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Development of Phagotrophic Algae-based Process for Conversion of Waste Organics to Algae Lipids as Potential Feedstock for Biofuel

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Honors Abstract Addendum

Interest in sustainable feedstock for biofuel has encouraged research on the production of phagotrophic algae from bacteria using a two-staged continuous-flow system. Bacteria cultures were fed the content of a waste stream from The J.M. Smuckers Company containing high concentrations of sugar. In the design, the bacteria effluent overflowed into the algae tank which was kept at a set volume. Several methods were developed to assist in maintaining the system at steady state and minimize the impact from contaminants. A rigorous cleaning protocol was implemented for both tanks, effectively eliminating protozoa from the bacteria system. The protozoa were decreased in the algae tank by decreasing the dissolved oxygen to 5% or less. Fungi were removed from the algae tank by filtering through a mesh wire. An effective method for maintaining steady state was established and implemented during research to reduce time spent in lab. Data was collected for optical density, cell concentration, and dissolved oxygen to assess the health of the system. Results and observations suggest the feasibility of this method for producing phagotrophic algae, but since it was difficult to maintain the system at steady state, more trials need to be conducted to gather data for analysis.

Executive Summary

In response to the increasing demand for alternative and renewable energy sources, biofuel has emerged as a viable option. Research has been focused on identifying a sustainable feedstock that would make biofuel competitive with petroleum-based fossil fuels. Continued use of current feedstocks raise concerns regarding the use of arable land and an increase in the price of crops. A promising biomass is algae, specifically phagotrophic algae which grows by consuming other microorganisms such as bacteria. Initial research suggests harvesting phagotrophic algae from a two-staged continuous-flow system using some waste product as the substrate for bacteria growth.

The focus was to optimize algae concentration by stabilizing bacteria, performing batch cultivation of phagotrophic algae, and to set-up and complete experiments with a two-staged bacteria-algae continuous culture system. The initial stage of the research was to determine an efficient set-up of all required materials and equipment. A custom bacteria tank was designed that allowed the volume to remain at 400-mL and the effluent to be directed into the algae tank. The main nutrient supplied to the system was a waste product provided by The J.M. Smuckers Company and had a high concentration of sugar. The other nutrient supplied to the system was a concentrated salt solution.

There were several obstacles encountered in the first months of research related to maintaining the bacteria tank in steady state and preventing contamination from other microorganisms. These difficulties led to the development of several methods and protocols that can be used in future research. Protozoa was eliminated from the bacteria tank through the implementation of a rigorous cleaning protocol. Protozoa was minimized in the algae tank by decreasing the dissolved oxygen percentage to less than 5%. This was supported by data that was obtained, showing a correlation between the percentage of microorganisms that were protozoa

compared to the amount of dissolved oxygen. An outlet pump was also placed on the bottom of the algae tank that allowed heavier particles, such as fungi and protozoa, to be periodically removed. To remove fungi, the contents were filtered through a wire mesh during each cleaning.

The continuous system was operated without interruption from November 4th, 2020 until February 15th, 2021. While there was a significant amount of quantitative data collected, there were many qualitative aspects that added to this project's complexity. Observations and data collected were equally important when making decisions on the direction of the project. Cell dry weight, dissolved oxygen, and optical density were used when analyzing the health and productivity of the system.

There are several implications based on this research. First, a sustainable feedstock would encourage corporations and governments to invest more into biofuels and renewable energies, spurring new research. Second, if a continuous-flow process for the production of healthy algae is established, this can be marketed to industries that produce waste capable of being reduced through the use of microorganisms. Immediate implications are new methods to reduce and prevent contamination from other microorganisms from taking over an open biosystem.

It is highly recommended that this project to be continued. The continuous-flow system established showed success related to maintaining bacteria production in steady state, however, it was difficult to sustain healthy algae growth for longer periods of time. Batch algae experiments should be conducted to determine what is limiting its growth and then to use those results to adjust the system design for optimal algae production. A significant obstacle was the time required to maintain the system, as well as the sheer number of variables and parameters that needed to be observed and considered. If this project is continued, there should be at least two students, if not more, assigned to it in order to achieve maximum results.

Introduction

The demand for alternative and renewable energy increases each year and remains an attractive option for investment from both governments and corporations. The ability to produce energy without harmful emissions or damage to the environment are just two components of the equation. Other important aspects that should be considered include sustainability and practicality. A viable option is biofuel, a form of renewable energy that is produced from geological processes in much shorter cycles than compared to fossil fuels [1]. The global market is expected to grow substantially from $141 billion in 2020 to over $300 billion by 2030, encouraging further research [2]. Current feedstocks used for the production of biofuels include corn, sugar cane, animal fats, and vegetable oils. The continued use of these feedstocks raises concerns regarding use of arable land, excessive fertilization, and an increase in the price of crops and their products [3].

In response to these concerns, microalgae have emerged as the biomass to supplement and potentially replace other feedstocks. It requires less arable land for cultivation and produces more oil than alternatives [1]. According to the University of Michigan, “algae could potentially produce 10 to 100 times more fuel per acre than other crops” [3]. The production of algae has been primarily focused on utilizing a photosynthetic process, but there are several obstacles that prevent it from being an economically viable option. Some of the challenges include growing large amounts of phototrophic algae outdoors in variable conditions, invasive species competing for nutrients, and a lack of light penetration as concentration of algae increases [4].

In the past few years, an alternate method of algae cultivation using phagotrophic algae has gained interest among researchers. Phagotrophic algae consumes bacteria in order to grow and does not require arable land [5]. A promising, experimental design was analyzed and evaluated by Lu-Kwang Ju, chemical engineering professor at The University of Akron, and Cong Li in 2014

[5]. The two-staged design utilized a continuous-flow system to cultivate bacteria from organic matter found in wastewater. Since 2014, there have been further attempts to improve upon this process and establish phagotrophic algae as a competitive feedstock for biofuel production.

To support the main goal of achieving sustainable biofuel production, this research project's focus was to optimize algae concentration generated in a continuous system. This can be accomplished by completing the following three objectives. The first is to stabilize bacteria growth in a continuous-flow process and to evaluate the continuous-flow process at various operating conditions. The second is to perform batch cultivation of phagotrophic algae and identify ideal conditions that promote healthy algae growth. The third is to set-up and complete experiments of a two-staged bacteria-algae continuous culture system.

This project is supported by current and past research conducted at The University of Akron by Dr. Lu-Kwang Ju. During the summer of 2020, an undergraduate student working with Dr. Ju established parameters that were used as a control for the continuous process. The student determined the optimized nutrient concentrations and settings for the pump to dispense those nutrients. Based on the status of both Dr. Ju and the student's research, this research project followed the suggested path presented by the results and conclusions.

Continuing research on the development and cultivation of a phagotrophic algae process is necessary to achieve the long-term goal of producing biofuels reliably and sustainably. Utilizing algae and bacteria biomass as the feedstock to support and replace both renewable and non-renewable energy sources requires fewer resources than the production of other feedstocks, including fuel crops or animal fat. An additional benefit and potential outcome of this research is the development of a process to be marketed to companies producing wastewater that has a high

concentration of organics. The cultivation of phagotrophic algae deserves further research as it is related to biofuels, which will remain a central part of renewable energies in the future.

Background

History of Biofuels

Despite the current excitement and interest centered around biofuel, this renewable energy source has an ancient history. From their earliest days, humans have been burning wood to attain warmth and nourishment. Jumping forward several thousand years, it was well known that alcohol was a viable and efficient fuel, however, it was not competitive with the government supported, petroleum-based fossil fuels [6]. Significant strides in engine and automotive development were brought to realization through the use of fuel mixtures containing alcohol, however, biofuels lost their prominence as governments incentivized the use of oil and gas. It was not until the 1990s that a strong desire to develop and promote cleaner, more sustainable energy sources emerged. As environmental concerns increase and the global oil supply remains unstable, biofuels will continue to be of interest.

Microorganisms

A central component of this research project was the use of microorganisms to convert organic materials into simpler substances. The two-staged continuous system took advantage of some special characteristics of both bacteria and algae, while other methods were used to eliminate protozoa and fungi.

Bacteria

Bacteria are small, single cell organisms that form a domain consisting of prokaryotic cells. Bacteria are one of the earliest life forms to have existed on Earth and are extremely resilient,

capable of living in extreme environments. They obtain their carbon through either an inorganic compound such as CO_2, or an organic compound such as glucose [7]. As a result of their metabolism, bacteria are capable of breaking down organic materials and perform many essential functions for almost all life-forms. They are prevalent in various industries including the preparation of fermented food, treatment of wastewater, and production of fertilizers. Bacteria are fast-growing organisms, capable of reproducing within minutes, which makes it an ideal microorganism to use for the first stage of this project.

Algae

Algae belong to the domain Eukarya and include both unicellular and multicellular species. Most algae are phototrophic, but there are some that are able to obtain energy from both photosynthesis and from the absorption of carbon substances, which is known as phagotrophic. The growth characteristics will vary depending on environmental conditions but expected doubling time can be from 7-20 hours [8]. Algae cultivation is more susceptible to contamination from other microorganisms than fast growing bacteria since they have a longer doubling rate.

Fungi

Fungi is a kingdom of life that contains eukaryotic organisms such as yeast and mold. Fungi are saprophyte heterotrophs, meaning that they consume dead or decomposing organic compounds as their energy and carbon sources [9]. Fungal organisms can reproduce asexually through budding and production of spores and some species can reproduce both asexually and sexually through alteration of generations. Fungus comes in different shapes and sizes and can range from single cell organisms to branched, filamentous structures.

Protozoa

Protozoa are also eukaryotic, single cell organisms found in aquatic habitats [10]. Protozoa can be either phototrophic or heterotrophic and a defining characteristic is that they are highly mobile due to their flagella. They feed off of bacteria and are seen as competition to be eliminated in this particular project.

Wastewater Treatment

A prevalent problem that occurs globally is the contamination of wastewater due to organic material. These organic pollutants include pesticides and herbicides used in agriculture, oil and food from manufacturing of food and beverage products, and other industrial compounds [11]. The presence of these contaminants is dangerous to both the environment and humans, but can be removed through the use of chemical, centrifugal, filtration, and other methods [12]. A natural wastewater treatment known as biodegradation utilizes bacteria, fungi, and other plants to transform wastewater organics into harmless substances. This process can be harnessed for commercial use and could even support an increase in biofuel production. In addition to the fast-growing bacteria consuming the organics found in the wastewater, as it grows it can be harvested for phagotrophic algae cultivation.

Previous Research

This project was based on the research of Dr. Lu-Kwang Ju and supported by two of his publications, of which he was the primary author. The first publication was titled "Conversion of wastewater organics into biodiesel feedstock through the predator-prey interactions between phagotrophic microalgae and bacteria" [5]. The article shares a new cultivation method of phagotrophic algae using wastewater for the carbon source consumed by bacteria, which will then be consumed by the algae and harvested for biodiesel feedstock. It discusses a two-staged continuous-flow process that takes advantage of the fast-growing characteristics of bacteria and

the phagotrophic nature of a certain species of algae, *Ochromonas danica*. Cultivation conditions for this alga were determined and included a pH range, dissolved oxygen percentage, and agitation speed. The continuous-flow process was evaluated with wastewater to determine the retention times for both tanks. Many of the results and conclusions drawn in this article were used throughout the set-up of this project's experimental design and it was referenced to compare observations made on the system in order to identify trends in the data collected.

The second publication was titled Growth and Lipid Production of a Phagotrophic Alga Feeding on *Escherichia coli* Cells: A New Approach for Algal Biomass and Lipid Production from Wastewater Bacteria" [8]. The purpose of this report was to utilize the continuous system established in the previous experiment and to determine if it was a feasible application for wastewater treatment. The research concluded that *O. danica* has the potential for cell growth on *E. coli* under various conditions. This article will be useful for lipid extraction from algae if the project is continued with another student and the ideal conditions for healthy algae growth are established.

Experimental Methods

Experimental Design

The first step to conducting research on a continuous system for bacteria and algae production was to determine an efficient set-up of all required materials and equipment. There was limited space in the lab and it needed to be confined to the available counterspace. The final configuration is shown in

Figure 1 depicts the final set-up for the bacteria-algae continuous biosystem. The process starts with two pumps on the right side supplying the required nutrients to the bacteria

tank. A custom beaker with a glass tube extension is placed above the algae tank so that the effluent can be continuously supplied. A pH probe is placed in each tank and a base solution is pumped into the bacteria tank to maintain the pH and both an acid and base solution are pumped into the algae tank to maintain the pH within a specific range. Two outlet pumps are used to remove algae biomass and collected as waste to be disposed of properly.

. Most of the equipment was obtained from the lab, but the bacteria tank was a custom design. Based on a preliminary sketch of the final configuration, the bacteria tank dimensions were calculated to ensure that it would be at an adequate height above the algae tank so that the effluent could overflow. A 1000-mL beaker was taken to the glassblower located in the Goodyear Polymer building on The University of Akron's campus, along with a sketch of the design. The request was to attach a glass tube to the outside of the beaker. The desired retention time was 6-hrs which required the volume of the bacteria tank to be 400-mL. The glass tube was to be attached at the 400-mL mark so that the volume would remain constant as nutrients were continuously added and the effluent of bacteria cells overflowed to the algae.

Once the modified beaker was completed, the system was pieced together. In **Figure 1**, the process begins on the right side of the image and proceeds to the left. There are three pumps located to the right of the bacteria tank. Two are for delivering nutrients to the bacteria tank which includes a concentrated salt solution (CSS) and the carbon source, which was waste obtained from The J. M. Smucker Company. The third pump delivered a base solution to maintain the bacteria tank at a pH of 7, since it tended to be more acidic.

The bacteria tank was kept on top of stir plate and within the tank there was a stir bar to agitate the contents and ensure a well-mixed system. The stir plate was kept at level 7 as suggested by Lu and Li [5]. An air stone controlled by a flow meter provided the system with the required

oxygen. A pH probe was submerged in the tank contents to ensure that the bacteria system remained at the desired pH level.

The algae tank had several inputs, outputs, and monitoring devices. As the nutrients were added to the bacteria tank, the effluent would overflow into the algae tank when the volume exceeded 400-mL. This was the only nutrient provided to the algae and any disturbances that occurred that affected the bacteria growth, would in turn also affect the algae growth. The algae tank was kept on a stir plate and the stir bar agitated the system at level 7 to ensure it was well-mixed. For both the bacteria and algae tank, it was important to not exceed level 7 on the stir plate so as to avoid damaging the cells [5]. Instead of an air stone, tubing attached to a flow meter was placed directly in the contents of the tank. This was to provide gentle aeration and avoid damaging the algae cells. Another pH probe was submerged in the tank contents and to keep the pH between a specified range. Since there was both a lower and upper limit to the pH range, there were two separate pumps to supply an acid solution in addition to a base solution. The volume of the algae tank was kept constant through the use of two outlet pumps. One outlet tubing was kept on the bottom of the tank and periodically removed a few milliliters. This was one of the methods developed during the project to assist in the removal of protozoa and other contaminants. Since they were heavier than the bacteria and algae, they would settle at or near the bottom of the tank, allowing the pump to remove them. The second outlet pump was kept at the desired volume level and was operated constantly to remove any excess liquid. Both of the outlet pumps were directed into a waste tank, where the collected biomass was autoclaved and then disposed of accordingly.

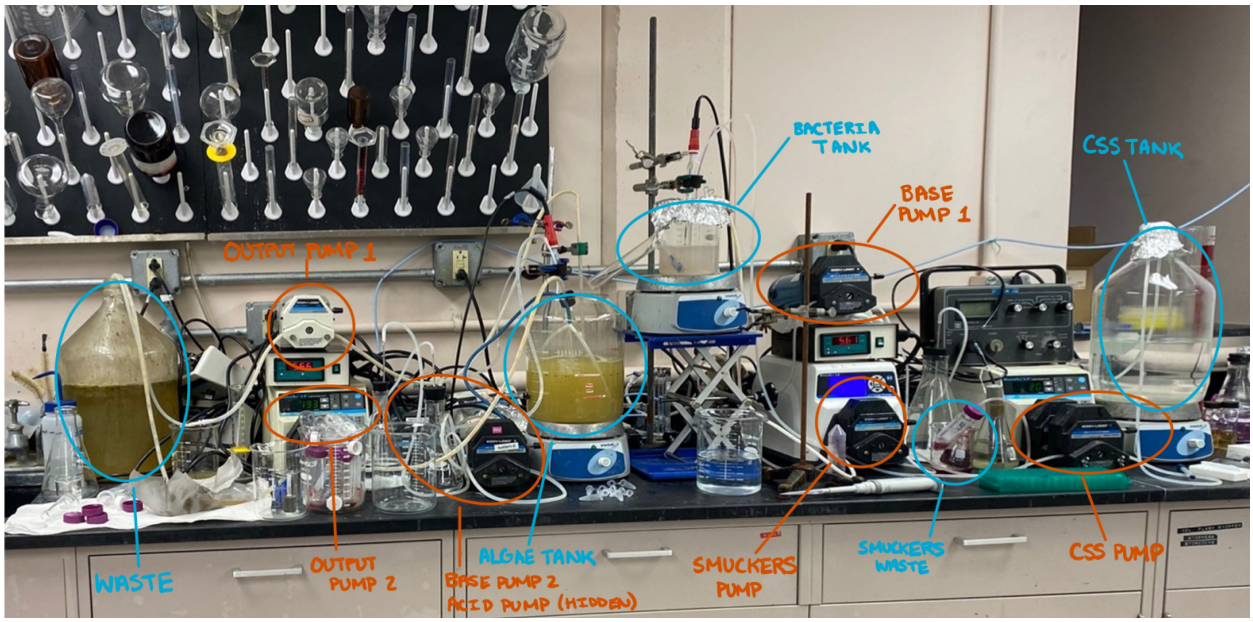

Figure 1 *depicts the final set-up for the bacteria-algae continuous biosystem. The process starts with two pumps on the right side supplying the required nutrients to the bacteria tank. A custom beaker with a glass tube extension is placed above the algae tank so that the effluent can be continuously supplied. A pH probe is placed in each tank and a base solution is pumped into the bacteria tank to maintain the pH and both an acid and base solution are pumped into the algae tank to maintain the pH within a specific range. Two outlet pumps are used to remove algae biomass and collected as waste to be disposed of properly.*

Cleaning and Maintenance

Steady State

When operating the continuous system, it had to run for several days before reaching steady state and once that had been achieved, it was maintained at those conditions to record values and observations for further analysis. A significant amount of time was required to perform the daily maintenance on the system. To minimize the time spent in lab, an efficient approach to accomplishing the daily tasks was created and is listed in **Table 6**. This checklist was developed over many weeks and hours spent in lab. It provides a systematic approach to reduce the amount of time spent on maintaining the system.

When arriving at lab, the first task was to take a picture of the entire system. This was an efficient way to capture a lot of information that could be easily referenced if data related to pumps, pH, and tank color was needed. Next, the algae pH, tank color, and liquid level was recorded. A

sample of the algae was obtained using a pipette and placed on a glass microscopic slide for viewing. Once the microscope has been adjusted and the contents of the slide were visible, a picture was taken. The composition of the algae sample was also recorded based on visual observation. The percentage of algae, fungi, and protozoa present in relation to each other was visually determined and recorded.

Moving to the bacteria tank, a short video of the contents was recorded so that the thickness, density, and color of the contents can be referenced. The bacteria pH and tank color were recorded and as with the algae sample, a small amount of bacteria sample was viewed underneath the microscope and checked for contamination. The bacteria used in this project was difficult to see with the lab's microscope, but the contaminants were larger in size and would be visible in the sample if present in the tank.

Next, the dissolved oxygen (DO) percentage of both tanks were measured. If the DO% was not within the desired range, then the flow meters connected to the airlines were adjusted. To minimize contamination, the algae and bacteria tanks were cleaned almost every day. After the tank cleaning was completed, three 0.200-mL samples were taken from the bacteria tank and dispensed in three cuvettes to test the optical density (OD). One 10-mL sample was also taken from the bacteria tank and dispensed into a centrifuge tube to be used for cell dry weight. After the cell dry weight sample had been prepared and the OD measurements completed, the remaining system checks listed in **Table 6** were verified. For the algae tank, the outlet pumps were checked to be operational to avoid exceeding the desired volume, the contents of the acid and base flasks were checked to ensure there was enough solution to keep the algae pH within the desired range, and the air flow was checked to ensure adequate aeration. If the flow meter had been adjusted, the DO% should be remeasured here.

The final checks for the bacteria tank included observing that the air stone was supplying oxygen, the stir bar was spinning, the sugar line and CSS lines were clear, and that the sugar flask was refilled. The final configuration shown in **Figure 1** had the Smuckers' waste contained in a 50-mL flask. This was meant to prevent the carbon source from being depleted and to avoid any negative affects it might have on the algae production and growth.

Weekly Maintenance

The previous section consisted of the daily tasks that needed to be accomplished in order to maintain steady state. There were a few tasks that were done throughout the week and included calibrating the pH probe, preparing CSS vials, preparing Smuckers supernatant, and disposing of the waste properly. These tasks usually took place every 3-4 days, but there were checks included in **Table 4** to ensure the system would remain operational until the next day.

Tank Cleaning Protocol

Throughout the completion of this project, it became evident how important thorough cleaning was to minimizing contamination of the system. The following bacteria and algae tank cleaning protocols were developed in the lab and once implemented, there was a significant decrease in contamination.

To clean the bacteria tank, the following materials were needed: one 1-L beakers, one 600-mL beaker, wire mesh, soap, ethanol, DI water, and paper towels. First, the pH probe was removed from the bacteria tank and secured in the spare test stand with the 600-mL beaker placed beneath it. The pH probe was sprayed with water and gently wipe with a wet paper towel to remove built-up residue. It was then sprayed with ethanol and patted dry. Next, the Smuckers sugar line and CSS line were cleaned by dismantling them from both the bacteria tank and their pumps. Dismantling either line should be done when there is enough time remaining on their respective

pump, but if the time is going to reach zero, press pause until the lines are reassembled. The sugar and CSS lines were brought over to the lab sink and had one end of the tubing placed in the faucet head and the other end pointed at the drain. The faucet was turned on slowly and a high stream of water was forced through the tubing, removing built up sugar or salt that could cause blockages and prevent nutrients from being delivered. A paper towel sprayed with ethanol was used to wipe the outside of the lines before re-attaching them to the system and pumps. Next, the air stone was removed from the bacteria tank and any biomass and contaminants were cleaned off with water in the sink. A dry air stone (new or previously cleaned) was attached to the air line and set in the 1-L beaker. The stir plate was turned off and the contents of the bacteria tank were gently poured through a wire mesh into the beaker. Once emptied, the bacteria tank and stir bar were washed with soap in the sink and rinsed. The tank was then wiped with an ethanol-soaked paper towel. After drying, the container was placed back on the stir plate with the stir bar. The air stone and pH probe were moved back to their positions in the tank. The bacteria were poured back into the tank and the stir plate was turned on to level 7. The system should be observed for a few seconds to ensure that the start-up was successful.

To clean the algae tank, the following materials were needed: three 1-L beakers, 600-mL beaker, wire mesh, soap, ethanol, and paper towels. First, the pH probe was removed from the algae tank and secured in the spare test stand with the 600-mL beaker placed beneath it. The pH probe was sprayed with water and gently wipe with a wet paper towel to remove built-up residue. It was then sprayed with ethanol and patted dry. Next, both outlet tubing was removed from the tank and place in the sink. The double ended airline was removed and wiped down with ethanol and an end was placed in two of the 1-L beakers. The stir plate was turned off the contents of the algae tank were gently poured through the wire mesh, into each of the 1-L beakers. If too much

flock is gathered on the wire mesh, it should be rinsed off in the sink and repeated until the 1-L beaker is filled. The algae tank contents were poured through the wire mesh until all algae tank is drained. If more than two 1-L beakers were in use, the airline was occasionally moved from beaker to beaker to ensure that the algae were properly aerated. The algae tank was then washed in the sink with soap. The tank was dried with paper towel and sprayed with ethanol. After it had dried, it was returned to the stir plate with the stir bar. The double ended airline was moved from the algae filled beakers to the algae tank. The contents of the beakers were gently poured into the algae tank and the stir plate was turned on to level 7. If the algae outlet pipes had significant blockage, they were dismantled and cleaned following the same procedure for the sugar and CSS lines. If there wasn't biomass backed-up within the tubing, then they were wiped down with ethanol and returned to their positions in the algae tank. As with the bacteria tank, the system should be observed for a few seconds to ensure that the start-up was successful.

Waste Disposal

Since the system being operated was a continuous system, the two outlet pumps placed within the algae tank were constantly removing bio-waste. Waste containing biomass (bacteria, algae, fungi, etc.) cannot be disposed of by pouring in the lab sink, since it can lead to contamination of other water sources and treatment facilities. To dispose of the waste properly, two large 10-L tanks were obtained and when one tank was filled halfway with algae waste, it was switched with the empty 10-L tank. The half-filled waste tank was transported to the autoclave and sterilized, killing all microorganisms. The tank was transported back to the lab and the contents were dumped down the sink.

Contaminant Removal

Two issues that occurred repeatedly during research were contamination of the bacteria and algae tanks from fungi and protozoa. The presence of these microorganisms negatively impacts the health of the systems and must be removed. Several methods and protocols were created to remove the fungi and protozoa, which were based on observations and trends identified from collected data. The methods and protocols were adjusted as the continuous system was operated to determine what was most effective at removing the contaminants.

Fungi was the most prevalent source of contamination in the algae tank. In Dr. Ju's lab, fungi and bacteria are grown in the same shared space and since this system was open to the environment, fungal spores were likely to enter. **Figure 2** compares two microscopic images of a non-contaminated algae sample taken from an algae seed flask and a contaminated algae sample taken from the algae tank. In the algae tank there are three microorganisms present: algae, fungi, and protozoa. There were two different fungi structures found in the algae tank and the one captured in **Figure 2** shows fungi with a splinter-like structure. The other type of fungi that was observed can be seen in **Figure 3**, which are shown to be a massive, branched structure.

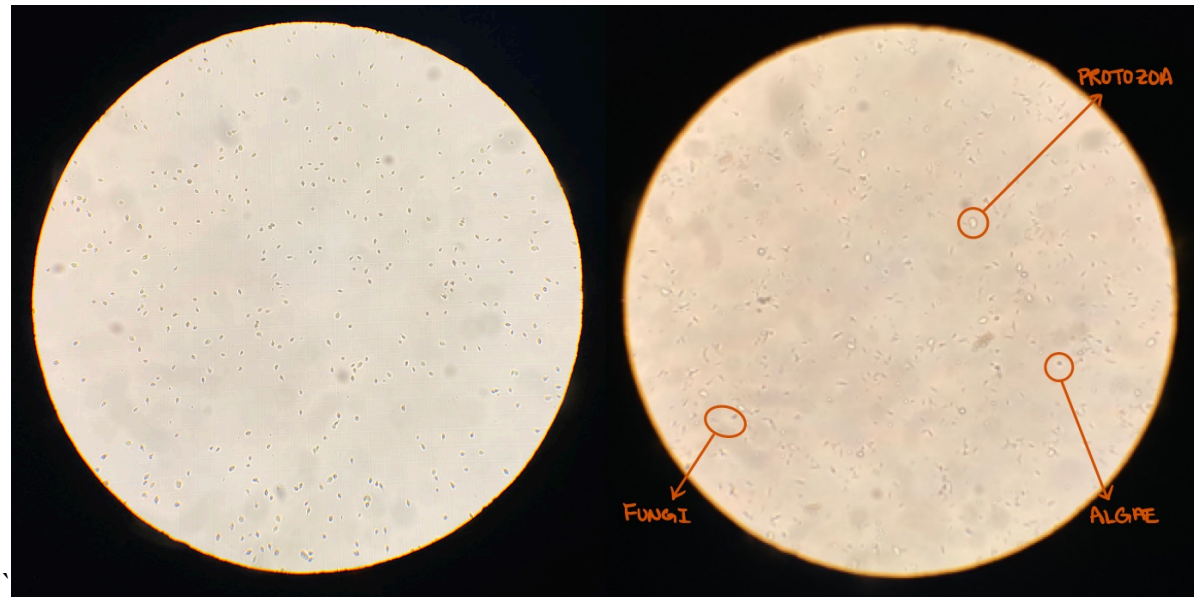

Figure 2 *shows two microscopic images of algae. The image on the left was taken of an algae seed batch kept in the lab on a stir-plate. It was given abundant nutrients and remained covered to prevent contamination. The image on the*

right was taken from the two-staged continuous system. There are two contaminant microorganisms present. The algae can be identified as the smaller black dots, while the protozoa are noticeably larger and translucent. The fungi captured in this particular sample had a splinter-like shape versus the branched structure shown in ***Figure 3****.*

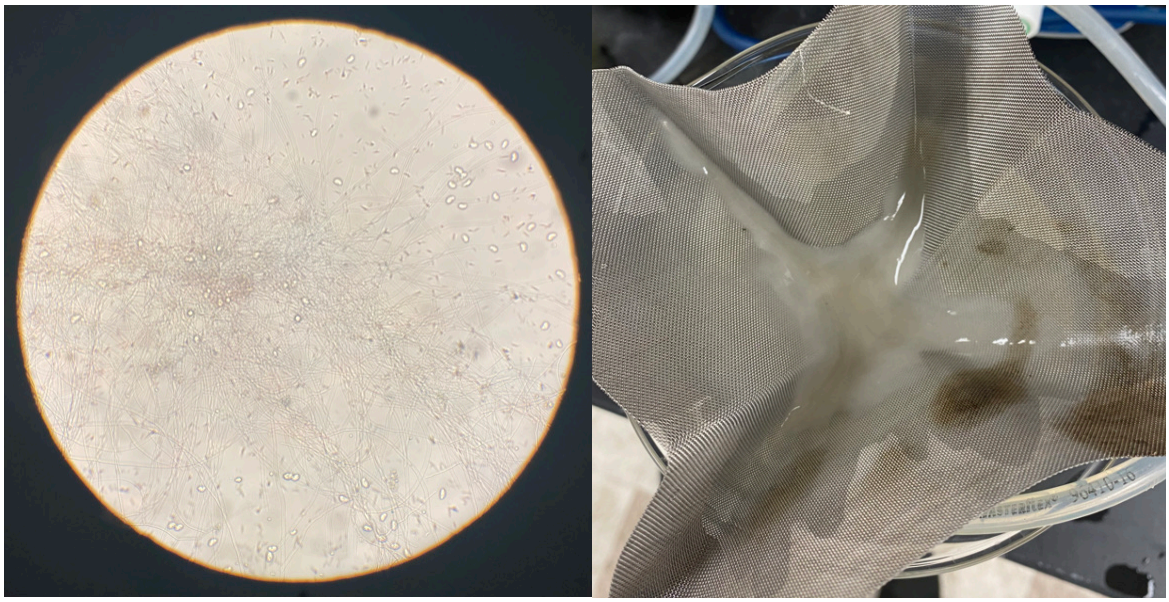

Figure 3 *shows the microscopic image (on the left) of branched fungi that contaminated the algae tank system and the fungi that was collected by draining the contents of the algae tank through a wire mesh (on the right).*

The method for fungi removal was incorporated into the cleaning protocol for the algae tank. While the tank was being cleaned, the contents of the algae tank were poured through a wire mesh into a 1-L beaker. The wire mesh was able to collect most of the branched fungi, but the splinter-like fungi was smaller than the mesh and passed through. The image on the right in **Figure 3** shows the fungi collected by the wire mesh after the algae tank contents had been filtered through it. After filtering the algae tank contents through the wire mesh, several samples were taken from different areas in the tank and viewed underneath the microscope. There were no large networks of branched fungi visible. Although the branched fungi would reappear in a few days, using this technique each time the tank was cleaned kept the growth to a minimum.

Protozoa was another common contaminant present in both the bacteria and algae system. To combat its presence in the bacteria tank, the best method was to follow the cleaning protocol written in detail above. As mentioned before, this system was open to the environment, but there

were several steps to help minimize the competition between microorganisms. Since the bacteria has a shorter doubling time than protozoa, it was important to counter any contamination early so that the bacteria would remain the dominant organism in the tank. During the earlier months of this project, protozoa levels remained high and were negatively impacting bacteria growth. Once a rigorous cleaning protocol was implemented, the bacteria population increased and was able to outperform the protozoa. In addition to cleaning, the tank was covered with a sheet of foil to prevent contamination from air particles and spores.

Protozoa was also found in the algae tank and since the algae's doubling time is significantly slower than bacteria's, once protozoa entered the system, it was able to increase its population quickly. An effective strategy to eliminating the protozoa from the algae tank was to keep the DO level below 5%. According to Ju, phagotrophic algae could survive at levels as low as 1% but were much more efficient at 5% [5]. The data gathered from weeks of observation support this statement, and it was shown that lowering DO levels was an effective way to decrease the amount of protozoa present in the system. Another method was incorporated into the system design. Instead of one outlet pumping algae content from the top to keep it at the desired volume, another outlet was placed on the bottom of the tank. It was observed that the protozoa and other contaminants settled to the bottom since they were heavier than the algae. Periodically, the second outlet pump would remove a few milliliters of the bottom algae tank contents thought to be higher in concentration of contaminants.

Preparation of Systems

Bacteria Seed

While the system parameters were actively being observed and recorded, batch systems of bacteria and algae were maintained in order to revitalize the tanks if the concentration of the cells dropped to unhealthy levels.

There were two methods used to reactivate the bacteria system. The first involved using bacteria culture frozen in a glycerol medium. Several 1-mL samples of healthy bacteria culture were preserved in small tubes using a glycerol medium to protect cells from crystallization that can occur while freezing. To reactivate the system, a frozen bacteria culture sample was removed from the freezer and placed on the counter to thaw. All reagents listed under the "Stage 1" column of **Table 1** were added to a small vial and autoclaved, excluding the bacteria sample. Then, following proper hood techniques, the 1-mL bacteria culture sample was added to the autoclaved vial containing the other reagents. The vial was then placed in an incubator with the cap loosely placed on top to allow some airflow to the growing bacteria. The vial contents were monitored and left undisturbed for 8-12 hours. At the end of the incubation period, the inoculated vial contents were added to an autoclaved flask containing the reagents listed under the column "Stage 2." This flask was placed in one of the lab's shakers at a temperature of 25°C and 200 rpm. It was monitored for another 6-10 hours. The inoculum from stage 2 is added to the bacteria tank along with the other reagents in the column "Stage 3". The other nutrients were supplied, and the system was operated as it would be if in steady state. While monitoring the system until it stabilizes at steady state, the pH was checked every 1-2 hours. If it dropped below 6.5, 0.01-g of Na2CO3 was added.

Table 1 *shows the reagents required at different stages of reactivation. If preparing a flask to grow bacteria in a shaker from bacteria culture taken from the system, the column "Flask" is followed. Each flask containing all reagents should be autoclaved before adding the bacteria culture.*

Bacteria Flask Contents				
Reagents	**Flask**	**Stage 1**	**Stage 2**	**Stage 3**
DI Water	87.17 mL	4.3585 mL	39.2265 mL	305.095 mL

Smuckers Supernatant	0.334 mL	0.0167 mL	0.1503 mL	1.169 mL
CSS	2.5 mL	0.125 mL	1.125 mL	8.75 mL
Bacteria Culture	10 mL	5 colonies from agar plate or 1 mL of thawed seed culture	4.5 mL of inoculum from (i)	35 mL of inoculum from (ii)
Na_2CO_3	0.01 g	0.0005 g	0.0045 g	0.035 g
Total Volume	**100 mL**	**5 mL**	**45 mL**	**350 mL**

Another method to reactivate an unhealthy or dead bacteria system was to use bacteria colonies grown on agar plates. The same process that was described above is followed, but instead of using the 1-mL of thawed bacteria culture, 6 bacteria colonies were transferred from the agar plate to the small vial. The following steps were used to ensure there was no contamination of the vial contents when transferring the bacteria colonies. Using the hood located in the lab, the fan was turned on 15 minutes before use. The flame was lit and ethanol was used to wipe down all surfaces, including gloves. A small wire looped tool was heated in the flame and then placed to the side to cool. The autoclaved glass vial was opened, and the edges were heated in the flame. Opening the agar plate containing the bacteria colonies, the looped tool was used to scrape a colony and transfer it to the vial. The looped tool was heated and cooled after each transfer. It was recommended to use different colored bacteria colonies since a specific species of bacteria was not being used for the continuous system. After the transfer was complete, the vial opening was heated in the flame again and the cap was gently secured and placed in incubator.

Algae Seed

The reactivation and preparation of algae seed to be used in the continuous system was accomplished through the following procedures. The list of reagents necessary to prepare the algae seed flask can be found in **Table 2**. A 1-L flask that would be used to grow the algae seed was obtained and the following reagents were added: 20-mL of CSS, 5-mL of trace element solution,

20-mL of NaH2PO4, and 300-mL of DI water. Two of the reagents, glucose and amino acid solution, had to be prepared separately. The composition of the trace element solution can be found in **Table 10.** The trace element was typically prepared in bulk solution and kept in the fridge located in Lab 308. The CSS used for algae reactivation had a higher concentration of reagents than the CSS used in the continuous system. The composition of the CSS used for algae reactivation can be found in **Table 9.** This CSS was prepared as a bulk solution and kept on the lab bench in Lab 308. The 1-L flask, along with the glucose and amino acid solutions were autoclaved separately. Using proper hood techniques, the flasks were transferred to the hood workstation to prepare the algae reactivation flask. 20-mL of the amino acid solution and 30-mL of the glucose solution were added to the autoclaved 1-L flask containing the other reagents. A previously prepared biotin solution that was stored in the fridge was brought to the hood and 0.5-mL was added to the flask. Once all reagents had been added, a flask containing a previously prepared, healthy algae culture was transferred to the hood and 20-mL was measured and added to the reactivation flask. The flask was covered and removed from the hood and placed on a stir plate. Within a couple days, the algae culture would turn a vibrant green, indicating it was healthy and could be used to revitalize the system or to help maintain the desired levels of algae concentration.

Table 2 *provides the reagents used to prepare the algae seed culture. Concentrations are listed when available. When adding the reagents, all transfers should be done in the hood. The glucose and amino acid solutions must be prepared in separate flasks with DI water and autoclaved separately. If there is solution remaining for either reagent, it can be kept on the lab bench until the next algae seed culture is prepared. The flask is then placed on a stir plate for agitation.*

Algae Seed Culture				
Reagents	Location	Concentration	Amount (mL)	Notes
Glucose	Prepared	100 g/L	30	Autoclave separately
Amino Acids	Prepared	18 g/L	20	9g/L Yeast Extract, 9g/L Tryptone Autoclave separately

Concentrated Salt Solution	Bench 308		20	Autoclave together in flask
Trace Element	Fridge 308		5	
NaH_2PO_4	Bench 308	0.2mol/L	20	
DI Water			300	
Biotin Solution	Fridge 308		0.5	Add in hood
Previous Algae Culture			20	Add in hood

Smuckers Waste

As with all biosystems using a heterotrophic substrate such as bacteria, a carbon source is required. For this particular biosystem, the carbon source used was a waste product provided from The J. M. Smucker Company. It contained various Smucker products that have a high sugar content such as strawberry jam, grape jelly, and caramel ice cream topping. An inductively coupled plasma (ICP) mass spectrometry was conducted at Smuckers and the results were included with the waste. The analysis provided the elemental composition and concentrations, which are shown in **Figure 9** and **Table 7**. The elements present in the largest amounts were potassium, sodium, and calcium, respectively. A liquid chromatography test was conducted on Smuckers waste samples and the breakdown of sugars present in the jam are shown in **Table 3**.

Table 3 *contains information related to the sugar composition of the Smuckers waste used throughout the project.*

Concentration (g/L)				
	Sucrose	Glucose	Fructose	Total sugar
Smuckers	76.3	88.6	78.9	243.7

The Smuckers waste could not be added directly to the bacteria and had to first be diluted and centrifuged. Centrifugation was done using the Thermo Scientific Sorvall Legend X1R Centrifuge. When preparing the Smuckers solution to be supplied to the bacteria tank, the Smuckers waste was mixed with equal parts DI water. The Smuckers mixture was typically made in batches of 600-mL, with 300-g of Smuckers waste and 300-mL of DI water. The DI water and Smuckers waste

was then mixed with a magnetic stir bar on a stir plate for about three hours. Once the solution was well-mixed, it was poured into centrifuge tubes in about 40-mL increments. Using a balance scale, centrifuge tubes were balanced and placed directly across from one another in the centrifuge. The centrifuge was set to 12000 rpm for 15 minutes in order to separate the solids from the supernatant. When the centrifuge cycle was complete, the tubes were carefully removed, and the supernatant was pipetted into containers for storage in the refrigerator. About 35-mL of supernatant was extracted from the 40 mL tube each time and 5 mL of solids from the Smuckers mixture was disposed of properly.

Consecrated Salt Solution

One of the nutrients provided to the bacteria system was a consecrated salt solution. It was composed of several reagents which are listed in **Table 8**, along with the required concentration of each reagent per liter. CSS was prepared often prepared for in advance by measuring the dry reagents needed to prepare 4-L of solution. They were stored in small vials and kept on the counter by the system. Another CSS solution was prepared for use during algae seed preparation and the list of reagents and compositions are found in **Table 9.** This could be prepared in larger batches and kept in the lab for future seed preparation and other uses.

Analytical Methods

Cell Concentration

An important measurement recorded throughout this research project was cell concentration. There were two methods used to determine concentration: cell counting with a counting chamber and cell dry weight. Cell counting allowed for a quick, visual check on the health and current growth stage of a system. The cell count for a large volume could be quickly estimated through by using a counting chamber. The counting chamber consisted of a glass slide with

machined chambers for holding a specific volume and glass coverslips that had a small etched grid to use as a reference when counting. First, the coverslip was placed on top of the slide, forming a small chamber between the slide due to the slightly elevated sides of the machined glass. A 0.5-mL sample was taken of the algae and mixed with 0.5-mL glyceraldehyde in a small test tube. Using a pipette, a few milliliters of solution was transferred to the opening of the chamber. The liquid was drawn into the chamber between the glass slide and coverslip due to capillary action. The counting chamber is placed underneath the microscope and the lens was adjusted until the algae was clear and the etched grid was visible, as shown in **Figure 4.** Consistency was essential when counting cells. In **Figure 4**, notice the smaller grids arranged in a 5x5 square, each containing 16 of the smallest squares. Each of the smaller grids is separated by a series of lines etched in rows of three. Starting in the upper right corner, all cells on and within the three-line border were counted. Moving down to the next box this was repeated, but this time the top three-line border was not included. This is repeated until the bottom of the first column is reached. Moving to the right, the cells present in that smaller grid are counted, but this time the left three-line border is not included. Moving upward, this was repeated but leaving out both the left and bottom -line borders. This is continued until all the cells are counted and the total cell count is recorded. For the most accurate results, the counting process should be repeated three times for each sample taken and at least with two samples.

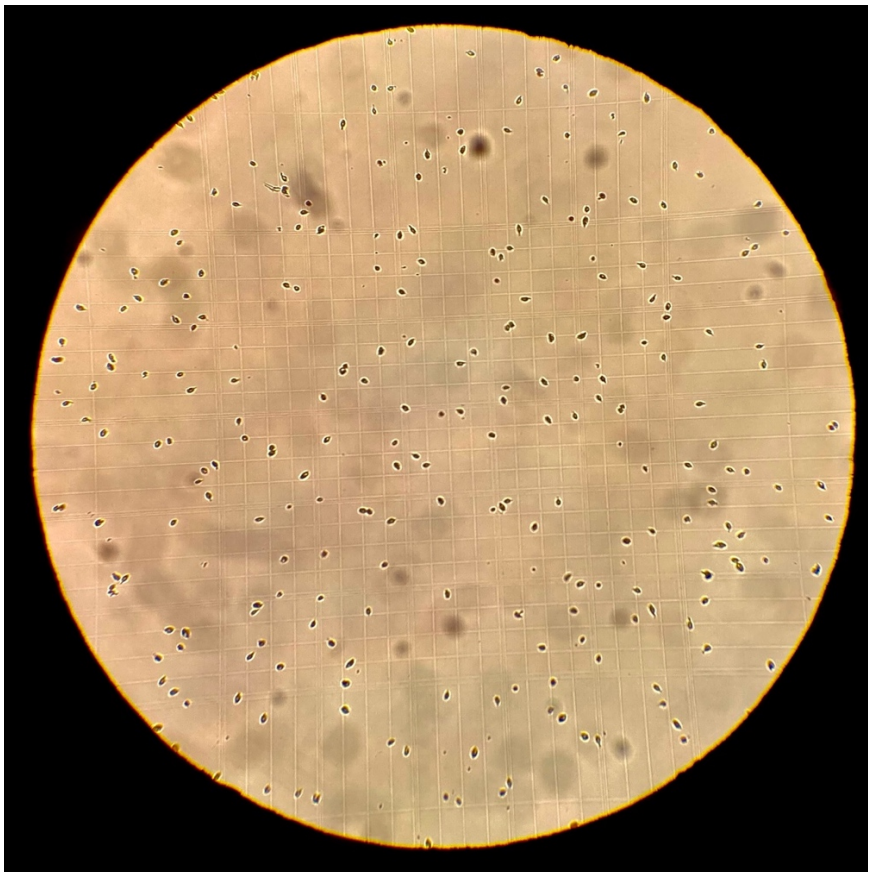

Figure 4 *shows the microscopic view of algae cells being counted using a counting chamber. There were microscopic lines carved into the piece of glass placed on top of the chamber. The area of this grid was 1 mm^2 and was made-up of a 5x5 square grid and each square contained 16 smaller squares.*

The other method for determining cell concentration is by cell dry weight. It takes more time to prepare samples, but it is more accurate than cell counting. To take the cell dry weight of a sample, a small amount of liquid was obtained from the system and dried in an oven to evaporate the water. The dried cells are weighed and the concentration of that particular organism can be calculated for the entire system. This method was only used for bacteria, since there was little contamination from other microorganisms. In the algae tank, the algae concentration calculated from cell dry weight would have been skewed with protozoa and fungi dry weights. To take the cell dry weight of the bacteria, 10-mL of solution was measured and placed in a centrifuge tube. Using a balance, 10-mL of water was measured and placed in second centrifuge tube until the scale was balanced. Using a centrifuge machine, the sample was centrifuged for 10 minutes at 12000

rpm. The bacteria tube was removed and using a pipette, the supernatant was removed and discarded. Another 10-mL of water was measured and added to the bacteria tube until it is balanced with the control tube. The sample is centrifuged for another 10 minutes. The wash process and centrifuging were repeated one more time. After the second wash, the supernatant was removed and discarded and 10-mL of DI water was added. The tube was shaken vigorously to ensure that the bacteria was not stuck to the sides of the tube. The weight of a small foil tray was measured and recorded. The contents of the bacteria were dumped into the tray and placed in a lab oven for 24 hours at 100°C. The next day the tray was removed and weighed with the dried contents.

Optical Density

A useful method for determining the amount of bacteria present in the tank and the overall health of the system was to measure the optical density (OD) using the Shimadzu UV-1601 Spectrophotometer. A 2-mL sample had to be prepared and placed into the spectrophotometer to obtain the OD value. To prepare the 2-mL sample, a combination of DI water and bacteria culture was used. Depending on the concentration of the bacteria, the sample could be diluted more or less, but for this project it was typically diluted 10 times. This required 0.2-mL of bacteria solution to be mixed with 1.8-mL of DI water in a cuvette. The mixed solution was inverted carefully five times before it was placed in the spectrophotometer. If the monitor reading was above 0.7 ABS, then the sample had to be diluted more than what it had been originally. The value was recorded, and the steps repeated for each sample taken. To obtain the optical density value used for data analysis, the value recorded from the monitor had to be multiplied by the dilution rate used.

Dissolved Oxygen

In addition to carbon and energy sources, aerobic biosystems require adequate oxygen to maintain healthy growth rates. The amount of oxygen present in the system is measured as the

percentage of dissolved oxygen (DO) by using a dissolved oxygen meter and probe. The DO meter was kept in a flask, submerged in water. First, the DO meter was turned on and the probe was rinsed with DI water. The end of the probe was gently dried with a Kimtech wipe. To calibrate, the probe was held vertically with the end pointed down in the air. The dial was turned until the machine read 100% for dissolved oxygen. Once calibrated, the probe was placed into the tank and when the measurement on the meter stabilized, it was recorded. The probe was rinsed and gently dried with a Kimtech wipe. This was repeated with the other tank and then the probe was submerged in the flask of water and the machine was turned off.

pH and Probe Calibration

An important measurement taken during this project was the pH of both the bacteria and algae tanks. It needed to be carefully controlled and monitored. In order for accurate pH measurements to be taken, the pH probes had to be recalibrated once a week. To calibrate, the pH probe was removed from the tank and clean with ethanol and Kimtech wipes. It was rinsed with DI water and patted dry with more Kimtech wipes. The monitor that was connected to the probe was set to "calibrate" mode. First, the probe was place in an acidic solution of pH 4 and the tip remained submerged until the monitor showed no changes in the pH reading. The probe was rinsed with DI water and then placed in the basic solution of pH 10. Once the monitor reading was no longer changing, the pH probe was removed, and calibration was complete. The pH probe was returned to its place within the system. The pH values recorded during observations were obtained from the monitor screen, which displayed the current pH value of the tanks at all times.

Results and Discussion

While the entire system consists of both the bacteria and algae tanks, it is useful to divide between the two when analyzing the data. The first component to be discussed is the bacteria tank. Over the course of the summer, the student previously researching in Dr. Ju's lab was establishing parameters for a batch system set-up for bacteria production. Based on these experiments, the appropriate set-up of the continuous system was determined. The system characteristics that were used from September until November are contained in **Table 4**.

Table 4 *lists the system characteristics used during the first set of experiments beginning in September of 2020. The specific goal of these experiments was to establish a continuous system for the bacteria tank that would supply the algae tank in a consistent and predictable manner. The two nutrient sources included the Smuckers jam and the consecrated salt solution (CSS). The retention time was set to 6 hours and the volume of the bacteria tank was 400 mL. The Smuckers pump supplied a volume of 0.5 mL every half hour and the CSS pump supplied a volume of 32.84 mL every half hour. The pumps are not synchronized due to them being paused to clean the tank and piping lines, but the expected flow rate of the effluent is 66.6 mL/hr, with no set off-time. As a continuous system, the tank will remain at 400 mL and as nutrients are supplied by the pumps, the effluent will continuously overflow into the algae tank whenever the volume exceeds 400 mL.*

System Characteristics		
Smuckers Pump		
Volume	0.5	mL
Flow Rate	3	mL/min
Off Time	1790	sec
Dispense Time	10	sec
CSS Pump		
Volume	32.84	mL
Flow Rate	16.4	mL/min
Off Time	1680	sec
Dispense Time	120	sec
Effluent		
Volume	33.3	mL
Flow Rate	66.6	mL/hr

Experiments were conducted over the course of many months, beginning in September and ending in February. The main goal remained the same throughout the research period, but the approach was altered. There were many days spent in the lab in order to perform the necessary tasks required to maintain a continuous biosystem. Results were collected for a wide range of

variables, to determine the state of the system and attempt to establish the ideal parameters for maximizing algae production. There were several problems encountered during the experiments, but as with all research, these can be expected, and the solutions can be used to help prevent them in any future research.

The data and results collected over the past year help to answer a few important questions that will further develop phagotrophic algae and its potential use as a feedstock for biofuel. The results indicate several interesting points that will guide the student or researcher that continues this project next.

First, the two-staged bacteria-algae continuous system was able to successfully run from December to mid-February. It was eventually shut-down due to unhealthy algae growth. Sustaining the healthy algae over long periods of time did not seem to work, but the bacteria remained in steady state over those months.

An effective cleaning procedure was established to help maintain the bacteria tank in steady state. Before the start-up of the steady state system in December, the bacteria tank encountered many issues and was not healthy. Once a cleaning procedure had been put in place and tailored to this particular set-up, the optical density only dropped in number due to interruption in delivery of nutrients from either the sugar or CSS solution.

While the fungi in the algae tank was never completely eliminated, a significant amount could be captured when straining it through a wire mesh. The fungi would return to its typical rates within a few days, but if kept on top of it helped the algae to grow.

When starting the algae tank from one of the previously made algae seeds, it would remain at healthy levels in both color and concentration for a couple days, but slowly the green color would fade to a light yellow. It is thought that the protozoa and fungi are producing toxins and

overtime, they gradually take over the system. Removing these or preventing them from contaminating the system would be essential for further research. If scaled-up, this would not be as much of a concern since it could be a closed system.

The continuous system ran from November 4th, 2020 until February 15th, 2021. Over the three and a half months of operating both the bacteria and algae tank, there were several trends that became obvious upon further investigation into the results.

As mentioned before, contamination from protozoa was prevalent throughout the entire research period. There were a few methods developed that helped to decrease the amount of protozoa found in the algae tank. One of those methods was to decrease the amount of dissolved oxygen being supplied to the algae tank. Algae is able to grow at rates less than 5%, but protozoa are suffocated. If protozoa were present and the dissolved oxygen was decreased, within a couple days the protozoa percentage (based on all the microorganisms present in the tank) would also decrease. This is supported by the data recorded and presented in **Figure 5.**

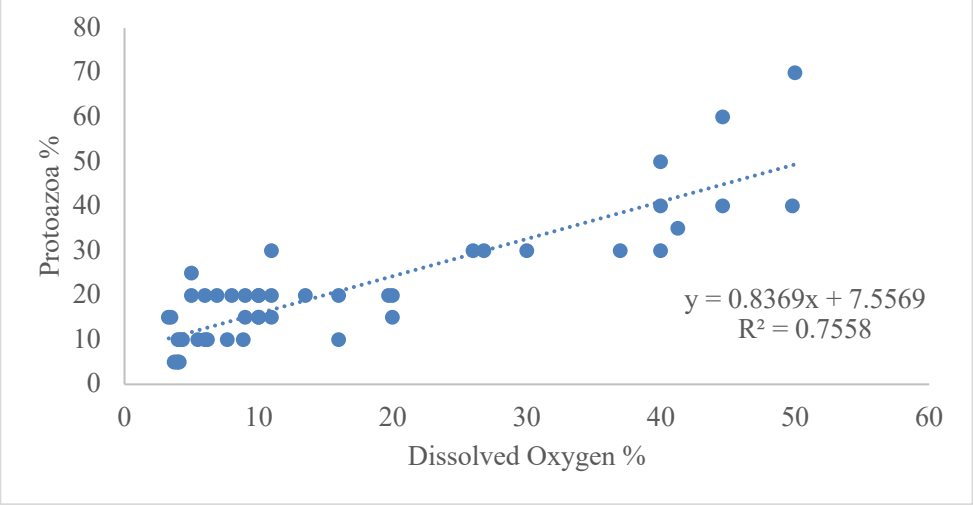

Figure 5 *shows several data points comparing the protozoa percentage out of all the microorganisms present to the dissolved oxygen % in the algae tank. The data was fitted with a linear trendline that has an* R^2 *value of 0.7558. From this figure, a general correlation between the protozoa % and DO % can be observed. This suggests that decreasing the DO % may be an effective strategy to eliminating protozoa from the algae tank. The standard deviation for the data collected for the percent of protozoa present in the tank was 14.1 and the standard deviation for amount of dissolved oxygen was 14.7.*

Figure 6 shows the correlation between the percentage of microorganisms in the algae tank that are algae compared to the dissolved oxygen percentage. It is suggested that lower dissolved oxygen rates allow for algae to dominate the system over fungi and protozoa.

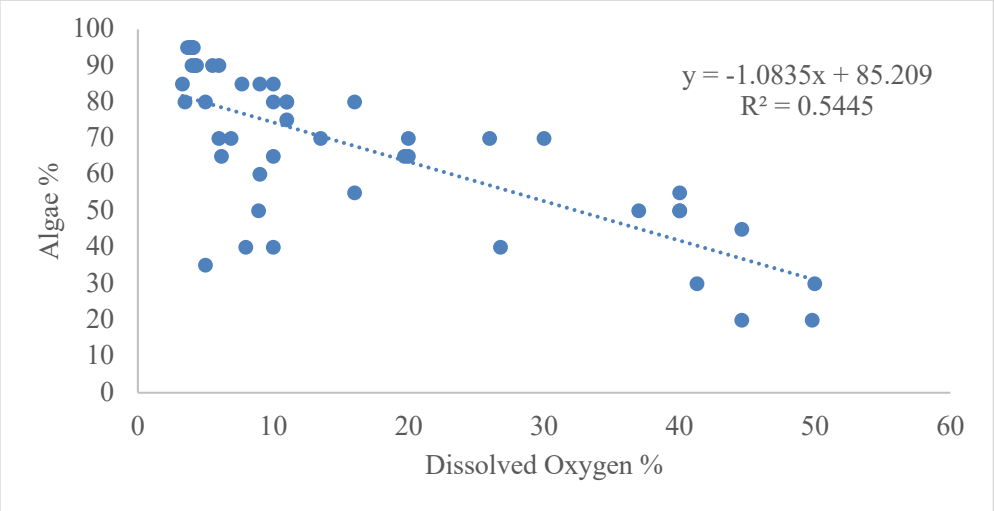

Figure 6 *shows several data points comparing the algae percentage out of all the microorganisms present to the dissolved oxygen percentage in the algae tank. The data was fitted with a linear trendline and the* R^2 *value was 0.5445. The correlation between the two variables is not as strong as the data presented in* ***Figure 5****, this graph suggests that decreasing dissolved oxygen helps algae become the dominant microorganism in the system. The standard deviation for the data collected for the percent of algae present in the tank was 21.6 and the standard deviation for amount of dissolved oxygen was 14.7.*

Another measurement taken several times to assist in the quantitative analysis of the continuous-flow system was cell dry weight. **Table 15** contains all the data for cell dry weight taken during the operation of the system. The bacteria produced a thick flock that would accumulate between each cleaning. To determine how much this affected the concentration of bacteria, the cell dry weight was taken before and after cleaning. **Table 5** shows the concentrations of bacteria before and after cleaning as well as the calculated percent difference.

Table 5 *provides data on the cell dry weight of the bacteria tank taken before and after cleaning. The percent difference was calculated and shows that there was significant different in the weight of the cells before the flock was filtered out. The data was taken from* ***Table 15*** *for the days that had cell dry weight samples taken before and after cleaning. The first four data points in the table below had two or more samples measured, while the remaining three*

had only one sample taken. For the days that had more than one sample taken, the standard deviation was calculated and included below.

Before Cleaning CDW (g/L)	After Cleaning CDW (g/L)	% Difference
0.36 ± 0.08	0.24 ± 0.01	40.0%
0.24 ± 0.08	0.23 ± 0.01	4.3%
0.42 ± 0.04	0.35 ± 0.04	18.2%
0.36 ± 0.04	0.29 ± 0.04	21.5%
0.46	0.37	21.7%
0.49	0.3	48.1%
0.7	0.52	29.5%

It was initially thought that a correlation could be established between the optical density of the bacteria tank and the cell dry weight taken from the same day. The reason this was of interest was to eliminate the time needed to prepare the cell dry weight samples. Taking three optical density measurements was much quicker. **Figure 7** compares these measurements taken from the bacteria tank on the same day. It is possible that a correlation exists, since optical density is measuring how much light is being absorbed in each sample, which should increase as the concentration increases. However, this graph is inconclusive and further investigation is required.

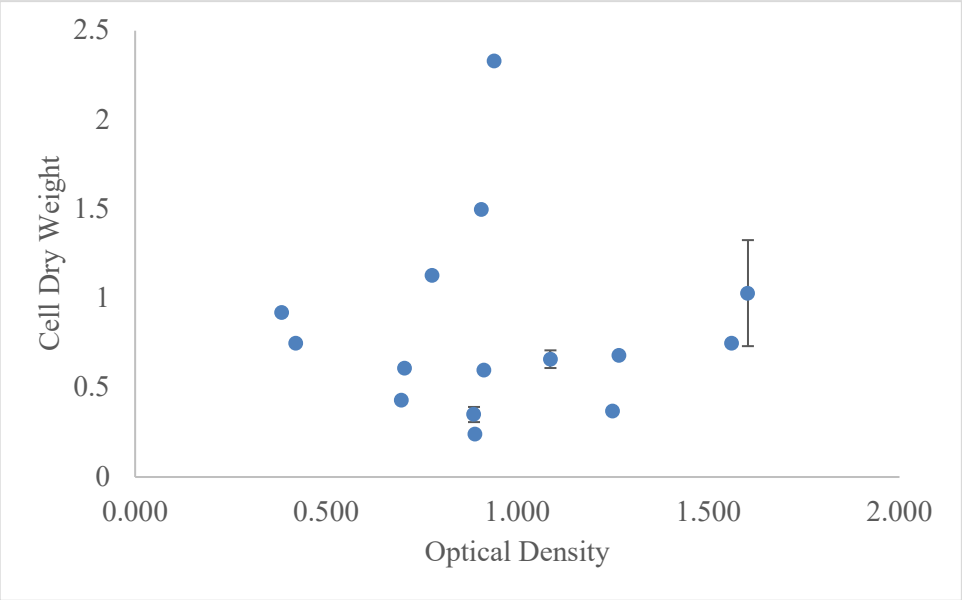

Figure 7 *compares the cell dry weight of bacteria to the average optical density value taken on the same day. The data provides no indication that a correlation exists between cell dry weight and optical density, but further research is suggested since the theory says the opposite. Standard error bars were added based on the standard deviation calculated at each point.*

An algae growth batch study was conducted to determine the baseline growth of algae when supplied a surplus of nutrients. With this baseline, the current cell count of algae could be evaluated to determine if it was reaching its full potential. The growth curve of two flasks of algae is shown in **Figure 8**. The data collected and used for graphing is included in the appendix as

Table 12.

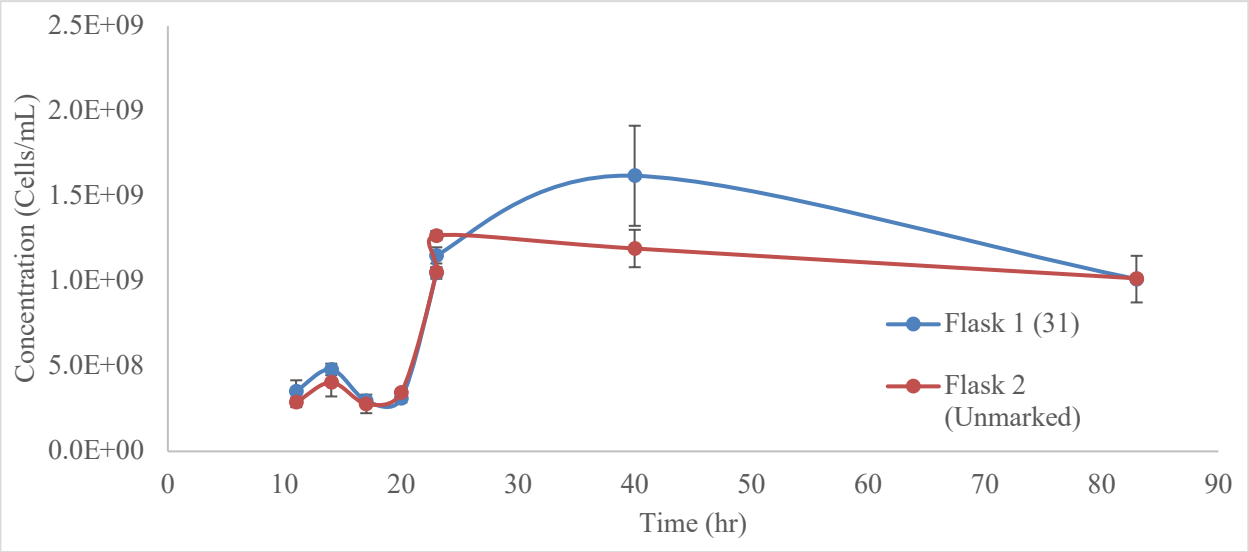

Figure 8 *depicts the growth curve of two flasks prepared from a previous algae seed. Over the course of several hours, the cell concentration was determined through the use of a counting chamber. The data used to create this graph can be viewed in*

Table 12*. Standard error bars were added based on the standard deviation calculated at each point.*

While many important conclusions can be drawn from observations and data collected over the past few months, the factor that prevented the research project from progressing to the next phase was the lack of healthy and sustainable algae growth. There are several reasons that could possibly explain why it was negatively impacted and might provide a reasonable starting place for continuing research. There were three different researchers maintaining and recording observations on the continuous-flow system. It is possible that there were inconsistencies present between system management and loss of information due to poor communication. Another reasonable explanation for lack of healthy algae growth would be from fungal and protozoa contamination. When competing for limited nutrients and oxygen, microorganisms will create toxins to harm competitors, which may have affected the algae. There were several occasions where the nutrient delivery was interrupted to the bacteria tank, which in turn affected the algae tank. If the algae weren't receiving enough bacteria in the effluent, then it is possible that protozoa or fungi were able to become dominant in the tank. The algae tank was cleaned almost every day,

which required the contents to be poured into smaller beakers and then back into the tank. The disturbance might have had a negative impact or there was left over soap residue on the tank which killed some of the algae. If this system were to be scaled-up for industrial use, it is recommended to filter the algae tank contents through a mesh to remove fungi constantly, instead of when cleaning the tank. It is possible that the splinter shaped fungi could be caught with a finer mesh and if it was constantly being filtered, time wouldn't be a constraint.

Considering next steps for this research, there are several batch studies that can be suggested. The first is to maximize bacteria growth by comparing bacteria prepared with Smuckers supernatant or a pure glucose substrate. This should be done to determine if the bacteria growth is being limited and therefore affecting algae growth negatively. The second batch of experiments should be to maximize algae growth and determine what the maximum concentration possible is for the algae seed. This can then be used to compare measurements of algae concentration made on the continuous system. The third is to prepare bacteria with either Smuckers supernatant or glucose and compare the concentration of algae grown in each substrate. It is possible that the sugar source is the limiting factor for algae growth. The final batch experiment suggested is to grow algae with only bacteria supernatant. This would show if bacteria is producing toxins that negatively impact the algae growth. These are just a few of the many possible batch experiments that would help move this project forward.

There were several difficulties encountered throughout this project that affected the completion of the listed objectives. They included the amount of time required to maintain a continuous system, preventing contamination in an open system, and the numerous variables that needed to be considered when deciding what to do next. When continuing this research, there are a few recommendations that would minimize the impact of these difficulties. In regard to the

amount of time required to maintain the system, a schedule of what tasks need to be done and when would reduce the amount of time spent in lab. The development of the checklist shown in **Table 6** made any time in lab more efficient. Contamination remained the biggest barrier to achieving further success with this project. Much of the time spent was combating fungi and protozoa. Based on the recorded observations, the protozoa were decreased significantly in the algae tank through various methods and eliminated completely in the bacteria tank through a rigorous cleaning protocol, both of which are found in Experimental Methods. It is recommended that these methods and protocols be followed and adapted to future research to prevent contamination and loss of time. The third difficulty faced during this project was related to the number of variables that needed to be considered. Much of results obtained from the past year have been gathered as observations. There were qualitative data collected and analyzed, but the majority of the conclusions made are based on the observations. It is recommended to start with algae and bacteria batch experiments to determine the limiting source for algae growth. Once identified, the next steps of the project will be easier to plan and prepare for successfully.

Conclusion

In pursuit of a more sustainable feedstock for biofuels, the research presented in this report showcases various methods developed and supportive data collected for constructing and maintaining a continuous-flow process for the production of phagotrophic algae. The objectives established at the beginning of this project were to sustain bacteria growth once achieving steady state, determine ideal conditions for healthy algae growth, and conduct experiments on a two-staged bacteria-algae culture system. Based on previous research and progress made on this project over last summer, the two-staged system was constructed. Through several experimental trials, the

bacteria tank was able to reach steady state and remained productive and healthy for several months. This accomplishment was preceded by the development of a rigorous cleaning protocol for the tank, nutrient tubing, and CSS tank. Establishing a healthy algae system presented more challenges and produced less successful results. As with any open biosystem, it is difficult to avoid contamination from other microorganisms and the algae tank included protozoa and different types of fungi. This led to the development of two methods for removing the contaminants. The first was to filter the algae tank contents through a wire mesh that would collect the branched fungi. This was to be done with each cleaning. If fungal contamination is encountered regularly during future research, it is suggested to create a filtering system within the algae tank to allow for constant fungi removal instead of only at cleaning. The second method is to combat protozoa by decreasing the dissolved oxygen percentage to about 5%. This slows the growth rate of algae but will eliminate protozoa. Based on observations made during the continuous culture trials, there are some batch experiments that were planned but did not get completed due to the time constraint. These batch experiments would help to identify the factors limiting algae growth and are recommended as the next steps in this project. They include maximizing bacteria growth with different sugar substrates, maximizing algae growth to determine a baseline, observing algae growth with bacteria and different sugar substrates, and observing algae growth using bacteria supernatant.

There were several difficulties encountered throughout this project that affected the completion of the listed objectives. They included the amount of time required to maintain a continuous system, preventing contamination in an open system, and the numerous variables that needed to be considered when deciding what to do next. While there was a significant amount of quantitative data collected, there were many qualitative aspects that added to this project's complexity. Going forward, there must be further research conducted on what ideal conditions are

necessary to support healthy algae growth. From there the continuous system can be re-started and evaluated. If the two-staged system is determined a success for algae production, the next phase of research that is related to processing the phagotrophic algae for lipids can be started.

Appendix

Table 6 *shows the formatted schedule that was used when completing daily tasks. It includes spaces for recording values and observations. The set-up allows you to complete tasks as you go down the column. Boxes in light gray serve as a reminder of equipment and components that need to be checked before leaving the lab such as checking that the pumps are running, levels of acid and base are adequate, and there are sufficient amounts of nutrients remaining. Boxes in white require value or observation to be recorded.*

		Week 5						
	Task	8-Feb	9-Feb	10-Feb	11-Feb	12-Feb	13-Feb	14-Feb
	Initial							
	Time							
ALGAE TANK	System Picture							
	Algae pH							
	Tank Color							
	Liquid Level							
	Microscope Picture							
	Algae, A%/F%/P%							
	DO (%)							
	Outlet Pump							
	Acid Flask							
	Base Flask							
	Air Flow							
BACTERIA TANK	3-second video							
	Bacteria pH							
	Tank Color							
	Microscope Picture							
	DO (%)							
	OD (3x)							
	Cell Dry Weight							
	Airstone							
	Stir Bar							
	Sugar Line Clear							
	Sugar Flask Refilled							
	Base Flask							
	CSS Tank Liquid Level							
	Sugar Pump							
	CSS Pump							
	No. of CSS Vials							
	Calibrate pH probe							

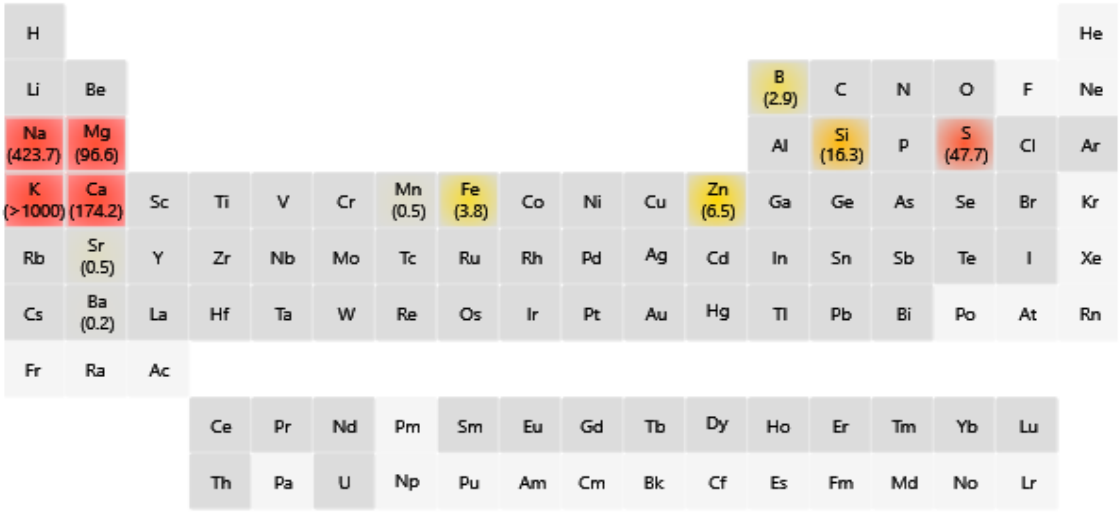

Figure 9 *depicts the concentration gradient of elements present in the Smucker's waste provided to UA in October 2020. The sample is a food waste containing a mixture of chocolate sundae syrup, strawberry jam, grape jelly, orange marmalade, honey, and caramel ice cream topping. The sample was completely digested prior to analysis. Results are a semiquantitative ICP screen based on a supplied calibration. The lighter yellow color indicates lower levels and the red color indicates higher levels of the highlighted element.*

Table 7 *provides the concentrations of the various elements present in the waste from Smuckers.*

B	Ba	Ca	Cu	Fe	K	Mg	Mn	Na	P	S	Si	Sr	Ti	Zn
mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
2.95	0.16	174.23	ND	3.80	1.05E+03	96.57	0.48	423.70	ND	47.68	16.30	0.52	ND	6.54

Table 8 *provides information on the composition of the concentrated salt solution prepared for the continuous system and supplied to the bacteria tank. The CSS could be prepared ahead of time and stored in small vials as a mixture of the dry reagents.*

Concentrated Salt Solution (Bacteria)		
Chemical Name	Chemical Formula	Concentration (g/L)
Potassium Phosphate Monobasic	KH_2PO_4	0.3
Magnesium Carbonate Hydroxide Pentahydrate	$(MgCO_3)_4$-$Mg(OH)_2$-$5H_2O$	0.1
Sodium Carbonate Anhydrous	Na_2CO_3	0.0126
Calcium Carbonate	$CaCO_3$	0.005
Ammonium Chloride	NH_4Cl	0.75
Magnesium Sulfate Heptahydrate	$MgSO_4$-$7H_2O$	0.1

Table 9 *provides information on the composition of the concentrated salt solution used for reactivating algae system and algae culture seed. The CSS could be prepared in bulk and was stored on the lab counter in room 308.*

Concentrated Salt Solution (Algae)		
Chemical Name	Chemical Formula	Concentration (g/L)
Nitrilotriacetic acid	$C_6H_9NO_6$	2.0
Potassium Phosphate Monobasic	KH_2PO_4	3.0
Magnesium Carbonate Hydroxide Pentahydrate	$C_4Mg_4O_{12}$-H_2MgO_2-$5H2_O$	4.0
Calcium Carbonate	$CaCO_3$	0.5
Ammonium Chloride	NH_4Cl	5.0
Magnesium Sulfate Heptahydrate	$MgSO_4$-$7H_2O$	1.0

Table 10 *provides information on the composition of the trace element solution used to prepare algae seed cultures and reactivate the algae system. It was stored in the fridge in room 308.*

Trace element solution		
Chemical Name	Chemical Formula	Concentration (g/L)
Disodium EDTA	Na_2EDTA	4.4
Boric Acid	H_3BO_3	0.97
Iron (III) Chloride Hexahydrate	$FeCl_3$-$6H_2O$	3.15
Manganese (II) Chloride Tetrahydrate	$MnCl_2$-$4H_2O$	0.18
Zinc Sulfate Heptahydrate	$ZnSO_4$-$7H_2O$	0.02
Cobalt (II) Chloride Hexahydrate	$CoCl_2$-$6H_2O$	0.01
Sodium Molybdate Dihydrate	Na_2MoO_4-$2H_2O$	0.006

Table 11 *provides the reagents used to prepare the bacteria seed culture. Smuckers supernatant was prepared using a 1 to 1 weight ratio of Smuckers waste and DI water. All reagents were combined into a flask except for the bacteria culture. The flask was autoclaved and then the bacteria was added underneath the hood and placed into a shaker for agitation.*

Bacteria Seed Culture				
Reagents	Location	Concentration	Amount (mL)	Notes
DI Water			87.17	Autoclave together in flask
Smuckers Supernatant	Fridge 311	300g/L	0.334	
Concentrated Salt Solution	Bench 308		2.5	
Na_2CO_3	311		0.01	
Bacteria Culture			10	Add in hood

Table 12 *includes all data obtained from the algae growth study completed in February. Two flasks were prepared from an algae seed culture according to the method described in Experimental Methods. The time indicates how many hours the recorded cell counting occurred after the previous algae seed was added to the flask. At the time each measurement was taken, the cell counting was repeated three times for each flask and the average value was calculated. The volume of liquid within the flask was 135-mL and based on the volume counted underneath the microscope, the concentration of cells and the total count for the flask could be calculated. The time is graphed against the concentration in* ***Figure 8****.*

Date	**Time**	**Time (hr)**	**Flask 1 (31)**			**AVG ± StDev**	**Cells (10^6/mL)**	**Count (10^8 cells)**
12-Feb	7:15 AM	11	29	21	29	26 ± 5	2.63 ± 0.46	3.56 ± 0.62
12-Feb	10:15 AM	14	33	38	36	36 ± 2	3.57 ± 0.25	4.82 ± 0.34
12-Feb	1:15 PM	17	21	23	22	22 ± 1	2.2 ± 0.10	2.97 ± 0.14
12-Feb	4:30 PM	20	22	22	25	23 ± 2	2.3 ± 0.17	3.11 ± 0.23
12-Feb	7:30 PM	23	75	80	78	78 ± 2	7.77 ± 0.25	10.5 ± 0.34
12-Feb	7:30 PM	23	89	82	85	85 ± 4	8.53 ± 0.35	11.5 ± 0.47
13-Feb	12:00 PM	40	130	95	135	120 ± 22	12 ± 2.2	16.2 ± 2.9
15-Feb	11:30 AM	83	77	64	84	75 ± 10	7.5 ± 1.0	10.1 ± 1.4
Date	**Time**	**Time (hr)**	**Flask 2 (Unmarked)**			**AVG ± StDev**	**Cells (10^6/mL)**	**Count (10^8 cells)**
12-Feb	7:15 AM	11	19	22	23	21 ± 2	2.13 ± 0.21	2.88 ± 0.28
12-Feb	10:15 AM	14	27	26	37	30 ± 6	3 ± 0.61	4.05 ± 0.82
12-Feb	1:15 PM	17	17	20	25	20 ± 4	2.07 ± 0.40	2.79 ± 0.55
12-Feb	4:30 PM	20	27	24	26	26 ± 2	2.57 ± 0.15	3.47 ± 0.21
12-Feb	7:30 PM	23	80	76	78	78 ± 2	7.8 ± 0.20	10.5 ± 0.27
12-Feb	7:30 PM	23	96	92	94	94 ± 2	9.4 ± 0.20	12.7 ± 0.27
13-Feb	12:00 PM	40	79	92	94	88 ± 8	8.83 ± 0.81	11.9 ± 1.1
15-Feb	11:30 AM	83	77	74	75	75 ± 2	7.53 ± 0.15	10.2 ± 0.21

Table 13 *shows data obtained from the steady state operation of the two-staged continuous system, specifically for the algae tank. The volume is the observed volume of the algae tank. The column labeled "Algae" is based on researcher observation of the concentration of algae cells compared to the known visual concentration of the algae seed. A visual observation on the percent of each microorganism, algae, fungi, and protozoa, was recorded.*

Date	Volume (L)	Algae	Algae (%)	Fungi (%)	Protozoa (%)	DO (%)
12/16/20	3.2	40	90	0	10	
12/17/20	2.8	30	85	0	15	3.3
12/18/20	2.8	30	85	5	10	7.7
12/19/20	2.9	30	90	0	10	5.5
12/20/20	3	30	90	0	10	4.35
12/21/20	3.4	30	95	0	5	3.7
12/22/20	2.9	30	95	0	5	4.1
12/23/20	2.5	50	90	0	10	4
12/24/20	2.8	40	90	0	10	4.2
12/25/20	3	40	95	0	5	3.8
12/26/20	3.1	45	95	0	5	4
12/27/20	3	50	95	0	5	4
12/28/20	2	70	85	0	15	10
12/29/20	2.5	75	80	0	20	11
12/30/20	3.3	70	80	0	20	5
12/31/20	2.5	65	80	0	20	10
1/1/21	2.7	60	70	10	20	20
1/2/21	2.5	60	70	10	20	6.9
1/3/21	2.5	60	60	20	20	9
1/4/21	2.5	60	55	25	20	16
1/5/21	2.5	50	40	40	20	8
1/6/21	2.5	45	40	40	20	10
1/7/21	3.2	35	35	40	25	5
1/8/21	2.7	30	70	10	20	6
1/9/21	2.5	30	80	10	10	16
1/10/21	2.5	50	90	0	10	6
1/11/21	2.5	20	80	0	30	11
1/12/21	2.5	20	30	0	70	50
1/13/21	2.5	25	50	0	50	40
1/14/21	2.5	30	70	0	30	26
1/15/21	2.5	20	70	0	30	30
1/16/21	2.5	30	55	5	40	40
1/17/21	2.5	25	50	20	30	37

1/18/21	2.5	30	50	20	30	40
1/19/21	2.5					34.4
1/20/21	2	40	70	10	20	13.5
1/21/21		35	65	20	15	10
1/22/21	2.5	70	80	5	15	3.5
1/24/21	2.5	60	85	0	15	9
1/25/21	2.4	60	75	10	15	11
1/26/21	2.4	50	65	20	15	20
1/27/21	2.6	30	65	25	10	6.2
1/28/21	2.4	30	50	40	10	8.9
1/29/21	2.4	35	65	15	20	19.7
1/30/21	2.2	20	20	40	40	49.8
1/31/21	2.3	20	20	20	60	44.6
2/1/21	2.4	40	45	15	40	44.6
2/3/21	2.9	40	40	30	30	26.8
2/5/21		10	30	35	35	41.3

Table 14 *provides the data on optical density collected from the operation of the continuous-flow system from the end of October the beginning of February. Every sample was diluted 10 times the original amount taken from the bacteria tank, which was 0.2-mL. Three samples were measured, and the average optical density was calculated by multiplying by the dilution rate. There was significant variance in optical density values, but many factors contributed to this fluctuation. Some of these might include the interruption of nutrients supplied to the tank because the sugar or CSS line became blocked, or the air stone was stopped and the contents were not well-mixed.*

Date	OD #1	OD#2	OD #3	Dilution	Avg. OD	StDev
30-Oct	0.036	0.04	0.039	x10	0.383	0.002
31-Oct	0.055	0.056	0.052	x10	0.543	0.002
18-Nov	0.036	0.042	0.062	x10	0.467	0.014
12-Dec	0.106	0.105	0.115	x10	1.087	0.006
14-Dec	0.129	0.136	0.115	x10	1.267	0.011
19-Dec	0.068	0.073		x10	0.705	0.004
27-Dec	0.09	0.088		x10	0.890	0.001
2-Jan	0.09	0.088	0.088	x10	0.887	0.001
13-Jan	0.13	0.12	0.125	x10	1.250	0.005
14-Jan	0.093	0.091	0.098	x10	0.940	0.004
19-Jan	0.093	0.089	0.09	x10	0.907	0.002
20-Jan	0.063	0.063	0.061	x10	0.623	0.001
22-Jan	0.145	0.191	0.145	x10	1.603	0.027
23-Jan	0.081	0.077	0.075	x10	0.777	0.003
25-Jan	0.07	0.07	0.069	x10	0.697	0.001

27-Jan	0.096	0.074	0.104	x10	0.913	0.016
29-Jan	0.049	0.046	0.031	x10	0.420	0.010
31-Jan	0.028	0.029	0.027	x10	0.280	0.001
2-Feb	0.158	0.157	0.153	x10	1.560	0.003
3-Feb	0.096	0.095	0.093	x10	0.947	0.002
4-Feb	0.09	0.089	0.091	x10	0.900	0.001
5-Feb	0.184	0.188	0.188	x10	1.867	0.002
6-Feb	0.083	0.084	0.085	x10	0.840	0.001

Table 15 *shares information regarding the cell dry weight of bacteria in the continuous-flow system. It is presented in two main section with one being the cell dry weight before cleaning the tank and the other after. The purpose of taking both of these measurements was to see how much of an impact cleaning the system had on the cell dry weight.*

	Before Cleaning Flock				**After Cleaning Flock**			
Date	**Tray**	**DW**	**Cells (g/L)**	**StDev**	**Tray**	**DW**	**Cells (g/L)**	**StDev**
30-Oct					1.2898	1.299	0.92	0.00
					1.2668	1.276	0.92	
16-Nov					1.2929	1.299	0.61	0.01
					1.269	1.2752	0.62	
1-Dec					1.2887	1.2951	0.64	0.08
					1.2875	1.2946	0.71	
					1.2671	1.275	0.79	
3-Dec					1.2795	1.284	0.45	0.01
					1.2735	1.2779	0.44	
12-Dec					1.2806	1.2872	0.66	0.05
					1.2902	1.2975	0.73	
14-Dec					1.2898	1.2966	0.68	0.00
					1.2868	1.2936	0.68	
19-Dec					1.2938	1.2999	0.61	0.01
					1.284	1.2903	0.63	
27-Dec	1.2865	1.2901	0.36	0.08	1.2852	1.2876	0.24	0.01
	1.277	1.2794	0.24		1.2901	1.2924	0.23	
28-Dec			1.33					
29-Dec			0.49				0.3	
30-Dec			0.87					
31-Dec			0.7				0.52	
2-Jan	1.2829	1.2871	0.42	0.04	1.2886	1.2921	0.35	0.04
	1.2826	1.2862	0.36		1.2904	1.2933	0.29	

5-Jan			0.7					
13-Jan	1.2819	1.2865	0.46		1.2779	1.2816	0.37	
14-Jan					1.2795	1.3028	2.33	
19-Jan					1.2677	1.2827	1.5	
22-Jan					1.2804	1.2907	1.03	0.30
					1.2782	1.2843	0.61	
23-Jan					1.2618	1.2731	1.13	
25-Jan					1.2656	1.2699	0.43	
26-Jan					1.289	1.2987	0.97	
27-Jan					1.2892	1.2952	0.6	
29-Jan					1.2892	1.2967	0.75	
30-Jan					1.273	1.2811	0.81	
2-Feb					1.2801	1.2876	0.75	

Table 16 *shares information regarding cell dry weight and optical density data for the operation of the continuous system between October 30th to February 6th. The average cell dry weight was calculated along with the standard deviation when there was more than one data point taken for a specific date.*

Date	OD	CDW (g/L)	Avg CDW	StDev
30-Oct	0.383	0.92	0.92	
31-Oct	0.543			
16-Nov		0.61	0.615	0.007
		0.62		
18-Nov	0.467			
1-Dec		0.64	0.713	0.075
		0.71		
		0.79		
3-Dec		0.45	0.445	0.007
		0.44		
12-Dec	1.087	0.66	0.695	0.049
		0.73		
14-Dec	1.267	0.68	0.680	0.000
		0.68		
19-Dec	0.705	0.61	0.620	0.014
		0.63		
27-Dec	0.890	0.24	0.235	0.007
		0.23		

29-Dec		0.3		
31-Dec		0.52		
2-Jan	0.887	0.35 0.29	0.320	0.042
3-Jan		0.52		
13-Jan	1.250	0.37		
14-Jan	0.940	2.33		
19-Jan	0.907	1.5		
20-Jan	0.623			
22-Jan	1.603	1.03 0.61	0.820	0.297
23-Jan	0.777	1.13		
25-Jan	0.697	0.43		
26-Jan		0.97		
27-Jan	0.913	0.6		
29-Jan	0.420	0.75		
30-Jan		0.81		
31-Jan	0.280			
2-Feb	1.560	0.75		
3-Feb	0.947			
4-Feb	0.900			
5-Feb	1.867			
6-Feb	0.840			

References

[1] Culaba, A. B., Ubando, A. T., Ching, P. M., Chen, W.-H., & Chang, J.-S. (2020). Biofuel from Microalgae: Sustainable Pathways. *Sustainability*, *12*(19), 8009. https://doi.org/10.3390/su12198009

[2] *Biofuels Market Size, Share, Growth, Trends, Report 2030*. Precedence Research. (n.d.). https://www.precedenceresearch.com/biofuels-market.

[3] *Biofuels Market Size, Share, Growth, Trends, Report 2030*. Precedence Research. (n.d.). https://www.precedenceresearch.com/biofuels-market.

[4] Randakovits, R., Jinkerson, R. E., Darzins, A., & Posewitz, M. C. (2010). Genetic Engineering of Algae for Enhanced Biofuel Production. *Eukaryotic Cell*, *9*(4), 486–501. https://doi.org/10.1128/EC.00364-09

[5] Li, C., and L. Ju. Conversion of wastewater organics into biodiesel feedstock through the predator-prey interactions between phagotrophic microalgae and bacteria. RSC Advances, 2014, 4, 44026-44029.

[6] Siegel, R. P. (2012, October 1). *A Brief History of Biofuels*. TriplePundit. https://www.triplepundit.com/story/2012/brief-history-biofuels/61986.

[7] Kadner, R. J., & Rogers, K. (n.d.). *Biosynthetic pathways of bacteria*. Encyclopædia Britannica. https://www.britannica.com/science/bacteria/Biosynthetic-pathways-of-bacteria.

[8] Lin, Z., and L. Ju. Growth and Lipid Production of a Phagotrophic Alga Feeding on Escherichia coli Cells: A New Approach for Algal Biomass and Lipid Production from Wastewater Bacteria. Environmental Engineering Science, 2016.

[9] Lumen. (n.d.). *Characteristics of Fungi*. Boundless Biology. https://courses.lumenlearning.com/boundless-biology/chapter/characteristics-of-fungi/.

[10] Laybourn-Parry, Johanna. “Protozoan.” *Encyclopædia Britannica*, www.britannica.com/science/protozoan.

[11] Zheng, Chunli, et al. “Treatment Technologies for Organic Wastewater.” *Water Treatment*, 16 Jan. 2013, pp. 249–286., doi:10.5772/52665.

[12] “Removal of Organic Matter from Wastewater.” *Ekoton*, 17 Oct. 2017, ekoton-corp.com/solutions/removal-of-matter-from-wastewater/.