ETD Archive

2014

Mineral Uptake Rates and Yield Coefficients of the Green Microalgae Scenedesmus Dimorphus

Grace N. Mahfouz
Cleveland State University

MINERAL UPTAKE RATES AND YIELD

COEFFICIENTS OF THE GREEN MICROALGAE

SCENEDESMUS DIMORPHUS

GRACE N. MAHFOUZ

Bachelor of Science in Chemistry

John Carroll University

May 2011

Submitted in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE IN CHEMICAL ENGINEERING

at the

CLEVELAND STATE UNIVERSITY

December 2014

We hereby approve this thesis for

Grace N. Mahfouz

Candidate for the Master of Science in Chemical Engineering degree for the

Department of Chemical and Biomedical Engineering

and the CLEVELAND STATE UNIVERSITY

College of Graduate Studies

_____
Thesis Chairperson, Dr. Joanne M. Belovich

_____Chemical and Biomedical Engineering Department_____
Department & Date

_____
Thesis Committee Member, Dr. Jorge E. Gatica

_____Chemical and Biomedical Engineering Department_____
Department & Date

_____
Thesis Committee Member, Dr. Orhan Talu

_____Chemical and Biomedical Engineering Department_____
Department & Date

_____
Thesis Committee Member, Dr. Xiang Zhou

_____ Chemistry Department _____
Department & Date

Student's Date of Defense: December 5, 2014

Mineral Uptake Rates and Yield Coefficients of the Green Microalgae Scenedesmus Dimorphus

Grace N. Mahfouz

ABSTRACT

The need for renewable sources of energy has become critical as fossil fuel resources, which include oil, coal, and natural gas, are known to contribute to climate change and will eventually be depleted. Biofuels that originate from the fat of plants, animals, microalgae, and bacteria are an attractive alternative. Microalgae, in particular, are strong candidates for biofuel production since they have a high growth rate and lipid content (up to 50% in some species). The goal of this research was to optimize 3N-BBM+V recipe (Bold Basal Medium with 3-fold Nitrogen and Vitamins) in an effort of reducing the amount of nutrients used in growing the green microalgae *Scenedesmus dimorphus*, and hence lower the cost of cultivation. Three runs were carried out on a 5L bioreactor in batch mode. The average growth rate of *S. dimorphus* was 0.17 ± 0.03 days^{-1}.

Inductively Coupled Plasma was utilized to monitor substrate concentration with time in the bioreactor which allowed the determination of yield coefficients in gdw biomass/g substrate for magnesium (253±50), calcium (341±84), potassium (61±15), phosphorous (60±13), and iron (5807±3000). These yields were utilized to calculate the optimal starting substrate concentration in the media in mg/L for magnesium (4.3±0.3), calcium (3.2±0.2), potassium (18±1), phosphorous (18±1), and iron (0.19±0.015). The

inverse of mass fraction for elements in the biomass was obtained in gdw biomass/g substrate for magnesium (309 ± 9), calcium (341 ± 84), potassium (98 ± 6), phosphorous (70 ± 5), and iron (8075 ± 2500). The average substrate consumption rates in the three runs for Mg, Ca, Fe, P, and K are 0.61 ± 0.41, 0.31 ± 0.64, 0.008 ± 0.03, 2.8 ± 1.6, and 1.3 ± 2.2 mg substrate/gdw biomass.day respectively. Potassium and phosphorous have the lowest yields which is consistent with them having the highest consumption rates.

The bead beater and mortar and pestle cell lysing methods were utilized to determine which is the most consistent and efficient in terms of lipid extraction. The average lipid content obtained using the bead beater and mortar and pestle were $9 \pm 2\%$ and $19 \pm 2\%$ respectively, making the mortar and pestle the cell lysing method of choice in this research. The lipid content on the last day of the three runs of the photobioreactor was 11-16%.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE

A600: Absorbance or optical density at 600 nm

DO: Dissolved oxygen

PBR: photobioreactor

CSTR: Continuously Stirred Tank Reactor

S. dimorphus: *Scenedesmus dimorphus*

S. obliquus: *Scenedesmus obliquus*

BBM: Bold basal medium

3N: 3-fold sodium nitrate concentration

ICP-OES: Inductively coupled plasma-optical emission spectroscopy

X: Biomass concentration in gdw/L

RO water: Reverse osmosis water, same as DI water

DI water: De-ionized or distilled water, same as RO water

BOD: biological oxygen demand

QC: quality control

Std: standard

Mg: Magnesium

Ca: Calcium

Fe: Iron

P: Phosphorous

K: Potassium

gdw: grams dry weight

OD: optical density

ATP: adenosine triphosphate

YX/s: yield coefficient

s: substrate

rs/ r'_s: rate of substrate consumption

t: time, specific day of the experiment

F: volumetric flow rate

s_0: substrate concentrations in the feed

s_f: substrate concentrations in the effluent

V: reactor working volume

D: Dilution rate

μ_{net}: specific growth rate

μ_{max}: maximum growth rate

K_s: Monod constant

X_{max}: maximum biomass concentration

C_i: concentration of element i

M_B: dry mass of biomass sample

t-clac: t-calculated, student's calculated t-value

t-table: tabulated t distribution critical values

CHAPTER I

INTRODUCTION

In this day and age, the need for renewable and green sources of energy has become critical as fossil fuel resources, which include oil, coal, and natural gas, will eventually run out due to our extreme dependence on them. Our daily activities are significantly influenced by the availability of fuel, making it a vital component in humanity's function. The use of fossil fuels generates a significant amount of greenhouse gases into the atmosphere, such as carbon dioxide, which are responsible for global warming. These greenhouse gases trap the sun's heat thus elevating the earth's temperature. Figure 1 shows the total U.S. greenhouse gas emissions by economic sector in 2011. In the United States, the burning of fossil fuels for electricity, transportation, and industrial purposes is the major source of greenhouse gases.
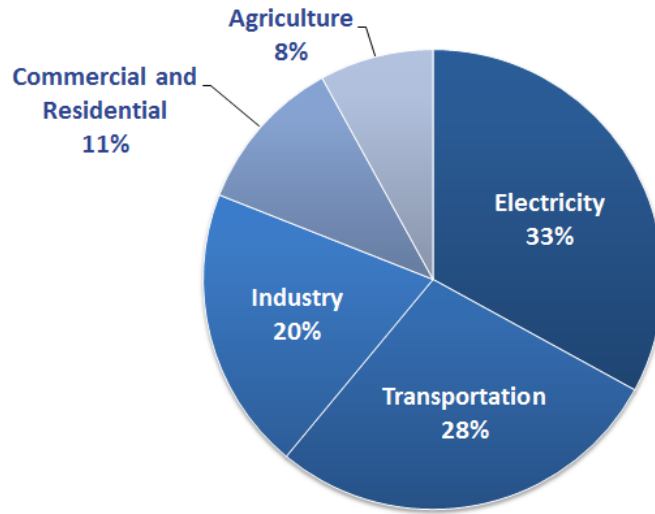
Figure 1: Total U.S. greenhouse gas emissions by economic sector in 2011 (http://www.epa.gov/climatechange/ghgemissions/sources.html#ref1).

Hubbert, a geologist who worked at Shell Oil, indicated that the petroleum production as a function of time takes the shape of a bell curve. In a paper he wrote in 1956, Hubbert predicted that oil production in the United States would peak sometime between 1965 to 1970. His claim was confirmed in 1970 (Willner, 2013). Therefore, to reduce the impact of this energy crisis, renewable, biodegradable, and environmentally friendly sources of energy are needed to produce biofuels that originate from the fat of plants, animals, microalgae, from bacteria and solid wastes (Velichkova et al., 2013).

The term algae can refer to macroalgae or microalgae. However, all the algae terms referred to in this thesis correspond to microalgae. Macroalgae are large aquatic plants usually called seaweeds. On the other hand, microalgae are microscopic aquatic plants. Microalgae can grow in fresh-, brackish-, sea- and waste-water, however, unlike a typical plant they do not have stems, leaves, or roots. They can flourish in a large number of climates and can grow in open ponds and photobioreactors all year long. Microalgae are strong candidates for biofuel production since they have high growth rate and lipid content (up to 50% in some species). They grow fast and are able to double their number of cells in one day. Their cultivation does not require arable land or fresh water that are otherwise needed for growing food crops. Thus, they do not compete with agriculture as they can be even cultivated in the desert. Microalgae consume carbon dioxide emitted by transportation, industry, as well as other sources, hence, reducing the impact of pollution. Furthermore, being capable of growing in waste water, microalgae can help purify it (allaboutalgae.com/benefits).

Microalgae serve as a feedstock for "green" fuels such as biodiesel, methane, hydrogen, and ethanol which are environmentally friendly and non-toxic (Velichkova et

al., 2013). The use of microalgae goes beyond them only being a source of biofuel, as they are utilized as an animal feed and a dietary supplement for humans. Algae are also included in some plastics, fertilizers, and cosmetics. Thus, the entire algae biomass is converted to valuable products. However, crops such as corn, sugar cane, rapeseed, soybeans, and sunflower result in a significant amount of biomass being wasted (allaboutalgae.com/benefits). Table 1 presents the productive capacity of biofuels from certain crops, in gallons of oil per acre per year. It can be deduced that algae produce the largest amount of biofuel for the same amount of land used by other crops, hence, they have a very high photosynthetic efficiency.

Table 1: Productive capacity of biofuels from certain crops, in gallons of oil per acre per year (Biodiesel, 2008).

Crop	Productive Capacity (US oil gal/acre*year)
Algae	8,000 (Schmidt, 2007)
Palm Oil	650
Corn (ethanol)	420 (Worldwatch Institute, 2013)
Jatropha	175
Mustard	140
Rapeseed	110-145
Soybean	40-50

The number of algae species is approximated to be something between 30,000 to more than 1 million. Algae belong to four kingdoms: Bacteria, Plantae, Chromista, and Protozoa (Guiry, 2012). *Scenedesmus dimorphus* or *obliquus* is a strain of green microalgae that is of interest due to its high lipid content of 16-40% compared to other strains (Velichkova et al., 2013). It has a crescent or bean shape as illustrated in Figure 2, is unicellular or can grow in colonies of four or more cells. It reproduces through cell division along the width of colony members resulting in a new colony containing the same number of cells as the original (www.algaedepot.org). A single *S. dimorphus* cell is

made up of a cell wall, a nucleus, lipid globules, starch granules and a pyrenoid (Letcher et al., 2013).
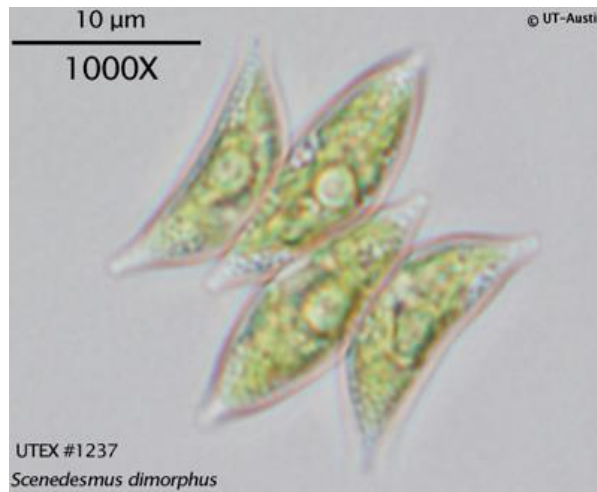
Figure 2: *Scenedesmus dimorphus* cells
(http://www.sbs.utexas.edu/utex/images/algae/1237%20scenedesmus%20dimorphus%202.jpg).

The composition of the 3N-BB media, on which the green microalgae *S. dimorphus* depends for growth, has a significant impact on the growth rate and lipid content. From an economic standpoint, it is best to limit the amount of nutrients used in the media to concentrations that would produce the highest possible growth rates, yields, and lipid content.

Little is known about the mineral composition of microalgae (x_i) and there is a limited amount of literature data about the yield coefficient ($Y_{x/s}$). The experiment on the 5 L PBR in batch mode was focused on monitoring the concentrations of magnesium, calcium, potassium, iron, and phosphorous with time in *S. dimorphus* using inductively coupled plasma optical emission spectroscopy (ICP-OES). Three similar experiments were conducted on the PBR with only a slight change in the growth conditions, as discussed in the Materials and Methods chapter of this thesis. The major goal of this set of experiments was to optimize the reactor system by determining the minimum amount

of nutrients needed in 3N-BB media and to find the consumption rate of the five key nutrients in an effort of lowering the cost of cultivation.

The yield coefficient for these nutrients was determined using two different methods, both involving the ratio of biomass concentration to substrate concentration. However, in one method the substrate concentration was found in the supernatant, while in the other it was determined in the biomass. Lipid extraction on the *S. dimorphus* biomass collected the last day of the experiment was also carried out, and the growth rate in each run was derived.

In another experiment, the growth rate in batch mode, of *S. dimorphus* cultivated in 2L bottles with a working volume of 1.5L in 3N-BB media was measured. The first experiment determined which biomass permeabilization technique, mortar and pestle or bead-beater, is more effective at breaking the *S. dimorphus* cell walls in order to facilitate lipid extraction. It was believed that the bead beater would produce a more homogeneous and finer algae powder compared to the mortar and pestle, and thus a higher lipid content. Lipid extraction was carried out using the Bligh-Dyer method with chloroform and methanol being the extraction solvents.

CHAPTER II

BACKGROUND

2.1   Biochemistry of Photosynthesis and Oil Production

2.1.1   Photosynthesis

Microalgae depend on photosynthesis to produce their own food, in which they require carbon dioxide, light, nutrients, and water to produce oxygen, lipids, and chemical energy as shown in Figure 3, which also includes the major steps needed in producing biodiesel starting with photosynthesis. The chemical reaction involved in photosynthesis is the following (Photosynthesis, 2014):

$$6CO_2 + 6H_2O + \text{light energy} \rightarrow C_6H_{12}O_6 + 6O_2$$
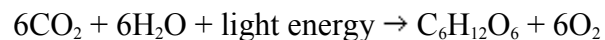
The chemical energy produced is in the form of carbohydrates (sugars). Some of the sugar resulting from photosynthesis is converted to lipids in the chloroplast. These lipids store energy and are made up of fatty acids. Photosynthesis occurs in the chloroplasts that contain chlorophyll a and chlorophyll b, which are green pigments that give microalgae

their color and capture light. Hence, microalgae are photoautotrophs since they utilize

carbon dioxide as their carbon source and light as their source of energy.
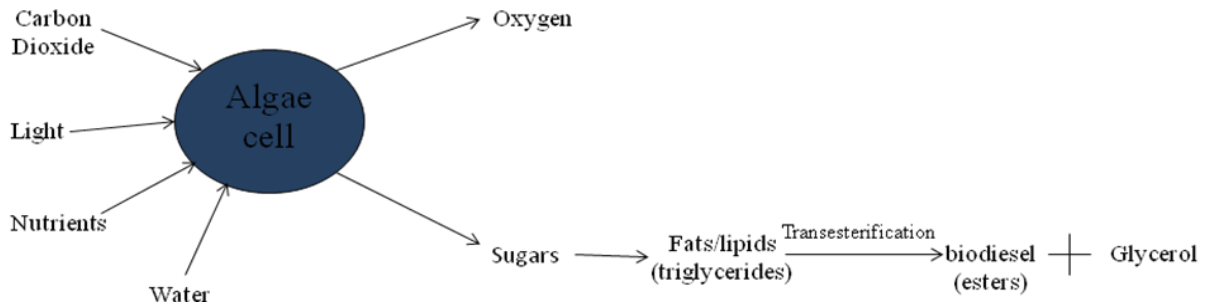
Figure 3: From photosynthesis to biodiesel

2.1.2 Converting Lipids to Biodiesel through Transesterification

The production and consumption of biodiesel is a "greener" alternative compared

to the utilization of fossil fuels. However, biodiesel still impacts the environment due to

the fertilizers used in cultivating the crops, its transesterification process, the harsh

solvents used in oil extraction, its purification, drying, and transportation.

Transesterification is the chemical reaction responsible for the production of biodiesel.

Fats/lipids (triglycerides) are reacted with alcohol, such as methanol or ethanol, which

converts them into biodiesel (esters) and a glycerol by-product. This reaction is usually

catalyzed by a strong base like sodium or potassium hydroxide. Figure 4 shows the

production of methyl esters using methanol (University of Strathclyde, Scotland, Energy

Systems Research Unit, 2014).
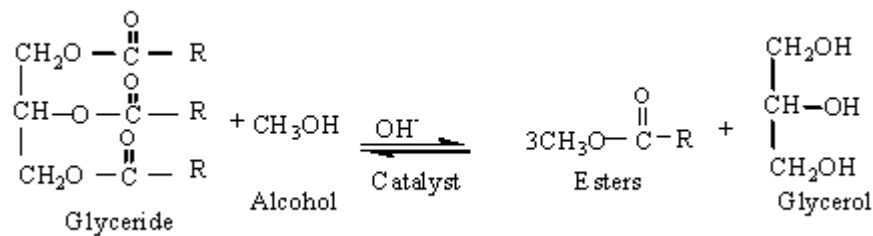
Figure 4: Transesterification reaction of triglyceride to produce methyl esters (http://www.esru.strath.ac.uk/EandE/Web_sites/02-03/biofuels/what_biodiesel.htm).

8

Glycerine is the base molecule for triglyceride which also has three fatty acid chains. Transesterification is a reversible reaction; therefore, the alcohol must be in excess in order to ensure that the forward reaction is favored and all the triglycerides are used up. When the reaction is complete, the glycerol layer separates from the ester layer. Glycerol can be further purified and used, for example, in the pharmaceutical and cosmetic industries (University of Strathclyde, Scotland, Energy Systems Research Unit, 2014).

2.1.3 Cell Permeabilization

The main steps involved in recovering lipids from algae are physical cell lysing or permeabilization and chemical lipid extraction processes. During cell lysing, the cells are broken up releasing lipids and other cell components. Lab-scale cell lysing methods include grinding, bead-beater, sonication, microwaves, autoclaving, and osmotic shock. Chemical lipid extraction procedures require the use of solvents such as chloroform and methanol used in the Bligh and Dyer method. Lee et al. (2010) performed almost all of the above permeabilization methods on *S*. *dimorphus*. The bead-beater shattered cells by means of high speed spinning for 5 minutes using fine beads (0.1 mm diameter). Sonication cracked cell walls and membranes as a result of cavitation. Microwaves broke up cells using high frequency waves (2450 MHz) at 100°C for 5 minutes. Autoclaving was done at a high temperature (125°C) and pressure (1.5 MPa) for 5 minutes. Osmotic shock used a 10% sodium chloride solution to crack cells through osmotic pressure for 48 hours with a vortex of 1 minute. Results using these methods (Lee et al. 2010) are summarized in Figure 5.

The mortar and pestle and bead beater cell lysing methods were compared in this research to determine which is capable of enhancing lipid recovery and giving more reproducible results. Previous research like that done by a colleague, Bahareh Kanani (2013), showed that the mortar and pestle gave the most consistent results among the various cell lysing methods. However, there is a limited amount of literature data concerning the usage of the bead beater in comparison to the traditional mortar and pestle method. Hence, the effectiveness of the bead beater in comparison to mortar and pestle, in terms of algae permeabilization, was investigated here.
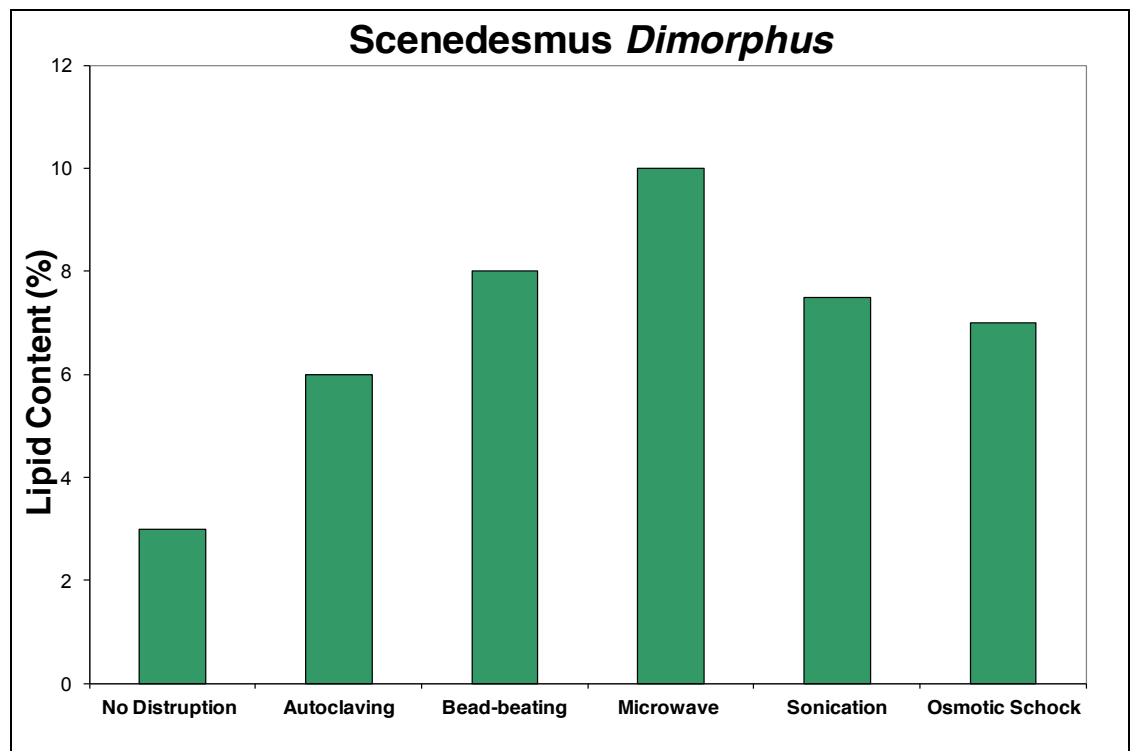
Figure 5: Comparison of lipid content using different cell lysing methods for *S. dimorphus* (Lee et al., 2010). All lipid extractions were performed using the Bligh-Dyer method with a 1:1 volumetric ratio of chloroform/methanol.

2.2   Importance of Phosphorous, Magnesium, Potassium, Calcium, and Iron in

Microalgae Metabolism

a) <u>Phosphorous</u>. Phosphorous plays an important role in algae growth and cell division. It

is present in many organic and inorganic compounds in the algae cells such as the DNA

and RNA. It is also a significant constituent of phospholipids that form the algae cell

membranes. Phosphorous is present in ATP (adenosine triphosphate) shown in Figure 6,

a molecule used for short-term chemical energy storage (Shuler and Kargi, 1992;

Martinez et al., 1999).
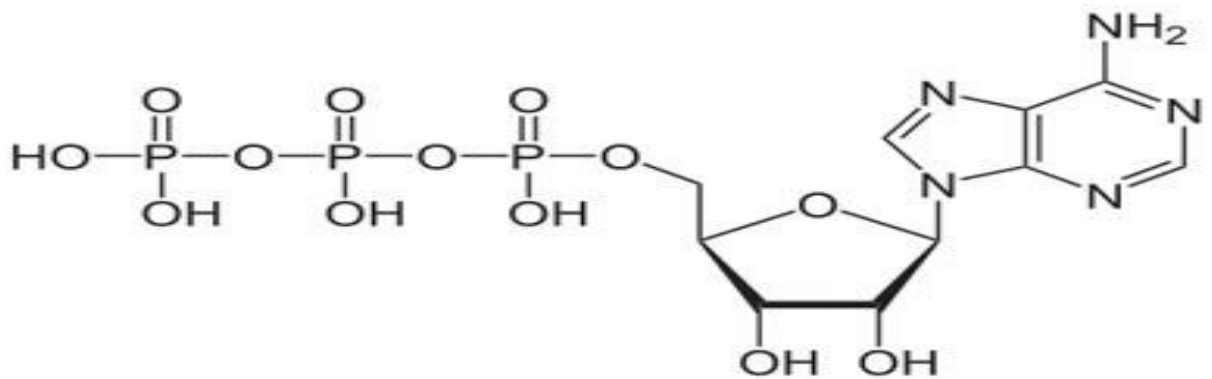
Figure 6: Adenosine Triphosphate (ATP)
(http://www.daviddarling.info/encyclopedia/A/ATP.html).

b) <u>Magnesium</u>. Algae cells need magnesium to synthesize chlorophyll (in the chloroplast)

which has a magnesium ion at its center (Figure 7). Chlorophyll is responsible for

absorbing sunlight for photosynthesis. Magnesium is present in many enzymes, and is a

co-factor for other enzymes.   Moreover, it is present in the cell walls and membranes

(Shuler and Kargi, 1992).

Figure 7: Chlorophyll a (http://scifun.chem.wisc.edu/chemweek/chlrphyl/chlrphyl.html).

c) Potassium. Potassium is a major cytoplasmic cation that affects the activity of enzymes since it is an enzyme cofactor. It is also needed for carbohydrate metabolism (Shuler and Kargi, 1992).

d) Calcium. Calcium is a constituent of algae cell walls, which gives them strength and support. Figure 8 shows the Ca^{2+} bridges between the pectin molecules within the cell wall. Furthermore, calcium is a cofactor for amylases and some proteases (Shuler and Kargi, 1992).

Figure 8: The presence of calcium in cell walls (http://www.mhhe.com/biosci/pae/botany/uno/graphics/uno01pob/vrl/images/0032.gif).

e) <u>Iron</u>. Iron plays an important role in algae growth as it is necessary for the synthesis of chlorophyll which is needed for photosynthesis (Figure 9). The enzymes made during the production of chlorophyll contain iron. Iron is also found in proteins such as ferredoxins and cytochromes (Shuler and Kargi, 1992; BioGro, Inc., 2013).
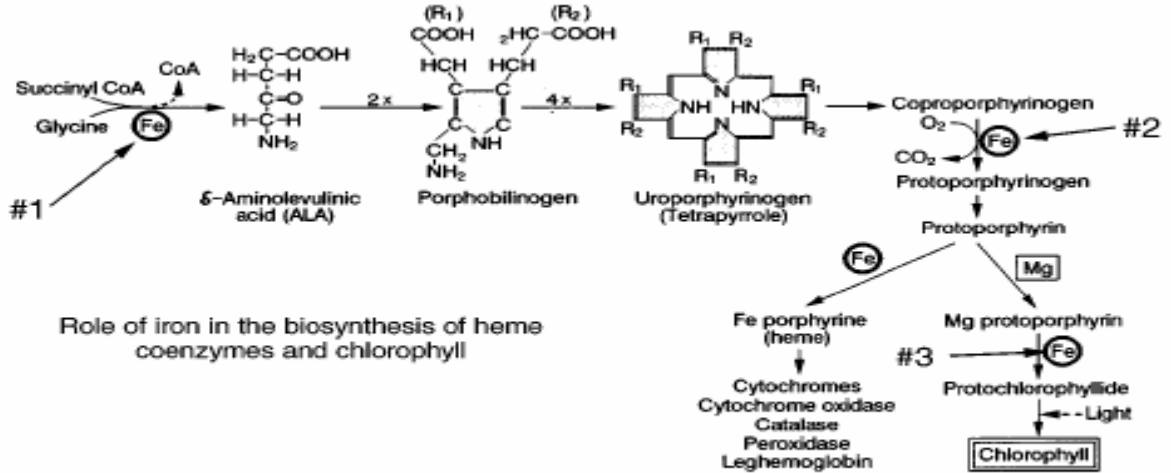
Figure 9: The use of iron in the formation of chlorophyll ("Mineral Nutrition of Higher Plants," Horst Marschner, Academic Press, page 315).

2.3 Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)

ICP-OES is an analytical technique used to determine the concentration of trace metals in liquid solutions. It is a powerful method as it allows analysis of multiple elements at a relatively low cost and with low detection limits. The high temperature of the argon plasma makes it inert and stable (not reactive), thus it reduces matrix inferences. It is important to ensure that the algae sample fed to the ICP is centrifuged well to prevent the presence of any organic particles that can affect the accuracy of the measurements. Phosphorous, magnesium, potassium, calcium, and iron were the elements of interest in this research. The elemental composition of the media in which *S. dimorphus* was cultivated in a 5 L photobioreactor was monitored with time using ICP.

13

Moreover, the algae biomass at the end the experiment was analyzed for elemental content by ICP after being digested with nitric acid.

The sample is introduced to the nebulizer of the ICP via a pump where it is converted into a mist. The tiny droplets are carried by the argon gas into the plasma, whereas large droplets exit the system into a waste container. The argon plasma has an average temperature of 7000 to 8000°C. It first desolvates the sample, vaporizing it into gas molecules, which in turn get atomized forming individual gaseous atoms and ions in the excited state. When these return to the ground state, they emit photons with certain energies and at a specific wavelength characteristic of a particular element. These photons reach the detector after passing through the grating (Figure 10). The number of photons or emission intensity is directly related to the concentration of elements based on the calibration curve prepared for each element (Hou and Jones, 2000).
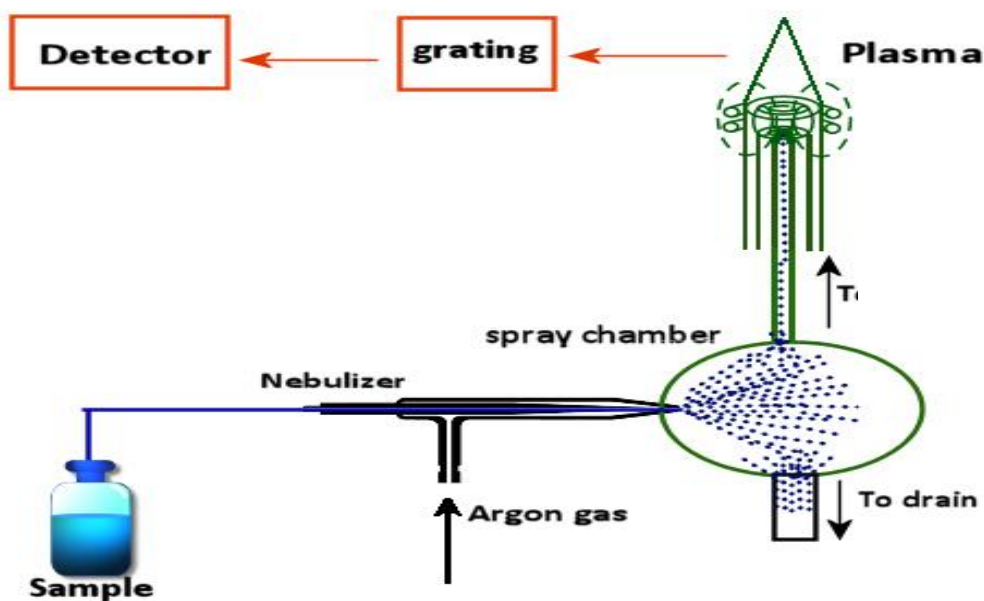
Figure 10: Schematic of ICP operation (http://www.chemiasoft.com/chemd/node/52).

2.4   Operational Parameters in Algae Culture

2.4.1   Measuring the Optical Density of the Green Microalgae *S. Dimorphus* at 600 nm

Chlorophyll absorbs light in the blue (maximum absorbance at 440 nm) and red (maximum absorbance at 675 nm) regions of the electromagnetic spectrum. Moreover, it absorbs significantly below 400 nm. The spectral curve of *Microcystis Aeruginosa* in Figure 11 exhibits this trend. However, chlorophyll reflects green light causing microalgae to appear green. Green light has a wavelength of about 510 nm. In general, 600 nm was found to be the most appropriate wavelength to use in monitoring microalgae growth using a spectrophotometer (Held, 2011).
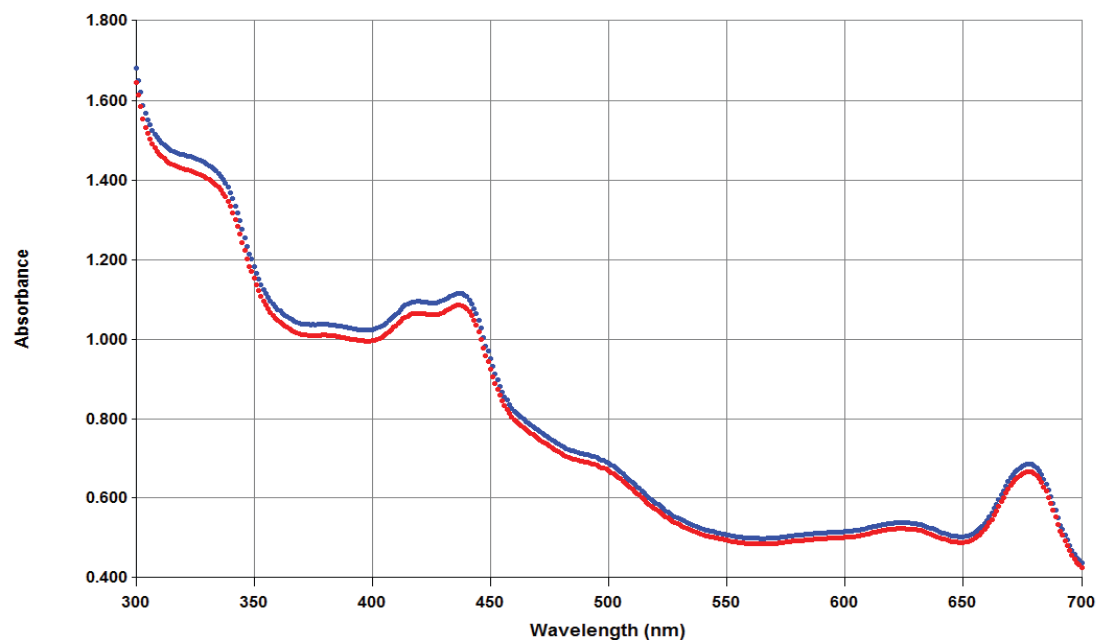
Figure 11: Absorbance Spectral Curve of *Microcystis Aeruginosa* Suspension Cultures (http://www.biotek.com/resources/articles/monitoring-of-algal-growth-using-intrinsic-properties.html).

15

2.4.2 Effect of Carbon Dioxide on the Growth of Microalgae

A paper by Greque and Costa in 2007 showed that the growth rate of *S. obliquus* is the highest at 6% CO_2 as opposed to 0% and 12% CO_2. When cultivating microalgae in the PBR or growth bottles, carbon dioxide was added with air (5% of the air supplied, v/v) to promote growth. The percentage of supplied carbon dioxide was increased when a lower culture pH was desired, since carbonic acid is formed when carbon dioxide is mixed with water.

2.4.3 Effect of Light on the Growth of Microalgae

Light is essential for photosynthesis since without it this reaction cannot take place. Light oxidizes water generating electrons. However, excess light causes electron buildup resulting in the formation of reactive oxygen species (ROS). These species are harmful to the algae culture leading to photo-inhibition and oxidative stress in which the photosynthetic activity of the microalgae drops significantly (Packer et al., 2010). The effect of light on microalgae depends upon the depth of the vessel in which algae is growing and the concentration of the cells. Deep vessels and high cell concentrations require greater light intensity to be able to reach the entire algae culture. The medium in which the algae is being cultivated is usually continuously agitated using different techniques to ensure that all the algae is getting a similar light exposure (Ramos Encarnación et al., 2010).

When growing microalgae in the 5 L photobioreactor, light intensity emitted by each light stand placed around the reactor was measured via a light meter (in foot-candles) midway between the light fixtures at the surface of the bioreactor. The three light stands consist of two Coralife 6700 K, 24 W fluorescent bulbs placed around the bioreactor at about 120° from each other and at approximately the same distance from it. A fourth additional stand having two Coralife 10000 K, 24W fluorescent bulbs was used in the

third run. This was done with the intention to increase the growth rate and reduce light

limitation which becomes significant as the absorbance goes up.

The measured light intensity will be different than that experienced by each cell in the

algae suspension. That is because the algae cells feel the effect of light coming from all light

stands, as well as from the reflection of light. Covering the benchtop on which the bioreactor

is placed with aluminum foil enhances light reflection and increases the amount of light

received by the algae cells. Moreover, culturing algae in 2 L bottles with the light stand

mounted on top of the bottles influences the amount of light captured by the algae cells

differently (Sorokin and Krauss, 1958). The light intensity during direct sunlight is 10000

ft-candles and 0.01 ft-candles with a full moon (Lashen, 2011).

Scenedesmus obliquus has a constant growth rate when exposed to a light

intensity between 500 and 2000 foot-candles (Figure 12, from Sorokin and Krauss,

1958). This range of light is known as light saturation intensity. It should be noted that

this range of intensities depends on the type of the light source, the geometry of

illumination, and type of culture vessel.
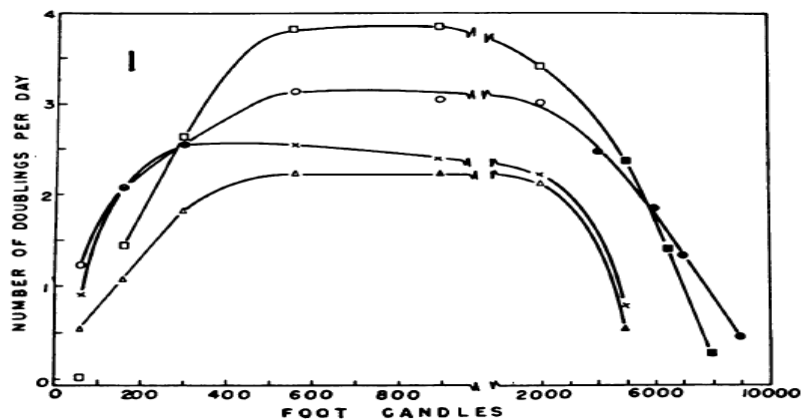
Figure 12: The effect of light intensity on the growth rate of select algae strains. S. obliquus is represented by triangles (Sorokin and Krauss, 1958).

17

Natural sunlight is 5000-6000 Kelvin. Fluorescent light bulbs are 6700 K and therefore

they were used in this research to create an atmosphere similar to that found in nature.

"Color temperatures" above 5000 K are bluish-white, and values of 2700-3000 K are

yellowish-white. Thus, the lower the "color temperature" the more red the light will be,

while higher "color temperatures" make the light more bluish (Energy & Automation,

Inc., 2013).

2.4.4   Effect of Temperature on the Growth of Microalgae

An appropriate temperature is essential for microalgal growth and for normal

metabolic activities to take place in the microalgal cells. Figure 13 shows the results

obtained by Xin et al. for growing *Scenedesmus* sp. LX1 at different temperatures. The

growth rate is highest at 25 and 30°C. Thus, an optimal temperature for the cultivation of

this species should fall within this range. A low temperature will reduce the growth rate

of microalgae, which is undesirable, and a really high temperature can lead to culture

collapse. However, the lipid content of microalgal cells seem to be higher at lower

temperatures of 10 and 20°C compared to 25 and 30°C (Xin et al., 2011).
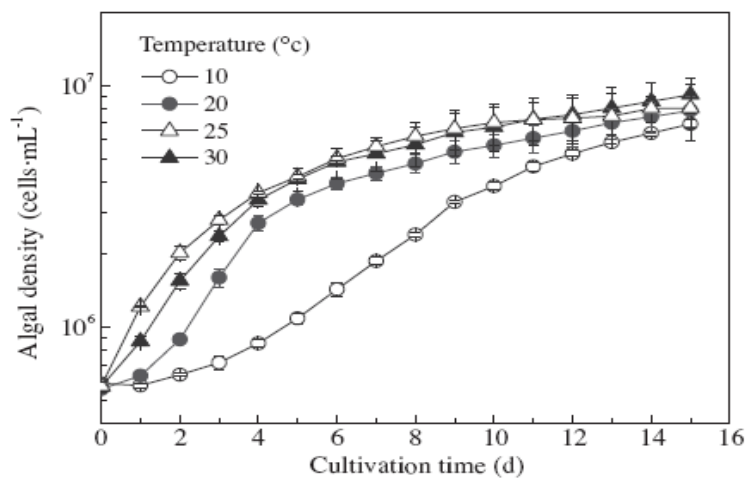
Figure 13: Growth curves of *Scenedesmus* sp. LX1 at different cultivation temperatures (Xin et al., 2011).

18

2.4.5 Effect of Agitation on the Growth of Microalgae

S. dimorphus is denser than water and settles out fast, forming sediments when left without agitation. Hence, it should be continuously mixed when growing to prevent the disruption of its growth (www.algaedepot.org). Agitation is also important as it ensures that the environment is uniform throughout the reactor. High agitation speeds can result in shearing forces that split up the cell colonies into individual cells. For the 5L PBR, the best mixing rate was found to be 300 RPM; however, 250 RPM also seemed to be a reasonable speed and thus it was used in this research (Malik, 2012).

2.4.6 Effect of pH on the Growth of Microalgae

The pH of 3N-BB media prepared for the three experiments using the PBR was about 6.4. This was expected since according to Bischoff and Bold (1963), 3N-BB media's pH is expected to be between 6.4 and 6.8. Three species of *Scenedesmus* demonstrated maximum growth rate at a pH value of 6.5 (Nalewajko et al., 1996). Therefore, the target pH value in the PBR was 6.5 in the second and third runs of this research.

Failure to maintain an acceptable pH during microalgae cultivation causes a number of cellular processes to be disrupted. This will most likely lead to the collapse of the entire culture (Lavens and Sorgeloos, 1996). pH is also important because it affects nutrient transport. Moreover, precipitation of the salts in the media, such as phosphate, can take place at a high pH (Wong and Tam, 1998). If a higher starting pH is needed during microalgae cultivation, 0.5 M sodium hydroxide can be added in order to increase the pH to the targeted value, as was done in the second and third runs.

The growth media used in this research for the cultivation of *S. dimorphus* is a modified recipe of Bold Basal Medium (BBM) with a three-fold higher sodium nitrate concentration and vitamins (3N-BBM+V). This media is highly buffered due to the phosphate buffer (KH_2PO_4/K_2HPO_4) where $H_2PO_4^-$ is the acid and HPO_4^{2-} is the conjugate base. This buffering system is able to resist significant changes in pH.

2.4.7 Dissolved Oxygen (DO) and Microalgae Cultivation

In open bodies of water, dissolved oxygen is introduced through diffusion from air, in addition to the photosynthesis carried out by aquatic plants. The amount of dissolved oxygen (DO), in percent, in the microalgae suspension is measured using a DO probe. DO refers to the amount of dissolved oxygen in the algae culture if the solution is completely saturated with air. The DO may change over the course of microalgae cultivation due other chemical reactions that can be taking place in the microalgae culture such as the decomposition of organic matter by microorganisms.

For a healthy ecosystem, it is desirable to have high DO levels to ensure the survival of the living organisms in water. Organic waste, such as that originating from industry, lowers the DO levels due to the consumption of dissolved oxygen by microorganisms and bacteria. Furthermore, warm water tends to have lower DO amounts compared to cold water, and salt water holds less compared to fresh water (Cary Institute of Ecosystem Studies, 2014; Fondriest Environmental Inc., 2014).

2.5 Growing Microalgae in a Photobioreactor

Cultivating algae in a photobioreactor has many advantages. It provides a sterile environment for microalgae to grow and allows a significant amount of control over culture parameters including light intensity, pH, carbon dioxide flow rate, agitation,

temperature, and the concentration of nutrients. It also ensures that only the microalgae strain of interest is growing, thus, removing the possibility of any invasive species. In this research, a 5 L New Brunswick BioFlo/CelliGen 115 Benchtop Fermentor and Bioreactor was used to grow *S. dimorphus* in 3N-BB media containing vitamins. The bioreactor had a water jacket and a heater that kept it at about 28ºC. It was also connected to a controlling unit equipped with peristaltic pumps, gas flow, pH, DO, and foam/level controllers.

2.6   Yield Coefficient ($Y_{X/s}$) and Mass Fraction (x_i)

The yield coefficient is defined as the amount of algae produced over the amount of substrate consumed. To determine the yield coefficient ($Y_{X/s}$), the biomass concentration (X in gdw/L) versus substrate concentration (s in g/L) should be plotted over the course of the experiment for each substrate, and the negative of the slope gives the yield coefficient:

$$Y_{X/s} = -\frac{\Delta X}{\Delta s} \qquad (1)$$

- where X (gdw/L) = 0.50*A_{600} (Kanani, 2013)

- ΔX is the mass of new cells formed

- Δs is the mass of substrate consumed

The mass fraction (x_i) of an element i is the grams of i in the biomass over gdw biomass. The biomass left at the end of the experiment was digested with nitric acid to determine x_i. $1/x_i$ is the amount of biomass produced over the amount of substrate present in the biomass. It is a snapshot of $Y_{X/s}$ on the last day of the experiment. Ideally, $Y_{X/s}$ and $1/x_i$, of the elements studied here (Mg, K, P, Fe, Ca) should be equal to each other, as it is

21

not expected that the cells secrete these elements as waste products. Table 2 presents the literature data of $1/x_i$ and $Y_{X/s}$.

Table 2: Literature data for the inverse of mass fraction and yield coefficient for *S. dimorphus*. Jacob Schwenk's yield was obtained from growing *S. dimorphus* in 2 L bottles with a working volume of 1.6 L.

Element	Mass fraction, *x*, from Mandalam & Palsson, 1998, for *S. dimorphus*	1/*x* from Mandalam & Palsson, 1998, for *S. dimorphus*	Yield (*Y*x/s), from Schwenk, 2012, for *S. dimorphus*
Phosphorus	0.01-0.02	50-100	N/A
Potassium	0.0085-0.0162	62-118	N/A
Magnesium	0.0036-0.008	125-277	476
Iron	0.0004-0.0055	181-2,500	8549
Calcium	0.00005-0.0008	1250-20,000	247

2.7 Substrate Consumption Rate and Optimal Media Composition

Substrate balance in a batch reactor that includes the rate of substrate consumption (r_s), where s is a particular substrate is:

$$\left(\frac{ds}{dt}\right) = -r_s = -r'_s X \tag{2}$$

r'_s is defined as:

$$r'_s = \frac{\mu_{net}}{Y_{X/s}} \tag{3}$$

Rearranging Equation 2:

$$r'_s = -\left(\frac{1}{X}\right)\left(\frac{ds}{dt}\right) \tag{4}$$

$$r'_s = -\left(\frac{s_{n+1} - s_{n-1}}{2X_n}\right)\frac{1}{\Delta t} \tag{5}$$

Where n is a specific day of the experiment. Equation 5 is the central difference, second order approximation to the first derivative ds/dt and is utilized in this research to calculate the rate of substrate consumption for magnesium, calcium, potassium, phosphorous, and iron with $\Delta t = 1$ day.

Substrate balance in a CSTR is shown in Equation 6, where F is the volumetric flow rate, s_0 and s_f are the feed and effluent substrate concentrations respectively, and V is the reactor working volume.

$$Fs_0 - Fs_f - r'_s\, X\, V = V\left(\frac{ds}{dt}\right) \tag{6}$$

At steady state $\left(\dfrac{ds}{dt}\right) = 0$:

$$F\,(s_0 - s_f) = r'_s X\, V \tag{7}$$

Substituting in r'_s:

$$F\,(s_0 - s_f) = \frac{\mu_{net}}{Y_{X/s}}\, X\, V \tag{8}$$

Dilution rate (D) is related to volumetric flow rate:

$$D = \frac{F}{V} \tag{9}$$

Substituting $D\,V$ for F in Equation (8):

$$D\,V\,(s_0 - s_f) = \frac{\mu_{net}}{Y_{X/s}}\, X\, V \tag{10}$$

At steady state, $D = \mu_{net}$ from the biomass balance. In substrate limited growth, the Monod equation for μ_{net} can be utilized:

$$D = \mu_{net} = \frac{\mu_{max}s}{K_s + s} \tag{11}$$

μ_{max} is the maximum growth rate when $s >>> K_s$, where K_s is the Monod constant.

Substituting μ_{net} for D into Equation 10 and solving for X and s_0 yields Equations 12 and

13 respectively. Equation 13 is applied to find the optimal substrate concentration in the

media that would help reduce the cost of growing microalgae. X_{max}, the maximum

biomass concentration the final day of the experiment is substituted for X in Equations 12

and 13.

$$X_{max} = (s_0 - s_f) \, Y_{X/s} \tag{12}$$

$$s_0 = \frac{X_{max}}{Y_{X/s}} + s_f \tag{13}$$

s_f was taken as 1% of s_0, thus aiming for a low substrate concentration by the end of the

experiment.

$$s_0 = \frac{X_{max}}{0.99 Y_{X/s}} \tag{14}$$

Rearranging Equation 11 and solving for s:

$$s = \frac{DK_s}{\mu_{max} - D} \tag{15}$$

Substituting Equation 15 for s_f in Equations 12 and 13:

$$X_{max} = (s_0 - \frac{DK_s}{\mu_{max} - D}) \, Y_{X/s} \tag{16}$$

$$s_0 = \frac{DK_s}{\mu_{max} - D} + \frac{X_{max}}{Y_{X/s}} \tag{17}$$

24

Equations 16 and 17 assume the yield coefficient to be constant. With all variables

known in a CSTR, s_0 can be calculated as the optimal starting substrate concentration in

the media by applying Equation 17 (Shuler and Kargi, 1992).

CHAPTER III

MATERIALS AND METHODS

3.1 General Experimental Procedures

3.1.1 Media Preparation

Tables 3 a, b, and c show the recipes of stock solutions used in making 3N-BB+V media (Bischoff and Bold, 1963; UTEX, The Culture Collection of Algae). All media components were purchased from Sigma Aldrich. All stock solutions were stored at room temperature, and were well mixed before use. In preparing the P-IV trace metal solution, Na_2EDTA was first dissolved in some DI H_2O in a 1000ml bottle containing a stirring rod and placed on a stir plate. The remaining components were added in order and diluted to the 500ml mark with DI H_2O. The vitamin solutions were prepared separately in 50 ml plastic centrifuge tubes covered with aluminum foil to prevent their interaction with light. They were stored at 4ºC until use.

Table 3a: Stock solutions used in making 3N-BB+V media.

Nutrient	Stock Solution Concentration	Concentration in Stock Solution (M)
$NaNO_3$	12.5g / 500ml DI H_2O	0.294
$CaCl_2*2H_2O$	0.5g / 200ml DI H_2O	0.017
$MgSO_4*7H_2O$	1.5g / 200ml DI H_2O	0.0305
K_2HPO_4	1.5g / 200ml DI H_2O	0.0431
KH_2PO_4	3.5g / 200ml DI H_2O	0.0129
NaCl	0.5g / 200ml DI H_2O	0.043

Table 3b: P-IV trace metal solution preparation.

Component	Stock Solution Concentration
Na_2EDTA	0.375g / 500ml DI H_2O
$FeCl_3*6 H_2O$	0.0485g / 500ml DI H_2O
$MnCl_2*4 H_2O$	0.0205g / 500ml DI H_2O
$ZnCl_2$	0.0025g / 500ml DI H_2O
$CoCl_2$	0.001g / 500ml DI H_2O
$NaMO_4$	0.002g / 500ml DI H_2O

Table 3c: Vitamins recipe.

Component	Stock Solution Concentration
H (Biotin)	0.0016g / 40ml of 50mM HEPES Solution
B_1 (thiamine)	0.0442g / 40ml of 50mM HEPES Solution
B_{12} (cyanocobalamin)	0.0063g / 40ml of 50mM HEPES Solution

The quantities of the stock solutions necessary to prepare 1.0 L of 3N-BB media, and the final concentrations, are shown in Table 4. The P-IV metal solution was placed on a stir plate for about five minutes before use to ensure uniform composition, since the metals are not very soluble and tend to settle out when the solution is stored for a long time. The media bottles were loosely capped and autoclaved (Steris, Mentor, OH) at a temperature of 121ºC and pressure of 0.5 psig. The autoclave takes one to two hours depending on the cycle chosen. The bottle caps were tightened after cooling in the autoclave, and left to cool to room temperature. The bottles were sprayed and rubbed with a 70% ethanol solution and then placed in a laminar flow sterile hood. Using a

syringe attached to a 0.2 μm sterile filter, 1 ml of each vitamin stock solution (H, B_1, and B_{12}) was added to the media, and the bottle was capped instantly to keep it sterile.

Table 4: 3N-BB media recipe. <u>Note:</u> 8.82×10^{-3} M $NaNO_3$ = 750ppm $NaNO_3$ = 547ppm NO_3^- = 123.529ppm N.

Nutrient	Amount of Stock Solution (ml)	Concentration in Media (M)
$NaNO_3$	30	8.82×10^{-3}
$CaCl_2$	10	1.70×10^{-4}
$MgSO_4$	10	3.04×10^{-4}
K_2HPO_4	10	4.31×10^{-4}
KH_2PO_4	10	1.29×10^{-3}
NaCl	10	4.27×10^{-4}
P-IV Trace Metal	6	

3.1.2 ICP Standards and Quality Controls

The analytical balance was calibrated before any solutions were made to prevent any errors in weighing. All clean glassware used were rinsed with RO water to avoid the addition of extra ions to the standards, quality controls, and samples analyzed by the ICP. A liter of 3N-BB media was carefully prepared to make the standards and the quality controls. New standards and quality controls were made from this media in the second and third runs on the PBR. The standards and quality controls were prepared in 50 ml volumetric flasks by diluting them with RO water from 3N-BB media. To each of these, 200 μL of 16 M trace grade nitric acid were added so that their nitric acid concentration was 0.064 M. A 100 ml solution containing 2 times the media concentration of all the nutrients (standard 8) was prepared, and 400 μL of the 16 M trace grade nitric acid were added to it.

Tables 5a and b show the concentrations of Mg, Ca, Fe, P, and K in each of the standards and quality controls. Table 6 indicates the volume of 3N-BB media needed to prepare the standards and quality controls in 50 ml volumetric flasks. These solutions

were stored in glass BOD (biological oxygen demand) bottles at 4ºC in order to avoid

evaporation.

Table 5a: Nutrient concentration in the ICP standards.

	Std1 (ppm)	Std2 (ppm)	Std3 (ppm)	Std4 (ppm)	Std5 (ppm)	Std6 (ppm)	Std7 (ppm)	Std8 (ppm)
Factor of 3N-BB Media	0 (DI water+HNO_3)	1/8	1/6	1/4	1/3	1/2	1 (Media)	2
Mg	0	0.925	1.233	1.85	2.467	3.7	7.4	14.8
Ca	0	0.85	1.133	1.7	2.267	3.4	6.8	13.6
Fe	0	0.015	0.02	0.03	0.04	0.06	0.12	0.24
P	0	6.625	8.833	13.25	17.667	26.5	53	106
K	0	10.5	14	21	28	42	84	168

Table 5b: Nutrient concentration in the ICP quality controls.

	QC1 (ppm)	QC2 (ppm)	QC3 (ppm)
Factor of 3N-BB Media	1/16	3/16	3/4
Mg	0.4625	1.3875	5.55
Ca	0.425	1.275	5.1
Fe	0.0075	0.0225	0.09
P	3.3125	9.9375	39.75
K	5.25	15.75	63

Table 6: Volume of 3N-BB media needed to prepare the standards and quality controls.

	ml of 3N-BB media needed in a 50 ml volumetric flask
Std1	0
Std2	6.25
Std3	8.334
Std4	12.5
Std5	16.666
Std6	25
Std7	50
Std8	Made separately
QC1	3.124
QC2	9.374
QC3	37.5

3.1.3   Algae Seed Culture Preparation

Two liters of 3N-BB+V media in a 2 L glass bottle were prepared. The metallic inoculating loop was sterilized with a lighter or bunsen burner until glowing red, then left to cool. A plastic, sterile, single-use inoculating loop could also be used. The inoculating ring was used to scrape some of the *S. dimorphus* algae from the agar (UTEX culture collection, ID#1237, Proteose Medium, University of Texas), and placed into the media bottle. The bottle was sealed with an autoclaved rubber stopper, containing a sampling port, vent, and a tube with a 0.2 μm filter for CO_2/air feed. A small piece of cotton was placed in the vent tube, the sampling port was clamped, and the CO_2/air feed line at 5% CO_2 (v/v) was connected to the tube upstream of the 0.2 μm filter. The seed jar was placed in a shaker bath (Labline, Mumbai, India), at 30 ± 1ºC and 132 RPM to prevent the microalgae from forming sediments. The shaker bath was equipped with seven Coralife 6700 K, 24 W fluorescent light bulbs that were turned on for a 24 hour light cycle. The light intensity was about 1600 foot-candles at the liquid surface, measured with a light meter (Sper Scientific, model# 840021, Scottsdale, Arizona).

3.1.4   PBR Operation

Bioreactor experiments were conducted in a New Brunswick BioFlo/CelliGen 115 Benchtop Fermentor and Bioreactor (Eppendorf, Hamburg, Germany)**,** with 5 L working volume. Before starting a run, the PBR's condensers and tubes were cleaned with a vinegar-Sparkleen detergent solution to make them much more efficient. *S. dimorphus* was growing in 3N-BB+V media held at about 28ºC, agitated at 250 RPM with an air flow rate of 1 LPM and a starting carbon dioxide flow rate of 0.1 LPM. A light cycle of 16 hours light/8 hours dark was set with an average light intensity of

approximately 1269 ft-candles. The desired media and culture pH values were about 7 and 6.5 respectively (Nalewajko et al., 1996).

3.1.5 PBR Lighting

Three to four light stands each containing two 24 W/6500 K Coralife fluorescent bulbs, were placed around the PBR at about 120° from each other and at approximately the same distance from it. These light bulbs were cleaned with paper towel before the start of the experiment, to remove any dust on them. Their light intensity was measured midway between each pair of light stands, at the liquid surface of the bioreactor with a light meter. This was done several times during the experiment to make sure that the light intensity was consistent.

3.1.6 Measuring the Evaporation Rate of the PBR

Evaporation causes absorbance to be higher than what it should be. It also exaggerates substrate concentrations to the same extent. In the experiments run on the PBR, evaporation was affected by the room's temperature which was not constant and changed especially during summer weekends when the air condition was turned off. Moreover, the autoclave is present in the same room as the bioreactor. Thus, to reduce the heat evolved by the autoclave, when possible, the lab's door was kept open with a fan on to facilitate heat transfer to the outside.

In the first run on the PBR, on day 0 when the reactor was inoculated and the first sample taken, the level sensor was positioned just touching the algae suspension or slightly higher (close to the 5 L mark) so that pump 3, which was used for makeup RO water addition, started adding water at a setpoint of 100% and assignment "level 2 (or 1) dry." The setpoint is the percent of time the pump is on. This pump went on and off

frequently since the agitation was on and the algae moved up and down touching the sensor sometimes and not touching it at other times. The total volume of water added in one day was recorded by resetting the pump starting at 0 ml and noting the exact time. 24 hours later, the total volume of water added for evaporation compensation was noted, at about 55 mL.This should be roughly how much makeup RO water must be added daily to the reactor to account for evaporation. For the remaining duration of this experiment, 0.03805 ml/min of makeup RO water was added to the reactor by changing the setpoint of pump 3 to 1.4% and its assignment to "None."

Before the second experiment on the PBR, the evaporation rate was measured with tap water by running it for five days. One liter of RO water was placed in a 2 L bottle using a 1000 ml graduated cylinder. This bottle was used for makeup water addition to account for evaporation. The level sensor in the reactor was just touching the water when the experiment started. As water evaporated and its level dropped below that of the level sensor, makeup water was automatically added. At the end of the 5 day-period, the amount of water left in the bottle was measured using a 1000 ml graduated cylinder to be 719 ml. Therefore, a total of about 281 ml of makeup water were added to the reactor during the five days, which is close to 282 ml recorded by the control unit. Hence, the evaporation rate was found to be about 56 ml per day, equivalent to 0.0389 mL/min. Pump 3 was used for makeup water addition with a setpoint of 1.4% for the 2nd experiment in the PBR, the same value used for the first experiment. In the third experiment on the PBR, the evaporation rate was taken as the average of the evaporation rates from the first two runs to be about 0.039 ml/min.

3.1.7 PBR Sampling

A 10 ml sample was obtained at approximately the same time daily, after discarding the first 50 ml of cell suspension sitting in the tubes. About 2 ml of the sample was used for recording absorbance at a wavelength of 600 nm. If it was above 1.0 OD, the sample was diluted and an adjusted absorbance was reported. Therefore, about 8 ml were left to be analyzed with ICP. The inoculum added nutrients to the reactor, but this was fine since the initial nutrient content on day 0 was analyzed with the ICP. The reactor temperature and pH were recorded daily in addition to absorbance.

3.1.8 Metal Concentration Measurement in PBR Supernatant

The samples to be tested with ICP were first centrifuged for 5-10 minutes at 1500 RPM shortly after the sample was retrieved. To each 8 ml of supernatant, 40 μL of 16 M trace grade nitric acid were added, so that it became acidic (pH = 1.1). The supernatant was stored in glass centrifuge tubes at 4ºC. Cleveland State University chemistry department's Inductively Coupled Plasma Optical Emission Spectrometer (Perkin Elmer, Waltham, Massachusetts) was used for the analysis of samples to track nutrient consumption with time of Mg, Ca, Fe, P, and K.

Stock solutions of $MgSO_4*7H_2O$, $CaCl_2*2H_2O$, K_2HPO_4, and KH_2PO_4 were made beforehand, but the lab's stocks of $NaNO_3$, NaCl, and trace metals were used. The standards were run on the ICP each time prior to measuring samples in order to obtain a calibration curve for each element. Also, three quality controls (QC's) were run every time the ICP was operated. Two replicates were measured for each standard and sample and an average ICP response was reported. Testing the samples for potassium required

diluting them by a factor of 4 so that the potassium concentration fell within the calibration curve. Refer to Appendix A for detailed instructions on use of ICP.

3.1.9 Biomass Digestion to Determine Biomass Composition

The analysis of dried biomass for elemental composition was done at the end of the experiment by doing three replicates with about 0.5 g of dried algae per replicate. The purpose was to calculate $1/x_i$, the inverse of the mass fraction of an element i in the biomass (g Biomass / g element i). This required a large sample volume which depended on absorbance. At the end of each experiment, the algae suspension remaining in the reactor were stored in glass bottles at 4ºC until the experimenter was ready to process them. This allowed the algae to settle to the bottom of the bottles. The supernatant was discarded leaving only a small amount of it to dissolve the algae settling at the bottom of the bottles. The algae solution was then placed into 50 ml plastic centrifuge tubes that were centrifuged for twenty minutes at 1500 RPM. The supernatant was removed and the algae remaining at the bottom of the test tubes were rinsed with 0.125 M ammonium bicarbonate solution and then placed in an oven at about 40ºC for 5-7 days to dry completely and reach a constant weight (checked within 4-6 hours).

The algae were ground in a mortar and pestle for about 10 minutes, and about 0.5 g per replicate (M_B) were transferred to a 50 ml Erlenmeyer flask to be digested by adding 5 ml of 16 M trace grade nitric acid. The flasks were placed on a hot plate in the fume hood. The ground algae and the 16 M nitric acid turned brown/reddish in color and were left to boil for 20 minutes in the fume hood while brown fumes were being evolved. If they were running dry, 1-2 ml of RO water were added. After boiling for 20 minutes, 15-20 ml of RO water were added to each flask, causing the mixture to turn yellow in

color. After cooling down, the mixture was filtered into a 50 ml volumetric flask through

Whatman number 2 filter paper (9 cm diameter) using a glass funnel. Before filtration,

the filter paper was wetted with RO water to stay in place. If any algae were left in the

beaker, enough RO water was added and the mixture was filtered. The filter paper was

rinsed with RO water and enough RO water was added to the volumetric flask to bring

the volume to the 50 ml mark. This solution was diluted by a factor of 10 and measured

for Mg, Ca, Fe, P, and K concentrations with ICP, in mg/L (see Appendix A for details).

The mass fraction xi of an element i in $\dfrac{g, i}{gdw\ Biomass}$ can be calculated by the following

equation:

$$xi = C_i \times \left(\frac{0.05L}{M_B}\right) \times \left(\frac{1\,g}{1000mg}\right) \qquad (18)$$

where Ci is the concentration of element i in $\dfrac{mg}{L}$; M_B is dry mass of biomass sample, in

g.

3.1.10   Biomass Concentration and Specific Growth Rate Determination

The absorbance of the algae sample at 600 nm was measured using either

Spectronic Genesys 5 Spectrophotometer (Milton Roy Company, USA) or Genesys 10S

UV-Vis Spectrophotometer (Thermo Scientific, Waltham, Massachusetts). The

absorbance is linearly correlated with cell concentration up to an absorbance of 1.

Therefore, if the measured absorbance was above 1, the sample was diluted by ½ using

DI water and its absorbance was measured again. This process was repeated until the

absorbance was just below 1. The "adjusted absorbance" was obtained by multiplying the

A_{600} by the dilution factor. The biomass concentration (X) was calculated from absorbance using the following equation (Bahareh Kanani, 2013):

$$X \text{ (gdw algae/L)} = \text{Absorbance} \times 0.5 \text{ (gdw algae/L)} \quad (19)$$

The specific growth rate of the culture (μ_{net}), in divisions per cell per unit time, is a measure of the ability of *Scenedesmus Dimorphous* species to adapt to their environment as it measures the increase in the number of microalgae cells with time. For a batch culture, it is given by the following differential equation expressing the mass balance on biomass concentration and t is time in days (Shuler and Kargi, 2002):

$$\mu_{net} = \left(\frac{1}{X}\right)\left(\frac{dX}{dt}\right) \quad (20)$$

Rearranging and taking the integral of this equation with respect to X and t, results in:

$$\ln(X) = \ln(X_o) + \mu_{net}(t\text{-}t_o) \quad (21)$$

X_o is the biomass concentration at t_o, just after inoculation, while X is the biomass concentration at time t. The linear region (exponential growth phase) slope for the plot of natural log of biomass concentration versus time is the specific growth rate.

3.1.11   Lipid Extraction of S. Dimorphus

All lipid extractions were performed using a modified version of the Bligh-Dyer method (Bligh,E.G. and Dyer,W.J., 1959). Please refer to Appendix A for the detailed procedure. The following equation was used to calculate the lipid content:

$$\% \text{ Lipids} = \frac{\text{Lipid Weight(g)}}{\text{Starting Biomass Weight(g)}} \times 100\% \quad (22)$$

3.1.12 Statistical Analysis

The student's t-test, with a 95% confidence interval, was used to compare the yield coefficient and $1/x_i$ for each element. t-calculated (t-clac) was compared t-table and if it was greater than t-table, it meant that there is a statistically significant difference between the two values compared. t-table refers to tabulated t distribution critical values found using the tinv function in excel; where t-table = tinv(probability, DOF). t-clac was determined by the following equation:

$$\text{t-calc} = \frac{|\mu_1 - \mu_2|}{s_{M,unpool}} \tag{23}$$

Where μ_1 and μ_2 are the two values being compared and $s_{M,unpool}$ is the unpooled standard error.

$S_{M,unpool}$ can be calculated by the following formula:

$$S_{M,unpool} = \sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}} \tag{24}$$

The degrees of freedom used in finding t-table were obtained by the following relationship:

$$\text{DOF} = \frac{\left(\dfrac{s_1^2}{n_1} + \dfrac{s_2^2}{n_2}\right)^2}{\dfrac{\left(\dfrac{s_1^2}{n_1}\right)^2}{n_1 - 1} + \dfrac{\left(\dfrac{s_2^2}{n_2}\right)^2}{n_2 - 1}} \tag{25}$$

Where s_1, s_2, n_1, n_2, σ_1^2 and σ_2^2 are the standard deviations, number of determinations, and variances of methods 1 and 2 respectively.

3.2 Comparison of Algae Permeabilization Methods

A volume of 1200 mL of *S. dimorphus* cell suspension, with a concentration of about 1.2 gdw/L, was divided equally into six Erlenmeyer flasks. The suspension from each Erlenmeyer flask was divided into 4 50 ml plastic centrifuge tubes and centrifuged for 10-15 minutes at 1500 RPM using IEC HN-SII Centrifuge. The supernatants were discarded and the biomass pellets were rinsed with a 0.125 M solution of ammonium bicarbonate $[(NH_4) HCO_3]$ to combine them into one tube and centrifuged again. Rinsing the biomass pellets with ammonium bicarbonate helped remove any residual salts and solids coming from the media, and hence precludes overestimation of the biomass. The supernatant was discarded. Three of the samples were extracted using the mortar and pestle/Bligh-Dyer method, while the other three samples were extracted using the bead beater/Bligh-Dyer.

The samples to be permeabilized using mortar and pestle were dried in an oven at approximately 40ºC for 5-7 days until they reached a constant weight (checked within 4-6 hours). The dried pellets were ground into a fine homogeneous powder and placed in pyrex centrifuge tubes. If lipid extraction was not performed immediately, the pyrex centrifuge tubes containing the ground samples were filled with nitrogen, capped tightly, and wrapped with parafilm. They were then stored at -20ºC to prevent the oxidation of lipids, until lipid extraction was carried out.

3.2.1 Bead Beating Method

The biomass was partially dried overnight in an oven at about 40ºC to remove the majority of the water present in it and facilitate its transfer to the bead beater vial. The moist algae paste was transferred to the 2 ml polypropylene bead beater vial, which was

half filled with 0.7 mm zirconia beads. The empty space remaining in the vial was filled with 0.125 M ammonium bicarbonate solution to prevent any air gaps. The vial was inverted about 5 times to make sure that the beads were wetted, as they can entrap air. However, some air space was kept to allow room for the mixture to go back and forth in the vial. The vial was placed in the arm assembly of the bead-beater and the safety cover was closed. A speed of 4800 RPM (displayed as 48) and a time of 60 seconds (displayed as 6) were selected, and finally the green button was pressed to start bead beating.

The sample was disrupted by bead beating using four 1-minute bursts. Heat was generated at a rate of 10ºC per minute; hence, samples were cooled for one minute between bursts using cold water based on a recommendation from the manual of Mini-BeadBeater-1 (BioSpec Products, Bartlesville, OK). At the end of this process, the beads quickly settled to the bottom of the vial. The supernatant with the biomass was removed using a glass pipette and transferred to a new pre-weighed 50 ml glass centrifuge tube. The 0.125 M ammonium bicarbonate solution was used to rinse the beads several times and the resulting solution was added to the same glass centrifuge tube. This tube was centrifuged at 1500 RPM for 1 hour (or 30 minutes) until the biomass was concentrated at the bottom of the tube, after which the supernatant was removed. This wet biomass was dried under nitrogen for about 5 hours at room temperature until a constant weight was reached. The Bligh and Dyer method was then used to extract the lipids from the biomass.

3.3 Nutrient Consumption Rates and Biomass Content

The purpose of this experiment was to measure consumption rates (Equation 5) using ICP and determine yield coefficients (Equation 1) for calcium, magnesium,

phosphorous, potassium, and iron. It is desirable to optimize the reactor system, especially the media composition to reduce nutrient waste, especially phosphorous due to its limited global supply, and ultimately lowering the cost of cultivation. The optimal substrate concentration in the media was calculated based on Equation 14. Lipid extraction was performed at the completion of each experiment using the Bligh-Dyer method on about 0.2 g of dried algae per replicate (Appendix A). Please refer to the New Brunswick BioFlo/CelliGen 115 Benchtop Fermentor and Bioreactor's manual (Eppendorf, Hamburg, Germany) for its operating and cleaning instructions such as calibrating the pumps, DO, and pH probes, and controlling temperature and agitation.

3.3.1   Inoculation Process

Before inoculation into the experimental reactor, an algae sample of the seed culture bottle was observed to evaluate the culture health under the Nikon Eclipse E400 microscope (Nikon Corporation, Tokyo, Japan). A healthy culture contains algae cells growing in colonies, with crescent shape, and no evidence of bacteria or fungi or algae other than *S. dimorphus*. The algae should not exist as individual, round cells, which indicates that the cells have stopped growing and are accumulating lipids instead. The desired absorbance of this sample should be greater than or equal to 1.0 OD (optical density).

Before inoculating the PBR, the pH of the 3N-BB media was noted to be about 6.5, therefore, no 0.5 M sodium hydroxide solution was added. On day 0 of the experiment, a specific amount of media was first removed from the reactor through the harvest port. Vitamins (5 ml of H, B_1, and B_{12}) were added via a sterile 0.2 μm filter through the side tubing used for adding the inoculum. The inoculum was then pumped

through tubes connected to the headplate, and the bottle containing it was continuously

stirred by hand to prevent it from settling as the inoculation process was taking place. The

starting absorbance in the PBR was targeted to be about 0.15 OD. This starting

absorbance was higher than the usually used value of 0.05 OD, so that the consumption

of nutrients could be better monitored.

3.3.2 pH Adjustment in the 5 L PBR

No pH control was attempted in the first run in the 5-L PBR. In the second run,

after autoclaving the 5 L of media in the bioreactor, its pH was measured with the

calibrated pH meter and was found to be 6.44. Since it was below 7, a sterile solution of

0.5 M sodium hydroxide was added dropwise via a syringe and needle through a septum

in the reactor headplate, until the pH reached 6.97 (about 5 ml of 0.5 M sodium

hydroxide was added). After inoculation and turning on the carbon dioxide flow rate to

about 0.1 LPM on day 0 of the experiment, the pH dropped to 5.83 which was below the

target pH of 6.5. On days 1 and 2 of this experimental run the pH was about 6.03,

therefore, to bring it up to about 6.5 about 47 drops of 0.5 M sodium hydroxide were

added. However, the pH did not change much and only went up to 6.04. To avoid

overshooting the pH, no more sodium hydroxide was added as the pH probe might not

have been responding well. From day 19 onward, the CO_2 flow rate was increased to 0.15

LPM to allow the pH to be close to 6.5.

In the third run on the PBR, the media had a pH of 6.37. With the air/CO_2 flow

turned off, sodium hydroxide was added dropwise and this was accompanied with a

noticeable increase in the pH. A total of about 35 ml of NaOH was added with the pH

reaching 6.65. When the air/CO_2 was turned on, the pH dropped to 6.22. However, when

the CO_2 supply was turned off again with only air at 1 LPM being sparged to drive out

any trapped CO_2 through the condenser's filter, the pH went up to 8.221. The air/CO_2 was

then turned on and the pH went back to 6.22. After inoculation with *S. dimorphus* to an

optical density of 0.179 (the target was 0.15), the pH reached 6.3 within an hour of

inoculation. During the course of the experiment, if the pH got higher than 6.5 the CO_2

flow rate was increased as needed to bring it down.

3.3.3   Difference between the Experimental Runs on the 5 L PBR

In the second and third runs, new standards and quality controls were made with

the same RO water batch used in the reactor media. This same RO water batch was also

used in the water bottles used to account for evaporation. The resistivity of this water was

noted to be 17.7 Megaohm.cm. This was done to ensure the most accurate measurements

of the metal concentrations in the ICP. The samples tested with ICP were also run from

the last day of the experiment towards day zero, that is, from low concentration to high

concentration, as were the standards.

The third experimental run had a higher culture and media pH compared to the

first two runs. Moreover, a fourth light stand was added having two 24 W/10000 K

Coralife fluorescent bulbs. This was done in an effort of increasing the growth rate and

reducing light limitation which becomes significant as the absorbance goes up. 900 mL of

autoclaved 0.56 M sodium hydroxide solution was connected to the reactor for manual

NaOH addition. Table 7 summarizes the major differences between the three runs on the

PBR.

Table 7: F in conditions between the three runs on the PBR.

Run	1	2	3
Initial pH after inoculation with air/CO2 sparging	5.76	5.83	6.3
NaOH addition	None	5 mL of 0.5M, prior to inoculation	35 mL of 0.5M, prior to inoculation
pH control	None	pH was maintained at about 6.5 via manual manipulation of CO_2 flow rate	pH was maintained at about 6.5 via manual manipulation of CO_2 flow rate
CO_2 Flowrate	Constant at 0.1 LPM	Increased to 0.15 LPM on day 19	Increased several times
ICP Samples	Ran from high to low conc.	Ran from low to high conc.	Ran from low to high conc.
Light	3 light stands	3 light stands	4 light stands
RO Water	Different batches used	Same batch used	Same batch used
Evaporation Rate	Measured over the course of one day (55 ml/day)	Measured over the course of five days before inoculation (56 ml/day)	Not measured
Evaporation Control	RO water added at rate of 0.03805 mL/mn	RO water added at rate of 0.0393 mL/mn	RO water added at rate of 0.0387 mL/mn

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Comparison of Algae Permeabilization Methods

Algae biomass was lysed with either the mortar and pestle or the bead beater in an effort to determine which technique is the most consistent and efficient in terms of lipid extraction (Section 3.2). The initial results obtained are summarized in Figure 14a with all samples dried under N_2. This experiment was repeated, however only one sample was extracted using each method resulting in 15% and 18.4% lipids using the mortar and pestle and bead beater respectively (Figure 14b). The algae suspension used was sitting in the hood for 5-7 weeks with no CO_2, light, or agitation, however, it was the same algae suspension used before. Also, air was used to dry the lipids and not N_2.

Due to the lack of confidence in these results, cell lysing was carried out on a fresh *S. dimorphus* batch. The average lipid content obtained using the bead beater and mortar and pestle were $9 \pm 2\%$ and $19 \pm 2\%$, respectively, by performing three replicates using each method (Figure 14c).

The bead beater results agree with Lee et al. (2010) who determined that *Scenedesmus* sp. have a lipid content of about 8% as measured also using bead beater. It was expected that the bead beater would result in a more homogeneous algae powder compared to the mortar and pestle. The mortar and pestle gave higher lipid content compared to the bead beater, thus it was used in all the lipid extractions performed in this research.
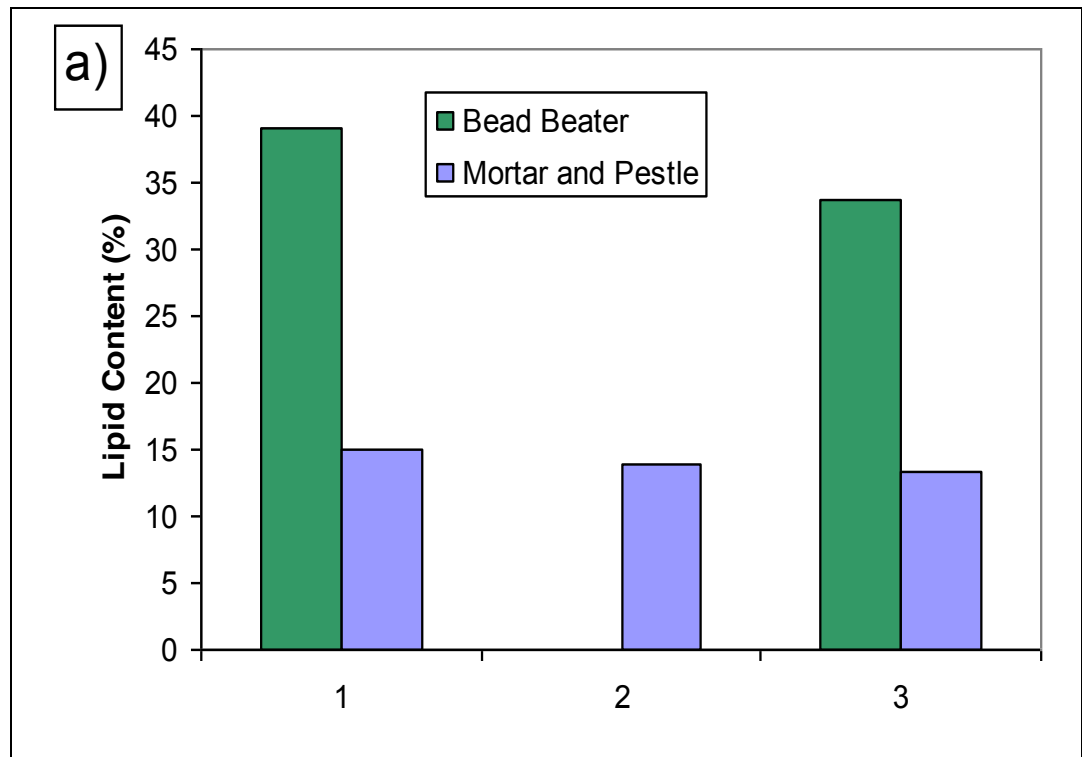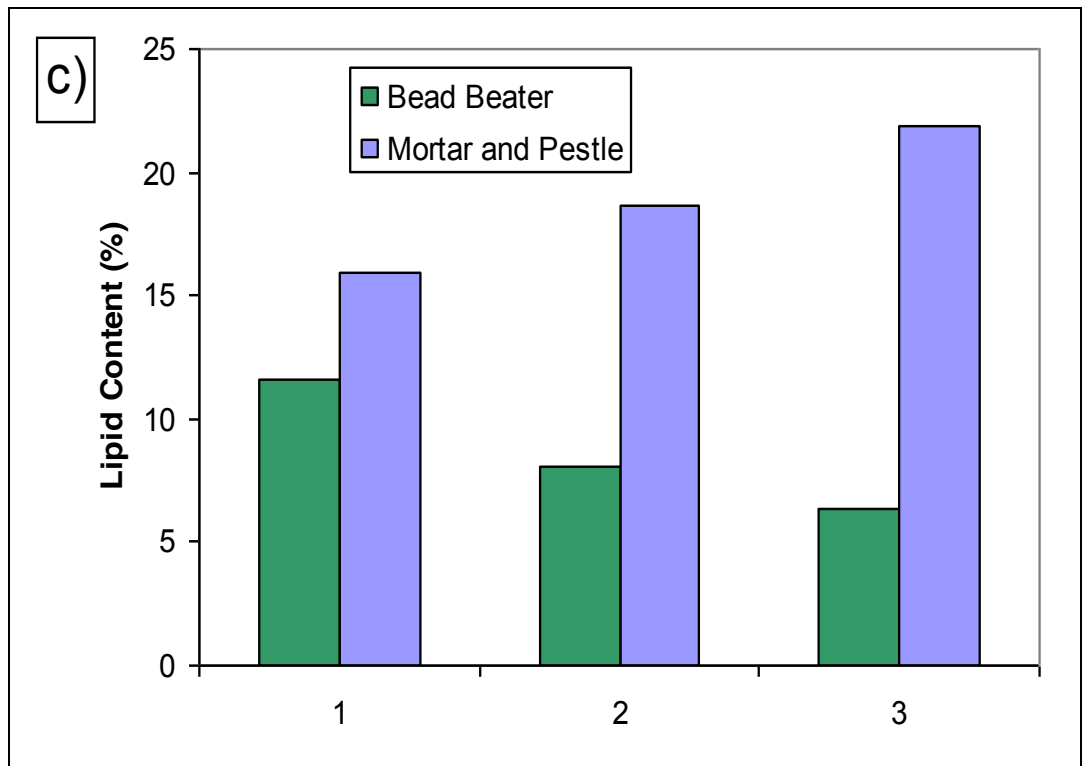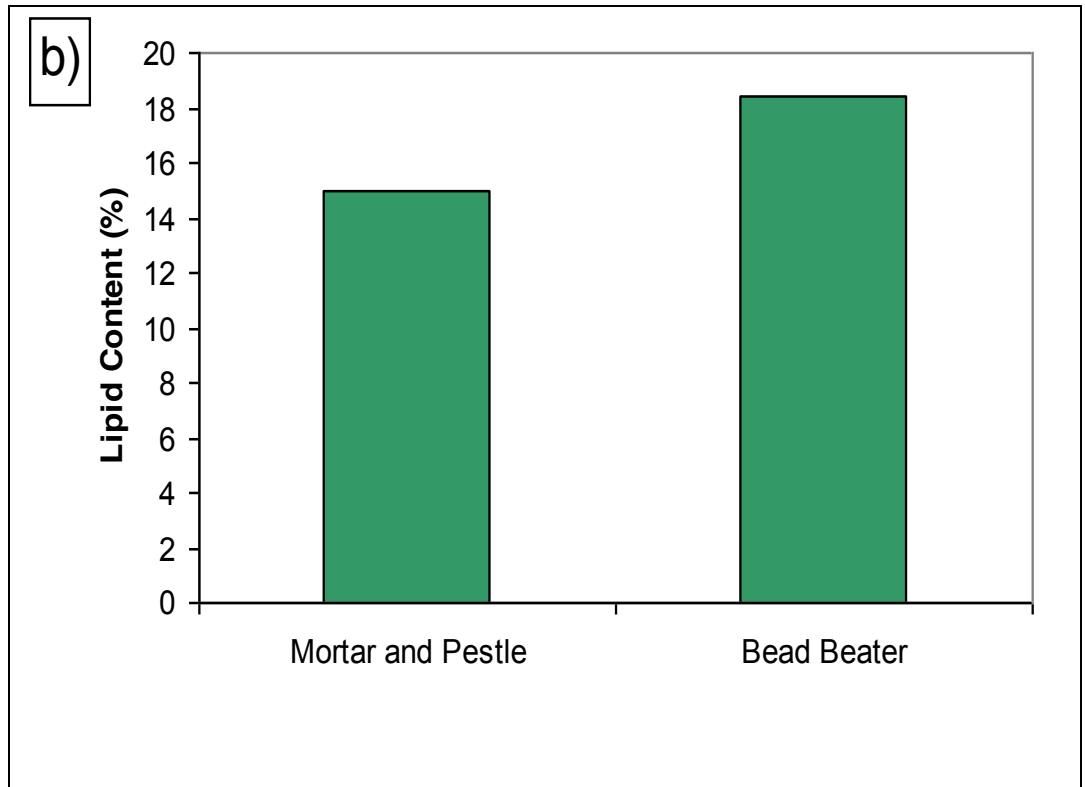
Figure 14: Comparison of lipid content obtained using the bead beater and mortar and pestle methods for permeabilization. A fresh batch of algae was used in Fig. 14c as opposed to Fig. 14a and b.

4.2   Growth Rates of *S. Dimorphus* in 2 L Bottles

The purpose of this experiment was to measure the growth rate in batch mode, of *S. dimorphus* growing in four 2 L bottles with a working volume of 1.5 L in 3N-BB media. The growth curves are shown in Figure 15. Sample collection alternated between the four bottles up to day 7 of the experiment, after which samples from bottles 2 and 4 were taken in the same day. The next day samples from bottles 1 and 3 were taken and so on. The large increases in absorbance after days 10-11 might have been due to evaporation, as it was not accounted for due to the inaccurate volume labeling on the bottles. The microalgae cells did not reach the stationary phase during the sixteen days of the experiment in bottles 1, 2, and 4. Growth was disrupted after day 13 in bottle 3 as its optical density started going down, possibly due to culture contamination or light limitation. Moreover, the CO_2/air flow rates might not have been constant in the four bottles, thus affecting their growth. For example, some vents might have been less effective than others, causing slight increase in pressure and higher CO_2 concentrations in some bottles.
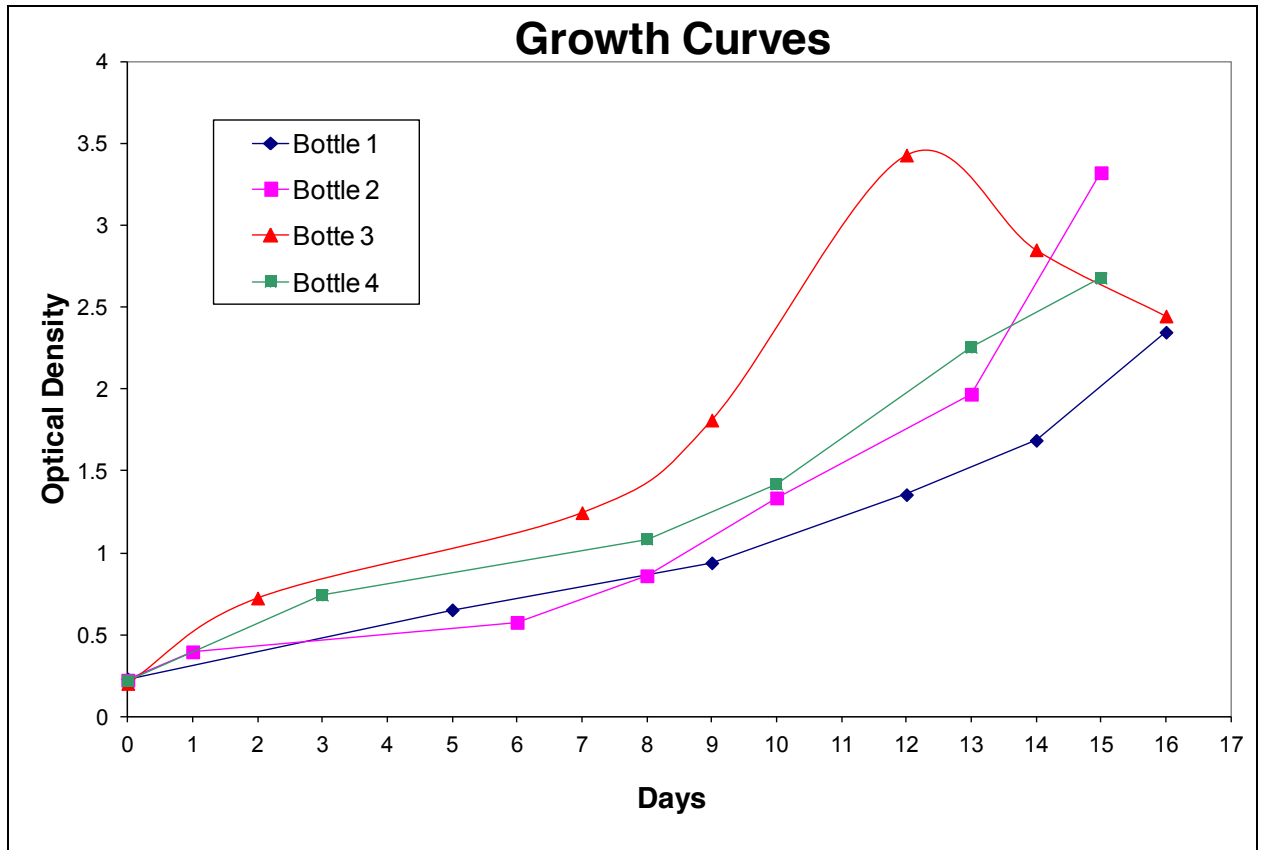
Figure 15: Growth curves for *S.dimorphus* growing in the four bottles having a working volume of 1500 ml each.

Larger sample volumes were obtained every few days from the bottles, for measurement of biomass concentration via dry weight measurement. The graph of biomass concentration versus absorbance measured at 600 nm for the four bottles combined is shown in Figure 16, and the average slope of the best-fit line was determined to be 0.58 ± 0.01 gdw/L*A_{600}. This slope can then be used to relate future absorbance measurements to biomass concentration:

$$\text{Biomass concentration (g/L)} = \text{Factor (slope)} \times A_{600} \qquad (26)$$
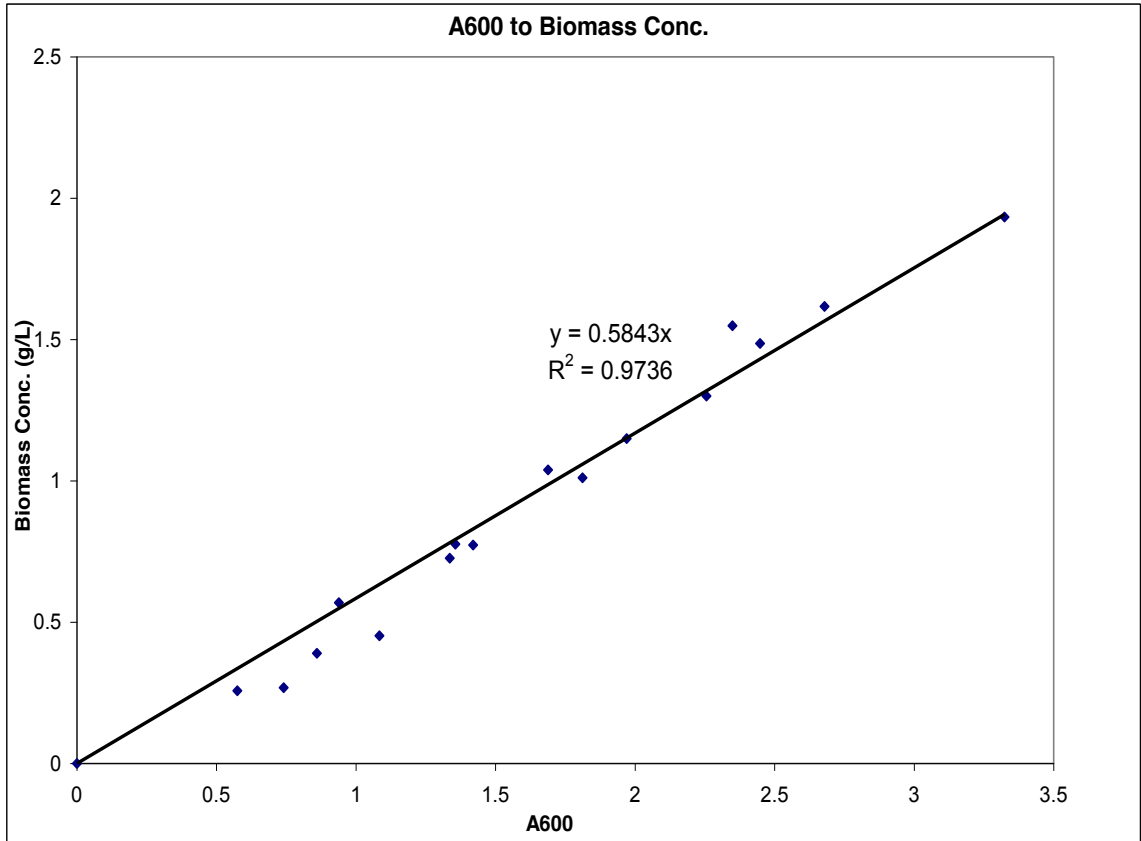
Figure 16: Combined growth data for *S. dimorphus* in the bottles having a working volume of 1500 ml.
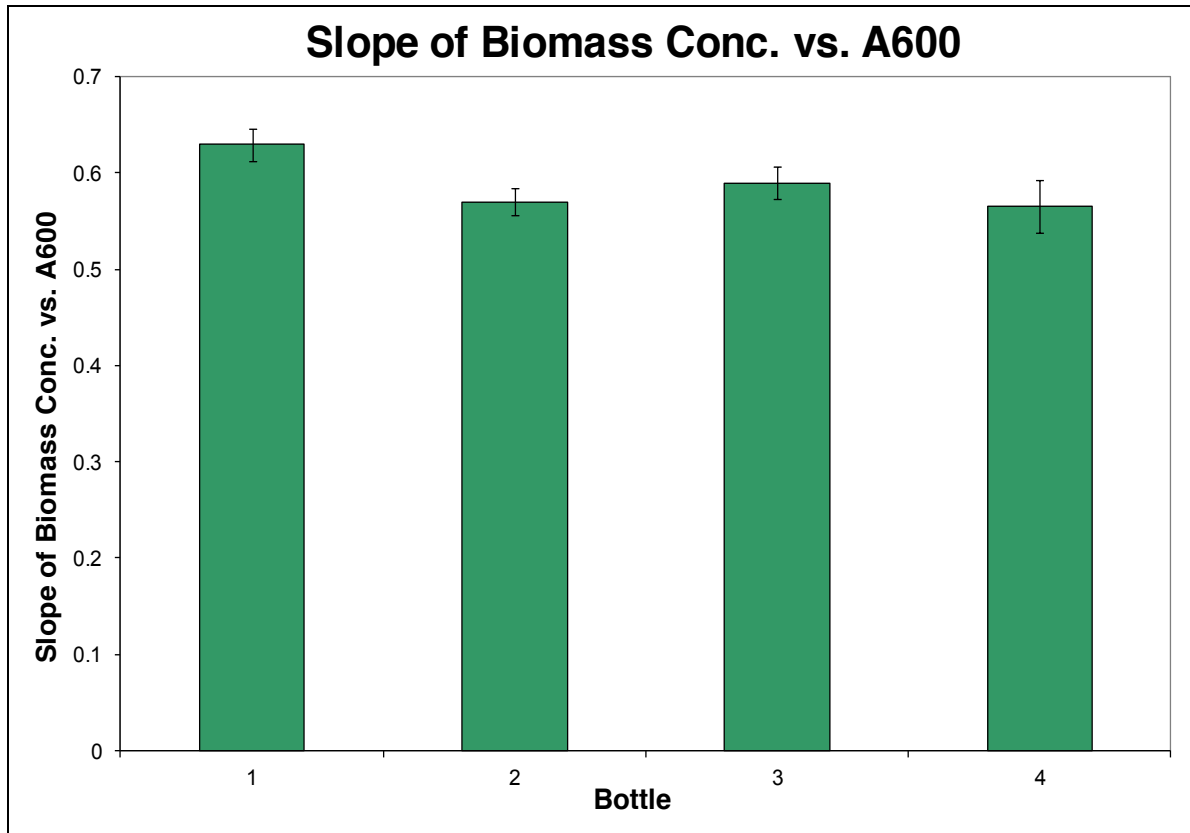
Figure 17: Slope of biomass concentration versus absorbance for each of the growth bottles. Average slope was 0.59 ± 0.03 gdw/L*A_{600}. The error bars are standard errors calculated using Excel's Linest function.

Bahareh Kanani (2013) measured this slope in 40-250 mL Erlenmeyer flasks of 150 mL working volume and found it to be 0.50 ± 0.03 gdw/L*A_{600}. The factor of 0.50 rather than 0.58 was used in this research to convert A_{600} to biomass concentration since Kanani's slope was based on a larger number of data points. The importance of this result lies in the fact that absorbance is much faster to measure and requires only a small sample volume, compared to determining biomass concentration.

The optical density measurements of Fig. 15 are plotted on a log scale in Fig. 18a for determination of specific growth rates during the exponential growth periods, according to Equation 21. The growth rates were calculated from the linear region of each graph, and are shown in Fig. 18b. The student's t-test indicated that there is a

statistically significant difference between the growth rates in bottles 1 and 2, and bottles 2 and 4 with bottle 2 being an outlier. However, there is no statistically significant difference between the growth rates in bottles 2 and 3. The average growth rate among the four bottles was 0.13 ± 0.03 days^{-1}. Despite the effort of trying to make the growth conditions in the four bottles as close as possible to each other, they differed slightly in their nutrient content, CO_2/air flow rate, pH, contamination, and illumination, hence affecting their growth. Schwenk found this growth rate to be 0.321 ± 0.009 days^{-1} in a batch experiment performed in 2 L bottles (Schwenk, 2012). Kanani determined this growth rate to be 0.27 ± 0.14 days^{-1} (Kanani, 2013). Bottle 2 seems to be the only bottle with a "normal" growth curve, despite a little long lag phase. However, the average growth rate obtained in this experiment of 0.13 ± 0.03 days^{-1} is within Kanani's range.
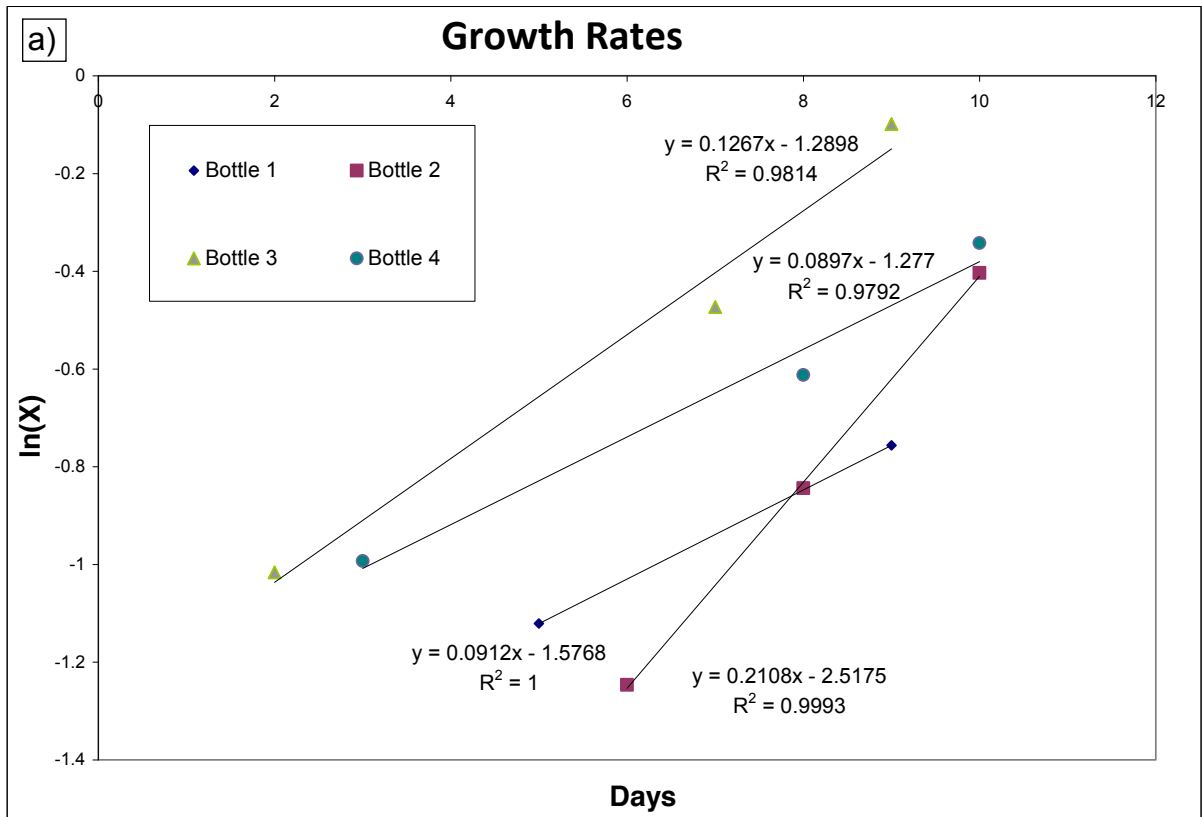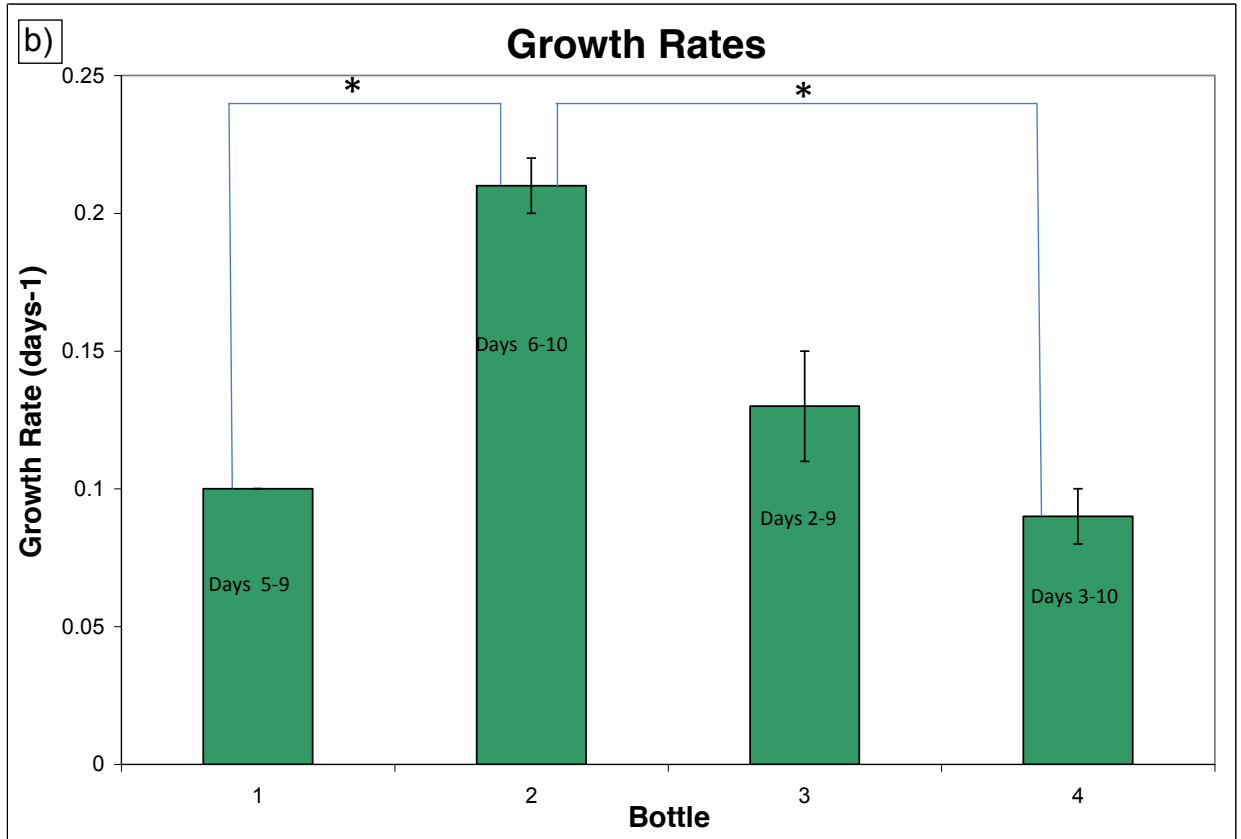
Figure 18: Growth rates for *S. dimorphus* growing in 3N-BB+V media in four bottles with a working volume of 1500 ml. Average growth rate was 0.13 ± 0.03 days^{-1}. The error bars correspond to standard errors given by Excel's Linest Function. "Days" refer to the day of the experiment starting day 0. *Denotes a statistically significant difference.

4.3   Nutrient Consumption Rates and Biomass Content

This experiment was carried out on *S. dimorphus* grown in 3N-BB+V media in a 5 L photobioreactor, as described in Section 3.3. The objective was to measure consumption rates, yields, and biomass content. This data is useful for determining the optimal concentration of magnesium, calcium, potassium, iron, and phosphorous in the media to avoid waste.

4.3.1   Growth Curves and pH Data

The optical density (which is proportional to biomass concentration) and pH data are shown in Figures 19 and 20 respectively.
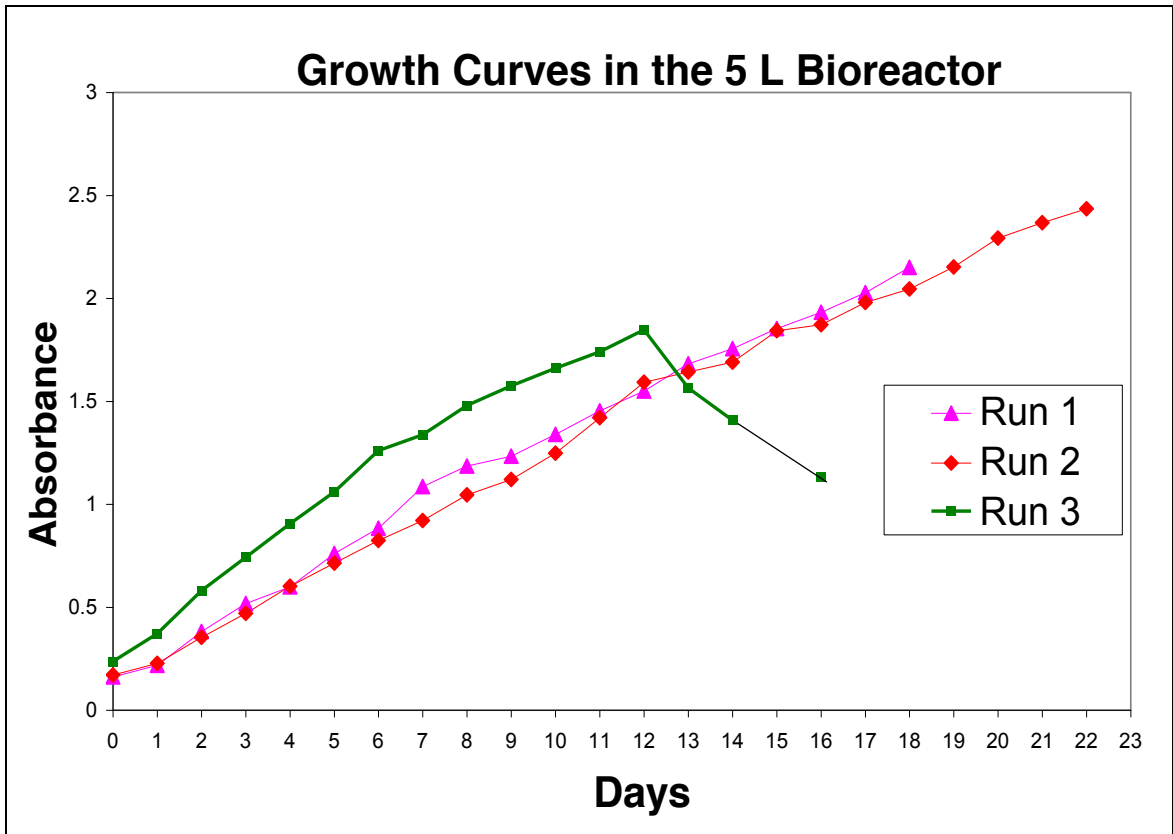
Figure 19: Cell growth data for *S*. *dimorphus* in 3N-BB+V media in a 5 L bioreactor. Run 3 had four light stands as opposed to runs 1 and 2 with three light stands.
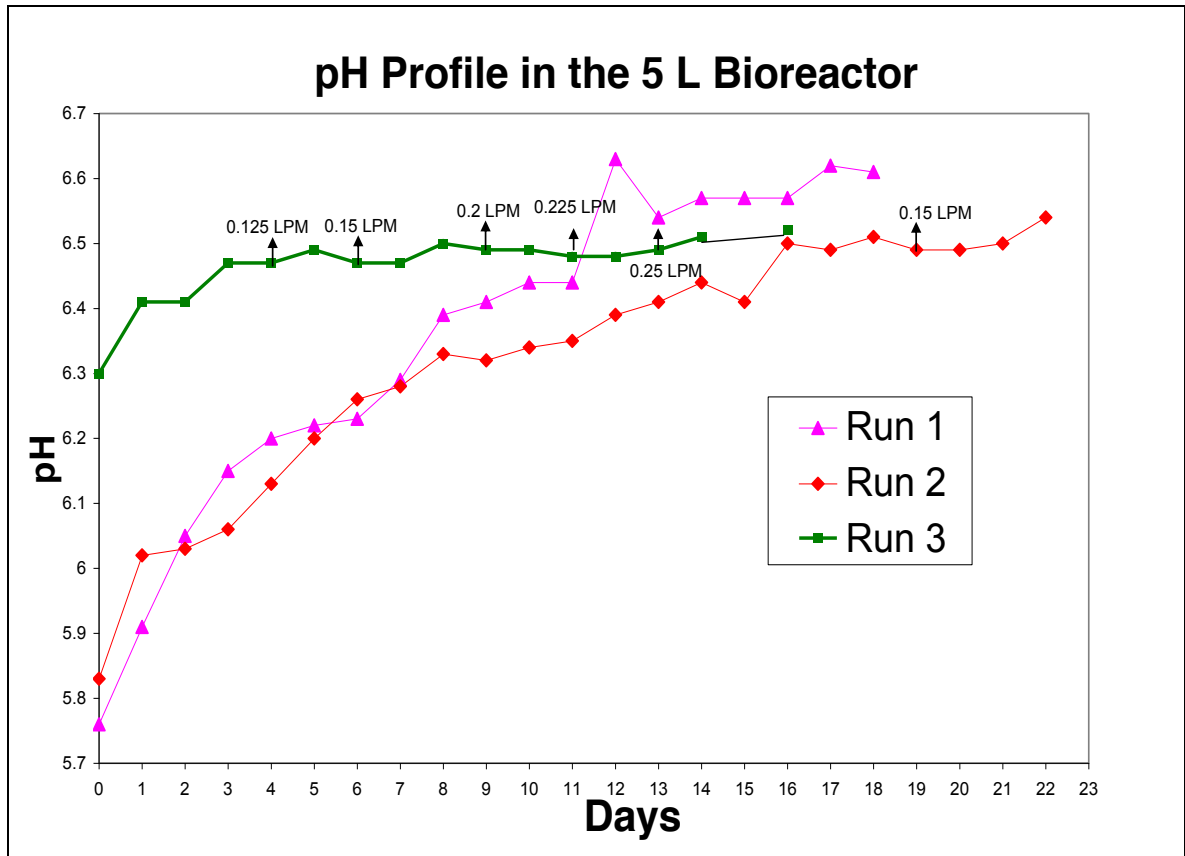
Figure 20: pH profile for cultivating *S. dimorphus* in 3N-BB+V media in a 5 L bioreactor. In runs 2 and 3, sodium hydroxide was added to the media only at the beginning to increase its pH. pH was also maintained at about 6.5 via manual manipulation of CO_2 flow rate as shown by the arrows, starting at 0.1 LPM.

The growth phases in the three runs are linear and not exponential as expected signifying slower growth rates due to light limitation. The absorbance of the *S. dimorphus* culture started to go down beginning day 13 of run 3. This decrease in the biomass concentration in suspension in the fluid, is attributed to biofilm accumulation on the reactor walls which increased notably with time. When the algae were removed from the reactor at the end of this run, about 5.8 L of tap water were added in an effort of cleaning the reactor. After agitating the water for sometime along with increasing the agitation speed, a sample was taken to check the absorbance of the resulting suspension. It was found to be 0.715, which corresponds to 0.358 gdw/L; meaning that a total of about 2.1 g of biomass or about 50% of the total biomass adhered to the surface.

Microalgae stickiness in run 3 is likely the result of stress due to excess sodium and/or low magnesium concentration. Magnesium concentration in run 3 was lower at day 13 (2.1 ppm), compared to the end of runs 1 and 2 of 4.6 ppm (day 18) and 3.3 ppm (day 22), respectively. It has been shown (Guerrini et al., 1998) that under nutrient limitation, algae generate more polysaccharides which cause the cells to adhere together and to surfaces.

When microalgae concentration is very low, the CO_2 consumption rate is very low. Hence, CO_2 stays at steady state value resulting from a balance between CO_2 delivery and consumption rate and the pH drops as carbonic acid forms. This explains the low pH at the beginning of growth in runs 1 and 2. As the microalgae biomass concentration increases, CO_2 consumption rate goes up. As a result, the CO_2 concentration decreases, a lower amount of carbonic acid is formed, and the pH increases.

4.3.2 Specific Growth Rate

The specific growth rate for each run was obtained by plotting the natural log of cell concentration versus days, as presented in Figure 21. Specific growth rates were 0.14-0.19 days^{-1} as shown by the bar plots in Figure 22. According to the student's t-test, there is a statistically significant difference between the growth rates obtained in runs 1 and 2, and well as between runs 2 and 3. However, there is not a statistically significant difference in the growth rate of runs 1 and 3. Hence, pH control did not seem to have an effect on growth rate, unlike the previously reported results where the highest growth rate was reached at an optimal pH value of 6.5 (Nalewajko et al., 1996).

The average growth rate of *S.dimorphus* in the 5 L bioreactor (0.17 ± 0.03 days^{-1}) was not significantly different from that measured in the 2 L bottles (0.13 ± 0.05 days^{-1}). In fact, one would expect a smaller growth rate in the 5 L bioreactor, since it has a smaller surface area to volume ratio compared to the 2 L bottles, hence reducing the amount of light reaching the reactor's interior as it is being blocked by the algae cells. Therefore, algae in the 5 L bioreactor have to accumulate a larger amount of chlorophyll to absorb light.
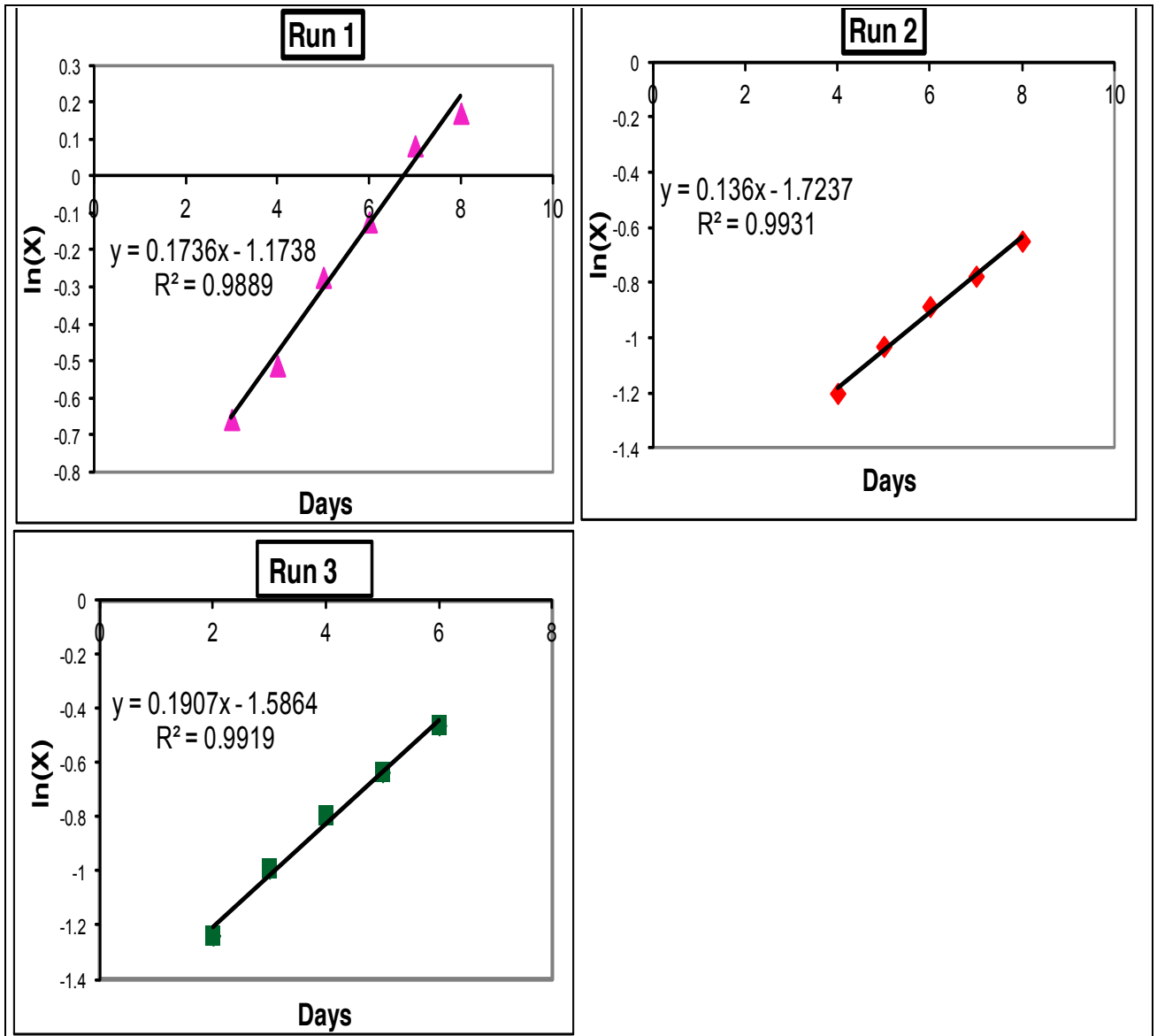
Figure 21: Specific growth rates for *S. dimorphus* in runs 1, 2, and 3 in the 5 L photobioreactor were calculated from the exponential growth phases of the data in Figure 19. Windows of growth varied between runs: days 3-8 for run 1, days 4-8 for run 2, and days 2-6 for run 3.
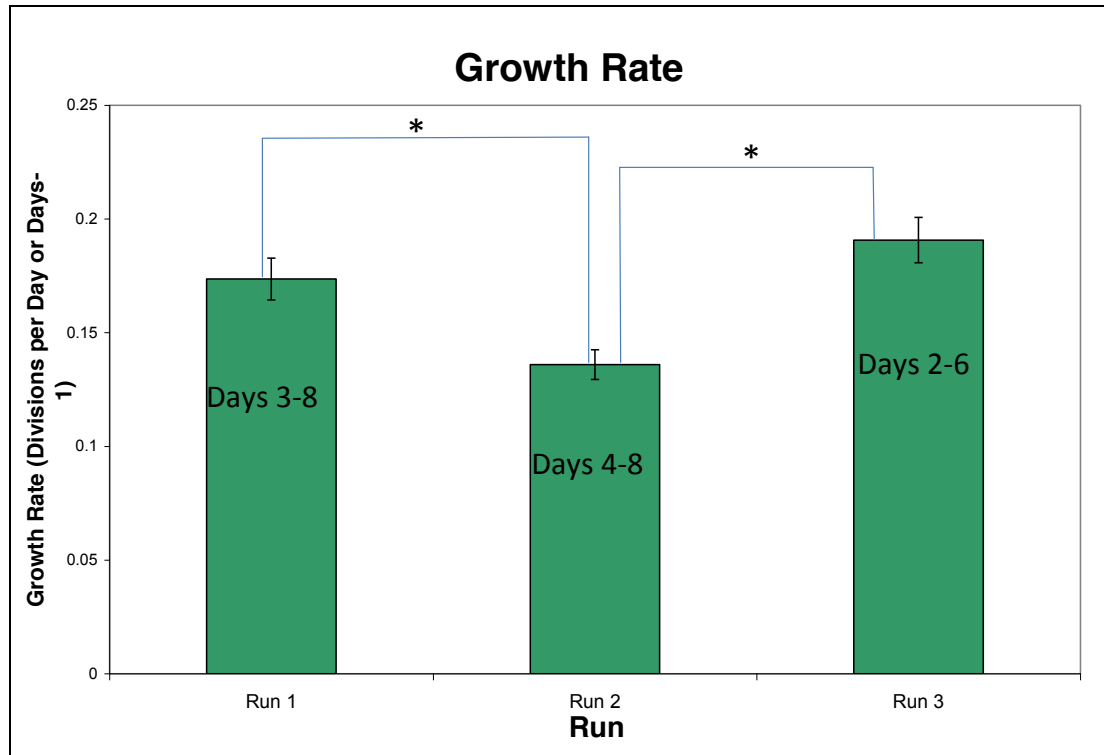
Figure 22: Summary of all the growth rates obtained with the 5 L PBR. Average growth rate was 0.17 ± 0.03 days[^-1]. The error bars represent the standard error obtained from Excel's Linest Function. *Denotes a statistically significant difference (p<0.05).

4.3.3   Lipid Content

The lipid content on the last day of runs 1, 2, and 3 of the PBR was 11-16% as displayed in Figure 23. According to the student's t-test, there is no significant difference between the lipid content obtained on the last day of runs 2 and 3. However, the lipid content in run 1 was significantly greater than both runs 2 and 3. The lower lipid content obtained from runs 2 and 3 was most likely due to lipid oxidation as the algae was ground and the tube's headspace was filled with nitrogen, capped, and placed in the -20ºC freezer until the next day when the lipid extraction was carried out. While the sample from run 1 was extracted on the same day as the drying was completed. Therefore, it is best to perform the Bligh-Dyer method the same day algae is ground. Another reason for the

lower lipid content in runs 2 and 3 could have been over drying the algae for 1-2 days
before cell lysing.
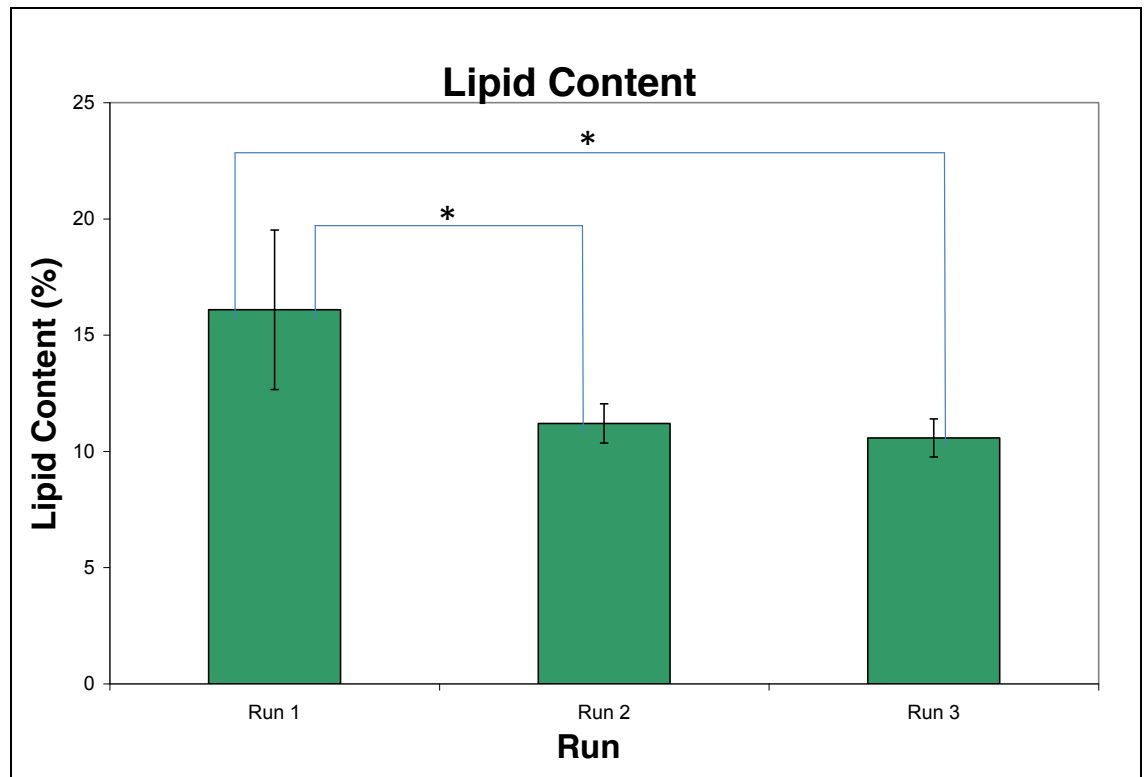
Figure 23: Lipid content of biomass harvested at end of the experiment. The percentage reported is lipid content in the dried biomass, which was permeabilized via the mortar and pestle. Average lipid content was 13 ± 1%. Error bars represent standard error, N=3 (number of measurements). *Denotes a statistically significant difference ($p < 0.05$).

4.3.4   Change in Elemental Concentration with Time using ICP

The concentration profiles of magnesium, calcium, phosphorous, potassium, and iron, measured by ICP, are displayed in Figure 24. In general, the concentrations of magnesium, phosphorous, and calcium decreased with time, as expected. There was a negligible change in the potassium concentration with time in runs 1 and 2; however, in run 3 there was a noticeable decrease in the potassium concentration over the course of the experiment.

Iron was consumed in the three experiments; however, in run 1, the measured iron concentration dropped to negative values at day 10. This might mean that all the iron in the media was consumed after day 10, and the negative values were reflecting scatter around the detection limit of the ICP. Or, the values in general may not have been accurate. The plots of iron intensity versus wavelength were examined for several samples. The iron peaks looked normal, and appeared at the appropriate wavelength of 238.204 nm as depicted in Figure 25, indicating that the ICP was functioning properly. In run 1, the media was prepared separately from the standards and quality controls, using different water batches. The water utilized in the media might have had a lower iron content than that present in the standards and quality controls. This was an issue only for iron as it is present in trace amount in the media compared to the other elements which are present at a much higher concentration. To avoid getting negative iron concentrations in runs 2 and 3, the same water batch was used for making the media, standards, and quality controls.
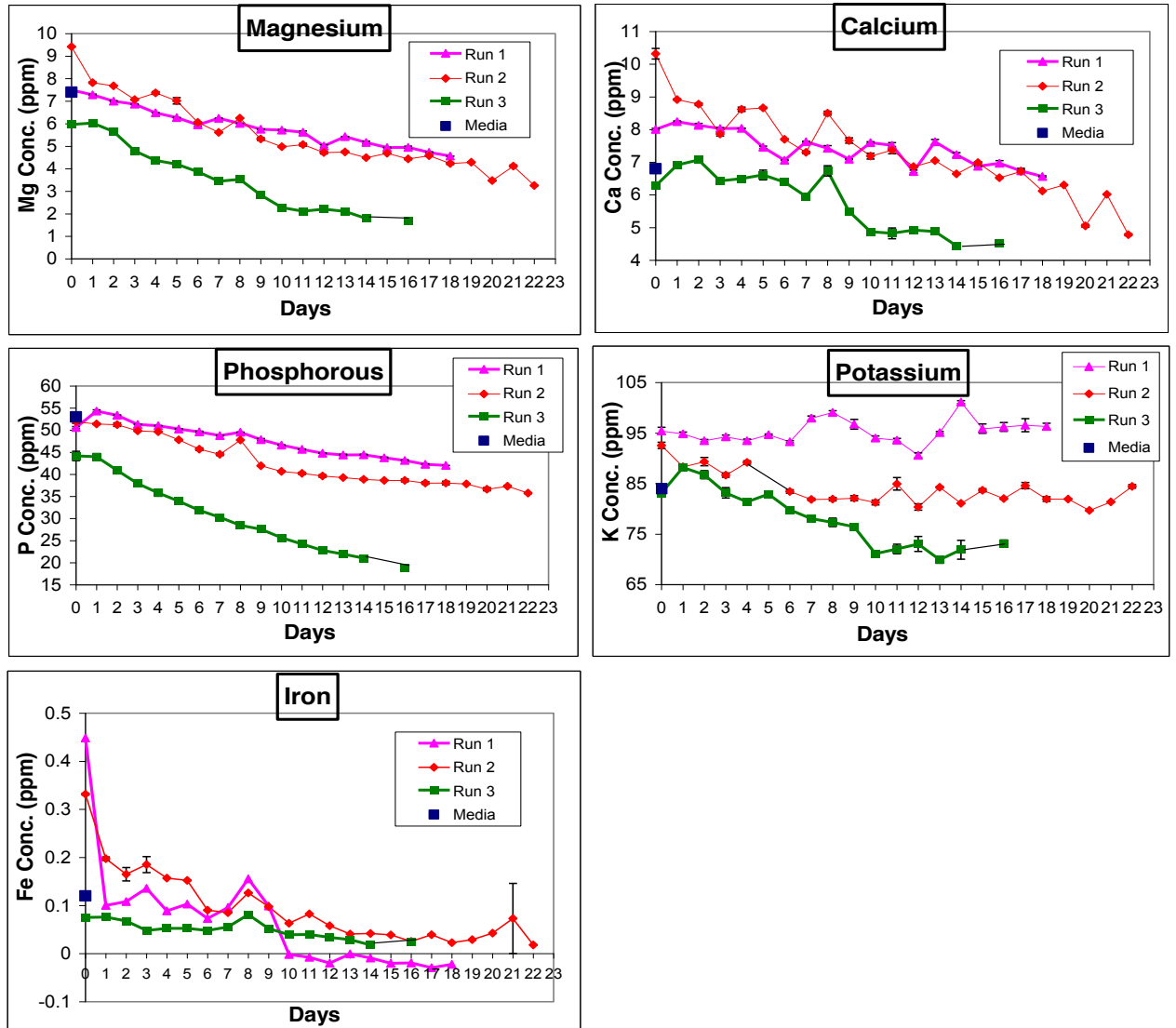
Figure 24: Concentration profiles of the five elements in the liquid during the course of the three batch cultures in the 5 L bioreactor. Potassium concentration showed little change with time in runs 1 and 2. Error bars (often not visible due to the very low errors) are standard deviations from two measurements at each time point.
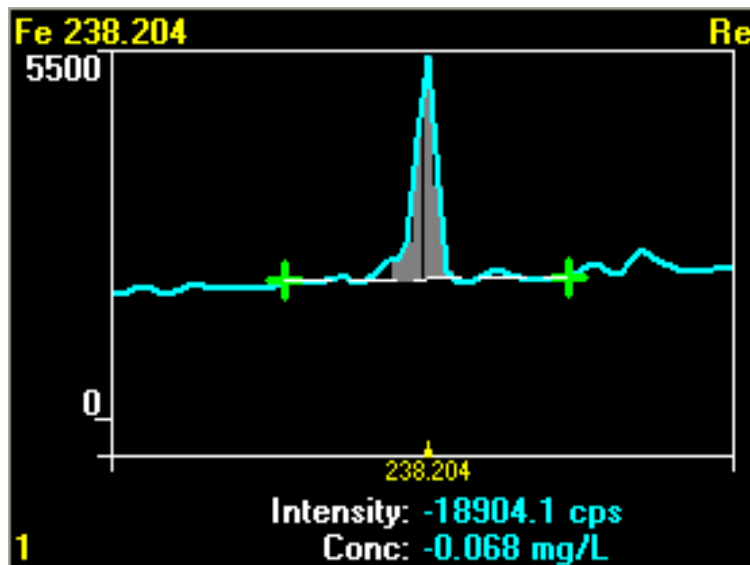
Figure 25: Iron peak for a sample in run 1.

4.3.5   Yield Coefficient ($Y_{X/s}$) and Mass Fraction (x_i)

Figures 26 a, b, and c show how the yield coefficient was obtained for each element in runs 1, 2, and 3 (respectively), based on Equation 1, from the negative of the slope of biomass concentration versus substrate concentration. Biomass concentration was calculated by multiplying the optical density by a factor of 0.5 g/L, while the substrate concentration was measured by ICP.

a) Run 1
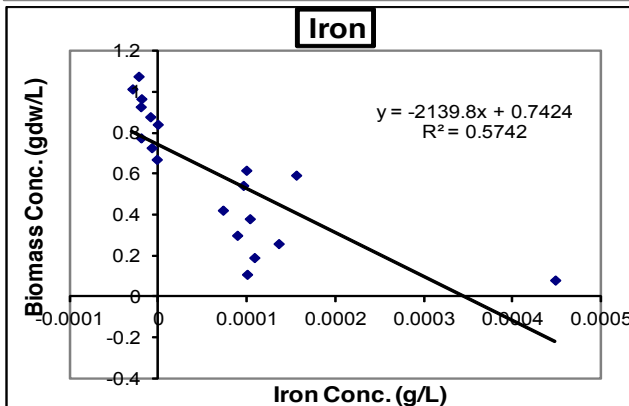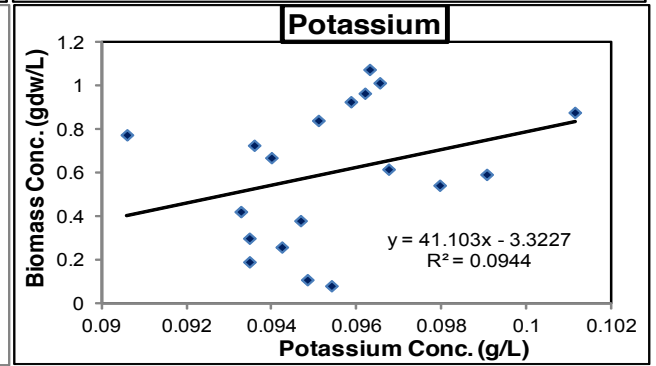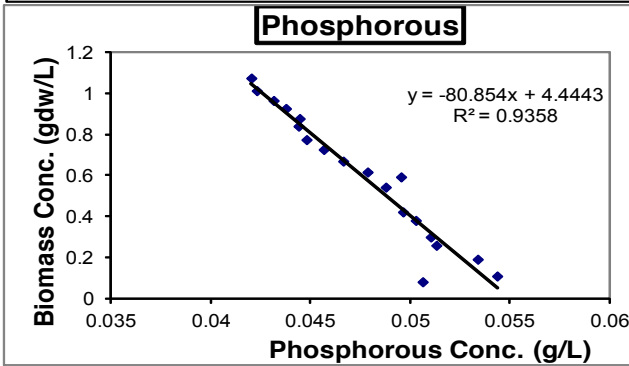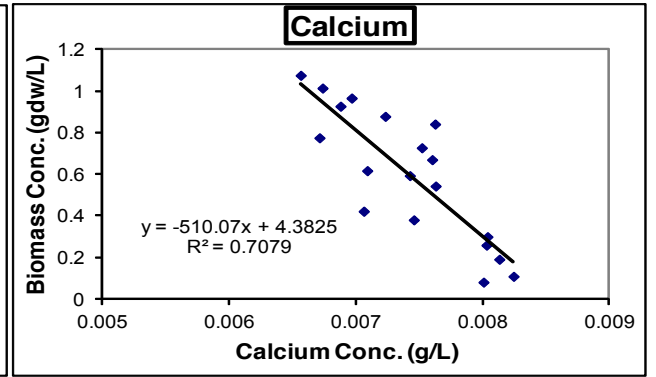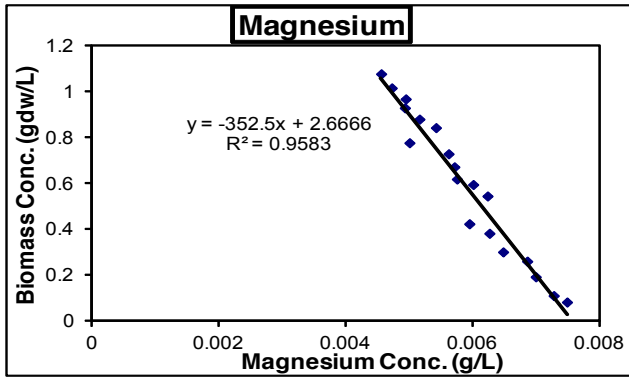
b) Run 2
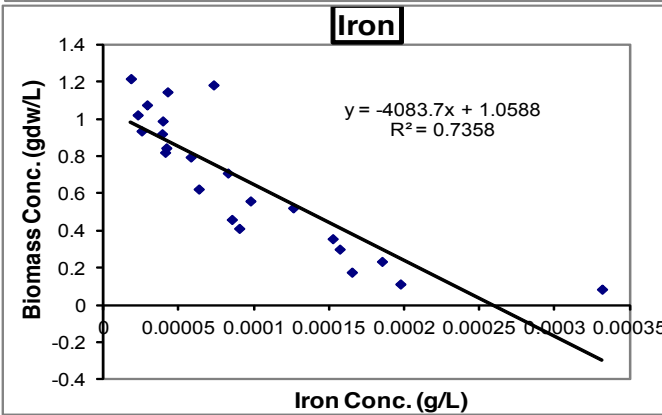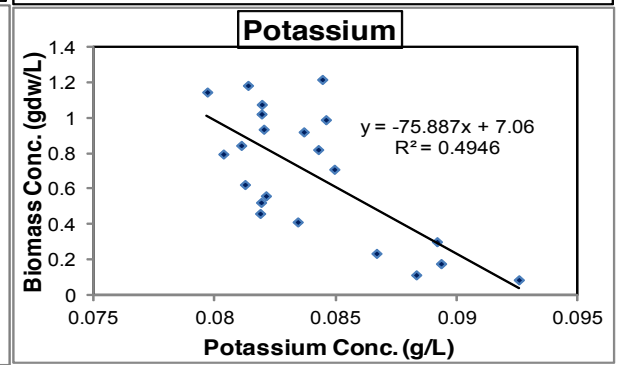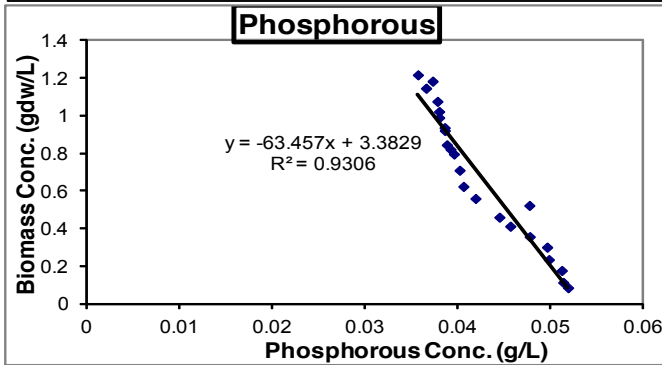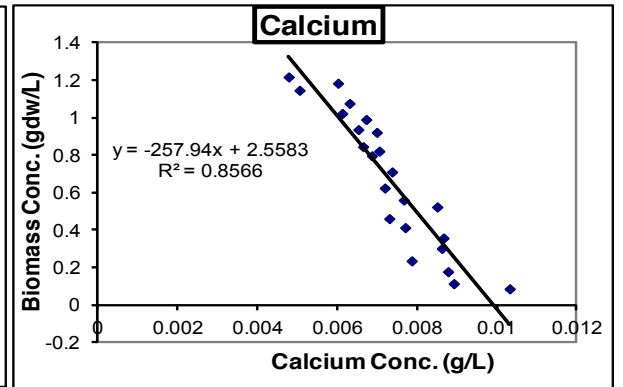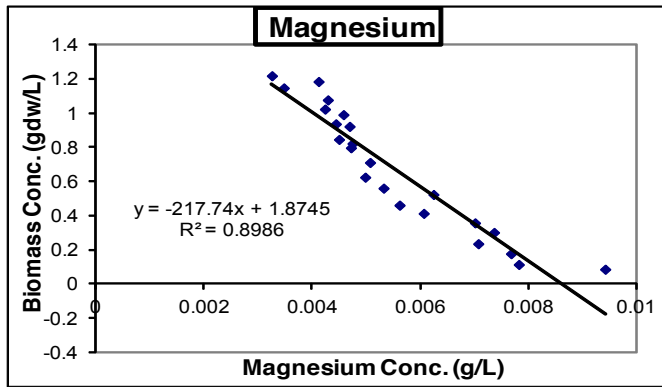
c) Run 3
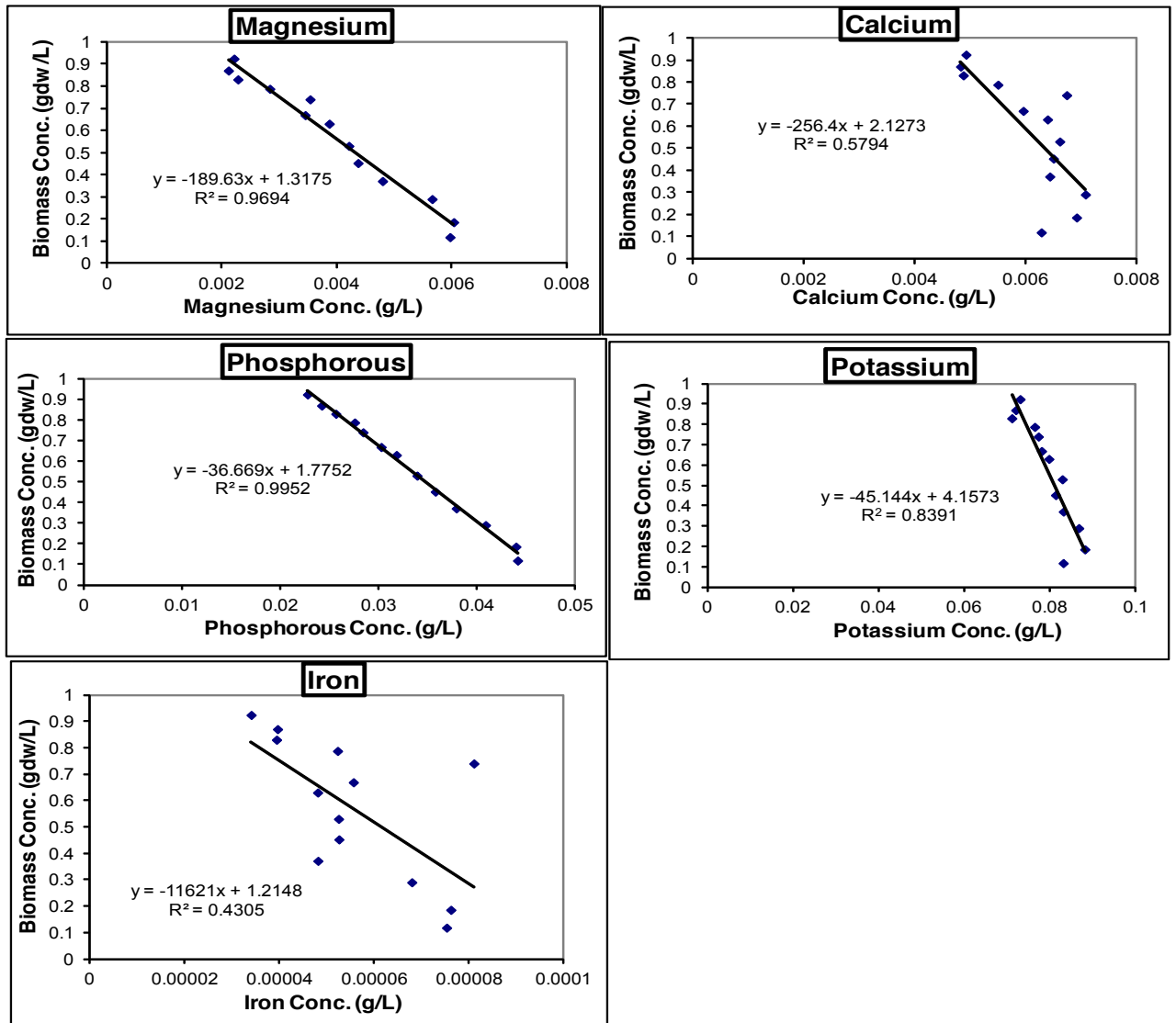
Figure 26: Yield coefficients were obtained from the negative of the slope of biomass concentration (gdw/L) versus substrate concentration (g/L) plots for days 0-18 in run 1, days 0-22 for run 2, and days 0-12 for run 3. The data for potassium in run 1 showed a very low correlation between biomass and potassium concentrations ($R^2 = 0.0944$).

Figure 27 shows a comparison of the yield coefficient ($Y_{X/s}$) to the inverse of mass fraction ($1/x_i$), while Figure 28 presents a comparison of mass fraction (x_i) to the inverse of the yield coefficient ($1/Y_{X/s}$). Table 8 compares yields and inverse mass fractions obtained in this research to literature values.
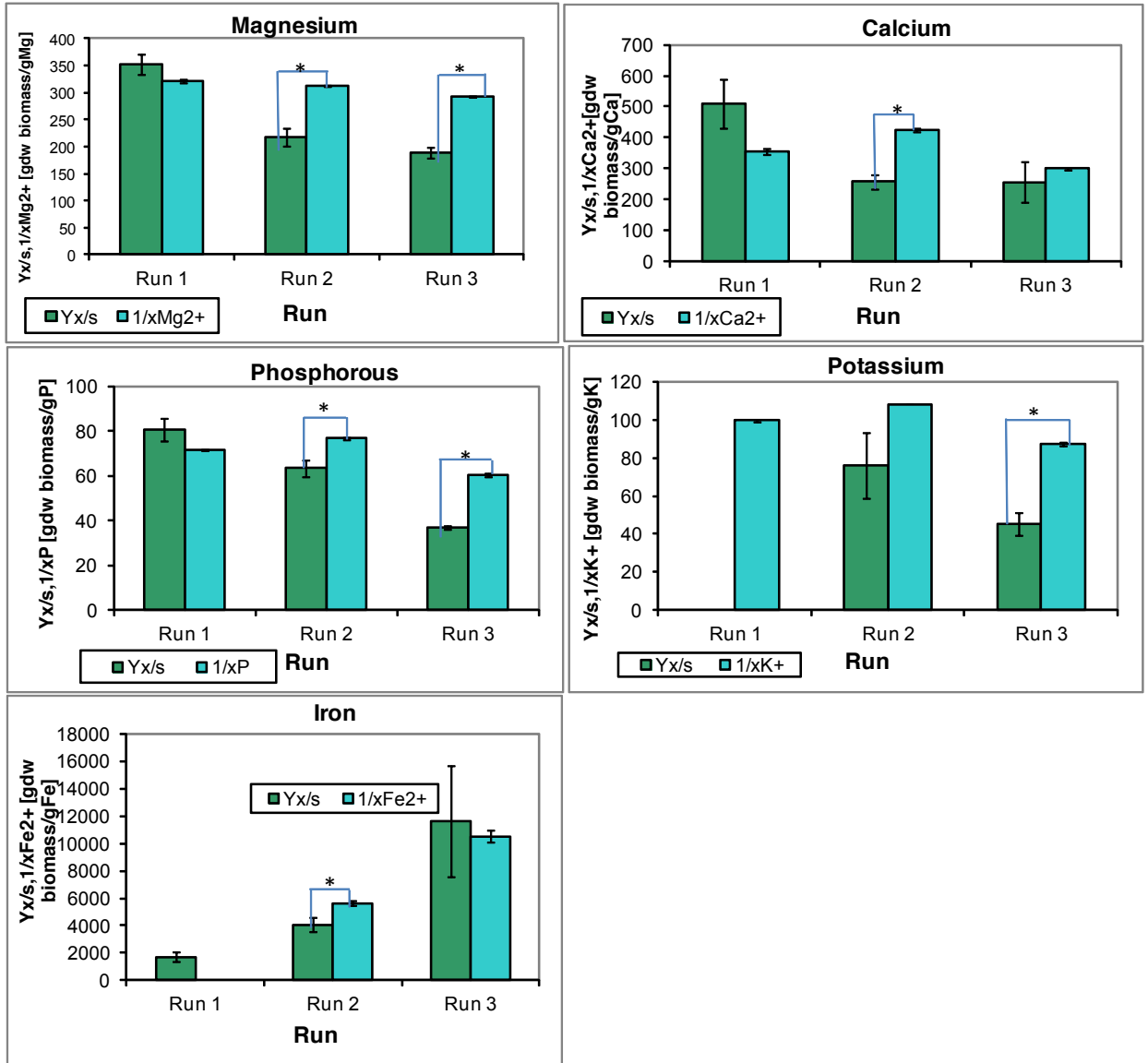
Figure 27: Comparison of the yield coefficient ($Y_{X/s}$) and the inverse of mass fraction ($1/x_i$). Potassium had a negative yield in run 1 because of anomalous data and hence it is not shown. Iron yield in run 1 is not considered reliable due to problems in measurements as described in section 4.3.4. Error bars represent standard errors; for $Y_{X/s}$, they are obtained from Excel's Linest Function. The standard errors in $1/x_i$ are calculated from standard deviations, N=3. *Denotes a statistically significant difference.
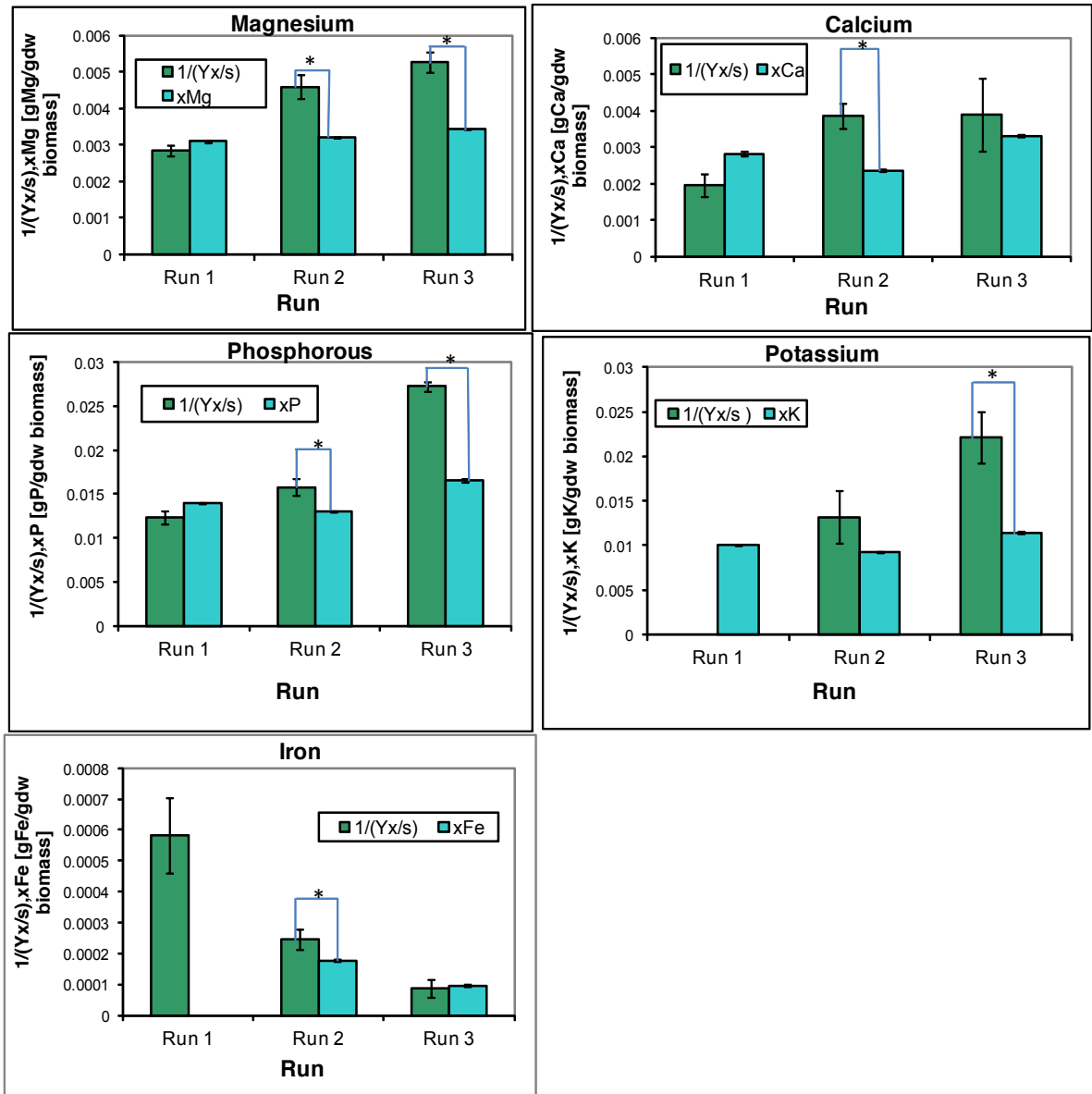
Figure 28: Comparison of mass fraction (x_i) to the inverse of the yield coefficient (1/ $Y_{X/s}$). Error bars represent standard errors. *Denotes a statistically significant difference.

Table 8: Yields and inverse of mass fractions (gdw biomass/gsubstrate), measured for *S. dimorphus*. The mean yields and inverse of mass fractions are for the three runs in the 5 L photobioreactor. Schwenk's (2012) yields were obtained from growing *S. dimorphus* in 2 L bottles with a working volume of 1.6 L.

Element	$Yx/s \pm$ SEM	Yield (Yx/s) from Schwenk, 2012	$1/xi \pm$ SEM	$1/xi$, from Mandalam & Palsson, 1998
P	60±10	N/A	70±5	50-100
K	61±15	N/A	100±6	62-118
Mg	250±50	476	310±10	125-277
Fe	6000±3000	8549	8000±3000	181-2,500
Ca	340±84	247	360±35	1250-20,000

The mass fraction (x_i) in % is the lowest to highest for Fe (0.01), Ca and Mg (0.3), K (1), and P (1.5). The average content of a typical plant (% of dry plant weight) for Fe, P, Mg, Ca, and K are 0.01, 0.2, 0.2, 0.5, and 1 % respectively (Mahler, 2004). A low biomass fraction indicates that a small amount of substrate ends up in the biomass. Iron is the best example for this as it is present in trace amount in the media; hence a miniscule amount will make it to the biomass.

With respect to the yield coefficient, $Y_{X/Fe} > Y_{X/Ca} > Y_{X/Mg} > Y_{X/K} \sim Y_{X/P}$ (Table 8). Moreover, the starting concentration of these elements in the media is ranked as follows [Fe]<[Ca]<[Mg]<[P]<[K]. Iron has the highest yield as it present in trace amounts in the media and is found in a negligible amount in plants. Potassium and phosphorous have the lowest yields, and thus their starting concentrations in the media are the largest. In run 1, there is no significant difference between the yield and the inverse of the biomass fraction for Mg, Ca, and P. In run 2, there is no significant difference between $Y_{X/s}$ and $1/x_i$ only for K. While in run 3, $Y_{X/s}$ and $1/x_i$ are about the same for Ca and Fe (Figure 27).

Table 8 presents the literature and measured yields and mass fractions ($Y_{X/s}$ and $1/x_i$) for *S. dimorphus*. Comparing Schwenk's yields ($Y_{X/s}$) to those obtained in this research, his magnesium yield was higher, his iron yield was within the range found in this research, and his calcium's yield was very close to the lower bound of the range determined this research. Phosphorous and potassium mass fractions ($1/x_i$) were within the ranges previously reported. Magnesium mass fractions were close to literature. Iron and calcium mass fractions were within an order of magnitude of the previously reported values, with iron's mass fraction being too high while calcium's being too low.

Theoretically, all the elements of Mg, Ca, Fe, K, and P that were consumed should accumulate in the biomass since they were used for the purpose of biomass production only (assuming no cell maintenance). Therefore, one would expect equivalence between the yield coefficient ($Y_{x/s}$) and the inverse of the biomass mass fraction ($1/x_i$). $Y_{X/s}$ for P, K, and Mg are within Mandalam & Palsson, 1998 $1/x_i$ range (Fig. 27).

In some circumstances (runs 2 and 3 only), $1/Y_{X/s}$ was greater than x_i (Fig. 28), indicating that more substrate was consumed from the media than showed up in the biomass.

The inverse of the yield coefficient can be interpreted as:

$$\left. \frac{1}{Y_{X/s}} \right|_{actual} = \frac{\Delta s_{actual}}{\Delta X_{actual}} \qquad (27)$$

Where Δs is defined as the change in substrate concentration in the media, while ΔX is the change in biomass concentration.

Similarly, we can define the measured yield coefficient by:

$$\left.\frac{1}{Y_{X/s}}\right|_{measured} = \frac{\Delta s_{measured}}{\Delta X_{measured}} \quad (28)$$

If we assume that the concentration measurements in the fluid are accurate, then:

$$\Delta s_{actual} = \Delta s_{measured} \quad (29)$$

However, significant level of biofilm accumulation on the bioreactor walls was noticed in runs 2 and 3, therefore the true amount of biomass produced is greater than that measured by OD in the liquid, thus:

$$\Delta X_{actual} > \Delta X_{measured} \quad (30)$$

Combining Equations 27 and 28 yields:

$$\left.\frac{1}{Y_{X/s}}\right|_{actual} < \left.\frac{1}{Y_{X/s}}\right|_{measured} \quad (31)$$

Therefore, the inverse of the measured yield is overestimated compared to x_i as was observed for Mg, Ca, P, and Fe in run 2 and Mg, P, and K in run 3. The measured yield is underestimated compared to $1/x_i$ in these same situations (Figure 27). Moreover, the measured inverse yield for runs 2 and 3 is larger than that of run 1 for all elements except iron as was expected due to biofilm formation.

4.3.6   Substrate Consumption Rate and Optimal Media Composition

The rate of substrate consumption r'_s in mg substrate/gdw biomass.day is calculated based on Equation 5. Figure 29 and 30 present the results obtained in the three runs in the bioreactor. The average substrate consumption rates ± standard error in the three runs during exponential growth (days 2-8) for Mg, Ca, Fe, P, and K are 0.61±0.41,

0.31±0.64, 0.008±0.03, 2.8±1.6, and 1.3±2.2 mg substrate/gdw biomass.day respectively (Figure 30).
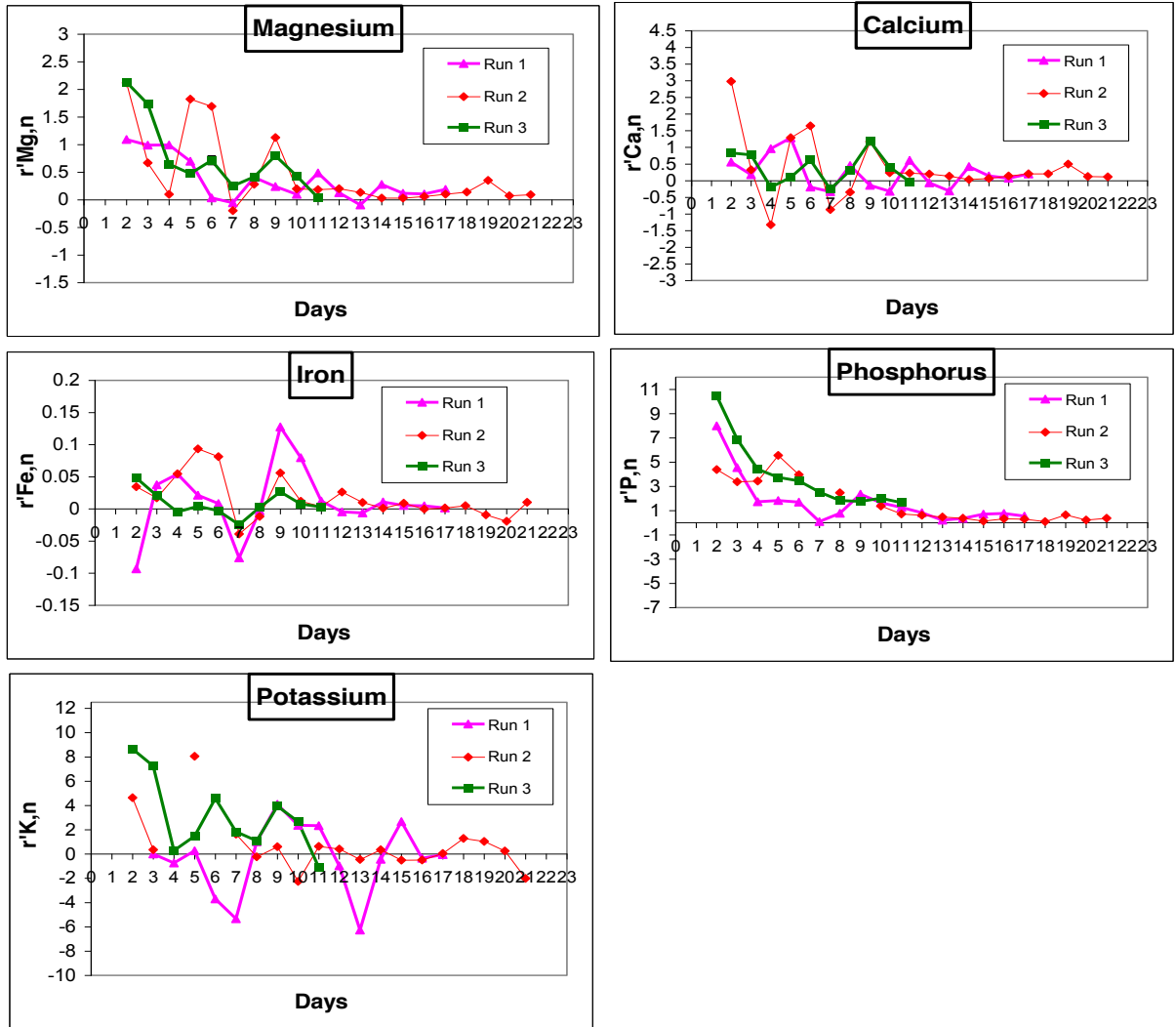
Figure 29: Profiles for substrate consumption rates during the course of the three batch cultures in the 5 L bioreactor.
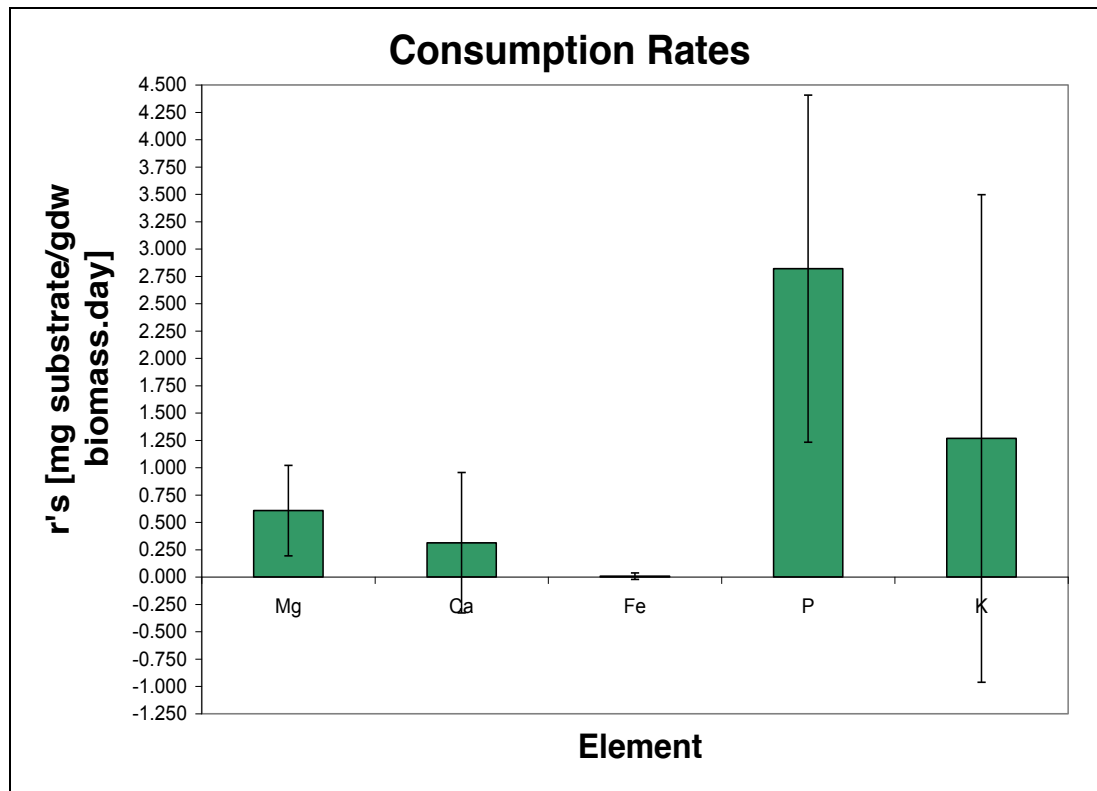
Figure 30: Average substrate consumption rates of the three batch cultures in the 5 L bioreactor during exponential growth (days 2-8). Error bars represent standard errors.

Calcium, iron, and potassium's consumption rates seemed to be almost constant during the course of the experimental runs. However, magnesium and phosphorous consumption rates decreased with time. No literature data was found for the consumption rates of Mg, Ca, Fe, P, and K. Magnesium and calcium have about the same consumption rates. Potassium and phosphorous have the lowest yields, which is consistent with them having the highest consumption rates. The importance of phosphorous lies in its presence in DNA, RNA, and ATP. Iron has the highest yield which agrees with it having the lowest consumption rate. Potassium's consumption rate is not very accurate in runs 1 and 2 since there was a negligible change in its concentration with time in these runs. This explains the negative consumption rates for potassium. However, in run 3 there was a

noticeable decrease in the potassium concentration over the course of the experiment and therefore its consumption rate is reasonable.

Table 9 compares 3N-BBM's substrate composition to a suggested composition derived from Equation 14 for the five elements studied.

Table 9: Suggested optimal substrate concentration in media reported as average±std error.

Element	Substrate Conc. in 3N-BB Media (mg/L)	Optimal Substrate Conc. in Media (mg/L)
Magnesium	7.4	4.3±0.3
Calcium	6.8	3.2±0.2
Phosphorous	53	18±1
Potassium	84	18±1
Iron	0.12	0.19±0.015

The optimal substrate concentrations are lower than that in 3N-BBM except for iron which is a little higher. Reducing the concentration of these elements will help drive down the cost of growing microalgae, hence making it economically feasible to grow microalgae for biofuel production.

4.3.7   Light Meter Issues

Table 10 displays the light intensities, in foot-candles, measured by light meters directly facing each lamp at the middle of the bioreactor's surface. The Sper Scientific light meter did not seem to be functioning well on day 8 of run 3 as its readings fluctuated significantly. This was most likely because its battery was running out. When the battery was replaced on day 9, the light meter's readings were lower than expected compared to day 0 and the previous two experiments. To verify the light intensity values obtained with the Sper Scientific light meter, two other light meters were used for comparison on day 16 of run 3, with the data obtained summarized in Table 10. The light intensity measurements by the Sper Scientific and Extech Instruments light meters seem

to be in agreement with each other. However, they are a little different from the measurements obtained by the Milwaukee light meter. The discrepancy between the light intensity values obtained by the Sper Scientific meter after the battery was changed in run 3 and before it was changed in runs 1, 2, and 3 cannot be explained. However, the microalgae in run 3 definitely had more light exposure.

Table 10: Light intensities, in foot-candles, measured directly facing each lamp at the middle of the bioreactor's surface.

	Run 1			Run 2				Run 3					
												Extech Instruments, Model #407026	Milwaukee SM 700
	Sper Scientific light meter												
									New battery			Day 16	Day 16
Lamp	Day 0	Day 7	Day 14	Day 0	Day 8	Day 15	Day 22	Day 0	Day 9	Day 13	Day 16		
1	1434	1412	1387	1477	1164	1794	1697	1599	614	695	528	542	459
2	1168	1209	1153	1265	927	1244	1153	1859	694	762	722	727	576
3	1312	1217	1133	1374	1246	1701	1621	1583	652	641	591	599	496
4								1645	688	651	634	649	541
average	1305	1279	1224	1372	1112	1580	1490	1672	662	687.25	619	629	518

75

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1   Conclusions

- The mortar and pestle cell lysing method was found to be the most consistent and efficient, producing the highest lipid content of about $19 \pm 2\%$ compared to the bead beater of $9 \pm 2\%$ (Figure 14).

- The three runs performed on the 5 L photobioreactor resulted in *S. dimorphus* lipid content of 11-16% (Figure 23).

- pH control did not seem to have an effect on growth rate (Figure 22).

- The decrease in biomass concentration in run 3 was attributed to biofilm accumulation on the reactor walls causing a loss of about 50% of the total biomass. The algae were stressed due to excess sodium and/or low magnesium concentration (Figure 19).

- The inverse of the measured yield is overestimated compared to x_i as was observed for Mg, Ca, P, and Fe in run 2 and Mg, P, and K in run 3 (Figure 28).

- The inverse yield for runs 2 and 3 is larger than that of run 1 for all elements except iron as was expected due to biofilm formation (Figure 28).

- In some circumstances, more substrate was consumed from the media than showed up in the biomass, attributed to biofilm accumulation (runs 2 and 3):

$$xi < \frac{1}{Y_{X/s}}$$

- Yield coefficients in gdw biomass/g substrate for magnesium, calcium, potassium, phosphorous, and iron are 253 ± 50, 341 ± 84, 61 ± 15, 60 ± 13, and 5807 ± 3000 respectively. A high substrate yield is desirable in achieving the highest growth with the lowest amount of nutrients possible (Figure 27).

- The biomass fraction is the lowest to highest respectively for Fe, Ca and Mg, K, and P $[x_{Fe} < x_{Ca} \sim x_{Mg} < x_K < x_P]$ (Figure 28). These compositions are similar to those of a typical plant. A low biomass fraction indicates that a small amount of substrate ends up in the biomass.

- The average substrate consumption rates during exponential growth in the three runs for Mg, Ca, Fe, P, and K are 0.61 ± 0.41, 0.31 ± 0.64, 0.008 ± 0.03, 2.8 ± 1.6, and 1.3 ± 2.2 mg substrate/gdw biomass.day respectively (Figure 30).

- The optimal substrate concentration in the media in mg/L for magnesium, calcium, potassium, phosphorous, and iron are 4.3 ± 0.6, 3.2 ± 0.4, 18 ± 2, 18 ± 2, and

0.19±0.026 respectively. These optimal concentrations are lower than that of 3N-BBM except for iron (Table 9).

5.2 Recommendations

- Run the experiment in the 2 L bottles and in the 5L bioreactor for a longer time until the absorbance drops or levels off instead of it continuing to go up with time.

- Calculate the maintenance coefficient, that accounts for the maintenance needs of microalgae cells, and which affects substrate consumption rates.

- Analyze the lipids obtained using analytical techniques such as GC-MS (gas chromatography–mass spectrometry) and LC-MS (liquid chromatography–mass spectrometry) to determine the lipids responsible for biofuel production.

- Investigate the effect of increased light exposure on *S. dimorphus* growth.

- Scale up *S. dimorphus* cultivation to verify the growth data obtained in the lab scale experiment.

- Use the suggested concentrations of iron, magnesium, calcium, potassium, and phosphorous in the media by modifying 3N-BBM's composition and run an experiment on the 5L bioreactor similar to the ones carried out in this research. The effect of this modified media recipe on the growth rates and lipid content should be noted.

- Perform a cost analysis to determine the cost reduction involved in using the suggested modified version of 3N-BBM with lower substrate concentrations.

- Look at the possibility of reducing the concentration of sodium nitrate in 3N-BBM.

- Monitor the lipid content profile as a function of time with 3N-BB media as well as with the suggested modified 3N-BBM recipe.

- Run an experiment in the 5L bioreactor in continuous mode, after optimizing the media composition.

REFERENCES

1. "Algae basics: benefits." <u>All about Algae.</u> 2014.
   <http://allaboutalgae.com/benefits/>.

2. "Better than corn? Algae set to beat out other biofuel feedstocks." <u>World Watch Institute</u>. 2013. <http://www.worldwatch.org/node/5391>.

3. "Biodiesel." <u>Energy Justice Network.</u> 2008.
   <http://www.energyjustice.net/biodiesel>.

4. Bischoff, H. W. & Bold, H. C. <u>Some Soil Algae from Enchanted Rock and Related Algal Species.</u>, Austin, Texas: University of Texas Publication No. 6318, 1963.

5. Blanch H. W, Clark D. S. (1996). *Biochemical Engineering*. Marcel Dekker, Inc.

6. Bligh, E.G. and W.J. Dyer. "A rapid method for total lipid extraction and purification."
   <u>Canadian journal of biochemistry and physiology</u> 37.8 (1959): 911-917.

7. "Bold 3N medium." <u>UTEX The Culture Collection of Algae</u>. 2014. <
   http://web.biosci.utexas.edu/utex/mediaDetail.aspx?mediaID=24>.

8. Converti, Attilio, et al. "Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel
   production." <u>Chemical Engineering and Processing: Process Intensification</u> 48.6 (2009):
   1146-1151.

9. "Dissolved oxygen." <u>Cary Institute of Ecosystem Studies</u>. 2014.
   <http://www.caryinstitute.org/sites/default/files/public/downloads/curriculum-project/1C1_dissolved_oxygen_reading.pdf>.

10. "Dissolved oxygen." <u>Fondriest Environmental Inc.</u> 2013.
    <http://www.fondriest.com/science-library/water-quality/dissolved-oxygen>.

11. Elbeling, James, et al. "Engineering analysis of the stoichiometry of photoautotrophic,
    autotrophic, and heterotrophic removal of ammonia-nitrogen in aquaculture systems." <u>Aquaculture</u> 257 (2006): 346-358.

12. Greque de Morais, M. and J. A. Vierira Costa. "Biofixation of carbon dioxide by Spirulina sp. and Scenedesmus obliquus cultivated in a three-stage serial tubular photbioreactor." <u>Journal of Biotechnology</u> 129 (2007): 439‑445.

13. Guerrini, Franca, et al. "Bacterial-algal interactions in polysaccharide production." <u>Aquatic Microbial Ecology</u> *15* (1998): 247-253.

14. Guiry, Michael D. "How many species of algae are there?." <u>Journal of Phycology</u> 48.5 (2012): 1057-1063.

15. Held, Paul. "Monitoring of algal growth using their intrinsic properties: Use of a Multi-Mode Monochromator-based Microplate Reader for Biofuel Research." <u>BioTek.</u> 2011. <http://www.biotek.es/es/resources/articles/monitoring-of-algal-growth-using-intrinsic-properties.html>.

16. Hou, Xiandeng and Bradley T. Jones. "Inductively coupled plasma/optical emission spectrometry." *Encyclopedia of Analytical Chemistry*. Chichester: Ó John Wiley & Sons Ltd, 2000. 9468–9485.

17. Kanani, Bahareh. Master's Thesis: Lipid production by *Scenedesmus Dimorphus*: Comparison of extraction techniques and effect of sodium nitrogen concentration. Cleveland State University. 2013.

18. Lavens, Patrick, and Patrick Sorgeloos. <u>Manual on the Production and Use of Live Food for Aquaculture.</u> Ghent, Belgium: University of Ghent, 1996.

19. "LED colour." <u>Energy and Automation</u>. 2013. <http://www.energyandautomation.com/assets/e-a-led-advanced-led-colour.pdf>.

20. Lee, Jae-Yon, et al. "Comparison of several methods for effective lipid extraction from microalgae." <u>Bioresource Technology</u> 101.1 (2010): S75-S77.

21. Letcher, Peter M., et al. "Characterization of amoeboaphelidium protococcarum, an algal parasite new to the cryptomycota isolated from an outdoor algal pond used for the production of biofuel." PLoS ONE 8.2 (2013): e56232.

22. Long, R.D. and E. Abdelkader. "Mixed-polarity azeotropic solvents for efficient extraction of lipids from Nannochloropsis microalgae." <u>American Journal of Biochemistry and Biotechnology</u> 7.2 (2011): 70-73.

23. Mahler, Robert. "Nutrients plants require for growth." <u>University of Idaho, College of Agricultural and Life Sciences</u>. 2004. <http://www.cals.uidaho.edu/edComm/pdf/CIS/CIS1124.pdf >.

24. Malik, Haider. Master's Project: Microalgae growth kinetics: An investigation of nitrate limited growth kinetics of *Scenedesmus Dimorphus* in a continuous reactor. Cleveland State University. 2012.

25. Martinez, et al. "Influence of phosphorus concentration and temperature on growth and phosphorus uptake by the microalga Scenedesmus obliquus." Bioresource Technology 67.3 (1999): 233-240.

26. Nalewajko, Czeslawa, Brian Colman, and Mary Olaveson. "Effects of pH on growth, photosynthesis, respiration, and copper tolerance of three Scenedesmus strains." Environmental and Experimental Botany 37.2-3 (1997): 153-160.

27. Packer et al. "Growth and neutral lipid synthesis in green microalgae: A mathematical model." Bioresource Technology 102.1 (2011): 111-117.

28. "Photosynthesis." Clermont College. 2014. <http://biology.clc.uc.edu/courses/bio104/photosyn.htm>.

29. Ribita, Daniela. Master's Thesis: Quantification of *Scenedesmus Dimorphus* growth and substrate kinetics for continuous photobioreactor design. Cleveland State University. 2011.

30. "Scenedesmus dimorphus". Algae Depot. 2010. <http://www.algaedepot.org/algae-reference/algae-depot-wiki/doku.php?id=strains;scenedesmus_dimorphus&rev=1283452653>.

31. Schmidt, Charles. "Biodiesel: Cultivating alternative fuels." Environmental Heath Perspectives 115.2 (2007): A86–A91.

32. Schwenk, Jacob. Master's Thesis: Effect of magnesium, digestate, and other inorganic nutrients on the phototrophic growth of the green microalga *Scenedesmus Dimorphus*. Cleveland State University. 2012.

33. Shuler, Michael L., and Fikret Kargi. Bioprocess Engineering: Basic Concepts. Englewood Cliffs, NJ: Prentice Hall, 1992.

34. Shuler, Michael L., and Fikret Kargi. Bioprocess Engineering: Basic Concepts. Englewood Cliffs, NJ: Prentice Hall, 2002.

35. Sorokin, C. & Krauss, R. W. "The effects of light intensity on the growth rates of green algae." Plant Physiology 33.2 (1958): 109−113.

36. Sostaric, M., et al. "Growth, lipid extraction and thermal degradation of the microalgae *Chlorella vulgaris*." New Biotechnology 29.3 (2012): 325-331.

37. "Typical foot candle (FC) and LUX ratings." <u>Lashen Electronics</u>. 2012. <http://www.lashen.com/vendors/pelco/Typical_Light_Levels.asp>.

38. "Using a chlorophyll meter to improve foliar fertilizer efficiency & customer returns." <u>BioGro, Inc</u>. 2013. < http://www.biogro.com/resources/CFSM/BG%20CFS%2005%20-%20Chlorophyll%20Meters%20and%20Foliar%20Fertilizers.pdf>.

39. Uusitalo, Jarmo. "Algal carbon uptake and the difference between alkalinity and high pH ("alkalization"), exemplified with a pH drift experiment." <u>Scientia Marina</u> 60 (Suppl. 1) (1996): 129–134.

40. Velichkova, K., I. Sirakov, G. Georgiev. "Cultivation of *Scenedesmus dimorphus* strain for biofuel production." <u>Agricultural, Science and Technology</u>. 5.2 (2013): 181-185.

41. "What is biodiesel?." <u>University of Strathclyde</u>. 2014. <http://www.esru.strath.ac.uk/EandE/Web_sites/02-03/biofuels/what_biodiesel.htm.>

42. Willner, Andrew. "Hubbert's peak, the economy, and war for oil." <u>Andrew Willner</u>. 2013. <http://www.andrewwillner.com/2013/03/hubberts-peak-the-economy-and-war-for-oil/>.

43. Wong, Yuk-Shan and Nora F.Y. Tam. <u>Wastewater Treatment with Algae.</u> New York: Springer, 1998.

44. Xin, L., H. Hong-ying, and Z. Yu-ping. "Growth and lipid accumulation properties of a freshwater microalga Scenedesmus sp. under different cultivation temperature." <u>Bioresource Technology</u> 102.3 (2011): 3098-3102.

Appendix A: Protocols

A1.   Method of Preparing Bottle Stoppers with a Vent, Sampling Port, and CO$_2$/Air Feed

Make 3 holes in a rubber stopper. Each hole has a steel tube with about 1cm sticking out from each side. One hole is used as a vent to remove the excess CO$_2$ supplied. It has a Masterflex silicone tube with a small piece of cotton. The second hole is attached to a Masterflex silicone tube with a T-connector. Two tubes come out of this T-connector, one is used as a sampling port and the other has a 0.2 μm sterile filter to which a syringe filled with air is connected. Its purpose is to push any algae suspension in the tubing back into the growth bottle when sampling, thus protecting the algae from any contamination by the bacteria present in air. The third hole has a Masterflex silicone tube coming out of it with a 0.2 μm filter which will be attached to a CO$_2$/air feed. Through this hole, a long steel tube goes into the bottle with its end connected to a small Masterflex silicone tube with a pipet tip, thus facilitating the formation of small gas bubbles. The CO$_2$/air feed consists of 5% CO$_2$ mixed with air, which is provided to each of the algae growth bottles using a manifold having flow meters that can be adjusted to ensure the same gas flow rate into the growth bottles. The rubber stoppers with all the tubing and filters are autoclaved with saturated steam before starting an experiment in order to sterilize them. They are wrapped with autoclave paper and tape prior to sterilization.

A2.   Operating Procedure of Genesys 10S UV-Vis Spectrophotometer by Thermo Scientific

The optical density (OD) or absorbance (A_{600}) of the algae suspension is measured at a wavelength of 600 nm.

a) Turn on the spectrophotometer and make sure there is no blank or sample in it.
b) Place the blank (RO water cuvette) in the "B" compartment marked inside the spectrophotometer, with its clear side facing the laser beam.
c) Close the spectrophotometer's lid and hit measure blank. The absorbance is now set to zero for the blank.
d) Place 2 ml of the algae sample in a cuvette using a micropipette and set it in the compartment labeled "1."
e) Close the lid and hit measure sample

The spectrophotometer is accurate up to an absorbance of 1, since there is a linear relationship between absorbance and biomass concentration for $A_{600} \leq 1$. Therefore, if the measured absorbance is above 1 dilute the sample by ½ using DI water and measure its absorbance again. Repeat this process until the absorbance is just below 1. Multiply by the dilution factor to obtain an adjusted absorbance. The dilution can be carried out in a plastic cuvette using a micropipette. For example, place in the cuvette 1000 µL of algae suspension and 1000 µL of DI water. Mix the solution by withdrawing and suspending it several times using the micropipette.

A3. Operating Procedure of Nikon Eclipse E400 Microscope to Examine Algae Samples

OC View computer program is used in conjunction with the microscope in order to observe the microalgae sample on a large screen. This allows the user to take a photo or record a video via the Optix Cam Summit Series camera. After launching this program, place a check mark in the auto-expose box.

Uncover the microscope and turn it on. Clean the hemocytometer with soap and DI water then dry it with a Kimwipe. There are two isolated compartments, fill either one

with the algae sample using a micropipette. Place a glass square over the two compartments and make sure it is centered and is pressing down the sample. Orient the hemocytometer such that the sample is under the microscope light. A zoom of 10X, 20X, and 40X can be used, however, the 20X zoom is usually utilized. There are two knobs for focusing, one on the right (results in a smaller effect) and one on the left (results in a larger effect). Moreover, there are two knobs at the bottom to move the sample right/left or forwards/backwards. The "opticam piece" at the top of the microscope can be adjusted to level out the squares of the hemocytometer. Also, there is a metal bar on the side that controls the light. When it is not pulled out, all the light is received by our eyes. When it is pulled out halfway, half of the light goes to our eyes and the other half goes to the camera. However, when the metal bar is pulled all the way out, all of the light is directed to the camera and nothing can be viewed directly from the microscope.

A4.   Operating Procedure of IEC HN-S II Centrifuge

The centrifuge can hold up to six samples in 50 ml centrifuge tubes, hence, make sure that they are balanced and are within 0.5 g from each other. Turn the power switch to on/timed. The speed dial is always set at ¾ which corresponds to about 1500 RPM as shown on the tachometer. Finally, set the timer for the minutes needed to centrifuge your sample.

A5.   Operating Procedure of Steris Autoclave

The autoclave is always in standby mode. To use it, press cancel once and wait a little since it takes some time to respond. If it did not respond, press cancel again as needed and wait for a response. Place in it the items you need to autoclave and close the door. Choose the cycle needed, and let it run. The sterilization is performed at a

temperature of 121ºC and at a pressure of 0.5 psig. It usually takes one to two hours depending on the cycle chosen.

The following is a list of cycles used in this research:

a) Dry Rapt: sterilizes solids such as stoppers, tubings, and pipette tips

b) Liq 250: sterilizes up to 250 ml liquid volume

c) Liq 1000: sterilizes up to 1000 ml liquid volume

d) Liq 2000: sterilizes up to 2000 ml liquid volume

A6.   Protocol for Use of PerkinElmer ICP
Based on Dr. Xiang Zhou's Protocol (Director of the Instrumentation Center, Chemistry Department, Cleveland State University)

SPECIAL CAUTIONS FOR ICP:
- Be sure the exhaust vent is in operation, and the shutter is open
- It is critical to run particle-free samples; well filtrate and/or centrifuge your samples if any particles exist. The algae samples in this research were centrifuged.
- Avoid too high sample concentrations (ppm or lower is recommended)
- It is recommended to prepare samples in DI water with 2% nitric acid
- Use fresh DI water as solvent. Distilled water and old DI water may have precipitation.
- Never run oil samples
- Sample pumping flow rate of 1 ml/min is recommended
- Never connect to wrong gas tanks (be sure argon line to argon tank and nitrogen to nitrogen)
- The waste liquid collection container should be less than ¾ full

1. Clean and organize the working area, and then login using the ICP Logbook.

2.
a) Nitrogen is supplied by a generator using a wall line. Be sure the two shut-off valves in the N_2 wall line are open
b) Open the main valve for the argon tank; do not adjust the regulator

c) Change gas tank when the tank pressure is below 300 PSI
- Procedure to change tank: a) close the valve for the tank to be replaced; b) switch the regulator onto a full tank; label this full tank with "In Use" and the replaced tank with "Empty"
- Must be "Ar regulator to Ar tank" and "N_2 regulator to N_2 tank"

- Always secure tanks

3. Be sure the cooler beneath the ICP is on, and then switch on ICP

4. Switch on and Login to PC. The username and password are ICP.

5. Generate your folders in PC if you do not have one:
   - Generate a file folder under C, by going to "my computer," "C-Drive," and create a new folder there: (YourName-FacultySupervisor'sName).
   - Generate an ICP Workspace folder under C:\PE\Xiang\Workspaces\ (YourName-FacultySupervisor'sName).

6. Open WinLab32. Wait a couple of minutes for communication.
Note: Press F1 for help with the software.

7. Do 7A, 7B, and then 7C; if you will use a previous method saved in your Workspace, skip to Step 10.

7A. 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
   e. Do modifications following Section 7B-a to 7B-j

7B. Generate Method from Default Method
   - Usually generate method by modification of an existing method as Section 7A
   - 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 ml/min, 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 (used here) or Internal Standard
      - Settings: Normal for wavelength above 190 nm; Auto; Delay Time: 20 sec;
        Replicates: 2 usually (measurement done twice then averaged); Measured by: Element

- Spectral Windows: do nothing
  b. Sampler
    - Plasma: 20 sec; Same for All Elements; Wet; Instant
    - Peristaltic Pump: 1.0 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 (No for this research)
    - Internal Stds Checks: do nothing

  d. Calibration
    - Define Standards: Do not check Method of Additions; Calib Blank1 given B1(ID) and 1 (A/S Location); Calib Std1 given S1 and 2; Calib Std2 given S2 and 3; etc
    - Calib Units and Concentrations: give Units and the concentrations
    - Blank Usage: B1
    - Equations and Sample Units: Lin, Calc Int; unit; 3 (max decimal places); 4 (max sig figs)
    - 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 test Reports, Calibration Summary; Spectral Data
  h. Save the Method: File – Save As – Method - Method Name: Your Initials-yymmdd-ID–Ok (This saves the new method replacing the old one)
  i. Check Method (workable or not): Edit – Check Method ("Method is ok" is the message you get)
  j. Exit the Method Editor Win

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

8. Open Working Sub-Windows: Plasma; Manual; Spectra; and 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) – Save

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

11. a. Setup Peristaltic Pump
- Left track for inlet tubing (yellow tubing-in which the sample goes) and right track for outlet tubing (white tubing-to waste container)
- The flow goes clockwise: the earlier pathway at lower side and later pathway at upper side
- The screws are set at ~ 3 mm position (do not change usually)
- Be sure each tubing is in the center of each track, and then lock the tracks
- Place the Inlet tubing in a test tube containing fresh DI water
- It is ok to have bubbles in the waste line but not in the sample uptake tubing
- If the tubes are connected in a wrong way, bubbles will form in the DI water

b. Start the flow
- Click pump under plasma control
- Check the flow rate to be sure the flow is normal. The water level in the test tube should go down at a rate if 1ml per minute.
- If there is no flow, adjust the tubing position and/or the screws; in most (over 90%) of the cases, no flow can be resolved by such adjustments
- Changing the tubing (expensive) is the last solution for resolving no flow

12. Start Plasma
- Click on Plasma in the plasma control box. A blue flame will be seen in the plasma viewing window.
- Place the inlet tubing in fresh DI water (washing solution). The flow rate can be badly affected if air enters the tubing.

13. Generate Data 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 for ~ 20 seconds, then click Analyze Blank; Put inlet in washing solution (DI water). The blank contains DI water and 0.064 M trace grade nitric acid only.
- Put inlet in the calibration standard S1; click Analyze Standard; put inlet in washing solution; repeat this step with other standards
- Run samples in a similar manner by clicking Analyze Sample. You can give each sample an ID (for example sample 1).

15. Clean the system in the end. This step is important to get rid of contamination from the instrument.
    - Pump the cleaning solution (10% nitric acid) for about 15 minutes
    - Wash the system for 5 minutes using fresh DI water

16. (Optional) Click off Plasma and Pump during long breaks (ICP consumes a lot of gas).

17. Generate report and print out data; backing up your data is important.
    - 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-yymmdd-ID; Save
    e. Open the PDF or Word file in your file folder; print it
    f. If you want to print all the results including the elements' intensities, click on the results tab, then go to File-Print-Active Window

18. Finishing with the Instrument
    - Click off Plasma
    - Close the main valve for the argon tank; leave the shut-off nitrogen valves in the wall line open.
    - Unlock the tracks for the pump; loosen the tubing from the higher side
    - Exit WinLab32; shutdown the PC
    - Switch off ICP

19. Logout the Logbook

A7. Bligh-Dyer Lipid Extraction Method

Always wear a lab coat, goggles, and viton gloves (latex or nitrile gloves underneath), and perform all the lipid extractions in the hood. To perform three replicates

of lipid extraction on an algae suspension that was centrifuged and dried do the following

(200 ml of algae with an absorbance above 1 is usulally used per replicate):

a) Clean three 50 ml glass centrifuge tubes and three glass tubes with a flat bottom using soap and DI water, then rise with methanol and let them air dry. Cover with foil until ready to use.

b) Pre-weigh a 50 ml glass centrifuge tube and add to it 0.2 g of ground and dry biomass (amount targeted). The mortar and pestle was used to grind the dried biomass pellets into a fine powder. If using 0.1-0.5 gdw of biomass use the amount of solvents stated below throughout the extraction. Weigh the glass tube with the ground algae to find its mass by difference.

c) If lipid extraction is not carried out the same day algae was ground, purge the glass centrifuge tube with nitrogen and store it at -20ºC. Lipid extraction should preferably be carried out the next day.

d) Add 7.5 ml of methanol/chloroform solution (2:1, v/v) and mix the mixture for 1 minute then vortex it for 1 minute. The tube can be capped and stored at -20ºC if lipid extraction will be carried out soon, otherwise store it at -80ºC until the next step.

e) Add to it 7.5 ml of 100% chloroform and mix for 1 minute.

f) Then add 5 ml of 20% w/w $CaCl_2$ solution and shake for 30 seconds.

g) Centrifuge at 1500 RPM for 5-10 minutes until a clear separation of layers is observed. The lipids will be extracted into the lower organic layer composed mainly of chloroform and methanol.

h) Use a Pasteur pipette to remove the lower organic layer.

i) Filter the organic layer through chloroform resistant paper (hydrophilic PVDF, 0.45 μm, Millipore Durapore Membrane Filters) using a glass funnel (or polypropylene plastic funnel) into a pre-weighed 50 ml glass tube with a flat bottom.

j) Add 5 ml of 100% chloroform solution to the residual biomass (aqueous layer), and mix for 1 minute. Centrifuge at 1500 RPM for 5 minutes.

k) Remove the lower organic layer with a Pasteur pipette and filter it through chloroform resistant paper into the same 50 ml glass tube previously used, thus combining the filtrates.

l) Dry the filtrate to a constant weight under the hood with a nitrogen purge connected to a glass pipette (about 5-6 hours). This process consumes a significant amount of nitrogen. Therefore, another way is allowing the organic layer to dry in the hood on its own (while the hood is on).

m) Record the weight of dried lipids and glass tube, then find the mass of dried filtrate (lipids) by difference.

n) Add about 5 ml of toluene to the lipids in the glass tube and swirl it around to dissolve them. Store the tube at -20°C for further lipid analysis using gas chromatography in order to determine the type of fatty acids present.