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Investigating the feasibility of growing algae for fuel in southern Nevada

Faegheh Moazeni
University of Nevada, Las Vegas, moazenif@UNLV.Nevada.edu

INVESTIGATING THE FEASIBILITY OF GROWING ALGAE FOR FUEL IN SOUTHERN NEVADA

By

Faegheh Moazeni

Bachelor of Science in Chemical Engineering
Sharif University of Technology, Tehran –Iran
2005

Master of Science in Chemical Engineering
Sharif University of Technology, Tehran –Iran
2007

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Faegheh Moazeni

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Nader Ghafoori, Ph.D., Committee Chair

Henry Sun, Ph.D., Committee Member

Mohamed Kaseko, Ph.D., Committee Member

Daniel Gerrity, Ph.D., Committee Member

Spencer M. Steinberg, Ph.D., Graduate College Representative

Tom Piechota, Ph.D., Interim Vice President for Research &
Dean of the Graduate College

May 2013

ABSTRACT

Investigating the Feasibility of Growing Algae for Fuel in Southern Nevada

by

Faegheh Moazeni

Dr. Nader Ghafoori, Examination Committee Chair
Professor of Civil and Environmental Engineering and Construction
University of Nevada, Las Vegas

Dr. Henry Sun, Research adviser
Assistant Professor at Division of Earth and Ecosystem Science
Desert Research Institute, Las Vegas

Microalgae capable of growing in waste are adequate to be mass-cultivated for biodiesel, avoiding fertilizers and clean water, two obstacles to sustainability of the feedstock production. This study replaces fertilizers and clean water with waste products. The investigated wastes include (1) the liquid fraction of sewage after solids and particles are removed, known as centrate, and (2) algal biomass residue, i.e. the algae remaining at the end of the lipids extraction process at biofuel plants. These wastes contain sufficient amount of nitrogen and phosphorus required for algal growth. This study proposes a system in which centrate would be used as an initial source of water and nutrients for microalgal growth. The generated biomass waste can be continuously recycled, serving as a fertilizer. If so desired, the centrate can be reverted back into the system from time to time as a nutrition supplement and as a make-up water source, particularly in open ponds that face evaporation. Of the six studied algae, i.e. *Chlorella sorokiniana, Encyonema caespitosum, Nitzschia thermalis, Scenedesmus* sp., *Synechocystis* sp., and *Limnothrix* sp., mostly isolated from the habitats influenced by municipal wastewater in

and around the Las Vegas Valley, two green algae were eligible. In the laboratory, the green algae *C. sorokiniana* and *Scenedesmus* sp. grew in the media composed of centrate or algal residue faster than in the mineral medium BG11, optimized for algal growth. The enhanced productivity is mainly attributed to the photosynthesis known for mixotrophic process and the presence of organic carbon in the waste which serves as an extra source of energy. Tolerance for hard water and strong light and, in the case of *C. sorokiniana*, an unusually high optimum temperature between 32 and 35°C are also attributing factors to the enhanced productivity of algae. These studied species are particularly suited for cultivation in their native southwestern United States, particularly Southern Nevada, where warm climate, non-arable land, and wastewater are available.

In addition, this study examines the variations in lipid content of algae, which affects the overall oil productivity at biofuel plants. The results demonstrated that lipid content is a dynamic property, negatively correlated to growth rate. Under varied environmental conditions, where growth rate can vary, lipid content also varies, but in an opposite direction. Therefore, the conditions that support a high rate of growth may not necessarily cause lipid content to increase. As a result, the tradeoff between growth rate and lipid content becomes critical at biofuel plants where the overall oil productivity must be optimum. If the efficiency of a biodiesel production process is 100%, the total productivity is equivalent to the amount of lipids produced by each cell multiplied by the total number of cells in the culture. Consequently, the oil productivity would be directly influenced by the outcome of the interactions between lipid content and growth rate. This research presents a universal pattern that elaborates the relationship between lipid content and growth rate of algae under varied environmental conditions.

Key words: native algae, algal biodiesel, municipal wastewater, algal biomass residue, lipid content, growth rate, mixotrophy

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OVERVIEW

1.1. General Introduction

It is broadly recognized that the era of fossil fuels is coming to an end. The global consumption of these fuels cannot continue at its current rate, because of accumulation of carbon dioxide in the atmosphere drastically increases global temperature. Man must explore "clean" and renewable sources of energy that minimize dependence on fossil fuels. While our need for electricity can potentially be met with wind, geothermal, and solar (using photovoltaic) energy sources, there is also a need for carbon-neutral transportation fuels, meaning that carbon is drawn from and returned to the *current* atmosphere, causing no net change. Biofuels are considered to be carbon-neutral. Biofuels are obtained from a wide-range of alternatives, with corn being the main source of ethanol (one type of biofuel) in the United States. However, corn ethanol creates a number of serious issues, including competing with the food industry, increasing food prices, and converting the use of agricultural land (Searchinger *et al.*, 2008; Farrell, 2006). Hence, cellulosic ethanol and algal biodiesel have become the most promising options on the horizon. Cellulosic ethanol is ethanol produced by turning the sugars in cellulose into alcohol fuel. Cellulose refers to the material comprising the cell walls of any green plant, including agricultural residues such as wood and grass. However, the presence of lignin, which has evolved to protect plants from biodegradation, interferes with fermentation, i.e. the process of ethanol production. Ongoing studies into plant

biology are seeking ways to grow plants with less lignin. Besides cellulosic ethanol, biofuel can also be produced from aquatic species, specifically algae. These photosynthetic microorganisms grow fast, and are rich in fatty acids and triglycerides, precursors to biodiesel. Algae yield as much as 6,280gal of algae/acre/year, which is about 60 times higher than the oil produced by the most prevalent biodiesel feedstock in the world, canola (Dropcho *et al*., 2008; Yang *et al*., 2011). As algae are aquatic microorganisms, algae farms can be built in deserts or on land that is otherwise of little value. (Amaro *et al*., 2011; Chisti, 2007; Dismukes *et al*., 2008; Li *et al*., 2008; Pienkos *et al*., 2009).

Interests in algal oil, as a substitute for petroleum, began in 1970s. It arose as a response to the oil embargo imposed on the United States by the Arab countries in retaliation for its support of Israel during the Fourth Arab-Israel War. At that time, the technology for turning lipids into diesel, a chemical process known as esterification, was already well established. So the challenge became determining what organisms to use for biodiesel and how to grow them on an industrial scale. Microscopic algae, which grow much faster and contain more lipids than higher plants on a whole-plant basis, naturally became the focus of different studies (Benemann *et al*., 1977; Golueke and Oswald, 1959). In response to this challenge, a major new program, named the Aquatic Species Program (ASP), was established in 1978 by the U.S. Department of Energy. Over the next 18 years, as a result of this program, significant progress was made in three areas. First, several thousands of microalgae were cultured from various habitats across the United States and screened to yield over 300 fuel-producing candidates rich in lipids. Second, genetic engineering methods for improving lipid production were developed in

previously isolated, well studied model algae, although the methods had yet to be applied to the new isolates. Third, outdoor pilot tests were conducted in Hawaii, Southern California, and New Mexico. Among these, the test at the latter site was the most successful in that high, year-round productivity was maintained continuously for two years. The test showed that open, shallow ponds of raceway design served as an economical and effective production system. The results provided a basis for comparing production costs with petroleum. The estimated cost for one barrel of algal biodiesel, assuming the entire process is optimized, could be as low as $59. At that time, a barrel of petro-diesel sold for about $20. This comparison indicated that, with further technological developments, algal diesel could well become a reality. Unfortunately, the ASP program was terminated in 1996 due to budget cuts (Sheehan *et al.*, 1998).

Algal biodiesel again attracted attention in 2007, when the U.S. Congress passed a bill called the Energy Independence and Security Act (EISA). The law mandated that 20% of gasoline consumption must be replaced by renewable resources by the year 2017. In addition, the renewable substitutes were dictated to have, from cradle-to-grave, at least 50% less greenhouse gas (GHG) emissions than fossil fuels. Hence, while the mandate stimulated general interests in biofuels, the ambitious target in respect to GHG emissions raised challenges for biofuels. The first result of the mandate was the re-evaluating of ethanol production from corn. New life cycle analyses revealed that such ethanol is not as "green" as it was promised. Its GHG emission was estimated to be only slightly less than that from fossil fuels (Farrell, 2006; Searchinger *et al.*, 2008). This added to the previously existing problems of corn ethanol, impacting food production and agricultural land-use. Hence, interests were again toward on algal biodiesel. Even though the idea of

algal biofuel was scientifically sound, its production on an industrial scale still faced important issues. Its main obstacles were identified as feedstock production and chemical conversion of algal oil into diesel (Clarens *et al*., 2010). Conventional practice in conversion of lipids to biodiesel relies on transesterification, through which triglycerides react with methanol to produce methyl esters of fatty acids, i.e. biodiesel, and glycerol. Transesterification is catalyzed by acids, alkalis, or lipase enzymes. Finding suitable catalysts is the topic of many studies in this area. Lipase enzymes offer important advantages, but they are not considered feasible because of their high cost. Alkali catalysts carry out the reaction about 4000 times faster than acidic catalysts, but are more expensive (Fukuda *et al*., 2001). An alkali-catalyzed reaction requires a temperature of 60°C under atmospheric pressure, and about 90 minutes to complete, while a higher temperature combined with a higher pressure could speed up the reaction, resulting in higher costs. Currently, chemists are trying to invent affordable and efficient, heterogeneous and homogenous, catalysts to expedite the reactions. In addition to the transesterification process, feedstock production also presents challenges to the commercialization of algal biodiesel. The feedstock production process consists of two steps, growing and harvesting algae. Harvesting the biomass is hard due to the microscopic size of the algal cells, ranging from 3-30 µM (Grima *et al*., 2003). The process of biomass recovery is a multi-step solid-liquid separation carried out by centrifugation, filtration, or in some cases gravity sedimentation. Filtration and gravity sedimentation are inefficient for mass harvesting because they are very slow. For cultures of a very small volume, sedimentation is suitable when enhanced by flocculation. For larger volumes, centrifugation would be the most rapid and efficient choice, yet this

method is expensive. In order to improve sedimentation, new flocculants, such as multivalent metal salts, polyferric sulfates, polyelectrolytes, and electroflocculants, are being explained. Such flocculants can neutralize the negative charges being carried by algal cells, thus letting the cells coagulate and easily settle (Grima *et al*., 2003). The other important element of feedstock mass-production is cultivating algae, which also poses serious challenges. This research addresses some of the challenges associated with the cultivation of algae in Southern Nevada. The findings could also be applied to other regions of the Southwestern U.S.

The cultivation of microalgae as biodiesel feedstock requires an enormous supply of growth medium. Sustainability considerations argue against meeting this need at the expense of clean water and fertilizers for at least two reasons. First, clean water and fertilizers may not be available in sufficient quantity, especially in the regions considered ideal as production sites. In the Southwestern United States, algae farms could be built on non-arable land where the year-round warm climate permits efficient, uninterrupted production, but the region has limited water resources. For instance, in Southern Nevada, according to the Southern Nevada Water Authority (SNWA), the Colorado River, i.e. the only water resource of this region, is facing the worst drought on record and the water level of Lake Mead, serving as a reservoir, has dropped more than 100 feet since January 2000 (www.snwa.com). The situation with fertilizer is no better. While nitrogen fertilizers can be synthesized, phosphorus fertilizers must be mined from finite, non-renewable reserves. According to recent estimates, the global consumption of phosphate rocks due to agricultural food production alone may peak in another 50-100 years (Cordell *et al*., 2009). In addition, the biogeochemical cycle of phosphorus is already

"broken" without algae aquaculture. Heavy use of fertilizer in agriculture has not only depleted the global reservoirs of phosphorus, but excess fertilizers have also leached into lakes and oceans, where they fuel algal blooms. Both of these issues would be exacerbated considerably by the addition of another fertilizer-dependent industry. Second, even if clean water and fertilizers could be allocated for biofuel production, they are expensive and incur GHG emissions (Clarens *et al*., 2010). The latter are associated with fertilizer synthesis, mining, and transportation which are currently powered by fossil fuels. Hence, algal aquaculture for biofuel will not be feasible if it relies on clean water and fertilizers.

To mitigate the above-mentioned concerns, this study substitutes water and fertilizers with waste including centrate, i.e. the liquid fraction of sewage, and algal biomass residue, i.e. the algal cells after lipids are extracted. Unlike clean water, centrate is abundant and renewable. The large cities existing close to the future biodiesel production sites could provide a sufficient volume of "dirty" water for algae aquaculture. For instance, in Southern Nevada, the city of Las Vegas alone generates over 600 million liters of municipal wastewater a day, which could supply water for approximately 2 hectares of algae farms every day (Lien and Roessler, 1986). In addition, centrate is also a rich source of nutrients, i.e. nitrate and phosphate. Nutrients that are currently being removed at a cost at treatment facilities could be made available to algae farms free of charge. However, centrate also contains materials that could be toxic to algae, e.g. heavy metals, which should be identified and removed from centrate before it is used as a growth medium. Centrate is a very complex material and all its constituents and their effects on algal growth have not been identified. Even if the sources of toxicity are

determined, removing them would be economically infeasible. One solution is to dilute the centrate with fully treated sewage to a level at which the toxicity disappears. Yet, it should not be over diluted so that the nutrients become insufficient for algal growth. Only a properly diluted centrate could support sufficient growth. Another potential source of nutrients is lipid-free algal biomass remaining at the end of the biodiesel production process. It contains nitrogen and phosphorus in the form of proteins and nucleotides that could be recycled to algae farms, serving as fertilizers. Because algae are not capable of utilizing such polymers and big molecules, biomass residue must be decomposed before it is used as a nutrient source. This study investigated biotic degradation, via composting, which has been successfully used for years in agriculture for plant residue degradation. It is slow, but economical. Compost tea, i.e. the product of composting, serves as growth medium. Whether (or not) the target algae are able to grow in such medium is yet to be investigated. In addition to biological degradation, the waste biomass can be decomposed chemically as well. This approach would be fast, but energy intensive. Through chemical degradation, the polymers (e.g. protein) that are not usable by algae convert to the monomers (e.g. amino acids) that contained free nutrients, which could be consumable by algae. However, such free nutrients are organic and are not usually usable by algae. While algae, like higher plants, prefer inorganic nutrients, e.g. nitrate, some marine diatoms utilize the organic nitrogen of amino acids to grow (Liu and Hellebust, 1974; Nilsson and Sundback, 1996). Therefore, it is possible that "fuel-producer" species are also capable of using this organic nutrient. Hence, this study investigates the feasibility of growing the target algae on amino acids, as the sole source of nitrogen. In short, this author believes that growing feedstock on waste products,

when feasible, not only eliminates the use of clean water and fertilizers but also transforms waste from being a "cost" to a "benefit".

Centrate and algal biomass residue are not only sources of nitrogen and phosphorus but also contain an abundance of organic carbon. Some algae are able to utilize organic carbon as a source of energy in the presence, or absence, of light. When there is light, organic carbon could be consumed as an additional source of energy for photosynthesis (known as mixotrophy), while in the absence of light, it can serve as the only source of energy (known as heterotrophy). In algae farms, during the day, mixotrophic conditions could stimulate growth due to the extra available energy. The conditions at night would be more beneficial in contrast to the autotrophic growth mode that is halted at night. Heterotrophic conditions allow for 24-hour nonstop growth, leading to an increase in total biomass density. This study suggests that taking advantage of the organic carbon present in waste will improve biomass productivity in algae farms. However, there are some concerns. First, not all algae are able to utilize organic carbon. Like higher plants, algae receive their required energy from light, and thus are usually not able to utilize organic carbon. But some species, acting like plants and bacteria at the same time, consume both organic carbon and light as sources of energy. Second, even organic-utilizing algae may not be capable of consuming all types of organic carbon. For instance, an alga that consumes acetate may not consume glycerol. Third, some algae require light to utilize a particular source of organic carbon. For example, some species can utilize glycerol only in the presence of light, mixotrophically, but not heterotrophically (Liang *et al*., 2009). Hence, to take full advantage of the waste in algal

farms, it is essential to identify the types of species capable of consuming the organic compounds of waste both under light and in darkness.

This study postulates that, even if all the stated issues and inefficiencies are improved or resolved, only indigenous species are adequate to produce biodiesel feedstock, at least for cultivation in open ponds. They are well adapted to the chemistry of water and climate of the region, and thus able to tolerate some moderate degree of salinity and temperature, which are desirable for algae aquaculture. This was illustrated by a two-year pilot test conducted outside Roswell, New Mexico, where crops of introduced species were quickly displaced by invasive local species (Sheehan *et al*., 1998). In light of this finding, the present research began with isolating and culturing algae from urban streams and lakes in and around the Las Vegas Valley, the algal habitats that are heavily influenced by treated municipal wastewater.

Last of all, to accomplish a high oil yield, biomass density and lipid content of algae must be tuned together. They both are influenced by growth conditions, but not in the same direction. Under optimum growth conditions, growth rate is maxima, so is biomass density, i.e. the number of cells in a culture. However, such conditions may not support high lipid content, i.e. the quantity of lipids per cell, and in fact, could cause lipid content to decrease. Indeed, unfavorable and stressful growth conditions sometimes increase lipid content. Therefore, there is a "tradeoff" between growth rate and lipid content, the outcome of which determines total oil productivity. This study investigated both growth rates and lipid content simultaneously against variations in different growth parameters. It provides enough data points to develop a pattern that explains the

interactions between growth rate and lipid content across the major algal groups, i.e. green algae, cyanobacteria, and diatoms.

1.2. Current State of Knowledge

1.2.1. Wastewater as the Growth Medium

The idea of using wastewater for feeding algae farms is not new. In 1977, Benemann *et al.* suggested that algal biofuel plants could be combined with wastewater treatment facilities. Since then, different types of wastewater, including animal urine, dairy manure, winery wastewater, industrial wastewater, partially treated sewage, and raw municipal wastewater, have been tested in place of, or as supplements to, the growth medium. The current study chose to use sewage because the location of the research, i.e. Southern Nevada, does not have farms or wineries, or any other sources of wastewater. The following is a brief review of previous work on this topic, which discusses the effects of different wastewaters on growth rate, on biomass productivity, and on lipid contents of algae. Although the purpose of some studies was not biofuel (e.g. fish fed, human nutrient supplements, pharmaceutical, etc.), their findings are still useful for this industry.

Tsukahara and Sawayama (2005) grew *Botryococcus braunii* in a secondary treated sewage, on a laboratory scale, in a batch and a continuous bioreactor system. Results showed that, in both systems, this alga could remove nitrogen and phosphorus from wastewater and grow. After 9 days, in the batch system, the hydrocarbon content reached 53% of cell weight, a relatively high number compared to the usual amount, i.e. ~20%.

Kim *et al.* (2007) grew algae in a mineral medium and partially treated swine urine. The mineral medium amended with 3% fermented swine urine enhanced the growth rate of algae in a mixed culture of *Scendesmus acutus*, S. *spinosus*, and S. *quadricauda*. In such a synthetic medium, all the biomass dry weight, the algal growth rate, photosynthetic rate, total carotenoids, and fatty acids increased by 2.5, 3, 1.4, 3.1, and 0.2 times, respectively, compared to the mineral medium. The higher biomass and lipid contents suggest that adding urine to the growth medium could be an effective way to grow algae.

Shen *et al.* (2008) grew *Botryococcus braunii* in a batch system with various concentrations of livestock wastewater. The highest growth occurred in the half strength wastewater with the biomass concentration of 2.543 g/L and the lipid content of 20% by weight. After 14 days incubation, 88% of total nitrogen and 98% of total phosphorus of the wastewater was removed. They suggested that algal growth can be used for the purpose of both biofuel production and animal wastewater treatment.

Kong *et al.* (2010) grew *Chlamydomonas reinhardtii* in three types of municipal wastewater: raw, centrate, and treated. The least growth occurred in the culture that contained treated wastewater due to the low concentrations of nutrients. To find the best concentration of wastewater for growing algae, centrate was diluted to the three concentrations of 50%, 75%, and 100%, among which 100% centrate delivered the highest cell density, but showed about 4-5 days of lag phase. The culture containing raw wastewater supported growth, but it showed slight inhibition. It appeared that there was some inhibitor in the centrate and raw wastewater, which was removed by, or became tolerable by, the algae after a few days.

Wang *et al.* (2010) grew *Chlorella* sp. in different dilutions (10, 15, 20, and 25 times) of digested dairy manure. In the first 7 days, slower growth occurred in the least diluted manure samples significantly. After 21 days of incubation, the algae removed 100% of ammonium, 75.7-82.5% of total nitrogen, 62.5-74.7% of total phosphorus, and 27.4-38.4% of COD from the cultures. This showed that high concentration of ammonium, 81-178 mg/L, was not toxic or inhibitory to the algae.

In a separate study, Wang *et al.* (2010) used undigested dairy manure, diluted to 20 times, to grow *Chlorella vulgaris* in a semi-continuous system. The biomass increased more than twice in 4 days of the experiment, higher than the number they achieved with growing the algae in the digested dairy manure in the same period of incubation. Also, nutrient and COD removal efficiency increased compared to those obtained in the digested manure culture. This implies that the higher biological load, containing high amounts of organics, enhanced growth. In this case, the undigested manure had a higher BOD than the digested one. However, if the loading rate had gone beyond a certain level, the nutrient buildup could have been lethal to the algae.

Woertz *et al.* (2009) grew an algal community including green algae, cyanobacteria, and diatoms in municipal wastewater and diary manure, in two separate experiments. The municipal wastewater was the effluent of the primary clarifier and the dairy manure was the free-stall barn flush water which was already treated in an anaerobic digester before the experiment. The dairy manure experiment was conducted outdoors and in batch cultures, while the municipal wastewater experiment was conducted indoors in semi-continuous cultures with a 2-4 day hydraulic resident time.

Both cultures were supplemented with CO_2. Two media containing 25% and 10% dairy manure were tested, in which the culture with more concentrated manure (25%) showed better results. In the 25% manure culture, after 6 days, the lipid content reached its maximum, i.e. 14-29% with a productivity of 17 mg/day/L, and after 12 days, ammonium and phosphorus were removed almost completely. In the municipal wastewater culture, after 3 days, lipid productivity reached its maximum, i.e. 24 mg/day/L, and almost 100% of ammonium and phosphorus were removed.

Xin *et al.* (2010) grew 11 algal species in a medium that contained secondary treated municipal wastewater, among which *Scenedesmus* sp. grew best. After 10 days, about 98% of inorganic nutrients of the wastewater were removed and the lipid content of *Scenedesmus* sp. increased from 14% to 31%. The highest biomass density and lipid content of *Scenedesmus* sp. was measured as 0.11 g/L and 31-33%, respectively.

Yang *et al.* (2011) grew *Chlorella ellipsoidea* in three different secondary treated wastewaters: 1) activated sludge (AS) effluent, 2) anaerobic-anoxic-oxic (A^2O) effluent, and 3) oxidation ditch (OD) effluent. All three wastewaters supported the algal growth. While the amount of nitrogen and phosphorus was only 5% and 1% of the nitrogen and phosphorus in the mineral medium, the biomass concentration was only one order of magnitude lower than the density obtained in the mineral medium. In all three cultures, the highest lipid content was achieved when the cells entered the stationary phase, i.e. 35-40% compared to 10-15% during the growth phase.

Sydney *et al.* (2011) tested secondary treated sewage to grow twenty algal species. More than 80% of the species could grow in such water, among which *Chlorella vulgaris* and *Botryococcus braunii* were identified via DNA sequencing. Of the

wastewater-utilizing isolates, *B. braunii* showed the highest nitrogen and phosphorus removal (80% and 100%, respectively), the highest biomass density (1.88g/L), and the highest lipid quantity (36.14%), which makes this species a very promising candidate for biofuel production. *C. vuglaris* removed only 54% of nitrogen and 50% of phosphorus of the wastewater. The reason for this low efficiency was not discussed in the study.

Chinnasamy *et al.* (2010) grew a consortium of fifteen algal species in a medium composed of 85-90% carpet industry wastewater, supplemented with 10-15% municipal sewage, in open ponds with raceway design. The results showed that the algae grew in such water much better than in the mineral medium, even when the levels of nitrogen and phosphorus were lower than those in the mineral medium.

Mutanda *et al.* (2011) grew *Chlorella* spp. in the mineral medium BG11 amended with various amounts (100, 80, 60, 40, 20, 0%) of municipal wastewater. The highest growth rate and biomass density occurred where the culture contained 60% wastewater. However, the oil yield was almost constant in all the cultures. These researchers also measured the growth rate in a culture that contained post-chlorinated wastewater, serially diluted from 0.2 mg/L to undetected chlorine. The highest growth rate, biomass density, and lipid yield occurred in the least diluted culture with 0.2 mg/L chlorine. As chlorine was increased from 0 to 0.2 mg/L, the biomass density and the lipid yield increased by ~ 8 times. However, chlorine concentrations higher than 0.4mg/L were found to be inhibitory.

Feng *et al.* (2011) grew *Chlorella vulgaris* in artificial wastewater in a column aerated photobioreactor, under batch and semi-continuous conditions. High lipid content of 42%, with high productivity of 147 mg/L/d, was achieved. Also, the highest removal

of COD, ammonium, and total phosphorus recorded during the experiments were 89%, 97%, and 96%, respectively.

Zhou *et al.* (2011) isolated a variety of microalgae from wastewater and non-wastewater environments in Minnesota and compared their growth potential in sewage liquor centrate. The fastest-growing strains, mostly species of *Chlorella* and *Scenedesmus*, originated from wastewater habitats. They reported that on day 3 of the incubation, total organic carbon (TOC) concentration of the wastewater decreased significantly, by 82.27% to 96.18%, while the biomass increased from 0.48 to 1.08g/L, meaning the algae utilized the organic carbon of the wastewater. The algae showed lipid contents of 27.5% to 33.53%, which make them suitable candidates for biofuel.

In a similar study to this work, Bhatnagar *et al.* (2011) isolated a strain of *Chlorella minutissima* from sewage oxidation ponds in India. This isolate prefers a sewage formulation to the mineral medium BG11. Results showed that in the half-strength wastewater, i.e. the sewage diluted by half with tap water, *C. minutissima* produced more cells that exceeded the maximum growth achieved in mineral medium.

1.2.2. Algae Residue as a Source of Nutrients

Algae residue contain proteins composed of polypeptides, polymers of amino acids linked by strong bonds of peptides between the carboxyl and amine groups. Amino acids are composed of an amine group and a side-chain containing the key elements of carbon, hydrogen, oxygen, and nitrogen. However, whether (or not) algae are able to utilize such organic nitrogen is yet to be investigated. Prior studies in oceanography showed that some marine diatoms could utilize some amino acids as a nitrogen source. This study examined the capability of the studied green algae for assimilating amino

acids. Below is a brief review of the work that has been done on this topic in marine systems. Although the purpose of these studies was not biofuel, the results still can be applied to the subject of biofuel.

Liu and Hellebust (1974) used amino acids as a source of nitrogen to grow marine diatom *Cyclotella cryptica*. Cells grew on arginine and glutamine as well as on nitrate. Also, glutamate, proline, ornithine, and asparagine supported growth very well. But, isoleucine was not consumed. In the presence of nitrate, no inhibition occurred by any amino acid at the tested concentration of 0.1mM.

Admiraal *et al.* (1984) investigated the ability of marine diatoms *Navicula salinarum* and *Amphiprora paludosa* to assimilate nitrogen from a variety of sources, e.g. ammonia, nitrate and amino acids. Both cultures utilized amino acids in the presence or absence of inorganic nitrogen sources, i.e. ammonium and nitrate. The rate of consumption, however, was higher in nitrogen-deprived cultures that were supplemented with amino acids than in those containing both inorganic nitrogen and amino acids.

Nilsson and Sundback (1996) studied the uptake of dissolved amino acids in microalgal communities. They added a mixture of amino acids to sediment samples collected from a sandy bay on the west coast of Sweden, where benthic diatoms were dominated. About 1 to 44% of algal biomass could assimilate amino acids.

1.2.3. Organic Carbon as a Source of Energy

Acting as plant and bacteria, mixotrophic algae can consume simple organic carbon sources as an extra source of energy and grow faster. Similar to bacteria, in the absence of photosynthesis, heterotrophic algae can use organic carbon as the sole source

of energy and grow in the dark. Below is a short review of several studies that tested these characteristics in algae.

In 1958, Samejima and Myers tested the ability of algae to grow on organic carbon. They showed that *Chlorella pyrenoidosa* can grow on glucose, galactose, and acetate in the dark, i.e. heterotrophic growth. Additionally, they demonstrated that a number of different nitrogen sources (nitrate, ammonia, or urea) that the growth rate of *C. pyrenoidosa* was similar if there was glucose in the culture. These authors supplemented the mineral growth medium with various sugars and sugar-alcohols including arabinose, sucrose, lactose, xylose, ribose, glucose, mannose, galactose, fructose, maltose, cellobiose, glycerol, mannitol, *i*-inositol, in the darkness. Only glucose and galactose supported growth. Neither sugar phosphate (e.g. phosphoglycerate, glucose-1-phosphate, gluose-6-phosphate, fructose phosphate, and fructose-1:6-diphosphate) nor organic acids, e.g. acetic acid, supported growth. Samejima and Myers (1958) suggested that cell permeability restrictions in *Chlorella pyrenoidosa* were the reason for the lack of versatility in the use of organic compounds.

Goulding and Merrett (1967) tested various sources of organic carbon including alcohols, amino acids, organic acids and sugars, for growing *Pyrobotrys stellate* in the light and the dark. Only acetate, under light, supported the growth.

Khoja and Whitton (1971) reported the ability of 24 filamentous cyanobacteria to grow on 0.01M sucrose, heterotrophically. Out of 24 species, only 7 did not grow on this sugar when kept in darkness. Out of these 7 algae, two species were still alive after 3 months and started to grow when transferred under light. In all cases, the heterotrophic growth rate of an alga was lower than its mixotrophic growth rate. For instance,

Chlorogloea fritschii grew heterotrophically at a rate 0.2 times the mixotrophic growth rate.

Ingram *et al.* (1973) showed that *Nostoc* sp. can grow heterotrophically on glucose, fructose, or sucrose. When grown mixotrophically, *Nostoc* cells could also use glycerol and acetate as carbon sources. In the mixotrophic growth mode, when there was high light (300 ft-ca) and 1% CO_2, adding an organic source did not stimulate the growth. When *Nostoc* cells were grown under a low light level (80 ft-ca) and ambient air, the presence of organic carbon in the culture stimulated growth significantly. However, the biomass density obtained in such stimulated growth condition never exceeded the biomass density that was obtained under high light and 1% CO_2.

Cero´n Garci´a *et al.* (2000) grew diatom *Phaeodactylum tricornutum* on different initial concentrations of glycerol (0-0.1M) mixotrophically. Results revealed that the highest biomass density and biomass productivity (16.2 g/L and 61.5 mg/L/h, respectively) occurred in the culture that contained 0.1 M initial glycerol.

Wood *et al.* (1999) grew 8 different algal species on glycerol, glucose, and acetate. The results showed that, for some of the trial species, growth was stimulated in the presence of glycerol, although in some cases the stimulation was not significant. None were stimulated by acetate and only one species showed stimulation by glucose.

Bouarab *et al.* (2004) grew *Micractinium pusillum* in a mineral medium supplemented with glucose and acetate, under both light and dark concentration. The highest growth occurred in the culture that contained glucose, under light, followed by glucose supplemented culture under dark, and then acetate supplemented cultures under light and under dark, and finally in the mineral culture with no organic compound under

light at the rates of 0.94 d^{-1}, 0.79 d^{-1}, 0.58 d^{-1}, 0.52d^{-1}, and 0.38 d^{-1}, respectively. No growth occurred in cultures that contained no organics in the absence of light.

Xu *et al.* (2006) grew *Chlorella protothecoids* autotrophically and heterotrophically to produce high quality biodiesel. Under heterotrophic growth, they added glucose and corn powder hydrolysate (CPH) separately to a mineral medium. Results revealed that *C. protothecoids* could grow heterotrophically on both glucose and CPH much faster than in the autotrophic culture. Also, heterotrophic cells accumulated more lipids (55.2%) and carbohydrate (15.4%) than autotrophic cells (14.57% lipids and 10.62% carbohydrate). Moreover, *C. protothecoids* showed slightly higher growth rates on CPH than on glucose. This can reduce the final cost due to the low price of CPH. After 144h of incubation, the cell density reached 3.92 g/L in the culture that contained CPH compared to 3.74 g/L in the culture with glucose. Also, the lipid content of *C. protothecoids* was similar in the presence of both CPH (54.7%) and glucose (55.3%).

Xiong *et al.* (2008) grew *Chlorella protothecoides* heterotrophically on glucose. Results showed that the biomass density increased as the glucose concentration was increased from 15 to 60 g/L, above which glucose was inhibitory.

Sun *et al.* (2008) tested various sugars including glucose, mannose, fructose, sucrose, galactose, and lactose to grow *Chlorella zofinginesis* heterotrophically. Of these sugars, glucose and mannose showed the best results: the highest specific growth rate (0.28 h^{-1}) and the highest dry biomass (10.6 gL^{-1}). The next best sugar was fructose, which showed a high specific growth rate and the dry biomass (0.027 h^{-1} and 9.44 gL^{-1}, respectively). Sucrose and galactose resulted in very low numbers, 0.018 h^{-1} and 5.46 gL^{-1} for specific growth rate and dry biomass productivity, respectively. Lactose

was the poorest carbon source with a very low specific growth rate and dry biomass, 87% lower than those obtained in the glucose-supplemented culture.

Liang *et al.* (2009) showed that *Chlorella vulgaris* reached their highest biomass density when they were grown in a medium supplemented with 1% (w/v) glucose, under light. Also, the highest amount of carbohydrate, lipids, and protein was achieved in cultures containing 1% glucose, 2% glycerol, and 1% glycerol, respectively.

Xiong *et al.* (2010) used a photosynthesis-fermentation approach (PFM) to grow *Chlorella protothecoides*. First, cells were grown under illumination without any organic source for 120h in the photosynthetic mode (PM), and then the photosynthetic grown cells were entered into a fermenter to grow on organics heterotrophically for another 120 h (FM). In the PFM, the lipid yield was 69% higher than the yield obtained in the FM, which already was higher than the yield obtained in the PM. When photosynthetic grown cells were transferred from autotrophic cultures (PM) into the heterotrophic cultures (FM), the color started to change from green to yellowish, meaning that the cells were losing their chlorophyll. The cytoplasm was full of big oil droplets, over 1 μM in diameter. In the PFM, sugars were consumed completely after 48h, i.e. the cells adapted to glucose by sequential transfer to a glucose-enriched medium until chlorophyll was no longer detectable; the "glucose-adapted" cells left about 2g/L of glucose unused when they were grown heterotrophically. More importantly, in the PFM, the consumed sugars were converted to lipids, resulting in a 69.32% higher lipid yield than in the FM. In both stages of photosynthesis and fermentation, CO_2 was fixed, and this "double fixation" increased the amount of carbon converted from sugar to oil.

Kamjunke *et al.* (2008) showed that *Chlamydomonas acidophila* can grow on glucose mixotrophically.

O´Grady and Morgan (2011) grew *Chlorella protothecoides*, in the cultures that contained glycerol as the sole carbon source and in a 9:1 glucose:glycerol combination, mixotrophically. The growth rate in the culture with only glycerol was 0.105 d^{-1}, while this number was 0.104 d^{-1} in the culture containing glucose-glycerol. The biomass yield was also higher in the sole glycerol culture (0.56 g biomass/g substrate) than that in the mixed culture (0.45 g biomass/g substrate). But, the lipid productivity in the sole glycerol culture was lower (0.079 g/L/d) than it was in the mixed glucose-glycerol culture (0.104 g/l/d).

Heredia-Arroyo *et al.* (2010) showed the effect of different carbon sources on the biomass concentration and lipid content of *Chlorella protothecoides*, purchased from UTEX. Glucose, glycerol, and acetate were added to the mineral medium as sole or mixed carbon sources, among which the culture that contained only glucose showed the highest cell density. The biomass concentration increased, though not significantly, as the initial glucose concentration was increased. But the lipid content did not change with the glucose concentration. Growth occurred in the presence of glycerol and acetate as well, but the cell density in those cultures was lower than that in the culture with glucose. Also, combinations of glucose-glycerol and glucose-acetate did not increase the biomass density or lipid contents.

In a separate study, Heredia-Arroyo *et al.* (2011) added different concentrations of glucose, glycerol, and acetate to a culture of *Chlorella vulgaris*, purchased from UTEX, and measured the biomass and lipid content. The biomass and fatty acids

production increased as the glucose concentration was increased. Yet, the specific growth rate remained constant. Unlike glucose, glycerol inhibited the growth. It only stimulated the growth when it was combined with glucose. The highest biomass and lipid contents were achieved in the culture containing 80:20% glucose:glycerol. At this ratio, the biomass productivity was 4 times the biomass productivity in the culture with only glycerol as the carbon source. The same result was reported for the glucose-acetate combination. The highest biomass and lipid content were obtained at 80:20% of glucose:acetate.

Liu *et al.* (2010) compared the growth characteristics of *Chlorella zofingiensis*, purchased from the American Type Culture Collection (ATCC), under photoautotrophic and heterotrophic modes. Photoautotrophic cells grew very slowly (0.233 d^{-1}) compared to the heterotrophic growth (0.769 d^{-1}), in the presence of 30g/L glucose. The photoautotrophic biomass density (1.9 g/L) was also lower than the heterotrophic one (9.7 g/L). The photoautotrophic grown cells of *C. zofingiensis* accumulated less lipids (25.8%) than the heterotrophic grown cells did (51.1%). The ratio of neutral lipids to the total lipids in the autotrophic grown cells was 29.4% compared to 80.9% in the heterotrophic grown cells. Overall, Liu et al. showed that *C. zofingiensis* grew faster, produced more biomass, and accumulated more neutral lipids if they were grown heterotrophically on glucose than when they were grown autotrophically.

Wan *et al.* (2011) compared the mixotrophic and autotrophic growth of three algae *Nannochloropsis oculata*, *Dunaliella saline*, and *Chlorella sorokiniana* on glucose. The growth of all three was stimulated on glucose and their biomass productivity was 1.4 to 4.2 times the biomass productivity obtained in the autotrophic growth. In all three

algae, the protein and lipid content increased with the glucose concentration. However, at glucose concentrations higher than a certain level, both lipid and protein contents declined. This critical level for *Chlorella sorokiniana*, a similar species to the one used in this dissertation study, was 25 g/L.

1.2.4. Lipid Content Variation

The amount of lipids varies in response to environmental and growth conditions. Proper understanding of the cause of such variations guides biofuel plants to adopt effective and efficient ways to increase oil yield. Earlier studies suggested that when nutrients are deficient, or growth conditions are stressful, lipid content increases. Almost all of these findings are qualitative. Moreover, there is no consistent trend for the variations of lipid content. The findings also differed from one alga to another, with no regular pattern even within the same group. As such, it is hard to speculate the lipid content response of the target algae to the fluctuations of environmental conditions. Through quantitative studies, the current work sought to develop a universal pattern that explains the fluctuations in lipid content of all types of algae in response to various environmental conditions. Below is a summary of previous research on this topic.

Merzlyak *et al.* (2007) showed that the fatty acid content of green alga *Hamematococcus pluvialis* increased when it was grown in a nitrogen free medium.

Zhekisheva *et al.* (2002) also showed that the fatty acid content of green alga *Hamematococcus pluvialis* increased when it was grown in a nitrogen free medium. In a separate experiment, these researchers grew the alga in BG11, under high light (350 $\mu mole/m^2.s$). After two days incubation, neutral lipid content increased by 80 times. Results revealed that both nitrogen deficiency and high light increased the lipid content.

Pruvost *et al.* (2009) showed that nitrogen deficiency decreased the growth rate and increased the total lipids content in *Neochloris oleoabundan.* Total lipid productivity was the highest when cells were grown in medium containing sufficient amount of nitrogen.

In separate studies James *et al.* (2011), Chen *et al.* (2010), and Rodolfi *et al.* (2009) showed that when there was no detectable or insufficient amounts of nitrogen in their cultures, *Chlamydomonas reinhardti, Dunaliella teriolecta, Chlorella sp., Scenedesmus sp., Tetraselmis suecica,* and *Nannochloropsis* accumulated lipids.

The same observation was reported for phosphorus deficiency. Khozin-Goldberg *et al.* (2006) showed that the lipid content of *Monodus subterraneus* increased when it was grown in a low phosphate medium. Also, in a phosphate free culture, triglycerides content increased 6 times, though the growth rate was low in the free/low phosphate cultures.

Tornabene *et al.* (1983) showed that in a nitrogen deficient culture, cells of *Neochloris oleoabundans* accumulated 35-45% (DW) lipid content, 80% of which was triglycerides.

Feng *et al.* (2011), however, reported a different observation. They showed an increase in lipid content of *Isochyrysis zhangjiangensis* when they were grown in an extremely high nitrogen medium, about 100 times the nitrogen level in a normal medium. The authors considered this level of nitrogen as a stressful condition for algae.

Chen *et al.* (2010) also observed different findings. They showed that in a P-limited culture, the lipid content of *Dunaliella teriolecta* did not increase or decrease. These authors hypothesized that intracellular phosphate storage provided the needed

phosphorus for the cells. In fact, cells were not under phosphorus deficiency conditions at all, so cell divisions occurred along with photosynthesis, resulting in no change in lipid content.

1.3. Research Significance

1) As mentioned by Sheehan *et al.* (1998), southwestern regions of the United States are ideal locations to establish the algal biofuel plants. Yet, no study has been conducted to investigate the feasibility of producing such biofuel in Southern Nevada. While some general information can be used from data and analyses obtained from prior studies, future algal farms cannot be developed based on previous literature alone. Each region possesses a unique climate and geological characteristics (such as sunlight intensity, water chemistry, temperature, land, etc.) that affect the biology and ecology of that region. For instance, species from Alaska would require different growth parameters than species from Arizona. Hence, the current study provided the information necessary for producing biodiesel feedstock in Southern Nevada.
2) According to a two-year pilot test conducted by DOE outside Roswell, New Mexico, only indigenous algae are adequate for cultivation as the feedstock for biofuel. This study conducts all experiments with the species isolated from the lakes and channels in and around Las Vegas. These isolates have never been studied before in the context of this research. Since, even under similar environmental and growth conditions, algal species could grow differently, results of the experiments with other species cannot be utilized for the isolates of this study.

3) Earlier studies showed that wastewater can support algal growth. However, not all types of wastewater are suitable for growing any and all varieties of algae. For example, swine urine might be able to support only a few particular species. Hence, it is essential to know what species can grow in the target wastewater. Even from one single source of wastewater, various strengths of such water impact the growth dissimilarly, ranging from inhibiting to stimulating the growth. For instance, fully-treated sewage, i.e. the weakest strength, might not support growth at all, while a mixture of raw-treated sewage (1:1 v/v), i.e. half strength, could be superior to the mineral medium, reaching a growth rate higher than the rate accomplished in the mineral medium. Therefore, this study conducted several experiments with various strengths of centrate to find the best concentration supporting the most growth. Moreover, besides the nutritious components, wastewater also contains inhibitors and toxins, e.g. heavy metals. The presence of such substances could hurt the growth of algae. This study investigated all aspects of growing algae in the sewage of Las Vegas, including potential nutritional and inhibiting sources.

4) This study proposes algal residue as a source of nutrients. However, the nutritional components of this waste are in the form of polypeptides and polysaccharides. Algae cannot utilize polymers and large molecules. Hence, biomass must be decomposed first. This study attempted to use biological degradation, i.e. composting, to break down the strong bonds of peptides in order to free nutrients. The feasibility of growing the isolates in the compost

tea, i.e. product of such biotic degradation, must be examined. In addition to the biological approach, chemical degradation is an alternative method of breaking the big molecules and polymers of biomass into monomers, e.g. amino acids. Nonetheless, the nature of the librated nutrients are organic, and although algae prefer inorganic nutrients, such as nitrate, some marine diatoms were able to consume amino acids as a source of nitrogen. Hence, this study investigated the ability of the isolates to assimilate amino acids as the sole source of nitrogen.

5) This author suggests that growing eligible algae in the presence of a proper source of organic carbon, existing in different wastes, can enhance biomass productivity due to night-time heterotrophic growth and day-time mixotrophic growth. Previous studies showed that mixotrophic and heterotrophic algae are able to consume simple organic carbon as a source of energy. Yet, which algae could consume organic carbon sources has not been accurately identified; at times contradicting data have been reported. For instance, Liang et al. showed that *Chlorella vulgaris* consumed glycerol, while Heredia-Arroyo et al. showed that the same alga, *Chlorella vulgaris*, could not consume glycerol, with contradictory results for the same species and the same organic compound. Due to such confusion, and because there are no guidelines in the selection of organic carbon sources by different algae, this study investigates the capability of its isolates for utilizing selected organic carbon sources, under light and in darkness.

6) In order to maximize the total oil yield, it is critical to know how the lipid content of various algae correlate with growth rate under different environmental and growth conditions. Though, there were some uncertainties, earlier findings showed that unfavorable and stressful growth conditions, such as nutrient-deficient conditions, increased the lipid content of algae. First, the findings were qualitative, not quantitative. For instance, lipid content was reported "high" under "low" nitrate concentration with no quantification. Second, total oil quantity is directly related to both growth rate and lipid content, while the prior data reported only lipid content variations. This dissertation studies quantitatively both lipid content and growth rate against gradients of growth parameters. In prior studies, there was not a clear pattern available for the variations in lipid content under different environmental conditions. The earlier findings were not consistent, not even within the same algal group and under the same condition. Hence, based on previous data, it was difficult to predict both the lipid content and growth rate of a given alga under various environmental conditions. This dissertation renders a universal model, valid for all types of algae, which describes the relationship between oil content and growth rate for various growth variables.

1.4. General Problem Statement

Algal biofuel is scientifically sound, but it is yet to become a reality. It is not sustainable, as it relies on finite, nonrenewable resources of fertilizers and clean water for feedstock production. Moreover, it is not economically feasible, for it is too expensive to compete with petroleum. More than 30% of the estimated price of this biofuel is

associated with fertilizers and clean water. Hence, the needs for nutrients and water have to be satisfied from affordable, renewable resources, not from fertilizers and clean water.

1.5. Research Objectives

The main objectives of this study are as follows:

1) Growing the studied algae in municipal wastewater
2) Growing the studied algae in the medium composed of recycled nutrients from waste algal biomass.
3) Developing a universal pattern that explains the interactions between lipid content and growth rate, in response to various environmental conditions, and for all types of algae

The first two objectives aim to reduce the need for freshwater and fertilizers, along with increasing the biomass productivity. The third objective intends to find the best condition under which oil productivity is the highest, controlled by both lipid content and growth rate.

1.5.1. Sub-objectives

To accomplish the above-mentioned objectives, the sub-objectives are as follows:

1) To collect samples from the waters influenced by wastewater in the Las Vegas Valley, where the experiments are being held
2) To isolate diverse algal species from major groups, i.e. green algae, cyanobacteria, and diatoms
3) To optimize the growth of isolates with respect to temperature, light, major nutrients (nitrate and phosphate), and salinity

4) To determine the ability of isolates to grow in municipal wastewater and to find the best concentration of wastewater to accomplish the highest growth

5) To determine the ability of isolates to grow on amino acids as the only source of nitrogen

6) To grow isolates in extracts from the algal composts as the growth medium

7) To determine the ability of isolates to grow on simple organic compounds such as glucose, lactate, acetate, and glycerol in the dark and in the light

8) To investigate the cause of variations in oil content of algae by studying the effects of nutrient concentrations (N and P) and light intensity on lipid content and growth rate simultaneously

9) To develop a uniform relationship, valid across all algal groups, between growth rate and lipid content.

1.6. Chapter Breakdown

Chapter 1– Overview. This chapter includes general introduction, problem statement, objectives, background, and literary review.

Chapter 2– Isolation, culturing, and growth optimization. In this chapter, characteristics of the studied algae are described. In addition, this chapter demonstrates the optimum temperature, light and nutrient requirements, and the salinity tolerance threshold for all the isolates.

Chapter 3– Wastewater as the growth medium. This chapter discusses negative and positive effects of wastewater (as a source of water, inorganic nutrients, and organic carbon) on algal growth. It demonstrates the growth of the isolates in centrate diluted with the Wash water at serial dilution rates. It displays the optimum strength of centrate,

at which the algae grew most. Moreover, in order to take advantage of the organic carbon present in the wastewater, this chapter seeks the organic compounds consumable by the studied algae under light and in darkness.

Chapter 4– Recycling algal biomass residue as a nutrient source. This chapter investigates the feasibility of using algal biomass residue as the source of nutrients, nitrogen and phosphorus. It shows whether (or not) the studied algae are able to utilize the organic nitrogen of amino acids in absence of nitrate. This chapter also explains the results of biotic degradation, i.e. composting, of the algal biomass, to break down the polymers and free the nutrients. It presents the result of growing the studied isolates in compost tea, a product of composting. It introduces the challenges and difficulties of growing the algae in such medium, and the way this study addresses these.

Chapter 5– Lipid content of algae, variable of growth rate, and photosynthesis efficiency. This chapter displays a pattern that shows the relationship between lipid content and growth rate, across the major algal groups in response to varied environmental conditions.

Chapter 6– Conclusions and future recommendations. This chapter summarizes the findings and recommendations of this study.

Appendix A– Diatom cell walls as a source of silica. This chapter demonstrates that silica existing in the dead cell walls of diatoms can be recovered and re-used in their growth medium.

Appendix B– Amino acids uptake by green algae. This chapter discusses about the potential mechanisms that green algae use to consume amino acids. In this chapter,

three different mechanisms are proposed for three different green algae; *C. sorokiniana*, *Scenedesmus* sp., and *E. gracilis*.

Appendix C– Raw data. This chapter displays all the data used in this dissertation.

References

CHAPTER 2

ISOLATION, CULTUR, AND GROWTH OPTIMIZATION

2.1. Introduction

Isolation and culturing of microalgae is a laborious, time-consuming process. To save time and costs, most previous studies took advantage of existing culture collections, where a large number of well described species, representing major algal taxa, are available for a relatively low cost. These existing resources permitted studies where the source of the algae is not critical. For instance, they have been used to improve lipid measurement methods, test various methods of lipid extraction (Murray and Thomson, 1977; Matsumoto *et al.*, 2009; Wang *et al.*, 2009), study how flue gas might be used to stimulate growth (Sheehan *et al*., 1998), and how nutrient starvation and light stress might be employed to raise the lipid content of algae (Liu *et al*., 2008; Imamoglu *et al*., 2009; Mandal and Mallick, 2009; Pruvost *et al*., 2009; Dragone *et al*., 2011).

Recently, it has been recognized that there is a need to go beyond the existing culture collections and initiate new isolation efforts targeting algae that live in specific environments at specific production sites. This recognition in part came as a result of a field pilot test conducted near Roswell, New Mexico, as part of the Aquatic Species Program. In that test, algae isolated from the southern U.S., e.g. Alabama, were grown in an open pond cultivation system with domestic wastewater as a source of nutrients. Within a couple weeks, local algae invaded the ponds and displaced the inoculated algae (Sheehan *et al*., 1998).

Several recent studies suggest that when isolating and culturing for fuel-producing algae, one should take into consideration not only the production site where the isolated algae are to be mass cultivated, but also what kinds of water and nutrients that are to be used to grow them. If municipal wastewater is to be used as a nutrient medium, then algae that live in environments where they are exposed to wastewater should be targeted for isolation. Indeed, Bhatnagar *et al.* (2009) showed that *Chlorella minutissima* isolated from sewage oxidation ponds thrived in domestic wastewater, growing faster than in standard mineral nutrient medium. Kong *et al.* (2010) found that such wastewater loving algae are not limited to oxidation ponds, but also present in streams and rivers into which wastewater is released after treatment.

In light of these recent insights, the present isolation effort targeted algae that grow in urban streams and lakes in the Las Vegas Valley, including the Las Vegas Wash (the Wash hereafter) where 85% of the water is discharge from local sewage treatment facilities. Although other streams do not receive treated wastewater, the presence of some amount of feces and urine from wild and domesticated animals is expected. It is hypothesized that the algae that live in these urban waters are not only well adapted to local water chemistry and local climate, they may also be able to thrive in an optimized, sewage-based medium.

This chapter has three specific objectives: 1) to isolate and culture local microalgae and purify the isolates to the axenic state, i.e. one alga in one test tube and free of any bacteria; 2) determine the taxonomic identity of the isolates by means of cell morphology and DNA-based molecular methods; and 3) define the optimum growth

condition of each isolate in terms of temperature, light level, salinity tolerance, and major nutrient requirement.

2.2. Methodology

2.2.1. Sites Description

Chlorella sorokiniana and *Limnothrix* sp. were isolated from the Wash in June 2009. The green alga *Scendesmus* sp. was isolated from the man-made Lake Las Vegas. In March 2010, when it was sampled, an algal bloom dominated by the golden alga *Prymnesium parvum* had broken out, indicating a trend towards eutrophication. The cyanobacterium *Synechocystis* sp. and the diatom *Encyonema caespitosum* were isolated from the Flamingo Wash and the Pittman Wash in June 2009. Flows in these urban streams are small and shallow but year-round, and have formed from urban runoff and storm water. They both ultimately drain into the Wash. The diatom *Nitzschia thermalis* was isolated from the Echo Bay Marina, Lake Mead in June 2009. The marina is located at the northern end of Lake Mead, on Overton Basin. Echo Bay Marina is located in a part of the reservoir where the water is primarily from the Colorado River and away from the influence of the Wash. Figure 2.1 shows the sampling sites.

2.2.2. Isolation

Samples were collected in sterile plastic bottles and sent back to the laboratory. In the laboratory, the samples were centrifuged and concentrated to increase the biomass density. Concentrated samples were transferred into petri dish plates containing agar-BG11 (the mineral medium) for enrichment and placed under light. After about one week, dominant algal colonies were selected under the microscope, transferred, and streaked by loop, as is shown in Figure 2.2, onto fresh agar-BG11 plates to grow.

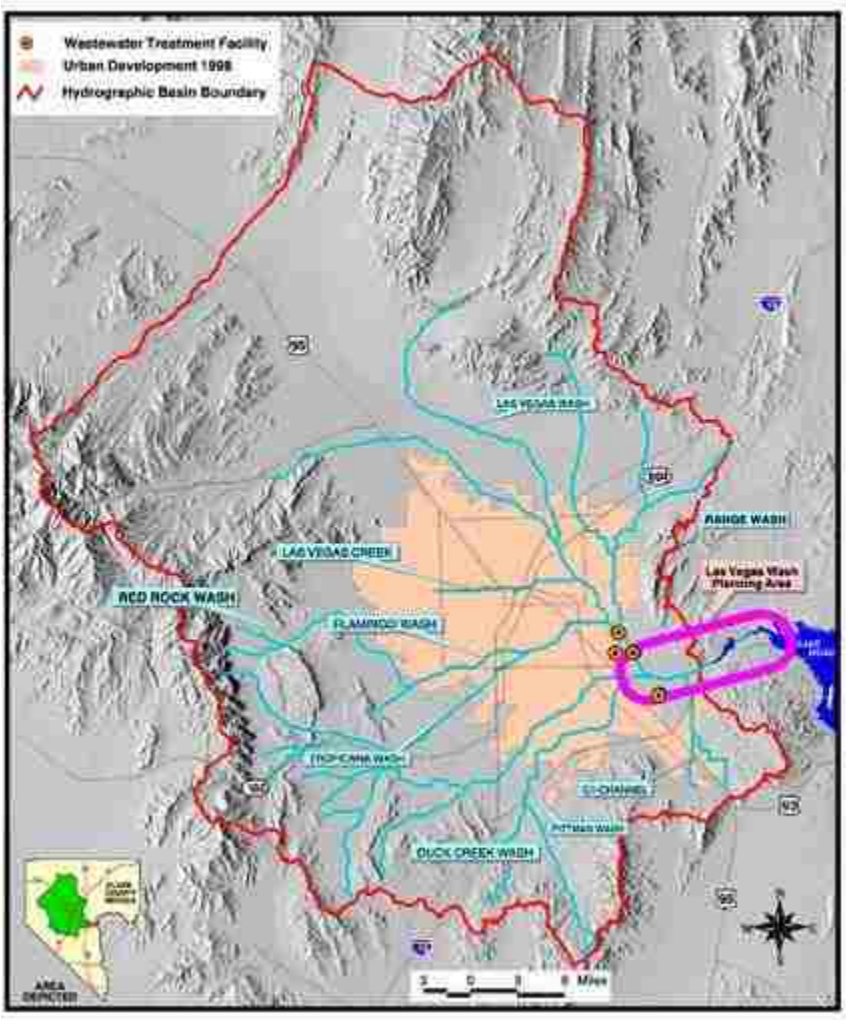

Figure 2.1. Las Vegas Valley hydrographic basin. Yellow circles indicate the locations of wastewater treatment facilities.

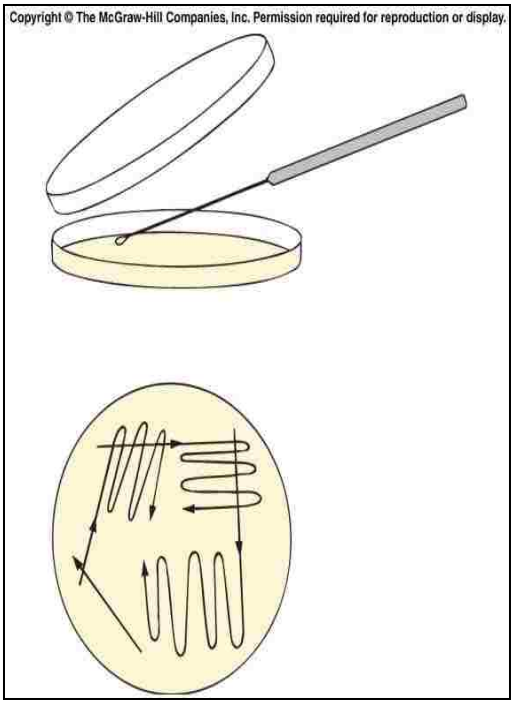

Figure 2.2. Inoculation and streaking with loop on a petri dish plate containing agar-BG11 medium.

The ultimate goal was to obtain axenic cultures. To accomplish this goal, serial dilution plating was used. The serial dilution protocol had three steps: counting the cells, diluting the cultures, and plating the diluted cultures. To count the cells after they grew, one colony was picked up from a plate, placed under a hood, and suspended in 1mL sterile Wash water. Afterwards, 10µl of Gentian violet, a triarylmethane dye, was added to 20µl of the sample, to make the cells visible, and 170µl of nano-pure water were added to bring the volume to 200 µl. One drop of the suspension was transferred onto a hemocytometer composed of several counting chambers (Figure 2.3). Under the microscope, stained microorganisms of about 20 different chambers were counted, and averaged. This procedure was repeated three times. The calculated average number was multiplied by the reciprocal volume of the chamber (1/4000 mm^3) and by 10 (because the

sample was 10 times diluted at first). The obtained number was reversed, resulting in total number of cells per mL. For instance, if the average number is 70, the final number of cells per mL would be $[1/ (74 * ¼ *10^{-6})] *10 = 5.4 * 10^5$ cells/mL.

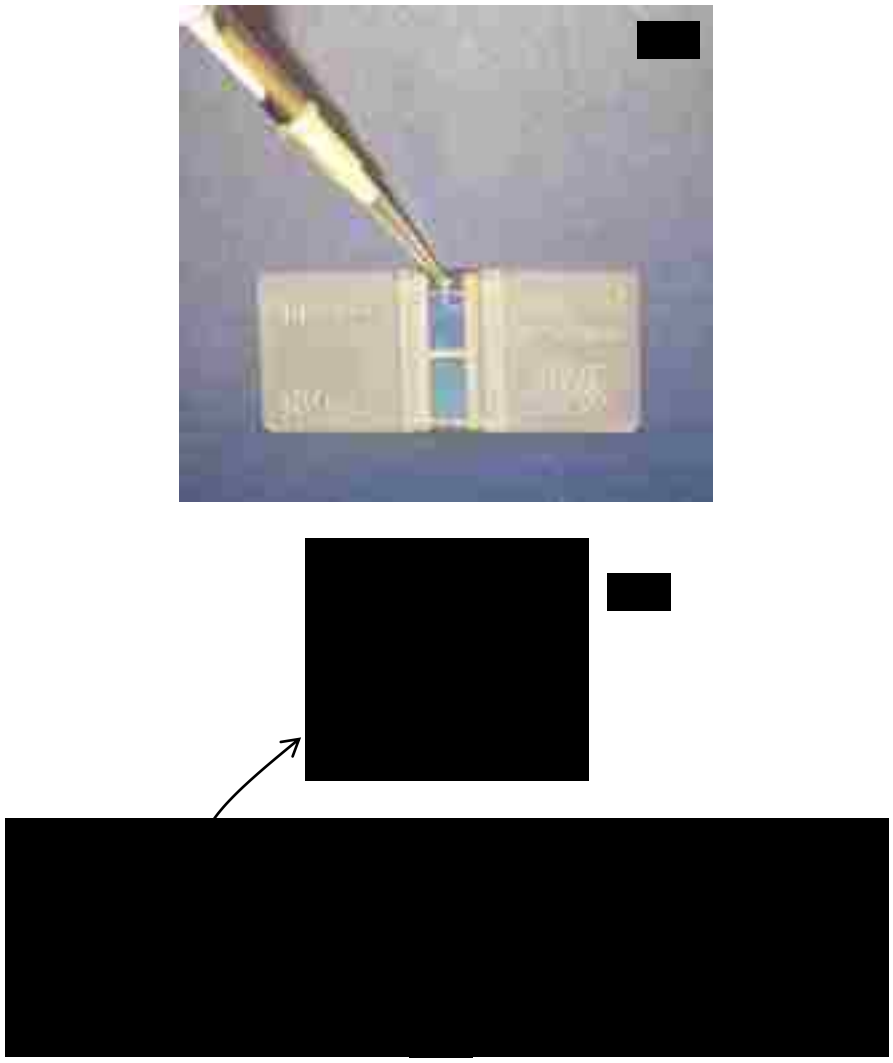

Figure 2.3. Hemocytometer: A) Transferring a small amount of culture with a pipette aid. B) Components C) Counting chambers.

After the number of cells in one milliliter was estimated, serial dilutions was performed; 1 mL of the original culture was diluted consecutively into serial sterile tubes, each of which contained 9 mL sterile Wash water and reached 10 mL after 1 mL of the culture was added, until the number of cells came to less than 10 per mL in a tube. For instance, if the culture contained 5.4 * 10^5 cells/mL, after 5 dilutions, the last tube contained ~ 5 cells (Figure 2.4). At the end, the diluted cultures were plated. From each tube, 100µL of the culture was transferred onto an agar-BG11 plate and was spread with a sterile triangle spreader to cover the entire area of the plate (Figure 2.4). In order to sterilize and prevent contamination, the triangle spreader was dipped in an ethanol solution, sterilized by a flame, and then cooled before spreading the cultures on each plate.

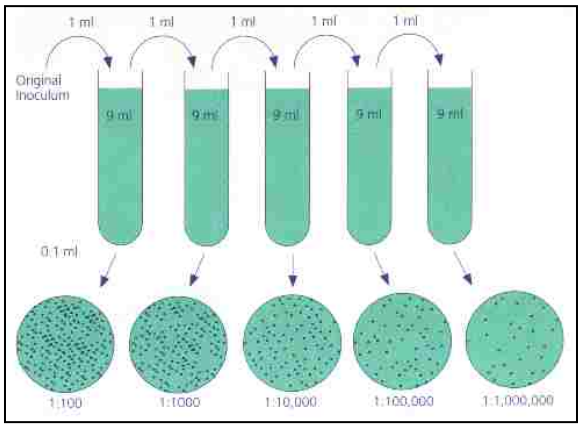

Figure 2.4. Serial dilution and plating.

After about one week, various colonies of microorganisms grew on the plates, among which one type was the target. The target colony was picked up under microscope and the whole of the serial dilution procedure, described above in three steps,

was repeated until only one type of algal colony and no other microorganism was found on the plates. This procedure took 6 months to yield axenic cultures. In some cases, novel modifications were added to the original procedure. For example, to isolate *N. thermalis*, a motile diatom, the whole plate was covered with a piece of paper and only one small spot was exposed to light. The diatoms moved toward that spot to receive light while other microorganisms remained in place, making it easier to isolate a pure diatom colony. Another example was the use of a particular antibiotic that could kill the bacterial cells but not algal cells. By adding a low dosage of this antibiotic to the surface of the medium in the plates, part of bacteria was killed.

2.2.3. Growth Media

C. sorokiniana, *Limnothrix, Scendesmus* sp., and *Synechocystis* sp. were grown in BG11 and diatoms *E. caespitosum* and *N. thermalis* were grown in Diatom Media (Table 2.1). Both media are pH 7. Plates were incubated at 25°C and under the illumination of 50 $\mu mole \cdot m^{-2} \cdot s^{-1}$ (Figure 2.5). The isolated strain was transferred from the plate into a liquid medium, the same medium that the alga was grown in, and incubated under the same conditions of temperature and light, with continuous shaking (Figure 2.6). Liquid cultures served as the source of inoculums for the designed experiments.

2.2.4. Taxonomic Identification

The isolated strains were identified through two methods: microscopy and molecular biology. Morphological features were determined by light microcopy with an Axioscop2 Plus microscope. Also, the genomes of isolates were identified based on their DNA.

Table 2.1. Constituents of mineral growth media BG11, used for green algae and cyanobacteria, and DM, used for diatoms.

BG11		**DM (Diatom Medium)**	
Component	Final Concentration, µM	Component	Final Concentration, µM
$NaNO_3$	17,600	$Ca(NO_3)_2.4H_2O$	85
K_2HPO_4	220	KH_2PO_4	91
$MgSO_4.7H_2O$	30	$MgSO_4.7H_2O$	101
$CaCl_2.2H_2O$	200	$NaHCO_3$	221
Citric Acid.H_2O	30	EDTAFeNa	3.065
Ammonium Ferric Citrate	20	$EDTANa_2$	3.022
$Na_2EDTA.2H_2O$	2	H_3BO_3	13.4
Na_2CO_3	180	$MnCl_2.4H_2O$	2.34
H_3BO_3	46	$(NH_4)_6Mo_7O_{24}.4H_2O$	1.68
$MnCl_2.4H_2O$	9	Cyanocobalamin	$9.84*10^{-3}$
$ZnSO_4.7H_2O$	0.77	Thiamine HCl	0.039
$Na_2MoO_4.2H_2O$	1.6	Biotin	0.054
$CuSO_4.5H_2O$	0.3	$NaSiO_3.9H_2O$	218
$Co(NO_3)_2.6H_2O$	0.17		

Figure 2.5. Cultures on plates, under light.

Figure 2.6. Liquid cultures on the shaker under light.

Such identification, of which Dr. Gaosen Zhang at the Desert Research Institute took the lead, were conducted based on phylogenetic analysis of the nucleotide sequence of the 18S ribosomal RNA gene. Genomic DNA was extracted by the use of an UltraClean soil DNA isolation kit (MoBio). The 18S gene was amplified by way of polymerase chain reaction (PCR) using EukA and EukB primers (Medlin *et al*., 1988). PCR product was purified by the use of an UltraClean GelSpin DNA purification kit (MoBio). Nucleotide sequence was determined by Functional Biosciences, Inc. (Madison, WI). Sequences of closely related species were identified by a BLAST search of the GenBank database (Altschul *et al*., 1990). Sequences were aligned using CLUSTAL W 2.0 software (Larkin

et al., 2007). Phylogenetic trees were generated using neighbor-joining methods available in MEGA 3.1 software and based on 1,000 bootstrap re-samplings (Felsenstein, 1985; Saitou and Nei, 1987; Kumar *et al*., 2004).

2.2.5. Growth Rate Determination

In order to achieve the optima for growth parameters, a growth rate specific to each parameter was calculated. A series of media, containing various amounts of a target parameters, were prepared. Each culture, representing only one variable, grew at a particular rate. The highest growth rate determined the optimum amount of that particular parameter. Growth rate, *k*, expressed as number of doublings per day, was calculated according to the following Sorokin and Krauss (1958) equation (Eq. 2.1):

$$K = (\log_2(N_2 / N_1)) / t \qquad \text{(Eq. 2.1)}$$

Where N_2 and N_1 are biomass density at the beginning and end of a time interval (t) in the logarithmic growth phase. The biomass density was measured either by optical density at 600 nm (OD_{600}), with Spectronic $20D^+$ spectrophotometer, or by protein concentration, quantified by the use of the Folin's phenol reagent (Lowry at al., 1951). Briefly, 1mL of Lowry stock solution, made of a 49 mL solution of 2% Na_2CO_3 in 0.1M NaOH, 0.5 mL of 1% $CuSO_4$ in dH_2O, and 0.5 mL of 2% sodium potassium tartrate, was added to cell pellet. The tubes were left for 30 minutes in a hot water bath. After that, 100 µL of Folin's reagent (1N) was added to each tube, and they were incubated for another 30 minutes at room temperature. Absorbance was read in PharmaSpec UV-1700 SHIMADZU spectrophotometer at the wavelength of 595nm. The protein concentration was quantified by applying a standard calibration curve, using Proteose Peptone No.3 (DIFCO).

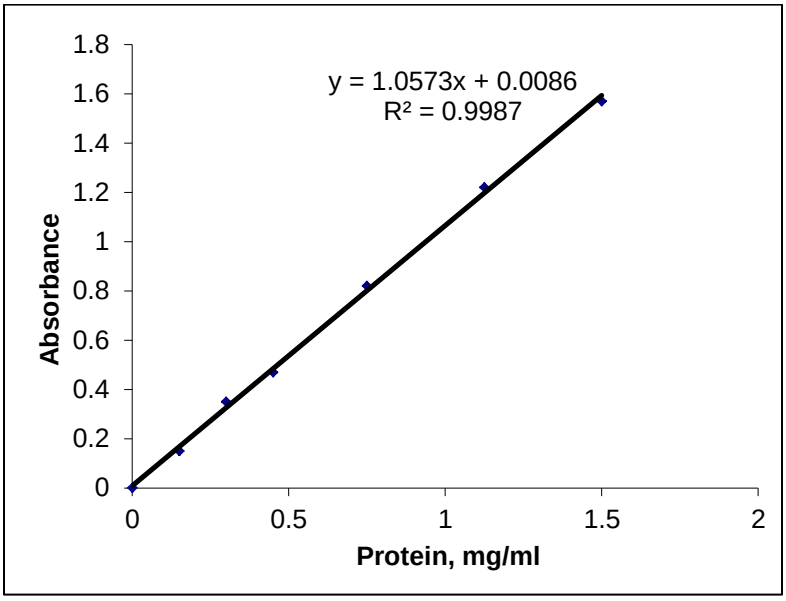

Figure 2.7. Standard calibration curve for protein content assay.

2.2.6. Lipid Content Measurement (gravimetric method)

The gravimetric procedure of Bligh and Dyer (1959) was applied. Results were used for the discussion. Cells from 100 ml of late-log-phase culture were collected, dried at 60ºC, and weighed. Pellet was placed in a solvent containing 2.5 ml of methanol, 2.5 ml of chloroform, and 1.25 ml of deionized (D.I.) water, and agitated vigorously for two minutes. After centrifugation, the lipid phase was collected. The algae residues were extracted two more times, and the three extracts were combined, dried, and weighed.

2.2.7. Determination of Temperature Optima

This measurement used a thermal gradient table, a 50cm by 50cm by 2cm aluminum plate that was heated at one end, and cooled at the other with circulating water from two thermostatically-controlled, high-precision (±0.1°C) water baths (Halldal and French, 1958). The table sat atop a shaker to provide mixing (Figure 2.8). After being

inoculated with the test alga, six 50mL Erlenmeyer flasks containing 15ml of liquid medium were placed on the table along the temperature gradient. A bank of fluorescent light tubes overhead (4 tubes, PAR 38) supplied illumination, each of which provided 1,250 lumens. Thermocouples in a row of water-filled flasks were used to maintain culture temperatures.

2.2.8. Determination of Light Optima

Test tubes containing algal culture were wrapped in layers of wire mesh to create different illumination levels. The source of illumination was provided from the side by 4 incandescent light bulbs, each of which provided 1,260 lumens and 100W, with a 10L/14D cycle.

To prevent the heating of cultures, the test tubes were placed in a small water bath at the optimum temperature of the species. Before the experiments started, the light intensity that each tube could receive underneath the mesh layers was detected by a flat model L1-1400 light meter and data logger. The LI-1400 is a highly sensitive current meter able to measure currents from 0±250 µA, with a resolution as low as 7.6 picoamps. This makes the LI-1400 ideally suited for accurately measuring photo voltaic and other type sensors that produce very small currents. Most commonly, the current channels are used to measure LI-COR radiation sensors (pyranometers, quantum, and photometric sensors). The highest light intensity, received by the tube with no mesh layer, was comparable to sunlight intensity around noon.

2.2.9. Nutrients Requirement

Growth rates as a function of nitrate concentrations were determined. Series of liquid media containing various amounts of nitrate were inoculated, while all other

parameters were sufficient. Each culture grew at a rate different from other cultures. Similarly, phosphate requirement was studied by varying the amounts of phosphate in a set of liquid media, while other parameters were sufficient.

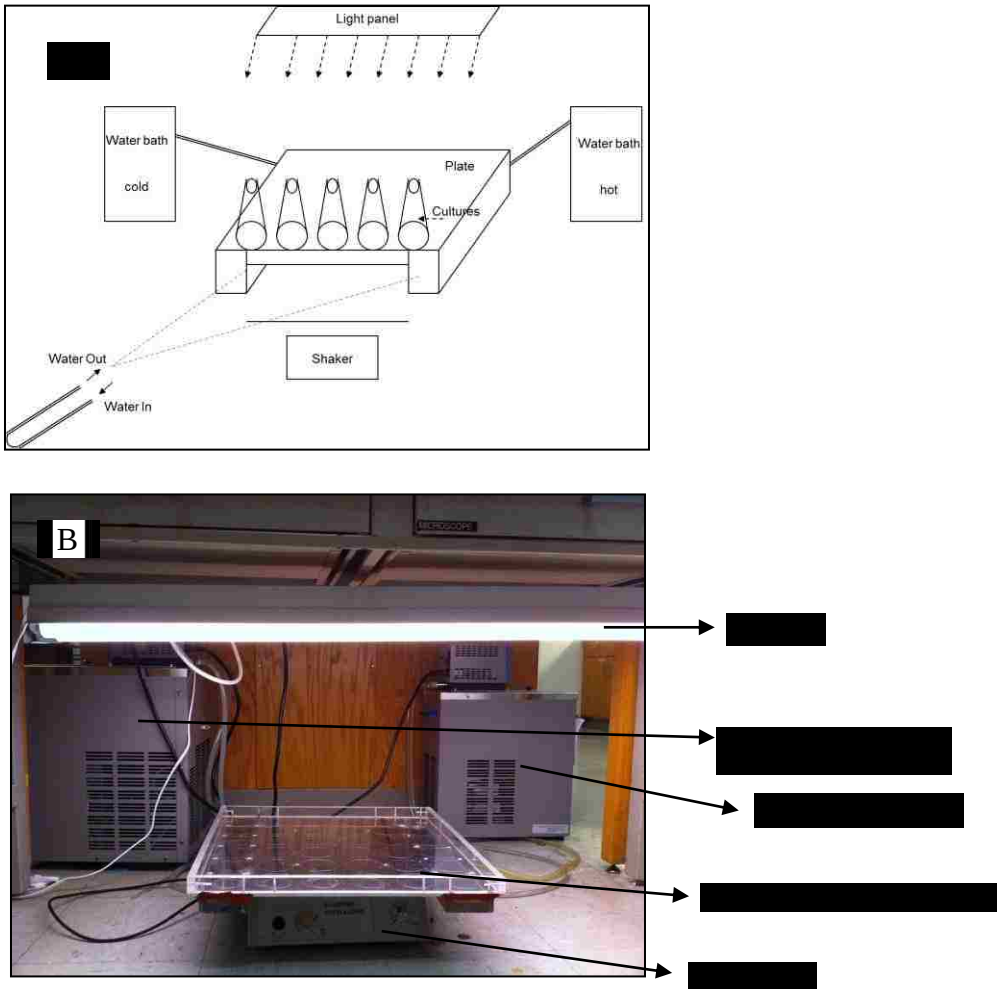

Figure 2.8. A) Schematic design of the temperature experiment. B) Picture of the temperature table, two water baths, and light.

2.2.10. Salinity Tolerance Determination

Like the nutrient requirements, salinity tolerance was investigated by supplementing various amounts of NaCl to the prepared liquid media. Though different types of salts present in a system would affect salinity, they all were calculated as equivalent sodium chloride. Thus, for the sake of simplicity, NaCl represented the total salts in the system.

2.3. Results

2.3.1. Taxonomic Identity

Error bars in all figures of this dissertation represent the standard error (*SE*) calculated from the standard deviation of each sample ($SE = \sigma/\sqrt{n}$, where σ is the standard deviation of the population and n is the size of the sample). Each data point is the average of four replicates.

Figure 2.9 shows the pictures of the two green algae, *Chlorella sorokiniana* and *Scenedesmus* sp., taken by an optical microscope. The phylogenetic relationship was constructed on the basis of the nucleotide sequence of the 18S rRNA gene, between our isolates and closely related algae (Figure 2.10 and 2.11). Based on this comparison, the six new isolates are designated as strains of *Chlorella sorokiniana*, *Encyonema caespitosum*, and *Nitzschia thermalis*, and species of *Scenedesmus*, *Synechocystis*, and *Limnothrix*.

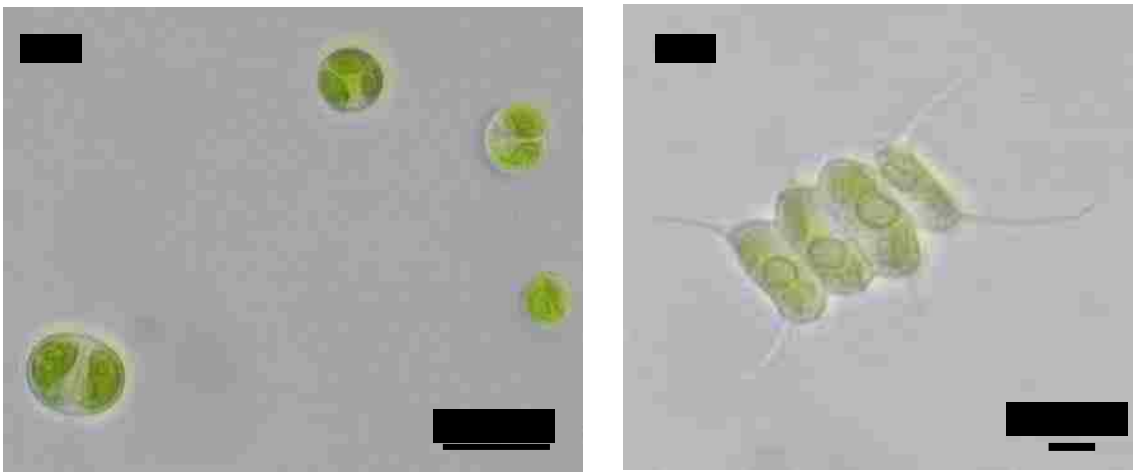

Figure 2.9. Light micrograph of the two green algae, (A) *C. sorokiniana* and (B) *Scenedesmus* sp., taken by AxioCam MRC, magnified 1000 times.

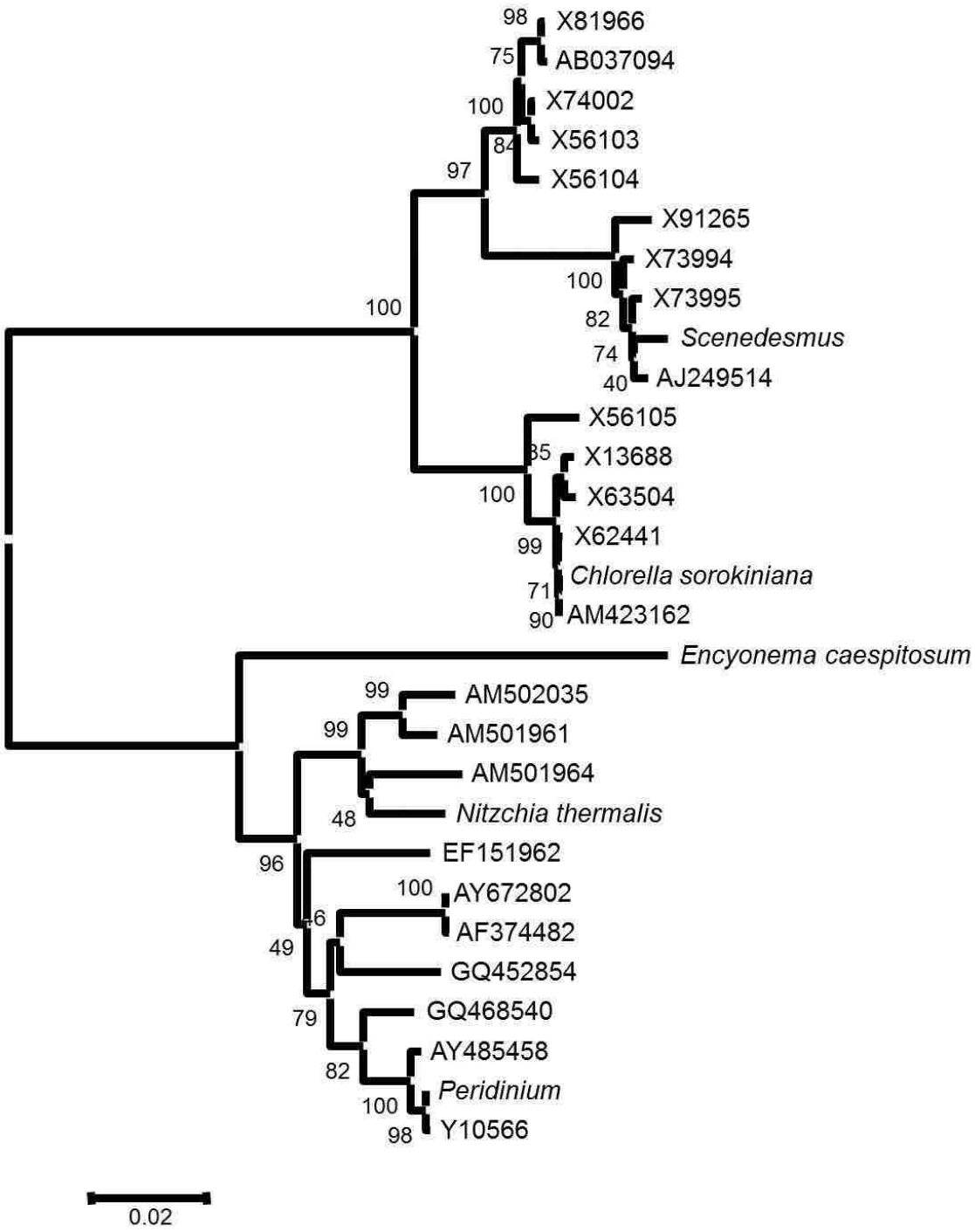

Figure 2.10. Neighbor-joining trees of 18S rRNA nucleotide sequences depicting phylogenetic distances between the new isolates and known species. Bar represents percentage change per nucleotide position.

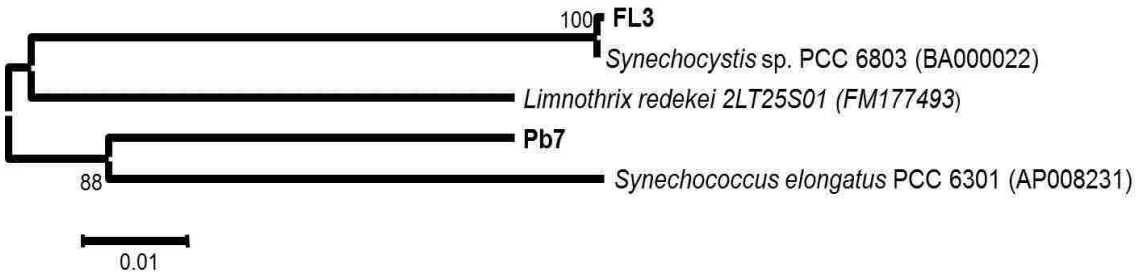

Figure 2.11. Neighbor-joining trees of 18S rRNA nucleotide sequences depicting phylogenetic distances between the new isolates and known species. Bar represents percentage change per nucleotide position.

2.3.2. Optimum Temperature

Temperature-growth curves were fit to a second degree polynomial diagram with a maximum point or plateau (Figure 2.12). The optimum temperatures of diatom *E. caespitosum* and *Scenedesmus* sp. were around 27 ºC and 22ºC, respectively (Fig. 2.12 F and B). Every other studied organism showed an optimal growth temperature at around 32 ºC (Fig. 2.12 ACDE).

2.3.3. Light Determination

Light-growth curves of the two green algae *C. sorokiniana* and *Scenedesmus* sp. consist of a light sensitive part where the growth rate rises steeply with photo flux, and a light insensitive part where the growth rate increases only gradually with light. Neither alga shows photoinhibition in the studied light range (Figure 2.13 A,B). The two cyanobacteria, in contrast, are organisms of dim light. *Synechocystis* grow well from 2 to 50µMole/m^2.s. At higher light levels, growth rate plummets (Figure 2.13 C). *Limnothrix* grow well at an extreme low light intensity of 2µMole/m^2.s. Higher light intensity causes inhibition, but the decrease in growth rate with light is gradual (Figure 2.13 D).

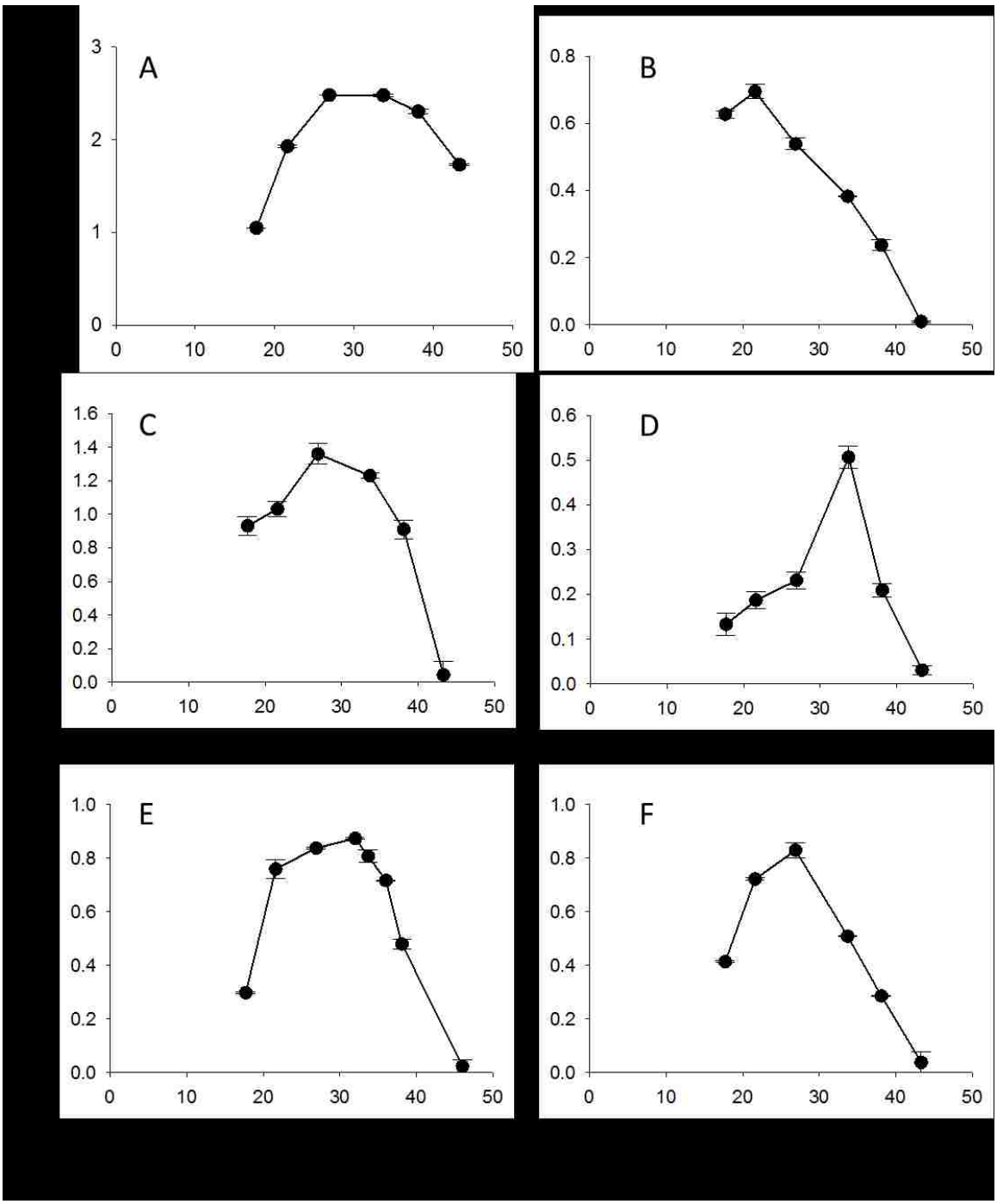

Figure 2.12. Partial temperature-growth curves of A) *C. sorokiniana*, B) *Scenedesmus* sp., C) *Synechocystis* sp., D) *Limnothrix* sp., E) *N. thermalis*, and F) *E. caespitosum*. Except for *Scenedesmus* sp. which had an optimum temperature at ~20°C.

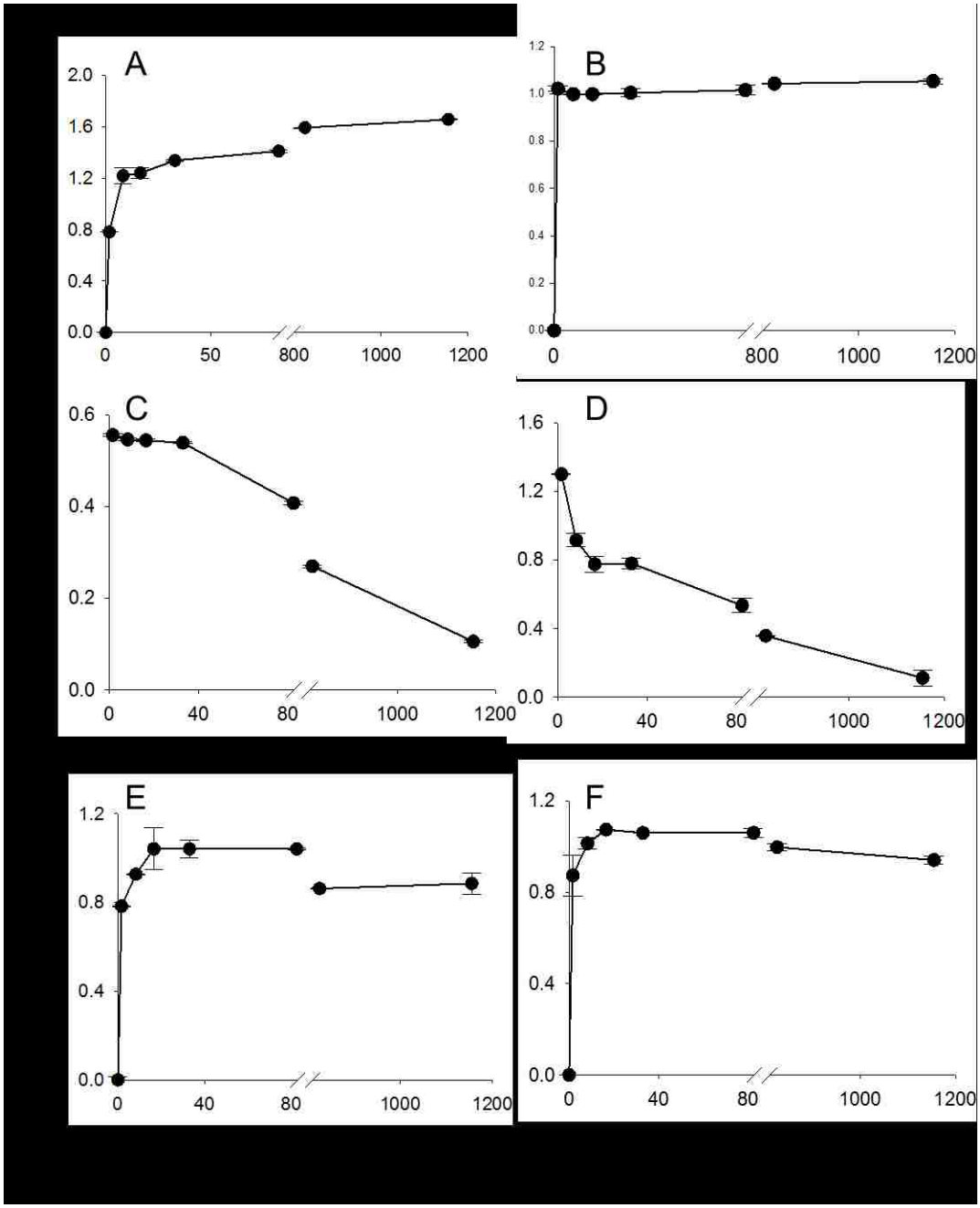

Figure 2.13. Light requirement for the growth of A) *C. sorokiniana*, B) *Scenedesmus* sp., C) *Synechocystis* sp., D) *Limnothrix* sp., E) *N. thermalis*, and F) *E. caespitosum*. Only the two green algae grow well under high light intensity, comparable to sunlight during a normal day in Las Vegas. Cyanobacteria grow best at low light intensities, close to darkness, and become inhibited at high light intensities. Diatoms grow most under intermediate light. The growth rate increases as the light is increased until it reaches a critical level,~800µMol.m-2.s-1. Beyond this level, growth becomes inhibited.

The diatoms *E. caespitosum* and *N. thermalis* are similar to the green algae in the low-moderate light range. However, they do not flourish at high light as the green algae do (Figure 2.13 E and F).

2.3.4. Nitrate and Phosphate Requirement

Figures 2.14 and 2.15 show the effects of nitrate and phosphate on growth rates, respectively. Each curve is resolved into a nutrient-sensitive phase where the growth rate rises rapidly with nutrient availability and a nutrient-saturated phase where the growth rate no longer changes with nutrient concentration. No growth occurred if either nitrogen or phosphorus was omitted from the medium. No inhibition happened to any of the species in the presence of nitrate as high as 500 μM or phosphate as high as 300 μM.

2.3.5. Salinity Tolerance

No inhibition occurred to either the green algae or the cyanobacteria until sodium chloride (NaCl) reached about 25 mM. Beyond this point, more salt led to slower growth, and 90 mM NaCl stopped the growth completely (Figure 2.16). In contrast, the two diatoms were sensitive to the salinity level. A salinity level as low as 3 mM caused depression in growth. For reference, the Wash water and seawater contain 12 mM and 599 mM NaCl, respectively.

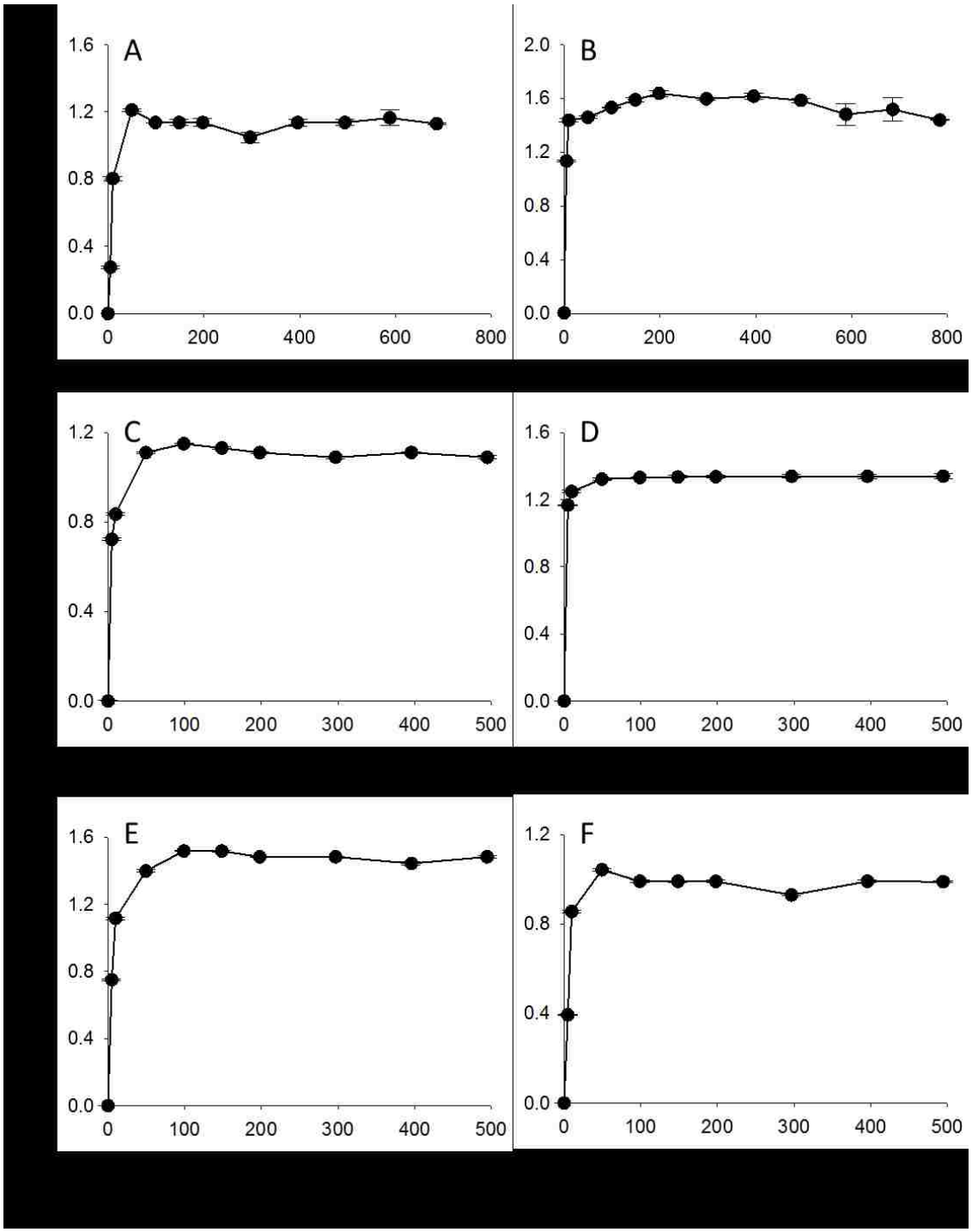

Figure 2.14. Nitrate requirement of A) *C. sorokiniana*, B) *Scenedesmus* sp., C) *Synechocystis* sp., D) *Limnothrix* sp., E) *N. thermalis*, and F) *E. caespitosum*. The growth rate increased as the nitrate concentration was increased until it reached the saturation level, ~100µM. Beyond this, more nitrate did not increase the growth rate. No inhibition occurred.

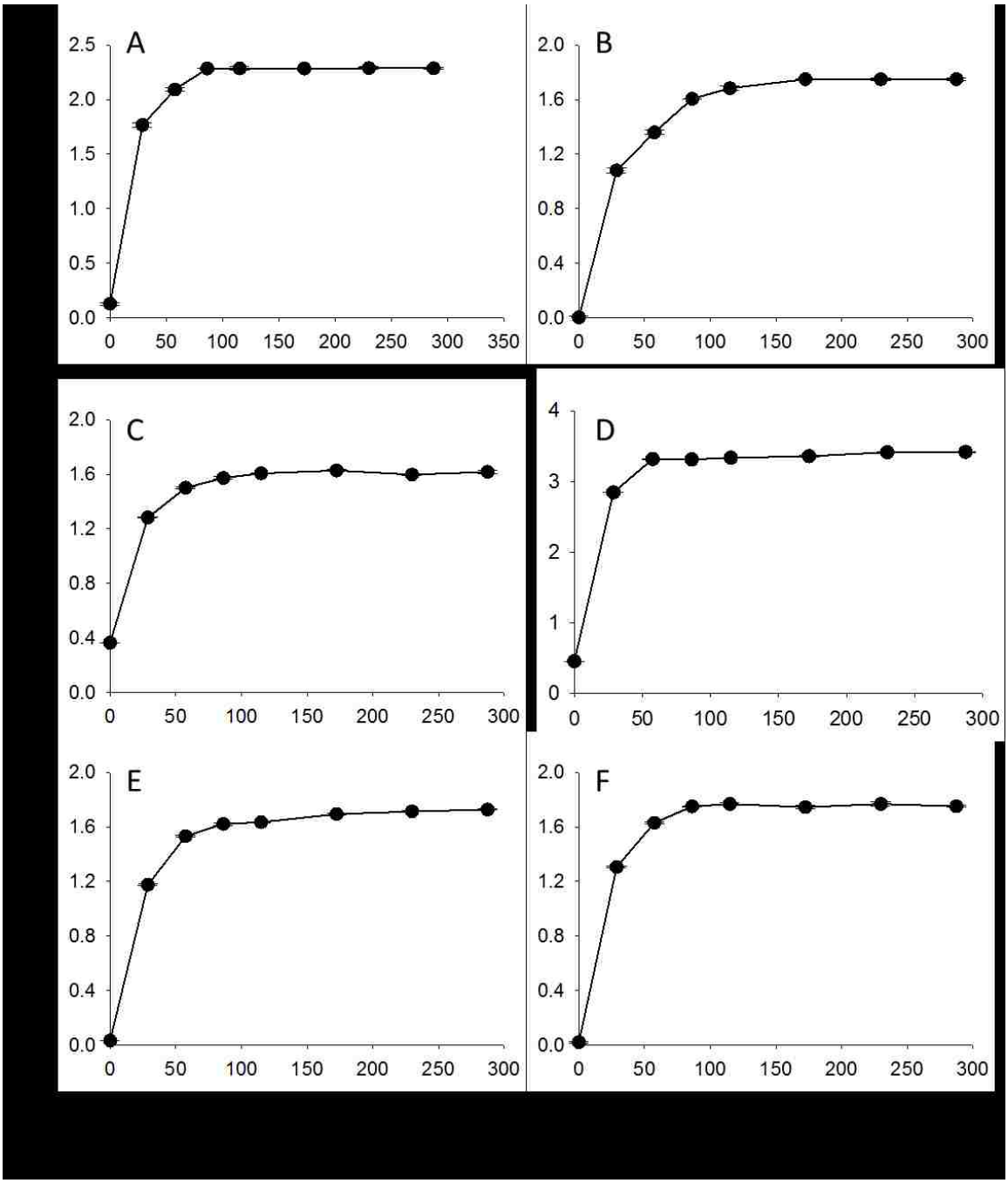

Figure 2.15. Phosphate requirement of A) *C. sorokiniana*, B) *Scenedesmus* sp., C) *Synechocystis* sp., D) *Limnothrix* sp., E) *N. thermalis*, and F) *E. caespitosum*. The growth rate increased as the phosphate concentration was increased until it reached the saturation level, ~70µM. Beyond such, more phosphate didn't increase the growth rate. No inhibition occurred.

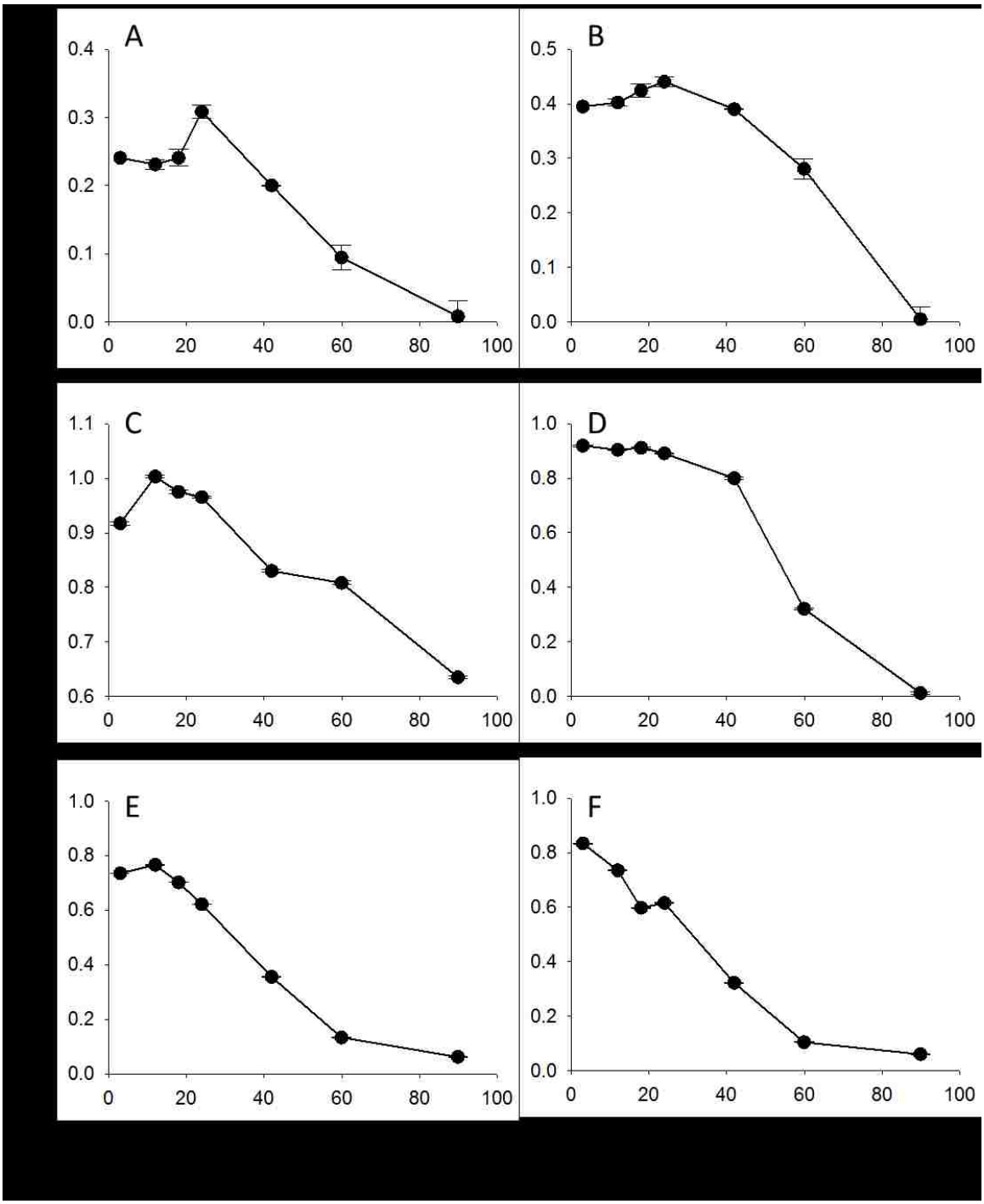

Figure 2.16. Salinity tolerance of A) *C. sorokiniana*, B) *Scenedesmus* sp., C) *Synechocystis* sp., D) *Limnothrix* sp., E) *N. thermalis*, and F) *E. caespitosum*. Except for (F), all the isolates could tolerate some degree of salinity, ~15mM. No growth occurred at 90mM of sodium chloride, comparable to 15% of the sea salts.

2.4. Discussion

This study provides evidence for the suggestion that, everything else being equal, local microalgae are preferable for fuel producing crops. As a result of long-term ecological adaptation, they would thrive in the local environment and local water chemistry. For instance, *C. sorokiniana*, which was isolated from the Wash in the summer, has an unusually high temperature optimum, between 27 and 33°C. From June to August, the average water temperature in the Wash is 25°C (State of Nevada: Division of Environmental Protection, 1987). Daily maximum in excess of 30°C are not uncommon. In contrast, *Senedesmus* sp., which originated from Lake Las Vegas in the spring, has a much lower temperature optimum, around 22°C, indicating that the temperature requirements are related to the season in which they grow in nature. This result has important practical implications. It suggests that within the local species, some are better suited as summer crops, while others may be suited for production in winter and spring.

The isolates used in this study also show evidence of adaptation to local water chemistry. Water in the Las Vegas Valley has high hardness level 288 parts per million (ppm) (www.lvvwd.com). Not surprisingly, the isolates, all of which are fundamentally freshwater species, can, to various extents, tolerate salinity. This characteristic is also of practical value. For example, the green algae and cyanobacteria isolates can tolerate salinity ten times that of the Wash. This suggests that even in open pond cultivation systems the spent medium can be re-used for many cycles despite the inevitable increase in salinity associated with evaporative water loss.

The light levels preferred by algae vary widely among major taxonomic groups. Cyanobacteria, for instance, generally prefer low light levels and suffer from exposure to high light levels, a phenomenon known as photoinhibitation (Konopka and Schnur, 1980; Konopka, 1983). Green algae, in contrast, prefer high levels and generally resistant to photoinhibition (Li *et al.*, 2010). The results with the Las Vegas isolates confirmed this generality.

The diatoms appear to be different from both the cyanobacteria and the green algae. The light dependent part of their growth curve is similar to that of the green algae. However, unlike the latter, the diatoms are not tolerant of extraordinary high light levels (comparable to summer sunlight at noon). Thus, from a light as well as a temperature perspective, the green algae are best suited for production in the summer, whereas the cyanobacteria and diatoms may best take advantage of the lower temperature and lower light level of the winter time.

At this stage of the investigation, there is insufficient information about the lipid content or lipid quality of the tested isolates in order to evaluate their suitability in these regards. However, it is known that cells of *C. sorokiniana* and *Scenedesmus* sp. do contain 19.0% and 29.8% lipids respectively, when the organisms are growing logarithmically. Compared to the literature values reported for other algae, these lipids concentrations are low and moderate. However, as will be shown in Chapter 5, the lipid contents could be improved by manipulating the growth conditions.

CHAPTER 3

WASTEWATER AS THE GROWTH MEDIUM

3.1. Introduction

The idea of growing algae in wastewater is not new. In 1977, Benemann *et al.* suggested that algae farms can be combined with municipal wastewater treatment facilities. In this system, algae would remove the nutrients from wastewater to grow, decreasing fertilizers and clean water usage by algae farms and the cost of nutrient removal processes at wastewater treatment plants.

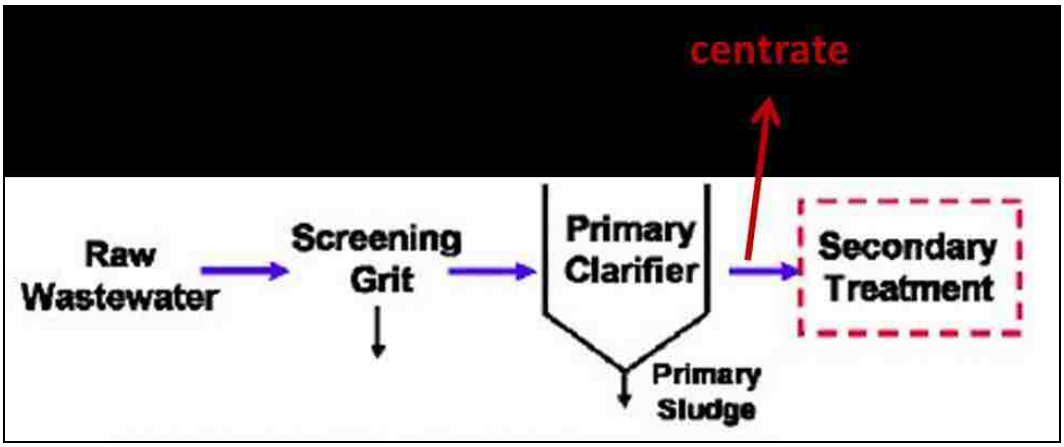

Figure 3.1. Typical wastewater treatment schematic in municipal wastewater plant.

Figure 3.1 demonstrates the general scheme of a wastewater treatment facility. There are three main stages of wastewater treatment: primary, secondary, and tertiary (or advanced). Primary and secondary treatments are mandatory in the United States, but the tertiary process is used only under special circumstances because it can be very expensive, often doubling the cost of the secondary treatment. Primary treatment removes materials that are either floating or readily settle out by gravity. In "primary

settling tanks" or "primary clarifiers", the sludge settles, while the grease and oil float to the surface to be skimmed off. The sludge is mechanically collected by a hopper at the bottom of the tank to be pumped out. Primary treatment removes about 60% of total suspended solids (TSS) and about 35% of biological oxygen demand (BOD). The liquid fraction of the primary clarifiers, also known as centrate, enters the secondary treatment process, performed by activated sludge or oxidation ponds. In the activated sludge stage, organic material is removed by aerobic microorganisms. The microorganisms also convert ammonia to nitrite and nitrate, and eventually, to nitrogen gas, a process called denitrification. Oxidation ponds, also called stabilization ponds or lagoons, are large, shallow ponds designed for bacterial and algal photosynthetic activities. In the ponds, algae remove inorganic compounds, i.e. nitrate and phosphate, and release oxygen needed by aerobic bacteria. The bacteria degrade organic compounds and reduce nitrogen via denitrification. Secondary treatment removes more than 85% of both TSS and BOD. If more than 85% must be removed, or if nitrate and phosphate levels are still greater than the authorized level, tertiary treatment methods are used. While removing nutrient is currently performed at a cost and with the use of energy at treatment facilities, in a combined system, i.e. a wastewater treatment facility/algae farm, the secondary treatment stage could be completely replaced by algal cultivation ponds. This would result in nutrient removal free of charge, as algae are capable of removing 85% to100% of the nutrients (Shen *et al*., 2008; Wang *et al.*, 2010; Woertz *et al*., 2009).

However, existing oxidation ponds cannot be directly used for cultivation of algae for biofuel. Although these lagoons support algal growth, the growth of algae are not be sufficient for the purpose of biofuel production. Plus, the growing strains may not

necessarily be the 'fuel-producing' species. The ponds configuration and growth conditions should be designed and optimized specifically to fulfill the highest growth of selected algae. Moreover, a proper dilution of wastewater, at which the growth of algae is adequate, should be exploited. This study tries to resolve the latter issue. Even though centrate can stimulate the growth of some species, it might be too concentrated for others, leading to growth inhibition. For example, Kong *et al*. (2010) reported inhibition when *Chlamydomonas reinhardtii* was grown in raw centrate. Wastewater is a complex medium; while it is a source of nutrients, it contains some compounds that may be toxic to algae. Therefore, the sources of inhibition and toxicity should be identified and adds costs before wastewater is used as the growth medium.

This study examines the feasibility of growing *C. sorokiniana* and *Scenesemus* sp. in locally generated centrate. In order to determine the best concentration of centrate, various strengths of the centrate were explored. In addition, this study seeks to identify potential sources of toxicity in the centrate. Instead of removing the toxins, which may not be economically viable, the centrate was diluted to a level at which toxicity disappeared, while the nutrients levels are still sufficient for algal growth.

In addition to inorganic nutrients, centrate is also a rich source of organic carbon that can provide extra energy for algae (Zhou *et al*., 2011). However, the ability of algae to consume these organic compounds needs to be investigated. Algae are photosynthetic organisms utilizing the energy of the sun to fix CO_2 and produce biomass. They convert light energy into chemical energy, fueling algal activities. However, some algae are able to use organic carbon as an alternative source of energy. When there is light, such algae obtain energy from both resources of organic carbon and photosynthesis. This

mixotrophic condition stimulates algal growth during the day. In the absence of light, these algae are able to acquire their required energy from organic carbon. Such heterotrophic ability supports algal growth during the night. Hence, the presence of organic carbon offers continued biomass production at higher rates.

However, heterotrophic/mixotrophic algae are not able to consume all types of organic carbon resources. Depending on their origin and what organic compounds they have been exposed to, different algae have evolved to assimilate various sources of organic carbon. This effect becomes evident when different studies sometimes report completely opposite results for the same species. For example, Liang *et al*. (2009) showed that *Chlorella vulgaris* consumed glycerol while Heredia-Arroyo *et al*. (2011) showed that the same alga, *C. vulgaris,* did not consume glycerol. This is most likely because *C. vulgaris* was isolated from two different regions, in one of which the cells were exposed to glycerol while in another they were not. Therefore, it is crucial to determine what algae can assimilate what organic compounds. This study tests the ability of its two isolates, *C. sorokiniana* and *Scenedesmus* sp., to assimilate four organic compounds: glucose, acetate, glycerol, and lactate. The rationale for choosing glucose and acetate, in addition to their availability in different wastewaters, is their simplicity. These two are the most common organic molecules being consumed by microorganisms. Glycerol has been chosen because it is the main by-product of biofuel plants. Ideally, all the waste products and by-products of a biofuel plant would be re-used. Finally, lactate has been selected because it is found in various types of wastewaters such as milk, diary, and ice-cream factories. Eventually, the goal should be to utilize all the organic substrates from waste sources. For instance, winery wastewater containing glucose,

fructose, lactate, acetic acid, citric acid, and malic acid, could be added to the centrate and serve as an organic supplement (Malandra *et al*., 2003).

3.2. Methodology

3.2.1. Centrate and Treated Wastewater

Centrate was obtained from the Henderson wastewater treatment facility. The Las Vegas Wash water represented the treated wastewater (TWW). Serial liquid media synthesized from TWW amended with centrate, resulting in ratios of 0, 10, 15, 30, 35, 40, 50, 70, 80, and 100% centrate. The synthesized medium was filtered and stored in sterile containers in a refrigerator.

3.2.2. Effect of Ammonium on Growth

C. sorokiniana was inoculated in serial tubes containing nitrogen-free BG11 supplemented with various concentrations of ammonium, the same amount that is available in the centrate. In a separate experiment, the same gradients of ammonium were added to BG11 in serial tubes, in which *C. sorokiniana* was inoculated. The experiments were terminated when the first green pallets became visible. Pallets collected with centrifuge were used for protein measurement.

3.2.3. Effect of Urea on Growth

Three concentrations of 0.1, 0.01, and 0.001% (w/v) of urea were added to 4 mL of BG11. After *C. sorokiniana* was inoculated, the cultures were placed on the shaker under the optimum light condition. The optical density (OD_{600}) was measured with a Spectronic 20D+ spectrophotometer twice a day until the cells entered the stationary phase.

3.2.4. Effects of Organic Components on Growth

Tubes containing 4ml of BG11 supplemented with 1% (w/v) of glucose, glycerol, acetate, or lactate were placed on a shaker, under the optimum light condition of 50 $\mu mole \cdot m^{-2} \cdot s^{-1}$ with 10:14 light dark cycle. For the heterotrophic growth experiments, tubes were wrapped fully in aluminum foil and placed on the shaker. To avoid any photosynthetic activity, growth measurements were all conducted in full darkness. Two series of tubes containing 4 mL of BG11 were put in the dark and light, representing the reference conditions for the heterotrophic and mixotrophic growth, respectively. Growth was measured by recording the optical density (OD_{600}) with the Spectronic 20D+ spectrophotometer on a daily basis.

3.3. Results

3.3.1. TWW and Centrate Characteristics

Table 3.1 shows the characteristics of the two water sources. Note that treated wastewater is nearly devoid of phosphate, but its nitrate content is comparable to that of centrate.

3.3.2. Growth in TWW and Centrate

The highest growth rates, exceeding rates achieved in BG11, occurred in the half-strength centrate. The two algae grew well in treated wastewater (TWW) amended with 20 to 70% centrate, significantly better than growth in BG11 (Figure 3.2).

Table 3.1. Characteristics of treated wastewater (TWW) and centrate used in this study.

Nutrient	Centrate	TWW
NH_3-N, µM	1,802	4
NO_3-N, µM	686	1,023
TKN, µM	2,754	8
PO_4-P, µM	168	NA
TP, µM	NA	4
BOD, mg/L	234	NA
Salinity, mM	NA	2

TWW amended with less than 20% centrate supported less growth, apparently due to phosphate insufficiency. Amendment greater than 70% was counterproductive, resulting in slower growth of *Scenedesmus* sp. and a long lag phase for *C. sorokiniana*. To discover the cause of such inhibition, the effects of ammonium, urea, and arsenate on growth of *C. sorokiniana* were explored.

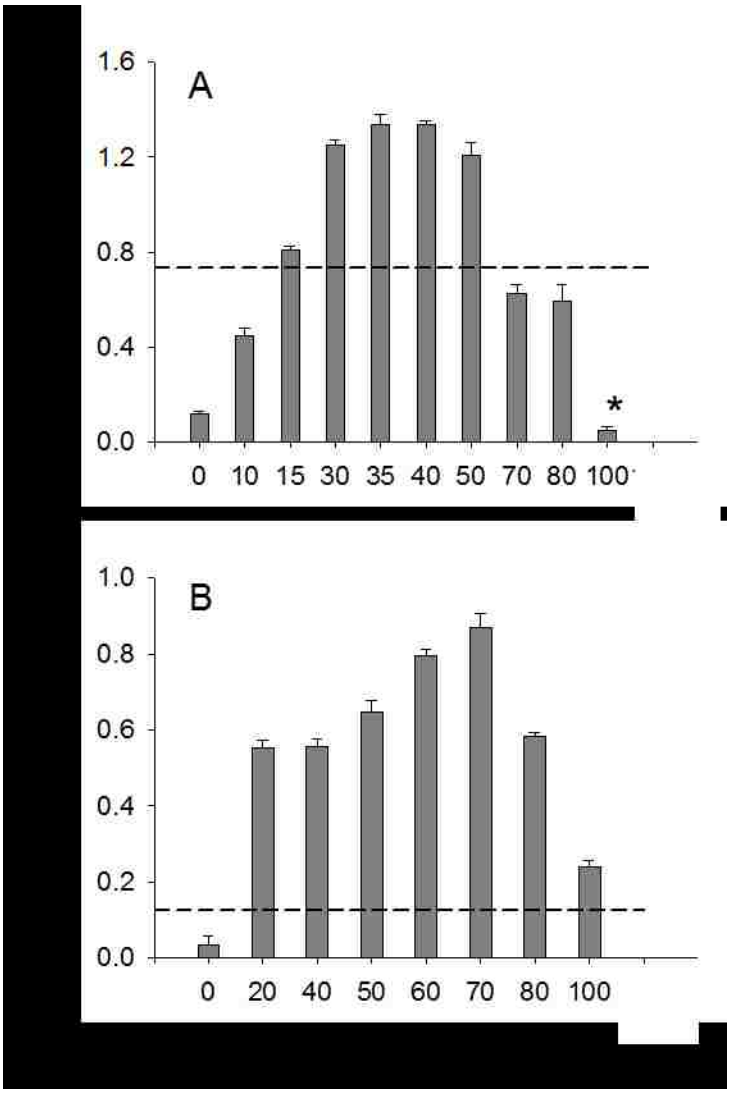

Figure 3.2. Log-phase growth rates of *C. sorokiniana* (A) and *Scenedesmus* sp. (B) in Wash water with different amounts of centrate (sewage liquor). Asterisk denotes an exceptional situation where the organism does not enter lag-phase for two to three days.

3.3.3. Effect of Ammonium on Growth

A slight inhibition occurred when ammonium was the only nitrogen source in the medium. In contrast, in the presence of nitrate, no inhibition occurred (Figure 3.3).

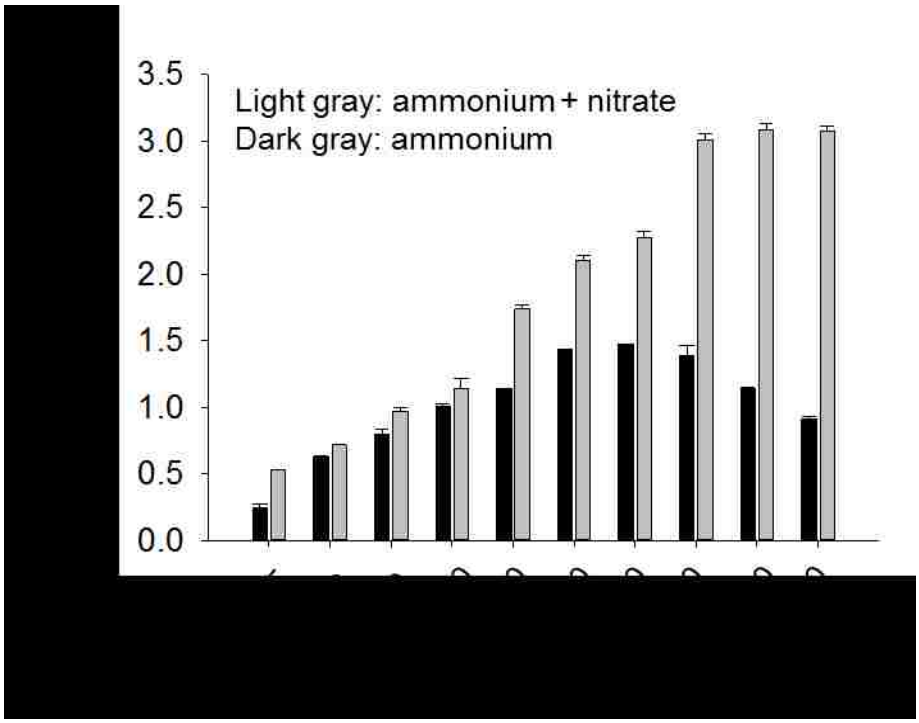

Figure 3.3. Effect of ammonium chloride on growth rate of *C. sorokiniana*, in nitrogen-replete (light grey columns) and nitrogen-omitted (dark grey columns) BG11. Only if ammonium was the only source of nitrogen, a slight inhibition occurred in the cultures that contained >200µM ammonium. Ammonium did not inhibit the growth if nitrate was present. As there is always sufficient amount of nitrate in the centrate, ammonium cannot cause inhibition.

3.3.4. Effect of Urea on Growth

Different concentrations of urea caused completely opposite impacts on the growth rate. Below 1 gram of urea per liter of medium (0.1% of urea), the growth was stimulated. At 0.1% and above (data are not shown), growth was inhibited and cells showed a long lag phase, ~ 50 hours to grow (Figure 3.4). When cells entered the lag phase, they grew at a rate relatively equal to the rate they achieved in the BG11. However, the biomass density in the culture with BG11 was significantly higher than that in the culture containing 0.1% urea (Figure 3.5B). Figure 3.5 compares the growth in the

culture containing 100% centrate to the culture containing BG11 supplemented with 0.1% urea.

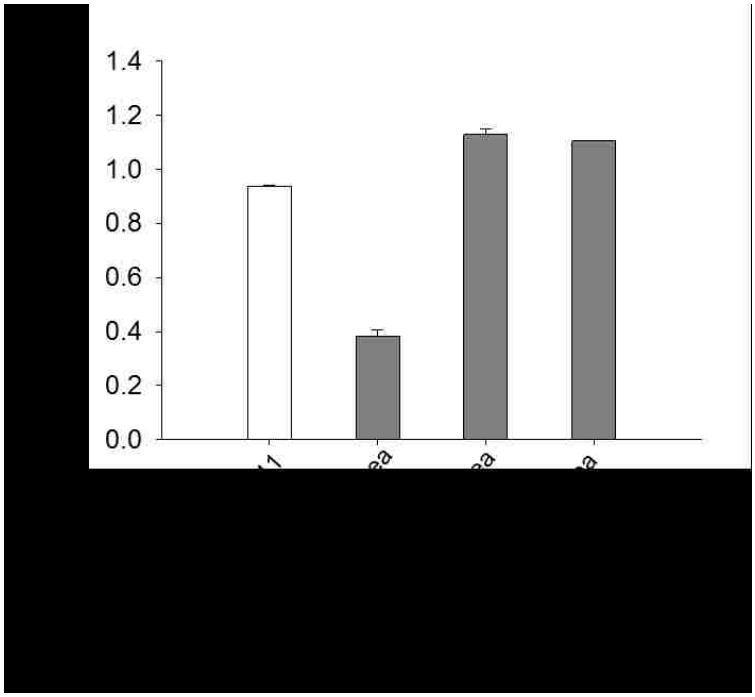

Figure 3.4. Effect of various concentrations of urea on growth rate of *C. sorokiniana*. While urea levels below 0.1% stimulated the growth, beyond such was inhibitory.

3.3.5. Utilization of Organic Compounds

Both of the algae grow mixotrophically. The number of organic carbon that can be utilized as a substrate is slightly different for the two studied algae. *C. sorokiniana* grew on glucose and acetate, but not on glycerol and lactate (Figure 3.6A). *Scenedesmus* sp. grew on glucose, acetate, and glycerol, but not lactate (Figure 3.6B). In the heterotrophic mode, in the dark, both isolates grew on glucose and acetate. Neither grew on glycerol nor lactate (Figure 3.7).

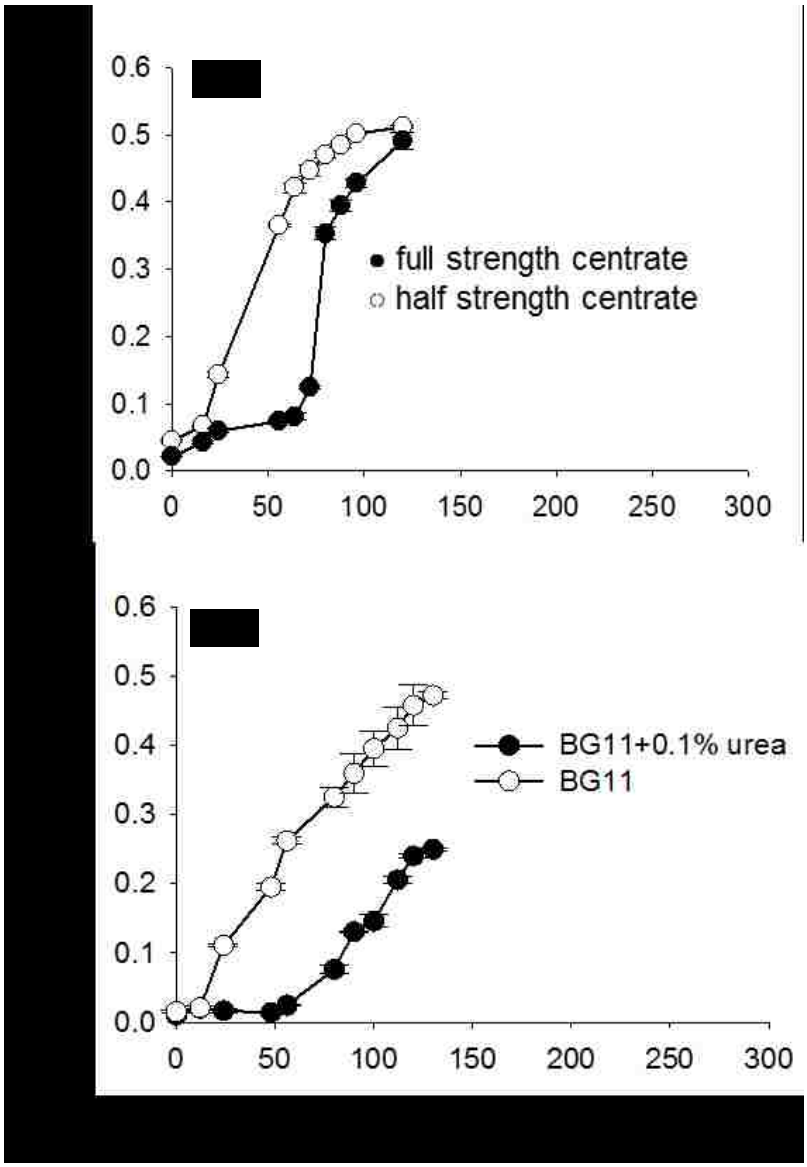

Figure 3.5. Delayed but not slowed: A) the growth of *C. sorokiniana* in the full-strength centrate versus the half strength, B) the growth of *C. sorokiniana* in the BG11 with and without 0.1% urea. Similar to the full-strength centrate, urea added to BG11 delayed the growth, but growth rate was still comparable to the growth rate of BG11. The similarity between the growth in the urea-plus culture and the full-strength centrate implies that urea could be an important inhibitor in the centrate.

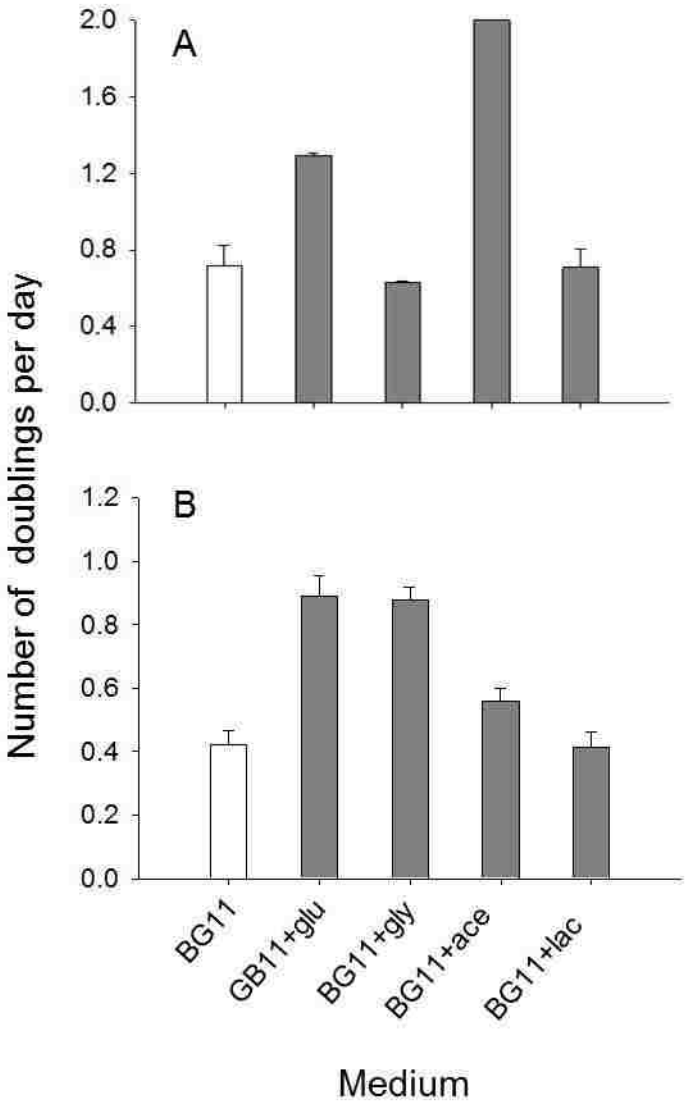

Figure 3.6. Mixotrophic growth rates in the BG11 without and with glucose (glu), glycerol (gly), acetate (ace), and lactate (lac). A) *C. sorokiniana*, B) *Scenedesmus* sp. In both species, presence of glucose and acetate enhanced the growth rate. In Scenedesmus sp., glycerol also stimulated the growth as well as glucose.

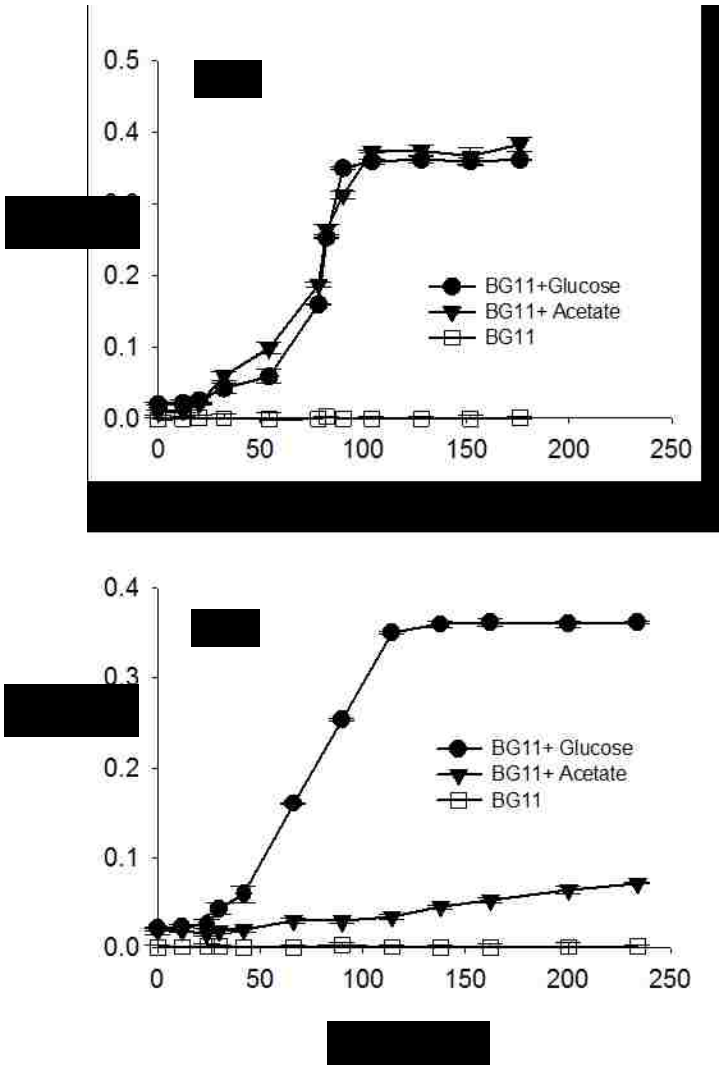

Figure 3.7. Heterotrophic growth occurred only in the cultures that contained BG11 supplemented with either glucose or acetate. Neither lactate nor glycerol supported the growth under the dark (data is not shown). A) *C. sorokiniana*, B) *Scenedesmus* sp.

3.4. Discussion

The results of growing the isolates in different strengths of centrate are similar to those obtained by Bhatnagar *et al.* (2009), who studied a strain of *Chlorella minutissima* isolated from oxidation ponds in wastewater treatment facilities in India. Like the strains investigated in this study, their isolate grew faster in half-strength centrate than in BG11.

Full-strength centrate also supported lower growth than BG11. Thus, it has been demonstrated that for at least these three algae prefer municipal wastewater to a freshwater-based medium.

The results of this study also answer two important questions. First, why does the centrate need dilution? Second, what makes the centrate better than the standard growth medium, BG11? Centrate is a complex material containing a wide range of organic and inorganic substances. While some substances, such as nitrate, phosphate, and possibly organics are beneficial; others could be suppressive or toxic. The results from chapter 2 indicate that inhibition does not come from excessive salt, as the two green algae could tolerate a moderate degree of salinity. Another potential toxin is ammonium. Ammonium is a source of nitrogen for algae, but it can be toxic in high concentrations (Abeliovich and Azov, 1976; Azov and Goldman, 1982). However, the results exclude the potential toxicity of ammonium. In response to ammonium gradients, *C. sorokiniana* did not show any types of inhibition when there was adequate nitrate in the medium. Given the presence of sufficient nitrate in the centrate, inhibition could not be imposed by ammonium.

The last candidate for a source of toxicity is urea. Domestic sewage usually contains some amount of urea, which varies from one location to another. Urea metabolism in green algae is permitted either by urease or by ATP-urea amidolyase (Hodson and Thompson, 1969; Bekheet and Syrett, 1977). Both enzymes degrade urea to NH_3 and CO_2 (Eq. 3.1).

$$CO(NH_2)_2 + H_2O \rightarrow CO_2 + 2\,NH_3 \qquad \text{(Eq. 3.1)}$$

While it is well known that green algae can utilize urea as a nitrogen source (Bekheet and Syrett, 1977), the results reveal significant inhibition when the dosage of urea goes beyond 1 g/L. At this concentration, urea suppressed growth to slightly below pre-stimulation levels. This result replicated the inhibitive nature of undiluted centrate, in which *C. sorokiniana* was suppressed for the first two to three days of incubation. The stimulation of growth by urea at low concentrations most likely comes from the carbon source (CO_2), while the inhibition at high concentrations almost certainly comes from NH_3. In order to avoid inhibition, the current study tried to adapt the algae to urea before transferring them into the centrate. The cells were exposed to urea during the pre-adaptation phase to produce urea-degrading enzymes, i.e. either urease or ATP-urea amidolyase. Hence, the already induced enzymes would degrade urea right after the algae were transferred into the centrate. The results were unsuccessful as the cells could not adapt and the growth curve duplicated the previous result.

The optimum centrate dilutions, 20% to 70%, represent a tradeoff between diluting toxins to a non-toxic level, without overly diluting nitrate and phosphate to sub-saturation levels. In contrast to the toxic characteristics of full-strength centrate, properly diluted centrate outperforms BG11 because it contains, in addition to mineral nutrients, organic compounds that provide additional energy for growth. As demonstrated by the results from this chapter, both isolates grew mixotrophically and heterotrophically on glucose, acetate, or glycerol, indicating that the isolates are able to take advantage of organic compounds present in the centrate. Although it is not known what organic compounds are exactly present in the tested centrate (responsible for the BOD), it is likely that some of them are available to the isolates, given their broad

mixotrophic/heterotrophic capabilities. This finding adds to a growing body of literature that many algae, including *Chlorella* and *Senedesmus* species, are able to utilize organics for growth either under heterotrophic conditions or under mixotrophic conditions (Samejima and Myers, 1958; Karlander and Krauss, 1966; Kamjunke and Tittel, 2009; Liang *et al.*, 2009; Gao *et al.*, 2010; Heredia-Arroyo et al., 2010; Xiong *et al*., 2010; Heredia-Arroyo *et al*., 2011; O'Grady and Morgan, 2011).

CHAPTER 4

RECYCLING ALGAL BIOMASS RESIDUE AS A SOURCE OF NUTRIENT

4.1. Introduction

An algal biofuel plant must function as a closed system, in terms of nutrients, if it is intended to be sustainable. One key step towards this goal is to supply input material, including nutrients, from the generated waste at the plant. One major waste would be algal biomass residue, i.e. the algae remaining at the end of biofuel production processes. After lipids are extracted, algal cells still contain nitrogen and phosphorus in the form of nucleotides and proteins, which can be recycled and re-used in place of fertilizers. Nucleotides are composed of nitrogenous bases, five-carbon sugars, and phosphate groups, all potential nutrients and energy sources. Proteins present in the cell wall are polymers of amino acids, i.e. a source of nitrogen. However, such nutrients are not biologically available to algae. In nucleotides, phosphate groups form strong bonds with the carbon sugar. Similarly, in polypeptides (i.e. polymers of the protein) amino acids are held together by strong peptide bonds. Hence, it is impossible for algae to consume the existing substrates unless the biomass is broken down to small and simple molecules. Two possible ways to accomplish this are biological and chemical degradation. The current study performs the former, biotic decomposition, via composting. Although composting is slow, it is economical. Also, it has been successfully practiced for a long time for purposes of recycling agricultural residues. Through this biological process, bacteria convert the big molecules, which algae cannot utilize, into substrates that algae can potentially use. Algal biomass composting is followed by extracting the dissolved

nutrients, known as compost tea, which can be used as a growth medium. However, the feasibility of growing the selected algae in compost tea needs to be explored.

In order to determine the most efficient method of composting, which would also support the highest growth rate in compost tea, aerobic and anaerobic conditions were tested. While the anaerobic condition generated higher amounts of compost (out of the same base of biomass) than the aerobic condition, the production occurred at a lower pace. Under both conditions, composting with and without paper as a source of cellulose was also tested. The paperless compost is preferred because it requires fewer additives, making the process more economical and sustainable. However, the feasibility of composting without a cellulose source is ambiguous. Finally, after the composts are ready, the compost teas with and without the supplementing of glucose (as a simple organic carbon source) were tested to determine whether additional organic carbon enhances growth. Overall, eight varieties of compost tea were examined to grow the two isolates *C. sorokiniana* and *Scenedesmus* sp.: tea from aerobic compost without paper, aerobic compost with paper, anaerobic compost without paper, and anaerobic compost with paper, and all other mentioned teas supplemented with glucose.

Chemical degradation is another way of degrading polymers and large molecules (e.g. proteins) to the monomers (e.g. amino acids) possibly consumable by algae. Such monomers are sources of 'organic' nutrient, such as organic nitrogen from amino acids. While algae prefer inorganic nitrogen, some algal species are known to assimilate amino acids, especially if inorganic nitrogen is not available (Liu and Hellebust, 1974; Admiraal et al., 1984; Admiraal et al., 1987; Nilsson and Sundback, 1996).

After centuries of being exposed to amino acids dissolved in the oceans, marine diatoms have evolved to utilize amino acids as a source of nitrogen. But whether 'fuel-producer' species are also capable of utilizing this type of nitrogen is yet to be investigated. Even though this study does not perform chemical decomposition, it examines the feasibility of growing two green algae, *C. sorokiniana* and *Scenedesmus* sp., on a potential product of such a process, i.e. amino acids. The tested amino acids, including aspartic acid, glutamic acid, alanine, and leucine, serve as the sole sources of nitrogen in the medium. These are the most common amino acids present in the algal cell wall (Punnett and Derrenbacker 1966), therefore they are expected to be present in the biomass residue as well.

4.2. Methodology

4.2.1. Growth Experiments

Growth experiments were conducted at 25ºC, under 50 ìmole·m^{-2}·s^{-1} light with 10:14 light dark cycle, and continuous shaking. Four L-amino acids, including aspartic acid, glutamic acid, alanine, and leucine, were examined. For each experiment, cells were grown in three types of media: amino acid-positive, reference, and blank. The amino acid-positive medium was made of the nitrogen-free BG11 supplemented with one amino acid, the reference medium contained the standard mineral medium (BG11), and the blank medium was made of nitrogen-free BG11, without any type of nitrogen source. Each experiment was performed with four replicates.

4.2.2. Growth Curves

The optical density of the cultures was measured daily by Spectronic 20D+ spectrophotometer, at the wavelength of 600 nm (OD_{600}). This shows the algal growth over the time course of the experiment, providing the data points for the growth curve.

4.2.3. Amino Acid Uptake Determination

A 50µl sample was collected, on a daily basis, from the amino acid-positive cultures under sterile conditions to keep the cultures axenic, and then centrifuged. The supernatant was prepared for the amino acid detection assay. Amino acids were derivatized with OPA-NAC and analyzed with HPLC (Agilent 1100) as previously described with Zhao and Bada (1995). The derivatives of 4 L-amino acids were separated on a Luna 5u C18 column (Phenomenex). However, since each injected sample carried only one amino acid at a time, only one single strong peak was detected. The mobile phases were (A) methanol and (B) 50 mM sodium acetate (pH 7.5). The column was equilibrated with 5% A and 95% B for 5 min after injection. A gradient was started to change the mobile phase to 94% in 6 min, 94% in 7 min, 88% in 8 min, 81% in 9 min, 75% in 10 min, 70% in 11 min, 65% in 12 min, 60% in 13 min, 55% in 14 min, 50% in 15 min, 45% in 16 min, and 40% in 18 to 24 min. It finally switched back to 95% B in 25-30 min, as the system was ready for the next injection. The flow rate was 1 mL/min. The column effluent was monitored with a Ga321A fluorescence monitor at an excitation wavelength of 340nm and an emission wavelength of 450nm. The actual quantities of amino acids in samples were calculated from peak-area integration by using the peak area determined from a known amount of authentic standard.

4.2.4. Algae Compost

Naturally grown biomass of macroscopic green alga *Cladophora,* collected in Summer 2009 from the Flamingo Wash, was decomposed under aerobic and anaerobic conditions and with or without paper as a source of carbon (C:N ratio 4:1). Soil was added as the microbial source. During the incubation time, the biomass kept moist and the internal temperatures of composts were monitored on a daily basis with a thermometer placed deep inside the composting buckets, located in an incubator with the temperature set at 25ºC. When the temperature started dropping, indicating a decrease in microbial activities, the experiment was terminated. After three weeks, the compost was extracted in an equal volume of water. The resultant extracts were centrifuged and autoclaved, ready to be used as growth medium for the studied isolates, either with glucose (1g/L), or without, as a source of energy. A small portion of each extract was saved for chemical analysis. The two algae, *C. sorokiniana* and *Scenedesmus* sp. were inoculated in the sterile compost teas. The cultures were placed at 25ºC under 50 $\mu mole \cdot m^{-2} \cdot s^{-1}$ lights with 10:14 light dark cycle, and with continuous shaking. Optical density was measured twice a day with a Spectronic 20D+ spectrophotometer, at the wavelength of 600 nm (OD_{600}).

4.3. Results

4.3.1. Consumption of and Growth on the Amino Acids

Scendesmus sp. consumed the four tested amino acids (Figure 4.1). In all the experiments, as the amino acid concentration decreases, the cell density of the culture increases until the concentration of the amino acid approaches zero. After this, the algal

cells enter the stationary phase and the cell density no longer changes; the growth rate becomes almost zero. In the cultures containing aspartic acid and alanine, the growth is stimulated compared to the reference.

Unlike *Scendesmus* sp., *C. sorokiniana* consumed only leucine and aspartic acid, but not alanine nor glutamic acid (Figure 4.2). In the cultures containing aspartic acid and leucine, the cell density increases as the concentration of the amino acid decreases, and becomes constant as amino acid concentration approaches its minimum level. In the culture with aspartic acid, a slight stimulation in growth is observed, compared to the reference curve which is the growth curve in the nitrate-containing BG11 (shown by triangles). In the cultures that contained alanine or glutamic acid, the concentration of the amino acid does not change, indicating no uptake. The growth curve of these cultures is identical to the growth curve of the blank, lying far below the reference curve.

The amino acids did not inhibit growth in any of the experiments. A slight growth in the blank cultures might be due to the nitrogen contamination.

4.3.2. Growing the Isolates in the Extracts from Algal Compost

The compost teas supported the growth of both *C. sorokiniana* and *Scendesmus* sp. The extract from the aerobic compost, with or without paper, supported a robust growth rate of *C. sorokiniana* when supplemented with glucose (Figure 4.3A, columns 2 and 4 from left). For *Scendesmus* sp., even without glucose supplement, the tea from the aerobic compost with paper could also support a significant growth rate (Figure 4.3B, column 3 from left).

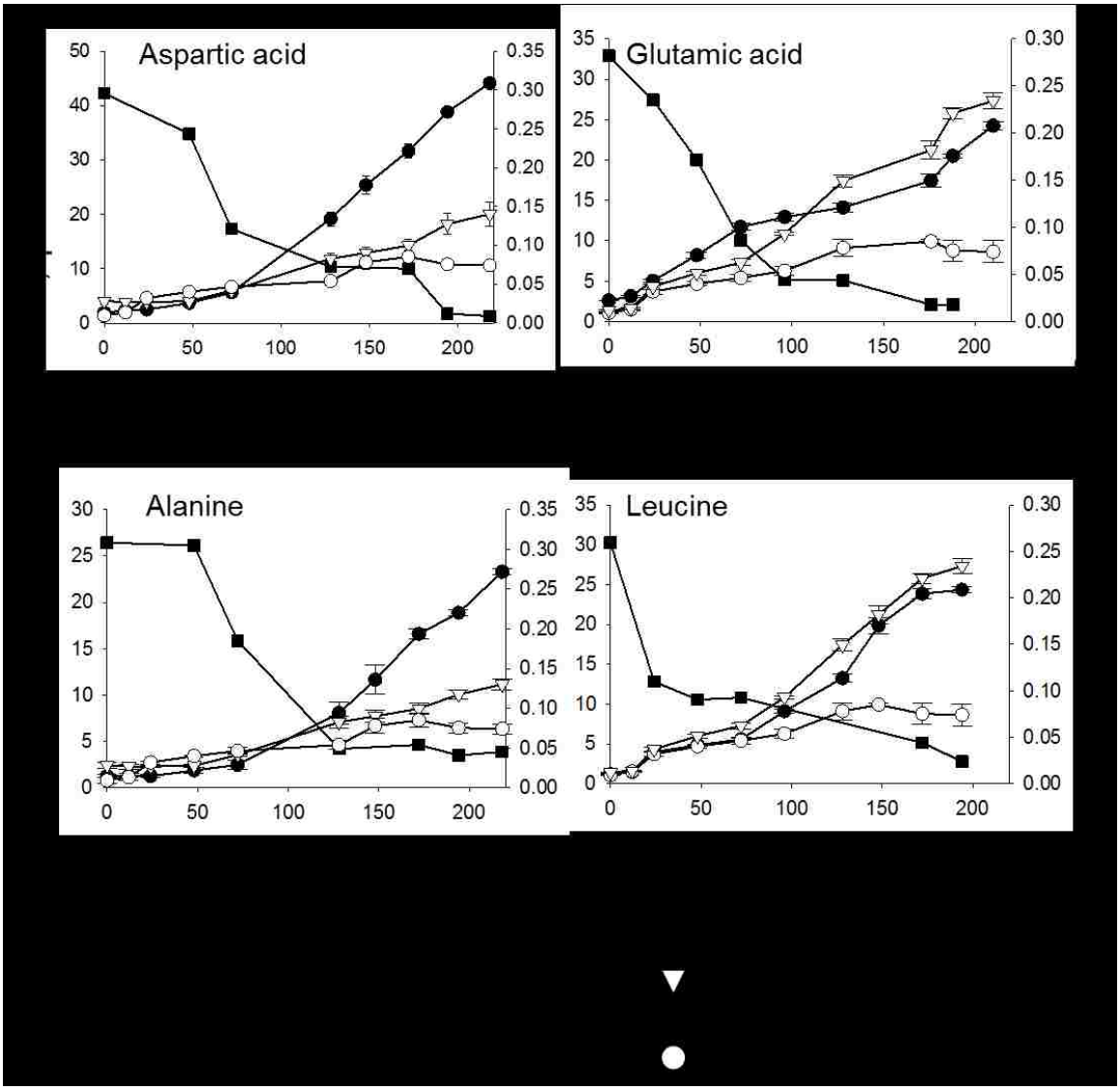

Figure 4.1. Growth curves of *Scendesmus* sp. in the amino acids-positive (filled circles), the reference (open triangles), and the blank (open circles) media, along with the amino acid uptake diagram (filled squares). Amino acid is the sole nitrogen source in the amino acid-positive media, nitrate is the nitrogen source of the reference, and no nitrogen source exists in the blank.

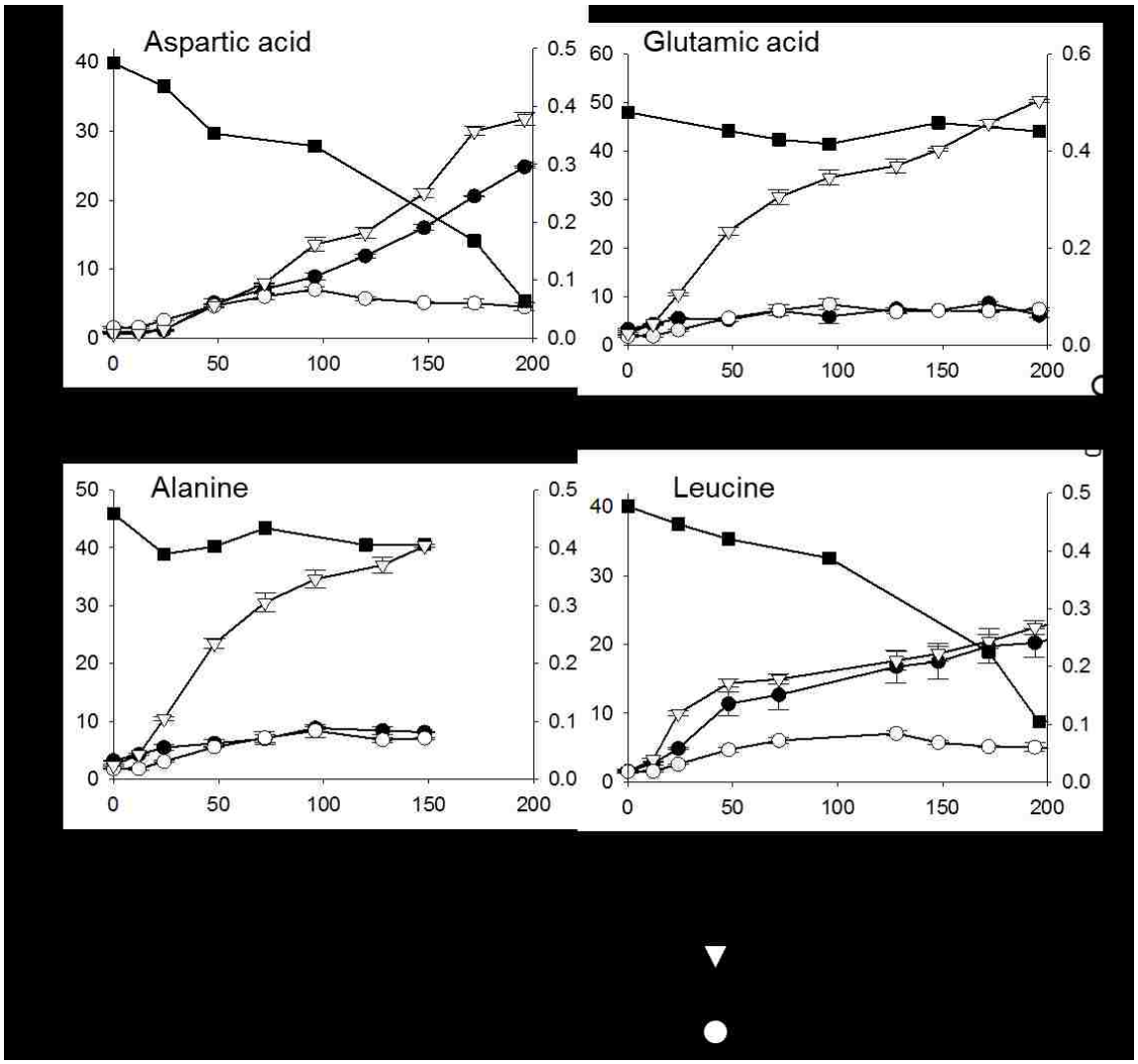

Figure 4.2. Growth curves of *C. sorokiniana* in the amino acids-positive (filled circles), the reference (open triangles), and the blank (open circles) media along with the amino acid uptake diagram (filled squares). Amino acid is the sole nitrogen source in the amino acid-positive media, nitrate is the nitrogen source of the reference, and no nitrogen source exists in the blank. *C. sorokiniana* consumed aspartic acid and leucine, but not glutamic acid and alanine. Also, growth occurred only in these two cultures, meaning that cells gained the required nitrogen from amino acids. In the glutamic acid and alanine cultures, cells didn't grow as they were not able to obtain their needed nitrogen from amino acids, although there was not any other nitrogen source in the cultures.

Tea from aerobic, paper-less compost supported some growth (Figure 4.3A&B, first column from left). *C. sorokiniana* showed signs of stress, in the form of clumping, and *Scenedesmus* sp. showed a very long lag phase, ~172h. The growth of *C. sorokiniana* was not registered in optical density due to cell clumping. Adding glucose rescued the algae (Figure 4.3 A&B, second column from left). When, instead of receiving glucose, the tea was treated with H_2O_2 (1:1 v/v), the toxicity also disappeared (Table 4.1), suggesting that the potential source of toxicity is organic in nature, possible pheophytin, an oxidation product of chlorophyll.

Tea from anaerobic, paper-containing compost did not support any growth (Figure 4.3 A&B, first and second columns from right). In contrast, tea from the compost that is also anaerobic, but paperless, supported algal growth equivalent to BG11, horizontal dashed lines (Figure 4.3 A&B, fourth column from right). Adding glucose to this tea reduced the growth rate of *C. sorokiniana* but stimulated the growth of *Scenedesmus* sp. (Figure 4.3 A&B, third column from right).

The highest growth rate for *C. sorokiniana* occurred in the extract from aerobic compost supplemented with glucose, with or without paper. For *Scenedesmus* sp., the highest growth happened in the extract from the aerobic compost with paper, but no glucose. Comparing their highest growth rates, *C. sorokinian*'s was slightly higher than that of *Scendesmus* sp.

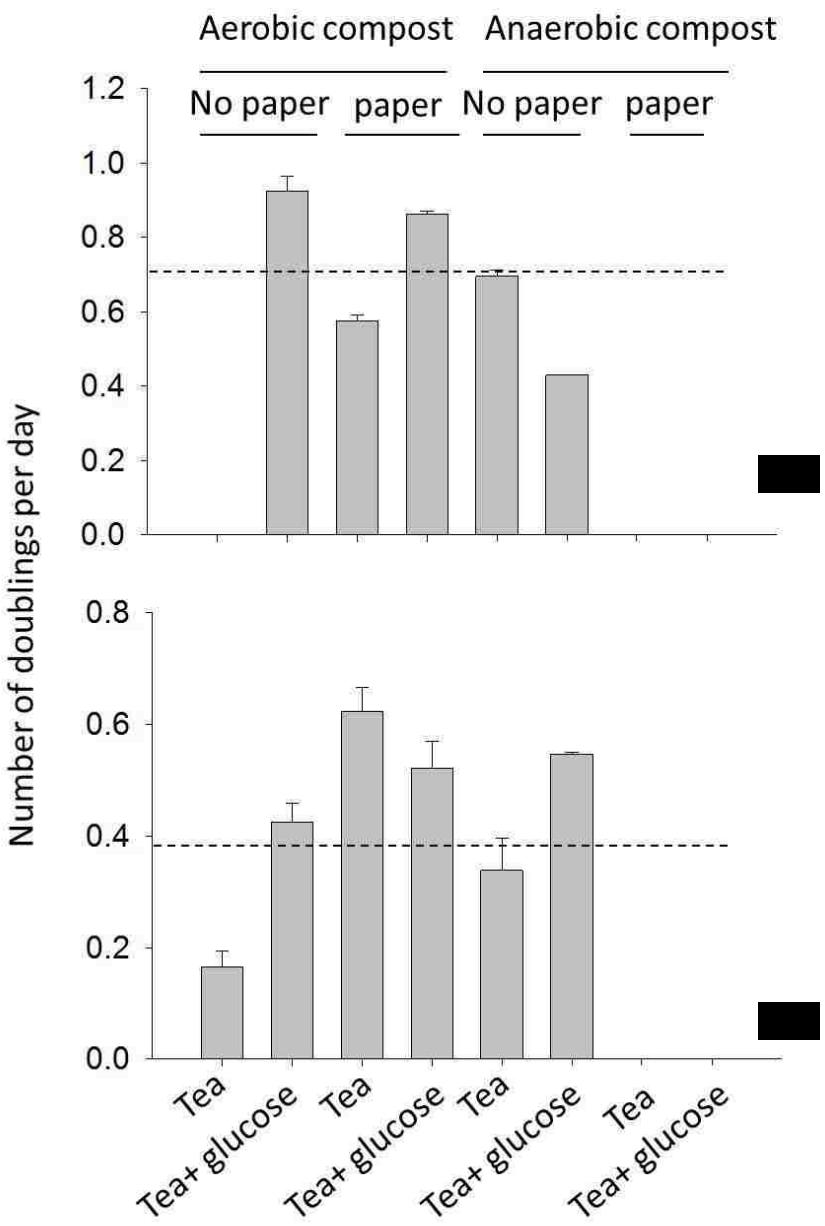

Figure 4.3. Growth rate of (A) *C. sorokiniana* and (B) *Scenedesmus* sp. in 8 extracts of algal composts. The highest growth rate of *C. sorokiniana* occurred in the extract from aerobic compost without paper, with glucose. For *Scenesedmus* sp., the highest growth rate was detected in the extract from aerobic compost with paper, without glucose. This means that presence of paper, as a source of carbon, turned on the mixotrophic growth mode, stimulating growth.

Table 4.2 compares chemical characteristics of anaerobic paper-containing compost tea to BG11. As highlighted in gray, the extract contains large quantities of nitrogen and phosphorus, orders of magnitudes greater than those in BG11. Plus, the tea contains a high amount of acetate, a source of organic carbon that could stimulate algal growth.

Table 4.1. Growth rate (number of doublings per hour) of the two isolates, *C. sorokiniana* and *Scendesmus* sp., in different compost teas. O_2 represents oxygen, C represents carbon, G represents glucose, and plus and minus signs represent the presence and absence, respectively. For example, O_2(+)C(+)G(-) means aerobic compost with paper without glucose. NG means no growth. * The growth rate in the extract after treated with H_2O_2.

Compost tea	*C. sorokiniana*	*Scendesmus sp.*
O_2(+)C(-)G(-)	0.022*	0.0096
O_2(+)C(-)G(+)	0.0385	0.0177
O_2(+)C(+)G(-)	0.024	0.026
O_2(+)C(+)G(+)	0.036	0.0217
O_2(-)C(-)G(-)	0.029	0.01408
O_2(-)C(-)G(+)	0.0179	0.02277
O_2(-)C(+)G(-)	NG	NG
O_2(-)C(+)G(+)	NG	NG

Table 4.2. A comparison between the chemical components of the extract from anaerobic algal compost with paper without glucose (O_2(-)C(+)G(-)) and the mineral medium of BG11. ND and NA mean non detectable and not available, respectively. The highlighted components point out the potential nutritious and inhibiting substances to the algal growth present in this compost tea.

Characteristics	O_2(-)C(+)G(-)	BG11
NH_4, µM	1191.0	20
SiO_4, µM	2252.2	NA
NO_2+NO_3, µM	86.5	NA
Lithium , µM	6.8	NA
Sodium , µM	8494.6	17.600
Ammonium , µM	881.5	20
Potassium , µM	21446.1	110
Magnesium , µM	8594.0	30
Calcium , µM	87947.4	200
Fluoride , µM	ND	NA
Chloride , µM	14244.6	105
Nitrite , µM	ND	NA
Bromide , µM	ND	NA
Nitrate , µM	ND	17,600
Phosphate , µM	935.2	220
Sulfate , µM	334.3	15
Acetate , µM	152223.6	NA

4.4. Discussion

The results of this study revealed the potential of growing *Scenedesmus* sp. and *C. sorokiniana* in medium composed of algal biomass residue, either biologically or chemically decomposed. Amino acids served as the sole source of nitrogen. In the cultures containing amino acids, the decrease in amino acid concentration and the

increase of algal cell density are nicely synchronized, implying that the algae obtained their required nitrogen from amino acids. Cells grow fast as long as there is enough amino acid in the culture, yet by the time that the amino acid is depleted, cells stop growing. No growth was observed in cultures in which uptake was not detected. The results demonstrate a considerable stimulation in the growth of *Scenedesmus* sp. in the presence of aspartic acid and alanine, indicating the mixotrophic growth. It appears that the algal cells consumed both the nitrogen and carbon components of these two amino acids, the former as nutrient and the latter as an extra energy source. Likewise, the growth of *C. sorokiniana* was stimulated in the presence of aspartic acid, representing the mixotrophic growth mode.

The inflexibility of *C. sorokiniana* cells in metabolizing different amino acids, i.e. consuming only two amino acids, led to their death in some cases due to the lack of nitrogen. It is hypothesized that the alga forms L-amino acid oxidases (LAAOs) specific to the type of amino acid outside the cell. For instance, the LAAOs that are formed to oxidize L-aspartic acid may not oxidize L-glutamic acid, and so on. Two of the oxidation products, ammonium and α-keto acid, are recycled as carbon and nitrogen, while the third one, hydrogen peroxide, is converted to oxygen and water. To determine if this hypothesis is correct, additional experiments were performed in the presence of D-amino acids and with an additional green alga, *Euglena gracilis*. Since this is not directly related to biofuel and was conducted purely for the sake of science, the results are presented separately, in the Appendix B.

The compost teas, i.e. the products of biotic degradation, supported the algal growth. The results of this study revealed significant stimulation in the growth of *C.*

sorokiniana and *Scenedesmus* sp. in the tea from the aerobic compost supplemented with glucose (with or without paper), indicating mixotrophic growth.

Stress signs of *C. sorokiniana* and, less severely, of *Scenedesmus* sp. grown in tea from the aerobic compost without paper or glucose, went away after being treated with hydrogen peroxide. Since the olive-gray color of the compost tea disappeared after the treatment, pigments that caused such color are considered to be the source of stress and inhibition. Pheophytin generates such color. Pheophytin is a chlorophyll molecule lacking a central Mg^{+2} ion that can be produced from chlorophyll. The reason why pheophytin inhibits the algal growth is not known. However, in a cancer-related study, chlorophyll groups (chlorophyll, pheophytin, pyropheophytin, and phophorbide) significantly inhibited cancer developments (Chernomorsky *et al*., 1999). Although this information cannot justify the inhibitory effects of the chlorophyll group on algae growth, it can help to understand it. It is speculated that the chlorophyll family could form complex molecules with the substances that are essential to algal growth and thus reduce or totally eliminate the bioavailability of those compounds, the same way they acted against the cancer mutagens. To assure that phaophytin caused the stress, it would be useful to determine the pheophytin concentration of the compost tea before and after H_2O_2 treatment. The level of pheophytin is expected to be reduced significantly after the treatment. This experiment required fluoresce spectrometer that was not available during this study. However, even if it is proved that the inhibition comes from the chlorophyll group, the mechanism of such inhibition would still remain undiscovered. This necessitates more studies in the level of molecular biology to understand the exact effects of these molecules on the structure of the cells.

Inhibition also occurred in the tea from the anaerobic compost with paper, with or without glucose. Table 4.2 presents the components of this compost tea, some of which are much higher than the potential sources of inhibition in BG11. First, relatively high levels of sodium chloride could cause osmotic stress and thereby growth suppression. However, the results from chapter 2 rule out this possibility. The chapter 2 results also showed no growth rate reduction in response to sodium chloride until it reached 40mM. The second possibility could be the presence of ammonium in the absence of nitrate. Still, the results from ammonium experiments in chapter 3 showed only a slight growth inhibition. Therefore, this level of ammonium could not cause a complete inhibition on growth. Finally, excessive amounts of calcium existing in paper used as the cellulose source could cause the inhibition. Calcium carbonate, i.e. the most common additive being used as whitening agent in common papers (Sundara-Rajan *et al*., 2004), remains in paper and leaches into the compost and then into the compost tea. Calcium is a chaotropic agent. In the Hofmeister series (shown in the box), calcium followed by magnesium was identified as the second strongest chaotropic cations, after guanidinium. Chaotropic agents destabilize the enzymes and denature the protein (Von Hippel and Wong, 1965; Herberhold et al., 2004; Zhang and Cremer, 2006). In contrast, kosmotropic agents can stabilize the enzymes. In the anion series of Hofmeister, species to the left of chloride are kosmotropic, with SCN^- being the strongest. Sufficient amounts of kosmotropes in a system can detoxify the chaotropes.

Hofmeister series

Kosmotropes (detoxifier) ← ----------→ Chaotropes (toxin)

Anion series 

Cation series 

Earlier studies have speculated that chaotropic ions affect the bulk property of water (Zhang and Cremer, 2006). Recent evidence contradicts this early notion. Instead, chaotropic effects appear to rise from direct interactions between such ions and enzymes. Functional enzymes are folded, typically with the hydrophobic core of the center. Hydrogen bonds are the key to the folding. Emerging evidence indicates that chaotropic agents such as calcium and magnesium have a "salt in" effect, disrupting hydrogen bonds and causing the hydrophobic core to collapse. Conversely, kosmotropic agents have the effect of "salt out", helping stabilize protein folding (Herberhold et al., 2004; Zhang and Cremer, 2006). To shed some light on this, an additional experiment was conducted in which BG11 was supplemented with 10 mM calcium chloride. As it is shown in Figure 4.4, the algal growth was significantly inhibited by this amount of calcium. Also, in the presence of calcium, the *C. sorokiniana* cells became stressed (Figure 4.5, tube on the right); instead of a healthy suspension (Figure 4.5, tube on the left), the cells aggregated into clumps and grew attached to the wall of the test tube. Such stress signs and inhibition occurred in the presence of only 10 mM of calcium, while the concentration of calcium present in the extract is almost 10 times higher, ~90mM, which could cause more severe damage to the cells and possibly completely inhibit growth. As stated before, kosmotropic agents can detoxify chaotropic cations. Based on Hofmeister seris, sulfate is

a strong kosmotrope. The compost tea contain sulfate, yet its concentration is too low to be able to detoxify calcium.

The above discussion might raise this question: why such serious inhibition was not observed in the aerobic compost teas containing paper? It is believed that anaerobic conditions reduce the level of sulfate significantly, most likely due to the presence of sulfur bacteria. Unlike these compost teas, the aerobic compost teas contain quite high amounts of sulfate, sufficient for detoxifying calcium.

Other elements, including trace metals, shown in Table 4.2 did not contribute to toxicity because they are not considered chaotropic agents or toxic.

Considering the above evidence, the author is of opinion that the source of toxicity in the compost teas is the high concentration of calcium. Hence, paper was not a good choice as a cellulose source.

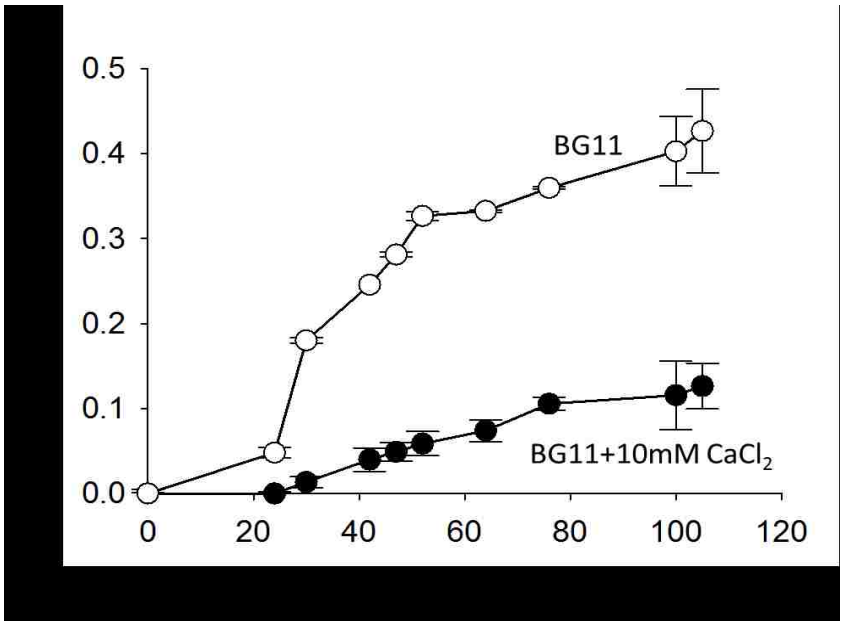

Figure 4.4. Effect of calcium on growth of *c. sorokiniana*. Even a small amount of calcium (10mM) could significantly inhibit growth. This is about 9 times smaller than the amount of calcium existing in the O_2(-)C(+)G(-) extract, which was completely inhibitory to growth.

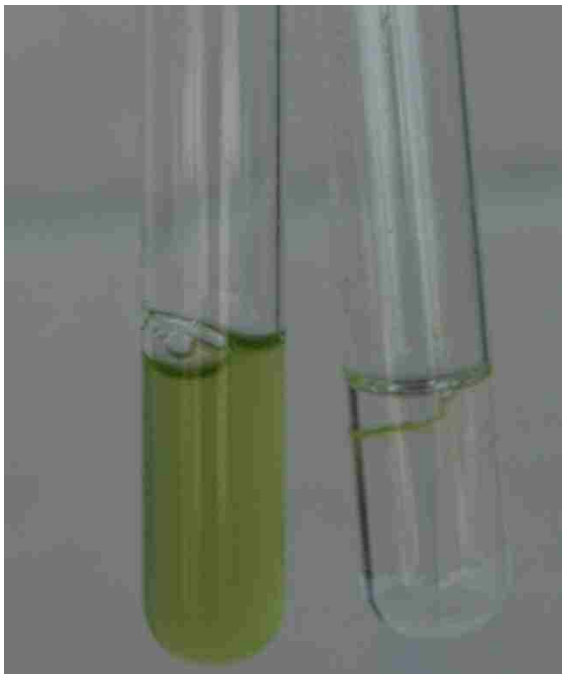

Figure 4.5. Algal growth in medium containing BG11 (on the left) and 10mM of calcium (on the right). While cells are nicely suspended in the BG11, they are attached to the wall in the presence of calcium.

CHAPTER 5

MANIPULATION OF GROWTH CONDITIONS FOR HIGHER LIPIDS PRODUCTION

5.1. Introduction

The amount of lipids in an algal cell is a dynamic feature, at least in some species. This was first shown by Spoehr and Milner (1948) working on the green alga *Chlorella pyrenoidosa* in an attempt to understand how the growth environment affects the relative proportions of its lipid, protein, and carbohydrate content. The authors expressed these proportions in a simple measurement– the amount of molecular oxygen required to oxidize a given amount of biomass to carbon dioxide, expressed as the R-value. Because lipids contain less oxygen than proteins and carbohydrates, cells rich in lipids require more oxygen to oxidize. In other words, the R-value is an approximation of lipid content. The relationship between the R-value and several environmental factors, including the level of CO_2, nutrient starvation, temperature, and light, was investigated. Cells raised at 3% CO_2 had a higher R-value than those raised at 0.04% CO_2 (i.e. ambient air). However, higher CO_2 concentrations, 5% or 10%, resulted in little further increase in the R-value. Differences in growth temperature, from 15 to 30°C, had a little effect on the R-value. When a culture was aged for 112 days, the R-value increased by 12%, due to nutrient starvation. The effect of nitrogen starvation on lipid content in another *Chlorella* species, *C. sorokiniana*, was also investigated by Richardson *et al.* (1969). In batch culture, fatty acids rose from 57 to 137.8 mg/g dry weight, which was attributed to nitrate depletion. In continuous culture, in contrast, the differences in nitrate levels (20, 10, and 5 mM) had no effect on fatty acid content, likely because none of these levels were limiting. While Spoehr and Milner (1948) showed that, in contrast, for the green alga *C.*

pyrenoidosa growth temperature does not affect lipid content, Kleinschmidt and McMahon (1970) showed that in the red alga *Cyanidium caldarium* temperature does affect lipid content. In this case, cells grown at 55°C, the optimum temperature for the organism, contained 20 mg lipids per gram dry weight. Cells grown at 20°C contained 50 mg lipids per gram dry weight, a 2.5-fold increase.

Most of the studies conducted after the 1980s focused on increasing lipid production through manipulating two factors of nutrient level and light intensity because, from the algal biodiesel production standpoint, these two are more practical to be varried than other environmental factors. Lien and Roessler (1986) demonstrated that lipid content of green algae *Chlorella* sp. and *Ankistrodesmus* sp. and golden alga *Chryosphyte* sp. can be raised two- to three-fold via nitrate limitation. James *et al.* (2011) showed that when green alga *Chlamydomonas reinhardtii* was transferred from a nitrate-sufficient medium to a nitrate-omitted medium, cells division was halted but lipid bodies started to appear. Merzlyak *et al.* (2007) also reported that, after one month of incubation, lipid content of green alga *Parietochloris incise* grown in a nitrate-omitted medium was one order of magnitude higher than that in cells grown in a nitrate-sufficient medium. Zhekisheva *et al.* (2002) demonstrated that in green alga *Hamematococcus pluvialis*, astaxanthin (mostly mono-esterified) and triglycerides, i.e. esters derived from glycerol and fatty acids, increased when cells were grown in a medium with no nitrate. Under nitrate deprivation, production of each pico-gram of astaxanthin was accompanied by production of 5 pico-grams of fatty acids. After 8 days of incubation, while only a slight growth occurred in the nitrate-free medium, fatty acids level increased by 30%. In contrast, in the nitrate-sufficient medium, no change in fatty acid content was observed,

over the same time course of incubation. Pruvost *et al.* (2009) demonstrated that lipid content of green alga *Neochloris oleoabundans* increased from 17% to 37% of dry weight during progressive nitrate deprivation. However, Feng *et al.* (2011) found an increase in lipid content of marine alga *Isochrysis zhangjiajiensis* under excessive nitrate. They compared the lipid content of cells grown in three batch cultures: one with daily nitrate replenishment, another with nitrate replenishment in every two days, and the other one with nitrate replenishment in every three days. The highest lipid content was attained in the culture with daily nitrate replenishment. Even though these authors believed that such high lipid content was due to extra nitrate, it is also possible that it was due to phosphate depletion. Phosphate could be depleted quickly because nitrate was abundant and thus growth was fast. Indeed, Khozin-Goldberg and Cohen (2006) showed that phosphate limitation caused higher lipid content in *Monodus subterraneus*. They reported that in the cultures containing 52.5 and 17.5 µM phosphate, fatty acid content was twice as high as that of the 175 µM phosphate and was 2.7 times higher in the absence of phosphate. However, Chen *et al.* (2010) reported a different result. Lipid content of marine alga *Dunaliella teriolecta* grown in a phosphate-free Erdschreiber medium was similar to the lipid content in cells grown in a standard Erdschreiber medium. They speculated that cells had internal phosphorus storages sufficient for their need during the experiment. In this author's opinion, it is possible that, under some circumstances, this type of alga stored the excess carbon in a form different from lipids, e.g. starch. As a part of the same study, Chen *et al.* (2010) reported consistent results with other studies in respect to nitrogen deficiency. In the nitrate-free medium, lipid

content of *D. teriolecta* peaked by the third day and then dropped down by the day 7, whereas cells grown in the standard medium showed their largest lipid content on day 7.

Based on the literature published so far, there is little doubt that once algae run out of nutrients and stop multiplying in numbers, they begin accumulating lipids and triglycerides. What is unclear from the past experiments, because of the way they were carried out, is exactly which nutrient ran out causing this change. For example, when two cultures, one with limited nitrogen and one with abundant nitrogen, are both allowed reaching the stationary phase, as is customarily done, they may not be entirely comparable. In the former case, the onset of the stationary phase may indeed be caused by nitrogen depletion. The same is not necessarily true of the latter, however. Because it contains more nitrogen without other ingredients being proportionally raised, something else, for example phosphorus, is more likely to be depleted first. This is a limitation associated with the batch system. A better alternative is a flow through culture where the nutrient concentrations are kept constant throughout the experiment.

Lacking access to a flow through system, this study attempts to use the batch culture in an improved way, by making the lipid measurements at the logarithmic growth phase. At this early stage of the growth, lipid content, as well as growth rate, is still controlled by the starting nutrient concentration. In addition, for each of the algae under study, the saturating nitrate and phosphate concentrations are known from the chapter 2. The methodological improvement, plus the diversity of algae available, allows the effects of nutrient limitation and of different genetics on algal lipid content to be better defined.

This study also demonstrates a universal pattern in which lipid content is negatively correlated to the growth rate. It is hypothesized that changes in the growth rate are accompanied by changes in lipid content, but in an opposite direction. Therefore, growth rate and lipid content of the six isolates, including green algae *Chlorella sorokiniana* and *Scenedesmus* sp., diatoms *Encyonema caespitosum* and *Nitzchia thermalis,* and cyanobacteria *Synechocystis* and *Limnothrix,* are determined in response to changes of nitrogen and phosphorus.

After nutrients, light intensity has the most influence on lipid content. Spoehr and Milner (1948) showed that while growth rate of *C. pyrenoidosa* increased with light power, in a range of 25 to 100 Watt, the R-value decreased and reached down a minimum level and then increased again. Within this range, the R-value changed more quickly under stronger light: the stronger light, the shorter time was needed to reach the minimum R-value. For example, under 100 Watt light power, the R-value reached the minimum level in 9 days, while under 40 Watt, it reached the same minimum level after 32 days. Zhekisheva *et al.* (2002) demonstrated that in green alga *Hamematococcus pluvialis,* cells grown under 350 $\mu mole.m^{-2}.s^{-1}$ contained higher lipid content than those grown under 75 $\mu mole.m^{-2}.s^{-1}$. Under both conditions, the maximum lipid content was attained on day two of the 6 days incubation. Runagsomboon (2011) studied the lipid content of green alga *Botryococcus braunii* under various intensities of 0.3, 87.5, 200, and 538 $\mu mole.m^{-2}.s^{-1}$. Under the highest intensity (538 $\mu mole.m^{-2}.s^{-1}$), the highest lipid content was obtained. However, cultures exposed to low light intensity of 87.5 $\mu mole.m^{-2}.s^{-1}$ showed the highest biomass. Yeesang and Cheirsilp (2011) found that in four strains of *Botryococcus* grown under 33, 49.5, and 82.5 $\mu mole.m^{-2}.s^{-1}$, the highest

lipid content of the four strains were obtained under 49.5 µmole.m^{-2}.s^{-1}. All four strains showed photoinhibition under high light intensity, 82.5 µmole.m^{-2}.s^{-1}.

Prior findings revealed that the effect of light on algal growth is as complicated as on lipid production of algae. While algae require the energy from light to grow, they sometimes become suppressed by high light levels, a phenomenon known as photoinhibition. Jones and Kok (1966) verified that strong light can cause the loss of all chloroplast activities in some algae. Such high light level can damage photosystem I and II and deactivate some of the light-dependent processes, yet the actual mechanism remains unclear. Algae across the major groups respond to light differently. Most green algae grow well under strong light, while cyanobacteria prefer dim light. Li *et al.* (2010) showed that growth rate of green alga *Pseduchlorococcum* sp. increased with light, experiencing no photoinhibition. Also, Sorokin and Krauss (1961) demonstrated that the growth of green alga *C. pyrenoidosa* was proportional to light intensities, ranging from zero to 3000 ft-ca, facing no photoinhibition. In contrast, cyanobacteria *Merismopedia tenuissima* and *Oscillatoria* sp. grew well under low light intensity and became inhibited by high light intensities (Konopka and Schnur, 1980; Konopka, 1983).

In spite of all the above-mentioned complexities, this study offers a uniform, simple pattern that describes and explains variations in lipid content, as well as in growth rate. It is hypothesized that when light level changes, there is a *negative* correlation between growth rate and lipid content, a pattern similar to what is proposed for nutrients. Therefore, growth rate and lipid content of the six isolates of this study is determined simultaneously in response to a light gradient.

5.2. Methodology

5.2.1. Growth Rate Determination

Logarithmic growth rate, *k*, expressed as number of doublings per day, was calculated according to the following equation of Sorokin and Krauss (1958):

$$K = (\log_2(N_2 / N_1))/t \qquad \text{(Eq. 5.1)}$$

Where N_2 and N_1 are the biomass quantity at the beginning and end of a time interval (t). The biomass quantity was measured in protein per volume culture, quantified by the use of the Folin's phenol reagent (Lowry *et al*., 1951). 1mL of Lowry stock solution, made of 49 mL solution of 2% Na_2CO_3 in 0.1 M NaOH, 0.5 mL of 1% $CuSO_4$ in distilled water, and 0.5 mL of 2% sodium potassium tartrate, were added to cell pellet. The tubes were left for 30 min in a hot water bath. After that, 100 µL of Folin's reagent (1 N) was added to each tube, and incubated them for 30 min at room temperature. Absorbance was read in a spectrophotometer at 595nm. The protein concentration was quantified by applying the standard calibration curve.

5.2.2. Lipid Content Measurement

Neutral lipids were determined by the improved Nile Red method of Chen *et al*. (2009). Briefly, 1 mL of 25% (v/v) DMSO and 50µL of NR reagent (10 µg/mL NR in acetone) were added to cell pellet, which was then kept at 35C for 10 min. Fluorescence intensity was measured in a fluorometer at 530 nm excitation and 575 nm emissions. To calculate the lipid content (amount of lipid per cell), each number obtained here was divided to its relative protein amount (refer to chapter two for the protein measurement protocol).

5.2.3. Light Experiment

Test tubes containing algal culture were wrapped in layers of wire mesh to create different light levels. The source of illumination was provided from the side by a bank of 100W incandescent light bulbs, with a 10L/14D cycle. To prevent the heating of cultures, the test tubes were placed in a small water bath at their optimum temperature. Before the experiments started, the light intensity that each tube could receive, underneath the mesh layers, was detected by a flat model L1-1400 light meter and data logger, able to detect all the photosynthetic range. The highest light intensity, received by the tube with no mesh layer, was comparable to summer sunlight intensity around noon.

5.2.4. Nutrients Experiment

Growth rates as a function of nitrate concentrations were determined. Series of liquid media containing various amounts of nitrate were inoculated, while all other parameters were sufficient. Each culture grew at a rate different from other cultures. Similarly, phosphate requirement was studied by varying the amounts of phosphate in a set of liquid media, while other parameters were sufficient.

5.3. Results

5.3.1. Effect of Nitrate on Lipid Content

The six studied isolates displayed a similar pattern of oil content variations in response to nitrate gradients. The lipid content varied only if nitrate was limiting, but not if it was sufficient. In the cells grown under various degrees of nitrate deficiency, i.e. 0-100µM, the lipid content was inversely correlated to the nitrate level and the growth rate. As nitrate was raised from near zero to the saturation level (~100µM), the growth rate

increased, but the lipid content decreased. At higher nitrate levels, the growth rate became constant, as did the lipid content (Figure 5.1 A-F).

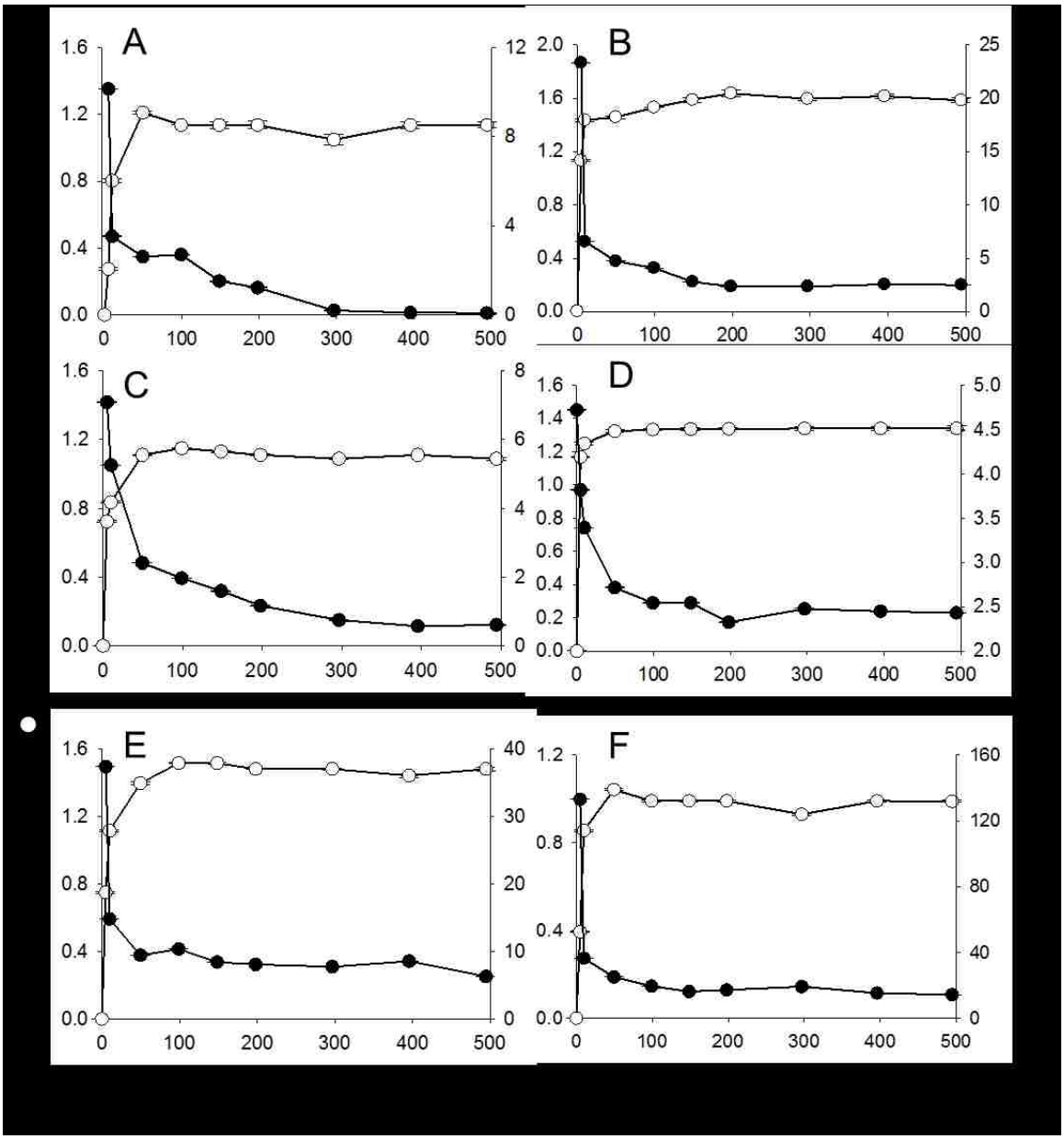

Figure 5.1. Negative correlation between growth rate and oil content, in response to nitrogen gradients. While nitrate was limited, in a range of zero to the saturation level (~100µM), lipid content was inversely related to growth rate. Beyond such level (>100µM), the growth rate did not change, neither did oil content. (A) C. *sorokiniana*, (B) *Scenedesmus sp.*, (C) *Synechocystis* sp., (D) *Limnothrix* sp., (E) *E. caespitosum, and* (F) *N. thermalis.*

5.3.2. Effect of Phosphate on Lipid Content.

In five of the organisms, including *C. sorokiniana, Scendesmus* sp., *Limnothrix* sp., *E. caespitosum*, and *N. thermalis*, the lipid-phosphate relationship was nearly the same as the lipid-nitrate relationship. The growth rate varied only if phosphate was below the saturation level, ~100µM (Figure 5.2 A,B,D,E,F). Within this range, the oil content was negatively correlated with the phosphate concentration and the growth rate. As phosphate concentration was increased from zero to below the saturation level, the cells divided faster, but contained less lipids. Beyond the saturation point, more phosphate did not lead to a higher or a lower growth rate. At this point, the lipid content no longer varied. The only exception was *Synechocystis* sp., in which the lipid content did not change with the phosphate level at all, yet the growth rate did (Figure 5.2C).

5.3.3. Effect of Light Level on Lipid Content.

Both lipid-light and growth-light relationships were dissimilar among the cultures of the different algal groups. In the two green algae, the light curve consisted of a light sensitive part where the growth rate and lipid content changed rapidly with the light, and a light insensitive part where the growth rate increased and lipid content decreased slowly as light level increased largely. No photoinhibition occurred (Figure 5.3 A,B). Unlike the green algae, the two cyanobacteria grew most under low light and contained the least amount of lipids. In *Synechocystis* sp., photoinhibition occurred at a relatively moderate level of light, i.e. 50 µmole/m^2.s, below which both growth rate and lipid content were constant. Beyond this critical threshold, the growth rate decreased rapidly while lipid content spiked (Figure 5.3C). *Limnothrix* sp. required a light intensity close to darkness (2 µmole. $m^{-2}.s^{-1}$), as even low intensities were too high for the cells. This made it hard

to get data points below 2 µmole. $m^{-2}.s^{-1}$ (Figure 5.3D). In this cyanobacterium, the growth rate decreased with the light level, while lipid content increased. In contrast to the green algae and the cyanobacteria, the diatoms preferred the moderate light levels. The light curve comprised of a light sensitive phase, at which the growth rate increased and lipid content decreased rapidly with the light, a light insensitive phase, at which both growth rate and lipid content were constant, and a photo-inhibited phase, where growth was suppressed but lipid content increased (Figure 5.3 E,F).

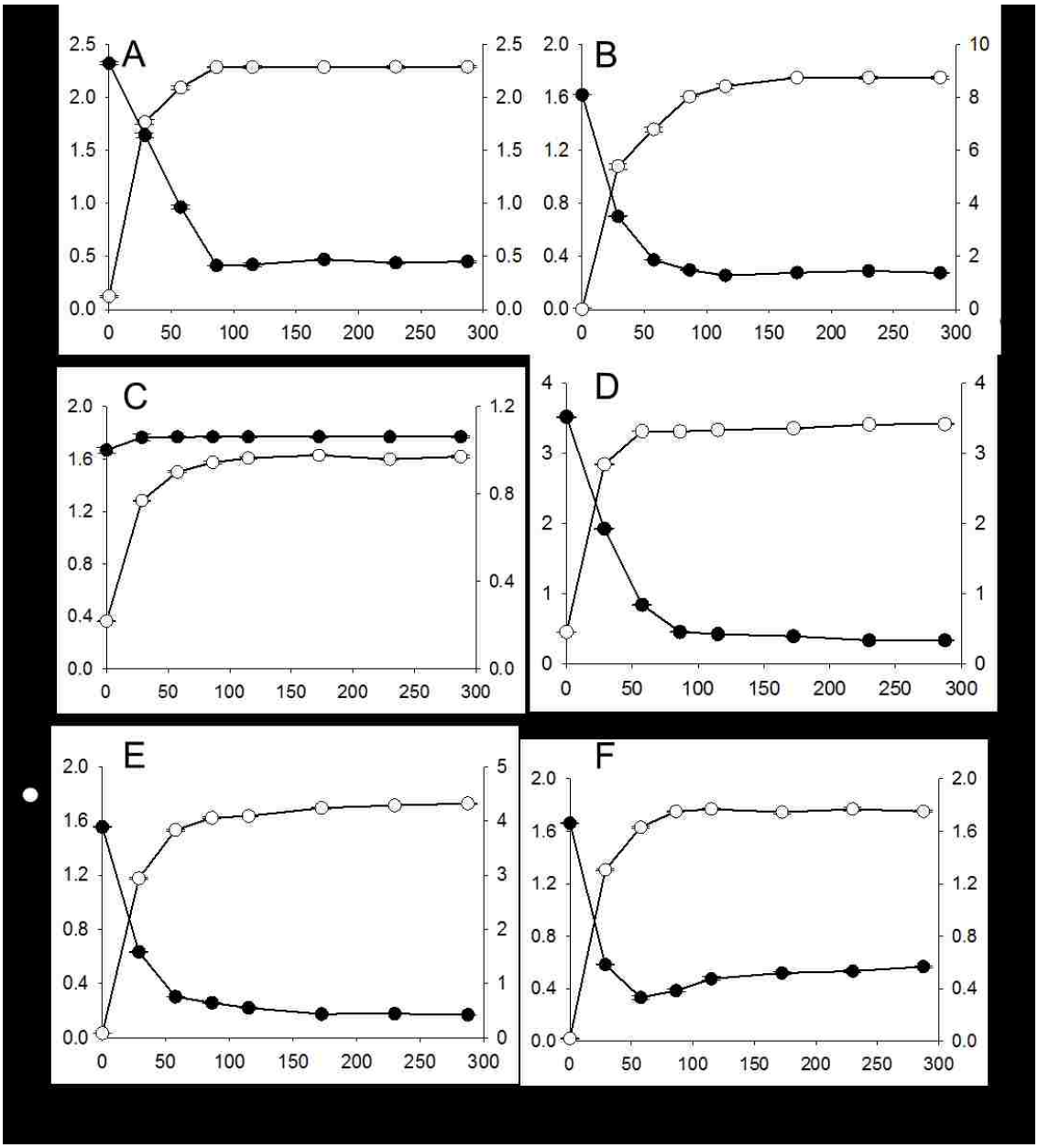

Figure 5.2. Except for (C), in which oil content was constant, oil content of other species was negatively correlated to growth rate, as long as phosphate was limiting, i.e. <100µM. Above this level, growth rate did not change, neither did oil content. (A) C. sorokiniana, (B) Scenedesmus sp., (C) Synechocystis sp., (D) Limnothrix sp., (E) E. caespitosum, and (F) N. thermalis.

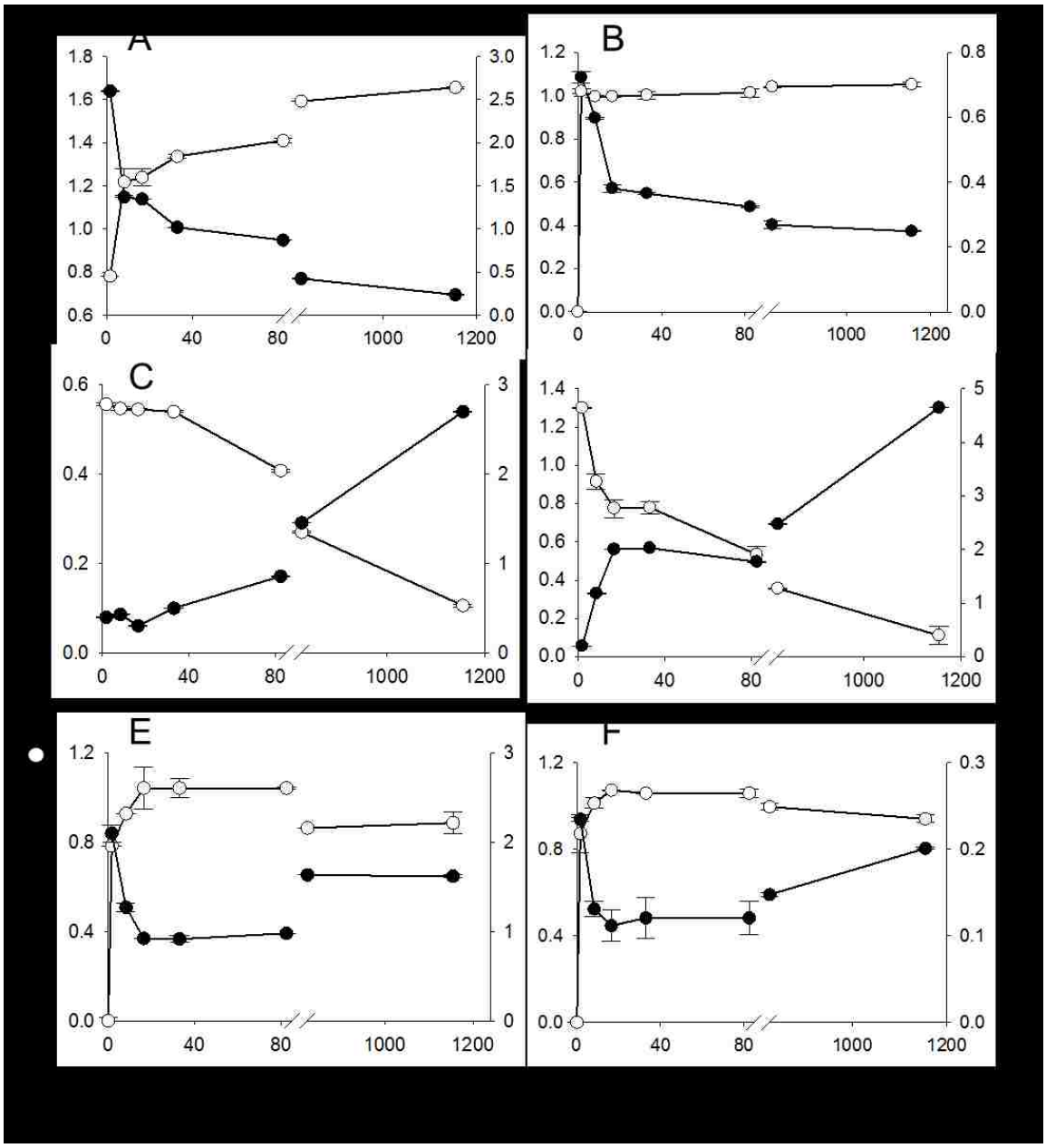

Figure 5.3. Correlation between oil content and growth rate, in response to the light variations. In the green algae (A) C. sorokiniana and (B) Scenedesmus sp., growth rate increased with light, reaching the highest amount at high intensities. In an opposite direction, lipid content was maximal at low light and decreased with light until it reached its lowest amount at high light intensities. In the cyanobacteria (C) Synechocystis sp. and (D) Limnothrix sp., growth rate decreased with light intensity, became inhibited with high light, comparable to the Summer sunlight at noon. But, lipid content increased. In the diatoms (E) E. caespitosum, and (F) N. thermalis, growth rate increased with light, became constant, and then decreased with light. In an exact opposite direction, lipid content decreased with light, stayed constant, and then increased with light.

5.4. Discussion

Except for the cyanobacterium *Synechocystis* sp. under varied phosphate concentration, lipid content in the other studied algae varied with nitrate and phosphate concentrations. Below the saturation level, where growth rate significantly changed with nutrient concentration, lipid content was negatively correlated to growth rate. This finding illustrates that the amount of lipids in the studied algae is a dynamic property.

The results of this study indicate that algae grown under nutrient-limitation, but not nutrient-starvation, accumulate lipids. In their logarithmic phase of growth, cells raised in a medium with inadequate amount of nutrient contained more lipids than those grown in a nutrient-sufficient medium. However, lipid content of *Synechocystis* sp. neither increases nor decreases in response to phosphate gradient. Although this result was not expected, it can be explained. Excess carbon can be stored inside a cell in different forms of lipid, starch, or polysaccharide. It is possible that this cyanobacterium, under phosphate limitation, used starch or polysaccharide for storage of carbon. This phenomenon is known in cyanobacteria. Konopka and Schnur (1980) and Konopka (1983) demonstrated that the cyanobacteria *Merismopedia tenuissima* and *Oscillatoria rubescenes* accumulated polysaccharide when they were grown under stressful growth environments. Polysaccharide storage under starvation has also been observed in some green algae. For instance, the green alga *Pseudochlorococcum* sp. used starch as a primary storage product of carbon. During the time course of the experiment, starch content gradually decreased and lipid content increased until on day 10 that all the starch disappeared and lipids became predominant (Li *et al.*, 2011). This finding suggests that there are some species capable of producing both starch and lipids. On the other hand,

there are some algae that are not able to store excess carbon inside the cell in any form; they excrete the excess carbon in the form of polysaccharide (Hellebust, 1965; Mehta and Vaidya, 1978; Jensen, 1984). This is not the case in *Synechocystis* sp., as it did accumulate lipids in response to nitrate and light gradients. Indeed, this species might use different forms of storage under various circumstances.

How exactly nutrient limitation can cause an increase in lipid content is not clear. It is speculated that when at least one major nutrient is not sufficient, cells do not divide well and thus do not consume all the photosynthetically generated reduced carbon for the purpose of cell wall synthesis. Therefore, carbon starts to build up and is stored as starch, polysaccharide, or lipids.

In response to light, lipid content in all the studied species varied. Such variations were accompanied by variations in growth rate. Growth was mostly slow under high light levels, which are stressful for most of the algae, or low light levels, which are inadequate to support growth. In either case, light intensity impacted the lipid content. Indeed, lipid content was negatively correlated to growth rate, similar to what was observed for nutrients. Though, not all the organisms demonstrated the same pattern. In the two green algae, at low light, cells did not grow fast, likely due to inadequate energy. Yet for some unknown reason, they generated excess carbon, more than what they needed for cell wall synthesis. It could be because photosynthesis efficiency is high at low light, meaning that the number of fixed CO_2 per photon is higher (Flameling and Kromkamp, 1998). But such fixed carbon was not utilized for the growth; thereby it went into the storage. At high light levels, cells grew well and thus almost all the generated carbon was utilized, producing little excess carbon. In the case of

cyanobacteria, however, the scenario was different. At low light levels, cells grew very well, consuming nearly all the generated carbon which resulted in a very low excess carbon. At high light levels, growth was so or halted so that practically all the generated carbon remained unused and thereby stored as lipids. Diatoms appeared to act similarly to both cyanobacteria and green algae. Similar to green algae, cells division was slow at low light levels, leading to a high excess carbon. Similar to cyanobacteria at high light, photosynthetic reaction centers became saturated, causing a decrease in growth rate. Hence, almost all the fixed carbon accumulated as lipids.

To summarize, lipid content of an alga is a function of the exact growth rate relative to the maximum growth rate that potentially could be accomplished under optimum growth conditions. Consequently, any environmental factor, e.g. nutrient limitation or low/high light level, that negatively impacts the growth rate can cause an increase in lipid content.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1. Introduction

This study affirms that growing native algae in the southwest of the U.S. does not require clean water or chemical fertilizers. Fertilizers could be replaced by the nutrients obtained from two waste sources: centrate (the liquid fraction of sewage) and algal biomass residue (algae after lipids are extracted). The idea is to use centrate as an initial source of water and nutrients, i.e. nitrogen and phosphorus, to start producing biomass. Afterwards, the recycled biomass by itself, once lipids are extracted, can continuously provide the required nutrients. In fact, in terms of nutrients, a biodiesel plant would function as a closed system, fed by its own waste. The nutrients existing in the biomass residue, along with the used water would be returned to the algal cultivation unit, serving as the growth medium. Meanwhile, centrate supplements the culture from time to time, boosting growth of the algae and bringing fresh water into the system. The used water often needs to be replaced by the fresh water, as salt accumulating in the overly used water could increase the salinity beyond the tolerance threshold of the algae, particularly in open ponds facing evaporation. In addition to nitrogen and phosphorus, centrate and algal biomass residue contain organic carbon that serves as an additional source of energy because it stimulates growth and increases feedstock production. In summary, using centrate amended with the recycled substrates from biomass residue mitigates the use of fertilizers and clean water and, in addition, it enhances the biomass productivity (Figure 6.1).

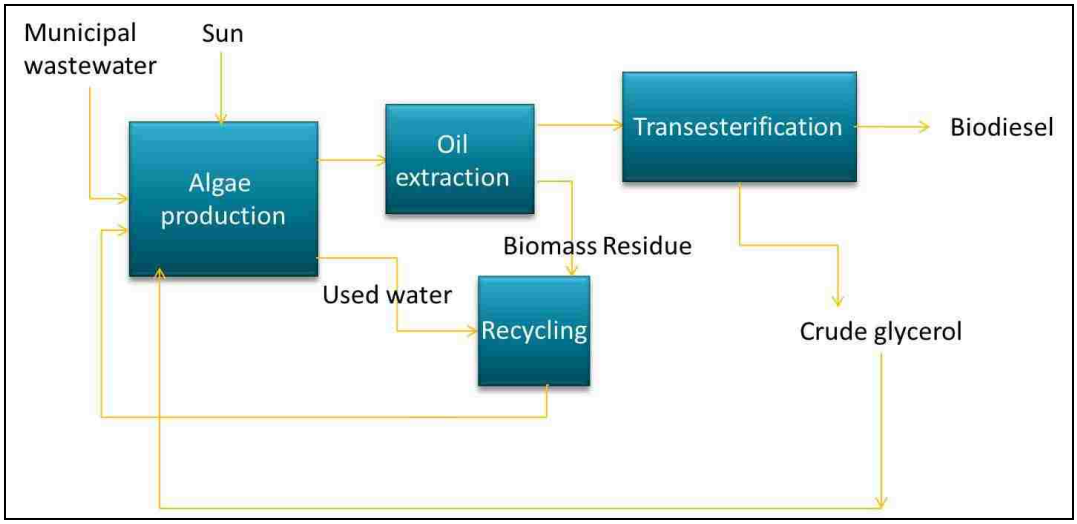

Figure 6.1. A schematic design of an algal biofuel plant, emphasizing the recycling of generated waste.

6.2. Conclusions and Discussions

6.2.1. Isolation, Culturing, and Growth Optimization

Chapter 2 provides an introduction to the biology of the studied algae. It serves two objectives. First, to isolate species indigenous to Southern Nevada which are suitable for the biofuel feedstock. Second, to optimize the growth of the promising isolates in terms of nutrients, light, and temperature. Previous efforts to develop algal biodiesel in the Southwestern U.S. indicated that, everything else being equal, native algae are preferable. Specifically, in an outdoor pilot test conducted outside Roswell, New Mexico, inoculums of algae originating elsewhere were quickly invaded and replaced by native species (Sheehan *et al*., 1998). The present study shows that native species might provide better crop stability. Both *C. sorokiniana* and *Scenedesmus* species can tolerate salinity, excessive light, and elevated temperatures, suggesting that they are well-adapted to the local hard water and to the local climate conditions.

Additionally, they both contained relatively high lipid contents; *C. sorokiniana* and *Scenedesmus* sp. contained 19.0% and 29.8% lipids respectively, making them capable of being biofuel feedstock. *C. sorokiniana* grew best at 32-35ºC, while *Scenedesmus* sp. grew best at 22ºC. Hence, this study suggests utilizing *C. sorokiniana* for cultivation in summer, and *Scenedesmus* sp. for other seasons.

6.2.2. Wastewater as the Growth Medium

The goal of chapter 3 was to investigate the possibility of using centrate as a source of nutrients and water to grow algal feedstock. In addition to inorganic nutrients, centrate is also a good source of organic compounds. Some algae are able to use such organic carbon as an additional source of energy and grow even faster. This chapter also investigated the ability of the studied isolates to take advantage of the organics present in centrate. However, centrate is a complex material containing a wide range of organic and inorganic substances. While some substrates are nutritious, others can be suppressive or toxic. Inhibitors should be eliminated or diluted to a non-toxic level. This chapter sought a proper concentration of centrate, at which toxins are detoxified, whereas at the same time the nutrients are still adequate for algal growth.

6.2.2.1. Results and Discussions

For both isolates, *C. sorokiniana* and *Scenedesmus* sp., the highest growth rates occurred in 20 - 70% centrate, and these rates were higher than those in BG11. This agreed with the results of growing algae in different strengths obtained by Bhatnagar *et al* (2009), who studied a strain of *Chlorella minutissima* isolated from oxidation ponds in wastewater treatment facilities in India. Our results also showed that the Las Vegas Wash water with less than 20% centrate was suboptimal, and inferior to BG11,

presumably due to insufficient phosphate. The Wash water containing more than 70% centrate was counterproductive, and the two isolates responded differently. *Scenedesmus* sp. grew at a reduced rate for the entire duration of the experiment. *C. sorokiniana*, in contrast, overcame the inhibition after two to three days. At the log phase that followed, its growth rate was high, in fact higher than that reached at optimum centrate concentration, i.e. 50%. A similar situation was previously observed with *Chlamydomonas reinhardtii* in undiluted centrate (Kong *et al*., 2010). As a result, at least the four algae, *C. sorokiniana*, *Scenedesmus* sp., *C. minutissima, and C. reinhardtii*, prefer municipal wastewater over a freshwater-based medium.

As pointed out earlier, in addition to the nutritious substrates, centrate also carries toxic compounds. Further investigation tentatively identified four potential sources of inhibition in the centrate: high nutrients concentration, ammonium, heavy metals, and urea. The results from chapter 2 rule out the possibility of inhibition caused by high nutrient concentrations. No evidence of inhibition was observed from nitrate as high as 800 μM, or from phosphate as high as 300 μM, the upper concentration limits to nitrate and phosphate in centrate. The next possible inhibitor would be ammonium. Ammonium is a source of nitrogen to algae, but it can be toxic in high concentrations (Abeliovich and Azov, 1976; Azov and Goldman, 1982). This is also the case with *C. sorokiniana*. Ammonium inhibited growth when it was the only source of nitrogen. This inhibition was not observed if the organism was grown in nitrate-replete instead of nitrate-omitted BG11. Centrate contains both nitrate and ammonium. Therefore, the ammonium toxicity observed in the nitrate-omitted BG11 is unlikely to be the cause of inhibition observed in centrate. The other potential source of toxicity would be heavy

metals. Centrate is known to contain arsenate/arsenic and other toxic heavy metals (Mohan and Pittman, 2007). Yet, the presence of heavy metals in high concentration in sewage is very improbable. Therefore, heavy metals cannot be the source of toxicity. The last possibility would be urea, which is usually present in typical domestic sewage. The results indicate that there is a critical level for urea, above which it is toxic and below which it is nutritious. The growth rate of *C. sorokiniana* in nitrate-replete BG11 was significantly enhanced by low-level urea amendments, 0.001% and 0.01%. At a higher concentration of 0.1%, urea suppressed growth to slightly below pre-stimulation level. This result replicated the inhibitive nature of undiluted centrate. *C. sorokiniana* could overcome the inhibition of 100% centrate after two to three days of incubation. This result is shown in more details in Figure 3.5A, by comparing the growth dynamic in 100% centrate, where inhibition exists, and in 50% centrate, where inhibition does not exist. The same result was observed when *C. sorokiniana* was grown in BG11 with 0.1% urea (Figure 3.5B). In the absence of urea, the organism displayed virtually no lag phase. In its presence, in contrast, it showed a long lag phase.

Utilization of urea by green algae as a nitrogen source is well documented (Bekheet and Syrett, 1977). It is also known that urea metabolism in green algae is enabled either by urease or by ATP-urea amidolyase (Hodson and Thompson, 1969; Bekheet and Syrett, 1977). Both enzymes degrade urea to NH_3 and CO_2 (Eq. 6.1).

$$CO(NH_2)_2 + H_2O \rightarrow CO_2 + 2\,NH_3 \qquad \text{(Eq. 6.1)}$$

The stimulation of urea at low concentrations most likely comes from the carbon source (CO_2), while the inhibition at high concentrations almost certainly comes from NH_3. To avoid inhibition, an attempt was made to adapt *C. sorokiniana* to urea before being

transferred into the centrate. The rationale was to expose the cells to urea during the pre-adaptation phase in order to induce urea-degrading enzymes, i.e. either urease or ATP-urea amidolyase. It was hoped that the induced enzymes would degrade urea right away, once the alga is transferred into the centrate, negating or shortening long lag phase. Ultimately, the result of this experiment resulted in a long lag phase occurred.

As mentioned previously, properly diluted centrate not only supports growth, but also outperforms the mineral medium BG11 due to the presence of organic substrates, which provide an additional source of energy for the algae. The growth of *C. sorokiniana* was enhanced by glucose and acetate, but not by glycerol and lactate (Figure 3.6A). The growth of *Scenedesmus* sp. was stimulated by glucose, acetate, and glycerol, but not by lactate (Figure 3.6B). The results also illustrated the heterotrophic abilities of the two algae, growing on organic carbon in darkness. Both algae could consume glucose and acetate in the dark, but not glycerol and lactate (Figure 3.7). This result is consistent with previous findings that many algae, including *Chlorella* and *Scenedesmus* species, consumed organic carbon as an energy source in the dark or under light (Samejima and Myers, 1958; Karlander and Krauss, 1966; Kamjunke and Tittel, 2009; Liang *et al.*, 2009; Gao *et al.*, 2010; Heredia-Arroyo *et al*., 2010; Xiong *et al*., 2010; Heredia-Arroyo *et al*., 2011; O'Grady and Morgan, 2011). The heterotrophic abilities of the isolates permit uninterrupted growth, in contrast to the phototrophic growth which is halted during the night. The continuous growth enriches biomass density. Centrate has a significant biological oxygen demand or BOD (Table 3.1). While it is not known what organic compounds are exactly responsible for the BOD, it is likely that some of them are available to the isolates, given their broad mixotrophic/heterotrophic capabilities. Such

substances support night-time growth and stimulate day-time growth, improving total biomass productivity.

The rational of choosing glucose and acetate for the hetero/mixo-trophic experiments was that they are the most common organic carbon sources that microorganisms can utilize. Glucose is a simple monosaccharide found in plants, a primary source of energy, and a metabolic intermediate for most organisms. Acetate, also, is the most common building block for biosynthesis. Hence, it was likely that algae could utilize these two carbon sources. Lactate was adopted due to its availability in the wastewater of dairy and ice-cream factories. The notion was to amend the growth medium with such organic-rich wastewaters to boost growth. Glycerol was also tested, as a growth stimulator, due to its excessive production and abundance in the market (as a byproduct of biodiesel production).

6.2.3. Recycling Algal Biomass Residue as a Source of Nutrient

Chapter 4 discusses the feasibility of replacing fertilizers with the nutrients recycled from algal biomass residue, i.e. the algal cells remaining at the end of the lipid extraction process. However, such nutrients are bonded in complex molecules such as proteins, nucleotides, or carbohydrates. Algae are not able to utilize such large molecules. Hence, waste biomass must be decomposed first. Biological degradation was carried out through "algal composting", similar to plant composting in agriculture, in which bacteria convert the polymer-like molecules into substrates that algae can utilize. Through algal composting, dissolved nutrients were extracted. The resulting liquid-like extract is commonly referred as 'compost tea". The compost tea was used as growth medium to examine its suitability to grow the studied algae. The alternative, chemical

approach, decomposes the biomass faster and simpler, but it incurs energy cost. During this process, polymers (e.g. proteins) would break down to monomers (e.g. free amino acids), possibly consumable by algae. However, such monomers are sources of *organic* nutrients. Although algae, like higher plants, prefer inorganic forms of nutrients, e.g. nitrate, some marine diatoms can utilize the organic nitrogen of amino acids as well (Admiraal *et al*., 1984; Admiraal *et al*., 1987). Thus, it is possible that some 'fuel strains' also could assimilate amino acids as a source of nitrogen. The present study tested the would-be product of such a chemical degradation, i.e. amino acids, to grow algae. Consequently, the ability of the studied isolates to consume four amino acids, including aspartic acid, glutamic acid, alanine, and leucine, as their sole source of nitrogen, was examined. These are the most common amino acids present in the algal cell wall (Punnett and Derrenbacker, 1966) which are also expected to be present in the waste biomass.

The rationale for preparing composts under aerobic (moist, daily turnover) and anaerobic (submerged in water, no mixing) conditions was to find the most efficient of the two. The anaerobic condition generates more compost than the aerobic condition out of the same base biomass, but at a slower process rate. Under each condition, algae were decomposed with a cellulose source in the form of office papers, or without it. Composting without paper is preferred, if it is viable, because fewer additives make the composting more economical. All composts were extracted in the same amount of water. The extracts, or teas, with or without supplemented glucose (as a simple organic carbon source) were tested to grow the studied algae. Overall, eight types of teas were used to

grow the two isolates *C. sorokiniana* and *Scenedesmus* sp. Growth in BG11 was a point of reference.

6.2.3.1. Results and Discussions

The results illustrate the ability of the two studied algae, *C. sorokiniana* and *Scenedesmus* sp., to grow in compost tea at a rate comparable to, or higher than that obtained in BG11. The tea from aerobic compost, with or without paper, supported a robust growth rate of *C. sorokiniana* when supplemented with glucose, most likely due to mixotrophy. For *Scendesmus* sp., the tea from aerobic paper-containing compost, without glucose supplement, could also support a significant growth rate. Such enhancement could be due to the organic carbon sources, such as acetate, already present in the tea. The occurrence of growth demonstrates that compost tea, i.e. the product of biologically degraded biomass, can be used in place of growth medium.

However, no growth, for either alga, occurred in the teas from anaerobic paper-containing composts, with or without glucose. In retrospect, the use of office papers as a source of cellulose was a poor choice. Office papers contain calcium carbonate, added during the whitening process of paper during commercial production (Sundara-Rajan *et al*., 2004), which leads to calcium toxicity. According to the Hofmeister series, shown in the box, calcium is the strongest chaotropic cation after guanidinium. Chaotropic agents denature protein (Von Hippel and Wong, 1965; Herberhold et al., 2004; Zhang and Cremer, 2006), which in this case would be the protein of the algae. Calcium toxicity could be remedied by a strong kosmotropic anion. The Hofmeister series shows that sulfate and fluoride are the strongest kosmotropes. Kosmotropic ions, opposite to chaotropes, stabilize protein, thus these can detoxify the chaotropic agents (explained in

detail in chapter 4). Even though sulfate was detected in the tea, its quantity was not enough to detoxify the high concentration of calcium, ~90mM.

Hofmeister series

Kosmotropes (detoxifier) ←----------→ Chaotropes (toxin)

Anion series $F^- \approx SO_4^{2-} > HPO_4^{2-} >$ acetate $> Cl^- > NO_3^- > Br^- > ClO_3^- > I^- > ClO_4^- > SCN^-$

Cation series $NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+} >$ guanidinium

The toxicity of calcium to algae was also revealed by the results of our preliminary experiments, in which the presence of 10mM calcium, in the culture, suppressed the growth of *C. sorokiniana* significantly (Figures 4.4 and 4.5).

In contrast, tea from the compost that is also anaerobic but paperless supported algal growth, equivalent to BG11 (Figure 4.3 A, B–horizontal dashed lines).

Tea from aerobic, paper-less composting supported some growth. The growth of *C. sorokiniana* was not measured by optical density due to cell clumping. Clumping itself is an indication of stress response. Adding glucose rescued the algae. Such partial inhibition, was also eliminated after treatment with hydrogen peroxide (data not shown). The olive-grey color of the tea and inhibition disappeared simultaneously, implying a single cause for both and suggesting an organic source of toxicity such as pheophytin. Pheophytin is an organic substance creating such color. Pheophytin is a chlorophyll molecule lacking a central Mg^{+2} ion that can be produced from chlorophyll under high temperatures, i.e. > 100 °C. This could have happened during autoclaving. How exactly this molecule suppresses growth is yet to be investigated.

Tea from the paper-present aerobic compost supported growth of *C. sorokiniana* at about 80% of BG11 and of *Scenedesmus* sp. better than BG11, suggesting that the presence of cellulose provided the energy needed to oxidize pheophytin. If supplemented with glucose, the same tea became superior to BG11, for both species. This is probably because both algae are mixotrophic, utilizing glucose as an extra source of energy. Here, although paper was present, there was no calcium toxicity to either alga, perhaps due to aerobic conditions promoting sulfate formation instead of its removal (i.e. sulfate reduction).

To summarize, aerobic decomposition is fast, yet has the drawback of producing pheophytin. However, such toxicity could be prevented by adding a source of cellulose during composting. Anaerobic decomposition, in absence of paper, is without the problem of pheophytin. However, the rate of decomposition is slow.

In addition to compost tea, the two algae consumed amino acids, i.e. hypothetical products of chemical biomass decomposition, as the sole source of nitrogen (Figures 4.1 and 4.2). A decrease in the amino acid concentration, accompanied by an increase in the cells density, specifies that the algae obtained their required nitrogen from the amino acids. This implies that chemically decomposed biomass can also be used instead of fertilizers.

Figure 6.1 demonstrates a very general schematic design of what is explained above. It introduces three benefits. One, it provides an efficient, sustainable way to dispose of a large amount of waste (biomass and glycerol) that will be generated at biofuel plants. Second, it makes biofuel plants independent from fertilizers and clean water. Third, it enhances biomass productivity at no extra cost.

6.2.4. Manipulation of Growth Conditions for Higher Lipids Production

The aim of chapter 5 was to provide a better understanding of the cause of variations in lipid content as it impacts the overall productivity of lipids. Lipids serve as storage for excess carbon, the content of which fluctuates with inflow and outflow carbon. The inflow represents the generated carbon, via photosynthesis, while the outflow symbolizes the utilized carbon, through growth. Hence, lipid content is the outcome of interactions between photosynthesis efficiency (the number of fixed CO_2 divided by the number of photons absorbed at irradiances which are sub-saturating to photosynthesis) and growth rate. When light is consistent, growth rate alone controls lipid content. On the other hand, lipids productivity changes with both lipid content and biomass density. In order to achieve a high lipid yield, both variables of 'lipids quantity per cell' and the 'total number of cells in the culture' must be optimized, according to the following:

*Total lipids per volume of culture= (the amount of lipids produced per cell) * (total number of cells per volume of culture)*

Prior studies showed that lipid content increased under unfavorable growth conditions, e.g. nutrient deficiency (Zhekisheva *et al*., 2002; Khozin-Goldberg and Cohen, 2006; Merzlyak *et al*., 2007; Pruvost *et al*., 2009; Rodolfi *et al*., 2009; Chen *et al*., 2010; James *et al*., 2011). Yet, there were some issues that needed to be clarified or optimized. First, the approach of performing the experiments invited uncertainties to the results. For instance, where the effect of nitrate deficiency on lipid content was tested, there is a high possibility that phosphate had also become limiting. Although the experiments began with sufficient amounts of phosphate, phosphate had become limited

later, at the time that lipid content was measured; in batch cultures, in general, all the chemicals change over time. Hence, nitrate concentration may not have been the only variable in the system affecting the lipid content. To improve that issue, this research used a gradient of concentrations. Parallel experiments were begun at the same time and under equal conditions, but with different initial concentrations of the target variable. All the experiments were terminated before other parameters became limiting. As a result, the lipid contents were compared among the cells experiencing the same phase of growth, i.e. log phase, while all parameters, except the target, were still sufficient.

Previous results reported in the literature were sometimes contradictory. Different findings were reported for one given parameter, in species belonging to one algal group. This situation made it almost impossible to predict the trend of lipid content variations in response to a given variable. This study developed a universal pattern that shows the relationship between growth rate and lipid content in response to varied environmental conditions.

6.2.4.1. Results and Discussions

Irrespective of the environmental and growth conditions, test results indicate that lipid content is negatively correlated to growth rate (Figures 5.1, 5.2, 5.3). This finding suggests that while unfavorable growth conditions increase the amount of lipids inside the cell, they do not necessarily increase the total lipid production.

One exception to the proposed trend was *Synechocystis* sp. in response to phosphate gradients, in which growth rate varied but lipid content did not (Figure 5.2C). In this case, the excess carbon was likely stored as starch. This agrees with Li *et al*. (2010) results, showing that *Pseduchlorococcum* sp. used starch as a primary storage

product of carbon, not lipids. These findings indicate the importance of genetics, in addition to physiology, in lipid production. Some species are able to store excess carbon in different forms, i.e. starch, polysaccharide, and lipids, under various conditions. It is evident from the results of this study that *Synechocystis* sp. stored lipids under nitrate-limitation conditions and high light intensities while producing starch or polysaccharide under phosphate-limitation circumstances. On the other hand, some algae are not genetically capable of storing any forms of excess carbon inside the cell: some, mostly cyanobacteria, excrete the excess carbon in the form of polysaccharide into the culture while some others store polysaccharide outside the cell and use it when it is needed (Hellebust, 1965; Mehta and Vaidya, 1978; Jensen, 1984). However, neither of these types of algae nor the starch-producers are suitable candidates for biodiesel.

In contrast to light intensity and nutrient concentration, temperature did not change the lipid content (data are not shown). It agrees with the findings of Spoehr and Milner (1948) in which lipid content of *C. pyrenoidosa* did not change with temperature. The reason could be that temperature affects both photosynthesis and growth similarly and simultaneously, increasing or decreasing both jointly. In other words, the difference of the generated carbon and the utilized carbon is always constant and thereby, lipid content stays constant. Similar to the growth-temperature curve (Figure 2.12), the photosynthesis-temperature relationship also forms a bell curve. Photosynthesis is a series of chemical reactions influenced by temperature, like all other chemical reactions. At low temperatures, the enzymes responsible for photosynthesis do not have adequate kinetic energy to function. Increasing temperature, which generates more kinetic energy, causes net photosynthesis to increase. More kinetic energy leads to more collisions

between the reactants in photosynthesis, until net photosynthetic activity reaches its peak, above which too much heat denatures those enzymes responsible to catalyze photosynthesis. This condition causes photosynthesis to become severely inhibited. While this study did not determine the photosynthesis rate in relation to temperature, including this study would not have changed the conclusions.

6.3. Future Recommendations

6.3.1. Effect of Carbon Dioxide on Growth Rate

In addition to nutrients and light, algae also require carbon dioxide to grow. Due to unavailability of the proper instruments, this study was unable to investigate growth against various concentrations of carbon dioxide. However, the preliminary results of this study, conducted with one concentration at a time against ambient air as a reference, portrayed an increase in the growth rate of *C. sorokiniana* in the presence of additional CO_2 (Figure 6.2). It can be speculated that, if all other growth parameters are sufficient, an additional CO_2 can speed up the photosynthetic rate, thereby increasing biomass production (Eq. 6.2).

$$n\,CO_2 + n\,H_2O \rightarrow biomass + n\,O_2 \qquad \text{(Eq. 6.2)}$$

However, beyond a certain level, carbon dioxide may become ineffective due to the pH falling below the tolerance threshold of algae. Carbon dioxide dissolves slightly in water and forms a weak acid, H_2CO_3 (Eq. 6.3).

$$CO_2 + H_2O \rightarrow H_2CO_3 \qquad \text{(Eq. 6.3)}$$

Carbonic acid in water forms a hydronium cation, H_3O^+, and bicarbonate ion, HCO_3^-, reducing the pH of the culture (Eq. 6.4).

$$H_2CO_3 + H_2O \rightarrow HCO_3^- + H3O^+ \qquad \text{(Eq. 6.4)}$$

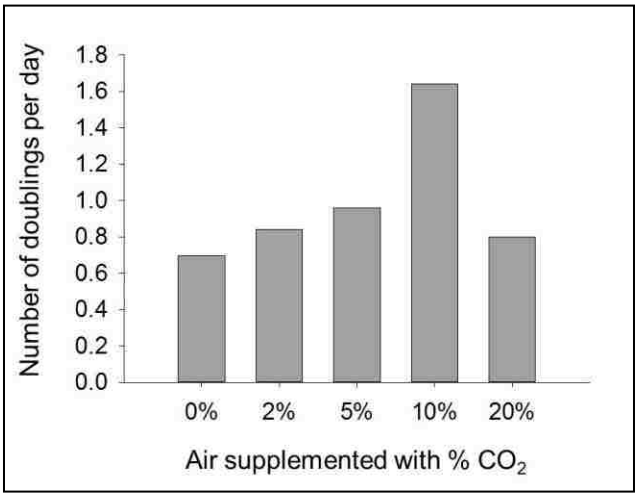

Figure 6.2. Effect of carbon dioxide on growth rate of *C. sorokiniana*. Growth rate increases as carbon dioxide is increased. Above 10% CO2, the growth rate dropped down due to the acidic environment.

Nevertheless, the removal of CO_2 by algae increases pH and decreases the partial pressure of CO_2. Therefore, the pH of the culture depends on the rate of CO_2 consumption and the rate of CO_2 diffusion. As such, it is crucial to monitor the pH of the culture continuously and adjust it to the level at which growth is optimal. Similar to other growth parameters, the pH tolerance level of algae is species-specific. The preliminary results of this study suggest that the optimum pH for *C. sorokiniana* was around 8 while it could tolerate a pH in the range of 6.5-9. Practically, no growth occurred at pH of 4 or less (Figure 6.3). A study to determine the pH tolerance range of the species and the maximum allowed CO_2 concentration is recommended.

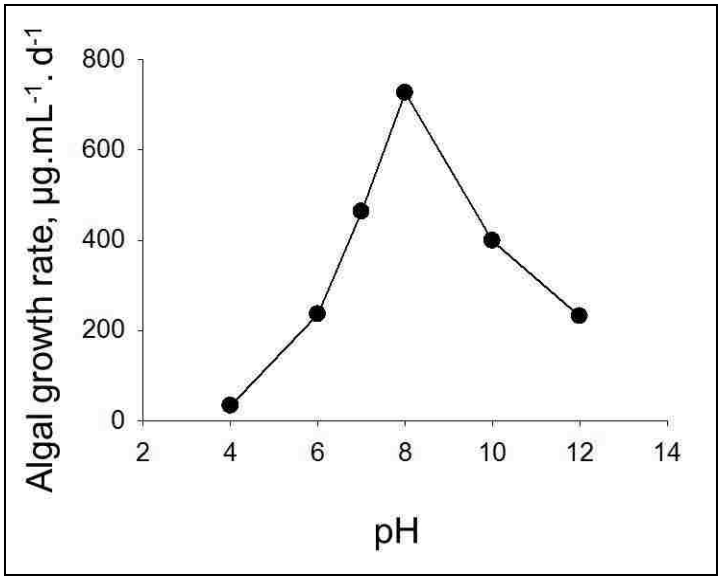

Figure 6.3. pH tolerance of *C. sorokiniana* The highest growth occurred at pH = 8.

6.3.2. More Collection and Screening

Since the algae must be able to utilize wastewater, it would be a good idea to collect samples from oxidation ponds in wastewater treatment facilities. Also, in order to grow them on waste biomass, species must be chosen from amongst those that are able to consume organic nutrients, e.g. amino acids. Additionally, the isolates should have mixotrophic and heterotrophic capability to take advantage of the organic compounds available in wastewater and waste algal biomass.

Among diverse types of algae, diatoms contain high lipid content. Two of them originated from the Las Vegas Wash (shown in Figure 6.4) and possess oil droplets full of neutral lipids in addition to membrane lipids. The droplets fluoresce yellow (excitation-emission 517-584 nm) after staining with Nile Red, a lipophilic dye, indicating the presence of neutral lipids. One of them was designated as *Stephanodiscuss* sp. This study never succeeded in bringing either diatom into the pure cultures to do

experiments. Particularly in the culture of *Stephanodiscuss* sp., there were many small single cell diatoms and different bacteria attached to this large filamentous diatom, which grew so fast that the slow fat diatom never dominated the nutrient uptake. Other microorganisms existing in the culture flourished, causing the large diatom to die.

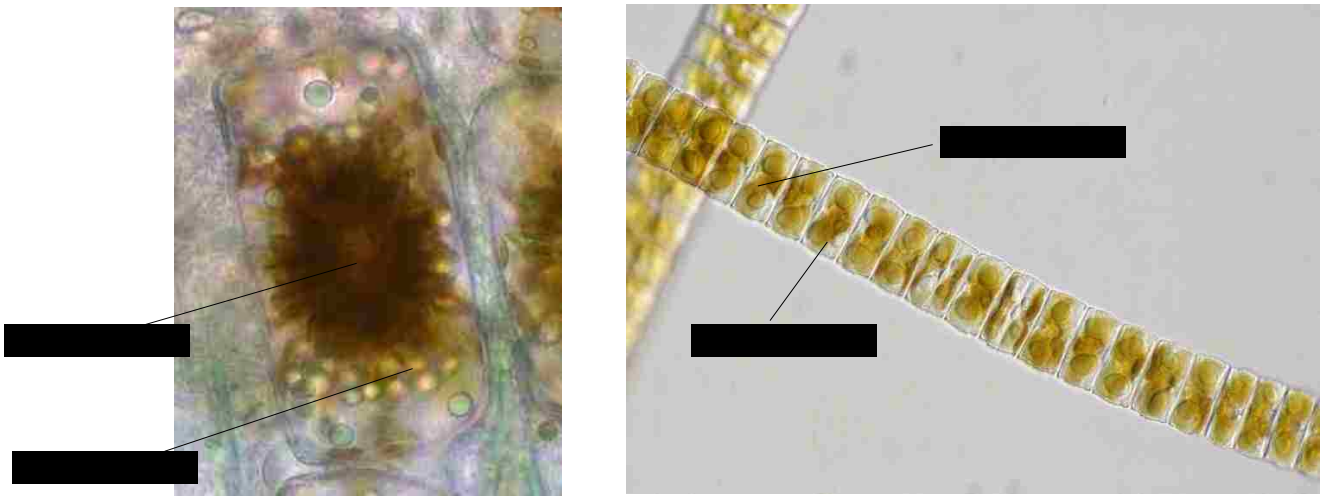

Figure 6.4. Oil rich diatoms, native to the Las Vegas Wash. *Stephanodiscus* sp. on the left and unidentified diatom on the right. In addition to the lipid membrane, all types of algae possess, these diatoms contain additional oils, fluoresced yellow and confined in the droplet shaped storages.

In general, the challenge with growing diatoms on an industrial scale is their need for silicate. The silicate is required for cell wall synthesis. In this study, for the first time, the silica of the dead diatom cell walls was used as a nutrient source (explained in Appendix A). As a result, diatom *N. thermalis* grew well in a medium made of the recycled silica. The second issue with the oil-rich diatoms is their slow growth. The low growth rate decreases biomass and lipid productivities. This could be resolved by applying genetic engineering methods in which the diatom genes responsible for oil

production would be transferred to a fast growing alga, like *Chlorella*. This approach is not yet developed, and currently is an on-going research topic in genetic engineering.

6.3.3. Determining Toxins in Centrate

Heavy metals could impose a serious threat to algal growth if they are present at high concentration, i.e. millimolar. Wastewater is known to contain heavy metals, the concentrations of which vary based on the water chemistry and geological characteristics of the region. A study to detect the concentration of the heavy metals in the wastewater before it is used in algal farms is recommended. Moreover, to determine the safety margin of centrate concentration in algal farms, it is recommended that an investigation to determine suitable concentration of urea in the centrate be carried out.

6.3.4. Improving the Methods of Algal Biomass Decomposition

In order to break down waste algal biomass and to free the nutrients, the present study used biodegradation, i.e. composting the algae. Yet, inhibition occurred in some compost teas. To eliminate the inhibition caused by calcium toxicity of papers in the anaerobic compost teas, other sources of cellulose, such as waste wood, should be examined. In order to figure out the exact source of stress and inhibition that occurred in the extract from aerobic compost without paper or glucose, it is recommended that the concentration of chlorophyll group molecules, particularly pheophytin, before and after the hydrogen peroxide treatment be determined. A considerable reduction in any of these molecules designates them as the source of toxicity. Additionally, it would be helpful to do a growth experiment in BG11 supplemented with chlorophyll or pheophytin, to observe whether any inhibition occurs.

Even though composting is an economical way to decompose the algal biomass, it is slow. As mentioned earlier, chemical approach is an alternative in which high heat and pressure would break the polymers. This approach is fast, but is energy-intensive. The other option for degrading the biomass could be a two-step process: fermentation and chemical decomposition. Through fermentation, methane gas is produced while converting polymers into bio-accessible substrates. The generated methane gas can provide the required energy for the subsequent chemical process to break down the rest of the biomass residue.

6.3.5. Other Waste Organic Carbon Sources

In addition to centrate and algal biomass residue, wastewater from sugar factories, breweries, wineries, dairy factories, and etc. could also be tested as a source of organic carbon. If these nutrient-rich wastewaters are found suitable for and consumable by algae, they can be added to the waste-based growth medium to stimulate algal growth. To improve efficiency of biofuel plants, exploitation of glycerol-utilizing algae (such as *Scenedesmu*s sp.) as feedstock is highly recommended.

6.3.6. Calculating Overall Lipid Productivity

In order to calculate overall lipid productivity, it is necessary to determine the exact quantity of lipids produced in an algal cell. While the results of this study revealed a uniform trend of lipid content in response to various environmental factors, they cannot be used for lipid productivity calculation. For this reason, it is highly recommended to either quantify lipid content with a standard calibration curve, in place of reporting relative numbers, or determine lipid content with a gas chromatography–mass spectrometer (GC-MS), instead of the nile red method used in this study.

APPENDIX A

DIATOM CELL AS A SOURCE OF SILICA

Silicate, as well as nitrate and phosphate, is a major nutrient for diatoms, one of the main groups of algae. Silica, i.e. the primary source of silicate, is most commonly found in nature as sand or quartz, i.e. finite, non-renewable resources. Providing this compound in a large scale for algal farms could cause economic barriers. Therefore, it is important to find renewable and economical sources of silicate. One source could be the cell walls of dead diatoms. Diatom cell walls possess a complicated structure made of nano-patterned silica, SiO_2 (Kröger and Poulsen, 2008). While the structure of the cell wall is species-specific, its material is similar within all the diatoms. Antonides, 1998 verified that about 80% of oven-dried diatom cell walls are made of silica. This study suggests that after all the useful material of a diatom (including lipids, nutrients, organic carbon, etc.) is extracted, the cell can be completely burned until only silica is remained. The obtained silica in reaction with hot sodium hydroxide would generate sodium silicate that can be used in diatom medium (DM). However, the feasibility of this idea and the ability of diatoms to consume such source of silicate are yet to be investigated. Hence, this study tries to grow the diatom *N. thermalis* in a DM contained recycled silica as the sole source of silicate.

Cells of the diatom *Stephanodiscus* sp., collected from the Las Vegas Wash, were baked in an oven at 400°C for three hours (Figure A.1).

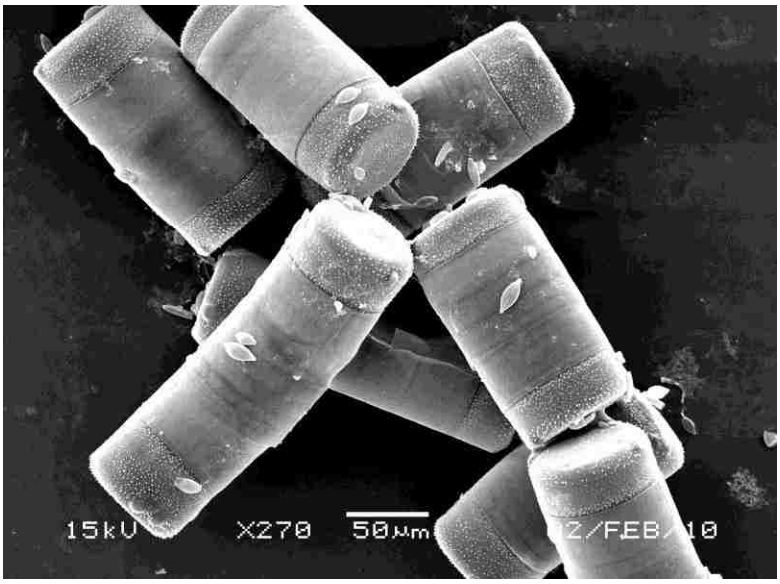

Figure A.1. After baking at 400˚C for 3 h, frustules' structures of *Stephanodiscus* sp. without the organic components of the diatom cells observed by scanning electron microscopy.

One gram of the baked diatoms was added to about 1.5g "molten" sodium hydroxide, at 320°C, to produce sodium silicate (Eq. A.1).

$$2\ NaOH + SiO_2 \rightarrow Na_2SiO_3 + H_2O \qquad \text{(Eq. A.1)}$$

Sodium silicate could also be generated from adding hot aqueous NaOH, instead of molten. The whole procedure was performed under a hood and inside a ceramic container.

The generated sodium silicate then was added to the silicate-free DM, while all other gradients were the same as the DM recipe. The new medium was autoclaved and then cooled down in room temperature. Thereafter, the diatom *N. thermalis* was inoculated. As a reference, the diatom was also inoculated into the standard DM. After five days, both cultures became turbid, showing golden color which is the color of the inoculated diatom. Cells were harvested by centrifugation and the protein content was

measured based on the Lowry assay. One milliliter of the culture composed of the recycled-silica contained 1.10 mg protein while the same volume of the reference culture contained 1.41 mg protein. This finding shows that the diatom cells actually could consume the recycled-silica to grow.

SEM Preparation Procedure

Cells were fixed in 3% glutaraldehyde solution buffered with 0.1% phosphate buffer (pH 7) for 2 hours. After centrifugation, the cells pellet was rinsed with nano-water for three times. The pallet then was dehydrated with 100% ethanol for five minutes. Ethanol was dried and the cells were rinsed with nano-water for five times. The wet cells were baked at 400°C for three hours to burn all the organics.

APPENDIX B

AMINO ACIDS UPTAKE BY GREEN ALGAE

This appendix discusses the findings produced by additional experiments with amino acids, in addition to the experiments presented in chapter 4. In order to understand the mechanism of consuming amino acids by green algae, two native algae, *C. sorokiniana* and *Scenedesmus* sp., plus one additional green alga, *Euglena gracilis*, purchased from the UTEX, were grown separately on D- or L-enantiomers of aspartic acid, glutamic acid, alanine, and leucine, serving as the sole source of nitrogen. The three species showed three distinct uptake patterns. *Scenedesmus* species consumed both D- and L-enantiomers of the four studied amino acids (Figure B.1, A and B). *C. sorokiniana* utilized only L-aspartic acid and L- alanine, but no D-amino acid (Figure B.2, A and B). *E. gracilis* did not take up any D- or L-amino acid (Figure B.3 and B.4). It is hypothesized that two mechanisms are involved, racemization and producing outer-cell amino acid oxidases, specific to each amino acid. Through racemization, organisms convert D-amino acids (D-AAs) to L-amino acids (L-AAs). D-AAs are a source of toxicity. The fact that *Scenedesmus* sp. could consume all four D-AAs as well as L-AAs to grow suggests the presence of racemization enzymes in this species. Dr. Sun, the supervisor of this research, in his recent under-revision paper suggested that in racemic mixtures, the organism first exhaust L-AAs and then, without interruption, begins to use D-AAs, already converted to L- form. To find out if *Scenedesmus* sp. possesses racemases, a mixture of D- and L-enantiomers of each amino acid was added to the concentrated culture of *Scenedesmus* sp. Uptake was monitored by high performance liquid chromatography analysis of enantiomers remaining in the medium. If the uptake

of D- and L-amino acids were identical, there would be a very high chance of the racemization presence.

As demonstrated in Figure B.1, A and B, *Scenedesmus* sp. consumes L-glutamic acid and L-alanine at the rates comparable to D-glutamic acid and D-alanine, respectively. In the culture containing DL-glutamic acid, L-glutamic acid uptake starts immediately but D- is not consumed for about 40 hours. The delay in the onset of the activity could be because D-AAs have to wait for the L-enantiomers to be exhausted first. However, at hour forty, the cells consumed the D-glutamic acid at a rate that could meet their need for the L-AAs (Figure B.5). This suggests that D-glutamic acid is taken up, converted, and metabolized as L-glutamic acid. Zhang and Sun, in their recent under-revision paper, proposed three scenarios for racemization, all of which correspond to the current findings. In scenario one, an organism with sufficient rasemase activity would consume both D- and L-AAs in equal rates, the case of DL-alanine. In scenario two, the organism would also take up both enantiomers, but with rate kinetics that favor L-enantiomers, the case of DL-aspartic acid and DL-leucine. In scenario three, the organism with limited racemase activity would consume the two forms sequentially, first the L-enantiomers, then the D-enantiomers, the case of DL-glutamic acid.

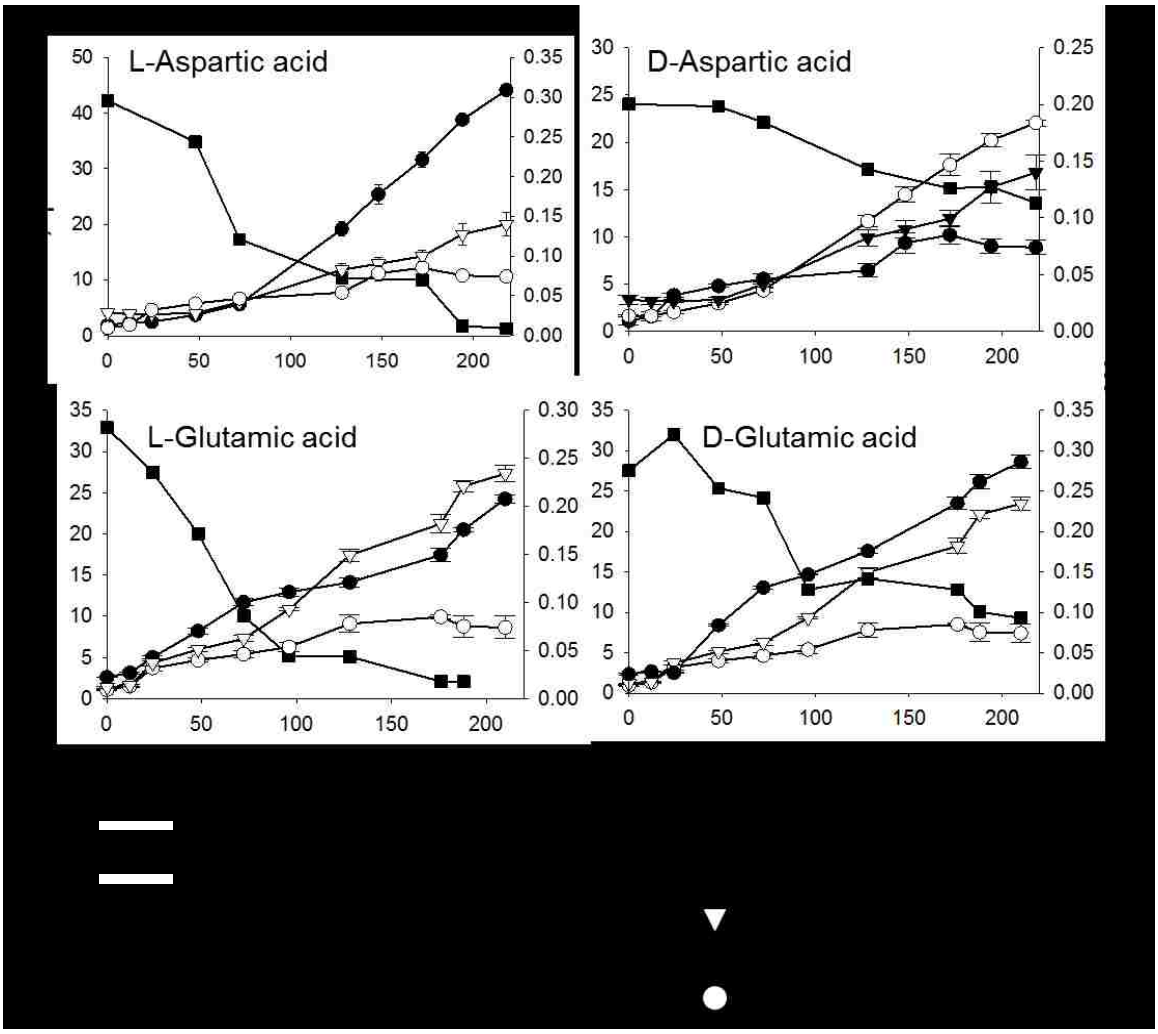

Figure B.1. A. Growth curve of *Scendesmus* sp. in the media composing of nitrogen-free BG11 supplemented with L- or D- amino acids, aspartic acid or glutamic acid, (filled circles), in the standard BG11 (open triangles), and in the nitrogen-free BG11 (open circles) along with the amino acid uptake diagram (filled squares). In consort with growth, all the L- and D-enantiomers were utilized, indicating that the alga obtained its nitrogen requirement, for growth, from the amino acids.

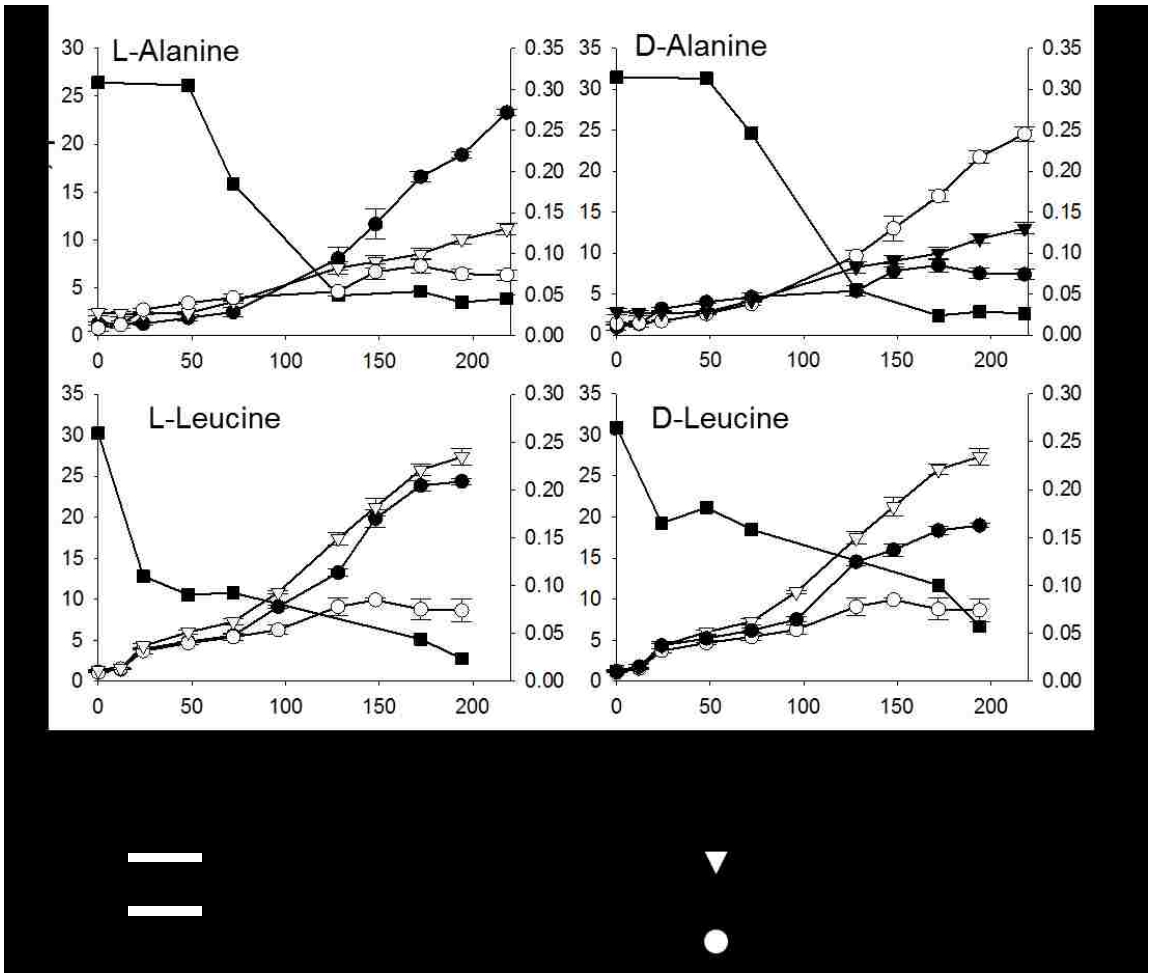

Figure B.1. B. Growth curve of *Scendesmus* sp. in the media composing of nitrogen-free BG11 supplemented with L- or D-amino acids, alanine or leucine, (filled circles), in the standard BG11 (open triangles), and in the nitrogen-free BG11 (open circles) along with the amino acid uptake diagram (filled squares). In consort with growth, all the L- and D-enantiomers were utilized, indicating that the alga obtained its nitrogen requirement, for growth, from the amino acids.

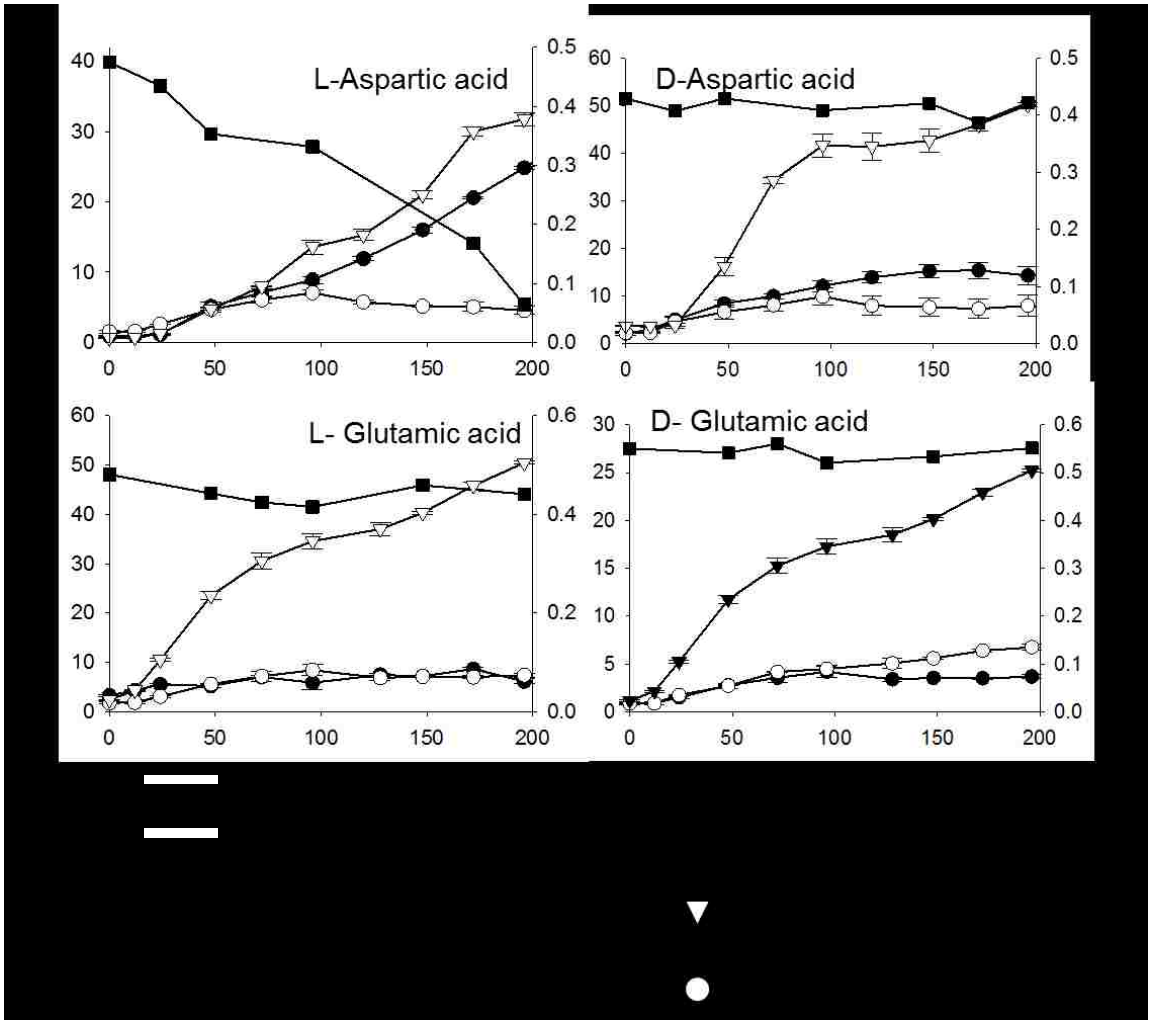

Figure B.2. A. Growth curve of *C. sorokiniana* in the media composing of nitrogen-free BG11 supplemented with either L- or D-amino acids, aspartic acid or glutamic acid, (filled circles), in the standard BG11 (open triangles), and in the nitrogen-free BG11 (open circles) along with the amino acid uptake diagram (filled squares). Only L-aspartic acid and L-Leucnine were utilized, accompanied with growth. The unutilized enantiomer did not support nor inhibit the growth.

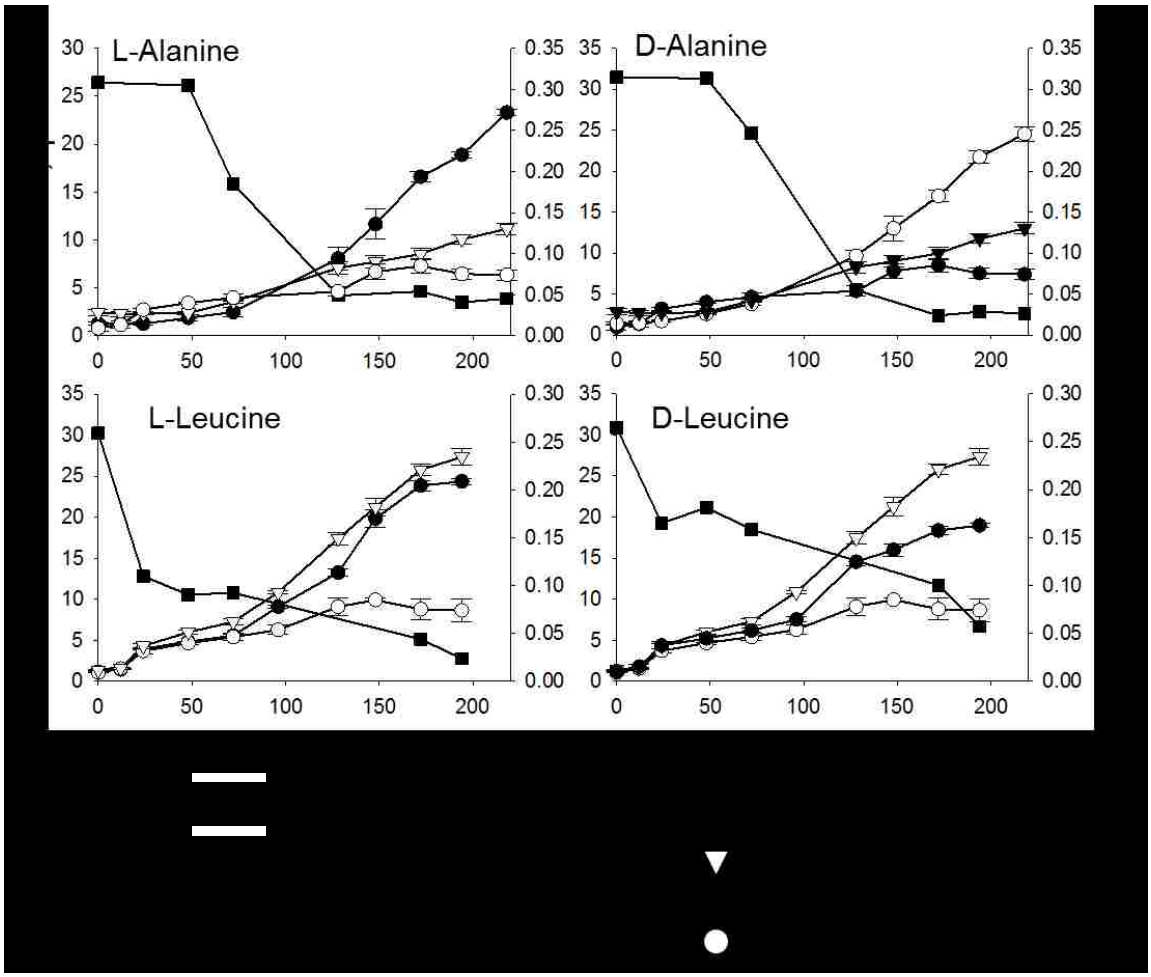

Figure B.2. B. Growth curve of *C. sorokiniana* in the media composing of nitrogen-free BG11 supplemented with either L- or D-amino acids, alanine or leucine, (filled circles), in the standard BG11 (open triangles), and in the nitrogen-free BG11 (open circles) along with the amino acid uptake diagram (filled squares). Only L-aspartic acid and L-Leucnine were utilized, accompanied with growth. The unutilized enantiomer did not support nor inhibit the growth.

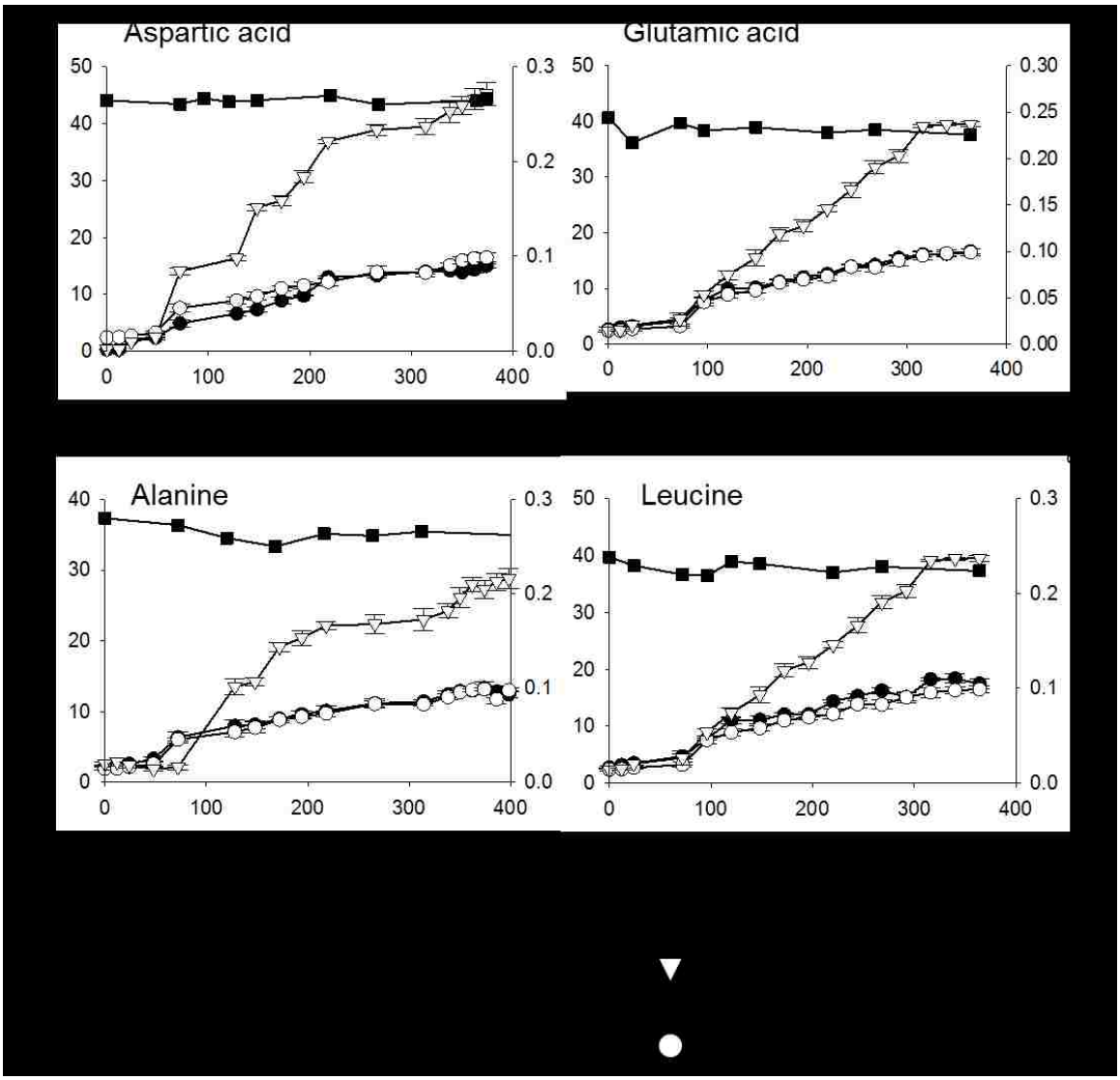

Figure B.3. Growth curve of *Euglena gracili*, UTEX 367, in the media composing of nitrogen-free P49 supplemented with either L-amino acids (filled circles), in the standard P49 (open triangles), and in the nitrogen-free P49 (open circles) along with the amino acid uptake diagram (filled squares). No amino acid was utilized.

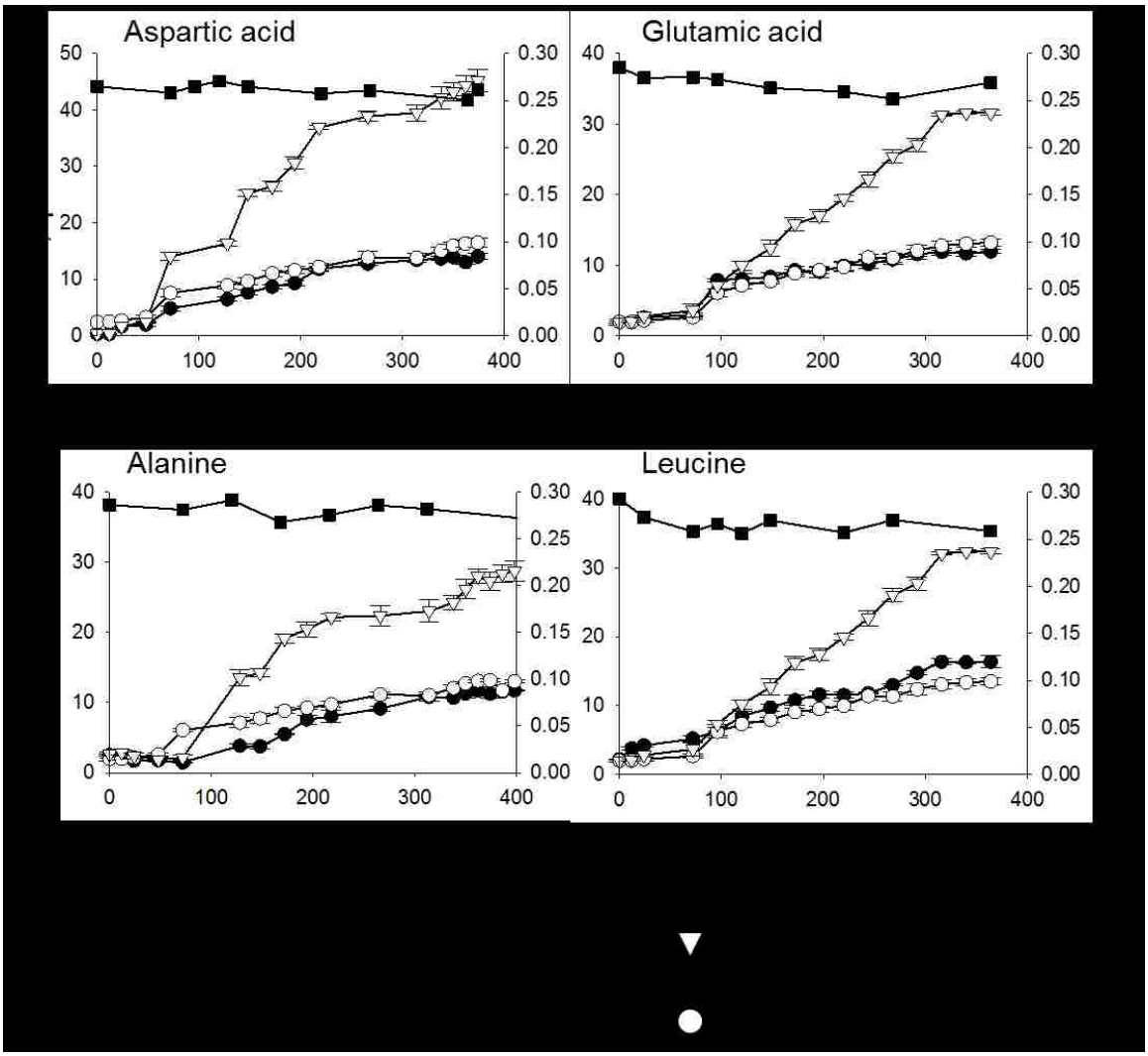

Figure B.4. Growth curve of *Euglena gracili*, UTEX 367, in the media composing of nitrogen-free P49 supplemented with either D-amino acids (filled circles), in the standard P49 (open triangles), and in the nitrogen-free P49 (open circles) along with the amino acid uptake diagram (filled squares). No amino acid was utilized.

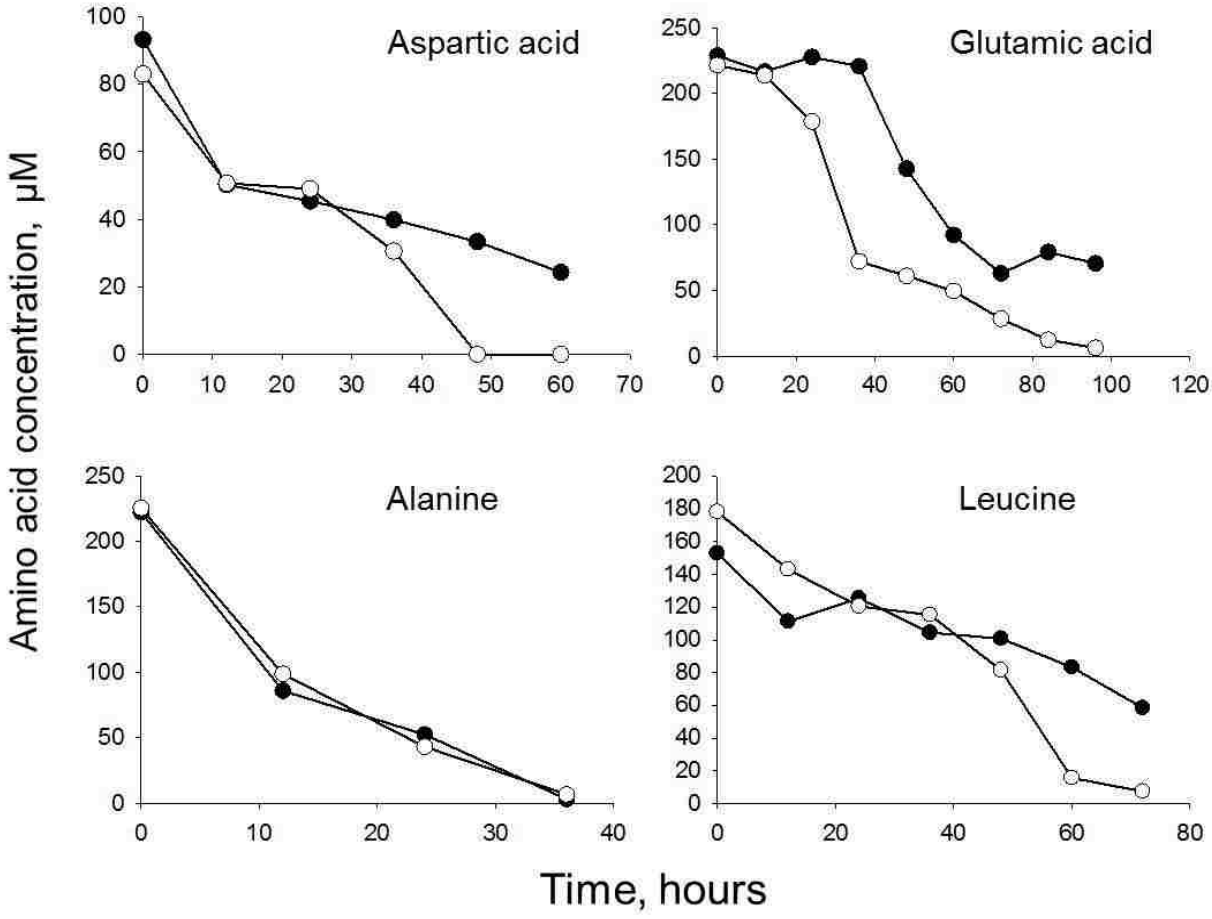

Figure B.5. Kinetics of the D- (filled circle) and the L- (open circles) enantiomers of the amino acids consumption by *Scenedesmus* sp., when the two enantiomers were supplied as a mixture, with no nitrate added.

For *C. sorokiniana*, the situation was different. Cells consumed only two amino acids, aspartic acid and leucine, and only the L- forms. This indicates that this alga does not possess racemases. Two potential situations are proposed. First, D-AAs were toxic to the alga but the toxicity did not appear due to the lack of nitrogen source in the medium. Since the cells are not able to grow in the absence of nitrogen any way, the potential inhibition was not exposed. Hence, further experiments were conducted in

which the D-AAs were added to the media that contained sufficient amount of nitrate. In this case, no growth would mean suppression by D-AAs. The results ruled out this possibility (Figure B.6A, B.7A). Cells grew in presence of D-AAs as well in the mineral medium BG11, in the absence of D-AAs.

In another additional experiments, L-aspartic acid and L-leucine were added to the media containing nitrate. No uptake occurred (Figure B.6B, B.7B). That *C. sorokiniana* consumed these two L-AAs only in the absence of nitrate but not in the presence of it suggests that the cells express the responsible enzymes only when the amino acids are required for growth. It is speculated that only when it is required, cells secrete L-AA oxidases (LAAOs) into the culture, outside the cell. This enzyme is specific to the type of the amino acid, thus LAAOs associated to aspartic acid cannot metabolize glutamic acid. This could be the reason why *C. sorokiniana* assimilated only two amino acids. The enzyme also is enantiomeric- specific, meaning LAAOs cannot oxidize D-AAs and vice versa. It is postulated that the cells produce LAAOs only in the demanding situations because of energy cost associated with enzyme production. For instance, they produce LAAOs when they need to consume the nitrogen source of amino acids to divide. The LAAOs oxidize the amino acids into ammonium, α-keto acid, and hydrogen peroxide, the first two of which are recycled as nitrogen and carbon and the third one converts to oxygen and hydrogen.

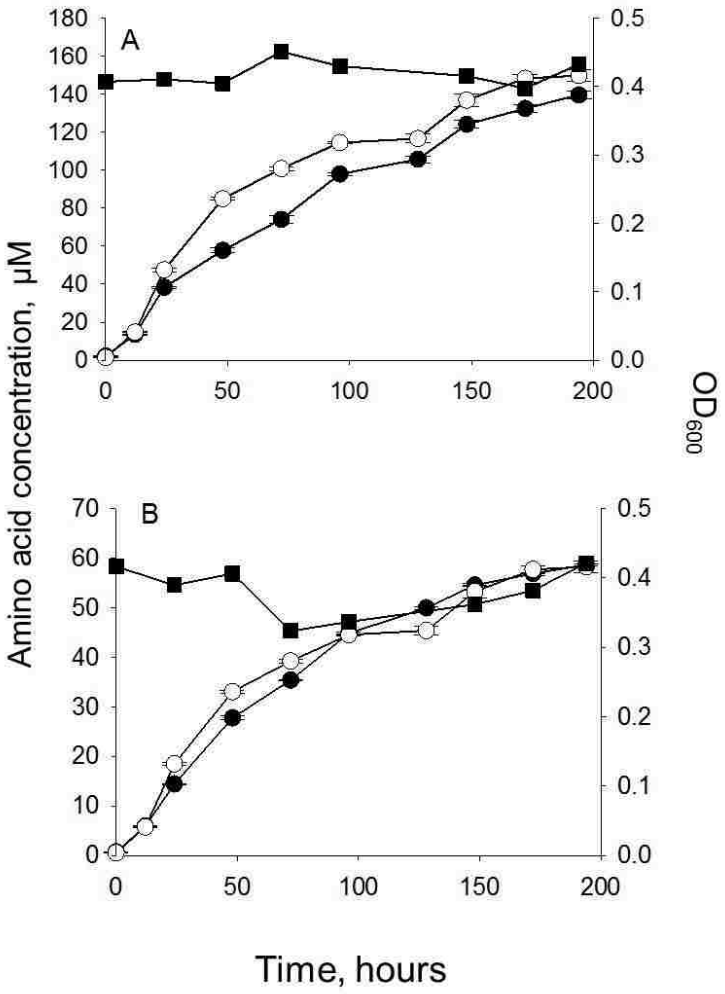

Figure B.6. Growth curve of *C. sorokiniana* in the media containing the standard medium BG11 added with either D- or L- enantiomers of aspartic acid (filled circles), and in the standard BG11 (open circles) along with the amino acid uptake diagram (filled squares). A) D-aspartic acid, B) L-aspartic acid. None were utilized. No inhibition occurred.

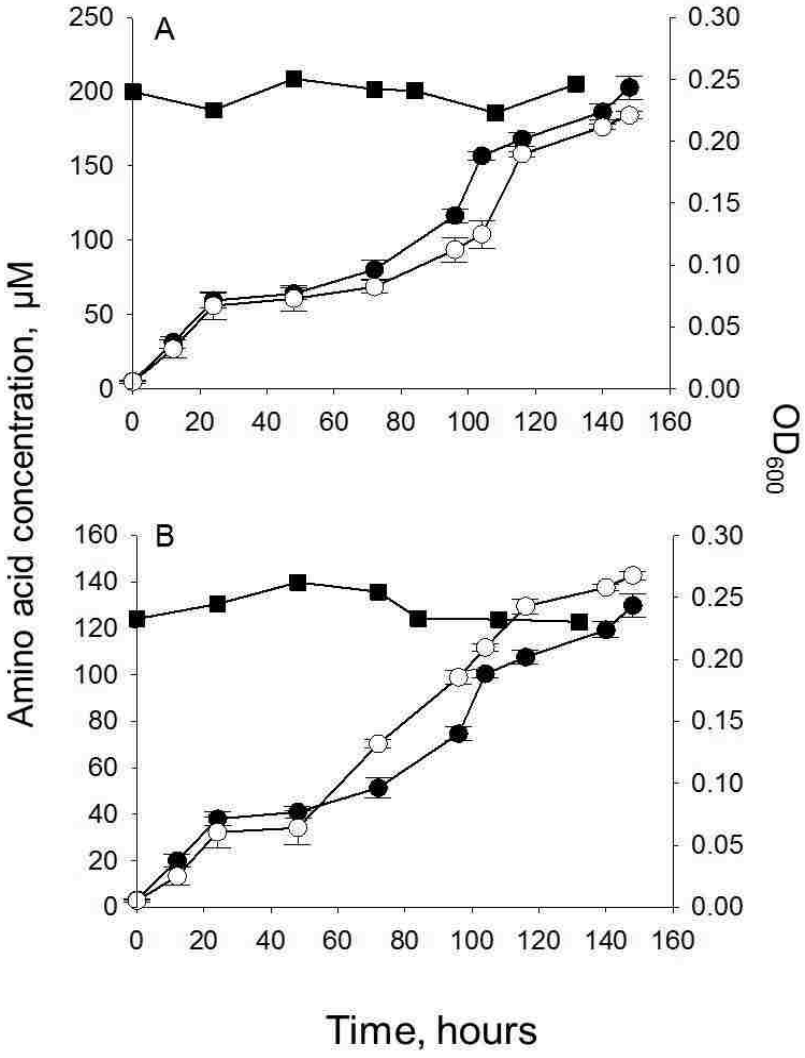

Figure B.7. Growth curve of *C. sorokiniana* in the media containing the standard BG11 added with either D- or L- enantiomers of leucine (filled circles), and in the standard BG11 (open circles) along with the amino acid uptake diagram (filled squares). A) D-leucine, B) L-leucine. None were utilized. No inhibition occurred.

Hence, the results suggest that *C. sorokiniana* cells produce only LAAOs corresponding to aspartic acid and Leucine. They do not produce any DAAOs.

APPENDIX C

RAW DATA

Nitrate Requirement

Chlorella sorokiniana		
Nitrate, µM	Rate	Error
0	0	0
4.9	0.27	6.98E-03
9.9	0.80	0.0123
49.4	1.21	9.31E-03
98.9	1.13	6.65E-04
148.3	1.13	0.0185
197.8	1.13	0.0233
296.7	1.04	0.0305
395.6	1.13	0.0211
494.5	1.13	0.0174
588	1.16	0.0461
686	1.12	6.34E-03
784	1.12	4.02E-03
Scenedesmus sp.		
Nitrate, µM	Rate	Error
0	5.22E-03	0
4.9	1.1339	6.98E-03
9.9	1.4372	0.0123
49.4	1.4589	9.31E-03
98.9	1.5318	6.65E-04
148.3	1.5895	0.0185
197.8	1.6372	0.0233
296.7	1.5971	0.0105
395.6	1.6165	0.0211
494.5	1.5852	0.0174
588	1.4814	0.079
686	1.518	0.0868
784	1.4389	2.63E-03

Synechocystis sp.		
Nitrate, µM	Rate	Error
0	0	2.02E-03
4.9	0.7234	4.99E-03
9.9	0.8356	5.33E-03
49.4	1.1111	4.70E-03
98.9	1.1504	4.09E-03
148.3	1.1313	4.77E-03
197.8	1.1111	4.77E-03
296.7	1.0896	5.72E-03
395.6	1.1111	4.59E-03
494.5	1.0896	7.85E-03
Limnothrix sp.		
Nitrate, µM	Rate	Error
0	0	8.54E-04
4.9	1.16	2.59E-03
9.9	1.24	7.07E-03
49.4	1.32	8.13E-03
98.9	1.33	4.66E-03
148.3	1.33	7.63E-03
197.8	1.33	6.14E-03
296.7	1.33	0.0111
395.6	1.33	0.0123
494.5	1.34	0.0136
Nitzschia thermalis		
Nitrate, µM	Rate	Error
0	0	1.26E-03
4.9	0.75	5.31E-03
9.9	1.11	7.43E-03
49.4	1.39	8.34E-03
98.9	1.51	2.60E-03
148.3	1.51	4.97E-03
197.8	1.48	3.12E-03
296.7	1.48	3.77E-03
395.6	1.44	0.0109
494.5	1.48	0.0104

Encyonema caespitosum		
Nitrate, µM	Rate	Error
0	0	4.08E-04
4.9	0.39	2.02E-03
9.9	0.85	4.99E-03
49.4	1.04	5.33E-03
98.9	0.99	4.70E-03
148.3	0.99	4.09E-03
197.8	0.99	4.77E-03
296.7	0.93	4.77E-03
395.6	0.99	5.72E-03
494.5	0.98	4.59E-03

Phosphate Requirement

C. sorokiniana		
Phosphate, µM	Rate	Error
0	0.1266	0.0105
28.7	1.7672	0.0211
57.4	2.0931	0.0174
86.2	2.286	7.90E-04
114.9	2.2866	0.0168
172.4	2.2869	2.63E-03
229.9	2.2894	0.0123
287.3	2.2892	9.31E-03
Scenedesmus sp.		
Phosphate, µM	Rate	Error
0	2.20E-03	0.0105
28.7	1.08	0.0211
57.4	1.36	0.0174
86.2	1.61	7.90E-04
114.9	1.68	0.0168
172.4	1.75	2.63E-03
229.9	1.75	0.0123
287.3	1.75	9.31E-03
Synechocystis sp.		
Phosphate, µM	Rate	Error
0	0.36	8.54E-04
28.7	1.28	2.59E-03

57.4	1.50	7.07E-03
86.2	1.57	8.13E-03
114.9	1.61	4.66E-03
172.4	1.63	7.63E-03
229.9	1.6	6.14E-03
287.3	1.62	0.0111
Limnothrix sp.		
Phosphate, uM	Rate	Error
0	0.45	1.70E-03
28.7	2.84	1.19E-03
57.4	3.32	2.96E-03
86.2	3.31	3.43E-03
114.9	3.33	2.52E-03
172.4	3.36	6.61E-03
229.9	3.41	5.30E-03
287.3	3.42	6.89E-03
N. thermalis		
Phosphate, µM	Rate	Error
0	0.03	1.26E-03
28.7	1.17	5.31E-03
57.4	1.53	7.43E-03
86.2	1.62	8.34E-03
114.9	1.63	2.60E-03
172.4	1.69	4.97E-03
229.9	1.71	3.12E-03
287.3	1.72	3.77E-03
E. caespitosum		
Phosphate, µM	Rate	Error
0	0.02	5.45E-03
28.7	1.31	5.35E-03
57.4	1.63	5.45E-03
86.2	1.75	3.47E-03
114.9	1.77	0.0105
172.4	1.74	0.0109
229.9	1.77	0.0135
287.3	1.75	5.98E-03

C. sorokiniana		
Sodium chloride, mM	Rate	Error
2.995	0.24	0
11.98	0.23	6.98E-03
17.97	0.24	0.0123
23.96	0.31	9.31E-03
41.93	0.20	6.65E-04
59.9	0.09	0.0185
89.85	7.95E-03	0.0233
Scenedesmus sp.		
Sodium chloride, mM	Rate	Error
2.99	0.39	0
11.98	0.40	6.98E-03
17.97	0.42	0.0123
23.96	0.44	9.31E-03
41.93	0.39	6.65E-04
59.9	0.28	0.0185
89.85	4.39E-03	0.0233
Synechocystis sp.		
Sodium chloride, mM	Rate	Error
2.995	0.91	2.74E-03
11.98	1.00	2.21E-03
17.97	0.97	2.68E-03
23.96	0.96	2.02E-03
41.93	0.83	2.81E-03
59.9	0.81	3.38E-03
89.85	0.63	2.90E-03
Limnothrix sp.		
Sodium chloride, mM	Rate	Error
2.995	0.92	3.30E-03
11.98	0.90	3.52E-03
17.97	0.91	1.89E-03
23.96	0.89	2.35E-03
41.93	0.80	5.04E-03
59.9	0.32	3.94E-03

89.85	0.01	5.24E-03
N. thermalis		
Sodium chloride, mM	Rate	Error
2.995	0.73	4.79E-04
11.98	0.76	8.17E-04
17.97	0.70	2.89E-04
23.96	0.62	4.08E-04
41.93	0.35	9.46E-04
59.9	0.13	8.54E-04
89.85	0.06	1.08E-03
E. caespitosum		
Sodium chloride, mM	Rate	Error
2.995	0.83	1.47E-03
11.98	0.73	1.08E-03
17.97	0.59	1.11E-03
23.96	0.61	1.80E-03
41.93	0.32	1.66E-03
59.9	0.10	1.89E-03
89.85	0.06	2.25E-03

Temperature Requirement

C. sorokiniana		
T,C	Rate	Error
43.3	1.72	0.0105
38.1	2.30	0.0211
33.7	2.48	0.0174
26.9	2.48	7.90E-04
21.6	1.92	0.0168
17.7	1.04	2.63E-03
Scenedesmus sp.		
T,C	Rate	Error
43.3	0.62	0.0105
38.1	0.69	0.0211
33.7	0.53	0.0174

26.9	0.38	7.90E-04
21.6	0.23	0.0168
17.7	9.72E-03	2.63E-03
Synechocystis sp.		
T,C	Rate	Error
43.3	0.04	0.0829
38.1	0.91	0.0539
33.7	1.23	0.0157
26.9	1.36	0.0608
21.6	1.03	0.045
17.7	0.93	0.0549
Limnothrix sp.		
T,C	Rate	Error
43.3	0.03	0.01
38.1	0.20	0.0147
33.7	0.51	0.025
26.9	0.23	0.0185
21.6	0.18	0.0196
17.7	0.13	0.0248
N. thermalis		
T,C	Rate	Error
46	0.02	0.025
38.1	0.48	0.0194
36	0.71	2.33E-03
33.7	0.81	0.0238
32	0.87	4.03E-03
26.9	0.84	2.56E-03
21.6	0.76	0.0342
17.7	0.3	3.00E-03
E. caespitosum		
T,C	Rate	Error
43.3	0.04	0.039
38.1	0.29	2.22E-03
33.7	0.51	9.57E-04
26.9	0.83	0.0281
21.6	0.72	4.35E-03

17.7	0.41	3.68E-03

Light Requirement

C. sorokiniana		
Light intensity, µmole/m^2.s	Rate	Error
1155	1.65	4.08E-03
825	1.59	8.66E-04
82.5	1.41	0.0122
33	1.34	7.50E-03
16.5	1.24	0.0408
8.25	1.22	0.0629
1.65	0.78	1.18E-03
0	0	9.46E-04
Scenedesmus sp.		
Light intensity, µmole/m^2.s	Rate	Error
1155	1.05	0.0119
825	1.04	2.17E-03
82.5	1.01	0.0206
33	1.00	0.0171
16.5	0.99	2.25E-03
8.25	0.99	2.83E-03
1.65	1.02	0.0108
0	0	1.80E-03
Synechocystis sp.		
Light intensity, µmole/m^2.s	Rate	Error
1155	0.10	2.90E-03
825	0.27	2.81E-03
82.5	0.41	3.28E-03
33	0.54	2.17E-03
16.5	0.54	2.46E-03
8.25	0.55	3.64E-03
1.65	0.56	3.71E-03
Limnothrix sp.		
Light intensity,	Rate	Error

µmole/m^2.s		
1155	0.11	0.0466
825	0.35	2.72E-03
82.5	0.53	0.0409
33	0.78	0.0315
16.5	0.77	0.0474
8.25	0.91	0.0397
1.65	1.3	3.16E-03
N. thermalis		
Light intensity, µmole/m^2.s	Rate	Error
1155	0.88	0.0479
825	0.86	8.17E-04
82.5	1.04	2.89E-03
33	1.04	0.0408
16.5	1.04	0.0946
8.25	0.93	8.54E-04
1.65	0.78	1.08E-03
0	0	0.0147
E. caespitosum		
Light intensity, µmole/m^2.s	Rate	Error
1155	0.94	0.018
825	1.00	0.0166
82.5	1.06	0.0189
33	1.06	2.25E-03
16.5	1.07	1.89E-03
8.25	1.01	0.0239
1.65	0.87	0.0887
0	0	1.22E-03

Centrate Amendment

C. sorokiniana		
Time	0%	Error
0	0.031	8.54E-04
16	0.032	1.25E-03
24	0.039	1.32E-03
56	0.037	3.20E-03

64	0.042	5.76E-03
72	0.044	6.76E-03
80	0.048	4.79E-03
88	0.046	4.50E-03
96	0.057	5.64E-03
120	0.055	4.65E-03
10%	Error	15%
0.04	2.27E-03	0.041
0.061	2.18E-03	0.063
0.093	3.52E-03	0.162
0.156	0.0196	0.271
0.172	0.0176	0.316
0.201	7.04E-03	0.329
0.204	6.51E-03	0.336
0.234	5.36E-03	0.365
0.262	0.0189	0.382
0.286	0.0154	0.399
Error	25%	Error
3.18E-03	0.04	1.89E-03
4.29E-03	0.06	2.74E-03
3.82E-03	0.19	2.06E-03
7.96E-03	0.27	0.0241
0.0169	0.35	6.26E-03
8.63E-03	0.4	5.20E-03
9.29E-03	0.41	5.65E-03
4.89E-03	0.43	8.26E-03
7.27E-03	0.45	7.85E-03
9.29E-03	0.46	9.31E-03
35%	Error	40%
0.049	4.92E-03	0.044
0.077	1.89E-03	0.069
0.19	4.27E-03	0.24
0.32	8.73E-03	0.34
0.41	0.0315	0.44
0.44	0.0245	0.47
0.45	0.0279	0.47

0.47	0.0168	0.49
0.46	0.0223	0.50
0.48	0.0148	0.52
Error	50%	Error
2.00E-03	0.036	1.02E-03
1.32E-03	0.05	3.85E-04
4.25E-03	0.15	5.43E-03
2.17E-03	0.34	6.57E-03
7.32E-03	0.45	0.024
7.72E-03	0.47	0.0128
4.92E-03	0.49	0.0122
3.15E-03	0.49	0.0149
2.50E-04	0.51	0.0122
2.94E-03	0.52	0.011

BG11	Error
0.021	6.67E-04
0.039	1.68E-03
0.11	0.014
0.28	5.81E-03
0.33	8.88E-03
0.37	0.018
0.39	0.0227
0.43	0.0208
0.47	0.0169
0.48	0.0154

Centrate Percentage	Rate	Error
0	0.12	0.0131
10	0.44	0.0352
15	0.81	0.0188
30	1.25	0.0178
35	1.34	0.0427
40	1.33	0.0169
50	1.21	0.0543
70	0.62	0.0402
80	0.59	0.0657

100	0.049	0.0169

Scenedesmus sp.				
Time	0%	Error	20%	Error
0	9.00E-03	4.08E-04	9.00E-03	1.15E-03
12	9.75E-03	4.79E-04	0.012	2.50E-03
36	0.016	2.50E-04	0.029	2.66E-03
60	0.021	1.19E-03	0.069	8.90E-03
84	0.031	1.19E-03	0.13	0.0199
132	0.049	2.63E-03	0.32	0.0174
156	0.069	2.96E-03	0.36	0.0179
180	0.073	2.46E-03	0.38	0.0255
204	0.082	5.95E-03	0.40	0.0288
228	0.085	8.26E-03	0.43	0.0226
278	0.092	3.86E-03	0.48	9.47E-03
300	0.092	2.75E-03	0.49	7.59E-03
40%	Error	50%	Error	60%
9.00E-03	4.08E-04	0.01	6.29E-04	0.011
0.012	1.89E-03	0.013	1.32E-03	0.016
0.030	2.18E-03	0.032	1.47E-03	0.036
0.074	3.28E-03	0.066	3.88E-03	0.060
0.17	0.0141	0.143	3.17E-03	0.123
0.36	0.0195	0.351	2.93E-03	0.370
0.39	0.0185	0.381	5.07E-03	0.408
0.43	0.0173	0.403	0.0134	0.441
0.46	0.0127	0.437	5.85E-03	0.469
0.48	9.85E-03	0.462	5.94E-03	0.486
0.52	3.95E-03	0.511	6.46E-03	0.525
0.53	3.95E-03	0.522	5.23E-03	0.533
Error	70%	Error	80%	Error
1.32E-03	0.015	3.66E-03	0.014	7.07E-04
2.16E-03	0.018	4.32E-03	0.017	4.79E-04
1.55E-03	0.033	4.71E-03	0.036	2.50E-03
2.50E-03	0.052	4.44E-03	0.054	1.68E-03
0.0112	0.094	0.0146	0.089	4.37E-03
0.0166	0.314	5.76E-03	0.279	0.0114
0.0178	0.391	0.0318	0.377	6.65E-03

0.0149	0.41	2.84E-03	0.420	0.0105
0.0171	0.454	0.0239	0.443	8.02E-03
0.019	0.469	0.0228	0.462	7.39E-03
0.0151	0.487	0.022	0.507	9.67E-03
0.0132	0.503	0.0182	0.519	0.0106
100%	Error	BG11	Error	
0.017	1.50E-03	0.010	8.00E-04	
0.019	1.32E-03	0.012	8.90E-04	
0.037	2.46E-03	0.02	1.03E-03	
0.050	2.25E-03	0.023	4.33E-04	
0.065	3.42E-03	0.028	1.51E-03	
0.154	0.0153	0.077	9.90E-03	
0.217	0.0137	0.099	0.0148	
0.267	0.0204	0.130	0.0203	
0.325	0.0393	0.161	0.0263	
0.368	0.0361	0.192	0.0307	
0.427	0.0209	0.27	0.0411	
0.432	0.0215	0.280	0.0398	

Centrate Percentage	Rate	Error
0	0.033	1.36E-03
20	0.554	0.0231
40	0.557	0.0232
50	0.647	0.027
60	0.795	0.0331
70	0.868	0.0362
80	0.581	0.0242
100	0.24	0.01

Growth of *C. sorokiniana* in Presence of Different Urea Percentages

Time	0.10%	Error	0.01%	Error	0.001%
0	0.01	0	0.011	7.07E-04	0.012
12	0.02	0	0.045	1.55E-03	0.085
24	0.017	3.46E-03	0.171	2.12E-03	0.162
48	0.014	1.41E-03	0.287	1.19E-03	0.255
56	0.024	4.95E-04	0.357	7.07E-04	0.337

80	0.076	5.44E-03	0.392	0.0289	0.388
90	0.130	1.05E-03	0.422	0.0411	0.420
100	0.146	9.19E-03	0.479	0.0255	0.473
112	0.205	6.01E-03	0.531	1.55E-03	0.519
120	0.24	3.54E-03	0.532	4.31E-03	0.529
130	0.249	2.12E-03	0.539	3.97E-03	0.539

Error	BG11	Error
1.44E-03	0.015	2.50E-03
2.90E-03	0.021	2.14E-03
0.02	0.111	2.00E-03
8.66E-04	0.195	5.17E-03
2.86E-03	0.262	5.45E-03
0.0173	0.325	0.0135
4.25E-03	0.359	0.028
0.0142	0.395	0.026
1.32E-03	0.424	0.0298
6.49E-03	0.457	0.0287
6.77E-03	0.472	5.17E-03

Medium	Rate	Error
BG11	0.963	3.87E-03
BG11+0.001	1.104	0.0212
BG11+0.01	1.128	0.0197
BG11+0.1	0.394	2.86E-03

Growth of *C. sorokiniana* in Presence of Arsenate

Time	Rate with As	Error	Rate in BG11	Error
0	0.032	0	0.029	7.07E-04
12	0.048	0.012	0.061	1.41E-03
60	0.067	0.0198	0.224	9.19E-03
68	0.081	0.0127	0.261	2.12E-03
76	0.097	8.49E-03	0.304	4.95E-03
84	0.130	0.0219	0.317	0.0184
92	0.198	0.0141	0.344	0.0191
118	0.236	0.012	0.381	0.0184

142	0.307	0.012	0.432	0.0283
214	0.315	0.012	0.438	0.0226
226	0.318	8.49E-03	0.431	0.0148
250	0.330	0.0233	0.428	7.78E-03

Mixotrophic Growth

C. sorokiniana		
	Rate	Error
BG11	0.717	0.1066
glucose	1.295	0.0101
glycerol	0.626	0.0113
acetate	1.899	0.0119
lactate	0.708	0.097
Scenedesmus sp.		
	Rate	Errors
BG11	0.420	0.0466
glucose	0.888	0.0659
glycerol	0.879	0.0384
acetate	0.56	0.0388
lactate	0.41	0.0512

Heterotrophic Growth

C. sorokiniana				
Time	BG11+glucose	Error	BG11+acetate	Error
0	0.021	1.89E-03	9.50E-03	4.11E-03
12	0.023	3.54E-03	0.011	2.06E-03
20	0.026	4.48E-03	0.021	1.93E-03
32	0.043	6.60E-03	0.060	6.12E-03
54	0.06	9.59E-03	0.099	7.44E-03
78	0.16	1.11E-03	0.187	3.93E-03
82	0.25	1.19E-03	0.264	7.20E-03
90	0.35	1.71E-03	0.313	4.74E-03

104	0.36	3.37E-03	0.373	2.40E-03
128	0.362	4.01E-03	0.374	7.03E-03
152	0.360	4.01E-03	0.367	0.0126
176	0.362	1.72E-03	0.383	9.32E-03
Only BG11				
0				
1.00E-03				
2.00E-03				
1.25E-03				
2.50E-04				
5.00E-04				
3.50E-03				
1.00E-03				
7.50E-04				
7.50E-04				
1.00E-03				
2.00E-03				
Scenedesmus sp.				
Time	BG11+glucose	Error	BG11+glycerol	Error
0	0.021	1.89E-03	0.014	8.17E-04
12	0.023	3.54E-03	0.014	1.55E-03
24	0.026	4.48E-03	0.013	1.38E-03
30	0.043	6.60E-03	0.014	1.85E-03
42	0.06	9.59E-03	0.013	8.54E-04
66	0.160	1.11E-03	0.012	9.13E-04
90	0.253	1.19E-03	0.012	8.54E-04
114	0.350	1.71E-03	0.012	5.00E-04
138	0.36	3.37E-03	0.012	4.79E-04
162	0.362	4.01E-03	0.012	1.11E-03
200	0.360	4.01E-03	0.012	6.78E-04
234	0.362	1.72E-03	9.00E-03	2.22E-03
BG11+ acetate	Error	Only BG11		
0.018	3.20E-03	0		
0.018	1.03E-03	1.00E-03		
0.016	1.97E-03	2.00E-03		
0.019	2.61E-03	1.25E-03		

0.02	1.96E-03	2.50E-04
0.03	2.69E-03	5.00E-04
0.03	2.53E-03	3.50E-03
0.034	2.72E-03	1.00E-03
0.045	2.25E-03	7.50E-04
0.053	3.15E-03	7.50E-04
0.064	4.45E-03	1.00E-03
0.072	1.17E-03	2.00E-03

Amino Acids Uptake

Scenedesmus sp.				
Aspartic acid				
Time	L/rate	Error	D/rate	Error
0	0.013	4.08E-04	0.014	8.54E-04
12	0.016	1.47E-03	0.014	1.03E-03
24	0.017	7.07E-04	0.017	1.18E-03
48	0.025	1.55E-03	0.025	1.03E-03
72	0.039	4.67E-03	0.036	1.70E-03
128	0.134	9.08E-03	0.097	4.74E-03
148	0.177	0.0118	0.120	6.66E-03
172	0.221	9.28E-03	0.147	9.50E-03
194	0.272	6.60E-03	0.168	5.78E-03
218	0.309	3.08E-03	0.184	2.69E-03
266	0.325	1.26E-03	0.224	2.94E-03
314	0.330	1.19E-03	0.235	2.32E-03
BG11-N/rate	Error	BG11/rate	Error	
9.25E-03	3.50E-03	0.028	3.94E-03	
0.013	4.25E-03	0.027	3.30E-03	
0.032	1.93E-03	0.027	1.44E-03	
0.04	1.32E-03	0.028	1.93E-03	
0.046	4.52E-03	0.041	2.02E-03	
0.054	5.95E-03	0.082	7.32E-03	
0.078	9.12E-03	0.09	7.42E-03	
0.085	8.22E-03	0.1	7.56E-03	
0.075	6.42E-03	0.127	0.0138	
0.074	6.20E-03	0.14	0.0154	

0.073	3.49E-03	0.2	0.0111	
0.074	6.41E-03	0.208	8.44E-03	

Time	L concentration	D concentration
0	42.23	24.03
48	34.81	23.8
72	17.28	22.11
128	10.31	17.13
172	9.96	15.13
194	1.67	15.31
218	1.23	13.6
266	1.44	4.89
314		2.22

Glutamic acid

Time	L/rate	Error	D/rate	Error
0	0.022	4.79E-04	0.023	9.57E-04
12	0.027	1.11E-03	0.027	7.50E-04
24	0.043	1.29E-03	0.025	7.07E-04
48	0.070	3.22E-03	0.084	1.47E-03
72	0.10	3.75E-03	0.13	2.40E-03
96	0.11	4.31E-03	0.15	8.66E-04
128	0.12	4.64E-03	0.17	3.12E-03
176	0.15	7.26E-03	0.23	7.69E-03
188	0.17	2.29E-03	0.26	9.26E-03
210	0.21	4.61E-03	0.28	8.28E-03

BG11-N/rate	Error	BG11/rate	Error
9.25E-03	4.79E-04	0.01	7.50E-04
0.013	1.38E-03	0.014	9.46E-04
0.033	2.69E-03	0.037	2.68E-03
0.04	2.04E-03	0.051	1.89E-03
0.046	3.57E-03	0.062	3.20E-03
0.054	4.82E-03	0.093	1.68E-03
0.078	9.25E-03	0.149	6.46E-03
0.085	2.25E-03	0.182	9.41E-03
0.075	0.0112	0.221	5.60E-03

0.074	0.0117	0.234	8.59E-03

Time	L concentration, µM	D concentration, µM
0	32.88	27.513
24	27.45	31.983
48	20.04	25.333
72	10.00	24.159
96	5.20	12.780
128	5.087	14.150
176	2.083	12.794
188	2.083	10.045
210		9.262

Alanine

Time	L/rate	Error	D/rate	Error
0	0.015	2.02E-03	0.014	1.78E-03
12	0.015	1.44E-03	0.014	1.04E-03
24	0.015	2.17E-03	0.017	1.47E-03
48	0.022	3.28E-03	0.026	1.65E-03
72	0.029	5.73E-03	0.038	1.83E-03
128	0.094	0.0134	0.096	6.99E-03
148	0.136	0.0184	0.13	0.0153
172	0.193	6.51E-03	0.169	7.17E-03
194	0.22	3.94E-03	0.217	7.58E-03
218	0.27	3.75E-03	0.245	9.12E-03
266	0.28	1.41E-03	0.287	3.90E-03
314	0.29	1.32E-03	0.297	3.07E-03

BG11-N/rate	Error	BG11/rate	Error	
9.25E-03	3.50E-03	0.028	3.94E-03	
0.013	4.25E-03	0.027	3.30E-03	
0.032	1.93E-03	0.027	1.44E-03	
0.04	1.32E-03	0.028	1.93E-03	
0.046	4.52E-03	0.041	2.02E-03	
0.054	5.95E-03	0.082	7.32E-03	

0.078	9.12E-03	0.09	7.42E-03	
0.085	8.22E-03	0.1	7.56E-03	
0.075	6.42E-03	0.117	5.60E-03	
0.074	6.20E-03	0.13	7.18E-03	
0.073	3.49E-03	0.195	8.40E-03	
0.074	6.41E-03	0.208	8.44E-03	

Time	L concentration	D concentration
0	26.42	31.43
48	26.13	31.31
72	15.87	24.60
128	4.18	5.46
172	4.63	2.37
194	3.49	2.84
218	3.88	2.62
266	4.12	1.62
314	3.87	2.01

C. sorokiniana				
Aspartic acid				
Time	L/rate	Error	D/rate	Error
0	0.010	1.02E-03	0.018	3.20E-03
12	0.011	1.15E-03	0.025	3.57E-03
24	0.013	1.68E-03	0.042	6.86E-03
48	0.061	6.96E-03	0.071	6.07E-03
72	0.084	5.67E-03	0.083	4.66E-03
96	0.106	6.00E-03	0.10	9.55E-03
120	0.142	3.71E-03	0.12	9.65E-03
148	0.190	5.05E-03	0.13	0.0115
172	0.245	1.33E-03	0.13	0.0138
196	0.296	2.04E-03	0.12	0.0159
220	0.347	3.91E-03	0.12	0.0162
244	0.371	7.73E-03	0.12	0.0287
268	0.368	1.92E-03		
292	0.372	4.73E-03		
BG11-N/rate	Error	BG11/rate	Error	
0.018	2.04E-03	7.50E-03	6.46E-04	

0.018	1.76E-03	8.50E-03	8.66E-04	
0.031	3.85E-04	0.014	1.19E-03	
0.056	3.79E-03	0.056	1.78E-03	
0.072	5.55E-03	0.095	1.32E-03	
0.084	4.37E-03	0.161	0.0119	
0.07	4.00E-03	0.182	9.11E-03	
0.06	1.33E-03	0.250	7.04E-03	
0.060	7.34E-03	0.357	7.67E-03	
0.054	6.58E-03	0.378	0.0116	
0.052	4.07E-03	0.435	5.82E-03	
0.050	3.42E-03	0.443	6.98E-03	
0.051	3.42E-03	0.440	6.98E-03	
0.054	3.42E-03	0.443	6.98E-03	
Time	L concen.	D concen.		
0	39.87	51.48		
24	36.50	48.90		
48	29.67	51.50		
96	27.85	49.04		
172	14.11	50.49		
196	5.36	46.49		
244	3.52	50.66		
292	4.05	51.19		

Glutamic acid				
Time	L/rate	Error	D/rate	Error
0	0.032	2.02E-03	0.018	3.15E-03
12	0.043	1.08E-03	0.018	2.50E-03
24	0.055	1.41E-03	0.035	1.71E-03
48	0.052	3.66E-03	0.055	5.24E-03
72	0.070	4.03E-03	0.084	7.07E-04
96	0.059	0.0145	0.090	6.88E-03
128	0.074	3.07E-03	0.101	0.0106
148	0.072	1.84E-03	0.112	2.25E-03
172	0.086	3.25E-03	0.128	3.23E-03
196	0.061	5.42E-03	0.135	5.69E-03

BG11-N/rate	Error	BG11/rate	Error	
0.018	1.25E-03	0.024	1.44E-03	
0.018	2.29E-03	0.043	2.02E-03	
0.031	1.83E-03	0.105	2.93E-03	
0.056	8.54E-04	0.235	7.84E-03	
0.072	0.0108	0.305	0.0158	
0.084	0.011	0.346	0.0154	
0.068	4.35E-03	0.37	0.0141	
0.071	1.75E-03	0.40	2.74E-03	
0.070	2.66E-03	0.46	6.54E-03	
0.074	3.80E-03	0.504	2.90E-03	
Time	L concentration	D concentration		
0	48.067	27.49		
48	44.21	27.05		
72	42.41	28.00		
96	41.49	26.02		
148	45.88	26.68		
196	44.0743	27.54		

Alanine				
Time	L/rate	Error	D/rate	Error
0	0.032	1.11E-03	0.018	1.32E-03
12	0.043	1.73E-03	0.025	2.17E-03
24	0.055	4.57E-03	0.037	2.74E-03
48	0.062	6.80E-03	0.063	1.04E-03
72	0.070	6.87E-03	0.075	2.21E-03
96	0.088	2.16E-03	0.083	8.54E-04
128	0.084	7.04E-03	0.085	3.71E-03
148	0.082	1.32E-03	0.087	2.40E-03
BG11-N/rate	Error	BG11/rate	Error	
0.018	1.25E-03	0.024	1.44E-03	
0.018	2.29E-03	0.043	2.02E-03	
0.031	1.83E-03	0.105	2.93E-03	
0.056	8.54E-04	0.235	7.84E-03	
0.072	0.0108	0.305	0.0158	
0.084	0.011	0.346	0.0154	
0.068	4.35E-03	0.37	0.0141	

0.071	1.75E-03	0.403	2.74E-03	
Time	L concentration	D concentration		
0	45.88	44.63		
24	38.91	40.57		
48	40.21	46.42		
72	43.384	40.72		
120	40.472	46.65		
148	40.574	44.31		

Leucine				
Time	L/rate	Error	D/rate	Error
0	0.02	3.40E-03	0.027	3.88E-03
12	0.032	2.17E-03	0.048	1.44E-03
24	0.058	1.58E-03	0.068	2.39E-03
48	0.135	0.0213	0.097	3.70E-03
72	0.151	0.0253	0.090	3.69E-03
128	0.199	0.0282	0.072	3.24E-03
148	0.208	0.0311	0.047	0.013
172	0.235	0.03	0.028	3.47E-03
194	0.241	0.0248	0.035	5.82E-03
218	0.26	0.0288	0.052	0.0142
266	0.262	0.0295	0.038	0.0152
314	0.275	0.0252	0.041	0.0151
338	0.296	0.0302	0.053	0.0135
350	0.300	0.0278	0.054	0.0142
362	0.315	0.0242	0.063	0.0114
374	0.329	0.0193	0.066	0.0165
386	0.331	0.02	0.070	0.0165
398	0.33	0.018	0.058	0.0114
410	0.34	0.0184	0.067	0.0186
BG11/rate	Error	BG11-N/rate	Error	
0.017	1.26E-03	0.018	2.04E-03	
0.038	2.50E-03	0.018	1.76E-03	
0.118	4.17E-03	0.031	3.85E-04	
0.171	7.08E-03	0.056	3.79E-03	
0.178	8.26E-03	0.072	5.55E-03	
0.21	0.0159	0.084	4.37E-03	

0.222	0.0129	0.068	4.00E-03	
0.243	0.0122	0.061	1.33E-03	
0.267	0.0124	0.060	7.34E-03	
0.287	0.0144	0.054	6.58E-03	
0.286	0.0109	0.052	4.07E-03	
0.306	0.0195	0.050	3.42E-03	
0.316	0.0175	0.049	3.42E-03	
0.321	0.0171	0.054	3.42E-03	
0.345	0.0185	0.072	4.37E-03	
0.353	0.0206	0.086	4.00E-03	
0.356	0.0184	0.061	1.33E-03	
0.364	0.0178	0.074	7.34E-03	
0.365	0.0167	0.063	6.58E-03	
Time	D concentration	L concentration		
0	74.153	40.08		
24	67.436	37.48		
48	71.351	35.32		
96	68.072	32.55		
172	71.119	18.93		
196	55.99	8.733		
244	66.13	9.637		
292	69.23	4.323		
386	68.37	4.757		
410	71.25	4.3		

E. gracilis
Aspartic acid

Time	L/rate	Error	D/rate	Error	No N/rate	Error	with N/rate	Error
0	2.00E-03	0	2.00E-03	0	0.0145	0	2.00E-03	0
12	2.00E-03	0	2.00E-03	0	0.015	0	2.00E-03	0
24	0.0125	1.76E-03	0.01	0	0.016	0	0.01	0
48	0.0145	3.07E-03	0.012	1.32E-03	0.019	1.89E-03	0.014	4.79E-04

72	0.0293	3.47E-03	0.029	3.03E-03	0.045	4.59E-03	0.084	4.11E-03
128	0.034	2.29E-03	0.039	2.02E-03	0.053	3.63E-03	0.098	2.55E-03
148	0.044	2.86E-03	0.046	3.08E-03	0.058	4.02E-03	0.151	3.23E-03
172	0.053	3.48E-03	0.052	1.78E-03	0.066	2.95E-03	0.159	5.74E-03
194	0.059	6.29E-04	0.056	2.38E-03	0.069	3.81E-03	0.184	6.34E-03
218	0.078	1.41E-03	0.071	2.27E-03	0.073	3.72E-03	0.22	2.02E-03
266	0.08	8.54E-04	0.076	1.55E-03	0.083	6.25E-03	0.23	5.60E-03
314	0.084	2.58E-03	0.080	2.87E-03	0.083	4.34E-03	0.27	8.43E-03
338	0.085	1.80E-03	0.082	2.00E-03	0.090	3.17E-03	0.25	0.0118
350	0.083	2.25E-03	0.083	2.29E-03	0.095	3.67E-03	0.259	9.65E-03
362	0.086	3.47E-03	0.078	1.87E-03	0.098	4.05E-03	0.266	0.0111
374	0.09	1.63E-03	0.084	2.90E-03	0.099	4.59E-03	0.271	0.0123

Time	L concentration	D concentration
0	44.07	44.146
72	43.41	43.031
96	44.40	44.172
120	43.811	45.125
148	44.086	44.088
220	44.884	42.905
268	43.357	43.455
364	44.01	41.763
374	44.442	43.592

Glutamic acid

Time	L/rate	Error	D/rate	Error	No N/rate	Error	with N/rate	Error
0	0.016	2.35E-03	0.014	3.28E-03	0.014	1.19E-03	0.014	0
12	0.018	3.65E-03	0.014	3.66E-	0.014	1.11E-	0.015	9.46E-

				03		03		04
24	0.019	1.55E-03	0.019	2.33E-03	0.016	1.44E-03	0.020	1.55E-03
72	0.025	3.54E-03	0.022	1.41E-03	0.019	2.40E-03	0.027	7.44E-03
96	0.048	2.81E-03	0.059	1.49E-03	0.045	3.88E-03	0.053	4.17E-03
120	0.06	4.13E-03	0.06	5.52E-03	0.053	3.38E-03	0.074	5.11E-03
148	0.061	5.28E-03	0.062	5.07E-03	0.058	2.52E-03	0.093	8.41E-03
172	0.067	2.33E-03	0.069	4.56E-03	0.066	3.97E-03	0.118	6.90E-03
196	0.072	2.95E-03	0.068	5.79E-03	0.069	2.90E-03	0.127	6.41E-03
220	0.076	1.11E-03	0.074	4.53E-03	0.073	4.95E-03	0.146	3.45E-03
244	0.084	1.32E-03	0.076	5.31E-03	0.083	3.82E-03	0.166	8.08E-03
268	0.085	1.32E-03	0.081	5.64E-03	0.083	4.61E-03	0.191	7.11E-03
292	0.093	2.55E-03	0.086	5.33E-03	0.090	6.20E-03	0.202	6.98E-03
316	0.097	2.17E-03	0.089	4.25E-03	0.095	4.99E-03	0.234	1.38E-03
340	0.097	1.93E-03	0.087	2.66E-03	0.098	3.87E-03	0.237	1.60E-03
364	0.1	3.14E-03	0.089	2.10E-03	0.1	3.84E-03	0.24	2.97E-03
Time	L concentration	D concentration						
0	40.66	38						
24	36.18	36.56						
72	39.71	36.62						
96	38.34	36.35						
148	38.94	35.1						
220	37.98	34.57						
268	38.5	33.55						
364	37.61	35.88						

Alanine

Time	L/rate	Error	D/rate	Error	No N/rate	Error	with N/rate	Error
0	0.016	2.43E-03	0.019	1.08E-03	0.014	1.78E-03	0.02	2.14E-03
12	0.018	2.04E-03	0.017	5.77E-04	0.015	1.58E-03	0.021	2.50E-03
24	0.019	1.35E-03	0.013	1.08E-03	0.016	1.03E-03	0.018	3.24E-03
48	0.025	1.91E-03	0.013	1.25E-03	0.019	1.85E-03	0.014	2.35E-03
72	0.048	6.20E-03	0.011	4.08E-04	0.045	1.25E-03	0.016	2.66E-03
128	0.06	7.00E-03	0.029	2.56E-03	0.053	5.24E-03	0.101	8.68E-03
148	0.061	3.79E-03	0.028	2.72E-03	0.058	5.95E-03	0.106	4.29E-03
172	0.067	1.89E-03	0.041	9.46E-04	0.066	3.24E-03	0.143	4.84E-03
194	0.072	3.57E-03	0.057	4.96E-03	0.069	2.04E-03	0.153	8.01E-03
218	0.076	5.92E-03	0.060	6.17E-03	0.073	3.30E-03	0.166	3.84E-03
266	0.084	4.80E-03	0.068	2.84E-03	0.083	3.28E-03	0.168	0.0105
314	0.085	2.33E-03	0.081	5.02E-03	0.083	1.76E-03	0.173	0.0119
338	0.093	3.82E-03	0.08	5.77E-04	0.090	3.40E-03	0.182	7.51E-03
350	0.097	3.09E-03	0.085	5.12E-03	0.095	1.04E-03	0.196	0.0105
362	0.097	2.21E-03	0.088	3.55E-03	0.098	1.61E-03	0.21	7.43E-03
374	0.1	6.91E-03	0.0843	4.65E-03	0.099	1.80E-03	0.204	9.50E-03
386	0.096	6.60E-03	0.0863	2.02E-03	0.088	4.33E-03	0.212	9.35E-03
398	0.093	3.55E-03	0.088	8.66E-04	0.098	2.02E-03	0.216	0.0108
410	0.103	3.01E-03	0.091	1.80E-03	0.106	2.84E-03	0.221	0.01
422	0.108	1.26E-03	0.099	3.33E	0.107	2.18E	0.224	9.34E-

			3	-03		-03		03
434	0.109	1.26E-03	0.1073	4.91E-03	0.107	1.04E-03	0.227	0.0115
446	0.112	3.55E-03	0.1087	4.19E-03	0.107	1.04E-03	0.23	0.0107
458	0.115	5.77E-03	0.109	4.33E-03	0.107	1.04E-03	0.233	9.81E-03
470	0.115	5.77E-03	0.107	3.46E-03	0.105	3.79E-03	0.231	0.0115
482	0.116	6.14E-03	0.097	4.27E-03	0.1	4.58E-03	0.232	0.0132
Time	L concentration	D concentration						
0	37.33	38.16						
72	36.362	37.465						
120	34.53	38.845						
168	33.342	35.71						
216	35.124	36.73						
264	34.84	38.105						
312	35.44	37.591						
408	34.92	36.197						
482	35.215	38.423						

Lucine

Time	L/rate	Error	D/rate	Error	No N/rate	Error	with N/rate	Error
0	0.0165	1.32E-03	0.016	6.67E-04	0.0145	1.19E-03	0.014	0
12	0.019	2.48E-03	0.028	1.92E-03	0.0148	1.11E-03	0.0153	9.46E-04
24	0.021	2.45E-03	0.031	1.02E-03	0.0163	1.44E-03	0.0205	1.55E-03
72	0.028	2.29E-03	0.038	3.71E-03	0.0195	2.40E-03	0.0268	7.44E-03
96	0.047	8.54E-04	0.046	6.67E-03	0.0455	3.88E-03	0.0535	4.17E-03
120	0.066	3.66E-03	0.062	4.91E-03	0.0535	3.38E-03	0.0743	5.11E-03
148	0.067	3.94E-03	0.071	4.16E-03	0.058	2.52E-03	0.0928	8.41E-03
172	0.072	2.25E-03	0.079	5.29E-03	0.0663	3.97E-03	0.1185	6.90E-03
196	0.073	2.66E-03	0.085	3.71E-	0.0695	2.90E-	0.1273	6.41E-

				03		03		03
220	0.086	1.89E-03	0.084	3.67E-03	0.073	4.95E-03	0.1458	3.45E-03
244	0.092	2.46E-03	0.086	1.02E-03	0.083	3.82E-03	0.1663	8.08E-03
268	0.097	2.99E-03	0.095	1.33E-03	0.083	4.61E-03	0.1908	7.11E-03
292	0.091	4.15E-03	0.108	2.31E-03	0.09	6.20E-03	0.2028	6.98E-03
316	0.109	1.65E-03	0.12	3.01E-03	0.09	4.99E-03	0.2348	1.38E-03
340	0.110	5.02E-03	0.119	1.15E-03	0.098	3.87E-03	0.2368	1.60E-03
364	0.105	5.72E-03	0.12	6.68E-03	0.099	3.84E-03	0.237	2.97E-03

Time	L concentration	D concentration
0	39.707	40.057
24	38.224	37.350
72	36.631	35.323
96	36.448	36.388
120	38.926	35.040
148	38.555	36.904
220	37.041	35.114
268	38.024	36.960
364	37.281	35.344

Kinetics of Aspartic Acid Uptake by *Scenedesmus* sp. When Both Enantiomers were Present, With No Nitrate Added.

Time	D concentration	L concentration
0	93.22	82.99
12	50.252	50.68
24	45.398	49.05
36	39.832	30.566
48	33.315	0
60	24.312	0

Growth of *C. sorokiniana* in the Media Supplemented With Aspartic Acid and Leucine , in Presence of Nitrate

Aspartic acid

Time	D/rate	Error	L/rate	Error	BG11/rate	Error
0	5.75E-03	5.98E-05	5.25E-03	1.07E-04	4.00E-03	7.50E-04
12	0.037	6.24E-04	0.042	2.90E-04	0.041	9.46E-04
24	0.106	1.49E-03	0.103	7.11E-04	0.132	2.68E-03
48	0.160	3.76E-03	0.198	3.24E-03	0.236	1.89E-03
72	0.206	5.40E-03	0.253	9.90E-04	0.28	3.20E-03
96	0.272	2.25E-03	0.32	1.71E-03	0.318	1.68E-03
128	0.294	5.19E-03	0.357	2.02E-03	0.324	6.46E-03
148	0.345	5.74E-03	0.389	1.88E-03	0.38	9.41E-03
172	0.368	5.12E-03	0.406	2.11E-03	0.412	5.60E-03
194	0.388	5.58E-03	0.419	1.63E-03	0.416	8.59E-03

Time	D concentration	L concentration
0	146.581	58.282
24	147.825	54.511
48	145.748	56.85
72	162.373	45.29
96	154.76	47.14
148	149.61	50.65
172	143.11	53.48
194	155.72	58.96

Lucine

Time	D/rate	Error	L/rate	Error	BG11/rate	Error
0	5.75E-03	5.98E-05	5.25E-03	1.07E-04	4.00E-03	7.50E-04
12	0.037	6.24E-04	0.042	2.90E-04	0.041	9.46E-04
24	0.106	1.49E-03	0.103	7.11E-04	0.132	2.68E-03
48	0.160	3.76E-03	0.198	3.24E-03	0.236	1.89E-03
72	0.206	5.40E-03	0.253	9.90E-04	0.28	3.20E-03
96	0.272	2.25E-03	0.32	1.71E-03	0.318	1.68E-03
128	0.294	5.19E-03	0.36	2.02E-03	0.324	6.46E-03
148	0.345	5.74E-03	0.39	1.88E-03	0.38	9.41E-03
172	0.37	5.12E-03	0.41	2.11E-03	0.412	5.60E-03
194	0.388	5.58E-03	0.42	1.63E-03	0.416	8.59E-03

Time	D concentration	L concentration
0	146.581	58.28
24	147.825	54.51
48	145.75	56.85
72	162.37	45.29
96	154.76	47.14
148	149.61	50.65
172	143.11	53.48
194	155.73	58.96

Lipid Content Variation

Variable Nitrate

C. sorokiniana		
Nitrate, µM	lipid content	Error
4.9	10.146	6.98E-03
9.9	3.521	0.0123
49.4	2.609	9.31E-03
98.9	2.699	6.65E-04
148.4	1.513	0.0185
197.8	1.217	0.0233
296.7	0.192	0.0305
395.6	0.096	0.0211
494.5	0.082	0.0174
Scenedesmus sp.		
Nitrate, µM	lipid content	Error
4.9	23.33	6.98E-03
9.9	6.582	0.0123
49.4	4.756	9.31E-03
98.9	4.105	6.65E-04
148.4	2.818	0.0185
197.8	2.384	0.0233
296.7	2.385	0.0105
395.6	2.58	0.0211
494.5	2.522	0.0174
4.9		
9.9		

49.4	lipid content	Error
98.9	7.09	3.40E-03
148.4	5.25	5.56E-03
197.8	2.40	6.42E-03
296.7	1.96	9.49E-03
395.6	1.59	9.12E-03
494.5	1.16	6.34E-03
4.9	0.75	6.25E-03
9.9	0.58	2.12E-03
49.4	0.61	8.98E-03
Limnothrix sp.		
Nitrate, µM	lipid content	Error
4.9	4.719	3.45E-03
9.9	3.818	5.33E-03
49.4	3.391	2.87E-03
98.9	2.715	6.02E-03
148.4	2.543	2.65E-03
197.8	2.543	3.45E-03
296.7	2.326	3.97E-03
395.6	2.476	7.88E-03
494.5	2.45	5.12E-03
N. thermalis		
Nitrate, µM	lipid content	Error
4.9	37.45	0.01
9.9	14.83	0.0112
49.4	9.453	2.06E-03
98.9	10.37	2.10E-03
148.4	8.426	3.19E-03
197.8	8.089	5.45E-03
296.7	7.73	5.35E-03
395.6	8.561	5.45E-03
494.5	6.300	3.47E-03
E. caespitosum		
Nitrate, µM	lipid content	Error
4.9	133.078	7.85E-03
9.9	36.4	5.52E-03

49.4	25.02	3.40E-03
98.9	19.51	5.56E-03
148.4	16.20	6.42E-03
197.8	17.18	9.49E-03
296.7	19.17	9.12E-03
395.6	15.29	6.34E-03
494.5	14.22	6.25E-03

Variable Phosphate

C. sorokiniana		
Phosphate, µM	lipid content	Error
0	2.322	0.0105
28.7	1.64	0.0211
57.4	0.96	0.0174
86.21	0.41	7.90E-04
114.9	0.42	0.0168
172.4	0.472	2.63E-03
229.9	0.441	0.0123
287.3	0.453	9.31E-03
Scenedesmus sp.		
Phosphate, µM	lipid content	Error
0	8.104	0.0105
28.7	3.513	0.0211
57.4	1.872	0.0174
86.21	1.493	7.90E-04
114.9	1.284	0.0168
172.4	1.392	2.63E-03
229.9	1.459	0.0123
287.3	1.386	9.31E-03
Synechocystis sp.		
Phosphate, µM	lipid content	Error
0	1.001	0.0123
28.7	1.059	0.0136
57.4	1.061	3.45E-03
86.21	1.062	5.33E-03

114.9	1.062	2.87E-03
172.4	1.062	6.02E-03
229.9	1.062	2.65E-03
287.3	1.062	3.45E-03
Limnothrix sp.		
Phosphate, µM	lipid content	Error
0	3.52	6.40E-03
28.7	1.93	6.28E-03
57.4	0.84	6.01E-03
86.21	0.456	2.02E-03
114.9	0.42	1.87E-03
172.4	0.39	2.50E-04
229.9	0.33	2.89E-04
287.3	0.334	4.55E-03
N. thermalis		
Phosphate, µM	lipid content	Error
0	3.884	0.0109
28.7	1.581	1.04E-02
57.4	0.754	4.94E-03
86.21	0.642	0.01
114.9	0.548	1.12E-02
172.4	0.435	2.06E-03
229.9	0.441	2.10E-03
287.3	0.419	3.19E-03
E. caespitosum		
Phosphate, µM	lipid content	Error
0	1.661	5.12E-03
28.7	0.582	4.29E-03
57.4	0.332	1.23E-02
86.21	0.384	1.18E-02
114.9	0.475	1.52E-02
172.4	0.518	1.33E-02
229.9	0.533	8.54E-03
287.3	0.567	9.12E-03

Variable Light Intensity

Scenedesmus sp.		
Light intensity, µmole/m^2.s	lipid content	Error
1155	0.249	0.0119
825	0.269	2.17E-03
82.5	0.324	0.0206
33	0.366	0.0171
16.5	0.381	2.25E-03
8.25	0.598	2.83E-03
1.65	0.724	0.0108
C. sorokiniana		
Light intensity, µmole/m^2.s	lipid content	Error
1155	0.239	1.19E-03
825	0.425	2.17E-03
82.5	0.871	2.06E-03
33	1.02	1.71E-03
16.5	1.347	2.25E-03
8.25	1.371	2.83E-03
1.65	2.598	1.08E-03
Synechocystis sp.		
Light intensity, µmole/m^2.s	lipid content	Error
1155	2.695	2.90E-03
825	1.452	2.81E-03
82.5	0.853	3.28E-03
33	0.496	2.17E-03
16.5	0.3	2.46E-03
8.25	0.42	3.64E-03
1.65	0.39	3.71E-03
Limnothrix sp.		
Light intensity, µmole/m^2.s	lipid content	Error
1155	4.64	4.09E-03
825	2.47	5.12E-03

82.5	1.77	4.74E-03
33	2.026	3.97E-03
16.5	2.00	4.66E-03
8.25	1.18	2.72E-03
1.65	0.20	3.16E-03
N. thermalis		
Light intensity, µmole/m^2.s	lipid content	Error
1155	1.61	0.0479
825	1.633	8.17E-04
82.5	0.979	2.89E-03
33	0.914	0.0408
16.5	0.921	0.0946
8.25	1.27	8.54E-04
1.65	2.098	1.08E-03
E. caespitosum		
Light intensity, µmole/m^2.s	lipid content	Error
1155	0.20	0.0119
825	0.15	2.17E-03
82.5	0.120	0.0206
33	0.121	0.0171
16.5	0.112	2.25E-03
8.25	0.131	2.83E-03
1.65	0.235	0.0108

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VITA
Graduate College

University of Nevada, Las Vegas

Faegheh Moazeni

Degrees:

Bachelor of Science, Chemical Engineering, 2005

Sharif University of Technology, Tehran, Iran

Master of Science, Chemical Engineering, 2007

Sharif University of Technology, Tehran, Iran

Special Honors and Awards:

UNLV Graduate Student Renewable Energy Scholarship, 2011

Abgrad2011, NASA astrobiology grad students travel award, 2011

DRI Graduate Research Assistantship, 2009-2012

Publications:

Moazeni, F., Zhang, G., Sun, H. 2010. Imperfect asymmetry of life: Earth microbial communities prefer D-lactate but can use L-lactate also. Astrobiology 10: 397-402.

Sun, H., **Moazeni, F.,** Zhang, G. 2012. Ideal fuel algae from an ideal production location: *Chlorella* and *Scenedesmus* from southern Nevada that thrive in municipal wastewater (in review).

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Moazeni, F., Mirzaee, F., Mohajer, P. 2003. Stimulation of fluid's behavior in hydrocarbon's reservoir with PVTi softeware. The 4thChemical Engineering & 2nd Petroleum Engineering Conference. Tehran, Iran, Sep 2003.

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Dissertation Examination Committee:

Chairman, Nader Ghafoori, Ph.D.

Committee member and research adviser, Henry J. Sun, Ph.D.

Committee member, Moses Karakouzian, Ph.D.

Committee member, Mohamed Kaseko, Ph.D.

Graduate Faculty Representative, Spencer M. Steinberg, Ph.D.