DEVELOPING OPTIMAL GROWTH PARAMETERS FOR THE GREEN MICROALGAE *NANNOCHLORIS OCULATA* AND THE DIATOM *NITZSCHIA SP.* FOR LARGE SCALE RACEWAY PRODUCTON

A Thesis

by

PHILLIP RYAN LUEDECKE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2011

Major Subject: Biological and Agricultural Engineering

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Approved by:

Chair of Committee,	Ronald E. Lacey
Committee Members,	Raghupathy Karthikeyan
	Antonietta Quigg
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ABSTRACT

Developing Optimal Growth Parameters for the Green Microalgae *Nannochloris oculata* and the Diatom *Nitzschia sp*. for Large Scale Raceway Production. (August 2011)

Phillip Ryan Luedecke, B.A., Texas A&M University

Chair of Advisory Committee: Dr. Ronald E. Lacey

Microalgae produce large quantities of lipids that can be used for biofuel feedstock. The goal of this project was to determine the effect of several engineering and management parameters on the productivity of microalgae cultivated in large, outdoor facilities. The specific objectives were focused on the effects of inoculation ratios; the effects of light, temperature, and culture depth on growth; and the minimum circulation velocity necessary to maintain growth and minimize settling in open ponds.

Microalgae must first be cultured in smaller quantities before the raceway is inoculated for optimized growth. Concentration ratios are defined as the ratio of the volume of microalgae inoculum to the volume of new growth media. The microalgae species used was *Nannochloris oculata* (UTEX #LB 1998). Inoculation ratios studied varied from 1:1 to 1:32 and were grown in 500 mL Erlenmeyer flasks. The study found that 1:16 and 1:32 were too dilute, while the 1:8 concentration had the largest growth rate.

Determination of the effects of temperature, light intensity, and cultivation depth is critical to maintaining healthy cultures. Excess light intensity can result in

photoinhibition and temperatures above the maximum growing tolerance can have detrimental effects. These factors can affect growth and evidence suggests an interaction that exacerbates these effects. In an outdoor culture there are few practical control variables other than pond depth. As cultivation depth increases, the algae undergo "self-shading" and the increased cultivation volume hinders temperature changes. Scaled raceway ponds were maintained at 10.16 cm (4 inch) and 13.97 cm (5.5 inch) depths. The species used was *Nannochloris oculata* and it was found to grow best at 785 μmol m^{-2} s^{-1}, 20°C, and 10.16 cm.

Diatoms are attractive because of high growth rates, faster lipid production, and greater cell density. The latter promotes rapid settling once mixing has stopped. Because of the silica cell wall composition, diatoms are believed to be more susceptible to shear forces which can result in lysis. Determining the natural settling rate to the minimum channel velocity relationship in cultivation ponds was the objective. No flocculants/coagulants were added which created a case of "natural" settling. Four pennate *Nitzschia sp.* and one centric diatom were tested in a jar tester. There was no significant difference in settling times between the species. The mean settling time was 4.55 cm min^{-1} and the minimum channel velocity was determined to be 10.12 cm min^{-1}.

DEDICATION

To

My Parents

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Ronald E. Lacey, for providing the guidance and support throughout this process, as well as being there to answer any questions I might have had and to provide the means to complete this research. I would also like to thank my committee members, Dr. Karthikeyan and Dr. Quigg, for their guidance and support during the course of this research.

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NOMENCLATURE

AFDW	Ash Free Dry Weight
ANOVA	Analysis of Variance Test
DO	Dissolved Oxygen
DW	Dry Weight
LS Means	Least Square Means
OD	Optical Density
PAR	Photosynthetically Active Radiation
PPM	Parts per Million
PSU	Practical Salinity Units
RPM	Revolutions per Minute
UTEX	The Culture Collection of Algae at The University of Texas at Austin
WT4	Weight 4
WT5	Weight 5

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CHAPTER I

INTRODUCTION

This microalgae research was divided into three different sections that relate to how both green microalgae and diatoms grow and behave under different conditions and how to overcome these effects for determining ideal growing conditions. The first part of this research began with determining microalgae inoculation ratios for biofuel production for raceway ponds. The second section pertains to the interaction effects of solar irradiance [light], temperature and pond depth, while the last section focused on the natural settling of diatoms and how this natural settling relates to the mixing velocity needed in a microalgae production raceway pond.

Microalgae have been known to produce large quantities of oil, from approximately 16 to 75 percent oil dry weight depending on algae species (Chisti, 2007). This oil can be used to produce biofuels in an effort to reduce America's dependence on foreign oil. Large scale microalgae production in outdoor raceway ponds for the purpose of creating biofuels is a relatively new process which found light in the Department of Energy's Aquatic Species Program: Biodiesel from Algae from 1978 to 1996 (Sheehan et al., 1998). Currently there is little published information on what the optimal beginning concentration ratios of algae and growth media should be. As the production scale increases, the microalgae must first be cultured in smaller quantities and then the raceway must be inoculated or seeded with the correct amount of microalgae for

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This thesis follows the style of *Transactions of the ASABE*.

optimized growth. The microalgae cannot simply be placed into a raceway pond and expected to grow as desired. Concentrations too dilute will result in solar bleaching, contamination, nutrient toxicity, and increased time for the microalgae to reach a harvest concentration.

The inoculation rate of cultured microalgae into the raceway pond depends on the size of the raceway. A 0.50 ha raceway would require more inoculant per volume than a 0.10 ha raceway pond. The objective of this study was to determine an optimal concentration ratio for raceway ponds such that the maximum and the utilization of resources are obtained.

Growing microalgae in open raceway ponds depends on the local environmental conditions. Determination of the effects of temperature, light intensity, and cultivation depth is critical to maintaining healthy cultures under the weather extremes typically seen in the desert southwest U.S.A. It is important to have algae strains that are suited to the climate or develop a control process in order to mitigate the effect of the extreme changes in the environment the microalgae may experience. Excess light intensity can result in photoinhibition or even cause damage to the algal cells. Likewise, temperatures above the maximum growing tolerance can have detrimental effects on the growth of microalgae as well. Both of these factors can yield lower than expected growth or kill the culture in the raceway pond. While it is known that each of these factors can affect algal growth there has been some anecdotal evidence that there is an interaction effect between temperature and light intensity that exacerbates the effects. In an outdoor culture there are a few practical control variables but the one most often employed is

pond depth. As the cultivation pond depth is increased, the algae provide “self-shading” and the increased thermal mass dampens temperature changes in the growth media. The objective of this study was to determine if there was an interaction effect on algal growth between light intensity and temperature and if increasing the depth of the culture would mitigate these effects.

There are many types of microalgae including green algae, cyanobacteria, and diatoms. Diatoms have attributes that make them attractive as a biofuel source including high growth rates, faster production of lipids, and greater particle density, which may assist in harvesting. The increased density also presents a challenge, as diatoms tend to settle out of suspension rapidly once mixing has stopped. Because of the silica cell structure, diatoms are also more susceptible to shear forces and excess mixing can result in cell damage and lysis. Natural settling is important since no flocculants or energy consuming machinery (e.g. centrifuge or dissolved air flotation) would be needed to separate the diatoms from the media. Adding flocculants, coagulants, and other means are costly and time consuming and if the diatoms will naturally settle, this leads to input costs being reduced. This study focuses on the determination of the natural settling rate of diatoms and the relationship to minimum channel velocity in cultivation ponds.

Objectives

The overall objective was to improve the efficiency of producing microalgae on a large scale by improving the growth parameters and minimizing the energy and material inputs. Specifically, the objectives of this research were:

1. Find the optimal inoculation concentration ratio(s) that reduce the amount of culturing time so more time and resources can be devoted to the final production ponds.
2. Determine the effects of light intensity, temperature and pond depth on algae growth and determine if there is an interaction effect between these factors.
3. Determine the rate and overall effectiveness of natural settling for a diatom species and estimate the minimum circulation velocity required for an open raceway pond.

CHAPTER II

LITERATURE REVIEW

Tedesco and Duerr (1989) performed an experiment with a 25-fold dilution (1:25 ratio) using *Spirulina platensis* UTEX 1928 in a 200 mL culture vessel. They varied the irradiance from 170 to 1400 μmol photons m^{-2} s^{-1} and the temperature from 25 to 38°C. The tests where run either testing the temperature or light separately for a predetermined period using a nitrogen rich media. After the growing time was reached, the cells were washed with nitrogen deficient media and recultured with the nitrogen deficient media. The algae was then placed in standard growing conditions at 37 ± 0.5°C with continuous light at 600 ± 33 μmol photons m^{-2} s^{-1} of light. The goal of this study was to test what conditions are needed to improve the fatty acid content and composition of the microalgae. The high light irradiance and temperatures are comparable with those in the desert southwest U.S.A. It was found that light intensity affected the growth rate and total lipid content but had little effect on the fatty acid content. The total lipid as percent dry weight decreased as light intensity increased, except at the highest irradiance and there was no change in total fatty acid content. At the highest irradiance tested, there was a slight increase in total lipids noted. Tedesco and Duerr (1989) suggest not to shade outdoor mass cultures because of this result in order to obtain the highest possible fatty acid percentage. If increasing the lipid content of the microalgae is goal, this could be the way to achieve this. They reported that above 33°C (standard light conditions), there was a sharp decrease in cell growth as well as the fatty acids and percent of total lipids.

However, 33°C showed the most rapid growth rate for *Spirulina platensis*. It was also reported that temperatures over 39°C resulted in cells bleaching white within 48 h. Since there was no test on the temperature and irradiance together, it would be reasonable to believe that outdoor cultures would have to be shaded if grown in areas where the irradiance and temperature exceed the high limits tested here.

Each strain of microalgae has a specific set of growing conditions that will yield the most biomass. Cho et al. (2007) did a study on the optimum temperature and salinity conditions for growth of green algae *Chlorella elliposidea* and *Nannochloris oculata*. They found that the optimal temperature for *Nannochloris oculata* was 25°C for growth and it occurred at two different salinities, 10 and 30 [PSU]. Since there are two salinities that yield comparable results, it is possible that salinity is not as important as the temperature for *Nannochloris oculata*. Cho et al. (2007) found a maximum density of *Nannochloris oculata* at high temperature (25 and 30°C) rather than low temperature (15 and 20°C) at all salinities tested in this study indicate that high temperature is better for achieving the maximum density of *Nannochloris oculata.*

Latala et al. (1991) performed a similar study on growing conditions on green [micro] algae. They tested nine different species and looked at the influence of salinity, temperature and light. The species tested covered a wide range of conditions but their results confirmed several statements on algal cell behavior. First, was that decreasing the salinity negatively affects cell behavior. It significantly decreases the metabolic rate and consequently cell volume. This was confirmed in six species by a cell volume decrease of 30% and with a 64% cell volume decrease in *Scenedesmus armatus*. Abu-Rezq et al.

(1999) also did a study where salinity was a factor being tested on growing conditions. This test was to determine an ideal growing salinity range. The salinities found in the Latala et al. (1991) study and by Abu-Rezq et al. (1999) were different because they were looking at different strains; which proves that each strain has its own optimum conditions. Secondly it was found that at low temperatures, all biochemical processes proceed at a slow rate, inhibiting metabolic activity and growth. High temperatures could result in enzyme inactivation and soluble protein coagulation. The tests of Latala et al. (1991) confirmed the inverse relationship between cell size and temperature. As temperature increases, the cell size decreases. Latala et al. (1991) reported *Scenedesmus acuminatus* had the largest cell volume change due to temperature at 42%. Finally, up to a certain light intensity algal biomass increased. However, once beyond that value, cell division decreased and eventually ceased as a result of light saturation of growth or photoinhibition. Their study was comparable with previously published irradiance values for *Chlorella vulgaris*. Also, this study confirmed the relationship that the size of algal cells increases with increasing light intensity. The highest correlation that Latala et al. (1991) obtained was 35% with *Oocystis parva.* These relationships are very important and can be applied to other algal strains. There are various other works that pertain to determining optimum growing conditions of algae. These works include but are not limited to Brand and Guillard (1981), Wetz et al. (2004), and Tadros and Johansen (1988). Both the Wetz et al. (2004) study and the Tadros and Johansen (1988) study use diatoms instead of green algae.

The inoculation ratio, the ratio of the volume of inoculum to volume of fresh media, is critical in designing algal growth systems and determining the numbers and sizes of ponds and reactors needed in the inoculum chain. A large inoculation ratio (e.g. 10:1) will result in more resident time in each pond in the inoculum chain while reaching a concentration to be able to inoculate the next pond in the chain. Generally this is desired since it requires fewer ponds and increases the utilization efficiency for each pond in the chain. However, a large inoculation ratio also has the potential for solar bleaching and contamination, particularly under open, outside conditions. Another important consideration is nutrient toxicity. Very dilute concentrations of algae subjected to normal media levels can be poisoned by the nutrients just like terrestrial plants can be with too much fertilizer. Therefore, there is a need to balance the inoculation ratio against the effects of light and temperature to maintain growth. Depth is a factor since it is one means of mitigating solar intensity is to use deeper ponds and attenuate the light.

Tedesco and Duerr (1989) performed their experiment inoculating 200 mL culturing vessels. They also did not report any problems with the 1:25 ratio being too dilute at the 1400 μmol photons m^{-2} s^{-1}. The Cho et al. (2007) study that was performed with *Nannochloris oculata* used a different method for cell concentration. Cho et al. (2007) used a hemacytometer to count cells for that inoculation. A cell concentration of 100 x 10^4 cells/mL was used to inoculate 250 mL flasks for their study. A light intensity of only 31μmol photons m^{-2} s^{-1} was used in that study.

Nitzschia sp. diatoms are among the most abundant diatoms in the ocean and large naturally occurring mass flocculation of diatom blooms have been recorded settling

to the ocean floor (Alldredge and Gotschalk, 1989). Diatoms contain a heavy silica shell that acts as the cell wall in place of the cellulosic cell wall of green algae. According to Ramachandra et al. (2009), "The specific weight of a diatom test [silica shell] has been reported to be 2.07…the specific weight of seawater usually ranges from 1.020 to 1.028. It is evident that diatoms are heavier than seawater and will ordinarily sink rapidly…" As a diatom sinks it flocculates with other diatoms and the settling rate increases exponentially as the floc diameter increases (Alldredge and Gotschalk, 1989). Alldredge and Gotschalk (1989) also follow the premise that a certain amount of cell collision must occur for the cells to collide together to start forming chains. However, excess mixing can cause cell damage and cell lysis (Michels et al., 2010). The silica shell is fragile which limits the stress it can endure. Shear stress from pumping and rotational mixing can break the cell, resulting in cell death and non-viable cultures.

Diatom health is another important factor related to settling. Eppley et al. (1967) reports that after experimentation, cells from non-growing cultures sink at about four times faster than cells from growing cultures. This would indicate that there is metabolic function that viable cells have to help keep themselves in suspension or neutrally buoyant. Waite et al. (1992) created an experiment to test this theory. They proposed that if an intracellular, energy-requiring ionic pump governs settling rates, then sinking rates should be predictable by the amount of energy available to the cell via photosynthesis and respiration. They found that the lowest settling rates occurred when cells were grown under saturating light and the highest settling rates occurred when cells were placed in the dark or treated with a respiratory inhibitor.

Eppley et al. (1967) and Waite et al. (1992) differ on the settling rate relation to cell size. Eppley et al. (1967) found that settling rates appeared to increase with cell diameter. Waite et al. (1992) reported that small cells sank more rapidly than large cells under saturation light, but under severe energy limitation settling rates were directly proportional to cell volume. This could be due the reason that smaller cells are denser since they contain less cytoplasm. A smaller cell also has less surface area so with it being denser and having less surface area than a larger cell, it could sink at a faster rate.

CHAPTER III

MICROALGAE INOCULATION RATIOS FOR BIOFUEL PRODUCTION IN RACEWAY PONDS

Introduction

Large scale microalgae production in outdoor raceway ponds for the purpose of creating biofuels is a relatively new process. Currently there is little published information on the optimal beginning concentration ratios of algae and growth media. This directly affects production time and operating costs. The microalgae cannot simply be placed into a raceway pond and expected to grow as desired. Concentrations too dilute will result in solar bleaching, contamination, nutrient toxicity, and increased time for the microalgae to reach a harvest concentration.

Microalgae must first be grown in small quantities and scaled stepwise to the production raceway. Currently the Texas AgriLIFE Research microalgae research group uses a 1:1 inoculation concentration ratio. This is the ratio of algae inoculum to new growth media. However, this takes a lot of time to culture enough microalgae to inoculate a 0.40 ha raceway pond. The needed 0.20 ha worth of inoculum requires a series of successively increasing ponds and culturing devices. In order to get to production size, the microalgae have to be grown in smaller quantities. Currently a transfer into a larger culturing device or pond is made at a 1:1 concentration ratio when the algae reach a concentration of 1 g L^{-1}, i.e. a 1 L system at 1 g L^{-1} is put into a 2 L system and fresh media is added until the concentration is 0.5 g L^{-1} and 2 L volume is

reached. This process is repeated until the final production pond size is reached. The addition of CO_2 increased the rate of growth of the microalgae since the microalgae utilized the CO_2 similarly to terrestrial plants. A pH outside the optimal growth range can have detrimental effects on the growth of the microalgae and can lead to inconsistencies within the data. Having the pH that high also helps to discourage contamination from other microalgae strains, viruses and bacteria. This process directly affects production time and operating costs. The microalgae cannot simply be placed into a raceway pond and expected to grow as desired. Concentrations too dilute will result in solar bleaching, contamination, nutrient toxicity, and increased time for the microalgae to reach a harvest concentration.

Objective

The objective of this study was to determine an optimal inoculation concentration ratio for raceway ponds such that the maximum algae production rates and the utilization of resources are obtained.

Materials and Methods

A single strain of algae was grown in 500 mL Erlenmeyer flasks at six different concentration ratios. Each concentration ratio was tested in replicate. The inoculation ratios of inoculum to new growth media studied were 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32. Each flask was inoculated with *Nannochloris oculata* (UTEX LB# 1998) in a modified Erdschreiber's growth media. The inoculum was previously grown to the start of the

study. The concentration of the density of the inoculum was determined based on the current weekly transfer cycle. This study was started when the inoculum was ready to be transferred into a new culturing vessel, in this case the testing flasks. It should be noted that a pseudo replicate was performed with this experiment. This means that the inoculum was grown in one culturing vessel and then split among the testing flasks and the testing replicates were started at the same time. There was 24 h of light provided by four 15 W grow lights for a total of 60 W for each system. These provided 65 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) measured with a handheld quantum light meter (Model QMSW, Apogee Instruments, Logan, UT). Ambient air and CO_2 were delivered by aquarium air pumps to provide the CO_2 for each flask as well as the required agitation to keep the microalgae in suspension. The microalgae contained in each flask were immersed in a water bath to maintain the temperature at 27°C. Figure 1 shows the Erlenmeyer flask experimental setup. The experimental setup is the same as used to grow the inoculum. The light and temperature conditions were kept the same for the study as those used to previously grow the inoculums.

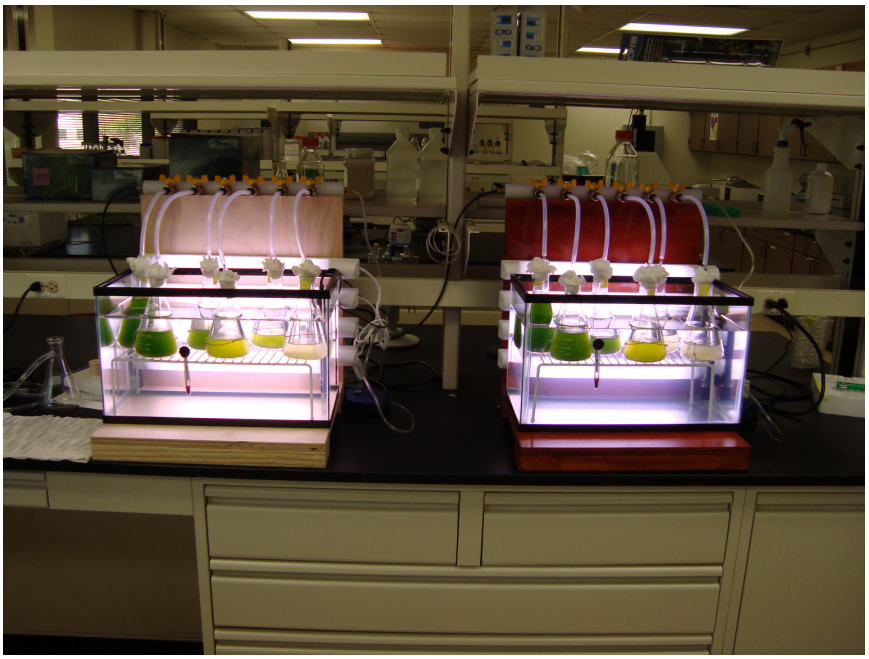

Figure 1: Erlenmeyer flask setup.

In order to determine microalgae concentration, a Spectrophotometer (Model Spectronic 20D^{+}, Milton Roy, USA) was used to measure the optical density (OD) reading at 750 nm. A growth curve of OD versus time was created. This test was performed in accordance with the standard operating procedures used at the Texas AgriLIFE Microalgae Research Facility in Pecos, TX. The procedures used are as follows:

Measurement Methods

For the transmittance measurement by the spectrophotometer, three wavelengths are used. The wavelengths are 745, 750, and 755 nm. For each wavelength, the spectrophotometer must be calibrated and the sample being tested must be diluted into the linear range of the machine. A 10X dilution for *Nannochloris oculata* will be

adequate. After recording the values for each wavelength, take the averaged transmittance value from the three wavelengths and use this value for the transmittance to OD calculation.

The transmittance to OD calculation is next (Stein, 1973, pp. 331-338). The readings provided by the spectrometer cannot be used as is. They must be converted from a transmittance value to an optical density measurement. The conversion used for this study is as follows:

$$O.D. = \log(I_o / I) \quad (1)$$

where,

I = the transmission of the growth media (no microalgae cells) and

I_o = transmission of concentration ratio sample (later adjusted to read 100%.

Results and Discussion

This study originally began with six different concentration ratios being tested in 500 mL flasks: 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32. Figure 2 shows the combined averaged OD values from each inoculation ratio of the flask study.

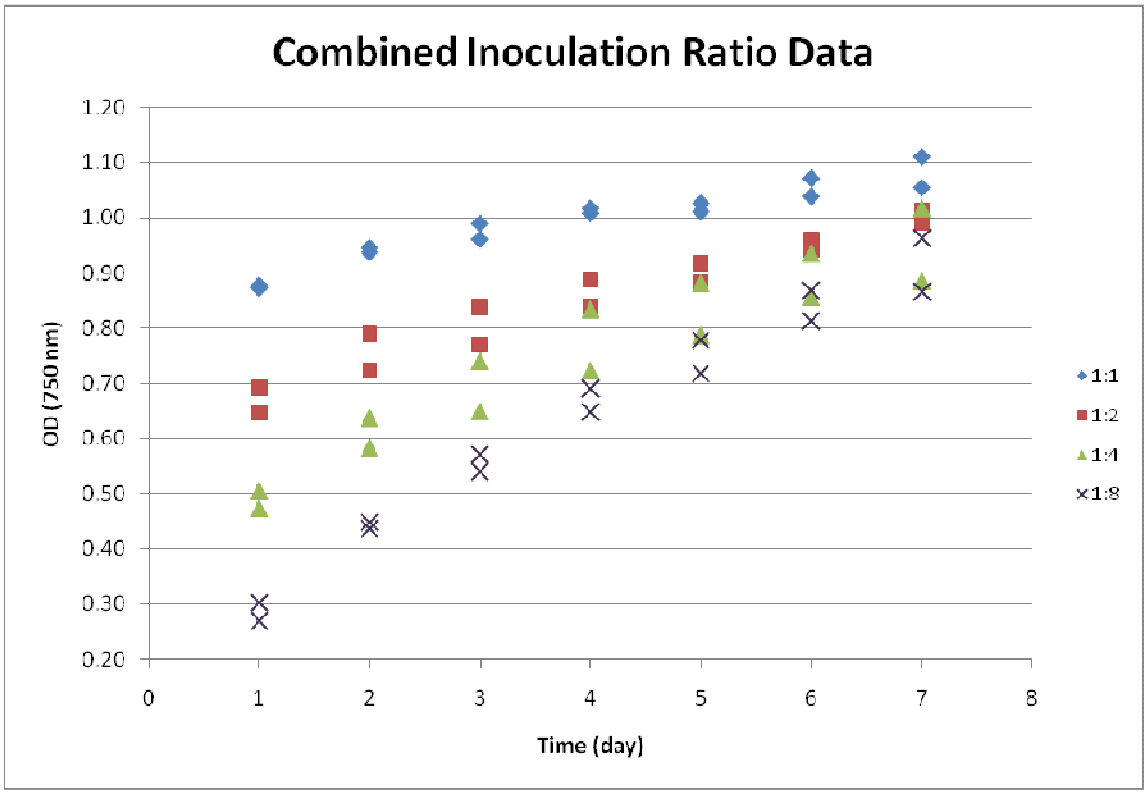

Figure 2: Optical density data from growth study of *N. oculata* in 500 mL flasks.

The 1:16 and the 1:32 concentration ratios did not grow, which can be attributed to the inoculation ratio being too dilute for the light intensity and temperature. It is known from previous work that microalgae will undergo photoinhibition when exposed to high amounts of light irradiation (Vonshak and Guy, 1988; Jensen and Knutsen, 1993). According to Chisti (2007), "Above a certain value of light intensity, a further increase in light level actually reduces the biomass growth rate in a phenomenon known as photoinhibition." Photoinhibition usually results in permanent damage to the cell. Greater concentrations of inoculum in the culturing vessel creates a condition where the algae cells shade themselves as they are mixed with the culturing vessel; thus providing a net reduction in the overall light intensity received by each cell. Henceforth, the denser

the inoculation ratio is, the greater possibility the culture will not experience photoinhibition. Since these two inoculation ratios were so dilute in relation to the other ratios being tested, another important topic that could have resulted in their demise is nutrient toxicity. The amounts of the nutrients were in such excess that the cells underwent tremendous stress and could not tolerate the amount of nutrients. Lorenz et al. (2005) suggests for poor inocula, improved recovery may be obtained by using a less-defined medium in combination with the standard mineral medium. So using a growth medium with decreased nutrients would have allowed theses cells in these two flasks to possible grow but that would not have been the objective of the study. However, since the total amount of light and PAR light was not excessive for this study, it is more likely that the reason why the 1:16 and 1:32 ratios died was a result to nutrient toxicity based on the media components. Table 1 shows the media components used in the modified Erdschreiber's growth media.

Table 1: Media components used.

Chemical	g/L
KCl	0.7500
$CaCl_2*2H_2O$	0.6500
$MgSO_4*7H_2O$	7.6200
$NaNO_3$	0.8500
H_3BO_3	0.0340
$NaH_2PO_4*H_2O$	0.0400
Tru-Soft (NaCl)	21.0000
Trace Metals	0.0034

The slope of the linear trend line is the growth rate for each concentration ratio. The larger the slope, the faster the microalgae grew. Table 2 provides the averaged growth rates derived from the slope of concentration versus time for each of the concentration ratios.

Table 2: Averaged concentration ratio growth data.

Conc. Ratio	Growth Rate (ΔOD/d)
1:1	0.0320
1:2	0.0531
1:4	0.0750
1:8	*0.1027*

The 1:8 concentration ratio has the largest slope. This could be explained by the fact that at a less dense concentration, more light can penetrate the volume of the sample. This could also explain why the 1:1 concentration ratio grew the slowest. There were more microalgae cells in the flask and having more cells did prevent the light from fully penetrating the flask depth. Table 3 shows how the test will be run and analyzed in Design Expert 8 (2010). Table 3 also provides the growth rate for each ratio and trial as well as the biomass concentration from an OD vs. ash-free dry weight correlation performed after the study was completed. Appendix A provides this correlation plot. The daily productivity also is based on the lighted surface area of the culturing flask used for each ratio. As the table clearly shows, the 1:8 inoculation concentration ratio is the best ratio to inoculate with.

Table 3: Design Expert data table and concentration ratio performance.

Std	Run	Block	Factor 1 A: Conc. Ratio	Response 1 Growth Rate (ΔOD/d)	Biomass Conc. (g/L)	Productivity (g/L·m²·d)
7	*1*	*Trial 1*	*1:8*	*0.0972*	*0.194*	*6.965*
5	2	Trial 1	1:4	0.0687	0.180	6.142
3	3	Trial 1	1:2	0.0473	0.169	5.294
1	4	Trial 1	1:1	0.0291	0.160	4.165
8	*5*	*Trial 2*	*1:8*	*0.1082*	*0.199*	*7.145*
6	6	Trial 2	1:4	0.0813	0.186	6.347
4	7	Trial 2	1:2	0.0589	0.175	5.482
2	8	Trial 2	1:1	0.0349	0.163	4.243

The statistical software program Design Expert 8 (2010) was used for an ANOVA and Least Squares (LS) Means analysis on the concentration ratio model and to compare the linear regressions for each of the four concentration ratios. This regression analysis determined that each of the four concentration ratios are significant ($\alpha = 0.05$).

Table 4: ANOVA table for the inoculation concentration ratios model.

Response	**1**	**Growth Rate**			
ANOVA for selected factorial model					
Analysis of variance table [Classical sum of squares - Type II]					
	Sum of		**Mean**	**F**	**p-value**
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**
Block	2.101E-004	1	2.101E-004		
Model	5.500E-003	3	1.833E-003	396.96	0.0002
A-Conc. Ratio	*5.500E-003*	*3*	*1.833E-003*	*396.96*	*0.0002*
Residual	1.385E-005	3	4.618E-006		
Cor Total	5.724E-003	7			
Std. Dev.	2.149E-003		R-Squared	0.9975	
Mean	0.066		Adj R-Squared	0.9950	
C.V. %	3.27		Pred R-Square	0.9821	
PRESS	9.852E-005		Adeq Precisior	47.647	

Table 4 provides the ANOVA output and it shows that the model is significant because the p-value is so small. The model includes all of the concentration ratios so it is not possible to determine which ratio is significant or not from this output. A LS Means test will need to be applied to the concentration ratios to determine the significant or not significant ratios. A LS Means tests the fitted regression of each concentration ratio and compares it to the other concentration ratios. Table 5 provides the LS Means output from Design Expert 8 (2010).

Table 5: LS Means output for the concentration ratios.

Treatment	Mean Difference	df	Standard Error	t for H_0 Coeff=0	Prob > |t|
1 vs 2	-0.021	1	2.149E-003	-9.82	0.0022
1 vs 3	-0.043	1	2.149E-003	-20.01	0.0003
1 vs 4	-0.071	1	2.149E-003	-32.90	< 0.0001
2 vs 3	-0.022	1	2.149E-003	-10.19	0.0020
2 vs 4	-0.050	1	2.149E-003	-23.08	0.0002
3 vs 4	-0.028	1	2.149E-003	-12.89	0.0010

Table 5 displays the LS Means results for comparing the linear regression of each concentration ratio with each of the concentration ratios. This table expresses the concentration ratios in the treatment column, where treatment 1 is the 1:1 ratio, treatment 2 is the 1:2 ratio, treatment 3 is the 1:4 ratio and treatment 4 is the 1:8 ratio. In order to determine significance, if the value in the Prob > |t| column is less than 0.0500 then the difference between the two concentration ratios tested is significant. Since all of the comparisons are significant, this LS Means concludes that each concentration ratio was significantly different. This means that each inoculation ratio will cause the culturing vessel used to grow at its own specific rate depending on such factors including light penetration, available nutrients and contamination. However, once a certain inoculation concentration ratio is used in an outdoor raceway pond, the environmental factors begin to take more control of the outcome of the growth of the pond.

Conclusions

This study examined the effect of inoculation ratio on growth rate and the impact on commercial algae facility design. Test results showed a significant impact of inoculation ratio on growth rate and that the less dense, 1:8 inoculation ratio produced the greatest growth rate. However, if the inoculation ratio is more dilute, photoinhibition can occur and the cells may die. This could become an important factor in open raceway ponds in the desert southwest where solar irradiances are very high. Also, nutrient toxicity is another potential cause for pond loss and is likely the reason for the loss of the 1:16 and 1:32 ratios. Nutrient toxicity for algae is an area that needs more focus.

In order to obtain an accurate and precise inoculation concentration ratio, the scale up testing has to take place. Scale up testing in lab scale and in large scale raceway ponds are critical in determining an ideal inoculation concentration ratio. Future work will include scale up from 500 mL flask to scaled raceway ponds to ultimately large scale production raceway ponds. There are also other factors that need to be considered which include environmental changes, strain selection and production timelines. Future studies may be expanded to additional testing of concentration ratios not already tested as well as different levels of irradiances and media mixtures. This can have the potential to dramatically increase production time and decrease the cost of producing fuels derived from microalgae. Based on these results, a 1:8 concentration ratio looked to be the best candidate for raceway pond inoculations. By using this concentration, more time can be spent growing the microalgae in the final production growth ponds than in culturing the inoculum to be used in inoculating the production ponds. The more dense

an inoculation ratio used, i.e. more inoculum to new media, the more time and capital costs are needed in producing the required inoculum volume thus taking away from producing microalgae and the required lipids for biofuel production.

CHAPTER IV

IRRADIANCE, TEMPERATURE, AND POND DEPTH GROWTH STUDY ON *NANNOCHLORIS OCULATA*

Introduction

Microalgae are known to produce large quantities of lipids that can be used as a feedstock for biofuels. Large scale microalgae production in outdoor raceway ponds for the purpose of creating biofuels is a new process. Currently there are few data on large scale microalgae production and there are several hurdles to creating an algal biofuel that is cost competitive with traditional petroleum fuels. One of these hurdles is to maximize the cultivation process to create a high growth rate regardless of environmental influences.

Growing microalgae in open raceway ponds for biofuel production must rely on freely available sunlight, adjust to daily and seasonal variations in light levels, and adapt to wide temperature changes (Chisti, 2007). Specific growing conditions for each strain of microalgae vary and must be considered when selecting a strain or geographical region for producing microalgae. Jensen and Knutsen (1993) showed that important limiting factors were pH, contaminates, culture depth, and stirring rate. High light intensities led to photoinhibition causing a loss as high as 30% of the potential production rate. Vonshak and Guy (1988) reported that shading outdoor cultures sensitive to photoinhibition resulted in a daily photosynthetic rate increase of up to 35% for *Spirulina* strains.

Previous work (Cho et al., 2007; Terlizzi and Karlander, 1980) reported that ideal temperature to achieve maximum growth for *Nannochloris oculata* fell in the temperature range of 20-25°C. Increasing the pond depth will slow the media from heating up too rapidly above the optimal growing temperature by increasing the thermal mass and slowing the response to environmental changes. Chisti (2007) reports that the only cooling a raceway undergoes is achieved by evaporation. During times of very high irradiance and temperatures, evaporative losses can be significant. An increase in the salinity of the raceway pond can have harmful effects on the microalgae. A deeper pond slows the rate of increase of total solids concentration as the fresh water is evaporated. Conversely, reducing the pond depths by one half, from 15.0 to 7.5 cm, increased the algae production (Richmond and Grobbelaar, 1986). Thus, there is a balance between optimizing depth for maximum algae growth and mitigating environmental effects.

Determination of the effects of temperature, light intensity, and cultivation depth is critical to maintaining healthy cultures under the weather extremes typically seen in the desert southwest of the U.S.A. Annually, air temperatures can vary from below 0°C to above 45°C while the solar intensity can reach as high as 10 kWh m^{-2} day^{-1} during the summer months (National Renewable Energy Laboratory, 2011). Either of these factors can suppress or kill the microalgae. As part of the overall control of algae biomass production, it is necessary to determine the effects of solar intensity and temperature for the specific species under consideration for biofuel development and to determine if there are interaction effects between the parameters. Adjusting pond depth has been

shown to mitigate the effects of high temperatures and solar radiance by enhancing self-shading of the algae and increasing the thermal mass of the pond.

Objectives

The objective of this research was to determine how light intensity and temperature factors affect the growth rate of a species of algae used in biofuels research and if adjusting cultivation depth could mitigate these effects. Additionally, the effects would be determined under conditions that would mimic an outdoor production process; i.e. an open, raceway pond with a volume of approximately 40 L. The objectives were to:

1. Determine if there were effects of temperature and light intensity on a large volume of an algal species currently under consideration for algal-based biofuels production.
2. Determine if changes in culture depth would have a mitigating effect on the response of algal growth to environmental extremes.

Materials and Methods

Experimental Plan

Temperature and light reportedly play a critical role in the growth and health of growing microalgae in outdoor production settings. Temperatures that exceed a species upper limit of growth will harm the microalgae and reduce the production rates. High light intensities can have unfavorable effects on microalgae that are irreversible for the

most part. Having one of these factors exceeding an algal strain's growth limits can be detrimental, however if they are combined, the results could be much worse. Both temperature and irradiance have their own effects on microalgae growth. Combining them together might increase the problems they pose on the microalgae and create new ones. It seems that the only option if the light irradiance and/or temperature ever increase over the optimal growing conditions, the only feasible option would be to increase the pond depth. Covering the ponds with a shade cloth or UV filter would be too costly on a large scale. The increased depth would act as a self-shading mechanism for the algae. Lorenz et al. (2005) reports that in dense cultures, self-shading can have significant effect on the intensity of light reaching an individual cell. Increasing the pond depth would allow for decreased solar penetration on the algal cells providing less stress on the cells due to irradiance. During times of very high irradiance and temperatures, evaporative losses can be significant. Design Expert 8 (2010) is the statistical software package that will provide the analysis and determine the interaction effects on temperature, irradiance, and pond depth. Table 6 provides the testing factors and levels for this study. As Table 6 shows, there are three testing factors, temperature, irradiance, and depth. There are also two levels for each factor. The temperatures looked at will be 38°C and 20°C. The high and low irradiance values were approximately 785 and 220 $\mu mol\ m^{-2}\ s^{-1}$ respectively. Finally, the depth levels looked at is 10.16 cm (4 inch) and 13.97 cm (5.5 inch).

Table 6: Testing factors and levels for this study.

Test #	Temp (°C)	Irradiance	Depth (cm)
1	38	High	10.16 13.97
2	38	Low	10.16 13.97
3	20	High	10.16 13.97
4	20	Low	10.16 13.97
5	38	High	10.16 13.97
6	38	Low	10.16 13.97
7	20	High	10.16 13.97
8	20	Low	10.16 13.97

Standard Growing Conditions

A single strain of algae was grown separately prior to the start of this study and for each separate test under the standard growing conditions. The standard growing conditions used for *Nannochloris oculata* in raceways were 21-25°C, 172 μmol m^{-2} s^{-1} on a (16h/8h light/dark cycle), pH 9, paddle wheel speed of 12 rpm, and 10.16 cm (4 inch) pond depth. Figure 3 shows the micro raceway ponds used in this study. Each raceway in the figure is 40 L at a 10.16 cm (4 inch) depth. The pH is maintained by an automatic CO_2 system (American Marine Inc., Pinpoint pH controller and probe, Azoo CO_2 solenoid and regulator) that injects CO_2 when the pH rises above a preset pH set point. The media used for the standard conditions and all of the experimental treatments was modified Erdschreiber's growth media.

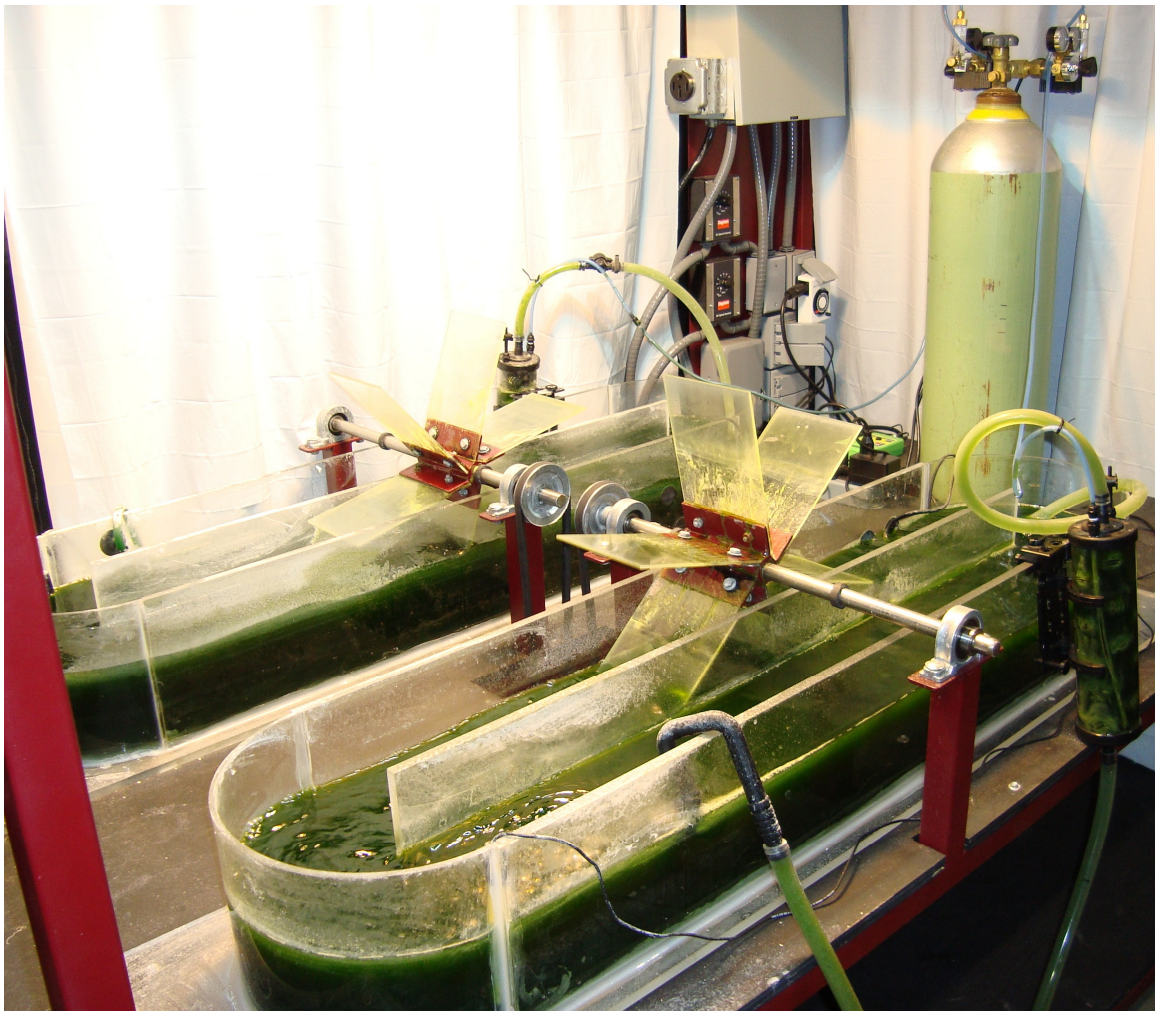

Figure 3: Micro-raceway algae ponds used for light and temperature effects studies.

Experimental Apparatus

Two raceways were placed in an M36 Precision growth chamber from Environmental Growth Chambers. The growth chamber allowed for increased control over lights, temperature, and humidity. The relative humidity was kept high to mitigate evaporative losses in the ponds caused by the high temperature and irradiances. The humidity was kept constant at 80%. One of the two raceways in the growth chamber was

kept at the 10.16 cm (4 inch) depth and the second raceway was maintained at 13.97 cm (5.5 inch) depth. The increased pond depth tested whether or not the irradiance could penetrate the full depth of the culture and if at high irradiances, there was a benefit or not from self shading. The sides of the raceways were covered with black cardstock allowing light to only reach and penetrate the surface of each raceway.

The high and low irradiance values were approximately 785 and 220 μmol m^{-2} s^{-1}, respectively and were constrained by the limits of the growth chamber. Irradiance was measured daily with a handheld quantum light meter from Apogee Instruments (Model QMSW, Apogee Instruments, Logan, UT, USA). Each test lasted for 5 d and included a photoperiod of 16 h of light and 8 h dark. The temperature and irradiance were held constant during each test based on the testing parameters in Table 5. In addition to the Apogee meter, a multi parameter weather station from HOBO (U30 Station, Onset, USA; PAR Sensor Part # S-LIA-MDD3; 12-Bit Temperature Sensor Part # S-TMB-M0XX) was placed in the growth chamber to provide data monitoring of ambient air temperature, temperature of the algae in each pond, the irradiance penetrating the algae in each pond and the irradiance reaching the surface each pond.

Each pond was inoculated using a 1:1 concentration ratio using previously grown algae under standard growing conditions. Also, pseudo replications were done for these tests as well. Since the inoculum was split between the two testing raceways and the testing raceways were started at the same time. The water level was also checked each day and the appropriate amount of DI water was added to replace evaporative losses and keep the desired depth in each pond. To keep everything uniform, the water was added

after samples were taken. It was assumed that the salts, nutrients and algal cells stayed thoroughly mixed with evaporation and water additions. The fill water was also kept at the same temperature as the test being conducted to prevent any thermal shock. Daily samples were measured for ash free dry weight (AFDW), dry weight (DW), optical density (OD), dissolved oxygen (DO), conductivity, pH, nitrate/nitrite utilization and lipid content. The lipid analysis was used to determine if the algae were undergoing stress and producing lipids at any point during each test. The DO, conductivity, pH, and nitrate/nitrite tests were used for determining the health of the algae but were not used for the statistical analysis of this study.

The OD measurements were conducted using a UV-Vis Spectrophotometer (T80+, PG instruments Ltd.). To make sure the sample fell within the linear range of the machine, a 10x dilution was performed every time a measurement was taken. Three wavelengths were used for each measurement 745 nm, 750 nm, and 755 nm with an average value taken for the OD reading. The DW and AFDW measurements were adapted from methods given by Zhu and Lee (1997) and Eaton (2005) for microalgae samples. For dispensing samples on the filter, if the density is greater than 1 g/L then filter 20 mL of algae sample and if the density is less than 1 g/L then filter 40 mL of algae sample. In calculating the DW and AFDW, the following two equations are used.

DW and AFDW Calculations:

i. $$DW, g/L = \frac{(WT4 - WT3)}{Sample\ volume,\ mL} \times 1000 \quad (2)$$

ii. $$AFDW, g/L = \frac{(WT4 - WT5)}{Sample\ volume,\ mL} \times 1000 \quad (3)$$

For the pH, conductivity, and dissolved oxygen (DO) measurements, a multimeter analytical instrument was used for this (Beckman Coulter, model # pHi 570, Fullerton, CA). Nitrate and Nitrate test strips (Lamotte Insta Test, 0 to 50 PPM and 0 to 10 PPM, Model # 2996) were used for the nitrogen uptake of the microalgae. These testing strips were not reliable and could not provide an accurate measure of nitrogen utilization. The lipid analyses were conducted with a scanning fluorescence spectrophotometer (PTI QuantaMaster, Photon Technology Intl., Canada). The sample was diluted to the same concentration as that used for the OD measurement and 30 μL of Nile Red solution was added to 10 mL of correctly diluted algae sample. The lipid analysis data has not been processed and was not used for any statistical analysis.

Results and Discussion

Exponential growth phase was assumed during an initial two-day growth period for each test and was used in estimating the various parameters for this experiment. The OD values were normalized for every test conducted in this experiment as well. Design Expert 8 (2010) was used for the statistical analysis of this test. The OD vs AFDW,

AFDW, and OD were analyzed separately for significance and interaction effects between the testing factors.

The ANOVA first performed was the OD vs AFDW data. OD and AFDW data were compiled to create OD vs AFDW plots for each test. This was accomplished by combining the OD values with the AFDW values for each day in a plot to get a growth rate for that test. This would allow for a more precise growth rate and give a correlation between OD and AFDW. Two data points were removed from the analysis, 4-38-H-1 and 4-38-H-2. The point 4-38-H-1 was omitted from all of the Design Expert 8 (2010) ANOVAs because this pond failed the very first night of testing since the CO_2 supply line had collapsed on itself causing the pH to raise too high and thus killing the algae before it could be fixed. The point 4-38-H-2 was removed since the growth rate was a statistical outlier. No transformation was needed for this ANOVA. Table 7 provides the Design Expert 8 (2010) output of OD vs AFDW ANOVA output table.

Table 7: OD vs AFDW ANOVA output table.

ANOVA for selected factorial model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	1.31	4	0.33	1.23	0.3647
A-Irradiance	*0.29*	*1*	*0.29*	*1.08*	*0.3258*
B-Temperature	*1.654E-003*	*1*	*1.654E-003*	*6.203E-003*	*0.9389*
C-Depth	*0.23*	*1*	*0.23*	*0.84*	*0.3823*
BC	*0.52*	*1*	*0.52*	*1.96*	*0.1950*
Residual	2.40	9	0.27		
Lack of Fit	*0.12*	*2*	*0.061*	*0.19*	*0.8335*
Pure Error	*2.28*	*7*	*0.33*		
Cor Total	3.71	13			

As the table shows, there are no significant factors or interactions with this method of combining growth rates. Not all of the interactions were included since they had negligible contributions to the ANOVA. The only good aspect of this is that the lack of fit is not significant so the model fits the data.

Next was the AFDW data that was taken each day from the ponds and analyzed. Table 8 shows the Design Expert 8 (2010) output of AFDW ANOVA table. Design Expert 8 (2010) recommended a square root transformation of the data (constant = 0.0528). Not all of the interactions were included in the ANOVA since they had negligible percent contributions.

Table 8: AFDW ANOVA output table.

ANOVA for selected factorial model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	0.89	5	0.18	2.21	0.1527
A-Irradiance	*5.174E-004*	*1*	*5.174E-004*	*6.387E-003*	*0.9383*
B-Temperature	*0.63*	*1*	*0.63*	*7.72*	*0.0240*
C-Depth	*0.018*	*1*	*0.018*	*0.22*	*0.6516*
BC	*0.12*	*1*	*0.12*	*1.45*	*0.2634*
ABC	*0.11*	*1*	*0.11*	*1.33*	*0.2814*
Residual	0.65	8	0.081		
Lack of Fit	*0.018*	*2*	*9.199E-003*	*0.088*	*0.9172*
Pure Error	*0.63*	*6*	*0.10*		
Cor Total	1.54	13			

Two data points were also omitted from this analysis. The data points removed from this ANOVA were 4-38-H-1 and 5.5-20-H-1. Data point 5.5-20-H-1 had a growth rate value that was a statistical outlier from the data set and was removed as a result. As Table 8 shows, there was only one significant factor which was temperature. The model was not significant since the p-value was so large, so there is no basis to reject the null hypothesis that there is a difference between the inoculation ratios. For a factor or the model to be significant, the p-values would need to be less than the 0.0500. Factors need to be greater than 0.1000 to be considered significant. Based on these results alone, temperature appears to be a likely candidate for limiting the growth of microalgae. The lack of fit for this ANOVA was also not significant relative to the pure error which was ideal. With the lack of fit being so large, the model fits the data well.

Finally, the OD growth rates were analyzed in an ANOVA. Table 9 provides the Design Expert 8 (2010) output of the OD ANOVA table. Interactions that not significant were excluded. The 4-38-H-1 data point was missing because of a failure in the raceway.

Table 9: OD ANOVA output table.

ANOVA for selected factorial model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	0.46	4	0.12	10.40	0.0014
A-Irradiance	*0.32*	*1*	*0.32*	*28.38*	*0.0003*
B-Temperature	*1.690E-003*	*1*	*1.690E-003*	*0.15*	*0.7051*
C-Depth	*0.15*	*1*	*0.15*	*13.76*	*0.0040*
AC	*0.033*	*1*	*0.033*	*3.01*	*0.1135*
Residual	0.11	10	0.011		
Lack of Fit	*2.540E-003*	*3*	*8.467E-004*	*0.054*	*0.9819*
Pure Error	*0.11*	*7*	*0.016*		
Cor Total	0.57	14			

The overall model is significant ($p = 0.0014$). Irradiance and depth were significant factors, while temperature was not significant. Lack of fit was also not significant. Based on these results, the 20°C temperature, high irradiance and 10.16 cm (4 inch) pond depth were the best conditions for growing this strain of green algae. At the deeper pond depth, the light could not fully penetrate the microalgae to allow for full light exposure.

After all of analysis was complete, the interactions that were expected did not appear. Several reasons could account for this occurrence. Lab conditions don't always resemble real world, outside growing conditions. The light intensity in the growth chamber could not fully reach the irradiance levels that are common in the summer months in tropical and desert regions were microalgae facilities are currently located. The upper limit of the temperature range was a close approximation to outside conditions but again, did not fully reach the extreme high temperatures seen in the outdoor production. Not being able to compare the absolute irradiance and temperature extremes that are experienced in outdoor production may have been the reason why the expected interactions were not seen in the ANOVA analyses. If testing could take place during those weather conditions that are normally seen, there will be a greater chance of interactions between the factors tested. Performing this experiment again in outdoor production conditions would be ideal but that is not available; increasing the irradiance and maximum temperature would be a requirement. If there are truly no interactions between temperature, irradiance, and depth, then temperature and irradiance should have no combined effect on the productivity of microalgae growth. Also, the depth of the culture doesn't matter and light should be able to penetrate the culture. However, this is already known not to be true. Richmond and Grobbelaar (1986) found by using *Spirulina platensis* that having a shallower pond depth was beneficial. They discovered in their testing that reducing the pond depths by one half, from 15.0 to 7.5 cm, it greatly increased the algal concentration to maximum productivity.

Conclusions

Upon completion of this study, it was found that *Nannochloris oculata* grew the best at a light irradiance of 785 μmol m^{-2} s^{-1}, 20°C, and 10.16 cm (4 inch) pond depth. Irradiance and pond depth were found to have significant effects but there were no interaction effects noted. Temperature effects were not shown to be significant.

Additional testing needs to be performed to determine the exact temperature, irradiance and pond depth that would be optimal for growing this particular strain of microalgae. Additional factors can also be tested like pH, salinity and mixing rate among others. Once the ideal conditions are found, this will provide valuable information in regards to growing this strain and provide a stepping stone for other strains as well. Having the ideal growing conditions known to maximize productivity, and knowing how to counter the effects of the extreme conditions will help to bring the cost of algal based biofuels down. For the following tests, more intermediate temperatures need to be tested as well expand the upper limit of the temperature range. Tropical and desert regions where large scale microalgae facilities are located, temperatures reach greater than 38°C. Also, having greater irradiance intensities closer to actual conditions is needed in forthcoming studies. By both increasing the temperature and irradiance, pond depth should also be looked at more closely, both decreasing and increasing the depth.

CHAPTER V

DETERMINING THE MINIMUM MIXING VELOCITY FOR PENNATE AND CENTRIC DIATOMS

Introduction

Microalgae are the subject of current research as a source of biomass and lipids for conversion to biofuels. There are many types of microalgae including green algae, cyanobacteria, and diatoms. Diatoms are of particular interest because they are fast growing, contain large amount of lipids, and are easily harvested (Ramachandra et al., 2009). Diatoms contain a silica cell wall as opposed to a cellulosic cell wall similar to plants and green algae. The silica cell wall is more dense and once mixing has stopped, the diatoms are easily separated from the growth media by natural settling. The specific weight of the diatom cell wall was found to be 2.07 versus 1.02 for seawater (Ramachandra et al., 2009).

Natural settling is important since no flocculants or energy consuming machinery (e.g. centrifuge or dissolved air flotation) would be needed to separate the diatoms from the media. Adding flocculants, coagulants, and other means are costly and time consuming and if the diatoms will naturally settle, this leads to input costs being reduced.

The silica shell does make diatoms more susceptible to damage. Processing and transporting diatoms and with centrifugal pumps as it is done for green algae, can result in cell damage and lysis (Michels et al., 2010). This is will reduce growth and lipid

production and may even result in the death of the cell. Consequently, it is necessary that the minimum amount of energy needed for mixing be used in diatom cultivation so that cell damage is minimized or eliminated. However, sufficient mixing is necessary to support gas exchange, nutrient uptake, and access to sunlight so that the diatoms maintain a desired growth rate as well as to prevent thermal stratification (Becker, 2008).

Objectives

The objectives of this study were to evaluate several species of diatoms under consideration for biofuel production and determine the minimal channel velocity needed to maintain mixing and desired growth rates. Specifically the objectives of this study were to:

1. Develop the methodology to measure the settling velocity of monocultures of diatoms without added flocculants or coagulants.
2. Determine the natural settling velocity for several species of diatoms at different stages of health.
3. Derive the minimal channel velocity required for cultivation of diatoms in open, outdoor raceway ponds.

Materials and Methods

Four strains of the pennate diatoms from the *Nitzschia sp.* and one centric diatom were selected for the natural settling tests of this study. These strains were isolated by

General Atomics in San Diego, CA, the Texas AgriLIFE Experiment Station in Lubbock, TX and at the Texas AgriLIFE Research Facility in Pecos, TX.

A four basin jar tester from Phipps and Bird is the device that was utilized for this study. Phipps and Bird have a method for settling that is used in the wastewater treatment industry (Hudson and Wagner, 1981). This method was adapted for use for these experiments. Each basin has a 2 L capacity with a sampling spout 10 cm below the 2 L fill mark. Samples were taken immediately after the premixing time and at elapsed time following premixing of 1 min, 2 min, 5 min, and 10 min. Samples were taken from the sampling spout and run in the UV-Vis (Thermo Scientific, Genesys 20, Model #4001/4) to determine the amount of diatoms that had settled at each time period. The OD was taken at 750 nm. Before each test began, the basins being utilized were filled and mixed with the jar tester at 50 rpm for 10 min to ensure there was a homogenous mixture in each basin. This is completely natural diatom settling, no flocculants or coagulants were added to the diatoms.

Premixing Test

The premixing time and rpm were determined experimentally. The rpm was chosen to provide adequate mixing while inducing minimal sheer to the diatom cells. The time used allowed the cell interaction to dissipate since it is believed that there is an interaction that causes the diatoms to flocculate together. This is evident from observations as diatoms settle and when they are settled. The cells form large flocs and require either a longer mixing time or a greater mixing speed to disrupt the surface

charge. To determine the mixing time, the jar tester was used to compare 4 different times to see which time resulted in the best settling data. The Diatom 1 strain was used with premixing times of 5, 10, 15, and 20 min respectively. The Diatom 1 strain was used for this test since that was the only strain that there was enough volume to fill each basin of the jar tester. Figure 4 below shows a composite graph of the four premixing times.

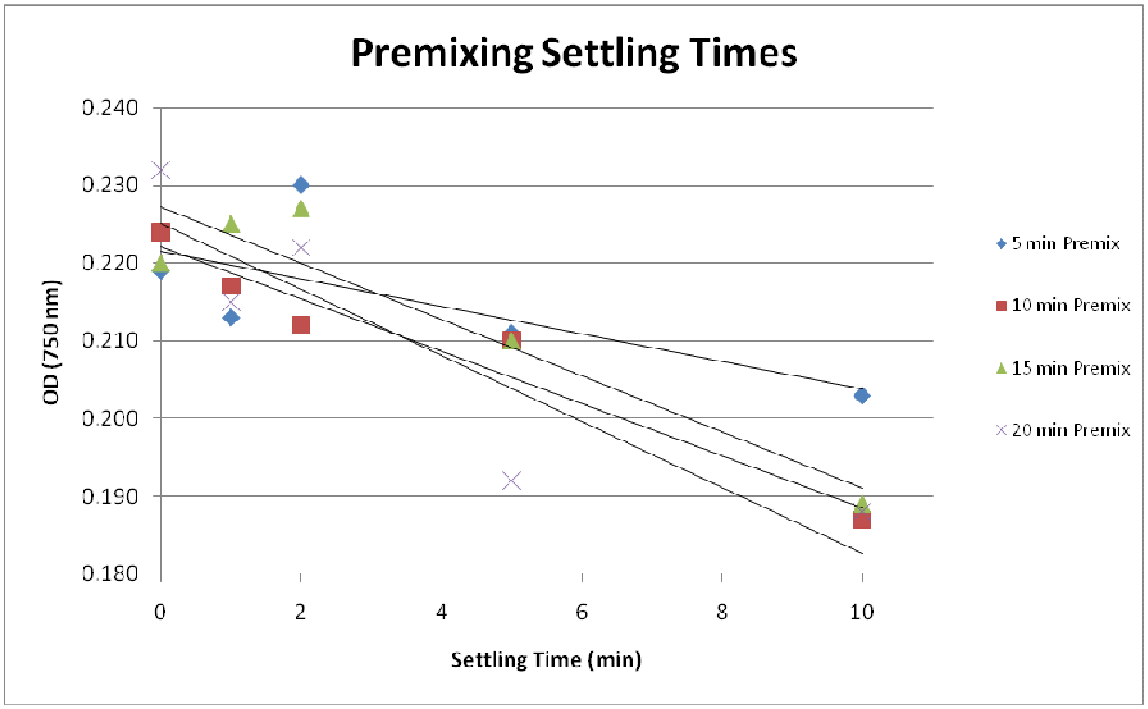

Figure 4: Four premixing settling times graph.

To better represent all of the settling rates (slope of the curve) and fits of the linear regression line (R^2) values, Table 10 shows these values.

Table 10: Premixing settling times graph data.

Time (min)	Settling Rate	R^2
5	-0.0018	0.5063
10	-0.0034	0.9455
15	-0.0036	0.8881
20	-0.0043	0.8079

Based on the settling rate (slope of the curve), the fit of the linear regression line, and the OD at the 10 min mark it was concluded that the 10 min premixing rate was best for premixing before each settling test. The 10 min time had the best regression fit and one of the best settling rates. At the end of the test, the 10 min premixing test had the lowest OD which translates to the least amount of diatom cells left in solution.

Settling Tests

The five strains were tested in accordance with the methodology given by Phipps and Bird (Hudson and Wagner, 1981). At the end of the premixing period, the sample spout was flushed and an initial sample was taken which was run in a UV-Vis spectrophotometer (Thermo Scientific, Genesys 10S). This process of flushing the sample spout of settled diatoms and taking a sample at the end of each time interval was carried out throughout each test. The reason why there was a different UV-Vis used for the settling tests than was used for the premixing tests is because the settling tests were carried out at the Texas AgriLIFE Research Facility in Pecos, TX and the premixing tests were performed at Texas A&M University in College Station, TX.

Not all strains could be tested with a replicate since volume quantities were very limited and it was found that retesting the same sample did not yield similar results. The sample in the basin was only tested once and then discarded accordingly. The native centric diatom from Pecos, TX (Diatom 2) was the only strain that there was enough volume to make numerous testing replications of both healthy and unhealthy (stressed) cultures. The three replications of Diatom 1 were all unhealthy, the Diatom 3 was unhealthy, the Diatom 4 was healthy and the Diatom 5 was unhealthy as well.

Results and Discussion

Table 11 shows how each strain was tested and classified. Batch is the culturing vessel that particular test came from. The basin column designates which one of the four basins of the jar tester were used for that test. There was enough culture from batch 2 for a replicate as well as from batch 3. For this particular test, health was determined visually. The diatoms were determined to be healthy or not healthy (under stress) based on a visual inspection of their color. The mean settling velocity values came from determining the mean OD of each batch and then using that calculated value to solve for the corresponding settling velocity using the linear regression equation obtained by plotting OD vs settling velocity for each batch test. The median value was found by determining the median OD value and then solving for the corresponding settling velocity. OD values were normalized before being used in their respective calculations.

Table 11: Strain testing factors and responses.

Factors					Responses	
			Health		Settling Velocity (cm min^{-1})	
Strain	Batch	Basin	Healthy	Unhealthy	Mean	Median
Diatom 1	1	A		Unhealthy	4.46	5.36
	2	A		Unhealthy	4.51	4.36
	3	A		Unhealthy	4.46	4.16
Diatom 2	1	A		Unhealthy	4.50	4.50
	2A	A	Healthy		4.55	4.21
	2A	B	Healthy		4.53	2.95
	2A	C	Healthy		6.00	6.00
	2B	A	Healthy		4.63	3.05
	2B	B	Healthy		4.20	0.87
	2B	C	Healthy		3.75	7.50
	3A	A		Unhealthy	4.64	3.73
	3A	B		Unhealthy	4.56	5.19
	3A	C		Unhealthy	4.10	6.10
	3A	D		Unhealthy	4.65	3.36
	3B	A		Unhealthy	4.53	5.71
	3B	B		Unhealthy	4.60	3.71
	3B	C		Unhealthy	4.33	3.00
	3B	D		Unhealthy	5.00	5.00
Diatom 3	1	A		Unhealthy	4.49	4.11
Diatom 4	1	A	Healthy		4.49	7.21
Diatom 5	1	A		Unhealthy	4.50	5.51

An ANOVA was performed in Design Expert 8 (2010) to test the significance of each of the two responses listed in Table 11. It was found that each of the responses were not significant on the 95% confidence interval. Table 12 provides the ANOVA output (Design Expert 8, 2010) for the mean settling velocity. The model p-value equaled 1.0000, implying that the model was not significant.

Table 12: Response 1 - mean settling velocity (cm/min).

Response 1	Mean Settling Velocity				
ANOVA for selected factorial model					
Analysis of variance table [Classical sum of squares - Type II]					
	Sum of		Mean	F	p-value
Source	Squares	df	Square	Value	Prob > F
Model	0.052	7	7.364E-003	0.029	1.0000
A-Strain	*0.013*	*4*	*3.153E-003*	*0.012*	*0.9997*
B-Batch	*3.275E-003*	*2*	*1.638E-003*	*6.406E-003*	*0.9936*
C-Health	*3.430E-004*	*1*	*3.430E-004*	*1.342E-003*	*0.9713*
Residual	3.32	13	0.26		
Lack of Fit	*8.282E-004*	*1*	*8.282E-004*	*2.991E-003*	*0.9573*
Pure Error	*3.32*	*12*	*0.28*		
Cor Total	3.37	20			
Std. Dev.	0.51		R-Squared	0.0153	
Mean	4.55		Adj R-Squared	-0.5150	
C.V. %	11.12		Pred R-Square	N/A	
PRESS	N/A		Adeq Precisior	0.549	

The median settling velocity ANOVAs yielded not significant results as well. Since none of the models are significant, none of the testing factors are significant and also there no interaction affects that exist between the factors. The reason why there are no significant strains is due to the fact Diatom 2 has a very small size as compared to the Diatoms 1, 3-5 strains and is prone to floating. Also the remaining strains are all closely related so one would expect them to behave similarly. Since none of the testing factors are significant, a new set of testing factors for determining significance like cells with a greater set of different particle sizes, a larger range of strains and species, and having a quantified value for the lipid content for each test would be beneficial. In theory, the

more lipids each cell contains, the more buoyant they become and should resist settling once mixing has stopped. This is based simply on the fact that lipids, like oil, are lighter than water (Sargent and Falk-Petersen, 1988).

In order to combat the effects of settling in a raceway pond, a similar test should be performed with the strain of diatoms being grown and the settling rate of that particular strain should be determined. Once that is known, the velocity of the raceway should be kept higher than the diatom settling velocity to keep the diatoms in suspension as the diatoms travel around the length of the raceway until the diatoms reach the paddlewheel where they can be mixed thoroughly by the paddlewheel. For example, if the mean settling velocity of an algae strain was found to be 4.55 cm min^{-1} then the minimum mixing velocity of the raceway should be kept at 4.55 cm min^{-1} for neutral buoyancy or slightly higher to make sure all of the cells stay in suspension. Since the settling velocity was a mean value, theoretically only half of the cells will float so using two standard deviations to achieve a 95% suspension rate, the mixing velocity in the raceway should be maintained at 10.1 cm min^{-1} throughout the raceway. For a 99% suspension rate, three standard deviations of the mean settling velocity should be used for a mixing velocity of 15.2 cm min^{-1}.

If the raceway has only one paddlewheel, then the raceway velocity will need to be higher than it would be if there were more paddlewheels in the raceway. Having more paddlewheels reduces the length between mixing periods and the length diatoms need to travel before they will settle out of suspension. There are very large velocity drops through the bends in a raceway and so the mixing speed of the paddlewheel needs to be

increased to compensate for this or having additional mixers may be necessary. The ponds can also benefit from having baffles placed in the bends of the raceway to increase the channel velocity by dividing the channel into smaller cross-sections. Increased channel velocity in the raceway bends will allow for greater mixing capability with a lower paddlewheel speed and thus reducing the overall operating cost and shear placed on the diatoms. Careful consideration must be taken when choosing a mixing speed so that the induced shear does not exceed the tolerable levels for the strain being grown. Also, having excessive mixing over the minimal mixing rate needed to maintain cell suspension increases operating costs.

Since the 10 min time mark was the last data point taken, it should theoretically have the least amount of diatoms remaining in suspension and subsequently be the maximum settling velocity. This maximum value is then 10 cm min^{-1} which means for every minute, a diatom cell falls 10 cm within the basin. The basin is static so there is no movement or mixing within the basin once the settling test started. However, in a production setting, like a raceway pond, the mixing velocity, which is the flow rate of medium, necessary to ensure all the effects required for optimal algal growth, varies primarily depending on the settling rate of the specific algal cells (Becker, 2008). Each species of algae has its own specific settling velocity. In order to keep the algae in suspension, the mixing velocity must be maintained at a greater horizontal velocity throughout all points of the raceway to overcome the settling rate of the algae. According to Becker (2008), it is assumed that a velocity of 10 cm s^{-1} (600 cm min^{-1}) is generally sufficient to avoid deposition of cells. However, an average value of 20 cm s^{-1}

(1200 cm min^{-1}) is often used because of unavoidable fluctuations of the velocity, particularly at the bends of the horizontal raceway ponds. In practice, most of the ponds are operated at a speed between 10 and 30 cm s^{-1} (60 and 1800 cm min^{-1}). Since all of the mean and max settling velocities of the diatoms tested are below the published minimum design pond velocity, there should be no diatom settling at any point in the raceway path if the published values are used. As it is known, the paddlewheel causes shear stresses on the diatoms which cause damage. Having excessive rotational speed can induce unnecessary shear stresses so determining the exact settling velocity of the strain being grown and matching the pond velocity accordingly to keep the algae in suspension is necessary for optimal growth and to reduce the costs associated with operating paddlewheels.

Conclusions

As the lipid potential of diatoms is being studied for biofuel production, it is becoming important to effectively culture these organisms for maximum productivity levels. The natural settling of diatoms is one aspect of that. The mean, maximum, and median settling velocity ANOVAs all yielded not significant results partly due to the fact the species tested were very similar in physiology and the only different strain favored floating once mixing had ended. As a result, it was determined that the mixing velocity of the raceway being used to grow the diatoms should be kept at least two standard deviations higher than the mean settling velocity to ensure that 95% of the diatoms will stay in suspension. It is important to know the natural settling velocity of each algal

strain being grown in a raceway so the appropriate mixing velocity can be used to help keep electrical costs to a minimum as well as the shear forces exerted on the diatoms.

The next step for this experiment is to test more strains, both healthy and unhealthy and have sufficient volumes of each strain to have enough replicates. It is also important to know exactly how much biomass is being removed and left in the basins during the tests. Performing cell counts at each OD with a hemacytometer is one method that is being looked at. More testing with this method will need to occur for more accurate results and it is also possible to correlate cell counts with AFDW measurements. Both cell counts at each OD and cell counts being correlated with an AFDW will take place in future work.

CHAPTER VI

CONCLUSIONS

These studies each represent one aspect in developing optimal growth parameters for the green microalgae *Nannochloris sp.* and the diatom *Nitzschia sp.* for large scale raceway production. Each study focused on a unique aspect of effectively producing microalgae. These studies play into the larger role of cultivating microalgae both efficiently and to its fullest potential. The conclusions below summarize the findings of each study and how they can be used to benefit microalgae production.

The results from the inoculation ratio test showed a significant impact of inoculation ratio used and on growth rate and that a less dense, 1:8 inoculation ratio produced the greatest growth rate. However, if the inoculation ratio is more dilute, photoinhibition can occur as well as nutrient toxicity, and the cells may die as observed with the two most dilute ratios. Knowing this type of information is critical in the proposed ideal areas where microalgae facilities are located since the amount of available sunlight or solar irradiance can be detrimental.

The amount of available solar irradiance plays a large role in the outcome of the results for the second study. After the testing was completed, it was found that the microalgae grew the best at a light irradiance of 785 μmol m^{-2} s^{-1}, 20°C, and with a pond depth of 10.16 cm (4 inch). This correlated to the highest irradiance value tested and lowest temperature and pond depth tested. This was significant because based on the limitations of the experiment; a high irradiance value coupled with a shallow pond depth provided ideal conditions over having a deeper pond depth to help mitigate the damages

incurred from intense solar intensities and temperature extremes. This is definitely an experiment that needs to be duplicated outdoors so real world growing conditions can be experienced and also scaled up to production ponds as well.

Finally, the settling study showed that there were no significant factors in settling times between the species or the growth phase of the diatoms, i.e. strain health. This is attributed to the fact that the species tested were very similar in physiology and the only different strain favored floating once mixing had stopped. The mean settling time was found to be 4.55 cm min^{-1} and the minimum channel velocity was determined to be 10.12 cm min^{-1} based on the second standard deviation. For any algal strain being considered for growth in a raceway pond, this same settling test needs to be performed so the settling velocity can be determined. Once that is known, the mixing velocity of the raceway can be determined so minimal energy can be used to keep the algae in suspension as well reduce the shear stress on the algae.

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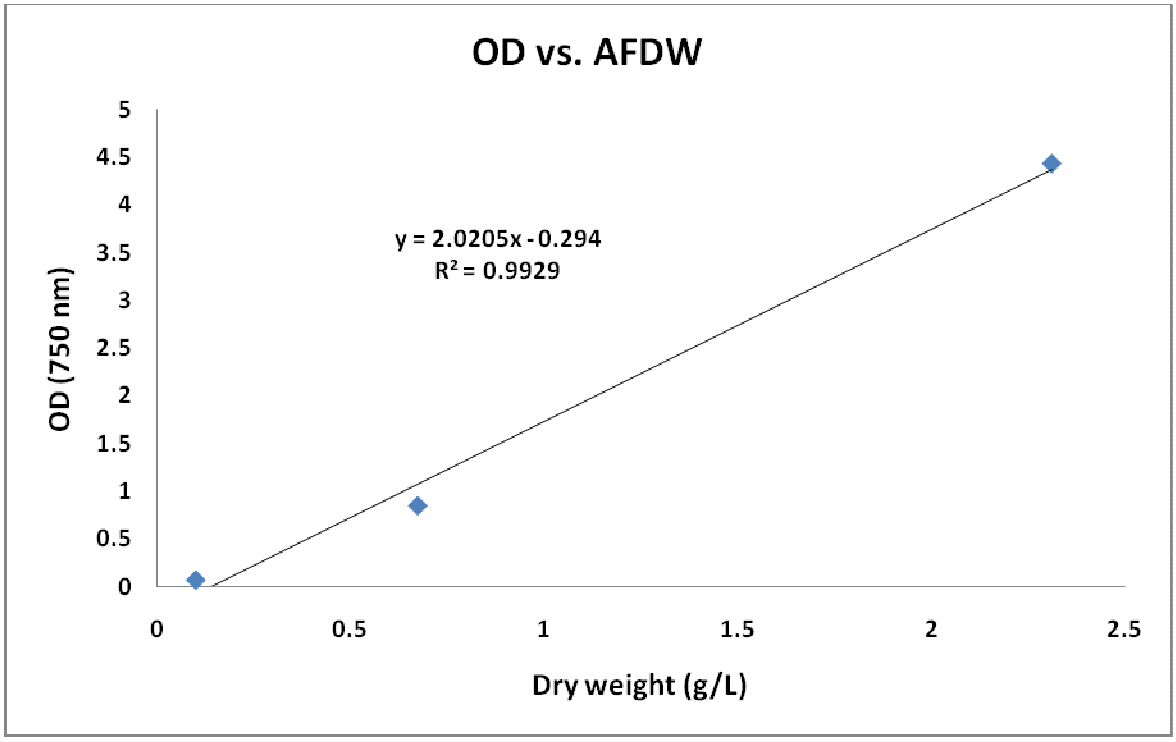
APPENDIX A

OPTICAL DENSITY VS. ASH-FREE DRYWEIGHT CORRELATION CURVE



Note: This curve was constructed using the data from dry weights taken from the growth curve. Each point was made with triplicates. (Unpublished data from Andrea Garzon).

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