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# An Alternative for the Future: Growth and Lipid Production in Extremophilic Algae

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# An Alternative for the Future: Growth and Lipid Production in Extremophilic Algae

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**Abstract:**

Microscopic algae are a potential source of renewable fuels. Determining what conditions are most favorable to the growth and lipid production of specific algal strains can aid in the search for an alternative to fossil fuels. Desert and polar strains of *Bracteacoccus bullatus* were grown on different media and tracked for their growth rates over a month. In another experiment, the same strains were frozen for two hours, grown for several weeks, and subsequently harvested. The cellular lipids were chemically extracted and analyzed using a GC/MS. The results suggested that the polar strains grew best in nutrient-enriched media while the desert strains grew best in the nutrient-poor media. In response to freezing, total lipid content increased in the desert strains and decreased in the polar strains. This suggested major physiological differences between the desert and polar strains of the same species. The polar strains were better acclimated to the freezing and nutrient stress than the desert strains, which could be explained by adaptations to different environments.

**Introduction:**

Human civilization has grown significantly in size over the past century and with it has come a boom in industrialization and technological innovation. For technology and infrastructure to work or be made, something has to power it. For example, transportation is largely powered by fossil fuels, natural fuels that were geologically formed over millions of years, which are considered a reliable energy source. Fossil fuels, like oil, natural gas, and coal, are burned to provide heat, generate electricity, and power vehicles (Fossil Fuels [date unknown]). While humans have benefited greatly from fossil fuels, the world's oil supply will eventually be exhausted, and the constant burning of these fuels has been an issue that is rapidly magnifying in

scale. In the words of literary writer Margaret Atwood, “We’re hooked on oil, and without it we can’t do much of anything. And since it’s bound to run out eventually, and since cheap oil is already a thing of the past, we ought to be investing a lot of time, effort, and money in ways to replace it” (Atwood 2015). The dependence humanity has on fossil fuels, particularly petroleum, has led to disastrous effects like climate change-and its major component, global warming, which have (and will) only worsen over time. When fossil fuels that are rich in carbon are burned by combustion, carbon dioxide gas and other greenhouse gases are released into the atmosphere, specifically the troposphere (Fig. 1). Oil, natural gas, and coal constitute 45, 32, and 29% of greenhouse gas emissions in the U.S (Fossil Fuels [date unknown]).

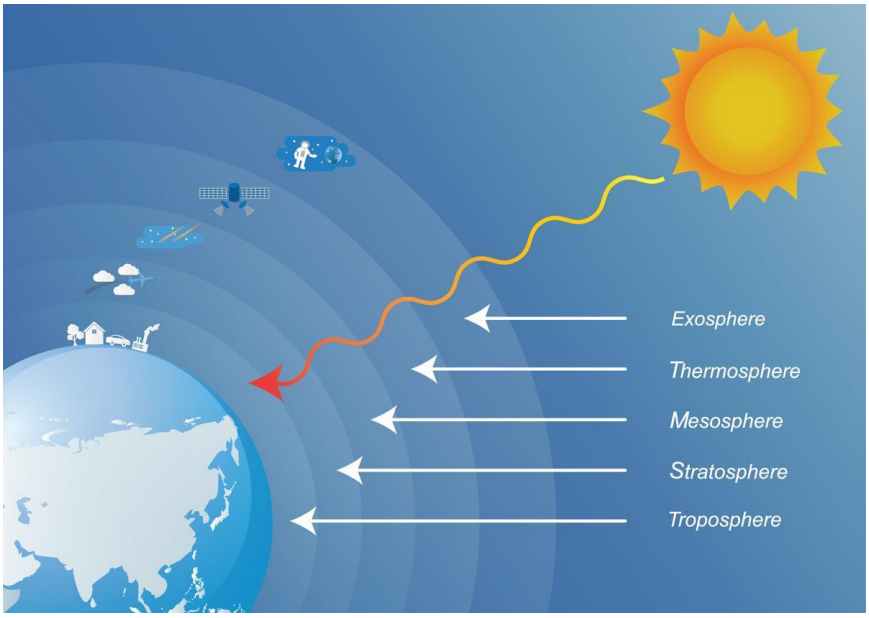


Figure 1. Layers of the Earth’s Atmosphere (https://sciencestruck.com/atmosphere-layers-facts)

The atmosphere is mostly made up of the elements nitrogen, oxygen, and argon ($N_2$, $O_2$, and Ar), none of which absorb infrared rays. Carbon dioxide and methane are the most abundant greenhouse gases but together add up to less than 0.05% of the atmosphere. Nitrogenous gas derivatives like nitrous oxide are also found in the air and can act as greenhouse gases. Carbon dioxide in particular has been increasing in the atmosphere for centuries because it is being

released faster than it can be removed by photosynthesis, and therefore it has the greatest concentration in the air out of the greenhouse gases. During the start of the Industrial Revolution, the concentration of carbon dioxide was 280 ppm, and the 400 ppm threshold was crossed in 2013. However, it has been predicted that by 2100, the carbon dioxide concentration is supposed to hit 970 ppm, which is more than triple the concentration before the Industrial Revolution (Eneji et. al 2017). Methane is produced naturally from bacterial decomposition, plants, and animals but also comes from coal mining and natural gas (Eneji et. al 2017). The gases then accumulate in the troposphere and form a layer that traps and concentrates heat from the sun's rays, which in turn, causes the warming effect (Nunez 2019). Global warming is more complex and can be further explained by the Albedo effect. As the sun's rays hit the Earth, the Earth absorbs a portion of those rays and emits heat in the form of infrared rays. These rays then hit the molecules of the gas layer and cause the molecules' bonds to stretch or bend as they absorb the infrared energy. Simultaneously, the molecules disperse the heat in different directions which leads to the intense concentration of heat within the atmosphere (Eneji et al. 2017). The effects of this heating can result in rising sea levels from melting glaciers, a rise in unseasonal weather like droughts or hurricanes, and an increase in disease carriers like mosquitoes (NASA 2019). For example, there has been a documented increase in heat waves since 1980, when globalization and the utilization of fossil fuels started to advance. In addition to that, the increased temperature allows the atmosphere to retain more moisture. As a result, the increased moisture leads to heavy precipitation for short periods of time which contribute to mass flooding, but also drought at other times (Loiy Al-Ghussain 2018 ). While this paints a bleak picture of the world's present and near future, there is hope in the potential of biological organisms to provide a renewable

alternative to fossil fuels and improve the health of the planet. Scientists are now studying algae as a potential biofuel of the future.

**What are Algae?**

Algae are a diverse group of typically aquatic organisms that can conduct photosynthesis, a sunlight-powered process that in addition to creating oxygen from water molecules also takes carbon dioxide from the atmosphere and incorporates it into sugars. The term “algae” is broad, encompassing many organisms from kelp to plankton. Algae inhabit both freshwater and saltwater habitats, but their adaptability as organisms also allows them to survive on land in desert crusts, polar terrain, tree trunks, and animal fur (Vidyasagar 2016). The three major groups of algae, which are green algae, red algae, and brown algae, are classified by their pigments and evolutionary origins. The phylum Chloroplastida encompass green algae that are related to plants (Embryophyta), while the Phaeophyta, also called kelps, and Rhodophyceae, brown and red algae respectively, are groups containing large seaweeds (Fig. 2).

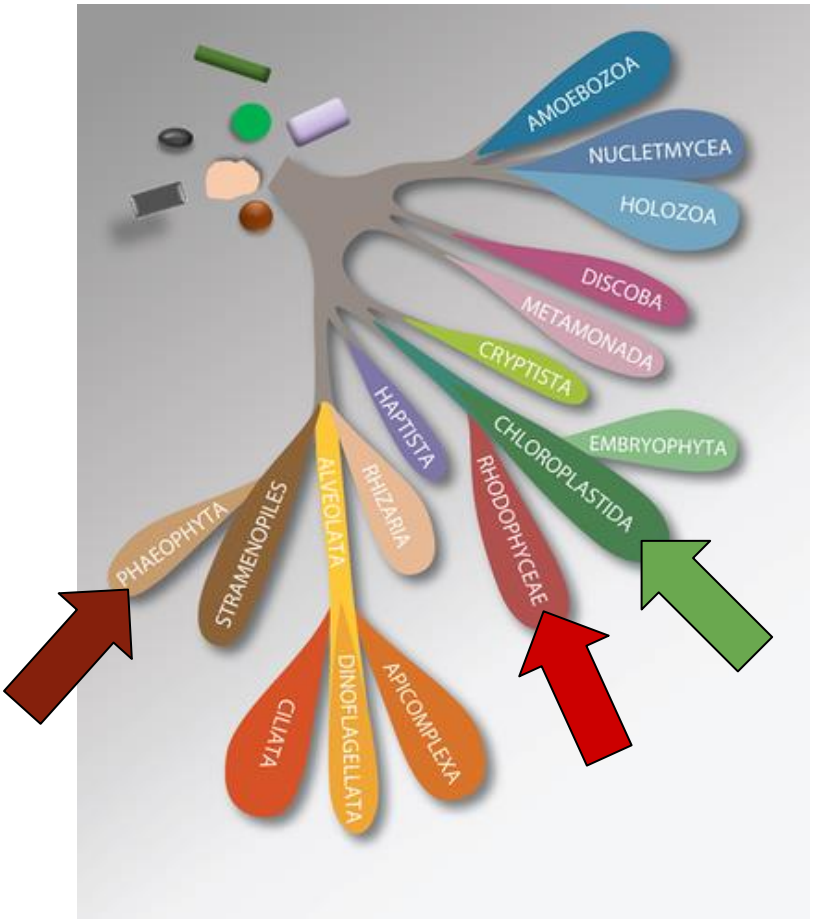


Figure 2: Evolutionary Tree of Eukaryotes inferred from molecular data (Adl et al. 2018): The brown algae are indicated by the brown arrow. The red algae are indicated by the red arrow. The green algae are indicated by the green arrow. The group containing animals is Holozoa, and the group containing land plants is Embryophyta.

Other groups of microscopic algae are known as well – dinoflagellates, cryptophytes, haptophytes and others (Lenntech). Algae come in a variety of shapes and sizes. Many species are microscopic and unicellular. Others, like leafy kelp and other seaweeds, have plant-like bodies that can be seen with the naked eye. Even the large-bodied algae, however, lack the more complex structural features of plants like roots, stems, leaves, and a vascular system to transport water and nutrients. Despite this difference, algae can be considered “miniature plants” and, even more importantly, potential alternatives to fossil fuels. The key to their usefulness as a biofuel is

their lipid content, as many algae store the carbon fixed through photosynthesis in the form of lipids.

**What are Lipids?**

Lipids are biological compounds that act as structural parts of membranes and energy storage molecules for cells. They are comprised of carbon and hydrogen atoms, whose combustible nature makes them desirable for burning for fuel. Algae's adaptability to many environments has led to evolutionary variation in their lipids. The main classes of algal lipids are phospholipids, glycolipids, and neutral, non-polar, lipids. They are characterized as mostly hydrophobic, but some classes of lipids can have both hydrophobic and hydrophilic parts (Fahy et al. 2011) (Fig. 3).

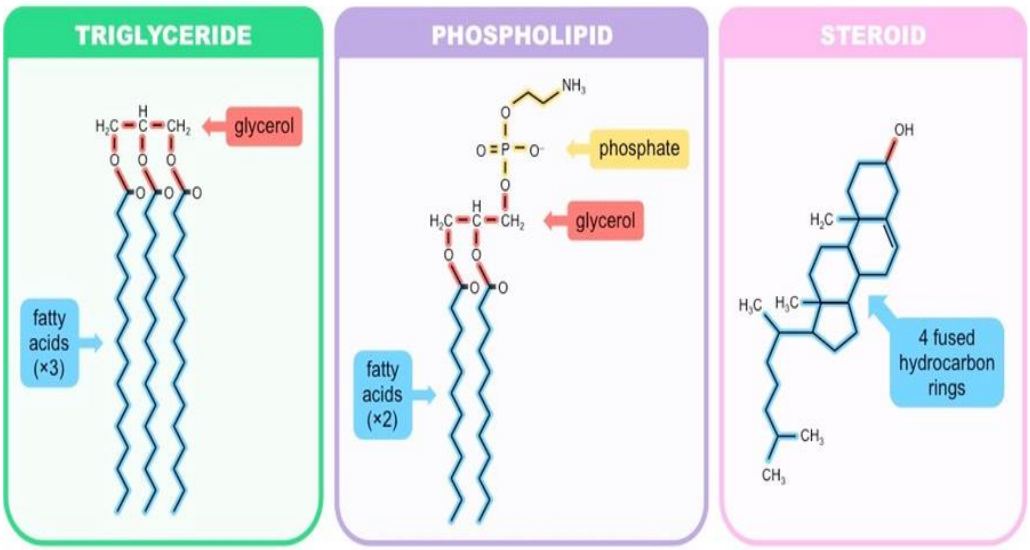


Figure 3. Types of Lipids (https://ib.bioninja.com.au/standard-level/topic-2-molecular-biology/21-molecules-to-metabolism/organic-polymers.html)

Lipids are crucial for cell survival in part because of their role in the cell membrane, a structural component that surrounds the cell whose proteins traffic what enters and exits the cell. Cell membranes follow the fluid mosaic model to be functional, which characterizes a fluid lipid bilayer membrane with proteins embedded within the membrane (Fig. 5). Around fifty percent of the membrane is made up of phospholipids, which have hydrophilic head groups that face the outside of the membrane and hydrophobic fatty acid tails that face inside the membrane. If the tails lack double bonds, they are referred to as saturated and, therefore, less likely to move. When fatty tails have double bonds, they are called unsaturated (Fig. 4).

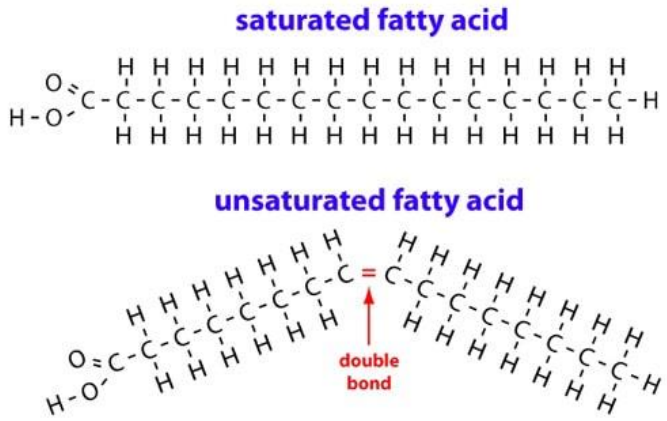


Figure 4. Saturation (top) and Unsaturation (bottom) of a Fatty Acid (https://www.healthline.com/nutrition/saturated-fat-good-or-bad)

Algal lipids have a higher degree of unsaturation than plant lipids which allows the algal membrane to be more fluid (Kumari et al. 2013).

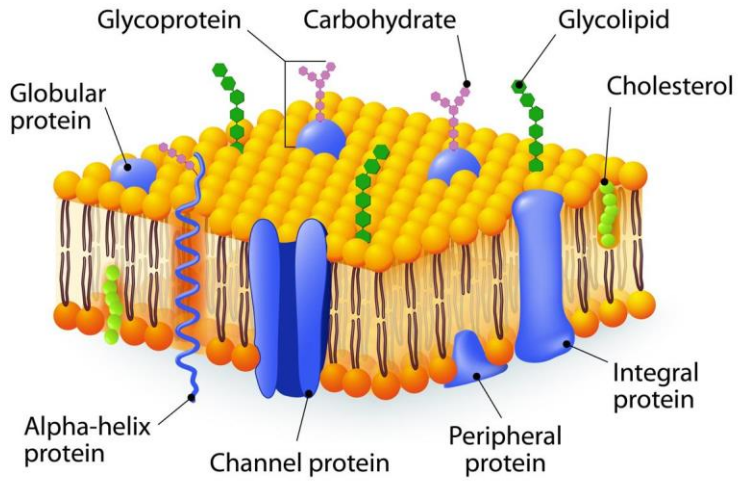


Figure 5. Fluid Mosaic Model of the Cell Membrane (https://biologywise.com/fluid-mosaic-model)

The fluidity and mobility of the membrane depends on the lipid composition which can vary based on temperature. Colder temperatures trigger the cell to "swap" saturated fatty acids with unsaturated fatty acids so that the cell membrane remains fluid and still retains its function (Alberts et al. 2002). The thickness of the membrane varies according to the saturation and length of the fatty acid chains. Longer fatty acids are harder to move than shorter fatty acids. Overall, the lipid bilayers are asymmetric and dynamic (Pratt and Cornely 2014). My project aims to address how to find an alternative biofuel to fossil fuels using microalgae by exploring the lipid content in selected algal strains.

**Why use Algae?**

The global supply of crude oil, otherwise known as petroleum, which is extracted from ancient algal, plant, and animal deposits will be exhausted by 2050. Thus, the search for an alternative to fossil fuels has recently started to gain momentum with biodiesel, which has become a central focus for many researchers (Hannon et al. 2010). Biodiesel is a preferable form

of energy because even though burning the hydrocarbons from biomass releases greenhouse gases, plants can remove carbon dioxide from the atmosphere and convert it into glucose through a renewable cycle. Scientists have subjected terrestrial plants to biofuel experimentation but an economic debate over whether crops should be utilized for fuel or human/livestock consumption has stifled much of the research. Another area of controversy has centered on whether the land that could be used to grow crops should be designated for biofuel cultivation. Additionally, growing the number of crops needed to be converted into fuel would be an expensive and resource-depleting endeavor. So, researchers have turned to a more promising source for biofuels: algae (Mondal et al. 2017).

Algae have an array of benefits that make them more suitable for biofuel production than plants. They are adaptable because they can be grown in almost any environment, including aquatic environments. Unlike plants, algae can produce a lot of biomass in relatively little time which from an economic standpoint generates more yield in less time. Growing algae also offers less risk of excess fertilizer runoff. With greater diversity and longer ancestry than plants, algae offer more strains or species for production. Like plants, algae can also be bioengineered thus making them the more agile candidate for conducting research. Algae can also be grown on land and they can remove toxins from water if they are cultured aquatically (Hannon et al. 2010). Both plants and algae are autotrophic, which means that algae can convert carbon dioxide into other biological compounds that are essential to the cell through the process of photosynthesis. By removing carbon dioxide from the atmosphere, the algae can reduce existing carbon dioxide concentrations (Fischer 2015). The removed carbon dioxide is then converted into either lipids, carbohydrates, or proteins in the algae (Mondal et al. 2017). Despite their promising nature and

importance to global ecology, algae are greatly understudied organisms that are starting to garner the attention of scientists.

**Approaches to algal research**

Biofuel can be made from algae either through bioethanol production or through transesterification. Bioethanol involves the alcoholic fermentation of usually simple carbohydrates like sucrose from algal biomass. Generally, this process is conducted on crops like sugar cane, corn, and wheat. The sugar is extracted from the biomass and mixed with yeasts and nutrients that promote sugar fermentation. After fermentation, the hydrolysate containing the fermented products are then distilled and dehydrated to form anhydrous ethanol (Gnansounou and Dauriat 2005). There has been growing concern regarding the ethics of growing crops for bioethanol that are also used for food. The alternative method for fuel is transesterification, also known as biodiesel. Researchers expose a specific class of lipid called triglycerides to an alcohol in the presence of a catalyst to make fatty acid methyl esters. Triglycerides are generally favored for these reactions over types of lipids due to their high fatty acid content. The triglycerides are then converted into diglycerides and monoglycerides. The monoglycerides are split into esters and a single glycerol molecule. Fatty acid methyl esters, abbreviated as FAME, are the final product that is used for energy (Mondal et al. 2017). In algal research, scientists are aiming to determine what conditions best optimize algal growth and lipid content. They have determined that the main factors of algal growth are light and the amount of nutrients the algae receives. Lipid content is now tracked through algal databased collections of lipids called lipidomes so that researchers can improve biofuels. The frontiers of bioengineering have allowed scientists to genetically modify algal strains to create a stronger strain (Hannon et al. 2010).

**Freezing stress**

Many researchers have conducted research to test how well specific species of algae respond when exposed to certain environmental stresses. Experiments with plants can also reveal something about algae since algae, specifically green algae, and plants respond similarly to environmental stresses. Wang et al. conducted a study where *Arabidopsis thaliana*, the "model" plant, was exposed to freezing treatments at various cold temperatures to test how the plant adapted to the change in temperature. Lipids were extracted from the plants and analyzed using Electrospray Ionization Mass Spectrometry (ESI-MS/MS). Their results indicated that when the plant was in a cold environment there was a drastic change in the amount of cell membrane lipids and that enzymes like phospholipases were activated. The increase in unsaturated fatty acids and glycerolipids led to the conclusion that lipid metabolic pathways adapted to desaturate the membrane. Desaturation is the process of adding more lipids with fatty acid tails that have more double bonds and therefore can move easily (Wang et al. 2006).

In another study, Valledor et al. exposed a species of green algae called *Chlamydomonas reinhardtii* to cold temperatures to analyze changes in protein, carbohydrate, and lipid composition. The results indicated that total lipid content was reduced but more fatty acids were unsaturated after cold exposure. As in the previous study, the researchers attributed increased fatty acid desaturation to the algal cells' adaptation during the experiment. They found a noticeable increase in the enzyme acyl-CoA thioesterase and acyl-CoA transferase which promoted fatty acid synthesis. Their adaptation, termed "reprogramming", resulted in an increase of C16:2 and C18:2 fatty acids, both of which are unsaturated, and a decrease in long, saturated fatty acids like C18:0. The researchers also proposed that the increased desaturation was necessary for the cell membrane to remain fluid in cold temperatures, since the function of the

membrane depends on its fluidity (Valledor et al. 2013). Moreover, polyunsaturated fatty acids have other economical promise, being a desirable commodity in nutraceutics, which are products with physiological benefits that can be alternatives to drugs and medications.

**Different Media**

Another approach researchers have taken to understand how algal lipids and algal growth rates are affected is to grow algae in different media. Chia et al. tested the changes in biochemical composition and growth rate in the algae *Chlorella vulgaris* when it was grown on semi-continuous cultures of Chu 10, WC, or LC Oligo media. These media have varying amounts of nutrients like nitrogen and phosphorus that can stimulate algal growth. In another experiment by Chia, the WC media had a higher concentration of $K_2HPO_4$ than the other two media and the LC Oligo media was made of more nitrates than the other media (Chia 2012). After extracting the lipids and analyzing them through an Iatroscan TLC-FID MK6S chromatographic analysis system, the results showed no significant difference in lipid class among the three medias. Examination of the lipid composition showed that acetone mobile polar lipids (AMPL) and phospholipids (PL) were the most abundant types of lipids in the Chu 10 media. This led them to conclude that an abundance of polar lipids contributes to the production of polyunsaturated fatty acids, which promotes algal growth. Total lipid production was lowest in the WC media which could be attributed to the high nitrogen to phosphorus ratio in the WC media in comparison to the Chu 10 and LC Oligo medias. The algae grown in LC Oligo had a significantly higher growth rate than cells grown in the other two medias (Chia et al. 2013).

A similar experimental method was used in another study conducted by Hala Yassin El-Kassas. The microalga *Picochlorum* sp. was grown in normal and nutrient stressed conditions for

nitrogen and phosphorus to determine the growth rate and the fatty acid profile. Just like the protocols of previous research, the lipids were extracted and analyzed with gas chromatography/ mass spectrometry (GC/MS). It was determined that nitrogen and phosphorus deficient media stimulated algal growth and increased algal lipid content. The fatty acid profile revealed that the lipid content after nutrient treatments was comprised of polyunsaturated and monounsaturated fatty acids, indicating that *Picochlorum* sp. could be a viable biodiesel candidate (Yassin El-Kassas 2013).

**Polar Algae**

Although much of researchers' attention is on aquatic microalgae, terrestrial polar algae have some interesting qualities that make them suitable for cultivation. This type of algae can survive in seemingly inhospitably cold temperatures. Their environment, which in the northern hemisphere spans northern regions of Europe, Asia, and North America, and in the southern hemisphere Antarctica, is mostly covered in sea-ice. The rough permafrost soil, characteristic of the polar climate, contains these algae which have adapted to the harsh conditions. The polar regions pose solar, nutrient, and osmotic problems for the algae, but the algae have evolved adaptations to survive amidst such conditions. Polar algae can produce greater amounts of polyunsaturated fatty acids to protect the cells from freezing damage. In cold temperatures, these algae increase the amount of fatty acids with unsaturated bonds so the cell membrane can remain fluid and functional (Lyon and Mock 2014). In one study, two species of the Antarctic green alga *Stichococcus* were isolated and grown at various temperatures to observe its response. It was determined that a lot of unsaturated fatty acids were found when the strains were grown at 4-15℃. However, the researchers concluded that the strains' cold adaptation was not limited to

fatty acid unsaturation due to the increase in $\alpha$-linolenic acid (C18:3 n-3) from 4 to 15 °C (Chen et al. 2011).

**Desert Algae**

While researchers have uncovered some of the adaptive capabilities of polar algae, some have taken a different route altogether and have uncovered the potential of desert algae. The desert climate is in some ways the opposite extreme to the polar climate. The lack of rainfall and high evaporation levels contribute to the dry, hot temperatures commonly associated with the desert. This arid climate can also experience cold temperatures during the night. The biodiversity of the desert has adapted to these harsh conditions. For example, desert plants like cacti store water for long periods during the drought season (WWF 2019). Many researchers have discovered terrestrial green algae within the desert soil crusts. Two researchers isolated and phylogenetically categorized desert algae classified within the *Scenedesmus* genus from microbiotic desert crusts. They determined that the algae were phylogenetically diverse and morphologically similar. The researchers also obtained sequence data that separated the algae *Scenedesmus obliquus* into 2 distinct groups called clades (Lewis and Flechtner 2004). This shows that the desert algae are evolutionarily diverse despite inhabiting a difficult environment. Several studies have indicated that the algae in desert soil crusts belong to the Chlorophyceae and Trebouxiophyceae classes of green algae. In isolating the algae *Pleurastrosarcina terriformae* from the soil crusts of the Atacama Desert in Chile, the researchers documented the conditions of the environment. The desert's air humidity was 60-70% and 80-85% during the day and night respectively, which indicated that the algae most likely their water uptake from the air over actual precipitation which was rare and less than 13 mm (Darienko et al. 2019). In another

study, researchers determined that the desert algal isolates from southwestern U.S desert soil crusts used in the Lewis and Lewis 2005 study and Lewis and Flechtner 2004 study survived desiccation for 4 weeks after being dried in the dark and light. Additionally, they found that after one hour of rehydration, the desert algae were able to restart photosynthesis quickly after rehydration, quicker than their aquatic relatives. This research demonstrates how desert algae are physiologically capable of enduring the dry, desert climate because the algae may potentially protect their photosynthetic structures from drying out. Additionally, the photosensitivity of the desert algae indicated that they potentially inhabit protective microsites within the crusts that slightly differ from their environment (Gray et al. 2007).

**My Approach**

Building upon previous research, the experiments that were performed examined both growth rate and lipid content and composition in algae. The proposed approach modeled the experiments conducted by Yassin El-Kassas. Two different experiments were conducted: algae were 1) grown in different growth media of increasing nutrient concentration and 2) exposed to freezing temperatures and then grown at normal conditions and exposed to a freezing and nutrient stress simultaneously.

This research used different strains found in polar and desert regions of the world that are within one species of algae called *Bracteacoccus bullatus*. The genus *Bracteococcus* is a green, spherical coccoid algae that can be around 3-24 μm in diameter and can have many nuclei and plastids. Algae within the *Bracteococcus* genus reproduce asexually using zoospores. *Bracteacoccus bullatus* share the same characteristics of its genus. It has a thin cell wall and the algal cells are characterized by a lateral bulge, hence its name. Their plastids lack pyrenoids, or

compartments, and older cells have an orange pigment. These algae are found in a variety of environments, including polar and desert regions (Fučíková et al. 2013). Because they adapted to specific climates, desert and polar algae strains have evolved different local adaptations, but in this case are still members of the same species. Growing these five strains in different enriched media and growing them after a freezing treatment should present some insight on how nutrient abundance and freezing affect algae of different climates. By using both types of strains, I hope to show which ones are more productive to help identify which kinds of algae can be utilized for biofuel.

## Methods:

### Algal Maintenance:

Research was conducted on strains of the green alga *Bracteococcus bullatus*. The polar strains were KF72, KF69, KF80 (all from northern latitudes). The desert strains were BCP-CNP3-VF20 and BCP-UT826. KF72, KF69, and KF80 originated from northern Siberia, Svalbard (Spitsbergen), and northern Canada respectively. The polar environment, from which the strains were collected, is dry and cold. BCP-CNP3-VF20 and BCP-UT8-26 were isolated from desert soil crusts in Utah, USA. BCP-CNP3-VF20 was specifically found in the Island-in-the-Sky District of Utah, which is sandstone-based (Island in the Sky 2019).The desert from which these algal strains were isolated experiences high temperatures during the day, colder temperatures at night, and low yearly precipitation. While all the strains are of the same species, there is some genetic variation among them, as assessed previously by Fučíková et al. (2013) using the *rbc*L and ITS genes. The polar strains are all very closely related to each other despite their different locations. BCP-CNP3-VF20 and BCP-UT8-26 are not as closely related (Fig. 6).

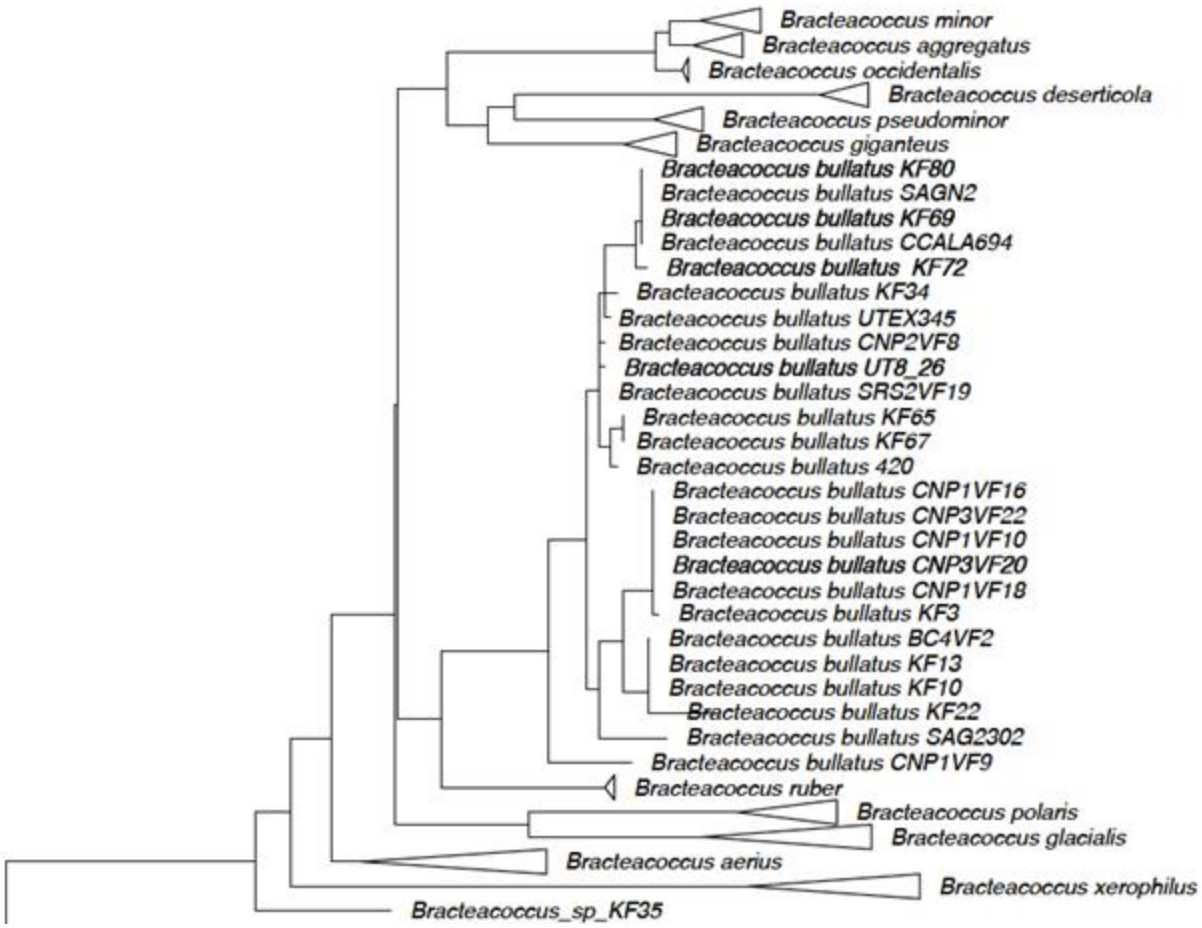


Figure 6. Phylogenetic tree based on rbcL data from Fučíková et al. 2013. Triangles represent groups of 2 or more strains of the same species. The strains that were used are in bold.

The algae were cultured on solid and liquid growth media (Bischoff & Bold 1963, Kan & Pan 2010). When growing, the algae were placed in a GrowLab growth rig with 40W fluorescent lights at 15°C and 2000 to 4000 lux until they were extracted for their biomass. The rig followed a 12:12 light and dark cycle.

**Freezing/Nutrient Enrichment Stress Experiment:**

250 ml of solid BBM media with carbendazim (control media) and 250 ml of solid BBM media with carbendazim and goat manure extract (enriched media) were prepared. BBM media with carbendazim was made with of $NaNO_3$, $MgSO_4$, $7H_2O$, NaCl, $K_2HPO_4$, $KH_2PO_4$, $CaCl_2$, $2H_2O$, in concentrations specified by Bischoff & Bold (1963), as well as 0.01 g of carbendazim (Kan & Pan 2010). BBM media with goat extract and carbendazim was made with the same amounts of supplements, except 25 ml of goat manure were added to the media, and the final volume was adjusted to the total 250ml. After both batches of media were autoclaved, ampicillin and cefotaxime were added to the media to prevent bacterial contamination (Kan and Pan 2010). Each of the five strains were inoculated onto 2 BBM plates and 2 BBM with goat manure plates, for a total of 20 plates. The duplicates of each type of media (10 plates) were placed in Ziploc bags and put in a freezer at -20 °C for 2 hours post-inoculation. The remaining 10 plates were immediately placed under the growth lights. After 2 hours, the 10 duplicate plates were removed from the freezer and the Ziploc bags and placed under the growth chamber. After the algae reached substantial growth on the plates, which took about 2 to 3 weeks (depending on the strain), the algal biomass was harvested, and its lipids were extracted and transesterified according to a standard NREL protocol (Wychen et al. 2015).

The same procedure was repeated with normal BBM agar plates without goat manure enrichment that were grown in triplicate to test the freezing effect only.

0.015 grams or higher of wet algal biomass was harvested and suspended in GC vials with methanol. The vials were placed in a glass desiccator overnight with their caps off with drierite as the desiccant. After weighing the vials for their dry mass (optimum dry mass above 5 mg), transesterification was conducted using 2:1 $CHCl_3$: Methanol, 0.6M HCl: methanol, and

C13:0 methyl ester internal standard. After being heated at 85°C for an hour on a hot plate, hexane was added to each vial (Wychen et al. 2015).

The same procedure was conducted for the second freezing experiment except the vials were sealed with parafilm before heating to prevent evaporation of the reagents and burning the lipids. Additionally, the vials were heated at the same temperature and time in a water bath instead of a hot plate.

All lipid analysis was quantitated by Varian CP-3800 Gas Chromatography and Saturn 2000 Mass Spectrometry. The GC analysis was conducted using a Phenomenex ZB-WAX capillary column. The lipid content,composition, and saturation indexes were graphed in Excel. The percent total lipid content of the samples was calculated using the initial dry mass of the algae after they were desiccated. Based on total lipid content, estimated percent composition of various fatty acids were determined. The equation used to calculate unsaturation index (∑ (amount of each unsaturated fatty acid x number of double bonds in fatty acid chain)) was modified from the equation used in Saber et al. (2018).

**Growth Rate Experiment:**

The listed algal species were transferred from media slants with inoculation loops and suspended in eppendorfs of 500 µl of sterile water. The average number of cells was determined using a hemocytometer for each algal strain, which was used to make stock solutions with sterile water from the original suspended cell solution. After each strain's stock solution was prepared, 100 µl of each stock solution were pipetted into 25 ml of liquid BBM media and 25 ml of liquid BBM with goat manure extract in T-25 flasks. UT8-26 liquid BBM media was inoculated with 200 µl of its stock solution due to low cell concentration in the stock. Liquid BBM and BBM

with goat manure extract media were made with the same components as the solid media without the agar. The flasks were placed under a growth rig with Sylvania F40/GRO/AQ lights at 2000 to 4000 lux and their cell concentration was measured using a hemocytometer every two days for about a month. The cell concentrations over the course of 36 days were graphed in Excel and the growth rates for each strain were determined using the slope of the trendline for each strain's media. The growth rates were individually graphed according to each strain and their type of media.

**Results:**

Growth rate was measured over the course of 36 days using a hemocytometer to count the number of cells thrice each week. The polar strains (KF72, KF80, KF69) grew faster with goat manure extract (GME) enrichment, but the desert strains (BCP and UT8) responded negatively to the enrichment. On average, there was a 190,846 difference in cells/ml between the desert and polar strains with nutrient enrichment. Instead, the desert strains grew faster with normal BBM media (Fig.7).

Lipid content and composition of the algae was quantified by GC/MS analysis after the lipids were chemically extracted and transesterified. The freezing stress affected each strain's total lipid content more than the goat manure enrichment stress as demonstrated by the decreased lipid content in KF80, KF72, and BCP and increased lipid content in UT8. The polar strains have three times as much C18:3 n-3 under each treatment than the desert strains (Fig. 8).

In the second experiment where only the effect of freezing was tested, the freezing stress increased total lipid content in the desert strains and decreased total lipid content in the polar strains. The average total lipid content for the desert strains was 2.8% and 2% for the polar

strains, which was a 0.8% decrease. When they were not frozen, the total lipid content for the desert and polar strains were 1.15% and 5.8% respectively (Fig. 9). Additionally, freezing overall increased the unsaturation indexes of all the strains by about 1.49, except for KF69. The desert strains had overall lower indexes than the polar strains. The average unsaturation indexes of the desert strains were 1.18 frozen and 0.96 not frozen while the average indexes of the polar strains were 1.67 frozen and 1.58 not frozen. BCP had the greatest difference in index from freezing and no freezing (Fig. 10). The polar strains had a different fatty acid composition than the desert strains. There was more C18:3 n-3 in the polar strains and more C18:1 cis-9 in the desert strains. On average, the polar strains were 40.17% C18:3 n-3 and the desert strains were 12.25% C18:3 n-3 for both treatments. The desert strains were about 13.5% C18:1 cis-9 and the polar strains were 3.33% C18:1 cis-9. The desert strain lipid composition varied more between each other than the polar strains. Generally, lipid composition in all the strains did not change with the freezing stress with regard to the top five lipids, except in BCP. Even though the proportions of these fatty acids varied between strain and treatment, C18:1 cis-9 (oleic acid), C18:1 trans-11 (vaccenic acid), C18:3 n-3 (alpha-linolenic acid), C18:2 (linoleic acid), and C16:0 (palmitic acid) were consistently present in most of the strains (Fig. 11).

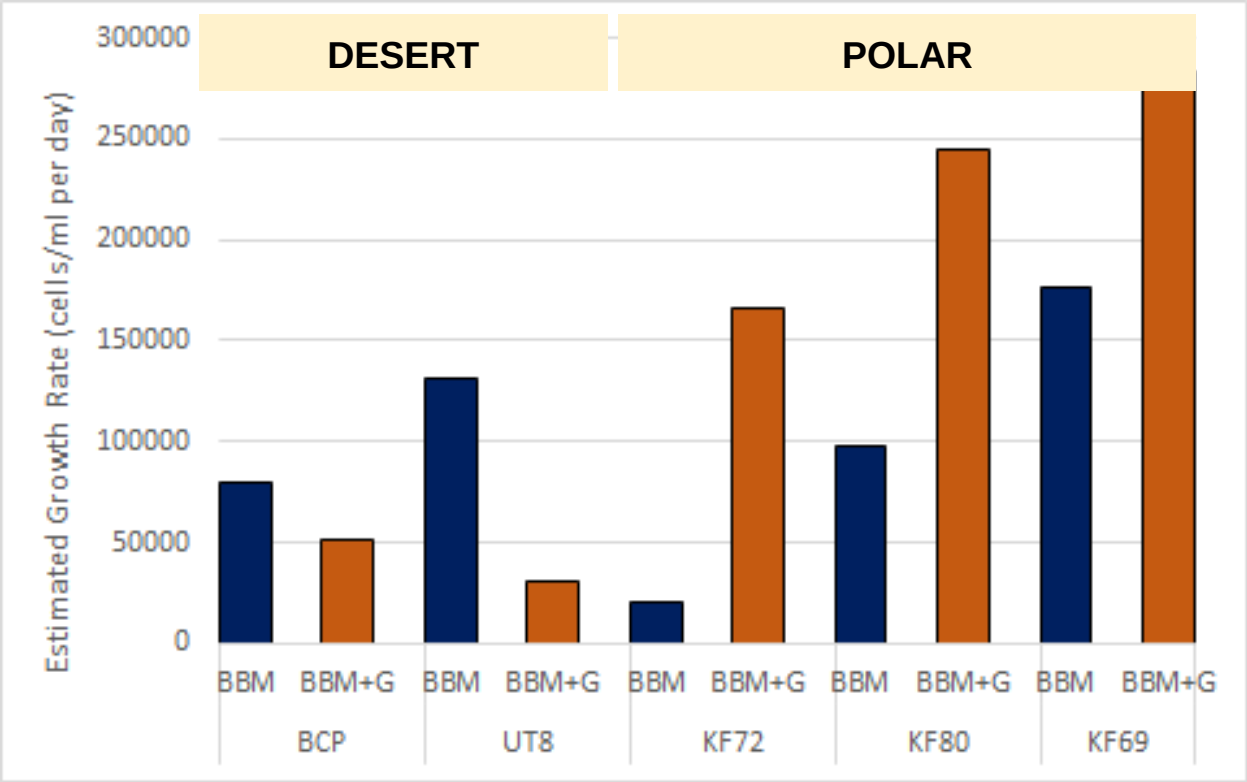


Figure 7. Growth rates of the 5 strains of *Bracteococcus bullatus* in normal liquid BBM media (BBM) and liquid BBM media with goat manure (BBM+G) after 36 days assuming linear growth rate. BCP and UT8 are desert strains and KF72, KF80, and KF69 are polar strains.

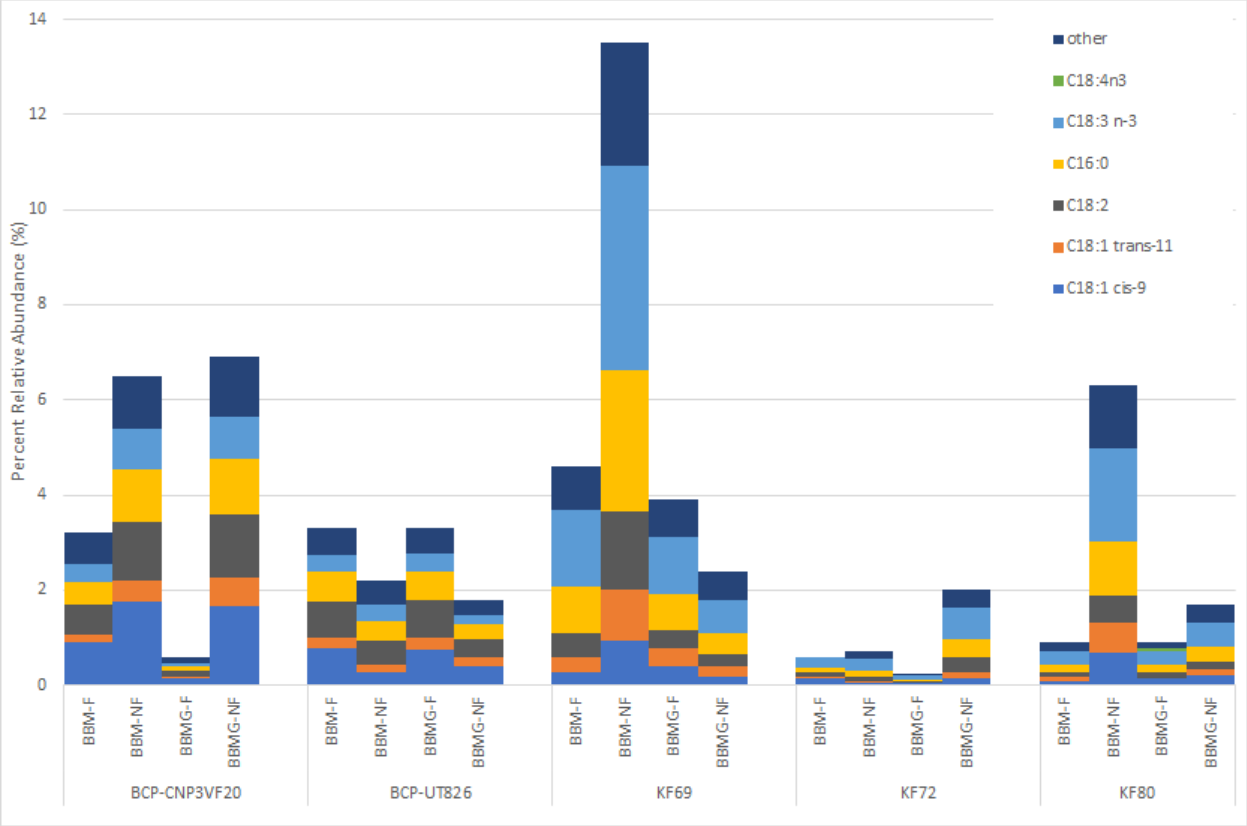


Figure 8. Total lipid content and composition found in each strain under different growth regimes. BBM was the standard growth media – agarized Bold's Basal Medium (Bischoff & Bold 1963); BBMG stands for growth media enriched with goat manure extract. F stands for initial freezing treatment of freshly plated cultures; NF stands for not-frozen – control treatment without freezing.

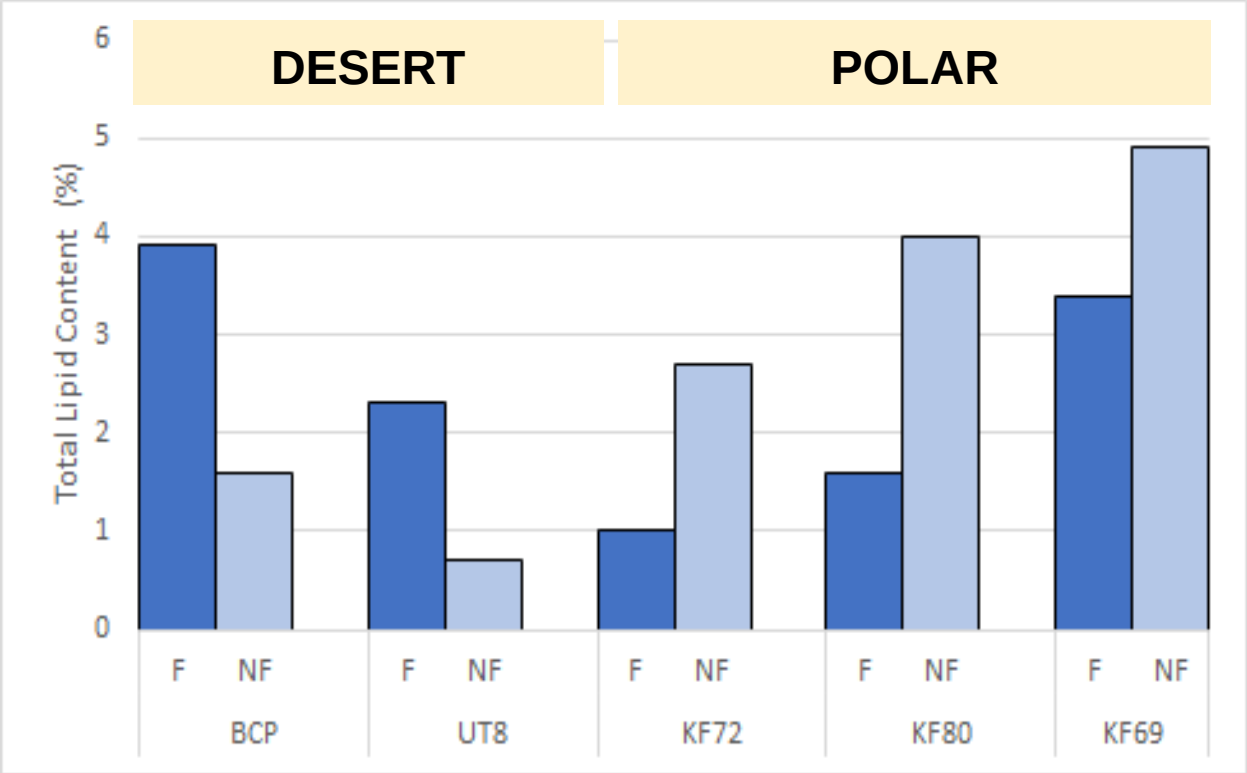


Figure 9. Total lipid content of the five strains after the initial two hour exposure to freezing temperatures (F) compared to the control non-freezing (NF) treatment based on percent dry mass. All strains were subsequently grown for 3 weeks, regardless of freezing treatment. BCP and UT8 are desert strains and KF72, KF80, and KF69 are polar strains.

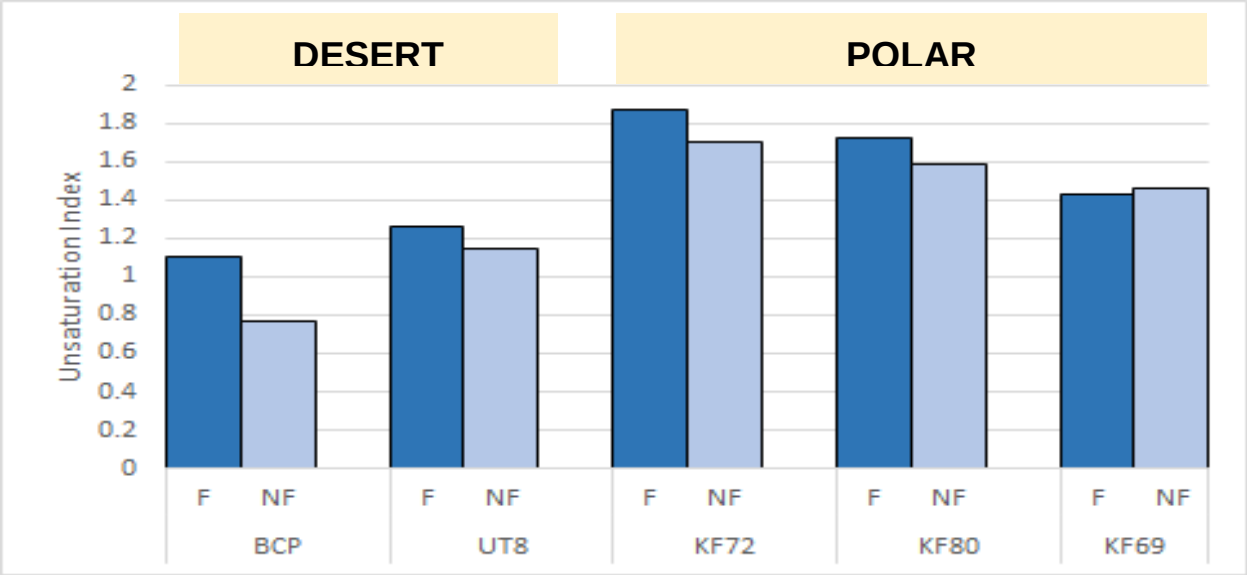


Figure 10. Unsaturation indices of the 5 strains grown after initial two hour exposure to freezing temperatures (F) compared to the control (non-freezing, NF) treatment. BCP and UT8 are desert strains and KF72, KF80, and KF69 are polar strains.

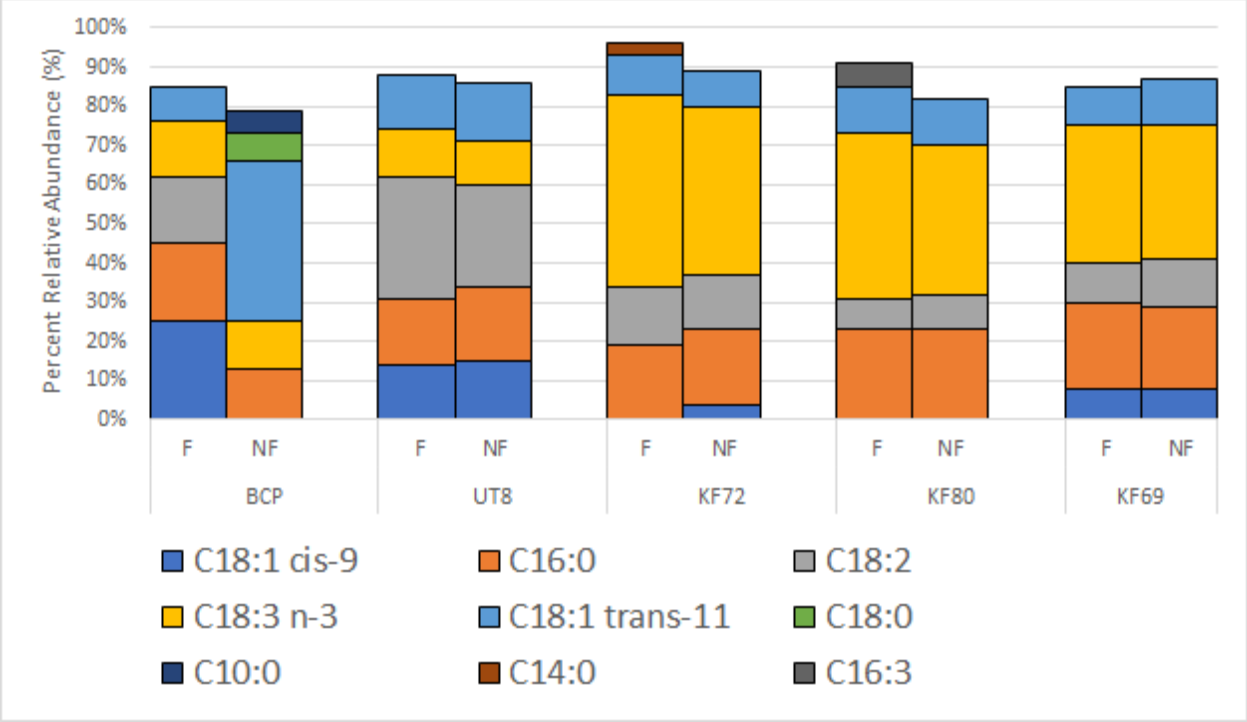


Figure 11. Relative abundances of the most common lipids found in each strain grown after initial two hour exposure to freezing temperatures (F), compared to control (non-freezing, NF) treatment. BCP and UT8 are desert strains and KF72, KF80, and KF69 are polar strains.

**Discussion:**

The goal of this study was to determine how algae of the same species from different environments respond to temperature and nutrient stresses by analyzing their variations in growth rate and lipid content and composition.

In the growth rate experiment, the polar algae responded better to the goat-manure enrichment as demonstrated by their higher growth rates when compared to the desert strains. Goat-manure was an added source of nitrogen and phosphorus, two nutrients that generally stimulate algal growth, as well as other possible micronutrients, minerals, and vitamins. The desert strains thrived on minimal nutrient enrichment. The results indicate that the polar algae's response is opposite to that of the desert algae. Similar to the desert algae, the green alga *Picochlorum* was grown in media with varying $NaNO_3$ concentrations. The study had shown that biomass and growth rate were negatively affected in increased nitrate concentrations (Yassin El-Kassas 2013).The fact that both the desert strains and *Picochlorum* could not thrive in nitrate-rich environments indicated that their original environments may not be that heavy in those nutrients. The desert algae's response differed from *Chlorella vulgaris* of the Chia et. al study when grown in nutrient-rich media, which experienced a higher growth rate in the nitrate-rich LC Oligo media. The high cell density of *Chlorella vulgaris* for that treatment also demonstrated that the nitrate heavy media stimulated biomass production which is a good indicator of growth (Chia et al. 2013). The difference could be attributed to different locations from which both algae were originally isolated. The desert environment is extremely harsh and characterized by a semiarid climate. The soil crusts from which the desert strains were isolated from is often desiccated, irradiated by the sun's rays, and water deficient. These desert strains' crusts, particularly soil crusts of Southwest United States, have high organic carbon and organic

nitrogen content. The desert species of genus *Bracteococcus* have been isolated from diaphanous substrata of the soil crusts that have scatterings of quartz and chalcedony minerals so nitrogen may be lacking in the desert strains' specific environment. Other organisms like nematodes and mites live in the same environment so they could potentially compete for nutrients and resources like the algae. Since nitrates and phosphorus compounds are inorganic, these algae may grow by utilizing the organic content more than the inorganic components in the crusts, which indicates that they could be mixotrophic (Cameron and Blank 1966).

Opposite to the desert algae, the polar strains acted like *Chlorella vulgaris* from the Chia et al. study. The higher growth rates of the polar strains could be attributed to the algae's ability to utilize the nutrients to bolster growth. Polar algae especially rely on carbon and other nutrients whether the algae are embedded with sea ice or not (Hopes and Mock 2015). The results of the polar algae growth rates are comparable to those of another study involving arctic algae isolated from the Resolute Passage in Canada. The researchers in that study exposed the algae to different media with added nitrogen, phosphorus, and silicon separately and combined. They determined that nitrogen was the limiting reactant for the algal growth and biomass as they observed a drastic increase in biomass from the nitrogen enrichment (Smith et al. 1995). The reason that polar algae seem to respond positively to higher nitrogen concentrations could stem from the algae's ability to utilize nutrients they have already been exposed to previously. Their environment can be heavy in nitrate, phosphate, and silica concentrations to maintain certain metabolic processes (Hopes and Mock 2015). The soil crusts of Svalbard, the location of the KF69 strain, are high in carbon and nitrogen which contributes to the acidity of the soil so the algae are acclimated to these harsh conditions (Borchhardt et al. 2017). Overall, the polar strains were potentially more evolutionarily equipped to handle a higher nutrient environment than the

desert strains given the conditions they were originally exposed to. Another explanation to these results could stem from how the light from the growth rig was potentially scattered by the color of each media, since the BBM media was clear while the BBM+G media was brown and cloudy.

After freezing and growing the algal strains on different enriched media simultaneously in the first lipid experiment, it was determined that freezing affected the strains' total lipid content more than the goat manure enrichment. When the second experiment was conducted to further test the effect of the freezing stress alone, there was an increase in total lipid content in the desert strains and a decrease in total lipid content in the polar strains. This result seems to contradict the results found in the Cid-Aguero et al study concerning the polar algae. They also found a high total lipid content in Antarctic algae *Chlamydomonas* sp. after it was grown at lower temperatures of 12 ± 2℃ (Cid-Aguero et al. 2017). The polar strains in this experiment may be better acclimated to the freezing stress than the desert strains so they may not have to overcompensate and expend more energy to make more lipids with unsaturated fatty acids. The polar strains also could be degrading some of the lipids with longer or saturated fatty acid chains in response to freezing. This degradation occurs from enzymes, like phospholipases that cleave lipids at specific sites to render them inefficient (Tan and Lee 2016). The desert strains, which are not usually exposed to freezing conditions, may have increased their unsaturated lipid content as a stress response, which in turn increases total lipid content. Differences in total lipid content between the two types of strains can stem from their evolution to have different physiological adaptations to freezing. Desert algae are typically exposed to stresses in the form of high temperatures, low nutrients, UV radiation, and high pH so they would not be as equipped to adapt as quickly the freezing stress as the polar algae (Perera et al. 2018). The unsaturation indexes increased in all the strains after freezing, with higher unsaturation indexes for the polar

strains when compared to the desert strains. The polar and desert strains may have produced more unsaturated lipids but the polar strains are adapted to a colder climate so they would make more unsaturated lipids. These results coincide with how the cell membrane maintains its fluidity and mobility through unsaturation. Unsaturated fatty acids ensure membrane fluidity at colder temperatures because the presence of double bonds in the fatty acid chains make the lipid more fluid at lower temperatures. That in turn allows the membrane to remain fluid, flexible, and functional to the cell at low temperatures (Pratt and Cornely 2014). Oleic acid (C18:1 cis-9), palmitic acid (C16:0), α-linolenic acid (C18:3 n-3), linoleic acid (C18:2), and vaccenic acid (C18:1 trans-11) were the top five most abundant fatty acids in both the polar and desert strains. Phosphotidylglycerols, a type of phospholipid found predominantly in green algal cell membranes, have high amounts of *n*-3 fatty acids like alpha-linolenic acid. Phospholipids also tend to have a high oleic and palmitic acid content to ensure membrane structural integrity and fluidity (Kumari et al. 2013).

Two potential explanations for the results of the *Bracteococcus* strains' responses to freezing are natural selection and epigenetic modifications. All the algae that were analyzed for both the first and second freezing experiment were the descendants of the cells (parental generation) that were exposed to the freezing stress. In other words, the lipid profiles in figures 7-11 represent descendants of the stressed cells, not the stressed cells themselves.

If epigenetics were affecting the response, the algae of the parental generation that were subjected to a freezing stress responded by methylating their genes, which turned certain genes off. The descendants that were used in the experiments had this epigenetic response passed down in order to survive when frozen. On the contrary, if natural selection was the root cause of the results, then some cells in the parental generation already possessed genetic mutations that were

selected for to survive over the algae that did not have the genetic predisposition to survive against the freezing stress. The effects of epigenetics and selection were expanded upon in Kronholm et al in which the researchers exposed the four different strains of the algae *Chlamydomonas* to various stressed and control conditions and resequenced the genomes and methylomes to determine how epigenetics affected gene methylation in selection environments. They determined that the algae underwent epigenetic changes when grown in high NaCl and $CO_2$ rich environments to adapt to those conditions. They concluded that epigenetics plays a role in adaptation that depended on the environment that selects for certain adaptations (Kronholm et al. 2017).

For future experiments, the freezing stress could be repeated on solid BBM agar plates in triplicate to further support these current observations. Conducting a successful triplicate experiment would allow the patterns that were determined from the lipid experiments to be subject to statistical analysis. To determine whether the algae exhibit these same patterns at different growth phases, the freezing portion of the lipid experiment would be repeated post inoculation and after 2 weeks or when all the algae grew substantial biomass on the plates. The growth rate experiment could be conducted by first freezing the algae in the liquid BBM media and then re-exposing them to the normal growth conditions to determine how freezing affects growth. Another approach could be to grow the strains in a different media enriched with one nutrient like nitrates, phosphates, or calcium to determine the effects of specific nutrients on growth. Epigenetic and natural selection experiments could be modeled like those of the Kronholm et al. study. The strains would be grown in different conditions, freezing and no freezing. The not frozen batch of strains would act as controls that would be grown in the same lab environment. Then to grow new generations, the original algae that were frozen and not

frozen would be inoculated onto new plates for a designated period of time and rexposed to the freezing conditions (Kronholm et al. 2017). In addition to testing freezing, we could test the effect of heating on lipid content/composition as well as growth rate.

These results can help determine what specific conditions are necessary to produce a greater yield of lipids for biofuel. These algae can be manipulated using stresses to either grow faster and accumulate biomass in a short amount of time or to make more lipids that can be converted into fuel. This research also aimed to understand the biological and evolutionary background of these polar and desert algae in order to find which type could be a suitable candidate for algal farming. Information gathered from the study can be used to decide what media is needed to grow either type of algae on a mass scale. The polar and desert strains of the same species responded differently to different treatments. The results of the polar and desert algae demonstrate that there is a significant difference on what kind of algae one uses and what treatment it is exposed to. These same treatments can also be applied to other species of algae to compare which species are more productive at achieving both high growth rates and lipid content when stressed. The hope is that, with more funding and research dedicated to this discipline, we can find an alternative source of fuel that does not take up as many resources as plants. Growing plants requires time and land, both of which are not in abundance. The current efforts to use bioethanol from crops like corn has been met with debate because corn is used for food (Khan et al. 2018). Additionally, algal lipid composition has a considerable effect on the quality and characteristics of biodiesel. For example, saturated methyl esters have higher freezing points than unsaturated methyl esters. Unsaturated methyl esters have low ignition quality (cetane) as well as a lower fuel stability and higher nitrous oxide emissions than saturated methyl esters (Conley and

Tao 2006). While there are many challenges to overcome with research, algae is projected to become a promising alternative to fossil fuels in the near future.

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