Reduction of Three Major Bottlenecks Limiting Current Commercial Microalgae Production: Light Utilization, Waste Nutrient Utilization, and Harvesting

by

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ABSTRACT

Microscopic algae have been investigated extensively by researchers for decades for their ability to bioremediate wastewater and flue gas while producing valuable biomass for use as feed, fuel, fertilizer, nutraceutical, and other specialty products. Reports of the exciting commercial potential of this diverse group of organisms started appearing in the literature as early as the 1940's. However, nearly 80 years later, relatively few successful commercial microalgae installations exist and algae have not yet reached agricultural commodity status. This dissertation examines three major bottlenecks to commercial microalgae production including lack of an efficient and economical cultivation strategy, poor management of volatile waste nutrients, and costly harvesting and post processing strategies. A chapter is devoted to each of these three areas to gain a better understanding of each bottleneck as well as strategies for overcoming them.

The first chapter demonstrates the capability of two strains of *Scenedesmus acutus* to grow in ultra-high-density (>10 g L^{-1} dry weight biomass) cultures in flat panel photobioreactors for year-round production in the desert Southwest with record volumetric biomass productivity. The advantages and efficiency of high-density cultivation are discussed. The second chapter focuses on uptake and utilization of the volatile components of wastewater: ammonia and carbon dioxide. *Scenedesmus acutus* was cultured on wastewater from both municipal and agricultural origin and was shown to perform significantly better on flue gas as compared to commercial grade CO_2 and just as well on waste nutrients as the commonly used BG-11 laboratory culture media, all while producing up to 50% lipids of the dry weight biomass suitable for use in biodiesel. The third chapter evaluates the feasibility of using gravity sedimentation for the

harvesting of the difficult-to-separate *Scenedesmus acutus* green algae biomass followed by microfluidization to disrupt the cells. Lipid-extracted biomass was then studied as a fertilizer for plants and shown to have similar performance to a commercially available 4-6-6 fertilizer. Based on the work from these three chapters, a summary of modifications are suggested to help current and future microalgae companies be more competitive in the marketplace with traditional agricultural commodities.

DEDICATION

In everliving memory of “The Wizard of Ooze,”

Dr. Milton Sommerfeld

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1. INTRODUCTION

1.1 The Untapped Potential of Microalgae

Hold your breath for one second. Now breathe normally and imagine skipping every other breath. That is what life on Earth would be like without algae. It is a well-documented but seemingly little known fact that algae—both macroscopic and microscopic—are responsible for about half of the oxygen that we breathe each day. Although planktonic algae is a mere 0.8% of the average areal concentration of terrestrial plant life (O. Pulz 1998), researchers estimate that due to the higher photosynthetic efficiency of such microscopic algae, they are responsible for more than 99% of the total photosynthetic activity in the world's oceans (Field et al. 1998) . Most people would likely assume that trees, grass, and other higher plant life produce most of the oxygen we breathe, because they are the main producers we observe or cultivate in our terrestrial environment. However, because approximately three-quarters of the Earth's surface is covered by ocean that provides habitat for trillions of microscopic phytoplankton, algae take the center stage when discussing carbon dioxide fixation or oxygen evolution from a global perspective.

When hearing the word "algae", most people probably picture pond scum or macroscopic edible seaweeds such as nori, wakame, or kombu. Few people likely know many of the microscopic algae including various diatoms, green or red algae, and cyanobacteria. These are among some of the oldest living organisms found in the fossil record and they are quite ubiquitous in nature (Andersen 1992). They have carved out niche habitats across the globe and in doing so developed the genetic diversity to survive and adapt to many adverse environments (Stibal et al. 2007). All this while achieving superior biomass yields in comparison to traditional crops (Dismukes et al. 2008) and

simultaneously producing valuable compounds including but not limited to various antioxidants (Xia et al. 2013), pigments (Borowitzka 2013), fatty acids (Cohen et al. 1992), carbohydrates (Branyikova et al. 2011) and other bioactive molecules (Coates RC 2013).

These microscopic phytoplankton, dubbed "microalgae" have tremendous potential and have become regarded as the next frontier in agriculture (Carnegie 2017). They have been studied closely by NASA-funded projects (Harris et al. 2013) with the intent of someday providing a way of recycling nutrients in space (Niederwiesera et al. 2018). Microalgae have been the focus of massive research efforts funded by U.S. agencies such as the Department of Energy (DOE), Department of Defense (DOD), and Defense Advanced Research Projects Agency (DARPA), Environmental Protection Agency (EPA), United States Department of Agriculture (USDA) and many others for use as biofuel and/or bioremediation (Resnik et al. 2004).

Research centers for microalgae have been long established internationally in places such as the Czech Republic (Masojídek 2009) and Israel (Masojídek and Prail 2010) and more recently extended to countries all over the globe including research conducted on all seven continents. Power utilities and manufacturing facilities have investigated microalgae in recent years as a way to generate biofuel while bioremediating wastewaters (Chan et al. 2014) or flue emissions (Li et al. 2011). Also, many private companies have invested in startup ventures and partnered with universities on projects pursuing microalgae for omega-3 fatty acids (Fedorova-Dahms et al. 2011), astaxanthin (Butler et al. 2017), lutein (Vaquero et al. 2014), or nutritional supplements (Xia et al. 2013). They can be regarded as single-cell "cellular factories" producing a plethora of biomolecules naturally. Research teams are just beginning to understand the molecular

techniques required to tune these small factories genetically which makes the possibilities practically limitless (Liu et al. 2009). However, despite their amazing potential and the amount of work that has been completed, microalgae has only seen limited success in agriculture due to a number of factors, namely the industry's limited knowledge of cultivating, harvesting, and processing algae as a crop. Better understanding these fundamentals to algaculture will someday unlock the full potential of microalgae and make wide scale commercial algaculture a reality.

1.2 Classification of Algae

Algae refer to a very diverse group of organisms first appearing in the fossil record over 3.5 billion years ago, and possibly being some of the first life forms on Earth. Because of such extensive evolutionary history, it can be difficult to accurately define algae. According to (Graham and Wilcox 2000):

> "Algae are (with numerous exceptions) aquatic organisms that (with frequent exceptions) are photosynthetic, oxygenic autotrophs that are (except for the kelps) typically smaller and less structurally complex than land plants."

Such a broad definition is necessary to describe a group of organisms belonging to the kingdom Protista that, depending on definitions, spans two different domains of life including division Cyanophyta, or blue-green algae, coming from the domain Eubacteria and six taxonomic divisions from the domain Eukarya including Charophyta, Chlorophyta, Chromophyta, Rhodophyta, Dinoflagellata, Euglenophyta (Andersen and Lewin 2018). New classifications based on molecular and structural data have recently surfaced in the literature dividing Eukarya into seven supergroups, including Archaeplastids (Rhodophyta, Chlorophyta, Charophyta); Excavates (Euglenophyta);

Table 1. 1**:** Estimated algal species described to date and total estimated species from the major divisions of algae (modified from Guiry 2012).

Division:	Described species:	Estimated Total species:
Cyanophyta	5,000	8,000
Charophyta	6,000	9,000
Chlorophyta	8,000	13,000
Chromophyta	12,500	21,000
Rhodophyta	7,000	14,000
Dinoflagellata	2,500	3,000
Euglenophyta	2000	3,000
Other Divisions	918	1536
Total:	43,918	72,536

Alveolates (Dinoflagellata); Stramenopiles (Chromophyta); Amoebozoa; Opisthokonts; Rhizaria and an nameless seventh supergroup (Cryptophyta among others) (Worden et al. 2015). Literature estimates suggest thousands of species for each major division of which only a fraction have been described to date (Andersen 1992; Guiry 2012). Table 1.1 shows the total estimated algae species in comparison to species already described by the literature as given by Guiry 2012. The category entitled "other divisions" includes species from the smaller divisions of Glaucophyta, Cryptophyta, Perclozoa and Choanozoa.

This demonstrates the diversity and success of algae in evolutionary history and offers a rich genetic pool from which to select new agricultural crops. Commercial applications, current or potential, exist for representative species from all seven divisions of algae presented.

Historically, algae have been difficult for taxonomists because of the amount of diversity they possess. In the 1830's they were grouped by their color which included

blue-green, green, brown, and red algae and throughout the years were further classified using mostly morphological features that were again refined with electron microscopy (Andersen and Lewin 2018). With the advent of molecular techniques, some of these classifications have been stood on end and taxonomists still debate the proper classifications of some algae. Cyanobacteria, which have been known as blue-green algae for decades, have recently been left out of the discussion when talking about algae because some modern phycologists do not define them as true algae, having their origins in the Eubacteria domain (Andersen and Lewin 2018). They will be discussed in this work, however, as this work focuses primarily on the applied, commercial applications of microalgae. *Spirulina sp*. and *Aphanizomenon sp*. are two such blue-green algae species produced as health supplements and are therefore major contributors to current total world production of microalgae.

Also important to note is that although the overview of the algae given below is limited to a division level classification in this work, many phycologists are not in complete agreement on the classifications at the division level or higher, although most are in agreement with classifications made at the order levels (Andersen and Lewin 2018). Each major division of algae has its own unique properties and classes of compounds, which means that many commercial niches have the potential to be fulfilled by algae or algae byproducts.

1.2.1 Division Cyanophyta

Representative members of the division Cyanophyta, better known as blue-green algae, are not considered by contemporary phycology as true algae because of their simplistic nature, lacking membrane bound organelles found in eukaryotic algae and instead sharing more characteristics with prokaryotic bacteria. Nevertheless, *Spirulina*

sp. is a member belonging to this division and is one of first microscopic organisms successfully cultivated on a commercial scale (Soni et al. 2017) with production having slowly ramped up over the past few decades. Other important organisms from this class include filamentous strains like *Anabaena sp*. and *Aphanizomenon sp*. that exhibit heterocyst formation capable of fixing atmospheric nitrogen. Experts estimate total global biological nitrogen fixation at 175 to 240 million tons per year from both bacterial and cyanobacterial activity in the root nodules of plants and crops, soil crusts, lichens, freshwater sources and up to 100 million tons originating from marine cyanobacterial blooms (Bezdicek and Kennedy 1998; Biswas and Gresshoff 2014; A et al. 2014). The activity of this group of organisms has a tremendous impact on the nitrogen cycle and is essential for the production of many agricultural commodities such as legumes or any products derived from them (Graham and Vance 2003). Cyanobacteria also form symbioses with a number of different organisms that allow them to survive almost everywhere in nature, from lichens on trees or rocks to root nodules in higher plants to symbioses with other algae like diatoms (Foster et al. 2011). Some nitrogen fixing stains directly excrete up to 60% of the fixed nitrogen into the surrounding environment for use by other organisms and others have the ability for chromatic adaptations that allow them to adjust their pigment concentration to adapt to a changing light source (Graham and Wilcox 2000). Chlorophyll a, b, c, d, and f are produced by different members of Cyanophyta as are blue and red phycobilin proteins, several xanthophylls, and carotenoids such as β-carotene (Komárek et al. 2014; Chen et al. 2012).

Chloroplasts, the photosynthetic organelles found in eukaryotic organisms, are thought to have originated from an endosymbiotic event where a eukaryotic cell engulfed a prokaryotic cyanobacteria that was, over time, integrated into the cell. There is substantial evidence to support this including the similarity of chloroplast DNA, which is

circular and organized into operons like cyanobacterial DNA. In addition, some chloroplasts have remnants of cyanobacterial structure like a peptidoglycan layer in the membrane common in cyanobacteria. The passage of time and evolution since this endosymbiotic event has increased the differences between chloroplasts and their cyanobacterial ancestors. Unlike chloroplasts found in eukaryotic algae, the thylakoids are structured differently in cyanobacteria, not organized into stacks within a chloroplast organelle. They produce a form of starch and cyanophycin, a polypeptide compound, both stored in cytoplasmic granules. Reproducing asexually, by means of binary or multiple fission, cyanobacteria are quite successful on a global scale, being responsible for the formation of many algal blooms in lakes and reservoirs. They are responsible for up to 60% of the chlorophyll a found in or around the tropics and are found almost everywhere including the extreme environments of hot springs or as black zones on beaches (Barsanti and Gualtieri 2006). Other important members of Cyanophyta include *Nostoc sp*. which is already in commercial production and the model organism *Synechocystis sp*. that was the first photosynthetic organism to have a completely sequenced genome (Kaneko et al. 1996).

1.2.2 Division Charophyta

Members of Charophyta include the stonewort *Chara sp*., one of the oldest living macroscopic representative organisms of this division for many years thought to be the sister group to land plants (Embryophyta). This is due to the presence of a phragmoplast type of cell division in *Chara sp*. similar to that of land plants in addition to the presence of phytohormones and more complex transcriptional regulation as found in in higher plants (Nishiyama et al. 2018). More recently, evidence has surfaced in the literature suggesting Zygnematophyceae of the Charophyta division might be the sister group to

land plants (Delwiche and Cooper 2015). This topic will be further refined as more molecular data is discovered to provide a better understanding the phylogeny of these organisms during such an important event in evolutionary history.

1.2.3 Division Chlorophyta

Chlorophyta is the division of green algae with members having a chloroplast containing chlorophyll a and/or b, a trait also shared with higher plants. Members of this division produce and store starch inside their chloroplasts (Andersen and Lewin 2018) and can shift their metabolism to produce lipids when placed in unfavorable conditions with limited nutrients (de Jaeger et al. 2014). The class Chlorophyceae includes several freshwater strains including *Chlamydomonas sp,* the model species used for study on algal genetics. This class also contains *Chlorella sp*. and *Dunaliella sp*., two algae currently in commercial production (Hulatt et al. 2012). Other important classes in this division include Pleurastrophyceae that includes *Tetraselmis sp*., Prasinophyceae that includes *Micromonas sp*., and Ulvophyceae which includes *Ulva sp*. commonly known as sea lettuce (Andersen and Lewin 2018).

1.2.4 Division Chromophyta

Chromophyta is a very diverse division that includes by far the largest class Bacillariophyceae, or diatoms, with an estimated 12-15,000 species. Diatoms live in freshwater, marine and soil environments and have characteristic cell walls composed of silica called frustules that are highly ornate (Andersen and Lewin 2018). They are known as prominent bloom formers in aquatic environments. Their presence in soil or water samples can be used as a sort of forensic fingerprint to identify where samples originated by the types and amount of organisms present. However, they are most well

known among algal biofuel experts for their ability to accumulate large amounts of oil and have been the focus of many studies. Liberation of oil droplets from the cells of certain diatoms can be accomplished by only the weight of a coverslip on a microscope slide, making them attractive candidates for economical oil extraction.

Besides diatoms, Chromophyta also includes Eustigmatophyceae which are small green spherical algae known for oil production as well. *Nannochlorpsis sp.* has been the focus of many research projects for either jet fuel or omega-3 production. Phaeophyceae or brown algae or seaweeds include 1500 species one of which is *Saccharina sp.* and *Ascophyllum sp.* both of which are currently commercially cultivated for food, fertilizer, iodine, etc. Other members include Chrysophyaceae, or golden algae, of which the microalga *Ochromonas sp.* is a member. *Ochromonas sp.* has been studied extensively as a high yielding oil crop exceeding 70% of its weight in oil (Ru et al. 2010). Also included in this division are the Xanthophyceae, or yellow-green algae. Most members of Chromophyta produce chlorophyll a with carotenoids and a special beta- 1, 3-linked polysaccharide stored around the chloroplast (Andersen and Lewin 2018).

1.2.5 Division Euglenophyta

Euglena sp. is the star member of this division being studied intensely for its commercial application in natural α-tocopherol (vitamin E) production as well as paramylon, or β-1,3 glycan, a polysaccharide known for its immune stimulating effects (Grimma et al. 2015). *Euglena sp.* as well as other genera in the division produce paramylon starch stored in the pyrenoid outside the chloroplast and make use of chlorophyll a as well as chlorophyll b (Andersen and Lewin 2018).

1.2.6 Division Dinoflagellata

Dinoflagellates are an interesting group of organisms making up this division of mostly unicellular flagellates that exhibit an array of interesting features from having ejectile trichocysts just under the cell surface to being bioluminescent to being hosted as symbionts in corals, jellyfish, etc. (Andersen and Lewin 2018). Some members of this group are well known for their ability to produce toxins that kill aquatic life or people from consumption of contaminated shellfish and research has focused on ways of controlling this exposure (Castaing et al. 2011).

1.2.7 Division Rhodophyta

Rhodophyta, or red algae, are primarily filamentous producing chlorophyll a and sometimes d along with phycocyanin and phycoerythrin pigments stored in phycobillisomes. Starch is synthesized and stored outside the chloroplast (Andersen and Lewin 2018). Red algae are responsible in part for many of the beautiful coral formations around the globe, with crustose coralline red algae cementing much of the carbonate foundations for coral reed (Matsuda 1989). Some more recent studies have demonstrated an anit-fouling effect of coralline red algae, preventing other organisms from ocean currents and boats passing by from taking over (Keats et al. 1997). Also *Porphrya sp*., known to many as "nori," and *Gracilaria sp*. are produced on a commercial scale for food and agar, collectively totaling to 21% of the global seaweed production in 2014 (Buschmann et al. 2017).

1.3 History of Algae Use

The present-day use of the word algae is more likely to be heard in the households of some countries i.e. Japan as compared to others, and this is due in part to history. However, the word is becoming more commonplace as people all over the world realize how much of their diet comes from algae. While algae are many times not directly found in ingredient lists, there are compounds from algae that are found in everyday products ranging from cottage cheese to ice cream to beer and many other processed foods like jellies, jams, candy bars, etc. In addition, the impact on daily life does not stop at the dinner table as algae-based ingredients find their ways into cosmetics, pharmaceuticals, and many other household items that everyone uses on a daily basis. This impact did not happen overnight, but over millennia as man has become more aware of algae—both macro and micro—and all of the useful substances they produce naturally using only minimal inputs for production and energy from the sun.

1.3.1 Macroalgae in History

Macroalgae, large multicellular organisms reaching up to 100 meters in length also show up in the historical record as far back as 300 A.D. in Japan and 500 A.D. in China where nori was first used in food. While some macroalgae had been cultivated on small scale centuries prior, there was no significant cultivation of macroalgae until the mid-1600's when agar production began on a mass scale, soon followed by iodine and soda production from brown macroscopic algae in the 1700's (Pulz and Gross 2004).

Large scale industrial algae biotechnology, as shown in Figure 1.1, was first realized with macroscopic algae in the early 1900's but did not peak until after World War II, giving rise to production of nutritional supplements with assumed health benefits such as fucoxanthin (Terasaki et al. 2009). Alginic acid has been used in products from food additives (White and Wilson 2015) to lithium ion batteries (Chen et al. 2017) to aquaculture (Gioacchini et al. 2010).

Today five major species of macroalgae including *Saccharina, Undaria, Porphyra, Eucheuma/Kappaphycus* and *Gracilaria* –make up 98% of the total global seaweed production (Pereira and Yarish 2008; Suo and Wang 1992) with most production (23 million tons) coming from China and Indonesia (FAO 2016).

Figure 1. 1: Current commercial, multi-trophic aquaculture along the eastern coast of China. This type of aquaculture produces 800,000 tons (24 tons $acre^{-1}yr^{-1}$) of seaweed in the 130 km^2 Sanggou Bay area along with other seafood (Buschmann et al. 2017). Photo credit: Max Troell.

Eucheuma/Kappaphycus is cultivated for carrageenan and *Gracilaria* for agar, while *Saccharina, Undaria,* and *Porphyra* are grown for human grade food (FAO 2016).

1.3.2 Microalgae in History

The first known use of microalgae dates to over 2,000 years ago, where a blue-green alga presently known as *Nostoc* was used in China as alternate nutrition during periods of food shortage. For the last 1,000 years, *Aphanizomenon* and *Spirulina*, two other blue-green algae strains, have been used as food by peoples around the world, mostly from natural habitats that support vigorous growth of these organisms. Such natural places include the alkaline waters of Africa's Lake Chad, Mexico's Lake Texcoco, the ocean beaches at Hawaii and Japan, Klamath Lake in North America, and Lake Titicaca in South America (Jensen et al. 2001).

During the 1500's, Spanish conquistadors documented the Aztec people of present day Mexico using nets and bowls to harvest what they called "Techuitlatl" using boats and skimming the surface of the alkaline lakes including present day Lake Texcoco (Jensen et al. 2001). Likewise, the Kanembu tribe near Lake Chad have been preparing S*pirulina sp*. algae into dried cakes dubbed "Dihé" to use as food or for trading for centuries as shown in Figure 1.2. This was first described by Dangeard, a French phycologist visiting the area in 1940 and noticing stacks of these dried cakes in the markets surrounding Lake Chad (Batello et al. 2004). The centuries-old tradition is still alive and well for the Kanembu people who still harvest and sell the algae to this day. The Aztecs were however plagued with smallpox and conquered by the Spanish conquistadors in the 1500's, who eventually drained the natural lake areas and started soda production. It was not until the 1960's when Hubert Durand-Chastel, serving as director of a local company in the area, observed the cyanobacteria *Spirulina* clogging pipes at the soda

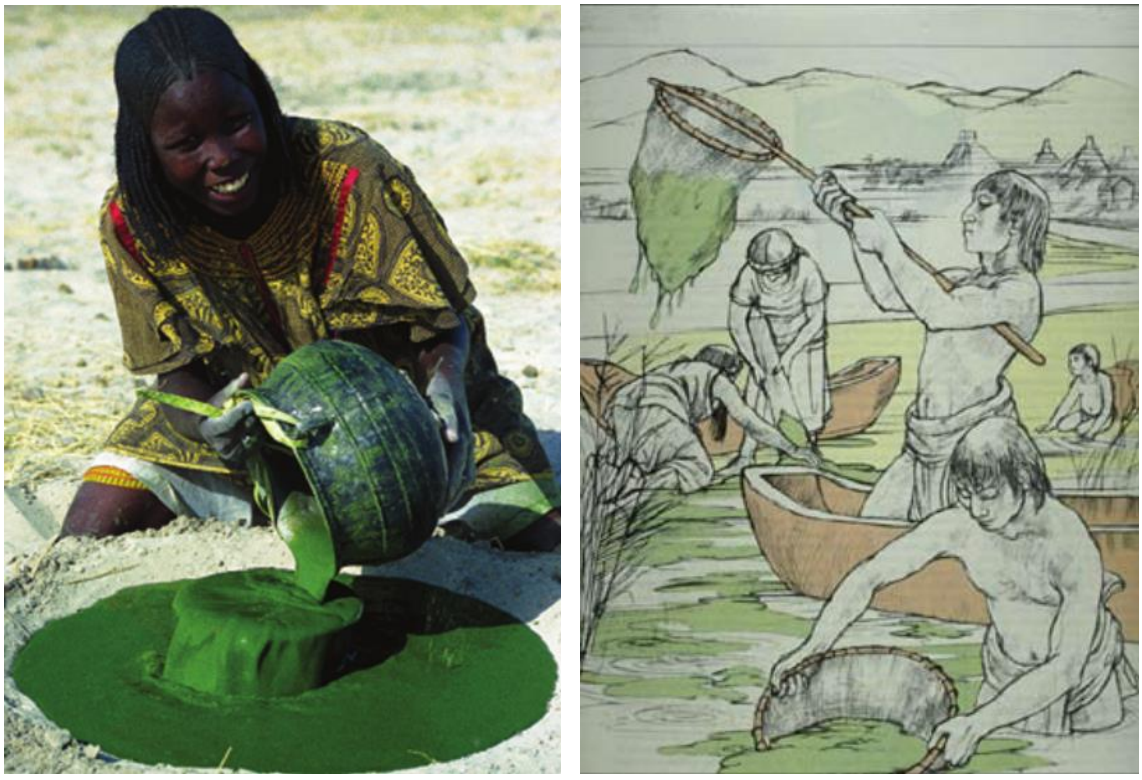

Figure 1. 2: Left image shows the Kanembu women harvesting *Spirulina sp*. referred to as "Dihé" from Lake Chad (Batello et al. 2004). Photo credit: Marzio Marzot.

Figure 1. 3: Harvesting of *Spirulina sp.* called "Techuitlatl" by the Aztecs from lakes in the present-day Mexico on right. Illustration from Human Nature, March 1978 by Peter T. Furst (Hamed 2016).

plant at Lake Texcoco. Rediscovering that these microalgae had been a staple of the Aztecs some 400 years ago in the area, he urged the soda company to start a *Spirulina* farm in Mexico that became the largest *Spirulina* farm in the world in the 1980's. Soon after supplement companies producing *Spirulina* began to arise (Henrikson 2010).

Even though microalgae were discovered by humans over 2000 years ago and have been utilized throughout the centuries, it was not until the 1940's that their cultivation began to be investigated by researchers with an ever-increasing curiosity,

particularly for biofuel (Hu et al. 2008). While early attempts were made in the 1800's to cultivate diatoms, Germany saw the first research of diatom cultivation as a means of alternative biofuel production during World War II. In the 1950's microalgae made a more prominent appearance in peer reviewed journals fueled by the hope of unveiling a new source of protein for the world's growing population (O. Pulz 1998). Microalgae were studied as an alternative source of bioactive compounds since the 1950's (Borowitzka 1995) but also as an energy source during the energy crisis in the 1970's (Borowitzka 1995, Sheehan et al. 1998, Pulz and Gross 2004). By the 1950's centers for algae research were established in the Czech Republic, Israel, U.S.A., Germany, and Japan some of which remain active to this day (Masojídek and Prail 2010). Microalgae have been a prominent topic in academic literature from the U.S.A. from the first major research funding initiative called the Aquatic Species Program in 1978 (Sheehan et al. 1998) to the current Advanced Algal Systems Program (DOE 2018).

1.4 Current Algaculture Production

To better develop algae as an agricultural commodity it is important to first consider the current scale of production in relation to other agricultural crops. According to the Food and Agriculture Organization of the United Nations (FAO 2016), worldwide seaweed production during 2015 was 30 million tons, with much of the production coming from shallow coastal waters surrounding China and Indonesia. While this may sound like a lot, current terrestrial-based agricultural production is estimated at over 10 billion tons per year—an order of magnitude higher than current seaweed production—and at an annual growth rate of 6%, it is not expected to reach the 10 billion tonnethreshold for 80 years, which would effectively be doubling the world's agricultural output. New cultivation strategies for macroalgae are being tested with

Table 1. 2: Plant gate pricing for agricultural commodities (Barrientos and Soria 2018) in relation to algae (Buschmann et al. 2017; Chavan et al. 2018)

Commodity:	Price ($US MT^{-1}):
CARBOHYDRATES:	
Soybeans	442
Corn	176
Wheat	214
Rice	451
Sugar	270
OILS:	
Peanut Oil	1,330
Coconut Oil	1,140
Rapeseed Oil	794
Soybean Oil	827
PROTEIN:	
Soybean Meal	473
Fishmeal	1,578
FERTILIZERS:	
Diammonium Phosphate	411
Potassium Chloride	216
Rock Phosphate	103
Triple Superphosphate	325
Urea	224
MACROALGAE:	
Seaweed	400
MICROALGAE:	
Spirulina	10,804

minimal inputs and production costs averaging US $400 per metric tonne (MT) with potential yields up to 80 MT per acre (Buschmann et al. 2017). Table 1.2 shows how average plant gate prices for microalgae and macroalgae (seaweed) stack up against current agricultural commodities.

Table 1. 3: Global yield estimates (MT yr^{-1}) for the main commercial microalgae strains. Aquaculture strains include *Tetraselmis, Isochrysis, Pavlova, Phaeodactylum, Chaetoceros, Nannochloropsis, Skeletonema* and *Thalassiosira* involved in aquaculture (Hemaiswarya et al. 2011).

Algae:	Yield (MT yr^{-1}):	Country of Origin:	Source:
Spirulina spp.	11,336	China, India, United States, Myanmar, Japan	(Chavan et al. 2018)
Chlorella spp.	5,000	Taiwan, Germany, Japan	(Lundquist et al. 2010)
Dunaliella salina	1,200	Australia, Germany, Japan	(Spolaore et al. 2006b)
Aquaculture strains	1,000	United States, Japan, China	(Hemaiswarya et al. 2011)
Aphanizomenon spp.	500	United States	(Spolaore et al. 2006)
Haematococcus spp.	300	United States, India, Israel, China	(Spolaore et al. 2006)
Total:	19,336	Global	

Average seaweed prices are on par with other agricultural commodities pricewise, however microalgae are not. *Spirulina sp.* is one microalgae with a relatively low cost of production however, it commands prices over 25 times the average-selling price of seaweed. There is clearly a major hurdle for microalgae biotechnology to overcome to become a significant contributor as an agricultural commodity for the world. Because of the substantial cost of production, microalgae cultivation remains yet another order of magnitude lower than that of macroalgae with current estimated world production levels below 20,000 tons worldwide as displayed in Table 1.3 above. Most of the current world

estimate of microalgae cultivation comes from the production of *Spirulina sp.*, which along with *Haematococcus sp.*, has seen considerable growth in China in recent years.

1.4.1 Production Systems

Microalgae production systems suffer from many of the same issues of macroalgae production systems, typically being plagued by high capital, labor, or energetic costs. Macroscopic algae circumvent many of these issues by simply being cultivated in the open ocean water where the yields might not be quite as high but the associated production costs are substantially lower. Microalgae would diffuse into the open ocean currents and drift away. Most microalgae need some sort of vessel to contain them. In contrast, macroalgae can attach to rocks or other substrates during cultivation and be easily harvested. The vessel of choice for commercial cultivation of microalgae is typically a raceway pond due to its low cost. However, a variety of photobioreactors has been developed that achieve record productivities and culture control.

1.4.1.1 Raceways Ponds

Traditional raceway ponds for microalgae cultivation, often called Oswald ponds after first being pioneered by William J. Oswald in the 1950's, are shallow flat channels with semi-circular ends that allow for economical mixing of algal cultures to enhance growth (Chisti 2013). Traditional raceways are typically 0.25 to 0.30 m deep channels mixed by a paddlewheel that propels the water down the channel at 0.25 to 0.3 ms^{-1} to keep the microalgae in suspension while ensuring nutrient delivery and gas exchange. Some more advanced designs have special geometries at either end to illuminate dead zones—places where microalgae collect and settle out (Sompech et al. 2012). The mixing helps to bring algae in and out of the light to enhance photosynthesis while preventing

Figure 1. 4: Commercial *Spirulina sp*. production at Earthrise Nutritionals, LLC. in California, USA using traditional paddlewheel driven open raceways (Henrikson 2010).

Figure 1. 5: Commercial *Spirulina sp*. production at Cyanotech, Inc. in Hawaii, USA with modern paddlewheel driven open raceways used to minimize dead zones in the culture (Cyanotech 2018).

inhibitory levels of O_2 and ensuring adequate CO_2 delivery. Raceway configurations are some of the most economical cultivation setups available for commercial cultivation of microalgae. A paddlewheel is the main energy requirement for mechanical mixing of the cultures. There are three types of open raceways used: traditional (Oswald), circular, and cascade (gravity flow). Despite being the most economical approach for algae production, the main trade-off to these types of systems is lowered productivity and contamination by competing species or debris from the wind.

Figure 1. 6: Circular pond with center pivot mixer. Photo Credit: A. Ben-Amotz, National Institute of Oceanography, Israel.

Figure 1. 7: Třeboň-type thin layer cascade reactor with a 224 m^2 production area cultivating *Chlorella sp*. in Czech Republic. Adopted from Masojidek et al. (2011).

Traditional raceway systems experience some of the lowest productivities in the literature with any peak areal productivity values reported over 30 $gm^{-2}day^{-1}$ dry weight biomass suspect and highly unlikely over prolonged production periods (communication with industry experts). This is because paddlewheel-driven raceways require the momentum of the water to overcome the frictional losses of the moving water around the raceway, and the required momentum is only achievable by the raceway having significant depth, which creates a problem with light penetration into the culture. A raceway with a depth of 7cm typically is the minimum depth for typical raceway configurations to ensure proper mixing of algae. However, a depth in the range of 15 to 30cm is more appropriate for paddle driven raceways (unpublished data). Depth of culture is a critical factor that is especially important to ensure delivery of light into the

culture on a time scale appropriate for photosynthesis. This is what has been referred to in the literature as the flashing light effect or light-dark cycling, and for pond depths in the range of 0.25 to 0.3 m the frequency is on the order of seconds, much too long for optimal photosynthetic activity with timescales in the range of milliseconds.

Light-dark cycling, or a given algae cell moving into and out of the range of photosynthetically available radiation (PAR) is much more pronounced in a shallow raceway than in a deeper raceway. This is because the average distance to the photic zone where PAR is available for photosynthesis is much smaller. The photic zone, as described in Gitelson et al. (1996), is in the range of a few millimeters thick in dense algal cultures. This is what inspired the elevated raceway ponds known as Třeboň-type cascade units that were developed in the Czech Republic with pioneering work from Ivan Šetlík in the 1950's (Masojídek 2009). This pioneering concept has since been modified over the years to realize some of the best productivities observed for open raceway systems, even out-performing the productivity of many high-yielding photobioreactor systems.

These special raceways called cascade raceway systems rely off gravity and a centrifugal pump to mix the algae instead of a paddlewheel requiring a certain depth to work properly. These raceways are able to take productivity to the extreme with photosynthetic yields of 10% (based on PAR), while using culture depths as low as 6-7 mm. They also use significantly less water than traditional raceways leading to reduced harvesting costs and production costs as low as 20% of that of traditional raceways (Doucha and Livansky 2009; Masojídek et al. 2011).

Cascades systems come with their own unique issues, as some algae cannot withstand the high shear stress of constant pumping. There is the fact that the algal

culture, albeit much less volume than in traditional raceways, must be constantly pumped up a 1-2% incline to allow gravity to pull it back down (Doucha and Livansky 2006). This consumes considerably more energy than traditional paddlewheel driven raceways but in a recent review it was suggested that this added cost was more than compensated for by the extraordinary productivity realized by such a thin culture depth (Badvipour et al. 2016). The culture density and productivity of cascades has been shown to be significantly higher than that of traditional raceways with yields reaching 55 g $m^{-2}day^{-1}$ dry weight algal biomass (Masojidek et al. 2011). In addition, cascade and other high-density production systems benefit from reduced harvesting costs as well as reducing the water requirement for cultivation (Doucha and Livansky 2006).

1.4.1.2 Photobioreactors

Due to the low biomass yields realized in traditional raceway systems, a whole class of culture vessels called photobioreactors were developed in hopes of achieving the full autotrophic potential of microalgae. There has been a plethora of devices made over the years with some interesting results, however many designs remain expensive to build and maintain as well as being too energetically costly for anything other than high value nutraceutical or pharmaceutical production, although they are also useful for algal research and field-testing.

The major classes of photobioreactors include tubular, dome-shaped, flat panel, column, bio-film, coil, as well as various thin film bag designs. Most designs are circulated by centrifugal pumps, static mixers or by airlift and are semi-enclosed

Figure 1. 8: Acrylic column photobioreactors producing *Scenedesmus acutus* on Arizona Center for Algae Technology and Innovation (AzCATI) field site on Arizona State University Polytechnic campus in Mesa, AZ. Photo credit: M. Villaseñor, AzCATI.

Figure 1. 9: Acrylic flat panel photobioreactor array producing *Scenedesmus acutus* on AzCATI field site. Photo credit: M. Villaseñor, AzCATI.

Figure 1. 10: Glass tubular photobioreactors (left) growing *Haematoccoccous sp*. for astaxanthin production in Beijing Ginkgo Group's production facility in China. This is one of the world's largest tubular systems covering 80 acres and producing 2 tons pure astaxanthin per year (BGG 2018).

Figure 1. 11: Dome photobioreactor (right) stressing *Haematococcous sp*. for astaxanthin accumulation adopted from Olaizola and Huntley (2003). Photo credit: A. Diffley, Micro Gaia, Inc.

systems, capable of unialgal production unlike open systems that can collect algae blown into the cultures by the wind. Thin film bag designs have become popular in recent years for the possible deployment on lakes or the ocean and their low-cost design. Glass tubular photobioreactors, as shown in Figure 1.6, growing *Haematoccoccous sp.* for astaxanthin production in Beijing Gingko Group's (BGG) production facility in China. This is one of the world's largest tubular systems covering 80 acres and producing 2 tons per year at the Beijing Gingko Group facility in China (BGG 2018). Tubular photobioreactors have some of the highest productivities reported Olivieri et al. (2014) and a large system is currently in use in China for commercial astaxanthin accumulation on the upper right. Dome photobioreactor stressing *Haematococcous sp.* for production of aplanospores containing red astaxanthin pigment as shown in Figure 1.9 above. Biofilm reactors and vertical systems are prized for maximizing areal footprint of the system and achieving some of the best areal yields observed photoautrophically (Liu et al. 2013b). The capital and energetic costs of such systems are prohibitive in order to realize microalgae as an agricultural commodity. While the productivity of many systems is appealing, much of the commercial production today is accomplished using open ponds to produce biomass economically.

1.5 Products from Microalgae

Probably the most exciting features of microalgae is the amount of different chemical species they create, which is comparatively limited in seaweeds. Microalgae stains accumulate fatty acids, an array of accessory pigments, carbohydrates, protein, as well as nutraceutical, pharmaceutical or other specialty products. While some stains can be shifted from production of one product to another i.e. starch to lipid (de Jaeger et al. 2014) other strains simply produce a lot of one product. For example, *Spirulina maxima*

can accumulate up to 70% of its dry weight as protein, *Chlorella sp*. reaching 70% starch dry weight, or oleaginous strains such as *Botryococcus sp*. reaching over 70% lipid dry weight (Dos Santos et al. 2016; Doucha and Lívanský 2008; Boussiba et al. 1987). Each of these main classes of algae products will be explored in more detail below with examples from each class of biomolecules.

1.5.1 Lipids

The ability of microalgae to shift their metabolism during times of stress and accumulate large amounts of lipid has long been known. Oleaginous strains are capable of reaching up to 75% lipid in the dry weight biomass as displayed by Table 1.4. There is even evidence microalgae contributed to the formation of present day oil and gas reserves such as the Colorado and North Sea oil deposits that are possibly derived from *Botryococcuss sp*. and coccolithic algae respectively, and present-day Lake Baikal *Botryococcuss sp*. blooms in where it releases enough oil to skim off the lake's surface (Andersen and Lewin 2018).

Algaenan, an aliphatic, high molecular weight compound from the cell wall of algae being present in today's oil fields is strong supporting evidence that microalgae gave rise to present-day oil and gas deposits (Zhang et al. 2016). This is not surprising since planktonic algae are major contributors to marine snow, the precipitation of organic matter including but not limited to the algae biomass itself to the bottom of the ocean. Oxygenic respiration continues breaking much of this biomass down on the seafloor. However, in areas of high productivity such as continental margins, biomass deposition overwhelms microbial degradation and geologic processes take over instead. This buries large deposits of carbon deep in the earth and is an important part of the carbon cycle on earth (Graham and Wilcox 2000).

Table 1. 4**:** Lipid content of cultivated microalgae as percentage of dry weight as well as the corresponding volumetric productivities. Modified from (Varfolomeev and Wasserman 2011) using data from (Rodolfi et al. 2009; Metzger and Largeau 2005; M Renaud et al. 1999; Mata et al. 2010; Chisti 2007; Richmond 2004).

Microalgae Species:	Lipid %	Lipid ($mg\ l^{-1}day^{-1}$)	Biomass ($g\ l^{-1}day^{-1}$)
Ankistrodesmus sp.	24.0–31.0	–	–
Botryococcus braunii	25.0–75.0	–	0.07
Chaetoceros muelleri	33.6	21.8	0.07
Chaetoceros calcitrans	14.6–39.8	17.6	0.04
Chlorella emersonii	25.0–63.0	10.3–50.0	0.036–0.041
Chlorella protothecoides	14.6–57.8	12.14	2.00–7.70
Chlorella sorokiniana	19.0–22.0	44.7	0.23–1.47
Chlorella vulgaris	5.0–58.0	11.2–40.0	0.02–0.20
Chlorococcum sp.	19.3	53.7	0.28
Crypthecodinium cohnii	20.0–51.1	–	10
Dunaliella salina	6.0–25.0	116	0.22–0.34
Dunaliella primolecta	23.1	–	0.09
Dunaliella tertiolecta	16.7–71.0	–	0.12
Dunaliella sp.	17.5–67.0	33.5	–
Elipsoidion sp.	27.4	–	7.7
Euglena gracilis	14.0–22.0	–	0.05–0.06
Isochrysis galbana	7.0–40.0	–	0.32–1.60
Monallanthus salina	20.0–22.0	–	0.08
Nannochloris sp.	20.0–56.0	60.9–76.5	0.17–0.51
Nannochloropsis	22.7–29.7	84.0–142.0	0.37–0.48
Nannochloropsis sp.	12.0–53.0	37.6–90.0	0.17–0.43
Nitzschia sp.	16.0–47.0	–	–
Pavlova lutheri	35.5	40.2	0.14
Phaeodactylum sp.	18.0–57.0	44.8	0.003–1.9
Porphyridium cruentum	9.0–60.7	34.8	0.36–1.50
Scenedesmus obliquus	11.0–55.0	–	0.004–0.74
Scenedesmus sp.	1.9–18.4	35.1	0.19
Skeletonema sp.	13.3–31.8	27.3	0.09
Skeletonema costatum	13.5–51.3	17.4	0.08
Spirulina platensis	4.0–16.0	–	0.06–4.3
Thalassiosira sp.	20.6	17.4	0.08
Tetraselmis sp.	12.6–14.7	43.4	0.3

Table 1. 5: Comparison of the properties of algal diesel from *Chlorella*, diesel fuel and the American Society for Testing and Materials (ASTM) biodiesel standard (Xu et al. 2006).

Property	Algal biodiesel	Diesel fuel	ASTM biodiesel standard
Density (g cm^{-3})	0.864	0.838	0.86–0.90
Viscosity (mm^2 s^{-1}; 40°C)	5.2	1.9 - 4.1	3.5–5.0
Flash point (°C)	115	Min. 100	Min. 100
Solidifying point (°C)	-12	-50 -10	-
Cold filter plugging point (°C)	-11	-3.0	Summer max 0 Winter max< –15
Acid value (mg KOH g^{-1})	0.374	Max. 0.5	Max 0.5
Heating value (MJ kg^{-1})	1.81	1.81	-

Numerous studies have demonstrated the technical feasibility of using algae oil for biofuel production as shown in Table 1.5 above which shows the fuel properties for algal biodiesel made from *Chlorella sp*. oil in comparison to regular diesel fuel and the American Society for Testing and Materials (ASTM) standards for biodiesel currently produced using other oil crops. It is notable the cold filter plugging point is lower with the algae biodiesel which is an advantageous characteristic for colder climates (Xu, 2006). Table 1.6 below compares the relative yields of current oil crops in terms of annual areal yields of liters per hectare and square meters per kilogram of oil. It is notable that microalgae can accumulate a significantly higher percentage by weight of oil in the biomass as compared to traditional oil crop. The projected areal yields are thus much higher and subsequently the projected land use is much lower. However, it is important to note that since these numbers for microalgae oil yields have yet to be

Table 1. 6**:** Comparison of current agricultural crops produced for oil in comparison with low (50% by wt) and high (70% by wt) microalgae crops. Yields are given in liters per hectare per year and square meters per kilogram of oil per year. Modified from (Priyadarshani and Rath 2012)**.**

Oil source:	Oil %	Oil yield ($L\ ha^{-2}\ year^{-1}$)	Land use ($m^2\ kg(oil)^{-1}\ year^{-1}$)
Hemp	33	363	**31**
Soybean	18	636	**18**
Jatropha	28	741	**15**
Palm	36	5 ,366	**2.0**
Microalgae low oil	50	97,800	**0.1**
Microalgae high oil	**70**	**136, 900**	**0.1**

verified by real world commercial scale production they remain potential projected numbers until the relative costs associated with microalgae cultivation can be reduced to make biofuel from microalgae a reality.

Besides the accumulation of lipids as storage compounds for energy, many microalgae also accumulate large amounts of essential fatty acids, including omega 3, 6, 7 and 9 fatty acids which are all polyunsaturated fatty acids (PUFAs) (Harwood and Guschina 2009; Aurora 2014). Strains are being produced commercially for a special type of omega-3 called docosahexaenoic acid (DHA) that is important for healthy brain formation and function as well healthy skin and eye function. *Spirulina sp.* and *Porphyridium sp.* are commercial strains producing high amounts of γ-Linolenic acid and arachidonic acid, respectively. There are two important precursor fatty acids to the production of either DHA or EPA.

Many microalgae strains produce DHA with *Crypthecodinium sp*. and *Schizochytrium sp*. being the most widely cultivated DHA-rich strains, currently being produced commercially under heterotrophic conditions (Harwood and Guschina 2009). Another important omega-3 fatty acid produced by some microalgae is eicosapentaenoic acid (EPA) which has been studied extensively for its effect in stopping platelet aggregation. EPA-producing stains include *Nannochloropsis sp*., *Nitzschia sp*., and *Phaeodactylum sp*. (Harwood and Guschina 2009). EPA is a precursor to DHA and also prostaglandin-3, and has, along with DHA been shown to lower triglyceride levels in the blood while taken as a supplement (Simopoulos 2002).

While all microalgae have a certain level of omega-3 production, the highest producing strains such as *Nannochloropsis sp., Isochrysis sp., Tetraselmis sp.*, etc. and numerous other marine microalgae are produced commercially for the aquaculture industry (Pulz and Gross 2004;Spolaore et al. 2006). These strains are typically produced photoautotropically in photobioreactors for quality and contamination control and the expense of production usually limits their use as a feed to the nursery stages of aquaculture. These strains are especially important for bi-valve, mollusk, and shrimp production and are typically grown on site to ensure a steady feed supply for the hatcheries although commercial algae pastes do exist (Duy et al. 2015).

Microalgae, being at the bottom of the aquatic food web, are the main producers of polyunsaturated fatty acids (PUFAs). All wild caught seafood contains PUFAs that have been concentrated up the food chain (Pulz and Gross 2004; Spolaore et al. 2006). Consumers are finding that algal oil makes a superior replacement for fish and krill oil as a health supplement that can sometimes have a fishy aftertaste and that carries the added risk of mercury or environmental toxin exposure. This can be by-passed by

consuming algae oil directly. The demand for PUFAs is expected to increase annually by 13.8% from a market size of $US 9.94 billion (as of 2015) as people learn about their importance to overall health (MarketsandMarkets 2016).

1.5.2 Carbohydrates

Although much of the literature is focused on replacement of fossil fuels using oil from microalgae, several strains have demonstrated the ability to accumulate large amounts of starch or other carbohydrates with biofuel potential (Takeshita et al. 2014; Zhu et al. 2014). Green algae are known to accumulate large amounts of starch in excess of 70% dry weight around structures called pyrenoids (Griffiths 1970). This accumulated starch is very prominent in electron microscope images of some algae. Starch granules around the pyrenoid until much of the cell is full of white starch granules. Green algae have been intensely studied to elucidate the biosynthetic pathway of starch to lipid conversion initiated by environmental stressors (Ji et al. 2014). This research has been greatly aided by the formation of starch-less mutants (de Jaeger et al. 2014; Li et al. 2010; Takahashia et al. 2018). Cyanobacteria have been studied to make cellulose for biofuel production (Branyikova et al. 2011; Nobles and Brown 2008).

Most algae-derived carbohydrates available on the market today come from macroalgae, including agar, carrageenan, and alginate. However, microalgae produce a number of unique carbohydrate sources that the cells utilize for short-term energy storage to survive dark periods. Cyanobacteria typically store carbohydrates as glycogen (Schneegurt et al. 1994; Cano et al. 2018) unlike green algae that produce starch (Rengel et al. 2018). Diatoms produce chrysolaminarin, a special β-1,3-linked glucan stored in vacuoles outside the chloroplast (Zhang et al. 2018). This special carbohydrate has proven anti-tumor properties and inhibitory activity against human colon cancer cells

(Kusaikin et al. 2010). Red algae that typically store an α-polyglucan called floridean starch stored outside the chloroplast in grains (Andersen and Lewin 2018). Euglena has also been studied for its ability to produce a β-1,3-linked glucan called paramylon (Muchut et al. 2018) which has demonstrated anti-fibrotic activity in mice (Kusmic et al. 2018).

Even though biofuel production has historically been the major driving force behind commercial algaculture, the specialty carbohydrate energy stores microalgae produce would potentially be more valuable in feed markets which generally command higher prices than biofuels (Romano 2018; Peiretti and Meineri 2008). Besides the widespread use of macroalgal polysaccharides as thickeners and emulsifying agents, especially for everyday foods (Ioannou and Roussis 2009), microalgae polysaccharides are becoming known for numerous other uses, including nutraceutical and pharmaceutical applications. These include anti-viral and anti-HIV (Schaeffer and Krylov 2000; Gustafson et al. 1989; Tziveleka et al. 2003; Hayashi et al. 1996), anti-inflammatory (Matsui et al. 2003), and antioxidant activity (Geresh et al. 2002). There is truly a lot of potential for a variety of products from microalgal carbohydrates as new compounds and activities are uncovered over time.

Microalgae can be induced to overproduce their respective carbohydrate storage compounds easily using various environmental manipulations, which typically involves adjusting nutrients in the culture medium, typically depriving them of sulfur (Varfolomeev and Wasserman 2011; Jerez et al. 2016a). Many of these algae produce exopolysaccharides that are secreted outside the cells for easy extraction (Soanen et al. 2016; Giraldo Calderón et al. 2018). Exopolysaccharides are thought to be very

important in desert crust formations and may be useful for slowing soil erosion (Mazor et al. 1996; Lan et al. 2018; Zheng et al. 2018b).

1.5.3 Protein

Spirulina sp. is the single most widely cultivated microalga on Earth to date and it contains 65-70% protein content that along with its vitamin and mineral content makes it one of the world's best superfoods. The protein from *Spirulina* contains all essential amino acids and is readily digestible (Henrikson 2010). Other microalgae, including *Chlorella sp.* and *Scenedesmus sp.* have demonstrated the ability to make considerable protein reserves as well but none of them have been studied as extensively as *Spirulina sp.* as both a human dietary supplement and animal feed.

Numerous studies have been conducted to determine digestibility and performance of various microalgae, including *Spirulina sp.* and *Chlorella sp.* for use in livestock feed for poultry (E. and W. 1990), swine (Kajan et al. 1991; Svoboda et al. 2010), dairy (Kotrbacek et al. 2013), and even rabbits (Zotte et al. 2013) among other animals with generally promising results.

Microalgae could one day serve as an alternative source of protein with less water, fertilizer and land requirements than traditional legume crops with high protein content like soy or alfalfa. Table 1.7 below shows a comparison of the nutrient profiles of three high protein microalgae currently being cultivated for commercial or research purposes. These include *Spirulina maxima, Chlorella sp.*, and *Scenedesmus acutus*, which are easily cultivated with minimal contamination issues and good productivity in the field. All are potential candidates as high protein commodity crops for widespread

Table 1. 7: Compositional comparison of microalgae and high-protein agricultural commodities. Values are given as g per 100 g except for amino acid and vitamin values that are noted accordingly. Modified From García-Garibay et al. (2014) with additional values taken from Council and Agriculture (1971) and Bontems et al. (2018).

Compound	*Spirulina maxima*	*Chlorella sp.*	*Scenedesmus acutus*	Alfalfa hay	Soybeans (flaked)
Crude protein (N X 6.25)	55–71	40–58	46–64	23-26	35.5
True protein	48–61	-	44–48	22-25	-
Amino acids (g 16 g^{-1} N)					
Alanine	5.0–6.1	4.2–7.4	5.3–10.4	5.2	4.3
Arginine	4.5–9.3	5.8–10.2	4.6–7.1	4.6	7.3
Aspartic acid	6.0–15.2	6.9–8.8	6.5–11.1	10	11.1
Cystine	0.6–2.2	0.3–0.9	0.6–1.6	1.2	1.5
Glutamic acid	8.2–21.8	8.0	5.3–10.7	9.6	17.6
Glycine	3.2–4.0	4.9–5.5	3.4–7.0	4.6	4.3
Histidine	0.9–1.6	1.4–3.0	1.5–2.3	1.8	2.7
Isoleucine	3.7–4.5	3.1–6.4	2.2–4.9	4.3	4.7
Leucine	5.6–7.7	6.8–9.7	5.0–10.6	7.6	7.5
Lysine	3.0–4.5	4.9–9.4	5.0–6.4	4.9	6.2
Methionine	1.6–2.2	1.0–2.0	1.4–2.7	1.4	1.4
Phenylalanine	2.8–4.0	3.2–5.1	3.6–6.4	4.9	5.1
Proline	2.7–3.2	2.2–6.4	3.1–6.1	4.2	5
Serine	3.2–4.3	3.0–4.1	3.2–5.4	4.1	5.3
Threonine	3.2–4.5	3.6–4.7	3.0–5.8	4.1	4.1
Tryptophan	0.8–1.2	1.0–1.5	0.3–1.8	1.4	1.3
Tyrosine	3.9	2.6–4.1	2.0–4.6	3.4	3.6
Valine	4.2–6.0	4.8–6.0	4.7–7.4	6	4.8
Lipids	4–7	6–16	8–14	3.3	18.2
Carbohydrates	13–16	-	-	4.4	15.9
Minerals	4–9	6–9	6–17	13	5.3
Vitamins (mg 100 g^{-1})					
Thiamin	5.5	0.6–2.3	1.2–8.2	0.7	1.2
Riboflavin	4.0	2.0–6.0	3.4–36.6	1.7	0.3
Pyridoxine	0.3	0.1–3.2	1.1–2.5	-	1.2
Nicotinic acid	11.8	10–22	12–16.7	4.1	2.5
Pantothenic acid	1.1	1–10	1.5	4.4	1.7
Folic acid	0.05	0.1–4.0	0.7	-	0.4
Biotin	0.04	0.015–0.064	0.02–0.2	-	0.03
Cyanocobalamin	0.02	traces	0.04–0.44	-	-
Ascorbic acid	-	18–370	165–181	-	-
b-Carotene	0.17	-	-	16.5	-
γ-Tocopherol	19.0	26–33	14–18.5	-	4.1

use as animal feed or human dietary supplements, which have already demonstrated promise in various research studies.

While the possibility exists for supplementing animal and human feedstocks with protein derived from microalgae, the current cost of production for microalgae in comparison with traditional high-protein crops like alfalfa or soy make microalgae protein economically unattractive. With more efficient production technologies for microalgal culture, algae protein may one day take center stage in animal and human nutrition alike as resources like fresh water and arable land—resources that microalgae do not depend on—become more in demand.

1.5.4 Pigments

Algae produce many pigments used for light energy capture in photosynthesis. Pigments are the starting point for the conversion of light energy to chemical energy via photosynthesis. The primary photosynthetic pigment is chlorophyll a, but accessory pigments such as carotenoids, phycobilliproteins, and chlorophyll b, c, d are also critical for light capture. Accessory pigments help build antennae to pass on energy they absorb to the chlorophyll a reaction center where the process of photosynthesis begins. Pigments are produced in a great abundance in many microalgae, accumulating several percent of the cell dry weight in content. Certain strains, such as *Nostoc sp*. or *Phormidium valderianum,* produce up to 20% dry weight of phycocyanin, a phycobilliprotein (S and G 1998; H et al. 1989). *Spirulina*, the most widely cultivated microalgae to date is able to accumulate up to 25% dry weight as phycocyanin, a pigment protein gaining support for its immunomodulating and anti-tumor activities (Zeng et al. 2012; Boussiba and Richmond 1979; Sarada et al. 1999; Bharathiraja et al. 2018) Phycoerythrin, a red phycobiliprotein, is accumulated in *Porphyridium cruentum,* a red

algae with commercial attention. Phycoerythrin, like phycocyanin, is in widespread demand as a fluorescent probe in analytical techniques (Bermejo Román et al. 2002), but also as a food dye (Varfolomeev and Wasserman 2011), cosmetics (Parmar et al. 2011), and as an antioxidant (Hirata et al. 2000).

Many researchers believe that several microalgae overexpress carotenoid production to survive adverse conditions, much as the oil-producing strains do. Actually much of the carotenoids end up dissolving in oil globules in the cell due to their hydrophobic nature (Zhekisheva et al. 2002). Both oil and carotenoid production are thought to be ways of shunting excess energy from the photosynthetic reaction centers into long-term energy stores for the cells. There is evidence that the oil globules are able to migrate to the periphery of the cell, bringing the carotenoids along to provide shade to the photosynthetic apparatus, especially under high irradiance levels of blue light (Peled et al. 2012). In the case of *Dunaliella sp*. the carotenoid β-carotene is greatly overproduced up to 14% of its dry weight to help survive nutrient deprivation combined with salt stress up to three times the salinity of sea water. *Haematococcus sp*., a green mesophylic alga, produces astaxanthin up to 8% of its dry weight to survive salt stress or high light, low nutrient conditions (Kang et al. 2005; Metting 1996). Astaxanthin is a carotenoid with one of the highest anti-oxidant activities in the world and researchers hypothesize that algae use this pigment to survive free radical damage to its photosynthetic apparatus. The green psychrophilic snow algae *Chlamydomonas nivalis* experiences especially high irradiances within snow banks and astaxanthin may help protect the cell against photooxidative stress *(Leya et al. 2009)*. This green alga is responsible for creating "watermelon" or "blood" snow on the Sierra Nevada mountain range in California. *Haematococcus sp*. has become an important commercial strain of microalgae due to its ability to produce this bright red pigment typically used in salmon

farming and other aquaculture (Wathne et al. 1998). Other green algae such as *Scenedesmus almeriensis* produce large amount of lutein, another highly potent anti-oxidant already sold as a nutraceutical product for preventing disease such as macular degeneration (Sánchez et al. 2008).

1.5.5 Specialty Products from Microalgae

The use of microalgae as a platform for the production of bioactive molecules has been a focus of many studies in recent years. To date, microalgae have been studied for their potential use in cosmetics, sunblock, plant fertilizer, as well as for production of stable isotope-labelled organic molecules for use in research, to name a few. This list continues to grow as more valuable compounds from microalgae are discovered.

Stable isotope-labelled products from microalgae is a particularly attractive platform for production of these high-value compounds because photosynthetic algae can simply be fed inorganic forms of the isotopes in a highly controlled environment to ensure quality. Labelled amino acids, carbohydrates, nucleic acids and lipids are in constant demand for research projects where either structural determination of molecules (Radmer 1996; Apt and Behrens 1999; Acién Fernández et al. 2005) or elucidation of metabolic pathways is underway (Hanson et al. 2014). They may also be used to investigate and diagnosis disease (Zachleder et al. 2018). Table 1.8 above shows 2015 prices in US$ for such compounds.

Macroalgae have long been a focus of the food, nutraceutical, and cosmetic industries for many years and microalgae have just begun to attract attention. While there have been microalgal-based nutraceutical and dietary supplements on the shelves of supermarkets for years, the diversity of products is increasing in these industries and branching more heavily into the cosmetics industry. *Spirulina* protein in hair products (IGK 2018) is already hitting the market and other microalgae are being investigated for

Table 1. 8: Market price (US$) of Chlorella biomass in comparison to isotope-labelled compounds for research purposes. Prices were from 2015 adopted from Zavřel (2015) with exception of the price of ^{13}C-DHA taken from [1]Spolaore (2006).

Product	Market Price (US$)
Chlorella powder	60 / kg
^{13}C-mixed fatty acids	530 / g
^{15}N-alanine	940 / g
$^{2}H_7$, ^{13}C, $^{15}N_4$-arginine	6,200 / g
dATP-CN	37,000 / g
^{13}C-DHA (>95%)	38,000 / g
^{2}H-DHA / ^{2}H-EPA	280 / mg

use as in creams for skin moisturizer, sunblock, anti-wrinkle and collagen-forming products as presented in Table 1.9 below.

Table 1. 9: Cosmetic products derived from microalgae strains modified from **Ariede** et al. (2017).

Microalga	Chemical form	Cosmetic effect	Galenic form	Reference
Spirulina sp. Chlorella sp	Protein and Peptides (dry powder)	Gloss and moisture on the skin Smoothness, moisture and gloss on the hair	Skin & Milky lotion Skin cream Body soap & Shampoo Rinse Bath agents	(Hagino and Saito 2003)
Coccoid and Filamentous	Extract	Enhancing skin barrier, collagen formation Anti-aging effect	Emulsion Lotion Powder product	(Einarsson et al. 2010)
Phaeodactylum tricornutum	Extract	Protecting against UV exposure Preventing and/or delaying the appearance of skin aging effects	Cream Emulsion Emulsion-Gel	(Nizard et al. 2007)
Spirulina, Dunaliella, Hematococcus, Nannochloropsis, Tetraselmis	Cell algae	Sunscreen	Gel, Emulsion (Water/Oil and Oil/Water)	(Lotan 2012)
Prototheca sp., Chlorella or Parachlorella	Cell algae or extract	Sun protection, hydration, anti-aging, exfoliant to skin or hair	Cream, soap, lotion, Shampoo, facial wash	(Schiff-deb and Sharma 2015)
Cyanobacteria	Extract	Sunscreen	Cream	(Huner et al. 2004)

Current commercially relevant microalgae species include *Chlorella sp., Spirulina sp., Phaeodactylum tricornutum, Nannochloropsis sp., Tetraselmis* sp., *Haematococcus sp*. and *Dunaliella sp*. *Schizochytrium sp*.to name a few. The literature covers the commercial potential of many more strains, however real commercial production of microalgae is limited by a number of factors. Some of these factors are strain-specific, but most apply to production of all microalgae, such as dealing with inefficient cultivation techniques or processing and handling strategies. Understanding and improving these areas which are fundamental to microalgae biotechnology could offer a breakthrough in getting more economically viable products from microalgae to market.

1.6 Bottlenecks to Commercial Microalgae Production

The idea of widespread commercial micro-algaculture was first reported in the literature in the early 1940's (Myers and Burr 1940). Since then there has been a tremendous amount of research devoted to finding more economical ways of culturing algae on a mass scale with a major focus on testing all types of photobioreactors including tubular, dome-shaped, thin film bags, coil, flat panel, biofilm, and a variety of optimized pond designs over the years as described previously in Section 1.4.1. Despite these intensive efforts, relatively few commercial algae companies exist to date even with the overwhelming body of evidence showing the utility of microalgae for commercial applications (Borowitzka 2017; Chisti 2013; Quinn et al. 2011).

Many research review papers have suggested this has to do with not finding the right strain, the strain not being genetically optimized for production, not having the right photobioreactor, or algae just not being economically viable for a particular application (Del Campo et al. 2007; Salama et al. 2018; Nedbal et al. 2008). After reviewing a wide variety of papers focusing on commercial agriculture, there seem to be

three overarching areas of research that need attention to realize the full potential of microalgae as a crop. These include:

- Optimize a micro-environment better suited for photosynthesis.
- Optimize utilization waste nutrient sources for efficient biomass production.
- Optimize harvesting and disruption techniques for a given strain being cultivated.

Macroalgae have experienced greater success as a cultivated crop as they are not held back by these economic constraints as much. For instance, many seaweed can be cultured directly in the ocean as shown in Section 1.4, without the need of a containment system such as artificially made ponds or plastic bags. They also get their nutrients from the ocean and rely on wave action for timely delivery. Microalgae cultivation has typically required some sort of mechanical mixing, some type of containment, and some type of harvesting and disruption process. All three of the requirements can have significant economic considerations that need to be taken into account in the final price of production. Only after more information is obtained in these three areas of basic algae biotechnological research can new methods and techniques be developed for general field use. Simplified and efficient techniques or methods will provide the quickest avenue to making sustainable widespread commercial algaculture a reality.

1.7 Unlocking Microalgae Potential

A significant portion of literature concerning commercial algaculture today focuses on how to genetically enhanced or modified microalgae to produce a more commercially viable strain (Matsuoka et al. 2001; Fukuda et al. 1994; Vermaas 1996; Hoi et al. 2018). That was my initial thought in pursuing a PhD in molecular and cellular biology studying algae, but I soon came to realize that there is tremendous natural

genetic diversity at our disposal as researchers. There are over 43,000 recognized species of algae according to Guiry (2012) and even more differentiation when it comes to individual strains. Adopting random mutagenesis in the lab as a strategy to search for a superior mutant (Wang et al. 2018) or venturing down the long and tedious path of site-directed mutagenesis can be invaluable for laboratory experiments for studying photosynthesis (Bulychev et al. 2018), or other metabolic pathways (Breuer et al. 2014) but such projects typically have had limited success in field applications to date (Marcus et al. 2011; Takahashia et al. 2018; Song et al. 2018).

With the amount of natural biodiversity in algae one could select organisms that can be adapted from freshwater to saltwater and back (Youngs et al. 1998), or that accumulate glycerol up to 17% of their dry weight in order to survive growth in hypersaline environments up to 3 times the salinity of seawater (Saha and Murray 2018). Other algae are known to withstand significant changes in pH (Moraes et al. 2013), high temperatures (Abu-Ghosh et al. 2018), high sunlight (Cuaresma et al. 2009; de-Bashan et al. 2008) or high levels of CO_2 or nitric oxide commonly found in flue gas (Varshney et al. 2018). These are valuable traits to have for the production of these strains as crops in an agricultural setting, which may experience large environmental variations over time.

With open production systems in current mainstream use for microalgae cultivation, it is also imperative to find strains that out-compete the native algal strains in order to achieve and maintain a monoalgal culture given the right culture conditions (Wang et al. 2013). Just by changing the environment the cells are experiencing by adjusting culture pH, salinity, nitrogen concentration, or temperature, huge shifts in biochemical composition can be achieved in a given microalgae crop. For instance, accumulating as much as 70% biomass by weight as either starch (Griffiths 1970),

protein (Henrikson, 2010), or lipid (Metzger, 2005). Strains such as *Isochrysis* or *Haematococcus* are capable of producing fatty acids or carotenoids, accumulating as much as 8% of the total biomass (Metting, 1996) in the case of *Haematococcus sp.* producing astaxanthin or in the case of *Isochrysis sp.* almost 2% DHA fatty content in the biomass (Liu et al. 2013a).

While these numbers could likely be enhanced by genetic manipulations, researchers are only slowly gaining control over the secondary effects created by the genetic modifications. For instance, by diverting energy into one metabolic pathway, energy is likely diverted from another, which could actually put the organism at a disadvantage in real world conditions. Many times, but not always, one quality or advantageous trait of an organism is traded off for another. There is much yet to learn about genetic engineering of microalgae especially considering researchers have yet to reach an agreement on how to most efficiently cultivate the algae as an agricultural crop. Should a photobioreactor or pond be used? What culture conditions for which strain? How should it be harvested, etc.? These are the more fundamental questions to be answered in order to allow microalgae to make an impact as a global agricultural commodity and support the costly genetic engineering and strain optimization research projects that will further enhance its success. To answer these fundamental questions, a strain that produces a desired product and has proven to be competitive in its environment is first selected. The next step is to take that strain to the field to learn as much as possible about its cultivation in order to make its production as efficient as possible. Only then can more elaborate (and costly) research projects produce useful applied technology for use in the field. Most agricultural crops had general husbandry and cultivation strategies outlined before the more recent advent of genetic manipulation techniques. Microalgae need to go through this process as well.

Experts believe teosinte, a distant relative of corn from Mexico, gave rise to modern corn over a process of artificial selection by man for approximately 10,000 years ago. The majority of the differences between the two stems from differences in only 5 genes (GSLC 2013). It was developed through selective breeding over years and years of culturing so that the corn plant itself is considered a construct of breeding The know-how of cultivation and working with the plant is likely equally if not more valuable in obtaining record yields as selecting for the genetic traits we have come to know and utilize today. The genetics of corn can make it colorful, sweet, drought tolerant, or insect and fungus resistant, but without first knowing how to grow the plant efficiently in a field, by optimization of soil conditions, climate, seeding rate, row spacing, timing etc., the development of genetically modified crops is somewhat useless for commercial production until we know an efficient cultivation strategy. In the history of corn, the genetics and husbandry of the crop developed slowly over the years until the advent of modern analytical techniques, technology including machinery. It is interesting to see how history unfolds for microalgae as a crop with much more advanced techniques and technology in agriculture and in genetics, etc. compared to when corn was first developed from teosinte.

Before even sequencing many of the microalgae that are commercial prospects, it is apparent that they exhibit a lot of potential for shifting metabolic pathway with simple environmental cues. It seems that the fastest approach to making commercial algaculture economical would be to better understand the cell biology and ecology of the best-suited strains. This includes husbandry practices for the new crop such as stain-specific culture conditions and strategies that lead to the most efficient biomass production. There have been a number of interesting projects to date using molecular techniques to produce GMO algae (Wang et al. 2014; Nakamura and Gowans 1964;

Takahama et al. 2003), but few have demonstrated commercial viability because many mutants take energy from one biosynthetic pathway to support overproduction in another.

This means that even though many mutants do something better than their wild-type form, they will still likely have a harder time competing with native strains. This is why mutant stains many times revert to wild type on the field and the effect is lost. There is a trade-off on the molecular level of mutants that has not yet been tested by evolution in a natural environment. Shunting more energy into a certain pathway many times makes the organism less fit for its natural environment. It is not until we better understand algal ecology, physiology, and genetics that we will be able to make stable GMO algae economically viable for commercial production. Instead, it may be more productive to turn our attention to the more pressing areas of research that need to be better understood first in order to realize economically viable widespread commercial algae production. Such areas include light and cultivation strategies, utilization of waste nutrients, and harvesting and disruption of biomass, which will each be discussed in more detail below.

1.8 Light and Cultivation Strategies

Techniques employed in algae cultivation are in their infancy at best, having almost exclusively been done in open raceways up until the late 20th century. This is largely because the key parameters critical for algal cultivation are only starting to be understood. Although algae are very adaptable and can survive a wide range of environmental conditions, a given culture has certain parameters that must be maintained in spec in order to achieve the best possible yields. This obviously includes physical and chemical parameters like temperature, pH, salinity, conductivity, nutrient

concentrations, etc. that play an important physiological role in the life cycle of a given strain of algae (or any life form for that matter). However, since algae are primary producers relying on solar radiation for photosynthesis, light penetration and utilization play a critical role in efficient biomass growth. Productivity of an algal culture is really a function of more complex interactions between biomass density, mixing, light intensity, concentration of organics in the medium, etc. (Mathimani et al. 2018; Hindersin et al. 2013; Leupold et al. 2013).

Only in the last few decades have researchers started to grasp the importance of these types of interactions, namely the critical importance of getting light efficiently into the culture (Gordon and Polle 2007). A wide variety of photobioreactors have appeared in the literature over the last few decades (Ranjbar et al. 2008; Pruvost et al. 2017; Chang et al. 2017) with the purpose of more efficiently delivering photosynthetically active radiation (PAR) to algal cultures. This has helped explore more possibilities in terms of algal cultivation techniques and helped us get a better understanding of the physiology of select strains of algae (Borowitzka 2017). However, light delivery into an algal culture remains a significant roadblock for widespread commercial algae production.

1.8.1 Photosynthesis and Light Utilization

Photosynthesis is a complex metabolic pathway that evolved billions of years ago in prokaryotic life forms and is responsible for the reduction of carbon dioxide into glucose and the simultaneous oxidation of water to produce the oxygen we breathe. The process of oxygenic photosynthesis begins as light is captured by specialized molecules in the thylakoid membranes within chloroplasts known as light harvesting complexes (LHCs). LHCs are special proteins-pigment antennae molecules responsible for

absorbing light energy and through a complex series of interactions, pass the electronic energy to the reaction center of the photosystem where charge separation can occur and light energy is successfully transferred into chemical energy. Secondary reactions occur down an electron transport chain to convert this energy into a chemical form of energy the cell can use (Blankenship 2008).

In oxygenic photosynthesis, the reaction center consists of either a P680 or P700 chlorophyll dimer molecule that passes an electron down a series of energy carriers collectively known as the classic Z-scheme model of photosynthesis. As a end result, $NADP^+$ is reduced to NADPH and water is oxidized into H^+ and O_2. While O_2 diffuses across the membrane out of the cell, the H^+ is held within the lumen of the thylakoid membranes, creating a pH differential that drives production of ATP and NADPH that serve as energy currency within the cells (Blankenship 2008).

Photosynthesis varies from species to species but possibly the biggest difference is the way in which the electrons flow in the process. Some anoxygenic photosynthetic bacteria use cyclic electron flow in which the electronically excited chlorophyll passes an electron to a series of secondary carrier molecules and eventually back to the original chlorophyll pigment. The speed of the secondary reactions passing the electron must be much faster than the competing reverse reactions to ensure stabilization of the charge separation. This cyclic electronic movement is used to pull H^+ ions across the membrane to create a proton gradient that can be used as energy currency to drive cellular processes. Additionally, in oxygenic photosynthetic organisms, there is also non-cyclic electron flow. Electrons flow from the hydrolysis of water down a series of electron carriers with energy input from two different photosystem protein-pigment complexes

(PSI & II) to create the reduced energy currency within the cell (NADPH) required to fix CO_2, evolve O_2 and drive all life process (Blankenship 2008).

In contrast to photosynthetic organisms, heterotrophic cultures are typically cultured under sterile conditions and thrive in the absence of light, relying solely on an organic substrate as their energy source. This includes most cultures of bacteria, yeast, or sometimes algae. Martek Bioscience Corp. cultivates the alga *Schizochytrium sp*. for production of DHA in this manner (Fedorova-Dahms et al. 2011). This mode of production means algae never see the light of day and instead utilize an organic energy source that can be continuously added and distributed throughout the culture to obtain record areal and volumetric productivities with minimal capital and energetic inputs. Since heterotrophic culture requires glucose or some organic substrate, this type of production is limited to high value products only and relies continually on some photoautotrophically-produced agricultural commodity.

Photoautotrophic algae production differs in that light must be supplied to the culture, preferably on a timescale consistent with photosynthesis, which according to current literature is in the order of milliseconds. Concepts such as the "light-dark cycle" which appeared in the literature in the early 1900's before commercial algae production existed. It wasn't until the 1950's that the development of the traditional (Oswald) raceway and the Třeboň-type cascades helped illustrate the integral relationship between biomass density, depth, mixing, and average light intensity. Researchers soon realized the complexity of finding an economically viable light delivery solution. For example, increasing the mixing rate, can increase productivity, but at an energetic cost (Chiaramonti et al. 2013). Operating at a higher cell density and thinner path length can

offer a better productivity and lead to more cost effective cultivation and harvesting, however high cell densities can exhibit culture inhibition (Tian-Yuan et al. 2013).

1.9 Waste Nutrient Utilization and CO_2 Mitigation

Another current roadblock to commercial algaculture is the use of waste nutrient streams to feed an algal culture. The gaseous nutrients of nitrogen in the form of ammonia and carbon in the form of carbon dioxide are particularly the most challenging. It is a delicate balance to supply both of these nutrients to the culture medium simultaneously as a result of differing pKA's (Eustance et al. 2016), however a large portion of the current literature boasts that algae can be grown on wastewater utilizing waste carbon and ammonia from a variety of sources (Zheng et al. 2018a; Gao et al. 2018; Hom-Diaz et al. 2017; Lee and Han 2016). While this is may be true, few papers focus on the feasibility, economics, and efficiency of actually delivering these nutrients on a commercial scale. For instance, how can the gases or nutrients be captured or delivered efficiently? What are the limiting nutrients from the waste streams to be utilized? These questions should be addressed more thoroughly by literature.

More data on this aspect of algaculture will be necessary to assess the economics of using these nutrients in their gaseous form. There needs to be a more in depth look at culture feeding regimens to minimize loss, including timing, dosing, and mode of delivery. Admittedly, this information is inherently somewhat strain specific so it needs to be collected for a stain with known biofuel potential such as *Scenedesmus sp*. This will help the industry better understand how close it is to the ultimate goal of biofuel production from algae.

1.10 Harvesting and Biomass Processing

Finally, the third major obstacle for commercial algaculture is harvesting and post processing of the biomass after the culture process is over. Dewatering can be a significant energetic cost for the production of biomass, especially for biofuels (Passell et al. 2013). Many different options exist including centrifugation (Passell, 2013), dissolved air flotation (Nguyen et al. 2013), membrane filtration (Castaing et al. 2011), coagulation (Oliveira et al. 2018) and flocculation with a variety of chemical compounds (Choy et al. 2018), however the energetic cost of active harvesting limits biofuel production for many of these proposed methods. Wastewater treatment plants use flocculation with microbes and auto-flocculation has been documented for some algal strains (Christenson and Sims 2011), where algae can form flocs and fall out of solution without the addition of chemicals. Morphological features including cell size and shape can also provide a low energetic means of harvesting algal biomass, including strains with biofuel potential such as *Scenedesmus acutus*.

After harvesting the biomass, there are several post-processing options for the biomass depending on its end use. For high value products this includes energy intensive processes such as spray drying or freeze drying then extracting the bioproducts (Ceron-Garcia Mdel et al. 2010), while research is still ongoing for lower value commodities like fuel, with current emphasis on gasification, pyrolysis and cracking techniques (Duan et al. 2018; Pujan et al. 2017).

1.11 Goal of my Dissertation

The purpose of this work is to identify and determine ways around three specific roadblocks to present-day commercial microalgae production including light utilization,

waste nutrient utilization, and biomass harvesting and post-processing. To accomplish this, a ubiquitous, high-yielding alga, *Scenedesmus acutus*, with potential for use as a biofuel source or as animal feedstock will be used throughout the work to follow the process from start to finish. The primary objective is to shed light upon each of these three areas and suggest ways to minimize inputs of water, land area, fertilizer, and other capital inputs while maximizing yields.

The ultimate goal of this work is for the recommendations in the following three chapters to help guide future commercial production or research endeavors so that more of microalgae's full potential as a global agricultural commodity may be realized. Chapter 1 gives a more in depth look at the importance of light availability in an algal culture on a timescale that works in conjunction with the mechanics of photosynthesis. Strategies are offered for dealing with the issue of bringing more light into the culture. Chapter 2 gives a thorough look at the dilemma of delivering gaseous waste nutrients along with aqueous waste nutrients into the culture medium, while exploring options for making waste nutrient delivery and utilization more efficient. Finally, Chapter 3 explores the option of using gravity sedimentation followed by mechanical disruption (microfluidization) of the wet biomass. Gaining valuable information on these areas will help reduce the current bottlenecks to commercial microalgae cultivation.

2. MANUSCRIPT 1: ULTRA-HIGH CELL DENSITY YIELDS OF TWO STRAINS OF *SCENEDESMUS ACUTUS* GROWN IN THIN FLAT PANEL REACTORS FOR YEAR-ROUND PRODUCTION

Manuscript Information Page

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2.1 Abstract

Record photoautotrophic biomass yield—up to 30g $m^{-2}day^{-1}$ –was obtained for two prolific green algae strains common to wastewater streams of the desert Southwest using acrylic flat panel photobioreactors with a short path length (<1.3 cm) to achieve record light utilization and photosynthetic efficiency (>2% total insolation) for these strains. Identified via ITS1 sequencing as two unique strains of *Scenedesmus acutus*, these ubiquitous freshwater green microalgae have a documented potential not only for use in human food, but also a feedstock for livestock and/or biofuel production. Laboratory growth on a temperature gradient suggests the two strains could provide year round production of biomass via crop rotation between winter and summer. The advantages of ultra-high cell density growth (>10 g L^{-1} dry weight biomass) include less susceptibility to contamination, less water use, ease of harvest, and reduced operating cost. Limitations and challenges in the design of a flat panel system for algaculture are discussed along with future recommendations for improved cultivation strategies.

2.2 Introduction

Microalgae have recently taken the center stage for the next promising frontier in agriculture—dubbed algaculture—with the potential to serve as feedstocks for many commodity markets. Such markets include aquaculture and animal feed (Svoboda et al. 2010; Peiretti and Meineri 2009; Supamattaya et al. 2005), nutraceutical and pharmaceutical applications (Jha et al. 2017; Eggersdorfer and Wyss 2018) (Dewi et al. 2018; Murugan and Iyer 2013), EPA and DHA fatty acid production (Sahin et al. 2018; Pia Steinrückena 2018), as well as other specialty product production (Fu et al. 2017; Fu et al. 2015; Coates RC 2013). Additionally, select strains have been used in the production of protein (Sankaran et al. 2018; Morançais et al. 2018), carbohydrate (Julius

2018; Roussel et al. 2015), and for use as fertilizer (Vaishampayan et al. 2001; Marks et al. 2017; Solovchenko et al. 2016; Mulbry et al. 2005). However, most people would associate algal biotechnology as a potential platform for biofuel production to replace declining fossil fuel reserves (DOE 2018; Shi et al. 2018; Sankaran et al. 2018). This is logical given the evidence that certain oil deposits, namely the Colorado oil shales and North Sea oil deposits are thought to have been formed by specific strains of microalgae including coccolithophore microalgae and relatives of present-day *Botryococcus sp.*, respectfully (Andersen and Lewin 2018). Today *Botryococcus sp.* produces enough oil to be skimmed from Lake Baikal in Russia for use as a biofuel (Andersen and Lewin 2018). Even with such great potential and diverse utility, many of these prospective uses for microalgae remain only distant dreams demonstrated in laboratories with relatively few strains being translated into actual commercial ventures with economic impact.

Numerous algae startup companies have come and gone over the past two decades, leveraging millions in funding from government sponsors and venture capitalists alike (Wesoff 2017). Those that have found lasting success over the years typically rely on extremophiles capable of thriving in culture conditions toxic to other organisms. This includes organisms such as *Spirulina sp.* which is grown in alkaline culture media (Soni et al. 2017) or *Galdieria sp.* and lutein-*rich Coccomyxa sp.* which thrive in acidic environments (Henkanatte-Gedera et al. 2015; Vaquero et al. 2014). Also extreme salinity has been used in culturing the halotolerant organism *Dunaliella sp.* (Zhu et al. 2018). In addition to extremophilic organisms, most companies with sustained economic impact typically utilize outdated, low-yielding traditional raceway production systems that have seen limited advancements for decades (Cyanotech 2018; Chen et al. 2015). Such a strategy of using extremophilic algae strains coupled with simplistic production methods such as raceway ponds has allowed for some commercial

production of microalgae, albeit an order of magnitude below macroalgae and traditional agricultural commodities.

The true productivity potential of many microalgae stains, as demonstrated in laboratory settings has yet to be duplicated in real world outdoor environments on a scale with commercial viability. This is primarily due to the economic considerations of maintaining those ideal conditions in an outdoor setting. Raceway ponds are used to keep production costs relatively low at the sacrifice of biomass yield and photosynthetic efficiency. Since the yield is low, production must be spread over vast open areas that are easier to maintain in monoculture by use of extremophilic strains. This excludes many promising strains from bringing new potential products into commercial production. The use of low-yielding traditional open raceway systems does not maximize the yield of a given strain to improve upon the economics of production.

The only economical and viable route to date for developing microalgae as a commercial crop is through production of high-value microalgal products (Wesoff, 2017). This is because the labor, energetic, and capital costs of production are limiting the realization of microalgae as an agricultural commodity. With microalgae production costs in the $1,000's per metric tonne of biomass as compared to $100's per metric tonne for most agricultural commodities, many companies struggle to survive (Buschmann et al. 2017; Chavan et al. 2018; Barrientos and Soria 2018). The lack of an efficient and cost-effective microalgae production system that pushes the photosynthetic yield seen in the field to levels observed in laboratories is severely bottlenecking the emerging algae industry. Considering that just a handful of the 40,000 plus strains have been fully exploited for their commercial potential, the development of such a system to

maximize the yields of microalgae and lower production costs could really bring a lot of new products to the market with positive economic and environmental impacts.

2.2.1 History of Microalgae production

While microalgae has been utilized by centuries by different cultures, including the Chinese over 2,000 years ago, the Japanese, Africans in the Lake Chad region, and the Aztecs of the present day Lake Texcoco region (Priyadarshani and Rath 2012; Jensen et al. 2001) to name a few, it wasn't until the mid-20th century that commercial algaculture—including seaweed—really blossomed (Pulz and Gross 2004). Macroalgae, or seaweed, are generally what come to most people's minds when algae is being discussed. This is because macroalgae have seen a greater success in the market due to the low cost of production in open ocean waters, which requires very little energetic or capital investments, fertilizers, labor or other inputs. Current world production of macroscopic algae strains is estimated at approximately 30 million metric tons (FAO 2016) and is used in all sorts of products from ice cream to cosmetics or even biofertilizers (Buschmann et al. 2017; Renuka et al. 2018). Microalgae production has not seen this success and current world production estimates is at less than 20,000 metric tons (Hemaiswarya et al. 2011; Spolaore et al. 2006a; Lundquist et al. 2010; Chavan et al. 2018), an order of magnitude below macroalgae production.

The reason for this boils down to one major difference between microalgae and macroalgae: multicellularity. Macroalgae are multicellular and create structures called holdfasts that anchor them at sea and allow for easy harvesting while single cell microalgae would be impossible to cultivate in open sea without some type of containment system that is capable of withstanding ocean tide and currents. Microalgae have demonstrated superior growth rates than many macroalgae or higher plants, while

producing carbohydrates, protein or oils for nutritional or biofuel applications. Many are also adaptable to a wide range of environments, not just the open sea as in the case of most seaweeds. Even with all this potential, microalgae, being microscopic in nature, cannot be cultivated in open seawater efficiently without substantial losses to the environment via predation or sedimentation. Microscopic algal cultures require some sort of a containment system such as a photobioreactor or traditional raceway pond to contain the culture and support systems such as aeration or cooling systems to mitigate environmental fluctuations for the culture. This infrastructure greatly enhances the productivity of microalgae as compared to natural unmixed ponds but the energetic and capital costs associated with many systems are cost prohibitive.

2.2.2 Raceway and photobioreactor development

Most production systems are classified as either raceways or photobioreactors. For raceway ponds, there are three main types, including traditional or Oswald ponds, circular ponds and cascade or gravity-fed system. Photobioreactors have a lot more variability and are generally more complex. They have a higher capital and operating cost attributed to aeration and circulation pumps whereas ponds typically use an energy efficient paddlewheel for mixing. Photobioreactor designs include flat panel, column, dome, coiled, thin-film and biofilm reactors. Some designs are more cost effective than others but overall they generally produce yields an order of magnitude larger than most raceway configurations, with the added costs of production.

Oswald ponds are the traditional raceway design that was pioneered by William J. Oswald in the 1950's. They consist of shallow, flat raceways that generally have semi-circular ends that allow for economical mixing of algal cultures to enhance growth (Chisti 2013). Traditional raceways are typically 0.25 to 0.30 m deep channels mixed by

a paddlewheel that propels the water down the channel at 0.25 to 0.3 ms^{-1} to keep the microalgae in suspension while also ensuring nutrient delivery and gas exchange. A raceway with a depth in the range of 15 to 30cm is common for paddle driven raceways, as this depth is needed to ensure proper mixing for the algal cultures. Unfortunately this is a reason why many raceway ponds suffer from such low productivity and why many photobioreactor, which have lower culture depths or light paths, attain higher photosynthetic efficiencies than traditional raceways.

Depth of culture is a critical factor that is important to ensure efficient delivery of light into the culture on a time scale appropriate for photosynthesis. This is what has been referred to in the literature as the flashing light effect or light-dark cycling, and for pond depths in the range of 0.25 to 0.3 m the frequency is on the order of seconds, much too long for optimal photosynthetic activity with timescales in the range of milliseconds. This is what inspired the elevated raceway ponds known as Třeboň-type cascade units that were developed in the Czech Republic with pioneering work from Ivan Šetlík in the 1950's (Masojídek 2009). Light-dark cycling, or a given algae cell moving into and out of the range of photosynthetically active radiation (PAR) is much more pronounced in a shallow raceway than in a deeper raceway. This is because the average distance to the photic zone where PAR is available for photosynthesis is much smaller and therefore the cycle will be on average faster. The photic zone, as described in Gitelson et al. (1996), is in the range of a few millimeters thick in dense algal cultures.

This pioneering gravity-fed raceway concept has since been modified thinner and thinner over the years to realize some of the best productivities observed for open raceway systems, even out-performing the productivity of many high-yielding photobioreactor systems as well (Masojídek 2009). These special raceways as shown in

Figure 2.2 below are called cascade raceway systems rely off gravity and a centrifugal pump to mix the algae instead of a paddlewheel requiring a certain depth to work properly. These raceways are able to take productivity to the extreme with photosynthetic yields approaching 10% (based on PAR), while using culture depths as low as 6-7 mm and all while using significantly less water than traditional raceways leading to reduced harvesting costs and production costs as low as 20% of that of traditional raceways possible (Doucha and Livansky 2009; Masojídek et al. 2011). The main drawbacks to this production system is the requirement of constantly lifting water against the force of gravity, which is energetically intensive if carried out all day long. Special positive displacement pumps are required for pumping algal culture so as to not damage the cells with the level of shear stress experienced in more centrifugal pumps. The system also remains open, which does nothing to protect the cultures from contamination by invading species or predation or to maintain a level of CO_2 within the culture. The gravity fed system does allow for much higher yielding cultures than traditional raceways buy using the force of gravity to overcome the mixing problems experienced by a raceway operating at depths below 15 cm.

In more recent years, a special class of photobioreactors called biofilm reactors has demonstrated, on a lab or pilot scale, the best microalgae photosynthetic efficiencies and biomass yields to date. One such system achieved 80 $gm^{-2}day^{-1}$ growing *Scenedesmus obliquus* in an outdoor setting (Liu et al. 2013b). Also *Spirulina sp*. which typically yields 10-15 g $m^{-2}day^{-1}$ dry weight biomass in ponds was able to achieve yields of 60 g $m^{-2}day^{-1}$ dry weight biomass on a biofilm production system as shown in Figure 2.3 below (Zhang et al. 2015). Like cascade reactors, these systems also require constant pumping of water which can prove energy intensive when pumping against the head created by gravity all day long. Also like cascade systems the culture is open with

extreme exposure to air which greatly enhances evaporation rates and contamination risks.

Another type of system that has seen many iterations of promising design in recent years is thin film photobioreactor systems which are made by typically heat welding two thin films of thermoplastic together to create a very low-cost yet durable containment system. Vertical bag systems as depicted in Figure 2.4 below suffer from the same dilemma as cascade or biofilm systems, constantly moving water against the force of gravity to accomplish mixing, nutrient delivery and temperature control. Running the bag horizontally as seen in Figure 2.5 alleviates much of the head pressure created by gravity, however the system should be designed to eliminate dead zones, areas where algae cells settle out and putrefy due to inefficient mixing. Either system offers a semi-enclosed design that allow CO_2 levels to be raised within the culture media without escaping to the atmosphere. At the same time a semi-enclosed culture does present the issue of how to degas the culture effectively removing oxygen while maintaining CO_2 levels, which can present a challenge. Semi-enclosed cultures does help mitigate the risk of contaminating species which can help bring those strains that are not extremophilic but possess the biology to deliver new products to market.

Thin film reactors are relatively inexpensive compared to column, flat plate or tubular reactors that require much more plastic per unit biomass produced or more expensive structural materials that stand up to the elements and vertical head pressure of those systems placed upright. All enclosed systems share the problem of dealing with biofouling and dust and debris settling on the lighted surface of the reactor, both having negative effects on the rate of photosynthesis and typically leading to more labor costs to be considered which have historically been the biggest cost with these systems

accounting for up to 2/3 the total cost of production in some cases (Cheng-Wu et al. 2001).

2.2.3 Getting the same yield in the field

Much of the excitement surrounding the potential productivity of microalgae has been based on laboratory results or small studies that can be difficult to reproduce in the field. This is due in part to unpredictable and uncontrollable nature of an outdoor field test environment, and in part to assumptions that are being made when transitioning the given study to the field. Much more attention has to be paid to all the fine details and provisions have to be made for unforeseen circumstances in the field. Sometimes experiments are lost and have to be repeated, and this is much more common in an outdoor setting. That being said, sensors, equipment and technology have progressed to the point that many variables can at least be monitored if not controlled, even remotely.

This greatly enhances the reproducibility of field site tests, nonetheless researchers must still be diligent and creative when transitioning experiments to the field, and the results compiled should be taken in light of the whole picture. For instance, light utilization is hard to compare from an indoor to outdoor study if the cells indoors are subject to constant light of 200 $\mu Em^{-2}\ s^{-1}$ with the optimized wavelengths and intensity and cells outdoors are subject to full spectrum sunlight at >2000 $\mu Em^{-2}s^{-1}$ along with fluctuating temperature, pH, nutrient levels, potential predators and contamination risks. Understanding the interactions of all these parameters in an ever-changing field site is a challenging task but must be undertaken in order to arrive at an economically viable methodology for commercial microalgae production.

Figure 2. 1: Attached vertical biofilm reactor (right) culturing *Spirulina sp*. with yields of 60 $gm^{-2}day^{-1}$ (Zhang et al. 2015)

Figure 2. 2: Thin layer (6-7 mm) cascade raceway system at the Laboratory of Algal Biotechnology in Třebon, Czech Republic (left) yielding up to 55 $gm^{-2}day^{-1}$ *Chlorella sp*. biomass (Algatech 2019; Masojidek et al. 2011).

Figure 2. 3: Low-cost horizontal thin film plastic bag (right) for high-density algal growth (Photo credit: Photon 8, Inc.).

Figure 2. 4: Low-cost vertical thin film plastic bag (left) for high-density algal culture (Photo credit: Coastal Biomarine, LLC).

Whether an alga cell is grown in a traditional raceway pond or in a photobioreactor, the cell must experience an environment with the proper pH, temperature, salinity, dissolved oxygen, carbon dioxide, and nutrient concentrations among other things for each specific strain to achieve the best possible growth in the field. To accomplish this, some form of mixing will need to happen to not only dissipate evolved oxygen from photosynthesis but also ensure proper delivery of nutrients including carbon dioxide while also maintaining temperature stability. Since the cell culture is made up of billions of cells constantly shading each other, mixing is also critical to ensure optimum light exposure. This is more often than not short flashes of sunlight at photoinhibitory levels. All of these parameters must be allotted for in the design of an efficient outdoor production system capable maximizing photosynthetic yield to arrive at economically viable commercial microalgae production. Since photosynthesis is the rate-limiting step of this entire process, it is critical that photobioreactor systems operate in accordance with the principles of photosynthesis.

2.2.4 Timescale of photosynthesis

Photosynthesis is the driving force behind practically all agricultural commodities. In most cases, crops are produced with minimal inputs by working with nature and ambient conditions to keep costs as low as possible. And the biology of traditional crops is what handles the aspects of production that makes microalgae cultivation both challenging and expensive in relation to other crops. For instance, traditional crops manage cell temperature via evapotranspiration or in the case of seaweeds by being submerged in seawater. This is passive cooling as compared to microalgal cultures requiring chillers, pumps, or paddlewheels needed for mixing and

temperature control. Another example is light utilization. Traditional crops including seaweeds manage light by growing vertically and supporting thin overlapping layers of leaves or leaf-like structures to efficiently harvest light. The structure is non-existent for microscopic algae cells that must be contained by some type of vessel to maintain culture. Most crops are simply use open ground or seabed as support whereas most algacultural production systems uses metal and/or plastic support structure and vessels to produce biomass. This is costly and the main reason microalgae biomass remains in the $1000's per MT whereas most agricultural commodities are in the $100's, an order of magnitude lower.

Despite these production challenges, there are straightforward approaches for bringing production costs of microalgae closer to that of agricultural commodities. Using passive heating and cooling by using seawater or lake water to cool the algal culture (Quinn et al. 2012; Harris et al. 2013) with minimal or no pumping would eliminate the need to evaporate large amounts of fresh water while giving algae an advantage over terrestrial crops that rely on freshwater for cooling via evapotranspiration. Likewise, it would reduce the energetic requirement of complex chilling systems requiring a lot of energy and maintenance. Another approach using nature as an example would be to bring the average culture depth experienced by the cells closer to the examples provided by nature. For example an average corn plant has its photosynthetic cells arranged in a leaf structure that is only 200 microns thick (Research Bulletin 1916). This is on the order of many biofilm systems (Liu et al. 2013b; Zhang et al. 2015; Xu et al. 2017) and thin layer cascade systems (Jerez et al. 2016b; Silva Benavides et al. 2017) common in the literature in recent years yielding 50 to 80 g m^{-2} day^{-1}. Most open raceways operated at 15 cm or more depth require the movement of a lot of water continuously to capture 10% of the yields of more advanced systems.

This is directly related to the timescale of photosynthesis. Oxygen-evolving photosynthetic organism have a non-cyclic electron transfer chain with two photochemical reaction center complexes and depicted in Figure 2.5 below both energetically and structurally. These reaction centers called photosystem I and II make the Z-scheme of photosynthesis and are embedded within the chloroplast thylakoid membranes. When excited with photosynthetically active radiation (PAR) of 380-700 nm a network of pigment passes the energy to the chlorophyll in the given reaction

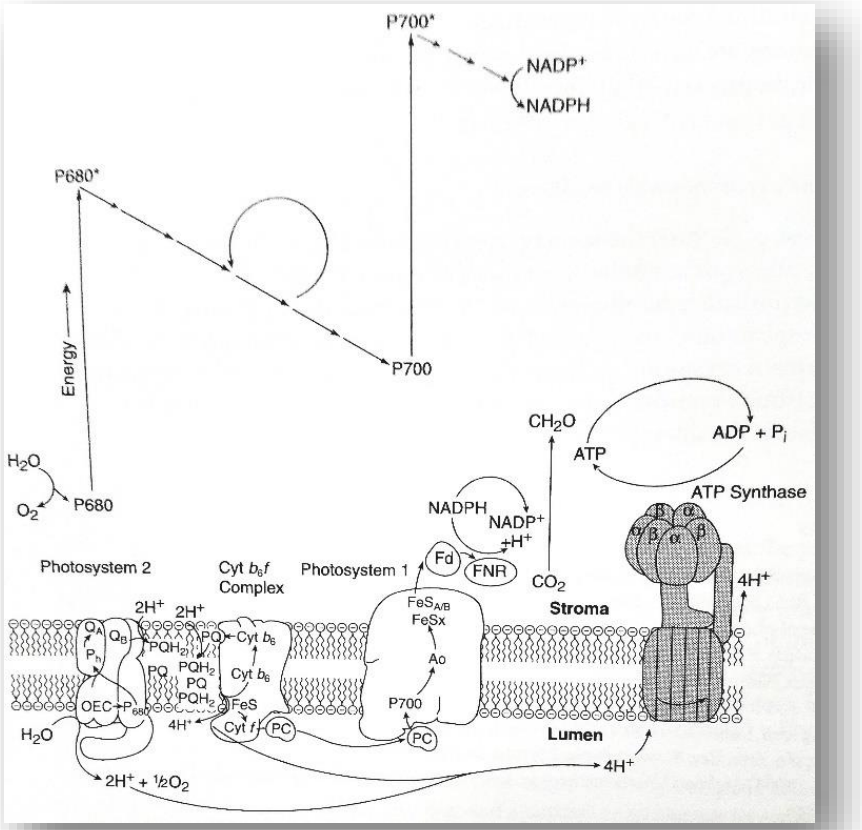

Figure 2. 5: Non-cyclic electron flow also known as the Z-scheme of photosynthesis. Top illustrations shows the energy flow in the system and the bottom shows the spatial and structural arrangement within the thylakoid cross-section. Adapted from (Blankenship 2008).

center where charge separation occurs which strips water of its electrons and passes them down the electron transport chain to be donated to Photosystem I. A second excitation event in Photosystem I leads to another charge separation that reduces the intermediate electron acceptor NADPH. The act of Photosystem II oxidizing water creates molecular oxygen that diffuses out of the cell and hydrogen protons that combine with additional protons created by the electron being passed down the electron transport chain. These protons collect in the lumen of the thylakoids creating a pH gradient and when transported across the membrane drive ATP synthase that phosphorylates ADP into ATP. The phosphate bond energy and the NADPH generated by Photosystem I is used to reduce carbon dioxide into phosphorylated sugars that are exported from the chloroplast (Blankenship 2008).

Previous work (Burda 2007) has shown that most of the enzyme-driven reactions involved with photosynthesis occur in the timescale of a few μs but 1 ms is required to evolve oxygen. This has formed the basis of many projects investigating the use of light-dark cycling to improve photosynthetic yields (Janssen 2002; Sforza et al. 2012). Increasing the frequency of light-dark (L/D) cycling from 10s to 10ms for the same given L/D stimulated photosynthetic rates by 2.1 fold and when the dark period were twice the light period (2D/1L) the rate increased 6.7 times (Grobbelaar 2009). An increase of Reynold's number, a measure of turbulent flow for a given system, has shown to achieve record biomass productivities yet typically at a high energetic cost by supplying more air or pumping velocity to the culture. Also a number of projects involving fiber optics, specialty lighting or even fluorescent or phosphorescent particles have been completed to demonstrate higher photosynthetic yields (O. Pulz 1998) however many of these approaches are not economically feasible when taken to commercial scale. One route

that has seen success in increasing photosynthetic yields without comprising the commercial scalability of the culturing system is the use of a smaller path length as seen in the biofilm and thin layer cascade production systems.

2.2.5 Goal of this work

The primary objective of this work is to characterize two strains of Scenedesmus suitable for year-round production in the desert southwest and determine if adopting a thinner path length (width) for an acrylic flat panel production system can show enhanced photosynthetic activity and/or cut the associated production costs. A reduction in the path length is hypothesized to increase both areal and volumetric biomass output while offering a reduction in terms of systems cost and energetic requirement for operation.

2.3 Materials and Methods

2.3.1 *Scenedesmus* acutus strains

Two strains of *Scenedesmus acutus*, LRB-AZ-0414 and LRB-AZ-0424, were utilized for all experiments. The strains were isolated on BG-11 agar plates and maintained on monoalgal BG-11 agar plates in the laboratory. All experiments used cultures that had been acclimated to grow outdoors in the desert southwest. This was to identify any acclimatization effects experienced by the cultures when changing the environment from an indoor setting with fluorescent lighting to an outdoor setting with full spectrum, full intensity sunlight and large temperature swings. This was accomplished by scaling the cultures in the lab (using round 800 mL glass tubes D=4.6cm, L=61cm) and then transferring into two acrylic vertical flat panels in the

outdoor photobioreactor array. These two stock panels were maintained on the modified BG-11 culture medium described below and were used as inoculum for all experiments.

2.3.2 Modified BG-11 culture medium

A modified version of the standard laboratory BG-11 culture medium consisting of one fourth of the nitrogen (375 ppm $NaNO_3$) and no additional magnesium sulfate, calcium chloride, or sodium bicarbonate was utilized for these outdoor experiments. These changes were made because lowering the nitrogen concentration led to a faster stress response and the tap water utilized outside already has sufficient micronutrients to replace these ingredients. Analyzed tap water at ASU's AzCATI field site (33°18'14.2"N, 111°40'21.5"W) had an average hardness of 280 ppm as $CaCO_3$ with 180 ppm Na^+, 75 ppm Ca^{2+}, 70 ppm SO_4^{2-}, 24 ppm Mg^+, and 8 ppm K^+. Final composition of the modified growth medium included 375 ppm $NaNO_3$ (Alfa Aesar), 9.58 ppm K_2HPO_4 (BDH), 5.24 ppm of Ammonium Ferric Citrate (Alfa Aesar), 1.31 ppm Citric Acid Monohydrate (Sigma-Aldrich), 0.71 ppm H_3BO_3 (Sigma-Aldrich), 0.445 ppm $MnCl_2{\cdot}4H_2O$ (Sigma-Aldrich), 0.10 ppm $Na_2MoO_4{\cdot}2H_2O$ (Sigma-Aldrich), 0.06 ppm $ZnSO_4{\cdot}7H_2O$ (Sigma-Aldrich), 0.02 ppm $CuSO_4{\cdot}5H_2O$ (Sigma-Aldrich), 0.1 ppm $Co(NO_3)_2{\cdot}6H_2O$ (Sigma-Aldrich).

2.3.3 Flat panel photobioreactor array

All high-density growth experiments conducted on the Arizona Center for Algae Technology and Innovation (AzCATI) outdoor field site in Mesa, AZ utilized a north-south facing array of 48 vertical acrylic flat panel photobioreactors. Some panels were modified from the original design as described below to shorten the path length of the photobioreactors.

2.3.3.1 Acrylic flat panel photobioreactors

The flat panel photobioreactors were constructed of 13 mm thick acrylic sheets. They were aligned in four rows of twelve panels with sufficient spacing to minimize shading (~1.2 m). Tank dimensions are 1.17m by 1.17m by 3.8cm giving an internal volume (accounting for flex of acrylic) of 60L and a working volume (accounting for aeration volume) of 50L.

2.3.3.2 Modified flat panels for high-density growth

Four tanks were disassembled and modified to test the effects of culture depth and light intensity on growth. The tanks were reduced in thickness: two to 1.27cm and two to 0.95cm. Due to the narrow space in the tanks cooling was accomplished by culture being airlifted through and external cooling jacket made of galvanized conduit and PVC and 6.4mm OD tubing was used with 0.8mm holes drilled every 3.8cm across the bottom of the tank to provide adequate mixing and aeration.

2.3.3.3 Aeration blower and CO_2 dewar

Aeration was provided via 13mm PVC pipe with 0.8mm holes drilled every 3.8 cm across the bottom of the PVC pipe in a staggered arrangement to promote mixing. This supplied the cultures with 0.5 vvm aeration. Commercial grade carbon dioxide (CO_2) was obtained from Praxair dewar located on site and plumbed into the photobioreactor array. The CO_2 was fed into the inlet of a 2 H.P. Sweetwater blower at maintain at a feed rate of 1.5% CO_2 from sunrise to sunset daily.

2.3.3.4 Cooling tower and reservoir

The tanks were cooled by placing 13mm stainless steel cooling loops into tanks and circulating cooling water from a 2000L underground reservoir cooled using two GPC-25 Glacier evaporative pool coolers set on a Johnson Controls thermostat to regulate the reservoir temperature to 24±3°C for the LRB-AZ-0414 strain and 29±3°C for the LRB-AZ-0424 strain. A 2 HP 3 phase pool pump circulated chilled reservoir water during the day to regulate culture temperature in the photobioreactor array.

2.3.4 Indoor temperature gradient setup

For determining optimum temperature range for each strain, laboratory cultures of both LRB-AZ-0414 and LRB-AZ-0424 were scaled and placed in 1 L flasks along a temperature gradient, ranging from 15 to 45°C to better define the optimum temperature range for the two strains. The gradient was created by a heating element and refrigeration unit running together underneath an aluminum table surface. Due to limitations of the system, a flat-bottomed Erlenmeyer flask was used to provide sufficient contact with the heating elements on the table. For this reason, only a small daily sample was permitted so optical density was used to monitor growth as opposed to AFDW. One mL of culture was removed from each flask daily, diluted 10x with 18 Ohm water and analyzed in a 96 well plate at 750 nm to access growth rate at the different temperatures. Mixing was provided by bubbling compressed air mixed with 1.5% CO_2 via submerged glass capillary tubes. Lighting was provide by florescent lights mounted approximately 0.6m above the cultures.

2.3.5 Culture monitoring

A Neptune apex datalogger (Neptune Systems, LLC.) was used to monitor the pH and temperature of the culture in the tanks. While this system is capable of controlling in addition to monitoring, the required equipment was not available for the course of these experiments. The system did however serve as a redundant monitoring system capable of sending SMS alerts if tank pH or temperature out of range. A Li-1400 datalogger (Li-COR Biosciences) with two Li-190 sensors monitored incoming PAR. Since the photobioreactors are vertical and have only one side incident to direct sun, one sensor was placed on the south side to measure the maximum direct radiation and the other was placed in the shade to measure non-direct radiation on the north side, although in summer months the north facing side receives more incident sunlight.

2.3.6 Nutrient Analysis

2.3.6.1 Flow injection analysis

Nitrate, ammonia and phosphate data were analyzed using a QuickChem 8500 Flow Injection system. Standards and reagents were prepared as per manufacturer's recommendations and a standard curve with R-correlation value ≥ 0.95 was generated for each ion analyzed. The samples, collected in duplicate, were prepared by centrifugation at 1000g then subsequent filtering through 0.2 µm membrane filter to remove any remaining particles. The samples were diluted 1:7 with 18 MOhm filtered water to bring the concentration of all analytes within the realm of the standard curve. Samples were collected in 2 mL centrifuge tubes (VWR) and centrifuged at 1000g to remove all suspended cells then preserved in cold room at 4°C until each experiment was complete and ready for analysis.

2.3.7 Culture density assessment

A daily sampling regimen was undertaken to monitor biomass density via ash-free dry weight (AFDW) by filling one 50 mL centrifuge full from top of tank after top-off water was added from tap to account for evaporation. For the temperature gradient experiment using 1 L flasks, a 1 mL samples was removed for optical density measurement after topping of flask with DI water to the line.

2.3.7.1 Gravimetric analysis

Culture density was measured via gravimetric analysis using ash-free dry weight (AFDW) to track growth of the biomass over the course of each growth trial. Pre-weighted, pre-ashed (500°C for four hours) 1.2µm glass microfiber filters (VWR 696) were used in vacuum funnels to collect suspended algae cells in 5-20 mL cultures samples in duplicate. The samples of algae biomass collected on the filters wash subsequently washed to remove excess salt, bacteria, or other contaminates. The filters were then dried at 105°C for eight hours before transferring to a desiccator to cool to room temperature. The filters containing the dried samples were then weighted and moved to a muffle furnace held at 500 ± 25°C for four hours. The samples were transferred to the desiccator and allowed to cool to room temperature before recording the final weight of the remaining ash from the algae samples. All weights were recorded to 0.0001g precision and the weight of the filter and ash was subtracted from the total weight to determine the algae cell weight per sample volume (g/L).

2.3.7.2 Optical density

Optical density (OD) measurements were taken using a Molecular Devices Spectamax 190 spectrophotometer at 750 nm. Culture was read in triplicate and corrected using a blank of BG-11 culture media. Cultures were diluted 10-fold after

reaching an OD reading of 1.0 to reduce error as this is outside of the linear range based on previous data.

2.3.8 DNA amplification and sequencing

Colony PCR was used to amplify the genomic DNA for the 18s ribosomal subunit utilizing the 360FE: 5′-CGGAGARGGMGCMTGAGA-3′ forward and 1391RE: 5′-GGGCGGTGTGTACAARGRG-3′ reverse primers (Dawson and Pace 2002). The cells were boiled for 10 minutes in 2X Phusion GC buffer after which the primers, dNTPs, Phusion DNA polymerase (Finnzymes) were added along with water. Final concentrations for the primers, dNTPs, Phusion DNA polymerase was 0.5 mM, 0.2mM, 0.01 U, respectively, and the Phusion GC buffer was 1X. Denaturation in the thermocycler was at 98°C for first 30s, then 29 cycles of 98°C for 10s, 56°C for 30s, 72°C for 2 minutes and then 72°C for 10 minutes. Electrophoreses on a 0.8% agarose gel of the amplified product was purified using a QIAquick gel extraction kit from Qiagen and was then sequenced with the above primers on a Applied Biosystems 3730 capillary sequencer.

2.3.9 Bomb calorimetry

Bomb calorimetry was used to measure the heat of combustion of log and nutrient limited biomass samples. A 1341 Parr bomb calorimeter was used in accordance with the operating instructions. A calibration was carried out using benzoic acid. Algae sample pellets were compressed prior to combustion and fuse wire was weighted and corrected for.

2.3.10 Microscopy

2.3.10.1 Transmission electron microscopy (TEM)

In preparation for TEM, the cells were first fixed for 1 hr in a solution of 2% glutaraldehyde in a 0.025 M phosphate buffer with pH 7.0. In order to transfer the cells to 0.5% veronal-buffered osmic acid for post-fixation they were centrifuged at 2000 g for 5min, then rinsed two times using0.025M phosphate buffer and transferred to the buffered osmic acid left at 47°C overnight. The cells were then taken through an alcohol series containing uranyl acetate (70–90% alcohols), to quickly dehydrate and then placed in a 1:1 alcohol–acetone mixture briefly before leaving in pure acetone overnight. Spurr's resin was used to embed the cells and once cured, ultrathin sections were obtained on the microtome and stained with uranyl acetate and lead citrate. The stained sections with embedded cells were imaged using a Philips CM12S (Philips Electronic Instruments Co., Mahwah, NJ) scanning transmission electron microscope.

2.3.10.2 Light, DIC, epifluorescent, and scanning confocal microscopy

Light microscope images of cells were obtained using an Olympus BH-2 light microscope with 20X or 40X objectives. Epifluorescent and DIC images of cells in stationary growth phase were captured using a Nikon Elipse TE300 inverted video microscope on a Hamamatsu Orca 3CCD camera by use of BODIPY as a stain to image neutral lipid bodies (green). Scanning confocal images were obtained using a Leica SP8 scanning confocal microscope. The cells were stained with BODIPY for 15 mins to visualize neutral lipid bodies using excitation of 495 nm for BODIPY (green) and 568 nm for chlorophyll autofluorescence (red).

2.3.11 Experiment 2.1: Nitrogen replete growth of LRB-AZ-0414 in pond vs. flat panel

Strain LRB-AZ-0414 was cultivated on modified BG-11 media (as described above) containing full nitrogen (247 mg/L N-NO_3) to test maximum growth rate in a 7.62 cm pond, 3.80 cm panel, 1.27 cm panel and 0.95 cm panel. For panels, aeration air was supplied at 0.5 vvm with 1.5% CO_2 (v/v) for mixing, pH control and carbon supply. Pond CO_2 was supplied was added through a Sweetwater diffusion stone (30 cm X 5 cm) at 5 L/min. This generally kept culture levels at a pH of 8.0 ± 0.5 for optimum growth. Dry weight data was collected every other day over six days of growth using ash-free gravimetric analysis and initial dry weight was approximately the same volumetric density. The growth data was compared to local (Queen Creek) AzMET data (Azmet Weather Data 2018) to calculate photosynthetic efficiencies assuming an energetic content of 6.49 ±0.49 kWh kg^{-1} energetic content for the biomass and that available PAR is 42% of incoming insolation.

2.3.12 Experiment 2.2: Nitrogen deplete growth of LRB-AZ-0414 in pond vs. flat panel

In order to test growth in a nitrogen-limited environment that is typically used to induce lipid production, strain LRB-AZ-0414 was cultivated under the same culture conditions as Experiment 2.1 except that the modified BG-11 media (as described above) contained only 25% of the nitrogen (61 mg/L N-NO_3). Also due to circulation issues with the 0.95 cm panel, this panel size was omitted and instead only carried out using a 7.62 cm pond, 3.80 cm panel, and 1.27 cm panel. All other parameters remained the same to Experiment 2.1.

2.3.13 Experiment 2.3: Nitrogen replete growth of LRB-AZ-0424 in flat panel with decreasing path length

Strain LRB-AZ-0424 was cultivated on modified BG-11 media (as described above) containing full nitrogen (247 mg/L N-NO_3) to test maximum growth rate for lipid production in a 3.80 cm panel, and 1.27 cm panel and 0.95 panel in duplicate. For panels, aeration air was supplied at 0.5 vvm with 1.5% CO_2 (v/v) for mixing, pH control and carbon supply. Pond CO_2 was supplied was added through a Sweetwater diffusion stone (30 cm X 5 cm) at 5 L/min, keeping levels at a pH of 8.0 ± 0.5 for optimum growth. Dry weight data was collected every day over the course of thirteen days using ash-free gravimetric analysis and initial dry weight was approximately the same areal density. Growth data was compared to local (Queen Creek) AzMET data (Azmet Weather Data 2018) to calculate photosynthetic efficiencies assuming an energetic content of 6.49 ±0.49 kWh kg^{-1} energetic content for the biomass and that available PAR is 42% of incoming insolation.

2.3.14 Experiment 2.4: Temperature gradient for LRB-AZ-0414 and LRB-AZ-0424

The temperature gradient was set up (as described above) with Erlenmeyer flasks aerated with 0.5 vvm air enhanced with 1.5% CO_2 (v/v) was used to test the optimum temperature for strains LRB-AZ-0414 with flask temperatures of 20.85^{0}C, 22.65^{0}C, 24.6^{0}C, 26.6^{0}C, 28.75^{0}C, 30.2^{0}C, and 31.95^{0}C and LRB-AZ-0424 with flask temperatures of 20.95^{0}C, 25.95^{0}C, 30.6^{0}C, 34.3^{0}C, 38.6^{0}C, and 39.8^{0}C. Optical density at 750 nm was recorded daily as a quick way of tracking growth.

2.4 Results and discussion

2.4.1 Strain characterization

ITS-1 sequencing of two strains of *Scenedesmus* isolated in the Phoenix area revealed 99.9% identity to two strains of *Scenedesmus acutus*, a ubiquitous and robust

green alga commonly found in wastewater streams and areas of nutrient pollution. One strain, named LRB-AZ-0414 (GenBank KR904912) was isolated from dairy wastewater from the Phoenix area and maintained in Arizona State University's Laboratory for Algal Research and Biotechnology lab (LARB) for years. It has proven to be a robust algae strain capable of quickly adapting to high light, a variety of nutrient sources, and up to 10% seawater, all while out-competing other algal species and out-producing predators (unpublished data). It has also been shown to produce almost 50% of its dry weight oil and substantial protein or carbohydrate reserves as well (Eustance 2015).

TEM images depicted in Figure 2.6 and 2.7 below show some of the ultrastructural details of the LRB-AZ-0414 strain with white starch granules encasing the pyrenoid and numerous oil droplets in the periphery of the late stationary cell. The cells have a thick microfibrillar inner cell wall characteristic of this algae class and large oil and starch deposits are typical for this strain after being deprived of nitrogen for a few days. Cells of this algal strain are oblong apparently 2-3 micron across and 8-10 microns in length as evidenced by TEM imaging of the cells. The cells exhibit latitudinal wrinkles common to this class of algae and when grown in non-turbulent culture conditions form four cell coenobia, attaching to each other longitudinally, and breaking apart under turbulent conditions (Baudelet et al. 2017).

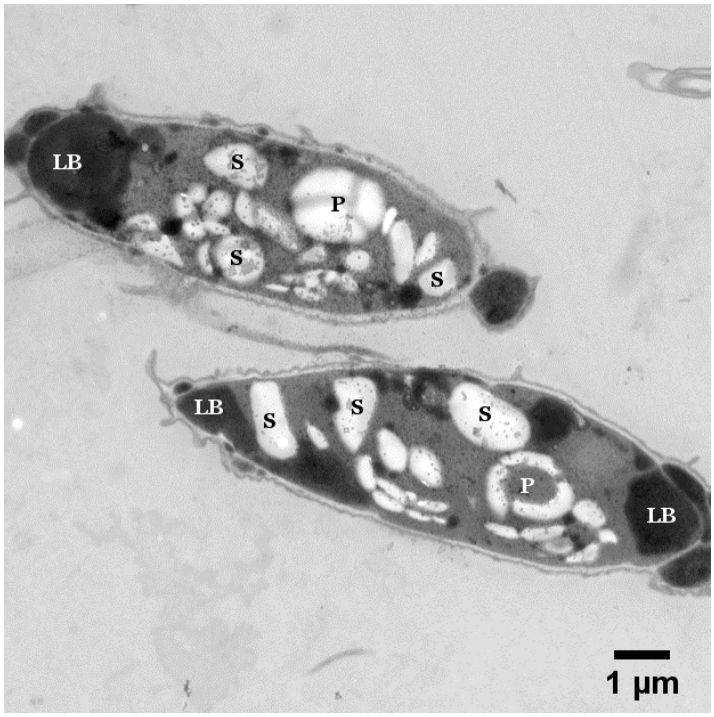

Figure 2. 6: Transmission electron micrograph of *Scenedesmus acutus* LRB-AZ-0414 showing starch granules (S) and lipid bodies (LB) and pyrenoid (P).

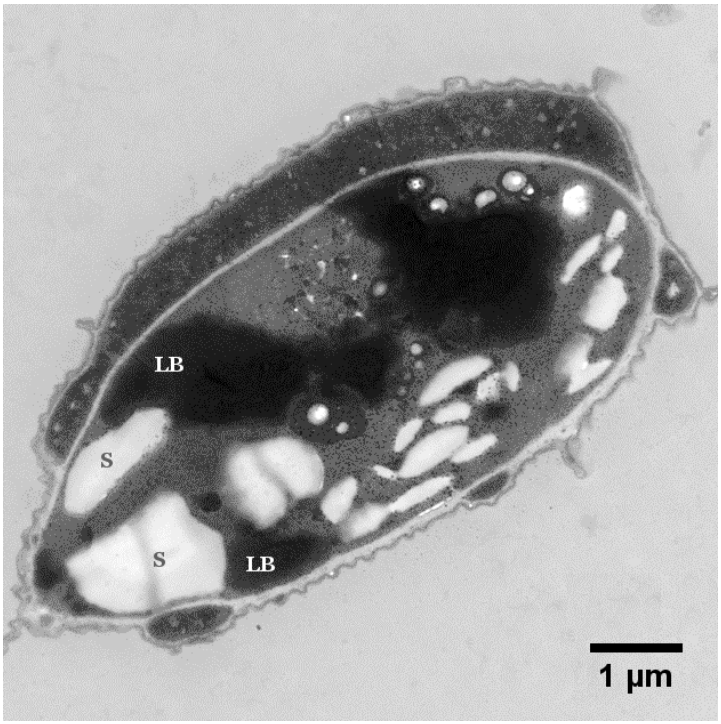

Figure 2. 7: Transmission electron micrograph of *Scenedesmus acutus* LRB-AZ-0414 showing starch granules (S) and lipid bodies (LB).

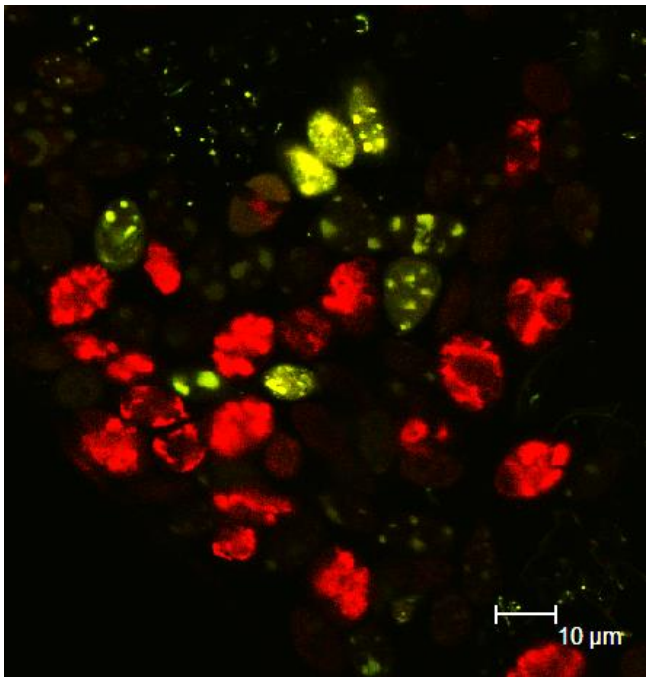

Figure 2. 8: Scanning confocal overlay image of early stationary phase *Scenedesmus acutus* strain LRB-AZ-0414 showing chlorophyll autofluorescence (red) and bodipy stained lipid bodies (green) with overlap in yellow.

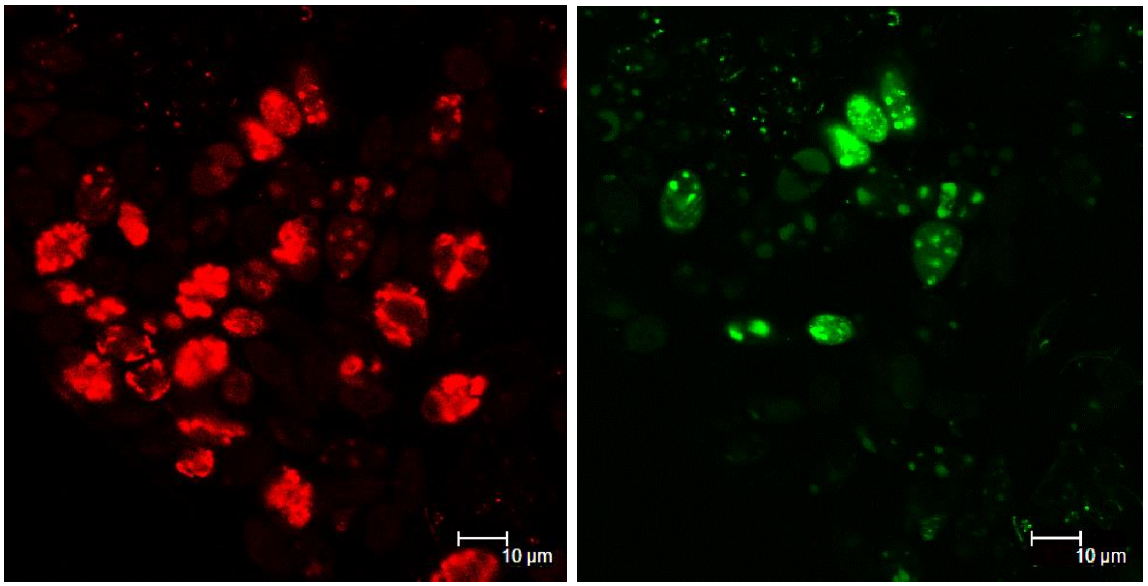

Figure 2. 9: Scanning confocal stack image (left) of early stationary phase *Scenedesmus acutus* strain LRB-AZ-0414 showing chlorophyll autofluorescence.

Figure 2. 10: Scanning confocal stack image (right) of early stationary phase *Scenedesmus acutus* strain LRB-AZ-0414 showing bodipy stained lipid bodies.

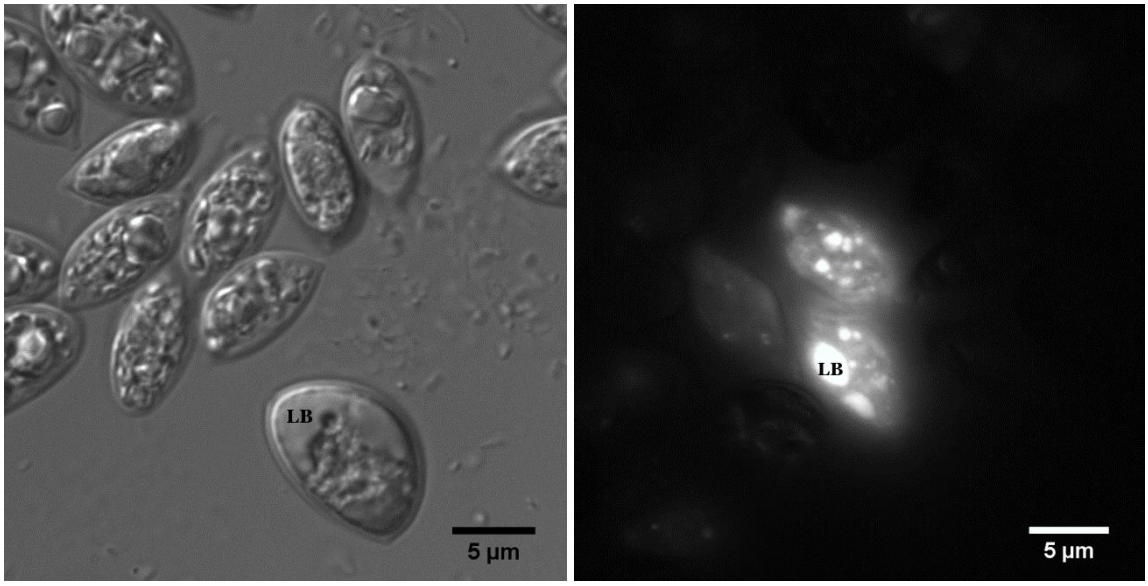

Figure 2. 12: Stationary phase *Scenedesmus acutus* strain LRB-AZ-0414 (left) on DIC displaying lipid bodies (LB).

Figure 2. 11: Epifluorescent image of *Scenedesmus acutus* strain LRB-AZ-0414 (right) showing lipid bodies (LB) stained with BODIPY.

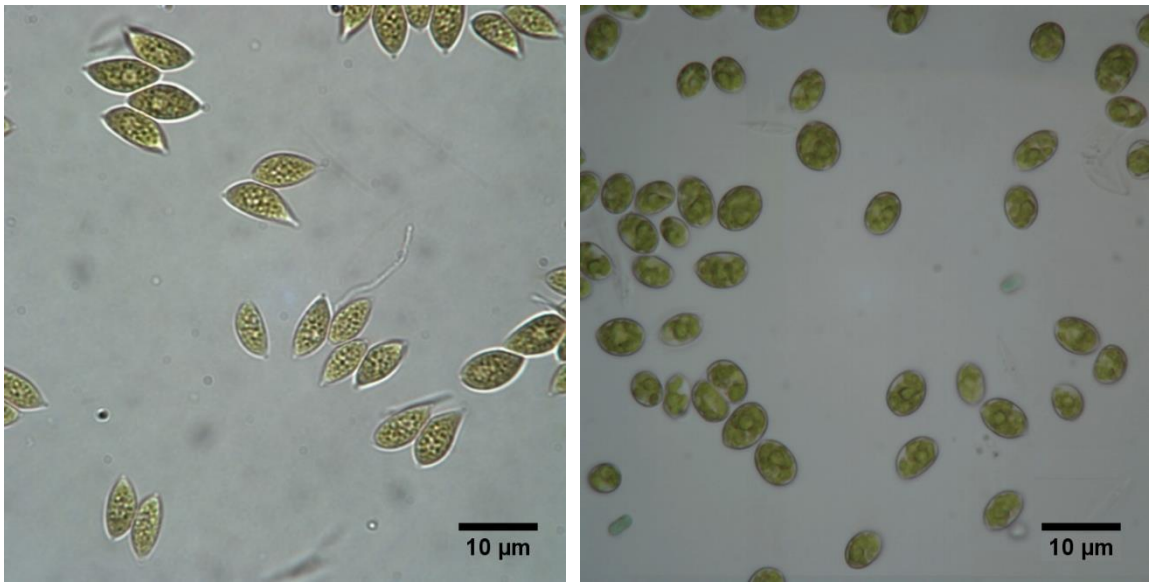

Figure 2. 13: Late log phase *Scenedesmus acutus* strain LRB-AZ-0414 (left).

Figure 2. 14: Late log phase *Scenedesmus acutus* strain LRB-AZ-0424 (right).

Figures 2.8, 2.9 and 2.10 above show scanning confocal microscope images of strain LRB-AZ-0414 displaying oil droplets within the cells stained with BODIPY (green) and chlorophyll autofluorescence (red) which highlights the plate-like chloroplasts common for this alga. The image stack overlay shows the yellow where the oil and chlorophyll fluorescence overlap. Figures 2.11, 2.12, and 2.13 show strain LRB-AZ-0414 under DIC, epifluorescent and bright field microscopy, respectfully. These images display the thick cell and large lipid bodies that can accumulate in late stationary phase for this strain.

Strain LRB-AZ-0424 as depicted under bright field imaging in Figure 2.14 above shows the slightly different morphology as compared to strain LRB-AZ-0414. This strain was also isolated locally when it inoculated a flat panel photobioreactor that had been heat stressed prior to a haboob in 2014 in the Phoenix area. Unfortunately, very little strain characterization has been completed to date for this strain aside from the ITS-1 sequencing, however preliminary data (unpublished) suggests it is similar in composition to the LRB-AZ-0414 strain and performs better during the Phoenix summer in comparison. Aside from the growth studies presented here, the LRB-AZ-0424 strain warrants further investigation as a summer complement to the LRB-AZ-0414 strain to enable year-round production in the desert Southwest region or areas with mild winters.

2.4.2 Experiment 2.1: Nitrogen replete growth of LRB-AZ-0414 in pond vs. flat panel

Experiment 2.1 compared volumetric and areal productivity of strain LRB-AZ-0414 grown using modified BG-11 in a pond with a 7.62 cm light path (culture depth) and panels with decreasing ligth path lengths of 3.9 cm, 1.27 cm, and 0.95 cm. It was expected that the decreased culture depth would increase both the volumetric and areal

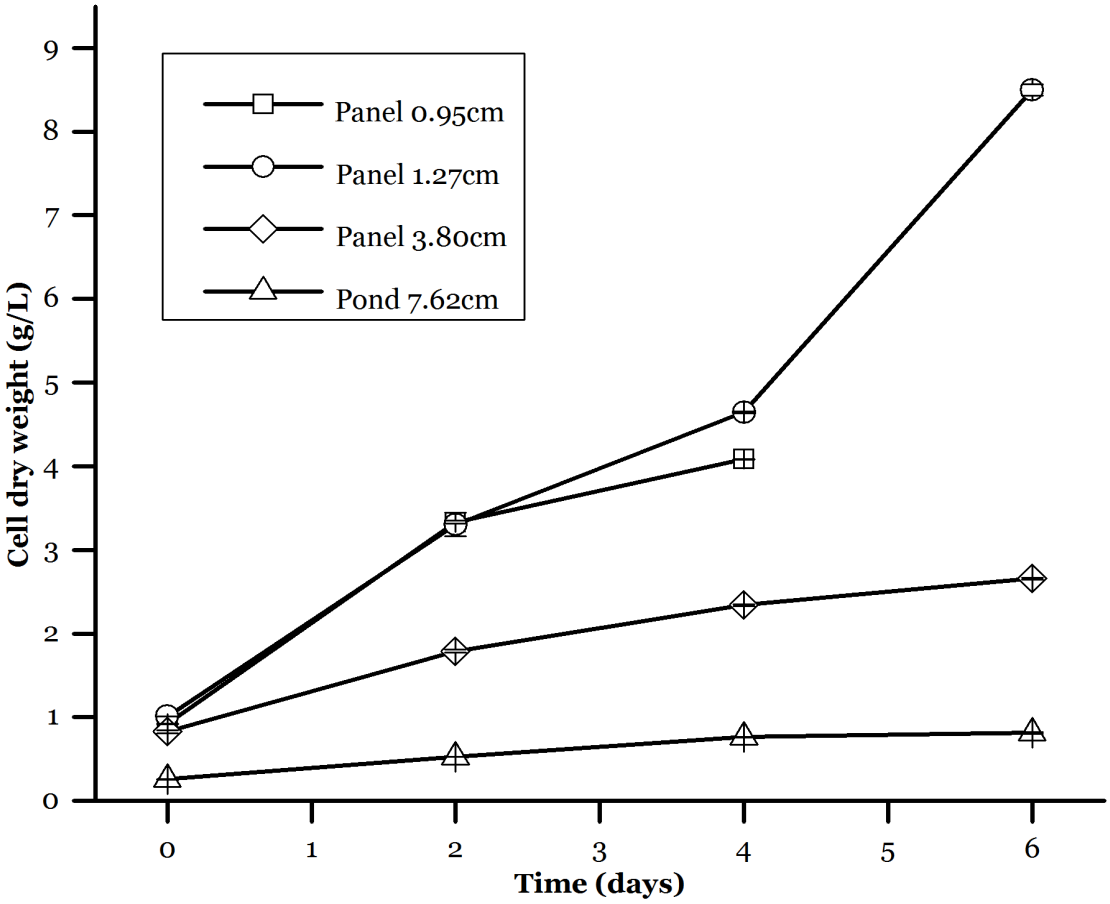

Figure 2. 15: Cell dry weight (g/L) during the first six days of growth of *Scenedesmus acutus* LRB-AZ-0414 on modified BG-11 media in 7.62 cm pond, 3.80 cm panel, 1.27 cm panel and 0.95 cm panel. Vertical bars denote standard error.

productivity by increasing the light-dark cycling experienced by the cells creating an environment more conducive of photosynthesis.

Figure 2.15 illustrates the change in cell dry weight over the course of six days of growth with sampling every other day. The results illustrate the potential of strain LRB-AZ-0414 to be cultured at high cell density effectively by reducing the path length of the given reactor. Despite the small difference in starting volumetric cell density (to achieve similar initial areal density of biomass), the change in volumetric cell density between the different path lengths became apparent over the course of the experiment.

Unfortunately, due to the extreme narrow channel of the 0.95 cm panel, only four days of growth data was obtained before the panel crashed due to insufficient cooling and mixing. Internal ¼" stainless steel cooling loops proved too large to fit in the tank and allow for adequate cirulation to cool the culture and simultaneously too small to provide flow for aqueate cooling. An external airlift was developed for future experiments with this panel in order to provide cooling and circulation, and eventually outfitted to all thin tanks as aeration lines were all that could easily fit in the tanks. In addition, the small volume of both the 1.27 cm and 0.95 cm panel, when operated at a significantly higher cell density were subject to greater loss of biomass at the top of the reactor by the action of aeration bubbles bursting while exiting the culture, splattering algae on the sides of the tank. While the amount lost may be similar to the 3.8 cm tanks in terms of volume of culture, the amount of biomass lost was inherently greater due to a higher operating cell density of the thinner tanks (both visibly and in accordance with data).

Circulation proved to be an issue for both the 1.27 cm and 0.95 cm tanks with some biomass settling daily which required manual agitation. A two-tailed student t-test found the volumetric productivity levels significantly different ($p \leq 0.1$) for the 7.62 cm pond vs. 1.27 cm panel but not significant between any of the other treatment or in terms of areal productivty. These results are promising considering the challenges of transitioning to a thin tank system, however this study would need futher modificaitons and system improvements to provide more consistent data with statisical significance, namely more consistent mixing, a better cooling apparatus, and more data point with dry weight taken at least once if not twice daily.

Table 2. 1: Areal productivity and photosynthetic efficiency for five days of growth of LRB-AZ-0414 strain on BG-11 in different light path lengths. A traditional raceway pond of operated at 7.62 cm depth and three different acrylic flat panel photobioreactors with path length of 3.8 cm, 1.27 cm and 0.95 cm were used. Assuming biomass with 6.49 ±0.49 kWh kg^{-1} energetic content, PAR is 42% of total insolation and an average total insolation of 7.92±0.43 kWh $m^{-2}day^{-1}$as taken from AzMET database.

Path length:		**Areal productivity:**		**Photosynthetic efficiency:**	
		g m^{-2} day^{-1}	kWh m^{-2} day^{-1}	% of Total	% of PAR
7.62 cm-Pond	AVG	7.02±0.94	0.04±0.01	0.57±0.08	1.35±0.18
	MAX	10.2± 0.22	0.07±0.00	0.82±0.02	1.95±0.04
3.8 cm-Panel	AVG	10.47±1.03	0.07±0.01	0.84±0.08	2.01±0.20
	MAX	17.82±0.31	0.11±0.00	1.43±0.02	3.41±0.06
1.27 cm-Panel	AVG	19.21±3.23	0.12±0.02	1.55±0.26	3.68±0.62
	MAX	29.65±1.10	0.19±0.01	2.39±0.09	5.68±0.21
0.95 cm-Panel	AVG	9.13±0.28	0.06±0.00	0.73±0.02	1.75±0.05
	MAX	13.92± 0.20	0.09±0.00	1.12±0.02	2.67±0.04

Table 2.1 below outlines the average and maximum areal productivity of the given cultures and photosynthetic efficiencies as a percentage of total sunlight (from AzMET database) and photosynthetically active radiation (PAR) which has been assumed to be 42% of total sunlight. Although not statistically significant, the avg and max areal productivities are inversly proportional to the path length of the culture increasing up to a max productivity of 29.65±1.10 g m^{-2} day^{-1} for the 1.27 cm path length. The 0.95 cm panel crashed and was the only treatment not following the trend. It would be expected to have peak production above 30 g m^{-2} day^{-1} once better optimized for a more stabilized system. Overall, these results are in general supportive of the fact that decreasing the path length of a given system could potentially produce gains in photosynthetic

efficiency (PE) and areal productivity as well as significant gains in volumetric productivity as well. Volumetric productivity cannot be overlook in this system because the energetic and capital investments into the system increase significantly with

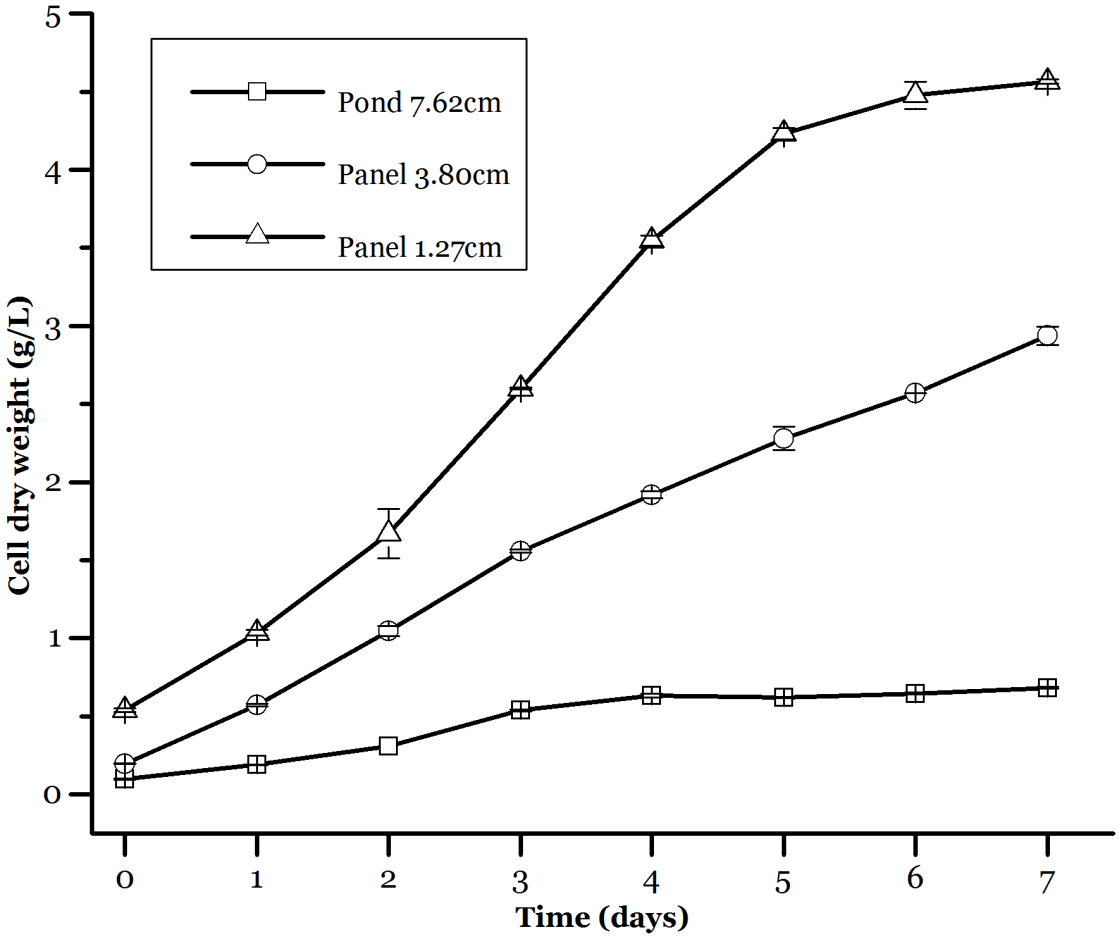

Figure 2. 16: Cell dry weight (g/L) during seven days of growth of *Scenedesmus acutus* LRB-AZ-0414 on modified BG-11 media in 7.62 cm pond, 3.80 cm panel, and 1.27 cm panel. Vertical bars denote standard error.

increasing culture volume. In example, thicker gauge metal and thicker acrylic need to be used to support the weight of the water in larger tanks as compared to thinner tanks. Also culturing in a 0.95 cm reactor as compared to a 7.62 cm pond reduces water usage 8 fold and transitioning from a 3.8 cm panel to a 1.27 cm or 0.95 cm panel offers a 3-fold to four-fold reduction in culture volume and water usage as well as aeration costs.

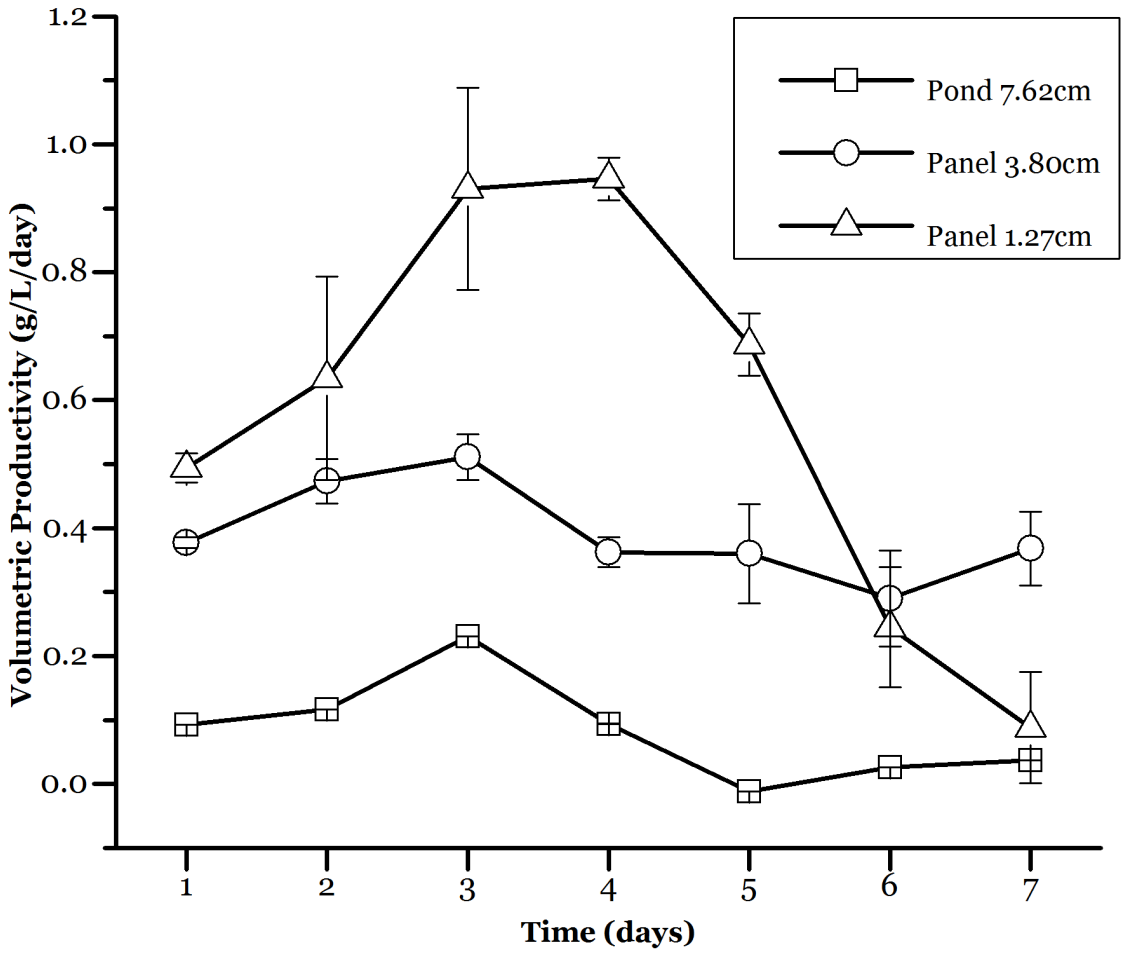

Figure 2. 17: Volumetric productivity (g/L/day) during seven days of growth of *Scenedesmus acutus* LRB-AZ-0414 on modified BG-11 media in 7.62 cm pond, 3.80 cm panel, and 1.27 cm panel. Vertical bars denote standard error.

2.4.3 Experiment 2.2: Nitrogen deplete growth of LRB-AZ-0414 in pond vs. flat panel

Experiment 2.1 was conducted in April 2014 using full strength BG-11 to show maximum log growth rates for biomass with sufficient nitrogen. Experiment 2.2 in September 2014 used the same conditions as experiment 2.1 except for dropping the initial nitrogen concentration to ¼ of the full BG-11 and removing the 0.95 cm panel from the study. Using the 3.8 cm panels, strain LRB-AZ-0414 typically grows from 0.25 g/L inoculation density to 3.5 -4.0 g/L biomass with 48-50% total lipid by dry weight in

10 days (unpublished data). This is consistent with the growth curve as shown in Figure 2.16 below. Notice the 1.27 cm panel has already reached stationary phase by day 7. This is also evident in the volumetric productivity as shown in Figure 2.17. The 7.62 cm pond and 3.8 cm panel seem to have somewhat consistent growth rates during the course of the study and the 1.27 cm panel peaks and the daily productivity decreases. A two-tailed student t-test revealed volumetric productivities of all treatments were statistically different for the first five days of production: 7.62 cm pond vs 3.8 panel ($p \leq 0.01$), 7.62 cm pond vs. 1.27 cm panel ($p \leq 0.05$), and 3.8 cm vs. 1.27 cm panels ($p \leq 0.1$).

Areal productivity results also showed statistical significance between the pond and panel but not between the two panels: 7.62 cm pond vs 3.8 panel ($p \leq 0.05$) and 7.62 cm pond vs. 1.27 cm panel ($p \leq 0.05$). The 1.27 cm panel used the available nitrogen reserves rapidly, which made a quick spike of productivity and not enough data points to show statistically significant results for areal productivity. Future studies will need to collect data points for growth throughout the day and/or increase the available nitrogen concentration to extend the growth over more days. Table 2.2 summaries the areal productivities for the given path lengths over the course of the first four days of the study, upon which the 1.27 cm panel starts to enter early stationary phase after using up the available nutrients that contributed to a lower average productivity than the 3.8 cm panel. The available nitrogen 60 mg $N\text{-}NO_3/L$ was apparently insufficient to maximize areal production in the study. The experiments of this study used the same volumetric concentration of nutrients (60 g $N\text{-}NO_3/L$), not areal dosing of nutrients, which is something to consider for future studies, as each would offer unique growth information.

2.4.4 Experiment 2.3: Nitrogen replete growth of LRB-AZ-0424 in flat panel with decreasing path length

Experiment 2.3 demonstrates similar results with the LRB-AZ-0424 strain. Figure 2. 18 below displays the cell dry weight (g/L) during the 13 days of growth of *Scenedesmus acutus* LRB-AZ-0424 on modified full strength BG-11 media in duplicate

Table 2. 2: Areal productivity and photosynthetic efficiency for four days of growth of LRB-AZ-0414 strain on BG-11 in different light path lengths. A traditional raceway pond of operated at 7.62 cm depth and three different acrylic flat panel photobioreactors with path length of 3.8 cm, 1.27 cm and 0.95 cm were used. Assuming biomass with 6.49 ±0.49 kWh kg^{-1} energetic content, PAR is 42% of total isolation and an average total insolation of 4.98±1.56 kWh $m^{-2}day^{-1}$as taken from AzMET database.

Path length:		**Areal productivity:**		**Photosynthetic efficiency:**	
		g m^{-2} day^{-1}	kWh m^{-2} day^{-1}	% of Total	% of PAR
7.62 cm-Pond	AVG	8.57±1.93	0.06±0.01	1.12±0.25	2.67±0.60
	MAX	11.18± 1.84	0.07±0.01	1.46±0.24	3.48±0.57
3.8 cm-Panel	AVG	15.78±2.66	0.10±0.02	2.06±0.35	4.90±0.83
	MAX	18.72±2.69	0.12±0.02	2.44±0.35	5.81±0.83
1.27 cm-Panel	AVG	11.56±3.44	0.08±0.02	1.51±0.45	3.60±1.07
	MAX	14.84±2.54	0.10±0.02	1.94±0.33	4.62±0.79

for 3.80 cm panel, and 1.27 cm panel, 0.95 cm panel. The starting cell density was adjusted slightly higher than previous experiments because it was conducted during the end of June in Phoenix with intense sun. However, the starting density was slightly off target for the 1.27 cm and 0.95 cm panels due the small volumes of the tanks and/or mixing of the inoculum as it was placed in the tanks. With the addition of the external airlift cooling loops these thin tanks, the circulation and cooling worked better for this study than in previous studies, however a couple tanks did lose some biomass during the

last two days of the experiment. A two-tailed student t-test revealed statistically significant results for 3.8 cm vs 1.27 ($p \leq 0.05$) and 3.8 vs 0.95 ($p \leq 0.05$) but not for 1.27 vs 0.95 panel and no significant difference for areal productivities between the panels.

The areal productivities, as shown in Table 2.3 below, peaked during the first four days of production. The four-day average and max yields were again inversely proportional to the path length of the panels with maximum productivity at 26.76 ±0.65 g m^{-2} day^{-1} and 5.39 ±0.13% photosynthetic efficiency (based on PAR) for the 0.95 panel.

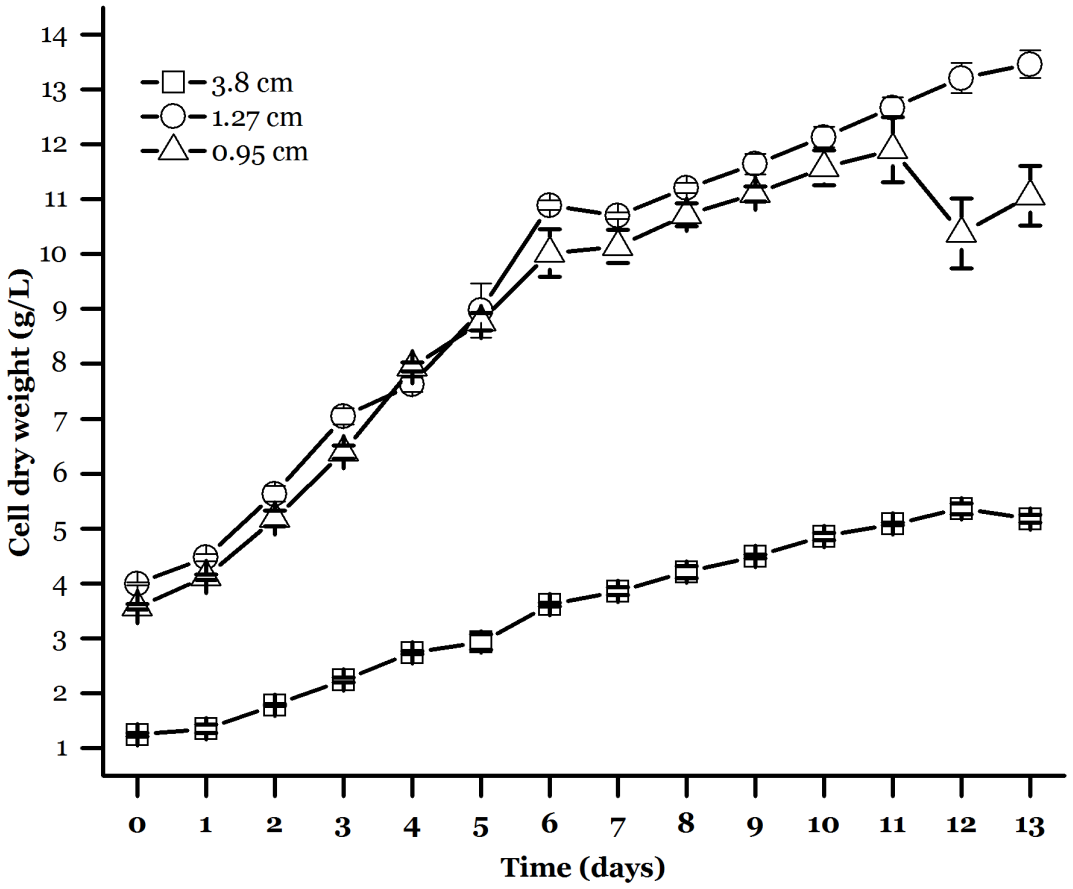

Figure 2. 18: Cell dry weight (g/L) during thirteen days of growth of *Scenedesmus acutus* LRB-AZ-0424 on modified BG-11 media in duplicate for 3.80 cm panel, and 1.27 cm panel, 0.95 cm panel. Vertical bars denote standard error.

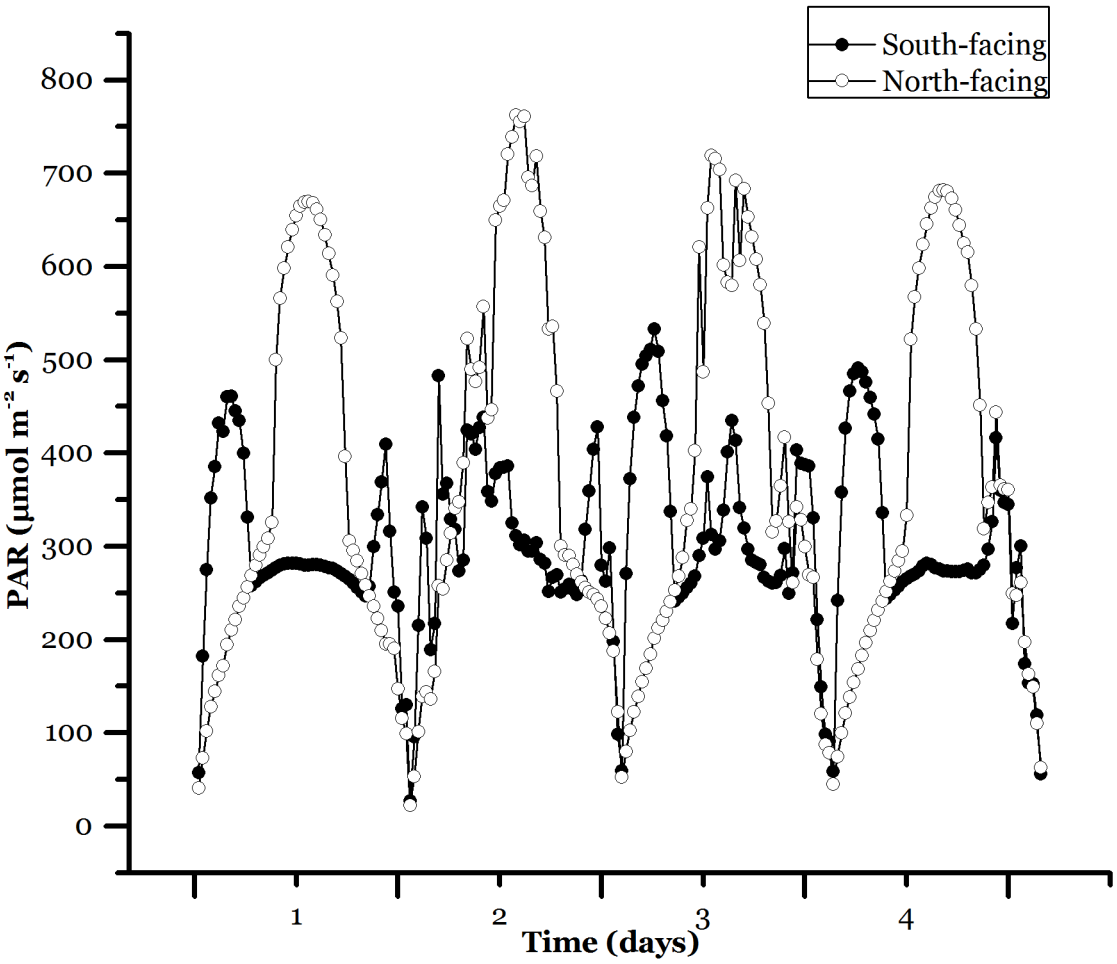

Figure 2. 19: Photosynthetic active radiation (PAR) µmol m^{-2} s^{-1} from the North and South facing side of the flat panel photobioreactor array cultivating strain LRB-AZ-0424 during June 26th to July 2nd.

This is promising considering the panels, arranged vertically were only able to capture about 30 (integral of area under Figure 2.19) of the almost 60 mol photons (from AzMET data) delivered each day. The PAR sensors were attached to the north and south-facing sides of the reactors to create the graph as displayed in Figure 2.19. This pattern is typical for the summer months when the sun passes overhead, coming at a southeastern angle in the morning, passing overhead and the intense afternoon sun coming in from a northwest angle to the flat panel array. A different pattern exists for the winter months when the sun comes mainly from the south and does not directly radiate on the north

face. This data demonstrates a disadvantage of vertical oriented systems that capture much less sunlight than horizontal systems. The cooling load may be somewhat reduced for summer months as compared to a horizontal system but at the expense of missing about half the incoming light. To better understand the energetics of producing these

Table 2. 3: Areal productivity and photosynthetic efficiency averages over four-day growth period for *Scenedesmus acutus* strain LRB-AZ-0424. Average total insolation was 7.55 ±0.24 kWh m^2 day^{-1} from AzMET data. PAR is assumed to be 42% of total insolation.

Path length		**Areal productivity**		**Photosynthetic efficiency**	
		g m^{-2} day^{-1}	kWh m^{-2} day^{-1}	**% of Total**	**% of PAR**
3.8 cm	AVG	14.19 ±3.46	0.09 ±0.02	1.20 ±0.29	2.86 ±0.70
	MAX	19.01 ±1.29	0.12±0.01	1.61 ±0.11	3.83 ±0.26
1.27 cm	AVG	14.79 ±2.53	0.2 ±0.05	1.25 ±0.21	2.98 ±0.51
	MAX	22.15 ±1.50	0.29 ±0.03	1.87 ±0.13	4.46 ±0.30
0.95 cm	*AVG*	*15.04 ±0.93*	*0.2 ±0.03*	*1.27 ±0.08*	*3.03 ±0.19*
	MAX	26.76 ±0.65	0.27 ±0.01	2.26 ±0.05	5.39 ±0.13

algae strains which may one day be utilized as an energy crop, bomb calorimetry was used to determine the heat of combustion of algae samples in log and nutrient limited phase for strain LRB-AZ-0414. The values were in line with other literature values for oleaginous green algae at -23.38±1.75 MJ kg^{-1} for green log growing culture (Schlagermann et al. 2012). The value was slightly higher for high oil algae, which is not surprising given the energetic content of oil. This value is slightly higher than previous assumptions used for calculations involving this strain (Eustance 2015). This is fortunate considering the high energy requirement (E_{areal}) for this acrylic flat panel array previously calculated at between 1.08 to 1.28 kWh m^{-2} d^{-1} depending on the season (Badvipour 2015).

In order to break even energetically using this flat panel array there would have to be a 4-fold increase in the best yields seen in this study or this energy requirement

Table 2. 4: Heat of combustion of LRB-AZ-0414 and LRB-AZ-0424 strains in exponential and stressed states in both MJ kg^{-1} and kWh kg^{-1}.

Strain	Physiological state of biomass	Heat of combustion (MJ/kg)	
		MJ kg^{-1}	kWh kg^{-1}
LRB-AZ-0414	log	-23.38±1.75	-6.50±0.49
LRB-AZ-0414	nutrient limited	-25.63±2.16	-7.12±0.60
LRB-AZ-0424	nutrient limited	-25.36±0.02	-7.05±0.00

would have to be greatly reduced. For instance, reducing to path length from a 3.8 panel to a 0.95 cm would be a 4-fold reduction in water usage while simultaneously cutting aeration costs by 75% since there is 4 times less volume to aerate. Aeration and chilling were the biggest energetic costs for the acrylic array with the highest coming in the middle of summer at E_{aerate} = 0.71 kWh m^{-2} d^{-1} and E_{chill}=0.57 kWh m^{-2} d^{-1} for a total E_{areal}=1.28 kWh m^{-2} d^{-1}. A four-fold reduction in E_{aerate} would reduce E_{areal} by 42% to 0.75 kWh m^{-2} d^{-1} which is still and order of magnitude larger E_{areal} for raceways coming in at just 0.051 kWh m^{-2} d^{-1} for 7.5 cm ponds (Badvipour 2015). Combined with other methods like reduction of aeration at night that could easily reduce energy required for aeration another 45% to 0.097 kWh m^{-2} d^{-1} which is much more in line with value for open raceways. Efforts would then be better focused on reduction of E_{chill}.

2.4.5 Experiment 2.4: Temperature gradient for LRB-AZ-0414 and LRB-AZ-0424

A promising way to save on chilling cost is to utilize strains better adapted to higher temperature. Experiment 2.4 was conducted using a temperature gradient apparatus constructed for finding the ideal temperature for a given algae quickly. Both

strains of *Scenedesmus acutus* were subjected to a number of temperatures as shown in Figure 2.20 and 2.21 below to determine the optimum temperature for each strain. The cultures were put on the temperature gradient plate and aerated with 1% CO_2 in an Erlenmeyer flask while being sampled for 3 days to track OD750 to monitor growth. LRB-AZ-0414 had best growth performance in the temperature range of 22.65°C to 24.6°C with tolerance up to 26.6°C; however, performance of the culture dropped significantly above this point. LRB-AZ-0424 had a wider temperature range 20.95°C to

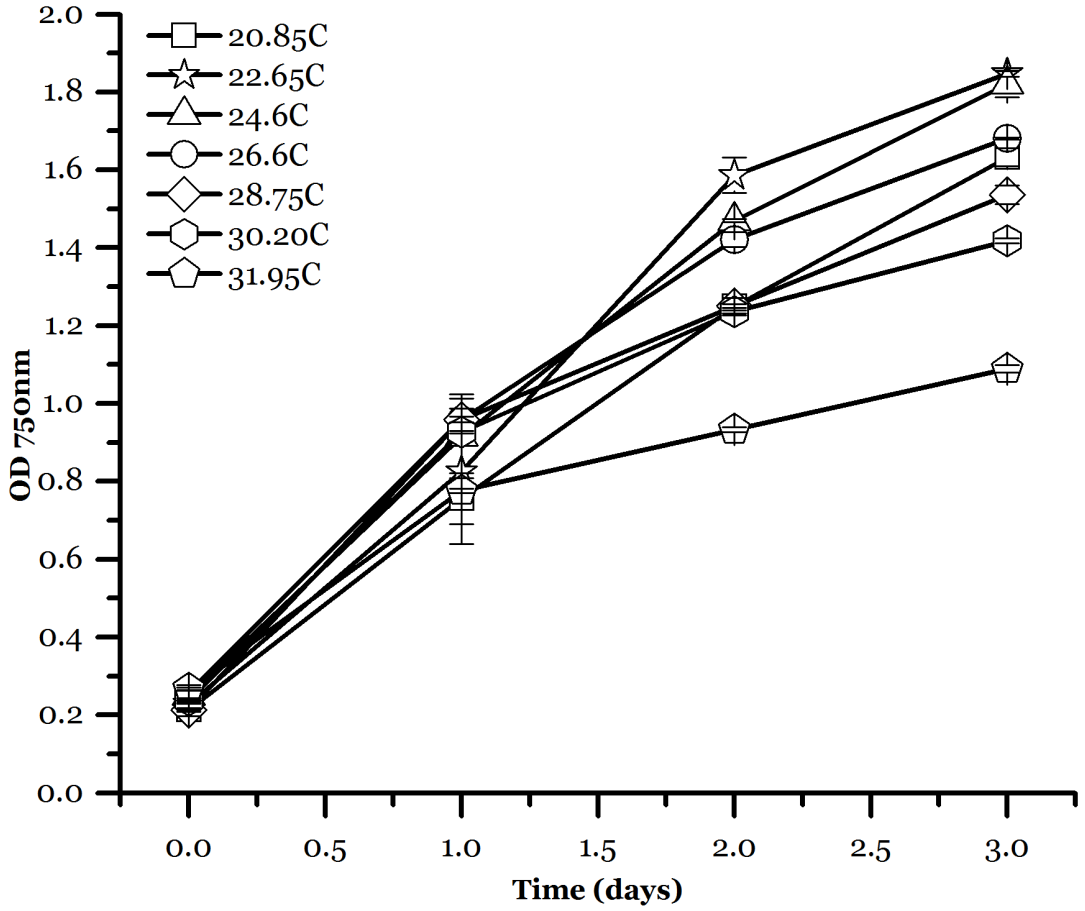

Figure 2. 20: Optical density measurements at 750 nm demonstrating growth of *Scenedesmus acutus* strain LRB-AZ-0414 on BG-11 media across a temperature gradient. Vertical bars denote standard error.

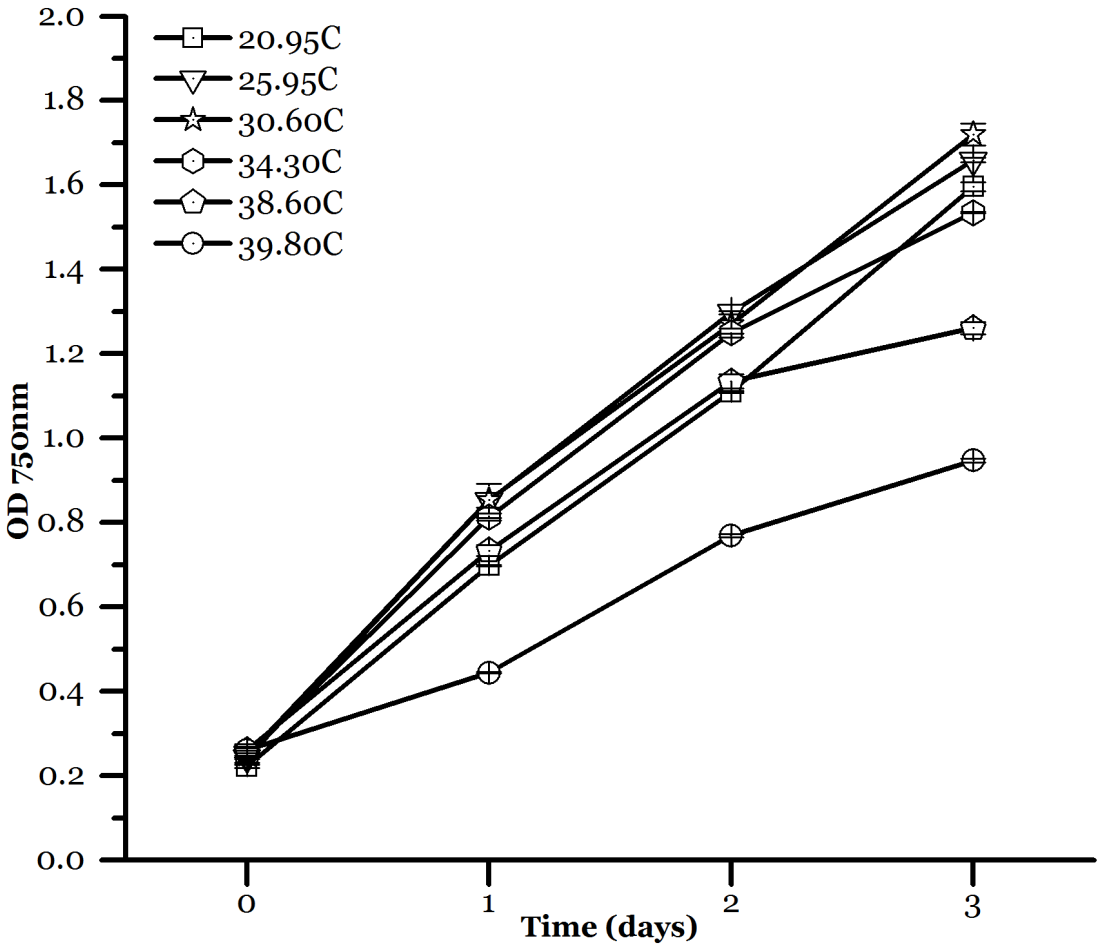

Figure 2. 21: Optical density measurements at 750 nm demonstrating growth of *Scenedesmus acutus* strain LRB-AZ-0424 on BG-11 media across a temperature gradient.

34.3°C with optimum temperature around 30.6°C. Having two similar strains with different temperature requirements is promising for providing year-round production as a crop.

These results resonate with other literature values for optimal temperatures for growing this alga. A strain identified in the literature as *Scenedesmus acutus* was found to grow best at 30°C and up to 40°C, however the relative amount of poly-unsaturated

fatty acids dropped significantly with higher temperature (El-Sheekh et al. 2017). This is similar in results to the LRB-AZ-0424 strain, which demonstrates a much larger temperature range. Another strain identified as *Scenedesmus acutus* shares a temperature range similar to LRB-AZ-0414, with an optimal temperature range between 23-25°C (Doria et al. 2011). The stain also produces similar lipid content to LRB-AZ-0414, with almost 50% lipid dry weight, most of which is oleic acid (Damiani et al. 2013).

With the potential to produce protein(45%), carbohydrates (30%), or lipids (50%) and shown in (Eustance 2015) while also growing well on dairy waste strains like LRB-AZ-0414 and LRB-AZ-0424 have been discussed in a recent review for use as a dairy ration (Badvipour et al. 2016). Having access to strains that can perform well in different seasons is critical to lowering chilling costs or to achieve maximum productivity when using raceways and other systems that rely on passive heating and cooling.

2.5 Conclusions

One major bottleneck limiting the commercial production of microalgae as an agricultural commodity is lack of an efficient and effective cultivation system. Knowing the biology of the crop being produced is critical to arriving at such a production system that can create the specific environmental conditions required for opitimal yields that lower the overall cost of production. Most commercial microalgae production systems in use today are traditional raceways that operate at depths where cells seldom see the light and spend most of the time in the dark. Despite the efficiency of an algal culture where all cells are photosynthetic, this obstacle is a major bottleneck for microalgae production. New, high-yielding production systems such as thin layer cascades and biofilms have one thing in common: they are all getting thinner, approaching the thickness of a leaf—a structure which all of agriculture relies upon. Designing and testing systems that operate

with the timescales of photosynthesis in mind is key to removing this bottleneck for the emerging algae industry.

This study has demonstrated the ability of two stains of *Scenedesmus acutus* (LRB-AZ-0414 and LRB-AZ-0424) to be cultured in narrow flat panel photobioreactor at high cell density and at differing temperature ranges for year-round production of biomass in the desert Southwest. The data collected demonstrates that decreasing the light path of a flat panel photobioreactor will accelerate growth towards the stationary phase and oil production while increasing both the volumetric and areal output of the algal cultures. Higher frequency of light-dark cycling that is more suitable for photosynthesis is experienced by algae cells within a thinner light path. This is consistent with the findings compiled on a recent review of a number of different algal production systems (Olivieri et al. 2014). This study also demonstrates that high-density algae cultivation has the potential to reduce aeration costs for flat panels four fold or more while reducing water requirements by the same factor.

The aerated, acrylic flat panel photobioreactors used in this study, while being great for research, are costly to operate and maintain. Future work should be focused on developing low-cost, high-yielding production systems that use passive heating and cooling and acknowledge the timescale of photosynthesis. Thin film bags placed horizontally have several attractive features for the development of a new thin culture system requiring minimal energy input and should be further investigated. Additionally, other low-cost, passive techniques of bringing light to the culture such as phosphorescent dyes capable of delivering visible light into the culture are promising. With the advent of simpler and more economical production systems designed with

photosynthesis in mind, this bottleneck to commercial algaculture will soon be eliminated.

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3. MANUSCRIPT 2: BIOREMEDIATION POTENTIAL OF *SCENEDESMUS ACUTUS* CULTURES GROWN OUTDOORS IN FLAT PANEL PHOTOBIOREACTORS

Manuscript Information Page

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3.1 Abstract

Scenedesmus acutus, a ubiquitous microalgae common to wastewaters of the desert Southwest was field tested on various waste nutrient streams including flue gas from a natural gas-fired boiler at a semiconductor wafer fabrication plant, digester centrate from a municipal wastewater treatment facility, and dairy lagoon water from a local dairy. Wastewaters were characterized in terms of macro and micronutrients for comparison to BG-11 laboratory culture media. The alga performed well on most wastewaters, and a comparison of the advantages of each is discussed in more detail. Overall, the alga showed promising growth on wastewaters containing ammonium when culture pH was maintained properly. The cultures demonstrated significantly higher growth on flue gas containing ~40 ppm NO_x as compared to commercial grade CO_2. Based on these findings, recommendations on best nutrient utilization strategies for each given waste stream were made to maximize the bioremediation potential of this alga.

3.2 Introduction

Algae, both microscopic and macroscopic, are responsible for producing the oxygen in every other breath we breathe on Earth. In fact, researchers estimate that microscopic algae alone are responsible for more than 99% of the total photosynthetic activity in the world's oceans (Field et al. 1998), even though planktonic algae are on average a mere 0.8% of the average areal concentration of terrestrial plant life (O. Pulz 1998). They are one of the most important and prolific groups of primary producers in food webs across the globe, and, as such, are great scavengers of waste or excess nutrients in both our air and waterways. In cases of minor nutrient pollution, algae are known to efficiently bioremediate waters and remove nitrates, ammonium, and phosphates from waters along with a number of trace metals including heavy metals and in general having a positive environmental impact. However, when sufficiently fertilized

by nutrients from agricultural run-off, waste from municipalities, or other anthropogenic sources, algae of all kinds blooms out of control, wreaking havoc on ecosystems. Certain algae capable of producing toxins can grow out of control killing fish and wildlife in the process and if left unchecked result in huge dead zones.

3.2.1 Harnessing the bioremediation potential of algae

With such capability for removing nutrient pollution, algae—microalgae in particular—have been studied for decades as a potential means of bioremediation of various wastewater streams. This includes agricultural waste streams from field run-off or confined animal feeding operations (CAFO's) that release large numbers of nutrients into the environment(Joshi and Wang 2018; Hou et al. 2015), much of which in the United States ends up in the Mississippi River Delta and the Gulf of Mexico creating vast dead zones and sometimes toxic algae blooms(Flood and Burkholder 2018). Other major watersheds have been plagued by the same issue including the Great Lakes(Soontiens et al. 2018), Lake Okeechobee in Florida(J. Kramer et al. 2018), Lake Baikal in Russia(Medvezhonkova et al. 2018), Chao Lake in China(Villa et al. 2012), and many other water bodies in mainly industrialized nations of the world. While the bioremediation potential of algae can wreak havoc on the environments if left unchecked, if harnessed properly, algae can make a powerful bioremediation option for industries producing major nutrient pollution streams if the algae biomass can be captured and utilized directly instead of decaying on a mass scale in our waterways and further degrading these ecosystems.

The literature has been primarily focused on treatment of high throughput, high nutrient wastewater sources like those coming from municipalities. To keep maintenance and operational costs low, traditional raceways ponds with relatively low

operational costs are the main systems used to date. Due to the low productivity of such raceway systems, it would take a vast area to treat even a small municipality. This required scale makes such systems cost prohibitive and algae has come to be known as means of only tertiary treatment or polishing of wastewater streams in most cases. Flocculating bacterial suspensions requiring large volumes of aeration (and a lot of energy) have proven the most efficient for removing bulk, partially digested organic matter from a relatively high-throughput water stream. Wastewater sources high in organic matter also tend to increase water turbidity and reduce light penetration into the water column, further reducing productivity. Also, there is the aspect of capturing volatile nutrients like CO_2 and ammonia, and finally how to cost effectively capture and remove the algae biomass prior to discharging the purified water. Nevertheless, the current means of wastewater treatment adopted by many municipalities is costly and not applicable for many other industries such as agriculture, which creates vast amounts of nutrient pollution that is mainly left to native algae in waterways to treat.

3.2.2 Purpose of this work

In order to take full advantage of the bioremediation potential of microalgae, one must consider all three of the major roadblocks to commercial algaculture, namely the development of a cost-effective production system, harvesting process, and a means of nutrient capture and delivery for volatile nutrients such as CO_2 and ammonium. The third roadblock is the focus of this work. Many publications have focused on various aspects of this work including CO_2 capture from flue gas(Ekendahl et al. 2018), NO_x uptake from emissions (Radmann et al. 2011), or nutrient uptake from various wastewater streams. However, few studies have collected data on all of these topics for a single alga species. This work attempts to show the diverse bioremediation potential of

Scenedesmus acutus while laying the groundwork for future studies, including the challenges of efficiently capturing each of these waste nutrient streams.

3.3 Materials and Methods

3.3.1 *Scenedesmus acutus* strains

Scenedesmus acutus, LRB-AZ-0414 and was utilized for all experiments. The strains were isolated on BG-11 agar plates and maintained on monoalgal BG-11 agar plates in the laboratory. All experiments used cultures that had been acclimated to grow outdoors in the desert southwest. This was to identify any acclimatization effects experienced by the cultures when changing the environment from an indoor setting with fluorescent lighting to an outdoor setting with full spectrum, full intensity sunlight and large temperature swings. This was accomplished by scaling the cultures in the lab (using round 800 mL glass tubes D=4.6cm, L=61cm) and then transferring into two acrylic vertical flat panels in the outdoor photobioreactor array. These two stock panels were maintained on the modified BG-11 culture medium described below and were used as inoculum for all experiments.

3.3.2 Modified BG-11 culture medium

A modified version of the standard laboratory BG-11 culture medium consisting of one fourth of the nitrogen (375 ppm $NaNO_3$) and no additional magnesium sulfate, calcium chloride, or sodium bicarbonate was utilized for outdoor experiments conducted in batch mode for oil production. The only exception was for cultures in cultivated in continuous log mode that received 1500 ppm NaNO3 the full amount of nitrogen in BG-11 and cultures for the phosphate experiment defined in more detail below.

These changes were made because lowering the nitrogen concentration led to a faster stress response and the tap water utilized outside already has sufficient micronutrients to replace these ingredients. Analyzed tap water at ASU's AzCATI field site (33°18'14.2"N, 111°40'21.5"W) had an average hardness of 280 ppm as $CaCO_3$ with 180 ppm Na^+, 75 ppm Ca^{2+}, 70 ppm SO_4^{2-}, 24 ppm Mg^+, and 8 ppm K^+.

Final composition of the modified growth medium included 375 ppm $NaNO_3$ (Alfa Aesar), 9.58 ppm K_2HPO_4 (BDH), 5.24 ppm of Ammonium Ferric Citrate (Alfa Aesar), 1.31 ppm Citric Acid Monohydrate (Sigma-Aldrich), 0.71 ppm H_3BO_3 (Sigma-Aldrich), 0.445 ppm $MnCl_2{\cdot}4H_2O$ (Sigma-Aldrich), 0.10 ppm $Na_2MoO_4{\cdot}2H_2O$ (Sigma-Aldrich), 0.06 ppm $ZnSO_4{\cdot}7H_2O$ (Sigma-Aldrich), 0.02 ppm $CuSO_4{\cdot}5H_2O$ (Sigma-Aldrich), 0.1 ppm $Co(NO_3)_2{\cdot}6H_2O$ (Sigma-Aldrich). For Experiment 3.4 on phosphate using the BG-11 components above the levels of phosphate were reduced from 6.81 mg P/L to 3.41 (50%), 1.70 (25%), 0.85 (12.5%), and 0.43 (6.25%) while simultaneously lowering nitrate levels from 246 mg P/L to 61 (25%). The control was kept at full BG-11 nutrient levels for comparison.

3.3.3 Flat panel photobioreactor array

All high-density growth experiments conducted on the Arizona Center for Algae Technology and Innovation (AzCATI) outdoor field site in Mesa, AZ utilized a north-south facing array of 48 vertical acrylic flat panel photobioreactors.

3.3.3.1 Acrylic flat panel photobioreactors

The flat panel photobioreactors were constructed of 13 mm thick acrylic sheets. They were aligned in four rows of twelve panels with sufficient spacing to minimize

shading (~1.2 m). Tank dimensions are 1.17m by 1.17m by 3.8cm giving an internal volume (accounting for flex of acrylic) of 60 L and a working volume (accounting for aeration volume) of 50 L.

3.3.3.2 Mobile flat panel array

A mobile flat panel array containing three 1.17m by 1.17 m by 3.8 cm tanks was spaced at approximately 1.17 m was used for growth tests on boiler flue gas from a local semiconductor wafer fab plant. The array was placed on the rooftop via crane and connected directly to the flue stack of the boilers. The mobile unit has its own cooling 1/3 HP Arctica chiller (JBJ Chillers), circulation pump (Little Giant) and a model LA-120 air pump (Medo). An acrylic dewatering box was used to remove excess water from the flue gas and a flow meter was set at 1.5% CO_2 during the duration of the experiments.

3.3.3.3 NO_x capture column

A column 4.57 m high was constructed from 4 cm ID polycarbonate tubing and PVC fittings. The column consisted of two 4 cm tubes connected by PVC fittings at the top and bottom such that, once filled with water, one column could be airlifted into the other to provide a counterflow of water to keep NO_x bubbles coming up the other tube down in the solution longer as to extend contact time with the given media. This design was adapted from Nagase et al. (1997). Air for the counterflow was supplied at 0.5 vvm. A similar horizontal column was also tested.

3.3.3.4 Aeration blower and CO_2 dewar

Aeration was provided via 13mm PVC pipe with 0.8mm holes drilled every 3.8 cm across the bottom of the PVC pipe in a staggered arrangement to promote mixing.

This supplied the cultures with 0.5 vvm aeration. Commercial grade carbon dioxide (CO_2) was obtained from Praxair dewar located on site and plumbed into the photobioreactor array. The CO_2 was fed into the inlet of a 2 H.P. Sweetwater blower at maintain at a feed rate of 1.5% CO_2 from sunrise to sunset daily.

3.3.3.5 Cooling tower and reservoir

The tanks were cooled by placing 13mm stainless steel cooling loops into tanks and circulating cooling water from a 2000L underground reservoir cooled using two GPC-25 Glacier evaporative pool coolers set on a Johnson Controls thermostat to regulate the reservoir temperature to 24±3°C for the LRB-AZ-0414 strain and 29±3°C for the LRB-AZ-0424 strain. A 2 HP 3 phase pool pump circulated chilled reservoir water during the day to regulate culture temperature in the photobioreactor array.

3.3.4 Culture monitoring

The cultures were monitored with a Neptune apex datalogger from Neptune Systems, LLC to track changes in temperature and pH of the culture in the tanks. While this system is capable of controlling in addition to monitoring, the required equipment was not available for the course of these experiments. The system did however serve as a redundant monitoring system capable of sending SMS alerts if tank pH or temperature get out of range.. A Li-1400 datalogger (Li-COR Biosciences) was used to monitor incoming PAR from each side of the tanks in the array. For the experiments conducted at the semiconductor wafer fab plant, a CR-1000 datalogger from Campbell Scientific, Inc. was used to track culture pH and temperature during the course of the experiments conducted in the mobile flat panel tanks on the roof of the semiconductor wafer fab plant. A Dräger X-am 5600 portable gas monitoring unit was used to track CO_2, NO_x at

the wafer fab plant in the boiler flue gas. It was also used to track ammonia off-gassing from cultures grown on wastewater.

3.3.5 Nutrient Analysis

Nutrient analysis was conducted using a QuickChem 8500 Flow Injection system for analysis of nitrogen and phosphorous and a Thermo Scientific ICP-OES for analysis of trace elements.

3.3.5.1 Flow injection analysis

Nitrate, ammonia and phosphate data were analyzed using a QuickChem 8500 Flow Injection system. Standards and reagents were prepared as per manufacturer's recommendations and a standard curve with R-correlation value ≥ 0.95 was generated for each ion analyzed. The samples, collected in duplicate, were prepared by centrifugation at 1000g then subsequent filtering through 0.2 μm membrane filter to remove any remaining particles. The samples were diluted 1:7 with 18 MOhm filtered water to bring the concentration of all analytes within the realm of the standard curve. Samples were collected in 2mL centrifuge tubes (VWR) and centrifuged at 1000g to remove all suspended cells then preserved in cold room at 4°C until each experiment was complete and ready for analysis.

3.3.5.2 ICP-OES analysis

A Thermo Scientific ICP-OES was used to analyze trace elements. The reagents, standards, and standard curve for all analytes were generated using manufacturer's recommendations and a standard curve with R-correlation value ≥ 0.95 was generated for each ion analyzed. The samples were first filtered using a 0.2 micron cellulose nitrate

syringe filter diluted 1:10 with 18 MOhm filtered water while simultaneously acidifying to 2% w/v nitric acid. This was done to fully dissolve all metals and bring the concentration of all analytes within the realm of the standard curve.

3.3.5.3 Gravimetric Lipid Analysis

Lipid analysis was conducted using a Dionex Accelerated Solvent Extraction (ASE) 350 with a modified extraction protocol adopted from (C Bigognoa 2002). Using the ASE five mL extraction cells, 150 mg pre-weighed and freeze-dried algae samples were transferred onto the ASE and extracted with five min heated cycles using 10% DMSO in methanol and 1:1 diethyl ether and hexane. After the samples were completed 15 mL of MQ water was added along with inverting several times. Next samples were centrifuged at 1000 rpm for ten mins. Then add approximately two mL hexane-diethyl ether (1:1) and knock down any white emulsions with approximately two mL ethanol then centrifuge again at 1000 rpm for 10 min and repeat this process until all green color is gone (four or five times). Evaporate the 40 mL aliquots of hexane under purge air (N2) and use hexane to move lipid fraction to pre-weighed two mL microcentrifuge tube, dry down again with purge air and then freeze the samples in -80°C and freeze dry before determining total lipid weight for calculation.

3.3.6 Culture density assessment

A daily sampling regimen was undertaken to monitor biomass density via ash-free dry weight (AFDW) by filling one 50 mL centrifuge full from top of tank after top-off water was added from tap to account for evaporation.

3.3.6.1 Gravimetric analysis

Culture density was measured via gravimetric analysis using ash-free dry weight (AFDW) to track growth of the biomass over the course of each growth trial. Pre-weighted, pre-ashed (500°C for four hours) 1.2µm glass microfiber filters (VWR 696) were used in vacuum funnels to collect suspended algae cells in 5-20 mL cultures samples in duplicate. The samples of algae biomass collected on the filters wash subsequently washed to remove excess salt, bacteria, or other contaminates. The filters were then dried at 105°C for eight hours before transferring to a desiccator to cool to room temperature. The filters containing the dried samples were then weighted and moved to a muffle furnace held at 500 ± 25°C for four hours. The samples were transferred to the desiccator and allowed to cool to room temperature before recording the final weight of the remaining ash from the algae samples. All weights were recorded to 0.0001g precision and the weight of the filter and ash was subtracted from the total weight to determine the algae cell weight per sample volume (g/L). Ash content was determined by placing the algae sample in a pre-weight ceramic crucible, placing in muffle furnace held at 500 ± 25°C for four hours, then removing from oven, cooling and weighing the crucible plus ash.

3.3.7 Light Microscopy

Light microscope images of cells were obtained using an Olympus BH-2 light microscope with either 20X or 40X objectives and 10X ocular lenses.

3.3.8 Experiment 3.1: Batch growth on flue gas

The ability of strain LRB-AZ-0414 to grow on flue gas from a natural gas-fired boiler at a local semiconductor wafer fabrication facility was tested with a 14-day batch growth experiment using a mobile rack and support equipment (as described above) with three 1.17 m X 1.17 m flat panel acrylic tanks. Flue gas was mixed into an air line at 1.5% (v/v) delivering 0.5 vvm air for culture mixing and pH control to 8.0 ± 0.5 in modified BG-11 culture media (with ¼ nitrate or ~60 mg/L N-NO_3) . Pictures of the tanks were taken on day 0, 7, and 14 as were micrographs of the cultures to show the oil accumulation process, along with nitrate levels in the media and total lipid content of the biomass. Measurement of daily ash-free dry weight was used to track the growth of the cultures. It was hypothesized that strain LRB-AZ-0414 would show no significant difference in growth rate or lipid productivity for cultures grown in similar conditions on CO_2 and flue gas from a natural gas-powered fab plant boiler.

3.3.9 Experiment 3.2: Continuous growth on flue gas

Strain LRB-AZ-0414 was cultivated in continuous cultivation mode using modified BG-11 (with full nitrate or 246 mg/L N-NO_3) using the same experimental setup as in Experiment 3.1. Approximately 66% of the culture volume was harvested and replaced with fresh Bg-11 media every other day to test growth on boiler flue vs. commercial grade CO_2. Using a mobile rack and support equipment (as previously described) with four 1.17 m X 1.17 m flat panel acrylic tanks, flue gas and commercial grade CO_2 were mixed into two separate airlines, each supplying two tanks at 1.5% (v/v) CO_2 and delivering 0.5 vvm air for mixing and pH control to 8.0 ± 0.5. . The growth data was compared to local (Mesa) AzMET data (Azmet Weather Data 2018) to calculate

photosynthetic efficiencies assuming an energetic content of 6.49 ±0.49 kWh kg^{-1} energetic content for the biomass and that available PAR is 42% of incoming insolation.

3.3.10 Experiment 3.3: NO_x removal

To determine whether strain LRB-AZ-0414 was able to remove NO_x emissions from flue gas from a natural gas-fired boiler, a 4.57 m tall contact column was constructed (as previously described) and a 9.14 m long horizontal column with 1.0% slope was similarly constructed alongside. Simulated flue gas (containing ~40 ppm NO_x and 1.5% CO_2) was sparged directly into the water in order to test scrubbing or NO_x removal efficiency. The effect of column height on NO_x removal efficiency was tested using only tap water at 1.22 m and 4.57 m, followed by testing at 4.57 m for both 2.0 g/L culture of LRB-AZ-0414 in modified BG-11 media and also with a 5 mM solution $FeCl_3$ in tap water. The horizontal tube was tested using 5 mM Fe(III)EDTA. A simulated gas flow rate of 0.5 L/min was used for all tests with bubble size ranging from 2mm to 5 cm.

3.3.11 Experiment 3.4: Growth on wastewater

Strain LRB-AZ-0414 was tested on wastewater media created from local waste streams to evaluate its growth and lipid accumulation on the various wastewater media. Wastewater was collected from a number of sites including ananaerobic digester centrate and secondary effluent from municipal wastewater treatment plant as well as dairy lagoon waste. Available nutrients and trace metals were characterized via flow injection and ICP-OES analysis for comparison to levels in BG-11 medium. Based on these results, which inicated wildly varying nitrogen and phosphorous levels, a series of experiments were conducted on LRB-AZ-0414 to show growth and lipid accumlation on varying level of nitrogen and phosphate. First an experiment was conducted to explore the culture of

LRB-AZ-0414 on different N:P ratios and its effect on growth and lipid productivity. Then a second experiment was conducted to compare the growth, areal productivity as well as lipid and protein content of this strain when grown on different wastewater sources. Turbidity was characterized and was reduced by treatment with lime and dilution of the wastestreams.

3.4 Results and Discussion

3.4.1 Experiment 3.1: Batch growth on flue gas

The progression of Experiment 3.1, a 14-day batch experiment on strain LRB-AZ-0414 to evaluate growth performance on flue gas from a natural gas-fired boiler, is depicted on day 0, 7 and 14 in below Figures 3.1, Figure 3.2, and Figure 3.3, respectively. The evolution of change in both color and density is consistent with other studies involving this strain in a batch growth experiment with limited nitrogen (unpublished data). For LRB-AZ-0414, modified BG-ll media containing only 60 mg/L N-NO_3 is sufficient to increase cell density from approximately 0.5 g/L to 3.5 g/L in 10 days as shown in Figure 3.4 Below.

Cells exposed to nitrogen-limited conditions experience morphological and color changes as shown at day 0, 7, and 14 of growth on flue gas in Figures 3.5, 3.6, and 3.7 below, respectively. These are consistent with previous findings for this strain and based on the images collected, the use of flue gas in the cultivation of this strain appears to have not delterious effects on a macroscopic or microscopic level.

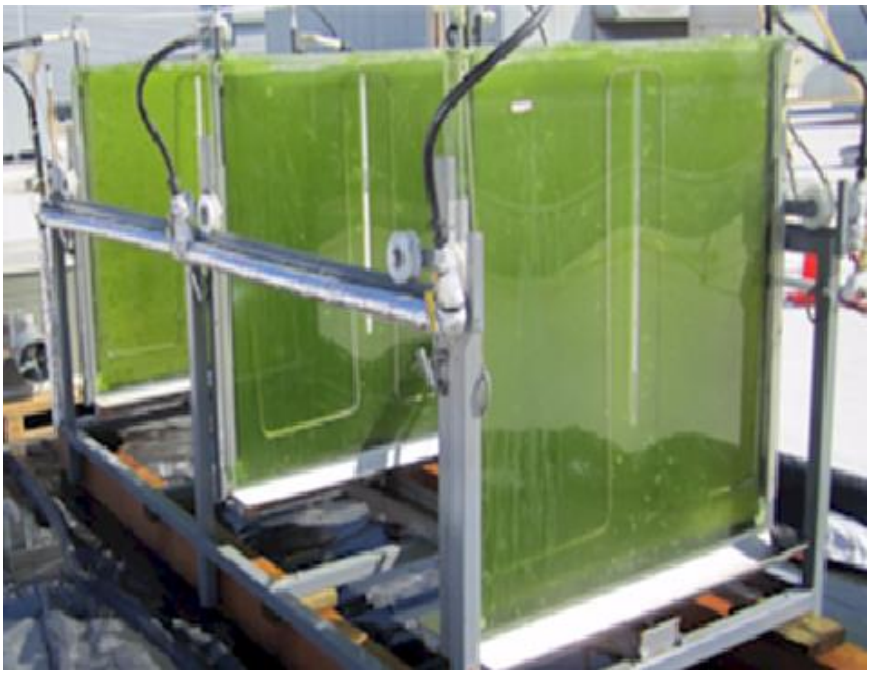

Figure 3. 1: Flat panel photobioreactors on a mobile rack growing *Scenedesmus acutus* through a batch growth experiment at Day 0 on flue gas from a natural gas-fired boiler.

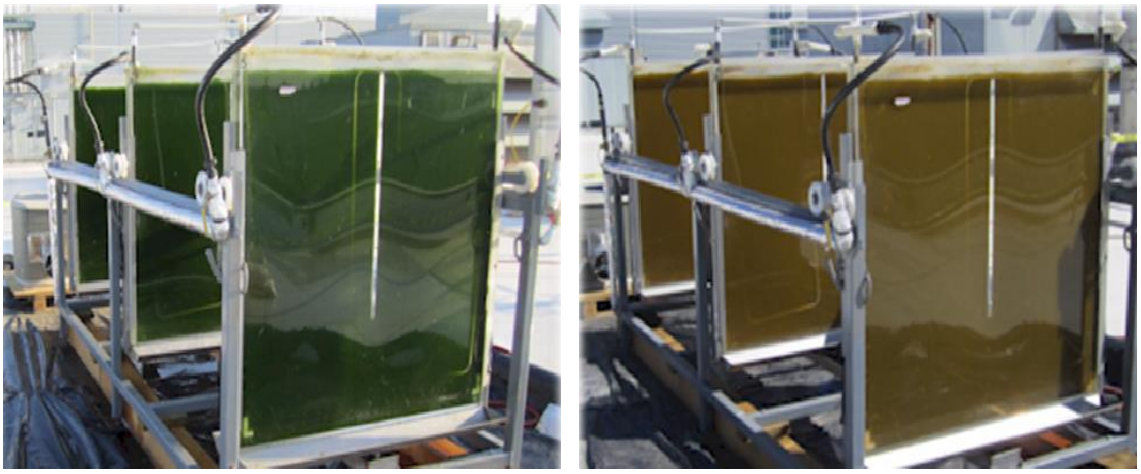

Figure 3. 2: Photo of tanks showing the accumulation of oil in *Scenedesmus acutus* through a batch growth experiment at Day 7 (left). -

Figure 3. 3: Photo of high oil *Scenedesmus acutus* strain LRB-AZ-0414 in tanks on grown using flue gas from a natural gas-fired boiler at Day 14 (right).

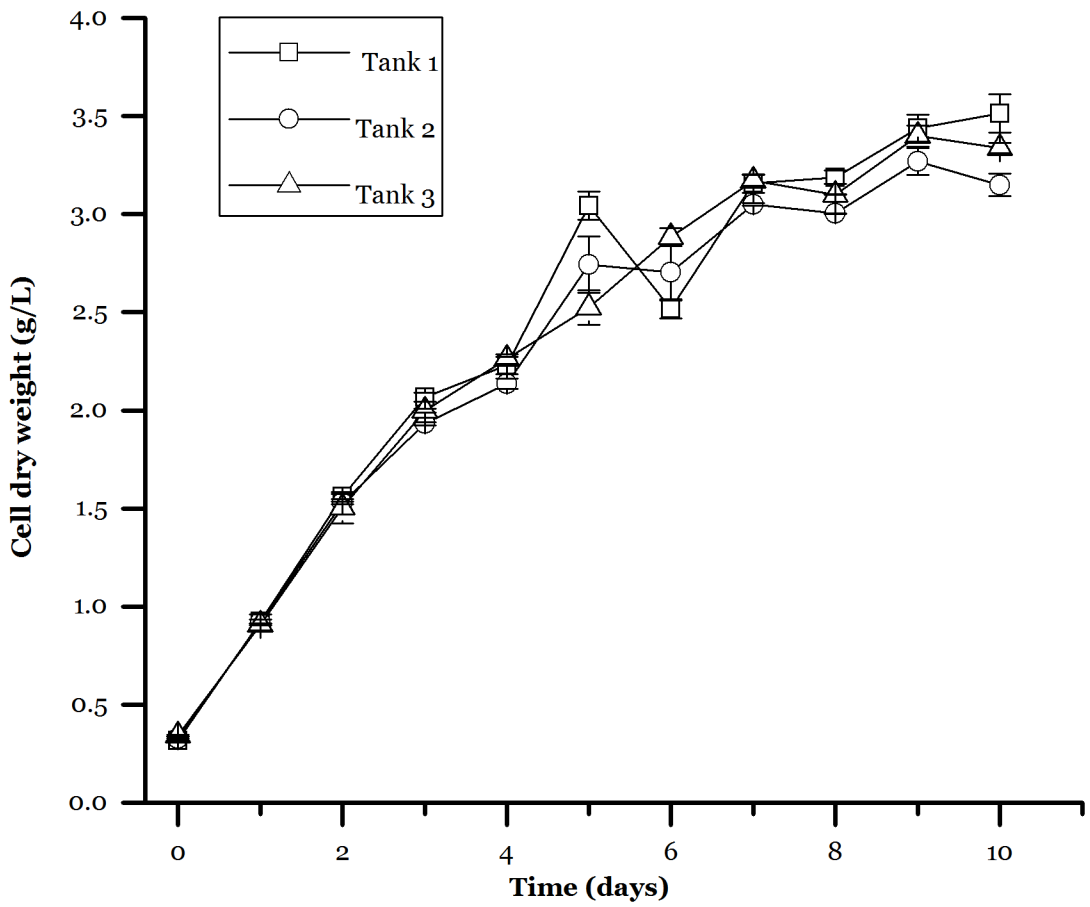

Figure 3. 4: Dry cell weight of *Scenedesmus acutus* throughout a batch growth experiment on flue gas from a natural gas-fired boiler. Vertical bars denote standard error.

The volumetric nitrate consumption for strain LRB-AZ-0414 in an active growing state has been shown in previous works to approach up to 40 mg/L N-NO_3/day when grown on BG-11 with full levels of nitrate (246 mg/L N-NO_3) (Eustance et al. 2015a). These results indicate a slightly higher initial rate of consumption approaching 60 mg/L N-NO_3/day for biomass grown in batch mode and entering a period of nitrogen starvation to induce lipid biosynthesis, as demonstrated by Figure 3.8 below.

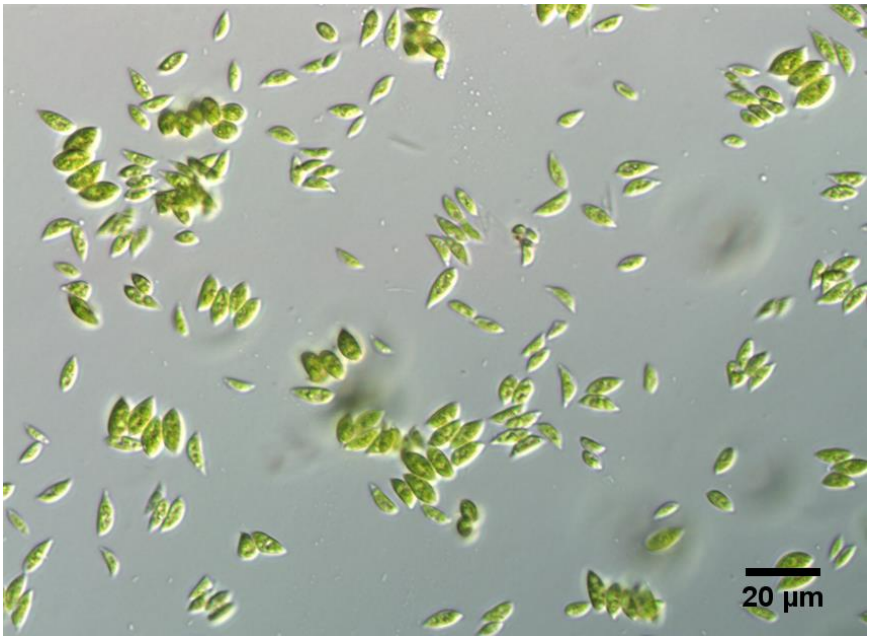

Figure 3. 5: *Scenedesmus acutus* strain LRB-AZ-0414 innoculum biomass at Day 0 of batch growth experiment on flue gas from a natural gas-fired boiler.

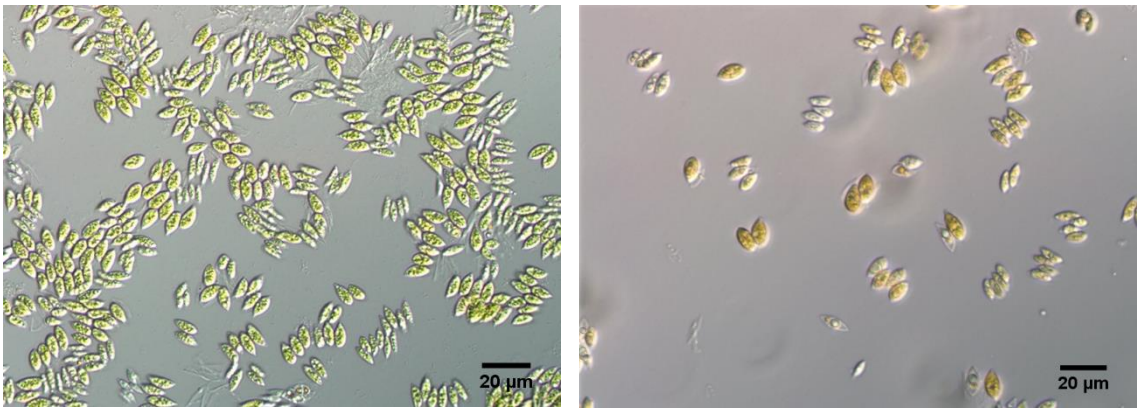

Figure 3. 6: Day 7 of the batch growth experiment on flue gas from a natural gas-fired boiler (left).

Figure 3. 7: Day 14 showing the accumulation of oil and morphological changes in the biomass cultivated on flue gas (right).

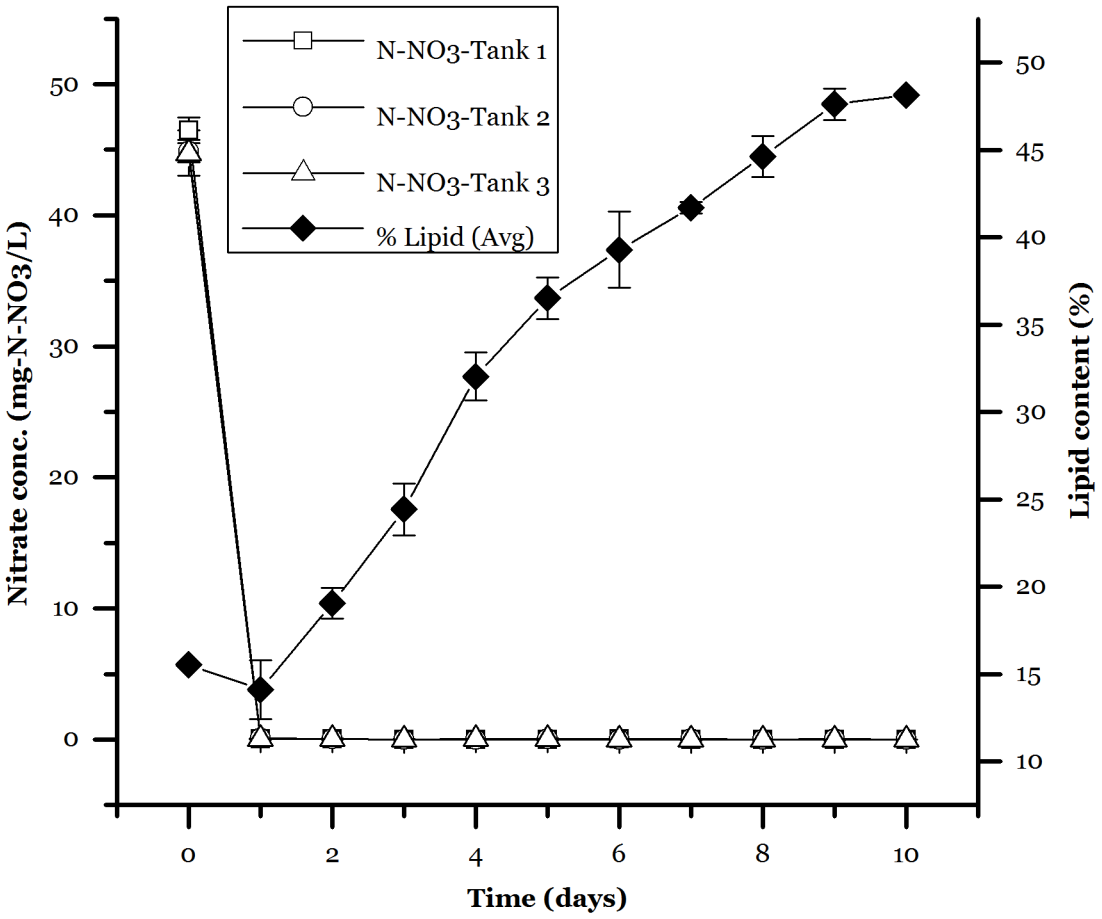

Figure 3. 8: Nitrate uptake and lipid accumulation of *Scenedesmus acutus* grown in batch mode on flue gas from a natural gas-fired boiler. Vertical bars denote standard error.

The nitrogen is depleted from all three tanks upon which the average lipid content drops slightly lower on the first day after which the nitrate in the culture media is completely depleted and the lipid content rises with the cell dry weight until reaching a maximum at day 10 at approximately 3.5 g/L dry weight while approaching 50% total lipid content. These results are consistent with LRB-AZ-0414 biomass cultivated in batch mode using commercial grade CO_2 in place of flue gas from previous studies on this organism. The average nitrogen consumption per gram biomass was determined to be 18.04±0.99 mg N/g biomass for batch cultivation mode in this study. This value is

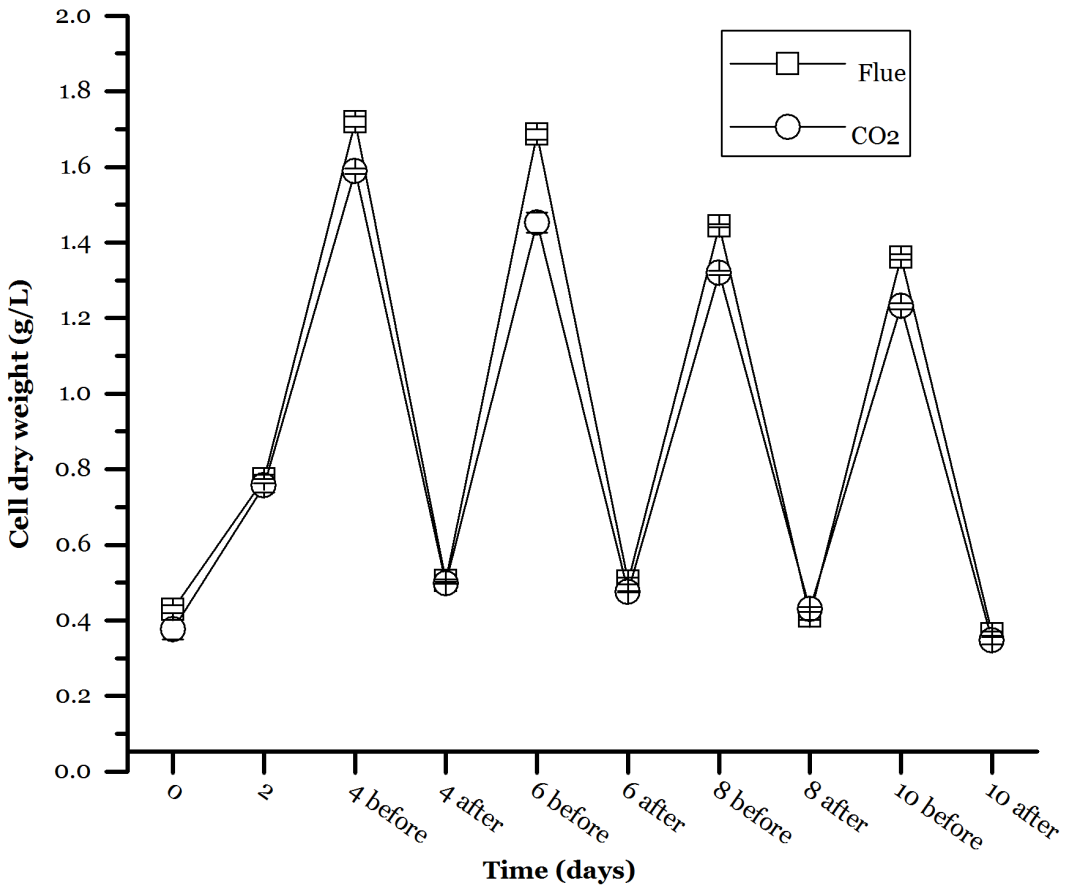

Figure 3. 9: Cell dry weight for *Scenedesmus acutus* biomass in continuous cultivation mode for ten days before and after harvesting 66% of culture volume every other day to determine maximum productivity. Vertical bars denote standard error.

much lower thanconsumption of 77 mg N/g biomass previously reported for this strain in continuous growth mode (Eustance 2015), but is consistent with biomass with extreme nitrogen deprivation.

3.4.2 Experiment 3.2: Continuous growth on flue gas

To test the continuous growth performance of the strain on flue gas vs commercial grade CO_2 an additional panel was added and two replicates were fed aeration air with 1.5% CO_2 from either a commercial grade CO_2 cylinder or flue gas from the stack. The cultures were cultivated on BG-11 and allowed to reach approximately 1.5

g/L cell dry weight density before harvesting 66% of the culture and replacing with fresh modifided BG-11 media. The cell dry weight allowed to reach above 1.5 g/L density and was first harvested on day 4 as depicted in Figure 3.9 above. Cell dry weight was monitored before and after harvest and harvested every other day thereafter for the duration of the 10 day cultivation period conducted in late August.

A two-tailed student t-test on the results shows a significant difference in volumetric productivity ($p \leq 0.1$) after the initial lag phase up until day 4 of the experiment. This is the point where the cell density first reached 1.5 g/L whish is an optimal production density for this strain in this 3.8 cm panel to maximize light utilization (unpublished data). This is also when the first harvest took place and volumetric productivity held between approximately 0.4 to 0.5 g/L/day for both treatments for the remainder of the experiment as shown in Figure 3.10 below. Other studies comparing growth of algae on flue gas in comparison to comercial grade CO2 have noted improvements to growth by using flue gas (John Burgess 2011). Emissions like SO_x from coal-fired power plants has been known to acidify the culture and kill many algae stains but other emissions such as NO_x adding nitrogen to the culture media to increase production when grown on flue gas as compared to just commercial-grade CO_2 alone. The natural gas fired boilers used in this study average 9.7% CO_2 in their emissions steams and 37 ppm NO_x with 0 ppm SO_x.

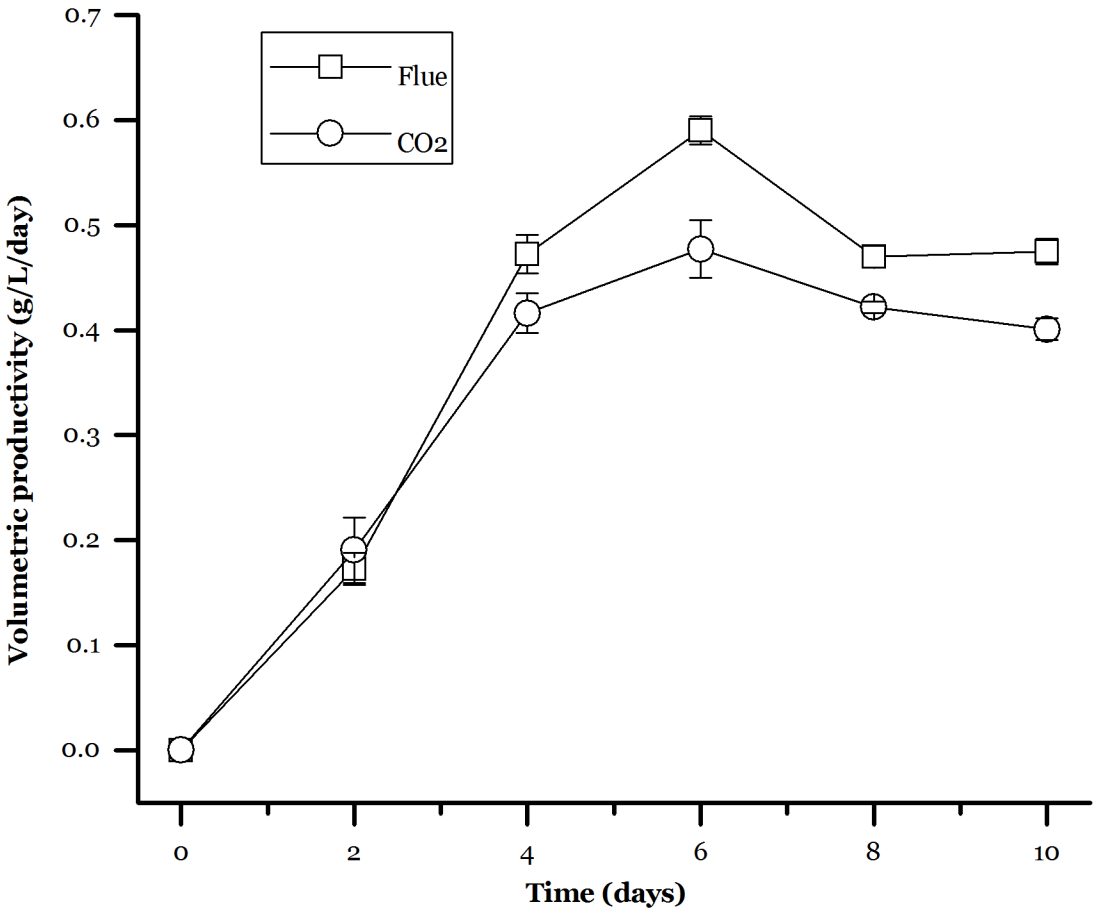

Figure 3. 10: Volumetric productivity of *Scenedesmus acutus* grown using CO_2 from both flue gas from a natural gas fired boiler and commercial grade CO_2. Vertical bars denote standard error.

The areal productivity and photosythetic efficiency based on total solar radiation from AzMET data at the nearby Mesa station also demonstrates slightly better performance of the cultures grown on flue gas in comparison to commercial grade CO2 which at their peak on day 6 averaged 21.62±0.49 g m^{-2} day^{-1} and 17.48±1.01 g m^{-2} day^{-1}, respectively. The photosynthetic efficiency based on photosynthetically active radiation (PAR) was 4.96±0.11 for the flue gas tanks on day 6, assuming PAR was 42% of total

Table 3. 1: Areal productivity and photosynthetic efficiency for strain LRB-AZ-0414 cultivated on boiler in continuous cultivation mode by removing 66% of culture volume every other day to achieve maximum biomass yields. AzMET data was used to calculate photosynthetic efficiencies assuming an energetic content of 6.49 ±0.49 kWh kg^{-1} energetic content for the biomass and that available PAR is 42% of incoming insolation.

	Areal Productivity				**Total Irradiance**		**Photosynthetic Efficiency**			
Day	g m^{-2} day^{-1}		kWh m^{-2} day^{-1}		kWh m^{-2} day^{-1}*		% Total		% PAR	
Flue										
2	6.32	±0.56	0.04	±0.00	6.28	±0.79	0.65	±0.06	1.56	±0.14
4	17.31	±0.67	0.11	±0.00	6.68	±0.11	1.68	±0.06	4.00	±0.15
6	21.62	±0.49	0.14	±0.00	6.74	±0.09	2.08	±0.05	4.96	±0.11
8	17.21	±0.38	0.11	±0.00	6.5	±0.09	1.72	±0.04	4.09	±0.09
10	17.39	±0.44	0.11	±0.00	5.89	±0.16	1.92	±0.05	4.57	±0.12
CO_2										
2	6.98	±1.14	0.05	±0.01	6.28	±0.79	0.72	±0.12	1.72	±0.28
4	15.24	±0.69	0.1	±0.00	6.68	±0.11	1.48	±0.07	3.53	±0.16
6	17.48	±1.01	0.11	±0.01	6.74	±0.09	1.68	±0.10	4.01	±0.23
8	15.45	±0.20	0.1	±0.00	6.5	±0.09	1.54	±0.02	3.67	±0.05
10	14.69	±0.37	0.1	±0.00	5.89	±0.16	1.62	±0.04	3.86	±0.10

*Based on AzMET data.

radiation. Also, with the addition of a fourth tank in Experiment 3.2 the panel spacing was reduced from approximately 1.37 m which is more than the height of the tank to minimize shading to 0.69 m which increase tank to tank shading and results in slightly lower productivity values. Also, the roof was coated with a white reflective layer that could have increased light delivery to the cultures slightly enhancing productivity. Nevertheless, record volumetric and areal productivites were observed for both experiments, demonstrating suitable growth and lipid productivity for strain LRB-AZ-0414 on flue gas from natural gas-fired boilers.

3.4.3 Experiment 3.3: NO_x removal

To further test the potential removal efficiency of NOx for a culture of strain LRB-AZ-0414 grown on modified BG-11 media, a 4.57 m contact column made of 4 cm polycarbonate tubing was errected to attempt to enhance the scrubbing capability of the culture, because the contact time of aeration bubbles spent in an acrylic flat panel with a culture depth of approximately 1.1 m was insufficient to remove NO_x fed in the simulated flue gas stream at 40 ppm concentration. Based off previous literature, 4.57 m was chosen as the colum height and ports were made to allow testing at 1.22 m. Counter flow was created by placing two columns side-by-side then airlift media from on tube to the other to create a continuously flowing loop of water. Excess air was removed at the top of the column and the simulated flue gas was injected into the column with a downward countercurrent flow to increase contact time for the bubbles to approximately 20 seconds at a sparge rate of approximately 0.1 vvm. The column was tested first on tap water alone at the two heights, then with stain LRB-AZ-0414 innoculated at approximately 2.0 g/L density in modified BG-11 media, and then 5 mM $FeCl_3$ in tap water as summarized in Table 3.2 below. Other conditions tested include bubble size and operation of the column in horizontal orientation.

Overall, the results indicate that a number of parameters increase removal efficiency of NO_x, including increasing contact time for the gas in the contact vessel, decreasing average bubble size, adding actively growing culture or adding 5 mM $FeCl_3$ to the column or 5mM Fe(III)EDTA. Increasing the retention time in the column by adding countercurrent flow and increasing the contact time to 20 sec (for 4.57 m) or 10 sec (for 1.22 m) as compared to 1-2 sec of contact time which is typical for the 1.17 m tall acrylic

Table 3. 2: NO_x removal efficiency from simulated flue gas with ~40ppm NO_x sparged into inlet of 4.57 m scrubber in various medias.

Bubble path (cm)	Contact vessel:	Media	Bubble Size (mm)	NO_x Removal Efficiency (%)
121.9	Vertical column	Tap water	10-15	3
121.9	Vertical column	Tap water	2-3	10
457.2	Vertical column	Tap water	2-3	52
457.2	Vertical column	Algae in BG-11 media	2-3	58
457.2	Vertical column	5mM $FeCl_3$ in tap water	2-3	100
457.2	Vertical column	5mM Fe(III)EDTA in tap water	2-3	93.3
914.4	Horizontal tube	1.8mM Fe(III)EDTA in tap water	20-50	40

production panel increased the removal efficiency from 3% (production panel) to 10% (tap water at 1.22 m) and 52% removal (tap water at 4.57 m). Adding $FeCl_3$ at 5mM concentration increased removal rate to 100% at 4.57 m. By decreasing average bubble size from 1-1.5 cm to 2-3 mm there was a 3-fold increase in NO_x removal efficiency for the column, however the approximate 3-fold increase in added inlet pressure required to accomplish this might be prove cost prohibitive for such large scale commercial applications with boilers requiring large air flows to operate.

For this reason, the horizontal tube is suggested for future studies involving NO_x removal via microalgae, reducing the pressure required for operation to only 1-2 psi, which is acheivable with a low pressure commercial blower connected to a flue stack as

Figure 3. 11: Flue gas stack with sampling port at local semiconductor wafer fabrication plant.

Figure 3. 12: NOx capture column 4.57 m in height capable of capturing 40ppm NOx from boiler flue gas stream.

shown in Figure 3.11 above. Previous research has suggested NO_x removal efficiency is a function of how well the NO_x becomes dissolved in the aqueous state as well as exposure to oxygen for efficient oxidation to occur (Nagase et al. 1997). Ultimately, NO_x has to be oxidized into a form algae can take up such as nitrate. More recent detailed studies involving NO_x capture with a bubble column have eulicidated the potential chemistry behind the use of iron in a bubble column, which identified FeO is a by-product and acid as a constant input to the system to adjust pH down (Lefan et al. 2012). The study, which utilized iron filing as an iron source, also suggests an optimum oxygen concentration for a given pH, temperature, and NO_x flow rate.

According to the results of the present study, decreasing bubble size or contact time directly impacts dissolution of NO_x into the aqueous phase. The Fe(III) likely helps

catalyze a photochemical oxidation of NO_x and EDTA chealate the oxidized Fe(III) to keep it from precipitating out of solution. Nagase et al. (1997) first described the approach of using counter flow to increase contact time as adopted by this study. The vertical setup seems logical, but the use of a column as shown in Figure 3.12 above may not prove economically feasible on commercial scale due to the high pressures required to overcome the water column. The shear volume of air flow required for proper boiler operation makes a longer horizontal tube, albeit less efficient, a more attractive option for scrubbing NO_x from industrial emissions.

Recommendations for future studies would involve injection of flue gas containing NO_x into ultra thin panels that maximizes light penetration into the culture while containing dense algal cultures to maximize oxygen evolution via photosynthesis into the medium. Meanwhile, bubble size has to be sufficiently small to provide adequate surface area and time for dissolution of NO_x into the media, but not so small as to impinge air flow from high flow boiler flue stacks. The results of this study provides additional evidence to existing literature on algae-based scrubbers as a NO_x-mitigating technology. Further studies on horizontal tube setups might be conducted in light of the new findings reported by Lefan (2012) concerning further optimization of system conditions including pH, temperature, NO_x flow rate and iron concentration. Also, NO_x capture using the Aqua Ammonia Process described by Resnik et al. (2004) where NO_x is oxidized then absorbed by ammonia for simultaneous capture of CO_2, NO_x and SO_2 in flue gas. This an option worth revisiting in light of the information obtained by this study.

3.4.4 Experiment 3.4: Growth on wastewater

Wastewater samples were collected and characterized from a number of wastewater streams as summarized in Table 3.3 below. These included secondary treated effluent from the Chandler Airport Water Reclamation Facilty (AWRF), secondary treated effluent from Johnson Utilities at the Anthem, SanTan and Pecan Creek locations, anaerobic digester centrate from the Greefield Water Reclamation Plant (WRP), and Van Rijn dairy lagoon. The collected wastewater sources were characterized in term of macronutrients including nitrate, ammonia and phosphate levels via flow injection analysis and also run on OES-ICP to determine trace metal levels. The values collected were compared to modified BG-11 with ¼ (~60 mg N-NO_3/L) nitrogen content but all other nutrient levels unchanged. While some nutrients were in excess of BG-11 levels such as Ca^{2+} or Mg^{2+} across the board, others like Mn^{2+} and Mo^{2+} were difficient for all secondary treated municipal wastewater samples. Difficiency of these micronutrients likely posed little issue for using these waste streams as algal culture

Table 3. 3**:** Comparison of nutrient profiles of common wastewaters to modified BG-11 media (with ¼ nitrogen content). Values in bold denote possible deficiencies.

Element	Chandler AWRF Effluent	Anthem Effluent	San Tan Effluent	Pecan Creek Effluent	Digester Centrate	Dairy	¼ BG-11
	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)
B	0.433	0.422	0.465	0.365	1.05	1.048	0.124
Ca	73.49	94.74	56.89	59.5	371.6	377.8	2.004
Co	0.008	**0.001**	**0.00**	**0.00**	0.008	0.005	**0.003**
Cu	0.026	0.011	0.047	0.014	0.035	0.089	0.005
Fe	**0.003**	**0.00**	**0.036**	**0.00**	0.202	0.273	**0.279**
K	21.52	21.71	18.18	18.00	-	-	4.301
Mg	23.81	19.25	11.33	11.16	-	-	0.182
Mn	**0.009**	**0.024**	**0.011**	**0.008**	0.113	0.331	0.124
Mo	**0.007**	**0.002**	**0.006**	**0.003**	**0.03**	**0.009**	**0.038**
N	**6.00**	**3.41**	**2.88**	**5.13**	500	270	61.8
Na	339.3	252.7	193.1	157.4	-	-	102.5
P	3.421	0.194	2.625	2.531	4.05	3.5	1.704
S	121.4	46.65	26.74	25.13	376	379	0.24
Si	8.438	13.67	12.91	15.11	123.6	140.3	-
Zn	0.053	0.064	0.07	0.059	0.028	0.074	0.013

media because they are very low to begin with. However, the iron levels were low across all wastewater samples tested. This includes the centrate and dairy lagoon waters conidering they would have to be diluted significantly to bring nitrogen and phosphate levels in check while simultaneously addressing turbidity issues with these two given nutrient waste streams. The secondary treated wastewaters samples from the muncipal wastewater treatment facilities had insufficient nutrients to cultivate a dense algal culture. The bacterial flocculation and polishing techniques remove much of the available nitrogen and phosphorous content. The focus for the remainder of the study was turned towards the more concentrated nutrient sources such as anaerabic digester centrate and dairy lagoon water.

Turbidity caused by dissolved organic substances is known to reduce photosynthetic performance of cultures. Table 3.4 below outlines the optical density of common waste water stream at 450 and 750 nm. It is important that particles absorbing through the photosynthetically active radiation (PAR) band be kept in check if similar levels of production are expected from algal cultures grown on wastewater media. This matter is many times overlooked in wastewater studies involving algae but is likely of

Table 3. 4**:** Optical density (OD) at 450 and 750 nm for various wastewater media

Media	**OD 450**	**OD 750**
Distilled water	**0.049**	**0.051**
BG-11 medium	**0.054**	**0.053**
Centrifuged anaerobic digester sludge (no polymer)	3.732	1.9
Settled anaerobic digester sludge	1.679	0.9
Centrifuged anaerobic digester centrate (polymer)	0.193	0.091
Settled dairy	0.818	0.175

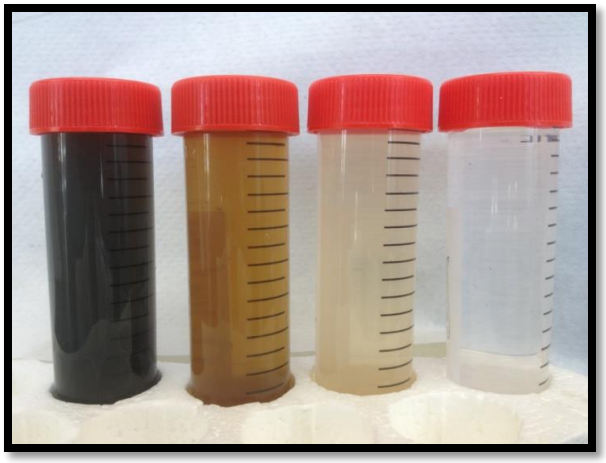

Figure 3. 13: Comparison of relative turbidity of wastewater samples in comparison to BG-11 medium. Left to right: Anaerobic digester centrate, settled dairy lagoon effluent, polymer treated centrifuged digester centrate, and BG-11 medium.

similar importance to the given nutrient profiles and ratios in the given wastewaters. Based on the given results, the wastewaters collected in the present study either needed additional filtration, dilution, or to be utilized in a culture with a short light path such as an acrylic flat plate panel. For comparison, Figure 3.13 demonstrates the relative turbidity of the anaerobic distester centrate, settled dairy lagoon effluent, polymer treated centrate, and BG-11 culture media for comparison. A previous study determined lime as an economical means to treat dairy wastewater, but at a much higher rate of 5wt%. At this rate, the study suggests treatment could be accomplished for $0.06 per cow (E Sheffield et al. 2010). However, the lime functions by raising the pH which volatilzes ammonia and precipitates the phosphate and organic matter. Ammonia volatilization is a problem in algal cultures where photosynthesis constantly removes available CO_2 from the media, driving up the pH (Eustance et al. 2016).

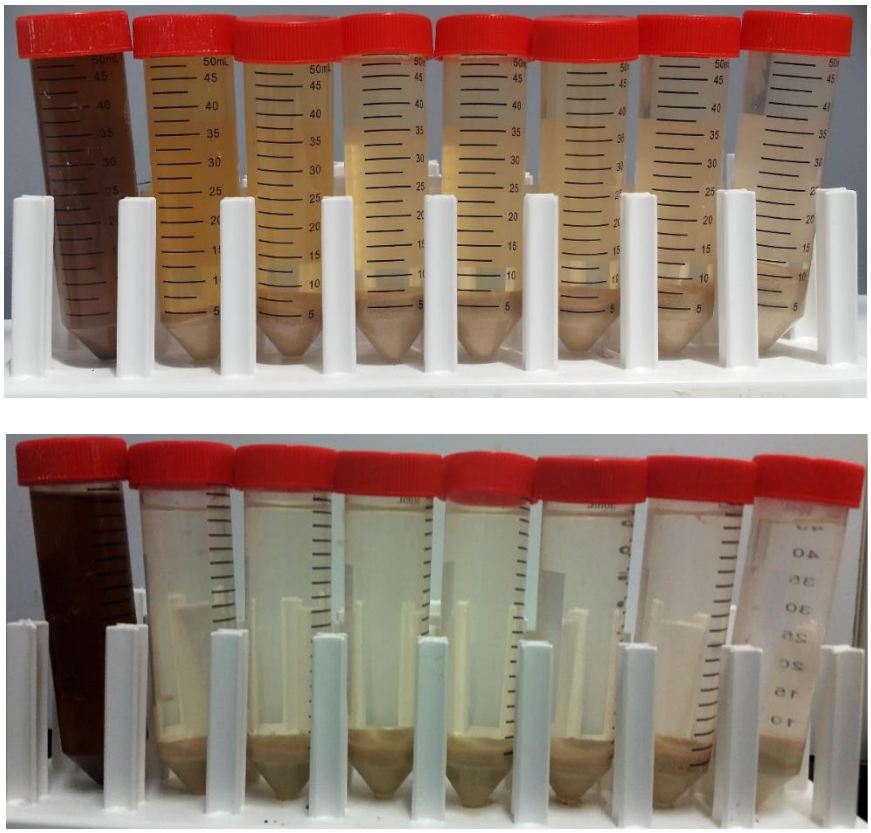

Figure 3. 14: Treatment of dairy lagoon water with lime to reduce turbidity. Top left to right: Untreated dairy, 1.25wt% Lime, 1.5wt% Lime, 1.75wt% Lime, 2wt% Lime, 2.25wt% Lime, 2.5wt% Lime, and 5wt% Lime. Bottom picture shows samples after 1 week of treatment.

Equation 3.1 below shows how CO_2 can help capture and store ammonia and vice versa by creating ammonium bicarbonate. This is a promising concept for CO_2 abatement as described in the literature (Resnik et al. 2004). The process could also be used to capture CO_2 and ammonia emissions form CAFOs to grow algae. Figure 3.14 shows untreated dairy alongside dairy treated with increasing wt% of agricultural lime. This preliminary study shows promise in being able reduce the lime dosing levels found

Equation 3.1:

$$NH_3(g) + CO_2(g) + H_2O(aq) \rightarrow NH_4HCO_3(aq)$$

by Sheffield (2010), possibly below the four-fold reduction to 1.25wt% found in this study. LRB-AZ-0414 was successfully cultivated on lime-treated water with added BG-11 nutrients. This is promising as future studies might explore the use of dense algal cultures used in conjunction with lime to precipate the organics and nutrients out of the water so that some lagoon water could be recycled for washing dairy lanes, etc. The development of using lime to improve the turbidity of wastwater streams was beyond the scope of this study since the available ammonia and phosphate would be stripped from the water and additional processing and/or capture would be necessary to make use of the nutrients. To deal with the issue of turbidity for this study, dilution with tap water was used as a means to bring nutrient and turbidity levels in check (in the case of digester centrate and dairy).

In addition to wastewater characterization, Experiment 3.4 includes growth studies on strain LRB-AZ-0414 to work toward development of a wastewater media for this alga. The first growth study sought to determine minimum phosphate required for growth as well as the nitrate and phosphate levels potential impact on lipid production as noted in the literature (Bajhaiya et al. 2017). The second was a comparison of growth between the secondary treated effluent, diluted digester centrate, and diluted dairy wastewater to determine the relative performance of the strain on the given wastewaters in comparison to modified BG-11 with ¼ nitrate levels (~60 mg/L N-NO_3).

As depicted in Figure 3.15 below, the acrylic flat panels were used to cultivate six cultures of LRB-AZ-0414 with different N:P ratios to determine the effects on growth rate, nitrate uptake, phosphate uptake, and lipid productivity to better understand the physiology of the strain when exposed to different nutrient levels. The control with full BG-11 nitrate (246 mg N/L) and phosphate levels (6.81 mg P/L) performed the best in

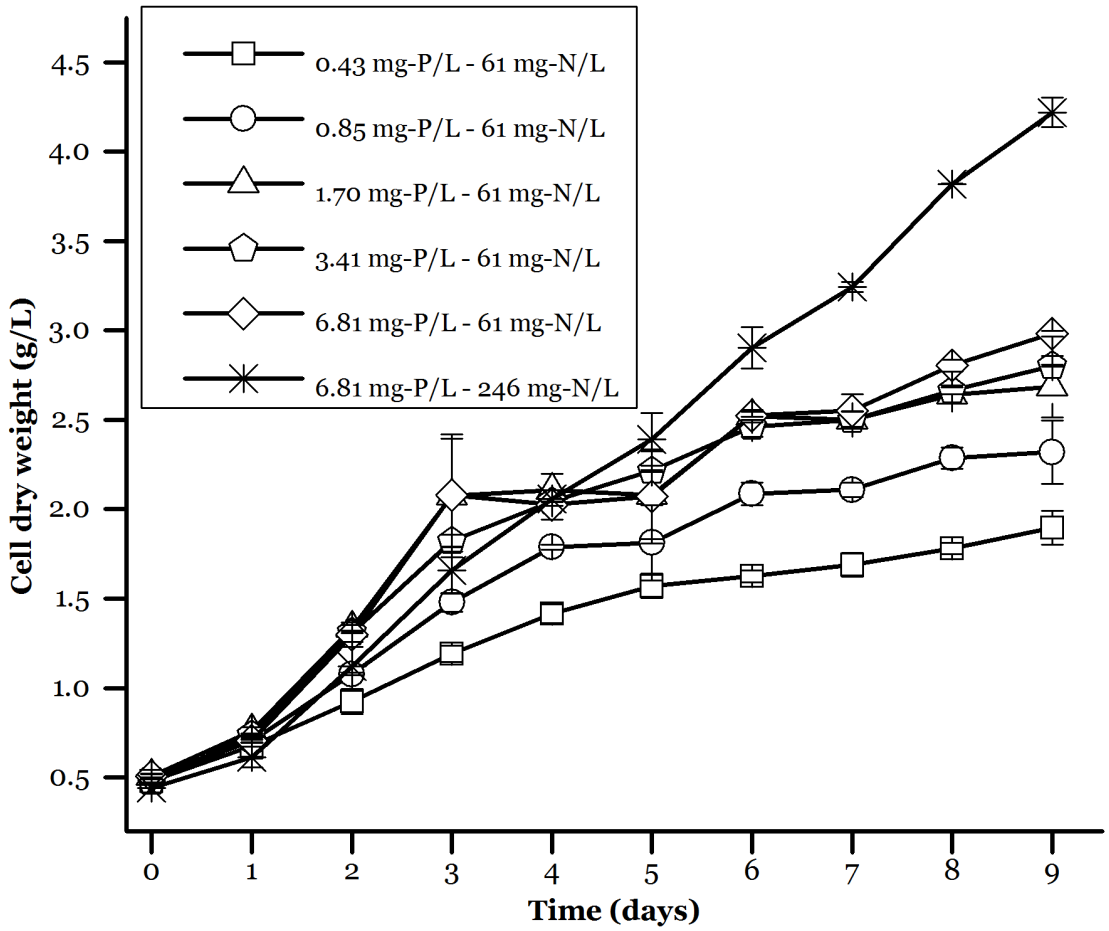

Figure 3. 15: Cell dry weight of *Scenedesmus acutus* strain LRB-AZ-0414 grown on modified BG-11 media with different levels of phosphate and nitrogen. Vertical bars denote standard error.

terms of growth rate and worst in terms of lipid accumulation in the biomass. This was to be expected as nitrogen limitation is strongly correlated with lipid accumulation in the biomass, typically reaching 50% in this strain (unpublished data). All cultures with limited nitrogen demonstrated significantly lowered productivity after exhausting their nitrate reserves by day 4. When comparing growth rate within only the nitrogen deprived cultures there was a direct correlation to decreasing phosphate level from the full BG-11 levels of 6.81 mg N/L to lower amounts. This became a significant difference

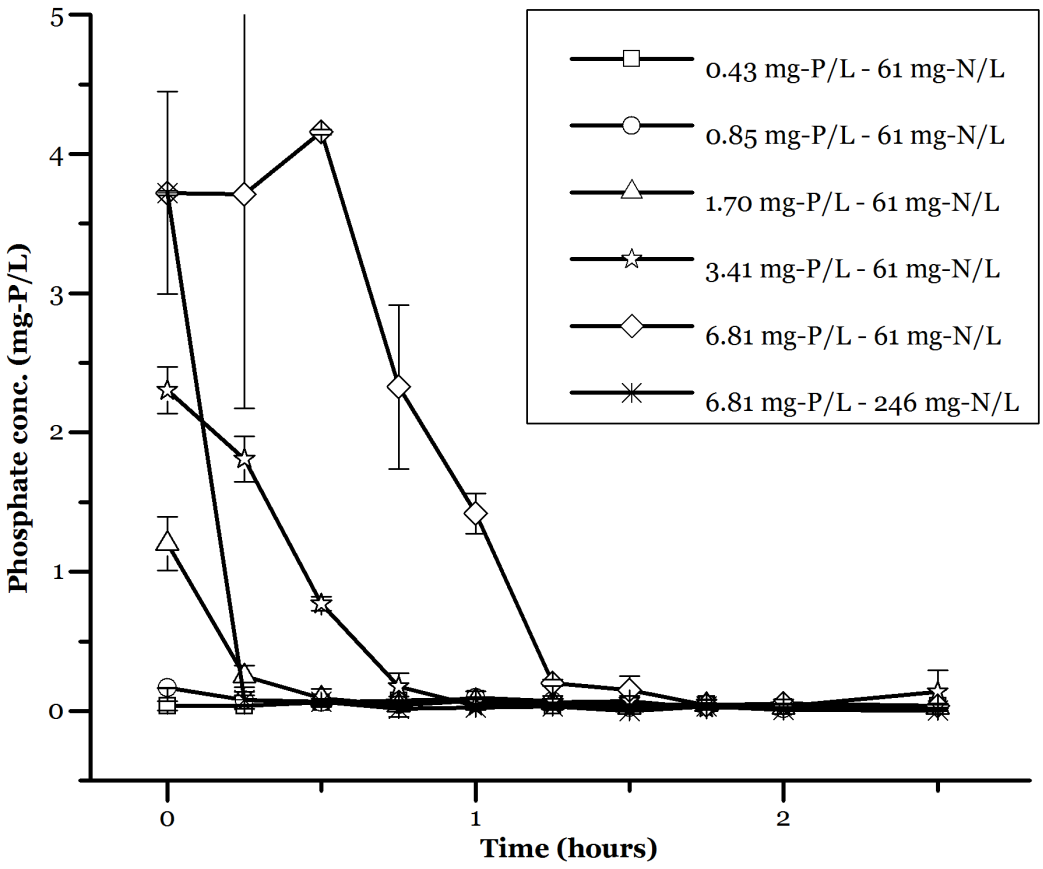

Figure 3. 16: Phosphate uptake by *Scenedesmus acutus* strain LRB-AZ-0414 on different starting levels of phosphate. Vertical bars denote standard error.

via a two-tailed students t-test at 0.85 mg N/L ($p \leq 0.05$) and 0.43 mg N/L ($p \leq 0.01$) levels, which had markedly reduced growth rates.

Phosphate from the culture media is absorbed extremely fast from the culture medium as demonstrated by Figure 3.16 above. This fact required the sampling interval to be adjust to 15 min intervals to capture the uptake, which ranged from 0.03-0.08 mg P L^{-1} min^{-1} for the nitrogen-deprived cultures but jumped to 0.45 mg P L^{-1} min^{-1}. Having sufficient nitrogen levels apparently increases phosphate uptake over 6 times more than for nitrogen deficient cultures. All cultures removed practically all phosphate from the media by 1.5 hours at a starting cell density of approximately 0.5 g/L.

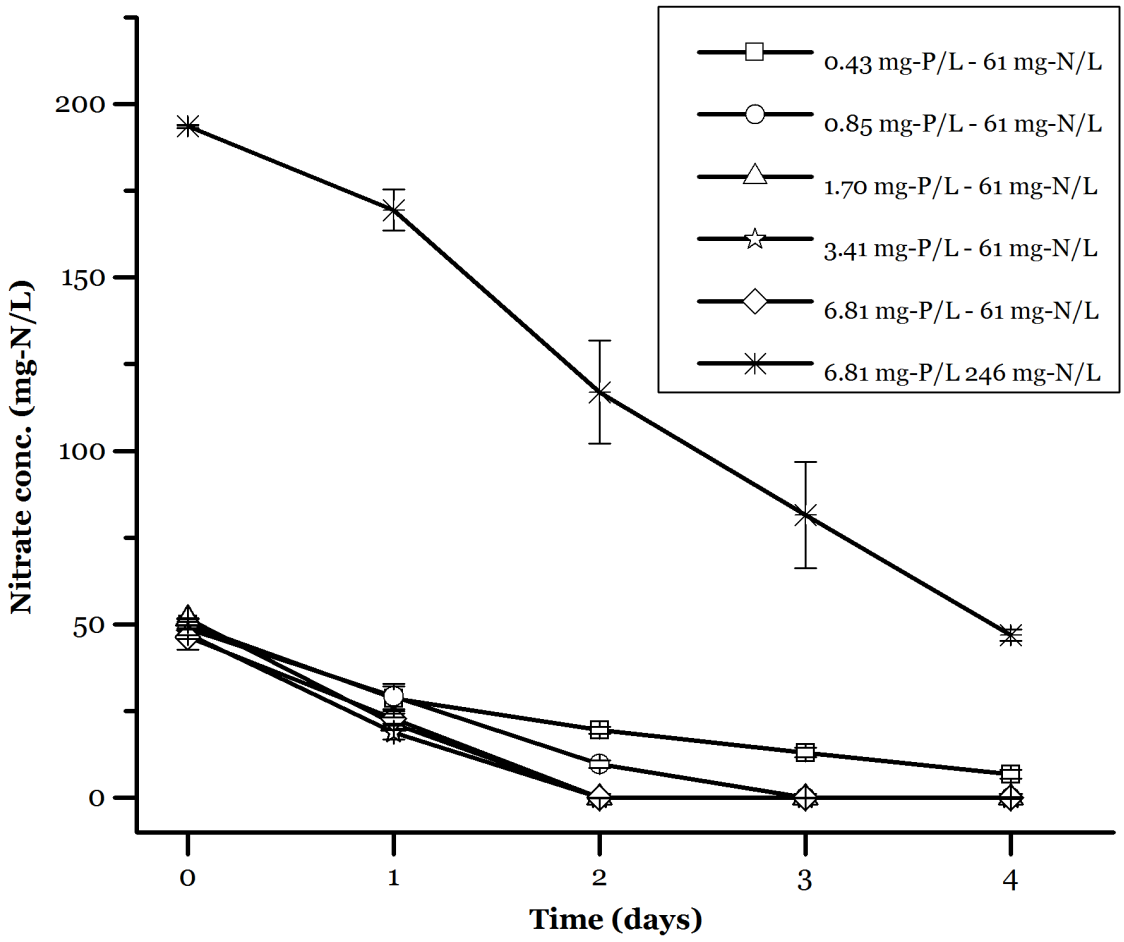

Figure 3. 17: Nitrogen uptake by *Scenedesmus acutus* strain LRB-AZ-0414 on different starting levels of phosphate. Vertical bars denote standard error.

Likewise, the nitrate uptake in Figure 3.17 demonstrates an influence of initial phosphate levels in the media. All cultures with reduced nitrogen levels had depleted most of the nitrate in the media by day 4 with the exception of the control that started with 246 mg N/L and surprisingly the tanks with the lowest level of phosphorous which had about 10% of its initial nitrate level left on day 4. Both the 0.43 and 0.85 mg P/L treatments that showed a significantly lowered cell density and phosphate uptake also had a reduced nitrogen uptake at 12 and 20 mg N L^{-1} day^{-1}, respectively. The 1.7, 3.41,

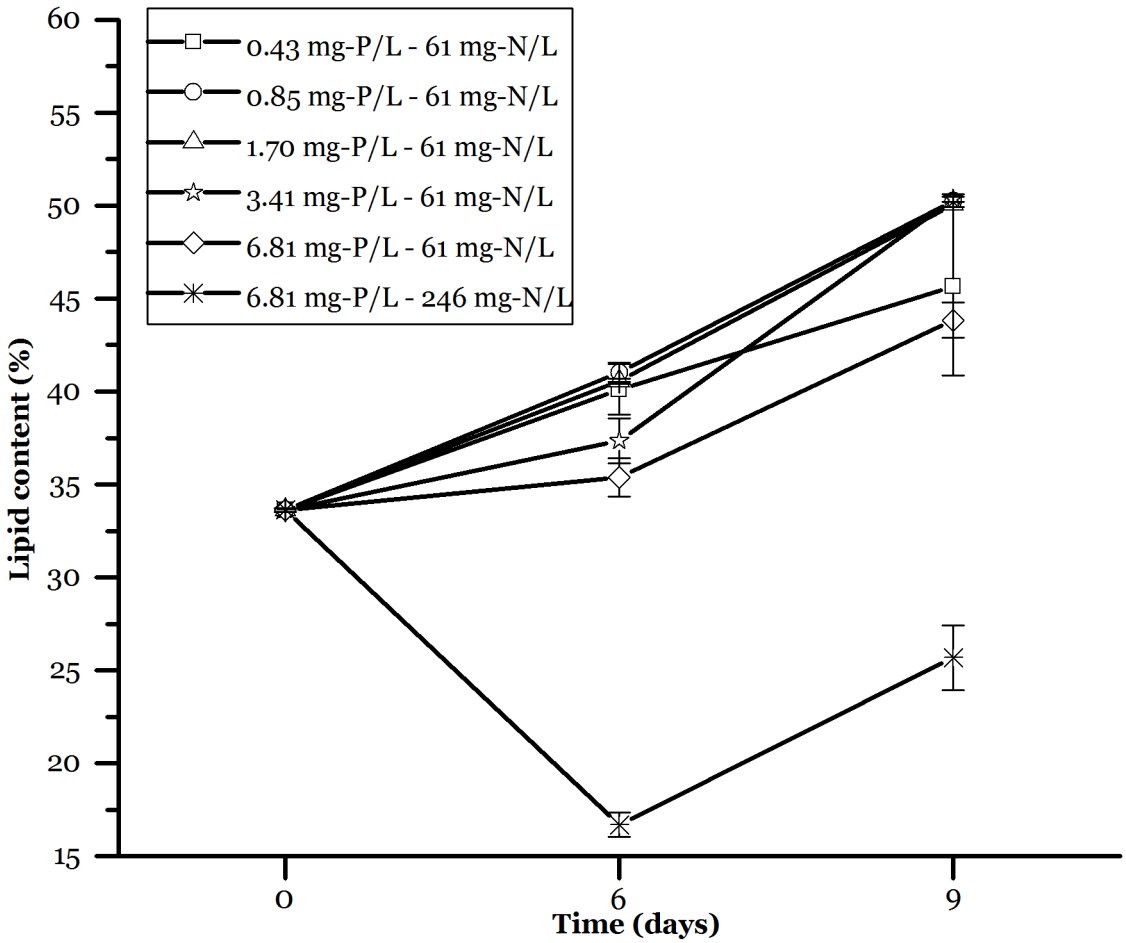

Figure 3. 18: Lipid percentage (wt) for strain LRB-AZ-0414 grown on different levels of nitrate and phosphate. Vertical bars denote standard error.

and full 6.81 mg P/L treatments with reduced nitrate levels all depleted nitrate by day 2 with an average uptake rate of 31 mg N L^{-1} day^{-1}, similar to previous values reported for this strain (Eustance, 2015). The control with full BG-11 nitrate and phosphate levels averaged 50 mg N L^{-1} day^{-1} uptake over the first four days.

Nitrate and phosphate levels demonstrated an inversely proportional trend to lipid accumulation and directly proportional to biomass accumulation that is typical for LRB-AZ-0414 and many other green algae. Lipid content in Figure 3.18 shows the control tanks dropping to a lower lipid content than in the original inoculum. This is

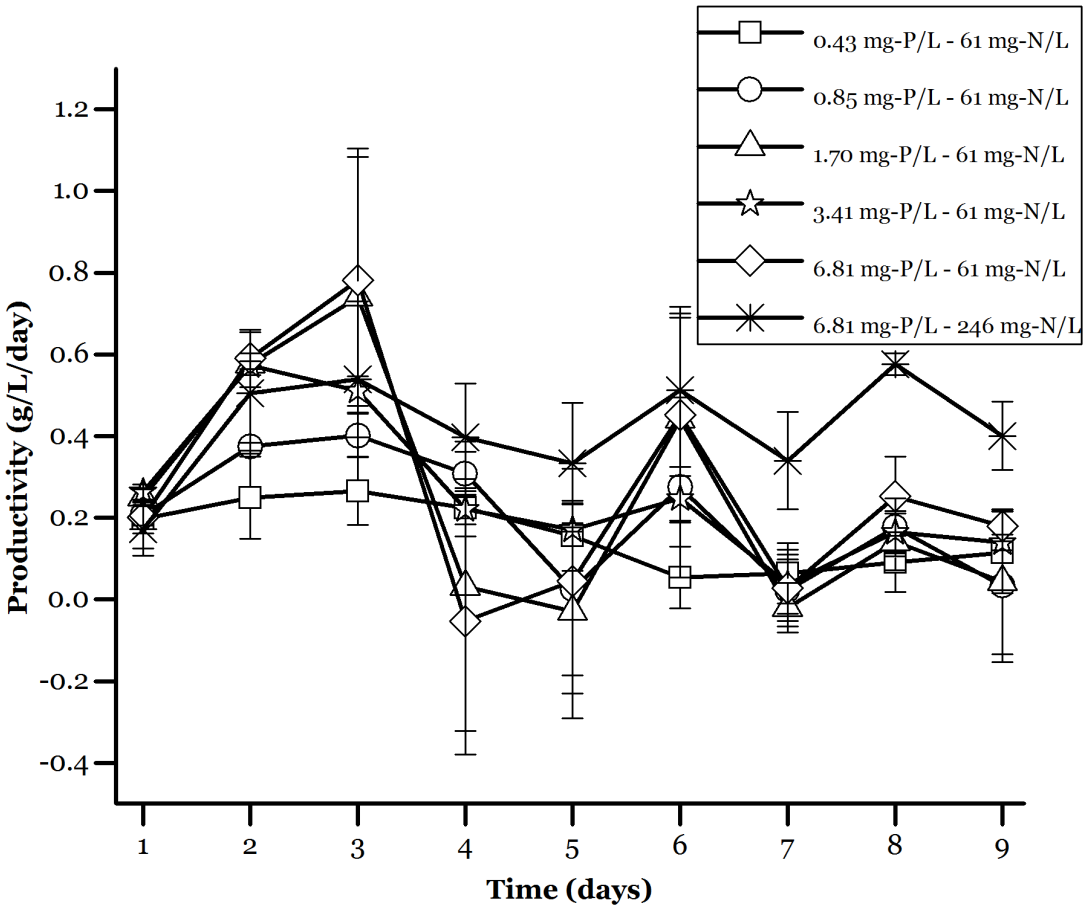

Figure 3. 19: Volumetric productivity (g L^{-1} day^{-1}) of *Scenedesmus acutus* strain LRB-AZ-0414 on different starting levels of phosphate. Vertical bars denote standard error.

consistent with high growth rates where very little energy is routed into oil biosynthesis. Figure 3.19 shows the volumetric productivity with the nitrogen replete tank averaging around 0.4 g/L/day throughout the experiment. The nitrogen deplete tanks had productivity over the first 6 days while the nitrogen reserves are exhausted, then productivity trails off as lipid accumulation commences. With the exception of the lowest initial phosphate level of 0.43 mg P/L, the observed trends show a slightly higher lipid content by decreasing phosphate levels from 6.81 to 0.85 mg P/L but with no statistical significance. However, lipid values were significantly different ($p \leq 0.01$) between the two levels of nitrate tested.

Considering the molar N:P ratios for the given treatments in this study, ranging from 314 to 20, it appears 0.43 and 0.85 mg P/L treatments with N:P ratios of 214 and 159, respectively, had a significantly reduced growth rate among the nitrate limited cultures. Nevertheless, there was no significant decrease in lipid accumulation in the biomass. In fact, the data suggests a slight increase in lipid accumulation in all groups with limited nitrogen with N:P ratios ranging from 40 to 314 with peak oil accumulation coming from the N:P range between 40 to 159. BG-11 media was created for blue-green algae and has an N:P ratio at 80, which appears to be high given the results of this experiment. The Redfield N:P ratio is only 16, but this value may not give the best growth performance. Other studies have suggested optimal N:P ratios for growth may be in range of 20-50, which is consistent with the findings of the present study (Geider and La Roche 2002). The highest total growth of the nitrogen-limited tanks was the 6.81 g P/L tank with 2.98 g/L biomass, which is 6% higher than the 3.41 g P/L tanks. However, the total lipid yield was greatest in the 3.41 g P/L tank (N:P of 40) with a total lipid yield of 1.41 g/L, which is 7% higher than the 6.81 g P/L (N:P of 20) nitrogen-limited tank. This is inconsistent with literature results showing that reducing the N:P ratio towards 1:1 increases lipid production (Ruiz et al. 2013).

The findings of this study suggest both nitrogen and phosphorous limitation increase lipid production at the cost of biomass production, which is important to consider when creating a wastewater media for algaculture. The best N:P ratio was determined to be 40 to maximize oil production for stain LRB-AZ-0414 which reached 50% total lipid. Further refinement of these results is necessary to verify this as the optimum ratio. Also more work needs to be completed to determine the maximum N:P ratio for log growth on wastewaters containing excess nitrogen and phosphates. Literature values for N:P ratios for microalgae range from less than 5 to more than 100

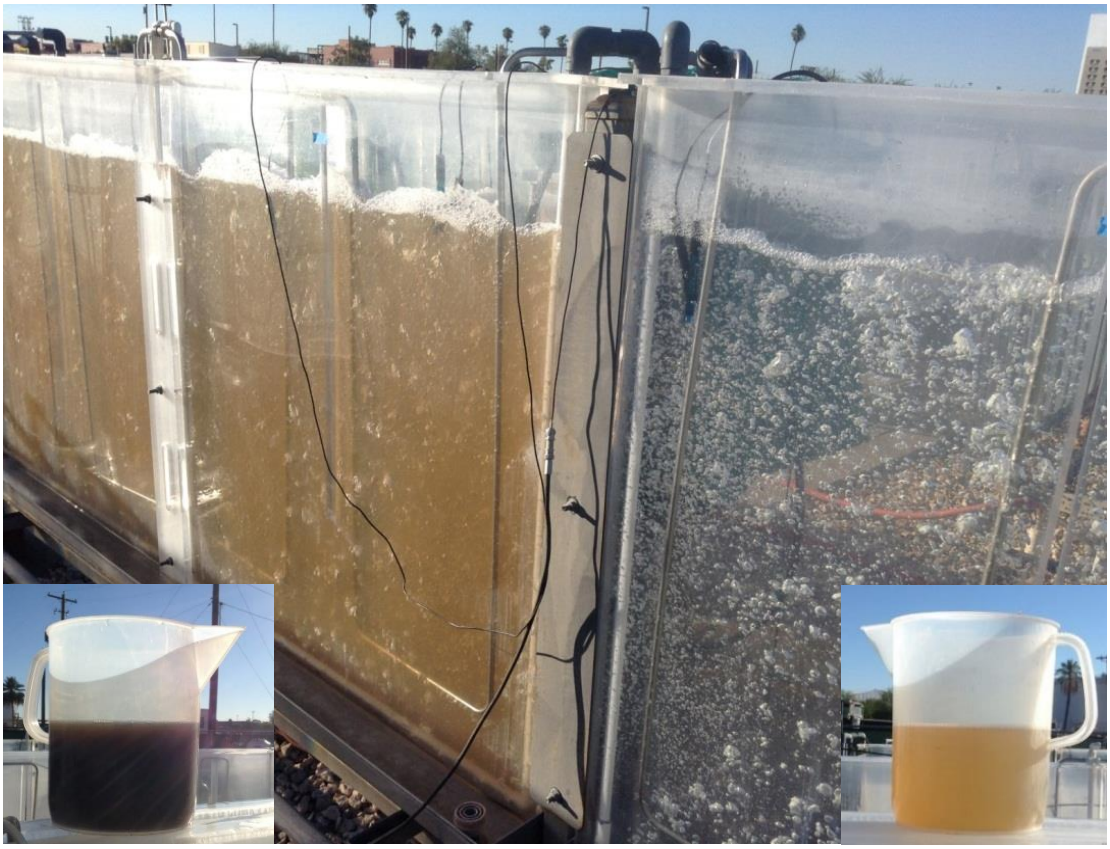

Figure 3. 20: Dairy wastewater (left) and digester centrate (right) diluted 1:10 with tap water as culture media for *Scenedesmus acutus* strain LRB-AZ-0414.

(Geider and La Roche 2002). Earlier studies on *Scenedesmus sp*. have shown the critical N:P ratio, the ratio at which neither phosphorous or nitrogen are limiting is 30 for *Scenedesmus* similar to the findings of the present study (Rhee 1978).

The final study of Experiment 3.4 was to assess the side-by-side productivity of several wastewater streams including digester centrate from a municipal wastewater anaerobic digester, dairy wastewater from a lagoon, and secondary treated wastewater (2° effluent) from a wastewater treatment facility. As discussed previously, the turbidity of the dairy and centrate was unacceptable as an algal culture media as demonstrated in Figure 3.20

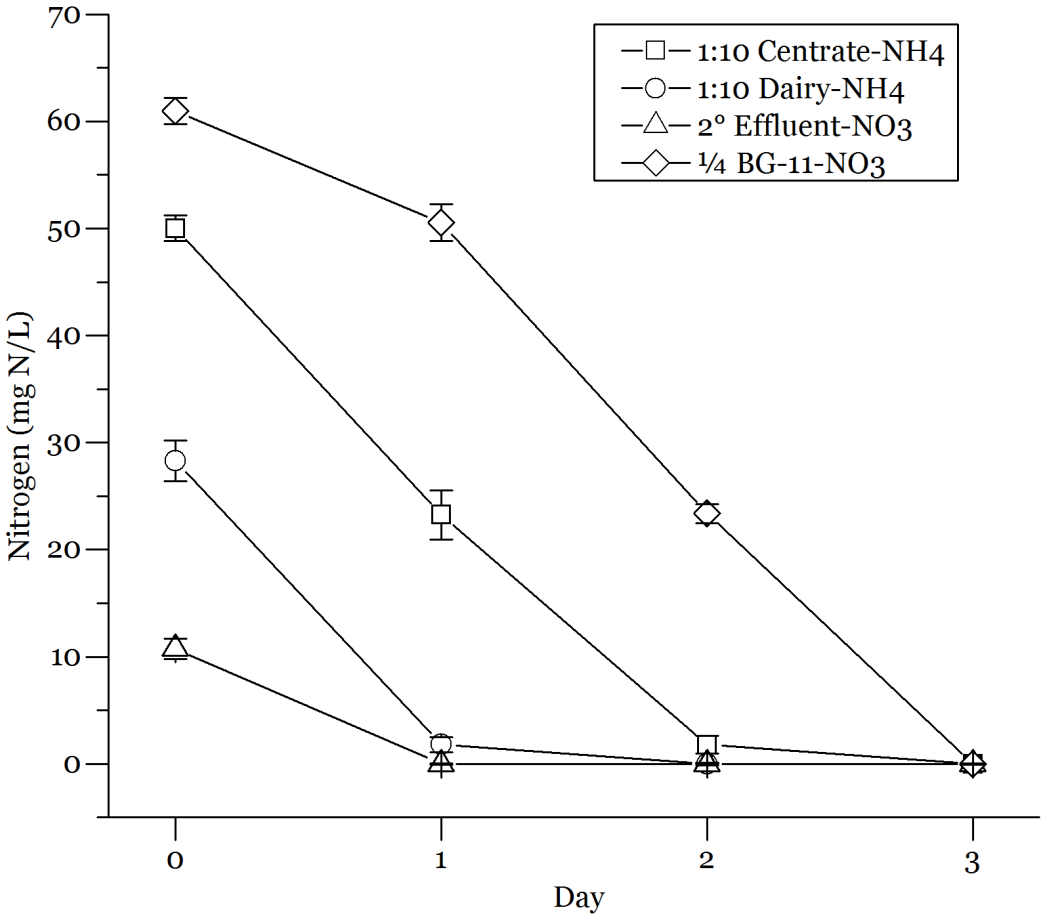

Figure 3. 21: Uptake of nitrogen by *Scenedesmus acutus* strain LRB-AZ-0414 from various wastewater media in comparison with modified BG-11 media. Vertical bars denote standard error.

above. The centrate water was treated with a polymer that strips some of the phosphate from the treated water. Alternatively, treatment with lime could be used but both will remove more nutrients from the wastewater, including ammonia and phosphate. The focus was instead aimed at developing a simple, low cost utilization of the available waste streams. Dilution was used as a method of reducing turbidity (and excess ammonia). A ten-fold dilution with tap water was adopted to test the wastewaters at their respective nutrient levels, as shown in Figure 3.21 for comparison with the modified BG-11 media

with ¼ (61 mg N/L) nitrate content. The dairy and centrate both had predominately ammonium as a nitrogen source. The municipal 2° effluent had a small amount of remaining nitrate that still poses a problem with wastewater maintenance crews as algae grow on and clog equipment. Strain LRB-AZ-0414 has shown similar growth performance on both ammonium and nitrate as nitrogen sources provided the pH is maintained around or below 7.5 to prevent volatilization of the ammonia as a gas due to its pKa as reviewed previously (Eustance et al. 2016).

Table 3. 5: Lipid, Protein and Ash content of biomass produced on wastewater media in comparison to modified BG-11 (61 mg N/L).

Biochemical Composition (wt%)				
	Centrate	**Dairy**	**Effluent**	**¼** BG-11 (NO_3^-)
Lipid	34.9%	39.4%	39.2%	34.4%
Protein	21.0%	17.4%	16.5%	24.0%
Ash	0.63%	0.75%	1.64%	1.77%

Table 3. 6: Comparison of areal productivity from modified laboratory grade BG-11 media to media derived from anaerobic digester centrate, dairy lagoon water, and municipal treatment plant effluent.

Areal Productivity (g m^{-2} day^{-1})					
Growth phase	BG-11 (NO_3^-)	BG-11 (NH_4^+)	1:10 Centrate	1:10 Dairy	Effluent
Log	17.80±0.71	17.92±0.87	17.2±0.69	12.1±0.61	7.5±0.26
Nutrient limited	6.37±0.43	6.01±0.36	8.5±0.38	5.5±0.19	4.0±0.18
Overall	11.87±0.83	11.04±0.95	12.3±0.55	9.1±0.45	7.30.36

Figure 3.21 shows the nitrate was taken up from all cultures within the first three days of cultivation, with the effluent and dairy being the first to exhaust their smaller nitrogen reserves and begin to stress and accumulate oil. Areal productivities are averaged over the course of the experiment for log growth phase and stationary (nutrient limited) phase with an overall average as presented in Table 3.5, which includes productivity of tanks cultivated on BG-11 with ammonium as the nitrogen source as taken from another study on strain LRB-AZ-0414. Table 3.6 summarizes the biochemical composition as percentage of dry weight as protein lipid or ash. It is important to note the increase in lipid and decrease in protein because these cultures had too few nutrients. Also noteworthy is the small amount of ash dairy and centrate as compared to BG-11.

Overall, the 1:10 dilution of centrate out-performed BG-11 (both 61 mg N/L nitrate and ammonium) in terms of overall average productivity. This source of wastewater has the highest available nutrients and least turbidity but was not expected to outperform standard BG-11 media. Further work with this strain will be required to develop a feasible wastewater treatment strategy, but the preliminary results are promising and match the successful cultivation of algae on centrate presented in numerous other studies(Hughes et al. 2018; Ren et al. 2017; Bohutskyi et al. 2015; Morales-Amaral et al. 2015). The strain is also fully capable of growing on dairy and 2° effluent as well. In this study the dairy had a rather low amount of ammonia at 270 mg N/L which, once diluted 10-fold to reduce the turbidity, was insufficient. Sampling of local dairy lagoons has also shown the wide range of ammonia levels possible, with much higher starting concentrations available by finding the right sampling place and time. Also, municipalities handle their treatment systems differently and some release more nutrients than others depending on a number of factors. However, operators are

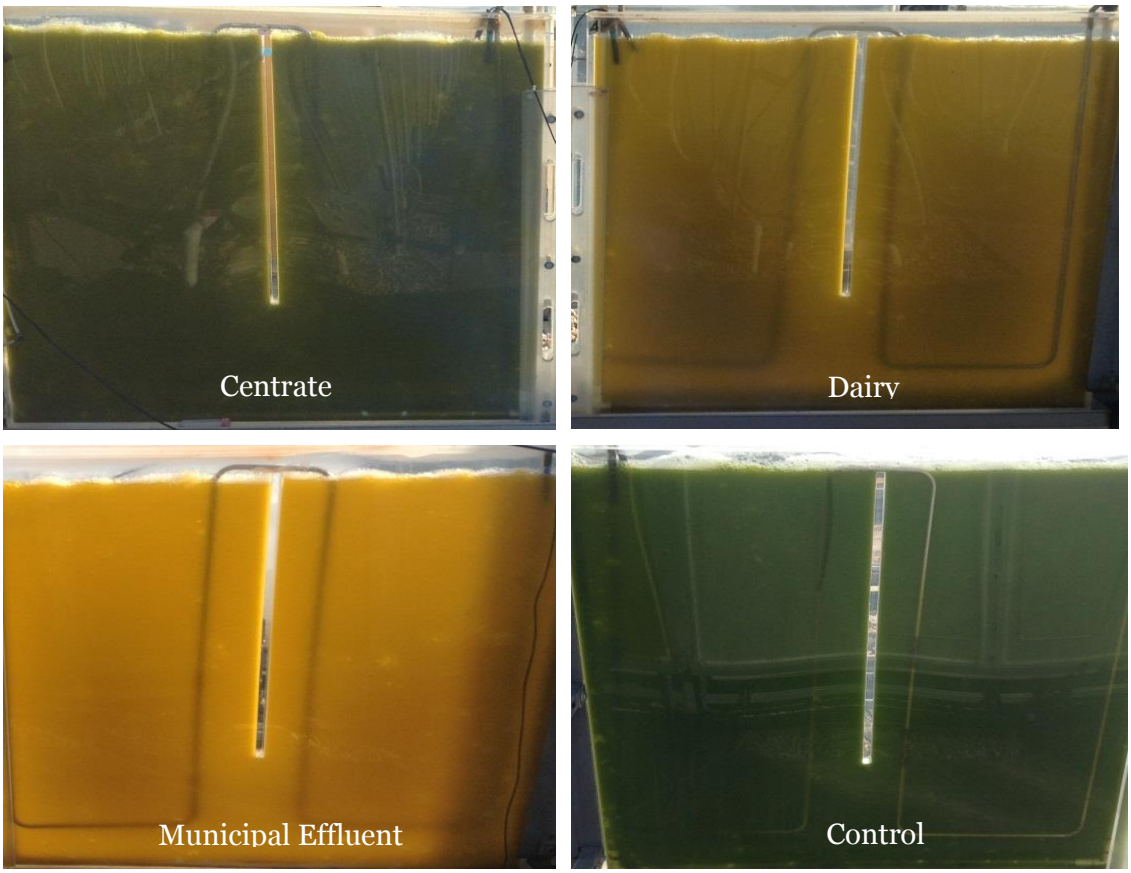

Figure 3. 22: LRB-AZ-0414 grown on 1:10 diluted anaerobic digester centrate (top left), 1:10 diluted dairy lagoon water (top right), secondary treated municipal effluent (bottom left) and modified BG-11 as control (bottom right).

becoming proficient at removing the waste quickly and keeping waste nutrients in discharge low. This makes 2° effluent more suitable for a high-throughput attached or biofilm algal production system placed on site more realistic. In addition, the amount of nutrients currently released would not support a dense algal culture, although the municipal treatment process could likely be modified to release more nutrients.

Figure 3.22 shows the algal cultures grown in three wastewater media in comparison with BG-11 media. The color change is consistent with the nutrient levels and productivity data for this study. LRB-AZ-0414 quickly loses its green color as much

of its chlorophyll degrades and lipid accumulation begins which is evident in the low nutrient dairy and 2° effluent tanks that quickly used their nitrogen reserves and changed color. Future effort should be focused on further refinement of a given wastewater source as an algal culture media, however this study demonstrates the ability of *Scenedesmus acutus* strain LRB-AZ-0414 to grow successfully on a variety of common wastewater streams while producing valuable lipid and protein.

3.5 Conclusions

The knowledge of how to effectively utilize waste nutrient streams for microalgae biomass production is a current bottleneck of realizing widespread commercial microalgae production as an agricultural commodity. Characterization of the given waste nutrient stream (aqueous or gaseous) as well as characterization of the strain to be grown on wastewater is of upmost importance to remove this bottleneck. This study demonstrates that *Scenedesmus acutus* strain LRB-AZ-0414 can be successfully cultivated in acrylic flat panel reactors using flue gas from a natural gas-fired boilers on a semiconductor fab plant. The strain demonstrated significantly better growth achieving up to 4.96±0.11 % PE (based on PAR) for the flue gas tanks and 4.01±0.23 % PE when grown on commercial grade CO_2. Future work should be devoted to developing an efficient means of delivery of gaseous components into an aqueous system, while minimizing losses to atmosphere. Development of passive gas contact devices and/or closed production systems could go a long way towards solving this problem.

Additionally, the strain has demonstrated the ability to be grown on a number of wastewater streams including muncipal effluent and anaerobic digester centrate from a municipal wastewater treatment plant as well as dairy lagoon water. Centrate performed better than BG-11 media in acrylic flat panel reactors producing an overall 12.3±0.55 g m-

^{2} day^{-1} and 34.8% total lipid. Municipal anaerobic digester centrate and dairy lagoon wastewaters make good culture media for strain LRB-AZ-0414 but have high turbidity that blocks light into the culture. Turbidity can be reduced with use of lime or polymers, but nutrients are removed. Dilution is another option for reducing turbidity of wastewaters and proved an effective measure in this study. Strain LRB-AZ-0414, a high lipid strain was also found to accumulate a significant amount of lipid when grown on wastewater of varying nitrogen and phosphorous content. This study determined that a N:P ratio of 40 was optimal to produce the highest lipid yield amongst nitrogen deprived cultures. Future studies should focus on optimization of nutrient ratios in waste streams while simultaneously balancing with dilution rates to achieve the best balance of nutrients and light penetration into the culture. Decreasing the path length of the given photobioreactor system may also provide a passive approach to dealing with turbidity issues common to many wastewater streams.

Microalgae have the potential to capture and utilize NO_x emmissions from flue gas provided the gas can be oxidized and solubilized into a useable form such as nitrate. This study demonstrated NO_x emmissions can be captured and used by algal cultures. A 4.57 m tall column captured 100% of the simulated 40 ppm NO_x emmissions with addition 5mM $FeCl_3$ but requiring signifant energy to do so. Algae at 2.0 g/L alone removed 58% of NO_x emissions, and capture with ultra high-density cultures (>10 g/L) should be investigated further due to increased O_2 concentration seen in these cultures that could drive the oxidation and capture of NO_x further. A horizonatal NO_x contact reactor was shown to decrease the required energy input and demonstrated up to 40% NO_x removal with the addition of 1.8 mM Fe(III)EDTA to drive the oxidation of NO_x and increase the capture rate.

With better knowledge of appropriate capture strategies and techniques, as well as better characterization of the given waste streams and algal cultures being cultivated, the bottleneck of not being able to efficiently and economically utilize waste nutrients streams can be reduced or eliminated, thereby making commercial microalgae production a reality. Future work should be devoted to better understanding how to manage these issues of nutrient capture or utilization for production of a given alga.

3.6 Acknowledgments

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4. MANUSCRIPT 3: GRAVITY SEDIMENTATION AND MICROFLUIDIZATION OF *SCENEDESMUS ACUTUS* BIOMASS FOR USE AS BIOFUEL AND FERTILIZER.

Manuscript Information Page

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4.1 Abstract

The realization of efficient commercial microalgae production as an agricultural commodity to be used as feedstocks for food, fuel, and fertilizer relies on the development of energy efficient harvesting and post-processing techniques for the biomass. This study investigates the use of gravity sedimentation of *Scenedesmus acutus* biomass as a harvest technique in comparison to harvesting via centrifugation. Additionally, a Microfluidics M110-EH microfluidizer was investigated as a means of post-processing for cellular disruption of the biomass and was found to be a potential method of liberating high value biomass components, such as lutein and astaxanthin, which were both found in this alga strain. The use of microfluidization as a post-processing option was determined to only be applicable to production of high value products due to the relatively large energy demand for biomass processing. Settling as a means of harvesting, which has a much smaller energy footprint, has much wider applicability as a harvesting technique if the process is optimized for each given strain. Ideas for adapting a lamella settler to harvest microalgae are discussed. Also lipid-extracted *Scenedesmus acutus* biomass was tested for its suitability as nutrient source for the growth of cucumber (Cucumis sativus) and corn (Zea mays). With comparable results to a commercially available 4-6-6 fertilizer, the extracted biomass was shown to have potential as a slow-release organic fertilizer for plants after the high value products like oil or carotenoids were extracted from the biomass.

4.2 Introduction

The idea of farming algae on a commercial scale for the production of biofuels and other bioproducts is not a novel idea with attempts being made as early as 1931. The roots of algaculture extend even farther back with the first laboratory culture of

microalgae described in scientific literature over 140 years ago (M. Borowitzka 2013). Despite its more recent renaissance as a biofuel crop, algae—both macro and micro—are rather old crops first being harvested from seawater in Asia over 2000 years ago (Enamala et al. 2018). Also, people harvested spirulina for centuries from Lake Chad in Africa (Batello et al. 2004) and Lake Texcoco in Central America (Soni et al. 2017).

Even with its prior use and cultivation throughout history, knowledge of how to efficiently cultivate and harvest microalgae on a commercial scale has not progressed to the degree other traditional terrestrial crops have. This is unfortunate considering the average yields from algae can be 5-10 times that of higher plants. Algae are some of the most efficient photosynthetic organisms on the planet, producing biomass with up to 90% of its weight in valuable lipids (Metting 1996), 70% protein (Priyadarshani and Rath 2012), or 70% carbohydrates (Becker 1994). In addition, the biomass can be shifted to produce more of a particular bio-compound at will by manipulating environmental conditions while simultaneously having a much shorter life cycle than terrestrial crops. Algae can also be much more adaptable and produce a wide variety of compounds in concentrations terrestrial crops cannot compete with. This includes astaxanthin and other valuable carotenoids (Butler et al. 2017; Vaquero et al. 2014; Wichuk et al. 2014), EPA and DHA fatty acids (Allemann and Allen 2018; Lu et al. 2001; Liu et al. 2013a), beta carotene and glycerol (Zhu et al. 2018), carbohydrates (Ho et al. 2013; Jerez et al. 2016a), and of course triacylglycerides from which we can make biofuels (Duong et al. 2012; Amer et al. 2011).

Unfortunately, techniques in algae cultivation are in their infancy at best, with production almost exclusively being done in open raceways up until the late 20th century (O. Pulz 1998). This is largely due to the fact that the key components in algal culture are

only starting to be understood. Although algae are very adaptable and can survive a wide range of environmental conditions, a given culture has certain parameters that must be maintained in within a specific tolerance in order to achieve the best possible yields. This includes physical and chemical parameters like temperature, pH, salinity, conductivity, nutrient concentrations, etc. that play an important physiological role in the life cycle of a given strain of algae. However, since algae are producers relying on solar radiation to complete photosynthesis, light penetration and utilization plays a critical role in efficient biomass growth. Productivity of an algal culture is really a function of more complex interactions between biomass density, mixing, light intensity, concentration of nutrients in the medium, etc.

Only in the last few decades have researchers started to grasp the importance of these types of interactions, and narrowed the focus to areas requiring greater attention. The following are critical roadblocks to commercial algaculture that must be addressed in order for the full potential of microalgae to be realized:

- Optimize a micro-environment better suited for photosynthesis.
- Optimize utilization waste nutrient sources for efficient biomass production.
- Optimize harvesting and disruption techniques for a given strain being cultivated.

These three areas must be addressed in order to bring microalgae on board as an agricultural commodity. Current prices for agricultural commodities are in the $100's per MT and microalgae biomass in an order of magnitude higher in the 1000's per mo. This is because the innate biology of macroscopic algae and higher plants makes them more easily adapted to commercial scale farming. For instance, both higher plant and seaweed create thin leaves or leaf-like structures to better manage photosynthesis and

light utilization. They both also have structures like roots or holdfast for efficient uptake of nutrient or that provide anchoring to the ground. Additionally, their structure provides for easy harvesting of the biomass: instead of collecting many small individual cells, one can collect many cells all at once. These issues are simple in nature, but focusing on ways to make microalgae competitive in these specific areas will help spur the realization of microalgae as an agricultural commodity.

This work is aimed at identifying potential solutions to the long-standing issue of harvesting and post-processing of microalgal biomass. Harvest of microalgae is challenging because microalgae, unlike macroalgae, are on the order of a few microns in size which inherently creates an energy intensive harvesting scenarios. For example, energy consumption of harvesting via centrifugation has been shown to range from 20 kWh/m^3 to 0.80 kWh/m^3 when the flow rate through the centrifuge varied from 0.94 L/min to 23 L/min, respectively. However, increasing the flow rate decreased the % recovery of the algal biomass from 94% to 17% (Dassey and Theegala 2013). The longer the culture stays in the bowl of the centrifuge, the longer it has to sediment out on the sides of the bowl, increasing efficiency of harvest but also increasing energy input. Because of this, centrifugation is not energetically or economically feasible in the production of algae as an agricultural commodity, especially as a biofuel crop.

Even though production of a potential microalgae energy crop is highly sensitive to significant energy inputs like those involved in the harvest of biomass, there is little consensus on a widespread harvesting practice for microscopic algae (Mata et al. 2010). A number of other methods exist including dissolved air flotation, flocculation, auto-flocculation, membrane filtration, and gravity sedimentation (Milledge and Heaven 2012). While dissolved air flotation, membrane filtration, and centrifugation are all very active, energy-consuming processes, both auto-flocculation and gravity sedimentation

are two mostly passive techniques of algae harvest that offer high throughput rates with minimal capital and operational costs. Gravity sedimentation relies on the very slight difference in density between algae and water and the force of gravity is used to settle out the biomass. Auto-flocculation is usually a species-specific phenomenon where the algae flocculate and fall out of the culture due to some environmental stimuli, typically high pH. Many strains cannot auto-flocculate, leaving gravity sedimentation as the best possible means to economically harvest a wide range of microalgae biomass.

Lamella settlers used in wastewater treatment for years have been suggested to be adapted to microalgae harvesting (Janelt et al. 1997; Letourneau et al. 1988). However, few if any designs are in commercial service to date as much of the production in the field has been on high value products where a centrifuge can be utilized and most research projects over the years have been concerned with growth rates and high performance strains rather than cost-effective harvesting (Mata et al. 2010). The potential of the lamella settler, along with information about settling characteristics could go a long way in reducing the harvesting cost for a given algae strain. This work is aimed at identifying the feasibility of using gravity sedimentation to reduce the harvesting cost of *Scenedesmus acutus* biomass.

Additionally, adopting appropriate post-processing techniques and defining valuable uses for residual biomass components can both go a long way to making a positive economic impact on a given microalgae crop. This ultimately depends on the final end use and perceived value of the biomass. In order to use the intracellular components, the cell must first be ruptured. This is typically done mechanically or chemically. Besides investigation into settling characteristics of *Scenedesmus acutus* biomass, this study aims to determine the feasibility of using a microfluidizer to

mechanically disrupt biomass for oil and or carotenoid extraction while the spent biomass is used as a plant fertilizer. Doing this adds additional value to the biomass beyond the production of the primary oil or carotenoid product. A mineralization study was carried out to show release of nitrogen from the algae biomass into the soil. Also, a growth study on cucumber (*Cucumis sativus*) and corn (*Zea mays*) is conducted to show the feasibility and potential application rates of the extracted algae biomass as a fertilizer as adapted from a previous study (Mulbry et al. 2005). Obtaining this information for strain LRB-AZ-0414 with help remove the harvesting as a post-processing roadblock currently limiting its commercial production.

4.3 Materials and Methods

4.3.1 *Scenedesmus acutus* strains

Two strains of *Scenedesmus acutus*, LRB-AZ-0414 (KR904912) and LRB-AZ-0424, were utilized for all experiments. The strains were isolated on BG-11 agar plates and maintained on monoalgal BG-11 agar plates in the laboratory. All experiments used cultures that had been acclimated to grow outdoors in the desert southwest. This was accomplished by scaling the cultures in the lab (using round 800 mL glass tubes D=4.6cm, L=61cm) and then transferring into two 50 L acrylic vertical flat panels in the outdoor photobioreactor array. These two stock panels were maintained on the modified BG-11 culture medium described below and were used as inoculum to grow biomass for all harvesting and post-processing experiments.

4.3.2 Modified BG-11 culture medium

A modified version of the standard laboratory BG-11 culture medium consisting of one fourth of the nitrogen (375 ppm $NaNO_3$) and no additional magnesium sulfate,

calcium chloride, or sodium bicarbonate was utilized for producing the biomass utilized in this study. These changes were made because lowering the nitrogen concentration led to a faster stress response (oil accumulation) and the tap water utilized outside already had sufficient micronutrients to replace these ingredients. Analyzed tap water at ASU's AzCATI field site (33°18'14.2"N, 111°40'21.5"W) had an average hardness of 280 ppm as $CaCO_3$ with 180 ppm Na^+, 75 ppm Ca^{2+}, 70 ppm SO_4^{2-}, 24 ppm Mg^+, and 8 ppm K^+. Final composition of the modified growth medium included 375 ppm $NaNO_3$ (Alfa Aesar), 9.58 ppm K_2HPO_4 (BDH), 5.24 ppm of Ammonium Ferric Citrate (Alfa Aesar), 1.31 ppm Citric Acid Monohydrate (Sigma-Aldrich), 0.71 ppm H_3BO_3 (Sigma-Aldrich), 0.445 ppm $MnCl_2{\cdot}4H_2O$ (Sigma-Aldrich), 0.10 ppm $Na_2MoO_4{\cdot}2H_2O$ (Sigma-Aldrich), 0.06 ppm $ZnSO_4{\cdot}7H_2O$ (Sigma-Aldrich), 0.02 ppm $CuSO_4{\cdot}5H_2O$ (Sigma-Aldrich), 0.1 ppm $Co(NO_3)_2{\cdot}6H_2O$ (Sigma-Aldrich).

4.3.3 Flat panel photobioreactor array

Experiments in this study were conducted using biomass cultivated on the Arizona Center for Algae Technology and Innovation (AzCATI) outdoor field site in Mesa, AZ that utilized a north-south facing array of 48 vertical acrylic flat panel photobioreactors. The flat panel photobioreactors were constructed of 13 mm thick acrylic sheets. They were aligned in four rows of twelve panels with sufficient spacing to minimize shading (~1.2 m). Tank dimensions are 1.17m by 1.17m by 3.8cm giving an internal volume (accounting for flex of acrylic) of 60L and a working volume (accounting for aeration volume) of 50L. Aeration was provided via 13mm PVC pipe with 0.8mm holes drilled every 3.8 cm across the bottom of the PVC pipe in a staggered arrangement to promote mixing. This supplied the cultures with 0.5 vvm aeration. Commercial grade carbon dioxide (CO_2) was obtained from Praxair dewar located on site and plumbed into

the photobioreactor array. The CO_2 was fed into the inlet of a 2 H.P. Sweetwater blower at maintain at a feed rate of 1.5% CO_2 from sunrise to sunset daily. The tanks were cooled by placing 13mm stainless steel cooling loops into tanks and circulating cooling water from a 2000L underground reservoir cooled using two GPC-25 Glacier evaporative pool coolers set on a Johnson Controls thermostat to regulate the reservoir temperature to 24±3°C for the LRB-AZ-0414 strain and 29±3°C for the LRB-AZ-0424 strain. A 2 HP 3 phase pool pump circulated chilled reservoir water during the day to regulate culture temperature in the photobioreactor array.

4.3.4 Harvesting via centrifugation

Harvesting of algae biomass was accomplished using a 1.5 hp Lavin 12-413v centrifuge by AML Industries, Inc. at 3000 g. Flow rate of algal cultures through the centrifuge was regulated to 6.5 to 7.5 LPM to ensure efficient harvesting of biomass.

4.3.5 Light Microscopy

Light microscope images of cells were obtained using an Olympus BH-2 light microscope with 20X or 40X objectives to access relative disruption efficiency of microfluidized cells after each pass through the machine.

4.3.6 High Performance Liquid Chromatography (HPLC) analysis

A Beckman Ultrasphere C18 column (250 mm long, 4.6 mm ID.; 5 μm; Beckman Instruments, USA) was used for quantification of pigments in nutrient limited algae biomass by HPLC at 25°C. The algae cells were freeze-dried and extracted in 25:75 (v/v) solution of dichloromethane and methanol. Solvent A, the mobile phase, was a 5.0:85.0:5.5:4.5 (v/v) solution of dichloromethane, methanol, acetonitrile, water

respectively. Solvent B was a 25.0:28.0:42.5:4.5 (v/v) solution of dichloromethane, methanol, acetonitrile, and water, respectively. Peaks on the chromatograph were measured at a wavelength of 480 nm and a flow rate of 1.0 mL min^{-1}in order to facilitate detection of the different astaxanthin species, canthaxanthin, lutein, and β-carotene. The peaks for chlorophyll a and chlorophyll b were measured at 450 nm in accordance with (Yuan et al. 1997). Comparison of the retention times of known standards or other spectra from published data were used for peak identification.

4.3.7 Ion Chromatography (HPAE-PAD) analysis

Carbohydrate analysis was completed with the use of a Thermo Scientific—Dionex ICS-5000+ Analytical HPIC System equipped with a PA-20 column. Sample prep was completed by TFA digestion of 10 mg samples of freeze-dried cell free culture media with 153 µL of 12.98 M TFA 847 µL 18 MOhm filtered water and digested at a temperature of 100°C for 6 hours. The TFA is evaporated under purge gas followed by resuspension and a 1:50 dilution using 18 MOhm water and subsequent filtering using a 0.2 µm nylon filter. The samples were then analyzed using high pressure anion exchange liquid chromatography using pulsed amperometric detection (HPAE-PAD).

4.3.8 Carbon Hydrogen Nitrogen (CHN) analysis

CHN analysis was completed using approximately 2.5 mg of freeze-dried algae samples into a tin cup for combustion at 1760°C, upon which halogens are chemically scrubbed and the resulting gas is separated on a GC column and analyze via a thermal conductivity detector to calculate total C, H, and N in the samples to determine C:N ratios of the samples.

4.3.9 Microwave digestion

Microwave digestion of the algae samples was completed using a CEM microwave digester prior to trace metal analysis. Algae samples of 0.5 g were transferred to the digestion vessels upon which 10 mL of nitric acid was added and gently swirled to dissolve the samples. The vessels were allowed to react for 15 mins upon which the digestion vessels were closed, placed in the microwave and held at 200°C and 800 psi for 15 mins. The clear digested samples were diluted to 50 mL with MQ water for ICP-OES trace metal analysis.

4.3.10 Inductively Coupled Plasma (ICP-OES) analysis

A Thermo Scientific ICP-OES was used to analyze trace elements. The reagents, standards, and standard curve for all analytes were generated using manufacturer's recommendations and a standard curve with R-correlation value ≥ 0.95 was generated for each ion analyzed. The samples were first filtered using a 0.2 micron cellulose nitrate syringe filter diluted 1:10 with 18 MOhm filtered water while simultaneously acidifying to 2% w/v nitric acid. This was done to fully dissolve all metals and bring the concentration of all analytes within the realm of the standard curve.

4.3.11 Flow injection analysis

Nitrate, ammonia and phosphate data were analyzed using a QuickChem 8500 Flow Injection system. Standards and reagents were prepared as per manufacturer's recommendations and a standard curve with R-correlation value ≥ 0.95 was generated for each ion analyzed. The samples, collected in duplicate, were prepared by

centrifugation at 1000g, then subsequently filtered through a 0.2 μm membrane filter to remove any remaining particles. The samples were diluted 1:7 with 18 MOhm filtered water to bring the concentration of all analytes within the realm of the standard curve. Samples were collected in 2mL centrifuge tubes (VWR) and centrifuged at 1000g to remove all suspended cells then preserved in cold room at 4°C until each experiment was complete and ready for analysis.

4.3.12 Experiment 4.1: Gravity sedimentation

Gravity sedimentation rates for *Scenedesmus acutus* strain LRB-AZ-0414 was determined using both 4.6 cm and 2.5 cm diameter glass columns to observe sedimentation rates for the organism at different physiological states, including log phase, stationary phase, and late stationary phase biomass. The 2.5 cm columns were fitted with polypropylene disks fitted at 2.5 cm intervals along a glass rod and submerged into the culture tube to determine if a lamella settler setup could effectively decrease the settling time. Columns were visually inspected on 3 hour intervals for the course of the 24 hour experiment, with photos being taken to capture significant points in time of the settling process.

4.3.13 Experiment 4.2: Cellular disruption

A Microfluidics M110-EH microfluidizer was used to study the mechanical cellular disruption of strain LRB-AZ-0414 biomass. This machine uses shear force and 30,000 psi hydraulic pressure to disrupt cells using a 200 μm ceramic (H30Z) ceramic auxiliary processing module in line with the 87 μm (G10Z) diamond interaction chamber. The algae was diluted to approximately 15% solids and passed through the machine for multiple passes to measure cell disruption of the biomass. The resulting

biomass was inspected under a bright field microscope to access the relative amount of cells remaining intact after each treatment. Biomass was also analyzed for carotenoid and sugar content.

4.3.14 Experiment 4.3: Mineralization study

A local alkaline soil, Gilman loam, was used in a mineralization study to determine the rate of release of N from algae biomass applied to the soil. Algae strain LRB-AZ-0414 was grown in 3.8 cm acrylic flat panels and cultivated approximately one week in modified BG-11 media with depleted nitrogen levels in order to produce biomass with a lipid content of 35 to 40 dry wt%. This biomass was harvested via centrifugation and freeze-dried to preserve. Some of the algae was then extracted for oil with an algal solvent extraction method adapted from literature (Zhang et al. 2002). The remainder of the high oil biomass was not extracted and left in raw form for use as a baseline for comparison.

The soil obtained was air-dried, crushed, sieved and weighed out in 100 g samples. Each 100 g sample was then placed into a separate flask. The soil in the flasks was amended with either 0.0, 0.75, or 1.5 g of extracted or raw algae biomass. The soil in the flasks was moisture adjusted using DI water to approximately -33 kPa. The flasks were then capped with aluminum foil to reduce moisture loss, and incubated at 25^{o} C for 42 days in the dark. During the incubation period, the flasks were weighed once a week and distilled water was added to maintain the moisture level at approximately field capacity (-33 kPa). Triplicates were run for each treatment. Samples were taken from the flasks on day 0, 7, 21 and 42 for N and P analysis. A moisture analysis was determined on samples dried at 105^{o} C overnight. All the filtrates were obtained by filtering extractants through a 0.45 μm filter and analyzed using flow injection analysis (Lachat Instruments,

Milwaukee, WI). For nitrate-N and ammonium-N, 25 ml 2 M KCl was added to centrifuge tubes containing 5 g soil samples. The tubes were placed on a rotary shaker at 200 oscillations min^{-1} at 20° C for 60 minutes and then allowed to settle for one hour before filtering.

4.3.15 Experiment 4.4: Fertilizer studies

In order to test the feasibility of using extracted *Scenedesmus acutus* biomass A plant growth study was completed to compare plant growth and nutrient uptake by cucumber (*Cucumis sativus*) and corn (*Zea mays*) in Earthgro potting mix either not amended, amended with extracted algae, non-extracted algae, or a commercial grade fertilizer (4-6-6 Garden-tone).

For the cucumber study, 100 g of potting mix were placed in 10 cm plastic pots and amended with 0.0, 2.8, 5.5, and 11 g of either extracted or non-extracted algae. The application rates for the commercial fertilizer were either 1.25 or 2.5 g to yield a comparable N loading rate to the non-extracted algae. The potting mixture was stirred to distribute the fertilizer throughout the pot. Five cucumber seeds were planted in each pot and the pots were placed in a greenhouse. DI water was used as needed to water the plants with a leaching rate of approximately 10%. On day 27, the cucumber plants were cut at potting mix level, the biomass dried at 70° C for 48 h, and weighed.

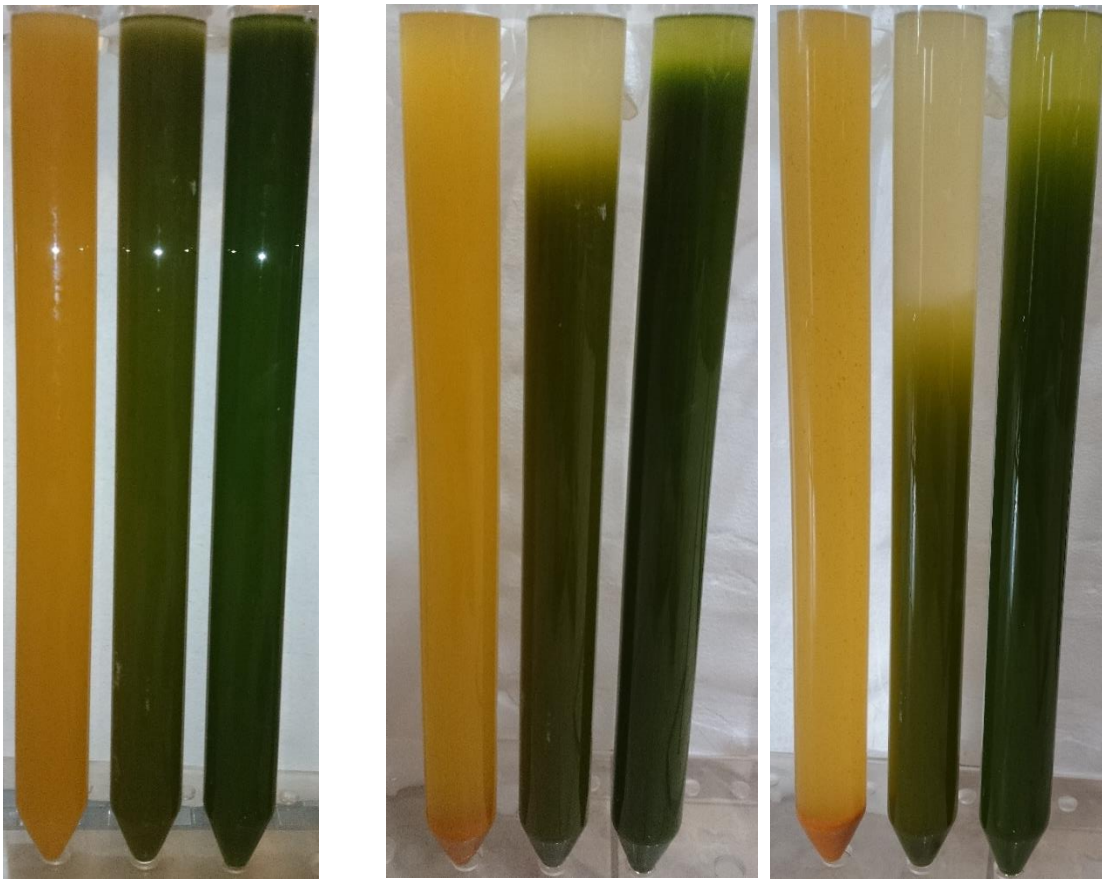
For the corn study, 15 cm plastic pots were filled with 400 g of potting mix and amended with either 0.0, 10, 20 g of extracted or non-extracted algae. The application rates for the commercial fertilizer were 5.5 and 11 g to yield a comparable N loading rate to the non-extracted algae. The potting mix was stirred and planted with five seeds. The pots were moved to the greenhouse and the plants were grown using natural lighting.

The plants were watered, with leaching, using DI water. After 29 days, the corn plants were cut at potting mix level and the biomass dried at 70° C for 48 h and weighed.

4.4 Results and discussion

4.4.1 Experiment 4.1: Gravity sedimentation

Biomass of *Scenedesmus acutus* strain LRB-AZ-0414 at three different physiological states was obtained by maintaining two acrylic flat panels on modified BG-11 media with full nitrate (246 mg N/L) and subsequent inoculation of two additional



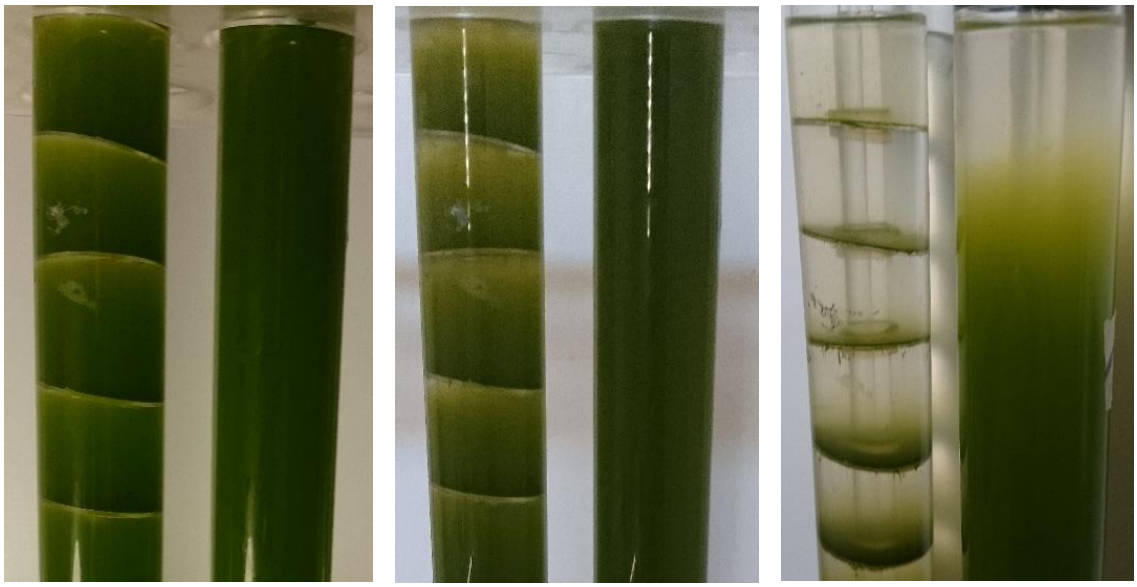
0 hours 3 hours 12 hours

Figure 4. 1: Settling rate of LRB-AZ-0414 biomass in 4.6 cm glass columns at zero, three and twelve hours. Left tube in each picture is late stationary phase, middle tube is stationary phase (high oil), and right tube is log phase biomass.

tanks with reduced nitrogen (61 mg N/L) each a week apart. This biomass was used in Experiment 4.1 to determine the settling rates of the strain at different physiological states as Figure 4.1 demonstrates at 0, 3, and 12 hours after filling the tubes. A layer formed in the culture tubes, separating the dense algal culture below from the relatively clear culture media above as the biomass began the sedimentation process. This layer was followed down the column over the course of 24 hours to track how far down the column (cm) the cells moved over time in order to determine sedimentation rates for the alga in differing physiological conditions.

The results indicate a significant difference ($p \leq 0.05$) for sedimentation rates of all three biomass states, with rates significantly increasing with increasing age of the cells. The log phase cells being approximately 1 week old cultures, while the stationary cells being 2 week old and late stationary being 3 weeks all three of which can be easily distinguished by color. The values for log phase biomass of 0.78 ±0.05 cm hr^{-1} appear to correlate well with literature values for other *Scenedesmus spp.* sedimentation rates of less than 1 cm hr^{-1} (Choi 2006). This is likely due to cell size and weight but may also be related to changes in structures on the cell wall. For instance, bristles of *Desmodesmus spp.* have been shown to greatly affect buoyancy when removed (Baudelet et al. 2017). The early stationary phase shows an improvement in sedimentation performance with a rate of 2.29 ±0.23 cm hr^{-1} while still leaving relatively clear culture media. However, by late stationary phase some of the old cells have begun to lyse and degrade into the water making efficient separation likely impossible without chemical flocculants.

The rate of sedimentation is closer to rates expected for feasible harvesting techniques being almost 20 times the rate of sedimentation for log phase biomass as displayed in Table 4.1 below. However, this stage would likely never be harvested and if



0 hour 1 hour 4 hours

Figure 4. 2: Gravity sedimentation of *Scenedesmus acutus* LRB-AZ-0414 in 2.5 cm glass tubes at zero, one, and four hours. The tube on the left has plastic disks to divide the column into 2.54 cm sections.

it were harvested, careful attention would have to be made to harvest before lysis of cell starts. With the observance of slightly cloudy culture media starting in early log phase, suspicions were raised about the presence of exopolysaccharides (EPS) known to be produced in some *Scenedesmus spp*. To investigate this further, the cells were removed from the media via centrifugation at 3000 g, which was then freeze-dried, weighed, and subjected to TFA digestion and analysis via HPAE-PAD to produce the values shown in Table 4.2 below. Together the sugar content accounts for almost 75 wt% of the roughly 0.5 g/L solids freeze-dried from the cell-free supernatant of the spent culture media.

Table 4. 1: Sedimentation rate of *Scenedesmus acutus* strain LRB-AZ-0414 at different physiological conditions.

Physiological state	Color	Rate (cm hr^{-1})
Log	green	0.78 ±0.05
Stationary	brown	2.29 ±0.23
Late stationary	orange	19.01 ±1.71

Table 4. 2: Sugar content (wt%) in the supernatant of centrifuged culture media of high oil *Scenedesmus acutus* strain LRB-AZ-0414 culture.

Sugar	% Dry weight
Mannitol	2.40 ± 0.85
Fucose	**15.68 ± 4.12**
Rhamnose	**10.82 ± 2.73**
Arabinose	2.34 ± 0.93
Glucosamine	1.98 ± 0.74
Galactose	4.91 ± 1.21
Glucose	**8.94 ± 1.49**
Mannose	**10.99 ± 1.56**

These sugars are consistent with many of the sugars found in TFA-hydrolysable fractions of other *Scenedesmus sp*. with the exceptions of mannitol, arabinose, and glucosamine (Baudelet et al. 2017). These could have originated from a contaminating species, which has recently been investigated for another *Scenedesmus acutus* strain (Schambach 2017). Little is known about this carbohydrate fraction found in the spent culture media after harvesting cells, however it offers another potential co-product for algae grown for biofuel production.

Figure 4.2 shows LRB-AZ-0414 sedimentation rates with and without 1 in plates in order to show how a lamella settler may help decrease harvesting times. Lamella

settlers have been suggested as a means to cost-effectively and efficiently harvest microalgae biomass (Janelt et al. 1997; Letourneau et al. 1988). However, many times this is in conjunction with a chemical flocculant. Due to the relatively low settling rates of this strain the lamella plates would likely have to be closer together and the retention times longer in order to harvest microalgae with are effectively neutrally buoyant particles a few microns in length. A sedimentation rate below 1 cm hr^{-1} is unacceptable for efficient harvesting. This is due to the fact that in harvesting algae biomass, speed is of upmost importance as the biochemical composition of the biomass can change significantly over the course of a few hours as demonstrated previously for this strain (Eustance et al. 2015b).

For this reason, most algae facilities like AzCATI use a centrifuge to harvest. AzCATI uses a Lavin 12-413V capable of producing 3000 g that was used to harvest biomass for comparison in this study. The centrifuge uses 1.12 kWh to harvest 1.57 kg of biomass for strain LRB-AZ-0414 with 90% removal efficiency. Assuming a calorific content of 6.5 kWh/kg biomass this would require 11% of energetic content of the biomass just to harvest it. This was also tested for strain LRB-AZ-0424, a heat tolerant *Scenedesmus acutus* strain, which came to 12% of the energy content of biomass. For this reason, centrifugation is only feasible for high-value products from algae and not algae for use as an energy crop. Sedimentation via lamella settler is a mostly passive process with minimal energy required for slow pumping of the culture through the settler. Lamella sedimentation of microalgae is an attractive approach to remove the bottleneck of harvesting and allow for commercial production of microalgae.

The results of Experiment 4.1 demonstrate the slow sedimentation rate of strain LRB-AZ-0414 along with the effect of physiological state on this rate and that lamella plates

used to divide the vertical distance of the cultures may help significantly reduce culture harvest time. Future studies should focus on design of a lamella device suitable for harvesting this strain with special attention on defining optimized parameters including plate spacing, angle, and loading rates. Development of such a device would remove the obstacle of finding an efficient, yet cost effective harvesting techniques that is mostly passive and requires minimal energy input.

4.4.2 Experiment 4.2: Cellular disruption

Experiment 4.2 utilized strain LRB-AZ-0414 biomass in early stationary phase to test the relative efficiency a Microfluidics microfluidzer model M110-EH at cellular disruption of the biomass. Cell walls of *Scenedesmus spp*. are robust and difficult to break to liberate cellular components of the biomass, such as the lipid content. This is due to a thick cell wall durably impregnated with polymers practically impervious to degradation, such as algaenan (Baudelet et al. 2017). This polymer is impervious to most enzyme attacks and is commonly found in ancient oil deposits having stood the test of time. The robust cell wall of these algae is thought to serve as grazing deterrent (Van Donk et al. 2010).

This also makes the algae difficult to extract for its valuable components. Extraction methods either use hazardous chemicals or are energetically or economically costly. This is the case of enzyme treatment that may one day provide a cost-effective breakthrough for this issue, especially if the enzymes can be recovered for multiple use (Gerken et al. 2013). Looking to nature for ideas may prove useful as a parasite specific to *Scenedesmus spp*. has recently been discovered without a clear mechanism of its mode of entry into the cell (Baudelet et al. 2017).

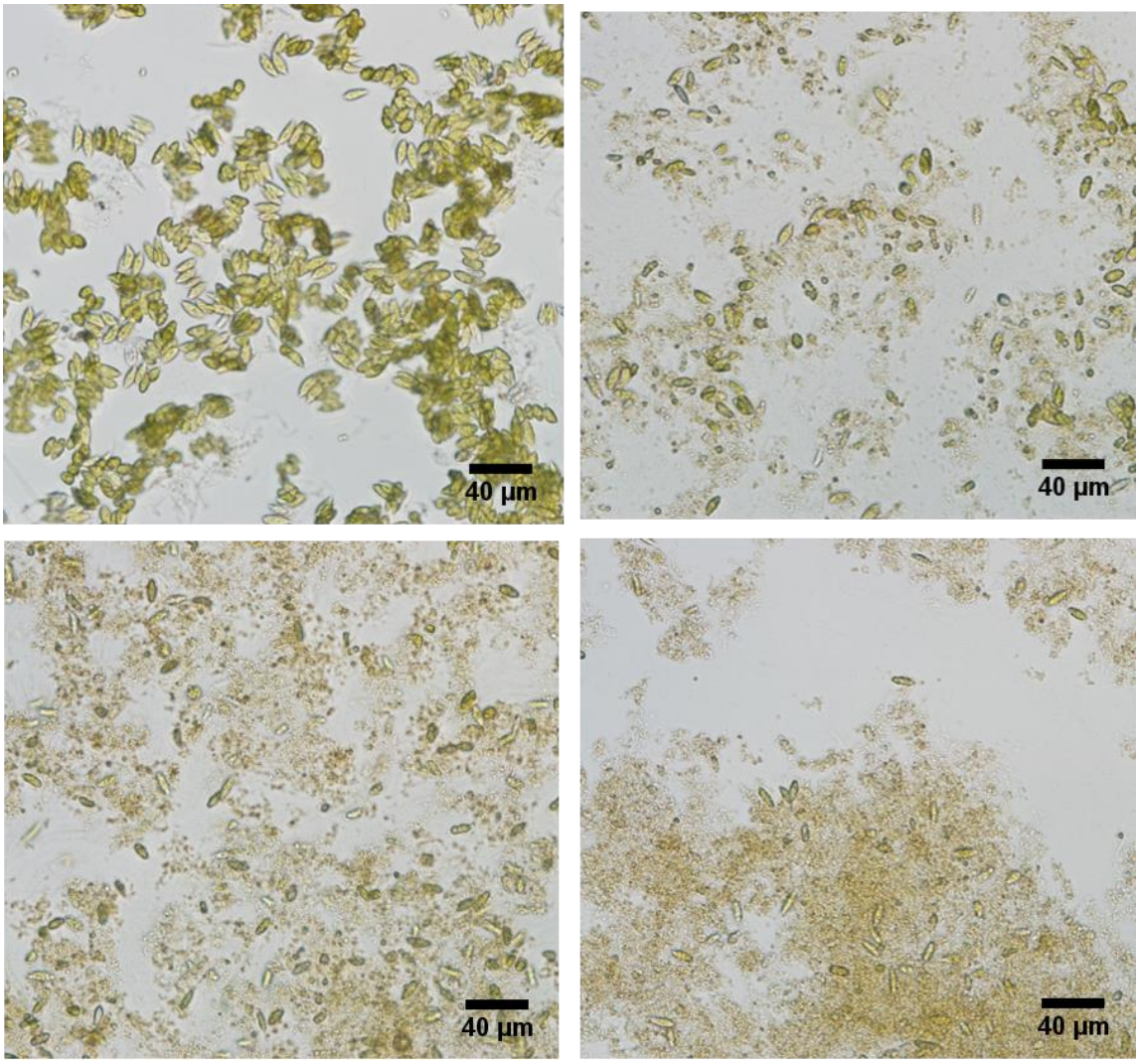


Figure 4. 3: Microscopic examination of microfluidized biomass at 30,000 psi through the diamond disruption chamber of the Microfluidics M110-EH. Top left is the raw biomass, top right is after one pass, bottom left after two passes, and bottom right after three passes.

Microfluidics is known for making laboratory scale microfluidizers for cellular disruption in labs, however the M110-EH is a larger unit capable of continuous duty one might expect at a commercial algae production facility. As shown in Figure 4.3 above, the M110-EH effectively disrupted strain LRB-AZ-0414 with 3 passes at 30,000 psi

through the machine, leaving a minimal amount of cells intact. This is invaluable for releasing the high-value components from the biomass such as lutein or other carotenoids, oil, or for other general research purposes that require a significant sample volume. However, the machine requires 3.73 kW of power to process 325 mL per minute at a maximum 15% solids for strain LRB-AZ-0414. At this loading rate the machine can process 2.93 kg/hr and 0.78 kg/kWh. This energetic consumption would require approximately 20% of the energetic content of the biomass (assuming 6.5 kWh/kg) with each pass through the machine, which means this method of cell rupture would not be feasible for use on energy crops. However, use of this technology could prove useful for extraction of high-value components from the biomass such as carotenoids, carbohydrates, etc. LRB-AZ-0414 was analyzed via HPLC to determine relative content of pigments in early and late stationary phase biomass. The results, summarized in Table 4.3 below, show a number of high value carotenoids from the algae albeit much lower than other strains reported in the literature (Galarza et al. 2018; Liu et al. 2014; Jeon et al. 2014). Still these carotenoids may serve as a valuable co-product with the right extraction technique.

4.4.3 Experiment 4.3: Mineralization study

Experiment 4.3, the soil mineralization study, was completed to quantify the net mineralizable N of extracted and non-extracted algae applied to Gilman loam soil as adapted from (Mulbry et al. 2005). According to CHNO/S combustion analysis shown in Table 4.4 below, the total N content per gram of algae biomass was higher for the extracted than for the non-extracted algae. This is likely a direct result from the relatively greater reduction of carbon content as compared to nitrogen in the process of extracting the oil. This effectively reduced the C:N ratio of the extracted biomass. The N loading

Table 4. 3: Carotenoid and chlorophyll content as percentage of dry weight for LRB-AZ-0414 strain harvested from flat panel photobioreactors in BG-11 culture medium.

Pigment	% Dry weight	
	Early Stationary	Late Stationary
Lutein	**0.068 ± 0.000**	**0.015 ± 0.001**
Canthaxanthin	0.046 ± 0.000	0.048 ± 0.000
B-Carotene	0.025 ± 0.000	0.017 ± 0.001
Astaxanthin Monoester	0.001 ± 0.000	0.003 ± 0.000
Astaxanthin Diester	0.015 ± 0.000	0.024 ± 0.000
Free Astaxanthin	0.009 ± 0.000	0.004 ± 0.000
Total Astaxanthin	0.036 ± 0.015	0.031 ± 0.000
Chlorophyll a	**0.226 ± 0.000**	**0.019 ± 0.001**
Chlorophyll b	**0.160 ± 0.160**	**0.004 ± 0.000**

Table 4. 4: CHNO/S analysis summary of *Scenedesmus acutus* strain LRB-AZ-0414 biomass with C:N ratio before and after lipid extraction.

Biomass state:	Carbon (wt%)	Nitrogen (wt%)	C:N
Raw biomass	55.34 ±3.82	6.17 ±0.37	12.9±3.84
Extracted Biomass	47.52 ±0.55	4.30 ±0.06	7.7±0.55

rates for this incubation study using 0.75 g and 1.5 g extracted algae were 463 and 926 mg kg^{-1} soil. The corresponding N loading rates for non-extracted algae were 323 and 646 mg kg^{-1} soil.

On day 0, the control, which was Gilman loam without amendments, tested with the highest level of mineral N at 59 mg N kg^{-1} soil as displayed in Table 4.5 below. However, by day 7 the soil amended with extracted algae showed a significant increase in mineral N while the control showed a slight decline in mineral N. By day 7, the soil amended with non-extracted algae showed a significant decline in mineral N. From day 7 to the end of the testing on day 42, the treatments with extracted algae had the mineral N available, mostly in the form of nitrate.

The treatment with 1.5 g continually had the highest levels of mineral N although the level on day 21 dropped somewhat. While the majority of available nitrogen was in the form of nitrate for all treatments, there was a spike on day 7 for the treatments with extracted biomass where ammonium release spiked. The reason for this is unknown and deserves further investigation. The treatments with non-extracted biomass had the least amount of mineral N available. The treatment with 1.5 g continually had the least level of mineralized N ending with 29.59 mg N kg^{-1} on day 42. All the treatments showed an increase in mineral N between day 21 and day 42. Overall, these results suggest that both extracted or non-extracted algae acts best as a slow-release N fertilizer for plants with the extracted biomass having more available N from the beginning while the non-extracted biomass likely has a lot of intact cells that have to first degrade in the soil in order to release nitrogen reserves.

The decrease in available soil N could be due to soil microbes utilizing scarce nitrogen resources in the beginning until the non-extracted biomass degrades enough to start supplying N to the soil. This process seems to start well beyond 30 days following application to soil. The soil N only started to increase by day 42 of the mineralization

Table 4. 5: Mineralization (mg N kg^{-1} soil) of non-extracted and extracted *Scenedesmus acutus* LRB-AZ-0414 biomass in Gilman loam.

Amendment	Applied N	Day 0	Day 7	Day 21	Day 42
0 (Gilman loam)	0	59.00±7.83	56.00±2.39	44.12±7.73	79.06±1.54
750 (extracted)	463±0.28	48.98±5.83	97.22±10.0	112.47±8.45	161.29±1.73
1500 (extracted)	926±0.55	45.91±6.43	**150.17±13.3**	**130.27±18.8**	**198.31±3.26**
750 (non-extracted)	323±1.20	45.82±4.43	8.77±1.47	16.46±2.75	36.72±2.07
1500 (non-extracted)	646±2.39	42.23±1.00	**1.87±1.06**	**7.57±1.81**	**29.50±3.22**

study. The extracted biomass supplies regular N levels with a slight dip in N levels only seen at day O.

The *Scenedesmus acutus* strain LRB-AZ-0414 used for this study is a unicellular microalga with a thick cell wall. The extraction process used to remove oil from the algae disrupts the cell wall of the algae, selectively removing the lipid fraction. The process removes more C than N, which may account for the extracted algae containing 61,700 mg kg^{-1} of N versus 43,000 mg kg^{-1} of N in non-extracted algae as seen in Table 4.6 above. In addition, the disrupted cell walls of the extracted algae likely accounts for the initial faster N mineralization rate. However, the algae biomass was not examined to confirm if non-extracted algae had more intact cells, which is an area in need of more refinement in future studies.

Table 4. 6: Nutrient Content (mg kg^{-1}) of extracted and non-extracted *Scenedesmus acutus* biomass in comparison to Garden-tone 4-6-6 fertilizer.

Constituent	Extracted algae	Non-extracted algae	Garden-tone 4-6-6 Fertilizer
N	61,700	43,000	48,000
P	13,000	14,500	25,200
K	2,147	5,125	49,800
Ca	27,790	51,720	30,000
Mg	7,029	1,951	5000
Fe	728	149	10,000
Al	654	187	n.d.*
	148	207	500
Zn	113	74	500
Cu	5,829	4,720	500
Cd	0.10	.02	n.d.
Pb	50.90	10.5	n.d.
Ni	3.09	7.0	n.d.
Mo	0.68	n.d	5

* n.d. is not determined

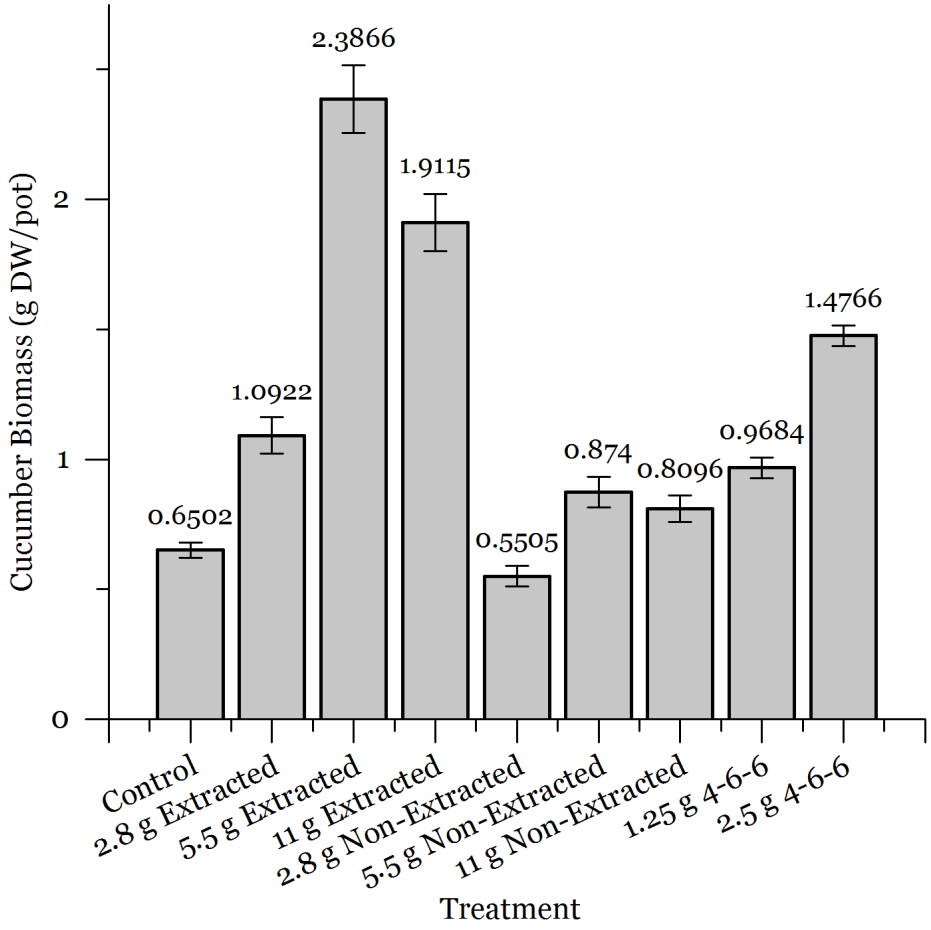


Figure 4. 4: Cucumber biomass production in dry weight (g/pot) over 27 days for algae fertilizer treatments in comparison to a commercially available 4-6-6 fertilizer. Vertical bars denote standard error.

4.4.4 Experiment 4.4: Fertilizer studies

Experiment 4.4 was a plant growth experiment completed to compare the growth and nutrient uptake of cucumber (Figure 4.4) and corn plants (Figure 4.5) grown in Earthgro potting mix amended with algae biomass or a commercial fertilizer (Garden-tone 4-6-6). The cucumber plants, grown with 5.5 g of extracted algae yielded the most dry weight (DW) biomass at 2.9 g pot^{-1}. The plants grown on 2.8 g non-extracted algae had the least DW biomass at 0.6 g pot^{-1}which was slightly less than the control

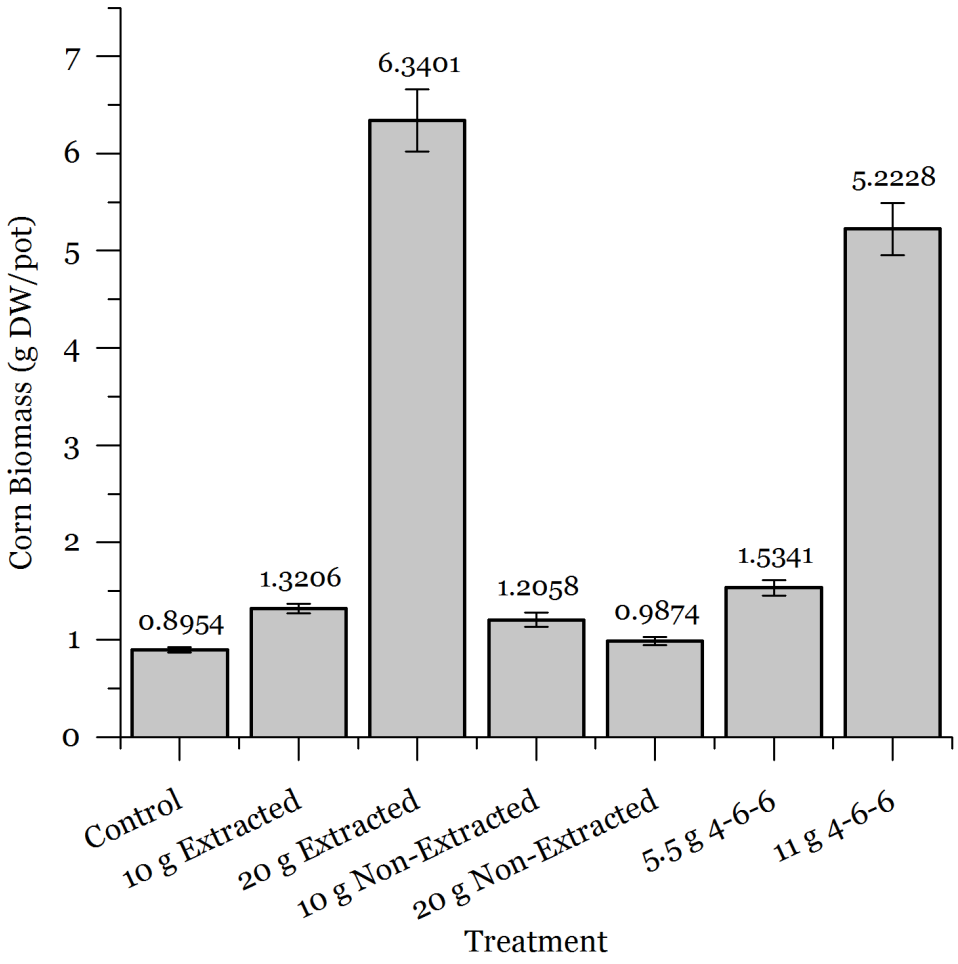


Figure 4. 5: Corn biomass production (g/pot) over 27 days for algae fertilizer treatments in comparison to a commercially available 4-6-6 fertilizer. Vertical bars denote standard error.

(0.7 g pot^{-1}). This correlates with the findings of available N taken from Experiment 4.3, the mineralization study. The amount of cucumber plant biomass produced on the treatment with 2.8 g of extracted algae fell between the treatments with 1.25 g and 2.5 g of 4-6-6 fertilizer. The treatments with 5.5 g and 11 g of extracted biomass showed significantly more biomass production than all the other treatments as clearly demonstrated in Figure 4.6 below.

The corn plants grown with 20 g of extracted algae produced the most DW corn biomass with a yield of 6.3 g pot^{-1}. The commercial 4-6-6 fertilizer yielded 5.2 g pot^{-1} DW of corn plant biomass. All other treatments produced significantly lower amounts of plant biomass ranging from 0.9 to 1.5 g DW^{-1} pot as displayed by Figure 4.7 below. Corn, being a crop with a high requirement for nitrogen, was a perfect crop to test algae biomass as a fertilizer. Not only was a relatively large amount of algae required to effectively meet the nutrient requirement for emerging corn (20 g) but the biomass must certainly be extracted to provide adequate nutrients to the newly emerging corn as evidenced by the poor growth performance of the 20 g treatment with not-extracted algae biomass.

This is especially important to note when considering the use of a microalgae-based fertilizer. Based on these preliminary growth results and the results of the mineralization study it is likely extracted biomass acts more like an inorganic fertilizer. This is likely due to the fact the cells have chemically weakened or ruptured cell walls, making nutrients readily available for plant growth. Non-extracted algae biomass would seem to work more as a long-term slow-release organic fertilizer that releases nutrients into the soil overtime, although more research will be required to confirm this.

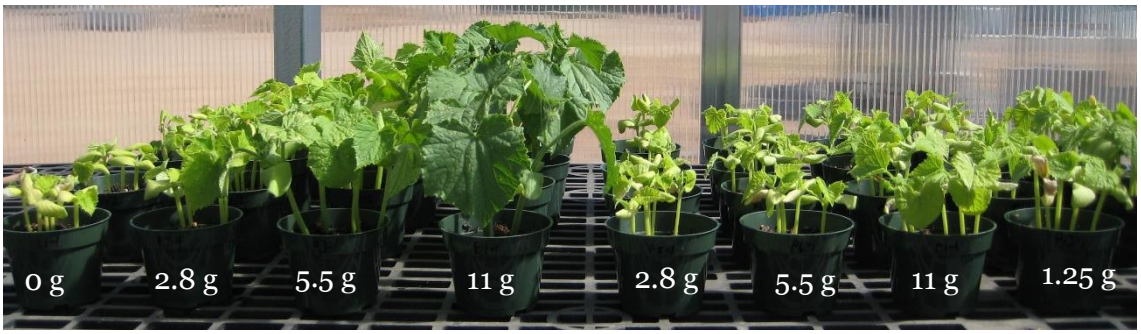


Figure 4. 6: Cucumbers grown for 27 days on 0 g ext, 2.8 ext, 5.5 g ext, 11 ext, 2.8 g non-ext, 5.5 g non-ext, 11 g non-ext algae biomass and 1.25 g 4-6-6 fertilizer from left to right, respectively. (ext=extracted, non-ext=non-extracted)

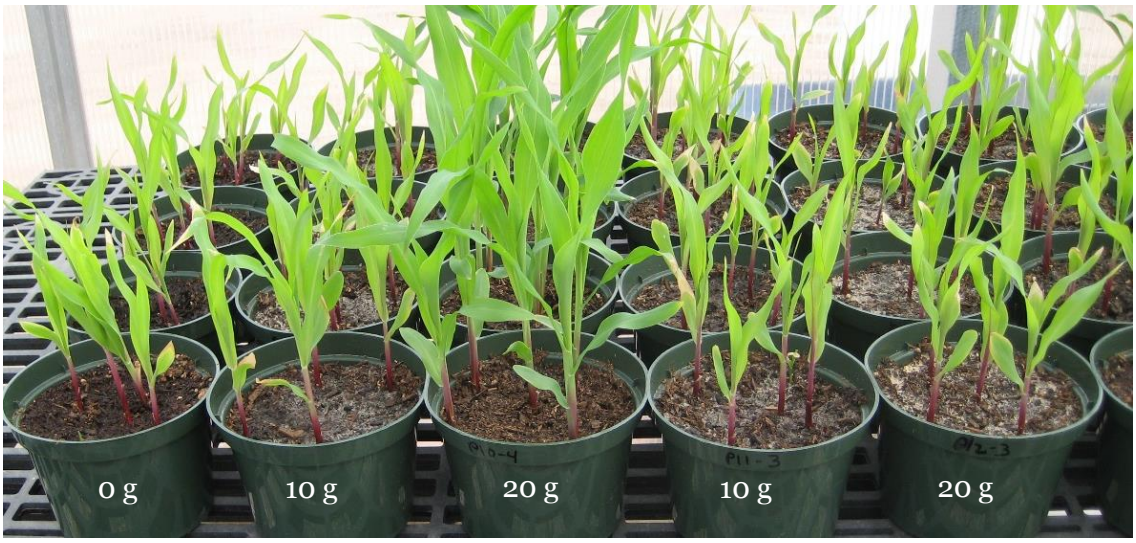


Figure 4. 7: Corn grown for 27 days on 0 g, 10 g ext, 20 g ext, 10 g non-ext, 20 g non-ext from left to right, respectively (ext=extracted, non-ext=non-extracted).

4.5 Conclusions

In order to make commercial algaculture a reality, harvesting and post-processing techniques must be energetically and economically feasible for the given product being produced. Additionally, is important to consider all available potential co-products from algae cultivation, including all products isolated from the biomass or culture medium or those left over after extraction of valuable components of the

biomass. This will help to improve the overall economic feasibility of producing an alga strain.

This study has demonstrated that *Scenedesmus acutus* strain LRB-AZ-0414 is plagued by low sedimentation rates (<1.0cm hr^{-1}) which significantly increases with the age or physiological state of the biomass, but not enough to quickly and effectively harvest the biomass, especially green log phase biomass. This makes harvesting this strain a difficult and costly venture for an algae company. Centrifugation of LRB-AZ-0414 is an energetically costly with the harvest process consuming up to 11-12% of the energy content of the biomass itself. While this may be feasible for microalgae currently commanding high market values as nutraceuticals or other specialty products, this does not open the doors for algae to become an agricultural commodity or biofuel crop.

Using lamella settler principles as inspiration, a settling tube was designed with plate spacing of 2.54 cm, much narrower than traditional lamella settlers. Data collected demonstrates the potential of the design to greatly reduce time and energy required to harvest high-oil LRB-AZ-0414 biomass. The inherent settling rate is not reduced but the distance and time required to settle out of the water suspension is controlled by plate spacing. Lamella settlers for algae would be mostly passive in design and would only require the use of a low flow pump to pass the culture over the settling plates and a sludge pump to gather the concentrated biomass. More research should be devoted to developing such simple and mostly passive harvesting techniques to reduce the burden of harvesting for emerging microalgal ventures.

After the biomass is harvested, microalgae must generally be treated to release the desired biomass components. Microfluidization of strain LRB-AZ-0414, as investigated in this study, lead to nearly complete cellular disruption of high oil biomass

after 3 passes through the unit at 30K psi. This, however, has a high energetic cost consuming up to 20% of the energy content of the biomass per pass and therefore would not be feasible for production of low-value microalgal products and definitely not feasible for use with biofuel crops unless the biofuel was by-product of production for something more valuable. High oil LRB-AZ-0414 biomass was shown to contain trace amounts of lutein, astaxanthin, canthaxanthin. Also, the freeze-dried spent culture media was shown to be rich in TFA-hydrolysable sugars including fucose, mannose, rhamnose, and glucose. These could serve as by-products to lower the overall cost of production if commercial production of this strain as an animal feed or biofuel crop one day becomes a reality.

Another potential use of strain LRB-AZ-0414 was to use the extracted biomass as a fertilizer amendment for plants after oil extraction. A soil mineralization study demonstrated the release of predominantly nitrate but also ammonium from algae biomass into the soil for use as a plant fertilizer. The lipid-extracted algae biomass shows a slight decrease in nitrogen content from 6% to 4% and a corresponding decrease in C:N ration after lipid (C) is removed. Non-extracted algae biomass first depletes soil nitrogen upon incorporation of biomass followed by slow release over a period of weeks, similar to a slow-release organic fertilizer. Extracted algae shows immediate release of nitrogen into soil, followed by a sustained gradual increase up to 198 mg N kg^{-1} soil by day 42.

Plant growth trials of both corn and cucumber seedlings demonstrated the potential of lipid-extracted algae biomass as a N fertilizer to corn and cucumber plants, with similar performance to a commercial grade 4-6-6 fertilizer. Extracted biomass increased availability of N in the soil faster than non-extracted biomass, which

temporarily decreased available soil N for the emerging seedling, thereby stunting its growth. Non-extracted algae might be blended with extracted algae to create extended or continuous release fertilizer.

4.6 Acknowledgments

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5. CONCLUSIONS AND FUTURE WORK

The purpose of this work was to identify and find ways to reduce three major bottlenecks of current commercial microalgal culture. These three main obstacles include the following areas concerning current microalgae biotechnology:

- Optimize a micro-environment better suited for photosynthesis.
- Optimize utilization waste nutrient sources for efficient biomass production.
- Optimize harvesting and disruption techniques for a given strain being cultivated.

All three of these areas have been addressed with a chapter devoted to each and the main conclusions summarized below. Recommendations and ideas for future research endeavors are discussed in more detail for each chapter summarized below.

5.1 Achieving higher yields in the field

Chapter 2 attempted to debunk a long-standing debate about best practices for light utilization for microalgal cultures grown outdoors in a field environment. Is the productivity and photosynthetic efficiency of photobioreactors the best way to go or is it the economy of raceway ponds that is needed for commercial algaculture. This chapter was intended to shine light on the underlying principles that should guide decisions on how microalgae is produced in the field. The following is a summary of the major points demonstrated in Chapter 2:

- The ability of two strains of *Scenedesmus acutus* to be cultured in narrow flat panel photobioreactors at high cell density and at different temperature ranges for year-round production of biomass in the desert Southwest.

- The reduction of light path in an algal culture accelerates its growth rate and oil production, and increases volumetric and areal output.
- Aeration, temperature control, and mixing can be an issue for very thin flat panels.
- Biomass in stationary phase cultures of *Scenedesmus acutus* has higher energetic content compared to cultures in log phase.
- High-density algae cultivation has the potential to reduce aeration costs for flat panels 4 fold or more while reducing water requirements by the same factor.

All of the findings for Chapter 2 support a growing trend in the literature on algae biotechnology over the years towards production systems with thinner light paths and higher density cultures. Record areal and volumetric biomass yields and photosynthetic efficiencies are being observed as the culture thickness of a given production system approaches that of a leaf. For example, some biofilm reactors are only a couple mm thick and the Czech cascade systems take raceway design to the extreme with 6-7 mm thick cultures. Cultures can now be measured in % solids instead of g/L. With the momentum of algae research articles increasing in recent years and thousands of new articles getting published each year, the field is destined to see some innovative approaches coming soon.

New photobioreactor designs should rely more on passive systems whenever possible. For example, the use of sunlight as opposed to artificial lighting and thinner light paths for dense cultures or turbid waters. Also, the use of passive heating and cooling systems such as geothermal, lake or ocean water, etc. could greatly improve the feasibly of algal production systems and help remove this roadblock to commercial production. Thin film plastic bag reactors such as OMEGA or the system designed by

Photon 8 are promising economical and flexible designs capable of integrating may passive systems into one. Horizontal systems that can be deployed on the ground with no supports or greenhouse enclosures, etc. are also attractive in the fact they minimize pumping costs associated with vertical systems or cascade runways that rely on gravity moving cultures down a slope to mix the culture. Every time that lifting water against the force of gravity can be minimized, energy efficiency will be gained in the system. Circulating suspended phosphorescent particles within the culture is another idea of passively bringing light into the culture to increase production and deserves future attention from researchers. The field and future research endeavors should avoid complicated systems requiring most high capital or operational costs. Simplicity and good design working with the mechanics of photosynthesis is key.

5.2 Utilizing wastewater and flue gas

Utilizing waste nutrient streams such as flue gas or wastewater is important to realize microalgae's potential as an agricultural commodity. This is particularly important in order to lower the costs of producing microalgae crop as the current cost of production is an order of magnitude above all other agricultural commodities. The purpose of Chapter 3 was to explore the possibility of growing a given alga strain on a number of wastewater media to come up with recommendations on how such waste streams could potentially be utilized to grow the strain as a crop. The main conclusion of Chapter 3 include:

- *Scenedesmus acutus* strain LRB-AZ-0414 can be successfully cultivated in acrylic flat panel reactors using flue gas from a natural gas-fired boiler on a semiconductor fab plant.

- LRB-AZ-0414 demonstrated significantly better growth achieving up to 4.96±0.11 % PE based on PAR for the flue gas tanks and 4.01±0.23 % PE grown on commercial grade CO_2.
- NO_x emmissions can be captured and used in algal cultures and a 4.57 m column captured 100% of the simulated 40 ppm NOx emmissions with addition 5mM $FeCl_3$ but requiring signifant energy to do so. Algae at 2.0 g/L alone removed 58% of NOx emissions.
- A horizonatal NO_x contact reactor decreases the energy input and demonstrates up to 40% NO_x removal in 1.8 mM Fe(III)EDTA.
- An N:P ratio of 40 was found to produce the highest lipid yield amongst nitrogen deprived cultures.
- Municipal anaerobic digester centrate and dairy lagoon wastewaters make good culture media for strain LRB-AZ-0414 but have high turbidity that blocks light into the culture.
- Turbidity can be reduced with lime or polymers, but nutrients are removed in this process Dilution is another option for reducing turbidity of wastewaters
- Centrate performed better than BG-11 media in acrylic flat panel reactors producing an overall 12.3±0.55 g m^{-2} day^{-1} and 34.8% total lipid.

The conclusions drawn from Chapter 3 show that strain LRB-AZ-0414 is a versatile oleaginous algae able to be cultured on a variety of wastewater sources. Chapter 3 also emphasizes the challenges of dealing with volatile nutrients such as CO_2, NH_3 or NO_x, which are tricky to deal with on a commercial scale. There are many ideas that have been proposed that merit further investigation including the aqua ammonia process for CO_2 capture, where the ammonium bicarbonate produced would able to be utilized as

a nutrient source for cultures. Also simple aquarium diffusers for injection of CO_2 into cultures in order to increase uptake efficiency or covered dairy lagoons as a means of capturing ammonia and CO_2 emissions for use in algal cultures would be worthwhile projects. Alternatively, the use of lime to precipitate PO_4 and to collect the volatilized ammonia in an acid trap to feed dose-wise to an algal culture is another possibility. There are countless ideas to be explored and many possibilities for improvement.

5.3 Economical Harvesting

In order to make commercial algaculture a reality, harvesting and post-processing techniques must be energetically feasible for the given product being produced. Additionally, all available co-products from algae cultivation should be considered to improve the overall economic feasibility of producing an alga strain. This study has demonstrated the following about *Scenedesmus acutus* strain LRB-AZ-0414:

- LRB-AZ-0414 is plagued by low sedimentation rates (<1.0cm hr^{-1}) which significantly increases with the age or physiological state of the biomass.
- A lamella settler designed with plate spacing of 2.54 cm has potential to greatly reduce time and energy required to harvest high-oil LRB-AZ-0414 biomass.
- Centrifugation of LRB-AZ-0414 is an energetically costly harvest process consuming 11-12% of the energy content of the biomass itself.
- Microfluidization of strain LRB-AZ-0414 lead to nearly complete cellular disruption of high oil biomass in 3 passes through machine at 30Kpsi, but with a high energetic cost consuming 20% of the energy content of the biomass per pass.
- High oil LRB-AZ-0414 biomass was shown to contain trace amounts of lutein, astaxanthin, and canthaxanthin that are valuable by-products.

- Freeze-dried spent culture media was shown to be rich in TFA-hydrolysable sugars including fucose, mannose, rhamnose, and glucose.
- Soil mineralization studies show release of predominantly nitrate but also ammonium from algae biomass into the soil for use as a plant fertilizer.
- Lipid extracted algae biomass shows a slight decrease in nitrogen content from 6% to 4% and a corresponding decrease in C:N ration after lipid (C) is removed.
- Non-extracted algae biomass first depletes soil nitrogen upon incorporation of biomass followed by slow release over a period of weeks.
- Extracted algae shows immediate release of nitrogen into soil, followed by a sustained gradual increase up to 198 mg N kg^{-1} soil by day 42.
- Lipid-extracted algae biomass has value as a N fertilizer to corn and cucumber plants, with similar performance to a commercial grade 4-6-6 fertilizer.
- Extracted biomass increased availability of N in the soil faster than non-extracted biomass, which temporarily decreased available soil N for starting seedling.
- Non-extracted algae might be blended with extracted algae to create extended or continuous release fertilizer.

The finding of Chapter 4 demonstrates the importance of selecting the appropriate harvesting and post-processing techniques when considering growing a given microalga as a new crop. For instance, if one uses a centrifuge to harvest high value products like carotenoids or DHA, etc., then investing in the high energetic and capital costs of the centrifuge might be the way to go. However, if one is growing the algae for livestock feed or energy crop then this does not make economical or practical sense, as the harvesting costs are far too high. Chapter 4 was successful in demonstrating this concept and outlining potential options for stain LRB-AZ-0414.

Constant iterations of design and consideration of multiple paths for harvesting or processing will help progress the field in a direction to eventually remove this roadblock to commercial algaculture.

5.4 Future outlook

By combining efficient, low-cost microalgal production systems with the knowledge of how to best utilize light and waste nutrient streams while optimizing harvesting and processing for a given alga strain, the roadblocks preventing wide scale microalgal culture can be removed. This would allow many exciting new crops to hit the marketplace, with new services to be offered, etc. To get there, improvements must be realized across all three areas simultaneously. However, this goal is attainable with the focus of diligent researchers across the globe continuing to investigate promising strains, cultivation strategies, harvesting and post-processing techniques. This will drive the cost of microalgal production down to the level of other agricultural commodities

Many people currently picture algae as a potential biofuel source. Only when the cost of microalgal production is significantly reduced could microalgal biofuel be economically feasible. This would likely be in conjunction with other forms of renewable energy such as solar, wind, etc. A rapidly expanding knowledge base for microalgal husbandry will continue to fuel new technological advances in algal production, in both fields and research labs, bringing algaculture continually closer to mainstream agriculture where its true potential can finally be realized.

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