Effect of Temperature Control on Green Algae grown under Continuous Culture

Carolann M. Knutson[‡†], Evelyn M. McLaughlin[†], Brett M. Barney[‡†]

[‡] Biotechnology Institute, St. Paul, MN 55108-6130

[†]Department of Bioproducts and Biosystems Engineering, 1390 Eckles Avenue, St. Paul, MN 55108-6130, 612-626-8751, bbarney@umn.edu.

* Correspondence to B. M. Barney

Keywords: *Algae, Scenedesmus dimorphus, Neochloris oleoabundans, Turbidostat, Temperature*

Abstract

The large-scale cultivation of algae of commercial value requires a variety of important energy-related inputs to achieve the proposed yields required for economic viability. One specific energy input of interest is temperature control and the associated costs of this control, both in terms of initial capital investment and costs associated with continued cooling or heating. In this work, the green algae *Neochloris oleoabundans* and *Scenedesmus dimorphus* were grown in tightly-controlled turbidostat-based photobioreactors to determine the potential benefits of temperature control for biomass production of these organisms versus allowing these cultures to experience temperature fluctuations similar to what would be found in uncontrolled, outdoor open ponds or closed bioreactors. The results of these studies indicate stark differences between these two strains, with *N. oleoabundans* yielding improved growth rates under conditions of stringent temperature control at 22˚C, while *S. dimorphus* yielded slightly higher growth under conditions where temperature was allowed to fluctuate based on modeled natural temperature profiles. Further analysis reveals that the utilization of non-conventional temperature profiles could enhance growth yields further for *N. oleoabundans*, allowing it to overcome the detrimental effects of the natural temperature fluctuation. These results and a discussion of the potential for turbidostat-based algal culture growth are presented.

Introduction

Microalgae comprise an important class of photosynthetic organisms that are able to fix carbon through photosynthesis and have the potential to serve as a source of pigments, lipids, proteins, and carbohydrates (1, 2). The large-scale commercial culture of algae for utilization as a source of either commodity or high-value products will require a variety of important capital cost investments and ongoing energetic inputs to achieve the yields required for economic viability (1, 3). For this reason, studies to elucidate the importance of various energy intensive inputs are invaluable in assessing the viability and economic feasibility of a particular strain of algae for commercial use. These potential high-cost inputs may be evaluated through a variety of low-cost approaches to determine how essential each input is to the culture productivity and evaluate the associated cost for growing that strain, both monetarily and energetically.

One specific energy input of interest is associated with the requirements for temperature control, as it has been shown to have a dramatic impact on algal growth (4, 5). Most large-scale algal bioreactors and open ponds or raceways are designed to operate outdoors, where the culture could be exposed to large fluctuations in internal temperature as a result of ambient air temperature, evaporation and internal heating of the culture related to radiant thermal energy absorption. While various approaches to bioreactor or pond design could mitigate issues associated with temperature fluctuations in the culture, the importance of these design parameters need to be assessed for each strain. Evaluations of the effect of temperature on other algal strains such as *Phaeodactylum tricornutum* (6) and *Dunaliella salina* (7) have been completed under batch growth conditions, while considerably less information is currently available related to growth in turbidostat grown reactors.

In this report, continuous culture experiments were conducted to determine the importance and role that temperature control played in the culture of two strains of green algae with potential commercial value. The two strains of algae selected for this study were *Neochloris oleoabundans* and *Scenedesmus dimorphus*, as the first has been targeted for lipid production and biofuels (8, 9), and the second, grown for various high-value co-products such as chlorophyll (10). To accomplish the goals of this study, side by side photobioreactors, operated as turbidostats, were constructed to provide simple, programmable and strictly-controlled laboratory experiments. Additionally, the studies described in this report illustrate the use of a turbidostat as a potential tool to evaluate algal growth under non-typical temperature profiles. Turbidostats are a valuable

tool for many non-photosynthetic organisms, where they have been used to assist in research related to bacterial antibiotic resistance and in synthetic circuit characterization (11, 12). Application of this combined turbidostat/photobioreactor system to algae has allowed us to grow strains under static and highly fluctuating temperature conditions where energy inputs could be altered and their effect characterized. The studies described in this report utilized controlled, turbidostat-operated, photobioreactors monitored over the duration of several days to assess the effects of maintaining a constant temperature versus allowing the culture to experience large temperature fluctuations. The potential benefits in elucidating the effects of temperature of this simple reactor system are described.

Materials and Methods

Algae Strains and Media

Neochloris oleoabundans UTEX 1185 and *Scenedesmus dimorphus* UTEX 417 were obtained from the UTEX Culture Collection of algae. Strains were grown on a low nitrogen freshwater medium containing 1.85 mM K_2HPO_4, 0.35 mM $MgSO_4 \cdot 7H_2O$, 0.15 mM $CaCl_2 \cdot 2H_2O$, 0.61 mM NaCl, 1.51 mM Na_2SO_4, 0.07 mM Ferric Ammonium Citrate, 5.88 mM $NaNO_3$, and adjusted to pH 7.6. Trace elements were added at a concentration of 2 mL/L of the freshwater medium. The composition of the trace element solution (per L) was 1 g boric acid, 1 g sodium EDTA, 200 mg $MnCl_2 \cdot 4H_2O$, 20 mg $ZnCl_2$, 15 mg $CuCl_2 \cdot 2H_2O$, 15 mg $Na_2MoO_4 \cdot 2H_2O$, 15 mg $CoCl_2 \cdot 6H_2O$ and 10 mg KBr. Several drops of polypropylene glycol (approximately 50 μL) was added to each liter of medium to minimize foaming. All chemical reagents were obtained from Sigma Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Pittsburgh, PA), unless stated otherwise.

Growth Conditions

Cultures were maintained as pure stocks by multiple passages on solid medium prior to transfer via sterile loop to the initial medium for inoculation of the reactor through the medium input line (Figure 1). Algal strains were subsequently grown in side-by-side, turbidostat operated, 1.2-L glass tubular reactors (51-mm inner diameter) with 1-mm inner diameter glass capillary tubes to provide constant aeration at a flow rate of 0.3 L air per minute per L of culture medium. Compressed air combined with 0.2% CO_2 was provided via mass flow controllers (Alicat, Tucson, AZ). The flow of the gases provided CO_2, gas exchange and mixing to the culture. Additional 1-mm inner diameter glass capillary tubes were used to provide fresh medium required for dilution of each culture. Each culture was provided light by three independent fluorescent light banks containing two T8 fluorescent lamps (2 ft length) in a stepwise fashion intended to mimic natural lighting conditions, and were placed on a timer to deliver a 14:10-hour light:dark cycle. The step function used for the three light banks is depicted on each figure. Reactors and light banks were held in place by a custom polycarbonate and wood structure and covered by white curtains to block external environmental lighting and reflect internal lighting. Under full artificial lighting, the surface of the reactors were receiving approximately 200 μmols

m^{-2} s^{-1} of light (MQ-200, Apogee Instruments, Logan, UT), with each light bank providing roughly 1/3 of the light.

Temperature control was provided via water circulation through the reactor jackets by two programmable water baths (VWR, Radnor, Pennsylvania). Initially, two temperature conditions were selected, termed constant and sinusoidal; the sinusoidal temperature profile was modeled after outdoor data collected from an open raceway pond operated during the months of May through July in Logan, Utah, so as to mimic natural temperature fluctuations. A constant temperature of 22°C was selected as a control, as these cultures are typically maintained at room temperature in the laboratory. After observing differences in growth rates between the constant and sinusoidal profiles for *N. oleoabundans*, further optimization was investigated with this specific culture. The optimal growth temperature of *N. oleoabundans* and *S. dimorphus* were determined through a temperature optimization experiment, in which the strain was subjected to a range of temperatures based on an 8 hour dark/light cycle; where each 8-hour period consisted of a four-hour dark period followed by a four-hour light period. Cultures were maintained at that temperature for at least three consecutive dark/light cycles, following one cycle to allow the culture to acclimate to the current temperature setting before taking any measurements. Based on the findings from the temperature profile of *N. oleoabundans*, we developed two rapid temperature profiles for this strain, termed *Rapid 1* and *Rapid 2*. In each of the laboratory experiments presented, excluding the temperature optimization test, cultures were grown for multiple days, with each day serving as a single replicate from 0 to 24 hours. In most cases, the first day of data was excluded from the analysis, as it was used to allow the culture to synchronize to the light and temperature cycles.

Turbidostat Control System

Reactor parameters were controlled and monitored via LabVIEW (National Instruments, Austin, TX) and operated under continuous culture conditions as a turbidostat, in which a constant density was targeted. To accomplish this, the LabVIEW program controlled the supply of power to a constant delivery peristaltic pump (Cole Parmer, Vernon Hills, IL) and, if culture density exceeded the set point, the culture would undergo dilution. The amount of fresh medium used for dilution was determined by storing the fresh medium reservoir on a balance, as depicted in Figure 1. To obtain *in situ* density measurements of the culture, an external “clamp” was designed in Tinkercad (Autodesk, San Rafael, CA) and 3D printed (MakerBot, New York City,

NY). Five white LEDs were utilized in conjunction with a photodetector, as shown in Figure 2, to measure the culture density through the reactor wall once every minute. Density readings were averaged over 30 events to reduce fluctuations in the data. The clamp was positioned on the neck of each reactor, above the jacketed region and secured with wire wrapped around the “anchor posts”, to keep the clamp in place around the tube during the experiments.

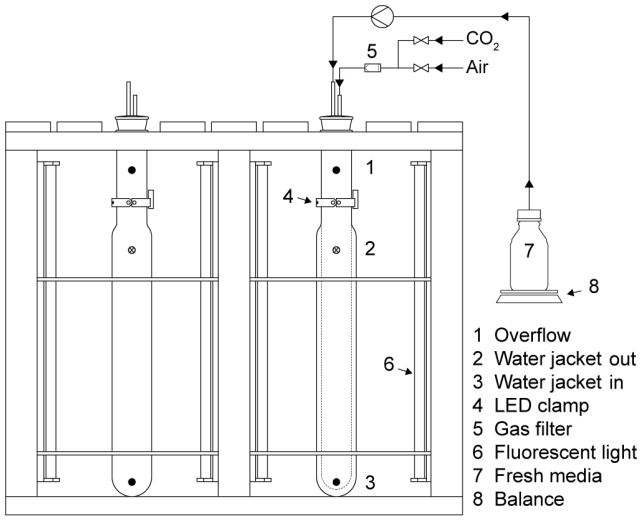

Figure 1. Schematic of the turbidostat system.

Cell Counts, Optical Density and Dry Weight Determinations

Optical density (OD) was measured at 600 and 750 nm and cell counts were correlated to density readings taken using the 3D printed clamp. Density set points were chosen such that each culture was maintained at a similar density. Cell counts were used to verify that the side by side reactors were comparable in growth and in agreement with OD readings. For each reactor, the overflow solution was collected from the reactor in an autoclaved collection bottle placed on ice. Algal cells in solution were then measured using a hemocytometer following the directions of the manufacturer (Hausser Scientific, Horsham, PA). OD scans, which were used to compare the quantities of algae in different samples, were performed from 200 nm to 800 nm on a Varian Cary Winuv 50 Bio UV-Visible Spectrometer with 1 mL of sample in a 1-cm path length quartz cuvette. Set points for the light sensor readings made with the clamps were adjusted so that each reactor yielded a similar optical density at 750 nm and similar cell numbers.

Dry weights were determined by collecting approximately 50 mL of culture and spinning the cells at 3500 g for 2 minutes. Supernatant was removed and cells were transferred to a pre-weighed 2 mL microfuge tube, and centrifuged again at > 10,000 g for 2 minutes. Supernatant was removed and cell pellets were frozen. Cells were lyophilized overnight, and the dry weights determined by measuring the centrifuge tube and dried cells. Final weight was determined by dividing the total mass of dry cells by the amount of culture collected for each sample.

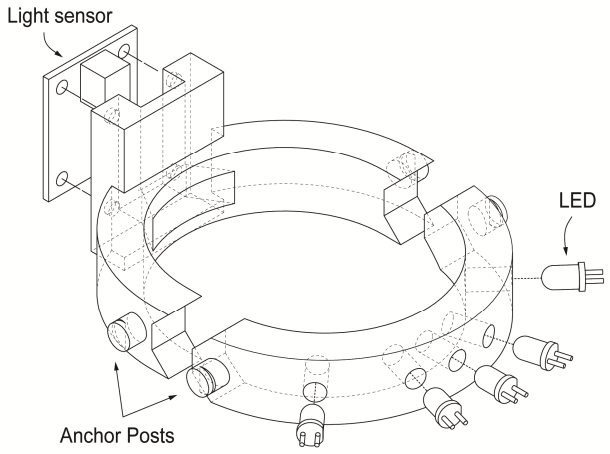

Figure 2. Features of 3D printed clamp used to take density measurements of algal cultures.

Results

Effect of temperature control versus temperature fluctuation

The two algae, *Neochloris oleoabundans* and *Scenedesmus dimorphus*, were grown in a custom turbidostat system where growth rate was measured as the volume of liquid introduced into the turbidostat to maintain a constant cell density. Data are presented as the fraction of the bioreactor culture medium exchanged. The fraction of medium exchanged is the quantity of fresh medium required for dilution relative to the total working volume of the reactor. Side by side reactors, as depicted in Figure 1, were utilized throughout this study to minimize the effects of variable external environmental lighting, and reactors were generally operated simultaneously with one under constant and the other under sinusoidal temperature conditions and were then switched such that any potential differences between the two reactors could also be accounted for in the combined data analysis.

The results from the growth of each of these algae at two different temperature profiles are presented in Figure 3. Based on the dilution curves, approximately 15% more medium was required for dilution of *N. oleoabundans* under a constant temperature of 22°C than what was required for growth following a sinusoidal temperature curve (Figure 3A). *S. dimorphus* showed an approximate 5% decrease in medium required for dilution under 22°C constant temperature versus what was required for growth following the sinusoidal temperature curve, illustrating a substantial difference between the two strains cultured in either manner.

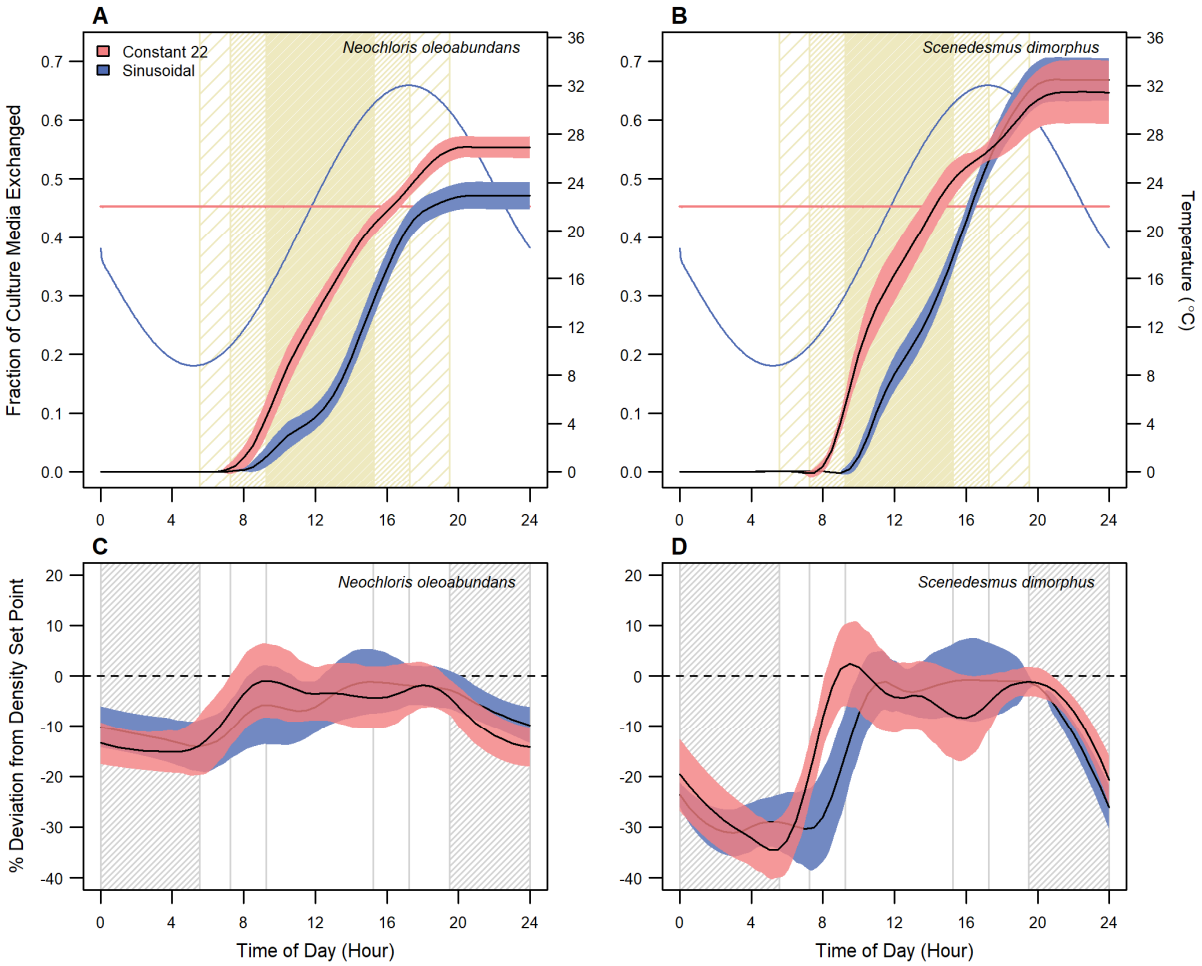

Figure 3. Fraction of culture medium exchanged and corresponding turbidity of *Neochloris oleoabundans* and *Scenedesmus dimorphus*. (A, B) Growth of each culture expressed as the fraction of culture medium exchanged over a 24-hour period. Yellow fill regions indicate the three intensities of light with full intensity in the center. (C, D) Density of each culture expressed as the percent deviation from the density set point. Grey fill regions represent periods of dark. Curves and standard deviations were generated in R using a LOESS regression with spans of 0.2 and 0.3 for *N. oleoabundans* (n = 14) and *S. dimorphus* (n = 8), respectively.

Following the initiation of the daily light cycle, a delay was observed before additional medium was required to maintain the culture density for both temperature conditions with each algal strain, as shown in Figures 3A and 3B. This delay between the initiation of the light cycle and required medium addition was assumed to be related to the loss of cell density during the dark cycle, and indicated a diurnal delay in dilution where the culture required recovery before reaching the target cell density. Both the constant and sinusoidal temperature profiles for *N. oleoabundans* and the constant temperature profile for *S. dimorphus* required approximately two hours to recover once the lights were triggered, whereas the sinusoidal profile for *S. dimorphus* required four hours for recovery. Since measurements using the clamp shown in Figure 2 provide an indication of cell density throughout the day, we were also able to track biomass losses through the dark period. As shown in Figure 3C and 3D, both strains showed a decline in measured optical density starting at hour nineteen, corresponding to when the lights were turned off. However, *S. dimorphus* showed a much more rapid and immediate decline than what was observed for *N. oleoabundans*. The sinusoidal conditions for *S. dimorphus* resulted in a small plateau in the loss of biomass starting at hour three, while the constant temperature for *S. dimorphus* continued to drop.

A further analysis of cell dry weight was made for the *S. dimorphus* culture at hour 5 and at hour 15, to determine if this drop in biomass obtained from the clamp optical density measurements correlated with the cell mass obtained at each condition. The *S. dimorphus* culture grown at constant temperature (22°C) resulted in a loss of ~26% of the dry cell weight over the dark cycle, while the sinusoidal culture resulted in a loss of ~42% of the dry cell weight (Supplemental Figure S1), indicating that the measured cell density using the custom clamp does correlate to a drop in cell mass for *S. dimorphus*. The constant temperature for *S. dimorphus* promptly began to increase in turbidity upon light introduction, while the sinusoidal curve required two hours before the recovery stage was initiated in this culture (Figure 3D). Following the initial decline, both of the densities for *N. oleoabundans* reached a stable plateau in loss of between 10-15%. Once the light was reintroduced, both profiles for *N. oleoabundans* recovered their density quickly and started growing immediately, even under low light, indicating that the influence of temperature on the recovery of the culture following dark period losses was not as pronounced as what was seen for *S. dimorphus*.

The measurement of fresh medium addition over time resulted in a plot of the growth rate for each culture (Figure 3A and 3B). For *N. oleoabundans* grown at constant temperature, there were three distinguishable rates of growth that could be fit to the overall growth plot between hours eight and nineteen. The initial rate of medium addition was the most rapid, starting when the lights were still at 67% of maximum illumination, and continued for about three hours, before slowing between hours eleven and fifteen when the light intensity dropped back to 67% of maximum intensity, and the growth rate dropped again. For *N. oleoabundans* grown under the sinusoidal temperature profile, the initial rate of growth was slower than what was found for constant temperature (seen in both Figure 3A and 3C), but switched to a period of maximum growth rate starting at about hour thirteen, when the temperature rose above 22°C, and then remained at this rate even as the light intensity dropped back to 67% of maximum intensity. A similar trend to what was observed for *N. oleoabundans* at constant temperature was seen for *S. dimorphus* at constant temperature, with three similar and distinct rates of growth over the course of each day. For the sinusoidal temperature profile, *S. dimorphus* started out with a rapid growth rate, which slowed for a short period between hours twelve and fourteen, before recovering and growing at a similar rapid rate between hours fourteen and nineteen. As described above, although there was a lag of two additional hours before medium addition began for *S. dimorphus* grown under the sinusoidal temperature curve versus constant temperature, the sinusoidal culture was able to overcome this delay and achieve a slightly higher final daily dilution total. *N. oleoabundans* was never able to overcome this gap. Additionally, both temperature conditions of *S. dimorphus* achieved a higher overall fraction of medium exchanged than what was seen for either growth condition with *N. oleoabundans*.

Optimization of biomass production for N. oleoabundans through temperature control

Due to the differences that were observed between the constant and sinusoidal conditions for *N. oleoabundans*, we expanded our studies with this particular strain to include additional temperatures profiles. These additional profiles were selected to determine if rapidly lowering the temperature during the dark phase of growth would limit biomass loss, and result in a higher biomass yield each day. To test this, we altered the temperature profile to include step-like functions in which overnight temperatures were maintained at either 4 or 8°C and rapidly increased to 24°C during the light cycle. An optimal temperature of 24°C for *N. oleoabundans* was selected based on a temperature optimization study (Supplemental Figure S2). Both reactors

were operated simultaneously under the same conditions for the temperature optimization experiments so that a comparison could be made between the two reactors.

Figure 4 shows the five different temperature profiles and the fraction of medium that was exchanged for each condition. The first two profiles (sinusoidal and constant 22) are those that were employed for the comparison study shown in Figure 3A. The third temperature profile shown used a constant temperature of 24°C which resulted in a slight improvement (almost 3%) versus constant temperature 22°C. The fourth temperature profile represented the first step function experiment, where initially the temperature was 8°C and starting at hour three, was rapidly warmed to 24°C over a three hour period and then held there until hour twenty, where over another three hour period, it dropped back down to 8°C (*Rapid 1*). This condition showed further improvement over the constant temperature profile at either 22°C or 24°C (almost 6% improvement over constant temperature 24°C). The final temperature profile represented a more dramatic step function, where the temperature was rapidly lowered over a one hour period down to 4°C to see if this would lead to an even more pronounced improvement in cell growth (*Rapid 2*). The culture did not respond as well to this temperature profile, achieving total cell yields similar to what was seen for constant 24°C. A comparison of the growth rates and drop in cell density overnight is shown in Figure 5, where *Rapid 1* showed a gradual loss in cell density, but then plateaued between 0 and 6 hours, while *Rapid 2* continued to drop, indicating that the further drop in temperature was more detrimental to this culture under these enhanced cooling conditions.

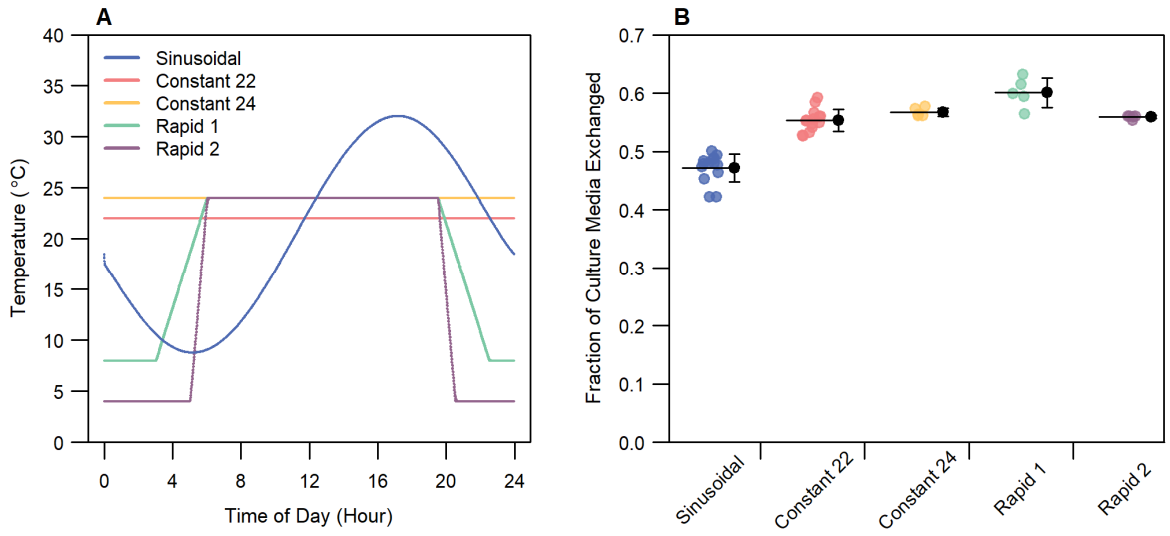

Figure 4. Comparison of temperature profiles and normalized total daily fraction of culture exchanged for *N. oleoabundans* under various temperature conditions. (A) Temperature profiles and (B) total daily fraction of culture exchanged for each reactor (average ± standard deviation, n ≥ 5). Sinusoidal and Rapid 1 were found to differ from all other conditions and one another (statistically significant with $p \leq 0.05$ by Welch's t-test for all combined samples in both reactors).

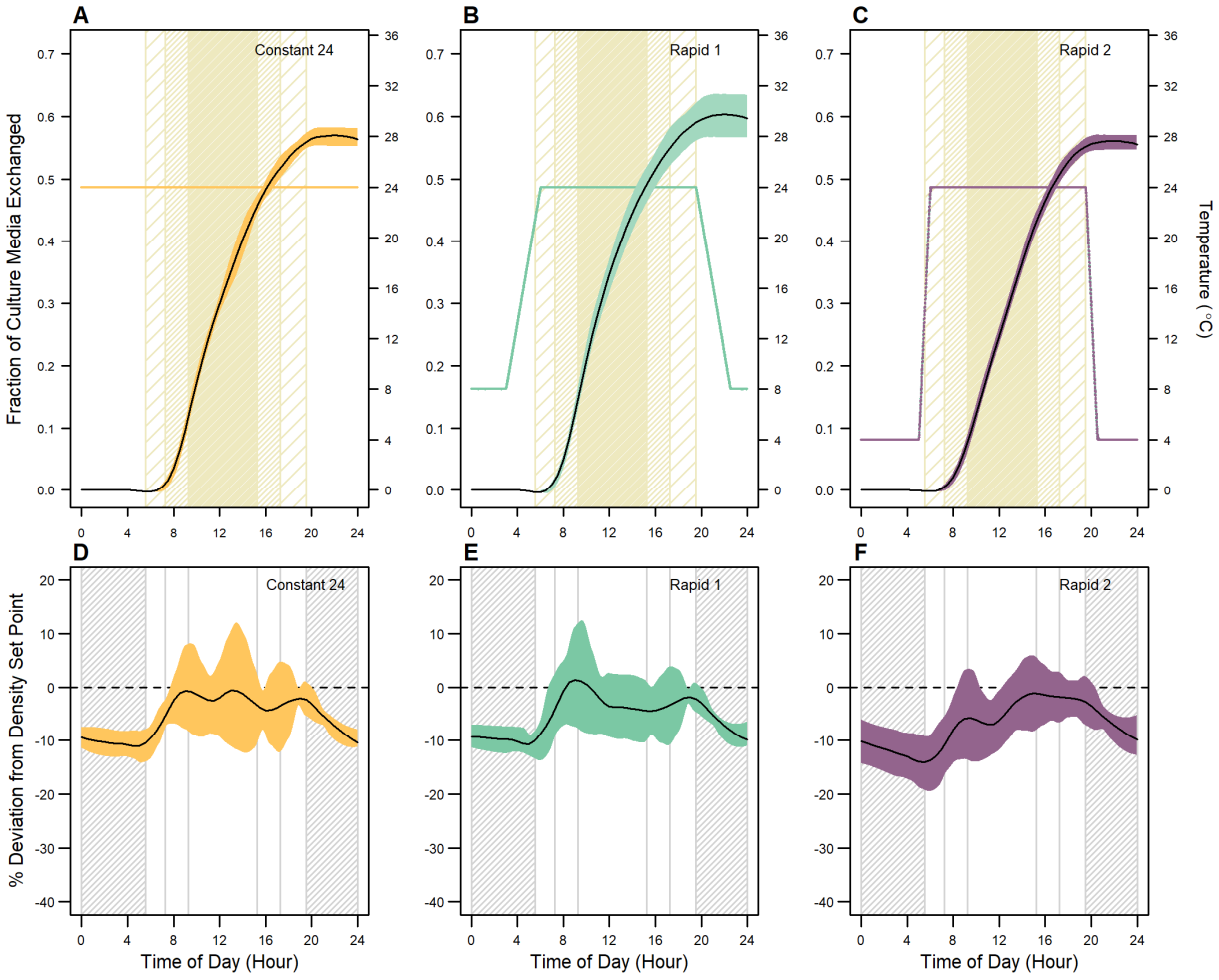

Figure 5. Fraction of culture medium exchanged and corresponding turbidity of *Neochloris oleoabundans*. (A-C) Growth expressed as the fraction of culture medium exchanged over a 24-hour period for the three alternative growth conditions (Constant 24, Rapid 1 and Rapid 2). Yellow fill regions indicate the three intensities of light with full intensity in the center. (D-F) Density expressed as the percent deviation from the density set point for the corresponding conditions in A-C. Grey fill regions represent periods of dark. Curves and standard deviations were generated in R using a LOESS regression with a span of 0.3 (n = 5).

Discussion

The primary goal of these experiments was to compare and contrast the effect of differing temperature control on the growth of two species of green algae when grown under continuous culture conditions. *Neochloris oleoabundans* and *Scenedesmus dimorphus* were selected for these studies as they have been target strains in multiple studies for the production of various high-value bioproducts or biofuels and are model algae that have been cultivated in our laboratory for many years (8-10, 13, 14). The first step in these studies was to construct a photobioreactor system that allowed us to test two different growth conditions in parallel and maintain the growth of photosynthetic strains with an automated system operated as a turbidostat, where culture density was maintained below a constant threshold through dilution of the culture. Turbidostat systems provide an added degree of control over batch culture, and are optimized for delivering the maximum culture yield at a specific cell density. The ability to concurrently test different temperature profiles in similar reactors allowed us to probe the effect of maintaining a constant temperature versus a fluctuating temperature, similar to what has been found for algae grown in open ponds outdoors with or without minimal temperature control. The fluctuating, or sinusoidal, temperature profile utilized in this study was based on data obtained from cultures grown outdoors in Logan, Utah in either open ponds or closed bioreactors with minimal temperature control. The 22°C control temperature was selected as it mirrors the laboratory conditions in which these strains have been maintained for more than 5 years, though we acknowledge that the optimal growth temperatures for both strains (24°C for *N. oleoabundans* and 26°C for *S. dimorphus*, respectively, Figures S2 and S3) differs from this initial setting. The benefit of using a laboratory based system was that it allowed us to replicate these experiments without concern for the potentially large fluctuations in light intensity that could occur if the experiment were performed outdoors under natural lighting, and allowed for taking measurements year round.

Comparisons of growth rates with two model green algae using conventional temperature profiles

In these experiments, two strains of algae utilizing identical growth parameters were selected to see how these strains might differ in their behavior under similar temperature regimes. Both strains showed a more immediate and rapid rate of growth following initiation of the light cycle each day when grown at a constant temperature. When grown with a sinusoidal temperature

profile, where the light was introduced near the coldest point in the temperature profile, both cultures showed some degree of a slower growth response, as expected, though this manifested itself differently for each strain. For *N. oleoabundans*, the culture responded to the initiation of light with an immediate increase in biomass, although the rate of biomass production was slower than what was found for the constant temperature condition (Figure 3A and 3C). This rate remained lower until hour 13, when the sinusoidal temperature had surpassed the constant temperature, after which time the rate of growth increased rapidly until the light intensity dropped to 33% of maximum intensity. Comparatively for the *S. dimorphus* culture, growth lagged 2 hours behind light introduction (Figure 3D), but once biomass production began, it continued at a rate very similar to what was seen when grown at a constant temperature and outperformed the constant temperature culture by the end of the day. This indicates that maintaining the 22°C constant temperature provided no real benefit to the *S. dimorphus* culture versus allowing the culture temperature to fluctuate for this specific strain.

The results obtained from the constant temperature growths were counter-intuitive to what one might expect based on available photons. We anticipated that the growth rate would correlate more tightly with levels of light intensity, with maximum growth occurring during the six-hour period of maximum light intensity around midday. However, both strains appeared to experience maximum growth starting in the earlier hours of the light cycle when the culture was still cycling between 67% of maximum and maximum light intensity. These results could be interpreted several ways. The culture could be experiencing growth inhibition due to the elevated light levels (photo-inhibition). Alternatively, the culture may have depleted a key internal metabolite or cofactor, or reached a point where maintenance of the photosynthetic apparatus is limiting cell growth and replication from what was seen during the initial phase of growth, and had instead become unable to take advantage of the additional available light to maintain a maximal growth rate for the entire day. A final option is that this is a preprogrammed aspect of the synchronous cell cycle. Since the light levels provided to the culture are well below what would be found for full sunlight if grown outdoors (approximately 10% of full outdoor sunlight under full lighting), we favor one of the other two potential scenarios as the more plausible explanations. This was supported by the fact that both strains experienced a further decline in growth rate between hours 15 and 17, when the light intensity returned to the same level (67% of maximum intensity) that was seen earlier in the day during the period of maximal growth

(between 8 and 10 hours, Figure 3A and 3B). Based on these results, it seems that the cultures were only able to take advantage of the maximal rate of growth for a few hours at the start of the day, before additional factors other than temperature or available light limited continued biomass production at the maximum rate.

Potential of unconventional temperature profiles to improve growth rates with Neochloris oleoabundans

Building from the findings of the experiment testing two temperature profiles (Figure 3), we sought to further determine if alternative profiles could potentially result in an increase in cell growth. Since the cultures were grown under photoautotrophic conditions with a light and dark cycle, it is quite likely that the culture shifted to a heterotrophic metabolism overnight when light availability was minimal, and the cell relied upon internal energy reserves to maintain metabolism. One consequence of this shift in metabolism is that the cell would deplete energy reserves that were synthesized during the light cycle, which should have limited the ability of the culture to immediately begin to replicate once light was again available. These postulates are supported by the fact that a drop in biomass density was seen for both cultures over the dark period each day (Figure 3C and 3D). We predicted that by rapidly lowering the temperature, versus the slow sinusoidal temperature profile that was used in the experiments to model natural fluctuations shown in Figure 3, we might be able to slow down this depletion of energy reserves during the dark cycle, and as a result, achieve a higher total daily cell yield. Since the *N. oleoabundans* had shown a significantly lower total daily cell yield under the sinusoidal growth curve, we selected this strain as our test case. By utilizing a more rapid decrease in temperature and then holding a steady temperature of 8 °C throughout most of the dark hours (*Rapid 1*), we achieved a small increase in total volume exchanged versus growth at constant temperature of 24 °C (Figure 4). An alternative profile, *Rapid 2*, containing a further drop to 4 °C and a more rapid change in temperature between dark and light cycles, had a negative effect, resulting in a decrease in total daily cell yield versus *Rapid 1*. We interpreted this result to indicate that while lowering the metabolism during the dark did result in a positive effect at 8 °C, a lower temperature might inhibit key biosynthetic processes necessary to prepare the culture to take maximum advantage of the following light cycle. A more thorough analysis of the data from the rapid experiments (Figure 5) showed that all three of the alternative conditions tested (Figure 4)

showed similar losses in cell density during the night, but each showed a different rate of growth once lights were initiated the following morning. These results also highlight that biomass could be increased further by using alternative, and non-conventional temperature control strategies, which will be studied in further detail in the future.

Considerations and limitations of turbidostats for the culture of photoautotrophs

One drawback of a turbidostat system for the growth of algae or other photoautotrophs is that turbidostats operate by diluting the culture when the cell density exceeds a specific threshold. However, the turbidostat is not capable of maintaining this density during the dark cycle without the addition of some sort of cell-concentrating step, which would be difficult to maintain since the bioreactor working volume is fixed in our system as it would be for most large-scale outdoor bioreactors or open/raceway ponds. As the system was still capable of measuring cell density during this dark cycle, there was still significant data available for interpretation during this dark period, as we show in Figure 3C and 3D. The analysis of this loss in cell density during the dark hours could be beneficial in studies of key parameters to maximize cell yield in a turbidostat, and these key measurements should also be taken into account as part of the data interpretation for any photoautotroph grown with light/dark cycles.

Implications of this study and relationship to prior studies of temperature control

The primary objective in this study was to compare the benefits or drawbacks of temperature control versus allowing a culture to follow a sinusoidal temperature curve that would be expected if growing outdoors with minimal temperature control. The ability to heat or cool a culture comes with an added cost in energy which will fluctuate drastically based on the surrounding environment where the culture is being cultivated. Two general costs would be associated with maintaining specific temperatures in the culture. The first would be the initial capital investment associated with design and incorporation of the temperature control system. The second would be due to the addition or removal of thermal energy from the culture, which would be a continual cost associated with each growth. While the first cost is a one-time expenditure associated with maintenance of the system, the energy input cost will vary based on time of year, geographical location, and constantly changing weather conditions.

Algorithms to model the various factors that contribute to culture temperature in both outdoor bioreactors and outdoor raceway ponds have been developed and refined (15-19), and in some cases, these models have been validated by long-term measurements that collect real-world data (18). Based on these algorithms, costs can be calculated for the operation of bioreactors or raceway ponds in a variety of geographical locations. The sinusoidal temperature profile selected here was based on real-world measurements obtained from a bioreactor operated outdoors in Logan, Utah between May and July. However, this curve would likely need to be adapted based on the physical parameters and location of any proposed outdoor system meant to culture algae. By utilizing accurate models to predict the temperatures that the culture will experience based on the design parameters of the bioreactor, the geographical location and the time of year, a simulated temperature profile could be generated, which could be incorporated into a simple turbidostat reactor such as was employed in these studies. The combination of these tools would allow one to determine the most effective strains to operate under those conditions, and minimize the potential costs associated with heating and cooling the bioreactor, or develop non-conventional heating and cooling strategies to maximize growth by comparing the results from incremental changes from this natural fluctuation.

By using a turbidostat, we received immediate feedback about the growth rate and by inference, the overall health of the bulk culture. Using an automated system that controls as well as allows for simple modifications of parameters such as temperature, light, and gas composition and delivery, we could test cells under conditions not generally found in nature. This makes it possible to further test approaches to improve yield without incurring large costs associated with a large-scale culture. The use of photobioreactors that also operate in a turbidostat mode is not as common as batch reactor systems, though they have been used previously to test the effects on photosynthetic efficiency using unnatural lighting schemes (20). Our results demonstrate potential benefits that could be obtained using unnatural temperature profiles with one specific strain, and also point toward additional phenomena that could be tested in the future using unnatural lighting schemes that could only be achieved in a laboratory setting.

Acknowledgements

This work was supported in part through a career award to B.M.B. (RC-0007-12) from the Initiative for Renewable Energy and the Environment, a program of the Institute on the Environment at the University of Minnesota, supported by the State of Minnesota and Minnesota electricity ratepayers, Minnesota Agricultural Experiment Station MIN-12-070 and MIN-12-081 and the United States Department of Energy DE FG3608GO18161. We thank the Microbial Engineering program at the University of Minnesota for additional support to C.M.K. We thank Jack O'Brien and Leland Martineau for efforts related to initiation of the construction of the turbidostat reactor and preliminary studies related to temperature constraints with these two strains. We thank Yaniv Brandvain for helpful suggestions related to statistical analysis of the data.

Contributions

C.M.K and E.M.M constructed the turbidostat bioreactor system, ran experiments and wrote the manuscript. B.M.B conceived initial experiments, supervised the project and wrote the manuscript.

References

1. **Hariskos I, Posten C.** 2014. Biorefinery of microalgae - opportunities and constraints for different production scenarios. Biotechnol J **9:**739-752.
2. **Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A.** 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J **54:**621-639.
3. **Wijffels RH, Barbosa MJ.** 2010. An Outlook on Microalgal Biofuels. Science **329:**796-799.
4. **Guedes AC, Amaro HM, Pereira RD, Malcata FX.** 2011. Effects of Temperature and pH on Growth and Antioxidant Content of the Microalga *Scenedesmus obliquus*. Biotechnol Prog **27:**1218-1224.
5. **Juneja A, Ceballos RM, Murthy GS.** 2013. Effects of Environmental Factors and Nutrient Availability on the Biochemical Composition of Algae for Biofuels Production: A Review. Energies **6:**4607-4638.
6. **Santos-Ballardo DU, Rendón-Unceta MD, Rossi S, Vázquez-Gómez R, Hernández-Verdugo S, Valdez-Ortiz A.** 2016. Effects of outdoor cultures on the growth and lipid production of *Phaeodactylum tricornutum* using closed photobioreactors. World J Microbiol Biotechnol **32:**128.
7. **Wu Z, Duangmanee P, Zhao P, Juntawong N, Ma CH.** 2016. The Effects of Light, Temperature, and Nutrition on Growth and Pigment Accumulation of Three *Dunaliella salina* Strains Isolated from Saline Soil. Jundishapur J Microbiol **9:**9.
8. **Li YQ, Horsman M, Wang B, Wu N, Lan CQ.** 2008. Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. Appl Microbiol Biotechnol **81:**629-636.
9. **Pruvost J, Van Vooren G, Cogne G, Legrand J.** 2009. Investigation of biomass and lipids production with *Neochloris oleoabundans* in photobioreactor. Bioresource Technol **100:**5988-5995.
10. **Ferreira VS, Pinto RF, Sant'Anna C.** 2016. Low light intensity and nitrogen starvation modulate the chlorophyll content of *Scenedesmus dimorphus*. J Appl Microbiol **120:**661-670.
11. **Takahashi CN, Miller AW, Ekness F, Dunham MJ, Klavins E.** 2015. A Low Cost, Customizable Turbidostat for Use in Synthetic Circuit Characterization. ACS Synth Biol **4:**32-38.
12. **Toprak E, Veres A, Yildiz S, Pedraza JM, Chait R, Paulsson J, Kishony R.** 2013. Building a morbidostat: an automated continuous-culture device for studying bacterial drug resistance under dynamically sustained drug inhibition. Nat Protoc **8:**555-567.

13. **Jiang YL, Zhang W, Wang JF, Chen Y, Shen SH, Liu TZ.** 2013. Utilization of simulated flue gas for cultivation of *Scenedesmus dimorphus*. Bioresource Technol **128:**359-364.
14. **Villa JA, Ray EE, Barney BM.** 2014. *Azotobacter vinelandii* siderophore can provide nitrogen to support the culture of the green algae *Neochloris oleoabundans* and *Scenedesmus sp.* BA032. FEMS Microbiology Letters **351:**70-77.
15. **Béchet Q, Shilton A, Fringer OB, Muñoz R, Guieysse B.** 2010. Mechanistic Modeling of Broth Temperature in Outdoor Photobioreactors. Environ Sci Technol **44:**2197-2203.
16. **Béchet Q, Shilton A, Guieysse B.** 2014. Full-Scale Validation of a Model of Algal Productivity. Environ Sci Technol **48:**13826-13833.
17. **Béchet Q, Shilton A, Guieysse B.** 2016. Maximizing Productivity and Reducing Environmental Impacts of Full-Scale Algal Production through Optimization of Open Pond Depth and Hydraulic Retention Time. Environ Sci Technol **50:**4102-4110.
18. **Béchet Q, Shilton A, Park JBK, Craggs RJ, Guieysse B.** 2011. Universal Temperature Model for Shallow Algal Ponds Provides Improved Accuracy. Environ Sci Technol **45:**3702-3709.
19. **Endres CH, Roth A, Brück TB.** 2016. Thermal Reactor Model for Large-Scale Algae Cultivation in Vertical Flat Panel Photobioreactors. Environ Sci Technol **50:**3920-3927.
20. **Vejrazka C, Janssen M, Streefland M, Wijffels RH.** 2012. Photosynthetic efficiency of *Chlamydomonas reinhardtii* in attenuated, flashing light. Biotechnol Bioeng **109:**2567-2574.