Understanding precision nitrogen stress to optimize the growth and lipid content tradeoff in oleaginous green microalgae

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

Keywords: biodiesel; biofuel; nitrogen trigger; stress physiology.

1 Introduction

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

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

If N deficiency is to be an effective tool for the production of algal lipids, we must have a thorough understanding of the resulting tradeoff between growth and lipid accumulation. The executive summary of the Aquatic Research Program (Sheehan *et al.*,

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

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

Little is known quantitatively about the interactions of growth and lipid content, the time course of lipid accumulation and the magnitude of nitrogen deficiency required to

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

2 Materials and Methods

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

2.2 Semi-Continuous Culture Study: Experimental design and setup are described here; apparatus and measurement details are described in the sections that follow. A seven day semi-continuous culture study was done with *N. oleoabundans* to compare lipid

productivity by this method with batch culture. There were four N treatments that were defined by the initial concentration of N in the media and the concentration of N in the refill media: 11, 5.5, 2.8, and 1.4 mM N. The N treatments were designed to supply N in a range from abundant to severely limiting for growth. There were two replicate air-lift, glass bioreactors per N treatment. Partial culture removal and media renewal were done once daily during the dark period in response to growth. For each reactor, culture was removed at a volume calculated to bring transmission at 750 nm to 1% (0.43 g L^{-1}) after renewal of media, thus minimizing effects of differential light absorption among treatments with different growth rates. Daily measurements of cellular N and lipid content were taken on harvested biomass. Solution N concentration was measured daily during the dark period on all reactors.

2.3 Algal Strains: *Chlorella sorokiniana* (UTEX #1602); *Chlorella vulgaris* (UTEX #265); *Chlorococcum oleofaciens* (UTEX #105); *Neochloris oleoabundans* (UTEX #1185); *Scenedesmus dimorphus* (UTEX #417); *Scenedesmus naegelii* (UTEX #74).

2.4 Culture Apparatus: Algal cultures were grown in glass, air-lift bioreactor tubes that were autoclaved before use. The bioreactors had an outer diameter of 50 mm, inner diameter of 45 mm and were filled to an approximate height of 75 cm, giving a culture volume of 1.2 L. The bioreactor tubes were placed in a plexiglass water tank maintained at 25˚C. CO_2-enriched (1%), filtered air (Whatman PolyVENT 0.2 μm PTFE filters, L#639) was bubbled into the bottom of each bioreactor through a 1 mm glass capillary tube at a rate of 0.5 L min^{-1}. For the semi-continuous study, the bioreactor tubes were altered with

valves fixed to the reactor bottoms, for simple and clean removal of culture. Light was supplied by banks of fluorescent tubes that ran perpendicular to the bioreactor tubes, completely covering one side. The photosynthetic photon flux (PPF) was 300 μmol m^{-2} s^{-1} (300 μE m^{-2} s^{-1}) for the batch cultures and 350 μmol m^{-2} s^{-1} for the semi-continuous cultures, both with a 16-h photoperiod. (The Einstein, commonly used in algae literature, is defined as a mole of photons, but is not an SI unit and does not facilitate comparison with the photochemical, photobiology and plant biology literature.) A 16-h photoperiod was chosen to approximate the natural photoperiod of summers days in the mid-latitudes and to provide a regenerative dark period. Growth of photosynthetic organisms is best determined by the daily integrated PPF (Bugbee & Monje, 1992), which was 17.3 mol m^{-2} d^{-1} in the batch cultures and 20.2 mol m^{-2} d^{-1} in the semi-continuous cultures. This is less than 50% of the average daily PPF of 45 to 55 mol m^{-2} d^{-1} in the summer months in North America. For conversion of measurements made by volume (e.g. g L^{-1}) to a unit of area (e.g. g m^{-2}), the illuminated area of the bioreactor tubes containing algae was used as a conversion factor as follows: 1.2 L / πrh = 1.2 L / (3.14 x 2.25 cm x 75.5 cm) x (1 m^2 / 100^2 cm^2) = 22.5 L m^{-2} and 22.5 L m^{-2} x g L^{-1} = g m^{-2}.

2.5 Media Composition and Preparation: To make media, all ingredients other than P were added to deionized water in 20 L carboys. The carboys were autoclaved, maintaining a liquid temperature of 130˚C for one hour. A P stock solution was autoclaved separately and added to the media after cooling. The freshwater culture media was a custom recipe with the following composition: 0.61 mM $CaCl_2 \cdot 2H_2O$; 0.63 mM $MgSO_4 \cdot 7H_2O$; 0.34 mM KH_2PO_4; 21.4 μM DTPA-Fe; 11.4 μM H_3BO_3; 7.0 μM $MnCl_2 \cdot 4H_2O$; 0.79 μM $CuSO_4 \cdot$

$5H_2O$; 3.1 μM $ZnSO_4 \cdot 7H_2O$; 0.16 μM $Na_2MoO_4 \cdot 2H_2O$; 0.34 μM $CoCl_2 \cdot 6H_2O$. For all N treatments, 1 mM N was added as $NaNO_3$ and the remaining N was added as KNO_3. For the high N stress treatment (4 mM N): 1 mM $NaNO_3$ and 3 mM KNO_3. For the low N stress treatment (11 mM N): 1 mM $NaNO_3$ and 10 mM KNO_3 (except in the case of *C. sorokiniana*, where 15 mM KNO_3 was added for a final concentration of 16 mM N). The media had the following elemental concentrations: 4 mM N (high N stress) or 11 mM N (low N stress); 0.34 mM P; 1.34 mM K (high N stress; low as a counter ion to N) or 10.34 mM K (low N stress; high as a counter ion to N); 0.61 mM Ca; 0.63 mM Mg; 0.64 mM S; 1.2 mM Cl; 1.0 mM Na; 21.4 μM Fe; 11.4 μM B; 7.0 μM Mn; 3.1 μM Zn; 0.79 μM Cu; 0.16 μM Mo; 0.34 μM Co. In the semi-continuous culture of *N. oleoabundans* the same media was used, but in each treatment (11, 5.5, 2.8, and 1.4 mM N) N was split evenly by moles between $NaNO_3$ and KNO_3. Potassium was adequate for normal growth in both low and high N stress treatments; a K effect on growth or lipids is not expected.

2.6 Growth/Algal Density Measurement: Measurements of culture density were made spectrophotometrically at 750 nm with a Shimadzu UV-2401 PC, UV-VIS recording spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Dilution of algal suspensions was made if absorbance measurements exceeded a value of 1.0. Spectral measurements were converted to dry mass densities by relationships developed individually for each species. Dry mass was determined by filtering 10 mL suspensions of algae with Whatman GF/C filters that were dried for one to two days at 105˚C. Virtually all increases in dry mass are necessarily due to photosynthetic production, whether in the form of lipids or

cellular components. This work focused on photosynthetic production. Cell counts were not made, limiting the ability to make conclusions on rates of cell division.

2.7 Biomass Harvesting and Drying: Cells suspended in media were concentrated for harvest with a Sorvall RC6 Plus centrifuge (ThermoFisher Scientific, Waltham, MA). Spinning speed and duration varied somewhat among species, depending on pelleting characteristics. Generally, a speed of 7500 rpm for 5 minutes was sufficient for full recovery of the algae. Following centrifugation the biomass was loaded into 15 mL plastic sample vials and frozen at -80˚C. Frozen biomass was freeze-dried with a Labconco Freezone 4.5 freeze-drier (Labconco, Kansas City, MO).

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

2.9 Lipid Extraction, Conversion to FAME and Quantification: Simultaneous conversion and extraction of algal lipids to fatty acid methyl esters (FAME) was done by the method of Wahlen *et al.* (2011). This method effectively converts to FAME the fatty acids contained in membrane phospholipids and glycolipids, as well as free fatty acids and storage lipid

triglyceride. The lipid or FAME content of 100 mg freeze-dried algal samples was determined with a gas chromatograph (Model 2010, Shimadzu Scientific, Columbia, MD) equipped with a programmable temperature vaporizer (PTV), split/splitless injector, flame ionization detector (FID) (GCMS-QP2010S, Shimadzu Scientific, Columbia, MD), and autosampler. Analytes were separated on an RTX-Biodiesel column (15 m, 0.32 mm ID, 0.10 μm film thickness, Restek, Bellefonte, PA) using a temperature program of 60˚C for 1 min followed by a temperature ramp of 10˚C per minute to 360˚C for 6 min. Constant velocity of helium as a carrier gas was set at 50 cm s^{-1} in velocity mode. Sample sizes of 1 μL were injected into the PTV injector in direct mode that followed an identical temperature program to that of the column. The FID detector was set at 380˚C. Each sample contained octacosane (10 μg mL^{-1}) as an internal standard. FID detector response to FAME was calibrated using methyl tetradecanoate (C14:0), methyl palmitoleate (C16:1), and methyl oleate (C18:1) at concentrations ranging from 0.1 mg mL^{-1} to 1 mg mL^{-1} and tripalmitin at concentrations ranging from 0.05 mg mL^{-1} to 0.5 mg mL^{-1}. Standards were obtained as pure compounds (Nu-Chek Prep, Inc., Elysian MN) and were diluted with chloroform to obtain the needed concentrations. A standard (GLC-68A, Nu-Chek Prep, Inc.) containing methyl esters ranging from methyl tetradecanoate (C14:0) to methyl nervonate (C24:1) was used to identify the retention time window for FAME peak integration. Peaks within this region were integrated using GC solution postrun v. 2.3 (Shimadzu) and concentrations were determined by linear regression analysis.

3 Results & Discussion

3.1 Introduction

This is an analysis of progressive N deprivation in batch cultures of six species of oleaginous green microalgae at two N supply rates, keeping other nutrients constant between treatments. The treatment with a lower supply of N was designed to apply significant N stress while allowing reasonable growth and is hereafter referred to as "high N stress." The treatment with a higher supply of N was designed to apply only minimal N stress and is hereafter referred to as "low N stress." We also investigated the effectiveness of semi-continuous culture for yielding high productivity of high-lipid content biomass. In studies of the effect of N on algal lipids it is common to quantify lipids by a gravimetric method, a portion of which is lipid soluble material. To ensure an accurate representation of the lipid production potentials of the algae and cultural methods studied here, the *in situ* transesterification method of Wahlen *et al.* (2011) was used to extract and convert lipids to FAME for quantification by gas chromatography. This method effectively converts to FAME the fatty acids contained in membrane phospholipids and glycolipids, as well as free fatty acids and storage lipid triglyceride. Because algae rapidly consume and store nitrogen (luxury consumption (Dortch et al., 1984)), measurements of cellular N were necessary to gauge the magnitude of N stress in the cells. Cellular N was thus used in analysis of lipid and growth responses.

3.2 Growth and Lipid Content Tradeoffs in Batch Culture

Nitrogen stress generally had disproportionate effects on growth and lipid content, with profound differences among species (Figure 1). Conceptually, the organisms can be

separated into three categories of response (comparing from low to high N stress): 1) the increase in lipid content exceeded the decrease in growth, resulting in higher lipid content and productivity with high N stress; 2) the decrease in growth exceeded the increase in lipid content, resulting in higher lipid productivity with low N stress and higher lipid content with high N stress; and 3) the increase in lipid content was offset by the decrease in growth, resulting in approximately equal lipid productivity with high or low N stress but higher lipid content with high stress.

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

These categories are somewhat arbitrary, as species may be categorized differently depending on the exact N supply rates tested. However, the differences among species

identified here may signify differences in N metabolism and/or strategies for handling N deficiency. First category species may have a panic response, making significant physiological sacrifices to generate adequate lipids to supply energy and C needs for times of hardship. *N. oleoabundans*, a first category species, was apparently first isolated in an arid, sand dune environment in Saudi Arabia (Chantanachat & Bold, 1962) and would be expected to have adaptations for managing prolonged periods of resource scarcity. In the event of N deficiency, second category species may directly divert energy and C intended for growth into lipids. In third category species, cellular function and thus growth may be particularly sensitive to severe N deficiency; when N deficiency is less severe, the photosynthetic mechanism may be functioning and capable of high rates of lipid production.

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

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

3.3 Stress Quantification and the Timing of Lipid Accumulation

There were tremendous differences among the species in the magnitude of N stress required to stimulate the onset of lipid accumulation. Figure 2 shows how the lipid content of the cells varied as a function of total N in algae (cellular N concentrations are used here as a proxy for levels of N stress). The level of N stress at which lipid accumulation was triggered was unique for every species, ranging from minimal to severe stress. *N. oleoabundans* began accumulating lipids with the application of minimal stress (~7.5% cellular N), just following exhaustion of exogenous N; *C. vulgaris*, *C. sorokiniana*, *C. oleofaciens*, and *S. dimorphus* were intermediate (~4 to 5% cellular N); and *S. naegelii* required the most severe N deprivation (~3% cellular N). The coincidence of these lipid development trends as a function of cellular N in independent N treatments provides strong evidence for the roll of N in the response, even for species requiring only minimal N stress. In general, the species that accumulated the highest lipid content were the species that responded to the least stress.

A response to minimal N stress was less well correlated to high rates of lipid productivity, however, due to species differences in growth cessation with stress. There was a three-fold variation among species in the cellular N content at which growth stopped, from ~1% to 3% N. The algae that stopped growing with higher cellular N presumably employed a more conservative growth strategy, to preserve their physiological state at a higher level of function (e.g. maintenance of higher cellular protein concentrations). But early cessation of growth resulted in stalled rates of lipid productivity, and not all species continued to accumulate lipids in the stationary phase. With a finite supply of N as in batch cultures, where cellular N decreases with growth, the range in cellular N over which lipid

accumulation occurred is proportional to the amount of growth and lipid accumulation that occurred concurrently. Among the six species in this study, those that exhibited the highest values of lipid productivity were the species in which there was more concurrent growth and lipid accumulation. This trend is evident by comparing lipid productivities in Table 1 with the ranges in cellular N where lipids accumulated in Figure 2. At the extremes, some algae grow first and then accumulate lipids, while other species grow and accumulate lipids at the same time.

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

Selection of species as lipid feedstock crops should be broadened to include concurrent lipid accumulation and growth, a characteristic that resulted in higher lipid productivity in this study. The most promising biodiesel feedstock species will combine

this characteristic with the ability to accumulate high lipid content. The data indicate that the species capable of accumulating higher lipid content generally form lipids in response to more minimal N stress.

3.4 Extrapolating to the field

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

the equator of 40 mol m^{-2} d^{-1} gives 0.036 g mol^{-1} of photons. This makes oil production by oil palm about 20% of what we achieved in this study with algae, per unit light. Our values are similar to the peak values reported by others (Amaro et al., 2011). The challenge is to achieve high lipid productivity at scale.

3.5 Semi-Continuous Culture

Because *N. oleoabundans* required the least N stress to prompt lipid accumulation in batch cultures, this species was chosen to compare its lipid productivity in batch and semi-continuous cultures. Semi-continuous culture yielded relatively low lipid content at all levels of N limitation tested and, in fact, lipid content and lipid productivity were inversely related (Figure 4). To attain lipid productivity approximately equivalent the peak productivity in batch culture, N replete conditions were required, a condition in which there was likely no storage lipid accumulation. Rather, lipid productivity directly mirrored biomass productivity and the production of structural lipids.

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

however, as suggested by the research of Hsieh *et al.* (2009), may be capable of high productivity of high-lipid content biomass in semi-continuous culture.

4 Conclusions

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

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

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

Figure 3a (The Timing of Lipid Accumulation): The impact on lipid productivity of the timing of lipid accumulation relative to the timing of growth is evident in comparing *C. sorokiniana* and *N. oleoabundans*. The vertical, dotted line in each graph signifies the point

at which lipid accumulation began. *C. sorokiniana* grew and then accumulated lipids—only 15% of growth occurred as lipids were accumulating. *N. oleoabundans* grew and accumulated lipids at the same time—75% of growth occurred as lipids accumulated. Concurrent growth and lipid accumulation resulted in higher lipid productivity.

Figure 3b (Time-Series Data): Time-series data on growth, cellular N, lipid content, and lipid productivity for six species of oleaginous green algae. The data shows wide variation in the extent to which each species combined or separated their growth and lipid accumulation phases. The importance of harvest timing is clear, as an optimal harvest window may be brief, just prior to growth and lipid-accumulation cessation.

Figure 4 (Semi-Continuous Lipid Production): Because it required the least N stress to prompt lipid accumulation in batch cultures, *N. oleoabundans* was chosen to evaluate the effectiveness of semi-continuous culture for production of high-lipid content biomass. A limiting supply of N resulted in drastically diminished growth and only small increases in the lipid content of the biomass. In fact, lipid content and lipid productivity were inversely related across the scope of N supply rates studied. The data suggests that this cultural method is unsuitable for production of high-lipid content biomass.

Table 1 (Peak Values and Calculations): Peak values and calculations on algal density, lipid content and lipid productivity in 12 day batch cultures. Nearly three-fold differences among species were observed in growth between the high and low N stress treatments. Lipid content increased over baseline levels up to 377% and as little as 222% with high N stress. Even with low N stress, lipid increased from 46 to 302%. Lipid productivities reflect the independent effects of N supply on growth and lipid content on each species. To convert "per L" to "per m^2," multiply by 22.5 L m^{-2}.

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

All figures should be reproduced in color on the web and black and white in print.

Figure 1

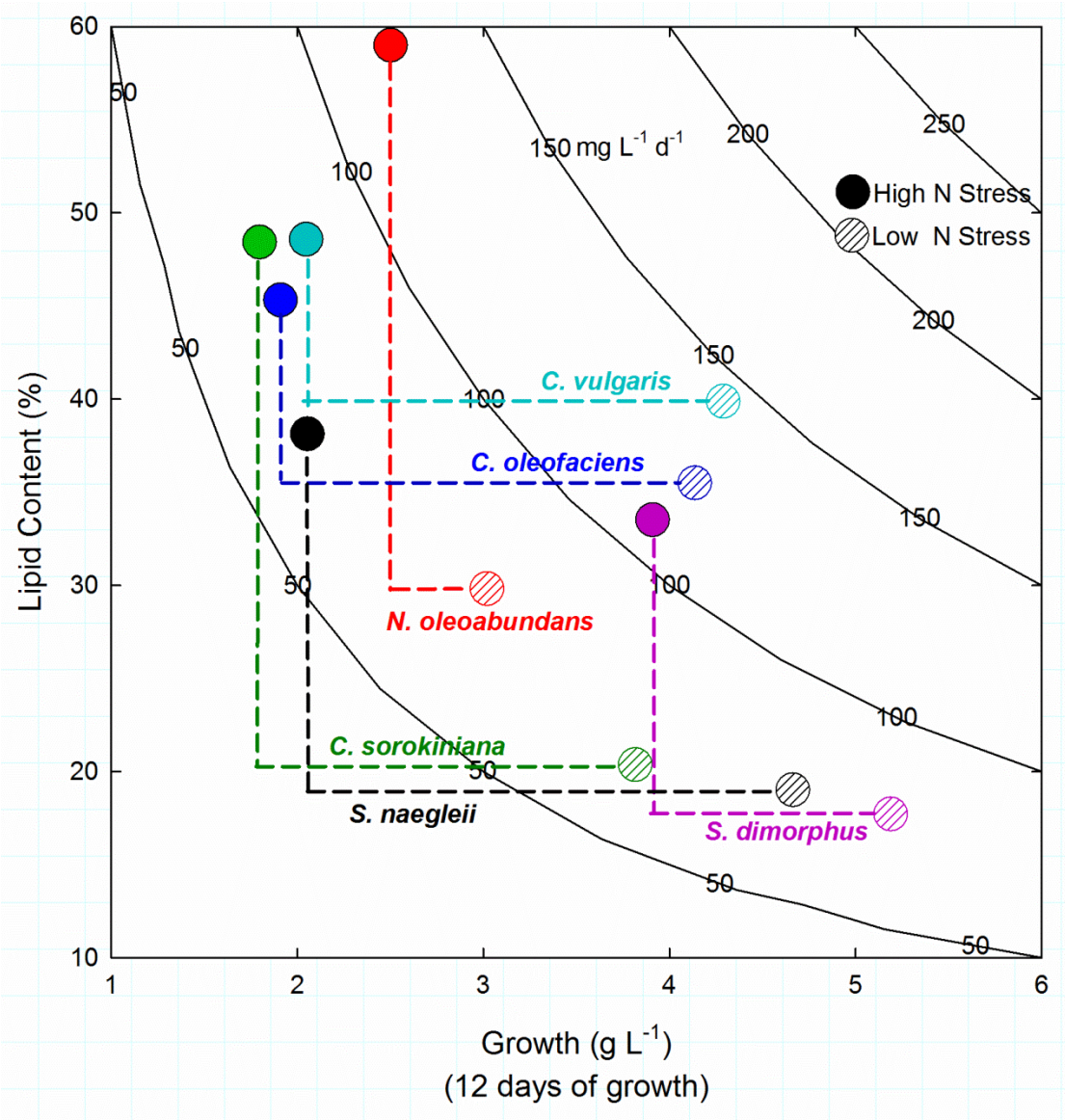


Figure 2

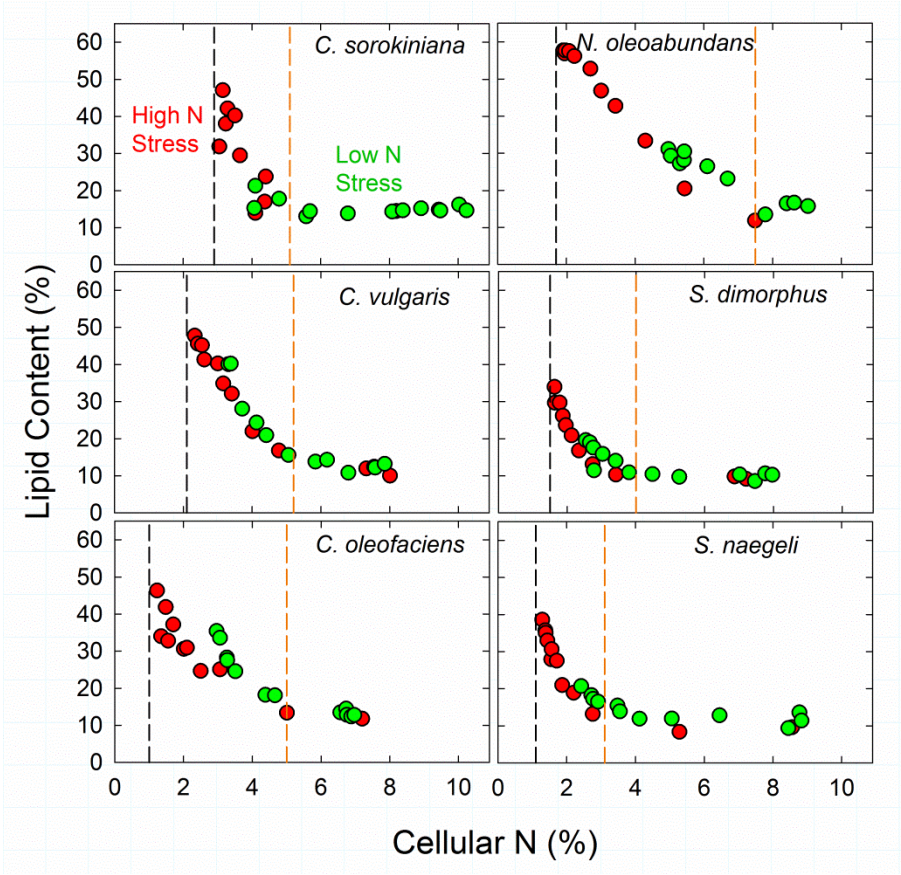
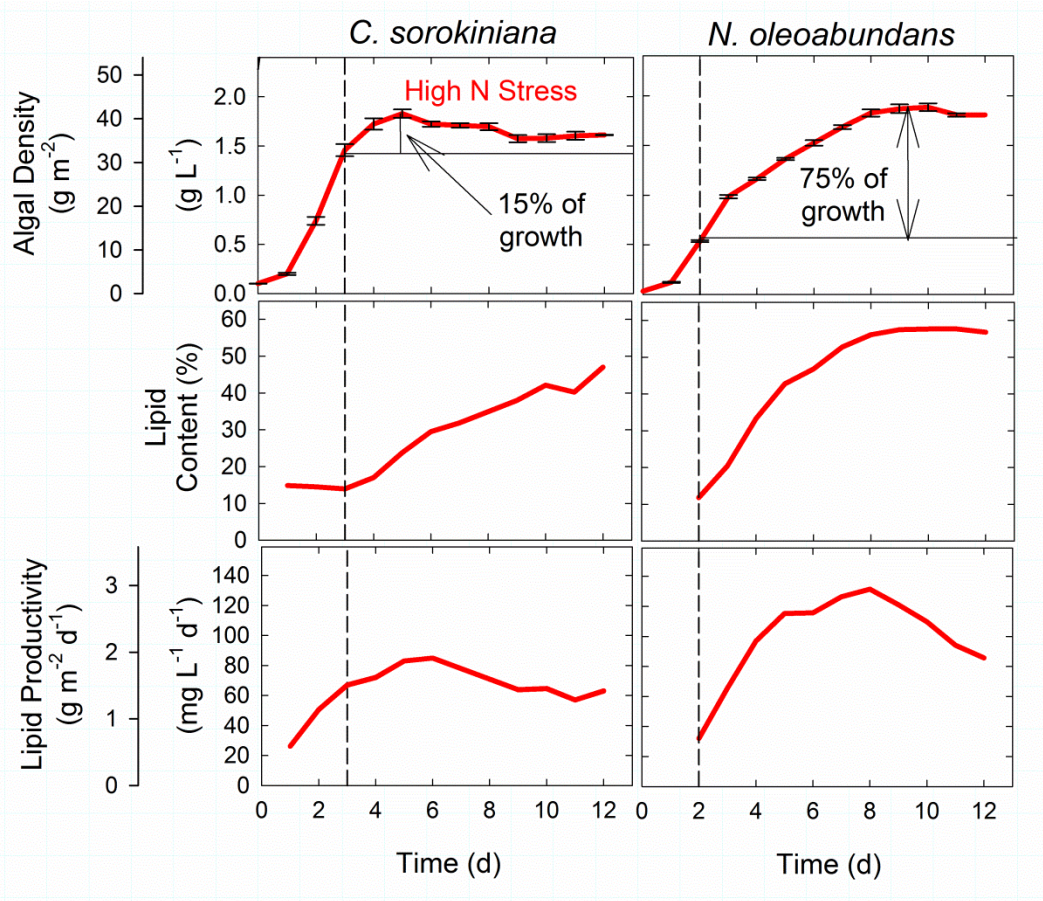

C. sorokiniana
N. oleoabundans
C. vulgaris
S. dimorphus
C. oleofaciens
S. naegeli
High N Stress
Low N Stress
Lipid Content (%)
Cellular N (%)
0
10
20
30
40
50
60
0
2
4
6
8
10

Figure 3a


C. sorokiniana
N. oleoabundans
High N Stress
15% of growth
75% of growth
Algal Density (g m-2)
(g L-1)
Lipid Content (%)
Lipid Productivity (g m-2 d-1)
(mg L-1 d-1)
Time (d)
Time (d)