

INVESTIGATING ALAKLI-TOLERANT MICROALGAL MONOCULTURES AND
POLYCULTURES FOR INDUSTRIAL CULTIVATION

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ABSTRACT

The use of algae to create high value products from the biomass grown is not a novel idea. However, problems with culture stability, productivity, and resource costs have limited the large-scale production of algae. The ability for growth in alkaline conditions allows for stability of the culture's pH and a more efficient capture of atmospheric CO₂. This thesis explores the use of high pH and high alkalinity tolerant algal strains both as monocultures and in polyculture combinations to produce lipids and other high value products. The algae used were a green alga (strain ALgE), a cyanobacterium (blue-green 'alga', strain SLcyaH) and a diatom (strain SLdC) that were obtained from a Soda Lake chain near Spokane, WA. The monocultures, two-member cocultures, and the three-member polyculture were compared for their product spectrum and productivities. While not as productive as monocultures, in every combination, algal polycultures incorporating the diatom SLdC produced similar amounts of lipids ($4.23 \times 10^{-2} \pm 1.84 \times 10^{-2}$ grams of lipids per liter per day) and biomass ($0.357 \pm 3.85 \times 10^{-2}$ grams of biomass per liter per day) as the monoculture of the diatom, while having lower input costs due to the lower amount of silica used in the media. Moreover, because the different organisms produce different lipids, the choice of strain or strain combination has the potential to affect the type of lipids produced. The diatom for instance produces high value polyunsaturated lipids while the green alga produces lipids more suitable for use in fuels, biodiesel, and foam production. The cyanobacterium does not produce many lipids but rather carbohydrates (sugars, starches, etc.). hence, the potential benefits of using polycultures are their culture stability and ability to affect lipid speciation with little to no loss in productivity.

CHAPTER ONE

INTRODUCTION

Take a deep breath and now thank algae as they produce roughly half of the world's breathable oxygen (Chapman, 2013). In addition to the oxygen, algae are also primary producers that act as a food source for larger organisms by performing photosynthesis and fixating CO₂ into longer carbon chain molecules (Häder, 2022). The compounds produced by algae can provide renewable sources of energy, bioproducts, and biobased chemicals (U.S. Department of Energy, 2016). For example, some algae can produce astaxanthin, an antioxidant in the food and drug industry (Yang, et al. 2021).

The use of algae presents challenges, one challenge being the limited biomass and product yields, due to shifting pH levels during the growth of algae (Yang, et al. 2021). Another problem that large scale algae farms face is the competition for land near a CO₂ source as the cost of compression and delivery of the gas can be 30 percent of the farm's operational cost (Quiroz, et al. 2023). A third problem is the inevitable contamination of industrial open-air algal ponds. These contaminants can be competitive, parasitic, or even predatory to the alga (McBride, et al. 2014).

A potential solution to all three of these problems is the cultivation of algae in growth medium with a high alkalinity buffer system. The pH levels of these media can be 10 or even higher, this provides an environmental selection gradient against many potential pests like algae grazers and other predators (Miller I., et al. 2024). The high pH also creates a natural sink that converts dissolved CO₂ or carbonic acid into bicarbonate and carbonate (**Figure 1**); bicarbonate is readily bioavailable for algal consumption (Vadlamani, et al. 2017). The solubility of

bicarbonate and carbonates is generally much greater than the solubility of CO_2 at atmospheric pressure, and bicarbonate appears to be as good as a source of inorganic carbon for photosynthesis as CO_2 (Vadlamani, et al. 2017). An equimolar solution of bicarbonate and carbonate will create a buffer system around the pK_a value of approximately 10.3. Such a buffer system can provide large amounts of inorganic carbon, a fairly stable pH, and improve the growth of algae tolerant to this pH level (Vadlamani, et al. 2017). As the dissolved inorganic carbon (DIC) is consumed, more CO_2 is drawn into solution and converted into carbonic acid or directly into bicarbonate - a process that is enhanced through the nucleophilic attack of hydroxyl ions (Pinsent and Roughton. 1951).

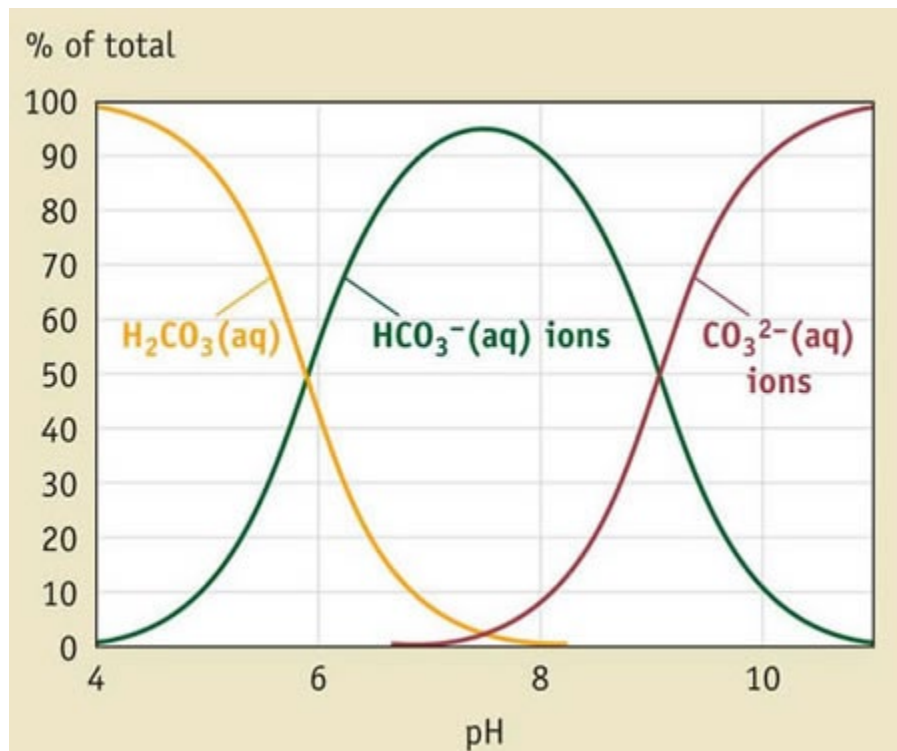


Figure 1: A graph showing the distribution of dissolved carbonate species across changing pH. Image from Shrestha et al. (2023).

In natural systems, high alkalinity bodies of water have been reported to have the most productive photosynthetic systems in any aquatic species (Melack, 1981; Lerman and Stumm,

1989; Sorokin et al, 2014). The species used in this work come from Soap Lake and Alkali Lake in Eastern Washington State, USA, near Spokane. These lakes have a pH ranging from 9.5 to 9.9 and an alkalinity equivalent measured in moles of calcium carbonate per liter of solution ranging from 9.4 to 177.3.

In this work, growth of alkali-tolerant algae was investigated at low and high alkalinity conditions to first describe differences observed in the algal species due to the different alkalinity levels. Then the growth of algal polycultures was compared with each other and their monoculture counterparts.

Background on Algal Kingdoms

The algae used in this work comes from two different Kingdoms of Eukarya and a Prokarya species. A green alga (Kingdom Plantae) referred to as species ALgE throughout this thesis, a diatom (Kingdom Chromista) referred to as species SLdC, and a cyanobacteria referred to as species SLcyaH. The next few paragraphs will provide some background information on each, followed by **Table 1** summarizing each of the species. The rationale for using the different species is that each species uses, or can use, different wavelengths of light from each other, and may fill different niches to better use the incoming energy from the light efficiently for their growth.

Cyanobacteria.

Cyanobacteria are the evolutionary oldest photosynthetic organisms. They are a gram-negative bacterium that contain photo-active pigments such as chlorophyll-a which all cyanobacteria have. They also have other pigments such as phycocyanin that give them a blue pigment and their common classification of blue-green algae (Whitton and Potts. 2012). Some species of cyanobacteria are

also able to fix nitrogen to make it bioavailable. These species are often associated with other species in a symbiotic relationship, providing the bioavailable nitrogen to the host. A common cyanobacterium that most people have interacted with unknowingly is *Arthrospira* commonly known as “Spirulina” that is used in dietary supplement (Whitton and Potts. 2012). Cyanobacteria also only accumulate roughly 5% of their biomass as lipids and instead accumulate more starch (Somrack et al. 2007).

Diatoms.

Diatoms are eukaryotic single cell photosynthetic organisms that unlike green algae, have a cell wall that is composed of amorphous silica (Bozarth, et al. 2009). They also uniquely have four membranes that separate their plastid from their cytoplasm (Gibbs, 1979). They are known for their accumulation of lipids primarily their polyunsaturated fatty acids such as omega-3s, commonly known as fish oil (Bozarth, et al. 2009). They were also first recorded by Antonie van Leeuwenhoek in 1703 when he called them “animalcule” meaning tiny animal (Williams and Kociolek. 2011). They also are referred to as brown algae due to the pigment fucoxanthin within their chloroplasts giving them a brown or yellow color (Kumar, et al. 2015).

Green Algae.

Green Algae are eukaryotic photosynthetic group of organisms that have a double membrane envelope around their stacked thylakoids. They contain pigments chlorophyll *a* and *b* and sometimes others depending on the species (Arora and Sahoo. 2015). They also have a cell wall made of cellulose like terrestrial plants (Lewis and McCourt. 2004) This class of algae is extremely diverse in the details of their structure and where they can grow. Their size can range from being microscopic to a meter at the largest (Lewis and McCourt. 2004). Their morphology can range from single unicells and colonies to branching and filaments, some motile and others not. The best way to classify greens is by if they grow planktonically (suspended in the water

column) or benthic (attached to a surface primarily) (Pröschold and Leliaert. 2007). An industrial use of a green algae is the growth of *Dunaliella salina* for beta-carotene, an orange dye and vitamin C supplement (Priyadarshani. and Rath, 2021).

Table 1: Summary of Different Algal Species

Species	Cyanobacteria	Diatom	Green
Pigment	phycocyanin	fucoxanthin	Chlorophyll
Wavelength of light used by pigment	Orange and Red 620 nm	Blue-green and yellow-green 510-525 nm	Blue and Red 430 nm and 662 nm 453 nm and 642 nm
Industrial Use Example	Food additive/supplement (protein)	Omega 3s	Food additive/supplement (vitamin C)

Huyen Bui and Sandra Rincon enriched 29 algae species from the Soap Lake region and determined their growth rate and biofuel production potential. From these species, three were selected for study within this work. Selections were made based on the highest lipid content and fastest growth rate for each of the three types of algae.

Overview of Chapters

Chapter Two will describe the materials and methods used throughout the study with subsections for abiotic and biotic analyses. Chapter Three investigates first monocultures of each species grown under high and low alkalinity, followed by the results of polyculture experiments. Chapter 4 provides the conclusions and summary of the results and suggests future research opportunities for the organisms used.

CHAPTER TWO

MATERIALS & METHODS

Media Used

The media used for these experiments was Bold's Basal Media or BBM (Deason and Bold. 1960) (175 mg/L KH_2PO_4 , 25 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 75 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 250 mg/L NaNO_3 , 75 mg/L K_2HPO_4 , 25 mg/L NaCl , 11.42 mg/L H_3BO_3 , 1 mL/L of a solution of 50 g/L Na_2EDTA and 3.1 g/L KOH , 1 mL/L of a solution of 4.98 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 1 mL/L concentrated H_2SO_4 , and 1 mL/L of a solution of 8.82 g/L of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.44 g/L of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 710 mg/L of MoO_3 , 1.57 g/L of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 490 mg/L of $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$). In addition to the standard media recipe, 1 mL/L of a 2 mg/L stock solution of Vitamin B12 and 1 mL/L of Vitamin S3 mix (500 mg/L myo-inositol, 300 mg/L Thymine, 50 mg/L of vitamin B1, 10 mg/L of niacin, 10 mg/L of calcium pantothenate, 1 mg/L of *p*-Aminobenzoic acid, 0.1 mg/L of vitamin H, and 0.2 mg/L of folic acid) was added. For high alkalinity media 4.2 g/L of NaHCO_3 and 5.3 g/L of Na_2CO_3 were added. For the media used to grow diatoms either 568.4 mg/L or 1.14 g/L of $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ was added for either 2 mM or 4 mM respectively.

Strains and Culturing Conditions

Cultures used were allowed to grow for at least two growth cycles in baffled 250 mL shake flasks with the experimental media condition before being transferred into 1.25 L tube reactors. A growth cycle is allowing the algae to grow until nitrate depletion before being transferred into fresh media of the same type. The tube reactors are glass 1.25L tubes with a

rubber stopper at the top that has a sample port, a glass pressure relief line, and a glass air sparge line that feeds humidified air with a pump at a rate of 0.4 liters per minute (see **Figure 2**).

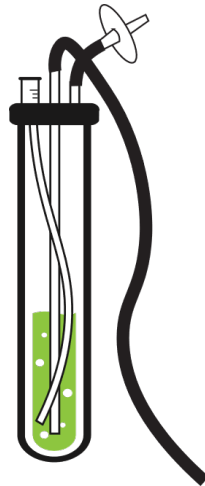


Figure 2: Graphic of tube reactor setup. Image created by Jill Story

The tubes and shake flasks were illuminated with T5 fluorescent lights at an intensity between 140 and 120 PPFD (photosynthetic photon flux density in units of $\mu\text{mol}_{\text{photons}} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) on a 14:10 light cycle. At the time of inoculation, the appropriate volume of starting culture to reach an initial cell density of 10^6 cells/mL was spun down at 847 rcf for ten minutes. The supernatant was discarded; the pellet was resuspended in fresh medium and added to the tube reactor.

Harvesting

At the end of the experiment the cultures were collected in 50 mL centrifuge tubes and spun down at 847 rcf for 10 minutes. Fifty (50) mL of the supernatant was stored for later use in other assays and the rest was discarded. The remaining pellets were lyophilized and stored at -20°C .

Culture Analysis

Microscopy direct counts

Direct counts were performed to estimate the cell concentration of algal cultures. A Bright-Line® hemocytometer with a 0.1 mm deep chamber was used, and cells were counted within the four 1 mm² corner regions of the grid until approximately 100 cells were counted. These regions are divided into a 4x4 grid of 0.25 mm squares. As cells were counted the number of grid squares were also counted. Using the ratio that 16 grid squares are equal to 10⁻⁴ mL the cell counts were converted into cells per mL.

Optical Density and Spectra

For optical density and spectra readings 200 µL of culture was pipetted into a 96 flat-bottom well plate and read on a plate reader at 750 nm and from 300 nm to 800 nm at 10 nm increments. The estimated path length for absorbance measurements was 0.565 cm.

Chlorophyll Measurements

1 mL of sample was spun at 3,000 x g for ten minutes. The supernatant was discarded, and the samples were stored at -20 °C until time of extraction. The extraction was performed using a protocol from Ritchie et al. (2006). Methanol (950 µL) was added to the pellet before vortexing to disrupt the pellet. The sample was incubated in the dark at 4°C for 24 hours. The resulting supernatant was collected and measured using a spectrophotometer with pure methanol as a blank with 1 cm cuvettes. Total chlorophyll content was estimated using an equation (see below) obtained from Ritchie et al. (2008) using absorbances measurement at 632, 652, and 665 nm.

$$Total\ Chl\ \left(\frac{mg}{L}\right) = [27.9603(ABS\ 632nm) + 12.9241(ABS\ 652nm) + 1.0015(ABS\ 665nm)] * dilution\ factor$$

Nile Red Fluorescence

Nile Red fluorescence was obtained by diluting 250 μL of culture with 750 μL of deionized water and adding 80 μL of a 25 $\mu\text{g/mL}$ of Nile Red solution in DMSO to achieve a final Nile Red concentration of 2 $\mu\text{g/mL}$. The solution was vortexed and 200 μL was pipetted into the wells of a black walled, flat bottom 96 well plate in triplicate. The plate was then incubated in the dark for ten minutes before being read after excitation at 530 nm and emission at 575 nm. Blanks were made in the same fashion with supernatants from spun down culture and fresh media.

Lipid Extraction

Transesterification for FAME Analysis. Lyophilized biomass was transesterified to obtain fatty acid methyl esters (FAMES) from the lipids contained in the cells. A recorded mass (~ 2 mg) of biomass was placed into a screw top test tube with a cap that has a Teflon septum. To each sample 1 mL of toluene and 2 mL of sodium methoxide were added using glass syringes. This suspension was vortexed and then heated at 90°C for 30 minutes, vortexing every 10 minutes. The sample was removed and allowed to cool to room temperature before adding 2 mL of boron trifluoride. Again, the sample was vortexed and heated for 30 minutes at 90°C, vortexing every 10 minutes. Once cooled to room temperature 0.8 mL of hexane was added using a glass syringe, 0.8 mL of a saturated salt solution (14% NaCl) was also added to the test tube. The sample was vortexed and heated at 90°C for ten minutes and then vortexed again. To aid in the separation of the organic and aqueous layers the sample was centrifuged for 2 minutes. The top organic layer was transferred to GC vials.

FAME Analysis using GC-MS. From the transesterified lipids in GC vials, samples were diluted in hexane as necessary to fit within a standard curve made from Supelco 37 component FAME

mix (#CRM47885) before an aliquot of approximately 100 μL was placed into a GC vial with a 0.3 mL insert. As an example, samples obtained from the final timepoints were diluted by 1:10 with hexane before running. Gas chromatography-mass spectrometry (GC-MS) analyses were conducted using a Trace 1310 GC coupled to a ISQ 7000 MS, both by ThermoFisher Scientific. Samples or standards (0.5 μL) were injected using a ThermoFisher AS 1310 autosampler and ThermoFisher AI 1310 injector using a split of 10:1. The column, a DB-23, 60 m long, inner diameter 0.250 mm, film thickness 0.15 μm was operated at 1.0 mL/min. The injector temperature was 240 $^{\circ}\text{C}$, the GC-MS interface 230 $^{\circ}\text{C}$, and the ion source temperature was 250 $^{\circ}\text{C}$. The column temperature was initially held at 50 $^{\circ}\text{C}$ for one minute before ramping to 175 $^{\circ}\text{C}$ at a rate of 25 $^{\circ}\text{C min}^{-1}$, followed by another ramp at 4 $^{\circ}\text{C min}^{-1}$ to a final temperature of 230 $^{\circ}\text{C}$. This temperature was held for 10 minutes before the termination of the run. The MS is a single quadrupole positive polarity that uses electron impact ionization to fragment the compounds. Using an electron lens voltage of 5 V gives the electrons an energy value of 70 eV and an emission current of 50 μA . The software used for data analysis was Chromeleon (Ver. 7.2.10). Compounds were identified through library searches combined with quantification via quantitation and confirmation peaks. FAME-like unknowns identified from their fragmentation patterns were quantified by surrogate standard quantification from the response peak and the closest eluting calibration standard.

Carbohydrates

Starch and carbohydrates from biomass samples were quantified as described in Beck et al. (2018). The anthrone reagent was prepared fresh by dissolving 1 g of anthrone in 25 mL of pure ethanol and 475 mL of 75% sulfuric acid. A sample of 0.5-1.0 mg of algal biomass was suspended in 1 mL of deionized water in a test tube with a screw top cap that contains a Teflon septum. To this sample 5 mL of the anthrone reagent was added. The samples were then boiled at 98°C in a water bath for ten minutes. After boiling, 200 μ L of the sample was placed into a flat-bottom 96 well plate and the absorbance of the sample was measured at 625 nm. The samples were compared to a standard curve created using glucose to determine starch content.

Elemental composition (CHNS/O)

Elemental composition (Carbon, Hydrogen, Nitrogen, Sulfur (CHNS) and Oxygen (O)) of dried biomass was determined using a Flash 2000 organic elemental analyzer from CE Elantech (Lakewood, NJ). Tin cups were used for analyzing CHNS and silver cups were used for analyzing O composition. Between 2 mg and 3 mg of sample were weighed into the cups and the cups were closed securely before the exact weight was recorded. BBOT (2,5-bis(5-tert-butyl-2-benzo-oxazol-2-yl) thiophene) was used as a standard with weights ranging from 2-5 mg of the compound. The temperature for the CHNS reactor was set to 950 °C, for the O reactor to 1060 °C. The secondary pressures of the carrier gases of Helium and Oxygen were set to 40 psi. Flow rates of the gases were 250 mL min⁻¹ for Oxygen and when used as a carrier, the flow rate of Helium was 140 mL min⁻¹, the reference flow of Helium was 100 mL min⁻¹ the GC oven temperature was set to 65 °C. The two GC columns used were CE Elantech #26008215 for the CHNS and #26007900 for the Oxygen analysis.

Analysis of Media Components

pH

The pH of media and samples that were filtered with 0.2 μm PES filters was measured using an Orion Star A211 pH meter and a Thermo Scientific Orion Economy Series (cat. 911600) that was calibrated daily using buffers of 7.0, 10.01 and 12.46.

Nitrate assay

Nitrate concentrations within the media were measured in two ways. One method for immediate results and another for confirmation later. For determining when to harvest the cultures nitrate was quantified using a colorimetric assay using HACH Test'n Tube™ kit #53 that uses sulfuric acid and a NitraVer® reagent. This was done by first combining one packet of the reagent to 900 μL of nanopure water into a microcentrifuge tube and vortexing to dissolve the reagent. Samples of algal cultures (1 mL) were centrifuged at 10,000 rpm for 2 minutes. To a new microcentrifuge tube 15 μL of the supernatant from the algal culture and 85 μL of the reagent solution was added. From the provided acid vials in the kit 750 μL of the acid was also added to the new microcentrifuge tube. The solution was then vortexed and allowed to react for five minutes. To measure absorbance, 200 μL of each sample were placed in triplicate into a 96 flat-bottom well plate. The absorbance of these samples was measured at 410 nm and compared to a standard curve ranging from 0 to 2.94 mMol of sodium nitrate made in Bolds Basal medium.

Anion isocratic chromatography was used for post-experiment confirmation of nitrate concentrations, using a Dionex ICS1100. The mobile phase used is the Dionex AS22 eluent concentrate from Thermo Fisher (#063965), the 100x concentrate contains 0.45 M carbonate and 0.14 M bicarbonate. The analytical column used was the Dionex IonPac AS22 standard bore 250 mm x 4 mm (Thermo Fisher #064141) and guard column Dionex IonPac AG22 standard bore 50

mm x 4 mm (Thermo Fisher #064139). When operating the flow rate of the eluent is 1.2 mL/min. Each sample was filtered with a 0.22 μ m filter and if stored was immediately frozen after filtering at -20 °C. The standards used during the runs were diluted from a certified Dionex 'Combined Seven Anion Standard 1' (Thermo Scientific #056933). The sample loop used during analysis was 25 μ L.

DIC/TIC/DOC/TOC

Carbon concentration within the media was determined by using a SKALAR Formacs^{HT} TOC/TN analyzer that uses an IR detector for CO₂ at 0.1-5000 mg C/L detection range. The standard used were made from dilutions of a stock solution containing 1000 mg C/L made by dissolving 0.442 g of sodium carbonate and 0.3500 g of sodium bicarbonate in 100 mL of nanopure water. Samples were filtered with 0.2 μ m cellulose nitrate filters. The acid used when determining dissolved inorganic carbon was 4% phosphoric acid.

Methods Specific to Each Species

ALgE

Strain ALgE was cultivated and sampled once a day around the halfway point of the light cycle, until harvested where the biomass collected was analyzed for TSS and AFDW along with lipid composition.

SLcyaH

Strain SLcyaH was cultivated and sampled twice a day, approximately one hour before the light cycle switched. Samples for TSS and AFDW were taken. Cultivation and sampling continued until three days after the depletion of nitrate, where the resulting biomass was analyzed for lipid composition.

SLdC

Strain SLdC was also cultivated and sampled twice a day, approximately one hour before the light cycle switched. Samples for TSS, AFDW, and biomass for lipid concentration were done at each timepoint. Sampling continued until a rise and then a stabilization of the pH occurred as nitrate never reached a depletion point.

Methods Specific to Polyculture Experiment

The lights and their intensity used in the following section were changed to best mimic outdoor lighting conditions in Kona, HI. One of these changes was the switch from T5 fluorescent lights to SpiderFarmer® SF600 full spectrum LED lights to better emulate the wavelengths of light in natural sunlight see **Figure 3**. The intensity was also changed to $300 \mu\text{mol}_{\text{photons}} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PPFD to match the average summer intensity of Kona, HI to the depth of the media in the shake flasks.

Figure 3: (top left) fluorescent light intensity spectrum of Accupro™ T5 lights, (top right) SpiderFarmer® Full spectrum LED light intensity spectrum, (bottom) Natural Sunlight intensity spectrum highlighting the visible range 400 nm to 750 nm image from (Baird).

Due to the different sizes of the cyanobacteria, green algae and diatoms, the polyculture experiments were not inoculated with equal cell numbers but rather with approximately equal mass concentrations for each strain based on the OD 750 nm-AFDW correlations for each species in **Table 2**. All cultures started at a mass concentration of 30 µg/mL.

Table 2 OD 750 nm to AFDW correlations

Species	OD 750 nm / AFDW (mL/ μ g)
SLdC	5.68×10^{-4}
ALgE	1.28×10^{-3}
SLcyaH	8.96×10^{-4}

CHAPTER THREE

RESULTS AND DISCUSSION

ALgE

Strain ALgE, tentatively identified as a *Desmodesmus* sp., has a higher productivity under high alkalinity (150 mM total alkalinity) than under low alkalinity conditions. The HA culture exhibited a generation time of approximately 21.6 hours while the LA culture had a generation time of approximately 38.4 hours. As shown in **Figure 4**, the species grows in stacked groups along their longer sides, and once they reach the stationary phase, they are mostly found as single cells.

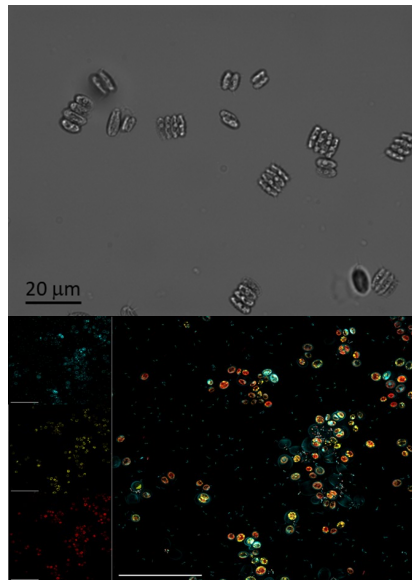


Figure 4: (top) Bright field image of species ALgE, (bottom) Microscopy image of strain ALgE grown in HA conditions. Blue is a DAPI stain for DNA, yellow is Bodipy, red is chlorophyll autofluorescence. The scale bar is 30 micrometers. The blue speckles are likely bacteria associated with the species microbiome

Images of species ALgE growing in tube reactors are shown at two timepoints in **Figure 5**. Under high alkalinity conditions, the consumption of nitrate lasted only four days while the low alkalinity conditions took nine days to reach nitrate depletion (**Figure 6**).

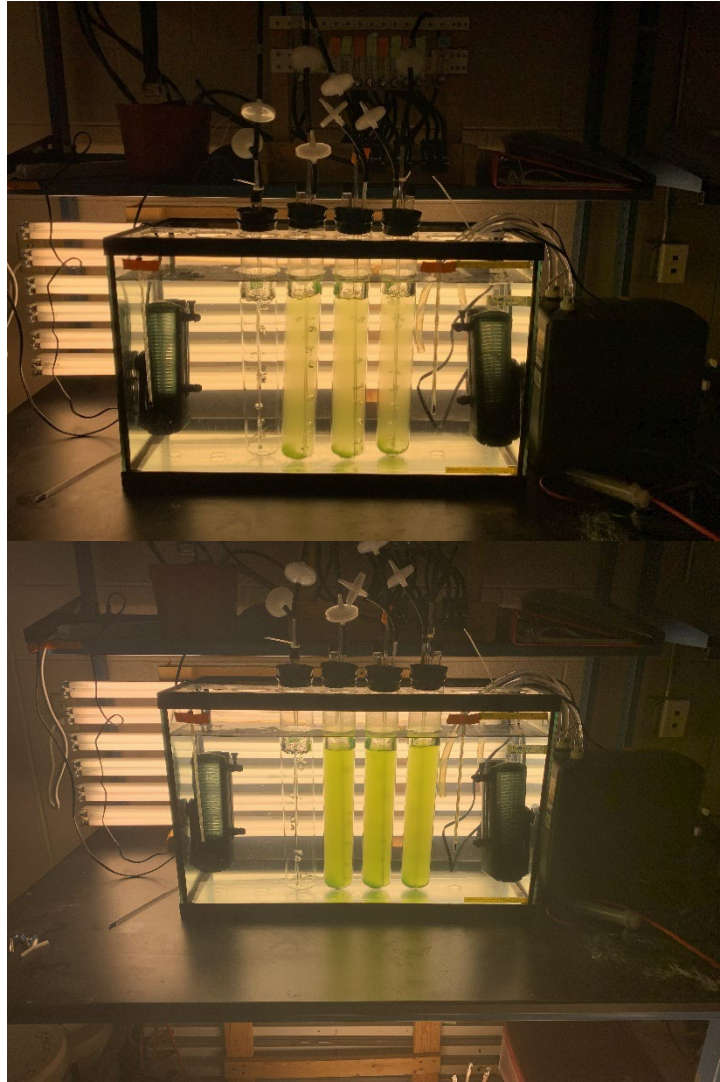


Figure 5: Photos of Tube reactors of species ALgE at 24 hours (top) and at 96 hours (bottom)

The pH of the high alkalinity condition was very stable and showed little increase over time, while the low alkalinity condition's pH increased from 8 to almost 10. Also shown in **Figure 6** are the cell concentrations and optical density (OD = absorbance) at 750 nm over time. Comparing the two graphs, the absorbance in both conditions increased after nitrate depletion

while cell counts did not significantly increase, suggesting that the cells are becoming larger due to the accumulation of storage compounds.

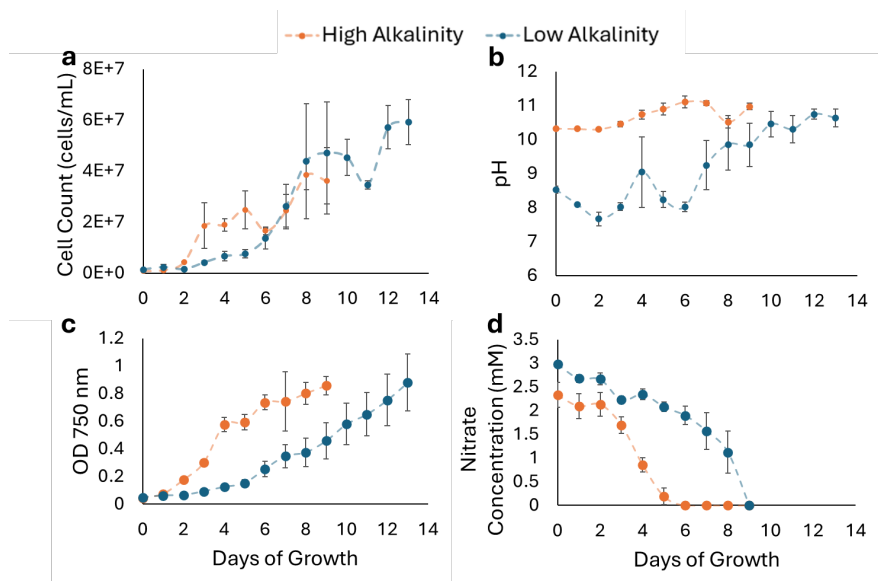


Figure 6: (a) Cell concentration from direct counts over time for species ALgE. (b) pH levels of ALgE cultures over time. (c) OD 750 nm over time for high and low alkalinity cultures of strain ALgE. (d) Change in the nitrate concentration in the media over time

This is also supported by **Figure 7** where the Nile Red fluorescence, indicative of hydrophobic regions inside the cells, increases after nitrate depletion in both conditions.

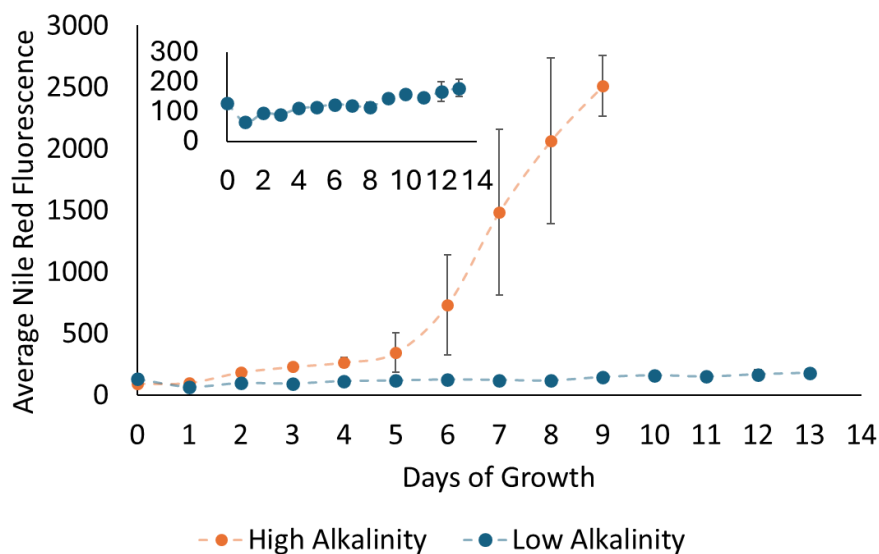


Figure 7: Comparison of the average Nile Red Fluorescence for species ALgE under high and low alkalinity conditions.

The absorbance spectra of the cultures at nitrate depletion and three days later at harvest display that the absorbance of the cultures at the range of wavelengths suggest an increase in cell size and or storage compounds within the cells (**Figure 8**). The spectra also have no substantial change in the absorbance pattern meaning that the overall pigmentation of the cells likely did not change after nitrate depletion.

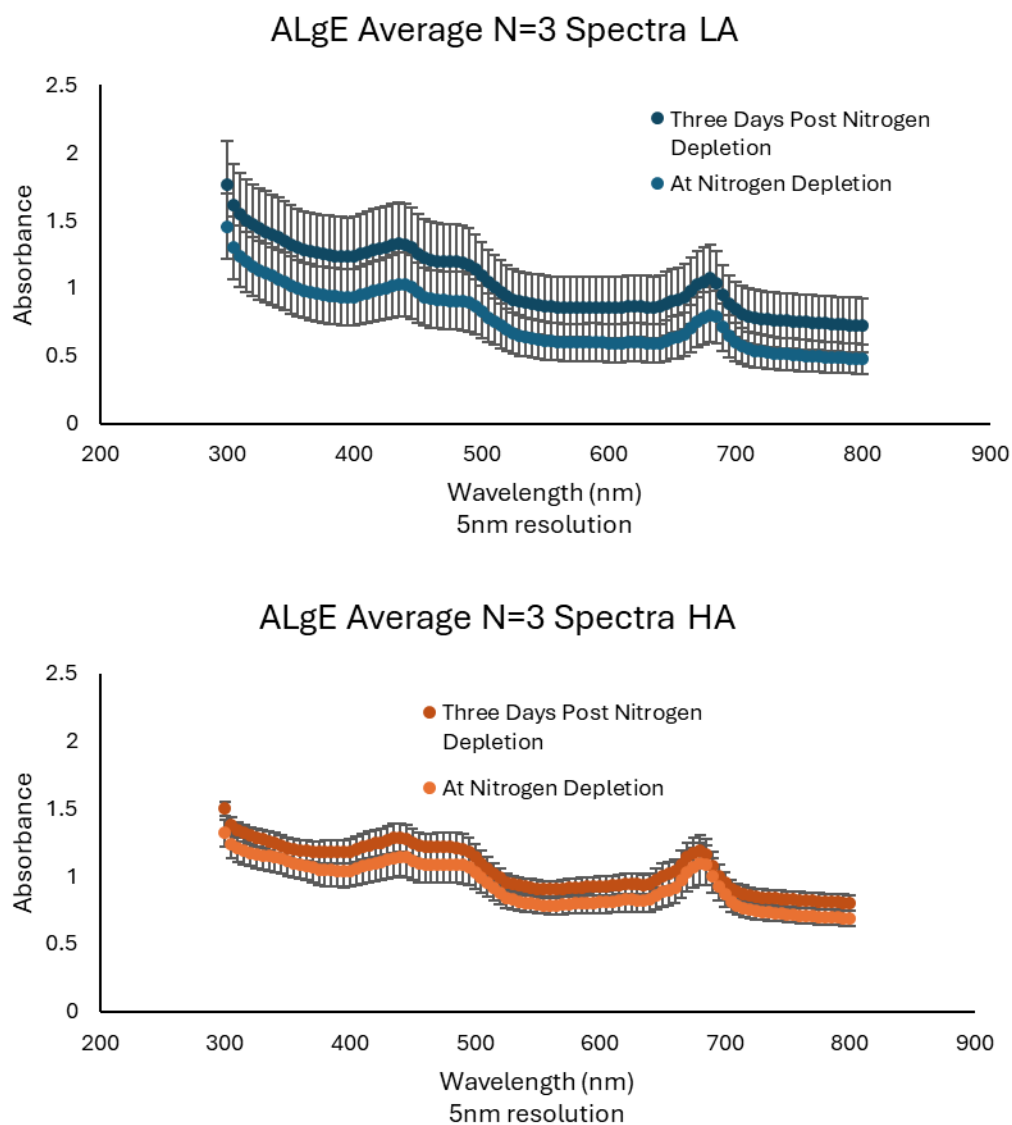


Figure 8: Comparison of absorbance spectra at and three days after nitrogen depletion of species ALgE, cultivated in low and high alkalinity conditions. Values are averaged over three biological replicates.

ALgE (**Figure 9**) accumulated more more total FAMES, (15.28% w/w of biomass) under the high alkalinity conditions compared to low alkalinity conditions (11.76 % w/w). Moreover, the speciation of those FAMES shifted to longer chains or more unsaturated FAMES under high alkalinity conditions. The FAME productivity of the low alkalinity condition was determined to

be 6.59 mg/(L*day) on average over the 13-day cultivation period and reached 85.7 mg/L total FAMES, while the high alkalinity condition resulted in an average rate of 15.6 mg/(L*day) over the nine-day cultivation period and reached 140 mg/L total FAMES.

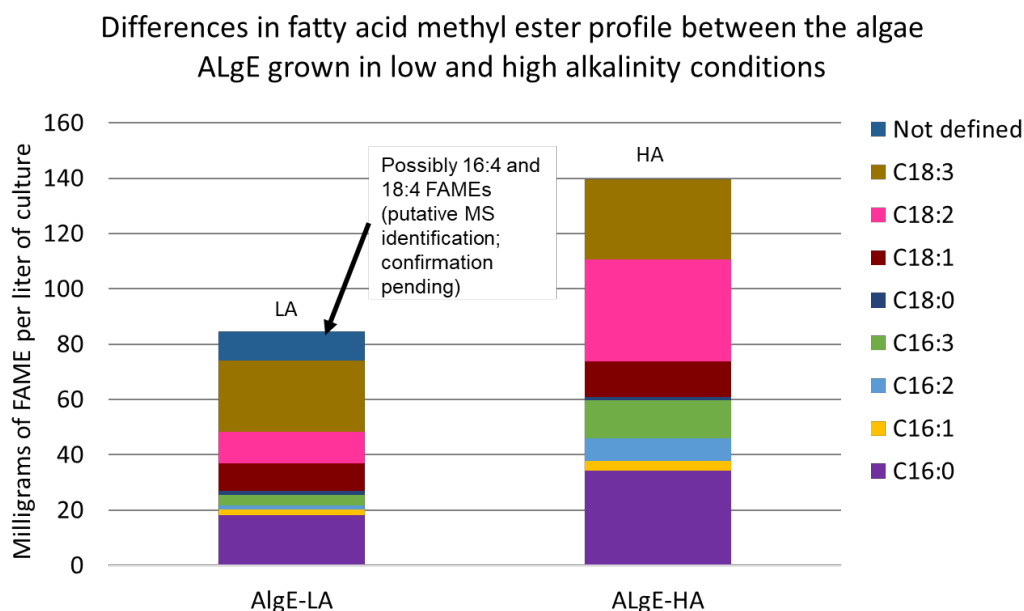


Figure 9: FAME profile and volumetric yield of species ALgE under low and high alkalinity conditions.

SLcyaH

Growth in high and low alkalinity systems did not appear to differ significantly based on OD readings or cell concentration for SLcyaH up to hour 232 (**Figure 10c and 10a**). After hour 232, the high alkalinity system was harvested but due to having nitrate left in the media, the low alkalinity culture was allowed to grow for longer. This resulted in the final cell concentration of the low alkalinity cultures to be significantly different than the final cell concentration in the high alkalinity cultures. Using a two tailed unequal variance T-test a p-value of 0.001 was obtained indicating a statistically significant difference between cell concentrations of SLcyaH in low and high alkalinity media in the employed tube reactor systems at harvest. When comparing the

cultures at the same timepoint (hour 232), using the same statistical test, a p-value of 0.11 was obtained, indicating no significant difference in the cell concentrations. The slight difference in cell concentrations but lack of significant difference in OD 750 nm measurements suggests that under high alkalinity conditions the cells of SLcyaH are larger than the cells grown under low alkalinity conditions. A possible explanation for this is the accumulation of compounds such as starch to maintain osmotic pressure in the cells.

The growth rates of the cultures were determined by fitting an exponential curve to the cell concentrations of each culture over time. The R^2 values for the fits range from 0.84 to 0.94, and the growth rates for the high alkalinity cultures were $0.0164 \pm 2.52 \cdot 10^{-4}$ OD 750 nm*hr⁻¹ or a generation time of 42.4 ± 0.661 hours, while the low alkalinity cultures were $0.0185 \pm 2.52 \cdot 10^{-3}$ OD 750 nm*hr⁻¹, or a generation time of 37.5 ± 5.92 hours. The rates in the different media were not statistically significant with a p-value of 0.28 when using a two tailed T-test with unequal variance.

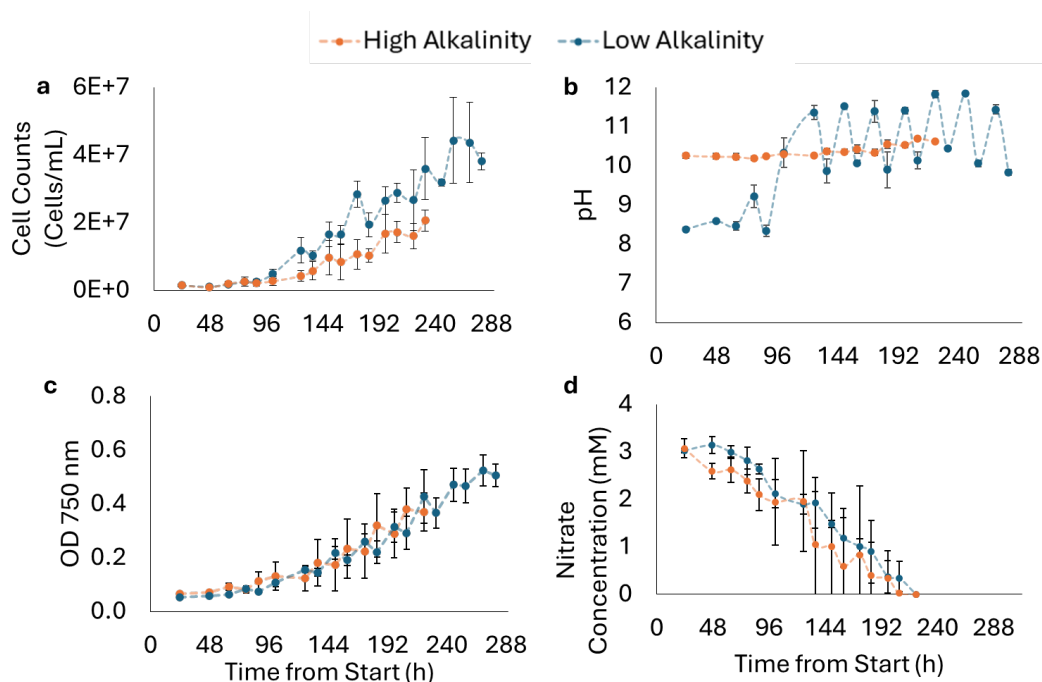


Figure 10: a) Cell concentrations of species SLcyaH grown in high and low alkalinity conditions. b) pH of the cultures over the experiment. c) OD 750 nm of species SLcyaH grown in high and low alkalinity. d) Average nitrate concentration (mM) for each culture. Error bars represent $\pm$ one standard deviation of triplicate treatments

As shown above in **Figure 10d**, the average nitrogen consumption rates for both conditions were similar, and when the approximate exponential consumption rate was compared using a two-tailed T test the rates, between conditions were not significantly different with a p-value of 0.39.

The pH of the cultures in the high alkalinity environment was stable throughout the experiment while the pH of the low alkalinity cultures increased from just above pH 8 at the beginning of the experiment to significantly higher pH values (**Figure 10b**). Indeed, the low alkalinity cultures experienced clear fluctuations in pH, which were correlated with the light cycle. The pH in the low alkalinity treatments would increase during the light periods due to the fixation of CO_2 and bicarbonate (HCO_3^-) producing hydroxyl ions (OH^-), and the pH would

decrease during dark periods due to net in-gassing of CO₂ and net internal respiration. pH values of well above 11.5 were observed during the light periods and even during the dark periods, the pH did not always drop to below pH 10, indicating that these systems were deficient in dissolved CO₂ relative to the equilibrium concentrations (based on Henry's Law).

Growth in all reactors was first observed by biomass accumulating near the bottom of the reactor (**Figure 11**, left). Once substantial growth occurred, biomass appeared to be more homogeneously distributed throughout the reactors (**Figure 11**, right).

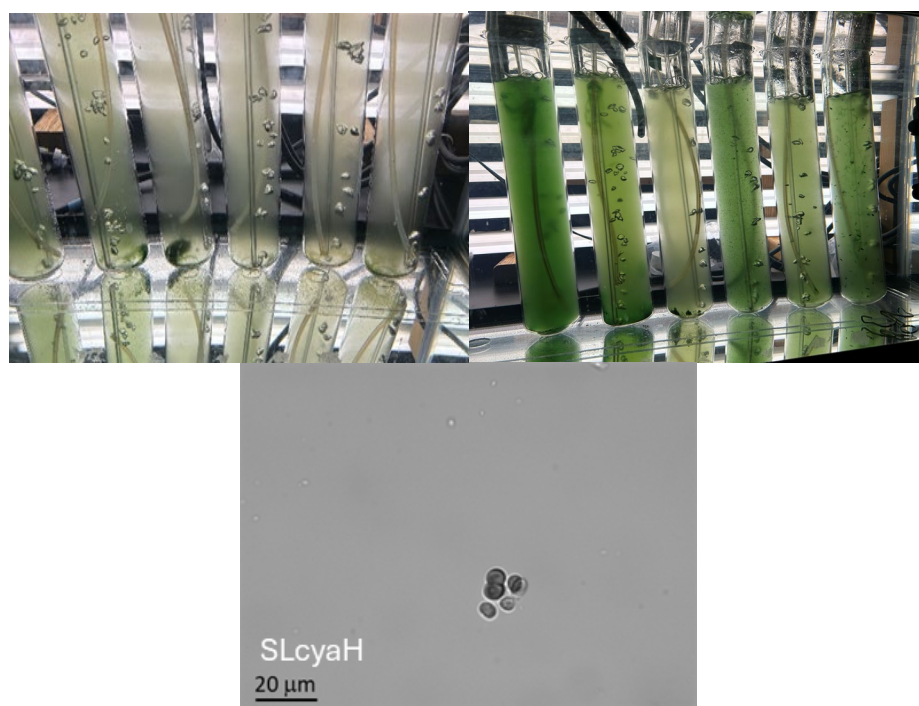


Figure 11: (left) Reactors after 2 days (hour 48) of the experiment. (right) Picture of the reactors during the experiment at hour 102. From left to right, tubes 1, 2, and 3 are high alkalinity and tubes 4, 5, and 6 are low alkalinity. (bottom) Microscopy image of species SLcyaH.

Interestingly within the conditions themselves there was an observable variation. Within the low alkalinity condition, reactors 4 and 6 contained numerous visible clumps of

cyanobacteria. Reactor 5, however, did not contain visible clumps. A similar phenotypic variation was visible in the high alkalinity reactors 1, 2, and 3. Reactor 3 contained large clumps present at the bottom of the reactor and very few throughout the reactor. However, reactors 1 and 2 contained biomass throughout the reactor and large suspended clumps. The tendency to clump together or remain attached to surfaces can be a strategy of organisms to deal with stressful environmental conditions such as light saturation or as defense against potential predators (Miller et al. 2024). This strategy can be both beneficial and detrimental for industrial growth; for instance, if the biomass clumps the operational costs for harvesting can be lower as more robust filtering (larger pore size) can be used to separate the liquid media from biomass. The downside of clumping is potentially a decreased overall photosynthetic activity through shading, and the possibility that clumps can physically obstruct mixing equipment, pipes, valves, etc. and thus possibly reduce the overall productivity of an algal farm. **Figure 11** (bottom) displays a microscopy image of species SLcyaH and how the organism is approximately 4 μm in diameter and clusters into tetrads or larger.

A FAME content of approximately 5% was observed for SLcyaH, which is consistent with what was observed by Somrak et al. (2007) for cyanobacteria. The concentration of the lipids was on average $0.537 \pm 3.69 \times 10^{-2}$ mg/L for high alkalinity conditions and $0.304 \pm 3.57 \times 10^{-2}$ mg/L for low alkalinity conditions. FAMES in both conditions are similar chain lengths were reported to be associated with the cell walls of the cyanobacteria (Wada and Murata. 1998). Interestingly, the appearance of both, *cis*- and *trans*-forms of the 18:2 FAMES, were observed when SLcyaH was grown in high alkalinity conditions. Other FAMES were detected such as *cis*-18:1 but were less than one percent of the total FAMES and are, for simplicity, not included in **Figure 12**.

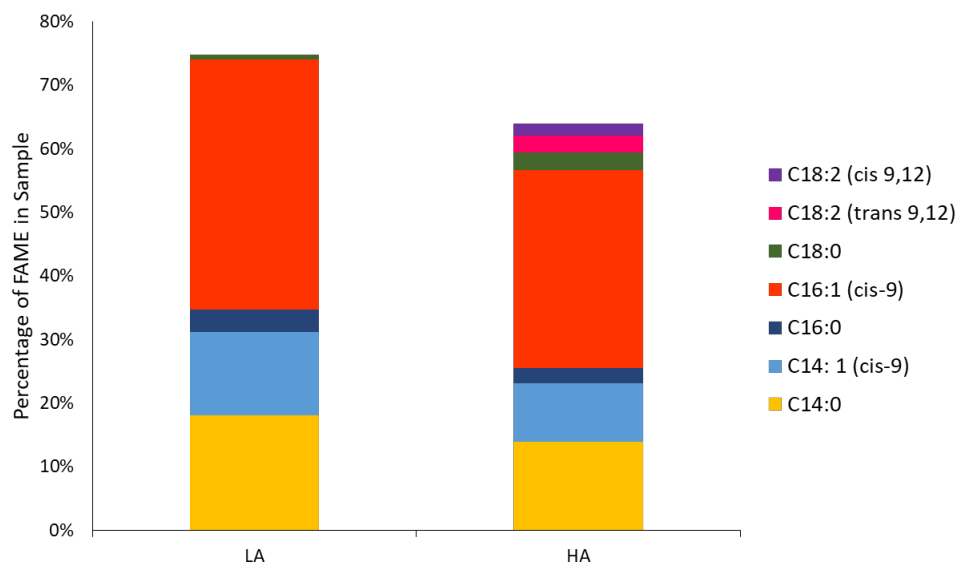


Figure 12: FAME distribution of species SLcyaH when grown under high and low alkalinity. The total FAME content is around five percent of biomass (w/w). Other FAMES were detected but account for less than 2% of the total and were not included in the figure.

SLdC

The culturing of species SLdC in tube reactors presented some challenges due to its preference for benthic growth until a certain culture density was obtained. This behavior is best presented in **Figure 13**. This preference resulted in measurements that represent growth to be highly variable but from a qualitative observation the HA culture of SLdC reached a homogeneous mixture faster than its LA counterpart.

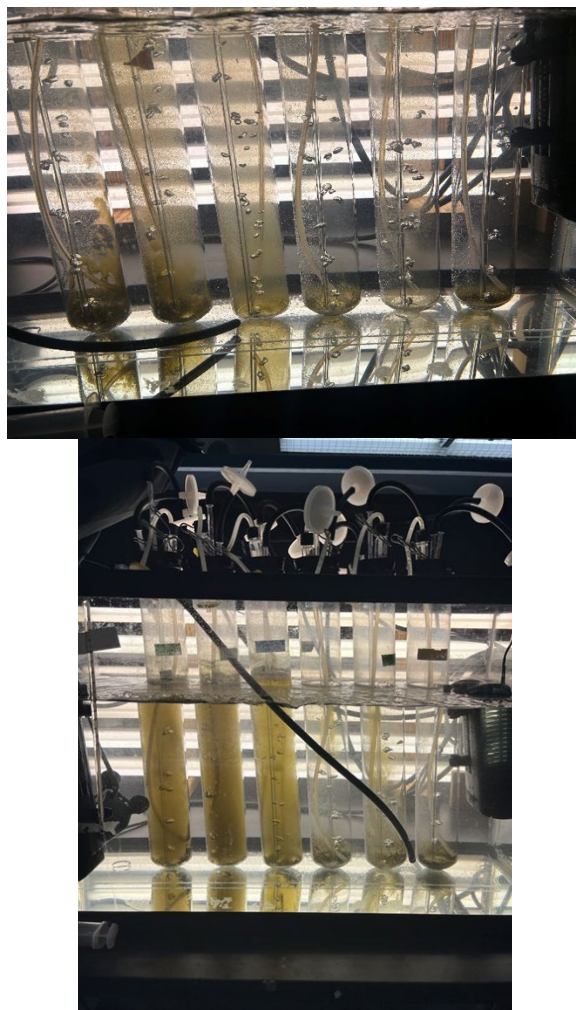


Figure 13: Photos of species SLdC throughout the tube reactor experiment displaying preference for benthic growth. 67 hours (top) and 146 hours (bottom). High alkalinity reactors are on the left and low alkalinity reactors on the right (three replicates each).

As shown in **Figure 14 (c)** the OD 750 nm of the cultures reached a maximum of approximately 0.3 excluding the two measurements around 200 hours for the high alkalinity condition. These readings and others can be explained by the preference for benthic growth by SLdC and the design of the reactors, as for some samples large amounts of biomass that had recently detached were sometimes acquired and other times little to no biomass would be collected. This highlights the importance of having well-mixed cultures for both future research

on this organism and potentially for its use industrially. Alternatively, biomass measurements should be developed that can more reliably estimate biomass concentrations representatively for organisms that tend to aggregate or attach to reactor surfaces. Cell counts were performed for these cultures and are shown in **Figure 14 (a)**. Again, the cell counts are highly variable due to the benthic growth pattern.

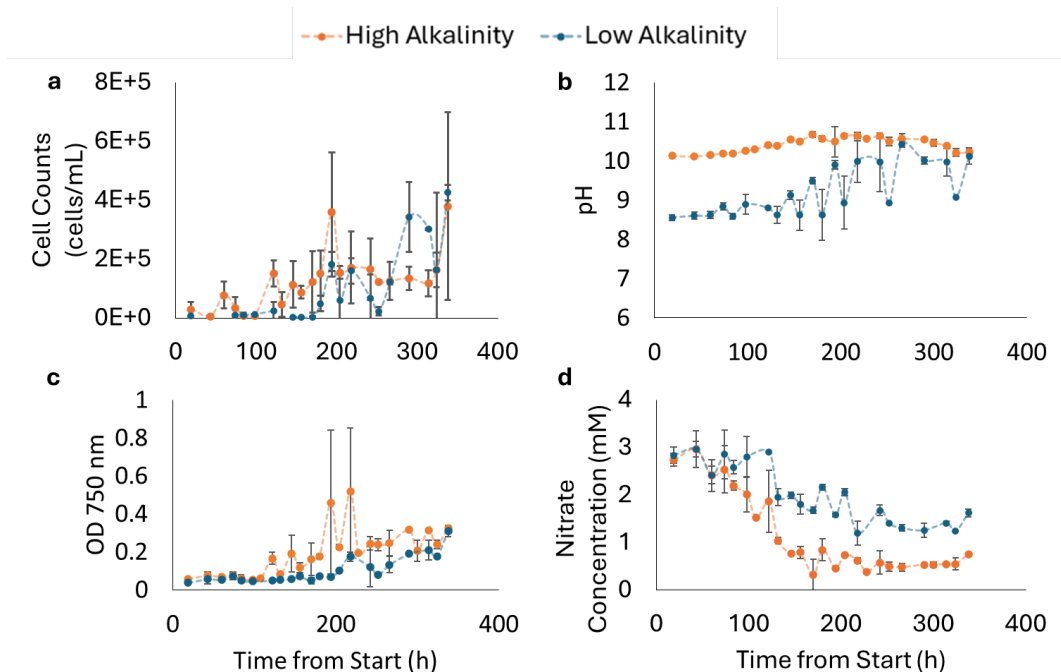


Figure 14: a) Cell counts of SLdC over time. b) The average pH of the two alkalinity conditions. c) comparison of the absorbance at 750 nm of SLdC cultures in high and low alkalinity cultures. d) Average concentration of nitrate over time in the two conditions.

The pH of the LA cultures fluctuated with pH values generally high when the lights were on and lower then the lights were off. The alkalinity of the HA culture allowed the pH to remain relatively stable around pH 10.5, though increasing slightly over time indicating a net consumption of inorganic carbon in the HA treatments (**Figure 14, b**). The consumption of nitrogen in both conditions was reflective of their growth rates as the high alkalinity cultures consumed nitrate faster. However, neither culture appeared to reach a point of nitrogen limitation

as shown in **Figure 14 (d)**. The hypothesis is that both conditions were limited in silica, as the molar ratio of silicon to nitrogen in the media was 1:1.47, when the media recipe is 2 mM of silica to 2.94 mM of nitrate. Data for SLdC growth with more silica are provided below (in the Polyculture section). Interestingly, more nitrate remained unconsumed in the low alkalinity conditions suggesting that the high alkalinity is incorporating the nitrate into more biomass (e.g., proteins), storing it within the cells, or utilizing it to produce extracellular polymeric substance to enable the establishment of the more benthic cells. The diatom is roughly 10 times the size of the green alga (ALgE) and the cyanobacterium (SLcyaH) used in this study; the diatom reaches a length of about 40 μm along the major axis and a width of about 14 μm (**Figure 15**).

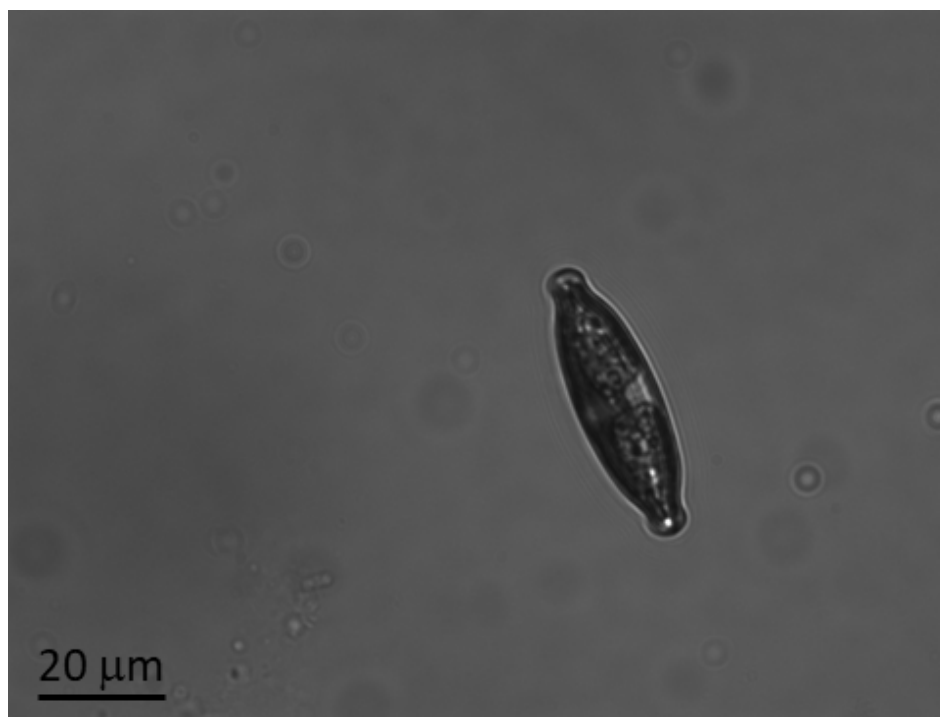


Figure 15: Microscopy image of species SLdC. The major axis of the cell is approximately 40 μm and the minor axis is approximately 14 μm .

Lipid contents of 29.9 ± 5.24 % of biomass as FAMES or 253 ± 92 mg/L of FAMES per liter of culture, and 18.37 ± 6.29 % of biomass as FAMES or 91 ± 1.7 mg/L of FAMES per liter of culture, were observed for the HA- and LA-SLdC treatments, respectively (**Figure 16**). SLdC produces longer carbon chain poly-unsaturated fatty acids such as C20:3, C20:5, and C22:6. These compounds are often more valuable than the C18 and C16 FAMES. As an example, comparing the cost of C18:3 to C20:5 through Fisher Scientific™, two compounds found within the SLdC culture's FAME profile, the C20:5 costs roughly 1900 times more per milligram than the C18:3.

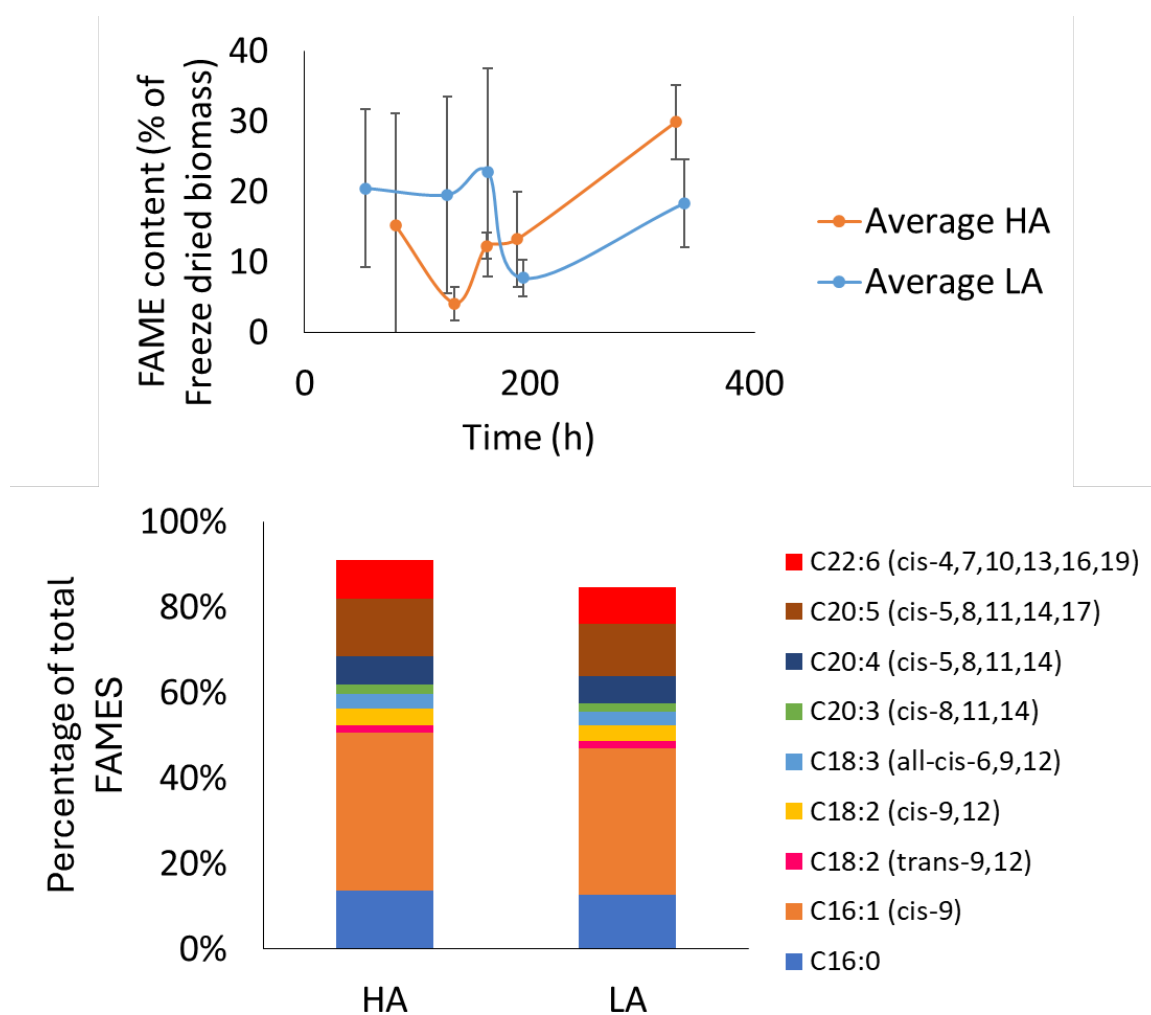


Figure 16: FAME content and composition in HA and LA culture conditions of SLdC

Polyculture

Silicate Tolerance Screening

In preparation for a full-factorial design polyculture experiment, the growth of each strain (AlgE, SLdC, and SLcyaH) was evaluated in the presence of 4mM sodium metasilicate to assess potential inhibition of strains, specifically AlgE or SLcyaH, by the elevated silicate concentrations necessary to reach nitrogen depletion in the diatom cultures (**Figures 17-20**). As expected, the diatom SLdC was unable to grow in the absence of a silica source; the growth rate in the presence 4mM of silicate was estimated to be $0.0227 \pm 1.4 \times 10^{-3}$ OD 750 nm*hr⁻¹. SLcyaH showed a significant change in growth rate with a p-value of 5.31×10^{-5} with a two tailed equal variance T-test, when in the presence of 4 mM sodium metasilicate. The rate increased from $0.0212 \pm 4.04 \times 10^{-4}$ OD 750 nm*hr⁻¹ to $0.0292 \pm 6.43 \times 10^{-4}$ OD 750 nm*hr⁻¹. It appeared though as if the lag period, before significant growth (OD increase) occurred, decreased in the presence of 4 mM sodium metasilicate for SLcyaH. It is possible that at the earlier stages of growth that the formation of silica gels that can form at high pH values are contributing to light absorbance or scattering resulting in a higher OD 750. One way to test this is comparing the OD 750 of abiotic media at high alkalinity of two conditions one with 4 mM silica and one without. AlgE exhibited a significant decrease in growth rate when comparing the changes in OD 750 measurements between treatments with a p-value of 0.008 using a two tailed unequal variance T-test. The estimated growth rate decreased from $0.0186 \pm 8.54 \times 10^{-4}$ OD 750 nm*hr⁻¹ on average without silicate to $0.0118 \pm 1.7 \times 10^{-3}$ OD 750 nm*hr⁻¹ with 4 mM added silicate. These results are further exemplified in the images of the shake-flasks (**Figures 18-20**).

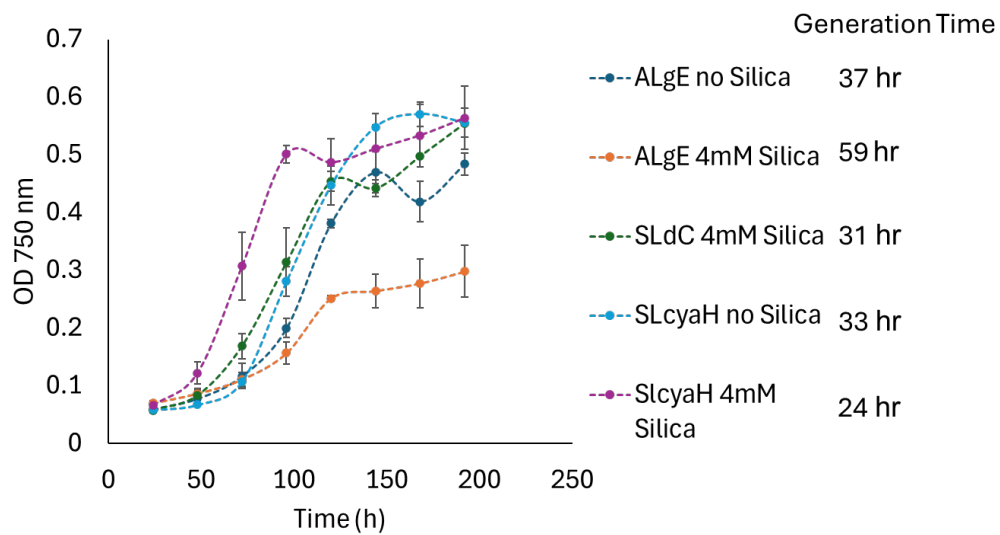


Figure 17: Average OD 750 nm of species SLdC, ALgE, and SLcyaH with and without 4 mM sodium metasilicate

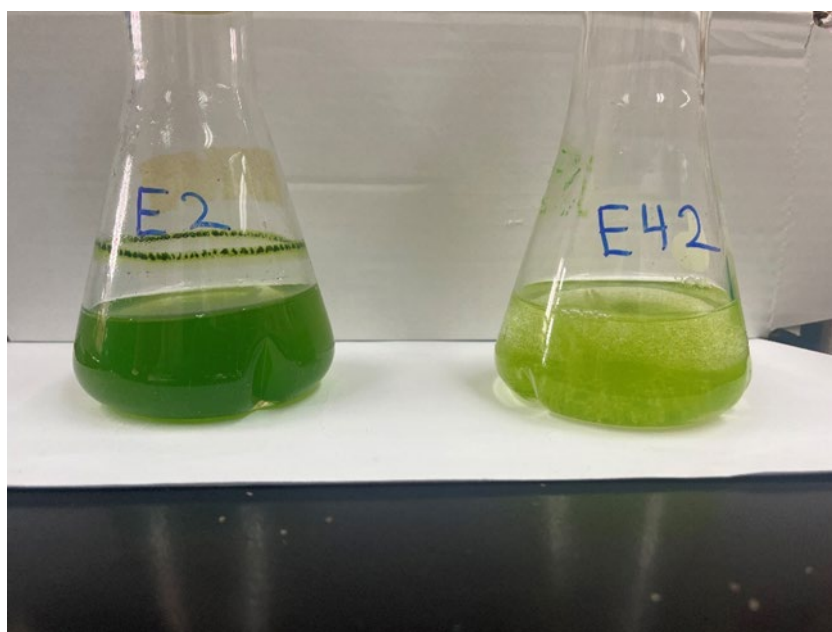


Figure 18: Representative photos of shake flasks of strain ALgE without (left) and with (right) 4 mM silicate

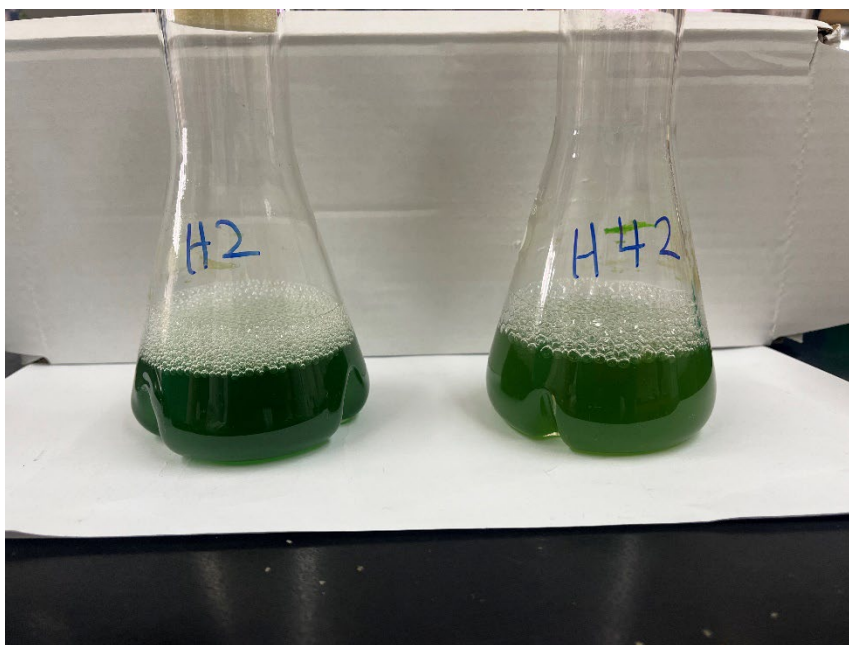


Figure 19: Representative photos of shake flasks for strain SLcyaH without (left) and with (right) 4 mM silicate

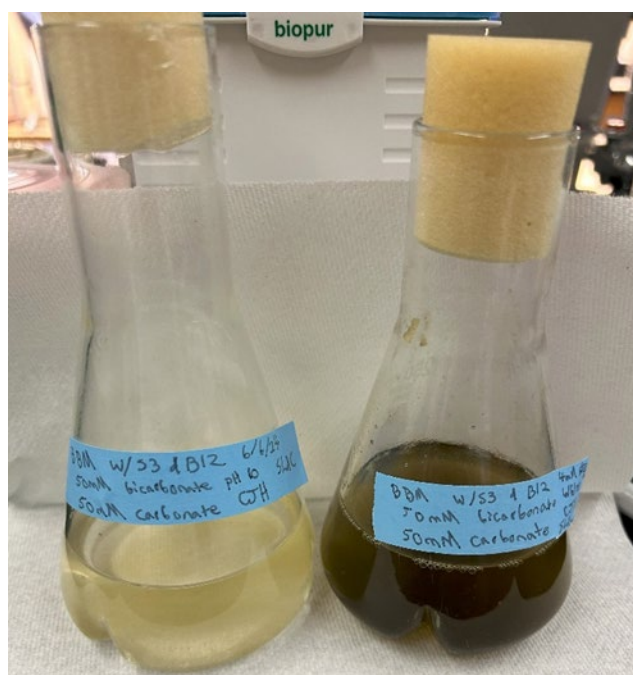


Figure 20: Representative photos of shake flasks of SLdC without (left) and with (right) 4 mM silicate

This silica testing experiment was important to determine the amount necessary for the growth of cocultures with SLdC. As a reference source Agrizon.com charges \$45 for a 25 kg bag of sodium metasilicate pentahydrate that is 57.7% purity. To achieve a concentration of 4 mM for a one-acre algal pond that is 0.2 m deep it would cost roughly \$2,150 USD before shipping. If similar results from the algae can be achieved at a lower silica concentration, then a significant reduction in operating costs can be achieved.

Polyculture Experiment Results

The growth rates of the monocultures of SLdC were statistically significantly different from the growth rates of the coculture of species ALgE and SLcyaH. A Tukey's HSD test resulted in a p value of 0.027 between the two conditions growth rates. As shown in **Figure 21** all cultures tested reached a final OD 750 nm absorbance value of 0.496 ± 0.0591 . Significant differences (based on 95% confidence) were found for the final OD 750 nm values between the monocultures of SLcyaH and ALgE ($p=0.043$), ALgE and the polyculture of SLdC and SLcyaH ($p=0.003$), and SLdC and SLcyaH to the culture of ALgE and SLcyaH ($p=0.022$). The growth rates of each of the cultures are graphically displayed in **Figure 22** and in **Table 2** numerically. Growth rates were calculated by fitting an exponential regression curve to the OD 750 nm data over the linear range when plotted on a semi-log plot. It is important to note that for cultures containing the species SLdC sodium metasilicate nonahydrate was added at a concentration of either 2 mM for polycultures or 4 mM for the monoculture as silica is a necessary nutrient for the diatom to grow.

Figure 21: (Left) Average OD 750 nm of monocultures, (Right) Average OD 750 nm of polyculture combinations. Error bars represent +/- one standard deviation

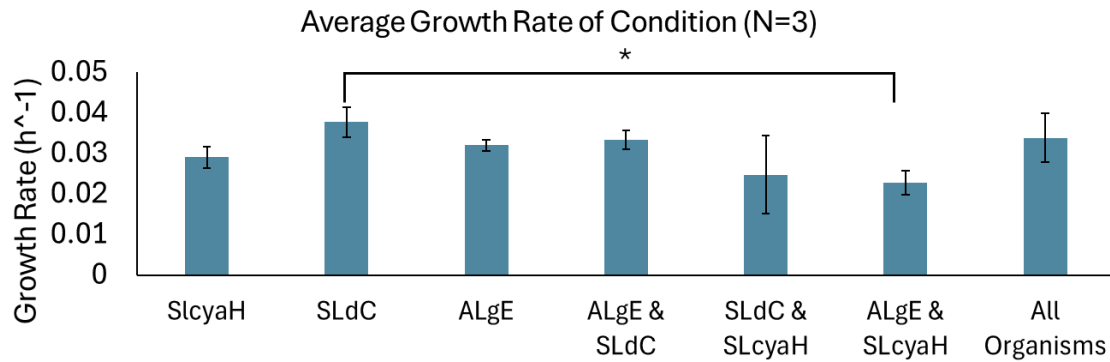


Figure 22: The average growth rates of each culture across three replicates with standard deviations. Asterisks show significant difference between conditions (only the growth rate of monocultures of SLdC were statistically significantly different from the growth rate of the coculture of ALgE & SLcyaH).

Table 3 Growth rates and corresponding the variances of mono- and polycultures obtained from the “Polyculture” experiment

Species Present	average growth rate (h ⁻¹)	average growth rate (day ⁻¹)	sample variance (h ⁻¹)
SlcyaH	0.02903	0.6968	0.002702
SLdC	0.03770	0.9048	0.003764
ALgE	0.03197	0.7672	0.00135
ALgE & SLdC	0.03330	0.7992	0.002261
SLdC & SLcyaH	0.02473	0.5936	0.009577
ALgE & SLcyaH	0.02277	0.5464	0.003027
All Organisms	0.03380	0.8112	0.006022

Also shown in **Table 4** is a comparison of the generation times of the monocultures under fluorescent lights and under full spectrum LED lights. Each species saw at least a 25 percent reduction in their generation time when grown under full spectrum LED lights. It is unclear if this is due to the only the changing of the light spectrum used or the different intensity of the light.

Table 4: Comparison of generation times with standard deviations of monocultures under different light sources

Species Present	Generation Time Fluorescent (h)	Generation Time Full Spectrum LED (h)
SLcyaH	33 ± 0.61	24 ± 2.1
SLdC	31 ± 2.0	18 ± 1.9
ALgE	37 ± 1.7	22 ± 0.92

The change in pH across cultures was expected and agrees with previous results obtained using BBM high alkalinity media (**Figure 23**). The pH starts around 10 and as nitrate is consumed and algal growth occurs through photosynthesis, the pH increases to around 11.5 to 12. The pH then levels off or begins to decrease back towards the buffer point of ~10.

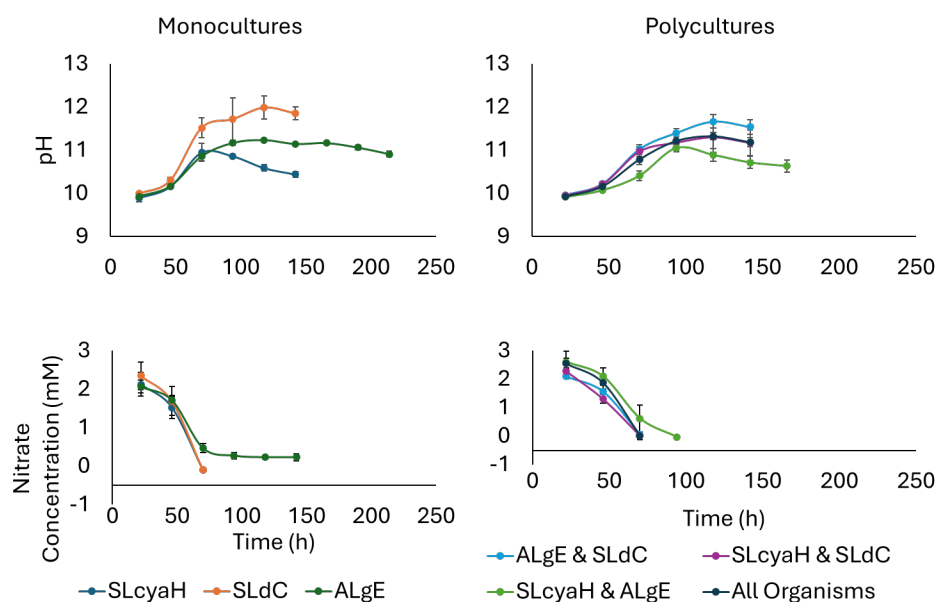


Figure 23: Time plot of the pH each day for the different cultures and their standard deviation (top row). The nitrogen (nitrate) concentration of cultures determined by the HACH assay kit with standard deviations (bottom row).

The biomass concentration (TSS, g/L) of the cultures at their point of harvest reached over 0.7 g/L, with most cultures maintaining or exceeding that value also at the time of nitrogen depletion with the exception of SLcyaH cultures (**Figure 24**). SLcyaH cultures experienced a reduction from 0.76 to 0.30 g/L (*cf.* **Figure 24 top**). ALgE cultures generally exhibited the lowest productivity of all conditions until the point of nitrogen depletion. Indeed, the productivity of ALgE monocultures was statistically significantly different ($p < 0.05$) from all cultures except the cocultures of ALgE & SLdC, and ALgE & SLcyaH. It should be noted that the coculture of ALgE & SLcyaH took one more day to reach nitrogen depletion compared to the other cultures (*cf.* **Figure 23**), and that the monoculture of ALgE did not reach a point of nitrate depletion but was determined to be done growing after 3 days of nitrate measurements with no significant difference between the three (one-way ANOVA analysis p value 0.787). The TSS productivity of the monoculture of SLdC was significantly higher than the cocultures of SLdC & ALgE, and ALgE & SLcyaH.

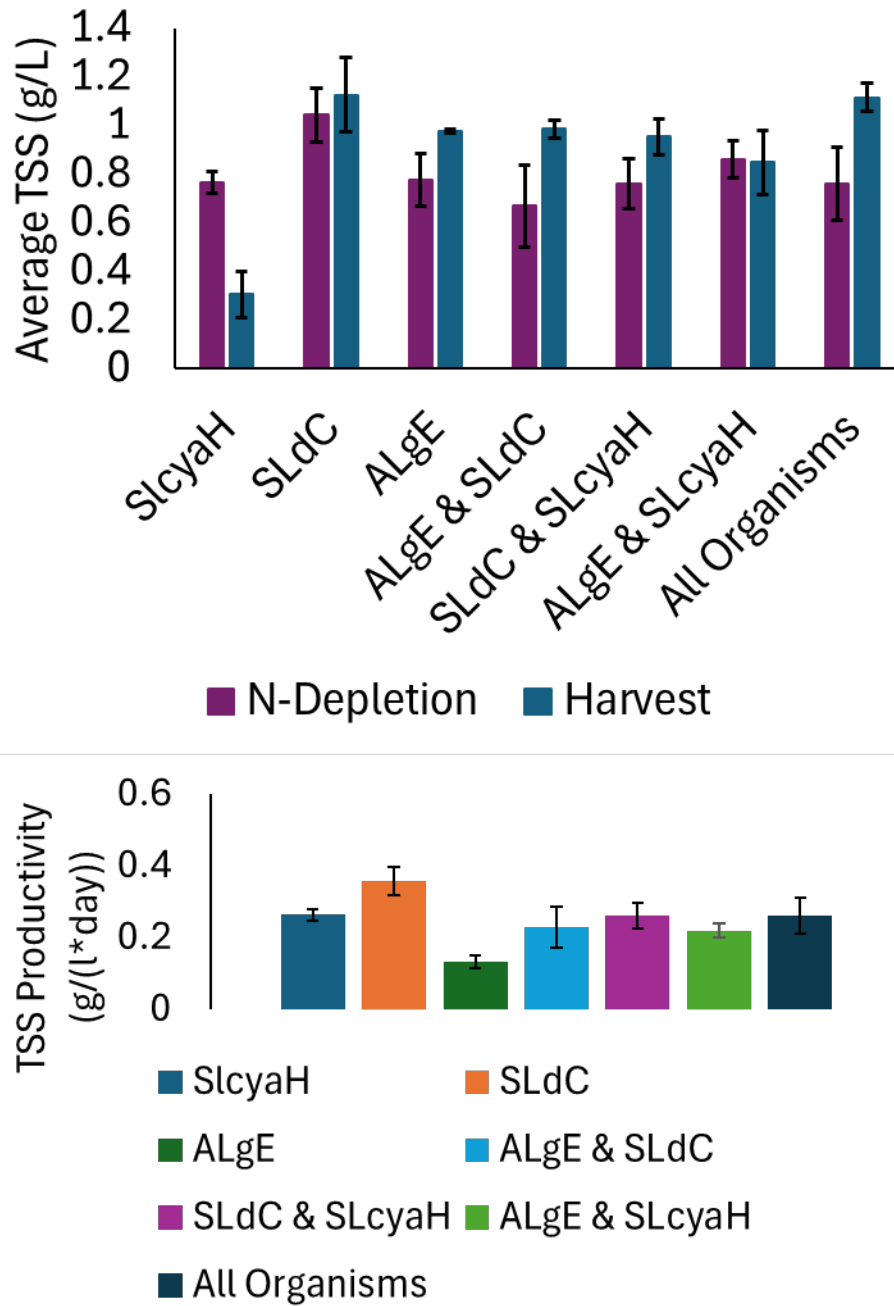


Figure 24: (top) Average TSS values of cultures at time of nitrogen depletion and at time of harvest. (bottom) Average daily TSS productivity during the consumption of nitrate.

When looking at the carbon storage compounds of the cultures, as expected and shown in other cyanobacteria in Somrak et al. 2007, the culture of SLcyaH increased in starch content after nitrogen depletion with the p value of the two tailed t-test being 0.0252 when compared to its nitrogen depleted sample, while the other cultures stayed around the same starch content percentage of biomass between the time of nitrogen depletion and harvest (**Figure 25**).

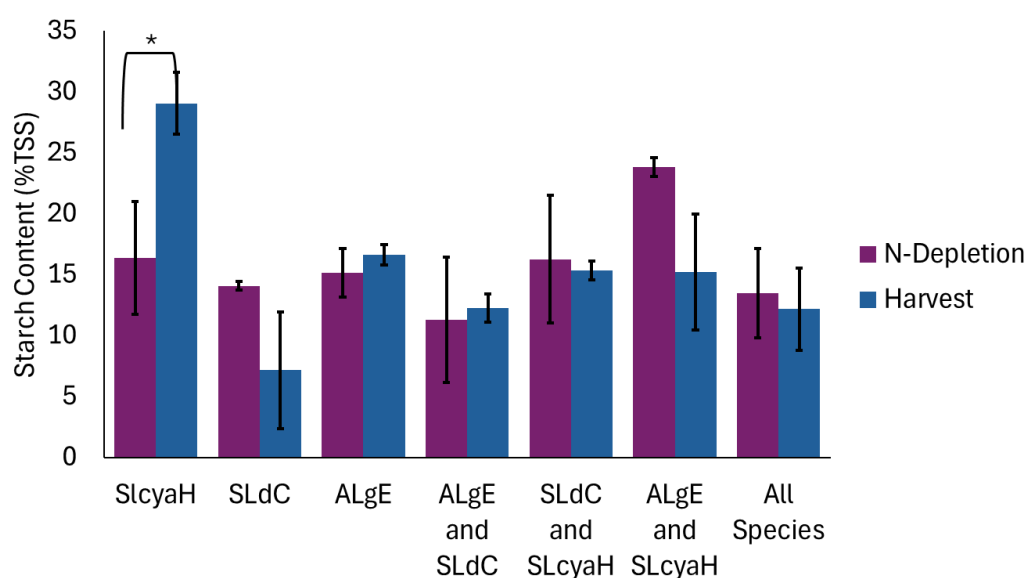


Figure 25: Average starch content at time of nitrogen depletion and at harvest. The asterisk denotes a statistically significant difference ($p < 0.05$)

The accumulation of lipids (estimated as fatty acid methyl esters - FAMES) within the cultures resulted in yields above 15 percent AFDW, except for SLcyaH, which yielded only about five percent of biomass as FAMES (**Figure 26a**). The FAME productivity on a per liter per day of cultivation basis was the greatest when the diatom SLdC was part of the community, with average FAME productivities of $0.0372 \pm 3.48 \times 10^{-3}$ g/(L*day) with no significant difference between the conditions containing SLdC (**Figure 26b**). For comparison with a commonly used

industry standard, the volumetric productivities (in $\text{g}/(\text{L} \cdot \text{day})$) were converted into areal productivities in $\text{g}/(\text{m}^2 \cdot \text{day})$ using the surface area of the liquid-gas interface in the shake flasks and the volume of the culture (**Figure 26c**).

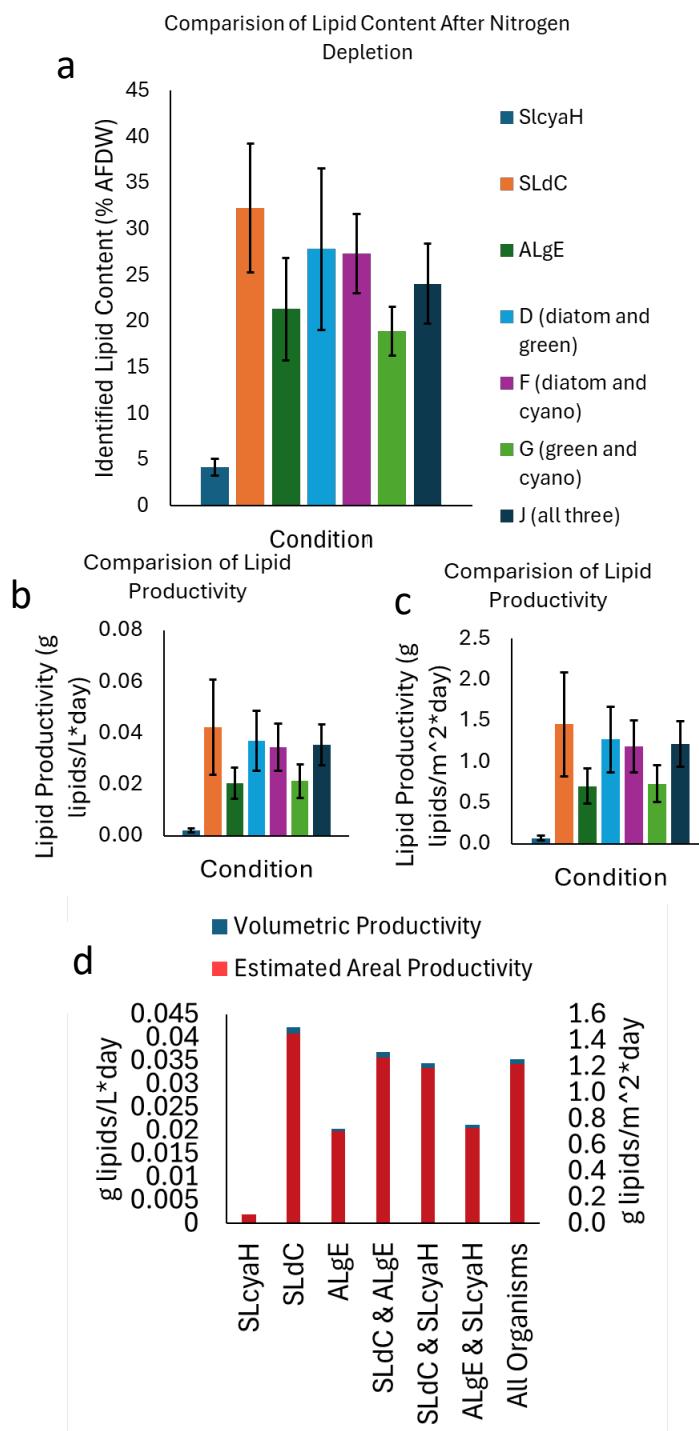


Figure 26: a) The lipid content from monocultures and polycultures. b) The volumetric productivity of lipids from monocultures and polycultures. c) The estimated areal productivity of lipids from monocultures and polycultures. d) A comparison between the estimated areal and volumetric productivity.

A comparison between the lipid content (assessed as FAMES) of the cultures at the approximate time of nitrogen depletion to the lipid content at the time of harvest is shown in **Figure 27**. All cultures except SLcyaH showed an increase in lipid content of five percent or more when normalized to AFDW over the three-day period between nitrate depletion and harvest. This increase indicates that SLdC and ALgE accumulated lipids during the three-day nitrogen starvation phase.

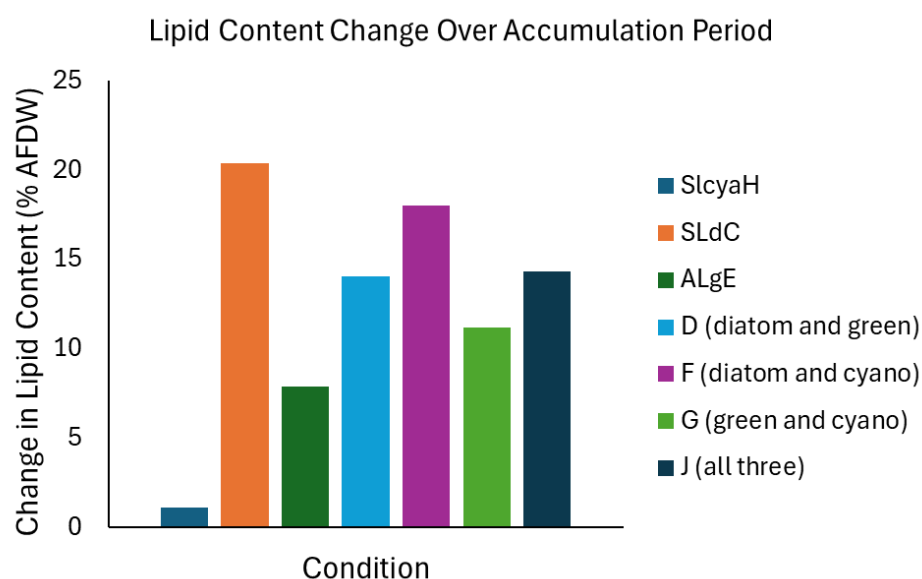


Figure 27 The change in lipid content of cultures in percent AFDW over the three-day period between nitrate depletion and harvest.

As discussed above, the four cultures containing the diatom SLdC contained silicate, as it is necessary for the growth of the diatom; the monoculture contained 4 mM of silicate at inoculation, while the polycultures contained only 2 mM of silicate. The 2 mM of silicate for the co-cultures were chosen based on the preliminary experiments and in an effort to balance the diatom's need for silica and the inhibition of ALgE growth in the presence of 4 mM silicate (*cf.*

Figure 17). A higher concentration of silicate (4 mM) was chosen for the SLdC monocultures as it was observed in preliminary experiments (*cf.* **Figure 13**) that nitrogen depletion was not achieved when the starting silicate concentration was 2 mM. However, in polycultures, multiple strains are competing for nitrogen, resulting in nitrate depletion before silica depletion even in 2 mM silicate containing cultures if the diatom is present (**Figure 28** (left)).

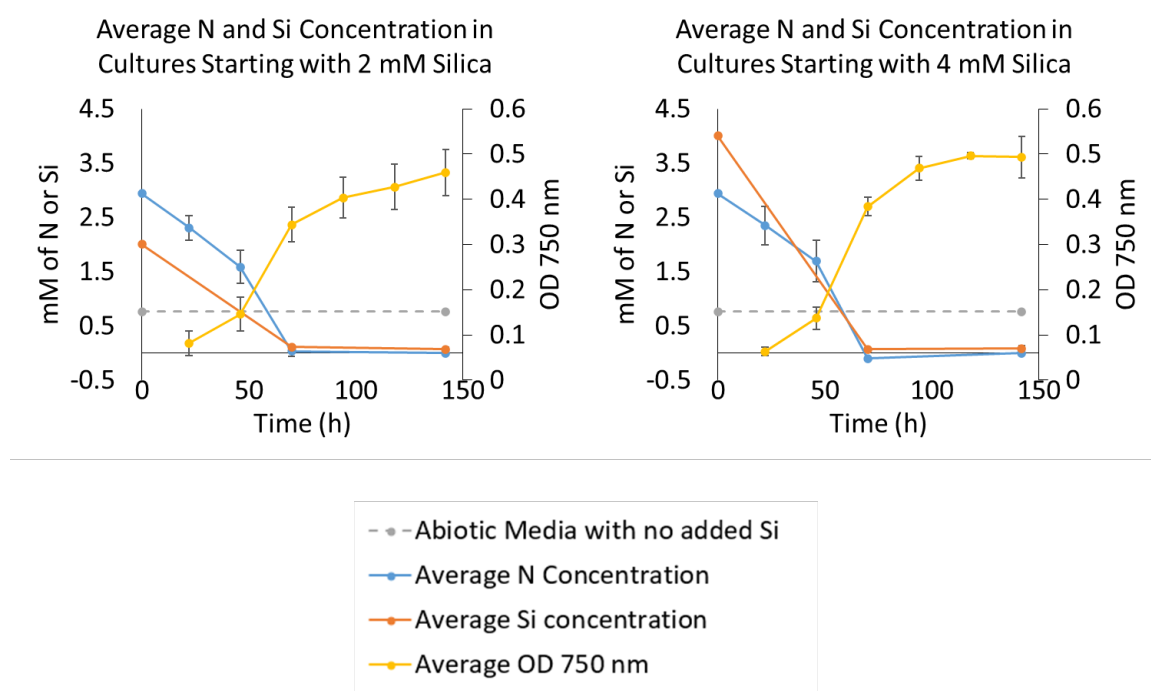


Figure 28: A comparison of Si and N concentrations in polyculture media separated by their starting condition. The horizontal gray-dashed line represents the silicon concentration of abiotic media made without added silica but was kept in a glass container, indicating leaching of silicates from glass.

Samples to determine the silica content were taken at the point of N-depletion and at final harvest to confirm that silica was not a limiting factor for the cultures containing diatoms. The

silica content of the cultures at the point of nitrogen depletion was on average $0.100 \pm 3.28 \times 10^{-2}$ mM (**Figure 28**). When compared to the average nitrogen concentration of those cultures the silica concentration was significantly higher with a p-value of 0.002. Due to algae- and silicate-free medium having silicon present at a larger amount than diatom-containing media at all times, it is suggested that the diatom cultures in the glass shake flasks -while possibly silica limited- were likely never completely depleted in silica due to the leaching continuous leaching of silicates from glass. The potential combined effect of nitrogen depletion and silicon starvation for the diatoms will have to be investigated further in the future. Photos of the cultures at different points in their growth cycle are shown in **Figures 29** through **32**.

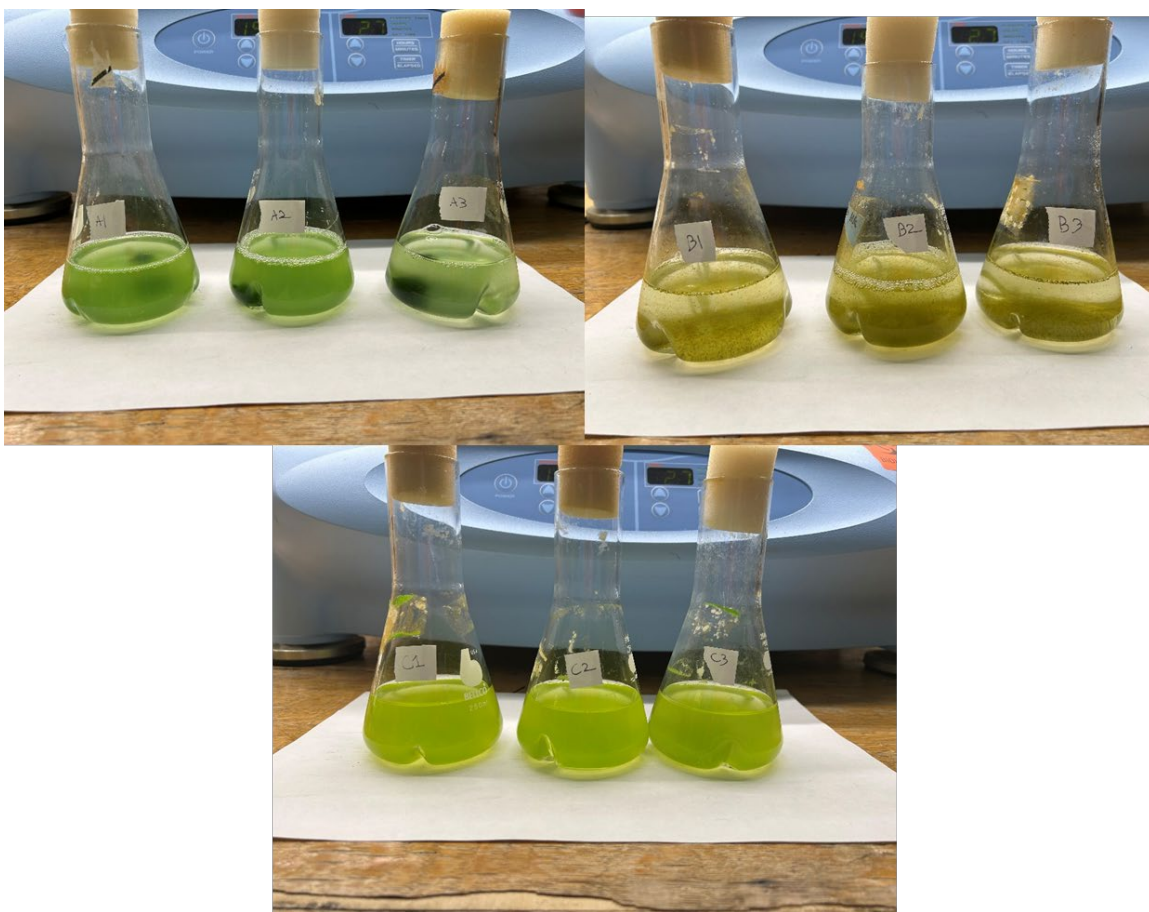


Figure 29: Photos of cultures on day 2 of the experiment cultures a through c: monoalgal cultures of SLcyaH, SLdC, and ALgE.

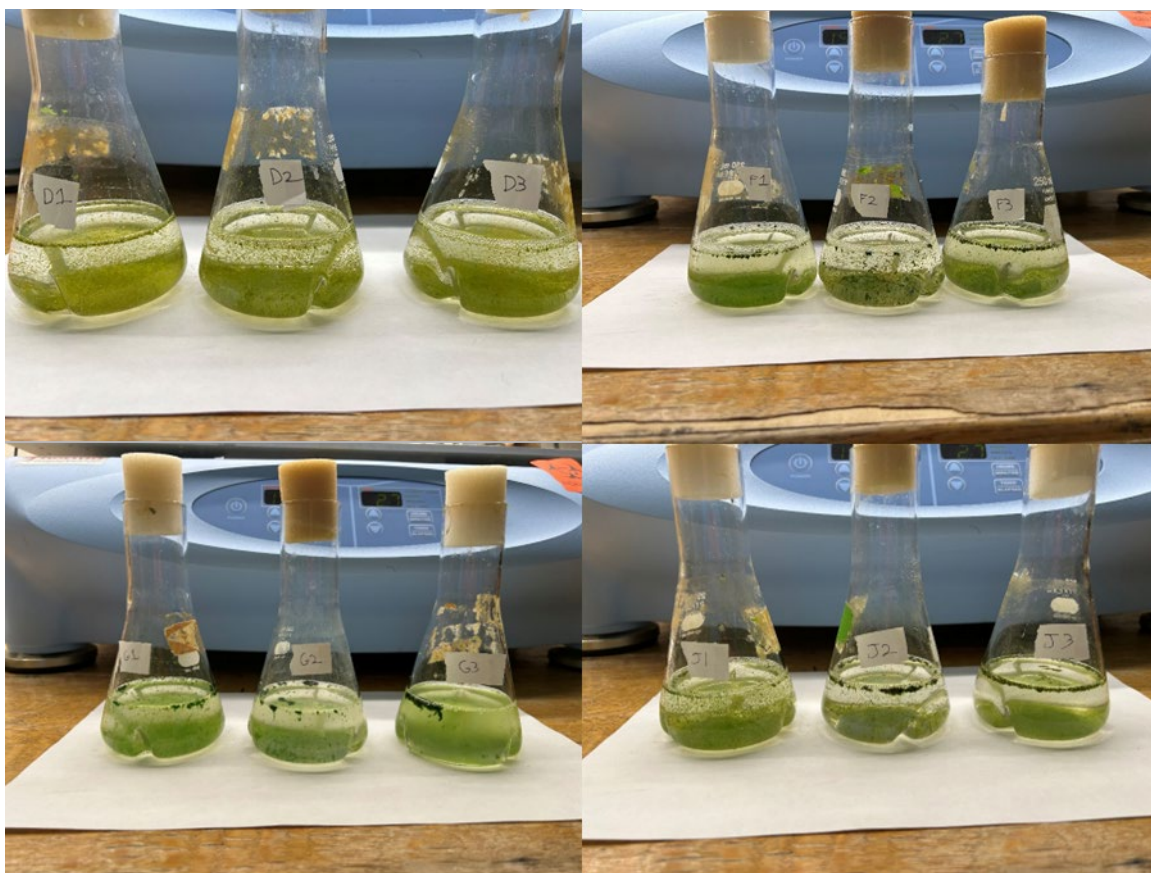


Figure 30: Photos of cultures on day 2 of the experiment cultures D, F, G, and J: SLdC & ALgE, SLdC & SLcyaH, ALgE & SLcyaH, all three species.

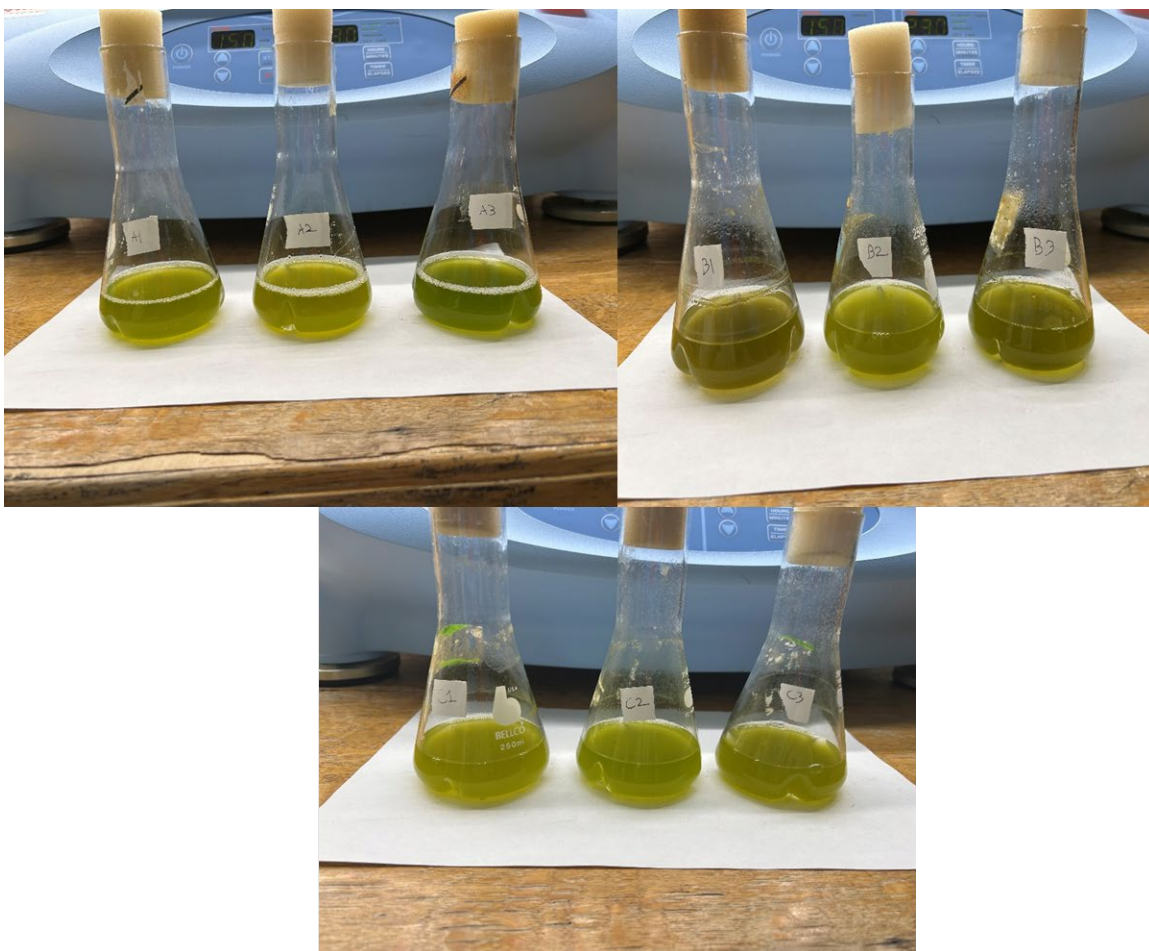


Figure 31: Photos of cultures on the day of harvest of the experiment cultures a through c: monoalgal cultures of SLcyaH, SLdC, and ALgE.

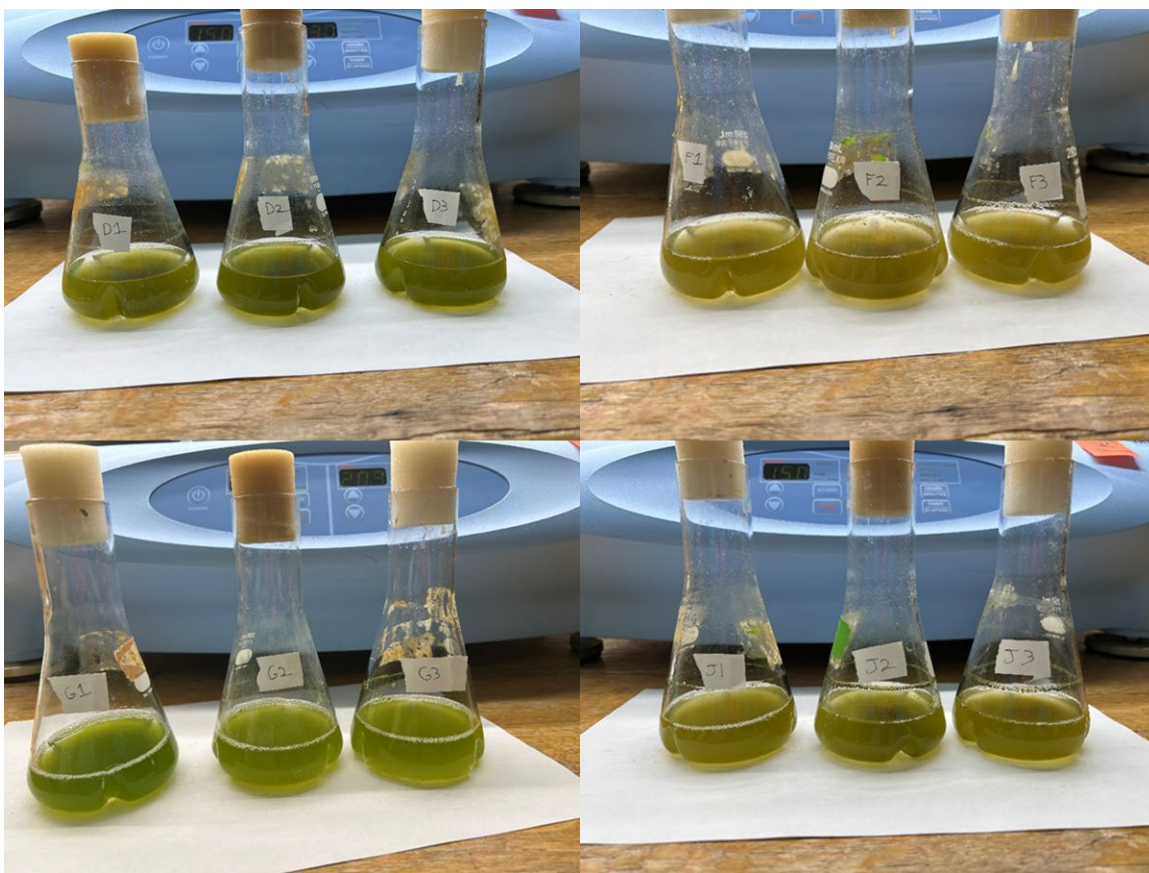


Figure 32: Photos of cultures on the day of harvest of the experiment cultures D, F, G, and J: SLdC & AlgE, SLdC & SLcyaH, ALgE & SLcyaH, all three species

The cell populations of each coculture and the polyculture at nitrate depletion and at harvest are shown in **Figures 33-37**. In each condition a shift in the relative abundance of each species present in the culture suggests that some species survive longer under nitrate depletion than others.

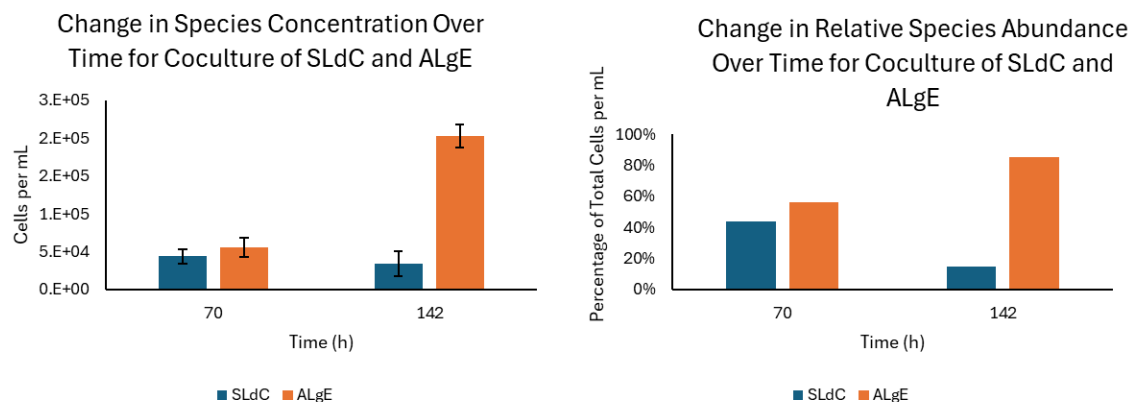


Figure 33: Species concentrations and relative abundances for each species in the coculture of SLdC and ALgE, 70 hrs is the approximate time of nitrogen depletion.

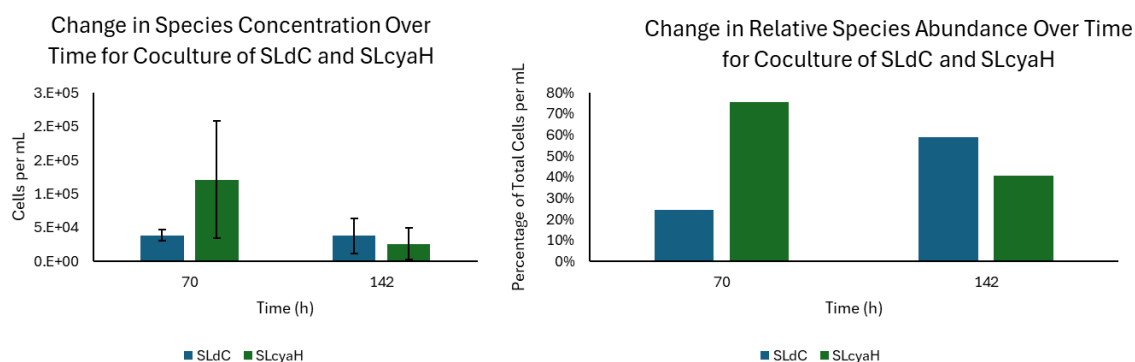


Figure 34: Species concentrations and relative abundances for each species in the coculture of SLdC and SLcyaH, 70 hrs is the approximate time of nitrogen depletion.

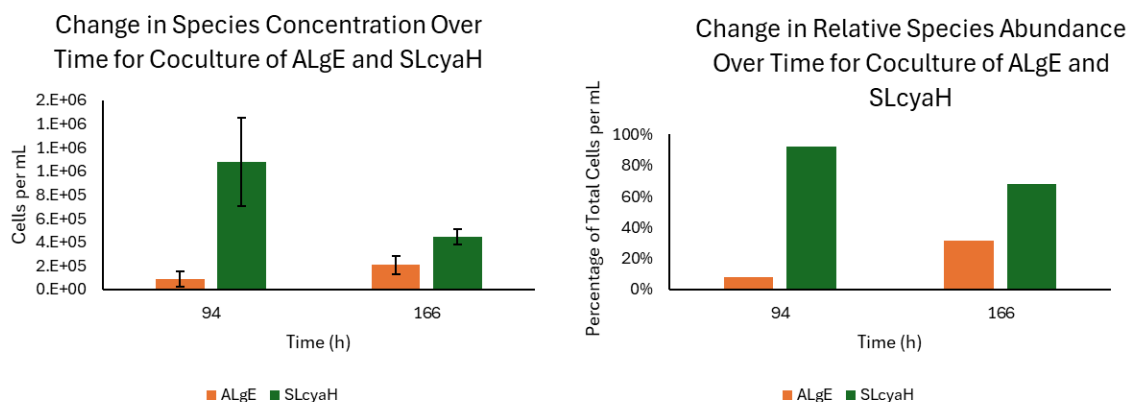


Figure 35: Species concentrations and relative abundances for each species in the coculture of ALgE and SLcyaH, 94 hrs is the approximate time of nitrogen depletion.

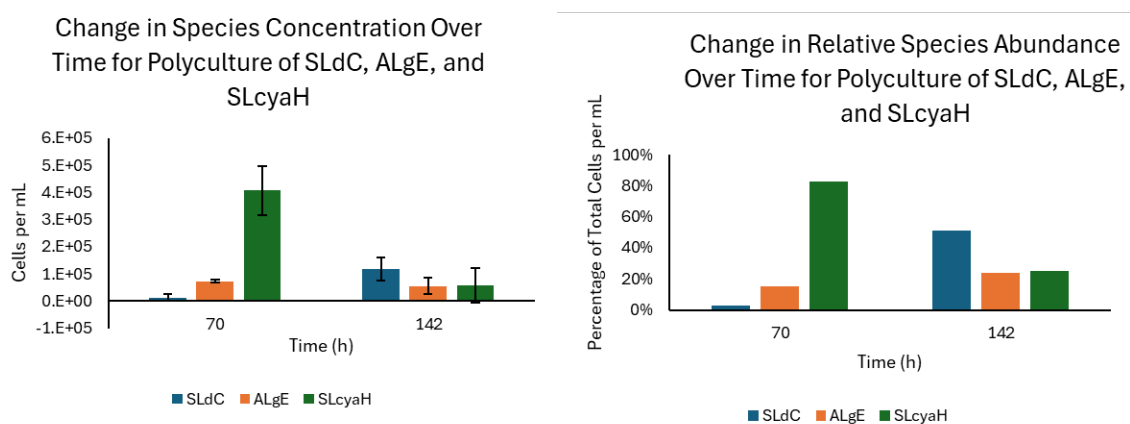


Figure 36: Species concentrations and relative abundances for each species in the polyculture, 70 hrs is the approximate time of nitrogen.

CHAPTER FOUR

CONCLUSIONS

The production of high value and commodity products by algae and other photoautotrophic organisms, such as plants, has great potential. Indeed, microalgae (including diatoms, green algae, and cyanobacteria (aka as blue-green algae and included here for ease as ‘algae’)) are responsible for most of the petroleum (rock-oil) currently used by the world’s population just created millions of years ago. Cultivating algae and turning the grown biomass into fuels, chemical feedstocks, etc. would reduce the need for drilling, fracking, etc. to produce fuels and materials essential to our economy.

This thesis evaluated the growth of three different algal strains obtained from high pH environments, which demonstrated a tolerance and even a preference for higher alkalinity growth conditions, which can provide a growth environment that is not rate-limited by the availability of inorganic carbon (i.e. bicarbonate (HCO_3^-) and carbon dioxide (CO_2)). Enrichment cultures dominated by the diatom SLdC (from Soap Lake, WA), the green alga ALgE (from Alkali Lake, WA), and the cyanobacterium SLcyaH (from Soap Lake, WA) were selected from a total of more than 20 different enrichment cultures based on their biomass and lipid content and productivity, and then made the subject of an in-depth study with the goal to optimize biomass and high value product (*e.g.*, carbohydrate and lipid) generation, as well as optimize cultivation conditions and costs. Initially, monoalgal cultures (monocultures) were investigated, before polyalgal cultures (polycultures) were compared. All strains used were xenic, meaning they were cultivated in the presence of their microbiomes (*e.g.*, bacteria).

Growth of Monocultures in High and Low Alkalinity

Strain ALgE tentatively identified as a *Desmodesmus* species, when grown under high alkalinity conditions, results in a higher productivity for its storage compounds due to the increase in nitrogen consumption. When this species is grown under high alkalinity conditions, more storage compounds are observed as indicated by Nile Red fluorescence and the accumulation of FAMES in the cell. C16 and C18 dominate the FAMES assembled with varying degrees of saturation. In general, ALgE performs similarly to *Chlorella* sp. and has some tendency to clump or become attached to surfaces.

Strain SLcyaH tentatively identified as a *Geminocystis* species, was found to grow slower, but be more abundant in terms of cell counts when grown under low alkalinity conditions compared to high alkalinity conditions. However, when compared at similar times, the conditions do not show a significant difference in growth metrics. The low alkalinity conditions did have more cells at the time point of 232 hours but not significantly different from the high alkalinity conditions and did not have a difference in OD 750 nm measurements. This suggests that under high alkalinity conditions, the cell size of species SLcyaH is larger than for SLcyaH cells grown at low alkalinity conditions. The FAME content was similar between conditions, around 5% of biomass, but the high alkalinity condition indicated the presence of C18:2 FAMES, while the low alkalinity condition did not. This could be a result of an adaptation to higher alkalinity (and thus salinity), as more unsaturated membrane lipids lend more fluidity to the membrane (Mikami and Murata, 2003).

Strain SLdC tentatively identified as a *Craticula* species, created major challenges when grown in the tube reactor design, as its preference for benthic growth made collecting representative samples hard. However, SLdC produces the most lipids out of the three tested,

around 0.5 g/L, and the profile of the FAMES shows that the species likely creates oils made from containing long-chain polyunsaturated fatty acids such as C20:3, C20:4, and C20:5, which have an order-of-magnitude higher commercial value than oils containing shorter and more saturated fatty acids. Strain SLdC, however requires silica in the growth medium, and the associated costs for providing silicates have to be a consideration for the large-scale growth of the species. The results obtained in the monoculture experiments under fluorescent lights were conducted under a silica limitation (but likely not nitrogen limitation) and further testing and characterization of the cultures might be necessary to better understand the interplay between nitrate as the limiting nutrient and not silica. Part of this work was conducted as part of the polyculture experiments.

Growth of Polycultures

The comparison between polyculture combinations and their monoculture members showed that strain SLdC, when grown as a monoculture with 4 mM silica, had the fastest growth rate but the growth rate was not statistically, significantly different from the polycultures that contained this species and only had 2 mM silica. In terms of productivity of storage compounds, the monocultures did not show a significant difference from the polycultures, except when comparing the lipid accumulation of the SLcyaH monoculture. The use of polycultures has the potential to allow for some control over the product spectrum by promoting the growth of the species with the product spectrum closest to the desired one to be dominant. Also, considering that only half of the silica is used to cultivate polycultures containing SLdC, it is recommended to have a polyculture containing the diatom when growing on an industrial scale due to the lower cost of the media components, while maintain approximately the same level of productivity. Polycultures appear to have a greater tendency to clump together, and while this could result in

reduced photosynthetic efficiency, it may also provide significant benefits in terms of protection from predators and allow for a more effective harvesting method without the use of flocculants. Future work will investigate the microbiome of all conditions by being sequenced for comparison of their members and their potential effects on axenic cultures.

Summary of Findings

Under high alkalinity growth, strain ALgE had a faster generation time of 0.9 days and lipid productivity of 15.6 mg/(L*day) over nine days than its low alkalinity counterpart. Strain SLcyaH was found not to have a significant difference between high and low alkalinity conditions when comparing at the same time points. SLcyaH also had a generation time of 42.4 ± 0.661 hours under high alkalinity conditions and 37.5 ± 5.92 hours under low alkalinity conditions. The OD 750 nm and cell counts (concentration of cells) do suggest that under high alkalinity conditions, the cyanobacterium grows into larger cells. Also, under high alkalinity conditions, the cyanobacterium produces unsaturated C18 lipids that are not seen in the low alkalinity condition, possibly as a result to stress experienced under high alkalinity conditions. The growth of the diatom SLdC did not show a significant difference between high and low alkalinity, but due to the tendency to prefer benthic (*i.e.*, attached) growth, causing variability in sampling efficiency and representativeness, a possible silica limitation more work needs to be performed (where the culture and samples are more homogeneous and have excess silica). The diatom produced the most lipids out of all three species at 0.50 ± 0.023 g/L under low alkalinity conditions and 0.82 ± 0.56 g/L under high alkalinity conditions. The lipids produced were longer and more unsaturated than the lipids found in the other two species. This is important because lipids like C20:5 are more valuable as nutraceuticals than for use as an energy source (*e.g.*, fuel). Under high alkalinity conditions, the pH of all species did not fluctuate as much as it did under

low alkalinity conditions. When grown with 4 mM silica, the diatom SLdC had a generation time of 30.5 ± 2 hours, and when growing as a polyculture with the other members of this study, SLdC did not show a significant difference to its growth as a monoculture in terms of growth rate and productivity. On average, the polycultures containing SLdC produced 37.2 ± 3.48 mg lipids/(L*day) compared to the diatom monoculture producing 42.2 ± 18.4 mg lipids/(L*day). Considering that the diatom culture had double the amount of silica, the 13% increase in productivity might not be worth the additional cost of the silica input for the media to just grow the diatom. The polycultures exhibited a higher tendency to clump together, which can aid in the harvesting of the culture in industrial settings. Clumping may also provide other benefits, such as resistance to predation or oversaturation of light.

Future Work

Further research into the potential benefits of using polycultures is needed and the mechanisms that create these benefits. One thought is the microbiome of each algal species is naturally selected to best support the community and may provide better resistance to potential culture crashing bacteria and viruses. Research also needs to be done on species SLdC to determine the differences between nitrate depleted lipid accumulation and silica depleted lipid accumulation, and to evaluate whether a combined silica and nitrate depletion could enhance the accumulation of lipids. This potential work was explored in another diatom species by Moll et. al. (2014).

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APPENDIX

ADDITIONAL DATA ON SPECIES ALGE, SLDC, AND SLCYAH

Table 5: Elemental analysis results from algae harvested after lipid accumulation compared to Redfield Ratio

Moles of in 1g	C	N	H
SLdC	3.03E-02	2.13E-03	5.68E-02
AlgE	3.75E-02	2.51E-03	6.52E-02
SLcyaH	3.47E-02	4.90E-03	6.68E-02
Redfield Ratio	106	16	

Species SLdC and ALgE after lipid accumulation resulted in a carbon to nitrogen ratio that is double the Redfield Ratio of 6.625. Species SLcyaH resulted in a carbon to nitrogen ratio of 7.