DEVELOPMENT, OPTIMIZATION, AND ECONOMIC EVALUATION OF A NOVEL ATTACHED GROWTH ALGAE CULTIVATION SYSTEM

by

MICHAEL THOMAS JOHNSON

A dissertation submitted to the

Graduate School – New Brunswick

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Environmental Sciences

Written under the direction of

Dr. A.J. Both

And approved by

_____

_____

_____

_____

New Brunswick, New Jersey

October 2017

ABSTRACT OF THE DISSERTATION

Development, Optimization, and Economic Evaluation of a Novel Attached Growth Cultivation System.

By MICHAEL JOHNSON

Dissertation Director:

Dr. A.J. Both

The focus of this work was to evaluate a novel attached cultivation system developed by AL-G Technologies Inc. through lab-scale experiments for its ability to produce algae biomass and then to utilize these learnings to model its commercial operation at large-scale. The intention of conducting this work was to determine if this unique cultivation platform had potential advantages over traditional open raceway ponds and photobioreactor systems as these traditional systems are limited in their financial viability. It was demonstrated that several algae and cyanobacteria species (*Parachlorella kessleri*, *Tetraselmis chuii*, *Botryococcus braunii*, *Thalassiosira sp.*, *Chaetoceros calcitrans and Oscillatoria sp.*) could be cultivated on the system and that *P. kessleri* could achieve potential large-scale productivities exceeding 10 grams of dry biomass m^{-2} d^{-1}. Through cultivation experiments, it was shown that freshwater algae species tend to grow better than marine algae and

substantial salinity gradients retard growth rates. Contrary to the original hypothesis, it was demonstrated that attached growth on the system does not significantly alter the nutritional composition of algae in a favorable manner and that while the nutritional composition of the nutrient solution can be rapidly changed to remove a nutrient constituent (e.g., nitrogen), this does not result in as rapid of an accumulation of lipids as it does in traditional suspended growth systems. Through the cultivation experiments, it was also demonstrated that algae on the system are carbon limited and their growth rates increase with supplemental carbon provided they are continually harvested and dense biofilms do not form that limit irradiation and gas diffusion.

Through basic research experiments it was shown that our current mechanical harvesting approach is not operationally or financially scalable and thus we evaluated an irrigation-based harvesting system that was able to achieve a harvest concentration of 1.4 grams of dry biomass L^{-1}. These findings were combined to model the financial viability of the system where it was demonstrated that the system could achieve a production cost of \$7.53 to \$16.17 kg^{-1} of algae, which would be in suspension requiring subsequent dewatering and processing. This production cost varied based on the location of the system within the United States and the production was primarily driven by temperature because it drove the cost of heating the greenhouse that contained the system, as well as by the productivity of the algae. Based on the high production cost, the system would be limited commercially to the production of a few nutritional products (e.g., astaxanthin, omega-3 fatty acids, β-carotene) and fishery feed. Therefore, future research efforts with the system should focus on the growth of marine algae species, limiting salinity gradients, and minimizing contamination. However, there are currently no substantial benefits of this

system in comparison to traditional systems as it costs 3 to 8 times more to construct and up to 35 times more to operate to produce the same quantity of algae biomass per area under ideal conditions compared to traditional systems.

Acknowledgements

My PhD work has led me down a path of discovery and growth that I could never have anticipated and I will always look fondly on the profound impact this project and my PhD experience had on me personally and professionally. Working on my PhD while working multiple jobs and launching a biotech company was an incredible challenge and I could not have done it without the constant support and love of my wife Kassandra. She was always there for me and put up with the late nights and early mornings that it took to accomplish so much in such a short period of time. Along the way, I met and had the pleasure of working with numerous mentors such as Dr. A.J. Both, Dr. Gaston Picard, Dr. Jim Simon, Mike De Luca, Dr. Stephen Suh, John Miller, Dr. Jerry Batista, Dr. Kevin Jordan, Dr. Uta Krogmann, Dr. Daniel Gimenez, Dr. Chris Uchrin, Dr. Peter Strom, Dr. Eric Lam, Dr. Linda Anthony, Eduardo Merkovich, Dr. George Heinrich, Dr. George Merz, Mike Wiley, Dr. Carolyn Salafia, Dr. Scott Kachlany, Dr. David Crockett, and Dr. Mark Pierce who have helped me grow and prosper as a researcher, entrepreneur, and person. I am incredibly fortunate to have had Dr. A.J. Both as my PhD advisor as he encouraged me to obtain an interdisciplinary set of skills and to always be open to new challenges and projects. This driving force led me to human waste ponds in Argentina, building CT scanners, and numerous opportunities that changed my perspective on the world and showed me how we can use science to solve the BIG problems. Without this freedom and encouragement, I certainly would not have achieved so much in such a short period of time and would have never launched Visikol Inc. I would also like to acknowledge the National Science Foundation for funding my dissertational work through an NSF IGERT Biofuels Fellowship. This program provided me the flexibility and

autonomy to jump well outside of my field and to become an expert in bio-imaging and I am sincerely grateful to the NSF for this opportunity.

This research was funded by the National Science Foundation Interdisciplinary Graduate Education and Research Traineeship Biofuels Fellowship program (NSF DGE 0903675), the United States Department of Agriculture (USDA Rural Business Enterprise Grant No. 00-08), Creation of Jobs and New Economic Benefits in South Jersey from Aquaculture, the Rutgers University Department of Marine and Coastal Sciences, the Rutgers School of Environmental and Biological Sciences, the New Jersey Agricultural Experiment Station and the Rutgers New Use Agriculture and Natural Plant Products Program. I want to give special thanks to Mike De Luca, Charlotte Fuller, Piotr Nawrot, AL-G Technologies, Inc. and Dr. Gary Taghon.

Contents

List of Tables

List of Illustrations

Introduction

As the world's population continues to grow and the overall quality of life improves for people around-the-world, the demand for resources and energy will continue to increase. Due to the finite amount of resources on the earth and the negative environmental impact of generating energy through fossil fuel combustion, it is desired that this demand can be met in a sustainable manner that leaves future generations with the same amount of resources that we currently have access to. This general theme is the motivation for this work wherein this work investigates a novel algae production system that was thought to produce more biomass and in a more sustainable manner than current algae production systems. Algae includes diverse groups of organisms that can convert light energy into chemical energy through photosynthesis and can do so while not requiring arable land or potable water, thus making them an ideal candidate for biofuel and biomass production. Producing biomass and potentially biofuel while not requiring potable water or land that could be used to produce crops, has made algae very attractive candidates for sustainably producing resources compared to the practice of using food crops such as corn and sugarcane, because that practice increases food prices, eutrophication, deforestation, and has several other indirect consequences.

Traditionally, either algae are cultivated in a large open raceway pond (ORP) that mimics the natural environment for algae in aquatic systems or they are cultivated in closed translucent vessels referred to as photobioreactors (PBR). Large ORP systems are typically designed as shallow (approximately 1 m deep) ellipsoid loops wherein algae and the nutrient solution are circulated using a paddle wheel (Demirbas, 2010). At commercial-

scale, hundreds of ORP systems could be placed side-by-side in a region that is not suitable for farming but has high light insolation and a sufficient supply of clean water. While these systems are simple to construct and relatively inexpensive, they have several disadvantages:

- **Contamination:** Because ORPs are maintained as large open systems, they are susceptible to contamination by heterotrophs (e.g., rotifers) that will consume algae as well as contamination by other algae species (Ashokkumar and Rengasamy, 2012; Slade and Bauen, 2013). If a researcher is attempting to cultivate a specific type of algae because of the extractable compounds (e.g., high lipid content, high astaxanthin content), it is likely that the algae species cultivated are not the most ideal algal species to thrive in that set of conditions. For example, an algae species that does not produce large quantities of high-value nutritional products might grow much more rapidly than a target species in that environment, leading to a situation in which the targeted algal species can be outcompeted within a short period of time, and the culture would no longer be comprised of the ideal algae. This contamination can occur rapidly and can be caused by something as simple as a bird carrying algae from one pond to the next.
- **Low productivity:** Optimal algae productivity depends on several factors including irradiation levels, access to CO_2, nutrient solution composition (e.g., nutrients, pH, salinity) and temperature (Handler et al., 2012). In an ORP, CO_2 can be added via supplemental CO_2 pumping or bicarbonate addition and the nutrient solution composition can be controlled. However, for the ORP it is challenging to achieve and maintain optimal algae irradiation and temperature levels throughout

the course of a day. During the night, an ORP system can cool down considerably, leaving the algae at sub-optimal productivities until the algae solution is warmed by the sun. Additionally, solutions in a shallow ORP can be over-heated during the day, causing sub-optimal productivities. Another problem affecting the productivity of algae grown in an ORP is that although the ORP is directly exposed to solar radiation, this light only reaches a few centimeters into dense algae solutions (James and Boriah, 2010) and thus much of the algae in the solution do not experience optimal light intensity. This problem worsens as the algae concentration increases, but can be addressed by increasing the mixing rate of this system. However, adding more energy to the system to increase mixing will increase the energy expenditures and must be balanced with the corresponding financial return resulting from increased productivity (Weissman et al., 1988).

- **Water loss:** ORPs are open to the environment and as such they can lose considerable volumes of water in the form of evaporation. The rate of this water loss depends on climate and weather conditions but can be substantial (>1 L m^{-2} d^{-1}) (Weissman and Goebel, 1987). This is problematic because it causes large quantities of water to be lost and it leads to increasing salinity gradients when culturing marine algae, thus retarding growth. This water loss has been estimated to be at least 40 mL of water per gram of dry biomass produced if the system achieves a growth rate of 25 g dry biomass m^{-2} d^{-1}.
- **Low effluent concentration:** While the ORP system uses a considerable amount of water and even at the highest of productivities, the overall effluent concentration from the system is low (approximately 0.5 g dry biomass L^{-1}) (Vandamme et al.,

2013). This necessitates the use of time consuming and expensive dewatering systems such as flocculation, settling, and centrifugation (Uduman et al., 2010) that are required to concentrate the algae solution so that the algae biomass can be further processed into valuable products.

Conversely, PBRs are closed systems that address many of these disadvantages, but are limited to translucent vessels that are considerably more expensive than excavating an oval raceway and filling it with water. However, PBRs are able to achieve greater productivities (up to 125 g m^{-2} d^{-1}) than ORP systems (up to 25 g m^{-2} d^{-1}) while producing higher biomass effluent concentrations and limiting water loss (Davis et al., 2011). The major disadvantages of the PBR system are listed below:

- **Cost:** Constructing hundreds or thousands of individual PBRs is very expensive because the building materials are expensive as well as their construction. Davis et al. (2011) indicated that the capital cost for a 10,000 m^2 ORP and PBR would be $112,900 and $319,200 (excluding the cost of land, lipid extraction facility and dewatering), respectively.
- **Energy:** A closed PBR with algae, nutrient solution, sunlight, CO_2, and an ideal temperature requires mixing and aeration to be a productive system because otherwise oxygen gradients will rapidly form and algae productivity will decrease (Huang et al., 2014; Vejrazka et al., 2013). Additionally, without aeration algae will settle out of solution and the overall productivity of the system will be poor. Therefore, PBRs require constant mixing to keep algae in solution as well as to remove oxygen gradients and to ensure that algae are continually illuminated and in contact with sufficient CO_2. This energy expenditure adds to the cost to operate

the system compared to an ORP system that utilizes a single paddle wheel to mix an entire system.

- **Overheating:** Because PBRs are closed systems that are translucent, they have a tendency to store heat and they can potentially overheat, causing reductions in overall productivity (Pate, 2018; Harris et al., 2013). Therefore, in some climates it is necessary to cool PBRs using a system of pumps and active or passive cooling techniques (Tu et al., 2016). This process can be costly and requires substantial operational energy. Overheating can also be managed by movable shading, but this would result in reduced solar radiation and productivity of the system.
- **Algal agglomeration**: Many algae by their nature are sticky; they have a tendency to secrete a dense polysaccharide matrix allowing them to adhere to surfaces. The problem with this tendency is that the adherence of algae to the translucent walls of a PBR reduces the overall productivity of the PBR system. This creates a substantial engineering problem as either materials need to be used that algae cannot adhere to or there has to be a method for cleaning the inside of the PBRs. This is a challenge at the large-scale because there would be thousands of PBRs in a system. Devising a system to effectively clean all of these surfaces is challenging and having to remove all of the PBRs to clean their surfaces with a solvent or other detergent would be time consuming, expensive, and would reduce productivity.

Because of these limitations, a substantial amount of the research in the algae cultivation field is aimed at optimizing algae production systems by minimizing the effect of these problems while maximizing productivity and reducing energy and costs. Recently, researchers have been working on a third type of system which is referred to as an attached

cultivation system (ACS) wherein algae grow attached to a substrate instead of in a suspension. The principle behind this approach is that algae have a tendency to stick to surfaces as previously described and that algae can adhere to a substrate that can then be irrigated and illuminated. The motivation for investigating these systems is that they have several unique potential advantages:

- **Lower water usage:** ACSs rely on algae adhering to substrates and not on maintaining algae within large suspended solutions. This reduces the overall quantity of water required for cultivating algae.
- **High effluent concentration:** Because algae are attached to a substrate instead of being suspended within a suspended solution, higher concentrations of algae effluent can be removed from the system (Ozkan et al., 2012; Shen et al., 2009), reducing energy requirements for dewatering compared to ORP and PBR systems (Johnson and Wen, 2010). These higher effluent concentrations are achieved as algae can be removed from substrates by way of scraping, pressing, or in some cases shaking.
- **Ability to grow acidophilic algae species:** Because CO_2 is limiting in suspended growth systems, it must be added to these system by way of dissolving it into the solution. To effectively dissolve CO_2 into a solution, the pH of the solution is typically made alkaline, or a sufficient quantity of CO_2 will not diffuse into the solution. With ACSs, algae are suspended in air and thus CO_2 is not thought to be limiting since it diffuses directly from the air into the algae that are attached to the substrates.

- **No energy required for mixing solution:** Because thin layers of algae are suspended in air, they are in contact with the CO_2 in the air and it is thought that oxygen can readily diffuse away from the algae (Pengfei et al., 2017). The stationary nature of the algae on the system therefore removes the need for energy to mix the algae, which is a major energy expenditure for traditional systems.

While ACSs have shown some potential advantages over traditional suspended growth systems, much is still unknown about their specific operation. The purpose of the work described here was to investigate a novel ACS that was developed by AL-G Technologies, Inc. The system was originally developed by Dr. Gaston Picard in the early 1990's for wastewater treatment, but was later deemed too expensive for that purpose (Kaya and Picard, 1995). While ACSs have been investigated in the literature by several other researchers (Lutzu et al., 2017; Kiperstok et al., 2017; Cheng et al., 2014; Gross et al., 2013), this specific type of system has not yet been investigated in depth and there are many uncertainties about it:

- **Water loss:** It had been proposed that ACSs reduce the amount of water required for algae production. While it is true that ACSs do not grow algae in suspension and there is less water present in the system at any given time, this does not necessarily translate into the ACS using less water overall compared to traditional systems. ACSs rely on placing many square meters of irrigated substrates in a single square meter of floor space. While this allows for similar productivities on a per area basis compared to traditional systems, this drastically increases the effective surface area of the system and the potential amount of water that can be lost from the system by way of evaporation. Although ACSs would be housed within a

controlled growth environment at large-scale (e.g., greenhouses), these systems are not completely closed and they exchange their air volume with the outside environment and in some climates require increased air exchange rates to reduce greenhouse temperatures. Therefore, it is possible that this ACS could lose more water to the environment than ORP systems.

- **Salinity gradients:** One of the main challenges associated with water loss from ACSs or any algae cultivation system is that if marine algae are being cultivated, then any loss in water due to evaporation will result in dramatically increasing the salinity of the nutrient solution. This is problematic because increasing the salinity can raise it above the ideal concentration for the growth of a particular algae species and will cause reductions or cessations in productivity. Ultimately, the success of the ACS described here will depend on its ability to cultivate marine algae species because these species produce the most valuable co-products (Reitan et. al., 1994; Fernandex-Reiriz et al., 1989).
- **Nutritional composition:** It has been hypothesized by AL-G Technologies, Inc. that the growth of algae on an ACS will increase oxidative stress because oxygen can more readily diffuse and interact with algae, since they are no longer in a solution. The proposed commercial application of this theory is that increased oxidative stress could lead to an increased production of antioxidant components. This would be commercially advantageous as antioxidant products (e.g. astaxanthin) have substantial commercial value compared to using algae biomass as feed or fuel.

- **Cultivation of various species:** Several species have been shown to grow on ACSs, but many of these species have little commercial value. Because the system is very different from most algae's natural suspended growth growing conditions, it is thought that some species of algae will not be able to adhere to the substrates and simply will not thrive on the ACS. Experiments were conducted to determine which algae species of value can successfully grow on the ACS because this will dictate the potential commercial products and commercial viability of the system.
- **Algae adherence:** While some species of algae can adhere to substrates very well and thrive on their surface, the adherence mechanism is not completely understood. Obtaining a better understanding of how and why algae adhere to the substrates might allow for the optimization of algae species that are known to poorly adhere. Conversely, it is also important to understand how best to remove algae from the substrates to improve algal harvesting efficiency.
- **Lipid accumulation:** In traditional systems, increasing the lipid content of algae can be achieved through inducing nutritional stress (Zhu et al., 2014; Machado et al., 2016). Experiments were conducted to investigate whether this approach can be used for the ACS described here. This will dictate the commercial products that can be produced on the system and if nutrient solution changes can be used to alter the production capabilities of the system.
- **Supplemental CO_2:** Although it is thought that algae on the ACS are able to obtain a sufficient quantity of CO_2 through diffusion from ambient air, this has yet to be demonstrated for this system. It is possible that the algae growing on the ACS are

still limited by CO_2 availability and thus will increase their productivity when the atmospheric CO_2 concentration is increased.

- **Harvesting:** One of the major challenges in scaling up the ACS is the removal of algae from the substrates. At small-scale, algae can be removed by using a scraper or press, but ultimately both mechanisms are challenging to implement at large-scale and both can degrade substrates, leading to increased maintenance costs over time. A simple harvesting procedure is desired that is rapidly scalable, achieves a concentrated harvest effluent, is economical, and requires little labor.

Investigating the fundamental characteristics of the ACS formed the basis for Chapters 1 and 2 of this dissertation and Chapter 3 focuses on using the results to investigate the financial viability of an ACS at scale. The aim of Chapter 3 was not to investigate how or why algae grow on an ACS, but if it is practical to use this approach for the cultivation of algae and what it would cost to do so. The approach for Chapter 3 was to compare the ACS with traditional systems to show areas of improvement as well as compare production on a cost basis ($\$ kg^{-1}$ of biomass). This dissertation describes the results of basic research on a novel ACS, as well as financial modeling to determine where future research on this system should focus to best develop a commercially viable ACS.

References

- Ashokkumar, V., & Rengasamy, R. (2012). Mass culture of *Botryococcus braunii* Kutz. under open raceway pond for biofuel production. *Bioresource Technology, 104,* 394-399.
- Cheng, P., Wang, J., & Liu, T. (2014). Effects of nitrogen source and nitrogen supply model on the growth and hydrocarbon accumulation of immobilized biofilm cultivation of *B. braunii*. *Bioresource Technology, 166,* 527-533.
- Davis, R., Aden, A., & Pienkos, P. T. (2011). Techno-economic analysis of autotrophic microalgae for fuel production. *Applied Energy*, *88*(10), 3524-3531.
- Demirbas, A. (2010). Use of algae as biofuel sources. *Energy Conversion and Management, 51*(12), 2738-2749.
- Fernández-Reiriz, M. J., Perez-Camacho, A., Ferreiro, M. J., Blanco, J., Planas, M., Campos, M. J., & Labarta, U. (1989). Biomass production and variation in the biochemical profile (total protein, carbohydrates, RNA, lipids and fatty acids) of seven species of marine microalgae. *Aquaculture*, *83*(1-2), 17-37.
- Gross, M., Henry, W., Michael, C., & Wen, Z. (2013). Development of a rotating algal biofilm growth system for attached microalgae growth with in situ biomass harvest. *Bioresource Technology, 150,* 195-201.
- Handler, R. M., Canter, C. E., Kalnes, T. N., Lupton, F. S., Kholiqov, O., Shonnard, D. R., & Blowers, P. (2012). Evaluation of environmental impacts from microalgae cultivation in open-air raceway ponds: Analysis of the prior literature and investigation of wide variance in predicted impacts. *Algal Research, 1*(1), 83-92.
- Harris, L., Tozzi, S., Wiley, P., Young, C., Richardson, T. M. J., Clark, K., & Trent, J. D. (2013). Potential impact of biofouling on the photobioreactors of the Offshore

Membrane Enclosures for Growing Algae (OMEGA) system. *Bioresource Technology, 144*, 420-428.

- Huang, J., Li, Y., Wan, M., Yan, Y., Feng, F., Qu, X., & Wang, W. (2014). Novel flat-plate photobioreactors for microalgae cultivation with special mixers to promote mixing along the light gradient. *Bioresource Technology*, *159*, 8-16.
- James, S. C., & Boriah, V. (2010). Modeling algae growth in an open-channel raceway. *Journal of Computational Biology*, *17*(7), 895-906.
- Johnson, M. B., & Wen, Z. (2010). Development of an attached microalgal growth system for biofuel production. *Applied Microbiology and Biotechnology*, *85*(3), 525-534.
- Kaya, V. M., & Picard, G. (1995). The viability of Scenedesmus bicellularis cells immobilized on alginate screens following nutrient starvation in air at 100% relative humidity. *Biotechnology and Bioengineering*, *46*(5), 459-464.
- Kiperstok, A. C., Sebestyén, P., Podola, B., & Melkonian, M. (2017). Biofilm cultivation of *Haematococcus pluvialis* enables a highly productive one-phase process for astaxanthin production using high light intensities. *Algal Research*, *21*, 213-222.
- Lutzu, G. A., Zhang, L., Zhang, Z., & Liu, T. (2017). Feasibility of attached cultivation for polysaccharides production by *Porphyridium cruentum*. *Bioprocess and Biosystems Engineering*, *40*(1), 73-83.
- Machado, M., Bromke, M., Júnior, A. P. D., Vaz, M. G. M. V., Rosa, R. M., Vinson, C. C., & Willmitzer, L. (2016). Comprehensive metabolic reprograming in

freshwater *Nitzschia palea* strains undergoing nitrogen starvation is likely associated with its ecological origin. *Algal Research, 18,* 116-126.

- Ozkan, A., Kinney, K., Katz, L., & Berberoglu, H. (2012). Reduction of water and energy requirement of algae cultivation using an algae biofilm photobioreactor. *Bioresource Technology, 114,* 542-548.
- Pate, R. (2008). Biofuels and the energy− water nexus. *AAAS/SWARM, Albuquerque, NM*.
- Pengfei, C., Yan, W., Qiyong, Y., & Tianzhong, L. (2017). Comparison of growth, hydrocarbon accumulation and metabolites of *Botryococcus braunii* between attached cultivation and aqueous-suspension cultivation. *International Journal of Agricultural and Biological Engineering*, *10*(1), 134.
- Reitan, K. I., Rainuzzo, J. R., & Olsen, Y. (1994). Effect of nutrient limitation on fatty acid and lipid content of marine microalgae. *Journal of Phycology*, *30*(6), 972-979.
- Shen, Y., Yuan, W., Pei, Z. J., Wu, Q., & Mao, E. (2009). Microalgae mass production methods. *Algae*, *6*(4), 1.
- Slade, R., & Bauen, A. (2013). Micro-algae cultivation for biofuels: cost, energy balance, environmental impacts and future prospects. *Biomass and Bioenergy*, *53*, 29-38.
- Tu, Q., Lu, M., Thiansathit, W., & Keener, T. C. (2016). Review of water consumption and water conservation technologies in the algal biofuel process. *Water Environment Research*, *88*(1), 21-28.

- Uduman, N., Qi, Y., Danquah, M. K., Forde, G. M., & Hoadley, A. (2010). Dewatering of microalgal cultures: a major bottleneck to algae-based fuels. *Journal of Renewable and Sustainable Energy*, *2*(1), 012701.
- Vandamme, D., Foubert, I., & Muylaert, K. (2013). Flocculation as a low-cost method for harvesting microalgae for bulk biomass production. *Trends in Biotechnology*, *31*(4), 233-239.
- Vejrazka, C., Janssen, M., Benvenuti, G., Streefland, M., & Wijffels, R. H. (2013). Photosynthetic efficiency and oxygen evolution of *Chlamydomonas reinhardtii* under continuous and flashing light. *Applied microbiology and Biotechnology*, *97*(4), 1523-1532.
- Weissman, J. C., Goebel, R. P., & Benemann, J. R. (1988). Photobioreactor design: mixing, carbon utilization, and oxygen accumulation. *Biotechnology and Bioengineering*, *31*(4), 336-344.
- Weissman, J. C., & Goebel, R. P. (1987). *Design and analysis of microalgal open pond systems for the purpose of producing fuels: a subcontract report* (No. SERI/STR-231-2840). Solar Energy Research Inst., Golden, CO (USA).
- Zhu, S., Huang, W., Xu, J., Wang, Z., Xu, J., & Yuan, Z. (2014). Metabolic changes of starch and lipid triggered by nitrogen starvation in the microalga *Chlorella zofingiensis*. *Bioresource Technology*, *152*, 292-298.

Chapter 1: Evaluation of Algal Biomass Production on Vertical Aeroponic Substrates

This Chapter was originally published in Algal Research: Johnson, M., Villani, T. S., Azmat, A., Simon, J. E., & Both, A. J. (2015). Evaluation of algal biomass production on vertical aeroponic substrates. Algal Research, 10, 240-248. This work is presented here with only minor edits.

Michael Johnson[1], Tom S. Villani[2,3], Aisha Azmat[4], James E. Simon[2], A.J. Both[1,]*

[1] Department of Environmental Sciences, Rutgers University, New Brunswick, NJ 08901

[2] New Use Agriculture and Natural Plant Products Program, Department of Plant Biology and Pathology, Rutgers University, New Brunswick, NJ 08901

[3] Department of Medicinal Chemistry, Ernest Mario School of Pharmacy, Rutgers University, Piscataway, NJ 08854

[4] School of Environmental and Biological Sciences, Rutgers University, New Brunswick, NJ 08901

Abstract

Large-scale algal biomass production has focused primarily on the Open Raceway Pond (ORP) and Photobioreactor (PBR) systems, but to date neither system has been able to produce algae biofuel in a financially viable manner. For this reason, a novel attached cultivation system (ACS) was evaluated to determine if it could produce substantial quantities of biomass without a negative impact on the lipid productivity and fatty acid profile compared to the two traditional systems. This ACS produced high per area yields (exceeding 10 g dry biomass m^{-2} floor space d^{-1}) assuming a 1:1 small-scale to large-scale conversion and substantial harvest concentrations (exceeding 3.3 g biomass L^{-1}), resulting

in reduced energy inputs and increased financial return. In addition to productivity increases, the aeroponic nature of this substrate system did not negatively affect the fatty acid composition of the cultivated biomass, thus demonstrating the promising potential for using ACSs to produce biofuel, nutraceuticals, and feed for fisheries and various other applications.

Introduction

For the production of algal biomass, much focus has been placed on selecting algal strains, as well as downstream biomass processing. However, major innovations in algae cultivation techniques have remained elusive. To cultivate algae for large-scale biofuel production, the per area productivity (g dry biomass m^{-2}) and harvest concentration (g dry biomass L^{-1}) must be sufficient so that the chemical energy output exceeds the energy that is required for cultivation, harvesting, mass transfer, and downstream chemical transformation (Batan et al., 2010). Additionally, for a sustainable system to be able to produce biomass in an energetically positive manner, it is essential that the system does not require unsustainable quantities of raw materials (e.g., water, nutrients) (Batan et al., 2010).

Open Raceway Pond (ORP) and Photobioreactor Systems (PBR)

Current platforms for cultivating algae are quite diversified but can be predominantly categorized as ORPs or PBR systems. An ORP system is comprised of a race track shaped shallow canal that is typically circulated by a paddle wheel and thus ORP systems are often referred to as *raceway ponds* (Chaumont, 1993). In contrast, the PBR system is a closed translucent system comprised of flasks, bags, tubes or other translucent containment vessels (Chaumont, 1993). These systems are similar in that they both are

typically maintained at an alkaline pH to accommodate elevated CO_2 concentrations, but have various advantages and disadvantages in comparison to one another.

The ORP system is very simple, requires minimal maintenance to operate, and has low investment costs, yet has many disadvantages in relation to performance (Chaumont, 1993; Mata et al., 2010). ORP systems have lower productivities (g biomass m^{-2} d^{-1}) than PBR systems and have comparatively dilute harvest concentrations (g biomass L^{-1}) necessitating high mass transfers to transport and subsequently dewater algae (Mata et al., 2010). ORPs are open to the environment, allowing for contamination and experience a typical evaporative loss of approximately 1 liter of water per gram of biomass produced (Slade and Bauen, 2013).

The PBR offers a closed system that minimizes loss from evaporation and contamination, but the closed system requires the use of temperature control and constant maintenance to minimize algae agglomeration (Mata el al., 2010). Algae have a tendency to accumulate on surfaces and this accumulation eventually leads to the reduced productivity of PBRs without constant maintenance. While the PBR system has greater per area productivity compared to ORPs (10 g m^{-2} d^{-1} for OPs, 20 g m^{-2} d^{-1} for PBRs) and harvest concentrations (0.5 g L^{-1} for ORPs and 3.0 g L^{-1} for PBRs), the PBR system requires a substantial energy input to mix the algae suspension and to remove oxygen gradients (Slade and Bauen, 2013; Rogers et al., 2014; Richardson et al., 2014). For these reasons, neither of these systems is ideal, and both have challenges to overcome before they can be developed into commercially viable systems for large-scale biomass production.

Attached Cultivation Systems

Because of the inabilities of ORPs and PBRs to produce financially viable biofuel at scale, AL-G Technologies Inc. designed a novel cultivation system that is able to increase algal productivity as well as harvest concentration in comparison to ORP and PBR systems (US Patent Application number: 20140127776). The AL-G system is based on the principle of attached growth that utilizes specific species of algae that have a tendency to adhere to substrates and create dense biofilms (Johnson and Wen, 2010). This system is comprised of multiple vertical aeroponic substrates that are suspended from a scaffolding system such that at commercial scale ten, one-meter squared substrates would be placed within a square meter of floor space. The substrates are suspended from a scaffolding system using simple loops or hooks so that the substrates can be easily removed. At commercial scale, the substrates would be spaced 8 to 10 cm apart from one another and would be mounted within a greenhouse in long rows of hundreds of substrates. The substrates themselves are composed of a simple 50/50 (%) cotton and polyester weave, are approximately 5 mm thick and readily retain water. Using this approach, algae are grown up to a density of approximately 5g L^{-1} in a PBR system and then the substrates are either removed from the scaffolding system and soaked in the inoculum or remain on the scaffolding system and are sprayed with the inoculum. Following inoculation, the substrates on the scaffolding system are continuously irrigated with a nutrient solution (water and nutrients) and the algae begin to grow on the substrates.

The spent nutrient solution is collected from the bottom of the substrates and can be replenished with nutrients and reused or disposed of. Over a period of a few weeks the inoculum takes hold on the substrates and forms a dense biofilm that can subsequently be

harvested. The algae-covered substrates are typically harvested every 3-4 days using a simple mechanical roller press. The substrates can be removed from the scaffolding system and pressed elsewhere or can remain attached to the scaffolding system and be pressed in-place. The moist substrates are simply passed through a mechanical roller press that removes approximately 50-70% of the algae from the substrates and leaves the remaining algae on the substrate for regrowth. Because the substrates contain minimal water, the effluent concentration from this process is high (as high as 3.3 g L^{-1}) which minimizes downstream dewatering. The substrates can maintain this harvesting and regrowth process for long periods of time and can produce substantial per area biomass yields with a dense harvest concentration. This entire process can be described and illustrated in four major steps: 1) Stock Solution (inoculum production), 2) Inoculate Substrate, 3) Algae Growth on Substrate and 4) Harvest (**Figure 1**). Following steps 1 and 2, steps 3 and 4 are conducted as a continuously repeated process until the substrates are damaged from mechanical harvesting or removed from the system for other reasons.

For the experiments described here, the commercial scale AL-G system was scaled down to allow for sufficient replicates within the budgetary and space constraints. This scaled down system is illustrated in **Figure 2** and shows 15 by 25 cm growth substrates housed within a controlled environment chamber and flanked by two dummy substrates to provide more equal lighting distribution. **Figure 2** also shows the LED lighting system as well as the individual irrigation drippers at the top of every substrate and the collection bin for the excess irrigation water dripping from the bottom of the substrates. In this chapter, we report on the evaluation of this ACS for the growth and development of the marine alga

Tetraselmis chuii and the freshwater alga *Parachlorella kessleri*. *T. chuii* and *P. kessleri* both create resilient biofilms, allowing them to grow continuously on the substrates.

T. chuii was chosen since it has a great economic benefit due to its ability to provide food cultures for use in shellfish hatcheries that produce shellfish for human consumption and for its nutritional profile, which is dense in essential fatty acids (Brune et al., 2009). In contrast, *P. kessleri* does not have a favorable composition for use in hatcheries or as biofuels since it contains minimal lipids by weight (0-29%) (Mizuno et al., 2013; Fernandes et al., 2013). Rather, *P. kessleri* was selected because of its rapid growth rate and ability to grow under varying conditions, as well as its similarity to *Chlorella vulgaris*, a commercially relevant algal strain (Fernandes et al., 2013). While both of these algae grow successfully on the substrates, the objective of this research was to compare the impact of aeroponic culturing of these different algal species on biomass productivity and fatty-acid composition using the ACS.

Materials and Methods

Evaluating algae production for aquaculture applications

For our studies, *T. chuii* was obtained from the Institute of Marine and Coastal Sciences (Rutgers University, New Brunswick, NJ) and *P. kessleri* was obtained from AL-G Technologies Inc (Charny, Quebec, Canada). Both species were initially transferred to 2 L Erlenmeyer flasks containing their respective nutrient solution and placed 20 cm below an LED lighting array with a light intensity of approximately 200 µmol m^{-2} s^{-1} (16 hours light, 8 hours dark). The nutrient solution used for *P. kessleri* was as follows: KNO_3 200 mg L^{-1}, NH_4Cl 106 mg L^{-1}, $CaCl_2 * 2\ H_2O$ 25 mg L^{-1}, $MgSO_4 * 7H_2O$ 25 mg L^{-1}, K_2HPO_4 25 mg L^{-1}, $Na_2EDTA * 2\ H_2O$ 1.1 mg L^{-1}, $FeSO_4 * 7\ H_2O$ 1 mg L^{-1} and the nutrient solution used for *T. chuii* was F/2 (Guillard and Ryther, 1962;

Olaizola et al., 1991). The nutrient solution for *P. kessleri* had been optimized by AL-G Technologies Inc. for growth on the ACS. The *P. kessleri* nutrient solution was made using New Brunswick, NJ tap water and brought to a pH of 5.5 using 0.8 M HCl while the *T. chuii* nutrient solution was made using 35 parts per thousand (ppt) seawater collected from Corson's Inlet (Cape May, NJ), which was autoclaved prior to use. The inoculation flasks were aerated with an aquarium air pump (Active Aqua Air Pump, AAPA78L) at a rate of 2 L^{-1} m^{-1} (2:1 v/v) with ambient air and following 10 days of growth, the biomass concentration was sufficient to begin experimentation.

To model traditional non-aeroponic growth, the controls for both *T. chuii* and *P. kessleri* were placed into three 1 L Erlenmeyer flasks in which 50 mL of inoculum was added to 950 mL of their respective nutrient solution. The flasks were capped with a one inch Styrofoam plug and covered with aluminum foil to minimize water loss due to evaporation. Throughout the experiment, the appropriate nutrient solution was added to the flasks to compensate for water lost to evaporation and the temperature was maintained between 24-27ºC. These control flasks were aerated at a rate of 2 L^{-1} m^{-1} (2:1 v/v) with ambient air and were placed 20 cm below an LED lighting array with a light intensity of approximately 200 µmol m^{-2} s^{-1} (16 hours light, 8 hours dark). The ¼" aeration tubing was placed at the bottom and center of the flasks to provide thorough mixing.

In parallel to the control flasks being inoculated, 1 L of the *T. chuii* inoculum and the *P. kessleri* inoculum was used to inoculate ten proprietary cotton-like aeroponic substrates (15 by 25 by 0.75 cm) that were made of a proprietary material (AL-G Technologies, Inc). To inoculate the substrates with algae, they were soaked in 200 mL of the inoculum for 20 seconds and then rolled through a mechanical roller press (ATLAS,

AT01, Winnipeg, Canada) to allow for infiltration of the inoculum throughout the substrate structure. The effluent from the mechanical press was captured in a bin and reused so that approximately 100 mL of inoculum was used for each substrate. These substrates were then hung from a stainless-steel scaffolding system using plastic loops such that the substrates were placed 1 cm below a 1/16" irrigation dripper and spaced 5 cm apart from one another. In addition to the ten substrates that were inoculated, an additional substrate was placed at either side of the ten substrates so that the ten substrates experienced equal lighting conditions. The substrates were illuminated with an LED array consisting of seven 60 cm LED strips (Mouser Electronics, 901-SB-6500-TR) spread 3 cm apart and placed 8 cm above the substrates. The LED strips provided a light intensity of approximately 200 μmol m^{-2} s^{-1} measured at the top of the substrates using a 16 hour on 8 hours off light cycle. The set of ten *T. chuii* substrates and ten *P. kessleri* substrates were placed on separate shelves within a 75 by 75 by 40 cm growth chamber with an air exchange rate of 1 volume h^{-1} and a temperature set point of 24-27^{o}C and resulting relative humidity of 96-99%. The two sets of ten substrates were irrigated with 5.7 liters of their respective nutrient solution per day using a peristaltic pump (Masterflex, HV-07522-20). The volume from the pump to the sets of ten substrates was 4 mL min^{-1}, which was dispensed intermittently (12 seconds on, 168 seconds off) at a flow rate of 60 ml min^{-1}.

Following inoculation, the substrates were harvested using the mechanical roller press every 3-4 days and the effluent was collected for subsequent evaluation. For this experiment the harvest data used from each substrate system was from the 34th day since inoculation for the *T. chuii* and from the 50th day since inoculation for the *P. kessleri*. These days were selected because the systems reached a plateau in biomass productivity (g m^{-2} d^{-}

^{1}) based on data collected during previous experiments conducted under the same experimental conditions (results not reported here). For the control treatments of *T. chuii* and *P. kessleri*, both samples were taken on day 10 when the systems both reached their growth plateau. All harvested algae was immediately centrifuged (4,000 rpm, 5 min, 5˚C) and transferred to a freezer (-80˚C). These samples were then freeze dried, and stored at -20˚C for subsequent analysis. In addition to the biomass being harvested every 3-4 days, 5 mL of effluent was collected from each set of substrates twice a week to determine the quantity of algae falling off of the substrates (non-adherent biomass) and the amount of exopolysaccharides being produced.

Carbon Sequestration

To determine the total productivity of an algae production system, the total mass of carbon sequestered needed to be determined. Carbon sequestration is the measure of how much carbon is fixed by algae from the air and describes overall productivity. In the case of these experiments, they were all conducted at an ambient CO_2 concentration of approximately 400 ppm and the CO_2 that was sequestered was from the air within the growth chambers. The total carbon sequestration could not be determined as is typically done for traditional algae systems using only the optical density (OD) of the algae solution. Instead, the total productivity of the aeroponic system needs to be determined based on three separate components: 1) Biomass harvest, 2) Non-adherent biomass, and 3) Polysaccharides. The biomass harvest and non-adherent biomass components were determined using OD_{680} in accordance with **Equation 1** (Watanabe et al., 2005, Meiling et al., 2010):

$$Yield_B \text{ (g biomass } L^{-1}) = 0.379 \times OD_{680} \ (R^2 = 0.9652) \quad \textbf{(1)}$$

This standard curve was constructed for *P. kessleri* whereas a new standard curve needs to be created for all algae species as the slope will vary. Additionally, algae was agitated through using sonication prior to creating this standard curve as algae aggregates would reduce the accuracy of this optical density approach. Using two volumes, the harvest volume and total volume of nutrient solution used per day, the biomass per day from each one of these components was determined. To determine the quantity of polysaccharides produced, the effluent used to determine non-adherent biomass was filtered through a 0.20 µm filter and diluted 2:1 with deionized water. The samples were then analyzed using a phenol-sulfuric acid assay with a standard curve constructed using a serial dilution of glucose as depicted in **Equation 2:** (Dubois et al., 1956).

$$Yield_C \text{ (mg of carbon } L^{-1}) = 29.468 \times OD_{490} + 1.827 \ (R^2 = 0.9818) \quad \textbf{(2)}$$

To determine the total mass of carbon sequestered per day, the biomass harvest and non-adherent biomass was converted into the mass of carbon (mg) using the percentage of carbon in each type of biomass for each algae species. The percent of carbon was determined from three replicate samples of freeze-dried biomass of *P. kessleri* and *T. chuii*, using a Carlo Erba NA1500 series 2 Elemental Analyzer. The total percent carbon of *P. kessleri* and *T. chuii* were 49.8% and 23.6%, respectively.

Extraction and Derivatization of Fatty Acids

Heptadecanoic acid (3.884 mg mL^{-1} dissolved in toluene) was selected for use as an internal standard (this fatty acid was not detected in any algae samples) in order to validate experimental recovery and instrumental precision. Supelco 37 component fatty acid methyl esters (FAMEs) mix (10.0 mg mL^{-1} in CH_2Cl_2) was used as a reference material for the identification and quantification of derivatized fatty acids within the algal samples.

The only FAME not contained in the 37 component mix, which was detected in the algal samples, was stearidonic acid (18:4). To quantify this fatty acid, the response factor for alpha-linolenic acid (18:3) was multiplied by the ratio of molecular weight of stearidonic to alpha-linolenic.

Fresh HCl in methanol was prepared as follows: 20 mL anhydrous methanol (fresh from SIGMA ALDRICH®) was added to a dry Erlenmeyer flask; 2 mL acetyl-chloride was added drop-wise. Culture tubes (2x20 cm), a 25 mL volumetric flask, and a 50 mL Erlenmeyer flask were rinsed with methanol and dried in an oven at 90˚C overnight. The glassware was removed from the oven and placed in a $CaCl_2$ desiccator and cooled down to room temperature before use. Then 97.1 mg of heptadecanoic acid (17:0) was diluted to 25 mL with toluene (3.884 mg mL^{-1}). Fatty acid methyl esters (FAMEs) were prepared as follows. Approximately 50 mg of freeze-dried algal sample was added quickly to each culture tube, followed by 1.00 mL heptadecanoic acid internal standard solution (3.884 mg mL^{-1} in toluene), and 1.00 mL toluene. For the marine algae *T. chuii*, the freeze dried algal mass took into account the mass of the 35 ppt salinity that was present in the nutrient solution as well as the mass of the nutrient constituents. Next, 3.00 mL freshly prepared methanolic HCl was added, followed by a magnetic stirbar. The samples were very gently vortexed to avoid splashing sample on the side of the vials. The culture tubes were flushed with N_2 (atmospheric pressure) and sealed tightly. The sealed culture tubes were placed in a water bath at 80˚C for 3 hours with magnetic stirring. Periodically the reaction vials were visually checked for leaks. Tubes that leaked were not included in subsequent analyses. The reaction tubes were removed from heat and conditioned to room temperature. Next, 5 mL of Na_2CO_3 solution (6% m/v) was added, followed by 2 mL of toluene. The resultant

biphasic mixture was thoroughly vortexed (~30 seconds). The tubes were centrifuged to settle the emulsion. The organic phase was separated and diluted to 5 mL volume. Approximately 2 mL of the clear brown/yellow upper layer (toluene layer) was aliquoted and dried over Na_2SO_4. The dried solutions were placed in amber autosampler vials and analyzed immediately by Gas Chromatograph with a Flame Ionization Detector (GC-FID). All samples were repeated in triplicate.

Gas Chromatography Flame Ionization Detector Analysis for Fatty Acid Methyl Esters

A volume of 0.5 µL toluene solution was injected on an HP1125 GC-FID, using a 1:25 split injection. The column used was an Alltech EC-WAX, 30 m, 0.25 mm I.D., 0.25 µm film. The inlet temperature was set at 220˚C, Helium carrier gas flow was set at 71.8 kPa, 23.7 mL min^{-1}. The following temperature gradient was employed which provided adequate separation of all fatty acids: Initial temperature, 60˚C, hold 1 min; 10 ˚C min^{-1} to 220˚C, hold 23 min, 5˚C min^{-1} to 240˚C, hold for 16 minutes.

Results and Discussion

Biomass Productivity

It is challenging to make a biomass productivity comparison between the control flask and the ACS since the substrate system is not a traditional suspended growth system that can be easily described. However, a practical approach to use when comparing these two types of systems is to compare them in terms of per area productivity (g biomass m^{-2} floor space day^{-1}) and in volumetric productivity (g biomass L^{-1} day^{-1}). The ACS used for this experimentation covered a floor space of 675 cm^2 (15 by 45 cm), whereas the flask based control systems covered 201 cm^2 (8 cm radius), thus making their conversion factors to 1 m^2 of floor space 14.8 and 49.8, respectively. Such a relationship ignores scaling factors associated with larger scale systems, although it can be used as a means for

comparison. To compare the two systems volumetrically, the amount of nutrient solution retained within the ACS was used, which was 21 mL per substrate. This equates to a volume of 4.7 L m^{-2} and thus the per area productivity for the ACS can be converted into a volumetric productivity as presented in **Table 1**. Following inoculation, the control *P. kessleri* grew initially at a quicker rate than the ACS while the *T. chuii* grew more quickly on the substrate based system than the control (**Figure 3**). However, following a 34 day acclimation period, the substrate based *P. kessleri* achieved a quicker growth rate than the *P. kessleri* control. When comparing the *T. chuii* and *P. kessleri* growth on the substrates, we observed that both require an acclimation period before a resilient biofilm forms. Following this acclimation period (11 days for *T. chuii*, 34 days for *P. kessleri*), both *T. chuii* and *P. kessleri* maintained linear biomass production of 2.78 g m^{-2} d^{-1} (R^2 = 0.9958) and 7.95 g m^{-2} d^{-1} (R^2 = 0.9937), respectively (**Table 1**). As well as increasing biomass production, cultivating *P. kessleri* and *T. chuii* on the substrates increased their harvest concentration in comparison to the control flasks. The reason that *P. kessleri* grew better than *T. chuii* on the substrates may be in part because marine algae are more prone to desiccation on the substrates compared to freshwater algae and salinity gradients reduce overall growth rates. Regardless, both species showed increased productivity on the substrates, including increased biomass concentrations, since the substrates contained minimal quantities of water and yet provided improved irradiance and gas exchange. This ability to increase algal concentration can reduce energetic and financial expenditures associated with the dewatering and transportation of algae.

Because of the challenges of comparing the productivity of the ACS to the control system, it is important to compare these results to the productivities reported in the

literature for similar systems. Ozkan et al. (2012) conducted research on a novel algae biofilm PBR that was oriented horizontally, using *Botryococcus braunii* and achieved a per area productivity of 0.71 g m^{-2} day^{-1}. For *B. braunii* grown in raceway ponds, flat-plate PBRs and tubular PBRs, the per area growth rates were 11, 27 and 25 g m^{-2}, respectively (Ozkan et al., 2012). While depending highly on the system, conditions and algal species, generally the per area productivity of an ORP system is described in the literature to be approximately 10 g m^{-2} d^{-1} and the per area productivity of a PBR system is approximately 25 g m^{-2} d^{-1} (Ozkan et al., 2012; Christenson and Sims, 2012). These productivities vary considerably in the literature as many researchers report maximum theoretical large-scale productivities based upon small-scale experiments and few have conducted long-term large-scale experiments to substantiate these claims. When comparing the AL-G system to these values as reported in the literature, it appears that the AL-G system has a lower productivity than the PBR systems and only slightly outperforms the ORP system. However, it is inherently difficult to convert small-scale results to larger-scale systems and the AL-G system has not yet been tested at a larger scale, nor has it been optimized.

While the AL-G system is patented and is unique in many aspects, there are other ACSs such as the system evaluated by Ozkan et al., (2012) that are currently being evaluated by other researchers. A more common type of ACS is the rotating algal biofilm (RAB) system that has been shown to have a biomass productivity of 3-31 g m^{-2} d^{-1} (Gross et al., 2013; Christenson and Sims, 2012). These RAB systems achieve similar biomass productivities to the AL-G system and have the same major benefit of greatly increasing harvest concentration (Christenson and Sims, 2012). The major difference between the two systems resides in their ability to scale up. While both systems have similar productivities,

the infrastructure required to hang substrates is thought to be simpler and less expensive than the infrastructure required to have large spinning discs or wheels. Overall, we conclude that the AL-G system is able to achieve substantial productivity, but that the system must be further evaluated with respect to productivity and optimization in order to compare it to current production systems.

Carbon Sequestration

The total cumulative biomass for the control system is displayed in **Figure 3** and is representative of carbon sequestration achieved on the system. Compared to the control system, the ACS is not a closed system because large quantities of sequestered carbon are lost from the system in the form of non-adherent biomass and exopolysaccharides. In **Figure 4**, the biomass harvested from the ACS is shown to be only 46% for the *P. kessleri* and 14% for the *T. chuii* of the total carbon sequestered from the system. For the *P. kessleri* 12% of the sequestered carbon was in the form of exopolysaccharides and 42% was in the form of non-adherent biomass while for *T. chuii* 70% was in the form of exopolysaccharides and 16% was in the form of non-adherent biomass. This partitioning of the carbon can be explained based on the growth data of the two algal species and their respective environments. The overall carbon sequestration of the *P. kessleri* is greater since it has a faster growth rate than *T. chuii*, but the amount of exopolysaccharides produced by the *T. chuii* culture is greater since the *T. chuii* nutrient solution is maintained at 35 ppt salinity and the *T. chuii* requires this additional polysaccharide matrix to prevent desiccation (Sutherland, 2001; Mishra and Jha, 2009). Since the substrates are suspended in air, they are vulnerable to gradients of increasing salinity due to evaporation. Although the relative humidity of the growth chambers was maintained between 96-99%, there was an exchange rate and air was lost from the controlled growth environment to the outside

air that has a lower humidity. The nutrient solution used for *T. chuii* had a salinity of 35 ppt and the loss of water from the system resulted in salinity gradients. For example, the periphery of the substrate was measured to have a salinity of up to 47 ppt. These results indicate that for both *T. chuii* and *P. kessleri* the non-adherent biomass was similar to the amount of biomass produced on the substrates. This correlated with the *P. kessleri* having a faster growth rate and greater overall production.

In comparison to the quantity of polysaccharides being produced by algae on the ACS, most algae species produce minimal quantities of exopolysaccharides under normal conditions in traditional suspended growth systems (Gross et al., 2013). For this reason, algae from the substrates were investigated under the microscope and it was observed that in addition to the exopolysaccharides produced by the algae, there were also fungi producing exopolysaccharides. These fungi appear to live in tandem with the algae and aid in producing an exopolysaccharide biofilm that helps prevent desiccation. Spontaneous symbiotic relationships between fungi and algae have been demonstrated in the literature and it is possible both the fungi and algae are contributing to the total exopolysaccharide matrix (Hom and Murray, 2014). Such a relationship can be mutualistic, but requires further study to investigate the complex interplay between the autotroph (algae) and heterotroph (fungi) (Hom and Murray, 2014). The loss of carbon from the ACS represents a major deficiency that needs to be rectified as this greatly reduces the total usable biomass from the system if the nutrient solution dripping off the substrates is not collected. Future research on the system needs to investigate the capture and placement of exopolysaccharides and non-adherent biomass back onto the substrates. This type of

carbon recycling loop could increase the frequency by which the substrates can be harvested and would increase the total biomass collected from the system.

In addition to comparing the carbon sequestration between *T. chuii* and *P. kessleri,* the impact of fungi on carbon sequestration was also investigated using mature cultures of *P. kessleri* that had been cultivated for over 55 days on the system using the methodology previously described. The objective of this work was to determine to what extant algae or fungi were producing polysaccharides on the system. This was achieved over a 21 day experiment (6 harvests) by using the anti-fungal carbendazime to remove fungi from the substrates. It was demonstrated (**Figure 5**) that the substrates with carbendazime achieved a similar algae productivity to the control substrates without carbendazime, but that the substrates without fungi had substantially more algae fall off of the substrates and produced more polysaccharides. This result is illuminating as it demonstrates that fungi play an important role on the system and also that the growth of algae on the system and the productivity of the system as a whole is limited by some factor. We are able to conclude this as the control system is capable of producing more total algae biomass than the fungi-free system as more algae fall off of the substrates, but both maintain similar algae populations on the substrates. This means that some factor (e.g., light, CO_2, O_2) is limiting the total amount of algae that can be on the system at any one given time and that if this factor is mitigated, then the overall amount of algae harvested from the system can be increased. It is speculated that this is due to the biofilm reducing diffusion rates and overall algal illumination.

Fatty Acid Composition

The absolute content of fatty acids was determined as a percentage of freeze dried biomass, allowing for a quantitative comparison of fatty acid (FA) content and composition

between experiments. A comparison between the fatty acid content of *T. chuii* and *P. kessleri* grown using substrates and in flask is presented in detail in **Table 2**; the same data is represented graphically in **Figure 6**. Additionally, the statistical significance of the results was determined using a two-tailed, unpaired t-test; p values are depicted in **Table 2**. For results deemed significant ($P < 0.05$), the difference between the average FA content of substrate biomass and flask biomass was calculated.

On the surface, it appears that for *T. chuii*, there was little impact on fatty acid content due to growth on the substrate, as there was no significant difference in the total content of omega-3, polyunsaturated, monounsaturated, or saturated fats. However, on closer scrutiny, there was a significant difference in 6 fatty acids: an increase of oleic acid (18:1n9), linolenic acid (18:2n6), and eicosapentaenoic acid (EPA, 20:5n3) on the substrate biomass, and a decrease in myristoleic (16:1n7), stearidonic (18:4n3) and eicosenoic acid (20:1n9). Importantly, there was an increase in 20:5n3, one of the most important FAs in nutrition and aquaculture. Interestingly, the 0.111% increase in content of 20:5n3 in substrate biomass was balanced by a corresponding 0.150% decrease in 18:4n3. The changes in composition were slight, and cumulatively affected no significant change in the total compositions of any major class of FAs. As such, this data confirms that the growth of *T. chuii* on substrates provided a slight overall benefit to fatty acid profile, and most importantly, provided no measurable disadvantage with respect to FA composition. This illustrates the capability of aeroponic substrate growth for use in aquaculture of *T. chuii*.

In contrast, the impact of substrate growth on the FA content of *P. kessleri* was dramatic. There was a highly significant difference determined for nearly every fatty acid detected in substrate-grown *P. kessleri* biomass compared to suspension-grown. There was

a substantial increase in omega-3, polyunsaturated, and mono-unsaturated FAs. Accordingly, there was a 2-3x increase in every major fatty acid detected in substrate grown *P. kessleri* biomass except for palmitic acid (16:0). Total fatty acid content was 4.830 ± 0.533% in suspended growth *P. kessleri* biomass, which increased to 10.395 ± 0.173% on the substrate, whereas polyunsaturated FAs nearly tripled from 0.914 ± 0.143% to 2.269 ± 0.037%.

While these results demonstrated a favorable impact of substrate-grown *P. kessleri* biomass with respect to FA content, the underlying reasoning behind the substantial differences demands more thorough explanation. A comparison of the conditions used to grow *P. kessleri* to the conditions used to grow *T. chuii* is illustrative since substrate grown *T. chuii* showed minimal change in FA composition. The critical variable is pH: the pH of the nutrient solution used to grow *P. kessleri* was maintained at 5.5, whereas for *T. chuii*, the pH was maintained at 8.2. This nutrient solution has been historically used for *P. kessleri* growth since it minimized contamination and *P. kessleri* appears to thrive at this pH. From the acid-base equilibrium equation for carbonate species, it can be shown that concentration of carbonate species in nutrient solution is dependent on pH. At pH less than 5, total CO_2 content is constant and at a minimum—the predominant species is aqueous CO_2 (in the form of carbonic acid) and is limited by the diffusion of CO_2 into nutrient solution by Henry's law (Mata et al., 2007). A graph representing total CO_2 solubility in water at 25˚C at various pH values is shown in **Figure 7**. As can be seen in the figure, there is a substantial increase in the solubility of CO_2 in water as the pH increases.

In order to better understand the difference in FA productivity in *P. kessleri* and the absence of such a difference in *T. chuii*, it is important to examine the solubility of

CO_2 at the pH of each nutrient solution used. Using the equation to calculate the CO_2 solubility at a pH of 5.5, the nutrient solution used for growing *P. kessleri*, a solubility of 0.013 mM CO_2 was calculated, just slightly above the Henry's law imposed minimum of 0.012 mM CO_2 content. The nutrient solution used to grow *T. chuii* had a pH of 8.2, resulting in a solubility of 0.865 mM CO_2. This indicates that the *T. chuii* was exposed to a much higher content (>60-fold increase) of dissolved CO_2 than the *P. kessleri*. The difference in solubility of CO_2 in the nutrient solution can be used to explain the differences in FA content between the growing systems and species. It is known that FA content increases with increased CO_2 content and decreased nitrogen content (Tsuzuki et al., 1990). It appears that *T. chuii* was exposed to saturating CO_2 content in the substrate system as well as the flask, since the FA content remained relatively consistent. Since there is a high content of dissolved CO_2 available at a pH of 8.2, CO_2 was therefore not a limiting factor in lipid synthesis, and at atmospheric levels, the substrate offered only a slight benefit in FA production due to increased exposure of *T. chuii* to CO_2. It therefore follows that since *P. kessleri* was exposed to low levels of CO_2 in the flask system, that CO_2 was a limiting nutrient, and therefore lipid synthesis was reduced in flask-grown *P. kessleri*. In substrate-grown *P. kessleri*, the greater surface area of algae exposed to CO_2, coupled with the effective increase in concentration of algae on the substrate, led to a compensation for the decreased solubility of CO_2 in acidic nutrient solution, resulting in a substantial increase in FA synthesis in substrate grown *P. kessleri*.

Conclusions

Because of increased oxidative stress, it was originally hypothesized that an aeroponic system would allow for increased biomass productivities while positively affecting FA content and increasing harvest biomass concentrations. Our results indicate

that *P. kessleri* and *T. chuii* appear to have greater productivity on the ACS than traditional systems and that both *P. kessleri* and *T. chuii* showed increases in biomass harvest concentration. This was surprising since it was originally thought that marine species would grow poorly on the system due to salinity gradients. However, this assumption is based on scaling the productivity of the lab-scale system linearly to a large-scale system.

It appears that the overall FA content of algae grown on the substrates would not necessarily change and that the main reason the FA content of *P. kessleri* differed from the control was that the growth conditions were fundamentally changed between the two systems. It is suspected that even though the ACS is contained in air, that the algae on the system are essentially within a suspended growth environment as water is contained within the pores of the substrates and exopolysaccharides prevent substantial desiccation. Therefore, the FA content of most species on the ACS are suspected not to differ from a suspended growth system. In regards to carbon sequestration, this ACS is highly amenable for this task as it can be placed in greenhouses where CO_2 is passively provided instead of needing to be pressurized and pumped into a solution.

With routine mechanical harvesting, this ACS was able to maintain a stationary phase for extended periods of time (> 6 months) without requiring cleaning or maintenance. From these results, we conclude that this ACS can produce substantial quantities of biomass and that cultivation on the substrates does not substantially alter the nutritional profile of algae. Based upon the volumetric and per area productivity of the ACS achieved at small scale, this system is potentially capable of productivities in the same range as the ORP and PBR traditional systems.

While the ACS does have benefits in comparison to traditional systems, it must be noted that there are some considerable downsides to this ACS approach. Since the substrates are suspended in air within a humid and hot environment, contamination by heterotrophs and other algae is a major concern. Growing extremophile algae species on the system can avoid some of this contamination, but this represents a major drawback for the system and makes it difficult for the system to grow a highly pure product. This could potentially limit the system in its ability to be used as a source of feed for fisheries as marine fish larvae are very vulnerable to contamination and it greatly reduces their productivity (Makridis et al., 2006). Additionally, if ten square meter substrates are grown within 1 m^2 of floor space, then the effective surface area per square meter of floor space is 20 m^2. Even though the substrates are housed within controlled environments (e.g., greenhouses) this surface area is 20 times greater than the surface area of an ORP and thus this system can potentially lose considerable amounts of water in comparison to traditional systems. While the ACS does have disadvantages, it can still serve as a useful cultivation process even if it is not more successful than ORPs or PBRs for all applications as it could be used in tandem with traditional systems to provide a concentrated inoculant. Regardless of its future commercial application, this ACS does show value in its ability to produce dense effluent concentrations and to grow unique algae species and as such warrants future study.

Figures and Tables

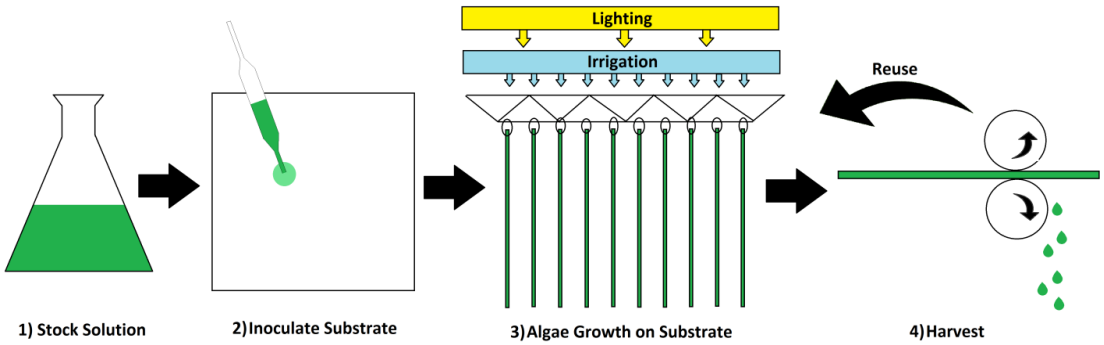

Figure 1. Aeroponic substrate based cultivation system overview. The process from growing inoculum through harvesting is comprised of four distinct steps: 1) Stock Solution, 2) Inoculate Substrate, 3) Algae Growth on Substrate and 4) Harvest.

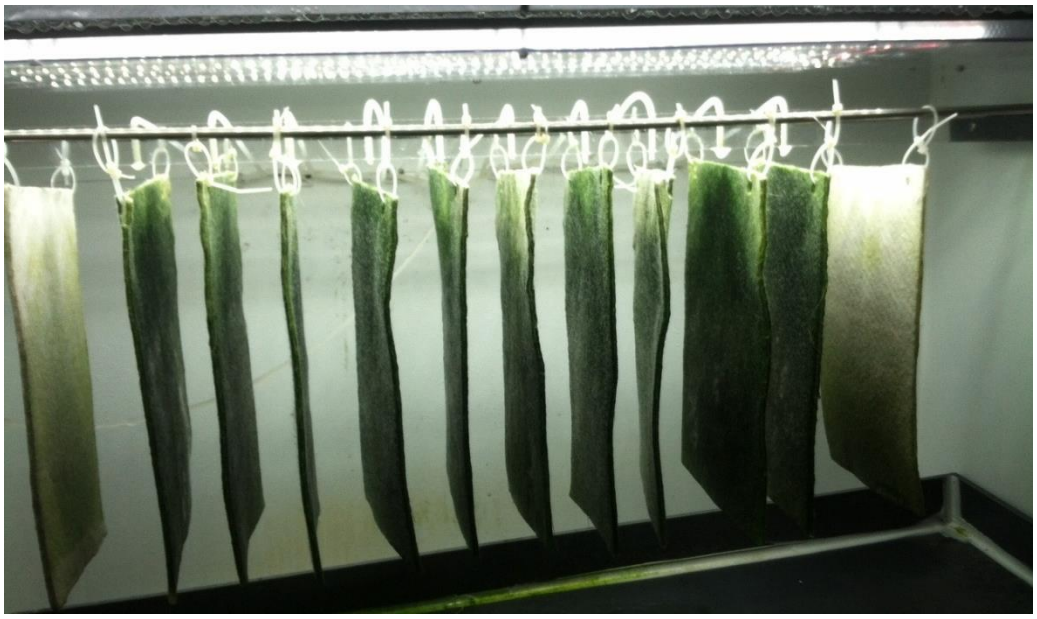

Figure 2. Small scale aeroponic substrate based system which was inoculated with *P. kessleri* and irrigated for three weeks. The left most and right most substrates are not inoculated or irrigated and serve to maintain consistent lighting amongst all ten growth substrates. At the top of each substrate a small 1/8" irrigation dripper can be seen and the nutrient solution that drips off the bottom of the substrates is collected and used for analysis. The substrates are contained within a closed chamber and are illuminated with an array of LED lights on a 16 hour on and 8 hour off light cycle.

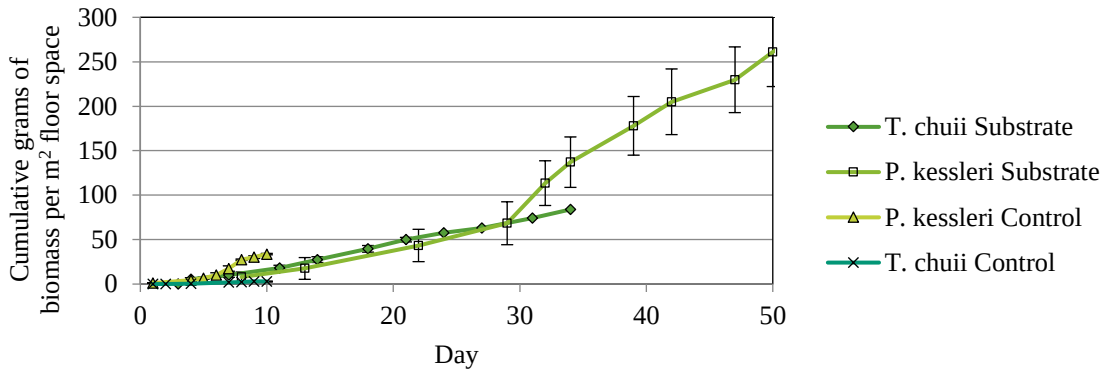

Figure 3. Cumulative grams of biomass produced from *T. chuii* and *P. kessleri* using substrate and control PBR flask system. The experiments were terminated when growth rates peaked based upon findings from preliminary data. The inflection point seen for *P. kessleri* substrate growth represents the point in time when a dense biofilm rapidly formed on the substrates. The vertical bars represent the standard deviation of the data from three replicate trials.

Table 1. Maximum growth rate and maximum biomass concentration achieved during the duration of experiments. The Control in the table represents the flask PBR system that was used for testing and the biomass represented in grams is dry biomass. The maximum growth rates were determined based upon the maximum growth rate achieved during the duration of the testing and these units are represented in square meters of floor space.

	Max. Growth Rate ($g\ day^{-1}\ m^{-2}$)	Max. Growth Rate ($g\ L^{-1}\ day^{-1}$)	Standard Deviation (n=3)	Max. Conc. ($g\ L^{-1}$)	Standard Deviation (n=3)
P. kessleri Control	6.43	0.20	± 2.53	0.68	± 0.02
P. kessleri Substrate	7.95	2.15	± 2.07	3.30	± 0.87
T. chuii Control	0.78	0.02	± 0.22	0.06	± 0.01
T. chuii Substrate	2.78	0.49	± 0.22	1.40	± 0.29

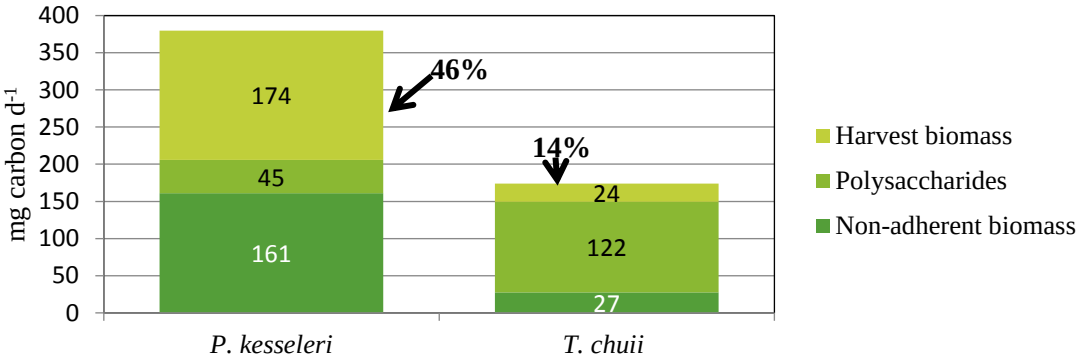

Figure 4. Carbon partitioning within *P. kessleri* and *T. chuii* cultures cultivated on the substrate based system. The non-adherent biomass is defined as the algae that did not adhere to substrate and fell off the substrate with the effluent drip from the bottom of the substrate. Non-adherent biomass was separated from polysaccharides in substrate effluent for analysis using centrifugation and harvest biomass was determined directly following mechanical harvesting. The percentages seen at the top of both bar graphs represent the percentage of carbon sequestered in the harvested biomass for *P. kessleri* and *T. chuii*.

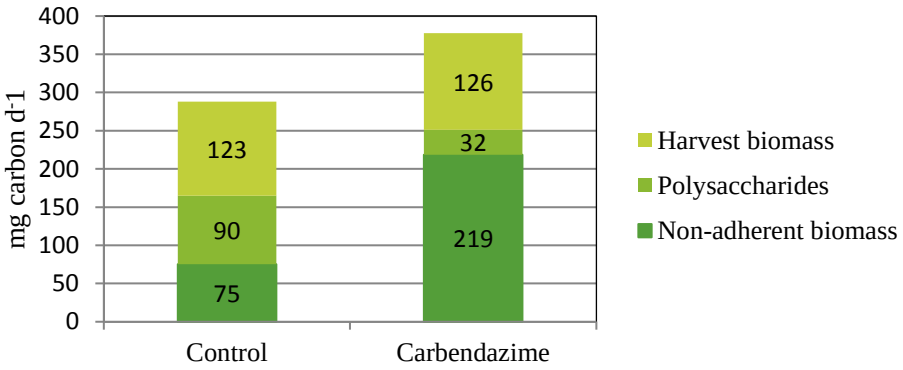

Figure 5. Impact of fungi on the production of polysaccharides on the system. Carbendazime was used to remove the fungi present on the system while not disrupting the algae.

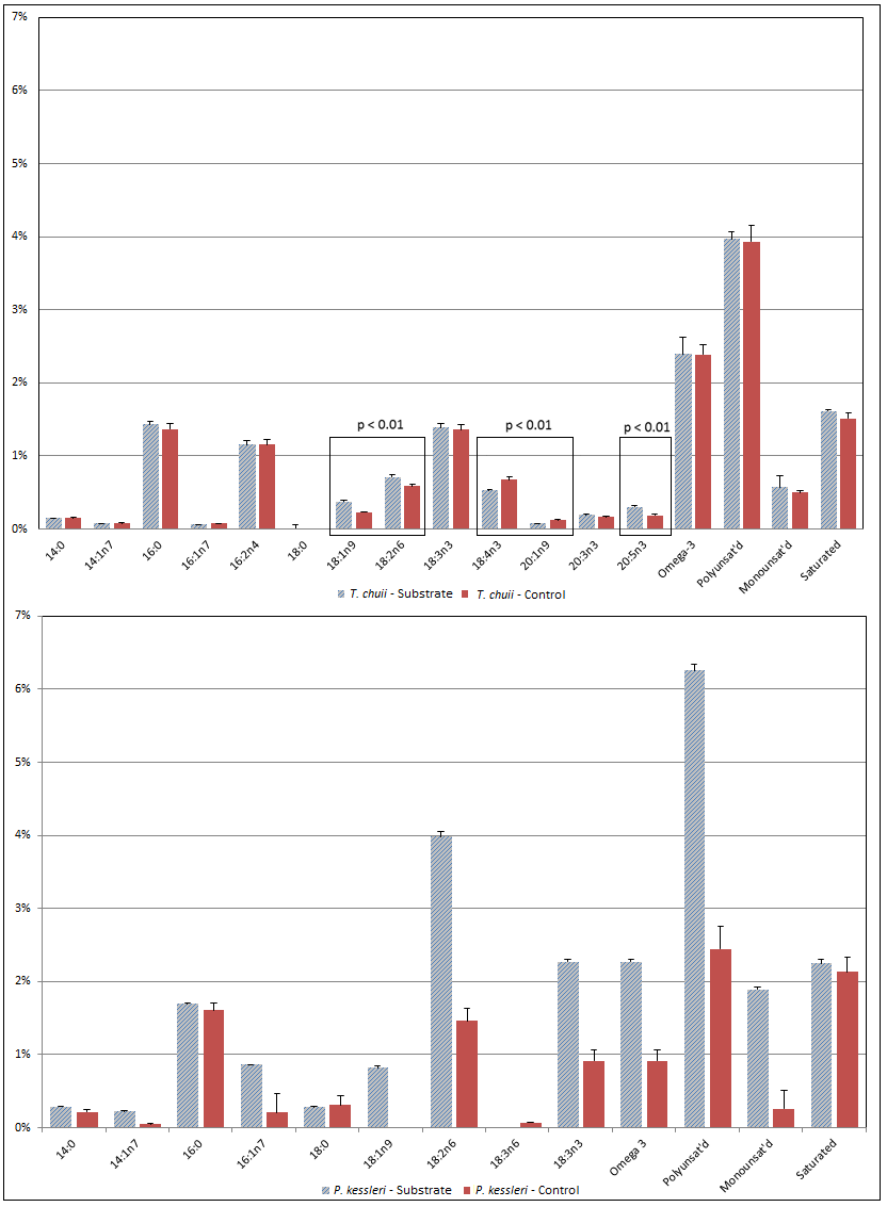

Figure 6. Fatty acid content (as % of freeze dried mass) for *Parachlorella kessleri* and *Tetraselmis chuii* grown on the substrate based system compared to grown in the control photobioreactor system. Fatty acid description: 18:4n3 – "18" is the number of carbons, "4" is the number of double bonds, "3" is location of the double bond. The vertical bars represent the standard error (standard deviation) and the three boxes represent where the fatty acid components are significantly ($p < 0.01$) different from one another for *T. chuii*.

Table 2. Absolute fatty acid content of *T. chuii* and *P. kessleri* grown on substrates and in flasks. Bold p values are statically significant ($p < 0.05$).

Fatty Acid	***T. chuii* – substrate**	***T. chuii* – control**	**p Value (n=6)**	**Change due to substrate**
14:0	0.147% ± 0.003%	0.148% ± 0.010%	0.8203	N.S.
14:1n7	0.074% ± 0.001%	0.075% ± 0.006%	0.8099	N.S.
16:0	1.434% ± 0.041%	1.365% ± 0.071%	0.2217	N.S.
16:1n7	0.055% ± 0.005%	0.074% ± 0.004%	**0.0070**	-0.018%
16:2n4	1.158% ± 0.048%	1.152% ± 0.070%	0.9098	N.S.
18:0	0.032% ± 0.031%	N.D.	0.1484	N.S.
18:1n9	0.365% ± 0.026%	0.227% ± 0.012%	**0.0011**	0.138%
18:2n6	0.710% ± 0.027%	0.583% ± 0.03%	**0.0056**	0.126%
18:3n3	1.383% ± 0.056%	1.358% ± 0.078%	0.6701	N.S.
18:4n3	0.528% ± 0.019%	0.677% ± 0.039%	**0.0038**	-0.150%
20:1n9	0.070% ± 0.005%	0.121% ± 0.007%	**0.0004**	-0.052%
20:3n3	0.187% ± 0.015%	0.161% ± 0.009%	0.0533	N.S.
20:5n3	0.300% ± 0.016%	0.189% ± 0.01%	**0.0006**	0.111%
Total	6.442% ± 0.228%	6.13% ± 0.344%	0.2601	N.S.
Omega-3	2.398% ± 0.100%	2.384% ± 0.136%	0.8965	N.S.
Polyunsaturated	3.966% ± 0.163%	3.931% ± 0.226%	0.8399	N.S.
Monounsaturated	0.564% ± 0.024%	0.497% ± 0.027%	**0.0330**	N.S.
Saturated	1.612% ± 0.031%	1.513% ± 0.081%	0.1182	N.S.
Fatty Acid	***P. kessleri* Substrate**	***P. kessleri* Control**	**p Value (n=6)**	**Change due to substrate**
14:0	0.285% ± 0.013%	0.213% ± 0.038%	**0.0365**	N.S.
14:1n7	0.220% ± 0.013%	0.049% ± 0.005%	**< 0.0001**	0.171%
16:0	1.690% ± 0.021%	1.609% ± 0.093%	0.2168	N.S.
16:1n7	0.857% ± 0.006%	0.209% ± 0.253%	**0.0114**	0.648%
18:0	0.276% ± 0.023%	0.307% ± 0.134%	0.7094	N.S.
18:1n9	0.812% ± 0.031%	N.D.	**< 0.0001**	0.812%
18:2n6	3.986% ± 0.069%	1.459% ± 0.169%	**< 0.0001**	2.527%
18:3n6	N.D.	0.070% ± 0.003%	**< 0.0001**	-0.070%
18:3n3	2.269% ± 0.037%	0.914% ± 0.143%	**0.0001**	1.354%
Total	10.395% ± 0.173%	4.830% ± 0.533%	**0.0001**	5.565%
Omega 3	2.269% ± 0.037%	0.914% ± 0.143%	**0.0001**	1.354%
Polyunsaturated	6.255% ± 0.106%	2.374% ± 0.311%	**< 0.0001**	3.881%
Monounsaturated	1.890% ± 0.050%	0.258% ± 0.258%	**0.0004**	1.632%
Saturated	2.251% ± 0.057%	2.129% ± 0.265%	0.3823	N.S.

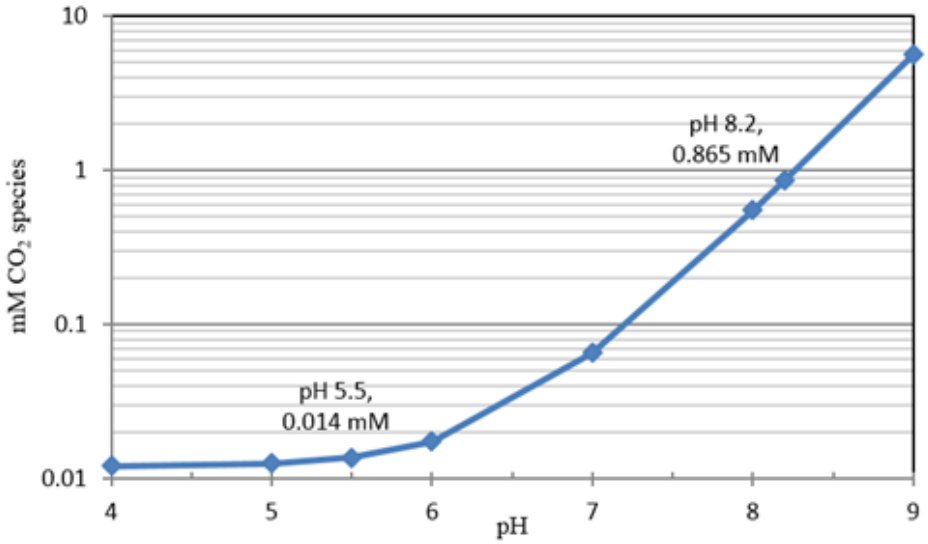

Figure 7. Total CO_2 content in water at atmospheric pressure and background CO_2 levels (0.0400% atmospheric content), showing non-linear pH dependency. This relationship is based upon aquatic CO_2 speciation as described by Morel and Hering in Principles and Applications of Aquatic Chemistry (1993). $mMCO_2$ can be converted to mg CO_2 per liter by multiplying by 44.

References

- Batan, L., Quinn, J., Willson, B., & Bradley, T. (2010). Net energy and greenhouse gas emission evaluation of biodiesel derived from microalgae. *Environmental Science & Technology*, *44*(20), 7975-7980.
- Chaumont, D. (1993). Biotechnology of algal biomass production: a review of systems for outdoor mass culture. *Journal of Applied Phycology*, *5*(6), 593-604.
- Christenson, L. B., & Sims, R. C. (2012). Rotating algal biofilm reactor and spool harvester for wastewater treatment with biofuels by-products. *Biotechnology and Bioengineering*, *109*(7), 1674-1684.
- Collet, P., Lardon, L., Hélias, A., Bricout, S., Lombaert-Valot, I., Perrier, B., & Bernard, O. (2014). Biodiesel from microalgae–Life cycle assessment and recommendations for potential improvements. *Renewable Energy*, *71*, 525-533.
- Da Silva, T. L., Reis, A., Medeiros, R., Oliveira, A. C., & Gouveia, L. (2009). Oil production towards biofuel from autotrophic microalgae semicontinuous cultivations monitorized by flow cytometry. *Applied Biochemistry and Biotechnology*, *159*(2), 568-578.
- Dubois M., Gilles K.A., Hamilton J.K., Rebers P., & Smith F. (1956). Colorimetric method for determination of sugars and related substances, *Analytical Chemistry* 28(3) 350-356.
- B Fernandes, B., Teixeira, J., Dragone, G., Vicente, A. A., Kawano, S., Bišová, K., & Vítová, M. (2013). Relationship between starch and lipid accumulation induced by nutrient depletion and replenishment in the microalga *Parachlorella kessleri*. *Bioresource Technology*, *144*, 268-274.

- Gross, M., Henry, W., Michael, C., & Wen, Z. (2013). Development of a rotating algal biofilm growth system for attached microalgae growth with in situ biomass harvest. *Bioresource Technology*, *150*, 195-201.
- Hom, E. F., & Murray, A. W. (2014). Niche engineering demonstrates a latent capacity for fungal-algal mutualism. *Science*, *345*(6192), 94-98.
- Guillard, R. R., & Ryther, J. H. (1962). Studies of marine planktonic diatoms: I. Cyclotella Nana Hustedt, and Detonula Confervacea (CLEVE) Gran. *Canadian Journal of Microbiology*, *8*(2), 229-239.
- Johnson, M. B., & Wen, Z. (2010). Development of an attached microalgal growth system for biofuel production. *Applied Microbiology and Biotechnology*, *85*(3), 525-534.
- Makridis, P., Costa, R. A., & Dinis, M. T. (2006). Microbial conditions and antimicrobial activity in cultures of two microalgae species, *Tetraselmis chuii* and *Chlorella minutissima*, and effect on bacterial load of enriched *Artemia metanauplii*. *Aquaculture*, *255*(1), 76-81.
- Mata, T. M., Martins, A. A., & Caetano, N. S. (2010). Microalgae for biodiesel production and other applications: a review. *Renewable and Sustainable Energy Reviews*, *14*(1), 217-232.
- Meiling, H., Qing, H., Jianrong, H., & Zufu, L. (2010). A rapid determination of chlorella biomass [J]. *Hebei Fisheries*, *4*(003).
- Meseck, S. L., Alix, J. H., & Wikfors, G. H. (2005). Photoperiod and light intensity effects on growth and utilization of nutrients by the aquaculture feed microalga, Tetraselmis chui (PLY429). *Aquaculture*, *246*(1), 393-404.

- Mishra, A., & Jha, B. (2009). Isolation and characterization of extracellular polymeric substances from micro-algae Dunaliellasalina under salt stress. *Bioresource Technology*, *100*(13), 3382-3386.
- YMizuno, Y., Sato, A., Watanabe, K., Hirata, A., Takeshita, T., Ota, S., & Kawano, S. (2013). Sequential accumulation of starch and lipid induced by sulfur deficiency in *Chlorella* and *Parachlorella* species. *Bioresource Technology*, *129*, 150-155.
- Morel, F. M., & Hering, J. G. (1993). *Principles and Applications of Aquatic Ahemistry*. John Wiley & Sons.
- Olaizola, M., Duerr, E. O., & Freeman, D. W. (1991). Effect of CO2 enhancement in an outdoor algal production system usingTetraselmis. *Journal of Applied Phycology*, *3*(4), 363-366.
- Ozkan, A., Kinney, K., Katz, L., & Berberoglu, H. (2012). Reduction of water and energy requirement of algae cultivation using an algae biofilm photobioreactor. *Bioresource Technology*, *114*, 542-548.
- Pokoo-Aikins, G., Nadim, A., El-Halwagi, M. M., & Mahalec, V. (2010). Design and analysis of biodiesel production from algae grown through carbon sequestration. *Clean Technologies and Environmental Policy*, *12*(3), 239-254.
- Richardson, J. W., Johnson, M. D., Zhang, X., Zemke, P., Chen, W., & Hu, Q. (2014). A financial assessment of two alternative cultivation systems and their contributions to algae biofuel economic viability. *Algal Research*, *4*, 96-104.
- Rogers, J. N., Rosenberg, J. N., Guzman, B. J., Oh, V. H., Mimbela, L. E., Ghassemi, A., & Donohue, M. D. (2014). A critical analysis of paddlewheel-driven

raceway ponds for algal biofuel production at commercial scales. *Algal Research*, *4*, 76-88.

- Slade, R., & Bauen, A. (2013). Micro-algae cultivation for biofuels: cost, energy balance, environmental impacts and future prospects. *Biomass and Bioenergy*, *53*, 29-38.
- Sutherland, I. W. (2001). Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology*, *147*(1), 3-9.
- Tsuzuki, M., Ohnuma, E., Sato, N., Takaku, T., & Kawaguchi, A. (1990). Effects of CO2 concentration during growth on fatty acid composition in microalgae. *Plant Physiology*, *93*(3), 851-856.
- U.S. Patent No. 20140127776. Washington, DC: U.S. Patent and Trademark Office.
- Watanabe, K., Takihana, N., Aoyagi, H., Hanada, S., Watanabe, Y., Ohmura, N., & Tanaka, H. (2005). Symbiotic association in Chlorella culture. *FEMS Microbiology Ecology*, *51*(2), 187-196.

Chapter 2: Effects of Nitrogen, CO_2 and Harvesting Method on *Parachlorella kessleri* Grown on a Vertical Attached Growth System

Michael Johnson[1], Thomas S. Villani[2,3], James E. Simon[2], A.J. Both[1]

[1] Department of Environmental Sciences, Rutgers University, New Brunswick, NJ 08901

[2] New Use Agriculture and Natural Plant Products Program, Department of Plant Biology and Pathology, Rutgers University, New Brunswick, NJ 08901

[3] Department of Medicinal Chemistry, Ernest Mario School of Pharmacy, Rutgers University, Piscataway, NJ 08854

Abstract

We investigated a simple and inexpensive attached algae cultivation system that allows algae to grow on vertically mounted substrates. We have previously demonstrated that this system is capable of producing high biomass yields (in some cases exceeding 10 g dry biomass m^{-2} floor space d^{-1}), and report here on how *Parachlorella kessleri* responded to changes in nutrient solution composition (N) and gas phase carbon dioxide concentration. In addition, we investigated the effect of evaporation rate on salinity gradients and whether an alternative harvesting method would be an improvement compared to a mechanical press method. We found that in addition to *Parachlorella kessleri* and *Tetraselmis chuii*, which have been previously shown to grow on the substrates, *Botryococcus braunii*, and to a lesser extent *Thalassiosira sp., Chaetoceros calcitrans*, and the cyanobacteria *Oscillatoria sp.*, can also be grown on this system. Increasing the CO_2 concentration from 402 to 3,000 ppm was found to increase growth rates of *Parachlorella kessleri*, but further increasing the CO_2 concentration to 9,000 ppm

did not further increase growth rates. Inducing nitrogen stress by removing nitrogen from the nutrient solution did not result in as rapid of an increase of the lipid content of *Parachlorella kessleri* as is typically seen in traditional suspended growth algae production systems. Finally, we determined that mechanical harvesting will be prohibitive at commercial production scale and suggest that alternative harvesting techniques should be investigated.

Introduction

Commercial algae production is typically conducted for biomass production (e.g., for use as a biofuel) and/or for the production of specific chemical compounds (e.g., for use as nutritional supplements or pigments). Different growing systems are used to produce algae at scale and these include traditional systems such as open raceway ponds (ORPs) and photobioreactors (PBRs), as well as attached cultivation systems (ACSs). Unlike algae growing in a suspension, algae grown on an ACS are primarily immobile, form dense biofilms that are surrounded by air, and can be harvested mechanically by scraping or squeezing the substrate to form dense harvest concentrations. It has been demonstrated that the traditional ORP and PBR systems used for algae production are limited in their production capabilities and financial viability due to their operational and capital costs (Gross et al., 2015; Sheehan et al., 1998). As a result, researchers have been evaluating other cultivation platforms such as ACSs (e.g., Zhuang et al., 2016; Su et al., 2016; Hoh et al., 2016; Gross et al., 2013) to determine if their unique properties (e.g., biofilm growth, minimal water use) would allow for more economically viable algae production. The ACS we used here is similar in design to previously described ACSs (e.g., Cheng et al., 2013), with the major difference that we used inexpensive cotton/polyester substrates instead of multilayered and complex growing surfaces. Cotton

is an effective substrate material, especially when combined with polyester for durability (Gross, 2013). In our ACS, the substrates were hung vertically and irrigated with drippers placed just above the substrates. This ACS at large-scale (Johnson et al., 2015) can be placed in a greenhouse or growth chamber and consists of a mounting system, nutrient solution supply with pump, and the substrates. This system was originally developed by AL-G Technologies, Inc. (Charny, Quebec, Canada) and was designed to be simple, scalable, and rapidly deployable (Kaya and Picard, 1995).

Algae are photosynthetic organisms that usually live in aqueous (either saltwater or fresh water) environments and can adapt to a range of environmental conditions, including salinity, pH, temperature, light intensity, and nutrient concentrations. In traditional ORP and PBR systems, the growth characteristics, nutritional requirements, and chemical composition of various algae species are well understood and have been researched extensively (e.g., Mata et al., 2010; Schnurr et al., 2013). However, it has been demonstrated in several studies that the growth characteristics of algae vary dramatically when comparing traditional ORP and PBR systems with ACSs. When grown in traditional ORP and PBR systems, several algae species rapidly accumulate lipids as a result of low nitrogen availability (Li et al., 2013; Choi et al., 2011), but when grown in an ACS, *Botryococcus braunii* (Gross et al., 2013), *Scenedesmus obliquus,* and *Nitzschia palea* (Schnurr et al., 2013) only slowly accumulated lipids under nitrogen starvation. In order to evaluate how several algae species performed when grown on this ACS, we studied responses to: 1) removal of nitrogen from the nutrient solution, 2) increasing the CO_2 concentration, and 3) changing the harvesting method. We also evaluated the formation of salinity gradients on substrates irrigated with saltwater as well

as the amount of water lost from the system through evaporation. The overall goal of our work was to determine the suitability of our ACS for algae production at scale in a controlled environment such as a greenhouse. To determine the economic feasibility of this system, detailed information about production characteristics and attainable yields was critical.

Materials and Methods

Botryococcus braunii (UTEX 572) was procured from the University of Texas Algae Collection (Austin, TX, USA) and *Tetraselmis chuii* (UTEX 232), *Thalassiosira sp.* (UTEX 2054), *Chaetoceros calcitrans* (CCMP 1315). and *Oscillatoria sp*. (EE10) were procured from Rutgers University, Department of Marine and Coastal Sciences (New Brunswick, NJ, USA). The *Parachlorella kessleri* used for this work was provided by AL-G Technologies, Inc. (Charny, Quebec, Canada).

Culture Conditions and Sampling

The substrates used for this work were all made from Pellon® 50/50 Light Blend with Scrim Batting (Clearwater, FL, USA), which is comprised of 50% polyester and 50% cotton. This material was chosen because other research had shown the effectiveness of cotton substrates (Gross, 2013) and this blend of cotton and polyester was resistant to degradation from mechanical harvesting. To inoculate the substrates for all experiments, 1 L of inoculum was grown in Erlenmeyer flasks and the suspensions were emptied into polycarbonate bins. Ten substrates (15 by 25 by 0.5 cm; width by length by thickness) were then soaked in the algae suspension in the bins. The substrates were then pressed through a mechanical roller press (ATLAS, Model AT01, Winnipeg, Canada) so that the algae could infiltrate the substrates. This process of soaking and

pressing was repeated twice for all ten substrates and then the substrates were suspended from a scaffolding system placed inside a controlled environment chamber and irrigated at a rate of 4 mL min^{-1} with a nutrient solution (**Table 3**) using a peristaltic pump (Masterflex, Model HV-07522-20, Vernon Hills, IL, USA) with pumping head (Masterflex, Model EW-77200-60). A single pumping head was connected to ten individual 1.6 mm inner diameter Tygon® tubes of equal length that delivered 0.4 mL min^{-1} of nutrient solution on top and in the center of each substrate. Each set of ten substrates was mounted in a single row during the experiments and were flanked at the beginning and end of the row by an additional substrate (not irrigated) to provide uniform illumination for all ten substrates. This substrate configuration and irrigation system were used to cultivate algae during all of the experiments described here.

Algae harvested from the system were evaluated for biomass accumulation (g dry biomass L^{-1}) using an optical density approach wherein a standard curve was utilized that correlated dry algae mass to absorption at a wavelength of 680 nm (Griffiths et al., 2011). To create this standard curve, a known volume of algae solution from three different sets of ten substrates was diluted with de-ionized water and sufficiently agitated by mixing and vortexing to remove algal aggregates. These three solutions were then serially diluted into ten different concentrations of algae solution that were utilized to create the standard curve. Each of these ten solution's absorbance was measured with a spectrophotometer (Beckman, Model DU® -64, Jersey City, NJ, USA) to determine its absorbance at a wavelength of 680 nm. Additionally, to determine the algal concentration of each solution, a known volume of each solution was filtered through a pre-weighted 0.45 µm GF/C filter membrane (Whatman, England). The filter membranes were then oven dried

at 100^{o}C for 12 hours and subsequently cooled to room temperature to measure dry weight and to determine the algal concentration (Cheng et al., 2013). The dry mass for the algae was corrected for the impact of dried salt and dried nutrients through using the known volume of the solutions and the concentration and mass of salt and nutrients. The resulting standard curve had an R^2 value of 0.99 for each algae species and the process of determining a standard curve was repeated each week throughout the course of experimentation. This approach of using optical density to measure biomass productivity was used for this work for two separate reasons. First, we wanted to utilize a simple approach for measuring productivity that could be deployed to rural cultivation locations where drying ovens are not readily available. The second reason was that for future large-scale research on this system, we plan to develop an automated nutrient monitoring system that uses real-time optical density readings to determine nutrient usage and system productivity.

For all growth experiments, substrates were kept in a growth chamber at ambient laboratory conditions (402 ppm CO_2, 24-27^{o}C, 97-99% relative humidity) for 70 days prior to the start of experimentation, during which the substrates were harvested every 3-4 days using a mechanical roller press (Johnson et al., 2015). The ambient CO_2 concentration for all experiments was determined using a CO_2 sensor (iSense, Model AZ-0004, Ormond Beach, FL, USA) and was allowed to fluctuate with ambient laboratory conditions. An LED lighting system (Mouser Electronics, Model 901-SB-6500-TR, Mansfield, TX, USA) was used for all experiments and its photosynthetically active radiation (400 – 700 nm) intensity was 246 µmol m^{-2} s^{-1} (16 hours light, 8 hours dark) which was measured with a spectroradiometer at 8 cm below the LED lighting array

(Apogee Instruments, Model PS-300, Logan, UT, USA). The substrates were hung from a support system and 5 cm apart from one another using plastic loops so that their tops were positioned at 8 cm below the LED lighting array. The light intensity in between the substrates was measured, and it was shown that at the substrate center line the light intensity decreased 10% (SD = 2.7%, n=9) for every cm moving down from the top of the substrate. The light spectrum for the LED lights is shown in **Figure 8** and was measured using the spectroradiometer, placed so that its sensor was 20 cm below the LED lighting array. The light intensity at the top of the substrates was maintained at a relatively low level (246 µmol m^{-2} s^{-1}) for all experiments reported here because at the small-scale used, higher light intensities resulted in algal photo-oxidation to some areas of the substrates as the algae were non-mobile and thus constantly illuminated. At a larger scale, higher light intensities can be used as the ratio of substrate surface area to floor area (20 m^2 per m^2 of floor space) is greater than the small-scale system (11 m^2 per m^2 of floor space). The higher substrate surface area to floor area ratio could not be maintained at the small-scale because it would have obstructed visualization and when the substrates are wet and close together (2 cm), they tend to bend and stick to one another.

Nitrogen Stress

Ten substrates were cultivated with *P. kessleri* for 70 days according to the growth conditions previously described with an irrigation rate of 4 mL min^{-1} using the *P. kessleri* nutrient solution (**Table 3**) that contained 200 mg L^{-1} NH_4Cl and 106 mg L^{-1} KNO_3 as nitrogen sources. At the start of the treatment, the nutrient solution pumped to the substrates was replaced with a fresh solution lacking NH_4Cl and KNO_3. Following

this removal of nitrogen from the nutrient solution, the substrates were harvested every 3-4 days for 17 days (5 harvests) and the resulting biomass was collected for subsequent analysis. This 17-day depletion of nitrogen scheme was conducted three consecutive times using the same ten substrates with 24 days of standard nutrient solution applied in between treatments. The 24-day time period was used as a conservative acclimation period since it had been demonstrated during preliminary testing (data not shown here) that *P. kessleri* returned to normal lipid content (± 3.1% lipid content, SD = 1.1%, n=14) after approximately 14 days. The biomass concentration (g dry biomass L^{-1}) of the harvested algae was evaluated using the optical density approach previously described.

The samples were then centrifuged for four minutes at 3,000 RPM (DCF = 8,000) at 5^{o}C to remove the supernatant before being immediately freeze dried and stored in a freezer at -80^{o}C for subsequent lipid analysis. Lipid content was determined using a gravimetric approach (Folch et al., 1957). At each harvest (0, 3, 7, 10, 14, 17 days), 60 mg of freeze dried algae were placed in a pre-weighed 30 mL glass vial in triplicate, while being careful not to expose samples to air for a long period. Freeze dried algae were stored in sealed falcon tubes prior to weighing and these falcon tubes were re-sealed throughout all processing steps using parafilm. These vials were sonicated for 20 minutes and then centrifuged. Next, 0.8 mL of chloroform followed by 0.4 mL of methanol were added to recover the supernatant liquid phase. The solvent was then washed with 250 µL of deionized water and vortexed for 20 seconds. The mixture was centrifuged at 2,000 RPM (DCF = 5,333) to separate the two phases and the upper phase was removed with a

micro-pipette. The lower phase was evaporated under vacuum using a rotovap and the vials were then weighed to determine the dry mass of lipids in the algae samples.

Increasing the CO_2 Concentration

Ten substrates inoculated with *P. kessleri* were cultivated for 70 days according to the growth conditions previously described to establish a growth rate at ambient CO_2 concentration (402 ppm CO_2, SD =12 ppm over 70 days) using the last five harvests. After 70 days, the CO_2 concentration in the chamber was gradually increased to 2,000 ppm CO_2 (±4 ppm) over a period of 32 hours (in steps of 50 ppm CO_2 per hour) using a CO_2 controller (iSense, Model CM-0045-NM-SP) and bottled CO_2 gas (Airgas, Model CGA 320, Industrial Grade, 99.5% purity, Manalapan, NJ, USA). The substrates were maintained at 2,000 ppm for 17 days (5 harvests) and then the CO_2 concentration was gradually increased to 3,000 ppm (±6 ppm) over the course of 20 hours. Again, the concentration was maintained for 17 days (5 harvests) before the CO_2 concentration was gradually increased to 6,000 ppm (±12 ppm) over 60 hours. The system was maintained at 6,000 ppm for 17 days (5 harvests) before the CO_2 concentration was gradually increased to 9,000 ppm (±18 ppm) over 60 hours and was maintained at 9,000 ppm for 17 days (5 harvests). The 5 harvests at each CO_2 concentration were analyzed using the optical density approach to determine the biomass concentration (dry grams of biomass L^{-1}) and growth rate (dry grams of biomass day^{-1}) (Choi et al., 2011).

The sampling schedule and CO_2 concentrations were chosen based upon previous work that had investigated the use of CO_2 to increase biomass productivity on ACSs (Blanken el al., 2014; Gross et al., 2013). Unlike traditional suspended growth systems, it had been shown that increasing the CO_2 concentration to 3,000 ppm in a rotating attached

biofilm (RAB) system had a minimal impact on the growth rate of *Chlorella* (Blanken et al., 2014). Therefore, for *P. kessleri* we wanted to investigate the growth of this algae species over a wider CO_2 concentration range. CO_2 concentrations of 2,000 and 3,000 ppm represent manageable and cost effective targets that are below the Occupational Safety and Health Administration's (OSHA) guideline of 5,000 ppm (OSHA, 2016). Concentrations of 6,000 and 9,000 ppm are above this OSHA guideline and were used to investigate the impact of higher CO_2 concentrations even though these concentrations would not be feasible at scale due to worker health concerns. Safe working conditions were achieved by maintaining these elevated CO_2 concentrations within small controlled growth chambers that were vented into a room of substantial volume that had an ambient CO_2 concentration and constant CO_2 monitoring (instrument described previously) for lab personnel safety.

Alternative Harvesting Method

To test harvesting (i.e., removing) algae from the substrates by recirculating the nutrient solution instead of using a mechanical press, ten substrates were inoculated with *P. kessleri* and were cultivated for 70 days according to the growth conditions previously described. The collected effluent from the substrates contained 0.109 to 0.154 g L^{-1} of non-adherent biomass that was gravity drained into an effluent bin and collected via an effluent tube. After 70 days of acclimation and harvesting every 3-4 days, the substrates were harvested and then the alternative harvesting experiment was initiated. The substrates were left to grow for three days prior to the start of testing so that they would have their maximum biomass load for testing. Evaluation of the ability to remove algae by recirculating the nutrient solution was conducted during a 1-hour experiment and a 12-

hour experiment in order to determine the kinetics of algae removal. Prior to the start of this test, a 250 mL Erlenmeyer flask was filled with 120 mL of *P. kessleri* nutrient solution (**Table 3**) so that it could serve as an effluent reservoir from which samples could be taken as the concentration of algae increased (**Figure 9**). Additionally, all remaining nutrient solution from the tubing system (33 mL) was drained so that only the 120 mL of nutrient solution from the effluent reservoir was used for testing. This was done so that the volume of nutrient solution being recycled would be known. At the start of the test (time = 0), the effluent tube from the effluent collection bin was placed into the Erlenmeyer flask reservoir so that the effluent could be recycled by the pump that was used for substrate irrigation (**Figure 9**).

The overall pumping rate to the ten substrates was increased to 50 mL min^{-1} from 4 mL min^{-1} so that the nutrient solution could be recycled 25 times each hour. At time zero and every three minutes thereafter for a total time of one hour, a one mL sample was taken from the reservoir and stored for subsequent analysis. The one mL of nutrient solution that was removed from the reservoir was replaced with one mL of stock nutrient solution and the concentration change created by this exchange was corrected for using a mass balance. Following one hour of nutrient solution recirculation, the substrates were harvested and the system was irrigated under normal conditions for three days. The above nutrient solution recirculation experiment was then repeated twice with a three-day interval of normal nutrient solution circulation in between. After three tests were conducted to evaluate the 1-hour nutrient solution recycling scheme, the same experimentation was conducted for 12-hour nutrient solution recycling using the same set of substrates and three days of normal conditions in between triplicate trials. For these

12-hour tests, one mL samples were taken from the system every hour for analysis. The concentration of algae (g dry biomass L^{-1}) within samples was determined using the previously described optical density technique.

Salinity and Water Loss Measurement

One of the major concerns for cultivating marine species of algae on the ACS was that salinity gradients would form on the substrates due to non-uniform irrigation and evaporation. To examine the presence of salinity gradients on the system, ten substrates were placed in one of the growth chambers and 35 parts per thousand (ppt) saltwater was used to irrigate the ten substrates at a combined rate of 4 mL min^{-1}. After two weeks of irrigating the substrates with saltwater, the salinity from three of the ten substrates was measured using a refractometer (Extech, Model RF20, Portable Brix Refractometer, Nashua, NH, USA) every two days over an eight-day period. These measurements were taken by extracting two drops of solution from the substrates with a pipette. To determine the sampling locations, a 5 by 5 cm grid was laid on top of the 15 by 25 cm substrates, and 24 measurements were taken from each of the 3 selected substrates at the vertices of the grid.

To quantify the volume of water being lost from the substrates, ten substrates were inoculated with *P. kessleri* and were cultivated for 70 days according to the growth conditions previously described. These substrates were contained within a growth chamber that had an exchange rate of 1.2 volumes hr^{-1} that exchanged its volume with its laboratory surroundings (21-23^{o}C, 40-50% relative humidity). This exchange rate was determined by temporarily increasing the CO_2 concentration of the chamber to 5,000 ppm and measuring the reduction in the CO_2 concentration over time using a CO_2 controller

(instrument described previously). To determine the loss of water from the system, a known volume of nutrient solution (6 liters) was placed in the system's irrigation reservoir after 70 days of cultivation and the irrigation lines were purged of any remaining nutrient solution. The system was then left to utilize this nutrient solution for 24 hours and the nutrient solution that dripped from the substrates was collected. After 24 hours, all of the nutrient solution in the reservoir, collection reservoir and tubing was weighed to determine the mass of water lost to the environment. This water loss measurement was conducted fourteen times over a 21-day period.

Cultivating Various Species

To analyze algal growth for several algae species on the ACS, *P. kessleri*, *B. braunii*, *T. chuii*, *Thalassiosira sp.*, *C. calcitrans*, and the cyanobacteria *Oscillatoria sp.* were placed in 1 L Erlenmeyer flasks with appropriate nutrient solutions (**Table 3**). All saltwater used was collected from Corson's Inlet (Cape May, NJ, USA) at low tide (35 ppt salinity) and was autoclaved prior to use, while all freshwater used to create the nutrient solution was municipal tapwater (pH = 7.8, New Brunswick, NJ, USA). Dilutions of saltwater were created using deionized water. The flasks were placed in a growth chamber 20 cm below the LED lighting array at ambient laboratory conditions. The flasks were capped with a 3 cm long polystyrene plug and the flasks were aerated using an air pump with a single 6 mm tube and no diffuser with ambient laboratory air at a rate of 2 L min^{-1}. After three weeks of growth in the flasks and sufficient biomass accumulation (more than 1 g dry biomass L^{-1}), the solution from the flasks was utilized to inoculate ten substrates for each species of algae. Species of algae were placed in individual enclosures within a controlled environment growth chamber to avoid

contamination between different species and were irrigated at a combined rate of 4 mL min^{-1} using the appropriate nutrient solution (**Table 3**). Algae were left to grow on the substrates for 7 days, or until adherence was visually noticeable, and were then harvested for the first time using the mechanical roller press. Subsequently, the substrates were harvested every 3-4 days and harvest samples were collected for biomass analysis using the optical density technique. A standard curve was created for each of the species to determine biomass concentration (g dry biomass L^{-1}) as previously described. The algae were allowed to grow on the substrates for 70 days, or until the growth of algae on the substrates ceased based on visual inspection when the harvest solution contained minimal algae. On a weekly basis throughout the course of all studies, cultures from the substrates were observed under a microscope (10X) to monitor for contamination. We had previously demonstrated that through routine cleaning and the use of controlled growth chambers, we could maintain cultures on the substrates that had no substantial change in chemical or nutrient composition over extended periods of time (Johnson et al., 2015).

Results

Nitrogen Stress

Over a period of 17 days of nitrogen depletion, the growth rate of the *P. kessleri* was reduced from 5.4 g dry biomass m^{-2} floor space d^{-1} to a final growth rate of 2.9 g dry biomass m^{-2} floor space d^{-1}, which represents a 45% decrease in growth rate (**Figure 10**). Concurrently, the lipid content of the *P. kessleri* increased from 3.78% to 33.36% of dry weight (**Figure 10**). The lipid production rate peaked at day 14 after nitrogen depletion (Day 21 in **Figure 10**) and began to decline thereafter because while overall lipid content (%) increased, lipid production (g dry lipids d^{-1}; data not shown) decreased with the decreasing growth rate.

CO_2 Concentration

During the CO_2 enrichment trials, the growth rate of *P. kessleri* increased as the CO_2 concentration increased from 402 to 3,000 ppm, but declined between 3,000-9,000 ppm of CO_2 (**Figure 11**). However, only the change in growth rates between 402, 2,000 and 3,000 were statistically significant (n=5, paired T-test). The growth rate at 3,000 ppm was approximately 60% higher compared to that of the ambient CO_2 concentration of 402 ppm.

Alternative Harvesting Method

The 1-hour test that started with recycling the substrate effluent is presented in **Figure 12A**. After the initial increase in biomass removal, the effluent concentration linearly increased over time. The 12-hour test (**Figure 12B**) shows that over this period of time the removal of algae from the system also exhibited a linear relationship ($y = 0.0016x + 0.277$, $R^2 = 0.989$). This trend correlates well with the trend ($y = 0.0017x + 0.4086$, $R^2 = 0.948$) observed during the last 21 minutes of the 1-hour recirculation tests. After 12-hours, the system reached a concentration of 1.4 g dry biomass L^{-1} using nutrient solution recirculation.

Salinity and Water Loss Measurement

The salinity measurements across the substrates show (**Figure 13**) gradients such that the periphery can reach a salinity of 45 ppt when the input nutrient solution had a salinity of 35 ppt. The impact of these salinity gradients is illustrated in **Figure 14**, which shows an unsuccessful preliminary experiment where *Arthrospira sp.* was irrigated with one dripper (**Figure 14A**) and with three drippers (**Figure 14B**) supplying a 25 ppt saltwater nutrient solution of F/2 nutrient solution (Guillard, 1975). The *Arthrospira sp.*

was able to grow for a short period within a narrow salinity range (approximately 25-30 ppt) and therefore grew on the substrates in small bands where the salinity fell within that narrow range (Bartley et al., 2013; Adenan et al., 2013). Over time, the salinity on the substrates increased and the *Arthrospira sp.* ceased growing on the substrates all together after three weeks. By measuring the water loss from the system, we determined that 2.6% (SD = 0.3%, n=14) of the water that was circulated through the system was lost to evaporation. In our small-scale system, we irrigated 0.375 m^2 of substrates with 5.76 L d^{-1} (0.75 m^2 of total surface area). If this relationship is linearly extrapolated to a large-scale system with ten, one meter squared substrates per square meter of floor space (20 m^2 of total surface area), the system would lose 4 L m^{-2} d^{-1} to evaporation.

Cultivating Various Species

In addition to *P. kessleri* which was used for the studies described above, we also cultivated *B. braunii, T. chuii, Thalassiosira sp., C. calcitrans,* and *Oscillatoria sp.* The maximum growth rates at ambient conditions for these species varied from 1.3 to 11.8 g dry biomass m^{-2} floor space d^{-1}. The growth rates per meter squared of substrate area were extrapolated from the per area productivities of these algae species on the small-scale system. The small-scale system had a total substrate area of 0.75 m^2 in a 0.068 m^2 footprint (equivalent to 11.1 m^2 substrate area m^{-2} floor space) and the productivity per meter squared of floor space was calculated by multiplying the small-scale system productivity by 14.8. This extrapolation assumed that the small-scale substrate productivity could be linearly scaled to the large-scale productivity and that the increased surface area (20 m^2 of substrate area m^{-2} floor space) would not negatively impact overall productivity.

Of all the species tested, *P. kessleri* had the greatest maximum productivity of 11.8 g dry biomass m^{-2} floor space day^{-1} followed by *B. braunii*, *T. chuii*, *Thalassiosira sp.*, *Oscillatoria sp.*, and *C. calcitrans* which had maximum growth rates of 9.7, 4.5, 2.3, 2.2, and 1.3 g dry biomass m^{-2} floor space day^{-1}, respectively. The maximum growth rates were the greatest productivities achieved over the 70-day growth periods that included 20 harvests. *P. kessleri*, *B. braunii*, *T. chuii*, *Thalassiosira sp.*, *Oscillatoria sp.*, and *C. calcitrans* had average growth rates during this time of 7.9, 7.7, 2.5, 1.5, 1.7, and 1.0 g dry biomass m^{-2} floor space day^{-1} with standard deviations of 3.7, 1.2, 1.0, 0.3, 0.3, and 0.2 g dry biomass m^{-2} floor space day^{-1} (n=20), respectively.

Discussion

Nitrogen Stress

While there are several species of algae that have been shown to grow on the ACS described here, most of our work has focused on *P. kessleri* and thus, its growth characteristics are best understood. *P. kessleri* consistently produces low lipid concentrations (less than 10%) and therefore makes a good candidate for evaluating the impact of nutrient stress on lipid accumulation (Li et al., 2013). Nitrogen was chosen as the nutrient to induce stress since its impact on lipid accumulation in *P. kessleri* grown in PBR systems has been the most substantial of the different nutrients that have been evaluated (Li et al., 2013). In addition, other researchers have evaluated the impact of nitrogen starvation on lipid accumulation in other ACSs (Cheng et al., 2013; Gross et al., 2013; Schnurr et al., 2013). Using a PBR system, Li et al., (2013) showed that the dry mass lipid content of *P. kessleri* can be increased from approximately 5% to 60% when the nutrient solution is depleted of nitrogen over five days. Therefore, *P. kessleri* was used for nitrogen starvation tests on our ACS due to existing data on nitrogen starvation

in other ACSs and the ability to compare the effectiveness of nitrogen starvation to a traditional suspended growth system.

The long-term goal for most algae production systems is to be able to produce biofuel and high value co-products (Brune et al., 2009; Rodolfi et al., 2009). To produce biofuel from algae, lipids and fatty acids need to be removed using an organic solvent, followed by transesterfication using an alcohol (e.g., methanol) to produce fatty acid methyl esters (FAMEs) (Montes et al., 2011). The remaining portion of the algae (e.g., proteins, sugars) consists primarily of lower value products that are used as animal feed or other low value commodity products (Scott et al., 2010). Because of the low value of the remaining biomass, much focus has been placed on increasing the lipid fraction of algae prior to harvesting and subsequent processing (Mata et al., 2010; Scott et al., 2010). A method of initiating such an increase in lipid content has been achieved through nutrient depletion (Li et al., 2013) where removing a nutrient (e.g., nitrogen) from the nutrient solution induces metabolic stress (Li et al., 2013). Many algae species will reduce their growth rate, but will increase their lipid concentration as a protective means to efficiently store chemical energy (Li et al., 2013). However, harnessing this mechanism requires a delicate balance between increasing lipid content and reducing overall biomass productivity and we felt it was important to evaluate this process for this ACS since it would demonstrate its potential value for lipid production.

While this lipid accumulation strategy has been well studied, it is difficult to exploit at large-scale in OP or PBR systems because for it to work, the concentration of a nutrient in a large volume of water needs to be reduced. This can be accomplished by precipitating that nutrient out of solution, allowing algae to consume all of the nitrogen,

or by collecting the algae and replacing the entire water volume. The water cannot simply be diluted because this would cause the concentration of algae biomass to be reduced, and this would subsequently increase the volume of solution that needs to be processed. The process of altering the nutrient solution at scale can be time consuming, energy intensive, and will reduce the overall productivity of the growing system (Scott et al., 2010). For ACSs, the nutrient solution used to irrigate the substrates can easily be changed such that a complete nutrient solution can be replaced with, for example, a nutrient solution lacking nitrogen. We hypothesized that the ability of our ACS to rapidly decrease the concentration of nitrogen in the nutrient solution would result in a substantial increase in lipid content. We increased the lipid content under our specific set of conditions, but observed that the effect was much less substantial than was reported for *P. kessleri* in a PBR system (Li et al., 2013). The same result has been observed with other algae produced on other ACSs (Schnurr et al., 2013) and could be due to the fact that the algae biofilm retained nutrients (Berner et al., 2015), or that the stationary nature of ACSs and the low light intensity used here led to a reduction in photosynthetic yield and lipid production. Future work is needed to determine the cause of these different results. For now, it appears that our initial hypothesis that the ACS allows for improved lipid accumulation does not hold.

Increasing the CO_2 Concentration

One of the goals of this research was to determine the effect of increased CO_2 concentrations on *P. kessleri* grown on the ACS. Previous work in rotating algae biofilm (RAB) systems indicated that increasing the CO_2 concentration above ambient (around 400 ppm) has a minimal impact on increasing algal growth rates (Blanken et al., 2014).

We hypothesized that increasing the CO_2 concentration would increase growth rates, but that the cumulative effect would not be as great as for suspended growth systems where the supply of carbon is typically more limiting. However, our results showed a mixed impact of the CO_2 concentration on growth rates. Some studies have shown that increasing the CO_2 concentration to 3,000 ppm has a minimal impact on growth rates (Gross et al., 2013), while other studies have shown that optimum biomass productivities are only achieved at elevated CO_2 concentrations (Ji et al., 2014). Our results showed an initial increase in productivity followed by a decline. Because of the nutrient solution's low pH (5.5) that was optimized for the growth of *P. kessleri*, the increase in the CO_2 concentration did not lead to a significant drop in the nutrient solution pH, which would have decreased growth rates.

We suspect that the reduction in growth rate was not a result of the algae slowing their growth rate at concentrations of 3,000 ppm and above, but instead a manifestation of our harvesting procedure. The substrates by nature have a carrying capacity and the faster the growth rate of the algae the more quickly the substrates reach this carrying capacity. We estimate that at a 3,000-6,000 ppm CO_2 concentration the substrates reach their carrying capacity and this slowed the growth rate of algae on the system. When harvesting the substrates exposed to 3,000-6,000 ppm of CO_2 it was observed that the algal biofilm on the substrates was thick and secreting a dense extracellular polymeric substance matrix. This dense mat of algae at the carrying capacity of the substrates can retard growth by reducing nutrient and CO_2 availability, irradiance, and by causing oxygen gradients to form, which can reduce growth rates (Singh et al., 2011). We hypothesize that substrate productivity on this system could potentially increase well

above the 3,000-6,000 ppm CO_2 range when coupled with an increased harvesting frequency, proportional to the increase in algal productivity. However, this indicates that the algae on the system are carbon-limited as adding CO_2 to the system does increase overall growth rates. This could be directly due to increased algae biomass production and/or the increased production of exopolysaccharides that cause more algae to adhere to the substrates.

Alternative Harvesting Method

As ACSs are scaled up, some of the major limiting factors are not biological, but rather the physical and engineering characteristics of the system. ACSs are comprised of substrates that, over time, can slowly be degraded by the harvesting methods used to remove the algae from their surface (e.g., scraping, pressing, washing) (Blanken et al., 2014; Cheng et al., 2013). This physical degradation of the substrates necessitates that they are periodically replaced, which creates a capital expenditure for materials and labor.

For our ACS, research to date has focused on mechanical harvesting that squeezes the vertical substrates between opposing rolling presses to achieve high (>15 g L^{-1}) harvest concentrations. While this process is amenable to a laboratory scale system, the physical removal and re-attachment of one square meter substrates (approximately 5 kg) for mechanical harvesting is cumbersome at scale and takes two technicians approximately five minutes to manage. It is desirable that the harvesting of this system can be automated such that technicians are not required to manually process substrates. In addition to automating the harvesting process, it is desirable that the harvesting system used for this ACS is non-destructive and does not use a mechanical system that is prone to damaging substrates. Algae have been shown to continuously wash off the ACS and

the concentration of this non-adherent biomass is approximately 50-100 times less than the biomass that can be harvested from the system using the mechanical pressing technique. We hypothesized that if the effluent containing this non-adherent biomass was recycled numerous times through the substrate, its concentration would increase to a point where it is equal in algal biomass concentration to that of a typical harvest concentration. Instead of disposing of the effluent from the substrates, the effluent would be collected and fed back through the irrigation pump to the top of the substrates. Since the same volume of nutrient solution is used over-and-over, its algal concentration will increase since non-adherent biomass is continually washed off the substrates. We hypothesized that the nutrient solution could be recirculated to achieve a higher biomass concentration and that this process could potentially reduce complexities associated with system scaling and mechanical harvesting. This type of harvesting would allow for the system to be harvested without the use of manual labor and could potentially reduce operating and capital costs.

The importance of improving the harvesting process at scale can be demonstrated by showing the time and cost associated with harvesting the substrates. If a one-hectare algae production system was constructed (80,000 substrates, 80% space utilization) and each substrate was harvested twice per week, it would require approximately 27,000 hours of labor per week as each substrate takes two technicians approximately 5 minutes to harvest. This would result in an operating cost of $270,000 per week for harvesting ($10 hr^{-1} wages) whereas a one-hectare facility with a very high productivity (25 g dry biomass m^{-2} d^{-1}) would need to sell the dry algae product (1,400 kg $week^{-1}$) at a cost of $193 kg^{-1} just to cover the cost of labor to harvest the substrates. Conversely, the

approach of harvesting the substrates by continually recirculating the substrate effluent would require the use of 37 one-horse-power pumps (Delat, SKU #D1162180, Torrance, CA, USA) to pump approximately 108 million liters of nutrient solution $week^{-1}$ (1,350 L m^{-2}). Combined, these pumps would require 6,870 kWh $week^{-1}$ that would cost $825 $week^{-1}$ at an electricity price of \$0.12 kWh^{-1}.

This operating cost would only require a price of \$0.59 kg^{-1} dry algae to cover the cost of harvesting. The cost of mechanically harvesting the substrates at scale is prohibitive, but it is still unclear from the results (**Figure 12**) whether the alternative method described here would be economically feasible. The main reason is that lipid extraction techniques require that the moisture content of the algae is reduced to 80-90% whereas the alternative approach described here had a moisture content of 99.8% (1.4 g dry biomass L^{-1}). This high moisture content could potentially be reduced by decreasing the total volume of nutrient solution being recirculated or using novel amendments that more easily remove algae from the substrates. However, without improvements, neither one of these harvesting techniques will be a viable method as both would require dewatering following harvesting which negates one of the main proposed benefits of ACSs.

Salinity and Water Loss Measurement

One of the concerns in developing this simple ACS was that salinity gradients would form on the substrates due to evaporation and that marine algal species would not be able to effectively grow. While this ACS would be operated in a controlled environment, typical greenhouses and other controlled environments (e.g., growth chambers) are not fully closed systems since they exchange air with their surrounding

environment. The exchange rate for typical greenhouses is at a minimum around one unit of their internal volume with the surrounding air every hour for winter conditions, and as high as one air exchange per minute for summer conditions (ANSI/ASAE, 2003). Evaporative water loss from the system can potentially create problems for cultivating marine algae since algae have specific salinity ranges in which they grow and non-uniform salinity on the substrates could lead to sub-optimal production. This is a major concern for the viability of this system because marine species of algae tend to produce higher value products compared to freshwater species and because cultivating marine species reduces the environmental impact of needing to use freshwater for cultivation (Pulz and Gross, 2004; Wang, 2013). Therefore, during our evaluation of this system it was deemed particularly important to determine to what extent salinity gradients formed on the substrates and how they would impact growth. It was seen through this experimentation that salinity gradients can form on the substrates (**Figure 13**) in a relatively short period of time. While many algae species can withstand salinity gradients, a 10 ppt salinity gradient is considered important and will greatly reduce the productivity of many marine algae species (Bartley et al., 2013; Adenan et al., 2013).

One of the most commonly cited benefits of ACSs outside of their high productivities and dense effluent concentrations is their minimal use of water (Cheng et al., 2013). While it is true that these systems do not utilize suspensions of algae for biomass production, it is possible that they require larger quantities of water for operation than traditional systems. The reason for this is that by placing many double-sided substrates suspended in air within a small floor space, the effective surface area of the system per unit of floor area is increased an order of magnitude or more compared to the

ORP or PBR systems. Even though these ACSs would be placed in controlled environments, these environments, as previously discussed are not closed systems. Therefore, before this proposed benefit can be attributed to the system described here, the actual amount of water lost from the system had to be quantified. A linear scaling relationship was used to determine the amount of water that the system might lose at large-scale and it was determined that it would lose approximately 4 L m^{-2} d^{-1} to the environment. While scaling the small-scale data to a large-scale system relies upon several assumptions, this result is surprising as ORP systems are reported to lose 1.6 to 11 L m^{-2} d^{-1} to the environment and are completely open systems (Lundquist et al., 2010; Borowitzka and Moheimani, 2013). The exact relationship between the small-scale system described here and the large-scale system will differ based on several factors, but this extrapolation illustrates an important point which is that ACSs are not housed in entirely closed systems and this water loss needs to be thoroughly investigated for any large-scale configuration. The water loss in ACSs is further increased as more substrates are used and when high light intensities necessitate high air exchange rates necessary for cooling. Ultimately, these salinity and evaporation issues may make this ACS less appealing compared to ORP and PBR systems as this was one of the originally proposed potential benefits of the system.

Cultivating Various Species

While a wide range of algae grow on ACSs (e.g., *Botryococcus braunii*, *Cyanobacteria isolates*, *Nitzschia palea*, *Scenedesmus obliques*, *Nanochlorposis occulata*), we sought to identify which species would grow on this specific type of ACS (Cheng et al., 2013; Wijihastuti et al., 2016). The freshwater algae that were selected for

our studies included: *Botryococcus braunii* and *Parachlorella kessleri*, and the marine species were: *Tetraselmis chuii, Thalassiosira sp., Chaetoceros calcitrans,* and *Oscillatoria sp*. These species were chosen for their nutritional profile and growth rates and because they are commonly cultivated at the New Jersey Aquaculture Innovation Center and are well documented in the literature (Brennan et al., 2010; Napolitano et al., 1990; Meseck et al., 2005; Fernández-Reiriz et al., 1989). More marine species were chosen than freshwater species because they tend to have more valuable nutritional profiles and some of the species described here can also be utilized as high value feed in aquaculture operations (Volkman et al., 1989).

When the ACS described here was originally developed, it was designed not for its use as a cultivation platform for high value products or biofuel, but to treat wastewater (Kaya and Picard, 1995). Because of this original purpose, species of algae were utilized that could survive under varying conditions and were able to grow quickly and remove undesired chemical constituents from wastewater. The most successful species of algae to grow on the system was *P. kessleri* because it grows quickly under varying conditions and forms dense biofilms that adhere well to the substrates (Johnson et al., 2015). *P. kessleri* was found to thrive at a low pH, which minimizes competition from other algae and grazers (e.g., ciliates, rotifers) (Juárez et al., 2011). While these important characteristics allow *P. kessleri* to thrive on the substrates, it has little commercial value because under normal environmental conditions it produces primarily low value starches and only small quantities of lipids (Li et al., 2013). Because of the low value of *P. kessleri*, we examined which other species of algae can grow on the system and selected species that under normal conditions produce high value products such as phenylalanine,

botryococcenes, xanthophylls, docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), linoleic acid and arachidonic acid (AA) (Ducat et al., 2011; Pulz and Gross, 2004). Based on preliminary experimentation and previous work conducted on ACSs (Johnson et al., 2015; Cheng et al., 2013; Gross et al., 2013; Blanken et al., 2014), we hypothesized that several algae species would be able to grow on the substrates, and that their growth kinetics could potentially vary widely from traditional systems.

With only one replicate for these trials, the only conclusion that can be drawn is that four species in addition to *T. chuii* and *P. kessleri*, first described in earlier work (Johnson et al., 2015), can grow on the substrates. These species should be evaluated during future work to better understand their productivities on this ACS. Our work on cultivating species of algae and a cyanobacteria indicates that multiple species can be grown on the ACS and that an array of high value products can be produced (Pulz and Gross, 2004; Brennan and Owende, 2010; Banerjee et al., 2002). While some species did not perform well (e.g., *Arthrospira sp.*) and others did not grow at all (e.g., *Nannochloropsis sp.* (UTEX 2379), *Amphora montana* (UTEX SP2) and *Isochrysis galbana* (UTEX LB987) during preliminary experiments, our results are only based on a specific set of growth conditions and it is possible that other environmental conditions can increase growth rates or allow other species to successfully grow on the substrates. These results are important since they begin to elucidate some of the characteristics of the ACS and they highlight that species other than *P. kessleri* can be grown successfully on this system. While *P. kessleri* had the greatest productivity, with continued optimization we believe the productivities of the other species can be increased and these other species of algae can produce higher value products than *P. kessleri*. However, we speculate that

the overall growth rates of marine algae species will be hindered based on the salinity gradient experiment described.

Conclusions

The ACS described here is a novel type of ACS that was evaluated to determine its specific properties. Results from our experiments show that algae species such as *P. kessleri* can be cultivated on the system and with further optimization several additional algal species can potentially grow and produce valuable commercial co-products. From our observations, a few conclusions can be drawn about the nature of this ACS and why some algae species grow successfully on the system and why others do not. First, for an algae species to be successful on the system it needs to have a relatively rapid growth rate. Even when algae are growing successfully on the substrates and have formed a dense biofilm, a certain percentage of the algae wash off the substrates during irrigation. When the substrates are initially inoculated, the quantity of algae that falls off the substrates is even greater and if the algae do not have a fast enough growth rate, they can be washed off the substrates before they can establish a dense biofilm. Another important conclusion that can be drawn from this work is that freshwater species of algae generally grow well on the substrates and appeared more suited to this system than the marine species we evaluated. The work described here identifies challenges with cultivating marine algae species due to the formation of salinity gradients. In evaluating the system for its potential to produce algae biomass for biofuel production, it was shown that the system has a major drawback in its ability to respond to nitrogen stress to stimulate lipid production. We showed that there is a carrying capacity for algae biomass on the substrates and that when this capacity is reached, the overall production stagnates and then can decline.

Therefore, harvesting frequency should be correlated to growth rates and should be altered proportionately to changes in growth rate. While the experiments with different algal species, nitrogen stress, and CO_2 enrichment elucidated several key aspects of this ACS, the most important outcome from the work resulted from the experiments on an alternative harvesting strategy as we feel this to be the greatest operational barrier to scaling the system. These tests demonstrated that at scale the system could potentially be harvested using an irrigation based approach if removal rates could be improved. This approach could potentially remove the need for tedious and expensive manual harvesting which is not economically viable at large-scale. Additionally, we demonstrated that the system does lose a considerable amount of water to the environment and future analyses of this system needs to pay close attention to the interplay between system exchange rates, humidity, and effective surface area. Results presented will assist in shaping the direction of future research by focusing efforts on cultivating freshwater species at larger scale and developing nutrient solution amendments that can chemically remove algae biomass from the substrates for improved irrigation-based harvesting. Improvements in harvesting and water usage are needed for this ACS to be economically viable at large-scale.

Figures and Tables

Table 3. List of the different algae species grown on the ACS and the type and composition of the applied nutrient solutions. F/2 and Modified Bold 3N are nutrient solutions commonly used to cultivate algae in ORP and PBR systems. Salinity is expressed in parts per thousand (ppt = g kg^{-1}).

Saltwater Species			
Species	**Salinity**	**Nutrient Solution**	**pH**
Tetraselmis chuii	35 ppt	F/2 (Guillard, 1975)	8.2
Thalassiosira sp.	15 ppt	F/2 (Guillard, 1975)	8.2
Chaetoceros calcitrans	15 ppt	F/2 + 350 µL Silica (Guillard, 1975)	8.2
Oscillatoria sp.	25 ppt	F/2 (Guillard, 1975)	8.2
Freshwater Species			
Botryococcus braunii	-	Modified Bold 3N (Tang et al., 2011)	7.4
Parachlorella kessleri	-	*P. kessleri* nutrient solution (Johnson et al., 2015)	5.5

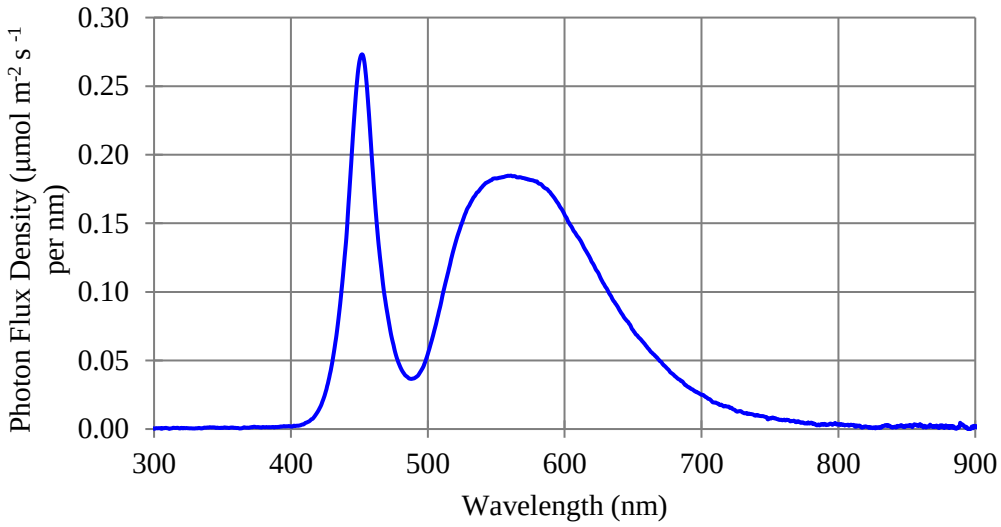

Figure 8. Photon flux produced by the LED lighting array measured at its center and 20 cm below the array across the 300-900 nm waveband in 0.5 nm increments. The spectrum shows two characteristic peaks: one narrower peak in the blue waveband peaking at 452 nm (0.273 µmol m^{-2} s^{-1}) and another wider peak in the green waveband peaking at 560 nm (0.185 µmol m^{-2} s^{-1}). The overall PAR (400-700 nm) at 20 cm below the LED array was 32 µmol m^{-2} s^{-1}. The blue peak and green peak had full widths at half maximum of 23.5 nm and 130 nm, respectively.

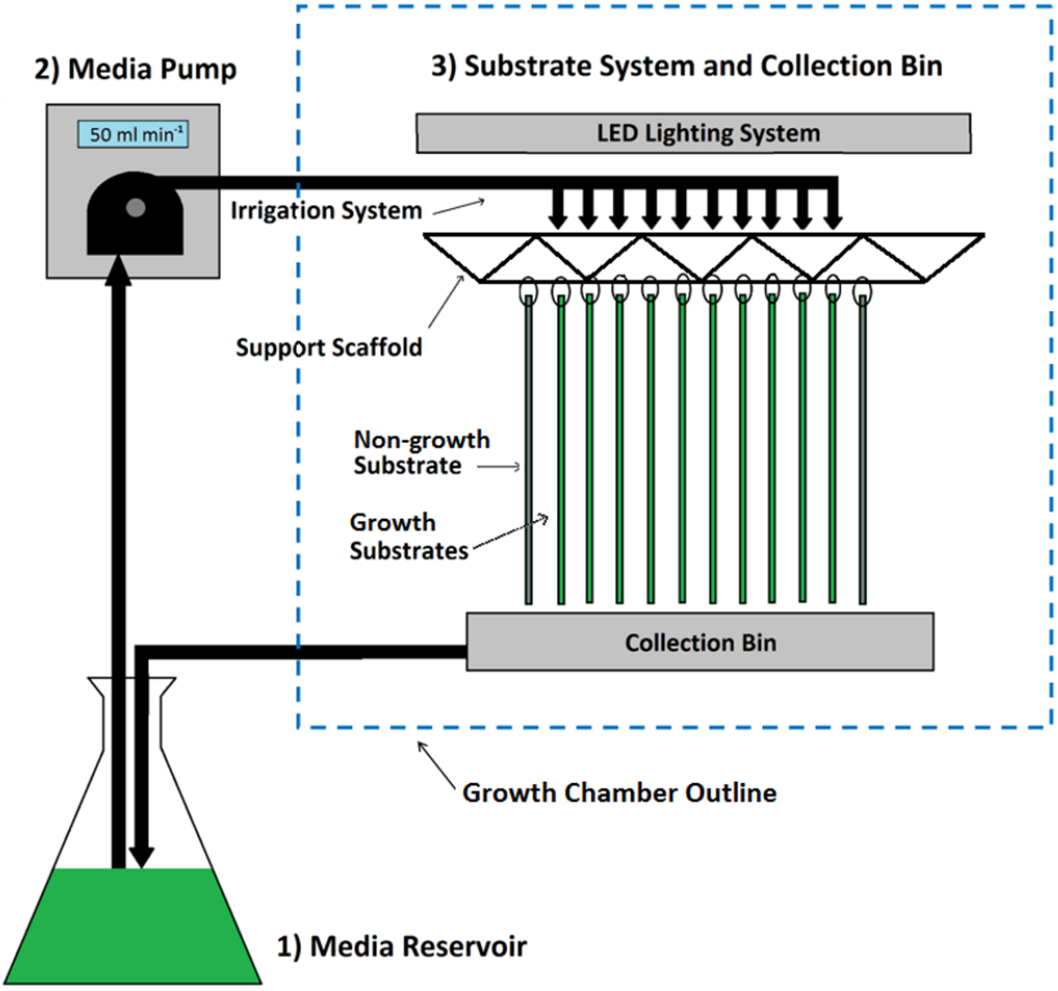

Figure 9. For the nutrient solution recirculation experiments, the effluent from the substrates was collected in a polycarbonate collection bin that gravity drained into an Erlenmeyer flask that acted as the nutrient solution reservoir (1). The effluent from this flask was then pumped using a peristaltic pump (2) to the irrigation system (3) that distributed the effluent over the substrates. The pump was operated continuously with the goal of increasing the algae concentration in the effluent. The idea of this approach was that the algae could be more easily harvested from the effluent compared to mechanically harvesting the substrates. In this diagram, the blue dashed line depicts the growth chamber that the system was contained within and in this experiment there were ten growth substrates and two non-growth substrates, one on either end of the row of substrates.

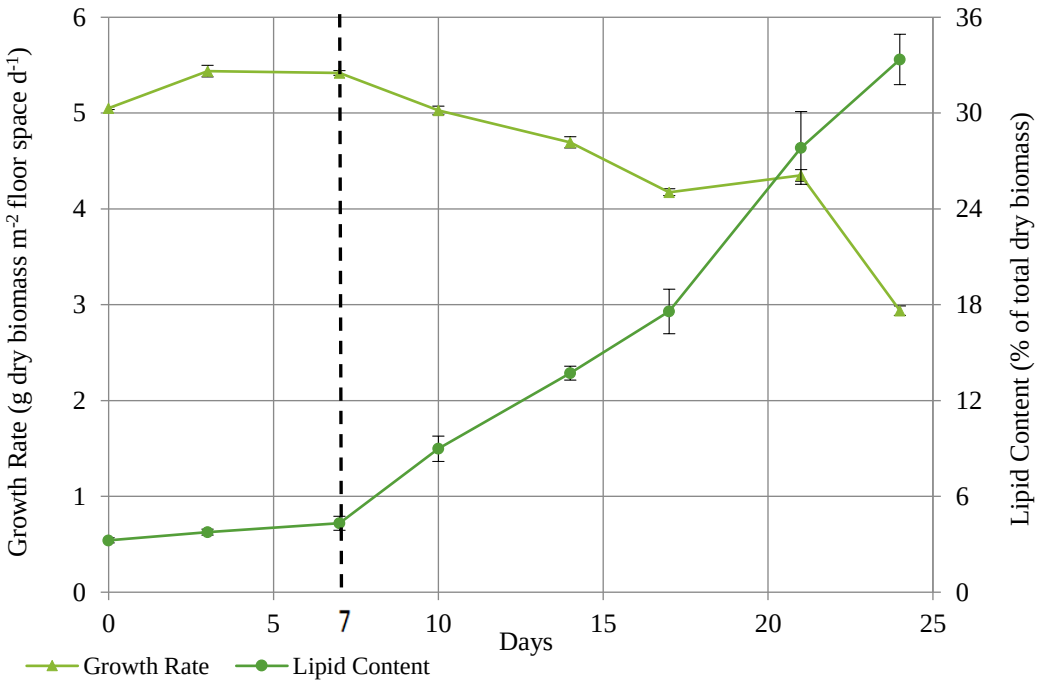

Figure 10. Growth rate (g dry biomass m^{-2} floor space d^{-1}) and lipid content of *Parachlorella kessleri* grown over a 24-day period during which nitrogen was removed from the nutrient solution starting at day 7. The error bars show the standard deviation of the measurements conducted on three samples taken at each harvesting time.

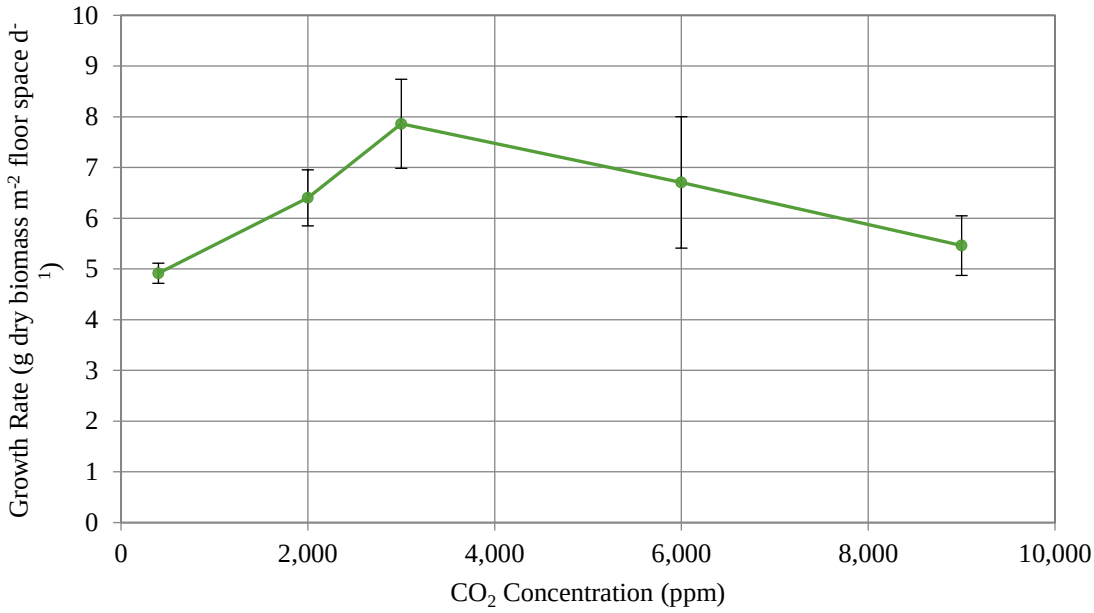

Figure 11. Growth rate (g dry biomass m^{-2} floor space d^{-1}) of *Parachlorella kessleri* when exposed to increasing CO_2 concentrations from 402 to 9,000 ppm. The error bars represent the standard deviations of the measured growth rates (n=7). The growth rates at 400, 2,000 and 3,000 ppm were statistically different from one another. Additionally, the growth rate at 9,000 ppm was statistically different (p value $\leq$ 0.05) than the growth rate at 400 or 3,000 ppm)

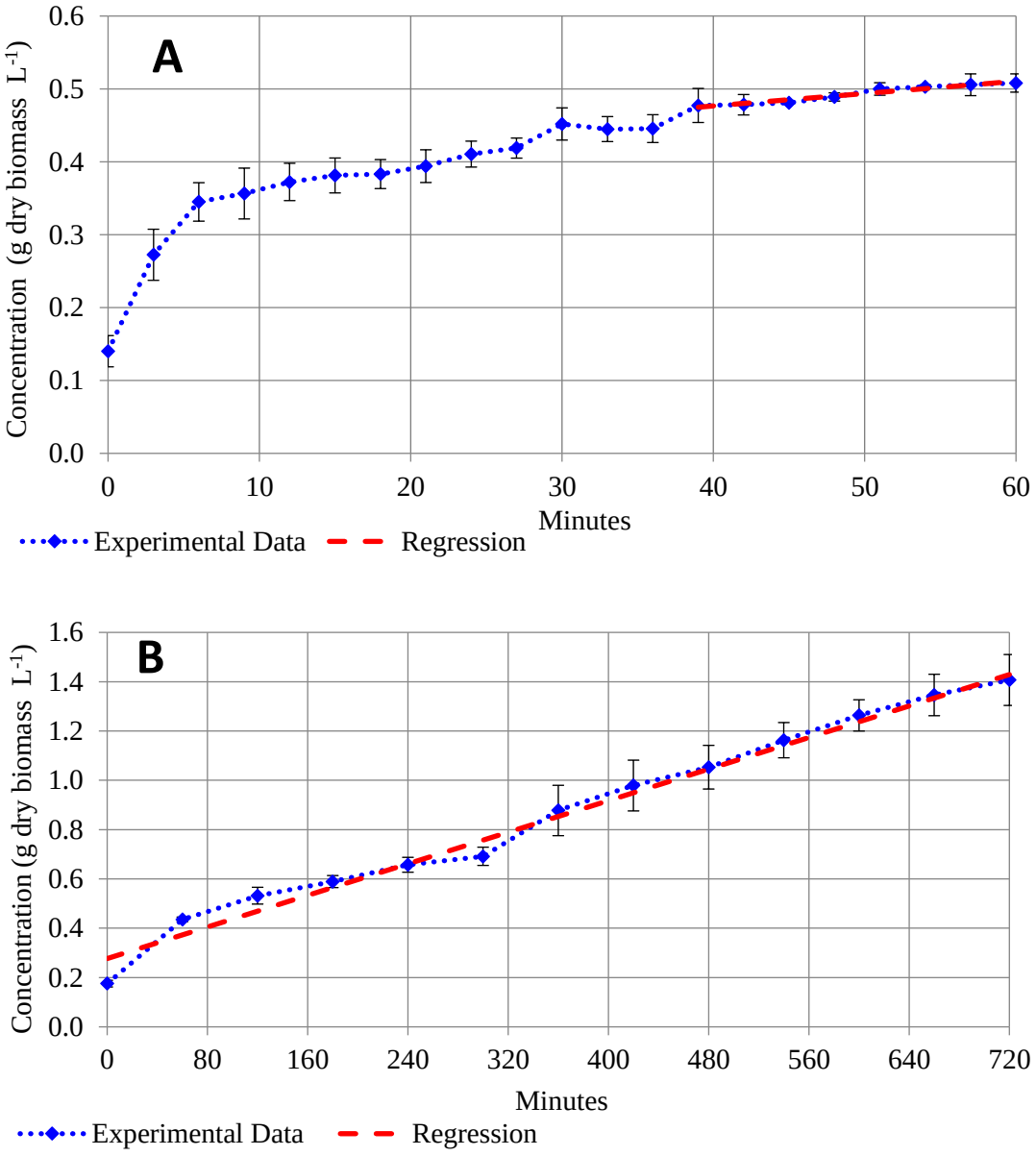

Figure 12. Effluent concentration of *P. kessleri* (g dry biomass L^{-1}) during the 60-minute (**A**) and 12-hour (**B**) recirculation experiments. Data points were derived from three replicate experiments and the error bars represent the standard deviations. The slope for the final 21 minutes (0.0017 g dry biomass L^{-1} min^{-1}) of the 60-minute experiments is similar to the slope of the 12-hour experiments (0.0016 g dry biomass L^{-1} min^{-1}) and points to a linear increase in biomass accumulation (R^2 = 0.948 and 0.989, respectively) after the initial 39-minute period.

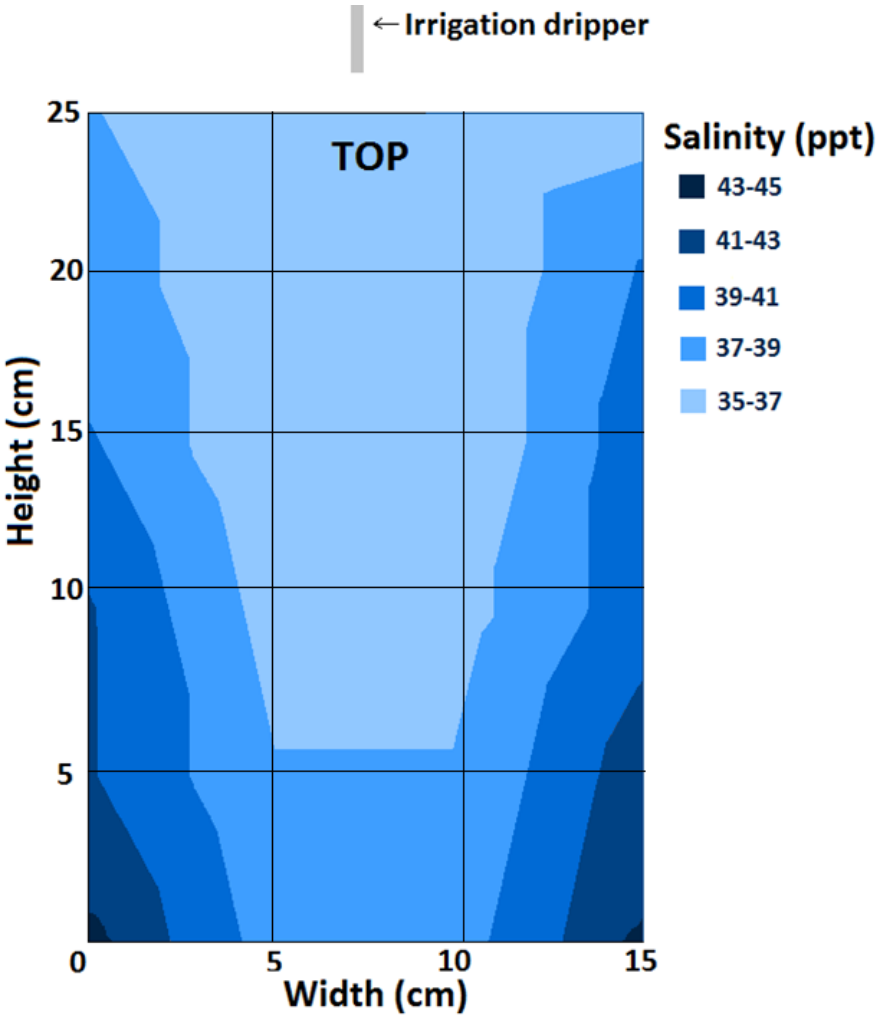

Figure 13. Graphical representation of the salinity gradient across substrates that were top-irrigated with 35 ppt (g kg^{-1}) seawater from a single dripper at the top center of the substrates. This representation is from the 24th day of substrate irrigation (standard deviation = 1.6 -3.5 ppt; n=3). Excess seatwater was allowed to drip from the bottom of the substrate and was collected in the collection bin (as described in **Figure 9**).

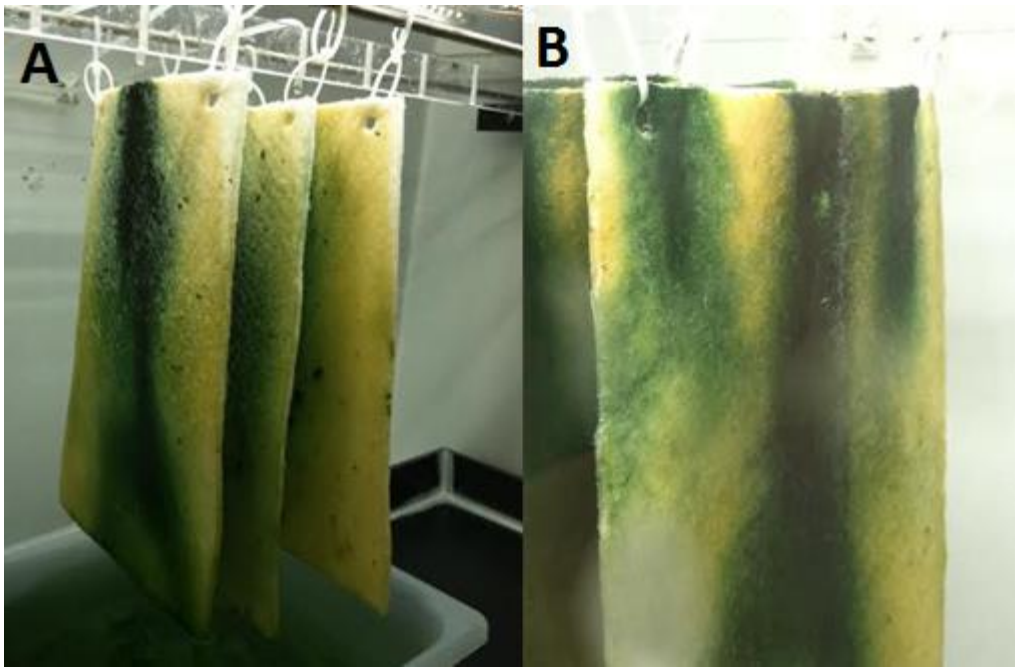

Figure 14. Algae (*Arthrospira sp.*) grown on vertical substrates for two weeks following inoculation and top-irrigated with 25 ppt salinity F/2 nutrient solution, with (A) a single irrigation dripper located just above the top center of the substrate, and (B) with three evenly spaced irrigation drippers located just above the top of the substrate. The greener (darker) the color of the substrate, the more algae biomass. The uneven plumes of algae biomass are thought to result from an uneven flow of nutrient solution throughout the substrates and consequently uneven salinity gradients. Only a few substrates are shown for clarity.

References

- Adenan, N. S., Yusoff, F. M., & Shariff, M. (2013). Effect of salinity and temperature on the growth of diatoms and green algae. *Journal of Fisheries and Aquatic Science*, *8*(2), 397.
- ANSI/ASAE (2003) Engineering Practice 406.4: Heating, Ventilation and Cooling Greenhouses: American Society of Agricultural Engineers. Michigan, USA.
- Bartley, M. L., Boeing, W. J., Corcoran, A. A., Holguin, F. O., & Schaub, T. (2013). Effects of salinity on growth and lipid accumulation of biofuel microalga *Nannochloropsis salina* and invading organisms. *Biomass and Bioenergy*, *54*, 83-88.
- Blanken, W., Janssen, M., Cuaresma, M., Libor, Z., Bhaiji, T., & Wijffels, R. H. (2014). Biofilm growth of *Chlorella sorokiniana* in a rotating biological contactor based photobioreactor. *Biotechnology and Bioengineering*, *111*(12), 2436-2445.
- Brennan, L., & Owende, P. (2010). Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews*, *14*(2), 557-577.
- Brune, D. E., Lundquist, T. J., & Benemann, J. R. (2009). Microalgal biomass for greenhouse gas reductions: potential for replacement of fossil fuels and animal feeds. *Journal of Environmental Engineering*, *135*(11), 1136-1144.
- Borowitzka, M. A., & Moheimani, N. R. (2013). Sustainable biofuels from algae. *Mitigation and Adaptation Strategies for Global Change*, *18*(1), 13-25.

- Cheng, P., Ji, B., Gao, L., Zhang, W., Wang, J., & Liu, T. (2013). The growth, lipid and hydrocarbon production of *Botryococcus braunii* with attached cultivation. *Bioresource Technology*, *138*, 95-100.
- Choi, G. G., Kim, B. H., Ahn, C. Y., & Oh, H. M. (2011). Effect of nitrogen limitation on oleic acid biosynthesis in *Botryococcus braunii*. *Journal of Applied Phycology*, *23*(6), 1031-1037.
- Ducat, D. C., Way, J. C., & Silver, P. A. (2011). Engineering cyanobacteria to generate high-value products. *Trends in biotechnology*, *29*(2), 95-103.
- Fernández-Reiriz, M. J., Perez-Camacho, A., Ferreiro, M. J., Blanco, J., Planas, M., Campos, M. J., & Labarta, U. (1989). Biomass production and variation in the biochemical profile (total protein, carbohydrates, RNA, lipids and fatty acids) of seven species of marine microalgae. *Aquaculture*, *83*(1-2), 17-37.
- Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry*, *226*(1), 497-509.
- Griffiths, M. J., Garcin, C., van Hille, R. P., & Harrison, S. T. (2011). Interference by pigment in the estimation of microalgal biomass concentration by optical density. *Journal of Microbiological Methods*, *85*(2), 119-123.
- Gross, M., Henry, W., Michael, C., & Wen, Z. (2013). Development of a rotating algal biofilm growth system for attached microalgae growth with in situ biomass harvest. *Bioresource Technology*, *150*, 195-201.
- Gross, M. (2013). Development and optimization of algal cultivation systems. Dissertation, Iowa State University.

- Gross, M., Jarboe, D., & Wen, Z. (2015). Biofilm-based algal cultivation systems. *Applied Microbiology and Biotechnology*, *99*(14), 5781-5789.
- Guillard, Robert RL. "Culture of phytoplankton for feeding marine invertebrates." *Culture of Marine Invertebrate Animals*. Springer US, 1975. 29-60.
- Hoh, D., Watson, S., & Kan, E. (2016). Algal biofilm reactors for integrated wastewater treatment and biofuel production: A review. *Chemical Engineering Journal*, *287*, 466-473.
- Ji, B., Zhang, W., Zhang, N., Wang, J., Lutzu, G. A., & Liu, T. (2014). Biofilm cultivation of the oleaginous microalgae *Pseudochlorococcum sp*. *Bioprocess and Biosystems Engineering*, *37*(7), 1369-1375.
- Johnson, M., Villani, T. S., Azmat, A., Simon, J. E., & Both, A. J. (2015). Evaluation of algal biomass production on vertical aeroponic substrates. *Algal Research*, *10*, 240-248.
- Juárez, Á. B., Vélez, C. G., Iñiguez, A. R., Martínez, D. E., Rodríguez, M. C., Vigna, M. S., & de Molina, M. D. C. R. (2011). *A Parachlorella kessleri* (*Trebouxiophyceae, Chlorophyta*) strain from an extremely acidic geothermal pond in Argentina. *Phycologia*, *50*(4), 413-421.
- Kaya, V. M., & Picard, G. (1995). The viability of Scenedesmus bicellularis cells immobilized on alginate screens following nutrient starvation in air at 100% relative humidity. *Biotechnology and Bioengineering*, *46*(5), 459-464.

- Li, X., Přibyl, P., Bišová, K., Kawano, S., Cepák, V., Zachleder, V., & Vítová, M. (2013). The microalga Parachlorella kessleri—A novel highly efficient lipid producer. *Biotechnology and Bioengineering*, *110*(1), 97-107.
- Lundquist, T. J., Woertz, I. C., Quinn, N. W. T., & Benemann, J. R. (2010). A realistic technology and engineering assessment of algae biofuel production. *Energy Biosciences Institute*, 1-178.
- Mata, T. M., Martins, A. A., & Caetano, N. S. (2010). Microalgae for biodiesel production and other applications: a review. *Renewable and Sustainable Energy reviews*, *14*(1), 217-232.
- Meseck, S. L., Alix, J. H., & Wikfors, G. H. (2005). Photoperiod and light intensity effects on growth and utilization of nutrients by the aquaculture feed microalga, *Tetraselmis chuii* (PLY429). *Aquaculture*, *246*(1), 393-404.
- D'Oca, M. G. M., Vieˆgas, C. V., Lemoes, J. S., Miyasaki, E. K., Morón-Villarreyes, J. A., Primel, E. G., & Abreu, P. C. (2011). Production of FAMEs from several microalgal lipidic extracts and direct transesterification of the *Chlorella pyrenoidosa*. *Biomass and Bioenergy*, *35*(4), 1533-1538.
- Napolitano, G. E., Ackman, R. G., & Ratnayake, W. (1990). Fatty acid composition of three cultured algal species (*Isochvysis galbana*, *Chaetoceros gracilis* and *Chaetoceros calcitrans*) used as food for bivalve larvae. *Journal of the World Aquaculture Society*, *21*(2), 122-130.
- Occupational Safety and Health Administration (2017) Gaseous Exposure Limits. https://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=STANDARDS&p_id=9992ago. Accessed 25 March 2017

- Pulz, O., & Gross, W. (2004). Valuable products from biotechnology of microalgae. *Applied Microbiology and Biotechnology*, *65*(6), 635-648.
- Rodolfi, L., Chini Zittelli, G., Bassi, N., Padovani, G., Biondi, N., Bonini, G., & Tredici, M. R. (2009). Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnology and Bioengineering*, *102*(1), 100-112.
- Schnurr, P. J., Espie, G. S., & Allen, D. G. (2013). Algae biofilm growth and the potential to stimulate lipid accumulation through nutrient starvation. *Bioresource Technology*, *136*, 337-344.
- Scott, S. A., Davey, M. P., Dennis, J. S., Horst, I., Howe, C. J., Lea-Smith, D. J., & Smith, A. G. (2010). Biodiesel from algae: challenges and prospects. *Current Opinion in Biotechnology*, *21*(3), 277-286.
- Sheehan, J., Dunahay, T., Benemann, J., & Roessler, P. (1998). A look back at the US Department of Energy's aquatic species program: biodiesel from algae. *National Renewable Energy Laboratory*, *328*.
- Singh, A., Nigam, P. S., & Murphy, J. D. (2011). Mechanism and challenges in commercialisation of algal biofuels. *Bioresource Technology*, *102*(1), 26-34.
- Su, Y., Mennerich, A., & Urban, B. (2016). The long-term effects of wall attached microalgal biofilm on algae-based wastewater treatment. *Bioresource Technology*, *218*, 1249-1252.
- Volkman, J. K., Jeffrey, S. W., Nichols, P. D., Rogers, G. I., & Garland, C. D. (1989). Fatty acid and lipid composition of 10 species of microalgae used in

mariculture. *Journal of Experimental Marine Biology and Ecology*, *128*(3), 219-240.

- Liu, T., Wang, J., Hu, Q., Cheng, P., Ji, B., Liu, J., & Gao, L. (2013). Attached cultivation technology of microalgae for efficient biomass feedstock production. *Bioresource Technology*, *127*, 216-222.
- Wijihastuti, R. S., Moheimani, N. R., Bahri, P. A., Cosgrove, J. J., & Watanabe, M. M. (2016). Growth and photosynthetic activity of *Botryococcus braunii* biofilms. *Journal of Applied Phycology*, 1-12.
- Zhuang, L. L., Azimi, Y., Yu, D., Wang, W. L., Wu, Y. H., Dao, G. H., & Hu, H. Y. (2016). Enhanced attached growth of microalgae *Scenedesmus*. LX1 through ambient bacterial pre-coating of cotton fiber carriers. *Bioresource Technology*, *218*, 643-649.

Chapter 3 - Design and Simulation of an Algae Vertical Attached Growth System for the Production of High-Value Co-Products

Michael Johnson[1], Thomas S. Villani[2,3], A.J. Both[1]

[1] Department of Environmental Sciences, Rutgers University, New Brunswick, NJ 08901

[2] New Use Agriculture and Natural Plant Products Program, Department of Plant Biology, Rutgers University, New Brunswick, NJ 08901

[3] Department of Medicinal Chemistry, Ernest Mario School of Pharmacy, Rutgers University, Piscataway, NJ 08854

Abstract

Commercial algae production is typically used to produce biomass (especially lipids for biofuels), and/or to produce valuable co-products (e.g., astaxanthin, omega-3 fatty acids, and β-carotene). To produce algae there are three major classes of production systems that can be used: open raceway ponds (ORP), photobioreactors (PBR), or attached cultivation systems (ACS). The construction and operating costs for these systems can be substantial, but production can be year-round if the systems are placed inside controlled environments (e.g., greenhouses). Based on previous laboratory scale experiments, we designed and simulated the production capabilities of a commercially scaled greenhouse-based ACS system and modeled the economics of operating such a system at various locations throughout the United States. We determined this ACS can produce a low-concentration (<50 grams dry biomass L^{-1}) algae slurry at a cost of $14.74 to $32.24 kg^{-1} of dried algae depending on its location and these cost variations were primarily affected by heating costs, water costs and algae productivity. While these production costs and subsequent dewatering and extraction make biodiesel production

impractical, the system can produce *Chlorella sp*., omega-3 fatty acids, β-carotene, and fishery feed in a financially viable manner based on their current market prices with production margins ranging from 22 to 99%. Therefore, current market prices make the ACS modeled here economically attractive for the production of several high value co-products.

Introduction

Over the last century, an increased demand for resources has generated substantial interest in new feedstocks for energy and food production that are renewable and have a minimal environmental impact. Of the possible options, algae production has been viewed favorably as it does not require arable land or potable water for cultivation and can be used to produce a wide-range of bio-based products including biodiesel, nutraceuticals, and animal feed (Brennan and Owende, 2010; Harun et al., 2010). However, to date, large-scale commercial cultivation of algae for biodiesel production has yet to become profitable due to the low direct cost of fossil-fuel derived energy. Therefore, commercial algae cultivation has mostly focused on the production of high-value bio-based products (Borowitzka, 2013; Pulz and Gross, 2004). This strategy allows for the economically viable production of algae biomass while continuing optimization with the long-term goal of increasing productivity and efficiency to a point where algae-derived fuel becomes competitive with fossil fuel.

Algae biomass is produced primarily using three different types of systems: open raceway ponds (ORP), photobioreactors (PBR), and attached cultivation systems (ACS). Most research has been focused on ORP and PBR systems. ACSs represent a different type of algae cultivation system that relies on the adherence of algae to substrates instead

of growth in a suspension (Gross et al., 2013; Christenson and Sims, 2012). ACSs have advantages compared to traditional suspended growth systems (ORP, PBR) as algae can be removed from the system in a dense biofilm, decreasing the need for energy intensive dewatering (Gross et al., 2013). Additionally, in ACSs algae are exposed directly to air which removes the need for increasing CO_2 concentration in the nutrient solution and enables acidophilic algae to be cultivated because an alkaline nutrient solution is not required. An alkaline nutrient solution is typically required to increase the solubility of CO_2 into the nutrient solution. However, ACSs are limited in their ability to produce large quantities of lipids because nutrient stress in ACSs does not lead to substantial increases in lipid concentration as it does in ORP and PBR systems (Schnurr et al., 2013; Cheng et al., 2013). Algae will typically have a relatively low lipid content and the common practice to increase lipid content prior to harvest is to induce nutrient stress which results in increased lipid composition. In Chapter 2, it was shown that this effect is less substantial than what is seen in suspended growth systems.

The purpose of this study was to evaluate a specific ACS developed by AL-G Technologies, Inc. (Charny, Quebec, Canada) to produce high-value co-products. We wanted to determine if this system could be used by rural farmers as a year-round revenue stream because it is a simple and potentially scalable system that can be easily implemented. This study focused on high-value bio-based products instead of the production of lipids for biodiesel because it has been demonstrated that under favorable conditions ACSs (e.g., rotating algal biofilm reactor) are only able to achieve a price of $11.90 per gallon of biodiesel (Barlow et al., 2016). This work leveraged the data acquired through lab scale experiments on the AL-G Technologies ACS (Johnson et al.,

2015) to model the performance of this system at a larger scale as well as through algae growth data for various temperatures (Ňancucheo and Johnson, 2012) and light intensities (Chang and Yang, 2003). This data was combined with greenhouse modeling using the USDA Virtual Grower program (USDA, 2016), greenhouse construction costs (Robbins, 1999), nutraceutical pricing data (Vigani et al., 2015), and algae composition data (D'Alessandro and Antoniosi, 2016) to determine the products that could be produced with this system. The discussion of this system for larger scale production focuses on areas of optimization, generated products, and a financial analysis.

Materials and Methods

This section defines the scope of the economic analysis, the specific research questions that were addressed, and the methodology that was used to model the system. A simulation model was developed to evaluate the cost of producing an algae slurry (<50 grams dry biomass L^{-1}), but subsequent dewatering and extraction were ignored as the efficiency and cost of these processes are determined by the individual products that are derived from algae (e.g., dry algae, fatty acid methyl esters). It was assumed that the algae slurry was sold on a per kilogram liquid basis, realizing that each kilogram of dry biomass would be contained in suspension. The analysis used a one-hectare ACS with an estimated (maximum) algal productivity of 25 g dry biomass m^{-2} d^{-1} and focused on the financial impact of geographic location, heating, and algal growth rates. It was assumed that the system had an operational life of ten years and that after this period the system would require substantial repairs. This analysis allowed for the determination of which high value products can be produced on the system and their specific financial viability.

Locations

Five geographic locations were chosen to model the ACS: Tucson, AZ, Houston, TX, Newark, NJ, Spokane, WA, and Anchorage, AK. These locations were chosen to illustrate the viability of the system in locations across the United States that have optimal and sub-optimal lighting and temperature parameters. Tucson, AZ represents an optimal location with high light intensities and warm temperatures, while Anchorage, AK represents a very poor environment for algal growth based on its low light intensities and low temperatures.

Capital Expenditures

The first component of building a financial model for this ACS was to determine the cost of the overall system because capital expenditures (CAPEX) drive the overall financial viability of the system. The basic premise of the ACS described here is that a series of scaffolds holding algae growth substrates are housed in a greenhouse such that ten 1 m^2 substrates are placed in a square meter of floor space for a total of 20 m^2 of substrate surface area per square meter of floor space. Our economic model focused on the simplest of greenhouse designs; a gutter connected qounset greenhouse covered by two layers of air inflated polyethylene film (0.152 mm thick). This qounset greenhouse was comprised of ten, 10 x 100 m bays that were attached to one another to form a 100 x 100 m footprint (Supplementary Materials **Figure 1**). The floor of the greenhouse was comprised of dirt covered with landscape fabric and the substrates were hung from a pressure treated wood scaffolding systems and irrigated and harvested with the same irrigation system. This approach is still being optimized with respect to irrigation based harvesting because it is considerably less energy intensive than mechanically harvesting the individual substrates (Johnson et al., 2015). We assumed that at large-scale the system

would be harvested using the irrigation system instead of mechanical harvesting due to the projected cost benefits. The cost of constructing this greenhouse was based on the work by Robbins (1999) from which 2017 prices were extrapolated using a 2.1% annual inflation rate. Each one of the ten sections of the greenhouse had two exterior doors in the end walls and two connecting doors (or one additional exterior door if the section included an exterior wall) (Supplementary Materials **Figure 2**), and four, 29,000 cfm fans (Model: American Coolair #NBFA54M, Greenhouse Megastore, Danville, IL) for ventilation and cooling. The fans were mounted on one end of the greenhouse sections on opposite sides of the door to the exterior. On the opposite side of the greenhouse, there were four, 1.22 m x 1.22 m exhaust ducts. For all locations chosen for this study the ASAE (2003) recommended ventilation rates of 0.006 $m^3 s^{-1} m^{-2}$ for the winter and 0.06 $m^3 s^{-1} m^{-2}$ for the summer were used. Additionally, 200,000 BTU gas-fired heaters (Model: PTP 200 Unit Heater #HT-PTP200, Modine, Buena Vista, VA, 80% efficient) were used in each greenhouse section. The number of heaters in each greenhouse depended on the amount of heating required based on the greenhouse's location. Construction labor was calculated to cost $48,000 because it would take 4 workers 6 weeks at \$50 hr^{-1} for forty hours per week. The electrical work was estimated to cost \$9,600, and this cost would be billed at \$80 hr^{-1} for an electrician who would work 40 hours per week for three weeks to install all of the electrical components.

To determine the cost of materials to construct the greenhouse, the model from Robbins (1999) was used and all of the materials not required for the ACS were removed. The total cost of constructing a 10,000 m^2 qounset greenhouse was estimated to be \$385,578 to \$436,342 (**Table 4**) depending on the number of heaters used. This cost was

inclusive of building rates and materials but did not consider the price of property as it was assumed that the system was constructed on reclaimed or underutilized land that was available at no cost.

Once the greenhouse costs were modeled, the substrate scaffold system could be placed within the greenhouse and the system's costs were modeled as a whole. The large-scale substrate scaffold system was constructed from 2.2 m^2 units that were constructed primarily out of pressure treated 2" x 4" wood (Douglas Fir) (Supplementary Materials **Figure 3**) and cost \$88.15 each (\$44.08 m^{-2}) in materials to construct (**Table 5**). For each 10 m wide section of the qounset greenhouse, there were seven rows of these units with four 0.575 meter walkways in between (Supplementary Materials **Figure 2**) and a 1.5 m walkway at the end of each section as well as a one meter wide walkway across the middle of the section, connecting it through doors to adjacent sections. This layout represented a 67.2% (336 substrate scaffolds per bay with 20 m^2 of growth area each) space utilization for the greenhouse floor area, resulting in a total of 67,200 substrates and allowed for all substrates to be serviced for maintenance from the walkways. The overall cost in materials to construct these units was \$296,184 (**Table 7**). Because each one of these units takes approximately 1 hour to build and set up and the average wage for such manual labor is \$50 hr^{-1}, it cost approximately \$175,000 to put these together.

In addition to the cost of the scaffolding, there was also the associated cost of installing all the irrigation tubing on each unit. For each unit, there was one irrigation tube that splits into 20 lines (Supplementary Materials **Figure 4**) that each had three drippers per substrate and the cost of materials for this irrigation system was \$81,312 (Supplementary Materials **Table 1**). It was assumed that it takes 30 minutes to setup each

irrigation system for each scaffold so that it cost approximately $87,500 to install these at a labor rate of $50 hr^{-1} (**Table 5**).

In addition to these costs are the CAPEX for the substrates, nutrient monitoring system, greenhouse monitoring systems, computer, and irrigation pumps. The system would use one, 20 horsepower electric pump (Berkeley B70221, Farmingdale, NJ, USA) and 67,200 substrates and a similar pump in stand-by back-up mode ($3,823 each). (Supplementary Materials **Table 2**). The pump is capable of pumping 1,000 L min^{-1} at a total head of 10 meters which is sufficient for the entire system that requires approx. 700 L min^{-1} of capacity. The automated nutrient monitoring system would measure the nutrient concentration (i.e., nitrogen, phosphorous) and pH of the nutrient solution that is collected in a sump to make real time adjustments to the nutrient concentration and pH. The sump would be a recessed hole in the ground where the nutrient solution collects and the pumping system recirculates the solution from. The sump would be 500 liters in volume and would be constructed from poured concrete at a total cost of $1,200 (including: labor, materials). An Anderson (Muskogee, OK, USA) nutrient control system (JP101-3) and pH control system (07922/A3-VCP) would be used to control these nutrient parameters at a cost of $5,846. The greenhouse monitoring and control system would measure and control the temperature of each individual greenhouse bay at a total cost of $20,000, and $3,000 would be spent on computers and software to run the nutrient monitoring system. There would be one temperature sensor mounted at substrate height in the center of each greenhouse bay (included in system price) and each greenhouse would have a target temperature of 30^{o}C. Additionally, $8,000 would be allocated for shipping all the materials to the building site for construction.

When these costs are combined, the total CAPEX for the one hectare system ranged between $1,100,834 and $1,191,372 to construct and install, depending on location. This total cost was paid for through a ten-year, 8 percent interest loan ($160,273 to $173,456 yr^{-1}) and it was assumed that the system requires a complete replacement after ten years.

Operating Expenses

It was assumed that the operational expenses (OPEX) for the system described here were minimal throughout its operation and that two full-time employees (e.g., Manager and a Technician) can keep the system operational for its ten-year expected term of use (Supplementary Materials **Table 2**). This is based on the assumption that minimal effort is required to operate the system and that these two full-time employees are primarily responsible for optimizing the system and ensuring that it is running at optimal efficiency. The two major OPEXs for the system were the salary for these two employees which were $140,000 combined excluding overhead expenses of 30%, and the ongoing repayment of the loan for the construction of this system, which had an 8% annual interest rate. Variable OPEXs were from heating of the system, which was important, but depended on the geographic location of the system and the associated outdoor temperatures. It was assumed that municipal water was used for the system at a cost of $0.001 L^{-1}, maintenance costs would be 10% of OPEX costs and that the electrical costs were $0.111, $0.113, $0.163, $0.082 and $0.172 kWh^{-1} for Tucson, AZ, Houston, TX, Newark, NJ, Spokane, WA and Anchorage, AK, respectively (EIA, 2017). Additionally, 10% of the OPEX was allocated for contingency costs in case there was

substantial damage to the system from a storm or if equipment needed to be replaced (**Table 6**).

Cooling

For the purposes of this model, it was assumed that the greenhouse system did not have an evaporative cooling system in place to reduce the temperature of the greenhouse. Instead, the ASAE (2003) recommended ventilation rates of 0.06 m^3 s^{-1} m^{-2} for the summer and 0.006 m^3 s^{-1} m^{-2} for the winter were used for cooling. However, increasing the ventilation rate to cool the system relies on energy and mass balances and the temperature of the greenhouse can only be reduced to the temperature of the outside air and doing so can result in substantial increases in the amount of water that is lost from the system as well as any supplemental CO_2 that is being used.

Algae Productivity

To model the growth of algae on this ACS, *Chlorella sp.* was used as a model species as it has been previously demonstrated that fresh water algae are best adapted to ACSs (Johnson et al., 2015). Algae productivity on the system as a function of temperature and daily light integral (measured as photosynthetically active radiation across the 400-700 nm waveband) was modeled using **Equations 1-3** in Supplemental Materials.

Water Loss

One of the main proposed advantages of the system described here is that it greatly reduces the amount of water that is lost to the environment in comparison to traditional open systems. While the system relies on placing 20 m^2 of substrates (front and back) per 1.1 square meter of floor space and significantly increases the effective

surface area for evaporation, the controlled growth enclosure was assumed to greatly limit water loss by way of evaporative cooling. To calculate the water lost from the system, the water contained within the outside air and air inside the greenhouse needed to be calculated as well as the ventilation rate. Because the ACS had a substantial effective surface area, it was assumed that the relative humidity within the greenhouse is maintained at approximately 80% throughout the course of all cultivation efforts. The amount of water in the system at 80% RH was calculated using **Equation 3** (Tetens, 1930) to determine saturation vapor pressure and **Equation 4** (Tetens, 1930) to determine the water vapor concentration.

$$P_s = 610.78 \times \exp(T / (T + 238.3) \times 17.2694) \quad \textbf{(3)}$$

Where:

P_s = Saturation vapor pressure (Pascals)
T= Temperature (°Celsius)

The output of **Equation 3** was then used in **Equation 4** to determine the mass of water in a cubic meter of air.

$$W_c = 0.002166 \times ((P_s \times R_h)/100)/ (T +273.15) \quad \textbf{(4)}$$

Where:

W_c = Water concentration (kg m^{-3})
R_h = Relative humidity (Percent)

To determine the total amount of water lost from the system, the infiltration rate and ventilation rates were combined throughout the year and a mass balance was used to determine how much water was lost to the outside environment.

Results

Productivity

The system was able to achieve a production of 84%, 76%, 70%, 66% and 51% compared to the optimal productivity for Tucson, AZ, Houston, TX, Newark, NJ, Spokane, WA, and Anchorage, AK.

Dried *Chlorella sp.* and *Spirulina sp.*

This model assumed that all algae species grown on the system would behave similarly to *Chlorella sp*. However, *Chlorella sp.* has a relatively low commercial market value and thus it was assumed that other algae species could be grown on the system with different nutritional and commercial properties. Dry *Spirulina sp.* and *Chlorella sp.* have current market prices of $8 and $19 kg^{-1}, respectively when sold as powders, which leaves a margin of -84% and 22.4% after production in Houston, TX (Vigani et al., 2015) that can be used for subsequent dewatering and processing. When these figures are estimated for other regions, it is demonstrated that none of the locations can produce *Spirulina sp.* in a financially viable manner and in addition to Houston, TX only Tucson, AZ could produce *Chlorella sp.* in a viable manner with a production margin of 18%.

Astaxanthin, β-carotene, Omega-3-Fatty Acids and Fishery Feed

While *Chlorella sp.* cannot produce many high value products, marine algae can and it was assumed that marine algae could be grown on the system with similar growth characteristics to *Chlorella sp*. As shown in **Table 7**, the gross profit margins remaining to dewater, process, and distribute the astaxanthin, omega-3 fatty acids, β-carotene, and fishery feed after cultivation in Houston, TX, at market prices of $700, $61,208, $238,

and \$363 kg^{-1} (Vigani et al., 2015; Coutteau and Sorgeloos, 1993; Coutteau and Sorgeloos, 1992) were -\$110, \$60,913, \$132, and \$348 kg^{-1}, respectively.

Water Loss

Using the ASAE exchange rates and a greenhouse relative humidity of 80%, the ACS loses on average 40, 14, 28, 42 and 37 L m^{-2} d^{-1} for Tucson, AZ, Houston, TX, Newark, NJ, Spokane, WA and Anchorage, AK, respectively.

Discussion

The overall productivity of the ACS varied widely based on the location of the greenhouse structure and the time of year. Using the model described above, the average yearly cost per ton of dry algae biomass for Tucson, AZ, Houston, TX, Newark, NJ, Spokane, WA, and Anchorage, AK, were \$15,596, \$14,743, \$19,319, \$22,264, and \$32,243, respectively. These figures assumed that the greenhouses are all heated to their optimal temperatures (30^{o}C) and that there is no supplemental lighting of any kind. If the system reached optimal productivity year-round with no supplemental heating or lighting in Tucson, AZ, the production costs of algae would \$10.85 kg^{-1}. Supplemental lighting could have increased the productivity of the system, but would have increased CAPEX costs and put the construction of the system well outside the reach of a farmer. Instead, it is preferred to use an optimal location like Tucson, AZ that achieves 84% of the optimal productivity without any supplemental lighting. However, other factors need to be taken into account as Arizona has the highest productivity, but a higher production cost than Houston, TX due to electrical costs associated with ventilation and increased water loss. Though the results reiterate that the technology could not produce low value commodity products (e.g., biofuels) in a financially viable manner, it does indicate that several higher

value products could potentially be produced economically on the system based on model calculations.

Productivity

The productivity of the system was calculated using **Equation 3** in the Supplemental Materials that uses temperature and light integral data that were acquired from the U.S. Naval Observatory (2017) and the National Weather Service (2017). First, the effect of the greenhouse design on the inside temperature was determined. Subsequently, it was determined how much energy was needed to raise the greenhouse temperature to 30^{o}C throughout the course of the day. Once this was determined, the productivity of the algae was determined using 30^{o}C and the daily light integral inside the greenhouse. It was assumed that 1/3rd of the solar radiation went to directly heating the greenhouse.

By multiplying the resulting number (0-1) by the maximum productivity (25 g dry biomass d^{-1}), the projected biomass production for the system was determined. Though the system modeled here could achieve high productivity yields (Supplementary Materials **Figure 2**), this model makes several optimistic assumptions. These include that the system would be able to achieve a 25 g dry biomass d^{-1} productivity, which would be a two-fold increase over current productivities and would assume that the system was able to continually achieve this productivity. Many publications have cited problems with maintaining axenic cultures and preventing culture crashes and this will be a major challenge for the system described here at large-scale (Bruggeman et al., 2015). Additionally, the system has yet to be optimized for harvesting as it currently is harvested using a tedious and time consuming mechanical approach that results in less than desired

effluent yields (<3.3 g L^{-1}) or an irrigation based approach that has yet to achieve high harvest concentrations and currently only achieves approximately 1.4 g L^{-1} (Johnson et al., 2015). This results in the system producing algae biomass at a low concentration, which would require substantial detwatering and shipping costs to a processing facility.

Financial Viability

Because of the high costs ($14.74 to $32.24 kg^{-1} of dry algae) of producing an algae slurry using this approach, there are only a few high-value products that can be produced using this system: *Chlorella sp*., omega-3-fatty acids, β-carotene, and fishery feed (**Figure 15**). These products have current prices that range from $238 to $61,208 kg^{-1} which makes them particularly well-suited to be produced using this approach (Vigani et al., 2015; Coutteau and Sorgeloos, 1993; Coutteau and Sorgeloos, 1992). While dried *Chlorella sp.* and *Spirulina sp.* are sold as a nutritional supplements or in specialty drinks (Naked Juice Company, 2017), only *Chlorella sp.* could be potentially produced in a financial viable manner in two of the locations chose. Though *Chlorella sp.* and *Spirulina sp.* have high production volumes and are freshwater species, one of their limitations is that they have a relatively low commercial value compared to higher value products like astaxanthin, omega-3 fatty acids, β-carotene, and fishery feed that have current market prices of approximately $700, $61,208, $238, and $363 kg^{-1}, respectively (Vigani et al., 2015). However, these high value nutraceutical products all are derived primarily from marine algae species; astaxanthin is produced by *Haematococcus pluvialis* and omega-3-fatty acids, β-carotene, and fishery feed can be produced by a wide-range of marine algae species (Kiperstok et al., 2017).

It has been shown that *Haematococcus pluvialis* generally accumulates up to 2.5% astaxanthin by dry weight (Kiperstok et al., 2017, Olaizola, 2000) and it has been recently shown that it can achieve high biomass yields on ACSs (Kiperstok et al., 2017). As shown in **Table 7**, the margins remaining to dewater, process, and distribute the astaxanthin, omega-3 fatty acids, and β-carotene after cultivation were -$110, $60,913 and $132, respectively. While the lipid content of algae species varies considerably (D'Alessandro and Antoniosi, 2016), it was assumed that the average lipid content of algae species cultivated on the system would be 10% and that the average concentration of omega-3 fatty acids in the algae are 50% of dry mass as this also varies considerably between species and conditions (D'Alessandro and Antoniosi, 2016; Saumya et al., 2016, Yongmanitchai and Ward, 1991). It was assumed that *Dunaliella salina* would be grown for β-carotene production and 14% of the dry weight would be β-carotene (D'Alessandro and Antoniosi, 2016). While the market price for astaxanthin is much higher than β-carotene, the content of β-carotene (approximately 14%) on a mass basis in algae biomass is substantially higher than that of astaxanthin (approximately 2.5%) (D'Alessandro and Antoniosi, 2016; Kiperstok et al., 2017, Olaizola, 2000). This large difference in content accounts for the inability to produce astaxanthin in a financially viable manner on the system.

In addition to these products, it is also shown the system can produce algae for fishery feed with a margin of $348 kg^{-1}. However, from a biological point of view, using the ACS for this application is challenging because the system is open to the indoor environment and can quickly be contaminated with heterotrophs (e.g., ciliates, bacteria) and other species of algae. Even if the contamination is only a small percentage of the

overall harvested biomass, it has been shown to have a detrimental impact on fisheries as it substantially reduces their productivity (Coutteau and Sorgeloos, 1993; Coutteau and Sorgeloos, 1992). Therefore, from a financial and practical point of view, it is unlikely that this system could be used for this purpose unless the contaminant problem can be mitigated. It was illustrated through the financial breakdown (**Table 7**) that the biomass produced by this system is an unsuitable feedstock for the production of biofuel and low value commodity products (e.g., animal feed) and that only a few products can be produced economically on the system.

Cooling and Water Loss

The main driver for water loss is the outside temperature as the ventilation rate will drive how much water is lost from the system. In addition, cold and dry climates will cause more water to be lost from the system than warmer more humid climates. This water loss rate is substantially greater than the water loss rate for ORP systems (1.6 to 11 L m^{-2} d^{-1}) (Lundquist et al., 2010; Borowitzka and Moheimani, 2013; Richardson et al., 2012) even though this system is housed in a controlled environment and the ORP system is completely open to the environment. This finding indicates that the large-scale production of algae using this methodology will potentially use a greater quantity of water than ORP systems. Therefore, the proposed benefit of using less water does not appear valid at the large-scale and while not a suspended growth system, this ACS appears to utilize an equivalent amount of water as ORP systems. This is due to the high humidity within the greenhouse caused by the high effective surface area of the substrates (20 m^2 m^{-2} floor space) and the water loss due to the ventilation rate.

Conclusions

It was determined that dry algae biomass and several high value nutraceutical products can potentially be produced in a financially viable manner depending on the cost of subsequent dewatering and processing. The purpose of this analysis was to provide guidance on the future focus of this ACS and to compare the economic viability of producing different commercial products. Because the system is open to the environment, it is susceptible to culture crashes and shading from clouds that will reduce its productivity over the course of the year. Because of the low profitability of freshwater species, it is suggested that future efforts to optimize the system focus on the cultivation of marine algae species. While astaxanthin could be produced on the system, its low concentration in biomass causes it to have a low profit margin when its downstream processing (e.g., dewatering, extraction, purification) is considered. Therefore, the focus of future work should be on the cultivation of algae species that produce omega-3 fatty acids and β-carotene as well as algae that can be used as fishery feed. However, additional research will need to focus on how to reduce contamination on this system so that high quality products such as fishery feed can be produced.

The model described here illustrates that the ACS can potentially produce algae feedstock in a financially viable manner if the subsequent dewatering and processing costs are reasonable. This is based on numerous assumptions which include the system having an optimal productivity of 25 g m^{-2} d^{-1}, minimal maintenance and the ability to avoid culture contamination and various other large-scale operational issues. The system has not yet demonstrated long-term productivities of 25 g m^{-2} d^{-1} at scale and reductions in this optimal productivity will reduce the financial viability of the system for producing lower value commercial products. Additionally, the system has been shown in its current

configuration to retard marine algal growth (Johnson et al., 2015). While the use of this system appears to be plausible from a commercial perspective, the supporting infrastructure required to make the system commercially viable needs to be considered. The scope of the analysis described here was only on generating algae slurry and substantial CAPEXs and OPEXs are required for the dewatering and processing of algae for commercial use. For this system to become financially viable, this processing equipment needs to be created and operated. Therefore, it is suggested that the first application of the technology is to produce algae slurry for fishery operations. If algae contamination can be controlled, even at low productivities (2.5 g m^{-2} d^{-1}), *T. chuii* could be used in a financially viable manner since it was demonstrated that 2.28 g m^{-2} d^{-1} could be produced (Johnson et al., 2015) on this ACS at small-scale.

Fishery feed does not require dewatering or extraction and the only cost following harvesting would be the transportation of the liquid slurry to a fishery operation. This system would be particularly well-suited for this application as fishery operations require year-round feed and take place in many geographic regions where year-round production of algae using ORPs or PBRs is unsuitable outside. This commercialization path would allow for revenue to be reinvested into dewatering operations and subsequently for the investment in extraction equipment for omega-3-fatty acids and β-carotene. This three-stepped commercialization approach would reduce risk and would reduce the initial CAPEX required to create an operational controlled environment ACS.

Figures and Tables

Table 4. Financial overview of CAPEX associated with the construction of the 10,000 m^2 greenhouse used for this model including materials and installation. The cost for the heaters varied based upon location as colder climates required more heating capacity than warmer climates. It was assumed that the land used for this facility will be flat and will require minimal preparation and the design plans for the system will be generated by an academic institution and open sourced for the builder. The permits and zoning for the facility were excluded from the calculation as these will be similar for all locations and can be ignored if being constructed by a state or federal entity.

Greenhouse Capital Expenditures	Cost
Galvanized steel with posts spaced 1.5 m apart	$157,500
Ventilation fans (29,000 cfm)	$64,125
Gas unit heater (200,000 BTU) (20-64 units)	$23,120 to $73,984
Greenhouse installation and labor	$48,000
Two layers of 0.15 mm polyethylene film	$20,747
Assorted heater equipment	$18,034
Doors to exterior	$15,400
Outer side wall material	$10,752
Electrical installation	$9,600
Landscape fabric for ground cover	$9,000
Shipping	$8,000
Sump construction and materials	$1,200
Total Cost	$385,578 to $436,342

Table 5. CAPEX required to produce 3,360 substrate scaffold units for the ACS.

Substrate System Capital Expenditures	Cost
Materials for scaffolding system	$296,140
Construction of scaffolding system	$175,000
Installation of irrigation system (labor)	$87,500
Irrigation system materials	$81,312
Substrates	$33,600
Greenhouse monitoring system	$20,000
Water pumps	$7,646
Automated nutrient monitoring system	$5,846
Computers and software	$3,000
Total Cost	$709,842

Table 6. Operating expenditures (OPEXs) associated with the operation of the ACS. This overview comprises all of the OPEXs associated with the operation of the system. The electricity cost assumed a per kWh cost of $0.111, $0.113, $0.163, $0.082 and $0.172 for Tucson, AZ, Houston, TX, Newark, NJ, Spokane, WA and Anchorage, AK, respectively (EIA, 2017). For all locations, the CAPEX loans were at an 8% annual interest rate over the ten-year period. Inflation was assumed to be 2.1% yr^{-1} throughout the operation of the system and it was assumed that the greenhouse covering needed to be replaced every three years.

	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7	Year 8	Year 9	Year 10
Maintenance	$50,082	$50,823	$54,399	$52,352	$53,141	$56,947	$54,768	$55,608	$59,659	$57,340
Manager salary	$85,000	$86,785	$88,607	$90,468	$92,368	$94,308	$96,288	$98,310	$100,375	$102,483
Lab Tech salary	$55,000	$56,155	$57,334	$58,538	$59,768	$61,023	$62,304	$63,613	$64,948	$66,312
Benefits and overhead	$42,000	$42,882	$43,783	$44,702	$45,641	$46,599	$47,578	$48,577	$49,597	$50,639
Covering replacement			$28,194			$30,008			$31,939	
Nutrient solution and pH control	$3,360	$3,431	$3,503	$3,576	$3,651	$3,728	$3,806	$3,886	$3,968	$4,051
Contingency costs	$55,091	$55,906	$59,839	$57,588	$58,455	$62,642	$60,245	$61,169	$65,625	$63,074
CAPEX Repayment										
Tucson, AZ	$160,273	$160,273	$159,552	$159,552	$159,552	$159,552	$159,552	$159,552	$159,552	$159,552
Houston, TX	$161,172	$160,450	$160,450	$160,450	$160,450	$160,450	$160,450	$160,450	$160,450	$160,450
Newark, NJ	$165,067	$164,345	$164,345	$164,345	$164,345	$164,345	$164,345	$164,345	$164,345	$164,345
Spokane, WA	$167,164	$166,442	$166,442	$166,442	$166,442	$166,442	$166,442	$166,442	$166,442	$166,442
Anchorage, AK	$173,455	$172,733	$172,733	$172,733	$172,733	$172,733	$172,733	$172,733	$172,733	$172,733
Water, Electricity and Heating										
Tucson, AZ	$337,848	$344,943	$352,187	$359,583	$367,134	$374,844	$382,715	$390,752	$398,958	$407,336
Houston, TX	$244,363	$249,495	$254,734	$260,083	$265,545	$271,122	$276,815	$282,628	$288,563	$294,623
Newark, NJ	$367,454	$375,171	$383,049	$391,093	$399,306	$407,692	$416,253	$424,994	$433,919	$443,032
Spokane, WA	$430,117	$439,149	$448,372	$457,787	$467,401	$477,216	$487,238	$497,470	$507,917	$518,583
Anchorage, AK	$517,381	$528,246	$539,339	$550,665	$562,229	$574,036	$586,091	$598,399	$610,965	$623,795
Total Expenses										
Tucson, AZ	$788,654	$801,198	$847,398	$826,359	$839,710	$889,651	$867,256	$881,467	$934,621	$910,787
Houston, TX	$645,986	$655,104	$696,444	$675,405	$685,878	$729,880	$707,486	$718,633	$765,465	$741,632
Newark, NJ	$687,972	$697,890	$740,047	$719,842	$731,166	$776,037	$754,531	$766,584	$814,341	$791,453
Spokane, WA	$858,005	$868,083	$909,685	$889,647	$901,142	$946,187	$924,861	$937,096	$985,040	$962,341
Anchorage, AK	$1,070,732	$1,081,039	$1,123,610	$1,104,564	$1,117,070	$1,163,149	$1,142,877	$1,156,189	$1,205,232	$1,183,655

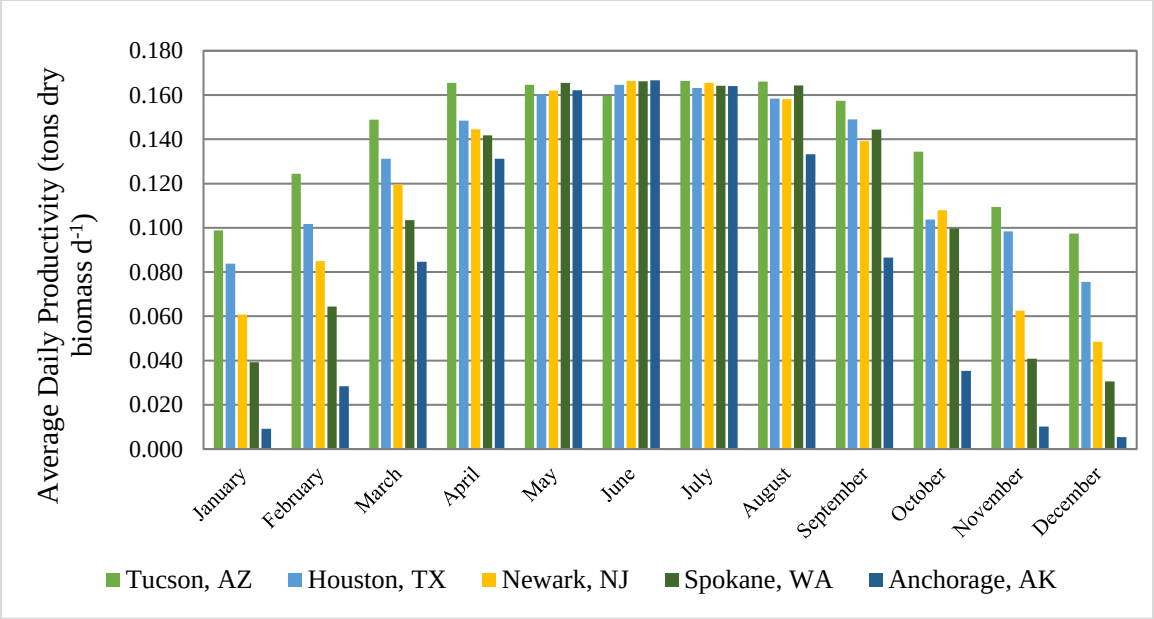

Figure 15. Simulated average daily productivity of the ACS in five different locations across the United States during the course of a year. The maximum productivity of the system was 0.17 tons of biomass d^{-1} based on the optimal daily light integral (40 mol m^{-2} d^{-1}) and temperature (30°C).

Table 7. Sensitivity analysis of the effect of growth rate on financial viability. This model used Houston, TX as an ideal location and assumed a year-round set point temperature of 30°C. This model shows the product profitability ($ kg^{-1}) of each kilogram of algae that is produced on the system excluding the cost for dewatering and processing.

Product Profitability ($ kg-1)							
Percent of Maximum Growth Rate (%)		***Spirulina sp.***	***Chlorella sp.***	**Astaxanthin**	**Omega-3 Fatty Acids**	**β-Carotene**	**Fishery Feed**
	100	-$6.74	$4.26	$2.76	$3,045.66	$18.51	$348.26
	90	-$7.54	$2.36	$1.01	$2,739.62	$15.18	$311.96
	80	-$8.34	$0.46	-$0.74	$2,433.58	$11.86	$275.66
	70	-$9.14	-$1.44	-$2.49	$2,127.54	$8.53	$239.36
	60	-$9.94	-$3.34	-$4.24	$1,821.50	$5.21	$203.06
	50	-$10.74	-$5.24	-$5.99	$1,515.46	$1.88	$166.76
	40	-$11.54	-$7.14	-$7.74	$1,209.42	-$1.44	$130.46
	30	-$12.34	-$9.04	-$9.49	$903.38	-$4.77	$94.16
	20	-$13.14	-$10.94	-$11.24	$597.34	-$8.09	$57.86
	10	-$13.94	-$12.84	-$12.99	$291.30	-$11.42	$21.56

References

- Barlow, J., Sims, R. C., & Quinn, J. C. (2016). Techno-economic and life-cycle assessment of an attached growth algal biorefinery. *Bioresource Technology*, *220*, 360-368.
- Borowitzka, M. A. (2013). High-value products from microalgae—their development and commercialisation. *Journal of Applied Phycology*, *25*(3), 743-756.
- Borowitzka, M. A., & Moheimani, N. R. (2013). Sustainable biofuels from algae. *Mitigation and Adaptation Strategies for Global Change*, *18*(1), 13-25.
- Bruggeman, A. J., Kuehler, D., & Weeks, D. P. (2014). Evaluation of three herbicide resistance genes for use in genetic transformations and for potential crop protection in algae production. *Plant Biotechnology Journal*, *12*(7), 894-902.
- Chang, E. H., & Yang, S. S. (2003). Some characteristics of microalgae isolated in Taiwan for biofixation of carbon dioxide. *Botanical Bulletin of Academia Sinica*, 44.
- Cheng, P., Ji, B., Gao, L., Zhang, W., Wang, J., & Liu, T. (2013). The growth, lipid and hydrocarbon production of Botryococcus braunii with attached cultivation. *Bioresource Technology*, *138*, 95-100.
- Christenson, L. B., & Sims, R. C. (2012). Rotating algal biofilm reactor and spool harvester for wastewater treatment with biofuels by-products. *Biotechnology and Bioengineering*, *109*(7), 1674-1684.

- Coutteau, P., Sorgeloos, P. (1992). The use of algal substitutes and the requirement for live algae in the hatchery and nursery rearing of bivalve molluscs: an international survey. *Journal of Shellfish Research*, 11, 467-467.
- Coutteau, P., Sorgeloos, P. (1993). Substitute diets for live algae in the intensive rearing of bivalve mollusks—a state of the art report. *World Aquaculture*, *24*(2), 45-52.
- Rankings: Average Retail Price of Electricity to Residential Sector, January 2017 (cents/kWh). Retrieved April 29, 2017, from https://www.eia.gov/state/rankings/?sid=US#/series/31.
- Green Machine. Retrieved February 24, 2017, from http://www.nakedjuice.com/our-products/green-machine/.
- Gross, M., Henry, W., Michael, C., & Wen, Z. (2013). Development of a rotating algal biofilm growth system for attached microalgae growth with in situ biomass harvest. *Bioresource technology*, *150*, 195-201.
- Harun, R., Singh, M., Forde, G. M., & Danquah, M. K. (2010). Bioprocess engineering of microalgae to produce a variety of consumer products. *Renewable and Sustainable Energy Reviews*, *14*(3), 1037-1047.
- ANSI/ASAE. (2003). Heating, Ventilating and Cooling Greenhouses. EP406.4 January.
- Johnson, M., Villani, T. S., Azmat, A., Simon, J. E., & Both, A. J. (2015). Evaluation of algal biomass production on vertical aeroponic substrates. *Algal Research*, *10*, 240-248.

- Kiperstok, A. C., Sebestyén, P., Podola, B., & Melkonian, M. (2017). Biofilm cultivation of Haematococcus pluvialis enables a highly productive one-phase process for astaxanthin production using high light intensities. *Algal Research*, *21*, 213-222.
- Lundquist, T. J., Woertz, I. C., Quinn, N. W. T., & Benemann, J. R. (2010). A realistic technology and engineering assessment of algae biofuel production. *Energy Biosciences Institute*, 1-178.
- Ňancucheo, I., & Johnson, D. B. (2012). Acidophilic algae isolated from mine-impacted environments and their roles in sustaining heterotrophic acidophiles. *Frontiers in Microbiology*, 3.
- National Weather Service Corporate Image Web Team. NWS Tucson. Retrieved February 24, 2017, from http://w2.weather.gov/climate/index.php?wfo=twc.
- Natural Gas Prices. U.S. Energy Information Administration: Independent Statistics & Analysis. Retrieved February 24, 2017, from https://www.eia.gov/dnav/ng/ng_pri_sum_dcu_nus_m.htm.
- Virtual Grower 3.0. (2016). USDA. Retrieved April 27, 2017, from https://www.ars.usda.gov/research/software/download/?softwareid=309.
- Olaizola, M. (2000). Commercial production of astaxanthin from Haematococcus pluvialis using 25,000-liter outdoor photobioreactors. *Journal of Applied Phycology*, *12*(3-5), 499-506.
- Pulz, O., & Gross, W. (2004). Valuable products from biotechnology of microalgae. *Applied Microbiology and Biotechnology*, *65*(6), 635-648.

- Richardson, J. W., Johnson, M. D., & Outlaw, J. L. (2012). Economic comparison of open pond raceways to photo bio-reactors for profitable production of algae for transportation fuels in the Southwest. *Algal Research, 1*(1), 93-100.
- Robbins, J. (1999). Starting a Greenhouse Business (Part 1) Some Basic Questions. University of Arkansas: Division of Agriculture. Retrieved February 24, 2017, from http://www.uaex.edu/publications/pdf/FSA-6051.pdf.
- Saumya, D., Kannan, D. C., & Dhawan, V. (2016). Understanding Urea Assimilation and its Effect on Lipid Production and Fatty Acid Composition of *Scenedesmus sp*. *SOJ Biochem, 2*(1), 7.
- Schnurr, P. J., Espie, G. S., & Allen, D. G. (2013). Algae biofilm growth and the potential to stimulate lipid accumulation through nutrient starvation. *Bioresource Technology*, 136, 337-344.
- Sun or Moon Rise/Set Table for One Year. Retrieved February 24, 2017, from http://aa.usno.navy.mil/data/docs/RS_OneYear.php.
- Tetens, O. (1930) Uber einige meteorologische Begriffe. *Zeitschrift fur Geophysik*, 6:297.
- Vigani, M., Parisi, C., Rodríguez-Cerezo, E., Barbosa, M. J., Sijtsma, L., Ploeg, M., & Enzing, C. (2015). Food and feed products from micro-algae: Market opportunities and challenges for the EU. *Trends in Food Science & Technology, 42*(1), 81-92.
- Yongmanitchai, W., & Ward, O. P. (1991). Growth of and omega-3 fatty acid production by *Phaeodactylum tricornutum* under different culture conditions. *Applied and Environmental Microbiology, 57*(2), 419-425.

- Brennan, L., & Owende, P. (2010). Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews*, *14*(2), 557-577.

Supplementary Materials

Figures and Tables

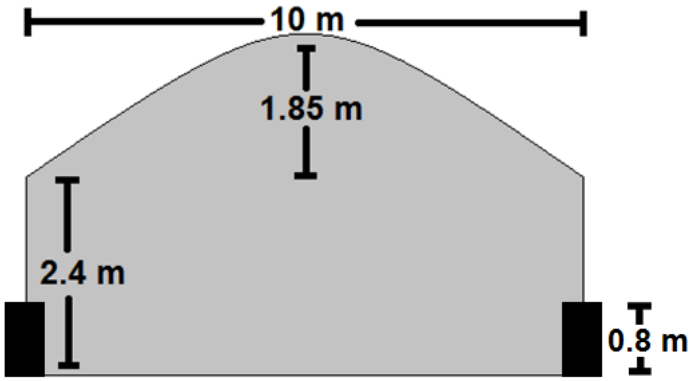

Figure 1. Depiction of greenhouse structure that was used to contain the ACS. The facility was comprised of ten 10 x 100 m gutter-connected bays for a total footprint of 10,000 m^2. The outer most perimeter of the greenhouse had a 0.8 m high concrete block curtain wall.

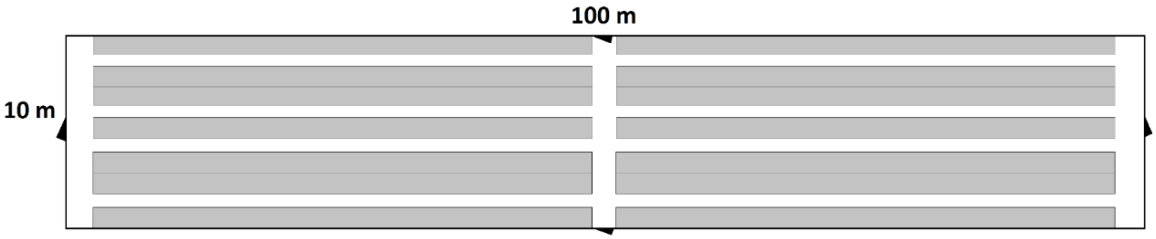

Figure 2. Overhead layout of one of ten greenhouse sections. Each greenhouse section had 7 rows of 1.1 m wide scaffolds (gray) separated by four walkways that were 0.575 m wide each. In the middle of the section there was a one meter wide walkway that connected a section to an adjacent section and at either end of the section there was a 1.5 m wide walkway that connects all four walkways to the exit doors. Additionally, two exit doors were envisoned in the end walls. The outer wall consisted of a 0.8 m high concrete block wall and all of the other covering material on the greenhouse was air infiltrated double layer polyethylene film. Each greenhouse bay achieves a 67.2% area utilization as there are 336 scaffold units in each bay and each unit covers 2.2 m^2 of floor space with 20 m^2 of growth area.

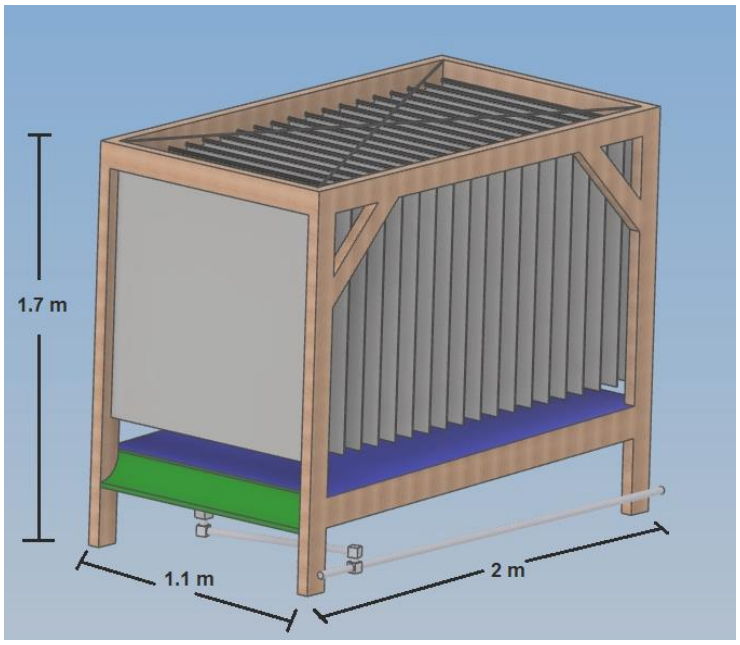

Figure 3. Depiction of a 2 by 1.1 m substrate scaffold unit that contained twenty, one square meter substrates. This substrate scaffold was comprised primarily of pressure treated wooden 2 by 4's that were screwed together by four 4" screws at each joint. The ACS used for this model contains 3,360 of these units arranged side-by-side and in rows to achieve a 67.2% per area utilization in the one hectare greenhouse with 67,200 total substrates. In addition to this wooden structure, there were two cross wires on top of the structure to provide additional torsional support and there was a plastic board that sits on top of the bottom angled 2 by 4. This plastic board collects the dripping nutrient solution. The substrates were held from this structure by using three clamps that were attached to ¼" aluminum rods that were mounted to the scaffoldthrough ¼" drill holes in the side of the scaffold system. The effluent from the substrates dripped onto the plastic board and traveled down into a 3" PVC gutter that drained into a ½" PVC pipe that flowed into a sump. All the effluent from the substrates was collected in a sump in the floor of the greenhouse for subsequent nutrient replenishment and recirculation. These scaffold units were placed back to back to one another such that the two halves of the 3" irrigation collection pipe connected to one another.

Table 1. The ACS described in this model was comprised of 3,360, 2 by 1.1 m substrate scaffold units that were constructed primarily out of pressure treated wood. The individual components and costs are described for each individual scaffold unit. Each wood piece was joined to another wood piece using four 4" screws and there are 20 joints in each scaffold. The 1/8" metal wire was used to provide cross-bracing support and the aluminum rod was used to hold up the individual substrates and was chosen as it would not corrode over time. Retail prices from The Home Depot were used for cost estimation.

Scaffold Materials Per Unit			Unit Cost	Total Cost
Screws for construction (4")	80	Screws	$0.20	$16.00
Length of 2 x 4 pressure treated wood	22.8	M	$1.18	$26.90
Length of wire (1/8")	4.72	M	$0.20	$0.94
Plastic board (corrugated)	2	m^2	$4.04	$8.08
Aluminum rod (1/4")	22	M	$1.05	$23.10
1/2" PVC piping	3	M	$0.45	$1.35
1/2" PVC elbows	2	Units	$0.30	$0.60
3" drainage Tubing	1	M	$5.17	$5.17
Clamps to hang substrates from rods	60	units	$0.10	$6.00
			Total Per Scaffold	$88.15
			Total Cost	$296,184

Table 2. Each of the 3,360 scaffold units had irrigation tubing attached to it in order to effectively irrigate the substrates. There were 20 irrigation lines placed above the 20 substrates in each scaffold such that each substrate was irrigated with three drippers that were placed evenly across the substrates top. These irrigation lines were capped at one end by an end cap and were connected to one another using “L” and “T” fittings such that all irrigation lines connected to a single tube that connected to the major irrigation line that runs to all of the substrates.

Irrigation Materials Per Unit			Unit Cost	Total Cost
L and T fittings	20	units	$0.45	$9.00
1/2" polyethylene tubing	20	m	$0.21	$4.20
End cap fittings	20	units	$0.25	$5.00
Drippers	60	units	$0.10	$6.00
			Total Per Unit	$24.20
			Total Cost	$81,312

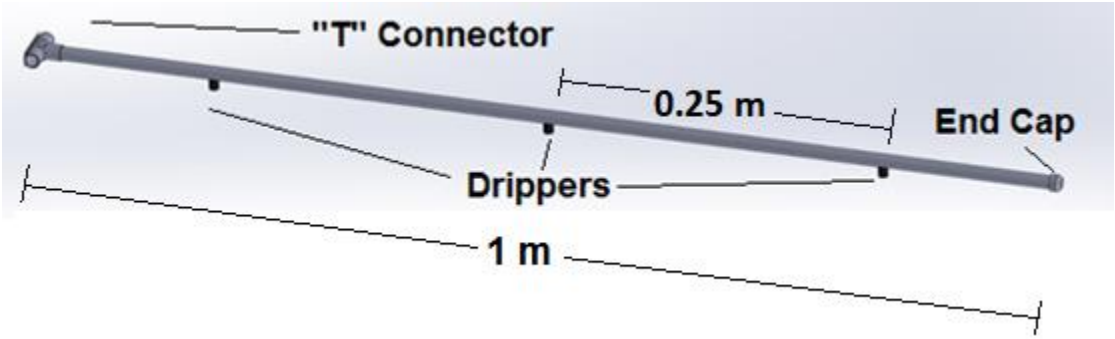

Figure 4. Depiction of one of the twenty irrigation units that was used to irrigate the 20 individual substrates that are in each scaffolding system. This irrigation unit had three drippers (black) that are spaced 25 cm from one another with the central dripper directly in the center of the substrate. This irrigation unit was connected to the other 19 units and to the main irrigation system. This irrigation tube is capped on one end by an end cap and on the other end by a “T” or “L” fitting for the end substrate. The pipe is ½” inch in diameter and is 1 m long. The drippers have an approximately 200 µm by 4 mm slit from which the nutrient solution drips.

Table 3. Equipment required for the operation of the ACS. The ACS used one Berkley B70221 water pump and had a backup pump in case this one fails or requires maintenance. The substrates required for the system cost $0.50 each and the nutrients in the system were monitored and augmented using an Anderson nutrient monitoring system that could adjust the pH of the nutrient solution as well as the overall nutrient concentration. The greenhouse was operated by a greenhouse control system that consisted of ten separate zones with independent control and monitoring of temperature, humidity, fan speed, exchange rates, and heating. This system cost approximately $20,000 and the computers and software required to control the nutrient monitoring system cost approximately $3,000.

Irrigation and System Materials			Unit Cost	Total Cost
20 hp water pump- Berkeley B70221	2	pumps	$3,823.15	$7,646
Substrates	67,200	substrates	$0.50	$33,600
Anderson nutrient monitoring system (JP101-3)	1	system	$5,846	$5,846
Greenhouse monitoring system	1	system	$20,000	$20,000
Computers and software	1	system	$3,000	$3,000
			Total Cost	$47,092

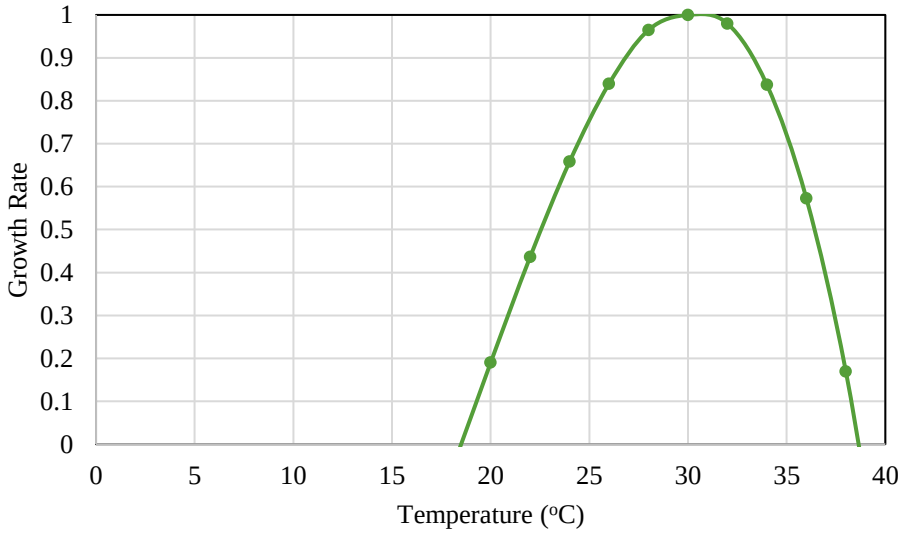

Figure 5. Graphical representation of the normalized relationship between temperature and growth rate for *Chlorella* on the ACS (Ňancucheo and Johnson, 2012). Growth Rate = $-0.000334T^3 + 0.01912T^2 - 0.2392T - 0.014$.

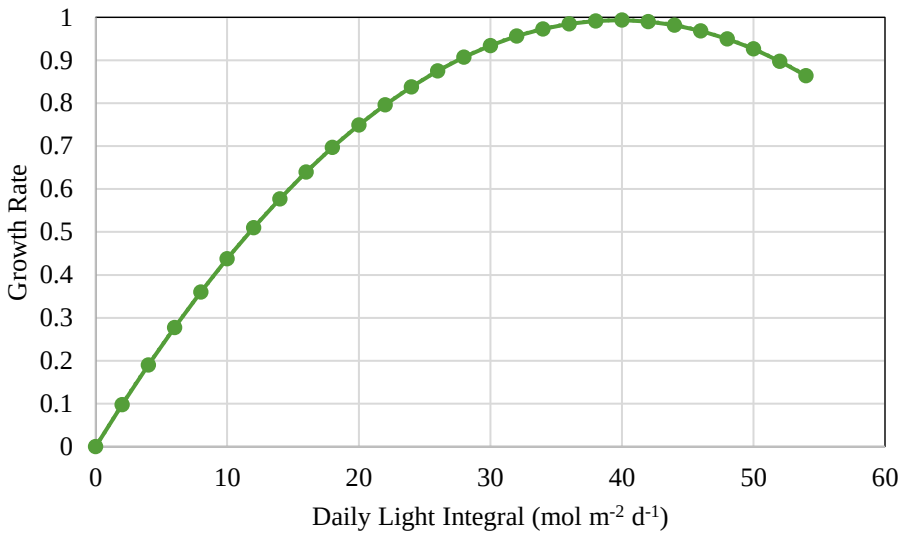

Figure 6. Graphical representation of the normalized relationship between daily light integral and growth rate for *Chlorella sp.* grown on the ACS (Chang and Yang, 2003). Growth Rate = -0.0006308L^2 + 0.05007L.

Equations

Algae Productivity

To determine the effect of temperature on the growth rate of *Chlorella sp.*, growth rate data was used from research conducted by Ňancucheo and Johnson (2012). Their work showed growth rate data for operating temperatures of 20-35ºC and from their data the following growth curve was established (**Figure 5**):

$$U = -0.000334T^3 + 0.01912T^2 - 0.2392T - 0.014 \qquad (R^2 = 0.99) \qquad \textbf{(1)}$$

Where:

U = Growth Rate (unitless) – normalized (0-1)
T = Ambient Temperature (ºC)

The growth rate units were originally described as doubling time by Ňancucheo and Johnson (2012) and this doubling time was normalized so that *Chlorella sp.* was assumed

to have a maximum growth rate (U = 1) at 30^{o}C. **Equation 1** was only used for temperatures between 20-35^{o}C as these are the temperatures that will yield the highest productivity and will consequently be the only temperatures that allow for a financially viable system. The second component of the three-dimensional model was the relationship between daily light intensity and growth rate. The fundamental relationship between these two parameters was determined by Chang and Yang (2013) using a PBR system for *Chlorella sp*. They demonstrated that the growth rate increased rapidly because of increasing light intensity and then plateaued (Chang and Yang, 2003). This relationship between light intensity and growth for *Chlorella sp*. can therefore be modeled using a second order equation. As with the temperature equation, this equation was normalized so that the maximum growth rate was given a growth rate value of "1." This equation was adapted for the ACS described here by using empirical pilot scale data compiled by AL-G Technologies, Inc. that demonstrated that the peak growth rate was achieved at approximately 40 mol m^{-2} d^{-1} over a 12 hour photo period and light integrals greater than this resulted in a reduction in growth rate due to photo-oxidation (**Figure 6** in Supplemental Materials).

$$U = -0.0006308L^2 + 0.05007L \qquad (R^2 = 0.97) \qquad \textbf{(2)}$$

Where:

U = Growth Rate (unitless) – normalized (0-1)
L = Daily Light Integral (mol m^{-2} d^{-1}), measured over the 400 – 700 nm waveband

In order to use this equation, the daily light integral was used (National Renewable Energy Laboratory, 1991) where it was assumed that the growth rate

throughout the course of the day was constant. To combine the light intensity and temperature equations into one equation, the two equations were multiplied to create **Equation 3**. This approach assumes that the impact of both variables on growth rate were equivalent.

$$U = (-0.0006308L^2 + 0.05007L) \times (-0.000334T^3 + 0.01912T^2 - 0.2392T - 0.014) \quad \textbf{(3)}$$

This equation can be used to determine the growth rate of algae on the system using both the temperature of a given environment and the light intensity as shown in **Figure 6** in Supplemental Materials. For the model described here, it was assumed that the maximum growth rate of the system was 25 g dry biomass m^{-2} d^{-1} and that under ideal conditions (30ºC, 40 mol m^{-2} d^{-1}) the system will achieve this productivity. In small-scale studies, growth rates approaching 25 g dry biomass m^{-2} d^{-1} have been achieved, but this production has yet to be demonstrated consistently at large-scale and will be affected by several factors not present at small-scale including, angle of irradiation, substrate shadowing, and clouds. This model also assumed that the impact of temperature and daily light integral on growth rates were independent of one another as we did not have the resources to investigate the combined or independent impact of each one of these parameters using our system.

Energy Flux

To determine the effect of varying location on greenhouse temperature, heating costs and algae productivity, a model was created for the greenhouse. The USDA has created a simulation model for greenhouses called *Virtual Grower* (USDA, 2016) that

formed the basis for the model described here. This model calculated heat loss using structural heat loss (conduction and convection), infiltration (air movement) and perimeter heat loss (along outside walls). Transfer of energy by way of structural heat loss depends on the heat transfer coefficient of a material, the surface area involved and the temperature difference between the air inside and outside the greenhouse (**Equation 4**). The rate at which the greenhouse loses energy to the environment is contingent upon the heat transfer coefficient (U value) of the greenhouse covering material as a single layer of glass will lose energy an order of magnitude more quickly than a 2" thick piece of Styrofoam (R=10) and the double layer of polyethylene and concrete wall used in this study had U values of 14.4 and 10.2 kJ hr^{-1} m^{-2} $^{o}C^{-1}$, respectively (USDA, 2016). The second component of the greenhouse model is unintended air infiltration which is the amount of energy that is lost to the environment in the form of air exchanging between the greenhouse and outside environment when the ventilation system is turned off. Though greenhouses tend to be thought of as closed systems, they are quite open and will exchange air between the greenhouse and the environment. Many well-built greenhouses will exchange an entire volume with the outside air every hour (USDA, 2016). This exchange is due to greenhouses not being completely sealed systems and their tendency to lose air to their surroundings over time. The equation used to model the loss of energy due to air exchanges is described below (**Equation 5**). This equation assumes that the humidity of the air inside the greenhouse is the same as the humidity of the air outside the greenhouse as there is no correction factor for differences in specific heat. This might cause an underestimation of heat loss for systems that operate in dry climates as a volume of very humid air in the greenhouse at a given temperature has more energy contained

within it than that same volume of very dry air. The third component for energy loss from the system is perimeter heat loss. Perimeter heat loss can occur when the warm greenhouse floor comes in contact with cold (and wet) outside soil/dirt and forms a conduit for heat loss. Adding insulation can prevent/reduce perimeter heat loss. This energy flux is estimated in **Equation 6** shown below.

Structural Heat Loss

$$Q_{structural} = U \times A \times (T_{inside} - T_{oustide}) \quad \textbf{(4)}$$

Infiltration Heat Loss

$$Q_{infiltration} = 0.5 \times V \times C \times (T_{inside} - T_{outside}) \quad \textbf{(5)}$$

Perimeter Heat Loss

$$Q_{perimeter} = H \times P \times (T_{inside} - T_{outside}) \quad \textbf{(6)}$$

Where:

U = Heat Loss Coefficient = 14.4 for double layer air-inflated polyethylene film and
A = Area = 11,488 m^2 (Double layer polyethylene), 320 m^2 (Concrete block wall)
T_{inside} = Inside Temperature (^oC)
$T_{outside}$ = Outside Temperature (^oC)
V = Greenhouse volume = 33,250 m^3
C = Air infiltration rate = 2 air exchanges hr^{-1}
H = Perimeter Heat Loss Factor = 5.4 kJ hr^{-1} m^{-1} $^oC^{-1}$
P = Perimeter = (400 m)
10.22 for concrete block wall (kJ hr^{-1} m^{-2} $^oC^{-1}$)

These three heat loss components and the overall total were calculated as:

$Q_{structural}$ = 167,452 kJ hr^{-1} $^oC^{-1}$ or 46,513 W $^oC^{-1}$
$Q_{perimeter}$ = 6,536 kJ hr^{-1} $^oC^{-1}$ or 1,815 W $^oC^{-1}$
$Q_{infilitration}$ = 33,250 hr^{-1} $^oC^{-1}$ or 9,236 W $^oC^{-1}$
Q_{total} = 208,477 kJ hr^{-1} $^oC^{-1}$ or 57,910 W $^oC^{-1}$

The model used here assumed that $1/3^{rd}$ of the irradiance from the sun per area of floor space was converted to sensible heat (USDA, 2016). The total energy required for

heating or cooling were based on the energy (kJ hr^{-1}) the energy required to increase the temperature of the greenhouse (kJ hr^{-1} $^oC^{-1}$). For this model, the efficiency of the natural gas heaters was assumed to be 80% and the cost of the natural gas was assumed to be equivalent to \$34.5 MWh^{-1} (US EIA, 2017). This cost is variable throughout the course of the year and by location and was therefore assumed to be constant to minimize complexity.

Heating

The impact of heating on algae production was modeled because temperatures below 30^{o}C would result in reductions in overall algae productivity as it was assumed the optimal productivity would occur at 30^{o}C. The greenhouse used for this model was a double layered polyethylene quonset greenhouse that had a total heat loss of 208,477 kJ hr^{-1} $^oC^{-1}$ (**Equations 4-6**) where the loss of energy from the system is dependent upon the difference between the temperature inside and outside the greenhouse. To calculate the heating required to maintain a temperature of 30^{o}C and achieve optimal productivity, the effect of the greenhouse structure on the temperature within the greenhouse was determined. This was calculated using **Equation 7** showing that the energy required to increase the temperature of the greenhouse is based on the set point temperature of the greenhouse and the heat loss of the greenhouse on an hourly basis.

$$H = ((T_I - T_o) \times Q) - (1/3 \times L_a \times A) \quad \textbf{(7)}$$

Where:

T_I = Inside Set Point Temperature (^{o}C)
L_a = Solar Radiation (kJ hr^{-1} m^{-2})
A = Greenhouse Floor Area (m^2)
Q = Greenhouse Heat Loss (kJ hr^{-1} $^oC^{-1}$)
T_o = Outside Temperature (^{o}C)

H = Additional Heating Required (kJ hr^{-1})

This equation assumed that 1/3rd of solar radiation went towards heating the greenhouse (USDA, 2016) and can be used to determine the increase in temperature. The ventilation rate was also assumed to be zero during periods of heating. The solar radiation and outside temperature data were acquired from the National Weather Service (2017) and was used on an hourly basis. This equation was used to calculate the amount of energy required to heat the greenhouse to the 30^{o}C optimal temperature. This equation was also used to determine the total cost of heating the greenhouse and whether improving covering materials or other greenhouse parameters had a positive return on investment while using a cost of natural gas of 34.5 $ MWh^{-1} and a heater efficiency of 80%. Based on this equation and the maximum energy required to heat the greenhouse throughout the year, it was determined that for Tucson, AZ, Houston, TX, Newark, NJ, Spokane, WA and Anchorage, AK, that 20, 23, 36, 43, and 64 heaters would be required, respectively.

References

- Horticultural Engineering - Rutgers University. (n.d.). Retrieved June 26, 2017, from http://horteng.envsci.rutgers.edu/

Chapter 4: Conclusions and Applications

The work described here focused on addressing a single question: "Is the ACS developed by AL-G Technologies, Inc. a financially viable production system and how does it compare to traditional open raceway ponds (ORP) and photobioreactor (PBR) systems for the production of algae biomass?" While this is a relatively complex question, the work described in this dissertation focused on addressing it through three-parts, including Chapter 1: Investigation of the system from a basic research perspective, Chapter 2 Investigation of opportunities for optimization of the system, and Chapter 3: Development of an economic model.

Chapter 1

In Chapter 1, the basic principles involving the attached cultivation system (ACS) were investigated through three distinct questions:

1) What growth rates (g biomass m^{-2} d^{-1}) can be achieved on the ACS?
2) Does the ACS change the nutritional composition of algae compared to traditional systems?
3) Why do algae stick to the substrates used for an ACS?

Addressing these questions was crucial to understanding the fundamental aspects of the ACS and how it operated. It was determined that both *P. kessleri* and *T. chuii* could be grown on the ACS and that their per area productivity varied greatly, but was similar to traditional suspended growth systems, such as the ORP and PBR systems. AL-G Technologies originally hypothesized that their system and ACSs in general can potentially increase the quantity of antioxidants that algae produce. The rationale for this hypothesis was that algae adhered to substrates had easier access to oxygen compared to algae suspended in a nutrient solution and thus would need to produce more antioxidants to

mitigate oxygen free radicals. Though algae produce oxygen and are exposed to oxygen, it was thought that the high concentration of oxygen in air would increase overall productivity. It was determined in Chapter 1 that this is not necessarily true and algae appear to have similar nutritional profiles when grown on substrates or in liquid culture. It is thought that while algae are adhered to a substrate exposed to air, they would be able to maintain a semi-suspended growth system because they are continually irrigated. While the thin interface would certainly increase the diffusion of CO_2, the dense algae biofilm that forms on the substrates would slow down gas diffusion and potentially cause growth-limiting oxygen gradients to form as well. It was described in Chapter 1 that algae adhere to the substrates through excreting a polysaccharide matrix that allows them to stick as well as hold water to prevent desiccation. It was also determined that *P. kessleri* grows symbiotically with fungi on the substrates, as they also produce polysaccharides and assist *P. kessleri* with adherence.

The initial research indicated that the ACS would not be able to be used as a unique tool for stimulating anti-oxidant production. The research also showed that many algae species could potentially be cultivated on the substrates so long as they could maintain adherence through the generation of a dense biofilm. However, the research also illustrated that contamination on the substrates will be a challenge as algae are producing a polysaccharide matrix that serves as a carbon source for a wide array of heterotrophs. Additionally, algae grown on the substrates might have lower overall productivities than algae grown in suspensions because a portion of the energy that is fixed by algae on the ACS needs to go to polysaccharide production that is simply washed off during irrigation cycles.

Chapter 2

After demonstrating that several marine and freshwater algae could both be grown on the system, the research focus of this work shifted toward optimizing the production of algae and determining which additional species could be grown. The purpose of the research described in Chapter 2 was to better understand the advantages and limitations of the system and to determine where there might be opportunities for improvement. Ultimately, the financial viability of an algae production system is based upon its productivity (g dry biomass m^{-2} d^{-1}) and this was thoroughly evaluated as described in Chapter 2.

There are additional downstream factors such as dewatering and extraction, but these were not included in the scope of work. To investigate the productivity of algae on the system, a better understanding was needed about how algae growth was affected by increased CO_2 concentration. It has been shown in algae production systems that increasing the CO_2 concentration can have a variable effect on productivity based on several factors (e.g., nutrient solution pH, carbon limitation) and it was necessary to investigate if increasing CO_2 concentration could improve the productivity of algae on the ACS. It was demonstrated that increasing the CO_2 concentration can improve growth rates, but that the substrates have a carrying capacity and if they are not harvested more frequently as the rate increases, then the productivity will decline. While increasing CO_2 was shown to have a positive effect on growth rates, it is unclear whether the introduction of CO_2 at scale would be feasible as it would require the facility to be co-located next to a power plant or other large CO_2 emitter that generates flue gas without unwanted particulate or chemical (e.g., mercury, lead) constituents.

Throughout the experiments, the substrates were harvested using a mechanical roller press that was very effective in removing algae from the substrates without using large quantities of water. Though this approach is effective, it is not feasible at large-scale with tens of thousands of substrates in a hectare-sized greenhouse and each one weighing more than 5 kg. Therefore, to reduce the cost of production and the use of mechanical harvesting at scale, the use of the existing irrigation system to wash algae from the substrates was investigated. By recirculating the nutrient solution numerous times, it was thought that a dense algae effluent could be created. While the experiments only resulted in the production of a 1.4 g L^{-1} solution, it demonstrated that this could potentially be a method for algae harvesting if the process could be improved. Improving the harvesting process represents a major challenge for this ACS and without optimizing the irrigation based harvesting procedure, it is unlikely that this system could ever be scaled to a large commercial system because the operational costs would be substantial. Future work should focus on adding amendments (e.g. salts, surfactants) to the harvesting solution that help with removing algae from the substrates. With the addition of chemical amendments to the harvesting process, it is possible that the effluent concentration could be increased and economically replace mechanical harvesting. However, irrigation-based harvesting has other challenges that must be mitigated such as the clogging of irrigation lines due to algal aggregation.

In cultivating algae, researchers and companies have relied on inducing metabolic stress through the removal of specific nutrients (e.g., nitrogen) to induce algae to produce more lipids. These lipids can be used to produce biodiesel and high value products and thus it was important to understand how algae responded to nutritional stress while grown on

the ACS. It was shown that due to the formation of a biofilm the removal of nitrogen from the nutrient solution did not result in a rapid increase in lipids as it does in traditional systems. This finding is similar to what was found for other types of ACSs and shows that these systems cannot increase lipid content as rapidly as traditional suspended growth systems. This is thought to be due to the biofilm storing nitrogen. Therefore, the types of products that the ACS can produce will be limited if no other method for inducing lipid accumulation can be found.

To understand what types of products can be produced on the ACS, we introduced several algae species on the system and determined that various algae species can be grown at varying levels of success. Though the research did not focus on optimizing production for each individual species of algae, it did illustrate that the system can be used to grow several species and thus a wide array of commercial products could potentially be produced. However, it was shown that marine algae species tend to grow poorly on the system and subsequent work showed that salinity gradients form on the substrates. The research also showed that considerable volumes of water can be lost from the system even though it is contained within a controlled growth environment. The major conclusion from the research was that harvesting needs to be optimized for the system to be viable at large-scale and that the use of marine algae species might be severely limited unless halo-tolerant algae species can be specifically optimized for growth on the system. The inability to grow marine species on the system would significantly limit the financial viability of the ACS as it would require the use of freshwater, as well as limit the system to produce lower value commercial products derived from freshwater algae.

Chapter 3

After conducting basic research to better understand the properties of the ACS, a large-scale production system was designed and simulated. While this model idealized production and assumed that the system could grow marine species, maintain high productivities, and be easily harvested at scale, it showed that the system is severely limited in its financial productivity with production costs of \$18.02 to \$36.95 kg^{-1}, depending primarily on geographic location. Houston had the most optimal productivity due to its temperature and its use of electricity which was less than Tucson due to less need for ventilation. These production costs only considered the price of producing a dense effluent slurry and did not consider dewatering, extraction, processing, or distribution. Thus, in this ideal state, the system is primarily limited to the production of marine algae species and to the production of high-value products that could potentially be produced using other traditional production systems, but at a lower cost. The main driver for the low productivity of the system is that the system is fundamentally light limited and produces less biomass per area compared to traditional systems while requiring more capital and greater operating costs. According to the technoeconomic analysis presented by Davis et al. (2011), the capital cost for a 10,000 m^2 ORP and PBR would be \$112,900 and \$319,200 (excluding the cost of land, extraction and dewatering) while the operating costs would be \$12,720 and \$18,900 yr^{-1}, respectively. Conversely, the ACS described here has an initial capital cost of \$1,095,508 and an operating cost of \$659,596 yr^{-1} for a 10,000 m^2 system size. Therefore, even if the system were able to achieve a similar biomass productivity compared to ORP and PBR systems at the large-scale, its cost would have to be reduced an order of magnitude to be competitive with traditional systems. Cost reductions could be achieved if the system did not need two full-time employees to operate and/or the greenhouse was

removed. Removing the greenhouse would reduce the capital costs of the system to a similar level to PBRs, but would result in substantial increases in water loss and contamination. These problems could be further mitigated by locating the system in a very humid climate (e.g., Puerto Rico) and using extremophile algae species to limit contamination, but this would greatly reduce the applications of the system and its potential commercial products.

Future Direction

While it has been shown that the system can be used to produce algae biomass and that under ideal circumstances it can be financially viable, the question arises whether the system should be utilized for commercial algae production and pursued further. From the original proposed benefits of the system (e.g., higher productivities, lower water usage, increased production of high value products), it appears that there are no substantial advantages over traditional systems regarding these factors and it is plausible that the ACS will lose more water and have lower productivities compared to traditional systems. Additionally, the system currently has the disadvantages of high contamination risk and being limited in effectively growing marine algae species. Therefore, unless other benefits can be elucidated or the production cost ($ kg^{-1} dry biomass) can be substantially reduced, there are few viable applications for the ACS.

At this time, it is thought that there is potentially one other benefit of this ACS that was not investigated here which should be further evaluated. The focus of this work was to evaluate the photosynthetic yield of the system on the basis of cost ($ kg^{-1}) and productivity (g biomass m^{-2} d^{-1}). However, it is possible that the wrong question was asked about the system from the beginning and the wrong frame of reference was chosen. This ACS system by its nature costs as much as a PBR system to construct on a $ m^{-2} basis and

requires the additional capital expenditure of a greenhouse to allow for environmental control and the reduction of water loss. Because an algae system's overall productivity is capped by the number of photons m^{-2} received and the photosynthetic yield of algae, it is unlikely that the system could ever achieve a greater productivity than a well-mixed and well-designed PBR regardless of how many square meters of growth substrates the ACS has per square meter of floor space. Therefore, the question that should have perhaps been asked is "how can we make this system produce more biomass?", instead of attempting to compare its productivity directly to traditional systems. A method to accomplish such an increase could be to utilize mixo-trophic/heterotrophic algae that produce high value products and to add a carbon substrate (e.g., glucose) to the nutrient solution. Applying this nutrient solution would require the use of several amendments (e.g., antibiotics, antifungals) to prevent unwanted heterotroph production, but could result in a substantial increase in overall algae biomass production. At large-scale, the top of the substrates would be well-illuminated, but the light intensity would decrease down the substrates. Therefore, the majority of the substrate would be poorly illuminated and while these areas would be poor for autotrophic production, they would form the ideal environment for heterotrophic production of algae as there would be an abundant carbon source as well as an electron acceptor (e.g., O_2) for aerobic respiration. This is one of the areas that should be further investigated as it has the potential to drastically improve the production of the system and the amount of biomass that can be produced. While moving toward heterotrophic growth combined with the need for a carbon source for production might reduce the sustainability of the system compared to autotrophic production, this could have a substantial positive impact on the commercial viability of the system. This suggestion and its ramifications

illustrate that optimizing an algae production system for sustainability and profitability can lead towards opposing outcomes.

Appendix 1– Small Scale Algae Production Protocols

Harvesting Procedure

The substrates were hung from the scaffolding system with two, 4 cm plastic loops that go through a whole punched at the top corners of the substrates. These loops can be made from any non-corroding material, but the easiest to use for this purpose are Zip-Ties. The growth chambers were designed so that the scaffolding system that the substrates hang from can be removed from the growth chamber. When this scaffolding system is removed, the substrates hanging from the plastic loops can simply be slid off for harvesting, cleaning, or evaluation. To harvest the algae from the small-scale system, the 12 substrates (including 2 dummy substrates at either end) are removed and the 10 substrates utilized to cultivate algae are placed in an autoclaved polycarbonate bin. Great care should be taken not to contaminate the substrates with other algae species being cultivated. Then 400 mL of the same nutrient solution used for cultivation is added to the bin with the substrates; this additional water aids in saturating the substrates and allowing algae to more easily be pressed off. Algae adheres to the substrates using a dense polysaccharide matrix and this nutrient solution solvates the polysaccharides, allowing for the algae to be more easily removed from the substrates. However, this additional nutrient solution dillutes the algae harvest concentration by increasing the algae harvest volume. This volume can be reduced to increase the final effluent concentration, but this will result in less algae being removed from the substrates. More algae can be removed from the substrates by increasing the pressure of the mechanical roller press, but this will result in permanently compressing the substrates, thus reducing porosity and overall productivity.

After the nutrient solution has been added to the bin so that all of the substrates are covered, the substrates are flipped and moved around. Once they are all saturated, the

substrates can then be mechanically harvested. One substrate at a time is removed from the bin and pressed through a simple mechanical roller press. The bin containing the remaining substrates is placed under the roller press such that all of the algae harvested from the substrate drains into that bin. The substrates are then all pressed one at a time until all ten have been pressed. The ten substrates are then placed back in the bin to again be saturated with the nutrient solution, which is now dense with algae biomass. The substrates are again one by one rolled through the mechanical roller press and the effluent is allowed to drain into the bin. The substrates are all then rolled through the mechanical roller press a third time without re-saturating them in the solution so that the rest of the solution within the substrates can be removed. The substrates are then placed back on the scaffolding system and loaded back into the growth chamber while making sure that the irrigation drippers are lined up with each substrate. This process leaves a bin of dense algae effluent behind and the next step is to determine the volume of this effluent so that the overall productivity can be determined. It must be noted that the harvesting procedure will combine the algae from multiple substrates and that any contamination of a single substrate will be spread to all ten growth substrates through this process. This process has not been extensively optimized and can be further improved to reduce overall harvest volume while increasing the harvested biomass concentration.

Inoculating Substrates

Currently, there are two separate procedures for inoculating substrates and an inoculation procedure has not yet been determined that is ideal for all algae species. However, both processes require the use of a PBR. The desired algae is grown to a high concentration of 1 g L^{-1} in a PBR (e.g., Erlenmeyer flask, beaker) using that algae's ideal nutrient solution. In the first method, this inoculant solution is then placed in a bin and the

substrates are soaked in the solution. After the substrates are all saturated, they are then pressed through the mechanical roller press twice to make sure that the algae have infiltrated the substrates before placing them on the scaffolding system. The second method is to utilize an inoculant PBR that has a large head space. Instead of saturating the substrates and pressing them through a roller press, the substrates are placed within the PBR. As the algae concentration within the photobioreactor grows, the algae infiltrate the substrates. Once the algae concentration in the PBR has peaked, the substrates can then be removed and hung from the scaffolding system. Both approaches have been tried, but it has not yet been concluded which one is more successful in producing a dense algae biofilms more quickly and inexpensively.

Cleaning Tubing

The flow rate for the small-scale substrate system was very low (5.76 L d^{-1} for ten substrates) and peristaltic pumps were used to control this growth rate. While these pumps and tubing can provide a very accurate volume of nutrient solution, the low flow rate and nature of the pump require that the tubing utilized for this application had a very small diameter (1.59 mm). If a larger diameter tube is utilized, the pressure in the tubing will be low and substrates will receive varying quantities of nutrient solution depending on their orientation and length of tube. This can create a problem because a small diameter tube is vulnerable to clogging. Though it is desired to keep the nutrient solution sterile, bacteria and contaminants will certainly get into the nutrient solution and they will tend to grow in the tubing, which is translucent and full of nutrients. Non-translucent tubing could reduce this growth, but would make it challenging to determine where in an irrigation line a clog forms. Additionally, the peristaltic tubing does break down over time because of the nature of the pumping head and pieces of the tubing can clog the small tubing. The reason for this

accelerated tubing breakdown is that as the tubing begins to clog with algae and other biological aggregates, the effective cross-sectional area of the tubing is decreased and the pressure within the tubing increases. This increase in pressure causes the tubing to break down prematurely in the pumping head and for small pieces of the tubing can further exacerbate this problem. Eventually, the tubing will become so clogged that the increase in pressure in the tubing causes the tubing in the pumping head to rupture or for one of the connections in the tubing to fail. This cessation in pumping will lead to substrates becoming dry and algae rapidly becoming non-viable if not quickly rectified.

Cleaning Irrigation Tubing

Because the tubing has a tendency to clog, no tubing segment should be longer than a few feet. Segments should be attached to one another by connections so that they can easily be separated and cleaned. To clean out the tubing for shorter sections, a 1.59 mm piece of wire can be used to push out any clogs. For longer pieces, a 40 ml syringe can be placed against the tubing and pressed to force any clogs out with air pressure. Following removal of clogs, a syringe should be used to squirt water through the tube to remove any additional material. Another problem is that clogs occur at the irrigation drippers. When harvesting the algae and working on the system, great care should be taken to not let the substrate contact the irrigation tubing. If these two surfaces touch one another, the algae will begin to grow inside of the irrigation drippers. This will cause the irrigation rate to become irregular. To fix this problem, the irrigation tubing can also be removed and cleaned. However, when cleaning the tubing it should be noted that ceasing the pumping of nutrient solution for a an extended period of time will lead to a proliferation of biological aggregates in the tubing and further problems with clogging as these aggregates then dry and harden. If there are multiple clogs in the system the whole system should be taken apart

and cleaned of clogs and then submerged in bleach and rinsed before reinstalling. This same procedure should be followed when switching a growth system from one species to another to avoid cross contamination.

Appendix 2 – Production and Nutrient Analysis

NO_3^- Determination (AL-G Technologies Inc. Protocol)

This protocol was originally designed by AL-G Technologies Inc. and was amended it for this work.

In evaluating the performance and characteristics of the ACS described here, it is crucial to have a real-time method for determining biomass productivity. The average elemental composition of an algae species can be measured and the relationship between a single element (e.g., nitrogen) and overall biomass can therefore be determined. For example, in the case of algae species that utilize only nitrate as their nitrogen source, the relationship between nitrogen utilization and biomass production can be determined. Therefore, a real-time methodology for measuring nitrate in solution can be used to determine the productivity of algae on the substrates. The method described below was developed by AL-G Technologies, Inc. to measure the concentration of nitrate in nutrient solution for experiments where real-time productivity data was required for species that only used nitrate as their nitrogen sources.

Reagents

- Stock solution of NO_3^- at 100 mg/L (KNO_3 at 722 mg/L). Prepare with distilled DI water.
- Solutions for NO_3 standard curve (these can be kept in a refrigerator for several months): 8.0 mg L^{-1} NO_3^{-1}, 4.0 mg L^{-1} NO_3^{-1}, 2.0 mg L^{-1} NO_3^{-1}, 1.0 mg L^{-1} NO_3^{-1}, 0.5 mg L^{-1} NO_3^{-1}, 0.25 mg L^{-1} NO_3^{-1} and 0.0 mg L^{-1} NO_3^{-1}.

Alternatively, one can just prepare 50 mL of the 8.0 mg/L NO_3^{-1} and do serial dilutions of 25 mL into 25 mL in Falcon tubes (50 mL) to get these 2x dilutions.

Standard Curve

This method used a spectrophotometer to measure the amount of nitrate in a sample. The first step in using this method is to create a standard curve using the reagents with known concentrations that were prepared previously. Prior to developing the standard curve, the spectrophotometer needed to be turned on for at least 30 minutes so that its bulb could warm up. Additionally, all readings using the spectrophotometer needed to be taken at the same temperature. After zeroing the instrument with DI water, 1 mL of each standard curve solution was transferred into a 1 cm long light path crystal cuvette (compatible for UV) and the absorbance at 220 nm was determined. The crystal cuvette is essential for this assay as the traditional polystyrene cuvettes will not work effectively at this wavelength. Once the standard curve was created, three readings of the cuvette filled with nutrient solution were taken by removing and replacing the cuvette in the spectrophotometer. This is necessary as the cuvette can slightly move in the cuvette holder and you want to make sure it is always in the same position. At the start and finish of all readings, a measurement of the blank (0.0 mg L^{-1} $N\text{-}NO_3^-$) should be taken. This measurement assures that the spectrophotometer did not drift during the readings. The standard curve should be prepared every time the spectrophotometer is used and if the standard curve differs from the previous standard curve the stock solutions should be replaced.

Preparation and reading of the samples

- The samples were all filtered from the ACS harvest solutions with a luer-lock 0.45 μm filter mounted on an appropriate syringe. The filter was used to prevent any non-microscopic matter (e.g. substrate fibers) from contaminating the sample.

- The proper dilution factor was determined that is appropriate in order to obtain values of absorbance that are within the range of the standard curve. The expected NO_3^- concentration needs to be within the standard curve range that has been created.
- Three measurements needed to be taken for each sample by removing and replacing the cuvette within the sample slot.
- The readings were always started and finished by measuring a blank sample (confirming the absence of drift for the spectrophotometer).

Phenol Sulfuric Acid Assay for Polysaccharides

This method mentioned in Chapter 1 was based on the methods described in the papers listed below:

1. Nielsen, S. Suzanne. "Phenol-sulfuric acid method for total carbohydrates." *Food Analysis Laboratory Manual*. Springer US, 2010. 47-53.
2. Albalasmeh, A. A., Berhe, A. A., & Ghezzehei, T. A. (2013). A new method for rapid determination of carbohydrate and total carbon concentrations using UV spectrophotometry. *Carbohydrate Polymers*, *97*(2), 253-261.

For the determination of polysaccharides in the effluent solution, a rapid sulfuric acid-UV method was employed. This method was based upon the reaction of carbohydrates with concentrated sulfuric acid in which furfural derivatives were produced. Further reaction with the phenol in the solution creates the color that enables a quantification of the carbohydrates. To use this method, an effluent solution of 2 mL from the ACS was filtered through a 0.45 µm leur-lock filter. This 2 mL volume of solution was then mixed with a 1 mL solution of 5% aqueous phenol. This solution was then immediately mixed

with 5 mL of concentrated sulfuric acid in a test tube. As a note, be careful once the sulfuric acid and phenol solution are mixed together as the temperature within the mixture will rapidly rise. This test tube is left to sit for ten minutes and then the test tube was vortexed for 30 seconds and then placed for 20 minutes in a water bath at room temperature to allow for the color to form for the spectrophotometric test. The test tube is then poured into a quartz cuvette and the absorption is measured on a spectrophotometer at 490 nm. Much care needed to be given to not spill the solution on or within the spectrophotometer as it will damage the device's plastic internal components. The phenol solution used for this experiment needed to be prepared with DI water immediately prior to the start of experimentation. In a similar fashion to the nitrate test (described above), a standard curve needed to be constructed to determine the concentration of polysaccharides in a sample. For the standard curve utilized for this analysis, seven concentrations of glucose were utilized within the expected polysaccharide range to develop the standard curve.

Appendix 3 – Greenhouse Model

Greenhouse Model

The third chapter of this dissertation focused on the economic analysis of the attached growth system for five separate locations and was used to draw conclusions about which products could be produced on the system in a financially viable manner. This ultimately led to the conclusion that the focus of further developments should be on the optimization of marine algae species specifically for the production of fishery feed followed by the production of β-carotene due to its high commercial value. However, Chapter 3 only focused on evaluating this system under a specific set of conditions wherein changing the conditions can substantially impact these conclusions. Therefore, an easy-to-use and readily adaptable model (spreadsheet) was developed to allow for the changing of

system parameters to evaluate how they affect the financial viability and productivity of the system. This model was developed in Microsoft Excel® such that it can be easily adapted and optimized based on a researcher's needs. However, before the model is described in detail, it is important to understand how it is designed and what specifically it is modeling.

The greenhouse model "AL-G Technologies, Inc. System Model" was designed to model the productivity and financial viability of a 10,000 m^2 qounset greenhouse ACS facility as described in Chapter 3. The model has been designed so that the major properties of the system can be altered as per the users' needs. As described in Chapter 3, the main drivers of productivity for the system are the energy flux of the greenhouse and the growth curve of the algae. The energy flux of the system is based on the input parameters and it is assumed that the algae on the system have an optimal productivity at 30^{o}C. The purpose of the model is to depict the cost of producing algae slurry, but the cost of dewatering and subsequent processing is not included in the model predictions.

Underlying Assumptions

- The number of greenhouse heaters required was based on the 99% design temperatures for the five locations as defined by the Virginia Plumbing Code Appendix D (2006). All five locations have a different number of heaters that are required and this is the major variable between the locations.
- The algae on the system will grow similarly to *Chlorella* with regards to temperature and daily light integral with an optimum growth rate at 30^{o}C and a 40 mol m^{-2} d^{-1} daily light integral.

- The polyethylene film covering the greenhouse will need to be replaced every three years as it will be damaged by the wind and UV radiation over time. Other materials chosen in the model (e.g., glass) would not need to be replaced.
- The humidity within the greenhouse will be maintained at 80%. This humidity level was selected because of the large surface area of moist substrates per square meter of floor space. Water evaporating from the system as a result of air movement will not be collected by way of condensers.
- The system is operational for ten years and then needs to be completely replaced.
- The CAPEXs are paid for through a ten-year loan that has a user-selectable annual interest rate.
- There is a curtain wall comprised of concrete blocks or another material (e.g., polycarbonate) along the perimeter of the greenhouse that is 1/3 the height to the gutter.
- Two employees (e.g. Manager, Lab Technician) are capable of operating a 10,000 m^2 greenhouse system.
- Accessory buildings (e.g., offices, bathrooms, break room) are not included in the model.
- The OPEXs increase each year at a fixed rate of inflation (2.1% yr^{-1}).
- The model uses average hourly outdoor temperatures and light intensities for heating calculations and daily average outdoor temperatures and light integrals for productivity calculations.
- When the system is increased in size from its 10,000 m^2 preset value it is assumed that the system scales linearly (i.e., no bulk discounts) such that 2 employees (e.g.,

Manager, Lab Technician) are required for every 10,000 m^2, and similarly for the number of heaters, pumps, and the cost of installation.

- The electrical costs for the five locations are based on the US Energy Information Administration 2017 report and can only be changed through changing the values in the red "Electricity" tab.
- The ventilation rate will vary between the maximum and minimum levels that are presented in the model and the greenhouses infiltration rate is fixed at 2 exchanges per hour.

Usage

The model has two major blocks on the tab "Input and Output", which are where the user will interact primarily with the program. The first block is the input block where the user can enter all of the parameters that control the output of the model. The default values for the system are the values that were discussed in Chapter 3 and are defined on the red "reset" tab. The user has the ability to change any of the values in the blue boxes in the input form. Once the user begins to change values (**Figure 16**), they will notice that the output box (**Figure 17**) will begin to change.

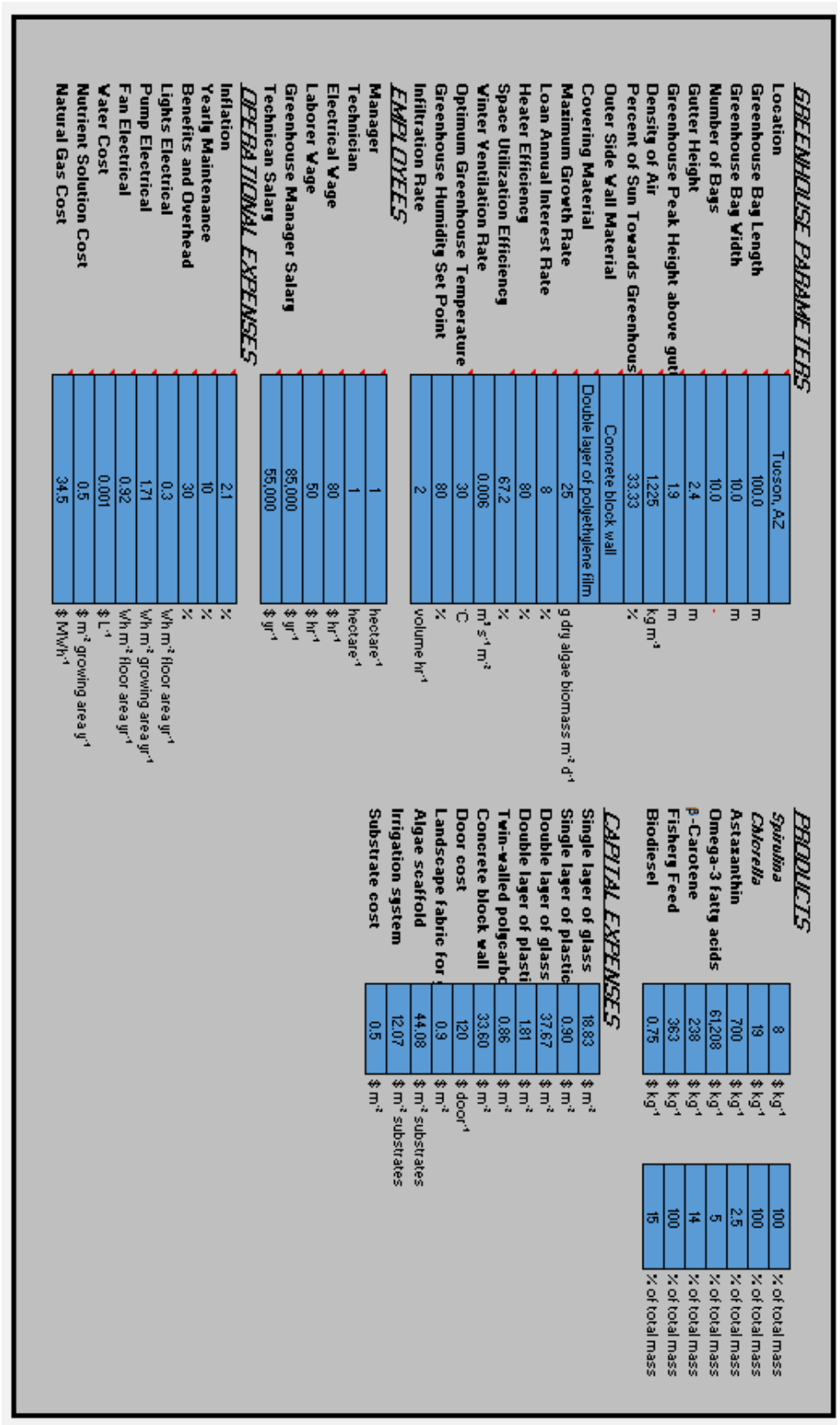

Figure 16. Visual representation of the input page generated by the Excel model.

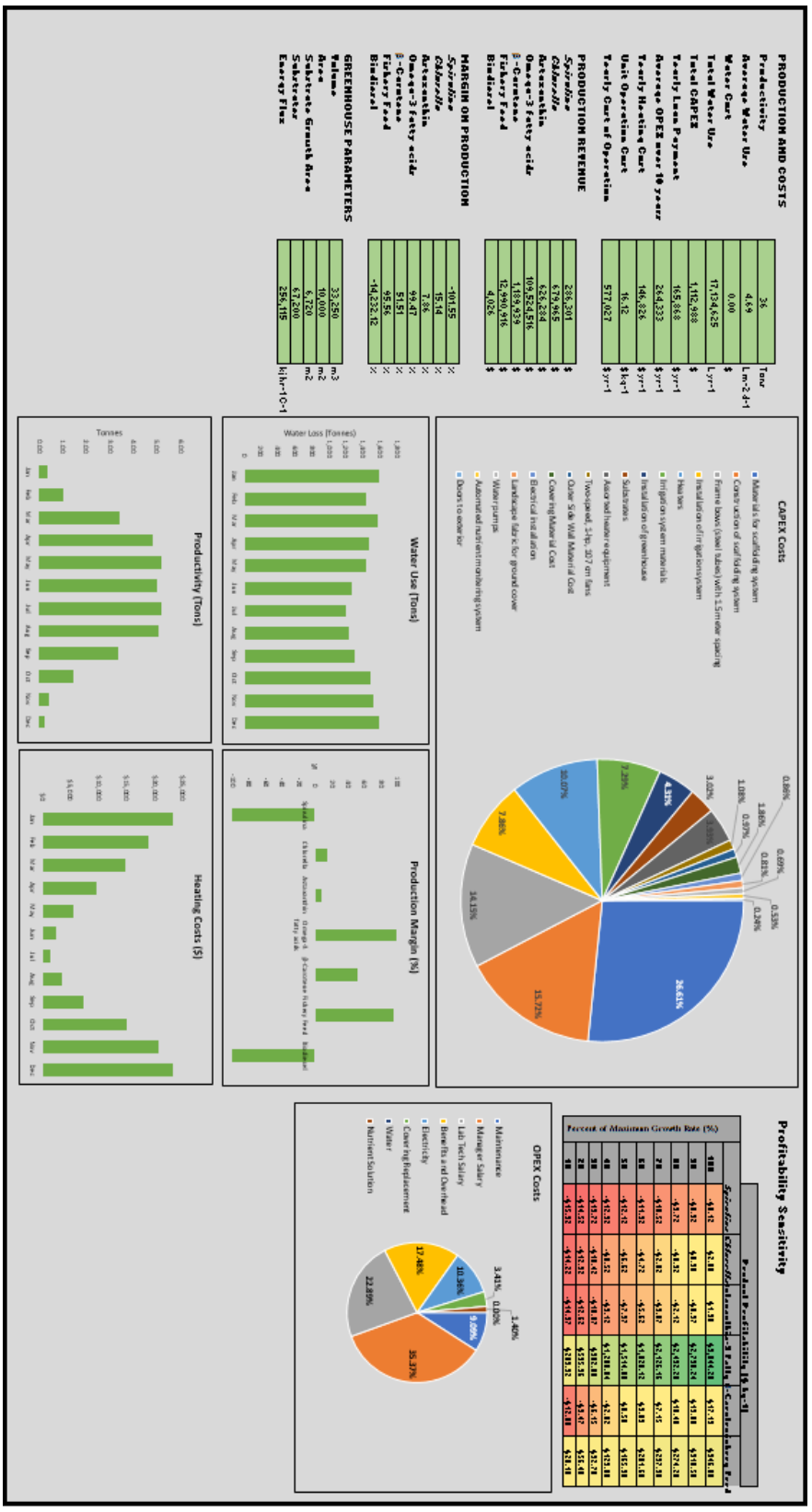

Figure 17. Visual representation of the output page generated by the Excel model.

The output page will automatically update and the user can visualize the specific outputs for the system including product profitability, water usage, and heating costs. The red tabs are used for the calculations and can also be changes as needed.

How the Model Works

The model uses the equations and methods described in Chapter 3 to calculate the productivity of the system and its other operational parameters (e.g. water loss). The financial numbers are driven primarily by the CAPEX costs of the system and the OPEX costs to run the system over its ten-year life cycle. The CAPEX costs are variable based on location and the number of heaters that are purchased. These CAPEX costs are paid for through a loan whose payment is calculated through the "loan" tab in the model. This loan tab uses the "solver" function in Microsoft Excel® to determine the annual payment on the loan using the "calculate" button to initiate a macro.

How to Use the Model

The purpose of this model is to allow future researchers to determine the economic impact of research on the system and in what direction to go with future research. For example, a researcher can now determine which species of algae should be cultivated on the system based on the theoretical productivities and the market price of the commercial co-products. Additionally, the overall economic impact of specific improvements can be quantified and decisions about commercially implementing the system can be thoroughly evaluated.

Sensitivity Analysis

In working to optimize the system, a sensitivity analysis was performed to evaluate the impact of improving specific parameters on the overall financial viability. Several key

parameters were evaluated independently to determine where efforts should be focused to improve the system through evaluating the relationship between changes in a parameter and the financial viability of the system. Using the model, it is shown that there are a few key parameters that drive the financial viability of the system (**Table 8**) and that the system is substantially more sensitive to certain factors (e.g. growth rate, electricity) than it is to others (e.g. interest rate, inflation).

Table 8. Individual impact of percentage improvements to system parameters and their effect on system production cost.

	Change in Production Cost ($ kg^{-1})						
Improvement (%)	**Water Cost**	**Natural Gas Cost**	**Electricity Cost**	**Interest Rate**	**Laborer Hourly Wage**	**Growth Rate**	**Inflation**
20	3.76%	2.95%	4.67%	1.13%	1.02%	16.70%	0.97%
40	7.47%	5.85%	9.29%	2.26%	2.04%	28.57%	1.93%
60	11.22%	8.75%	13.96%	3.38%	3.06%	37.49%	2.90%
80	14.93%	11.65%	18.64%	4.51%	4.08%	44.47%	3.87%

The most important factor that drives viability is the growth rate (**Figure 18**) of algae on the system as it directly defines the relationship between the systems parameters (e.g., temperature, daily light integral) and production margins. Growth rate is followed in importance by electricity and water costs for the system. However, through optimizing several parameters in parallel (**Figure 18**) it is possible that the overall viability of the system can be improved dramatically as shown by the cumulative improvement line (**Figure 18**) that shows the combination of all improvements.

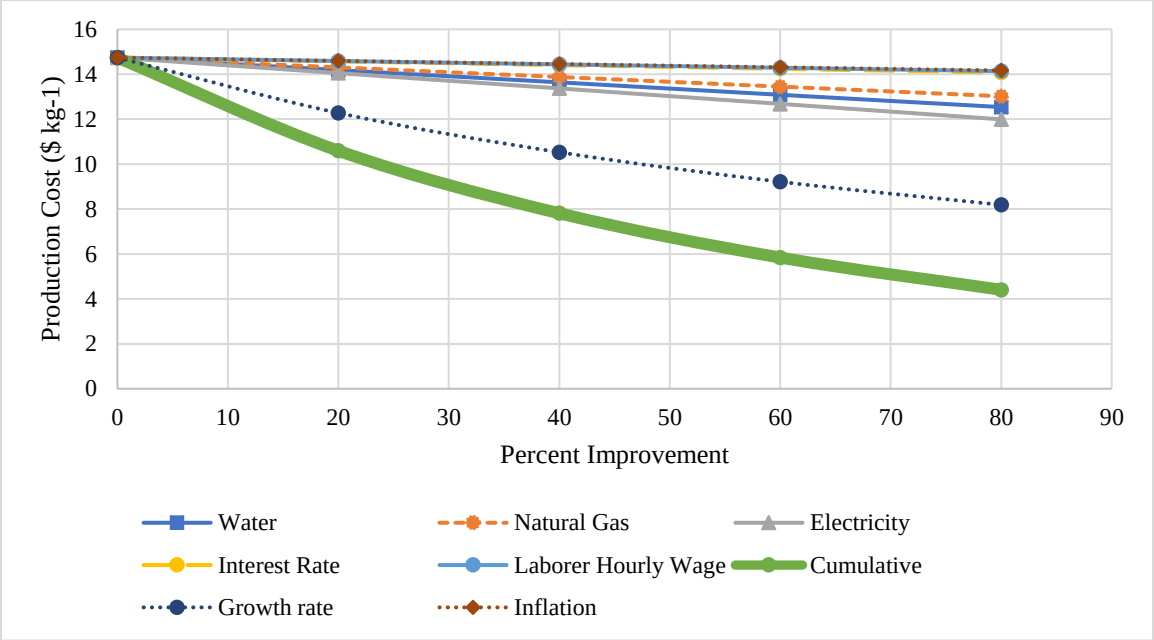

Figure 18. Sensitivity analysis showing the effect of changing several key inputs on the financial viability of the system (production cost). The figure shows the impact of improving each parameter by a specific percentage from its original value as previously described in Chapter 3. This improvement is either an increase in the case of growth rate or a decrease in the case of electricity costs. The impact of these combined factors is shown in a bold dashed line titled "Cumulative."

Equations and Model Usage

The equations that were used to construct this model are presented in the red tabs in the AL-G Technologies Inc System Model that can be requested from Michael Johnson at m.johnson.36.80@gmail.com. This model is open access and all of the equations and input values for the model can be altered by the user so that the model can be updated over time based upon changes in any of the model's parameters (e.g., growth rates, temperature). The equations that support this model leverage the equations and insights that have been detailed throughout this manuscript.