FINAL REPORT

Marine Engine Testing and Emissions Laboratory (METEL)

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Algae Based Glycerin Fuel Project

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Abstract

Climate change and global warming are a worldwide concern, and utilizing alternative fuels is one way to reduce carbon dioxide emissions to the atmosphere. Microalgae are promising as a potential replacement for fossil fuels via biofuels. This study was aimed to show that the microalga *Dunaliella tertiolecta* is a viable species for biofuel production in the form of glycerin. Glycerin is an inexpensive fuel of moderately high viscosity and is a potential fuel replacement of heavy fuel used in large diesel engine.. The project developed experimental methods, calibration protocols, culture media and equipment to grow *Dunaliella tertiolecta* and study its glycerin production properties. Both batch style benchtop and continuous through flow systems were developed at model scale as part of this effort. The continuous flow models were used to model a typical farm operation by flowing the media continuously though a system, which can monitor growth rate, glycerin production and feed nutrients to the "farm" pond. The benchtop system was used for fundamental studies on the growth rate and glycerin production rate. This system was used to study the effects of salinity shocking on glycerin production, which the study concluded as an effective method for boosting glycerin production. These results could be applied to an algae farm for increasing production and profitability.

Motivation and Background

Alternative sources of energy are being studied to mitigate climate change and global warming caused by greenhouse gases. According to the Environmental Protection Agency (EPA) 65% of carbon dioxide (CO_2) emissions in 2010 were produced by the burning of fossil fuels and since the 1970s, CO_2 emissions in the atmosphere have increased 90% (EPA, 2017). In 2008 the United States alone was using $19.5x10^6$ barrels of oil per day (Hannon et al., 2010). Creating alternative fuels such as biofuel or biodiesel, which are fuel products derived from living biomass, (e.g. corn oil), is important for limiting the world's current and future greenhouse gas emissions. Biofuels lessen CO_2 emissions because they use carbon that is found in our natural carbon cycle, rather than releasing carbon that has been buried for centuries. The ability to use microalgae for biofuel production is a step in the right direction to lessen fossil fuel usage, as well as limit CO_2 emissions (Hannon et al., 2010). Optimizing the amount of hydrocarbon or oil produced is an important first step for being able to utilize a microalga for biofuel (Lee, 2018).

Microalgae offer many advantages to crops traditionally used to produce biofuel, such as corn and rapeseed. Biofuels can be synthesized using a variety of lipids, including those from vegetable oils (Mata et al., 2010). Oils for biofuel can include anything from corn oil, to oil produced by microalgae. A major difficulty with using land-based crops (e.g. corn oil or rapeseed) for biofuel production is the lack of arable land to grow the crops (Mata et al., 2010) whereas microalgae can be grown all year round, as well as on land that is not suitable for land-based crops (Brennan, 2010; Mata et al., 2010). Microalgae also reproduce very rapidly compared to land-based crops, meaning they will yield a product much more efficiently (Hannon et al., 2010).

One challenge of utilizing microalgal-based biofuels is making them economically feasible; however, there are advantages to using biofuels, specifically microalgae-derived

biofuels. According to Hannon et al. (2010) the price of biofuels dwarfed fossil fuels in 2009, being nearly 30 times higher. But even a microalga with low oil content produces a greater volume of oil compared to any land based oil-seed crop (Mata et al., 2010). A land based oil-seed crop such as corn is, on average composed of 44% oil in terms of its biomass whereas a high oil content microalga is, on average 70% oil in terms of its biomass. This translates to a biodiesel productivity of a high oil content microalga of approximately $1.2x10^5$ kg biodiesel per ha^{-1} $year^{-1}$; alternatively, a crop like corn would only produce approximately 152 kg biodiesel per ha^{-1} $year^{-1}$ (Mata et al., 2010). Microalgae are also more efficient because they require less space and fewer nutrients in order to grow successfully. Microalgae utilize carbon very efficiently and are responsible for approximately 40% of carbon fixation in the world; this coupled with the fact that they can be grown in a wide array of environments make them a good alternative to land-based crops, or fossil fuels (Hannon et al., 2010). The minimal land use of the microalgae, compared to an oil-seed crop, like corn would save money and resources, as well as space for food crops (Mata et al., 2010).

Hydrocarbons produced by microalgae are viable for synthesis of biofuel and other materials. Glycerol is a lipid byproduct of some microalgae, and is one such hydrocarbon that that can be utilized for production of biofuels or other goods such as food products and pharmaceuticals (Chow et al., 2013, Chow and Ng, 2015). Worldwide demand for refined glycerol has grown from 0.9 million metric tons in 2009, to 2.0 million metric tons in 2015 (Chow and Ng, 2015). *Dunaliella tertiolecta* is a green microalga that has the ability to adapt to external changes in the environment by producing glycerol to act as a buffer to protect its cells. This glycerol can be harvested and used in the synthesis of biofuel and other products (Chow et al., 2013). *D. tertiolecta* has a flexible cell wall, allowing the cells to expand or shrink when in hyper or hypo-osmotic shock (Tafreshi and Shariati, 2009, and Chow et al., 2013). *D. tertiolecta* cells can do this instantaneously, allowing for rapid glycerol production following an osmotic shock. (Tafreshi and Shariati, 2009).

There are a number of viable species of microalgae that can be utilized for biofuel production, including *Dunaliella spp.* (Tafreshi and Shariati, 2009). Hydrocarbons are found in all algae, but at small amounts in some (*Chaetoceros calcirtans*) according to Mata et al. (2010), approximately 2% of their dry biomass (Lee, 2018). However, other such as *D. tertiolecta* can have up to 50% of their dry biomass composed of hydrocarbons (Chow et al., 2013). *D. tertiolecta,* can be induced to secrete more glycerol byproduct for harvesting and utilization purposes. Abiotic factors that cause *D. tertiolecta* to produce glycerol include changes in temperature, salinity, carbon content, nutrient availability, and variations in cell concentrations (Chow et al., 2013). Sustaining the proper nutrients and carbon concentrations are key variables for maximizing glycerol production by *D. tertiolecta* (Chow et al., 2013). There are two ways *D. tertiolecta* has been shown to produce glycerol: through photosynthesis or through starch storage (Goyal, 2007). Both of these glycerol production pathways require optimal conditions for photosynthesis and require nutrients (i.e. nitrogen and phosphorus) to be replete. Optimal conditions for photosynthesis of *D. tertiolecta* include having proper, light, nutrients, temperature, and salinity in order for the cells to be able produce glycerol.

Project Overview

The thrust of this project is demonstrating the feasibility of a marine based bioreactor capable of producing, extracting and purifying glycerol from the microalgae species, Dunaliella tertiolecta. Dunaliella tertiolecta, a saltwater microalgae species, has been shown to dedicate the majority of its fixed carbon dioxide to extracellular glycerol production. The leakage of glycerol across the cell membrane into the growth medium raise the possibility of extracting glycerol while maintaining a healthy algae culture – a major necessity for economical biofuel production from microalgae. Microalgae represent a potentially huge opportunity in offshore renewable energy generation and storage. Microalgae produce energy rich biofuels year round in marginal environments at rates in excess of 10 times that of other plants. If microalgae could be cultured and maintained remotely a huge quantity of biofuels that could be produced economically without displacing conventional agricultural products. The project studies the production of glycerin from algae with the intention of scaling up this system to farm scale production process which can produce commercial quantities of biofuel in a sustainable and efficient manner. Protocols for growth and production condition, including inexpensive growth media, fixation issues, glycerin monitoring systems and glycerin separation methods are issues being studied in this project.

There were several different efforts studied in these effort which are outlined in this report. These are:

- Development of a baseline growth media for the algae based on the species environmental conditions and availability of economical ingredients to fabricate an "artificial seawater" which could be used in a production farming operation.
- Development of cost effective instruments which could be used to infer glycerin content, salinity and carbon dioxide content in the media while the algae is producing. Refractive index, conductivity and CO2 sensors were selected as the indirect method of measurement.
- Development of a benchtop test systems and protocols to conduct fundamental research on the conditions required for glycerin production, including shocking effects using salt and other compounds.
- Development of a scale model through flow "farm" system which implements a continuous monitoring loop capable of measuring glycerin and key compounds such as salinity and CO2.
- Studies on the effects of salinity shocking on production as a method of enhancing glycerin production in algae farms.

The results of these studies produced the following results:

- A cost effective culture media was developed and utilized and the recipe reported.
- Instruments were procured for measuring refractive index, conductivity and CO2 were procured and used to generate calibration maps for monitoring glycerol percentage, salinity and CO2 in the growth media in real time.
- A scale model "farm" system was developed with through flow/continuous flow capabilities for monitoring media parameters previously described with a computer data

acquisition system. The system also explored capability to inject compounds to change salinity and enhance CO2 as a basic growth control system.

- Benchtop studies were conducted on the effects of salinity and NaOH shocking with results showing greatly enhanced glycerol production over producing glycerol in high salinity media held constant over time.

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Project History and Progression

Beginnings as a STEM project

Preliminary work on the development of an algae based glycerin fuel project was begun as an extension of the Diesel/Glycerin fuel project to develop a middle school science STEM project for use by teachers. The project uses a special algae which can produce and excrete significant quantities of glycerin. The Marine Engine Testing and Emissions Laboratory (METEL) at Maine Maritime Academy (MMA) acquired cultures of this algae and is developing a system to study the growth of this algae in a "Farm" situation in order to produce glycerin biofuel. The STEM project is developing a low cost, easy to use Algae farm kit, which students can use to study an optimize the production of this biofuel, processing into fuel and then burn it in a simple engine. Figure 1 shows our bioreactor for culturing the algae and the resulting culture.

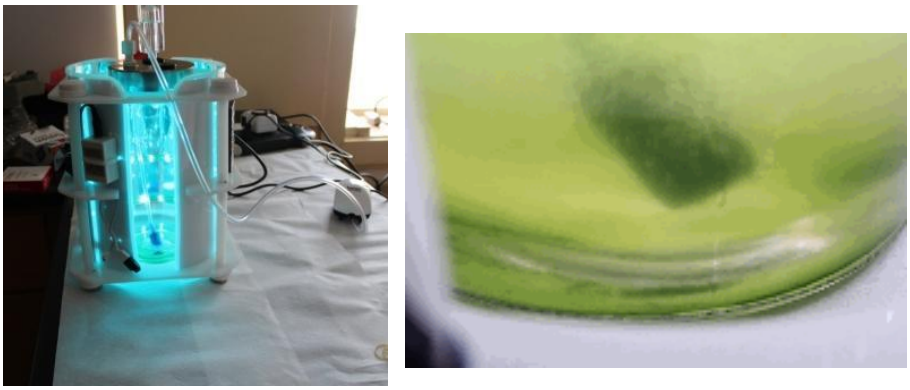

Figure 1: Algae Biofuel reactor and Algae culture

The project, though simple, touched on many important STEM areas and contemporary issues. The system promoted careful laboratory study techniques, requires the use of dimensional analysis to scale up the number to a real farm size, connects biological science with engineering, acts as a conduit to studying global warming using data and analysis as well as studying global energy security. The projects intent was to motivate students that by being good scientists and engineers they can solve major real world issues and make a difference to the planet.

Progression to a Research Project at METEL

After the success of the STEM project a formal research project was initiated ate METEL to determine whether glycerol, produced by microalgae, can be an energy positive (*i.e.* produce more energy than they consume) and economically competitive transportation fuel. Both points rely

heavily on minimizing the energy required to grow the algae and extract and purify fuel molecules.

Preliminary benchtop experiments

The first efforts on this research project was to obtain the algae and culture media from a standard supplier and start preliminary studies on the growing of the algae and producing glycerol.

Dunaliella tertiolecta, a saltwater microalgae species, was selected for the studies as it had been shown to produce glycerol extracellularly from fixed atmospheric CO_2. The leakage of glycerol across the cell membrane allows for the extraction of glycerol from the culture medium, without killing the algae. This was demonstrated on the small scale in Figure 2 for *Dunaliella tertiolecta*. Once separated, the glycerol-containing medium could be decanted off and replenished with fresh media.

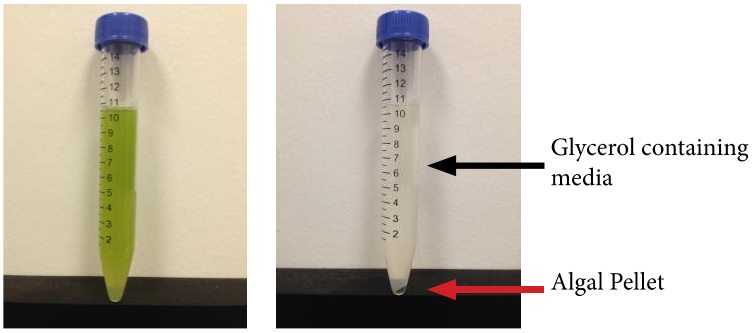

Figure 2. Test tube of mature culture of *Dunaliella Tertiolecta* in UTEX media before and after centrifugation.

Once refreshed with new media the remaining algae continue to grow in the new media and produce glycerol.[i] How the separation affects the rate of glycerol production is still under investigation, but assuming glycerol production is proportional to cell concentration this is a very positive result. It means that the targeted molecules can be separated from the algae by simple physical processes like centrifugation or membrane microfiltration and without a large expenditure of energy.

These Initial experiments focused on manipulating the algal chemical environment to realize glycerol production at minimum cost. Two hundred milliliters of 4 different solutions (UTEX, Provasoli's Modified Seawater, Duna Base and sterilized Penobscot Bay seawater) were inoculated with algae from 20ml of stock algae solution that had been chilled and centrifuged to remove the stock culture medium. Cultures were put in a sterile 500ml Erlenmeyer flask, covered with a gas permeable lid and placed under a grow lamp. Initially we have focused on media that did not contain significant fertilizing additives. A 17hr/7hr light/dark cycle at $160\ \frac{W}{m^2}$ illumination was maintained throughout the experiment. From the outset it was clear from qualitative observation that certain media (Provasoli's and PB Seawater) did not promote healthy culture growth. This was supported by measurements of algal cell concentrations, shown in Figure 3.

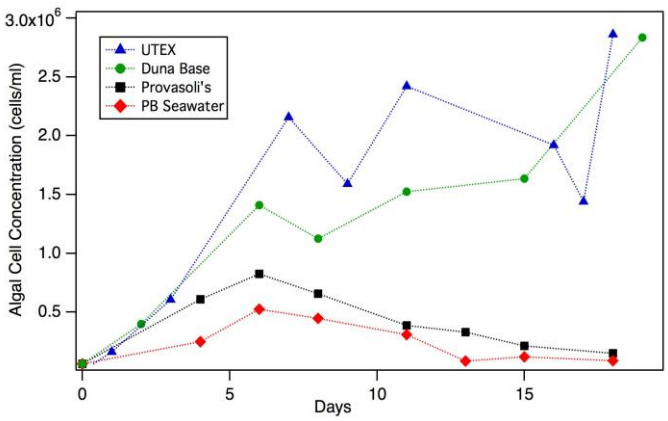

Figure 3. Hemocytometry measurements of algal cell concentrations in three standard microalgae cultures and sterilized Penobscot Bay Seawater.

Duna Base and UTEX medium proved to be significantly better than any of our other formulations. Interestingly Duna Base also proved to be chemically very simple. The lack of an active CO_2 source (besides atmospheric diffusion) and the presence of sodium bicarbonate ($NaHCO_3$, baking soda) in Duna Base targeted Duna for a second round of experiments. Figure 4 compares Duna Base with and without $NaHCO_3$. The results speak for themselves. Algae populations in the media without $NaHCO_3$ never took off. Experiments tweaking media formulations continue.

Unfortunately, little information was gleaned from these experiments in terms of the relationship between algal cell population and glycerol concentration. Based on our reading of the literature and due to instrumental constraints glycerol was quantified using a published chemical spectroscopic assay.[ii] A calibration curve was reproduced from the paper and the assay initially seemed to produce reasonable results, however about a month into experiments it was clear that glycerol measurements were not correct. An assay of the media, including UTEX and Duna Base, indicated significant glycerol concentrations confirming the suspected lack of assay specificity.

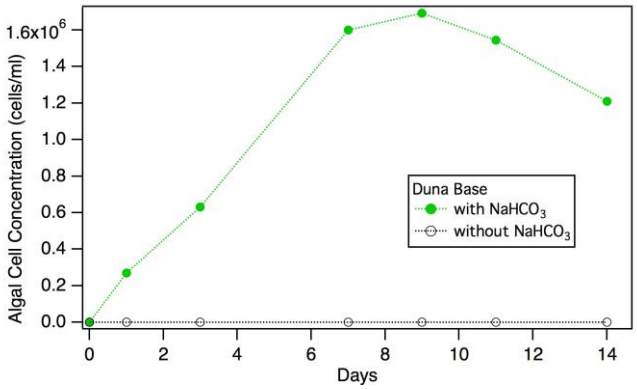

Figure 4. Graph plotting algal cell concentration versus time for Duna Base media with and without sodium bicarbonate (Baking Soda)

Development of indirect methods of assaying glycerol content

Desire for a simple practical method to assay the glycerol content in the media during these initial efforts directed the project towards identifying simple measurements of physical properties like viscosity and index of refraction could be correlated with glycerol concentration in spite of other changes that may be occurring in solution.

Experiments were carried out with the intent of generating experimental plots of viscosity and refractive index of extracellular solution versus glycerol concentration. Cells were first separated from the growth media by centrifugation. Viscosity of the resulting solution was then recorded manually using a Zahn cup (#1) and measuring the solution's efflux time. Refractive index was measured using a digital refractometer (Sper Scientific). Because of the difficulties in measuring glycerol concentration an experimental plots of refractive index and viscosity as a function of extracellular glycerol were not generated. Calibration plots of viscosity and refractive index over the range of extracellular glycerol concentrations reported in the literature, however, suggest that it is possible (Figure 5).

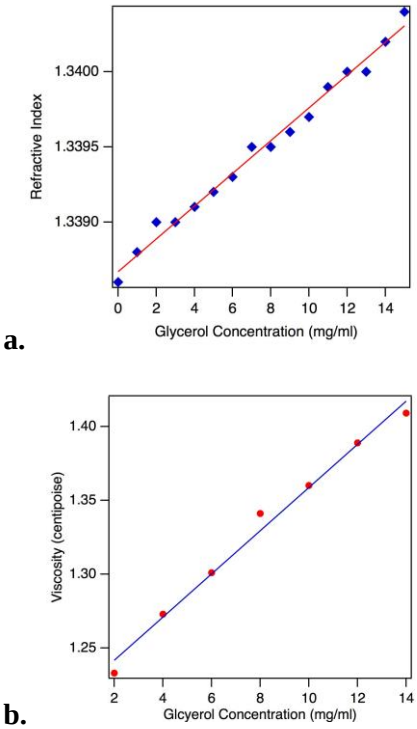

Figure 5. Plot of **a.** refractive index and **b.** of seawater media as a function of glycerol concentration.

Development of an NMR based Glycerol Assay Method

Initial experiments in this project focused on quantifying the amount of glycerol that could be produced and correlating those concentrations with easily measured physical properties of the algae culture like cell density, refractive index and viscosity. Those experiments continue. Attempts to utilize a spectrophotometric assay used for glycerol quantification was not reliable. A process was developed using a new Nuclear Magnetic Resonance (NMR)-based procedure for glycerol quantification that is both more sensitive (~10 micromolar/0.01 mg/ml), more reliable and requires less sample. To quantify the glycerol a 500µL sample of the algae is centrifuged in an eppendorf tube to separate the algae and the media. The media is decanted into another tube and centrivaped to drive off the water. The glycerol is then resuspended in deuterated water (D_2O) and spiked with a known concentration of pentachloroethane (PCE). Comparison of the PCE and glycerol proton peak integration is then used to quantify glycerol concentration.

Collaboration effort with Bigelow Laboratory

METEL also starting working with Bigelow Laboratory for Ocean Science's National Center for Marine Algae (NCMA). NCMA has the largest and most diverse collection of marine algae in the world and is an immense resource for algae culturing techniques on the small and large scale. NCMA is providing the culture and media for METELs experiments and consulting on METEL's algae maintenance, handling and culturing procedures.

The potential of this collaboration was significant. Many of the cultures that NCMA maintains secrete other small, oxygenated hydrocarbon molecules to the extracellular medium such as acetate and pyruvate and succinate (Fig. 6) as well as mono- and polysaccharides. METEL and NCMA are initiating a project to do a comprehensive chemical analysis of the NCMA's chemical library to identify and quantify these molecules and profile their potential as diesel fuel additives.

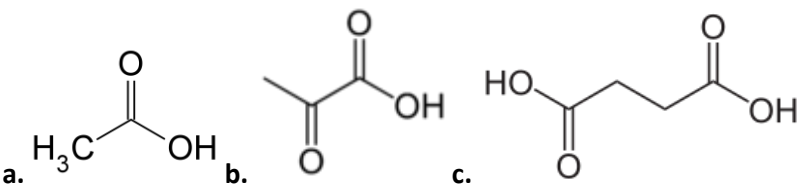

Figure 6. Skelatel structures of the protonated forms of **a.** acetate **b.** pyruvate and **c.** succinate.

Development of a continuous flow glycerol measurement system

What differentiates our approach from other groups working on algae biofuels is our focus on small molecules, like glycerol, that are expelled from the algae into the extracellular media. The goal of the project is to develop instrumentation for quantifying, characterizing and extracting small molecules without killing the microalgae culture. An initial step forward in instrumentation was the development of small-scale bioreactor programmed to automatically measure the temperature, conductivity and refractive index of the culture. Graphs of these variables, taken every twenty minutes for the first ten days of a culture of *Dunaliella tertiolecta*, are shown in Figure 7. Refractometry is a common method determining solute concentrations since refractive index (n) changes with solute concentration (C). The refractive index, however, also changes with salinity and temperature (T), as does the degree of change $\left(\frac{dn}{dC}\right)$. We are monitoring salinity, temperature and refractive index and in an effort to develop an algorithm for glycerol concentration. In the meantime a Nuclear Magnetic Resonance (NMR)-based procedure was developed for glycerol quantification that is both more sensitive (~10 micromolar/0.01 mg/ml), more reliable and requires less sample than conventional spectrophotometric assays.

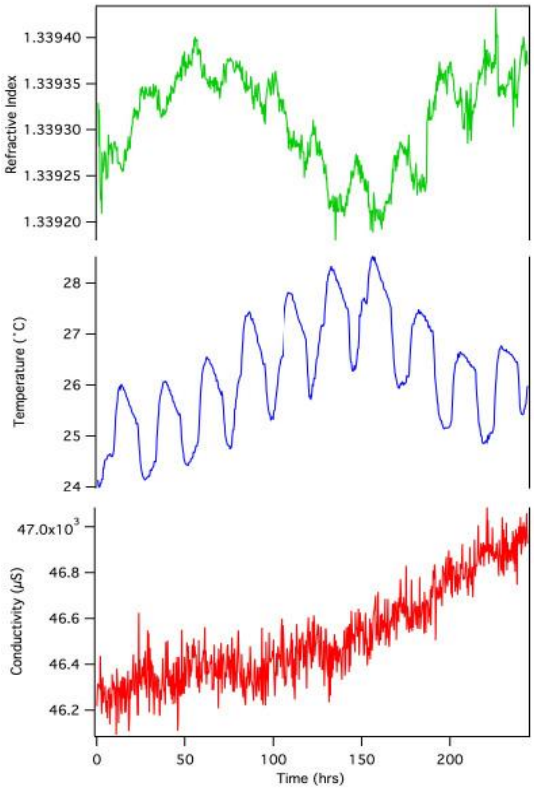

Figure 7: Data from instrumented METEL microalgae bioreactor. The periodic changes in temperature correspond to the 14/10 hr daily lighting schedule for the culture.

Figure 8 shows a calibration curve for Nuclear Magnetic Resonance (NMR)-based procedure for quantification of extracellular hydrocarbons.

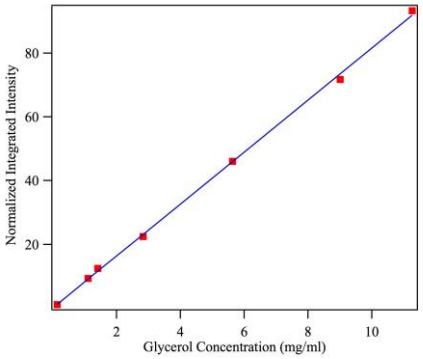

Figure 8. Normalized integrated intensity of peaks in NMR spectra attributable to glycerol versus glycerol concentration.

Algae production studies using various media formulations

Optimal environmental conditions for promoting the production of extracellular hydrocarbons in microalgae cultures remain elusive. Figure 9 shows a graph of glycerol production in two cultures of Dunaliella tertiolecta grown in L1 medias (https://ncma.bigelow.org/media/pdf/NCMA-algal-medium-L1.pdf) with differing nitrate and phosphate concentrations. However, even in fertilized cultures, glycerol concentrations have been inconsistent and significantly lower than what has been reported in the literature.

For example, Chow *et. al*. have investigated extracellular glycerol production in *Dunaliella tertiolecta* (UT Austin strain: LB-999) and reported concentrations as high 6 mg/ml using a modified ATCC–1174 DA containing 2M NaCl.

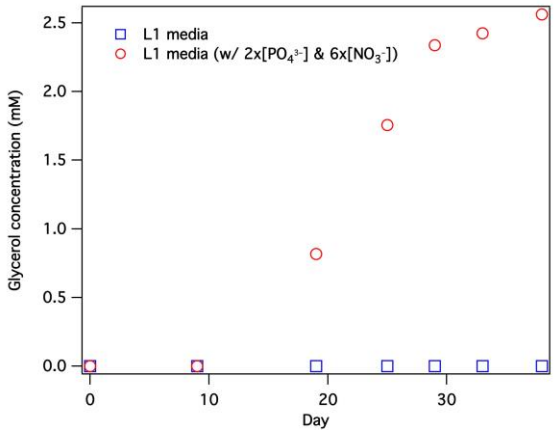

Figure 9. Concentration of extracellular glycerol versus age of microalgae culture (*Dunaliella tertiolecta)*

Our efforts to reproduce these results using the identical strain (National Center for Marine Algae: CCMP364) have not been successful. Various salinities (0.5-2.0M), phosphate and nitrate concentrations($[PO_4^{3-}] = 0.4 - 2 \times 10^{-4}M, [NO_3^-] = 0.9 - 5 \times 10^{-3}M$) and light intensities have been investigated, but the highest glycerol concentrations remain <1 ppm. A follow up publication from the same research lab suggests that the large differences in observed glycerol concentrations are not likely attributable to micronutrient ($e.g. Mn^{2+}, Cu^{2+}, Zn^{2+}, etc.$) concentrations.

Algal cell concentrations, however, have been shown to have a large effect on glycerol concentration. Our cultures have had issues maintaining high cell counts beyond 2-3 weeks. To this point little has been done to control dissolved CO_2 concentration, pH and temperature, which are all potential culprits. Cell counts from recent temperature controlled experiments are shown in Figure 10.

Calibration Refractive index and Ph vs. Glycerol content in base growth media

Figure 11 shows the calibrations for refractive index and pH for the concentration of glycerin in the algae growth media. This data was used to calibrate instruments in the algae farm system for continuous monitoring of glycerin production in the media in real time.

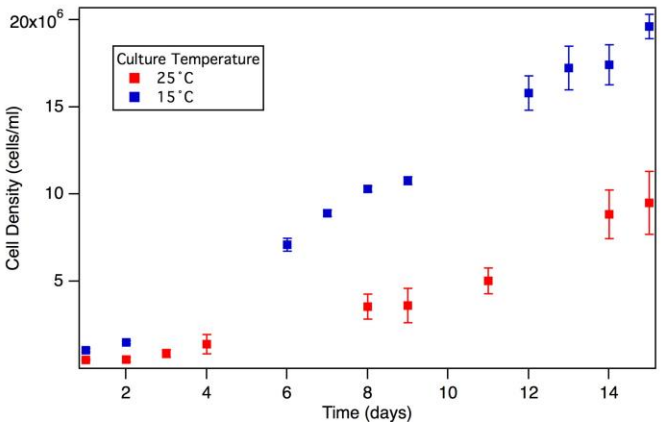

Figure 10: Cell density for *Dunaliella tertiolecta* cultures (identical chemical environments) measured at two different temperatures.

The use of the calibrations methods using refractive index, conductivity and pH (RI/S/pH)are being developed and calibrated against known samples using the base media and validated using a high end GCMS systems due to arrive at MMA in December 2017. The RI/S/pH system is a vital part of the development of a farm grade monitoring system for detecting glycerin that is simple,

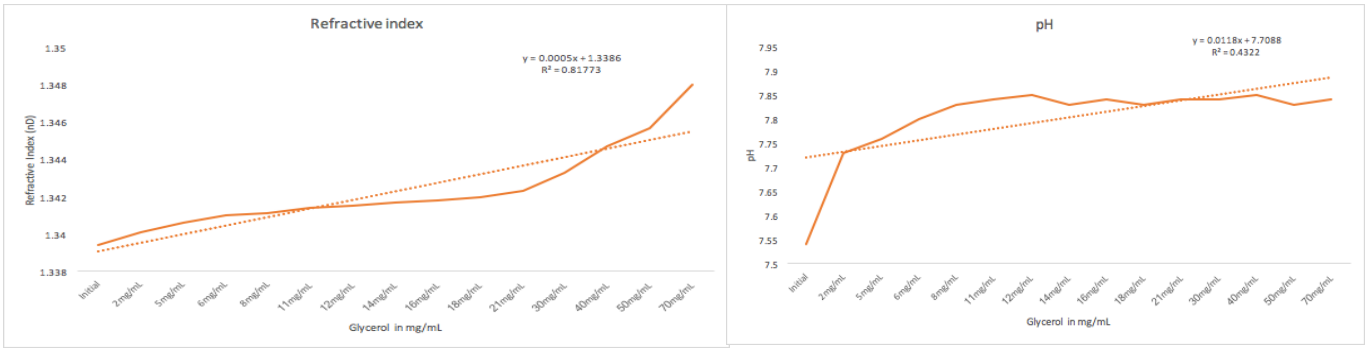

Figure 11. Measurements of refractive index & Ph to media samples with the addition of pure

Development of a scale model continuous flow algae "farm" system

Figure 12 shows the mock farm style system with continuous data monitoring and carbonate control system. Shown also in figure 12 is the control schematic for this system. This system allows us to move away from the flask based laboratory experiments to a small scale algae farm production system for development and optimization of the algae glycerin production on a system that could be scaled to a larger agricultural scale.

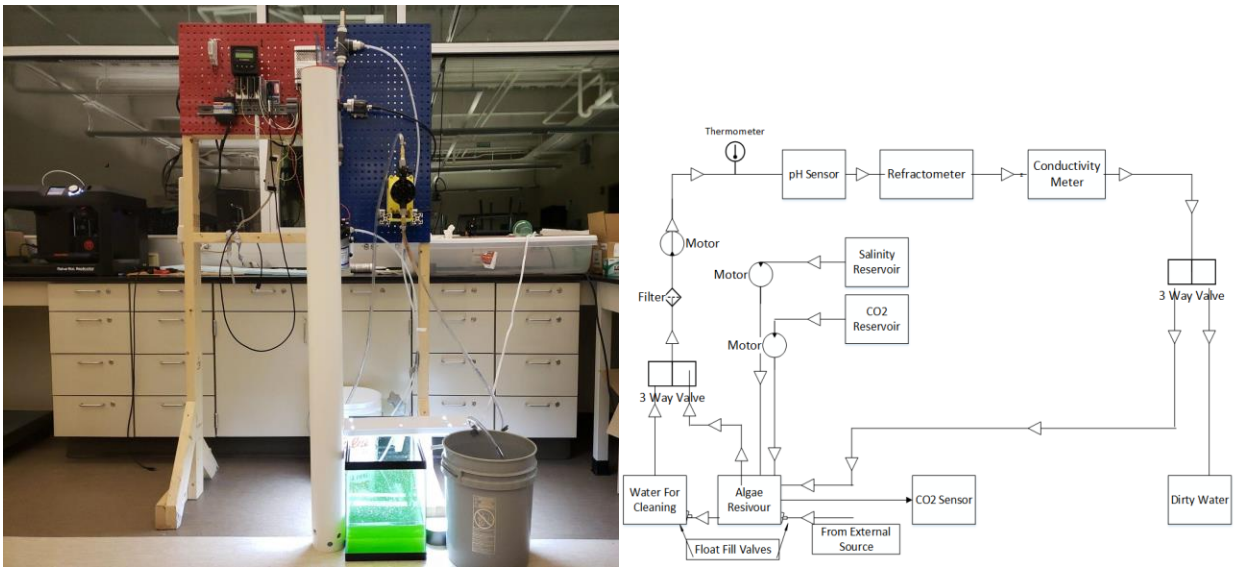

Figure 12: Mock "farm" style Algae system with continuous monitoring system Actual system shown with control schematic.

The system includes PH monitoring for CO2 measurement, conductivity for salinity measurement and Refractive index for Glycerin content estimation and allows studies on CO2 effects, salinity effects and osmotic shocking experiments and control of the glycerin harvest intervals.

Quantifying glycerol concentrations produced by the marine microalga, Dunaliella tertiolecta, under hyperosmotic conditions

Experiment Overview

Figure 13: Osmotic shocking test setup

. The microalga *D. tertiolecta* was chosen for this study because of its previously demonstrated rapid glycerol production in response to hyperosmotic shocking. Figure 13 shows the culture setup for the experiment. The microalga *D. tertiolecta* was cultured and hyperosmotically shocked over a 4 hour period to induce glycerol production. The glycerol was measured and quantified utilizing the Nash method of glycerol oxidation. Hyperosmotic shocking solutions of 1 M, 3 M, and 5 M NaCl with and without $NaHCO_3$ to provide additional carbon were applied to the algal cultures. *D. tertiolecta* produced glycerol under hyperosmotic conditions and the glycerol production increased over

time for all three NaCl treatments without additional $NaHCO_3$. The glycerol produced reached 2-3 mg mL^{-1}, similarly to other studies of the same nature. This mass of glycerol production is promising for future biofuel production and warrants further research. The microalga *D. tertiolecta* is a viable candidate for biofuel synthesis

There is evidence that hyperosmotic shocking increases the glycerol production by *D. tertiolecta* (Goyal, 2007; Chow et al., 2013). Hyperosmotic shocking appears promising for obtaining the highest volume of glycerol from *D. tertiolecta* (Goyal, 2007). According to Goyal (2007), glycerol is produced by *D. tertiolecta* under salt stress during photosynthesis, as well as starch breakdown. Goyal (2007) indicated that glycerol production by *D. tertiolecta* during salt stress can occur in the first 10 minutes after hyperosmotic shocking, and that within an hour of hyperosmotic shocking *D. tertiolecta* production of intracellular glycerol increases from less than 10 µmol mg^{-1} to nearly 50 µmol mg^{-1}. If one can optimize growth conditions and utilize osmotic shocking to produce glycerol from *D. tertiolecta*, the glycerol could then be used in the production of biofuels (Chow et al., 2013).

In this study, glycerol production by *D. tertiolecta* was measured and quantified under hyperosmotic conditions with and without additional carbon to aid carbon fixation during photosynthesis. The additional carbon can be used by *D. tertiolecta* because it is photoautotrophic, which means it uses CO_2 and bicarbonate (HCO_3^-) as inorganic carbon sources (Tafreshi and Shariati, 2009). Utilizing this additional carbon has been shown previously to allow for greater glycerol production during hyperosmotic shocking (Chow et al., 2013). Hyperosmotic shocking is cost effective and easily achieved, and NaCl salt is less expensive and easier to come by than potential nutrients (e.g. nitrogen or phosphorous) that are also used to induce glycerol production (Chow and Ng, 2015). This study aimed to quantify the change in glycerol production over time after hyperosmotic shocking of *D. Tertiolecta*. The range of NaCl concentrations in which *D. tertiolecta* can survive is 0.1 M – 5 M (Chow et al., 2013); thus treatments of 1 M, 3 M, and 5 M were chosen to assess glycerol production. A separate set of treatments of 1 M, 3 M, and 5 M NaCl contained $NaHCO_3$ to determine whether additional carbon in the system had an effect on glycerol production.

Methods

Culturing

A culture of *D. tertiolecta* was obtained from the National Center for Marine Algae and Microbiota (NCMA) at the Bigelow Laboratory for Ocean Sciences. This culture has been maintained at the Marine Engineer Testing and Emissions Laboratory (METEL) at Maine Maritime Academy since September, 2018 and has been utilized for this project. These microalgae were cultured in a 0.5 M NaCl solution and the chemical and physical growth conditions were modified from Chow et al. (2013). *D. tertiolecta* was cultured in 500 mL beakers, with 100 mL of the medium. The beakers were used in order to optimize light utilization by the *D. tertiolecta*. The culture medium was prepared to mimic ocean water.

Culture Medium

L1 culture media (Table 1) was made in 1 L of deionized (DI) water. This media was used as a stock solution to grow the *D. tertiolectas*. The media was autoclaved and sodium

bicarbonate ($NaHCO_3$) was added following the autoclaving. The sodium bicarbonate was added for additional carbon that could be utilized by the *D. tertiolecta* for carbon fixation. The cultures were illuminated using cool white LED lights, under a 16h/8h - Light/Dark cycle (light intensities were not measured). This photoperiod was meant to be the optimal sunlight condition imitation and was slightly modified from Chow et al. (2013) who had used a 17h/7h period.

Table 1: L1 Media components used for the growth of *D. tertiolecta.*

Media components (Per 1 L)	Volume (mL)	Mass (g)
$NaNO_3$ (8.82×10^{-4} M)	6	N/A
$NaH_2PO \cdot H_2O$ (3.62×10^{-5} M)	3	N/A
Vitamin Solution	1	N/A
Trace Metal Solution	1	N/A
Ocean Pure	N/A	34
DI water	1000	N/A
Sodium Bicarbonate	N/A	6.8

Cell Concentration

Cultures of *D. tertiolecta* were inoculated with 25 mL of algae from a stock culture and 75 mL of the L1 medium. The cultures were grown for two weeks prior to osmotic shocking. Cell counts were conducted to monitor the cell growth over the two week period using a compound light microscope (Nikon) at 400x magnification. Cells were counted by placing 1 µL of the algal culture onto a hemocytometer slide with a drop of Evans blue dye and a drop of Lugol's (iodine). Lugol's caused death for the *D. tertiolecta* and Evans blue dye stains the cells for better visibility on the microscope.

Osmotic shocking

To test salt stress on glycerol production, media with NaCl concentrations of 1, 3, and 5 M were prepared according to Table 2. The algal cultures were shocked by adding 25 mL of the algal culture to 75 mL of each shocking treatment. Samples of 2 mL were taken from each of the shocking treatments every 20 minutes for an hour, one at 2 hours, and for a final time at 4 hours. There were 6 shocking treatments made, a 1 M, 3 M, and 5 M NaCl treatment with no additional salts, and 1 M, 3 M, and 5 M NaCl treatment each with additions of sodium bicarbonate ($NaHCO_3$) (Table 2). The additional carbon was utilized into order to determine if excess carbon would aid in and increase glycerol production. For each of the six shocking solutions three replicates were taken at each sampling time point. Once hyperosmotic shocking was complete, samples were centrifuged to extract the supernatant for glycerol measurements. The samples were stored in a freezer (-18°C) to halt the glycerol production and for later glycerol testing.

Table 2: Hyperosmotic shocking solutions used for hyperosmotic shocking the microalga *D. tertiolecta.* Made with Di water, NaCl, and Na HCO_3^-.

Hyperosmotic shocking Media concentrations (Per 1 L)	NaCl (g)	Sodium Bicarbonate Na HCO_3 (g)
Initial	32	6.8
1 M	58.4	6.8
3 M	175.2	6.8
5 M	292	6.8
1 M + Na HCO_3	58.4	13.6
3 M + Na HCO_3	175.2	13.6
5 M + Na HCO_3	292	13.6

Glycerol oxidation technique and measurement

Methods described by Bompelly and Skaf (2014) were used to measure glycerol by oxidation and spectrophotometry. Components required for this technique (Table 3) included: 0.05 M acetic acid, 2.0 M ammonium acetate, 5 mM sodium periodate, 40 mM potassium iodide, and 80 mM sodium thiosulfate. The oxidation of glycerol to formaldehyde, and finally to diacytldihydrolutidine (DDL) were measured with a spectrophotometer. The first step to accomplish the glycerol oxidation was to add 1 mL of 5 mM sodium periodate to 2 mL of supernatant containing glycerol. The solution of glycerol and sodium periodate was shaken for 5 minutes in a 30°C water bath. Immediately after shaking, 1 mL of 40 mM potassium iodide and 1 mL of 80 mM sodium thiosulfate were added to the glycerol and sodium periodate solution. Two mL of this solution was removed and added to 2 mL of the 0.2 M acetyl acetone solution. The acetyl acetone solution (Table 4) was made using 37.5 g of ammonium acetate, 0.75 mL of glacial acetic acid, and 0.50 mL of acetyl acetone. The ammonium acetate, glacial acetic acid, and acetyl acetone was mixed with 250 mL of DI water (Bompelly and Skaf, 2014). The solution was heated in a vial for 10 minutes in a 60°C water bath to develop the yellow diacytldihydrolutidine (DDL) analyte. The solutions were cooled in water to stop the reaction. According to Bompelly and Skaf, (2014) the glycerol oxidation reaction produces two formaldehyde molecules for each glycerol molecule. The reaction also produces two iodate anions, from two periodate anions, and one formic acid molecule:

$$C_3H_8O_3 + IO_4^- \rightarrow HCHO + CH_3COOH + IO_3^- + H_2O$$

$$CH_3COOH + IO_4^- \rightarrow HCHO + HCOOH + IO_3^-$$

Table 3: Glycerol oxidation materials were utilized to oxidize the glycerol and produce diacytldihydrolutidine which was then measured using a spectrophotometer.

Glycerol oxidation materials (in 10 mL of DI water)	Volume (mL)	Mass (g)
5 mM Sodium Periodate	N/A	0.0106
40 mM Potassium Iodide	N/A	0.0664
80 mM Sodium Thiosulfate	N/A	0.1265

Table 4: Acetyl acetone solution components, was used during the glycerol oxidation.

Acetyl Acetone Solution (in 250 mL DI water)	Volume (mL)	Mass (g)
2 M Ammonium Acetate	N/A	37.5
0.05 M Glacial Acetic Acid	0.75	N/A
0.02 M Acetyl Acetone	0.50	N/A

Absorbance measurements

The samples of the glycerol oxidation reaction were measured using a spectrophotometer (Thermo Scientific, model: 335903-006). The acetyl acetone solution was used to blank the spectrophotometer for the absorbance measurements. Absorbance measurements were performed using a UV/VIS spectrophotometer set at 410 nm. Bompelly and Skaf (2014) noted error in absorbance measurements can be introduced by certain monosaccharides that can be produced by a microalgae; specifically dextrose, fructose, galactose, sorbitol, and tagatose can all produce a yellow color when this method of glycerol measurement is used. To account for this source of error Barfoed's Test for monosaccharides was conducted to determine if the solution contains monosaccharides previously mentioned.

Barfoed's Test for monosaccharides

Barfoed's Test for monosaccharides employs a solution of 70 g of copper acetate, and 10 mL of glacial acetic acid in 1 L of DI water (Barfoed's reagent). The Barfoed's reagent (Table 5) was mixed with medium taken from the cultures of *D. tertiolecta* in a 3 to 1 ratio: 30 mL of Barfoed's reagent to10 mL of the algal medium. Reducing monosaccharides were oxidized by the copper ions in solution, to produce carboxylic acid, as well as copper oxide, which took the form of a red precipitate:

$$RCHO(aq) + 2Cu^{2+}(aq) + 2H_2O \rightarrow RCOOH(aq) + Cu_2O\downarrow + 4H^{+}(aq)$$

The mixture was boiled, and observed to determine whether there was a change in color and formation of copper oxide. A solution with fructose and DI water was also prepared to use as a positive control for comparison to the samples of the L1 medium from the *D. tertiolecta* cultures.

Table 5: Barfoed's Reagent was used to assess if the microalga or medium had monosaccharides which could possibly have produced false positives during the glycerol oxidation phase.

Barfoed's Reagent (in 1L of DI water)	Volume (mL)	Mass (g)
Copper Acetate	N/A	70
Glacial Acetic Acid	10	N/A

Creating formaldehyde and glycerol standard curves

A formaldehyde standard curve was made in order to determine to the concentration of formaldehyde via DDL produced by the cultures of *D. tertiolecta.* The formaldehyde curve was made by creating solutions of 0.1 mM - 1 mM formaldehyde (which is approximately the amount to be produce by the glycerol oxidation reaction) and mixing with the acetyl acetone solution, which produced DDL to measure using a spectrophotometer. A glycerol standard curve (Figure 14) was made to compare to the samples collected and tested from the *D. tertiolecta* medium. Glycerol was added to media made for the algal cultures and the glycerol oxidation procedure was conducted on the samples.

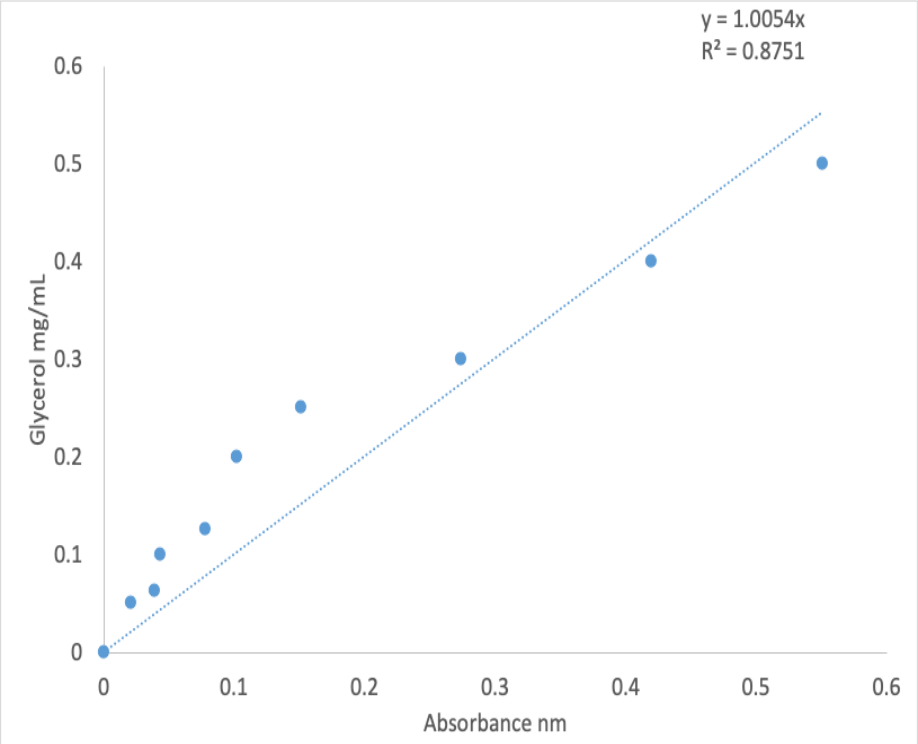

Figure 14: A glycerol standard curve was made using the L1 medium and glycerol. It was then oxidized, using the method of Bompelly and Skaf (2014). The samples were then measured using a spectrophotometer, and a standard curve was made to estimate glycerol concentrations from the *D. tertiolecta* shocking samples.

Statistical analysis

SPSS was used for all statistical analysis. A 2-Way analysis of variance (ANOVA) was conducted in order to compare the change over time for each different NaCl concentration treatment. It was also used to determine if there was a difference between the time and concentration on glycerol production.

Sources of error

Potential sources of error include contamination of a culture or improper sterilization techniques. The autoclave is used to mitigate any contamination that could affect the cultures of *D. tertiolecta*. The glycerol oxidation can also potentially introduce error. Bompelly and Skaf (2014) noted error can be caused by certain monosaccharides which produce a yellow color when this method of glycerol measurement is used. However, a test for monosaccharides was utilized in order to determine whether there were monosaccharides in the media. The presence of monosaccharides is measured very quickly using this technique; if there were disaccharides in solution they take much longer to reduce. If the reaction is run too long the disaccharides may be measured, rather than the monosaccharides. The glycerol standard curve was not linear, and could account for error in the estimation of glycerol produce by *D. tertiolecta*, however the R^2 value = 0.8751, which was acceptable for this study.

Results of hyperosmotic shocking studies

Osmotic shocking of *D. tertiolecta* yielded glycerol. The greatest amount of glycerol was produced between two and four hours from the 1, 3, or 5 M NaCl shocking solutions (Figure 15). Samples with $NaHCO_3$ showed high glycerol concentrations at time zero (Figure 16). While the cultures that were hyperosmotically shocked, the base culture showed no production of glycerol. The greatest amount of glycerol was produced overall by the *D. tertiolecta* shocked with 5 M NaCl and additional $NaHCO_3$ at time zero. The production was approximately 3 mg mL^{-1} (Figure 4).

Levene's test of variances was conducted and resulted in $p<0.001$, which meant the data were not homogenous. However, there is no other test that is comparable to a 2-Way ANOVA for such a data set, and the 2-Way ANOVA can still be applicable. The tests between the

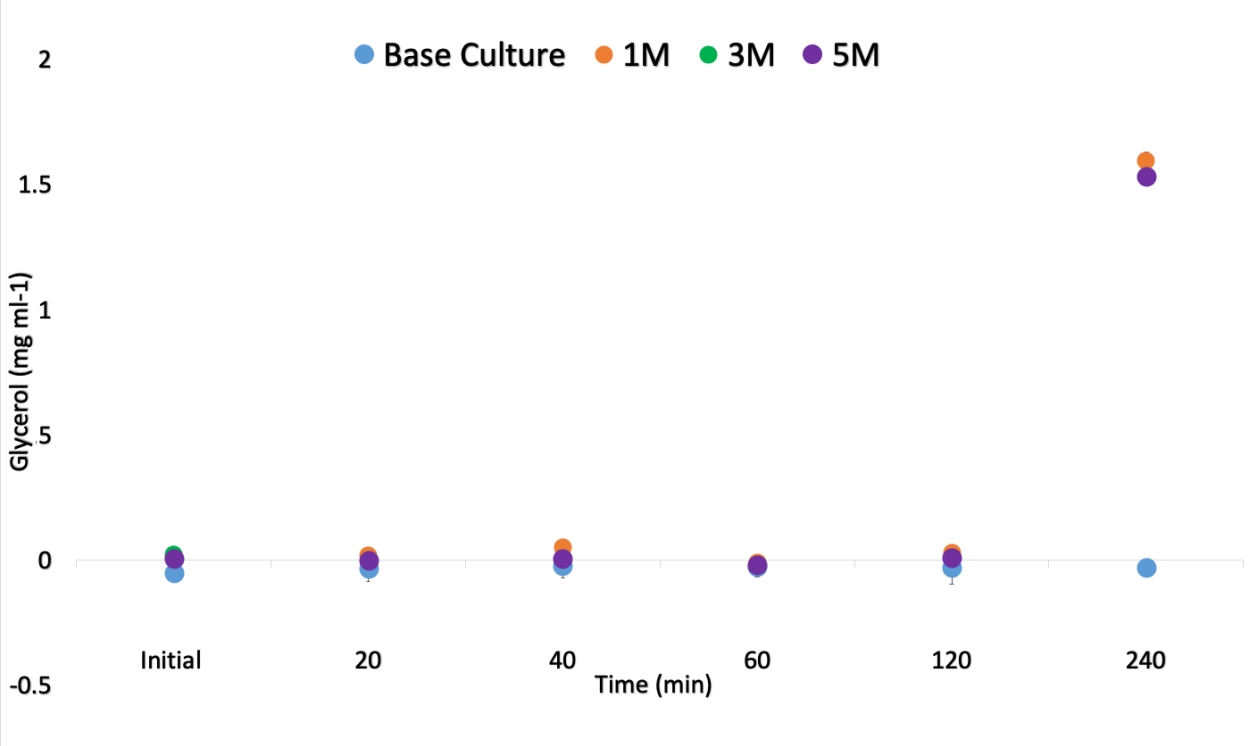

Figure 15. Glycerol produced during hyperosmotic shocking of *D, tertiolecta*. There was a significant difference in glycerol production over time, over the different treatments, as well as treatments over time. This means that a treatment can be selected to produce the greatest amount of glycerol in as well as a certain amount of time can be chosen to maximize the glycerol production. (2-Way ANOVA: $F_{5,30}= 128.435$, $p<0.001$, there is a significant difference between the different times in which glycerol was produced. Tests between subjects for treatment, $F_{6,30}= 14.028$, $p<0.001$, there is a significant difference between the treatments on production of glycerol by D. tertiolecta. Tests between subjects for time*treatment: $F_{30,30}= 42.752$, $p<0.001$, there is a significant difference between glycerol production over both time and treatment.)

different times showed that over time, there was a significant difference between the glycerol produced between time 0 and time 240 min ($F_{5,30}$= 128.435, p<0.001).

Tests across treatments showed that the different shocking treatments demonstrated a significant difference between the all shocking treatments on production of glycerol by *D. tertiolecta* compared to the base culture samples: ($F_{6,30}$= 14.028, p<0.001). The base culture showed no change over the four hours. The treatments that were of the same subsets (NaCl or NaCl + $NaCHO_3$) showed no significant difference between glycerol concentrations.

Tests between subjects for time*treatment showed a significant difference between glycerol production over both time and treatment: ($F_{30,30}$= 42.752: p<0.001). However, only the shocking treatments with NaCl only showed a significant difference over all times*treatments. The shocking treatments with $NaHCO_3$ showed a significant difference at time 0*treatment, and at no other intervals.

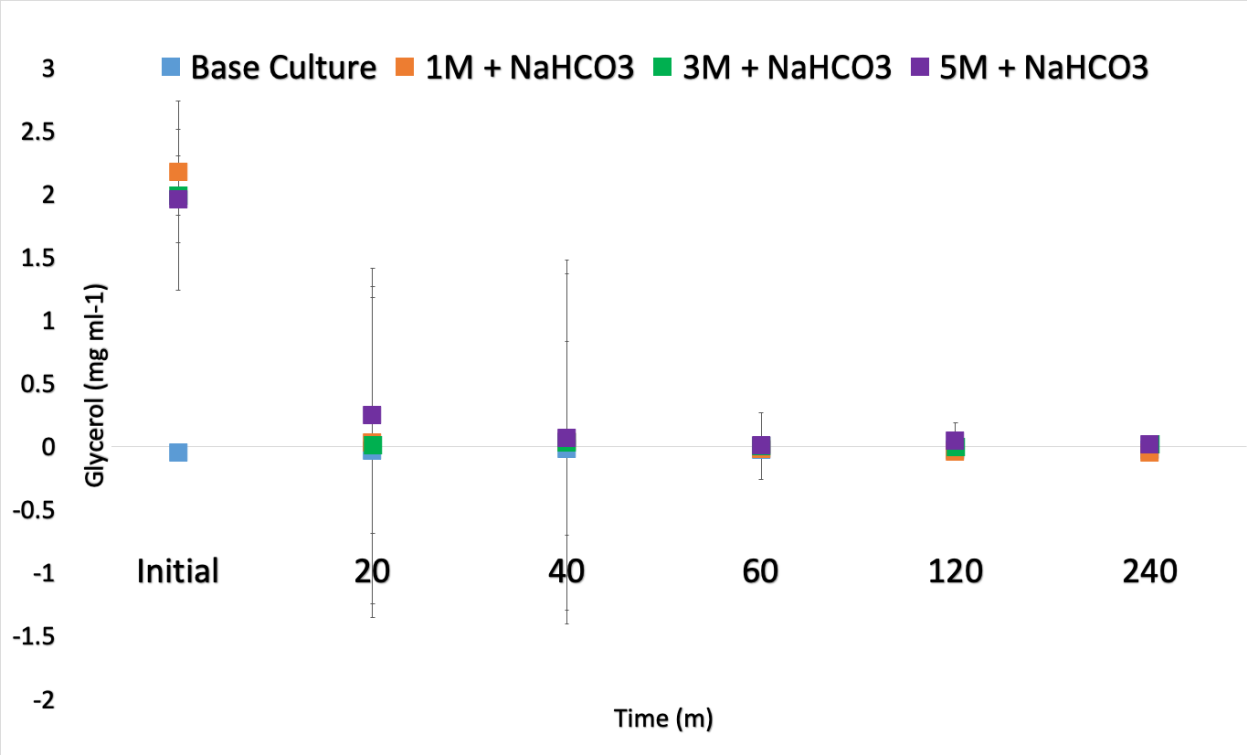

Figure 16. Glycerol produced during hyperosmotic shocking of *D, tertiolecta* with additional $NaHCO_3$. There was a significant difference in glycerol production over time, over the different treatments, as well as treatments over time. This means that a treatment can be selected to produce the greatest amount of glycerol in as well as a certain amount of time can be chosen to maximize the glycerol production. (2-Way ANOVA: $F_{5,30}$= 128.435, p<0.001, there is a significant difference between the different times in which glycerol was produced. Tests between subjects for treatment, $F_{6,30}$= 14.028, p<0.001, there is a significant difference between the treatments on production of glycerol by D. tertiolecta. Tests between subjects for time*treatment: $F_{30,30}$= 42.752, p<0.001, there is a significant difference between glycerol production over both time and treatment.)

Discussion of hyperosmotic shocking study results

The microalga *D. tertiolecta* was successful in producing glycerol over time under hyperosmotic conditions (Figure 15) with the exception of the cultures shocked with additional $NaHCO_3$ (Figure 16). The base culture did not show a change over time, as there was no glycerol being produced. This is a good indication that indeed the hyperosmotic shocking is crucial for production of glycerol by *D. tertiolecta*. The amount of glycerol produced is promising for
future utilization for biofuel synthesis. The fact that there was no real difference in glycerol produced between the different NaCl treatments, both with and without $NaHCO_3$, means that any of these treatments could be used for hyperosmotic shocking of *D. tertiolecta*. It would be beneficial to conduct a shocking procedure using only 1 M NaCl, because this would require less product being used to induce the same results.

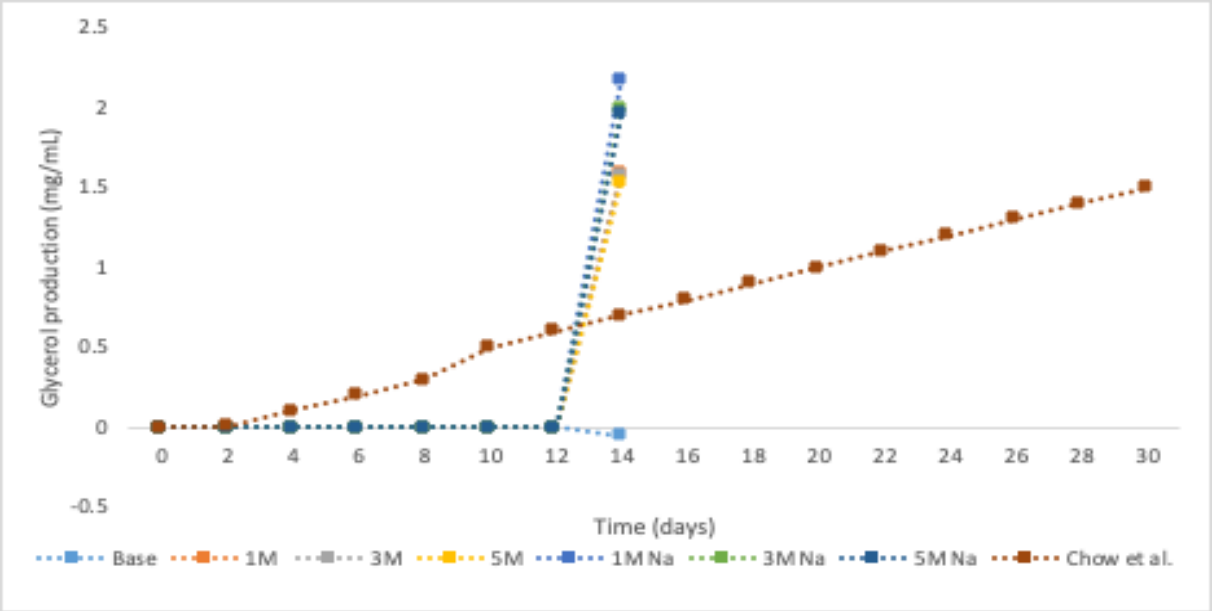

Figure 17. Comparison of glycerol production over a 30 day time period, one utilizing sudden hyperosmotic shocking (this study), and the other growing the microalga *D. tertiolecta* under hyperosmotic conditions throughout the experiment (Chow et al., 2013). The microalgae that was suddenly shocked showed rapid glycerol production, and in a much shorter

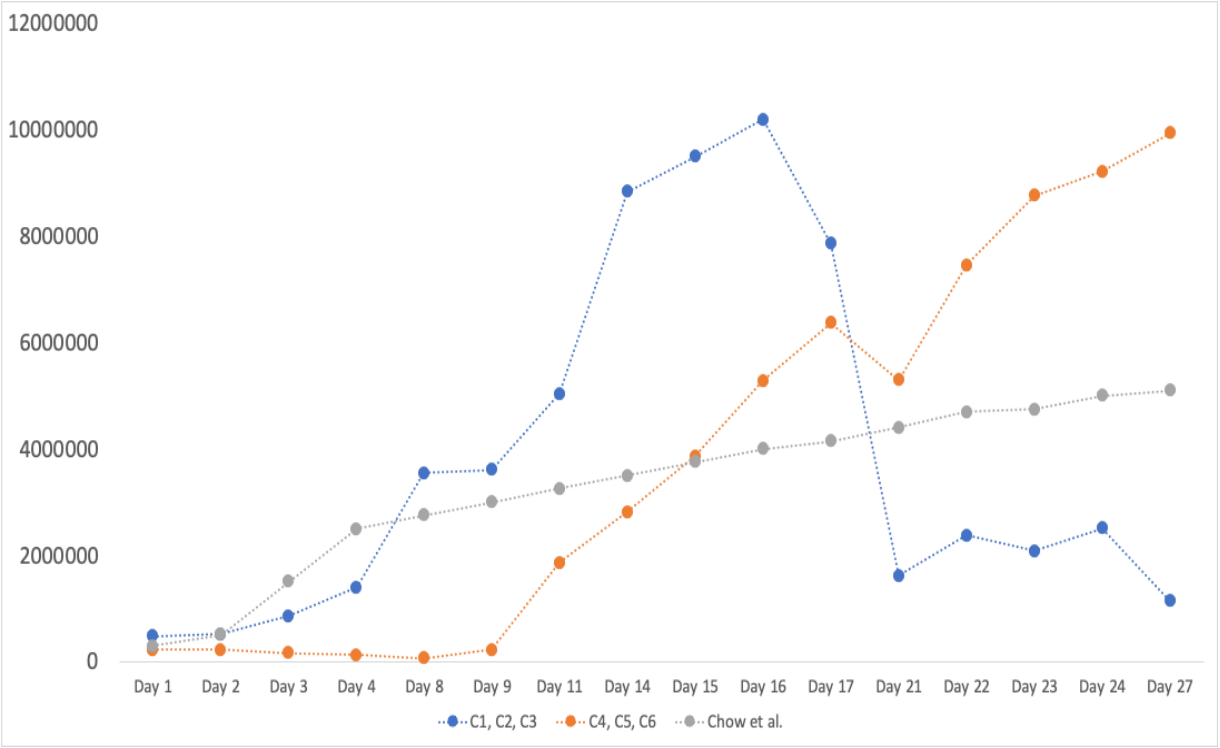

Figure 18. Cell concentrations were measured for cultures in order to estimate their cellular concentrations, to assess a good time to hyperosmotic shock the microalga *D. tertiolecta.* The microalga showed a high growth rate, maximizing between day 11 to day 27. For this study the cultures had been growing for approximately 14 days prior to hyperosmotic shocking, and this study showed faster growth rates for the microalga, compared to Chow et al., 2013, the growth conditions were the same aside from the continuous growth in 2 M NaCl, compared to this project

The glycerol produced reached approximately 3 mg mL^{-1}, which is similar to glycerol production other studies have shown (Figure 17), (Chow et al., 2013). The fact that glycerol is being produced in a short time period between 2 and 4 hours is a good sign for the ability to use the microalga for glycerol production. The higher cell concentrations (Figure 18), along with more rapid glycerol concentrations compared to Chow et al., 2013, is a good sign that this microalga is viable for utilizing as a producer of glycerol. It would be prudent to see if this increase would continue over a longer period of time, or if the glycerol concentrations decrease. *D. tertiolecta* has the ability to reuptake glycerol from the surrounding medium, and could potentially secrete glycerol, and then reabsorb it (Lin et al., 2013).

The *D. tertiolecta* that was shocked using the treatments with $NaHCO_3$ may have reabsorbed the glycerol as a response to stress, Lin et al. (2013) found that as a response to stress additional glycerol added to a shocking solution could be reabsorbed by the *D. tertiolecta.* This could account for the lack of glycerol shown by the *D, tertiolecta* shocked with NaCl and $NaHCO_3$.

There is evidence that the excess CO_2 in the treatments with $NaHCO_3$ allowed the microalga to synthesize glycerol very rapidly. Tafreshi and Shariati (2008) suggest that sodium hydroxide (NaOH) acts as a flocculant causing *D. tertiolecta* to clump together. The excess $NaHCO_3$ could have caused the alga to behave in a similar way, as the additional Na^+ may have created additional NaOH in solution, which may have induced production of glycerol instantaneously and not over time as predicted. The result of the shocking treatment with $NaHCO_3$ was not anticipated; however, it may be a useful way of extracting glycerol from these microalga. If they are made to flocculate, and the excess CO_2 also allows them to synthesize

glycerol more readily and quickly, perhaps this method could be used when attempting to extract glycerol from the algal medium.

Mata et al. (2009) estimated the potential production of different microalgae, including *D. salina* and *D. tertiolecta.* The estimates for *D. salina* are very similar to the amount produced by *D. tertiolecta. D. salina* produced approximately 116 mg of lipid L^{-1} day^{-1} (Mata et al., 2009). *D. tertiolecta* produced approximately 125 mg of glycerol L^{-1} day^{-1}. The productivity estimate of *D. salina* for biomass in g m^{-2} day^{-1} is approximately 1.6-3.5/20-38 (Mata et al., 2009). *D. tertiolecta* appears to be a medium oil content microalga, which has the potential to produce 97,800 L oil ha^{-1} $year^{-1}$. Knowing the amount of glycerol that could be produced under the correct conditions is crucial to utilizing *D. tertiolecta* for biofuel synthesis.

Land use for microalgal production of biofuels necessary for large scale production. Estimates by Hannon et al. (2010) suggest that 30 million acres of land could be required in order for biofuel production to compete with petroleum products. Finding the space for such production could prove to be a challenge. A difficulty faced by scientists attempting to grow microalgae for biofuel production is creating a photobioreactor (PBR) that is large enough for large scale production, but not unfeasibly expensive or difficult to run or build (Hannon et al., 2010). Another issue those creating biofuel face using microalgae is an inexpensive way to extract lipid byproducts from the culturing solution in which the mircoalgae are being grown (Hannon et al., 2010) Improving the engineering behind biofuel production is an important step for being able to utilize microalgae in the future for biofuels. Biologists, chemists, and engineers must be able to work together and find a microalgae that can grow in an open system with a practical oil yield, that can grow in an inexpensive setting (Hannon et al., 2010). Being able to optimize a large scale system for biofuel production using microalgae is crucial for future utilization of biofuels.

Biofuels are efficient, and they can reduce greenhouse gas emissions; however they are still more expensive than comparable fossil fuels or land-based oil fuels. If algae-based fuels price can be lessened it could create much more biofuel than any land based crop. There are certain attributes that can make a microalgae more economically viable for using a biofuel source. Microalgae have many advantages compared to land based plants, primarily because microalgae are single celled organisms they reproduce are cultures grow much more quickly than land-based plants (Hannon et al., 2010). Some other favorable characteristics include salt tolerance, growth at high pH, improved nutrient usage, and the ability to be harvested in an efficient way e.g. flocculation (Hannon et al., 2010). *D. tertiolecta* has demonstrated these characteristics according to Young et al. (2001) the microalga can be cultured in a pH up to 9.5. It has also been shown that the microalga can withstand salinity up to 5 M NaCl (Chow et al., 2013). Studies have also shown that *D. tertiolecta* can produce greater amounts of glycerol when in the presence of certain nutrients, Chow and Ng (2015) found that Mg utilization by *D. tertiolecta* increases glycerol production over time. The flocculation of *D. tertiolecta* cells in the presence of NaOH could potentially be useful for harvesting the microalga for biofuel synthesis (Tafreshi and Shariati, 2008). The ability of *D. tertiolecta* to adapt and survive these different conditions makes it a good candidate for large farm growth, and biofuel production.

Conclusion for hyperosmotic shocking study

Further study of *D. tertiolecta* would include longer treatment periods greater than four hours for samples, and an increase in the number of samples over time. Due to time constraints the number of samples between two and four hours were limited in this study. However, due to the increase in glycerol during hyperosmotic shocking between two and four hours there should be further study into glycerol production during that time frame. It would also be beneficial to test additional shocking solutions, with lower NaCl concentrations to see if there is a difference at smaller concentrations, and to see if there is a certain threshold at which there is a maximal glycerol production, as well as survival of the *D. tertiolecta* cells.

The results are promising, and suggest the need for future studies for optimizing the time during growth cycles of *D. tertiolecta* for hyperosmotic shocking. Ultimately utilizing hyperosmotic shocking causes glycerol production to increase more rapidly than growing the microalga in a higher NaCl molar concentration. It could serve as a method to quickly cause the microalga to excrete glycerol, and it can be done at any time point during the growth cycle, however it may be more effective during certain phases of growth. It warrants further study into when would be the best time after inoculating a culture to hyperosmotic shock, and after the shocking, could the microalga be salvaged and continued to grow, and shocked again at a later time.

Concluding Remarks:

This work achieved the following summarized results:

- Development of growth media and benchtop culturing methods for *D. tertiolecta*
- Development Glycerol assay method using NMR
- Development of indirect glycerol measurement using refractive index, pH, conductivity
- Development of a scale model continuous flow "farm" with in situ instrumentation
- Demonstrated that osmotic shocking can increase glycerol production

Ultimately there are challenges faced when using a microalgae to synthesize biofuels; however the benefits are innumerable. Not only could microalgae-based fuels help reduce greenhouse gases, they could also lessen the carbon footprint by taking the place of farms which require maintenance which undoubtedly use fossil fuels. Microalgal-based fuels would allow for farms to produce agriculture for food rather than for fuel, and could effectively replace fossil fuels if this research is continued. Using a microalga such *D. tertiolecta* would be prudent because of the ability of microalgae to rapidly grow, and produce high volumes of glycerol, byproduct which can be used as a fuel source. Continuing to study and adapt microalgal-based fuels is crucial for mitigating climate change, and protecting the planet from future harm due to climate changes.

Literature Cited

Bompelly, R. and Skaf, D. 2014. Optimization of a colorimetric test method for quantifying glycerol in an aqueous solution. J Am Oil Chem Soc, 91: 1605-1610.

Brennan, L. and Owende, P. 2010. Biofuels from microalgae-A review of technologies for production, processing, and extractions of biofuels and co-products. Renew & Sust En Rev, 14: 557-577.

Chow, Y., Gon, S., Su, Z., Ng, D., Yuen Lim, C., Lim, N., Lin, H., Fang, L., Lee, Y. 2013. Continual production of glycerol from carbon dioxide. Biores Technol, 136: 550-555.

Chow, Y., Ng, D. 2015. The role of micronutrients and strategies for optimized continual glycerol production from carbon dioxide by *Dunaliella tertiolecta*: Micronutrients Strategies for Glycerol Synthesis. Biotechnology and Bioengineering.

Environmental Protection Agency. 2017. Global Greenhouse Gas Emissions Data. EPA.gov. Website accessed on April 8, 2018.

Goyal, A. 2007. Osmoregulation in *Dunaliella*, Part II: Photosynthesis and starch contribute carbon for glycerol synthesis during salt stress in *Dunaliella tertiolecta.* Plant Physiology and Biochemistry, 45: 705-710.

Hannon, M., Gimpel, J., Tran, M., Rasala, B., Mayfield, S. 2010. Biofuels from algae: challenges and potentials. Biofuels, 5: 763-784.

Lee, R. 2018. Phycology. Cambridge University Press, 5th Edition.

Lin, H., Fang, L., Low, C., Chow, Y., Lee, Y. 2013. Occurrence of glycerol uptake in *Dunaliella tertiolecta* under hyperosmotic stress. FEBS Journal, 280: 1064–1072.

Mata, T., Martins, A., Caetano, N. 2010. Microalgae for biodiesel production and other applications: A review. Renewable and Sustainable Energy Reviews, 14: 217-232.

Tafreshi, A., Shariati, M. 2009. *Dunaliella* biotechnology: methods and applications. Journal of Applied Microbiology, 107: 14-35.

Young, E., Beardall, J., Giordano, M. 2001. Inorganic carbon acquisition by *Dunaliella tertiolecta* (Chlorophyta) involves external carbonic anhydrase and direct HCO3- utilization insensitive to the anion exchange inhibitor DIDS. European Journal of Phycology, 36: 81-88.

B. Petrusevski, G. Bolier, A.N. Van Breemen, G. J. Alaerts, "Tangential flow filtration: a method to concentrate freshwater algae." *Water Research*. vol. 29(5).pp.1419–24. *1995.*

P. Bondioli and L. Della Bella, "An alternative spectrophotometric method for the determination of free glycerol in biodiesel" *Eur. J Lipid Sci. Tech. vol. 107, p.153-157, 2005.*