Fall 12-2019

EFFECTS OF TEMPERATURE AND NITROGEN DEPLETION ON BIODIESEL PRODUCTION UNDER SALINE CONDITIONS BY A MIXED ALGAL COMMUNITY

Sarah J. Kintner
University of New Mexico

Sarah J. Kintner

Candidate

Nanoscience and Microsystems Engineering

Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

Dr. Andrew Schuler, Chairperson

Dr. David Hanson

Dr. Abhaya Datye

Dr. Kerry Howe

EFFECTS OF TEMPERATURE AND NITROGEN DEPLETION ON BIODIESEL PRODUCTION UNDER SALINE CONDITIONS BY A MIXED ALGAL COMMUNITY

BY

Sarah J. Kintner

B.S. Mechanical Engineering, Villanova University, 1982

DISSERTATION

Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctorate of Philosophy

Nanoscience and Microsystems Engineering

The University of New Mexico

Albuquerque, New Mexico

December, 2019

ACKNOWLEDGMENTS

I heartily acknowledge Dr. Andrew Schuler, my advisor and dissertation chair, for continuing to assist me through the years of research and the long number of years and months writing and rewriting these chapters. His guidance and professional style will remain with me as I continue my career.

I also thank my committee members, Dr. Abhaya Datye, Dr. David Hanson, and Dr. Kerry Howe, for their valuable recommendations and technical assistance pertaining to this research. Gratitude is extended to the GAANN Grant, CREST Grant, and UNM for the funding provided to me to pursue this research. I particularly thank my two wonderful Nanoscience and Microsystems Engineering advisors Heather Armstrong, and Linda Stewart.

I thank my brother Eric, for his encouragement to finish this research, and his tropical fish tank, 45 years ago, that held the algae giving me the motivation to perform this research. I thank my best friend, Pam Harris, for the many years of support and to my friends, Duke, Dana, Rick, John, Sarah, John, Leiv, Trish, Ken, Dave, Mary Anne, Linda and Tom who gave me immeasurable support over the years of study and laboratory work.

EFFECTS OF TEMPERATURE AND NITROGEN DEPLETION ON BIODIESEL PRODUCTION UNDER SALINE CONDITIONS BY A MIXED PHOTOTROPHIC COMMUNITY

By

Sarah Kintner

B.S., Mechanical Engineering, Villanova University, 1982

PhD, Nanoscience and Microsystems Engineering, 2019

Abstract

The overall objective addressed in this research was to gain a better understanding of how native mixed cultures of phototrophic microorganisms from a saline system may be used for biodiesel production. Laboratory batch reactors were grown under variable temperatures and nitrogen concentrations with original inoculum from a hypersaline pond in central New Mexico. Growth at 40 C° led to lower lipid production, lower nitrate consumption, and less diverse communities than growth at 20° C. All communities were dominated by the cyanobacteria *Plectonema* except the in second experiment, where they were only 30% of the culture. In a second experiment with variable initial concentrations, a high nitrate medium (179 mg N/L) led to significantly higher growth rates than a low nitrate medium (75 mg N/L) even before nitrate was depleted in the low nitrate reactors. Lipid production was similar under both conditions. A final experiment determined that optimal mixed community storage

conditions were determined to be -196 deg C in 5% DMSO. This research helped to identify conditions for biodiesel production in saline media, which could improved its viability in full scale systems.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Abbreviation	Definition of Abbreviation
bp	base pairs
BB	bead beaten
Chl *a*, Chl *b*, Chl *c*	chlorophyll *a*, chlorophyll *b*, chlorophyll *c*
CN	cetane number
CPA(s)	cryoprotective additive(s)
DMSO	dimethylsulfoxide
FAMEs	fatty acid methyl esters
Ga	billion years ago
GM	genetically modified
HN	high nitrate
HT	high temperature
KOH	potassium hydroxide
l or L	liter
LN	low nitrate
LQN	liquid nitrogen
m or M	meter
Ma	million years ago
MEOH	methanol
μg	microgram 1 x 10^{-6} g
mg	milligram 1 x 10^{-3} gram
μl or μL	microliter, 1 x 10^{-6} L
ml or mL	millileter, 1 x 10^{-3} L
μm	micrometer 1 x 10^{-6} M
μmol	micromole 1 x 10^{-6} mol
NB	not bead beaten
nm	nanometer 1 x10^{-9} M
NM	New Mexico
NO3-	nitrate
PAR	photosynthetically active radiation (400 - 700 nm)

Abbreviation	Definition of Abbreviation
pg	pico gram 1 x 10^{-12}
pres	preservant
rDNA	ribosomal DNA
RPM	revolutions per minute
RT	room temperature
± SE	plus or minus standard error
TAG(s)	Triglyceride(s) or triacylglycerol(s)
UVA	ultra violet light wavelengths between 320 - 400 nm
UVB	ultra violet light wavelengths between 280–320 nm
UVC	ultra violet light wavelengths less than 280 nm
UVR	ultra violet light wavelengths less than 400 nm
%V/%V or v/v	volume percent per total volume
WT	wild type

GLOSSARY

Name	Definition
axenic	Relating to or denoting a culture that is free from living organisms other than the species required
cyanobacteria	are a group of photosynthetic bacteria
cryoprotective additive	A chemical additive used in the cold storage of microorganisms
photoautotrophic	Any organism that derives its energy from light by converting light energy into chemical energy to capture carbon from the atmosphere
photoinhibition	Photoinhibition is damage caused to the photosynthetic apparatus by excessive light levels.
planktonic	Small organisms floating as single cells in water or medium
reconstitution	Process of thawing and reviving a frozen algae culture
wild type	A species as it is found in nature, not genetically modified
xenic	A culture medium containing one or more unidentified organisms, which may be bacteria, fungus or other organisms than the species required

Chapter 1 : Introduction and Background

1.1 WHY SELECT A MIXED ALGAL COMMUNITY?

The selection of an algae community for this research surfaced from the knowledge that biodiesel production occurs in arid desert lands capitalizing on abundant sunlight. Raceway ponds require constant movement to keep algal cell bathed in the nutrients that will make them produce the lipids as a precursor to the ultimate goal, biodiesel.

The lack of available freshwater in arid climates may direct the need to find alternative water sources from saline, brine, brackish waters or potentially produced water. High saline waters naturally deter invasions by freshwater algae species and freshwater grazers since their adaptation hinges on a freshwater environment. The mixed algal community grown for this research were selected from a high light and high temperature brine pond, knowing that any algal community in the conditions found at the brine pond would likely have many of the characteristics necessary for successful biodiesel cultivation in dry climates. Invading algal species and other grazing organisms would not be tolerant of the high temperatures.

The research of the mixed algal community created the same conditions in the laboratory as those found at the pond site. Unable to reach the highest PAR value (2014, Albuquerque incident PAR 1602 µmols photons m^{-2} sec^{-1}) from the cool white light set up in the laboratory, the maximum achieved was 1400 µmols photons m^{-2} sec^{-1} ,and illuminated all the algal reactors for the 3 research

experiments. 1400 µmols photons m^{-2} sec^{-1} is considered an extremely high PAR intensity when working with algal species. Experiment 1 used temperature that reflected the highest temperature in Albuquerque in 2014 of 40 °C (104 °F).

The media for all 3 experiment used a synthetic brine medium formulated to match the high saline composition of the San Acacia Brine Pond [1, 2] detailed in Table 2.2 with a final TDS greater than seawater**.**

1.2 BIODIESEL PRODUCTION

The current primary fuel for diesel cars and trucks in the U.S. Grade No. 2 petro diesel (No. 2 petro diesel) extracted from wells and hydraulic fracturing. Biofuels offer sustainable alternatives to petroleum based fuels and include feedstocks from corn, soybean oil, animal fats, canola oil, and grease. Biofuels are renewable and have lower emissions than petro diesel for carbon monoxide (CO), carbon dioxide (CO_2), particulate matter (PM), hydrocarbons (THC), and sulphate. [3] Fermented corn produces bioethanol and is used as an additive to gasoline. Soybean oil, fats and other oils are transesterified and added at 5% to 20% v/v to petro diesel for diesel fuel blends.

Microalgae biomass is an attractive feedstock for biodiesel production, in part due to rapid growth rates. Growing microalgae in media with limited nitrogen concentrations can induce the sequestration of TAGs (triacylglycerols) creating greater biodiesel yields. Advantages of green algae, diatoms and cyanobacteria produced biodiesel include emissions that are far lower than petro diesel, they do not contain sulfur, use far less land than farmed biodiesel crops, and turn over a

crop in 1 to 2 weeks. Compared to No. 2 petro diesel, 100% algal biodiesel (B100) has 50% less CO, 70% less PM, and 40% less THC. [4] , but critical challenges to cost-effective production include: water availability, research scale-up to commercial ponds, and competition from other plant based biodiesel sources.

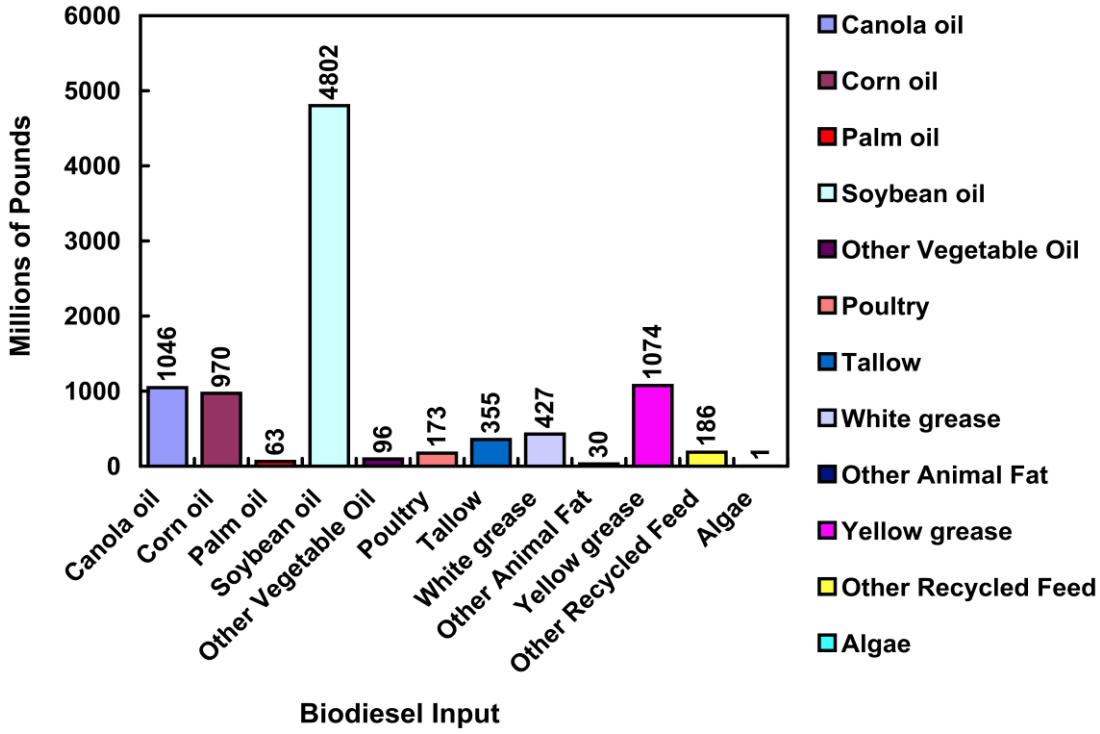

Figure 1.1 2014 Biodiesel production in the United States
The 2014 biodiesel production and sources came from the U.S. Energy Information Administration (eia) input received from suppliers.[5] Soybean oil is the highest biodiesel available for blending with petro diesel in the U.S. market. The algal biodiesel is the number "1" in the lower right hand corner of the graph frame.

In 2006 the U.S. EPA reduced the sulfur content of No. 2 petro diesel from 500 to 15 ppm to improve catalytic conversion of pollutants [6], but this also reduced the .engine lubricity of the diesel fuel.[7] Biodiesel has essentially no sulfur content, and biodiesel blended with ultra low sulfur diesel fuel (ULSD) No. 2 petro diesel fuel has been found to increase lubricity.[8, 9] Biodiesel has been shown

to increase diesel engine combustion and performance when the biodiesel contains more saturated fatty acid methyl esters (FAMEs) than unsaturated FAMEs.[9] Biodiesel has increasingly been used as an additive to No. 2 petro diesel fuel, with U.S. and European manufacturers permitting 2% to 20% biodiesel content dependent on the diesel engine requirements.[10]

In 2014 U.S. petro diesel refineries produced 1.7 billion barrels per year of No. 2 diesel fuel [11] , while biodiesel derived from soybean oil, the largest biodiesel source in the U.S., Figure 1.1, was 15 million barrels. In 2014, the U.S. algal biodiesel production was only 1800 barrels/year, or 0.01% of the soybean oil biodiesel production. [11] No. 2 Petro diesel is refined from crude oil in a distillation column. The No. 2 petro diesel is removed from the distillation column level with a 250 - 350 °C temperature. At this level the distilled No. 2 diesel contains 75% aliphatic hydrocarbons $C_{10}H_{20}$–$C_{15}H_{28}$. which are similar to the FAMEs found in algal biodiesel (FAMEs C14:0 to C24:0).

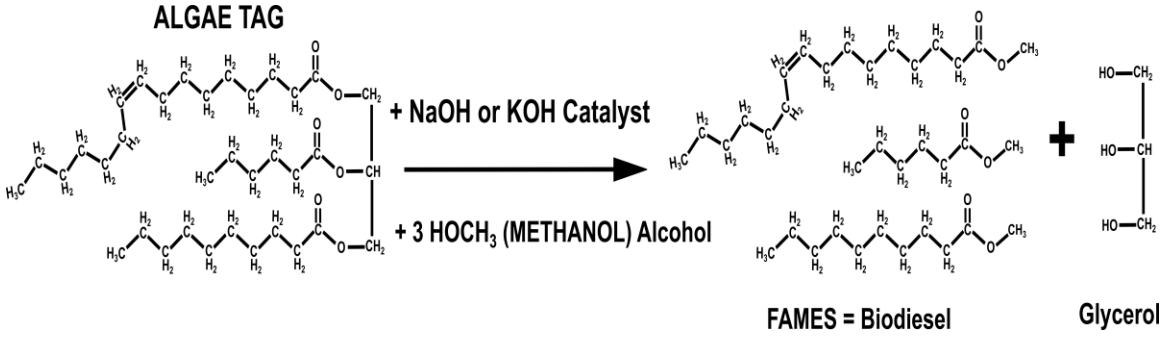

Figure 1.2 Transesterification of a phototrophic algae produced triacylglycerol (TAG)
Algal phototrophs create TAGs for long term storage, which are converted into biodiesel.

Cyanobacteria, diatoms and algae create TAGs by repeatedly incorporating fatty acids into one of three sites on the glycerol backbone of the TAGs. This creates three hydrocarbon chains which the algal cell use for long term storage. TAGs are transesterified (Figure 1.2) to produce biodiesel, which are fatty acid methyl esters (FAMEs).

1.3 Major Barriers to algal biodiesel production

Major barriers to algal biodiesel production include the cost to produce biodiesel, the scale-up costs, raceway pond construction, cost of photobioreactors design and installation. Finding species that produce enough biomass to harvest in a short period of time (1-2 weeks), which involves research to determine their biodiesel production capacity and the number of days to harvest.

Additionally, single algal species cultures (monocultures) at scale-up can fail due to colony collapse, which stems from internal culture competition for nutrients, high temperature, high light intensity, high salinity, algal toxins, waste accumulation, and programmed cell death [12-14]. Any one of these conditions can sweep through a single species algal culture during scale-up resulting in complete death.

One of the largest barriers depends on the price of No. 2 Petro diesel per gallon compared to the cost per gallon of a gallon of algal biodiesel even if the algal biodiesel is used as an additive for blending with No. 2 Petro diesel. Competition from existing biofuels can be steep such as that from soybean oil,

which is the largest oil biofuel produced having a large production base and distribution infrastructure. Water availability can be a barrier for the production of biodiesel. Saline ground water and produced water may be attractive water sources [15] for biodiesel production from halotolerant phototrophic green algae, diatoms and cyanobacteria. An article assembled a GIS-based model to estimate the availability and costs of providing water from a range of saline groundwater sites and found that there were 3,036 sites where the water would only cost 5% of the produced biodiesel value. The model used water consumption and biofuel production rates in conjunction with the viable water options and the most cost-effective source for each site. [15]

1.4 PHOTOTROPHIC MICROORGANISMS

Algae are defined here as aquatic photosynthetic phototrophic eukaryote green algae, prokaryote cyanobacteria and eukaryote diatoms.

The generic word "algae" encompasses a large diverse group of organisms from microalgae 1µm to 20 micrometers (µm) at the smallest end of the algae dimensional spectrum to the macroalgae kelp, (Macrocystis pyrifera), reaching lengths of 150 to 180 meters. In nature, microalgae live in communities, not only with other algae, but also in biotic relationships with bacteria.[16] Algae have different morphologies: unicellular, colonial, filamentous, or composed of simple tissues.[17] The number of green algae and cyanobacteria species published in the Algae Database included an estimated 72,500 species. The number of

diatom species are estimated to be over 200,000, It is likely that many unknown algal species still exist in the world.[17]

Photosynthetic prokaryote cyanobacteria, eukaryote diatoms and eukaryote green algae are global prime producers forming the lowest trophic level, which are food for the levels above.[18]

1.4.1 CYANOBACTERIA

The earliest oxygenic photosynthetic prokaryotes are generically named cyanobacteria (phyla) or blue-green algae. Cyanobacteria are phototrophic prokaryotes fixing carbon while releasing oxygen.[19] Cyanobacteria produce chlorophyll *a* (Chl *a*), but not chlorophyll *b* (Chl *b*). [20]

Cyanobacteria are phototrophic gram-negative microorganisms and are considered one of the oldest the oxygen evolving organisms present in the fossil record dating back 3.9 billion years (early Archean era.[20]

The origins of cyanobacteria adaptation started in an environment where challenges included direct ultraviolet solar radiation: UVA (320-400 nm), UVB (280-320 nm), UVC (wavelength <280 nm) and an atmosphere containing 90% nitrogen, 5% carbon dioxide, (5% methane + ammonia), and 0.01% oxygen.[21, 22]

One specific family of cyanobacteria, Oscillatoria, inherited the cyanobacteria adaptive responses from the Archean era [23] and populate all oceans and continents, living in hot, cold, and hypersaline environs, where cyanobacteria have acclimated to these surroundings.[24] The Oscillatoria family is comprised of filamentous species performing oxygenic photosynthesis using pigments:

Chl *a*, phycocyanin, allophycocyanin, phycoerythrin, β-carotene and xanthophylls. The Oscillatoria cyanobacteria multiply by fragmentation where a new individual organism can start from a single shed cell or a cleaved strand from the filamentous original.[20] Present day cyanobacteria survive in freshwater or marine environments.

The cyanobacteria gram-negative double layer cell wall consists of an outer and inner membrane. The outer membrane has two opposing phospholipid micelle layers where the hydrophilic phospholipids micelle heads sit on opposite sides of the lipid bilayer (outer membrane) in contact with the environment, and the hydrophobic tails protrude into toward the cell wall center. The entire outer membrane thickness is 8 nm. A peptidoglycan layer located below the outer membrane formed from sugars and amino acids gives structure to the cell wall. The peptidoglycan layer allows the passage of nutrients and other components into and out of the cell. Cyanobacteria produce starches for short-term storage. The starches are converted into fatty acids and then turned TAGs for long term storage.[25]

1.4.2 Diatoms

Diatoms fall under the Eukaryota kingdom and are oxygenic phototrophic algae appearing in the fossil record at 135–240 Ma (Mesozoic) and currently encompass 200,000 known species.[17, 24] Diatoms live in both marine and freshwater habitats and are the most numerous algae on the earth and account up to 40% of the primary production in the oceans.[26] Diatoms have two hinged frustules (outer silica shells) which overlap on the two opposite edges. Diatoms

contain thin chloroplasts, mitochondria attached to the inner wall of the frustules. Diatoms produce photosynthetic pigments Chl *a* and chlorophyll *c* (Chl *c*), β-carotene, and xanthophylls. The frustule silica weight pulls the diatom down into the water bottom sediment, a preferred diatom environment. Diatoms store both their short and long term energy products in spherical lipid oil bodies. The oil bodies contain triacylglycerols (TAGs) and often in the dark, the diatom cell will remove parts of one or more of the three TAG branches to desaturate them and create unsaturate lipids for thylakoid and other cellular uses [27].

1.4.3 GREEN ALGAE

Green algae are eukaryotes and populate worldwide fresh and marine water environments. Green algae (phylum: Chlorophyta) represent a large autotrophic diverse microalgae group and trace back 350 - 425 million years ago (Ma) (Paleozoic).[28] The green algae have may several forms: unicellular, multicellular, or colonial which may include thousands of independent algal cells and identified green microalgae species number over 8000. Green algae are purported to have descended from cyanobacteria, but display a larger number of specialized organelles than cyanobacteria.[17] Most eukaryotes have a nucleus, plastids, chloroplasts, mitochondria, golgi bodies, and endoplasmic reticulum. The nucleus houses the green algae DNA and cytoplasm contains the balance of the rest of the organelles. The cell walls of green algae are typically composed of cellulose, but depending on the species, the cell walls may be made of hemicellulose, pectic compounds, and glycoproteins. Green algae produce a variety of photosynthetic pigments: chlorophyll *a, b* and *c*, β-carotene, other

carotenes and xanthophylls.[29] The typical green algae stores short term energy products as sugars, starch and excess starches can be converted to TAGs.[30]

1.5 Green algae, diatoms and Cyanobacteria TAGs production

Green algae, diatoms and cyanobacteria, create TAGs on a glycerol backbone synthesizing three hydrocarbon chains. All three of the hydrocarbon chains can be any mix of single or double hydrogen bonds (saturated and unsaturated). The hydrocarbon chains can be of any length depending upon the cyanobacteria species that created the lipids. TAGs are compact stable hydrophobic lipid molecules with a melting temperature determined by the attached three fatty acid chains. Triglycerides with more saturated bonds have a higher melting temperature while triacylglycerols with more unsaturated bonds have a lower the melting temperature.[25, 31] When TAGs are transesterified, the three fatty acid chains detached from the glycerol backbone form fatty acid methyl esters (biodiesel).

1.5.1 Algal production for biodiesel

It is obvious that large raceway ponds and photobioreactors will grow and produce algal biomass for biodiesel, but the harvesting stage remain hidden behind the scenes, but is presented in this section.

The path to biodiesel starts with a halophilic algal community grown in a small brine water filled raceway pond with a paddle wheel moving the algal community around the serpentine waterway. When the algal concentration in the pond met a

specified biomass value, the entire pond was pumped into a flocculation tank where the algal water was pretreated with sodium methylate, heated and pH adjusted.

The algal biomass flocculates and dropped to the bottom of the tank where the excess water was returned to the pond. The algal flocculant was pumped to another tank where a non-polar solvent (hexane) was added to remove the neutral lipids from the flocculant. The entire flocculant with the neutral lipids was pumped into a tank half filled with water to separate out the neutral lipids in the non polar solvent from the algal biomass. The biomass was sent to a collection tank to be dried. The non-polar solvents and neutral lipids were run through a distillation tank with the no-polar solvent recovered for reuse and the neutral lipids (biocrude) sent into a secondary tank for later transesterification.

1.5.2 PURE CULTURES AND MIXED CULTURES

Pure algal cultures raised in the laboratory have difficulty transitioning to outdoor facilities for the commercial production of biodiesel because wild algae, grazers and pathogens easily contaminate them. Monocultures invaded by microbes and invertebrates are more susceptible to culture death from the invading species in less than a day. [32] Single specie cultures must adapt to fluctuating temperatures and one of the best survival traits would be to adapt to changing temperatures year round. The collapse of monocultures can stem from predation particularly if the monoculture specie is considered a preferred prey.

The advantages of mixed algal cultures stems from diversity traits and stability. The mixed algal cultures will exhibit these traits in several ways by

including ranges in cell diameters and different morphologies including filamentous and those with benthic tendencies. Smaller algal cell sizes may make predation a more difficult process and place pressure on incoming invasive species to find an algal niche population that will sustain their nutrient needs. Mixed algal cultures is less competitive for any one micronutrient and survive on lower concentrations of nitrates, phosphorous, other mineral and vitamins. The relationship between diversity and biodiesel productivity can be positive since the species assortment of species will weather more of the extremes in temperature, grazers and micronutrients. The diversity among the community causes invaders to spend time seeking a specie that they desire to consume, thus slowing down the loss of the culture overall. Mixed communities with lost species tend to have a replacement species available to fill the niche.[32]

1.5.3 Open (raceway), Closed (tube reactor) systems

Two common open pond systems for growing algae and they are raceway and circular ponds. Wild algae, grazers and pathogens mentioned above, contaminate raceway ponds easily. The raceway ponds are oval shaped or serpentine in shape and the channel are constructed of concrete with depths of 15-40 cm deep. To prevent sedimentation, a continuously turning paddle wheel, propeller, or air-lift pump agitates the algal culture. A velocity of 10 - 20 cm / sec keeps the algae dispersed and prevents them from attaching to surfaces. A cell concentration of up to 1 g / L (dry weight) yield of the for raceway pond.

Circular ponds are typically 45 meters in diameter with a bottom depth of 30 to 70 cms with a central rotating arm agitating and mixing the algae in water.

Energy consumption to run the paddle wheels, propellers and air lift pumps must be considered and weighed against the algal biodiesel energy consumed in the process. A raceway pond with 100 m^2 open surface area had a paddle wheel motor that consumed 600 watts compared against a 85 m^2 raceway pond using an air-lift pump that used 195 watts. The air-lift pump used less energy. Water evaporation can be a concern in raceway ponds, for 1 Ha of water (10,000 m^2) could lose 100,000 liters of water per day.[4]

Closed tube photobioreactors are transparent containers or vessels assembled to promote the light available for algal cells to grow in a tubular reactor with wall thicknesses of 2 - 4 cms. Photobioreactors have large surface to volume ratios and have the advantage of a totally enclosed reactor preventing contamination and providing planned productivity. Depending upon the design of the bioreactor they can be deployed in arid high light deserts or in urban areas where they are displayed as living walls. A desert-installed photobioreactor has a lower land foot print than a raceway pond, Photobioreactors capital costs are high, but the reactor size can have a small while still producing a large biomass nearly as large as a raceway pond. One benefit of the photobioreactor provides flexible and modular designs to allow for total process control. One disadvantage of the photobioreactor is the control of heat entering the tubes. Certain algal species are difficult to grow in raceways ponds and because of the controls added to a photobioreactor, very productive algae species can be grown to high biomass levels.[4]

Both the raceway open pond model and the closed photobioreactor models have advantages and disadvantages. The selection of the system depends entirely on the intended use of the reactor. [33]

1.6 Environmental effects on Algal lipid production

The main environmental challenges to algal lipid production are high light, photosynthetic active radiation (PAR), high temperatures, limited nitrogen, and high salinity medium. Green algae, diatoms and cyanobacteria, employ specific internal responses to environmental challenges to ensure their survival. One of the most common responses to environmental challenges is the conversion of short-term energy sources (glycogen, starches) into TAGs. The creation of TAGs expends large amounts of algal cellular energy, which stresses the green algae, diatoms and cyanobacteria by slowing or even curtailing biomass growth, cell division, and other lipid metabolisms.[34]

1.6.1 Light intensity

Sunlight initiates the algal photosynthesis cycle by biologically converting photon energy into electron energy in the chlorophyll molecule. The captured electron supplies the energy necessary to split atmospheric carbon dioxide into gaseous oxygen and carbon. The algal cell creates short term energy storage products from the carbon (sugars, glycogen, starches, oil bodies) for immediate use. The excess short term energy products are turned into fatty acids and

incorporated into triacylglycerols for long term storage. These triacylglycerols are vital for the creation of biodiesel.[30]

At night the green algae, diatoms and cyanobacteria enter a dark respiration cycle that shuts down the photosynthetic processes, and performs cellular maintenance of proteins, creates biomass, replaces macromolecules, regulates cell volume and repairs the motility apparatus. The dark activities require a small consumption of the stored energy products and small amounts of oxygen, while discharging carbon dioxide. [34]

When cultivating green algae, diatoms and cyanobacteria, the preferred algal light intensities drive quick algal growth producing large amounts of biomass, but do not injure the algal cell or impair the photosynthesis process. Depending upon the species of algae and growth conditions, the best light intensity can vary widely.[25]

Algal light harvesting antennae, Photosystem II and Photosystem I contain chlorophylls. In order to capture a larger part of the solar spectrum, chlorophyll *a* has two absorption bands: blue (430 nm) and red absorption (680 nm).

When intense light saturates algal cells, and the photons cannot be diverted through the cellular methods described earlier, the algal cell experiences photoinhibition which impairs carbon fixation and ultimately decreases the manufacture of lipid energy needed for TAGs.[35]

1.6.2 TEMPERATURE EFFECTS

Algal biochemical processes are extremely sensitive to environmental temperatures. Twelve algal strains when grown in a large study increased the

temperature over time to a temperature higher than their physiological temperature limits of each strain using temperatures 22 °C, 30 °C, and 35 °C. The twelve marine algal strains were kept at each temperature for 15 to 20 days. When the algal cells were counted, if they were statistically higher, then the temperature was increased to the next temperature. If the cell counts were not greater than they were initially, it was inferred that they could not adapt to the temperature.

The survival of the strains under the increasing temperatures was first attributed to physiological acclimation, then genetic mutation if the strains could not physiologically adapt to the higher temperatures and finally selection. The protocol estimated the maximum adaptation of the phytoplankton. Growing a number of generations of the algal strains often produced an adaptation to the higher temperatures. *Scenedesmus intermedius* successfully adapted from 22 °C to 40 °C increasing from an initial 15 generations at 22 °C to 135 at 40 °C. [36]

Algal immersed in a medium at high temperature will transfer heat into the algal cell through the cell wall. High and low temperatures affect most of the processes and components in the algal cell including: enzymes, biochemical interactions, photosynthesis, and cell membranes.

The optimum algal temperature occurs where the algal species grows rapidly producing biomass containing substantial lipids. The stress of hot or cold temperatures may suppress the photosynthetic rates and ultimately lower the algal culture lipid synthesis.[37]

The algal cell walls for both green algae and cyanobacteria respond rapidly to temperatures above and below their optimal temperatures. Algal cell walls react to high temperature by increasing the saturated lipids incorporated in the cell walls, which increases the cell wall stiffness. Conversely when the algal culture environment drops below the optimal temperature, the algal cell walls will incorporate unsaturated lipids promoting wall fluidity.[30]

1.6.3 NITROGEN CONCENTRATION EFFECTS

All algae create proteins from nitrogen, incorporating the proteins in cellular structures such as: cell walls, membranes, gas vesicles, chloroplasts, plastids, and thylakoid stacks. Algal communities typically consume nitrogen from the medium in the form of nitrate (NO_{3-}) or ammonia. At the onset of low nitrogen concentrations, when nitrogen is depleted in batch cultures, TAGs synthesis increases and is often called a long-term algal storage strategy.[38, 39]

If the algal community comes from freshwater or saltwater, the inclusion or exclusion of various medium constituents are suggested to generate algal biomass. Several of the minerals and vitamins often added to algal medium are phosphorous, nitrates, sodium, potassium, ammonium, calcium, iron, boron, and vitamins: B12, B1 and B7 biotin. Algal cultures require nitrogen sources to produce amino acids and proteins and algal medium typically contains nitrates or ammonia.

In the United States Department of Energy follow-up report, “A Look Back at the Aquatic Species Program, A Closeout Report” [38] the project researchers searched for the "lipid trigger" to initiate microalgae cells to store TAGs. The

Aquatic Species Program cultivated many species of algae and discovered, that while algae were stressed under nitrogen starvation, the algal cells increased TAGs production. Algae species under nitrogen limited culture conditions increase TAGs accumulation, but lowered overall cell division and growth. The lipid energy stored in the cells was diverted from the rest of the cellular processes. Biomass did not increase under nitrogen nutrient starvation.[38]

1.6.4 Salinity effects

Green algae, diatoms and cyanobacteria growing in high salinity environments are call halophytes. Halophytes immersed in high salinity media face external hydrostatic water pressure outside their cell walls, constantly. The internal algal cell pressure is called turgor pressure. The algal cells must keep a specific turgor pressure within the cell, which must match or exceed the external applied pressure to prevent loss of intracellular conditions: ionic composition, pH, and metabolite pools.[40] However, for the algal cell to grow larger, the internal pressure must increase to expand the cell volume.[41]

In addition to turgor pressure, the cell must control the ion gradients from ions: sodium, potassium, chloride, magnesium, calcium and sulphate, across the cell wall and membranes. When the algal cell controls the ionic channels and pores to change cell pressure to control the turgor pressure, the algal cell expends the least amount of cellular energy. [42] The algal cell regularly produces osmoregulatory substances called osmoticants to divert, control, and breakdown the influx of salinity. The production of osmoticants requires high inputs of cellular

energy, making the production of osmoticants less desirable than the lower energy methods for salinity control. Some types of algal cell osmoticants produced in the algal cell are: polyols: glycerol, mannitol, galactitol, sorbitol, sugars, maltose, and amino acids: proline.[41]

Green algae, when in a saline medium are particularly subject to continuous maintenance of the internal turgor pressure. Marine algal species, if tolerant to high salinity, will have a preferred salinity range where the marine species will survive.

1.6.5 METHODS FOR PRESERVATION

Preservation of algal cultures may be useful for replicating experiments and for repeated inoculations in industrial applications. Retaining the algal community in a frozen state can prevent culture changes and contamination to the algal community. Holding algal samples in a frozen cell bank provides a source for reconstituting viable algal populations.

Freeze drying, also known as lyophilization, is defined as a controllable method of dehydrating labile products by vacuum desiccation which has been used to store algae. Lyophylization begins by freezing the algal cells in water or suitable solution that will be sublimated initially during the freezing process. The freeze drying eventually retains only the algal cells in a desiccated state. Restoring the algae to a viable state must be done slowly to allow rehydration in a growth medium.[43, 44]

Freezing algae with compatible cryopreservants may increase the viability of the final reconstituted algal species. Green algae, diatoms and cyanobacteria

can be frozen at various temperatures with different cryoprotective additives (CPAs).[45] CPAs vary widely and include: DMSO (dimethylsulfoxide), glycerol, peptone, glucose, MEOH (methanol), sorbitol, ethylene glycol, sucrose, dextran and honey.

Specific CPAs that have rapid penetration of into green algae, diatoms and cyanobacteria within 30 minutes and these are DMSO, methanol, ethanol, ethylene glycol, and propylene glycol. DMSO and MEOH penetrate both the algal cell wall and cell membrane.[45, 46]

1.6.6 Benefits of algal diversity

Mixed algal communities exist in many environments. Greater algal diversity can increase the sustainability, biomass, and function within the cultures.[47] Much of the previous research on biodiesel production from algae has focused on monocultures.[48] Algal monocultures are often susceptible colony collapse due to competition for nutrients within the same species, excess light competition for light exposure, and predation by invasive species. Algal cultures with a planned diversity and a strong lipid producing algal specie can improve biodiesel production and stability.[49, 50]

A controlled algal diversity study asked whether there is a link between diversity and lipid production.[50] The study grew cultures of 22 algae species (from several culture collections) and 3 mixed natural phytoplankton samples from eight Bavarian lakes and ponds. The experiments were performed to determine whether different types of algal sources (culture collections or natural water bodies) and the number of species in the test cultures would affect the lipid

production. [50] Monocultures from a culture collection species were also grown alongside diversified culture collections over 7 days. The results for the monoculture culture collection algae lipid content were lower (3.26 x10^6 picogram (pg) mL^{-1}) when compared to the larger lipid content of the diverse culture collection 1.08×10^7 pg mL^{-1}.

Similarly, the natural algal samples from the ponds and lakes were cultured in the same way as the 22 algae culture collection species. The average algal lipid content from the lake phytoplankton community (species richness = 10) was lower (1.10 x10^5 pg mL^{-1}) when compared to the pond community (species richness = 19) containing (5.17 x10^6 pg mL^{-1}) lipid content. The study greater diversity contributed to the larger lipid production.[50]

Bacteria co-exist with algae in nature, where bacteria recycle algal waste products to basic level nutrients such a minerals, vitamins (B12) and carbon dioxide. Algal communities excrete carbon, oxygen and other metabolic byproducts, which bacteria readily consume. The bacteria break down the algal products to basic nutrients [51] with algae absorbing the bacterial nutrients, completing the biocycle.

1.7 RESEARCH NEEDS

The increasing demand for new fuel sources drives the need to look at sustainable options to supplement petroleum based diesel fuel. Biofuel sources like corn or soybean crops require three to five months to harvest, while an entire crop of microalgal biodiesel can be turned over in one or two weeks. [52, 53]

Growing interest in photoautotrophic microalgae has unlocked a surge of scientific research over the last decade focusing on their use for biofuels and biodiesel. The application of microalgae for green fuels holds promise, as the required resources and acreage for microalgae production are much smaller than other biofuel feedstocks like soybean and corn. Microalgae do not compete with food cropland and can exist in environments where most food crops cannot be grown such as deserts.[12]

Other microalgae research has genetically modified (GM) specific species to produce certain products or developed effects within the microalgae. The unintentional release of GM algae into the local biome can be a disaster when the algae can survive and then insert their GM genes into other algae with possible negative consequences.[54] The GM traits added to the algae to enhance their commercial viability frequently render the algae incompetent when competing with wild type species.

Natural mixed wild type algal communities have advantages for large scale algal production due to their greater robustness than that of a single species system and prior adaptation to challenging environments [50]. The biomass production by an algal community was reported to be higher and more stable than that of a single culture.[55]

A quote from the United States Government report, “A Look Back at the Aquatic Species Program, A Closeout Report”,[38] points to a compelling reason for the selection of a wild type (WT) algal community for research studies:

"An important lesson from the outdoor testing of algae production systems is the inability to maintain laboratory organisms in the field. Algal species that looked very promising when tested in the laboratory were not robust under conditions encountered in the field. In fact, the best approach for successful cultivation of a consistent species of algae was to allow a contaminant native to the area to take over." [38]

To date, most algal biofuel research has focused on monocultures, although monocultures are difficult to maintain in an outdoor setting where they are contaminated from aerial contamination by wild type strains, grazers and pathogens. [32] There has been little research on growth of algal mixed algal communities in saline media for biofuel production. As noted, water is often a limiting factor in phototroph production, and saline sources, such as produced water and saline groundwater, which are not suitable for agriculture or other uses, may be usable for phototroph production. There is therefore a research need to determine factors affecting phototrophic algal growth and lipid production in saline media, with an environmental (mixed algal community) inocula. Once a mixed algal community has proven successful in the field, the need to preserve the mixed algal community genetic composition intact becomes obvious. Preservation of the community algal species, can be met by a cryogenic freezing method. Freezing the mixed algal community in liquid nitrogen can hold the algal community protected from changing due to bacteria contamination. Reconstituting the frozen mixed algal culture in media, restores the algae viability and growth.

There is a need to research mixed algal communities for biodiesel and a need to understand how a mixed algal community diversity changes, and the best methods to grow and optimize their biodiesel output. The mixed algal communities should have origins from a location receiving intense sunshine, containing high saline water with nutrients capable of supporting several algal species. When harvested for research, the mixed algal community would have prior adaptation the intense environmental conditions.

1.7.1 WHAT IS UNKNOWN

There is a knowledge gap in the understanding of the benefits from growing a high saline mixed algal community culture for biofuel production. The reaction of the high saline mixed algal community to various challenging environmental conditions including temperature, a limited nitrogen source and freezing for preservation is unknown. Most algae research investigates single specie monocultures that have issues during scale-up to larger outdoor growing environments.

1.8 OBJECTIVES AND EXPERIMENTAL APPROACH

Objective 1 was to determine the effects of high and low temperatures on a highly saline algal community, including the types and quantities of Chl *a* and Chl

b produced, community diversity, and FAME content for biodiesel production. This objective was addressed in Experiment 1, Chapter 3.

Objective 2 was to determine the effects of limited nitrogen media compared to a higher nitrogen media on a high a highly saline algal community including the nitrogen effects on the quantities of Chl *a* and Chl *b* produced, community diversity, and FAME content for biodiesel production. This objective was addressed in Experiment 2, Chapter 4.

Objective 3 was to determine the effects of cold preservation at 4 temperatures of a highly saline algal community with two cryoprotective additives DMSO and MEOH. Agitated inoculum was added to one seed reactor and the second seed reactor received undisturbed inocula. The effects of agitation on the algal cultures before and after freezing will be assessed and compared to algal cultures with non- agitated inoculum. After freezing algae cultures in 32 tubes, the thawed algal cultures were added to (32) 50 mL reactors with 30 mL of brine media and 50 mL cultures and grown. The initial results included cataloging the viable algal growth after freezing, calculating the Chl *a*, and identified highly saline algal community genera present after re. This objective was addressed in Experiment 3, Chapter 5.

The methods, materials and analytical measurements used in the three experiments are described in Chapter 2. Some of the methods are shared between experiments 1, 2 and 3.

Experimental Approach, Experiment 1: The experimental system used in Experiment 1 were batch reactors (500 mL flasks), which were illuminated and monitored for 14 days. Total FAMEs mg / L concentrations (biodiesel) were quantified each day. These systems were inoculated with a NM algae community taken from a brine pond located in an environment with unobstructed high intensity sunlight (San Acacia Brine Pond, Soccoro, NM).

Experimental Approach, Experiment 2: The experimental systems used in Experiment 2 were batch reactors (500 mL flasks) which were illuminated and monitored for 0 to 12 days. Total FAMEs mg / L concentrations (biodiesel) were quantified each day. These systems were inoculated with a NM algae community taken from a brine pond located in an environment with unobstructed high intensity sunlight (San Acacia Brine Pond, Soccoro, NM).

Experimental Approach, Experiment 3: The experimental systems used in Experiment 3 were 32 batch reactors (50 mL flasks with media) which were illuminated and monitored for days 0 to 13. These systems were inoculated with a NM mixed algal community taken from a brine pond located in an environment with unobstructed high intensity sunlight.(San Acacia Brine Pond, Soccoro, NM) The 2 seed cultures were mixed with 2 different CPAs and frozen at 4 temperatures. After reconstituting the culture, the algae were analyzed for viability and genera counts.

Prior to the start of the first study, a native WT NM algal community was pulled from a natural brine pond located in an environment under high temperature with unobstructed high intensity sunlight, and living in high saline brine.

Chapter 2 : Methods, Materials and Analytical Measurements

2.1 EXPERIMENT 1 : INOCULUM

The xenic algal community (inoculum) was obtained from a non-shaded brine pond (>35,000 TDS mg /L of brine) [56] in the Sevilleta National Wildlife Refuge located in San Acacia, New Mexico (NM)**,** Figure 2.1. A synthetic brine medium was formulated to match the high saline composition of the San Acacia Brine Pond, which was based upon a master's thesis that recorded the San Acacia Brine Pond mineral composition, Table 2.5. [1, 2]

Figure 2.1 San Acacia Brine Pond
San Acacia Brine Pond, San Acacia, NM, is located in the Sevilleta National Wildlife Refuge. The San Acacia Mesa to the left of the pond feeds minerals to the pond from underneath. The San Acacia Diversion, a branch from the Rio Grande, does not provide any water to the brine pond. This is the origin of the research algal culture. The X designates the location where the San Acacia Mixed Algal community were harvested.

To create the first generation seed culture, 1 mL of the original San Acacia xenic NM algal community was added to 75 mLs of the synthetic brine medium in

a 250 mL reactor (an Erlenmeyer flask). Three first generation seed reactors were created at the same time. The first generation seed culture reactors were positioned on a VWR orbital shaker table, Model 3500, Cat No. 89032-096, (150 rpm) illuminated by (4) T5 Plusrite cool white fluorescent lights at room temperature located on a laboratory table. These first generation seed culture reactors supplied the inoculum for all 3 Experiments.

Over time, the first generation reactors were split periodically into two reactors, by pouring 1/2 of the original reactor into a new autoclaved reactor. Then both reactors received sufficient new media to attain the original 75 mL volume. Both seed culture reactors were placed back on the shaker table to continue growing.

2.2 EXPERIMENT 1 : DESIGN AND OPERATION

Experiment 1 consisted of two studies: RT (room temperature) and HT (high temperature). The Experiment 1 RT study was performed first, over a 14-day period and later, the Experiment 1 HT study was executed afterwards over 14 days.

Each of the RT and HT reactor sets consisted of 6 reactors divided into two three-reactor sets**,** Figure 2.2. The 500 mL Erlenmeyer flask reactor contained 250 mL of medium. Each of the six reactors were inoculated with 3.3 mL seed culture described first generation inoculum section above. At the beginning of each study, immediately after inoculation (9)1 mL samples were taken from the first generation seed reactor and frozen at -20 °C for 454 pyrosequencing

analysis. The 9 seed reactor samples were taken to determine what genera were present in the first generation algal community

Both reactor sets were incubated in a temperature controlled incubator set at 25 °C for the RT flasks, and 40 °C for the HT flasks (more temperature details in section 2.2.2 below). The flasks were placed on an orbital shaker table, set at 150 rpm and illuminated by cool white fluorescent lights with more detail found in Section 2.3.1, below. The Experiment 1 reactors sets were operated as batch reactors inside the temperature-controlled incubator.

The Experiment 1 reactor sets 1 and 2 were sampled alternately every second day per Table 2.1. For both of the Experiment 1 studies (RT and HT), the reactor set 1 sampling occurred on days: 2, 6, 10, and 14, and for reactor set 2, the sampling was taken on days: 4, 8, and 12. The two sets of 3-reactors were placed next to the cool white fluorescent bulbs, Figure 2.2. The samples were removed from the batch cultures without replacement with fresh media, so the reactor volumes decreased over the course of the experiments.

2.2.1 EXPERIMENT 1 : DAILY SAMPLING

The 6 reactors used in each of the RT and HT studies were divided into sets of 3, which were each sampled every 4 days on a staggered schedule (set one on days 2, 6, 10, and 14, set two on days 4, 8, and 12). The constituents that were tested and the associated sample volumes are shown in Table 2.1.

Table 2.1 lists the Experiment 1 samples taken for each parameter. Samples were taken from RT and HT cultures from reactor set 1 or reactor set 2 on the sample day. The average, standard deviation, standard error of the mean and

test for significance between to sets of sample data were calculated for each of the (3) experiments except for data from algal community populations analysis.

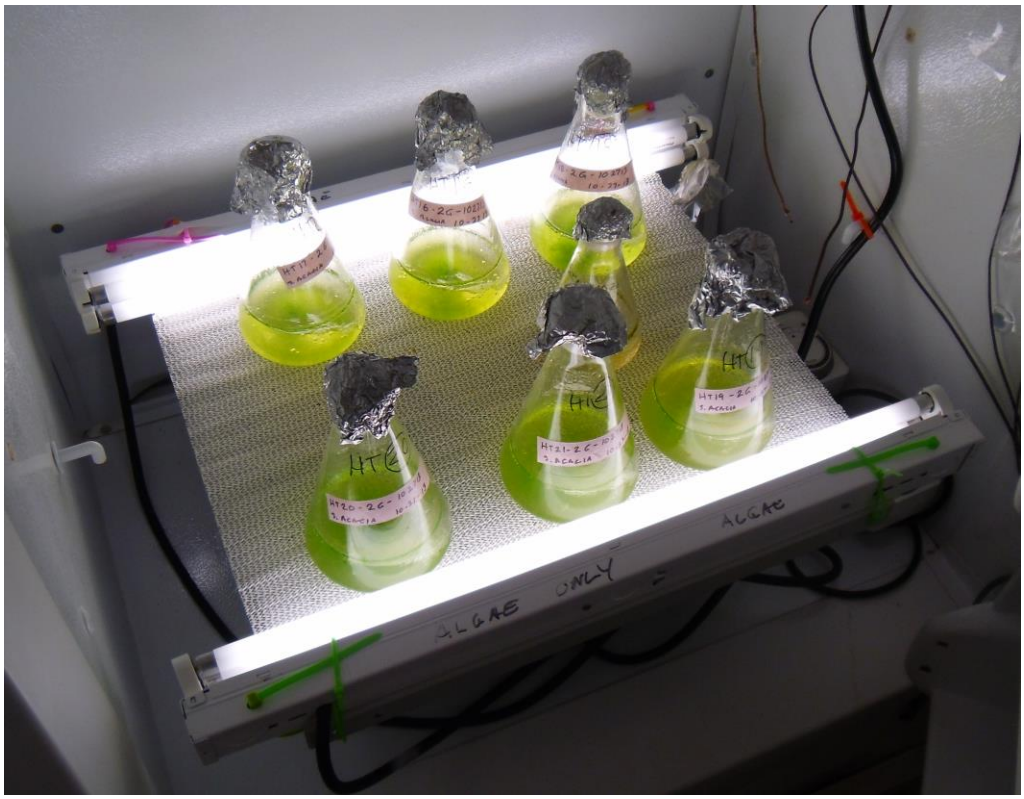

Figure 2.2 Two 3-reactor sets on shaker table inside of incubator

Table 2.1 Experiment 1 sample parameters, number of samples and volume per reactor.

Parameters evaluated from samples taken on sampling day	Sample volume per each reactor volume mL	Samples per each reactor per day	Total volume sampled for three reactors
Frozen samples for DNA pyrosequencing	1.0 mL for DNA pyrosequencing	3 DNA samples per reactor	9 mLs
Frozen samples for NO_3 mg/L NO_3 mg/L	1.0 mL for NO_3	1 NO_3 frozen sample per reactor	3 mLs frozen for NO_3 mg/L
Samples for Chl *a* and *b* mg/L	3.0 mL	3	9.0 mLs
FAMEs for identification and concentration mg/L	10 mL	3	30.0 mLs
Algal images on hemocytometer	1.0 mL	3	3.0 mLs

2.2.2 Experiment 1 : culture temperatures

During the Experiment 1, the RT study culture 2 reactor sets were maintained at 25 °C light phase / 20 °C dark phase in the incubator and in the HT study, the culture reactor sets were maintained at 40 °C light phase / 35 °C dark phase.

2.3 Experiment 1 : Methods

2.3.1 Experiment 1 : Lighting hours, shaker table, and PAR intensity

All 3 Experiments used these parameters for growing the algal cultures inside a Fisher Scientific Incubator, Model 307C. Reactors were exposed to 14.5 hours of light and 9.5 hours of darkness while continuously mixed on a VWR orbital shaker table Model DS-500, 57018-754, rotating at 150 revolution per minute (RPM). Two sets of stacked (4) F14 T5 Plusrite cool white fluorescent lights were mounted on both sides of the shaker table to insure equal lighting of all reactors. All the reactors were positioned 6.4 mm from the light source, which provided an average PAR level of 1400 µmol photons m-2 s-1, which is approximately 87% of the 2014 summer average daily PAR (1602 µmol photons m-2 s-1) in Albuquerque, New Mexico. [57, 58] The PAR measurements were taken at 6.4mm distance from the cool white fluorescent bulbs along their length using a Spectrum Technologies Field Scout Light Sensor Reader, 3415FX, with the attached Quantum Light Sensor, 36681.

2.3.2 Experiment 1 : Chlorophyll *a* and *b* concentration

Chl *a* and Chl *b* concentrations (µg / mL), were extracted in methanol from a 3 mL algal sample. The absorbance was measured at three wavelengths: 652.0,

665.2 and 750.0 nm with a Cary 50 UV-Visible Spectrophotometer in 3 mL quartz cuvettes. The calculation for the Chl *a* and Chl *b* concentration (μg / mL methanol) applied the three absorbance values to the 3 equations listed in the publication. [59]

2.3.3 EXPERIMENT 1 : SYNTHETIC NITRATE BRINE MEDIUM

Instant Ocean, a commercial saltwater mixture, was added to 0.95 liter of Milli-Q water, creating a seawater base for the synthetic medium. The nitrate brine medium chemicals are listed in Table 2.5. The media contained no carbon source.

Table 2.2 Experiment 1 synthetic brine medium with nitrate and chemicals

Contains 179.2 mg nitrate / L

No.	Concentration mg / L of medium	Chemical	No.	Concentration mg / L of medium	Chemical
1	35000	Instant Ocean	11	0.06	Manganous chloride, 4-hydrate
2	170	Boric acid	12	0.73	Sodium molybdate dihydrate
3	1100	Calcium chloride dihydrate	13	0.025	Biotin
4	3.16	EDTA disodium salt dihydrate, 0.1M volumetric solution	14	0.135	Vitamin B12
5	84.99	Sodium nitrate	15	200	Strontium nitrate
6	0.05	Cobalt nitrate hexahydrate	16	57	Lithium chloride
7	0.09	Cupric sulfate pentahydrate	17	22	Potassium bromide
8	0.58	Zinc sulfide heptahydrate	18	10	Calcium sulfate
9	80	Potassium phosphate monobasic	19	13	Ammonium iron (III) citrate
10	1.10	Vitamin B1			

Three other dry salts, four mineral solutions, and one vitamin solution were added to the 0.95 L of medium base and brought up to 1 L with Milli-Q water to complete the medium. The medium pH was adjusted to 8.4 with potassium hydroxide (KOH) before and after autoclaving. The medium was stored at 4 ºC and warmed to room temperature before use. The medium contained 179.2 mg NO_{3-} per liter.

2.3.4 EXPERIMENT 1 : FAMES EXTRACTION, TRANSESTERIFICATION AND GAS CHROMATOGRAPH QUANTIFICATION

Samples were taken from RT and HT cultures from reactor set 1 or reactor set 2 on the sample day. FAME concentrations were determined as follows: from the Table 2.1, the row labeled "FAMEs for identification and concentration" 10 mL sample, was added to a 15 mL centrifuge tube. The 10 mL algal sample was centrifuged into a pellet. The algal sample pellet was extracted with a solution of methanol, chloroform, and formic acid (20:10:1 v/v/v) and vigorously shaken for 5 minutes. After shaking, the extraction solution (0.2M phosphoric acid and 1M potassium chloride salt solution) was added to algal cells and lightly vortexed, then centrifuged to separate the mixture into water and chloroform layers. The algal fatty acids were dissolved in the chloroform phase at the bottom. [60]

The bottom chloroform phase was removed and 0.3 mg pentadecanoic acid as an internal standard (Sigma, C15:0, P6125) C15:0 (150 uL of 2.0 mg C15:0 /1 mL hexane) was added to a glass tube and dried. A solution of methanol and 8% HCl w/v was added to the tube and sealed with a Teflon line cap. The tube was held in water bath at 45 ºC for 14 hours. [61]

The FAMEs were extracted from the transesterification solution by adding 1 mL of hexane and 1 mL of water and the glass tube was gently shaken. The hexane upper layer, containing the FAMEs, was pipetted into a 1.7 mL septum capped glass vial and stored at -20 ºC. FAMEs were measured using a gas chromatograph (Shimadzu 2010) with a J&W, DB-FFAP column: 30M length inner diameter of 0.25 – 0.32 mm, and a 0.25 – 1.00 µm thick nitroterephthalic-acid-modified polyethylene glycol (PEG) film. The column temperature limits were (°C) 40 – 250 °C.

The calibration standard was formulated with the Supelco FAMEs 37 Mix (18919-1AMP) in hexane at six dilutions. The gas chromatograph resolved each identified FAME concentration to a resolution of 0.01 mg FAMEs / L / d. Only the experiment samples run on the gas chromatograph that matched the peaks of the external standard FAMEs 37 Mix were analyzed for the experimental FAMEs data.

2.4 Experiment 1 : Analysis

2.4.1 Experiment 1 : Algal community analysis

The room temperature (RT) and high temperature (HT) culture algal communities were characterized by 454 pyrosequencing of 23s ribosomal DNA (rDNA) genes. The DNA was extracted from two frozen algal culture samples for each study day. The DNA purity was assessed by the nucleic acid $A_{260/280}$ absorbance ratio [62] on a NanoDrop spectrophotometer (Thermo Scientific, ND-1000), with a minimum ratio of 1.8 and the sample with the best nucleic acid and

absorbance ratio was sent for analysis. The Roche 454 pyrosequencing was performed by Research and Testing Laboratory, Lubbock, Texas (RTL) using the plastid 23s rDNA forward primer p23SrV_f1 (5' GGA CAG AAA GAC CCT ATG AA 3') and reverse primer p23SrV_r1 (5' TCA GCC TGT TAT CCC TAG AG 3') [62] which occur in the Eukaryota, Plantae, and Cyanobacteria algal populations [63]. The average read length was 403 (RT) and 396 (HT) base pairs per sample. The sequences were aligned against an algal rDNA proprietary nucleotide database compiled by RTL. The RTL minimum base pairs (bp) read length was 250 which is one-half of the maximum read length of the GS Flex + 454 Titanium [64]**.** The minimum RT and HT read lengths were both 250 bp. The maximum RT and HT read lengths were 770 bp and 653 bp respectively. RTL reported taxonomy and counts for each submitted DNA sample.

2.4.2 EXPERIMENT 1 : ANALYSIS OF NITRATE CONCENTRATION

The method for determining the nitrate (NO_{3-}) concentration in the medium was adapted from the Standard Method 4500-NO_{3-} B. [65] The algal culture samples were filtered (0.2 µm pore size), diluted with Mill-Q water (1:100), centrifuged to collapse microbubbles (5000 rpm, 2 minutes), and measured on a Cary 50- UV-Visible spectrophotometer at two absorbance wavelengths, 220 nm and 275 nm. The NO_{3-} concentration calibration was based on 6 NO_{3-} standard concentrations prepared from the 1000X NO_{3-} concentration solution mixed in the no-nitrate synthetic brine medium and a negative no-nitrate medium control verified a negative NO_{3-} measurement.

2.4.3 Experiment 1 : Analysis of algal culture images

On every sampling day, multiple images were taken of the Experiment 1 RT and HT algal cultures. Each algal culture image was taken at either 100X or 400X magnification on an Olympus BX51 microscope with a microscope top mounted Olympus digital camera (DP71). The algal images were taken on a hemocytometer to provide an algal cell reference size. Occasionally an image was taken at 1000X magnification to distinguish internal algal organelles. On the sampling day, each of the three reactors were imaged. The images were analyzed later to determine types of green algae, diatoms and cyanobacteria present.[18]

2.5 Experiment 2 : Inoculum

2.5.1 Same as Experiment 1, Section 2.1 Inoculum

2.6 Experiment 2 : Design and operation

2.6.1 Experiment 2 culture daily sampling

Experiment 2 had two studies: the HN (high nitrate) and LN (low nitrate). The HN study was performed first over a 12-day period, and the LN study was executed later over a separate 12-day period.

At the start of Experiment 2 in each study, (9) 1 mL samples were taken from the first generation seed reactor and immediately frozen at -20 °C for Illumina Miseq analysis and population evaluation described below in section 2.7.4.

The Experiment 2 design for the each of the LN and HN studies contained 6 reactors (500 mL reactors, 300 mL of medium, 3.3 mL inoculum), divided into two 3-reactor sets**,** Figure 2.2. Each of the six reactors were inoculated with the previously described first generation NM algal community reactor in section 2.1. The Experiment 2 reactors sets were operated as batch reactors inside a temperature-controlled incubator, placed on an orbital shaker table at 150 RPM, and illuminated with 4 cool white fluorescent bulbs as described with more detail in Section 2.3.1.The samples were removed from the batch cultures without replacement with fresh media, so the reactor volumes decreased over the course of the experiments.

Table 2.3 Experiment 2 LN and HN sampling parameters, number and volume per reactor.

Parameters evaluated from samples taken on sampling day	**Sample volume per each reactor volume mL**	**Samples per each reactor per day**	**Total volume sampled for three reactors**
Frozen samples for DNA pyrosequencing	1.0 mL for DNA pyrosequencing	3 DNA samples per reactor	9 mLs
Fresh samples for NO_3 mg/L NO_3 mg/L	1.0 mL for NO_3	1 NO_3 frozen sample per reactor	3 mLs frozen for NO_3 mg/L
Samples for Chl *a* and *b* mg/L	3.0 mL	3	9.0 mLs
FAMEs for identification and concentration mg/L	10 mL	3	30.0 mLs
Algal images on hemocytometer	1.0 mL	3	3.0 mLs

The sampling schedule for Experiment 2 HN study, reactor set 1, occurred on days: 1, 3, 5, 7, 9, 11, and for HN reactor set 2, the samples were taken on days: 2, 4, 6, 8, 10, 12. In the LN study the reactor set 3 sampling occurred on days: 1, 3, 5, 7, 9, 11, and for reactor set 4, the samples were taken on days: 2, 4, 6, 8,

10, 12. Table 2.3 lists the LN and HN sampling day plan details for each reactor set.

2.7 Experiment 2 : Methods

2.7.1 Experiment 2 : Lighting hours, shaker table, and PAR intensity

Same as Experiment 1 Section 2.3.1 Lighting hours, shaker table, and PAR intensity

2.7.2 Experiment 2 : Chlorophyll a and b concentration

Same as Experiment 1, Section 2.3.2 Chlorophyll *a* and *b* concentration

2.7.3 Experiment 2 : LN and HN culture temperatures

Both the LN and HN study maintained 28 °C light phase / 23 °C dark phase in the incubator and all culture reactor sets were held at the study temperatures for the entire study. The LN and HN temperatures selected represent a better fit to the optimum temperature ranges of the algal community to prevent adverse reactions at low or high temperatures in the LN and HN cultures.

2.7.4 Experiment 2 : LN and HN no-nitrate medium base

To know the exact NO_{3-} concentration in the Experiment 2 medium required a method to create a medium without any NO_{3-} (no-nitrate medium, Table 2.4) for both studies, where the balance of the medium constituents remained the same as the medium in the Experiment 1 studies.

The (LN and HN) no-nitrate synthesized brine medium base, Table 2.4, has the same chemical constituents as the RT and HT media, except the 3 NO_{3-}

compounds (sodium nitrate, strontium nitrate and cobalt nitrate hexahydrate) which were removed and placed into a separate single 1000X NO_{3-} concentrated solution, Table 2.4.

Table 2.4 Experiment 2 Synthetic no-nitrate brine medium and chemicals

(The no-nitrate medium contained 0 mg nitrate / L)

No.	Concentration mg / L of	Chemical	No.	Concentration mg / L of	Chemical
1	35000	Instant Ocean	9	0.73	Sodium molybdate dihydrate
2	170	Boric acid	10	0.025	Biotin
3	1100	Calcium chloride dihydrate	11	0.135	Vitamin B12
4	3.16	EDTA disodium salt dihydrate, 0.1M	12	57.0	Lithium chloride
5	0.09	Cupric sulfate pentahydrate	13	22.0	Potassium bromide
6	0.58	Zinc sulfide heptahydrate	14	10.0	Calcium sulfate
7	1.10	Vitamin B1	15	13.0	Ammonium iron (III) citrate
8	0.059	Manganous chloride, 4-hydrate	16	80.0	Potassium phosphate monobasic

Table 2.5 1000X Nitrate solution

1mL of the 1000X Nitrate Solution added to 0.999 liter of no-nitrate medium produces a concentration of 179.2 NO_{3-} mg / L, HN medium.

Component Number	Solution Concentration 1 mg /mL of medium	Nitrate mg/mL of medium	Chemical
1NS	84.99	62.001	Sodium nitrate
2NS	0.050	0.021	Cobalt nitrate hexahydrate
3NS	200.00	117.187	Strontium nitrate
Totals	**285.04**	**179.2**	

One mL of the 1000X NO_{3-} solution added to 0.999 liter of the HN no-nitrate medium produced an exact concentration of 179.2 NO_{3-} mg per liter of medium. The LN medium contained 75.0 mg of NO_{3-} per liter and was created by adding 0.419 mL of the NO_{3-}1000X solution to 0.9996 liter of the no-nitrate medium.

2.7.5 EXPERIMENT 2 : FAMES EXTRACTION AND QUANTIFICATION

Same as Experiment 1, Section 2.3.4

2.8 EXPERIMENT 2 : ANALYSIS

2.8.1 EXPERIMENT 2, LN AND HN ALGAL COMMUNITY ANALYSIS

LN culture frozen algal samples were pulled from the -20 °C freezer and sent to RTL for DNA extraction and sequencing by synthesis on the Illumina MiSeq. The Illumina MiSeq system applied the same rDNA 23s plastid primers [63] as those used in Experiments 1 and 3 algal community analysis.

The Illumina MiSeq performed the sequencing by bridge amplification with the results presented in read files. The two double end read files were demultiplexed and produced two double end paired sequences files in the FASTQ format. The demultiplexed paired end reads were split into two FASTQ files, with the forward primer FASTQ file stored the first half of the paired end reads and the reverse primer FASTQ stored the second half of the paired end reads. Both LN and HN paired end reads FASTQ files (first half) had an average 250 base pairs and the (second half) had an average of 250 base pairs. RTL compared the FASTQ base

pair files to an internal proprietary algal sequence database and supplied the taxonomy and counts for each of the ten algal samples.

2.8.2 EXPERIMENT 2 : LN AND HN COMMUNITY ANALYSIS FROM IMAGES

Microscopic examination of cell morphologies was applied to estimate quantities of particular groups of microorganisms. The genera counts per mL of culture in Figure 4.1, LN (4.1A) and HN (4.1B), came from 100X magnification microscope captured images taken from each of the three reactor cultures on each sample day. The Olympus BX51 microscope focused on a glass hemocytometer with a finely etched dimensional grids and 100X recorded two images grid squares from each of the three culture reactors. The hemocytomer 1 mm^2 square multiplied by the depth of 0.1 mm represents a 0.1 mm^3 volume between the coverslip to the lower etched hemocytomer surface where the algae settle. The algal cell length and width can be measured against the etched hemocytomer dimensions and algal cell counts per mL were calculated over the 1 mm^3 volume.

A free interactive segmentation software program, ILastik (version 0.5.12) which classifies different algal features and places them on different layers. using a random forest classifier. Each ILastik layer was turned into a black and white image and run on an ImageJ macro program, which determined the algal cell counts, size and cross-sectional area. Algal cell counts per mL were calculated with the data from the ImageJ results and the hemocytometer grid size. The ILastik program provides a method for training so that similar images can be

batch processed with the first Ilastik file layer results. A Image-J macro converted the Ilastik layers into filled outlines of the algal cell and counted them.

2.9 Experiment 3 : Inoculum

2.9.1 Experiment 3 : NB and BB reactors inoculum

Two reactors 250 mL reactors containing 100 mL of the Experiment 1 synthetic brine medium were inoculated with the first generation NM algal community source described previously (Section 2.1). The green algae, diatoms and cyanobacteria cultures were grown, frozen with CPAs, and reconstituted to determine the surviving algal genera present.

2.10 Experiment 3 :Design and Operation

One mL of the first generation inoculum NM algal community algal seed reactor inoculum (Section 2.1) the first reactor and was labeled NB Figure 2.3. The second 1 mL of inoculum, from the same first generation seed reactor, was agitated in a bead beater (no beads) at 2500 rpm for 2 minutes prior to adding to the second 250 mL reactor. The second reactor was labeled BB Figure 2.3. After 6 days of growth, (8)1 mL samples were taken from both of the NB and BB reactors and placed separately in two labeled plastic bags, and put in the -20 upright freezer for DNA evaluation of the algal seed reactors by pyrosequencing.

2.11 Experiment 3 : Methods

2.11.1 Experiment 3 : NB and BB temperature and growth conditions

Both of the inoculated NB and BB study reactors were placed in an upright temperature controlled incubator held at 25 °C light phase / 20 °C dark phase to grow. The NB and BB 250 mL reactors were placed on a rotary shaker table rotating at 150 rpm. The fluorescent bulbs had the same PAR value as stated in section 2.3.1.

During the 6 days of growth, on days 1, 4 and 6, the Chl *a* and Chl *b* were measured to determine if there was significant difference between the non-agitated inoculum and the agitated inoculum. (8)1 mL samples were taken from both of the NB and BB reactors and placed in two separate plastic bags labeled "NB" and "BB". The labeled bags were put in the -20 upright freezer for DNA evaluation of the algal seed reactors by pyrosequencing.

2.11.2 EXPERIMENT 3 : CHLOROPHYLL *A* AND *B* CONCENTRATION

Same as Experiment 1, Section 2.3.2 Inoculum

On day 7, the NB and BB inocula cultures were removed from the incubator to supply the algal seed culture for mixing with the DMSO and MEOH on a volume percentage of the final volume total, just prior to freezing, as described below in Tables 2.6 and 2.7.

2.11.3 EXPERIMENT 3 : SPECIFIC EXPERIMENTAL DESIGN

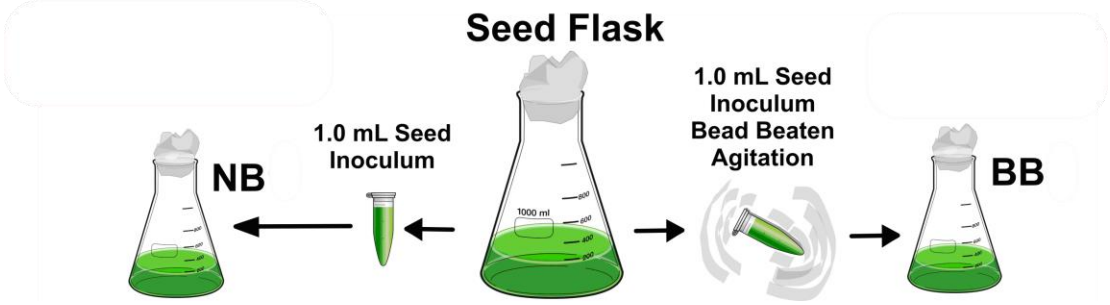

Figure 2.3 Experiment 3 NB and BB inoculum without and with agitation.
The NB and BB seed reactor source was a first generation NM algal community.

2.11.4 EXPERIMENT 3 : DMSO AND MEOH 5% AND 10% SOLUTIONS

Eight 15 mL centrifuge tubes were prepared to create the CPA percentages for freezing down with NB and BB NM algal community cultures. Four 15 mL NB tubes were labeled (NB: DMSO 5%, NB: DMSO 10%, NB: MEOH 5%, NB: MEOH 10%). Each of the four tubes were filled with the corresponding NB algal culture volumes listed in Table 2.6 and then loaded with the Table 2.7 NB DMSO or MEOH volumes corresponding to 5% DMSO and 5% MEOH or 10% DMSO and 10% MEOH.

The four BB tubes were labeled (BB: DMSO 5%, BB: DMSO 10%, BB: MEOH 5%, BB: MEOH 10%) and filled with the corresponding Table 2.6 BB algal culture and DMSO or MEOH volumes.

After filling all eight 15 mL centrifuge tubes with the DMSO or MEOH and NM algal cultures, the tubes were gently rocked manually to mix the DMSO or MEOH with the algal cultures. Once mixed, the 0 °C and -20 °C NB and BB DMSO or MEOH/ algal cultures were pipetted into 16 pre-labeled 1.7 mL microcentrifuge

tubes per Table 2.7 and placed immediately in the corresponding 0 °C or -20 °C freezer, Figure 2.4.

The NB and BB, -80 °C and -196 °C mixed DMSO or MEOH with algal cultures were pipetted per Table 2.7 into the 16 pre-labeled cryovials and then placed into a freeze-down container (Mr. Frosty, Nalgene, 1°C /min freezing rate) for passive cool down at -80 °C for 4 hours, Figure 2.5.

2.11.5 EXPERIMENT 3 : FREEZING EQUIPMENT

Three freezers and a liquid nitrogen dewar held the frozen algal culture microcentrifuge tubes and cryovials mixed with DMSO or MEOH. The three freezers were: a standard household refrigerator with a 0 °C top freezer; a -20 °C was a standard upright Electrolux freezer, Model FFH17F7HWC, and a top load - 80 °C VWR laboratory freezer, Model 5608. The -196 °C cryovials were immersed in a 30 liter liquid nitrogen Union Carbide, model LR-30 liquid nitrogen dewar

Table 2.6 Experiment 3 Matrix NB and BB algal cultures mixed with DMSO or MEOH percentages

NB Culture	CPA DMSO 5% Volume mL	CPA DMSO 10% Volume mL	CPA MEOH 5% Volume mL	CPA MEOH 10% Volume mL
0 °C	X	X	X	X
-20 °C	X	X	X	X
-80 °C	X	X	X	X
-196 °C	X	X	X	X
BB Culture	**CPA DMSO 5% Volume mL**	**CPA DMSO 10% Volume mL**	**CPA MEOH 5% Volume mL**	**CPA MEOH 10% Volume mL**
0 °C	X	X	X	X
-20 °C	X	X	X	X
-80 °C	X	X	X	X
-196 °C	X	X	X	X

Table 2.7 Experiment 3 NB and BB algal cultures mixed with DMSO or MEOH volumes

The DMSO or MEOH were added to the 15 mL centrifuge tubes (4) NB tubes and (4)BB.

NB Culture	DMSO 5% Volume mL	DMSO 10% Volume mL	MEOH 5% Volume mL	MEOH 10% Volume mL
CPA Volume	0.35	0.70	0.35	0.70
Algal Culture Volume	6.65	6.30	6.65	6.30
Total Volume mL	7.0	7.0	7.0	7.0
BB Culture	**DMSO 5% Volume mL**	**DMSO 10% Volume mL**	**MEOH 5% Volume mL**	**MEOH 10% Volume mL**
CPA Volume	0.35	0.70	0.35	0.70
Algal Culture Volume	6.65	6.30	6.65	6.30
Total Volume mL	7.0	7.0	7.0	7.0

Table 2.8 Experiment 3 NB and BB freezing tube / cryovial volumes

The filling sources are the (8) 15 mL tubes containing the DMSO or MEOH mixed with the NM algal community. All microcentrifuge tubes 1.7 mL and 2.0 mL cryovials were labeled with NB or BB, freezing temperature, DMSO or MEOH and percentage.

NB Culture Freezing Temperature	DMSO 5% + Algae Mix mL	DMSO 10% + Algae Mix mL	MEOH 5% + Algae Mix mL	MEOH 10% + Algae Mix mL
0 °C 1 .7 mL tube	1.5	1.5	1.5	1.5
-20 °C 1.7 mL tube	1.5	1.5	1.5	1.5
-80 °C 2.0 mL Cryovial	2.0	2.0	2.0	2.0
-196 °C 2.0 mL Cryovial	2.0	2.0	2.0	2.0
BB Culture Freezing Temperature	**DMSO 5% + Algae Mix mL**	**DMSO 10% + Algae Mix mL**	**MEOH 5% + Algae Mix mL**	**MEOH 10% + Algae Mix mL**
0 °C 1 .7 mL tube	1.5	1.5	1.5	1.5
-20 °C 1.7 mL tube	1.5	1.5	1.5	1.5
-80 °C 2.0 mL Cryovial	2.0	2.0	2.0	2.0
-196 °C 2.0 mL Cryovial	2.0	2.0	2.0	2.0

After four hours, the (8) -80 °C frozen cryovials stayed in the Mr. Frosty freezing container, while the (8) -196 °C cryovials were removed from the Mr.

Frosty container and were lowered into the liquid nitrogen (LQN) dewar, Figure 2.5.

The frozen DMSO or MEOH / algal mix microcentrifuge tubes and cryovials were held for one week at their freezing temperatures, and then thawed per the procedure below.

2.11.6 Experiment 3 thawing (0 °C, -20 °C) tubes, and (-80 °C, 196 °C) 2.0 mL cryovials

1. The water in a shaking incubator was pre-warmed to 37 °C. The frozen algal / DMSO or MEOH microcentrifuge tubes and cryovials were inserted in the 37 °C water bath to gently agitate during thawing and left in the water bath until all ice had melted (generally under 2 minutes).
2. The 0 °C and -20 °C thawed algal / DMSO or MEOH microcentrifuge tubes were centrifuged at 2000 rpm for 2 minutes to pellet the green algae, diatoms and cyanobacteria and then a pipette removed the supernatant. Proceed to step 4.
3. The -80 °C and -196 °C thawed cryovials contents were immediately transferred to a 2.0 mL microcentrifuge tube. The 2.0 mL algal / DMSO or MEOH microcentrifuge tube was centrifuged at 2000 rpm for 2 minutes to pellet the green algae, diatoms and cyanobacteria and the supernatant was removed by pipette. Proceed to step 4.
4. Each algal mix in a 1.7 ml tube or 2.0 mL tube was filled with fresh room temperature Experiment 1 synthetic brine medium (section 2.4.2) equal to

the volume of the original algal culture / DMSO or MEOH mix volume (Table 2.7). The tubes were rocked twice manually to mix and resuspended the pellet in the new medium, diluting the DMSO or MEOH. The tube with the resuspended pellet was left undisturbed for 2 minutes. After the wait period, the algal tubes were centrifuged at 2000 rpm for 2 minutes again to pellet the algae and the supernatant liquid was removed as before.

5. Fresh room temperature Experiment1 synthetic brine medium was added to the algal tubes again equal to the original amounts amount added in step 4 and gently rocked manually to resuspend the algae pellet.

6. For each labeled microcentrifuge tube, 1 mL of the suspended algal pellet was transferred to 30 mL of Experiment1 synthetic brine medium in the corresponding pre-labeled 50 mL reactors. The reactors were placed on two VWR shaker tables operating with parameters described previously in section "Experiment 3, (NB and BB) Temperature and Growth Conditions" (Section 2.10.2).

Figure 2.4 NB and BB 0 °C and -20 °C, algal culture and DMSO or MEOH
The NB and BB microcentrifuge tubes were directly placed upright in the corresponding 0 °C or -20 °C freezers.

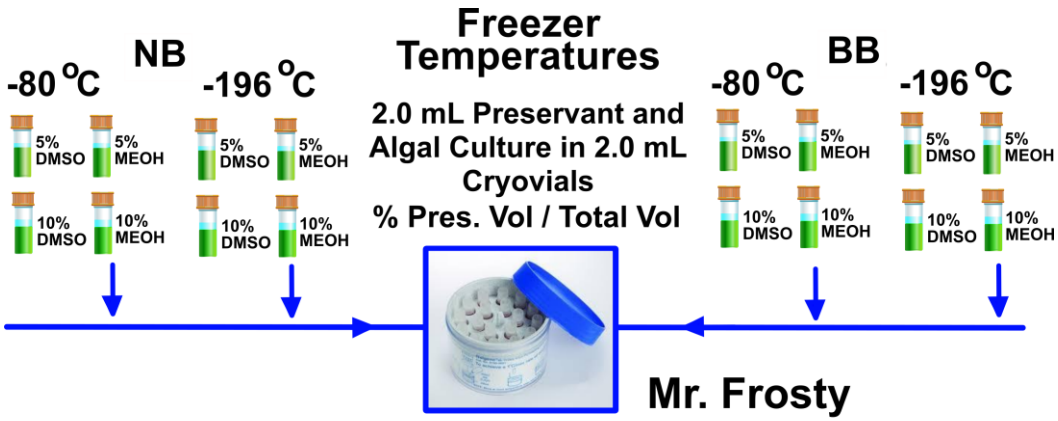

Figure 2.5 NB and BB -80 °C and -196 °C, algal culture with DMSO or MEOH
Mr. Frosty is a passive freezing unit for the -80 °C and -196 °C cryovials and slows the freezing temperature to increase the chances of successful thawed and revived frozen algal culture (reconstituted). [66] [61] The -80 °C cryovials were left upright in the -80 °C freezer and the -196 °C cryovials were placed in a basket and lowered into the LQN dewar.

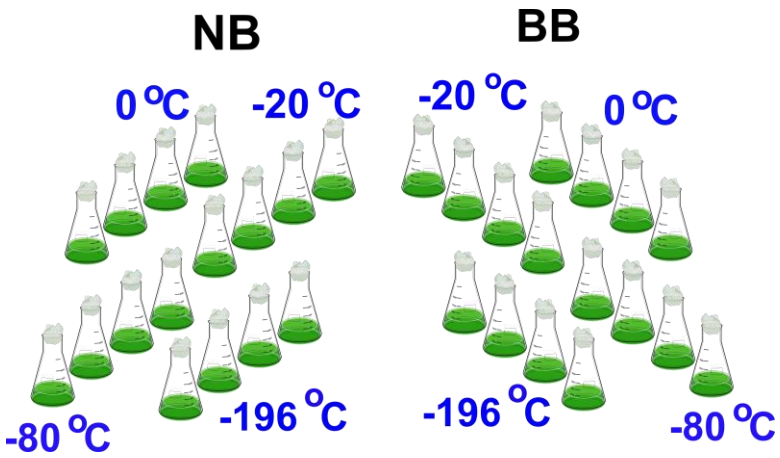

Figure 2.6 NB and BB reconstituted NM algal community in 50 mL reactors
The 32 post freezing NB reactors were placed on one shaker table and the BB reactors were placed on a second shaker table. Both sets of reactors were grown on the shaker tables.

The (32) 50 mL reactors, Figure 2.6, were broken into 2 (16) 50 ml reactors groups (NB and BB) and placed on the two VWR shaker tables to grow for 13 days. The two VWR orbital shaker tables details are listed in sections 2.1 and

2.3.1. On each day, an observation of green growth in every reactor was cataloged on the growth score sheet Figure 5.2.

2.12 Experiment 3 : Analysis

On the 13th day, final growth day, (8) 1 mL samples were taken from each of inoculum NB and BB 50 mL flasks including all the and stored in labeled plastic bags (labeled with freezing temperature, and DMSO or MEOH volume percentages), which were placed in the -20 freezer for later 454 pyrosequencing rDNA 23s analysis.

The frozen samples with DMSO 5% and frozen at -196 °C were selected for pyrosequencing analysis based upon the number of algal species observed in the 400X images taken from each of the (32) 50 mL reconstituted flasks.

2.12.1 Experiment 3 : reconstituted Algal community analysis

Same as Experiment 1 : Algal community analysis, Section 2.4.1

Chapter 3 : Experiment 1, The Effects of Temperature

3.1 KNOWLEDGE GAP

One algal temperature study analyzed whether the algal groups chlorophyte or cyanobacteria could tolerate high temperatures and still have high growth rates. Each flask started with an inocula containing 50 µg / L Chl *a*. and the daily growth rates were assessed using Chl *a* concentration changes. The medium was a low mineral concentration WC recipe [67]. The study had a low illumination level of 80 µmol photons m^{-2} s^{-1} and provided light 16:8 hours light/dark cycle.

The optimum temperature was defined as the highest incubation temperature that yielded the highest growth rate. Six different temperatures were tested (20 - 35°C). At the maximum temperature (35°C), which was also the optimum temperature for the cyanobacteria, *Synechococcus elongatus,* the mean growth rate was 0.91 ± 0.01 SE. At the optimum temperature for the chlorophyte, *Scenedesmus maximus,* the mean growth rate was 0.86 ± 0.03 SE. The maximum growth rates at the highest temperatures were very close for the cyanobacteria and chlorophyte.[37]

These study results may not be the same for highly saline medium with a much higher illumination intensity. Locating articles that push the temperature extremes are difficult to find and none have been located for the analysis of a mixed algal community.

Temperature has a negative effect on algae particularly when temperatures exceed the optimum ranges for green algae, diatoms and cyanobacteria. [68]

To date, little research has been performed to discover how natural mixed algal communities sourced from a hot and highly saline pond can enhance their ability to produce biodiesel at different temperatures.

Experiment 1: Objective 1 was to determine the effects of temperature on a highly saline algal community, including the biomass tracked through Chl *a* community diversity, and FAMEs content for biodiesel.

Experimental Approach, Experiment 1: The experimental system used in Experiment 1 was batch reactors (500 mL flasks) which were illuminated and monitored for days 0 to 14. Total FAMEs mg / L concentrations (biodiesel) were quantified each day. These systems were inoculated with a NM algal community taken from a highly saline pond located in an environment with unobstructed high intensity sunlight (San Acacia Brine Pond, Soccoro, NM).

3.2 RESULTS

3.2.1 AVERAGE CHL *A* AND *B* CONCENTRATIONS

The average Chl *a* concentrations in the room temperature (RT, 25 deg C) and high temperature (HT, 40 deg C) reactor sets over the 14 day Experiment 1 were shown in Figures 3.1A and 3.1B. Because the biomass dry weight measurements were very low (less than 1 mg from a 10 mL sample volume), the average Chl *a* concentrations were used to quantify algal growth. Previously, Chl *a* concentrations have been shown to correlate to algal growth rates [69].

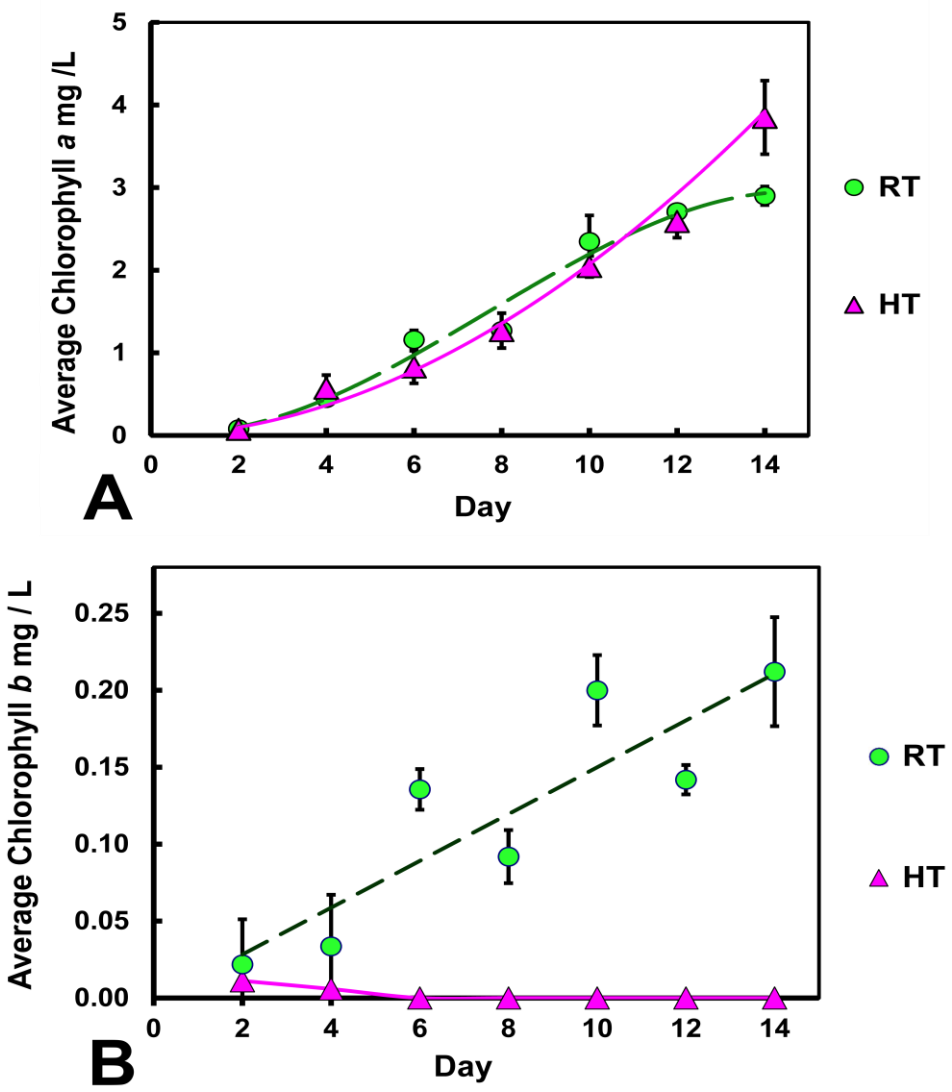

Figure 3.1 Average Chl *a* and Chl *b*
Figure 3.1A RT and HT average Chl *a* mg / L and Figure 3.1B RT and HT average Chl *b* mg / L, n = 3, ± Standard Error (±SE) bars are shown on each data point.

Figure 3.1A indicates similar average Chl *a* production rate and therefore similar growth rates of the RT and HT culture biomasses. A regression between the RT and HT culture Chl *a* had a $R^2 = 0.96$ and ($p < 0.001$). On the last day of the experiment (day 14) the RT Chl *a* concentrations were 25% less than the HT concentrations.

In contrast, the average Chl *b* concentration on day 0 was 0 mg / L in the HT culture and the average Chl *b* for days 0 to 4 was 0.0134 mg / L. Over day 4 to day 14, the average Chl *b* concentrations were 0 mg / L.

Both the RT and HT culture Chl *a* concentrations per day increased nearly identically until day 14. The RT Chl *a* concentration increased slightly from day 12 (Chl *a* 2.7 mg / L) to day 14 (RT Chl *a* 2.9 mg / L, but was less than the day 14 HT Chl *a* concentration 3.8 mg / L. On last day of the experiment the RT average Chl *a* concentration was 25% of the HT average Chl *a* concentration. This difference may have been caused by the RT medium NO_{3^-} concentrations, Section 3.2.3, which had been completely consumed by day 14. During the analysis of the HT algal community composition, Section 3.2.2, it was discovered that only cyanobacteria were present, which do not produce Chl *b,* and would explain the absence of Chl *b*. [20]

3.2.2 ALGAL COMMUNITY COMPOSITIONS

The RT and HT algal genera count percentages of the total counts per day are shown in Figures 3.2A and 3.2B. The genera data were determined by 454 pyrosequencing of 23s rDNA plastid genes. [63] The RT and HT algal cultures genera population changed over the course of the two 14-day experiments. The RT seed culture, Figure 3.2A, day 0, contained algal genera populations, which were held at RT temperature prior to inoculation of the (6) 500 mL reactors.

One day prior to the start of the HT culture experiment, the HT algal seed reactor was held in an incubator at an intermediate temperature (35 °C for 12

hours) to acclimate the HT algal seed cultures to the intermediate temperature prior to the start of the HT experiment. At the same time, six 500 mL reactors, filled with HT media, were warmed to the 35 °C.

At the start of the HT experiment, on day 0, the temperature acclimated HT algal seed reactor culture inoculated the six pre-warmed media reactors. The six inoculated reactors were placed in the incubator set 40 °C.

On day 0, both the RT and HT algal high saline seed cultures were sampled and later analyzed by 454 pyrosequencing. The 454 pyrosequencing returned genera counts for the RT and HT seed cultures. The day 0 RT and HT community seed cultures were graphed as a percentage of the daily total genus counts (Figure 3.2) which were the algal used to inoculate for RT and HT cultures (Figures 3.2A and 3.2B).

The HT inoculum containing a much higher fraction of the Spirulina genus than the RT inoculum, likely because one day prior to the start of the HT culture experiment the inoculum was held in an incubator at 35 °C for 12 hours to acclimate the cultures to higher temperatures prior to the start of the HT experiment.

The day 0 compositions differed for the RT and HT cultures (Figures 3.2A and 3.2B, respectively, with the HT inoculum containing a much higher fraction of the *Spirulina* genus than the RT inoculum), likely because one day prior to the start of the HT culture experiment the inoculum was held in an incubator at 35 °C for 12 hours to acclimate the cultures to higher temperatures prior to the start of the HT experiment.

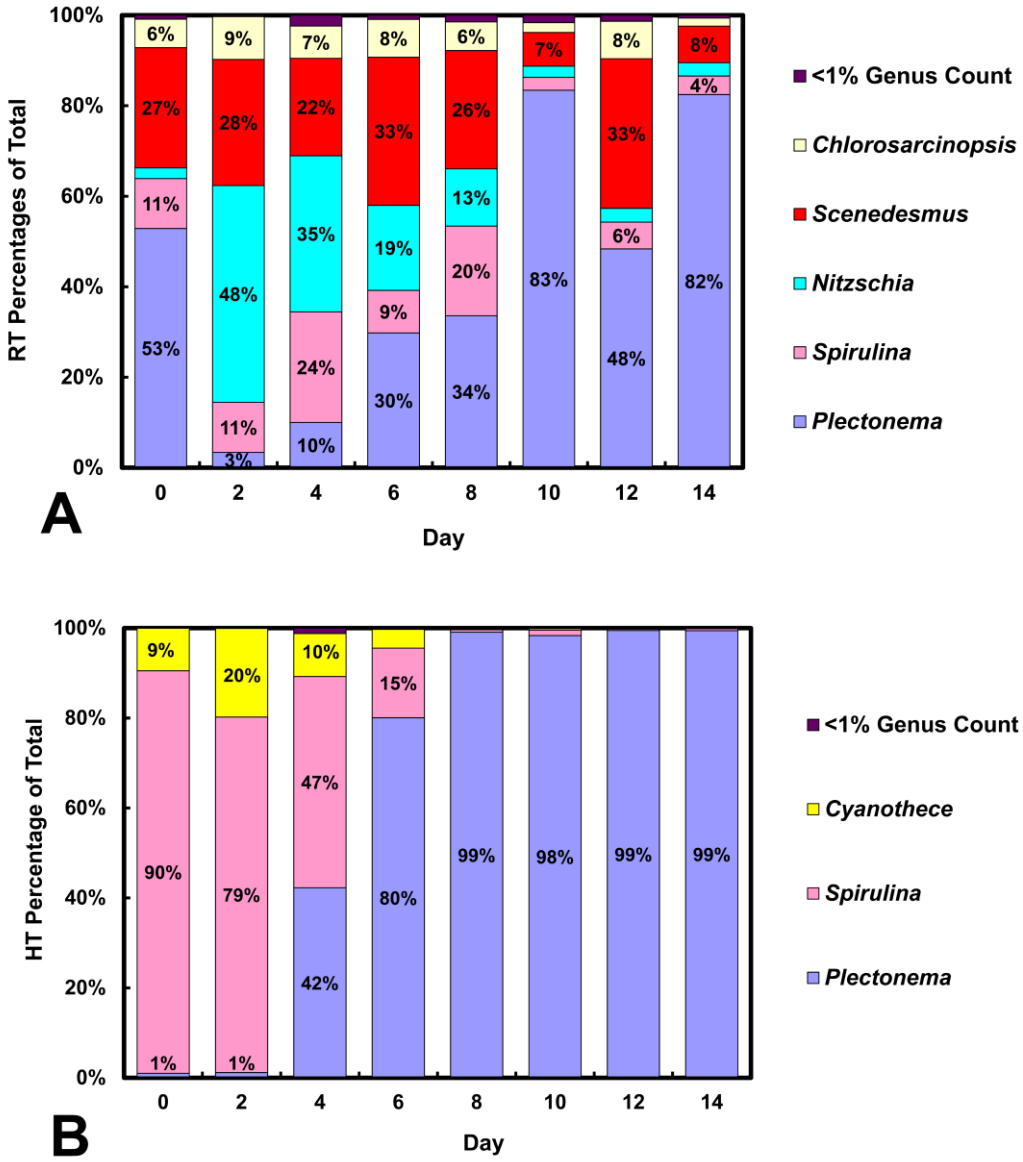

Figure 3.2 Algal populations at genus level determined by 454 pyrosequencing

RT 3.2A and HT 3.2B population composition genera are expressed as percent of total genera counts per day.

RT and HT genera percentages less than 1% of the total counts are in Appendix 7.1.1 in Figures RT 7.1A and HT 7.1B.

The algal population cultures were sampled regularly as described in Chapter 2 over the course of the experiments. In order to identify the algal genera present in the 23s rDNA gene sequences determined by 454 pyrosequencing were compared to an algal database (Figure 3.2).[63] In the RT culture reactor, the C*hlorosarcinopsis* genus (Kingdom: Plante, Class: Chlorophyceae, Family: Chlorosarcinales) percentage was 6% on day 0. From days 2 to 8, the *Chlorosarcinopsis* genus average was of 7.8 ± 0.7%, and over days 10 to 14 the average had decreased to 4.1 ± 2.1% .

Chlorosarcinopsis is a terrestrial sublithic eukaryotic algae inhabiting desert crusts and brine ponds worldwide. [70, 71] Microscopic observations (phase contrast, 400X) indicated a phenotype consistent with *Chlorosarcinopsis* at approximately 12 *μ*m length and moving rapidly across the slide propelled by four flagella that they wrap along the side of their oblong bodies when at rest. Some *Chlorosarcinopsis* genera have survived dry desert conditions in a dormant state for weeks where they revived with rainfall. [70, 71]

In Figure 3.2A, day 0, the *Scenedesmus* genus RT culture reactor pyrosequencing data percentage was 27.0% (Kingdom: Plante, Class: Chlorophyceae, Family: Scenedesmaceae). The Scenedesmus (Figure 3.2A) average genus count percentage for days 2 to 14 was 22.4 ± 4.1%. *Scenedesmus* genera exist as unicellular green algae, and have been found in fresh and salt water with the ability to live in desert soils. [72, 73]

On day 0, Figure 3.2A, the *Nitzschia* genus (Kingdom: Eukaryota, Class: Bacillariophyceae, Family: Bacillariaceae) in the RT culture seed reactor

pyrosequencing percentage was 2.4%. On day 2, the RT *Nitzschia* pyrosequencing data percentage peaked at 48% and then dropped until day 10. From day 10 to 14 the RT *Nitzschia* average genus percentage leveled out to 2.8% ± 0.2% SE. The *Nitzschia* species is a pennate, motile marine diatom (25 *μ*m length) encased in a silica frustule.

The day 0, Figure 3.2A, the *Spirulina* genus (Kingdom: Bacteria, Class: Cyanophyceae, Family: Oscillatoriales) RT seed reactor pyrosequencing data genera percentage was 11.0%. For days 2 to 8, the RT culture reactor average genus percentage was 16.2% ± 0.1% SE. Over days 10 to 14, the RT culture reactor genus percentage dropped to an average of 4.3% ± 0.9% SE. Spirulina genus are filamentous marine algae and have been researched extensively for their ability to produce biodiesel lipids.[74, 75]

The RT *Plectonema* culture day 0 seed genus percentage of the total counts was 53% (Kingdom: Bacteria, Class Cyanophyceae, Family: Oscillatoriales) and increased rapidly in the RT to a final presence in the culture of 82%. The *Plectonema* genus is a filamentous, endolithic microboring cyanobacterium ubiquitously found around the world in almost all oceans, deserts, fresh water lakes and coral barrier reefs. [76] The extremely adaptable *P. terebrans* have been found at depths of one hundred meters where the *P. terebrans* bore into carbonate and limestone substrates at very low light conditions. [77]

The 40 °C HT culture pyrosequencing results of the 14-day study in Figure 3.2B, identified three algal genera with counts greater than 1% of the total. Two genera from the RT culture reactors also grew in the HT culture reactors,

Spirulina. and *Plectonema,* with one new HT reactor culture genus, *Cyanothece*. The other three genera from the RT reactor culture, *Scenedesmus, Nitzschia* and *Chlorosarcinopsis,* did not survive in the HT culture.

The HT culture reactor pyrosequencing results identified the *Cyanothece* genus (Kingdom: Bacteria, Class Chroococcales, Family: Cyanobacteria), which is a unicellular marine algae species. The *Cyanothece* genera in the RT culture was present in the RT culture at a percentage less than 1% and is graphed in Figure 7.1 (Appendix 7.1.1). In the HT culture, the *Cyanothece* count percentage represented a small portion of the HT culture total count percentage. The *Cyanothece* genera increased to a peak on day 2 and then fell below 1% of the total count from days 8 to 14. In the interval from day 0 to 6 the *Cyanothece* genera average count percentage was 10.7 ± 3.2%. *Cyanothece* genera can fix nitrogen directly from the atmosphere (diazotroph) and convert it into ammonia through intracellular heterocysts situated between their internal thylakoid membranes. [78]

The HT culture seed reactor *Spirulina sp.* genera count percentage was a maximum on day 0 and decreased to less than 1% of the total by day 8, with the *Spirulina sp.* genera count percentage remaining less than 1% until day 14. The average *Spirulina sp.* genera count percentage for days 0 to 6 was 57.7 ± 16.8%. The *Spirulina* genus is not a diazotroph and does not fix nitrogen from the atmosphere.

The HT reactor *Plectonema* genera increased from 1% to 99% over the course of the experiment in the HT culture reactors. The average *Plectonema*

genera percentage for days 6 to 14, was 95.3 ± 3.8%. *Plectonema* is another diazotrophic alga, has the capacity to fix nitrogen, but only during low oxygen periods. Unlike *Cyanothece sp*., *Plectonema* can only fix nitrogen at night when the algal energy comes from respiration.

The algal community did not exist entirely planktonically during the study, but large algal clumps formed, around the filamentous cyanobacterium *Spirulina* and *Plectonema,* leaving the medium clear. Shaking the cultures before sampling resuspended the algal clumps to disperse the green algae, diatoms and cyanobacteria cultures into a planktonic state. At the end of 4 days, the genera *Nitzschia*, *Scenedesmus*, and *Chlorosarcinopsis* were loosely attached to the filamentous cyanobacteria clumps.

Table 3.1 compared the RT and HT culture genera experimental temperatures ranges to the typical literature temperature ranges. [68]

For the RT culture, *Chlorosarcinopsis* genera, the low end of experimental temperature range was slightly lower (20 °C) than the typical literature temperature range low end, 25 °C. The RT *Chlorosarcinopsis* genera literature high temperature (35 °C) was higher than the experiment high temperature (25 °C).

The RT experimental temperature range for the *Scenedesmus* and *Nitzschia* genera fell completely inside the literature temperature ranges. Both the genera *Scenedesmus* and *Nitzschia* grew continuously in the RT culture and had a presence over all 14 days.

The 2 RT culture cyanobacteria genera also found in the HT culture were *Spirulina* sp. and *Plectonema* with both their literature temperature ranges greater than the experimental RT culture temperature ranges. At the end of the RT study, the *Spirulina* genera reached 4% of the total algal count percentage and the *Plectonema* attaining 82%. The RT culture lower temperature (20 °C) apparently did not appear to have any negative effect on the growth of these two cyanobacteria genera.

During the HT culture experiment, the effects of the higher culture temperature ranges were evident by the death of three algal genera: *Chlorosarcinopsis, Scenedesmus,* and *Nitzschia*. The 3 HT culture genera surviving the higher temperatures were *Cyanothece*, *Spirulina*, and *Plectonema*.

Table 3.1 RT and HT cultures typical algal growth temperature ranges. Literature temperatures were compared to the experimental growth temperature ranges.

RT Culture Algae Genus > 1%	**Literature Temperature Range º C**	**RT Experimental Temperature Range º C**
Chlorosarcinopsis (Chroococcales) [79]	25 - 35	20 - 25
Scenedesmus (Chlorophyceae) [74]	20 - 32	20 - 25
Nitzschia (Bacillariophyceae) [80]	20 - 35	20 - 25
Spirulina (Cyanophyceae) [81]	25 - 35	20 - 25
Plectonema (Cyanophyceae) [82]	35 - 45	20 - 25
HT Culture Algae Genus > 1%	**Literature Temperature Range º C**	**HT Experimental Temperature Range º C**
Cyanothece (Cyanophyceae) [78]	30 - 38	35 - 40
Spirulina (Cyanophyceae) [81]	25 - 35	35 - 40
Plectonema (Cyanophyceae) [83]	35 - 45	35 - 40

In the first 4 days of the HT culture (Figure 3.2B), there were three Chl *b* producing HT genera with low counts: *Scenedesmus*, C*hlorosarcinopsis* and

Nitzschia, which initially produced small amounts of Chl *b.* All three of these RT genera died off in the elevated temperature by day 6, which stopped all the Chl *b* production in the HT culture.

3.2.3 CULTURE NO_{3-} CUMULATIVE CONSUMPTION

The RT and HT cultures average NO_{3-} cumulative consumption curves are shown in Figure 3.3A and 3.3B. The initial RT and HT culture medium NO_{3-} concentrations were both 179.2 mg / L and on the final day, the NO_{3-} cumulative consumption for the RT and HT cultures were respectively 175 mg / L and 126 mg / L . The average NO_{3-} cumulative consumption rate in the RT culture reactors was 13.2 NO_{3-} mg / L / d, which is larger than the HT culture reactors average 9.42 NO_{3-} mg / L / d.

On day 14, the RT culture had consumed all the NO_{3-} in the medium, while the HT culture had 53.0 NO_{3-} mg / L remaining in the medium.

In Figure 3.3B, the average RT and HT culture NO_{3-} consumption rates were normalized to Chl *a* concentrations, NO_{3-} / Chl a / d. For both the RT and HT cultures, the normalized rates were very high on day 2.

In Figure 3.3B, on day 2 due to a very low Chl *a* mg value in the RT and HT cultures the NO_{3-} produced a large NO_{3-} / Chl *a* mg / L (183.6 ±19.7 and 144.1± 16 SE mg / L / d) . Over days 4 to 14, the RT culture average NO_{3-} / Chl *a* / d

was 59.55 ± 4.74 SE, and the HT reactors average NO_{3-} / Chl *a* / d was an average 40.51 ± 2.47 SE.

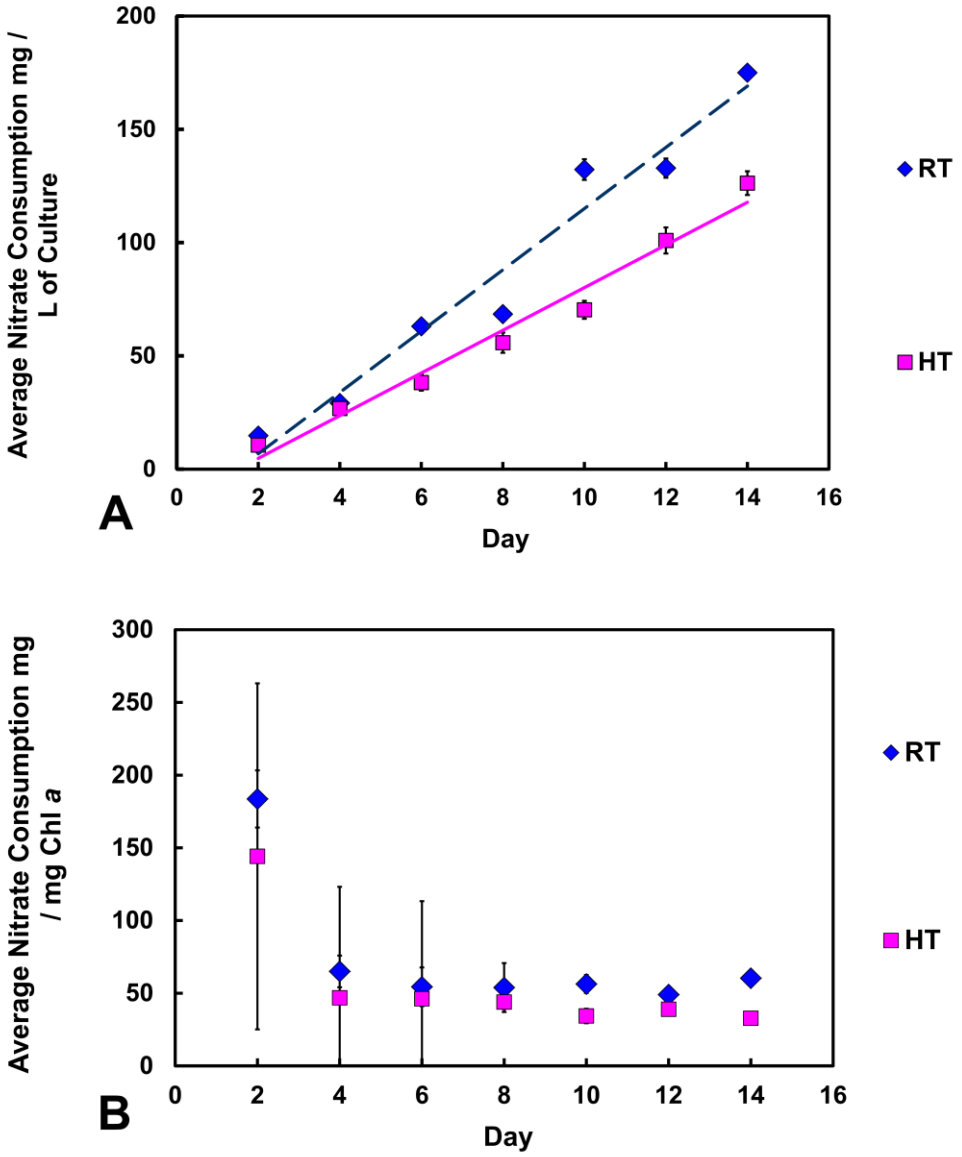

Figure 3.3 RT and HT average NO_{3-} cumulative consumption

Figure 3.3A graphs the RT and HT cultures the average NO_{3-} cumulative consumption mg /L /d. Figure 3.3B graphs the RT and HT culture average cumulative algal NO_{3-} consumption mg per mg Chl *a* / d. n = 3. ±SE bars are shown on each data point.

The RT algal culture consumed all the NO_{3-} in the medium. The HT culture, Figure 3.3A, consumed the NO_{3-} at a lower rate than the RT culture. The lower HT consumption rate may stem from the fewer algal genera present in the HT culture. Each of the three surviving HT cyanobacteria genera may have just had a lower NO_{3-} consumption rate at the higher temperature.

Two of the HT Cyanobacteria genera, *Cyanothece* and *Plectonema*, Figure 3.2B, can fix atmospheric nitrogen to ammonia (diazotrophs) as an often preferred nitrogen source over NO_{3-} [82]. The *Cyanothece sp*. fixes nitrogen in a heterocyst incorporated inside the cell and *Plectonema* genus fixes nitrogen through a non-heterocystous pathway. If the *Cyanothece sp*. and *Plectonema* genus fixed atmospheric nitrogen to ammonia during the HT study, as an alternate nitrogen source, the NO_{3-} consumption would have been measured at a lower concentration during the ammonia fixation times. Since the ammonia consumption was not measured, the ammonia may have been consumed by the HT algal culture instead of the NO_{3-}. Nitrogen fixation may have become part of the cellular nitrogen source in the algal community and was not exclusively provided by the NO_{3-} in the medium.

For the *Plectonema* genus, nitrogen is not fixed during photosynthesis, light phase, since oxygen poisons the Plectonema genus nitrogenase enzyme [78]. Twenty to eighty percent of the ammonia can leak through the *Plectonema* genus cell membrane, which becomes readily available to other green algae, diatoms and cyanobacteria in close proximity. Non-heterocystous nitrogen fixation in the

Plectonema genus can only occur at night (dark phase). The nitrogen fixed by cyanobacteria inside the cell is converted to ammonia (EQ 7) [82]. The nitrogen dark cycle for *P. terebrans* only lasts for six hours each night.

$$N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2 \text{ [84]} \qquad \text{EQ (7)}$$

The *Plectonema* genus fixes nitrogen after the daylight levels diminish. Daylight stops nitrogenase activity, but in darkness *Plectonema* converts sufficient atmospheric nitrogen to ammonia for cell synthesis and leaks a large portion of ammonia into the surrounding medium. [85].

From daily microscopic observation, the San Acacia community motile unicellular algal cells often adhered to the long intertwined filaments of the *Plectonema* genus, to possibly avoid the high light irradiance and capitalize on the leaked ammonia as a nitrogen supply. In the dark cycle, the Plectonema hosted unicellular algal populations benefited when the medium NO_{3^-} concentrations were depleted by absorbing the *Plectonema* leaked ammonia as a nitrogen source. During the phase transition from the light to dark, the hosted green algae, diatoms and cyanobacteria continued their oxygen consumption around the filaments, which allowed the *Plectonema* genus to start nitrogen fixation sooner during the dark phase

3.2.4 TOTAL IDENTIFIED FAMES

Fatty acids extracted from both the RT and HT algal culture samples were transesterified producing FAMEs, which are also known as biodiesel. The RT

and HT cultures FAMEs were analyzed in a Shimadzu 2010 gas chromatography system with a DB-FFAP column.

An external FAMEs standard containing 37 different known FAMEs of specific concentrations (FAMEs mg / mL hexane), was run through the gas chromatograph to quantify the 37 FAMEs peak areas (the peak area is correlated to the FAMEs concentration) and to determined elution time for each of the FAMEs, which is the time (minutes) the FAME takes to move through the column. With the 37 known FAMES peak area concentrations (FAME mg / L hexane) and the column elution times, the experimental algal FAMEs could be identified and quantitatively measured.

The external 37 FAMEs standard peaks and elution times were compared to the gas chromatograph RT and HT culture experimental FAMEs peak elution times and peak areas. The RT and HT experimental FAMEs that matched the 37 FAMEs standard peaks and elution times were then called "identified FAMEs".

The RT and HT culture average total identified FAME concentrations were separately summed and graphed, Figure 3.4A. The RT culture produced FAMEs starting on day 2, but the HT culture FAME production lagged until day 8, Figure 3.4A. On day 14, the RT culture average total FAMEs measured 25.8 mg FAMEs / L, and the HT culture average total FAMEs was 11.7 mg FAMEs mg / L. The RT culture reactors produced more than twice the total average FAMEs than the HT culture reactors. The HT culture required the 6 days to initiate significant lipid production. The HT culture total FAMEs increased steadily from days 8 to 14.

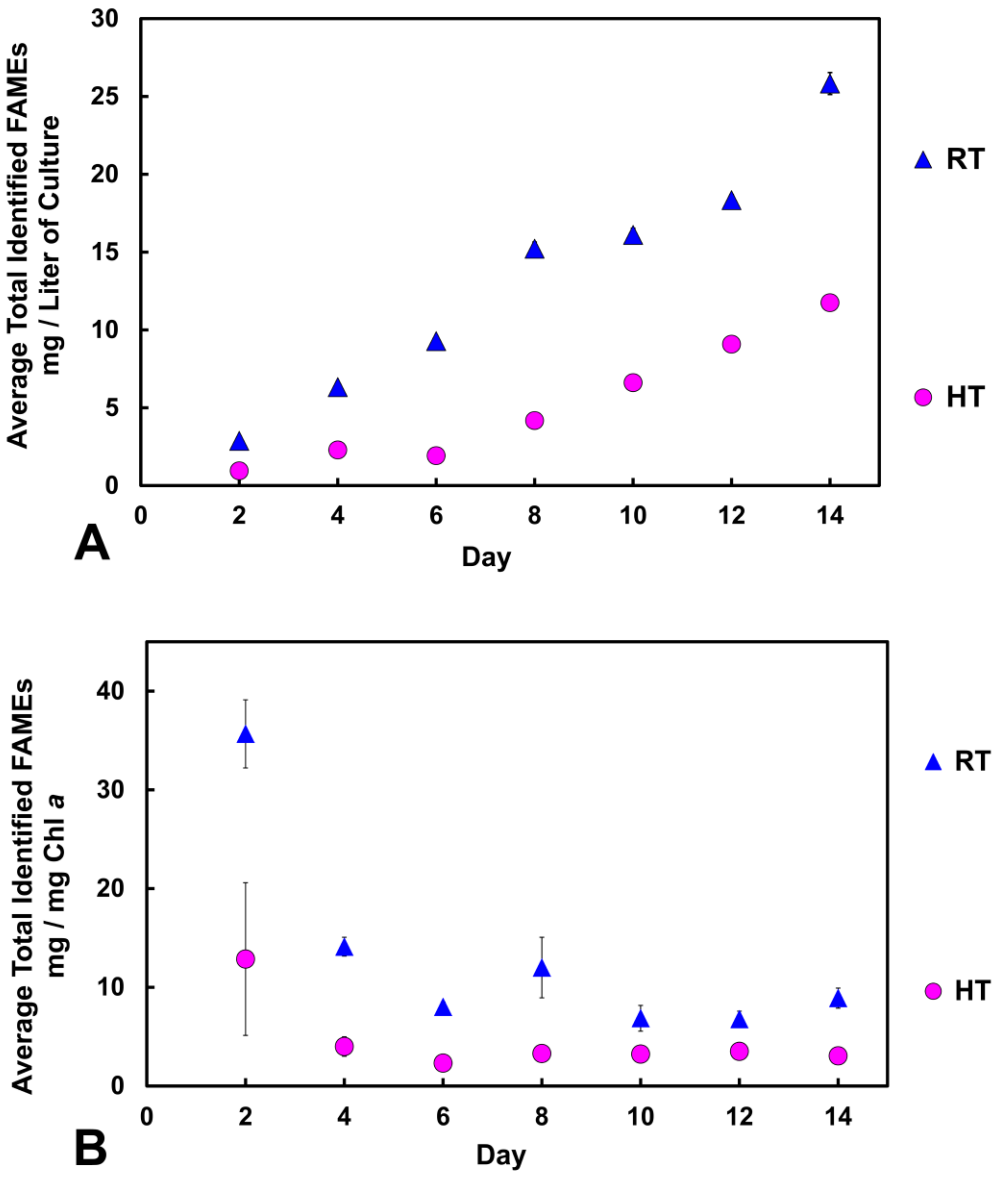

Figure 3.4 Total average identified FAMEs
The Figure 3.4A graph plots the RT and HT average total FAMEs mg / d. The Figure 3.4B graph plots the RT and HT average total FAMEs mg / Chl *a* mg / d. n=3, ±SE bars shown on each data point.

In Section 3.2.2, Figure 3.2B, the HT culture algal populations, for days 8 to 14, the *Plectonema* genus count percentages were 98% to 100% of the total counts. In Figure 3.4A, over days 4 to 14, the RT culture total average FAMEs

was 9.44 FAMEs mg / Chl a mg ± 1.22 SE / d and the HT culture total average FAMEs was 3.23 FAMEs mg / Chl *a* mg ± 0.23 SE / d.

The total FAMEs produced by the RT algal community increased rapidly while the HT culture produced a low amount of total lipids until day 8 when it began increasing lipid production. Three algae, *Scenedesmus, Nitzschia* and *Chlorosarcinopsis,* from the RT NM algal community died when introduced to HT temperatures and possibly when the 3 algae expired, their lipids spilled into the HT medium where other algae species within the culture harvested the available lipids for their own storage.[51]

In other research, *P. terebrans* proved to be capable of degrading crude oil directly [86]. Produced water often contains petroleum oils, and may make *P. terebrans* a desirable species to break down petroleum oil. *P. terebrans* could decompose petroleum oils found in produced and reuse water, while converting the decomposed lipids into TAGs for biodiesel.

3.2.5 SATURATED AND UNSATURATED FAMES

The *Plectonema* genus created the HT culture total average FAMEs on the days 8 thru 14, but the HT total average FAMEs was only 34% of the RT culture average Fames. The most plentiful saturated FAME in both the RT and HT cultures was methyl palmitate C16:0 on day 14. The RT algal community synthesized 8.64 mg / L of C16:0, while the HT culture C16:0 produced 5.07 mg / L.

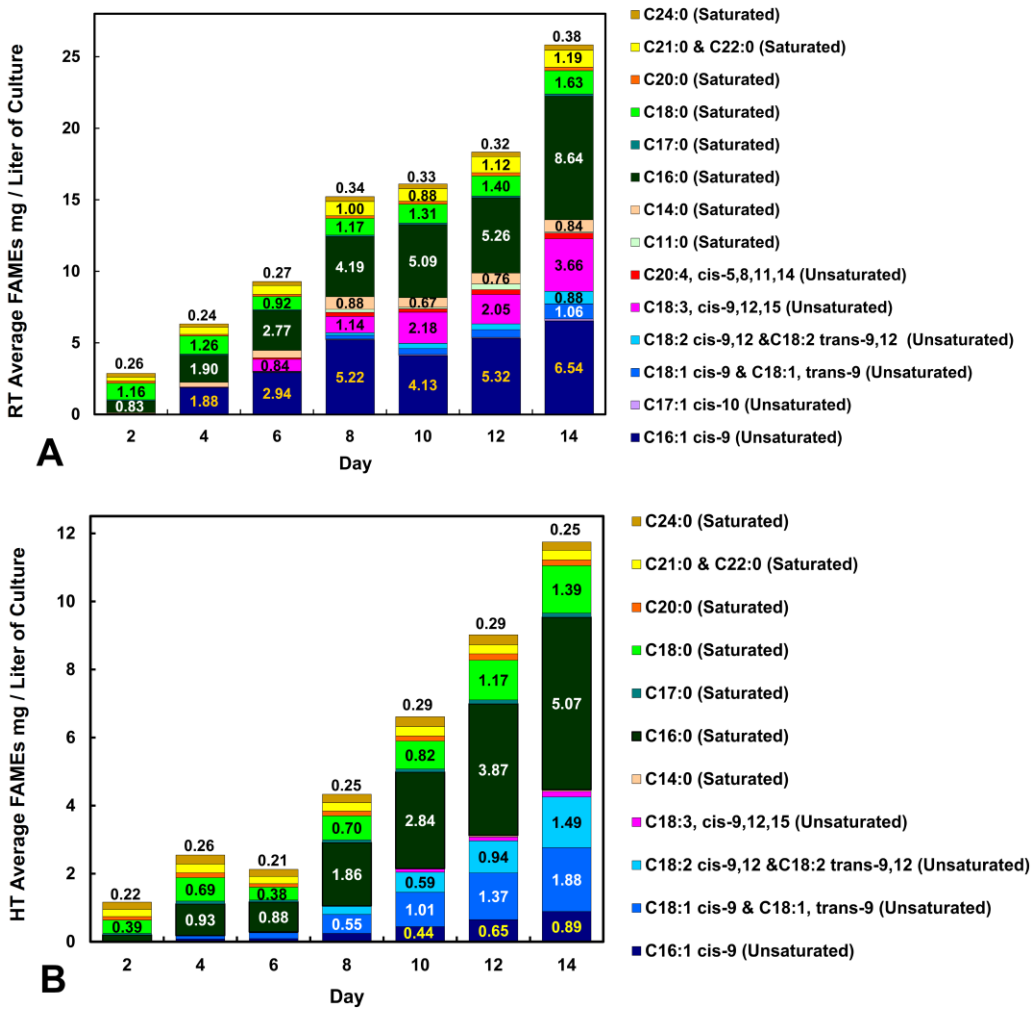

Figure 3.5 Average saturated and unsaturated FAMEs
The RT (3.5A) and HT (3.5B) n=3,

The highest RT unsaturated FAME was methyl palmitoleate (C16:1, day 14), while the highest HT unsaturated FAME was methyl oleate (C18:1, day 14). The average RT and HT FAMEs, stacked graphs FAMEs SE's, are in Appendix 7.1, and listed in Tables 7.1, and 7.2. RT and HT average FAMEs mg / L that are too small to label on Figures 3.5A and 3.5B are shown in Appendix 7.1 on Tables 7.1, and 7.2.

Biodiesel commercial production focuses on the quantity of lipids that can be made in a week of growth. The NM algal community cultivated at the RT temperatures out performed the HT due to the HT loss of lipid producing species unable to survive in the higher temperature of 40 °C.

The HT culture may have an advantage over the RT culture when the temperature exceeds the survivable limits of the genus *Chlorosarcinopsis*, *Scenedesmus sp*., and *Nitzschia* since the HT culture genera can still produce biodiesel lipids although at a lower lipid production rate. A commercial algal facility could plan to operate with a culture similar to the RT culture and in the RT culture temperature range (20 °C to 25 °C) in spring and early summer. Then during higher temperatures in mid to late summer (35 °C to 40 °C), the facility could still grow the RT culture knowing eventually there would be fewer algal cells, but the HT cyanobacteria would still produce lipids.

3.2.6 FAMES CORRELATIONS

Three pairwise regressions were performed: comparing the daily FAME concentrations RT-to-RT FAMEs, HT-to-HT FAMEs, and RT-to-HT FAMEs. The RT-to-RT FAMEs FAMEs concentrations (mg / L / d) were correlated with the same RT culture FAME concentrations for every day of the 14 day study. The HT-to-HT FAME concentrations (mg / L / d) were correlated with the same HT culture FAME concentrations for every day of the 14 day study.

All regressions were calculated with a 99.9% confidence level.

The RT-RT, Figure 3.6A, the regression analysis compared the RT 14 different individual FAME concentrations with each of the other. The RT FAME concentrations generated over the 14 day experiment produced 89 R^2 values total.

The HT-HT Figure 3.6B regression analysis compared the HT 11 FAME concentrations /d with the corresponding HT FAMEs resulting in 57 R^2 values. .

The RT-HT Figure 3.6C regression analysis compared the 11 HT FAME concentrations /d to the corresponding RT FAMEs finding 11 R^2 values total. The RT culture contained three more FAMEs than the HT culture, so that the RT-HT correlation comparison excluded these three RT FAMEs.

The R^2 values in the RT to RT and HT to HT correlation maps greater than 0.90 had *p* values less than 0.001.

In Figure 3.6A, RT culture, there was a high correlation between three unsaturated RT FAMEs: C18:1, C18:2, and C18:3 with each having an $R^2 > 0.91$, $p < 0.001$.

In Figure 3.6A, RT culture, one saturated FAME C16:0 had a medium correlation to these 4 unsaturated RT FAMEs: C16:1, C18:1, C18:2, and C18:3 with a R^2 value between $0.89 > R^2 > 0.86$.

In Figure 3.6B, HT-to-HT, HT culture FAMEs had a high correlation between the HT culture FAME C16:0 and FAMEs C16:1, C18:1, C18:2. $R^2 > 0.97$. Additionally, a high R^2 correlation was found between the HT culture three FAMEs: C16.1, C18:1, and C18:2, $R^2 > 0.94$.

In the RT to HT FAME regressions, Figure 3.6C, none of the RT-HT R^2 coefficients had any R^2 over 0.799 indicating relatively low correlation between the RT and HT FAME values (mg / L / d) over the two 14 day studies. In Figure 3.6C the low correlation values demonstrate that the RT culture and HT culture FAME production were independent of each other.

The FAME correlation (3.6C) between the RT and HT FAME concentration was performed to prove there was only a weak correlation between the two RT to HT studies

The RT-to-RT FAME correlations had similar correlations between three unsaturated RT FAMEs: C18:1, C18:2, and C18:3.

In the HT-to-HT FAME correlation there was a high correlation between FAME C16:0 and FAMEs C16:1, C18:1, C18:2. R^2 >0.97.

The differences between the two can be traced back to the difference in the genera in RT and HT cultures.

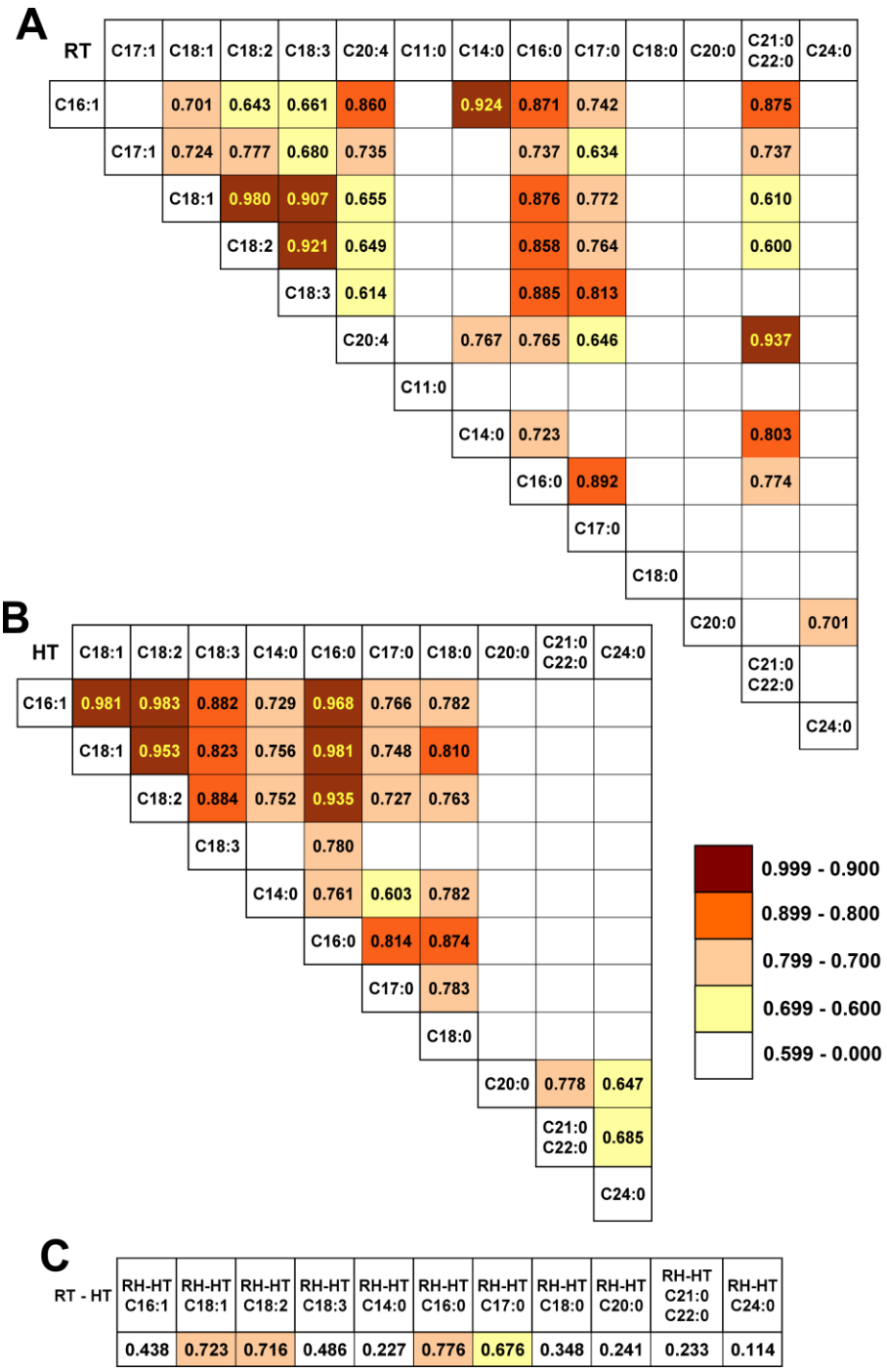

Figure 3.6 Heat maps from pair-wise R-squared regression
RT (3.6A), HT (3.6B), and RT to HT (3.6C) The R^2 heat map values legend is located on the lower right side of the page. The R^2 heat map values legend is located on the lower right side of the page.

3.2.7 FAMES FOR BIODIESEL

The cetane number (CN; unitless) represents the quality of the No. 2 petro diesel fuel with an acceptable minimum CN of 40 per ASTM D 975 - 07b, Standard Specification for Diesel Fuel Oils, Grade No. 2-D SI5. No. 2 petro diesel fuel CN uses an ASTM cetane reference fuel for calibration, hexadecane, which is equivalent to a CN of 100. [87] The minimum biodiesel CN per the ASTM D 6751 standard for biodiesel is 47. [88]

The higher the CN, the shorter the delay time will be to ignition. A low CN implies poor fuel quality and the compression ignition will be retarded slowing down the engine speed. A low CN may cause misfiring, poor combustion, and roughness in the diesel engine.

The No. 2 petro diesel blended with 20% biodiesel will produce rapid ignition in the diesel engine. When a diesel engine injector vaporizes the biodiesel blend, it spreads over the piston face autoigniting, and produces a smoother and more powerful stroke.

RT and HT saturated and unsaturated (S%/U%) FAME ratios, RT and HT Figure 3.7, directly affect the cetane number. The higher the saturated % to unsaturated% FAME ratio, the higher the CN of the FAME mixture. [89] On day 2, RT Figure 3.7A, the S%/U% FAMEs was 98%:1%, and by day 14, the S/U FAMEs ratio decreased to 51:49.

On day 0, the HT FAME S%/U% ratio starts at 100:0 and drops to 62:38 on higher HT S/U ratios.

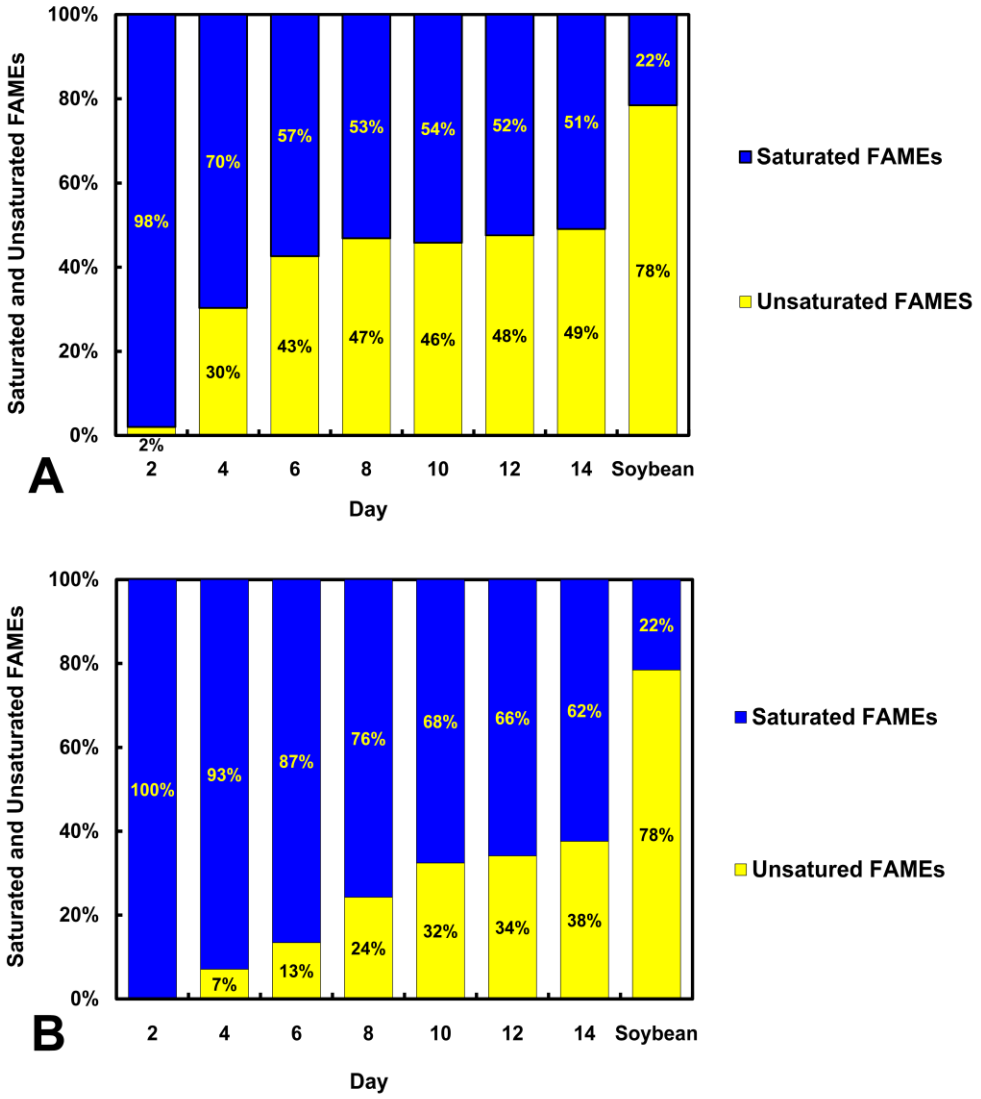

Figure 3.7 Saturated and unsaturated FAMEs
Fames are shown as percentages of total values in the RT (3.7A), HT (3.7B), and soybean (A and B). [90]

Both the RT and HT saturated FAME ratios exceeded the soybean (S%/U%) 0.28:1 ratio, and so the algal FAME CN's were much higher than the CN of the soybean oil.

A rough calculation for the derived cetane number (DCN) of the RT FAME and HT FAME mixed biodiesel can be performed using EQ(1), based on the individual FAME weight fraction of the total mixed FAMEs (single FAME mg / FAMEs mg total) and multiplied by the CN of the single FAMEs obtained from published CN data. [91] All individually weighted CN values are added together to obtain the DCN.

DCN = (1st FAME weight fraction of total) × (1st FAME CN) + (2nd FAME fraction of total) × (2nd FAME CN) + … (last FAME Fraction) x (last FAME CN) EQ (1)

The DCNs for the RT and HT culture algal FAMEs were calculated and listed in EQ 2 and EQ 3. Both the RT and HT DCN values are larger than the minimum biodiesel CN 47 required in the ASTM D 6751 standard [88]. The RT and HT DCN calculation tables are located in Appendix 7.1, Tables 7.5 and 7.6.

Blended RT FAMEs DCN = 60.6 EQ (2)

Blended HT FAMEs DCN = 63.4 EQ (3)

The San Acacia algal community was the single source for both the RT and HT cultures. The San Acacia original algal community adapted better to the RT experimental temperature ranges (RT, 25 °C), which may be an advantage for

biofuel use. The RT FAME production was larger than the HT production in almost all cases.

Though the HT culture is not as productive as the RT culture, the HT culture has an advantage over the RT culture when the temperature exceeds the lower survivable limits of the Chlorophyta and Bacillariophyceae, two productive RT culture genera. At the elevated temperatures, the HT algal culture can still produce a larger percentage of biodiesel lipids. The lower HT culture production rate may still be economically viable when operating a commercial facility in high temperature locations.

3.2.8 SHANNON DIVERSITY INDEX

The Shannon diversity index (H) is a measure of population diversity (Equation 4) [92]. The original Shannon diversity came from number theory as a decoding method to predict the entropy of letters in encrypted messages. The entropy index resulted from noting the location of each letter and how often the letter occurred. [92]

In the Shannon Diversity Index, H EQ (4) the variables are: S = number of species in the community, p_i = proportion of each S made from the *i*-th species, n_i = abundance of *i*-th species, N = total number of individuals from all species in the community.

The Shannon diversity index calculations included all the RT and HT species and their counts from the pyrosequencing results for each day of the experiment.

The Shannon Index accounts for all the algal species (S) and the abundance (n_i) within each specie. Higher H values indicated a more diverse community.

$$Shannon\ Diversity = H = -\sum_{i=1}^{S} p_i \bullet \ln p_i \quad Where \quad p_i = \frac{n_i}{N} \qquad \text{EQ (4)}$$

$$H_{Max} = ln(S) \qquad \text{EQ (5)}$$

Equation five, EQ (5), provides a method to calculate the possible maximum Shannon Index, H_{Max} for each day. The higher the experimental H value compared to the calculated possible H_{Max}, the greater the diversity of the culture for that day.

The RT culture H values increased from 1.3 on day 0 to 1.61 on day 8, and there after, decreased to 0.72 by the end of the experiment. The HT culture H values were consistently lower than the RT values.

The HT culture H value on day 0 increased from 0.37 to 1.01 on day 4 and decreased to 0.04 by the end of the HT experiment. On day 0, the HT algal culture reactors Shannon index H value on was 28% of the RT culture reactors value. In the HT algal culture, the H value on day 8 was only 3.9% of the RT algal culture H value.

To maximize the Shannon diversity index, H, in a culture all species in the culture must maintain a similar species abundance ($n_{i)}$ and number of species

(S). If any one species has excessive abundance or the species number (S) or drops extremely, then the Shannon diversity index will also drop.

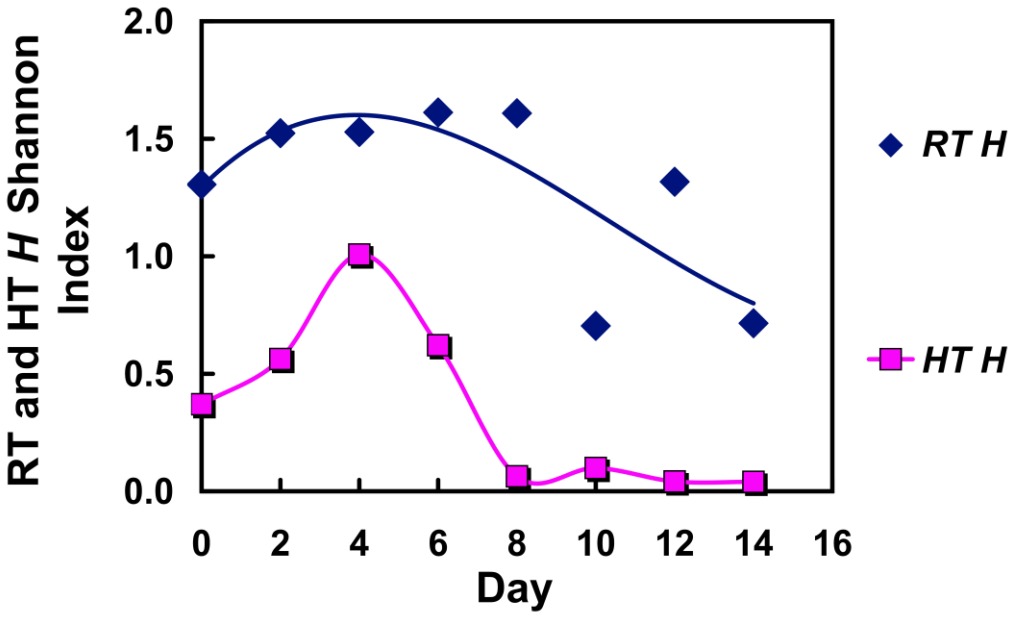

Figure 3.8 Shannon diversity index per day
Figure 3.8 graphs the RT and HT algal species reactor Shannon diversity index *(H)* per d and The Shannon diversity was calculated from all the RT and HT pyrosequencing results, including species with less than 1% of the total algae counts.

The Shannon diversity index (Figure 3.8) for RT reactor species (S) maintained a stable average diversity H for the period from days 2 to 8 days of 1.57 ± 0.02 SE. Beyond day 8, one specie, *P. terebrans*, continued to grow rapidly with a species abundance ($n_{i)}$ equal to 99% resulting in a final diversity of H = 0.72. The low H resulted from the lack of culture diversity directly due to the increased growth of the specie, *P. terebrans*.

The HT culture Shannon diversity index H increased initially to 1.01 on day 4. On day 4, the two highest HT culture species present were *Spirulina sp* and

P. terebrans, which accounted for 47.0 % and 42.3% of the total specie respectively. The similar percentages of the two species created a better diversity index H. On day 8 HT culture diversity index dropped to 0.063. Over days 8 to 14, when the HT culture average diversity index was 0.061 ± 0.014 SE. The HT culture low diversity index H resulted from the nearly total species dominance of *P. terebrans* from day 8 to 14 with an average of 99.0% ± 0.3% SE. When the HT culture *P. terebrans* species predominated all other algal species, the diversity dropped to near zero.

3.3 CHAPTER 3 EXPERIMENT 1 CONCLUSION

The Chl *a* from the RT and HT cultures increased continuously from day 2 to day 14. On last day of the experiment the RT average Chl *a* concentration was 23% of the HT average Chl *a* concentration. This difference may have been caused by the RT medium NO_{3^-} concentrations, Section 3.2.3, which had been completely consumed by day 14.

In the algal community composition, during the RT study, there were 5 genera greater than 1% of the total identified: 2 green algae, Scenedesmus and Chlorosarcinopsis. Also identified were two cyanobacteria: Spirulina and Plectonema and 1 diatom: Nitzschia.

During the HT culture experiment, the effects of the higher culture temperature ranges were evident by the death of three algal genera: *Chlorosarcinopsis, Scenedesmus,* and *Nitzschia*. In the HT culture 3 algal genera survived, the higher temperatures: *Cyanothece*, *Spirulina*, and *Plectonema*.

The RT algal culture consumed all the NO_{3-} in the medium. The HT culture, Figure 3.3A, consumed the NO_{3-} at a lower rate than the RT culture. The lower HT consumption rate may stem from the fewer algal genera present in the HT culture. Each of the three surviving HT cyanobacteria genera may have just had a lower NO_{3-} consumption rate at the higher temperature or the Plectonema may have produced ammonia internally offsetting the consumption of the NO_{3-}.

When the nitrate consumption was normalized to the Chl *a,* over days 4 to 14, the RT culture average NO_{3-} / Chl *a* / d was 59.55 ± 4.74 SE, and the HT reactors average NO_{3-} / Chl *a* / d was an average 40.51 ± 2.47 SE. The NO_{3-} and Chl *a* had a ratio between them. Both the NO_{3-} and Chl *a* were cumulative processes.

The total FAMEs produced by the RT algal community increased rapidly while the HT culture produced a low amount of total lipids until day 8 when it began increasing lipid production. Three algae genera from the RT NM algal community died when introduced to HT temperatures and possibly when the algal cells expired, their lipids spilled into the HT medium where other algae species within the culture harvested the available lipids for their own storage. The ingestion of the spilled lipids precluded the need for the culture to make lipids over the first 3 days.

The RT and HT cultures contained 14 and 11 identified FAMEs, respectively. The RT culture included two identified average unsaturated FAMEs (C17:0, C20:4) and one average saturated FAME (C11:0) not found in the HT culture. The HT culture C16:0 FAME mg / L was only 58% of the RT culture C16:0 FAME mg / L.

The NM algal community average saturated and unsaturated FAMEs cultivated at the RT temperatures out performed the HT due to the HT loss of lipid producing species unable to survive in the higher temperature of 40 °C.

In FAMEs correlation heat map, the RT-to-RT FAME correlations had similar correlations between three unsaturated RT FAMEs: C18:1, C18:2, and C18:3.

In the HT-to-HT FAME correlation there was a high correlation between FAME C16:0 and FAMEs C16:1, C18:1, C18:2. R^2 >0.97.

The minimum biodiesel CN per the ASTM D 6751 standard for biodiesel is 47. [88] The higher the CN, the shorter the delay time will be to ignition. The calculated derived Cetane Number (DCN) for the RT and HT FAMEs are respectively: 60.6 and 63.4, which are well above the ASTM D 6751 minimum requirement of 47.

The Shannon diversity index (Figure 3.8) for RT reactor species (S) maintained a stable average diversity H for the period from days 2 to 8 days of 1.57 ± 0.02 SE. Beyond day 8, one specie, *P. terebrans*, continued to grow

rapidly with a species abundance ($n_{i)}$ equal to 99% resulting in a final diversity of H = 0.72. The low H resulted from the lack of culture diversity directly due to the increased growth of the specie, *P. terebrans*.

The HT culture low diversity index H resulted from the nearly total species dominance of *P. terebrans* from day 8 to 14 with an average of 99.0% ± 0.3% SE. When the HT culture *P. terebrans* species predominated all other algal species, the diversity dropped to near zero

Chapter 4 : Experiment 2, Nitrogen Limited Medium

4.1 KNOWLEDGE GAP

All algae create proteins from nitrogen, incorporating the proteins in cellular structures such as: cell walls, membranes, gas vesicles, chloroplasts, plastids, and thylakoid stacks. Cultured algal communities typically consume nitrogen from the medium in the form of NO_{3-}.

Nitrogen deficiencies were studied in *Spirulina platensis* cultures. A study compared lipid production of *S. platensis* at different N concentrations in culture medium. Nitrogen (N) limiting conditions were in fact reported to increase the lipid fraction significantly of many microalgae. Nitrogen (NO_{3-}) was limited by 50% and 100% to determine the effects on lipid production. In this study, the NO_{3-} deficiency was limited to 50% and 100% NO_{3-} in a *Spirulina* culture medium, which increased the total lipid content by 13.66 and 17.05% and reduced the protein by 53.5 and 5.6%. [93]

. To date, little research has been performed to discover how natural mixed algal communities grown in media with different nitrate concentrations can produce biodiesel at higher concentrations.

Experiment 2 objective was to determine the effects of NO_{3-} concentrations on FAME production, community diversity, and biomass growth in a highly saline algal community.

Experimental Approach: The experimental systems used in Experiment 2 were batch reactors (500 mL flasks) which were illuminated and monitored for days 0 to 12. Total FAMEs mg / L concentrations (biodiesel) were quantified each day. The initial NO_{3-} concentrations for the low and high NO_{3-} media were respectively 75.0 mg / L and 179.2 mg / L. These systems were inoculated with a NM algal community taken from a brine pond located in an environment with unobstructed high intensity sunlight (San Acacia Brine Pond, Soccoro, NM).

4.2 RESULTS

4.2.1 CULTURE CHL A AND CHL B CONCENTRATIONS

In both the LN and HN culture studies, the Chl *a* and Chl *b* data were taken from the alternating culture reactor sets.

Daily data from the two different culture reactor sets (LN 3, 4 and HN 1, 2) were each combined into a single plot (Figure 4.1) for Chl *a*, and Chl *b*. The Chl *a* concentrations from the LN and HN cultures (Chl *a* mg / L / d) were used to normalized the other LN and HN culture parameters: nitrate consumption and total FAMEs concentrations.

The Chl *a* concentrations for the LN and HN cultures reactor sets in Figures 4.2A increased similarly over 12 days of the experiment with a final Chl *a* mg / L very close to each other. A regression of the LN and HN cultures (all data points) Chl *a* mg/ L / d, over 12 days, indicated a high correlation between the two studies ($R^2 = 0.93$, $p < 0.001$). In Figure 4.2A, the LN culture, on days 11 and 12, the Chl *a* concentrations were 2.04 and 1.88 Chl *a* mg / L respectively.

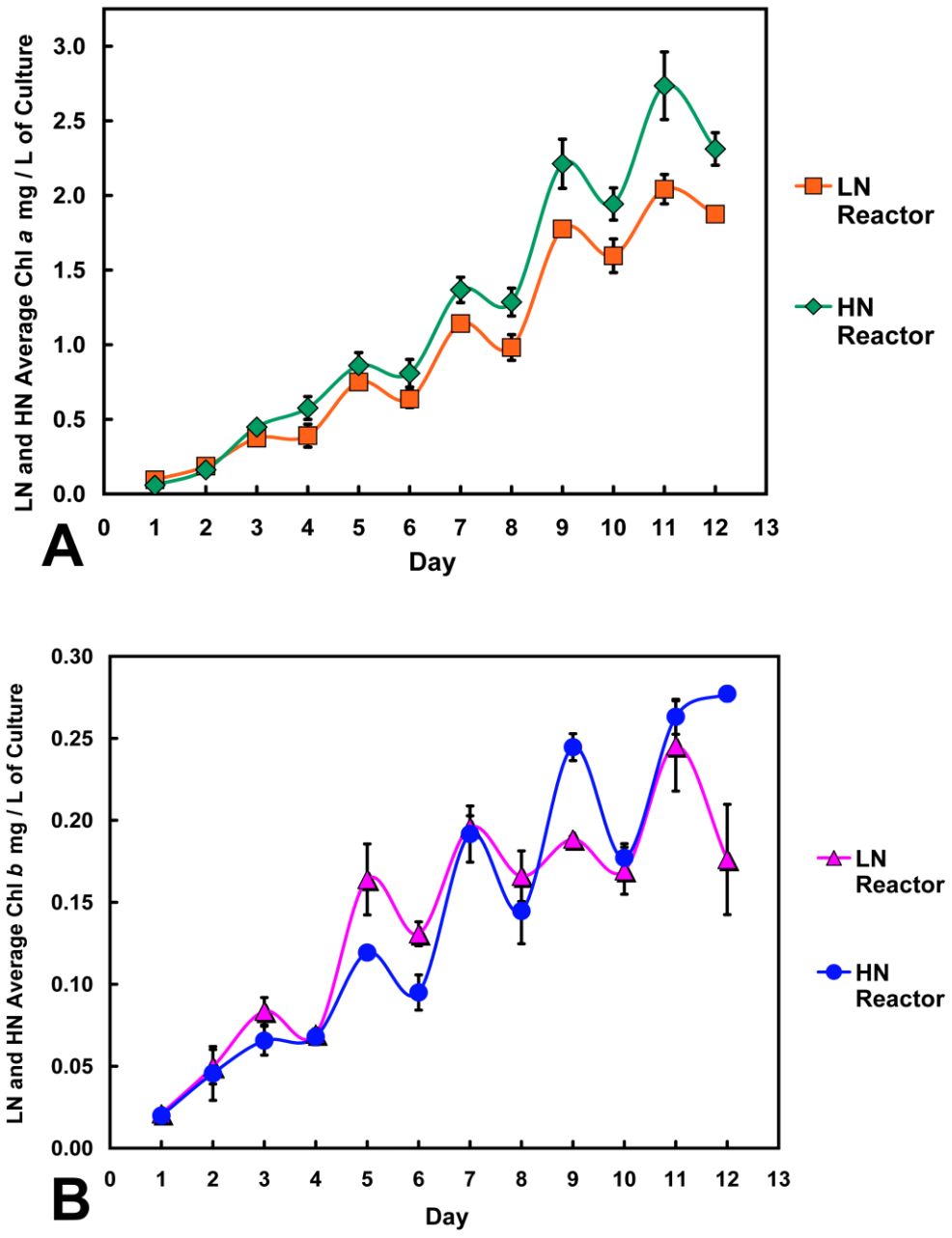

Figure 4.1 LN and HN Chl *a* and Chl *b*
Chl *a* concentrations for the LN and HN experiments. The LN 4.1A and HN 4.1B graphs display the average Chl *b* mg / L / d. n = 3, ±SE bars shown on each data point. Reactors 1, 2, 3, and 4 represents subsets of 3 flasks sampled every 2 days. n = 3, ±SE bars are shown on each data point.

The HN culture Chl *a* concentrations on days 11 and 12, Figure 4.2A, were respectively 2.74 and 2.31 Chl *a* mg / L. The HN culture Chl *a* concentration on

days 11 and 12 exceeded the LN culture Chl *a* concentrations for day 11, by 25% and day 12 by 19%.

. The LN culture Chl *b* plot in Figure 4.1B began with an increasing average Chl *b* concentration. The LN culture reactor increased the Chl *b* concentration production from day 1 to a peak on day 11 (0.25 mg Chl b / L) and on day 12 dropped to 0.18 mg Chl b / L) . The LN culture reactor Chl *b* regression line slope for days 1 to 12 had a rate of 0.017 Chl *b* mg / L / d and a $R^2 = 0.78$.

The HN culture reactor Chl *b* average concentration increased linearly (Figure 4.1B) with a regression line rate for days 1 to 12 of 0.023 Chl *b* mg / L / d with a $R^2 = 0.90$.

On day 12, the HN culture reactor had a maximum average Chl *b* concentration larger than the LN culture maximum average Chl *b* concentration on day 11.

The LN and HN cultures Chl *a* concentrations (mg / L / d) increased at similar rates with high correlation between the two, though, the final HN Chl *a* concentrations were higher by 19% than the LN Chl *a* concentrations. The Chl *a* growth did not stop during the entire study and was key to the continued algal energy production. The LN culture with the lower nitrate concentrations had a lower Chl *a* concentration compared to the HN culture Chl *a* concentration.

The LN and HN cultures contained green algae, diatoms and cyanobacteria, but cyanobacteria do not synthesize Chl *b.* [20] The green algal genera in LN and HN cultures created the Chl *b* and *Chlorosarcinopsis* was the predominant algal genera from days 1 to 12. The Chl *b* concentrations on day 12 from the two HN

reactor sets (Figure 4.12B) were higher than the Chl *b* concentrations from two LN reactor sets (Figure 4.12A).

4.2.2 ALGAL COMMUNITY COUNTS FROM IMAGES

The algal counts, Figures LN 4.2A and HN 14.2B, on each captured image were separated by the algal morphology and are referred to as unicelluar and filamentous algal cells in the graphs.

The unicelluar algal cell outlines were distinct and readily counted. When the filamentous cells were intertwined they were counted a one cell.

Overall, the LN culture unicellular and filamentous cell counts (counts x 10^6 / mL of culture) were lower than the HN culture unicellular and filamentous cell counts. Both LN and HN culture unicellular counts continuously increased over the 12 days. The LN and HN culture unicelluar algal growth rates from two linear regressions were:

- LN 0.3931 x 10^6 cell counts / d (R^2 =0.80)
- HN 0.9942 x 10^6 cell counts / d (R^2 =0.88)

The LN reactor growth rate is 40% of the HN reactor growth rate.

Coincidentally on day 7 for both the LN and HN studies, the filamentous cell counts were nearly the same (LN filamentous cell counts = 4.3 x 10^6, HN filamentous cell counts = 4.6 x 10^6).

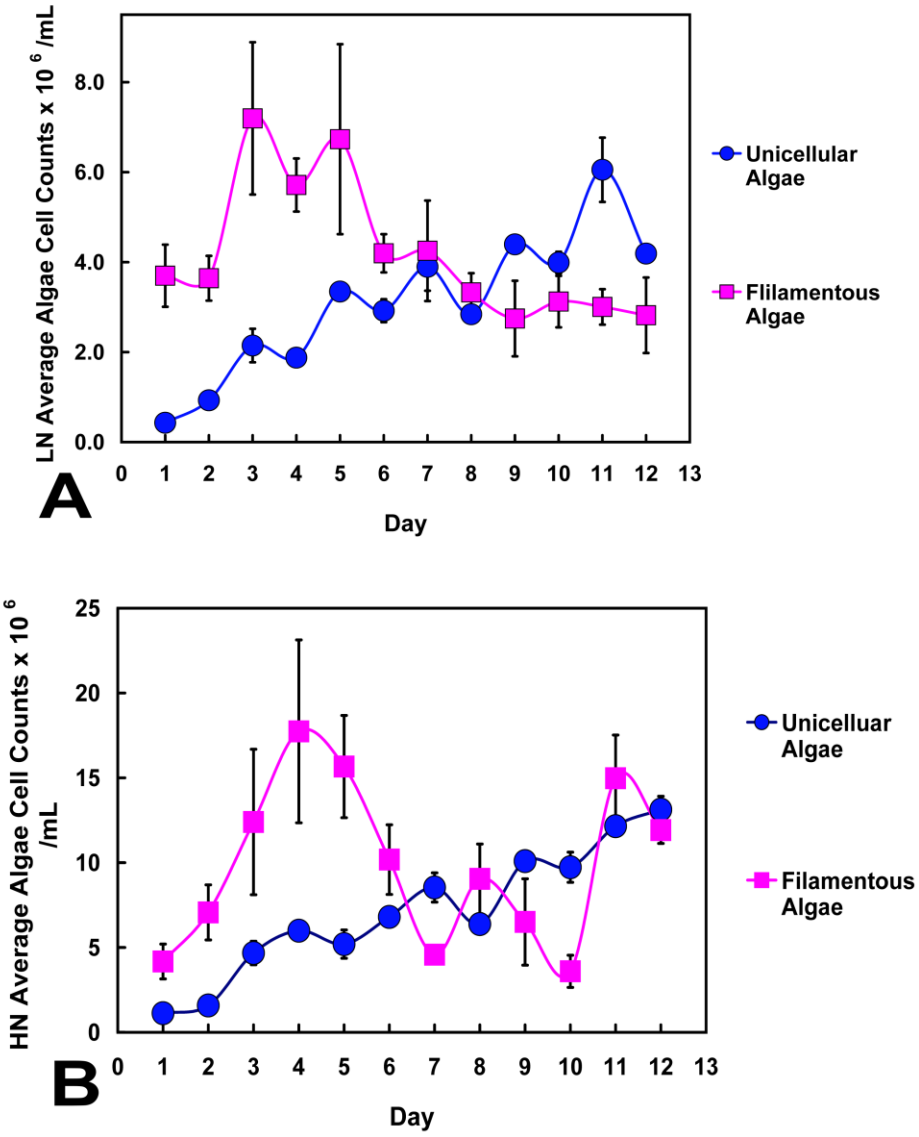

Figure 4.2 LN and HN algae community counts

Figure LN 4.2A and HN 4.2B displays the LN and HN average counts respectively for the unicelluar and filamentous algae per mL of culture. The images were taken on a hemocytometer with a Olympus BX51 microscope at a magnification of 100X, and analyzed with two free open source programs: ILastik and ImageJ. n = 3, ±SE bars are shown on each data point.

4.2.3 NO_{3-} AVERAGE CUMULATIVE CONSUMPTION

The NO_{3-} average cumulative consumption of the LN and HN cultures indicated ongoing assimilation of the media NO_{3-} by the algal communities.

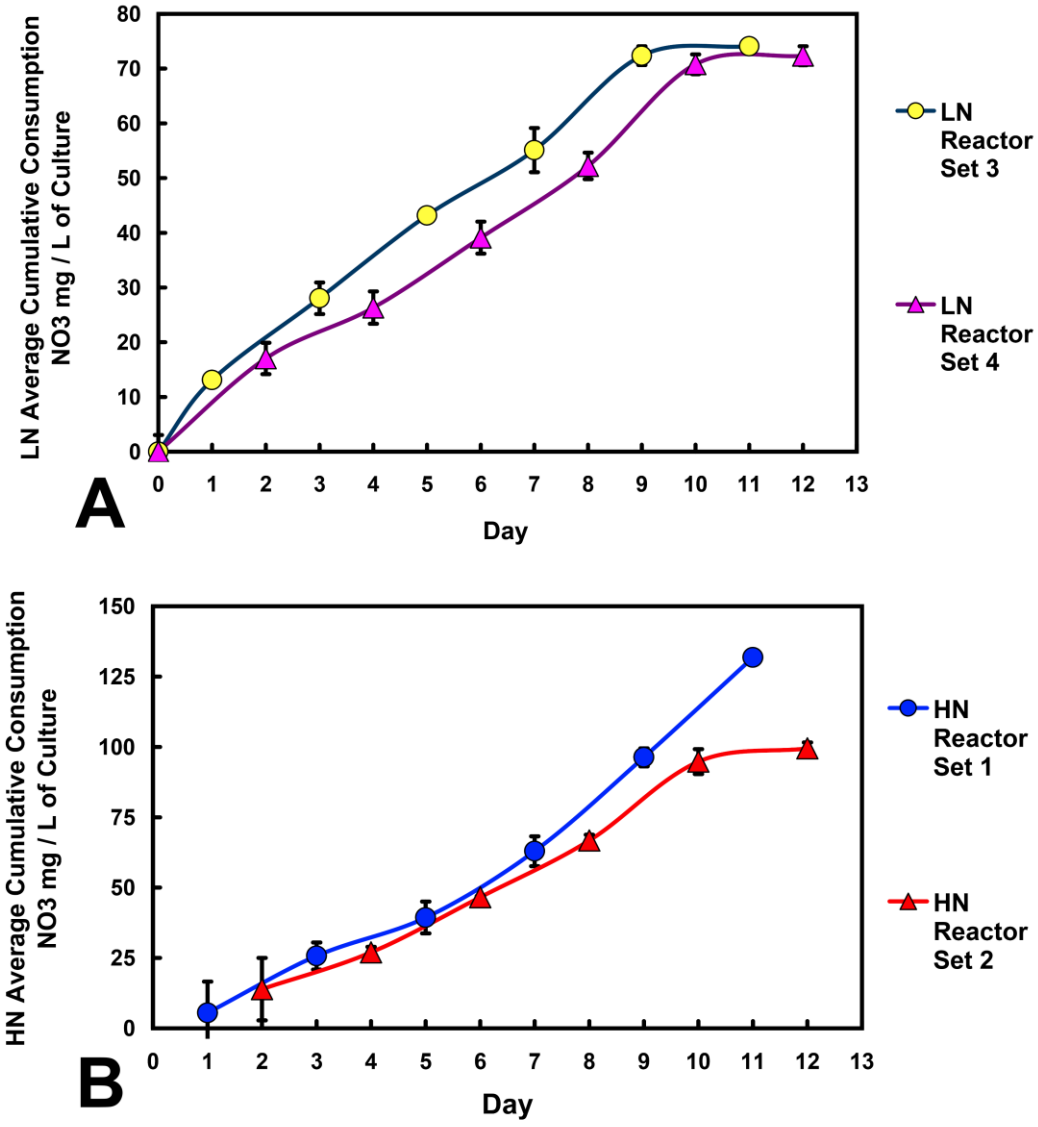

Figure 4.3 LN and HN algal average NO_{3-} cumulative consumption

Figure 4.4A displays the LN average NO_{3-} cumulative consumption mg / L / d. Figure 4.4B graphs the HN average NO_{3-} cumulative consumption mg / L / d. n = 3. ±SE bars shown on each data point.

The starting average NO_{3-} concentrations (day 0) for the LN and HN media were 75 mg NO_{3-} / L and 179.2 NO_{3-} mg / L respectively.

A linear regression line through days 1 to 9 for the RT reactor set 3 of NO_{3-} (Figure 4.3A) had an average consumption slope of 7.28 NO_{3-} mg / L / d with a $R^2 = 0.99$. The regression line through the LN reactor set 4 (Figure 4.3A) for days 1 to 9 had a average consumption slope of 6.67 NO_{3-} mg / L / d and a $R^2 = 0.99$.

The LN reactor set 3 consumed all the NO_{3-} by day 10 and the LN reactor set 4 by day 12. Both of the LN cultures in reactor sets 3 and 4 consumed 99% and 96% respectively of the initial average NO_{3-} concentration (75 mg NO_{3-} / L) from the medium in each reactor.

The (Figure 4.3B) regression for the HN culture NO_{3-} reactor set 1 over days 1 to 9 had a slope of 10.9 NO_{3-} mg / L / d and $R^2 = 0.97$. The (Figure 4.3B) regression of the HN culture NO_{3-} reactor set 2 for days 2 to 10 resulted in a slope of 10.1 mg NO_{3-} / L / d and $R^2 = 0.98$. Neither of the HN reactors sets 1 and 2 consumed all the NO_{3-} in the media.

The final LN culture average NO_{3-} cumulative consumption for reactor set 3 on day 11 was 74.1 NO_{3-} mg/ L / d and for the reactor set 4 on day 12 the average cumulative consumption was 72.3 mg / L / d. The final HN NO_{3-} average culture cumulative consumption for reactor set 1 on day 11 was 131.8 NO_{3-} mg/ L / d

and on day 12 the HN culture reactor set 2 final average NO_{3-} consumption, was 99.5 NO_{3-} mg / L / d.

As the NO_{3-} was consumed, other algal community parameters changed due to the decreasing nitrate concentrations, particularly algal biomass. The cultures in the two HN reactor sets 1 and 2, did not consume all the average NO_{3-} concentration. By day 11 the HN reactor set 1 had consumed 74% of the original NO_3 concentration (179.2 mg NO_{3-} / L) and by day 12 HN reactor set 2 had consumed only 56% of the original NO_{3-} concentrations.

The LN culture average NO_{3-} consumption of the two LN reactor sets clearly indicated there was a very low concentration at the end of the LN study. The LN culture average NO_{3-} consumption compared to the HN culture in the reactor sets 1 and 2 had consumed only a 1/2 and 3/4 of the average NO_{3-} concentrations respectively.

The LN and HN cultures contained the *Plectonema* genera, a nitrogen fixing diazotroph, which can pull in atmospheric nitrogen and convert it to ammonia in the algal cell and may have added ammonia to the reactor sets 1 and 2. It is possible that the HN culture consumed all the NO_{3-} that was necessary for growth over the 12-day experiment.

4.2.4 AVERAGE NO_{3-} CUMULATIVE CONSUMPTION PER CHL A

As the LN and HN cultures average Chl *a* concentrations increased, so did the average NO_{3-} cumulative consumption concentration. In Figure 4.4, both the LN

and HN culture daily cumulative NO_{3-} concentrations were divided by the corresponding average Chl *a* concentrations (NO_{3-} mg / Chl *a* mg / d). A stable ratio was observed between the NO_{3-} and Chl *a* concentrations except for the first two days. The day 1 and 2 LN and HN culture NO_{3-} concentrations were normalized to the corresponding very low Chl *a* concentrations, which resulted in ratios which greater than 230 NO_{3-} mg / Chl *a* mg and 594 NO_{3-} mg / Chl *a* mg respectively.

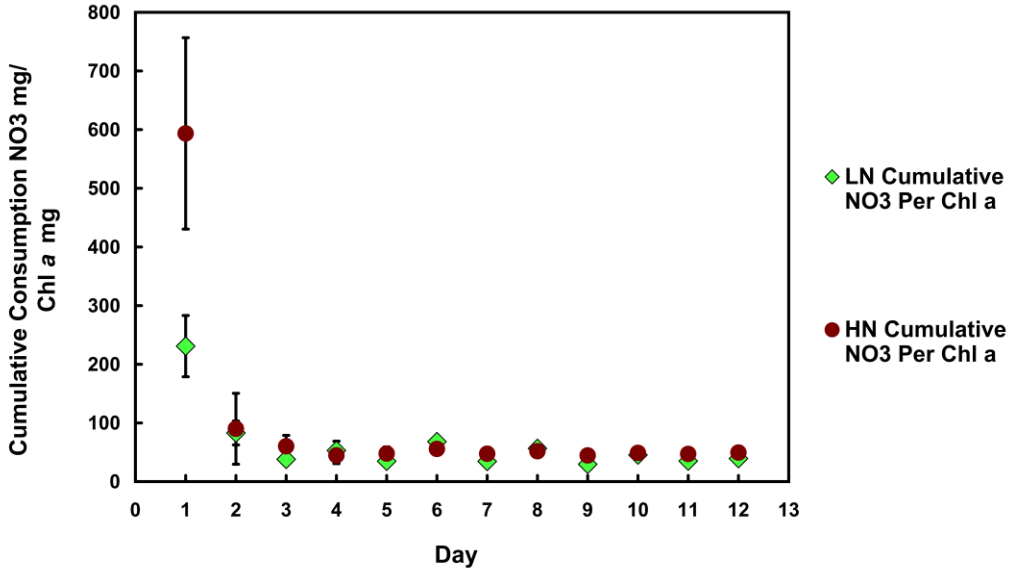

Figure 4.4 LN and HN average cumulative consumption NO_{3-} per Chl *a*
n = 3, ±SE bars are shown on each data point.

Over days 3 through 12, the average ratio between the LN culture cumulative NO_{3-} consumption concentration and Chl *a* concentration was 43.42 mg ±3.85 SE (NO_{3-} mg / Chl *a* mg / d). For the HN culture days 3 through 12, the ratio between theNO_{3-} average cumulative consumption concentration and average Chl *a* concentration was 49.79 mg ±1.55 SE (NO_{3-} mg / Chl *a* mg / d). The LN

culture cumulative consumption concentration ratio to the Chl a concentration was lower than that of the HN.

The LN and HN Chl *a* average concentrations were normalized to the corresponding NO_{3-} cumulative consumption concentrations. The first two days of the LN and HN experiments had low Chl *a* average concentrations, which produced high ratios over 200 NO_{3-} mg / Chl *a* mg / d. For the days 3 to 12 the average ratio between the LN culture cumulative NO_{3-} concentrations and the Chl *a* average concentrations was 43.42 mg ±3.85 SE (NO_{3-} mg / Chl *a* mg / d). The HN culture NO_{3-} concentration normalized to the Chl *a* concentration had a average ratio, for the days 3 to 12 L was 49.79 mg ±1.55 SE (NO_{3-} mg / Chl *a* mg / d). The LN and HN average NO_{3-} to Chl *a* ratios indicated a correlation between the cumulative consumed NO_{3-} and the production of Chl *a.*

4.2.5 TOTAL AVERAGE FAMES

During the creation of the LN and HN culture biomass, the algal communities also increased the synthesis of lipids required for membranes, cellular energy and cell walls. The average total FAMEs of the LN and HN cultures, Figure 4.5, were calculated separately. Only the identified FAME concentrations were summed for each day.

On day 1, the LN culture total average FAMEs increased from 1.43 (mg FAMEs / L / d) to a peak value of 2.03 (mg FAMEs / L / d) on day 5. On day 1, the HN culture total average FAMEs increased from 2.10 mg (FAMEs / L / d) to a peak value of 3.23 mg FAMEs / L / d, on day 4. The HN culture total average

FAMEs peaked sooner and to higher total average FAMEs value than the LN culture.

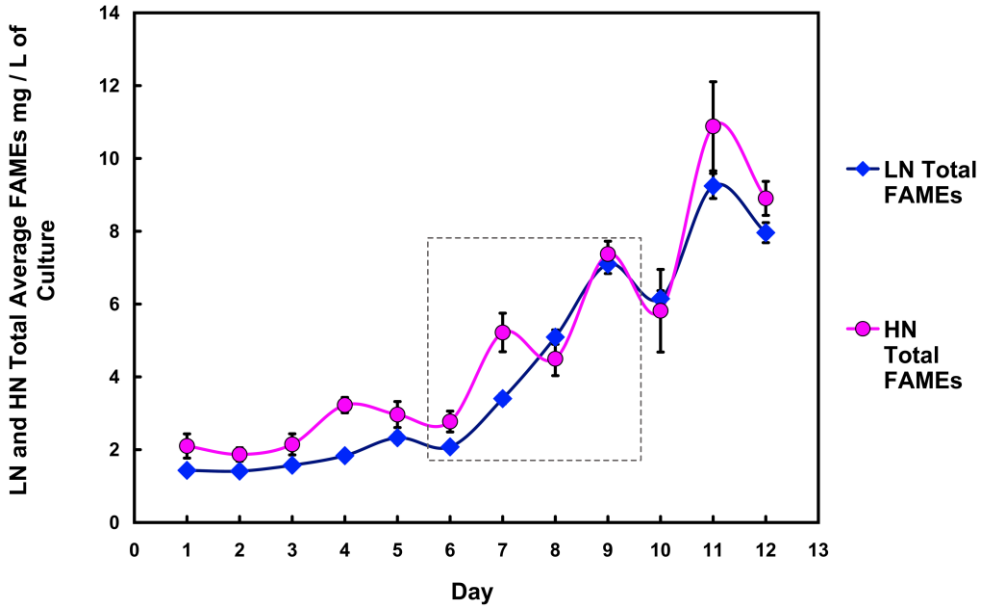

Figure 4.5 LN and HN total average FAMEs
Figure 4.6 graphs the total average of the LN and HN FAMEs cultures mg / L / d. n = 3, ±SE shown on all data points. The area enclosed in the dashed square captures the graphical segments of the LN and HN cultures with increasing FAMEs concentrations.

In Figure 4.6, the LN culture for days 6 to 9, the regression line R^2 value was 0.99 , indicating that the LN culture average total FAMEs data points were a good linear fit for each of the 4 days. This was an important, unexpected result.

The LN culture regression line slope describes a common lipid synthesis rate for the two LN algal reactor sets (3 and 4) over the 4 days. The HN line regression for the same 4 days had an R^2 value of 0.79 and does not have a good linear fit of the HN culture average total FAMEs data points.

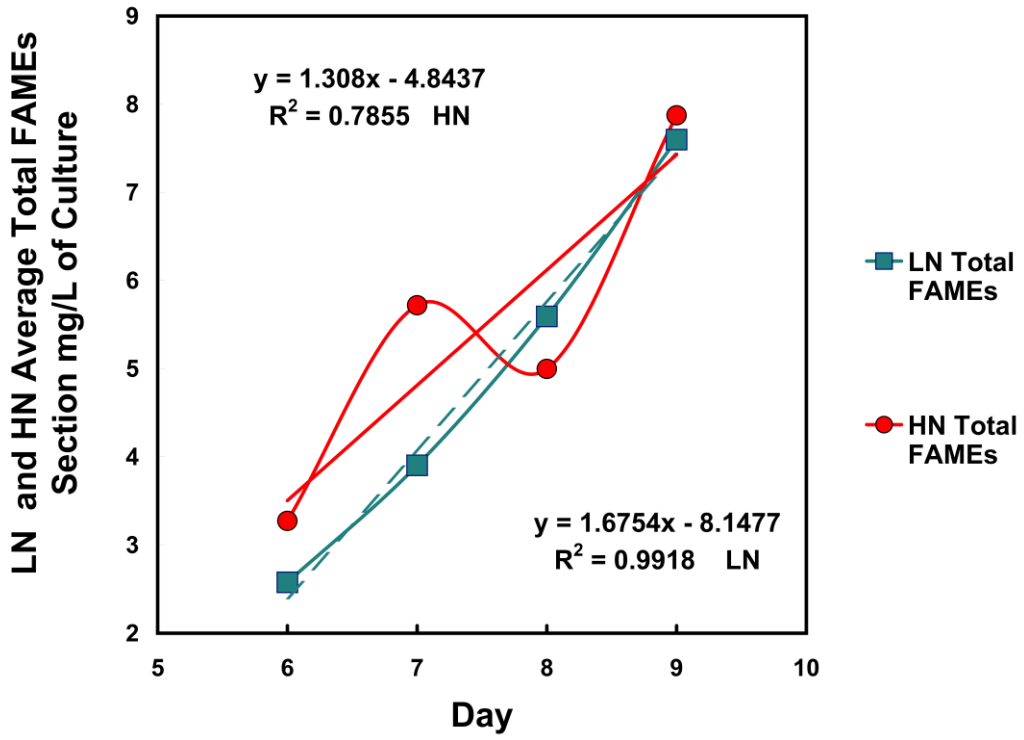

Figure 4.6 LN and HN enlarged total FAMEs section
This enlarged graph section displays the LN and HN cultures total average FAMEs concentration mg / L / d containing data from both of the LN and HN two reactor sets over study days 6 to 9. The LN regression line is the teal dotted line with the regression equation and R^2 value located in lower right portion of the graph. The HN regression line is the straight solid red line with the regression equation and R^2 value located in upper left portion of the graph.

The total average FAMEs in Figure 4.6 indicates something occurred on day 6 in the LN culture which pushed the two independent flask sets 3 and 4 to begin to simultaneously producing larger amounts of total FAMEs till day 9. The average total FAMEs production in both flask set had the same rate of production from the slope value of the regression line = 1.68 mg FAMEs / day.

4.2.6 Total average FAMEs per Chl a

The LN total average FAMEs per Chl *a,* Figure 4.7, over days 3 to 12, had an average ratio of 1.18 FAMEs mg / L / Chl *a* mg ± 0.19 SE. The HN total average

FAMEs per Chl *a,* over days 3 to 12, had an average ratio of 1.44 FAMEs mg/ L / Chl *a* mg ± 0.25 SE. The HN average total FAMEs per Chl *a* ratio was larger than the LN ratio average total FAMEs per Chl *a* ratio.

The solar energy captured by the Chl *a* molecule was converted to chemical energy and must be present for FAME synthesis.

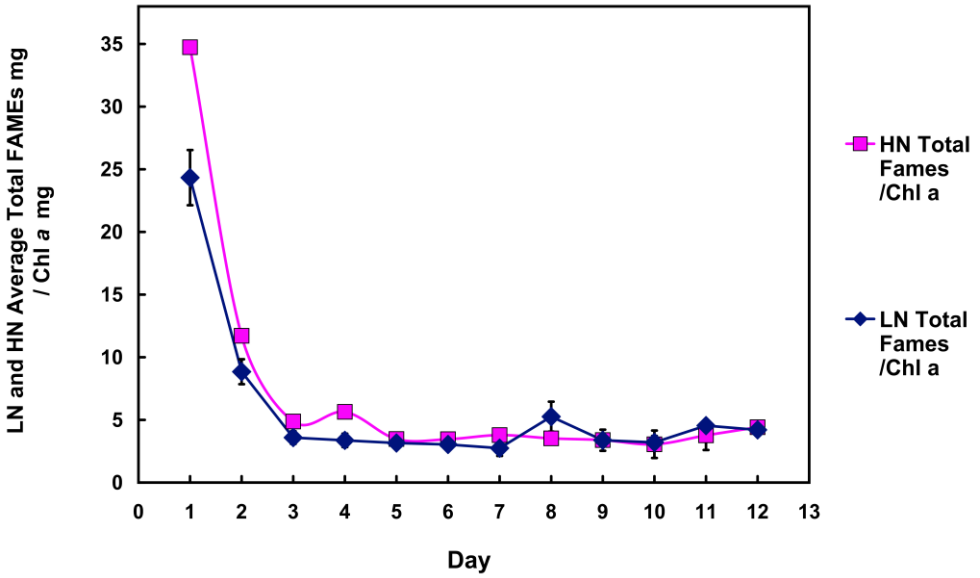

Figure 4.7 LN and HN total average FAMEs per Chl a
The LN and HN cultures total average FAMEs day 1 value peaks resulted from very low Chl a concentration (mg/ L / d). n =3, ±SE bars shown on each data point.

The normalization of the LN and HN total average FAME mg / Chl *a* mg discloses a relationship between the total average FAME production and the Chl *a* mg/ L. The LN and HN culture total average FAMEs were graphed from the summation of the average identified FAME peaks (mg / L) for each of the LN and HN culture studies. The LN study total FAMEs on day 1 was 1.43 mg FAMEs / L / d, and increased to a peak of 2.03 mg FAMEs / L / d on day 5

. During the LN growth, Figure 4.8, a section of LN culture total average FAMEs for days 6 to 9 resulted in a very linear section with a positive slope of 1.68 mg total FAMEs / L / d. When the increase in the LN FAME concentration was compared to the HN culture FAME concentration for the same days 6 to 9, the HN slope of 1.31 mg total FAMEs/ L / d, was less than that of LN culture.

The LN culture on day 6 reached a point where the LN culture NO_{3-} concentration contained only 35.1 mg NO_{3-} / L and on day 9 the LN NO_{3-} concentration dropped down to 1.72 mg NO_{3-} / L. The precipitous drop in the LN culture NO_{3-} concentration triggered an immediate stress to store lipids on the culture as evidenced by the increasing LN FAME concentration (higher linear slope). After day 9, the LN culture continued to grow similarly to that of the HN culture.

4.2.7 AVERAGE SATURATED AND UNSATURATED FAMES

The LN and HN saturated, and unsaturated lipid biosynthesis had distinct FAME production Figure 4.8 patterns over the 12-day experiments.

Not all the individual FAMEs listed in the legend were found in the LN and HN cultures from day 1 to the end of each experiment.

On day 7, the LN and HN cultures started producing 3 unsaturated FAMEs (C18:1, C18:2, 18:3), where the production lasted until day 12.

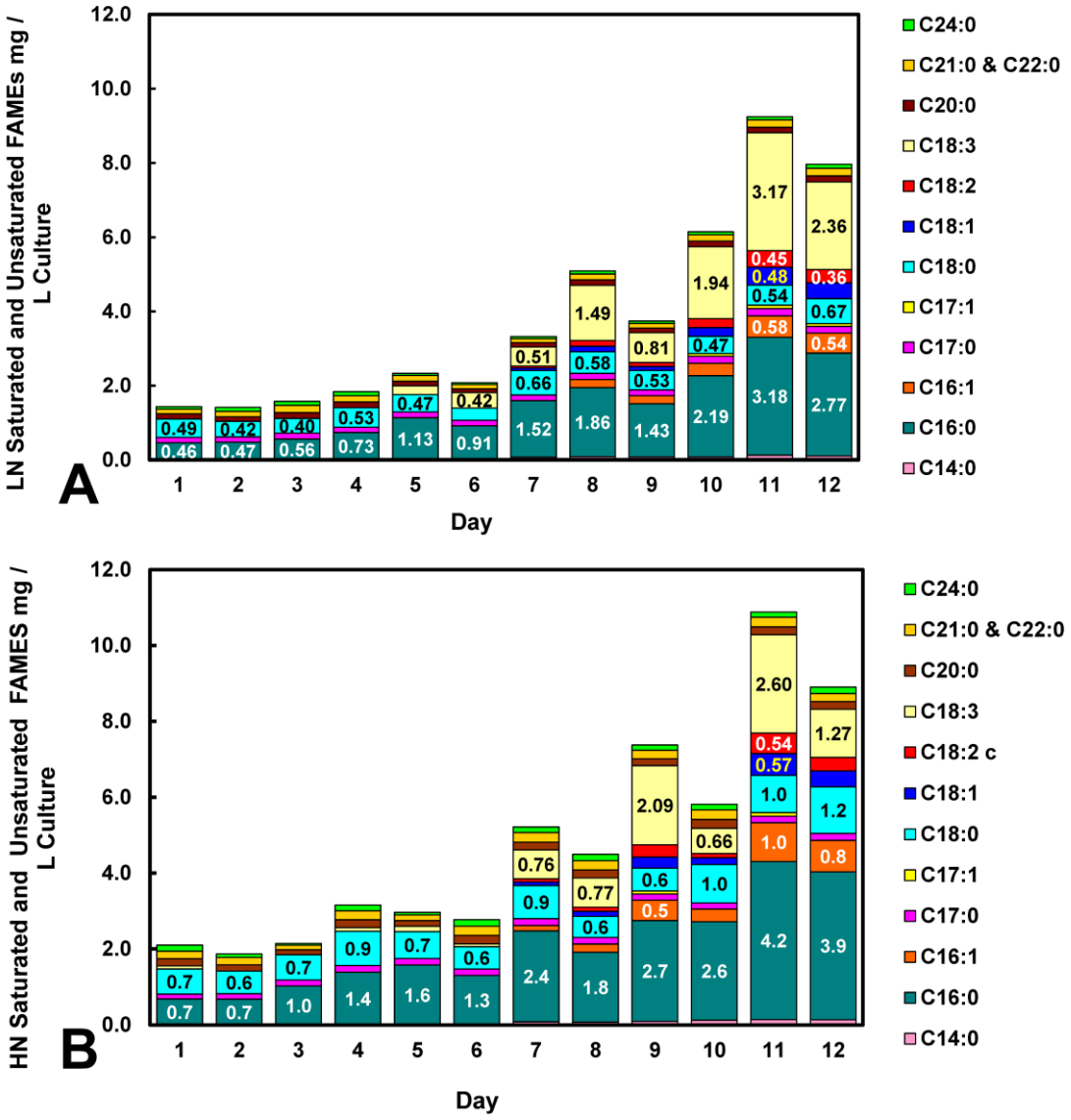

Figure 4.8 LN and HN average saturated and unsaturated FAMEs
Figure 4.9A stacked column chart contains all the LN identified FAMEs mg/ L / d and Figure 4.9B stacked chart contains all the HN identified FAMEs mg / L / d. Both LN and HN had n = 3.

Both the LN and HN studies, the algal cultures synthesized a specific set of FAMEs. Both the LN and HN cultures created 6 saturated FAMEs from day 1 to day 12 and they were C16:0, C17:0, C18:0, C20:0, C21:0&C22, and C24:0. The saturated FAMEs C21:0 & C22 were co-eluted from the gas chromatograph column and listed as one FAME peak.

Over the LN and HN cultures 12-day study, the LN culture C16:0 FAME concentrations were lower than those of the HN culture C16:0 FAME. The LN culture C16:0 FAME reached the largest concentration of 3.18 mg / L / d on day 11 and the HN culture attained highest concentration for FAME C16:0 of 4.17 mg / L on day 11. The HN culture C16:0 concentration on day 11 was 0.99 mg / L / d larger than the LN culture C16:0 concentration.

The LN culture produced the largest unsaturated FAME, C18:3, with a concentration of 3.17 mg / L on day 11. The highest unsaturated HN culture, FAME 18:3, had a concentration of 2.60 mg / L / d, which also occurred on day 11. The LN culture C18:3 FAME concentration exceeded the HN culture C18:3 FAME concentration by 0.57 mg / L / d.

The LN and HN cultures produced the same types of FAMEs, but the daily production rate of each FAME varied widely. The LN and HN cultures FAMEs C17:0 and 18.0 did not increase over the 12 days, but both FAMEs formed a horizontal plot with what appears to be small consumptions and then syntheses every day. These 12-day FAME concentrations were averaged to better represent their inconsistent growth.

The LN culture C17:0 FAME average concentration over the 12 days was 0.16 mg ± 0.005 SE. and the HN culture C17:0 FAME average concentration over the 12-day study was 0.17 mg ± 0.005 SE. The LN culture C18:0 FAME average concentration over the 12 days was 0.25 mg ± 0.030 SE and the HN culture C18:0 FAME average concentration was 0.78 mg ± 0.062 SE.

Both LN and HN culture C16:0, and C18:3 FAME concentrations increased until day 11. The LN and HN average C16:0, C18:0, and C18:3 FAME concentrations had the largest percentages of the saturated and unsaturated FAME concentrations (LN culture C16:0 34.8%, LN C18:0 8.5% and LN C18:3 C 29.6%) and the (HN culture C16:0 43.8%, HN C18:0 13.8.%, and HN C18:3 14.3%).

On study day 12, the LN and HN culture FAMEs: C14:0, C17:0, C17:1, C18:1, C18:2, C20:0, C21:0, C22:0, and C24:0 had less than 5% of the total FAME concentrations.

The HN FAME synthesis, in general, produced larger FAME concentrations than the LN FAMEs except for the C18:3 unsaturated FAME, which had a larger concentration on day 11 compared to the HN FAME 18:3.

4.2.8 FAMES FOR BIODIESEL

The FAME ratio of saturated to unsaturated percentage of the total determines how the well the FAMEs will burn in a compression ignition diesel engine.[9]

The average saturated to unsaturated ratio (S/U) for the LN culture over the first 7 days was 2.05, and over the same days, the HN culture had an average S/U ratio of 2.02 Figure 4.9.

Both the LN and HN culture S/U ratios on day 8 were the same at 1.03. The average LN culture S/U ratio for the days 9 through 12 was 0.55 and for the same days, the average HN culture S/U ratio was 0.60.

The importance of the ratios of the saturated to unsaturated FAMEs in both the LN and HN cultures relates to two characteristics of the FAMEs when blended with No.2 petro diesel, combustion performance, and lubricity.

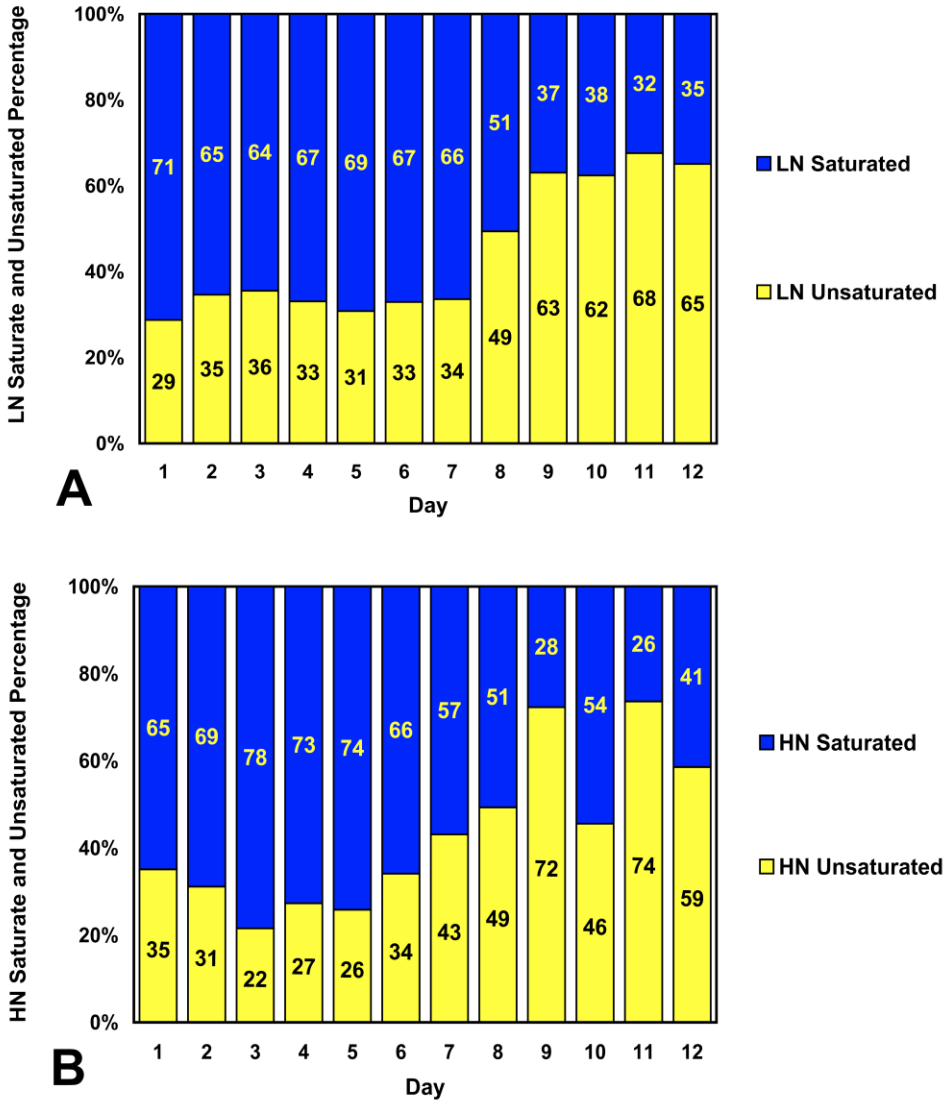

Figure 4.9 LN and HN average saturated and unsaturated FAMEs percentage

Figure4.9A displays the LN culture average saturated and unsaturated FAMEs percentage of the total FAMEs / d and figure 4.10B displays the HN average saturated and unsaturated FAMEs percentage of the total FAMEs / d.

Fatty acids do not perform favourably when added directly into diesel engines, but the transesterified FAMEs (biodiesel) do. Long straight saturated FAMEs chains burn rapidly in a diesel engine.[94]

The more unsaturated double bonds in the straight FAMEs chain, the lower the CN due to the additional heat required to break the double bonds during autoignition (carbon single bond, 347 kj /mol, carbon double bond, 611 kj /mol). [95]

Long chain unsaturated FAMEs in biodiesel have been shown to impart lubricity when blended with No.2 petro diesel. The FAME 18:3 (methyl linolenate) was shown to provide sufficient lubricity when added at a volume of 1% to No.2 petro diesel and tested on a High-Frequency Reciprocating Rig (HFRR). The test used a weighed steel ball pushed against a stationary steel disk immersed in the diesel fuel mixture which vibrated at 50 HZ for 75 minutes. The diameter of the wear scar left on the ball is measured under a microscope. International Standards Organization (ISO) requires that the ball wear scar be a maximum of 0.45 mm to pass when used in standard diesel engines. [7]

4.3 ALGAL COMMUNITY COMPOSITIONS

The LN culture samples were analyzed for days 0, 4, 5, 11 and 12. The LN culture on days 4 and 5 were selected to capture the community present when the lipid production reached the first LN lipid peak.

The HN culture samples were analyzed for days 0, 3, 4, 11, and 12. The HN for day 3 and 4 samples were taken to determine the algal culture community present at the first highest value, Figure 4.10.

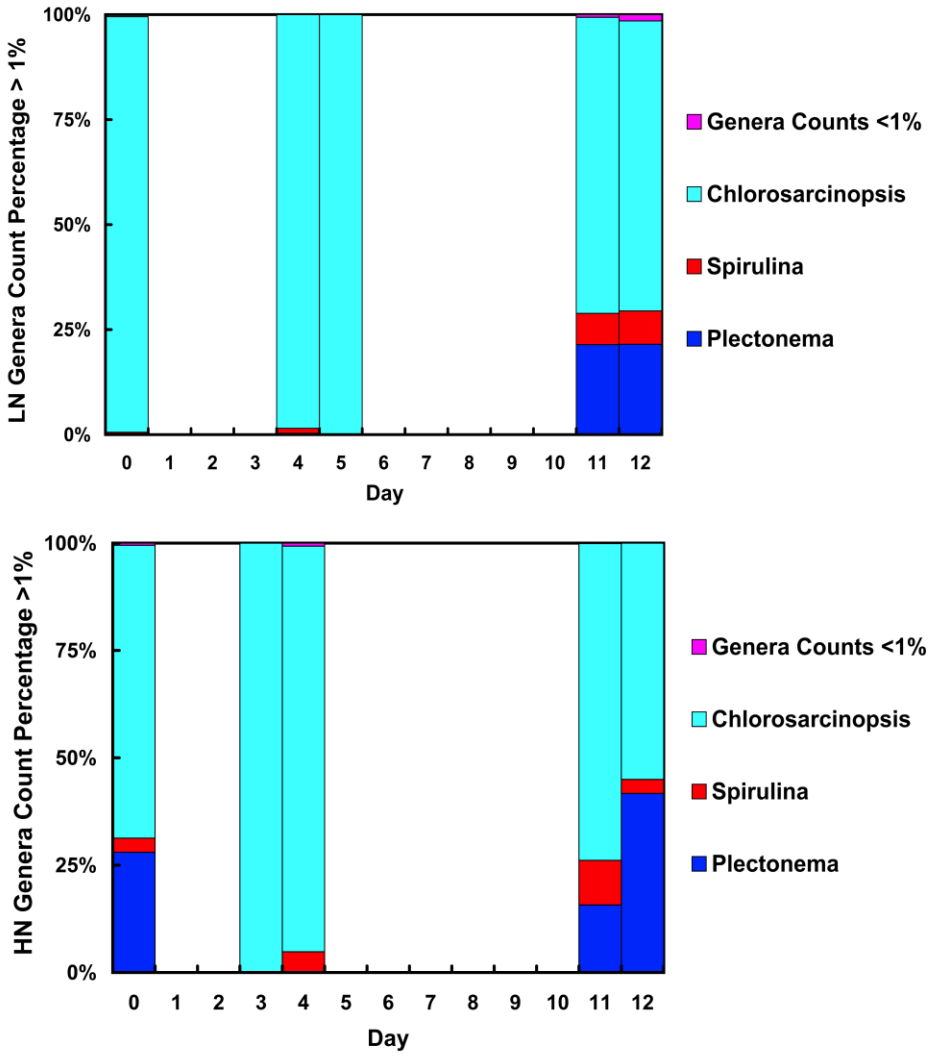

Figure 4.10 LN and HN culture algal community genera by Illumina MiSeq
Figure 4.10 LN and Figure 4.10 HN community composition genera expressed as a percentage of total genera count for each day**.** Day 0 represented the percentage of genus counts in the algal community seed culture used to inoculate the reactors. Genra less than 1% of the total counts are presented in Appendix Chapter 7.2.1 LN and HN genus percentage counts less than 1%.

All LN and HN culture genera percentages over 1% were calculated for each sample day. Each genera sample percentage count was calculated by dividing the genera sample count by the summed total of all counts. Each genera percentage was plotted on a stacked bar graph by day.

The *Chlorosarcinopsis* genera percentages of the total were higher than any other genera in both the LN and HN culture studies over all sampled days.

On days 0, 4, 5, 11, and 12 the LN culture *Chlorosarcinopsis* genera percentages were respectively 99.0, 99.5, 99.8, 70.5, and 69.1. The LN *Plectonema* genera percentages for days 0, 4 and 5 were less than 1% of the total, but the percentages on days 11 and 12, were 21.4 and 21.5 respectively. The LN culture *Spirulina* genera percentages on days 0, 4, and 5 were each less than 1% of the total for each day, although on days 11 and 12 the percentages were 7.4 and 7.9 correspondingly. The genera percentage named (Genera Counts < 1%) on all sample days were less than 1% except for day 12, where the LN culture percentage was 1.5.

The HN culture *Chlorosarcinopsis* genera for days 0, 3, 4, 11, and 12 revealed percentages of 68, 99.8, 94.5, 73.8, and 55.0 respectively of the total genera counts. The HN culture *Plectonema* genera percentages on days 0, 11, and 12 were 28.0, 15.7, and 41.7 respectively. The HN culture *Spirulina* genera percentages on days 0, 4, 11, and 12 were respectively 3.3, 4.82, 10.4, and 3.27.

In both the LN and HN cultures there were only 3 identified genera greater than 1% of the total population: *Chlorosarcinopsis, Plectonema,* and *Spirulina sp.* with the *Chlorosarcinopsis* genus having the largest percentage of the total

genera counts over the all 12 days of the LN and HN studies. On day 0, the HN culture *Plectonema* genera counts had 28% of the total counts, and on day 12 the *Plectonema* genera counts increase to 42% of the total counts. In the LN culture, the *Plectonema* genera counts were not identified until days 11 and 12 with a count percentage of 21% and 22%. For the LN and HN studies, the *Spirulina sp*. genera counts were a small percentage of the total on days 0, 4, 11, and 12.

4.3.1 CULTURE SHANNON DIVERSITY INDEX

The Shannon diversity index (Shannon index H) calculations, Figure 4.11, for the LN and HN cultures included all the algal species counts greater than 1% alnd less than 1% present for each day. On day 0 the LN Shannon index was low (H_{LN} = 0.04), then increased slightly on day 4 (H_{LN} = 0.09). For more explanation of the Shannon diversity index and variables, see Experiment 1, Section 3.2.8. The reason the LN and HN diversity day 0, seed cultures Shannon diversity was different stems from the genera present in the cultures when sampled. The *Chlorosarcinopsis* genera was the largest in the cultures and drive the Shannon index to very low value. Compared to the HN culture, the *Chlorosarcinopsis* genera held a substantial number counts, but were there was a large presence of *Plectonema* and *Spirulina.*

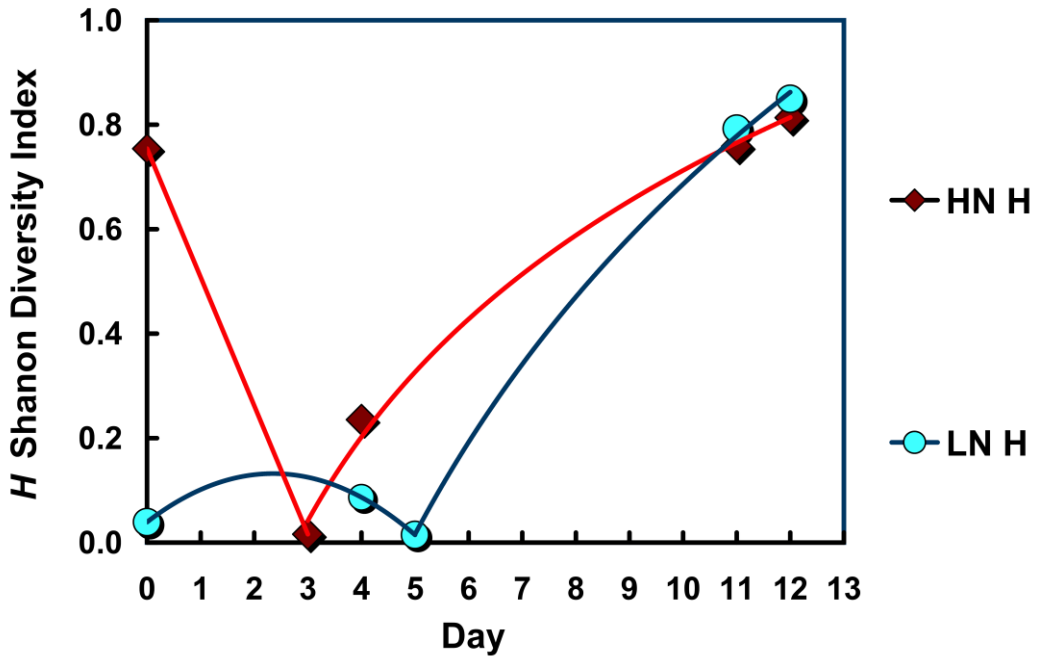

Figure 4.11 LN and HN Shannon diversity index
The LN and HN Shannon diversity index calculated from Chapter 2, EQ (4) and EQ (5). The LN and HN algae data came from the Illumina MiSeq, next generation algal DNA sequencer, which produced the algal cell counts and identified the algae species from the algal cell reassembled DNA sequences.

On day 5 the HN cultures Shannon index (H) fell to 0.01, indicating a significant loss of species to only 3: *P. terebrans, Spirulina sp.,* and *Chlorosarcinopsis*.

Six days later on day 11, the LN culture (H_{LN}) increased to 0.80 and on the final experiment day 12, the (H_{LN}) reached 0.85 with a species count of 11.

On day 0 the HN culture Shannon index was high (H_{HN} = 0.75), but on day 3, the HN culture Shannon index dropped to 0.016. The next day, the HN culture Shannon index increased to H_{HN} = 0.24. On the last two days (11, 12) of the HN study, the Shannon indices increased to 0.76 and 0.81 respectively. On day 12 the number of species was 7.

The Shannon diversity index assigns a number for the species count (S) in a population and sums all the individuals (N) in each specie. The number of species found in both the LN and HN culture fluctuated and came from the species representing less than 1% of the total counts as determined by the MiSeq Illumina Sequencing next generation sequencing. The less than 1% populations often have many species that fall just above or below the cutoff limits required to declare the species present in a sample.

The data for the LN culture Shannon diversity index came from the algal community genera obtained from the LN and HN algal population analysis described in Section 2.3.4. On day 0, the LN culture Shannon diversity index was low, H_{LN} = 0.04, with S = 5 and total N = 69181 present. Later on day 4 in the LN culture the Shannon diversity index grew to H_{LN} = 0.09 with S = 5 and N = 99799. On day 5 the Shannon diversity index dropped to H_{LN} = 0.015 with only S = 3 and N = 49888. The Shannon diversity index increased to H_{LN} = 0.85 on day 12 as the number of species increased S = 10 and N = 59731.

On day 0 the HN culture Shannon diversity index was H_{HN} = 0.75 and the S =10 with total species N = 110431. On day 4 of the HN culture the Shannon diversity index dropped to H_{HN} = 0.24 due to species loss (S = 4) and N = 147937. The Shannon diversity index HT culture on day 12 rose to H_{HN} = 0.81 with an increase in species (S = 7) and N = 77273.

Both the LN and HN Shannon diversity indices varied across the two studies and reflected the large culture population dynamics in the reactor sets.

4.4 CHAPTER 4 EXPERIMENT 2 CONCLUSION

The LN and HN cultures Chl *a* concentrations (mg / L / d) grew at similar rates, but the final HN Chl *a* concentrations were higher by 19% than the LN Chl *a* concentrations.

The low nitrate concentration in the LN cultures produced less biomass than the HN.

The LN culture on day 6 reached a point where the LN culture NO_{3-} concentration contained only 35.1 mg NO_{3-} / L and continued to drop until day 9, when the LN NO_3 concentration dropped down to 1.72 mg NO_{3-} / L.

The LN culture over 6 to 9 had a positive linear of 1.68 mg total FAMEs / L / d, which was larger than the HN culture slope of 1.31 mg total FAMEs / L / d for the same days.

In both the LN and HN cultures there were only 3 identified genera greater than 1% of the total population: *Chlorosarcinopsis, Plectonema,* and *Spirulina sp.* with the *Chlorosarcinopsis* genus having the largest percentage of the total genera counts over the all 12 days of the LN and HN studies.

The LN reactor growth rate was 40% of the HN reactor growth rate. Coincidentally on day 7 for both the LN and HN studies, the filamentous cell

counts were nearly the same (LN filamentous cell counts = 4.3 x 10^6, HN filamentous cell counts = 4.6 x 10^6).

The cultures in the two HN reactor sets 1 and 2, did not consume all the average NO_{3-} concentration. By day 11 the HN reactor set 1 had consumed 74% of the original NO_3 concentration (179.2 mg NO_{3-} / L) and by day 12 HN reactor set 2 had consumed only 56% of the original NO_{3-} concentrations.

The LN culture average NO_{3-} consumption of the two LN reactor sets clearly indicated there was a very low concentration at the end of the LN study. The HN culture average NO_{3-} in the reactor sets 1 and 2 consumed only a 1/2 and 3/4 of the average NO_{3-} concentrations respectively.

For the days 3 to 12 the average ratio between the LN culture cumulative NO_{3-} concentrations and the Chl *a* average concentrations was 43.42 mg ±3.85 SE (NO_{3-} mg / Chl *a* mg / d). The HN culture NO_{3-} concentration normalized to the Chl *a* concentration had a average ratio, for the days 3 to 12 was 49.79 mg ±1.55 SE (NO_{3-} mg / Chl *a* mg / d).

The total average FAMEs in Figure 4.6 indicates something occurred on day 6 in the LN culture which pushed the two independent flask sets 3 and 4 to begin to simultaneously producing larger amounts of total FAMEs till day 9. The average

total FAMEs production in both flask set had the same rate of production from the slope value of the regression line = 1.68 mg FAMEs / day.

The normalization of the LN and HN total average FAME mg / Chl *a* mg discloses a relationship between the total average FAME production and the Chl *a* mg/ L. The LN and HN culture total average FAMEs were graphed from the summation of the average identified FAME peaks (mg / L) for each of the LN and HN culture studies.

The HN FAME synthesis, in general, produced larger FAME concentrations than the LN FAMEs except for the C18:3 unsaturated FAME, which had a larger concentration on day 11 compared to the HN FAME 18:3.

The average saturated to unsaturated ratio (S/U) for the LN culture over the first 7 days was 2.05, and over the same days, the HN culture had an average S/U ratio of 2.02 Figure 4.9.

In both the LN and HN cultures there were only 3 identified genera greater than 1% of the total population: *Chlorosarcinopsis, Plectonema,* and *Spirulina sp.* with the *Chlorosarcinopsis* genus had the largest percentage of the total genera counts over the all 12 days of the LN and HN studies. On day 0, the HN culture *Plectonema* genera counts had 28% of the total counts, and on day 12 the *Plectonema* genera counts increase to 42% of the total counts.

The data for the LN culture Shannon diversity index came from the algal community genera obtained from the LN and HN algal population analysis described in Section 2.3.4. The Shannon diversity index for both the LN and HN data was low due to the low number of species present in the culture.

Chapter 5 : Experiment 3, Cold Preservation of Algae

5.1 KNOWLEDGE GAP

Preservation of algal cultures is important to improve reproducibility from one study to the next, and to provide identical inocula for full-scale operations, such as biofuel production. [96] While storage of pure cultures of microorganisms is well established, [67] little research has been performed on the preservation of a mixed algal communities with bacteria. Successful cryopreservation can be measured by the percentage of the viable algal cells after freezing that resume normal growth. [96]

Algal cells often are exposed to turbulence from natural water bodies or mechanical pumping. Turbulence can come from pumps, wind shear and agitation. Algal cells are sensitivity to small shear forces and the highest sensitivity occurs in diatoms, then less so in cyanobacteria and the least in green algae. [97] There has been little research determining the effects of agitation on mixed algae communities.

Few research articles describe the freezing, thawing and growing of algal cells in a mixed community in order to determine their viability and identifying the algal genera present with next generation DNA sequencing. Positive identification of the surviving genera provides detailed information about the freezing survival rates and those genera that died.

Cryopreservation can be defined as storage of a living organism at ultra low temperatures lower than -130 °C. Cryopreservation of algal cells is an empirical

practice because algal species often respond differently during freezing and thawing.[96] CPAs added to the assist in protecting the algal cells when frozen and thawed. Two CPAs, DMSO and MEOH, passively move through the plasma membrane providing equilibration between the extracellular solution with the cell interior. [66]

Experiment 3 objective was develop a protocol for cold preservation protocol applicable to four temperatures (0 °C, -20 °C, -80 °C and -196 °C) mixed with algal communities, including calculation of Chl a before and after freezing, after freezing determine the most viability of the algal cells visually and their corresponding temperatures and CPAs. Compare the effects freezing on the two seed cultures with and without agitation based upon the viable genera counts after pyrosequencing.

Experimental Approach, Experiment 3: These systems were inoculated with a NM algal community taken from a brine pond located in an environment with unobstructed high intensity sunlight. (San Acacia Brine Pond, Soccoro, NM). One seed culture inoculum was agitated and the 2nd was not. Each inoculum was added to a separate 250 mL reactor containing 100 mL of media to grow for 6 days. After the 6 days, the mixed culture in each reactor was blended with 4 different CPAs at two percentages and frozen at 4 temperatures Table 5.1. The post freezing experimental systems used in Experiment 3 were 32 batch reactors (50 mL flasks / 30 mL media) which were illuminated and monitored for days 0 to

13. Additional Experiment 3 description and figures of the inoculum, design and operation, methods, freezing equipment, analysis are found in Sections 2.9, 2.10, 2.11, 2.12 and 2.13 respectively.

After reconstituting the culture, the algal biomass was observed for viability and analyzed to determine the effects of the CPAs DMSO and MEOH and their volume percentages in each 50 mL reactor.

Table 5.1 Experiment 3 NB and BB plan for algal cultures
mixed with DMSO or MEOH percentages

NB Culture	CPA DMSO 5% Volume mL	CPA DMSO 10% Volume mL	CPA MEOH 5% Volume mL	CPA MEOH 10% Volume mL
0 °C	X	X	X	X
-20 °C	X	X	X	X
-80 °C	X	X	X	X
-196 °C	X	X	X	X
BB Culture	**CPA DMSO 5% Volume mL**	**CPA DMSO 10% Volume mL**	**CPA MEOH 5% Volume mL**	**CPA MEOH 10% Volume mL**
0 °C	X	X	X	X
-20 °C	X	X	X	X
-80 °C	X	X	X	X
-196 °C	X	X	X	X

Table 5.1 Matrix describes the NB 4 freezing temperatures (4 rows) and the 2 CPAs with 2 volume percentages in each columns. The matrix also describes the BB 4 freezing temperatures (4 rows) and the 2 CPAs with 2 volume percentages in each columns. Each X describes the seed culture (NB or BB) mixed with the CPA DMSO or MEOH with one of two volume percentages (5% or 10%) and the freezing temperature. Example: (NB, 0 °C, DMSO 5%)

5.2 NB AND BB RESULTS

5.2.1 NB AND BB INOCULA CULTURES CHLOROPHYLL A AND B CONCENTRATIONS

The two seed reactors labeled NB and BB, Figure 5.1, grew over 6 days before sampling and analyzing the Chl *a* and Chl *b*. The Chl a increased in both reactors with a final concentration in the NB and BB reactors of 3.8 mg / L and 4.2 mg / L respectively. The NB and BB reactor Chl b final concentrations were 0.35 mg / L and 0.35 mg / L respectively.

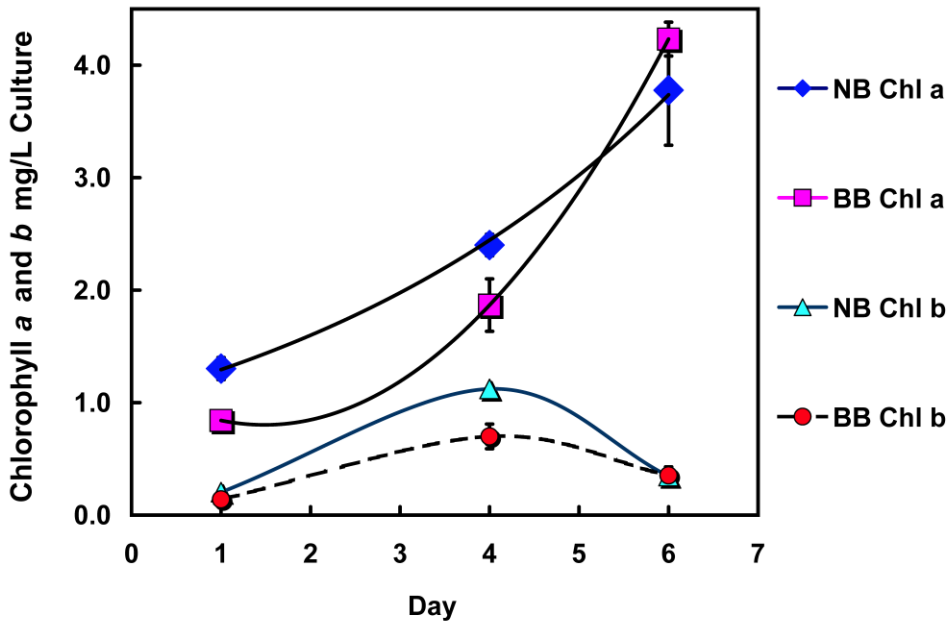

Figure 5.1 NB and BB algal community cultures Chl *a* and Chl *b*
Figure 5.1 captures the increasing Chl *a* and Chl *b* concentrations (mg / liter / d) of the two cultures, which indicates increasing growth rates. n = 3, ±SE bars are shown on each data point.

The two inoculated reactors (NB and BB) had similar Chl *a* concentrations for the 6 days and when both were compared to each other, a T-test indicated that

there was no statistical difference between the NB and BB Chl *a* concentrations. If the algal Chl *a* of the two reactors had been significantly different it might have been due to the agitation of the inoculum (BB), but that was not the case. The graph of the NB and BB Chl *a* concentrations for the reactors were very similar. The decrease in the Chl *b* on day 6 likely resulted from a larger percentage of culture containing cyanobacteria in the flask, which do no make Chl *b*.

The NB and BB experimental design is described in detail in Chapter 2, section 2.5.3 Experiment 3 Specific Experimental Design.

5.2.2 POST FREEZING ALGAL GROWTH SCORECARD ANALYSIS

The thawed frozen NB and BB cultures were transferred to 50mL reactors containing 30 mL of media shown in Figure 5.2 and described in Chapter 2 section 2.11.7 Experiment 3 Thawing. The 50 mL algal reactors holding the reconstituted algal cultures were allowed to grow and initially appeared as fuzzy white algal clumps when illuminated on the lighted shaker table.

Assessment of the algal growth scorecard results were performed visually. After an adaptation period of 5 days, the cultures began growing, turning a bright green. None of the 50 mL algal reactors on the lighted shaker table had any viable growth for first 5 days with the all the algal cultures remaining white.

The NB and BB cultures with DMSO 5% and DMSO 10%, had the best reconstitution survival at all temperatures. Overall, the lower temperatures, -80 °C and -196 °C produced more visible growth earlier for days 6 to 13 than the

two higher temperatures (0 °C and -20 °C) where initial growth was observed from days 7 to 14. [42]

Additional analysis of the post freezing reconstituted NB and BB cultures with CPAs in are in Chapter 7 : Appendix, section 7.4 "Chapter 5 - Cold preservation of algal growth score card analysis with 6 subsections". Each subsection describes the results of the culture growth after freezing in Figure 5.2 NB and BB algal growth scorecard after reconstitution.

The reconstituted NB and BB cultures frozen with cryoprotective additive CPA concentrations methanol 5% (MEOH 5%) and MEOH 10% were less likely to have viable growth at 0 °C and -20 °C temperatures. The MEOH in the cultures may have caused issues within the cell or created a toxic mixture with the components in the saline brine media.

The NB and BB cultures with dimethylsulfoxide 5% (DMSO 5%) and DMSO10%, had the best reconstitution survival at all temperatures. The DMSO at the lower two temperatures, -80 °C and -196 °C, viable growth onset was earlier viable algal community growth for days 6 to 13 than the two higher temperatures (0 °C and -20 °C). [42]

NB and BB Growth Scorecard

Thirteen days of growth after reconstitution

Day 0 Cultures Started

--	No Algae Growth	+	Algae Growth	+?	Algae Growth Small Clump

NB		Day	1	2	3	4	5	6	7	8	9	10	11	12	13
0° C	DMSO	5%	--	--	--	--	--	--	--	+?	+	+	+	+	+
	DMSO	10%	--	--	--	--	--	--	--	--	+	+	+	+	+
	MEOH	5%	--	--	--	--	--	--	--	--	--	--	--	--	+?
	MEOH	10%	--	--	--	--	--	--	--	--	--	--	--	--	+?

NB			1	2	3	4	5	6	7	8	9	10	11	12	13
-20° C	DMSO	5%	--	--	--	--	--	--	+	+	+	+	+	+	+
	DMSO	10%	--	--	--	--	--	+	+	+	+	+	+	+	+
	MEOH	5%	--	--	--	--	--	--	--	--	--	--	--	--	--
	MEOH	10%	--	--	--	--	--	--	--	--	--	--	--	--	--

NB			1	2	3	4	5	6	7	8	9	10	11	12	13
-80° C	DMSO	5%	--	--	--	--	--	--	+	+	+	+	+	+	+
	DMSO	10%	--	--	--	--	--	+	+	+	+	+	+	+	+
	MEOH	5%	--	--	--	--	--	+	--	+	+	+	+	+	+
	MEOH	10%	--	--	--	--	--	--	+	+	+	+	+	+	+

NB			1	2	3	4	5	6	7	8	9	10	11	12	13
-196° C LN	DMSO	5%	--	--	--	--	--	+	+	+	+	+	+	+	+
	DMSO	10%	--	--	--	--	--	+	+	+	+	+	+	+	+
	MEOH	5%	--	--	--	--	--	+	+	+	+	+	+	+	+
	MEOH	10%	--	--	--	--	--	+	--	--	+	+	+	+	+

BB			1	2	3	4	5	6	7	8	9	10	11	12	13
0° C	DMSO	5%	--	--	--	--	--	--	--	+?	+	+	+	+	+
	DMSO	10%	--	--	--	--	--	--	--	--	+	+	+	+	+
	MEOH	5%	--	--	--	--	--	--	--	--	--	--	--	--	+
	MEOH	10%	--	--	--	--	--	--	--	--	--	--	--	--	--

BB			1	2	3	4	5	6	7	8	9	10	11	12	13
-20° C	DMSO	5%	--	--	--	--	--	+	+	+	+	+	+	+	+
	DMSO	10%	--	--	--	--	--	+	+	+	+	+	+	+	+
	MEOH	5%	--	--	--	--	--	--	--	--	--	--	--	--	+
	MEOH	10%	--	--	--	--	--	--	--	--	--	--	--	--	+

BB			1	2	3	4	5	6	7	8	9	10	11	12	13
-80° C	DMSO	5%	--	--	--	--	--	+	+	+	+	+	+	+	+
	DMSO	10%	--	--	--	--	--	--	--	--	--	--	--	--	+?
	MEOH	5%	--	--	--	--	--	+	+	+	+	+	+	+	+
	MEOH	10%	--	--	--	--	--	--	--	+	+	+	+	+	+

BB			1	2	3	4	5	6	7	8	9	10	11	12	13
-196° C LN	DMSO	5%	--	--	--	--	--	+?	+	+	+	+	+	+	+
	DMSO	10%	--	--	--	--	--	+?	+	+	+	+	+	+	+
	MEOH	5%	--	--	--	--	--	+	+	--	+	+	+	+	+
	MEOH	10%	--	--	--	--	--	+	--	--	+	+	+	+	+

Figure 5.2 NB and BB algal growth scorecard after reconstitution

Figure 5.2 captures the freezing protection that the CPAs enabled the algal community to endure freezing and still result in algal growth after reconstitution. The +? symbol in a square, was not counted as an day of normal growth and excluded from growth day counts.

5.2.3 Reconstituted 5% DMSO NB and BB Chl *a* concentrations

The NB culture Chl *a* concentrations was lower than the BB culture Chl *a* concentrations (Figure 5.3) except for the NB culture frozen at 0 °C. The BB cultures for the freezing temperatures of -20 °C, -80 °C and -196 °C had more green biomass growth than the NB.

The Chl *a* mg / L concentration data in the Figure 5.3 bar graph came from the reconstituted DMSO 5% NB and BB cultures on day 13.

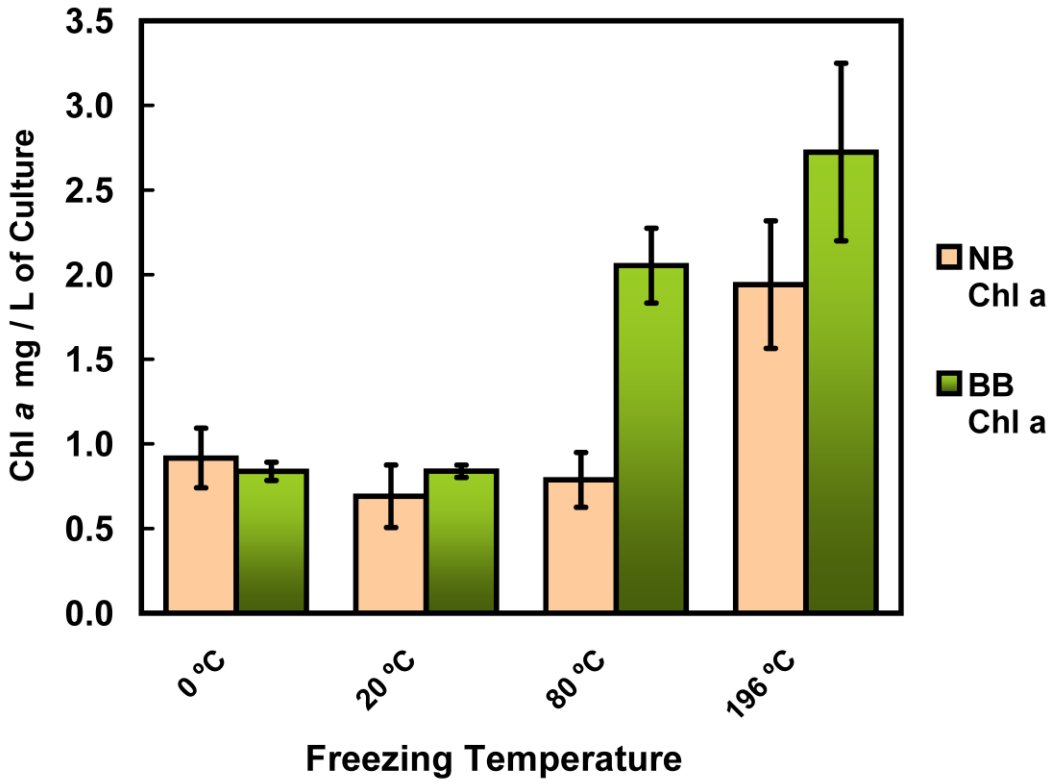

Figure 5.3 Reconstituted DMSO 5% NB and BB Chl *a* concentrations
Only data for the DMSO 5% NB and BB Chl a mg / L were taken during Experiment 3. The Chl a concentrations from the NB and BB cultures with CPA DMSO 5% were analyzed on day 13 for the 8 samples. n = 3, ±SE bars are shown on each data point..

The NB culture Chl *a* mg / L concentration frozen at 0 °C temperature had a slightly higher Chl *a* concentration than the BB culture by (Chl *a* 0.08 mg / L).

The BB culture frozen at -20 °C temperature had a Chl *a* concentration larger than the NB -20 °C frozen culture by Chl *a* 0.15 mg / L. The BB cultures frozen at -80 °C and -196 °C temperatures had Chl a concentrations that exceeded both of the corresponding NB cultures concentrations.

A t-test between (Figure 5.3) the NB and BB Chl *a* concentrations at temperatures -20 °C, -20 °C, -80 °C, -196 °C indicated there was no significant difference between the data.

Both the NB and BB cultures for all 4 freezing temperatures were analyzed for Chl *b* concentrations and only 3 NB resulted in positive Chl *b* concentrations. The 3 positive Chl *b* mg / L concentrations were: NB, 0 °C freezing temperature, Chl *b* 018 mg / L; NB, -196 °C freezing temperature, Chl *b* 0.031 mg / L; BB, -196 °C freezing temperature, Chl *b* 0.26 mg / L.

The DMSO 5% or MEOH 5% worked the best to protect the algal cell from freezing damage. The synthesis of Chl *a* was correlated to the algal biomass growth for a specific freezing temperature with the 5% DMSO. The NB culture Chl *a* concentration was lower than the BB culture Chl *a* concentrations except for the NB culture held at 0 °C. The BB cultures for the freezing temperatures of -20 °C, -80 °C and -196 °C had more green biomass growth than the NB.

5.2.4 POST FREEZING ALGAL GENERA POPULATION COUNTS GREATER THAN 1%

Figure 5.4 displays the reconstituted NB and BB algal genera with counts greater than 1% of the total genera counts per each CPA. Only the -196 °C, frozen samples from the reconstituted 50 mL reactors were sent out for 454

pyrosequencing. The DNA was extracted from the -196 °C frozen algal samples listed here:

NB: NB Seed, NB 5% DMSO, NB 10% DMSO, NB 5% MEOH, NB 10% MEOH

BB: BB Seed, BB 5% DMSO, BB 10% DMSO, BB 5% MEOH, BB 10% MEOH.

The extracted DNA was analyzed using Roche 454 pyrosequencing, which identified the algal genera for each of the samples above. The pyrosequencing procedure is described in Chapter 2, section 2.2. subsection 2.2.3. Algal Community Analysis.

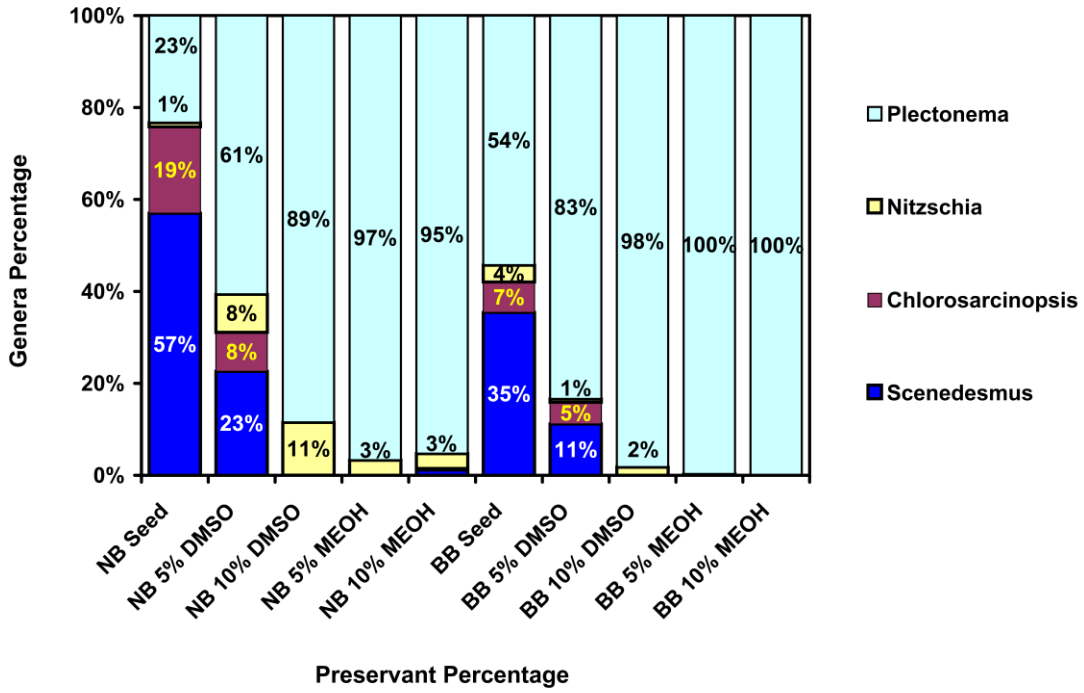

Figure 5.4 Algal community genera percentages greater than 1%
Only the -196 °C NB and BB genera with different CPAs at two percentages were analyzed from pyrosequencing data. The analysis included the NB and BB seed cultures to determine the initial genera of the inocula for the NB and BB cultures.

Figure 5.4 graphs the NB and BB seed cultures and revealed the effects of the agitation on the green algae, diatom and cyanobacteria genera count percentages greater than 1% in the NB and BB seed cultures. The BB seed culture *Scenedesmus* was 22% less than the seed NB *Scenedesmus*. The BB seed culture *Chlorosarcinopsis* was 12% less than the seed NB. *Chlorosarcinopsis.* The NB seed culture genera *Nitzschia* was 3% less the BB *Nitzschia* and the seed culture NB genera *Plectonema* was 31% less than the BB *Plectonema.*

Two NB culture genus (*Scenedesmus* and *Chlorosarcinopsis)* frozen in the 10% DMSO were not present after reconstitution. Two NB culture genus (*Scenedesmus* and *Chlorosarcinopsis)* frozen in 5% MEOH, and not present after reconstitution. One NB culture genus (*Scenedesmus)* frozen in the 10% MEOH after reconstitution had a of percentage of 1.2% and was not shown in Figure 5.4 and the *Chlorosarcinopsis* genera was not present.

Two BB culture genera *Scenedesmus* and *Chlorosarcinopsis.* were frozen in the 10% DMSO, but not present after reconstitution. Two BB culture genus (*Scenedesmus* and *Chlorosarcinopsis)* were frozen in the 5% MEOH, but not present after reconstitution. Three BB culture genus (*Scenedesmus*, *Chlorosarcinopsis* and *Nitzschia*) were frozen in the10% MEOH CPA, but not present after reconstitution.

One BB culture genus, *Plectonema,* was frozen in two separate tests with 5% MEOH and a 10% MEOH, and after reconstitution, each test had the highest *Plectonema* genus percentages (100%). The BB culture *Plectonema* genus

frozen in the 5% and 10% DMSO after reconstitution had percentages 83% and 98% respectively.

The primary genus in the BB culture is *Plectonema*, which is from the Oscillatoriales Family. The Oscillatoriales Family genera reproduce by asexual filament fragmentation where a single fragment can form a whole new organism. The BB first generation inoculum was agitated before adding to the BB seed culture reactor. The agitated BB inocula caused fragmentation of the *Plectonema* genera, creating significant quantities of new cells. [98] The additional cells in the BB culture would have increased the algal cell counts and outnumbered those of the NB *Plectonema* and *Spirulina* biomass.

The green algae, diatoms and cyanobacteria were affected by the BB agitation differently. The green algae (*Scenedesmus* and *Chlorosarcinopsis*) were damaged or died from the agitation. The *Nitzschia* was damaged by the agitation, but not to the extent of the green algae, which allowed the Nitzschia to recover, but with fewer numbers. The *Plectonema* genus, which is a cyanobacteria, benefited from the agitation by increasing their numbers. The *Plectonema* after reconstitution multiplied rapidly and dominated their NB or BB cultures.

5.2.5 POST FREEZING GENERA POPULATION COUNTS LESS THAN 1%

Figure 5.4 graphed the reconstituted (post freezing) algal community genera from only the -196 freezing temperature with pyrosequencing counts less than 1 percent of the total. The BB seed culture genera percentages had 4.4% *Trebouxia*, 3.3% *Tryblionella*, 70.7% *Spirulina*, and 9.8% *Synechococcus*, and

12.1 *Leptolyngbya*. The BB 5% MEOH CPA had no algal genera, while the BB 10% MEOH had a low *Spirulina* count.

The BB 5% DMSO genera percentages contained 5.6% *Trebouxia*, 11.1% *Tryblionella*, 72.2.% *Spirulina*, and 11.1% *Cyanothece*. The BB 10% DMSO genera percentages had 84.6% *Spirulina*, 5.0% *Leptolyngbya* and 9.3% *Cyanothece*. The NB seed culture genera percentages had 17.2% *Trebouxia*, 62.1% *Spirulina*, and 20.7% *Synechococcus*. The NB 10% MEOH culture had genera percentages 92.9% *Tryblionella*, and 7.1% *Synechococcus*.

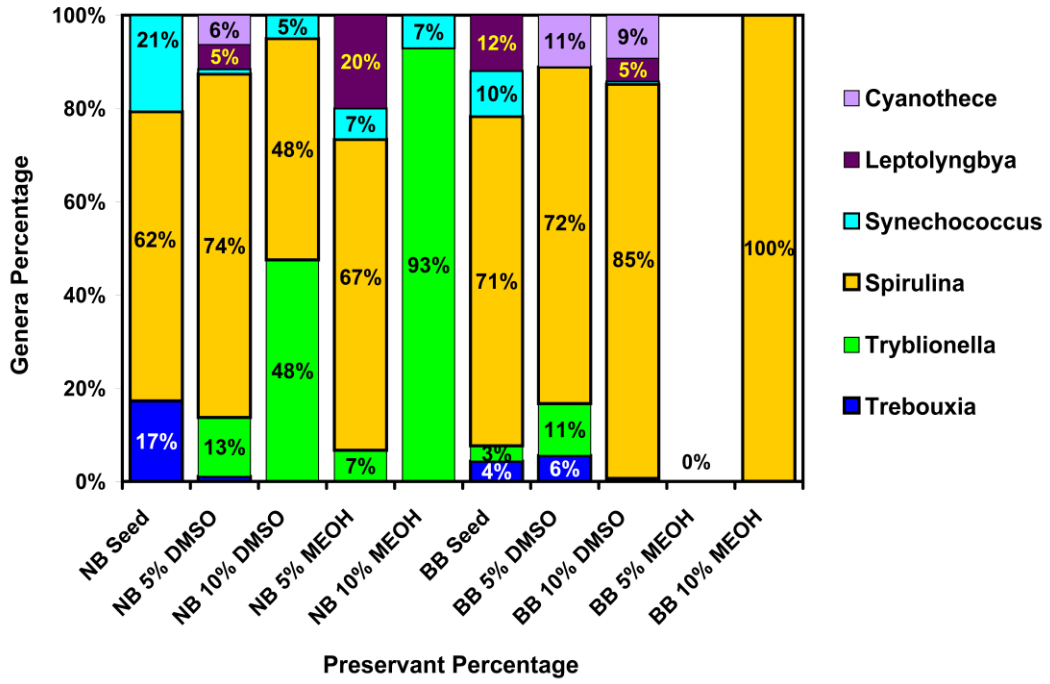

Figure 5.5 Algal community genera percentages less than 1%
Only the -196 °C NB and BB genera with different CPAs and percentages were analyzed from pyrosequencing data. The NB and BB seed cultures were analyzed to determine the NB and BB source culture genera.

The NB 5% MEOH culture had genera percentages 6.7%% *Tryblionella*, 66.7% *Spirulina*, 6.7% *Synechococcus* and 20.0% *Leptolyngbya*. The NB culture DMSO 10% genera percentages were 47.5% *Tryblionella*, 47.5% *Spirulina*, and 5.0% *Synechococcus*.

The NB culture DMSO 5% count percentages were 1.1% *Trebouxia*, 12.6% *Tryblionella*, 73.7% *Spirulina*, and 1.1% *Synechococcus*, 5.3% *Leptolyngbya*, and 6.3% *Cyanothece*.

The two inocula reactors displayed a normal growth for the 6 days. The graphed Chl *a* and Chl *b* concentrations for the NB and BB reactors were similar.

The dominant genus for the algal community genera less than 1% of the total was the cyanobacteria, *Spirulina*.

The presence of low genera counts in the both the BB and NB cultures occurs in nature during seasonal cycles when the environmental parameters change continuously such as nutrient availability, temperatures, weather, and predators. The low population genera counts may or may not increase when condition are favorable to their growth. [99]

5.3 Chapter 5 Experiment 3 Conclusion

Little research has focused complexity of maintaining specialized mixed cultures for research inoculations or for seeding pond start-up cultures to large commercial raceway ponds or photobioreactors. This study implemented a matrix that froze a mixed algal population at four different temperatures in two different

CPA percentages (v/v) to discover which sample freezing temperatures and CPA combinations with the best algal cell species recovery.

The agitated NM algal community seed culture inoculum (Bead Beaten "BB") and one inoculum without agitation (Not Bead Beaten "NB"), were added to two different 250 mL reactors containing HT medium to grow the NM algal community for 6 days before freezing. The agitation did not have any effect on the Chl a concentrations of the 6 day grow period as the two reactors had similar Chl *a* curves indicate healthy cultures suitable for freezing.

The algal cultures were grown, frozen with CPAs, and reconstituted to determine the surviving algal genera present. A growth scorecard for the large matrix pointed to two temperatures where the reconstituted algal cultures survived better (-80 °C and -196 °C) and one CPA (5% DMSO) had more of the algal culture survive than any of the the MEOH concentrations and freezing temperatures. Survival after reconstitution at the two lower temperatures was (-80 °C and -196 °C) more likely.

The post freezing Chl a concentrations (mg / L / d) from the NB and BB 5% DMSO cultures The NB culture Chl *a* concentration was lower than the BB culture Chl *a* concentrations except for the NB culture held at 0 °C.

The *Plectonema* genus reproduces by asexual filament fragmentation where a single fragment can form a whole new organism. The BB agitating prior to freezing may have contributed to the highest post freezing genus counts in the *Plectonema* genera. The BB culture post freezing *Plectonema* genus counts outnumbered those of the NB *Plectonema* and *Spirulina* biomass.

Some post freezing genera counts for the BB seed culture were less than the NB seed culture genera counts and survived better because they had not been subjected to the agitation prior to freezing.

Chapter 6 : Conclusions

6.1 CONCLUSIONS

The Chl a for both the RT and HT cultures were the same per t-test results.

Temperature had a large effect on the cultivation of the mixed algal cultures from a high saline brine pond.

The RT and HT culture Chl *b* concentrations differed extensively. The RT culture Chl *b* concentrations formed an increasing straight line, however the HT culture Chl *b* concentrations were nearly zero due to the fact that cyanobacteria do not produce Chl *b*.

The RT community composition contained 2 green algae, a diatom and 2 cyanobacteria genera. The HT community had three cyanobacteria genera.

In the HT cultures, two green algae and one a diatom genera were not found in the HT cultures held at 40 °C temperatures. The HT cultures contained 3 cyanobacteria genera.

The RT culture consumed all the media average NO_{3-} concentration 175 mg / L, but the HT only consumed 126 mg / L. The HT culture may not have needed the all the NO_{3-} since there were only 3 cyanobacteria genus present.

The HT culture total average identified FAMEs on the final day was 45% of the RT culture total average identified FAMES. The higher temperature of the HT culture reduced the FAMEs produced compared to the RT culture.

The LN and HN culture Chl *a* concentration were very similar. The low nitrate concentration medium produced significantly lower LN culture growth when compared to the HN culture grown in the high nitrate concentration medium.

The LN culture NO_{3-} concentration was sufficiently low on day 6 to something happened to the LN algal culture to produce a TAGs sequestration event, but only produced more lipids than the HN culture till day 9. Thereafter the increased LN culture lipid production ceased and the HN culture total FAMEs production was higher.

Preservation of algal cultures is important to improve reproducibility from one study to the next, and to provide identical inocula for full-scale operations, such as biofuel production.

Algae often are exposed to turbulence from natural water bodies or mechanical pumping. Turbulence can come from pumps, wind shear and agitation.

Algal cells are sensitivity to small shear forces and the highest sensitivity occurs in diatoms, then less so in cyanobacteria and the least in green algae.

The two inoculated reactors (NB and BB) had similar Chl *a* concentrations for the 6 days and when both were compared to each other, a T-test indicated that there was no statistical difference between the NB and BB Chl *a* concentrations

The (32) 50 mL algal reactors holding the reconstituted algal cultures were allowed to grow and initially appeared as fuzzy white algal clumps when illuminated on the lighted shaker table.

The *Plectonema* genus reproduces by asexual filament fragmentation where a single fragment can form a whole new organism. The BB agitating prior to freezing may have contributed to the highest post freezing genus counts in the *Plectonema* genera.

A growth scorecard for the large matrix pointed to two temperatures where the reconstituted algal cultures survived better (-80 °C and -196 °C) and one CPA (5% DMSO) had more of the algal culture survive than any of the the MEOH concentrations and freezing temperatures. Survival after reconstitution at the two lower temperatures was (-80 °C and -196 °C) more likely.

The post freezing Chl a concentrations (mg / L / d) from the NB and BB 5% DMSO cultures The NB culture Chl *a* concentration was lower than the BB culture Chl *a* concentrations except for the NB culture held at 0 °C.

Chapter 7 : Appendix

7.1 Chapter 3 RT and HT

7.1.1 RT and HT Additional Graphs and Tables

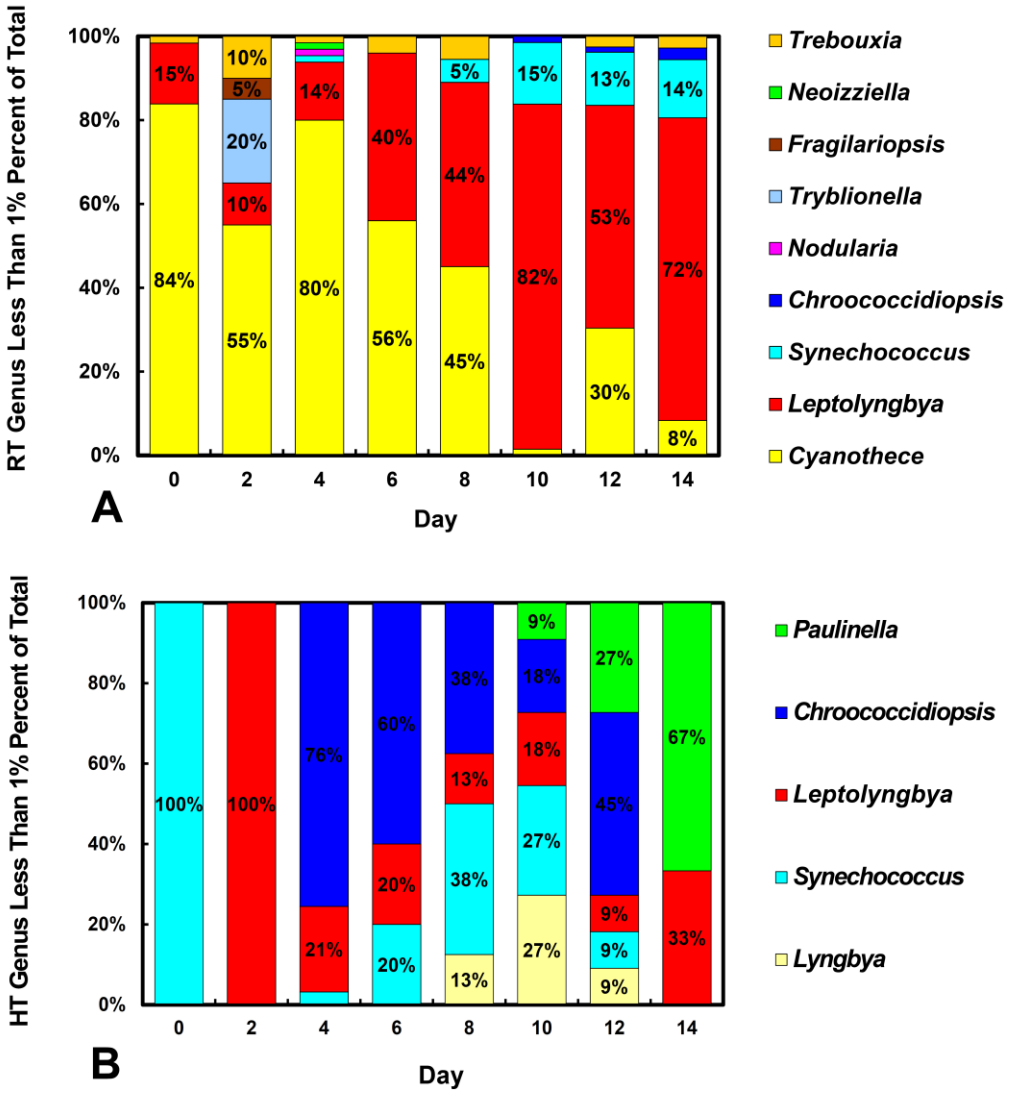

Figure 7.1 RT and HT community genus percentage counts Less than 1%
The RT (7.1A) and HT (7.1B) 100% stack column charts plot the reactor genus percentage of the total genus counts less than 1%. Day 0 bar represents the genus counts less than 1% of the total algal seed culture.

7.1.2 CHAPTER 3 RT AND HT PYROSEQUENCING SPECIES PERCENTAGES

On day 0, the RT seed culture pyrosequencing data identified two different C*hlorosarcinopsis* species: C*hlorosarcinopsis eremi (C. eremi)* and C*hlorosarcinopsis sp*. with species percentages of 99% and 1% respectively.

On day 0, the RT seed reactor pyrosequencing data listed two species: *Scenedesmus sp. and Scenedesmus rotundus* (*S. rotundus)* with species count percentages of 95.1% and 4.8% respectively.

The day 0, RT culture reactor pyrosequencing specie data listed two species: *Nitzschia laevis (N. laevis), and Nitzschia sp*. with species count percentages of 35.7% and 64.3% respectively.

The RT reactor pyrosequencing species analysis detected only the *Spirulina sp*. present in the culture.

The RT and HT species counts were required for the Shannon Diversity Index calculations in Chapter 3.

7.2 CHAPTER 3 RT AND HT FAMES NOT LABELED IN GRAPH

Table 7.1 RT FAMEs not labeled in graph

RT FAMEs concentrations (mg/ liter of culture) not labeled in Figure 3.5A, (not labeled data has bold italicized entries.) A dash (-) are data labeled on Figure 3.5A.
("a" indicates no algal FAMEs were produced.)

RT FAMEs	C16:1	C17:1	C18:1	C18:2	C18:3	C20:4	C11:0	C14:0	C16:0	C17:0	C18:0	C20:0	C21:0 / C22:0	C24:0
Day														
2	0.06	a	a	a	a	a	a	0.06	-	0.07	-	0.16	0.28	-
4	-	a	0.001	a	0.04	a	a	0.33	-	0.08	-	0.12	0.49	-
6	-	0.01	0.02	0.02	-	0.10	a	0.52	-	0.07	-	0.16	0.61	-
8	-	0.05	0.25	0.17	-	0.29	0.21	0.88	-	0.11	-	0.18	-	-
10	-	0.06	0.42	0.36	-	0.23	0.11	0.67	-	0.12	-	0.20	-	-
12	-	0.06	0.54	0.41	-	0.34	0.39	0.76	-	0.12	-	0.23	-	-
14	-	-	-	-	-	0.41	0.09	0.84	-	0.14	-	0.24	-	-

Table 7.2 HT FAMES not labeled in graph

HT FAMEs concentrations (mg/ liter of culture) not labeled in Figure 3.5B (not labeled data has bold italicized entries.) A dash (-) are data labeled on Figure 3.5B.
("a" indicates no algal FAMEs was produced.)

HT FAMEs	C16.1	C18:1	C18:2	C18:3	C14:0	C16:0	C17:0	C18:0	C20:0	C21:0 / C22:0	C24:0
Day											
2	a	a	a	a	0.001	0.20	0.05	-	0.10	0.21	-
4	0.08	0.10	0.001	a	0.001	-	0.08	-	0.14	0.26	-
6	0.09	0.17	0.02	a	0.001	-	0.06	-	0.10	0.21	-
8	0.25	-	0.24	0.01	0.001	-	0.08	-	0.14	0.25	-
10	-	-	-	0.10	0.01	-	0.09	-	0.15	0.28	-
12	-	-	-	0.11	0.04	-	0.12	-	0.19	0.27	-
14	-	-	-	0.16	0.05	-	0.12	-	0.17	0.28	-

Table 7.3 RT Blended FAMEs DCN

Calculations analyzed with EQ (1) adapted to multi-FAMEs algal blended DCN.

RT FAMEs	FAMEs Fraction of Total mg	FAMEs Total mg	Experimental Cetane Number	DCN
C14:0	0.839	24.682	66.2	2.250
C16:0	8.644	24.682	74.3	26.021
C18:0	1.630	24.682	75	4.953
C20:0	0.238	24.682	100	0.964
C21:0 / C22:0	1.194	24.682	68.8	3.328
C16:1	6.541	24.682	51	13.516
C18:1	1.055	24.682	45	1.923
C18:2	0.881	24.682	43	1.535
C18:3	3.660	24.682	41	6.080
Total DCN				**60.6**

Table 7.4 HT Blended FAMEs DCN

Calculations analyzed with EQ (1) adapted to multi-FAMEs algal blended DCN.

HT FAMEs	FAMEs Fraction of Total mg	FAMEs Total mg	Experimental Cetane Number	DCN
C14:0	0.050	11.377	66.2	0.291
C16:0	5.070	11.377	74.3	33.111
C18:0	1.392	11.377	75	9.174
C20:0	0.171	11.377	100	1.503
C21:0 / C22:0	0.277	11.377	68.8	1.677
C16:1	0.888	11.377	51	3.982
C18:1	1.879	11.377	45	7.430
C18:2	1.487	11.377	43	5.622
C18:3	0.163	11.377	41	0.587
Total DCN				**63.4**

7.3 CHAPTER 4 LN AND HN APPENDIX

7.3.1 ADDITIONAL GRAPHS AND TABLES

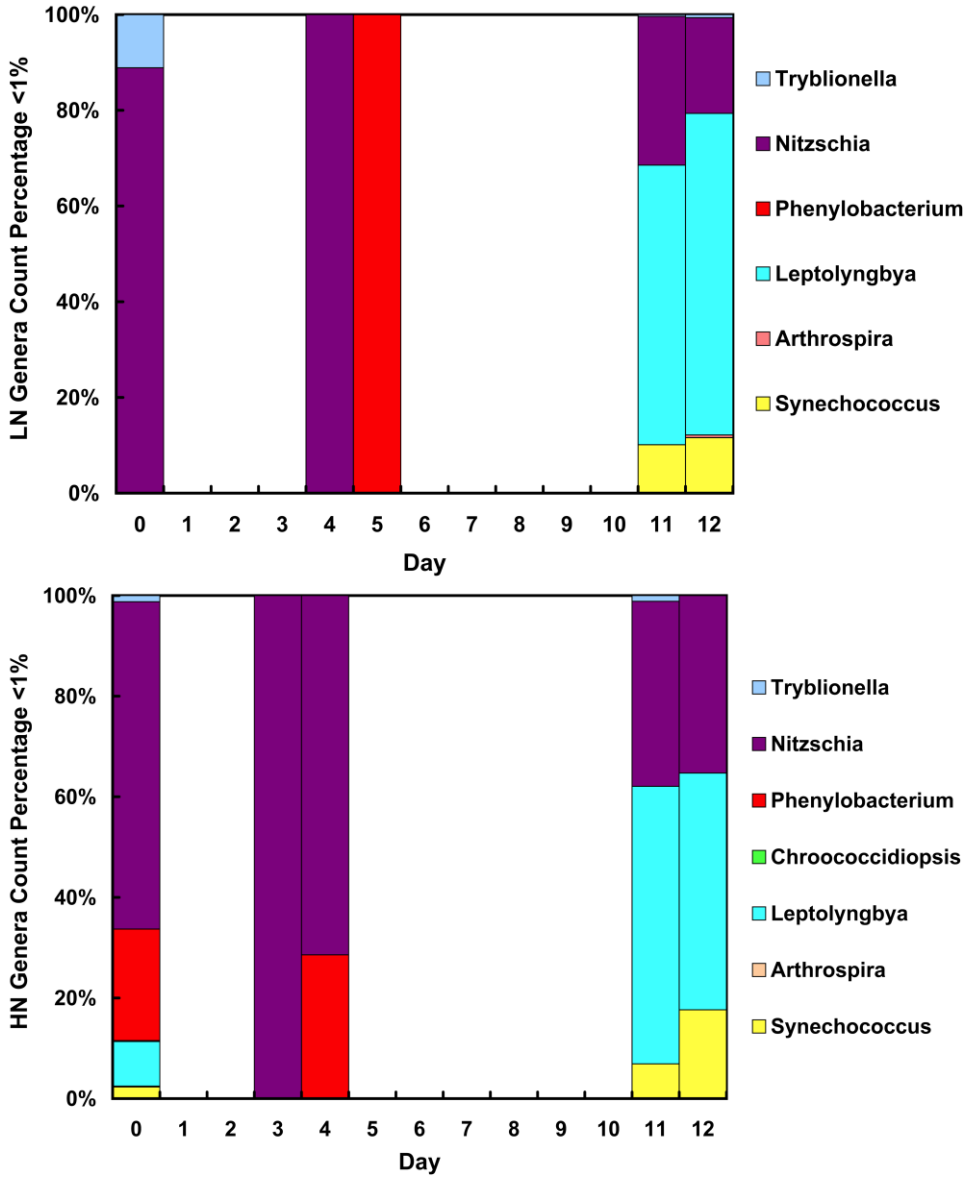

Figure 7.2 LN and HN genus percentage counts less than 1%
LN 7.2(A) and HN 7.2(B) The 100% stack column charts plot the reactor genus percentage of the total genus counts less than 1%. Day 0 bar represents the genus counts less than 1% of the total algal seed culture.

Table 7.3 RT Saturated and Unsaturated Percent FAMEs SE's

(Figure 7.3 A) as a percentage of the average identified unsaturated and saturated FAMEs (n=3) (mg / liter of culture)

Day	C11:0	C14:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:4	C21:0 b and C22:0	C24:0
2	a	60.6	56.0	0.0	22.9	a	44.7	a	a	a	4.7	a	1.7	5.1
4	a	8.5	7.6	8.8	9.7	a	15.2	a	a	60.2	19.8	a	5.1	16.4
6	a	13.3	9.9	11.3	11.1	32.6	10.4	23.3	82.3	5.3	15.4	27.4	9.5	8.3
8	22.4	19.2	8.3	18.6	5.5	7.8	4.3	20.9	9.0	19.6	15.0	21.9	10.8	10.9
10	58.6	11.4	6.7	10.9	2.8	43.6	6.1	3.5	8.1	2.3	3.9	31.3	19.6	7.4
12	28.5	12.6	12.0	12.4	7.1	34.0	18.4	21.4	19.4	9.1	18.7	17.5	10.4	5.2
14	43.3	15.5	7.2	20.3	10.8	5.5	13.1	24.4	17.5	21.6	12.9	16.2	3.2	8.8

a Average algal FAME resulted in less than 0.1% SE of average identified FAMEs .
b C21:0 and C22:0 co-eluted from gas chromatograph column.

Table 7.4 HT Saturated and Unsaturated Percent FAMEs SE's

HT (Figure 7.3 B) FAMEs SE as a percentage of the average identified unsaturated and saturated FAMEs (n=3) (mg / liter of culture)

Day	C14:0	C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C18:3	C20:0	C21:0 b and C22:0	C24:0
2	a	41.9	a	19.3	27.5	a	a	a	10.7	1.5	9.6
4	a	12.8	28.3	5.7	4.2	64.5	a	a	11.0	9.3	15.4
6	a	9.1	21.3	4.5	3.8	33.3	16.8	a	17.0	15.7	17.8
8	25.0	13.8	11.4	6.2	12.9	18.1	28.1	62.1	22.5	18.7	9.6
10	84.2	8.0	7.1	6.3	4.6	9.5	5.0	10.9	14.5	12.4	13.1
12	10.5	5.0	9.1	4.0	4.6	9.4	5.9	24.8	4.0	3.9	5.4
14	28.4	12.9	22.0	6.2	8.4	21.0	32.7	35.3	7.3	4.5	5.8

a Average algal FAME resulted in less than 0.1% SE of average identified FAMEs .
b C21:0 and C22:0 co-eluted from gas chromatograph column.

7.4 CHAPTER 5: SIX ADDITIONAL COLD PRESERVATION OF ALGAE SCORE CARD ANALYSES

7.4.1 SCORECARD FREEZING TEMPERATURES 0° C AND -20 °C, CPA MEOH

The NB culture from the freezing temperatures of 0° C and -20 °C with CPA concentrations of either 5% MEOH or 10% MEOH, did not show any growth. The BB culture freezing at 0° C with a CPA concentration of 5% MEOH was only viable on d 13. The BB culture from the freezing temperature at -20 °C with CPA concentrations of 5% MEOH or 10% MEOH were viable only on day 13.

BB cultures from the freezing temperature -20 °C containing CPA concentrations of 5% MEOH or 10% MEOH did not grow until day13.

7.4.2 SCORECARD FREEZING TEMPERATURES 0° C AND -20 °C, CPA DMSO

The reconstituted NB and BB cultures from the freezing temperature 0° C, with CPA concentrations of 5% DMSO and 10% DMSO, both had viable growth starting on d 9 continuing through d 13. The NB cultures from the freezing temperature -20 °C, with concentrations of 5% DMSO and 10% DMSO showed green algal growth on days 8 and 9 respectively.

The BB reconstituted cultures from freezing temperature 0° C, with CPA concentrations of 5% DMSO and 10% DMSO 0° had green culture cell growth starting on day 9 and continuing to day 13.

The two BB cultures, from the freezing temperature -20 °C containing concentrations of 5% DMSO and 10% DMSO, both had viable algal growth starting on day 6 and continued growing to day 13.

7.4.3 Scorecard freezing temperature -80° C, with CPA MEOH

The NB reconstituted cultures frozen at -80 °C with a CPA concentration of 5% MEOH had green algal growth starting on day 8 and continuing to day 13. The NB reconstituted cultures frozen at -80 °C with a CPA concentration of 10% MEOH growth began on day 7 and continued to day 13.

The BB reconstituted cultures frozen at -80 °C with a CPA concentration of 5% MEOH had green algal growth starting on day 6 and continuing to day 13. The BB reconstituted cultures frozen at -80 °C with a CPA concentration of 10% MEOH growth began on day 8 and continued to day 13.

7.4.4 Scorecard freezing temperatures -80° C, CPA DMSO

The NB reconstituted cultures frozen at -196 °C with a CPA concentration of 5% DMSO had green algal growth starting on day 8 and continuing to day 13. The NB reconstituted cultures frozen at -196 °C with a CPA concentration of 10% DMSO growth began on day 7 and continued to day 13.

The BB reconstituted cultures frozen at -196 °C with a CPA concentration of 5% DMSO had green algal growth starting on day 6 and continuing to day 13. The BB reconstituted cultures frozen at -196 °C with a CPA concentration of 10% DMSO growth began on day 8 and continued to day 13.

7.4.5 Scorecard freezing temperatures -196 °C, CPA MEOH

The NB reconstituted cultures frozen at -196 °C with a CPA concentration of 5% MEOH had green algal growth starting on day 8 and continuing to day 13. The NB reconstituted cultures frozen at -196 °C with a CPA concentration of 10% MEOH growth began on day 7 and continued to day 13.

The BB reconstituted cultures frozen at -196 °C with a CPA concentration of 5% MEOH had green algal growth starting on day 6 and continuing to day 13. The BB reconstituted cultures frozen at -196 °C with a CPA concentration of 10% MEOH growth began on day 8 and continued to day 13.

7.4.6 Scorecard freezing temperatures -196 °C, CPA DMSO

The NB reconstituted cultures frozen at -196 °C with a CPA concentration of 5% DMSO had green algal growth starting on day 8 and continuing to day 13. The NB reconstituted cultures frozen at -196 °C with a CPA concentration of 10% DMSO growth began on day 7 and continued to day 13.

The BB reconstituted cultures frozen at -196 °C with a CPA concentration of 5% DMSO had green algal growth starting on day 6 and continuing to day 13. The BB reconstituted cultures frozen at -196 °C with a CPA concentration of 10% DMSO growth began on day 8 and continued to day 13.

Chapter 8 : References

[1] A.J. Williams, L.J. Crossey, K.E. Karlstrom, D. Newell, M. Person, E. Woolsey, Hydrogeochemistry of the Middle Rio Grande aquifer system—Fluid mixing and salinization of the Rio Grande due to fault inputs, Chem. Geol., 351 (2013) 281-298.

[2] A.J. Williams, AN AQUEOUS GEOCHEMICAL AND HYDROLOGIC STUDY OF THE SPRINGS AND WELLS OF THE SEVILLETA NATIONAL WILDLIFE REFUGE: EVALUATING HYDROCHEMICAL PATHWAYS, Earth and Planetary Sciences, University of New Mexico, 2009, pp. 177.

[3] L. Batan, J. Quinn, B. Willson, T. Bradley, Net energy and greenhouse gas emission evaluation of biodiesel derived from microalgae, Environ. Sci. Technol., 44 (2010) 7975-7980.

[4] N.R. Council, Sustainable development of algal biofuels in the United States, National Academies Press2013.

[5] EIA, Monthly Biodiesel Production Report\ Table 1. U.S. Biodiesel production capacity and production, in: U.S.E.I.A.M.B.P. Report (Ed.), United States Government, Washington DC, 2015, pp. 1.

[6] EPA, Highway and Nonroad Diesel Fuel Standards, EPA, 2011, pp. 1.

[7] D.P. Geller, J.W. Goodrum, Effects of specific fatty acid methyl esters on diesel fuel lubricity, Fuel, 83 (2004) 2351-2356.

[8] A.K. Agarwal, L. Das, Biodiesel development and characterization for use as a fuel in compression ignition engines, Journal of engineering for gas turbines and power, 123 (2001) 440-447.

[9] A. Gopinath, S. Puhan, G. Nagarajan, Effect of unsaturated fatty acid esters of biodiesel fuels on combustion, performance and emission characteristics of a DI diesel engine, international Journal of Energy and Environment, 3 (2010) 411-430.

[10] N.B. Board, Biodiesel And The U.S. Diesel Vehicle Market: 2016, http://biodiesel.org, National Biodiesel Board, 605 Clark Ave., PO Box 104898, Jefferson City, MO 65110, Accessed: 2018, http://biodiesel.org/docs/default-source/ffs-basics/diesel-vehicle-list.pdf?sfvrsn=20.

[11] A. Barry, A. Wolfe, C. English, C. Ruddick, D. Lamber, National Algal Biofuels Technology Review, US Department of Energy, Office of Energy Efficiency & Renewable Energy, Bioenergy Technologies Office, (2016).

[12] D.J. Franklin, C.P. Brussaard, J.A. Berges, What is the role and nature of programmed cell death in phytoplankton ecology?, Eur. J. Phycol., 41 (2006) 1-14.

[13] A. Cohen, E. Sendersky, S. Carmeli, R. Schwarz, Collapsing aged culture of the cyanobacterium Synechococcus elongatus produces compound (s) toxic to photosynthetic organisms, PLoS ONE, 9 (2014) e100747.

[14] A. Zuppini, C. Andreoli, B. Baldan, Heat stress: an inducer of programmed cell death in Chlorella saccharophila, Plant Cell Physiol, 48 (2007) 1000-1009.

[15] E.R. Venteris, R.L. Skaggs, A.M. Coleman, M.S. Wigmosta, A GIS cost model to assess the availability of freshwater, seawater, and saline groundwater for algal biofuel production in the United States, Environ. Sci. Technol., 47 (2013) 4840-4849.

[16] L.A. Wal-fohd, Living Resources of the Sea, Soil Sci, 86 (1958) 228.

[17] M.D. Guiry, How many species of algae are there?, J. Phycol., 48 (2012) 1057-1063.

[18] F.J. Marquez, K. Sasaki, T. Kakizono, N. Nishio, S. Nagai, Growth characteristics of Spirulina platensis in mixotrophic and heterotrophic conditions, J. Ferment. Bioeng., 76 (1993) 408-410.

[19] N.-S. Lau, M. Matsui, A.A.-A. Abdullah, Cyanobacteria: photoautotrophic microbial factories for the sustainable synthesis of industrial products, BioMed research international, 2015 (2015).

[20] L. Graham, L. Wilcox, Algae, Prentice-Hall Inc., Upper Saddle River, NJ 07458, 2000, pp. 106.

[21] F. Garcia-Pichel, Solar ultraviolet and the evolutionary history of cyanobacteria, Origins Life Evol Biosphere, 28 (1998) 321-347.

[22] C.J. Allègre, S.H. Schneider, The evolution of the earth, Sci. Am., 271 (1994) 66-75.

[23] J. Newton, D. Tyler, M. Slodki, Effect of ultraviolet-B (280 to 320 nm) radiation on blue-green algae (cyanobacteria), possible biological indicators of stratospheric ozone depletion, Appl. Environ. Microbiol., 37 (1979) 1137-1141.

[24] J.W. Schopf, Microfossils of the Early Archean Apex chert: new evidence of the antiquity of life, Science, 260 (1993) 640-646.

[25] M.T. Madigan, Brock Biology of Microorganisms, 11th edn, SciELO Espana2006.

[26] K. Roberts, E. Granum, R.C. Leegood, J.A. Raven, Carbon acquisition by diatoms, Photosynthesis Res., 93 (2007) 79-88.

[27] T.Y. Eizadora, F.J. Zendejas, P.D. Lane, S. Gaucher, B.A. Simmons, T.W. Lane, Triacylglycerol accumulation and profiling in the model diatoms Thalassiosira pseudonana and Phaeodactylum tricornutum (Baccilariophyceae) during starvation, J. Appl. Phycol., 21 (2009) 669.

[28] P.G. Falkowski, J.A. Raven, Aquatic photosynthesis, Princeton University Press2013.

[29] R.A. Andersen, The microalgal cell, Handbook of microalgal culture: applied phycology and biotechnology, (2013) 1-20.

[30] A. Solovchenko, Physiological role of neutral lipid accumulation in eukaryotic microalgae under stresses, Russ J Plant Physiol, 59 (2012) 167-176.

[31] C.K. Zéberg-Mikkelsen, E.H. Stenby, Predicting the melting points and the enthalpies of fusion of saturated triglycerides by a group contribution method, Fluid Phase Equilib., 162 (1999) 7-17.

[32] D.T. Newby, T.J. Mathews, R.C. Pate, M.H. Huesemann, T.W. Lane, B.D. Wahlen, S. Mandal, R.K. Engler, K.P. Feris, J.B. Shurin, Assessing the potential of polyculture to accelerate algal biofuel production, Algal research, 19 (2016) 264-277.

[33] J.U. Grobbelaar, Factors governing algal growth in photobioreactors: the “open” versus “closed” debate, J. Appl. Phycol., 21 (2009) 489-492.

[34] R.J. Geider, B.A. Osborne, Respiration and microalgal growth: a review of the quantitative relationship between dark respiration and growth, New Phytol., 112 (1989) 327-341.

[35] S. Freeman, J.C. Sharp, M. Harrington, Biological science, in: GramNegCellWall.pdf (Ed.), Prentice Hall Upper Saddle River, NJ:, 2005.

[36] I.E. Huertas, M. Rouco, V. López-Rodas, E. Costas, Warming will affect phytoplankton differently: evidence through a mechanistic approach, Proc. R. Soc. B, 278 (2011) 3534-3543.

[37] M. LÜRLING, F. Eshetu, E.J. Faassen, S. Kosten, V.L. Huszar, Comparison of cyanobacterial and green algal growth rates at different temperatures, Freshwat. Biol., 58 (2013) 552-559.

[38] J. Sheehan, T. Dunahay, J. Benemann, P. Roessler, A look back at the US Department of Energy's aquatic species program: biodiesel from algae, National Renewable Energy Laboratory Golden, 1998.

[39] K.K. Sharma, H. Schuhmann, P.M. Schenk, High lipid induction in microalgae for biodiesel production, Energies, 5 (2012) 1532-1553.

[40] M.A. Bisson, G.O. Kirst, Osmotic acclimation and turgor pressure regulation in algae, Naturwissenschaften, 82 (1995) 461-471.

[41] K. Wegmann, Osmoregulation in eukaryotic algae, FEMS Microbiol. Rev., 2 (1986) 37-43.

[42] Z. Hubalek, Protectants used in the cryopreservation of microorganisms, Cryobiology, 46 (2003) 205-229.

[43] J.G. Day, G. Stacey, Cryopreservation and freeze-drying protocols, Springer Science & Business Media2007.

[44] O. Holm-Hansen, Factors affecting the viability of lyophilized algae, Cryobiology, 4 (1967) 17-23.

[45] R. Taylor, R.L. Fletcher, Cryopreservation of eukaryotic algae–a review of methodologies, J. Appl. Phycol., 10 (1998) 481-501.

[46] K. Kartha, Cryopreservation of Plant Cells and Organs. Ed, CRC Press, Boca Raton (F) USA, 276p1985.

[47] J. Ferrell, V. Sarisky-Reed, National algal biofuels technology roadmap, EERE Publication and Product Library, 2010.

[48] C.M. Godwin, A.R. Lashaway, D.C. Hietala, P.E. Savage, B.J. Cardinale, Biodiversity improves the ecological design of sustainable biofuel systems, GCB Bioenergy, (2018).

[49] A.A. Corcoran, W.J. Boeing, Biodiversity increases the productivity and stability of phytoplankton communities, PLoS ONE, 7 (2012) e49397.

[50] M. Stockenreiter, A.-K. Graber, F. Haupt, H. Stibor, The effect of species diversity on lipid production by micro-algal communities, J. Appl. Phycol., 24 (2012) 45-54.

[51] S.R. Subashchandrabose, B. Ramakrishnan, M. Megharaj, K. Venkateswarlu, R. Naidu, Consortia of cyanobacteria/microalgae and bacteria: biotechnological potential, Biotechnol. Adv., 29 (2011) 896-907.

[52] A. Singh, P.S. Nigam, J.D. Murphy, Renewable fuels from algae: an answer to debatable land based fuels, Bioresour. Technol., 102 (2011) 10-16.

[53] D.R. Erickson, Practical handbook of soybean processing and utilization, Elsevier2015.

[54] W.J. Henley, R.W. Litaker, L. Novoveská, C.S. Duke, H.D. Quemada, R.T. Sayre, Initial risk assessment of genetically modified (GM) microalgae for commodity-scale biofuel cultivation, Algal Research, 2 (2013) 66-77.

[55] B.J. Cardinale, K. Gross, K. Fritschie, P. Flombaum, J.W. Fox, C. Rixen, J. van Ruijven, P.B. Reich, M. Scherer-Lorenzen, B.J. Wilsey, Biodiversity simultaneously enhances the production and stability of community biomass, but the effects are independent, Ecology, 94 (2013) 1697-1707.

[56] W.M. Alley, Desalination of Ground Water: Earth Science Perspectives, USGS Numbered Series Fact Sheet 075-03, (2003) p 4.

[57] R.W. Thimijan, R.D. Heins, Photometric, radiometric, and quantum light units of measure: a review of procedures for interconversion, HortScience, 18 (1983) 818-822.

[58] W.E. Boyson, Albuquerque Solar Resource 2014, in: P.S.E. Laboratory (Ed.) Average highest temperature date: 07-26-2014 Sandia National Laboratories, Albuquerque, New Mexico, 2014, pp. Data file: 431906_431935.431909_431106.431962_432014.cvs.

[59] R. Porra, W. Thompson, P. Kriedemann, Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy, Biochimica et Biophysica Acta (BBA)-Bioenergetics, 975 (1989) 384-394.

[60] Z. Wang, C. Benning, Arabidopsis thaliana polar glycerolipid profiling by thin layer chromatography (TLC) coupled with gas-liquid chromatography (GLC), Journal of visualized experiments: JoVE, (2011).

[61] K.i. Ichihara, Y. Fukubayashi, Preparation of fatty acid methyl esters for gas-liquid chromatography, J. Lipid Res., 51 (2010) 635-640.

[62] K.R. Mitchell, C.D. Takacs-Vesbach, A comparison of methods for total community DNA preservation and extraction from various thermal environments, J. Ind. Microbiol. Biotechnol., 35 (2008) 1139-1147.

[63] A.R. Sherwood, G.G. Presting, Universal Primers Amplify a 23s rDNA Plastid Marker in Eukaryotic Algae and Cyanobacteria, J. Phycol., 43 (2007) 605-608.

[64] R.a.T.L. (RTL), Data Analysis Methodology08/01/2016, 2016, pp. 33.

[65] A. American Public Health, A. American Water Works, F. Water Pollution Control, Standard methods for the examination of water and wastewater, 4500-NO3 B. UltraViolet Spectrophotometric Screening Method, (1905) 4-120 & 124-121.

[66] J.G. Day, M.M. DeVille, Cryopreservation of algae, Cryopreservation and freeze-drying protocols, Springer1995, pp. 81-89.

[67] UTEX, UTEX Culture Maintenance Guides | UTEX Culture Collection of Algae, UTEX, 2019, Accessed: 07-09-2019, https://utex.org/pages/culture-maintenance.

[68] M. Ras, J.-P. Steyer, O. Bernard, Temperature effect on microalgae: a crucial factor for outdoor production, Reviews in Environmental Science and Bio/Technology, 12 (2013) 153-164.

[69] P.A. White, J. Kalff, J.B. Rasmussen, J.M. Gasol, The effect of temperature and algal biomass on bacterial production and specific growth rate in freshwater and marine habitats, Microb. Ecol., 21 (1991) 99-118.

[70] V.R. Flechtner, J.R. Johansen, W.H. Clark, Algal composition of microbiotic crusts from the central desert of Baja California, Mexico, The Great Basin Naturalist, (1998) 295-311.

[71] S. Watanabe, K. Mitsui, T. Nakayama, I. Inouye, Phylogenetic relationships and taxonomy of sarcinoid green algae: Chlorosarcinopsis, Desmotetra, Sarcinochlamys gen. nov., Neochlorosarcina, and Chlorosphaeropsis (Chlorophyceae, Chlorophyta), J. Phycol., 42 (2006) 679-695.

[72] L.A. Lewis, V.R. Flechtner, Cryptic Species Of Scenedesmus (Chlorophyta) From Desert Soil Communities Of Western North America1, J. Phycol., 40 (2004) 1127-1137.

[73] M. Evenari, L. Shanan, N. Tadmor, The Negev: the challenge of a desert, Harvard University Press1982.

[74] L. Xin, H. Hong-Ying, Z. Yu-Ping, Growth and lipid accumulation properties of a freshwater microalga Scenedesmus sp. under different cultivation temperature, Bioresour. Technol., 102 (2011) 3098-3102.

[75] T. Burton, H. Lyons, Y. Lerat, M. Stanley, M.B. Rasmussen, A review of the potential of marine algae as a source of biofuel in Ireland, Dublin: Sustainable Energy Ireland-SEI, 2009, pp. 30.

[76] K. Vogel, M. Gektidis, S. Golubic, W.E. Kiene, G. Radtke, Experimental studies on microbial bioerosion at Lee Stocking Island, Bahamas and One Tree Island, Great Barrier reef, Australia: implications for paleoecological reconstructions, Lethaia, 33 (2000) 190-204.

[77] J. Smoker, H. Owen, L. Lehnen, S. Barnum, Ultrastructure of the nitrogen-fixing, filamentous, nonheterocystous cyanobacterium, Plectonema boryanum, Protoplasma, 152 (1989) 130-135.

[78] K. Reddy, J.B. Haskell, D. Sherman, L. Sherman, Unicellular, aerobic nitrogen-fixing cyanobacteria of the genus Cyanothece, J. Bacteriol., 175 (1993) 1284-1292.

[79] Wongsnanslip, P. Tansakul, M. Arunyanar, Factors affecting growth of β-carotene content of Chlorosarcinopsis sp.(PSU/CHL20) in batch culture, Kasetsart Journal: Natural Science, 41 (2007) 153-157.

[80] W.S. Maddux, R.F. Jones, Some Interactions Of Temperature, Light Intensity, And Nutrient Concentration During The Continuous Culture Of Nitzschia Closterium And Tetraselmis Sp 1, Limnol. Oceanogr., 9 (1964) 79-86.

[81] J.-P. Jourdan, Grow your own spirulina, 2003, pp. 3-4.

[82] A. Herrero, A.M. Muro-Pastor, E. Flores, Nitrogen control in cyanobacteria, J. Bacteriol., 183 (2001) 411-425.

[83] P. Fay, The blue-greens (cyanophyta-cyanobacteria), Edward Arnold London1983, pp. 53-54.

[84] J.R. Gallon, A.E. Chaplin, An introduction to nitrogen fixation, London: Cassel1987, pp. 38-67,68-95.

[85] W. Stewart, Nitrogen fixation by photosynthetic microorganisms, Annual Reviews in Microbiology, 27 (1973) 283-316.

[86] C. Raghukumar, V. Vipparty, J. David, D. Chandramohan, Degradation of crude oil by marine cyanobacteria, Appl. Microbiol. Biotechnol., 57 (2001) 433-436.

[87] A. International, ASTM D1160 – 03 Standard Test Method for Distillation of Petroleum Products at Reduced Pressure1, D1160 – 03, ASTM International, 100 Barr Harbor Drive, PO Box C700, West Conshohocken, PA 19428-2959,, 2003.

[88] A. International, ASTM D 6751 - 07 -Standard Specification for Biodiesel Fuel Blend Stock (B100) for Middle Distillate Fuel, An American National Standard, ASTM International, 100 Barr Harbor Drive, West Conshohocken, PA, 19428-2959, 2007.

[89] G. Knothe, A.C. Matheaus, T.W. Ryan, Cetane numbers of branched and straight-chain fatty esters determined in an ignition quality tester☆, Fuel, 82 (2003) 971-975.

[90] FAO, SECTION 2. Codex Standards for Fats and Oils from Vegetable Sources, Food, CODEX-STAN 210 - 1999, Food and Agricultural Organinzation of the United Nations, 1999.

[91] J. Yanowitz, M. Ratcliff, R. McCormick, J. Tay, M. Murphy, Compendium of experimental cetane numbers, NREL Report: NREL/TP-5400-61693, 2014, pp. 6.

[92] C.E. Shannon, A mathematical theory of communication, ACM SIGMOBILE Mobile Computing and Communications Review, 5 (2001) 3-55.

[93] L. Uslu, O. Içik, K. Koç, T. Göksan, The effects of nitrogen deficiencies on the lipid and protein contents of Spirulina platensis, African Journal of Biotechnology, 10 (2011) 386-389.

[94] G. Knothe, Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters, Fuel Process. Technol., 86 (2005) 1059-1070.

[95] N.J. Tro, Chemistry: A Molecular Approach, New Jersey: Pearson Education Inc, Upper Saddle River, NJ. 07458, 2008.

[96] R.A. Andersen, Algal culturing techniques, Chapter 12, Elsevier2005.

[97] W.H. Thomas, C.H. Gibson, Effects of small-scale turbulence on microalgae, J. Appl. Phycol., 2 (1990) 71-77.

[98] H. Sluiman, R.E. Lee, Phycology, 2009.

[99] A.D. Rosemond, P.J. Mulholland, S.H. Brawley, Seasonally shifting limitation of stream periphyton: response of algal populations and assemblage biomass and productivity to variation in light, nutrients, and herbivores, Can. J. Fish. Aquat. Sci., 57 (2000) 66-75.