Algal Biofuels: The Effect of Salinity and pH on Growth and Lipid Content of Algae

by

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Dedication

This work is dedicated to the memory of my parents, Francisco and Hilda Gutierrez and to my children, Matt, Alex and Caleb.

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Abstract

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Cesar Carlos Gutierrez, M.A.

The University of Texas at Austin, 2009

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Co-supervisor: Mona Mehdy

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Supplies of nonrenewable fossil fuels are becoming more limited even as they continue to contribute to pollution and economic concerns. Alternative sources of energy must be developed that help minimize these problems. One potential source of energy is the production of biofuels from algae. Here we evaluate algae found in South Texas brackish water ponds used for aquaculture of fish as a possible source of biofuels. In particular, we examine the effects of salinity and pH on the growth and lipid content of the algae. Samples of algae from the ponds exhibited high levels of growth and lipid production at a salinity of 9 ppt and pH 7. These conditions are similar to the natural conditions of the ponds, indicating that they may be a good source of algal biofuels.

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Chapter 1: Introduction

We require massive amounts of energy to sustain our societies. Most of this energy is currently supplied in the form of fossil fuels, but fossil fuels cause pollution, are unequally distributed, and are not renewable. A major goal of energy research is to find alternative sources of energy that are sustainable, cost-effective and environmentally friendly. Biofuels are an alternative source of energy that already contributes to fuel supplies in the form of ethanol and biodiesel. Several agricultural crops, such as corn, soybeans, and sugar cane are grown specifically for biofuel production. One problem with these crops is that they can adversely affect food supplies and prices by using up land that would otherwise be used for food crops. Another problem is that the oil content of agricultural oil crops in comparison to their biomass is very small (Chisti, 2007). The use of algae as a source for biofuels helps eliminate some of these problems. The relatively high oil content and rapid growth rate of algae make them an attractive candidate for biofuel production. In addition, algae do not need to be grown on arable land and are not a major food source.

Biofuel algae can be grown in ideal conditions in different types of bioreactors, but the costs can be prohibitive. In contrast, the focus of our research is on harvesting algae from natural sources. There are several brackish water ponds in the Laguna Madre area of South Texas that are used as commercial fish hatcheries for food fishes such as tilapia. The ponds produce significant amounts of several species of algae. Our research involves the study of conditions in which these algae grow and the types and amounts of oil they produce. Our aim is to determine optimal conditions for growth of algae and production of usable oil. The objective of the research described in this paper is the

evaluation of the conditions of salinity and pH and their effect on the growth of algae and production of oil. A mixed culture of algae from the South Texas Ponds was inoculated into bubbler tubes and grown in two different sets of media. One set of media was prepared in the form of a salinity gradient and the other set was prepared in the form of a pH gradient. Total growth rates were measured and cell counts and observations were recorded for the various species of algae present. Oil was also extracted and measured from the algae in both the salinity and pH gradients.

Experimental salinity and pH gradients were used to help determine how these variables would affect growth of algae, types of algae, and amount and types of oil produced. The experimental gradients give an indication of conditions in nature that might be conducive to growth of algae that produce high yields of oil, particularly triacylglycerols (TAGs) that can be converted into biodiesel. The ultimate goal is to find locations in nature with similar conditions that can then be harvested for their algae and biofuel. In addition, natural sites could be inoculated with particular species of algae that are well-adapted to conditions in those sites and able to produce high lipid yields.

Chapter 2: Review of the Literature

General Review

The National Renewable Energy Laboratory prepared a report for the U.S. Department of Energy's Office of Fuel Development (Sheehan, *et al*., 1998). It provides a detailed and extensive look at the Office of Fuel Development's Aquatic Species Program (ASP). The program ran from 1978 to 1996 and had a focus of producing biodiesel from algae, using waste CO_2 from coal plants. It was one component of the DOE's Biofuels program, but funding was eliminated in 1995. The report examines the type of algae that work best as biofuel producers, lipid production triggers in algae, the role of molecular biology and genetic engineering, the use of open and closed bioreactor systems, and the availability of resources for the project.

The ASP focused on the study of microalgae because they produce natural oils that are useful in the production of biodiesel. The ASP report stated that, "Put quite simply, microalgae are remarkable and efficient biological factories capable of taking a waste (zero-energy) form of carbon (CO_2) and converting it into a high density liquid form of energy (natural oil)." Diatoms (*Bacillariophyceae*) and green algae (*Chlorophyceae*) store carbon in the form of natural oils, carbohydrates, and starch. They formed the bulk of organisms collected for the program. The algae collection contained over 3000 species, no one species emerged as the most productive in every environment (Sheehan, *et al*., 1998). Vasudevan and Briggs (2008) compared several sources of biodiesel and found that, "microalgae have become appealing because of the potential for significantly higher average photosynthetic efficiency than with typical land crops, due to their aquatic environment providing them with better access to water, CO_2, and

nutrients." They also cautioned that there are several challenges to using algae as an energy crop including costs of photobioreactors and the difficulty of maximizing oil production without limiting growth.

The ASP report (Sheehan, *et al*., 1998) and Vasudevan and Briggs (2008) both compared open "racetrack" algae farms and closed bioreactor designs. The ASP report focused on open systems because of their lower cost. They suggested placing algae farms near sources of waste CO_2, like coal plants, in order to recycle carbon and reduce the likelihood of global climate change. Vasudevan and Briggs (2008) stated that open systems tend to be contaminated by and taken over by algal strains with lower lipid content because they tend to grow faster than high oil content strains. They concluded that photobioreactors must be created at a much lower cost and that tying algal oil production to other products, such as use of waste algal biomass as fertilizer, and services, such as bioremediation of pollutants, can help make production more economical.

Algal Growth

Algae have the ability to grow at a very rapid rate, with most species easily doubling their biomass in 24 hours or less (Chisti, 2007). This gives them a significant advantage over other oil crops. The United States would require nearly 0.53 billion m^3 of biodiesel per year to replace petroleum based fuels at the current rate of consumption. In order to produce this amount of biodiesel from palm oil, 61% of U.S. agricultural land would have to be used. In contrast, algae could potentially produce this amount of oil using only 3% of U.S. agricultural land because of their high oil content and rapid growth. This would produce much less competition with food and fodder crops (Chisti, 2007). Theoretical yields of 30,000 liters of algae oil per hectare per year are 100 times greater than that of soybeans. Unfortunately, actual algal lipid yields in mass culture have

been 10 – 20 times lower than theoretical maximum yields. This is still ten times the production of terrestrial plants on an area basis (Hu, *et al*., 2008). Algae can grow in marginal land and water that are not suitable for conventional agriculture and aquaculture and can utilize nutrients such as nitrogen and phosphorous from wastewater sources (Hu, *et al*., 2008).

As with any living organism, many conditions can affect the growth of algae. Light intensity, availability of nutrients, temperature, turbidity, movement of water and presence of herbivores could all affect growth of different species of algae in various ways. Some studies have been done on the specific effects of salinity and pH on algal growth, the focus of this study. Kautsky & Kautsky (1989) studied algal species diversity in six areas on the coast of Sweden. The areas differed in levels of physical disturbance and stress. Physical disturbance factors included in the study were wave effects, ice scouring and grazing pressure. Stress was defined as decreasing salinity and light intensity. Areas that were under more stress and higher levels of disturbance had fewer species and a lower total biomass. Species that were more stress or disturbance tolerant were able to dominate in those areas. A similar study took place in an estuary in Portugal (Resende, *et al*., 2005). The authors used three sampling stations that were along a natural salinity gradient and studied the ecological preferences of over 70 diatom species. They found that the lower reaches of the estuary were dominated by marine species, while the less saline upper reaches were dominated by typical freshwater species. They found that seven environmental variables significantly affected diatom distribution. The most significant factor was salinity, followed in order of importance, by distance to the mouth of the estuary, temperature, tide, phosphate concentration, ammonia concentration and pH. This study implies that salinity has much more effect on growth of diatoms than pH does. A major difference in these two studies compared to the present study is that they

took place in natural gradients rather than artificial ones created in a lab. In the lab, the algae were probably subjected to much more rapid changes in salinity and pH than they would be in nature.

Hansen (2002) found that a particular fjord in Denmark exhibited a profound seasonal variation in pH levels. He stated that uptake of inorganic carbon by phytoplankton during photosynthesis can increase pH levels in the surrounding water. This effect is stronger in freshwater than seawater because of the natural buffers found in seawater. Hansen found that the seasonal variation in pH had an effect on the succession of algal species throughout the year. There was some suggestion that, in general, dinoflagellates can thrive at very high pH (>9.5) while diatoms prefer lower pH. Taraldsvik & Myklestad (2000) found that *Skeletonema costatum*, a marine diatom, grew very well over a wide pH range (6.5 – 8.5), but had a decreased growth rate at pH levels greater than 9.0. They also found evidence of decreases in rates of biochemical reactions and suggested that the decrease in growth rate was due to these metabolic changes along with changes in the cell membrane allowing more leakage of organic material.

Algae do not grow in isolation in nature. They are eaten by many herbivorous zooplankton such as rotifers and copepods and can produce defense responses. Guschina and Harwood (2006) report that diatoms produce at least 3 aldehydes that induce low hatching rates in copepods. The aldehydes are produced after mechanical wounding of the diatoms that mimics the crushing of cells by copepod grazers. Vardi, *et al*. (2006) examined diatom responses to decadienal, a highly reactive aldehyde that generates nitric oxide, a cause of cell death. The authors found that nitric oxide production varied among neighboring diatoms and that cells treated with low doses of decadienal followed by high doses had a higher survival rate than those simply treated with high doses. They reasoned that diatoms that escape herbivores might get an immunity effect from the aldehydes

released by their dying neighbors and that aldehydes act as infochemicals that monitor stress levels in diatoms. Van der Stap, *et al*., (2007) investigated food chain dynamics and found that formation of algal colonies may be an inducible defense against herbivory. *Scenedesmus acutus*, a species of green algae, forms four to eight-celled colonies in response to infochemicals released by cells being grazed upon by rotifers. Rotifers themselves can be induced to form spines that give them protection from carnivorous copepods. The authors found that inducible defenses in a food chain help prevent large population fluctuations.

Algal Lipids and Biodiesel

The most useful natural oil found in microalgae is in the form of triacylglycerols (TAGs), three long fatty acids with a glycerol backbone. TAGs are easily converted into biodiesel fuel (alkyl esters) through a reaction with simple alcohols (transesterfication) (Sheehan, *et al*., 1998). The reaction uses an alcohol (methanol or ethanol) and a catalyst to change TAGs into fatty acid alkyl esters with glycerol as a byproduct (Vasudevan & Briggs, 2008). Algae synthesize fatty acids primarily for use as membrane lipids, but under environmental stress, many algae alter their lipid biosynthesis towards the production and accumulation of neutral lipids, usually in the form of triacylglycerols (TAGs). TAGs store energy and carbon for the cell and can reach levels of 20 – 50% of dry cellular weight (Hu, *et al*., 2008). TAGs may also have a function in algal stress response. Experiments with *Porphyridium cruentum* grown under conditions of rapid temperature change indicate that fatty acids from TAGs are transferred to polar lipids and incorporated into chloroplastic membranes under this form of temperature stress (Cohen, *et al*., 2000).

Algal Species and Lipid Production

Much work has been published on the growth potential of different algal species and the factors that affect lipid production in them. Many studies have compared lipid production of different species. The ASP report (Sheehan, 1998) found that only a few strains, including *Spirulina*, *Dunaliella*, *Scenedesmus*, and *Chlorella* are suitable for mass culture, although many diatoms and green algae appear to be promising for their lipid content. Rodolfi, *et al*., (2008) evaluated 30 strains for lipid production potential and found that, in general, biomass productivity and lipid production were inversely related. The authors found that 3 marine species of *Nannochloropsis* provided the best combination of biomass productivity and lipid content. Freshwater *Chlorella* and *Scenedesmus* species also emerged as good producers.

Hu (2004) reviewed the effects of several environmental factors and found that they influence photosynthesis, biochemical composition and cellular metabolism. The cell composition varies greatly in different species grown under different conditions. Hu, *et al*. (2008) reported that low light intensities increase production of polar membrane lipids, especially those associated with chloroplasts, but high light intensities produce an increase in neutral storage lipids such as TAGs.

Temperature is another environmental factor that can affect lipid content and composition. In general, fatty acids become increasingly saturated as temperatures increase (Hu, 2004). A study of *Scenedesmus abundans* grown under different culture conditions showed that protein content was maximal at 15 °C, lipid content was maximal at 25 °C and carbohydrate content was maximal at 35 °C (Tahiri, *et al*., 2000). Temperature can affect total lipid content, but no general trend has been established (Hu, *et al*., 2008)

Nutrient limitation, especially limitation of nitrogen, has been shown in several studies to increase accumulation of neutral lipids and TAGs. Silicon, phosphate, and sulfate limitations have also been shown to increase lipid production. (Hu, *et al*., 2008). Kilham, *et al*., (1997) found that phosphorous and nitrogen limitation both reduced protein composition and increased lipid composition in *Ankistrodesmus facatus*. Both total lipids and triglycerides were highest under phosphorous limitation. Rodolfi, *et al*. (2008) differentiated between nutrient deficiency (starvation) and nutrient limitation. The authors found that nutrient deficiency causes cells to use endogenous reserves, eventually leading to a halt in growth. Under conditions of nutrient limitation, algae adapt to an environment of constant, but limited, supplies of nutrient. The authors compared three different strategies: nutrient sufficient cultures with high growth rates but low lipid content, nutrient limited cultures with lower growth rates but higher lipid content, and a two-phase strategy with a nutrient sufficient phase to induce growth, followed by a nutrient limited phase to induce lipid production. They found that in the two-phase strategy, the first few days of the second phase produced the best combination of biomass growth and lipid production. The study was not clear about the types of lipids being measured. The authors mentioned that lipids and fatty acids were analyzed, but the results were stated simply as lipid content or lipid productivity. A similar two-phase study (Chi, *et al*., 2008) examined the effect of dissolved oxygen (DO) on a marine alga, *Schizochytrium limacinum* SR21. Cells were first cultured with DO controlled at 50% and then, at different time points, samples were pulled and place in media at 10% DO. Biomass concentration was determined by both cell density and cell size and levels of docosahexaenoic acid (DHA), an important nutritional fatty acid, were measured. Cultures at 50% DO produced cell densities that were double that of the 10% DO cultures, but lipid accumulation was much higher at 10% DO. The authors found that

shifting cells from high DO to low DO at 40 hours gave the highest biomass and DHA production.

Increases in salinity may produce a slight increase in total lipids in algae because many algae accumulate small molecules like glycerol as a response to osmotic pressure (Hu, 2004). *B. braunii* is a slow growing strain of algae found in fresh and brackish water. Its growth rate was slowed by increasing salinity, but the effect on lipid concentration was minimal (Vazquez-Duhalt & Arredondo-Vega 1991) In contrast, Rao, *et al*., (2007) found that there was an increase in *B. braunii* lipid production at higher salinity levels. The authors concluded that the algae adapted to higher salinity by increasing lipid and carbohydrate synthesis. *Neochloris oleoabundans* responded to osmotic shock by breaking down polysaccarides into smaller, soluble carbohydrates, but total lipid content did not vary significantly from the control (Band, *et al*., 1992)

The effects of pH on algal growth and lipid content have not been widely studied. One study (Molina Grima, *et al*., 1992) looked at pH as one of several environmental factors affecting *Isochrysis galbana*, a marine alga. The authors found that growth was inhibited above pH 9. Below pH 8, the growth rate decreased as the pH was lowered. Total lipid content did not change, but the percentage of eicosapentaenoic acid (EPA – the food oil being studied) declined with pH. The authors concluded that cultures at pH 8 demonstrated the highest growth rate and best EPA production.

Raghavan, *et al*., (2008) studied the combined effects of temperature, salinity and CO_2 levels on *C. calcitrans*. They found that the optimal temperature for growth was between 20 and 25 °C and that the addition of CO_2 increased growth. Salinity did not have a significant effect on growth but did show a tendency towards higher biomass and lower cell density at lower salinity, possibly due to an unknown limiting factor reduced

by the dilution of seawater. Total lipid content was higher at low temperatures and at a salinity of 25 ppt. CO_2 levels did not affect lipid content.

Many studies have been completed on the growth of individual species and the conditions that promote lipid production. In general, the production of lipids is inversely related to the growth of algae because algae tend to produce more oil when they are under stressful conditions. The present study is aimed at examining the effects of salinity and pH on the growth and lipid production of algae from the South Texas Ponds. The expectation is that algal growth would be best in conditions of salinity and pH that mimic those found in the ponds. Conversely lipid production per cell would be highest under more extreme conditions that place cells under stress. The major aim of this study is to help find the conditions that generate the best combination of algal growth and cellular lipid production in order to create the highest possible yield of lipids from the South Texas Ponds.

Chapter 3: Methods

Preparation of Media

Most of the samples of mixed algae from the South Texas Ponds being used experimentally at the University of Texas have been cultured in a 1:2 ratio of F/2 seawater medium and Allen freshwater medium. This produces a salinity of approximately 10 ppt. that is similar to the salinity of the brackish water ponds being studied. The media used for the pH gradient was a mix of F/2 and Allen media in a 1:2 ratio. The media were buffered with Gly-Gly buffer to a pH of 9.02 and with MES buffer to a pH of 4.64. The pH was created for the gradient by mixing the buffered media and using hydrochloric acid and potassium hydroxide to adjust the pH. Media were produced at pH 5, pH 6, pH 7, pH 8, and pH 9. At the end of the experiment, pH was measured again for each sample.

Media for the salinity gradient were created by starting with Allen medium and then adding marine salts (Instant Ocean) to achieve salinities of 0, 3, 9, 15, and 27 ppt. Salts were added until the desired salinities were reached. A Pinpoint Salinity Monitor was used to measure salinity. All media were prepared under sterile conditions.

Source of Algae

The algae used in these experiments originally came from Pond 53 in the South Texas Ponds. The pond is used for aquaculture of tilapia for use as a food fish. A large mixed algae culture from Pond 53 is being grown at the University of Texas. A sample was collected from this culture in the first week of July and used in the experiments described below. The mixed sample included several species of algae. The predominant algal species present were a crescent-shaped algae tentatively identified as

Ankistrodesmus, a small spherical algae tentatively identified as *Nannochloropsis*, and an oval, *Navicula*-like algae that has not been identified yet. Other algae were also present. The Pond 53 sample was used to inoculate the salinity gradient medial and the pH gradient media. Experimental samples were inoculated on July 3, 2009 under sterile conditions with one mL of algae in 40 mL of media.

Experimental Growth Conditions

Algae were grown in large test tubes suspended in distilled water by a plexiglass rack in an aquarium. The temperature was maintained at 25-26 °C and light conditions were 12h light/12h dark. Air enriched with 5% carbon dioxide was bubbled slowly (about 1 bubble/sec) into each tube. Three trials of tubes at five different salinities (0 ppt, 3 ppt, 9 ppt, 15 ppt, 27 ppt) were kept in one aquarium, and three trials of tubes at five different pHs (pH 5, pH 6, pH 7, pH 8, pH 9) were kept in a second aquarium. Experimental data from each trial were pooled and the mean and standard error were calculated. Illustration 1 shows one of the growth chambers.

Illustration 1: Bubbler Tube Algal Growth Chamber

Growth Measurements

Overall algal growth throughout the experiments was measured using a Beckman DU 530 Spectrophotometer. Light absorbance was measured at a fixed wavelength of 678.0 nm. Measurements were taken daily at approximately 9:00 A.M. from Day 3 after inoculation through Day 11 (except for Day 8). Measurements were taken for all samples.

Another measurement of overall algal growth was dry weight. On Day 13 of growth, 1 mL samples were taken from each tube of algae. The samples were dried in a vacuum centrifuge and then weighed in an analytical balance.

Cell Counts and Identification

Cell counts were taken for each experimental sample using a Spencer Bright Line Hemacytometer. Cell counts were taken for each type of cell observed in each experimental sample. Cell counts were taken on Day 4 and Day 13 of the experiments. Cell identification was based on preliminary identifications by Dr. Jerry Brand and Michelle Randazzo at the UTEX Algae Culture Collection. Algal species from the South Texas Ponds are currently being isolated and identified by microscopy and DNA analysis in Dr. Brand's lab.

Lipid Extraction

Lipid extraction was based on a modified version of the Bligh-Dyer method (Bligh and Dyer, 1959). This method is used to extract neutral lipids. One mL samples of algae were centrifuged for 3 minutes at 10,000 rpm. The supernatant was removed and the algal pellets were resuspended in 1 mL of distilled water. Three samples from each experimental condition were combined at this point to give an initial sample volume of 3 mL. Chloroform (1 mL) and methanol (2 mL) were added, mixed and left undisturbed for 10 minutes. One more mL of chloroform was added, mixed and left undisturbed for 10

minutes. One mL of distilled water was added and mixed. The mixture was heated gently until there was a clear separation of the lipid layer (about 15 minutes). The lipid layer was extracted with a micropipette and dried in a vacuum centrifuge. The dried lipid was resuspended in 200 μL of a 6:1 chloroform to methanol mixture and stored in the freezer. The samples were analyzed using high performance liquid chromatography (HPLC) in Dr. Martin Poenie's lab in the Section of Molecular Cell and Developmental Biology in the School of Biological Sciences at the University of Texas. The analysis showed levels of TAGs and other neutral lipids that were extracted from the experimental samples. The results were measured in μg of lipid per mL of algal sample. This measurement was converted to percent lipid of dry weight by multiplying by the ratio of final resuspension volume (0.2 mL) to original sample volume (3 mL) and dividing by the measured algal dry weight.

Chapter 4: Results and Data Analysis

Initial Measurements

The experimental sample from the Pond 53 culture was tested on July 22, 2009. The resulting pH was 7.27, and the salinity was 2.95 ppt. The salinity reading was somewhat lower than previously observed in the South Texas Ponds. Most of the salinity readings from the ponds have been approximately 10 ppt. The difference is because the culture being grown in Austin is being replenished with tap water. Initial cell counts were taken on July 3, 2009. They are shown in Figure 1.

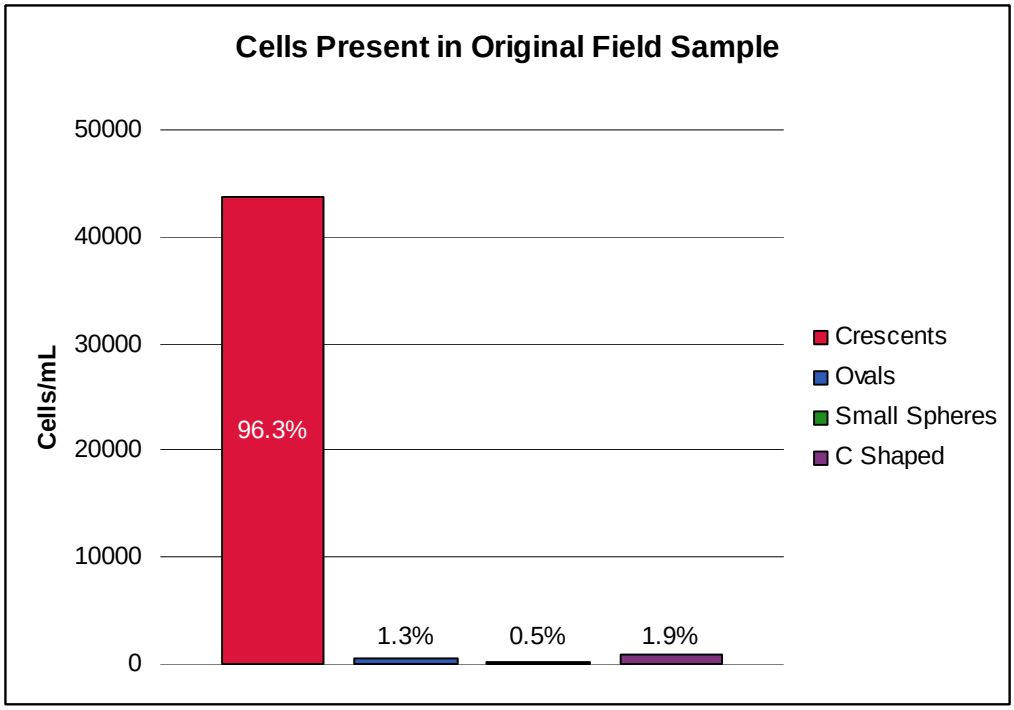

Figure 1: Cells Present in Original Field Sample

The predominant algal species found in the field sample was a light green, crescent shaped algae tentatively identified as *Ankistrodesmus*. *Ankistrodesmus* is a type

of green algae whose cells are long, thin and tapered. The observed species was approximately 12 – 15 μm in length and 1 – 3 μm in width. It also had a white structure that made it appear to be notched in the center. Crescent shaped *Ankistrodesmus* made up 96.3% of the total cell count. Three other types of algae were observed, but they each made up less than 2% of the total cell count.

An oval or barrel shaped species was observed. This species was approximately 6 μm in length and 3 μm in width. Each cell had two dark round spots that took up much of the interior volume. This species remains unidentified but appears to be related to *Navicula*. Illustration 2 shows a mixture of crescent shaped and oval algae that were observed at 400X.

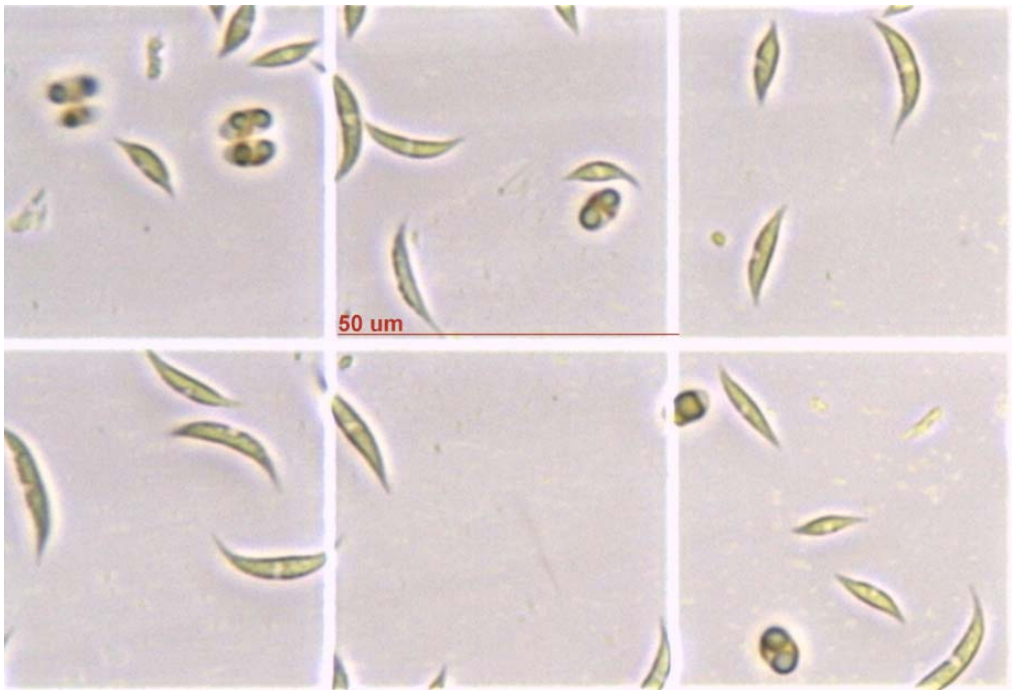

Illustration 2: Crescent (*Ankistrodesmus*) and Oval Shaped Algae (400X)

Another species that was found in small numbers was one that was shaped like a "fat C." This species has been tentatively identified as Selenastrum, another type of green

algae. Two of these are shown in Illustration 3 at 400X. The final species that was observed in the original field sample consisted of algae that had a small (1 – 2 µm) spherical shape. These were the most difficult to identify and count under the microscope because of their small size and the fact that they could not be easily distinguished from debris or from crescents viewed on end. This species could possibly be *Nannochloropsis*, but DNA analysis has not been completed.

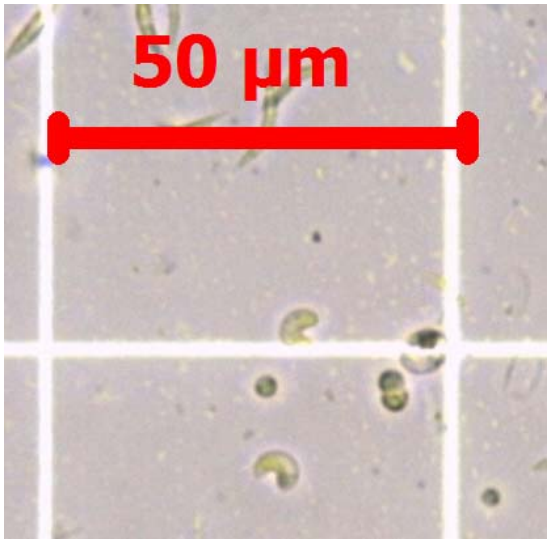

Illustration 3: C Shaped Algae (*Selenastrium*) (400X).

Salinity Gradient Results

Algal Growth

Algal dry weights were measured for each sample grown in the salinity gradient. The dry weights were measured after 13 days of growth. Dry weight was measured for 1 mL samples, and the data from 3 trials at each salinity level were averaged. The dry weights are shown in g per mL of sample in Figure 2.

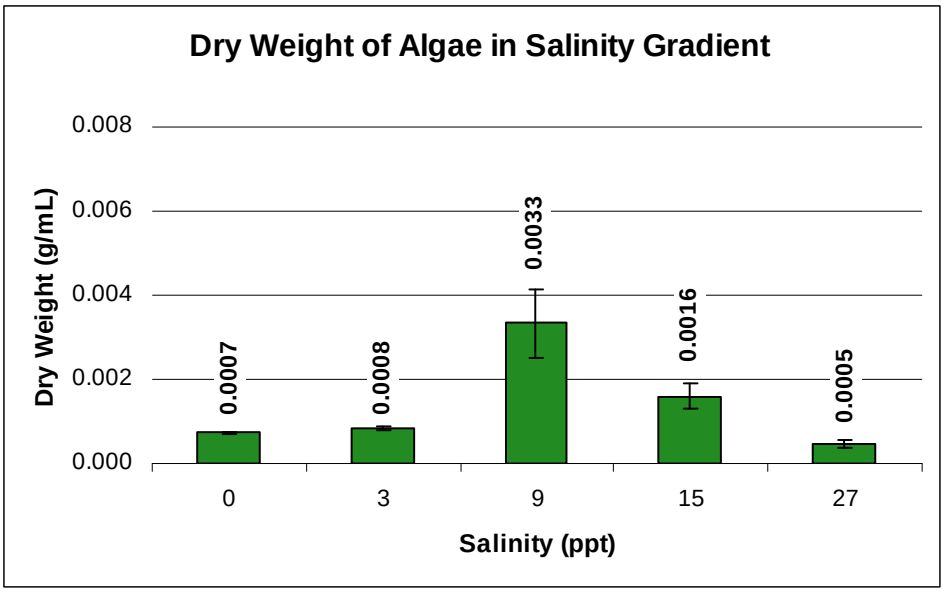

Figure 2: Dry Weight of Algae in Salinity Gradient

Algae from Pond 53 grew best at the experimental salinity of 9 ppt. The mean dry weight of algae grown at a salinity of 9 ppt was 0.0033g/mL of culture. This was twice the weight of algae that grew in a salinity of 15 ppt and four times the weight of algae that grew at each of the other salinities. The higher growth at 9 ppt was also reflected in light absorbance data taken throughout the experiment (Figure 3). Light absorbance peaked on days 9 and 11 at about 0.75 AU in the 9 ppt trials. None of the other trials ever exceeded 0.3 AU.

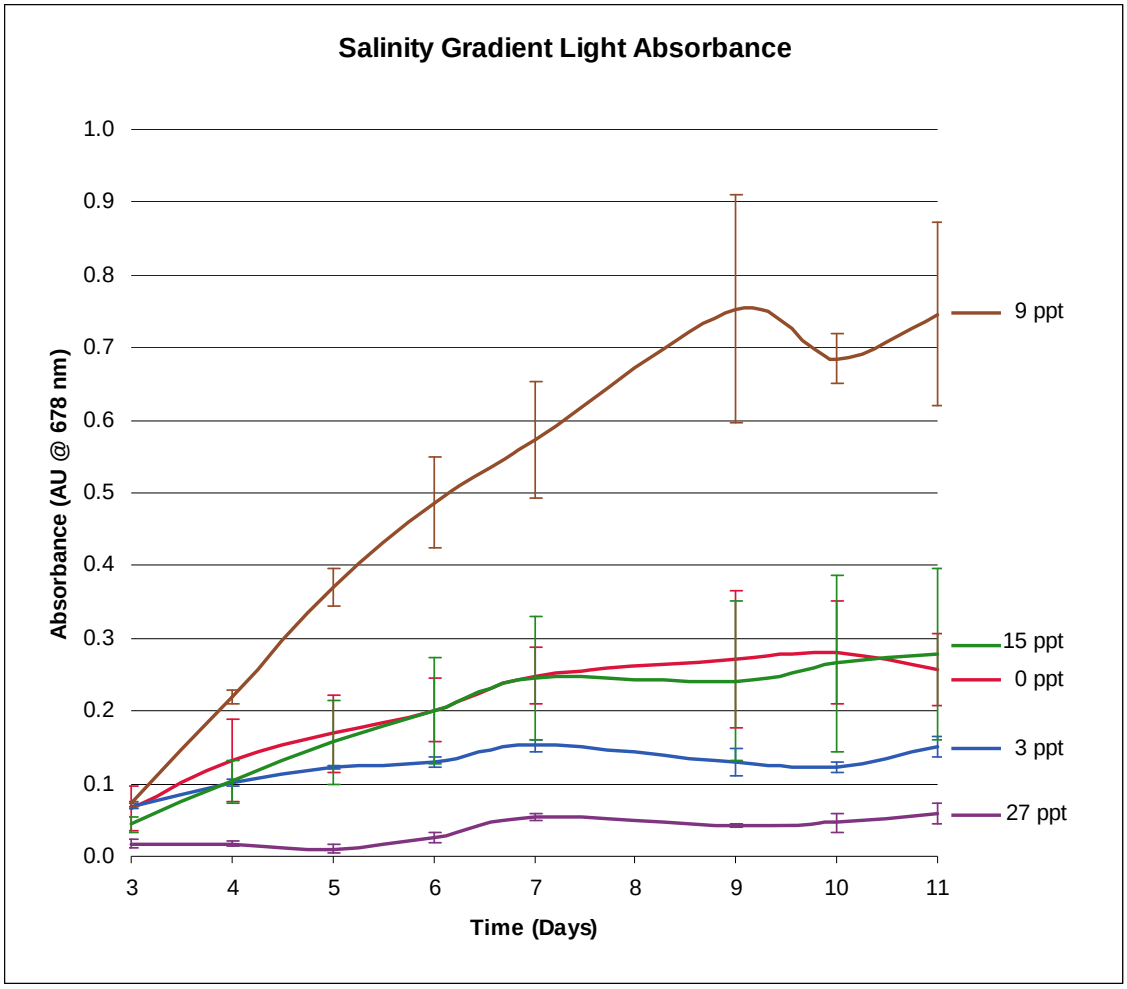

Figure 3: Salinity Gradient Light Absorbance

Algal Species

Cultures growing in the salinity gradient were observed after 4 and 13 days of growth. After 4 days of growth, the same species were found that were present in the original field sample. After 13 days, two new species were observed. A long, pennate diatom was seen. It is similar in appearance to the diatoms, *Navicula* and *Nitschia*. This species was clear with some golden color inside and was about 20 µm in length (Illustration 4).

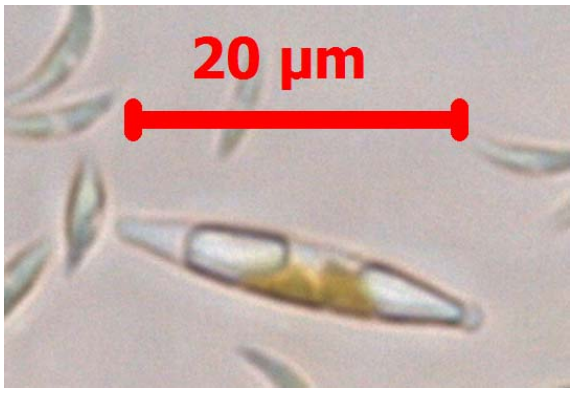

Illustration 4: Boat Shaped Diatom (1000X)

Another new species observed at day 13 was a large, dark green, spherical alga. It often appeared in pairs and had a diameter of about 10 µm (Illustration 5). It could belong to *Chlorophyta* (a division of green algae) or *Xanthophyta* (yellow-green algae).

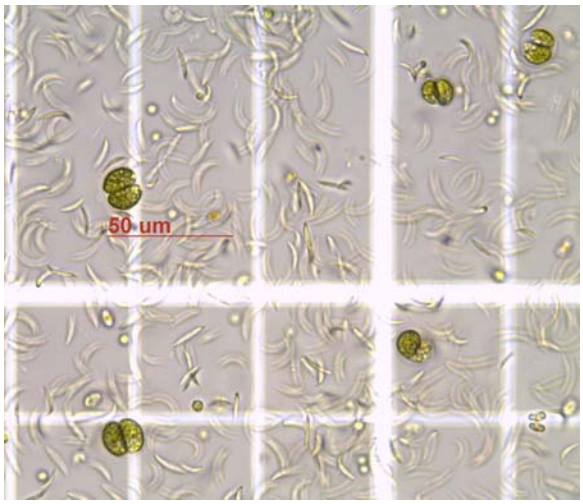

Illustration 5: Large Spherical Algae (400X)

The crescent shaped *Ankistrodesmus* dominated most of the cell counts, but did not grow as well at the seawater concentration of 27 ppt and became slightly less dominant overall at the later stages of the experiment. It dominated most completely at 9

ppt, accounting for 91.2% of cells on day 4 and 86.6% of cells on day 13. At 27 ppt on day 13, the oval shaped species accounted for 54.7% of cells, and the small spheres (*Nannochloropsis*) accounted for 35.5% of cells. Ankistrodesmus had dwindled to 5.2%. The small spheres (*Nannochloropsis*) also made up significant percentages of the cells at 0 ppt (23.1%) and 3 ppt (43.2%) on day 13. Algal percentages for days 4 and 13 are shown below. Both graphs are shown to illustrate some of the changes taking place in algal percentages as the experiment ran its course.

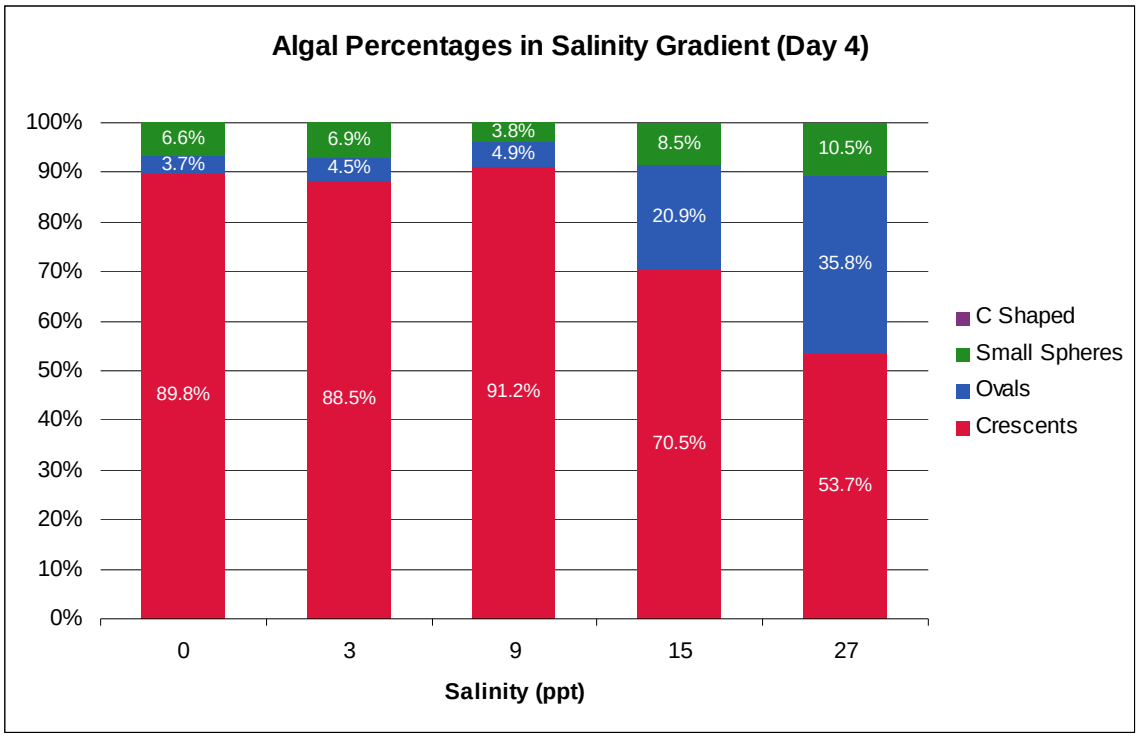

Figure 4: Algal Percentages in Salinity Gradient (Day 4)

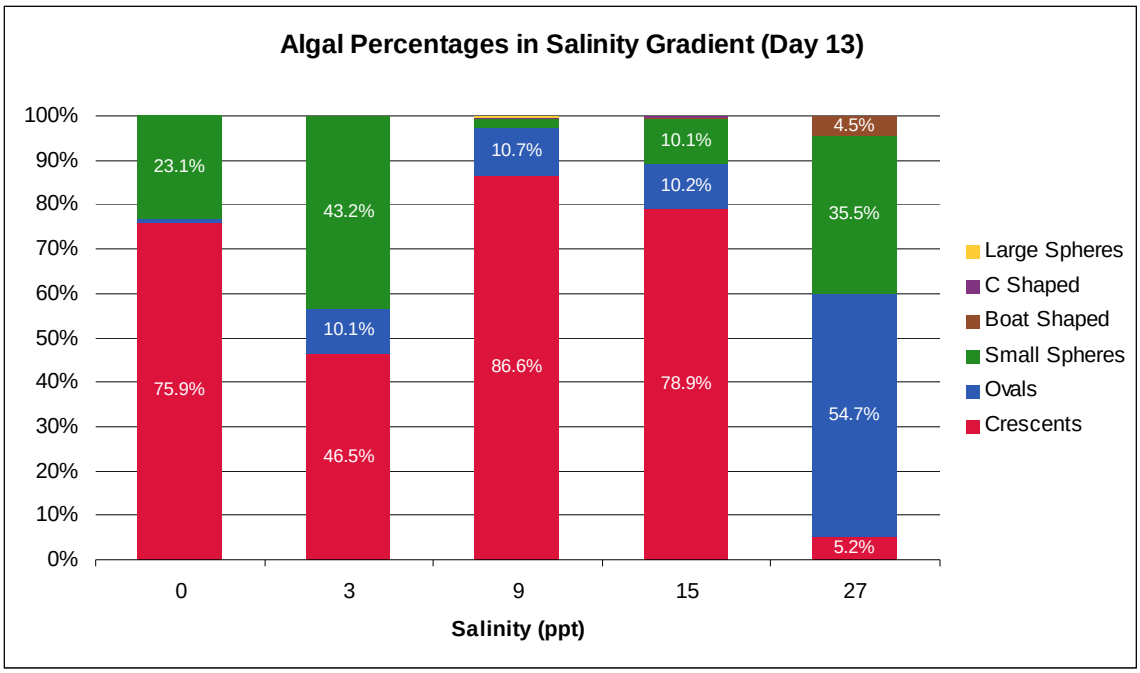

Figure 5: Algal Percentages in Salinity Gradient (Day 13)

With one exception, all species grew best at the experimental salinity of 9 ppt. The crescent shaped *Ankistrodesmus* grew to much higher numbers than other species at all salinities, except 27 ppt. It had a density of 1.51 x 10^8 cells/mL at 9 ppt on day 13. The next highest cell density was 1.87 x 10^7 cells/mL for the oval species at 9 ppt on day 13. The oval shaped algae grew best at 9 ppt and grew very little at 0 ppt. The small spheres (*Nannochloropsis*) seemed to be least affected by salinity. They grew best at 3 ppt (1.14 x 10^7 cells/mL) but were above 1 x 10^6 cells/mL at all salinity levels. The C shaped *Selenastrum,* the boat shaped diatom and the large spheres also grew best at 9 ppt, but their overall growth was much lower with even their best cell densities being less than 1 x 10^6 cells/mL. *Selenastrum* was not observed at all at 27 ppt. The boat shaped species (*Navicula* or *Nitschia*) was better adapted to higher salinities and made up 4.5% of cells

at 27 ppt. Cell counts in the salinity gradient are shown below in Figure 6. Note that the Y –axis is on a logarithmic scale.

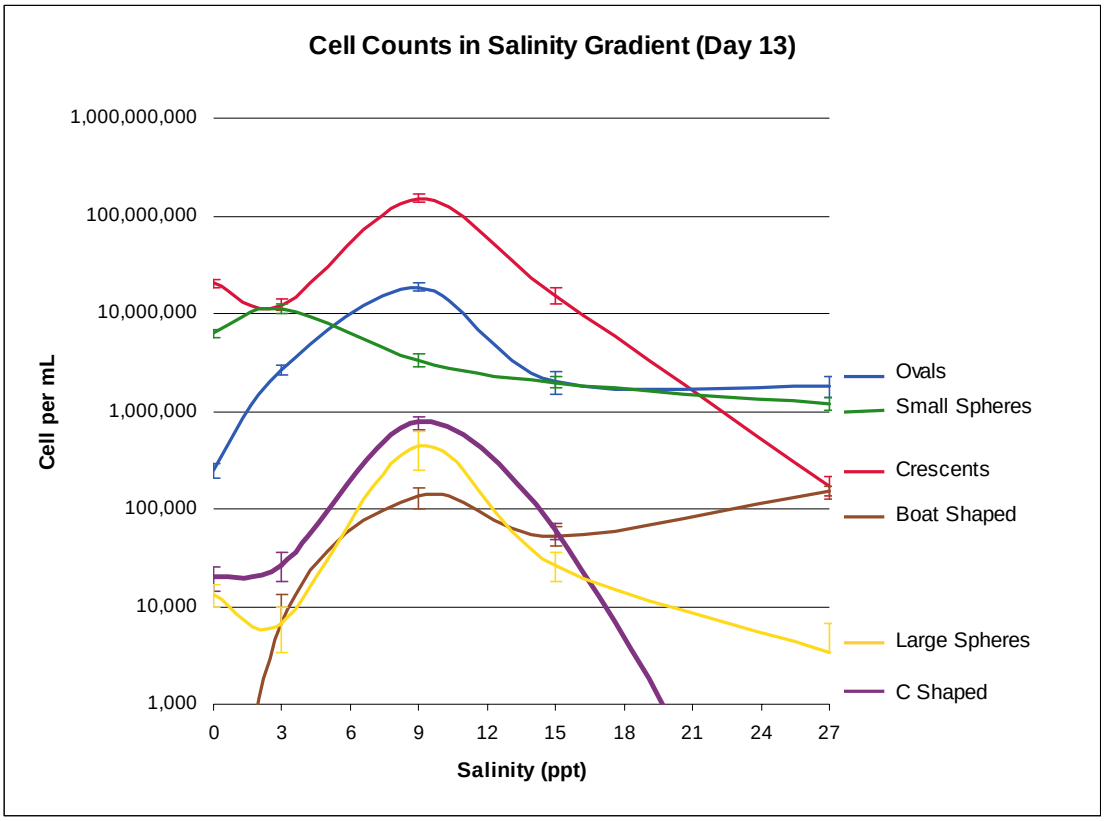

Figure 6: Cell Counts in Salinity Gradient (Day 13)

Lipid Content

On day 12 of the experiment, neutral lipids were extracted from the cultures growing in the salinity gradient. Results are shown in Figure 7 and are somewhat difficult to interpret because of the varying percentages of each algal species at different salinities. Results show lipid content as a percentage of the dry weight of the algal sample at each point in the salinity gradient. TAG levels were highest at 9 ppt (2.30 %) and at 0 ppt (2.13%). Total neutral lipids were highest at 27 ppt (5.08%) and at 0 ppt (4.43%).

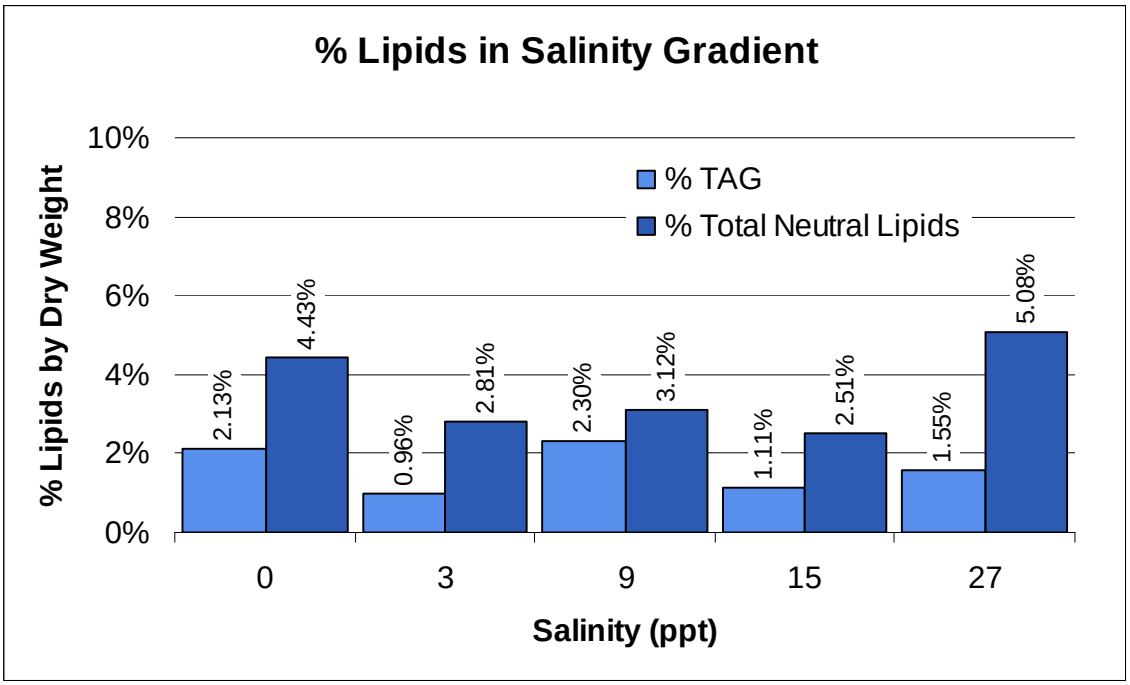

Figure 7: Percent Lipids in Salinity Gradient

pH Gradient Results

Algal Growth

Algal dry weights were measured after 13 days of growth for each experimental sample in the pH gradient. Dry weight was measured for 1 mL samples, and the data from 3 trials at each pH gradient were averaged (Figure 8). The highest dry weight mean was at pH 8, but pH 6 and pH 7 also grew well and were within the standard error of pH 8. Algae at pH 5 and pH 9 had less than half the dry weight of the other pH conditions. Overall growth in the pH gradient was much higher than in the salinity gradient. The best growth in the salinity gradient was 0.0033 g/mL at 9 ppt. This was about half the growth rate of the samples grown in pHs 6, 7, and 8. The samples in the pH gradient were grown

in media that had a salinity of approximately 10 ppt, which was shown to be near the optimal salinity as determined by the salinity gradient experiment.

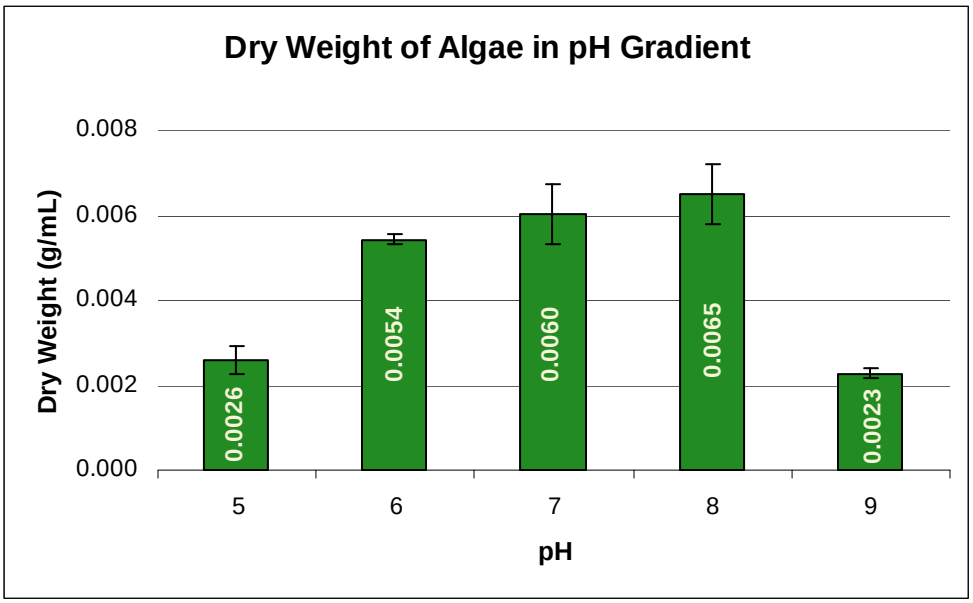

Figure 8: Dry Weight of Algae in pH Gradient

Data from daily light absorbance measurements showed very similar results to the dry weight measurements. Data are shown in Figure 9. The growth curves for pH 6, 7, and 8 were very similar. They all had a peak on day 7 between 1.0 and 1.2 AUs, followed by a slight decline in growth for 2 days. On day 10, there was a slightly higher peak at about 1.2 AUs. Growth at pH 5 was interesting in that there was almost no growth for the first 7 days of the experiment, but rapid growth after day 7 that peaked at 0.76 AUs on day 10. The pH 5 cultures became a clear, bright green color. All the other cultures were murkier and had more yellow and brown color. Growth at pH 9 had a small peak (0.44 AU) on day 9.

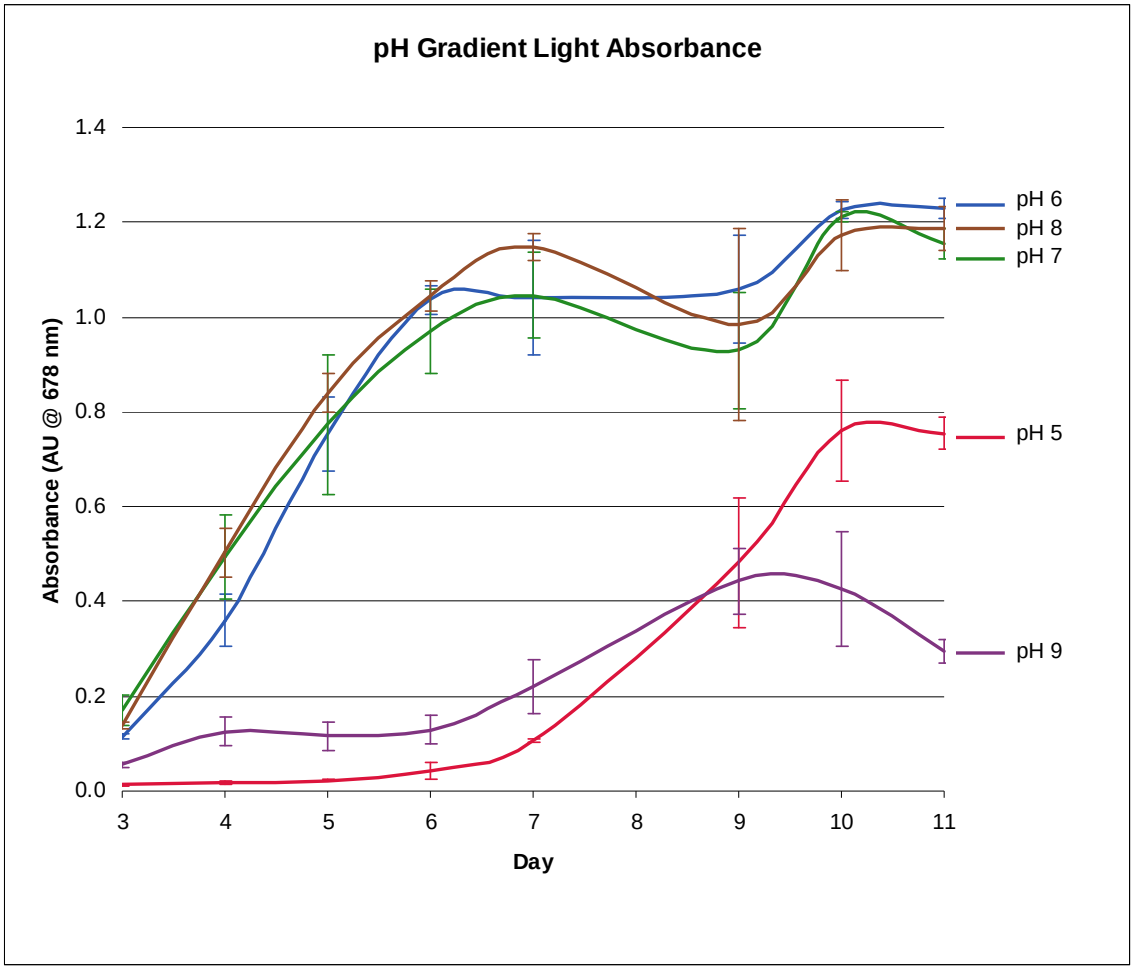

Figure 9: pH Gradient Light Absorbance

Algal Species

Cultures in the pH gradient were observed after 4 and 13 days of growth and cell counts were taken. Algal species observed in the pH gradient were the same as those present in the salinity gradient, except that the boat shaped pennate diatoms were not observed. Crescent shaped *Ankistrodesmus* was the dominant species in all but one

culture condition (pH 5). It was especially dominant at pH 6, making up 98.8% of cells on day 13. The percentage of *Ankistrodesmus* in pH conditionss 7 – 9 ranged from 84.2% to 88.0% on day 13. The late surge in growth in the pH 5 cultures was primarily made up of the large spherical algae (*Chlorophyta* or *Xanthophyta*). The large spheres made up 76.3% of the cells at pH 5. The oval *Navicula*-like species made up 14.9% and 10.9% of the cells at pH 7 and pH 8, respectively, on day 13. The small spheres, possibly Nannochloropsis, made up 13.8% and 10.6% of cells at pH 5 and pH 9, respectively, on day 13. Algal percentages for days 4 and 13 are shown below.

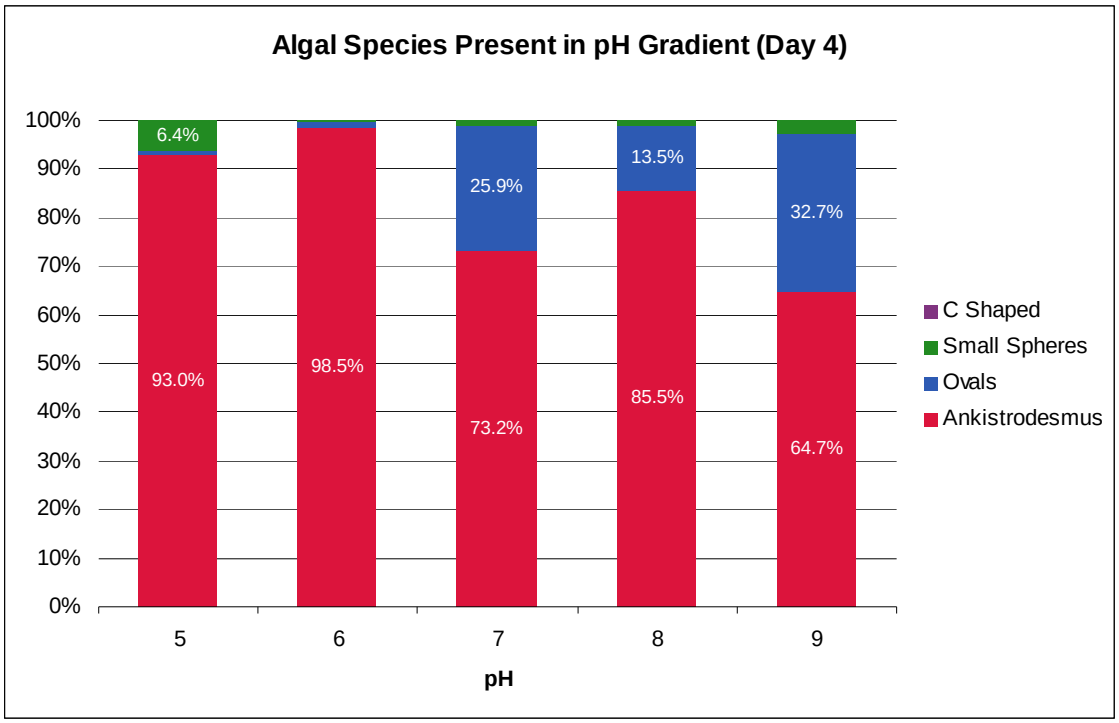

Figure 10: Algal Percentages in Salinity Gradient (Day 4)

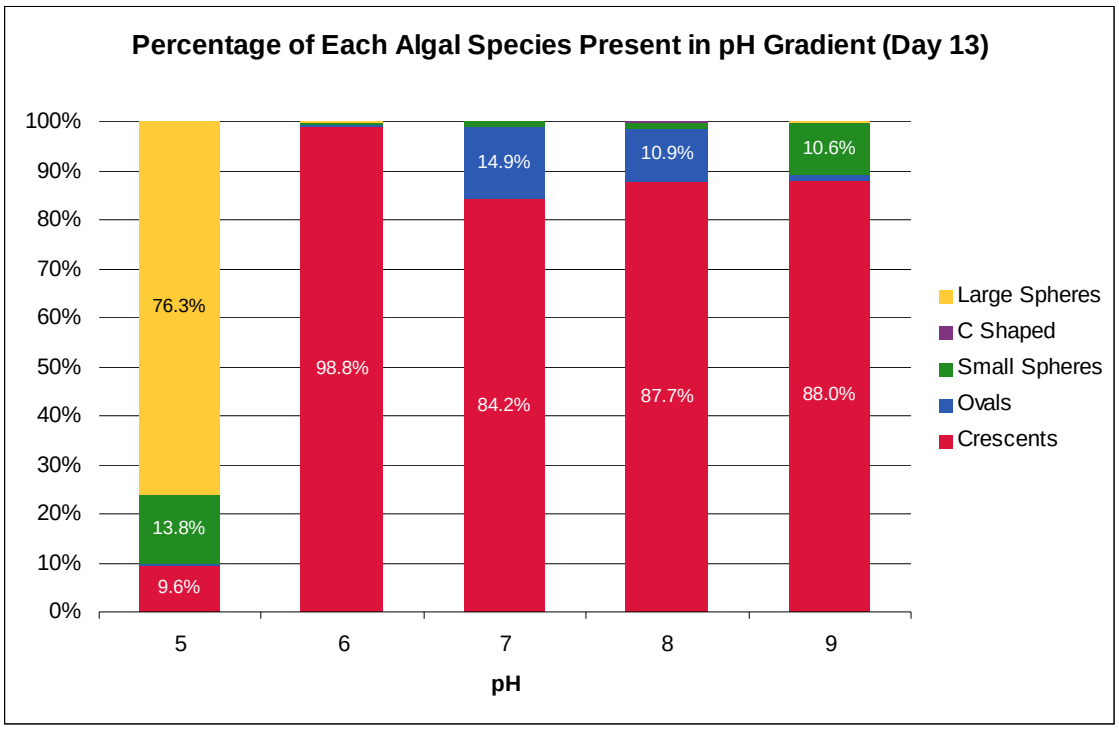

Figure 11: Algal Percentages in Salinity Gradient (Day 13)

Cell counts showed that *Ankistrodesmus* crescents grew well at all pHs except pH 5. The counts at pH 6 – 8 were all over 10^8 cells/mL *Ankistrodesmus* counts were at least an order of magnitude higher than all other species. Note that the Y-axis in Figure 12 is on a logarithmic scale. The oval *Navicula*-like species grew well at pH 7 and pH 8, but growth dropped off sharply outside of this range. The small spheres seemed least affected by pH, but did grow slightly better at higher pH values. The larger spheres were acidophilic and grew well at pH 5. C shaped Selenastrum had modest growth at pH 8 but in general, grew in significantly lower numbers than all other species.

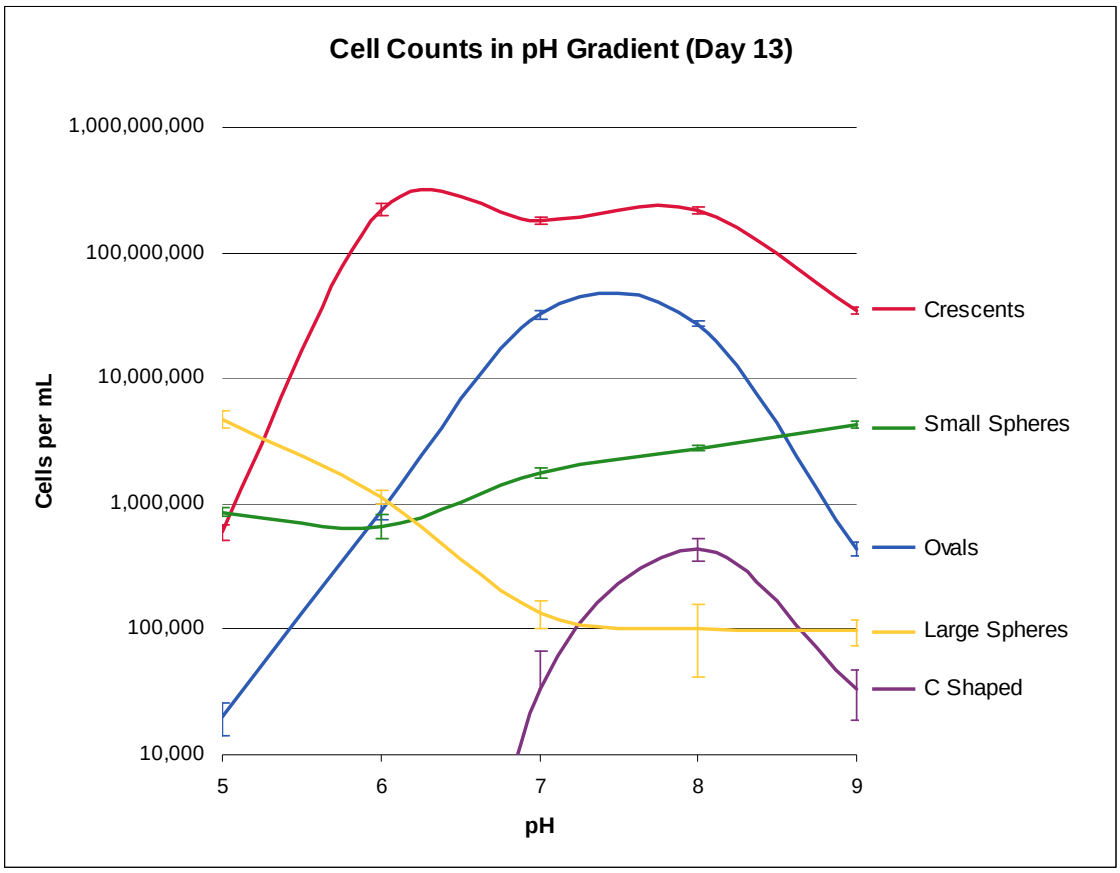

Figure 12: Cell Counts in pH Gradient (Day 13)

Lipid Content

On day 12 of the experiment, neutral lipids were extracted from the cultures growing in the pH gradient. Results are shown in Figure 13. TAG content was highest at pH 7 (6.94% by dry weight), but was also high at pH 8 (5.30%). TAG content was very low at pH 5 and pH 6 (well below 1%). The percentages for total neutral lipids were very similar, because TAGs made up most of the peaks in the HPLC analysis.

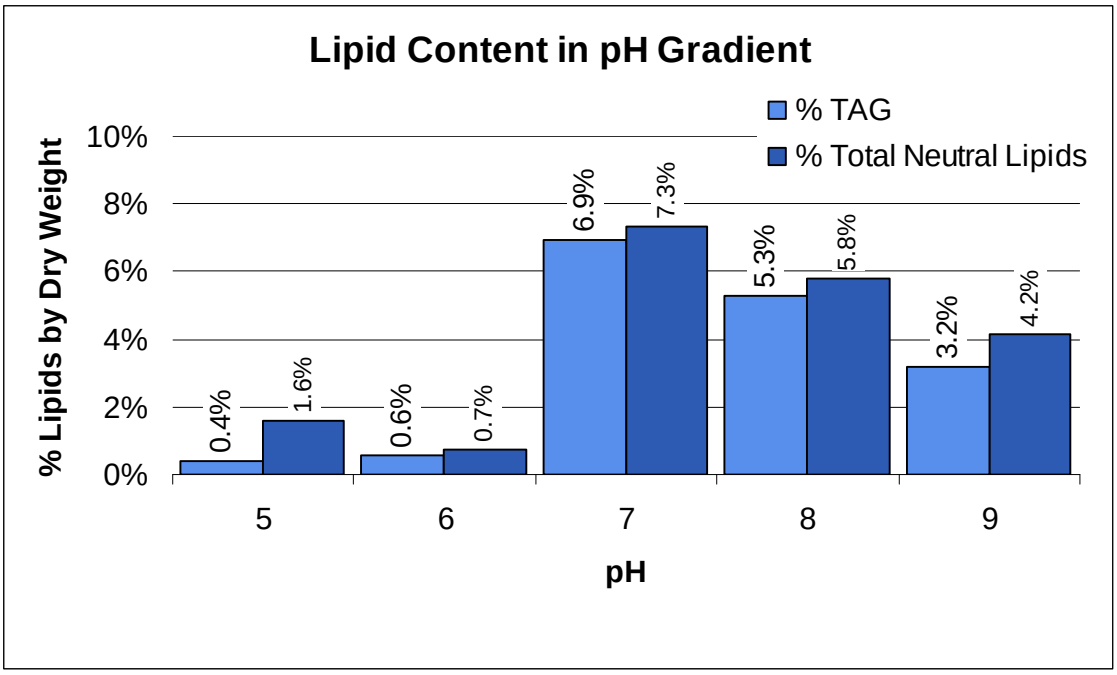

Figure 13: Lipid Content in pH Gradient

pH Changes

One interesting aspect of the pH gradient was that the pH of the cultures changed as the experiment progressed. The original culture was at pH 7.27. All of the cultures in the pH gradient seemed to converge on this original pH as the growing algae altered the conditions in their growth media. The addition of CO_2 to the air being bubbled into the cultures could also be affecting the pH. The pH 5 and 6 media became more basic and the pH 8 and 9 media became more acidic. The media that started at pH 7 ended up at an average pH of 7.26 on day 13, almost identical to the original culture. Data are summarized in Table 1.

	pH	pH	pH	pH	pH
Day 1	5.00	6.00	7.00	8.00	9.00
Day 13 Mean	5.43	6.24	7.26	7.66	7.94

Table 1: Initial and Final pH Values in pH Gradient

Chapter 5: Conclusions

Preliminary Conclusions

The objective of this research was to begin to determine some of the optimal conditions for growth of algae and production of lipids in the South Texas Ponds. Salinity and pH gradients were created in the lab, and algae from Pond 53 were cultured in these gradients and examined in several ways. One of the difficulties in drawing firm conclusions from this experiment is that so many variables were involved. Some of this variability is due to the nature of studying conditions and interactions in a natural ecosystem. Ultimately, our goal is to find out which conditions in nature will produce algae with high lipid content. Our laboratory experiments have some innate imitations, but they did produce some interesting data.

Data on overall growth were fairly unambiguous in both experiments. Algae grew best at a salinity of 9 ppt and a pH between 6 and 8. This result is expected because these conditions are similar to the natural conditions in the South Texas Ponds. The data on lipid content is much more difficult to interpret because we used a mixed culture. Experimental data showed that TAG lipid content was highest at 0 and 9 ppt and pH 7 and 8. The problem is that the percentages of each algal species varied under different conditions of salinity and pH. Therefore, lipids were extracted from a mixed group of algae that varied in each experimental condition. If oil is ever extracted commercially from algae in the South Texas Ponds, it will be from a mixture of several species, so these results are still useful. The fact that the experimental conditions that were optimal for growth and lipid production were similar to the natural conditions found in the South Texas Ponds is encouraging.

One experimental condition produced a nearly isolated culture of algae. At pH 6, 98.8% of the algae were crescent shaped *Ankistrodesmus*. This leads to the preliminary conclusion that the *Ankistrodesmus* species in Pond 53 produces only 0.57% TAG by dry weight at pH 6, 25 °C and salinity of 10 ppt. This is slightly lower than the value of 1.93% TAG described in Paik, *et al*., 2009 for *Ankistrodesmus braunii*. In contrast, the TAG value of 6.94% at pH 7 was the highest of any of the experimental conditions. The algae at pH 7 still consisted mostly of *Ankistrodesmus* (84.2%), but the oval *Navicula*-like species comprised a considerable percentage of the total (14.9%). This large difference in TAG value between pH 6 and 7 indicates that the oval species may produce significant amounts of TAGs and that Ankistrodesmus may produce more lipids under higher pH conditions. The values at pH 8 also support these conclusions with a 5.30% TAG value and 10.9% of the algae being the oval species. TAG values at pH 9 were lower at 3.18%, but the significant species other than *Ankistrodesmus* was the small sphere (possibly *Nannochloropsis*) at 10.6% of the total. The large spheres that dominated the culture at pH 5 produced very small amounts of TAG lipids (0.42%).

Lipid content in the salinity gradient was more difficult to interpret. The entire gradient only ranged from 0.96% to 2.30% TAG content and the algal percentages varied a great deal. The salinity gradient could be compared to the pH gradient in one instance. The species percentages at 9 ppt were almost identical to those at pH 8 in the pH gradient, but the TAG content was less than half (2.30% compared to 5.30%). A fact that further complicates the analysis is that algae under certain stresses like nitrogen deprivation, salt stress and low light and temperature produce higher lipid percentages, but at the expense of lower growth rates (Herman, 1991 and Hu, *et al*., 2008). It is possible that the algae at pH 8 were under more stress than those at 9 ppt and therefore produced more oil, but this is not supported by the fact that overall growth was higher at

pH 8 than at the salinity of 9 ppt. There was a relatively high percentage of the oval species at 27 ppt (35.8%) mixed in mostly with *Ankistrodesmus*. This mixture produced a modest 1.55% TAG value, but had a relatively high value of 5.08% for total neutral lipids. This is a further indication that the oval species is worthy of additional study.

It is apparent that there are a number of interacting variables that contribute to algal growth and production of lipids in a natural ecosystem. It is very difficult to tease out the effects of different variables. In addition, algae that store energy as oil rather than carbohydrates tend to have a slower reproduction rate than algae with lower oil contents and thus will tend to be dominated by low oil species in mixed systems (Vasudevan & Briggs, 2008). Clearly, it is important to isolate algal species and test their individual lipid production under different conditions, but, in reality, if algae are harvested from the South Texas Ponds or other similar ecosystems, they will be a mixture of species. Therefore it is important to continue working with samples that come directly from the location under consideration. Results from these tests can show a simple correlation between environmental conditions and overall lipid production even if all the mechanisms and interactions leading to lipid production are not completely understood.

Sources of Error

The largest potential source of error in these experiments is my own inexperience in some of the lab techniques. Cell counts were particularly difficult because I had to learn to distinguish the different species before I could accurately count them. I have more confidence in my later cell counts than in those done earlier in the experiment. I am least confident in my counts of the small spherical algae because they were easily obscured by larger species and they could be mistaken for debris or for other species viewed on end. I also had to use different microscopes on different days due to availability.

There were some issues with the bubbler tanks as well. One of the tanks ran at a slightly higher temperature than the other (about 0.5 °C). This probably did not make too much difference later in the experiment because all the pH trials were in one tank and all the salinity trials were in a different tank, but for the first few days some of the pH tubes had to be placed in the salinity gradient tank due to availability of space in the tanks. It was also difficult to control the amount of air (and CO_2) being bubbled into the tubes. I tried to use a rate of one bubble per second, but I'm sure there was much variation. More bubbling also causes more mixing and movement of the algae and its media. The addition of CO_2 to the media probably affected the pH of the media, as well.

Another potential source of error is the amount of mixing of the cultures prior to taking measurements for absorbance, cell counts and dry weights. Algae tends to settle in the bubbler tubes and the cultures must be stirred or mixed in some manner before taking measurements. I tried to mix the cultures in a consistent fashion, but it is possible that some of the cultures may not have been evenly mixed.

Future Research

Rising fuel prices, diminishing oil reserves, and environmental deterioration make algae attractive as an alternative fuel source, but many basic biological, environmental and engineering questions must be answered before production of biofuels from algae can be economically feasible (Hu, *et al*., 2008). If the work in this project is to be continued, a first step would be to repeat the pH and salinity gradient experiments to see if similar results are produced. The experiments would be more accurate if experimental samples were taken directly from the South Texas Ponds instead of from an intermediate culture. Isolation and identification of the various algae in the ponds would allow independent tests of growth response and lipid production under various conditions of pH and salinity. An additional aspect that could be examined is the morphological change of the various

species in different conditions. For instance, I noticed that the oval shaped species looked fatter at pH 7, where it had its best growth, than it did at other pH values, but I did not take precise measurements to confirm this.

Salinity and pH are only two of many factors that will determine the feasibility of harvesting oil from algae in the South Texas Ponds. Other factors that could be examined experimentally in the lab and in the field include the effects of temperature, light, nutrients, stress and seasonal variation on algal growth and lipid production. Experiments similar to the *C. calcitrans* study (Raghavan, *et al*., 2008) where multiple conditions are varied at once could also be done. Two-phase nutrient limitation studies as in Rodolfi, *et al*, (2008) look promising as well. Relationships in the ecosystem are another area that needs study. Different species of algae may affect each other if they are competing for resources and species that feed on the algae may also affect growth and lipid content. The study of inducible defenses and interspecies dynamics may lead to ways to strengthen and stabilize algal ecosystems. The interaction of all these conditions in a natural ecosystem is very complex. Some knowledge of the optimal conditions for growth and lipid production would allow us to alter conditions to favor species with high lipid content or to direct harvesting of algae during those times of year when favorable conditions are present. Even the algae themselves could be altered through genetic engineering to grow better or produce more lipids in existing conditions. One of the key recommendations in the D.O.E. Report on the Aquatic Species Program is to "start with what works in the field." Native strains in particular locations are most likely to be successful and should be the starting point for maximizing lipid production and beginning genetic engineering (Sheehan, 1998).

Algal biofuel production in the South Texas Ponds will not solve the energy crisis, but the success of the algae project could lead to the discovery of other places

where naturally occurring algae could be harvested for their oil. A better understanding of optimal conditions and their interactions will facilitate the discovery of other ecosystems that can contribute to biofuel production.

Chapter 6: Applications to Practice

When I began the UTeach Master's Program, I did so without any particular professional goal in mind. I already had a successful career as a science teacher, and was starting my job as a science curriculum specialist. Most of my knowledge of teaching and learning came from direct experience in the classroom, and I was confident in my abilities. My foremost objectives for the UTeach Program were to update my science content knowledge and to gain a more formal understanding of science curriculum and pedagogy. I was ready for new learning experiences outside of my own classroom, and the dual nature of the UTeach Master's, with instruction in both science and education, appealed to me.

My education classes helped me understand that curriculum is what happens in the classroom, not what is written in a textbook or a curriculum guide. Curriculum is the combination of what is written, what is taught, how it is taught, and how students learn. The ultimate goal in the classroom should always be to increase student learning and understanding. Teachers sometimes focus so much on their teaching that they forget about student learning.

Much of what I learned in my education classes came from interactions and discussions with other students in the science and math cohorts. The members of the cohort represented a good mix of practical experience and youthful enthusiasm. It was a wonderful experience to be able to work with the best and the brightest teachers around. Group projects, arguments and presentations were always stimulating and educational. The best debates were usually about theory versus practice. I was glad to see that no one

in either cohort was afraid to teach students in a rigorous way that challenged their thinking. I was also thankful that our professors were willing to challenge our beliefs and expectations about teaching and learning.

As much as I benefited from my education classes, I truly enjoyed my science classes and my science research experience. I learned biology at a level that I will probably never teach, but the content knowledge will make me a better teacher and curriculum specialist. It is important to be able to understand content at a higher level than you intend to teach. A good understanding of content gives you more confidence as a teacher or curriculum specialist. It allows you to ask questions at a higher level and to answer questions more correctly. Our science classes were very challenging and the pace was hectic. We learned to read scientific papers carefully and critically. We learned to reason and write at a high level. We learned that biology is an evolving and complex science with many intertwined branches. Reading, writing, thinking, analyzing, justifying, synthesizing and seeing relationships and connections in knowledge are all skills that we try to teach our students through science. If we can't use these skills at a high level, then we have little hope of teaching them.

My research with Dr. Sathasivan and Dr. Mehdy was very valuable in that it gave me the experience of thinking and working like a scientist. The combination of studying literature, planning and executing an experiment, interpreting results and writing a report requires a multitude of skills. Persistence in the face of mistakes, setbacks and deadlines is one of the most important skills and is an important one to pass on to other students and teachers. I learned science by doing science. I keep this in mind when I to write curriculum, teach my students, and coach other teachers. The experience of doing science is much more valuable to students than reading about it in a textbook. The scientific method is much more than a simple list of steps leading from hypothesis to conclusion. It

is a way of thinking that is best learned through experience. I gained a great deal of understanding about the scientific method in Dr. Sata and Dr. Mehdy's labs. I hope that I was able to contribute to their work.

The UTeach Master's Program helped me rediscover the joy in learning that convinced me to become a teacher in the first place. In my profession as a curriculum specialist, I have two main roles. One is to coordinate the writing of curriculum and assessments. The other is to mentor and support other teachers. My experiences in the UTeach Master's Program will improve my abilities in both of those roles.

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Vita

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