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Identifying, Monitoring, Quantifying and Converting Algae to Bio-Fuels in Bio-Reactors

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by

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ABSTRACT

Growing algae as a source for bio-fuels has become an area of interest due to concerns about global warming and the reliability and ecology of the production of fossil fuels. Dried algae harvested from a pilot water quality improvement technology at the Rockaway Wastewater Treatment Facility in New York were examined as a source of carbohydrates and lipids for the production of bio-fuels in bio-reactors. The length of storage time, storage conditions, sugar and lipid extraction processes, and fuel production were studied. The results show that if the algae is stored dry (<25% moisture), the algae stock can be used for up to a year with good conversion of carbohydrates to sugars using a 10% w/v of dried algae, yielding an average of 0.11 (g butanol/g sugar) from the bioreactors year-round from a wide range of diatoms and other microalgae used to treat wastewater. Similarly, lipids could be obtained from the stored algal with value of >0.015 g/g algae even after a year in storage. The types of algae harvested has an effect on the amounts of sugars and lipids extracted, so two different methods to identify, monitor and quantify algae grown in both open and closed systems were evaluated. Capillary electrophoresis single strand conformational polymorphism (CE-SSCP) was able to identify known algae samples in an environmental system by "fingerprint" comparison, but may be most useful as a fast, accurate method of monitoring changes in the species in closed systems. We also examined and found capillary electrophoresis single base extension (CE-SBE) to be an extremely fast and accurate method to quantify the algae DNA of *Chlorella vulgaris* and *Spirulina platensis* in a closed system photo-bioreactor. A primer was designed that allowed the accurate correlation of the algae DNA amounts with the area under the curve in an electropherogram. This primer also distinguished between and quantified each species. CE-SBE demonstrated great potential for quantification of algae with difficult morphologies, and algae grown in a co-culture photo-bioreactor.

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DEDICATION

This dissertation is dedicated to my wonderful husband, James Scott Jernigan, without whose support, physical, mental, emotional and especially computer, this long journey would never have been completed. I am the luckiest woman in the world to have such a loving partner.

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INTRODUCTION

Concerns about global warming and the effects that fossil fuels may have on the ecology of the earth as well as concerns over their eventual depletion, have led to the search for sustainable fuels. Alternative sources of energy are being explored and one of the sources that shows great potential for at least a partial replacement of fossil fuels is the exploitation of ubiquitous, rapid growth, photosynthetic algae. Algae use photosynthesis to create energy in the form of starches and lipids and, unlike higher plant forms, these energy sources are more readily available with less processing required for harvesting [1,2]. Algae, by their efficient use of solar energy and CO_2 to grow rapidly and store energy, with O_2 as a by-product, could negate at least some of the effects of global warming by reducing greenhouse gas emissions [3,4]. Because there are currently over 100,000 different recognized species of algae [5], they are a resource that has many potential uses besides bio-fuels. There are also many species that can be used as a secondary source for other beneficial products such as food, fertilizer, nutritional supplements, dyes, and pharmaceuticals [6-9].

However, there are advances required in several areas before algae can be recognized as an economical source of sustainable fuel. Some of the topics that have been considered are; algal speciation, the economics of using algae as a sustainable fuel, and growth and harvesting methods [10-16]. These are previous areas of significant study but, what has been less studied, are alternative methods of taxonomy for species identification both for open systems assessment or closed reactor monitoring. Of some concern is how quickly and accurately algal species can be monitored and identified, and preventing the overgrowth or contamination of a preferred species by undesirable opportunistic algae. It would be beneficial to quickly see the changes in

growth patterns that will allow for maintenance of the selected algal species, and to be able to quickly and easily identify different algal species.

The question of what is the best species of algae for producing bio-fuels has been discussed in several different ways. It is generally understood that there are several species of algae that produce high concentrations of lipids. *Chlorella* and *Nannochloropsis* are just two examples of lipid producing species that have been extensively researched [17]. However, these algae must be starved of nitrogen in order to increase their lipid production which in turn, decreases their metabolic rate. So the trade-off is a higher amount of lipid produced, but a lower amount of bio-mass to extract the lipid from. Rodolfi *et al.* screened thirty microalgal strains to find four (two freshwater and two marine) that were both high growth and had high lipid content [16]. They found that the best bio-mass producers had the lowest lipid content and vice-versa. Of the thirty strains tested those with the highest growth rate and the highest lipid content were three members of the marine genus *Nannochloropsis,* with a lipid content of 30% or higher and a lipid productivity ranging from 55 to 61 mg/L/day. When grown under conditions of nitrogen depletion, it was shown that *Nannochloropsis* increased its lipid production by approximately 35 to 40%. Trials were also conducted on two freshwater strains of *Chlorella* sp. and a strain of *Scenedesmus* sp. The results of these trials found that nitrogen deprivation decreased the growth rate to the point of the cessation of all cell division after 4 to 7 days, without any noticeable increase in lipid production [16]. Liang *et al.* studied *Chlorella vulgaris* using different autotrophic conditions (using light and photosynthesis to produce energy) and heterotrophic conditions (using organic compounds such as media supplemented with glucose, acetate and glycerol as carbon sources), with the intent of increasing the cell growth rate and the lipid production [18]. They found that the highest lipid content of algal cells was obtained in

autotrophic growth conditions under nitrogen depletion (38%). However the highest lipid productivity (defined as a product of total biomass and cell lipid content) was achieved when the cells were grown with a combination of autotrophic and heterotrophic conditions of 1% glucose in light.

Closed photo-bioreactors (PBRs) can be used to grow specific high value algal species as a pure culture. Some species of microalgae are edible, or may produce or be harvested for valuable compounds. Several algal species such as *Chlorella, Spirulina,* and *Scenedesmus* have high protein contents and are currently used as nutritional supplements for both humans and animals [6,19,20]. These microalgae are also sources of several major vitamins, polyunsaturated fatty acids (PUFAs), and carotenoids [6,19] . An algae species that will be harvested for food or pharmaceuticals must necessarily be grown in a highly controlled and regulated environment, which makes a closed photo-bioreactor system the only possible system [21,22]. This is not to say that photo-bioreactors cannot also be used for the culture of algae for bio-fuels. PBRs have also been used to grow high lipid producing algae for bio-fuels [15,16,23]. The main focus with these high value products from algae is the necessity of a controlled environment favoring the individual algal species of interest, the prevention of contamination and the ease of harvesting. The focus of many research groups has been the advancement of different designs of photo-bioreactors to facilitate algal growth and harvest [24-26].

Photo-bioreactors (PBRs) can be technically complex, or relatively simple. A bench-top or lab scale model is relatively easy to construct and works well for testing small batches of samples [27,28]. However, when discussing a scaled-up system on the order of thousands of liters, which is what would be required to make a photo-bioreactor a profitable venture for industrial use, the technical details are very important [11,24,25,29]. The major purpose of these

closed systems is to not allow atmospheric contaminants, opportunistic microbes, or grazers that may overwhelm the desired algal culture. This is an important consideration for algae products destined for food or health supplements. The closed system also prevents water loss, an important consideration if the reactor is sited in an arid region.

Closed systems also have some disadvantages. Light penetration is reduced due to the material used to construct the PBR and dust accumulation on the outside of the reactor [30,31]. With a dense cell concentration, the growth of the algae itself can causing fouling of tubing and light blockage. Chaumont *et al*. developed a system to continuously clean the inner surface of their tubular PBR [28]. Another problem that exists is the control of the oxygen concentration and overheating. Control of these parameters requires the expense of air lift and/or circulation pumps for mixing, heat exchangers for cooling, etc.[32,33] Much thought has been invested in developing the best materials and methods to economically run a PBR. Although many companies and researchers are developing newer and better designs, these issues currently make a PBR a capital intensive investment best suited for fine chemical product production.

An alternative to a closed PBR system is an open system. Historically, open lagoon and pond systems have been used in the treatment of wastewater [34-36]. By allowing native algae to seed and grow, the increased bio-mass produced can be converted to bio-fuel. While these native species are not high lipid producing, they have the advantage of being easy to grow in open ponds and raceways, negating the need for specialized culturing. One of the more economically successful ideas is to use algae to treat wastewater then harvest this algal growth for conversion to bio-fuel, thereby effectively benefitting both processes. Recently it has been suggested that algae growth could be enhanced by the addition of CO_2 from flue gases [37,38]. Coupling the removal of excess nitrogen, phosphorous and heavy metals from agricultural, residential and

industrial wastewater with the scrubbing of CO_2 would offset the cost of sustainable fuels. Doucha *et al.* cultivated *Chlorella* sp. in an outdoor open thin-layer bioreactor for their research on the utilization of flue gas as a carbon source [37]. They and other researchers have suggested that a coupling of anaerobic digestion of animal waste, which produces biogas, and the subsequent combustion and production of flue gas could be an economical feasible system for algal production of farm feedstock [39].

Algae have proven extremely useful for wastewater treatment, either in open ponds, raceways, or grown in bioreactors to produce other products of value as well as bio-fuels. The use of algae to treat wastewater has been examined many different ways since 1957 when Oswald and Gotaas first reported that algae could remove excess nitrogen and phosphorous from wastewater [35]. A reduction of nutrient and metal concentrations in wastewater effluent can be obtained by chemical and physical-based technologies, however these systems consume significant amounts of energy and chemicals, and in comparison to an algal based removal are more expensive [30,36]. With the current interest in sustainable fuels, the possibilities of using algae grown on wastewater as a feedstock for the production of bio-fuels has become an area of intense study. Several comparisons detailing the advantages and disadvantages of methods for growing and harvesting algal biomass from wastewater have been reported [12-14,34,36,40-47]. Open ponds and lagoons have been considered one of the most basic ways to treat wastewater using algae for many years. One particular type of system, called a high rate algal pond (HRAP), is a shallow oxidation pond designed to encourage the maximum amount of suspended algae. Detailed information on HRAPs is provided in an excellent review by Oswald [36].

However, open ponds have some serious disadvantages. They are subject to contamination from microbes and algal grazers which can consume the algae and compete for

the nutrients needed for the algae to grow [48]. Open ponds also require large amounts of space and are subject to both water loss due to evaporation and CO_2 losses from atmospheric diffusion as well as being susceptible to weather and temperature related issues. One of the largest energy requirements is the mixing that is needed for enhanced growth [29,30,49]. This can be done by several different methods, such as paddle wheels, pumps, and gravity flow. Open ponds also require energy to harvest the algae. The difficulty is in the separation of the biomass from the water they were grown in. Conventional processes used to separate algae include centrifugation, membrane filtration, and flocculation.

One method of treating wastewater that is more economical is by an algal turf scrubber (ATS™) [50]. An ATS™ system is a raceway built on an incline that uses gravity flow for nutrient diffusion, and allows for easier harvesting by cessation of water flow and the removal of the attached algae by either scraping or vacuuming of the raceway [43,51,52]. Once the algal growth is removed, it can be placed on screens and dried in a greenhouse with fans. Since harvest of the biomass is not perfect, not all of the algae are removed from the ATS™ and new growth will come from the previous culture, giving a somewhat continuous culture during the growing season. ATS™ systems have been successfully used to treat wastewater from agricultural and municipal effluent streams [44,52-56]. If these economically grown algae are to be used for biofuel production, other avenues of conversion to bio-fuels may be necessary, since the most common high growth rate algae are not high lipid producers. One focus of some researchers has been on harvesting the algae grown on wastewater, and using the starches and sugars they contain as a feedstock for a bio-reactor. These bio-reactors can produce ethanol or butanol that can be used as a bio-fuel.

Several different research groups have successfully used starches and sugars to produce ethanol or butanol in fermentation bioreactors [57-60]. Ellis *et al.* used algae grown in a wastewater lagoon to feed an acetone, butanol, and ethanol (ABE) fermentation reactor [59]. Potts *et al.* used macroalgae harvested from the nutrient contaminated Jamaica Bay in New York to produce butanol from an ABE reactor [60]. All of these groups agree that this method shows possibilities for advancement of algae as a source of bio-fuels. However, in order to effectively transition from a fossil fuel supply to an algal-based bio-fuel, more economical procedures should be developed to lower the price per gallon of bio-fuel to equal the cost of a gallon of fossil fuel. One of the requirements needed for even a partial replacement of fossil fuels is a reliable continuous source of a feedstock for the bio-reactor.

A stable year-round supply of the feedstock for a bio-reactor is needed, but typically the majority of algal species grow abundantly only in a temperate climate. The question becomes; how would it be possible to produce biofuel year-round using algae as a feedstock for a bio-reactor producing bio-fuels? In Chapter 1 of this dissertation we discuss the effects of drying and storage on year-round production of butanol and biodiesel from algal carbohydrates and lipids, using algae from water remediation [61]. While this is promising, the lower oil and carbohydrate values indicate that even slight changes in the species might increase the overall production. Current techniques for the evaluation of algal species require skill, experience, technical proficiency as well as time, to properly identify algal species. A fast, simple way to differentiate the algae growing in a reactor or open system is becoming more desirable as the need for algal products and biofuels increases.

Traditional methods of identifying algae are by microscopic examination [62,63] and more recently, genotyping [64]. Both of these methods can be technically difficult, time-

consuming and, in the case of genotyping, expensive for numerous samples. Microscopic examination consists of examination of live or prepared samples under either a light phase microscope or for more detailed results, a scanning electron microscope (SEM) [63]. Characteristics such as morphology and coloration are used to determine the family and genus. SEM can be used with some samples to determine species.

Recently, genotyping has been very successful in determining many different species of algae [64-66]. These methods, while very useful, require sample preparation of a technical nature for good results. Many researchers choose to send these genotyping samples to commercial or university laboratories which specialize in producing reliable results. Needless to say, analyzing multiple samples can be cost prohibitive. A method that is fast, easy to prepare and inexpensive would be of particular benefit in the future for sustainable energy.

An alternative method of identifying algae samples called capillary electrophoresis (CE) with single strand conformational polymorphism (SSCP) was explored. SSCP has been used to gain a rapid overview of bacterial communities with some success [67-74]. It was first developed using flat plate gel electrophoresis with silver staining [73], but the use of fluorescently labeled primers and capillary electrophoresis make this technique faster, with better resolution, higher throughput, sensitivity and reliability [75]. The method uses a piece of DNA that is common to all algal species. The genomic DNA is extracted from the algal samples and the region of interest is amplified by polymerase chain reaction (PCR). A dilute sample of the amplified DNA is heated to break the hydrogen bonds, giving two single strands of DNA. These single strands are snap-cooled in a non-denaturing, dilute solution, which causes them to fold in on themselves in a conformation dictated by their nucleotide sequence. These unique conformers are separated by CE and will form unique electropherogram patterns of peaks that can be identified by their

elution time, shape and size. These patterns, called “fingerprints”, are repeatable and should be specific to the nucleotide sequence, and thereby to the specific species. Therefore this method could allow for a fast and inexpensive method to determine differences in the genetic code of the species in the sample. What is required to use CE-SSCP as a method for the determination of species is a DNA sequence that is common to all algal DNA, and a known sample to compare the “fingerprint” pattern to.

CE-SSCP has been used to obtain a rapid overview of the different compositions of microbial communities in environmental samples such as the ocean [76,77], and also soil communities [78,79]. More recently, CE-SSCP has been used to monitor harmful algal blooms of dinoflagellates off the coast in Baja California Sur, Mexico [80]. CE-SSCP was found to be a fast, species specific method that allows high-throughput automation. We have explored this method of algae identification in both pure cultures and environmental samples in Chapter 2 of this dissertation.

While CE-SSCP lends itself as a rapid method for monitoring changes in an environmental sample or culture, it has not proved useful for quantification. A method that would allow for the quantification of the relative amounts or the number of cells in an algal sample would be beneficial to operators of photo-bioreactors. When changes are being made in the conditions of the reactor, or when multiple species are being grown in the same reactor, a simple, fast, inexpensive method for noting changes in growth would be valuable. A method, called capillary electrophoresis (CE) single base extension (SBE), is more rapid than CE-SSCP, but has a more narrow application.

Some algal species are relatively simple to quantify. They are single cells and can be counted using a hemocytometer and a light microscope, and will give a concentration of cells per

milliliter of fluid [81-83]. Some expertise and practice is required to be consistent, but it is not a difficult method to teach or learn. This microscopic counting technique can be time-consuming and a uniform mixture of cells and liquid is a necessity for accurate results [83]. This method of counting cells is made simpler if the cells are larger and in a fairly dilute solution to facilitate ease of counting and repeatability [81]. Such is not the case in a reactor where it is desirable that the cells be in a dense concentration for easier harvest. However, many algal cells are in the form of colonies or filaments, and these are much more difficult to quantify [82,84]. Some colonies can be separated by either chemical or mechanical means, however, there are some colonies where neither of these methods is possible and counting the cells in these colonies can be difficult and is often based on estimates [84]. This is where the possibility of using a molecular method becomes more feasible.

Capillary electrophoresis single base extension (CE-SBE) uses a common primer that has a single nucleotide base difference between algal species at the position following the primer. Using the incorporation of a single fluorescently labeled dideoxyribonucleotide (ddNTP) that has a different fluorescent dye for each nucleic acid, a comparison of the four different fluorescent peaks produced by the DNA can be analyzed. It is limited to four variations due to the limitations of the four regions of fluorescence (dyes) recognized by the laser induced fluorescence (LIF) of the CE sequencer. In order to recognize more than four separate species, a multiplex of different primers would be required. This quantity of DNA can be related to an area under the curve of the dye peak on the electropherogram and a calibration curve can be developed to evaluate the concentration. The advantages of this method are its extremely rapid determination, specificity and sensitivity. This method has been successfully used for single nucleotide polymorphisms (SNP) genotype determination [85]. Analysis of SNP markers can be used to identify genes and

mutations and are used as genomic markers for phylogeography and evolutionary ecology. SNPs are the most common of genetic markers throughout the genome and are more evolutionarily stable. Batley and Hayes estimated that there is one SNP every 80 bp in cyanobacterial genomes [86]. They used a variation of CE-SBE to differentiate between variants of *Nodularia* using three different loci. Wu *et al.* also used a variation of this technique to identify and quantify the relative abundance of *C. raciborskii* subspecies, an invasive and sometimes toxic cyanobacterium [87]. In Chapter 3 of this dissertation we have developed a method to quantify two different species of algae grown in a PBR. One species *Chlorella vulgaris* is a eukaryote while the second species *Spirulina platensis* is a prokaryote. Both of these algae are edible and are commonly used in food and health supplements.

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CHAPTER 1: EFFECTS OF DRYING AND STORAGE ON YEAR-ROUND PRODUCTION OF BUTANOL AND BIODIESEL FROM ALGAL CARBOHYDRATES AND LIPIDS USING ALGAE FROM WATER REMEDIATION

ABSTRACT

Algae harvested from a pilot water quality improvement technology at the Rockaway Wastewater Treatment Facility in New York were examined as a source of carbohydrates and lipids for the production of biofuels. Dried stocks of algae harvested during a six month period were used to feed the bioreactors, and the process to extract sugar from the natural wastewater grown algae was optimized. The length of storage time, storage conditions, sugar extraction process, and fuel production were studied. The results show that if the algae is stored dry (<25% moisture) the algae stock can be used for up to a year with good conversion of carbohydrates to sugars using a 10% w/v of dried algae. These optimized conditions extracted the maximum amount of sugar, which yielded an average of 0.11 (g butanol/g sugar) from the bioreactors year-round from a wide range of diatoms and other microalgae used to treat wastewater. Similarly, lipids could be obtained from the stored algal with value of >0.015 g/g algae even after a year in storage. These results demonstrate the potential for year round production of fuel from algae harvested as part of a water reclamation process.

INTRODUCTION

Algae are a versatile group of organisms capable of growth in fresh or salt water with a variety of different nutrient contents. Algae have been explored for their potential to reclaim water, produce biofuel, and capture CO_2 through re-use of the carbon in biofuel. In fact, work by Groom *et al.* has shown that algae are the fastest growing organism with the best carbon capture capacity of any of the feedstocks for liquid biofuels [22]. While significant emphasis has been placed on studying the environmental and fuel aspects of algae individually, the current work explores the potential of combining algae for water reclamation with production of biobutanol from algal sugars and biodiesel from algal lipids. The algae samples for this study were harvested from an algal turf scrubber® (ATS™)[23,24] receiving partially treated sewage from the Rockaway Wastewater Treatment Plant in Queens, New York as part of a pilot demonstration of the ATS™ funded and directed by the New York City Department of Environmental Protection. Specifically, the effects of storage conditions on sugar and lipid content, variables affecting optimum sugar extraction, and end-product production were explored. The ability to store algal material and still achieve high recovery of the raw materials for year-round fermentation of sugar or oil extraction presents a significant benefit for regional, small-scale systems. This study represents a unique perspective on the potential for coupling water reclamation with year-round biofuel production. Further, we show that through the use of ATS™ derived algal biomass, we were able to dry and store algae from six months to a year, a phenomenon that to date has been relatively unexplored.

The use of algae for water reclamation was first published by Oswald and Gotaas in 1957 [18]. Since then, there have been many studies reporting the ability of algae to achieve a high level of treatment for both domestic and agricultural wastewater, as well as natural waters, with

algal treatment driving the removal of nitrogen, phosphorous, and metals from the effluent stream [25-31]. The benefits of this process are two-fold: using wastewater as a source prevents the cost of chemical removal of these inorganic compounds, and in turn, increases the growth rate of the algae resulting in a higher biomass with the possibility of recovery and reuse as a low cost animal feed or fertilizer [32]. Many species of algae need nitrogen and phosphorous as a nutrient source and if not grown on wastewater or nutrient rich natural waters, these compounds must be added to the source water to achieve high growth rates of biomass [33]. Algae also have the ability to utilize CO_2 as a carbon source, which makes the possibility of using flue gases to increase growth rates an attractive option as well [16,17,34]. Several studies have evaluated the costs and benefits of different types of installations with models based on bench scale batch and continuous systems [26,29], while other groups focused on open pond harvesting [20,28] and algal turf scrubbers [25,27,31]. Chinnasamy *et al.* demonstrated some success growing algae in industrial wastewater from the carpet industry mixed with municipal wastewater [35]. Since the levels of nitrogen and phosphorous tend to be lower in industrial wastewater and the organic toxins higher, the growth rates are typically lower [36].

Optimal algae strains and conditions have been explored for biofuel production. Rodolfi *et al*. explored the maximization of lipid productivity and biomass by selecting strains that had the highest amount of lipid with a reasonable growth rate and then growing those strains under nitrogen deprivation conditions in a photo-bioreactor [11]. Mulbry *et al*. examined biomass production and lipid content from an algal turf scrubber open system using agricultural waste, either with or without added CO_2 [37]. They found, in agreement with other studies [38,39], that lipid-rich strains show lower biomass productivity and therefore are not as desirable in bioremediation.

Since high lipid content coupled with a high growth rate present significant challenges, the use of algal carbohydrates has been explored as an alternative or complementary option to increase biofuel output. The cultivation of algal species that have high starch/cellulose contents can be used to produce sugar as a substrate for ethanol or butanol fermentation [20,21,40,41]. Currently, the most widely used biofuel is ethanol produced from fermentation of feedstock crops such as sugar cane, sugar beet, corn and wheat [42]. However, the possible impact on food supply and the amount of arable land and water they require are significant drawbacks [43-45]. Several species of algae have high starch content which can be converted to sugars. Kim *et al.* used algal hydrolysates containing a mixture of sugars to produce ethanol from marine algae [40], while Ellis *et al.* used a mixture of high starch producing fresh water algae in an acetone, butanol, and ethanol (ABE) fermentation to produce butanol [20].

Algae can be grown in open systems exposed to environmental conditions or in a controlled environment such as a bioreactor. Most slow growing, high lipid producing algae are preferentially grown in a photo-bioreactor to reduce the possibility of contamination by fast growing opportunistic species [11]. This closed system also reduces the risk of predation, and the conditions and nutrient levels can be better controlled [46,47]. In an open pond or raceway system, only extreme conditions of pH or salinity have shown the capacity to keep a native mix from out-growing the more desirable cultivated species [48]. While a photo-bioreactor may seem the best method for cultivating algae, it is not without its own unique set of difficulties. The optimum growth conditions (pH, light, temperature) must be carefully controlled [11,16]. Stephenson *et al.* concluded that cultivation in a photo-bioreactor consumed significantly more energy than cultivation in raceways [47]. As an alternative to the photo-bioreactor, a native mix of algae can be grown on either a shallow pond or a raceway (commonly called an algal turf

scrubber, ATS™) [23]. An ATS™ system supplies a solid support for the growth of algae that adhere to a surface. This is a useful property in two respects: (1) there is no need to separate the algal biomass from the pond effluent since the algae remain attached to the raceway, and (2) if the apparatus is built on an incline, the wave surge used to distribute the wastewater down the floway prevents diffusion limited depletion of nutrients [25,27,32]. A shallow pond or raceway also allows the maximum light to reach the cells, is much less expensive to build, and facilitates algae harvesting [25,30,49,50]. While this is a much more economical set up, the slower growing, high lipid producers are likely to be a much smaller percentage of the native algae biomass [11,28]. The open pond system also has issues of predation on the microalgae. Sturm *et al*. minimized predation using a top-down ecological control of algae grazers via the introduction of zooplanktivorous fish [51].

Most algae grow best in a temperate climate which means that production and growth of the algae slows in the winter months [52]. Since algal harvesting will often be seasonally limited by the regional climate, the possibility of a year-round fuel conversion using fermentation of extracted sugars from stored materials to produce biofuels would be desirable. This study examined the possibility of harvesting the algae from an ATS™, using gravity draining and air drying for the algae, and then using the dried stock as a year-round supply for a biofuel reactor. Previous work by Mulbry *et al*. air dried samples using electric fans then ground and stored them for solvent extraction of fatty acids [53]. However no studies were completed on how (or if) the storage affected the use of algae as a feedstock for biofuels. The work presented here studies the storage and processing conditions and how they affect the production of biofuel by ABE fermentation. In addition, the potential for small-scale processing of algae grown on an ATS™

receiving partially treated sewage from Rockaway Wastewater Treatment Plant has been explored.

MATERIALS AND METHODS

Algae Growth, Harvest, and Storage

The algal production system piloted in New York City was located at the Rockaway Water Pollution Control Plant in Rockaway, Queens. The project was funded by the New York City Department of Environmental Protection (NYCDEP) and was part of a larger effort testing a variety of pilot efforts and environmental technologies for the restoration of Jamaica Bay, New York City. The algal production was based on an ATS™ system designed for testing the nutrient removal efficiencies that could be expected from a much larger multiple acre scale-up at the site for wastewater remediation. It consisted of dual elevated floways, each 107 m by 0.3 m in size set at a slope of 0.5%. The floways were operated with wastewater surged at an influent box pumped from secondary treatment at the Rockaway Water Pollution Control Plant with loading rates initially set at approximately 75 L/min and 151 L/min. The project began in September 2010 and the research continued through April of 2013. Algae for this study were harvested by mechanical scraping from upstream to downstream on a one to two week schedule with weekly harvests during the highest growth period. Upon harvesting, algae were gravity drained in a nylon mesh bag for at least 10 min then stored in an onsite drying shed under fans circulating air for a period of at least 72 h before it was considered "air dried." It was then packed and sent to the University of Arkansas for fuel production.

Algae Pretreatment

Upon receipt of algae samples at the University of Arkansas, all the samples were placed in an autoclave at 121°C and 20 psig steam pressure for 1 h. The dried sterilized algae lots were

then ground and stored in air tight plastic bags, and samples were taken for moisture analysis and reducing sugar analysis. The moisture analysis procedure consisted of weighing a small aluminum pan and adding 5 g of the ground algae sample [54]. The weight was recorded and the sample heated overnight in an oven at 125°C. The sample and pan were then removed from the oven and allowed to cool, then weighed again. The percent moisture was calculated from the weight lost.

Reducing Sugar Analysis

Before reducing sugar analysis, acid hydrolysis was performed using 5 g of the ground sample and 50 mL of either 2% or 4% (w/v) sulfuric acid (H_2SO_4) to extract sugar from the algae. This gave a 10% (w/v) ratio of ground algae to sulfuric acid. The mixture was put into 100 mL sealed serum bottles (Wheaton, Millville, NJ) and placed into a pressurized steam vessel at 121°C and 15 psig steam pressure for 1 h. The bottles were allowed to cool for several hours before the contents were placed into centrifuge tubes. The samples were centrifuged at 7000g for 15 min and the supernatant poured into fresh tubes for reducing sugar analysis.

To test for the algal sugars present in a sample the 3,5-dinitrosalicylic acid (DNS) test was performed [12,55,56]. The DNS solution was made by creating two solutions. The first solution was made by adding 1 g of DNS to 100 mL of 2N sodium hydroxide. This solution was heated and stirred until dissolved. The second solution was then made by solvating 100 g of sodium potassium tartrate with 250 mL of boiling water. The two solutions were combined while still hot to form the DNS solution. This solution was kept in a dark bottle at 4°C. The DNS sugar standards for the calibration curve consisted of 0.5, 1.0, 1.5, 2.0, and 3.0 g/L of glucose. Then 1 mL of each of the standard solutions (or sample solutions) was combined with 1 mL of the DNS solution. The calibration curve standards and the samples were placed in boiling water for

exactly 6 min, then removed from the boiling water and placed in ice water until cool enough to handle. Water (8 mL) was added to the 2 mL DNS/sugar solution to bring it to a total of 10 mL. The absorbance of each of the solutions was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Each solution was pipetted onto the NanoDrop pedestal and the absorbance at 470 nm was recorded. The concentration was then calculated from the standard calibration curve.

Algae Concentration Study

A study was done to determine the optimum amount of dried algae to sulfuric acid ratio. A series of algae samples were set up in 2 - 4% (w/v) sulfuric acid. The series was composed of 2%, 5%, 10%, 15%, and 20% (w/v) of dried ground algae to sulfuric acid. Then the sample mixtures were placed into sealed 100 mL glass serum bottles (Wheaton, Millville, NJ) and treated as described above in "Reducing Sugar Analysis." After the supernatant was poured off, the remaining wet algal material was weighed so that the water loss could be accounted for in the calculations.

Wet vs. Dry Storage of Algae and the Effect on Sugar for Butanol Production

Samples were divided into wet samples (30%, 50% or 85% moisture) and dry samples (15% moisture). All three sample sets were kept under the same conditions (light or dark) and at the same temperature (room temp, average of 24°C). The 5 g samples were weighed into 50 mL centrifuge tubes and clear creek water was added to bring the moisture content to 30%, 50% or 85% moisture. Since the algae samples had been previously sterilized, water from Scull Creek near the University of Arkansas campus was used to reintroduce microorganisms. Creek water was used due to health concerns in using samples of unsterilized sewage material. The creek is clear and swift flowing with several species of small fish. The water was taken in a clean plastic

jug from a deep, flowing area without silt or visible amounts of algae. Samples were kept in a drawer in the lab (for the dark samples) or in a window ledge (for the light samples). The light sample racks were turned daily so exposure to sunlight was kept the same for all samples. Samples were first taken daily for 12 days, and then once a week for 3 weeks. Sulfuric acid (25 mL of 4% w/v) was added to each sample tube and vortexed for ~30 s to thoroughly mix the sample with the acid solution. The sample was transferred to a 100 mL glass serum bottle. The bottles were capped with a 20 mm septum and crimp top cap. The septum was punctured to allow for pressure relief. The sample bottles were then sterilized at 121°C and 15 psig steam for one hour and allowed to cool at room temperature overnight. They were then centrifuged at 7000g for 15 min and a sample of the supernatant was used for reducing sugar analysis.

Inoculum Preparation for Acetone-Butanol-Ethanol (ABE) Fermentation

A pure culture of *Clostridium saccharoperbutylacetonicum* (ATCC 27021) was used for the ABE fermentation. The ATCC vial was used to inoculate a 20 mL commercial media (Anaerobe Systems, Morgan Hill, CA) filled test tube, and the test tube was incubated at room temperature. The pH was not adjusted allowing the bacteria to control the acidity. It was necessary to vent the headspace of the tube daily to prevent pressure build-up.

Each test tube was used to incubate two 80 mL culture bottles of sterile PYG (peptone at 6.5 g/L, yeast extract at 3.5 g/L, and glucose at 20 g/L), using about 1/3 of the test tube for each culture bottle. A single 100 mL culture bottle was used to inoculate a sterile 4 L bottle of PYG. The 4 L bottle was split weekly using sterile tubing, fittings and techniques as well as filtered nitrogen gas to prevent contamination and/or exposure to oxygen. The splits were grown until enough culture was obtained to charge several 14 L production reactors. All inoculum cultures

were monitored by microscopic examination (for live culture) and gas chromatography (for butanol production).

ABE Fermentation from Algal Media

The algal media was produced by combining 14 kg of ground algae with 14 L of 4% sulfuric acid and autoclaving for 3 hours at 121°C and 15 psig steam. This solution was allowed to cool and then poured through a large colander lined with a fiberglass screen into a large stock pot. This removed most of the large solids which were discarded. The solution was then adjusted to a pH of 6.5 with 6 N NaOH. The resultant solution was centrifuged at 7000g for 10 min to remove the precipitate. The supernatant was poured into a 20 L carboy fitted with a clamped dip tube and a vent tube with a bacteriological filter attached. These became the production reactors. The 11 L of algal solution was then re-autoclaved at 121°C and 15 psig steam for 1 h to sterilize it after the pH adjustment. After removal from the autoclave the solution was allowed to cool to approximately 30°C before inoculation with the culture. After inoculation, the reactors were warmed to approximately 35°C to induce the bacteria to switch to solventogenesis. In 3-5 days, the pH then dropped to 4.5-4.8 due to bacterial growth by-products.

Butanol Analysis by Gas Chromatography

Concentrations of solvents and organic acids were measured with a Shimadzu 2014 gas chromatograph with FID (Shimadzu Scientific Instruments, Houston, TX) fitted with a 30 meter by 0.32 mm by 0.25 µm FFAP column (Phenomenex, Torrance, CA). The injector and detector were held at 250°C and the column temperature was programmed to ramp from 40°C to 60°C at a rate of 6°C/min, then to 160°C at a rate of 20°C/min and had a final hold time of 3 min. With this temperature profile, the solvent peaks were well separated, as were the organic acids. The acetone displayed a retention time of 2.3 minutes, the ethanol at 2.9 min, the butanol at 5.2 min,

the acetic acid at 7.8 min, and the butyric acid was at 9.0 min. Three point calibration standards at 2, 5, and 10 g/L were made from HPLC solvents and ACS grade acids. Multiple injections of the standards gave a calibration curve for each standard with R^2 values greater than 0.98 for each standard.

Lipid Extraction and Fatty Acid Methyl Ester (FAME) Production

The lipid extraction process used approximately 3 g of ground algae mixed with 12 mL of hexanes in 20 mL disposable scintillation vials. The mixture was completely combined by tipping the vial upside down once. The extraction was allowed to permeate for 24 h and tipped once to remix. The sample was then filtered through a disposable funnel using 110 mm, hardened ashless circle filter paper to achieve a cleaner sample. Small aluminum weigh pans (158 mm at 4" W X 5/16" H) were used to catch the filtered solvent. A final solvent weight was recorded immediately following filtration and then again ten minutes after drying.

For evaluation of FAME production, the method of Abubakar *et al*. was used [57]. Briefly, the dried algal samples were ground with a mortar and pestle. Lipids were extracted by mixing the sample with two parts chloroform and one part methanol and allowing them to stand for 1 h. The samples were then incubated at 55°C for 1 h to allow the solvents to evaporate. The solid sample (20 mg) was then mixed with 2 mL of toluene and 2 mL sulfuric acid in dry methanol and then incubated overnight at 55°C. At this point the sample was added to 4 mL of saturated NaCl, vortexed with 2 mL of hexane, which was followed by the addition of 3 mL sodium hydrogen carbonate at 2% concentration. After mixing, the top layer was removed for analysis by gas chromatography (GC). Samples were analyzed using a Shimadzu 2014 gas chromatograph with FID (Shimadzu Scientific Instruments, Houston, TX), and fitted with a 30 meter by 0.32 mm by 0.25 µm FFAP column (Phenomenex, Torrance, CA).

RESULTS

Drying of Algae for Extraction of Carbohydrates and Lipids

In order for algae to be usable as a feedstock long term, several challenges remain. One of the first of these is harvesting and drying, which has been thought to require the equivalent of >40% of the energy contained within the algae. Algae were harvested from an ATS™ scrubber by mechanical means and "field dried" similarly to corn stover and other biomass feedstocks.

A previous study by Potts *et al*. has shown that when algae have a moisture content of ≤15%, it can be easily mechanically disrupted for removal of biofuel precursors [21]. While this moisture content can be primarily achieved through gravity draining of moisture, we assumed that an addition 5% would need to be removed through an air drying process. Using the latent heat of vaporization for water (2,260 kJ/kg), this leads to an energy consumption of 0.15 kJ/g for gravity drying followed by air drying in a greenhouse. Alternatively, for the case of forced drying, we assume that approximately 50% of the moisture would need to be removed, leading to an energy consumption of 3.2 kJ/g.

The results of the carbohydrate and lipid extractions are in **Figure 1** and **Figure 2**, respectively (88 samples total for Figure 1 and 33 for Figure 2). Over the 6 month harvest period, the average carbohydrate concentration was 10.9% (g sugar/g algae), ranging from a high of 24.9% to a low of 1.8% with a standard deviation of 5.7%. The samples consisted of a mix of diatoms with some species of green algae that favor brackish water. This is consistent with the reported range of 10% to 25% carbohydrates that was found in a mix of diatoms and green algae on an ATS™ system by Adey *et al.* over a 22 month period [50]. Other sources in the literature show an average range of 3% to 47% of total carbohydrates from pure cultures of diatoms grown

in bio-reactors [58-60]. As the amount of time the samples were stored increased beyond 200 days, the average amount of carbohydrates measured dropped by approximately 42%.

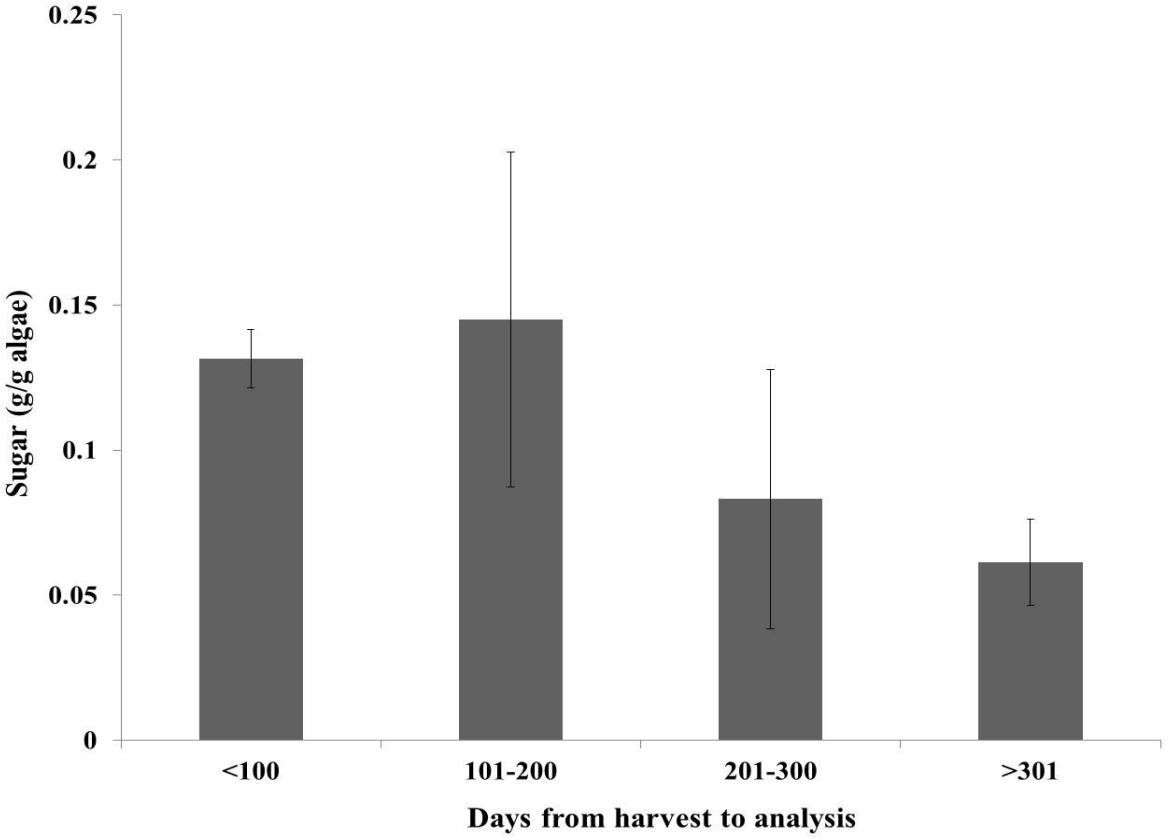

Figure 1. Sugar produced from acid hydrolysis (2% sulfuric acid) of dried algae. All samples were statistically different ($p<0.05$) except for <100 to 101-200 and 201-300 to 301.

The algal lipids had an average of 2.09% lipid (g lipid/ g algae), with a range between 0.62% and 4.39%, and while these percentages were highly variable (Figure 2), they did not show a significant difference up to 500 days (> 1 year) after harvest. This also was typical of the wide range of lipid amounts (0.5% to 9%) seen by Adey *et al*. [50]. Even though the data is more scattered, we see no significant difference after harvest. This suggests that as long as the algae samples are dried, they can be processed for a period of at least 6 months. To the best of our knowledge this is the first time long term storage of dry algae for biofuel production has been reported.

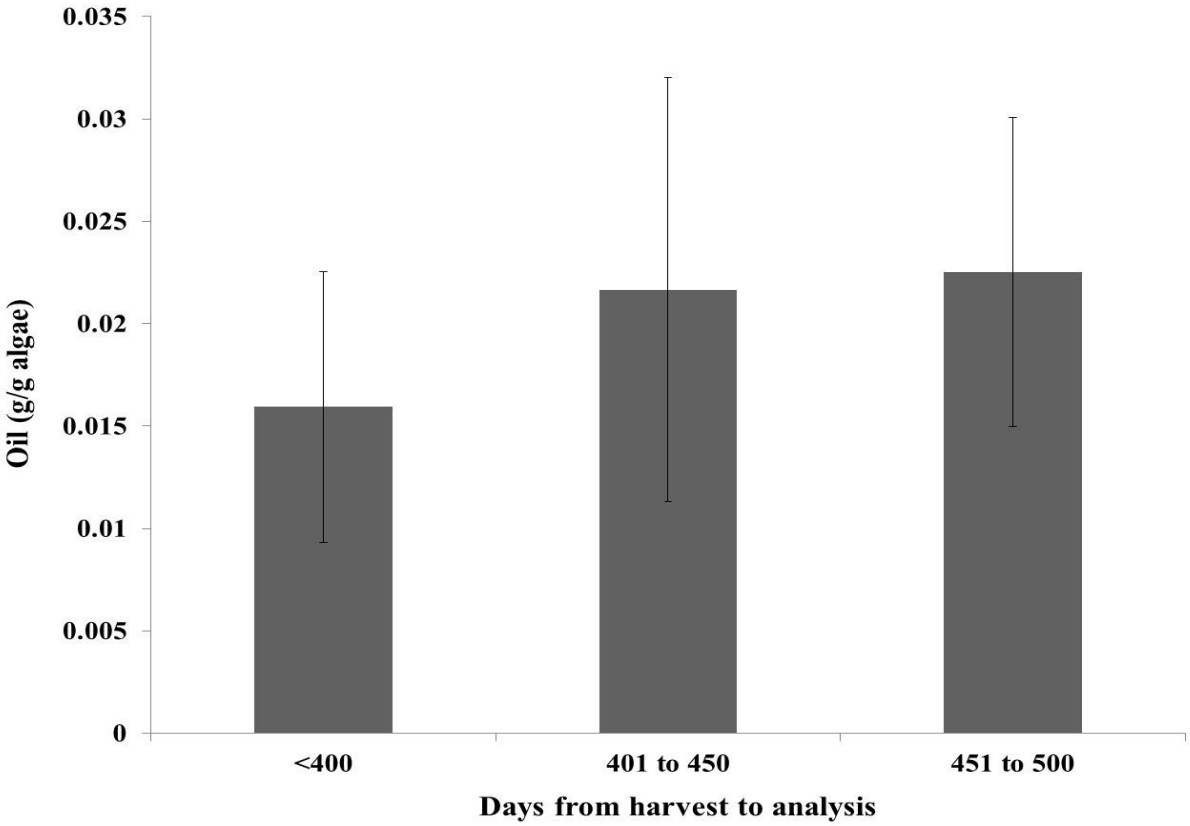

Figure 2. Oil produced from stored, dried algae over time. Samples remained statistically the same ($p<0.05$) over time.

It is important to note that the results presented above were for the typical dried sample which contained <25% moisture. Wet samples (35 - 85% moisture) received by the University of Arkansas were considered unusable for fuel production due to the presence of visible mold or, in some notable cases, methane production indicating significant decay. In either of these conditions, sugar recovery was extremely low or non-existent. Therefore, the effect of high moisture levels on sugar content by re-wetting the dried, stored samples was examined. **Figure 3** shows the effects on sugar recovery after dried samples were re-wetted. The mold associated with the fresh samples straight from the ATS™ was not observed in these samples, which suggested that algae samples getting wet (rain, water run-off) after being dried would not be an

issue even at levels up to 85% water. Interestingly, after a month under conditions of 85% moisture, the carbohydrate level only dropped by about 10-30%.

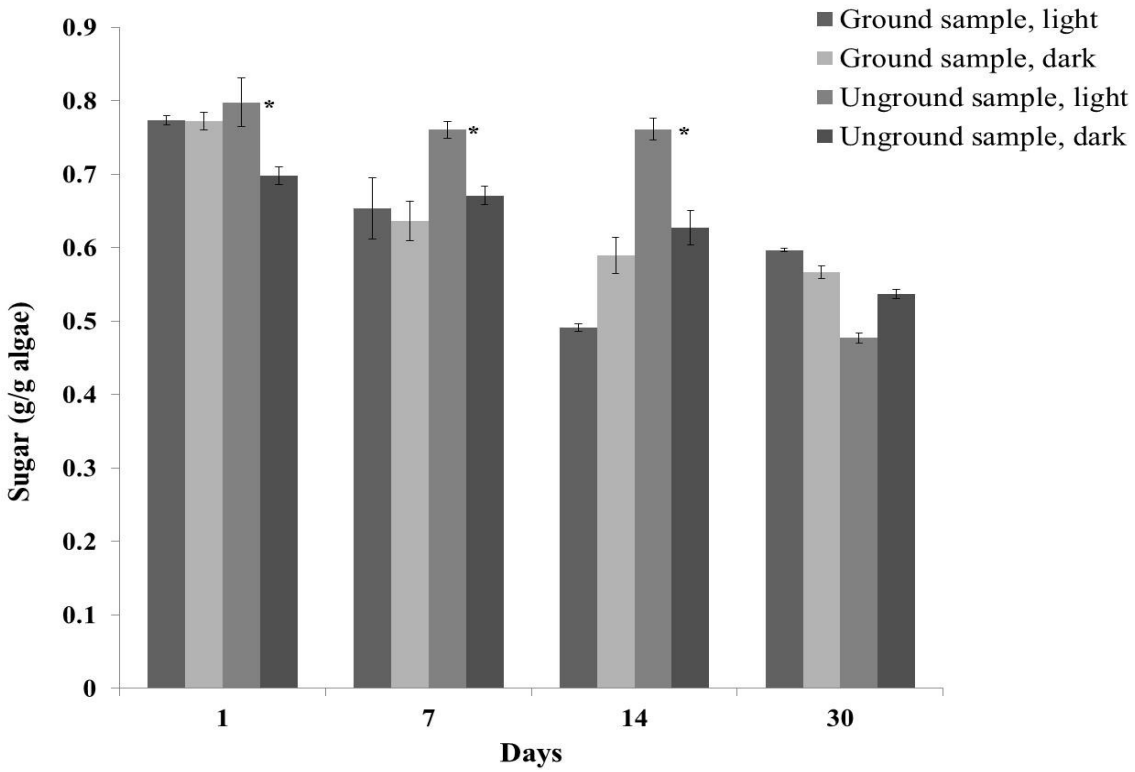

Figure 3. Storage of high moisture algae samples rewetted with creek water. All samples were of statistical significance ($p<0.01$) except for unground light samples day 1-14 (marked with *).

Since many of the same samples were analyzed for both carbohydrate and lipid content, we compared the carbohydrate and lipid values. As shown in **Figure 4,** samples that were higher in carbohydrates also tended to be higher in lipids (until about 0.20 g/g where lipid content dropped back down). While the lipid contents are low (compared to some single species high lipid producers), the data suggested that it would be possible to devise an ATS^{TM} system which: 1) treated wastewater, 2) produced usable carbohydrates for alcohol production, and 3) produced usable lipids for biodiesel production.

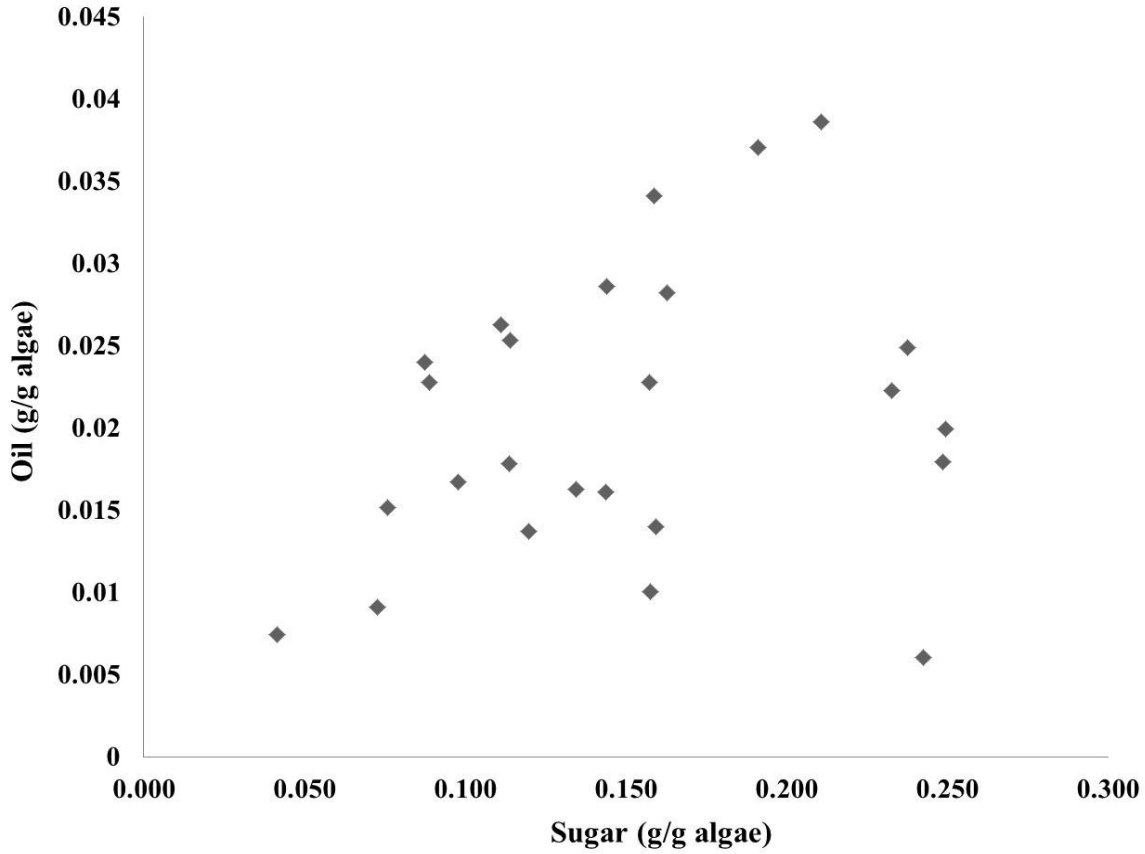

Figure 4. Comparison of oil production to sugar production from algae samples.

Effect of Processing Conditions

The conditions required to make sugars from carbohydrates are important to the production process. In an ideal case, one would like to use the least amount of sulfuric acid at the highest algae concentration possible in order to obtain the highest concentration of sugars in solution at the lowest cost. A previous study by Potts *et al*. on macroalgae found that hydrolysis time was not as important as the concentration of acid used for hydrolysis [21]. Since our study contained primarily microalgae, we investigated two sulfuric acid concentrations to determine if a similar effect would be seen. **Figure 5** shows a comparison of 2% and 4% sulfuric acid hydrolysis treatment. We found that 4% sulfuric acid doubled (or more) the amount of sugar (g/g

algae) than samples hydrolyzed with 2% sulfuric acid, even when samples were stored for more than 200 days.

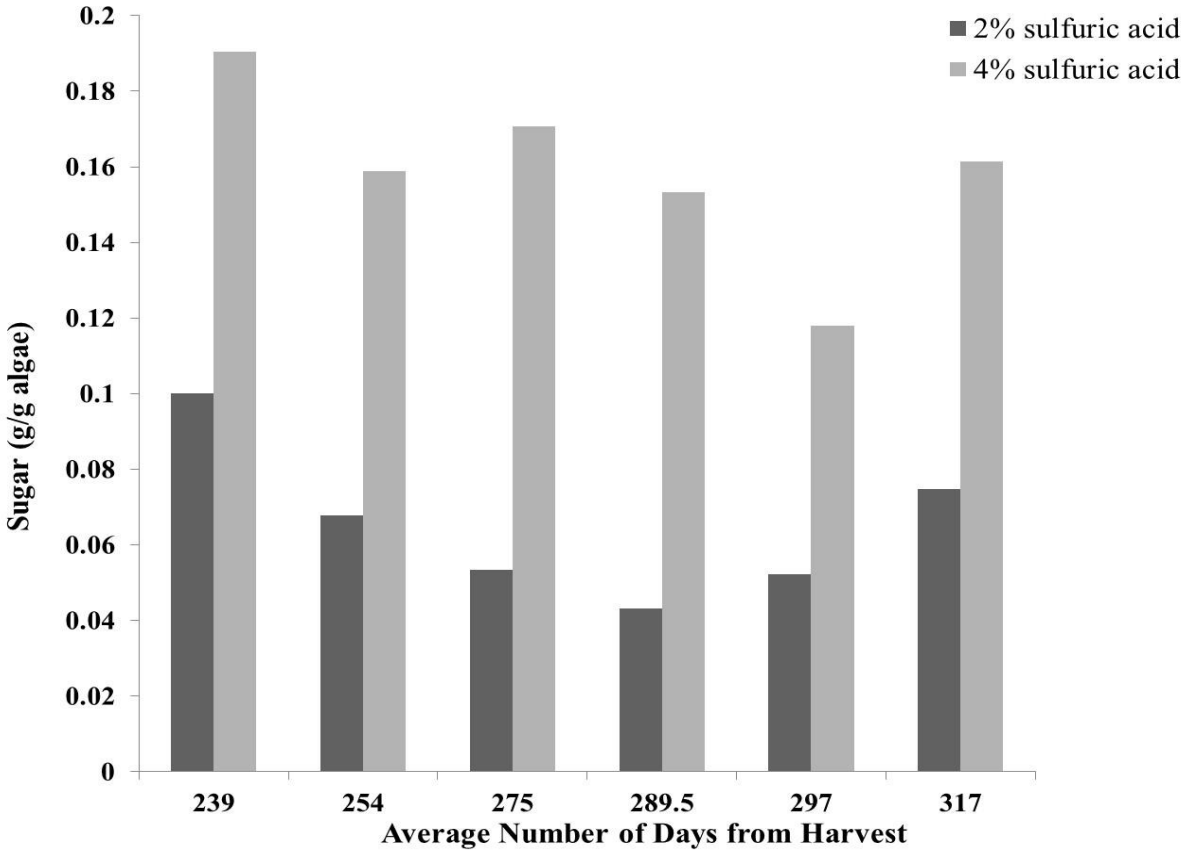

Figure 5. Effect of acid concentration on extracted sugar. For samples analyzed within 30 days of each other. Average number of days from harvest obtained as an average of days from harvest for each analysis.

Another important processing condition is the concentration of algae in the sulfuric acid. **Figure 6** shows the amount of carbohydrates that were produced from different algae to sulfuric acid ratios. Interestingly, at the lowest ratios of algae to sulfuric acid the most carbohydrates were recovered. Although the highest concentration of sugar was obtained for the highest algae concentration, the sample contained the least liquid and therefore significant amounts of sugar may have been retained in the wet algal material rather than being available in the supernatant.

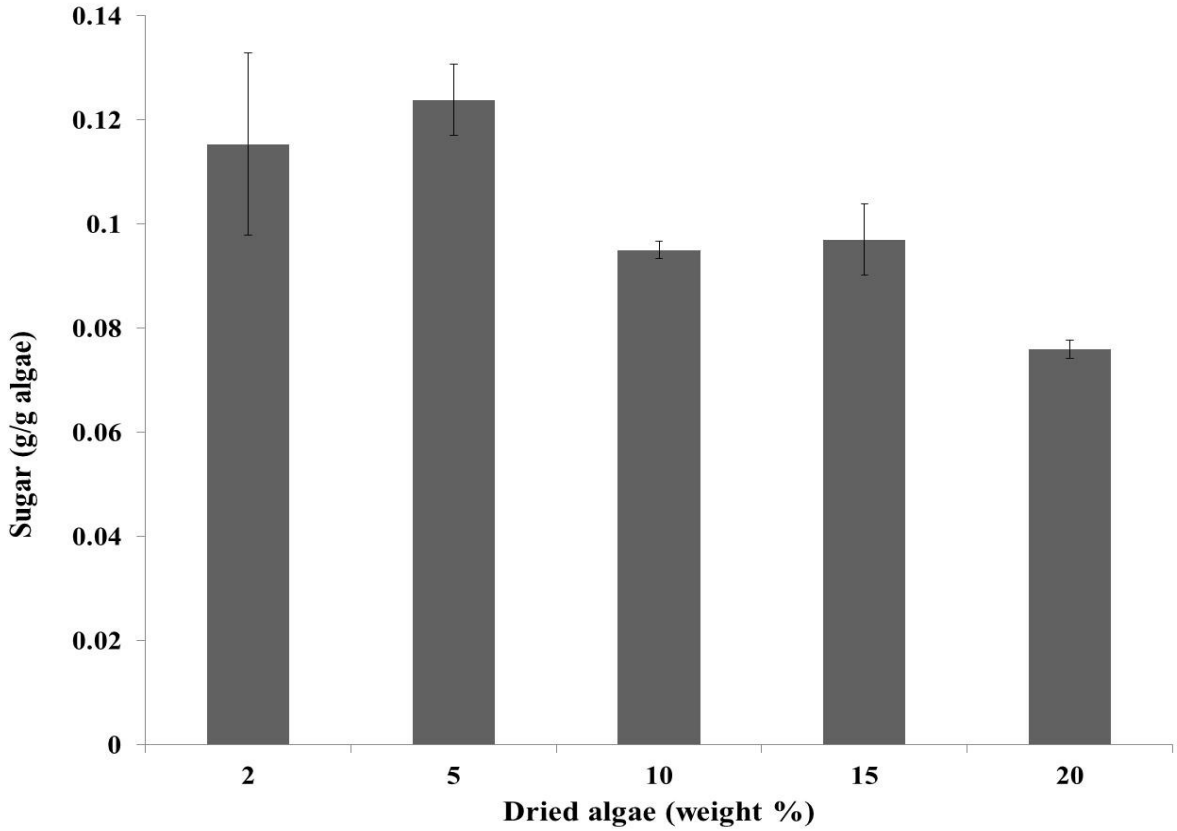

Figure 6. Effect of algae concentration in sulfuric acid on sugar recovery. The 2% algae concentration samples were quite variable and therefore were not statistically different from the other values. All remaining samples were statistically different ($p<0.05$) except for 10 to 15%.

In addition, at extremely high algae concentration (15 and 20%) a mechanical squeezing was necessary to extract the liquid which would increase the energy requirements of the process. However, while the total recovery was highest with the lowest algae concentration, it also produced the lowest concentration and therefore might require removal of liquid in order to increase the concentration to a desirable fermentation level. Based on these trade-offs, the 10% algae to liquid was considered the optimal method for the remainder of the experiments.

Biofuel Production

After optimizing the processing conditions, carbohydrates and lipids from the ATS™ algae were converted to biofuel. **Figure 7** shows the butanol production from 5 different lots

(>15 L) of algae produced with an ABE fermentation. The butanol yield was consistently between 0.1 to 0.13 g butanol /g sugar. In a traditional ABE process with glucose, it has been shown that approximately 0.30 g/g can be expected [20,21]. Based on these values, approximately 50% of the sugars from the DNS assay were usable for production of butanol. A DNS assay performed post-ABE fermentation indicated that the average carbohydrate concentration was 2.7% (g sugar/g algae), which was a reduction of the sugars by 75%. This indicated that a smaller amount of the sugars present in the algal sample were used to produce butanol than in a glucose-based system.

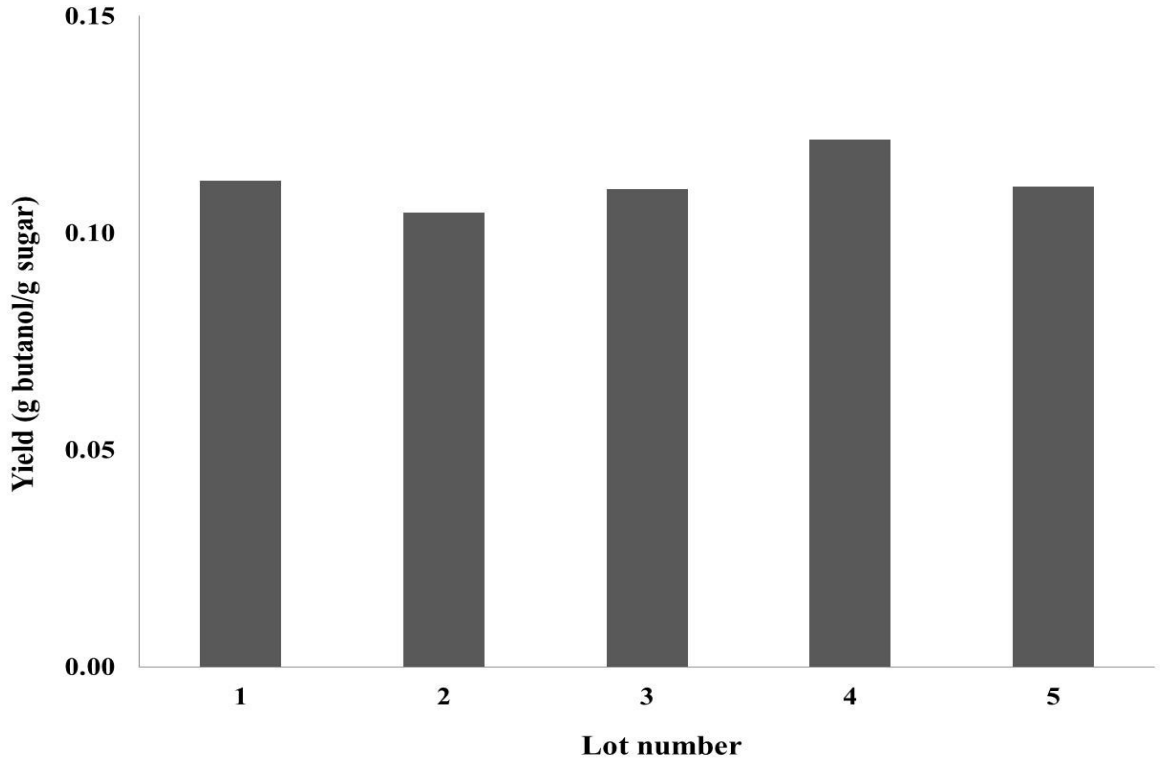

Figure 7. Yield of butanol for scaled-up production lots.

The lipid samples obtained from the algae were converted to biodiesel. The ratio of palmitic acid (C16:0) to steric acid (C18:0) to oleic acid (C18:1) over several samples was 4.7:0.8:1.0, respectively. However, only 8% of the oils were convertible to biodiesel and the rest

of the components were most likely heavy oils that would require further breakdown. This biodiesel was in the range that Wahlen *et al.* suggested as a good biodiesel blend [61]. Thus we have shown that properly utilized ATS™ algae can make carbohydrates for alcohol production and lipids for biodiesel production.

Case Study

A case study was performed for a 4 acre system located next to the Rockaway Wastewater Treatment plant as part of a larger effort testing pilot efforts and environmental technologies for the restoration of Jamaica Bay, New York City. The algal production was based on an ATS™ system designed for testing the nutrient removal efficiencies that could be expected from a much larger multiple acre scale-up at the site for wastewater remediation. This system would be built to help clean nitrogen and phosphorous from the wastewater as well as making biofuels. In a cost estimation given by Pizarro *et al*. for an ATS™ system treating dairy effluent, they found that nitrogen nutrient credits, from cleanup alone, were enough to pay for the ATS™ treatment system [62]. Thus, the incremental costs for clean-up of fuel would only involve drying and upgrading the sugars and lipids.

Based on algae growth and biofuel production data the following conditions were used: high growth of 30 g/m^2day, low growth of 15 g/m^2day; high sugar content of 20% of algae as usable sugars, low sugar content of 10% of algae as usable sugars; high lipid content of 4% of algae as usable lipids, low lipid content of 2% of algae as usable lipids; high butanol yield of 0.25 g/g, low butanol yield of 0.1 g/g; high biodiesel yield of 1 g biodiesel/ g lipid, low biodiesel yield of 0.2 g biodiesel/g lipid. In this case study, the low values represent consistent data and the high values represent the best data. Therefore it was estimated that the low would be obtainable without further optimization and the high would be possible with further research. Using these

assumptions, a 4 acre facility could remove between 9,700 to 18,000 pounds of nitrogen per year. As to biofuel production, it could make between 290 to 2900 gallons/year of butanol and 42 to 530 gallons per year of biodiesel. In terms of total biofuel yield, this gives a range of 83 to 850 gallons per acre year. This range gives a favorable comparison to soy (54 gallons per acre year), rapeseed oil (150 gallons per acre year), jatropha (270 gallons per acre year) and sugar beets (400-500 gallons per acre year) [43,44,63]. Although less than the extremely high growth potential of single species cultured algae, this process does not require sterile algal growth conditions, nutrient and carbon inputs, algae filtration from the growing media, or heated drying.

While the ATS™ algal production method was initially developed to improve water quality from impaired sources with the algal biomass as a natural byproduct, based on our studies it has additional potential to provide a feedstock for year-round biofuel production from stored algal material.

CONCLUSIONS

In this study, algae from an ATS™ system in New York City was harvested, dried, ground, hydrolyzed, and converted to butanol through ABE fermentation. Microalgae harvested from the ATS™ system gave an average sugar concentration of 10.9% by dry weight. As long as the microalgae from the ATS™ were dried to a value of less than 25% moisture, material could be stored for up to 200 days before a significant drop in sugar concentration was observed. This suggested that it would be possible to store algae as a more uniform feed for a fuel production facility. From these algae, a ratio of 0.13 g butanol /g algae sugar was obtained with ABE fermentation. Since this was about half the butanol that has been reported to be produced from ABE fermentation with glucose (0.25 g/g), it indicated that many of the sugars extracted from the algae were not fermentable to butanol. A case study suggested that an ATS™

system could produce values ranging from 83 to 850 gallons of fuel per acre, depending on assumptions. This estimate compares favorably to the many crop based approaches but is lower than monoculture algae. Since the primary purpose for this algal growth was to remove nitrogen and phosphorous from wastewater, the potential to also produce a significant amount of biofuel from material that can be stored over time represents a great potential side benefit.

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I (Christa Hestekin) certify that Alice Jernigan completed at least 51% of the work on "Effects of Drying and Storage on Year-Round Production of Butanol and Biodiesel from Algal Carbohydrates and Lipids using Algae from Water Remediation," a published article in the journal *Environmental Progress & Sustainable Energy*.

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CHAPTER 2: EVALUATION OF THE POTENTIAL FOR CAPILLARY ELECTROPHORESIS-SINGLE STRAND CONFORMATIONAL POLYMORPHISMS (CE-SSCP) AS A METHOD TO IDENTIFY AND MONITOR ALGAL SPECIES FROM PURE CULTURES AND ENVIRONMENTAL SAMPLES

INTRODUCTION

The two most frequently used traditional methods for taxonomic identification of algae beyond the genus level are by microscopic examination and genotyping [1-5]. Microscopic examination methods require skill and experience as they involve a thorough knowledge of the thousands of possible variations in the shape and morphology of the algae as well as familiarity with the algae ecology and reproduction [6]. To go beyond the genus level to species or sub strains of a species can require a scanning electron microscope (SEM), or other more complicated tests and techniques such as in the case of diatoms [7]. Even at this level, there may still be some ambiguity and discussion among the experts in the field of phycology.

Genotyping, in which the DNA is extracted and sequenced, is another method. While giving excellent results, it requires skill, takes time and if hired out to a laboratory that specializes in sequencing, can be costly. Genotyping requires primers that can isolate and amplify a portion of the genome of the species of interest. These amplified products and their primers are often sequenced by capillary electrophoresis. This sequence can then be compared to known sequences on file with the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST), and the most likely species matching the sequence can be determined.

Algae Speciation

Both genotyping and taxonomy are very useful tools to identify algae growing in a system. If the system is a pure culture that will be used for food or pharmaceutical purposes it is

critical to know if or when it becomes contaminated or mutates to a less valuable species. If the pure culture is a high lipid producing, but slower growing algae used to produce bio-fuels, it can be overgrown by unwanted species that will use up valuable resources. In both of these scenarios, it would be beneficial to know about the change in the system before too much time and resources are wasted. In some cases if caught quickly, it may be possible to reverse or stop the contamination of unwanted algal species. In a small lab-scale bio-reactor, this may not be an issue, but certainly, when dealing with large commercial reactors, it becomes a costly and time-consuming effort. So a method is needed that will allow a relatively fast and simple technique for identifying and monitoring a bioreactor's algal culture. If this method does not involve specialized personnel or shipping for lab analysis, both the cost and the time could be kept to a minimum.

In this research capillary electrophoresis single-strand conformational polymorphism (CE-SSCP) was explored to identify different algal species. This method has been previously used with success to identify and differentiate between bacterial species [8-15]. It is a relatively simple method that requires the amplification of a gene sequence that is common in all algae with a fluorescently labeled primer, followed by a method to distinguish the nucleotide differences in that gene. CE-SSCP uses heat denaturing to first separate the double stranded DNA into single strands, which is then snap cooled to cause those single strands to fold in upon themselves in a conformation unique to the nucleotide sequence. This results in a variation of the electrophoretic mobility of the DNA that has similar sizes but different sequences, allowing them to be differentiated by electrophoresis.

16S gene vs. 18S gene

Cyanobacteria, commonly known as blue–green algae are prokaryotes; therefore they have no higher cell structure such as cell organelles and a separate nucleus [6]. They can be identified, as can many bacteria, using the variable regions of the 16S rDNA gene which codes for the small subunit of the ribosome [8,14,16]. This small subunit of the ribosome is extremely important to the cell's ability to produce proteins and has critical structural constraints; therefore, portions of this DNA sequence are conserved and provide ideal regions for primer targeting. However, there are several highly variable regions that differ uniquely between species and have been used to differentiate, and determine species of bacteria and algae [17].

Most common algae, such as green algae are eukaryotes; therefore they do have higher cell structures such as chloroplasts, and a separate nucleus [6]. It is considered proven that millions of years ago, a eukaryotic algal ancestor was formed by a larger cell that incorporated a cyanobacterium into itself in a symbiotic relationship [18-21]. Some of the DNA from this cyanobacterium (which is now a chloroplast of the larger cell) may become incorporated into the nucleus of the larger eukaryotic cell. However, most of the DNA in the nucleus is different from the original cyanobacterium. Therefore, in order to distinguish different species it becomes necessary to examine the portion of the DNA that is in the nucleus of the eukaryotic cell. This is the 18S gene which codes for the small subunit of the ribosome in eukaryotes, and which may have some sequences in common with the 16S gene of the cyanobacteria (prokaryotic) algae [21]. However, due to the differences in the origin of the relative algal species and their difference in complexity, their variable regions are not exactly the same in number or sequence. The combination of a highly conserved area with a species dependent variable area is excellent for the purpose, as universal primers can be designed for the conserved areas and can be used to

amplify the intervening variable regions. The variable regions in these genes represent a potential method for identifying different species of algae.

Amplification by Polymerase Chain Reaction

Polymerase chain reaction is a technique that amplifies *in vitro* specific DNA sequences with a thermostable DNA polymerase enzyme isolated from *T. aquaticus* (commonly called Taq), and specific primers. Primers are oligonucleotides with 15-25 bases and their sequence is complimentary to the DNA sequence targeted for amplification. Template DNA, containing the sequence of interest, is placed in a tube with the oligonucleotide primers, each of the deoxyribonucleotide triphosphates (dNTPs), buffer, magnesium, and the thermostable DNA polymerase. The first step is to heat denature the double stranded DNA into single strands. The primers will then attach to the complimentary single strand DNA (annealing step) and the polymerase enzyme will begin to synthesize the complimentary nucleotide chain, beginning at the primer sequence (extension step). The thermal stability of the polymerase preserves the enzyme activity during the repetitive sequences of denaturing of the DNA. A cycle of denature-anneal-extension is repeated 25-35 times, with the newly synthesized molecules now acting as the templates in the next round. This process is automated in a thermocycler where products are amplified exponentially.

CE-SSCP

Electrophoresis is the use of an electric field to separate charged molecules based on a difference in electrophoretic mobility, which can result from differences in charge and/or shape or size. Capillary electrophoresis is advantageous in that it is electrophoresis performed in a 50 μm internal diameter silica tube, which allows the electrophoresis to be run at a much higher voltage while avoiding the adverse effects of Joule heating [22]. The increased voltage means a

faster run time, and the automation and multiple capillaries allows for a higher throughput of samples [22].

Single-stranded conformational polymorphism (SSCP) entails electrophoresis of single-stranded DNA fragments of suitable size through an entangled polymer. The double stranded DNA is heat denatured it until the hydrogen bonds holding the double helix backbone together disassociate and the DNA becomes two single strands in a dilute solution. These single strands of DNA are snap-cooled on ice causing the single strand to fold back on itself in ways that are dictated by its unique nucleotide sequence. This is called a conformer. Under conditions of low temperature and non-denaturing polymer the conformers will migrate according to their shape and will have a distinct mobility. SSCP performed on a capillary electrophoresis instrument gives the advantages of automated loading of samples and the use of laser-induced fluorescence detection, which allows for sensitive detection at low concentrations and repeatable results.

CE-SSCP has been used to successfully differentiate between mutated and non-mutated genes [14,23-25], and also to identify and differentiate bacterial species from such diverse samples such as blood, cheese, and soil [12,13,15,26], so it was considered possible to use this technique to determine the different species of algae growing in an environmental system. In 2012, Herrera-Sepúlveda used CE-SSCP to monitor activities of harmful algal blooms off the Baja California Sur Costal waters [27]. They successfully identified ten dinoflagellate species of different genera and have plans to implement this method for multiple samples as an early warning of harmful algal blooms without having the "pressure of counting with a taxonomic expert in phytoplankton taxonomy" [27].

The purpose of this chapter was to evaluate a method to identify and monitor the different species found in an ATS™ system. In Chapter 1 of this dissertation, we found that there was a

wide range of carbohydrates and lipids that were dependent on the dominate species of algae in the bio-mass that was the feedstock for a bio-fuel reactor. In order to maximize the production of bio-fuel, it might be necessary to change production or growing conditions, and therefore a method of evaluating the algal types in the feedstock was required. An examination of the potential for CE-SSCP as the method of choice was proposed. We had previously used CE-SSCP to monitor the bacteria in the bio-reactor for contamination, and thought this method would work equally well on algae. The goal was to be able to quickly identify multiple species from an environmental sample. In order to reach that goal, primers that would identify or differentiate between most species of algae were required. As stated previously, there is a body of research conducted on bacteria that has used the 16S gene with its seven different variable regions as that identifying gene. However, while this 16S gene is common to most prokaryotic species such as bacteria and cyanobacteria, only partial sequences are found in eukaryotic algae [19]. Therefore the 18S gene found in higher organized cells was also examined. The 18S gene has nine variable regions, so the CE-SSCP method was evaluated using two different variable regions of either the 16S gene of cyanobacterial algae or the 18S gene of eukaryotic algae. The method was first evaluated using "pure" culture samples to give us a "fingerprint" of the DNA that will allow us to identify the particular species of algae. Then the environmental samples were analyzed using the same primers and methods to determine if the algal species could be identified based on "fingerprints" from the pure cultures in the environmental samples. The environmental samples were also analyzed by taxonomic and genetic sequencing for confirmation.

MATERIALS AND METHODS

Pure Cultures

Pure cultures were obtained from Carolina Biological Supply Company (Burlington, NC, USA). The fifteen different pure cultures represented seven different types of algae including diatoms, brown algae, desmid, euglenoid, green algae and cyanobacteria. **Table 1** gives a list of the various types of algal species used for the fingerprint database.

Table 1: Pure Cultures for Fingerprint Database

Genus and Species	Class – Type	Source/catalog number
Chlorella vulgaris	Chlorophyceae - green algae	UTEX 395
Ulothrix fimbriata	Chlorophyceae - green algae	UTEX LB 638
Scenedesmus quadricauda	Chlorophyceae - green algae	UTEX B 614
Oedogonium foveolatum	Chlorophyceae - green algae	UTEX LB 933
Mougeotia sp.	Chlorophyceae - green algae	UTEX 758
Ulva fasciata	Chlorophyceae - green algae	UTEX LB 1422
Spirogyra communis	Chlorophyceae - green algae	UTEX LB 2462
Pandorina sp.	Chlorophyceae - green algae	UTEX B 2141
Micrasterias thomasiana	Chlorophyceae - green algae	UTEX LB 544
Peridinium sociale	Dinophyceae – dinoflagellate	UTEX LB1948
Ectocarpus sp.	Phaeophyceae - brown algae	UTEX LB 1433
Synedra radians	Bacillariophyceae – diatom	UTEX LB FD155
Anabaena inaequalis	Cyanophyceae - cyanobacterium	UTEX B 381
Nostoc muscorum	Cyanophyceae - cyanobacterium	UTEX B 486

The live pure cultures were used for DNA extraction within a week of arrival. DNA was extracted using either the PowerPlant® Pro DNA isolation kit from Mo Bio Laboratories (Carlsbad, CA. USA), or the DNeasy Plant Mini Kit from Qiagen (Germantown, MD, USA). The PowerPlant™ and the DNeasy Plant Mini Kit protocols were followed to obtain a clean DNA samples for PCR.

Environmental Samples

Environmental samples were taken from ATS systems: an experimental one in a laboratory at the University of Arkansas (Fayetteville, AR, USA), a pilot scale ATS system on a

local wastewater treatment plant (Springdale, AR, USA), and chilled samples were sent from a large scale ATS™ system (Rockaway, NY, USA). The environmental samples were used for DNA extraction within a week of sampling.

DNA Extraction

Two different methods of DNA extraction were used. The first method was the PowerPlant® Pro DNA isolation kit (Mo-Bio Laboratories, Carlsbad, CA, USA) that used bead beating in combination with chemical extraction for cell lysis and DNA release. Released genomic DNA was first precipitated with isopropanol and then captured on a silica membrane in a spin column format. DNA was washed and eluted from the membrane and ready for PCR and other downstream applications. This worked well on most normal algae samples, but samples that contained large amounts of diatoms which have silica cell walls were more problematic and required a different method.

For these more difficult samples (diatoms) the DNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA) utilized a combination of freezing and mechanical method for cell lysis and DNA extraction. After lysis, proteins and polysaccharides were salt precipitated. These contaminates were removed by a filtration and homogenization column. The cleared lysate was applied to a silica membrane spin column and pure DNA was washed and eluted from the membrane.

Reagents and Primers for PCR Amplifications

The PCR reagents 5x Go Taq Flexi Buffer, 25mM $MgCl_2$, 10 mM dNTP and Taq Polymerase were obtained from Promega (Madison, WI, USA) and all of the PCR primers used were obtained from Invitrogen (Carlsbad, CA, USA). Forward (sense) primers were fluorescently labeled with FAM and reverse (antisense) primers were fluorescently labeled with

HEX to enhance the ease of identification between the forward and reverse single strand peaks in the electropherograms. Once the primers were obtained, they were diluted to a stock concentration of 100 μM and this solution was used to make a working solution of 20 μM for the PCR reactions. After preparing the PCR mixture, it was placed in an Apollo ATC401 thermocycler (Ramsey, MN, USA). A fragment of either the 16S or 18S gene variable regions was amplified using the primer pairs shown in **Table 2.**

Table 2: Primer Information

Primer Name	Gene Amplified	Variable Region	Primer Sequence	Fragment Size*
V2F	16S	V2	GGCGAACGGGTGAGTAA	239 bp
V2R	16S	V2	ACTGCTGCCTCCCGTAG	239 bp
V3F	16S	V3	CCAGACTCCTACGGGAGGCAG	184 bp
V3R	16S	V3	CGTATTACCGCGGCTGCTG	184 bp
V7F	18S	V7	AACTTAAAGGAATTGACGGAA	156 bp
V7R	18S	V7	GCATCACAGACCTGTTATTGCCCC	156 bp
V9F	18S	V9	GTACACACCGCCCGTCGCACC	310 bp
V9R	18S	V9	TTCCGCAGGTTCACCTACGGA	310 bp

*fragment size estimated from NCBI (www.ncbi.nlm.nih.gov)

All the PCR reagents were mixed according to the standard PCR protocol shown in **Table 3.** After gently pipetting to mix and spinning the mixture for ~15 seconds to remove bubbles, the PCR mixture was subjected to the following amplification cycles on an Apollo ATC401 thermocycler, with temperature, time and cycle ranges based on an optimization of the program and the melting temperatures (T_m) of the different primer pairs (see **Table 4)**.

Table 3: Standard PCR Protocol

Reagent Name	Initial concentration of reagent	Required (or) Final Concentration of reagent	Amount in the PCR mixture
Go Taq Flexi Buffer	5 X		1.4 µl
MgCl2	25 mM	2 mM	1.6 µl
dNTP	10 mM	0.2 mM	0.4 µl
Taq Polymerase	5 units/µl	2.5 U	0.5 µl
Forward Primer	20 µM	0.5 µM	0.5 µl
Reverse Primer	20 µM	0.5 µM	0.5 µl
DNA Template	7 ng/µl		2 µl
Water			11.1 µl
Total			20 µl

Table 4: Amplification Protocol Ranges

Cycle (ranges)	**Step**	**Temperature (ranges in °C))**	**Time (ranges)**
1	1	95.0	2-5 minutes
30-35	1	95.0	30-45 seconds
30-35	2	$(T_m - 5)$	30-45 seconds
30-35	3	72.0	45-60 seconds
1	3	72.0	5-20 minutes

PCR Amplified DNA Samples Purification, Quantification and Storage

Amplified DNA fragments were purified using the QIAquick-spin PCR purification kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's instructions. Quantification of purified DNA was determined with a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) based on the absorbance of DNA at 260 nm. The purified samples were used as a stock solution for preparing SSCP samples and were stored at -20°C.

CE-SSCP Sample Preparation

The fluorescently labeled purified DNA stock solutions were diluted to 1 ng/µl with 10 mM Tris-HCL (pH 8.0) to obtain a 15 nM solution for SSCP. Ten µl of the diluted, purified

DNA was loaded per well into 4 wells of a 96-well plate followed by a denaturing step at 95°C for 3 minutes on the ATC 401 thermocycler. The samples were immediately snap-cooled on ice for 3 minutes before loading onto the ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Preparation of Polymer

For some capillary electrophoresis methods a separation matrix is required to provide resolution between the different peaks in the electropherogram. For CE-SSCP this matrix must be non-denaturing to avoid changing the conformers. The polymer used during capillary electrophoresis was polydimethylacrylamide (PDMA). The PDMA polymer was synthesized using the method of Albarghouthi *et al.* except with a decrease in the nitrogen bubbling time (from 16 hours to 4 hours) [28]. After adding the polymerization initialization compound V-50 (2,2-Azobis (2-amidino-propane) dihydrochloride; Wako Chemical, Richmond, CA, USA). Ultra high purity (>99.5%) monomer N,N-dimethyl acrylamide (DMA) (Monomer-Polymer and Dajac Labs, Feasterville, PA, USA) was diluted in deionized water to obtain a 4% (v/v) N,N-dimethyl acrylamide solution. The 4% (v/v) DMA solution was kept at 47°C in a water bath for 3 hours with ultra-pure nitrogen gas bubbling through the mixture. To initialize polymerization, 0.5 ml (per every 100 ml) of V-50 (60 mg/ml) solution was added to the solution, followed by 4 hours of polymerization under nitrogen gas in the 47°C water bath. After completion of the reaction, the polymer was purified by dialysis in de-ionized water using Spectra-Por cellulose ester dialysis membranes (Spectrum, Gardena, CA, USA), having a molecular mass cutoff of 1000 Da. The de-ionized water was changed 10 times in 5 to 10 days. After dialysis was complete, the polymer was frozen at -80°C for 48 hours and then recovered by lyophilization (Labconco, Kansas City, KS, USA). Dry polymers were dissolved in 1x TBE buffer + 10% glycerol (89 mM

Tris-base, 89 mM boric acid, 2mM EDTA and 10% glycerol) to obtain polymer concentrations of 3.5% (w/v). The diluted polymer was stored at room temperature.

Preparation of Samples for Sequencing

Samples were sent to the University of Arkansas for Medical Sciences (UAMS) Sequencing Core Facility for sequencing of the PCR product. The genomic DNA was amplified with the same primers as used in the amplification process for CE-SSCP, as shown in Table 2. However, primers used for sequencing were not fluorescently labeled. The amplified DNA was purified using a Qiaquick PCR purification kit (Qiagen, Germantown, MD, USA). The concentration of the amplified products was measured using a Nanodrop 1000 spectrometer (ThermoScientific, Wilmington, DE, USA), and were diluted to a final concentration of 10 ng/µl. The non-fluorescent primers and diluted purified PCR products were sent to University of Arkansas for Medical Sciences (UAMS) DNA Sequencing core Facility and sequencing results were returned.

RESULTS

Pure Culture "Fingerprints"

The results of CE-SSCP are called electropherograms, which are a graphical representation of the DNA sample in the form of a plot of the relative fluorescence of the sample versus the elution (or migration) time. The elution time is affected by the electrophoretic mobility of the sample, which is the effect of the shape, size and/or charge. To examine differences between species the electropherograms were examined for changes in the elution time and the shape and number of peaks for both forward and reverse DNA.

Figures 1a and **1b** show the electropherograms of the SSCP products of variable region 2 of the 16S gene of two prokaryotic algae species *Anabaena* and *Nostoc.* Because these samples

can produce multiple peaks, the graph was split into two sections and zoomed in on the peak areas to provide for easier viewing.

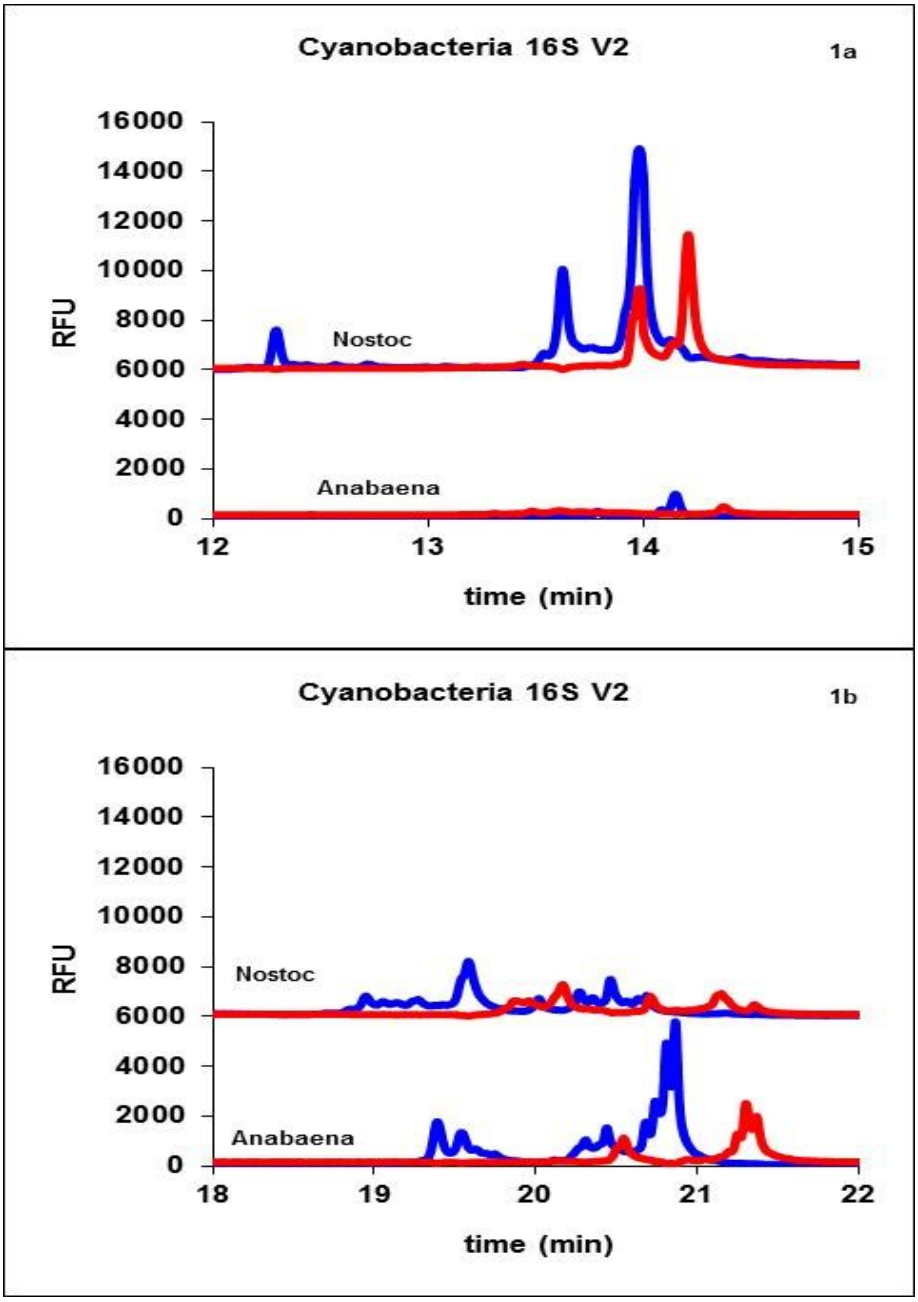

Figure 1: 1a and 1b is a split view of the electropherogram of 2 species of cyanobacteria (prokaryotes), showing the 16S gene variable region 2

The peak patterns in **Figure 1a** show a marked difference between the two species. There are three very strong forward (blue) and two reverse (red) peaks between the 12 to 15 minute

elution time for *Nostoc,* while *Anabaena* has only one very small forward and reverse peak during this same elution time. **Figure 1b** is a continuation of this graph and shows a different area between the 18 to 22 minute elution times. A more distinct difference in the peak patterns can be observed with ten small forward peaks and five small reverse peaks for *Nostoc*, while *Anabaena* has eight larger forward peaks and three reverse peaks. Many of these peaks such as the forward peaks for both *Nostoc* and *Anabaena* are in the form of multiple peaks that are clumped together. In an ideal electropherogram, there would be a single peak for each species. Multiple peaks with little to no resolution between them as is seen in Figure 1b indicate that there may be multiple sub-species with slight variations in the sequence, or there are multiple conformers of the same sequence. However, these are very distinct differences in the peak patterns between the two species even though both are cyanobacteria. Another variable region was investigated (shown in **Figure 2a** and **2b**) to compare these two species to determine if there was a more distinct and clear pattern.

Figure 2a and **2b** show the electropherogram for the variable region 3 of the 16S gene for the same two species of cyanobacteria. For early elution times (between 10 to 20 minutes) there are two very small forward and one reverse peaks for *Nostoc* while *Anabaena* has no discernable peaks. **Figure 2b** shows the 20 to 24 minute elution time period in which *Nostoc* has four larger forward peaks and five reverse peaks. The reverse strands of both *Nostoc* and *Anabaena* are at approximately the same elution time, but *Nostoc* has an extra peak of small height at the 22 minute mark. There are also differences in the height and width of the peak patterns. *Anabaena* has four forward peaks in the 20 to 24 minute elution time period.

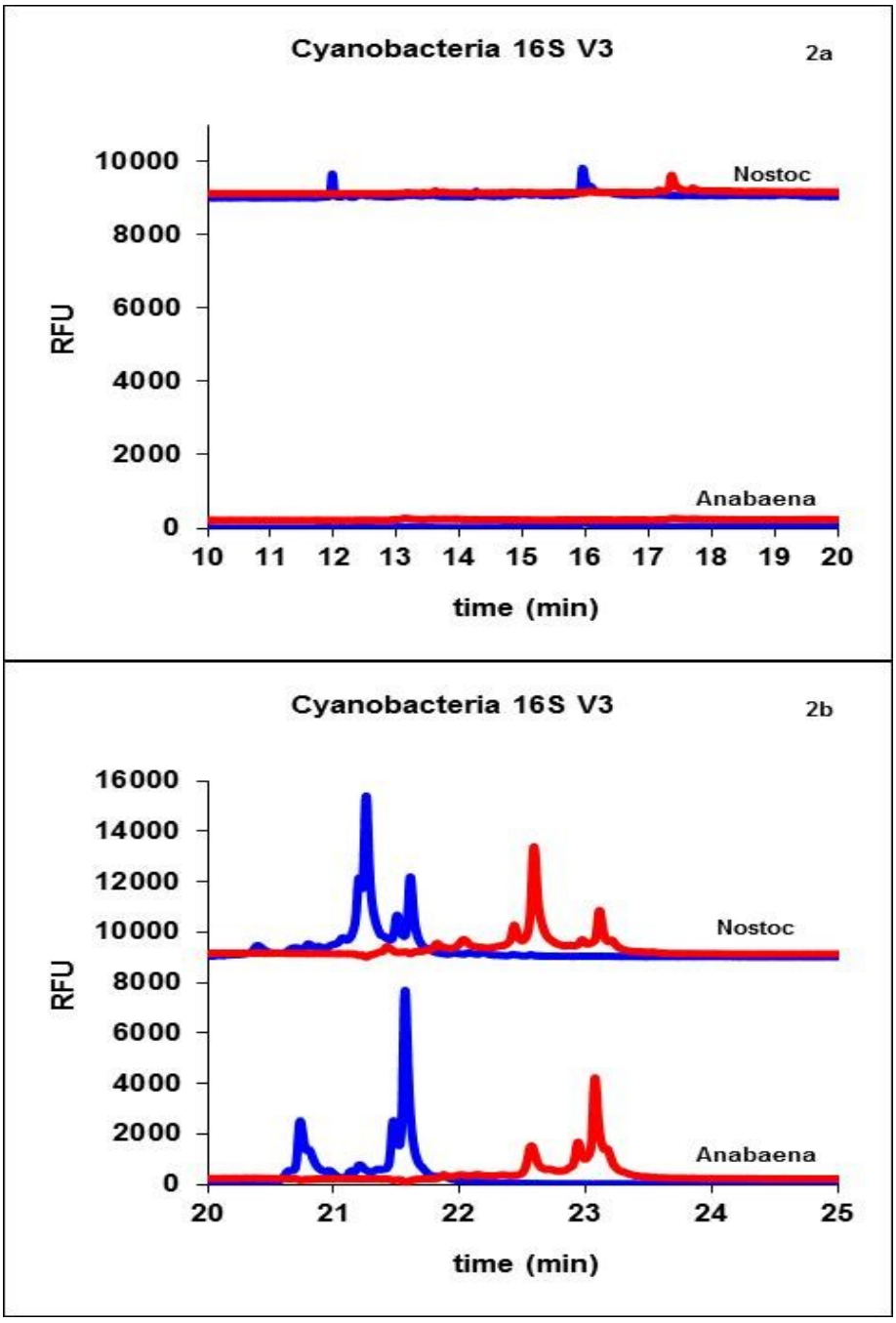

Figure 2: 2a and 2b is a split view of the electropherogram of 2 species of cyanobacteria (prokaryotes), showing the 16S gene variable region 3

These two peaks at 21.5 minutes look very similar to the same peaks for *Nostoc.* However, the first set of peaks for *Anabaena* at the 20.8 minute time has a difference in the elution time giving a slightly different pattern between the two species. The electropherograms

or “fingerprint” patterns produced by these two cyanobacteria are extremely stable and the differences between them are repeated in all of the duplicated CE-SSCP runs ($n \geq 4$). Therefore, these “fingerprint” differences clearly demonstrate how the electropherogram pattern could be used to differentiate between these two species. However, while the distinct pattern of peaks was very repeatable, the multiple peaks can create difficulties involved in interpreting these patterns as they can mask separate peaks and make the pattern difficult to read. Two different regions of each gene were examined to ensure verification of separate patterns for each species. For these two prokaryotic algae the 16S variable region 2 seems to be the most clearly differential pattern.

After comparing two species of cyanobacteria using two variable regions of the 16S gene, we looked at several species of eukaryotic algae. The eukaryotic algae are of a higher cell complexity and have an 18S gene in their nucleus. Therefore the DNA sample is from the 18S gene variable regions 7 and 9 respectively. Figure 3a and 3b are a comparison of four species shown on the same graph to highlight the differences between similar species of algae and how CE-SSCP can be used to distinguish between them. All four of these eukaryotic species belong to the phylum Chlorophyta and two belong to the class Chlorophyceae which is one of the classes of green algae [6]. Green algae are so called due to the dominance of the pigments chlorophyll *a* and chlorophyll *b* giving them a characteristic green color. Most members of this class have a rigid cell wall with an inner layer of cellulose and an outer layer of pectose, and typically store starch as an energy source in pyrenoids located in the chloroplast [6].

Figure 3a and 3b show the electropherogram patterns of four different species of green algae, *Chlorella, Ulothrix, Scenedesmus* and *Oedogonium.* These were the ‘fingerprint” patterns of variable region 7 of the 18S gene. As with the cyanobacterium, there are distinct differences in the patterns between the algal species. In **Figure 3a**, *Chlorella* has a small forward peak just

before the 14 minute elution time with a single, *Scenedesmus* has a small peak at 13 minutes and *Oedogonium* has multiple very small peaks from 13 to 15 minutes.

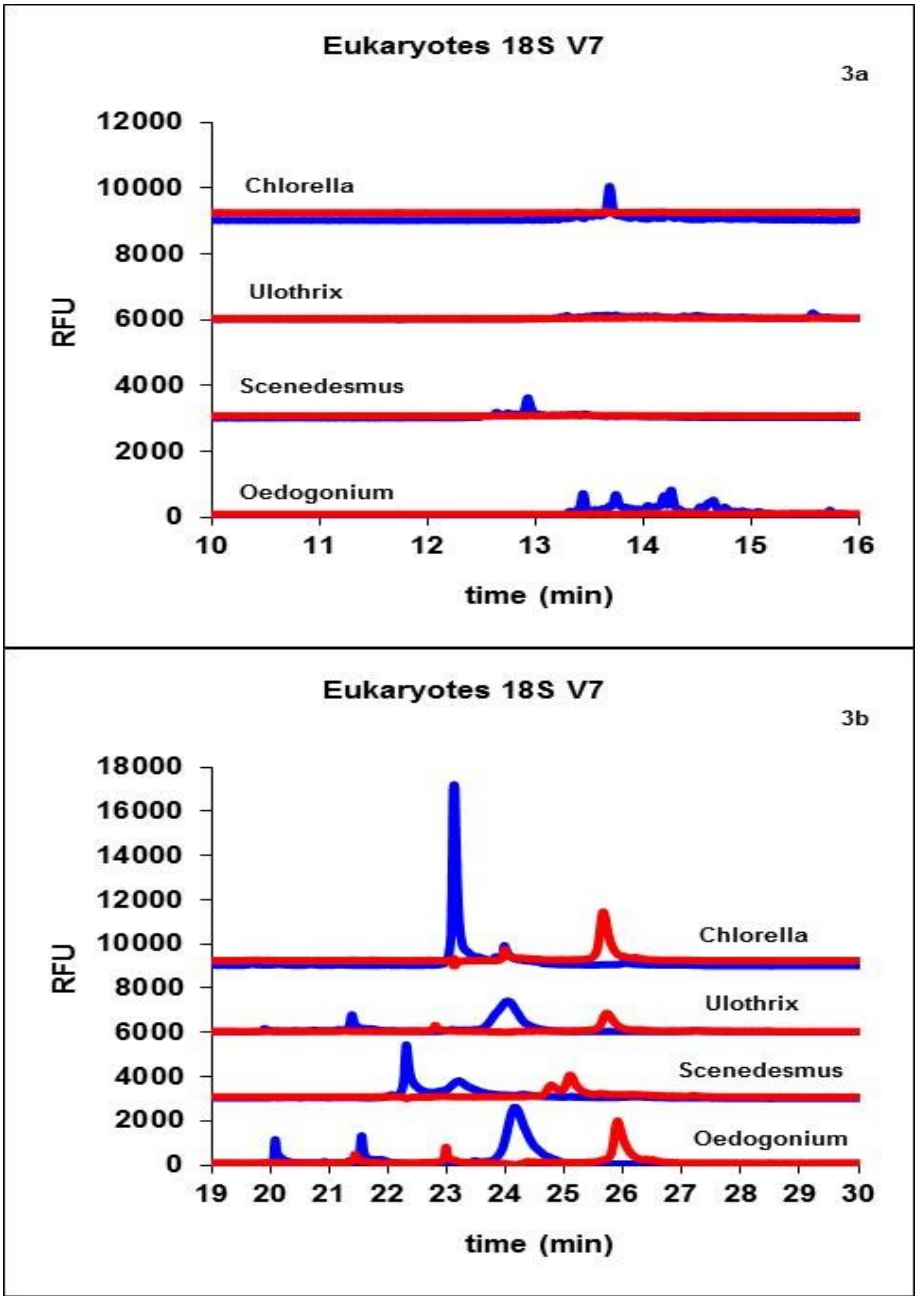

Figure 3: 3a and 3b is a split view of the electropherogram of 4 species of green algae (eukaryotes), showing the 18S gene variable region 7

In **Figure 3b** *Chlorella* has a large forward strand peak at the 23 minute elution point, while *Ulothrix* has two smaller peaks at 21 and 24 minutes, and another two slightly larger

forward peaks at 22 and 23 minutes, with three slightly larger forward peaks at 20, 21, and 24 minutes. The reverse strands of DNA (red) also show a difference between species, however, the different peaks are not as obvious as those of the forward strands of DNA, being close to the same elution time and not as obviously different in size. The forward and reverse strands of the DNA give a pattern that is repeatable but in some cases may not differentiate enough between very similar species. This is the reasoning behind using at least two different variable regions; if one region cannot differentiate decisively between the patterns a second region may be examined. In **Figure 4a** and **4b** we looked at a different variable region for these same four algae.

In **Figures 4a** and **4b**, the variable region 9 electropherogram, a completely different set of peak patterns is seen. *Chlorella* now has a small reverse peak at 12 minutes with a small then large reverse peak at 18.5 minutes, while *Ulothrix* has a single small multiple peaks at just past 19 minutes. *Scenedesmus* has three small reverse peaks just before and after 18 minutes, and *Oedogonium* has a small peak just before 12 minutes and two slightly larger peaks at 19 minutes. The forward strands of DNA also have quite different patterns. *Chlorella* has a small peak just after 13 minutes and a larger peak at 18.5 minutes followed closely by a triple peak pattern (somewhat obscured by the reverse peak overlaid on top of them) at just before the 19 minute elution time. *Ulothrix* has small multiple peaks just before and after the 18 minute mark, *Scenedesmus* has four small forward peaks through 17 to 18, while *Oedogonium* has a larger triple peak encompassing just before to just after the 18 minute elution time.

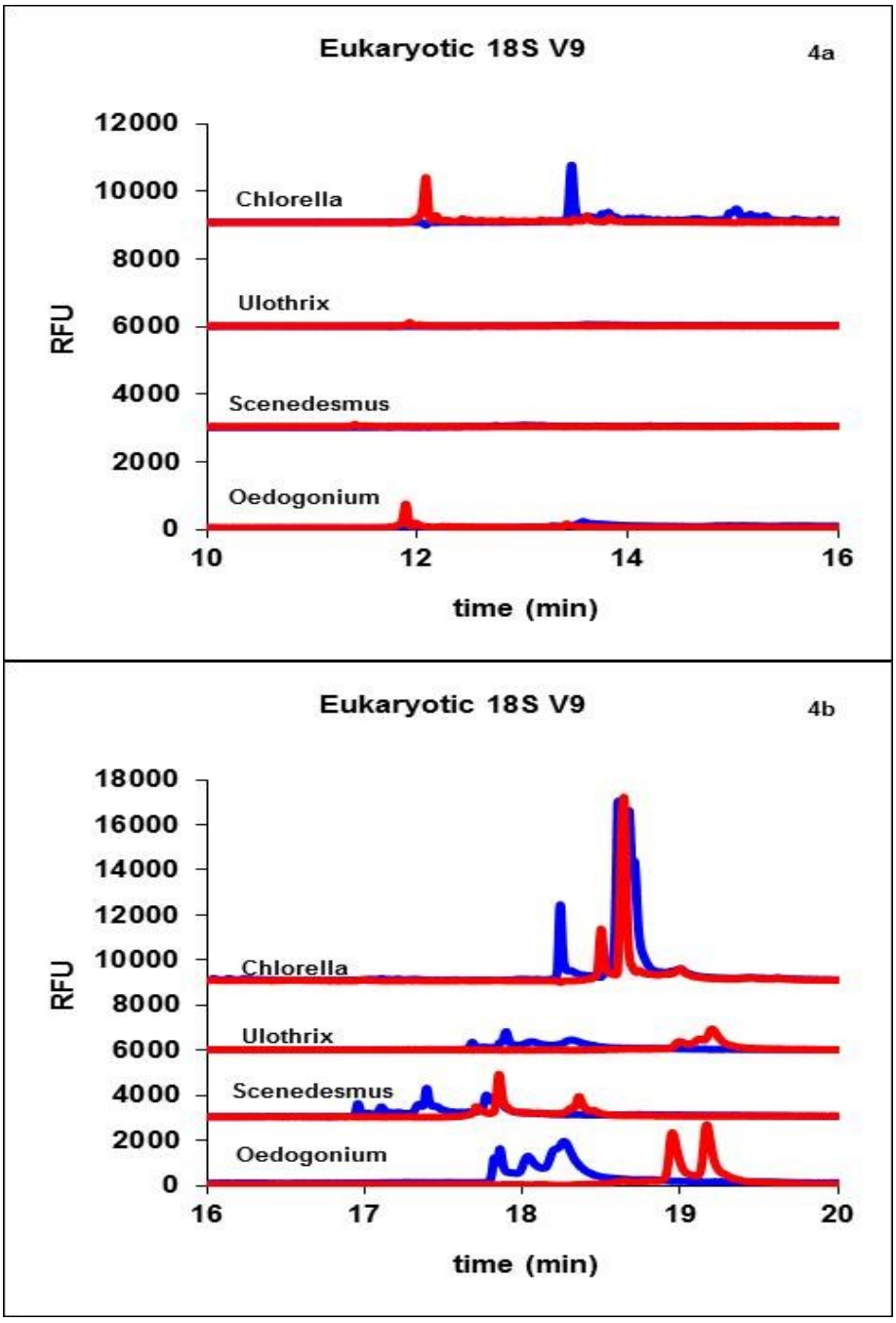

Figure 4: 4a and 4b are a split view of the electropherogram of 4 species of green algae (eukaryotes), showing the 18S variable region 9.

The advantages to this method are that it is fast and it is relatively easy to detect changes in the pattern by direct comparison. These changes in pattern can be used to detect changes in a culture or environment over time, effectively giving a "snapshot" of changes in either a closed or open system. The pattern is highly repeatable, in other words the nucleotide sequence determines

the shape and therefore the mobility of the conformers and this shape does in fact give a specific pattern that can be considered a "fingerprint". Using the two different variable regions we were able to show differences in the peak patterns of both prokaryotes and eukaryotes. Even very similar algal species had distinct, repeatable patterns. While the pattern or fingerprint is unique to the algal DNA sequence, there is not necessarily just one peak for each sequence. There are several sequences that give multiple peaks patterns. These peaks could indicate that the "pure" cultures are not genetically pure or more likely represent different conformers from the same sequence.

The multiple peak issues can be demonstrated using the MFOLD DNA folding algorithm program [29]. This program uses the Gibb's free energy amounts to predict the most likely folding pattern for any given nucleotide sequence. Glavač *et al*. explored the correlation of MFOLD predicted DNA secondary structures with CE-SSCP patterns at different temperatures and different lengths of DNA [30]. They found that shorter strands (52bp and 77bp) gave a positive correlation between the elution time shifts and the difference in free energy values of the secondary structures at all of the temperature ranges (25°C – 40°C). However, for longer sequences (193bp) and higher temperatures the differences were not as distinct and did not necessarily correlate with their secondary structures. Some of the uncertainties and multiple peak patterns of CE-SSCP could be designed out of the method with a more rigorous primer design and optimization of the run temperatures and other conditions.

Comparison of CE-SSCP to Traditional Methods in Environmental Samples

The pure cultures were used to prove the principle of the CE-SSCP method. Once the technique was shown to differentiate between pure cultures, the next step was to evaluate the possible potential of CE-SSCP as a valuable tool for identification of algal species in an

environmental sample including a validation of the identification of actual environmental samples using current methods. Two methods currently used to identify algae are taxonomic identification using a light phase microscope and visual examination, and genotype sequencing some portion of the algal sample genome using the Basic Local Alignment Search Tool (BLAST) function to identify possible algal species from a nucleotide sequence. The BLAST function is an algorithm that compares nucleotides of DNA sequences to a database of sequences on file with the National Center for Biotechnology Information (NCBI) which is part of the United States National Library of Medicine, a branch of the National Institutes of Health (NIH).

Table 5: Taxonomy and Sequencing Results

Sample	**Taxonomy Results**	**Variable Regions**	**Sequencing Results**	**SSCP Results**
LW043010-1	*Mougeotia*	V7 & V9	*Mougeotia sp.* >90% match	Figures 6 & 7
LW043010-2	*Mougeotia*	V7 & V9	*Mougeotia sp.* >90% match	Figures 6 & 7
LW062710	*Mougeotia*	V7 & V9	*Mougeotia sp.* >90% match	Figures 6 & 7

The environmental sample's predominate genus was identified first by taxonomic methods and then the portion of the DNA used for CE-SSCP was sequenced. In **Table 5** the results of these procedures are listed. Some of the results are only to the genus level as according to NCBI, the sequence could belong to multiple species. These samples were identified as *Mougeotia* sp. by taxonomic methods. *Mougeotia* are green algae (Chlorophyceae), found worldwide in freshwater habitats. The filaments are usually found as free-floating masses on sediments. The cells have unbranched thalli forming intertwining filaments. They are cylindrical, approximately 5 to 30 μm in diameter, and much longer than wide [6,31]. The nucleus is found halfway along the length of the cell and there is only one chloroplast per cell.[32]

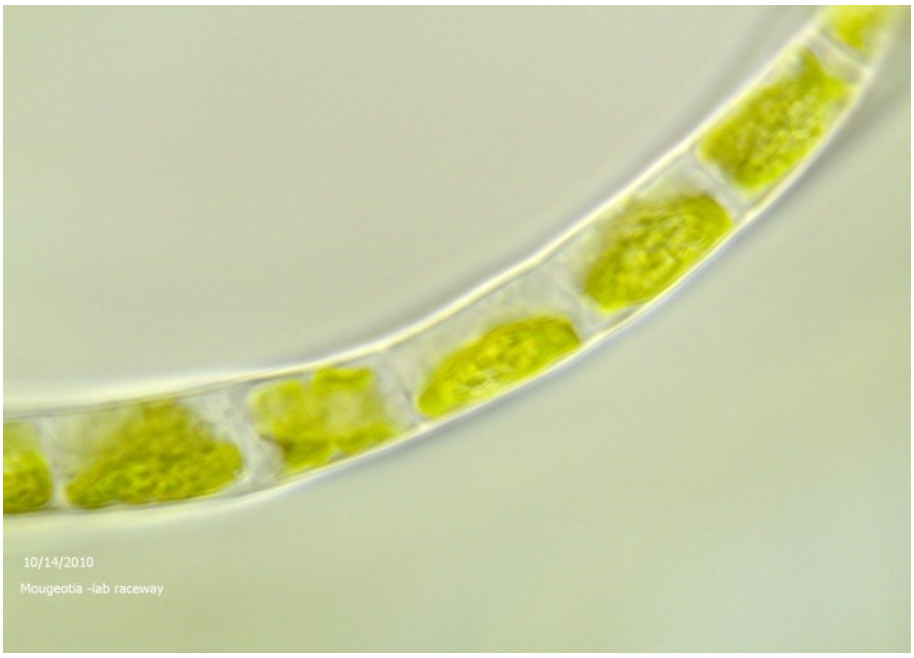

Figure 5: Digital image of sample taken from the Lab raceway (LW062710) used for taxonomy identification. The sample was identified as Mougeotia sp.

After the samples were identified as *Mougeotia* sp. by taxonomy, a sample of the amplified DNA was sent to the UAMS Core Sequencing Facility for sequencing. **Figure 5** shows a photograph of sample LW062710 taken from the Laboratory raceway at the University of Arkansas.

Two samples used for taxonomy and sequencing (LW043010-1 and LW043010-2) were taken at the same time, but treated as different samples for the purpose of demonstrating that the DNA fingerprint patterns were the same. The second sample, LW062710 was taken approximately two months later to look at the pattern to determine whether notable changes in the speciation of the raceway had occurred. The NCBI database was used to confirm the species and identification of the sequence.

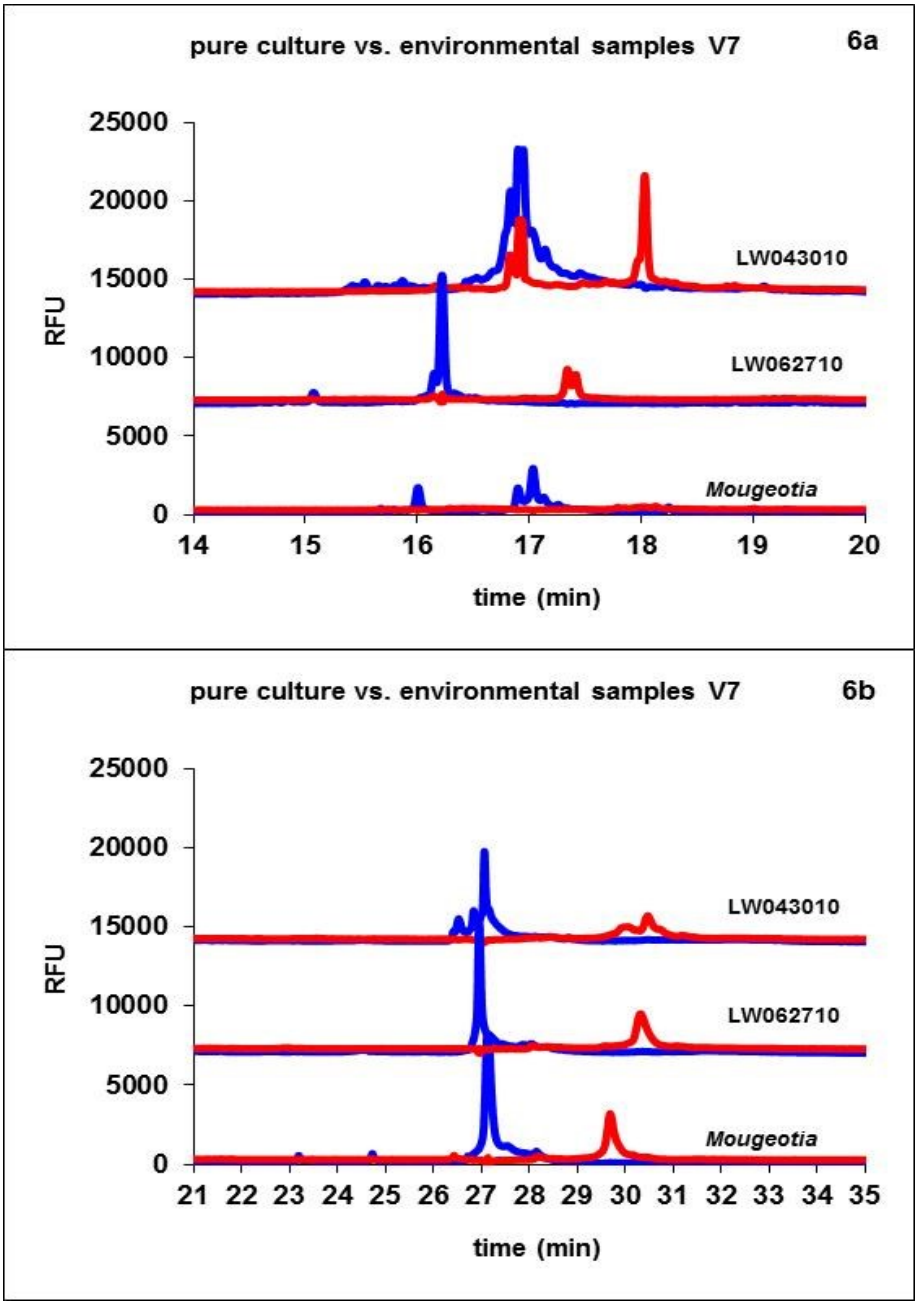

Figure 6: A split view of the variable region 7 comparisons of two environmental samples (identified by taxonomy and sequencing as *Mougeotia* sp.) and a pure culture sample of *Mougeotia* obtained from Carolina Biologicals.

The results of the CE-SSCP comparison are shown in **Figure 6a** and **6b** where the variable region 7 was compared between the two environmental samples and the culture of *Mougeotia* sp. (UTEX 758). The culture of *Mougeotia* sp. was from a culture originating in Bloomington, Indiana, USA and could not be guaranteed to be without sub species.

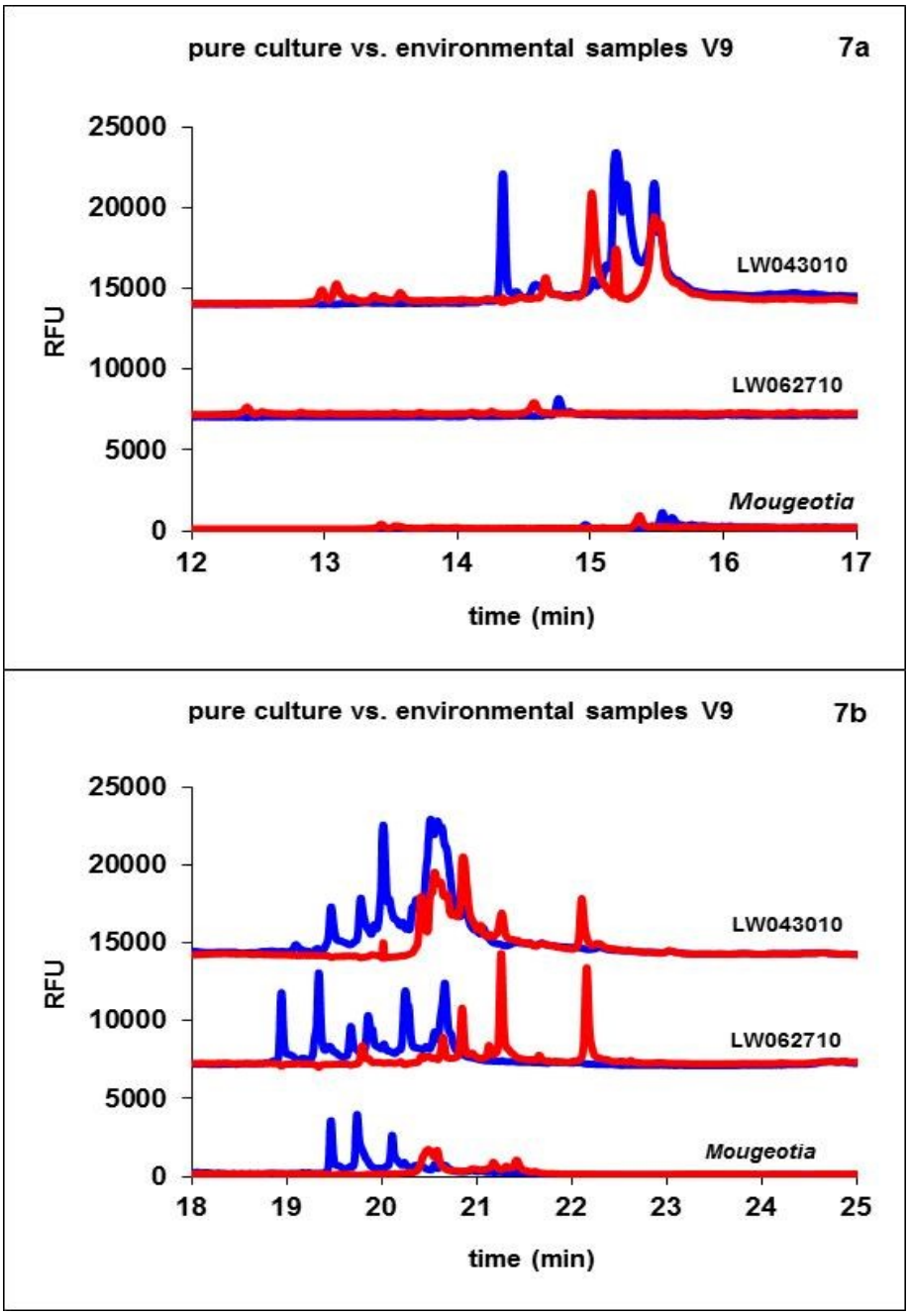

Figure 7: A split view of the variable region 9 comparisons of two environmental samples (identified by taxonomy and sequencing as *Mougeotia* sp.) and a pure culture sample of *Mougeotia* obtained from Carolina Biologicals.

Therefore, none of these three samples was considered to be a pure culture, so the differences that were seen in the electropherogram, such as the very distinct differences in patterns in **Figure 6a** could be explained by a mixture of other species or a sub species of

Mougeotia, while the similarities in the peak patterns seen in **Figure 6b** show where the nucleotide sequences are close with very small subtle differences in the peak shapes and elution times.

In **Figure 7a** and **7b**, where the variable region 9 is shown, there are quite distinct differences between all three samples. LW043010 has a large group of peaks in the 12 to 16 minute elution time (shown in **Figure 7a**) that is not seen in either the LW062710 or the *Mougeotia* samples. There are multiple large forward and reverse peaks in the 18 to 23 minute elution time range shown in **Figure 7b**, that could certainly be characteristic of the *Mougeotia* species, but while not an exact match, since they have differing numbers of peaks, they clearly show three forward peaks at 19 to 20 minutes that could be the same sequence for all three samples with different peak heights.

The graphs clearly demonstrate the possibility of monitoring changes over time. The two environmental samples were taken two months apart and demonstrate a change in the pattern possibly relating to a mutation or change in the species or changes in the lab growth environment. These graphs also show some of the disadvantages of CE-SSCP (*i.e.* the difficulties inherent in identifying unknown samples). Without an incredibly comprehensive library of fingerprint patterns representing all of the possible species that might be in the sample of interest, this method is of limited use as an identification system for unknown species. Multiple peaks were noted even in "pure culture" samples of algae, in the environmental samples, the multiple peaks make it easier for one pattern to mask peaks in another which would greatly limit how many different species can be analyzed simultaneously.

CONCLUSIONS

CE-SSCP is a relatively fast, easy and inexpensive method for determining changes in a small section of DNA such as a variable region of the 16S or 18S gene of the small subunit of the ribosome. CE-SSCP shows highly repeatable, unique fingerprint patterns of different algae through pure cultures. It also has the ability to monitor changes in environmental samples, but there are complications with multiple or not exact peak patterns. The limitations to CE-SSCP are that it cannot analyze large numbers of species at the same time because of these multiple and complex peak patterns. Therefore it is not as useful in identifying unknown species without using genotyping or taxonomy to first winnow the possibilities down to a reasonable number. This would eliminate the speed advantages of CE-SSCP and makes it more suited for a controlled environment than an open system. With the proper primer selection, one that was specific to a particular genus or species, CE-SSCP could be extremely useful for determining differences between particular species or sub types.

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CHAPTER 3: QUANTIFYING TWO SPECIES OF ALGAL DNA USING CE-SBE (CAPILLARY ELECTROPHORESIS – SINGLE BASE EXTENSION) IN A PHOTO-BIOREACTOR SYSTEM

INTRODUCTION

Quantifying Algae in Culture Samples

Most closed bio-reactor systems are used for single cultures of high value algae, such as *Nannochloropsis* which accumulates high levels of polyunsaturated fatty acids [1,2], useful for bio-fuel production or as a food supplement, and *Spirulina* and *Chlorella* which are used as food supplements and pigments [1,3,4]. Quantification of these species can be extremely difficult by traditional microscopy methods due to their very small size (e.g. *Nannochloropsis* 2-3μm)[5] or their complicated morphology (e.g. *Spirulina*, a filamentous cyanobacteria characterized by cylindrical, multicellular trichomes in an open left-hand helix) [5]. In order to quantify or identify different algal strains and species, light or electron microscopy is traditionally used. Identification of algae using traditional light microscopy methods is both time consuming and technically difficult, and requires significant experience and training.

Traditional methods for quantification by microscope have been in use for decades, and while they are time consuming, there has not been a more successful alternative until recent molecular methods were developed in the past twenty years. Microscopic counting methods can be relatively straightforward depending on the morphology and structure of the culture being studied. If it is a large single cell organism, it can be sampled and quantified using a hemocytometer and a light microscope [6]. It requires time, attention to detail and skill to accurately count and quantify the number of cells/mL of fluid. This does not include identification of similar species in this accounting, but only the numbers of cells present in the

sample. If, however, the morphology is very small or convoluted this type of quantification becomes technically very difficult and time consuming to accurately and repeatedly accomplish. Cells which grow in colonies need to be separated without damage and with most filamentous cells this is not possible [7,8]. With the more difficult cell types such as filamentous cyanobacteria, a molecular method solution is advantageous. Within the last fifteen years, molecular tools have proven to be extremely useful for the identification of algal species [9-13].

Polymerase chain reaction (PCR) has been successfully used to detect several toxic dinoflagellates in seawater samples [14,15]. The ribosomal DNA (rDNA) genes have been useful due to the dichotomy of a highly conserved nature for structural integrity of their product, yet they also have variable regions that can differentiate between species [9,16,17]. Different regions of the rDNA gene types, such as the small subunit, the large subunit and two internal transcribed spacers (ITS1 and ITS2) have been selected as targets for identification of algae. Galluzzi *et al*. developed a real-time PCR assay for detection and quantification of a dinoflagellate that causes toxic shell fish poisoning along the Mediterranean coast [14]. Real-time PCR allowed for the quantification of PCR product formation during the exponential phase of the reaction. The detection of PCR products was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded DNA. While this method was successful on the specific algal bloom samples tested, there were difficulties encountered in the purification of the genomic DNA that could lead to inhibition of the PCR and therefore give lower amounts. Godhe *et al*. also used real-time PCR to estimate the biomass of diatoms and dinoflagellates in coastal marine seawater [15]. Their method was different in that they were correlating the copy numbers of the rDNA in the cell with the cell volume. They also had issues with the DNA extraction negatively affecting their outcomes. Their solution was to look at the proportion of the

two types of algae and not the total amount of DNA, then using real-time PCR to quantify the proportion of dinoflagellates versus diatoms in a sample by estimating the cell volume or biomass. Another method that has been used to identify and quantify phytoplankton groups in lakes was a comparison of pigment analysis by high-performance liquid chromatography (HPLC) and microscopy. Schlüter *et al.* used a chemotaxonomic method involving analysis of the pigment content to determine the phytoplankton group composition [18]. This involved using a program called CHEMTAX to provide estimates of biomass based on the amount of chlorophyll *a* ratios for the phytoplankton groups detected pigments. This program however, required that the correct chlorophyll *a* ratios be entered into the program in order to get accurate results [19]. Some of the drawbacks of the traditional methods of microscopy can be addressed using these other methods however, these newer methods also have issues with inexact results, and certainly the HPLC method is also time consuming requiring the algae samples to be grown and tested for chlorophyll *a* content before the samples can be run and analyzed on HPLC.

Single base extension (SBE) is a specific and robust method that is both fast and accurate in determining the identity and quantity of two algal species in a bioreactor. In this method, a specific DNA primer is designed that anneals exactly one base before a locus that will positively identify and differentiate between two particular species of algae. The primer is designed for the two exact species and anneals with great accuracy. Due to its specificity and the fact that only one base pair is added during the reaction, SBE does not have the copying errors that can be found in real-time PCR. The DNA polymerase, in the presence of dideoxyribonucleic acids (ddNTP), which are chain terminating monomers, will attach a single fluorescently labeled nucleotide base to the 3' end of the DNA primer. Each base can be labelled with a different dye that is specific to the algal species and allows for a single reaction to identify all four nucleotide

bases. This provides a fast, easy identification using capillary electrophoresis (CE). The results, in an electropherogram, also provide a direct correlation to the amount of DNA in the sample. The quantity of DNA can be related to an area under the curve of the dye peak on the electropherogram and a calibration curve was developed.

SBE was developed to identify single nucleotide polymorphisms (SNPs) associated with mutations causing phenotypic traits and sometimes associated with certain diseases such as cancer [20-23]. One of the advantages to this method is the ability to identify all four possible nucleotide bases with a single reaction [20,24]. Batley and Hayes used single nucleotide primer extension to genotype three different loci of the cyanobacterium *Nodularia spumigena* [24]. This ability to identify the different nucleotide bases could allow for the identification of specific species or subspecies of algae with a specific primer. The quantification can be developed due to the primer only adding one nucleotide base for each copy of the DNA. We chose the small subunit of the ribosome as our template of choice due to its previous uses as an identifier in both microbial and algal systems [15,25-29].

In 1982, Richmond *et al.* detailed the effects of bicarbonate and carbon dioxide in a co-culture of *Chlorella vulgaris* and *Spirulina platensis* during a study on the interactions between the two species in eutrophic waters contaminated with phosphorous [30]. Currently, there is interest in these two species of algae as a source of bio-fuels as well as other high value products. Therefore, an interesting proposal was made to co-culture these two edible algae in the same photo-bioreactor. These two algae both will grow in an inexpensive media that does not contain any sugars (Zarrouk media), using bicarbonate as a carbon source, and under autotrophic (photosynthetic) conditions, with only light stirring to distribute light through the culture evenly and to remove dissolved oxygen. The high pH of the media helps prevent bacterial

contamination. This co-culturing of two pure species in a photo-bioreactor is considered unique, and the current methods of light microscopy used for quantifying the amounts of the two species in this culture were based on estimates due to their morphologies. This co-culture of *Chlorella vulgaris* and *Spirulina platensis* was considered an excellent proving ground for single base extension as a method of quantification of algae in a culture.

The first issue that needed to be addressed was the development of a specific primer that would allow for the distinction between *Chlorella vulgaris* and *Spirulina platensis*. The small subunit of the ribosome is coded for by the 16S gene in prokaryotes and the 18S gene in eukaryotes. *Spirulina* is a cyanobacterium that grows in a spiraling filamentous form, whose appearance is that of hair-like mats, while *Chlorella* is a single cell, small and round green algae [5]. The ability to quantify these two species is nearly impossible by traditional microscopic methods. When we started examining the possibility of genotyping as a solution, we found that using the 18S gene in *Chlorella* would not allow for the distinction between the two species due to only 23% query cover (max score 80.6) between the two species. There was 100% query coverage (max score 1460) to the 16S gene in the chloroplast of *Chlorella*. Therefore a primer was designed that would recognize the 16S gene in the chloroplast, without crossover to the 18S gene of the nucleus. The 16S regions of the two species were amplified before the SBE reaction was performed. This was the basis of the study for the identification and quantification of these two species co-cultured in a photo bio-reactor.

MATERIALS AND METHODS

Photo Bioreactor Cultures

Chlorella vulgaris

Chlorella vulgaris was purchased from the University of Texas (UTEX 26) as a pure algae lawn on agar slants. Initial cultures revealed the algae contained multiple bacterial contaminants such as bacillus. Therefore, *Chlorella vulgaris* was maintained in Zarrouk medium for 2 weeks at a pH of 9.5, a condition determined in our lab to kill the bacteria but not the *Chlorella vulgaris.* Zarrouk medium composition consists of 18.0 g $NaHCO_3$, 2.5 g $NaNO_3$, 0.5 g K_2HPO_4, 1.0 g K2SO_4, 1.0 g NaCl, 0.04 g $CaCl_2$, 0.08 g Na_2EDTA, 0.2 g $MgSO_4*7H_2O$, 0.01 g $FeSO_4*7H_2O$ and 1.0 mL trace elements (TE). Trace elements composition based on a g/L basis is H_3BO_3 2.86; $(NH_4)6Mo_7O_{24}$ 0.02; $MnCl_2*4H_2O$ 1.8; Cu_2SO_4 0.08; $ZnSo_4*7H_2O$ 0.22, from the formula published by Zarrouk in 1966 [31]. The cultures were grown in 500 mL clear glass bottles with 0.2 micron PVDF filters on outgoing port vents in 10 klux of light at 30^o C in a temperature controlled bench top greenhouse. The light source was a series of 48 inch 75 kW General Electric cool white light fluorescent bulbs controlled by an electronic timer regulating a 12 hour diurnal cycle. Bottles were stirred at least once per day to facilitate removal of dissolved oxygen.

After the initial acclimation of *Chlorella vulgaris* to Zarrouk media, the culture was transferred to a modified Zarrouk media more amenable to algae growth. This modified Zarrouk media is described by Richmond *et al.* and is appropriate for co-cultures with *Spirulina platensis* [30]. Since optimal growth for *Chlorella vulgaris* occurs within a pH range of 7-8, the modified Zarrouk media is adjusted to pH 8. In addition, since sodium bicarbonate has a negative effect on the growth of *Chlorella vulgaris*, it was decreased to 4.5 g/L and sodium chloride was increased

to 8.75 g/L to adjust for the change in osmolality. *Chlorella vulgaris* was maintained in the media for 3 months before use to allow the culture to acclimate to the new media conditions. In this culture system, *Chlorella vulgaris* created large clusters of cells and aggressive vortexing of samples (approximately 15 minutes) was required to break up these cell clusters before counting. The concentration of *Chlorella vulgaris* was determined by counting cells using a hemocytometer at 60X magnification on a light microscope.

Spirulina platensis

Spirulina platensis (UTEX LB 1926) was grown in the same photo bioreactor system in traditional Zarrouk medium as described above. *Spirulina platensis* is morphologically different from *Chlorella vulgaris* which grows in single cell clusters. *Spirulina platensis* is composed of long filament structures called trichomes. Trichomes are formed by a "stacking" of cells into a left-handed open helix in the range from 100 μm to 2000 μm in length and 2-7 μm in diameter [32]. These trichomes are typically viewed easily at 10X magnification. Since the shape of *Spirulina* is uniform and consistent, it was determined in our lab that each complete turn of the helix structure contained on average 58 cells (plus or minus 3).

Co-Cultures

Spirulina platensis and *Chlorella vulgaris* were co-cultured in the modified Zarrouk media at pH 8.0. The cell density was calculated with the use of a hemocytometer for *Chlorella*. The hemocytometer was also utilized for the *Spirulina platensis* with the modification of the procedure since trichomes were present. For *Spirulina platensis,* the individual spirals and number of helices within a given volume were counted and multiplied by the number of cells in a helical turn. This method allowed for a cell ratio to be determined for *Spirulina platensis* and *Chlorella vulgaris* in co-cultures

DNA Extraction

DNA was extracted from harvested algal cells using the PowerPlant® Pro DNA isolation kit (Mo-Bio Laboratories, Carlsbad, CA, USA). This method used bead beating in combination with chemical extraction for cell lysis and DNA release. The released genomic DNA was first precipitated with isopropanol and then captured on a silica membrane in a spin column format. DNA was washed and eluted from the spin column with purified water and was then ready for PCR amplification and other downstream applications.

PCR Amplification

The genomic DNA extracted from the algal cells and was amplified using primers to the section of the 16S gene which included the specific sites needed for the SBE primers. The two correlating sections from the algal species were on the 16S gene of *Spirulina platensis* and the 16S gene of the chloroplast in *Chlorella vulgaris.* Once this section of the 16S gene was amplified, the excess salts and unused primers were removed and the DNA was then used for SBE. The same SBE primer was used on both species of the algal DNA. This gave a common DNA sequence for comparison and calibration.

Table 1: Primers for gene amplification and SBE reaction

Primer Name	**Gene Amplified**	**Primer sequence (5' → 3')**	**Fragment Size***
AV-F	16S Chloroplast of *Chlorella*	GAGTACGGTAGGGGCAGAGGGAA	776 bp
AV-R	16S Chloroplast of *Chlorella*	GCGTGGCTAACTTCCATGGTG	776 bp
SP5-F	16S gene of *Spirulina*	TGAGGGACGAAAGCTAGGGG	690 bp
SP5-R	16S gene of *Spirulina*	TAGAGTAACGACTTCGGGCGT	690 bp
SBE	Primer + 1 bp	AGGCTGCAACTCGCCTGCATGAAG	26 bp

*Fragment size is estimated from NCBI (www.ncbi.nlm.nih.gov)

Table 1 shows the primers used for both the PCR amplification and the SBE reaction. The primers were generated using the Primer-BLAST feature of the BLAST program of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) using primer constraints of location, product length, GC content and melting point (T_m). This program generated 13 primer pairs to choose from that would isolate the portion of the 16S gene of the chloroplast for *Chlorella* and the 16S gene of *Spirulina* without amplifying any other portion of the genome. Of these 13 pairs, one pair for *Chlorella* and one pair for *Spirulina* gave excellent results and are shown in **Table 1**.

The PCR reagents 5X go Taq Flexi Buffer, 25 mM $MgCl_2$, 10 mM dNTP and Taq Polymerase were obtained from Promega (Madison, WI, USA) and all of the PCR primers used were obtained from Invitrogen (Carlsbad, CA, USA). Primers were diluted to a stock concentration of 100 µM and this solution was used to make a working solution of 20 µM for the PCR reactions. All of the PCR reagents were mixed according to the standard PCR protocol shown in **Table 2**.

Table 2: PCR Amplification Protocol

Reagent Name	**Initial concentration of reagent**	**Required (or) Final concentration of reagent**	**Amount in the PCR mixture**
Go Taq Flexi Buffer	5 X		1.4 µl
MgCl2	25 mM	2 mM	1.6 µl
dNTP	10 mM	0.2 mM	0.4 µl
Taq Polymerase	5 units/µl	2.5 U	0.5 µl
Forward Primer	20 µM	0.5 µM	0.5 µl
Reverse Primer	20 µM	0.5 µM	0.5 µl
DNA Template	7 ng/µl		2 µl
Water			11.1 µl
Total			20 µl

After gently pipetting to mix, and spinning the samples for 15 seconds at 10,000 x g to remove bubbles, the PCR mixture was amplified on an Apollo ATC401 thermocycler, with temperature, time and cycles based on an optimization of the program and the melting temperatures (T_m) of the different primer pairs. The amplification program started with an initial denaturation at 95°C for 1 minute, followed by 30 cycles of denaturing at 95°C for 30 seconds, annealing at 51°C for 30 seconds, extension at 72°C for 30 seconds, followed by a final extension at 72°C for 1 minute. The amplified DNA was purified using the Qiaquick-spin PCR purification kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's instructions. Quantification of the purified DNA was determined with a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) based on the absorbance at 260 nm. The purified samples were stored at -20°C.

CE-SBE

The protocol for single base extension (SBE) required an ABI Prism® SNaPshot™ Multiplex kit (Life Technologies, Grand Island, NY, USA) consisting of 5 µL of reaction mix, 3 µL of SBE primer, and 1µL of sample (purified 16S DNA), and 1 µL of nuclease-free water. After pipetting gently to mix, the samples were centrifuged for 15 seconds to remove bubbles and placed in an Apollo ATC401 thermocycler for 5 cycles consisting of 96°C for 2 seconds to denature, 69°C for 5 seconds to anneal, and 60°C for 10 seconds to extend. The samples were put on ice until processed for SBE. Samples were loaded into a 96 well plate with 0.5 µL of sample, 9 µL of Hi-Di™ Formamide (Life Technologies, Grand Island, NY, USA) and 0.5 µL of Genescan™ 120 LIZ® size standard (Life Technologies, Grand Island, NY, USA) in each sample well. The plate was then denatured at 95°C for 3 to 4 minutes and was kept on ice until loaded

into the ABI 3130 capillary electrophoresis machine (Applied Bioystems Inc. Carlsbad, CA, USA).

Capillary electrophoresis (CE) was conducted using an array of 4 capillaries that were 36 cm in length. The capillaries were filled with a 7% w/v PDMA polymer dissolved in 1X TTE buffer (50 mM Tris, 50 mM TAPS, 2mM EDTA). Samples were introduced into the capillary using a voltage of 2.0 kV (55 V/cm) for 20 seconds, followed by separation at 15 kV (417 V/cm) for 20 minutes. The capillaries were kept at 55°C during the analysis. Samples were detected by Laser Induced Fluorescence (LIF) of the dye-labeled ddNTP chain terminator. Electropherograms were produced from the raw data files using the Chromagna V0.9.8 conversion program, and the area under the curve was calculated using the OriginPro 9.0 graphing and analysis program.

SBE Primer Design

Pure cultures of *Chlorella vulgaris* and *Spirulina platensis* were evaluated using several possible SBE primers. A primer that would recognize the 16S gene of the chloroplast of *Chlorella* that would not match any other part of the genomic DNA, including the 18S portion of the nucleus, was needed. This primer also needed to match the 16S gene of *Spirulina*. The SBE primers were designed by using the BLAST program to align sequences and comparing the 16S of the chloroplast for *Chlorella* to the 16S of *Spirulina* and notating identical sequences between 20-25 bp in length with the following nucleotide being different for the two species. A partial picture of the sequence and alignment is shown in **Figure 1** with the differences between the two sequences highlighted in red.

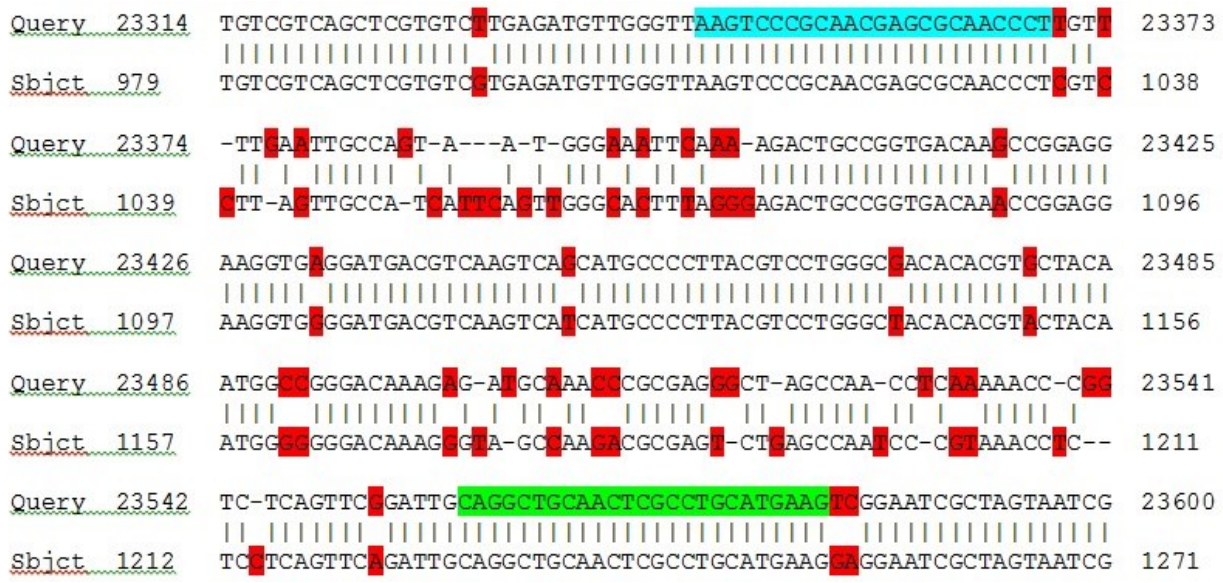

Figure 8: A partial sequence of the 16S gene of the chloroplast of *Chlorella vulgaris* aligned with the 16S gene of *Spirulina platensis*, including two SBE primers sites (SBE-2 and SBE-3).

There were many possible primer sites in the complete 16S gene sequence (complete sequence was not shown due to size constraints), but five possible sites were chosen that were spaced evenly throughout in gene sequence. These five primers, shown in **Table 3**, were analyzed and two of them (SBE-2 and SBE-3) were used for continued studies in DNA concentration.

Table 3: SBE test primers

Primer name	Sequence	T_m (^oC)	GC content	Length
SBE -1	GGGTGAAGTCGTAACAAGGTAGCCGTAC	60.81	54%	28 bp
SBE - 2	CAGGCTGCAACTCGCCTGCATGAAG	65.24	60%	25 bp
SBE - 3	AAGTCCCGCAACGAGCGCAACCCT	64.49	63%	24 bp
SBE - 4	AGCGGTGAAATGCGTAGA	51.52	50%	18 bp
SBE - 5	AGTGGCGGACGGGTGAGTAACACGT	62.25	60%	25 bp

The amplification of the 16S gene needed to have enough DNA to optimize the CE-SBE method and to prove the concept of DNA quantification with peak area calibration. When the

single base extension was tried on genomic DNA it failed to generate any peaks on the electropherogram. Our hypothesis was that the protocol did not allow enough time to find the exact primer match in the genomic DNA. Therefore it was decided to isolate the 16S gene and amplify it from each species prior to single base extension, which would allow for a shorter annealing period and prove the concept while optimizing the SBE protocol. Future work will include adapting the SBE method to genomic DNA, but it was necessary to have larger amounts of purified rDNA for the calibration studies.

RESULTS

Calibration Studies

Figure 2a shows an electropherogram of four different concentrations of *Chlorella* DNA run separately, and time adjusted to fit on the same graph without overlap. Using the same SBE primer (SBE-2), pure cultures of *Spirulina* were also analyzed. **Figure 2b** shows an electropherogram of four different concentrations of *Spirulina* DNA also run separately and time adjusted to fit on the same graph. It is clear that there is a correlation between the peaks and the concentration of the sample DNA. Analysis showed that the peak area, not the peak height is linearly correlated with the DNA concentration in the sample. **Figure 2b** also shows the limitations inherent in the use of fluorescence with the highest concentration (24.8 ng/μL) showing a characteristic flattening or cut off at the top of the peak. This is due to the saturation of the detector between 8000 and 10,000 RFU. In this range the peak area may not fit a Gaussian curve and the area calculation may be slightly inaccurate. The sample would need to be diluted if it was above the range of accurate analysis.

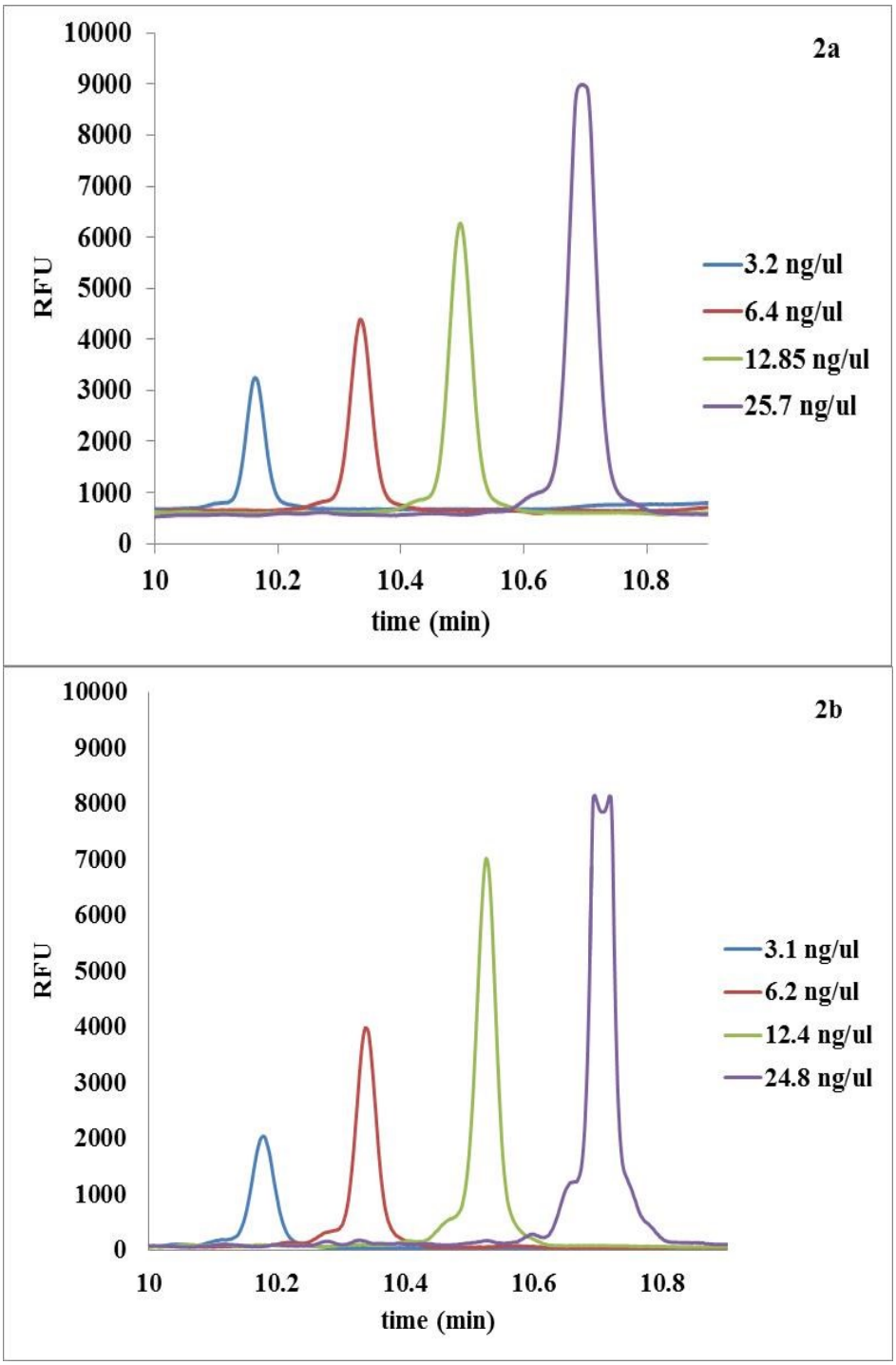

Figure 2a & 2b: Concentrations of *Chlorella* DNA from the 16S gene of the chloroplast (2a) and concentrations of 16S *Spirulina* DNA (2b).

DNA Calibration Curve

Four samples of each species at each concentration were used to develop the DNA concentration curve shown in **Figure 3**. Both species were plotted on the same graph to show the linear correlation of the area under the curve with the calculated DNA concentration. The area at the higher concentrations can give a less accurate Gaussian fit when analyzing for the peak area, causing a spread in the data. This is due to the saturation of the detector at the higher concentrations which causes a flattening at the top of the peak and results in slight inaccuracies in area calculations. There is also one outlier at the lowest concentration which may be due to slight inconsistencies in pipetting or an aged buffer or capillary.

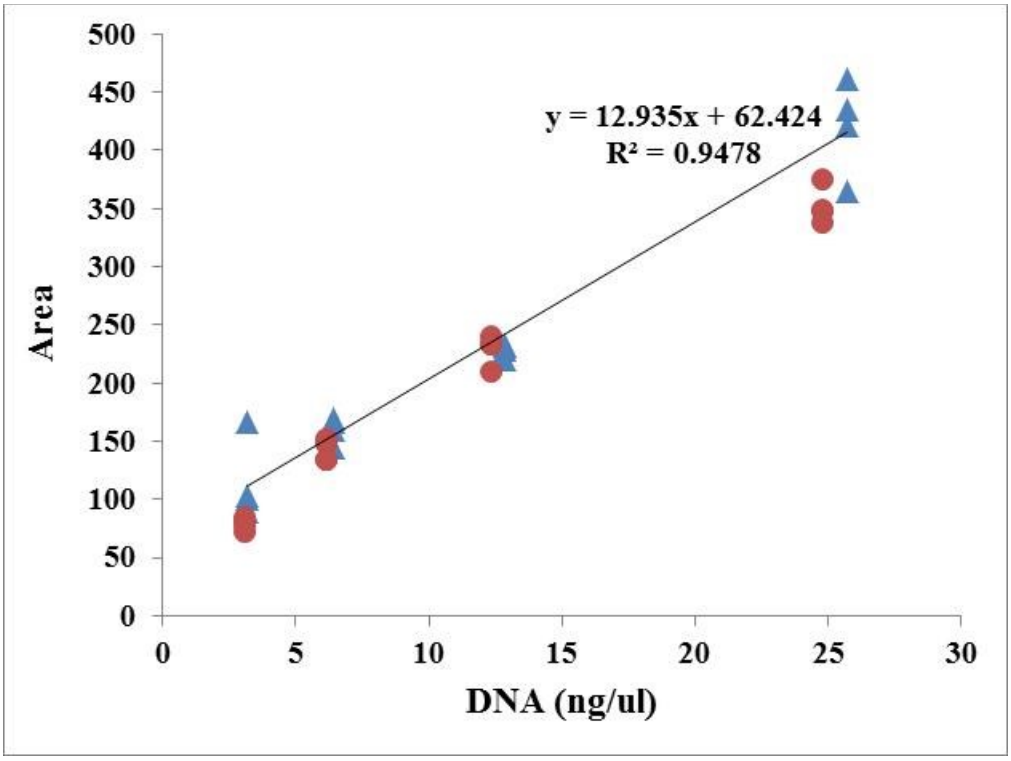

Figure 3: Area vs. DNA concentration curve. *Chlorella* (▲), *Spirulina* (●)

Mixed Cultures of *Chlorella* and *Spirulina*

A mix of *Spirulina* and *Chlorella* DNA was evaluated using the SBE primer. The following Figures (4, 5, and 6), show electropherograms establishing that the SBE primer recognized and distinguished the two different species when run in the same tube. A negative control (no algal DNA) and a positive control (from kit manufacturer) were also run at the same time (data not shown). The measured concentration of DNA of each species was determined by absorption at 260 nm and used as the basis for the mixtures.

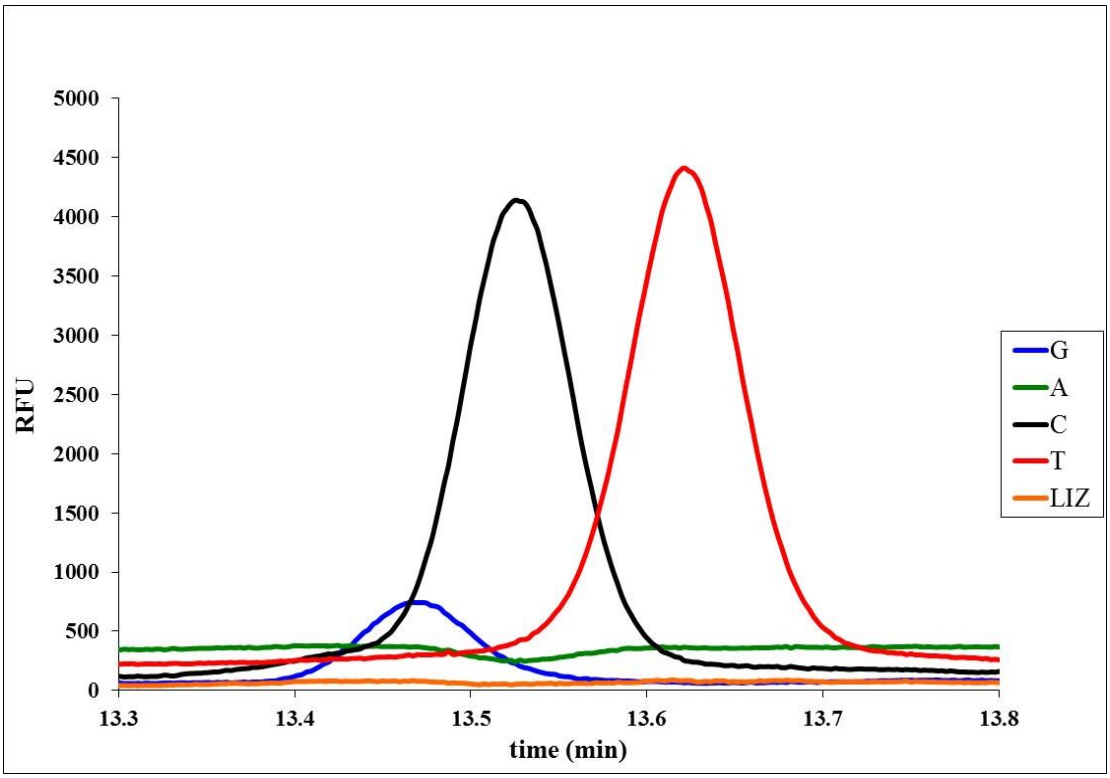

Figure 4: A 50:50 mixture of *Spirulina* (black) and *Chlorella* (red) DNA.

Figure 4 was the result of mixing two equal concentrations of *Spirulina* (black) and *Chlorella* (red). The resulting graph includes all four nucleotide bases as well as the 120 LIZ® sizing standard. The graph is zoomed in to the two peaks of interest which are between the third and fourth peaks of the 120 LIZ® sizing standard peaks (results not shown). The sizing standard

is run with the sample to account for changes in elution time due to polymer or capillary changes. When the peak area was calculated, *Spirulina* was 47.8% of the mixture and *Chlorella* was 52.2% with a standard deviation of 0.83. This was consistent throughout multiple repeats of the same sample mixture.

A mixture of approximately 80% *Spirulina* DNA was mixed with 20% *Chlorella* DNA for **Figure 5.** When the peak areas were calculated, the percentage of *Spirulina* was 71.5% and the percentage of *Chlorella* was 28.45% with a standard deviation of 0.9 which is reasonable agreement with the calculated values. **Figure 5** also shows all four nucleotides and the 120 LIZ sizing standard.

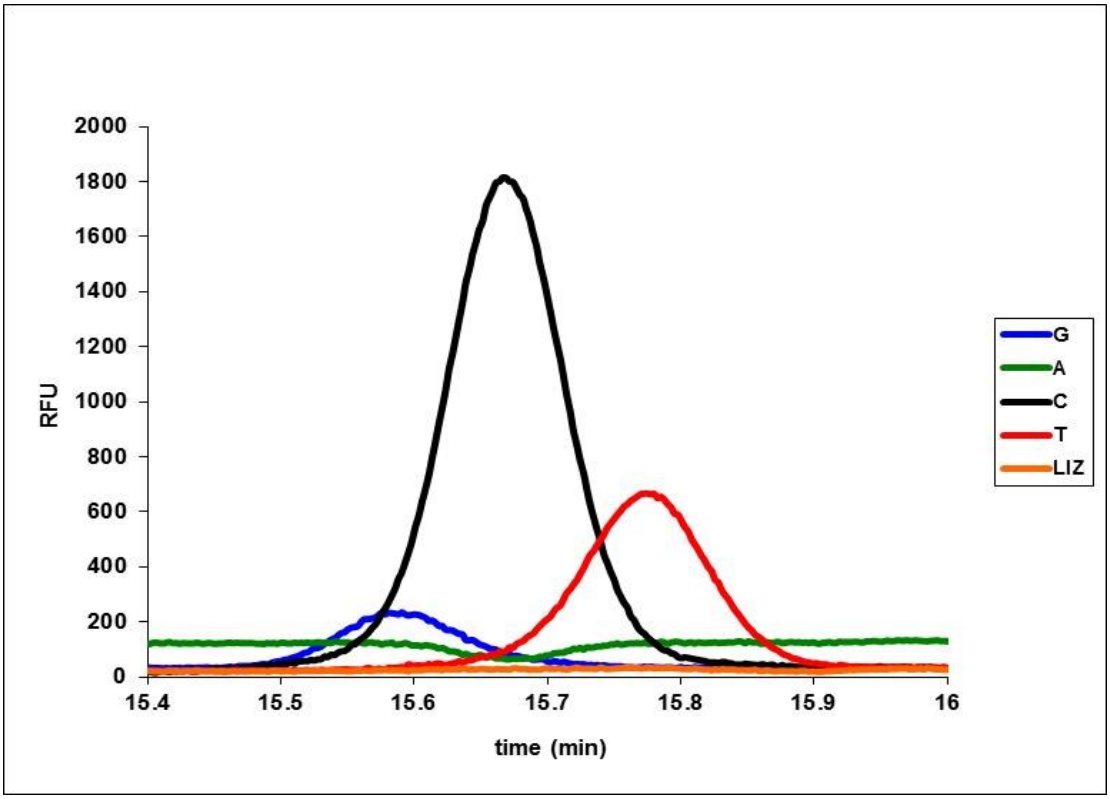

Figure 5: 80% *Spirulina* (black) and 20% *Chlorella* (red) DNA mixture

The opposite mixture of 20% *Spirulina* and 80% *Chlorella* was shown in **Figure 6** and the peak areas were calculated and gave a percentage of 23.5% for *Spirulina* and 76.5% for

Chlorella with a standard deviation of 0.129. These three mixed samples were tested with a second SBE primer (SBE-2 in Table 3) that also gave a good distinction between the two species without causing an artificial extra peak.

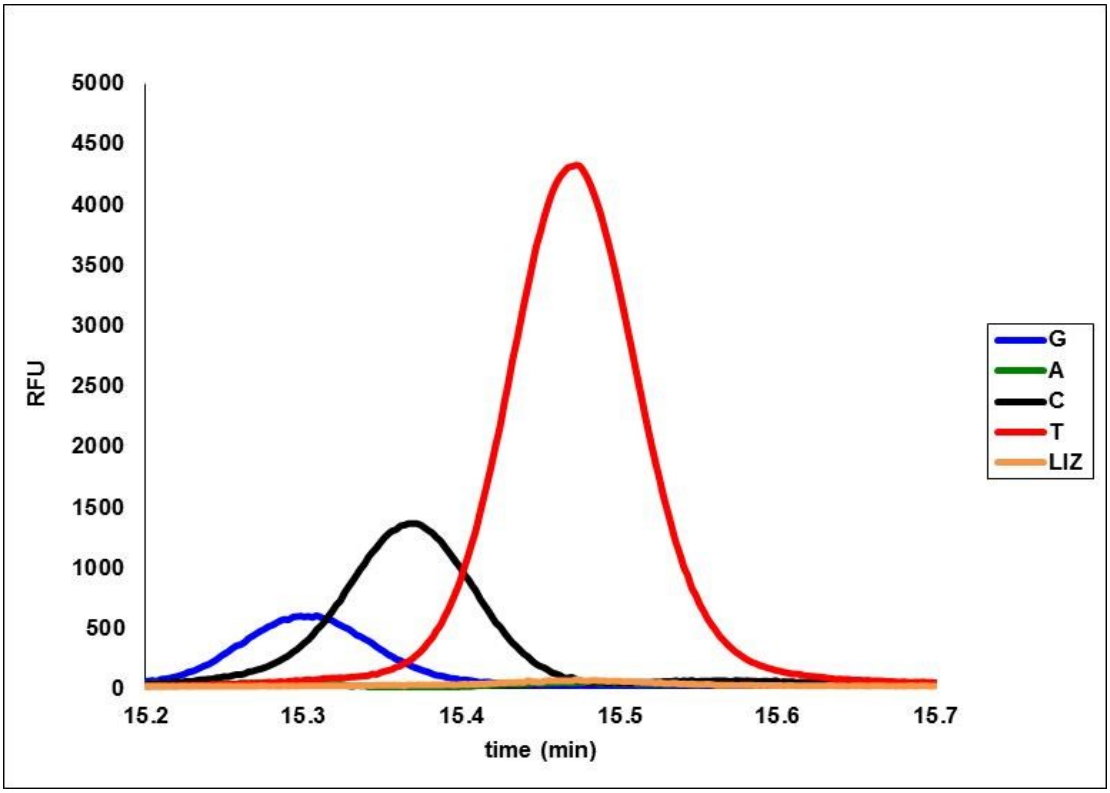

Figure 6: 20% *Spirulina* (black) mixed with 80% *Chlorella* (red) DNA

Seen in **Figures 4, 5** and **6** is an extra peak for the nucleotide "G", after multiple samples were tested with this SBE primer (SBE-3 in Table 3), it was noted that most electropherograms had this extra small peak. This was determined to be an artifact of the fluorescence detection of the SBE primer as it was not as strong in trials where the primer concentration was lower and became stronger as the primer concentrations were increased. This extra signal was independent of the species of algae in the sample and was visible in the negative control samples which did

not include DNA from the algae. Therefore, this primer was not recommended for further future use and was not used for the DNA calibration studies.

Verification of the Calibration Curve

A series of Spirulina and Chlorella samples were analyzed using this single base extension technique to determine if the calibration curve could be extended and to verify the calibration curve for an expanded set of samples. We found that the calibration curves are limited to a concentration less than 30 ng/µL and can only be extended by sample dilution. When the measured samples are greater than 30 ng/µL, the peak area becomes flattened at the apex which makes Gaussian calculations difficult due to the missing part of the peak. However the samples can be diluted and the original concentrations can be extrapolated from the diluted sample. These expanded samples and their calculated concentrations of DNA are shown in tabular form in **Table 4**.

Table 4: expanded samples of DNA with the percentage deviation from expected values

Algae sample	**Conc. (ng/µL)**	**Dilution Conc.**	**SBE exp. concentration (ng/µL)**	**Calculated concentration (ng/µL)**	**Percentage deviation (%)**
Spirulina-1	622	62 1:10	50.8	508	18.3
Spirulina-2	622	31 1:20	51.25	1025	64.8
Spirulina-3	622	21 1:30	23.25	697	12.1
Chlorella-1	479	48 1:10	21.2	212	55.7
Chlorella-2	479	24 1:20	21.2	424	11.5
Chlorella-3	479	16 1:30	17.5	525	9.6

Aside from the difficulty in obtaining an accurate measure of the peak area at higher concentrations, there was also noted some discrepancies on individual capillaries. Capillaries can

become blocked over time and will give only a very small peak, if any, and some troubleshooting techniques may be required to determine if the array should be replaced. The 120 LIZ® size standard was run so elution time differences could be accounted for. As the capillary aged the elution times became longer. In a "fresh" capillary the normal elution time for our primer was between 3 and 5 minutes (corresponding to just before the forth size standard peak). After months of use in these studies (over 150 runs), it was noted that the elution time was approximately 13 minutes. A new capillary restored the elution times to 3 to 5 minutes. Contributing to some of the differences noted between samples used for the calibration curve calculations and the expanded samples was the installation of a new laser on the capillary electrophoresis instrument. The new laser was calibrated and no gross differences have been observed, but the exact samples had been frozen during this time and may have had an impact on the repeatability studies.

DISCUSSION

SBE gives a direct correlation between DNA concentration and the area under the curve in an electropherogram. The best range of DNA concentrations are between 3 ng/µL and 25 ng/µL. Extension of that range can be obtained by accurate dilutions of samples that have higher DNA concentrations. The single SBE primer could successfully identify and quantify the rDNA of both *Chlorella vulgaris* and *Spirulina platensis*. In the case where the cultures were being grown in the same photo-bioreactor, this was an advantage as *Spirulina* is extremely difficult to accurately quantify due to its unique morphology. The next step in this project will be to correlate the amount of DNA with the number of cells in a given sample and then with SBE curves. Also we would like to be able to skip the amplification step required for 16S DNA. We were unable to successfully use single base extension on genomic DNA, but feel that with

optimization of the method, or possible slight modifications of the primers that it will be possible.

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CONCLUSIONS

The work in this thesis focuses on the storage and genotyping of algae for applications in biofuel production. The introduction presented an overview of the potential for algae in both wastewater remediation and biofuel production, as well as the importance for developing a rapid method that can monitor the type and quantity of algae produced. In Chapter 1, the potential for year-round production of biofuel from algae being used to remediate waste water was presented. Microalgae harvested from an ATS™ system used to treat wastewater gave an average sugar concentration of 10.9% by dry weight. A ratio of 0.13 g butanol/g algae sugar was obtained from ABE fermentation using algal sugars as the feedstock. This was approximately half the butanol reported to be produced from ABE fermentation results utilizing glucose (0.25 g/g). This indicates that many of the sugars extracted from the algae were not fermentable to butanol. The most important finding was that the algal feedstock needed to be dried to a value of less than 25% moisture for long term storage, and could be kept up to 200 days unground, before incurring a significant loss of sugar concentration. Therefore, dried algal feedstock can be used to supply a bio-reactor with carbohydrates for year-round production of bio-butanol. It was found that optimization of the acid hydrolysis for the type of algae in the feedstock was the key to increasing sugar production and that the dominant species has a direct effect on the amount of sugars and lipids. Therefore, a method called capillary electrophoresis single strand conformation polymorphism (CE-SSCP) was evaluated for its ability to quickly identify algal species.

In Chapter 2, CE-SSCP was determined to be a relatively fast, easy and inexpensive method for determining changes in a small section of DNA such as a variable region of the 16S or 18S gene coding for the small subunit of the ribosome. PCR amplification of the 16S V2 and

V3 regions for prokaryotes and 18S V7 and V9 regions for eukaryotes allowed the generation of highly repeatable, unique fingerprint patterns of different algae. Using this method we were able to show differences between the patterns of prokaryotes and eukaryotes as well as differentiating 4 different types of green algae. We were also able to compare the similarities and differences between environmental samples and a reference pattern. However, CE-SSCP does have limitations in that it is difficult to analyze large numbers of species in a sample at the same time due to multiple peaks leading to complex peak patterns. Large samples with a variety of algae leading to multiple overlapping peak patterns would require not only a large database of fingerprints, but also a complex computer program to separate out these potential overlapping peak patterns. Therefore, it might be easier to use traditional taxonomy to narrow down the options, or to use CE-SSCP in a system that has a more limited number of potential species. This leads to the conclusion that CE-SSCP could be an extremely useful method to monitor changes in a more controlled environment such as a photo-bioreactor rather than an open environmental system.

In Chapter 3, an SBE primer was designed that could be used to differentiate between *Chlorella vulgaris* and *Spirulina platensis* growing as a co-culture in a photo-bioreactor system. Quantification of some algal species such as *Spirulina* can be extremely difficult by traditional microscopic methods and therefore a genotyping method could be of great value. The usefulness of CE-SBE for quantification and identification of these two different species of algae was explored. CE-SBE was used to determine a direct correlation between the DNA concentration and the area under the curve in an electropherogram. The linear range for the correlation was between 3 – 25 ng/μL, with an extension of the range obtained by accurate dilutions of the samples that have higher DNA concentrations. The single SBE primer could successfully

identify and quantify the rDNA of both *Chlorella vulgaris* and *Spirulina platensis*. This method offers significant advantages over traditional methods of microscopic counting as *Spirulina* (which grows in small hair-like spiraling filaments) is an especially difficult cyanobacterium to count, and CE-SBE is an extremely fast method to identify and quantify algal species.

FUTURE WORK

Future work will include expansion of the data set for the CE-SBE calibration curve to explore and improve the accuracy of the correlation. We would also explore the absolute limits of the detection of fluorescently labeled DNA by the LIF detector and methods to improve the accuracy of the dilutions when DNA concentrations are out of the applicable range. In addition, fluorescent dyes can vary in their emission efficiency and/or electrophoretic mobility, which would be necessary to explore for increased applicability of the method.

Future work will also include optimizing the CE-SBE method to work on genomic DNA, without any amplification of a specific gene and correlating the DNA concentration with the number of cells. Currently, we are using an estimate of the number of cells per twist in the spiral of the filament for *Spirulina platensis.* If we can correlate the number of cells with the concentration of DNA per cell, we will be able to use CE-SBE to calculate the number of cells in a sample based on the DNA concentration. This will make the CE-SBE method a very fast means of identifying and quantifying algal species.

TO: Dr. Christa Hestekin

FROM: Linda Lancaster

DATE: January 7, 2014

RE: Biosafety Protocol # 10048

The referenced protocol, entitled "Identification of Algae Species via CE-SSCP for Biofuel Production," will expire 02/14/14. If you are no longer working on this project, please let me know so that I can terminate the protocol. If you are still carrying out research associated with this protocol, you must submit a new protocol prior to expiration. The forms are available on the Biological Safety website, http://ehs.uark.edu/FormsPage.aspx. If you have any questions or need assistance in completing the forms, you should contact the Biosafety Officer, Dr. Miriam Lonon, by phone or email (5-3597, mlonon@uark.edu).

Please complete all forms pertinent to your proposed activity and submit them to me electronically. Form 9 has been added for BSL2 protocols. The signature page of Form 1 must be signed by the investigator and either faxed or placed in campus mail. My email address is ibc@uark.edu and my FAX number is 575-3846. My campus address is: ADMIN 210.

Should you fail to resubmit your protocol for renewal it will be terminated. Until your resubmitted protocol has been approved by the Biosafety Committee, you may not continue to work under the original approval.

PLEASE **STATE SPECIFICALLY WHAT IS NEW IN THE E-MAIL OR ON A SEPARATE SHEET OF PAPER AND INCLUDE IT. THE COMMITTEE HAS ASKED THAT YOU PLEASE HIGHLIGHT THOSE NEW PARTS OF THE PROTOCOL IN YELLOW**. I WILL SEND IT TO THE COMMITTEE AS A RENEWAL. YOU MAY OR MAY NOT NEED TO ATTEND THE IBC MEETING. I WILL INFORM YOU OF THE COMMITTEE'S DECISION PRIOR TO THE NEXT MEETING.