# The Viability of Algal Biofuel in the Southwest

**A Thesis / Senior Capstone Project**

**Presented to the Southwest Studies Department**

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**By**

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**Abstract**

Algal biofuel shows incredible potential as a partial solution to our global energy problems, but whether algal biofuel will succeed in the Southwestern United States may depend on the ability of microalgae to effectively grow in water from brackish or saline aquifers. This study was designed to test how effectively algae can grow in water from these brackish aquifers. Experiments measured growth rates (determined by final chlorophyll content) of three algal cultures (*Chlorella vulgaris* and two locally collected cultures) in increasing concentrations of salt (NaCl), the growth of *C. vulgaris* in three types of salt found in southwestern aquifers ($MgCl_2$, NaCl, $Na_2SO_4$), and the ability of two species (*C. Vulgaris* and one local species) to produce more lipids when grown in a nitrogen deplete medium rather than a nitrogen replete medium (the "lipid trigger" theory). Data from the first experiment showed that increasing salt decreased overall growth in *C. vulgaris* and culture #1, but that culture #2 was salt tolerant. The second experiment showed that increasing concentrations of magnesium chloride and sodium chloride decreased growth overall, and that sodium sulfate increased growth overall. The third experiment showed that *C. vulgaris* had higher lipid content than culture #2, but that neither species significantly increased lipid production when deprived of nitrogen. Though cultivation of algae for biofuel is not currently profitable, utilization of one or more of these strains in brackish aquifer water may provide a viable means to produce biofuel in the future.

## Introduction

Finding viable and sustainable alternatives to fossil fuels will be one of the most important challenges of this century. Although the details of climate change and peak oil are still debated by reactionary consumerists, the majority of evidence suggests the necessity of a massive transformation of global energy production and consumption.

This thesis examines the possibility of microalgae to produce biofuel in the Southwestern United States. This region has plenty of open space and sunshine, but a lack of available fresh water. Algae can grow in brackish or saline aquifers in New Mexico and Texas that might otherwise be too salty for municipal or industrial use. Experiments were designed and conducted to test salt tolerance and oil content of local and nonlocal microalgae, to see whether this brackish aquifer water can be effectively utilized for production of algae based oil. Salt tolerances of two locally collected cultures were compared with a laboratory species (*Chlorella vulgaris).* The U.S. Department of Energy's Aquatic Species Program (ASP) found that the most effective way to grow algae cultures outdoors is to let a local "contaminant" dominate, rather than trying to keep the locally dominant strains out of the culture.[1] Other studies show that large outdoor algae cultures will inevitably contain several locally robust species, and that the resulting polyculture will be more stable and more productive than an algal monoculture.[2] Local species were not expected to grow the fastest in conditions without salt, or to have the highest lipid content, but they were expected to tolerate salinity better than *C. vulgaris*. Understanding the characteristics of local species will increase the potential for effective algal biomass operations.

**Biofuel**

It is clear that we need to find new ways to power our vehicles. While electricity may eventually provide a substantial portion of transportation energy, boats, planes, and freight vehicles will probably run on liquid fuel for a long time. Biofuel has shown some great successes and some massive failures. The controversy surrounding corn ethanol in the U.S. shows a great example of a quick fix gone wrong. Though some studies claim that corn ethanol is environmentally beneficial, most assert that corn ethanol is not economically or environmentally sensible. Despite this, 40% of the corn grown in the U.S. is being used for corn ethanol (another 40% is used as animal feed).[3] Perhaps the most poignant argument against corn ethanol is simply the food vs. fuels debate. It is hard to justify devoting good cropland to biofuel production when millions of people are starving. The amount of American-grown corn being diverted to ethanol production has driven food prices up, and has encouraged a farming system that diminishes ecological health (in part because growing a monoculture reduces ecological health, and in part because of the heavy loads of chemicals that are used on corn fields and end up damaging watersheds and ocean ecosystems). Corn ethanol is estimated to produce only 1.35 times the energy invested, and even that seems optimistic.[4] As the corn ethanol sagas continue to degrade ecosystems and drive up food prices, the quest for other biofuel feedstocks becomes more urgent.

Liquid biofuel typically comes in three forms: ethanol, bio-crude, and biodiesel. In 1900, Rudolph Diesel exhibited an engine that ran on peanut oil. Since then, the diesel engine has been modified slightly to run better on petroleum diesel, but diesel engines in tropical or very hot areas can use straight vegetable oil without serious problems. In

places where temperatures drop below 50 degrees Fahrenheit, the oil needs to be converted into biodiesel to help prevent coagulation inside the tank and engine. The oils are converted by transesterification. An easy and common process for making biodiesel is to react a fat or oil with alcohol (like methanol) and a catalyst (like sodium hydroxide) to produce glycerin (a byproduct) and methyl esters (biodiesel). Biodiesel can be used in most diesel engines, and is commonly mixed with petroleum diesel in various mixtures (B20 is 20% biodiesel, B80 is 80%) to prevent coagulation at cold temperatures. Biodiesel can be made from a variety of oils, including animal fats, used or unused vegetable oil, and oil from algae. Transportation in the United States consumes over 60 billion gallons of diesel fuel per year.[5] The National Biodiesel Board estimates that 94% of freight in the U.S. is moved using diesel, and 95% of transit buses and heavy machinery use diesel fuel.[6] Biodiesel can also be used in furnaces for home heating. In 2011, U.S. biodiesel production exceeded one billion gallons.[7] Clearly, biodiesel has the potential to be widely consumed in the United States and around the world.

Several terrestrial oil crops are currently the subjects of considerable research, as people continue to look for a feedstock that can produce high yields with low inputs and marginal land. Sorghum, switchgrass, and jatropha show much more promise than popular biofuel crops like soybeans, sugar cane, and corn because of their ability to produce biomass without large energy inputs, however their capabilities are often overestimated. Several companies have invested heavily in jatropha production in developing nations and have failed terribly.[89] Recently, Harry Stourton, a representative of Sun Biofuels, said, “The idea that jatropha can be grown on marginal land is a red herring. It does grow on marginal land, but if you use marginal land, you’ll get marginal

yields."[10] This is true for any terrestrial oil crop. It is possible that jatropha-based biodiesel will succeed on small scales in parts of the world where the economic and ecological conditions are right, but it is unlikely that it will succeed in meeting our energy demands on a global level. Second-generation biofuel feedstocks like sorghum, switchgrass, or jatropha may be viable someday, however their success remains to be seen.

**Microalgae: The ideal biofuel feedstock**

While more research and development are necessary in order for it to become truly cost competitive and sustainable, algae seem to hold the most promise of all the possible biofuel feedstocks. Its amazing growth rates, low energy requirements, and the fact that it does not require arable land or fresh water make algae a crop worthy of plenty more research. Algae can treat municipal wastewater and recycle carbon dioxide emitted from power plants. Many algae species are rich in oil, which can be extracted or pressed and then converted into biodiesel. The byproducts of algae oil production can also be useful. The high protein content of the byproduct make it a competitive animal feed. The algae material that remains after oil extraction can be fermented into bioethanol, pyrolyzed to produce biocrude, burned to help power the biodiesel conversion processes, anaerobically digested to produce biogas, or used for "co-firing" in power plants (burning biomass along with fossil fuels to produce electricity).[11]

Algae are unicellular or multicellular photosynthetic organisms that live in aquatic environments. Macroalgae, or "seaweed," can grow up to 60 meters long. Microalgae are microscopic algae, and include diatoms, green algae, blue-green algae, and golden algae.

Hundreds of thousands of microalgae species are known to exist. Cultures of microalgae can more than double their volume in one day. Some algae produce omega-3 fatty acids, and are cultivated for use as nutritional supplements and used in drinks like Odwalla Superfood™. Many algae produce oils called triacylglycerols (TAGs). TAGs comprise up to 60% of the dry weight of some oil rich species of microalgae, and these oils can be easily converted into biodiesel.

Much of the research into the viability of algae production is based on theory and laboratory findings, not on real production ventures, and different studies sometimes make drastically incongruous claims. One study claims that algae can produce 9,500 gallons of biodiesel per acre per year, which compares to 50 for soybeans, 125 for canola, and 635 for oil palm.[12] This estimate is probably too optimistic, but a thirty-year study conducted by the U.S. Department of Energy through the National Renewable Energy Laboratory (NREL) called the Aquatic Species Program (ASP) claims that algae are capable of producing up to thirty times the amount of oil per acre than terrestrial oilseed crops.[13] Petroleum giant Exxon Mobil has partnered with famous genomic scientist, Craig Venter and his company, Synthetic Genomics, to research and develop algal biodiesel. They hope to achieve yields of 2,000 gallons per acre and to be producing millions of gallons in five to ten years. This project will focus mainly on genetically engineering algae to maximize oil yields, but also on other engineering aspects of production, harvesting, and extraction.[14] Algae clearly have the potential to produce more oil than any other known crop, but the challenges of fertilizing and harvesting the cultures of algae, and extracting the algal oils continue to hold back the profitability of industrial production.

Economic research of algal biodiesel has yielded a variety of results, with projected break-even prices usually ranging from three to seven dollars per gallon. Most studies project the price to be between two and five dollars per gallon, however, one study estimates the cost to be around $20 per gallon.[15] [16] These studies suggest that algal biofuel production is not currently a profitable venture, however if oil prices exceed $110 per barrel, or gasoline prices exceed $5 per gallon, which seems very likely within the near future, the algal biodiesel industry could earn substantial profits.[17]

Algae's ability to grow in wastewater is well documented. Studies assert that algae ponds can treat municipal and industrial waste in more energy efficient and environmentally friendly ways than traditional electrochemical treatment plants.[18] [19] Many wastewater treatment plants emit treated water that can adversely affect ecosystems. Eutrophication of watersheds is a serious problem in this country and around the globe. Algal blooms in rivers, lakes, and oceans can kill the majority of species living there, thus classifying the bloom as a "dead zone." The dead zone in the Gulf of Mexico currently covers more than 8,500 square miles of ocean (about the size of New Jersey). The blame for this massive algal bloom is often placed upon corn and soy farming practices in the Midwest that use large amounts of fertilizers.[20] Rather than letting algae wreak havoc on ecosystems downstream, they should be utilized in controlled systems upstream, close to or in water treatment plants, to mitigate drastic eutrophication.

This thesis has built in many ways off of Zoe Keve's senior thesis for the Colorado College's Environmental Science Department entitled "Microalgae: A Systems Approach to Wastewater Treatment and Biodiesel Production." Zoe found that two species of algae each removed 100% of nitrate and phosphate from local wastewater, and

that the algae's growth rates were higher when grown in the wastewater rather than commercially produced algae growth media (which was also rather expensive).[21] Another study claims that algae can take out 99% of the ammonia, 88% of the nitrate, and 99% of the phosphate from wastewater.[22] Zoe's study supports suggestions that algae should be used in conjunction with water treatment plants. Algae growth is also enhanced by the addition of carbon dioxide. Carbon dioxide emitted from coal burning power plants or other carbon emitting facilities can be added to algae ponds to increase yields.[23] Integrating algae with wastewater treatment and power plants can seriously reduce cost, waste, and pollution. Algae clearly have the potential to produce massive amounts of biomass and reduce environmental damages, but the technologies required for algal cultivation require more development before it can be cost competitive.

**Algae in the Southwest**

The land and water use of industrial algae ponds are significant, despite the fact that algae use land and water much more efficiently than other biofuel feedstocks. One study concluded that the U.S. could eliminate 48% of transportation fuel imports with algae based fuel, but it would require 5.5% of the land in the continental U.S. and would consume about three times the amount of water the U.S. agriculture industry currently uses. If algae ponds were located in sunny and humid places to maximize yields while minimizing evaporation, they could replace 17% of transportation fuel imports while using 25% of the water currently used for irrigation.[24] Massive industrial algae facilities may not be best suited for dry areas like the Southwest U.S. because of evaporation, but it

may still be possible because of the great amount of solar radiation, unoccupied land, and brackish water.

The biggest challenge facing algae production in the Southwest is water availability. People continue to move to urban areas in Colorado, New Mexico and Arizona, while water availability is decreasing due to drought. Obtaining water rights is difficult and expensive, and there seems already to be barely enough water to go around. Farmers with good land and good water rights are selling land and rights at incredible rates, reducing the amount of food grown locally, thus increasing the distances that food needs to be shipped (another detriment to energy, economy, and environment). If anyone wants to grow anything on an industrial scale in this area, finding enough water is clearly the foremost challenge.

Brackish aquifers in the Southwest may be able to provide water for algae cultivation. There are many aquifers that have been deemed too salty for municipal or agricultural usage. Since algae grow well in wastewater, using one or both of these alternative water sources may provide new possibilities for energy production in the Southwestern U.S.

If algal biofuels become viable in the next several years or decades, its large scale implementation in the Southwest will likely depend on whether cultures can tolerate the concentrations of salts found in brackish aquifers. The work done for the ASP through NREL studied several thousand locally collected species, and focused salt and cold tolerance studies on several hundred of them, with this idea in mind. The program found several species that can tolerate cold and salt stress and also have high lipid content.[25] The ASP report asserted that New Mexico has between 2.5 and 5 million acre feet of

water in aquifers that may be too saline for agricultural or municipal use, and therefore could be used in industrial algae ponds. One piece of the report claims that the southwestern U.S. could potentially produce several quads (a quad= about 8 billion gallons of gasoline) of biodiesel.[26] Another report suggested that Texas would be a good candidate for cultivating algae because of the large amounts of brackish water in aquifers, sunlight, and power plants.[27]

A different report prepared for the U.S. DOE entitled "Evaluation of Available Saline Water Resources in New Mexico for the Production of Microalgae" examined six aquifers in New Mexico for their capacity to provide water for algae production. Chemical analyses of each aquifer were completed, and three were selected as potential candidates. The chemicals found in these analyses included sodium, chlorine, magnesium, sulfate, calcium, potassium, and bicarbonate.[28] Because of the relative abundance of these chemicals in the aquifers in New Mexico, the three chemicals used in this study on algae salt tolerance were sodium chloride, magnesium chloride, and sodium sulfate.

**Salt tolerance of algae**

Many species of algae can survive in brackish to saline water, but too much salt will diminish growth rates and eventually kill the algae.[29] This thesis focuses on the ability of three algal cultures to tolerate NaCl, the ability of *Chlorella vulgaris* to tolerate three salts (NaCl, MgCl2, and Na2SO4), and lipid production of two algal species. It was expected that *C. vulgaris* would show stimulated growth at low concentrations of NaCl (either up to .125 M or .25 M), but that it would not be able to tolerate .375 or .5 M NaCl.

Previous studies show these patterns. [30] [31] The locally collected species were expected to tolerate salt better than *C. vulgaris*, which is commonly grown in laboratories, because of their increased exposure to salts (particularly culture #1). Growth of *C. vulgaris* was expected to decrease with increasing concentrations of NaCl and MgCl2, but increase with increasing concentrations of Na2SO4. Previous studies show that chlorine is an algistat (a deterrent of algal growth) and that sulfate is often a resource for algae. [32] [33] [34] It was expected that nitrogen starvation would cause both species of algae to increase lipid production, and that *C. vulgaris* would have a higher lipid content than the locally collected species (*C. vulgaris* is known to have relatively high lipid content). Though the success of the "lipid trigger" is debated, previous studies have shown significant increases in lipid production due to nitrogen starvation.[35] [36] Overall, it was expected that one or more of the algal cultures examined would be good candidates for algal oil production in brackish aquifer water.

**Methods**

Three experiments were conducted for this thesis. The first experiment measured growth rates and final chlorophyll content of three different algae cultures grown in treated, sterilized wastewater with increasing concentrations of sodium chloride. The second experiment measured final chlorophyll content and growth rates of one species of algae grown in treated, sterilized wastewater with increasing concentrations of three separate chemicals (sodium chloride, magnesium chloride, and sodium sulfate) that are abundant in several aquifers in New Mexico. The third experiment measured lipid content in two species of algae when grown in nitrogen replete versus N replete media.

*Experiment 1*

This experiment was designed to test the salt (NaCl) tolerance of three cultures. *Chlorella vulgaris*, and two locally collected cultures were used. *Chlorella* is a high-performance alga commonly grown in laboratories and used in dietary supplements and "healthy" drinks like Odwalla Superfood. *Chlorella* is known to be very adaptive, and to have relatively high lipid content.[37] Employees at the National Renewable Energy Laboratory in Golden, CO provided one liter of replete *Chlorella* culture for the experiment. One culture, which will be referred to as Culture #1, was collected from a ditch adjacent to San Luis Lake, near Great Sand Dunes National Park in Colorado. There are high salt concentrations in the soil and water in and around San Luis Lake. Because of this, Culture #1 was expected to perform well in the growth media with high salt concentrations. Culture #2 was collected in Fountain Creek, just downstream from Widefield Water & Sanitation, a wastewater treatment plant in Colorado Springs. Because Fountain Creek receives effluent from several water treatment plants, and sterilized treated wastewater was used as the base of the growth media, Culture #2 was expected to perform well, since it is likely adapted to a wastewater environment. Treated wastewater effluent was collected from the release pipe at J.D. Phillips Reclamation facility in Colorado Springs, and sterilized in batches in an autoclave at 121 degrees Celsius for one hour.

Each culture received the same treatments. Algae were grown in media with no salt, and in salt concentrations of .125 M, .25 M, .375 M, and .5 M. Each treatment was triplicated, so there were 45 flasks of algae. In order to reduce the likelihood of instantly killing algae by adding salt directly, one half of the final volume with the salt was added

to one half the final volume with the algae. The half with the algae was measured using a spectrophotometer to be at .2 optical density, so that a final optical density of .1 would be the starting point for each sample. Optical density is absorbance at 700 nm relative to the growth medium. The samples were grown in 250 mL flasks with foam stoppers on a stir table under 24 hr/day white full spectrum grow lights.

Ideally, the growth rates of each species would have been measured the same way, with a hemocytometer, however Cultures #1 and #2 clumped together to such an extent that small samples gathered from the flasks could not be deemed representative. Temporal growth rates of these two cultures were measured by digital imaging. The flasks of algae were poured into a dish and placed on a scanner with a cardboard box over it to negate the effects of outside light, and scanned. Samples were scanned on day 2, day 3, day 5, and day 7. The images were analyzed in Adobe Photoshop by determining the ideal point on the "threshold tool" at which the algae (which were initially appearing as grey strings or dots) would appear black on a white background The number of black vs. white pixels was then measured with the histogram. Culture density of *Chlorella* was measured with a hemocytometer on day 1, day 2, day 3, day 5, and day 7.

After 7 days under the lights, the algae were filtered through pre-weighed Pall Corporation Binder Free glass fiber filters and dried. The weights of the filters with algae were recorded, and the weight of the filter without the algae was subtracted to obtain biomass measurements. The filters were then cut into .25 square centimeter pieces and placed in 50 mL plastic Falcon tubes and submerged in 10 mL of acetone. The tubes were then placed on a vortexer for 10 seconds, in a sonicator for 10 minutes, and in a liquid nitrogen bath for 5 minutes. The vortexer, sonicator, liquid nitrogen processes were then

repeated to ensure that the acetone extraction of chlorophyll was as complete as possible. The remaining filter paper was filtered out using pyrex wool, and the filtrate was measured in the spectrophotometer at 646 and 663 nm, and these measurements were used to calculate Chlorophyll-a and Chlorophyll-b. A dilution series was done separately to determine the effectiveness of this chlorophyll measurement, and the results showed a strong correlation. For this experiment, final chlorophyll content was ultimately the determinant of algal growth.

*Experiment 2*

The second experiment tested the tolerance of one alga to three different salts. *Chlorella vulgaris* was grown in increasing concentrations of sodium chloride, magnesium chloride, and sodium sulfate. The same concentrations of NaCl as in the first experiment (.125 M, .25 M, .375, and .5 M) were used, and concentrations of magnesium chloride and sodium sulfate were measured to match the molarity of the first salt. The concentrations of $MgCl_2$ had the same amount of chlorine as the concentrations of NaCl, and concentrations of $Na_2SO_4$ had the same amount of sodium as the concentrations of NaCl. Thus, the concentrations of $MgCl_2$ and $Na_2SO_4$ were .0625, .125 M, .1875 M, and .25 M. Three samples of *Chlorella* were grown without salts as a control. The same methods for growth were used as in the first (once again starting with an optical density of .1), except this experiment lasted six days instead of seven. Instead of measuring growth rates with a hemocytometer as in the first experiment, the spectrophotometer was used to measure cell density. 4 mL samples were taken from a culture with an autopipette, placed in a glass tube, vortexed for 5 seconds, then placed in a quartz cuvette

which was placed in the spectrophotometer. Absorbance at 700 nm was measured relative to the growth medium. Biomass and chlorophyll content were measured the same way as in the first experiment.

*Experiment 3*

This experiment was designed to test the "lipid trigger" theory on two species. *Chlorella vulgaris* and culture #2 were used. The algae were grown in two media. One was the commonly used Bristol medium, and the second was the same medium without the nitrate. Bristol medium contains 2.94 mM $NaNO_3$, .17 mM $CaCl_2 \cdot 2H_2O$, .3 mM $MgSO_4 \cdot 7H_2O$, .43 mM $K_2HPO_4$, 1.29 mM $KH_2PO_4$, and .43 mM NaCl.[38] After 2 days, 4 days, and 6 days, 5 mL aliquots of each sample were stained with BODIPY 515/505 fluorescent dye (diluted in DMSO) to achieve a final staining concentration of 5μM and .1% DMSO. This method was suggested by researchers at NREL, and referenced a recent study entitled *Visualizing "green oil" in live algal cells*.[39] The samples were then placed under a microscope, and photographs were taken of the algae cells under UV light and full spectrum light. The percentage of each cell that was fluorescing in the images was measured in Adobe Photoshop to determine lipid content.

Lipid content of algae can be measured in a number of ways, but the most common methods are pressing the dried algae and measuring the weight of the oil compared to the weight of the algae, or using fluorescent dye and a fluorescent microscope to roughly quantitate lipid content in live algae cells. For over 20 years, researchers have used a fluorescent dye called Nile Red to stain algae cells and measure the fluorescence emitted by lipid bodies under a microscope. Nile Red has shown

variability in its ability to enter cell walls of different algae, and requires high concentrations (30%) of dimethyl sulfoxide (DMSO), which can kill or damage cells. For this reason, BODIPY 505/515, a lipophilic fluorescent dye that requires only .02-2% DMSO and can effectively stain many kinds of algae, is recommended for staining live algae cells, and was used in this experiment.[40]

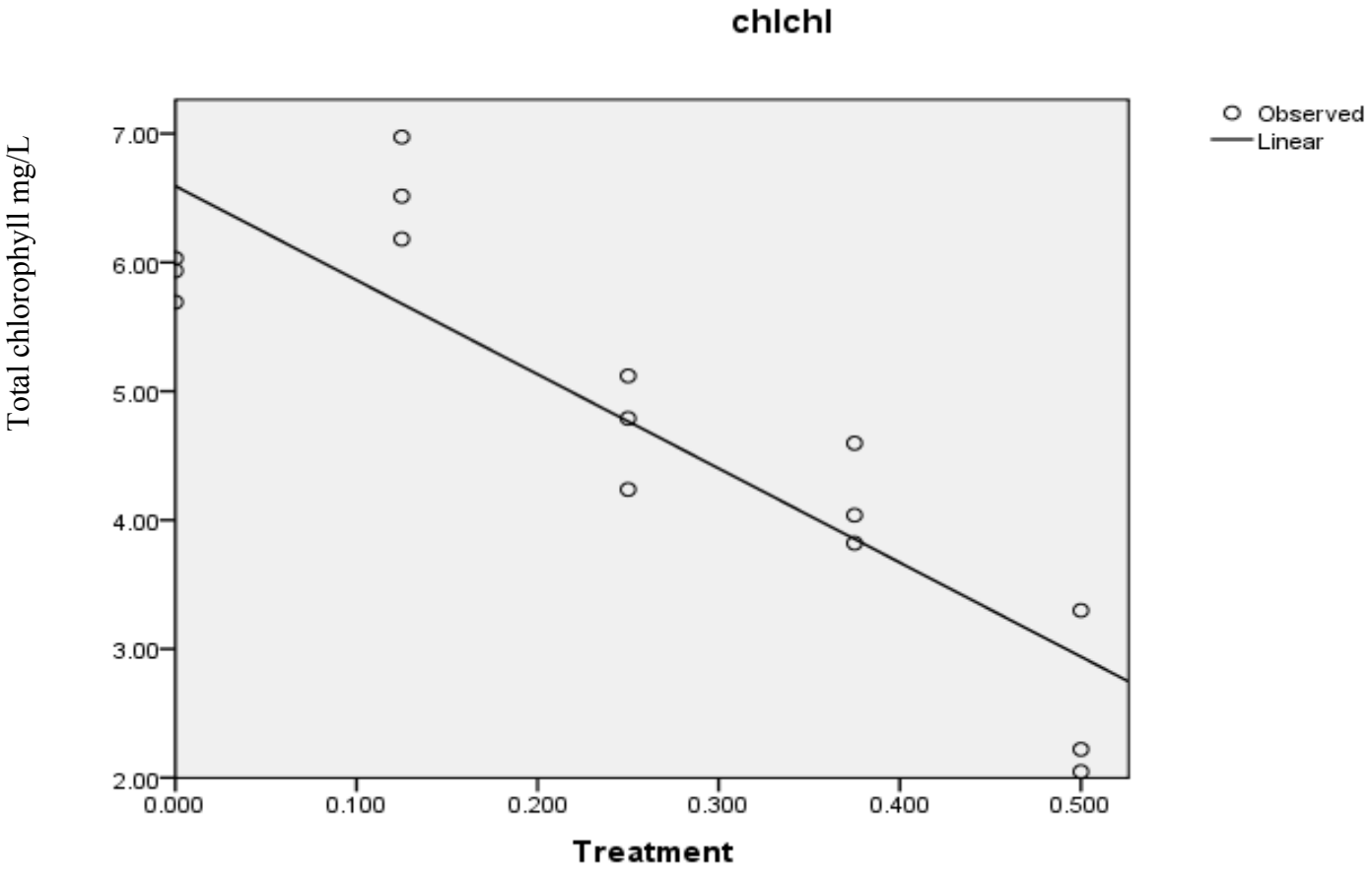
**Results**

Final chlorophyll content correlates well to overall algal growth, so it was used as the determinant for growth in the three cultures.

*Experiment 1*

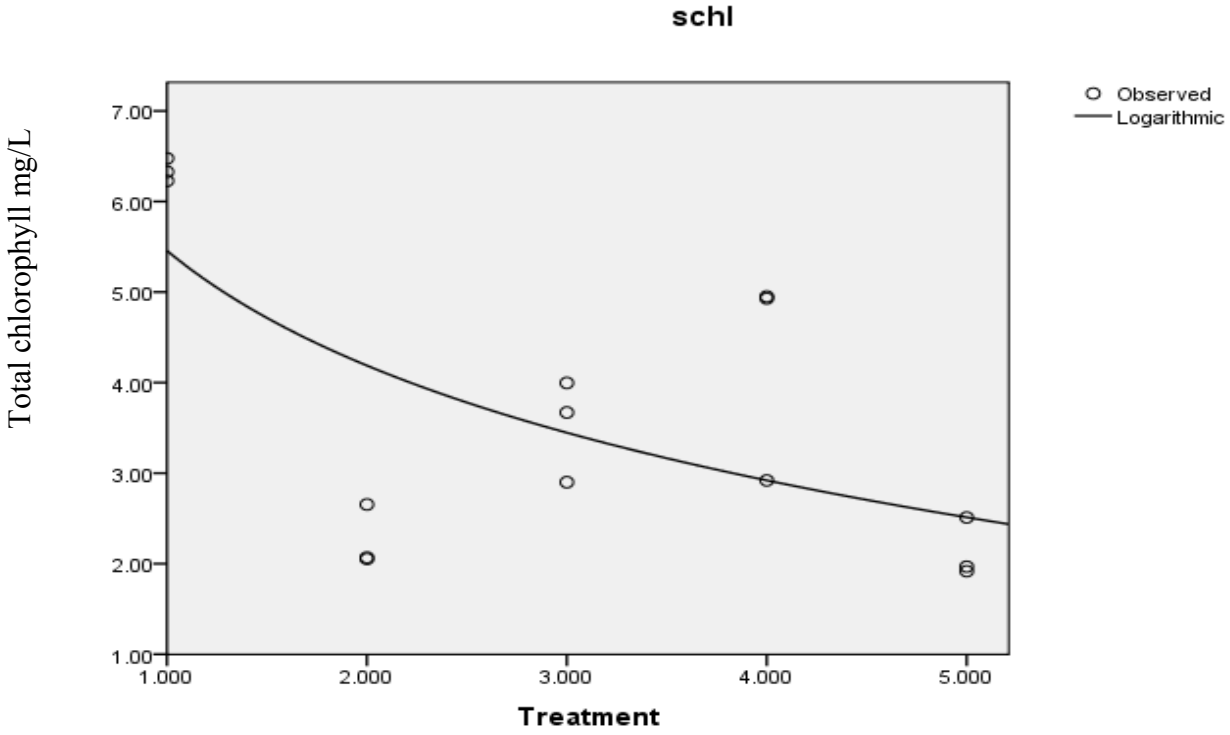
This experiment used final chlorophyll (chla) content of three algal cultures to determine the salt tolerances of each culture. Increasing salt concentrations decreased growth overall for both *C. vulgaris* ($R^2$=.791, n=15, p=.000, linear regression of final chla vs salt concentration) and culture #1. ($R^2$=.411, n=15, p=.010, logarithmic regression of final chla vs salt concentration). In contrast, culture 2 appeared to be more salt tolerant; growth did not decrease overall with increasing salt concentration. However, the relationship was slightly more complex when individual treatments were compared.

Chlorophyll content of *Chlorella vulgaris* vs. increasing salt - $R^2$=.791



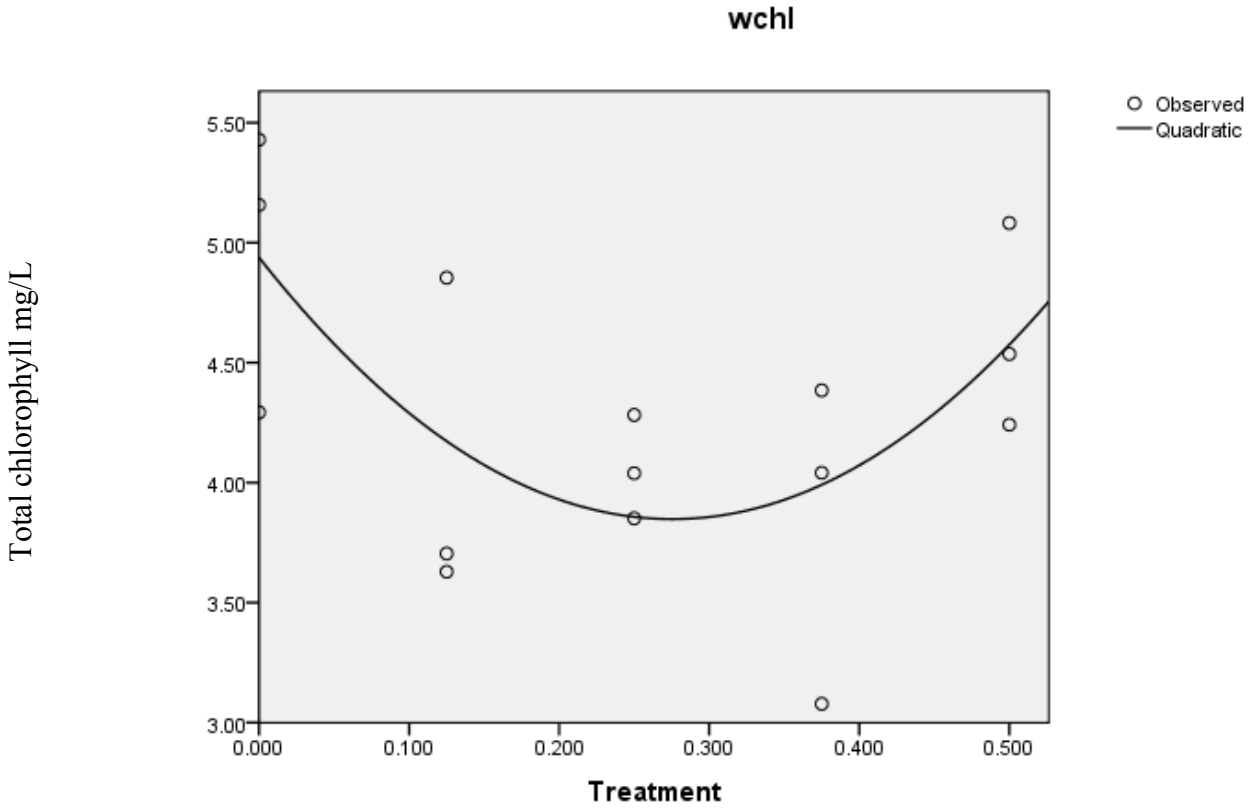

Examining the trends more closely shows that the growth of *C. vulgaris* increased slightly between no salt and .125 M NaCl (not significant at the .05 level), but then decreased dramatically with increasing concentrations of salt (F=36.757, df1=4, df2=10, p=.000). Culture density measured by counting cells in a hemocytometer (only performed for *Chlorella vulgaris*, because the other cultures clumped too much) fully supported the results derived from the chla analysis.

Chlorophyll content of Culture #1 vs. increasing salt - $R^2$=.411



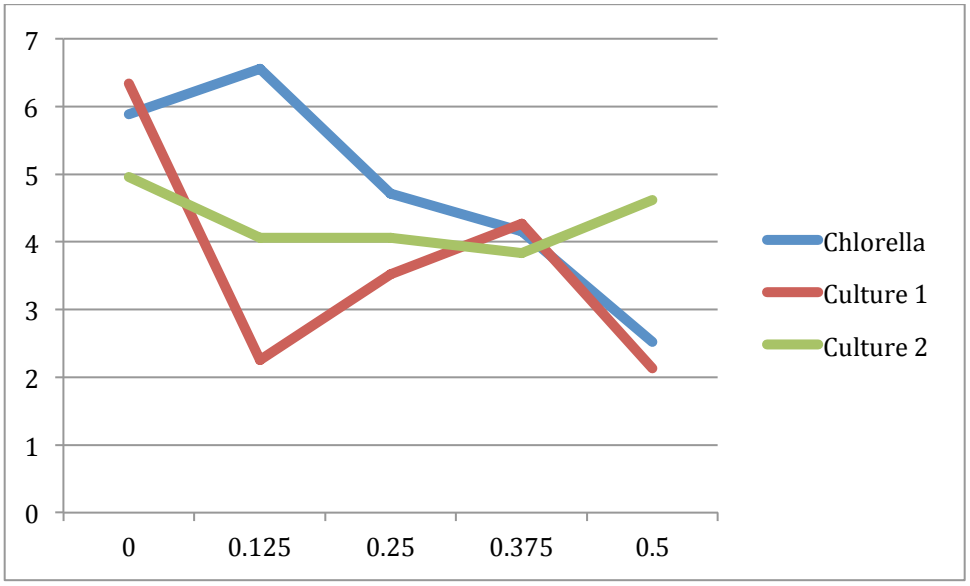

Culture #1 showed an overall decrease in growth when grown in salt. Chlorophyll content decreased significantly between treatments 1 & 2 (F=23.225, df1=1, df2=10, p=.000), then increased between treatments 2 & 4 (F=23.225, df1=1, df2=10, p=.003), and then decreased significantly between treatments 4 & 5 (F=23.225, df1=1, df2=10, p=.002).

Chlorophyll content of Culture #2 vs. increasing salt - $R^2$=.421




Culture #2 showed very little decrease in growth at increasing salt concentrations ($R^2$=.421, n=15, p=.038 quadratic regression of final chla content vs. salt concentration). The post-hoc statistics of a one-way ANOVA showed that growth of culture #2 that received treatment 4 (.375 M NaCl) was lower than growth of culture that received treatment 1 (no salt) (F=2.157, df1=4, df2=10, p=.031). Growth increased slightly from treatment 4 to treatment 5 (.5 M) so that the difference between growth in no salt and in .5 M NaCl was not significant at the .05 level.

Average final Chla content of 3 cultures vs. increasing salt concentrations




Comparisons were made between final Chla at the lowest salt concentration among the three cultues and the final chla among the three cultures at the highest salt concentration. At .125 M NaCl (ONEWAY ANOVA F=56.133, df1=2, df2=6, p<.0005) chlorella had the largest final chla, followed by culture 1, followed by culture 2 (the differences among all three cultures were statistically significant using LSD post-hoc comparisons). At the highest salt concentration (ONEWAY ANOVA F=21.477, df1=2, df2=6, p=.002), growth of culture 2 was significantly higher than that of the other two cultures.

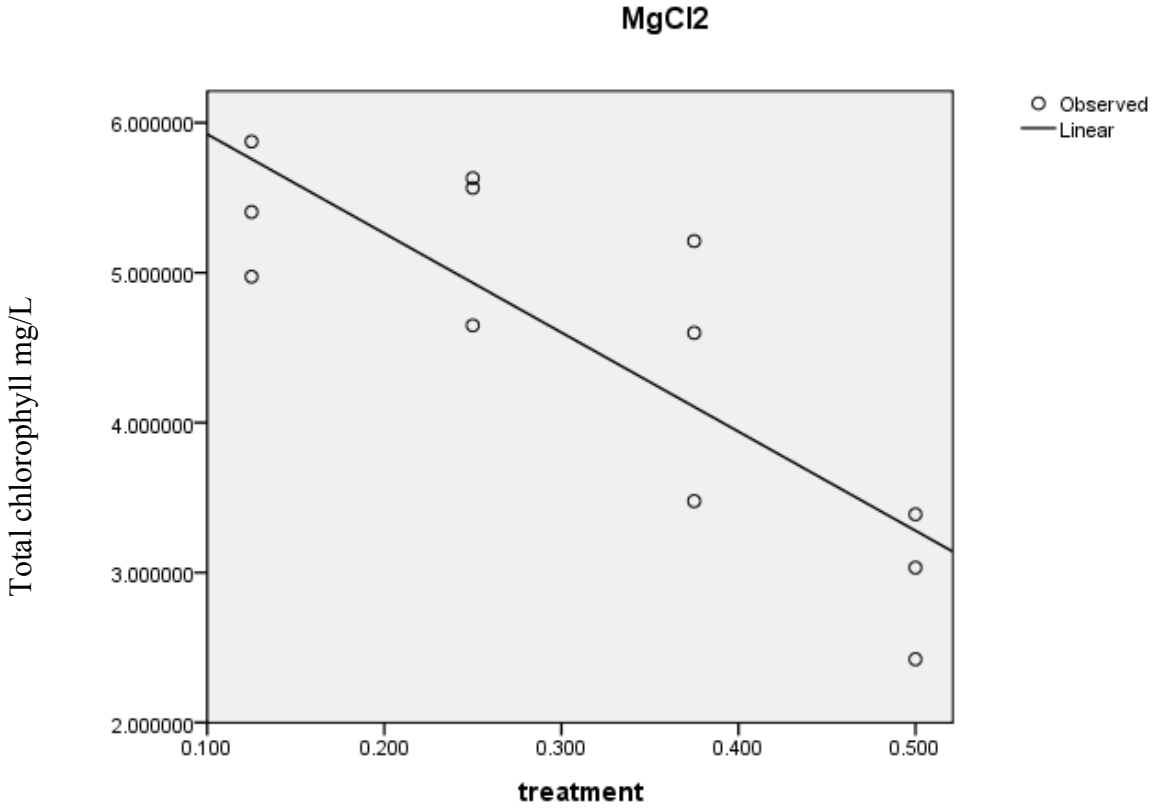
The Anderson-Darling test for normality showed that the departures were not large enough to reject the hypothesis of normality at the .05 level. Levene's test for homogeneity of variance showed that the hypothesis of equal variances could not be rejected for either *C. vulgaris* or Culture #2, but that Culture #1 had unequal variances.

*Experiment 2*

The purpose of this experiment was to determine the effects of three salts (NaCl, $MgCl_2$, and $Na_2SO_4$) on the growth *Chlorella vulgaris*. The concentrations of $MgCl_2$ had the same amount of chlorine as the concentrations of NaCl did, and the concentrations of $Na_2SO_4$ had the same amount of sodium as the concentrations of NaCl, so that comparisons could be made between chemicals. Increasing concentrations of both sodium chloride and magnesium chloride caused an overall decrease in growth ($R^2$=.546, n=12, p=.006 for linear regression of chla vs. increasing NaCl concentration; $R^2$=.700, n=12, p=.001 for linear regression of chla vs. increasing $MgCl_2$ concentration) and increasing concentrations of sodium sulfate stimulated growth at each increase ($R^2$=.917, n=12, p<.0005 for logarithmic regression of chla vs. increasing $Na_2SO_4$ concentration).

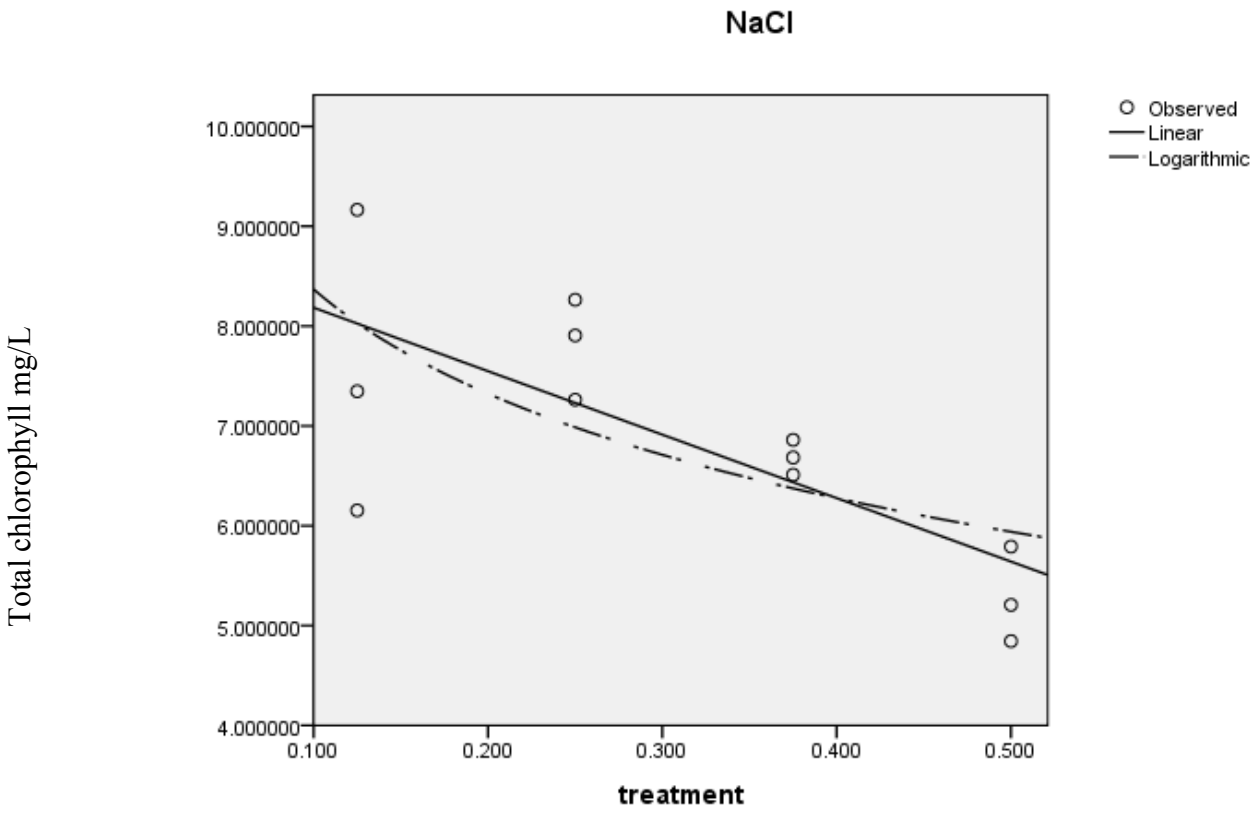
The three control samples (*C. vulgaris* grown without salt) appeared to be thriving for the first three days, but then suddenly died. Excluding this peculiarity, one-way ANOVA tests showed significant differences between means for culture density and chlorophyll content measurements for all three salts (NaCl: F=1.463, df1=3, df2=8, p=.031. $MgCl_2$: F=10.191, df1=3, df2=8, p=.004. $Na_2SO_4$: F=121.176, df1=3, df2=8, p=.000).

Chlorophyll content of *C. vulgaris* vs. increasing $MgCl_2$ - $R^2$=.700



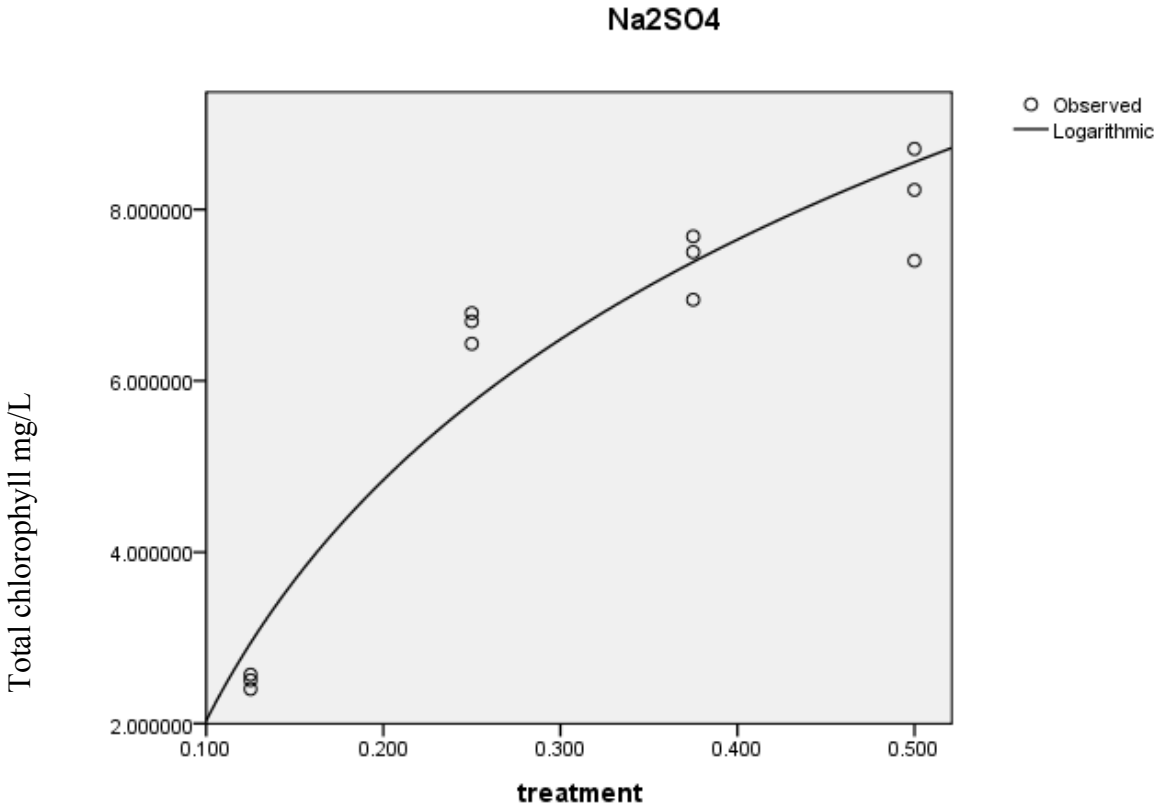

Increasing concentrations of magnesium chloride significantly reduced growth of *Chlorella vulgaris*.

Chlorophyll content of *C. vulgaris* vs. increasing NaCl - $R^2$=.546 for linear regression



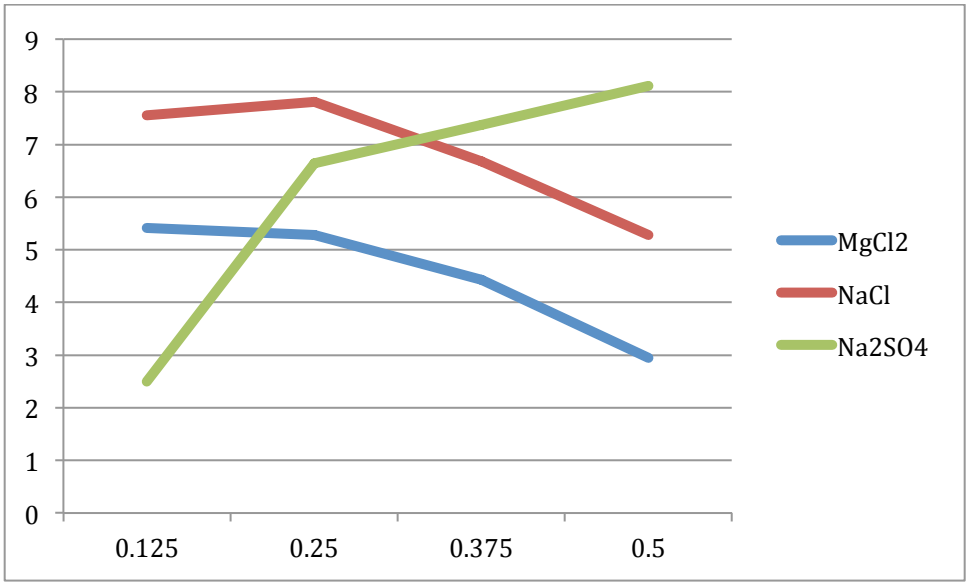

Increasing concentrations of sodium chloride also significantly reduced growth of *Chlorella vulgaris.*

Chlorophyll content of *C. vulgaris* vs. increasing $Na_2SO_4$ - $R^2$=.917




Chlorophyll content of algae grown in sodium sulfate increased significantly at each increase in concentration.

Average chlorophyll content of *Chlorella vulgaris* vs. increasing concentrations of 3 salts

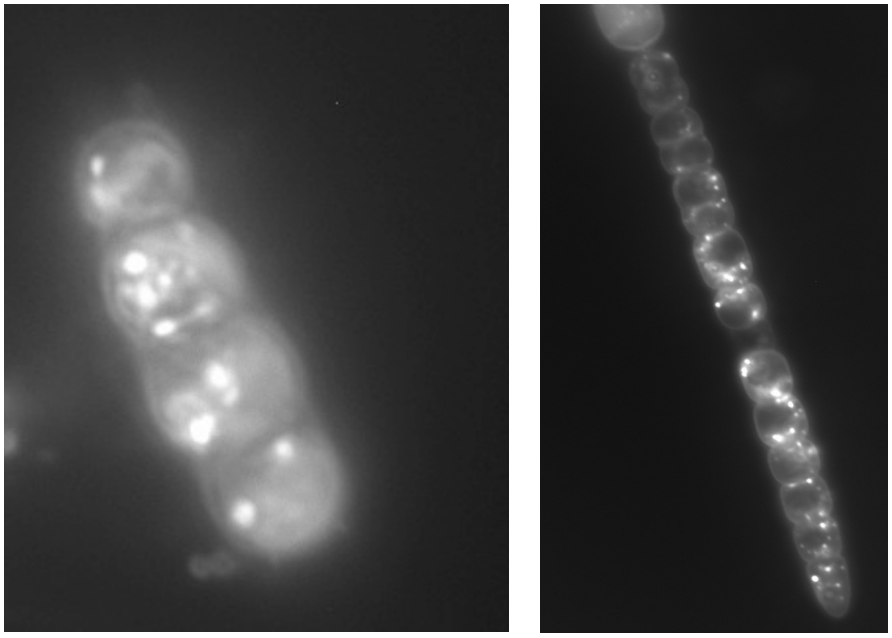



Chla content of algae in the lowest concentration of salts (.125 M) showed significant differences between each salt (ONEWAY ANOVA: F=23.152, df1=2, df2=6, p=.002). Chla content of algae in the highest concentration (.5) also showed significant differences between each salt (F=66.644, df1=2, df2=6, p<.0005). Chla content of algae grown in magnesium chloride was significantly lower than that of algae grown in sodium chloride at all levels of concentration.

The Anderson-Darling test for normality showed that the hypothesis of normality could not be rejected at the .05 level for growth of algae in any of the three salts. Levene's test for homogeneity of variances found that growth of algae in all three salts had equal variances.

*Experiment 3*

Algae grown in nitrogen deplete media did not show significantly increased lipid production. *Chlorella vulgaris* showed significantly higher lipid content than the algae in Culture #2. Average lipid content of *C. vulgaris* in N deplete media was .06% lower than in N replete media. Average lipid content of Culture #2 in N deplete media was 1.7% higher than in N replete media, but this difference was not statistically significant.



Microscope pictures of algal cells with fluorescing lipid bodies

*C. vulgaris* (left) averaged 24.4% lipid content in N deplete media on day 6.

Culture #2 (right) averaged 18.27% lipid content in N deplete media on day 6.

**Discussion**

The aim of this study was to determine how well algae can grow in water from brackish aquifers, and to examine lipid production of a local species versus the well-known lab performer, *Chlorella vulgaris*. Experiments measured growth of three algal cultures in increasing concentrations of sodium chloride, growth of *C. vulgaris* in increasing concentrations of three different salts (NaCl, $MgCl_2$, and $Na_2SO_4$), and the ability of *C. vulgaris* and a locally collected species to increase lipid production in a nitrogen deplete medium versus a nitrogen replete medium.

For the first experiment, it was expected that *Chlorella vulgaris* would achieve the highest growth rates in the lowest concentrations of salt, but that culture #1 would show more robustness to salt at higher concentrations. This is because *Chlorella* is known to be a good grower, but not notably salt tolerant, and because culture #1 was collected from an area with salty water and soil, and was therefore expected to tolerate salt well.[41] The results showed that culture #1 did not tolerate salt well, but that culture #2 (which was collected in Fountain Creek) did. *C. vulgaris* showed stimulated growth at low concentrations of salt, but diminished growth at high concentrations.

These findings supported the patterns found in much of the literature. One study shows that *Chlorella vulgaris* cannot adapt to salt concentrations above .5 M.[42] A study done for the *Journal of Algal Biomass Utilization* found that *Chlorella vulgaris* showed increased total chlorophyll content at 0.1 and 0.2M concentrations of NaCl, but reduced content at 0.3 and 0.4M NaCl. The study found a decrease in total protein content in all concentrations of NaCl, but increased proline content in all concentrations. Decreased capacity for protein synthesis has been shown to increase lipid and carbohydrate

production, potentially a good thing for algal biofuel production.[43] A study done for the *Journal of Medicinal Plants Research* claims that salt stress decreased growth rates, dry weight, and pigment content of algae species *Chlorella vulgaris, Spirulina platensis,* and *Scenedesmus* sp. at 0.1 M and higher concentrations of NaCl, but that lipid content of *S. platensis* was higher in concentrations up to 0.08 M NaCl compared to a control.[44] This research suggests that *Chlorella* and some other algae species can tolerate small to moderate levels of salt, and the findings in this thesis support these patterns.

A study examining the effects of NaCl and KCl on the unicellular green alga *Micrasterias denticulata* indicated that the ionic stress rather than the osmotic stress of the salt induced programmed cell death. The study found that prolonged salt stress (with salt concentrations of .2 M) significantly decreased photosynthetic activity in *Micrasterias* and other green algae.[45] Another study found that *Scenedesmus opoliensis* showed a decrease in dry algal biomass between no salt, 0.1 M NaCl, and 0.5 M NaCl, but that the difference between each salt concentration became smaller when the algae received more light. The algae in 0.1 M NaCl at medium light and the algae in 0.5 M NaCl at high light grew more than the algae in no salt at low light.[46] This indicates that although salt adversely affects algal growth, getting sufficient light is more important for the growth of the algae, and algae can still grow fairly well in slightly to moderately saline environments.

When stressed with salt, each culture showed a different growth pattern than the others. *C. vulgaris* grew better than the other cultures at the lowest concentrations of salt, and culture #2 grew better than the others at high concentrations of salt. Growth of culture # 2 in high salt concentrations was not significantly lower than its growth in no

salt, indicating that this was the most salt tolerant culture overall. Studies show that growth of *Chlorella* is often stimulated by low to moderate amounts of salt, but that growth will diminish significantly at high concentrations.[47] The data from my experiment show this pattern.

Contrary to expectation, culture #1 did not tolerate salt very well. This culture showed high levels of growth, similar to those of *C. vulgaris,* when grown in no salt, but growth was significantly lower in low and high concentrations of salt. This contradicted the expectations, since culture #1 was collected from an area with high levels of salt in the soil and water and was therefore expected to be salt tolerant. A possible explanation is that different species were growing in the same culture. It is possible that one species in the culture was not salt tolerant, and died immediately upon encountering the salt, and that growth of the other species in the culture was stimulated by the salt up to a point (.375M) and then began to die at increased concentrations. Either cohabitation or varying levels of contamination might have caused the surprising data for this culture. Identifying the species inside each culture would be beneficial, and could be the focus of future research.

The second experimented tested the hypothesis that growth of *C. vulgaris* would be inhibited by both sodium chloride and magnesium chloride, but enhanced by sodium sulfate. Previous studies show these patterns generally, and the results of this experiment support this hypothesis.[48] [49] [50] A study done for the American Journal of Botany found that while .42 M $MgSO_4$ reduced growth of *C. vulgaris* to 9.2% of the control, .2 M $MgCl_2$ reduced growth to 2.5% of the control.[51] It appears that magnesium is essential for *C. vulgaris* cultures to photosynthesize and grow, however high concentrations of

magnesium will deter growth.[52] Sodium sulfate is not generally toxic to algae. One report claims that the lowest found toxicity value for sodium sulfate on algae is 1900mg/L.[53] Sulfate can enhance growth up to a point, and has been shown to behave like a resource for algae. [54] [55] Sodium bicarbonate and sodium nitrate can enhance algal growth, and if the effects of sodium sulfate on algae are not detrimental, it might suggest that sodium is not largely detrimental to algal growth.[56] [57] Chlorine, on the other hand, is an algistat (a substance that inhibits algal growth).[58] Chlorine is used in swimming pools in part to kill algae. The effects of magnesium versus the effects of sodium can be compared in this study since the molarity of $MgCl_2$ will be measured so that the amount of chlorine matches the amount of chlorine in the measured NaCl.

The results indicate that *Chlorella vulgaris* can tolerate sodium chloride better than it can tolerate magnesium chloride, and that its growth is stimulated by high concentrations of sodium sulfate. This suggests that when monitoring the chemical composition of aquifer water for algal cultivation, keeping overall magnesium and overall chlorine concentrations low should be a top priority.

The third experiment was expected to show that algae produce more lipids when grown in nitrogen deplete media, and that *C. vulgaris* has a higher average lipid content than culture #2. This experiment was not able to prove the lipid trigger theory, however *C. vulgaris* showed significantly higher lipid content than culture #2 in both replete and deplete media for all three days of measurement. Though the ASP deemed nutrient depletion for triggering increased lipid production in algae not worthwhile because the increase in lipids was offset by decreases in growth rates, many researchers continue to study and support the "lipid trigger" theory.[59] [60] One study claims that putting algae in

nutrient deplete media immediately before harvesting can increase the lipid content 130-320%.[61] Another study supports the two-stage process, claiming that *Chlorella vulgaris* under various lipid trigger conditions can accumulate six times the lipid content of a control.[62] It is clear that different lipid trigger processes can have very different results, and that more research is necessary in order for this theory to be accepted or disproved.

**Problems with the study and areas for future research**

Several challenges arose in the middle of these experiments. Some of the initial measurement methods required modification in order to produce reliable results. In the first experiment, the wild cultures of algae clumped together in each flask. This clumping made it difficult or impossible to take a "representative" sample to measure with a hemocytometer in order to determine growth throughout the experiment. A method was improvised to continuously measure the growth of these cultures. The contents of each flask were poured into a pitri dish, placed on a scanner, and scanned. The photos were analyzed to determine the area that the algae covered. The results did not show comprehensible or patterned data. Variation within triplicates receiving the same treatments was very high, discounting the credibility of these measurement methods. Three-dimensional modeling of the images of algae cultures could potentially be used to determine culture size or density, however this could involve expensive or difficult methods. Final chlorophyll content provided clear and patterned data, but the need for better methods to determine daily growth of clumping algae is apparent.

Biomass measurements for experiment #1 did not show patterns. The filter paper caught or absorbed the high concentrations of salt present in some of the samples, and

this skewed the data. The weights showed high variation and very little pattern, even though some flasks clearly contained much more algae than others. For the second experiment, each sample was rinsed three times with mili-Q water to remove the salts. Unfortunately, the weights of the filter papers again showed high variation and not substantial patterns. The difficulty of weighing differences of thousandths of a gram suggests that increasing the size of each sample from 50 mL to 150-300 mL might yield more comprehensible biomass measurements.

The control samples used in the second experiment died unexpectedly after three days of rapid growth. The cultures may have died because of contamination or because the algae consumed all of the nutrients available in the medium very quickly. One study claims to "clearly indicate that *Chlorella* cells produce and liberate into the external solution a substance that tends to retard growth" when cultures reach higher densities. [63] It is unlikely that this process or a reaching of "stationary phase" caused this death, however, since it was very rapid and uniform. When algal cultures reach stationary phase they usually slow growth, rather than quickly die. [64] It seems more likely that this death was caused by contamination, which was a problem in all cultures of algae. Bacteria and nematodes were present in the cultures of Species #1 and #2, and were present to a lesser extent in some of the *Chlorella* samples despite efforts to filter them out of the collections and despite efforts to sterilize all growth media and containers. This contamination may have affected the results of the experiment.

In the third experiment, only thirty pictures were analyzed for each sample for each day. Perhaps more pictures would yield more significantly different data between treatments.

Future research should be done to determine at what point exactly each of these salts diminish growth of an outdoor algae culture. This study examined the general patterns of salt stress, but the range of concentrations was broad. More incremental studies may be able to find a more precise point at which growth media is too salty. Understanding that polycultures of algae are inevitable is crucial, but cultivation of algae for fuel in brackish water may benefit from the use of particularly salt tolerant species as inoculum, to reduce the amount of time required before harvesting the algae.

**Conclusion**

Algal biofuel presents a partial solution to our global energy and environmental problems. Their ability to grow in wastewater and moderately saline water from aquifers has been well documented, and utilizing one or both of these water sources could make algal biofuel industrially viable in the Southwestern United States. To further examine this possibility, this thesis tested the salt tolerance of three algal cultures (*Chlorella vulgaris* and two locally collected cultures), the tolerance of *C. vulgaris* to three salts ($MgCl_2$, NaCl, and $Na_2SO_4$), and the effect of nitrogen starvation on the lipid production of two algal species (*C. vulgaris* and a locally collected species). Results found that one local culture tolerated salt well, while growth of *C. vulgaris* and the other local culture were reduced in increasing salt concentrations. Growth of *C. vulgaris* was inhibited by both $MgCl_2$ and NaCl, and stimulated by $Na_2SO_4$. These patterns are consistent with the available literature. Neither *C. vulgaris* nor the local species produced significantly more lipid when grown in nitrogen deplete media nitrogen replete media, however *C. vulgaris* showed significantly higher lipid content than the local species. Based on the results of

this work, one or more of these cultures would be good candidates for algae cultivation in brackish aquifer water in the Southwest. Although the technologies necessary for cost-competitive algal fuel production require further development, it is clear that algae have the capacity to produce massive quantities of oil and reduce the negative effects that humans have on ecosystems around the world.

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