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Effects of Magnesium Sulfate, Digestate and Other Inorganic Nutrients on the Phototrophic Growth of the Green Microalga Scenedesmus Dimorphus

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EFFECTS OF MAGNESIUM SULFATE, DIGESTATE, AND OTHER INORGANIC NUTRIENTS ON THE PHOTOTROPHIC GROWTH OF THE GREEN MICROALGA

Scenedesmus dimorphus

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ABSTRACT

The individual components of the growth media, notably magnesium, have a profound impact on the growth and lipid production of the green microalgae, *Scenedesmus dimorphus*. The goal of this work was to investigate these effects and explain the causes via multiple experiments. It is important to understand the aspects of algal growth because they have a significant impact on the commercial viability of microalgal lipids as a potential feedstock for biofuel production. Digestate, derived from bovine waste and a potential cost effective nutrient replacement for conventional media types, was found to maximize growth and lipid concentration in *S. dimorphus* at 1.25% and 1.75% in water respectively and that increasing Mg concentration increased both growth and maximum cell density. The second experiment derived the Monod parameters $k_s$ (33 ± 7 mg/L) and $\mu_{\text{max}}$ (0.59 ± 0.04 days$^{-1}$) for magnesium limited growth. The third experiment utilized ICP spectral analysis to monitor nutrient consumption over time allowing for the calculation of biomass yields for Mg (476 ± 132), Ca (247 ± 57.8), Fe (8550 ± 824), Mn (5310 ± 1450) all in g/g.
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CHAPTER I

INTRODUCTION

The individual components of the growth media, notably magnesium, can have a profound impact on the growth and lipid production of the green microalga, *Scenedesmus dimorphus*. The goal of this work is to investigate and quantify these effects via experiments which either modify the initial composition of the growth medium or monitor the changes in the composition of the growth medium over time. It is important to understand these aspects of algal growth because they have a profound effect on the commercial viability of microalgal lipids as a potential feedstock for biofuel production.

1.1 Brief review of the history and taxonomy of *Scenedesmus dimorphus*

The genus *Scenedesmus* was first described in 1828 and only studied by light microscopy for over a century. *Scenedesmus* belongs to the family scenedesmoïdæ.
*Scenedemsus* is defined by coenobia of 2-32 cells in 1 or 2 rows and has characteristic, elongate cells (Hegewald, 1997).

*Dimorphus* falls under the genus, *Scenedemsus*, and the subgenus *Scenedemsus*. *S. dimorphus* is a green unicellular microalgae. *S. dimorphus* varies from many of other species of *Scenedemsus* in that it is spineless (Trainor & Burg, 1965). Figure 1, compares the spineless *dimorphus* with its spined relatives.

![Figure 1. From left to right, S. dimorphus showing no spines (Shen, et al. 2009), S. quadricauda with long spines and S. abundans with short spines (ProtistImages, 2011)](image)

There have been many changes to algal taxonomy over the years and *Scenedemsus* is no exception to this fact. Much research has been performed in the realm of microalgae and it is important to understand which specific species of algae is being investigated. *S. dimorphus* is not the only spineless *Scenedemsus* species. *S. obliquus* is also spineless, as is *S. acutus* (Marcenko, 1973). Are these different species of *Scenedemsus* altogether? The answer is, in short, no, they are not. They are taxonomically indifferent to *S. dimorphus*.

*Scenedemsus acutus* and *obliquus* are homotypic synonyms to *S. dimorphus* (AlgaeBase, 2011). A homotypic synonym is a nomenclatural synonym. These usually arise when a species has been moved from one genus to another or when the name is simply incorrect (Cactus, 2011). Apart from *acutus* and *obliquus*, there are at least two
other species of *Scenedensus* that are taxonomical synonyms to *dimorphus*. They are *S. rubescens* and *obtusus*. *Scenedensus rubescens* was commonly thought to be a *Chlorella* until DNA evidence said otherwise (Hegewald, 1997).

To conclude, *Scenedensus dimorphus* is no different than *S. acutus, obliquus, obtusus*, or *rubescens*. Some or all of these species will be referred to in the text, or used in works referenced in the text. It is important to remember that they are all taxonomically similar.

1.2 Microalgae and its many commercial applications

Microalgal culturing is now a commercially established industry and, while most culturing systems are relatively rudimentary, advances over the past half a century have led to the development of technologies such as closed photobioreactor systems which support many high-value products (Borowitzka, 1999). Open ponds are useful for lower value products such as biodiesel, but more stringent control methods are often necessary for higher value bio-pharmaceuticals.

Microalgae have a variety of commercial applications. Apart from biodiesel, algae can be grown to produce pigments, antioxidants, dyes, sugars (Mata, Martins, & Caetano, 2010), soil conditioners, food additives (Pulz & Gross, 2004), polyunsaturated fatty acids (PUFAs), and can even be incorporated in cosmetics (Spolaore, Joannis-Cassan, & Duran, 2006). Microalgae is produced as a food additive for animal feeds as well as a feed stock for aquacultural purposes (Spolaore, Joannis-Cassan, & Duran, 2006). *Scenedensus* and *Chlorella sp.* are widely used as a feed source for growing zooplankton in research scenarios. While *S. acutus* has not been known to develop any defense mechanism against grazing by zooplankton, *S. quadricauda* can (Flores-Burgos,
The oblong shape of *S. dimorphus* can make it unsuitable as a feed source for some herbivores when compared to other, more spherical, alga such as *Chlorella* (Renaud, Parry, & Thinh, 1994). Some species of algae even have inherent resistances to herbicides (Chalifour & Juneau, 2011).

Microalgae are widely used for development and risk assessment for environmental regulations concerning heavy metals because they are such sensitive indicators to environmental changes (Desouky, 2011). Related to this, algae are commonly used to remove heavy metals from wastewater or in other similar applications. *S. obliquus*, while it can remove copper and cadmium from wastewater environments, has stunted growth in the presence of heavy metals (Drbal, Veber, & Zahradnik, 1985).

As a health food for humans, algae is fairly well known. In fact, the first recorded use of microalgae was the use of Nostoc, a genus of filamentous cyanobacteria, as a foodstuff by the Chinese more than 2000 years ago (Spolaore, Joannis-Cassan, & Duran, 2006). It is difficult to enter a health foods store or a smoothie shop anymore and not see some species of algae, usually spirulina, being sold. Algal biomass as a healthfood has surged in recent decades. Production of spirulina was less than 100 tons in 1975. By 1999, spirulina production had surged to over 3500 tons annually (Pulz & Gross, 2004). This does not even consider the recent social pressures of ‘going green’ over the last decade either.

Algae has enormous biological and physiological potential as well. Research into pharmaceuticals from algae has led to advances towards treatments for brain development disorders and carpal tunnel syndrome. Anti-inflammatory and anti-HIV
drugs, as well immunoboosters and antifungal drugs have been considered as well (Pulz & Gross, 2004).

Beyond all of these, the most commercially profound of all of algae’s applications is that of biofuels.

1.3 Microalgae as a biofuel

Microalgae are viable as biodiesel feedstocks as well as for methane, hydrogen, and ethanol production. Microalgae are able to be grown in harsh environmental conditions which make them ideal as a potential fuel source (Mata, Martins, & Caetano, 2010). There are many important aspects of algal culturing for use as a biofuel. The most important of these is photosynthetic efficiency, biomass accumulation and growth rate, oil yield, and temperature and light tolerance (Csavina, Stuart, Riefler, & Vis, 2011). The combination of these, and other parameters, make algae ideal for this type of application as compared to other feedstocks for biofuel production. A study by Chisti (2007) found that to accommodate all the energy needs of the U.S. for one year, 61% of the area of the continental U.S. would need to be devoted to bioethanol from sugarcane. Conversely, if algae were used to produce biodiesel, only 3% of the area of the U.S. would need to be used. While 3% is still a large amount, it proves the viability of algal oil vs. other feedstocks such as sugar cane.

Growth. Algae grow fast. Some species of microalgae can double as many as 8 times daily (Csavina, Stuart, Riefler, & Vis, 2011) It is important that algae grow fast because, the faster they grow, the more lipid or other products can be harvested that much more often. Algal growth rates in phytoplankton vary greatly. It is commonly thought that growing faster is better and, in many cases, such as biofuel production, this is true.
But, in some cases, slow growing algae can have competitive advantages over faster growers when it comes to nutrient holding and resisting cell death (Flynn, 2009).

**Lipid yield.** Per unit mass, algae have one of the highest lipid contents of earth’s vast array of plantlife. Lipid percents per cell go hand in hand with fast growth. Under the right conditions, algae have been known to accumulate up to 85% lipid per dry weight (dwt) cells (Huang, Chen, Wei, Zhang, & Chen, 2010).

<table>
<thead>
<tr>
<th>Crop</th>
<th>Oil yield (L/ha)</th>
<th>Land area needed (M ha)</th>
<th>Percent of existing US cropping area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>172</td>
<td>1540</td>
<td>846</td>
</tr>
<tr>
<td>Soybean</td>
<td>446</td>
<td>594</td>
<td>326</td>
</tr>
<tr>
<td>Canola</td>
<td>1190</td>
<td>223</td>
<td>122</td>
</tr>
<tr>
<td>Jatropha</td>
<td>1892</td>
<td>140</td>
<td>77</td>
</tr>
<tr>
<td>Coconut</td>
<td>2689</td>
<td>99</td>
<td>54</td>
</tr>
<tr>
<td>Oil palm</td>
<td>5950</td>
<td>45</td>
<td>24</td>
</tr>
<tr>
<td>Microalgae</td>
<td>136,900</td>
<td>2</td>
<td>1.1</td>
</tr>
<tr>
<td>Microalgae</td>
<td>58,700</td>
<td>4.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

*For meeting 50% of all transport fuel needs of the United States.*

*70% oil (by wt) in biomass.*

*30% oil (by wt) in biomass.*

There are a few disadvantages to the use of microalgae as feedstock for biofuel production. One of the main disadvantages is the fact that most algal oils have lower fuel values than their petrol diesel counterparts. This seems to be a small price to pay for a renewable fuel source. Secondly, costs of microalgal cultivation and processing are quite high (Huang, Chen, Wei, Zhang, & Chen, 2010). There are many expensive elements of cultivation and processing that need to be optimized in order to reach a profitable process.
1.4 *Scenedesmus dimorphus* as a favorable microalgae for biofuel production

Of all of the species of microalgae, does *Scenedesmus dimorphus* hold up as a possible candidate for use in biofuels or any other industry? Maybe. While *S. dimorphus* does not showcase very high growth rates or very high lipid yields, these properties are almost always inversely proportional. A fast growing and high lipid accumulating microalgae is the holy grail for many algal biofuels companies. So, just because *S. dimorphus* does not grow particularly fast or accumulate significant lipid does not automatically disqualify it as a potential species for investigation. In fact, there are countless species of algae that do not grow nearly as fast or do not accumulate lipid at all.

One can focus on the strengths of the species.

- *S. rubescens* has been found to be a promising biofuel candidate because of its favorable fatty acid profile (Lin & Lin, 2011)
- *S. obliquus* has been found to be a viable grower in waste discharges with the intent of biofuel production (Mandal & Mallick, Waste utilization and biodiesel production by the green microalga Scenedesmus obliquus, 2011) (Park, Lim, & Lee, 2009)
- *S. obliquus* can accumulate up to 58% lipid by nutrient manipulation alone (Mandal & Mallick, Microalga Scenedesmus obliquus as a potential source for biodiesel production, 2009)
- *S. obliquus*, on a 20 day average, sustained growth rates of 1.24 days\(^{-1}\) with lipid content approaching 30% (Abou-Shanab, Matter, Kim, Oh, Choi, & Jeon, 2011)
• *Scenedemus sp.* is ubiquitous in freshwater environments. It is a dominant species for growth in piggery effluents, or other scenarios rich in both organic and inorganic nitrogen, phosphorus, and carbon sources. The genus excels due to its ability to grow and utilize N and P both phototrophically/mixotrophically and heterotrophically (Combres, Laliberte, Reyssac, & Noue, 1994)

• *Scenedemus sp.* and *S. obliquus* have been found to grow as well immobilized to certain substrates as they do freely (Fierro, Sanchez-Saavedra, & Copalcua, 2008)

• *S. obliquus* has been found to be an efficient algal strain for CO₂ removal with removal rates up to 390 mg/L/day (Ho, Chen, Yeh, & Chen, 2010)

• *S. obliquus* is a good source of vitamins as well as protein and carbohydrates with protein content reaching 51-58%, carbohydrate content at 12-17%, and lipid content at 14-22% (Spolaore, Joannis-Cassan, & Duran, 2006)

While *S. dimorphus* may not be the optimal species for all commercial aspects of algal biofuels, what species/strain is? Past research validates it as a competent species with which to investigate the important parameters associated with microalgal cultivation for use as a biofuel.

### 1.5 Importance of temperature on algal growth

Optimal growth temperatures vary between species, but generally range from 28-35°C (Park, Craggs, & Shilton, 2011). 30°C has been found to be an optimal temperature for growth of *S. obliquus* (Martinez, Jimenez, & El Yousfi, 1999). Low temperatures not
only stunt reproductive growth in *S. obliquus*, they also cause a decrease in overall cell size (Chen, Li, Dai, Sun, & Chen, 2011). Xin et al. (2011) found the ideal temperature range for *Scenedesmus sp.* growth to be between 25 and 30 °C. Additionally, reducing temperature to 20 °C as an environmental stress proved optimal for lipid production (Xin, Hong-ying, Ke, & Jia, 2010).a.

1.6 Importance of CO₂ on algal growth

The addition of CO₂ not only has a positive impact on algal growth as an essential nutrient, but also serves to mitigate pH inhibition in wastewater conditions (Park, Craggs, & Shilton, 2011). Studies have shown that growth rates in *S. obliquus* may increase with increasing CO₂ levels up to 20% (Ho, Chen, Yeh, & Chen, 2010). However, contradicting studies note the growth in *S. obliquus* is maximized 6% CO₂ levels as compared to 0% and 12% CO₂ (Greque de Morais & Vierira Costa, 2007). Another study found *S. obliquus* to have maximal growth rates at 60 μM CO₂ (Yang & Gao, 2003). CO₂ levels should be closely monitored because excess CO₂ can form carbonic acid with water and decrease the pH of the culture media to undesirable levels. Algae are sensitive to changes in pH. Growth in an acid-tolerate strain of *S. acutus* was found to be reduced by as much as 50% at pH 5 and growth stopped completely at pH 4.8 (Nalewajko, Colman, & Olaveson, 1997).

1.7 Determination of algal growth rate

There are multiple stages to microalgal growth. First, is a ‘lag phase’ wherein the cells do not grow appreciably. In the lag phase, the cells become acclimated to their new growing environment.
Second, is the growth phase which is broken into two separate steps: exponential growth and linear growth. Microalgal growth is most closely modeled as exponential growth, but as environmental constraints such as light/nutrient limitation become more apparent, algal growth slows and becomes more linear as it approaches the next phase of growth: stationary phase.

In the stationary phase of growth, cell multiplication stops as does biomass accumulation in most cases. This does not imply that all cell growth ceases, other metabolic pathways, such as those that govern lipid production, may continue to operate if conditions are appropriate. In other words, environmental stresses force some algae to switch carbon allocation from biomass growth to oil production (Csavina, Stuart, Riefler, & Vis, 2011).

The final phase of microalgal cell growth is cell death. If the cells can no longer support themselves in the stationary phase, they begin to die en masse. Cell death occurs, to some extent, in all the phases of growth. It is only most apparent once cell growth has stopped. Cell death takes place in multiple stages in microalgae. First, the cell membranes are compromised. Then the cells begin to lose their photosynthetic activities die to the degradation of chlorophyll and other photosynthetic pigments. Finally, cellular DNA begins to fragment (Veldhuis, Kraay, & Timmermans, 2001). Figure 2 depicts the stages of cell growth.
The growth rate is determined during the exponential stage of growth. By taking the natural log of the growth data during the exponential phase, one is left with data that constitutes a straight line. The slope of this line is the growth rate for that data.

1.8 Review of chlorophyll, magnesium importance, and Monod kinetics

In most cases, it is not economical to supply excess amounts of growth nutrients to the cells. In some cases, excess nutrient levels may even inhibit growth or other product formation. This makes it evermore important to supply the algal cells with just the right amounts of the nutrients they need to grow. While excess levels of magnesium have not been seen to limit growth in these trials, the cost-savings goal still applies.

Magnesium is important to microalgal cell growth. Chlorophyll, the main molecule responsible for light uptake in photosynthetic plants, consists of hydrocarbon phytol chain and a tetrapyrrrole ring structure surrounding a chelated Mg$^{2+}$ ion. This means that, for every one molecule of chlorophyll produced, one Mg$^{2+}$ is required.
Because of its importance in chlorophyll, green algae have higher magnesium contents than do red or brown microalgae. However, green algae have lower levels of sodium and potassium (Csikkel-Szolnoki, Bathori, & Blunden, 2000).

One method for evaluating the growth of a microorganism as a function of any limiting substrate is the Monod model for growth kinetics.

Equation 1. Monod equation

\[
\mu = \mu_{\text{max}} \frac{S}{(K_s + S)}
\]

\(\mu_{\text{max}}\) is the maximal growth rate and \(S\) is the substrate concentration. \(K_s\) is known as a ‘saturation constant.’ When \(S = K_s\), the growth rate is \(\frac{1}{2}\) the maximal growth rate. This model is semiempirical and assumes that the growth is only limited by \(S\), a single substrate (Shuler & Kargi, 2002). A graph of growth rate vs. substrate concentration can be seen below following the Monod kinetics.
A double reciprocal plot (Lineweaver-Burk plot) is used to graph growth \(^{-1}\) rate vs substrate \(^{-1}\) and \(K_s\) and \(\mu_{\text{max}}\) can be determined with better clarity than just estimating them graphically.

**Equation 2.**

\[
1/(\mu) = 1/(\mu_{\text{max}}) + (K_s/\mu_{\text{max}})^* (1/s)
\]

\(\mu_{\text{max}} = (y\text{-intercept})^{-1}\) and \(k_s = \text{slope}^* \mu_{\text{max}}\) on the Lineweaver Burke plot.

The Monod equation is a relationship between substrate concentration and growth rate. It allows one to determine an expected growth rate from a given substrate concentration. Alternatively, it can be used to determine a substrate concentration where growth is not limited. This is important in order to save on nutrient costs and not overfeed the nutrient.

**1.9 Brief overview of induced coupled plasma (ICP) spectroscopy**

Induced coupled plasma spectroscopy is a useful analytical technique wherein samples can be analyzed in the liquid state, also referred to as “wet” sampling. In optical emission spectroscopy (OES), the sample is vaporized into its elemental components in a core of inductively coupled argon plasma (ICP), which can reach temperatures of 8000°C. At such high temperatures, the individual elements become thermally excited
and emit visible light at their characteristic wavelengths. This light is then gathered by a
diffraction grating which resolves the light into constituent wavelengths. The
spectrometer then collects that light and converts it into some measured intensity. That
measured intensity can then be related to a physical concentration when calibration
standards are employed (Evans, 2012).

Individual atoms are measured in the ICP, not compounds. The super high heat
breaks all molecular bonds and leaves only individual atoms to be measured. This is
important to remember because there may be multiple sources of a single element in the
media at any one time. Cell debris or small organic compounds may be present in the
media during analysis that could skew the measurement results, which are assumed to
only contain supernatant.

1.10 Goal and scope of this work

The goal of this work is to investigate and gain a better understanding of the
effects of different nutrients, on the growth (and lipid accumulation) of Scenedemus
dimorphus; as well as to determine the nutrient/biomass yields for some of these
nutrients. Growth and lipid accumulation are two of the most important aspects of algal
growth as they pertain biofuel production (Csavina, Stuart, Riefler, & Vis, 2011).

The scope of this work spans three distinct experiments all related by the growth
of S. dimorphus and the investigation into the specific nutrients of the growth media and
their various effects on the growth, lipid production, and maximum cell density of S.
dimorphus. The three experiments are described here.
The first experiment pertains to the optimal digestate media design for growth and lipid production of *S. dimorphus*. Digestate refers to digested animal waste from agricultural sources. Agricultural wastes such as digestate can be obtained at prices much lower than what it would cost to create chemically-defined growth media. Past studies have shown that green algae grown in wastewater, either agricultural or industrial, is a promising method to produce lipids percentages up to 42% for biodiesel purposes (Feng, Chao, & Dawei, 2011). Additionally, although heavy metals such as Co and Zn may affect algal growth negatively, microalgae can be used to absorb heavy metals from industrial wastewater sources (Ajayan, Selvaraju, & Thirugnanamoorthy, 2011). This experiment focused on the effects on growth rate and lipid of *S. dimorphus* when the concentration of digestate and supplemented phosphorus and magnesium were changed. The results from this experiment confirmed the importance of magnesium in algal growth.

The second experiment seeks to determine the optimal magnesium concentration for growth using Monod growth kinetics. The importance of this experiment was to discover what amount of magnesium is necessary in the media so as not to have a
detrimental effect on the growth of the cells. To even better understand the effect of magnesium on cell growth, the consumption rate, and the yield of magnesium needed to be investigated.

The Monod experiment was a way to understand the effect of magnesium on cell growth without actually measuring any amount of magnesium beyond what was initially charged to the system. It was soon discovered that the capability to measure magnesium in solution, with great accuracy, was readily available by means of induced coupled plasma optical emission spectroscopy (ICP OES). With magnesium concentration data vs. time, understanding how much magnesium is consumed by the cells with time is achieved. Previously, only the initial amount of magnesium that needed to be charged to the system was known. Knowing the specific uptake of magnesium by the cells enables the ability to feed magnesium over time to the cells or even begin recycle the media to the system after the cells are harvested. Additionally, the ease of measurement of magnesium in the ICP spectrometer spawned the option of measuring many of the elements present in the media all at once.

This thesis is a story stemming from the discovered importance of magnesium in the first digestate experiment which grows into investigating the Monod growth kinetics of *S. dimorphus* with respect to magnesium and culminates in the ICP experiment which determines the yield of algal biomass to magnesium mass. Along the way, the importance of and growth yields from many other nutrients are uncovered as a supplement to this work.
CHAPTER II

MATERIALS AND METHODS

2.1 Algae culture

*S. dimorphus* was originally obtained from the UTEX culture collection (ID# 746 Austin, TX). Algae stock was thawed from liquid nitrogen (in 5% DMSO), and maintained in 1.5% agar prepared with modified triple nitrate Bold’s Basal Media (3N-BB). The 3N-BB medium contains the following components, per 1 L of DI water: 0.75 g NaNO₃, 0.025 g CaCl₂*2H₂O, 0.075 g MgSO₄*7H₂O, 0.075 g K₂HPO₄, 0.175 g KH₂PO₄, 0.025 g NaCl, 6 mL trace metal stock solution (containing 0.75 g/L Na₂EDTA, 0.097 g/L FeCl₃*6H₂O, 0.041 g/L MnCl₂*4H₂O, 0.005 g/L ZnCl₂, 0.002 g/L CoCl₂, and 0.004 g/L NaMoO₄), and 2 mL of each vitamin stock solution (H (biotin) at 0.04g/L, B₁ (thiamine) at 1.105 g/L, and B₁₂ (cyanocobalamin) at 0.1575 g/L, all in 50mM HEPES solution). All chemicals are from Sigma. Seed cultures for growth experiments were
prepared by inoculating cells from agar into a 250 mL Erlenmeyer flask with 3N-BB medium, sparged with 5% CO₂ in air at a flow rate of 0.1 LPM, and agitated on a stir plate at room temperature. Illumination was provided by 14 watt, 48” Accupro fluorescent bulbs on a 12 hours on/12 hours off cycle, with a measured intensity of 150-200 ft-candles at the liquid surface maintained in a sterile hood.

### 2.2 General growth conditions

While the experimental culture vessels varied in volume from experiment to experiment, the inoculum volume was determined so as to provide a starting \( A_{600} \) of about 0.1 - 0.2 in the experimental flasks. Illumination was provided by seven 14-watt, 24” fluorescent tubes (four Coralite Aquapro T-5/10,000K and three Accupro AFL/F14T5/14W/830) on a 12 hours on/12 hours off cycle, with a measured intensity of 515-550 ft-candles at the liquid surface. *S. obliquus* has been found to have a maximal growth rate at 500-600 ft-candles. Beyond that, growth plateaus until 2000 ft-candles where it begins to decline to zero growth at 6000 ft-candles (Sorokin & Krauss, 1957).

As a reference, direct sunlight is 10,000 ft-candles, full daylight is 1,000 ft-candles, and clear night with a full moon is only 0.01 ft-candles (Lashen, 2011).

Temperature and agitation control were provided by a Labline Orbital shaker bath at 33 ± 1 °C and 150 rpm of agitation. Gas (5% CO₂ in air v/v) was sparged into each flask through a 200 uL pipette tip at 0.1 ± 0.02 liters per minute. Flasks were vented through 1 µm syringe filters.

Each flask was sampled once daily by pipette and the absorbance at 600 nm measured (Spectronic Genesys 5). Absorbance was found to be linear with cell concentration for \( A_{600} < 1.0 \). Samples with higher cell concentrations (\( A_{600} > 1.0 \)) were
diluted with DI water, or parent media in the case of the digestate experiments, and the absorbance reported as “adjusted absorbance”.

2.4 Protocol for use of PerkinElmer ICP

See Appendix A

2.5 Determining optimal concentrations of digestate, MgSO₄, and KH₂PO₄/K₂HPO₄

A digestate slurry from dairy farm waste which had already undergone methane digestion was received along with chemical composition assays. Table 2 shows the concentrations of the important components for various dilutions of the stock digestate slurry, referred to simply as digestate from here, along with the base-line 3N-BB media.

Table 2. Elemental composition of dilutions of digestate (in water v/v), compared to standard 3N-BB medium.

<table>
<thead>
<tr>
<th>Component</th>
<th>1% digestate</th>
<th>1.5% digestate</th>
<th>2% digestate</th>
<th>5% digestate</th>
<th>3N-BBM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>0.0022</td>
<td>0.0033</td>
<td>0.0044</td>
<td>0.011</td>
<td>0.007</td>
</tr>
<tr>
<td>Chlorine</td>
<td>0.0098</td>
<td>0.0147</td>
<td>0.0196</td>
<td>0.049</td>
<td>0.027</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.0056</td>
<td>0.0084</td>
<td>0.011</td>
<td>0.028</td>
<td>0.007</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>0.032</td>
<td>0.048</td>
<td>0.064</td>
<td>0.160</td>
<td>0.124</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.0044</td>
<td>0.0066</td>
<td>0.0088</td>
<td>0.022</td>
<td>0.053</td>
</tr>
<tr>
<td>Potassium</td>
<td>0.131</td>
<td>0.197</td>
<td>0.262</td>
<td>0.656</td>
<td>0.118</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.0084</td>
<td>0.0126</td>
<td>0.0168</td>
<td>0.042</td>
<td>0.213</td>
</tr>
<tr>
<td>Sulfur</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.0098</td>
</tr>
</tbody>
</table>

Most of the component concentrations in the 3N-BB media in Table 2 fall within the range set by the 1% and 5% digestate dilutions in water. This range is the baseline for the media optimization.

The study was performed in three phases. In all phases, growth was carried out until day 15 or 16 whereupon stationary phase is approaching, firstly marked by a deviation from the exponential growth period and then by the halting of growth.
altogether. Lipid analysis was performed on the final day of experiment. All experiments were grown as described in section 2.2 unless otherwise noted.

The initial phase was a comparison of two digestate concentrations (2% and 5% digestate, v/v in water) with the standard research medium of 3N-BB. Digestate concentrations were chosen such that the chemical composition was around that of the 3N-BB media which has proven able to produce growth. In this first set of experiments, *S. dimorphus* was cultured in 2% and 5% digestate, in triplicate while algae culture in 3N-BB was in duplicate.

In phase 2 of experimentation, the effect of the solids in the diluted digestate media, on algal growth, was determined. Even at very low dilutions, less than 2% v/v, a significant amount of solids were present in the media. 2% unfiltered digestate (in duplicate), was compared to 2% and 1% filtered digestate (both in triplicate). Filtering was achieved through a 10 micron Watman filter.

Phase 3 of experimentation, was designed as a full-factorial design of experiment consisting of 16 flasks. The resulting design consisted of 3 factors (A: digestate%; B: mono- and di-basic phosphate, KH$_2$PO$_4$/K$_2$HPO$_4$; C: MgSO$_4$). Each factor was tested at two levels each with the inclusion of 8 center point flasks. The media used was 1% and 2% digestate, with a 1.5% centerpoint, in RO water (v/v) with and without supplementation of the aforementioned salts. All media were centrifuged to remove visible solids, instead of filtration. Each flask was supplemented with either 0 or 3 mL of 0.0305 M MgSO$_4$ and 0 or 3 mL of potassium phosphate solution containing 0.129 M KH$_2$PO$_4$, and 0.0431 M K$_2$HPO$_4$, with 1.5mL as the centerpoint value for both. The
design is shown in Table 3. This design produces the specific media compositions shown in Table 3 as well.

Table 3. Experimental design; Factors A, B, and C are defined as follows: A: % Digestate, at levels of 1% (-1) and 2% (1) with a center point value of 1.5% (0). B: mL of KH2PO4/K2HPO4 solution, at levels of 0 mL added (-1), 3 mL added (1), with a center point value of 1.5 mL (0). C: mL of 0.0305 M MgSO4 solution, at levels of 0 mL added (-1), 3 mL added (1), with a center point value of 1.5 mL (0). High and low levels for magnesium and phosphorus are based upon prior experiments with 3N-BB. Actual concentrations of Mg2+ and PO43- in each flask derived from additions of MgSO4 and KH2PO4/K2HPO4 solutions. Note that digestate already contains magnesium and phosphate, but there is considerable uncertainty in the exact amount in this particular batch of digestate. The concentrations shown in the table below do not reflect basal amounts contributed by the digestate.

<table>
<thead>
<tr>
<th>Flask #</th>
<th>CenterPt</th>
<th>Blocks</th>
<th>Factor A Digestate</th>
<th>Factor B KH2PO4/K2HPO4</th>
<th>Factor C MgSO4</th>
<th>Digestate%</th>
<th>PO43- (mM)</th>
<th>Mg2+ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>3.44</td>
<td>0.61</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
<td>1.72</td>
<td>0.305</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>2</td>
<td>-1</td>
<td>-1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0.61</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>2</td>
<td>-1</td>
<td>1</td>
<td>-1</td>
<td>1</td>
<td>3.44</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
<td>1.72</td>
<td>0.305</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
<td>1.72</td>
<td>0.305</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
<td>1.72</td>
<td>0.305</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>1</td>
<td>0</td>
<td>0</td>
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<td>10</td>
<td>1</td>
<td>1</td>
<td>-1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3.44</td>
<td>0.61</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
<td>1.72</td>
<td>0.305</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-1</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0.61</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
<td>1.72</td>
<td>0.305</td>
</tr>
<tr>
<td>14</td>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
<td>1.72</td>
<td>0.305</td>
</tr>
<tr>
<td>15</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
<td>1.72</td>
<td>0.305</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-1</td>
<td>2</td>
<td>3.44</td>
<td>0</td>
</tr>
</tbody>
</table>

Total lipid contents were measured on the final day of growth (day 15 for the digestate experiments and day 16 for the factorial design experiment). To measure lipid content, 75-100 mL of the algae suspension was divided into 50 mL centrifuge tubes and centrifuged for 1.5 hours at 2000 rpm. The supernatant was discarded and the pellets were rinsed with DI water, vortexed, and combined in a 1.5 mL microcentrifuge tube,
where they were centrifuged for 20 min at 14,000 rpm. The supernatant was discarded and the resulting pellets were dried in an oven at 45-50°C for 1-2 days. The dried pellet was ground to a fine powder in mortar and pestle and transferred to a glass test tube. 5 mL of isopropanol-hexane solution (2:3 v/v; Acros Co. NJ) were added to the powdered biomass, the samples were shaken at 200 rpm for 3 hours on a shaker platform, and the supernatant removed by pipette. The solvent extraction procedure was repeated with 5 mL of fresh solvent. After the 2nd shaking period, the solvent was removed by filtration through 0.65 μm pore filter paper (Micronsep, Honeoye, NY). The combined solvents and the extracted biomass were dried in the fume hood at room temperature and weighed to obtain the masses of the lipid and lipid-free fractions, respectively. These values can be used to calculate biomass concentration in the original sample (gdw/L), lipid % (w/w), and lipid concentration (g/L).

2.6 Determining Kinetics of Mg on Cell Growth in *Scenedesmus dimorphus*

8 - 250mL Erlenmeyer flasks with working volumes of 150mL were inoculated, grown, and sampled for nine days as described in section 2.2 with the exception that initial MgSO$_4$ was varied over all of the flasks (Table IV).

The seed for this experiment was grown under limiting magnesium conditions such that the inoculum volume would, ideally, impart no magnesium ions on the experimental flasks. This was achieved by growing a magnesium limited seed into late stationary phase. Results will show that this effect may not have been achieved entirely.
Table 4. The eight levels of Mg$^{2+}$ in the media as a percentage of the base Mg$^{2+}$ level in 3N-BBM media as well as a concentration in grams per liter.

<table>
<thead>
<tr>
<th>Flask #</th>
<th>Fraction of 3N</th>
<th>g/L (MgSO$_4$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>7.40E-02</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>3.70E-02</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>7.40E-03</td>
</tr>
<tr>
<td>4</td>
<td>0.1</td>
<td>7.40E-04</td>
</tr>
<tr>
<td>5</td>
<td>0.05</td>
<td>3.70E-04</td>
</tr>
<tr>
<td>6</td>
<td>0.02</td>
<td>1.48E-04</td>
</tr>
<tr>
<td>7</td>
<td>0.01</td>
<td>7.40E-05</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0.00E+00</td>
</tr>
</tbody>
</table>

The majority of the Mg concentrations are below that contained in 3N-BB media, in order to identify the concentrations that limit growth and to quantify the Monod parameters.

2.7 ICP Spectral Analysis to Determine Nutrient Uptake Rates in *S. dimorphus*

*S. dimorphus* was inoculated and grown under normal conditions in quadruplicate in 2 liter bottles with working volumes of 1.6L. A quadruplicate culture design was employed in order to establish standard deviations for all measurements and calculations. Air flow rates in the larger flasks were increased to compensate for larger culture volume. Due to limitations in the gas feed system, only flowrates of 0.4 - 0.5 L air/minute were achieved.

25 mL samples were taken daily and absorbance recorded for growth rate determination. Absorbance values in excess of 1.0 were diluted and an ‘adjusted absorbance’ calculated. The samples were centrifuged to pellet the biomass. The supernatant was then filtered through a 0.45 micron filter to ensure no algal or bacterial cells remained in the culture media. The samples were then refrigerated at 4$^\circ$C for 3 months until sample analysis was performed.
2.7.1 ICP analysis

Samples were analyzed using a Perkin-Elmer Induced Coupled Plasma Optical Emission Spectrometer (ICP OES). The measureable elements contained in the medium include sodium, calcium, chlorine, magnesium, sulfur, potassium, phosphorus, iron, zinc, manganese, cobalt, and molybdenum.

 Calibration solutions are necessary in order to get accurate results from the ICP. Table 5 outlines the concentrations of the 6 calibration standards as well the 3 quality control standards created to calibrate the ICP. Since the experimental cultures were to be grown in 3N-BB media, the range of concentrations for the calibration solutions was chosen as 2x, 1x, 1/2x, 1/4x, 1/8x, and 0x multiples of 3N-BB media.

Table 5. Shows the concentrations of the six calibration solutions and the three quality controls standards in ppm of each element by mass. The table also shows the elemental concentration of each calibration and standard as a multiple of its base level in 3N-BB media.

<table>
<thead>
<tr>
<th></th>
<th>Cal 1</th>
<th>Cal 2</th>
<th>Cal 3</th>
<th>Cal 4</th>
<th>Cal 5</th>
<th>Cal 6</th>
<th>QC 1</th>
<th>QC 2</th>
<th>QC 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>3N</td>
<td>x2</td>
<td>x1</td>
<td>x0.5</td>
<td>x0.25</td>
<td>x0.125</td>
<td>x0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na</td>
<td>428</td>
<td>214</td>
<td>107</td>
<td>54</td>
<td>27</td>
<td>0</td>
<td>161</td>
<td>40</td>
<td>13</td>
</tr>
<tr>
<td>Ca</td>
<td>14</td>
<td>6.8</td>
<td>3.4</td>
<td>1.7</td>
<td>0.86</td>
<td>0</td>
<td>5.1</td>
<td>1.3</td>
<td>0.43</td>
</tr>
<tr>
<td>Cl</td>
<td>31</td>
<td>16</td>
<td>7.8</td>
<td>3.9</td>
<td>1.9</td>
<td>0</td>
<td>11.7</td>
<td>2.9</td>
<td>0.97</td>
</tr>
<tr>
<td>Mg</td>
<td>15</td>
<td>7.4</td>
<td>3.7</td>
<td>1.8</td>
<td>0.93</td>
<td>0</td>
<td>5.6</td>
<td>1.4</td>
<td>0.46</td>
</tr>
<tr>
<td>S</td>
<td>20</td>
<td>9.8</td>
<td>4.9</td>
<td>2</td>
<td>1.2</td>
<td>0</td>
<td>7.3</td>
<td>1.8</td>
<td>0.61</td>
</tr>
<tr>
<td>K</td>
<td>169</td>
<td>84</td>
<td>42</td>
<td>21</td>
<td>10.5</td>
<td>0</td>
<td>63</td>
<td>16</td>
<td>5.3</td>
</tr>
<tr>
<td>P</td>
<td>107</td>
<td>53</td>
<td>27</td>
<td>13</td>
<td>6.7</td>
<td>0</td>
<td>40</td>
<td>10</td>
<td>3.3</td>
</tr>
<tr>
<td>Fe</td>
<td>0.24</td>
<td>0.12</td>
<td>0.06</td>
<td>0.03</td>
<td>0.01</td>
<td>0</td>
<td>0.09</td>
<td>0.022</td>
<td>0.008</td>
</tr>
<tr>
<td>Zn</td>
<td>0.03</td>
<td>0.01</td>
<td>0.007</td>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0.01</td>
<td>0.003</td>
<td>0.0009</td>
</tr>
<tr>
<td>Mn</td>
<td>0.14</td>
<td>0.07</td>
<td>0.03</td>
<td>0.017</td>
<td>0.009</td>
<td>0</td>
<td>0.05</td>
<td>0.013</td>
<td>0.004</td>
</tr>
<tr>
<td>Co</td>
<td>0.01</td>
<td>0.005</td>
<td>0.002</td>
<td>0.001</td>
<td>0.006</td>
<td>0</td>
<td>0.004</td>
<td>0.001</td>
<td>0.0003</td>
</tr>
<tr>
<td>Mo</td>
<td>0.03</td>
<td>0.013</td>
<td>0.006</td>
<td>0.003</td>
<td>0.002</td>
<td>0</td>
<td>0.009</td>
<td>0.002</td>
<td>0.0008</td>
</tr>
</tbody>
</table>

The three quality control standards were used as a double check for the accuracy of the calibrators and needed to be at concentrations that did not match those of the calibrators. Quality control standard concentrations were chosen to be 3/4x, 3/16x, and
1/16x multiples of 3N-BB media. The QC standards help to give more accuracy to the measurements around them as well.

Each measureable element has a number of wavelengths that the spectrometer can measure it at. The default wavelengths were chosen for each substrate to be measured, as shown in Figure 6. If the wavelengths are too close together, there may be inference in the measurement.

![Wavelengths of the Metals](image)

*Figure 6. Graphical representation of the default wavelengths of measurement for each element in the ICP.*
CHAPTER III
RESULTS AND DISCUSSION

3.1 Optimal digestate and inorganic nutrient supplementation levels

The initial objective of this experiment was to investigate how digestate, as a growth medium, affects the growth and lipid production of *S. dimorphus*. The experimental design allowed for including two more factors, magnesium and phosphorus, to be screened as well. Beyond that, the effect of filtering the digestate of its larger solids on growth and lipid content was investigated. Finally, the digestate media was supplemented with MgSO$_4$ and KH$_2$PO$_4$/K$_2$HPO$_4$ in a partial factorial design of experiments (DOE). Again, the effects of digestate and magnesium and phosphorus salts on growth and lipid content were observed.

The specific growth rates were calculated for each flask from the adjusted absorbance measurements during the exponential growth phase. The exact period of the
exponential growth phase was variable among each experiment, and the identification of this period affected the calculated growth rates.

Figure 7, Figure 8, and Figure 9 show the growth data at different digestate concentrations and 3N-BB media. Figure 10 shows the maximal biomass concentrations based on the final adjusted absorbance derived from conversion of 0.52 g/L biomass = 1.0 absorbance from previous experiments (Brittany Studmire, personal communication).
Figure 9. Growth curves for 3N-BB flask duplicate.

Figure 10. Maximal biomass concentrations comparing 2% digestate with 5% digestate and 3N-BBM (3N); values in gdw/L are calculated from maximal adjusted absorbance. Mean±s.d. (n=3 for 2% and 5%, n=2 for 3N). P values from t-test suggest significant differences between 2% and 5%, as well between 2% and 3N-BB, but insignificant differences between 5% and 3N-BB at the 95% confidence interval.

A two-sample t-test suggests statistically significant differences for the 2% and 5% digestate, as well as between 2% digestate and 3N-BB, but insignificant differences between 5% and 3N-BB at the 95% confidence interval. Figure 11 shows the maximum growth rates for each media type. Differing windows of growth are used in each case (days 3-7 for 2%, days 7-11 for 5%, and days 4-8 for 3N-BB), to capture the exponential growth phase in each flask. 5% digestate in water has the most growth much later in the
experiment possibly due to light limitation. Figure 12 and Figure 13 show the lipid results in the form of lipid concentration in the final cell suspension and the percent of lipids in the final dry biomass where 2% digestate clearly outperforms.

![Maximum Growth Rates](image)

Figure 11. Maximum growth rates. Windows of time vary per media type: days 3-7 for 2% digestate, days 7-11 for 5% digestate, and days 4-8 for 3N-BB media. Mean ± s. d. (n=3 for 2 and 5%; n=2 for 3N-BBM). Two-sample t-test in Microsoft excel showed that there is no statistical significant difference between the three growth means.

![Concentration of Lipids](image)

Figure 12. Concentration of lipids in final cell suspension measured on day 15. Results for each media type are from similar combined flasks to produce a sample size large enough for measurement, hence no error bars. (Data from colleague, Britany Studemire)
The fact that the 2% digestate outperformed the 5% in biomass and lipids could be due to that, at high concentrations of digestate light limitation became an issue. Indeed, 5% digestate is much more opaque than 2% and the transparent 3N-BB media. By all outcome measures, the 2% digestate performed as well as or better than the 5% and the 3N-BB media did.

Figure 14, Figure 15, and Figure 16 show the growth data for the experiments which investigate the effect of filtering the digestate solids out. Figure 15 and Figure 16 display late phase evaporation which did not affect the calculation of the growth rates for days 2-6. Figure 17 shows the biomass concentration from day 15 adjusted absorbance. Figure 18 shows the maximal growth rates. The days 2-6 window was used for all media types in this phase of the experiment. P-values from t-test suggest there is no statistically significant difference in the means of the growth rates between 2% unfiltered and 1% unfiltered on a 95% confidence interval, while the difference between the means of 2% unfiltered and 2% filtered growth is statistically significant. Figure 19 and Figure 20 contain the lipid results for this phase of experimentation.
Figure 14. Growth curves for 2% unfiltered flask duplicate.

Figure 15. Growth curves for 2% filtered flask triplicate; absorbance values are so high because significant evaporation was experienced in these flasks.
Figure 16. Growth curves for 1% unfiltered flask triplicate; the culture with the absorbance nearing 4.0 is so high because of significant evaporation.

Figure 17. Biomass concentration in grams per liter on day 15; the 1% unfiltered flask as well as all of the 2% filtered flasks that experienced an amount of evaporation has been excluded from this analysis. Mean±s. d. (n=2). P-values from t-test suggest there is no statistically significant difference between the two sets at the 95% confidence intervals.
Figure 18. Maximum growth rates with standard deviations measured during days 2-6. Mean±s.d. (n=2 2% unfiltered; others n=3). P values from t-test suggest there is no significant difference between 2% unfiltered and 1% unfiltered, and that there is a significant difference between 2% unfiltered and 2% filtered.

Figure 19. Concentration of lipids; 2% filtered has been omitted due to the uncertainty of the data from the effects of evaporation.
Filtering the media caused an increase in growth rate (Figure 18), most likely because the removal of solids increased the light availability to the algae. Reducing the digestate concentration from 2% to 1% had no effect on the final biomass concentration (Figure 17) nor on the growth rate (Figure 18), indicating that key components were present in adequate quantities in the 1% media. However, the 1% unfiltered had somewhat higher lipid content (22%) compared to the 2% digestate (17%), as shown in Figure 20, and slightly higher lipid concentration (Figure 19). Filtering the 2% digestate media resulted in an increase in lipid composition, from 17% to 22%, most likely because of the greater light transmission to the biomass, which increased energy available to accumulate lipid providing that CO₂ was supplied in excess.

Results from the next phase of experimentation (DOE supplementation of magnesium and phosphorus salts) are shown in Table 6 and Figure 21 - Figure 25.
Table 6. Growth rates were all calculated from days 3-7. Maximum cell density was calculated from the adjusted A600 using the standard conversion factor (0.52 g/L biomass = 1.0 absorbance at 600nm). The lipid concentration is calculated from the product of the lipid composition, and the actual biomass dry weight recovered (data not shown).

<table>
<thead>
<tr>
<th>Flask#</th>
<th>Growth rate (days 3-7), in day⁻¹</th>
<th>Maximum cell concentration(gdw/L)</th>
<th>Lipid composition(%)</th>
<th>Lipid concentration(g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.392</td>
<td>1.66</td>
<td>17.5</td>
<td>0.260</td>
</tr>
<tr>
<td>2</td>
<td>0.450</td>
<td>1.54</td>
<td>23.8</td>
<td>0.274</td>
</tr>
<tr>
<td>3</td>
<td>0.487</td>
<td>1.49</td>
<td>28.2</td>
<td>0.315</td>
</tr>
<tr>
<td>4</td>
<td>0.285</td>
<td>1.73</td>
<td>15.5</td>
<td>0.210</td>
</tr>
<tr>
<td>5</td>
<td>0.403</td>
<td>1.27</td>
<td>27.2</td>
<td>0.240</td>
</tr>
<tr>
<td>6</td>
<td>0.443</td>
<td>1.33</td>
<td>25.2</td>
<td>0.293</td>
</tr>
<tr>
<td>7</td>
<td>0.407</td>
<td>1.47</td>
<td>23.1</td>
<td>0.272</td>
</tr>
<tr>
<td>8</td>
<td>0.418</td>
<td>1.43</td>
<td>25.9</td>
<td>0.324</td>
</tr>
<tr>
<td>9</td>
<td>0.417</td>
<td>1.15</td>
<td>18.4</td>
<td>0.156</td>
</tr>
<tr>
<td>10</td>
<td>0.521</td>
<td>1.25</td>
<td>14.8</td>
<td>0.171</td>
</tr>
<tr>
<td>11</td>
<td>0.478</td>
<td>1.47</td>
<td>18.2</td>
<td>0.233</td>
</tr>
<tr>
<td>12</td>
<td>0.431</td>
<td>1.79</td>
<td>17.5</td>
<td>0.284</td>
</tr>
<tr>
<td>13</td>
<td>0.463</td>
<td>1.60</td>
<td>16.0</td>
<td>0.195</td>
</tr>
<tr>
<td>14</td>
<td>0.459</td>
<td>1.48</td>
<td>16.1</td>
<td>0.192</td>
</tr>
<tr>
<td>15</td>
<td>0.435</td>
<td>1.33</td>
<td>13.6</td>
<td>0.164</td>
</tr>
<tr>
<td>16</td>
<td>0.287</td>
<td>1.69</td>
<td>16.7</td>
<td>0.220</td>
</tr>
</tbody>
</table>

Figure 21. Growth curves for 2% digestate. Numbers in the legend refer to the flask number with varying levels of magnesium and phosphorus salts as seen in Table 3.
Figure 22. Growth curves for 1.5% digestate center point flasks. Numbers in the legend refer to the flask number (see Table 3).

Figure 23. Growth curves for 1% digestate flasks. Numbers in the legend refer to the flask number (see Table 3).
Growth rates and maximum cell densities are similar among all flasks, but conclusions are hard to draw from the graphs. Data analysis from Minitab is shown in Table VII and Figure 26.
Table 7. P-values for the four outcomes from ANOVA. Emboldened values (less than 0.05) are considered to be statistically significant on a 95% confidence interval; i.e. there is less than a 5% chance that these values occurred by chance.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Digestate %</th>
<th>PO₄³⁻</th>
<th>Mg²⁺</th>
<th>Dig%*PO₄³⁻</th>
<th>Dig%*Mg²⁺</th>
<th>Mg²⁺*PO₄³⁻</th>
<th>Center Pt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rate</td>
<td>0.000</td>
<td>0.756</td>
<td>0.000</td>
<td>0.303</td>
<td>0.270</td>
<td>0.887</td>
<td>0.003</td>
</tr>
<tr>
<td>[Max. biomass]</td>
<td>0.001</td>
<td>0.326</td>
<td>0.238</td>
<td>0.853</td>
<td>0.331</td>
<td>0.147</td>
<td>0.361</td>
</tr>
<tr>
<td>Lipid content</td>
<td>0.006</td>
<td>0.545</td>
<td>0.997</td>
<td>0.327</td>
<td>0.348</td>
<td>0.004</td>
<td>0.444</td>
</tr>
<tr>
<td>Lipid conc.</td>
<td>0.314</td>
<td>0.412</td>
<td>0.047</td>
<td>0.604</td>
<td>0.785</td>
<td>0.018</td>
<td>0.473</td>
</tr>
</tbody>
</table>

Figure 26. Contour plots for the results from the partial factorial DOE showing the relationships between digestate (A, the y-axis) and MgSO₄ (C, the x-axis). [a]growth rate for days 3-7; b)maximal cell density; c)lipid%; d)lipid concentration] Plots for growth rate on days 3-7 shows that growth rate increases as MgSO₄ increases and that a digestate value between 1%-1 on the y-axis) and the 1.5%(0 on the y-axis) results in optimum growth (figure a). Created from MiniTab ANOVA which produced polynomial models relating the four measured responses to both MgSO₄ and digestate.

The p-values in Table 7 indicate the significance of the parameter in terms of effect on the outcome. Values of p less than 0.05 indicate that there is less than a 5% chance that the results occurred by chance, or, the values can be considered statistically significant on a 95% confidence interval. Digestate % has a significant effect on growth.
rate, biomass, and lipid content. Magnesium concentration has an effect on growth rate and lipid concentration. The 2-way interaction between $\text{Mg}^{2+}$ and $\text{PO}_4^{3-}$ shows significance on lipid content and concentration. The contour plots of these predictions are presented in Figure 26, where the darker green represents higher values of the calculated outcome, while lighter green or blue represent the lowest values of the outcome. Recall that values of -1 represent the lowest value and +1 the highest value tested for each parameter. The ‘A’ term signifies digestate% and ‘C’ for magnesium level. Figure 26a, b, and d show that increasing concentrations of $\text{Mg}^{2+}$ has a positive effect on those responses (growth rate, maximal cell density, and lipid concentration). Figure 26c shows that lipid % is independent of magnesium concentration.

3.1.1 Discussion
The main result from the first phase of experimentation is the ability to screen out digestate % exceeding 2%. There is a marked decrease in the maximum cell density and lipid content at 5% digestate. This helped develop the next phase of experimentation. Also, it is important to note that 2% digestate performed as well as or better than 3N-BB in all scenarios.

The results from the second set of experiments suggest that the digestate solids do not contribute any critical nutrients for biomass growth and/or lipid accumulation, and may actually hinder both growth rate (Figure 18) and lipid accumulation. Therefore, no adverse effect should occur during large-scale pond operation if most of the solids settle to the bottom during operation. These results demonstrate that reducing the digestate concentration from 2% to 1% slightly improves overall performance. The results from these experiments are important because large scale filtration or centrifugation is
expensive. Moreover, it would be very difficult to keep the solids in suspension on a large scale.

The factorial design experiment provides much more insight into the importance of digestate on the responses explored in the previous experiments. From Table 7, it is evident that the % digestate (between 1% and 2% tested here) significantly affects the growth rate, the maximum biomass concentration, and the lipid content. Figure 26a indicates that growth rate is predicted to be maximized for digestate between 1.0 - 1.25%. Maximum biomass concentration occurs with the maximum (2%) digestate concentration (Figure 26b), which is in contrast to earlier results, in which biomass concentration was insensitive to digestate. Digestate has a significantly negative effect on lipid % (Figure 26c), which is consistent with results from both of the first phases of the experiment. These results from the fact that the digestate is the only source of nitrogen for the algae and it is well known that nitrogen-limitation promotes lipid accumulation. Interestingly, the most important outcome, lipid concentration, is relatively insensitive to digestate% (Table 7 and Figure 26d), since the effects on lipid content and biomass concentration appear to cancel out each other. This is consistent with results from the second phase of the experiment where only a slight increase in lipid concentration was obtained at the lower digestate concentration.

Magnesium has a significantly positive effect on growth rate (Table 7 and Figure 26a), most likely because magnesium is a component of chlorophyll. Although magnesium does not affect maximum biomass concentration or lipid content, it has a significant enhancement effect on lipid concentration (Table 7 and Figure 26d). If the cells become light limited, more chlorophyll will not help growth rate or maximal
biomass concentrations. For the concentrations tested, potassium phosphate did not significantly affect growth rate, maximum biomass concentration, lipid content or lipid concentration (Table 7).

The combination of magnesium sulfate and potassium phosphate negatively affects both lipid % and lipid concentration (Table 7). So while additional magnesium positively affects lipid concentration, it seems that the addition of both phosphate and magnesium has a negative effect. We cannot rule out the possibility that the inhibitory effect is due to the potassium ions rather than, or in addition to, the phosphate ions.

For maximizing lipid productivity 1.25% digestate, supplemented with MgSO₄ at a concentration of 0.61 mM is recommended. It is seen that digestate levels around 1.25% with higher levels of magnesium maximize growth rate. In addition, this lower level of digestate increases lipid content as well. Supplementation of the media with potassium phosphate has no effect.

While 1.25% digestate appears to be optimum, this actual value was never tested explicitly—only 1%, 2%, and 5%. It is noteworthy that additional experimentation should be conducted to determine if 1.25% does indeed maximize lipid productivity, or if this concentration can be decreased even further, and thus reduce raw material costs and possibly improve productivity. Magnesium positively influenced productivity up to the concentration tested (0.61 mM). Therefore, it would be worthwhile to examine the effects of even higher levels of MgSO₄, especially if cost is especially sensitive to this supplement. Alternatively, other sources of magnesium can be tested, such as MgCl₂.

Phosphorous is commonly used in fertilizer to promote plant growth. The digestate is a relatively poor source of phosphorous at the 1-2% digestate levels. While
the potassium phosphate used here had no positive benefits, perhaps because of inhibition from high levels of potassium which, at the 1% digestate level, already had a higher concentration than in that of the 3N-BB media, other inexpensive phosphate salts such as calcium phosphate may have beneficial effects. Testing the addition of other low-cost phosphorous sources (without potassium) as supplements to the digestate media is recommended.

3.2 Determining Kinetics of Mg on Cell Growth in *Scenedesmus dimorphus*

Magnesium has been shown to increase growth rates in *S. dimorphus* with increasing supplementation levels in tandem with digestate and phosphorus supplementation (Figure 26). This experiment seeks to better understand the individual effect of magnesium on the growth of *S. dimorphus*. By varying the initial magnesium concentration, its effect on overall algal growth rate can be observed. Additionally, a minimal level of magnesium can be established which still allows for maximal growth rate.

Figure 27 shows the growth data for *S. dimorphus* in 3N-BB media with different starting MgSO₄ concentrations.
Figure 27. Shows the growth curves for eight cultures of varying starting [MgSO₄] as a multiple of the [MgSO₄] in base 3N-BB media.

There is a clear difference in maximum cell density on day nine for each of the cultures. Higher initial Mg concentrations results in higher final cell densities. The same trend follows for growth rate.

Growth rate is graphed vs. Mg concentration to check for adherence to the Monod model in Figure 28.

Figure 28. Graph of growth rate (days⁻¹) vs. magnesium substrate concentration.
The maximum growth rate is estimated from the Lineweaver Burke plot to be $0.59 \pm 0.04 \text{ day}^{-1}$.

Lineweaver-Burk plot is shown as Figure 29.

![Figure 29. Lineweaver-Burk plot to determine $\mu_{\text{max}}$ and $K_s$.](image)

The parameter $k_s$ was found to be $0.009 \pm 0.002$, or $0.9\%$ of 3N-BB magnesium levels, roughly $33 \pm 7 \text{ mg MgSO}_4/\text{L}$. The predicted growth rate from MgSO$_4$ using the Monod kinetics $K_s = 33 \pm 7 \text{ mg MgSO}_4/\text{L}$ and $\mu_{\text{max}} = 0.59 \pm 0.04 \text{ day}^{-1}$ is shown in Figure 30.
Assuming that Mg was completely consumed in flasks 4, 5, 6, and 7 (10%, 5%, 2%, and 1% 3N-BB levels of Mg), because biomass concentrations are significantly less than maximal values in those flasks (the cells stop or begin to stop reproducing), a yield coefficient can be determined for each of these flasks where the yield coefficient \( Y_{X/S} \) for algal biomass \( X \) from a specific substrate \( S \) is:

**Equation 3.** Overall yield coefficient \((X=\text{biomass concentration}; s=\text{substrate concentration})\)

\[
Y_{X/S} = -\frac{\Delta X}{\Delta s} \quad \text{where } \Delta X = (X_{\text{final}} - X_{\text{initial}}) \quad \text{and} \quad \Delta s = (s_{\text{final}} - s_{\text{initial}})
\]

This method of determining the yield can only be applied when \( s_{\text{final}} \) is known, or in this case, can be assumed to be zero or close to zero. Using this method, the yield coefficients for algal biomass from magnesium for flasks 5-7 are found in Table 8 below.
Table 8. Table of yields over various concentrations of magnesium in multiples of the base 3N level. Yield data is shown for flasks 4-7 where final magnesium concentrations are assumed to be zero because biomass accumulation peaked in those flasks at day 9.

<table>
<thead>
<tr>
<th>Flask #</th>
<th>Fraction of 3N</th>
<th>g(biomass)/g(Mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.1</td>
<td>617.68</td>
</tr>
<tr>
<td>5</td>
<td>0.05</td>
<td>801.08</td>
</tr>
<tr>
<td>6</td>
<td>0.02</td>
<td>646.49</td>
</tr>
<tr>
<td>7</td>
<td>0.01</td>
<td>681.62</td>
</tr>
</tbody>
</table>

The mean and standard deviation of the yields from Table 8 is 687±81 g/g. The yield should be independent of magnesium concentration if magnesium was in fact depleted.

3.2.1 Discussion

Growth rates increase with increasing magnesium concentration as expected. Mg is a vital component of chlorophyll and, thusly, an important compound for the reproduction of phototrophic *S. dimorphus*. Growth rates peaked at roughly 0.6 inverse days for the 3N-BB level for Mg concentration and above. This is good news, indicating that the 3N-BB media recipe already has a fairly optimal amount of Mg for maximal cell growth. The 5x and 10x magnesium concentrations showed no increases in growth rate over the 1x level of Mg. At these levels, Mg was in excess and did not limit growth.

At the 0x level of Mg, the growth rate was 0.05 days⁻¹. While this value is very slight, one would think that it should have been zero growth. If no Mg²⁺ were present in the culture media, growth should have been zero. A value of 0.05 inverse days implies that this was not the case. One of two things had occurred. Either the algal cells were able to manage some small level of growth in an environment devoid of magnesium, or the environment was not devoid of magnesium. The second theory was probably the case. Inoculation of the experimental cultures was accomplished by taking a small volume sample from a sterile seed vessel and introducing it to the working culture media.
While steps were taken to reduce the total free Mg in the seed vessel by reducing the initial Mg concentration of the seed media and by growing the culture to stationary phase, some amount of Mg must have been introduced into the experimental flask during the inoculation step. It is important to understand this fact even though, for all intents and purposes, when compared to the other growth rate values, a growth of 0.05 inverse days for the 0x Mg flask might as well have been 0.

As Mg concentrations increased from 1% to 10% of 3N-BB base values, so too did the growth rates. The growth rate for flask 4 (10% of 3N-BB) dropped slightly and fell out of the trend established by the rest of the data. There is no explanation for this anomaly beyond that it must have be some sort of experimental error.

The growth rate data vs magnesium concentration fit the Monod model well. The sharp increase in growth rate vs substrate concentration means that $k_s$ should be very small.

3.3 ICP Spectral Analysis to Determine Nutrient Uptake Rates in S. dimorphus

The goal of this stage of experimentation was to determine consumption rates for all of the media components that are measureable through induced coupled plasma spectroscopy. Initially, only magnesium was to be measured, but due to the ease of measurement of many elements at once, the scope of the experiment quickly increased.

Being able to measure substrate over time as opposed to only knowing the initial substrates’s concentration effect on growth rate allows for the calculation of a yield coefficient of biomass to substrate. Previously, the best information acquired concerning magnesium’s effect on the growth of S. dimorphus was the minimum concentration of magnesium that would not limit the maximal growth of the cells over a certain period of
time. Gaining knowledge of the yield coefficient of magnesium for algal biomass allows for better control of nutrient feeding over time or more complex media schemes such as nutrient recycling.

3.3.1 Results

Samples were taken daily and optical absorbance at 600nm measured. Absorbance values in excess of 1.0 were diluted and an ‘adjusted absorbance’ calculated. The absorbance is graphed versus time in Figure 31.

![Biomass growth in 2-L Bottles](image)

The natural log of the absorbance values was taken and a growth rate was determined for the exponential period of growth. Figure 32 shows the natural log of the growth data from days 0-13. The lag phase can really be seen from days 0 to 1. The data begins to deviate from linearity past day 6 or so which means that it is exiting exponential growth at this point; growth rate only decreases past this point. It would appear that the maximal exponential growth rate should be evaluated from days 1 to 6.
Figure 32. Natural log of the mean of the growth data from day 0-13.

Figure 33 shows the natural log of the growth data over just days 1 to 6. Also included are days 0 and 7 to show how they deviate from the linear trend established over days 1 to 6. The growth rate was found to be $0.321 \pm 0.009$ (mean±se) day$^{-1}$ for days 1-6.

Figure 33. Ln of abs. vs. time for the ICP experiment. The growth rate was found to be $0.321 \pm 0.009$ inverse days over days 1-6 3N-BB media in 2L bottles. Error is standard deviation with N=4.
A significant amount of evaporation was experienced from days 13 onward. As the evaporation artificially inflated the biomass values, it also may have inflated the substrate concentrations to the same degree.

3.3.1.1 Calibration of the ICP

The calibrators and QC standards were run through the ICP and calibration curves generated. The calibration solutions have a known concentration as noted in the aforementioned Table 5. This information was given to the ICP analysis software and it related the measurements from the samples to the values assigned to them. The ICP does not have infallible accuracy for all types of elemental solutions or ranges of those solutions. Calibrating the machine allows the user to understand the accurate measuring ranges of the ICP spectrometer as well as to gauge if the machine has any accuracy for a particular element at all.

The calibration data is shown in Figure 34.
ICP responses were linearly related to elemental concentration for all elements measured except for sulfur and chlorine. Sulfur seems to have an exponential relationship between actual concentrations and measured ones. Chlorine was a long shot as a measureable element. Chlorine cannot be measured accurately in the ICP because it is not a metal. Sodium levels saturated the ICP at all concentrations greater than Cal 5. The calibration data for sodium is only valid within this range. Potassium also saturated the ICP at all concentrations greater than Cal 4. This means, that for both of these substrates, the ICP was not able to measure any change in their concentrations until they
decreased to these measureable levels. Diluting the calibrators, QC standards, and all of the samples to measureable levels would allow for concentration curves for these elements to be produced as seen for the other elements in the next section.

Figure 35 are the quality control standard actual concentrations vs. the measured ICP concentrations. Trends in this data are the same as that of the calibrators. There was poor accuracy in the measurement of chlorine or sulfur for any of the three QCs. Sodium measurements became more and more accurate as the concentration decreased and it moved away from saturation levels. As the QC concentration decreased, the accuracy of measurement for zinc diminished. The same held true for molybdenum. All other elements exhibited strong accuracy in concentration measurement over the entire range of QCs. This validated the trends from the calibrators.
Figure 35. Graphs of measured quality control standard concentrations vs the actual prepared concentrations.

The measured starting concentrations for all of the substrates fell within ±50% of what their actual starting concentrations should have been, based on 3N-BB media formulation, except for zinc and cobalt. Table 9 shows this comparison.
Table 9. The theoretical starting concentrations for each element compared to the actual, measured starting concentrations for each element in ppm from day 0 cell sampling. No data present for Na or K wherein the ICP was saturated. No data for S or Cl wherein the calibrator data did not exhibit a linear fit, nor for Na and K wherein the concentrations saturated the ICP.

<table>
<thead>
<tr>
<th>Element</th>
<th>Starting Concentrations in ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Theoretical</td>
</tr>
<tr>
<td>Ca</td>
<td>6.84</td>
</tr>
<tr>
<td>Mg</td>
<td>7.43</td>
</tr>
<tr>
<td>P</td>
<td>53.40</td>
</tr>
<tr>
<td>Fe</td>
<td>0.12</td>
</tr>
<tr>
<td>Zn</td>
<td>0.014</td>
</tr>
<tr>
<td>Mn</td>
<td>0.069</td>
</tr>
<tr>
<td>Co</td>
<td>0.0055</td>
</tr>
<tr>
<td>Mo</td>
<td>0.013</td>
</tr>
</tbody>
</table>

There is an explanation for why the actual, measured, initial values for zinc and cobalt deviate so far from their expected values. In order to make a 1000 mL solution of 3N-BB media, each salt (as detailed in section 2.1 Algae culture) is weighed out individually and added to the total volume of DI water. While in most cases, this mass of salt is considerable, this was not the case when preparing the trace metals solution. The mass of zinc chloride used to create the trace metals solution was only 0.0025g. On a scale that only measures to 4 decimal places, one begins to approach the lower limits of the scale. In the case of cobalt chloride, it got even worse. The mass of cobalt chloride needed for the trace metals solution was only 0.001 g, that was less than half of what was necessary for zinc. To put this into perspective, even manganese chloride required a mass of at least 0.0205g, almost ten times that of zinc. The fact that some these salts had become rock solid (they had become hydrated, further skewing proper weights) in their stock containers did not help to alleviate the concerns of trying to measure a precise, miniscule mass of any of these compounds. To again further complicate things, occasionally, when preparing the trace metals solution, it was very difficult to force all of the metals salts required for the recipe to fully dissolve in solution. The NaEDTA
increased metal solubility but only if it is added before all of the other metal salts are added.

3.3.1.2 Substrate Concentration with time

Except for sodium, sulfur, and chlorine, all the other substrates were determinable across the full range of the experiment. Graphs for substrate concentration vs time are presented in Figure 36.
3.3.1.3 Biomass concentration vs. substrate concentration

Biomass concentration (g/L) is plotted against substrate concentration (g/L) in Figure 37 for days 0-13. Because of evaporation experienced past day 13, that data was ignored for the purposes of yield calculation.
Figure 37. Graphs of biomass concentration (y-axis in g/L) vs substrate concentration (x-axis in g/L) for days 0-12 where yield coefficient is equal to the negative of the slope of the data.
There are two main conversions required to obtain yield coefficients in units of g/g. The first is to convert substrate concentration from ppm (by mass) to g/L as follows:

**Equation 4. Conversion from ppm to g/L**

\[
ppm \ s = \frac{1 g(s)}{10^6 g(H_2O)} \times \frac{957 g \ H_2O}{1 L \ H_2O} = \frac{g \ s}{L}
\]

The second is to convert biomass concentration from absorbance units to g/L:

**Equation 5. Conversion from absorbance units to g/L**

\[
\frac{g}{L} \ biomass = 0.52 \times absorbance \ (Brittany \ Studmire)
\]

From the graphs of biomass vs. substrate concentration above, the slope is:

**Equation 6. Slope**

\[
\frac{\Delta X}{\Delta s}
\]

The yield coefficient for algal biomass from a specific substrate is shown in

**Equation 7. Average yield coefficient**

\[
Y_{X/s} = -\frac{\Delta X}{\Delta s}
\]

The values and standard errors of the yield coefficients are found in Table X as are the p-values for the yield coefficients.
Table 10. Yield coefficients (g/g) for each element over days 0-13 along with standard error and p-values determined from Microsoft Excel simple linear regression analysis. P-values less than 0.05 mean that the slope is statistically significant, or rather that it was not achieved by chance on a 95% confidence interval.

<table>
<thead>
<tr>
<th></th>
<th>Yield (g/g)</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>247</td>
<td>57.8</td>
<td>7.82E-05</td>
</tr>
<tr>
<td>Mg</td>
<td>476</td>
<td>132</td>
<td>6.87E-04</td>
</tr>
<tr>
<td>P</td>
<td>-99.7</td>
<td>12.7</td>
<td>1.98E-10</td>
</tr>
<tr>
<td>Fe</td>
<td>8550</td>
<td>824</td>
<td>2.26E-14</td>
</tr>
<tr>
<td>Zn</td>
<td>464</td>
<td>515</td>
<td>3.72E-01</td>
</tr>
<tr>
<td>Mn</td>
<td>5310</td>
<td>1450</td>
<td>6.02E-04</td>
</tr>
<tr>
<td>Co</td>
<td>-148000</td>
<td>80000</td>
<td>6.95E-02</td>
</tr>
<tr>
<td>Mo</td>
<td>16600</td>
<td>11300</td>
<td>1.49E-01</td>
</tr>
</tbody>
</table>

3.3.2 Discussion

Larger yield coefficients imply that less mass of substrate is necessary to produce the equivalent amount of biomass. Yield coefficients for phosphorus and cobalt were negative. The standard error of the yield for zinc was greater than the yield itself. Similar results were found for the yield for molybdenum. The yields for zinc and molybdenum may not have been as reliable as the others.

Studies have shown, although cobalt is a heavy metal which is toxic at high enough levels like cadmium (Van Gemert, Ten Hoopen, Roels, & Fuchs, 1985), *S. obliquus* growth improves with the addition of cobalt up to 3ppm. This trend can be further increased to 4.5ppm in the presence of vitamin B2 or B6 (Desouky, 2011). Cobalt as a heavy metal did not appear to be consumed to any extent and or seem to have an important impact on the growth of *S. dimorphus*. Most heavy metals are merely bio-adsorbed into the cells and removed from the media.

Phosphorus showed a negative yield as well as positive trend in concentration with time (Figure 36) with a very low p-value. The ICP was unable to detect any
consumption of this nutrient. Again, unless evaporation was so severe over the course of the experiment as to drown out any trend, that would suggest phosphorus consumption (one of the most important elemental components of the algal cells) then there must have been some other effect at play. Aslan and Kapdan (2006) showed that phosphate removal for *C. vulgaris* in artificial wastewater decreased when light limitation became an issue. With 2L vessels used to culture this experiment, light limitation could very well have been a determining factor in the nutrient uptake rates of phosphorus and the other elements measured. Another study showed that *S. dimorphus* growth increased with increasing phosphorus level up to 0.5 mg/L at which point growth rates began to suffer (Chiou & Boyd, 1974). With initial concentration levels at 53 ppm by mass, or roughly 53 mg/L, starting concentration for phosphorus were 100 times what was observed to limit growth in the literature. If phosphorus was in fact fed in such extreme excess, it is not unreasonable that the ICP was unable to measure such small changes in such a large value. Alternatively, effects from phospholipid accumulation, variation from initial inoculum, or cell degradation with time could have affected this result. Table 11 shows the elemental composition of *Chlorella vulgaris*, another green microalgaes species, and the theoretical yield that those mass percents suggest. Assuming that all nutrient consumption goes directly toward biomass accumulation, the theoretical yield is simply the inverse of the mass percent.
Table 11. Elemental composition of C. vulgaris. Theoretical yield is determined by taking the inverse of the mass composition. Relevant yields from this experiment are seen in bold on the right. (Mandalam & Palsson, 1998)

<table>
<thead>
<tr>
<th>Element</th>
<th>Mass %</th>
<th>Theoretical Yields</th>
<th>Experimental Yields</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>51.4-72.6</td>
<td>1.37-1.94</td>
<td>N/A</td>
</tr>
<tr>
<td>Oxygen</td>
<td>11.6–28.5</td>
<td>3.51-8.62</td>
<td>N/A</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>7.0–10.0</td>
<td>10-14.3</td>
<td>N/A</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>6.2–7.7</td>
<td>13.0-16.1</td>
<td>N/A</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>1.0–2.0</td>
<td>50-100</td>
<td>N/A</td>
</tr>
<tr>
<td>Potassium</td>
<td>0.85–1.62</td>
<td>61.7-118</td>
<td>N/A</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.36–0.80</td>
<td>125-277</td>
<td>476</td>
</tr>
<tr>
<td>Sulfur</td>
<td>0.28–0.39</td>
<td>256-357</td>
<td>N/A</td>
</tr>
<tr>
<td>Iron</td>
<td>0.04–0.55</td>
<td>181-2500</td>
<td>8549</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.005–0.08</td>
<td>1250-20000</td>
<td>247</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.0006–0.005</td>
<td>20000-166667</td>
<td>464</td>
</tr>
<tr>
<td>Copper</td>
<td>0.001–0.004</td>
<td>25000-100000</td>
<td>N/A</td>
</tr>
<tr>
<td>Manganese</td>
<td>0.002–0.01</td>
<td>10000-50000</td>
<td>5307</td>
</tr>
</tbody>
</table>

Except for carbon, hydrogen, oxygen, and nitrogen, the “basic building blocks of life”, phosphorus content is the next highest on the list of elemental compositions. Phosphorus is commonly considered to be a common element of fertilizers and basic nutrient for plant growth so it would make sense that the cells would consume phosphorus with time. The experimental data herein does not support this observation.

The experimental growth yields for magnesium, iron, calcium, and manganese in S. dimorphus were are all within an order of magnitude of the theoretical yields derived from Mandalam’s findings (Mandalam & Palsson, 1998). The yield for zinc is not very close. This might be due in part to the large error associated with the calculated yield for zinc or may be due to the fact that a different species of algae can have widely differing nutrient needs.
CHAPTER IV
CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusions

This work has shown that the individual components of the growth media, notably magnesium, have a profound impact on the growth and lipid production of the green microalgae, *Scenedesmus dimorphus*.

4.1.1 Determining optimal concentration effect of digestate media and supplementation of MgSO$_4$ and KPO$_4$ on *Scenedensus dimorphus*

- Filtering the digestate seemed to have no noticeable effect on lipid accumulation but had a positive effect on growth rate (Figure 19, Figure 20)

- ~1.25% and 1.75% digestate in water were optimal concentrations for maximizing growth and lipid concentration respectively
4.1.2 Determining Kinetics of Mg on Cell Growth in *Scenedesmus dimorphus*  
- A minimum magnesium concentration of $33 \pm 7\text{mg/L}$ was found to not limit growth rate (Figure 28, Figure 29) in 250mL Erlenmeyer flasks  
- A yield of $687 \pm 81\text{ g Mg/g}$ biomass was determined from the flasks that were found to be growth limited by initial MgSO$_4$ concentrations (Table 8)

4.1.3 ICP Spectral Analysis to Determine Nutrient Uptake Rates in *S. dimorphus*  
- Nutrient yields for algal biomass were found for eight measureable elements (Ca, Mg, Fe, and Mn all had significant positive yields (g/g):  
  - Mg ($476 \pm 132$)  
  - Ca ($247 \pm 57.8$)  
  - Fe ($8550 \pm 824$)  
  - Mn ($5310 \pm 1450$)  
- When compared to literature values (Mandalam & Palsson, 1998), yields for all comparable elements (Mg, Ca, Fe, Zn, and Mn) except Zn were within an order a magnitude difference (Table 11)

4.1.4 Comparisons Among Experiments  
Maximal growth rates differed between experiments. This was due to differences in the growth vessel. Average growth rate in the ICP experiment in 2L bottles was $0.32 \pm 0.009\text{ days}^{-1}$. The “digestate” experiments and “Monod” experiments, using 250 mL Erlenmeyer flasks, showed maximal growth rates of 0.5 and 0.6 days$^{-1}$ respectively. Larger growth vessels have less surface area to total volume, and thus less light reaches the interior of the vessel because it has to pass through more cells suspended in media.

The effect of light limitation in the 2L bottles can also be seen in the differences in calculated yields for magnesium between the “Monod” and “ICP” experiments. Light limitation was less of an issue in the Monod experiment conducted in smaller 250 mL Erlenmeyer flasks. This may have allowed the cells to grow more efficiently by reducing
the amount of chlorophyll necessary for growth. Alternatively, the cells in the 2L bottles in the ICP experiment may have needed to accumulate more chlorophyll in order to maintain a certain level of light-limited growth. This would explain why the magnesium yield determined from the Monod experiment was higher than in the ICP experiment. Less chlorophyll was necessary for maintaining phototrophic growth in the Monod experiment, so less magnesium was required for cellular reproduction which in turn drove up the yield.

4.1.5 Conclusions
To conclude, microalgae have many profound applications in the realms of biofuels and biotechnology but commercial viability can only be achieved by optimizing process parameters with time and cost as objectives. When considering the application of algal lipid as a biofuel feedstock, the growth rate and lipid production are the two most important aspects affecting the profitability of the process. This work has shed light on discovering parameters for optimization of the phototrophic growth of the green microalgae Scenedesmus dimorphus.

As a potential cost saving measure (digestate) digested animal waste, was found to be useful as replacement for the more chemically-defined and expensive 3N-BB growth medium when supplemented with certain nutrients it was lacking. The minimum concentration of MgSO₄ that did not limit growth was found which could prevent that overfeeding of that nutrient. Finally, the yield for magnesium with respect to biomass was determined along with the yields for many other important nutrients for sustained algal growth. These yields allow one to precisely feed nutrient amounts based on a current biomass concentration, so as to not overfeed any one nutrient. To minimize the amount of nutrients fed, while not limiting growth, is the best one can do to optimize a
phototrophic medium. In time, as research and development progresses, biofuel production from microalgae may reach a point where it can out-compete energies derived from fossil fuels. That would bring us one step closer to a more sustainable and ‘greener’ future.

4.2 Recommendations

Further research is recommended, including:

- Continue to investigate higher and lower levels of digestate, Mg, and P to flesh out growth and lipid responses
- Derive Monod parameters for other substrates
- Determine yield values for sulfur, sodium, potassium, and chlorine


APPENDIX A

Protocol for Use of PerkinElmer ICP
Courtesy Dr. Xiang Zhou (Instrument Manager for Chemistry Dept. Cleveland State University)
(Revision 2011-06-23)

GENERAL ASPECTS
A. Must receive training and permission from Dr. Xiang Zhou (the Manager) for use of the instrument.
B. Have to follow the General Rules for Mass Spec Facility and Instrumentation Center
C. Book the Instrument in advance (particularly for classes)
D. Do not leave your items in the public areas after work
E. Have to follow the protocol

CAUTIONS:
- Be sure the exhaust vent is in operation, and the shutter is open
- Never connect to wrong gas tanks (must be Argon to Argon and Nitrogen to Nitrogen)
- Never place empty tanks in the “Full Tanks Only” area
- Secure the gas tanks; users must promptly move empty tanks to Stock Room
- Never run oil samples or unclear samples; filtrate or centrifuge samples if there are particles

0. Prepare the Following Things in Advance:
- Washing Solution (fresh distilled water) and Cleaning Solution (10% HNO₃ in distilled water)
- Your samples, calibration blanks and standards (must be clear; in 2% nitric acid recommended)
  - It is highly recommended that samples are prepared with 2% nitric acid in de-ionized water
- The ICP Waste Container must be at least 30% empty

1. Login ICP Logbook

2. Do 2a if nitrogen is from the wall line OR do 2b if the nitrogen is from tank
2a. Open the main valve for the argon tank
   - Be sure the two shut-off valves in the wall N₂ line are open (the valve hands in down position)
2b. Open the main valves for the argon and nitrogen tanks
   - Do not adjust the regulators
   - Change tanks (by users) when tank pressure is low (below 300 psi)
   - Procedure to change tanks: a) close the valve for the low pressure tank; b) switch the regulator from the low pressure to a full tank. Must be “Ar regulator to Ar tank” and “N₂ regulator to N₂ tank”
   - Never place empty tanks in “Full Tanks Only” area; Always secure tanks
   - Users must promptly move empty tanks to the Stock Room in “Empty” area
3. **Switch on ICP** (be sure the cooler is on also)
4. **Switch on / Log in PC**
5. **Generate Your Folders in PC if you do not have one**
   - Generate a file folder under C: (First Last Names)
   - Generate an ICP Workspace folder under C:\PE\Xiang\Workspaces\ (First Last Names)
6. **Open WinLab32.** Wait for a couple of minutes for communication

F1 for help with the software

**7A. Generate Method from Default Method (P. 2)**
- Usually generate method by modification of an existing method as Section 7B

**7B. Generate Method by Modification of an Existing Method (P. 2)**

**7C. Open a Method:** File – Open – Method – Select you method to open

**7A. Generate Method from Default Method**
- File – New – Method
- Select “Default and Plasma Conditions: Aqueous” in Create New Method Window
- Click Modify to verify the parameters (Aqueous, 15, 0.2, 0.8, 1300, 1.5, Wet, Instant);
  - Click OK, OK, and then work in Method Editor Win

  a. **Spectrometer**
     - Define Elements: click Periodic Table; right click an element; click a wavelength, Enter in Method; for Function, select Analyte or Internal Standard
     - Settings: Normal for wavelength above 190 nm; Auto; Delay Time: 20 sec;
       - Replicates: 3 usually; Measured by: Element
     - Spectral Windows: do nothing
  
  b. **Sampler**
     - Plasma: 20 sec; Same for All Elements; Wet; Instant
     - Peristaltic Pump: 1.5 ml/min; 0 sec
     - Autosampler: do nothing
  
  c. **Process**
     - Peak Processing: Peak Area; 7 Points
     - Spectral Corrections: None; 2-Points; Default values for BGC
     - Internal Standard: Yes or No depends
     - Internal Stds Checks: do nothing
  
  d. **Calibration**
     - Define Standards: Do not check Method of Auditions; Calib Blank1 given B1 and 1; Calib Std1 given S1 and 2; Calib Std2 given S2 and 3;
     - Calib Units and Concentrations: give Units and the concentrations
     - Blank Usage: B1
     - Equations and Sample Units: Lin, Calc Int; unit; 3; 4
     - Initial Calibration: Start by constructing new calibration curve
     - Multiline Calibration: Do not check
  
  e. **Checks:** do nothing (for auto sampler)
  
  f. **OK QC:** do nothing (for auto sampler)
  
  g. **Options:** check Analytical Header, Method Header, Sample Header, Short; Replicate Data, Means & Statistics, Matrix Reports, Calibration Summary; Spectral Data
  
  h. **Save the Method:** File – Save As – Method - Method Name: Your Initials-yymmdd-ID
  
  i. **Check Method (workable or not):** Edit – Check Method
  
  j. **Exit the Method Editor Win**

**7B. Generate Method by Modification of Existing Method**
a. File – Open – Method;
b. Select a reference method (e.g. All-training), then click OK
c. File – Save As – Method – Name: Your Initials-yymmdd-ID – Click OK
d. Click Method Ed icon (the 2nd)
e. Do modifications following the Section 7A-a to 7A-j

8. Open Working Sub-Windows: Plasma; Manual; Spectra; Calibration;

9. Save Workspace (a Workspace is a file including a set of working sub-windows)
   - File – Save As – Workspace – Double click your Workspace folder – File Name (initials-ID)

10. Open Workspace (a Workspace can be used repeatedly)
    - File – Open –Workspace – Double click your Workspace folder – Select one to open

11. Setup Peristaltic Pump
    - Left track for Inlet tubing and Right track for Outlet tubing
    - For the flow, the earlier pathway at Right-Lower side and later pathway at Left-Upper side
    - The screws are set at ~ 3 mm position (do not change usually)
    - Be sure the tubing are in tracks, and then lock the tracks
    - Place the Inlet tubing in Washing Solution

12. Click on Plasma
    - Be sure the flow is right after the pump starts to run
    - Adjust the screws as needed (do not over tighten)
    - Replace the tubing as needed
    - Click off Pump for saving samples
    - Click off Plasma for saving gases (ICP consumes two tanks of gas in 8 hours)

13. Generate Date File Name (all the results data are in the C:\PE\Xiang\Results\)
    - Click Open Results Data Set Name in Manual Analysis Control Win
    - Name: Initials-yymmdd-ID
    - Each set of experiments should have a separate data file name

14. Manual Analysis
    - Put Inlet in the blank B1; in ~ 20 seconds, click Analyze Blank; Put Inlet in Washing Solution
    - Put Inlet in the calibration standard S1; click Analyze Standard; put Inlet in Washing Solution; repeat the steps with other standards
    - Run samples in a similar manner by click Analyze Sample. You can give each sample an ID

15. Clean the System (this step is important to get rid of contamination from the instrument)
    - Pump the Cleaning Solution for 15 minutes

16. Wash the System in the End for 5 minutes using Washing Solution

17. Click off Plasma and Pump during Long Breaks

18. Generate Report and Print Out Data (P. 4); backup your data if important

19. Finishing with the Instrument
    - Click off Plasma
    - Do 20a or 20b with the gas supplies
    - Unlock the tracks for the pump; loosen the tubing from the higher side
    - Exit WinLab32; shutdown the PC
    - Switch off ICP
20. **Do 20a if the nitrogen is from the wall line OR do 20b if the nitrogen is from the nitrogen tank**
   20a. Close the main valve for the argon tank; leave the shut-off valves in the wall line open
   20b. Close the main valves for the argon and nitrogen tanks

21. **Must clean up and organize the working area:**
   Do not leave your own items in the public area
   Put the Facility and your items in assigned locations
   The desk, floor, cabinet, refrigeration, and sink should be clean and organized

   The items need to be handled:
   A. your samples, solvents, reagents, tips, vials, containers, etc.
   B. your wastes: gloves, Kimwiper, cleaning paper
   C. your data, articles, other printed out materials
   D. your data, articles, other printed out materials
   E. Facility tools, syringes, solvent, standard, accessories, washing bottles
   F. Logbook, manuals, and other documents

22. **Logout the Logbook**

**18. Generate Report and Print Out Data (P. 4)**

- File – Utilities – Data Manager
- In Data Manager Win: File - New Data Set Display Window - Click results - Open
- Select your Results Data Set Name; then click Report
- In Data Reporting Wizard, click Use Existing Design, Browse, select All-training to open, and then click Next 7 times (modify as needed)
- Save your report design (initials-ID);
- Click Preview; click the printing icon if you like the report design
- (Optional) If you want to save the data in PDF or Word file, in the Preview Win:
  a. Click Export (next to Printer icon)
  b. Format: PDF; Destination: Disk file; OK; OK
  c. Open your file folder under C:
  d. Type in the data file name: Data-yyyydd-ID; Save
  e. Open the PDF or Word file in your file folder; print it

**CAUTIONS:**

- **Log In / Out:** follow the instructions in the front page; use the booking section only for booking; fill in every column in the end
- **Samples:** never run oil samples; preparation of samples in 2% HNO₃ is highly recommended; must be particle-free; filtrate / centrifuge samples as needed
- **Tubing:** must loosen the pump tubing in the end
- **Cleanup:** do not leave any wastes after use; well clean and organize the work area

**Gas tanks handling:**

- Never connect to wrong tanks
  (must be the argon regulator to argon tank and the nitrogen regulator to nitrogen tank)
- Never place empty tanks in the “Full Tanks Only” area
- Users are responsible to move the empty tanks to the Chemistry Stock room promptly
  (to the empty tank area in the lab temporarily if the Stock Room is locked)
- Secure the gas tanks
- Do not adjust regulator
- Must turn off the tank valves in the end