The Pennsylvania State University
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A BIOPROCESSING COMPARISON OF HIGH DENSITY *BOTRYOCOCCUS BRAUNII*
AND *CHLORELLA VULGARIS* VERIFYING LIGHT LIMITED GROWTH

A Thesis in
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by

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ABSTRACT

High density cultures of *Chlorella vulgaris* and *Botryococcus braunii* were achieved in a trickle screen thin film photobioreactor. Both algae were grown under high light (525 µE/m²/s measured perpendicular to the screen and 280 µE/m²/s measured parallel to the screen), photoautrophic growth conditions in a semi-continuous reactor system where the dilution rate was set at 7.5% per day. Even though the doubling times of *Botryococcus*, 4-7 days, and *Chlorella*, 2-3 hours, are drastically different, essentially the same steady state Biomass Dry Weights, 20 gDW/L and 19 gDW/L respectively, and Biomass Productivities, 0.1 gDW/L/Photo-hr and 0.09 gDW/L/Photo-hr respectively, were achieved verifying light limited growth. Oil productivities between *Botryococcus* and *Chlorella* were quite different: 16.6 mg-oil/L/Photo-hr and 5.2 mg-oil/L/Photo-hr respectively. This difference in lipid accumulation is likely due to differences in the physiological roles of these lipids. Isoprene hydrocarbons are a structural component of the *Botryococcus* matrix that is inherent to the survival of the algae and fatty acids/triglycerides are a storage form of energy for *Chlorella*. 
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PREFACE

The work I completed during my graduate studies included seven reactor runs and genetic engineering work which are described in Appendix A and B. The focus of this thesis is to pull together data from two of those reactor runs to generate a draft of a research paper. This work represents the effort of more than a dozen students (both undergraduate and graduate students) with the work described in this thesis being largely my own effort yet only part of the total effort of the project. The second most significant piece of work I was part of (details in Appendix B) involved changing the mode of CO₂ delivery from 5% CO₂ in the gas phase to dissolving CO₂ in the evaporative liquid make-up stream. This work is still under analysis with the effort being passed on to another graduate student, Amalie Tuerk.
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And lastly, I would like to thank my family for their love and unconditional support throughout my education; I would not have made it to where I am today without them.

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Chapter 1 - Introduction and Background

The interest of algae for use as biofuels stems from several advantages of an algae based system. Compared to terrestrial plants, algae can be more productive. Algae productivity levels of 25 gDW/m²/day are routine, with reports achieving up to 100 gDW/m²/day [Lee, 2001; Pulz & Scheibenbogen, 1998]. This corresponds to 30 to 100+ tons of biomass/acre/year, which is several-fold greater than terrestrial plant productivity, for example switchgrass has typical productivities of 4 g/m²/day [FIKE et al., 2006; McLaughlin et al., 1999]. Algae like all other photosynthetic organisms are capable of using and recycling waste nutrients such as nitrogen and phosphorous from wastewater and CO₂/NOₓ from power plant emissions [Sheehan et al., July 1998]. Furthermore the higher productivities of an industrial algal processing system are amenable to be adapted for utilizing wastewater and emissions on a scale at which these wastes are present. Due to the diverse nature and ability of microalgae to adapt to environmental stresses faster than higher order plants, the use of seawater to cultivate algae has also been suggested [Sheehan et al., July 1998].

There are two algae strains that are of particular interest based on the intrinsic specific growth rate and class of lipids they produce. Botryococcus braunii, race B, has a doubling time ranging from four days to one week [Belcher, 1968; Yang et al., 2004] and produces isoprene hydrocarbons. Chlorella vulgaris has a doubling time of 2-3 hours [Sorokin, 1958], and produces fatty acids/triglycerides. Botryococcus is a colony-forming green microalgae, with each colony held together by an extracellular matrix of hydrocarbons [Knights et al., 1970; Maxwell et al., 1968], see Figure 1-1. Botryococcus is classified into three races (A, B, and L) depending on the type of hydrocarbon associated with its matrix [P. Metzger et al., 1985; P. Metzger et al., 1990]. For race B, the major matrix components are the polymethylated triterpene hydrocarbons, C30-
C37, which are referred to as botryococcenes and tetramethylsqualenes. The ‘A race’ accumulates straight chain alkadienes and alkatrienes (C25-C31), and the ‘L race’ accumulates tetraterpenes like lycopadiene [P. Metzger et al., 1985; P. Metzger et al., 1990]; [Templier et al., 1984; Templier et al., 1991]. Race B of Botryococcus is known to accumulate botryococcene and squalene derivatives up to 30–40% of their dry weight. As the greatest hydrocarbon productivity takes place during the active growth phase, these algal hydrocarbons are a growth-associated product [P. Metzger et al., 1985]; [Okada et al., 1995]. These hydrocarbons can be hydrocracked to yield smaller chain hydrocarbons for combustible grade fuels (67% of the converted oil to gasoline grade fuel, 15% to aviation fuel, and 15% to diesel fuel) [Hillen et al., 1982].

Botryococcus is an attractive algae for use as biofuel but due to its slow intrinsic growth rate has not received a lot of attention. Chlorella is a unicellular micro-algae that ranges in size from 5-10µm, Figure 1-1, and is known to accumulate large amount of lipids that can be transesterified to produce biodiesel. It has been reported that Chlorella can accumulate lipids anywhere from 20% to 60% of the biomass, depending on growth conditions and nitrogen availability [Piorreck et al., 1984]. While decreasing the nitrogen content of the media increases the lipid content, reducing nitrogen also limits biomass accumulation in a batch system [Piorreck et al., 1984]. Lipid accumulation is characterized as a growth dissociated product, where these lipids are accumulated during the stationary phase of batch growth [M. A. Borowitzka, 1988; Richardson et al., 1969]. This is in direct contrast to Botryococcus where it produces hydrocarbons while it is actively growing; Figure 1-2 illustrates the principles of growth-associated and growth-dissociated products. Due to the high lipid content of Chlorella and its fast doubling time it has received a lot of attention as use for biofuel. However, due to growth disassociated lipid production, fatty acid/triglyceride productivities are likely to be low. Gaining some insight into the impact of these different modes of lipid accumulation is a goal of this research.
Figure 1-1: (A) shows a microscope picture of *Chlorella vulgaris* and (B) a picture of *Botryococcus braunii*, race B (source noted in Materials and Methods).

![Microscope picture of Chlorella vulgaris and Botryococcus braunii](image)

Figure 1-2: Depicts growth dissociated lipid production of *Chlorella vulgaris*, where lipids are produced after a rapid period of growth (dashed line) and nitrogen depletion (dotted line), and growth associated hydrocarbon/isoprene production in *Botryococcus braunii*, race B.

Figure 1-2: Depicts growth dissociated lipid production of *Chlorella vulgaris*, where lipids are produced after a rapid period of growth (dashed line) and nitrogen depletion (dotted line), and growth associated hydrocarbon/isoprene production in *Botryococcus braunii*, race B.

In a batch system, producing biofuels by algae with a slow intrinsic growth rate (i.e. Botry) is severely limited because the slow growth rate limits productivity under non-light limited, exponential growth conditions. During batch non-light limited exponential growth, there are not enough cells to utilize all the available light during exponential phase, most of which passes through the culture. In running a continuous system, productivity is not limited by the intrinsic growth rate of an organism but is instead limited by the steady-state cell concentration and photon availability [Pipes & Koutsoyannis, January 1962]. It is logical to hypothesize that in such a photon flux limited system, the growth rate of all micro-algae would be the same. This can be shown by examining the specific growth rate equation, $\mu = \frac{1}{x} \frac{dx}{dt}$. Under light limited
conditions, the growth rate \((dx/dt)\) will be linear and since the steady state specific growth rate, \(\mu_{SS}\), is fixed by the dilution rate, the steady state cell density \((x_{SS})\) will also be fixed by the light limited growth rate. Under these conditions, the cell density is specified by the dilution rate and linear rate of growth:

\[
X_{SS} = \frac{(dx/dt)_{linear}}{D} = \frac{(dx/dt)_{linear}}{F/V}
\]

Where \(F/V\) is the daily replacement fraction of the culture, i.e. the dilution rate. An important constraint in achieving this steady state condition would be that the effective doubling time would have to be slower than the maximum intrinsic doubling time of the algae. To find the time in which the cell density is forced to double, \((t_d)\) and \(X_2 = 2 \cdot X_1\), for an operational dilution rate in a continuous system, the specific growth rate equation can be integrated and solved for time, \(t_d = \frac{\ln(2)}{F/V}\). In such a system, where the organism experiences no other limitations besides light, the productivity is dependent on the utilization of light/photons and not the organism's intrinsic specific growth rate. It is important to note that for this to be true, the dilution rate of the reactor must be lower than the intrinsic growth rate of the slowest growing algae (i.e. *Botryococcus*), otherwise the rate of removal of culture, the dilution rate, is greater than the growth of the organism and wash-out of the culture will occur. For example, at a dilution rate of 20% for *Botryococcus* culture, the doubling time, \(t_d\), of the reactor equals 3.5 days, calculated from the specific growth rate equation, \(t_d = \frac{\ln(2)}{F/V}\). The doubling time of the reactor is now faster than the double time of *Botryococcus*, doubling time of 4 – 7 days, and the cells will not double fast enough to keep up with the rate of removal. In order to achieve and maintain light limited growth in a chemostat or photobioreactor for a slow growing organism, ultra-high densities are required.
Besides the inability to utilize the diversity of algae and the low value compounds they make such as biofuels, current technology is limited by process economics to produce only high value products [Lundquist et al., October 2010]. To make an algal processing system economically feasible for biofuel production, improved bioreactor design operating principles are vital. Given the current technological status, a biofuel producing algal system is mainly plagued by low volumetric productivities and high hydraulic load (pumping, biomass separation, extraction efficiency). While open pond designs are cheap to operate, they do not yield high enough cell densities due to lack of light penetration. One the other hand, enclosed bioreactors have moderate productivities and provide protection against contaminants, though their high capital investment and operational costs are cost prohibitive [Sheehan et al., July 1998]. A more detailed comparison of some bioreactor systems is summarized in Table 1-1, table adapted from [M. Borowitzka, 1999; Pulz & Scheibenbogen, 1998].

Table 1-1: A qualitative comparison of bioreactor systems

<table>
<thead>
<tr>
<th>Reactor type</th>
<th>Mixing</th>
<th>Productivity</th>
<th>Cost</th>
<th>Light utilization efficiency</th>
<th>Temperature Control</th>
<th>Gas transfer</th>
<th>Species control</th>
<th>Scale-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstirred shallow open ponds</td>
<td>Very poor</td>
<td>Low</td>
<td>Low</td>
<td>Poor</td>
<td>None</td>
<td>Poor</td>
<td>Difficult</td>
<td>Very difficult</td>
</tr>
<tr>
<td>Open tanks</td>
<td>Poor</td>
<td>Low</td>
<td>Low</td>
<td>Very poor</td>
<td>None</td>
<td>Poor</td>
<td>Difficult</td>
<td>Very difficult</td>
</tr>
<tr>
<td>Circular stirred open ponds</td>
<td>Fair</td>
<td>Low</td>
<td>Low-Moderate</td>
<td>Fair-good</td>
<td>None</td>
<td>Poor</td>
<td>Difficult</td>
<td>Very difficult</td>
</tr>
<tr>
<td>Paddle-wheel open raceway ponds</td>
<td>Fair-good</td>
<td>Low</td>
<td>Low-Moderate</td>
<td>Fair-good</td>
<td>None</td>
<td>Poor</td>
<td>Difficult</td>
<td>Very difficult</td>
</tr>
<tr>
<td>Enclosed stirred tank reactor</td>
<td>Largely uniform</td>
<td>Low</td>
<td>Moderate-High</td>
<td>Fair-good</td>
<td>Excellent</td>
<td>Low-high</td>
<td>Easy</td>
<td>Difficult</td>
</tr>
<tr>
<td>Enclosed air-lift reactor</td>
<td>Generally uniform</td>
<td>Low</td>
<td>Low-Moderate</td>
<td>Good</td>
<td>Excellent</td>
<td>High</td>
<td>Easy</td>
<td>Difficult</td>
</tr>
<tr>
<td>Enclosed bag culture</td>
<td>Variable</td>
<td>Low-Moderate</td>
<td>High</td>
<td>Fair-good</td>
<td>Good</td>
<td>Low-high</td>
<td>Easy</td>
<td>Difficult</td>
</tr>
<tr>
<td>Enclosed flat-plate reactor</td>
<td>Uniform</td>
<td>High</td>
<td>High</td>
<td>Excellent</td>
<td>Excellent</td>
<td>High</td>
<td>Easy</td>
<td>Difficult</td>
</tr>
<tr>
<td>Enclosed tubular reactor</td>
<td>Uniform</td>
<td>High</td>
<td>High</td>
<td>Excellent</td>
<td>Excellent</td>
<td>Low-high</td>
<td>Easy</td>
<td>Reasonable - Easy</td>
</tr>
</tbody>
</table>
Ultra high density continuous cultures (greater than 20 g/L) allow for increased volumetric productivities as well as decreased hydraulic load. A thin film is required to grow high density cultures while maintaining high volumetric productivities because light penetration is a function of culture density. By increasing the culture density, the dark volume of the culture is also increased; this can be minimized in a trickle film photobioreactor. Figure 1-3 shows a schematic of a trickle screen photobioreactor and demonstrates the principles of increased light penetration and turbulent mixing for a thin film screen reactor. An ultra thin film (<1mm) allows for maximum light penetration and the perforated screen increases turbulent mixing. Based on the Beer-Lambert Law light can only penetrate a fraction of a millimeter through high density cultures; this is illustrated in Figure 1-4 where PAR stands for Photosynthetically Active Radiation, the range of solar radiation from 400 to 700 nm that photosynthetic organisms use for photosynthesis. The Beer-Lambert law would predict attenuation of full sunlight (2000 μE/m²/s) to the saturation point of typical algae (200 μE/m²/s) [Lloyd et al., 1977] proportional to the inverse of the optical density which corresponds to a culture depth of 0.2 mm at an OD=50, ~20 gDW/L for *Chlorella*. The issue of light penetration can be overcome by increasing the surface area to dark volume ratio (i.e. decreasing the culture depth). The turbulent mixing of a trickle screen reactor gives cells an equal opportunity to utilize the photons available and minimizes the dark volume.
Figure 1-3: (A) a schematic drawing of a trickle screen photobioreactor where (B) demonstrates the increased light penetration of a thin screen and (C) shows the increase in mixing on the trickle screen.
Figure 1-4: Light penetration in algae culture calculated by Beer-Lambert law for varying cell concentrations ($I=I_0*10^{-\alpha*L}$, $A=\alpha*L$ where $I$=intensity of the incident light, $L$=path length, $A$=absorbance, and $\alpha$=absorption coefficient)

We have achieved ultra high densities (30 gDW/L of total culture) and productivities (0.15 gDW/L/photo-hr) higher than those in literature on a trickle film photobioreactor. Our experiments were duplicated under the same operating conditions, light-limited and continuous operating mode, for *Botryococcus* and *Chlorella*, and we showed that their biomass accumulation and productivities were similar as hypothesized, even though the doubling time of *Chlorella* is 50 times faster than *Botryococcus*. 
Chapter 2 - Materials and Methods

Culture

The algae strain *Chlorella vulgaris* is indexed at UTEX as culture number 2714 [UTEX, ], and the algae strain *Botryococcus braunii*, race B was obtained from University of Kentucky (Berkeley, Showa strain) [Nonomura, 1988].

Media

To facilitate growth to high-density culture, a balanced media (WFAM) was developed based on comparison of existing algae formulations (Chu-13 and HS) as well as plant tissue culture media (MS and B5 salts). The rationale for utilizing plant based media is that composition of algae and higher plants should be similar for mineral nutrients and plant tissue culture has been used successfully for decades of serial heterotrophic culture to densities of 10-20 grams dry weight (gDW) without problems of accumulated toxic salts. It is worth noting that previous algae media were formulated for growth to 1-2 gDW/L and clearly had very high levels of some inorganics relative to the supported biomass that would not scale to the target of 20+ gDW/L media required for high-density culture. The composition of WFAM, used for *Botryococcus* cultures, included both ammonium and nitrate nitrogen sources, as is used in plant media to provide a balance of pH during nitrogen utilization. The basic composition for WFAM-3g is based on a nitrogen level of 0.3 gN/L, which could support biomass growth up to 3 gDW/L assuming 10% nitrogen by weight: KNO$_3$ (0.60 g/L), NH$_4$NO$_3$ (0.61 g/L), 1M phosphate stock of K$_2$HPO$_4$ (0.115 g/L) and KH$_2$PO$_4$ (0.045 g/L), Fe-EDTA (0.024 g/L), and 1 mL of WFAM micronutrient stock prepared as follows: H$_3$BO$_3$ (1.86 g/L), MnCl$_2$·4H$_2$O (0.54 g/L), ZnSO$_4$
(0.0371 g/L), Na$_2$MoO$_4$·2H$_2$O (0.031 g/L), CoCl$_2$·6H$_2$O (0.03 g/L), and CuSO$_4$·5H$_2$O (0.0075 g/L). After autoclaving, 1 mL of Mg$^{2+}$ and 0.88 mL of Ca$^{2+}$ solutions were added aseptically as 1M stocks (0.0588 g/L MgSO$_4$ and 0.0486 g/L MgCl$_2$; 0.132g/L CaCl$_2$·2H$_2$O). For media up to this concentration (WFAM-3g) there were minimal problems of precipitation, however to facilitate high density continuous feeding, media at higher concentrations was made without Mg$^{2+}$ or Ca$^{2+}$ and these solutions were added to an appropriate amount of media just before use from aseptic stocks.

WFAM media was slightly modified for growth of *Chlorella*. The composition of WFAMC, formulated for *Chlorella*, is similar to that of WFAM but included only nitrate as a nitrogen source with the use of acids for pH balance. The basic composition for WFAMC-3g is based on a nitrogen level of 0.319 gN/L: KNO$_3$ (2.2 g/L), 1M phosphate stock of K$_2$HPO$_4$ (0.150 g/L), KH$_2$PO$_4$ (0.059 g/L), Fe-EDTA (0.024 g/L), and 1mL of WFAM micronutrient stock prepared as follows: H$_3$BO$_3$ (1.86 g/L), MnCl$_2$·4H$_2$O (0.54 g/L), ZnSO$_4$ (0.0371 g/L), Na$_2$MoO$_4$·2H$_2$O (0.031 g/L), CoCl$_2$·6H$_2$O (0.03 g/L), and CuSO$_4$·5H$_2$O (0.0075 g/L). After autoclaving, 1 mL of Mg$^{2+}$ and 88 µL of Ca$^{2+}$ solutions were added aseptically as 1M stocks (0.0588 g/L MgSO$_4$ and 0.132 g/L Mg(NO$_3$)$_2$·6H$_2$O; 0.0132g/L CaCl$_2$·2H$_2$O). As with WFAM, to facilitate high density continuous feeding, media at higher concentrations was made without Mg$^{2+}$ or Ca$^{2+}$ and these solutions were added to an appropriate amount of media just before use from aseptic stocks.

WFAMC media was modified throughout the reactor run to account for pH changes in the culture. Media was modified as follows, the 1M phosphate stock was replaced with H$_3$PO$_4$ (0.126 g/L), with the same equivalence of phosphorous. This media was designated as Phosphoric acid WFAMC. Phosphoric acid WFAMC was further modified during the reactor run to control pH. Phosphoric acid WFAMC was made without KNO$_3$ (2.2g/L) where the equivalent amount of nitrogen (0.319gN/L) was added as HNO$_3$. This media was designated as Phosphoric/Nitric acid WFAMC.
**Lighting**

Experiments were carried out in a Conviron BDW120 walk-in incubator equipped with high-intensity lighting supplied by banks of Philips (#36881-1) 400W high-pressure sodium vapor and Philips (#34415-0) 400W metal-halide lamps in chilled loft that is constructed of plexiglass to decrease UV radiation by 20%. This environmental chamber allowed for mimicking 24-hr diurnal sunlight periods; the lights would come on at 1/3 power for the first and last hour of the 16-hr lighted photo-period. Local light intensities as photosynthetically active radiation (PAR) were measured with a LICOR LI-1400 data logger and LI-190SA quantum sensor. The studies carried out on the trickle film reactor occurred at an average light flux of 525 µE/m²/s measured perpendicular to the screen and at an average light flux of 282 µE/m²/s measured parallel to the screen. An average light flux of 282 µE/m²/s was used in subsequent calculations because measuring the light parallel to the reactor/screen is a more realistic view of how the light is actually hitting the screen.
### Light Flux measured parallel to the screen (µE/m²/s)

**FRONT of Screen**

<table>
<thead>
<tr>
<th>Inches down the screen</th>
<th>Right</th>
<th>Middle</th>
<th>Left</th>
<th>AVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>330</td>
<td>350</td>
<td>340</td>
<td>340</td>
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<tr>
<td>14</td>
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<td>28</td>
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<td>250</td>
<td>250</td>
</tr>
</tbody>
</table>

**BACK of Screen**

<table>
<thead>
<tr>
<th>Inches down the screen</th>
<th>Right</th>
<th>Middle</th>
<th>Left</th>
<th>AVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>350</td>
<td>330</td>
<td>340</td>
<td>340</td>
</tr>
<tr>
<td>14</td>
<td>280</td>
<td>290</td>
<td>270</td>
<td>280</td>
</tr>
<tr>
<td>28</td>
<td>180</td>
<td>190</td>
<td>200</td>
<td>190</td>
</tr>
</tbody>
</table>

**AVERAGE light flux across the screen (parallel), front and back (µE/m²/s):** 282

### Light Flux measured perpendicular to the screen

**FRONT of Screen**

<table>
<thead>
<tr>
<th>Inches down the screen</th>
<th>Right</th>
<th>Middle</th>
<th>Left</th>
<th>AVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>286</td>
<td>543</td>
<td>670</td>
<td>499.6667</td>
</tr>
<tr>
<td>14</td>
<td>600</td>
<td>600</td>
<td>600</td>
<td>600</td>
</tr>
<tr>
<td>28</td>
<td>580</td>
<td>550</td>
<td>570</td>
<td>566.6667</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>400</td>
<td>400</td>
<td>400</td>
</tr>
</tbody>
</table>

**AVERAGE light flux across the screen (perpendicular), front (µE/m²/s):** 525

Figure 2-1: Layout of light levels on the photobioreactor

**Temperature Control**

The environmental growth chamber temperature was controlled at 28°C during lighted hours and 25°C during dark hours with a linear temperature change during the lighting warm-up and cool down. Heat was removed from the enclosed CO₂ atmosphere trickle film reactor by passing the culture through a heat exchanger, Figure 2-2, and refrigerated circulator (more detailed description under the Trickle Film Reactor Set-up section).
Figure 2-2: A picture of the heat exchanger and humidification column for the trickle film photobioreactor set-up

Carbon Delivery

The reactor was supplemented with ~5% CO\textsubscript{2} v/v in air; the gas concentrations were obtained by mixing pure CO\textsubscript{2} from a gas cylinder and building air using rotameters set to achieve the appropriate gas concentration mixtures (Sho-Rate Model 1355E Rib Guided Tubes, Spherical Floats). CO\textsubscript{2} flow was set at 28 mL/min and air at 619 mL/min for a total flow of 647 mL/min. After the CO\textsubscript{2} and air were mixed, the gas line passed through a 0.2 μm Gelman Acro 50 gas vent. To minimize evaporative losses, the gas stream was humidified by passing through a 3” ID, 30.5” high glass column that was filled with distilled water and packed with stainless steel Berl saddles to improve gas-liquid contacting, see Figure 2-2, under Temperature Control. The CO\textsubscript{2}
concentration levels were measured at the inlet and outlet of the reactor with an Illinois Model 3750 Oxygen and Carbon Dioxide Analyzer.

The inoculum cultures, in shaker flasks, were also supplemented with ~5% CO$_2$ v/v in air by mixing pure CO$_2$ from a gas cylinder with building air using rotameters set to achieve the appropriate gas concentration mixtures. Evaporation was minimized by humidifying the gas stream by passing it through sintered glass spargers in a train of three 500-mL flasks containing distilled water. The gas was passed to the flasks cultures (arranged as a daisy chain of three flasks) through a 0.2 μm Gelman Acro 37TF gas vents at the flask inlets and sterilized non-absorbent cotton at the flask outlet.

**Growth Measurements**

Culture optical density for both *Chlorella* and *Botryococcus* was measured in 1-cm path-length cuvettes in a spectrophotometer at 550 nm (OD$_{550}$) since this wavelength is insensitive to changes in chlorophyll content, providing a biomass concentration independent of the chlorophyll present. All OD$_{550}$ measurements were diluted to provide OD $<$ 0.5 where response was more linear.

Because cultures of *Botryococcus* float and sometimes contain neutrally buoyant cells, the measurement of dry weight concentration could not be done using a rinsed centrifuged pellet; instead, a very meticulous freeze-drying procedure was used. Polypropylene microcentrifuge tubes (1.7 mL) were prepared to 4 decimal places and handled with clean-room gloves. Precise 1 mL aliquots of each whole culture and media filtrate (20 μm nylon mesh - Small Parts, Inc.; CMN-0020-D) were pipetted into the tubes; filter cake (cells retained on the 20μm mesh) was carefully transferred into a tube. All samples were stored in the -20°C freezer until they were freeze dried. The dry weight concentrations (Total Culture and Filtrate Dry Weights) were
determined from the final freeze dried weights and the initial volume placed into the tubes;
Biomass Dry Weight concentration was determined redundantly through both the filter cake method and by taking the different of the whole culture and media filtrate determinations.

Measurement of dry weight for *Chlorella* was done by two methods. The first involved the same freeze dried method used for *Botryococcus* where polypropylene microcentrifuge tubes (1.7 mL) were pretared to 4 decimal places and handled with clean-room gloves. Total/whole culture, supernatant and a rinsed centrifuged cell pellet were freeze dried. The weights of the Total Culture, Supernatant and Biomass (algae cell pellet) were determined by weight difference of the pretared tube. The centrifuged cell pellet was prepared as follows, in a pretared microcentrifuge tube a 1 mL total culture was spun down using an Eppendorf Centrifuge 5415C at 16,025 RCF for 5 min, the supernatant removed, 1 mL of tap water added and the pellet re-suspended, spun down again at 16,025 RCF for 5 min, supernatant removed and sample stored for analysis. The second method involved the use of a Baxter Scientific Products Muffle Furnace AFM-21 to determine salt DW and total organics DW from total culture and supernatant samples. Crucibles were pretared to 4 decimal places and handled with clean-room gloves. Crucible samples were then placed in a 70°C drying oven, for a minimum of two hours, until all liquid had evaporated from the sample. A scale weight of the sample was taken for dry weight of total culture and supernatant. Crucibles were then placed in a muffle furnace at 600°C overnight, for a minimum of 6 hours. Crucibles were removed from the muffle furnace and were allowed to cool to room temperature in a desiccated box after which the salt and organics dry weight were determined by weight difference. This crucible method was also performed on *Botryococcus* for salt and total organic dry weights.

The following definitions are used to describe the dry weight in the system:

**Total Culture Dry Weight (DW<sub>TC</sub>):** the concentration of the dehydrated mass of all solids in an algae culture, both suspended (cells and debris) and dissolved (salts, excreted polysaccharides,
other components); Biomass Dry Weight (DW_B), Botryococcus: the dehydrated weight of Botryococcus colonies, performed by taking the difference of the total culture dry weight and the dry weight of the filtrate (portion which passed through a 20µm mesh); Biomass Dry Weight, Chlorella: the dehydrated weight of the centrifuged and washed cell pellet that forms when the algae culture is centrifuged at 14,000 RPM on a microcentrifuge; Ash-free Dry Weight (DW_{AF}): determined by subtracting the weight of residual ash from the Total Culture Dry Weight after a dry total culture sample is combusted at 600°C overnight.

Chlorella cells were counted under a microscope with a hemocytometer. Since Botryococcus is colony-forming and aggregate sizes can vary significantly, it was not possible to accurately count algae cells using a hemocytometer. Cell counts were estimated based on a DNA analysis performed by collaborators at the University of Kentucky. This analysis was performed on previous Botryococcus shaker flask studies of similar density (18 gDW/L) where the average DNA density was 4427 ngDNA/mL. Assuming a proportionality between DNA concentration with increasing dry weight concentration, an estimate was made of the DNA density for the Botryococcus reactor culture. Using cell DNA content of 150 pg/cell (estimated from the genome size), an attempt was made to estimate the cell number concentration for Botryococcus.

For the analysis of DNA density (ng/mL) performed at the University of Kentucky, 500µL of total culture sample was diluted two-folds with Milli-Q water and sonicated twice at 60% power for 30 second increments. The sonicated lysates were filtered through a 0.2 µm filter to utilize for DNA analysis. The analysis was executed on a Hoefer DNAquant with Hoechst 33258 dye. DNA densities were determined by comparing readings to a calf thymus DNA standard.

The electrical conductivity of the culture filtrate was measured as a way to qualitatively monitor the inorganic ions in solution. The conductivity of both the Botryococcus and Chlorella runs was measured in a 1.5 mL microcentrifuge tube by equilibrating the sample to 25°C using a
MI-905 miniature conductivity probe (Microelectronics, Inc.) and a YSI model 35 conductance meter with the cell constant verified daily using a 0.01 N KCl standard. Conductivity was also measured with a VWR® Bench/Portable Conductivity Meter with a glass probe, VWR Catalog# 23226-504, (temperature compensation built in).

**Lipid extraction and analysis**

Hydrocarbon analysis on *Botryococcus* samples were performed by our collaborators at the University of Kentucky (see acknowledgements). Total culture samples (15-30 mg) was first vortexed at full speed for two minutes with acetone to break down the algae cells so the hydrocarbons could be more readily extracted. The algae-acetone mixture was then extracted with n-hexane, vortexed at full speed for two minutes, and centrifuged at 500-1000 RCF for 1 minute to separate the aqueous and organic phases. Hexane extraction was repeated on the aqueous phase to increase recovery of hydrocarbons. After concentration of the hexane extract under a stream of nitrogen, the dried oil samples were resuspended in n-hexane and analyzed with an HP 5890 GC-FID (Gas Chromatography with a Flame Ionization Detector) and a Restek Rtx-5ms column (30m, 0.25mmID, 0.25um df). The following program was used: initial temperature 220°C, hold for 1min, followed by a 20°C per min gradient until 280°C, then at a rate of 3°C per min until 320°C, hold at 320°C for 5min. Squalene calibration standards were included with every analysis for quantification purposes. Since hydrocarbon analysis was performed on the total culture, the oil data presented is on a total culture basis. It was estimated that about 95% of the total hydrocarbons were recovered in the two combined hexane extraction. This conclusion was based on the finding that when a samples was extracted three times with hexane, the first extract=1000ng of total oil, second extract=100ng of total oil, and the third extract=10ng of total oil. It was therefore concluded that each hexane extract successfully extracted about 90% of the
total oil in the sample. So in the case of the two pooled samples for the hydrocarbon analysis, 90% of the total oil was extracted in the first extract and 90% of the remaining 10% in the second extract. A conservative estimate of 95% of the total oil extracted was used as a recovery.

Total cellular lipid analysis on *Chlorella* samples were performed at NETL (National Energy Technology Laboratory) using their newly developed method of total cellular lipid extraction (see acknowledgements). Each sample was subject to direct transesterification and extraction in triplicate, with each replicate injected into the GC-FID in triplicate. Direct esterification was performed by reacting 3.5 mg of biomass sample with acidic methanol in an acid, at 120°C for 2 hours. Upon cooling, the sample was spiked with a known amount of a C_{13} FAME (Fatty Acid Methyl Ether), as an extraction recovery standard, and an equal volume of 20% CaCl$_2$ (w/w) was added, which serves as a catalyst. The mixture was extracted three times with hexane with all extracts were combined and dried over CaCl$_2$ and nitrogen. The resultant FAMEs were dissolved in 100 µl of toluene and analyzed by GC immediately. Spike recoveries, as FAME, were done to measure the completeness of the transesterification reaction as well as measure the extraction efficiency for removing the converted FAME from the reaction mixture. One-Way ANOVA analysis was performed on all of the samples at the 0.05 confidence level. The lipid content of the samples were compared with one another to see if the amount of lipid was truly a different amount or the result of a different mean produced by large differences in standard deviations. It was also determined if any single replicate or series of injections displayed statistical differences in the means. There were no statistically significant differences observed within replicates of the same sample, this suggested that any differences observed between samples were a result of differences in the individual sample themselves. Since lipid analysis was performed on the centrifuged cell pellet, the oil data presented is on a biomass basis.
Inoculum Preparation

*Botryococcus* inoculum was prepared from high density stock cultures supplemented with 5-10% CO₂. Cultures were grown up in a fed-batch manner, and then subcultured semi-continuously once a week at a 20-25% dilution rate, with increasing strength of media (up to 2x WFAM) and light (up to 500 µE/m²/s). Once the culture volume reached 125 mL in a 500 mL Erlenmeyer flask, any culture removed from the flaks was added to a secondary flask to amplify culture. The cultures were maintained at high density in shaker flasks at culture depth less than 1 inch, grown on a gyratory shaker (New Brunswick G10) at 122 rpm; this aided in heat removal from the culture.

Initial liquid cultures of *Chlorella* were inoculated in WFAMC from an axenic stock. These liquid cultures were started in 1/2x strength WFAMC, inoculated at an OD₅₅₀=0.02 from an axenic liquid culture, and grown up in a fed-batch manner where they were subcultured daily to twice daily, ranging from every 9 to 13 photo-hours, with increasing strength of media (5xWFAMC) and light (up to 500 µE/m²/s) to reach a high density culture quickly. Cultures were fed based on a mass balance rational and were given nutrients based on amount they grew between subcultures (assuming nitrogen content of 10%). Once cultures reached a high cell density (15-20 gDW/L) they were maintained with weekly subcultures, where the amount of culture removed was replaced with enough nutrients to maintain the concentration of media at 1xWFAMC strength assuming all previous nutrients had been depleted. This was employed until cultured were used in the trickle film reactor. This inoculum was also grown in shaker flasks at a culture depth less than 1 inch on a gyratory shaker (New Brunswick G10) at 122 rpm; this aided in heat removal from the culture. For the reactor 500 mL inoculum, in-order to force an even higher culture density, 700 mL of total culture (monoculture, not axenic) was spun down on in a Beckman Coulter Allegra® X-12R Benchtop Centrifuge at 2,000 RCF for 10 min. A total of 180
mL of supernatant was removed and the remaining culture resuspended by gentle mixing/inversion. 500 mL of this concentrated OD inoculum was measured out in an autoclaved glass cylinder.

**High-Density Culture Feeding Strategy**

To maintain cultures at high density, they were fed with highly concentrated nutrient solutions. To prevent build up of inhibitory levels of nutrients, a feeding strategy was developed to replace nutrients as they were consumed. The continuously running reactor was fed in a semi-continuous manner where it was subcultured once a day at 7.5% replacement rate. This feeding strategy was based on OD₅₅₀ measurements because they are immediately available at the time of each sampling and nitrogen was chosen as the component on which to base the quantitative feeding strategy. The measured OD₅₅₀ allowed an estimation of the biomass removed each day based on the average dry weight to OD₅₅₀ correlation, (gDW/L)/OD₅₅₀. For *Chlorella* this correlation was based on an average DWₐ to OD₅₅₀ ratio of 0.5 (gDWₐ/L)/(OD₅₅₀ @ 1cm). See Figure 2-3 for a schematic of the feeding strategy.

![Diagram](image)

Figure 2-3: Feed strategy based on calculated mass of nutrients removed, based on correlation with optical density and periodic dry weight correction
For the *Botryococcus* culture, there were dramatic changes in $D_{W TC}$ for the same $OD_{550}$, with the ratio ranging from 3.5 to 6.5 ($g_{DW TC}/L) / (OD_{550} @ 1cm$), and therefore there was a need to correct this ratio continuously during the reactor run. Since it takes a day or more to obtain freeze-dried weights, this ratio was updated typically using a prior 4-day average. By assuming a *Botryococcus* biomass nitrogen content of 5%, the amount of nitrogen that needed to be replaced could be immediately calculated, and using a 6x WFAM concentrate, an appropriate volume of concentrate and sterile water was prepared and added back to the culture.

In comparison to *Botryococcus*, the dry weight to $OD_{550}$ ($g_{DW TC}/L) / OD_{550}$ ratio of *Chlorella* stayed fairly constant throughout the reactor run, ratio of $0.5 \pm 0.05$ ($g_{DW b}/L) / (OD_{550} @ 1cm$). The ratio was still updated to account for any variation as freeze-dried weights became available. By taking biomass nitrogen content of 10% (in contrast to a 5% nitrogen content for *Botryococcus*, difference discussed in the Results and Discussion), the amount of nitrogen that needed to be replaced could be immediately calculated, and using a 20x WFAMC concentrate, an appropriate volume of concentrate and sterile water was prepared and added back to the culture.

**Trickle Film Reactor Set-up**

The reactor used in both experiments with *Botryococcus* and *Chlorella* was based off the photobioreactor model designed by Dr. Wayne Curtis, patent applied for [Curtis, April 2009].

The photobioreactor consisted of a single screen, 30.5” x 16.5” or 29.5” x 16.5”, thickness of 0.33 mm (measurement from Phifer manufacturer), enclosed in a clear plastic bag, 2 mil x 30”x 34”.

The screen material was a fiberglass window screen stock, manufactured by Phifer, whose perforations/wires were oriented at a 45° diagonal, this was done to increase resistance to flow and induce turbulence. Figure 2-4 shows a photograph of the operational reactor system with important components labeled. Total culture volume in the system was 500 mL and was
collected at the bottom of the screen to flow into a PYREX® 1L aspirator bottle with a bottom sidearm (product #1220-1L). The reservoir top was a silicone stopper penetrated by stainless steel ports (in/out gas port, liquid return, sample port, temperature probe; see Figure 2-5 for details). Culture was pumped through the system by a Watson-Marlow peristaltic pump 601S at (20-30 rpm, 0.48 L/min to 0.84 L/min) with neoprene tubing. The culture then passed through a 24” long piece of 3/8” OD stainless tube fashioned into a heat exchanger by passed through a set of 1.5” Tri-Clamp stainless steel Long-Tees with silicone stoppers holding the stainless tube in place. Cooling water for heat removal from the system was provided using a Fisher Scientific Isotemp Refrigerated Circulator, model 9100, which was set at 25°C. The culture then penetrated the clear head plate of the liquid distributor. The liquid distributor was constructed as a tube-within-a-tube to even out flow; the cultured entered both ends of the horizontal distributor into 1/4” inch tubing that was perforated with holes every 12 mm. This primary distributor was placed inside a second piece of 1/2” tubing that had holes drilled in a zig-zag array every 6mm.

The primary 1/4” distributor holes faced upward inside the larger secondary tube so that liquid would drain down onto the screen with minimal momentum. Where possible, clear vinyl tubing, 3/8” OD, for the reactor was used. For the Chlorella run, culture temperature was monitored with a thermometer, VWR® Digital Thermometer with Recorder Output # 77776-726 with type-K probe # 61161-305, and pH of the culture was monitored on-line with a Cole-Parmer® Standard Bulb, In-Line pH Electrode (EW-27001-90).
Figure 2-4: A photograph of the operational reactor system, depicting the culture path flow through the system and the monitoring system used in the Chlorella experiment (pH, temperature, and CO₂ monitoring)
Figure 2-5: Detailed picture of the reservoir and the silicon stopper ports
Chapter 3 - Results and Discussion

Under semi-continuous, light limited, photoautotrophic growth, our trickle film photobioreactor reached some of the highest volumetric productivities reported in literature. The photobioreactor design used in the experiments consisted of an enclosed, vertical screen upon which the algae culture flowed downward in a turbulent, thin film, which promoted mixing and increased light penetration. These design principles enabled our reactor system to achieve a high concentration of Dry Weight (DW) and high Productivities (P) in a semi-continuous system at a daily replacement rate (effective dilution rate) of 7.5% per day. The photobioreactor systems attained steady state Total Culture Dry Weights (DW\(_{TC}\)) and Total Culture Productivities (P\(_{TC}\)) of 31.6±1.0 gDW/L and 0.148±0.005 gDW/L/Photo-hr for *Chlorella* and 26.7±0.6 gDW/L and 0.125±0.003 gDW/L/Photo-hr for *Botryococcus*. The oil productivities of each system were 5.29±0.55 mg-oil/L/Photo-hr and lipid contents of 6.09±0.67 % (per cell biomass) for *Chlorella* and oil productivities of 16.64±3.23 mg-oil/L/Photo-hr and 13.20±2.70 % hydrocarbon content (per total culture) for *Botryococcus*. Table 3-1 is a summary of the dry weight data and Table 3-2 is a summary of the oil data. Figure 3-1 shows the time course of DW\(_{TC}\) and Biomass Dry Weight (DW\(_{B}\)) during the steady state periods compared between *Chlorella* and *Botryococcus*. The steady state time period for the *Chlorella* run was taken from day 13 through day 24 in the photobioreactor and for *Botryococcus* from day 16 through day 27. Both reactor cultures started out at a similar DW\(_{TC}\) of approximately 25 gDW/L and took 13 to 16 days to reach their respective steady state cell densities.
Table 3-1: Summary of dry weight data in the photobioreactor at steady state

<table>
<thead>
<tr>
<th>Algae Strain</th>
<th>Total Culture DW (gDW/L)</th>
<th>Total Culture Productivity (gDW/L/Photo-hr)</th>
<th>Ash Free Total Culture DW (gDW/L)</th>
<th>Biomass Dry Weight (gDW/L)</th>
<th>Biomass Productivity (gDW/L/Photo-hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorella</td>
<td>31.6 ± 1.0</td>
<td>0.148 ± 0.005</td>
<td>23.1 ± 1.3</td>
<td>18.5 ± 0.5</td>
<td>0.087 ± 0.002</td>
</tr>
<tr>
<td>Botryococcus</td>
<td>26.7 ± 0.6</td>
<td>0.125 ± 0.003</td>
<td>22.7 ± 0.6</td>
<td>20.5 ± 0.5</td>
<td>0.096 ± 0.002</td>
</tr>
</tbody>
</table>

Table 3-2: Steady state percent oil content and oil productivities for Chlorella and Botryococcus

<table>
<thead>
<tr>
<th>Algae Strain</th>
<th>Oil Concentration (g-oil/L)</th>
<th>Oil Productivity (mg-oil/L/Photo-hr)</th>
<th>% weight of oil (Biomass or Total Culture DW basis)</th>
<th>% weight of oil (Ash-free DW basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorella</td>
<td>1.1 ± 0.1</td>
<td>5.285 ± 0.551</td>
<td>6.1 ± 0.7</td>
<td>6.7 ± 0.9</td>
</tr>
<tr>
<td>Botryococcus</td>
<td>3.5 ± 0.7</td>
<td>16.640 ± 3.231</td>
<td>13.2 ± 1.6</td>
<td>15.7 ± 2.0</td>
</tr>
</tbody>
</table>

Figure 3-1: The time course of Total and Biomass Dry Weights in the photobioreactor. The steady state averages taken for the comparison of Chlorella to Botryococcus are marked as solid black lines.
Similar steady state dry weights and productivities were achieved in the system. A two-tailed T-test was performed on the dry weight and oil data to test the statistical significance of the steady state comparison between the Chlorella and Botryococcus runs; hypothesis tested, that the average dry weight and oil concentrations over the steady state are not significantly different.

The steady state concentrations of DW_{TC} and DW_{B}, oil concentration (g-oil/L), and weight percent of oil (calculated on both a total culture and ash-free culture mass basis) were each found to be statistically different at all confidence levels. This statistical difference is enabled in part due to the relatively small variance observed over the steady state period. The Ash-free Dry Weights (DW_{AF}) were, however, found to be statistically indistinguishable at a 99% confidence level. Equation 3-1 gives the list of equations used in the T-test and Table 3-3 is a truncated T-table for the degrees of freedom (DOF) used in the calculations over a range of confidence intervals. The steady state averages between the Chlorella and Botryococcus runs were likely only statistically indistinguishable for the DW_{AF} due to the disproportioned amount of salt in the culture between the two runs. This difference is illustrated in Figure 3-3 (page 39) and is discussed in more detail below.

\[
s_{1,2} = \sqrt{\frac{(n_1-1) \cdot s_1^2 + (n_2-1) \cdot s_2^2}{(n_1-1) + (n_2-1)}}
\]

\[
t_{\text{statistic}} = \frac{Y_{1,\text{avg}} - Y_{2,\text{avg}}}{s_{1,2} \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}
\]

\[
DOF = n_1 + n_2 - 2
\]

Equation 3-1: The \( t_{\text{statistic}} \), \( s_{1,2} \), and degrees of freedom (DOF) were calculated according to these equations.
Table 3-3: $T_{table}$ values for a range of confidence intervals

<table>
<thead>
<tr>
<th>Confidence interval</th>
<th>10%</th>
<th>25%</th>
<th>50%</th>
<th>75%</th>
<th>85%</th>
<th>90%</th>
<th>95%</th>
<th>99%</th>
</tr>
</thead>
<tbody>
<tr>
<td>alpha/2</td>
<td>0.45</td>
<td>0.375</td>
<td>0.25</td>
<td>0.125</td>
<td>0.075</td>
<td>0.05</td>
<td>0.025</td>
<td>0.005</td>
</tr>
<tr>
<td>$T_{table}, DOF=13$</td>
<td>0.7789</td>
<td>0.9187</td>
<td>1.2041</td>
<td>1.6398</td>
<td>1.9354</td>
<td>2.1604</td>
<td>2.5326</td>
<td>3.3725</td>
</tr>
<tr>
<td>$T_{table}, DOF=16$</td>
<td>0.7744</td>
<td>0.9126</td>
<td>1.1937</td>
<td>1.6189</td>
<td>1.9044</td>
<td>2.1199</td>
<td>2.4729</td>
<td>3.2520</td>
</tr>
<tr>
<td>$T_{table}, DOF=22$</td>
<td>0.7691</td>
<td>0.9055</td>
<td>1.1815</td>
<td>1.5949</td>
<td>1.8690</td>
<td>2.0739</td>
<td>2.4055</td>
<td>3.1188</td>
</tr>
</tbody>
</table>

Table 3-4: Summary of the T-test data performed on the steady state values of the *Chlorella* and *Botryococcus* runs

<table>
<thead>
<tr>
<th>Comparison</th>
<th>DOF</th>
<th>$s_{1,2}$</th>
<th>$t_{statistic}$</th>
<th>Outcome compared to $T_{table}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Culture DW</td>
<td>22</td>
<td>0.8159</td>
<td>14.6354</td>
<td>At all confidence intervals, averages are different</td>
</tr>
<tr>
<td>Ash Free DW</td>
<td>13</td>
<td>1.2387</td>
<td>0.5211</td>
<td>Averages the same at a 99% confidence level</td>
</tr>
<tr>
<td>Biomass DW</td>
<td>22</td>
<td>0.4807</td>
<td>9.9368</td>
<td>At all confidence intervals, averages are different</td>
</tr>
<tr>
<td>g-oil/L</td>
<td>16</td>
<td>0.4322</td>
<td>11.5932</td>
<td>At all confidence intervals, averages are different</td>
</tr>
<tr>
<td>% weight of oil (Culture DW basis)</td>
<td>16</td>
<td>1.1348</td>
<td>12.9696</td>
<td>At all confidence intervals, averages are different</td>
</tr>
<tr>
<td>% weight of oil (Ash-</td>
<td>16</td>
<td>1.4258</td>
<td>13.0705</td>
<td>At all confidence intervals, averages are different</td>
</tr>
</tbody>
</table>

It was demonstrated at a 99% confidence level that the steady state $DW_{AF}$ and Ash-free Productivities ($P_{AF}$) of *Botryococcus* and *Chlorella* were statistically indistinguishable and thus the two species, in the conditions specified for this photobioreactor system, can achieve similar ash-free cell densities regardless of their inherent specific growth rate. It is important to note again that for this to be true, the dilution rate of the reactor must be lower than the intrinsic
growth rate of the slowest growing algae (i.e. *Botryococcus*), otherwise the rate of removal of culture, the dilution rate, is greater than the growth rate of the organism and wash-out of the culture will occur. Previous shaker flask experiments with *Botryococcus* conducted in the Curtis Lab showed that a dilution rate of 7.5% is optimum for *Botryococcus* (Curtis Lab, unpublished). At a 7.5% dilution rate, the $t_d$ is calculated as 9.24 days, which is in fact slower than the doubling time of *Botryococcus* and the culture will not wash-out. While a chemostat for *Chlorella* could be run at much higher dilution rates due to the inherently faster doubling time of this algae, a dilution rate of 7.5% was chosen for two reasons. First, selecting the dilution rate of 7.5% allowed for a direct comparison of the photobioreactor performance between *Botryococcus* and *Chlorella*. Secondly, this dilution rate severely restricts the *Chlorella* growth rate, which was anticipated as a way to increase lipid content because lipid accumulation is growth dissociated. Furthermore, we suspect the productivities of the *Chlorella* to be the same at higher dilution rates due to the principles of light limited growth. This was observed in previous *Botryococcus* flask experiments, conducted by another lab member Waqas Khatri, where the dilution rate was varied from 5% to 15%. From the results of his experiments, a dilution rate of 7.5% was chosen as the optimum operating condition for *Botryococcus*.

Table 3-2 lists the steady state averages of the oil concentration. The oil productivity of *Chlorella*, 5.29±0.55 mg-oil/L/Photo-hr, is about 68% lower than the hydrocarbon productivity for *Botryococcus*, 16.64±3.23 mg-oil/L/Photo-hr. The percent oil content for *Chlorella*, 6.1±0.7 % per cell biomass basis and 6.7±0.9 % per ash-free biomass basis, is about 54% and 58%, respectively, lower than the percent oil content for *Botryococcus*, 13.2±2.7 % hydrocarbon content per total culture basis and 15.7±2.0 % per ash-free total culture basis. It was reported in literature that cell wall composition of *Chlorella* was 9.2% lipid (by gravimetric-methanol extraction) [Northcote & Goulding, 1958]. This reported value is considerably higher than the lipid content of *Chlorella* observed in our bioreactor, and there are several possible reasons for
this. First, it is possible that the lipid content observed in the reactor was mostly, if not all, cell membrane lipids and not storage lipids. The lipid content of *Chlorella* may be low due to forcing the system to grow at 10% nitrogen biomass content; this was imposed by feeding the system sufficient nitrogen to replace the biomass removed daily, assuming a 10% nitrogen content in the biomass. A 10% nitrogen biomass content for *Chlorella*, compared to a 5% nitrogen content basis for *Botryococcus*, was chosen based on taking a middle value for the nitrogen contents reported in literature for *Chlorella*, from 1.17 to 14.11% [Spoehr & Milner, 1949]. Additionally, the 10% nitrogen content by mass employed in the feeding strategy for *Chlorella* ensured that light, and not nutrients, was the limiting factor for growth. The same strategy was imposed on the *Botryococcus* cultures but a 10% nitrogen content was viewed as excess nitrogen because of the nitrogen free hydrocarbon extracellular matrix, which makes up a significant portion of the biomass, and colony formation of cells (where the inner most cell most likely do not see the same environment or nutrients as the cells at the boundary layer). Therefore, a 5% nitrogen biomass content was chosen for *Botryococcus*. This strategy may have resulted in forcing *Chlorella* to have a lower lipid content even with the severely stunted growth imposed by the 7.5% dilution rate per day. Although the imposed growth rate is very low in the chemostat, it is still growing and as previously noted, lipid accumulation is characterized as growth dissociated meaning the accumulation of lipids happens upon complete cessation of growth associated with nitrogen depletion. Batch experiments are planned for *Chlorella* to compare the growth dissociated lipid accumulation kinetics under both light-limited and non-light limited batch growth in bags. We will also be able to compare the biomass and oil productivities of a batch versus continuous photobioreactor operation. Overall, this batch experiment will enable us to examine the lipid content data over the course of a batch mode run and how light limitation affects lipid accumulation.
Another factor that could be contributing to the low observed lipid contents is the method of lipid content determination used in this work. Table 3-5 is a summary of dry weight and lipid data for batch and continuous runs of *Chlorella* and *Botryococcus* [Casadevall et al., July 1984; Chiu et al., 2008; Illman et al., 2000; Javanmardian & Palsson, 1991; Liu et al., 2008]. For a batch system, the important productivity is the oil produced from any given time point to the beginning of the batch culture period. In principle the instantaneous productivity is more indicative of what could be produced in a continuous system, however, these numbers are far more subject to error due to being a derivative. In cases where oil content is known as a function of time, both of these productivities could be calculated, but more commonly if only final oil content is known, then only the overall batch productivity can be calculated which will not necessarily be optimal even for batch as productivity will likely go through a maximum. The values in Table 3-5 are overall culture productivities based on final oil content. Another constraint in comparing to the literature is the methods used for oil analysis. Gravimetric analysis (extract and weigh) is the most widely used method to quantify lipids, but results in an overestimation of total lipid content because lipid is also co-extracted with non-lipid components such as proteins, carbohydrates, and pigments [Weyer et al., 2009; 2010]. In contrast, the method developed by NETL involves a direct transesterification of lipid species to their fatty acid methyl esters (FAMES) and these are quantified by GC-FID, ensuring that the lipid values determined are only lipids and not other cellular components. Therefore our lipid data is inherently lower than those reported in literature due to the method of lipid extraction. While the percent lipid content for *Chlorella* in our system, 6%, is lower than the reported values in Table 3-5, we achieved the second highest oil productivity at 4.84 mg-oil/L/Photo-hr and the highest culture productivity at 150 mgDW TC/L/Photo-hr. High lipid content accumulates to a high percentage in batch cultures (in a nitrogen limited system) but productivity of oil produced is low compared to a continuous system. In the continuous system of Botryococcus culture, we achieved culture
productivities an order of magnitude higher, 130 mgDW_{TC}/L/Photo-hr, than the reported value in literature and also higher oil productivities, 16.64 mg-oil/L/Photo-hr, even though the percent oil content was lower for our system.

Table 3-5: A comparative summary of algae growth and oil production in continuous and batch photoautotrophic photoreactor systems

<table>
<thead>
<tr>
<th>Organism</th>
<th>Mode of Operation</th>
<th>Reactor Type</th>
<th>Dilution Rate (per day)</th>
<th>gDW_{TC}/L</th>
<th>Cell Count (cells/mL)</th>
<th>Culture Productivity (mgDW_{TC}/L/hr)</th>
<th>Oil Productivity (mg-oil/L/hr)</th>
<th>% oil</th>
<th>Extraction Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorella vulgaris</td>
<td>continuous</td>
<td>Trickle Film Screen</td>
<td>0.075</td>
<td>31</td>
<td>1x10^9</td>
<td>150.0</td>
<td>4.84</td>
<td>6</td>
<td>Total Cellular Lipid - by NETL</td>
<td>Curtis Lab</td>
</tr>
<tr>
<td>Chlorella</td>
<td>batch to continuous</td>
<td>cylindrical glass</td>
<td>0.15</td>
<td>NA</td>
<td>8x10^8</td>
<td>63.0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Javanmardian 1991</td>
</tr>
<tr>
<td>Chlorella</td>
<td>continuous</td>
<td>cylindrical glass</td>
<td>0.5</td>
<td>1.4</td>
<td>NA</td>
<td>22.0</td>
<td>7.42</td>
<td>32-34</td>
<td>Gravimetric, methanol/chloroform</td>
<td>Chiu 2008</td>
</tr>
<tr>
<td>Chlorella emersonii</td>
<td>batch-low N media</td>
<td>stirred bioreactor</td>
<td>14 day batch run</td>
<td>1.11</td>
<td>NA</td>
<td>5.0</td>
<td>3.13</td>
<td>63</td>
<td>Gravimetric, methanol/chloroform</td>
<td>Illman 2000</td>
</tr>
<tr>
<td>Chlorella</td>
<td>batch-low N media</td>
<td>stirred bioreactor</td>
<td>15 day batch run</td>
<td>0.46</td>
<td>NA</td>
<td>2.1</td>
<td>1.17</td>
<td>57</td>
<td>Gravimetric, methanol/chloroform</td>
<td>Illman 2000</td>
</tr>
<tr>
<td>Chlorella vulgaris</td>
<td>batch-low N media</td>
<td>stirred bioreactor</td>
<td>16 day batch run</td>
<td>0.52</td>
<td>NA</td>
<td>2.3</td>
<td>0.93</td>
<td>40</td>
<td>Gravimetric, methanol/chloroform</td>
<td>Illman 2000</td>
</tr>
<tr>
<td>Chlorella vulgaris</td>
<td>batch</td>
<td>Flask</td>
<td>25 day batch run</td>
<td>0.43*</td>
<td>1.7x10^7</td>
<td>1.2</td>
<td>0.68</td>
<td>56.6</td>
<td>Gravimetric, methanol/chloroform</td>
<td>Liu 2008</td>
</tr>
<tr>
<td>*gDW/L estimated based in data given in the reference</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organism</th>
<th>Mode of Operation</th>
<th>Reactor Type</th>
<th>Dilution Rate (per day)</th>
<th>gDW_{TC}/L</th>
<th>Cell Count (cells/mL)</th>
<th>Culture Productivity (mgDW_{TC}/L/hr)</th>
<th>Oil Productivity (mg-oil/L/hr)</th>
<th>% oil</th>
<th>Extraction Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Botryococcus</td>
<td>continuous</td>
<td>airlift</td>
<td>0.25-0.3</td>
<td>27</td>
<td>NA</td>
<td>130.0</td>
<td>16.64</td>
<td>13</td>
<td></td>
<td>Casadeval 1984</td>
</tr>
<tr>
<td>Botryococcus</td>
<td>continuous</td>
<td>airlift</td>
<td>0.25-0.3</td>
<td>1.5</td>
<td>NA</td>
<td>32.1</td>
<td>6.93</td>
<td>27</td>
<td></td>
<td>Casadeval 1984</td>
</tr>
</tbody>
</table>

The photon yield efficiency was calculated for our 500 mL photobioreactor operated at 7.5% daily dilution rate. Photon efficiency calculations were based on an average light flux across the screen of 282 µE/m²/s, the average flux measured parallel to the plane of the screen. Utilizing an average of biomass productivity for both Chlorella and Botryococcus during steady state conditions, Table 3-6 summarizes photosynthetic efficiencies as calculated utilizing three different approaches as described in the table with varying results [Kirk, 1994; Nobel, 1999]. For Chlorella the photosynthetic efficiency was calculated at 7.00, 1.72, and 4.08 % and for Botryococcus 6.76, 1.66, and 3.94 %. The theoretical maximum photosynthetic efficiency is on the order of 18% [Kirk, 1994] indicating that there is considerable amount of the incident light that is not converted to biomass. A rough estimate of the efficiency of energy capture into oil
can be calculated based on these efficiencies of photon capture and the energy content of the algae oil. Utilizing the same estimates for photon flux and the calculated enthalpy of high and low heating combustion values, 41.121 to 43.859 kJ/g for botryococcene and 34.44 to 37.86 kJ/g for lipid, based on energy bonds mentioned in literature [Holman, 2000]; this represents an energy capture into the hydrocarbon oil of 1.50-1.60% of the incident PAR and an energy capture into the lipid oil of 0.40-0.44% of the incident PAR. It will be interesting to compare these values to the total energy captured as measured by bomb calorimetry (ongoing work). This also gives an idea of two important considerations. First, the energy captured into liquid fuels is quite small compared to the energy within solar radiation, and second, that unused energy along with non-photosynthetically active radiation represents thermal energy that has to be dissipated from a bioreactor (in this case by evaporation). Future work should also examine the water makeup rates to see how they compare to gain better insight into closing the overall energy balance.

Table 3-6: A summary of the photosynthetic efficiencies of *Chlorella* and *Botryococcus* with a summary of the approach and list of assumptions made

<table>
<thead>
<tr>
<th>PAR intensity = 282 uE/m2/sec</th>
<th>Approach</th>
<th>Assumptions</th>
<th>Photosynthetic Efficiency (%)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Chlorella</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Botryococcus</em></td>
<td></td>
</tr>
<tr>
<td>PAR required to convert 1 mol CO2 to carbohydrate equivalent</td>
<td>8 Einsteins of light required for reduction of 1 mole CO2 to starch equivalent</td>
<td>7.00</td>
<td>6.76</td>
<td>Kirk, 1994</td>
</tr>
<tr>
<td>Energy associated with photosynthetic conversion of one mole CO2 to its starch equivalent</td>
<td>0.24MJ/Einstein; Gibbs free energy released for oxidation of glucose to CO2=479 kJ/molC</td>
<td>1.72</td>
<td>1.66</td>
<td>Kirk, 1995</td>
</tr>
<tr>
<td>4 moles of NADPH and 5 moles ATP required for conversion of 1 mol CO2 to carbohydrate equivalent</td>
<td>0.24MJ/Einstein; 48 kJ required for the phosphorylation of 1 mole of ADP; 220 kJ required for reduction of 1 mole NADP+</td>
<td>4.08</td>
<td>3.94</td>
<td>Kirk, 1996; Nobel 1999</td>
</tr>
</tbody>
</table>
Table 3-7 is a summary of the cell count data of the *Chlorella* and *Botryococcus* cultures. There was a large difference, 5 orders of magnitude, in the cell count densities between *Chlorella* and *Botryococcus* cultures, $1.13 \times 10^9 \pm 1.37 \times 10^8$ and $4.38 \times 10^4 \pm 9.16 \times 10^2$ respectively. As a result, the data calculated from the cell counts, in Table 3-7, of cell productivity, cell weight, oil per cell and oil productivity also show large differences between *Chlorella* and *Botryococcus*. As stated in the Materials and Methods, a direct cell count with a hemocytometer was done on the *Chlorella* culture and not on the *Botryococcus* culture. This was due to the colony forming nature and aggregate size variation of *Botryococcus*. An effort was made by our collaborator at the University of Kentucky to estimate the cell number in *Botryococcus* cultures using previous DNA analysis results from *Botryococcus* shaker flask experiments (details under the Materials and Methods). At the steady state DW$_{TC}$ of 26.68 gDW/L, the concentration of DNA was estimated at 6562.6 ngDNA/mL (a correlation factor was used to account for the difference in dry weights between the bioreactor and prior shaker flask experiments). Based on a cell DNA content of 150 pgDNA/cell from the *Botryococcus* genome size (unpublished data, Chappell Lab), a rough estimate of cell density was made. The low cell count for the *Botryococcus* culture ($10^4$ compared to $10^9$ for *Chlorella*) could be attributed to the colonizing nature and method of DNA extraction used on the samples. Efforts by Amalie Tuerk to disrupt *Chlorella* cells for total organic carbon / total nitrogen analysis (TOC/TN) using sonication were unsuccessful; because of the colonial nature of *Botryococcus* in conjunction with the protective hydrocarbon matrix, it is highly anticipated that sonication of *Botryococcus* would also result in limited cell lysis and most likely only effective at the boundary layer of cells on the colony. Therefore, the estimate of the cell count for *Botryococcus* is expected to be artificially low because the DNA content of middle to inner most cells of the colony is probably not accounted for.
Table 3-7: Summary of steady state cell count data for *Chlorella* and *Botryococcus*

<table>
<thead>
<tr>
<th>Algae Strain</th>
<th>Cells/mL</th>
<th>Cell Productivity (Cells/mL/Photo-hr)</th>
<th>Cell Weight (ng algae DW/cell)</th>
<th>Oil per Cell (ng oil/cell)</th>
<th>Specific Oil Productivity (ng oil/cell/Photo-hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlorella</em></td>
<td>1.13E+09 ± 1.37E+08</td>
<td>5.27E+06 ± 6.43E+05</td>
<td>0.0167 ± 0.0019</td>
<td>0.0010 ± 0.0002</td>
<td>4.80E-06 ± 1.01E-06</td>
</tr>
<tr>
<td><em>Botryococcus</em></td>
<td>4.38E+04 ± 9.16E+02</td>
<td>2.05E+02 ± 4.30E+00</td>
<td>467.78 ± 4.14</td>
<td>83.55 ± 15.78</td>
<td>0.39 ± 0.07</td>
</tr>
</tbody>
</table>

The differences in cell/colony size between *Chlorella* and *Botryococcus* have many implications in the data and will be further discussed; refer back to Figure 1-1 for a visual of the difference in cell/colony size. In previous, semi-continuous shake flasks experiments executed in the Curtis Lab, *Botryococcus* was observed to have a very large variation in colony size and associated (gDW<sub>TC/L</sub>)/(OD<sub>550 @ 1cm</sub>) ratios. Waqas Khatri observed the colony size to vary from 0.3429 mm to 0.0914 mm over dilution rates of 5 % to 12.5 % per day respectively. This is in comparison to the cell size of *Chlorella*, known to have cell sizes ranging from 5-10µm.

*Botryococcus* colony size varied significantly and frequently throughout the bioreactor run, which affected the correlation of total culture dry weight to optical density in the system that was used in the feeding strategy. Since in this work we were operating at a single dilution rate, it was expected that the correlation of total culture concentration (gDW<sub>TC/L</sub>)/(OD<sub>550 @ 1cm</sub>) would remain sufficiently constant to implement the updated nutrient feeding strategy (described in the methods). This ratio ranged from 5.40 to 6.42 (gDW<sub>TC/L</sub>)/(OD<sub>550 @ 1cm</sub>) during the steady state and was updated every 3-4 days, typically using a prior 4-day average, as freeze dried dry weight samples became available; therefore, an estimate was made, based off this changing ratio, for the amount of nitrogen consumed and fed to the photobioreactor. This could have resulted in either too little or too much nutrients being fed to the culture over the course of the *Botryococcus* reactor run and have caused an imbalance in culture nutrients (either build up of toxic nutrients or
lack of nutrients). A progressive decline in the cell density was observed near the end of the run where the $DW_{TC}$ decreased from a steady state value of $\sim 26$ gDW/L to 8 gDW/L over the course of a 16 day wash-out period of the culture, Figure 3-2. This build up or lack of nutrients may have caused the steady decline in cell density after 27 days of operation in which no changes were made to the reactor system. By contrast, the dry weight to OD correlation for *Chlorella* only varied slightly during the steady state comparison from $0.49 \pm 0.01$ (gDW/L)/(OD$_{550}$ @ 1cm), and therefore the feeding strategy was based on a much more precise correlation.

![Dry Weight in Photobioreactor](image)

**Figure 3-2:** The time course of Total and Biomass Dry Weight in the photobioreactor over the entire run; the steady state averages taken for the comparison of *Chlorella* to *Botryococcus* are marked as a solid black line.

The breakdown of the total culture components of biomass, media, oil, inorganic salts in biomass and in media on a mass percentage basis is shown in Figure 3-3. An Anthrone assay is
currently under development in the Curtis Lab, which will be employed to quantify the polysaccharide content of the media fraction. Almost half the dry weight of the *Chlorella* culture media was salt and 20.3% of the biomass was salt. These salt levels are 4 times greater than the level in the *Botryococcus* media, 4.8%. There was an accumulation of 4.7% salt in the biomass of the *Chlorella*, in comparison the accumulation of salt in the *Botryococcus* was too low to measure and will have to be re-measured. An explanation for the excess amount of salt weight in *Chlorella* culture could be accounted for in the media recipe. The original media developed for high density cultures of algae, WFAM, was redesigned for culture of *Chlorella* due to pH instability displayed by cultures during growth on WFAM (results un-published, see Appendix B for further details on pH). It was observed that the uptake of ammonium in the levels present in 1X WFAM metabolically decreased pH to a level of ~3, leading to a cessation of growth in low density *Chlorella* cultures. In the original WFAM media, the ratio of nitrogen from ammonium to nitrogen from nitrate was set at 1:2 based on similar plant media compositions where ammonium and nitrate are used to provide a balance of pH during nitrogen utilization. Due to these instability issues of *Chlorella* cultures, the media was redeveloped (WFAMC) with KNO$_3$ as the only nitrogen source, which increased the potassium level by more than threefold from 0.297gK$^+$/L to 0.935gK$^+$/L. Since the uptake rate of potassium is an order of magnitude lower than the uptake of nitrogen (potassium uptake 0.137 to 0.210 gK$^+$/gN [Mandalam & Palsson, 1998] and nitrogen uptake 0.1gN/g-algae), over time the potassium in the culture media built up. The counter ion to K$^+$, NO$_3$ was consumed in culture, which was observed in pH changes as the nitrogen was assimilated. This build up of salt can be seen in Figure 3-4 where high salt levels are observed for the *Chlorella* culture. A marked difference in conductivity, 3 times higher, between the *Botryococcus* and *Chlorella* cultures can also be observed. In order to decrease the high salt level and control pH through nitrogen uptake, the culture was fed NH$_4$NO$_3$ and no KNO$_3$ (starting on day 40, little to no potassium was fed to the culture for over 45 days); we were able to
feed the cultures NH₄NO₃ without instability issues of pH due to the buffering capacity of the high density cultures in a 5% CO₂ environment. When a drop in OD and dry weight was observed from Day 41 – 63 (Figure 3-2), along with a drop in salt DW and conductivity over this same time-frame, Figure 3-4, we realized that the cells were suffering osmotic shock due to potassium depletion. (Note that the sudden drop in cell density during the Chlorella run after day 24 is unrelated to the potassium issue, and was instead due to a change in the carbon delivery method where low levels of carbon were delivered, see Appendix B for more detail. The culture was recovered with 5% CO₂ on day 28 and growth resumed). Prior to this we had tracked potassium feeding as if it were incorporated into the biomass, which meant that we were assuming that previously fed potassium ‘accumulated’ intracellularly would sustain the culture for a long time period after K⁺ feeding stopped. Potassium though, (acting in conjunction with sodium and chloride) is an electrolyte used to maintain ionic concentrations of the cell, and a minimum level of potassium is needed to maintain growth [Healey, 1973]; [Leigh & Wyn Jones, 1984]. To regain growth of the culture, KCl was added to re-establish potassium levels without increasing pH due to additional NO3 feeding, and potassium concentration was maintained at a level previous to the drop in cell density; this was done via a mass balance with an assumed potassium uptake rate of 0.210 gK/gN. KCl was added to the reactor culture to maintain minimum potassium levels while keeping the salt level reasonable, at 3-4 gDW/L of salt, and the conductivity around 5.0-6.0 millimhos.
Figure 3-3: Breakdown of the total culture components
Figure 3-4: Salt dry weight and conductivity levels for the *Chlorella* and *Botryococcus* photobioreactor runs; changes in media feed indicated for the *Chlorella* run.

Data from both the *Chlorella* and *Botryococcus* runs is currently being further analyzed for energy and carbon content by bomb calorimetry and Total Organic Carbon (TOC) analysis. These analyses will provide a more accurate comparison of energy and CO$_2$ captured by two strains of algae that have significantly different intrinsic growth rate. We suspect that the carbon fixation rate of both organisms to be similar because the total culture DW and productivities were similar. Preliminary data collected by Amalie Tuerk, show that the total energy captured by *Chlorella* and *Botryococcus* are different; *Botryococcus* total culture has twice the amount of energy per mass as *Chlorella*. The energy captured as oil, as non-oil biomass and as extracellular organics will give insight into understanding where the energy is being distributed within the culture.
Appendix A - *Chlamydomonas reinhardtii* Transformations

The goal of the algal transformations was to utilize a gene construct shown to be effect for engineering terpene metabolism in chloroplasts of terrestrial plants [Wu et al., 2006] and to master the algal transformation methodology proven successful for the cell wall deficient line of *Chlamydomonas reinhardtii*. The construct shown in Figure A-0-1 was introduced into the cw-15 cell-wall deficient line via the bead-beating methodology [Kindle, 1990]. When integrated into the algal genome, this cassette encodes for a hygromycin resistance marker (HPT) and directs a FPP synthase (FPS) and terpene synthase (PTS) to the chloroplasts compartments.

![Figure A-0-1. Plasmid Map for tpPTS+tpFPS gene](image)

Numerous attempts were taken to transform the cell-wall deficient line of *Chlamydomonas* and many challenges have been overcome. The bead beating transformation technique and PCR method of screening transformants have been optimized for the terpene plasmids. By varying the DNA concentration in the bead beating transformation, varying the hygromycin selection pressure, and optimizing PCR conditions, Figure A-0-2 shows a gel of PCR positive transformants for the tpPTS+tpFPS gene of interest. The PCR screened positive transformants are currently being analyzed for expression of the transgenes via RT-PCR assay.

In Figure A-0-2, lane 1 on the gel shows a band size of 661bp for the positive *E. coli* control with the tpPTS+tpFPS gene. Lanes marked 18 and 19 also show a band size of 661bp confirming the successful incorporation of the tpPTS+tpFPS gene in the cw-15 cell-wall deficient
Received 4 constructs from UKy, concentrated on tpPTS+tpFPS construct because it had the most success in tobacco. We have attempted multiple times to transform *Chlamydomonas*; we increased DNA concentration used in the transformation, redesigned primer used in PCR, and found that PCR results depended greatly on amount of DNA loaded (can lead to false negatives). The use of hygromycin as a selectable marker lead to breakthrough growth on negative control plates. Current work is being put into optimizing and redesigned plasmids and into choosing another selection marker (paramomycin).
Appendix B - Reactor Run Summary

Reactor Run #1 Summary

The first reactor run, Figure B-0-1, 03/12/09 - 04/24/09, was with Botryococcus on 5% CO₂ gas phase delivery. The goal of the run was to maintain steady-state high-density continuous culture of Botryococcus. This reactor run was used in the comparison of Botryococcus to Chlorella. Culture maintained a steady state DW_B of 20 gDW/L for 27 days on gaseous CO₂, after which the DW_TC and DW_B started to decline and washout, discussed further in the Results and Discussion.

![Dry Weight - Botryococcus, 5% CO2 delivery](image)

Figure B-0-1: Dry Weight in the photobioreactor for reactor run #1 with Botryococcus
Reactor Run #2 Summary

The second reactor run, Figure B-0-2, 04/26/09 - 05/21/09, was with algae species *Botryococcus* (with an unknown contaminating algae species) on 5% CO\(_2\) gas phase delivery. The goal of the run was to maintain a steady-state high-density continuous culture of *Botryococcus* and gain insight into instability of previous *Botryococcus* run. Culture maintained a steady state DW\(_B\) of 12 gDW\(_B\)/L for 10 days on gaseous CO\(_2\) before a contaminating, faster growing algae species took over the culture; by day 20 no *Botryococcus* remained in culture.

Figure B-0-2: Dry Weight in the photobioreactor for reactor run #2 with *Botryococcus*
Reactor Run #3 Summary

The third reactor run, 07/02/09 - 07/06/09, was with the algae species *Chlamydomonas* on 5% CO$_2$ gas phase delivery. The goal of the run was to maintain a steady-state high-density continuous culture of *Chlamydomonas*. Neither a high density culture nor a steady state were achieved in this run. The run was terminated after 3 days due to photobleached/dead culture. The photobleaching was later attributed to either low density inoculum and/or pH control issues. This spurred the investigation of issues growing *Chlamydomonas* to high density in shaker flask cultures and pH control issues.

Reactor Run #4 Summary

The fourth reactor run, Figure B-0-3, 07/27/09 - 09/21/09, was with algae species *Botryococcus* on 5% CO$_2$ gas phase delivery with a switch to liquid phase CO$_2$ delivery. The goal of the run was to maintain a steady-state high-density continuous culture of *Botryococcus* while delivering CO$_2$ in the liquid phase via the evaporative make-up water. The culture was maintained a steady state Biomass Dry Weight of 7-10 gDW/L for 26 days while delivering CO$_2$ in the evaporative liquid make-up stream. After the switch to liquid phase CO$_2$ delivery, there was an initial washout period and then a gradual recovery of *Botryococcus* culture density. The liquid CO$_2$ delivery system evolved throughout this run. The reactor run failed due to operator error and/or culture instability.
Reactor Run #5 Summary

The fifth reactor run, Figure B-0-4, 11/27/09 - 12/17/09, was with algae species *Chlorella* on 5% CO₂ gas phase delivery with a switch to liquid phase CO₂ delivery. A fast-growing algae species, *Chlorella*, was used to gain insight into photobioreactor dynamics because an inoculum could be prepared much faster as compared to an inoculum of *Botryococcus*. The goal of the run was to maintain a steady-state high-density continuous culture of *Chlorella* while delivering CO₂ in the liquid phase via the evaporative make-up water. The culture was maintained at a steady state Biomass Dry Weight of 20 gDW/L for 6 days on gaseous CO₂, then switched to liquid CO₂ delivery after which point the culture appeared to stop growing, began to wash out, and run reactor run was terminated. The reactor run failed due to operator error and/or because the culture crashed due to pH instability discovered in later reactor runs.
Figure B-0-4: Dry Weight in the photobioreactor for reactor run #5 with *Chlorella*; switch from 5% CO$_2$ to liquid delivery of CO$_2$ in the evaporative make-up stream

**Reactor Run #6 Summary**

The sixth reactor run, Figure B-0-5, 01/07/10 - 04/02/10, was with algae species *Chlorella* on 5% CO$_2$ gas phase delivery with a switch to liquid phase CO$_2$ delivery with pH monitoring. The goal of the run was to maintain a steady-state high-density continuous culture of *Chlorella* while delivering CO$_2$ in the liquid phase via the evaporative make-up water, with continuous monitoring of culture temperature, gas phase temperature and relative humidity, and culture pH. Pure CO$_2$ was dissolved in a pressurized vessel filled with distilled water. The amount of carbon delivered was controlled by the pressure at which the carbon was dissolved in water. The evaporative make-up stream was added at a flow rate of approximately 1 mL/min during day-light hours and its flow rate controlled by the diameter and length of HPLC tubing through which the dissolved CO$_2$ entered the culture. The culture maintained a steady state
Biomass Dry Weight of 20-30 gDW/L while on gaseous CO$_2$ over the course of the reactor run. The instability of culture pH was discovered with liquid CO$_2$ delivery and a pH control method was developed with nitrogen feed. This lead to the discovery of the pH control issues during liquid CO$_2$ delivery (due to loss of carbonate buffer) and then lead to the subsequent implementation of pH control via inorganic nitrogen feeding (potassium imbalance resulted with the new nitrogen feeding strategy).

Figure B-0-5: Dry Weight in the photobioreactor for reactor run #6 with *Chlorella*; switch from 5% CO$_2$ to liquid delivery of CO$_2$ in the evaporative make-up stream

Ammonium Uptake Studies, during reactor run #6

Prior to first liquid CO$_2$ switch in reactor run in reactor run #6, we tried to control the pH with acid (by replacing phosphorous in MR26Phosphate Solution with phosphoric acid and by replacing nitrogen in the media with nitric acid). When the switch to liquid CO$_2$ delivery was
made, the pH jumped from daytime pH of low 8’s to a pH of low-mid 9’s. Total Culture Dry Weight and OD declined at a rate faster than washout so the culture was recovered with gas delivery of CO₂ until the pH control strategy was developed. After this first attempt to switch the reactor to liquid CO₂ delivery, we developed and tested the theory of Chlorella’s ammonium tolerance (i.e. that cultures don’t die because of ammonium toxicity but because the conversion of ammonium to ammonia makes the media acidic and the pH kills the algae). The first two experiments were designed to vary the amount of NH₄ in WFAM media. These experiments were the basis for the nitrogen (NH₄-NO₃) feeding strategy for pH control.

For the first ammonium flask experiment, Figure B-0-6, the amount of ammonium in WFAM was taken as the basis at 1xNH₄: 1x, 1/2x, 1/4x, no NH₄ levels of NH₄ tested. 1x, 1/2x, 1/4x NH₄ flasks all stopped growing at an OD of ~0.4 and pH of ~3.5. The +control, no NH₄, was the only flask that continued to grow and whose pH was fairly constant.

![Figure B-0-6: pH and OD₅₅₀ of the First Ammonium Comparison Experiment](image)

For the second ammonium flask experiment, Figure B-0-7, lower levels of ammonium were tested since in the previous experiment the cultures grew but then stopped growing when the pH reached ~3.5, wanted to see the pH drop but be able to see the culture recover and come back up in pH and OD. Ammonium levels tested: 1/16x, 1/12x, 1/8x, and no NH₄. Saw little to no pH change in the 1/16x, 1/12x, and zero NH₄ flaks, but in the 1/8NH₄ flaks the pH dropped to ~4
and then came back up to the same pH as the other flasks; growth/OD off all flasks the same.

From this, we saw that *Chlorella* can grow in the presence of ammonium and that it actually preferentially eats ammonium compared to nitrate.

![Graphs](image)

**Figure B-0-7.** pH and OD\(_{550}\) of the Second Ammonium Comparison Experiment

Using the knowledge from these NH\(_4\) flasks experiments, the nitrogen feeding strategy was developed and implementing into reactor run #6; feeding was based on pH control by strategically balancing the NH\(_4\) and NO\(_3\) fed (while still on gas phase). To gain more reducing power from ammonium to further bring down the pH metabolically, NH\(_4\)Cl and (NH\(_4\))\(_2\)SO\(_4\) were used to bring down the pH; their use was conservative to avoid toxic effects of accumulating the counter ions of Cl and S. A mass balance was performed on nitrogen, potassium, sulfur and chloride to keep track of the concentration of each in the bioreactor. Sulfur limits were kept within that of 1xWFAM and chlorine levels at 1.8 gCl/L (based on NaCl salt adaptation studies at that time, these maximum levels have since increased).

**Reactor Run #7 Summary**

The seventh reactor run, Figure B-0-8, 04/10/10 - 08/13/10, was with algae species *Chlorella* on 5% CO\(_2\) gas phase delivery with a switch to liquid phase CO\(_2\) delivery and pH
control implementation. The goal of the run was to maintain steady-state high-density continuous culture of *Chlorella* while delivering CO₂ in the liquid phase via the evaporative make-up water with pH control by a nitrogen feeding strategy. The culture was maintained at a steady state Biomass Dry Weight of 25-35 gDW/L on gaseous and liquid delivery of CO₂ over the course of the reactor run. Due to the implementation of pH control with nitrogen feed, this enabled growth of *Chlorella* on low levels of carbon delivered in the evaporative liquid make-up stream for more than 100 days. Adaptation of algae to low levels of CO₂ and eventually to ambient levels was observed during liquid phase CO₂ delivery.

Figure B-0-8: Dry Weight in the photobioreactor for reactor run #7 with *Chlorella*; switch from 5% CO₂ to liquid delivery of CO₂ in the evaporative make-up stream, with a switch to ambient conditions, no CO₂
Other Details of Reactor Run 7

At the beginning of the run on day 4, an extra amount of media (and NH₄) was fed to the reactor in an attempt to not only bring down the pH but also to more quickly bring the culture up to steady state; this resulted in a sharp drop in pH to ~2.5, culture pH came back up to ~7 by adjusting the feeding to NO₃ till the desired pH was reached. OD and DW dropped below wash out the following day but then recovered the following day to the previous OD. After that, the pH at night was much lower and in a reasonable range for liquid CO₂ delivery where the buffering from the 5% CO₂ in bag is lost (sparged pH ~8.5).

The pH was under control and culture was growing so we made the switch to liquid CO₂, day 9 of run. Cultured started to wash out when switch was made to liquid delivery, but we kept with it (since washing out not completing crashing/dying like in previous run) hoping it would steady out at a lower density. The thought was the cells needed to adapt to the new lower CO₂ environment since on gas CO₂ they are exposed to excess CO₂. Culture continued to wash out for about 7 days after which it started to slightly grow but still not back to the OD from previous day before subculture. It slightly grew like this for an additional 8 days at which point the hold up on the screen was increased slightly by cutting some of the string hold the screens too tight together. For the next 5 days the culture grew and for the most part was able to maintain a steady state density of about 13 gDW/L. Over this time period the culture hold-up and coverage on the screen continued to increase as new techniques were discovered to increase the hold-up. Then, there was a mishap with the tubing/pump and an estimated 135 mL of culture was lost. For the first 5-6 days after, the culture maintained a steady state 9-10 gDW/L. As more techniques were learned to increase the hold-up on the screen, the culture density eventually reached the density it was on gas phase delivery of CO₂. Over the course of the reactor run, the amount of carbon in the evaporative make up stream was decreased in step changes. The culture was able to maintain and
actually increased in culture density as the amount of carbon in the liquid delivery system was decreased. The reactor was eventually switched to dissolving nitrogen in the evaporative make-up stream (removal of all supplemental CO$_2$ in the system and operation at ambient CO$_2$ levels only) to test the theory of whether they were capable of growing and maintaining their high cell density with the amount of carbon in the air. It was discovered that they were able to grow on air but were carbon limited because they were not able to maintain the previous steady state cell density, the dry weight in the photobioreactor steadied off at a Total Culture and Biomass Dry Weight 20% lower than the Dry Weight attained before the switch to ambient operating conditions.

The feeding of nitrogen for pH control with NH$_4$-NO$_3$ on liquid CO$_2$ delivery developed greatly over the course of this reactor run. The normal liquid delivery operating pH was maintained around 7.8-8 during daytime. All the media, minus all nitrogen, was fed at a single time when the reactor was sub-cultured, where 7.5% of the culture removed and the nutrients removed replaced with fresh media, (plus any KCl to maintain potassium level). The nitrogen was fed in 4-6 increments (increments of 20-25% of the total nitrogen) over the course of the remaining 24 hours. This incremental feeding was done to keep the pH above 7 as it consumed ammonium; a drop in pH for ammonium consumption was observed with an increase in pH as nitrate was consumed.
References


