The Pennsylvania State University

The Graduate School

College of Engineering

ACHIEVING PH CONTROL THROUGH STOICHIOMETRICALLY BALANCED MEDIA IN ALGAL PHOTOBIOREACTORS

A Thesis in

Chemical Engineering

by

Megerle L. Scherholz

© 2012 Megerle L. Scherholz

Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

December 2012
The thesis of Megerle L. Scherholz was reviewed and approved* by the following:

Wayne R. Curtis  
Professor of Chemical Engineering  
Thesis Adviser

Andrew Zydne
Professor of Chemical Engineering  
Head of the Department of Chemical Engineering

Esther Gomez  
Assistant Professor of Chemical Engineering

*Signatures are on file in the Graduate School.
ABSTRACT

High-density culturing methods and better operational strategies are needed to improve reactor productivities for algae-based biofuels at the commercial scale. Typical laboratory culture conditions for microalgae are nitrate-based media with elevated CO$_2$ gas supplementation such that the bicarbonate equilibrium mitigates the pH rise associated with nitrate consumption and additional pH control is not necessary. However, improving yield of CO$_2$ utilization is important to the economic success at the commercial scale, making it desirable to reduce gaseous CO$_2$ levels, which requires alternate culturing strategies to the nitrate/high CO$_2$ approach. Ammonium alone is rarely used in algae media due to a ‘toxic’ drop in culture pH from metabolism, but represents a unique approach to both media design and pH control when coupled with growth on nitrate.

Relying on stoichiometric theory for photoautotrophic growth, a mixed nitrogen source at 36%N-$\text{NH}_4^+$/64%N-$\text{NO}_3^-$ is anticipated to minimize the resulting proton imbalance from nitrogen metabolism such that growth can be sustained to higher densities in microalgae cultures. However, the microalgae *Chlorella vulgaris* and *Chlamydomonas reinhardtii* were shown to preferentially utilize ammonium when both ammonium and nitrate were provided in the medium. When ammonium-nitrogen was only 9% of the total nitrogen in the initial medium, the resulting proton imbalance from selective ammonium utilization caused the pH to drop too low to sustain further growth. However, providing incremental additions of ammonium in the presence of nitrate enabled growth to concentrations as high as 5 gDW/L on 0.3 gN/L, demonstrating that a fed-batch nitrogen strategy is necessary to balance the culture pH.
Although air-grown cultures demonstrated larger pH swings than observed on elevated CO₂ due to reduced bicarbonate buffering, the pH remained more balanced on a mixed nitrogen source than with the use of either ammonium or nitrate alone. Given a biological demand that exceeded the CO₂ transfer rate, carbon-limited *Chlamydomonas reinhardtii* cultures indicated a lack of pH control as the culture did not respond to ammonium addition. Subsequent studies will focus on understanding whether the lack of control with the media-based approach was an immediate result of carbon limitation or the cessation of growth at high pH when nitrogen would no longer be metabolized as the current experimental design makes this interpretation difficult. Although hard to achieve a lower pH during carbon-limitation, the addition of more ammonium prior to carbon limitation is expected to drive down the pH (well below 9) such that the confounding effects of carbon limitation and high pH on reactor dynamics will become distinguishable. However, it is anticipated that pH-balanced growth during carbon limitation can be achieved using a feed-forward strategy by considering both nitrogen metabolism and CO₂ transport, equilibrium and uptake kinetics within an adaptive control model to maintain the pH below 9 and avoid inhibitory pH levels.
# TABLE OF CONTENTS

LIST OF TABLES ........................................................................................................... viii
LIST OF FIGURES ......................................................................................................... ix
ACKNOWLEDGEMENTS ................................................................................................. xi

Chapter 1 – Introduction and Motivation ....................................................................... 1
   Overview of Commercial-Scale Microalgae Culturing Systems .................................. 1
   Selection of Algal Strains for Biofuels Production ....................................................... 4
   Photoautotrophic Growth of Microalgae ...................................................................... 4
      CO₂ Transport ........................................................................................................... 5
      Bicarbonate Equilibrium ........................................................................................ 7
      CO₂ Uptake Kinetics ............................................................................................... 9
   Carbon, Nitrogen, and Cell Mass Balances .................................................................. 13
   Photoautotrophic Growth requires pH Control ............................................................. 16
      Cellular metabolism causes changes to extracellular pH ........................................ 16
      Limitations of current pH control methods in high-density, commercial scale systems .................................................................................................................. 18
   Development of a Novel scalable pH control strategy .................................................. 19

Chapter 2 – Development of Stoichiometrically Balanced Growth Media ....................... 21
   Limitation of Current Stoichiometric Theory for Photoautotrophic Growth .............. 21
   Proton Imbalance must be reflected in Photoautotrophic growth equation ............... 23
   Achieving pH control with a Mixed Nitrogen source ................................................... 24
   Expansion of Photoautotrophic growth equation for product formulation ............... 28
   Discrepancy between Stoichiometric Theory and Experimental Mass Balances ......... 29

Chapter 3 – Materials and Methods .............................................................................. 33
   Algal Cultures ............................................................................................................. 33
   Algal Media Formulation ............................................................................................ 33
   Sampling Methods ..................................................................................................... 33
      Growth Measurement ............................................................................................. 33
      pH Measurement .................................................................................................... 34
   Nitrate Measurement by Ion Selective Electrode (ISE) .............................................. 35
   Measurement of the Mass Transfer Coefficient ......................................................... 35
Growth Chamber Light and Temperature ........................................................................ 36
Gas Delivery System for CO$_2$ supplementation ......................................................... 36
Shake Flask and Photobioreactor Setup ........................................................................ 37
Daisy-Chain Shake Flasks .............................................................................................. 37
Inoculum Cultures .......................................................................................................... 38
Loop Air-lift Photobioreactor .......................................................................................... 38
Trickle Film Photobioreactor ........................................................................................... 39
Chapter 4 - Inherent pH Instability in Microalgal Cultures due to Nitrogen Metabolism .............................................................................................................. 42
Literature Review of Nitrogen Metabolism in Microalgae ................................................ 42
Ammonium Assimilation ................................................................................................. 43
Nitrate Assimilation ....................................................................................................... 46
Nitrogen Metabolism is regulated by Carbon Availability ............................................... 49
Interactions between Nitrate and Ammonium Metabolism ............................................. 51
Experimental Results for Nitrogen Use in Algae ............................................................. 53
Proton imbalance in Microalgal cultures results from preferential NH$_4^+$ uptake ......... 54
Growth has a significant effect on culture buffering capacity ....................................... 61
Cellular regulation facilitates novel approach to pH control through nitrogen metabolism ......................................................................................................................... 63
Pulse addition of ammonium displays rapid pH drop in nitrate-grown culture ............. 64
Chapter 5 – Stoichiometrically balanced media requires fed-batch addition for pH control and sustained growth ......................................................................................... 67
Experiment Results for Fed-batch Nitrogen .................................................................... 68
Media-based pH control can be implemented during nitrogen-limited growth of *Chlamydomonas reinhardtii* ....................................................................................................................... 71
Chapter 6 – Carbon limitation reveals regulatory elements affecting pH control through nitrogen metabolism ................................................................. 75
Experimental Results and Discussion for CO$_2$-limited Culture .................................... 76
Adaptation of high-CO$_2$ grown Microalgal cultures to Air ........................................ 76
Bicarbonate equilibrium contributes significantly to the total buffering capacity of the culture ................................................................................................................................. 81
Carbon availability affects regulation of nitrogen metabolism and pH response predictability ................................................................................................................................. 84
Carbon availability dictates ability to utilized current media-based pH strategy ..... 88
Chapter 7 – Conclusions and Future Work................................................................................. 92
Appendix A – Re-evaluation of the Ammann Group Experimental Mass Balance ........ 96
Appendix B – Algal Culture Media Formulation ........................................................................ 100
Appendix C – Prediction of CO₂ Limited Growth Rates......................................................... 103
Appendix D – Nuclear Transformation of *Chlamydomonas reinhardtii* for
Isoprene Metabolism.............................................................................................................. 109
LIST OF TABLES

Table 1.1: Comparison of Closed-system Technologies for Algal Culturing .................. 3
Table 1.2: Apparent Growth Kinetics as a function of gaseous CO2 supplementation in photoautotrophic Chlamydomonas reinhardtii .......................... 10
Table 1.3: Kinetic parameters for CO2 fixation in photoautotrophic Chlorella vulgaris ........................................................................................................ 11
Table 1.4: Effect of Metabolic Processes on the pH of Plant Tissue Cultures ............ 17
Table 2.1: Stoichiometric Coefficients for Various Nitrogen Sources ....................... 22
Table 2.2: Nitrogen Content of Stoichiometrically Balanced Media for Photoautotrophic Growth ................................................................. 27
Table 2.3: Experimental and Theoretical Photosynthetic Quotients for Chlorella pyrenoidosa ......................................................................................... 30
Table 4.1: Kinetic parameters for Ammonium transporters on the plasma membrane ............................................................................................................. 45
Table 4.2: Kinetic parameters for Nitrate/nitrite transporters in Chlamydomonas reinhardtii ......................................................................................... 47
Table 6.1: Mass Transfer Coefficients and Carbon-limited Growth Rates ............... 75
Table 6.2: Growth rate and biomass yield in Chlamydomonas reinhardtii grown under high and low CO2 supplementation ........................................... 86
LIST OF FIGURES

Figure 1.1: CO$_2$ Dynamics in Photoautotrophic Cultures .................................................. 5
Figure 1.2: pH-dependent Distribution of the Bicarbonate buffering system ...................... 9
Figure 1.3: Proposed Model for Inorganic Carbon Movement in *Chlamydomonas reinhardtii* ........................................................................................................ 12
Figure 2.1: Effect of Nitrogen Distribution (Δ) on predicted Proton Flux (ϕ) ............... 28
Figure 3.1: Loop airlift photobioreactor used in Batch and Fed-batch pH experiments .................................................................................................................. 39
Figure 3.2: Trickle film photobioreactor used in Fed-batch pH experiments ................. 41
Figure 4.1: Nitrogen metabolism and regulation in *Chlamydomonas reinhardtii* ........ 43
Figure 4.2: Inherent pH stability in *Chlorella vulgaris* cultures under excess CO$_2$ .... 54
Figure 4.3: Inhibition of Nitrate transport by ammonium in photoautotrophic *Chlorella vulgaris* under excess CO$_2$ ................................................................. 57
Figure 4.4: pH Instability from Nitrogen metabolism in photoautotrophic *Chlamydomonas reinhardtii* under excess CO$_2$ ....................................................... 60
Figure 4.5: Effect of cell growth on culture buffering capacity ........................................ 62
Figure 4.6: Manipulation of cellular proton flux facilitates photobioreactor pH control through nitrogen metabolism ................................................................. 64
Figure 4.7: Inhibition of nitrate transport by ammonium addition in photoautotrophic *Chlamydomonas reinhardtii* under excess CO$_2$ ........................................... 66
Figure 5.1: Fed-batch addition of nitrogen for pH control in photoautotrophic *Chlorella vulgaris* grown under excess CO$_2$ .................................................... 69
Figure 5.2: pH response to pulse NH$_4$NO$_3$ feed during nitrogen excess and limitation .................................................................................................................. 72
Figure 5.3: pH control maintained during nitrogen limitation in photoautotrophic *Chlamydomonas reinhardtii* culture under excess CO$_2$ .............................. 73
Figure 6.1: Intrinsic growth rate of *Chlamydomonas reinhardtii* is independent of gaseous CO$_2$ level ............................................................................................. 78
Figure 6.2: Adaptation period required to adjust high CO₂ grown *Chlorella vulgaris* to air .......................................................... 81

Figure 6.3: Bicarbonate buffering affects the observed proton imbalance during nitrogen metabolism................................................................. 82

Figure 6.4: Carbon availability regulates predictability of pH response from nitrogen metabolism................................................................................... 85

Figure 6.5: Carbon availability dictates utility of media-based pH control in Chlamydomonas .............................................................. 90
ACKNOWLEDGEMENTS

I would like to acknowledge our collaboration with Dr. Joe Chappell and Stephen Bell at the University of Kentucky and thank them for their help with genetic engineering. I would also like to thank my fellow graduate students. I admire Trevor’s dedication to organization and planning as I tried to emanate this in my own work. Nymul’s help as the “Gas Man” was much appreciated during troubleshooting of the gas delivery system and mass transfer measurement. Sergio’s insight into molecular biology helped solve my PCR contamination problems. Mustafa’s music was always entertaining during late night sampling. Without Jeff’s mentoring and guidance, I would not have gained the confidence I needed to become a better leader and researcher. I enjoyed sharing an office with Salvador Barri as his positive energy was welcomed during my experimental marathons.

A special thanks goes to the current and old members of “Team Algae”. Justin Yoo and Jackie Guo, thank you for your help with experimental setup, sampling and culture maintenance. I appreciate the guidance from Lisa Grady and Waqas Khatri, who shared their algae knowledge with me so I could support and continue to expand their work. I value my friendship with Amalie Tuerk that helped me grow as a person as well as her invaluable academic advice. I appreciate Ryan Johnson’s work ethic and teamwork during our trickle film reactor experiments. Tim Miskimmin’s dedication and ability to get up to speed so quickly was extremely motivating in my final stretch of experiments. I value the guidance from Dr. Curtis and for continuing to believe in me. His devotion to research was something I tried to mimic as I took ownership of my own experiments and expressed pride in my work.

I would like to thank Manuel Escotet who kept me focused, motivated and encouraged through his unconditional love. I am also honored to have had the continuing support of my friends and family.

The work in this thesis was supported under the NSF Collaborative Grant No. CBET-0828648 titled “Development of a Sustainable Production Platform for Renewable Petroleum Based Oils in Algae” and NSF Grant No. DBI-0215923, which supported installation of the high-intensity lighted growth chamber.
Chapter 1 – Introduction and Motivation

Overview of Commercial-Scale Microalgae Culturing Systems

Algal-based biofuels have been gaining attention as a potential production platform for renewable and sustainable fuel sources. These algal systems offer advantages over terrestrial plant sources, such as higher productivities, avoid the direct use of food sources for biofuels production, and have the potential for utilization of both wastewater and saltwater (McLaughlin et al., 1999; Pulz et al., 1998). Additionally, under severally limited growth conditions, the lipid content of algae can be as high as 85% of its dry weight which exceeds most plants (Borowitzka, 1988). Another major advantage of microalgal systems is the direct conversion of CO₂ to biomass that can be achieved at a higher efficiency than with plants, representing not only an alternative production platform for fuels, but also the economic benefit of carbon sequestration from waste gas (Keffer and Kleinheinz, 2002). Heterotrophic microbial cultures have been shown to be more than an order of magnitude more productive than algal systems in terms of fuel production, but require sugar and much greater power input than photoautotrophic cultures to provide sufficient mass transfer (Eriksen, 2008). Despite these advantages over other biofuel technologies, the future success of algal-based systems depends on the economic feasibility, which motivates the development of improved bioreactor design and high-density culturing methods to maximize reactor productivity. Achieving higher reactor productivity through ultra-high culture densities will lead to proportionally reduced water pumping and lower dewatering costs as well as reduced downstream processing.
Early commercial-scale technologies focused on outdoor open pond systems to harvest light from the sun to support photosynthesis with demonstrated productivities ranging from 5 to 65 g/m²/day (James, 1990). However, evaporation was a significant problem and led to high water demands, which drove up the cost of biofuel produced in these systems. Such water loss results from maintaining temperature and the inherent inefficiency of photosynthesis. Attempts to use only photo-synthetically active radiation have resulted in unreasonably complex and expensive fiber optic photobioreactors.

Additional challenges associated with pond systems included low cell densities, settling from inadequate mixing, poor nutrient distribution, contamination by other organisms, and expensive downstream cell harvesting procedures (Richmond, 1987; Suh and Lee, 2003). As a result, more recent technologies have focused on the development of closed systems with the advantages of improved control over reactor conditions (temperature, pH, and CO₂ supplementation), higher culture densities, and reduced contamination by other organisms in comparison to algal ponds. These reactors still face challenges in light distribution, CO₂ mass transfer, and fluid dynamics (Molina Grima et al., 1999; Posten, 2009). Photoautotrophic growth is inherently challenged by self-shading of cells as not enough light becomes available for photosynthesis at higher densities (> 2 g/L).

However, improved photobioreactor designs have attempted to minimize the effects of light attenuation by reducing the path-length of which light must travel through the culture (Chen and Johns, 1994).

Many of the current commercial facilities for algal culturing use tubular photobioreactors with volumes as large as 700 m³. In these reactors, the culture is pumped through long transparent tubes. CO₂ depletion occurs down the length of the
reactor due to poor mass transfer, which poses a limit on further scale-up for photoautotrophic culturing in this design (Eriksen, 2008). Therefore, researchers are focusing on the development and scale-up of other closed-system bioreactor configurations for commercial algal culturing as presented in Table 1.1 (Eriksen, 2008; Suh and Lee, 2003). Of the reactor systems shown, the inclined slab photobioreactor led to the highest culture density as mass transfer was improved and light distribution in the culture was maximized as the culture formed a thin layer of cells in which light attenuation was lessened.

Table 1.1: Comparison of Closed-system Technologies for Algal Culturing

<table>
<thead>
<tr>
<th>Reactor</th>
<th>Light Source</th>
<th>Volume (L)</th>
<th>Algal Strain</th>
<th>Density (g/L)</th>
<th>Volumetric Productivity (g/L/day)</th>
<th>Areal Productivity (g/m²/day)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air-lift</td>
<td>Artificial</td>
<td>10</td>
<td>Chlorella</td>
<td>2.27</td>
<td>-</td>
<td>-</td>
<td>Ratchford,1992</td>
</tr>
<tr>
<td>Column</td>
<td>Sun</td>
<td>120</td>
<td>Tetraselmis</td>
<td>1.7</td>
<td>0.42</td>
<td>38.2</td>
<td>Zitelli, 2006</td>
</tr>
<tr>
<td>Column</td>
<td>Artificial</td>
<td>200</td>
<td>Nanochloropsis</td>
<td>-</td>
<td>0.506</td>
<td>76.2</td>
<td>James, 1990</td>
</tr>
<tr>
<td>Flat Panel</td>
<td>Sun</td>
<td>400</td>
<td>Chlorella</td>
<td>-</td>
<td>3.8</td>
<td>22.8</td>
<td>Doucha, 2005</td>
</tr>
<tr>
<td>Inclined Slab</td>
<td>Sun</td>
<td>6</td>
<td>Monodus</td>
<td>15.8</td>
<td>4.3</td>
<td>51.1</td>
<td>Hu, 1996</td>
</tr>
<tr>
<td>Tank</td>
<td>Artificial</td>
<td>5.6</td>
<td>Phaeodactylum</td>
<td>2.67</td>
<td>0.51</td>
<td>-</td>
<td>Yongmanitchai, 1992</td>
</tr>
<tr>
<td>Tubular</td>
<td>Sun</td>
<td>8000</td>
<td>Spirulina</td>
<td>1.2</td>
<td>0</td>
<td>25</td>
<td>Torzillo, 1986</td>
</tr>
<tr>
<td>Tubular</td>
<td>Sun</td>
<td>145</td>
<td>Spirulina</td>
<td>6.3</td>
<td>3.15</td>
<td>44</td>
<td>Torzillo, 1993</td>
</tr>
<tr>
<td>Tubular</td>
<td>Artificial</td>
<td>5.5</td>
<td>Spirulina</td>
<td>-</td>
<td>0.42</td>
<td>-</td>
<td>Converti, 2006</td>
</tr>
<tr>
<td>Tubular</td>
<td>Sun</td>
<td>146</td>
<td>Arthrospera</td>
<td>2.37</td>
<td>1.15</td>
<td>25.4</td>
<td>Carlozzi, 2000</td>
</tr>
<tr>
<td>Tubular</td>
<td>Sun</td>
<td>200</td>
<td>Phaeodactylum</td>
<td>4.1</td>
<td>1.52</td>
<td>25.3</td>
<td>Molina Grima, 2001</td>
</tr>
</tbody>
</table>
Selection of Algal Strains for Biofuels Production

Algal strains for biofuels production fall under two broad classifications: natural fatty acid producers for bio-diesel and hydrocarbon producers for bio-gasoline. We have chosen to work with the unicellular microalgae *Chlorella vulgaris* and *Chlamydomonas reinhardtii* as model organisms for the development of a photobioreactor operational strategy due to the ease of growth and relatively fast doubling times (4-6 hours) observed in our laboratory. *Chlorella vulgaris* is of particular interest to algal-based biofuels research because it naturally produces fatty acids up to 55% of its dry weight (Feng et al., 2011; Tsuzuki et al., 1990). *Chlamydomonas reinhardtii* does not naturally accumulate lipids or hydrocarbons to high levels, but serves as a ‘model’ organism for much algal genetic engineering research and is a promising host for transformation with the biosynthetic capabilities of *Botryococcus braunii*, a natural producer of unique C-34 branched hydrocarbons.

Photoautotrophic Growth of Microalgae

Algae require sufficient light, nutrients, and CO$_2$ to support photosynthesis. In addition to carbon, macronutrients include nitrogen, phosphorous, hydrogen from water, sulfur, calcium, magnesium, sodium potassium and chlorine (Mandalam and Palsson, 1998). These macronutrients as well as micronutrients are provided in the form of salts in defined growth media where the exact composition is dependent on the algal strain. When microalgal growth is achieved by photosynthesis, the bioreactor becomes a three-phase system as gaseous CO$_2$ is introduced. This creates an additional layer of complexity with respect to reactor operation compared to heterotrophic growth in which a
carbon source is dissolved within the growth media. Further obscuring photobioreactor
dynamics is the interactions of CO₂ kinetics and bicarbonate equilibrium with CO₂
transport from the gas phase into the culture medium, represented by the CO₂ ‘triangle’
as shown in Figure 1.1. The following sections discuss these relationships and the impact
on culture growth in more detail.

![Figure 1.1: CO₂ Dynamics in Photoautotrophic Cultures](image)

**CO₂ Transport**

CO₂ is first transported from the gas phase to the liquid medium to make it
available for carbon fixation by algal cells. Assuming light and other nutrients are not
limiting in the culture medium, gaseous CO₂ must be continuously supplied to maintain a
sufficient driving force for mass transfer to exceed the biological demand of the cells and
sustain intrinsic growth of the microalgae. The transfer rate of CO₂ (CO₂TR) from the gas
phase into the liquid culture is shown in Equation 1.1, where \( k_L \) = liquid phase mass
transfer coefficient, \( a \) = interfacial surface area per unit volume, \( C_L^{eq} \) = equilibrium
concentration of CO₂, and \( C_C^{eq} \) = bulk concentration of CO₂ in the liquid.

\[
CO₂TR = k_L[CO₂]a\left(C_C^{eq} - C_C^{eq}\right) \cdot V_{Culture}
\]

*Equation 1.1*
The mass transfer coefficient is a function of the bioreactor configuration and is the inverse of the resistance to mass transfer on the liquid side. Reactor designs aim to maximize $k_L[\text{CO}_2] a$, which is often achieved by increasing the interfacial area per unit volume (a). The gas phase resistance of CO$_2$ transfer into the liquid is assumed to be negligible. The mass transfer coefficient included in Equation 1.1 should be representative of the entire reactor configuration and not a local value, where significantly better mass transfer may be observed (i.e. in the film of a thin film reactor or in the riser of an air-lift reactor).

The equilibrium concentration of CO$_2$ in the liquid is a function of temperature and pressure, and can be calculated using Henry’s law as shown in Equation 1.2, where $y_{\text{CO}_2} =$ gas phase mole fraction, $P =$ pressure within the reactor, and $H_{\text{CO}_2} =$ Henry’s law coefficient for CO$_2$.

$$C_{\text{eq CO}_2} = \frac{y_{\text{CO}_2} P}{H_{\text{CO}_2}}$$

Equation 1.2

Typically, carbon limitation ($C^L_{\text{CO}_2} \approx 0$) is avoided in laboratory-scale photobioreactors by providing CO$_2$-enriched air at levels of 5-15% to increase the driving force for mass transfer by greater than 100-fold (Suh and Lee, 2003). At the laboratory scale, a source of CO$_2$ is often readily available, but becomes increasingly expensive and more difficult to meet the carbon demand upon scale-up of high-density cultures. Ideally, the reactor could be provided only air (0.039% CO$_2$) to maximize the CO$_2$ utilization yield of algal culturing systems and avoid the costs associated with supplemental CO$_2$, which has been estimated as high as $63-87$ per ton CO$_2$ (Black and NETL, 2010).
Sodium bicarbonate salts have also been suggested to replace gaseous CO₂ and alleviate associated gas-liquid mass transfer problems, but can cost more than three times per unit of carbon (Suh and Lee, 2003). Additionally, the conversion of inorganic carbon to fuel undermines the role of algal biofuels for reducing greenhouse gases.

**Bicarbonate Equilibrium**

CO₂ that dissolves into the liquid culture medium from the gas phase is hydrated to form carbonic acid, which dissociates into bicarbonate and further into carbonate at sufficiently high pH. Collectively, these inorganic carbon species are referred to as the bicarbonate buffering system. It is understood that the hydration reaction of aqueous CO₂ to carbonic acid ($CO_2 + H_2O \rightarrow H_2CO_3$) is the rate limiting reaction in the bicarbonate system. The carbonic acid that does forms is rapidly converted to bicarbonate; therefore, carbonic acid represents a minimal portion of the total aqueous inorganic carbon.

The equilibrium between these species contributes to the total buffering capacity of the culture and is proportional to the total dissolved inorganic carbon. When a pH change is imposed on the bicarbonate system, the distribution of inorganic carbon species will shift with the change in proton concentration and species will be dissociated or associated accordingly. Similarly, when aqueous carbon is removed from culture media due to growth, equilibrium will be reestablished according to Le Chatelier’s principle. The equilibrium reactions and equilibrium constants that define the bicarbonate equilibrium system are presented in Equation 1.3 thorough 1.5 (Edsall, 1969).
\[
CO_2(aq) + H_2O \rightleftharpoons H_2CO_3 \quad \text{Equation 1.3}
\]
\[
log K_{\text{Eq}_1}^o = -2.59
\]

\[
H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \quad \text{Equation 1.4}
\]
\[
log K_{\text{Eq}_2}^o = -6.35
\]

\[
HCO_3^- \rightleftharpoons CO_3^{2-} + H^+ \quad \text{Equation 1.5}
\]
\[
log K_{\text{Eq}_3}^o = -10.33
\]

At the temperature and pH of our systems, the majority of aqueous inorganic carbon exists as bicarbonate and the carbonic acid that forms from the hydration reaction is rapidly dissociated into bicarbonate. Therefore, the formation of bicarbonate can be expressed by summing the rate-limiting reaction of Equation 1.3 with the forward reaction of Equation 1.4 to yield Equation 1.6.

\[
CO_2(aq) + H_2O \rightleftharpoons HCO_3^- + H^+ \quad \text{Equation 1.6}
\]
\[
log K_{\text{Eq}_4}^o = -3.72
\]

The equilibrium between these species can be represented graphically as shown in Figure 1.2 where the distribution of these inorganic carbon species changes with pH (Oh-Hama and Miyachi, 1988). Due to the pH-dependence of the bicarbonate equilibrium, the amount of CO\(_2\) that can be transferred from the gas phase into the culture medium is also influenced by pH as more CO\(_2\) can be absorbed into the liquid medium under basic conditions (Fuggi et al., 1981b).
**Figure 1.2: pH-dependent Distribution of the Bicarbonate buffering system**

**CO₂ Uptake Kinetics**

Understanding carbon uptake by algal cells is important because biomass is approximately 50% carbon by weight. The kinetics of CO₂ fixation in photoautotrophic *Chlamydomonas reinhardtii* were evaluated to understand how photosynthesis is affected by culture pH and gaseous CO₂ supplementation as shown in Table 1.2 (Moroney and Tolbert, 1985). $K_S$ is associated with the CO₂ concentration at which the uptake rate is 50% of the maximum. $K_S$ is inversely proportional to the apparent affinity for CO₂ such that a high affinity is represented by a low $K_S$ value. To understand how uptake kinetics may relate to the bicarbonate equilibrium, the distribution of the inorganic carbon species for each pH was estimated using Figure 1.2 and are included in Table 1.2.
Table 1.2: Apparent Growth Kinetics as a function of gaseous CO$_2$ supplementation in photoautotrophic *Chlamydomonas reinhardtii*

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_S$ (µM)$^1$</th>
<th>Distribution of Inorganic Carbon Species$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Air</td>
<td>5% CO$_2$</td>
</tr>
<tr>
<td>5.95</td>
<td>4.0</td>
<td>35</td>
</tr>
<tr>
<td>6.45</td>
<td>1.8</td>
<td>62</td>
</tr>
<tr>
<td>6.95</td>
<td>4.9</td>
<td>110</td>
</tr>
<tr>
<td>7.45</td>
<td>5.8</td>
<td>350</td>
</tr>
<tr>
<td>7.95</td>
<td>15</td>
<td>875</td>
</tr>
<tr>
<td>8.45</td>
<td>27</td>
<td>3250</td>
</tr>
</tbody>
</table>

**NOTES**

1 – The $K_S$ of aqueous inorganic carbon was evaluated for cultures grown with both air-only and 5% CO$_2$ in air gaseous supplementation.
2 – The distribution of the inorganic carbon species for each pH was estimated using Figure 1.2.

The affinity for CO$_2$ and bicarbonate in air-grown cultures was greater than those grown on elevated CO$_2$. As the pH increased in the 5% CO$_2$-grown culture, $K_S$ increased by almost two orders of magnitude, corresponding to a significant decrease in carbon affinity. The shift in $K_S$ may be explained by the change in the inorganic carbon distribution with pH, as dissolved CO$_2$ and bicarbonate are believed to be the preferred carbon sources for photosynthesis in algal cells (Hogetsu and Miyachi, 1977; Imamura et al., 1983; Moroney and Tolbert, 1985; Suh and Lee, 2003). The air-grown culture also showed a decreasing affinity for photosynthesis with increasing pH, but the magnitude of this change was only 7-fold compared to almost 100-fold in 5% CO$_2$. The smaller change in $K_S$ can be explained by a reduction in the total aqueous inorganic carbon concentration associated with the lower driving force for mass transfer under air-only supplementation (0.039%). It is important to recognize that such kinetics based on growth represent a demand for carbon use which includes other physiological effects of pH that can be affected by the imposed proton gradients. Nonetheless, the influences of pH on the
carbonate buffering and growth are very clear. Similar kinetic behavior was observed in air-grown *Chlorella vulgaris* as shown in Table 1.3 (Hogetsu and Miyachi, 1977).

<table>
<thead>
<tr>
<th>pH</th>
<th>CO₂ Supplementation</th>
<th>Kₛ (%CO₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.6</td>
<td>Air-Grown</td>
<td>0.051</td>
</tr>
<tr>
<td>6.2-6.3</td>
<td>4% CO₂</td>
<td>0.150</td>
</tr>
</tbody>
</table>

The maximum photosynthetic rate achievable in air-grown cultures was believed to be less than those grown on elevated CO₂. This belief was associated with the emergence of different physiological states of *Chlamydomonas reinhardtii* that have increasing affinity for CO₂ and are dependent on the CO₂ level supplied: high (5.0 to 0.5%), low (0.4 to 0.03%), and very low (0.01 to 0.005%) (Vance and Spalding, 2005). However, microalgae and higher plants have the ability to concentrate CO₂ at the site of RuBisCO to ensure that CO₂ is available for carbon fixation. This carbon concentrating mechanism (CCM) allows air-grown cultures to achieve comparable photosynthetic rates to growth on elevated CO₂ while more efficiently utilizing inorganic carbon (Badger et al., 1980).

The excess carbon from the CCM is stored in the form of the bicarbonate ion within the cell. Since the substrate of the enzyme RuBisCO is CO₂, steps must be taken to convert the bicarbonate reserves into usable carbon ($CO₂(aq) + H₂O → HCO₃⁻ + H^+$). This process, as shown in the forward reaction of Equation 1.6, is rather slow at the cellular time scale. For such reason, cells require the presence of the enzyme carbonic anhydrase (CA), which catalyzes the conversion between CO₂ and bicarbonate. With carbonic anhydrase, cells can rapidly interconvert between the two carbon sources as
necessary to maintain internal carbon pools and avoid limitations at the site of RuBisCO.

Noting that carbonic acid rapidly dissociates \( H_2CO_3 \leftrightarrow HCO_3^- + H^+ \), one can effectively represent the carbonic anhydrase (CA) as the sum of this reaction and the catalyzed reaction \( H_2CO_3 \rightarrow CO_2 + H_2O \) from which water removal is the basis of this enzyme’s name. Without the presence of this enzyme, cells would otherwise require elevated \( CO_2 \) levels to provide sufficient aqueous \( CO_2 \) in the medium for transport by diffusion alone (Spalding et al., 1983). However, the active transport of the bicarbonate ion and its rapid inter-conversion of bicarbonate in the CCM provide an enhanced supply of \( CO_2 \) within the chloroplast as \( CO_2 \) is consumed by the RuBisCO-mediated photosynthetic reaction. Therefore, the appearance of distinct physiological states described above is likely due to extracellular mass transfer limitation, which would affect the ability of the CCM to maintain a sufficient intracellular level of bicarbonate. A proposed model for the \( CO_2 \) concentrating mechanism in *Chlamydomonas reinhardtii* is shown in Figure 1.3 (Spalding, 1998).

![Figure 1.3: Proposed Model for Inorganic Carbon Movement in Chlamydomonas reinhardtii](Adapted from Spalding, 1998)
Carbon, Nitrogen, and Cell Mass Balances

The amount of total aqueous inorganic carbon at any time during reactor operation is dependent on the amount of CO$_2$ transferred from the gas phase relative to the amount consumed by the cells or the biological CO$_2$ demand as shown in Equation 1.5, where $C_C^L$ = aqueous carbon concentration, $V_{Culture}$ = culture volume, CO$_2$TR = CO$_2$ transfer rate, Yield$_{Biomass}^{CO_2}$ = biomass yield on CO$_2$, and $X$ = biomass concentration.

\[
\frac{d(C_C^L \cdot V_{Culture})}{dt} = CO_2 TR - \left(\frac{1}{Yield_{Biomass}^{CO_2}}\right)\left[d(XV_{Culture})\right]
\]

Equation 1.5

Under elevated CO$_2$ supplementation when the CO$_2$ transport far exceeds the biological demand of the culture, the removal of inorganic carbon by the cells has a negligible effect on the total dissolved inorganic carbon concentration (Goldman et al., 1974). As with any reactive system, when the rate of supply greatly exceeds the demand, the concentrations approach equilibrium. At lower CO$_2$ levels, CO$_2$ consumption by the cells has a greater effect on the dissolved inorganic carbon concentration as the overall capacity of the bicarbonate buffering system is reduced. Similarly, ultra-high density algal cultures have an increased biological demand for carbon and result in a low level of inorganic carbon unless supplemental CO$_2$ is increased or mass transfer in the reactor is improved.

The cell and nitrogen mass balances are given in Equation 1.6 and Equation 1.7 for a culture with changing volume where $X$=biomass concentration, $V_{Culture}$ = volume of culture, $\mu$ = intrinsic growth rate, $C_N^L$ = concentration of exogenous ammonium or
nitrate, and \( \text{Yield}_{\text{biomass}} \) = biomass yield on ammonium or nitrate. Nitrogen was added to the reactor in pulse additions, which would be reflected by a jump in concentration at the time of addition. The total mass of nitrogen in the culture medium can be expressed as a simple instantaneous mass balance, \( C_N^L \cdot V_{Culture} = C_N^{Rxtr} \cdot V_{Rxtr} + C_N^{feed} \left( \frac{V_{feed}}{V_{Culture}} \right) \) where, \( C_N^{Rxtr} \cdot V_{Rxtr} \) is the unconsumed nitrogen remaining in the reactor medium, \( C_N^{feed} \cdot V_{Feed} \) is the mass of nitrogen in the feed, and \( V_{Culture} = V_{Rxtr} + V_{feed} \). This expression can be included in the mass balance at \( t_{feed} \), and the nitrogen balance could then be integrated over the time period between nitrogen additions.

\[
\frac{d(X \cdot V_{Culture})}{dt} = \mu X \cdot V_{Culture}
\]

Equation 1.6

\[
\frac{dC_N^L \cdot V_{Culture}}{dt} = - \frac{1}{\text{Yield}_{\text{biomass}}} \frac{d(X \cdot V_{Culture})}{dt}
\]

Equation 1.7

In an algal culture, the specific growth rate (\( \mu \)) is dependent on the inorganic nutrient concentrations, light, culture pH, temperature, and the presence of any inhibitors. Growth rate with respect to each of these parameters will be a standard Gaussian (bell-shaped) distribution. Models that incorporate these dependencies are not within the scope of the current work, but are required to model real world environmental fluctuations. The laboratory studies presented here focus on conditions where these are constant or well defined. The maximum intrinsic growth rate occurs at very specific culturing conditions and observed growth in reactor systems is often slower than this maximum rate. The growth rate of the culture for fixed environmental conditions can be given by the general form in Equation 1.8 where the effect of substrate (S) and inhibitor (I) concentrations are typically represented by Monod kinetics, but can follow other mathematical
dependencies. \( K_S \) and \( K_I \) are constants related to the affinity of the cells to a given substrate or inhibitor and incorporate all cellular metabolism that is affected by the particular substance. When the concentration of a given substrate is far greater than \( K_S \), the term for that substrate is dropped from Equation 1.8 as growth is not limited with respect to that substrate. As the concentration of an inhibitor increases, the growth rate is further reduced from the maximum rate.

\[
\mu = \mu_{\text{max}} \prod \frac{S_i}{K_{S,i} + S} \prod \frac{1}{1 + \frac{I_i^2}{K_{I,i}}}
\]

Equation 1.8

For typical laboratory photoautotrophic cultures, light is an important consideration to achieving and sustaining a predictable growth rate. Too much light can lead to photo-inhibition as photosystems become oversaturated with photons, while too little can yield light limitations that also have a negative impact on growth. The point at which this occurs is dependent on how much light the culture is receiving relative to how fast it is growing in a reactor system. For our experiment, we chose to provide the cultures with day and night cycles that included variable light intensities to avoid light inhibition and to mimic real-world daylight conditions. The culture pH is another factor that affects growth as most organisms have a range in which they can survive. Experimental results have demonstrated that growth becomes completely inhibited at pH of 10 or 11 when carbonate would become the primary inorganic species in the medium (Goldman et al., 1974). Prior kinetic data indicated that photoautotrophic growth of microalgae would likely be improved at pH of 7 where the preferred inorganic carbon species (i.e. bicarbonate, aqueous \( \text{CO}_2 \)) are present and \( \text{CO}_2 \) transport from gas to liquid would not be hindered by the presence of high levels of dissolved aqueous \( \text{CO}_2 \) (Table
1.2 and Table 1.3). However, a minimum pH at which growth becomes inhibited is also anticipated in microalgal cultures. It is worth noting that nitrogen limitation is often viewed as a method to induce lipid accumulation. This provides additional motivation to focus on gaining a greater understanding of the dynamics of nitrogen use.

**Photoautotrophic Growth requires pH Control**

Kinetic analysis has previously demonstrated the inherent complexity of CO$_2$ dynamics within a photobioreactor and the potential limitations to both transport and carbon availability under unfavorable pH conditions. Considering the decreasing affinity for CO$_2$ at high pH (Table 1.2 and Table 1.3), and the pH-dependent equilibrium of inorganic carbon species (Figure 1.2), a desirable pH for microalgae during photoautotrophic growth would likely be between 6 and 7 where equilibrium favors bicarbonate and low levels of dissolved CO$_2$. Further complicating the pH dynamics during microalgal growth is the relationship of metabolism with extracellular pH as protons play a key role in most cellular processes including the proton gradient generated to produce ATP during photosynthesis. Photobioreactor pH becomes a critical consideration in the development and application of a high-density culturing strategy as organisms often have a range of pH within which growth is optimal.

**Cellular metabolism causes changes to extracellular pH**

Since organisms typically contain mechanisms to maintain or balance internal pH, protons are taken up and excreted by cells as needed to support the cell cycle and growth.
Examples of processes that affect the external pH of plant cells are listed in Table 1.4 with similar results expected in microalgal cultures (Raven and Smith, 1974).

<table>
<thead>
<tr>
<th>Process</th>
<th>Direction of Change</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photosynthetic CO₂ fixation (CO₂ entering cell)</td>
<td>Increase</td>
<td>Raven, 1970</td>
</tr>
<tr>
<td>Photosynthetic CO₂ fixation (HCO₃⁻ entering cell)</td>
<td>Increase</td>
<td>Raven, 1970</td>
</tr>
<tr>
<td>Nitrate assimilation</td>
<td>Increase</td>
<td>Dijkshoorn, 1962</td>
</tr>
<tr>
<td>Ammonium assimilation</td>
<td>Decrease</td>
<td>Street and Sheet, 1958; Lycklama, 1963</td>
</tr>
<tr>
<td>NH₄OH assimilation</td>
<td>Decrease</td>
<td>Budd and Harley, 1962 a,b</td>
</tr>
<tr>
<td>Excess influx of cations over anions (K⁺ accumulation)</td>
<td>Decrease</td>
<td>Jackson and Adams, 1963</td>
</tr>
</tbody>
</table>

In addition to carbon, biomass contains a significant amount of nitrogen (~10%) and its metabolism is anticipated to play a significant role in photobioreactor pH dynamics. Ammonium is more energetically favorable to assimilate than its oxidized counterparts (nitrate, nitrite, urea or urines) because it can be directly assimilated by cells to form glutamate (Bloom et al., 1992; Fernández and Cárdenas, 1982; Florencio and Vega, 1983; Galván et al., 2000; Raven, 1985; Rexach et al., 1999; Tischner and Lorenzen, 1979). During growth on ammonium, a net efflux of protons from the cells is observed in the culture medium (Howitt and Udvardi, 2000; von Wirén et al., 2000). This proton efflux is related initially to the charge balance through membrane transporters, but also reflects an overall stoichiometry imposed on the longer time scale where nitrogen is typically bound to only one hydrogen in proteins. In contrast, microalgal growth on
nitrate leads to an increase in culture pH as protons are co-transported from the medium as required for transport and reduction of nitrate (Fuggi et al., 1981b) which requires 8 electrons.

**Limitations of current pH control methods in high-density, commercial scale systems**

The effect of nitrogen metabolism on pH has been implicitly recognized in the development of algal media and the selection of a pH control strategy. Ammonium is rarely used for growth because of its ‘toxicity’ associated with the inhibitory drop in pH from its metabolism (Britto and Kronzucker, 2002; Fuggi et al., 1981a; Howitt and Udvardi, 2000; Schlee and Komor, 1986; Troelstra and Dijk, 1985; von Wirén et al., 2000). Therefore, this ‘toxicity’ has led to the selection of nitrate as the primary nitrogen source in algal media. The ability to use nitrate as the sole nitrogen source despite the rise in pH associated with its metabolism is explained by the reliance on significant bicarbonate buffering in algal reactor systems and provide similar buffering as the direct addition of bicarbonate salts. As a result, elevated gaseous CO₂ acts to drive down pH from acidification during CO₂ adsorption into the culture media and to suppress the pH rise associated with nitrate. This pH buffering is not unlike the use of CO₂ incubators for mammalian cell culture. While this approach provides for pH buffering, the excess of CO₂ prevents high yield utilization in a photosynthetic system. Providing elevated CO₂ is also difficult to implement in a large-scale algal culture system and results in the release of unused CO₂ into the atmosphere. To improve both the economic feasibility of commercial scale systems and reduce greenhouse gas emissions, it is desirable to
maximize CO$_2$ yield, which will ultimately require reducing the gaseous CO$_2$

supplementation level supplied to the culture. The laboratory approach to pH control
through nitrate metabolism and elevated CO$_2$ has limited utility at larger scale and higher
culture densities.

Buffer solutions and acid/base addition are alternative pH control methods for
bench-scale reactors, but result in the accumulation of counter ions which can contribute
to ‘culture crash’ when operating continuously at ultra-high density (Tuerk, 2011)(Grady,
2010). The cost of pH buffer solutions in larger reactor volumes would be cost
prohibitive. Another alternative to pH control is through photoautotrophic growth on
urea, which has demonstrated a fairly constant experimental pH (Ammann and Lynch,
1965; Ammann and Lynch, 1967), but the use of urea does not offer the economic benefit
of carbon sequestration from waste gas streams.

**Development of a Novel scalable pH control strategy**

With the limited application of the current pH control methods to long-term high-
density growth, a novel pH control strategy has been proposed based on reducing pH
fluctuations that result from growth by providing a mixed nitrogen source with a
balanced degree of reduction. The major advantage of this media-based control strategy
is that nitrogen is already a critical nutrient for growth so that no further costs are
imposed. This approach also simultaneously represents an opportunity to reduce counter
ions by feeding a significant amount of nitrogen as ammonium nitrate in contrast to
nitrogen salts. This approach has already been implicitly utilized in plant tissue culture,
where growth to high densities is achievable as plant cells selectively consume
ammonium and nitrate to balance their external pH through the stoichiometry of nitrogen assimilation (Britto and Kronzucker, 2002; Curtis, 1999; Imsande, 1986; Kronzucker et al., 1999; Murashige and Skoog, 1962; Raven and Smith, 1974).

The work in this thesis discusses the development of the stoichiometrically balanced media for promoting growth to high density while simultaneously achieving pH control by balancing the reduction state of a mixed nitrogen source. Preliminary application of this media demonstrated an inherent pH instability that results from the dynamics of preferential uptake of ammonium over nitrate. Subsequent work focuses on the refinement of the feeding strategy to minimize pH fluctuations through incremental nitrogen addition and investigates the applicability of this pH control strategy under growth conditions that attempt to maximize the CO₂ utilization yield for future commercial scale-up work.
Chapter 2 – Development of Stoichiometrically Balanced Growth Media

A major challenge in microalgal culturing is the development of media, which promotes growth to higher densities by delaying nutrient limitation and avoiding the accumulation of inhibitory levels of counter-ions (Javanmardian and Palsson, 1992). One approach is through metabolic flux analysis that uses the stoichiometric rates of consumption in cellular processes, but is only as effective as our limited understanding of metabolism (Varma and Palsson, 1994). A more common approach to formulate balanced growth media is to supply all macro- and micronutrients to match the elemental composition of the biomass (Mandalam and Palsson, 1998). In this method, the amount of each element is considered in the media, but usually not the reduction state (reflected in the oxygen and hydrogen components) and does not address the energy balance. With a better understanding of the overall stoichiometry of nutrient growth including the generation of reducing power, it is anticipated that improved media design can simultaneously achieve the goals of ultra-high density algal cultivation while controlling the reactor pH through a mixed nitrogen source.

Limitation of Current Stoichiometric Theory for Photoautotrophic Growth

The generalized form for photoautotrophic growth that is typically presented in the literature when no extracellular products are synthesized as shown in Equation 2.1, where $N_i$ represents a single or mixed nitrogen source. This equation has been simplified to consider only carbon, hydrogen, oxygen, and nitrogen, which compose almost all of the biomass (~97-98%). From the overall growth equation, element balances (C, H, N, O)
can be written to determine the stoichiometric coefficients directly from a biomass composition (x, y and z). Table 2.1 is a compilation of these stoichiometric coefficients on various potential nitrogen sources under the base condition of no extracellular metabolite excretion. From these algebraic expressions, it becomes clear that the hydrogen and oxygen balances are affected by the degree of reduction of the nitrogen source in the media or the relative amounts of hydrogen (x) and oxygen (z).

\[ \alpha_i CO_2 + \psi_i N_i + \delta_i H_2O \xrightarrow{h\nu} CH_xN_yO_z + \lambda_i O_2 \]  

Equation 2.1

### Table 2.1: Stoichiometric Coefficients for Various Nitrogen Sources

<table>
<thead>
<tr>
<th>Nitrogen Source</th>
<th>Stoichiometric Coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\alpha)</td>
</tr>
<tr>
<td>N(_2)</td>
<td>1</td>
</tr>
<tr>
<td>NH(_3)</td>
<td>1</td>
</tr>
<tr>
<td>NH(_4^+)</td>
<td>1</td>
</tr>
<tr>
<td>NO(_3^-)</td>
<td>1</td>
</tr>
<tr>
<td>HNO(_3)</td>
<td>1</td>
</tr>
<tr>
<td>NH(_4)OH</td>
<td>1</td>
</tr>
<tr>
<td>(NH(_2))(_2)CO</td>
<td>(1 - \frac{y}{2})</td>
</tr>
<tr>
<td>(NH(_4))(_2)CO(_3)</td>
<td>(1 - \frac{y}{2})</td>
</tr>
<tr>
<td>(NH(_4))HCO(_3)</td>
<td>(1 - y)</td>
</tr>
</tbody>
</table>
Hydrogen for photoautotrophic growth is provided from the splitting of water for photosynthesis and by growth on a reduced nitrogen source. Based on the stoichiometry of Equation 2.3, this hydrogen must be absorbed into the biomass, as there is no “waste” hydrogen ions produced. Therefore, the biomass composition must change to compensate for differences in the degree of reduction of the nitrogen source provided. However, biomass composition is rather constant across organisms, indicating a major challenge to photosynthetic stoichiometry that must be addressed through an unappreciated correction of the overall growth equation to reflect proton use.

**Proton Imbalance must be reflected in Photoautotrophic growth equation**

As photons are absorbed by the chloroplast during photosynthesis, excited electrons are passed through the electron transport chain and water is split to replace these lost electrons in the chloroplast (Lee and Erickson, 1987). The splitting of water provides hydrogen for the reduction of electron carriers (\(\mathcal{Z}\)) such as ferredoxin or NADP\(^+\) as shown in Equation 2.2. A less efficient alga would require more reducing power, leading to a greater amount of water split and ultimately more “waste” hydrogen ions would accumulate. As extra reducing power is required, more light energy would be captured to account for the additional chemical energy needed to meet the energetic demands of biomass formation (Yang et al., 2000).

\[
2H_2O \stackrel{hv}{\rightarrow} O_2 + 4H^+(\mathcal{Z})
\]

Equation 2.2

When the overall growth equation is written as shown in Equation 2.1, differences in the energetic efficiency of organisms is not captured in the stoichiometry. The major
challenge of photoautotrophic stoichiometry is that the growth and energy balances cannot be decoupled as easily as can be done for heterotrophic or autotrophic growth by specifying a biomass yield on a particular substrate. For example, a less energetically efficient heterotrophic organism would require the combustion of more substrate to produce the same amount of biomass. For photoautotrophic growth, the energetic production reaction can be incorporated into stoichiometric theory by expanding the growth equation to account for the proton imbalance (or “waste” hydrogen production) as shown in Equation 2.3. In general, a positive value for $\phi_i$ would indicate an efflux of protons from the cell (i.e. growth on ammonium) whereas a negative value would represent a proton influx (i.e. growth on nitrate).

$$\alpha_i CO_2 + \psi_i N_i + \delta_i H_2O \xrightarrow{hv} CH_xN_yO_z + \lambda_i O_2 + \phi_i H^+$$  \hspace{1cm} \text{Equation 2.3}

This correction to the overall stoichiometry is reflected experimentally by the observed pH change associated with growth on different nitrogen sources. Attempts have been made to close the mass balance for algal growth, but have failed to account for this associated proton imbalance as buffering often obscures the true pH change (Burris, 1981; Erickson and Minkevich, 1979; Lee and Erickson, 1984; Lee and Erickson, 1987).

**Achieving pH control with a Mixed Nitrogen source**

The nature of $\phi_i$ for different nitrogen sources and its relation to energetics is the focus of media development that sustains ultra-high density growth while minimizing the inherent proton imbalance ($\phi_i = 0$) using a mixed nitrogen source of ammonium ($\phi_{NH4^+} \gg 1$) and nitrate ($\phi_{NO3^-} < 1$). The stoichiometric growth equation for co-provision of
ammonium and nitrate is shown in Equation 2.4A, where $\Delta$ is the molar fraction of nitrogen present in the form of ammonium given as $\Delta = [\text{NH}_4^+]/([\text{NO}_3^-]+[\text{NH}_4^+])$. Since the ammonium degree of reduction is large, $\Delta$ is expected to be less than 0.5. The corresponding elemental balances are shown in Equations 2.4B to Equation 2.4E.

\[
\alpha\text{CO}_2 + \Delta\psi\text{NH}_4^+ + (1-\Delta)\psi\text{NO}_3^- + \delta\text{H}_2\text{O} \rightarrow \text{CH}_x\text{N}_y\text{O}_z + \lambda\text{O}_2 + \phi\text{H}^+ \quad \text{Equation 2.4A}
\]

**Carbon:**
\[\alpha = 1 \quad \text{Equation 2.4B}\]

**Hydrogen:**
\[\delta = \frac{x}{2} - 2\Delta y \quad \text{Equation 2.4C}\]

**Nitrogen:**
\[\psi_{\Delta\text{N}} = y \quad \text{Equation 2.4D}\]

**Oxygen:**
\[\lambda = 1 + \frac{3y}{2} - \frac{5\Delta y}{2} + \frac{x}{4} - \frac{z}{2} \quad \text{Equation 2.4E}\]

From these elemental balances, three unknown stoichiometric coefficients ($\delta$, $\Delta$, and $\lambda$) result with only two associated equations (hydrogen and oxygen balances). Therefore, determination of the desired parameter ($\Delta$) requires an additional constraint to be specified. Direct experimental assessment of $\phi$ is the focus of future studies. In the absence of this data, we have taken an alternative approach to define a mixed nitrogen source. Our approach to obtaining a value for $\Delta$ to minimize the proton imbalance in algal growth uses the experimental observations and logic associated with growth on urea ($\phi_{\text{urea}} \approx 0$) (Ammann and Lynch, 1965; Ammann and Lynch, 1967). pH control is not required for growth on urea. This indicates that during growth on urea, cells effectively use all hydrogen ions for biomass formulation as neither excess protons are taken up by the cells nor “waste” hydrogen are produced.
The media-based pH control strategy will attempt to replicate this balanced degree of reduction achieved for photosynthetic growth on urea through the selection of the ammonium and nitrate distribution, which gives \( \phi = 0 \). This energetic rationale was the basis for equating the amount of carbon dioxide that is fixed relative to the amount of water that is split \((\alpha/\delta)\) for these two nitrogen alternatives. By setting \( \left( \frac{\alpha}{\delta} \right)_{\text{urea}} \) equal to \( \left( \frac{\alpha}{\delta} \right)_{(NH_4^+, NO_3^-)} \), \( \Delta \) can be expressed in terms of the biomass composition as given in Equation 2.5 when extracellular products are not considered.

\[
\Delta = \frac{4 - x}{4(2 - y)}
\]

Equation 2.5

The ash-free elemental compositions and corresponding biomass formulas, which vary depending on the relative lipid, carbohydrate and protein content of the cells are provided for *E. coli*, *Chlamydomonas reinhardtii* and *Chlorella pyrenoidosa* in Table 2.2. Excluding storage compounds such as lipids and carbohydrates, the composition of biomass is constant across organisms. For this reason, the biomass composition for *E. coli* has been used extensively for cellular energetic calculations (Lee and Erickson, 1984; Minkevich, 1973). Under some growth conditions, fatty acid production rises which increases the degree of reduction of the biomass (Oh-Hama and Miyachi, 1988; Spoehr, 1949). The biomass composition of *Chlorella pyrenoidosa*, which is also a natural fatty producer, has been used as a representative biomass composition in the absence of information for *Chlorella vulgaris*. 
### Table 2.2: Nitrogen Content of Stoichiometrically Balanced Media for Photoautotrophic Growth

<table>
<thead>
<tr>
<th>Element</th>
<th>E. Coli</th>
<th>Chlamydomonas reinhardtii&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Chlorella pyrenoidosa&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon Source</td>
<td>CH&lt;sub&gt;1.776&lt;/sub&gt;N&lt;sub&gt;0.165&lt;/sub&gt;O&lt;sub&gt;0.495&lt;/sub&gt;</td>
<td>C&lt;sub&gt;1&lt;/sub&gt;H&lt;sub&gt;1.82&lt;/sub&gt;N&lt;sub&gt;0.103&lt;/sub&gt;O&lt;sub&gt;0.065&lt;/sub&gt;</td>
<td>C&lt;sub&gt;1&lt;/sub&gt;H&lt;sub&gt;1.73&lt;/sub&gt;N&lt;sub&gt;0.065&lt;/sub&gt;O&lt;sub&gt;0.327&lt;/sub&gt;</td>
</tr>
<tr>
<td>Biomass Formula</td>
<td>Air</td>
<td>5% CO&lt;sub&gt;2&lt;/sub&gt; (v/v)</td>
<td>Ai&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Degree of Reduction&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4.29</td>
<td>4.15</td>
<td>4.88</td>
</tr>
<tr>
<td>Efficiency on Urea&lt;sup&gt;4&lt;/sup&gt; (MolCO&lt;sub&gt;2&lt;/sub&gt;/molH&lt;sub&gt;2&lt;/sub&gt;O)</td>
<td></td>
<td>1.14</td>
<td>1.21</td>
</tr>
<tr>
<td>Nitrogen Distribution (%N-NH&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>30%</td>
<td>29%</td>
<td>29%</td>
</tr>
</tbody>
</table>

**NOTES**

1 - The composition given for *Chlamydomonas reinhardtii* was determined for a photoautotrophic culture at 25°C with constant illumination at 65 µE/m<sup>2</sup>/s and grown on atmospheric CO<sub>2</sub> (Boyle and Morgan, 2009).

2 - The composition given for *Chlorella pyrenoidosa* was determined for photoautotrophic cultures grown on high and low CO<sub>2</sub> with continuous illumination at 200 watts (Oh-Hama and Miyachi, 1988; Spoehr, 1949).

3 - The degree of reduction was determined for each biomass composition with valences of C =4, H=1, O=2, and N=-3. The degree of reduction for nitrogen was taken as -3 rather than 0 as this is the valence of nitrogen in biomass (Erickson and Minkevich, 1979).

4 - The stoichiometric efficiency (α/δ) is determined from Equation 2.3 for growth on urea.

The average ammonium-nitrogen content (Δ) is 30% determined from Equation 6 using the biomass compositions presented in Table 2.2. This value of ammonium-nitrogen is consistent with the composition of MS media for plant tissue culture (Δ = 0.355), which have been arrived at empirically for growth without pH control (φ≈0). The predicted effect of changing the ammonium to nitrate ratio on the proton balance for *Chlorella pyrenoidosa* is demonstrated in Figure 2.1. Shifting the distribution from the balanced value of Δ = 0.29 will result in an observed change in culture pH. When the nitrogen distribution is shifted in favor of a more reduced ammonium nitrogen source, ‘waste’ hydrogen ions are generated as more protons are provided with ammonium-nitrogen than actually needed in the biomass (x). As a more oxidized nitrogen source
(nitrate) is provided, protons are taken up by cells to meet the hydrogen demand for energy production and biomass formation.

![Figure 2.1: Effect of Nitrogen Distribution ($\Delta$) on predicted Proton Flux ($\phi$)](image)

**Expansion of Photoautotrophic growth equation for product formulation**

Keeping in mind that the goal of this work is for high-density culturing of microalgae that produce biofuels, the media composition is anticipated to change as the amount of ammonium and nitrate needed to balance the degree of reduction would shift with the accumulation of a product that has a very high carbon to oxygen ratio ($r \ll z$) and no nitrogen ($q=0$). The accumulation of lipids in Chlorella can be as high as 55%, suggesting a significant change in the biomass composition as this product accumulates (Feng et al., 2011; Mallick et al., 2012; Sirisansaneeyakul et al., 2011). This product can be included in the stoichiometric growth equation as shown in Equation 2.6, where $\beta$ is
an experimentally determined yield for the product such as \( \% \text{l lipid} \left( \frac{\beta}{1+\beta} \times 100\% \right) \) after conversion from a mass to molar basis.

\[
\alpha CO_2 + \Delta \psi NH_4^+ + (1 - \Delta)\psi NO_3^- + \delta H_2O \xrightarrow{h\nu} CH_xN_yO_z + \beta CH_pN_qO_r + \lambda O_2 + \phi H^+ \quad \text{Equation 2.6}
\]

Considering lipid accumulation, there will be an increased demand for a reduced nitrogen source as the degree of reduction of the fuel product is greater than that of a non-fatty biomass. For example, a representative \textit{Chlorella vulgaris} lipid composition was determined from the literature data to be C\(_{16}\)H\(_{31.5}\)O\(_{1.9}\)4 (C\(_{1}\)H\(_{1.97}\)O\(_{0.121}\)) and has a degree of reduction of 5.728 (Yoo et al., 2010) compared to 4.15 to 4.88 for algal biomass (Table 2.2). Although the exact nitrogen distribution that will achieve a proton balance will be dependent upon the level and composition of products formed, an ammonium level of 36% of the total nitrogen (36\%N-NH\(_4^+\)) was chosen as the base media composition for photoautotrophic algal growth with pH balancing.

**Discrepancy between Stoichiometric Theory and Experimental Mass Balances**

A stoichiometric parameter of interest is the photosynthetic quotient (\(\lambda/\alpha\)), which is the amount of oxygen produced relative to the amount of carbon dioxide fixed. Greater O\(_2\) evolution is associated with the splitting of more water, the reduction of nitrate, and the formation of biomass that is in a more reduced state (Williams, 1991). Researchers have attempted to measure the photosynthetic quotient on different nitrogen sources in an effort to close the mass balance. For example, the photosynthetic quotient is given in Table 2.3 for \textit{Chlorella pyrenoidosa} grown on urea as determined experimentally during growth on 2\% CO\(_2\) (v/v) in air and by Equation 2.3 assuming a biomass composition of
CH$_{1.72}$N$_{0.067}$O$_{0.327}$ [determined during growth on 5% CO$_2$ (v/v) in air] and no proton flux ($\phi_{\text{urea}}=0$).

Using the experimental photosynthetic quotient of 1.11 mol O$_2$/ mol CO$_2$, the value of $\phi_{\text{urea}}$ was recalculated in Equation 2.3 to be -8.5 mol H$^+$/mol N-urea for the biomass compositions of *Chlorella pyrenoidosa* (Table 2.2), respectively. Experimental results for growth on urea have typically demonstrated a slight drop in pH, although far less significant than for growth on ammonium (Kirkby and Mengel, 1967). This discrepancy between theory and experimental work illustrates the difficulty of the application of stoichiometric theory to photoautotrophic growth as well as inadequacies associated with experimental closure of the mass balance.

The advantage of obtaining kinetics from a continuous steady-state system such as the work of Ammann et.al, is that long-term averages greatly improve data over dynamic batch experiments. However, flows in and out of the system complicate the mass balance (and associated stoichiometry). From the Ammann article, it was not clear whether a significant amount of carbon was leaving in the liquid overflow. Failing to account for this carbon would lead to a reduced photosynthetic quotient. To evaluate the cause for this discrepancy the mass balance performed by the authors was examined as

### Table 2.3: Experimental and Theoretical Photosynthetic Quotients for *Chlorella pyrenoidosa*

<table>
<thead>
<tr>
<th>Determination Method</th>
<th>PQ (Mol O$_2$/mol CO$_2$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stoichiometry</td>
<td>1.26</td>
<td>(Spoehr, 1949)</td>
</tr>
</tbody>
</table>
shown in Appendix A. The CO$_2$ leaving in the gas phase was determined to be far greater than that leaving in the liquid; therefore, the photosynthetic quotient presented in the paper appears to have been appropriately determined for this system growing *Chlorella pyrenoidosa*. Another potential cause for this difference is inaccurate measurement of the biomass composition that is used to determine the photosynthetic quotients by stoichiometry. Cells grown with different metabolism have different biomass compositions with oxygen showing the greatest variation between culture conditions (Chojnacka and Zielin, 2012).

A lower photosynthetic quotient may also be associated with an increase in photorespiration relative to photosynthesis (Burris, 1981). This metabolic inefficiency would increase the amount of required water splitting. However, the experimental culture was maintained at a meticulously monitored CO$_2$/O$_2$ conditions and thus an increase in oxygen relative to CO$_2$ would likely not be the cause for the lowered photosynthetic quotient. Another potential reason for the apparent increase in experimental efficiency is that the reducing power provided by photosynthesis may be more than sufficient to sustain biomass formation. The stoichiometric amount of water could actually be more than needed to support growth and could account for the lower photosynthetic quotient observed experimentally as the cells do not utilize all reducing power generated by photosynthesis. Under these circumstances, extra water would be split simply to satisfy stoichiometry with the balance of energy ‘wasted’ relative to the metabolic capability of the cells.

This unexplained discrepancy suggests the need for further refinement of the stoichiometric approach to media development and will be the focus of subsequent
studies. However, the stoichiometric approach to media development mimicking the logic of a balanced degree of reduction achieved during growth on urea is sufficient for a first attempt at media-based pH control through a mixed nitrogen source.
Chapter 3 – Materials and Methods

Algal Cultures

The algal strain *Chlorella vulgaris* with culture number 2714 was obtained from the UTEX culture collection and algal strain *Chlamydomonas reinhardtii* cc-1690 was obtained from the Chlamydomonas Resource Center (www.Chlamy.org).

Algal Media Formulation

The composition of WFAM is given in Appendix B and was originally designed for high-density growth of *Botryococcus braunii*. However, this media composition was sufficient for culturing of the microalgae *Chlorella vulgaris* and *Chlamydomonas reinhardtii*. The distribution of nitrogen between ammonium and nitrate can be adjusted according to the stock solution table shown in Appendix B. A second media for *Chlorella vulgaris* was developed with potassium nitrate as the only nitrogen source and reduced calcium levels. This media was referred to as WFAMC (WFAM for Chlorella) and the composition is given in Appendix B.

Sampling Methods

Growth Measurement

Optical density (OD) was measured using cuvettes with 1-cm path length in a Beckman Coulter DU 520 spectrophotometer. A wavelength of 550-nm (OD$_{550}$) was used to measure algal biomass growth because this wavelength is largely independent of the chlorophyll content of cells and primarily a measure of light scattering. The
spectrophotometer was referenced using tap water. To maintain linearity between OD and culture density, the measured OD<sub>550</sub> reading was kept below 0.4 by diluting samples with tap water.

To determine the dry weight (DW), 1-mL of well-mixed culture was added to a pre-tared 1.7-mL Eppendorf tube measured using an analytical balance to ±0.00001. The cells were pelleted in a microfuge (14,000 RPM, 10-min). The supernatant was removed without disturbing the pellet and the cells were rinsed with tap water. The Eppendorf tubes were stored in a -20°C freezer and transferred to a -80°C freezer for at least 30-min. The samples were placed with open lids in a Labocno freeze dryer and dried for 24 to 36-hr depending on the number of samples. Samples were re-measured to ±0.00001 using an analytical balance to determine the final weight of the tube and cell pellet.

**pH Measurement**

Cole-Parmer pH electrodes with double-junction BNC connectors were attached to pH control boxes that transmitted a voltage signal to a LI-COR LI-1400 datalogger, which converted the electrical signal to pH by calibrating with buffers (pH 4, 7, and 10). pH samples were taken every 30-s and averaged over 1 or 5-min periods for continuous online monitoring in the airlift and trickle film photobioreactors. Online pH samples were taken in shake flasks using Cole-Parmer extra long slender pH electrodes (Model EW-05990-45, 220 mm x 6 mm) with double-junction BNC connectors, which were interfaced to the LI-COR datalogger in the same way as the other Cole-Parmer electrodes. Offline pH samples were measured using a Metler Toledo SevenEasy pH Meter S20 that was calibrated using pH buffers 4, 7 and 10. Samples were degassed on a
gyratory shaker for 45-min to minimize the variability in offline pH readings due to degassing of samples during transport.

**Nitrate Measurement by Ion Selective Electrode (ISE)**

The Nico2000 Nitrate ISE (ELIT 8021) and liquid double junction reference electrode (ELIT 003) were used for offline measurement of exogenous nitrate concentration. The ISE and reference electrode were pre-conditioned in concentrated standard at 10 g NO$_3^-$/L for at least 30-min. The ion selective electrode was calibrated using three independent NaNO$_3$ standards at 10 g NO$_3^-$/L each serially diluted to 0.01 g/L. The ISE and reference electrode were left in the experimental samples until the electrical potential remained constant for 1-min. Due to the drift in electrical potential that occurs with use, the calibration standards and samples were measured the same day. Nitrogen-free media was used to determine the background contribution from interfering ions to adjust the baseline concentration to 0 g NO$_3^-$/L. Experimental media was used as the positive control for each treatment.

**Measurement of the Mass Transfer Coefficient**

The O$_2$ mass transfer coefficient, $k_{i,a}$, was measured using the unsteady-state sulfate addition method for each reactor configuration and converted to the CO$_2$ mass transfer coefficient using the relative diffusivities of O$_2$ and CO$_2$ in water. The details of this method and the associated calculations for the different photobioreactors types used for the work in this thesis are provided in Appendix C. The oxygen concentration was measured using a Metler Toledo InLab (R) 605 probe.
Growth Chamber Light and Temperature

All batch and fed-batch experiments were executed in a Conviron BDW120 walk-in incubator. High intensity lighting was supplied to cultures using Philips 400W high-pressure sodium vapor and Philips 400W metal-halide lamps. These lights were set to cycle on an 8-hr dark/16-hr light cycle to imitate sunlight. In the first and last hour of the photoperiod, the light intensity was 1/3 of the maximum. The temperature within the incubator was maintained at 28°C during the day and dropped to 25°C during the dark hours. This accommodated other plant growth studies within the incubator. Light measurements were made using the LI-COR LI-1400 datalogger paired with a LI-190SA quantum sensor to determine the photosynthetically active radiation (PAR) received by the culture.

Gas Delivery System for CO₂ supplementation

A pressurized tank at 800-1000 psi, which was down regulated to 12 psi, supplied CO₂ to the reactors. A solenoid valve connected to a power timer controlled when CO₂ was supplied and was subjected to the same cycling as the lights. The flow rate of CO₂ was controlled by a Sho-Rate rotameter (R-2-15 AAA, 1355E Rib Guided Tube, Spherical Float) which was calibrated inline using a Bubble-O meter to determine the volumetric flow rate. Air supplied to the gas delivery system was regulated at 12 psi. The flow rate was controlled by a Sho-Rate rotameter (R-2-15 A, 1355E Rib Guided Tube, Spherical Float) and calibrated inline by measuring the rate at which gas displaced liquid in an inverted graduated cylinder. The control rotameters for air and CO₂ were adjusted to provide the desired CO₂ supplementation level. Air and CO₂ were mixed at a T-
intersection before being divided into individual mixed-gas lines to the reactors and shake flasks. The mixed-gas rotameters were calibrated in series with the air control rotameter. To minimize fluctuations in gas flow rates due to changes in differential pressure upstream, an extra line was left open in the Conviron that also served as a sample port. A sample was taken by inserting a gas-tight syringe into the open end of the bleed line and analyzed using a SRI Multiple Gas Analyzer (Model MG#1) with a TCD.

**Shake Flask and Photobioreactor Setup**

**Daisy-Chain Shake Flasks**

Microalgal cultures were grown in 500-mL flasks at 120 RPM (0.75-in. stroke) on a New Brunswick Scientific G-10 gyratory platform shaker. Each flask was sealed with a silicone stopper with inlet and outlet gas lines. The flasks were connected in series through these gas lines. For each flask, a 0.2 μM Millipore filter was used on the inlet line and the outlet line was loosely plugged with cotton. These precautions were taken to minimize cross-contamination between flasks as well as outside contaminants when not connected to the gas supply. In some experiments, baffled Bellco flasks were used to improve mass transfer. When online pH measurement was needed in the shake flasks, a third hole was added to the silicone stoppers for insertion of the slender Cole-Parmer pH electrode.
Inoculum Cultures

*Chlorella vulgaris* and *Chlamydomonas reinhardtii* cultures were grown in WFAMC or 1/8x NH$_4^+$ WFAM (4.5%N from NH$_4^+$) under 5% CO$_2$ (v/v) supplementation in shake flasks. Cells were harvested by centrifugation (2000xg for 5 min at 24°C), washed in nitrogen-free media to remove extracellular nitrogen, and then centrifuged a second time. The cell pellet was re-suspended in less than 5-mL of nitrogen-free media to minimize osmotic shock during washing and re-suspension without introducing additional exogenous nitrogen.

Loop Air-lift Photobioreactor

The airlift bioreactors with working volume of 1.5-L were constructed from translucent polyethylene plastic tubing using a W-605A 24-inch Single Impulse heat-sealer with 5 mm seal (Recycle = 1, Congealing = 4, Sealing = 4) to form the bag configuration (Figure 3.1A). To create the baffle, paper was placed above and below the baffle on both sides of the plastic to prevent sealing. A ceramic sparger attached to plastic tubing was inserted into the reactor through a hole near the top of the bag to form the riser in the narrow side. A hole cut above the liquid level served as the inoculation and sample port. A Cole-Parmer pH electrode was inserted into the bag reactor in the opposite side of the sparger in the downcomer. The bag reactor was placed between two metal racks to limit the thickness to approximately 0.75-in while minimally blocking light. Gas was sparged into the reactor at 0.31 VVM with up to 5% CO$_2$ (v/v). The average light flux to the culture was 252 µmol/m$^2$/s over a total surface area of 0.11 m$^2$,
and was determined by holding the light sensor normal to the bag surface. The airlift photobioreactor is shown in Figure 3.1B.

![Diagram of Reactor Dimensions and Configuration](image)

**Figure 3.1: Loop airlift photobioreactor used in Batch and Fed-batch pH experiments**

**Trickle Film Photobioreactor**

The reactor consisted of two screens (fiberglass window screen stock) enclosed in a clear plastic bag (2 mil 30-in. x 34-in.) filled with gas at the desired CO$_2$ concentration. To increase the turbulence as the culture fell down the screen, two screen pieces (29.5-in. x 16.5-in.) were cut at a 45° diagonal to the wire mesh. The culture passed through a distributor and onto the screen as shown in Figure 3.2A. The bottom part of the distributor contained an inner tube (1/4”) with holes every 12 mm (holes facing up) and placed inside of a larger tube (1/2”) with holes every 6 mm in a zigzag pattern (holes facing down). The bottom part of the distributor was loosely wrapped in two pieces of screen (Figure 3.2B) to maximize distribution and minimize the momentum from which the culture exited the distributor and flowed down the large piece of screen.
After flowing down the screen, the culture was collected in a reservoir and pumped out the bottom through a sidearm using a Watson-Marlow peristaltic pump 601S with neoprene tubing in the pump for extended tubing life. The 1-L glass reservoir was sealed with a silicone stopper that was fitted with a gas port, the liquid return, sample port, and temperature probe. To maximize hold up between the screens and minimize the amount of culture in the reservoir, the liquid flow rate was adjusted between 0.5 to 1 L/min. Most fluid flow paths were 3/8” clear vinyl tubing. The culture was passed through a stainless steel heat exchanger (24” long, 3/8” OD) and cooled to 25°C using cooling water from a Fisher Scientific Isotemp Refrigerated Circulator, model 9100. CO₂ was supplied to the bag enclosure by passing gas through a 0.2 µM filter and then humidified by passing through a glass column (3” ID x 30.5” high) filled with distilled water and packed with stainless steel Berl saddles. The average measured light flux to the screen was 282 µmol/m²/s within the bag enclosure with an area of 0.3 m². The trickle film photobioreactor setup is shown in Figure 3.2C and has a working liquid volume of 500 mL.
Figure 3.2: Trickle film photobioreactor used in Fed-batch pH experiments
Chapter 4 - Inherent pH Instability in Microalgal Cultures due to Nitrogen Metabolism

Stoichiometrically balanced media containing a mixed nitrogen source is anticipated to minimize the proton imbalance that results from metabolism when the nitrogen provided has a balanced degree of reduction to achieve $\phi=0$. Understanding the metabolic pathways including any regulatory mechanisms proves a useful exercise to better appreciate the utility of stoichiometric theory for pH control and for the selection of a feed strategy. Included in this chapter is a description of nitrogen metabolism in microalgae and experimental data demonstrating an inherent dynamic proton imbalance in cultures due to the inability of microalgae to selectively consume ammonium and nitrate when provided a mixed nitrogen source. These results led to the modification of the pH control strategy to an incremental feeding approach in future experiments.

Literature Review of Nitrogen Metabolism in Microalgae

Figure 4.1 outlines the major steps and key features of nitrogen metabolism and regulation in a *Chlamydomonas reinhardtii* cell. The general pathways for nitrate and ammonium assimilation are anticipated to be essentially the same in *Chlorella vulgaris*; however, the genes included for transport and regulation are likely specific to Chlamydomonas.
Ammonium Assimilation

Ammonium is readily taken up by algal cells because it is has a lower energy requirement to metabolize than nitrate due to its higher degree of reduction (Bloom et al., 1992). Ammonium assimilation involves only transport steps across the plasma and chloroplast membranes prior to its incorporation into carbon skeletons within the chloroplast. In photoautotrophic cultures, ammonium uptake relies on ATP that is generated by photophosphorylation during photosynthesis to provide the energy needed for active transport across these membranes (Byrne et al., 1992).
Chlamydomonas reinhardtii has both high and low affinity ammonium transport proteins located on the plasma membrane to improve the efficiency of transport under various growth conditions (Franco et al., 1987). The low affinity transport systems (LATS) are constitutively expressed with high capacity for ammonium transport and is related to the passive K$^+$ channels associated with photosynthesis (Crawford and Forde, 2002; Ullrich et al., 1984; Wang et al., 1993b). The ammonium transport genes associated with the low affinity systems are maximally expressed in ammonium-containing media (Britto et al., 2001a; Britto et al., 2001b; Córdoba et al., 1987; Glass, 2003; González-Ballester et al., 2004; Wallsgrove et al., 1987; von Wirén et al., 2000).

The high affinity transport systems (HATS) of Chlamydomonas reinhardtii are negatively regulated by ammonium and are only active at very low concentrations of ammonium in the micro-molar range (Byrne et al., 1992; Franco et al., 1987; Ullrich et al., 1990). Genes associated with the HATS are maximally expressed in nitrate or nitrogen-free medium to improve capture efficiency under low levels of extracellular ammonium. Active transport of ammonium through the HATS is closely connected to the proton gradient generated during photosynthesis (Howitt and Udvardi, 2000; von Wirén et al., 2000). Therefore, uptake of ammonium in microalgal cells is coupled to an immediate excretion of K$^+$ which facilitates proton excretion as the exogenous ammonium concentration decreases and cells switch from LATS to HATS (Schlee and Komor, 1986). The kinetic parameters of the ammonium transporters of Chlorella vulgaris and Chlamydomonas reinhardtii are summarized in Table 4.1 (Franco et al., 1988; Schlee and Komor, 1986). The HATS of Chlorella vulgaris has a higher affinity reported for ammonium than Chlamydomonas reinhardtii.
Table 4.1: Kinetic parameters for Ammonium transporters on the plasma membrane

<table>
<thead>
<tr>
<th>Algal Strain</th>
<th>System</th>
<th>$K_S$</th>
<th>$V_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlamydomonas reinhardtii</em>^{(A)}</td>
<td>LATS</td>
<td>26-30 μM</td>
<td>11 μmol/mg chl·h</td>
</tr>
<tr>
<td></td>
<td>HATS</td>
<td>7.5 μM</td>
<td>2 μmol/mg chl·h.</td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em>^{(B)}</td>
<td>HATS</td>
<td>2 μM</td>
<td>500 μmol/h·ml p.c.</td>
</tr>
</tbody>
</table>

References

A – Franco et. al, 1988
B – Schlee and Komor, 1986

Once in the cytosol, ammonium is moved across the chloroplast membrane by active transport where it is assimilated into 2-oxoglutarate through the glutamate synthase (GS/GOGAT) cycle to form glutamate, which is an amino acid that connects the metabolic pathways of amino acids with carbohydrates and lipids (Lam et al., 1996; Torchinsky, 1987). The assimilation of ammonium can be represented by the two partial reactions as shown in Equation 4.1A and B with the overall reaction in Equation 4.1C (Britto and Kronzucker, 2002).

\[ \text{Equation 4.1A} \]
\[ NH_4^+ + \text{glutamate} + \text{ATP} \rightarrow \text{glutamine} + \text{ADP} + P_i + H^+ \]

\[ \text{Equation 4.1B} \]
\[ 2 - \text{oxoglutarate} + \text{glutamine} + H^+ + 2e^- \rightarrow 2 \text{glutamate} \]

\[ \text{Equation 4.1C} \]
\[ 2 - \text{oxoglutarate} + NH_4^+ + \text{ATP} + 2e^- \rightarrow \text{glutamate} + \text{ADP} + P_i \]

In unicellular algae such as *Chlamydomonas reinhardtii* and *Chlorella vulgaris*, cells need to be efficient at transporting very low extracellular concentrations of ammonium, but also able to excrete excess ammonium that is not assimilated (Wang et al., 1993a). This capability is unique to unicellular algae and is not necessary in plants where there is space between the plasma membranes of cells to dilute or concentrate ammonium as needed for growth. As a result, the plastidic transporters allow for bi-
directional transport of ammonium into and out of the chloroplast to prevent accumulation of excess nitrogen in the cells. In alga, ammonium is excreted from the chloroplast and eventually out of the cell through the transporters on the plasma membrane under carbon limitation, which is defined as the inadequate supply of 2-oxoglutarate in the chloroplast for the GS/GOGAT cycle (Azuara and Aparicio, 1983; Mariscal et al., 2004; Navarro et al., 2000; Rexach et al., 2000; Schlee and Komor, 1986).

**Nitrate Assimilation**

Nitrate assimilation requires two transport and two additional reduction steps prior to integration into carbon skeletons through the glutamate synthase cycle. As a result of the additional reduction steps required to produce nitrate-derived ammonium, nitrate is utilized at a rate 3-fold lower than ammonium (Fuggi et al., 1981a). Nitrogen-limited or nitrogen-starved *Chlamydomonas reinhardtii* cells were shown to regulate nitrate based on existing assimilatory capacity rather than increasing the flux of nitrate into the cell through additional transporters (Watt et al., 1993). This observation was later explained by the presence of high affinity transport systems (HATS) that scavenge low concentrations of nitrate and are operable under 250 μM (Galván and Fernández, 2001). In total, four nitrate transport systems, which have both high and low affinity for nitrate, have been discovered in *Chlamydomonas reinhardtii*. The kinetic parameters for these four transport systems are summarized in Table 4.2. The $K_S$ values for the nitrate transport systems are dependent on pH as a result of co-transport of protons with nitrate into algal cells, displaying increased transport rates as the media acidifies (Galván et al., 2000). This benefits stoichiometric balance as discussed in Chapter 2. Nitrate transport
eventually reaches a maximal rate as the transporters become saturated (Agüera et al., 1990; Llamas et al., 2002; Siddiqi et al., 1990).

### Table 4.2: Kinetic parameters for Nitrate/nitrite transporters in *Chlamydomonas reinhardtii*

<table>
<thead>
<tr>
<th>Transport System</th>
<th>Nitrate Transport</th>
<th>Nitrite Transport</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_S$ (µM)</td>
<td>$V_{max}$ (µmol/h·mg Chl)</td>
</tr>
<tr>
<td>I HANT/HANiT</td>
<td>1.8±0.3</td>
<td>9.0±1.1</td>
</tr>
<tr>
<td>II HANT</td>
<td>11.0 ±1.3</td>
<td>5.6±1.5</td>
</tr>
<tr>
<td>III LANT/HANiT</td>
<td>10 mM (B)</td>
<td>N/A</td>
</tr>
<tr>
<td>IV HANT/HANiT</td>
<td>40 µM (B)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

### References

A – Córdoba et al., 1986
B – Navarro et al., 2000
C – Rexach et al., 1999

System I is considered a high affinity transporter (HANT/HANiT), bi-specific for nitrate and nitrite. System II is also considered a HATS, but is specific to nitrate only (Galván et al., 1996; Rexach et al., 1999). In *Chlamydomonas reinhardtii*, the Nrt2;1 (System I) and Nrt2;2 (System II) transport proteins contain 12 hydrophobic membrane-spanning domains that form the channel for nitrate and co-transport of protons (Quesada and Galvan, 1994; Unkles et al., 1991). These two systems are constitutively expressed when carbon skeletons (2-oxoglutarate) are readily available in the chloroplast to synthesize glutamate. Additionally, these transporters allow bidirectional movement for excretion of excess nitrite from the cell (Navarro et al., 2000).

Unlike Systems I and II which are constitutively expressed, System III expression is induced when nitrate in the culture medium exceeds 1.1 mM (Watt et al., 1993).
System III is active under non-limiting carbon conditions and operates as a low affinity nitrate transporter, as well as a high affinity nitrite transporter (Galván et al., 1996; Rexach et al., 1999). This system has optimal activity at a slightly acidic pH of 6 (Rexach et al., 1999). System IV is a HATS bi-specific for nitrate and nitrite. Expression of System IV is also inducible, but is affected by carbon dioxide availability rather than extracellular nitrate levels. This system is only active when carbon is limited (2-oxoglutarate cannot be actively synthesized), and operates best at slightly basic pH of 8 (Rexach et al., 1999). However, this system becomes slightly inhibited when exogenous nitrate concentrations exceed 10 mM (Navarro et al., 2000). The inducible nitrate Transport Systems III and IV are likely regulated at translational or post-translation level (Watt et al., 1993). In Chlorella, evidence of two phases of nitrate uptake transport rates were also observed with a decrease in rate around 200 μM (Jeanfils et al., 1993; Tischner and Lorenzen, 1979). This change in transport rate likely corresponds to low and high affinity transporters similar to that of Chlamydomonas; however, the details of transport systems in Chlorella have not been fully characterized.

Upon entering the cytosol, nitrate is reduced to nitrite by nitrate reductase, where the electron donor is NAD(P)H. The assembly and activity of nitrate reductase is highly dependent on a molybdenum cofactor (Aguilar et al., 1992; Eilers et al., 2001; Galván and Fernández, 2001; Mendel and Bittner, 2006). Nitrate plays a regulatory role by binding to nitrate reductase, stabilizing it against breakdown and thus maintaining the integrity of the nitrate reductase complex (Florencio and Vega, 1983; Hipkin and Al-Bassam, 1980). The $K_M$ for nitrate reductase is 25 μM for Chlamydomonas (Córdoba et al., 1986). Morris and Syrett hypothesized that nitrate may act as an inducer to nitrate
reductase synthesis in *Chlorella vulgaris* (Morris and Syrett, 1963). However, nitrate uptake into the cell rather than the activity of nitrate reductase appears to be more important in regulation of the whole nitrate reduction pathway in *Chlorella vulgaris* (Tischner and Lorenzen, 1979). This observation is amenable to a simplistic stoichiometric model for growth and pH control.

Following reduction, nitrite is transported across the chloroplast membrane by active transport into the stroma. The plastidic nitrate transporters work with those on the plasma membrane to maximize nitrate assimilation efficiency, acting as regulators or sensors in addition to transporters (Rexach et al., 2000). Upon crossing the chloroplast membrane, nitrite is further reduced to ammonium by nitrite reductase in the stroma through a 6-electron step reduction with ferredoxin as the electron donor (Martinez-Rivas et al., 1991; Quesada et al., 1998; Thacker and Syrett, 1972). The reducing power for nitrite reductase is supplied by the electron transport chain in photosynthesis which generates the reduced form of ferredoxin (Huppe and Turpin, 1996), and is coupled to our simple stoichiometric logic via Equation 2.4. The close connection to photosynthesis explains why nitrite reductase is maximally expressed in light (Loppes et al., 1999; Martinez-Rivas et al., 1991; Quesada et al., 1998).

**Nitrogen Metabolism is regulated by Carbon Availability**

Nitrogen and carbon metabolism are closely linked due to the dependence on photosynthesis (or carbon fixation) to generate the reducing power and proton gradients needed to drive assimilation. More specifically, carbon and nitrogen assimilation are connected through amino acid synthesis (nitrogen-containing) in the chloroplast through the GS/GOGAT cycle, which links the carbohydrate (no nitrogen) and lipid (no nitrogen)
pathways (Lam et al., 1996; Torchinsky, 1987). In *Chlamydomonas reinhardtii*, regulation of nitrate transport genes are controlled by both carbon and nitrogen availability through the regulatory genes *CCM1* and *NIT2* (Mariscal et al., 2004; Rexach et al., 2000) as noted in Figure 1.3. *CCM1* is a central regulatory gene for carbon assimilation and is associated with the carbon concentrating mechanism that allows cells to concentrate carbon at the site of RuBisCO (Mariscal et al., 2006; Miura et al., 2004). When elevated CO$_2$ supplementation (5% gas-phase concentration) is provided to a photobioreactor, sufficient internal carbon for biomass formation can accumulate as the dissolved inorganic carbon level in the media will be sufficient to meet the biological demand of the cells, and the CCM limit nitrogen metabolism. If cells become carbon-starved in the chloroplast, there is no longer 2-oxoglutarate (carbon skeleton) and glutamate cannot be produced. Both ammonium and nitrate will initially be transported into the cell following carbon limitation, resulting in the intracellular accumulation of exogenously-supplied or nitrate-derived ammonium (Thacker and Syrett, 1972). The accumulation of ammonium in the chloroplast causes deactivation of nitrate reductase and has an additional effect on the plasma membrane transporters (Rexach et al., 2000). Coordination between the plasma membrane transporters with the nitrite transporters on the chloroplast membrane prevents excess nitrate from being transported into the cell and wasting energy to do so. Any exogenous or nitrate-derived ammonium that cannot be integrated into carbon skeletons is excreted from cells as a way to dissipate excess reducing power generated by photosynthesis (Galván and Fernández, 2001).
Interactions between Nitrate and Ammonium Metabolism

The presence of ammonium has been shown to inhibit nitrate transport in both *Chlamydomonas reinhardtii* and *Chlorella vulgaris*. Ammonium is preferred versus oxidized nitrogen compounds because its assimilation has less energetic cost (Fernández and Cárdenas, 1982; Florencio and Vega, 1983). When ammonium is present in the medium, the nitrate transport systems on the plasma membrane of *Chlamydomonas reinhardtii* become inhibited and nitrate is no longer transported into the cells (Fernández and Cárdenas, 1982; Florencio and Vega, 1983). Nitrate reductase becomes inactivated and begins to degrade. Complete inhibition of nitrate assimilation takes up to 10 minutes in *Cyanidium caldarium* and up to 5 minutes in *Chlorella vulgaris* (Fuggi et al., 1981a; Pistorius et al., 1978). In *Cyanidium caldarium*, nitrate reductase inhibition induced by ammonium was shown to decay exponentially with respect to time (Fuggi et al., 1981a). These findings suggest that it is not ammonium that directly inhibits nitrate transport and reduction, but rather the products formed following the assimilation of ammonium in the chloroplast (Crawford and Forde, 2002). A similar response to ammonium is observed in *Chlorella vulgaris* in which the presence of exogenous ammonium interferes with the nitrate transporters on the plasma membrane as well as the activity of nitrate reductase activity (Tischner and Lorenzen, 1979).

When ammonium-grown *C. reinhardtii* cells are transferred into a nitrate-containing media, nitrate consumption reaches a maximum rate after 60-90 min (Florencio and Vega, 1983). The delayed response in ammonium-grown cells is likely due to the fact that nitrate reductase levels are negligible and time is needed to produce enough nitrate reductase to regain normal activity (Hipkin and Al-Bassam, 1980). The
presence of ammonium in Chlamydomonas reinhardtii causes the degradation, rather than deactivation, of nitrate reductase through proteolytic breakdown and also results in negligible synthesis of this enzyme (Fernández and Cárdenas, 1982; Florencio and Vega, 1983; Morris and Syrett, 1963). Similarly, ammonium-grown Chlorella vulgaris cultures showed that the nitrate reductase activity was negligible in the absence of nitrate upon prolonged exposure to ammonium (Morris and Syrett, 1963; Pistorius et al., 1976; Pistorius et al., 1978). Nitrate-derived ammonium would be expected to have the same inhibitory effect as exogenous ammonium on nitrate transport. However, internal ammonium pools remain negligible as ammonium is rapidly incorporated into carbon skeletons when carbon is not limiting (Fuggi et al., 1981b; Thacker and Syrett, 1972).

Nitrate-grown Chlamydomonas reinhardtii subjected to 7 hr of nitrogen starvation returned to normal consumption rates after only 10-15 min (Florencio and Vega, 1983). Faster recovery of nitrate reductase was observed in nitrogen-starved cells because reactivation rather than synthesis of the enzyme was necessary. Reactivation of the nitrate reductase exhibited first order kinetics in Chlamydomonas reinhardtii with a lag time before reaching full activity (Fuggi et al., 1981a). The regulatory gene, Nit2 is responsible for the up-regulation at the transcriptional level of nitrate reductase in the presence of oxidized nitrogen compounds (González-Ballester et al., 2004; Rexach et al., 1999; Rexach et al., 2000). Additionally, this transcription factor is responsible for the deactivation of the nitrate assimilation pathway in the presence of ammonium.

In Chlamydomonas reinhardtii, the inhibition of nitrate transporters by ammonium was determined to apply only to nitrate transport Systems I-III when carbon is readily available in the chloroplast. However, under carbon-limited conditions when
only System IV is active, the presence of ammonium did not interfere with the nitrate assimilation pathway and nitrate could still be transported into the cells and assimilated (Galván and Fernández, 2001; Llamas et al., 2002; Rexach et al., 1999). Although the transport systems of *Chlorella vulgaris* are not as well characterized as in *Chlamydomonas reinhardtii*, ammonium was shown to prevent nitrate assimilation in the presence of excess carbon, but did not inhibit nitrate uptake when carbon was limiting (Smith and Thompson, 1971). These observations will become an important consideration when attempting to maximize the CO$_2$ utilization yield while controlling the pH through alternating metabolism stoichiometry of ammonium and nitrate. This regulatory mechanism may limit the application of this pH control strategy to culture conditions in which the dissolved inorganic carbon level is well above the biological demand of the cells.

**Experimental Results for Nitrogen Use in Algae**

While the literature review illustrates the complicated interaction of nitrogen assimilation mechanism, our interest is in achieving control, which means we need to understand how the cultures will predictably respond to ammonium and nitrate availability. Experimental results have demonstrated a proton imbalance in *Chlorella vulgaris* and *Chlamydomonas reinhardtii* cultures that results from complete inhibition of nitrate transport by exogenous ammonium when dissolved CO$_2$ exceeds the biological demand. This instability in pH occurs as microalgal cultures given ammonium and nitrate are unable to selectively consume either nitrogen source to balance extracellular pH.
Proton imbalance in Microalgal cultures results from preferential NH$_4^+$ uptake

*Chlorella vulgaris* cultures were grown in 1.5-L loop airlift photobioreactors (light path-length limited to 0.75-in.) under 5% CO$_2$ (v/v) in air with 0.3 gN/L provided as 0-36% from ammonium (0 to 0.108 g N-NH$_4^+$/L) and the balance as nitrate. The highest ammonium concentration corresponded to that of our stoichiometric growth media designed for un-buffered pH control. Only cultures provided with 0 and 4.5%N-NH$_4^+$ grew as shown in Figure 4.2A, whereas cultures given higher levels of ammonium ceased growth early in the experiment.

![Graph A](image.png)

**Figure 4.2:** Inherent pH stability in *Chlorella vulgaris* cultures under excess CO$_2$
The culture grown only on nitrate demonstrated a steady rise in pH over the growth period due to the net influx of protons required to support nitrate metabolism (Figure 4.1B). As the ammonium concentration increased above 9%, the proton drop became more significant until metabolism caused a significant enough efflux of protons to inhibit growth at a pH of 3.75. It is interesting to note that Chlorella was capable of surviving at pH of 4 where the bicarbonate equilibrium would be shifted almost entirely to aqueous CO$_2$ (Figure 1.2). In the culture grown on 4.5%N-NH$_4^+$ where the pH remained above 4, a pH drop followed by a pH rise was observed corresponding to sequential ammonium and nitrate metabolism. These observations demonstrated the inability of Chlorella vulgaris to balance extracellular pH to independently switch between nitrate and ammonium consumption to control the resulting proton imbalance from metabolism. The true minimum pH of the culture was likely not captured in the pH time course with offline samples taken at 3-hr intervals. Therefore, the change in external proton concentration during nitrogen transport was not determined.

To verify the observed pH swing in the surviving culture was due to preferential ammonium assimilation over nitrate, a second batch experiment was executed to compare the pH drop in Chlorella vulgaris cultures given ammonium with either nitrate and chloride as the counter-ion. Chloride as a counter-ion should not interfere with nitrogen metabolism in this experiment because CO$_2$ was provided in excess (Rexach et al., 1999). Both cultures were grown in 1.5-L loop airlift photobioreactors augmented with continuous online pH monitoring. Optical density was measured at 550 nm and converted to cell concentration using a biological conversion factor of 0.52 gDW/L/OD$_{550}$ determined experimentally.
Cultures were started on 0.0135 gN-NH$_4^+$/L (4.5% N as NH$_4^+$) and demonstrated nearly identical growth during the first 15 photo-hours (Figure 4.3). The culture grown on ammonium chloride ceased growth whereas the ammonium nitrate culture displayed a subsequent recovery of pH and continued growth. This observation is consistent with preferential utilization of ammonium over nitrate and is in alignment with previous reports that nitrate utilization is inhibited in the presence of ammonium when aqueous inorganic carbon maintained above the biological demand (Fernández and Cárdenas, 1982; Florencio and Vega, 1983; Tischner and Lorenzen, 1979). The cessation of growth in the NH$_4$Cl-grown culture is due to nitrogen depletion rather than inhibition of growth at low pH.

The average biomass yields during growth on ammonium and nitrate were 7.13 +/- 0.18 gDW/gN-NH$_4^+$ and 14.52 +/- 0.61 gDW/gN-NO$_3^-$, and are reversed from the expectation of higher yield on a more reduced nitrogen source. The observed lower biomass yield on ammonium is likely to be inverted from the expectation of higher yield on a more reduced nitrogen source due to faster assimilation (transport only) as exogenous ammonium is rapidly transported into the chloroplast and combined with 2-oxoglutarate to form glutamate without the reduction steps. Under these circumstances the uptake and utilization does not reflect an appropriate constant biomass yield.
The amount of protons removed from the medium during nitrate consumption appeared greater than the amount of protons excreted during ammonium consumption given that the culture started with an equimolar ratio of ammonium and nitrate. However, these results would be skewed by changes to the buffering capacity as well as shifts in bicarbonate equilibrium during growth. The change in extracellular proton concentration during ammonium consumption for both cultures was $0.136 \pm 0.015 \text{ mol H}^+$/mol N-NH$_4^+$, assuming only ammonium consumption during the initial pH drop and neglecting buffering. The net proton change is anticipated to be less significant in a culture grown on
NH₄NO₃ if nitrate and ammonium were simultaneously being transported into the cell due to the opposing direction of proton flux associated with these metabolic pathways.

Similar batch experiments were executed with *Chlamydomonas reinhardtii* to determine how this ‘model’ microalga responded to growth on mixed nitrogen sources. Photoautotrophic cultures were grown in 1.5L loop airlift photobioreactors under 5% CO₂ (v/v) in air on medium with 0.3 gN/L as 0-9% from ammonium (0 to 0.027 g N-NH₄⁺/L) and the balance nitrogen as nitrate. All cultures continued to grow during the 50-hr growth period as shown in Figure 4.4A. The biomass yields during growth on ammonium and nitrate in photoautotrophic *Chlamydomonas reinhardtii* cultures were determined to be 4.91 +/- 0.16 gDW/gN-NH₄⁺ and 6.10 +/- 0.13 gDW/gN-NO₃⁻, respectively. These yields indicate a cellular accumulation of nitrogen to over 16.4% by mass, compared to 9.6% for generic biomass (*E. coli*). The lower biomass yields on nitrogen for Chlamydomonas as compared to Chlorella are likely a result of the reduced accumulation of lipids in this algal strain.

*Chlamydomonas reinhardtii* grew at a pH as low as 3 as shown in Figure 4.4B compared to *Chlorella vulgaris* which did not sustain growth below a pH of 3.75. The minimum pH observed was proportional to the initial ammonium level provided. The nitrate concentration in the media was initially constant as measured by an ion selective electrode during ammonium metabolism and then began to decrease after the pH minimum as shown in Figure 4.4C, serving as further evidence for the preferential uptake of ammonium over nitrate. Although *Chlamydomonas reinhardtii* continued to grow under more acidic conditions, the ammonium concentration tested was still 4-fold less than that of our stoichiometric growth media. This observation lends to a modification of
the simplistic approach to pH control through a mixed nitrogen strategy and suggests a
fed-batch strategy would better overcome nitrogen regulatory mechanisms. Regardless of
the minimal pH achieved and varying level of nitrate, the same final pH was achieved in
all cultures, which is likely due to the increase in CO₂ absorption that can occur at higher
CO₂. This observation may indicate that the bicarbonate buffering not only masks
changes in proton concentration due to nitrogen metabolism, but also alters pH as a
function of CO₂ transport into the culture media and uptake rates by the cells.

Proton secretion in conjunction with ammonium metabolism can be calculated
from the observed pH drop and growth data. The change during ammonium transport
increased from 0.015 to 0.035 mol H⁺/mol NH₄⁺ as nitrogen ammonium increased from
6-9% (inset of Figure 4.4B). The large change in this ratio (ϕ/ψ in Equation 2.4) is
influenced by the buffering capacity of the medium including the contribution from the
bicarbonate equilibrium, which was not considered in the calculation of this ratio.
Understanding the combined role of nitrogen stoichiometry and pH dynamics is an
important step towards implementing media-based pH control strategy that is needed for
large-scale algal culturing that does not rely on buffering from high gaseous CO₂ or other
pH control methods.
Figure 4.4: pH Instability from Nitrogen metabolism in photoautotrophic *Chlamydomonas reinhardtii* under excess CO$_2$
Growth has a significant effect on culture buffering capacity

In carrying out these studies at different ammonium concentrations, it became apparent that the change in pH for a given amount of algal biomass growth varied considerably at different cell densities and at different stages associated with continuous culture. Titrations were performed to determine how cell density and media exhaustion affect the buffering capacity of the culture. Unfortunately, this study does not distinguish between the relative contributions of cell density and media exhaustion to the overall buffering capacity of the culture. To discern this difference, cells would have to be filtered from exhausted media and a titration performed to compare the buffering capacity to a total culture sample (cells and media).

To gain some insight into the extent to which buffering capacity of the cultures changes, photoautotrophic *Chlorella vulgaris* cultures were grown to final densities of 0.48, 0.79, and 0.95 gDW/L and the buffering capacity was evaluated. These culture were grown on 0.3 gN/L in shake flasks with 5% (v/v) CO$_2$ (v/v) in air. The cultures were grown on NH$_4$OH and KNO$_3$ with 0-9% N-NH$_4^+$, noting that NH$_4^+$ added as a base would have affected the initial pH (immediate response), but not the change in pH due to nitrogen metabolism associated with a longer time scale. These cultures were titrated against 0.014N HCl. The cultures were degassed to ensure the effects of the bicarbonate buffering system would be negligible prior to performing the titrations, and the results would demonstrate the ‘true’ buffering capacity of the culture. Fresh medium (0.3 gN/L with NH$_4$NO$_3$ and KNO$_3$ at 36%N-NH$_4^+$) was included as a comparison to the buffering capacity when cells were present at varying densities. Cultures at a moderate cell concentration of 0.48 gDW/L were shown to have a much greater buffering capacity than
fresh medium, requiring substantially larger additions of acid to reduce the pH change as shown in Figure 4.5. This buffering was also found to increase at higher cell concentrations, showing that a higher cell density exhibited a smaller pH change for a given amount of protons.

![Figure 4.5: Effect of cell growth on culture buffering capacity](image)

The increase in buffering capacity with longer-term continuous culturing suggests that it is not only elevated gaseous CO₂ supplementation that contributes to the total buffering capacity and that the culture itself plays an important role in its own buffering. Therefore, it is anticipated that a higher density culture can withstand growth on a greater amount of ammonium as the drop in pH is reduced with increased buffering capacity.
Cellular regulation facilitates novel approach to pH control through nitrogen metabolism

The ‘culture crash’ due to the preferential utilization of ammonium over nitrate suggests that the pH might be maintained if there was careful addition of ammonium (i.e. less than the stoichiometric amount). However, fluctuating between ammonium and nitrate utilization relies on the intracellular regulation of nitrogen metabolism to rapidly switch between pathways. The regulatory elements that give rise to the observed pH responses due to intra- and extracellular proton movement, and facilitate pH control through a mixed nitrogen source are presented in Figure 4.6. When ammonium is present, nitrate assimilation is inhibited and excess protons are excreted from the cells, as not all the hydrogen atoms from ammonium are required for biomass formation. Upon depletion of ammonium, nitrate assimilation can occur, which requires a net influx of protons into the cell for reduction. The success of the control strategy relies on manipulating the direction (and magnitude) of the proton flux by alternating between nitrogen sources to minimize the net effect on extracellular pH. The extent to which the extracellular pH changes at a certain density will help to define the frequency at which it is necessary to switch between nitrogen assimilation pathways. Additionally, maintaining sufficient dissolved inorganic carbon levels will be important to ensure CO₂ uptake kinetics, bicarbonate equilibrium, and gaseous transport do not interfere with regulation of the nitrogen pathways.
Pulse addition of ammonium displays rapid pH drop in nitrate-grown culture

A photoautotrophic culture of *Chlamydomonas reinhardtii* actively growing on nitrate was subjected to a pulse ammonium feed to understand if the culture would switch between nitrogen utilization pathways. This culture was given a single pulse of NH₄Cl after 12 photo-hours corresponding to a total of 0.3 gN/L of which 36% was from ammonium and the balance as nitrate provided as KNO₃. These cultures were grown in 1.5-L loop airlift photobioreactors under 5% CO₂ (v/v) in air. Both cultures grew comparably for 19 photo-hours until a biomass ratio of 10 gDW/gDW₀ as shown in

Figure 4.6: Manipulation of cellular proton flux facilitates photobioreactor pH control through nitrogen metabolism
Figure 4.7C, before an apparent decrease in cell density and photo bleaching. Two hours after the addition of ammonium, the nitrate concentration remained constant as nitrate assimilation became inhibited by the presence of ammonium as shown in Figure 4.7B. The pH dropped rapidly until leveling off at 3 as shown in Figure 4.7A. This suggested that there is sufficient expression of ammonium transporters during nitrate utilization (Franco et al., 1987) so that the incremental addition of ammonium can be used for periodic reduction of pH. The culture only sustained growth until the pH was 3.5, demonstrating that growth on 0.108 g N-NH₄⁺/L (equivalent to stoichiometrically balanced media) as a single pulse addition was inhibiting to growth. However, the ability to impose a change from nitrate to ammonium during active growth suggests that a fed-batch strategy that retains ammonium uptake capabilities could provide for pH control using alternating nitrogen assimilation stoichiometry with careful addition of nitrogen.
Figure 4.7: Inhibition of nitrate transport by ammonium addition in photoautotrophic *Chlamydomonas reinhardtii* under excess CO₂
Chapter 5 – Stoichiometrically balanced media requires fed-batch addition for pH control and sustained growth

Chlorella vulgaris and Chlamydomonas reinhardtii were incapable of selectively consuming either ammonium or nitrate to balance pH when provided a mixed nitrogen source. This inhibition by ammonium poses an operational challenge in algal photobioreactors, as algae cannot regulate its own metabolism to switch back to nitrate to avoid the ‘toxic’ drop in pH associated with ammonium assimilation. Since the pH response is closely coupled to nitrogen metabolism, it is anticipated that careful addition of nitrogen is a promising alternative to pH control given that ammonium is added in less than the stoichiometric amount in each feeding. This novel approach to pH control is advantageous both economically and with respect to cell growth as it minimizes the accumulation of inhibitory counter ion accumulation that occurs with traditional pH control through acid/base addition.

Relying on the observation that ammonium inhibits nitrate assimilation under excess carbon; all non-nitrogen components and KNO₃ can be added at the start of the experiment. NH₄NO₃ can be added as necessary to allow for periodic pH recovery by acidifying the media when nitrogen metabolism causes the pH to rise above 7. The targeted maximum pH is set at 7 because the preferred inorganic carbon sources (dissolved CO₂ and bicarbonate) for photosynthesis dominate at lower pH (Figure 1.2). To ensure sufficient inorganic carbon can be maintained within the cell to avoid a regulatory impact on nitrogen metabolism, 5% CO₂ (v/v) in air was provided to cultures. This excess CO₂ provided more buffering from the bicarbonate equilibrium, but offers the disadvantage of reduced CO₂ utilization yield. The purpose of the following
experiments was to demonstrate the utility of stoichiometric theory applied to media development for a simultaneous approach to both high-density culturing and long-term pH control.

**Experiment Results for Fed-batch Nitrogen**

Fed-batch NH₄NO₃ addition was successfully implemented *Chlorella vulgaris* in a trickle film photobioreactor grown under excess CO₂ with a 16-hr light/8-hr dark cycle. This reactor configuration was used to avoid the effect of light attenuation due to self-shading by minimizing film thickness and maximizing light penetration into the culture (Tuerk, 2011). The culture was initially grown on KNO₃ before NH₄NO₃ additions began after 6 hours. At the time of the first feeding, 0.09 g NO₃⁻/L remained in the medium and was maintained above 0.05 g NO₃⁻/L throughout the experiment until the final ammonium addition at which point all extracellular nitrogen was depleted (Data not shown). In the first addition, 10.8 mg N-NH₄⁺/L was added, which corresponded to 3.6% of the total nitrogen. Because the pH change was smaller than anticipated based on preliminary studies, subsequent ammonium nitrate additions were increased to 32.7 mg NH₄⁺/L while maintaining the pH well above 4 where growth becomes inhibited.

The pH was maintained between 7.0 and 7.8 during the light hours as shown in Figure 5.1A. Within this pH range, bicarbonate was the dominant dissolved inorganic carbon species and would be maintained well above the biological demand of the cells. Following the first four NH₄NO₃ additions, a pH drop was followed by a pH rise as ammonium was preferentially consumed over the nitrate in the culture medium. However, the pH response following the last feeding (red arrow in Figure 5.1A) did not display the characteristic recovery of pH following ammonium depletion despite nitrate
levels of 10 gNO₃⁻/L within the reactor, suggesting that other stoichiometric constraints might be encountered which affect pH control through nitrogen metabolism.

![Graph depicting pH control in photoautotrophic Chlorella vulgaris grown under excess CO₂](image)

**Figure 5.1:** Fed-batch addition of nitrogen for pH control in photoautotrophic *Chlorella vulgaris* grown under excess CO₂

It should be noted that the rise in pH that occurs during the dark culture hours resulted because the supplemental CO₂ was turned off ‘at night’. The rapid rise in pH occurred due to a shift in the inorganic carbon species to equilibrium at the reduced ambient CO₂ level (0.039%), noting that the mean gas residence time within the enclosed trickle film reactor bag enclosure was estimated at 6-min. This dark period increase in pH
illustrates the significant effect of CO\textsubscript{2} absorption and the bicarbonate buffering system on the observed culture pH that appears with elevated gaseous supplementation. Understanding the relationship between nitrogen metabolism, carbon availability, and pH dynamics will become especially important during high-density growth without supplemental CO\textsubscript{2}. As the carbon utilization yield increases, the bicarbonate buffering system is significantly reduced and is anticipated to result in greater pH fluctuations. For this experiment, the amount of inorganic carbon removed by the algal cells to meet the internal demand is far less than the total dissolved inorganic carbon present in the media which leads to significant buffering from the bicarbonate equilibrium system that masks the true proton flux from metabolism.

The accumulation of algal biomass during the 32-hr photobioreactor run during fed-batch nitrogen addition is shown in Figure 5.1B. This trickle film bioreactor run reached 5 gDW/L, which was a substantially higher density than observed in prior experiments with comparable feeding (0.3 gN/L). A possible explanation for the improved biomass yield is that maintenance of a more uniform pH during growth allowed the cells to more effectively utilize the energy available in the reduced nitrogen source. Alternatively it may be the result of the improved bioreactor design (thin film vs. air-lift photobioreactor). During the 24 hours of lighting, the culture grew at a specific growth rate of 0.088 hr\textsuperscript{-1} (doubling time = 7.88 hr). The observed exponential growth suggests that the culture had not become limited by light, CO\textsubscript{2} transport or inorganics during this period. The nitrate concentration was maintained above 0.05 gNO\textsubscript{3}\textsuperscript{-}/L until after the last addition of ammonium nitrate when nitrogen in the medium became depleted (Data not shown).
The change in proton secretion during ammonium uptake following an ammonium nitrate pulse was smaller at higher cell densities (Figure 5.1B inset). At the final density of 5 gDW/L achieved in this culture, the removal rate of CO₂ by the cells would be less than the transfer rate of carbon from gas supplementation. Therefore, the contribution of bicarbonate buffering to the total buffering capacity of the culture would likely remain constant and the apparent decrease in the value of $\phi/\Psi$ (protons/assimilated nitrogen) would be due to culture growth where higher cell concentrations increase buffering (Figure 4.5).

**Media-based pH control can be implemented during nitrogen-limited growth of Chlamydomonas reinhardtii**

Since nitrogen limitation is viewed as an important strategy to induce lipid accumulation in algae (Feng et al., 2011; Mallick et al., 2012; Sirisansaneeyakul et al., 2011), an experiment was carried out with Chlamydomonas under a N-limited feed rate. The culture was grown in a trickle film photobioreactor to avoid light-limited growth and 5% CO₂ (v/v) in air to provide excess carbon. The culture was initially grown on potassium nitrate with NH₄NO₃ additions started after 10 hours of growth. The base nitrogen level was doubled to 0.6 gN/L while retaining the overall total 36% nitrogen from ammonium and balance as nitrate. Initially, the nutrient feeding strategy maintained excess nitrogen, but was followed by growth where nitrogen became depleted between pulse additions. The dynamic pH response was measured before and after the culture reached nitrogen depletion (Figure 5.2). The pH response was the same regardless of the exogenous nitrate concentration in the reactor, displaying the characteristic decline during NH₄⁺ consumption and recovery during NO₃⁻, where the difference observed
under depleted conditions was a constant pH once exogenous nitrate was consumed. The amount of nitrogen from ammonium in each addition is given in the figure with units of mg NH$_4^+$/L.

![Figure 5.2: pH response to pulse NH$_4$NO$_3$ feed during nitrogen excess and limitation](image)

During the lighted 16-hour photoperiod of Days 1-4, the photobioreactor was maintained between 7.0 and 7.5 as shown in Figure 5.3A. These results demonstrate the utility of nitrogen feeding of a stoichiometrically balanced media as a pH control strategy while simultaneously inducing lipid accumulation and sustaining growth. Nightly pH swings were observed due to the removal of supplemental CO$_2$ at night. The pH reached approximately 9 each night (regardless of CO$_2$ supplementation), which is the pH when
bicarbonate and carbonate are present in equal amounts. The growth rate was not characterized prior to nitrogen limitation due to the small change observed in density during this phase of the experiment. Linear growth was observed at a rate of 0.082 gDW/L/hr as shown in Figure 5.3B. The biomass yield during nitrogen-limited growth was determined to be 10.3 gDW/gN corresponding to a nominal biomass composition of 10% nitrogen by weight.

Figure 5.3: pH control maintained during nitrogen limitation in photoautotrophic *Chlamydomonas reinhardtii* culture under excess CO₂
Under nitrogen-depleted conditions, the pH dropped, recovered and became constant, suggesting a nearly balanced proton secretion and uptake ($\phi / \Psi$) during the respective phases of ammonium and nitrate assimilation as illustrated by the Figure 5.3B inset. This change in pH with nearly identical magnitude but with opposite signs as ammonium and nitrate are consumed following NH$_4$NO$_3$ addition is consistent with a simplistic view of charge balance of proton flux during nitrogen ion uptake. The simplistic view of $\phi / \Psi$ may be valid when small rapid nutrient additions are made and metabolism frequently fluctuates between ammonium and nitrate metabolism. However, it must be remembered that the CO$_2$ buffering likely contributes significantly to this observation and will require more detailed study under lower CO$_2$ levels to quantify $\left( \frac{\phi}{\Psi} \right)_{NH_4^+}$ and $\left( \frac{\phi}{\Psi} \right)_{NO_3^-}$. 
Chapter 6 – Carbon limitation reveals regulatory elements affecting pH control through nitrogen metabolism

In keeping with the goal of maximizing CO₂ utilization, the next step in development of a high-density culturing strategy is to clarify the relationship between regulation of nitrogen metabolism and carbon availability. Understanding mass transfer with the reactor systems and having the ability to predict carbon limitation is important to the interpretation of air-grown culture growth and pH dynamics. A summary of the mass transfer coefficients and carbon-limited growth rates for the reactor systems used in this work is presented in Table 6.1. The maximum growth rate of *Chlamydomonas reinhardtii* during carbon limitation is provided for two gaseous CO₂ levels: ambient and 5% CO₂. The details of each reactor system are detailed in Chapter 2 and the mass transfer calculations are provided in Appendix C. From Table 6.1, the effect of mass transfer driving force (i.e. difference in concentration of CO₂ in gas phase and culture media) becomes clearer as the values given for 5% CO₂ are approximately 167-fold greater than the corresponding value for a culture grown on air-only supplementation.

<table>
<thead>
<tr>
<th>Reactor Type</th>
<th>Operating Conditions</th>
<th>kLa (1/hr)</th>
<th>CO₂TR (gCO₂/L/hr)</th>
<th>CO₂-Limited Growth Rate (gDW/L/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ambient CO₂</td>
<td>5% CO₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ambient CO₂</td>
<td>5% CO₂</td>
<td></td>
</tr>
<tr>
<td>Baffled shake flasks</td>
<td>75 mL in 500 mL flask, 130 RPM</td>
<td>48.33</td>
<td>2.17 x 10⁻²</td>
<td>3.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.23 x 10⁻²</td>
<td>2.05</td>
</tr>
<tr>
<td>Air-lift</td>
<td>VVM = 0.31, ceramic sparger</td>
<td>20.84</td>
<td>9.33 x 10⁻³</td>
<td>1.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.29 x 10⁻³</td>
<td>0.89</td>
</tr>
<tr>
<td>Trickle film</td>
<td>500 mL with 100 mL screen hold up</td>
<td>40.44</td>
<td>1.70 x 10⁻²</td>
<td>2.84</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>9.62 x 10⁻³</td>
<td>1.61</td>
</tr>
</tbody>
</table>
Under 5% CO$_2$ supplementation, the aqueous carbon concentration in baffled shake flasks and the trickle film photobioreactor remains well above the biological demand of *Chlamydomonas reinhardtii* during growth on 0.3 gN/L. In experiments discussed in Chapter 4 and Chapter 5, the observed linear growth rates are well below the carbon-limited growth rate presented in Table 6.1 and are likely a result of light limitation, which is outside the scope of this thesis. When the gaseous supplementation is reduced to air-only supplementation (0.039%), the driving force for mass transfer becomes 100-fold less and carbon limitation is anticipated to occur before the onset of light limitation in our reactor systems. Therefore, experiments with air-grown cultures first focused on understanding growth dynamics under ambient carbon supplementation to determine the effect of CO$_2$ level on growth rate and reactor productivity. The studies were then expanded to incorporate the effects of reduced buffering capacity and regulation of nitrogen metabolism on the ability to use media-based pH control with pulse addition of ammonium.

**Experimental Results and Discussion for CO$_2$-limited Culture**

**Adaptation of high-CO$_2$ grown Microalgal cultures to Air**

The intrinsic growth rate of *Chlamydomonas reinhardtii* cultures was evaluated to determine how low CO$_2$ affects growth. Cultures were grown in baffled shake flasks to determine if an acclimation phase to ambient CO$_2$ (0.039%) occurred when a 6% CO$_2$-grown culture was switched to air. This study was conducted in gyratory shake flasks where the mass transfer rate ($k_{L,a}$) could be accurately measured to verify the onset of carbon limitation as the cause for linear growth. The optical density was measured and
then converted to biomass density using a conversion factor of 0.53 gDW/L/OD\textsubscript{550} determined experimentally. The first experimental condition was grown for three days following the switch to lower CO\textsubscript{2} (air only) and then was used to start the subsequent experimental treatment at an initial density of 0.025 gDW/L in fresh medium. This procedure was repeated for a total of 8 sequential growth periods over a 24-day experiment. In this manner, growth kinetics were measured for an increasing duration of time when the algal cells were exposed to lower dissolved carbon concentrations, although the rate of CO\textsubscript{2} transport should be sufficient at all times to support growth. The growth rates of \textit{Chlamydomonas reinhardtii} for each increasing period of exposure to reduced CO\textsubscript{2} acclimation is shown in Figure 6.1. Cultures were grown on medium that contained Δ=4.5\%N-NH\textsubscript{4}\textsuperscript{+} (1/8x that of the stoichiometric growth media) to avoid dropping the pH too low under decreased bicarbonate buffering. The increase in nitrate relative to the base media was not expected to lead to an inhibitory pH.
Chlamydomonas displayed an ability to grow on air nearly independent of a period of adaptation. The observed growth rates suggested that an adaptation period would occur on the order of hours rather than days as comparable growth rates were achieved even for the initial transfers to air-grown cultures. The intrinsic growth rate of the control (6% CO₂) lines was 0.19±0.019 hr⁻¹ (doubling time = 3.66 hr) where that of the air-grown lines was 0.17±0.031 hr⁻¹ (doubling time = 4.13 hr). Performing a Student’s T test, these intrinsic growth rates were determined to be statistically the same at 95% confidence. This finding demonstrated that the growth of Chlamydomonas reinhardtii was likely independent of the elevated dissolved carbon provided by CO₂ supplementation when CO₂ mass transfer was sufficient. Intrinsic growth in air-grown Chlamydomonas reinhardtii was observed until an OD₅₅₀ of approximately 0.6 when
growth became linear at a rate of 0.0120 gDW/L/hr. The growth rate predicted during carbon limitation in the baffled shake flasks (k_l,a = 48 hr\(^{-1}\) at 75 mL culture, 120 RPM) was 0.0123 gDW/L/hr. The switch to linear growth was likely due to carbon limitation as the difference between the predicted and observed growth rates was only 4.75%. This means that carbon concentrating mechanisms in Chlamydomonas must be sufficiently expressed (or rapidly induced) to provide CO\(_2\) within the chloroplasts despite a greater than 100-fold reduction in dissolved carbon.

The same growth experiment was executed with *Chlorella vulgaris*. The results of this experiment are quite confusing but sufficiently interesting that the experiments should be repeated and investigated further. During the first three days of air exposure, the intrinsic growth rate in the air-grown Chlorella culture was 0.12 hr\(^{-1}\), which was approximately 60% of the rate observed in the culture with 5% CO\(_2\) supplementation. Following three days of growth (Day 1-3), this culture was then used to inoculated into fresh media at an initial density of 0.018 gDW/L. However, detectable growth over the next three day growth period (Day 4-6) was not observed. The adaptation experiment for growth on air was restarted after the culture was assumed to be no longer viable. Similar results were observed in this second attempt with a growth rate of 0.17 hr\(^{-1}\) (~85% of the control growth rate) during Days 1 to 3, and no growth during Days 4 to 6. This time, the experiment was not restarted, but rather the culture was spun down and these cells were inoculated into fresh media on Day 7. Again no growth was observed between Day 7 to Day 9 (Figure 6.2). The cells were spun down and used to start a new culture on Day 10, which demonstrated detectable growth at a rate of 0.12 hr\(^{-1}\) (~60% of control growth rate)! This delay in growth suggests an induction period may be necessary for the carbon
concentrating mechanisms of *Chlorella vulgaris* (see Figure 1.3). One could speculate as to the possibility of two parallel CCM; the first of which is initially expressed under high CO₂ conditions and decays following the switch to reduced aqueous carbon, followed by the induction of a second CCM that is induced only after extended exposure to low aqueous carbon concentrations. This observation is consistent with continuous high density experiments demonstrating washout in the trickle film reactor, but a subsequent recovery in growth after extended exposure (observed in our laboratory).

The doubling time of 6% CO₂-grown cultures in the baffled shake flasks was 3.56 hours \( (\mu = 0.19 \pm 0.02 \text{ hr}^{-1}) \). The optical density was measured at 550 nm and converted to biomass density using a conversion factor of 0.52 gDW/L/OD\(_{550}\). Interestingly, the cells remained viable as sufficient growth was observed in Line 4, but were not actively dividing in Lines 2 and 3. *Chlorella vulgaris* grew at its intrinsic growth rate until 0.34 gDW/L and then continued to grow linearly. The linear growth rate of *Chlorella vulgaris* cultures showed variability making it difficult to determine if mass transfer was the real cause for slowed growth. The linear growth rate was 0.034 gDW/L/hr compared to 0.012 gDW/L/hr as predicted by carbon limitation. The observed growth rate was almost 3-fold greater than the predicted value. An incorrect carbon biomass yield may have contributed to the error in these calculations as the biomass composition of Chlorella varies significantly under different growth conditions.
In all subsequent experiments relating to bicarbonate buffering and pH dynamics during carbon-limited growth, *Chlamydomonas reinhardtii* was selected as the model algae due to its ability to grow at the same rate regardless of CO$_2$ supplementation without an apparent adaptation period.

**Bicarbonate equilibrium contributes significantly to the total buffering capacity of the culture**

A batch pH experiment was executed in 1.5L airlift photobioreactors to demonstrate differences in the pH swings observed during nitrogen metabolism with varying degrees of buffering from bicarbonate equilibrium. *Chlamydomonas reinhardtii* was grown on 6%N-NH$_4^+$ (0.018 g/L N-NH$_4^+$) under 5% and 0.5% CO$_2$ (v/v) in air as this ammonium concentration did not inhibit growth previously under elevated CO$_2$. At
pH of 4, minimal total carbon absorbs into the culture medium as the bicarbonate equilibrium favors aqueous CO₂ (Figure 1.2). This leads to a small difference in the minimum pH achieved between CO₂ supplementation levels because the inclusion of ammonium in the batch growth medium results in a drop in pH where the CO₂ buffering capacity is minimal. In contrast, the largest difference between CO₂ supplementation levels was observed above pH of 7 when significantly more CO₂ can absorb into the medium, as the bicarbonate equilibrium would be shifted in favor of carbonate. The pH during nitrate consumption increased linearly; however, this does not necessarily correspond to a linear uptake of protons due to the logarithmic relationship with pH.

Figure 6.3: Bicarbonate buffering affects the observed proton imbalance during nitrogen metabolism
Linear growth at 0.0223 gDW/L/hr was observed on 0.5% CO\textsubscript{2} (v/v) as shown in Figure 6.3B. The predicted linear rate at carbon limitation was 0.095 gDW/L/hr given a mass transfer coefficient of 20.84 hr\textsuperscript{-1} (air-lift bioreactor at VVM=0.31, ceramic sparger). The details for the determination of \( k_{L,a} \) are given in Appendix C. The predicted linear growth rate was 4-fold higher than observed experimentally, which would indicate that the mass transfer in the airlift bioreactor was not measured accurately or did not represent the entire reactor system. The 5% CO\textsubscript{2}-grown culture grew at an intrinsic growth rate of 0.142 hr\textsuperscript{-1} (doubling time = 4.9 hours) for the first 20 photo-hours before growth became linear at 0.0514 gDW/L/hr, which was likely due to light limitation. Reduced affinity for aqueous carbon at high pH above 9 may also have contributed to slower growth observed during growth on 0.5% CO\textsubscript{2}.

The biological conversion factors differed between high and low CO\textsubscript{2}, which were determined experimentally to be 0.51±0.11 and 1.59±0.54 gDW/L/OD\textsubscript{550}, respectively. While OD can change significantly within cell size or differential refractive index of cells, these variances could also result from other systematic errors. A value of 0.5 for the biological conversion factor is more typical. The biomass yields on ammonium were also different between gas supplementation levels and were determined to be 4.30 gDW/gN-NH\textsubscript{4}\textsuperscript{+} for low CO\textsubscript{2} and 4.91 gDW/gN-NH\textsubscript{4}\textsuperscript{+} for high CO\textsubscript{2}. These yields were determined using experimental dry weight measurements during ammonium consumption (assuming total consumption) which was indicated by the drop in pH in Figure 6.3A. A slightly lower yield on nitrogen would be expected under carbon limitation. The observed difference is likely due to regulation of nitrogen metabolism by the carbon concentration
mechanism, which adjusts the transport rate of nitrogen into the cells to minimize energy utilization associated with over accumulation of internal nitrogen.

**Carbon availability affects regulation of nitrogen metabolism and pH response predictability**

As part of trying to assess effects of CO₂ buffering and transport, an experiment was carried out in trickle film photobioreactor with high light and CO₂ transport. The rough data is presented as it may provide an understanding of how the bicarbonate buffering affects the pH response from growth on a mixed nitrogen source.

*Chlamydomonas reinhardtii* was initially grown on 5% CO₂ (v/v) in air in a trickle film photobioreactor and then carbon limitation was forced by switching to only air supplementation. In the final mode of operation, the culture was supplied again with excess CO₂ to determine if the effects of carbon limitation on the pH dynamics and growth were reversible. Stoichiometrically balanced growth media (0.3 gN/L as 36%N-NH₄⁺ and the remaining as nitrate) was provided over the growth period with initial growth on KNO₃ followed by ten NH₄NO₃ additions. The nitrate concentration was maintained above 0.2 gNO₃⁻/L over the 70-hr growth period to avoid nitrogen-limited growth between feedings. Biomass growth presented in Figure 6.4B shows an increase in density in Phase I and Phase III, but a slight loss of culture density during Phase II. The optical density was measured at 550 nm and converted to density using a biological factor of 0.71 gDW/L/OD₅₅₀. This was determined experimentally using dry weight and optical density measurements from Phase I and Phase III growth data. The culture reached a final density of 3.4 gDW/L at 73-hr as shown in Figure 6.4B.
The growth rate and biomass yield data are given in Table 6.2. During Phase I, the doubling time was about twice that of Phase III, and likely resulted as the culture adjusted to growth in the trickle film reactor. In Phase II, the linear growth rate prior to 30-hours was used to back-calculate an overall mass transfer coefficient of 40.44 hr$^{-1}$ in the trickle film photobioreactor assuming growth was linear due only to CO$_2$ limitation and not to other nutrients (Appendix C). The lowest biomass yield was obtained during carbon limitation as the algal cells inefficiently utilized nitrogen and carbon to support growth.
Table 6.2: Growth rate and biomass yield in *Chlamydomonas reinhardtii* grown under high and low CO₂ supplementation

<table>
<thead>
<tr>
<th></th>
<th>Phase I: 5% CO₂</th>
<th>Phase II: Air</th>
<th>Phase III: 5% CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth Rate</td>
<td>( \mu = 0.0314 \text{ hr}^{-1} ) (( t_d = 22.1 \text{ hr} ))</td>
<td>0.0741 OD550/hr (0.0524 gDW/L/hr)</td>
<td>( \mu = 0.0601 \text{ hr}^{-1} ) (( t_d = 11.5 \text{ hr} ))</td>
</tr>
<tr>
<td>Yield</td>
<td>2.85 gDW/gN</td>
<td>1.12 gDW/gN</td>
<td>6.65 gDW/gN</td>
</tr>
</tbody>
</table>

During Phase I and Phase III of reactor operation, the pH was maintained below 7 and the pH response to NH₄NO₃ additions displayed the expected drop followed by rise as shown in Figure 6.4A. Backing off the gas supplementation to only air significantly reduced buffering as nitrogen additions led to larger pH swings that deviated from the characteristic fluctuation in pH. The absorption of CO₂ drives down the pH, explaining the more acidic conditions observed in Phases I and III. The change in proton concentration during ammonium assimilation is shown in the insets of Figure 6.4A for each phase. For Phase I, the change decreased by a factor of 4 as the culture density increased from 0.8 to 0.95 gDW/L and again by 4-fold in Phase III of the experiment, which is likely consistent with an increase in buffering capacity with growth. The pH dropped rapidly upon the addition of NH₄NO₃ to the culture at ~5 hours as shown in Figure 6.4A. The time scale of pH decline was too quick to correspond to growth, but the drop may be explained by the ability of the ammonium ion to readily give up a proton to form ammonia at this basic pH. The overnight pH of the culture in all phases of growth reached 9 as the bicarbonate system achieved equilibrium when the cells would no longer be fixing carbon in the dark. Bicarbonate would be the dominating inorganic carbon species at the pH range (Figure 1.1) of Phases I and III, indicating this excess bicarbonate is readily dissociated (Equation 1.5) to reach a new equilibrium between bicarbonate and...
carbonate at a pH of 9. The drops in pH following NH₄NO₃ addition and at night during Phase II cannot be as easily explained at this time.

During Phase II, growth ceased and the pH was not successfully controlled as it reached well above 9. Since the cells were no longer capable of growing during this phase, the effects of nitrogen addition on pH would no longer be a result of metabolism and cellular growth, but the direct addition of nitrate and ammonium to the medium. These results might initially suggest that the media-based pH control approach cannot be applied under carbon-limited conditions as this strategy relies on cells to be actively growing and capable of metabolizing nitrogen. However, the lack of success during implementation of the pH control strategy in Phase II is probably a result of no growth at high pH rather than carbon-limitation. As designed, the experiment makes it hard to interpret the data provided in Figure 6.4 due to the confounding effects of high pH (above 9) on growth during carbon-limitation.

Two explanations exists for the interpretation of no growth during carbon limitation with an emphasis on (1) a physiological effect to the cells and (2) the bicarbonate equilibrium. These alternative explanations justify further experimentation to verify that the lack of pH control is actually result of no growth at high pH rather than carbon-limitation. The first explanation for the cessation of growth in Phase II may be due simply to physiological inhibition of algal growth at high pH. With reference to the bicarbonate equilibrium system, all aqueous inorganic carbon would be present as carbonate, which is not anticipated to be a usable form of carbon by photosynthetic *Chlamydomonas reinhardtii*, at pH of 11. Therefore, the cessation of growth may occur as a result of the shift in the bicarbonate equilibrium away from dissolved CO₂ and
bicarbonate. Although CO₂ would continue to be absorbed into the media at this pH and may be taken up by cells before conversion to carbonate, this is hard to rationalize as a plausible explanation for sufficient growth at this pH.

Additional experiments are needed to separate the confounding effects of carbon limitation with high pH on growth dynamics before rejecting the hypothesis that media-based pH control can be achieved during carbon-limitation. This separation could be achieved by providing more ammonium to drive the pH lower prior to carbon limitation, which would alleviate both the physiological and bicarbonate equilibrium effects. In fact, a previous high-density continuous culture grown in the trickle film reactor demonstrated nitrogen uptake during minimal CO₂ feed when the pH was 9 (Grady, 2010). At this pH, bicarbonate would represent 50% of the total inorganic carbon, such that sufficient carbon would be provided by the bicarbonate equilibrium system in a usable form to support growth. Therefore, it is anticipated that the media-based pH control strategy can be utilized during carbon-limitation given that pH is maintained sufficiently low (<9) at the onset of carbon limitation.

**Carbon availability dictates ability to utilized current media-based pH strategy**

In keeping with the goal of maximizing CO₂ utilization yield in commercial-scale bioreactors, fed-batch addition of ammonium was demonstrated in an air-grown *Chlamydomonas reinhardtii* culture. This study was undertaken to not only reduce bicarbonate buffering, but also reveal any interactions between carbon and nitrogen metabolism that could jeopardize the utility of using a media-based pH control method. Carbon limitation was imposed by providing CO₂ transport at a rate, which eventually
became less than the biological demand for CO₂ (Figure 6.5A). The study was conducted in gyratory shake flasks where the mass transfer rate (kₐL) could be accurately measured. Prior to carbon limitation, the culture grew at an exponential growth rate of \( \mu = 0.17 \text{ hr}^{-1} \) (doubling time = 4.08-hr). Carbon-limited growth occurred at a rate of 0.0274 OD₅₅₀/hr (0.014 gDW/L/hr), which compared well with the predicted linear growth rate of 0.0307 OD₅₅₀/hr (0.0160 gDW/L/hr), calculated based on the CO₂ mass transfer rate (kₐL = 48 hr⁻¹, see Appendix C for calculation details). These growth patterns support the intended experimental conditions of excess carbon early in culture followed by carbon-limited growth after 29 photo-hours. The amount of nitrogen from ammonium is indicated in Figure 6.5B for each ammonium nitrate pulse.

It is worth noting in this experiment that the gradual rise of pH as the experiment progressed would result in a corresponding shift of the bicarbonate buffering towards bicarbonate (pKa = 6.4) and carbonate (pKa = 10.3) (Brown, 2008), thereby reducing the availability of the dissolved carbon that can be utilized for photosynthesis. Nonetheless, the transport of CO₂ can still occur through bicarbonate levels may not be sufficient for effective transport. More carbon-limited growth data is needed at lower pH to better understand how the pH control strategy will behave. However, this is difficult to achieve without the bicarbonate buffering system to drive down pH during CO₂ absorption into the media. The addition of more ammonium during carbon excess growth may provide enough proton excretion to drive down the pH and allow for better pH control under carbon-limited conditions. In this experiment, about 50% of the ammonium was provided prior to carbon limitation and was still not enough to drive down the pH to maintain bicarbonate equilibrium system in favor of the usable forms of inorganic carbon.
During exponential growth (0–29 hours), the pH was maintained between 6 and 8.5 with the expected pH decline and recovery following each NH$_4$NO$_3$ media addition (Figure 6.5B). The response to media addition became increasingly rapid as the culture density increased with fluctuations occurring on a scale of minutes rather than hours. In addition, the anticipated increase in buffering capacity at higher density justified increasing the NH$_4$NO$_3$ dosage at higher densities as carbon limitation was approached. The inset of Figure 6.5B shows the decline in proton secretion for growth on ammonium prior to the onset of carbon limitation, assuming no buffering capacity. After carbon limitation the pH dynamics became unpredictable and the pH ranged from 5 to 9. These
observations indicate that pH response and the success of the fed-batch nitrogen addition strategy are independent of the CO₂ supplementation as long as regulatory effects of carbon limitation are avoided and sufficient internal carbon is available to form glutamate.

A limitation of this experiment is that it would be preferred to maintained the pH below pH = 8 to maintain availability of CO₂ and HCO₃ transport since their relative roles are not clear. The unpredictable response under carbon limitation could be complicated by the fact that the pH was approaching 9. Not only could this impair carbon uptake, but an inhibition of growth would prevent nitrogen use and the associated drop in pH as ammonium is metabolized. The rapid decline in pH at 36 hours could have resulted in part from a pH-meditated growth limitation and associated accumulation at ammonium, which was finally overcome. In addition, the increased buffering capacity from elevated cells and total dissolved carbon would decrease the ability to facilitate a drop in pH. Once the pH did start to drop it would then proceed to utilize the accumulated ammonium ions. Unfortunately, the sampling of growth data in this experiment is too sparse to discern details.
Chapter 7 – Conclusions and Future Work

Growth on ammonium is energetically beneficial to the cell, but is rarely used due to the ‘toxic’ pH drop associated with prolonged growth on this nitrogen source. As a result, the selection of growth conditions have favored nitrate metabolism and elevated CO₂, which masks the pH increase from nitrate consumption and avoids the need for pH control. However, elevated CO₂ supplementation poses an economic bottleneck that must be eliminated prior to the successful commercialization of sustainable algal-based biofuels production. With the lack of bicarbonate buffering, pH control becomes an important consideration in the development of a bioreactor operation strategy for continuous high-density growth. Therefore, a scalable pH control strategy that is capable of adapting to the variable growth conditions that would be experienced in commercial outdoor reactor systems is necessary for pH maintenance within a range sustainable to growth. The most promising and novel alternative to traditional pH control methods is through the use of stoichiometrically balanced media containing a mixed nitrogen source that considers the overall degree of reduction of the biomass. The work in this thesis has made significant progress towards understanding the interactions between carbon and nitrogen metabolism for the development of an adaptive pH control strategy relying on the stoichiometry of nitrogen metabolism:

1. Algal media containing a single nitrogen source is not stoichiometrically balanced as ammonium and nitrate metabolism leads to a net efflux or influx of protons, respectively. The movement of protons compensates for the inability of a single nitrogen source to match the degree of reduction of biomass formation.
2. Using urea to represent a balanced degree of reduction and assuming comparable \( \text{H}_2\text{O/CO}_2 \) between nitrogen sources, the stoichiometric media was determined to contain 36% of the total nitrogen as ammonium with the remaining nitrogen as nitrate.

3. The amount of ammonium in stoichiometrically balanced media with \( \Delta=0.36 \) (0.108 g N-NH\(_4^+\)/L) cannot be added as a single pulse due to the preferential uptake of ammonium ions and associated pH drop, but an overall balanced pH results when ammonium is added incrementally. Excessive proton secretion or uptake that is observed on a single nitrogen source is avoided with this media, demonstrating the benefit of a balanced degree of reduction when fed-batch addition is implemented to both growth and energetic efficiency.

4. The frequency and magnitude of ammonium pulses affects both the short-term pH response and the overall pH change such that a periodic drip of ammonium nitrate into the culture medium is anticipated to provide \( \phi \approx 0 \) throughout the experiment rather than the larger fluctuations observed in this thesis.

5. The media-based pH control strategy for microalgae is insensitive to nitrogen limitation between ammonium additions. Carbon limitation appears to have a significant impact on the predictability of the pH response, although more detailed experiments that avoid elevated pH are needed to confirm. The actual cause for the lack of pH control during carbon limitation has not been determined and may be a result of a shift in bicarbonate equilibrium or a physiological impact on the cells that prevents growth at high pH. Without growth, nitrogen cannot be metabolized and the media-based pH control strategy cannot be implemented.
Therefore, the interactions of CO₂ transport, equilibrium, and kinetics become an important consideration in the implementation of this approach to pH control requiring a more complex model than the simplistic view in which only charge balance is considered.

6. The buffering capacity of Chlamydomonas cultures was shown to increase with high cell concentration, further supporting implementation of a constant slow drip of media addition to maintain $\phi \approx 0$ during growth. Therefore, the ultimate test will be to repeat prior ultra-high density continuous runs with this pH monitoring strategy.

7. The intrinsic growth rate of *Chlamydomonas reinhardtii* is independent of gaseous CO₂ supplementation provided mass transfer allows for a CO₂ transfer rate that exceeds the biological demand of the cells. It appears that the constitutive carbon concentration mechanism permits sufficient internal inorganic carbon to be maintained as long as extracellular inorganic carbon is present in the media. In contrast, the results from Chlorella cultures are complicated and require replication. *Chlorella vulgaris* displays both immediate growth as well as an intermediate acclimation period upon the switch from high to low gaseous CO₂ supplementation. If reproducible, this would indicate a different regulatory dynamic for Chlorella as compared to Chlamydomonas.

8. It is important to recognize that the pH control studied in this thesis was carried out under otherwise highly regulated conditions (light, temperature). For a real world application, it is anticipated that pH control cannot be controlled through simple addition of ammonium and requires an adaptive strategy to account for
changes in environmental conditions in addition to buffering capacity. The model will have to rely on predictable behavior such as nutrient consumption associated due to culture as well as stoichiometric changes that result from weather!

Future development of the pH control strategy will focus on quantifying the value of proton imbalance on different nitrogen sources (ϕ), which will allow further refinement of the stoichiometrically balanced media including the formation of biochemicals at a level that will alter stoichiometry. The pH-dependent buffering capacity of high-density cultures will determine the dynamics of nutrient addition, and it is quite likely that decisions on feeding will have to accommodate the dynamics of cellular biochemistry to maintain a cellular capacity for utilization of multiple forms of nitrogen. Together these results along with those presented in this thesis will allow for successful implementation of feed-forward pH control to further minimize the proton imbalance (ϕ=0) and achieve the goal of addressing the long-term photoautotrophic mass balance as part of a high-density culturing strategy.
Appendix A – Re-evaluation of the Ammann Group

Experimental Mass Balance

By our assessment, the NASA-supported work at Lockheed-Martin associated with the use of algae for life-support system represents some of the most detailed mass balances ever conducted on algae. A detailed analysis of that work is presented as it provides experimental insights on the stoichiometry of photosynthesis. The culture was grown under 2% CO₂ (v/v) in air at constant temperature and pH so that the solubility of gases would remain constant over the experiment. The gas was bubbled into the culture and kept at a constant flow rate by adjusting the pressure in the unit. The growth apparatus was run as a chemostat to maintain an ultra low constant density by adding fresh media as necessary with the overflow of culture collected in a carboy to maintain a constant culture volume. The culture was kept at low density to maintain sufficient spacing between cells to avoid self-shading and light energy gradients that can occur in higher density cultures. Oxygen evolution and CO₂ fixation were measured by the differences in the composition of the inlet and exit gas streams. The authors collected data over a 6-month period, extensively calibrated the gas analyzers, accounted for instrument drift, and changes to atmospheric pressure. The growth apparatus used in these experiments is shown in Figure A.1 (Ammann and Lynch, 1965; Ammann and Lynch, 1967).

The experimental photosynthetic quotient was the ratio between the observed changes in O₂ and CO₂ in the gas streams and determined to be 1.11 mol O₂/mol CO₂. The amount of CO₂ leaving in the liquid overflow was determined using information provided by the authors in a series of papers relating to gas exchange in the same algal culturing apparatus. The liquid flow rate was determined for photoautotrophic Chlorella pyrenoidosa with a doubling time of 25-hr (0.04 diversions per hour) in the chemostat with working volume of 700-mL (Ammann and Lynch, 1965; Ammann and Lynch, 1967).
The volumetric balance for CO\textsubscript{2} for the apparatus can be given by Equation A.1 and similar for oxygen in Equation A.2.

\begin{equation}
CO_{2\text{,inlet}} = CO_{2\text{,outlet}} + CO_{2\text{,consumed}} + CO_{2\text{,liquid}} \tag{A.1}
\end{equation}

\begin{equation}
O_{2\text{,inlet}} = O_{2\text{,unconsumed}} + O_{2\text{,produced}} + CO_{2\text{,liquid}} \tag{A.2}
\end{equation}

The average gas flow rate into the reactor is 102.5 mL/min at 2\% CO\textsubscript{2} (v/v) in air such that CO\textsubscript{2,in} = 2.05 mL/min. The amount of CO\textsubscript{2} consumed was determined to be
1.143 mL/min from Figure 1 in Ammann 1965, calculated by multiplying 0.9 mL CO₂/mL O₂ and the average oxygen evolution rate of 1.27 mL O₂/min. This means that the total gas leaving the growth apparatus and any dissolved CO₂ leaving in liquid overflow equals 0.907 mL CO₂/min.

The portion of CO₂ dissolved in the liquid can be determined for equilibrium with 2% CO₂ (v/v) in air. To do so, the liquid flow rate from the chemostat must first be determined. For this chemostat at steady state, the liquid flow rate can be determined from Equation A.2, where \( \mu = \) specific growth rate (hr\(^{-1}\)), \( x = \) culture density (g/L), \( D = \) dilution rate (hr\(^{-1}\)), \( Q_{\text{out}} = \) liquid flow rate leaving chemostat (mL/min), and \( V = \) chemostat working volume (mL). Given 0.04 diversions per hour in the growth apparatus for *Chlorella pyrenoidosa* (\( t_D = 25 \) hr per division), the liquid flow rate is determined to be 0.323 mL/min from Equations A.2 given \( \mu = 0.0277 \) 1/hr and \( V = 700 \) mL (Ammann and Lynch, 1965; Ammann and Lynch, 1967).

\[
\mu = \frac{1}{x} \left( \frac{dx}{dt} \right) = D = \frac{Q_{\text{out}}}{V} \quad \text{(A.3)}
\]

Once the liquid flow rate was determined, the amount of total carbon dissolved in the liquid assuming all species are in equilibrium at a pH of 5.91 and in excess of the biological demand for growth. This is valid as long as consumption rate is much lower than the gas liquid interface mass transfer rate. Using Henry’s Law (Equation 1.2), the equilibrium concentration of CO₂ in the culture was determined to be 6.72 \times 10^{-4} \text{ mol CO}_2/\text{L culture} \quad (\text{pH}=5.91, \text{T}=24.55^\circ\text{C}, \text{P} =1 \text{ atm}, \text{H} = 29.76 \text{ L}^*\text{atm/mol}). Using the equilibrium concentration of CO₂ and given the pH, the concentration of the other inorganic carbon species can be determined as shown below:

\[
\text{pH} = - \log[\text{H}^+] \rightarrow [\text{H}^+] = 10^{-5.91} = \frac{1.23 \times 10^{-6} \text{mol}}{\text{L}} \quad \text{(A.4)}
\]

\[
\text{CO}_2 \text{aq} + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3^* \quad \text{(A.5)}
\]

\[
k_1 = \frac{[\text{H}_2\text{CO}_3]}{[\text{CO}_2]} = 0.00159 \rightarrow [\text{H}_2\text{CO}_3] = 2.33 \times 10^{-5} \text{mol/L} \quad \text{(A.6)}
\]
\[ [H_2CO_3^+] = [H_2CO_3] + [CO_{2aq}] = 6.95 \times 10^{-4} \text{ mol L}^{-1} \quad (A.7) \]

\[ H_2CO_3^* \rightleftharpoons H^+ + HCO_3^- \quad (A.8) \]

\[ k_2 = \frac{[H^+][HCO_3^-]}{[H_2CO_3^*]} = 4.365 \times 10^{-7} \rightarrow [HCO_3^-] = 2.47 \times 10^{-4} \text{ mol L}^{-1} \quad (A.9) \]

\[ HCO_3^- \rightleftharpoons H^+ + CO_3^{2-} \quad (A.10) \]

\[ k_3 = \frac{[H^+][CO_3^{2-}]}{[HCO_3^-]} = 4.677 \times 10^{-11} \rightarrow [CO_3^{2-}] = 9.39 \times 10^{-9} \text{ mol L}^{-1} \quad (A.11) \]

The total dissolved carbon is \(9.42 \times 10^{-4}\) mol/L and can be converted to volumetric concentration using the ideal gas law to be 0.023 mL CO\(_2\)/mL culture. This concentration can then be multiplied by the liquid flow rate to determine the amount dissolved in the culture leaving in the liquid overflow to be 0.00743 mL CO\(_2\)/min, which is far less than that leaving in exit gas (0.8996 mL CO\(_2\)/min). The gas flow rate of CO\(_2\) was 1.143 mL/min determined by multiplying the photosynthetic quotient (0.9 mL CO\(_2\)/mL O\(_2\)) and the O\(_2\) evolution rate (1.27 mL O\(_2\)/min) from Figure 2 in Ammann, 1965 (Ammann and Lynch, 1965). Refer to Chapter 2 for a discussion of these results in reference to stoichiometry, particularly a comparison of the photosynthetic quotients predicted by Ammann’s experimental mass balance and stoichiometry theory for *Chlorella pyrenoidosa*. 
Appendix B – Algal Culture Media Formulation

B.1: Wayne’s Freshwater Algal Medium (1X WFAM)

<table>
<thead>
<tr>
<th></th>
<th>MW</th>
<th>[final]</th>
<th>[stock]</th>
<th>prep / L</th>
<th>/ 250 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>101.11</td>
<td>--</td>
<td>-na</td>
<td>0.60</td>
<td>0.15</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>80.04</td>
<td>--</td>
<td>-na</td>
<td>0.61</td>
<td>0.153</td>
</tr>
<tr>
<td><strong>MR26 Phosphates (50x, 1M) (pH 6.8) 1M</strong></td>
<td></td>
<td></td>
<td></td>
<td>1 mL</td>
<td>0.25 mL</td>
</tr>
<tr>
<td>K₂HPO₄ (dibasic)</td>
<td>174.18</td>
<td>0.115 g/L</td>
<td>115 g/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄ (monobasic)</td>
<td>136.09</td>
<td>0.045 g/L</td>
<td>44.9 g/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>pH to 6.8 with KOH or H₃PO₄</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WFAM MICRO nutrients (1000x)</td>
<td></td>
<td></td>
<td></td>
<td>1 mL</td>
<td>0.25 mL</td>
</tr>
<tr>
<td>H₃BO₃ (boric acid)</td>
<td>61.83</td>
<td></td>
<td></td>
<td></td>
<td>1.86</td>
</tr>
<tr>
<td>MnCl₂•4H₂O</td>
<td>197.41</td>
<td></td>
<td></td>
<td></td>
<td>0.54</td>
</tr>
<tr>
<td>ZnSO₄•7H₂O</td>
<td>287.56</td>
<td></td>
<td></td>
<td></td>
<td>0.066</td>
</tr>
<tr>
<td>ZnSO₄•H₂O</td>
<td>179</td>
<td></td>
<td></td>
<td></td>
<td>0.0411</td>
</tr>
<tr>
<td>ZnSO₄ (anhydrous)</td>
<td>161.47</td>
<td></td>
<td></td>
<td></td>
<td>0.0371</td>
</tr>
<tr>
<td>Na₂MoO₄•2H₂O</td>
<td>241.95</td>
<td></td>
<td></td>
<td></td>
<td>0.031</td>
</tr>
<tr>
<td>(NH₄)₂MoO₃•4H₂O</td>
<td>1235.86</td>
<td></td>
<td></td>
<td></td>
<td>0.0229</td>
</tr>
<tr>
<td>CoCl₂•6H₂O</td>
<td>237.93</td>
<td></td>
<td></td>
<td></td>
<td>0.030</td>
</tr>
<tr>
<td>CuSO₄•5H₂O</td>
<td>249.7</td>
<td></td>
<td></td>
<td></td>
<td>0.0075</td>
</tr>
<tr>
<td>Fe-EDTA•2H₂O (f)</td>
<td>403.1</td>
<td>0.024 g/L</td>
<td>4.0 g/L</td>
<td></td>
<td>6 mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(anhydrous)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>After autoclaving add Mg and Ca aseptically</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Magnesium Solution (1M, autoclaved/filter sterilized)</strong></td>
<td></td>
<td></td>
<td></td>
<td>1 mL</td>
<td>0.25 mL</td>
</tr>
<tr>
<td>MgSO₄•7H₂O</td>
<td>246.5</td>
<td>0.121 g/L</td>
<td>6.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO₄ (anhydrous)</td>
<td>120.0</td>
<td>0.0588 g/L</td>
<td>2.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgCl₂</td>
<td>95.21</td>
<td>0.0486 g/L</td>
<td>2.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Calcium Solution (1M, autoclaved/filter sterilized)</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.88 mL</td>
<td>0.22 mL</td>
</tr>
<tr>
<td>CaCl₂•2H₂O</td>
<td>147</td>
<td>0.132 g/L</td>
<td>7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCl₂ (anhydrous)</td>
<td>111</td>
<td>0.100 g/L</td>
<td>5.66</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* To make a concentrated form of WFAM (i.e. 4X WFAM), multiply the prep/L of solution by the concentration factor (use same stock solutions).

** Adding the Mg and Ca solutions aseptically to small batches of media from the original media can minimize precipitation in WFAM.
### B.2: Preparation Matrix for WFAM with varying Ammonium and Nitrate

**Preparation Matrix for WFAM Formulations based on 0.297 g N/L @ 1X**

This matrix gives the amounts of KN03 and NH4NO3 to weigh out for a 1L prep of varying permutations of WFAM where the basis is that a 1X overall concentration contains total of 0.297 g N/L. Permutations that result in overall POTASSIUM concentrations close to the 0.271 g K/L of WFAM are highlighted in red.

**NOTE:** the original WFAM formulation does NOT fall in this category, as it was designed to have 0.32 g N/L.

**NOTE:** using Mg for Chlorella will modify the total N concentration and the NH4 ratio as this solution contains MgNO3. This table is based on using the original Mg for Chlorella solution.

**HOW TO USE THE TABLES:** Knowing the overall desired strength (vertical axis) and desired NH4 strength (relative to the ORIGINAL WFAM - horizontal axis), the approximate amounts of each N source can be determined by the intersection of the row and column. For example, to make 2X strength 1.1x NH4, you need 3.420 g NH4NO3 and 8.538 g KN03 (shown in red).

---

#### NH4NO3 Grams per Liter Prep:

<table>
<thead>
<tr>
<th>NH4 Strengths: 0 NH4</th>
<th>0</th>
<th>1/8 NH4</th>
<th>1/6 NH4</th>
<th>1/5 NH4</th>
<th>1/4 NH4</th>
<th>1/2 NH4</th>
<th>0.5</th>
<th>0.25 X</th>
<th>0.375 X</th>
<th>0.5 X</th>
<th>1 X</th>
<th>1.5 X</th>
<th>2 X</th>
<th>5 X</th>
<th>10 X</th>
<th>15 X</th>
<th>20 X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall Strength</td>
<td>0</td>
<td>0.000</td>
<td>0.025</td>
<td>0.038</td>
<td>0.076</td>
<td>0.150</td>
<td>0.168</td>
<td>0.183</td>
<td>0.200</td>
<td>0.206</td>
<td>0.212</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25 X</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.375 X</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 X</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 X</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 X</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 X</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 X</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 X</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 X</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 X</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### KN03 Grams per Liter Prep:

<table>
<thead>
<tr>
<th>NH4 Strengths: 0 NH4</th>
<th>0</th>
<th>1/8 NH4</th>
<th>1/6 NH4</th>
<th>1/5 NH4</th>
<th>1/4 NH4</th>
<th>1/2 NH4</th>
<th>0.5</th>
<th>0.25 X</th>
<th>0.375 X</th>
<th>0.5 X</th>
<th>1 X</th>
<th>1.5 X</th>
<th>2 X</th>
<th>5 X</th>
<th>10 X</th>
<th>15 X</th>
<th>20 X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall Strength</td>
<td>0</td>
<td>0.000</td>
<td>0.025</td>
<td>0.038</td>
<td>0.076</td>
<td>0.150</td>
<td>0.168</td>
<td>0.183</td>
<td>0.200</td>
<td>0.206</td>
<td>0.212</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25 X</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.375 X</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 X</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 X</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 X</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 X</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 X</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 X</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 X</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 X</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### B.3: Wayne’s Freshwater Algal Medium for Chlorella (1X WFAMC)

<table>
<thead>
<tr>
<th></th>
<th>MW</th>
<th>[final]</th>
<th>[stock]</th>
<th>prep / L</th>
<th>/ 250 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>KNO₃</strong></td>
<td>101.11</td>
<td>--</td>
<td>-na-</td>
<td>2.2</td>
<td>0.55</td>
</tr>
<tr>
<td><strong>MR26 Phosphates (50x, 1M) (pH 6.8)</strong></td>
<td></td>
<td></td>
<td></td>
<td>1.3 mL</td>
<td>0.325 mL</td>
</tr>
<tr>
<td>K₂HPO₄ (dibasic)</td>
<td>174.18</td>
<td>0.150</td>
<td>115 g/L</td>
<td>1.3 mL</td>
<td>0.325 mL</td>
</tr>
<tr>
<td>KH₂PO₄ (monobasic)</td>
<td>136.09</td>
<td>0.059</td>
<td>44.9 g/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>pH to 6.8 with KOH or H₃PO₄</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>WFAM MICRO nutrients (1000x)</strong></td>
<td></td>
<td>g/L stock</td>
<td></td>
<td>1 mL</td>
<td>0.25 mL</td>
</tr>
<tr>
<td>H₃BO₃ (boric acid)</td>
<td>61.83</td>
<td>1.86</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MnCl₂*4H₂O</td>
<td>197.41</td>
<td>0.54</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZnSO₄*7H₂O</td>
<td>287.56</td>
<td>0.066</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZnSO₄*H₂O</td>
<td>179</td>
<td>0.0411</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZnSO₄ (anhydrous)</td>
<td>161.47</td>
<td>0.0371</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na₂MoO₄*2H₂O</td>
<td>241.95</td>
<td>0.031</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NH₄)₂MoO₄*4H₂O</td>
<td>1235.86</td>
<td>0.0229</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CoCl₂*6H₂O</td>
<td>237.93</td>
<td>0.030</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CuSO₄*5H₂O</td>
<td>249.7</td>
<td>0.0075</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe-EDTA*2H₂O <em>(f)</em></td>
<td>403.1</td>
<td>0.024</td>
<td>4.0 g/L (4 mg/mL)</td>
<td>6 mL</td>
<td>1.5 mL</td>
</tr>
</tbody>
</table>

After autoclaving add Mg & Ca solutions aseptically

<table>
<thead>
<tr>
<th></th>
<th>g / 50mL stock</th>
<th></th>
<th>1 mL</th>
<th>0.25 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Magnesium Solution (1M, filter sterilized)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg(NO₃)₂*6H₂O</td>
<td>256.41</td>
<td>0.132</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>MgSO₄*7H₂O</td>
<td>246.5</td>
<td>0.121</td>
<td>6.03</td>
<td></td>
</tr>
<tr>
<td>MgSO₄ (anhydrous)</td>
<td>120.0</td>
<td>0.0588</td>
<td>2.94</td>
<td></td>
</tr>
</tbody>
</table>

**Calcium Solution (1M, filter sterilized)**

<table>
<thead>
<tr>
<th></th>
<th>g / 50mL stock</th>
<th></th>
<th>0.088 mL</th>
<th>0.022 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂*2H₂O</td>
<td>147</td>
<td>0.0132</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>CaCl₂ (anhydrous)</td>
<td>111</td>
<td>0.0100</td>
<td>5.66</td>
<td></td>
</tr>
</tbody>
</table>
Appendix C – Prediction of CO$_2$ Limited Growth Rates

The mass transfer coefficient, $k_{La}$, for oxygen was determined using the unsteady state sulfite method (Ghaly and Kok, 1988; Materne et al., 2010). The dissolved oxygen (DO) was allowed to reach equilibrium before adding NaSO$_4$ and a cobalt catalyst to react out all DO in the reactor liquid. The oxygen concentration was measured using a Metler Toledo InLab (R) 605 probe to ensure that all DO was consumed by reaction (reaction rate $\gg$ mass transfer rate) before being reintroduced back into the culture by mass transfer (reaction rate $\ll$ mass transfer rate). Equation C.1 can be fit to a difference equation and used to solve for the mass transfer coefficient given experimental data, where $[DO_{eq}] = \text{DO concentration at equilibrium}$ and $[DO_L] = \text{DO concentration in liquid}$.

$$OTR = \frac{d[DO]}{dt} = k_{La}([DO_{eq}] - [DO_L]) \quad \text{C.1}$$

In a photoautotrophic algal culture, the mass transfer coefficient for CO$_2$ rather than oxygen is of interest. The mass transfer coefficient for DO was correlated to that of CO$_2$ using the relative diffusivities (D) as shown in Equation C.2. The liquid was anticipated to be sufficiently turbulent and well-mixed such that a negligible boundary layer would form at the gas-liquid interface. Therefore, an exponent of 0.5 was used for the ratio of diffusivities as based on Danckwert’s surface renewal theory or penetration theory (Gulliver, 2007; Perry and Green, 2008). The diffusivities of CO$_2$ and oxygen in water were used, as the culture is primarily water.

$$k_{LaCO_2} = k_{LaO_2} \sqrt{\frac{D_{CO_2}}{D_{O_2}}} \quad \text{C.2}$$

C.1: Growth Rate at Carbon limitation in Air-lift Photobioreactor

The mass transfer coefficient was calculated by fitting a difference equation (Equation C.1) using the dissolved oxygen data shown in Figure C.1.1. The mass transfer
The CO2 mass transfer coefficient was determined from Equation C.1.1 and the diffusivities for oxygen (2.41 x 10^9 m^2/s) and CO2 (2.00 x 10^9 m^2/s) in water at 25°C to be 20.84 hr^-1 (Geankoplis, 2003).

Using the mass transfer coefficient determined by Equation C.2, the growth rate at the onset of carbon limitation was calculated. The reactor and growth data needed for this calculation is shown in Table C.1.1.

**Table C.1.1: Growth and Airlift photobioreactor data**

<table>
<thead>
<tr>
<th>Volume</th>
<th>1.5 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Henry's Law Constant</td>
<td>29.41 L·atm/mol</td>
</tr>
<tr>
<td>Pressure</td>
<td>1 atm</td>
</tr>
<tr>
<td>Conversion factor</td>
<td>1.44 gDW/L/OD_{550}</td>
</tr>
<tr>
<td>Carbon yield</td>
<td>0.481 gC/gBiomass</td>
</tr>
</tbody>
</table>

The equilibrium concentration of CO2 ($C_{eq}^CO2$) as a function of the gas supplementation is determined by Henry’s Law as calculated by Equation C.1 and given.
in Table C.1.2, where $y_{CO_2} = \text{gas phase mole fraction}$, $P = \text{gas phase pressure}$, and $H = \text{Henry’s law constant}$.

$$C_{CO_2}^{eq} = \frac{y_{CO_2}P}{H} \quad \text{Equation C.1.2}$$

<table>
<thead>
<tr>
<th>$y_{CO_2}$</th>
<th>$C_{CO_2}^{eq}$ (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0003</td>
<td>4.49E-04</td>
</tr>
<tr>
<td>0.005</td>
<td>7.48E-03</td>
</tr>
<tr>
<td>0.01</td>
<td>1.50E-02</td>
</tr>
<tr>
<td>0.05</td>
<td>7.48E-02</td>
</tr>
</tbody>
</table>

When CO$_2$ is limited, the bulk liquid concentration is zero and the CO$_2$ transfer rate is at a maximum as the driving force between the gas and liquid phases is maximized. The CO$_2$ transfer rate ($CO_2TR_{max}$) is calculated by Equation C.1.3 and then converted to a growth rate using Equations C.1.4 and C.1.5 where $k_La = \text{mass transfer coefficient}$, $V_{Culture} = \text{volume of culture}$, $C_{carbon} = \text{concentration of carbon in liquid}$, $Yield_{DW/c} = \text{Biomass yield on carbon}$, $X = \text{biomass concentration}$, OD$_{550} = \text{optical density measured at 550 nm}$, and $g_{DW/L}$/$OD_{550} = \text{biological conversion factor between biomass concentration and optical density}$. The carbon-limited growth rates in the airlift photobioreactor are given in Table C.1.3.

$$CO_2TR_{max} = k_La \cdot C_{CO_2}^{eq} \cdot V_{Culture} \quad \text{Equation C.1.3}$$

$$\frac{d(Carbon \cdot V_{Culture})}{dt} = \frac{1}{Yield_{DW/c}} \frac{d(X \cdot V_{Culture})}{dt} \quad \text{Equation C.1.4}$$

$$\frac{d(OD_{550})}{dt} = \frac{1}{g_{DW/L}/OD_{550}} \frac{d(X \cdot V_{Culture})}{dt} \quad \text{Equation C.1.5}$$
Table C.1.3: Carbon-limited growth rates in airlift photobioreactor

<table>
<thead>
<tr>
<th>CO₂ Supplementation (v/v)</th>
<th>CO₂ TR max (gCO₂/hr)</th>
<th>CTR (gC/hr)</th>
<th>Growth (gDW/hr)</th>
<th>Growth (OD/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambient CO₂</td>
<td>1.40E-02</td>
<td>3.83E-03</td>
<td>7.96E-03</td>
<td>0.004</td>
</tr>
<tr>
<td>0.5% CO₂</td>
<td>2.34E-01</td>
<td>6.38E-02</td>
<td>1.33E-01</td>
<td>0.061</td>
</tr>
<tr>
<td>1% CO₂</td>
<td>4.68E-01</td>
<td>1.28E-01</td>
<td>2.65E-01</td>
<td>0.123</td>
</tr>
<tr>
<td>5% CO₂</td>
<td>2.34E+00</td>
<td>6.38E-01</td>
<td>1.33E+00</td>
<td>0.615</td>
</tr>
</tbody>
</table>

C.2: Growth Rate at Carbon limitation in Baffled Shake Flasks

The mass transfer coefficient, k_La, for oxygen was determined using the unsteady state sulfite method and was calculated by fitting a difference equation (Equation C.1) to the dissolved oxygen data shown in Figure C.2.1. The mass transfer coefficient was measured in a 500 mL baffled Bellco flask with 75 mL of culture at 25°C and on the shaker at 120 RPM.

![Figure C.2.1: DO in the baffled shake flasks](image)

The CO₂ mass transfer coefficient was determined from Equation C.1.1 using the diffusivities for oxygen (2.41x10⁹ m²/s) and CO₂ (2.00 x 10⁹ m²/s) in water at 25°C. The mass transfer coefficient for CO₂ was determined to be 48.33 hr⁻¹, which was used to
calculate the carbon-limited growth rate. The reactor and growth data needed for this calculation are shown in Table C.2.1. Using the equilibrium concentrations shown in Table C.1.2, and Equations C1.3, C1.4, and C1.5, the CO₂-limited growth rates were determined for both *Chlamydomonas reinhardtii* and *Chlorella vulgaris*. These growth rates are given in Table C.2.2. The carbon yield values presented were calculated from biomass composition.

**Table C.2.1: Data needed for mass transfer coefficient determination in baffled shake flasks**

<table>
<thead>
<tr>
<th></th>
<th><em>Chlamydomonas reinhardtii</em></th>
<th><em>Chlorella vulgaris</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>0.75L</td>
<td>0.75L</td>
</tr>
<tr>
<td>Henry's Law Constant, H</td>
<td>29.41 L·atm/mol</td>
<td>29.41 L·atm/mol</td>
</tr>
<tr>
<td>Pressure</td>
<td>1 atm</td>
<td>1 atm</td>
</tr>
<tr>
<td>gDW/L to OD_{550} Ratio</td>
<td>0.51</td>
<td>0.53</td>
</tr>
<tr>
<td>Carbon yield</td>
<td>0.48 gC/gDW</td>
<td>0.528 gC/gDW</td>
</tr>
</tbody>
</table>

**Table C.2.2: Carbon-limited growth of *C. reinhardtii* and *C. vulgaris* in baffled Bellco flasks**

<table>
<thead>
<tr>
<th>Culture</th>
<th>CO₂TR\textsubscript{max} (gCO₂/hr)</th>
<th>CTR (gC/hr)</th>
<th>Growth (gDW/hr)</th>
<th>Growth (OD/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air-grown <em>Chlamydomonas reinhardtii</em></td>
<td>1.63E-02</td>
<td>4.44E-03</td>
<td>9.22E-03</td>
<td>0.024</td>
</tr>
<tr>
<td>Air-grown <em>Chlorella vulgaris</em></td>
<td>1.63E-02</td>
<td>4.44E-03</td>
<td>8.40E-03</td>
<td>0.021</td>
</tr>
</tbody>
</table>

**C.3: Growth Rate at Carbon limitation in the Trickle Film Photobioreactor**

The mass transfer coefficient was back calculated using data obtained from carbon-limited linear growth on air in a *Chlamydomonas reinhardtii* culture as shown in Figure C.3.1. The growth rate was determined to be 7.41 x 10\(^{-2}\) OD\(_{550}\)/hr (4.81 x 10\(^{-3}\) gDW/hr) and translated to a CO₂ transfer rate of 8.49 x 10\(^{-3}\) gCO₂/hr.
Figure C.3.1: Linear growth data used to determine the trickle film reactor mass transfer coefficient

The mass transfer coefficient was determined using Equation C.3.1 and the data given in Table C.3.1 to be 40.44 hr\(^{-1}\). This value represents the overall coefficient for the reactor as the ratio of the screen holdup volume to the total culture volume was incorporated into these calculations. The local mass transfer coefficient on the screen was 206.7 hr\(^{-1}\), which is about 5-fold higher than the overall mass transfer coefficient for the reactor system.

**Table C.3.1: Trickle Film Reactor Data**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Culture Volume</td>
<td>467.5 mL</td>
</tr>
<tr>
<td>Screen Holdup</td>
<td>91.5 mL</td>
</tr>
<tr>
<td>Henry’s Law Constant, H</td>
<td>29.41 L*atm/mol</td>
</tr>
<tr>
<td>Pressure</td>
<td>1 atm</td>
</tr>
</tbody>
</table>

\[
k_L a = \frac{CO_2 TR}{C_L^{eq} \cdot V_{Culture}}\]

Equation C.3.1
Appendix D – Nuclear Transformation of *Chlamydomonas reinhardtii* for Isoprene Metabolism

D.1: Development of Selectable Marker Cassette

An objective of the NSF-sponsored grant on algal biofuels was to genetically engineer isoprene production in the model host *Chlamydomonas reinhardtii*. Although not successful, considerable effort and progress are documented here.

**Introduction**

Nuclear transformation of *Chlamydomonas reinhardtii* cc-503 was carried out using the patchouli synthase and farnesyl synthase gene construct for plastidic targeting developed by Wu as this led to the highest accumulation of patchouli in tobacco plants (Wu et al., 2006). The resulting transformants were limited in number and unstable as the Wu vector was designed for tobacco plants and not for use in algae. Additionally, a large number of untransformed hygromycin resistant colonies were present, which questioned whether the putative transformants had actually integrated the foreign genes. *Chlamydomonas* appears to have a transgene suppression mechanism, which prevents or limits transformants from expressing foreign genes to high levels. A vector designed for *Chlamydomonas* was used that had similar codon usage to facilitate expression.

The goal of the current transformation was to compare cell-wall mutant strains of *Chlamydomonas reinhardtii*, different antibiotic resistance genes as selectable markers, and the associated promoter to determine the conditions that produce the most stable transformants, maximize the transformation efficiency, and minimize untransformed resistant colonies. The results of these transformations will guide the selection of the selectable marker and promoter to be used in the next generation vector that will contain the FPS gene and transit peptide for manipulation of isoprene metabolism. The following cell-wall mutant *Chlamydomonas reinhardtii* strains were tested: cc-503, UVM-4, and UVM-11. UVM-4 and UVM-11, which are independent cell wall deficient strains generated by UV mutagenesis, were shown to overcome the regulation mechanism to
express genes to much higher levels than other cell wall deficient Chlamydomonas strains (Neupert et al., 2009).

A better selectable marker was necessary for more robust screening of putative transformants as hygromycin resulted in a large number of untransformed resistant colonies. The use of the paromomycin resistance gene, aphVIII is promising as a selectable marker because of the similar G+C content and codon bias to indigenous Chlamydomonas genes (Sizova et al., 1996). In the literature, paromomycin resistance resulted in fewer untransformed resistant mutants than other antibiotics (Hallmann and Wodniok, 2006; Sizova and Fuhrmann, 2001). For the best comparison between hygromycin and paromomycin resistance as selectable markers, these genes were directed by the same promoter, β2-tubulin. An additional vector containing paromomycin resistance directed by the chimeric HSP70A/RBCS2 promoter was also included in the comparison. These vectors are shown in Table D.1.1. The optimal selection pressure used for hygromycin resistance was determined from previous transformations to be 15 mg/L (Data not shown). The initial paromomycin resistance was based on the minimum inhibitory concentration (MIC) found in a literature search. The MIC of paromomycin determined for untransformed Chlamydomonas CC-849 (cell wall defective strain) and CC-125 (wild type) was 5 mg/L (Hallmann and Wodniok, 2006). Two to four times this concentration was used as the selection pressure.

Table D.1.1: Vectors used in Chlamydomonas transformation

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Resistance Gene Promoter</th>
<th>Resistance gene</th>
<th>Plasmid Size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKS-48&quot;-lox</td>
<td>β2-tubulin</td>
<td>hygromycin, aph48&quot;</td>
<td>4566 bp</td>
<td>Chlamy.org</td>
</tr>
<tr>
<td>pKS-aphVIII-lox</td>
<td>β2-tubulin</td>
<td>paromomycin, aphVIII</td>
<td>4308 bp</td>
<td>Chlamy.org</td>
</tr>
<tr>
<td>pSI103</td>
<td>chimeric HSP70A/RBCS2</td>
<td>paromomycin, aphVIII</td>
<td>4982 bp</td>
<td>Chlamy.org</td>
</tr>
<tr>
<td>tpPTS+tpFPS</td>
<td>P cav</td>
<td>hygromycin, CAMBIA</td>
<td>--</td>
<td>Wu 2006</td>
</tr>
</tbody>
</table>
Materials and Methods

Cultures

Axenic flasks of *Chlamydomonas reinhardtii* CC-503, UVM-4 and UVM-11 were subcultured into TAP at 2% (v/v) and grown for 24 hours. These flasks were then subcultured at 2% (v/v) into fresh TAP and grown for an additional 24 hours prior to transformation on the third day.

Algal Transformation by Bead Beating

Nuclear transformation of *Chlamydomonas reinhardtii* was performed by bead beating with glass beads (Kindle, 1990). Contamination tests were performed prior to transformation to verify that cultures were axenic. A 50-mL TAP culture was grown to 1-2 x 10^6 cells/mL for each treatment (OD_{550} = 0.1 to 0.2). Cells were harvested by centrifugation (2000xg for 5 min at 24°C) and re-suspended in 300-µL TAP. Harvested cells were then combined with 300 mg glass beads (Jencons Scientific, 0.45-0.5 mm diameter, neutralized in sulfuric acid and rinsed in distilled water), polyethylene glycol 6000 (20% v/v) and 10 µg of isolated DNA in an Eppendorf tube. The mixture was vortexed for 20 sec and then the liquid was transformed into 50-mL of fresh TAP and incubated under continuous light for 18 hours. Following recovery, cells were harvested by centrifugation (2000xg for 5 min at 24°C) in 400-µL TAP and then distributed onto TAP plates supplemented with antibiotics at the desired selection pressure. A control culture was subjected to the same transformation procedure without the addition of foreign DNA. The control culture was plated on TAP as a positive control to verify cell viability as well as TAP with selective agent to characterize the amount of untransformed resistant background. Transformants and control plates were grown on continuous light for 1 week prior to cell counts.
Results and Discussion

The transformation results are given in Table D.1.2. The number presented for Week 0 is from 7 days of growth immediately following transformation. These plates were subcultured and grown for 1 week from which a second count was performed (Week 1). Two counts were done to minimize mistaking non-viable green algal colonies as viable. These counts were done to determine the actual number of colonies that were transformed with foreign DNA and antibiotic resistant (Selection+) relative to the number of untransformed resistant colonies (URC). A Selection+/URC ratio of infinity indicates that there were no surviving URC. The Selection+/URC ratio was maximized for paromomycin resistance at 20 mg/L as fewer URC could grow and were consistent with the literature, which indicated that paromomycin was a better selectable marker. Additionally, a higher ratio was obtained with the UVM strains over cc-503. The number of transformants obtained when the chimeric HSP70A/RBSC2 promoter directed the paromomycin resistance gene was greater than the β2-tubulin promoter (Data not shown).

Table D.1.2: Comparison of different host strains and antibiotic resistance genes as selectable marker

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Strain</th>
<th>Selection</th>
<th>Primer</th>
<th>Selection+/URC</th>
<th>Selection+/URC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Week 0</td>
<td>Week 1</td>
</tr>
<tr>
<td>1</td>
<td>cc-503</td>
<td>10 mg/L Par.</td>
<td>HSP70A/RBSC2</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>UVM-4</td>
<td>15 mg/L Hyg.</td>
<td>β2-tubulin</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>UVM-4</td>
<td>20 mg/L Par.</td>
<td>β2-tubulin</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>UVM-4</td>
<td>20 mg/L Par.</td>
<td>HSP70A/RBSC2</td>
<td>8</td>
<td>∞</td>
</tr>
<tr>
<td>5</td>
<td>UVM-11</td>
<td>15 mg/L Hyg.</td>
<td>Pcv</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>UVM-11</td>
<td>15 mg/L Hyg.</td>
<td>β2-tubulin</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>UVM-11</td>
<td>10 mg/L Par.</td>
<td>HSP70A/RBSC2</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>UVM-11</td>
<td>20 mg/L Par.</td>
<td>HSP70A/RBSC2</td>
<td>10</td>
<td>∞</td>
</tr>
<tr>
<td>9</td>
<td>UVM-11</td>
<td>20 mg/L Par.</td>
<td>β2-tubulin</td>
<td>2</td>
<td>∞</td>
</tr>
</tbody>
</table>

The number of surviving colonies was counted each week to evaluate the stability of the transformants and normalized by the initial number of colonies (N) obtained as shown in Figure D.1. By Week 6, all untransformed resistant colonies had died off, which
suggested that these were not true mutants. The Wu transformants were the least stable and died off by Week 4 even in the UVM-11 host. Transformation of UVM-11 with the paromomycin resistant gene, *aphVIII* directed by the chimeric HSP70A/RBSC2 resulted in the most stable transformants. Based on the stability data, the number of resulting transformants, and the Selection+/URC ratio, this was the best case for transformation at a selection pressure of 20 mg/L (Treatment 8 in Table D.2). Transformation with the β2-tubulin promoter was also stable, but resulted in a lower number of transformants.

Expression of the antibiotic resistance gene was expected to be proportional to the selection pressure at which putative transformants could survive. The results of the
physiological test are shown in Figure D.1.2. UVM-4 and UVM-11 transformants were more stable at 60 mg/L paromomycin than cc-503. Additionally, the β2-tubulin promoter resulted in more stable transformants than the chimeric promoter for direction of the antibiotic resistance gene.

![Graph of %Survival over time for paromomycin and hygromycin](image)

**Figure D.1.2: Physiological test to evaluate selectable marker expression**

**Conclusions and Future Work**

Paromomycin resulted in less untransformed resistant background and gave the most stable transformants when the resistance gene was directed by the chimeric HSP70A/RBSC2 promoter, indicating it was a better selectable marker than hygromycin. Using 20-mg/L paromomycin as the selection pressure further minimized the untransformed resistant background in comparison to 10-mg/L paromomycin. The UV mutagens resulted in comparable transformation efficiencies (number of transformants normalized by initial culture density); UVM-11 was selected for the next generation.
transformation work. When the paromomycin concentration was increased to 2-3x the initial selection pressure, the β2-tubulin promoter resulted in fewer, but more stable transformants than the chimeric promoter. Similarly, hygromycin resistant colonies where the marker was under control of the β2-tubulin promoter were stable but represented by a low number of transformants. The β2-tubulin promoter was better in terms of stability once the cassette integrated into the genome, but the chimeric promoter led to integration in more cells. The efficiency and stability of transformation for vectors containing the paromomycin resistance gene (pSI103 vs. pKS-aphVIII-lox) could potentially be affected by the different backbones during bead beating.

In the isoprene metabolism vectors designed for use with Chlamydomonas, paromomycin resistance gene, *aphVIII*, will be used as the selectable marker. The chimeric promoter is expected to be a better promoter based on literature and confirmed in these transformation results. This promoter will be used to control the isoprene genes while the β2-tubulin promoter will be used to direct the selectable marker.

**D.2: Transformants for Farnesyl pyrophosphate synthesis**

**Introduction**

The ultimate goal of the transformation work is to engineer Chlamydomonas for high-level expression of terpenes through manipulation of the MVA (cytoplasm) and MEP (chloroplast) pathways. Previous algal transformations with the tobacco vector designed by Wu yielded little success, emphasizing the need for a vector containing Chlamydomonas-specific terpene genes. In the final transformants that will successfully alter the carbon flux for high-level terpene expression, multiple genes will be required. These genes include farnesyl pyrophosphate synthase (FPS), squalene synthase (SS), botryococcene synthase (BBS), and methyl transferases. Farnesyl pyrophosphate synthase is the first terpene gene to be incorporated in the vector used for Chlamydomonas transformation as the first step in manipulation of the terpene pathways. Chlamydomonas is known to limit expression of foreign genes, rationalizing the design of a synthetic FPS gene from the avian (chicken Gallus gallus) FPS protein
sequence for use in Chlamydomonas to overcome this regulation mechanism. The chimeric HSP70A/RBSC2 promoter directed the codon-optimized farnesyl pyrophosphate synthase, acFPS1.

The vectors containing the Chlamydomonas FPS cassette were denoted as pHRC-tpacFPS1-aphVIII (5564 bp) and pHRC-acFPS1-aphVIII (5423 bp), corresponding to constant with and without plastidic targeting, respectively. The vector map for plastidic targeting of the FPS gene is shown in Figure D.2.1. The pHRC vector obtained from The Chlamydomonas Resource Center was selected as the base vector for cloning because of its small backbone size. To direct expression of acFPS1 to the plastidic pathway, a transit peptide sequence is added (tpacFPS1). To avoid targeting FPP synthase to the chloroplast, the second vector did not contain the transit peptide sequence (Vector map not shown). Both vectors contained the paromomycin resistance gene, aphVIII, directed by the β2-tubulin promoter as the selectable marker. UVM-11 was selected as the host strain for transformation and 20-mg/L paromomycin was used as the selection pressure.

![Figure D.2.1: pHRC-tpacFPS1-AphVIII Vector map](image-url)
Results and Discussion

The results of transformation are shown in Table D.2.1. For all treatments, no untransformed resistant colonies were present. More putative transformants were obtained for cytosolic targeting (without transit peptide) than with targeting of gene expression to the chloroplast (transit peptide). Twenty-five transformants were selected at random from each treatment and maintained on 20-mg/L paromomycin for 30 weeks of which 100% survived. A physiological test was performed to understand how the selectable marker was expressed in these transformants and showed that all transformants survived at 60-m/L paromomycin for 17 weeks. Additionally, transformants were removed from selection for 10 weeks and then switched back to 20-mg/L paromomycin of which all colonies survived. The results of these three tests indicate that transformants were very stable with respect to integration of the selectable marker.

Table D.2.1: Comparison of cytosolic and plastidic targeting of FPP production in paromomycin resistant transformants

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Selectable Marker</th>
<th>Isoprene Metabolism</th>
<th>Transformants</th>
<th>Selection+/URC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Primer: β2-tubulin</td>
<td>Gene: aphVIII 20 mg/L</td>
<td>HSP70A/RBCS2</td>
<td>tPacFPS1</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>783</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>594</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td>1302</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td>1462</td>
</tr>
</tbody>
</table>

Putative transformants were screened by PCR to verify that the paromomycin resistance and farnesyl pyrophosphate synthase genes had been integrated into the genome of *Chlamydomonas reinhardtii*. The primer design is shown in Table D.2.2 and D.2.3. Primers for the FPS gene amplified the entire cassette from the chimeric promoter to the Rbcs2 terminator. Therefore, the amplicon length was larger by 141 bp when the transit peptide was present. Eight UVM-11 transformants with cytosolic targeting of FPS were screened of which seven were positive for the entire cassette. Nine UVM-11 transformants with plastidic targeting of FPS were screened for the cassette with eight putative transformants screening positive. The screening results are shown in Figure...
D.2.2. The PCR protocol is shown in Table D.2.3 and New England Biolabs Phusion High-fidelity DNA polymerase was used.

### Table D.2.2: Gene specific primers for FPS screening

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Tm (°C)</th>
<th>Amplicon Length (bp)</th>
<th>Dimers (kcal/mol)</th>
<th>Hairpins (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>GCTTGACATGATTGGTGCGT</td>
<td>53.3</td>
<td>1986</td>
<td>-0.4</td>
<td>none</td>
</tr>
<tr>
<td>Reverse</td>
<td>CGGAGGATCGTTACAACCAA</td>
<td>52.5</td>
<td>(2127)</td>
<td>1.7, 0.4, 1.7</td>
<td>none</td>
</tr>
</tbody>
</table>

Figure D.2.2: Screening results for FPS cassette in putative UVM-11 transformants

### Table D.2.3: PCR Protocol for FPS screening

1. Hot Start at 98°C, add samples after warm-up
2. 98°C 3 min (initial denaturation)
3. 98°C 10 sec (denaturation)
4. 63°C 20 sec (annealing)
5. 72°C 1 min, go to 2 35x (extension)
6. 72°C 5 min (final extension)
7. 10°C
8. 98°C 3 min

Conclusions and Future Work

Paromomycin resistance directed by the β2-tubulin promoter resulted in a high number of transformants that had stably integrated the selectable marker into the genome.
Additionally the majority of the antibiotic resistance transformants that were screened by PCR contained the FPS gene. These results suggest that the vector was not linearized at a restriction enzyme site in either the selectable marker or isoprene gene cassettes and were integrated as single fragments into the genome. Western blots and chemical analysis are necessary to confirm expression of FPS and synthesis of FPP to demonstrate that isoprene metabolism has been successfully been manipulated.
REFERENCES


