DISSERTATION

NITROGEN UTILIZATION IN HETEROTROPHIC *CHLAMYDOMONAS REINHARDTII*

Submitted by

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ABSTRACT

NITROGEN UTILIZATION IN HETEROTROPHIC *CHLAMYDOMONAS REINHARDTII*

The aim of this dissertation research is to bring better understanding to the process of nitrogen adaptation in heterotrophic *Chlamydomonas reinhardtii.* Microalgae are a diverse group of aquatic photosynthetic organisms that account for almost 50% of the photosynthetic productivity on Earth. There is immense interest in using the unique ability of microalgae to convert sunlight to triacylglycerides (TAGs) for industrial purposes. However, to date there has been little success in implementing these systems at scale and price parity with non-biological methods.

Microalgae can modify their metabolism to adapt to the surrounding environment. Under certain circumstances, including nutrient stress, microalgae divert carbon flow away from biomass production and into TAG accumulation. The most common nutrient stress used to trigger TAG accumulation is nitrogen stress, most often induced by transferring a cell from a nitrogen replete medium to a deficient one. The goals of this research were to understand this process, develop methods to manipulate the stress response, and ultimately, to find a way to decouple lipid production from nutrient depletion entirely.

Chapter 1 introduces the concepts and research referenced throughout the dissertation including: a background of the *C. reinhardtii* species, the cultivation techniques that have been applied to cultivation, the physiology behind nitrogen stress,

the mechanism that algae use to incorporate nitrogen into the cell, and finally an introduction to the global nitrogen regulator, PII. Chapters 2 through 4 present research into the nitrogen stress pathway and its modification. Chapter 2 discusses a simple method of cultivation used to bring about new insights into the nitrogen stress response, as well as a proposed technique for increasing cellular lipid production. Through differential nitrogen feeding, significantly different effects on cell growth were observed, demonstrating that the response to nitrogen availability is a continuous effect as opposed to an all or nothing “stress response”. Chapter 3 describes experiments in which *C. reinhardtii* was genetically modified to increase understanding of the nitrogen stress response. A nitrogen regulatory protein, PII, was downregulated via amiRNA. Cultures of a mutant strain with lower levels of PII exhibited slow adaptation to fresh nutrient-replete medium but achieved a higher final cell number, final mass concentration, and total neutral lipid content. Similar results were obtained in cultures shifted to nitrogen-free medium. Chapter 4 employs proteomics to identify differences in the specific protein expression pattern between a functional PII strain and a knock-down mutant. Chapter 5 demonstrates a unique approach to producing an engineered nutrient-limited environment in a continuous stirred tank bioreactor. Chapters 7 and 8 summarize the research findings and offer possible direction for future research.

Through this research work, new information was obtained on the effects of PII on the cellular response to nitrogen limitation. By increasing our understanding of this basic mechanism, we have proposed several processing conditions that may be implemented to increase microalgal productivity. Furthermore, the homology between microalgae and terrestrial plants suggests the possibility that the results discussed

within could give genetic engineers new targets for creating crops with decreased nitrogen demands and increased nitrogen-stress tolerance traits.

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Justin Sweeley

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DEDICATION

To my Snow Bunny, Poke, and Hen

I love you

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BACKGROUND

Microalgae

Microalgae are a diverse group of aquatic photosynthetic organisms that account for almost 50% of the photosynthetic productivity on Earth. The study of microalgae has enabled the discovery of many of the basic principles of eukaryotic life ranging from photosynthesis to the Calvin cycle. Algae represent many different types of organisms including cyanobacteria, green algae, diatoms, red algae, brown algae, coccolithophorids and dinoflagellates (Moroney, 2009). Microalgae are unique in that they inhabit an evolutionary place in between plants and animals. In *Chlamydomonas reinhardtii, d*etailed evolutionary genomics have revealed that green algae including *Chlamydomonas* and *Ostreococcus* diverged from the Streptophytes (land plants) over a billion years ago. And prior to the divergence from plants, *C. reinhardtii* diverged from opisthokonts (animals, fungi, and Choanozoa) (Merchant, Prochnik, & Vallon, 2007). Despite one billion years of evolutionary separation, the photosynthetic apparatus remains functionally the same between green algae and plants. For this reason, green algae became the foundation for what we now know about photosynthesis.

Among the many species of green algae to choose from, *Chlamydomonas reinhardtii* has become particularly useful because they can grow strictly on organic carbon, and because their photosynthetic apparatus is functional despite growing in complete darkness (Moroney, 2009). This means that phototrophic mutants can be studied even in the mutation completely removes photosynthetic capacity. These initial studies laid the foundation for the current status in microalgal biology, with *C. reinhardtii*

remaining the model organism for algal scientific studies (Harris, 2001). The past 50 years of study on *C. reinhardtii* had resulted in the creation of many mutant libraries that can be easily accessed (www.chlamycollection.org). There is a suite of tools developed to manipulate the cell in terms of reproductive cycle (Sager & Granick, 1954), genetic transformation (Bateman & Purton, 2000; Kindle, 1990; Kindle & Sodeinde, 1994; Sodeinde & Kindle, 1993), antibiotic selection (Bateman & Purton, 2000; Berthold et al. 2002), and gene knock-down (Burgesset al. 2012; Molnar et al., 2009). The development of these tools enabled great insight into the biology of microalgae, which also formed the basis to better understand photosynthetic plants. *C. reinhardtii* has thus proven to be a useful homolog for plant biology. More recently, microalgae have become important for the direct photosynthetic production of biofuels.

The characteristics that make *C. reinhardtii* desirable as a model organism do not translate to being a model microalgal biofuel strain. This issue has created a split between the genetic engineers who would prefer to use the strain with the greatest number of genetic tools (Radakovitset al. 2010), and the bioprospectors who prefer to search for a naturally occurring strain with the most desirable characteristics (Gouveia & Oliveira, 2009; Griffiths & Harrison, 2009) The most likely consensus is that the genetic engineering tools will be perfected in a model organism such as *C. reinhardtii* and will eventually migrate over to the other more desirable species identified by the bioprospectors. Indeed, this can be observed by the growing number of tools accessible to species beyond *C. reinhardtii* (Radakovits et al., 2012; Wu et al., 2012; Zaslavskaia & Lippmeier, 2001). To date, the bulk of biofuel achievements have been attained through the bioprospecting approach.

There is little doubt about the theoretical potential for microalgal biofuels to replace a significant portion of the United States fossil fuel demands (Carriquiry et al., 2011; Chisti, 2008; Dismukes, et al. 2008; Wijffels & Barbosa, 2010). However, with current technology, the life cycle assessment for microalgal production of biofuels shows that this route is less sustainable than biofuel production from lignocellulosic biomass (an exception is presented by (Clarens et al. 2010)). Economic sustainability for microalgal biofuels has also not yet been achieved. Despite the number of review papers demonstrating the theoretical potential of microalgal biofuels, the technology to date has fallen short of predictions, and there are no successful commercial-scale examples of microalgal biofuel production. Companies are now focusing on production of microalgal bioproducts to improve the process economics.

Microalgal bioproducts have significant potential because they are a medium to high-value product that can justify the expensive culturing conditions required for microalgal growth (Becker, 2007). Unlike biofuels, bioproducts have a long track record of successful commercial production (León-Bañares et al. 2004). Biomass from the microalgae *Dunaliella, Haematococcus,* and *Chlorela* have all been sold commercially. In addition, astaxanthin and β-carotene have been extracted from microalgae and sold by several companies (Olaizola, 2003). The success of these companies has created a growing market for these products as in many cases there are no other means to produce the molecules naturally found in microalgae. The estimated market for these products ranges from $1.2 B USD for health foods, to $5 M for isotopic products (Pulz & Gross, 2004). Despite these successes, there is still room for growth and improvement in the strains and processing as this remains a nascent field of research.

In addition to natural products, there is growing interest in using the genetic engineering tools to create heterologous proteins in microalgae for therapeutic protein production. It has been demonstrated that *C. reinhardtii* is capable of producing human proteins in the chloroplast, as well as various industrial enzymes and coproducts (Larkum et al. 2012; Rasala et al., 2012; Rasala et al., 2010). The range of potential products is large; however, the production of any algal product comes down to the same limitation. The single most challenging aspect of microalgal cultivation is the choice of reactor vessel and operational conditions.

Microalgal Cultivation

The large diversity of microalgae allows for a large diversity of growing techniques depending on the desired product and outcome. All algae can grow as phototrophs; however, some can grow as mixotrophs or heterotrophs. *C. reinhardtii* can grow as a heterotroph while maintaining a functional photosynthetic apparatus (Harris, 2001; Moroney, 2009). In addition to the growth conditions, one can vary the reactor as well to include both open and closed systems in a photobioreactor, and batch, fed-batch, multi-stage, or continuous operation. Each system has been designed to reach a specific end goal, and therefore the system changes for every project. From a cost basis, the open raceway pond is by far the most advantageous for commercial production. This point is emphasized by Stephenson et al., who demonstrated that not only are open raceways the best system, but a closed photobioreactor system is actually more costly than fossil fuels from an energy basis (Stephenson et al., 2010). Of the current commercial-scale implementations, the only processes which can be undergone economically are large open pond systems, open raceway ponds, and

closed heterotrophic systems (Eriksen, 2008). Despite the economic disadvantages of a closed system, the closed system remains the standard apparatus for scientific research into algal biology at the laboratory scale.

Heterotrophic Growth

Microalgae are unique in their ability to photosynthesize as a single-celled organism resulting in photosynthetic efficiencies that outcompete terrestrial land crops (Dismukes et al., 2008). However, the defining characteristic that leads to our understanding of photosynthesis is also a very interesting cultivation technique. Heterotrophic growth of microalgae has received a large amount of research for its many characteristics that outperform photosynthesis. As a general rule, heterotrophic cultivation is far cheaper, simpler to construct facilities, and easier than autotrophic cultivation to maintain on a large scale (Perez-Garcia et al. 2011). Additionally, by removing light-shading complications, high-cell density cultures can be maintained to dramatically increase productivity (Wang et al.2013). Heterotrophic culturing has been used to produce increased lipid and carotenoids (lutein) in *Chlorella* (Shi et al., 2002; Wang et al., 2013), or has been used in waste-water treatment applications to convert excess nitrogen and phosphorous streams into algal biomass (Di Termini et al.2011).

Mixotrophic growth is another area of study where microalgae can be cultivated in the presence of both organic carbon and light. The specific cultivation conditions have a large effect on cellular composition, and can thus be used to fine-tune outcomes such as specific fatty acid profiles in *C. reinhardtii* (El-Sheekh, 1993) or carbon use efficiency (Yanget al. 2000). The final goal will dictate the process conditions and there is beginning to be standardized best practices for the implementation of these regimes

(Bumbak et al. 2011). In the same way that medium conditions are dictated by desired product outcome, the reactor setup can be tailored to match the end-goal of the project.

Reactor Setup

There are four primary modes of cultivation: batch, fed-batch, multi-stage, and continuous. These modes are listed in order of increasing complexity, and there are benefits to each. Batch cultivations are simple allowing for large-scale screening analysis as a preliminary stage for experimentation (Gouveia & Oliveira, 2009; Griffiths & Harrison, 2009). Fed-batch cultivation can often allow for increased productivity in terms of biomass (Soletto et al., 2008), cellular components (De Swaaf et al.2003; Ren et al., 2009; Zou & Zhang, 2000), or as demonstrated in other organisms heterologous protein expression (Kazemi Seresht et al., 2011; Kim et al. 2012; Liu et al., 2013). Fed-batch systems are often used to alleviate nutrient toxicity through the controlled addition of a component which is necessary for growth but inhibitory at high concentrations. This is observed in *Arthrospira platensis* with the addition of urea to create significantly denser cultures than are possible in batch culturing (Morocho Jácome et al. , 2012). Similarly, *C. reinhardtii* cultures could be grown to 1.9-fold higher concentrations than obtained in batch culture (Zhang, et al., 1999). This same principle was applied to *Crythecodinium cohnii* with glucose added to fed-batch cultures to generate cell densities of 109 g/L including 61 g/L lipid content, 19 g/L of which were the highly valuable docosahexaenoic acid (DHA) variety (De Swaaf et al., 2003). Fed-batch systems are particularly useful when nutrient supplementation is required to increase productivity, however it cannot create completely different culture conditions depending on the growth status of the culture.

Multi-stage cultivation (most commonly two-stage) is beneficial when the cultures are expected to undergo two complete different phases of growth. In microalgae, this commonly refers to a biomass cultivation stage, followed by a bioproduct production stage (Koller et al., 2012). The transition between phases can be induced by altering exterior conditions (increased light flux, temperature change) (Kepekçi & Saygideger, 2011), or is commonly induced through the labor intensive process of nutrient depletion (Csavina et al., 2011). The process of nutrient depletion has been shown to increase lipid content by more than two-fold and will be covered extensively later in this review. The two-stage method has emerged as the procedure of choice to induce physiological change in microalgae.

The final method of microalgal cultivation is the continuous stirred tank reactor (CSTR, or chemostat). This system operates with a continuous fresh medium feed into the system and an equivalent effluent removal feed to maintain culture volume. In these systems, the dilution rate equals the growth rate at steady state, and thus the growth rate can be easily controlled (Droop, 1982). This process is significantly more complex in setup and operation but has advantages not afforded to the other cultivation systems.

Cultivating microalgae in a continuous system allows for precise control over nutrient levels, and cell growth rate. In this way growth and nutrient limitation can be tightly controlled in a steady-state environment (Takagi et al., 2000). CSTR studies have been carried out with *Dunalliella, Chlamydomonas, Chlorella,* and others (Csavina et al., 2011; Cunningham & Maas, 1978; Tang et al., 2012). The response of microalgal cultures under sustained nutrient stress is unique from the response seen in other organisms.

The microalgal cellular response to growth limitations in a CSTR is to enter into a stressed state often including an increase in lipid production (Kliphuis et al., 2012). This result is contrary to the findings of Meeuwse et al. with *Umbelopsis isabellina,*who found that the specific lipid productivity rate is independent of dilution rate and linked only to the culture density (Meeuwse et al., 2011). This is not to say that fungi are not affected by changes in growth rate, as there is a dramatic restructuring of *Saccharomyces cerevisiae* ribosomal content (Regenberg et al., 2006). Conversely, the response to microalgal growth and/or nutrient limitation is the opposite of the observations of Meeuwse et al. *Chlorella* and *Dunalliella* both exhibited an increase in specific stress levels when grown under growth limiting conditions.

In *Nannochloropsis,* cultivation in a CSTR under limited potassium yielded cells with significantly higher lipid content without any decrease in culture cell density (Takagi et al., 2000). In fact, a near universal response to decrease in cell growth is an increase in the cellular stress response, thereby making CSTR very useful for the investigation of nutrient-stress in microalgae (Álvarez & Acevedo, 2012; Dürrschmid et al., 2008; Saoudi-helis et al., 1994). The controlled stress response which can be elicited from cultivation in a CSTR makes it a powerful tool that can be used to study how microalgae adapt to nutrient-stress by removing the noise of transient batch cultures and replacing them with steady-state cultivation.

Stress Response

The four stages of cellular stress response

The nutrient stress response of microorganisms follows a characteristic pattern including four major processes: acquisition, mobilization, sparing, and recycling

(Merchant & Helmann, 2012). The content in this dissertation is entirely based around the phenomena which surround nitrogen limitation, and as such the review will focus on this area. However, the pattern and effective steps within the cell can be observed during many diverse forms of cellular stress that can occur in nature and the lab alike.

Acquisition involves the up-regulation of membrane transporter proteins with a higher binding efficiency (high-affinity uptake system, HATS), often through the input of ATP to facilitate the reaction, as opposed to the constitutively expressed passive channels (low-affinity uptake system, LATS) which dominate during times of nutrient excess. There is a direct relationship between the concentration of intracellular glutamine and the transcript levels for nitrogen-scavenging proteins (Merchant & Helmann, 2012). The pathway is so important that there are often multiple proteins with identical function, creating a redundancy to guarantee maximum efficiency in nutrient uptake (Shimizu, 2013). The changes during this process occur through transcriptional regulation of the nitrogen uptake proteins. This reaction is not as rapid as allosteric modifications that occur upon nitrogen stress; however, they are the first observable modifications in protein transcription (Matin & Auger, 1989). Matin et al. further deduced that starvation proteins are controlled at the transcriptional level through the observation that synthesis of starvation proteins is sensitive to rifampin (an RNA synthesis inhibitor).

In *Chlamydomonas*, the nitrogen-acquisition mechanism functions in the same inducible manner, with the added complexity that *C. reinhardtii* are able to incorporate both nitrate and nitrite (through the Nit pathway) as well as ammonium (through the Amt pathway) (Fernandez & Galvan, 2008). To further increase the uptake efficiency, *C. reinhardtii* has a dual regulation in which the presence of nitrate represses the

ammonium transporters and the inverse in the presence of ammonium (Rexach et al., 2002). Nitrogen uptake is a large focus of the research contained in this dissertation, and as such will be covered in detail later in the next section of this chapter.

The mobilization step includes the usage of internal nitrogen stores to supply the cell with nitrogen for critical cell activities until the nitrogen supply is returned. This strategy is observed the kelp *Laminaria longicruris* where seasonal variation of inorganic N concentration is ameliorated through high uptake during the N-replete winter months, to support rapid growth during the N-deficient summer months (Rosenberg et al., 1984). Osmotic imbalance dictates that nitrogen in its reduced form of NH_4^+ cannot increase to satisfactory levels, and therefore N must be stored in the form on nitrogen-rich amino acids. This commonly takes the form of glutamate (1 N), glutamine (2 N's), or asparagine (2 N's). It is hypothesized that the excess supply of these amino acids are transported to the vacuoles for storage; however, this has yet to be directly quantified. The common nitrogen sinks are well characterized in the cell and known to include protein, chlorophyll, and inorganic N (Adams & Bugbee, 2014). While the exact mechanism of storage, localization, and rates are not known, there has been extensive research into the rate of uptake during pulsed additions of nitrogen compared to continuous uptake during replete conditions (Naldi & Viaroli, 2002; Martínez & Rico, 2002; Smit, 2002; Teichberg et al. 2006). These experiments demonstrate the nitrogen pools are being conserved in nitrogen in order to mobilize them in nutrient-deficient conditions.

The nitrogen-sparing mechanism is another method of conservation implemented in the cells. There is a strong inverse correlation between the relative abundance of an

element in proteins that respond to nutrient limitation. For example, there is a characteristic shortage of sulfur atoms found in proteins that are responsible for the adaptation to sulfur-deficient conditions (Matin & Auger, 1989). It is readily understood that this would be an evolutionary advantage; however, the story is more complex for nitrogen starvation because nitrogen plays such a central role in all facets of cellular metabolism. This is precisely the reason that nitrogen elicits the strongest stress response. In addition to amino acid conservation, cellular metabolism will slow down in the form of protein turnover to decrease excess use of ATP for formulation of new proteins (Merchant & Helmann, 2012).

The final response to nutrient limitation is recycling, which involves the scavenging of “unnecessary” cellular components to recover necessary nutrients and repurpose them for survival. In the case of nitrogen, this results in the initiation of the selective autophagy known as ribophagy (Cebollero et al., 2012). As proteins are cellular component containing the most elemental nitrogen in the cell, they are a good place to begin the scavenging process. By evolving to selectively degrade ribosomes, the cell is able to scavenge large amounts of nitrogen without degradation of critical proteins for survival (Irr, 1972). In *E. coli,* nitrogen starvation was found to cause a rapid cessation of new protein synthesis with the exception of 20 identified putative starvation proteins that were upregulated upon starvation (Groat & Schultz, 1986). After the cessation of synthesis, protein degradation follows. The degradation rate of total *E. coli* cell protein (autophagy) increased six fold (from 1% to 6% total protein per hour) as growth of the culture ceased under nitrogen stress as well as other stresses (carbon, amino acids) (Matin & Auger, 1989). This process is so critical that in *S. cerevisiae* knock-out of a

ubiquitin gene necessary for ribophagy resulted in complete cell death upon nitrogen starvation (Kraft et al., 2008). In *Phaeodactylum tricornutum,* cellular nitrogen exhaustion lead to decreases in specific protein levels resulting from ribophagy as well as characteristic decrease in chlorophyll content (Mus et al., 2013).

The second observable phenomena of nutrient recycling in photosynthetic organisms is the dramatic reduction in the photosynthetic apparatus. Chlorosis is observed after 24 h of nitrogen depletion in *C. reinhardtii* as chlorophyll contains the largest fraction of nitrogen in the chloroplast (Chapin et al., 1987). This reduction in chlorophyll occurs in concert with the specific reduction in photosynthesis proteins enabling a complete metabolic shift from cell division and energy capture towards long-term nutrient-deficient cell survival (Blaby et al., 2013).

The process of adaptation to nutrient stress is both efficient and complex, involving many different regulatory levels and modifications to the cellular composition. The resulting effect is that a single-celled organism is able to adapt to ever changing environmental conditions rapidly and without the help of cellular differentiation that is afforded larger organisms. Studying this response can yield insight into the specific biology behind this adaptation as well as give insights into the potential areas for improvement in a controlled commercial setting.

Nitrogen stress in microalgae

The study of nitrogen stress in microalgae has received considerable attention in recent years because of their unique ability to accumulate large quantities of lipids in response to nitrogen stress. Lipids are valuable commodities with potential to be used in many diverse bioenergy and health related applications. As such, extensive research

has been taken to understand the specifics of the microalgal stress response (Hu et al., 2008). The nitrogen-stress response in microalgae follows the same pattern as in all microorganisms outlined previously, but the phenotypic response makes them particularly interesting. The process of adaptation in microalgae follows a distinct pattern: arrested cell growth, chlorosis, starch accumulation, and lipid accumulation.

The first response to nutrient depletion is the cessation of cell growth. In the common laboratory case of immediate nutrient removal, this process occurs after one cell doubling where the internal stores of nitrogen are used to complete the final cell division (Mus et al., 2013; Peltier & Schmidt, 1991; Work et al., 2010). In *C. reinhardtii,* Msanne et al. observed that the cell division stops after 48 h but the cell size continues to increase as the cells divert carbon towards storage as opposed to cell construction for division (Msanne et al., 2012). Miller et al. performed transcriptomics studies on *C. reinhardtii* cultures to analyze this process at an mRNA level and found that N deprivation activated a subset of control genes involved in gametogenesis while down regulating protein biosynthesis and photosynthesis (Miller et al., 2010). These gametogenesis results confirm the results observed by Sager and Granick in 1954 that nitrogen deprivation can control sexuality in *C. reinhardtii*, and the protein and photosynthesis regulation follow the observed pattern of chlorosis and ribophagy as an outcome of cell arrest (Sager & Granick, 1954). The phenomenon of cell arrest is well documented and is necessary for the carbon shift from growth to accumulation that creates the desirable lipid phenotype of microalgae under stress.

In microalgae, cell arrest is followed by storage carbon accumulation, first in the form of starch, and second as lipids in the form of TAG. Cells begin to accumulate

starch and lipids when the internal levels of carbon (as 2-oxoglutarate) become far in excess of the internal levels of nitrogen (as glutamine) (Griffiths et al., 2011). The immediate response is to accumulate starch starting as rapidly as 8 h after nitrogen stress and continuing until about 48 h post stress (Fan et al., 2012; Msanne et al., 2012). The lipid content follows a similar trajectory but appreciable accumulation does not occur until 16 h post stress and continued accumulation throughout the experiment. Garner et al. studied this effect and observed that starch does accumulate prior to TAG but is then degraded as maximum TAG was reached, suggesting that carbon is reallocated from starch-based to TAG based storage in the stress life-cycle (Gardner et al., 2013).

These data imply that starch accumulation is a precursor to lipid production. However, many starchless mutants have been isolated and characterized that have increased lipid production (Li et al., 2010). Additionally, Work et al. demonstrated that it is also possible to generate starch over-expression lines that are then able to assimilate all of their carbon storage in the form of starch with decreased levels of lipid accumulation (Work et al., 2010). Furthermore, these data demonstrated that overproduction of starch was energetically more efficient than lipid production, and while the starch-to-products pathway is more complicated than esterification to biofuels, this is still a more energetically favorable strategy. Taken as a whole, microalgae are able to synthesize either starch or lipids but have a preference towards starch in the short term and lipids in the long term. This is likely because the production of starch is more energy efficient and involves fewer proteins, making it a rapid response to changing conditions, while the lipid pathway creates the more energy-dense storage molecule at

the expense of slower-induction because of the required proteins and ATP to synthesize a TAG molecule.

Engineering a better TAG

Because TAG is such a desirable product, it has been the target of significant research in efforts to generate overexpression mutants. Some success has been achieved; for example, the previously mentioned starch mutant strains (Li et al., 2010), were observed to have significantly higher TAG contents. Even in this case, Work et al. demonstrated that it was coming at an energetic cost when compared to simply producing starch (Work et al., 2010). On the other hand, many attempts have not led to higher production rates, although new insights into the TAG synthesis pathway were obtained.

The DOE Aquatic Species Program included research to upregulate acetyl-coA carboxylase (ACCase) expression in *C. cryptica*. The effort yielded many insights into the structure of ACCase as well as the development of many tools for the genetic transformation of diatoms (Dunahay et al., 1996). But despite increased expression of ACCase, no increase in lipid content was observed (Sheehan et al., 1998). These results demonstrate the complexity of the lipid accumulation pathway. They may have been unsuccessful because ACCase is the first committed step of the TAG synthesis pathway, but increasing the flux into the pathway does not dictate flux through the pathway.

There has also been an attempt to modify the specific fatty acid composition within the cell to achieve a distribution that is more desirable for downstream processes. To that effect, *Phaeodactylum tricornutum* was modified through heterologous expression

of two thioesterases to bias fatty acid production towards lauric and myristic acid (Radakovits et al., 2010). They were able to demonstrate significant upregulation in shorter chain fatty acids and also that 75-90% of the synthesized fatty acids was further incorporated into TAG. This same strategy was successful in plants as modified lipid composition was carried out in *Brassica napus, Arabidopsis thaliana, Glycine max,* and *Nicotiana tobacum* (Yu et al., 2011).

Another avenue of research is modification of the Major Lipid Droplet Protein (MLDP) in microalgae. MLDP was identified through isolation of the lipid droplets in *C. reinhardtii* and then analyzed via mass spectrometry (Moellering & Benning, 2010). Repression of MLDP gene expression using and RNA interference approach led to increased lipid droplet size, but no change in TAG content or metabolism. This result was again confirmed in *Nannochloropsis* and *Arabidopsis*, demonstrating that this protein forms a monolayer around the lipid bodies to contain the hydrophobic TAG inside of the aqueous cytosol (Vieler et al., 2012). Again, the total production did not change through modification of these proteins.

Merchant et al. identified several potential targets for protein modification in the lipid synthesis pathway (Merchant et al., 2011). Among the candidates were the DGAT series of proteins comprising the last step in the TAG synthesis process. Russa et al. followed up with these targets through overexpression of three Type-2 DGAT genes in *C. reinhardtii* and demonstrated that an enhanced mRNA expression level did not boost the intracellular TAG accumulation or result in alteration of the fatty acid profiles (La Russa et al., 2012).

There is one area of success in algal lipid production, and it comes in the form of process development. While genetic modification of the lipid synthesis pathway has yielded poor results, altering the process conditions can result in significantly modified cellular composition and productivity. Growing cells in the presence or absence of light or organic carbon has a significant effect on cell composition (Boyle & Morgan, 2009).

It has also been observed that fed-batch carbon addition can generate significant results in terms of lipid productivity. Goodsen et al. demonstrated that a 20 mM acetate addition would result in cells so engorged with lipid bodies that they became buoyant (Goodson et al., 2011). Fed-batch culturing has shown the same result in *Nannochloris* where intermittent feeding of nitrate in growing but limited cultures resulting in an increase in lipid content (31.0 to 50.9%) while still accumulating identical levels of biomass (Takagi et al., 2000). These results demonstrate that the lipid content is not a fixed value that cannot be modified, but that our understanding of the mechanism is not strong enough to result in productive engineering of the system.

Nitrogen Cycle

Elemental Nitrogen

Nitrogen is an element with an atomic number of 7 and an atomic weight of 14.007 amu. It is most commonly found in the diatomic form N_2, in which two nitrogen atoms form a triple bond to create the stable N_2 molecule that comprises 78% of the earths' atmosphere (Lavoisier, 1965). The strong bond and stability found in molecular N_2 makes nitrogen a very difficult substrate with which to form organic compounds and the subsequent building blocks of life which are dependent on nitrogen: amino acids and nucleotides. The bioavailability of nitrogen is dictated by the nitrogen cycle, which

dictates the global transition of nitrogen between gaseous N_2 and the more bioavailable forms of nitrogen such as ammonia, nitrate, nitrite, and to a lesser extent urea (Bernhard, 2010).

Nitrogen Cycle

Converting diatomic N_2 to other molecules such as ammonia is extremely endothermic, and is therefore a rare event in nature. Over evolutionary history, only a limited number of species of bacteria and archaea have evolved the ability to convert N_2 to reactive nitrogen (Galloway et al., 2004). Because of the limited number of organisms that can fix nitrogen, nitrogen is often considered to be the element most often limiting to net primary production (gross production minus respiration of producers) in terrestrial and aquatic ecosystems (Vitousek & Howarth, 1991).

The process of nitrogen fixation requires eight electrons and 16 molecules of ATP to convert one molecule of N_2 into two ammonia molecules:

$$N_2 + 8\,H^+ + 8\,e^- \longrightarrow 2NH_3 + H_2$$

This high demand for energy is the reason that few organisms are able to carry out this process. Once incorporated into the more bioavailable form of ammonia, nitrogen can be passed between organisms at different levels of oxidation. This cycle continues on a closed loop with ammonium being incorporated into plants, and then released upon their eventual decomposition. Denitrifying bacteria return diatomic N_2 to the atmosphere. This reaction occurs in balance with the nitrogen fixing rate; however, the bulk of nitrogen flux occurs in the cycle with ammonia, biosynthesis to amino acids and nucleotides, and then back to ammonia (Bernhard, 2010).

Forms of bioavailable nitrogen (reactive nitrogen species)

Once nitrogen-fixing organisms have broken the triple bond of diatomic nitrogen, there are many forms of nitrogen that are readily accessed by non-fixing organisms. In all cases, organisms have developed a preference for one form over another, although many organisms can uptake several different types. The types of bioavailable or reactive nitrogen species that organisms can incorporate into biomass construction are reduced forms of N (e.g., NH_3, NH_4^+), inorganic oxidized forms (e.g., NO_x, HNO_3, N_2O, NO_3^-), and organic compounds (e.g., urea, amines, proteins, nucleic acids) (Galloway et al., 2004). The nitrogen is released from biomass constructs during the apoptosis phase of the cell cycle. Endocellular enzymes including hydrolases, oxidases, deaminases and lyases are activated in plants, animals, and microorganisms at this stage of the cell cycle (Hadas et al., 1992). Once released from the apoptotic biomass, the nitrogen can either be directly released into the environment, or can be further reduced by microbial cells via enzymes that can decompose the nitrogenous compounds, releasing excess nitrogen once cellular demands are met (Jones, et al., 2004). In higher plants and microalgae, the most commonly utilized forms of inorganic nitrogen are ammonia, nitrate, and nitrite.

Assimilation of nitrogen into cells

Diatomic nitrogen is the most prevalent molecule found in the earth's atmosphere. Additionally, organic nitrogen is a primary building block for life. Standing between the two functions is the slow process of nitrogen fixation in bacteria to convert one (N_2) into the other (NH_3). This energetic bottleneck means that nitrogen is the most common limiting resource in environmental systems, and therefore an organism that adapts a

more efficient mechanism of nitrogen assimilation will have a competitive advantage over others in its ecosystem.

The process of nitrogen assimilation in all organisms is a complex orchestration of many enzymes targeting several nitrogen sources at varying efficiencies and flux rates. In addition, organisms must be able to survive in environments in which nitrogen concentrations vary by as much as five orders of magnitude in concentration, from 10 μM to 100 mM (Crawford, 1995). The systems that have evolved for nitrogen uptake are generally divided into two distinct pathways: a high affinity transport system (HATS), and a low affinity transport system (LATS) (von Wirén et al., 2000). For example, in plants, ammonium and nitrate transport is dominated by LATS when concentrations are above 200 μM and dominated by HATS when below 200 μM (Glass et al., 2001).

In *Chlamydomonas*, this pathway is well studied from a genomic and phenotypic perspective. Fernandez et al. have constructed a diagram showing all 13 putative nitrate/nitrite transporters as well as the 8 putative ammonium transporters, including the expected locations within the cell (Fernandez & Galvan, 2007). Building on this framework, mRNA transcription has been quantified for all of the eight known ammonium transporters (González-Ballester et al., 2004) in both nitrogen-replete and -depleted conditions, as well as nitrate transporters in the HATS category (Rexach et al., 2002). It was observed that the passive transporters were expressed constitutively, even upon nitrogen depletion. The active transporters were quickly upregulated upon nitrogen stress in order to scavenge any remaining nitrogen in the medium. However, these HATS proteins are also saturable, with estimates of K_m typically below 100 μM (Howitt & Udvardi, 2000). In addition to the upregulation upon nitrogen stress, it also

appears that ammonium has a strong repression effect on the nitrate uptake pathway in *Chlamydomonas* demonstrating that ammonium is the preferred means for nitrogen assimilation in *C. reinhardtii* (de Montaigu et al., 2011; Franco et al., 1988).

In order to use nitrogen as a building block for cell components it must be converted into a bioavailable form, and for *C. reinhardtii* that form is ammonium. Nitrate is the most commonly available form of nitrogen in soil cultures and aqua-culture. For assimilation into cellular biomass, nitrate must be converted into ammonium. Once inside the cell, the nitrate is reduced by nitrate reductase (NR). NR catalyzes the reaction from nitrate to nitrite and is subject to strict regulation at the synthesis, activity, and degradation stages of the protein life-cycle (Kay et al., 1990). Once reduced, the nitrite passes through another transport channel leading to the chloroplast. Inside the chloroplast, the nitrite is then reduced again using nitrite reductase (NiR) into ammonia (Glass et al., 2001). These steps are specific to nitrate uptake, but once the nitrate is reduced to ammonium all pathways converge and the conversion into the various biosynthetic pathways is identical going forward.

GS/GOGAT and GDH

Ammonia can then be fixed to carbon by the action of the glutamine synthetase, glutamate synthase (GS/GOGAT) pathway via the carbon skeleton precursor 2-oxyglutarate (2-OG) (Howitt & Udvardi, 2000). The regulation of these genes is controlled within the cell through the abundance of 2-OG. In cyanobacteria, the nitrogen assimilation genes are activated by rising levels of 2-OG, indicating that nitrogen levels are low enough that the 2-OG cannot be converted to glutamine (Ohashi et al., 2011). The glutamine and glutamate derived from the GS/GOGAT reactions

function as N donors for the synthesis of most other amino acids and the other major N-containing compounds in the cell, including nucleic acids, cofactors, chlorophyll, and many secondary metabolites (Moorhead & Smith, 2003).

The GS/GOGAT pathway is the gatekeeper for biosynthesis. As ammonia enters the chloroplast, it is converted to glutamine through the interaction with a glutamate via the glutamine synthetase enzyme. Glutamine can then continue on to become the basis for nucleic acids. Alternatively, glutamine and its two nitrogen atoms can react with 2-oxoglutarate from the TCA cycle to make two glutamate molecules. Glutamate can be converted into the amino acids and then proteins, or the nitrogen can be incorporated into another ammonia molecule to begin the cycle anew (Andrews & Lea, 2004). Finally, glutamate dehydrogenase is able to either remove an ammonia molecule from glutamate, resulting in 2-oxoglutarate, or catalyze its incorporation into glutamate.

The importance of this pathway cannot be overstated, as this is the central feature of nitrogen regulation in cells. In *C. reinhardtii*, ammonium generation through the GDH pathway was stimulated in the presence of high glutamine (cellular surrogate for N), inhibited by the presence of high levels of intracellular carbon, and stoichiometrically related to loss of protein (Hipkin et al., 1982). High glutamine levels also repressed the expression of the HATS proteins in the ammonia pathway (Glass et al., 2001). In plants, the presence of high intracellular glutamine acts to regulation the expression of genes involved in nitrate and ammonium uptake and assimilation (Stitt & Krapp, 1999).

The understanding and manipulation of this pathway could be a powerful tool to controlling the nitrogen-stress response in *C. reinhardtii*. In many species from bacteria

to plants, the regulation of this pathway is controlled directly and indirectly by the global nitrogen regulator protein, PII.

PII, the Global Nitrogen Regulator

PII is a small (18 kDa) homotrimeric protein that controls the sensing and cellular response to changes in the availability of environmental nitrogen. PII was first identified as an adenylylation/deadenylylation protein for the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway in 1969 (Shapiro, 1969). The protein can be found in a large variety of species ranging from proteobacteria all the way up to higher plants (Arcondeguy et al., 2001).

The reason for the ubiquity of PII, despite the roughly one billion years of independent evolution, is because PII functions as the central regulator of the tightly controlled carbon:nitrogen balance within the cell (Williams et al., 2013). The PII protein has a very high homology among species (typically >50%) and all PII proteins perform the same primary function (N regulation). However, each species has evolved its own mechanism to modify and control regulation according to the surroundings.

The similarities of PII include size, conformation, some reaction pathways, and constitutive expression. The protein consists of three identical subunits each with a T-loop catalytic domain, and a separate ATP/ADP binding site for allosteric protein modification. In photosynthetic eukaryotes, PII is synthesized in the nucleus but localized to the chloroplast. For this reason, there is an additional localization tag added in the nucleus that is subsequently cleaved upon entry into the chloroplast (Ninfa & Atkinson, 2000). PII has been studied extensively since its discovery in 1969, and as

such has been outlined in detail through many review papers (Arcondeguy et al., 2001; Chellamuthu et al., 2013; Forchhammer, 2004).

PII functional mechanism

There are two primary functions of the PII protein upon sensing changing extracellular nitrogen levels: to increase the rate of uptake of new nitrogen (NO_3 or NH_4) and to shunt that nitrogen towards conversion to cell components in favorable conditions. The specific mechanisms are presented in detail in some excellent reviews (Arcondeguy et al., 2001; Chellamuthu et al., 2013; Forchhammer, 2004), and an abbreviated version is presented here.

PII in E. coli

PII was originally discovered in *E. coli* and most amount of research has been conducted on *E. coli* PII. As described above, nitrogen is incorporated into the cell and reduced to ammonia. From there it is incorporated into the cell via the GS/GOGAT pathway. At this point PII begins to monitor and control for the intracellular level of nitrogen through the concentration of 2-oxoglutarate (a product of citric acid cycle, and common carbon store in cells) (Ninfa & Atkinson, 2000)

There are two levels of control in *E. coli* PII in the regulation of nitrogen uptake: a covalent modification (uridylation) in response to intracellular glutamine levels, and an allosteric binding of 2-oxoglutarate correlating to the concentration of intracellular 2-oxoglutarate (Ninfa & Jiang, 2005). This bimodal regulation allows the PII protein to maintain strict control over the regulation of other genes and proteins further down in the metabolic cascade. In cases in which there is low glutamine (signaling low N status) as well as high concentrations of 2-oxoglutarate (signaling sufficient C), the PII protein

initiates the NRII signaling cascade which upregulates the HATS pathway of nitrogen acquisition (Jiang & Ninfa, 2007). In the case of NRII signaling, PII acts to inhibit the pathway as long as intracellular glutamine levels allow for UDP to remain covalently bound to the trimmers. Once nitrogen levels drop, the UDP dissociates and PII is free to activate NRII (Jiang et al., 2012). The two levels of control in the *E. coli* PII protein enable tight regulation on the nitrogen assimilation pathway.

PII in Cyanobacteria

In cyanobacteria, the role of PII is the same (incorporate nitrogen into the cell), but the mechanism is very different. Uridylation is replaced with phosphorylation and there is the addition of a new signaling molecule, PipX, that is unique to cyanobacteria (Espinosa et al., 2006). In addition to PipX, a second level of control is added where now PII controls not only nitrogen transportation, but also the integration of ammonia into amino acids and eventually proteins.

The cyanobacterial PII protein is modified by phosphorylation. With a sufficient nitrogen content (at low levels of 2-OG), PII stays in an unmodified state and forms complexes with the transcriptional co-activator PipX, breaking the stimulation of NtcA transcription factor by the latter and (2) with NAGK, promoting enzyme activity. Under conditions of nitrogen starvation (at high levels of 2-OG), the modified PII does not interact with NAGK, but is inhibited by arginine enzyme activity and PipX is released from the complex with PII, interacts with NtcA, and thus increases the expression of NtcA dependent genes. (Ermilova & Forchhammer, 2013) This mechanism means that it is now possible to control nitrogen flux in, as well as to direct nitrogen allocation. Cyanobacteria regulate ammonium transporter expression through inhibition in the

same way as *E. coli.* However, the regulation of NAGK is the opposite with allosteric interactions between PII and NAGK being a requirement for proper NAGK function (Osanai & Tanaka, 2007).

Despite the divergent evolution of PII in cyanobacteria, it is clear that the functions performed by this protein are critical. The transition continues through to higher plants.

PII in Higher Plants

The role of PII in higher plants is less clear than the highly specified role found in *E. coli*. Like cyanobacteria, higher plants regulate the internal concentration of nitrogen through allosteric interactions with 2-oxoglutarate (Huergo et al., 2013). PII binds 2-OG as a repressor in the same mechanism as cyanobacteria; however, the lack of PipX means that the mechanism that higher plants use to induce active nitrogen uptake upon nitrogen depletion is unknown (Radchenko et al., 2013). In addition to the nitrogen uptake, and arginine biosynthesis pathways, higher plants have evolved another regulatory pathway.

Pull-down assays of PII from *Arabidopsis* demonstrated that PII selectively binds the biotin carrier subunit of acetyl-CoA carboxylase, and when bound inhibits its activity (Feria Bourrellier et al., 2010). When intracellular carbon levels are low, PII remains bound to ACCase, keeping it inactive, but once carbon levels rise, PII releases its bond with ACCase and replaces with the now plentiful 2-oxoglutarate.

Another issue with eukaryotic phototrophs is protein localization. PII is translated in the nucleus and exported to the chloroplast through a localization signal. This makes the protein larger than usual after translation (22 kDa), but following transport across the

thylakoid membrane, the signal is cleaved to yield the 18 kDa highly homologous protein completely localized in the chloroplast (Ermilova et al., 2013). Structural analysis indicates that a charged C-terminus remains after cleavage and is likely another as yet discovered mechanism for cellular signaling. The evolution of PII has grown from one specific purpose (sensing N) into one of broad specificity leaving a large space for potential improvements through protein modification.

Phenotype of Engineered PII

One of the most powerful tools to discover how a protein works is genetic modification, and it has been implemented extensively to understand PII. Some of the first experiments were to verify the finding that PII is necessary for proper NAGK activity. Burillo et al. discovered that mutants without PII were unable to exhibit healthy NAGK functionality and therefore demonstrated that PII is a positive regulator of NAGK (Burillo et al., 2004). In *Synechocystis*, knockout of PII or NtcA was sufficient to completely remove the characteristic stress response in the mutant lines (Aldehni & Sauer, 2003). Further experiments in *Synechococcus* demonstrated that a PII null mutant exhibited no ammonium-dependent inhibition of nitrate and nitrite uptake; however, mutants with active PII that was deficient in phosphorylation showed a wild-type phenotype (Lee et al., 2000). These mutations demonstrate that PII directly affects the manner in which cells sense and respond to nitrogen levels, and that modifications to PII have repercussions that extend far beyond nitrogen assimilation.

Forchhammer et al. demonstrated global cellular restructuring as a result of knocking out PII in cyanobacteria. The PII deficient mutants had a 10% slower growth rate, significantly higher levels of glycogen, and significantly decreased levels of

chlorophyll a, phycocyanin, and total protein per cell (Forchhammer & Tandeau, 1995). The deficient lines also showed poor adaptability to new environments despite an increase in glutamine synthetase. These results were corroborated in *Anabaena* sp., for which PII deficiency also led to slower growth rates (Y. Zhang et al., 2007). Similar observations were made with *Arabidopsis*, in which PII-deficient mutants were unable to synthesize new amino acids after a brief period of nitrogen deprivation (Ferrario-Méry et al., 2006). In terms of adapting to nutrient stress, *E. coli* deficient in *glnB* (PII) and *glnK* were analyzed for their ability to recover after nitrogen stress. The researchers observed a normal recovery pattern in the PII deficient strains, but no recovery at all from the *glnK*-deficient mutants (Blauwkamp & Ninfa, 2002). These experiments are starting to uncover the mechanism behind PII and are demonstrating the potential that modification of this one global nitrogen regulator can have.

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CHEMOSTAT CULTIVATION OF CHLAMYDOMONAS REINHARDTII UNDER NITROGEN-LIMITED, HETEROTROPHIC CONDITIONS

Introduction

Microalgae are a diverse group of microorganisms adapted to a wide range of ecological habitats. The ability of these cells to quickly adapt to many different conditions for survival suggests the possibility that they can be used in a range of growth environments in industrial processes (Becker, 2007, Rasala et al., 2010). When the inherent advantages of microalgae are coupled with the rapidly growing genetic toolbox for their manipulation, we are left with a very viable alternative to the current platform organisms (e.g. *E. coli*, yeast, CHO cells). Microalgae can have the relatively high growth rate and ease of cultivation of microorganisms together with the ability to perform post-transcriptional and post-translational modification of plants (Potvin and Zhang 2010). Additionally, many species of microalgae are capable of using photosynthesis or organic carbon for growth. The ability to begin a process under heterotrophic growth and maintaining the option to switch to phototrophic growth is a great advantage of microalgae over other platform organisms.

Heterotrophic growth of microalgae is counter to the prevailing practice in algal cultivation because one of the principal benefits of microalgae above other platforms is their ability to incorporate carbon through photosynthesis (Greenwell et al. 2010). However, attempts to exploit phototrophic growth have not come to fruition because the cost and complexity of large-scale photobioreactors. A major advantage of microalgel advantage over industrial microorganisms lies in the ability of algae to use either

organic carbon or carbon dioxide. In this way, a process could be designed for heterotrophic conditions and then transitioned to phototrophic growth. This concept has been demonstrated with *Chlorella protothecoides*, for which heterotrophic cultures were exposed to limited light resulting in a 13-38% reduction in carbon dioxide production (Wang et al. 2013). The researchers observed that using low light increased carbon efficiency without compromising productivity. Heterotrophic growth has a multitude of advantages over phototrophic growth (higher cell density, even nutrient distribution, less expensive and efficient reactors) and one disadvantage (cost of the carbon source) (Bumbak et al. 2011) Leveraging the simplicity and current knowledge of heterotrophic processes is the best foundation to establish microalgae as an alternative to current industrial microorganisms. Changing the energy source is an important first step for microalgal production; however, the stress response to nutrient limitation must also be considered.

The most common microalgal stress response is nutrient depletion, which has long been used as a way of increasing lipid accumulation in microalgae, with nitrogen depletion typically proving most effective (Guschina and Harwood 2006). A large body of research from a phenotypic (Msanne et al. 2012), genotypic (Merchant, Prochnik, and Vallon 2007), and metabolomic standpoint (Boyle and Morgan 2009) has built a foundation of the cellular reaction to nutrient stress. This research has characterized the maximal lipid content and has provided insights into which metabolic pathways are modified when growth is restricted. The primary problem with the interpretation of these data is the growth system. Nitrogen stress is nearly always imposed on the cells in one of two ways: either cells are grown in nitrogen-replete medium and transferred to

nitrogen-free medium (Lee et al. 2012), or they are grown in nitrogen-limited medium and allowed to exhaust their supplies naturally. In both cases, cell growth must stop before the desired phenotype is achieved. Therefore, analysis in these conditions gives a static picture of a dynamic system. The literature currently available cannot elucidate the changes responsible for lipid accumulation as the cells transition to apoptosis. In order to understand how a cell transitions from growth to survival, its growth conditions must be locked in steady-state stress conditions.

Continuous stirred-tank reactor (CSTR) cultivations can maintain cells in steady-state nitrogen limitation. They are operated with a bioreactor containing a feed stream and an effluent stream at identical flow rates. The net effect is a constant liquid volume in the vessel and a defined medium composition. In CSTRs at steady state, the specific growth rate equals the dilution rate, meaning that growth can be controlled through the pump speed. This application has two benefits for a microalgal system: nutrient limitation can be tightly controlled, and productivity can be significantly increased over batch reactions (Kliphuis et al. 2012; Xu, Miao, and Wu 2006; Zhang, Chen, and Johns 1999). The substrate concentration in a steady-state CSTR for an organism whose growth is described by the Monod Equation is:

$$S = \frac{K_s * D}{\mu_{max} - D}$$

In this equation, the substrate concentration (S) is a function of the dilution rate (D), the Monod substrate affinity parameter (K_s), and the maximum specific growth rate (μ_{max}). Since K_s and μ_{max} are determined by the medium formulation and the microalgae being used, the only variable needed to control nutrient concentration is

dilution rate. Through tight control over dilution rate, we can modulate the level of nutrient limitation within the reactor, resulting in variable levels of stressed cell growth. Since nutrient limitation is widely viewed as a necessary trigger for lipid accumulation, the continuous, steady-state cultivation approach gives precise measurement of the trigger point where cell initiates what might be termed "survival growth." For a growth-associated product (such as a biomass component), productivity is maximal at the maximum dilution rate (D_{max}), which for an organism following Monod kinetics in a CSTR at steady state is calculated as (Shuler and Kargi 2002):

$$D_{max} = \mu_{max}\left(1 - \sqrt{\frac{K_s}{K_s + s_f}}\right)$$

Here, S_f is the concentration of the growth rate limiting nutrient in the feed. This equation holds true for phototrophic or heterotrophic growth, but the higher densities available in heterotrophic growth dramatically increase overall productivities (Bumbak et al. 2011). Combining high-density cultures with optimal productivity conditions will create a process that can compete with the traditional platform heterotrophic microorganisms. Before exploiting the advantages of CSTR production of algal biomass and bioproducts, we must first understand how an algal cell senses and adapts to a changing nutrient environment.

To date, there is a critical gap in our knowledge of microalgal stress response. Literature on lipid accumulation can neither identify a specific point of initiation nor the mechanism with which a cell identifies nutrient limitation. In this chapter, research into heterotrophic, nitrogen-limited CSTR cultivations of *C. reinhardtii* is described that

attempts to address this issue. The overall goal is to fill these knowledge gaps and demonstrate their applicability to industrial fermentations, beginning with establishing a means of using precise control over the cellular stress level to build a complete phenotypic picture of "survival growth."

The purpose of this project is to use controlled nutrient limitation in *Chlamydomonas reinhardtii* to induce a stress response within actively growing cultures. This project will demonstrate the phenotypic effects of "survival growth" (exponential growth under nitrogen-stress) in continuous, steady-state cultivations. The impact of this research is to support *Chlamydomonas* as a platform organism for bioproduct formation. Devising a system of controlled stress will allow a deeper understanding of the stress response. Learning how to manipulate the stress response can dramatically alter the productivity potential of continuous cultures.

Materials and Methods

Chlamydomonas strains and culture conditions

C. reinhardtii strain cc-503 (cw92 mt+) was obtained from the Chlamydomonas Resource Center (www.chlamycollection.org). One-hundred mL cultures of mother culture were prepared in modified 4X acetic acid TAP medium until late log-phase growth. To prevent mixotrophic growth, cultures were covered in aluminum foil until they were used to inoculate the reactors. Samples were kept at room temperature and shaken at 110 RPM before inoculation. All cultures were maintained on 1.5% agar plates in standard TAP medium before inoculation.

TAP Medium Formulation

Modified TAP medium was prepared as outlined by Gorman et al. (Gorman and Levine 1965), with acetate concentrations of 70 mM instead of the original 17.5 mM. The medium was also modified to contain 25% of the prescribed nitrogen content to ensure that nitrogen was the limiting nutrient and the cause of the end of log-phase growth. TAP medium was prepared in 12-L batches, and mixed in a 25-L carboy before being allocated to the four reactors. The remainder was left in the carboy, and used as the feed for continuous operation.

CSTR setup and running conditions

Four New Brunswick BioFlow III reactors with a 2-L total volume and 1.3-L working volume were used throughout these experiments. They were set up to run as a continuous system through use of attached peristaltic pumps for sterile feed input, and contained a passive effluent valve on the side of the reactor to ensure constant volume throughout the cultivation. The feed tank was set up in parallel with a single vessel feeding each of the four reactors. Separate effluent tanks were attached to collect the culture effluent. Vessels were maintained at room temperature with an agitation rate of 100 RPM. Dissolved oxygen was monitored in continuously and pH was measured off-line daily. The entire reactor was contained in aluminum foil to prevent mixotrophic growth. To minimize contamination, 25 ug/mL ampicillin was mixed into the feed medium and the reactor vessels prior to inoculation. Sampling was performed daily through the sample port with an initial 10-mL aliquot used to clean the sample lines, followed by a 5-mL aliquot used to daily samples. After two days of batch culture growth, the feed pumps were turned on and continuous culture was allowed to proceed.

Reactors were inoculated in a sterile hood through addition of 100 mL of mother culture through the input port.

Feed rates were calculated through individual pump calibration for each reactor. Pumps were run at 50% feed rate (10 s on/10 s off) for 2 h to determine flow rate. Dilution rate was calculated based on the published and observed heterotrophic growth rate of 0.035 h^{-1}. Flow rates were chosen to correspond to certain amounts of specific growth; 20% of maximum growth corresponded to 8.72 mL/h, 40% to 17.43 mL/h, and 60% to 26.15 mL/h.

Phenotype analysis

Cell composition analysis was performed daily for cell number concentration, lipid content, chlorophyll, and starch content, while added aliquots were taken on Day 3 and 7 for cell mass concentration (dry cell weight) and RNA extraction. Samples were taken daily for all analyses. All cultivations were performed in triplicate and results are reported as a mean with error bars corresponding to SD $\pm$ 1.

Cell concentration was determined by optical density at 750 nm and using hemocytometer measurements. OD750 was measured using a plate reader (BMG FLUOstar Omega) with clear, flat-bottomed 96-well plates (Fisher). At least two technical replicates were performed for all measurements. Hemocytometer measurements were performed in duplicate for each of the biological replicates. Dry cell weight was determined by filtering 15 mL of culture through pre-weighed 0.2-µm filters (EMD Millipore) under vacuum and placing the filtrate in a 60 °C oven for 36 h. Samples were cooled and reweighed to determine the dry cell weight per mL of culture. Specific growth rate was calculated according to the logarithmic growth equation

$\mu\ (hr^{-1}) = [\ln(X_2) - \ln(X_1)]/(t_2 - t_1)$

with the time reported as $t_{ave} = (t_2 + t_1)/2$.

Nile Red analysis was performed as a proxy for total neutral lipids within the culture. As outlined by Chen et al. (Chen et al. 2009), 100 µL of sample was combined with 100 µL of 2 µg/mL Nile Red in DMSO and incubated for 20 min in the dark. Fluorescence was measured with a plate reader (BMG FLUOstar Omega) at 530 nm excitation and 575 nm emission.

Chlorophyll content was determined as outlined by Porra (Porra 2002). Briefly, a 1000 µL sample of the culture was pelleted and resuspended in 80% acetone/20% methanol mixture. The sample was mixed thoroughly and repelleted at 13000 g for 10 min. A 200 µL portion of supernatant was then loaded onto 96-well plates and absorbance was measured at 646 nm (Chl a), 664 nm (Chl b), and 750 nm (control). The total chlorophyll content was then estimated using the relationship 17.76 * OD646 + 7.34 * OD664 - OD750 = ug/mL chlorophyll (Porra 2002).

The starch content of cells was determined using anthrone reagent as outlined by Work et al. (Work et al. 2010) and originally documented by Morris (Morris 1948). The protocol volumes were scaled down to a final total volume of 150 µl and fluorescence was measured on a plate reader (BMG FLUOstar Omega).

Results

Nitrogen limitation

Four different nitrogen formulations were tested for their ability to lead to nitrogen depletion in batch cultivations of *C. reinhardtii* (Figure 5.1). It was observed that the

standard formulation (10 mM NH_4Cl) as well as the 7.5 mM formulation resulted in similar growth patterns. When the initial nitrogen level was 5 mM NH_4Cl, the cells appeared to grow at a slightly slower rate, and reach a lower final optical density, but since these cultivations were not done in triplicate, a statistical analysis could not be carried out. Cells cultured in the medium containing 2.5 mM NH_4Cl stopped growth by Day 3 on and led to more dilute cultures at the end of the growth phase.

The medium with 2.5 mM NH_4Cl for the remainder of the CSTR experiments because the nutrient level has no effect on the steady-state outcome in a CSTR and therefore any limiting condition is sufficient to analyze nutrient-limited growth in a CSTR.

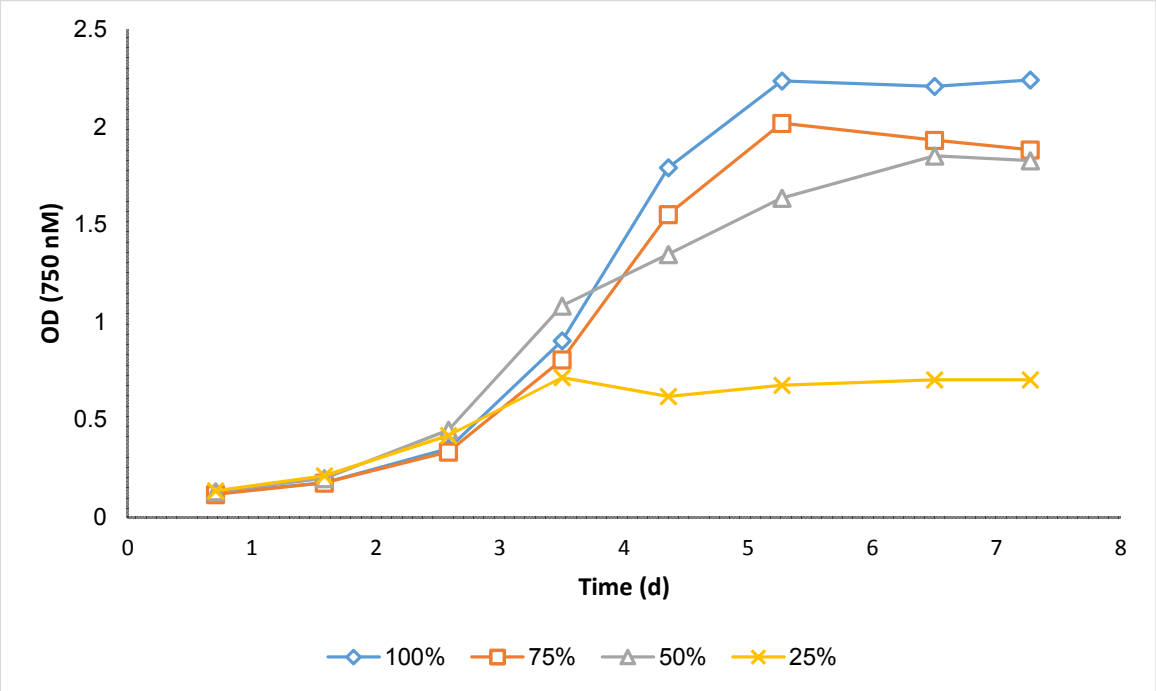


Figure 5.1: Nitrogen limitation experiment through modification of TAP medium to ensure complete nitrogen deprivation through normal culture growth. Single culture analysis only.

CSTR results

CSTR experiments were carried out in parallel with each of the four bioreactors operating at a different flow rate. The cultivations had a differential growth pattern that depended on the different flow rates. After seven days, the cultures reached steady state, and the mean of the final three days from each culture was taken to analyze statistical differences between the cultures. By Day 3, it became apparent that each of the four reactors was contaminated, even after the addition of ampicillin. Despite this, there was a statistical difference between the cultures operating at the highest flow rate and the next two slower flow rates. However, qualitatively the cells in the bioreactor with the shortest dilution time did not appear as healthy and the contamination appeared more prevalent. Without replicate bioreactors, one cannot determine if the effect was a random occurrence or an effect of the increased dilution rate.

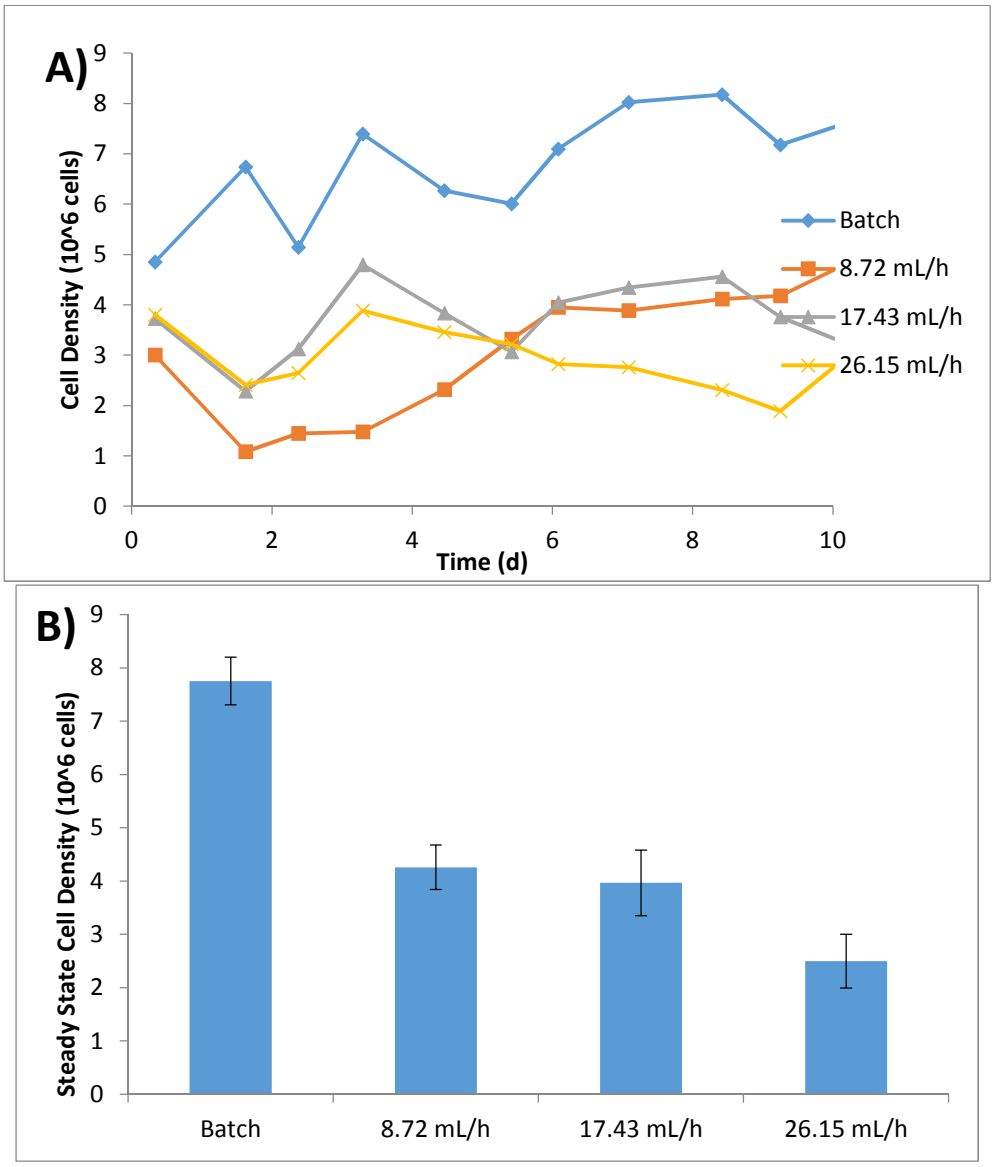
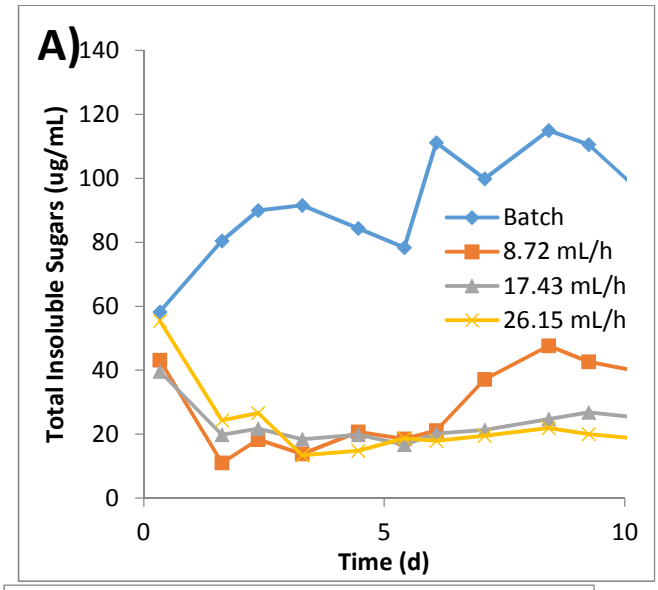
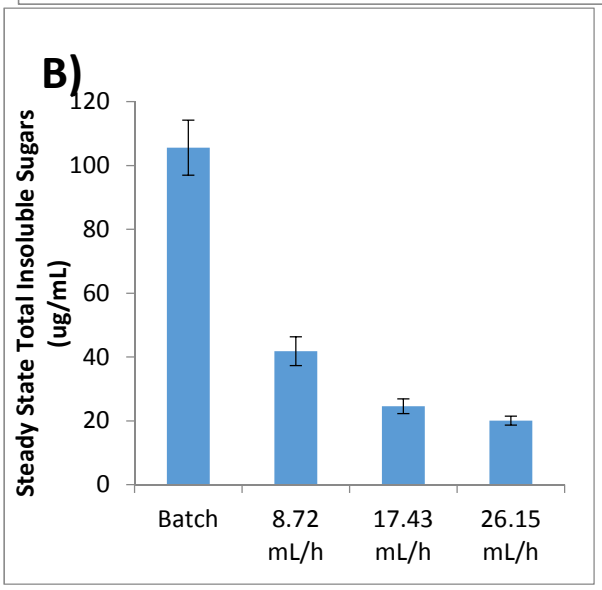


Figure 5.2: **A)** The cell density for each of the different reactors over the time course of the experiment. **B)** The averaged results from the steady-state stage of the experiment including Days 7-10.


A)
Total Insoluble Sugars (ug/mL)
140
120
100
80
60
40
20
0
0
5
10
Time (d)
Batch
8.72 mL/h
17.43 mL/h
26.15 mL/h


B)
Steady State Total Insoluble Sugars (ug/mL)
120
100
80
60
40
20
0
Batch
8.72 mL/h
17.43 mL/h
26.15 mL/h

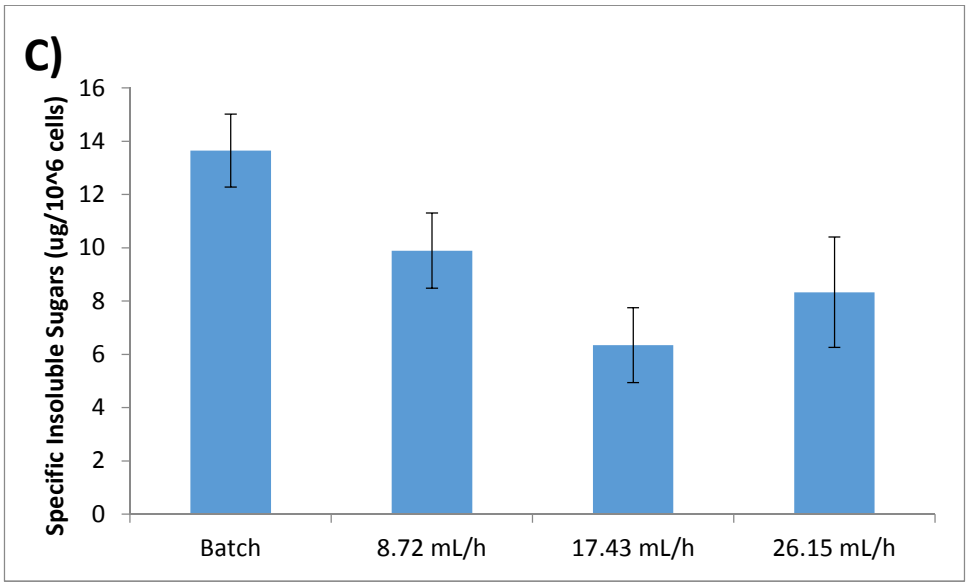


Figure 5.3: **A)** The total insoluble sugars for each of the different reactors over the course of the experiment. **B)** The averaged total insoluble sugars results from the steady stage of the experiment, including Days 7-10. **C)** Specific insoluble sugars from cultures at steady state.

The total insoluble sugars data show that nutrient-limited batch culture contained the highest starch concentration as well as at a specific (per cell) level. However, the 8.72 mL/h and 17.43 mL/h cultures had differential levels of starch per cell

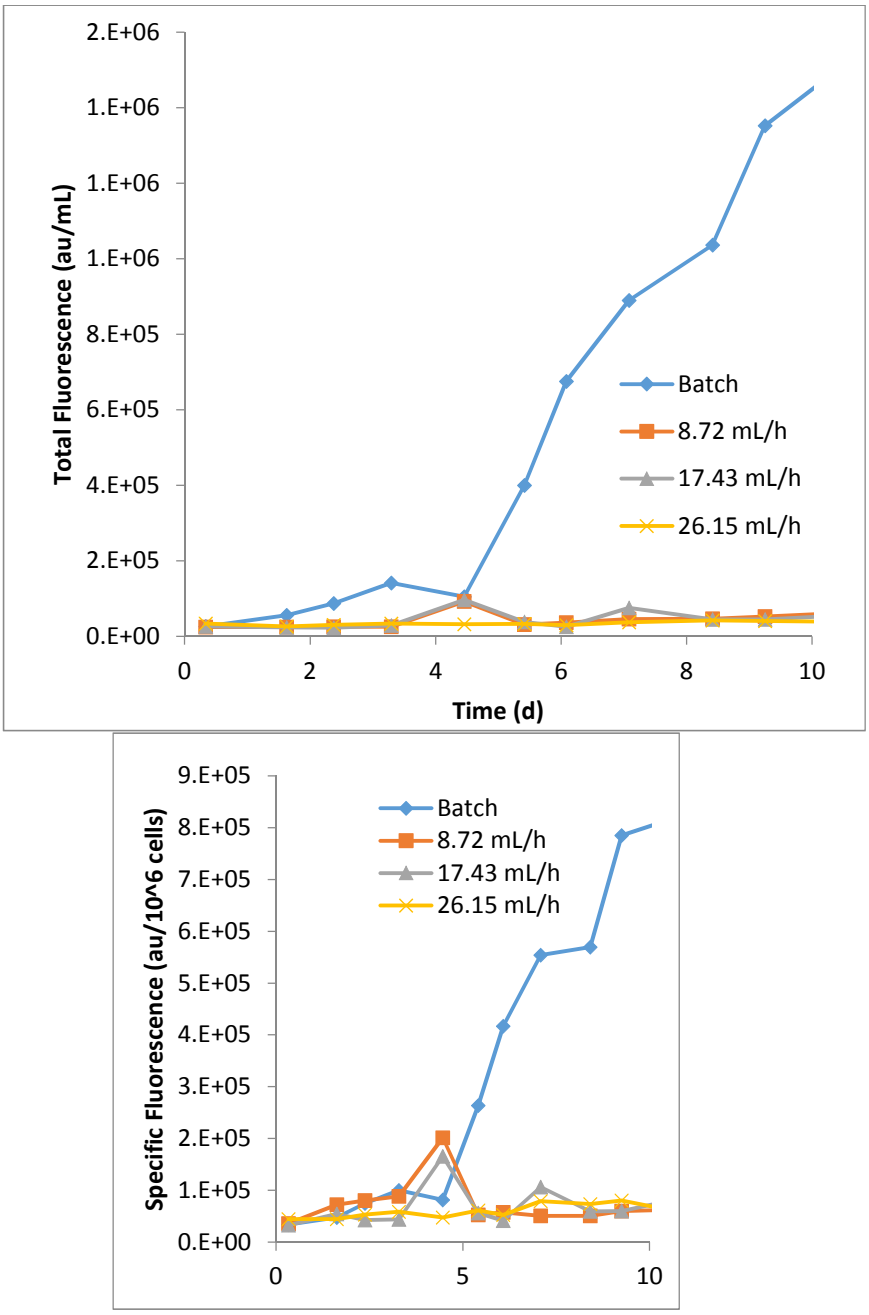
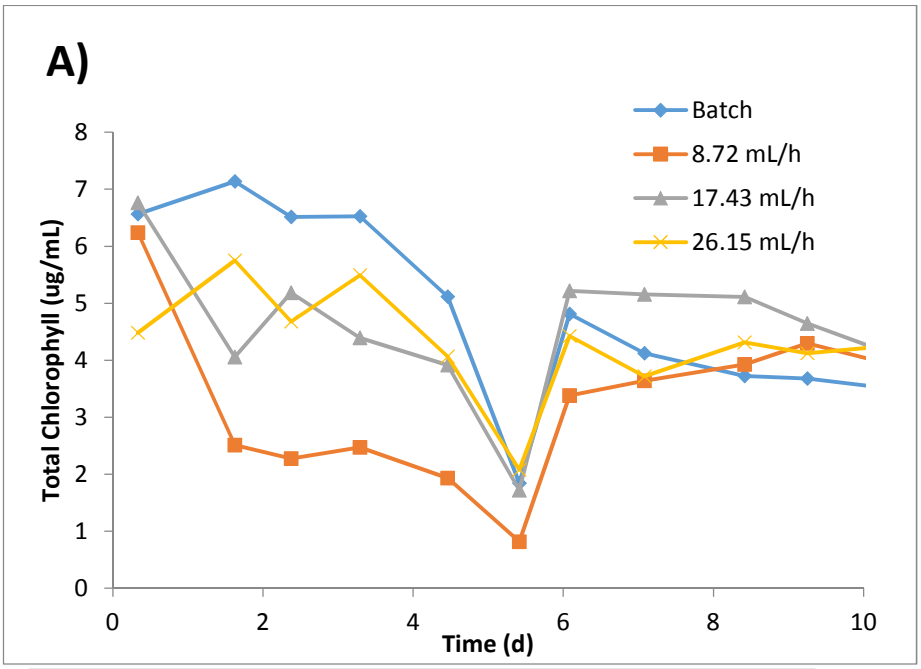
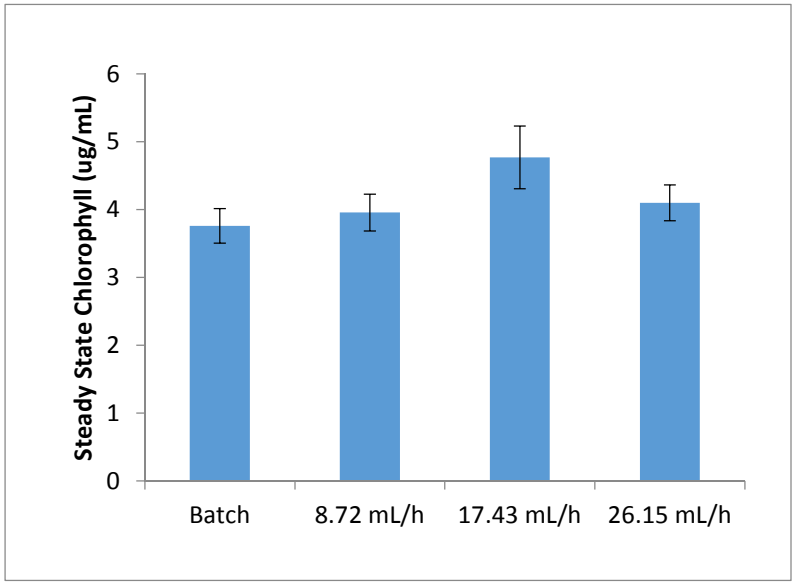


Figure 5.4: **A)** The total Nile Red fluorescence for each of the different reactors over the time course of the experiment. **B)** The specific Nile Red fluorescence each of the different reactors over the time course of the experiment.

The total Nile Red fluorescence data were less clear, with a significant increase observed in the batch culture and the same constant level in each of the continuous reactor cultures. For each of the continuous cultivations, the total and specific Nile Red fluorescence was statistically identical, indicating that the neutral lipid concentration remained constant for all of the actively dividing cells.





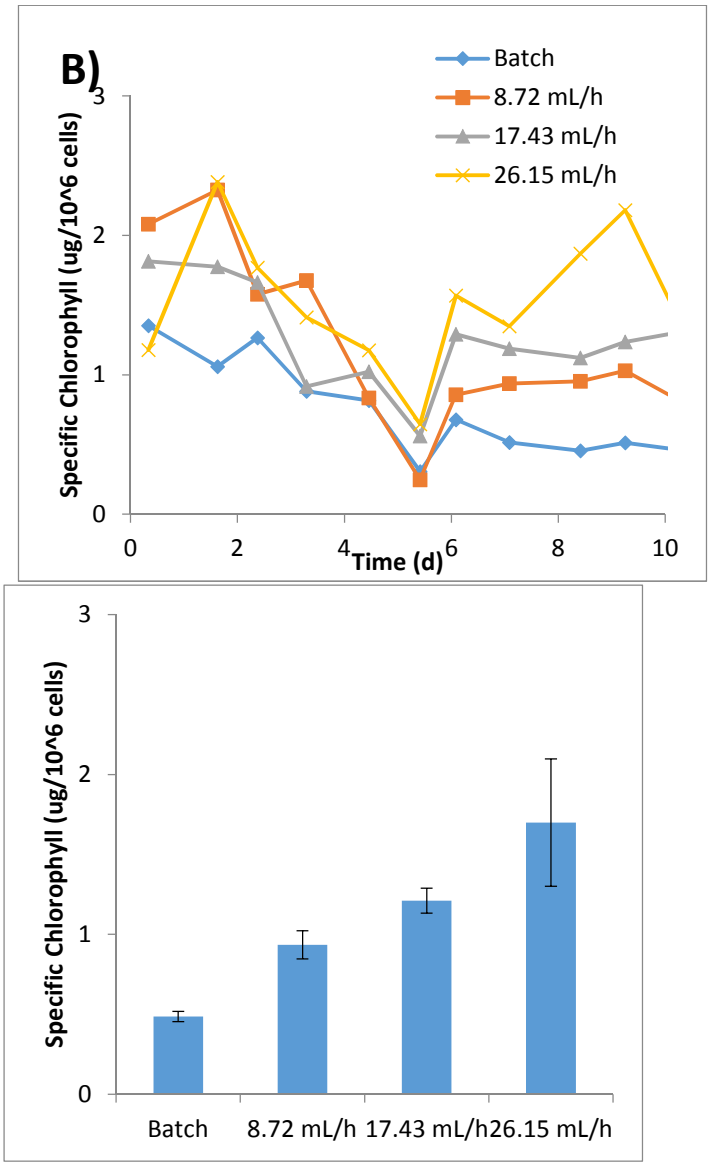


Figure 5.5: **A)** The chlorophyll content for each of the different reactors over the time course of the experiment, as well as the averaged results from the steady stage of the experiment including steady state days 7-9. **B)** The specific chlorophyll content for the time course and the average of steady state Days 7-9.

The chlorophyll results show a general trend downward with the cultures remaining close together in chlorophyll expression. When steady state was reached, only the 17.43 mL/h culture was significantly different from the batch reactor. On a specific level, the cultures had differential expression between each one, with a steady progression going from lowest in the batch to highest at the highest flow-rate.

Discussion

Experimental challenges

The data presented in this section came at the expense of surmounting several substantial obstacles required to operate with the available equipment. There were constant issues keeping the reactors operational, from losing power overnight to losing all temperature control, as well as inaccurate pH probes and highly variable dissolved oxygen readings. In addition, the feed rates varied within each specific reactor and the tubing constantly became clogged or damaged in some way.

In addition to the physical system issues, there were nearly constant culturing issues. In all reactors under all conditions, some level of contamination was present. At first, this was mitigated by the addition of antibiotic, but soon that became ineffective. The contamination caused a steady dilution of *C. reinhardtii* cells during the continuous phase of the experiment and, eventually, complete washout. As such, despite six separate attempts at performing this experiment under various different inoculation and growth conditions, only one set of reliable data were generated.

Nitrogen-limited growth conditions

The nitrogen-limited growth conditions demonstrated that there was a significant nitrogen deficiency at 2.5 mM NH_4Cl in the feed formulations, and that there may have

been a deficiency at the 5 mM level as well. These experiments were only carried out with one biological replicate, so, to ensure complete depletion, the more conservative level of 2.5 mM was chosen for the downstream experiments.

Effect of continuous nitrogen-limited growth on cells

The hypothesis for this experiment was that by culturing cells at several different flow rates (specific growth rates) in a continuous system, it would be possible to elicit varying degrees of stress response from the cells. Theoretically, each of the cultures should reach the same maximum cell concentration as dictated by the initial nitrogen concentration in the medium. Once in continuous mode, the cultures were expected to maintain their cell concentration and create new cells at the same rate that nitrogen and other medium components were fed into the system.

This expectation was not reflected in the steady state cell density. The batch control cultivation reached the highest cell concentration, and CSTR culture with the highest flow rate yielded the least dense culture. One explanation could be that the nitrogen was not completely consumed by the time the pumps were turned on, and as such the batch culture continued to divide (without volumetric dilution) while the continuous cultures could not increase beyond their initial cell concentration when continuous operation was initiated. Also, the bioreactor operated at the highest dilution rate appeared to be overcome by the contamination. If this is indeed the case, then that would explain the lower *microalgal* cell density, and thus only the 8.72 and 17.43 mL/h reactors should be analyzed. While there is not enough data to reach any conclusions, these two cultivations reached steady states that were identical to each other in the same manner that was expected from the experimental calculations.

The cellular starch data were revealing about the stress status of the cell. It was observed that starch levels were the highest in total culture concentration and in specific concentration for the batch reactor, with an exponential decrease in culture concentration and linear decrease in specific concentration for the CSTR cultures reactors operated at increasing dilution rates. These results directly correlate with the expected level of stress as a function of flow rate (lower flow = higher stress). From these data, it appears that it is possible to modulate stress through the specific control of flow rate in a CSTR.

Accumulation of TAG and starch are linked in *C. reinhardtii* (Wang et al. 2009) in that starch is produced first and TAG is produced subsequently. However, in all cases, both are shown to coexist in strains during nitrogen stress. The results from these CSTR experiments show that the batch cultures had greatly increased Nile Red fluorescence, while the three continuous cultures exhibited negligible fluorescence. There was no statistical difference between the three continuous cultures by the end of the experiment. This implies that while the starch reserves demonstrated a linear stress response, the TAG accumulation was binary, being “on” in the batch reactors, and “off” in the continuous ones. Unfortunately, there were some issues during the data collection that may have led to inaccurate results. It may be possible that the Nile Red reagent was beyond its effective lifetime and was not fluorescing in the cultures with lower neutral lipid content. Regardless of the cause, more experiments are necessary to investigate the validity of these findings, which indicate that nutrient-limited growth causes a linear increase in starch production but no measureable effect on lipid accumulation.

Chlorophyll was analyzed to quantify the known relationship between chlorosis and nitrogen stress. As expected, the batch cultures contained the lowest specific chlorophyll content, and as the flow was increased, there was a constant increase in the total chlorophyll content. This implies that the cells were less chlorotic as they increased their specific growth rate, exactly as would be expected, and exactly in line with the starch data observed above.

When taken together, the cell number concentration data, specific starch content, and specific chlorophyll content all suggest that the stress level can be modulated through the dilution rate of a continuous stirred tank reactor cultivating *C. reinhardtii* at steady state. The neutral lipid content did not follow the same trend, which may reveal insights about the nitrogen-stress mechanism or may be an artifact of the experimental conditions. Either way, further studies are necessary to both observe the change and to explain it.

CSTR contamination

Contamination became a serious impediment to reliable results. In some ways, this is a reflection upon the difficulty of continuous cultivations in general. However, these experiments were challenged by the microorganism used in this study. Heterotrophic *C. reinhardtii* has a doubling time of approximately one day. This means that running a CSTR with a dilution rate of 0.2 d^{-1} will have a reactor residence time of five days. When operating with the standard three residence times as an estimate of steady state, this means a cultivation of fifteen days before equilibrium can be reached. In the course of reaching equilibrium, there was a constant battle with contamination. The source of the contamination was never pinpointed, but the reactors being used had

many potential points of entry, and while they were sufficient for bacterial cultivations over an 8 h day, when run continuously for weeks at a time, the contamination could not be managed.

In many of the cultivations, the contamination reached a steady state and did not appear to increase in OD750 or cell count beyond a certain point. It was here that most of the data were collected, but this cannot be controlled for and undoubtedly led to some of the variation in the data. Also, the contamination would consume some of the nitrogen in the cultivation, leading to differing levels of algal cell concentration in each of the individual bioreactors. The contamination was a systemic problem within all reactors over the course of these experiments; however, it was most pronounced in the highest dilution rate condition regardless of the specific bioreactor used. This issue could not be overcome through the course of these experiments and was part of the reason for the discontinuation of this project before completion.

System optimization ideas

To properly test the hypothesis, a new set of bioreactors must be used. The bioreactor setup must have many of the systems not available for the experiments conducted in this study. At a minimum, this should include on-line monitoring of pH and dissolved oxygen, with accurate temperature control, and constant agitation. Also, the constant volume would be better controlled if the effluent were controlled in an active manner through a liquid removal tube that extended into the medium and a pump that was controlled by a level sensor, instead of the passive apparatus used in the experiments described here.

The medium formulation was useful, but further characterization could be carried out to determine the best level of nitrogen to produce dense cultures while still reaching nitrogen depletion. Switching to phototrophic conditions in a photobioreactor would alleviate some of the contamination concerns because the formulation could be adjusted to omit the acetic acid, the primary carbon source that supported the contamination.

Finally, it could be possible to use high-density heterotrophic conditions to enable the microalgae to outcompete any contamination while at the same time making the resulting changes more pronounced. Depending on the concentration used, this could also bring about a new variable to be tested since this would change the environmental conditions to which the cells are responding.

Conclusions

The purpose of these experiments was to demonstrate the ability of producing cultures growing at various stages of nitrogen stress. Through the analysis of these cultures at different levels of steady-state nitrogen stress, a stronger understanding of the nitrogen stress-response in *C. reinhardtii* was obtained without the noise inherit in a transient batch culture stress experiment. The experiments provided interesting clues towards confirming the hypothesis that nutrient stress is a variable condition that can be controlled through the dilution rate in a chemosat. However, the reactor conditions used were flawed and could not provide sufficiently consistent data to produce definitive results and conclusions.

In the future, this hypothesis can be better tested with a more robust reactor setup. In addition, there is no added benefit to having large cultivation volumes, although there

is a great benefit in replicates. Further experimentation should thus be carried out in smaller systems and with more replicates to form a true statistical average of the effect of culturing conditions.

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CONCLUSIONS

Overview

This dissertation presented on the nitrogen-stress mechanism of *Chlamydomonas reinhardtii*. The nitrogen-stress response was investigated via both process-control and genetic-modification to elicit new answers to old questions. The classification of "nitrogen-stress" was broadened from a binary on/off system to include a range of nitrogen concentrations through fed-batch experimentation much more similar to what would be encountered in the environment. This experimentation lead to the conclusion that there exists a nitrogen concentration below which a cell will not continue cell division but long before full nitrogen-depletion has occurred. However, the stress response is initiated as soon as the total nitrogen in medium level becomes inhibitory to maximum growth. This in turn demonstrated the tunable nature of nitrogen stress in *Chlamydomonas reinhardtii*. This tunable nature was further investigated in continuous reactors, and in terms of starch accumulation was shown to be controllable by dilution rate alone.

The stress response was further investigated from a genomic level through genetic knock-down of the nitrogen sensing protein PII. amiRNA techniques were implemented to knock-down PII transcript levels 13-fold. This procedure resulted in cultures which adapted to new environments more slowly, but which were able to grow to significantly higher cell number densities than the control cultures. Proteomic analysis confirmed the onset of nutrient-replete stress in PII knock-down cells and identified some target proteins to investigate in future work.

Stress in a Fed-Batch Culture

- Demonstrated that a fed-batch approach to nitrogen-stress in *C. reinhardtii* can yield significant changes in culture density and cell composition through control of total available nitrogen in the medium.

This work, explained in more detail in Chapter 2, demonstrated that the common methodology behind nutrient-stress in microalgal studies does not adequately frame a complex problem. The research presented in this dissertation is an attempt to fill in the story with the more realistic case of how a cell reacts to changing and growth-limiting environments. It was observed that the "stress" response could be observed at all levels of nitrogen feeding as long as the nitrogen level was low enough to limit cell growth. By modulating the concentration in the nitrogen feed we were able to successfully modulate the amount of "stress" expressed within the cell in terms of lipids, starch, and chlorophyll. This approach can yield a new approach towards implementing stress regimes in microalgal cultivations.

Unlike the continuous relationship observed in the stress response, the divide between cell division and chlorosis was binary. Either the cultures were actively dividing and thus contained healthy levels of chlorophyll, or the division was stopped, and chlorosis set in. We were able to roughly define the amount of nitrogen necessary to elicit this change, and prove that cultures growing on either side of the line are more effective at producing TAG and starch than the standard control cultures of nitrogen-replete or –depleted conditions.

These results bring about an interesting use case for large-scale cultivations. If cultivations are to be done in a continuous mode it is likely beneficial to limit nitrogen

thereby slightly decreasing growth but in turn increasing the cellular carbon allocation towards the more highly desirable TAG for commercial purposes. In addition, the standard two-stage cultivation techniques can be modified to include a fed-batch second stage to both increase final biomass concentrations as well as to generate a cell with significantly higher lipid content for harvesting.

PII regulates culture density and decreases revivability in *C. reinhardtii*

- Demonstrated increased cell weight density, culture lipid productivity, and revivability from exposure to nitrogen-free environments through genetic knock-down of the nitrogen regulator protein PII.

The details of this deliverable are discussed in Chapter 4. PII functions to control the cellular adaptation to changing nitrogen environments. By engineering decreased expression levels of PII in *C. reinhardtii* we created cultures which were able to grow into a significantly more dense population under both nitrogen-replete and –depleted culturing conditions. These results came at the expense of specific cellular lipid content, and an increased lag-phase when adapting to new conditions. These results imply that PII functions to regulate the cellular adaptation to new environments by controlling the nitrogen allocation within a cell. PII enables a more rapid uptake of nitrogen, likely through activation of high-affinity nitrogen transport proteins, giving the cell a competitive advantage amongst other organisms competing for the same resources. However, these effects come at the cost of decreased nitrogen efficiency in the culture. The cells containing lower amounts of PII were able to create more cells, more dry cell weight, and more lipids from a limiting amount of nitrogen. This is likely because the decreased rate of nitrogen consumption leads to more efficient nitrogen

usage allowing for more of the scarce resource to be allocated towards cell growth and carbon storage, and less to be lost in the cellular respiration and protein-turnover events than in the control cultures.

Interestingly, the cell response to recovery after prolonged nitrogen deficiency was heightened by a decreased level of PII. These results agree with literature which suggests that removal of PII in Arabidopsis does not impede revivability, however these results are the first to report a significant increase the revivability rate of a culture. This suggests that PII has evolved for the strict purpose of enable a cell to rapidly adapt to its environment at the cost of decreased ability to survive prolonged periods of nitrogen-depletion. The conclusion that can be drawn from these results is that the ability to adapt to new environments is more advantageous in nature than the ability to withstand prolonged periods of depletion.

Applying the effects of PII reduction to commercial cultivations could yield a strain more optimized for product formation. The cellular cost for decreased PII content, increased lag-phase and decreased cellular carbon storage, is critical for survival in the wild but are less important in terms of commercial production. However, the benefits of PII reduction: decreased cellular nitrogen demand, increased maximum biomass production, and increased neutral lipid production, comprise a valuable addition to the production of microalgae at scale. For example, decreasing nitrogen demand can have dramatic effects on the cost of medium formulation and therefore production cost.

Proteomic Demonstration of Stress-Response in Knock-Down Strains

- Identified the most affected pathways resulting from PII down-regulation in nitrogen-replete heterotrophic growth

The details of this deliverable are discussed in complete detail in Chapter 5. The proteomic analysis was able to identify proteins commonly associated with stress response during normal log-phase growth. These included several photosynthesis proteins being downregulated, along with ribosomal proteins which are typically downregulated to accommodate for ribophagy during stress. In addition to this we identified starch metabolism proteins which were up as would be expected from a “”stressed” cell. Interestingly, the lipid biosynthesis pathway was not significantly upregulated just as the specific lipids in the cells were not significantly different among the control and knock-down culture. It is believed that this occurred because the stress process in *C. reinhardtii* proceeds through starch synthesis before transitioning to lipid biosynthesis and it is possible that this hasn’t occurred yet, or that it will not occur as a result of the stress like pattern observed in the PII mutant.

Using proteomics as a tool to identify pathway modifications in genetic mutants is a powerful tool that gives insight into many pathways that could not be observed through experimentation alone. These data present a good process framework that can be used to analyze more mutants like this in the future.

Continuous Nitrogen-stress Response can be Controlled in a CSTR

- Showed the relationship between culture “stress” and dilution rate of *C. reinhardtii* in a continuous stirred tank reactor.

Building from the results observed in Chapter 3, the full explanation of these results is outlined in Chapter 6. Extensive work was taken to prove that “stress” cannot be a binary cellular output but must be a continuous relationship correlated to the extracellular nitrogen concentration. This baseline was established from Chapter 3, and

was further demonstrated in a continuous system to gain greater control over the specific state of stress which the cells were subjected to.

The continuous system was able to demonstrate multiple stressed states in terms of starch storage thereby supporting the hypothesis. However, the realities of cultivating a slow-growing heterotrophic culture on a system prone to contamination prevented reliable and repeatable data acquisition from being possible. The results from the experiments were close to expectations, but suffered from more variability than could be overcome under the systems available for the testing.

In this way, the research are did prove to be an interesting one with potential for new discoveries, however they must be undergone in a different type of system with stronger controls over process conditions and contamination to generate more reliable results. Additionally, the long growth cycles makes the standard three resonance time equilibrium period unrealistic and likely needs to instead be performed in triplicate with small reactors over a shorter time period to produce reliable and significant results.

FUTURE WORK

Introduction

The research presented in this dissertation opens the door for new methods and means which can be used to understand the process of nitrogen stress in *Chlamydomonas reinhardtii*. The results have demonstrated that there is more to be understood about the mechanism that microalgae use to adapt to changing environments. The data presented also demonstrate that the simplistic definition of "stress" is insufficient to explain the reaction to changing nitrogen levels. This chapter will suggest and briefly describe areas for further investigation to advance our understanding of nitrogen regulation in microalgae.

Expansion of PII experimental conditions

The experiments carried out in this dissertation represent a limited view of the potential operational conditions which are common in microalgal cultivations. The experiments could be repeated in different growth conditions. Namely, the same media could be used for mixotrophic growth thereby adding in photosynthesis to the equation. This would lead to much higher culture densities and has the potential to dramatically affect the outcome. It is known that cell composition characteristics vary significantly depending on the growth mode so exposure to mixotrophic as well as phototrophic growth would greatly increase our understanding of the role that PII plays in microalgae. In addition, this would likely be a better baseline for comparing PII in *C. reinhardtii* against other microalgae which are strict phototrophs.

Additionally, the source of nitrogen can be varied to determine its effect upon growth. Ammonium was chosen because of the simple pathway directly into the chloroplast in *C. reinhardtii* however this could be modified to incorporate nitrite, nitrate, or urea as nitrogen sources. In all cases the nitrogen is converted to ammonia within the cell, however that does not mean that the other molecules wouldn't have an effect on PII before their conversion and thus could be a new set of experiments to further understand the PII interaction. In *Arabidopsis*, PII knock-out was demonstrated to increase nitrate uptake eventually leading to higher ammonium levels inside the chloroplast. These studies could confirm or repute this finding in *Chlamydomonas* (Ferrario-Méry, Meyer, & Hodges, 2008).

Finally, the technique used to knock-down transcript level has some inherit flaws that could be mitigated through utilization of different techniques. First, a true knock-out would be preferable to the amiRNA technique implemented in this dissertation. However, this is not possible in *C. reinhardtii* so until more sophisticated techniques are developed this cannot be readily achieved. The second issue with the knock-down as presented was the marker gene used to confirm successful transformation. Following the standard protocol, the amiRNA was transformed with an antibiotic resistance gene conferring resistance to paromomycin. Growing on paromomycin could be producing some side-effects that would be complicating the results. This issue can be mitigated by instead using a recomplimentation strategy where an arginine deficient strain was transformed to regain the ability to make arginine, and then selected through medium without arginine supplementation. This would yield a strain truer to the wild-type for further study.

Explanation of revivability result

The observation that PII depleted cells were able to be revived after prolonged periods of nitrogen-depletion is one of the most vexing results discussed in this dissertation. The experiments performed thus far proved that there is a significant difference between the control strain and the knock-down, but they do not offer any explanation for why or how the cells achieve this result. Experiments must be carried out which follow culture phenotype through multiple cycles of replete and depleted nutrient exposure to explain this observation. Observations on the physiological characteristics during this transition will shed light on the changes going on during the cells transition between each environment.

Furthermore, the proteomic data revealed that in a nutrient-replete state the knock-down strains are operating in a state similar to nutrient-stress. A complete analysis of the proteome during nitrogen-stress is underway in the group currently and will help to shed light on the degree that PII effects the stress state in nitrogen-deplete conditions. A broader analysis of the replete results compared against the depleted results should help to shed light on how the cells are able to survive longer with PII knock-down strains.

Finally, it would be interesting to attempt to replicate this constant stress state created through PII knock-down by analyzing it against the constant-stress state observed by the 25% culture in the fed-batch experiments followed by nitrogen-depletion as in the other experiments. If the longevity is indeed caused by a partial shift towards a stress metabolism before true nitrogen-depletion occurs then it should be possible to replicate this effect through the analysis of the 25% strains.

Create PII overexpression strain

The experiments presented in this dissertation demonstrate the effect that decreased PII protein in *C. reinhardtii* can have on cell physiology. To build on this foundation it would be immensely useful to add in the effects of PII overexpression. If PII knock-down generates cells in a quasi-stressed state at all times, then it would be expected that overexpression would instead create cells which are resistant to any stress response at all.

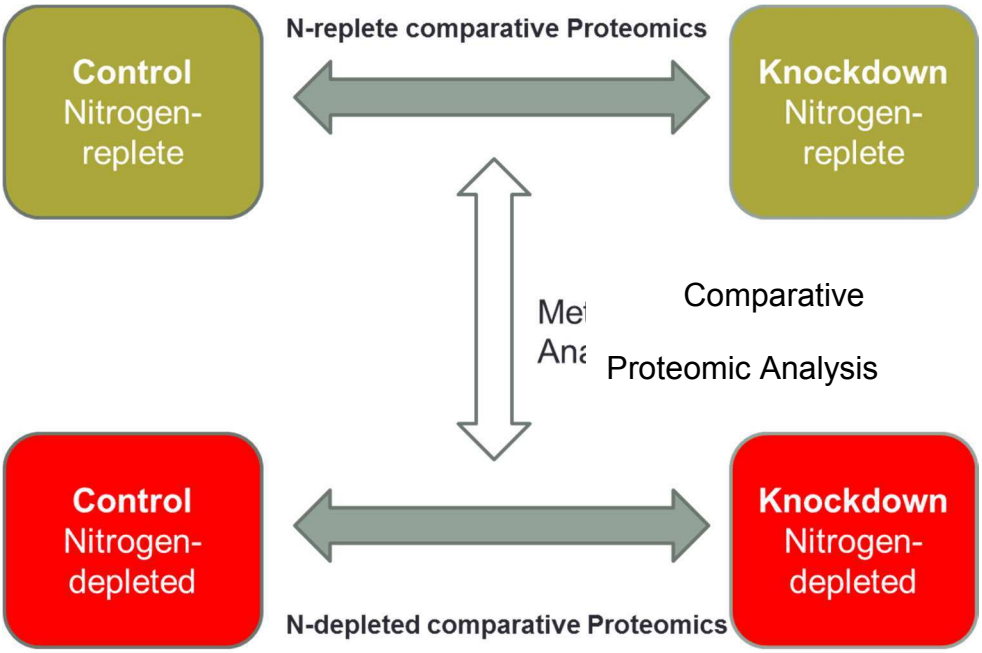
Overexpression of gene in *C. reinhardtii* is well documented and could be carried out through many different techniques. By studying the overexpression strain against both the wild-type and knock-down strain the role of PII could be further defined and explained. All of the data presented in this dissertation can be combined to conclude that “stress” is not a singular event with a defined set of cellular responses, and the ability to test both over and under expressing PII strains would give tremendous insight into the role that PII plays in the stress response. These data could then be used for downstream genetic manipulation to create cultures more favorable for industrial conditions, or to create unique operating conditions for the study of *Chlamydomonas* biology in general.

These studies could further benefit from the recommendation in the first section of this chapter where they can be subjected to different growth parameters. Analysis under hetero-, mixo-, and phototrophic growth would further help to complete the picture of PII function in microalgae.

Run differential proteomics on nitrogen-deplete condition

The proteome analysis discussed in this dissertation was limited to the nitrogen-replete condition. The presented data go a long way towards explaining the *in vivo* effect of PII in cultures growing in mid-log phase under standard nutrient-replete conditions. Owing to the complexity of proteomic analysis it was decided to limit the research presented to this condition alone, but further analysis must be performed to expand this research into the nutrient-depleted conditions as well.

A thorough analysis PII's effect in nutrient-depleted environments is critical to completing the understanding of PII's role in stress adaptation. We are currently in the process of completing this analysis, and will compare the outcomes against the results from Chapter 5. Through this analysis we will demonstrate the role of PII during nitrogen stress. The goal will be to build a picture of the stress response:



Completion of this meta-analysis will create a complete picture of the effect that PII has in both states of replete and depleted growth. It will also be the first time that such a diagnostic technique has been implemented in microalgae to elicit complex protein function through a knock-down analysis.

Build robust CSTR platform for stress testing/ Apply PII to CSTR

The purpose of the nitrogen-limited CSTR is to create a system allowing for steady-state tunable stress in microalgae. The data presented in this dissertation present the first insight into the realization of this goal. Unfortunately the systems need improvement before true experiments can be performed, but they did go a long way to demonstrate the potential of the system.

Further system optimization is needed to build a robust system capable of consistently growing microalgal cultures. The first step towards stronger system design would be to decrease the size of the reactors, and increase the number of reactors which are operating. Adding the ability to run biological replicates would greatly decrease the error in measurements and increase the accuracy of data obtained. A system must be designed to include at a minimum, accurate temperature control, consistent agitation, aseptic integrity, and consistent feed and effluent pump rates. Furthermore, the pump speeds will need to be highly tunable and accurate, particularly in the case of smaller reactors where the pump rates will be very small. One possible solution would be to implement a syringe pump mechanism owing to the exacting control of pump rates.

Once built and validated, these CSTR's would be a great resource for the study of stress response in microalgae. This research could move beyond wild-type strains and

could be expanded to include the PII strains as well. By taking advantage of the tunable stress level achievable in these reactors the PII effect could be studied in much greater detail, and begin to be optimized to achieve the desired result of that particular study.

Demonstrate Fed-batch at scale to increase two-phase production efficiency

One last series of experiments could simple be to understand how much of an effect fed-batch cultivation could achieve on a two-phase production model. The results presented in Chapter 3 demonstrated that there is an added effect to nitrogen-supplementation without the deleterious effect of putting the nitrogen to use for cell division, and could instead increase overall productivity.

It could also increase the time before apoptosis is initiated thereby giving the cultures more time to accumulate lipids which ends up being the limiting factor in many of these cultivations as fed-batch nitrogen could allow the cells to survive in stationary-phase for periods much longer than are typically seen during standard nitrogen-depletion experiments where chlorosis and apoptosis can be seen as early as 24 hours post nitrogen-depletion.