SarTec Corporation
PO 665
Anoka, Minnesota 55303
Phone 763-421-1072

Project Title: Flue gas carbon dioxide capture with rapid growth algae to produce biodiesel and other renewable fuels

Contract Number: RD3-2 **Report Number:** 23 **Report Date:** 11 July 2011

Principal Investigator: Clayton McNeff
763-421-1072 ext 12
claytonmcneff@sartec.com

Contract Contact: Tim Edman
612-330-2952
Timothy.J.Edman@xcelenergy.com

Congressional District: 6th Congressional District

Project funding provided by customers of Xcel Energy through a grant from the Renewable Development Fund

RD3-2 PROJECT FINAL REPORT

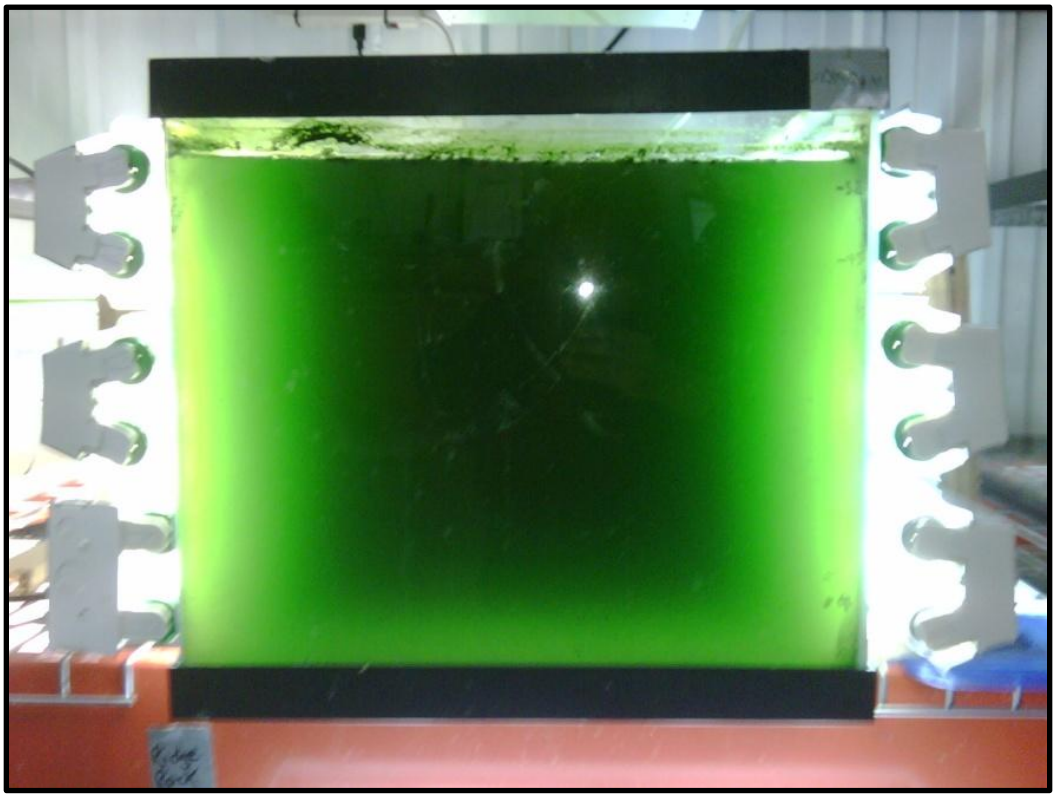


TABLE OF CONTENTS:

EXECUTIVE SUMMARY

Although there is much debate concerning global warming, atmospheric carbon dioxide accumulation and the burning of fossil fuels, there is a general consensus that the United States needs to find ways to use domestic sources of energy to decrease our dependence on foreign energy supplies. Coal is our most abundant energy resource and it is used extensively for generating electricity; though, burning coal produces large quantities of carbon dioxide. The energy industry is interested in technology that can capture carbon dioxide from coal combustion in an economical and sustainable manner. The industry is also interested in renewable fuels that can be used to displace fossil fuels. The Mcgyan® process uses a fixed bed metal-oxide reactor that rapidly produces biodiesel fuel from a variety of lipid feedstocks, such as cooking oil, cellulosic material and lipids from microorganisms.

This research project investigated a process for using solar energy, photosynthesis and rapid growth of algae to capture carbon dioxide (as is contained in flue gas) and produce biomass containing lipids that can be transformed into renewable biodiesel. Additionally, algal lipids (oil) can be extracted and converted to biodiesel (for use in combustion turbine plants or commercial sale). The biodiesel can be sold on the liquid fuels market to produce revenue to partially defray the costs associated with CO_2-capture. It also has the potential to create revenue for the power company from internally generated carbon credits. Overall this CO_2-capture/biodiesel process will partially close the carbon cycle at coal-fired plants and reduce fossil carbon emissions.

Algal species considered for this project were *Dunaliella parva, Dunaliella tertiolecta, Dunaliella salina* and *Nannochloropsis oculata*. Selection of algal cultures was based on initial research determining which species had a faster growth rate. Average growth rates in aquariums (55 gallons) were 0.0790 g/L/day for *D. tertiolecta*, 0.0446 g/L/day for *D. parva*, 0.0375 g/L/day for *D. salina* and 0.0556 g/L/day for *N. oculata* (Appendix C, Table 14). Algae with a faster growth rate capture higher amounts of carbon dioxide, which is why *D. tertiolecta* was chosen (Appendix C, Table 15).

Algae cultures of *D. tertiolecta* were scaled up in order to accumulate enough algae and, subsequently, algal oil for conversion into biodiesel. Cultures were moved from small, 1.5 liter photo bioreactors to 55 gallon aquariums (typically containing 190 – 200 liters of culture) and 275 gallon totes (typically containing 300 liters of culture). This allowed for a more realistic evaluation of the algae growth, carbon sequestration and accumulation of sufficient biomass from which lipids could be extracted.

One aquarium and two totes were maintained outdoors with natural light and an average mid-summer photoperiod of 15 hours of light and 9 hours dark within a 24 hour period. Three aquariums and one tote were maintained indoors with a 24 hour continuous light photoperiod. Indoor lighting was provided by fluorescent lights surrounding each aquarium. Artificial, fluorescent light is less intense than natural sunlight, but it is consistent from day-to-day. Photographs in Appendix B (Figures 9, 10) show examples of light penetration and how the cell density of the culture affects it. The growth rate of algae slows as the cell density increases due to self-shading of the algae.

All aquariums received approximately 5.6% (v/v) carbon dioxide in air at a flow rate of 200 ml/minute during light periods. The aquariums received air with no carbon dioxide at the same flow rate during periods of dark. Aeration of totes was similar to that of the aquariums, except the flow rate was 300 ml/minute.

It was noted that the size of the *D. tertiolecta* cells decreased as their growth rate increased. *D. tertiolecta* would divide into four, eight or sixteen daughter cells during increased growth rates. This rapid division resulted in smaller cells with a typical diameter of 3 to 5 microns. Cells typically divided into two daughter cells with a typical diameter of 8 to 12 microns during the original, slower growth rates.

Algae were harvested through centrifugation, dried, pulverized, and the lipids were extracted. Lipid profiles for all four species investigated (*D. tertiolecta, D. parva, D. salina* and *N. oculata*) and native, freshwater cultures (primarily Ankistrodesmus sp.) can be found in Appendix B, Figures 23-27. GC analysis has shown that the lipids are composed of primarily C16 and C18 carbon chains. Samples of residual material (post lipid extraction) for *D. tertiolecta, D. parva,* and *N. oculata* were analyzed for protein, carbohydrate, fiber, ash and moisture (Appendix C, Table 13). Tests performed by NMR (Nuclear Magnetic Resonance) showed the solidified lipids to consist of triacylglycerides and free fatty acids. NMR analysis of the fluid lipids showed them to consist primarily of free fatty acids.

Approximately 250 ml algae oil was accumulated throughout this project (Appendix B, Figure 19). The lab-scale Mcgyan® reactor was used to convert 189 grams (200 ml) of the oil into biodiesel. The optimal reactor operating conditions were 350º C and 2300 psi with a 30 second catalyst contact time (Appendix D, Figure 38). Some yield losses were incurred in the system and throughout the performance of the acid number tests. ASTM testing on the algal oil-based biodiesel fuel showed the fuel to be in compliance with the standards.

An experiment to evaluate the effects of artificial flue gas on growing cultures of *D. tertiolecta* was performed on four aquariums, two experimental and two controls. Experimental aquariums were supplied with artificial flue gas containing 106 ppm sulfur dioxide (SO_2) and 13% carbon dioxide. Control aquariums were supplied with compressed air containing approximately 13% carbon dioxide. No detrimental effects to the growth of algae with the artificial flue gas were observed in this experiment.

BASIS FOR THE STUDY

The basis of this study was to investigate fast growing algae strains for capturing carbon dioxide and converting the resultant algae into renewable fuels. The longer term areas of impact of the project relate to protecting the environment (increased level of carbon dioxide is associated with global warming); meeting future CO_2 emission requirements or taxes (carbon tax) and finding new sources of feedstocks for biofuels.

OBJECTIVES OF THE RDF GRANT

Grouped by Milestone numbers the objectives of the grant were:

- (M1) Review literature on existing methodologies for algae cultures. Construct a lab-scale bioreactor. Establish optimal operating parameters for the lab-scale bioreactor.
- (M2) Select and collect algae samples. Establish algae cultures and cultivate algae in the laboratory. Optimize the algae growth parameters. Analyze and profile algae compositions.

- (M3) Scale up cultivation of algae to a large scale. Analyze algae composition and test algae cultures. Develop algae preservation process.
- (M4) Develop algae oil extraction process, extract algae oil and produce biodiesel from the algae oil with the optimized Mcgyan® process.
- (M5) Test biodiesel and confirm that the fuel meets ASTM standards. Investigate alternative markets for the algal biomass and the residual fractions left after biodiesel production. Conduct data analysis and large-scale feasibility study of the CO_2 to algae to biodiesel process. Validate project results.

TECHNICAL PROGRESS

M1 Goal 1: Review Literature on Existing Methodology for Algae Cultures

A general literature review of algal production systems was performed and while there were a variety of methods for mass culturing algae outdoors, very few designs were successful on a large scale for long periods of time. Open systems are simple, have low capital and maintenance costs, and are the most prolific design to date. However, the small surface area to volume ratio, large area required, and high risk of contamination limits the areal productivity. Open systems typically consist of low-density cultures, which increases the downstream processing costs such as harvesting and dewatering. Closed systems have demonstrated an increase in the areal productivity and volumetric density over open systems, but the initial capital and maintenance costs are often too great for the system to be economically viable on a large-scale. Two-stage systems attempt to combine the cost benefits of an open system with the high productivity and ability to maintain a monoculture of closed system. Although the two-stage system shows great promise, it has yet to be demonstrated as an economically viable commercial system (please see Appendix A for the complete literature review).

M1 Goal 2: Design and Construct a Lab-scale Photo Bioreactor

SarTec has researched and developed a photo bioreactor (PBR) that allows for testing different growth parameters and algae species independently. Several PBR designs were constructed and tested. The final design was both relatively inexpensive and fully functional. Eight "final design" photo bioreactors in all were constructed; they are made from clear, thin-wall polycarbonate tubes mounted in a PVC base.

M1 Goal 3: Determine Optimal Operating Parameters for the Lab-scale Photo Bioreactor

The PBRs operate by inputting a gas mixture into the algae solution contained in each PBR. The gas mixture consisted of 1 part carbon dioxide and 9 parts air, giving it a carbon dioxide concentration of approximately 10% (v/v). Approximately 1.6 ml per minute of this mixture per PBR was used to sustain algae growth. Each PBR upon setup received an initial flow rate of 5 ml per minute (pumped through a fine bubble diffuser located at the bottom of each PBR) to ensure good circulation and prevent clumping of algae. The liquid suspension was sparged with 10% (v/v, CO_2 in air), however after carbon dioxide saturation was achieved, the majority of the gas mixture was released in the atmosphere. The algae were grown indoors under artificial LED

light with a daily cycle of 15 hours light and 9 hours dark. The temperature of the algae solution was maintained between 21 – 32^{o} C. After a PBR was established with an algae culture, algae cells were harvested at a rate of approximately 50% of the PBR volume per day. Nutrient growth media was used to replace the culture volume harvested. A growth media formulation for *D. tertiolecta* is contained in Appendix C, Table 12.

M2 Goal 1: Select and collect algae samples

Lipid content and growth rate were the main parameters examined in selecting algae species. Higher growth rates result in increased carbon dioxide sequestration. The consumed carbon can be used for lipid production, thus resulting in higher lipid yields and, consequently, more biodiesel.

The rationale for starting with the algae species *D. tertiolecta* was that the carbon captured by this species goes into a cell membrane (it doesn't have a cell wall) and supporting structures. With most other species, about 40% - 50% of the carbon goes into the cell wall. Extracting lipids from cell walls is more difficult than extracting lipids from membranes, thus making *D. tertiolecta* a desirable species for this project. Furthermore, multiple literature sources claim *D. tertiolecta* is a fast growing algal species. Several other species were selected and used in comparison to the *D. tertiolecta*, including *D. parva*, *D. salina* and *N. oculata*. The *Dunaliella* algae cultures were obtained from Dr. Arun Goyal. The *N. oculata* culture was purchased commercially from AlgaGen LLC (Vero Beach FL).

M2 Goal 2: Establish algae cultures and cultivate algae in the laboratory

Seed cultures of each species were cultivated in the laboratory to produce viable stocks. Some cultivating problems were encountered. For example, there were contamination issues (protozoa and bacteria) in the early stages of growing *D. tertiolecta* in 250 ml flasks. Flasks and stoppers were sterilized prior to adding the nutrient media and algae inoculum. At first, sampling and stirring tools were reused for multiple cultures. Switching to separate sampling pipettes and stirring tools for each flask helped reduce contamination. The lighting method was also altered from a fluorescent fixture hanging approximately 12" above the flasks to a fluorescent fixture 2" below the flasks thus directing the light up through the bottom. This resulted in increased growth rates and a decreased presence of protozoans. When algae secrete glycoproteins their growth is typically poor. Glycoprotein acts as a nutrient source for bacteria, and bacteria act as a nutrient source for protozoa. Maintaining adequate culture growth controlled most contamination issues.

M2 Goal 3: Optimize algae growth parameters

Algae cultures were transferred to photo bioreactors (PBRs) for growth rate optimization experiments. The following growth conditions were maintained: cultures were sparged with 5.6% CO_2 , 21 – 24^{o} C, 15 hours light/9 hours dark and pH 6.0 – 6.4. The light intensity of the LED emission was 1997 Lux. The slightly acidic pH was maintained so that most of the dissolved inorganic carbon (DIC) was available in the form of dissolved carbon dioxide.

The best overall composite growth rates in PBR's were seen in *D. salina* and *D. parva* followed by *N. oculata* then *D. tertiolecta*. However, when scaled up to larger volumes in the aquarium experiments, the *D. tertiolecta* exhibited the best growth rate when the algae were grown in 55 gallon aquariums (see Appendix C, Table 14). The average carbon dioxide capture rates of the species were calculated using average growth rates (see Appendix C, Table 15). The indoor fluorescent lights located around the aquariums emit 13,460 Lux on average and when measuring light from inside the aquariums, readings were approximately 4300 Lux. If the output of the fluorescent bulbs is measured with the Field Scout quantum light meter, readings of 280-300 $\mu mol/m^2$/second were observed.

In order to determine optimal growth conditions, several experiments were conducted for understanding effects of limiting light, nutrients, and different composition of growth media. All four species of algae showed greater growth rates in the 2L flasks compared to the photo bioreactors, indicating lighting was a limiting factor. The light intensity was approximately 2-3 times higher for the 2L flasks.

Effects of several different nutrient deficiencies algal growth were determined (ST31-28A-H). . Nitrate deprivation quickly halted algae growth and chlorophyll production. Iron deprivation caused the quickest and greatest inhibition of algae growth. A similar effect was observed with iron and phosphate deprivation. Phosphate deprivation alone did not cause cessation of growth as quickly as the other three deficiencies or hinder chlorophyll production as much. Most algae have a tendency of accumulating intracellular phosphate as polyphosphates. One of the primary reasons for delayed response of phosphate deprivation was likely to be presence of intracellular polyphosphates.

In the photo bioreactors (experiments ST31-28 J-R), five different media formulations were evaluated. The best overall composite growth rates were observed in regular 1% salinity medium.

Experiment ST31-30 A-F was conducted to compare medium containing iron chloride versus chelated iron. It also compared the amount of iron being added. It is known that chelated iron is a more stable form of iron that is easier for plants to absorb and is thought to pass through the cell membrane more easily [1]. At the beginning, the algae cells have excess iron bound to their surface which gets internalized over time. However, due to the metabolic rate of algae, the iron externally bound at the beginning would most likely be internalized during the first day [2]. Our observations show that iron chloride had a greater positive effect on the growth rate and therefore iron chloride is used in our medium. The final growth medium is presented in Appendix C, Table 12.

At one point, it appeared that *Chlorella* had taken over the vessels containing *D. tertiolecta*. Examination was made of a pure culture known to be *D. tertiolecta* where there was a mix of swimming adult cells and small, non-motile cells that resembled *Chlorella* [3]. Observations were also made of cells that were in the process of dividing [4]. It was confirmed that the small, round cells were a rapidly-dividing growth form of *D. tertiolecta*; the algae currently being studied exhibit this morphology.

Harvest methodology

Algae suspensions that have an A450 reading (absorbance at 450 nm wavelength using a spectrophotometer) of ≥ 1 or were centrifuged (Alfa- Laval model 104B solids-retaining centrifugal separator) at a flow rate of 1.0 – 1.5 liters/minute. The algae paste was collected from the bowl of the centrifuge, weighed and the percent dry matter was determined using a

Mettler-Toledo model HR83 moisture analyzer. Graphs depicting the harvest cycles of the algal species are presented in Appendix B, Figures 21-22.

Harvested algal pastes were dried in two manners. Initially, algal pastes were preserved in a freezer before being placed on metal trays lined with parchment paper and dried in a vacuum oven set at 85^{o} C for 6 or more hours. Starting in mid February 2010, the algae paste was dried in a food dehydrator immediately after harvesting for 18 – 20 hours at 57^{o} C. Dried algae specimens were pulverized in a blender for 5, 30 second sessions. Pulverized, dried algae were then placed in a ball mill and ground for 5 days.

M2 Goal 4: Analyze and profile algae composition

Extraction methodology

There are a significant number of methodologies for lipid extraction. In exploring for the ideal extraction method, we have tested the well-known Bligh and Dyer extraction as well as the Folch extraction which employs chloroform and methanol on wet algae paste. These methods have the benefit of allowing direct extraction of lipid from a wet sample, however, they call for a large quantity of extraction solution and create a significant amount of waste solvent. Since our goal is to be able to process large quantities of algae in an economical and environment-friendly way, our exploration eventually led us to the Soxhlet extraction method which utilizes hexane as the extraction solvent. Soxhlet extraction involves refluxing a solvent to repeatedly wash and extract the sample, and then the lipid is obtained by evaporating off the solvent. The benefits of using this extraction method include: (1) the extraction solvent (hexane) can be recycled and reused, (2) hexane is easier to handle than the more toxic chloroform and thus more environment friendly, and (3) the lipids obtained with this extraction method are neutral lipids that are ready for conversion into biodiesel via the Mcgyan® process. Samples of algae were harvested and prepared for lipid extraction (as described in the previous "harvest methodology" section). Sample preparation and procedures for extraction and GC analysis are detailed in Appendix D.

Gas chromatography-mass spectrometry (GC-MS) analysis

The gas chromatograph electron impact mass spectrometer is a powerful and extremely useful instrument that combines two techniques to allow a single method of analyzing mixtures of chemicals. Through gas chromatography, the components of a mixture were first separated; subsequently the separated components were characterized and identified individually by mass spectrometry. By combining these two techniques, this instrument not only allows qualitative and quantitative evaluation, but also identification of components in a mixture solution. Pertinent to this project, this instrument allows efficient identification of specific fatty acids contained in the various species of algae oil. In the future, this instrument will aid in qualitative and quantitative evaluation of the different chain length FAMEs in the biodiesel produced from the algae oil.

Results

The GC-MS results of five different species of algae oil are presented in Appendix B (Figures 23-27). Other than *N. oculata* which contains some fatty acid with a ten carbon chain, all algae lipids analyzed are primarily comprised of fatty acids that contain sixteen and eighteen length carbon chains with varying degrees of saturation. These observations are in agreement with published results on algae lipid profiles.

M3 Goal 1: Onsite demonstration of cultivation techniques to RDF administration

Algae cultivation and harvesting techniques were demonstrated to an RDF administration representative (Mark Ritter).

M3 Goal 2: Provide sample of algae biomass

Samples of algae paste (1) post centrifugation harvesting and (2) post drying and grinding were sent to an RDF administration representative (Mark Ritter).

M3 Goal 3: Algae composition analysis

An elemental analysis of a dried sample of *D. tertiolecta* was performed by Minnesota Valley Testing Labs (New Ulm, MN). The fat content of 4.02% is slightly low compared to the samples analyzed at SarTec on other occasions that typically ranged between 6% and 10%. Table 1 shows the elemental analysis composition results.

Table 1. Analysis of *D. tertiolecta* (centrifuged and dried), harvested 8-2-2010

Analysis	Result
Moisture	10.10 %
Ash	7.70 %
Calcium	1100 ppm
Carbohydrate	33.98 %
Copper	275 ppm
Fat (ethyl ether)	4.02 %
Fiber, crude	4.24 %
Iron	7520 ppm
Magnesium	3000 ppm
Manganese	92 ppm
Phosphorus	9600 ppm
Potassium	11 ppm
Protein	44.20 %
Sodium	9000 ppm
Zinc	113 ppm

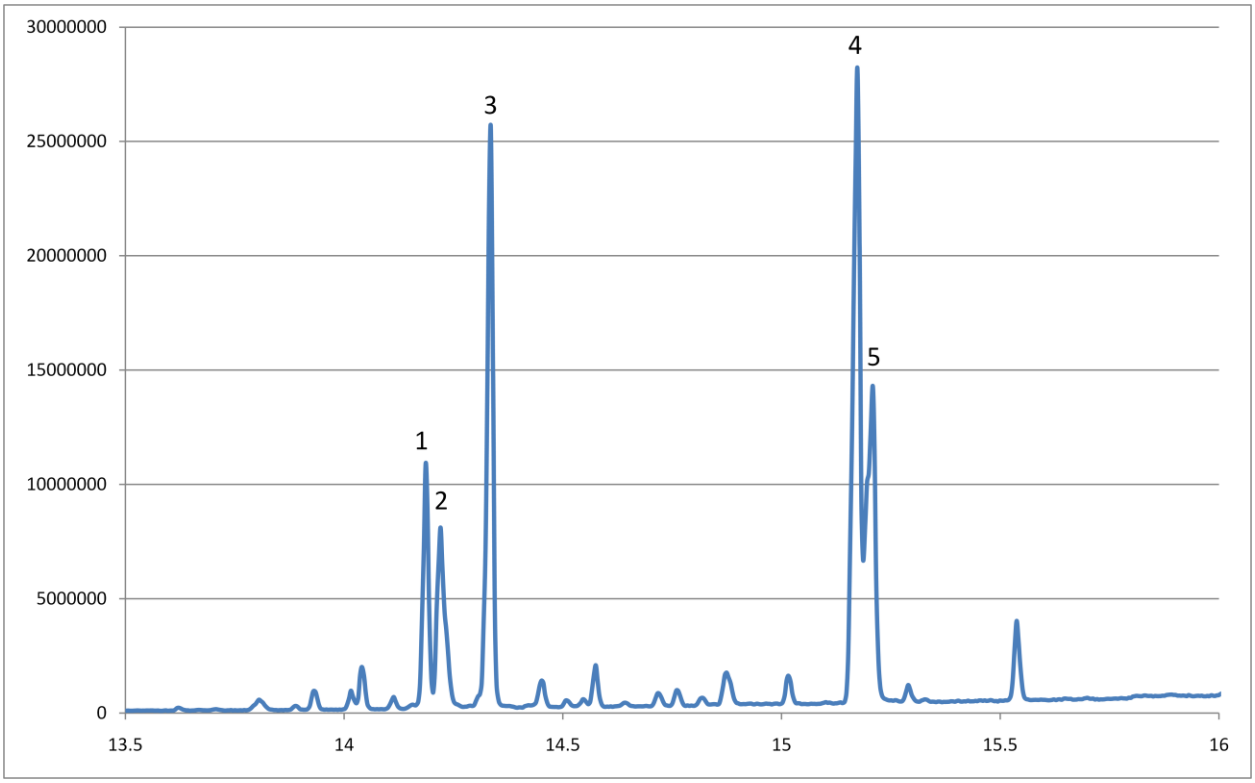


Figure 1. Lipid profile of *D. tertiolecta* separated and identified by GC-MS. Peak 1 : Hexadecadienoic acid methyl esters. Peak 2: Hexadecatrienoic acid methyl esters. Peak 3: Hexadecanoic acid methyl esters. Peak 4: Octadecadienoic acid methyl ester. Pea Peak 2: Hexadecatrienoic acid methyl esters. Peak 3: Hexadecanoic acid methyl esters. Peak 4: Octadecadienoic acid methyl ester. Peak 5: Octadecatrienoic acid methyl ester.

Lipids were extracted from the *D. tertiolecta* algae and prepared for gas chromatography-mass spectrometry analysis. The results are shown in Figure 1. The *D. tertiolecta* lipids are primarily comprised of fatty acids that contain sixteen and eighteen length carbon chains with varying degrees of saturation (Hexadecanoic acid and Octadecadienoic acid). These observations are in agreement with published results on algae lipid profiles. The extraction methodology and sample preparation are described in Appendix D. The GC/MS analyses of the different algae species profiled are shown in Appendix B, Figures 23 – 27.

<u>Things that affect lipid production and storage.</u>

Lipid composition and accumulation in algae are dependent on many variables, including species, growth cycle length, nutrients and nutrient deficiencies, salinity and diurnal light-dark cycles [5]. There are three general times when algae will synthesize triglycerides: when the carbon assimilation is greater than the immediate metabolic need of the cell; during nutrient starvation; and during other environmental stresses [6, 7].

Nitrogen and phosphorus deficiencies in growth medium for *D. tertiolecta* cells have been reported to not affect the lipid accumulation[8]. However, a more recent study claims that *D. tertiolecta* deprived of nitrogen (in the form of nitrate), iron or, to a lesser extent, cobalt accumulated substantially more lipid than *D. tertiolecta* that was not deprived of anything[9]. Additionally, both cell growth and lipid accumulation have been reported to increase as NaCl

concentrations rise from 0.5M (salt water) to 1M [10]. Cell growth drops quickly beyond 1M NaCl, and no data was available for lipid accumulation beyond 1M NaCl.

M3 Goal 4: Preliminary results of algae culture tests and preservation processes.

A total of 7.2 Kg (on a dry matter basis) of *D. tertiolecta* was produced for extracting algae oil for the next phase of the project (biodiesel production with the Mcgyan® process). The biomass was produced in mass batch cultures in 55 gallon aquariums and also in 275 gallon totes that were maintained at 300 liters of operating volume. This technique allowed for a more realistic evaluation of the algae growth, carbon sequestration and accumulation of sufficient biomass from which lipids could be extracted.

Outdoor lighting (average summer photoperiod of 15 hrs light/9 hours dark per 24 hour period) was used for growth in one aquarium and 2 totes. Indoor lighting was employed for three aquariums and one tote (Appendix B, Figures 11, 12) with a 24 hour continuous light photoperiod.

It was noted that the size of the *D. tertiolecta* cells decreased as their growth rate increased. During fast growth the cells would divide into four, eight or sixteen new cells. This rapid division resulted in smaller cells with a typical diameter of 3 to 5 microns (Appendix B, Figure 13). Prior to the rapid division (fast growth), the cells typically divided into two new cells that were larger with typical diameters in the 8 – 12 micron range. The change in cell division correlates with changes that were made in formulation of growth medium in late August, 2009. The change included decreasing the $MgCl_2$ $6H_20$ (50% of previous quantity) and reducing $CaCl_2$ $2H_2O$ (0.15 g/liter compared to previous 0.2 g/liter) and doubling the KNO_3 from 0.5 g/liter to 1.0 g/liter.

Productivity

D. tertiolecta was harvested from an indoor aquarium at three specific optical densities (O.D. = 0.4, 0.6 and 0.8) to compare the lipid content (Soxhlet extraction with hexane) and determine whether there is any benefit to harvesting earlier or later in the growth cycle. The results are presented in Table 2. A higher neutral lipid content (9.65%) was found for the algae harvested at O.D. = 0.8

Table 2. Analysis of algae residue after Soxhlet extraction of lipid sampled at different optical densities.

Sample ID	Dried Algae Mass (g)	Lipid Mass (g)	Neutral Lipid (%)	Optical Density
ST29-153	80.29	5.21	6.49	0.40
ST29-150	82.00	5.49	6.69	0.60
ST29-152	81.23	7.84	9.65	0.80

Carbon sequestration

The overall average growth rate for *D. tertiolecta* in these experiments (which is the sum of growth achieved over the duration of the experiment divided by the total days) was found to be 0.0790 grams/liter/day.

The potential for sequestering carbon dioxide by *D. tertiolecta* is shown in Table 3. The data is based on average growth rates in indoor vessels employing fluorescent lighting.

Table 3. Average carbon dioxide capture by *D. tertiolecta* algae.

Species	CO_2 Capture Based on Harvest (tons/acre year)	CO_2 Capture Based on Optical Density (tons/acre year)
D. tertiolecta	42.12	61.77

Based on: 365 days/year production, water depth = 0.30 meters and 100% capture.

Preservation processes

Algal cultures were maintained on solid supports using Agar. Cryopreservation allows for the indefinite storage of microalgae strains in an arrested state. However, this method is not suitable for *D. tertiolecta* because this species lacks a cell wall. Therefore, algae will be maintained by transferring organisms every 30 days and maintain slants under low light at 16/8 hours light/dark cycle. Algae from the slant tubes can be used to inoculate flasks of liquid growth medium when desired. This is a simple and reliable method for maintaining live algae cultures.

Freeze-drying is a method to preserve microorganisms through a two step process: freezing the sample with a cryoprotective substance, such as glycerol, then drying the sample, thus dehydrating and arresting the specimen's metabolism (anhydrobiosis). The temperature in each step is tightly regulated to control rates of dehydration and ice crystal formation, thereby reducing the amount on damage done to the specimen [11]. Advantages to freeze-drying cultures include: proven industrial success (both the American Type Culture Collection and National Collection of Type Cultures have used this method successfully for many years), forming an easily rehydrated product, and preserving the specimen for longer periods of time [11, 12]. However, this technique is costly, complex and detrimental to cell viability; typically less than 1% of the starting culture is viable after freezing [11, 13]. This means reliable revival requires a high concentration of cells, some recommendations being at least 10^7 cells ml^{-1} [12].

Cryopreservation allows for the indefinite storage of microalgae strains in an arrested state [14]. A cryoprotective substance that helps protect the living cell from damage due to freezing is added to the culture, and the culture is cooled to a subzero temperature. This allows for the dehydration of the cells, thus improving their preservation. Rapid cooling to a different storage temperature is the last step. The microalgae are then kept at the storage temperature until a desired thawing time. This is a popular method for preserving cultures for long periods of time. As with freeze-drying, viability levels of cultures are typically lower than before cryopreservation. Additionally, morphologically complex algae, most filamentous strains and those with larger cell size, cannot be cryopreserved successfully [14].

M4 Demonstration 1: Conduct onsite demonstration of oil extraction and biodiesel production techniques to RDF administration

An onsite demonstration of the algae oil extraction and biodiesel production techniques was performed on May 3rd, 2011 with RDF administration representatives Mark Ritter and Tim Edman attending.

M4 Goal 1: Develop algae oil extraction process, extract algae oil and produce biodiesel from the algae oil with the optimized Mcgyan® process

Methods

Tests were performed and known techniques were evaluated to determine a practical method of extracting oil (lipid) from the algae and separating the neutral and polar fractions of the oil. Pretreatment methods that might enhance lipid extraction from *D. tertiolecta* were examined. A review of the solubility of fatty acids in solvents showed that as the carbon chain length increases, the solubility (grams/liter at 20° C) decreases. For example, a C16 fatty acid has a solubility of 151 g/l in chloroform and 65 g/l in cyclohexane; a C18 fatty acid has solubility of 60 g/l in chloroform and 24 g/l in cyclohexane. Chloroform has a greater lipid solvation capacity than cyclohexane in this situation, however due to the higher toxicity and carcinogenic properties of chloroform, hexane and ethanol were used in these studies.

Small-scale lipid extractions were used to determine whether a combination of solvents or one single solvent would suffice and whether the reaction could benefit by vigorous stirring of the contents. The stirred samples yielded a higher lipid extraction content than the unstirred samples. Samples using straight hexane (a non-polar solvent) as the extraction solvent were compared to samples extracted using a 1:1 mix of hexane and ethanol (a polar solvent). The hexane/ethanol extractions yielded significantly greater amounts of neutral lipid than with just hexane alone. Based on this test, the dual solvent system was chosen as a good starting point.

D. tertiolecta saltwater algae cells have an outer membrane but not a cell wall. The membrane should make it easier to break the cells to improve access to the lipids. However, the membrane is flexible which can be seen when they are put into freshwater; the cells will swell but not lyse (split open).

Several procedures were examined to determine whether they would be useful in opening the cells to improve lipid yield when performing an extraction. Reconstituted algae paste was used for these studies. The methods employed were: homogenizing with a hand-held tissue grinder, homogenizing with a high-speed blender, placing a reconstituted sample in a microwave oven until boiling, and pushing a slurry of algae through a Yeda press under a pressure of 1400 psi nitrogen gas.

An additional experiment was performed by microwaving a sample of dried and pulverized algae for 2 minutes to determine if the moisture inside would further dismantle the cells. It appeared that it had some effect although not significant enough to seriously consider it an effective technique.

Algae subjected to the various pre-treatments intended to rupture the cell membranes were examined under a microscope. The only sample that showed evidence of significant disruption was the one that was microwaved; most of what was seen under the microscope appeared to be agglomerations of intact cells and cell fragments. The vast majority of the optically examined algae cells ranged from 2 to 5 microns in diameter. It appears that the small size of the algae cells makes it difficult to lyse them by mechanical methods.

A method involving supercritical fluid extraction of lipids with the aid of liquid CO_2 was also examined. This technology does not generate hazardous waste, which makes it environmentally friendly.

The use of an ultrasonication device in conjunction with hexane as an extraction solvent was under consideration but not tested due to time constraints and cost factors. This could be employed as a tool to increase the penetration of the solvent into the algae and therefore,

maximize the removal of lipids. The sonication of liquids generates high and low pressure cycles. Small vacuum bubbles are produced which collapse violently; this implosion is called cavitation. The shear forces from cavitation can rupture cells and aid in solvent penetration into the algae.

Enzymes are known to be useful for lysing algae cells but the cost is considered to be prohibitive. Enzymes such as a cell wall lytic enzyme (gamete wall-lysin) might be effective.

Scale up and technique for lipid extraction

For the purpose of extracting lipids from large batches of dried algae, a device for solvent extraction of lipids was assembled (Appendix B, Figure 17). Approximately 500 grams of dried algae was processed per batch using 5 liters of a hexane/ethanol mixture with constant agitation.

The first extractions resulted in the need to filter out large amounts of pigment from the solvent. Also, the lipid yields were variable (Table 4). The method was subsequently switched to a Soxhlet extraction technique (Appendix B, Figure 18) that employed hexane as the solvent.

In both cases, the solvent/lipid mixture was filtered through activated carbon to remove any pigment (mainly chlorophyll). Switching to the Soxhlet method seemed to reduce variations in lipid yield.

Table 4. Neutral lipid extraction comparison, 500g samples with the reflux apparatus.

Batch	**Solvent**	**Stirred & Heated**	**Neutral Lipids (%)**
1	Hexane/Ethanol	YES	0.45
2	Hexane/Ethanol	YES	0.80
3	Hexane/Ethanol	YES	0.29
4	Hexane/Ethanol	YES	1.29

Table 5. Comparison of lipid extractions from *D. tertiolecta*.

Method	**Batch Size (g)**	**Yield (%)**	Notes
Soxhlet June 2010	80	9.65	**Lipids solid at room temp (RT)**
Reflux	500	0.68	Batches 1-4 flour milled, lipids liquid at RT
Reflux	500	0.99	Batches 5,6,8 ball milled, lipids liquid at RT
Soxhlet	80*	3.52	Lipids solid at RT, *3 batches
Soxhlet	80**	0.85	Lipids liquid at RT, **11 batches
Supercritical CO_2	105.3	3.90	Lipids solid at RT
Supercritical CO_2	148	4.40	Lipids solid at RT

Other Notes:
-Solid lipids contain triglycerides and free fatty acids (NMR analysis)
-Liquid lipids contain free fatty acids only (NMR analysis)
-Algae pulverized by ball mill unless noted otherwise
-Soxhlet extractions performed using same equipment & same method
-Differences in yield unexplainable

Extraction Results and Discussion

Analysis of a dried sample of *D. tertiolecta* was analyzed by Minnesota Valley Testing Labs (New Ulm, MN). The fat content of 4.02% was low compared to the samples analyzed at SarTec on previous occasions which typically ranged between 6% and 10%.

Palmitic acids (C16) and stearic acids (C18) tend to be the most abundant neutral lipid components in algae oil. Saturated fatty acids tend to be solid at room temperature (21º C). Saturated palmitic acid has a melting point of 63.1º C and saturated stearic acid has a melting point of 69.6º C. Analyses performed on two samples of *D. tertiolecta* showed C16 compounds comprising 37.2% and 33.3% respectively and C18 compounds comprising 40.5% and 45.2% respectively of the total lipids (analysis by SDK Labs, Hutchinson KS).

For the extractions performed in 2010 and in spring, 2011, the neutral lipid yield has varied considerably (Table 5). When looking at the results from batches 1 – 4 compared to batches 5, 6 and 8, it appears that improvements in lipid yield can be achieved by more thorough pulverizing the algae in a ball mill compared to what is seen when the algae is ground in a flour mill (burr mill type). Using Soxhlet extraction tends to yield more consistent results than the reflux method but not always a greater amount of lipid. Where supercritical CO_2 was used to extract lipids yields ranged from 3.9% to 4.4%. It may be that CO_2 extraction is potentially better than Soxhlet and reflux.

Some of the neutral lipids extracted solidified upon cooling to room temperature whereas others remained fluid upon cooling to room temperature. Tests performed by H^1NMR (proton Nuclear Magnetic Resonance) showed the solidified lipids to consist of triacylglycerides and free fatty acids. NMR analysis of the fluid lipids showed them to consist of free fatty acids. The reasons for these differences have not yet been identified.

Lipid extraction was performed by two outside sources in an effort to verify the accuracy of the results obtained at SarTec. SRS (Solution Recovery Services, Dexter, MI) reported total lipid content to be 9.83% in a 100 gram (dry matter) sample. Generally, the neutral lipids from previous extractions at SarTec (Krohn 2009, internal correspondence) and others (Volkman et. al., Vanitha et. al.) comprised about 30% - 35% of the total; this would indicate a neutral lipid content of approximately 2.95% - 3.44% for this sample.

Cool Clean Technologies (Eagan MN) also performed an extraction on a sample of *D. tertiolecta* (105.3g dry matter). This extraction yielded 4.08g neutral lipid which equates to 3.9%. The process is expensive.

Small-scale Soxhlet extractions performed in June, 2010, yielded 6.49% to 9.65% neutral lipids. Based on those results, the expectation was for a yield of approximately 7% neutral lipids. The actual yield during milestone 4 has been closer to 3.9%. At this time, the reasons why the recent extractions have yielded less neutral lipid have not been confirmed.

Approximately 250 ml algae oil (Appendix B, Figure 19) was accumulated and available to be converted into biodiesel fuel.

Lipid Conversion to Biodiesel

The Mcgyan® Process (Appendix D, Figure 39) was used to convert a 200 ml sample of the algae oil into biodiesel in the lab-scale Mcgyan® reactor (Appendix D, Figure 38). Representative GC-MS data of the algae oil based biodiesel is shown in Appendix B, Figures 28 – 37. More information on the Mcgyan® process can be found at www.evercatfuels.com.

The optimal reactor operating conditions were 350º C and 2300 psi with a 30 second catalyst contact time. The as-made biodiesel fuel had an acid number of 5, which is above ASTM standards. As is typically done, the biodiesel was passed through the polishing section of the Mcgyan® process (EFAR) in the presence of DMC (dimethyl carbonate – methylating agent) to reduce the acid number to less than 0.5 (Appendix D, Table 17). After passing through the EFAR, the sample was distilled to remove excess DMC. Some yield losses were incurred in the system and throughout the performance of the acid number tests.

Biodiesel is a processed fuel derived from the esterification of free fatty acids (FFAs) and the transesterification of triglycerides (TGs) [15]. Both FFAs and TGs are lipids that come from renewable biological sources, such as plant oils and animal fats. There are four main methods of converting these lipids into fatty acid methyl esters (FAMEs): (1) base-catalyzed transesterification; (2) acid-catalyzed transesterification with simultaneous esterification of FFAs; (3) non-catalytic conversion via transesterification and esterification under supercritical alcohol conditions; and (4) heterogeneous catalyzed esterification and transesterification under supercritical conditions, Mcgyan process [15-17].

Base-catalyzed transesterification typically uses methanol and either KOH or NaOH as catalysts to convert TGs to FAMEs, liberating glycerol as a byproduct [18]. It is more widely used commercially than the other transesterification methods [16]. Disadvantages to base-catalysis include: (1) it is prone to unwanted byproduct formation; saponification takes place when FFAs and water are present, thus producing soap; (2) production of soap will lower the yield of FAMEs and makes the subsequent washing and purification steps much more difficult; (3) sensitivity to water and FFAs severely limits the type of feedstock that can be used to produce biodiesel, thus preventing the use of low-cost, waste oils and fats; (4) the catalysts, NaOH and KOH, are consumed in the process, thus increasing the total cost of the process; and (5) the final products must be neutralized, thereby generating a higher cost and requiring more time [15, 17, 19].

Some problems occurring from the sensitivity of base-catalysis to FFA content and water can be circumvented through an acid esterification pretreatment that involves FFAs reacting with alcohol and sulfuric acid [20]. While this method does make it possible to use low-cost feedstocks high in FFA content, it requires extra alcohol (for esterification then transesterification) and an additional neutralization step before continuing with transesterification [21]. In attempts to avoid these extra steps, acid-catalyzed esterificaiton of FFAs and simultaneous transesterification of TGs was proposed. However, acid catalysts will still be consumed in the process and they are not as efficient as base catalysts. Due to their lower activity during the reaction, higher concentrations of catalysts (acid and methanol) and higher pressure and temperature are required to accomplish similar yields to base-catalyzed transesterification [17].

Non-catalytic transesterification of TGs and simultaneous esterification of FFAs has been accomplished with supercritical methanol. Advantages to using supercritical methanol include: (1) supercritical methanol is fully miscible with oils; (2) there is a faster reaction rate; (3) it produces a higher yield if FAMEs; and (4) it does not require acids or bases as catalysts, and, therefore, does not require neutralization steps, thus resulting is much simpler purification steps [17, 22]. However, supercritical methanol must take place at high temperatures (350-400º C) and pressures (200-400 bar), which increases the operational cost [17].

The recently developed Mcgyan® process is a continuous, heterogeneous catalyzed process that simultaneously accomplishes esterification and transesterification with ultra-stable, porous, metal oxide microspheres and supercritical alcohol [23]. This process has a high yield, is not water

intensive, produces no soap nor significant waste streams, does not require continual addition of the catalyst, and the reaction takes place within minutes [15, 24].

Flue Gas Experiment

An experiment to evaluate effects of artificial flue gas on cultures of *D. tertiolecta* was performed. Two aquariums were supplied with artificial flue gas containing 106 ppm sulfur dioxide (SO_2) and 13% CO_2; these were compared with two control aquariums of *D. tertiolecta* that were supplied with a compressed CO_2/air mixture of approximately 13% CO_2.

The growth rate for the controls mostly ranged from 0.04 to 0.05 grams dry matter/L/day whereas the flue gas aquariums ranged from 0.03 up to 0.11 grams/dry matter/L/day. A growth rate comparison is shown (Table 6). There was no significant difference in total sulfur accumulation

Table 6. Comparison of growth rates (dry matter basis) using artificial flue gas (aquariums 29S, 29T) vs. compressed CO_2/air mixture (aquariums 29U, 29V).

		Growth Rate (grams per liter per day)			
Time Frame (days)	**Events**	**ST31-29S**	**ST31-29T**	**ST31-29U**	**ST31-29V**
day 0 - day 9	startup acclimation	0.0277	0.0237	0.047	0.0438
day 10 - day 23	growth to harvest	0.0396	0.0564	0.0512	0.0463
day 27 - day 37	grow to harvest, gas for 29S, 29T increased	0.0525	0.0702	0.0431	0.0491
day 37 - day 44	growth at 20ml/min. 29S, 29T	0.0611	0.0708	0.0332	0.038
day 44 - day 48	growth to harvest, 50ml/min. for 29S, 29T	0.0561	0.0778	0.029	0.038
day 48 - day 49	growth at 140ml/min. for 29S, 29T	0.0398	0.0531	0.0145	0.0193
day 48 - day 51	growth at 140 to 200ml/min. for 29S, 29T	0.0796	0.0987	0.0342	0.0447
day 49 - day 50	growth at 200ml/min. for 29S, 29T	0.0941	0.1122	0.0398	0.0507
day 49 - day 51	growth at 200ml/min. for 29S, 29T	0.0995	0.1158	0.0416	0.0543

The samples were also analyzed for their fatty acid profiles; the most abundant compounds contained 16 carbon atoms and 18 carbon atoms (see Table 7). These are neutral compounds suitable for conversion into biodiesel using the Mcgyan® process.

Table 7. Free Fatty Acids lipid composition in algae grown with artificial flue gas vs. control.

Identification	**C16 compounds**	**C18 compounds**	**Total**
Flue gas culture	37.2%	40.5 %	77.7 %
Control culture	33.3%	45.2 %	78.5 %

This experiment also focused on determining if there is a point at which the artificial flue gas inhibits algae growth. One concern was that the SO_2 infusion can decrease the pH of the liquid to a point at which the algae stop growing and/or die. The lowest pH recorded was 6.6. The flow rate of the artificial flue gas was gradually increased from 2 ml/minute to 200 ml/minute with no detrimental effects.

M5 Goal 1: Test biodiesel and confirm that the fuel meets ASTM standards.

Approximately 200 ml of algae oil was converted to biodiesel fuel followed by performance of ASTM tests. Results show that the fuel passed these ASTM D6751tests (Appendix D, Table 17)

M5 Goal 2: Investigate alternative markets for the algal biomass and the residual fractions left after biodiesel production.

Microalgae are already being used in the food, feed, pharmaceutical and cosmetic industries [25]. However, flue gas contains heavy metals that are toxic to any biological system. *D. tertiolecta* have ion transporters on the outer membrane (consultant, Arun Goyal) which enables them to move ions (monovalent, divalent, trivalent, etc.) into the cell. Due to the fact that algae can intracellularly concentrate heavy metals, the remaining cake after lipid extraction might be best used for pyrolysis (convert hydrocarbons to liquid form) or energy generation by burning. Pyrolysis could also be applied to the entire biomass but it yields a complex mixture of hydrocarbons which would not be amenable to fractionate out neutral lipids. This may qualify for the $35/ton carbon credit. Another possibility might be to put the algae through a bioconversion process to produce alcohols, organic acids or methane; this may or may not be economically viable as very little has been done in this area.

If heavy metals is not present in the algae cake, the potential uses would be greater in number. The amino acid content of some algae is reported to be similar to that of eggs and soybeans, and some of those amino acids cannot be synthesized by humans or animals [26, 27]. This assortment of amino acids would make microalgae a good source of protein, which is verified by the fact that 30% of algae produced worldwide is used in animal nutrition because of the protein [26]. Antioxidants are present in algae, and these can be used in food, pharmaceuticals and cosmetics [25]. Microalgae antioxidants are reported to be stronger than vitamin E and weaker than synthetic antioxidants [28]. Cosmetic companies have used algae extracts in products ranging from wrinkle reduction, anti-irritant, anti-aging, sun protection and hair care products [29].

M5 Goal 3: Conduct data analysis and large-scale feasibility study of the CO_2 to algae to biodiesel process.

Algae can be utilized for the production of many types of bio-fuels including bio-diesel, ethanol, biobutanol, gasoline, jet fuel, and others. Unlike conventional terrestrial plants such as soybeans, corn, sugar cane, and others, algae can be produced 365 days per year, can be grown in many climates, is not restricted by soil type, and does not compete with food crops. Simple cost-effective greenhouse enclosures have proven adequate in controlling the growing climate for productive light and temperature ranges.

When moving from small-scale to large-scale cultivation, one must consider a number of variables and make choices about how to proceed. Initially, data must be extrapolated to estimate how much CO_2 can be utilized and what level of productivity can be realistically achieved. This analysis assumes that individual ponds will have some downtime throughout the year and might only be operational 300 days/year. Here we use a rough number of 2g CO_2 per gram algal biomass (dry matter). Estimates of CO_2 consumption and algal growth are provided (Table 8).

Table 8 . CO_2 fixed and algal production, based on ponds 0.3 meters deep.

Growth rate	Tons CO_2 fixed/acre/yr	Acres/million tons CO_2	Tons biomass/million tons CO_2	Gallons biodiesel/million tons CO_2
0.123 g/L/day	100	10,000	500,000	12,500,000

Growing the algae outdoors in raceway type ponds is believed to be the most viable and economical method for algae cultivation; pond depth should be 0.3-0.4 meters. Surface area of each pond could range from 0.4 -1 hectare (1 – 2.47 acres); mixing dynamics, which haven't yet been reviewed, would most-likely factor into this determination. A sump (deep section) should be provided for fine bubble diffusers. Considering the rapid growth rate that is expected, light penetration may become the major issue if depth is greater. Any shading will cause respiration rather than carbon dioxide fixation. A power plant produces enough excess heat to run a system that would prevent freeze up during the winter. A system for pumping water to the ponds to make up for evaporation losses would be a necessity.

A combination of paddlewheel mixing and a gas diffuser chamber for saturating liquid media with carbon dioxide will be the optimum method. Gas–based mixing will result in a large scale release of carbon dioxide as the necessary flow rate would exceed what is needed for transfer of CO_2 to the water. As long as the dissolved CO_2 in the water is maintained at $\geq 0.1\%$, the algae will have more than enough CO_2 available to sustain their maximum growth rate (consultant, Arun Goyal).

Each ton of biomass is equal to about 2 tons of carbon dioxide; if calculating with conservative methods, about 50 tons biomass/acre/year could potentially be grown (based on 300 days production and favorable conditions), this means 100 tons CO_2 could be fixed per acre per year which will translate into about 10,000 acres per million tons of carbon dioxide per year. Lipid yields could potentially be converted into 1250 gallons of biodiesel/acre/year (27,000 lbs. total lipid, 9,000 lbs. neutral lipids).

Based on the majority of the lipid extractions performed during this study (3.9% neutral lipid), the yield of neutral lipids is estimated to be 3900 lb/50 tons dry matter (542 gals/acre/yr). This would be converted into 542 gallons biodiesel fuel.

The minimal growth medium is recommended; it consists of phosphate, nitrogen, magnesium and micronutrients; sufficient calcium should be available in the water. The cost should be no more than $1 per ton of biomass when ingredients are purchased in bulk. Most salts will be maintained except phosphate and nitrogen.

When determining the flow rate for flue gas to the ponds, several considerations must be taken into account. The liquid medium first needs to attain a saturation of at least 0.1% CO_2 after which the gas delivery rate would be regulated to maintain this level. The gas flow rate will be influenced by water depth and temperature, type of diffuser used, initial bubble size, actual transfer rate of CO_2 from the gas into the liquid and whether specific designs such as covering the gas delivery area are employed. The flow rate and these other factors will also affect how much of the gas and CO_2 rise to the water surface and get exhausted to the atmosphere. It is highly unlikely that all of the CO_2 in the flue gas can be transferred into the liquid medium. The flue gas flow should only be necessary during daylight hours when photosynthesis is occurring. Design calculations should be made when detailed engineering will be done. It is impractical to accurately predict these numbers from our current experience.

For a rough estimate of how much CO_2 might actually get used (Table 9), approximately 2g CO_2 is fixed/gram algal dry matter produced; the cell uses some of the internal carbon dioxide for respiration. For the cultures grown in the lab and supplied by artificial flue gas, it is estimated that 50.13 grams CO_2 were delivered/day and the algae fixed 6.75 g CO_2/day; therefore, approximately 86% of the CO_2 was exhausted to the atmosphere for this particular experiment.

Table 9. Estimated CO_2 utilization for imitation flue gas experiment.

Volume	Growth Rate	CO_2 Supplied	CO_2 Used	Approx. CO_2 Loss
32L	0.1054g/L/d	50.13g/d	6.75g/d	86%

Unlike small-scale vessels in which algae are grown under controlled and fairly optimal conditions, large scale pond systems will be less than ideal. Outdoors, there will be variations in light intensity, rain and potential infiltration by competing organisms such as bacteria, protozoa and rotifers. Protozoa and rotifers are known to graze on algae cells. In light of this, selection of an algae species that is hardy and has good productivity is important when considering outdoor culture. In order to avoid invasion, large-scale algae cultures must be maintained with very high cell density; adding fresh cultures from the pipeline would be a part of the ongoing maintenance.

Mixing in ponds or any other vessel is essential to achieving and maintaining the density of growth needed to produce a significant yield of biomass. Without it, cells will settle, clump together and cease to divide and grow. Using a diffuser system to provide mixing in an open pond system would require a large capital equipment cost. The mixing capabilities of a diffuser decrease as water depth decreases. Paddlewheel mixers are simple and much better suited to shallow water. Based on this, paddlewheels are recommended.

D. tertiolecta grows at 20° C and can remain productive up to 40 ° C. Consideration should be given to Minnesota climate seasonal variations and how to maintain temperature stability during the winter. The ponds need to be shallow to enable adequate exposure of the algae cells to the sunlight, so the surface area to volume ratio would not be favorable to heat retention during the colder months. Pumping heat to ponds from the power plant could be done to keep water temperatures between 25° – 35° C (75° - 95° F). Another consideration would be the paddlewheels to circulate the algae; in the winter, ice might build up on the paddles which may require a greater electric power demand for these pieces of equipment.

Harvesting algae is a process that can be expensive due to the small amount of biomass/unit volume of water. Electrolytic flocculation followed by sedimentation may be a less expensive method as it can flocculate and remove substantial quantities of algae cells in a short period of time. Several smaller units would be preferable over one large unit to provide some backup or redundancy when one or more units might be shut down for maintenance or repairs. Algae paste can be dried by using heat from the power plant.

Lipid extraction from the algae biomass is a process which has received a great deal of attention and research effort. Issues concerning the best way to extract large quantities of oil have not been settled although some entrepreneurs are proposing technologies that hold great promise. Solvent extraction using hexane works for extracting neutral lipids and can be scaled-up, however, it tends to be time-consuming and expensive. Pyrolysis is not widely used at this time but may become more viable; further separation of the lipid fractions, if feasible, would be

necessary with this process. Pyrolysis requires temperatures of 300° – 550° C (572° - 1022° F); it may be possible to use excess heat from the power plant to run this process.

Estimates of economic factors involved with the production, harvest, lipid extraction and conversion to biodiesel are listed in Table 10. Estimates of potential revenue streams from the harvested algae which could offset the total costs are listed in Table 11.

Table 10. Estimated costs based on 50 tons biomass/acre/yr and 1 acre pond.

Description	**Cost**
Electrolytic flocculation equipment	Unknown at this time
Electrolytic flocculation power cost	$410
Algae drying equipment	$20,000
Cost to run drying equipment	Unknown at this time
Pond construction	$40,000
Paddlewheel circulators (3)	$27,000
Growth medium ingredients ($1/ton biomass)	$50
Labor costs	Unknown at this time
Lipid extraction cost	Unknown at this time
Cost to convert lipid to biodiesel ($2 /gallon)	$2,500
Piping, pumps, other infrastructure	Unknown at this time

Table 11. Estimated potential income based on 50 tons biomass/acre/yr and 1 acre pond.

Description	**Price**
Estimated selling price of biodiesel ($3.80/gallon)	$4,750
Co-firing dry algae (8000 BTU/lb)	584,000,000 BTU
Co-firing algae oil (16,000 BTU/lb)	432,000,000 BTU
Sale of algae protein (if it is sellable) 22 tons/acre	$4,290
Sale of algae oil (if it is sellable) 13.5 tons/acre	$ 13,500

M5 Goal 4: Validate project results.

Biodiesel fuel testing was supervised and verified by Dr. Clayton McNeff, Dr. Ben Yan and Joel Schumacher, P.E. (see Figure 40, Appendix D).

A brief description of the most important ASTM D6751 tests follows [56].

Free & Total Glycerin

Free Glycerin is an indicator of improperly washed fuel for the traditional biodiesel process, triglycerides are the source of glycerin and with the Mcgyan process there is little if any glycerin produced. If the amount of free glycerin exceeds the ASTM specified limit of 0.020% by mass (0.240 % for total glycerin), then it is likely to separate or solidify over time. High glycerin in fuel can result in excessive deposits left on valves and engine components as well as clogging and fouling in fuel pumps, filters and lines. Free and total glycerin are tested for using gas chromatography (method D6584).

Sodium/Potassium and Calcium/Magnesium combined
These ASTM tests are designed to detect and measures metallic elements in fuel. Sodium, Potassium, Calcium and Magnesium can cause hard deposits on the piston crowns, valves and injectors of an engine. They can also poison emission control equipment and cause abrasion to engine components. Feedstocks that are high in these inorganic elements (contamination) should not be used as feedstocks for biodiesel production. These elements are tested for with an inductively coupled plasma (excitation) combined with optical emission spectroscopy (identification and quantification). The maximum permissible amount of sodium/potassium (combined) or calcium/magnesium (combined) allowed in ASTM spec grade biodiesel is 5 ppm (method EN14538).

Cloud Point
The cloud point represents the lowest temperature at which crystals begin to form in the fuel (the fuel begins to solidify). This is the most commonly used measure of low-temperature operability (fuels are expected to operate down to their cloud point) of the fuel. There is no temperature specification given on ASTM D2500, however the cloud point value of the fuel must be reported. The cloud point is determined via method D2500. There are several types of instrumentation that can measure cloud point successfully; typically the fuel is cooled slowly and optics are used to determine the temperature at which solidification begins.

Viscosity
Viscosity is the measurement of resistance of a fluid to flow. To ensure that adequate supply of fuel reaches the injectors and that there is a proper spray pattern of fuel from the injectors, the viscosity of the fuel must fall within a given range. The test limits for acceptable fuel viscosity are set at 1.9-6.0 cst. If a sample of fuel is poorly reacted (having a large percentage of triglycerides) it is likely to have a viscosity greater than 6.0 cst. At this high viscosity, a poor spray pattern and resulting incomplete fuel combustion is likely to occur. Viscosity is measured with a standard viscometer according to method D445.

Acid Number
The ASTM D664 test determines how acidic a particular sample of fuel is. It is entirely acceptable for fuel samples to be slightly acidic. The limit set for fuel acidity is 0.5 mg KOH/gram. This means that it takes 0.5 milligrams of potassium hydroxide to neutralize the acid in one gram of biodiesel. A higher acid number may indicate aged fuel that has developed insoluble organic compounds that could present a risk of polymerization and clogging of fuel lines. It can also signal residual free fatty acid molecules in the fuel which have not been converted into biodiesel. Acidic fuels have solvent properties that are more aggressive to metals than in specification processed biodiesel fuels. The acid number is typically measured by titration according to method D664.

Flash Point
The flash point is the lowest temperature at which the vapor of a combustible liquid can be made to ignite momentarily in air. The flash point of a fuel relates directly to the ignitability of the fuel. A low flash point can indicate residual methanol remaining from the conversion process. A high flash point can mean the reaction has not proceeded to completion. In a diesel engine, a low flash point can cause premature ignition, irregular timing, excessive fuel blow by into the crankcase oil, and excessive emissions. A high flash point can lead to poor ignition, resulting in inconsistent firing, and variable engine performance. A minimum flash point for biodiesel is required for safety. For B100 (100% biodiesel) the ASTM specification is a minimum of 93 ºC (200 ºF). Flash point is typically measured with a Pensky-Marten type flash point detector according to method D93.

Water & Sediment
This specification refers to free water droplets and sediment particles. The maximum allowable level for B100 is set at the same level allowed for conventional diesel fuel (0.050 % volume). Poor drying techniques during manufacturing or contact with excessive water during transport or storage can cause B100 to be out of specification for water content. Excess water can lead to corrosion and provides an environment for microorganisms. Fuel oxidation can also raise sediment levels, so this test can be used in conjunction with the acid number and viscosity tests to determine if fuels have oxidized too much during storage. Water and sediment are measured with a centrifuge according to method D2709.

Distillation
The D1160 method employs a method of determining the full range of volatility characteristics of a hydrocarbon-based liquid by progressively boiling off a sample under controlled heating. Biodiesel is fundamentally different than petroleum-based diesel, and this is particularly evident with distillation. Biodiesel has a fairly homogeneous consistency of straight chain hydrocarbons, typically in the 16 to 18 range. Consequently, it exhibits a boiling point rather than a distillation curve. The atmospheric boiling point of biodiesel is generally 626º to 675ºF (330º to 357ºC). A high distillation value may indicate the conversion reaction has not gone to completion, Low values indicate methanol carryover. High values lead to poor starting, especially in cold weather, whereas low values can lead to poor timing and seal material failure. Additionally, this specification was incorporated to ensure that fuels have not been contaminated with high boiling materials such as used motor oil. Distillation by the D1160 method involves the use of a distillation apparatus and measuring the temperature where 90% of the fuel is recovered (distilled). The maximum temperature allowed for this specification in 360 ºC (referred to as T90 – the temperature at which 90 % is recovered).

Oxidative Stability
This test measures the propensity of a fuel to oxidize over time. As a fuel oxidizes it tends to form insoluble compounds that may clog filters and injectors. In addition to this, it has a greater likelihood of polymerizing under the high temperatures and pressures of an injection system. This would result in a "varnish" buildup on the surfaces of engine parts exposed to the fuel. While to a large extent, oxidative stability is a function of feedstock (typically, the more saturated the fat or oil, the greater the oxidative stability) fuel production procedures have an effect as well. The minimum oxidation stability requirement is intended to ensure the storage stability of B100 and biodiesel blends and the specification is set at a minimum of 3 hours according to method EN14112.

Project Benefits

The process of capturing carbon dioxide from flue gas with algae and the subsequent conversion of the algal lipid into a high grade biodiesel fuel produces a valuable, renewable, universally consumed fuel and at the same time it reduces green house gas emissions from coal fired power plants. In this project, it has been demonstrated that flue gas can be used as a source of CO_2 for growing microalgae. This was moved beyond lab-scale and shown to work on a larger scale.

The current market for biodiesel in MN is partly based on a 5% biodiesel government mandate. Future demand for biodiesel in MN will be based on a government mandate of 10% biodiesel in 2012 and 20% in 2015. Diesel fuel consumption in Minnesota for the year 2000 was 631 million gallons. Currently, biodiesel demand is at least 31.5 MGY and will likely be greater than 126 MGY by the end of 2015.

- Carbon dioxide captured from power plant emissions and used to grow algae can produce feedstock oil for biodiesel fuel at a production rate per acre per year that can range from 25 - 300 times greater than that of traditional soybean oil productivity. Significant quantities of carbon were captured (fixed) by the algae in vessels where algal growth became very dense as a result of intensive culture.
- Biodiesel can be made from the algae oil and used by the power company to fuel trucks & cars or be sold. Algae produced feedstock can realistically be 27% - 30% oil, about 40% - 45% protein and the rest carbohydrate. Approximately 1/3 of the total lipids (oil) are neutral lipids (triglycerides and free fatty acids) and useful for making biodiesel. Conventional biodiesel production plants can only convert a part of this oil to biodiesel fuel because of algae's naturally high free fatty acid (FFA) content. However the Mcgyan® process will convert all of the neutral lipids to biodiesel. Because of its level of unsaturated fatty acids, algae oil makes a better winter biodiesel. Additionally the non-lignified carbohydrate fraction of the algae biomass can more easily be converted to ethanol than other more recalcitrant biomass such as switchgrass or corn residue. The remaining proteinaceous fraction could potentially be sold as animal feed or organic fertilizer to various agricultural markets or it can be burned at the plant as a renewable fuel.
- Environmentally it is sound practice to make biodiesel from algae lipid as it is a carbon-neutral fuel for which new petroleum is not required. Biodiesel from algae lipid (and in general), is a cleaner-burning fuel than petroleum diesel because it contains 11 percent oxygen by weight. This oxygen allows the fuel to combust more completely, so fewer unburned fuel emissions result and thus biodiesel reduces tailpipe emissions when compared to petroleum diesel[56].
- Taking the initiative to develop algae based biofuel technologies that will sustain our society in the future would be part of good corporate social responsibility. New, permanent jobs would be created in the process of commercializing this technology, including: jobs associated with providing feedstock lipid for conversion (growing algae and harvesting the lipid – engineers, management, plant operators and support personnel) and jobs associated with converting the lipid into biodiesel (plant engineers, management, operators and support personnel). For this latter component we have seen approximately 20 permanent jobs created for our 3 million gallon per year commercial production facility (Ever Cat Fuels, LLC). Additionally, temporary engineering and construction jobs for the manufacture of algae growth plants (on site near flue gas sources) and biodiesel production plants employing the Mcgyan process would be created.
- An algae based biofuel production system like this will not compete for farm grown food products in order to produce fuel. As a result, the "food vs. fuel" conflict is avoided. Furthermore the truck, rail and other traffic conventionally incurred to transport feedstocks from farms is eliminated when algal feedstock is produced on site.

Project Lessons Learned

- Although there are many species of algae that can grow fast and therefore, utilize larger amounts of CO_2, some are better than others. We found Dunaliella tertiolecta to be a fast growing species that is also known to yield greater amounts of lipids than many others.
- Manipulation of the growth medium influences the algal growth rate.
- Good light penetration & circulation of the cultures and sufficient, uninterrupted flow of CO_2 during the photoperiod are essential to maintaining a thriving algae population. The light source needs to include 430 nm and 680 nm wavelengths to support algae growth; natural sunlight is a reliable source. Artificial lights weaken in intensity over time and need to be replaced periodically.
- Growing algae outside can encourage high growth rates and greater CO_2 utilization, however, climate and variations in the local weather (low temperature, cloud cover) can significantly affect the overall growth rate of algae.
- Maintaining a pH that is neutral or somewhat acidic ensures more efficient utilization of CO_2 and higher growth rates. Lack of CO_2 allows the pH to increase to undesirable levels (8 – 9.5).
- Providing sufficient mixing to prevent algal cells from clumping in dense cultures; when cells clump together, they stop dividing and start to die.
- Outdoor culture of algae presents different challenges than indoor culture. Indoor culture is generally done in smaller vessels which can be clear on all sides which can increase light penetration. Outdoor culture in large scale vessels (ponds) only provides one surface through which light can penetrate; shallow ponds allow good light penetration. Outdoor cultures are more likely to have problems with competing organisms such as bacteria or algae grazers such as rotifers and protozoa. Daphnia (algae grazers) or planktivorous fish could also pose a problem in fresh water environments that are supplied by surface waters (lakes, rivers). These invasive organisms tend to thrive when the algae culture is not growing rapidly or is not healthy due to conditions such as lack of CO_2 or nutrient deficiency.
- Algal cells tend to age and precautions need to be taken to keep a continuous influx of new, healthy cells into the system. This is done by inoculating flasks of growth medium from the slant tubes followed by introducing this fresh material into the large scale system.
- Harvesting algae can be time consuming and expensive. The electric power costs for centrifugation can be a significant portion of the overall costs. Other techniques might be more efficient. Plasmalemma is released from the cells when passing through the electrolytic flocculator; this might be utilized as a food source for the algae if the medium gets recycled back into the aquarium. Also, harvesting at the most appropriate stage of growth helps to prevent algal self-shading and therefore enables the maintenance of high growth rates.
- There are limitations on re-using the post-harvest algae growth medium due to build up of metabolites such as glycoproteins, which inhibit algae growth and cause algae to stick to the walls of the vessel. Addition of fresh growth medium periodically discourages or mitigates this problem.
- Lipid extraction is slow and sometimes gives variable results. Neutral lipids, which comprise about 1/3 of the total lipids, are the only ones useful for making biodiesel. Better techniques will help make this more viable.

Usefulness of Project Findings

This project has demonstrated that it can be done; bringing the carbon emissions from a coal-burning power plant full-circle and creating a useful fuel in the process. If a portion of CO_2 emissions can be directed into growing energy crops such as microalgae, this may offset future enacted carbon taxes, which would help curtail future rate increases to consumers. The harvested algae have value for making biodiesel fuel or bio chemicals; the algal solids can also be burned to help fuel the generation of electricity. Algal components, such as protein, could potentially be sold as additives for animal feed, fertilizer or other products. These post-harvest uses for the algae can all help bring in revenue to offset any costs attributed to infrastructure needed to support this type of activity. Another result could be greater diversification in the Minnesota economy and creation of new jobs.

Ideas for Further Research

- Identifying how to concentrate and deliver CO_2; this might involve learning whether the CO_2 can be attached to a salt and pelletizing it or liquefying the CO_2.

- Exploring more cost effective methods of harvesting.

- Discovering ways to more completely dissolve the CO_2 so it doesn't bypass the algae and go into the atmosphere.

PROJECT STATUS

The project has been completed and is within budget.

ACKNOWLEDGEMENTS

This project has benefitted greatly from, and we are grateful for the contributions of: Dr. Arun Goyal, Dr. Mary Ann Yang, Nick Blixt, Robert Quigley, Joel Schumacher, Cool Clean Technologies, Solution Recovery Services, Water 360 LLC, Dr. Dan Nowlan, Dr. Ben Yan, Shane Wyborny, Julie Jenkins and Ever Cat Fuels, LLC.

Appendix A

Literature Review of Algal Production Systems

Introduction

Although there is much debate concerning global warming, atmospheric carbon dioxide accumulation and the burning of fossil fuels, there is mounting international pressure to reduce the global accumulation of carbon dioxide in the atmosphere[31]. In the United States this is also a growing concern and there is also a general consensus that the U.S. needs to find ways to use domestic sources of energy to decrease our dependence on foreign energy supplies. Coal is our most abundant energy resource and is used extensively for generating electricity but produces large quantities of carbon dioxide in the process. Currently, the energy industry has had an increased interest in technology that can capture carbon dioxide from coal combustion in an economical and sustainable manner.

Microalgae are photosynthetic microbes that show great CO_2 mitigation potential by biologically fixing carbon through photosynthesis at rates up to fifty times greater than the fastest growing terrestrial plant[32-34]. While the theoretical productivity of algae is impressive, practical large-scale algae production systems and techniques are still being developed. The wide variety of mass algae cultivation systems can be categorized into either open, closed, or two-phase systems each with its own benefits and limitations. This review will summarize the various outdoor naturally illuminated mass culturing systems that have been attempted.

In microalgal farming the key parameter in a system's viability is its areal productivity, dry biomass production per unit area and time, typically reported as g m^{-2} d^{-1}. Every algal production system strives to increase the areal productivity while reducing the capital and processing costs. The illuminated surface area ultimately limits productivity as long as the inorganic nutrients, nitrogen, phosphorus, potassium, carbon, and trace metals are sufficiently supplied. Open systems, such as ponds, lagoons, stirred raceways and circular ponds maximize the illuminated surface area to volume ratio by keeping the water depth shallow – 20 cm or less. To further increase the illuminated surface area closed systems such as tubular, airlift coils and columns, and flat plate photo bioreactors (PBR) were developed.

Open Algae Production Systems

Microalgae can be harvested from natural sources such as unmixed natural ponds, lakes, and lagoons. This approach is still practiced by a few health food companies around the world. In the U.S. and Asian countries *Aphanizomenon* and *Nostoc* are exclusively grown in natural waters for health food products. *Dunaliella,* which produces carotinoids as its primary product, is grown in 250 hectare and up to 50 cm deep lagoons in Australia and in smaller natural ponds in the Ukraine. Crater lakes in Myanmar (Burma) and Lake Texcoco in Mexico have been used to produce *Spirulina* [35]. The Lake Texcoco system has since shut down due to urban and industrial pollution. While these systems have virtually no construction costs, the productivity and quality of the products are low and highly variable[36]. Furthermore, feasible locations such as the alkaline crater lakes of Myanmar where villages can scoop out buckets of concentrated *Spirulina* are extremely rare. As a result, harvesting algae from natural sources is impractical for the mass production of most algal products.

To overcome the drawbacks of natural systems, man-made ponds were developed[37]. *Dunaliella, Chlorella,* and *Spirulina* are cultured in man-made systems in the U.S., China, Israel and other countries. To date only these three species have been successfully mass produced in monoculture and marketed commercially because they can be cultured under extreme conditions

(high salinity, high nutrients, and high alkalinity respectively), which limits contamination from competing organisms. Contamination from bacteria and other biological contaminants that reduce productivity or cause population crashes is a major shortcoming in open culture systems. In the twenty-year U.S. DOE Auquatic Species program attempts at monocultures in open raceway ponds were abandoned because of chronic contamination. The focus then shifted to wild native species that naturally took over the culture [38].

Raceway ponds are currently the predominant commercial system but circular ponds and cascade ponds are also in operation. Raceway ponds are racetrack shaped ponds of with a typical depth of 20 cm and a paddle wheel to circulate the culture. Larger ponds are divided into channels and cover an area of up to 1 ha [39]. The culture can be maintained at a cell density between 0.1 to 0.5 g L^{-1} with average productivity of 25 g m^{-2} d^{-1} during summer irradiances[37, 40]. Lower production rates of 10 g m^{-2} d^{-1} are widely reported during less ideal conditions (e.g. winter months) [38, 41]. Circular ponds, (used in Japan, Taiwan, and Indonesia) utilize a rotating radial scrapper to stir a culture of less than 5 cm deep. Circular ponds are limited in size by the strain of the water on the rotating motor [42]. Cascade ponds flow a thin film, less than 1 cm of culture, over a sloping glass surface [43]. Operated in the Czech Republic and for several years in Western Australia, the cascade pond achieves a higher cell density of 10g L^{-1} but has a comparable areal productivity of 25 g m^{-2} d^{-1}.

Closed Algae Production Systems

In order to maintain a pure algae culture without using extreme environments closed systems, called photo bioreactors (PBRs), are necessary. Closed systems have been around since the 1950's and are either tubular or flat-plate reactors. Tubular reactors come in a variety of designs: vertical airlift columns, horizontal tubes joined with U-bends, helical plastic tubing around a circular framework and α-shaped tubular reactors. Carvalho et al. provides helpful graphic diagrams of each system. The principles behind these designs are similar: a clear plastic or glass tube with a diameter of 2.5 to 40 cm increases the illuminated surface area to volume ratio allowing for high cell densities; turbulent gas is bubbled through the system to mix, and provide CO_2; a gas exchange or degasser system removes harmful O_2 build up; and the system is harvested and replenished with media continuously or semi-continuously [44-50]. Flat plate reactors utilize the same principles but the culture is contained between two clear panels a 2-4 cm apart [51]. The panels may be vertically oriented or tilted to face south to maximize exposure to sunlight [40].

Due to the higher illuminated surface area the cultures in PBRs can reach much higher densities and reportedly higher areal productivity. The cellular density can reach up to 20g L^{-1} with a volumetric productivity of 0.25-3.64 g L^{-1} d^{-1} [37]. The areal productivity of a PBR system is calculated from the footprint of the facility. Since the reactors are often vertical or tilted much higher areal productivity, up to 130 g m^{-2} d^{-1}, has been reported [52]. If the vertical systems are laid horizontally, the areal productivity is comparable to that achieved in open pond systems—25-27 g m^{-2} d^{-1} [37]. The increase in areal productivity, however, is accompanied by a significant increase in capital and maintenance costs; so much so that only small-scale systems are producing algae. While there have been numerous pilot plant studies, any commercial facilities attempted were closed within a few years due to the high costs.

Two-stage Algae Production Systems

Recently, two-stage production systems that couple PBRs with open ponds have shown promise in increasing areal productivity while maintaining monocultures under non-extreme

environments. A tubular or flat-plate PBR is used to cultivate a monoculture at high density. The culture is then used to inoculate an open pond where the algae rapidly grows and is harvested within a two days [53]. The added benefit of the sudden change in culture conditions is that it stresses the algae, which can stimulate the production of oils and other valuable bioproducts [54]. Huntley et al. demonstrated the system's feasibility on an industrial scale for several years, producing 36.4 27 g m^{-2} d^{-1} of *Haematococcus pluvialis*.

Conclusion

While there are a variety of methods for mass culturing algae outdoors, very few designs have been successful on a large scale for long periods of time. Open systems are simple, have low capital and maintenance costs and are the most prolific design to date. However, the small surface area to volume, large area required, and high risk of contamination limits the areal productivity. Open systems are also typically low-density cultures, which increases the down stream processing costs such as harvesting and dewatering. Closed systems have demonstrated an increase in the areal productivity and volumetric density over open systems, but the initial capital and maintenance costs are often too great for the system to be economically viable on a large-scale. Two-stage systems attempt to combine the cost benefits of an open system with the high productivity and ability to maintain a monoculture of closed system. Although the two-stage system shows great promise, it has not been demonstrated on as economically viable as a commercial system.

Appendix B

(a) (b)

Figure 2. Photo bioreactor (PBR) with LED light source and tubular photo shield (a) and with LED light source and tubular photo shield removed (b).

Figure 3. Photo bioreactor (PBR) showing LED light source in a spiral pattern on the internal side of the tubular photo shield.

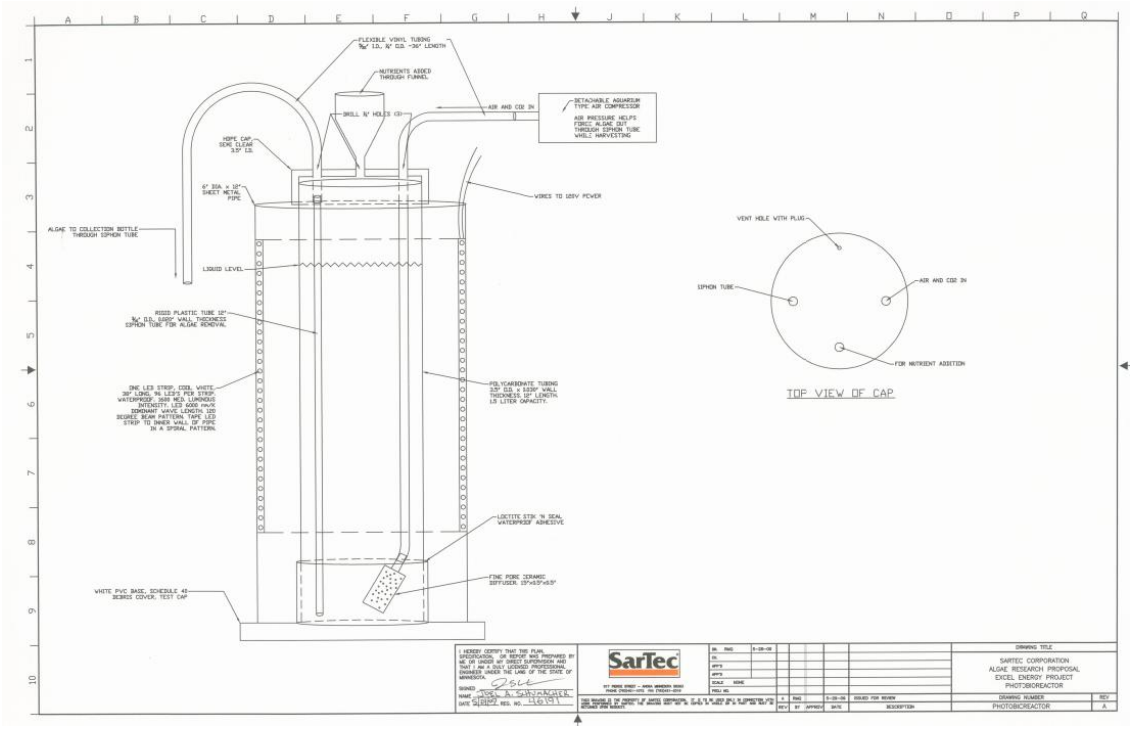


Figure 4. Engineers drawing of the photo bioreactor (PBR).

PBR Description:

The polycarbonate tube measures 3.5 inches in diameter by 12 inches in length and holds a volume of 1.5 liters. Thin walled (0.03 inch thickness) poly-carbonate material allows for high light penetration while still providing adequate containment. There is a HDPE, semitransparent cover on top with a siphon tube for removal of algae, and an airline going into vessel to supply a mixture of air and carbon dioxide. The air line runs internally to the bottom of the reactor and a fine pore ceramic diffuser is attached to the end of the line to disperse the air and carbon dioxide mixture into the solution, thus achieving greater solubility per volume of gas used. Culture and nutrient media can be added to the PBR through a small opening on the top. A 6 inch diameter by 12 inch length sheet metal pipe surrounds the polycarbonate tube in a concentric manner, and an opaque top cover in conjunction with the opaque PVC base allow one to minimize the amount of external light reaching the PBR. Cool white LED strip lights are mounted on the internal side of the metal pipe tubular shield in a spiral fashion. The PBR has been certified operational by Professional Engineer Joel Schumacher.

Aqueous carbon dioxide and algal carbon utilization:

Overall equation for carbon dioxide in water:

CO_2 (gas) $\leftrightarrow$ CO_2 (dissolved) + $H_2O \leftrightarrow H_2CO_3$ (carbonic acid) $\leftrightarrow HCO_3^-$ (bicarbonate) + $\leftrightarrow$ CO_3^{2-} (carbonate) + $H_2O + CO_2$

The quantity of CO_2 dissolved in water and the concentration of carbonic acid is directly proportional to the partial pressure of CO_2 in the gas phase. The hydration equilibrium constant at 25º C for dissolved carbon dioxide forming carbonic acid is $K_h = 1.70 \times 10^{-3}$.

Based on this relationship, the equation can be simplified to:
$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$ with an equilibrium of 4.30×10^{-7} and a $pK_a = 6.36$.

In biology, because the rate of transformation of CO_2 to bicarbonate is not fast enough for life processes, most all forms of life use a family of enzymes called carbonic anhydrases (CA) to greatly speed up this second reaction. Carbonic anhydrases have some of the fastest forward reaction rates known and are only limited by substrate diffusion.

Utilization of inorganic carbon by algae:

The primary enzyme of carbon dioxide fixation in photosynthetic organisms including algae is named Ribulose-1,5-bisphosphate carboxylase/oxygenase or Rubisco. Rubisco is a Bifunctional enzyme that fixes both carbon dioxide and oxygen, the relative rate depends on the availability of two gaseous substrates.

Algae contribute to more than 70% of global photosynthesis. Carbon concentration mechanism (CCM) plays an important role in the global climatic changes. A general working model for the DIC concentrating mechanism in unicellular green algae include isoforms of carbonic anhydrase (CA), membrane associated active transporters and P-type ATPase (both in the plasmalemma and the inner chloroplast envelope). Isoforms of CA have been well characterized, but the components of active DIC transporters have not.

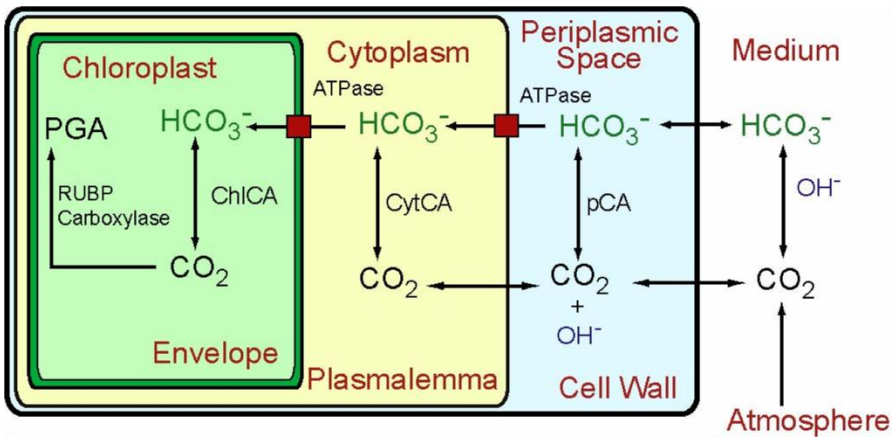


Figure 5. Carbon concentration mechanism in unicellular green algae.

Aquatic plants and algae also compensate for the CO_2/O_2 imbalance in the atmosphere through the use of carbon concentrating mechanisms (CCM) which bring inorganic carbon into the cells in ways which will allow elevated CO_2 levels in the cells.

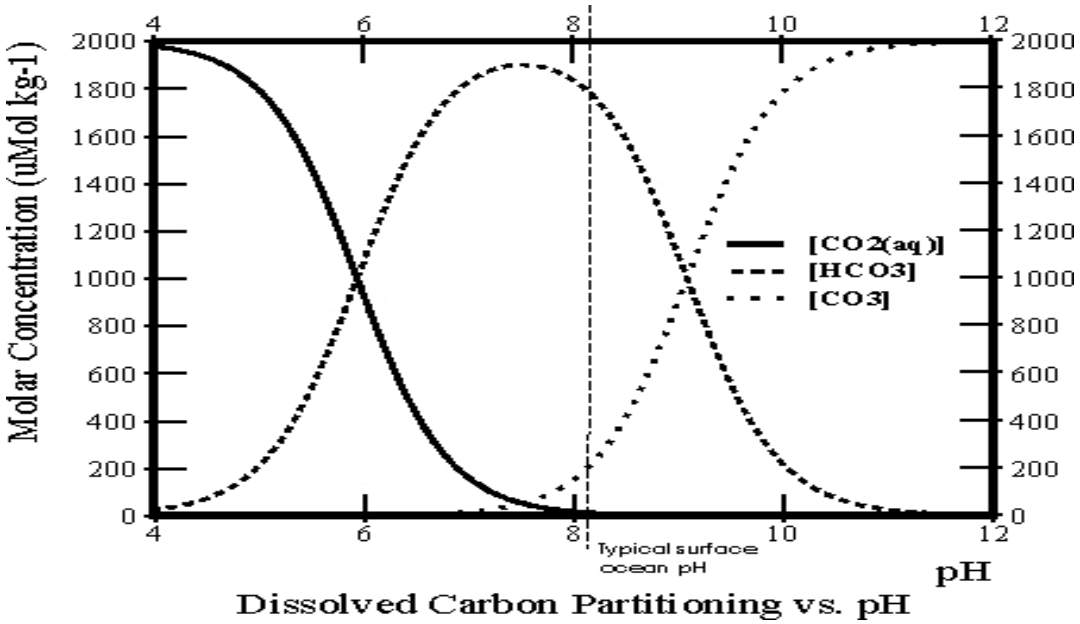


Figure 6. The effect of pH upon carbon partitioning.

Figure 7. Algae paste after harvesting with the centrifuge (not yet dried).

Figure 8. Picture of three aquariums. The *D. tertiolecta* aquarium (middle) and *D. salina* aquarium (right) are significantly darker than the *N. oculata* aquarium (left).

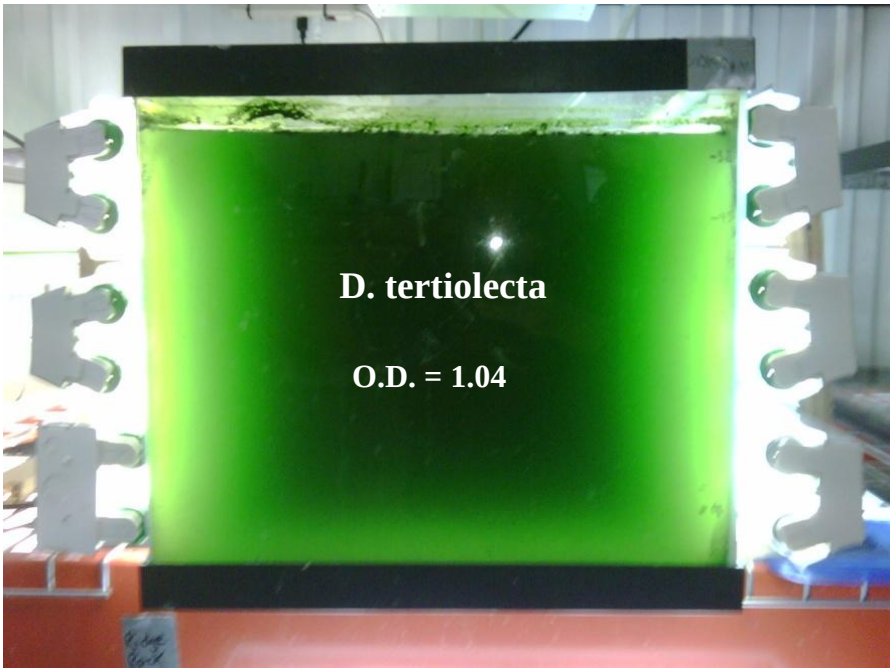


Figure 9. Picture showing light penetration into a *D. tertiolecta* culture with an OD=1.04.

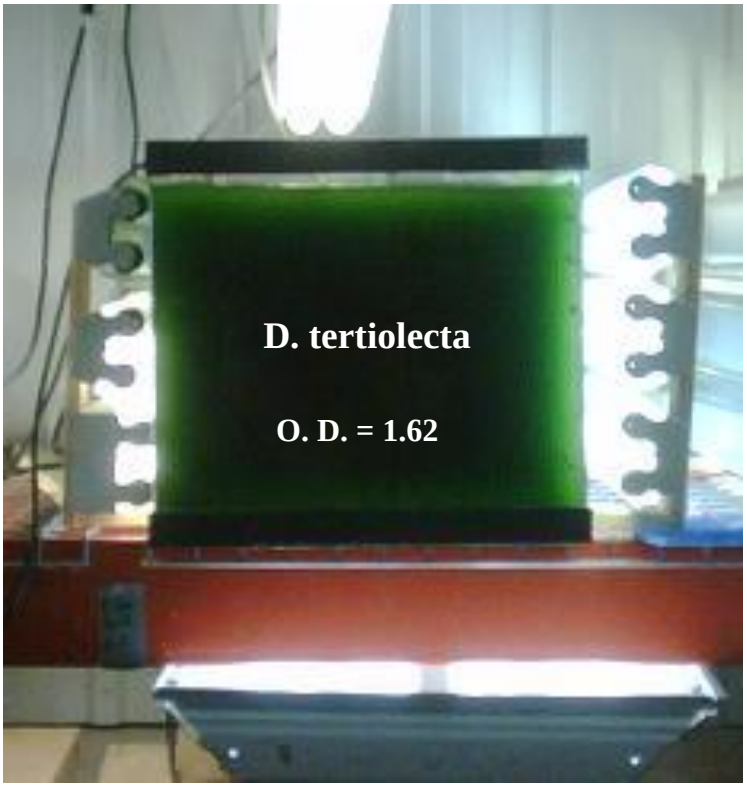


Figure 10. Picture showing the light penetration into a *D. tertiolecta* culture with an OD=1.62.

Figure 11. Photograph of *D. tertiolecta* growing in an outside aquarium and tote with natural sunlight.

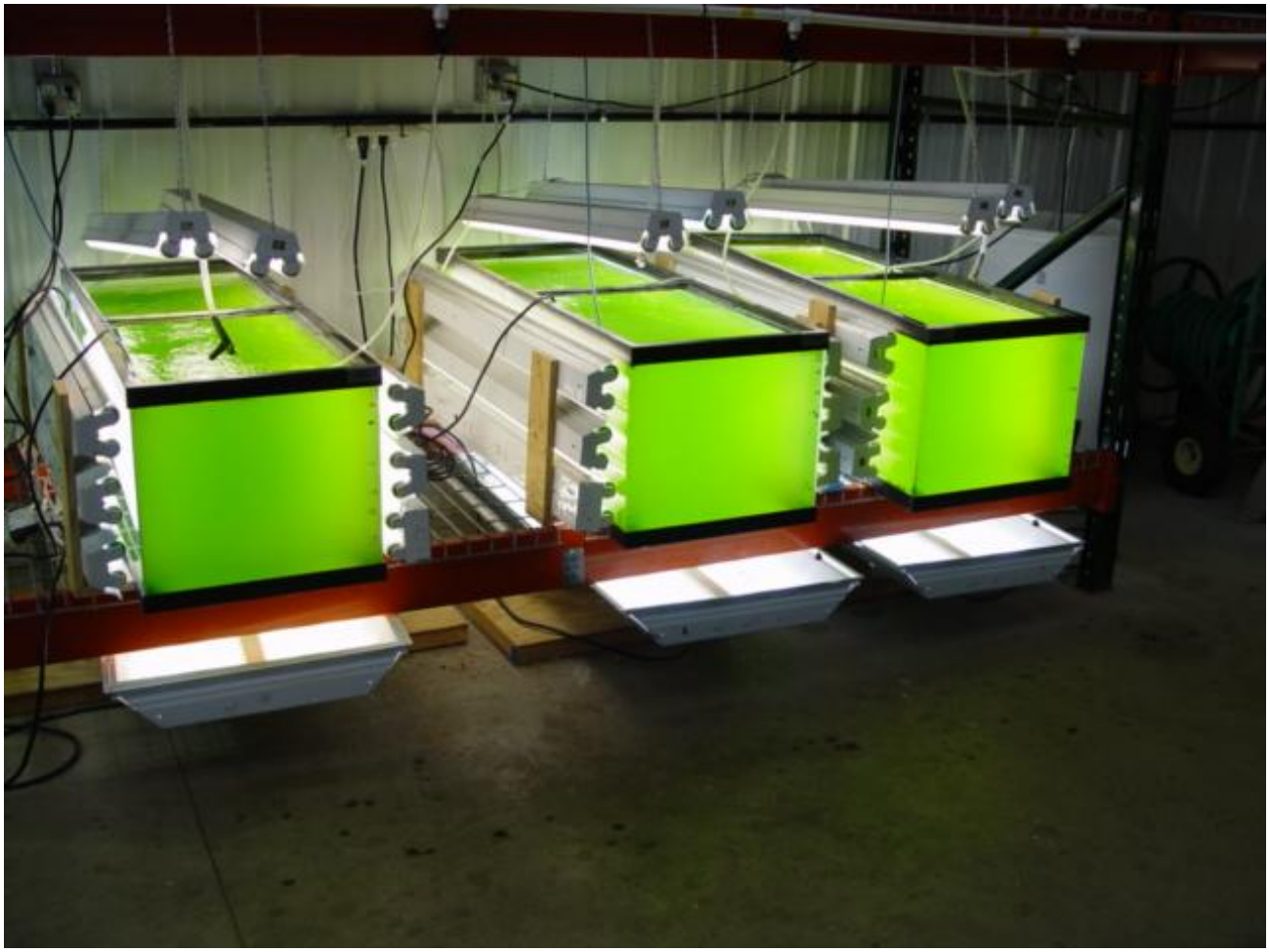


Figure 12. Photograph of three aquariums with *D. tertiolecta* growing inside with artificial light.

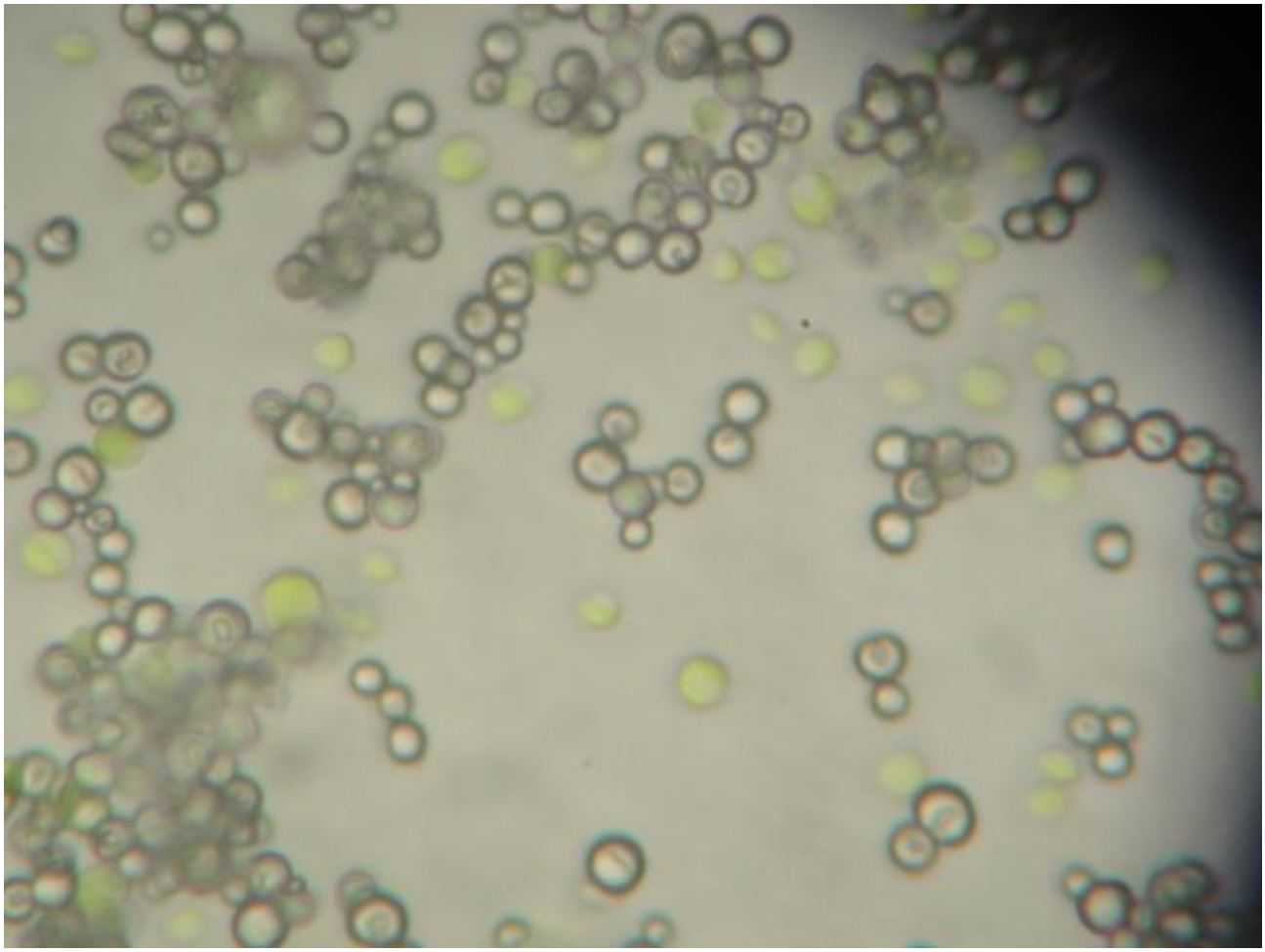


Figure 13. A microscope photograph of D. tertiolecta algae cells (green) and 3 micrometer diameter zirconia beads (clear/white). This shows the approximates size of the algae cells to be 3 microns in diameter.

Figure 14. Photograph of the mill used to pulverize dried algae.

Figure 15. Photograph of algae pulverized via the mill in Figure 14.

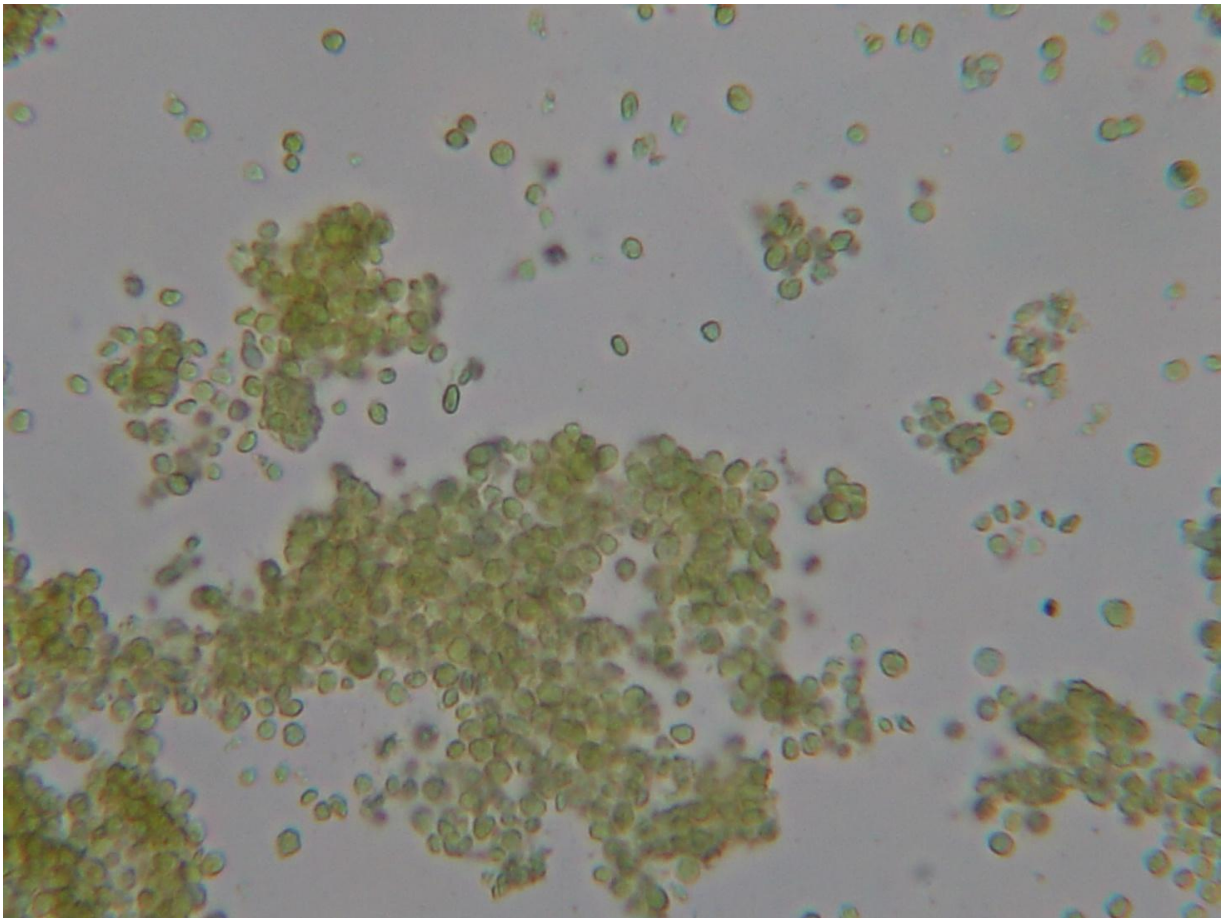


Figure 16. A microscope photograph of algae pulverized via the mill; cells are approximately 1-10 micrometers.

Figure 17. Reflux apparatus containing algae & solvent, with stirring and heat.

Figure 18. Soxhlet apparatus for extracting lipids from algae.

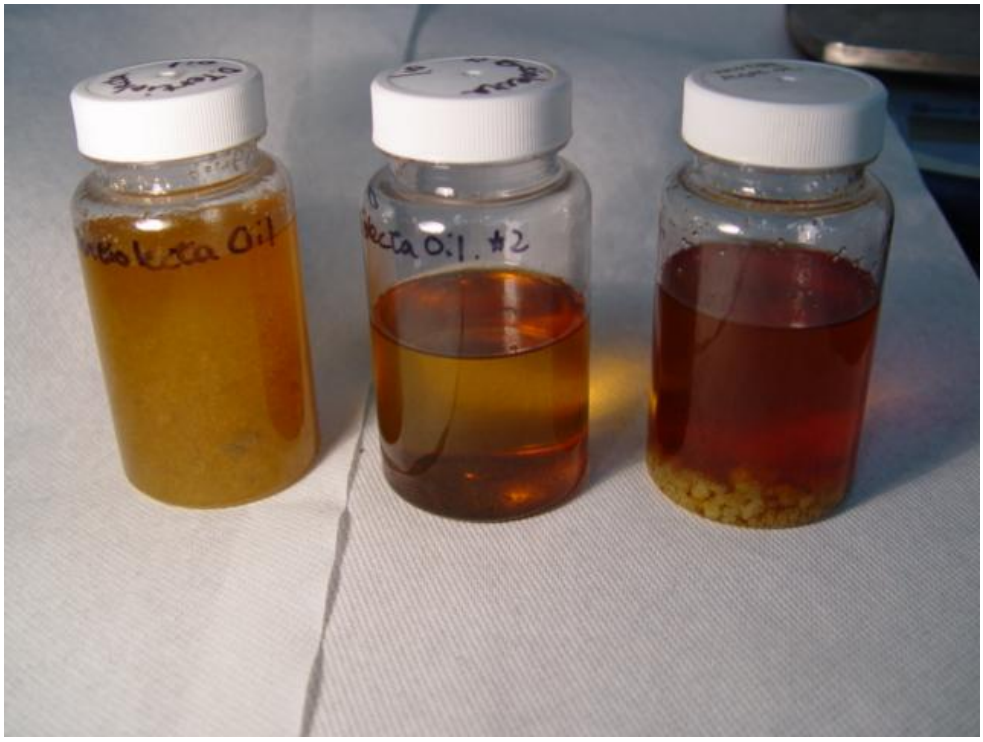


Figure 19. Neutral lipid fraction of algae oil.

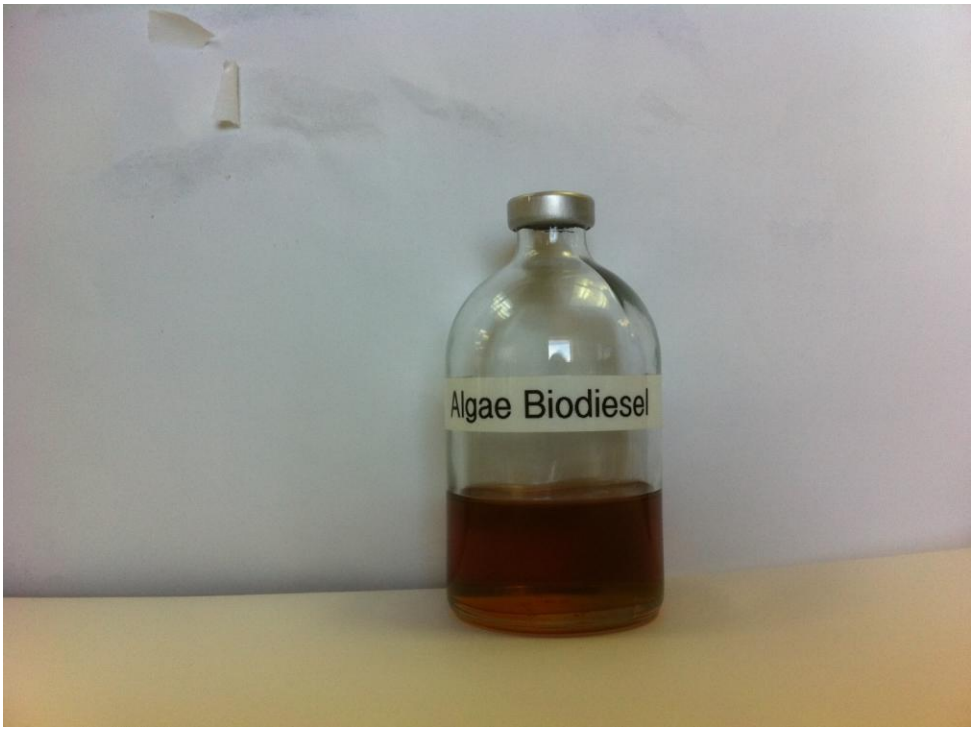


Figure 20. Biodiesel fuel made from *D. tertiolecta* oil.

The following graphs depict the growth of the algae through measurements of cell density using absorption of light by the algal suspension and total chlorophyll content in per unit volume of algal suspension respectively A_{450} and chlorophyll-a. Daily measurements show a pattern of algal growth.

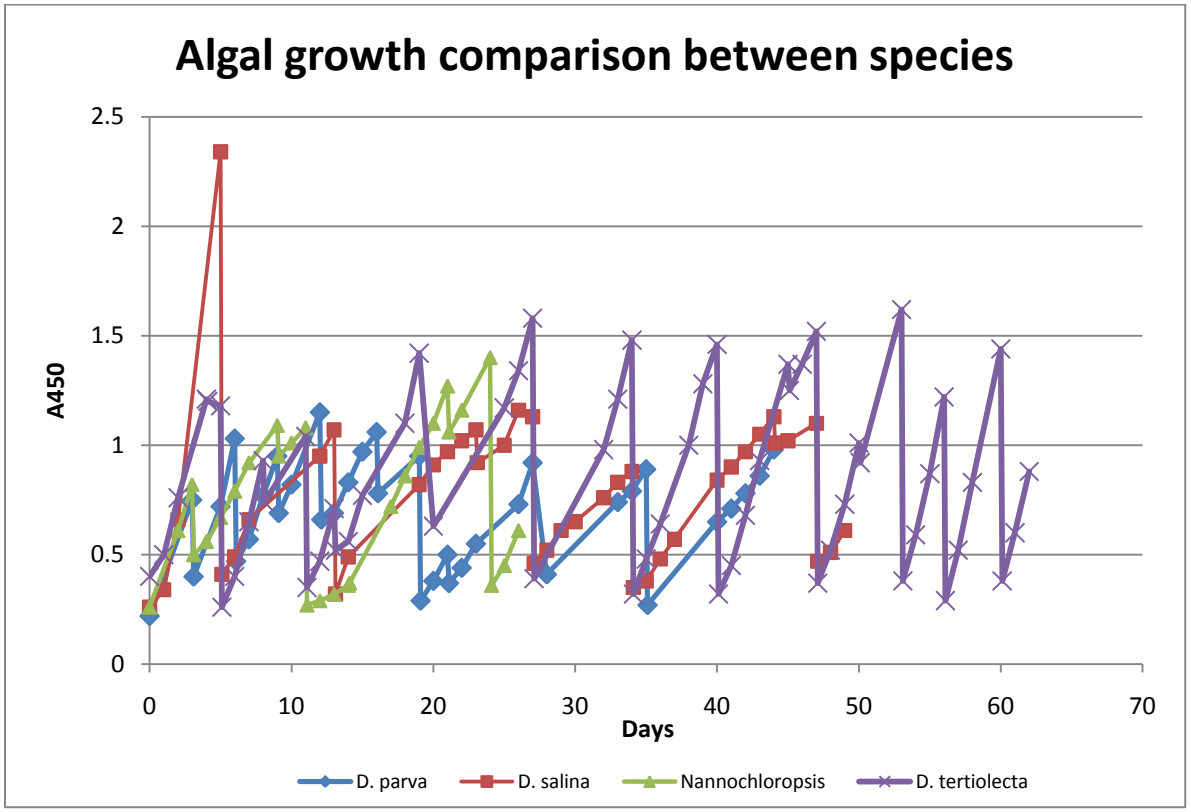


Figure 21. Graph comparing the growth rate of four different algal species.

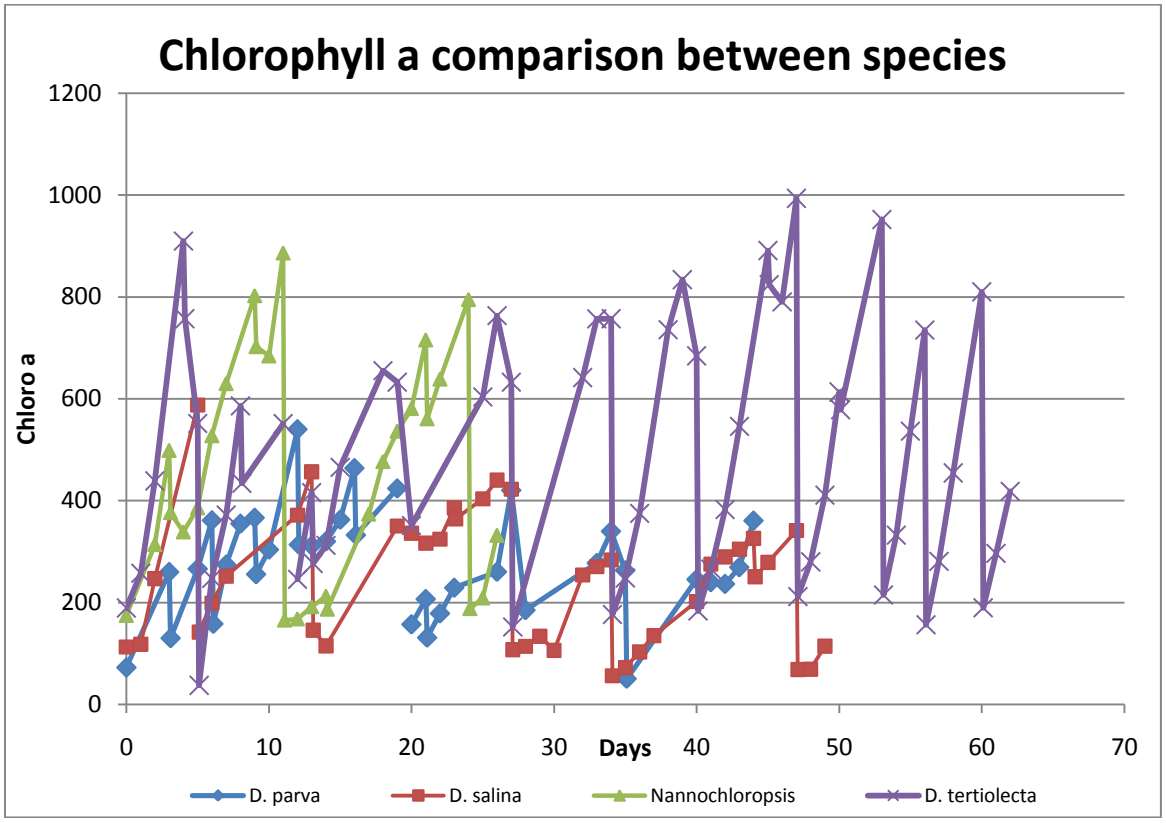


Figure 22. Graph comparing the chlorophyll-a levels over time for four different algal species.

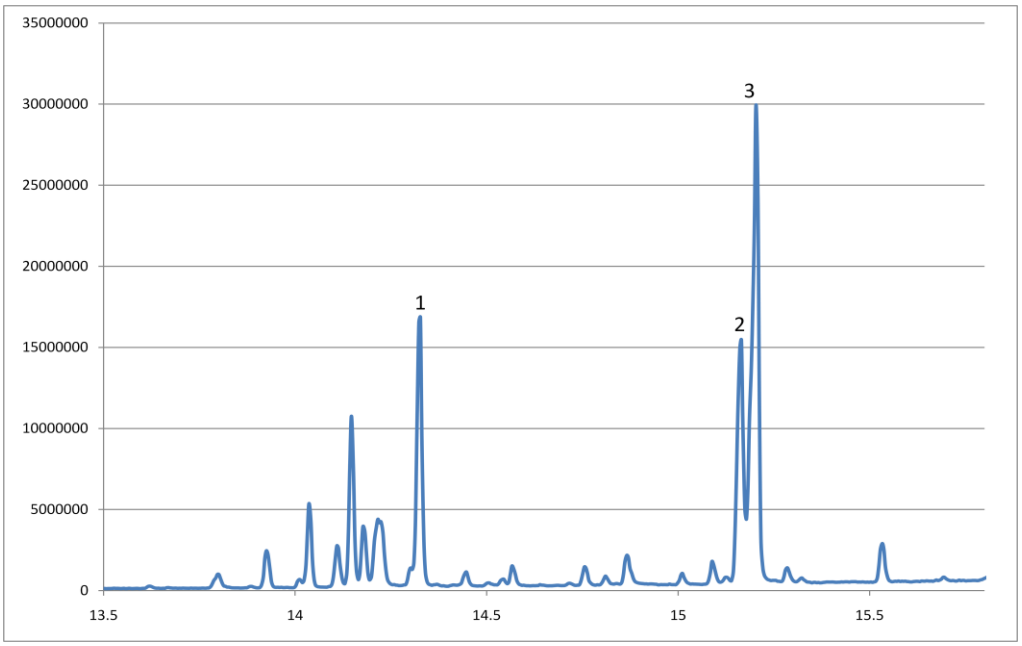


Figure 23. Lipid profile of D. parva separated and identified by GC-MS. Peak 1 : Hexadecenoic acid methyl esters. Peak 2: 12-Octadecadienoic acid methyl esters. Peak 3: 9,12,15-Octadecatrienoic acidmethyl esters.

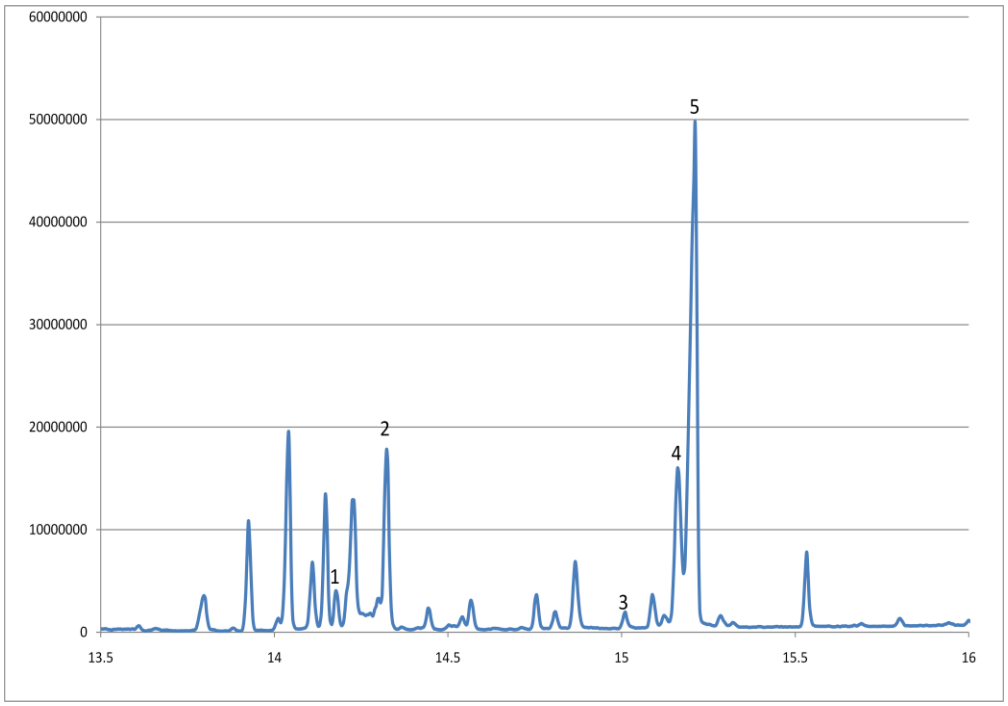


Figure 24. Lipid profile of D. salina separated and identified by GC-MS. Peak 1 : 7,10-Hexadecadienoic acid methyl esters. Peak 2: Hexadecenoic acid methyl esters. Peak 3: 6,9,12-Octadecatrienoic acid methyl esters. Peak 4: 9,12-Octadecatrienoic acid methyl ester.

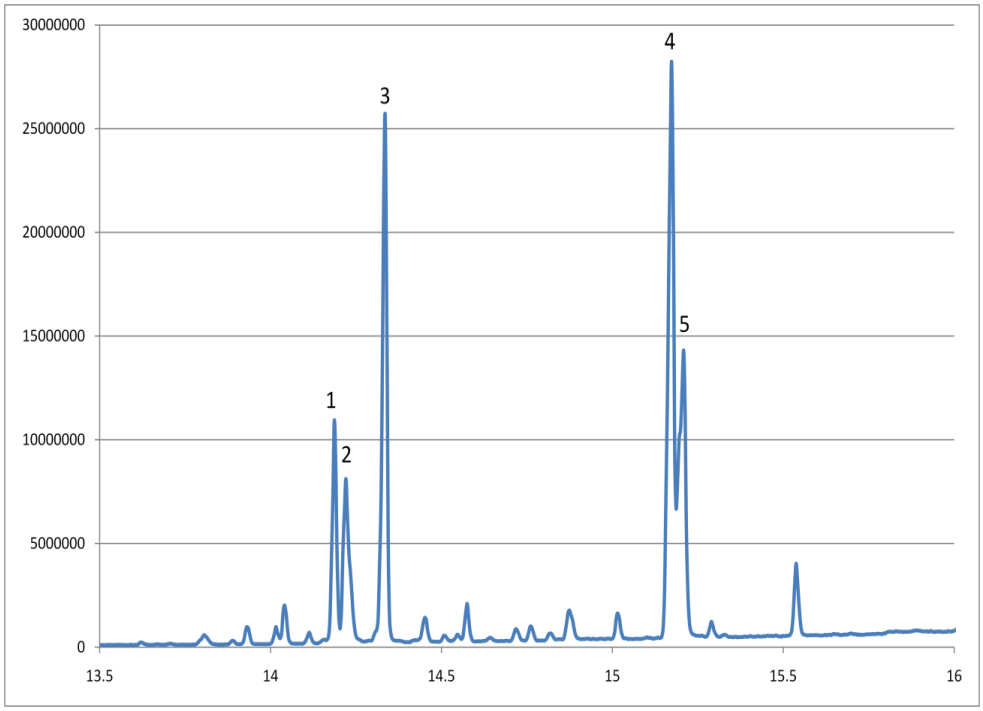


Figure 25. Lipid profile of D. tertiolecta separated and identified by GC-MS. Peak 1 : Hexadecadienoic acid methyl esters. Peak 2: Hexadecatrienoic acid methyl esters. Peak 3: Hexadecanoic acid methyl esters. Peak 4: Octadecadienoic acid methyl ester. Peak 5: Octadecatrienoic acid methyl ester.

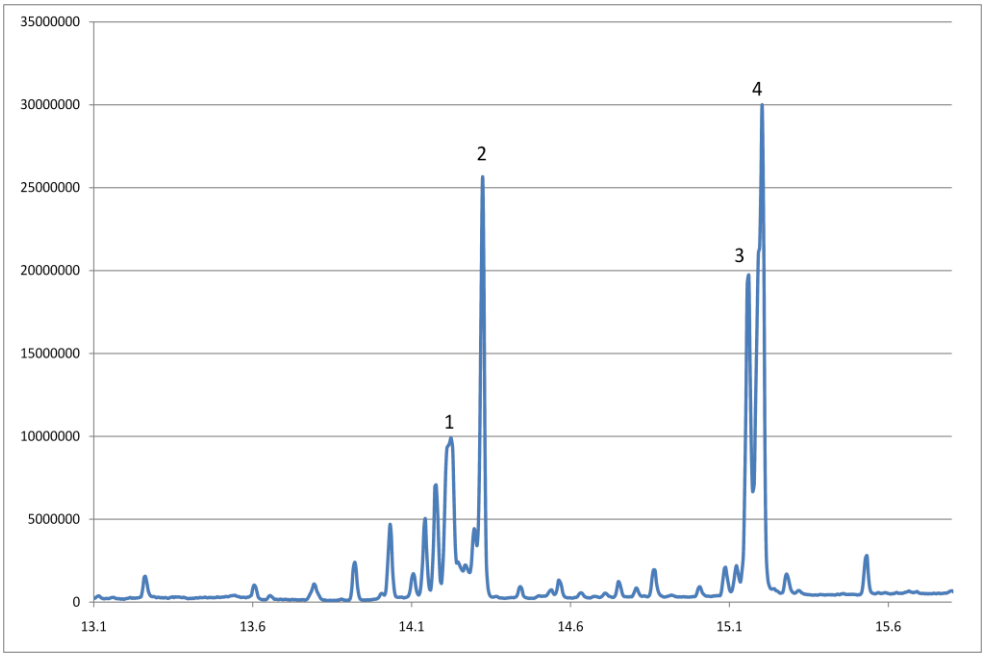


Figure 26. Lipid profile of wildtype algae separated and identified by GC-MS. Peak 1 : 9-Hexadecenoic acid methyl esters. Peak 2: Hexadecenoic acid methyl esters. Peak 3: 12-Octadecadienoic acid methyl esters. Peak 4: 12,15-Octadecatrienoic acid methyl ester. Peak 4: 12,15-Octadecatrienoic acid methyl ester. Peak 5: Octadecanoic acid methyl ester.

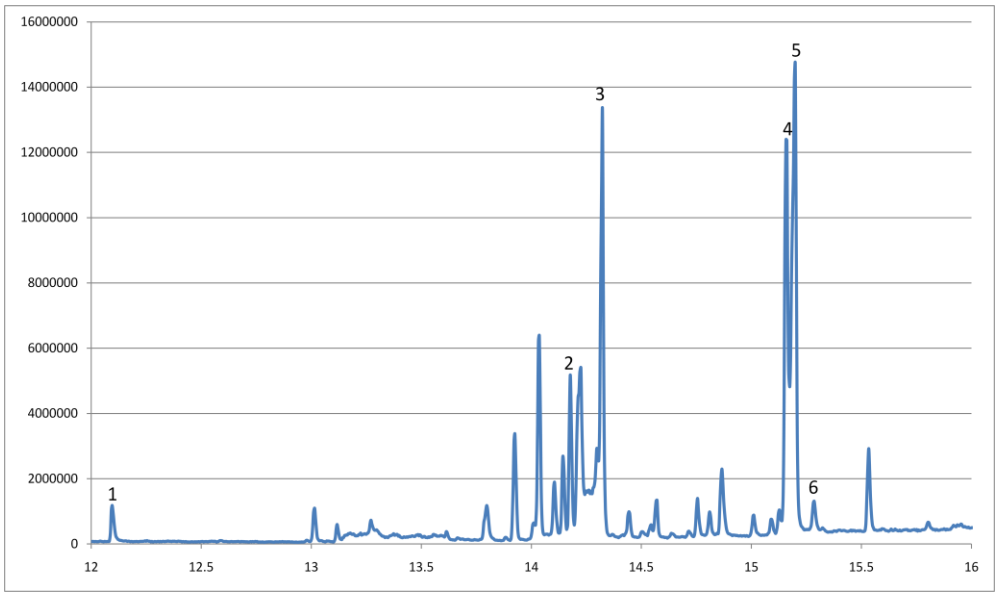


Figure 27. Lipid profile of N. oculata separated and identified by GC-MS. Peak 1 : Dodecanoic acid methyl esters. Peak 2: 7,10-Hexadecadienoic acid methyl esters. Peak 3: Hexadecanoic acid methyl esters. Peak 4: 9,12-Octadecadienoic acid methyl ester. Peak 5: 9,12,15-Octadecatrienoic acide methyl ester. Peak 6: Octadecanoic acid methyl ester.

Gas Chromatography Mass Spectrometry (GCMS) analysis of biodiesel produced from the neutral lipid fraction of algae oil:

GC-MS on Algae Biodiesel (ST39-21)

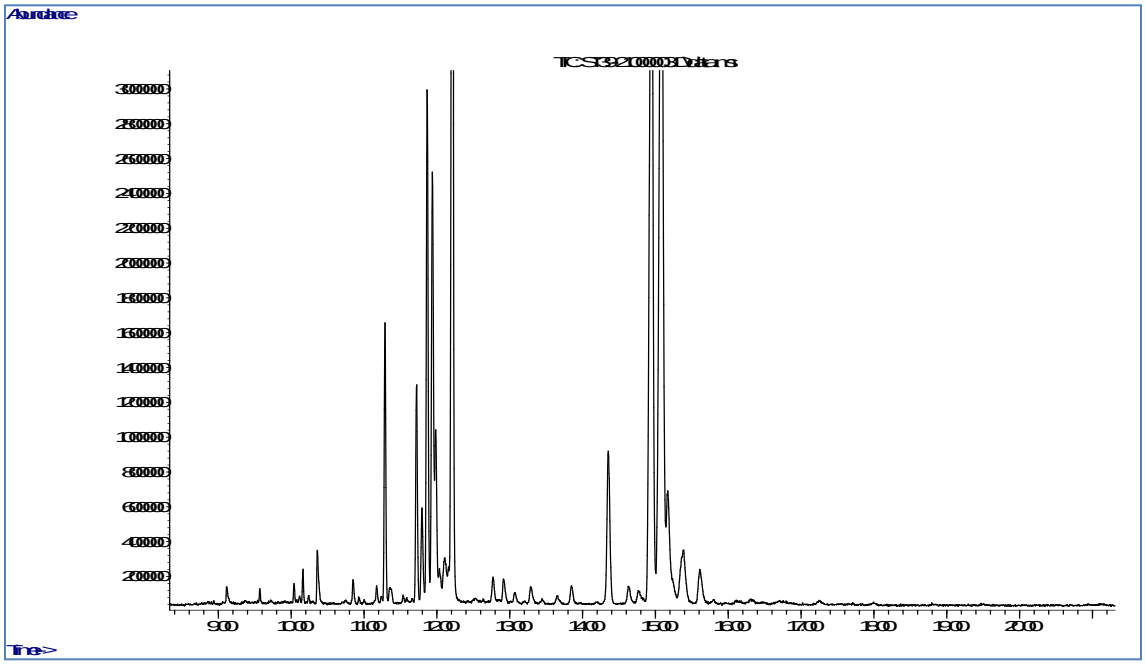


Figure 28. GC-MS spectrum of algae biodiesel.

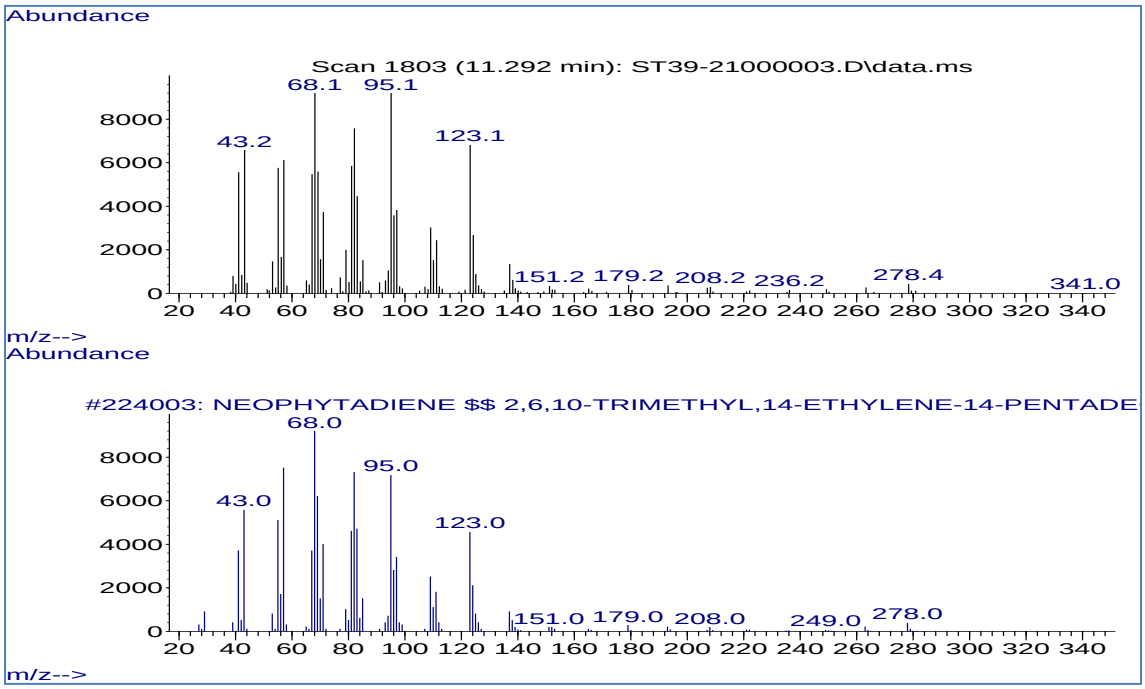


Figure 29. Peak at 11.292 min was identified as Neophytadiene.

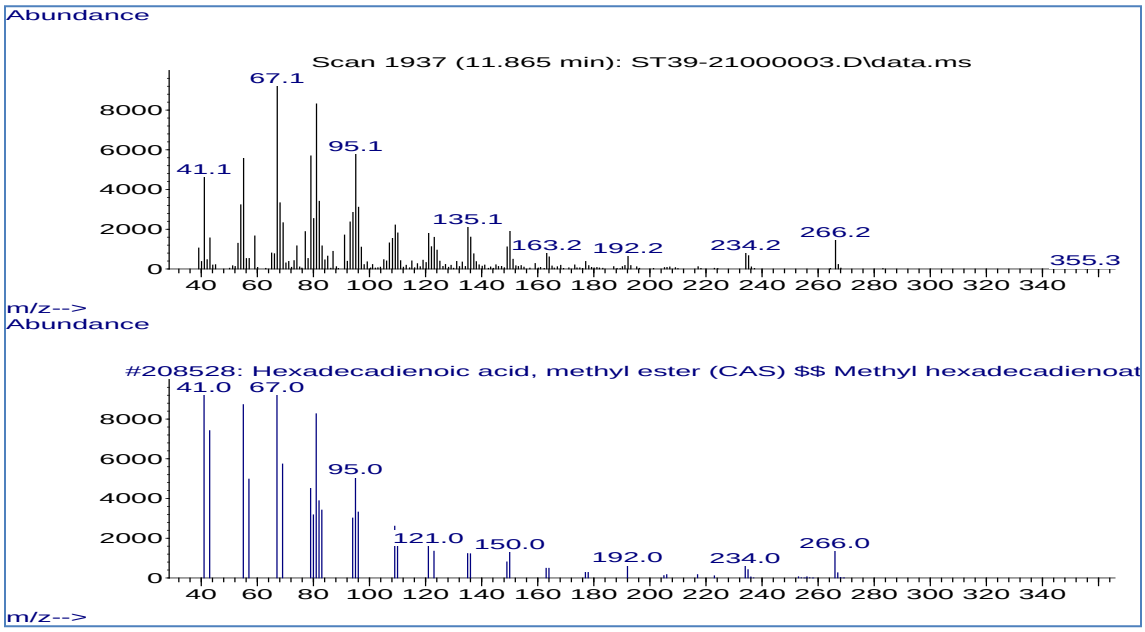


Figure 30. Peak at 11.865 min was identified as methyl hexadecadienoate.

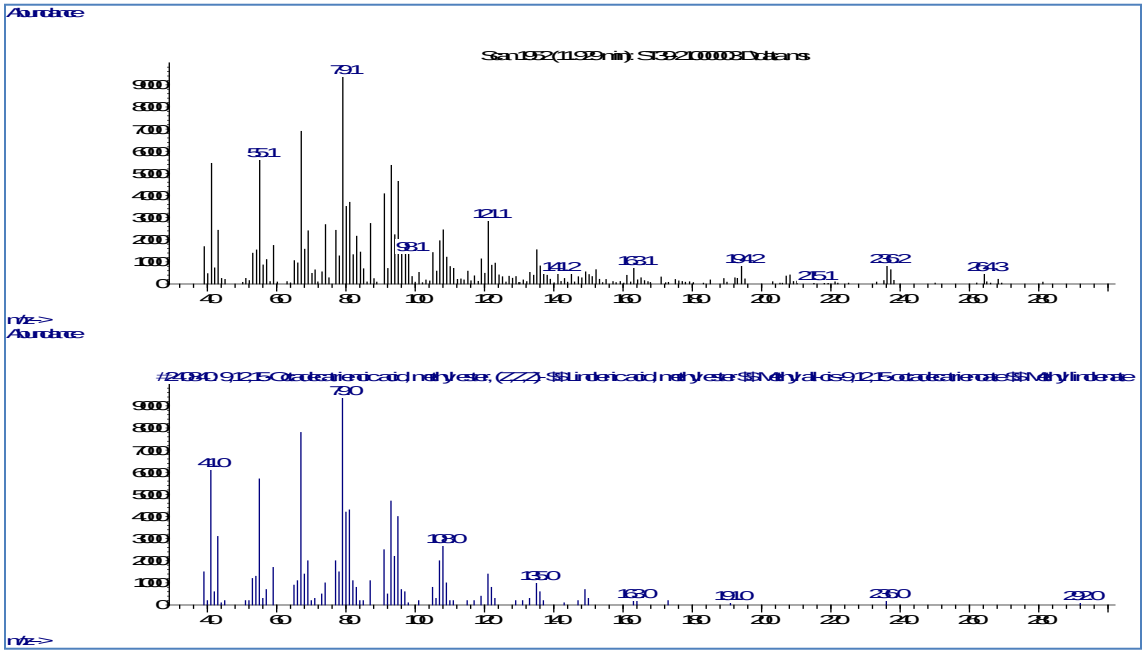


Figure 31. Peak at 11.865 min was identified as methyl hexadecadienoate.

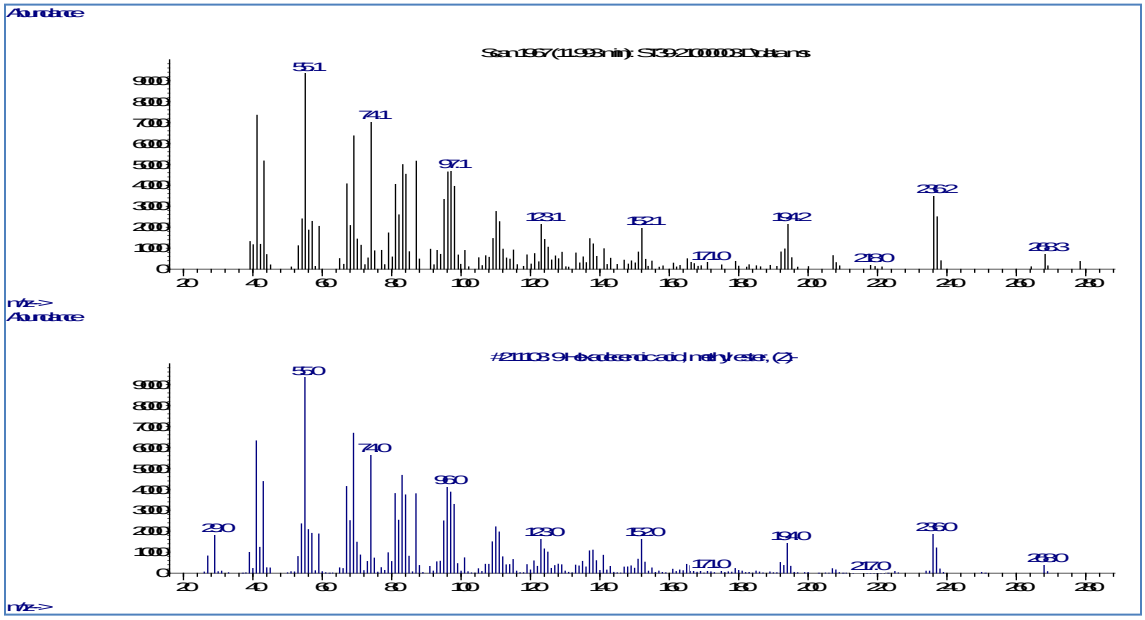


Figure 32. Peak at 11.993 min was identified as methyl 9-hexadecenoate.

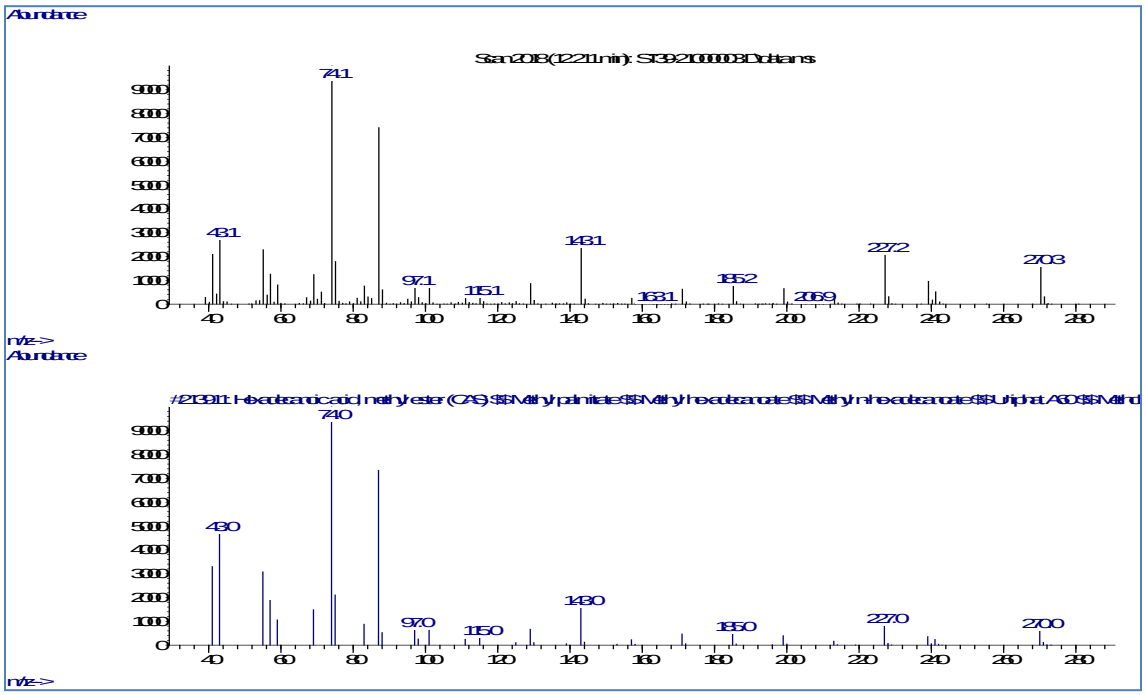


Figure 33. Peak at 12.211 min was identified as methyl hexadecanoate**.**

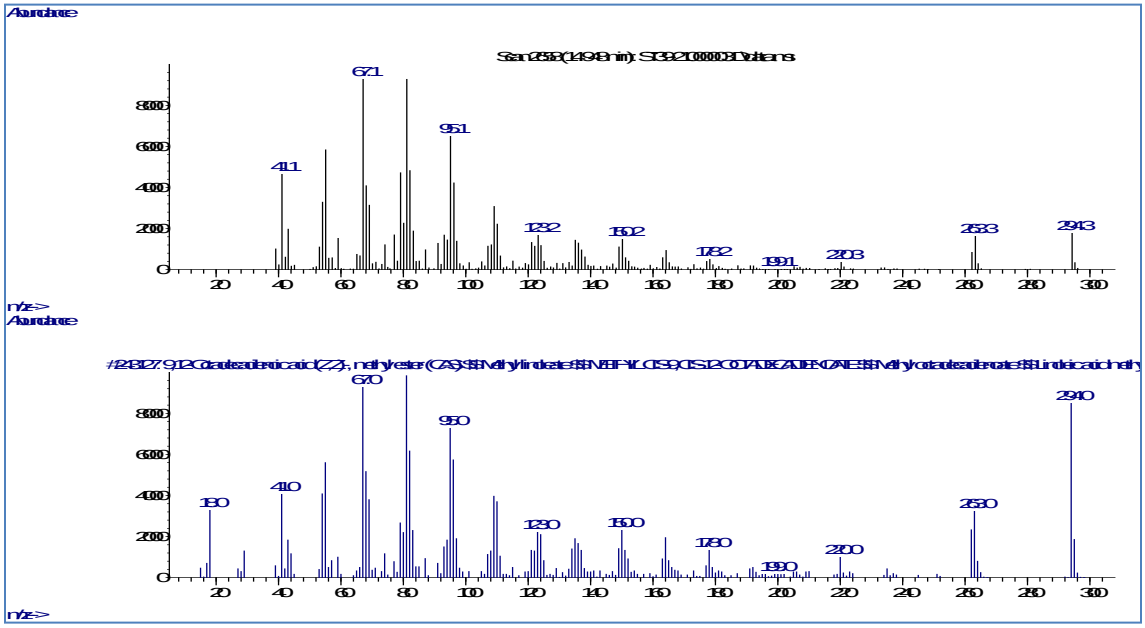


Figure 34. Peak at 14.948 min was identified as methyl linoleate.

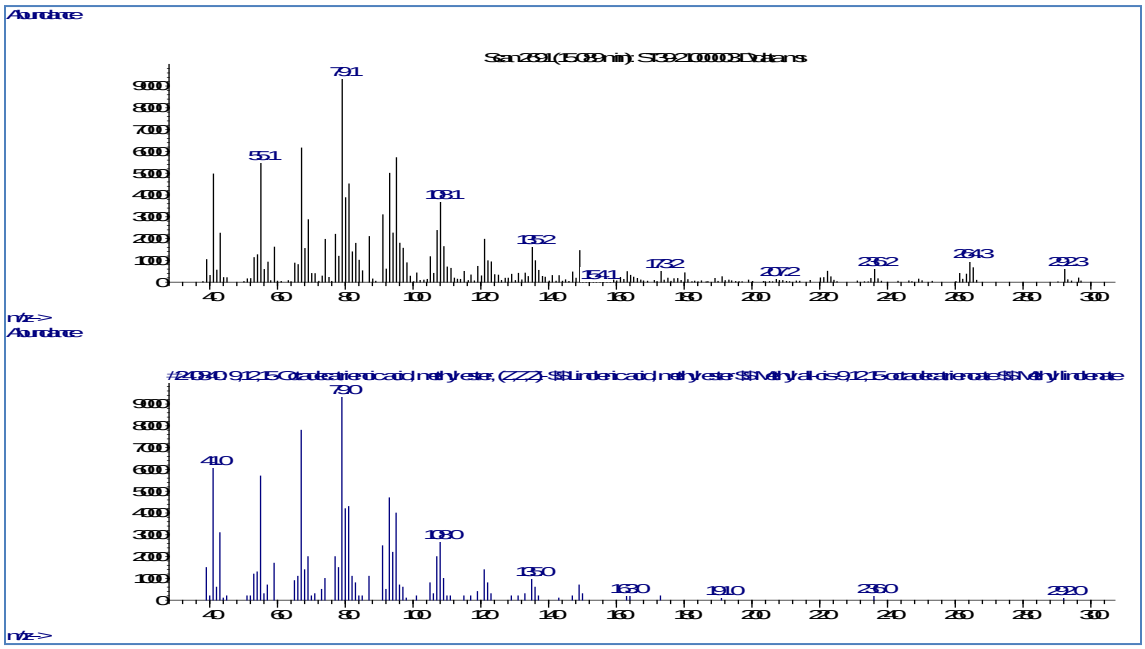


Figure 35. Peak at 18.089 min was identified as methyl linolenate**.**

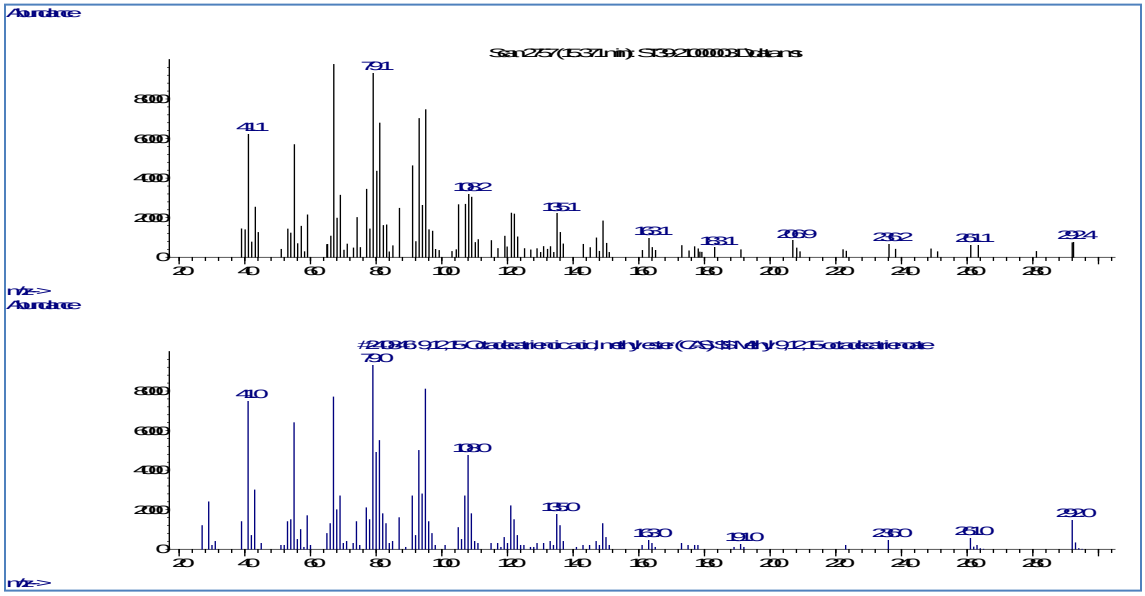


Figure 36. Peak at 18.089 min was identified as methyl, 9, 12, 15-octadecatrienoate.

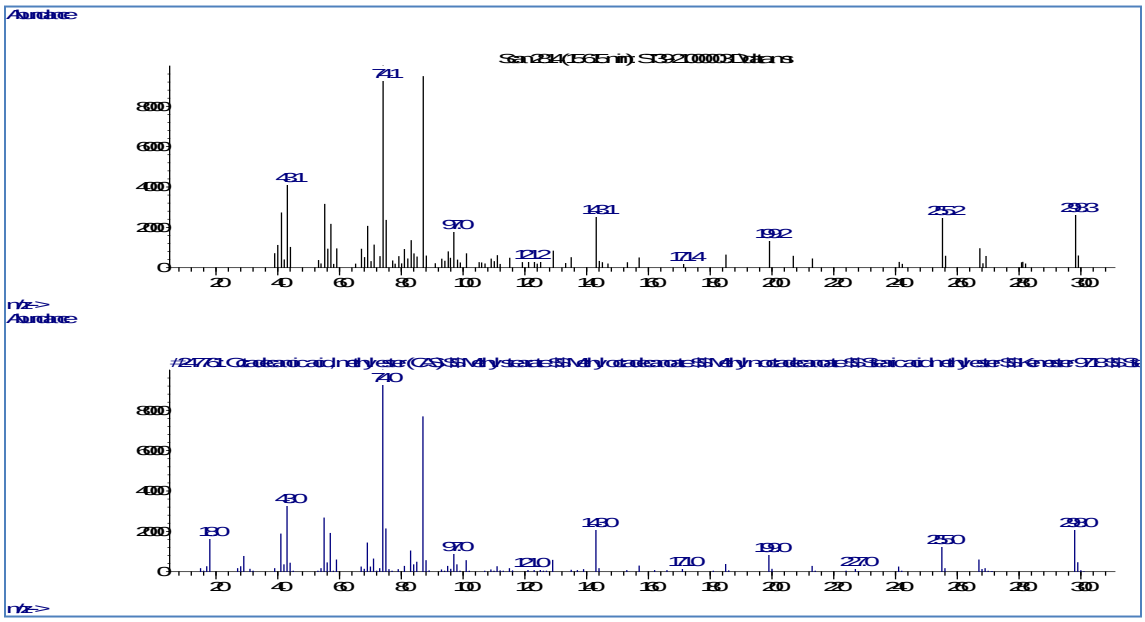


Figure 37. Peak at 15.615 min was identified as methyl octadecanoate.

Appendix C

Table 12. Final growth medium for *Dunaliella*.

Ingredient	**Amount (g/L)**
$MgCl_2$. $6H_2O$	0.75 g/L
$MgSO_4.7H_2O$ (Epson)	0.5 g/L
KCl	0.2 g/L
$CaCl_2.2H_2O$ (Dowflake)	0.1 g/L
KNO_3	1 g/L
NaCl	10 g/L
$FeCl_3$	0.05 g /L
1% KH_2PO_4 solution	5.0 ml/L
Micronutrient Stock Solution**	1.0 mL/L
**Micronutrient Stock Solution	
Salt	
EDTA-Na	1600 mg/L
$ZnCl_2$	40 mg/L
H_3BO_3	600 mg/L
$CoCl_2.6H_2O$	14.2 mg/L
$CuCl_2.2H_2O$	40 mg/L
$MnCl_2.4H_2O$	400 mg/L
$(NH_4)_6$ $Mo_7O_{24}.4H_2O$	380 mg/L

Note: $CaCl_2.2H_2O$ (Dowflake) was later removed due to sufficient levels in water.

Table13. Analysis of algal residue after Soxhlet extraction of lipid.

Analysis	**D.parva**	**D. tertiolecta**	**N. oculata**
Moisture	11.26%	5.54%	4.17%
Ash	17.54%	8.54%	8.31%
Carbohydrate	16.42%	28.24%	31.60%
Fat (ethyl ether)	1.28%	0.58%	1.32%
Fiber, crude	1.22%	5.14%	1.81%
Protein	53.50%	53.50%	54.60%

Table 14. Growth rates for different species of algae (the overall average growth rate is the sum of growth achieved over the duration of the experiment divided by the total days).

Algae Species	**Average Growth (g/L/day)***
D. tertiolecta	.0790
D. parva	.0446
D. salina	.0375
N. oculata	.0556

*The data are based on average growth rates, indoors and under fluorescent lighting. Growing the algae outdoors under natural sunlight has potential for higher growth rates and therefore greater amounts of carbon dioxide capture [55]. The output of artificial light is typically less than that of natural sunlight.

Table 15. Average carbon dioxide capture by algae species extrapolated to tons per acre per year.

ALGAE CARBON DIOXIDE CAPTURE (tons/acre/year)		
Species	**Based on Harvest**	**Based on Optical Density**
D. tertiolecta	42.12	61.77
D. parva	16.69	23.49
D. salina	15.91	16.23
N. oculata	26.09	37.96

*Based on: 365 days/year production, water depth = 0.30 meters, 100% capture efficiency

Appendix D

D. tertiolecta lipid extraction and preparation procedure for GC-MS

Extraction methodology

There are a significant number of methodologies for lipid extraction. In exploring for the ideal extraction method, we have tested the well-known Bligh and Dyer extraction as well as the Folch extraction which employs chloroform and methanol on wet algae paste. These methods have the benefit of allowing direct extraction of lipid from a wet sample, however, they call for a large quantity of extraction solution and create a significant amount of waste solvent. Since our goal is to be able to process large quantities of algae in an economical and environment-friendly way, our exploration eventually led us to the Soxhlet extraction method which utilizes hexane as the extraction solvent. Soxhlet extraction involves refluxing a solvent to repeatedly wash and extract the sample, then the lipid is obtained by evaporating off the solvent. The benefits of using this extraction method include: (1) the extraction solvent (hexane) can be recycled and reused, (2) hexane is easier to handle than the more toxic chloroform and thus more environment friendly, and (3) the lipids obtained with this extraction method are neutral lipids that are ready for conversion into biodiesel via the Mcgyan process. Samples of algae were harvested and prepared for lipid extraction.

Lipid extraction

For Soxhlet extraction, eighty grams of ground algae were placed in a Whatman cellulose extraction thimble and setup in a Soxhlet extractor. A one liter round bottom filled with 800ml of hexane and some boiling stones was then attached to the bottom of the extractor. This was followed by attaching a water cooling coil tube to the top of the extractor with cold water running through to help condense the rising hexane vapor. Lastly, heat was applied and the extraction was allowed to run until the extracting hexane appeared colorless (typically 48 hours). The lipid containing hexane solution was filtered through activated carbon to remove pigments from the algae and then the hexane was removed using a rotary evaporator. This resulted in neutral algae lipids being obtained that were transparent and ranged from light yellow to amber in color.

Sample preparation for GC analysis

HCl catalyzed methanolysis of algae lipid was performed to prepare the sample for GC-MS (Gas Chromatography Mass Spectrometry) analysis. Approximately 4-5 mg of algal lipid was added to a small Teflon capped vial (2 mL) with 0.2 mL methanol in it. Next, 0.8 mL of 5% acetyl chloride in methanol was added to the vial. The vial was tightly capped and heated in a sand bath at 60^{o} C for 6 hours. After cooling, the contents of the vial were transferred to a test tube containing 1 mL saturated $NaHCO_3$ and 2 mL pentane. The two layers were agitated by pipette until gas evolution ceased. The pentane solution was carefully decanted by pipette and transferred to another test tube and dried over a molecular sieve (4 Å). The pentane was evaporated by drying with a stream of air and the remaining residue was taken up in ~1 mL of HPLC grade methanol and analyzed by GC-FID (Flame Ionization Detector) and GC-MS.

Gas chromatography-mass spectrometry (GC-MS) analysis

The gas chromatograph electron impact mass spectrometer is a powerful and extremely useful instrument that combines two techniques to allow a single method of analyzing mixtures of chemicals. Through gas chromatography, the components of a mixture were first separated; subsequently the separated components were characterized and identified individually by mass

spectrometry. By combining these two techniques, this instrument not only allows qualitative and quantitative evaluation, but also identification of components in a mixture solution. Pertinent to this project, this instrument allows efficient identification of specific fatty acids contained in the various species of algae oil. In the future, this instrument will aid in qualitative and quantitative evaluation of the different chain length FAMEs in the biodiesel produced from the algae oil.

An Agilent 6890 gas chromatograph electron impact mass spectrometer was used for this analysis. One µL of sample solution was injected in splitless mode at a flow rate of 1.0 mL/min with helium as the carrier gas onto a 5% phenyl-methylpolysiloxane column (DB-5; 30m x .25 mm i.d.; 0.25 µm film thickness). The elution temperature program had an initial temperature of 50 °C and then linearly ramped to 180 ºC at 15 °C min^{-1}, then to 230 ºC at 2 °C min^{-1}, and finally to 310 ºC at 30 °C min^{-1}. The final temperature was held for 13.67 minutes (total run time = 50 min). Mass spectra were acquired using HP6890 MS software and peak identification was aided with the NIST MS library. The observed mass range was set from 37 to 800 amu to remove any solvent contribution.

Optimal Conditions for Algae Oil Conversion into Biodiesel

Feedstocks

- Lipid - 189g of algae oil was mixed with 1300 ml of n-Hexane (95%), flow rate 6.19 ml/min
- Alcohol – Methanol, flow rate 9.38 ml/min

Reactor

- Catalyst ratio - 50% Cat T (8.24g) & 50% Cat A (5.28g) (volume percentages)
- Pressure - Constant throughout at 2300psi
- Temperature - Constant throughout at350 ºC
- Contact time – 30 seconds

EFAR

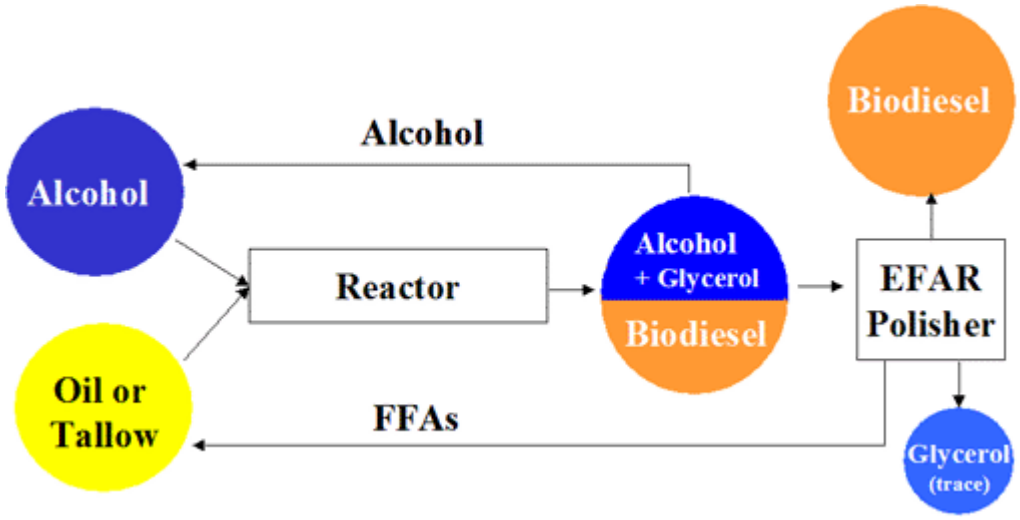
- Reactants - 126.5g of biodiesel was mixed with 6.32g DMC and 34.42g methanol, flow rate = 15 ml/min
- Catalyst ratio- 50% Cat T (8.24g) & 50% Cat A (5.28g) (volume percentages)
- Pressure - Constant throughout at 2400psi
- Temperature - Constant throughout at 290 ºC

Figure 38. Optimized conditions used for conversion of algae oil into biodiesel fuel.

The Mcgyan® Process

Description: The process is termed the Mcgyan® process after the names of the inventors (McNeff, Gyberg and Yan). The process performs a catalytic conversion of triglycerides and free fatty acids into fatty acid methyl esters (FAMES); in other words into biodiesel. In addition to the environmental advantages of producing a biofuel that replaces conventional fossil fuels (fossil fuels have detrimental effects on the environment since they release sequestered carbon compounds and other pollutants into the atmosphere, whereas bio-based fuels such as biodiesel are more environmentally friendly since their use recycles carbon through renewable biomass and because they burn cleaner than petroleum fuels), this new process offers several advantages over current biodiesel production methods.

The Mcgyan® Process A Simple Continuous Reactor



A general operational schematic of the Mcgyan® reactor is depicted above. Oil or tallow feedstocks and alcohol are converted into biodiesel. Once through the reactor, the excess alcohol is separated out and recycled back into the continuous process.

Figure 39. Description of the Mcgyan® Process.

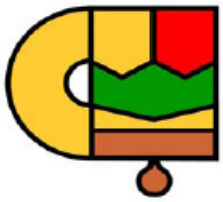
Table 16. Characteristics of the Mcgyan process.

PRODUCTION CHARACTERISTIC	PROCESS	
	Current State-of-the Art	Mcgyan®
Consumes catalyst	Yes	No
Uses large amounts of water	Yes	No
Produces waste products	Yes	No
Products soap byproducts	Yes	No
Requires large footprint	Yes	No
Sensitive to water	Yes	No
Sensitive to free fatty acid content	Yes	No
Uses large quantities of strong acids or bases	Yes	No
Conversion rate	Hours	Seconds
Converts free fatty acids to biodiesel	No	Yes
Can use a variety of feedstocks	No	Yes
Produces glycerol as a major byproduct	Yes	No
Is a continuous process	No	Yes

Process Benefits

1) Current waste products can be turned into fuel.
2) No use of strong acids or bases in the process.
3) Fast reaction times (seconds).
4) Cheap feedstocks such as waste grease and animal tallow as well as a variety of plant oils can be converted to biodiesel.
5) The metal oxide based catalyst is a contained in a fixed bed reactor thereby eliminating the current need to continuously add catalyst to the reaction mixture thereby reducing the amount of waste produced.
6) Unwanted side reactions with free fatty acids producing soaps are eliminated, thereby reducing the amount of waste that must be disposed of properly.
7) Insensitive to free fatty acid and water content of the feedstocks.
8) Flexible feedstock; animal or plant sources of lipids can be used.
9) The catalyst does not "poison" over time.

Table 17. Results of ASTM testing of biodiesel made from algal oil.



Mcgyan™ Biodiesel (ASTM Testing Results)

Date	5/31/2011	**Batch Number**	**ST39-21**
Contact Person	Ben Yan	**E-Mail Address**	Support@evercatfuels.com
Company	Ever Cat Fuels		
Business Address	100 Isanti Parkway NE, Isanti, MN 55040		
Phone Number	763-444-8444	**FAX Number**	763-444-3555

Test	ASTM Method	ASTM D6751-09 Specification	**Result**
Calcium &Magnesium, combined	EN 14538	5 ppm max	0.1
Flash Point	D93	93 °C min.	>93
Alcohol Control (one of the following must be met)			
1. Methanol Content	EN14110	0.2% (vol.) max	0.001%
2. Flash Point	D93	130 °C min.	>93
Water and Sediment	D2709	0.050% (vol.) max	0.001%
Kinematic Viscosity @ 40°C	D445	1.9 to 6.0 $mm^2/sec.$	5.8
Sulfated Ash	D874	0.02% (mass) max	0.012%
Sulfur	D5453	15 ppm max (S15 B100), 500 ppm max (S500 B100)	<15
Copper Strip Corrosion	D130	No. 3 max	1A
Cetane Number	D613	47 min.	53.4
Cloud Point	D5773	Report (°C)	-1.0
Carbon Residue	D4530	0.050% (mass) max	0.044%
Acid Number	D664	0.50 max	0.00
Cold Soak Filterability	D7501-09	360 seconds max	120
Free Glycerin	D6584	0.020% (mass) max	0.002%
Total Glycerin	D6584	0.240% (mass) max	0.125%
Monoglycerides	D6584		0.039%
Phosphorus Content	D4951	0.001% (mass) max	0.0003%
Distillation, T90 AET	D1160	360 °C max	352
Sodium/Potassium, combined	EN14538	5 ppm max	0.6
Oxidation Stability	EN14112	3 hours min.	>3

Remarks:

Mcgyan™ Biodiesel within all specifications.

8/4/2011
SarTec Corporation
617 Pierce St
Anoka, MN 55303
(800)472-7832

To whom it may concern,

The attached Certificate of Authenticity was produced by Dr. Ben Yan (or under his direct supervision), for Mcgyan fuel sample ST39-21, in the laboratory of Ever Cat Fuels, LLC. The methodology used was per ASTM D6751-09 and the individual testing methods shown. The testing shows that the sample meets or exceeds all specifications per ASTM D6751-09.

Joel Schumacher, P.E.
MN Licence No. 46191
Chemical Process Engineer
SarTec Corporation
cell: (612) 360-3539
plant: (763) 444-8444 ext. 116
fax: (763) 444-3555
email: joelschumacher@sartec.com
www.evercatfuels.com
www.sartec.com

Figure 40. Engineer's certification of ASTM D7651 testing.

References

1. Liu ZY, Wang GC. Effect of iron on growth and lipid accumulation in *chlorella vulgaris*. Bioresource Technology 2008;99(11):4717-22.

2. Paz Y, Shimoni E, Weiss M, Pick U. Effects of iron deficiency on iron binding and internalization into acidic vacuoles in *dunaliella salina*. Plant Physiology 2007;144(3):1407-15.

3. Oren A. A hundred years of *dunaliella* research: 1905-2005. Saline Systems 2005;1:2.

4. Borowitzka MA. The mass culture of *dunaliella salina*. Seaweed Culture 1990:180.

5. Greenwell HC, Laurens LML, Shields RJ, Lovitt RW, Flynn KJ. Placing microalgae on the biofuels priority list: A review of the technological challenges. Journal of the Royal Society Interface 2010;7:703-26.

6. Roessler PG. Environmental control of glycerolipid metabolism in microalgae: Commercial implications and future research directions. Journal of Phycology 1990;26:393-9.

7. Shifrin NS, Chisholm SW. Phytoplankton lipids interspecific differences and effects of nitrate, silicate and light-dark cycles. Journal of Phycology 1981;17:374-84.

8. Lombardi AT, Wangersky PJ. Particulate lipid class composition of three marine phytoplankters *chaetoceros gracilis, isochrysis galbana* (tahiti) and *dunaliella tertiolecta* grown in batch culture. Hydrobiologia 1995;306(1):1-6.

9. Salley SO, Chen M, Holland TC, Ma H, Ng KYS, Tang H. Effect of nutrients on growth and lipid accumulation in the green algae *dunaliella tertiolecta*. Bioresource Technology 2011;102:1649-55.

10. Takagi M, Yoshida T. Effect of salt concentration on intracellular accumulation of lipids and triglycerides in marine microalgae *dunaliella* cells. Journal of Bioscience and Bioengineering 2006;101(3):223-6.

11. Jansson JK, Bjerketorp J, Hakansson S, Belkin S. Advances in preservation methods: Keeping biosensor microorganisms alive and active. Current Opinion in Biotechnology 2006;17:43-9.

12. Morgan CA, Herman N, White PA, Vesey G. Preservation of micro-organisms by drying; a review. Journal of Microbiological Methods 2006;66:183-93.

13. McGrath MS, Daggett P, Dilworth S. Freeze-drying of algae: *Chlorophyta* and *chrysophyta*. Journal of Phycology 1978;14:521-5.

14. Day JG. Cryopreservation of microalgae and cyanobacteria. In: Cryopresercation and freeze-drying protocols. Totowa, New Jersey: Humana Press Inc; 2007. .

15. McNeff CV, McNeff LC, Yan B, Nowlan DT, Rasmussen M, Gyberg A. A continuous catalytic system for biodiesel production. Applied Catalysis A: General 2008(343):39-48.

16. Ma F, Hanna MA. Biodiesel production: A review. Bioresource Technology 1999;70:1-15.

17. Melero JA, Iglesias J, Morales G. Heterogeneous acid catalysts for biodiesel production: Current status and future challenges. Green Chemistry 2009;11:1285-308.

18. Demirbas A. Comparison of transesterification methods for production of biodiesel from vegetable oils and fats. Energy Conversion and Management 2008;49:125-30.

19. Wright HJ, Segur JB, Clark HV, Coburn SK, Langdon EE, Dupuis RN. A report on ester interchange. Oil and Soap 1944:145-8.

20. Azocar L, Ciudad G, Heipieper HJ, Navia R. Biotechnological processes for biodiesel production using alternative oils. Appl Microbiol Biotechnol 2010 Oct;88(3):621-36.

21. Ramadhas AS, Jayaraj S, Muraleedharan C. Biodiesel production from high FFA rubber seed oil. Fuel 2005;84:335-40.

22. Saka S, Kusdiana D, Warabi Y. Reactivity of triglycerides and fatty acids of rapeseed oil in supercritical alcohols. Bioresource Technology 2004;91:283-7.

23. Krohn BJ, McNeff CV, Yan B, Nowlan D. Production of algae-based biodiesel using the continuous catalytic mcgyan process. Bioresour Technol 2011 Jan;102(1):94-100.

24. Mcgyan Process [Internet]; c2011 [cited 2011 6/3]. Available from: http://www.evercatfuels.com/technology-McgyanProcess.asp.

25. Bonos E, Christaki E, Florou-Paneri P. Microalgae: A novel ingredient in nutrition. International Journal of Food Sciences and Nutirtion 2011:1-6.

26. Becker EW. Micro-algae as a source of protein. Biotechnology Advances 2007;25:207-10.

27. Gouveia L, Batista AP, Sousa I, Raymundo A, Bandarra NM. Microalgae in novel food products. In: Food chemistry research developments. Hauppauge, New York: Nova Science Publishers, Inc; 2008. .

28. Natrah F, Yosuff FM, Shariff M, Abas F, Mariana NS. Screening of malaysian indigenous microalgae for antioxidant properties and nutritional value. Journal of Applied Phycology 2007;19(6):711-8.

29. Spolaore P, Joannis-Cassan C, Duran E, Isambert A. Commercial applications of microalgae. Journal of Bioscience and Bioengineering 2006;101(2):87-96.

30. Weather Averages for Minneapolis, MN [Internet]; c2010 [cited 2011 6/29]. Available from: http://www.weather.com/wxclimatology/monthly/USMN0503.

31. Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M, Miller HL, editors. Climate change 2007: The physical science basis. New York City, New York: Cambridge University Press; 2007. .

32. Demirbas A. Oily products from mosses and algae via pyrolysis. Energy Sources, Part A 2006;28:933-40.

33. Li Y, Horsman M, Wu N, Lan CQ, Dubois-Calero N. Biofuels from microalgae. Biotechnology Progress 2008;24:815-20.

34. Nakamura DN. The mass appeal of biomass. Oil & Gas Journal 2006;104:15.

35. Pulz O. Photobioreactors: Production systems for phototrophic microorganisms. Applied Microbiology and Biotechnology 2001;57:287-93.

36. Barsanti L, Gualtieri P. Algal culturing. In: CRC press. Boca Raton, FL: ; 2006. .

37. Lee YK. Microalgal mass culture systems and methods: Their limitation and potential. Journal of Applied Phycology 2001;13:307-15.

38. Sheehan JT, Dunahay T, Benemann J, Roessler P. A look back at the U.S. department of Energy's aquatic species program: Biodiesel from algae. National Renewable Energy Laboratory; 1998. Report nr NREL/TP-580-24190.

39. Borowitzka MA. Culturing microalgae in outdoor ponds. In: R. A. Andersen, editor. Algal culturing techniques. Burlington, MA: Elsevier Academic Press; 2005. .

40. Borowitzka MA. Commercial production of microalgae: Ponds, tanks, tubes and fermenters. Journal of Biotechnology 1999;70:313-21.

41. Richmond A, Lichtenberg E, Stahl B, Vonshak A. Quantitative assessment of the major limitations on productivity of *spirulina platensis* in open raceways. Journal of Applied Phycology 1990;2(3):195-206.

42. Lee YK. Commercial production of microalgae in the asia-pacific rim. Journal of Applied Phycology 1997;9:403-11.

43. Setlik I, Veladimir S, Malek I. Dual purpose open circulation units for large scale culture of algae in temperate zones. I. basic design considerations and scheme of pilot plant. Algological Studies (Trebon) 1970;1:111-64.

44. Chaumont D, Thepenier C, Gudin C. In scaling up a tubular photoreactor for continuous culture of porphyridium cruentum - from laboratory to pilot plant. In: T. Stadler, J. Morillon, M. C. Verdus, W. Karamanos, H. Morvan, D. Christianen, editors. Algal biotechnology. London: Elsevier Applied Science; 1988. .

45. Fernandez FGA, Camacho FG, Perez JAS, Sevilla JMF, Grima EM. Modeling of biomass productivity in tubular photobioreactors for microalgal cultures: Effects of dilution rate, tube diameter, and solar irradiance Biotechnology and Bioengineering 1998;58:605-16.

46. Grima EM, Perez JAS, Camacho FG, Sanchez JLG, Fernandez FGA, Alonso DLJ. Outdoor culture of *isochrysis galbana* ALII-4 in a closed tubular photobioreactor. Journal of Biotechnology 1994;37:159-66.

47. Grima EM, Perez JAS, Camacho FG, Sevilla JMF, Fernandez FGA. Productivity analysis of outdoor chemostat culture in tubular air-lift photobioreactors. Journal of Applied Phycology 1996;8:369-80.

48. Hu Q, Richmond A. Optimizing the population density in *isochrysis galbana* grown outdoors in a glass column photobioreactor. Journal of Applied Phycology 1994;6(4):391-6.

49. Richmond A, Boussiba S, Vonshak A, Kopel R. A new tubular reactor for mass production of microalgae outdoors. Journal of Applied Phycology 1993;5(3):327-32.

50. Torzillo G, Pushparaj B, Bocci F, Balloni W, Materassi R, Florenzano G. Production of *spirulina* biomass in closed photobioreactors. Biomass 1986;11(1):61-74.

51. Hu Q, Guterman H, Richmond A. A flat inclined modular photobioreactor for outdoor mass cultivation of photoautotrophs. Biotechnology and Bioengineering 1996;51:51-60.

52. Lee YK, Low CS. Effect of photobioreactor inclination on the biomass productivity of an outdoor algal culture. Biotechnology and Bioengineering 1991;38:995-1000.

53. Huntley ME, Redalje DG. CO2 mitigation and renewable oil from photosynthetic microbes: A new appraisal. Mitigation Adapt. Strateg. Global Change 2007;12:573-608.

54. Rodolfi L, Zittelli G, Bassi N, Padovani G, Bonini N, Bonini G, Tredici M. Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnology and Bioengineering 2009;102:100-12.

55. Zitelli GC, Rodolfi L, Tedici MR. Mass cultivation of *nannochloropsis* sp. in annular reactors. Journal of Applied Phycology 2003;15(2-3):107-14.

56. Biodiesel Handling and Use Guide, fourth edition. National Renewable Energy Laboratory. NREL/TP-540-43672, September 2008.