Optimization of Growth Conditions for the Model Bacterium *Synechococcus sp.* PCC 7002 for Chemical Production Using Wastewater-Based Media

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ABSTRACT

The term “cyanobacterial biorefinery” refers to the application of genetically engineered cyanobacteria for production of chemicals that could serve as an alternative source to petroleum derivatives. Various life cycle assessments have indicated that nutrient sources for cell cultivation are a major limitation of the cyanobacterial biorefinery concept from both cost and environmental impact perspectives. Wastewater streams have previously been identified as possible promising sources of liquid macronutrients, but little work has investigated their use for axenic cyanobacterial culture. In these experiments, we grew the model cyanobacterium *Synechococcus* sp. PCC 7002 in media comprised of two municipal wastewater streams: anaerobic digester effluent (“GBF”) and secondary treatment clarifier effluent. Temperature, light intensity, and media composition effects on cytotoxicity and growth rate were investigated in two engineered strains of *Synechococcus* 7002. At 27°C, we were able to ameliorate most of the toxicity and growth inhibition observed in cells grown at high GBF concentration and 37°C, the latter which is considered optimal temperature for growth in minimal media. We also found significant interaction between factors, which indicates the importance of co-optimization of environmental conditions and suggests several possible causal mechanisms. This work represents an important first step towards developing sustainable alternatives for resource recovery from wastewater while reducing dependence on petroleum feedstocks for chemical production.
ACKNOWLEDGEMENTS

I would like to thank the many people who were instrumental in completing the work found in these pages and more broadly, my master’s degree.

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1 INTRODUCTION

The previous century saw a proliferation of uses for petrochemicals beyond high energy density fuels in practically all market sectors\(^1\). Since as early as 1905\(^2\), however, the public and scientific community have recognized the limitations of petroleum as a long-term source of these important compounds given the inevitability of eventual proven reserves depletion\(^3\) and, more recently, greenhouse gas emissions linked to their extraction, refinement, and use\(^4-8\).

Concurrently, wastewater treatment moved from a field primarily concerned with human health concerns to one that also tackled ecosystem impacts, namely surface water eutrophication. Driven in large part by the Federal Water Pollution Control Act of 1948, which was significantly rewritten as the Clean Water Act of 1972, wastewater treatment facilities have treated effluent to increasingly more stringent levels of nutrient removal over the last half century. More recently, municipal wastewater treatment has seen a rebranding and vision alignment as “water resource recovery” facilities as they strive to achieve energy neutrality\(^9\) and improve nutrient recovery\(^10,11\), as opposed to solely removal, in a localized circular economy\(^12\).

Addressing water stress, global climate change, and energy sources rank high for both the impact and probability of failure according to the World Economic Forum\(^13\). An appealing option to address petrochemical demand while reusing wastewater nutrients is the cyanobacterial biorefinery. Under this model, photoautotrophic bacteria capture energy from sunlight and fix carbon dioxide. Under some formulations, biocrude is produced by hydrothermal liquefaction, as in the case of some algae-based systems\(^14\). Alternately, bacteria can directly produce targeted
organic compounds ("bio-based chemicals"), which are then separated and feed into current chemical catalysis processes for production of a greater diversity of products\textsuperscript{15}.

By coupling the cyanobacterial biorefinery with wastewater treatment and post-combustion carbon sequestration, the system allows for the valorization of resource recovery from waste streams. This is accomplished while using less energy\textsuperscript{16}, nutrients, and water\textsuperscript{17} than separate algal biofuel production and wastewater treatment, which is positive from both economic and ecological perspectives\textsuperscript{18}.

The model bacterium \textit{Synechococcus sp.} PCC 7002, henceforth \textit{Synechococcus} 7002, has been utilized in a number of studies to investigate cyanobacterial metabolism and biotechnological applications\textsuperscript{19–26}, in large part due to its relatively rapid doubling time of 2.6 hours under optimal growth conditions.\textsuperscript{27} In addition, it is both euryhaline\textsuperscript{28} and has one of the highest tolerances for light intensity among cyanobacteria\textsuperscript{21}. These characteristics along with robust constitutive mechanisms to ameliorate oxidative stress\textsuperscript{27,29} make it a well-suited basis for a cyanobacterial biorefinery.

Previous work has explored algal cultivation on wastewater for nutrient removal and resource recovery\textsuperscript{30–34} and algae processes have entered pilot\textsuperscript{35} and full-scale\textsuperscript{36} wastewater treatment. Likewise, the use of photoautotrophic bacteria for waste stream treatment has been investigated\textsuperscript{37–40}. However, very little work has been dedicated to exploring the growth of axenic cyanobacteria for the same purposes\textsuperscript{41–43} and to our knowledge, none has explored the use of engineered strains. Thus, this work explores the feasibility of \textit{Synechococcus} 7002 growth in wastewater and the differences in growth and viability as compared to minimal media.
2 MATERIALS AND METHODS

2.1 Bacterial Strains

Two engineered strains derived from wild-type *Synechococcus* 7002, both constructed by Travis Korosh (University of Wisconsin-Madison), were used throughout for these experiments. *Synechococcus* 7002 ΔglpK GmR, henceforth *Synechococcus* 7002 GmR, constitutively expresses a gentamycin resistance cassette in the SYNPCC7002_A2843 (glpK) gene locus, but has growth rates which are indistinguishable from the wildtype (T. Korosh, personal communication, 2015). The other mutant used is *Synechococcus* 7002 ΔglpK UspA GmR, henceforth *Synechococcus* 7002 UspA. It incorporates copies of the *slr0670* gene cloned from *Synechocystis* sp. PCC 6803, encoding universal stress protein A (UspA) under control the IPTG-inducible P_{lac}094lac promoter system,

2.2 Media

2.2.1 Minimal Media Controls

*Synechococcus* 7002 grew in Medium A+ supplemented with 4 μg·L^{-1} vitamin B12 and pH adjusted to 8.0 (henceforth “Medium A+”) as a control in each experiment.

Medium A+ is buffered using the organic compound trisamonimethane, commonly known as “tris.” Given the importance of the nutrient profile of the culture medium to this work, it was important to determine that tris, which contains an amine, could not serve as a nitrogen source for *Synechococcus* 7002. Therefore, we conducted several experiments in which tris served as the only
nitrogen-bearing compound in the growth medium. In these experiments, sodium nitrate was excluded from Medium A+ to create what we termed “Medium A-”.

2.2.2 Wastewater-derived media

Since a primary objective of this work was to couple nutrient recovery with carbon capture, the majority of treatments were grown in a media comprised primarily of wastewater streams from Nine Springs Wastewater Treatment Plant (Nine Springs WWTP).

2.2.2.1 Source of Wastewater for Cultivation

Madison Metropolitan Sewerage District (MMSD) serves the majority of municipalities in Dane County, Wisconsin, including the city of Madison, from the Nine Springs Plant. The majority of its total flow of 40 million gallons per day (MGD) is discharged to Badfish Creek, which is a tributary to the lower Rock River. Both the Badfish Creek and Rock River are listed as impaired waters under 303(d) of the Clean Water Act and have phosphorus Total Maximum Daily Loads (TMDLs). A smaller portion of the plant’s flow returns to the other watershed that is within the District’s service area: Badger Mill Creek. While MMSD is regulated on ammonia, the effluent is not regulated on total nitrogen. These regulations inform both mainstream and sidestream characteristics.

Four of Nine Springs Treatment Plant’s five mainstream trains operate according to a Modified UCT (University of Cape Town) configuration, with one A/O (anoxic/oxic) mainstream train. This produces effluent with negligible soluble reactive phosphorus but moderate concentrations of nitrate (Table 2-1).
Table 2-1 Characteristics of Gravity Belt Filtrate and Secondary Clarifier Effluent time series, each spanning approximately 6 months.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Mean</th>
<th>Coefficient of Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBF Ammonia</td>
<td>919 mg NH₃ - N L⁻¹</td>
<td>22%</td>
</tr>
<tr>
<td>Nitrate</td>
<td>3.93 mg NO₃⁻ - N L⁻¹</td>
<td>43%</td>
</tr>
<tr>
<td>SRP</td>
<td>54.1 mg P L⁻¹</td>
<td>27%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Mean</th>
<th>Coefficient of Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secondary Clarifier Effluent Ammonia</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Nitrate</td>
<td>19.3 mg NO₃⁻ - N L⁻¹</td>
<td>6.6%</td>
</tr>
<tr>
<td>SRP</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

The Nine Springs Treatment Plant uses anaerobic digestion for solids stabilization, with the post-digester solids being land applied. To minimize unintended struvite production in the digester and maximize phosphorus recovery, waste activated sludge (WAS) enters an anaerobic chamber along with an acid sludge feed. Together, this causes phosphorus release from intracellular stores into solution, in a process termed “WASSTRIP”⁴⁷. The WAS then passes over a gravity belt thickener. The WAS solids and primary clarifier solids enter two phase digestion. Under acid digestion (HRT = 1.3 days), there is rapid production of volatile fatty acids (VFAs), some of which is fed to the WAS, per above. More complete digestion and methane production occur in a mesophilic anaerobic digester (HRT = 27 days). Effluent is dosed with an organic coagulant (Praestol K 144 L) and ferric chloride (710 mg FeCl₃ L⁻¹) before running over a gravity belt thickener for initial solids separation. The solids are further dewatered in preparation for land application, while the anaerobic digester gravity belt filtrate mixes with the WAS gravity belt filtrate in preparation for struvite harvesting. Effluent from the Ostara struvite harvesting process circulates to the head of the plant.
2.2.2.2 Wastewater Collection

Two streams within the Nine Springs Plant were used as a growth media in these experiments. The majority of macronutrients (i.e. nitrogen and phosphorus) were provided by the filtrate from the anaerobic digester gravity belt thickener (henceforth “GBF”), while effluent from the post-mainstream secondary treatment clarifier (hereafter “secondary effluent”) served as a diluent.

In these experiments, secondary effluent was collected one to four days before their commencement and held under refrigeration at approximately 2°C. All secondary effluent came from clarifiers fed entirely by modified UCT mainstream processes.

The GBF used in these experiments was collected as a single large batch, except as otherwise noted. Upon return to the lab, it was filtered through a paper filter to remove any exceptionally large flocs, then stored at -80°C until use. Characteristics of this GBF batch are in Table 2-2.
Table 2-2 Characteristics of GBF used for all cultivation experiments except as otherwise noted

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>1180 mg NH$_3$-N L$^{-1}$</td>
</tr>
<tr>
<td>Nitrate</td>
<td>7.48 mg NO$_3$-N L$^{-1}$</td>
</tr>
<tr>
<td>Soluble Reactive Phosphorus</td>
<td>78 mg L$^{-1}$</td>
</tr>
<tr>
<td>Chemical Oxygen Demand</td>
<td>735 mg L$^{-1}$</td>
</tr>
<tr>
<td>Total Suspended Solids</td>
<td>467 mg L$^{-1}$</td>
</tr>
<tr>
<td>Volatile Suspended Solids</td>
<td>17.8 mg L$^{-1}$</td>
</tr>
<tr>
<td>Total Solids</td>
<td>2.35 g L$^{-1}$</td>
</tr>
<tr>
<td>Total Solids, Glass Fiber Filtered</td>
<td>2.03 g L$^{-1}$</td>
</tr>
<tr>
<td>Total Solids, 0.45 μm membrane filtered</td>
<td>2.00 g L$^{-1}$</td>
</tr>
</tbody>
</table>

2.2.2.3 GBF Media Composition

Experimental media was comprised primarily of secondary clarifier effluent and GBF, combined in different proportions. Additional constituents were added according to Table 2-3. To ensure complete nutrient requirements, the media was augmented with trace metals at the concentrations found in Medium A+. The GBF from Nine Springs WWTP has an unusually low phosphorus concentration due to the WASSTRIP process, so potassium dihydrogen phosphate was added so there was a molar ratio of 1:32 soluble reactive phosphorus to bioavailable nitrogen (ammonia plus nitrate) based on the composition of Medium A+. Tris served as a buffering system during shake flask cultivation, but was not included for most experiments in pH-controlled reactors. All media was pH-adjusted to 8.0 with potassium hydroxide or hydrochloric acid before autoclaving.

After autoclaving, vitamin B12 was added at the same concentration as in Medium A+. Finally, gentamycin minimized growth of heterotrophs, and the inducer isopropyl β-D-1-thiogalactopyranoside (IPTG) was used with all strains, whether inducible or not.
Table 2-3 Constituents of wastewater-derived media. Tris was only included for shake flask cultivation

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Final Concentration</th>
<th>Volume per liter media</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBF</td>
<td>---</td>
<td>As dictated by percent (V/V)</td>
</tr>
<tr>
<td>Trace Metals Solution</td>
<td>Per Medium A+</td>
<td>10 mL</td>
</tr>
<tr>
<td>Tris</td>
<td>6.35 mM (per Medium A+)</td>
<td>10 mL</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>As dictated by 1:32 P:N molar ratio</td>
<td>&lt; 2.5 mL</td>
</tr>
<tr>
<td>Gentamycin sulfate</td>
<td>&gt;17,700 units L$^{-1}$</td>
<td>1 mL</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>2.95 x 10$^{-12}$ M</td>
<td>4 μL</td>
</tr>
<tr>
<td>IPTG</td>
<td>0.5 mM</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Secondary Clarifier Effluent</td>
<td>---</td>
<td>Remainder</td>
</tr>
</tbody>
</table>

2.3 Cultivation

*Synechococcus* 7002 was propagated using two different platforms in the described experiments.

The majority of experiments were conducted using 250 mL shake flasks filled to an initial volume of 75 mL media plus the appropriate volume of inoculum, between 1.5 and 2 mL. Each combination of media, strain, light intensity, and temperature was performed in triplicate. Flask replicates were distributed at fixed intervals in a chamber platform shaker (Kuhner ISF1-X). The shaker provided constant illumination from an LED panel (15,000 lux maximum), temperature, and 1% gas phase carbon dioxide concentration.

For experiments where nutrient uptake values were desired, batch reactors of 1 liter media volume (1 L PBR, henceforth) were used (Figure 2-2). Continuous illumination was provided by 6 cool white fluorescent bulbs (8 W each, T5), while a stir plate (Corning) paired with an egg-shaped stir bar (VWR) ensured completely mixed conditions. Carbon dioxide was mixed at 1% with atmospheric air and the mixture continuously bubbled at 0.3 SLPM air per liter of media into each reactor through a stainless steel line (1/8” outer diameter, 1/16” inner diameter). Fans moving
ambient air across the reactors provided cooling when indicated by a temperature probe (Elitech Stc-1000), while heat produced from the lighting ensured that only cooling was necessary even at temperatures higher than the experimental setpoint (data not shown). We found it necessary to prevent pH drift driven by nitrogen transport using 0.5 M sodium hydroxide or hydrochloric acid for media with ammonia or nitrate, respectively, as the predominant form of nitrogen. The pH was monitored and controlled at pH = 8.0 ± 0.15 by a meter (Pinpoint Marine) which would turn on a peristaltic pump (Masterflex) as necessary to provide the acid or base. Experiments were conducted in duplicate with this platform.

Cells for inoculation of each experiment were propagated in Medium A+ using the same platform used in each experiment as above. Inoculum cultivation was initiated at 37°C, 1% CO₂, and 100% light intensity. At least 24 hours before commencing each experiment, the temperature and light intensity were changed to the experimental conditions to avoid shock to the cells upon the experiment’s initiation.

At the start of each experiment, the cells were centrifuged (2000 RCF, 15 minutes), the spent media decanted, and the cells resuspended in tris buffered saline (TBS: 6.4

![Figure 2-2 Cut-away schematic of temperature- and pH-controlled batch photobioreactor. Fan, temperature controller, pH controller, and acid/base pumps not shown](image)
mM tris, 310 mM NaCl), for shake flask experiments, or each treatment’s media, for 1 L PBR experiments, to an approximate OD$_{730}$ of 2. After quantification of actual inoculum OD$_{730}$, each shake flask was inoculated with cells to achieve OD$_{730} = 0.05$.

### 2.4 Standard Analytes

Both the GBF and secondary clarifier effluent were characterized for a number of constituents. Soluble reactive phosphorus (SRP), ammonia, nitrate, and nitrite concentrations were determined for all secondary clarifier effluent and GBF samples utilized in these experiments. In addition, the large batch of GBF used for the majority of experiments was tested for total suspended solids (TSS), volatile suspended solids (VSS) total solids (TS), and chemical oxygen demand (COD).

Ammonia, SRP, and COD were determined by colorimetric tests using reagents from Hach. Nitrate and nitrite were determined using high performance liquid chromatography (Shimadzu) with a C18 column and photodiode array detector. Determination of TSS, VSS, and TS were performed according to Standard Methods\textsuperscript{53} 2540 D, 2540 E, and 2540 B, respectively, with 47mm diameter glass fiber filters (Whatman) used for TSS and VSS.

### 2.5 Cell State Quantification

Cell health and growth was quantified by two methods in this study. Absorbance of 730 nm light, OD$_{730}$, is widely used as a proxy for dry cell weight for *Synechococcus* PCC 7002\textsuperscript{54,55}. With the use of a plate reader, this is a high-throughput method to track cell growth across time and treatments. In addition, this study tracked the portion of cells that were live over time using a pair of fluorophores in conjunction with flow cytometry.
Flow cytometry was used to determine cell viability for several reasons. Staining coupled with bulk fluorescence of samples offers an alternative method, but is prone to method error, requires multiple washes to remove unreacted stain, and is only a relative indicator of cell death. Fluorescent staining and microscopy was also considered as a single cell method, but carried an additional time burden for a shallower sample depth.

2.5.1 Quantification of Cell Growth

To track cell growth, 200 μL of culture from each flask was pipetted in duplicate into a 96 well plate (Corning 3585). Samples of each media used in the experiment were also aliquoted into wells in duplicate. To ensure near linearity of absorbance with respect to dry cell weight, samples were diluted with Medium A+ or TBS as necessary for a target OD$_{730}$ reading between 0.25 and 0.5. Absorbance for each well was determined using a Tecan Infinite M1000 plate reader. Absorbance was captured at 5 nm intervals from 400 to 750 nm as well as at peaks corresponding to chlorophyll a (438 nm and 683 nm) and phycobilisomes (637 nm).

2.5.2 Quantification of Live Population

A sample volume equal to approximately 0.1 OD$_{730}$ mL was taken from each flask. After centrifugation (2 minutes, 5000 RCF$^{56-58}$), the media was decanted and cells were resuspended in 1 mL of filtered (0.22 μm), autoclaved TBS.

SYTO 59 (Life Technologies) was added to each sample for a final concentration of 1 μM. SYTO 59 is a membrane-permeant nucleic acid dye, so is able to stain all cells. After comparison to several other red spectrum SYTO series dyes, SYTO 59 was selected because it minimized peak spread while maximizing separation from debris (Appendix B). Stain concentration was optimized
in the same manner. Healthy *Synechococcus* 7002 cells have high autofluorescence at red spectrum excitation and emission wavelengths; previous flow cytometry studies have relied solely on red spectrum autofluorescence to identify cyanobacteria\(^{59-61}\). Thus, there was minimal difference between the tested dyes using relatively healthy cells. However, nitrogen-stressed cells have reduced autofluorescence. Therefore, SYTO 59 was used to ensure that all cells would have sufficiently high fluorescence for inclusion as cells when gated.

To identify membrane-compromised cells, the membrane-impermeant stain SYTOX Green (Life Technologies) was also added to each sample for a final concentration of 1 μM. SYTOX Green was chosen because *Synechococcus* PCC 7002 exhibits little autofluorescence that overlaps with SYTOX Green’s visualization. Optimum SYTOX Green concentration was determined as above (Appendix B).

At each sample time point, positive controls for cell death, unstained cells, and cells stained only with SYTO 59 were performed in triplicate. Triplicates were selected from three media to determine the breadth of fluorescence patterns for gating at each time point. Cells grown in Medium A+ served as negative controls for cell death.

As a positive control for cell death, a sample was taken according to the above procedure. The sample was centrifuged (2 minutes, 5000 RCF), media decanted, and cells resuspended in 1 mL of 190 proof ethanol to permeabilize the membrane\(^{56,62}\) if the sample volume was greater than 200 mL. For samples less than 200 mL, the culture was diluted to a total volume of 1 mL likewise with 190 proof ethanol. Samples rested for 1 minute before they were centrifuged (2 minutes, 5000 RCF), decanted, and cells resuspended in 1 mL TBS. Plating on Medium A+ agar plates (n = 3)
demonstrated complete cell death. The ethanol-treated controls were stained concurrently and with the same concentrations of SYTO 59 and SYTOX Green as other samples.

SYTO 59 and SYTOX Green are suspended in the organic solvent dimethyl sulfoxide (DMSO). Therefore, DMSO was added to the unstained controls at same volume as both stains (0.4 μL) while 0.2 μL DMSO was added to the samples stained with only SYTO 59.

Samples were vortexed and allowed to rest for at least 10 minutes in the dark at room temperature before proceeding to flow cytometry\textsuperscript{63,64}, which was initiated within 20 minutes. Flow cytometry was performed using a BD FACSCalibur. SYTOX green fluorescence was visualized using 488 nm laser excitation and emission area was read using a 530/30 nm bandpass filter. The 633 nm laser coupled with a 661/16 bandpass filter was used for SYTO 59 visualization.

Flow cell injection was performed at low pressure, with a target event rate of 1700-3500 events per second. Forward scatter, side scatter, and fluorescence channel voltages were calibrated to capture all cell events, both low and high fluorescence, based on unstained, live, and dead cell samples. No channel spillover besides autofluorescence was expected or observed, so no compensation was applied. To prevent cross-contamination, deionized water was run through the machine between each treatment. A minimum of 10,000 events gated as cells were captured for each sample.
Gating and data export, which was performed using CellQuest Pro\textsuperscript{65}, consisted of two stages: gating of total cells, and gating of live cells. Forward scatter, which is roughly a metric of cell size, was coupled with particle fluorescence in the red and green channels to gate for all cells and cell survival, respectively.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2-3.png}
\caption{Example total cell region creation. Top left is Medium A+ grown cells, top right is Medium A+ grown cells that were ethanol treated, bottom two panels are from GBF grown cells, with the initial (left) and final expanded regions (right).}
\end{figure}
A composite gate comprised of live and dead cells was constructed to measure total cells because there was some difference in degree of red fluorescence between live and dead cells. Each event for the ethanol-treated controls was plotted with forward scatter on the x axis and red channel fluorescence on the y axis. The region that captured the cells in all three replicates was selected (Figure 2-3). This procedure was repeated using the live controls, and then the area between the two was also included so that indeterminate cells would also be gated as cells. The area within the three regions comprised the gate for total cells.

A similar procedure was used to gate cells as live, dead, or indeterminate. For the ethanol-treated controls, each event gated as a cell was plotted on a graph with forward scatter versus green channel fluorescence. The area containing the vast majority of events, outliers notwithstanding, was drawn (Figure 2-4). Because some media conditions lead to patterns inconsistent with ethanol permeabilization, namely moderate shifts in forward scatter and greater fluorescence than the dead controls, this region was widened and the upper limit raised. A composite of the three dead control replicates was considered. This region comprised the dead cells gate.

Similarly, the positive controls’ events were plotted on the same axes as the dead controls with the region containing the majority of cells as the cell live region (Figure 2-4). This region was widened to account for discrepancies of forward scatter and extended the entire distance down to zero green channel fluorescence in the same manner as the dead cell region. This region comprised the live cell gate.

All events gated as cells but which did not fall within the live or dead gates were considered “indeterminate cells.” Cells continued to stain while in the queue for data capture on the flow cytometer, so there was a small shift in cells from the indeterminate population to the dead...
population with increased duration since staining (data not shown). No such shift was observed between live and indeterminate cells. Because of this, only the live portion of the population was evaluated in this research and the indeterminate and dead cells were treated as a single population.

Figure 2-4 Example live and dead region construction. The top left panel is the initial live cell region with Medium A+ grown cells, the top right is ethanol-treated Medium A+ grown cells. Bottom left shows a shift in GBF grown cells, and bottom right the final broadened gates.
2.6 Assay for Bacteriophage

Initial cultivation of *Synechococcus* 7002 in non-sterilized GBF and secondary effluent often caused apparent cell death based on greatly reduced OD$_{730}$ and/or inability to form colonies on Medium A+ agar plates (1.5% m/m, Bacto (Becton Dickinson)), so plaque assays were undertaken to determine the role, if any, of bacteriophages.

Medium A+ with 0.5% m/m agar was autoclaved, then allowed to cool to 40°C in a water bath. A mixture of 1 part healthy cells pre-grown in Medium A+ (OD$_{730}$ = 0.4) and 4 parts media was gently mixed in a tube, then 1.5 mL of various mixtures of non-sterilized GBF and/or secondary effluent, usually 0.45 μm filtered, were diluted in 14.5 mL of the Medium A+ and cell mixture. This was plated and incubated under lights at 37°C.

2.7 Data Analysis

All data were analyzed and plotted with either Microsoft Excel$^{66}$ or the R statistical language$^{67}$ with core functionality extended by a number of packages$^{68-86}$. Development for R was performed in the RStudio integrated development environment$^{87}$.

2.7.1 Fitting Parameters for Nitrogen Concentration

Several (multiple) linear regressions for total available nitrogen were considered to determine if assays already performed in monitoring *Synechococcus* 7002 could serve as a proxy for media nitrogen concentration. For this analysis, it was assumed that nitrate and ammonia comprised the sum of bioavailable nitrogen based on testing of tris as a bioavailable nitrogen source (Section 3.1) and non-detectable levels of nitrite.
All models were fitted to include parameter interactions, which is appropriate given that some interactions were statistically significant and furthermore, would be expected to be so. The simplest model only considered residual bioavailable nitrogen as a function of OD$_{637}$, with models increasing in complexity to include a number of other parameters: OD$_{683}$, OD$_{730}$, and initial nitrogen concentration. Statistical analysis of each model was performed according to the methods in part 2.7.3.

Nitrogen data from an experiment performed in the 1 L photobioreactors at 32°C, 1% carbon dioxide air stream, and varied GBF concentrations were used for this analysis. Reactors with 4% GBF encountered nitrogen limitation, while those with 12.5% GBF or Medium A+ did not, but samples from all reactors at the 24-, 48-, and 72-hour time points were included in this analysis.

### 2.7.2 Toxicity and Growth Rates

Both initial and chronic toxicities were calculated by linear regression as the time rate of change of the portion of live cells. Initial toxicity was conducted between inoculation and the first sample time, between 6 and 10.5 hours post-inoculation. Chronic toxicity regressions were calculated over the subsequent and final sampling times: 20 (±3) and 66 (±3) hours. Growth rates were similarly calculated by linear regression over the same time periods as the chronic toxicity.

### 2.7.3 Statistical Analysis

Multi-factor Analysis of Variance (ANOVA) was performed separately on initial toxicities, chronic toxicities, and growth rates in R using a linear model with factor interactions and the car package$^{88}$. Values reflect use of type III sum of squares because an initial ANOVA test using type I sum of squares suggested that many interactions between factors were significant. To avoid
significantly unbalanced design, only media and strain conditions tested under greater than one temperature and light intensity combination were utilized. To validate the varied effects of GBF concentration on growth and toxicity, all experiments in Medium A+ were excluded in a second set of ANOVA analyses. All results and discussion regarding ANOVA tests reference this second set of analyses unless explicitly stated otherwise.

Based on ANOVA results where each dependent variable (initial toxicity, chronic toxicity, growth rate) had either a significant main or interaction effect, treatments, with all four factors preserved, was tested for mean differences by the Tukey HSD test using the R package agricolae\textsuperscript{89}.

2.7.4 Change in Energy Utilization Calculations

The change in energy consumption for aeration during secondary treatment with the envisioned wastewater stream utilization as an alternative to the operation of separate systems was investigated. The base scenario was predicated on current operation parameters and wastewater characteristics at the Nine Springs WWTP.

Under the alternate scenario, the majority of effluent from the anaerobic digester gravity belt thickener was modeled to pass to a cyanobacterial cultivation process where it mixed with secondary clarifier effluent (Figure 2-5). The growth media was assumed to be comprised of 12.5\% GBF and 87.5\% secondary effluent, with nutrient characteristics as observed in these experiments. All liquid effluent from cyanobacterial cultivation was assumed to be recycled to the head of plant due to residual ammonia concentration in excess of Nine Springs WWTP’s WPDES (Wisconsin Pollutant Discharge Elimination System) permit. For simplicity, the ammonia concentration from
the cyanobacterial process was assumed to be 0 mg L\(^{-1}\). We assumed that wasted cyanobacterial biomass was used in an uncoupled process, which was not considered in this analysis.

To calculate the difference in energy represented by reduced futile ammonia cycling but increased internal flow to the head of plant, oxygen requirement and transfer calculations were performed. These calculations assume a three pass aerobic zone with relative volumes of 1/7, 3/7, and 3/7 for the first, second, and third aeration basins\(^90\). The cyanobacterial process separation performance was assumed to be sufficiently high such that the cyanobacterial cultivation process did not change carbonaceous BOD loadings to the head of plant.

The change in oxygen demand required for ammoniacal nitrogen oxidation was calculated according to Equations 2-2 and 2-3\(^91\), then distributed across the three aeration basins according to their relative volumes, and thus retention times (2-1).

![Figure 2-5](image-url) Alternate scenario plant configuration for Nine Springs WWTP. Bolded lines indicate new flows and processes, green process flow line indicates all cyanobacterial biomass and bio-based chemical product flow. All other process flow lines as in Figure 2-1.
\[ \Delta R_{0,n} = \Delta R_0 \frac{V_n}{V_{tot}} \]

Where:

\( \Delta R_{0,n} \) = change in oxygen transfer rate from the base scenario required for oxidation across aeration basin \( n \) (g d\(^{-1}\))

\( \Delta R_0 \) = change in oxygen transfer rate from the base scenario required for oxidation across entire aerobic zone (g d\(^{-1}\))

\( V_n \) = volume of aeration basin \( n \)

\( V_{tot} \) = total volume of aerobic zone

\[ \Delta R_0 = 4.33 \cdot \Delta \bar{N}_{ox} \]

\[ \Delta \bar{N}_{ox} = Q_{Ostara} \cdot NH_3,Ostara \]

Where:

\( \Delta \bar{N}_{ox} \) = change in influent nitrogen that is oxidized over the secondary treatment train (mg L\(^{-1}\))

\( Q_{Ostara} \) = current flow rate through Ostara process (m\(^3\) d\(^{-1}\)), assumed 0.9 MGD (3407 m\(^3\) d\(^{-1}\))

\( NH_3,Ostara \) = current ammonia concentration in Ostara effluent (mg L\(^{-1}\)), assumed 150 mg L\(^{-1}\)

A mass balance approach was used to determine the change in oxygen necessary to aerate each basin in the absence of oxidation\(^9\). Influent flow rate to basin one was calculated according to Equation 2-6. For influent to the first basin, primary clarifier effluent, return activated sludge (RAS), Ostara process effluent, and cyanobacterial cultivation process effluent were assumed to have a dissolved oxygen (DO) concentration of 0 mg L\(^{-1}\). Influent DO concentrations to basins two and three were the effluent concentrations from the immediately subsequent basin. Thus, the
reoxygenation mass balance across each basin is given by Equation 2-5, and the total actual oxygen transfer rate for each basin is then the sum of the reaeration rate and the oxidation rate (Equation 2-4).

\[
\Delta AOTR_{\text{tot},n} = \Delta AOTR_{\text{liq},n} + \Delta R_{O,n}
\]  
\[2-4\]

Where:
\[\Delta AOTR_{\text{tot},n} = \text{total change in Actual Oxygen Transfer Rate from base scenario to maintain assumed DO concentration across basin } n \ (g \ O_2 \cdot \text{d}^{-1})\]
\[\Delta AOTR_{\text{liq},n} = \text{change in Actual Oxygen Transfer Rate to reoxygenate basin } n \ (g \ O_2 \cdot \text{d}^{-1})\]

\[
\Delta AOTR_{\text{liq},n} = \Delta Q_{\text{tot}} \cdot (DO_{\text{in},n} - DO_{\text{eff},n})
\]  
\[2-5\]

Where:
\[\Delta Q_{\text{tot}} = \text{change in total flow rate through each aeration basin above the base scenario} \ (m^3 \ \text{d}^{-1}), \text{from Equation 2-7}\]
\[DO_{\text{in},n} = \text{influent DO concentration to basin } n \ (mg \ l^{-1})\]
\[DO_{\text{eff},n} = \text{effluent DO concentration from basin } n \ (mg \ l^{-1})\]

\[
Q_{\text{tot}} = Q + Q_{\text{RAS}} + Q_{\text{cyano}} + Q_{\text{Ostara}}
\]  
\[2-6\]

Where:
\[Q_{\text{RAS}} = \text{Return Activated Sludge flow rate} \ (m^3 \ \text{d}^{-1}), \text{assumed 0.5} \cdot Q\]
\[Q_{\text{cyano}} = \text{cyanobacterial process effluent flow rate} \ (m^3 \ \text{d}^{-1}), \text{from Equation 2-10}\]

Thus, the change in flow rate through secondary treatment is given by

\[
\Delta Q_{\text{tot}} = Q_{\text{cyano}} + Q_{\text{Ostara,final}} - Q_{\text{Ostara,initial}}
\]  
\[2-7\]
To conserve current precipitation of struvite in the Ostara process, a mass balance on ammonia across the Ostara process was calculated to determine its deficit in the combined flows excluding the GBF (Equation 2-8). The ammonia deficit was then balanced with a portion of the GBF flow. Ammonia demand in GBT for struvite precipitation, which is limited by phosphorus concentration at Nine Springs WWTP, was considered to be negligible. The remainder of GBT was assumed to enter the cyanobacterial process.

\[
\dot{A}_d = Q_{GBF}C_{A,GBF} - Q_{Ostara}C_{A,Ostara}
\]

Where:

\[
\dot{A}_d = \text{Ammonia mass flow rate deficit (g d}^{-1})
\]

\[
Q_{GBF} = \text{effluent flow rate from the post-anerobic digester gravity belt thicken (m}^3\text{ d}^{-1}), \text{ assumed 0.2 MGD (757 m}^3\text{ d}^{-1}, \text{ Matthew Seib, MMSD, personal communication, 2016)}
\]

\[
C_{A,GBF} = \text{GBF ammonia concentration, assumed 900 mg/L (Table 2-1)}
\]

\[
Q_{Ostara} = \text{Ostara process effluent flow rate (m}^3\text{ d}^{-1}), \text{ assumed 0.9 MGD (3407 m}^3\text{ d}^{-1}, \text{ M. Seib, personal communication, 2016)}
\]

\[
C_{A,Ostara} = \text{Ostara process effluent ammonia concentration, assumed 150 mg·L}^{-1} \text{ (M. Seib, personal communication, 2016)}
\]

\[
Q_{GBF,O} = \frac{\dot{A}_d}{C_{A,GBF}}
\]

Where:

\[
Q_{GBF,O} = \text{Flow rate of GBF to Ostara process under alternate configuration}
\]

\[
Q_{cyano} = [(Q_{GBF} - Q_{GBF,Ostara} + Q_{2eff}) \cdot \eta_{sep}]
\]

\[
Q_{2eff} = 7 \cdot (Q_{GBF} - Q_{GBF,Ostara})
\]
Where:

\( Q_{2\text{eff}} = \) flow rate from the secondary clarifier to dilute the GBF for the cyanobacterial process

\( \eta_{\text{sep}} = \) post cyanobacterial cultivation liquid separation efficiency (gallon liquid effluent \(
\cdot \) gallon liquid influent\(^{-1}\)), conservatively assumed 1

The field oxygen transfer was calculated for each basin (Equation 2-12)\(^9\) at an assumed temperature of 20°C. Standard oxygen transfer efficiency depends on basin geometry, diffuser type, degree of mixing, and water characteristics\(^9\), so previously determined standard oxygen transfer efficiencies for each of the three basins at Nine Springs WWTP were used.

\[
OTE_{20,n} = \alpha_{SOTE,n} \cdot \frac{DO_{sat} - DO_{eff,n}}{DO_{sat}} \quad 2-12
\]

Where:

\( OTE_{20,n} = \) field Oxygen Transfer Efficiency for basin n (unitless)

\( \alpha_{SOTE,n} = \) Standard Oxygen Transfer Efficiency for basin n (unitless), assumed 0.10, 0.18, and 0.18 for basins 1-3, respectively\(^9\)

\( DO_{sat} = \) DO saturation concentration at half the basin depth (mg L\(^{-1}\)), assumed 10.6 mg L\(^{-1}\)

Finally, the oxygen transfer rate was adjusted by the field oxygen transfer efficiency\(^9\) and converted to an atmospheric air flow rate (Equation 2-13) then summed across all three basins. After a conversion to standard CFM (SCFM, Equation 2-14)\(^9\), the annual required blower power was calculated (Equation 2-16)\(^9\).

\[
CFM_n = \frac{AOTR_{\text{total,n}}}{OTE_{20,n}} \cdot 9.06 \cdot 10^{-2} \cdot \frac{ft^3}{min \cdot d^{-1}} \quad 2-13
\]

Where:
CFM<sub>n</sub> = required air flow rate for basin <i>n</i> (ft<sup>3</sup> min<sup>-1</sup>)

\[
SCFM = \frac{CFM}{[P_{std}/(P_1 - P_{std}\Phi)] \times (T_{act}/T_{std})}
\]

\[
w = SCFM \cdot 0.0765 \frac{lb}{ft^2} \cdot \frac{1}{60 \text{ s}}
\]

\[
P_w = \left[ \frac{wRT_{act}}{550ne} \left( \frac{p_2}{p_1} \right)^{0.283} - 1 \right] \times \frac{0.746kW}{hp} \times \frac{8760h}{yr}
\]

Where:

SCFM = required airflow (SCFM)

P<sub>std</sub> = standard pressure (psi), 14.7 psi

p<sub>1</sub> = inlet pressure, (psi), 14.25 psi at the Nine Springs 877 ft elevation

Φ = blower air humidity (fraction of saturation), assumed 0.25

T<sub>act</sub> = actual inlet air temperature (°R), assumed 20° (527.7°R)

T<sub>std</sub> = standard temperature, (°R), assumed 520°R

w = weight of flow of air (lb · s<sup>-1</sup>)

P<sub>w</sub> = power requirement for blowers, kilowatt hours per year (kWh · yr<sup>-1</sup>)

R = ideal gas constant for air, 53.3 ft·lb (lb air)<sup>-1</sup>·°R

N = 0.283 for air and imperial units

e = blower efficiency (usual range is 0.70-0.90), Nine Springs WWTP calculated 0.63

p<sub>2</sub> = outlet pressure (psia), assumed 26.6 psia based on 16ft diffuser depth and 5 psi pressure loss
3 RESULTS

3.1 Growth on tris as a nitrogen source

We tested growth of *Synechococcus* 7002 on Medium A- to determine if nitrogen in tris is bioavailable. Both the GmR and UspA mutants of *Synechococcus* 7002 showed negative growth rates as measured by OD\textsubscript{730} on Medium A- (\(p = 0.0035\) and \(p = 1.51\times10^{-7}\), respectively), while they both demonstrated robust growth in Medium A+ (data not shown). These results indicate that tris cannot serve as an appreciable source of nitrogen, and thus could be added to media to serve as a buffering system without altering the bioavailable nutrient composition.

3.2 Bacteriophage Infection

Early experiments with non-sterilized media comprised of GBF and secondary effluent failed repeatedly because the cyanobacteria either did not grow or had minimal initial growth followed by collapse. Cells appeared to bleach and/or lyse after 12-36 hours when cultivated at 37°C, high light intensity and 0.04% (atmospheric) carbon dioxide. We initially hypothesized that cyanophage particles were present in one or both wastewater streams, causing cell lysis. Therefore, we aimed to detect the presence of viral particles in the wastewater culture medium. Viral plaque assays are a standard method for the determination of viral titer and can likewise serve for viral infection detection. We expected that regions of clearing would appear around area of infection when cells were lysed by phages. However, no such behavior was observed over multiple iterations with varied batches of GBF and secondary effluent. Thus, it was determined that phage infection was not the cause of cell death in these initial experiments.
### 3.3 Growth in Batch Photobioreactor

Photobioreactors were used for early experiments because batch cultures without a strong buffer (such as tris) suffered from rapid pH decline when the cyanobacteria were grown with ammonia in the medium. Batch photobioreactors were constructed to include automated pH control, as described in the methods section. Highly variable growth rates were observed in these reactors, including between pairs of reactors operated simultaneously under the same target conditions. This was especially true for cultures grown in wastewater-derived medium. However, this divergence between biological replicates was not as pronounced for cells grown in Medium A+.

No significant growth was observed at 32°C when atmospheric air was provided to the system, including in Media A+. Given previous successful cultivation in Medium A+ using shake flasks provided with atmospheric air (results not shown), it was determined that the aeration system did not allow sufficient inorganic carbon flux at such a low concentration. Previous work with diffusers had resulted in significant contamination by heterotrophic microorganisms (T. Korosh, personal communication, 2014), so subsequent experiments in the photobioreactors always used 1% carbon dioxide gas.

The variability, lack of biological replication, concern about aqueous total carbonate concentrations, and significant time burden for propagation in the 1 L photobioreactors led to examination of shake flasks as a reliable culturing platform. However, the photobioreactors were used for nutrient utilization experiments (Section 3.4).
3.4 Nitrogen Concentration Modeling

Previous work demonstrated a reduction in the ratio of OD$_{637}$ and OD$_{683}$ under nitrogen limitation in Medium A+ (T. Korosh, personal communication, 2014). Thus, OD$_{637}$ was investigated as a proxy for nitrogen limitation. The basis for this relationship is presumed to be degradation of the phycobilisomes, which have peak absorbance at 637 nm in *Synechococcus* 7002$^{22}$. Phycobilisomes are enriched in nitrogen as compared to the cell as a whole$^{95}$, and previous work showed that transcripts for the protein *nblA*, which is implicated in phycobilisome degradation, are greatly upregulated under nitrogen limitation$^{27}$. Thus, lower OD$_{637}$ would indicate depleted phycobilisomes and logically suggest nitrogen limitation.

Our analysis showed that OD$_{637}$ was a poor proxy for nitrogen concentration on its own. However, three models of varying complexity offered a reasonable proxy (Table 3-1). The model that included OD$_{637}$, OD$_{683}$, OD$_{730}$, and initial nitrogen concentration proved excellent. While offering lower fidelity, there was little deviance between a model that considered OD$_{637}$, OD$_{683}$, and OD$_{730}$ versus one that only considered the first two parameters.

Table 3-1 Summary of multiple linear regressions for development of proxy for nitrogen concentration

<table>
<thead>
<tr>
<th>Fitting Parameters</th>
<th>Adjusted R-squared</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD$_{637}$</td>
<td>0.2049</td>
</tr>
<tr>
<td>initial nitrogen concentration</td>
<td>0.5388</td>
</tr>
<tr>
<td>OD$<em>{637}$, OD$</em>{730}$</td>
<td>0.5173</td>
</tr>
<tr>
<td>OD$<em>{637}$, OD$</em>{683}$</td>
<td>0.7387</td>
</tr>
<tr>
<td>OD$<em>{637}$, OD$</em>{683}$, OD$_{730}$</td>
<td>0.7434</td>
</tr>
<tr>
<td>OD$<em>{637}$, OD$</em>{683}$, OD$_{730}$, initial nitrogen concentration</td>
<td>0.9563</td>
</tr>
</tbody>
</table>
3.5 Toxicity and Growth in Batch Shake Flask

Experiments to compare growth and toxicity were conducted at 1% gas phase carbon dioxide with varied media composition at combinations of two setpoints each of temperature and light intensity. Experiments were conducted with both the GmR and UspA strains of *Synechococcus* 7002.

3.5.1 Initial Toxicity

We defined “initial toxicity” to be the time rate of change portion of live cells during early stage of batch shake-flask cultivation.

Initial toxicity was impacted by main effects of strain, media, and light intensity ($\alpha = 0.05$). More interesting was the interaction effect between media, temperature, and light ($p = 4.5 \times 10^{-6}$), which also carried through to pair-wise interactions between each combination of the three factors.

Initial toxicity was substantial across most treatments, with the near-universal exception of those experiments with Medium A+ (Figure 3-1). However, several conditions that included GBF had initial toxicities that were not statistically different from cells grown in Medium A+. Initial toxicity clearly depended on GBF concentration under 50% illumination and 27°C and the dose-dependent response was also evident at 100% illumination 37°C (Figure 3-1). Initial toxicity was not functionally dose-dependent under the other two combinations of illumination and temperature.
Figure 3-1 Toxicity in each of two phases of cultivation. Treatments labeled with the same letter for a given phase are not statistically different (α = 0.05). Error bars indicate one standard deviation.
3.5.2 Chronic Toxicity

Like “initial toxicity”, we defined “chronic toxicity” to be the time rate of change of portion live cells, but during linear phase cultivation. Interaction effects on chronic toxicity were observed between all four factors ($p = 0.033$) as well as between media and temperature ($p = 1.9 \times 10^{-14}$). Media, temperature, and light all had statistical main effects on chronic toxicity.

![Image](image.png)

**Figure 3-2** Comparison of initial and chronic toxicity. Top panels show plot of three divergent toxicity patterns and their placement within the primary plot.

Strains grown in Medium A+ showed a characteristically low chronic toxicity, much like initial toxicity (Figure 3-1). Cells under most conditions that included GBF exhibited significant initial
toxicity, but there were two divergent patterns for cells grown in GBF-based media. Many saw a rebound, albeit at a slower rate than the initial die off as in the top middle panel of Figure 3-2, while others never recovered. This is illustrated in the top left panel of Figure 3-2 and manifested as lower overall growth rates among these treatments. Continued rapid decline was most evident when cells were grown at 37°C with media containing high GBF concentrations.

3.5.3 Growth Rate

Growth rate in minimal media was greatly influenced by light intensity, as has been demonstrated in previous work. However, a difference of 10°C had a non-significant effect for Medium A+ grown cells (Figure 3-3). No such relationship was universally evident for cells grown in GBF derived media. Cells grown in GBF derived media had generally lower growth rates than those grown in minimal media at 100% light intensity, though the growth rate of *Synechococcus* 7002 GmR cells grown in 6.25% GBF at 37°C was not statistically different than the conditions with the most rapid growth (Medium A+, 100% light, temperature and strain not significant).
Figure 3-3 Growth rate for each treatment. Treatments labeled with the same letter are not statistically different (α = 0.05). Error bars indicate one standard deviation.
3.6 Energy Utilization

Aeration in biological nutrient removal is used to maintain sufficient dissolved oxygen concentration for oxidation of reduced carbon and nitrogen found in the wastewater. In addition to oxygen used in BOD removal, wastewater treatment plants must aerate in order to meet effluent discharge standards to avoid hypoxia in the receiving water body.

We modeled the change in change in aeration demands during mainstream secondary treatment by examining the differences in ammonia loading and wastewater flow rates between the current aeration demands at the Nine Springs WWTP. This was compared to an alternate scenario in which sidestream ammonia was captured in a cyanobacterial cultivation process. The baseline model was previously found by Keene\textsuperscript{93} to be within 2\% of a 2014 report on energy use at the Nine Springs WWTP\textsuperscript{92}.

Futile nitrogen cycling from the sidestream to the mainstream represents a significant portion of BOD loading to the head of plant. Thus, we expected a significant modeled benefit associated with removing sidestream ammonia. Despite increased flow to the head of plant, and thus increased demand for reoxygenation, the addition of a cyanobacterial growth and separation process significantly reduced modeled aeration energy utilization. The increased reaeration demands due to elevated flow rate through secondary treatment was two orders of magnitude smaller than the savings for oxidation, and thus plays a minor role. According to the model, demand for electricity from aeration would decrease by 795,000 kWh per year. This translates to 54 kWh per MGD or roughly 2\% of the total energy required for collection, pumping and treatment of wastewater at the
Nine Springs WWTP and 6% of aeration energy use\textsuperscript{92}. At the current mean industrial electricity rate in Wisconsin\textsuperscript{97}, this represents $64,000 savings per year.
4 DISCUSSION

4.1 Environmental Optimization for *Synechococcus* 7002 Cultivation

In these experiments, we investigated the impacts of growth medium composition, light intensity, and temperature on growth rate and cell toxicity in two engineered strains of *Synechococcus* 7002. While cultivation of a cyanobacterial strain for bio-based chemical production would likely impact growth rate due to partitioning of organic carbon to the target compound\textsuperscript{98} and possibly altered photosynthetic efficiency\textsuperscript{99,100}, it serves as a useful proxy for carbon fixation rates\textsuperscript{54}. Likewise, bio-based chemical production may alter rates of cell mortality including by direct toxicity\textsuperscript{101}, altered internal oxidation state\textsuperscript{99,100}, or substrate toxicity sensitization\textsuperscript{102}. However, some of the factors studied in these experiments have large effect sizes, and thus are important to consider in future work with varied substrates and cyanobacterial strains.

4.1.1 Selection of optimal conditions

Multiple factors will dictate the optimal configuration of a given cyanobacterial biorefinery. While minimal cell toxicity is desirable, carbon fixation rate, as approximated by cell growth rate, is ultimately more important in bio-based chemical production. In balance, however, is the possibility that carbon partitioning to a product will impact cell survivability due to reduced growth rates\textsuperscript{98}. By selecting conditions where minimized cell toxicity over the entire cultivation period is concurrent with growth rate, we are able to select conditions most likely favorable for cultivation for bio-based chemical production. In both Figures Figure 4-2 and Figure 4-3, this ideal is located in the top right of each sub-plot. Unsurprisingly, cells grown in Medium A+ under standard conditions or reduced temperature best fit these criteria. For wastewater-grown cells, there appear...
to be two divergent choices; low concentration of GBF under standard conditions, or high concentration of GBF under reduced temperature and 100% illumination.

Figure 4-1 Comparison of initial and chronic toxicities grouped by treatment conditions. The top right of each plot represents the healthiest cells over the experiment duration.

The optimal selection between these disparate conditions, then, will be a function of other constraints in the cyanobacterial biorefinery. GBF was initially targeted as a nutrient source based on presumed ability to achieve high product yield in a relatively small volume, reducing the demands on separation techniques which can comprise over half of the production cost of bio-based chemicals\textsuperscript{15}. In addition, an elevated temperature of 37°C could prove relatively costly for systems open to the sun due to evaporative cooling\textsuperscript{103}, though an analysis needs to be performed to determine the effect degree. While 27°C is still well above the mean yearly temperature across the USA\textsuperscript{104}, it is nearer to realistic cultivation conditions for an open raceway system. Together,
these data and operational limitations suggest not only the viability of reduced temperature and high GBF concentration cultivation, but preferability of this option.

**Figure 4-2** Comparison of growth rate and initial toxicity grouped by treatment conditions. The top right of each plot represents conditions where cells saw mortality upon inoculation and grew rapidly.
Figure 4.3 Comparison of growth rate and chronic toxicity grouped by treatment conditions. The top right of each plot represents conditions where cells saw little mortality during linear phase and grew rapidly.

4.1.2 Possible causal mechanisms

The deviation between initial and chronic toxicity is useful to elucidate the causal mechanisms of cell toxicity. Initial toxicity will highlight death caused by apparent reaction rates that are with respect to media constituents, while chronic toxicity is likely to reflect pseudo n-order kinetics with respect to cells. In addition, initial toxicity greater than chronic toxicity may arise from constituents of GBF which are degraded by *Synechococcus* 7002 or spontaneously under cultivation conditions. Finally, variation between initial and chronic toxicity may arise from a regulatory response after which *Synechococcus* 7002 is better able to deal with environmental stress; the observed timeframe for toxicity inflection when present, between 6 and 24 hours, is consistent with response timeframe needed for cellular acclimation after perturbations in light intensity, temperature, and inorganic carbon concentration in various *Synechococcus* species.
The overall reduction in initial and chronic toxicity at lower temperature suggests possible causal mechanisms for toxicity. Ludwig and Bryant\textsuperscript{29} noted a marked decrease in \textit{nblA} transcripts when \textit{Synechococcus 7002} was exposed to 30°C and 22°C as compared to 37°C. This gene is instrumental in degradation of phycobilisomes\textsuperscript{105}, which are a light harvesting antenna. This is consistent with these results, where the ratio of OD\textsubscript{637} (peak absorbance for the phycobilisomes) to OD\textsubscript{683} (peak absorbance for chlorophyll \textit{a}) was statistically dependent on temperature after approximately 20 hours of growth, with cells at low temperature having a higher ratio.

In the same study\textsuperscript{29}, Ludwig and Bryant also noted that \textit{pntA, pntB, pntC}, which encode a membrane-bound transhydrogenase, were likewise downregulated. Excess electron flow without a readily available terminal electron acceptor such as oxidized carbon will lead to production of reactive oxygen species (ROS) by photoreduction of dioxygen\textsuperscript{106}, while the transhydrogenase is implicated in ROS quenching by production of NADPH; NADPH provides reducing equivalents necessary for regeneration of antioxidants pools such as reduced glutathione\textsuperscript{107}.

Together, these data suggest that there is significant intercellular oxidative stress at 37°C, which is supported by data from the same study that found little change in the global transcriptome of \textit{Synechococcus 7002} under oxidative stress at 37°C\textsuperscript{29}. A common response to oxidative stress in photoautotrophs is to reduce their light harvesting antenna. This was commonly observed in GBF-grown cells at 37°C, as visible in the relatively low peaks at 637 nm (phycobilisomes) and 683 nm (chlorophyll \textit{a}) in Figure 4-4. It is likely that GBF causes additional oxidative stress and under already stressful conditions, \textit{Synechococcus 7002} is unable to effectively quench or ameliorate the effects of ROS.
Another possible cause for increased cell survival at reduced temperature is a change in cell membrane composition. To maintain membrane fluidity at reduced temperatures, *Synechococcus* 7002 is able to reduce the degree of saturation of its membrane fatty acids\(^{29,108,109}\). This is instrumental in maintaining photosynthesis\(^{110}\) as well as for membrane structure and repair\(^{111}\). Specifically, the electron shuttle plastiquinone travels within the thylakoid membrane as an intermediate between photosystems I and II. If there is a net increase in membrane fluidity, *Synechococcus* 7002 would have membranes more resilient to damage.

A final mechanistic cause of toxicity may be precipitation of proteins by phenolic compounds. Wastewater carries significant levels of humic and fulvic acid-like organic matter\(^{112}\) as well as aromatic compounds\(^{113}\). Aromatics are widely used as antimicrobial agents\(^{114}\), though there is disagreement whether disruption of the cell membrane is important to their action\(^{115}\). Regardless,
their reaction kinetics are highly temperature-dependent, with a 3 to 5-fold change in reaction rate over a 10°C temperature change\textsuperscript{115}. Thus, it is likely that at least part of the observed toxicity effects across temperatures is a result of differing disinfection rates by aromatics. The observed difference in effect between GBF doses at 37°C versus 27°C may be due to the opposing rates of cell growth and aromatic disinfection; elevated nutrient concentrations are generally expected to drive increased growth rates\textsuperscript{116}.

Strain \textit{Synechococcus} 7002 UspA was tested against the GmR strain due to the role of universal stress protein \textit{A} in oxidative stress amelioration\textsuperscript{29}. The \textit{Synechococcus} 7002 native universal stress proteins at loci SYNPC7002\_A1587 and SYNPC7002\_A2690 were slightly upregulated under oxidative stress\textsuperscript{29}, so we expected some improvement in cell survival. This was not realized, however, likely due to protein production burden incommensurate with stress reduction. Additionally, IPTG has been reported to more negatively impact cell fitness as compared to lactose\textsuperscript{96,102}, likely due to excessively strong induction\textsuperscript{102}. Thus, reduced protein production burden by fine-tuning induction through reduced IPTG dose or use of lactose in lieu of IPTG may shift strain fitness dynamics.

\subsection*{4.2 Nitrogen Concentration Modeling}

Nitrogen concentration was modeled using parameters routinely collected for culture monitoring. This could be used as a process control parameter in both experimental and production facilities. Given the low time burden of nitrate and ammonia testing, direct determination of media nitrogen concentration should be used when possible. However, in situations where sample volume is limiting, such as shake flasks, or media constituents interfere with ammonia or nitrate analytical
methods, these data suggest that a modeled approach may be used. Additionally, it may be added as a post-hoc troubleshooting tool if nutrient composition was not monitored or implemented as a real-time monitor of nitrogen availability. Previous work shows that temperature alters nitrogen utilization in *Synechococcus* 7002\(^45\), phosphorus limitation alters carbon partitioning in *Synechocystis* sp. PCC 6803\(^117\), and we expect other environmental factors such as light intensity and overall growth media composition are likely impact the relative absorbance of light-harvesting complexes (OD\(_{637}\) and OD\(_{683}\)) with respect to cell biomass (OD\(_{730}\)). Therefore, we expect that this model would need to be calibrated to each set of environmental conditions and specific growth platform.

### 4.3 Energy Impacts of a Coupled Cyanobacterial Process

Significant work has focused on methods to reduce aeration energy demands, which routinely account for twenty to sixty-seven percent of energy utilized in collection and treatment of municipal wastewater in activated sludge plants\(^ {118,119}\). The relatively recent discovery of anammox fairly rapidly progressed from scientific discovery to a full-scale sidestream nitrogen removal process\(^ {120,121}\) and low DO aeration in the secondary biological treatment is an active area of research\(^ {122–124}\). Based on the aeration energy savings found in this work, it appears that the cyanobacterial biorefinery represents another method to tackle this costly process in municipal wastewater treatment. This must, of course, be balanced with whole system costs, impacts, and constraints, which have been investigated elsewhere\(^ {16–18}\) but were not evaluated in this specific setting.
5 RECOMMENDATIONS FOR FUTURE WORK

This research highlighted several areas where expanded knowledge would improve application of an eventual cyanobacterial cultivation process.

These experiments were conducted with a single aqueous nutrient source. A similar toxicity response was noted when *Synechococcus* 7002 was grown at 37°C in media comprised of manure digester effluent and deionized water (T. Korosh, personal communication, 2014). However, testing of effluent from various digesters is necessary to determine if this is a response common to anaerobic digestate. If such a response is common, it may be expedient to test methods for digestate treatment such as activate carbon sorption, biological pretreatment, or co-culture.

These results also suggest further exploration of the causal mechanism of GBF toxicity. Transcriptomic profiling of *Synechococcus* 7002 in varied GBF concentrations at 27°C and 37°C could elucidate the validity of the proposed causal mechanisms of GBF toxicity. This methodology, however, has some limitation because many pathways for oxidative stress amelioration are seemingly constitutively expressed in *Synechococcus* 7002\textsuperscript{27,29,125}. Thus, understanding of the pathways responsible for amelioration of GBF stress may be better investigated by testing growth and/or survivability of knockout transformed mutants, as in Xiong et al.\textsuperscript{126}. These tools together will identify key mechanisms for enhancing fitness when this framework is transferred to other cyanobacteria.

Finally, induction by lactose would likely prove a more cost-effective alternative to IPTG at production scale\textsuperscript{127} and reduced induction levels of genes responsible for oxidative stress amelioration could improve strain fitness by reduced protein production burden\textsuperscript{102}. Thus, further
fine-tuning of induction will be paramount to the success of any strain engineered for fitness in response to cultivation media and conditions stresses.
6 REFERENCES


68. Urbanek, S. *rJava: Low-Level R to Java Interface.* (2016).


94. SCFM versus ACFM and ICFM. *The Engineering ToolBox* Available at: http://www.engineeringtoolbox.com/scfm-acfm-icfm-d_1012.html. (Accessed: 19th November 2016)


### APPENDIX A : ANOVA FACTOR SIGNIFICANCE TESTING

**Appendix Table 1** Statistical significance of tested factors and factor interactions on determination of cell health metrics. Green highlighted values indicate statistically significant effects at $\alpha = 0.05$

<table>
<thead>
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Appendix Figure 1 Fluorescence density plots for SYTO 59. Concentrations (μM) from top to bottom: 0, 0.025, 0.125, 0.5, 2.5
Appendix Figure 2 Fluorescence density plots for SYTO 60. Concentrations (μM) from top to bottom: 0, 0.025, 0.125, 0.5, 2.5,
Appendix Figure 3 Fluorescence density plots for SYTO 61. Concentrations (μM) from top to bottom: 0, 0.025, 0.125, 0.5, 2.5,
Appendix Figure 4 Fluorescence density plots for SYTOX Green. Concentrations (μM) from top to bottom: 0, 0.5, 1, 2.5, 5