 mM $CaCl_2 \cdot 2H_2O$; 6.0 mM $MgSO_4 \cdot 7H_2O$; 300 mM NaCl; 0.5 mM KH_2PO_4; 0.20 mM $Na_2SiO_3 \cdot 9H_2O$; 7.5 μM Fe-NH_4 citrate; 7.5 μM DTPA-Fe; 15 μM H_3BO_3; 1.5 μM $MnCl_2 \cdot 4H_2O$; 0.08 μM $CuSO_4 \cdot 5H_2O$; 0.20 μM $ZnSO_4 \cdot 7H_2O$; 0.05 μM $Na_2MoO_4 \cdot 2H_2O$; 0.10 μM $CoCl_2 \cdot 6H_2O$; 1.5 nM vitamin B12; 4.1 nM biotin; and 150 nM thiamine.

[5] The media described here were designed to provide adequate levels of all essential nutrients, with the exception of N, for potential growth in our system. Particularly in seawater, however, P can precipitate and become deficient. We ran chemical speciation analysis on our seawater media using MINEQL+ version 4.6 (Environmental Research Software, Hallowell, ME) to determine P precipitation potential. This analysis indicated that all P would remain in solution in various free and complexed forms. Initially, for example, of 0.5 mM P supplied, only 2.2 x 10^{-7} mM would exist as the free PO_4^- ion while the remaining P would be in chemical complexes. With algal P uptake, we would expect complexed P to become available. There is also potential for P to become limiting in the later stages of growth with the higher N/P ratios tested here.

The media were autoclaved (P and Si stocks were autoclaved separately; vitamins were filter sterilized).

3.3.5. Cell Component Measurements

Protein, free amino acids and RNA/DNA were quantified on homogenates generated from cell suspensions taken daily from the first bioreactor in each N treatment series. Chlorophyll, total cellular N, and lipid content were quantified on dried algal cells of the same bioreactors. Cell suspensions were concentrated for drying by centrifugation (Sorvall RC6 Plus centrifuge, ThermoFisher Scientific, Waltham, MA) at 7500 rpm for 5 min; the biomass was loaded into 15 mL plastic sample vials, frozen at -80˚C, and lyophilized.

In the final analysis, assumptions had to be made on the N content of each N pool. The protein pool was assumed to contain 16% N, the N content of the BSA that was used as standard in protein measurements (Pierce Biotechnology, Rockford, IL). The free amino acid pool was assumed to have the same percent N as the protein pool. For DNA and RNA, the distribution of bases was assumed to be equal (i.e. A = T = C = G and A = U = C = G). Since the molar ratio of deoxyribose or ribose to base to phosphate is 1:1:1 in DNA and RNA, respectively, the total mass of N in each base was divided by the sum of the masses of the base, deoxyribose or ribose, and a phosphate group and the values for the four bases averaged for each nucleic acid (14.49% N for DNA and 13.95% N for RNA). Chlorophylls a and b were quantified separately, so the N content of each (6.27% N for a and 6.18% N for b) was applied separately then added together. The N content of each pool was divided by total cellular N determined by CHN combustion analysis

(described later) in determination of percent N recovery. Recovery efficiency of each N pool and total N was assumed to be constant among species.

3.3.5.1. Protein

Forty five mL of cell suspension was placed in a 50-mL centrifuge tube and centrifuged for 3 min at 7500 rpm at 5˚C to pellet the cells. The solution was decanted and the pellet resuspended in 20 mL of 100 μM NaCl solution by Vortex mixing. The sample was homogenated by three sequential runs through a French press (SLM Aminco French Pressure Cell Press Model FA-078, Urbana, IL) and immediately stored in a -80˚C freezer for measurement later. Once thawed, 2 mL of homogenate was subsampled and placed in a 15-mL centrifuge tube for trichloroacetic acid (TCA) precipitation of the protein (Waterborg 2002). To the homogenate, 0.2 mL of 3.6 mM deoxycholate (Sigma-Aldrich, St. Louis, MO) was added and mixed by Vortex. After standing at room temperature for 10 min, 0.2 mL of 4.4 M TCA (ThermoFisher Scientific, Waltham, MA) was added and mixed by Vortex. The homogenate was centrifuged for 10 min at 5000 rpm. The supernatant was decanted and stored at -80˚C for measurement of free amino acids at a later time. To dissolve the protein, 2 mL of a 4% SDS (Sigma-Aldrich, St. Louis, MO) was added to the biomass pellet and the centrifuge tube was placed in a boiling water bath for 15 min, mixing by Vortex every 5 min. Protein was measured on the solution with a BCA protein assay kit (Pierce Biotechnology, Rockford, IL), with BSA as the standard within the recommended concentration range given by the company.

3.3.5.2. Free Amino Acids

Free amino acids were measured by the fluorescamine method as described in Clayton et al. (1988), with slight modification, on supernatant collected from TCA-precipitated homogenate in the protein procedure. One mL of supernatant was placed in a 15-mL centrifuge tube and 3 mL of borate buffer were added. The buffer was prepared by making a 200 mM sodium borate solution that was adjusted to pH 9 with boric acid powder; heating in a water bath was necessary to dissolve all the material. A 0.5 mM fluorescamine solution was prepared by adding 14 mg of fluorescamine (Sigma-Aldrich, St. Louis, MO) to 100 mL acetone. One mL of fluorescamine was added to the buffered supernatant drop wise while Votex mixing. Fluorescence was measured on the mixture with a fluorescence spectrophotometer (Varian Cary Eclipse, Palo Alto, CA) with an excitation wavelength of 390 nm and emission wavelength of 480 nm. A standard curve was generated using glutamate (Sigma-Aldrich, St. Louis, MO), from 5 to 300 μg mL^{-1}.

3.3.5.3. RNA/DNA

RNA and DNA were quantified by the fluorometric, ethidium bromide method of Thoresen et al. (1983) with minor adaptations for our system. Thirty mL of cell suspension was placed in a 50-mL tube and centrifuged for 3 min at 7500 rpm at 5˚C to pellet the cells. The solution was decanted and the pellet resuspended in 29.76 mL of PBS and 0.18 mL of 15 μg mL^{-1} bentonite (Sigma-Aldrich, St. Louis, MO) solution. Bentonite is an inhibitor of ubiquitous RNase enzymes. The sample was homogenated by three sequential runs through a French press (SLM Aminco French Pressure Cell Press Model FA-078, Urbana, IL) and immediately stored in a -80˚C freezer for measurement

later. Once thawed, three, 2 mL samples of homogenate were subsampled and placed in 15-mL centrifuge tubes for measurement of DNA and RNA + DNA. To all three tubes, 0.25 mL of 200 μg mL^{-1} pronase from *Streptomyces griseus* (Sigma-Aldrich, St. Louis, MO) was added. To the DNA tube, 0.25 mL of 1 mg mL^{-1} RNase A from bovine pancreas (Sigma-Aldrich, St. Louis, MO) was added to degrade RNA, while PBS was added to the RNA + DNA tube. All tubes were incubated in a water bath at 37˚C for 20 min. The tubes were then centrifuged for 10 min at 5˚C at 7500 rpm. Two mL of supernatant from each tube was subsampled and placed in 5-mL glass tubes and 0.5 mL of 25 μg mL^{-1} ethidium bromide (Sigma-Aldrich, St. Louis, MO) solution was added. Three minutes were allowed for complete fluorescence development. Fluorescence was measured on a fluorescence spectrophotometer with an excitation wavelength of 365 nm and an emission wavelength of 590 nm. Separate RNA and DNA standard curves were prepared from yeast RNA Ambion, Life Technologies, Carlsbad, CA) and DNA sodium salt from calf thymus (Sigma-Aldrich, St. Louis, MO) at concentrations from 0.5 to 5 μg mL^{-1}.

3.3.5.4. Chlorophyll

Chlorophyll was extracted and quantified according to the method and equations of Wellburn (1994), with slight modifications for our system. Ten mg samples of dried algal tissue were weighed in glass vials and 10 mL of DMSO (Sigma-Aldrich, St. Louis, MO) was added. The vials were capped tightly, placed in an oven at 65°C for 1 h, and shaken about every 15 min. After removal from the oven, cell material was allowed to settle in the vials for 10 min. Two mL samples of DMSO solution were taken for spectral

determination of chlorophylls a and b (UV-2401 PC, UV-VIS recording spectrophotometer, 0.1 nm resolution, Shimadzu Corporation, Kyoto, Japan).

3.3.5.5. Total Cellular Nitrogen

Total C and N in algae were measured by combustion analysis (Model 2400 CHN analyzer, Perkin-Elmer, Waltham, MA) according to the standard methods published by the instrument manufacturer. EDTA was used as the standard.

3.3.5.6. Lipid Content

Simultaneous conversion and extraction of algal lipids to fatty acid methyl esters (FAME) was done by the method of Wahlen et al. (2011). This method effectively converts to FAME the fatty acids contained in membrane phospholipids and glycolipids, as well as free fatty acids and storage lipid triglyceride. The lipid or FAME content of 100 mg freeze-dried algal samples was determined with a gas chromatograph (Model 2010, Shimadzu Corporation, Kyoto, Japan) equipped with a programmable temperature vaporizer (PTV), split/splitless injector, flame ionization detector (FID) (GCMS-QP2010S, Shimadzu Corporation, Kyoto, Japan), and autosampler. The FID detector was used in this analysis. Analytes were separated on an RTX-Biodiesel column (15 m, 0.32 mm ID, 0.10 μm film thickness, Restek, Bellefonte, PA) using a temperature program of 60˚C for 1 min followed by a temperature ramp of 10˚C per min to 360˚C for 6 min. Constant velocity of helium as a carrier gas was set at 50 cm s^{-1} in velocity mode. Sample sizes of 1 μL were injected into the PTV injector in direct mode that followed an identical temperature program to that of the column. The FID detector was set at 380˚C. FID

detector response to FAME was calibrated using methyl tetradecanoate (C14:0), methyl palmitoleate (C16:1), and methyl oleate (C18:1) (Sigma-Aldrich, St. Louis, MO) at concentrations ranging from 0.1 mg mL^{-1} to 1 mg mL^{-1}. Standards were obtained as pure compounds (Nu-Chek Prep, Inc., Elysian MN) and were diluted with chloroform to obtain the needed concentrations. A standard (GLC-68A, Nu-Chek Prep, Inc.) containing methyl esters ranging from methyl tetradecanoate (C14:0) to methyl nervonate (C24:1) was used to identify the retention time window for FAME peak integration. Peaks within this region were integrated using GC solution postrun v. 2.3 (Shimadzu) and concentrations were determined by linear regression analysis.

3.3.6. Growth Parameter Measurements

Uniformity of growth among replicate bioreactors was monitored daily by measurements of optical density at 750 nm (UV-2401 PC, UV-VIS recording spectrophotometer, 0.1 nm resolution, Shimadzu Corporation, Kyoto, Japan). Measurements of cultural dry biomass density, cell counts, and cell volumes were made daily only on the first bioreactor in each N treatment series. Dry biomass density was determined by filtering 10 mL suspensions of algae (GF/C filters, Whatman, Kent, UK); the filters were dried for one day at 105˚C. Cell count and volume determinations were made microscopically (ICC50 microscope camera mounted on a DM750 microscope body, Leica, Buffalo Grove, IL) with a hemacytometer (Reichert Bright-Line, Hausser Scientific, Horsham, PA). Per sample, the hemacytometer was loaded twice with 15 μL samples of cell suspension, all cells in a 0.25 mm^2 grid were manually counted, and averaged between counts. Dilutions were made to keep cell numbers at approximately

300 or less. Images of the 0.25 mm^2 grid were taken for determination of cell volumes. Two 0.05 mm^2 boxes were chosen within the 0.25 mm^2 grid, this area was zoomed, images were printed, and the diameters of all cells were manually measured, normalized to the hemacytometer grid, and averaged. In data presentation, error bars showing standard deviations are given to describe variability in cell counts and volumes.

3.4. Results and Discussion

3.4.1. Metrics for Data Analysis

The lipid accumulation observed in N deficient microalgae may be signaled by reaching a critical low concentration of total cell N or certain pools of N (Flynn 1990). In assessing associations between algal N and lipid accumulation, the units of data summary are critically important as they impact the conclusions made. In single-celled, autonomous organisms, data summarized per unit biomass is primarily useful in assessment of population or ecological physiology; data summarized per cell is more appropriate for assessment of cellular physiology. Here we are interested in metabolic stress responses of the cell. Further normalizing the data to the volume of the cell facilitates comparison among cells of different volume, which ranges over nine orders of magnitude in algae (Finkel et al. 2010). As such, the data presented here is given per unit cell volume (e.g. mM N, g protein L^{-1}). This choice of units is validated by the lipid accumulation data (discussed later), which is presented both per unit biomass and per unit cell volume.

3.4.2. Cellular Nitrogen Uptake, Retention, and Distribution

Differences among species in growth parameters (cell density, cell mass, cell volume) and N uptake rate resulted in wide differences in total N uptake and retention (Fig. 3–1). *C. sorokiniana* had the broadest range in total N concentration from ~520 to 2050 mM N in the cell; *E. oleoabundans* ranged from 430 to 1230 mM, and *N. salina* from 350 to 1165 mM.

The cellular concentration of five major pools among which this N was distributed (protein, free amino acids, DNA, RNA, and chlorophyll) were measured for 10 days and their respective N contents were estimated. Fig. 3–2 displays the time-series trends in the N pools, for validation of the raw data per unit biomass. Fig. 3–3 displays the data normalized to cell volume, given as a function of the concentration of N in the cell. The daily percent N recovery was calculated as the sum of N in all measured pools—given the assumptions stated in the methods and materials—divided by the total N determined by combustion analysis (Table 3–1). In general, the concentration of each N pool decreased linearly with decreasing cell N. There were close relationships between total cell N and protein and RNA that were fairly consistent among species. Maximum and minimum concentrations of protein and RNA mirrored the unique concentration ranges of N taken up and retained by each species. The rate of decrease in RNA concentration with decreasing cell N was particularly dramatic, signifying the negative effect of N deficiency on cellular processes. Concentrations and patterns of change among the other pools of N varied more from species to species. The average cellular concentration of free amino acids was 4.5, 10.4, and 13.9 g L^{-1} (*N. salina, C. sorokiniana,*

and *E. oleoabundans*, respectively), though the rate of decrease in concentration with decreasing cell N was similar among species. DNA concentrations were relatively steady with decreasing N in *C. sorokiniana* and *E. oleoabundans*, but decreased to low levels in *N. salina* (this result was repeated twice on our original sample). Given that the cells were not synchronized, the integral function of DNA in the cell, and the data of Dortch et al. (1984) showing steady DNA levels with decreasing N, we expected the DNA concentration to remain fairly steady. DNA concentrations per cell for *C. sorokiniana* and *E. oleoabundans* were within the documented range reported in the Kew Royal Botanical Gardens Cvalues database (http://data.kew.org/cvalues/CvalServlet?querytype=6), but lower-end concentrations for *N. salina* were not. All these considerations provide cause for skepticism of the DNA values obtained for *N. salina.* The maximum concentration of chlorophyll and the rate of decline with decreasing cell N differed greatly among species, converging on a similar minimum concentration.

There were several notable differences among species in the fraction of N allocated to each pool as N deficiency progressed (Fig. 3–4). (This analysis is based on the linear regression of data presented in Fig. 3–3.) All species increased in the fraction of cell N held in protein, but to different extents: *C. sorokiniana* and *E. oleoabundans* experienced modest 6 to 7% increases, while *N. salina* had a 20% increase. In all species, protein N amounted to a minimum of 70% of total N; at the peak of N deficiency, 90% of total N was held in the protein pool in *N. salina*. Predictably, *N. salina* decreased in the fraction of N held in free amino acids, suggesting a priority of exporting free amino acids to build proteins. Interestingly, *C. sorokiniana* and *E. oleoabundans* increased in the

fraction of cell N held in free amino acids, suggesting that storage of free amino acids is a priority in these species. Free amino acid N constituted from 7 to 23% of total N. In one of nine algal species studied, Dortch et al. (1984) also reported a high value of percent free amino acid N of 26.4% in a N deprived condition. The fraction of cell N held in chlorophyll was fairly steady in *C. sorokiniana* and *N. salina*, but decreased somewhat in *E. oleoabundans*—the species with the highest overall chlorophyll concentration. This difference may suggest that *E. oleoabundans* overproduces chlorophyll when N supplies are sufficient to do so. Due to its relatively steady concentration in the cell, the fraction of N held in DNA increased (except in *N. salina*). The RNA pool, of course, decreased dramatically in the fraction of N held there.

Total N uptake and retention, N pool concentrations, and changes in N distribution with deficiency, as determined in this study, are generally consistent with the reports of Dortch et al. (1984) and McGlathery et al. (1996). As such, the data provide a sound basis for analysis of associations between N deficiency and lipid accumulation.

3.4.3. Associations between Cellular Nitrogen and Lipid Accumulation

In analysis of the effect of algal N on lipid accumulation, the data was initially summarized per unit biomass: lipids as a content (%), as a function of the N to C ratio of the biomass (Fig. 3–5, left). In all three species, there was a clear relationship between the N to C ratio and the initiation of lipid accumulation, as shown by the concomitant rise in lipid content in both N treatments. But large species differences were observed in the value of the N to C ratio at which lipid accumulation began, as well as the value at which accumulation (and growth) ceased. Normalizing this data to the volume of the cell greatly

reduced these species differences (Fig. 3–5, right). In this data, the ratio of minimum retained N to N at the initiation of lipid accumulation was fairly consistent at 0.5 to 1 (Table 3–2). This observation suggests a commonality among species in the magnitude of N deficiency that leads lipid accumulation.

Among five pools of N in the cell at the initiation of lipid accumulation (Table 3–3), the concentrations of protein, RNA, and their ratio were most similar among species with averages of 52 ± 8.6 g L^{-1}, 3.2 ± 0.26 g L^{-1}, and 16 ± 1.5, respectively. The most consistency among species was observed in RNA and the protein to RNA ratio, as shown by the coefficient of variation (standard deviation/mean *100) also listed in Table 3–3. Fig. 3–6 illustrates the convergence of the protein to RNA ratio at ~16 to 1 in all species at the initiation of lipid accumulation, despite differences in total N uptake and retention. In the remaining pools (free amino acids, DNA, chlorophyll), intrinsic species differences in concentration and disparate effects of N deficiency resulted in large dissimilarities among species at lipid accumulation. Other physiologically relevant ratios of the N pools were also highly inconsistent among species. As such, if lipid accumulation is signaled by reaching critical concentrations or ratios of specific pools of N, this data most implicates RNA and the protein to RNA ratio.

3.4.4. Use and Extrapolation of the Data

These results provide insight into the physiological drivers for lipid accumulation in N deficient algae. The information could guide biochemical and molecular studies that seek to elucidate signaling pathways and genetic regulation of lipid accumulation in response to N deficiency. The data could also provide information for models for

optimization of lipid production in commercial systems, and understanding production in nature.

3.5. Conclusions

These data suggest the initiation of lipid accumulation is associated with a commonality among species in the magnitude of total N deficiency. Among five pools of N in the cell, the data implicates critical levels of RNA and the protein to RNA ratio as potential signals for accumulation of lipids.

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	C. sorokiniana		*E. oleoabundans*		*N. salina*	
Time	Low N	High N	Low N	High N	Low N	High N
(d)	----------(% Nitrogen Recovery)----------					
1	114	109	103	119	135	110
2	104	87	96	129	77	90
3	110	82	107	95	75	60
4	103	78	119	83	84	78
5	105	75	122	92	93	67
6	94	75	107	84	94	74
7	99	67	101	89	100	77
8	95	86	104	85	76	81
9	93	75	107	80	77	84
10	99	67	106	87	84	71

Table 3–1: Percent Nitrogen Recovery: The sum of N (given assumptions stated in the methods and materials) in all measured N pools (protein, free amino acids, DNA, RNA, chlorophyll) divided by the total N determined by combustion analysis. Variation from 100% recovery reflects combined experimental error in determination of total N and each N pool, plus errors in estimating the N content of the N pools.

Species	Max N	Lipid Onset N	Min N	Min/Onset	Max/Onset
	----------(mM)----------			----------(ratio)----------	
C. sorokiniana	2050	950	520	0.55	2.2
E. oleoabundans	1230	910	430	0.47	1.4
N. salina	1165	730	350	0.48	1.6
Avg	1482	863	433	0.50	1.7
SD	493	117	85	0.041	0.41
CV*100	33	14	20	8.3	24

Table 3–2: Total N concentrations in the cell at their maximum (Max), minimum (Min), and at the initiation of lipid accumulation (Initiation). Maximum and minimum concentrations were measured, while the lipid accumulation initiation values were determined qualitatively according to the trends apparent in Fig. 3–5. A strong similarity among species was observed in the ratio of cell N at the minimum to the N at lipid accumulation initiation, with an average value of 0.5 ± 0.04. This commonality among species suggests that lipid accumulation was signaled by a common magnitude of cellular N deficiency. (Avg, average; SD, standard deviation; CV, coefficient of variation.)

Species	Prot	AA	DNA	RNA	Chl	AA/ Prot	Prot/ DNA	Prot/ RNA	RNA/ DNA
		----------(g L^{-1})----------				----------(ratio)----------			
C.s.	57	10	5.1	3.3	4.8	0.18	11	17	0.64
	(52, 61)	(8.4, 12)	(4.7, 5.5)	(1.9, 4.8)	(4.4, 5.2)				
E.o.	57	14.5	2.5	3.4	6.5	0.25	23	17	1.4
	(52, 62)	(13, 16)	(2.3, 2.7)	(1.7, 5.1)	(6.0, 7.1)				
N.s.	42	4.6	1.1	2.9	2.8	0.11	40	14	2.8
	(38, 46)	(4.0, 5.3)	(0.81, 1.3)	(2.2, 3.6)	(2.4, 3.1)				
Avg	52	10	2.9	3.2	4.7	0.18	25	16	1.6
SD	8.6	5.0	2.1	0.26	1.9	0.07	14	1.5	1.1
CV*100	17	51	71	8.2	40	40	59	9.2	68

Table 3–3: The cellular concentration of pools of N, and select ratios, at the initiation of lipid accumulation. The concentration of RNA and the protein to RNA ratio were most similar among species, as shown by low coefficient of variation, implicating critical levels of these parameters as potential signals for lipid accumulation. The table values were determined by a qualitative analysis of total N at initiation of lipid accumulation from Fig. 3–5, then calculation of N pool concentrations at those points from the regression lines in Fig. 3–3. The bracketed values are 95% confidence intervals for the regression lines at the lipid accumulation initiation points. Confidence intervals were calculated by SigmaPlot (Systat Software Inc., San Jose, CA) by the reduced chi-square method. (*C.s., C. sorokiniana; E.o., E. oleoabundans; N.s., N. salina*; Prot, protein; AA, free amino acids; Chl, chlorophyll; Avg, average; SD, standard deviation; CV, coefficient of variation.)

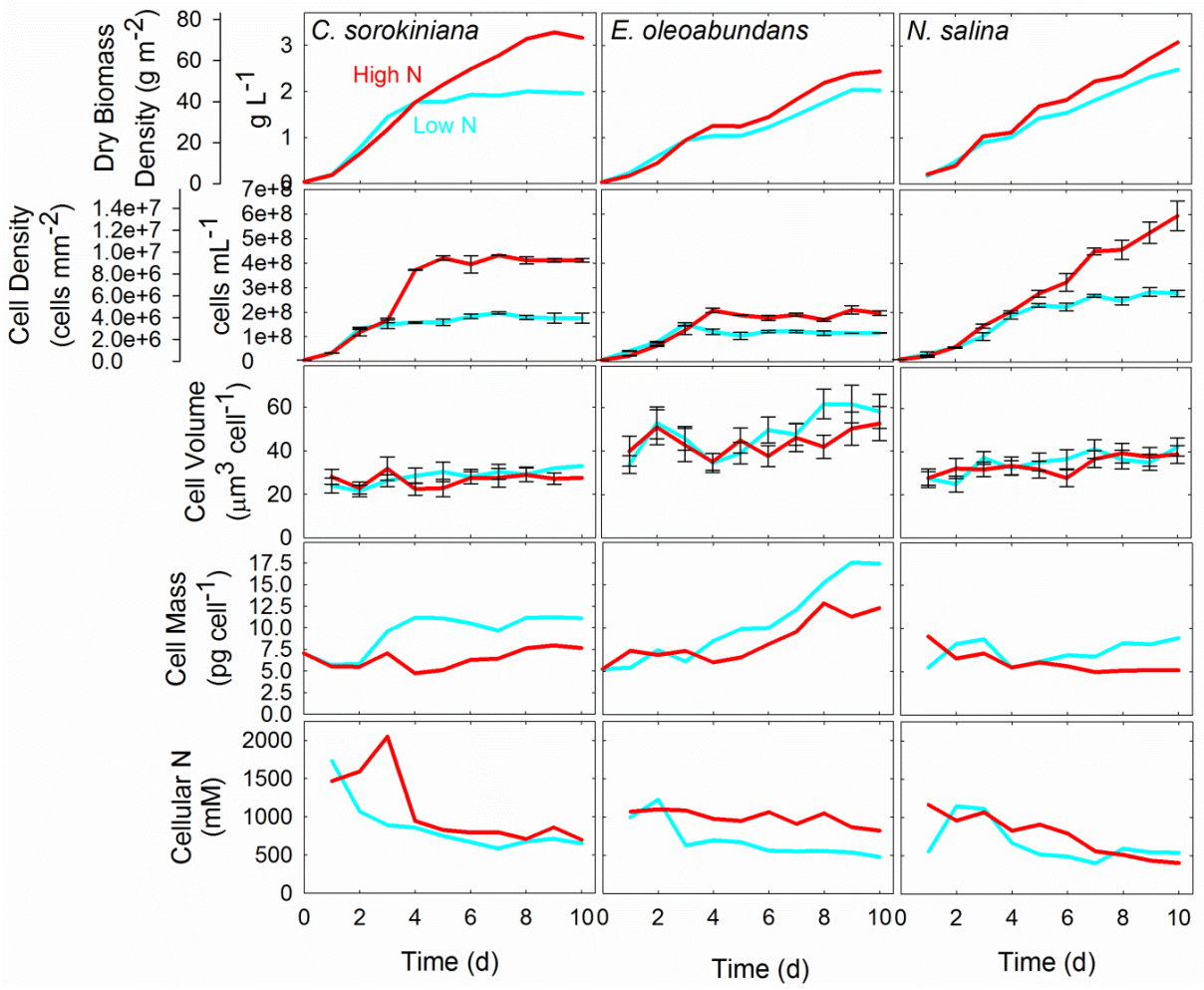

Fig. 3–1: Time-series measurements of growth parameters and cellular N concentration in two N treatments in cultures of three species of oleaginous algae. Data were collected once a day from independent bioreactor tubes (10 replicate tubes per N treatment; one tube processed per day). Time series trends are apparent in the data. Standard deviations are given for cell density and cell volume and show variability in these parameters within bioreactor tubes. (High N, 10 mM; low N, 4 mM.)

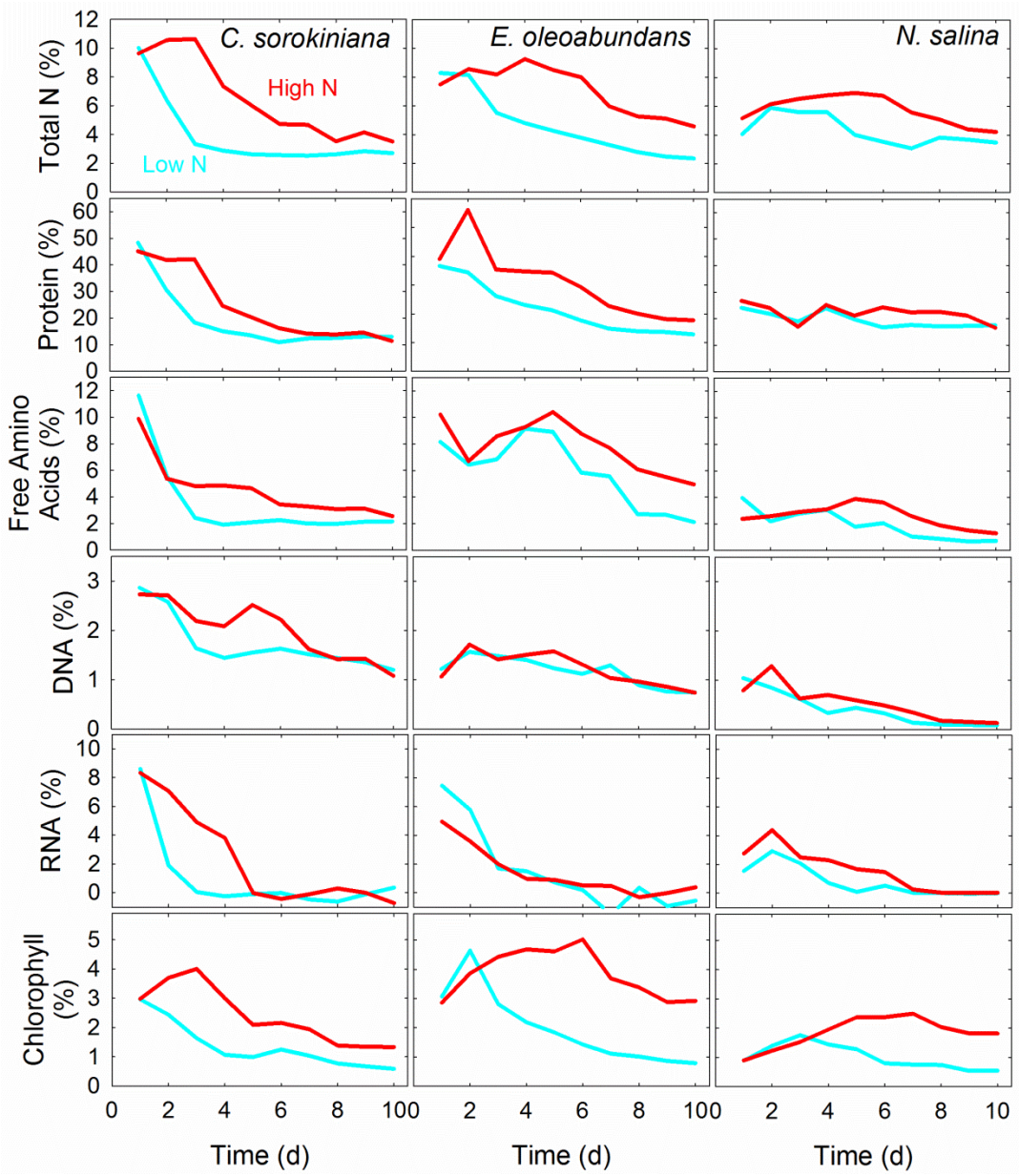

Fig. 3–2: Time-series measurements of the raw data for total biomass N and five cellular pools among which the N was distributed.

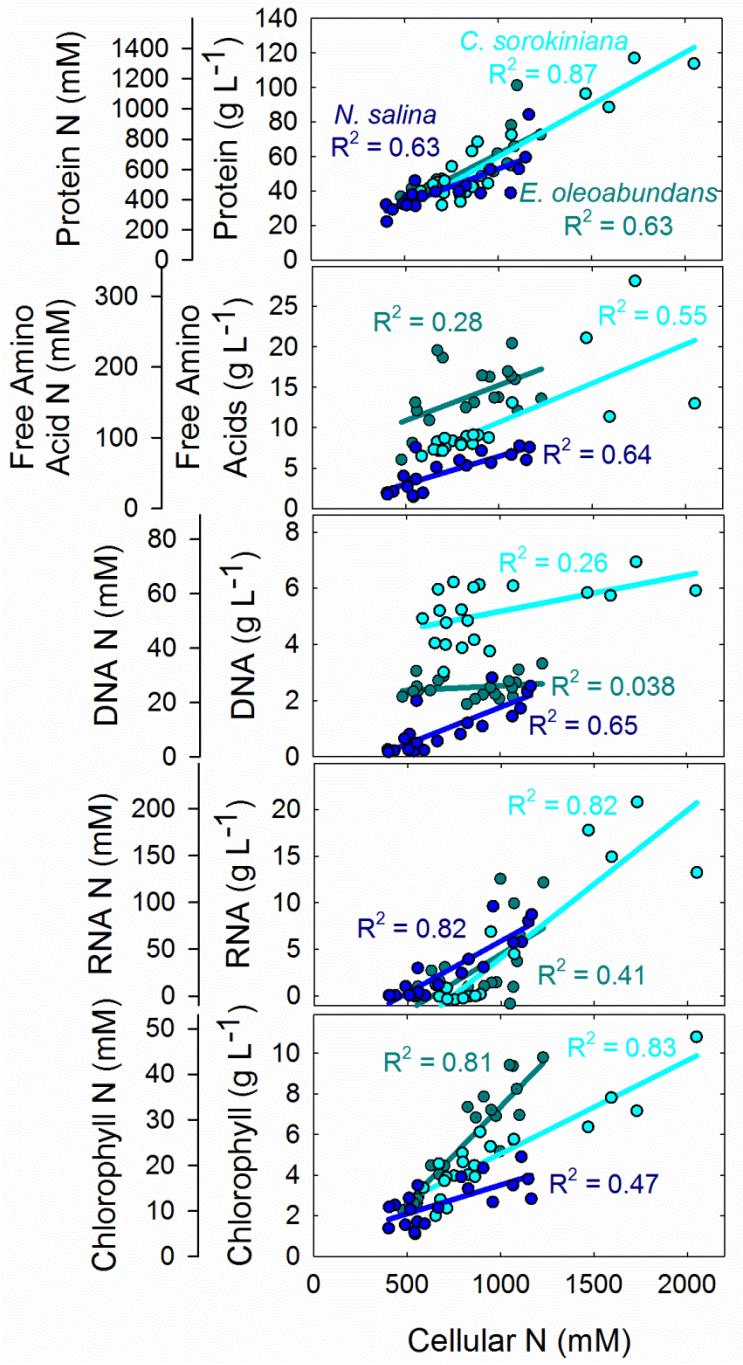

Fig. 3–3: The concentration of five major pools of N in the cell, including the cellular concentration of N in those pools (given assumptions stated in the methods and materials), as a function of total cell N. All data is given per unit cell volume, for relevance to the physiology of the cell. In general, the concentration of each pool decreased linearly with decreasing cell N. (Inner axis, pool concentration in cell; outer axis, pool N concentration in cell.)

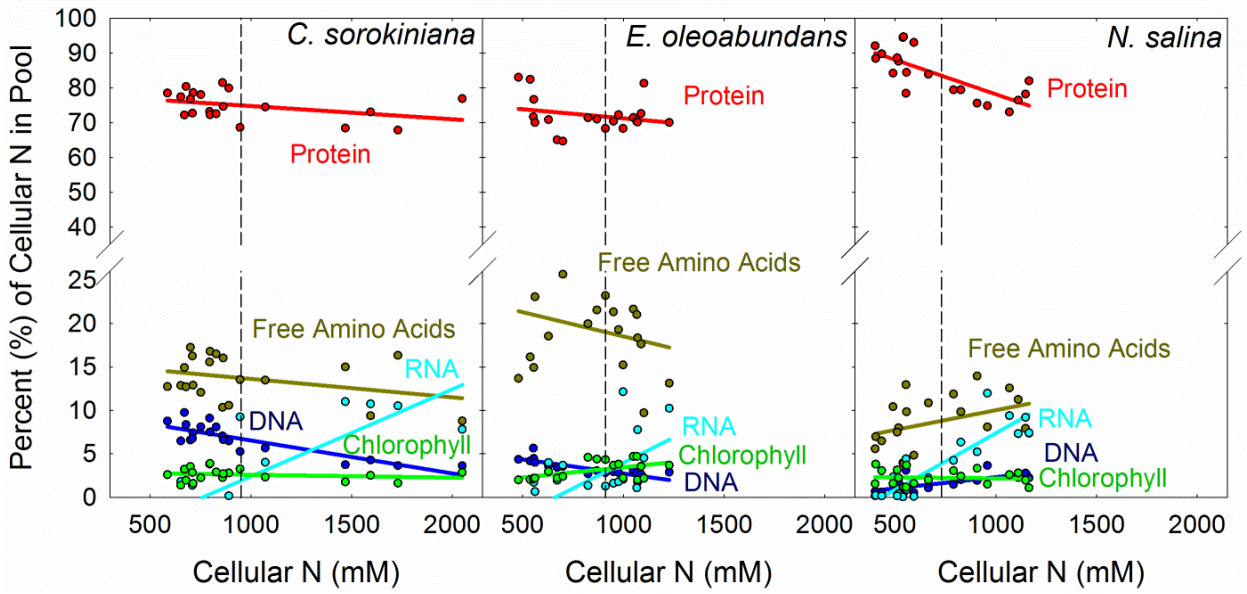

Fig. 3–4: The percentage of total N held in five cellular pools as a function of total N. The vertical, dotted line represents the initiation of lipid accumulation. As N deficiency progressed, all species increased in the fraction of N held in protein and most did in DNA. Predictably, all species decreased in the fraction of N in RNA. Patterns in free amino acids and chlorophyll varied more among species.

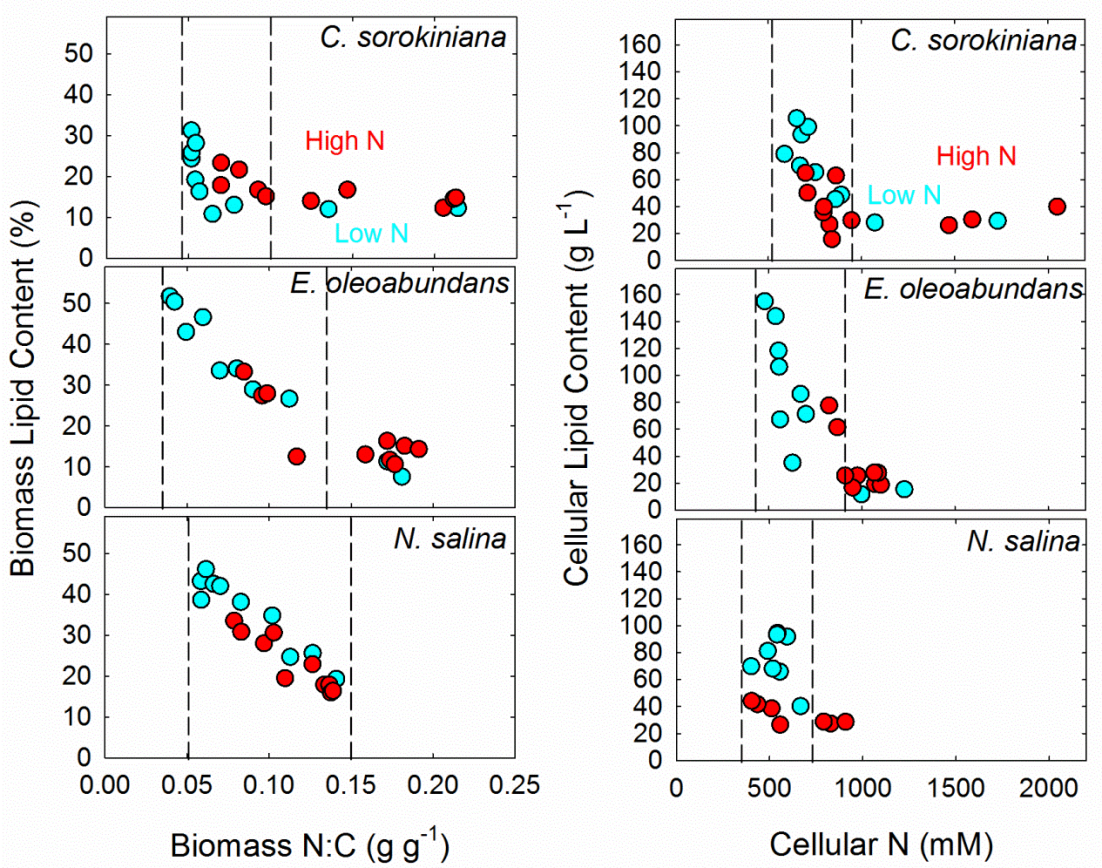

Fig. 3–5: Lipid accumulation per unit biomass as a function of the N to C mass ratio (left), and transformed data per unit cell volume as a function of cell N per unit cell volume (right). The initiation of lipid accumulation had a clear dependence on specific concentrations of total cell N in all three species, as shown by the concomitant rise in lipid content in two N treatments. The area between the vertical, dotted lines signifies the range in cell N over which lipids accumulated. Normalizing the data to the cell greatly reduced species differences in the value of cell N at which lipid accumulation began and ceased (High N, 10 mM; low N, 4 mM.)

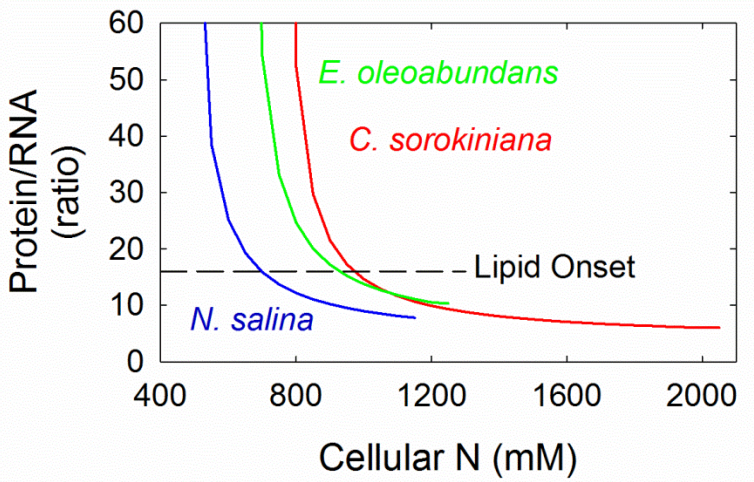

Fig. 3–6: Despite differences in total N uptake and retention, the protein to RNA ratio converged at ~16 to 1 in all species at the initiation of lipid accumulation. These trends are based on the regression lines for protein and RNA found in Fig. 3–3.

CHAPTER 4

ENHANCING LIPID PRODUCTION OF THE MARINE DIATOM *CHAETOCEROS GRACILIS*: SYNERGISTIC INTERACTIONS OF SODIUM CHLORIDE AND SILICON[6]

4.1. Abstract

Silicon deficiency is a lipid-promoting stress for many oleaginous diatoms. Literature reports suggest that reduced salinity in seawater, a primary component of which is sodium chloride, may inhibit metabolism of silicon in marine diatoms. We hypothesized that lowering sodium chloride below ocean levels may thus be effective in creating silicon stress and enhancing lipid productivity. We examined the interacting effects of silicon supply (0.05, 0.1, 0.2 and 0.8 mM) and sodium chloride concentration (50, 100, and 400 mM) on growth and lipid production in *Chaetoceros gracilis*. This was done in batch culture to facilitate the application of severe stress. Low levels of either sodium chloride or silicon resulted in at least 50% increases in lipid content. The synergy of simultaneous, moderate sodium chloride and silicon stress resulted in lipid content up

[6] The terminology for the different forms of silicon can be confusing and warrant a brief discussion. "Silicon" is the name of the element, found on the periodic table. "Silicate" is a general term for a large group of sparingly soluble or insoluble mineral salts that contain silicon. Silicate minerals are among the most abundant in the earth's crust. Most silicate compounds are oxides, but not all. "Silicic acid" generally refers to a specific silicate compound that is in solution, SiH_4O_4, and is the primary form of silicon available for biological uptake. Silicic acid is also a general name for a family of soluble silicon compounds containing oxide and hydroxyl groups. "Silica" refers to the solid, dioxide form of silicon and is synonymous with the term "silica glass." In this chapter we measured silicic acid by the QuikChem method 10-114-27-1-A developed by the Lachat company. To simplify our message, we refer to this only as silicon. This is simply done, as there is one mole of silicon per mole of silicic acid.

to 73% of dry mass and lipid productivity of 1.7 g m^{-2} d^{-1}; with a daily integrated photosynthetic photon flux of 17.3 mol m^{-2} d^{-1}, the efficiency of lipid synthesis was thus 0.1 g mol^{-1} of photons. Decreased silicon also resulted in a 5% shift in lipid chain length from C18 to C16 fatty acids. We observed a strong sodium chloride/silicon interaction on total and ash-free dry mass densities that arose because low sodium chloride concentrations were inhibitory to growth, but the inhibition was overcome with excessive silicon supply. This observation suggests that low levels of sodium chloride may have affected metabolism of silicon. The findings of this study can be used to enhance lipid production in oleaginous marine diatoms.

4.2. Introduction

4.2.1. Silicon and Sodium Chloride

Diatoms are a diverse and ecologically successful class of marine and freshwater algal organisms. Diatoms are the primary constituent of the marine plankton community, typically representing more than 70% of total plankton, and are estimated to contribute up to 40% of total oceanic primary production (Sumper and Brunner 2008). A primary driver of the ecological success of diatoms is their use of Si for formation of their exoskeleton, called a frustule. Some likely reasons for this are that it is energetically less costly to make a Si-based cell wall than a carbon-based cell wall and that, different from other limiting nutrients, diatoms have little competition from non-diatoms for Si (Martin-Jezequel et al. 2000). Huge diatom abundance has caused dissolved Si to be drawn down to low levels, however; there is an approximate average of 35 μM Si in surface waters (Hem 1985) and a global average of approximately 70 μM Si (Sumper and Brunner

2008). This has resulted in Si becoming a major limiting nutrient for diatoms in the oceans.

There are two primary Si pools in diatoms: cellular Si and frustule-associated or mineralized Si (Vrieling et al. 1999). At high concentrations, Si enters the cell by diffusion, but at low concentrations (< ~30 μM) Si is actively taken up by sodium (Na)-dependent Si transport proteins (Bhattacharyya and Volcani 1980, Hildebrand et al. 1997, Thamatrakoln and Hildebrand 2008). Cellular Si is mineralized to form the frustule in specialized intracellular compartments called Si deposition vesicles (SDVs). Cell division and Si metabolism are closely tied (Martin-Jezequel et al. 2000). In some species, including *Chaetoceros gracilis* (Lombardi and Wangersky 1991), the production of storage lipid (triacylglycerol or TAG) is stimulated when Si availability is limiting to cell division (Araujo et al. 2011, Hildebrand et al. 2012). Little is known, however, about the physiological mechanism by which deficient Si signals the accumulation of lipids (Merchant et al. 2012). Deficiencies of other nutrients, like N and P, have also been shown to promote lipid accumulation in diatoms, but several studies suggest that Si deficiency stimulates lipid formation more rapidly and can result in higher lipid content (Shifrin and Chisholm 1981, Enright et al. 1986, Taguchi et al. 1987, Mortensen et al. 1988, Parrish and Wangersky 1990, Lombardi and Wangersky 1991, McGinnis et al. 1997).

Some studies suggest that salinity may affect the metabolism of cellular Si in marine diatoms. At low salinity, Vrieling et al. (1999) and Tuchman et al. (1984) observed increases in total diatom Si concentration, with a larger cellular Si pool

accounting for most of the increase. At some level, these observations suggest inhibition by low salinity of Si movement from the cellular pool to the mineralized pool. In two diatoms grown at low salinity, Vrieling et al. (2007) observed nanostructural changes in mineralized Si, including increased Si density. The density of mineralized Si is affected by the size of coalescing Si particles inside the SDVs, with smaller particles resulting in a denser frustule. The authors therefore suggested that salinity affects intracellular Si transport to the SDVs or the function of the SDVs themselves (Vrieling et al. 1999, Vrieling et al. 2007). Currently little is known about the effect of salinity on these processes (Vrieling et al. 1999, Sumper and Kroger 2004, Vrieling et al. 2007). Observations of increased lipid content in marine diatoms grown at low salinity, including *C. gracilis*, may further suggest a connection between Si metabolism and salinity (Chelf 1990, Araujo et al. 2011).

4.2.2. Objectives and Hypotheses

The marine diatom *C. gracilis* is known to accumulate lipids when Si deficient and when cultivated at low salinity. These effects may have a connection, however, as studies suggest that low salinity may affect intracellular Si transport or mineralization. We thus hypothesized that reducing salinity (by reducing the concentration of NaCl) may be effective in enhancing lipid accumulation in *C. gracilis* at any given rate of Si supply. Testing combinations of wide ranges in Si and NaCl, we sought to characterize the respective effects of Si, NaCl and their interaction on lipid content, growth rate, and other parameters in batch culture. Our broad objectives were to provide useful information to

the lipid producer and, secondarily, to provide data suggesting the nature of the relationship between NaCl and the metabolism of Si in a marine diatom.

4.3. Methods and Materials

4.3.1. Experimental Outline

Experimental design and setup are described here; apparatus and measurement details are described in the sections that follow. Batch cultures of the marine diatom *Chaetoceros gracilis* (UTEX #LB 2658) were grown in glass, air-lift bioreactors to test combinations of three NaCl concentration treatments (50, 100 and 400 mM) and four Si supply treatments (0.05, 0.1, 0.2 and 0.8 mM). There were two replicate bioreactor tubes per combination of treatments, for a total of 24 tubes. The cultures were started by adding media and 100 mL of inoculum to the bioreactor tubes to a 1.2 L volume. The inoculum culture was pre-adapted to an intermediate NaCl concentration of 100 mM and a low Si supply of 0.1 mM. This was done by growing a culture at 100 mM NaCl and 0.1 mM Si for seven days, then transferring 100 mL to a new 100 mM NaCl/0.1 mM Si media and allowing it to grow for four days. An absorbance measurement was taken from each tube daily. Solution Si measurements were taken two days. The cultures were harvested when the absorbance of all treatments had plateaued (two days of consistent optical density). Terminal measurements were taken of total and ash-free dry mass, biomass lipid content and lipid chain length.

4.3.2. Culture Apparatus and Maintenance

The cultures were grown in glass, air-lift bioreactors that were autoclaved before use. The bioreactors had an outer diameter of 50 mm, inner diameter of 45 mm and were filled to an approximate height of 75 cm, giving a culture volume of 1.2 L. Under the same conditions and timeframe as the final experiment, we quantified Si leaching from the bioreactors and found that total leaching was 3.2 ± 0.3 μM Si (n=3), which is only 6% of the Si in the lowest treatment. As such, interference by Si leaching was expected to be minimal. The bioreactor tubes were placed in a plexiglass water tank that was maintained at a temperature of 25˚C. Filtered air (Whatman PolyVENT 0.2 μm PTFE filters, L#639) was bubbled into the bottom of each bioreactor through a 1 mm glass capillary tube at a rate of 0.5 L min^{-1}. Carbon supply and pH were managed by $NaHCO_3$ and CO_2 inputs, equally distributed to all bioreactors. pH was maintained between 7.2 and 7.5 (variation in pH among treatments did not exceed 0.15 pH units). pH was measured by a Mettler Toledo SG2 SevenGo pH electrode kit (Mettler Toledo, Columbus, OH). During the lag phase (the first two days of growth) 0.05 mM $NaHCO_3$ was added once daily. For the remainder of the experiment the air flow was enriched in CO_2; the enrichment rate increased over time in response to algal metabolism, up to ~1% CO_2. Light was supplied by banks of fluorescent tubes that ran perpendicular to the bioreactor tubes, completely covering one side. The photosynthetic photon flux (PPF) was 300 μmol m^{-2} s^{-1} (300 μE m^{-2} s^{-1}) with a 16-h photoperiod. A 16-h photoperiod was chosen to approximate the natural photoperiod of summers days in the mid-latitudes. Growth of photosynthetic organisms is best determined by the daily integrated PPF (Bugbee and Monje 1992),

which was 17.3 mol m^{-2} d^{-1} in this study. This is less than 50% of the average daily PPF of 45 to 55 mol m^{-2} d^{-1} in the summer months in North America. For conversion of measurements made by volume (e.g. g L^{-1}) to a unit of area (e.g. g m^{-2}), the illuminated area of the bioreactor tubes containing algae was used as a conversion factor as follows: 1.2 L / πrh = 1.2 L / (3.14 x 2.25 cm x 75.5 cm) x (1 m^2 / 100^2 cm^2) = 22.5 L m^{-2} and 22.5 L m^{-2} x g L^{-1} = g m^{-2}.

4.3.3. Media Composition and Preparation

Three media were prepared and autoclaved, differing in NaCl concentration: 50, 100 and 400 mM. Silicon was added separately to each bioreactor tube at four concentrations: 0.05, 0.1, 0.2 and 0.8 mM Si. Because of the effect of Si on pH, the pH of the bioreactor tubes was adjusted individually to 7.5 by addition of 1 M NaOH or 1 M HCl. The culture media had the following composition: 2.5 mM KNO_3; 0.9 mM $CaCl_2 \cdot 2H_2O$; 7.0 mM $MgSO_4 \cdot 7H_2O$; 6 mM KCl; 50, 100 or 400 mM NaCl; 1.5 mM KH_2PO_4; 0.05, 0.1, 0.2 or 0.8 mM $Na_2SiO_3 \cdot 9H_2O$; 14.3 µM ferric ammonium citrate; 25 µM H_3BO_3; 3.0 µM $MnCl_2 \cdot 4H_2O$; 0.25 µM $CuSO_4 \cdot 5H_2O$; 0.75 µM $ZnSO_4 \cdot 7H_2O$; 0.2 µM $Na_2MoO_4 \cdot 2H_2O$; 0.2 µM $CoCl_2 \cdot 6H_2O$; 1.5 nM vitamin B12; 4.1 nM biotin; and 150 nM thiamine. Differential addition of Na within each NaCl treatment—due to initial pH adjustment (NaOH) and the counter ions of Si ($Na_2SiO_3 \cdot 9H_2O$)—were expected to have a minimal effect. NaOH was added only to the 0.05 and 0.1 mM Si treatments, resulting in up to a 1.6 mM increase in Na. The counter ion Na that came with Si most affected the high Si treatments, up to 1.6 mM.

4.3.4. Algal Density Measurement and Harvest

Daily measurements of culture density were made spectrophotometrically at 750 nm with a Shimadzu UV-2401 PC, UV-VIS recording spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Ash-free dry mass densities were determined by filtering 10 mL suspensions of algae with Whatman GF/C filters. The filters were dried for one to two days at 105˚C, weighed to determine total dry mass density, then combusted at 550˚C for 15 min to determine ash. Cells suspended in media were concentrated for harvest by centrifugation at 7500 rpm for 5 min. Following centrifugation the biomass was loaded into 15 mL plastic sample vials, frozen at -80˚C, and lyophilized. For accurate determination of total dry mass (cellular mass, including ash), attempts were made to rinse the filters free of extracellular salts. This resulted in loss of algae from the filters. Therefore, ash content was determined by combusting 20 mg samples of lyophilized algae.

4.3.5. Solution Silicon Measurements

Samples of algal suspension (3 mL) were collected from each bioreactor tube every-other day and filtered with Whatman GF/C filters. Solution Si was measured on the filtered media with a Lachat QuikChem 8500 Automated Ion Analyzer using the QuikChem method 10-114-27-1-A that is available from the manufacturer (Lachat Instruments, Loveland, CO).

4.3.6. Lipid Extraction, Conversion to FAME and Quantification

Simultaneous conversion and extraction of algal lipids to fatty acid methyl esters (FAME) was done by the method of Wahlen et al. (2011). This method effectively converts to FAME the fatty acids contained in membrane phospholipids and glycolipids, as well as free fatty acids and storage lipid triglyceride. The lipid or FAME content of 100 mg freeze-dried algal samples was determined with a gas chromatograph (Model 2010, Shimadzu Scientific, Columbia, MD) equipped with programmable temperature vaporizer (PTV), split/splitless injector, flame ionization detector (FID) (GCMS-QP2010S, Shimadzu Scientific, Columbia, MD), and autosampler. The FID detector was used in this analysis. Analytes were separated on an RTX-Biodiesel column (15 m, 0.32 mm ID, 0.10 lm film thickness, Restek, Bellefonte, PA) using a temperature program of 60˚C for 1 min followed by a temperature ramp of 10˚C per min to 360˚C for 6 min. Constant velocity of carrier gas helium was set at 50 cm s^{-1} in velocity mode. Sample sizes of 1 μL were injected into the PTV injector in direct mode that followed an identical temperature program to that of the column. The FID detector was set at 380˚C. Each sample contained octacosane (10 μg mL^{-1}) as an internal standard. FID detector response to FAME was calibrated using methyl tetradecanoate (C14:0), methyl palmitoleate (C16:1), and methyl oleate (C18:1) at concentrations ranging from 0.1 mg mL^{-1} to 1 mg mL^{-1} and tripalmitin at concentrations ranging from 0.05 mg mL^{-1} to 0.5 mg mL^{-1}. Standards were obtained as pure compounds (Nu-Chek Prep, Inc., Elysian MN) and diluted with chloroform to obtain needed concentrations. Peaks were integrated using GC

solution postrun v. 2.3 (Shimadzu) and concentrations were determined by linear regression analysis.

4.3.7. Statistical Analysis

Analysis of variance was conducted using the general linear model (Proc GLM) in the SAS system (Statistical Analysis System, Cary, NC). Error bars in all plots represent the standard deviation.

4.4. Results and Discussion

4.4.1. Silicon Uptake, Growth, and Lipid Content

Despite decreased growth rates with both decreasing Si supply and NaCl concentration, the removal of Si was nearly complete in all NaCl/Si treatments (Fig. 4–1). Averaged across all treatments, 12 ± 2.8 μM Si was never recovered from solution. It is unclear whether this Si was available for Si uptake however, because soluble Si was measured by the molybdate assay in acidic conditions which could have solubilized polymeric forms of Si. Analysis of variance showed a strong interaction between NaCl and Si on total and ash-free dry mass densities, but that NaCl was the primary factor determining the outcome of these parameters (Table 4–1 and Fig. 4–2). The interaction arose because low NaCl concentrations were inhibitory to growth, but the inhibition was overcome with excessive Si supply rates. This suggests that low levels of NaCl may have affected the metabolism of Si.

In a synergistic effect, lipid content was positively affected by both decreasing Si and decreasing NaCl (Fig. 4–2). The relationships shown here are not perfectly linear,

however, as a result of the lipid content in the 50 and 100 mM NaCl treatments peaking at 0.2 and 0.1 mM Si, respectively, and decreasing with lower Si supply. We speculate that, due to the severe stress in these conditions, the relationships are not linear because lipids that were produced were consumed or degraded before measurement. Statistical analysis of the data showed that the relative effects of Si and NaCl on promoting storage lipids were similar—each increasing the lipid content by more than 50%—with Si having a slightly larger effect (Table 4–1). With combinations of low Si (0.05, 0.1 and 0.2 mM) and low NaCl (50 and 100 mM), lipid content increased to more than 70% of dry mass.

4.4.2. Lipid productivity

Lipid productivity is derived by multiplying total dry mass density by the lipid content and dividing by time (Fig. 4–2). Sodium chloride—because of its large impact on growth—and a significant NaCl/Si interaction were the dominant factors determining lipid productivity (Table 4–1). Lipid productivity peaked at a high NaCl concentration (400 mM) and a low rate of Si supply (0.05 mM), but was not significantly different than the productivity with intermediate levels of NaCl and Si (100 mM NaCl and 0.1 or 0.2 mM Si). These treatment combinations did differ in lipid content, however, with intermediate levels of NaCl and Si resulting in about 14% higher lipid content. Thus, from a lipid production standpoint, intermediate levels of NaCl and Si stress resulted in a more favorable outcome. The contour plots of Fig. 4–3 illustrate the tradeoffs in total dry mass density and lipid content as a function of NaCl concentration and Si supply.

4.4.3. Fatty Acid Chain Length Distribution

Decreased Si resulted in a 5% shift in lipid chain length from C18 to C16 fatty acids, but there was no effect of NaCl (Fig. 4–4). C14 fatty acids were unchanged. Together, C14, C16 and C18 fatty acids accounted for ~93% of all lipids.

4.4.4. Efficiency of Lipid Production

Radiation is the ultimate limiting factor in all photosynthetic systems and thus it is appropriate to compare productivities based on the input of light (Bugbee and Monje 1992). In this study, peak lipid productivity was about 75 mg L^{-1} d^{-1} over a 7-d growing period and the illuminated surface-to-volume ratio of the bioreactor tubes was 0.044 m^2 L^{-1}. This gives a lipid productivity of 1.7 g m^{-2} d^{-1}. The daily PPF integral was 17.3 $mol_{photons}$ m^{-2} d^{-1}, giving an efficiency of lipid production of 0.10 g mol^{-1} of photons. For comparison, this was about 50% of the best efficiency (0.19 g mol^{-1} of photons) found in green algae by using N deficiency to promote lipid accumulation in a recent publication using the same growth system (Adams et al. 2013).

4.5. Conclusions

Low levels of either NaCl or Si increased lipid content by about 50% each. The synergy of simultaneous, moderate NaCl and Si stress resulted in lipid content up to 73% with a lipid productivity of 1.7 g m^{-2} d^{-1}; with a daily integrated photosynthetic photon flux of 17.3 mol m^{-2} d^{-1}, the efficiency of lipid synthesis was thus 0.10 g $mole^{-1}$ of photons. The observation of a strong NaCl/Si interaction on total and ash-free dry mass densities suggests that low levels of NaCl may have affected the metabolism of Si. The

findings of this study can be used to enhance lipid production in oleaginous marine diatoms.

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Total Dry Mass Density			
Source	DF	F Value	Pr > F
Si	3	23.1	<.0001
NaCl	2	82.5	<.0001
Si*NaCl	6	8.9	0.0008
Error	12		
R^2	0.960		
Ash-Free Dry Mass Density			
Source	DF	F Value	Pr > F
Si	3	20.4	<.0001
NaCl	2	72.2	<.0001
Si*NaCl	6	11.0	0.0003
Error	12		
R^2	0.958		
Lipid Content			
Source	DF	F Value	Pr > F
Si	3	19.4	<.0001
NaCl	2	17	0.0003
Si*NaCl	6	2.1	0.1313
Error	12		
R^2	0.897		
Lipid Productivity			
Source	DF	F Value	Pr > F
Si	3	2.8	0.0834
NaCl	2	28.8	<.0001
Si*NaCl	6	11.9	0.0002
Error	12		
R^2	0.920		

Table 4–1: Analysis of variance in four response parameters (total dry mass density, ash-free dry mass density, lipid content and lipid productivity) for two factors and their interaction (Si, NaCl and Si*NaCl). DF = degrees of freedom. The F values were used in determination of the relative impact of each factor on the response parameters and in assigning statistical significance.

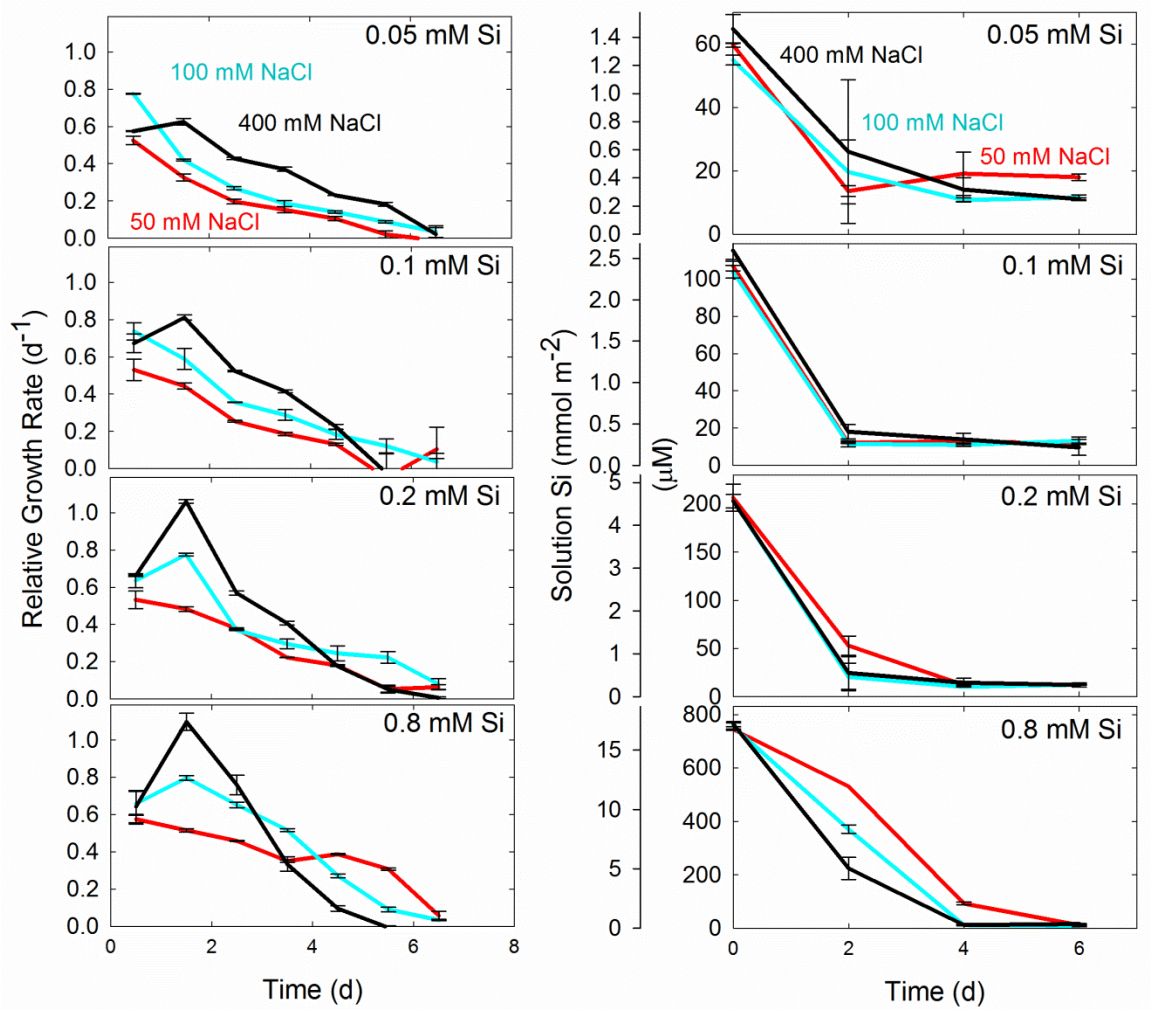

Fig. 4–1: Time series measurements of relative growth rate (on left) and Si in solution (on right) with combinations of four Si treatments (0.05, 0.1, 0.2 and 0.8 mM) and three NaCl treatments (50, 100 and 400 mM). The relative growth rate, determined by optical density at 750 nm, was negatively affected by decreasing NaCl concentration and Si supply rate. The diatom removal of Si was nearly complete in all treatments, with a fairly consistent, small amount of Si never recovered from solution: 12 ± 2.8 µM Si.

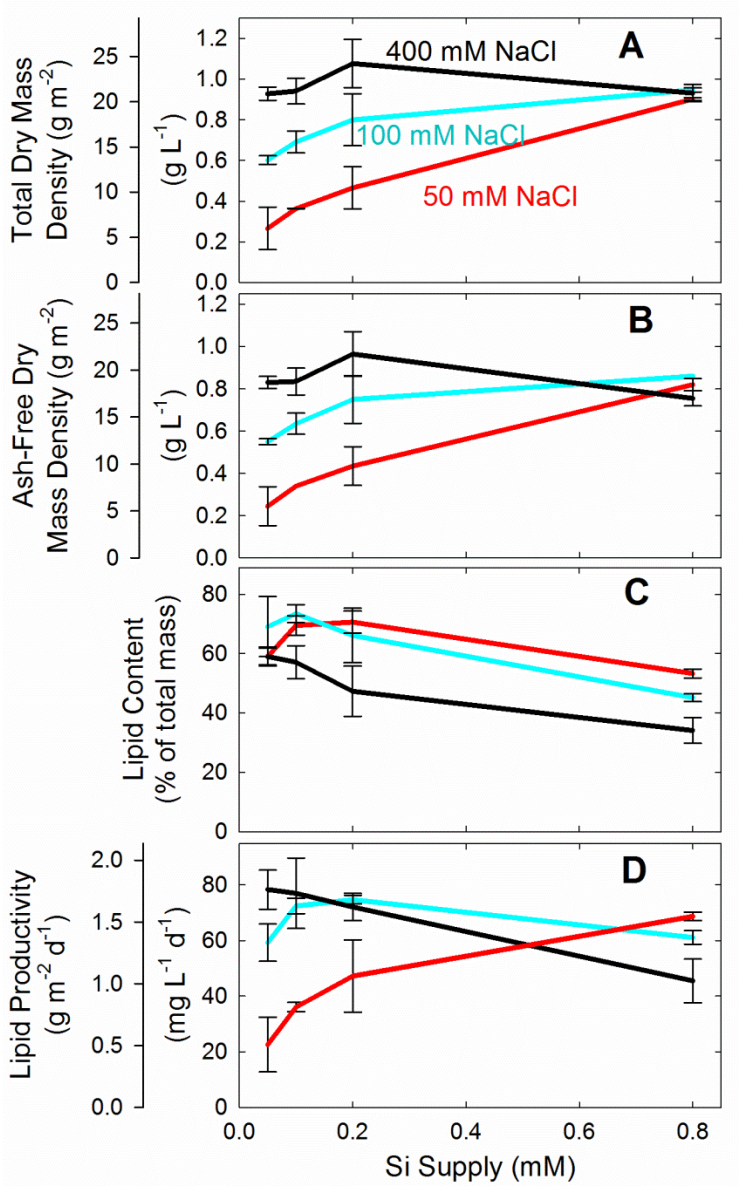

Fig. 4–2: Terminal measurements of total dry mass density, ash-free dry mass density, lipid content, and lipid productivity as a function of Si supply. Sodium chloride was the dominant factor determining total and ash-free dry mass yields (A and B), while NaCl and Si contributed approximately equally to the accumulation of storage lipid (C). Lipid productivity reflected the strong effect of NaCl on growth and a significant NaCl/Si interaction (D).

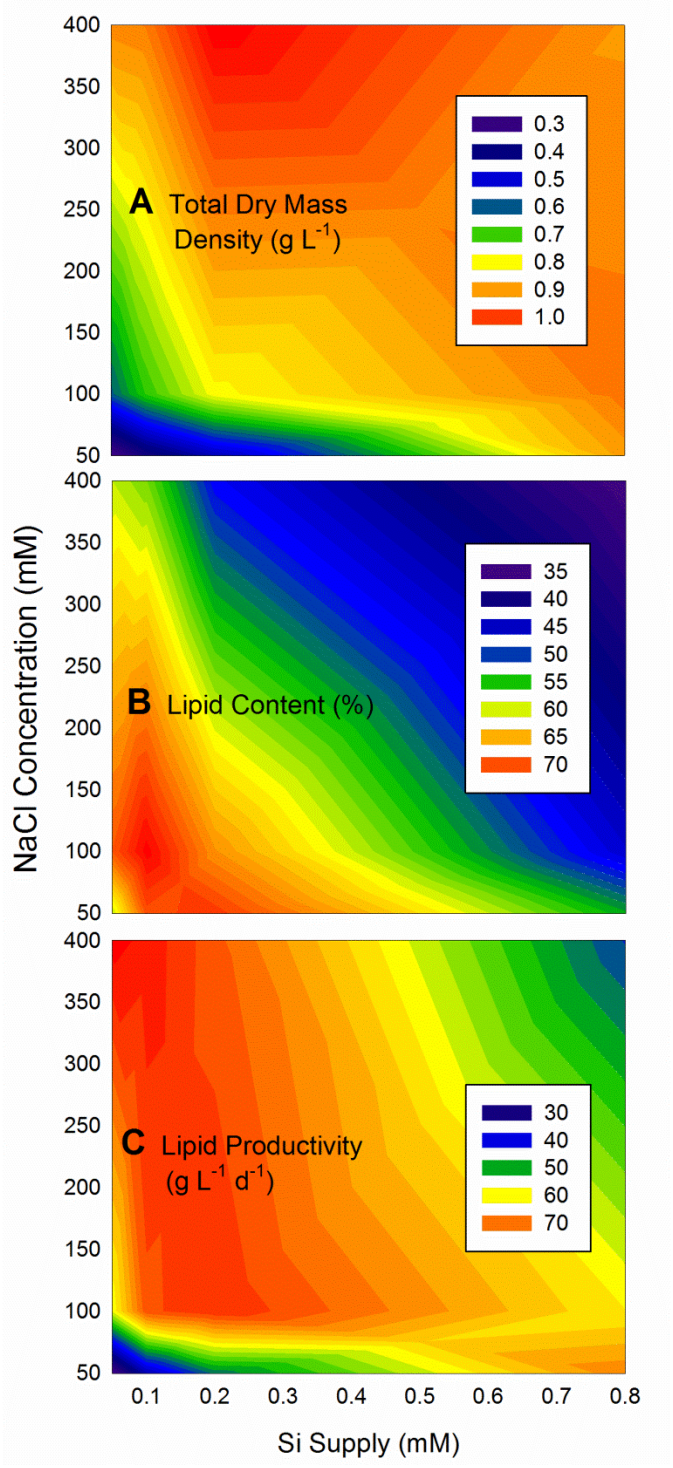

Fig. 4–3: Contour plots showing total dry mass density (A), lipid content (B) and lipid productivity (C) as a function of Si supply and NaCl concentration. The most favorable lipid production outcome—high lipid productivity and high lipid content—was observed with moderate levels of NaCl and Si stress

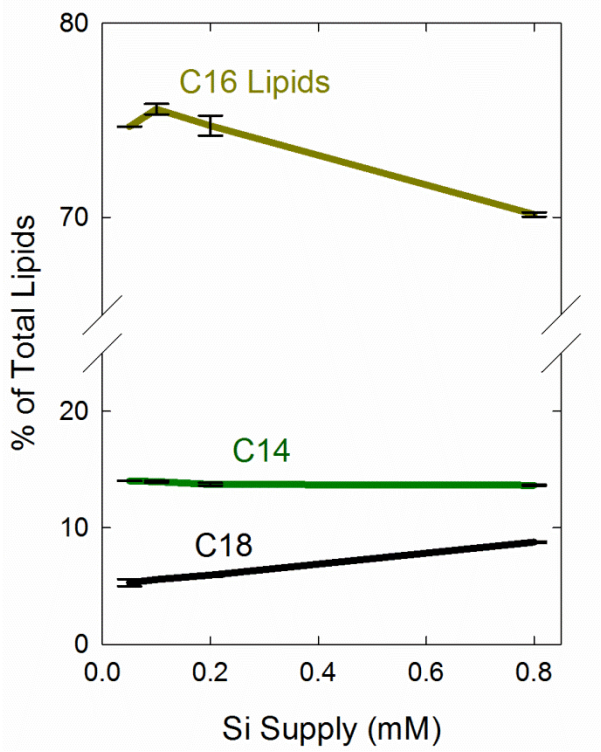

Fig. 4–4: The distribution in lipid chain length shifted with the rate of Si supply. An increase in C16 fatty acids with decreasing Si supply was offset by a similar reduction in C18 fatty acids. C14 fatty acids were unchanged. Sodium chloride had no significant effect on lipid distribution. The 50 mM NaCl treatment is shown here

CHAPTER 5

SUMMARY & CONCLUSIONS

5.1. This Research

Algae are increasingly being recognized as an important source of lipids. Owing to the wide diversity among species in native habitat and physiology, the types of lipids algae produce are many. This diversity in lipid products and their potential for high rates of lipid productivity make algae candidates for production of lipids for many applications. Accumulation of lipids generally occurs in stress conditions, including nutrient deficiency, but research is needed to understand the physiological drivers of the effect and how best to optimize the stress. The aim of the research summarized in this dissertation was to provide scientific insights into these problems.

Three sets of research were reported here: 1) An assessment of species differences in growth and lipid content tradeoffs with high and low level N deficiency; 2) An investigation of physiological drivers of lipid formation, by mass balance accounting of cellular N with progressing deficiency; 3) An examination of the effects of NaCl and Si on lipid production in a marine diatom.

1) Nitrogen deficiency typically had disproportionate effects on growth and lipid content, with profound differences among species. Optimally balancing the tradeoff required a wide range in N supply among species. Some species grew first and then accumulated lipids, while other species grew and accumulated lipids concurrently—a characteristic that increased lipid productivity. High lipid content generally resulted from a response to minimal stress.

2) Commonalities among species in cellular nitrogen at the initiation of lipid accumulation provided insight into the physiological drivers for lipid accumulation in nitrogen deficient algae. Total nitrogen uptake and retention differed widely among species, but the ratio of minimum retained nitrogen to nitrogen at the initiation of lipid accumulation was consistent among species at 0.5 ± 0.04. This suggests that lipid accumulation was signaled by a common magnitude of nitrogen deficiency. Among the cellular pools of nitrogen at the initiation of lipid accumulation, the concentration of RNA and the protein to RNA ratio were most similar among species with averages of 3.2 ± 0.26 g L^{-1} (8.2% variation) and 16 ± 1.5 (9.2% variation), respectively. This implicates critical levels of these parameters as potential signals initiating the accumulation of lipids.

3) In a marine diatom, low levels of either NaCl or Si resulted in at least 50% increases in lipid content. The synergy of simultaneous, moderate NaCl and Si stress resulted in lipid content up to 73%. There was a strong NaCl/Si interaction in total and ash-free dry mass densities that arose because low NaCl was inhibitory to growth, but the inhibition was overcome with excessive Si supply. This suggests that low NaCl may have affected metabolism of Si.

This research provides insight into how nutrient deficiencies promote lipid accumulation in oleaginous microalgae and how best to optimize the stress for production. The physiological reactions to nutrient deficiency that were identified provide an improved future basis for species selection, nutritional optimization, and discovery of the molecular mechanisms of algal lipid accumulation.

5.2. The Current State of Algal Cultivation for Lipids

A collaboration of partners in research, academia, and industry estimated the 2011 cost (a theoretical cost based on the state of research and technology) of extracted algal oil to be between \$10.87 and \$13.32 gallon^{-1} (Sun et al. 2011). These prices—which do not include downstream processing costs, such as conversion to biodiesel—far exceed current market values for most oils and oil products. The current state of algal cultivation for lipids shares many similarities with the former state of higher-plant agricultural production, prior to the agricultural revolution (Pienkos and Darzins 2009). Research and innovation in agriculture have resulted in enhanced plant material, improved management practices, more efficient processing equipment, better infrastructure, and the ability to care for a growing population. Continued work on algae would likely result in analogous advancements.

5.3. Future Research

A recent techno-economic analysis by Davis et al. (2011) helps put perspective on where research may be most critical in advancing lipid production by algae. A "sensitivity analysis" was presented in which the impact on production costs of reasonable changes in production parameters and inputs was assessed. According to their estimates, the parameter or input to which production cost was most sensitive was lipid content, followed by growth rate. The analysis indicated that these parameters were far more critical in determining product cost than inputs such as inorganic nutrients, flocculent for harvest, water, water and nutrient recycling processes, and CO_2 delivery. Davis et al. (2011) estimated that the cost of algal lipids could decrease by nearly \$4

gallon^{-1} if the biomass lipid content increased from 25% to 50%.This assessment validates the importance of the research summarized in this dissertation and indicates that more innovation is needed on this front. There is potential for identifying oleaginous species that are better suited for production systems, with the ability to maintain relatively high photosynthetic rates in stress conditions. Direct measurement of photosynthesis would be a powerful tool in understanding the impact of stress and in identifying superior species. There is room for continued work on nutritional optimization, to further hone lipid content-growth tradeoffs and to improve nutrient use efficiency. Broadening the focus beyond just lipids to also look at high-value co-products—such as protein, antioxidant molecules, and omega-3 fatty acids—will help to make algal cultivation cost effective and a more valued process. Molecular modifications will likely play a key role in enhancing and ensuring reliable algal production in the future. This could include, for example, imparting characteristics for cultural stability (e.g. tolerance to extreme conditions) and lipid accumulation without the need for stress.

5.4. References

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APPENDICES

APPENDIX A

SUPPLEMENTAL STATISTICAL ANALYSIS

FOR CHAPTERS 3 AND 4

A.1. Supplemental statistical analysis for chapter 3

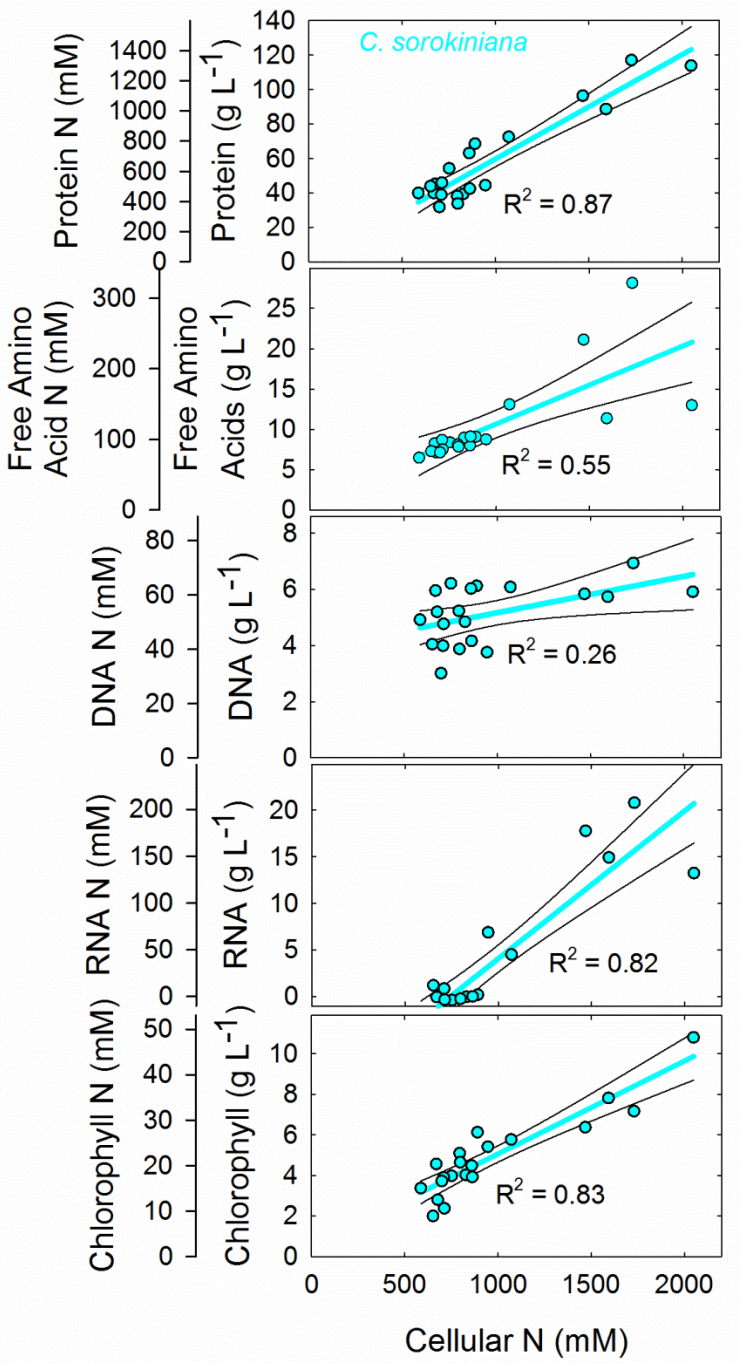

Fig. A–1: Measures of variability—R^2 and 95% confidence intervals—in determination of cellular N pool concentrations as a function of total cell N in batch cultures of the green alga *C. sorokiniana*.

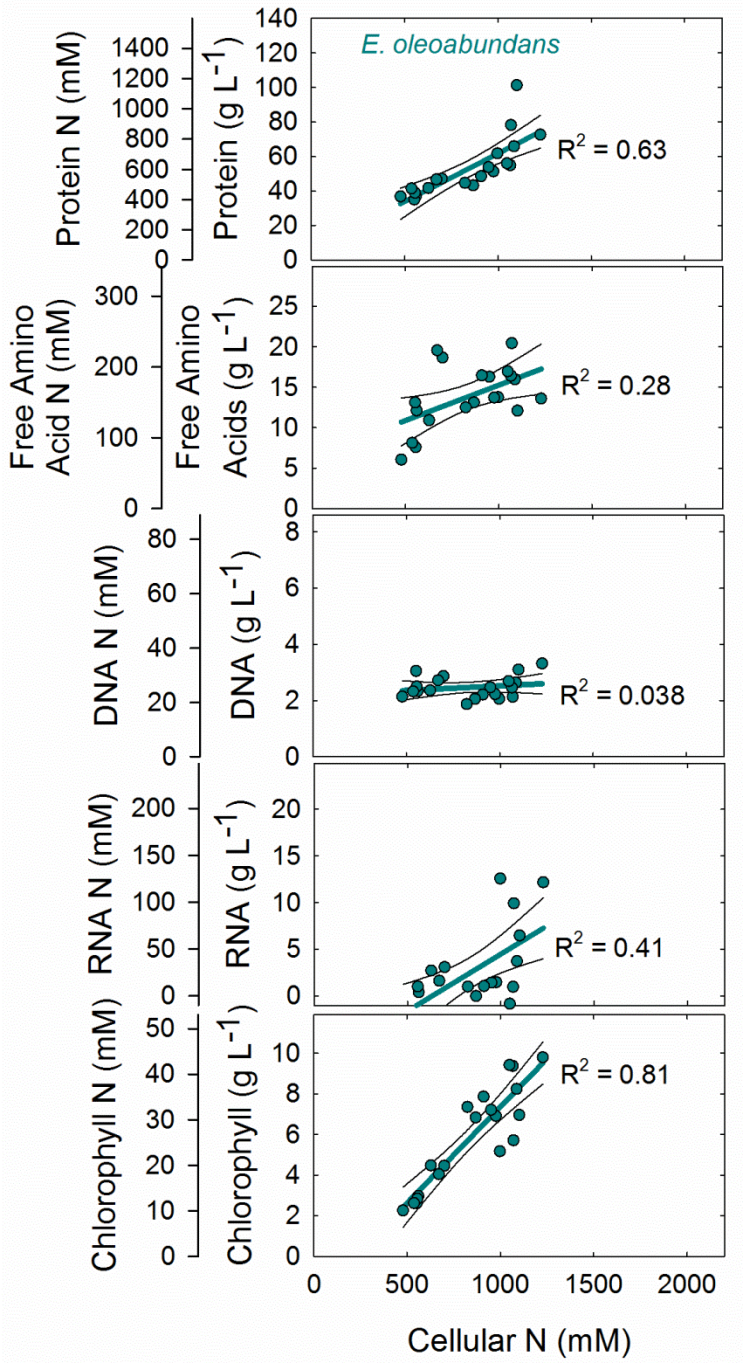

Fig. A–2: Measures of variability—R^2 and 95% confidence intervals—in determination of cellular N pool concentrations as a function of total cell N in batch cultures of the green alga *E. oleoabundans*.

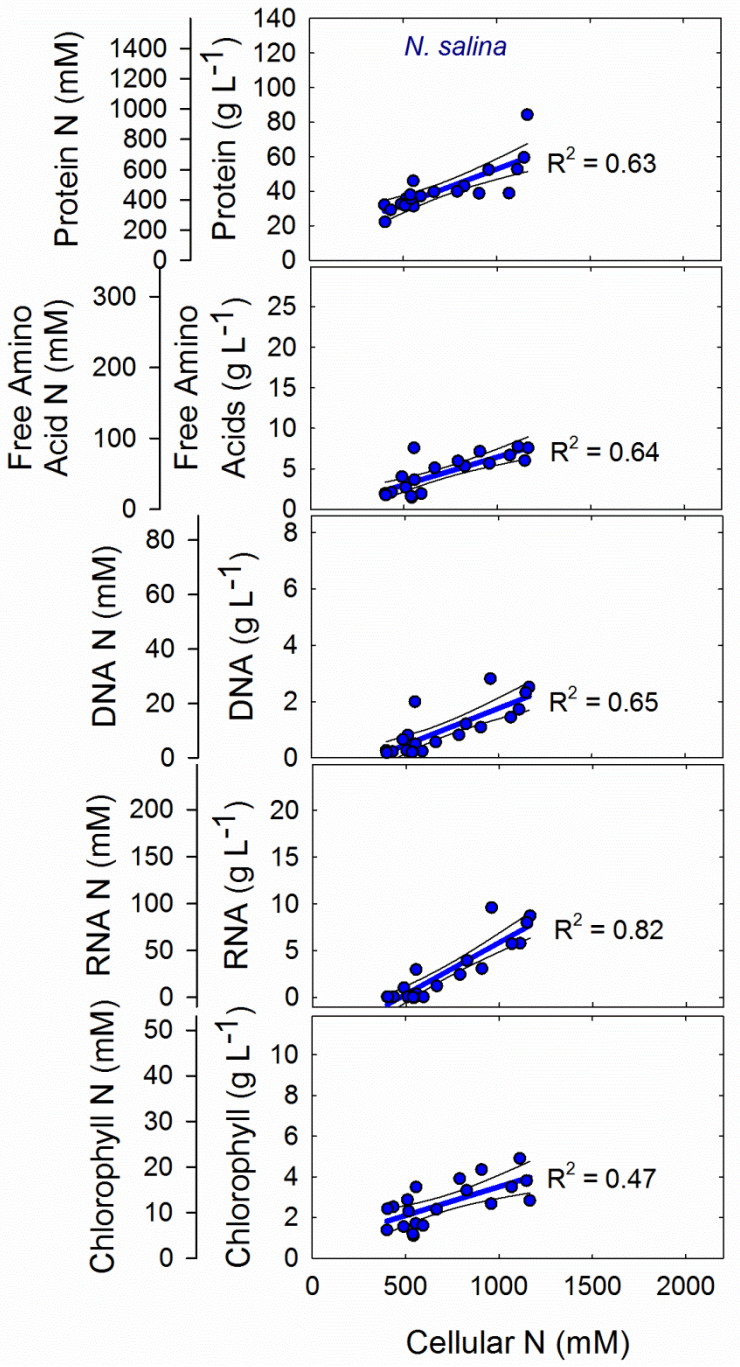

Fig. A–3: Measures of variability—R^2 and 95% confidence intervals—in determination of cellular N pool concentrations as a function of total cell N in batch cultures of the green alga *N. salina*.

A.2. Supplemental statistical analysis for chapter 4

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
Silica	4	0.05 0.1 0.2 0.8
Sodium	3	50 100 400

Number of Observations Read	24
Number of Observations Used	24

The SAS System

The GLM Procedure

Dependent Variable: TotalDryMass

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	1.44699046	0.13154459	26.15	<.0001
Error	12	0.06035950	0.00502996		
Corrected Total	23	1.50734996			

R-Square	Coeff Var	Root MSE	TotalDryMass Mean
0.959957	9.725917	0.070922	0.729208

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Silica	3	0.34830946	0.11610315	23.08	<.0001
Sodium	2	0.82973808	0.41486904	82.48	<.0001
Silica*Sodium	6	0.26894292	0.04482382	8.91	0.0008

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
Silica	4	0.05 0.1 0.2 0.8
Sodium	3	50 100 400

Number of Observations Read	24
Number of Observations Used	24

The SAS System

The GLM Procedure

Dependent Variable: AshFreeMass

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	1.14483333	0.10407576	24.73	<.0001
Error	12	0.05050000	0.00420833		
Corrected Total	23	1.19533333			

R-Square	Coeff Var	Root MSE	AshFreeMass Mean
0.957752	9.706484	0.064872	0.668333

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Silica	3	0.25890000	0.08630000	20.51	<.0001
Sodium	2	0.60785833	0.30392917	72.22	<.0001
Silica*Sodium	6	0.27807500	0.04634583	11.01	0.0003

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
Silica	4	0.05 0.1 0.2 0.8
Sodium	3	50 100 400

Number of Observations Read	24
Number of Observations Used	24

The SAS System

The GLM Procedure

Dependent Variable: LipidContent

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	3182.548737	289.322612	9.51	0.0002
Error	12	365.213451	30.434454		
Corrected Total	23	3547.762189			

R-Square	Coeff Var	Root MSE	FAMECont Mean
0.897058	9.406434	5.516743	58.64861

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Silica	3	1769.729205	589.909735	19.38	<.0001
Sodium	2	1032.122696	516.061348	16.96	0.0003
Silica*Sodium	6	380.696837	63.449473	2.08	0.1313

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
Silica	4	0.05 0.1 0.2 0.8
Sodium	3	50 100 400

Number of Observations Read	24
Number of Observations Used	24

The SAS System

The GLM Procedure

Dependent Variable: LipidProductivity

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	6894.205557	626.745960	12.48	<.0001
Error	12	602.733359	50.227780		
Corrected Total	23	7496.938916			

R-Square	Coeff Var	Root MSE	FAMEProd Mean
0.919603	12.10392	7.087156	58.55256

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Silica	3	426.218217	142.072739	2.83	0.0834
Sodium	2	2887.972161	1443.986081	28.75	<.0001
Silica*Sodium	6	3580.015179	596.669196	11.88	0.0002

APPENDIX B

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Signed Valerie Godfrey

May 17, 2013

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Dear Dr. Wahlen:

I am in the process of preparing my dissertation in the Department of Plants, Soils, and Climate at Utah State University. I hope to complete in the summer of2013.

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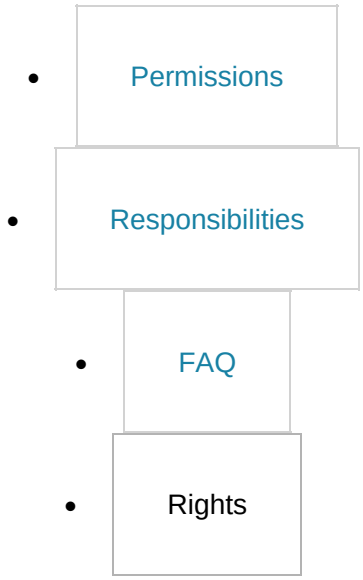

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Relevant Work Experience

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USU Turfgrass Studies, Logan, Utah

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AS in Graphic Art, August 2004, GPA 3.87
Utah Valley University, Orem, Utah

Refereed Publications (including those published, accepted, and in review)

Adams, C., Frantz, J., Bugbee, B. (2013) Macro- and micronutrient-release characteristics of three polymer-coated fertilizers: Theory and measurements. Journal of Plant Nutrition and Soil Science 176:76-88.

Adams, C., Godfrey, V., Wahlen, B., Seefeldt, L., Bugbee, B. (2013) Understanding precision nitrogen stress to optimize the growth and lipid content tradeoff in oleaginous green microalgae. Bioresource Technology 131:188-194.

Adams, C., Jacobson, A. Bugbee, B. (2013) Ceramic aggregate sorption and desorption chemistry: Implications for use as a component of soilless media. Journal of Plant Nutrition, *Accepted Manuscript*.

Adams, C., Bugbee, B. (2013) Potential signals for initiation of microalgal lipid accumulation are implicated by commonalities among species in cellular nitrogen. Journal of Phycology, *In Review*.

Adams, C., Bugbee, B. (2013) Enhancing lipid production of the marine diatom *Chaetoceros gracilis*: Synergistic interactions of sodium chloride and silicon. Journal of Applied Phycology, *In Review*.

Research Experience Descriptions

- Proposed an improved conceptual model of polymer-coated fertilizer (PCF) release mechanisms, better established the effect of substrate water content and temperature on release rates, and provided a comprehensive review of literature

- Investigated the sorption/desorption characteristics of a common aggregated clay substrate and interactions with plant nutrient uptake; toxic accumulation of Mn was observed in some plant species

- Investigated nitrogen status and partitioning in algae at the onset of lipid accumulation; identified commonalities among species that implicate critical low levels of protein and RNA as potential physiological signals for lipid accumulation

- Tested the utility of 1-MCP (1-methylcyclopropene) to block yield-limiting physiological responses to ethylene in water-stressed agronomic crop plants in containers; using a system of lysimeters and environmental instrumentation, stomatal conductance was derived from measurements of transpiration and vapor pressure deficit (VPD) and compared among treatments

- Assessed the environmental risk of transfer of the Roundup resistance transgene from turfgrass to wild grass species, including tests on pollen viability and genetic tests to verify transgene movement

- Identified species differences among oleaginous algae in the tradeoff in growth and lipid accumulation with nitrogen deprivation; described physiological characteristics that make some algae better lipid producers than others in this stressed condition

- Better established a connection between solution levels of sodium chloride and the metabolism of silica in a marine diatom; identified synergism between reducing supply of the two elements and lipid production

Professional Service

Peer reviewer for "Central European Journal of Chemistry"
Peer reviewer for "Biosystems Engineering"

Professional Presentations

Adams, C., Godfrey, V., Wahlen, B., Seefeldt, L., Bugbee, B. 2012. Understanding precision nitrogen stress to optimize the growth and lipid content tradeoff in green algae. Algae Biomass Summit, Denver, CO. Competitively selected speaker.

Adams, C., Bugbee, B. 2011. Nutrient management in steady-state algal cultures for biodiesel production. Intermountain Graduate Research Symposium, Logan, UT. Poster presentation.

Adams, C., Bugbee, B. 2010. The physical and nutritional environment of LADA. Space Dynamics Laboratory Conference on Plant Growth on the International Space Station, Logan, UT. Research report.

Adams, C., Franzt, J., Bugbee, B. 2010. Polymer-coated fertilizer application rate modeling based on phosphorus. NCERA-101 Conference on Controlled Environment Plant Studies, Park City, UT. Poster presentation

Adams, C., Bugbee, B. 2010. Effects of substrate and temperature on the nutrient release of three polymer-coated fertilizers. Intermountain Graduate Research Symposium, Logan, UT. Poster presentation.

Adams, C., Bugbee, B. 2009. Characterizing controlled-release fertilizers: Modeling for efficient use. NCERA-101 Conference on Controlled Environment Plant Studies, Madison, WI. Poster presentation.

Funded Grants

Understanding precision nitrogen stress to optimize the growth and lipid content tradeoff in green algae. Curtis Adams. 2012. Office of Research and Graduate Studies Graduate Student Travel Grant. $300 awarded.

Improving human nutrition: Domestic production of healthy oils by microalgae. Curtis Adams, Lance Seefeldt, Bruce Bugbee. 2011-2012. Utah Agricultural Experiment Station. $20,000 awarded.

Characterizing controlled-release fertilizers. Curtis Adams. 2009. Office of Research and Graduate Studies Graduate Student Travel Grant. $300 awarded.

Teaching Experience

Teaching assistant, Plant Nutrition (PSC 6430), USU Fall Semester 2010
Teaching assistant, Crop Physiology (PSC 5200), USU Spring Semester 2009

Fellowships & Scholarships

Apogee Instruments – Campbell Scientific Graduate Fellowship, 2013
USU Presidential Graduate Fellowship, 2008
USU Presidential Transfer Scholarship, 2004-2006
Rodney C. & Hilda Allred Memorial Scholarship, 2006
Rodney C. & Hilda Allred Memorial Scholarship, 2005
Utah Agricultural Leadership Scholarship, 2006
UVU Sterling Scholar Scholarship, 2000

Community and Educational Service Activities

Marshallese Translator, Logan School District, Logan, UT, 2012-2013
USU College of Agriculture Ambassador, 2006-2007