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Project and Report for Masters in Plant Science and Pest Management
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Total fatty acid production in golden alga *Prymnesium parvum* a potential bio-diesel feedstock

[Analyzing the fatty acids in select strains of *P. parvum* using gas chromatography]

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Contents

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Abstract

Developing renewable and sustainable feedstocks for biodiesel production is one strategy for reducing our dependence on petroleum-based fuel. Microalgae are attractive feedstocks for biodiesel due to their high lipid content and fast growth rate. In addition algae, can be cultured on marginal lands using saline water and thus avoiding competition with arable land. This study investigates the total fatty acid content in *Prymnesium parvum,* which has the potential as a feedstock for biodiesel production. *Prymnesium parvum* was cultured in artificial sea water with the salinity adjusted to 14 - 15 (psu) and a pH ranging from 7.8 - 8.5. The culture temperature was 25.7°C under florescent light with a 16:8 (L/D) cycle. Total fatty acids produced had a mean of 11.679 % ± 0.68671 (DW). These percentages were below the 22-38% previously reported by E. W. Becker 1994. Total algal biomass produced had a mean yield of 0.34mg ± 0.07204 (DW) / L. In comparison to other algal species used as feedstocks, *P. parvum* produced significantly less fatty acids and biomass. *Prymnesium parvum* also exhibited signs of self toxicity with frequent crash cycles. The self toxicity would have contributed to its inability to produce both lipids and dry biomass. Further studies for increasing fatty acid production in *P. parvum* may require using a continuous bioreactor system, controlling environmental conditions to relieve self-toxicity and adjusting nutrient concentrations. Exposure to different light/dark regiments along with increasing CO_2 concentrations should be explored to possibly increase yields.

***Keywords:* *Prymnesium parvum*; Biodiesel feedstock; Total fatty acids**

Introduction

Developing renewable and sustainable feedstocks for biodiesel production is one strategy for reducing our dependence on petroleum-based transportation fuels. Biodiesel produced from microalgae lipids are gaining popularity within the research community and have the potential to replace current feedstocks such as chicken fat if the technologies for growing algae and conversion of lipids to fuel are vastly improved (Chisti 2008).

Currently vegetable oils, animal fats and waste oils from cooking are used for biodiesel production. Microalgae produce fatty acids that can be converted into esters used in biodiesel synthesis, these esters are comparable to those produced from either animal and vegetable oils (Miyamoto 1997). These cellular lipids are membrane components, storage molecules and metabolites used in algal cell proliferation and buoyancy (Halver 1978). It is reported that the oil content of some algae species can exceed 80% (DW) in comparison to such crops as soybean or oil palm which produce less than 5 % of their total biomass as oil (Chisti 2008). In order to limit infrastructure, production and harvesting costs, technologies for converting microalgae feedstocks into biodiesel need improvement (Miao and Wu 2006).

In an algal comparison chart listed in the book entitled Microalgae: Biotechnology and Microbiology by E. W. Becker 1994, a strain of Algae *Prymnesium parvum* was listed as a potential species for bioenergy production with a high lipid percentage between 22-38% on a fresh or dry weight basis?. This same table was included in the 1997 - FAO Agricultural Bulletin 128 (Renewable Biological Systems for Sustainable Energy) and thus presented again as a potential species for biodiesel production (Miyamoto 1997).

There are no current studies that explicitly consider the lipid production of *P. parvum* under optimal growth conditions for use as a feedstock for biodiesel and it seems prudent to investigate its potential. The objectives of this project were to evaluate *P. parvum* as lipid producer. Growth rates were calculated to evualate the biomass production capability of this species.

Prymnesium parvum (Haptophyceae) is a golden alga that is highly toxic to fish and found in both marine and brackish waters worldwide. The distribution of *P. parvum* ranges from the Baltic Sea to South Africa; from China to Australia and from Canada to Texas. This wide distribution, makes *P. parvum* an ideal species to investigate biodiesel feedstock potential, since it is naturally adapted to temperate regions (Edvardsen and Larsen 2003).

Prymnesium parvum is extensively studied due to its toxicity in both benthic and pelagic organisms (Uronen et al. 2007). *Prymnesium parvum* was not listed in the U.S. Aquatic Species Program (ASP) in 1998 where 3000 algal strains were screened as potential candidates for bio-fuel feedstock. The program eventually narrowed the collection to roughly 300 species; mostly green algae (Chlorophyceae) and diatoms (Bacillariophyceae) for further evaluation (Sheehan et al. 1998).

The most likely scenario why *P. parvum* was not chosen as a candidate for the (ASP) is due to its potential invasive and toxic nature. If cultivated in outdoor raceway ponds it may contaminate nearby waters if released accidentally. The Aquatic Species Program concluded that outdoor raceway cultivation was highly productive if pH and CO_2 were optimized (Sheehan et al. 1998), but photobioreactors with recirculation systems have higher biomass production rates (Chisti

2007). *Prymnesium parvum* may have the potential for indoor cultivation if proper growth parameters for lipid synthesis are established. The current trend is to identify fast growing-high lipid containing algae for indoor/outdoor bioreactor systems. These algae must maintain long term growth and sustainability in controlled environments. Studies have been conducted on *P. parvum* evaluating optimal conditions for toxicity to other organisms in relationship to light, pH, temperature and salinity (Larsen and Bryant 1998) (Baker et al. 2007). However sustainability of growth in culture remains to be studied.

It has been shown that the toxicity of *P. parvum* is correlated with low phosphorus and nitrogen conditions that limit optimal growth condition (Johansson and Graneli 1999). Acute toxicity to fish was highest at the lowest salinity and temperatures , conditions not optimal for exponential growth of this species. These limiting growth factors combined with low pH contribute to the physiological stress on the algae, which released toxins into the environment. Maximum growth rates and highest cell mass of *P. parvum* were found under warm estuarine conditions (Baker et al. 2007). Algae under nutrient deficient conditions had the capacity to produce higher lipid percentages while overall growth was slowed (Sheehan et al. 1998). Maximum growth rate recorded for *P. parvum* was 1.4 divisions per day (Edvardsen and Larsen 2003).

Prymnesium parvum is suspected of producing an array of toxins but only two have been formally characterized and named: prymnesins 1 and 2. These toxins were reported to have potent hemolytic and ichthyotoxic properties (Igarashi et al. 1996).

Determining whether *P. parvum* is a good feedstock for bio-diesel production was the primary purpose of this report. Feedstock sources for biodiesel must be capable of producing large amounts of degradable biomass and contain large percentages of total fatty acids for easy conversion into biodiesel (Sheehan et al.1998).

An objective of this study was to determine whether the total fatty acid content between 22-38% quoted in FAO Agricultural Bulletin 128 (Miyamoto 1997) could be rountinely obtained in contained indoor culture. The second hypothesis to be tested is whether *P. parvum* could yield adequate to high biomass in addition to producing 22-38% fatty acids. The last objective was to determine whether *P. parvum* could sustain and thrive under optimal growth conditions in an indoor controlled environment.

This study examined growth and lipid contents of three strains of *P. parvum* grown under controlled nutrient, light, temperature and salinity conditions, in an attempt to optimize biomass and hydrocarbon production.

Life Cycle Analysis

Three major limiting factors in the production of algae for biodiesel are sustainable growth, biomass production and lipid content (Sheehan et al. 1998). Other hurdles exist when considering algae as a sustainable energy source. During outdoor cultivation, problems maintaining homogeneity among algal strains is effected by uncontrollable environmental conditions (Sheehan et al. 1998). Algae in controlled environments are hard to sustain over long periods without collapse or contamination (Hoff and Snell 2007). Some algae that have the

highest concentrations of lipids are freshwater algae (Li et al 2008) so a usable fresh water source is a factor to consider within the life cycle analysis.

Several factors to consider when using algae as a feedstock for biodiesel
1. Finding a sustainable algae feedstock that has a high lipid content
2. Finding a sustainable algae feedstock with a high biomass production rate
3. Growing algae in areas with an adequate water source
4. Growing the algae in areas where there is less of a economic & social concern
5. Market share - competing in the global market against big oil
6. Developing infrastructure for production and refining
7. Startup costs associated with production, transportation and refining
8. Disposal and recycling of production wastes – water, glycerol, etc
9. Research and development costs into new technologies and processes

The three concerns regarding *P. parvum* are: does this algae have a high enough fatty acid content; is it capable of producing high biomass and can it be sustainable during controlled cultural conditions?

Materials and Methodology

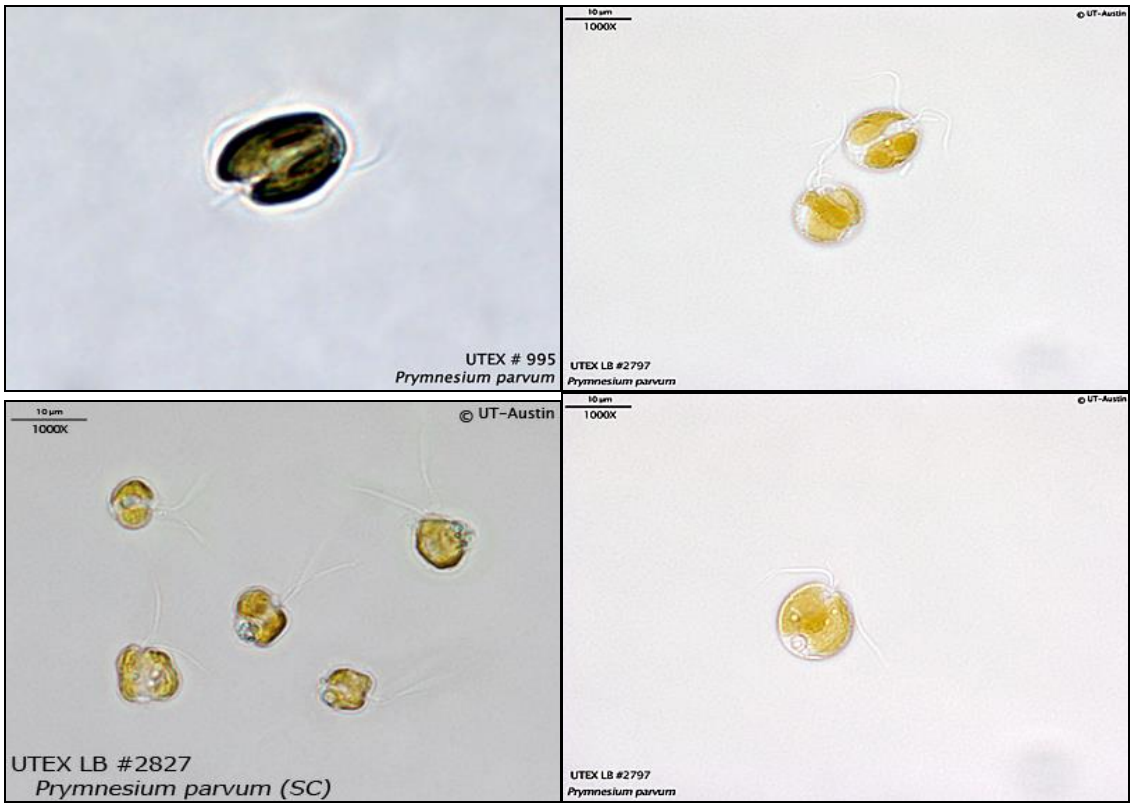
Experimental algae

Three isolated strains of *Prymnesium parvum* (Phylum Haptophyta) obtained from the Culture Collection of Algae at the University of Texas in Austin (UTEX) (Table 1 below) were used in this project. The three strains are adapted to diverse climates, with varying salinities and temperatures. Strain LB 995 was isolated in the United Kingdom and was incubated at UTEX in a soil and sea water medium. Strain LB 2827 (SC) and LB 2797 (TX) were incubated at UTEX on Erdschreiber's Medium. All three strains were axenic upon receipt and viewed under a microscope (Olympus BH-2 Brightfield) for cell viability and recognition. The three strains differ in morphology as shown below in Figure 1. These *P. parvum* strains are all unicellular, highly motile with two flagella and capable of coexisting with other organisms such as bacteria, considered mixotrophic (Larsen and Bryant 1998). Haptophyta algal cells are golden or yellow-brown due to the presence of accessory pigments (principally fucoxanthin) (Becker 1994) and three Chlorophylls C_1, C_2, and C_3 (Fawley 1989).

Table 1 - Algal strains being used in this study– www.utex.org

Species	Origin	Culture collection	Culture code	Isolated by, year
Prymnesium parvum	River Blackwater, Essex, England	UTEX	LB 995	R.W. Butcher (1952)
Prymnesium parvum	Oyster Rake Pond Charleston, SC	UTEX	LB 2827	J. Wolny (2002)
Prymnesium parvum	Texas Colorado River at US Hwy 183,TX	UTEX	LB 2797	J. Brand (4/01)

Figure 1 - Algal morphology from UTEX website – www.utex.org



Culture Media

The medium used was an Aquarium Grade Salt Solution (Instant Salts™, Aquarium Systems, Inc. please give the location) dissolved in de-ionized water. The artificial seawater was prescribed by the Plankton Culture Manual (Hoff and Snell 2007). Full strength Algae Culture Formula (Pro-Culture Professional F/2, Kent, location) was incorporated in the artificial sea water mixture for optimum nutrient density. The prescribed nutrient concentrations were 10 ml for every 20 gallons of part A and 10 ml for every 20 gallons of part B. The breakdown was 0.5 ml what? per gallon (3785 ml) deionized water? maximum nutrient density. Nutrients were added under sterile conditions (describe in detail) and after sterilization (how was this accomplished??) of artificial sea water to ensure optimum nutrient density for maximum growth

(what was the optimum nutrient density? Is nutrient density the same as concentration?) (Hoff and Snell 2007).

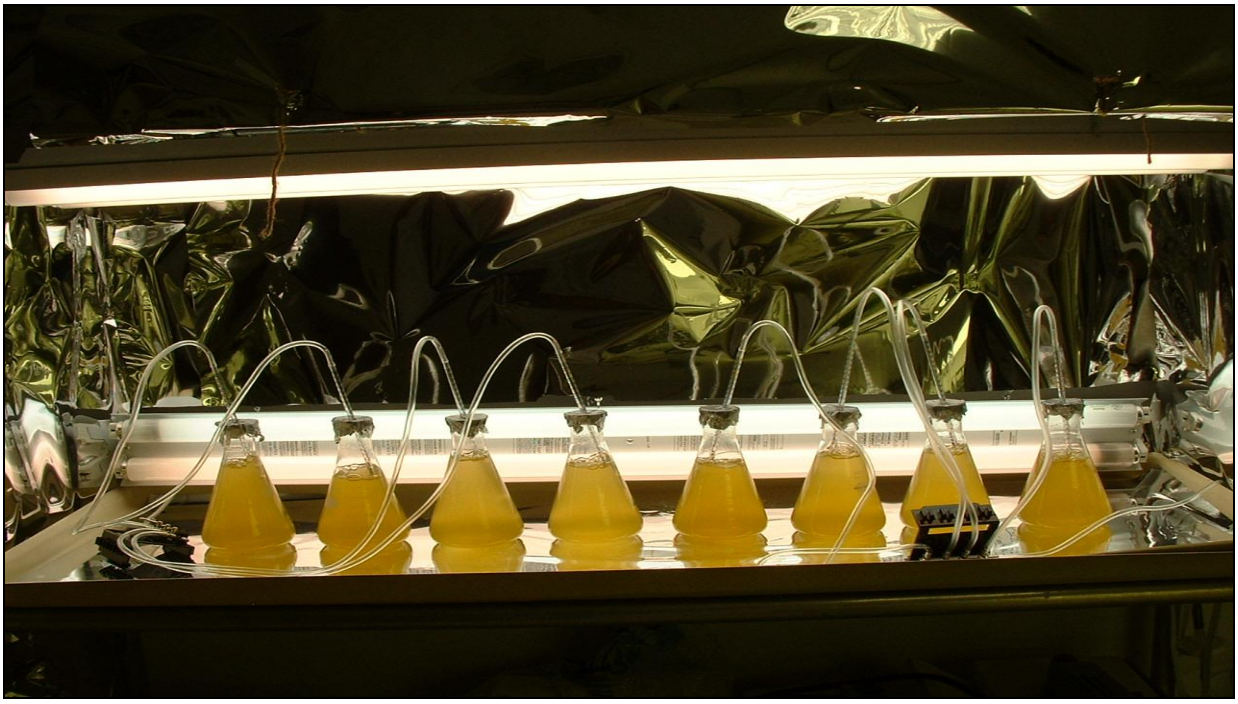
Culturing Method

The culturing method was the Modified Batch Culture Method, which allows the ability to scale up to higher quantities for optimum biomass production (what exactly does this mean, please describe the methode not how it can be used). This method insures the continuation of the initial cultures after leaving the lag phase and into an exponential growth phase (but what exactly is it?). After 8-days of exponential growth they what is they? can be divided into larger vessels and new inoculations can be derived from the exponential cultures. This method is used for a continuous culturing system like that in raceway ponds but not indoor bioreactors so is it the same or different then these two? What exactly is a bioreactor? (Hoff and Snell 2007). The down side to this method can be algae contamination or cultures that exhibit fatigue (how does algae show fatigue) over a long period of time. It is best to maintain stock cultures in an event of a colony collapse (Hoff and Snell 2007). Yes this sounds like common sense to me and a sound scientific principle

The axenic what does this mean?strains (20-25 ml) were transferred from the UTEX mediums what is this typle of media into 125 ml of prepared (autoclaved nutrient dense what does this mean?) room temperature what are you calling room temperature better to use degrees media under a Laminar Flow Hood. The sterile 500 ml flasks were placed in a special designed growth chamber (wouldn't call this a growth chamber, this means something else to most people,

Perhaps growth bench? (**Figure 2)** and cultures were suspended with forced filtered air under optimum light and temperature You must define what you mean by optimum. All transferring utensils, down tubes, flasks were sterilized using a GETINGE Castle 123 Multitherm Steam Sterilizer.

Figure 2: Reflective Mylar is used in enclosed growth chamber with optimal light and temperature control



Photoperiod, Light Intensity and Temperature

UTEX cultures were transferred to a sterilized culture media and subjected to a photoperiod of 16/8 L:D cycle (Hoff and Snell 2007). The growth chamber was 2 x 3 x 6 four sided rectangular structure and lined with Mylar to distribute light proportionally throughout the chamber and to block out any ambient light during the dark cycle (Demetropoulos and Langdon 2004). The chamber florescent lights used were a combination 48" F40 Ecolux Plant & Aquarium (that

contain a combination of phosphors which produce light rich in reds and blues) and a F40 Sunshine Light that are full spectrum. Six lights were alternately positioned above while two alternate lights were positioned on the lower back side of the chamber behind the growth flasks (**Figure 2).**

The Photon fluence rates were measured using a LI-COR Quantum/Radiometer/ Photometer – Model LI-250 Light Meter. Positions surrounding the flasks were measured and the mean light received was 114.85 µmol photons $^{m-2 \cdot s-1}$. This photon fluence rate is comparable to previous studies conducted by Larsen and Bryant 1998 and Baker et al. 2007.

Prymnesium parvum is very eurythermal (able to withstand extreme temperature variations) (Larsen and Bryant 1998). The cultures were maintained at an average temperature of 25.6 - 25.8° C during the light cycle and 23° C during the dark cycle. The temperature was measured using a hand-held YSI ® Model 63 pH, conductivity, temperature and salinity meter. Maximum growth potential for *P. parvum* was recorded at temperatures between 25° C and 30° C at moderate salinity (Baker et al. 2007).

Salinity and pH

Prymnesium parvum is extremely euryhaline (able to adapt to wide range of salinity) (Larsen and Bryant 1998). The cultures were maintained at ½ concentration of prescribed "Instant Salts" ™

in a range between 14 and 15 practical salinity units (psu). The optimal salinity was determined by Baker et al. 2007 and Larsen and Bryant 1998 at 22 psu. Most algae found in bay waters and brackish estuaries tolerate a salinity between 12-27 parts-per-thousand (ppt) which when converted is close to psu measurements not accounting for temperature and dissolved oxygen (Hoff and Snell). Toxic blooms have been associated with extreme low and high salinities but at lower temperatures than this experiments (Baker et al. 2007).

The pH measured ranged between 7.8 - 8.51 depending on the light and dark cycles. Optimal pH for salt tolerant algae in artificial seawater is between 7.5 and 8.5 (Hoff and Snell). Both the salinity and pH was measured using a hand-held YSI ® Model 63 pH, conductivity, temperature and salinity meter.

Algal Cell Counts, Harvesting, Drying and Lipid Analysis

Direct cell counts were taken to measure cell growth rates under an Olympus BH-2 Brightfield Microscope using a Neubauer hemacytometer at 10X objective (100X magnification). Cell counts were conducted before inoculation, during exponential growth phase, during a stationary growth phase and during a death or crash phase (Hoff and Snell 2007). Four counts total were taken per sample and averaged; then recorded using a hand held counter for accuracy. Counts were taken twice daily or once daily depending on the experiment run. The motile cells were killed using formalin; a mixture of formaldehyde, methanol and water to assure no cell movement. Cell counts are expressed as cells/ml (Hoff and Snell 2007).

Algal cells were harvested and poured into centrifuge tubes and centrifuged at 9000 rpm for 9 minutes. The algal cells were harvested and frozen at – 20° C until samples were able to be freeze dried over period between 72 – 96 hours. The freeze dryer used was a LABCONCO – Freezone ® 1 Liter Bench top Freeze Dry System. The freeze drying method assures that samples can be adequately weighed for total cell dry weight (Zhanyou et al 2007).

The fatty acid analysis was conducted using a SHIMADZU GC- 2010 Gas Chromatograph as outlined in a protocol developed by Indarti et al (2005). (See appendix **table 2** for fatty acid methyl ester preparation for GC). **Table 3** (appendix) shows the calculations created through excel to formulate the exact fatty acid percentages by weight of samples tested. All graphs and charts were created using Microsoft excel. Analysis of mean, standard deviation, and standard error were calculated using the statistical program from Microsoft Excel 2007 and checked by the JMP 7.0 program provided by Virginia Tech. Standard error was also checked using the following formula:

$$SE_{\bar{x}} = \frac{s}{\sqrt{n}}$$

Where *s* is the sample standard deviation (i.e., the sample based estimate of the standard deviation of the population), and *n* is the size (number of observations) of the sample.

Population doubling time (T_2), population growth rates (r) and divisions per day (k) were calculated using growth curve analysis equations developed by Guillard in 1973 and listed below (Andersen 2004).

Equation 1: $r = \ln (N_1/N_0) / \Delta t$

Equation 2: $k= r/0.6931$

Equation 3: $k= \log_2 (N_1/N_0) / \Delta t$

Equation 4: $T_2 = 0.6931/r$

Algae Growth Trials

Growth Trial One and Trial Two – Hampton Virginia Seafood AREC Summer 2008

During these two trials three strains of *Prymnesium parvum* (UK) LB 995, (SC) LB 2827, and (TX) LB 2797 were used. The growth conditions were different from the trials conducted in Blacksburg and real filtered seawater was used with salinity at 18 ppt. Medium temperature was 29° C and pH was between 7.8 and 8.53. Light intensity was not measured and all nutrients used were as described above (*Culture Media*) using Kent F/2 full strength solution. Water and utensils were autoclaved and the alga was suspended with forced filtered air. Algae was grown on a 12:12 L:D cycle as mentioned in Baker et al 2007. Algae was counted using a hemacytometer and recorded for growth analysis. No samples were collected for lipid analysis

during these two trials. Algae were transferred from 1 liter flasks to 5 gallon Carboys for a study injecting bottled CO_2 to measure pH variances and cell numbers. The trials were concluded earlier than expected and the data was used for analyzing doubling times of algae.

Growth and Lipid Analysis Trial Three, Four, Five – Biological Systems Engineering - Blacksburg Virginia (fall 2008 – spring 2009)

During these three trials three strains of *Prymnesium parvum* (UK) LB 995, (SC) LB 2827, and (TX) LB 2797 were tested. All environmental conditions are described in the material and methods. During **trial three** in Blacksburg; cell counts where **only** taken at harvesting time and analyzed for lipids. During trials four and five (once the algae grew out of the lag phase into the exponential growth phase) 1-liter of algae was distributed between eight-sterile 1-liter flasks at 125 ml per flask. The initial cell count before inoculation was recorded at 3,972,500/ ml. An additional 375 ml of media was added to each flask under sterile condition in a laminar hood. In trial four - cell counts were recorded each day for 12 days to determine if lipid percentages would increase or decrease over time and to determine each phase of the growth cycle. Before the trial ended cells from the first three flasks were transferred under sterile conditions to inoculate a new 1-liter flask to start the fifth trial (a modified batch method).

During trial five the initial cell counts before inoculation was recorded at 3,900,000/ ml. Once again 125 ml per flask was distributed into eight 1-liter flasks and additional 375 ml of media was added to each flask. In this trial cell counts were recorded each day of harvesting from day 7 - 14. The overall biomass was weighed after freeze drying and lipids were analyzed to see if

yields were lost or gained over time. Two trials were not conducted due to algae crashes in January and March, which will be discussed in the results section. The statistical analysis used is the same as stated above.

Results and Discussion

During all 5 trials three strains of *Prymnesium parvum* (UK) LB 995, (SC) LB 2827, and (TX) LB 2797 were tested for growth potential and lipid analysis. In both the Hampton and Blacksburg trials both (SC) LB 2827 and (TX) LB 2797 grew well in the media culture and showed some promising results although colony collapses did result with each strain, most likely due to self toxicity (Olli and Trunov 2007). Out of eleven algae samples delivered from UTEX, (TX) LB 2797 proved to be the most promising. Texas LB 2797 only failed to grow once; this after initial inoculation. Some start up cultures failed due to low cell density and inability to adapt to environmental changes.

The only lipid analysis of (SC) LB 2827 was conducted on samples from trial three in Blacksburg (**Graph 1- page 23**). Algal strain (UK) LB 995 did not grow in any of the trials and was discarded as a potential candidate for culturing. Out of five trials, lipid analysis was done on 18 replicates of - (TX) LB 2797 and only 2 replicates of - (SC) LB 2827 due to culture collapse. The growth parameters used in trials 1 and 2 are similar to 3, 4 and 5 but not exact so data should be viewed as a preliminary finding. The limited data makes it hard to substantiate the hypothesis concretely.

Growth Rate Trial One and Trial Two – Hampton Virginia Seafood AREC Summer 2008

Computation of population growth rates, divisions per day and population doubling times were conducted on Texas LB 2797 during trial one and two. Computation of population growth rates, divisions per day and population doubling times were conducted on South Carolina LB 2827 during trial one. South Carolina LB 2827 failed earlier in trial two and was used in a carbon Carboy trial not discussed in this paper since the trial was not completed.

Table 4 – Cell Count Results for Hampton Trials 1

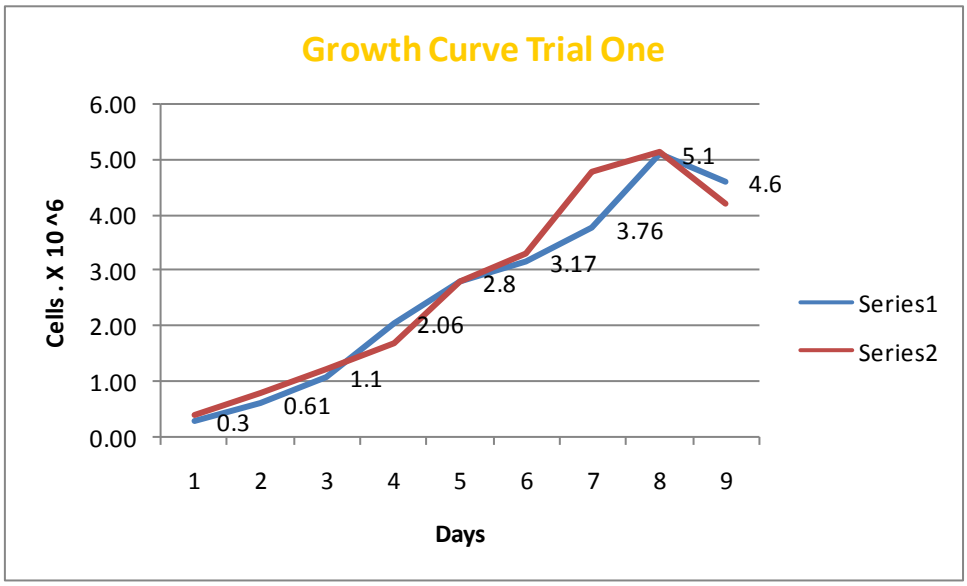
Sample ID – Date	Cell Counts at Harvest
Texas 2797 - July 3	610,000/ml
SC 2827 - July 3	800,000/ml
Texas 2797 – July 4	1,100,000/ml
SC 2827 – July 4	1,230,000/ml
Texas 2797 – July 5	2,060,000/ml
SC 2827 – July 5	1,710,000/ml

Table 5 – Cell Count Results for Hampton Trial 2

Sample ID - Date	Cell Counts at Harvest
Texas 2797 - July 11	760,000/ml
Texas 2797 - July 12	1,880,000/ml
Texas Carboy – July 12	1,080,000/ml
SC Carboy – July 12	960,000/ml
Texas 2797 – July 13	2,970,000/ml
Texas Carboy – July 13	2,260,000/ml

Texas 2797 – July 6	2,800,000/ml	**SC Carboy – July 13**	1,420,000/ml
SC 2827 – July 6	1,810,000/ml	Texas 2797 – July 14	3,120,000/ml
Texas 2797 – July 6	3,170,000/ml	Texas 2797 – July 14	3,820,000/ml
SC 2827 – July 6	2,810,000/ml	Texas Carboy – July 14	1,730,000/ml
Texas 2797 – July 7	3,760,000/ml	**SC Carboy – July 14**	1,670,000/ml
SC 2827 – July 7	3,310,000/ml	Texas 2797 – July 15	4,070,000/ml
Texas 2797 – July 8	5,350,000/ml	Texas 2797 – July 15	3,170,000/ml
SC 2827 – July 8	4,800,000/ml	Texas Carboy – July 15	2,200,000/ml
Texas 2797 –July 8	5,700,000/ml	Texas 2797 – July 16	3,680,000/ml
SC 2827 – July 8	5,150,000/ml	**SC Carboy – July 15**	1,840,000/ml
Texas 2797 – July 8	5,100,000/ml	**SC Carboy – July 16**	1,860,000/ml
SC 2827 – July 8	3,900,000/ml		
Texas 2797 – July 9	4,600,000/ml		
SC 2827 – July 9	4,200,000/ml		

Chart 1: Texas 2797 is series 1 with cell density labels; SC 2827 is series 2 with no listings



Population growth rate (r) for Texas 2797 was 0.46 (d^{-1}) over a 4-day period of exponential growth. Division's per-day (k) for Texas 2797 was 0.66 (d^{-1}) over a 4-day period of exponential growth. Population doubling time (T_2) for Texas 2797 was calculated at 1.51 days over a period of 4-days of exponential growth (see table below). In comparison, SC 2827 had a population growth rate of 0.36 (d^{-1}), a division's per-day rate of 0.51 (d^{-1}) and a population doubling time of 1.93 days over the same period.

Table 6 -Computation of population growth rate (r), divisions per day (k), and population doubling time (T_2)

Growth curve data for *P. parvum* TX 2797- trial 1									
	Day								
	0	*1*	*2*	*3*	*4*	*5*	*6*	*7*	
Cell number = n x 10^6 /mL	610000	1.1	2.06	2.8	3.76	5.35	5.7	4.6	
Time Interval (d)	***0-1***	***1-2***	***2-3***	***3-4***	***4-5***	***5-6***	***6-7***	***1-7***	***1-4***
N_2/N_1	**1.80**	**1.87**	**1.36**	**1.34**	**1.42**	**1.06**	**0.81**	**4.18**	**6.16**
ln (N_2/N_1)	**0.59**	**0.63**	**0.31**	**0.30**	**0.35**	**0.06**	**-0.21**	**1.43**	**1.82**
$\log_2(N_2/N_1)$	**0.85**	**0.90**	**0.44**	**0.42**	**0.51**	**0.08**	**-0.30**	**2.06**	**2.62**
$\Delta_1 = (t_2-t_1)$	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**6**	**4**
k (Eq.2)	**0.85**	**0.90**	**0.44**	**0.42**	**0.51**	**0.08**	**-0.30**	**0.34**	**0.66**
r (Eq.1)	**0.59**	**0.62**	**0.30**	**0.29**	**0.35**	**0.06**	**-0.21**	**0.24**	**0.46**
T_2 (Eq.4)	**1.17**	**1.12**	**2.31**	**2.39**	**1.98**	**11.6**	**-**	**2.91**	**1.51**

All equations used can be found under materials and methods – Andersen 2004

Table 7 – Growth percentages (d^{-1}) to determine mean, standard deviation and standard error for 4 trials

	Texas trial 1	Texas trial 2	Texas trial 4	S.C trial 1	Mean	Standard deviation	Standard error
Population growth rate (r)	0.46	0.56	0.23	0.36	0.40	0.14	**± 0.07**
Divisions per day (k)	0.66	0.81	0.33	0.51	0.58	0.21	**± 0.10**

Population doubling times (T_2)	**1.51**	**1.24**	**3.01**	**1.93**	**1.92**	**0.78**	**± 0.39**

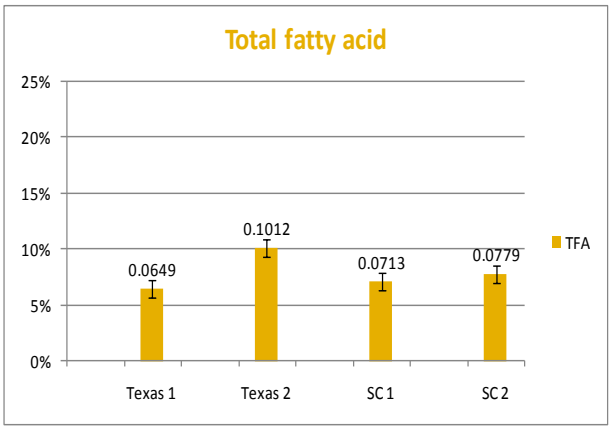
The divisions per day rate in these four trials were slightly higher when compared to research conducted by Larsen and Bryant 1998 in which *P. parvum* (using various strains) was grown in different combinations of salinity, light and temperature. The mean 0.58 ± 0.10 in this study (Table 7) is in comparison to 0.39 ± 0.06 reported by Larsen and Bryant (1998). The range of growth rates reported for *P. parvum* in previous studies ranged from 0.3-1.4 divisions per day (Edvardsen and Larsen 2003). Since most of the samples were counted during or after the stationary phase it would make sense why the numbers were slightly lower than average.

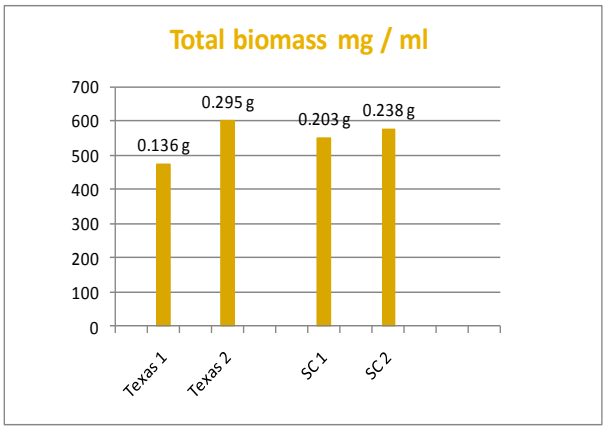
Biomass, cell densities and lipid analysis Trial Three – Biological Systems Engineering Blacksburg

Table 8 - Shows cell growth counts, TFA percentages and mass (DW) for (SC) 2827 and (TX) 2797 Trial 3 – Blacksburg

Sample ID	Cell Counts at Harvest	TFA %	Mass (DW)
Texas 2797 – 1	2,110,000/ml	6.50	0.136 g/475 ml
Texas 2797 – 2	3,130,000/ml	10.12	0.295 g/550 ml
SC 2827 – 1	2,170,000/ml	7.13	0.203 g/550 ml
SC 2827 -2	3,050,000/ml	7.79	0.238 g/575 ml

Graph 1 - left shows the total fatty acid percentage for Trial 3 with standard error bars – Blacksburg





Graph 2 right shows the total biomass (DW) per ml of solution at harvest for Trial 3 – Blacksburg

Table -9 Cell growth counts, TFA percentages and mass (DW) for (TX) 2797 Trial 4 & 5 – Blacksburg

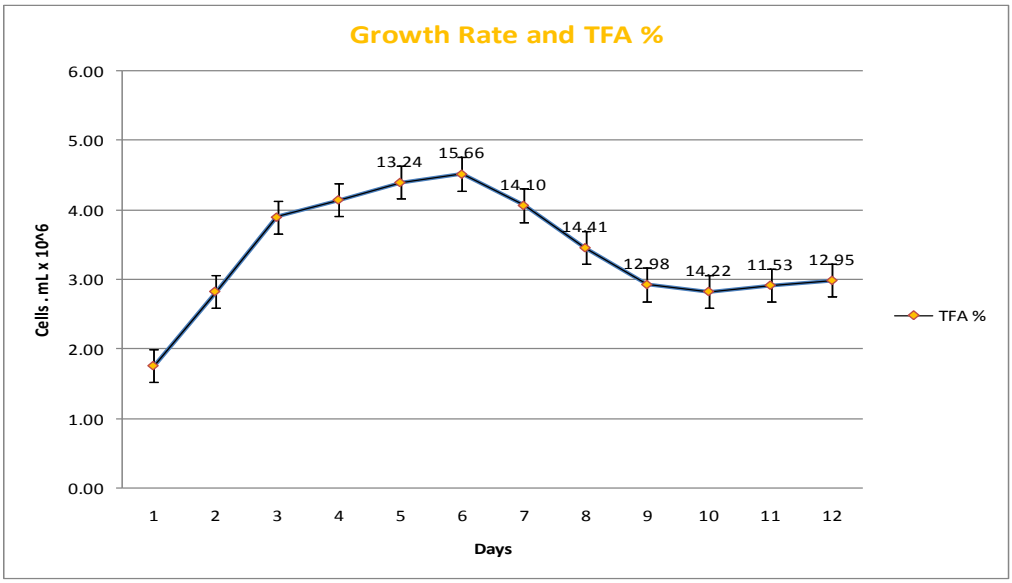
Sample ID	Cell Counts at Harvest	TFA %	Mass (DW)
Texas 2797 4-1	4,400,000/ml	13.24	0.103g/425ml
Texas 2797 4-2	4,520,000/ml	15.66	0.116g/400ml
Texas 2797 4-3	4,070,000/ml	14.10	0.108g/400ml
Texas 2797 4-4	3,460,000/ml	14.41	0.086g/375ml
Texas 2797 4-5	2,930,000/ml	12.98	0.094g/350ml
Texas 2797 4-6	2,830,000/ml	14.22	0.061g/340ml
Texas 2797 4-7	2,920,000/ml	11.53	0.064g/340ml
Texas 2797 4-8	2,990,000/ml	12.95	0.051g/330ml
Texas 2797 5-1	3,520,000/ml	14.67	0.096g/400ml
Texas 2797 5-2	3,350,000/ml	14.08	0.094g/400ml
Texas 2797 5-3	3,010,000/ml	8.79	0.087g/400ml
Texas 2797 5-4	2,730,000/ml	16.26	0.099g/400ml

Texas 2797 5-5	2,410,000/ml	12.94	0.056g/380ml
Texas 2797 5-6	2,300,000/ml	8.62	0.059g/375ml
Texas 2797 5-7	2,210,000/ml	8.07	0.051g/380ml
Texas 2797 5-8	1,980,000/ml	9.53	0.048g/350ml

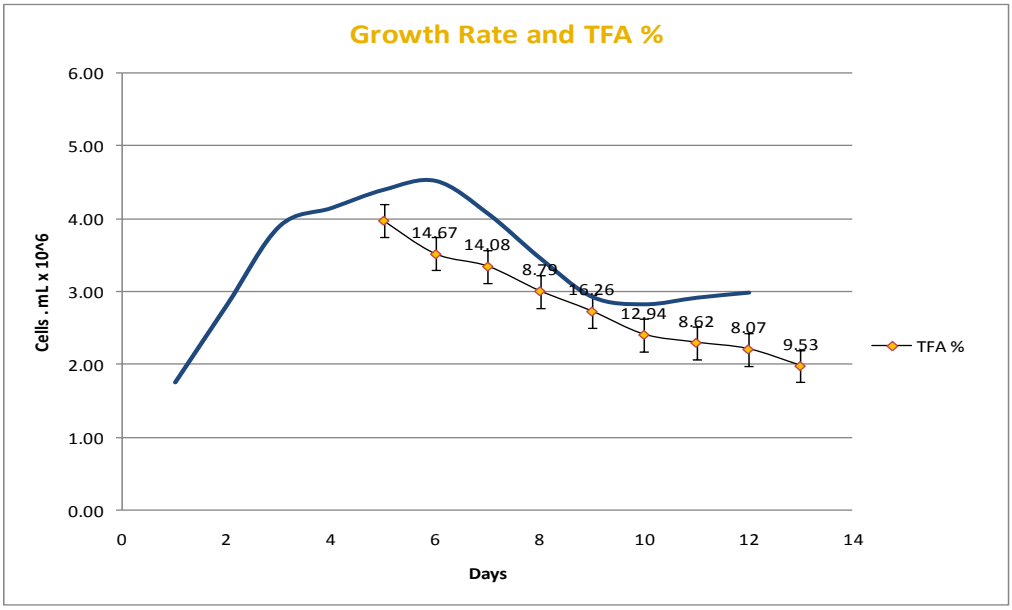
Cell densities and total fatty acid -Trial 4&5

Harvesting of algae began after day five for trial 4 and day six for trial 5. The purpose was to analyze total fatty acid (TFA) percentages during the stationary growth phase to determine the best harvesting period. The mean cell densities at harvest for trial 4 are $3.52 \text{ X } 10^6 \pm 0.25$ compared with $2.69 \text{ X } 10^6 \pm 0.20$ for trial-5. Maximum cell density peaked at day six of exponential growth in trial 4 at $4.52 \text{ x } 10^6$. The mean TFA % for trial 4 was 13.64% ± 0.43804 compared with 11.62% ± 1.13865 for trial 5. Maximum total fatty acid % (DW) was found at day six at 15.66 % for trial 4 and 16.26% at day nine for trial 5.

Graph 3 - Data from trial 4 from table 7: Cell counts over days with total fatty acid % at harvest



Graph 4 - Data from trial 5 from table 7: Cell counts over days with total fatty acid % overlying trial 4



Total fatty acids on average declined as algae went from a stationary phase into the death "crash" stage. This is most likely due to cell mortality rates. This is contrary to reports that algae increase lipid production near the end of their life cycle (Miyamoto 1997) due to nutrient

starvation. Nutrient levels may not be the limiting factor since nutrient levels for trial 5 were equivalent to trial 4, therefore decline may be contributed to loss of cell density as a result of self toxicity (Olli and Trunov 2007). Crashes and declines occurred in each trial in Hampton and Blacksburg. The crashes came during the third runs of each trial set in January and March in all strains suggesting that *P. parvum* may not be able to sustain itself under controlled lab conditions. In the Hampton trials when the algae were transferred to the Carboys the production rates declined as expected due to dilution factors and decreased light diffusion (Hoff and Snell 2007). In the small stock flasks numbers began to decline in both the SC and TX strains showing signs of imminent collapse. No Biomass was harvested during the Hampton trials, so no algae were analyzed for total fatty acids.

Table 10 – Total fatty acid percentage to determine mean, standard deviation and standard error for 3 trials

	Texas trial 3 mean	Texas trial 4 mean	Texas trial 5 mean	S.C trial 3 mean	Total mean	Standard deviation	Standard error
Total fatty acid	8.305 ± 1.815	13.63625 ± 0.43804	11.62 ± 1.13865	7.46 ± 0.33	11.679	3.07108	**± 0.68671**
C16:0 palmitic	14.09042 ± 1.14847	16.6961 ± 0.11006	16.9779 ± 0.30192	16.94075	16.55237	1.11289	**± 0.25531**
C18:0 stearic	**15.73147**	**15.17334 ± 0.36814**	**13.80517 ± 0.28356**	**14.79264**	**14.57176**	**1.11446**	**± 0.26268**

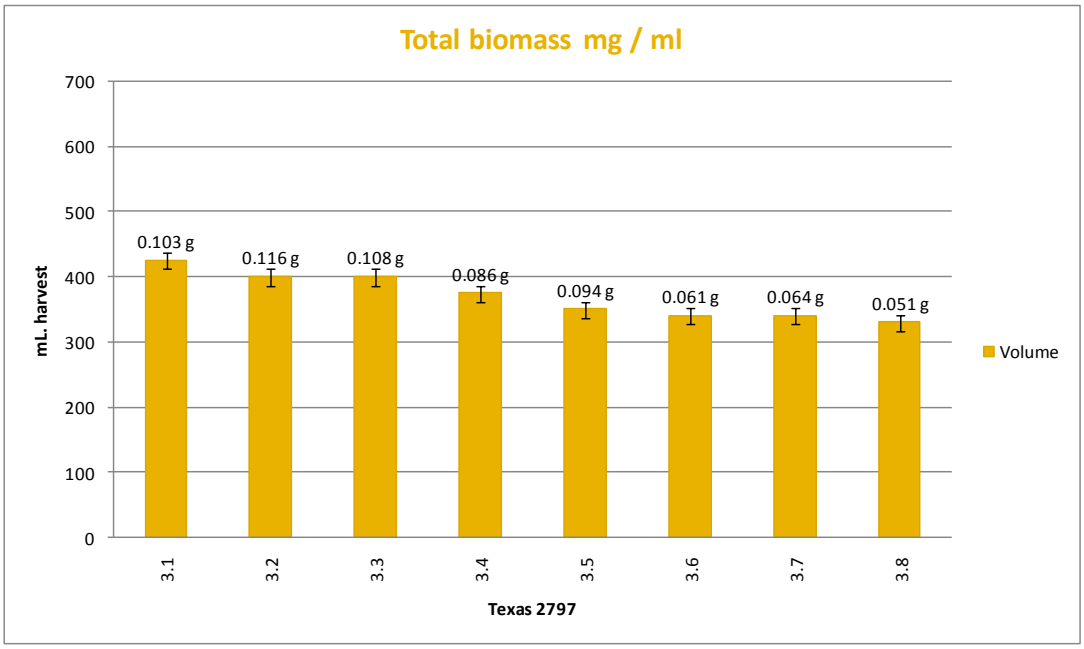
In order to determine the exact fatty acid composition from microalgae using Gas Chromatography, one must use a known set of prepared fatty acid standards. The above fatty acids C16:0 (Palmitic acid) and C18:0 (Stearic acid) were previously identified by Chi et al. in 2007 using the same gas chromatography method with a set of fatty acid standards and heptadecanoic acid internal standard C17:0. Retention times for C16:0 and C18:0 were recorded and compared with chromatograms of *P. parvum* standards. Two known fatty acids for *P. parvum* were then quantified (Table 10, Appendix). The two fatty acids C16:0 and C18:0 make up 30% of the total fatty acids in *P parvum*.(Table 10, Appendix).

Biomass Production -Trial 4&5

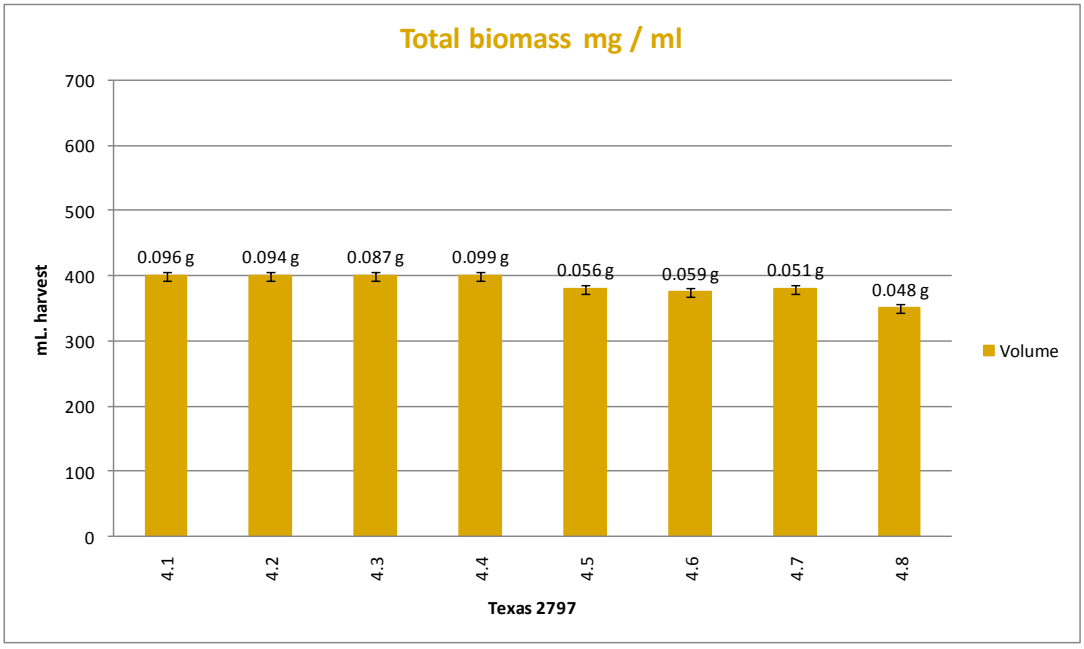
The mean dry weight (DW) of biomass for trial 4 was 0.09 g ± 0.01 in an average of 370 mL ± 12.43 of media at harvest time. The mean weight for biomass for trial 5 is 0.07 g ± 0.01 in an average of 386 mL ± 6.36 of culture at harvest time. All algae cultures combined mean biomass weight from trials 3, 4 & 5 is 0.17 g ± 0.03 in an average culture volume of 493 mL ± 40.13

In comparison to other algae species these preliminary results show that *P. parvum* in suspension culture had a very low biomass yield 0.34g (DW) / liter as compared with *Parietochloris incisa,* which ranged from 1.3 g / liter to 5.4 g / liter (DW). In comparison *Parietochloris incisa*, considered a high TFA alga, produced a TFA content as high as 60% (DW) (Bigogno et al. 2002).

Graph 5 represents total (DW) biomass from trial 4 in relationship to mL of solution at harvest time from table 7



Graph 6 represents total (DW) biomass from trial 5 in relationship to mL of solution at harvest time from table 7



Culture Sustainability

The question remains as to whether *P. parvum* can sustain itself in a controlled environment without self toxicity during a prolonged experiment. Toxic blooms in the wild are caused by factors such as low or high salinity, low temperature or nutrient stress (Baker et al. 2007). It was assumed that exoteric algae remain resistant to their own allopathic effects (Olli and Trunov 2007). *Prymnesium parvum* releases potent exotoxins, which have detrimental effects on a variety of aquatic species (Larsen and Bryant 1998). A previous study suggested that *P. parvum* cells lyse during the late logarithmic growth and stationary phases when their toxins were released under favorable conditions (Olli and Trunov 2007). This may explain why the cultures declined rapidly after the exponential phase and stationary growth phases, while overall collapse in the third leg of each trial occurred using the modified batch method. Toxins may have been transferred from old to new cultures.

Conclusion

The purpose of this study was to evaluate *P. parvum* as a potential feedstock for biodiesel. Three key components essential for microalgae to be considered as feedstocks are: overall biomass production, high fatty acid % (DW) and an overall ability to sustain itself in culture. This preliminary study suggests that *P. parvum* has potential as a high lipid producer but the overall ability to sustain itself in culture may be inhibited by autotoxicity. Therefore, biomass

production was limited by frequent crashes, although additional research is needed to confirm this hypothesis.

Future studies may include nutrient stability trials and stable monitoring using a Continuous-Culture Bioreactor System instead of the Modified Batch Method. Using a continuous culture system may inhibit toxicity if all growth parameters are controlled and harvesting is done before toxic buildup occurs . Increased levels of CO_2 may facilitate sustainability if pH is adequately monitored; this may or may not increase biomass production. Studies using DNA transformation technologies could reduce toxins and increase lipid production capabilities, making *P parvum* a candidate alga for biodiesel feedstock production.

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Appendix

Table 2 - Algae Fatty Acid Methyl Ester preparation for Gas Chromatography

Sample Preparation:

1. Weigh out approximately 20 mg of freeze-dried algal cells into glass test tube
2. Add 4 mL mixture of methanol, concentrated sulfuric acid, and chloroform (1.7:0.3:2.0 v/v/v) into each tube. This mixture should also contain 1 mg/sample of the internal standard C17:0 heptadecanoic acid. This translates into 1 mg per 4 mL of solution.
3. Tighten the lids very tightly onto the tubes (use plumbers tape). Place tubes in a 90C water bath for 40 minutes.
4. Remove the tubes from the water bath and let cool. 15 min.
5. Add 1 mL distilled water to each tube and vortex for 30 seconds.
6. After this, two phases should form. The bottom phase contains the fatty acid methyl esters.
7. Pipette the bottom phase into microcentrifuge tubes, add anhydrous Na2SO4 to the tubes.
8. Centrifuge the tubes at 10,000 RPM for 8 minutes.
9. Pipette the liquid from the tubes into glass GC vials for GC analysis.

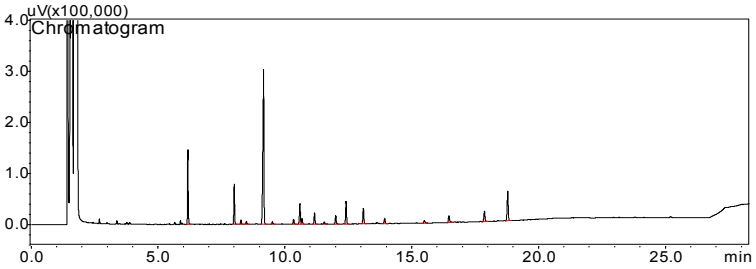
Table 3 – Shows a standard formula method used to determine the TFA% by weight of the sample harvested on Day 9 in Trial - 5 (8.79536 TFA % yield (DW))

Ret Time	Area	Area %	FA (mg)	FA (%)	TFA (mg)	Biomass (mg)	TFA Yield (%)
6.018	273841.7	9.4226	0.268263	14.52404	1.847026	21	8.79536
7.772	336377.2	11.5743	0.329524	17.84081			
8.016	20033.4	0.6893	0.019625	1.062534			
8.222	25198.7	0.8671	0.024685	1.336491			
8.882	1020796	35.1244	1	na			
10.031	11710.8	0.403	0.011472	0.621119			
10.277	255982.9	8.8081	0.250768	13.57685			
10.35	16367.6	0.5632	0.016034	0.868106			
10.825	70850	2.4379	0.069407	3.75775			
11.642	58997.5	2.03	0.057796	3.129116			
12.038	110949.9	3.8177	0.10869	5.884572			
12.706	79722.9	2.7432	0.078099	4.228352			
13.262	47382.7	1.6304	0.046417	2.513089			
13.541	48318.5	1.6626	0.047334	2.562722			
16.038	93866.4	3.2298	0.091954	4.978496			
17.426	137975.4	4.7476	0.135164	7.317954			
18.339	297861.3	10.249	0.291793	15.798			

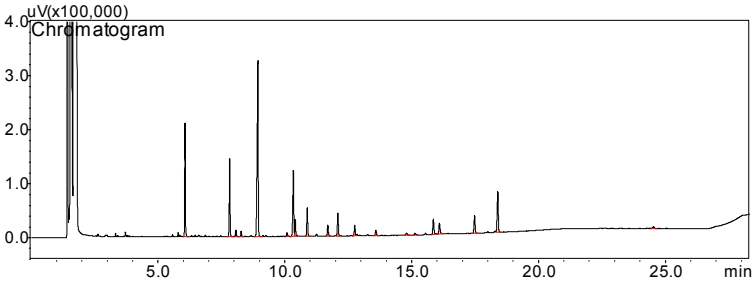
100

Chromatograms Set 1 – Shows the fatty acid profiles (Chromatograms) produced using Gas Chromatography for Trial 3 in Blacksburg TX 2797 Sample 1, 2 and SC Sample 1, 2.

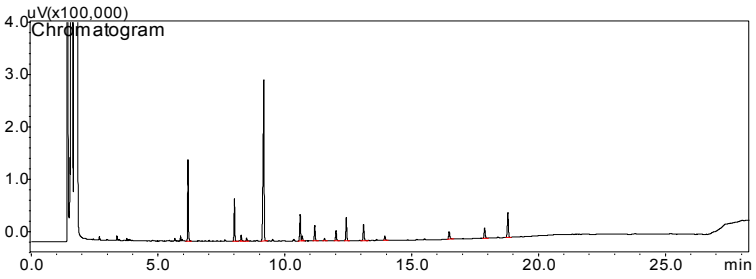
Sample One Texas 2797



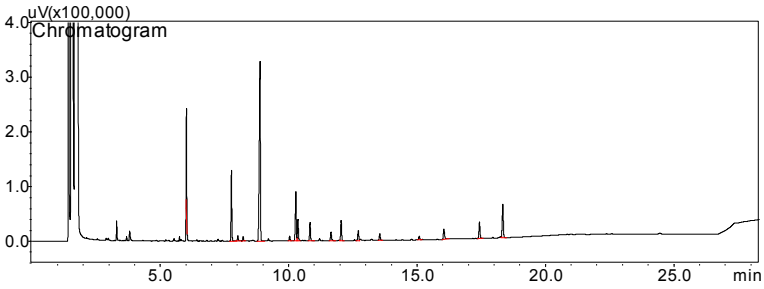
Sample Two Texas 2797



Sample One South Carolina 2827

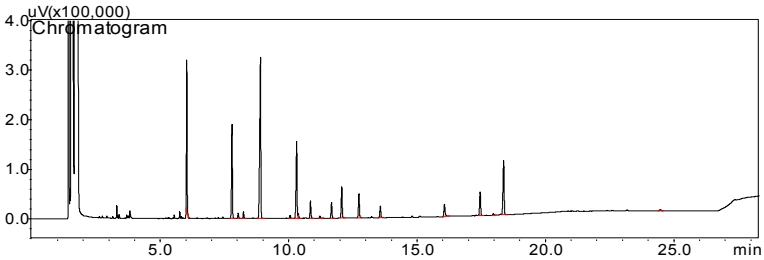


Sample Two South Carolina 2827



Chromatograms Set 2 – Shows the fatty acid profiles (Chromatograms) produced using Gas Chromatography for Trial 4 in Blacksburg TX 2797 Samples 1-8

Sample One



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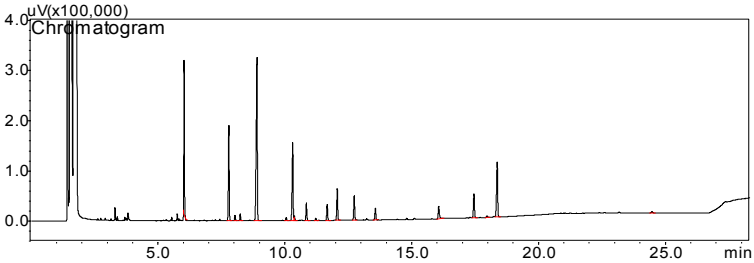

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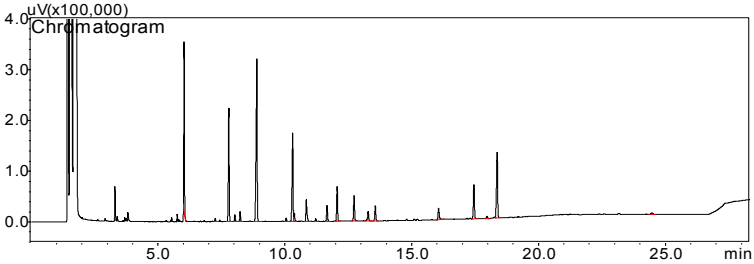
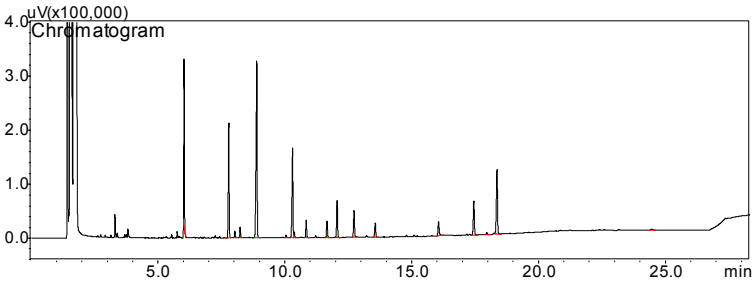
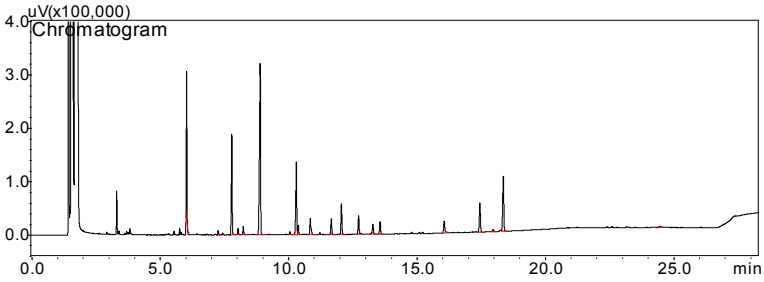


Sample Four



Sample Five



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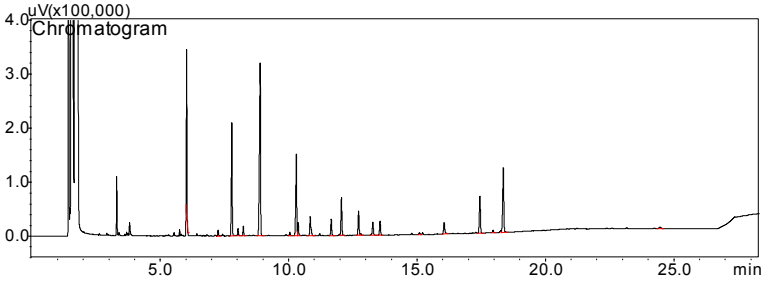

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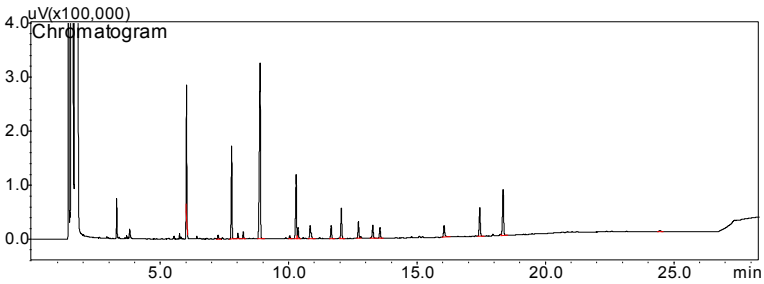
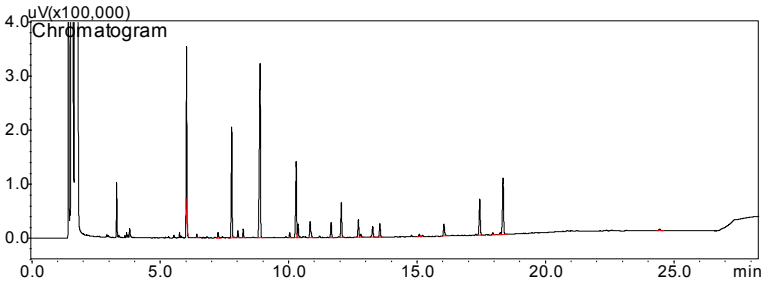
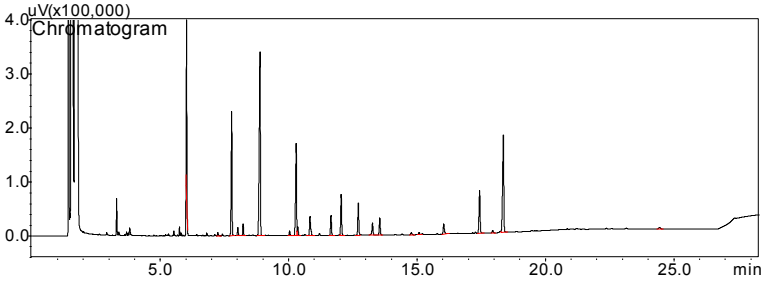


Sample Eight

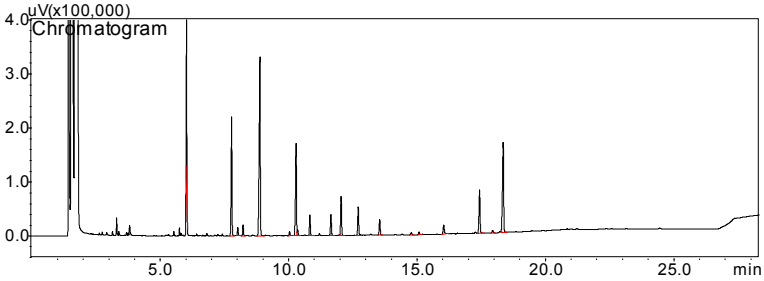


Chromatograms Set 3 – Shows the fatty acid profiles (Chromatograms) produced using Gas Chromatography for Trial 5 in Blacksburg TX 2797 Samples 1-8

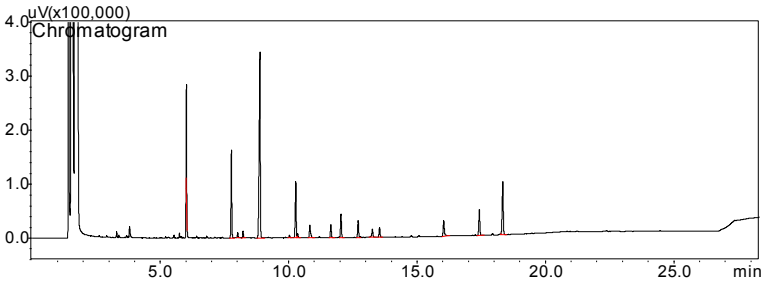
Sample One



Sample Two



Sample Three



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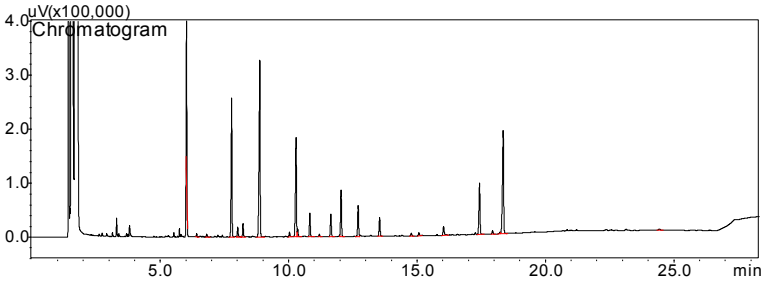

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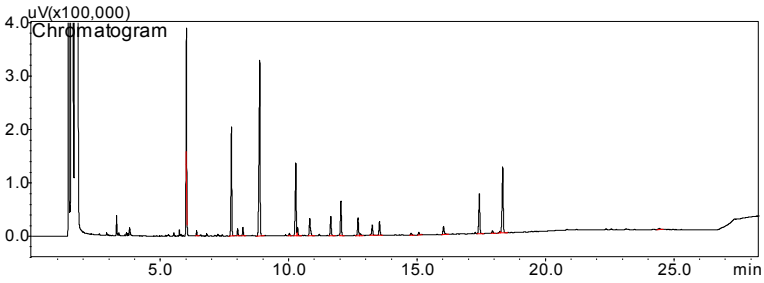

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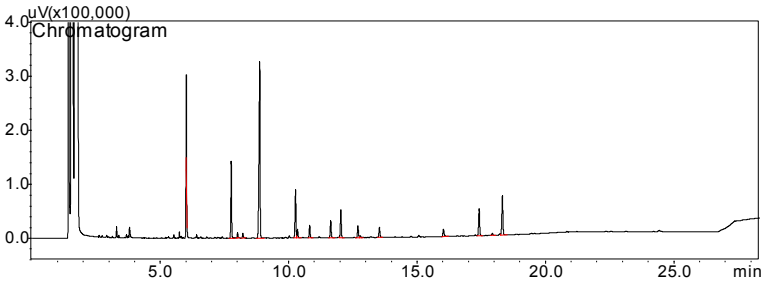
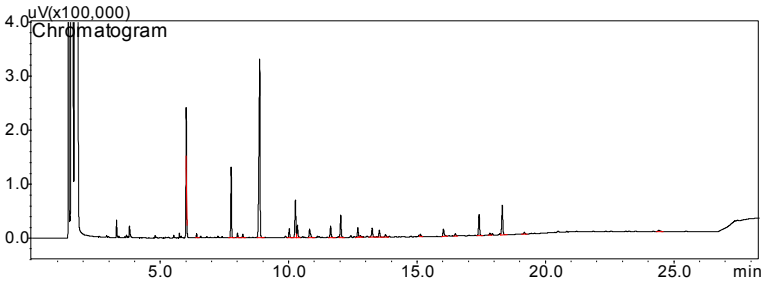
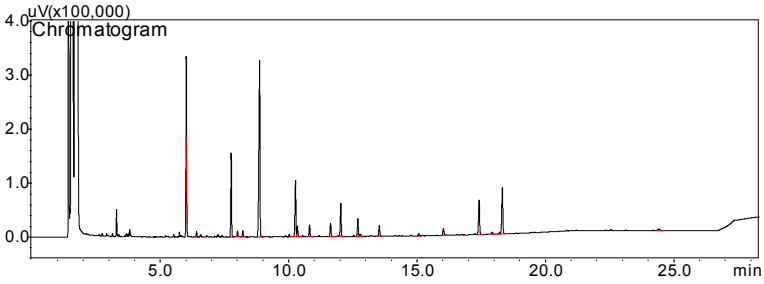


Sample Seven



Sample Eight



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