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Aquaria Final Report

Introduction

Eutrophication is defined as an increased load of nutrients, such as phosphorus and nitrogen, in a body of water that can cause dense overgrowth of plant and algal life along with the subsequent death of animal life. It contributes to significant changes in the microbial community and can create many consequences for ecosystems. Eutrophication involves the overabundance of algae and plants in which the excess eventually decomposes, producing large amounts of carbon dioxide, lowering the pH of water. This all ultimately affects wildlife negatively and leads to large amounts of death throughout the food web (1). In recent years particularly, eutrophication is causing huge issues. It is the leading cause of impairment in many marine ecosystems. This affects human life as well. Eutrophication can cause contaminated drinking water and hypoxia, an absence of oxygen (2). Another interesting fact about eutrophication is that it is able to decrease the light that is available in the areas it affects, and also can increase pH levels in the affected areas.

Eutrophication can be studied in a number of different ways, all of which demonstrate its effects. It is usually studied by measuring total phosphorus levels and total nitrogen levels in the water and comparing them to standard indexes. Phosphorus is known to be the most important

nutrient and may be used directly on its own as a measure of eutrophication (3). However, it is still important to look at other parameters involved such as temperature, pH, and salinity.

For this project, the sample used was taken from an ecologically diverse pond in Sunset, SC at approximately 9:00 am on February 1, 2024. The water temperature was recorded as 13℃ and the air temperature was recorded as 10℃. The ecosystem in the pond consists of multiple types of fish, birds, mammals, and plants. There are no industries or facilities around the pond, only three family homes. For the setup of this project, two tanks were used: one with the pond water only, as a control, and the other with the pond water with the additional supplement of phosphorus. Over a certain amount of weeks, pH, temperature, optical density (600 nm and 655 nm), nitrate concentration, and nitrite concentrations will be measured. This is significant particularly because since the pond is located in an area where there is not much contamination feeding into it, we would expect the control tank to show little to no signs of eutrophication based on the parameters being tested. In other words, we would expect the control tank to have very low nitrate and nitrate concentrations along with a fairly neutral pH. However, for the tank supplemented with phosphorus we would expect to see many signs of eutrophication, such as high nitrite and nitrate levels, and increasing pH and optical density.

Another way we are studying eutrophication in this project is with the creation of a Winodgradsky column. These columns are a type of enrichment cultures that are made by filling transparent cylinders with soil and incubating in light. Over time, microbial activity results in chemical and environmental stratifications from top to bottom. Light is the energy source for these columns and helps ensure that a structured microbial ecosystem develops in which nutrient cycling occurs efficiently (4). We used mud from the close surrounding area of the pond and enriched it with two whole eggs, including the shells. The eggs provide sulfur to the column, so

it is expected that there will be many sulfur cycle microbes in the column over time. The column should show color stratification with differentiated oxic and anoxic layers.

An additional way we are studying eutrophication in this project is with the creation of a cellulose enrichment beaker. Many bacteria, specifically ruminant bacteria, are known for cellulose system extraction. The purpose of the beaker in this project is to test for cellulose degrading bacteria like ruminants in order to indicate eutrophication. This makes sense because eutrophication can be influenced by agricultural processes, in which cellulase is typically used (5). We inoculated filter paper in a beaker with mud from the surrounding area of the pond and liquid medium to be observed throughout the experiment. Eventually, the filter paper should become macerated and the medium should be cloudy.

From the pond water sample, two separate colonies were selected as Isolate 1 and Isolate 2 and were maintained throughout the project. Throughout the weeks, the isolates were tested in many different ways including gram staining, spore staining, antibiotic susceptibility, carbohydrate response, 16s PCR, and more to try and determine their identities. These isolates are very important as they allow us to observe what kind of microbes grow and survive in these conditions and how they may adapt to their environment.

Results

Viable cell count (CFU/mL) at a dilution of 10^{-3}, pH, nitrite concentration, nitrate concentration, temperature, turbidity, and chlorophyll concentration were measured for both the experimental phosphate tank and control tank each week.

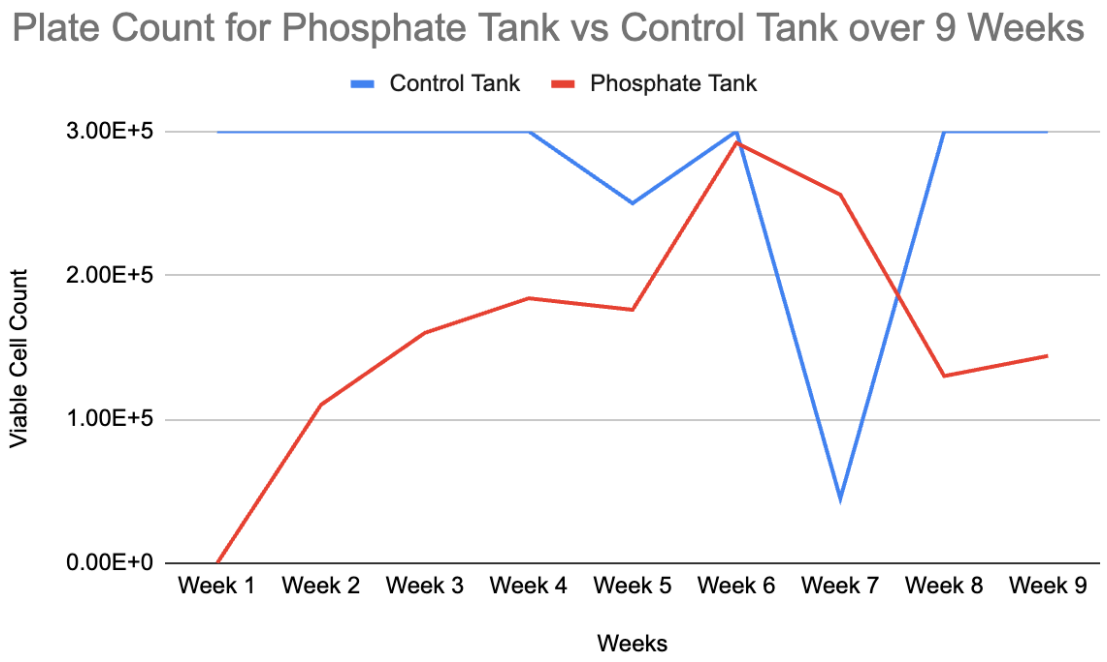


Figure 1: Average Plate Count and CFU/mL of Experimental and Control Tanks

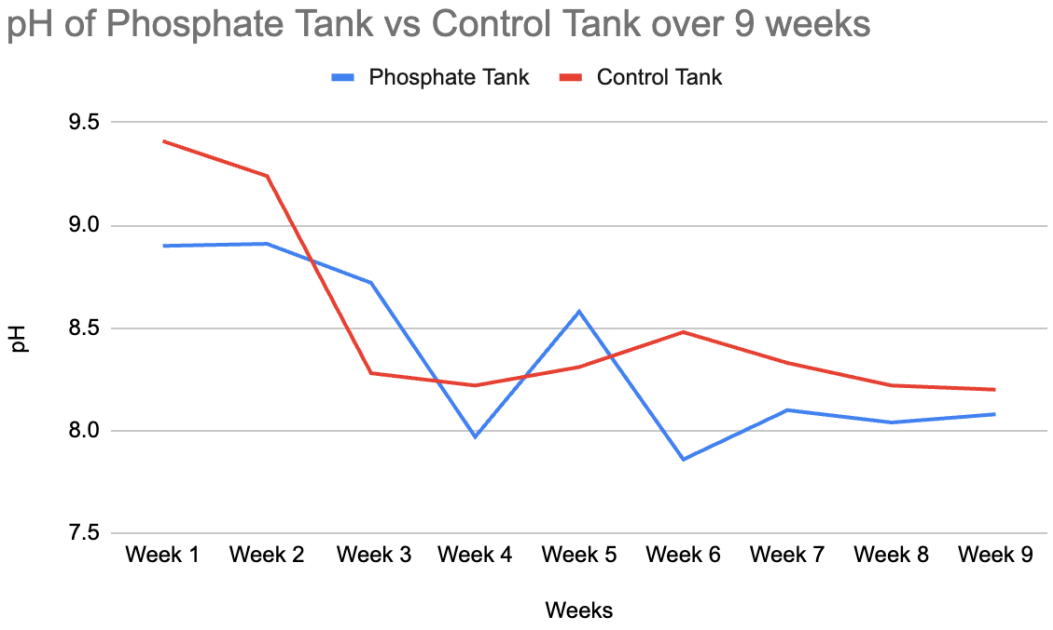


Figure 2. pH of Experimental and Control Tanks

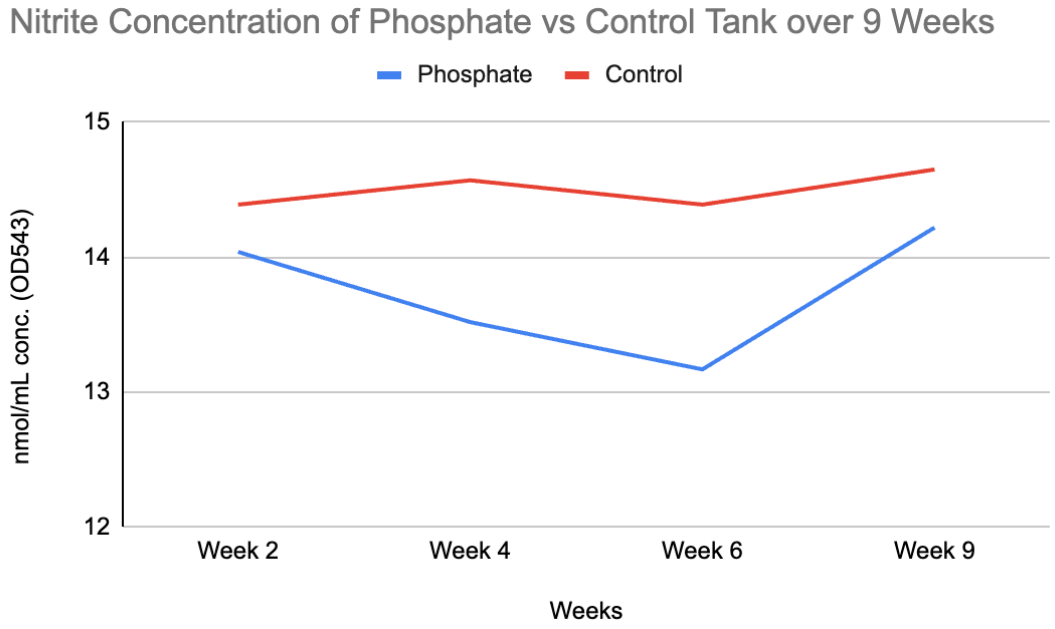


Figure 3. Nitrite Concentration of Experimental and Control Tanks

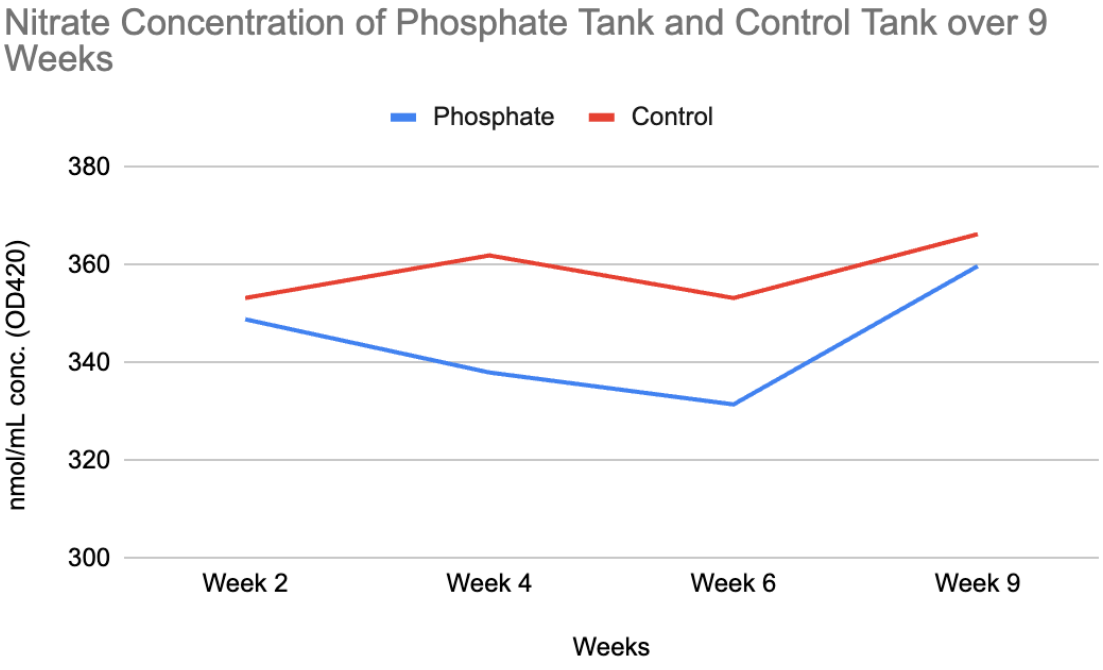


Figure 4. Nitrate Concentration of Experimental and Control Tanks

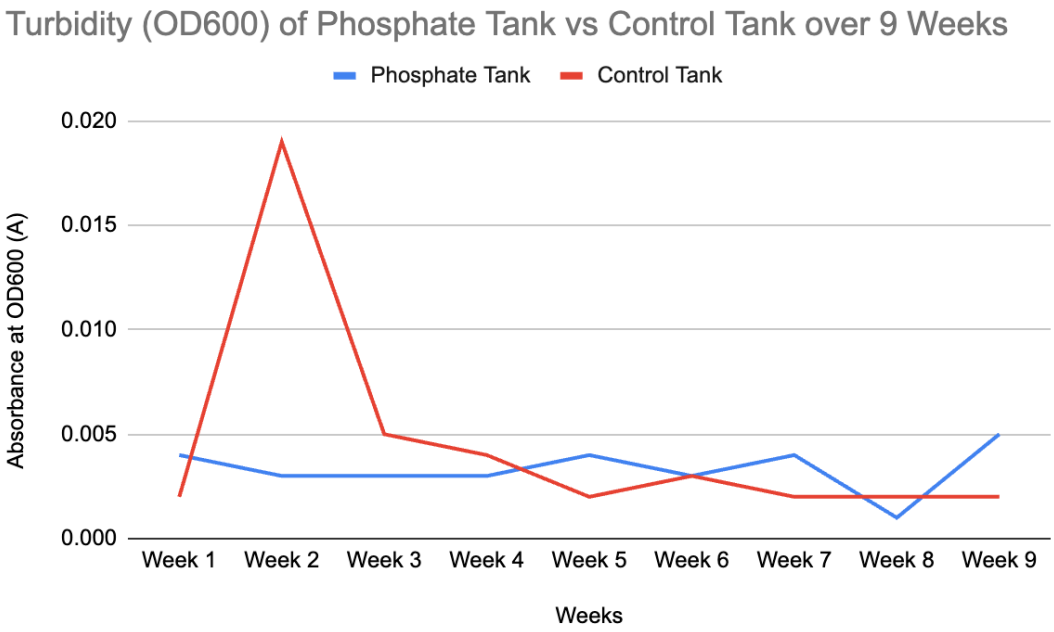


Figure 5. Turbidity of Experimental and Control Tanks

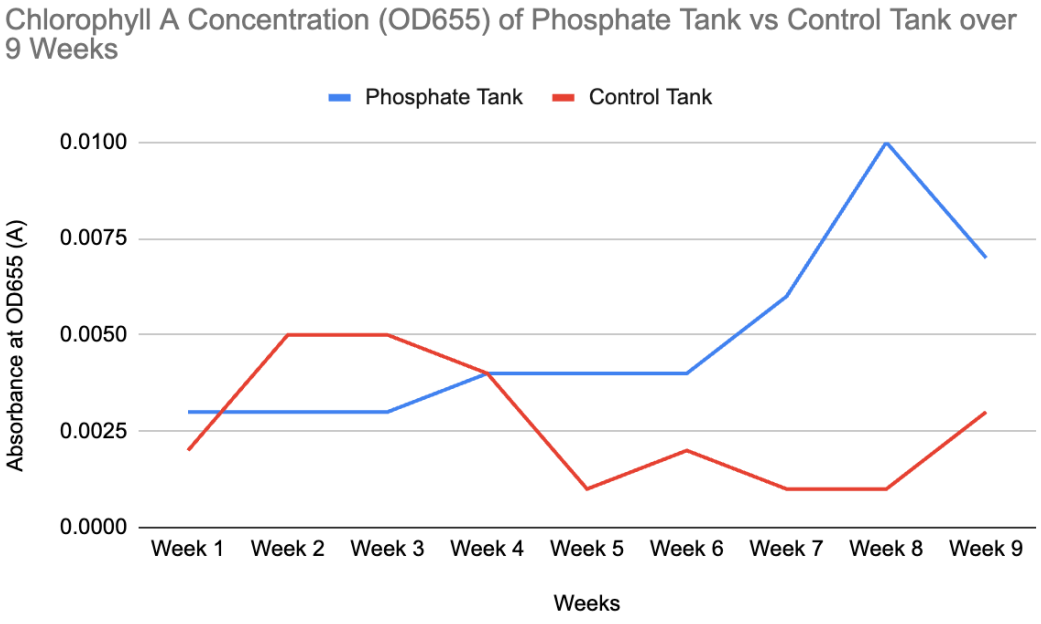


Figure 6. Chlorophyll A Concentration of Experimental and Control Tanks

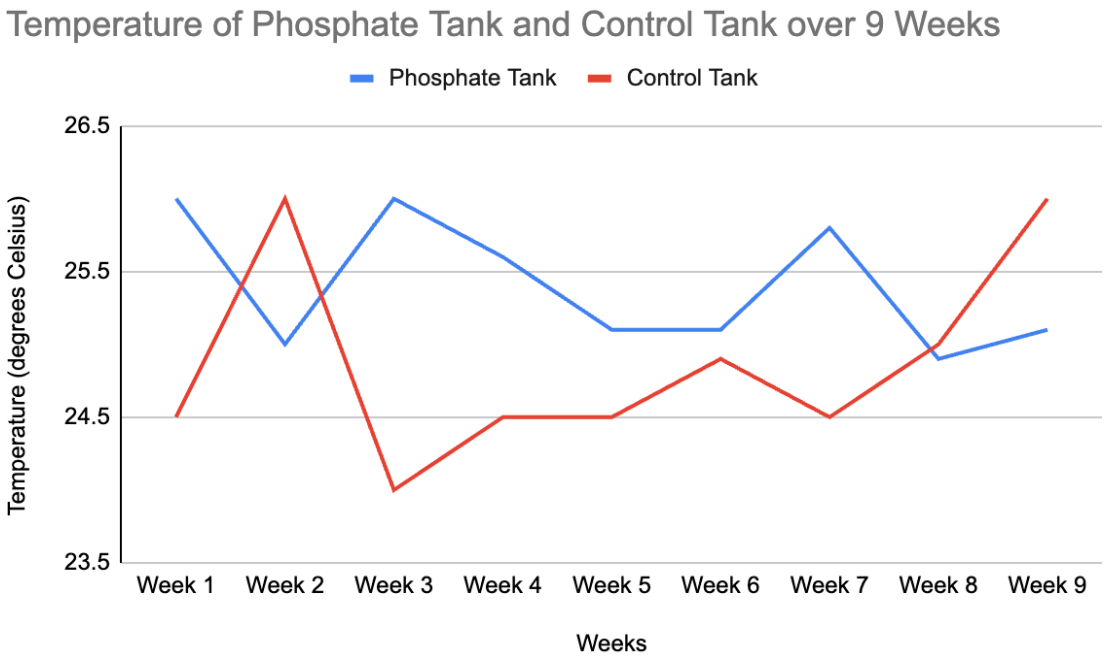
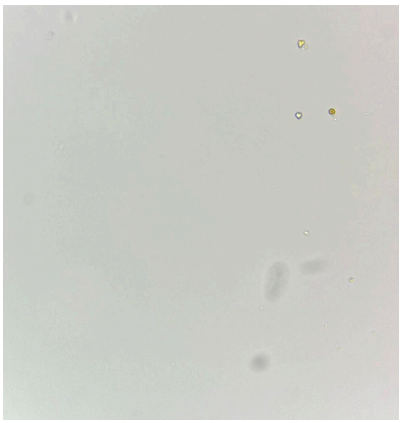


Figure 7. Temperature of Experimental and Control Tanks

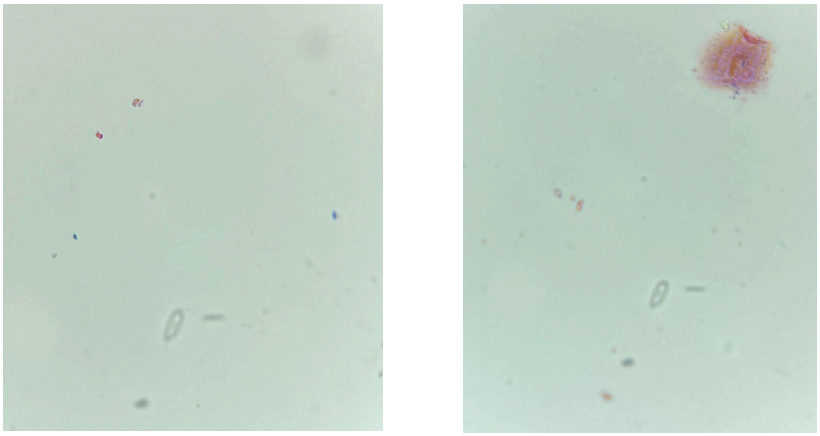
From the control tank, a wet mount slide (Figure 8) and gram stain (Figure 9) were created in order to observe the microbes present.

Figure 8. 1000x Magnification Oil Immersion Lens



The wet mount slide shows very minimal biodiversity. There are sparse green/yellow cocci.

Figure 9. 1000x Magnification Oil Immersion Lens



The gram stained slide shows both gram positive and gram negative organisms, but a very minimal amount of bacteria overall.

From the experimental tank supplemented with phosphate, a quadrant streak was performed on TSA without NaCl. After incubation, an isolate of unknown identity was selected, known as Isolate #1. The isolate was a milky shiny white and circular, and decently large in size, as seen in Figure 10. The isolate was then gram stained as seen in Figure 11.

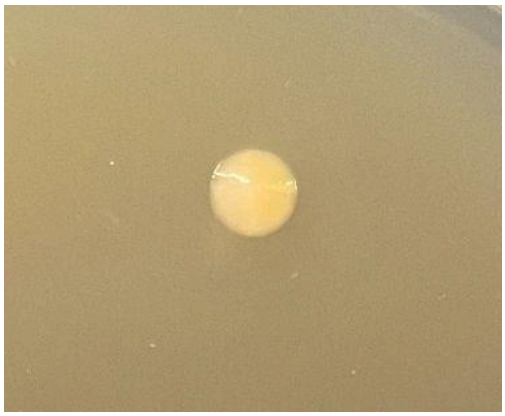


Figure 10. Isolate #1 Morphology on TSA agar without NaCl

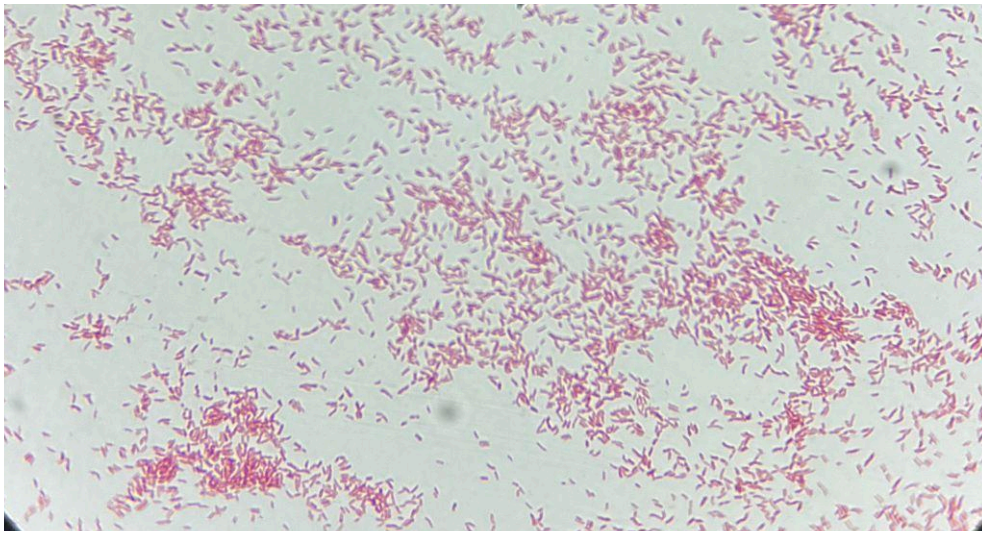


Figure 11. Gram stain of Isolate #1 on 1000x Magnification Oil Immersion lens

From the control tank, a quadrant streak was performed on TSA without NaCl. After incubation, another isolate of unknown identity was selected, known as Isolate #2. The isolate was a milky shiny white color and circular, and was large in size, as seen in Figure 12. The isolate was then gram stained as seen in Figure 13.

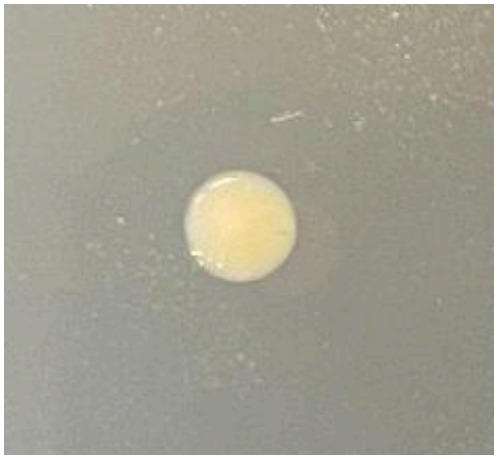


Figure 12. Isolate #2 Morphology on TSA agar without NaCl

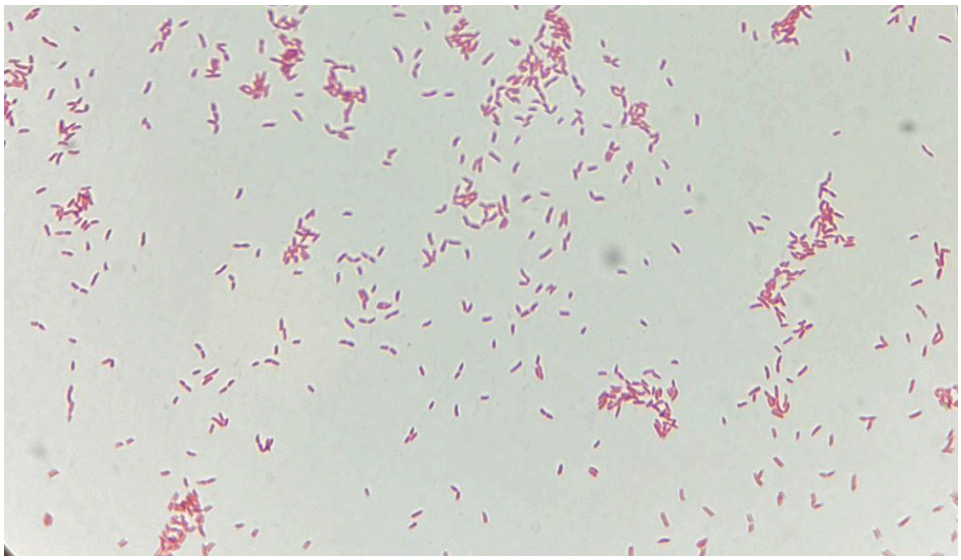


Figure 13. Gram stain of Isolate #2 on 1000x Oil Immersion lens

Both Isolate #1 and Isolate #2 were streak plated onto 10 different plates each containing a different carbohydrate in order to observe their growth. After incubation, growth was observed as either present (+) or not present (-) as seen in Table 2.

Table 1: Carbohydrate Growth Analysis

Type of Carbohydrate (%)	**Isolate 1**	**Isolate 2**
Rhamnose 0.1%	-	-
Cellobiose 0.1%	-	+
Mannose 0.1%	+	+

Lactose 0.1%	-	-
Xylose 0.1%	+	+
Fructose 0.1%	+	+
Sucrose 0.1%	-	-
Mannitol 0.1%	+	+
Dextrose 0.1%	+	+
Trehalose 0.1%	+	+

Both Isolate #1 and Isolate #2 were plated on agar with antibiotics to determine their susceptibilities to the antibiotics. After incubation, zones of inhibition were measured, as seen in Table 1.

Table 2. Antibiotic Susceptibility of Isolates

	Isolate 1 Zone of Inhibition	**Susceptible (R, I, S)**	**Isolate 2 Zone of Inhibition**	**Susceptible (R, I, S)**
CIP5	30 mm	S	35 mm	S
B10	X	X	X	X
AZM15	X	X	X	X

SAM20	X	X	X	X
AH10	X	X	X	X
E15	X	X	X	X
NA30	X	X	X	X
K30	X	X	20 mm	S
VA30	X	X	27 mm	S
TE30	17 mm	I	10 mm	R
S10	12 mm	I	X	X
OX1	X	X	X	X

Both Isolate #1 and Isolate #2 were pipetted into BioLog plates with results being shown in Table 3 and Table 4.

Table 3: Biolog Plate Results for Carbohydrate Utilization for Isolate 1

Water	β-Methyl-D- Glucoside	D-Galactonic Acid ɣ-Lactone	L-Arginine
Pyruvic Acid Methyl Ester	D-Xylose	D-Galacturonic Acid	L-Asparagine
Tween 40	I-Erythritol	2-Hydroxy Bezoic Acid	L-Phenylalanine
Tween 80	D-Mannitol	4-Hydroxy Benzoic Acid	L-Serine

α-Cyclodextrin	N-Acetyl-D-Glucosamine	ɣ-Amino Butyric Acid	L-Threonine
Glycogen	D-Glucosaminic Acid	Itaconic Acid	Glycyl-L-Glutamic Acid
D-Cellobiose	Glucose-1 Phosphate	α-Keto Butyric Acid	Phenylethyl-amine
α-D-Lactose	D,L-α-Glycerol Phosphate	D-Malic Acid	Putrescine

Table 4: Biolog Plate Results for Carbohydrate Utilization for Isolate 2

Water	β-Methyl-D- Glucoside	D-Galactonic Acid ɣ-Lactone	L-Arginine
Pyruvic Acid Methyl Ester	D-Xylose	D-Galacturonic Acid	L-Asparagine
Tween 40	I-Erythritol	2-Hydroxy Bezoic Acid	L-Phenylalanine
Tween 80	D-Mannitol	4-Hydroxy Benzoic Acid	L-Serine
α-Cyclodextrin	N-Acetyl-D-Glucosamine	ɣ-Amino Butyric Acid	L-Threonine
Glycogen	D-Glucosaminic Acid	Itaconic Acid	Glycyl-L-Glutamic Acid
D-Cellobiose	Glucose-1 Phosphate	α-Keto Butyric Acid	Phenylethyl-amine
α-D-Lactose	D,L-α-Glycerol Phosphate	D-Malic Acid	Putrescine

The isolates then both underwent 16s PCR tests in order to obtain more information about their identities as seen in Figure 14.

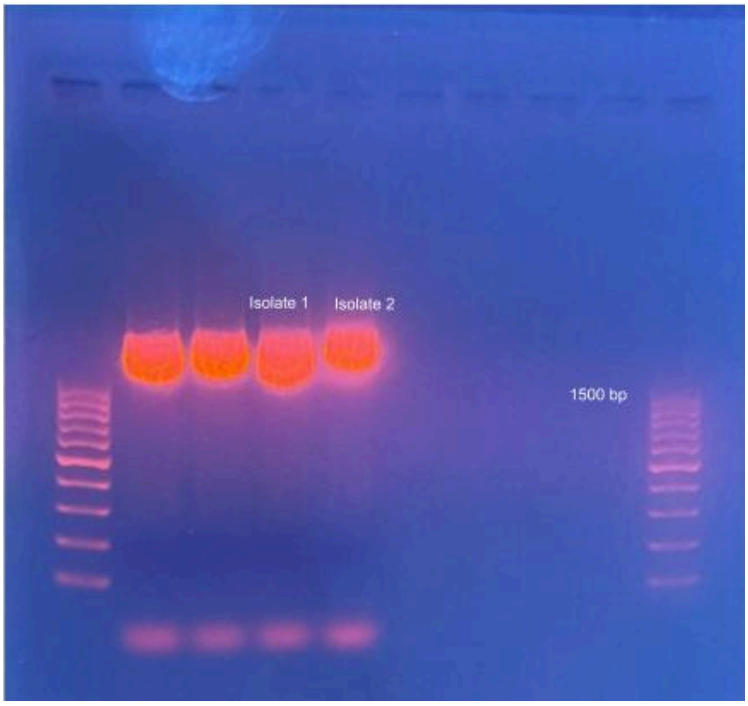


Figure 14: DNA gel electrophoresis results of 16s PCR. The DNA in well three represents isolate 1, while the DNA in well four represents isolate 2. Both bands are at or above the 1500 bp mark as seen by the 100 bp ladder used, demonstrating that the PCR products are about 1500 bp.

Table 5: 260/280 Ratios for DNA taken from Isolate 1 and Isolate 2

	260/280 Ratio
Isolate 1	3458.350
Isolate 2	3499.100

The sequences obtained were used in BLAST software to generate identity, sequence similarity, and query coverage of both isolates as seen in Table 6 and Table 7.

Table 6: 16s PCR BLAST Isolate 1

Result	Identity	Sequence Similarity (%)	Query Coverage (%)
1	*Pseudomonas grimontii* strain LG-4-3	96.95	92
2	*Pseudomonas rhodesiae* strain KBL5	96.87	92
3	*Pseudomonas rhodesiae* strain NL2019	96.87	93
4	*Pseudomonas fluorescens* strain KBL29	96.94	92
5	*Pseudomonas rhodesiae* strain BS2777	96.87	93

Table 7: 16s PCR BLAST Isolate 2

Result	Identity	Sequence Similarity (%)	Query Coverage (%)
1	*Pseudomonas grimontii* strain LG-4-3	96.22	98
2	*Pseudomonas rhodesiae* strain KUDC1808	96.15	98
3	*Pseudomonas* sp. KJ41	96.94	97
4	*Pseudomonas grimontii* strain LH-3-1	96.07	98
5	Uncultured bacterium clone NO24ab16s50	96.07	98

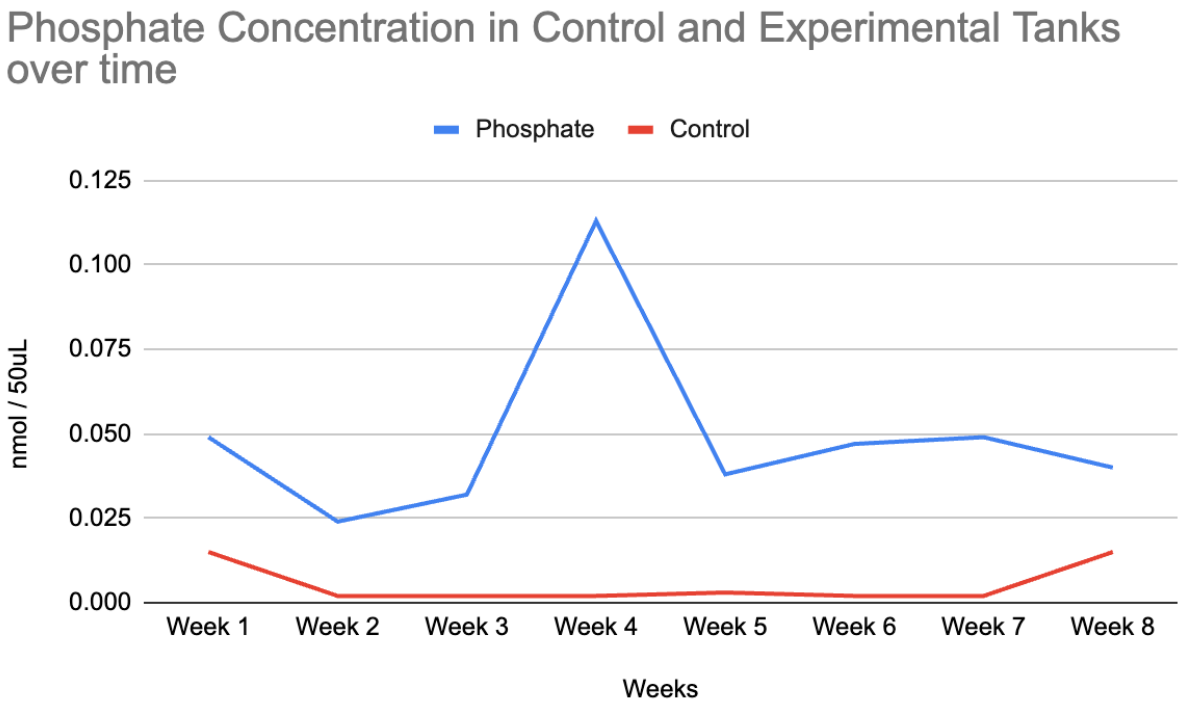


Figure 15: Phosphate concentration (nmol/50µL) in control vs experimental tanks

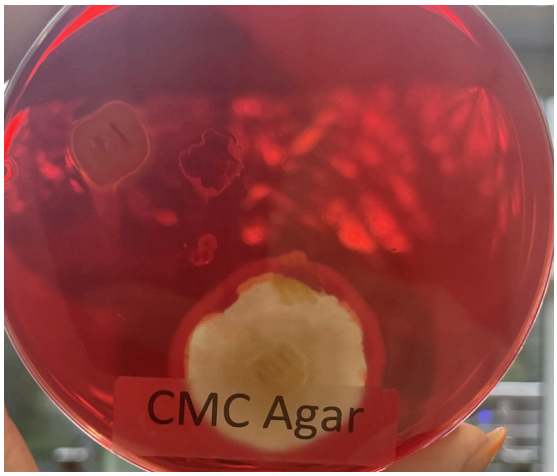


Figure 16: Morphology of three cellulose degraders stained with Congo Red dye and destained with NaCl. There is visible clearance around all three microbes on the agar, indicating cellulose degradation. The largest area of clearance is around the large fungal colony.

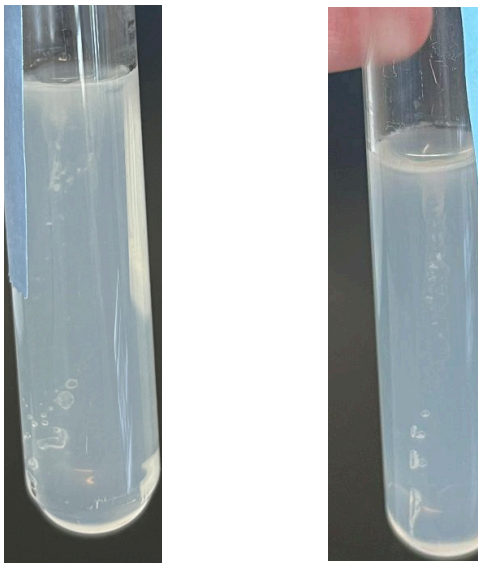


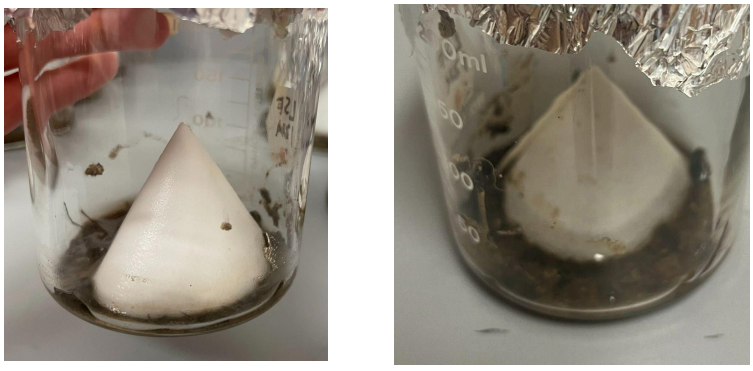
Figure 17: PYE agar deep of Isolates 1 and 2, respectively, in which isolate bacterial growth is present at the top of the agar deep.

Figure 18. Winogradsky Column

These two photos show the initial appearance of our Winodgradsky column on the left, supplemented with two eggs. There are no stratifications present. The soil is a dark brown

opaque color. On the right shows the most recent appearance of the Winodgradsky column. There is still no stratification present. There is visible gas production.

Figure 19. Cellulose Degradation Beaker



These two photos show the initial appearance of our cellulose degradation beaker on the left and most recent appearance on the right. The liquid has begun to infiltrate the filter paper but there is minimal degradation to be observed.

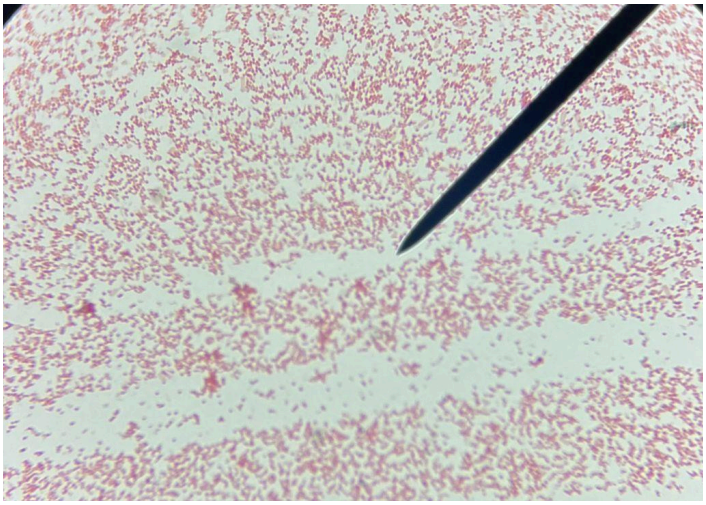


Figure 20: Spore stain of Isolate 1, in which no spores are observed. The spore stain will stain spores with malachite green and counterstain vegetative cells with safranin.

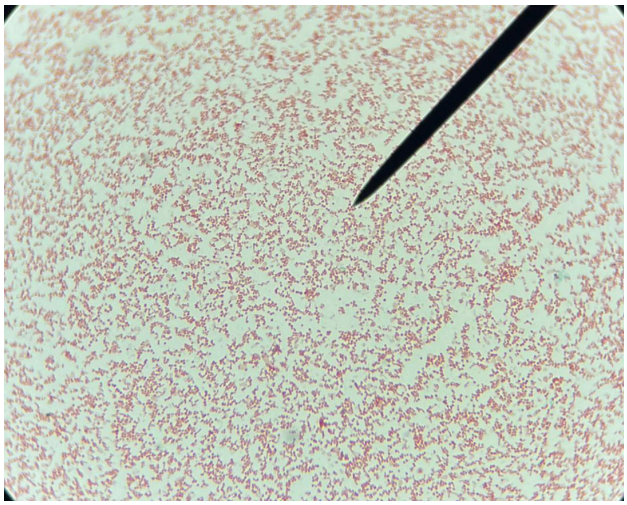


Figure 21: Spore stain of Isolate 2, in which no spores are observed. The spore stain will stain spores with malachite green and counterstain vegetative cells with safranin.

Discussion

Based on what we have learned about eutrophication throughout this semester, the experimental tank of pond water with phosphate added was expected to have higher levels of nitrite and nitrate, absorbances, and lower pH, as this would indicate eutrophication. Over the past 9 week period, it can be observed that the pH of the experimental tank has had an overall decline as expected. The control pH has had a very slight decline but has stayed pretty much the same. There has been a slight increase in both nitrite and nitrate concentration in the experimental tank, as expected, but it has stayed decently consistent. The control remained very consistent throughout the 9 weeks in terms of nitrite and nitrate concentrations, also as we expected. Further to be expected, the Chlorophyll A concentration shows an increase for the experimental tank while the control stayed generally the same. Somewhat unexpectedly, the turbidity had a very large spike early on but plateaued to a low consistent number in following

weeks. The control levels stayed very similar as expected. The temperature of both the experimental and control tanks has fluctuated, but stayed in a range of about 2 degrees. This is likely due to the room they are stored in and fluctuations in room temperature, and is to be expected. In terms of viable cell count, the experimental tank numbers show a general increase while the control shows a general consistency, aside from one downward spike outlier. This was expected as eutrophication over time provided an increase in these numbers for the phosphate tank. Overall, there is clear indication of the presence of eutrophication in the experimental tank.

Eutrophication has been known to occur naturally over very long periods of time, and it can be decades or even centuries before real measurable results are present (2). Therefore, it is not too far off base and is expected that after a short 9 weeks, while we see some signs of eutrophication, they are not extremely significant. We would not expect to see fully significant data to support that eutrophication is occurring in this short of a time period. However, the signs of it are definitely able to show early on. It is also important to note the appearance of the tank at the end of the 9 week period. The water in the tank became visibly murky with small particles floating around in it and there wawa some plant growth. We hypothesized that at the end of the project, there would be signs of eutrophication in the experimental phosphate enriched tank, and this hypothesis was supported.

It is also important to discuss the enrichment that the experimental tank had: phosphate. As mentioned in the introduction, phosphate is known to be arguably the most important nutrient and may be used directly on its own as a measure of eutrophication (3). This lines up with our data as our experimental tank showed significant differences both statistically and visibly in comparison to the control tank. As seen in Figure 15, the experimental tank had higher levels of phosphate from the start, which was expected. In large quantities, it can lead to eutrophication

and harmful algal growth, along with other water quality issues. Also, phosphorus generally occurs in small quantities in nature, therefore simple small increases can still negatively affect water quality and biological conditions in environments (7). This helps explain why phosphate was a good enrichment choice to measure eutrophication.

The antibiotic susceptibility test for Isolate #1 showed that it is susceptible to CIP5, TE30, and S10. The antibiotic susceptibility test for Isolate #2 showed that it is susceptible to CIP5, K30, VA30, and TE30, shown in Table 1. It has been found that microbes present in eutrophication processes tend to have or develop antibiotic resistance, with a wide resistance spectrum. Therefore, while the isolates are susceptible to the antibiotics listed above, this still leaves a large amount of antibiotics that they were resistant to, which was expected. This information also contributes to discovering the identities of the isolates.

Isolate #1 grew on the carbohydrate plates containing mannose, xylose, fructose, mannitol, dextrose, and trehalose, as seen in Table 2. Isolate #2 grew on carbohydrate plates containing cellobiose, mannose, xylose, fructose, mannitol, dextrose, and trehalose, as seen in Table 2. This information reveals more about both isolates and their properties, as both isolates show metabolism of carbohydrates. This was observed more using BioLog plates. Isolate #1 grew when in the presence of D-Xylose, D-Galactonic Acid ɣ-Lactone, D-Galacturonic Acid, L-Arginine, L-Asparagine, D-Mannitol, 4-Hydroxy Benzoic Acid, and ɣ-Amino Butyric Acid. Isolate #2 grew when in the presence of D-Galactonic Acid ɣ-Lactone, D-Galacturonic Acid, L-Arginine, L-Asparagine, D-Mannitol, and ɣ-Amino Butyric Acid. This was expected that the isolates would be able to grow in the presence of many carbohydrates, and it is also clear that the isolates are similar in where they can grow.

Sequences for Isolate 1 and Isolate 2 were obtained from 16s PCR. For Isolate #1, the top five BLAST results were different strains of species *Pseudomonas,* with *Pseudomonas grimontii* strain LG-4-3 being the top hit with 96.95% sequence similarity and a query coverage of 92%. The second hit was *Pseudomonas rhodesiae* strain KBL5 with a 96.87% sequence similarity and 92% query coverage. The third hit was *Pseudomonas rhodesiae* strain NL2019 with a 96.87% and a 93% query coverage. The fourth hit was *Pseudomonas fluorescens* strain KBL29 with a sequence similarity of 96.94% and 92% query coverage. The fifth hit was *Pseudomonas rhodesiae* strain BS2777 with a sequence similarity of 96.87% and query coverage of 93%. All five results have high sequence similarities and query coverages, indicating a high relation to the *Pseudomonas* species. For Isolate #2, the top four hits were different strains of species *Pseudomonas,* with *Pseudomonas grimontii* strain LG-4-3 being the top hit with 96.22% sequence similarity and a query coverage of 98. The second hit was *Pseudomonas rhodesiae* strain KUDC1808 with a sequence similarity of 96.22% and a query coverage of 98%. The third hit was *Pseudomonas* sp. KJ41 with a sequence similarity of 96.94% and query coverage of 97%. The fourth hit was *Pseudomonas grimontii* strain LH-3-1 with a sequence similarity 96.07% and a query coverage of 98%. It is very interesting to note that the fifth hit was an Uncultured bacterium clone NO24ab16s50 with a sequence similarity 96.07% and query coverage of 98%. It is also important to note that higher query coverage indicates high sequence alignment to the BLAST results, so all of these hits for both isolates show close alignment. This also demonstrates further the similarity between Isolate 1 and Isolate 2.

Regarding the agar deeps, both isolates grew at the top of the media as expected. This demonstrates their utilization of oxygen. Oxygen concentration is much higher at the top of the agar, which is why they grew there and also shows their aerobic qualities.

Regarding the spore stains, neither Isolate 1 nor Isolate 2 grew spores, which was expected in general.

After performing these tests and analyzing the data, the identities of Isolate 1 and 2 can be assumed. Isolate #1 and Isolate #2 were both determined to be *Pseudomonas grimontii* strain LG-4-3. Both isolates were gram-negative bacilli as shown by the gram stains. They also were both aerobic as shown by the agar deep growth. *Pseudomonas grimontii* is known to be a gram-negative bacillus that can complete respiratory metabolism, so it shares the same qualities (8). Also, *Pseudomonas* typically utilize amino and organic acids (9). The carbohydrates that both Isolate 1 and Isolate 2 metabolize are amino and organic acid compounds. Lastly, it was found that *Pseudomonas* typically do not form spores, as we also saw from both Isolates with the spore stain (10). Therefore, this classification as species *Pseudomonas* makes sense. Both isolates showed extremely similar results and characteristics throughout the duration of the project, so this was expected.

In terms of the cellulose enrichment beaker, there is a visible difference in the first and second pictures in which we can see the filter paper has become almost fully macerated and the liquid at the bottom has become much more cloudy, which was expected. This is due to the presence of certain microbes that are able to degrade cellulose in the soil. This was confirmed by the test of the Congo Red stain on CMC agar. All three microbes cultured demonstrated clear areas around them, deeming them as cellulose degraders. If this project was continued, we would expect the filter paper to be fully macerated and to see the liquid at the bottom as opaque (5).

In terms of the Winogradsky column, at the end of the 9 weeks we expected to see some visible stratifications reflecting microbial activity and nutrient cycles. Our hypothesis for this was not supported as there are no visible stratifications in the column. While it has been 9 weeks,

it is somewhat realistic that there are no stratifications yet (4). In fact, stratifications can take over 70 weeks to form (6). However, the amount of gas production seen at the top indicates that cycles such as anaerobic sulfate respiration may be occurring. The soil is a very dark color, which is likely due to the production of hydrogen sulfide (6). The use of two whole eggs as a sulfur source in the column allows us to assume that the reduction of sulfur has occurred. If this project were continued for a longer period of time, it can be assumed that there would definitely be stratifications that begin to appear.

Throughout this project, there were some limitations that should be addressed. The duration of the project was only 9 weeks, which in terms of eutrophication, Winogradsky column, and cellulose degradation is a very brief period.

Over the next few weeks as we observe and measure more, there will likely appear to be more signs of eutrophication in the experimental tank and cellulose enrichment beaker, as well as more stratifications in the Winodgradsky column. We also measured only one singular phosphate tank rather than multiple, which would provide more accurate and reliable results. Also, the tanks were kept in a university classroom which could have introduced contamination.

Based on these limitations, recommendations for the future would include a prolonged time period for the project to gather more significant data. Also, it would be good to increase the number of phosphate enriched tanks. Lastly, enriching other tanks with other elements would be interesting and provide a broader range of information about processes and microbial diversity in the tank.

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