Molecular analysis of microbial community structure in open ponds for algal biodiesel production

MOLECULAR ANALYSIS OF MICROBIAL COMMUNITY STRUCTURE IN OPEN PONDS FOR ALGAL BIODIESEL PRODUCTION

A Thesis by

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The following faculty members have examined the final copy of this thesis for form and content, and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science, with a major in Biological Sciences.

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DEDICATION

To my parents, my wife, my sister and Wichita friends

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ABSTRACT

Algal farming in open ponds can be done with simple technology and low capital expenditures. However, in relatively uncontrolled ponds the likelihood that microbial contamination that could affect algal yield is high. We are interested in understanding natural contamination as an ecological process to better control the trajectory of microbial community assembly. *Nannochloropsis salina* was grown in small outdoor open ponds (100 L; 10 cm deep) through three cycles of batch culture using a simplified brackish growth medium. Time-course samples were monitored via pigment analyses, and direct microscopic counts. Extracted metagenomic DNA was subjected to touchdown PCR for amplification of 16S rRNA genes with universal bacterial primers and 18S rRNA genes with algae-specific primers, both using GC-clamps. PCR products of similar lengths were separated by melting characteristics using denaturing gradient gel electrophoresis (DGGE). Contamination of the open ponds by algae was not observed over three two-week batch culture cycles, however, contamination by bacteria was observed. Salinity and pH were likely major factors behind limited algal contamination. DGGE bands from bacterial 16S rRNA gene amplifications were excised, eluted, reamplified, and sequenced, revealing a diverse consortium of bacteria including *Aeromonas, Loktanella, Marinobacter, and Pseudomonas.* Most of the bands were seen on third and fourth day of the batch culture. As the culture progressed, the number of bands seen with DGGE decreased. Band migration was measured and relative front values were calculated. A 2% overall window was used to analyze how closely one band is associated with another. Overall there were 22 individual bands designated as novel based on relative front values. A dendrogram of relatedness was created to compare time-course samples from triplicate ponds, supporting the conclusion that community assembly was more of stochastic than deterministic in nature.

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LIST OF ABBREVIATIONS

L	Liter
ml	Milliliter
µL	Microliter
cm	Centimeter
mm	Millimeter
nm	Nanometer
µm	Micrometer
d	Days
H	Hours
min	Minutes
s	Seconds
M	Molar
mM	Millimolar
g	Gravitational Acceleration
w/v	Weight by Volume
V	Volt
ºC	Degree Celsius
mmho	Micromho
T_m	Melting Temperature
ASP	Aquatic Species Program
ASW	Artificial Sea Water
AT	Adenosine-Thymine

LIST OF ABBREVIATIONS (continued)

BLAST	Basic Local Alignment Search Tool
CO_2	Carbon dioxide
DGGE	Denaturing Gradient Gel Electrophoresis
dNTP	Deoxyribonucleotide Triphosphate
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EPSCoR	Experimental Program to Stimulate Competitive Research
FA	Fatty Acid
Fe	Iron
GC	Guanine-Cytosine
MEGA	Molecular Evolutionary Genetics Analysis
N	Nitrogen
NREL	National Renewable Energy Laboratory
NTSYS	Numerical Taxonomy System
P	Phosphorus
PCR	Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid
Rf	Relative front
SAHN	Sequential Agglomerative Hierarchical Cluster Analysis
sp	Species
SSU	Small subunit

TAG	Triacylglyceride
TEMED	Tetramethylethylenediamine
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

Continuous use of petroleum-based fuels is bound to deplete its source in the future. Recent unexpected variations in oil prices and strong global concerns about climate change have driven scientists to search for alternative energy sources. The world is seeking a renewable source of transportation fuels that meets global energy needs and clean air standards. In the United States itself, two-thirds of all oil use is primarily for transportation (Briggs 2004). The use of hydrogen is considered as an alternative to oil, but it also brings serious problems as an automotive fuel. Biodiesel as a substitute for gasoline has drawn the attention of many people. Biodiesel is a fuel made from vegetable oils or animal fats and can replace the currently used diesel with no further changes (Briggs 2004).

According to the Energy Information Administration's statistics, the United States of America (USA) consumed around 134 billion gallons of gasoline in 2011 averaging 370 million gallons of gasoline per day. On the other hand the production of crude oil in the United States (US) was about 5 million barrels per day in the year 2011. Production of petroleum was about 8 million barrels per day in the year 2011. In the US, 71% of the fuel was consumed by transportation in 2011. The US maintains its supply by importing around 8 million barrels per day of crude oil from other countries. Total world production of oil in 2011 was 87 million barrels per day. The world consumed 87 million barrels per day of petroleum in 2010. This just shows how much we are dependent on oil and petrol. It is inevitable that oil will become a limited resource and we cannot always fulfill the demand. This demand has to be filled by something and that something is biofuel. Biodiesel production is currently based on food crop products like mustard, sunflower, safflower and soybean (Chisti 2007). These crops would be produced on large scale to satisfy the demand of the USA for gasoline. There are two general steps for producing biodiesel on a large scale – growing enough feedstock and transforming it into biodiesel. The first step itself is challenging as it is always doubtful whether or not it is possible to grow enough crops to provide oil for replacing the global demand of petroleum. These biodiesel crops have to overcome the demand of food crops. According to the US Department of Agriculture Economic Research Services, around 340 million acres of land was used to grow crops in 2008. Out of that, 74.6 million acreages were used for soybean harvesting. According to National Biodiesel Board soybeans contain about 18% oil. Average oil production per acre of soybean is about 49 gallons. Clearly the space used and the production amount cannot contribute enough to replace petroleum. Researchers have been working on another fuel source that is capable of meeting global demand for transportation fuel. Algae are photosynthetically efficient organism storing solar energy in the form of oil (Brown *et al.* 1994; Sheehan *et al.* 1998; Fukuda *et al.* 2001). Microalgae are a source that can be harvested with less effort, in less time, and with limited resources. The focus has not totally shifted towards microalgae for biodiesel production but has shown promising progress. A comparison of oil content in microalgae and conventional crops can be seen in Figure 1 (page 45) (Verma *et al.* 2010).

Extensive studies have been conducted around the world to produce biodiesel from microalgae. The US Department of Energy under the National Renewable Energy Laboratory (NREL) funded a program, Aquatic Species Program (ASP), which performed extensive study from 1978 to 1996. They focused on growing algae that can sequester carbon in CO_2 emitted from coal power plants. The researchers soon noticed the high-oil content algae and shifted their focus to growing algae for biodiesel. Scientists were successful in isolating 300 species of algae with high oil content from a collection of over 3000 strains (Sheehan *et al.* 1998). These algae were those which produced a lot of oil under a range of temperatures, pHs, and salinities. NREL was able to isolate a key molecule, acetyl-Coenzyme A that plays a major role in the synthesis of oil in algae (Sheehan *et al.* 1998). NREL also focused on the development of algal farms across the deserts of America. They demonstrated two major ways of cultivating microalgae in mass culture: open pond and closed pond. The program was able to highlight the difficulties of mass culture in those systems (Brown *et al.* 1994). The challenges mentioned in the report, if solved, would produce enough biodiesel on a relatively small amount of land. NREL's research, though closed abruptly, was able to demonstrate the potential of microalgae to replace petroleum (Sheehan *et al.* 1998).

1.1 Algal Taxonomy, a Brief Review:

Algae are eukaryotic unicellular organisms which are found either individually or in groups. Most of the algae fall under the obligate phototrophs, because they require light for their growth (Sheehan *et al.* 1998). With varying size, algae are classified as macroalgae or microalgae. Macroalgae are larger in size, multicellular, and are inhabitants of ponds. Microalgae are unicellular organisms that grow by means of photosynthesis (Sheehan *et al.* 1998). They have varying growth rates. A microalgal cell doubles every few hours during the exponential growth period (Chisti 2007; Johnson 2009). Algae easily adapt to growth conditions, thus having a high likelihood of growing in arid or semi-arid regions or saline water (Patil *et al.* 2008). NREL classified microalgae into several groups based on pigment composition, ultrastructure, biochemical constituents, and life cycle. Five groups of primary importance are described in brief:

1.1.1 Diatoms

Diatoms are dominant inhabitants of oceans and are also found in freshwater. High levels of fucoxanthin, a photosynthetic pigment makes the cells golden-brown. Pigments like chlorophyll *a*, chlorophyll *b*, and β-carotene are present at low levels. Their characteristic feature is a cell wall of polymerized silicon. Silicon deficiency results in accumulation of lipids in their cells. Diatoms are diploid during vegetative growth, a distinguishing factor from other haploid algae (Sheehan *et al.* 1998). Two examples are discussed below. *Nitzschia dissipata* is a diatom of the class Bacillariophyceae, order Pennales and family Nitzschiaceae (Barclay *et al.* 1986). Their cell size ranges from 40-50 µm x 6-8 µm. They grow at an optimum temperature of 30-36 ^{o}C with salinity ranging from 50-70 g TDS/L. *Chaetoceros muelleri* var *subsalsum* is a diatom of the family Chaetoceraceae, order Centrales, class Bacillariophyceae, and division Chrysophyta. Their cell size ranges from 4-7.5 µm x 6-12 µm and salinity range of 32.7-45.0 mmho/cm (1 mmho/cm = 640 TDS). These autotrophs have a temperature range of 17.7-42.8 ^{o}C (Johansen *et al.* 1987).

1.1.2 Green algae

Green algae are evolutionary progenitors of higher plants. These chlorophytes have chlorophyll *a* and chlorophyll *b*. They use starch as their primary storage component. Certain species accumulate lipid in nitrogen deprived environments.

1.1.3 Golden-brown Algae

Golden-brown algae are inhabitants of freshwater. They are similar to diatoms with similar pigment and biochemical compositions. They are commonly referred to as chrysophytes. They store carbon in the form of lipid and chrysolaminarin (Sheehan *et al.* 1998).

1.1.4 Prymnesiophytes

Prymnesiophytes are inhabitants of marine water and are known as haptophytes. Fucoxanthin gives a brown color to them. Their main storage products are lipids and chrysolaminarin (Sheehan *et al.* 1998).

1.1.5 Eustigmatophytes

Eustigmatophytes have very small cell size and therefore represent an important picoplankton component. Their major pigment component is chlorophyll *a*, although there are several xanthophylls (Sheenan *et al.* 1998). *Nannochloropsis* sp. falls under this group. *Nannochloropsis* are photoautotrophic organisms of class Eustigmatophyceae (chlorophyll *b*-lacking class) and order Eustigmatales (Rebolloso-Fuentes *et al.* 2001). Glucose is the dominant sugar in polysaccharides, whereas aspartate, glutamate and proline all predominate their protein content. These organisms are unicellular in nature with a cell size of 2.5 µm. Their growth rate is about 1 doubling per day with an optimum temperature of 24 ^{o}C and optimum salinity of 2-3%. Species of *Nannochloropsis* grow well at high pHs (9.0-optimum) (Barclay *et al.* 1986).

Microalgae are regarded as a superior feedstock for biodiesel production with respect to terrestrial vascular pants (Patil *et al.* 2008; Smith *et al.* 2010). Their existence in high salinity ranges, extreme growth rates, and capability of synthesizing and accumulating large quantities of neutral lipids and oils are a few key features of microalgae. Characters like higher oil content, ability to grow in both marine and freshwaters, and higher growth rate have made microalgae a potential target for new sources of biodiesel (Patil *et al.* 2008; Smith *et al.* 2010). Most of the algae produce oil in excessive amounts when nutrients are scarce. The average oil content for algae is between 20-50% of the dry weight and can reach up to 80% (Spolaore *et al.* 2006; Chisti 2007; Johnson 2009). Since algae lack definite non-photosynthetic structures like roots and shoots like those of terrestrial crops, they are easily cultivated in lands unsuitable for agriculture or any other uses (Chisti 2007). Wastewater bioremediation has also shown interest in algal farming as there occurs mutual benefits. Microalgae get desired nutrients like P, N and CO_2 etc. from wastewater (Smith *et al.* 2010).

1.2 Biochemistry of Algal Biodiesel:

Biodiesel is a fuel substitute derived from any biomass (Sheehan *et al.* 1998). Terrestrial plants like rapeseed in Europe and soybean in the US have been used as crops for production of biodiesel. Even Rudolph Diesel used peanut oil to demonstrate his diesel engine (Sheehan *et al.* 1998). The drawback of growing terrestrial plants is the space they consume and the time needed for them to grow. Algal culture has thus great advantages over terrestrial plants in terms of size and growth rate. Algae can easily replace terrestrial plants due to their high oil content and biomass productivity.

The process that can convert carbon dioxide into organic compounds with high energy content is photosynthesis. Algae uses naturally available components like water, sunlight, carbon dioxide and other nutrients to form biomass along with organic compounds (Sukahara & Awayama 2005; Sharma *et al.* 2012). Algal lipids consist of neutral lipids, polar lipids, wax esters, sterols and hydrocarbons along with prenyl derivatives like carotenoids, quinines, terpenes, tocopherols, and pyrrole derivatives (Sharma *et al.* 2012). Some algae modify their lipid metabolism in response to changes in environmental conditions (Thompson 1996). During optimal growth they produce large amounts of biomass but under unfavorable condition or stress algae alter their lipid biosynthetic pathways. They now start forming and accumulating neutral lipids mainly in the form of TAG (Triacylglyceride) to enable them to withstand the conditions.

Sharma *et al.* (2012) categorized algal lipids into two different types: storage lipids and structural lipids. Storage lipids are non-polar lipids whereas structural lipids are polar lipids. Structural lipids have a high content of polyunsaturated fatty acids and are major structural components of cell membrane. Non-polar lipids are mainly in the form of TAG. Microalgal TAGs are generally characterized by both saturated and monounsaturated fatty acids (FA). Generally TAGs are mostly synthesized in presence of light, stored in cytosolic lipid bodies and then reutilized for polar lipid synthesis in the dark (Thompson 1996). They are also easily catabolized to provide metabolic energy.

Algal oil consists of triglycerides made up of three fatty acid molecules esterified with a molecule of glycerol. When this triglyceride reacts with methanol a stepwise conversion of triglycerides into diglycerides, monoglycerides and then to glycerol occurs (Sukahara & Awayama 2005). This reaction step is called transesterification. The process is similar to hydrolysis but instead the displacement of an alcohol from an ester by another alcohol occurs. Thus the process is also called alcoholysis (Fukuda *et al.* 2001). Methanol is generally used because of its low cost. Alcoholysis also enhances the physical properties of biodiesel. Viscosity of triglycerides is highly reduced enhancing physical properties to improve engine performance (Clark *et al.* 1984).

The products formed are glycerol and methyl esters of fatty acids (biodiesel). The process is illustrated in Figure 2 (page 46). Thus three molecules of alcohol are utilized in converting one molecule of triglyceride to one molecule of glycerol and three molecules of methyl esters (Brown *et al.* 1994; Sheehan *et al.* 1998; Fukuda *et al.* 2001). In larger industrial reactors, six molecules of methanol are used for each molecule of triglyceride to ensure that the reaction occurs in the direction of methyl ester production (Chisti 2007). The process of transesterification is catalyzed by either acid or alkali. Alkali-catalyzed transesterification is about 4000 times faster than the acid catalyzed reaction. Alkali catalyzed transesterification is generally done at around 60 ^{o}C under atmospheric pressure keeping in mind that methanol boils at 65 ^{o}C at atmospheric pressure (Fukuda *et al.* 2001).

1.3 Algal culture:

Microalgae have to be grown in enormous bulk for industrial scale production of biodiesel. The basic steps include culturing algae, growing them and processing of algal biomass. Within these steps include the processes of mass culture, growth management, harvest, transport to plant, drying, product separation, recycling, waste management, and marketing (Sheehan *et al.* 1998; Chisti 2007). Microalgal farms are created for the mass culture of algae. The farms have inputs of sunlight, air, water, carbon-dioxide, and necessary nutrients. Thus compared to growing crops, it is believed to be an expensive process of production (Chisti 2007). Minimizing these costs eventually minimizes the price of the final product. Best available sources are used to achieve the necessity ingredients. Culture during daylight gives enough light from the sun for free. Many researchers have used wastewater treatment plant effluent to provide essential nutrients and carbon dioxide (Smith *et al.* 2010). Carbon dioxide released from fossil fuel-fired power plants are also sequestered for the plants growing algae (Patil *et al.* 2008; Smith *et al.* 2010). Mass algal cultivation is done either in open ponds or closed photobioreactors.

Open pond systems can be traced back to 1950s for mass algal culture (Spolaore *et al.* 2006). Studies conducted by NREL also used open-air pond systems. They are favorable because of lower capital costs compared to that of closed photobioreactors. The most common designs are raceway ponds and circular ponds (Sheehan *et al.* 1998; Verma *et al.* 2010). The most common algae strains used in open pond harvesting are- *Anabaena, Chlorella, Dunaliella,* *Haematococcus, Nostoc and Spirulina* (Chisti 2007; Verma *et al.* 2010). Open pond systems have issues with maintaining purity of culture, water availability, water evaporation, and climate conditions. They are always at a high risk of contamination by bacteria, fungi and protozoa (Patil *et al.* 2008). Increments in contaminant increase the competition for food. Food scarcity may result in depletion of expected cultured biomass (Amann *et al.* 1995).

Closed photobioreactors are more successful in commercial-scale cultivation. They are preferred over open ponds as they eliminate most of the general problems of open pond system. Working in a closed system reduces the chances of water loss and contamination. Temperature control is diurnal and seasonal in open pond systems (Chisti 2007). Closed systems have their own heat provider and thus do not have to depend on the sun for warmth. Christi 2007 stated that the algal biomass from photobioreactors is about 30 times as concentrated as the biomass from open raceway systems. Though closed photobioreactors eliminate many problems associated with open pond systems, they have their own issues too. One of the many problems associated with closed system is light penetration. In closed photobioreactors attachment of cells to the tube walls may prevent light penetration. Light penetration is inversely proportional to cell density and decreases exponentially with penetration depth (Johnson 2009). With the analysis of microbial communities of open pond systems, our research will find methods of controlling the production of desired algal strains over native microbial invaders.

1.3.1 Algal Growth in Wastewater Treatment Plants:

There is growing worldwide interest in treatment of domestic and industrial wastewater. Oswald began the use of algae as means of wastewater treatment in the late 1960s. Microalgae can easily grow in liquid wastes from our houses, industries and farms (Clarens *et al.* 2010). Algae have already been used in bioremediation of piggery wastewater efficiently oxidizing carbon and nitrogen from the wastewater (Godos *et al.* 2009). Nutrient removal from university's domestic wastewater in China was successfully conducted using algae and macrophytes. They were able to reduce wastewater discharge content to the required level avoiding eutrophication (Chen *et al.*, 2003). Abattoir Wastewater Management in rural South Australia also successfully removed nutrients using high rate algae pond treatment system (Evans *et al.* 2005).

With the growing increase in algae biodiesel production, a Kansas NSF EPSCoR-funded project is using wastewater treatment plant effluent through University of Kansas to grow algae. These environmental microalgae convert solar energy to heat energy which heats the wastewater. Aerobic and anaerobic oxidation or reduction takes place along with pH fluctuation which kills most of the pathogens (Clarens *et al.* 2010). Algae also use carbon dioxide and nutrients provided by the wastewater to help reduce the nutrient content of the wastewater (Oswald 1973; Oron *et al.* 1979). Potential burdens of providing CO_2 and fertilizers to algal growth can be thus overcome by wastewater (Clarens *et al.* 2010). This also provides nutrients and water for algal growth and helps in managing wastewater. The algae can be then harvested for biodiesel production.

1.4 Community analysis:

Microorganisms are ubiquitous in nature; from boiling water lakes to frozen Antarctica. Earth's freshwater accounts for less than 1% of total water (Morris *et al.* 2002). Despite years-long interests in microbiology and advancement in the field of technology, knowledge about freshwater microbial diversity is still incomplete. There are still many microorganisms that have not been characterized. There are some (e.g. magnetotactic bacteria) who have been identified but have not been properly cultured in the laboratory (Amann *et al.* 1995; Morris *et al.* 2002). A culture-independent technique using single subunit ribosomal ribonucleic acid (SSU rRNA) has been used as a major tool to reveal the unknowns. SSU rRNA is used as an evolutionary marker in many studies helping to construct a universal phylogeny of microbes based on genetic relationships without depending on phenotypic traits (Nold & Zwart 1998; Lozupone & Knight 2009). This universal phylogeny places organisms from various environments in classes. Amann *et al.* (1996) showed that the SSU rRNA genes clusters represent a close relationship between microbes isolated from various environments (Amann *et al.* 1996). Investigating various published papers, Nold and Zwart (1998) have revealed some patterns of microbial community structure based on their aquatic habitat. Every habitat has a dominant phylum whose organisms are found in abundant numbers compared to other organisms (Nold & Zwart 1998). Yannarell and Triplett (2003) demonstrated the composition of microbial communities within and between the lakes of northern and southern Wisconsin. Their study revealed that regional features do not lead to the development of similar bacterial communities (Yannarell & Triplett 2004).

Numerous studies on aquatic microbiology have been conducted at various ponds, lakes and rivers around the world. These studies use different techniques to classify organisms into different groups. The Proteobacteria are one of the dominant microorganisms of the aquatic habitat. They represent a large proportion of the community in different places like wastewater treatment plants, soil and groundwater. Based on SSU rRNA sequence these physiologically versatile microbes are classified into α, β, γ, δ, and ε subdivisions (Nold & Zwart 1998). Nold and Zwart (1998) explained that α-Proteobacteria and γ-Proteobacteria are both dominant in marine and freshwater environments, with the latter more dominant in marine water. They also dominate the wastewater treatment plants and soil. On the other hand, β-Proteobacteria are an important component of freshwater system but not of marine water. δ-Proteobacteria appear to be more dominant in benthic zone of marine water than freshwater habitats (Nold & Zwart

1998). Other phyla that have been isolated from fresh and marine water are within the Gram-positive bacteria. These organisms are present from the benthic zone to the pelagic zone of the water column. *Acidobacterium, Archaea, Cytophaga-Flexibacter-Bacteroides, and Fibrobacter* are a few of the other groups that have been isolated from freshwater ponds (Wise *et al.* 1997; Nold & Zwart 1998; Yannarell & Triplett 2004).

The ecology of freshwater systems does not end with the living components present in them. Physical and chemical parameters play a vital role in microbial diversity. The environmental variables influence the niches that microbial community can fill. Lindström *et al.* studied fifteen typical freshwater bacteria in fifteen lakes in Northern Europe. The report showed significant influence on microbial community by factors like pH, temperature and the theoretical hydrological retention time of the lakes (Lindström *et al.* 2005). Wise *et al.* (1997) worked on sediments of Carolina bay, a common neutral lentic ecosystem in Carolina coastal plains, to gain some insight into the types of bacteria in freshwater pond sediments. The isolation of diverse microbial populations in the bay revealed that bacterial population which can withstand low pH (Wise *et al.* 1997). Moheimani and Borowitzka (2007) researched the growth of the alga *Pleurochrysis carterae*, in outdoor raceway ponds. Their data supported the finding that outdoor contamination depends on the pH. Microbial contamination decreased when the pond's pH was higher, increasing the productivity of algae. When pH changed due to external factors like rainfall, microbial contamination was observed (Moheimani & Borowitzka 2007). Salinity is another physical factor that affects the growth of algae in aquatic systems. There are always species specific differences in the ability of microorganisms to acclimate to changes in salinity. *Nitzschia*, a genus of algae, have been seen growing at high salinities, whereas *Chaetoceros* sp. are abundant at low salinities (Zhang *et al.* 1999). Light intensity also affects the growth of microbial populations. Algae are photosynthetic organisms and thus tend to grow better in the presence of sunlight. Random laboratory cultures developed by Phillips and Myers (1954) suggested that cells that are on the surface receive more light than the cells in deeper regions of the culture. Natural water bodies like lakes are thus divided into different zones based on light intensity. Microbial populations in these zones vary according to light intensity penetration (Phillips & Myers 1954). A well engineered artificial open pond has an aerating system that mixes the culture evenly to provide even growth. Artificial ponds for algal culture have an aerating system that mixes the culture evenly (Brown *et al.* 1994). Organisms grow in an area where they have ample amounts of nutrients for their survival. Nutrients provide energy for the organism to thrive. Nutrient content has thus been a major entity affecting the microbial community. According to Smith *et al.* (2010), the growth of plants in a given ecosystem should be proportional to the rate of supply of the primary growth-limiting nutrients (Smith *et al.* 2010). Organisms get their organic carbon from photosynthesis in oceans and lakes. Aquatic biomass estimation is a classic method of indicating the tropic state of ponds. Counting and measuring algal cells microscopically is an old method of determining algal biomass and community composition. Algal pigment analysis is another method of measuring algal biomass (Aminot 2000). Chlorophyll *a* is the principal pigment in plants converting light energy to chemical energy. Chlorophyll is the most frequently measured pigment for estimating aquatic microalgal biomass. Light absorption bands of chlorophylls are mainly of two kinds: blue (<40 nm) and red (630-670 nm) (Aminot 2000). The determination of algal pigments depends on spectroscopic characteristics, i.e. light absorbance or fluorescence. Table 1 (page 61) shows the natural occurrence of the main pigments of the chlorophyll group. Chlorophylls along with phycobiliproteins and some carotenoids are measured on a whole cell basis.

Microbial community composition also varies depending on the presence of other microbial populations, predation and infection from viruses. Richard Blasco in 1965 performed experiments on the effect of bacterial contamination in open ponds growing *Chlorella pyrenoidosa* in mass culture. His research showed that bacterial numbers increased with increases in algal mass. He found symbiotic bacteria embedded in the surface of algal cells. He concluded in a good note that there is no relationship between gas-exchange in heavily polluted ponds and algal growth. The research showed that a certain amount of algal material is needed for the invader microorganisms to grow (Blasco 1965). Reimann *et al.* (2000) discusses a diatom bloom. The increase in diatom abundance decreased the bacterial population abruptly in the open-pond system. Along with the *Thalassiosora* sp., viruses and flagellates grew. They suggested that the decrease in bacterial numbers is an outcome of cell lyses and grazing. This decrease lasted only a couple of days as *Cytophagales* and α-Proteobacteria abundance increased in the pond water (Riemann *et al.* 2000). Few community analyses done in natural lakes showed positive viral influences on cyanobaterial community composition(van Hannen *et al.* 1999; Reimann *et al.* 2000; Zwart *et al.* 2005; Tijdens *et al.* 2008). Ewing *et al.* (1970) analyzed the interrelationship between nutrients and algal community composition artificial ponds. His research showed that variations in populations and species diversity were due to fluctuations in nutrient concentration and zooplankton populations. The increase in nitrate concentration in lagoons and ponds were due to increased grazing of algal species by zooplanktons (Ewing & Dorris 1970). *Bosmina* sp. grazed on algal species decimating their population and increasing the nitrogen content of the ponds. Through the analysis of microbial community structure in open pond systems, our research will find methods of enhancing the production of desired algal strains over allochthonous microbial invaders. Monitoring algal growth along with these contaminants in replicate troughs will provide insight into the trajectory of microbial community assembly.

1.5 Analysis of Species Composition

Analysis of microbial communities started with culture-dependent methods. Organisms were cultured in the laboratory and then analyzed. This was not an easy method of cultivation because there were problems in culturing organisms in a controlled environment. Selective cultivation and isolation of bacteria and lack of knowledge and techniques to create exact replicas of their niche in the laboratory led to our increased dependency on culture-independent techniques (Muyzer *et al.* 1993; von Wintzingerode *et al.* 1997; van Hannen *et al.* 1999; Morris *et al.* 2002; Ecrolini 2004). The pioneering work of Woese provided the clarification that organisms can be classified by comparative sequence analysis of their SSU rRNA (Woese 1987). This has helped researchers to use molecular genetic techniques like DNA isolation, characterization, rRNA sequencing, polymerase chain reaction (PCR) amplification of rDNA and functional genes, and in situ hybridization of rRNA oligonucleotides to analyze the community of microorganisms (Nakatsu *et al.* 1996).

1.5.1 Genetic Fingerprinting Technique:

Genetic fingerprinting is one of the culture-independent methods for classifying microorganisms. It provides a pattern or profile of the genetic diversity in a microbial community (Muyzer & Smalla 1998). Genetic fingerprinting analyzes rDNA genes in DNA extracted from natural habitats without prior cultivation and isolation of organisms. This gene is amplified using polymerase chain reaction and then its nucleotide sequence is determined (Muyzer *et al.* 1993). The basic steps include extraction of DNA from an unknown sample. DNA extracts are used as templates in PCR amplifications that amplify a particular region of interest in genomic DNA. Amplicons from different alleles are the same size, but have different primary sequences and can then separated using denaturing gradient gel electrophoresis (DGGE). This gives a specific fingerprint of the sample analyzed along with the series of bands relative to the microbes present in that sample (Ercolini 2004). DGGE is a commonly used culture-independent technique. This technique separates PCR amplicons of same length but with different primary sequences depending on their melting temperature (Muyzer *et al.* 1993; Muyzer & Smalla 1998).

1.5.2 Denaturing Gradient Gel Electrophoresis (DGGE):

DGGE is an electrophoretic technique which separates DNA fragments based on single base differences when run on a gradient difference gel (Ercolini 2004). Fisher and Lerman (1983) first described the separation of DNA with single base-pair substitutions when heat or chemical denaturants (formamide and urea mixture) were applied (Fischer & Lerman 1983). The melting is influenced by the presence of hydrogen bonds between the bases and the attraction between the neighboring bases of the same strand. Adenosine-thymine (AT)-rich regions of DNA fragments will have weaker bond strengths than guanine-cytosine (GC)-rich regions. The weaker bonds of AT regions have lower melting temperatures than GC regions. The temperature at which a strand melts is its melting temperature (T_m) and the discrete segments are called melting domains. Thus melting domains of different sequences of the DNA fragments will have different melting temperatures and will run a different distance in the gel since once melted the molecules move more slowly (Sheffield *et al.* 1989; Muyzer & Smalla 1998). Sequencing of bands will be helpful to differentiate organisms that give the same band position in the gel. This shows that the bands at same distance do not necessarily have same sequence (Sheffield *et al.* 1989; Top 1992; Muyzer & Smalla 1998).

Double stranded DNA is subjected to an increasing denaturant environment in DGGE. The melting DNA forms a branched molecule when the T_m of the lowest melting domain is reached. This branching governs the movement of fragments through the gel. The branched molecules get entangled in the polymerized acrylamide gel reducing the mobility of the fragment. DNA fragments of the same size but different base pair compositions will become retarded at different positions in the gel (Muyzer & Smalla 1998). DGGE has a risk of losing strands when they separate and could migrate completely out of the gel. This type of loss is avoided by the use of GC clamp. GC clamps are usually positioned adjacent to the highest melting point domain. GC pairing has strong hydrogen bonds, thus the clamps melt at higher temperatures than other regions of DNA fragments (Muyzer & Smalla 1998).

A GC clamp is an artificial GC-rich sequence added to the DNA fragments to improve the result of DGGE. A GC clamp usually has 40 nucleotide bases of guanine (G) and cytosine (C) which extends the 5' end of a primer. Two DNA fragments that differ by a single base change in the lowest melting domain will be separated from each other at the end of the gel (Top 1992). GC clamps serve as a stable part of the fragment making it possible to detect any variance at any part of the fragments attached to it (Sheffield *et al.* 1989; Top 1992).

1.6 Molecular Analysis of Species Composition

1.6.1 Ribosomal RNA: A Key to Phylogeny

Regular laboratory characterization of organisms is mostly restricted by their simplicity in morphology, ambiguous physiological traits and resistance in cultivation in artificial media. Nucleic acid sequence changes, especially ribosomal RNA (rRNA) genes, are relatively good indicators of evolutionary changes in an organism as they show a high degree of functional constancy yet change slowly in certain regions. This was demonstrated by C.R. Woese in the early 1980's. These changes in sequence occur in all organisms at different positions and in different directions (Woese 1987). Since then 16S and 18S rRNAs are widely used in mapping phylogenetic relationships.

16S rRNA genes are frequently used as molecular markers in the analysis of naturally occurring microbial populations at the genus and species level of prokaryotes. 16S rRNA gene sequences are about 1550 base pair long and are a major component of the small ribosomal subunit (Clarridge 2004). Similarly, for the eukaryotes 18S rRNA has been frequently used for the phylogenetic study. The 18S gene is part of the ribosomal functional core and is exposed to similar selective forces in all living beings (Meyer *et al.* 2010).

These molecular markers (rRNAs) perform protein synthesis and thus are functionally and evolutionarily homologous in all organisms. Due to their antiquity these rRNAs are extremely conserved in overall structures. Sequence information gathered from rRNAs is sufficient enough to conduct statistically significant comparisons. Using the regions of the molecule that display a greater rate of genetic drift, an evolutionary distance can also be compared (Sogin *et al.* 1986; Meyer *et al.* 2010). GenBank, a universal sequence database, holds over 40 million nucleotide sequences which can be used to compare unknown sequences with the known sequences (Clarridge 2004). Their repetitive arrangement within the genome provides excessive amounts of template DNA for PCR, even in the smallest organisms (Meyer *et al.* 2010). The degree of similarity in the sequences between two organisms can then be used to group them (Gray *et al.* 1984; Meyer *et al.* 2010).

1.7 Hypotheses:

In an open-pond algal culture system, the likelihood of invasion by native microbial contamination is very high. Researchers at NREL have shown that the invading organisms can negatively affect the total production of the desired algae, hence affecting the end product, biodiesel. Biological interactions like commensalism, mutualism, or antagonism are all likely to occur in our ponds. These interactions would bring competition for resources and thus affect the survival of the desired algal culture. If these common invaders are known, it is easier to develop measures to prevent their invasion. Thus replicate open-ponds were created near each other. Culture samples were collected and analyzed in the laboratory to identify some of the invading microorganisms. DNA extraction, PCR, and DGGE were done on the samples and organisms were identified by gene sequencing. Identification of organisms helps us work on preventive measures to protect our pure algal cultures from known invaders. Nearby replicates helps us compare and determine common invaders. Looking at the situations that would affect the growth of the desired algal culture, the following hypotheses are made:

- Wild algae invasion will change the structure of algal communities in open-air ponds

  Algae are mainly aquatic and an open pond is a good site for their proliferation. There is a high likelihood of invasion by wild algae in our open-pond systems. The desired inoculated algae then have to either grow in concerti with or fight against the invaders for survival. Though our algae are acclimatized to the laboratory environment, the native algae have a high possibility of outgrowing the desired algae. Our open-pond systems were close to each other and thus had less possibility of variation in invading algae although this may still be a stochastic process.

- Bacterial invasion may affect the community structure in open-air ponds

  Bacterial invasion in our open-pond systems is inevitable but a variation of bacterial invaders in them is likely. Some researchers have stated that the cause for changes in their algal community structures is bacterial dominance. It is likely that our pond system follows the same trend. The by-products of bacteria may affect the conditions in the pond water consequently affecting both the algal and bacterial communities.

- The trajectory of community assembly will vary in open-air ponds due to stochastic properties

  Open ponds are prone to microbial invasions. With the likelihood of bacterial, fungal, viral, and algal growth, there also are potential changes in open-pond conditions. Fluctuations in pH, turbidity, and dissolved oxygen may have an impact on community assembly. These properties along with some environmental determinants like temperature and evaporation may play a vital role in determining the presence of certain invaders and not others. The trajectory of community assembly thus may vary with time as these properties change. An organism present during the early days of startup of a culture may not be present over time. Variation in abiotic factors and microbial inoculation may make the community assembly process seem stochastic. Replicate ponds in a small area are not expected to develop the same microbial communities as the pure cultures become contaminated.

CHAPTER 2

MATERIALS AND METHODS

2.1 Algal Culture: Laboratory Scale

2.1.1 Pure Culture

Algal samples were obtained from the University of Hawaii ASP NREL algal collection. Erlenmeyer flasks with 100 ml of Artificial Salt Water (ASW) media (Tables 2-5; pages 62-65) was inoculated with 1 ml of the pure algal culture received from the NREL collection bank. A New Brunswick Scientific rotatry platform shaker at a speed of 150 rpm was used to continuously stir the culture under artificial light (cool white fluorescent of approximately 50 microEinsteins). The culture was maintained by transferring 1 ml into a new flask containing 100 ml of ASW media every 45 d. Samples were also stored in 15% and 50% glycerol in -80 ^{o}C.

2.1.2 Photobioreactor

Nannochloropsis sp. was grown in a 2-liter photobioreactor containing 1.5 L of ASW medium. Fifteen ml of 45-day-old pure culture of *Nannochloropsis* sp. was inoculated into the bioreactor. The bioreactor was continuously stirred with a magnetic stirrer attached to it. Continuous supply of air was provided to the bioreactor by sparging through water and filtering with a 0.2 µm filter to maintain axenic cultures. Bioreactors have air inlets and outlets made of sintered glass. The water-filtered air was passed in and out of the glass tubes. The bioreactors were set up at room temperature with fluorescent lights as above. After 15 days of incubation, samples were collected for analysis.

2.1.3 Algal Growth in Open Troughs

Empty glass troughs (baking dishes) of 2000 ml volume were autoclaved. Sterilized 1500 ml of ASW media was then poured into the autoclaved trough. Troughs were placed in a greenhouse and in the laboratory near a window for natural light. Daily monitoring and stirring of the trough was done to aerate and to prevent the cells from settling to the bottom. Fluctuations in pH, cell count, turbidity and chlorophyll content of the cultures were monitored every alternate day. Samples were collected in a 1.5 ml centrifuge tube. The trough was stopped after 15 d of culture. Algal biomass was collected after 15 d of culture in the bioreactor. Oakridge tubes of 30 ml were centrifuged at 8000 g for 10 minutes, where g is the gravitational acceleration. The liquid was poured off and the biomass was weighed. The collected algal biomass was sent to collaborators for further processing.

2.2 Pilot Scale

Pilot scale cultures sets were constructed in the Physical Plant section of Wichita State University campus. Three troughs (Two-ring inflatable pool 48" x 10", manufactured by Intex Corporations) that hold 1000-L volumes of ASW were set up in close vicinity. They were filled with clean ASW media. Laboratory-cultured *Nannochloropsis* sp. was then inoculated into these artificial ponds. Samples were collected on daily basis for cell count, turbidity measurement, pH, and DNA extraction. After 15 to 30 d of culture algal biomass was collected and analyzed.

2.3 Algal Cell Count

A Hausser Scientific Hemocytometer was used for the cell counts. Cells that fall in four squares of the grid were counted along with the center square. If the cell number was high, then the culture sample was diluted for feasible counts of algal cells. Hemocytometric cell count gives the number of cells per cubic millimeter.

2.4 Turbidity Measurement

Algal culture samples were collected and measured for turbidity by spectrophotometery. Plastic cuvettes were used for turbidity measurements. The machine was zeroed using uncultured

ASW media. Turbidity values at 500, 550, 600, and 650 nm were then measured. A graph was plotted based on the various absorbance readings to determine the turbidity at various intervals.

2.5 Chlorophyll Measurement

Chlorophyll extraction was based on a modified Arnon's whole cell extraction protocol. Chlorophyll concentration in whole cells was measured by modifying the light path of the spectrophotometer. Scotch (3M) tape was added to the light path after the cuvette in order to correct for light scattering by the cells. Here the culture itself was used without disrupting cells. The instrument was first zeroed using air. Absorbance of algal culture at different wavelengths (620, 678, and 750 nm) was then measured. The Chlorophyll 1.03 program was used to determine chlorophyll based on Arnon (1971) calculations.

2.6 DNA Extraction

A modified version of the DNA extraction protocol described by Tsai *et al.* (1991) was used for the extraction of DNA (Tsai & Olson 1991). Centrifuge tubes of 1.5 ml were filled with 1 ml of a pond sample. The tubes were centrifuged at room temperature for 15 minutes at 10,000 *g*. Pellets were re-suspended in 500 µL of 10 mM Tris (1 M, pH 8.0). The resuspended cells were submerged in liquid nitrogen for one minute and then immediately thawed in waterbath at 90 ^{o}C for another one minute. This cycle was repeated two more times and the centrifuge tube was shook in a cell disruptor Gene II at room temperature for 30 seconds. Freeze-thaw cycle was repeated again for three more sets. After the completion of sixth set, the tube was centrifuged at 10,000 *g* for 5 minutes. This precipitated the ruptured and unruptured cells and left the DNA suspended in the solution. The aqueous supernatant was transferred into a clean tube and stored at -20 ^{o}C.

2.7 PCR Amplification of 16S rRNA and 18S rRNA Genes

The DNA extracted from the water samples was amplified for analysis by denaturing gradient gel electrophoresis using PCR procedures. Universal bacterial primer set F357GC (5' -CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGA GGC AGC AG- 3') and R518 (5' -ATT ACC GCG GCT GCT GG -3') was the set of primers used for bacterial DNA amplification (Zwart *et al.* 2005; Nowak *et al.* 2008). Eppendorf Mastercycler was used for the amplification. Each reaction mixture (50 µL of final volume) contained 500 ng of DNA, 4 µL of dNTP mixture, 5µL of buffer containing magnesium ion, 1.25 µL of diluted primers and 0.25 µL of Ex-Taq were used. Ex-Taq, buffer, and dNTP mixes were obtained from Takara Bio, Inc. The temperature cycling conditions were as follows, after a preincubation at 94 ^{o}C for 5 min, a total of 35 cycles were performed. Denaturation was done at 94 ^{o}C for 30 s, followed by 1 min of annealing at 60 ^{o}C and 1 min of extension at 72 ^{o}C. This cycling was followed by 5 min of incubation at 72 ^{o}C. Electrophoresis was performed on 2% agarose gel. The gel was run in 1X TAE buffer containing Tris base, acetic acid and 0.5M EDTA for 90 minutes at 110 V.

A eukaryotic primer set Euk1A (5' -CTG GTT GAT CCT GCC AG- 3') and Euk516r (5'-ACC AGA CTT GCC CTC C-3') with a GC clamp 5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3' was used as eukaryotic-specific primers. PCR amplification was done with a different annealing temperature (56 ^{o}C) than that of the bacterial primer set (Diez *et al.* 2001). Non-specific sequence amplification was avoided by using a combination of eukaryotic primers and algae specific primers in touch-down PCR (Zwart *et al.* 2005). Algae-specific primers, Cya-b-F371 (5' -CCT ACG GGA GGC AGC AGT GGG GAA TTT TCC G- 3') and CyaR783 mod (5'-GAC TAC WGG GGT ATC TAA TCC C- 3') were used to eliminate the chances of fungal bands like that of yeast. The annealing temperature was set to 65 ^oC, which is 10 ^oC above the expected annealing temperature, and decreased by 1 ^oC every second cycle until a touchdown of 55 ^oC, at which 5 additional cycles were carried out (Zwart *et al.* 2005).

2.8 Denaturing Gradient Gel Electrophoresis (DGGE):

The products of both bacterial and eukaryotic primers were subjected to parallel denaturing gradient gel electrophoresis. Urea and formamide provided the denaturing conditions to the acrylamide gel. A solution of 100% chemical denaturant consists of 7 M urea and 40% formamide in water. Acrylamide gel of 8% (w/v, a mixture of acrylamide: *bis*-acrylamide; for bacterial primer products) containing denaturing gradients ranging from 40 to 70% were bottom-poured on 1.0 mm spaced casting. Acrylamide solution of 6% was used for eukaryotic primer products. Ammonium persulfate and TEMED were used as polymerizing agents. The sandwich was submerged in a DCode electrophoresis system (BioRad, Hercules, California) containing 7 L of 1X TAE buffer (Tris base, glacial acetic acid and EDTA). Fifteen µL of PCR sample was mixed with 10 µL of 2X loading dye (2% bromophenol blue, 2% xylene cyanol 100% glycerol and dH_2O). The mixture was loaded in the wells of the submerged gel. Electrophoresis was carried out at a constant temperature of 60 ^oC and a constant voltage of 60 V for 16 h. After a complete cycle, the gel was stained in SYBR Gold solution (1:100,000 dilution; dye:1X TAE) for 45 min. An image of the stained gel was then captured under UV transillumination and photographed and analyzed further accordingly.

2.9 Band Excision, Purification and Sequencing

Bands were excised using a fresh razor blade under UV transillumination and transferred to labeled micro-centrifuge tubes. They were then suspended in 500 µL of DNase-free water and stored in -20 ^{o}C overnight. Samples were thawed the next day and 2.5 µL was added to the PCR mixture. The sample was then electrophoresed again to verify the presence of the band and its purification.

2.10 Statistical Analysis:

Extreme end wells of DGGE were run with a standard positive control. The standards were *Nannochloropsis salina* for algal study and *Bacillus subtilis* for bacterial study. The trajectory of microbial invasion was observed using these two organisms as reference. Relatedness of microbial communities was determined using Quantity One-Versa Doc Software. A relative front (Rf) value was calculated by dividing the distance migrated by an unknown band by the distance migrated of a known standard organism mentioned above. During the study, bands migrating within 1% of each other were given a numerical value as their identification. The appearance and the absence of a certain band was converted into binary data for cluster analysis. A dendrogram of relatedness was created in NTSYSpc version 2.1 using Jaccard Similarity Coefficients where data were introduced as binary data (“1” for presence of a band and “0” for absence of a band).

Bands from DGGE were purified and sent for sequencing at University of Kansas, genetic sequencing facility. The obtained sequences were identified in GenBank using BLAST. After alignment in Clustal, MEGA 5.1 Beta4 used distance analysis by Jukes Cantor rules to generate phylogenetic trees.

CHAPTER 3

RESULTS

3.1 Preliminary Laboratory Algal Culture

The algae of interest, *Nannochloropsis salina*, obtained from University of Hawaii was successfully cultured in the laboratory using the artificial sea water medium recipe they provided (Table 2-5, pages 62-65). Various changes were made to reduce the total cost of algal culture for biodiesel production. Composition of the media was thus simplified; expensive chemicals were either replaced by regular tap water or simply excluded. *Nannochloropsis salina* showed normal growth even when the media composition was altered as seen in Table 6 (page 66). There were only a few cases when the algae of interest did not grow; in the absence of calcium chloride, potassium chloride or sodium nitrate. Some media components were substituted with less costly chemicals like the commercial plant food, Miracle-grow. Tap water only as the media for algal growth was also tested because of the presence of chemical ions in tap water. Most of these substitutes gave positive growth result for the algae of interest. Algae were constantly subcultured to maintain the stock culture.

Replicas of small-scale open pond were performed in laboratory and green house. In both cases glass baking dishes were used. Minimal evaporation of water was observed in the troughs (Table 7, page 67). Water level was maintained by adding tap water whenever necessary. Cell culture was periodically observed under the microscope to monitor contamination. DNA extraction and PCR was also performed on temporal samples. The trough pond did not show any algal contamination. Cell mass of *Nannochloropsis salina* increased in an expected pattern during the 14-d course of culture as seen in Figure 4 (page 48).

Algal growth was monitored using the following parameters: algal cell count method using hemocytometer and whole cell chlorophyll measurement method. Hemocytometer cell count showed that *Nannochloropsis salina* doubled to its maximum potential till around 14 d (Figure 3, page 47). Whole cell chlorophyll measurement also showed similar increment in cell number (Figure 4, page 48).

3.2 Pilot Scale Culture

Starter culture of *Nannochloropsis salina* of 1.5 L was grown in a 2.0-L bioreactor in the laboratory. Pilot-scale algal cultures (1000 L volume) prepared as open ponds was subcultured with 1 liter of starter culture. Within days the cultures showed significant increases in cell mass (Figure 5, page 49). Chlorophyll concentration was also measured to determine the growth of cell mass which showed significant increases as the days progressed (Figure 6, page 50).

Initial pH of the culture medium was 7.2 which increased gradually over time. The highest pH was measured on day 13 for pond 3 which reached 10.17 (Figure 7, page 51). At the end of the cycle the pH of all three ponds increased to around 10. There was also fluctuation in the level of media during the study. This decrease in volume observed during the time of sample collection (evening) was due to evaporation and was maintained by adding tap water to the marked depth of 10 cm.

Samples were collected from each pond regularly for two weeks. Sample collection was done using a sterile oakridge bottle for each pond after stirring the ponds with the same bottles (Figure 8, page 52). At the end of each batch the pond water was disposed properly. All three ponds had expected growth of the algae of interest during the entire incubation.

There were two control runs performed on DGGE gels. A PCR-amplified product if ran on two different DGGE gels should give the same pattern of bands and does (Figure 9, page 53).

The second control was a single sample that when PCR-amplified twice in two different reactions should give a similar band pattern and intensity (Figure 10, page 54). Both of these control DGGE runs gave acceptable results.

Molecular analysis showed that all three ponds did not show any detectable algal contamination (Figure 11, page 55). PCR amplicons of DNA extracts from pond samples using eukaryotic primers and algal primers only showed one band. DGGE showed that the band matched the band obtained with a control run of pure *Nannochloropsis salina* (Figure 11, page 55). The factors behind this are discussed later.

A great deal of bacterial contamination was observed by applying molecular analysis to the pond samples using bacterial primers. Samples from ponds I, II and III at different days had different numbers of bacterial contaminants. The pattern of bacterial community structure looked similar across the ponds of same set. Pond 1 had a total of 29 bands with the majority on days 3 and 4. Similarly, Pond 2 had a total of 30 bands with the majority also on days 3 and 4. Pond 3 showed only 21 bands, comparatively lesser than other two ponds. All three ponds had their maximum number of bands present on the fourth day after inoculation. Day 3 and 4 showed a higher number of contaminants than the days to follow. Samples from day 0 did not show any bands. Some bands appeared and disappeared while some stayed throughout the course of the culture as seen in Figure 12 (page 56). Bands C and F from S1P1 (Set1Pond1), bands B, D, and E from S1P2, and bands A, B, and C from S1P3 were the bands that were present throughout the course of culture. The bands were excised and re-run to verify the excision and elution process.

Out of numerous gels with same samples, the best quality gels were used for data analysis. Quantity One-Versa Doc software was used to detect bands and calculate relative front values for the bands (Figures 13, page 57). The images were manipulated to optimize the gel appearance. Bands were detected at a relative sensitivity 10.0 in the software. Due to intensity of bands and sensitivity of the software, some bands were added and some were deleted manually. Relative front values for the bands were manually calculated with help from the program (Tables 8-10, pages 68-70). A relative front value is the distance migrated by a band divided by the distance migrated by a control DGGE band from *Bacillus subtilis*.

Bacillus subtilis, the standard, migrated 8.14, 8.13, and 8.12 in gels with samples from pond 1, 2 and 3 respectively. Calculated relative front values ranged from 0.46 to 1.32. Bands were assigned alphabetical designations based on their relative front values. A 1% distance window on both sides of each band was used when numbers were designated. If a band fell within 2% of the relative front value of another band, these bands were considered to be the same and given the same numerical designation. There were total of 80 bands in three ponds which were grouped into 22 different types (a-v) based on their Rf values (Table 11, page 71).

NTSYSpc version 2.1 was used to make a dendrogram of relatedness. In any random day's sample, presence of a band was designated as "1" and absence of a band as "0". These binary data spreadsheet was used to calculate Jaccard Similarity Coefficients from the software. The alignment file was used as the input file for SAHN (Sequential Agglomerative Hierarchical Cluster Analysis) that uses cluster algorithms to create distance matrices. Tree Plot program was used at the end to obtain a dendrogram (Figure 14, page 58).

The dendrogram of relatedness (Figure 14, page 58) gave a lot of information about the microbial communities of three ponds. Samples with similar bands were closely associated in the dendrogram. Samples from the three pond samples separated into three different phenons as shown in (Figure 15, page 59). Phenon A had samples from pond 1, phenon B had samples from pond 2 and phenon 3 had samples from pond 3. Comparing the DGGE banding pattern and dendrogram, similar banding pattern showed in same phenons.

A phylogenetic tree created using the sequences of excised DGGE bands gave us some idea about the organisms present in the pond community. Most of these organisms were either *Alpha* or *Gammaproteobacteria* (Figure 16, page 60). With the help of the tree, organisms were identified to their genus level. *Loktanella* sp., *Marinobacter* sp. and *Pseudomonas* sp. were some genera identified. One common band that was present in each day was identified as the algae of interest, *Nannochloropsis salina*.

CHAPTER 4

DISCUSSION

DGGE is one of the commonly used molecular techniques that allows quick and easy comparison of microbial communities. This has played a major factor in culture independent ecological community assemblage studies. It is definitely time-saving as compared to clone libraries. An advantage of DGGE is that a selected band of interest can be sequenced to understand the phylotype in an environmental sample. Ferris *et al.* (1996) highlighted that DGGE can be used as a reliable method for determining diversity microbial community of a microbial mat community. They were able to discover populations of cyanobacteria using DGGE method that were not identified by cloning.

The use of the DGGE fingerprinting technique goes beyond environmental microbial communities. It is easy and quick comparison of profiles from related microbial assemblages has increased interests of different fields of researchers. This technique has been used in understanding microbial communities in the guts of human and animals, studying different disease conditions. Stebbings *et al.* (2002) used DGGE to demonstrate the complexity of intestinal microflora and its significance in the pathogenesis of spondyloarthritis. Helicobacter species from liver and stomach samples were detected by Stalke *et al.* (2005) using a DGGE technique. Miller *et al.* (1999) used a DGGE technique for the first time to differentiate and directly identify highly variable alleles in comparison with allele standards. This helped eliminate the need to sequence large numbers of clones to examine sequence variability.

One of the disadvantages of using DGGE analysis is the expense. Purchasing DGGE equipment along with the computer programs is very expensive. PCR primers and their specificity (primers with GC clamp) are always costly. The length of PCR products is also an important factor during DGGE analysis. DNA fragments larger than 1000 base pairs migrate slowly and thus are difficult to examine by DGGE (Sheffield *et al.* 1989). In this study PCR products of bacterial primer were approximately 200 base pairs where as the eukaryotic primer set gave 400-base pair fragments. Gels prepared for separation of these PCR products are tuned by changing the acrylamide/*bis*-acrylamide ratio. Acrylamide is an important chemical that crosslink's with *bis*-acrylamide and determines the pore size. According to the BioRad manual, variable gel percentage was used to run samples. A 6% gel was used for smaller fragments where as a 8% gel was used for fragments larger than 300 base pairs.

Using this molecular technique has limitations too. One of the major limitations to analysis of banding patterns using DGGE is the comparison between gels. In this study, a known organism was used as a control for the entire study. Since the analysis was based on the distance travelled by the bands from the top of the well, it was easier to use a known organism as a standard. Nowadays different DGGE markers can also be purchased.

DGGE is not able to properly analyze the community diversity of complex samples. In some cases environmental sampling at different time gives different band pattern suggesting the dominant population at that instant (Ferris *et al.* 1996). The total number of bands in a lane does not necessarily mean the diversity but means the dominant PCR amplicons population of that sample. Number of bands increased in DGGE gel when Gast *et al.* (2004) chose different method of analyzing same samples. Theoretically each DGGE band corresponds to individual species. This is not always true as explained by Gafan *et al.* (2005). They demonstrated that co-migration of different sequences occur during DGGE which when excised and re-ran on modified DGGE gave different bands.

Complex banding patterns observed in DGGE can be reduced by changing the PCR technique. Using eukaryotic primers would give a wide range of eukaryotic organisms such as fungi. For examination of algal dynamics in relation to the dynamics of other eukaryotic organisms nested PCR was performed. Nested PCR technique was first suggested by Muyzer *et al.* (1993). This technique was used by Zwart *et al.* (2005) to differentiate between heterotrophic bacteria and cyanobacteria. Eukaryotic primers amplified the broader range of eukaryotes from the pond sample. Same PCR product was reamplified using algal specific primers to get cyanobacteria only.

PCR product of pond samples with eukaryotic primer set and algae specific primer set only gave one band in DGGE. This showed that no other algal/cyanobacterial contamination occur in the ponds. This may be due to the high salinity of the artificial pond. As seen in Table 2 (page 62) the composition of media has high salt content. Also, the pH of the ponds increased gradually during the day as water is lost via evaporation. Abundance of algae is affected by pH as explained by the study done by Leavitt *et al.* (1999). When pH was lowered, they experienced increased in algal abundance. Pedersen and Hansen (2003) showed that growth of high pH tolerant species reduced when pH exceeded 9.0. Therefore, high pH could be one of the major reasons behind inhibition of algal growth.

Phylogenetic tree analysis gave us some idea about the excised bands representing pond community. It is not surprising that most of these bands represent bacteria that are seen in saline environment. It is already discussed that high salt environment is the major factor behind less contamination. Phylogenetic tree showed that one of the contaminant is *Loktanella* sp. Lau *et al.* (2004) traced the origin of one of the species of *Loktanella* to marine biofilms. It is not surprising to see some *Marinobacter* sp. and *Pseudomonas* sp. as our open pond contaminant.

CHAPTER 5

CONCLUSION

Algal cultivations done in open ponds often have contamination problems. Instead of a process of contamination, it is better to view it as a process of community assembly. The invaders are generally native to that habitat or that of a particular surrounding. Some abiotic factors also assist in the structuring of the community. The first hypothesis was based on the natural way that communities assembly. Our environment is full of micro- and macro- organisms which can easily move from one place to another or can move with the help of wind and other natural factors. It was expected that, while growing an algal pure culture in an open environment, there would be algal contamination. This study did not observe algal contamination in any of the ponds over the short course of these experiments. There was no change in the growth of alga of interest. The pond water resembled seawater with all the chemicals necessary to make it salty. This high salt may have acted as a protection against other algal invaders. The suggestion that higher the salt in the environment may lead to less diversity through selection pressure is one of the principles of ecology.

The second hypothesis stated that there would be bacterial contamination in the open ponds. Bacteria are found from the highest peak of the world to the deepest level of the ocean. Bacteria are everywhere and have broad survival ranges. Thus, bacterial invasion in the ponds was inevitable. Algal cultures in the ponds grew normally even in presence of bacterial contaminants. Molecular analysis revealed the presence of different bacteria but there were not that many taxa. High salt content and the increase in pH may have prevented the ponds from more widespread bacterial contaminants. Analysis showed that the bacteria that were present in the pond would also thrive in high-salt environments. Their presence did not affect the growth of algae in the pond. There may not have been enough time for bacterial products to effect algal growth.

The third hypothesis was that stochastic properties determine microbial community assembly. There were three ponds built. These ponds had the same depth, width, and medium composition. All three ponds were inoculated with same algal stock culture. Ponds were affected by similar environmental factors, as they were in close vicinity. Over time, the microbial communities assembled in the three ponds were different. This is supported by NTSys analysis where the three pond samples formed three distinct phenons (Figure 13, page 57). Communities assembled differently in all the ponds. None of the band patterns from in three ponds over the study period showed any identity. It is hard to predict microbial community assembly in the environment. Identical ponds close to each other might be assumed to assemble the same community. This suggests that in our community analysis stochastic properties such as unpredictable disturbance and random birth-death events in the community assembly process are more important than deterministic factors such as environmental filtering and species interaction.

Open pond algal growth for biodiesel production has always been a challenge because of its microbial contamination. In the current study, the alga of interest grew to give normal yield of cell mass even in presence of bacterial contaminants and when the medium components were replaced by household chemicals. Community assembly of three ponds at close vicinity was found to be different supporting the stochastic nature of assembly. Even then some deterministic properties cannot be ignored. The environment plays a major role in microbial community formation. Heat, cold, rain and wind cannot be controlled and have similar effect on community assembly. If these factors are considered and more detailed research is conducted, algal culture can be done in open pond with less expense. This eventually brings down the cost of biodiesel.

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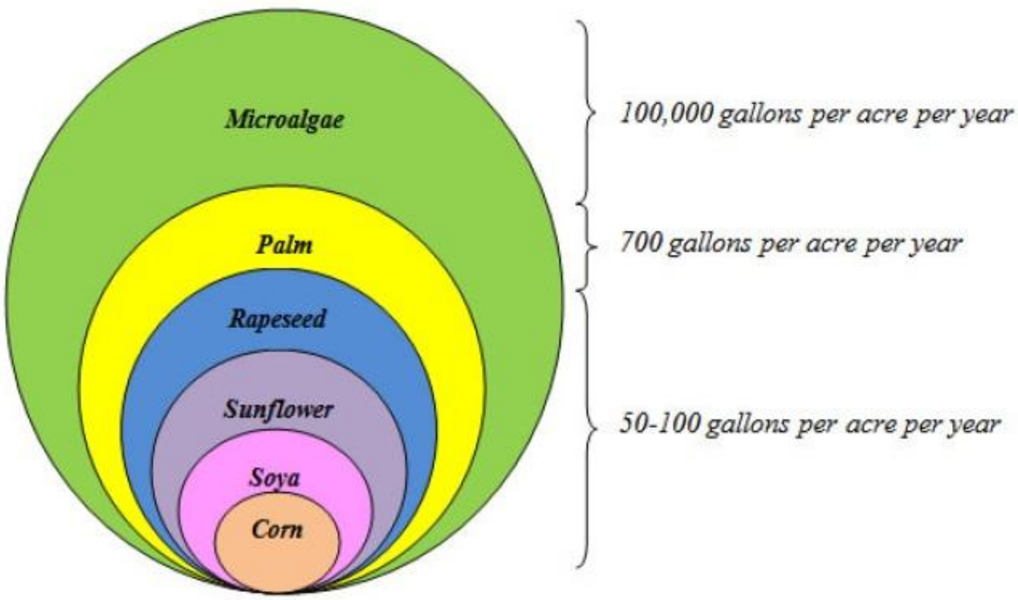
APPENDICES

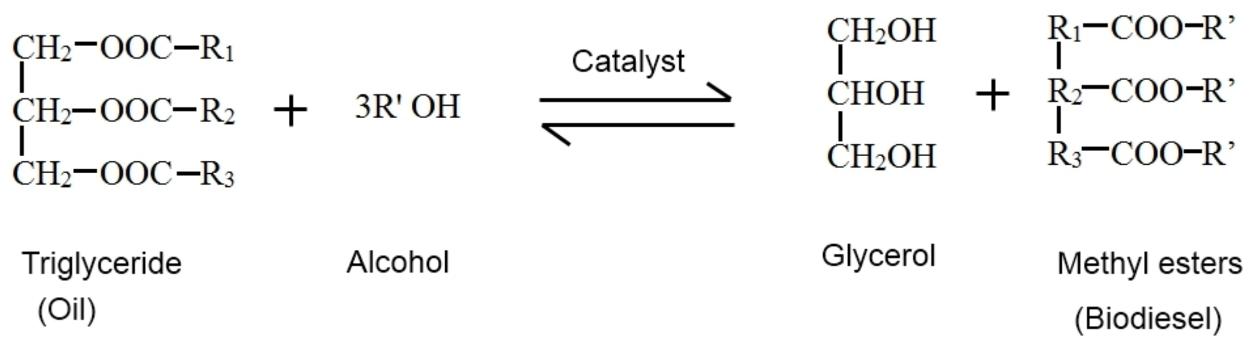
APPENDIX A

Figure 1: Feedstock for biodiesel production per acre



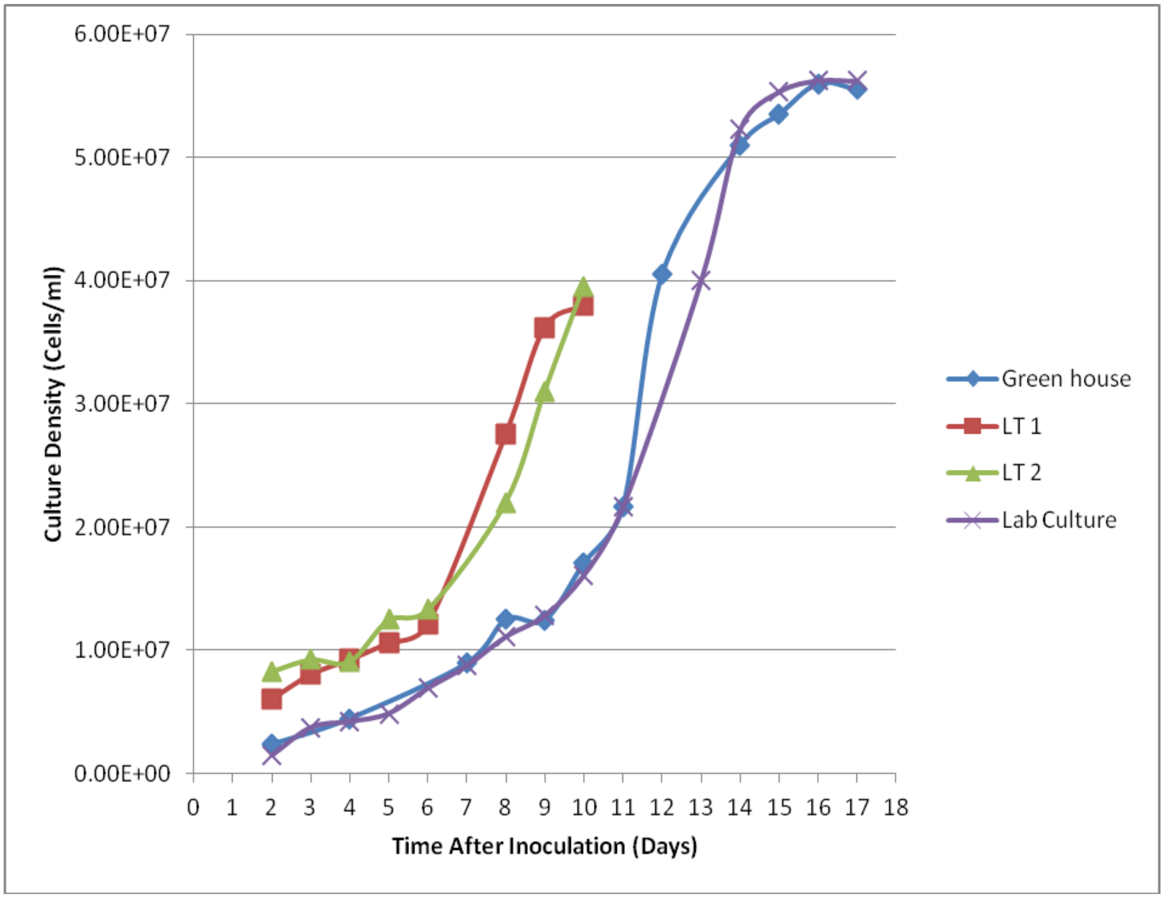
This figure compares the gallons of oil produced between microalgae and different conventional crops per acre per year (Verma et al 2010).

Figure 2: Biochemical reaction for transesterification



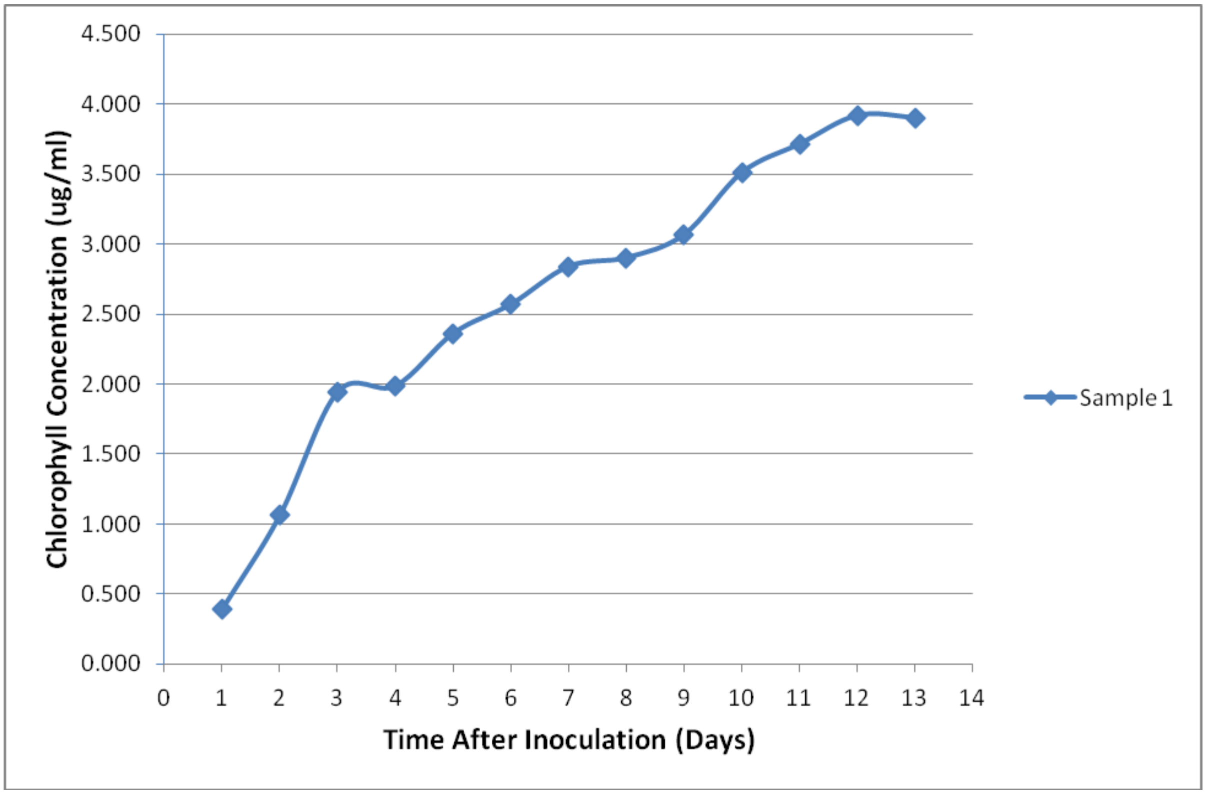
Triglyceride reacts with three molecules of alcohol to give biodiesel and glycerol.

Figure 3: Algal growth rate in preliminary studies using cell density



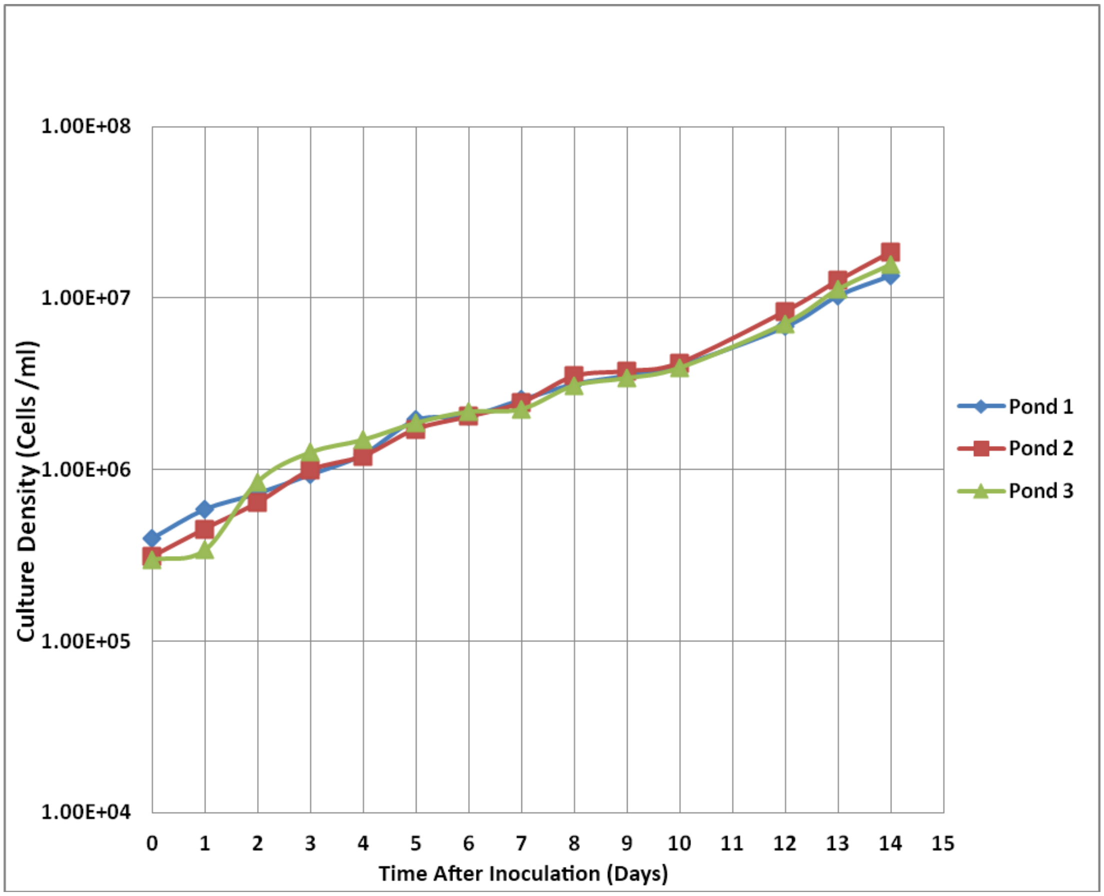
Algal growth in laboratory and green house cultures was measured. Measurement was done by counting cells using a hemocytometer. LT1= Lab Trough 1, LT2= Lab Trough 2.

Figure 4: Algal growth rate in preliminary laboratory culture using chlorophyll concentration



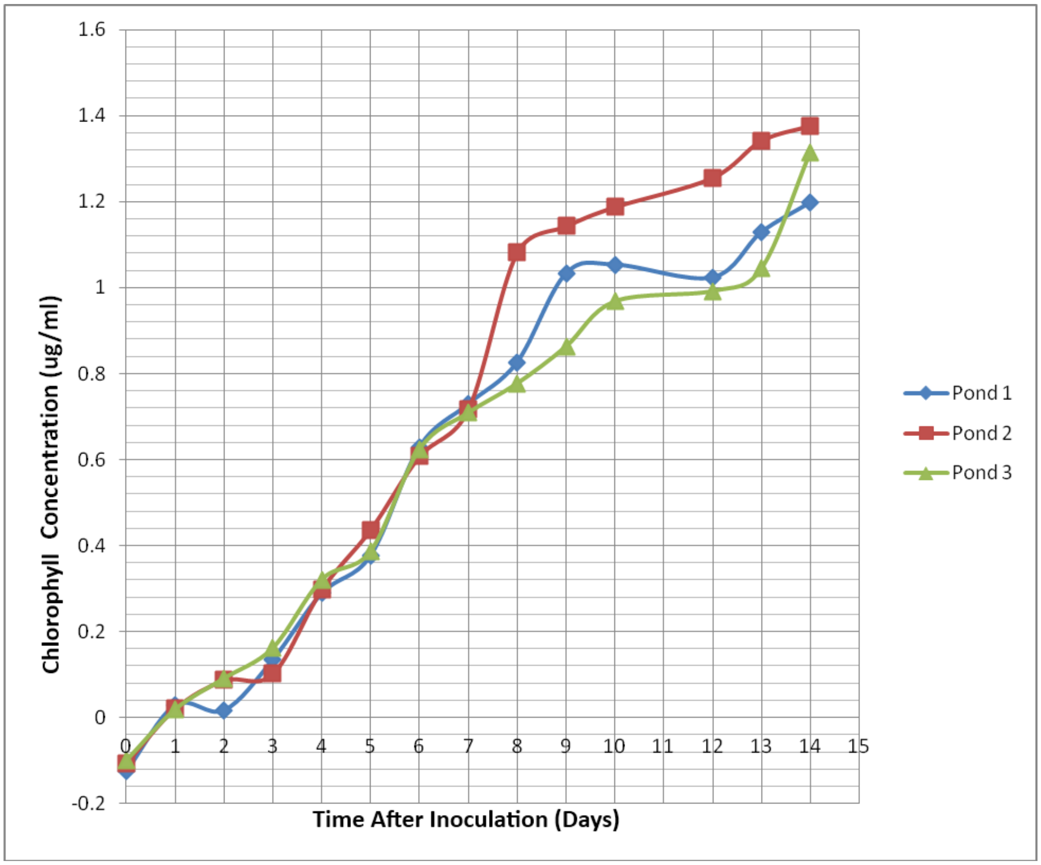
Algal growth in laboratory culture was measured using chlorophyll concentration from Day 1 to Day 13**.**

Figure 5: Semi-log graph of algal growth in three open-air ponds



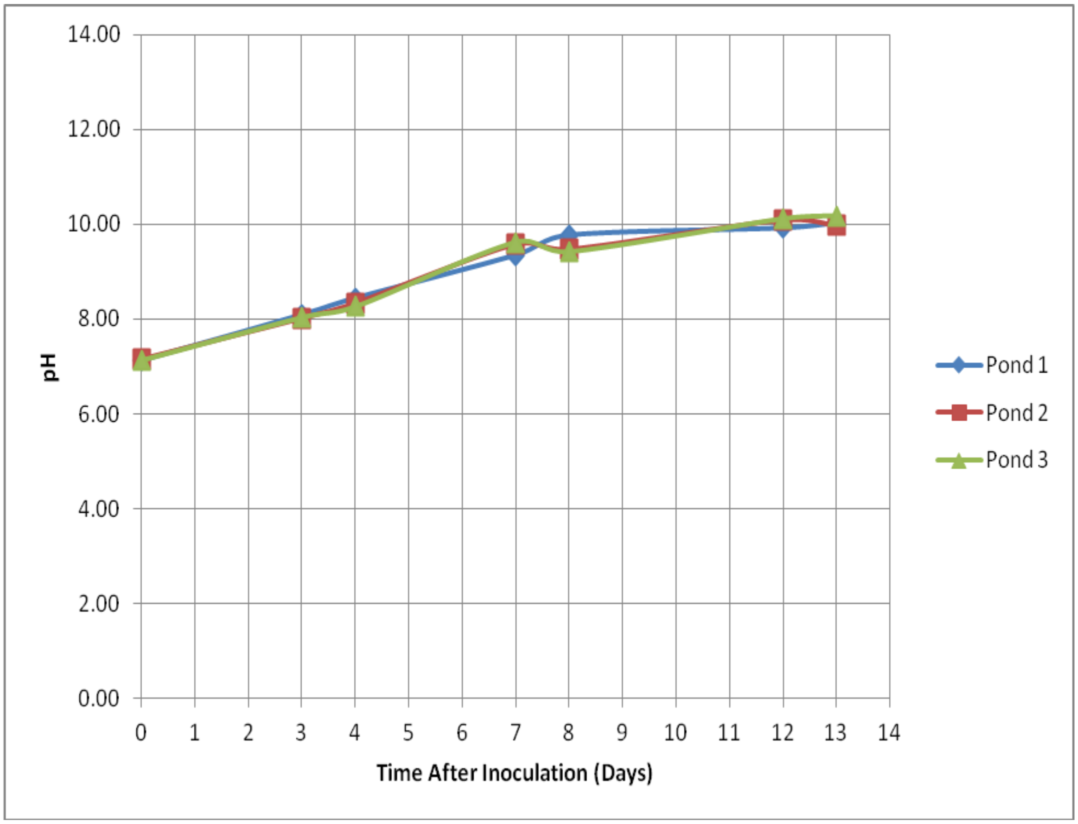
Cell density of three algal ponds was measured using hemocytometer.

Figure 6: Algal growth monitored in three different open-air ponds using chlorophyll concentration



Increase in chlorophyll concentration seen in three algal ponds.

Figure 7: Change in pH observed in three different open-air algal ponds

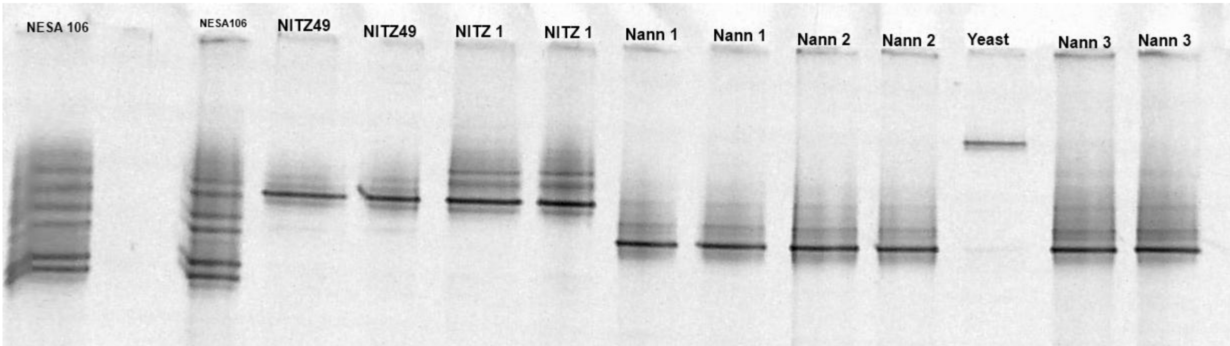


Change in pH observed during the course of algal culture in three different ponds.

Figure 8: Oakridge bottles used in collecting samples from open-air algal ponds

Oakridge bottles were autoclaved before samples were collected.

Figure 9: DGGE gel control run I

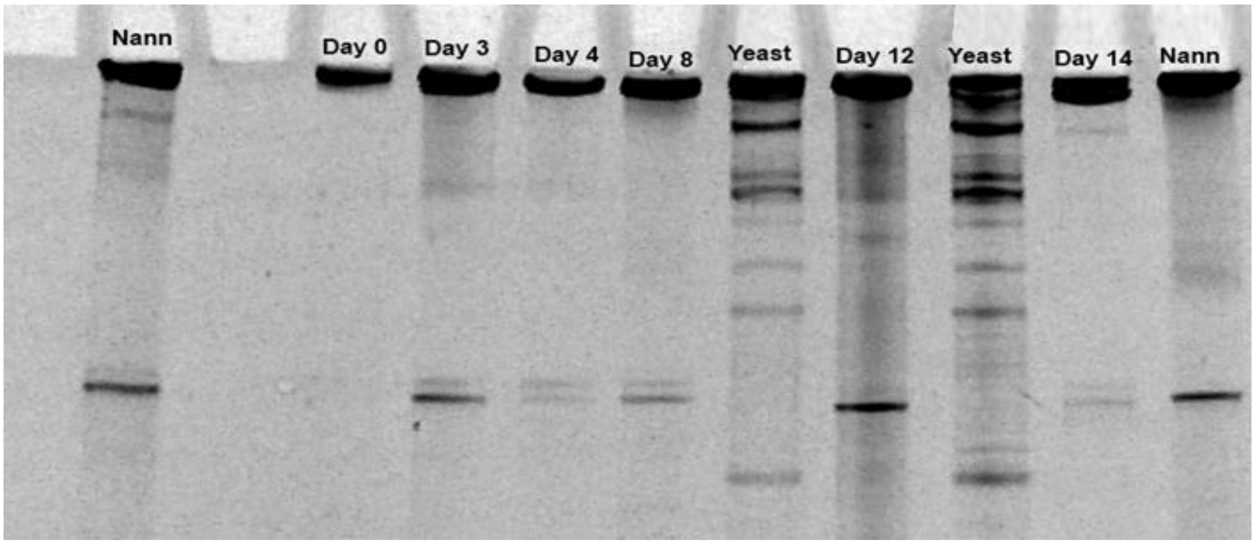


The same PCR-amplified products were run next to each other and gave same banding pattern. NESA 106 = sample from KU, NITZ49 = *Nitzschia* sp, NITZ1= *Nitzschia* sp., Nann1, Nann2, Nann3= *Nannochloropsis salina*

Figure 10: DGGE gel control run II

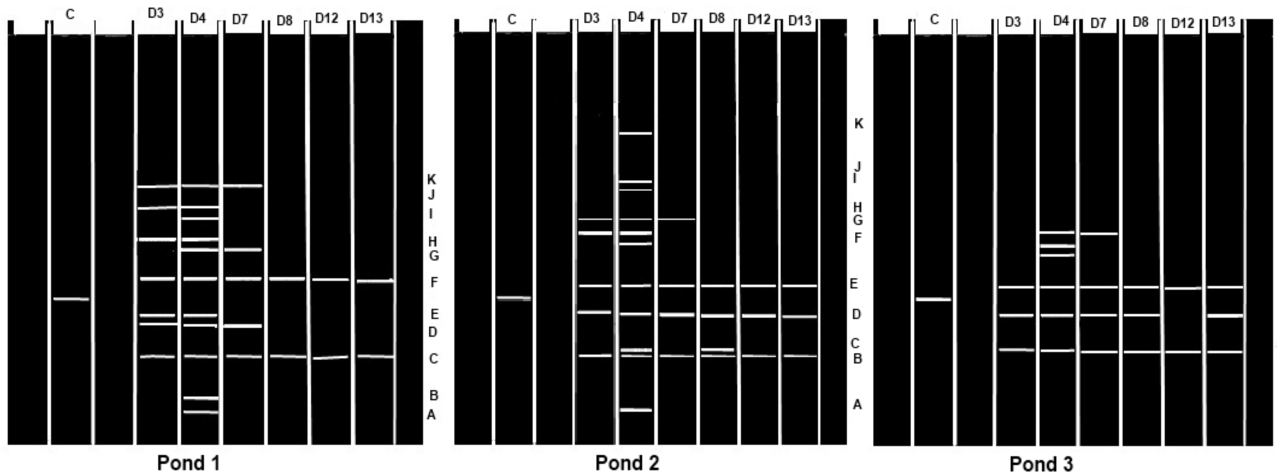
A single DNA extract (Pond 1 Day 4 – a and b) was PCR-amplified in different batches and run side by side. The DGGE control run showed similar banding pattern with similar intensities.

Figure 11: DGGE analysis showing algal contamination



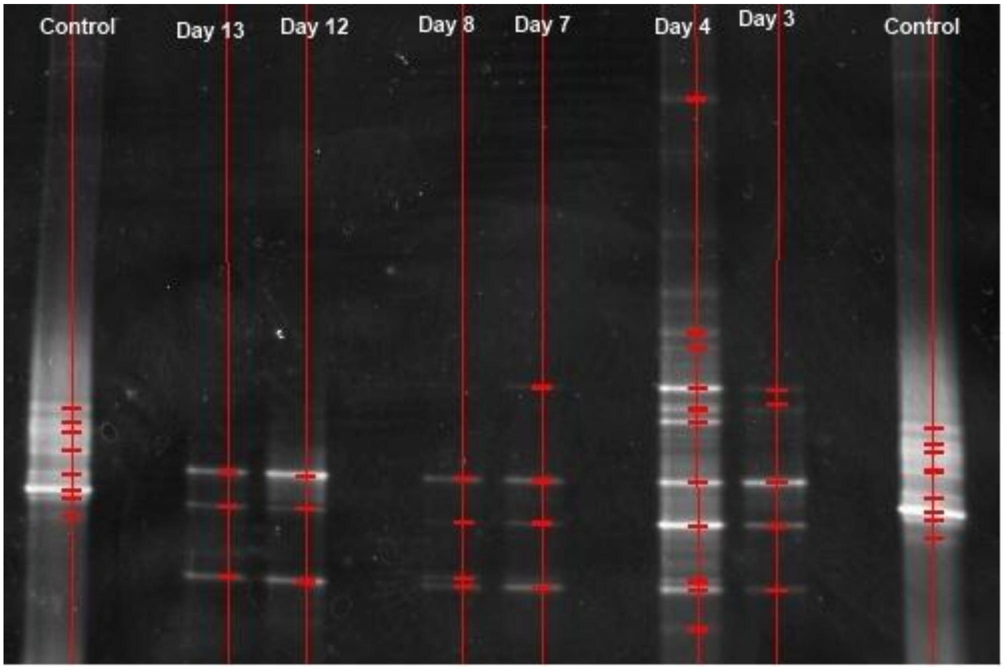
Algal contamination was analyzed using algal primers. This 6% DGGE gel only showed the alga that was inoculated. Nann= *Nannochloropsis salina*

Figure 12: DGGE analysis using bacterial primers



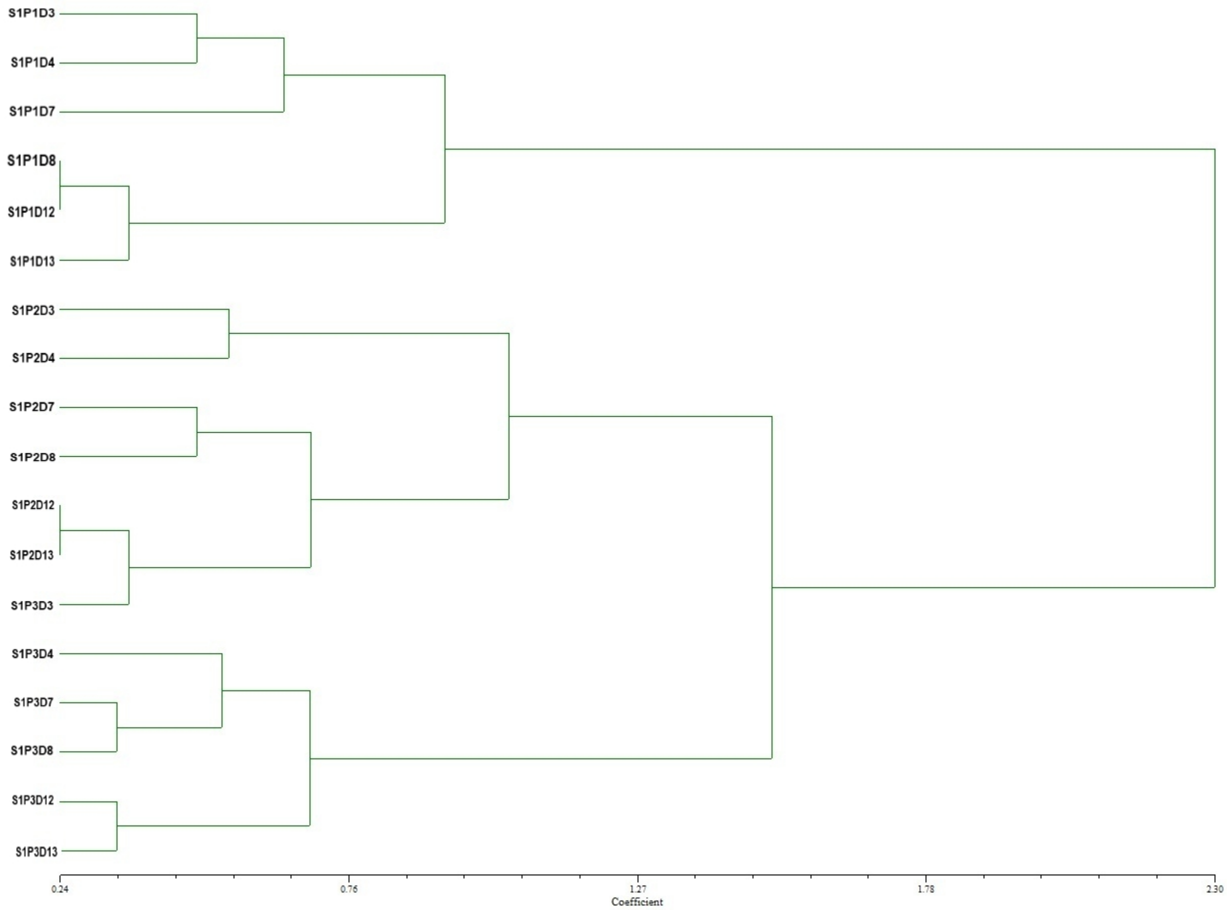
Graphical representation of DGGE gels showing position of bands and labels. C= control, D3= Day 3 sample, D4= Day 4 sample, D7= Day 7 sample, D8= Day 8 sample, D12= Day 12 sample and D13= Day13 sample

Figure 13: Analysis of DGGE gel



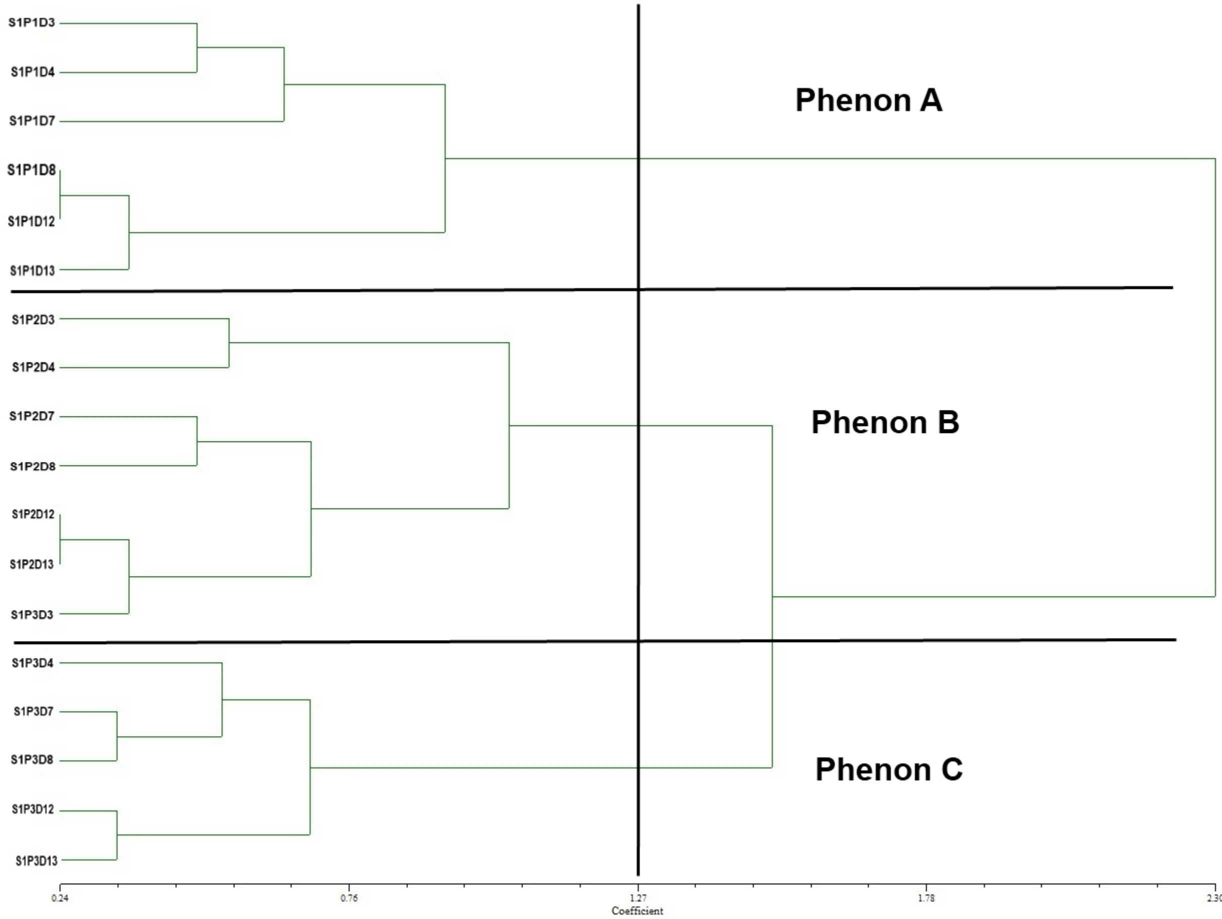
DGGE image of a gel of Pond 2 amplicons showing bands detected using Quantity One-Versa Doc software at a relative sensitivity 10.0.

Figure 14: Dendrogram showing the relatedness of bacterial communities in open ponds



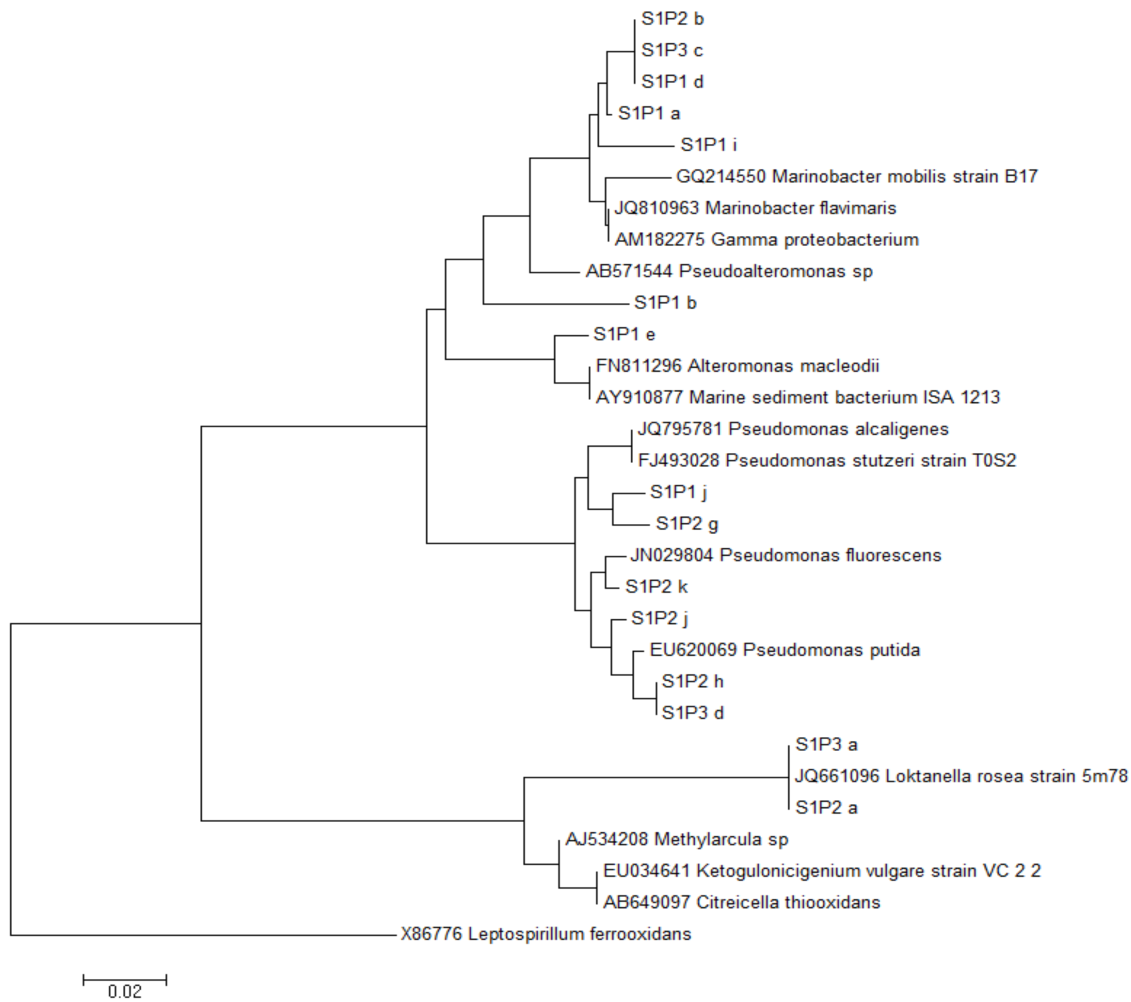
NTSys PC version 2.1 was used to make a dendrogram of relatedness. S1P1= Set1 Pond1, S1P2= Set 1 Pond 2 and S1P3= Set 1 Pond 3

Figure 15: Dendogram showing arbitrary phenons



The dendrogram was analyzed and the samples were placed into three different phenons. Each pond's samples fell into separate phenons. S1P1= Set1 Pond1, S1P2= Set 1 Pond 2 and S1P3= Set 1 Pond 3

Figure 16: Phylogenetic tree analysis of Gram-negative bacteria



Phylogenetic tree analysis was performed using MEGA 5 beta version. *Leptospirillum ferrooxidans* was used as the outgroup. Two different major clades of *Gammaproteobacteria* and *Alphaproteobacteria* can be seen.

APPENDIX B

Table 1: Natural Occurrence of the Main Pigments of the Chlorophyll Group

Pigment	Occurrence
Chlorophyll *a*	All photosynthetic algae (except prochlorophytes) and higher plants
Chlorophyll *b*	Higher plants, green algae, and symbiotic prochlorophytes
Chlorophyll *c*	Chromophyte algae, and brown seaweeds

Table 2: Artificial Sea Water (ASW) Media Composition

Compound	Stock g/L	ml of stock/L of medium				
		150% ASW	100% ASW	100% ASW/U	50% ASW	10% ASW
NaCl	186.4	225	150	150	75	15
$MgCl_2.6H_20$	520.5	15	10	10	5	1
$MgSO_4.7H_20$	356.2	30	20	20	10	2
$CaCl_2.2H_2O$	154.4	15	10	10	5	1
KCl	77.5	15	10	10	5	1
$NaHCO_3$	20.2	10	10	10	10	10
H_3BO_3	6	1	1	1	1	1
$Na_2SiO_2.9H_20$	60.82	5	5	5	5	5
$NaNO_3$	149.6	1	1	1	1	1
NaH_2PO_4	8.8	0.5	0.5	0.5	0.5	0.5
Fe/EDTA	See Below	0.5	0.5	0.5	0.5	0.5
Trace Metals	See Below	0.5	0.5	0.5	0.5	0.5
Vitamins	See Below	0.5	0.5	0.5	0.5	0.5
HEPES	See Below	5	5	5	5	5
Urea	60	-	-	1	-	-

Add ingredients to ca. 500 ml water. Adjust pH to 7.2 with 6N HCl. Bring volume to 1 L. Autoclave or filter sterilize, store in dark.

Table 3: Fe/EDTA Stock

Compound	Stock concentration g/L
$FeC_6H_5O_7$	5.71
$Na_2EDTA.2H_2O$	17.35

Dissolve ingredients in 800ml water, neutralize to pH 7 with 5N NaOH. Boil to dissolve, cool, adjust the volume to 1 liter. Store it in a dark bottle.

Table 4: Trace Metal Stock

Compound	Stock concentration mg/L
$CuSO_4.5H_2O$	3.92
$ZnSO_4.7H_2O$	8.8
$CoCl_2.6H_2O$	4
$MnCl_2.4H_2O$	72
$Na_2MoO_4.2H_2O$	2.52
$Na_2EDTA.2H_2O$	See on instruct

Make individual 200 ml of 1000X stocks of the first five components (these can be stored indefinitely at 4^oC). To make the trace metal stock, add 250 µL of each of the 5 stocks to 200 ml water. Add 0.41 g Na_2EDTA. Boil for 3 minutes. Cool and then adjust the volume to 250 ml. Store at room temperature in a dark cabinet, discard if precipitate forms.

Table 5: Vitamin Stock

First prepare primary stocks of B_{12} (11.4 mg/10 ml after adjustment for 12% water content) and biotin (10.4 mg/100 ml after adjustment for 4% water content). The biotin mixture may require adjustment to pH 10 to dissolve. Adjust stocks to pH 4.5 -5.0. Make to final volume, and store at -20 ^{o}C. To preparte the vitamin stock, use 1 ml biotin primary stock, 0.1 ml B_{12} primary stock and 20 mg thiamine-HCl in 100 ml water. Dispense in small aliquots and store at -20 ^{o}C.

Compound	Stock concentration mg/L
Thiamine-HCl	200
Biotin	1
B_{12}	1

HEPES stock: Dissolve 238.3 g HEPES in about 900 ml water. Adjust pH to 7.25. Bring to 1000 ml final volume. Autoclave and store at 4 ^{o}C.

Table 6: Altered Media and Algal Growth

Media composition	Growth	Media Composition	Growth
without NaCl	Yes	without $NaHCO_3$	Yes
without $MgCl_2.6H_2O$	Yes	without HBO_3	Yes
without $MgSO_4.7H_2O$	Yes	without $NaSiO_3.9H_2O$	Yes
without $CaCl_2.2H_2O$	No	without $NaNO_3$	No
without KCl	No	without NaH_2PO_4	Yes
without Fe/EDTA	Yes	without Vitamins	Yes
without Trace Metal	Yes	without HEPES	Yes
with Tap Water + KCl + $NaNO_3$ + $CaCl_2$ +HEPES	Yes	with Miracle-grow + NaCl+ $CaCl_2$	Yes
with Tap Water	Yes		

Regular artificial media were altered to reduce the cost of media. This table shows the changes in the ASW media and the growth response of algae.

Table 7: Volume of Water Added in Laboratory Scale Algal Growth

	Green House		Lab Trough	
Days	**Volume Added**	**pH**	**Volume Added**	**pH**
1	1500 ml	7.42	1500 ml	7.52
2	600 ml	7-7.5	200 ml	7-7.5
3	600 ml	7-7.5	100 ml	7-7.5
4	300 ml	7-7.5	200 ml	7.5-8.0
5	500 ml	7-7.5	150 ml	7-7.5
6	450 ml	8.5	300 ml	7.5-8.0
7	400 ml	7-7.5	100 ml	7-7.5
8	550 ml	7.5-8	200 ml	7-7.5
9	350ml	7.5-8	200 ml	7-7.5
10	650 ml	8-8.5	150 ml	7-7.5
11	300ml	7-7.5	200 ml	7.5-8.0
12	650 ml	8-8.5		
13	400 ml	7-7.5		
14	200 ml	7-7.5		

Table 8: Calculated Relative Front Values of Pond 1 Samples

Band Name	Distance by Band (cm)	Distance by Standard (cm)	Rf
S1P1D3 c	9.22	8.14	1.1327
S1P1D3 d	8.56	8.14	1.0516
S1P1D3 e	8.4	8.14	1.0319
S1P1D3 f	7.73	8.14	0.9496
S1P1D3 h	7.17	8.14	0.8808
S1P1D3 j	6.7	8.14	0.8231
S1P1D3 k	6.36	8.14	0.7813
S1P1D4 a	10.65	8.14	1.3084
S1P1D4 b	10.3	8.14	1.2654
S1P1D4 c	9.28	8.14	1.1400
S1P1D4 d	8.55	8.14	1.0504
S1P1D4 e	8.37	8.14	1.0283
S1P1D4 f	7.73	8.14	0.9496
S1P1D4 g	7.4	8.14	0.9091
S1P1D4 h	7.17	8.14	0.8808
S1P1D4 i	6.86	8.14	0.8428
S1P1D4 j	6.7	8.14	0.8231
S1P1D4 k	6.35	8.14	0.7801
S1P1D7 c	9.21	8.14	1.1314
S1P1D7 d	8.55	8.14	1.0504
S1P1D7 f	7.72	8.14	0.9484
S1P1D7 g	7.42	8.14	0.9115
S1P1D7 k	6.36	8.14	0.7813
S1P1D8 c	9.19	8.14	1.1290
S1P1D8 f	7.73	8.14	0.9496
S1P1D12 c	9.21	8.14	1.1314
S1P1D12 f	7.67	8.14	0.9423
S1P1D13 c	9.19	8.14	1.1290
S1P1D13 f	7.69	8.14	0.9447

Table 9: Calculated Relative Front Values of Pond 2 Samples

Band Name	Distance by Band (cm)	Distance by Standard (cm)	Rf
S1P2D3 b	8.93	8.13	1.0984
S1P2D3 d	8.26	8.13	1.0160
S1P2D3 e	7.82	8.13	0.9619
S1P2D3 g	7.03	8.13	0.8647
S1P2D3 h	6.82	8.13	0.8389
S1P2D4 a	10.71	8.13	1.3173
S1P2D4 b	8.94	8.13	1.0996
S1P2D4 c	8.84	8.13	1.0873
S1P2D4 d	8.27	8.13	1.0172
S1P2D4 e	7.82	8.13	0.9619
S1P2D4 f	7.20	8.13	0.8856
S1P2D4 g	7.02	8.13	0.8635
S1P2D4 h	6.81	8.13	0.8376
S1P2D4 i	6.28	8.13	0.7724
S1P2D4 j	5.82	8.13	0.7159
S1P2D4 k	3.78	8.13	0.4649
S1P2D7 b	8.93	8.13	1.0984
S1P2D7 d	8.26	8.13	1.0160
S1P2D7 e	7.81	8.13	0.9606
S1P2D7 h	6.81	8.13	0.8376
S1P2D8 b	8.94	8.13	1.0996
S1P2D8 c	8.85	8.13	1.0886
S1P2D8 d	8.28	8.13	1.0185
S1P2D8 e	7.83	8.13	0.9631
S1P2D12 b	8.92	8.13	1.0972
S1P2D12 d	8.19	8.13	1.0074
S1P2D12 e	7.80	8.13	0.9594
S1P2D13 b	8.91	8.13	1.0959
S1P2D13 d	8.20	8.13	1.0086
S1P2D13 e	7.80	8.13	0.9594

Table 10: Calculated Relative Front Values of Pond 3 Samples

Band Name	Distance by Band (cm)	Distance by Standard (cm)	Rf
S1P3D3 a	8.94	8.12	1.1010
S1P3D3 b	8.24	8.12	1.0148
S1P3D3 c	7.80	8.12	0.9606
S1P3D4 a	8.90	8.12	1.0961
S1P3D4 b	8.26	8.12	1.0172
S1P3D4 c	7.79	8.12	0.9594
S1P3D4 d	7.09	8.12	0.8732
S1P3D4 e	6.95	8.12	0.8559
S1P3D4 f	6.72	8.12	0.8276
S1P3D7 a	8.93	8.12	1.0998
S1P3D7 b	8.26	8.12	1.0172
S1P3D7 c	7.78	8.12	0.9581
S1P3D7 f	6.71	8.12	0.8264
S1P3D8 a	8.93	8.12	1.0998
S1P3D8 b	8.26	8.12	1.0172
S1P3D8 c	7.78	8.12	0.9581
S1P3D12 a	8.93	8.12	1.0998
S1P3D12 c	7.79	8.12	0.9594
S1P3D13 a	8.94	8.12	1.1010
S1P3D13 b	8.20	8.12	1.0099
S1P3D13 c	7.78	8.12	0.9581

Table 11: Bands Designation Based on Relative Front Value

Designation Based on Relative Front Values and Using a 2% Window on Either Side of Each Band											
S1P1D3	D	E	I	K	O	P	S				
S1P1D4	C	E	F	I	J	K	N	P	S	T	U
S1P1D7	D	J	K	P	S						
S1P1D8	K	S									
S1P1D12	K	S									
S1P1D13	K	S									
S1P2D3	F	H	L	M	R						
S1P2D4	A	B	C	F	G	I	L	M	Q	R	V
S1P2D7	F	L	M	R							
S1P2D8	L	N	Q	R							
S1P2D12	L	M	Q								
S1P2D13	L	M	Q								
S1P3D3	L	M	R								
S1P3D4	E	G	H	L	M	Q					
S1P3D7	E	L	M	R							
S1P3D8	L	M	R								
S1P3D12	L	R									
S1P3D13	L	M	R								