CHARACTERIZATION OF *RHODOPSEUDOMONAS PALUSTRUS* STRAINS FOR THE PRODUCTION OF FIXED NITROGEN FERTILIZER FOR MARS

by

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Project Abstract:

The purpose of this project was to characterize the potential of various strains of purple non-sulfur bacteria for the production of fixed nitrogen fertilizers for the manned Mars missions. Six strains of *Rhodopseudomonas palustris* (CGA009, CGA010, TIE-1, NifA*, and PB23) were all investigated. Through initial growth trial experiments, *R. palustris* NifA* and PB23 were selected for their engineered nitrogen fixation and rapid growth respectively. Growth curves and ammonium concentrations were collected over time in pilot scale batch photobioreactors (200 mL). Biomass production was then scaled up to benchtop photobioreactors (1.5 L) and then 14 L total volume for both NifA* and PB23. Cells were harvested via centrifugation, and nitrogen degradation was characterized using both volatile ammonia and soluble nitrogen arrays. While PB23 was found to grow more rapidly at small scale, NifA* performed better in scale up. Further studies will quantify rates of nitrogen degradation using the arrays developed in this Senior Design Project.

Acknowledgement:

We thank Dr. Ronald Sims for his guidance and invaluable advice during the completion of this project. We also acknowledge Dr. Lance Seefeldt and his graduate students, Mathangi Soundararajan and Kyle Valguardson for their assistance with laboratory work and for sponsoring the project.

Contents:

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Introduction:

The need for sustainable agriculture has become increasingly more important in recent years. A growing population continually encroaches rich farmland for residential purposes. Therefore, farmers need to grow more and more food on less and less land. The lack of land makes rotating crops more difficult, consuming the available nutrients and making the land barren. To combat this, commercial fertilizers are applied heavily to these farms. Overuse can result in ammonia and phosphorus pollution that can cause toxic algal blooms. In short, farmers have never been faced with a more challenging endeavor than to feed tomorrow's population.

One solution to this problem is to develop technologies that would allow agriculture on a planet more barren than any climate found on Earth: Mars. NASA's successful lunar missions catalyzed a technological revolution that has impacted nearly every facet of human existence.

This project aimed to produce fixed nitrogen and fertilizer from atmospheric nitrogen. Accomplishing this feat will allow the production of these critical nutrients from *in situ* resources found in the Martian atmosphere and regolith. It will also improve agricultural efforts on Earth, making it possible for farmers to create fertilizer from air and sunlight, instead of using expensive commercial fertilizers.

To accomplish this, a strain of *Rhodopseudomonas palustris*, a nitrogen-fixing bacterium with incredible metabolic diversity, was characterized. Testing of the strains was accomplished by performing degradation studies on the cell biomass to determine nitrogen availability.

Project Aims and Objectives:

The initial objective for this project was the classification and growth of the strains of *R. palustris* available to the team. The ideal strains produced robust colonies and grew quickly under nitrogen-fixing conditions.

Second, a baseline quantification of NH_4^+ production was completed for each strain. This was done by standardizing the growth media and bacterial inoculation for each strain and measuring soluble NH_4^+.

Third, the strains were grown under nitrogen fixing conditions, roughly simulating conditions that will be needed to grow on Mars. The ideal strain would be identified due to its performance under nitrogen fixing conditions.

Fourth, the best strains of *R. palustris* were selected and used for the degradation testing based on the results from the baseline quantification of NH_4^+ production and the growth curve.

Fifth, degradation trials were conducted to determine how quickly and rapidly the nitrogen is released from the cell biomass. Two trials were conducted, one to track volatile nitrogen in the form of ammonia, another to track soluble nitrogen release as ammonium, nitrite, and nitrate.

In summary, the project objectives were:

1. Grow each *R. palustris* strain and compare liquid colony growth curves and colony formation on solid media.
2. Compare each strain under nitrogen fixing conditions.

3. Quantify NH_4^+ production and secretion into culture media for each strain.
4. Select the top producer/most-workable bacterium for further testing and development.
5. Scale up the biomass production of the selected strains.
6. Conduct nitrogen degradation trials.

Evaluation Criteria:

Objective 1: Evaluate growth using the following criteria:

1. Speed to a specific optical density in liquid media
2. Colony formation on a plate
3. Light intensity requirements

Objective 2: Evaluate strains based on their ability to grow to high optical densities, and on NH_4^+ production in pilot scale batch reactors.

Objective 3: Quantify NH4+ production using a total nitrogen assay and NH_4^+ assays available in Dr. Seefeldt's lab.

Objective 4: Using the results from objective one, two, and three, identify the *R. palustris* strain that has the best growth characteristics, NH_4^+ production, and NH_4^+ excretion.

Objective 5: Characterize biomass production in benchtop scale photobioreactors.

Objective 6: Evaluate nitrogen degradation based on the concentration of nitrogen released from the sample, measuring both volatile and soluble nitrogen.

Constraints:

There are several constraints that are inherent to the design process that must be considered due to the collaboration with NASA CUBES. As the CUBES program is for use on Mars, the addition of weight is incredibly costly, and thus the design was to not constitute a significant increase in the payload.

Additionally, while it has been shown that *R. palustris* is capable of producing ammonia from gaseous nitrogen, it is vital that the fixed nitrogen be bioavailable for plant growth. In order for the fertilizer to be considered successful, the fixed nitrogen must be readily utilized by plants in an agricultural system. This means that any nitrogen in solution as ammonium as well as the nitrogen in cell biomass must be released from the system over time as soluble nitrogen such as nitrate, nitrite, and ammonium, and not volatile nitrogen such as ammonia.

Also, a serious concern for NASA is the health and safety of their astronauts. As those tasked with colonizing Mars will be working with the organism and applying the cell media and biomass directly to their food supply, it is important that the organism not be pathogenic and be safe to work with. The organism must represent a dependable source of nutrients, otherwise, the mission becomes risky for astronaut health. The bacteria being explored in the design do not show any significant risk to human health, and the design for growth is constructed in such a way that minimal contact with the bacteria will be needed by any people using it. This fits into the health constraints inherent with the use of this system by humans, and the constraints specified by the NASA CUBES group through Dr. Seefeldt.

The system must also fit into a strict vegetarian diet, a constraint of all Martian missions. The fertilizer will be used strictly to grow crops for human consumption, particularly nutrient-dense plants and fungi. All parameters inside the system must be self-sustainable on *in situ* resources, as NASA will be unable to send any additional supplies to the Martian astronauts. This need was demonstrated through the science fiction novel, *The Martian*. In this hypothetical case study, NASA could not respond quickly enough to save an astronaut from death in case of emergency if dependent on shipped supplies instead of *in situ* resources. To minimize the need for additional resources, this project uses a microbe that can be grown using acetate and the Martian atmosphere, two resources that are considered abundant to NASA and can be produced in quantities much greater than the project's needs require by other processes within the food production system proposed by the CUBES group.

Background and Literature Review:

A significant barrier to human survivability on Mars is becoming self-sufficient on *in situ* resources, as sending resources from Earth is cost-prohibitive.[1] To this end, a process must be developed to fix carbon and nitrogen from atmospheric gasses into usable materials for agriculture. The NASA Center for the Utilization of Biological Engineering in Space (CUBES) aims to accomplish these goals. Purple non-sulfur bacteria such as *Rhodopseudomonas palustris* have the capacity to fix carbon dioxide and nitrogen gas into biomass and ammonium (NH_4^+) respectively under anaerobic photoautotrophic conditions.[2] It has also been shown to fix nitrogen using waste compounds as carbon sources and electron donors, allowing wastewater to be used as a growth media.[3] However, a novel process developed by Dr. Matthew Kanan from Stanford

University has enabled the high yield conversion of carbon monoxide to acetate.[4] The MOXIE (Mars OXygen In situ resource utilization Experiment) device in development by MIT will split CO2 into oxygen and CO, which can then be converted to acetate using the process in development by Dr. Kanan. As such, acetate will be in abundance and carbon dioxide fixation will no longer be a requirement of the biological conversion system. This change in focus allowed the purple non-sulfur bacteria to be grown in photoheterotrophic nitrogen-fixing conditions, in lieu of photoautotrophic conditions. This change in growth conditions allows a greater yield of cell mass per time.

R. palustris has been shown to improve plant growth as a fertilizer.[5] However, the wild type strain is sensitive to NH_4^+ concentration in the media, and its nitrogenase will down-regulate when sufficient fixed nitrogen is present.[6]

This report works to characterize these natural features of *R. palustris* for their subsequent exploitation on the manned Mars missions expected to launch in the next few decades. First, the growth and baseline NH_4^+ production were characterized for the strains CGA009 [2,7], TIE-1 [8,9], NifA* [6], and a novel isolate, PB23 (unpublished). Based on various design criteria presented in this proposal, the ideal strain was selected considering both optimal conditions and under simulated Martian conditions: wastewater, infrared light, and low partial pressure nitrogen gas, as shown in Table 1.[10,11]

Table 1. *A comparison of conditions on Mars and Earth.*[10,11]

Conditions	Mars	Earth
Gravity (m/s^2)	3.71	9.80
Temperature (C, K)	-63, 210	15, 288

Surface Pressure (kPa)	0.636	101.4
Atmosphere (%)		
CO_2	95.32	400 ppm
N_2	2.7	78.08
Argon	1.6	-
O_2	0.13	20.95
CO	0.08	-

Microgravity conditions were not attempted, and the temperature was maintained at ideal conditions for the microbe. The strains were evaluated for their production of free ammonium and total nitrogen using validated methods. To the authors' knowledge at the time of writing, the characterization of ammonium production of these strains under the proposed conditions has not been previously attempted and will provide valuable data to the sponsor (NASA) as they prepare for manned missions to Mars.

It has been proposed that fixed nitrogen production in *R. palustris* could be improved using genetic engineering. As previously stated, the nitrogenase of the wild type *R. palustris* strain is sensitive to the level of NH_4^+ in the cell. The strain NifA* has a mutated *nifA* gene. The wild type *nifA* encodes an RNA polymerase sigma 54-dependent transcriptional activator. NH_4^+ prevents the transcription of *nifA*. It also structurally modifies any nifA protein, making the transcriptional activator unable to bind to its binding site and activating nitrogenase gene transcription (*nif*). NH_4^+ can also "switch off" nitrogenase through ADP-ribosylation mediated by DraT. The NifA* strain has a 48-nucleotide deletion in the *nifA* gene, which makes the transcriptional activator insensitive to the levels of NH_4^+, thereby producing the nitrogenase constitutively. This mutation has been leveraged to produce hydrogen biogas in ammonia-rich

media.[6] This strain was further engineered to express a modified nitrogenase capable of reducing carbon dioxide to methane.[12] It has been demonstrated in a closely related microbe, *Rhodobacter capsulatus,* that the "switch off" of nitrogenase can be circumvented by mutating the ammonium transporter AmtB.[13]

Originally, it was discussed to show the ability of the different strains of *R. palustris* to act as a fertilizer by conducting plant trials. However, after a discussion with Dr. Bruce Bugbee, it was determined that the final portion of the project would focus on quantifying the rate of available nitrogen that was released from the degradation of the bacteria. This was largely due to the known fact that the addition of bacteria to plants under nitrogen limiting conditions will enhance growth. It has been shown many times in research and several times by the Bugbee lab and the Seefeldt Lab, most recently using the bacterial strain *Azotobacter,* whose nitrogen-fixing capabilities are similar to *R. palustris* (See Figure 1). Due to this, and a discussion on the information that would be most valuable to Dr. Seefeldt, Dr. Bugbee, and the CUBES group, it was decided to develop a system in which the volatilized and solubilized ammonium could be captured and measured. This test would allow the quantification of the degradation rate of

nitrogen, informing the NASA scientists in charge of *in situ* agriculture.

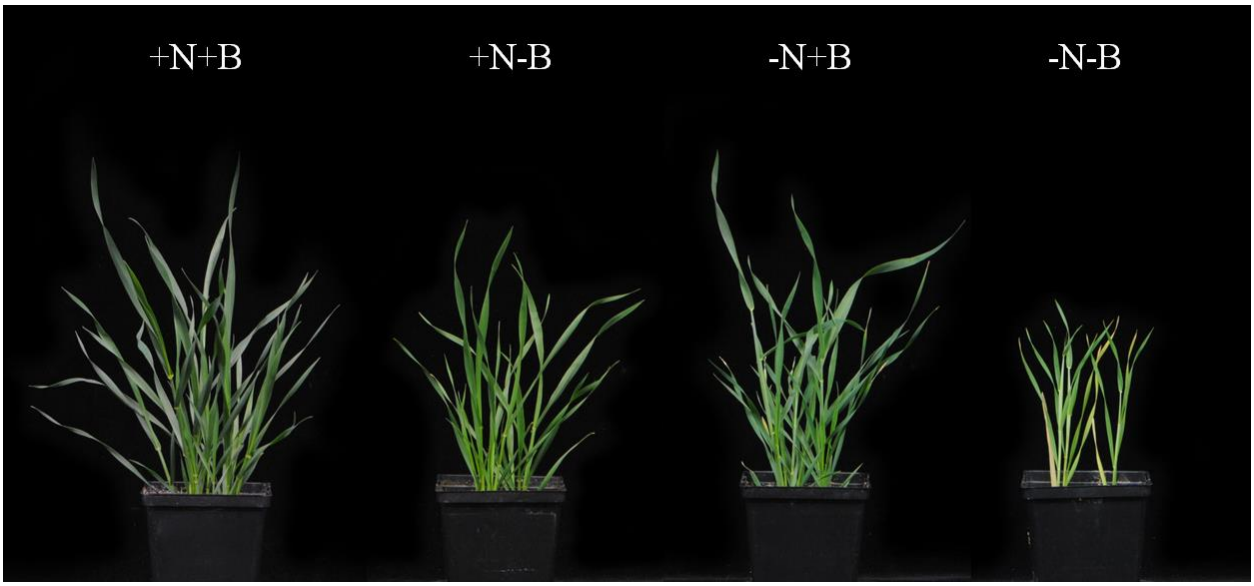

Figure 1. *Plant trial demonstrating the use of bacteria as fertilizer. N: traditional slow release nitrogen fertilizer. B: Azotobacter added as nitrogen source. Image credit: Paul Kusuma, Dr. Bruce Bugbee's Plant Physiology Lab, Utah State University.*

Approach:

Objective 1: For growth comparison of each strain, *R. palustris* was inoculated into liquid prepared anaerobic Rhodospirillaceae medium (RM media) and the optical density curve was developed. The most viable strain based on the growth curve was the bacterium that reached the late log phase most rapidly.

Objective 2: Using NASA's information on the Martian atmospheric conditions a workable bioreactor environment was calculated that could be produced on Mars. Using those

parameters, *R. palustris* was grown in scaled-up nitrogen-fixing conditions to show it is capable of being cultured on Mars.

Objective 3: While *R. palustris* is capable of nitrogen fixation, all fixed nitrogen is normally assimilated directly into proteins or other forms of biomass. To determine a baseline concentration of NH_4^+ production and excretion for each strain, a quantity of liquid culture was inoculated and allowed to grow to stationary phase. NH_4^+ production and secretion were then quantified by using an NH_4^+ fluorescent assay.

Objective 4: Using the results from Objectives 1, 2, and 3 the fastest-growing, most robust colony producing was selected for further development.

Objective 5: The strains of interest were grown in 1.5L benchtop scale photobioreactors. The cells were then harvested, and the average wet biomass yields per liter were quantified.

Objective 6: To determine the rate of degradation and release of nitrogen as NH_4^+ and any other form, degradation trials were conducted. Two different apparatuses were used, one to monitor release of nitrogen as NH_4^+, and one to monitor soluble nitrogen release as nitrate and nitrite.

Materials and Methods:

Growth Trials:

The first growth trial was conducted for five strains of *R. palustris*: NifA*, CGA009, CGA010, TIE1, and PB23. 200 mL sealed flasks were used with 75 mL of media under nitrogen-

fixing conditions. The media consisted of 30 mM sodium acetate, 12.5 mM disodium hydrogen phosphate, 12.5 mM potassium dihydrogen phosphate, concentrated base (1mL/L) , and Wolf's vitamins (5mL/L). The flasks were autoclaved and inoculated with *R. palustris* to an initial optical density of 0.05. Nitrogen fixing conditions do not have any available nitrogen in the liquid media. The only source of nitrogen is the N_2 gas in the headspace.

The flasks were placed in a temperature-controlled and illuminated shaking incubator. The exact position of each flask was randomized each time samples were taken to account for differences in light exposure based on flask position. Samples were taken three times a day for the first week, and then once a day for an additional week. The optical density of each sample was taken and recorded, and then each sample was centrifuged and was frozen at -80C for later use in the ammonium assay.

Ammonium Assay:

The supernatant of the frozen sample was used in an ammonium assay developed by the Seefeldt lab. The protocol is as follows: add 25 uL of the sample to 1 mL of reaction buffer in a cuvette, let it sit in the dark for 30 minutes, then use a spectrofluorometer to measure the fluorescence of the sample.

The reaction buffer is made from 270 mg of ortho-Phthalaldehyde dissolved in 5 mL of 190 proof ethanol. That mixture is then added to 25 uL of mercaptoethanol in 100 mL of 0.2 M Phosphate buffer at 7.3 pH. The excitation wavelength of the assay is at 410 nm and the emission is at 472 nm. Standards of known ammonium quantities (0, 5, 10, 15, 20, 30, 40 ug/mL) were also created and measured in duplicate so that a standard curve could be created.

The 25 uL of the sample came from the supernatant that was frozen in the 1.7 mL microcentrifuge tubes from the growth trial. Each sample tested with this ammonium assay was done in duplicate to improve accuracy.

Gas Chromatography:

The analysis of headspace gas compositions was made using a Shimadzu GC-8A gas chromatograph (Shimadzu Scientific Instruments, Inc.) with a thermal conductivity detector. The carrier gas used was argon (135 kPa), the injector/detector temperature was 100 ◦C and the column temperature was 60 ◦C. 200 μL of the headspace gas was sampled using a gas tight syringe and injected into the gas chromatograph. To quantify the amount of N_2, a set of standards were prepared with the required amount of water and known N_2 concentrations in the headspace. The corresponding peak was integrated for their area using the LabSolutions software (Shimadzu Scientific Instruments, Inc.), and a standard curve was derived. This was used to quantify the amount of N_2 in the culture headspace at different time points. The same traces were also used to analyze and quantify hydrogen production with an appropriate standard.

Scale Up:

Both PB23 and NifA* were used in scale-up. Fourteen 1.5 L test tubes were used for each strain. Each tube was filled with 1 L of the same media used in the growth trials. The tubes were fitted with rubber stoppers with two holes in them. In one hole was placed a small (~1.5”) hollow glass rod. In the other hole, a hollow glass rod was placed that reached all the way to the bottom of the tube. The silicone tubing was attached to the glass rods and covered in aluminum foil. All components were autoclaved.

After sterilization, Wolfs Vitamins were added using filter sterilization, along with 30 mL of culture at OD 1. The rubber stoppers were then wired to the tubes to prevent them from popping off under high pressure. Sets of one to three tubes were connected with the silicone tubing, short glass rod to tall glass rod. Nitrogen gas was connected to the first long glass rod in each set and bubbled through at 10 L/min at 10 psi. After 30 minutes the vent was clamped, and the system was pressurized until bubbles no longer exited the long glass rod. See Figure 1. The inlet was then clamped off to prevent oxygen from entering the system. The tubes were then placed in front of banks of 14 fluorescent bulbs and maintained at approximately 28 C. Temperature is approximate because it was dependent on the ambient temperature of the room, which fluctuated slightly based on the time of day, and the number of times the door to the room was opened. The cultures were allowed to grow for 12 days. This was the largest amount of time permitted for the cultures to grow, as the project deadline was approaching. Further research should be done to determine the most productive time to harvest cell mass. See Figures 2-4. Cells were harvested by centrifugation in 1 L bottles at 8000 rpm for 20 minutes. See Figures 5-7.

Figure 2. *Bubbling nitrogen through the tubes to remove oxygen, and to mix the vitamins and inoculum thoroughly.*

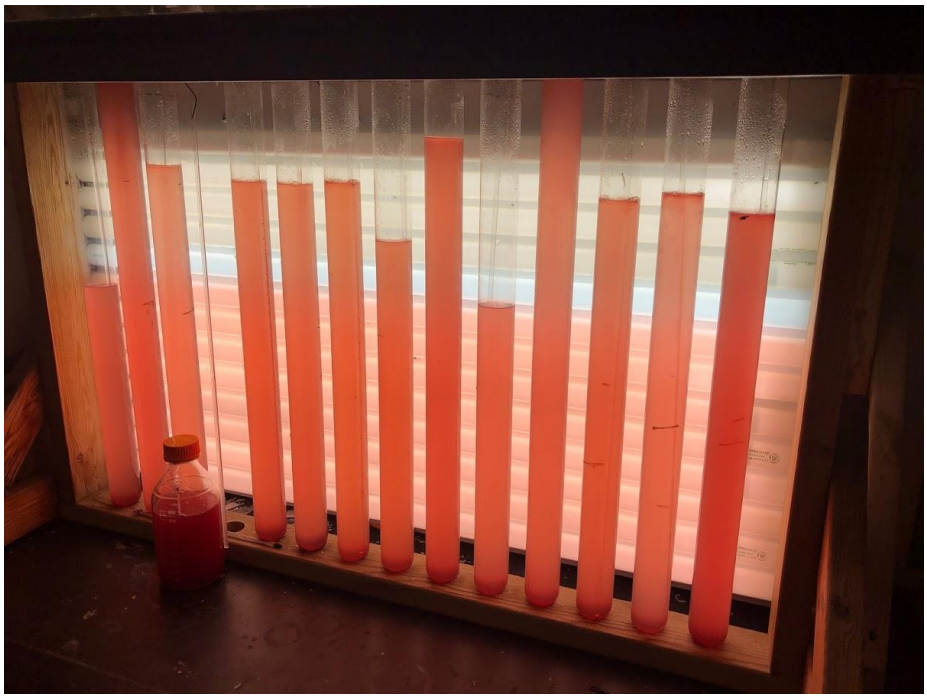

Figure 3. *Growth of the PB23 strain just before the cell mass was harvested. Note that the differences in liquid height come from slight pressure differences between the connected tubes. This allowed more pressurized tubes to push liquid into less pressurized ones. One tube shattered under high pressure, and the culture was salvaged by growing it in a 1 L media bottle.*

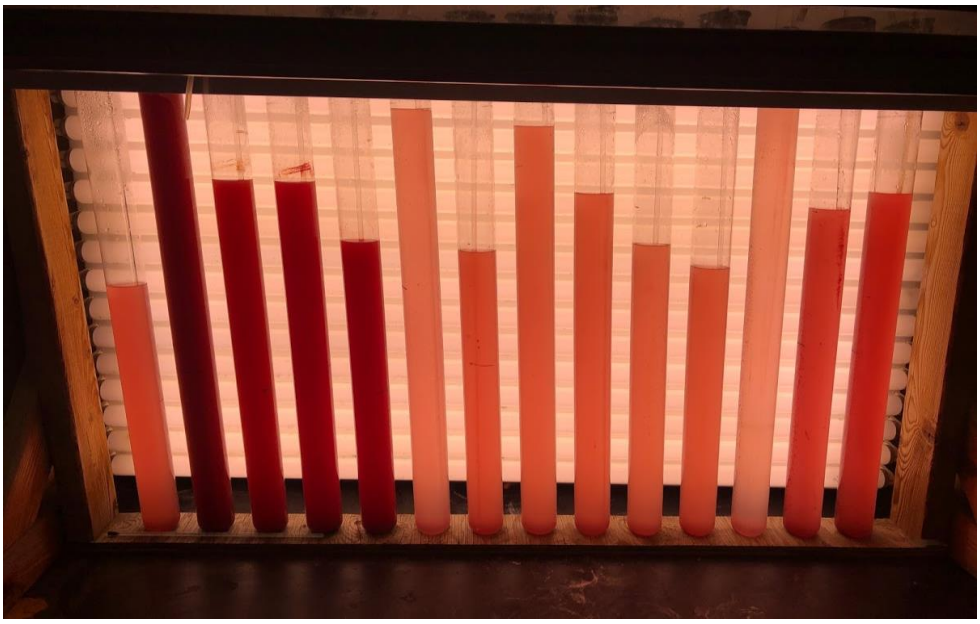

Figure 4. *Growth of the NifA* strain just before the cell mass was harvested.*

Figure 5. *Harvested R. palustris.*

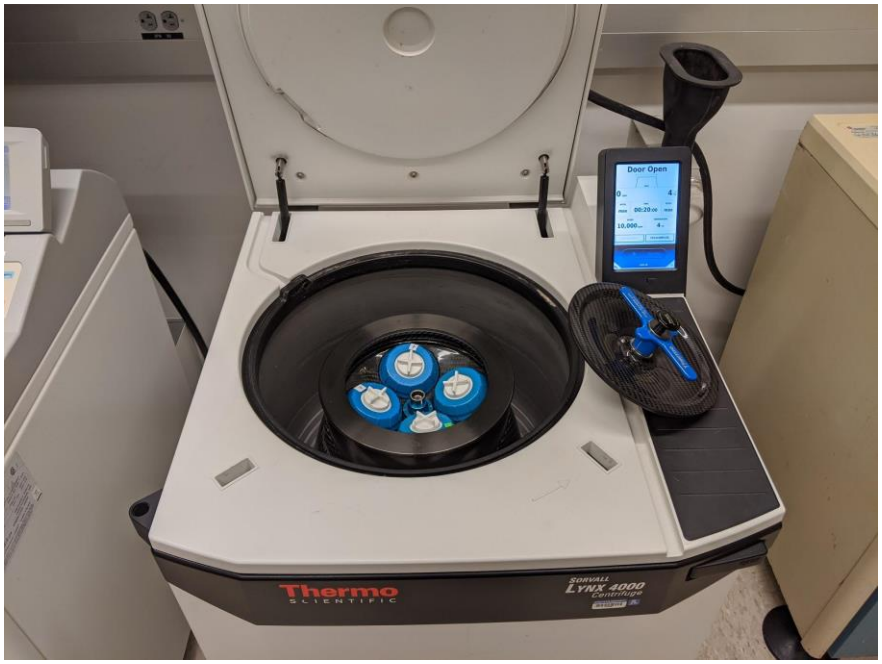

Figure 6. *Harvesting of R. palustris via centrifugation.*

Figure 7. *Cell pellet of harvested R. palustris*

Volatilized Nitrogen Degradation Study:

To determine the amount of nitrogen from biomass that is lost as volatile ammonia, a degradation apparatus was designed and constructed. As shown in **Figure 8** air was blown through several lengths of PVC pipe and beakers.

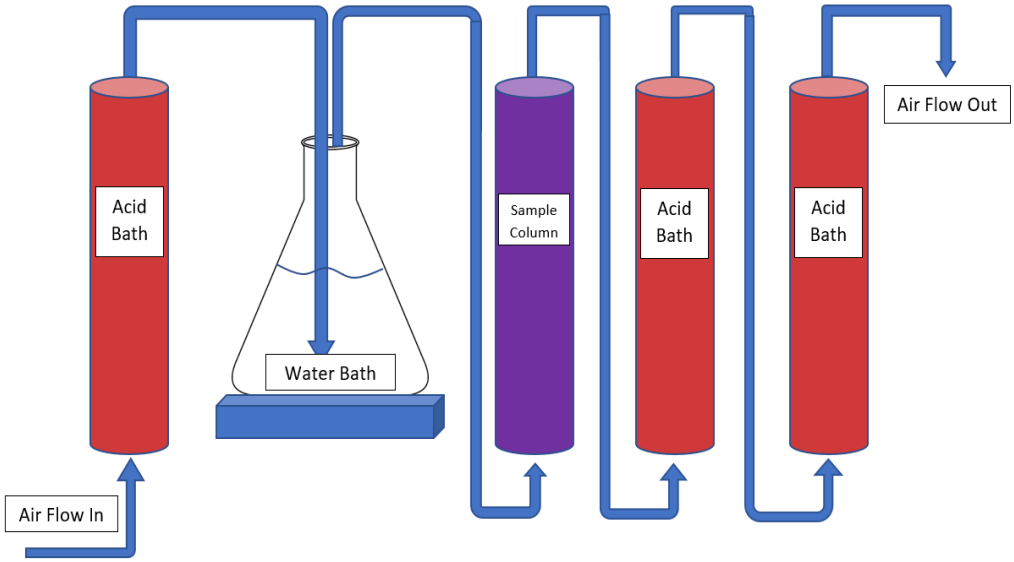

Figure 8. *A schematic illustrating the design of the volatile ammonia degradation system.*

The first pipe contained 50 mL of 2 M HCL and was used in the system is to remove any contaminating nitrogen from the atmospheric gas that is put into the system. The water bath then ensured the air was saturated with water, to prevent dehydration of the sample. The sample column contained 50 mL of coarse sand along with 50 mL of maximum OD cells mixed with media. As the biomass degraded, any volatile nitrogen was carried into the following acid bath containing 50 mL 2 M HCl.

Each of the acid baths was sampled at regular interviews using a colorimetric high range ammonium detection kit (and a spectrophotometer (LaMotte SMART3). 5 mL of acid was

removed for each sample, and a 5 mL volume of prepared 2 M HCl was added to replenish each column after sampling.

Soluble Nitrogen Degradation Study:

To determine the amount of nitrogen that is soluble in water and available for plants a trial was set up similar to a method developed by Dr. Bugbee and his lab.[15] Three groups of triplicates were set up. The groups were PB23, NifA*, and control. 11 grams of PB23 were mixed with 1200g of inert quartz sand and 11 g of NifA* were mixed with another 1200g sand. (See Figures 9-12) The sand/cell mixture was divided evenly between three PVC columns and, as described by Adams et. al., 400g of sand was placed in each of the triplicates for the control group. Deionized water was poured through the top of the column and the leachate was collected. The pH and electrical conductivity of the leachate was measured using the Hanna HI2209 pH meter and the Hanna DiST 4 respectively.

Figure 9. *Sand before adding bacteria for the soluble nitrogen degradation study.*

Figure 10. *Sand after adding R. palustris.*

Figure 11. *Sand after mixing in the R. palustris biomass.*

Figure 12. *The columns used in the soluble nitrogen degradation study.*

Timetable:

The first objective was the learning of the growth conditions and skills with *R. palustris* from Mathangi, which she has learned from experts in the Fixen Lab, and to start the growth of the strains for baseline NH_4^+ quantifications. All strains were grown in nitrogen fixing conditions and the ideal strain were selected before moving forward with the NifA* strain and PB23 strain. Follow up growth trials and ammonium assay analysis were done throughout the summer months for quantification of baseline growth and ammonium production of the two optimal strains. Due to the change in project objectives in September, there was a month break for planning while the procedures were developed for the scale up and degradation studies. Those portions of the experiment were completed from October through November, and the remaining time was

focused on the data analysis, formal paper, and review which were due in December. See Table 2 and Figure 13 for a more detailed timeline.

Table 2. *The timeline for the project, including objectives for the project and the time period that each task was worked on.*

TASKS	START	END	DAYS	STATUS
Literature Review	10/1	2/1	123	Complete
Agree on Objectives	1/1	2/1	31	Complete
Learn Culture Techniques	2/1	4/1	59	Complete
Growth Trials	4/1	8/1	122	Complete
Quantification of Baselines	6/1	8/1	61	Complete
Volitilized Nitrogen Study	9/1	12/1	91	Complete
Scale Up	10/1	11/1	31	Complete
Soluble Nitrogen Study	11/1	12/1	30	Complete
Data Acquisition and Analysis	11/1	12/5	34	Complete
Written Report	12/1	12/11	10	Complete
Presentation	11/15	12/2	17	Complete

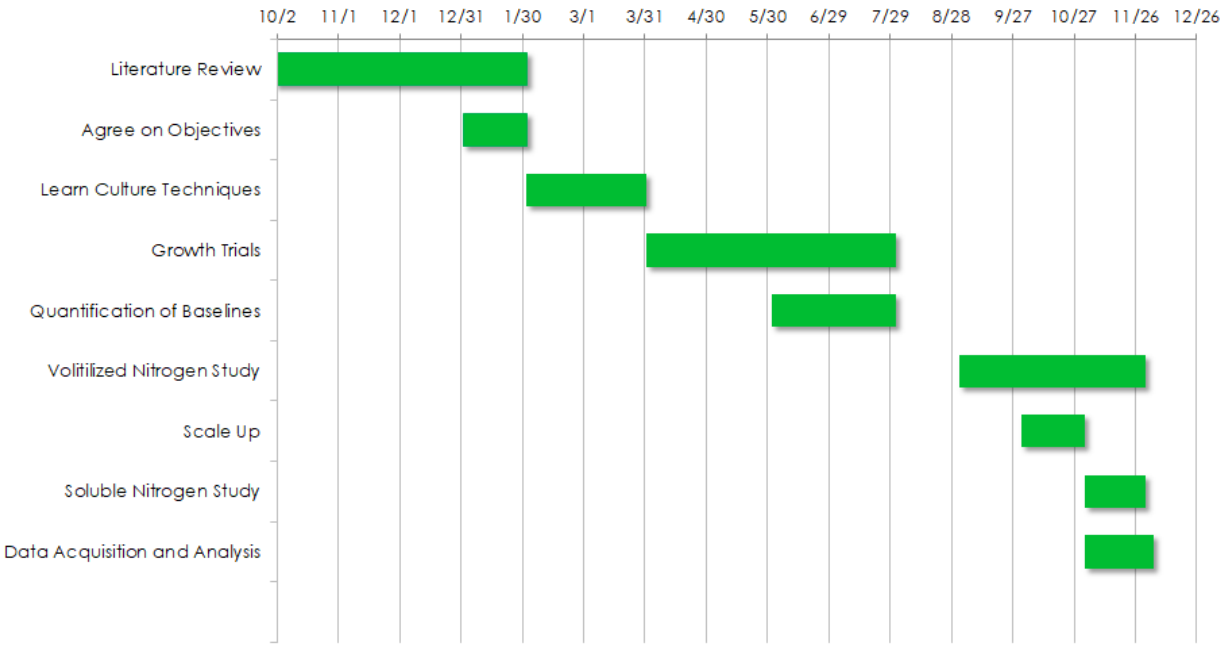

Figure 13. *A Gantt chart showing the timeline for the project and the individual tasks to be completed.*

Results and Discussion:

Growth Trials:

The optical density data collected in the growth trial experiments were analyzed using Google Sheets. The optical density data and the hour that samples were taken after time 0 were recorded. Due to having six conditions and five strains (negative control, NifA*, CGA009, CGA010, TIE1, and PB23), samples were not plotted individually. Instead, averages were calculated and plotted. Unfortunately, this first growth trial became contaminated due to poor aseptic technique during sampling. Using subjective observation, the uninoculated controls began to turn purple, indicating that at least one of the strains of *R. palustris* had contaminated the negative controls. This observation was confirmed by analyzing the optical density curve and found that the negative control indeed increased in optical density over time, instead of maintaining a zero baseline as desired as seen in Figure 14.

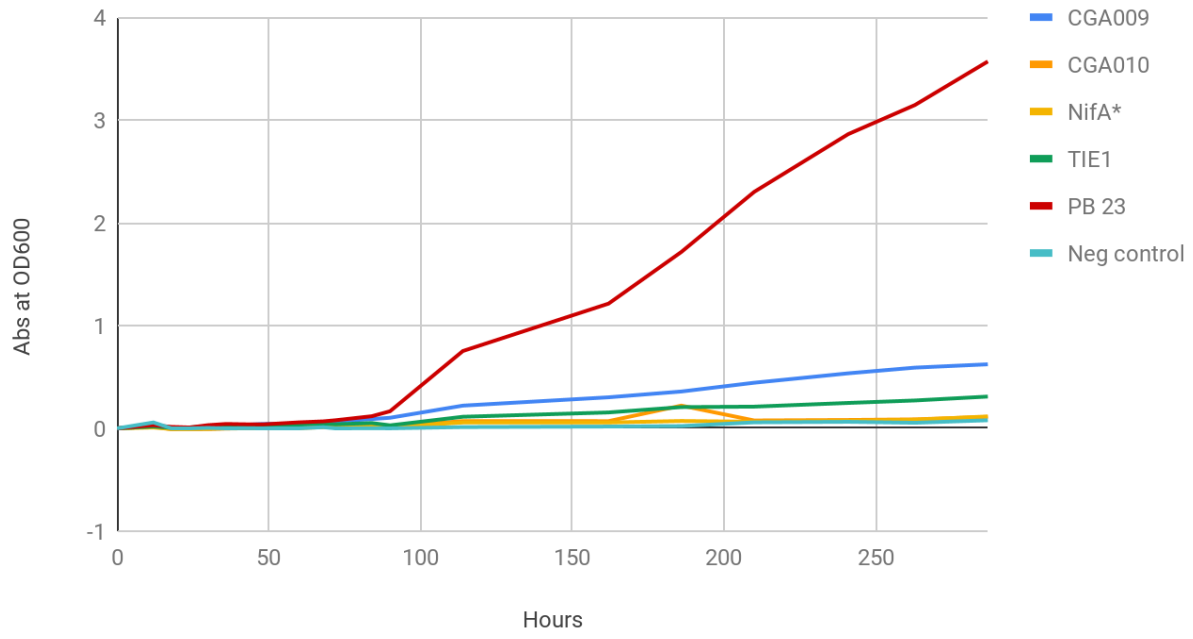

Figure 14. *Growth trial 1 of several R. palustris strains under nitrogen fixing conditions. PB23 was initially observed to be the fastest growing. Unfortunately, this trial became contaminated and data was unreliable.*

Additionally, it was observed that NifA* did not grow as reported, with very weak growth. PB23 (samples 13-15) was the only strain that grew well under these conditions but still did not grow as reported with no observable stationary phase.

To remedy this, the growth trial was repeated with a more rigorous aseptic technique and fewer samples. The collaborating lab (Dr. Lance Seefeldt and graduate students) reported no detectable ammonium production by CGA009, CGA010, or TIE-1 across several rigorous studies. In light of that, it was decided to focus on NifA* and PB23. NifA* is the only strain with reported detectable amounts of ammonia. PB23 is a novel isolate that has shown more robust growth in a wider range of conditions. In the second trial, only NifA* and PB23 were assayed.

We were advised that our controlled temperature shaking incubator was likely not providing sufficient light, nor was it necessary to shake the flasks. Due to this, an illuminated table available in our lab was used with much greater light, but less control over temperature. The temperature over the sampling period ranged from 37C to 40C, while ideal conditions for R. palustris growth is about 30C. Optical density was again sampled over time and the average was plotted in Google Sheets, as seen in Figure 15.

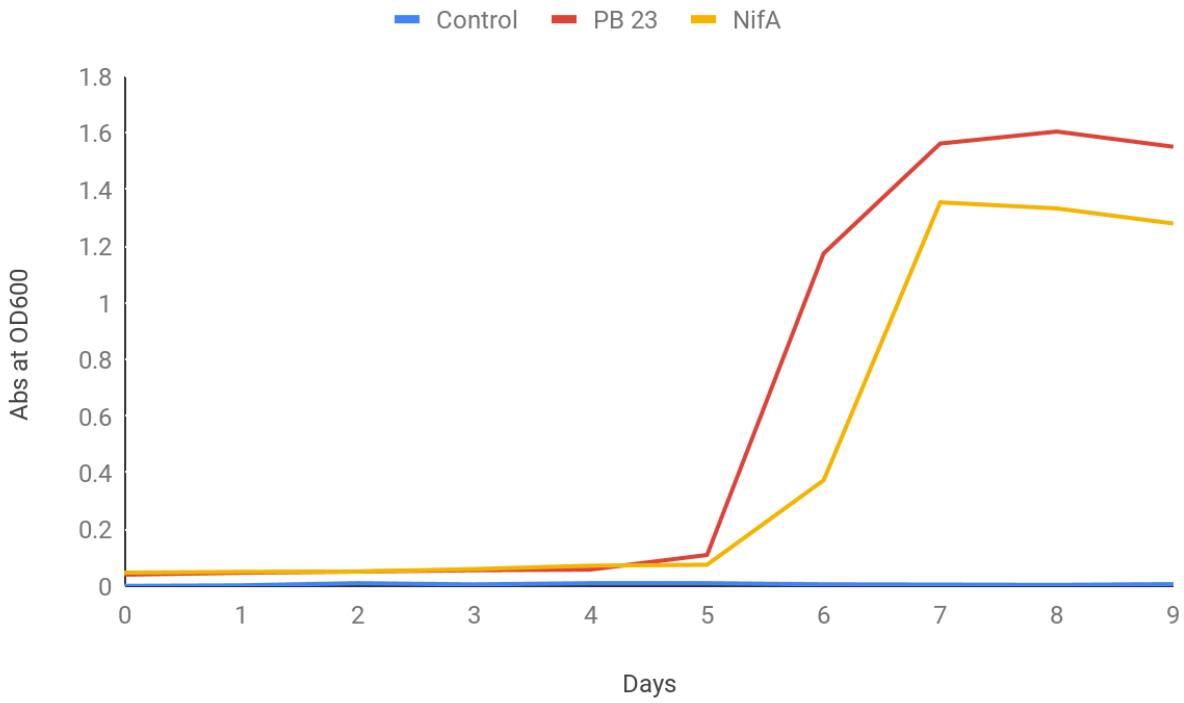

Figure 15. *Averaged OD600 Values for the Second Growth Trial under assumed N2 Fixing Conditions.*

In the second trial, the correct growth curve pattern was observed of both *R. palustris* PB23 and NifA*. However, observed that the maximum optical density was observed to be about half of the reported maximum in the Seefeldt lab. The photon flux of the three incubators were measured, two in our lab and one in the Seefeldt lab. See Table 3.

Table 3. *Growth conditions in each incubator used in this study.*

Growth trial:	Location	Photons: umol m^{-2} s^{-1}	Temperature: ℃
Trial 1	SER 117	131	30
Trial 2	SER 124	408	37-40
Trial 3	Seefeldt Lab	900	22-27

Using this method, it was realized that not only were the temperatures too high, but the cultures were not receiving nearly enough light. After learning this, it was not attempted to determine if the lack of light or shaking caused the poor growth of the microbes in Trial 1. It was observed that the cultures grew well when stationary and continued that method for Trial 3. As will be explained later, it was also realized that ammonium had unintentionally been added to the growth media in Trial 2. After learning this, the growth trials were repeated a final time in the Seefeldt lab under the correct temperature and light growth conditions.

The only observed detrimental effect of doing the growth trial in the Seefeldt lab was that the sampling periods were inconsistent. Access to the lab was limited to the working hours of 9-5. Therefore, the cultures were sampled at 9 am and 4 pm. That leads to a gap time between sampling of 7 hours and 17 hours respectively. That could have contributed to the slow growth curve observed during Trial 3, instead of the textbook growth curve observed in Trial 2. However, in Trial 3, the strains were successfully grown to the previously observed maximum optical density of between 2.5 and 3. Another error made was that optical density had previously been sampled at a wavelength of 600 nm. For Trial 3, samples were measured at 660 nm. See Figure 16.

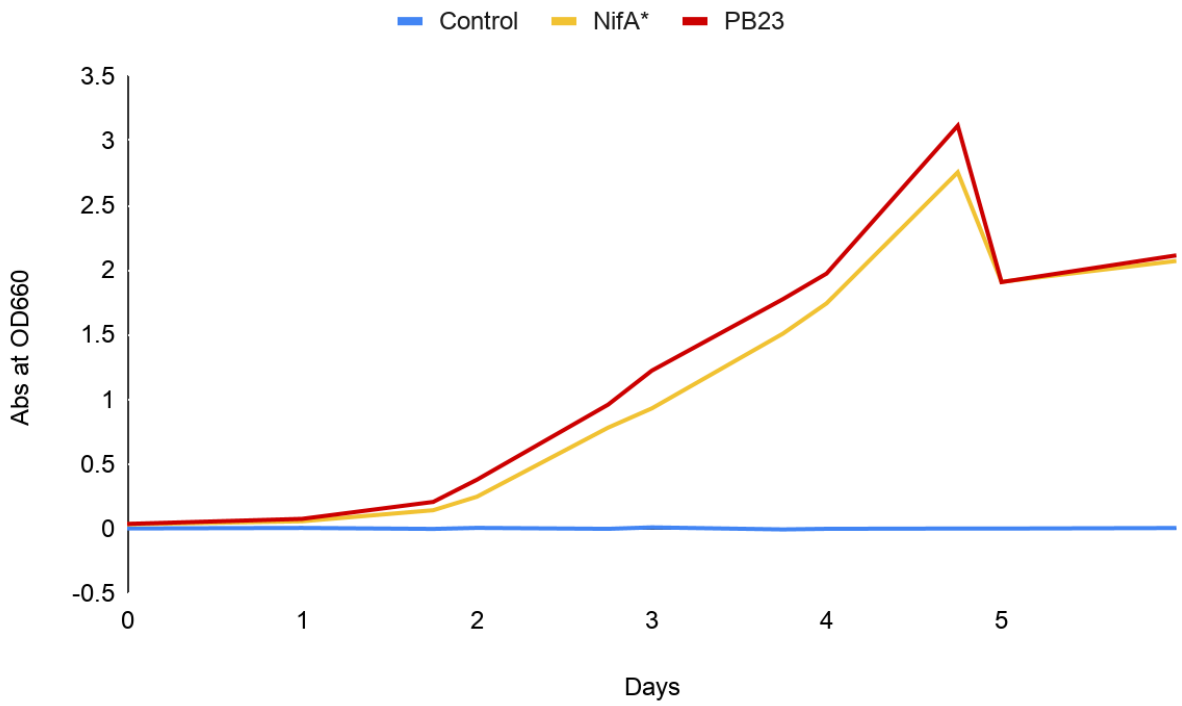

Figure 16. *OD660 of the third growth trial.*

Ammonium Assay:

From the samples collected during the first growth trials, an ammonium assay was completed. This was done using the samples taken in the afternoon of every other day during the growth trial. None of the samples had a reading much higher than 0.03, and from the standards a UV-Vis reading of 0.03 corresponds to a concentration of 5 micrograms per milliliter. This would be a positive sign, however as discussed above, the growth trial seemed to show some contamination to the vials, and even the negative control had UV-Vis readings upwards of 0.015, so the data from this assay trial was largely dismissed. See Figure 17.

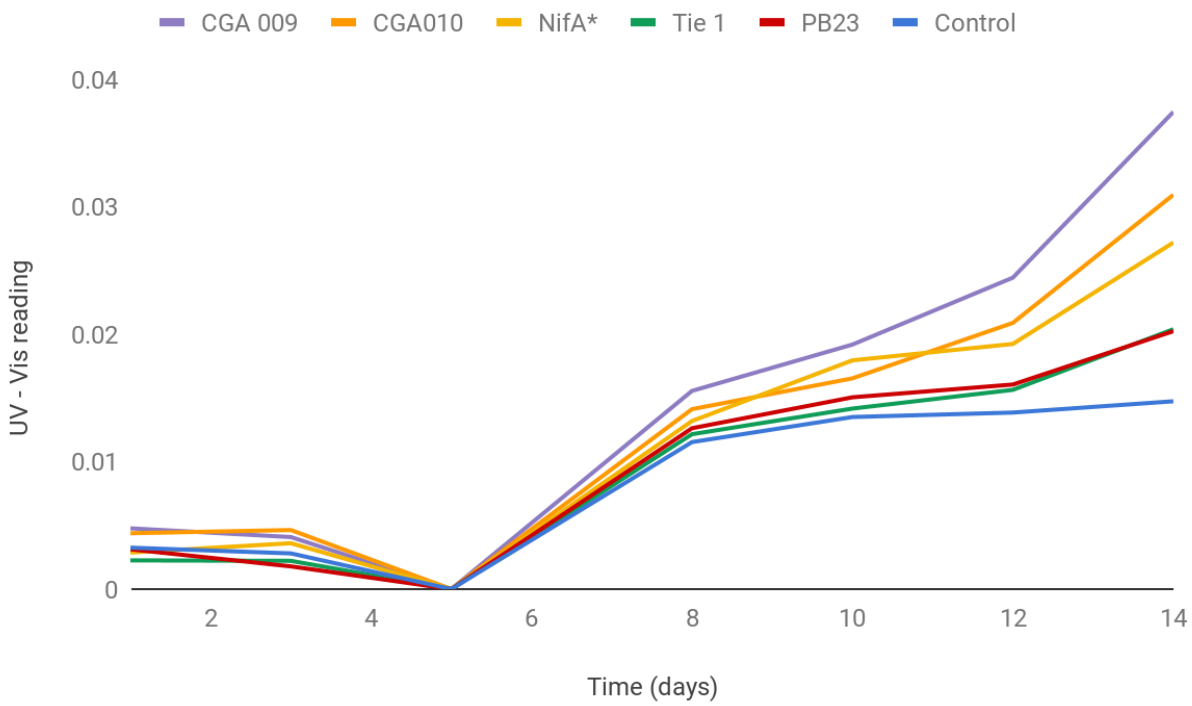

Figure 17. *Averaged ammonium assay data for growth trial #1.*

After completing Growth Trial #2, the ammonium assay was prepared. As the supernatant was mixed with the buffer, the vials changed to a dark yellow color and it was realized that even the uninoculated controls had ammonium levels well above the measurable range of the assay. It was then determined that the growth media had been inadvertently prepared with ammonium present, and therefore was not true nitrogen fixing conditions. As such, the ammonium assay was abandoned for the second growth trial. It was then determined to perform a third growth trial.

Growth Trial #3 was the only trial that was completed successfully. Only one error was made when transferring starter growths from ammonium rich conditions to ammonium deficient conditions, as the cells were not washed before being transferred. This led to the peak at time

zero observed in the following plot. Interestingly, NifA* did not excrete measurable amounts of ammonia into the media. It appears that both PB23 and NifA* consumed the residual ammonium from the cellular transfer, then fixed their own nitrogen without producing any extra that could be measured by our assay. See Figure 18.

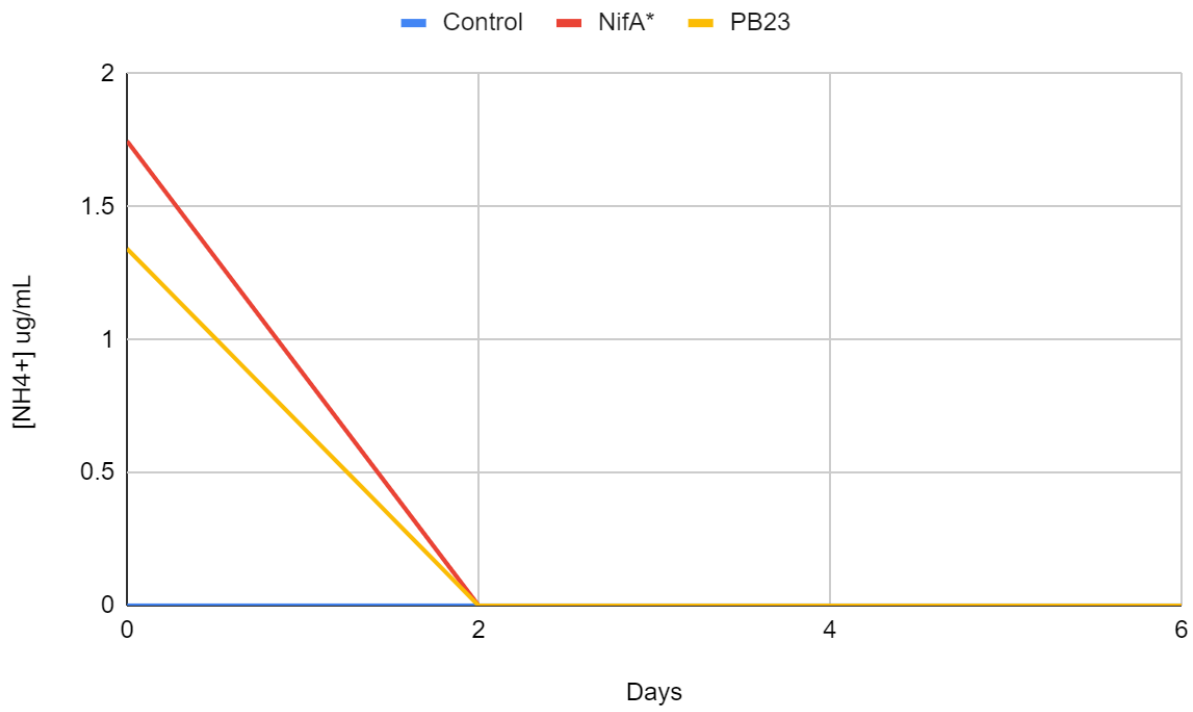

Figure 18: *Results of the ammonium assay from growth trial #3. Initial presence of ammonium is due to carryover from the seed cultures.*

Gas Chromatography:

Gas chromatography was used to ascertain nitrogenase activity. In this study, relative activity was not measured. Rather, presence or absence of hydrogen gas in the headspace was indicative of activity or inactivity respectively. Table 4 presents the results of the study.

Table 4: *Results of gas chromatography of the headspace*

Strain	H_2	N_2
PB23	+	+
NifA*	+	+
Control	-	+

These results indicate that the nitrogenase of PB23 and NifA* were both active during the third growth trial.

Decision Matrix:

From literature review it was expected that at the very least the NifA* strain would have produced a quantifiable amount of ammonium that would be excreted into the supernatant of the culture. Because this could not be reproduced in the growth trials done during the experiment, it was decided to move forward treating the cell culture as a whole and focusing on the nitrogen that would become available from the cell mass as it degraded. This wasn't the ideal method outlined in the original plan for the experiment, however, with the failure to produce any ammonium in the supernatant by any of the strains in the growth trial, this was the method that was chosen to continue with in order to still meet with the objective of the project and produce some usable nitrogen for plant growth.

Using the data from our growth trials and ammonium assay, a decision matrix was created to establish the two best strains of R. pal to use for scale-up and degradation trials. The matrix can be seen in Table 5**.** NifA* and PB23 were selected based off the matrix.

Table 5*. The decision matrix with weighting factors for determination of which strain of R. palustris to move forward with degradation testing. From this table, NifA* and PB23 were used for further testing.*

Category	Speed of Growth	Baseline Ammonium Production	Colony Formation	Light Intensity Requirement	Total
Weight	7	9	2	4	
NifA*	7	4	0	1	89
TIE-1	6	0	0	3	54
CGA009	6	0	0	3	54
PB23	9	0	5	6	97
CGA010	6	0	0	3	54

Scale Up:

From correspondence with Dr. Anna Doloman in Dr. Seefeld's lab, it was expected that the NifA* strain would have wet weight yields around 2 g/L. This data comes from her fed batch photobioreactor growing the NifA* strain. The yields from the scale up for this project were as follows: *R. palustris* PB23 had an average yield of 0.86 g/L and *R. palustris* NifA* average yield was 1.53 g/L. According to the data that was collected in the growth trials, PB23 was expected to have had a higher cell density than NifA*. However, as shown in Figure 4**,** there was not a uniform color between all the tubes for the NifA* strain. PB23 had a much more uniform color distribution as seen in Figure 3. These color differences are a possible reason that NifA* had a higher yield.

Another possible reason for the difference in yield is that one of the tubes for PB23 broke, and the media and cell mass were collected in a 1 L screw top flask. Nitrogen was bubbled through the flask to remove oxygen and the flask was sealed and then placed in front of

the lights. As seen in Figures 3 and 4 the spectrums of the fluorescent tubes were visually different between the two strains, which could have had an impact as well.

One other observation that was made during the scale up was that for at least the NifA* strain turbulent mixing inhibits growth. A few tubes did not seal properly and to prevent aerobicity nitrogen was continuously bubbled through the tubes, which caused turbulent mixing, and inhibited growth. It was assumed that the tubes would not grow, and so the nitrogen was not replaced after it ran out. Once the bubbling stopped, the tubes quickly flushed pink.

Volatilized Ammonia:

While much of the aforementioned project was considered my "Senior Design", the volatilized ammonia assay portion of the project was used as a special leadership opportunity to fulfill the requirements of my Capstone project. During this portion of the project I took the lead in designing, organizing, maintaining, executing and communicating on all aspects. With the help of my team, I developed a system for the quantification of volatized ammonia during a degradation trial using our bacteria.

After the development of the system for quantification of volatilized ammonium produced during the degradation, many different trials were needed in order to perfect the system. The system needed to be airtight in order to capture and direct any ammonia with the airflow, which was the only initial constraint that was applied on the design process. After the successful design of an airtight system that allowed airflow through the entire system, several trials with a positive control and a negative control were completed. For the negative control the sample column was loaded with uncultured growth media and allowed to run air through for

several days. After this trial the columns were each tested and returned results of a zero concentration of ammonium in all of the samples. A solution with a concentration of 3.28 ppm ammonium was made for the positive control and used to test the system. The initial trial worked as a positive control, showing that ammonia flowed into the first acid bath and was captured, and that the total ammonium in the system added to 3.26 ppm, which was within 0.002 ppm of the original addition. This difference was attributed to small errors in the calculation of the ammonium concentration with the colorimetric assay, rather than ammonium escaping as the seals were confirmed to be airtight on each component of the system. Although the positive control succeeded in verifying function within the system, it was seen that when sampling from the acid baths, the airtight sealing on the system caused that fluid would be pulled through the systems tubing. Due to this, flasks were added in between each fluid column to catch any flow through.

After the positive control the following trials were done using growth media with NifA* grown to an optical density of about 2. The next trial an acid flask was added before the system in order to eliminate any contamination from any available nitrogen in the air, as noted due to the greenhouse setting for the experiment, the air may have some nitrogen contamination. The third test trial using NifA* bacterial growth media produced better results, with an increase in ammonium captured in the acid baths the longer the trial endured, as can be seen in Figure 19.

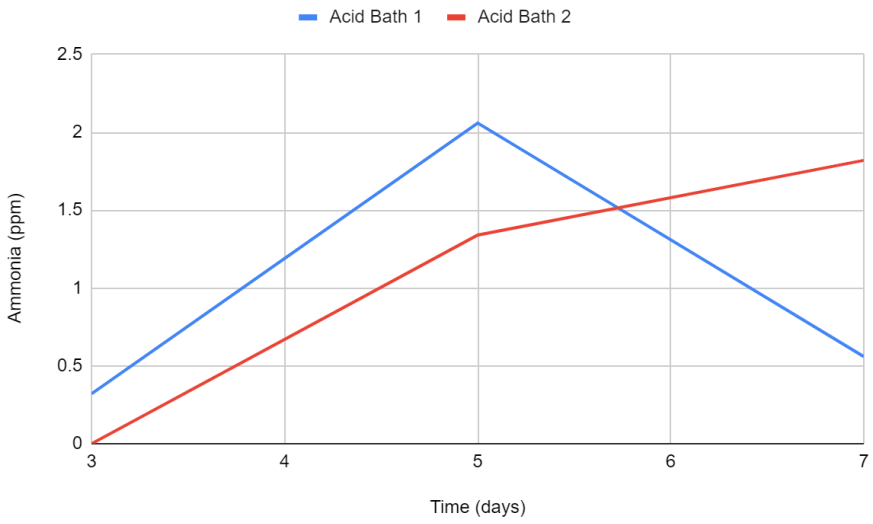

Figure 19. *Concentration of volatilized ammonia during the third test trial of the volatilized ammonia system with NifA* at OD 2.*

The success in the third trial led to the decision to move forward with the final degradation trials of each bacterial strain, however the time remaining for the project only allowed for a final degradation trial with the NifA* strain. As can be seen in Figure 20 the bacteria in the sample column took much longer than in the previous trials to start degrading and become volatilized. It is thought that this is due to the freshness of the media, and that, unlike in previous studies due to the time the culture spent outside of the incubator, there was a significant lag phase before degradation of the bacterial mass began. This noticeable lag phase lasted for the first six days before there is any real uptick in ammonia that is captured in the acid baths after release from the degrading sample. Due to time constraints samples could only be taken for ten days before data analysis for presentation of the results, however it is expected that the captured

ammonium would continue to increase if more samples were collected as time of the trial extended.

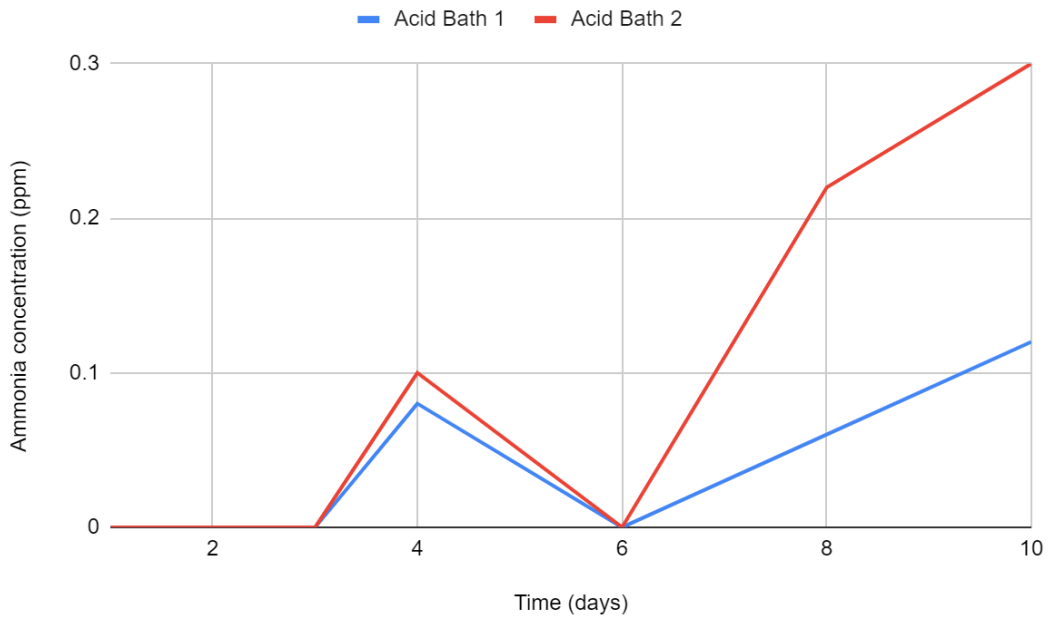

Figure 20. *The curve of the final degradation trial for NifA* bacteria over the course of ten days.*

While the final degradation trial was not as conclusive as desired, the many trials and testing that was done during the development and perfection of the system for measuring volatilized ammonia were very useful for a proof of concept and initial data to support the usefulness of the system. The resulting system will continue to be used by the Seefeldt and Bugbee labs in further analysis of *R. palustris* and any further exploration into the rate of nitrogen availability, and also provides a solid base for any desired modifications to the system for plant growth. The opportunity to lead this portion of the project also allowed for personal

growth, as honed my communication skills and worked to keep the group on task and on schedule.

Soluble Nitrogen:

Determining the amount of soluble nitrogen was not possible. Several challenges presented themselves that inhibited accurate results. The first challenge was that the cell biomass did not adhere to the sand as hoped and instead washed through the columns with the initial wash to remove any residual salts. To ensure that biomass was not lost, the leachate was collected and poured through the column every day instead of using fresh DI water. This reuse led to a solution of water and cell mass being left stagnant in between measurements. Evaporation of the water caused component concentrations to increase in the leachate. See Figure 21 and 22.

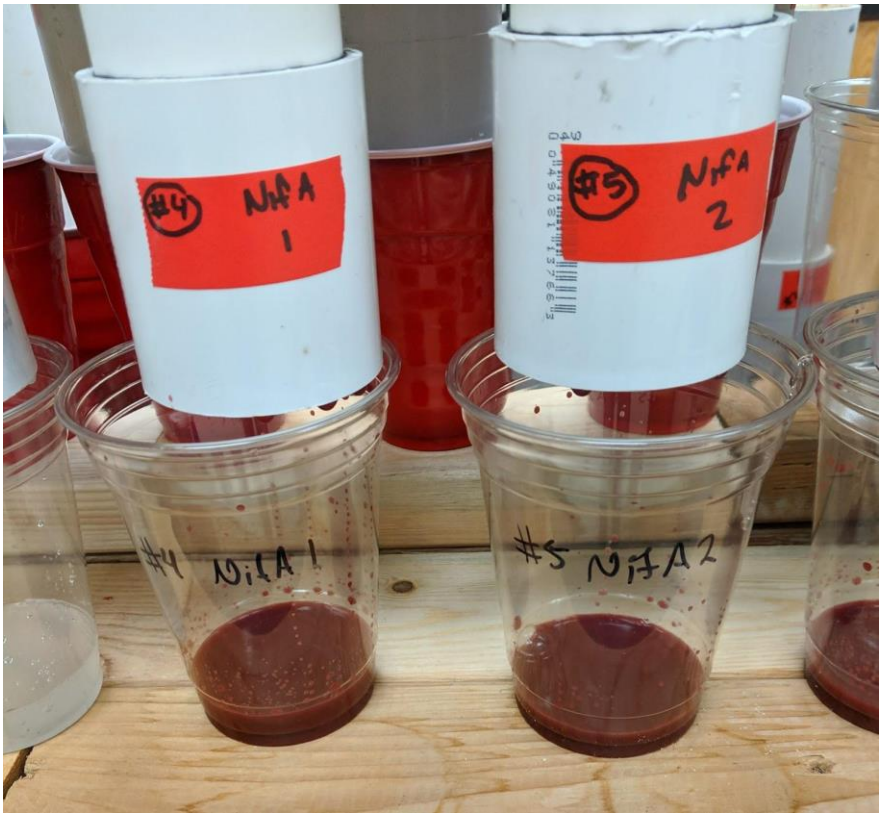

Figure 21. *Cell biomass captured in leachate after flowing through the column. In previous experiments testing the system, the leachate was clear*

Figure 22. *Sand after washing the cell biomass through the column. Compare to Figure 11 for an observation of the color difference before (Figure 11) and after (Figure 22).*

According to Dr. Bugbee and his students the pH of the leachate should have increased, and the electrical conductivity should have remained constant. However as can be seen in Figures 23 & 24, the pH stayed constant while electrical conductivity rose. It is believed that this is due to evaporation of the liquid increasing the concentration of ions. There was not sufficient time to redo the scale up growth and run another degradation trial. Further trials should be conducted to determine the amount of soluble nitrogen that is released.

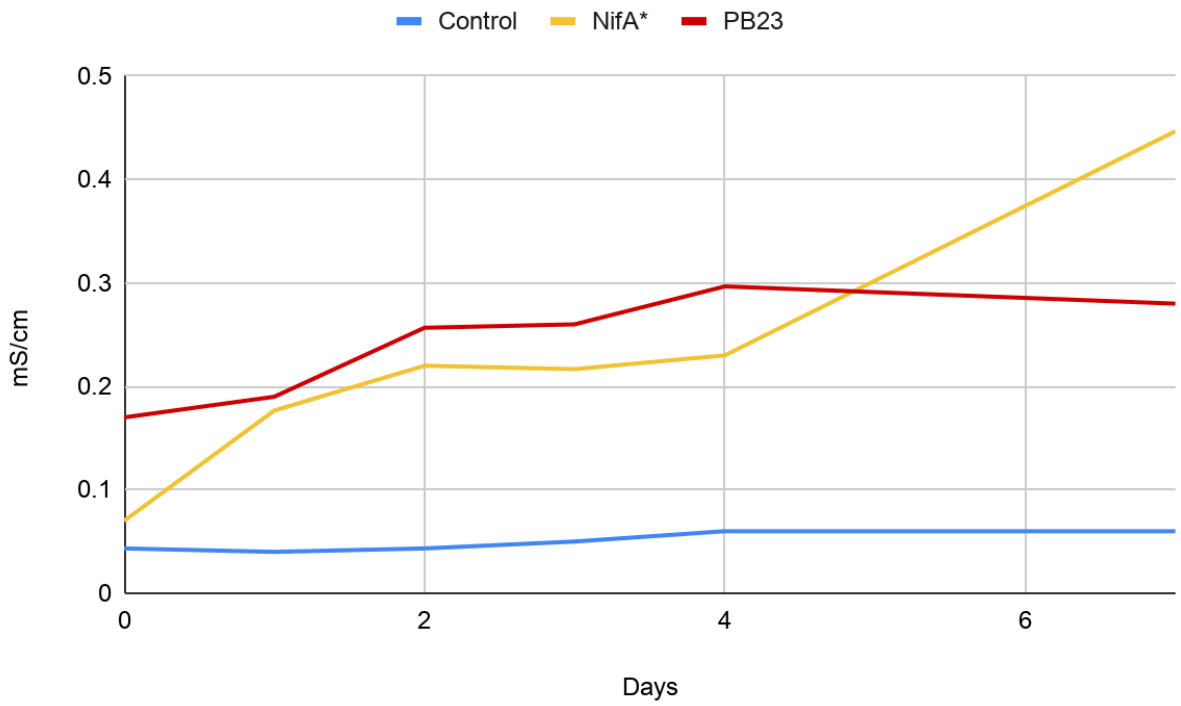

Figure 23. *Electrical conductivity of the NifA* and PB23 strains over a 7-day period for the soluble degradation study.*

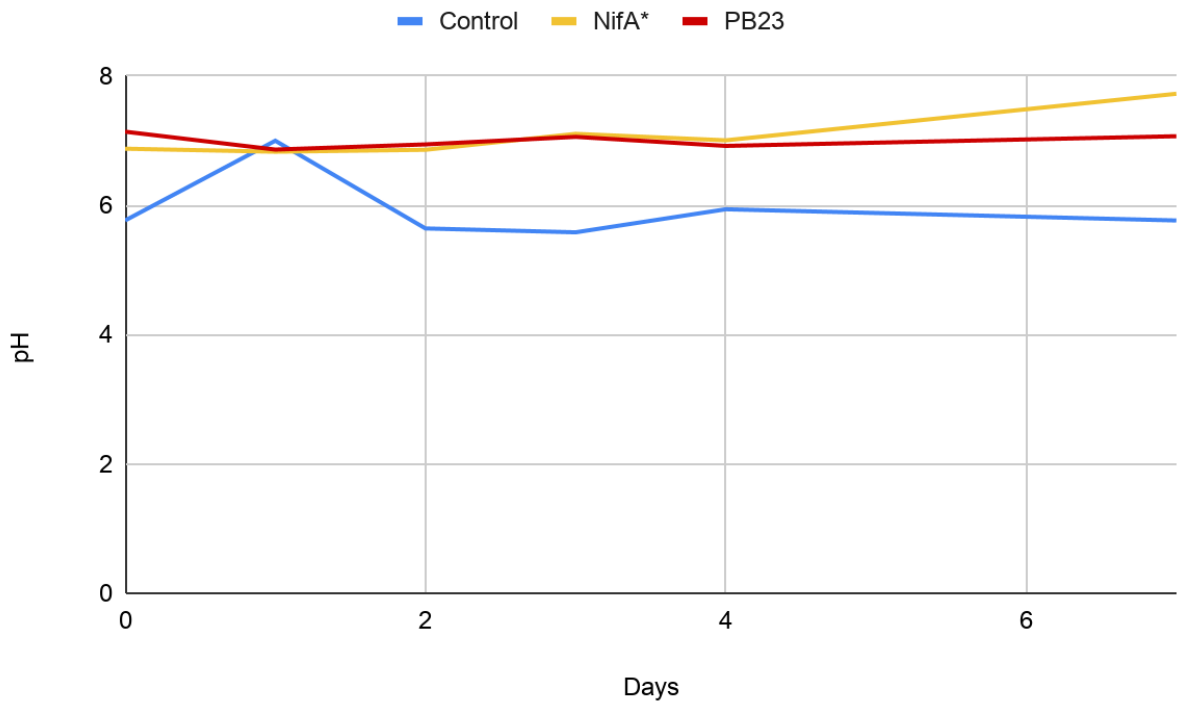

Figure 24. *The pH of the NifA* and PB23 strains over a 7-day period for the soluble degradation study.*

Communication of Results:

A poster was presented at the Intermountain Biological Engineering Conference (IBEC) at Utah State University on November 9, 2019. A formal presentation was also given to the senior design class on December 2, 2019 at the conclusion of the senior design course, and the presentation and report were given to Dr. Seefeldt and Dr. Sims on December 11, 2019.

Conclusions and Recommendations to Industry for Future Work:

At the conclusion of the project, the following items were accomplished/determined:

- Optimal growth conditions were identified for *R. palustris* strains.
    - 900 umol m^-2 s^-1 photons
    - 22-27℃
- Nitrogenase activity was verified by gas chromatography.
- Growth curves of *R. palustris* strains were obtained under various conditions.
- Ammonium was not excreted in detectable amounts in small batch cultures.
- Photobioreactors were successfully assembled and deployed.
- PB23 and NifA* were scaled up.
- Average cell mass yields were calculated.
    - PB23: 0.86 g/L
    - NifA*: 1.53 g/L
- Volatile nitrogen release = 0.1 mg L^{-1} day^{-1}.

It is recommended that studies be carried out to further determine optimal mixing for growth, most productive cell harvest time, the amount of soluble nitrogen from cell degradation, and the amount of volatilized ammonia. Plans are already being put into place to help members of the Seefeldt lab learn and use the methods determined in this project to carry out future studies.

Description of Personnel:

Dr. Lance Seefeldt is department head and professor at Utah State University in the Chemistry and Biochemistry Department who received his undergraduate and graduate degrees in Chemistry and Biochemistry. He is an expert on nitrogenase and nitrogen fixing in bacteria and is the faculty mentor for the project, as well as the representative for NASA.

Dr. Bruce Bugbee is a professor of Environmental Plant Physiology at Utah State University in the Plant, Soil, and Climate Department of the College of Agriculture. He received his PhD in Horticulture from Penn State University, and currently is working on the NASA CUBES team developing systems for plant growth on Mars.

Paul Kusuma is a plant physiology PhD student working under Dr. Bugbee and helping with the project through the CUBES organization. He was the liaison between the senior design group and the Bugbee lab, and worked with the group on the development of the degradation studies.

Mathangi "Mattie" Soundararajan is doing her PhD on the genetic engineering and characterization of *R. palustris* and is a graduate student working under Dr. Seefeldt. She has met with national experts on *R. palustris* and will help with the introductory instruction on growth conditions and procedures.

Daniel Kade Derrick currently works with Dr. Anhong Zhou on a cancer biomarker detection project and plans to pursue a career as a physician scientist. Kade has significant experience leading plant growth trials and is a highly talented experimentalist. He was selected

as the Outstanding Junior of the Year for the Biological Engineering Department for 2019, and was selected as the Scholar of the Year for the College of Engineering for 2020.

Caleb Walker currently is working in research with Dr. Randy Lewis and Dr. Justin Jones that focuses on the production of recombinant spider silk and hagfish proteins from genetically engineered bacteria. His experience centers in the production, purification, testing, and analysis of these proteins as filaments.

Drew Porter currently works with Dr. Miller to develop an open source synthetic biology educational kit using *Bacillus subtilis* and a DIY bioreactor. He hopes to work in the bioprocess/fermentation/automation fields after he graduates. Drew is skilled at reproducing protocols derived from literature and bioreactor design.

AJ Walters is currently pursuing many research aims. AJ hopes to pursue a Ph.D. in Biological Engineering focused on developing tools to more easily engineer complex cell types for various applications. He is skilled in DNA manipulation, culturing *R. palustris*, and experimental design.

Budget:

The budget in Table 6 was provided by the funding that is coming to Dr. Seefeldt's lab from the Microbial Media and Feedstock Division of CUBES and NASA. All materials and costs from any consumables or product characterization me out of the same funding.

Table 6. *Budget for project*

Supplies	Cost:
Reagents for growth media	$200
Culture Supplies	$150
Ammonium Assay Supplies and Reagents	$200
Molecular Biology Reagents	$500
Sand	$20
Fittings	$50
Light bulbs	$50
Total:	$1170

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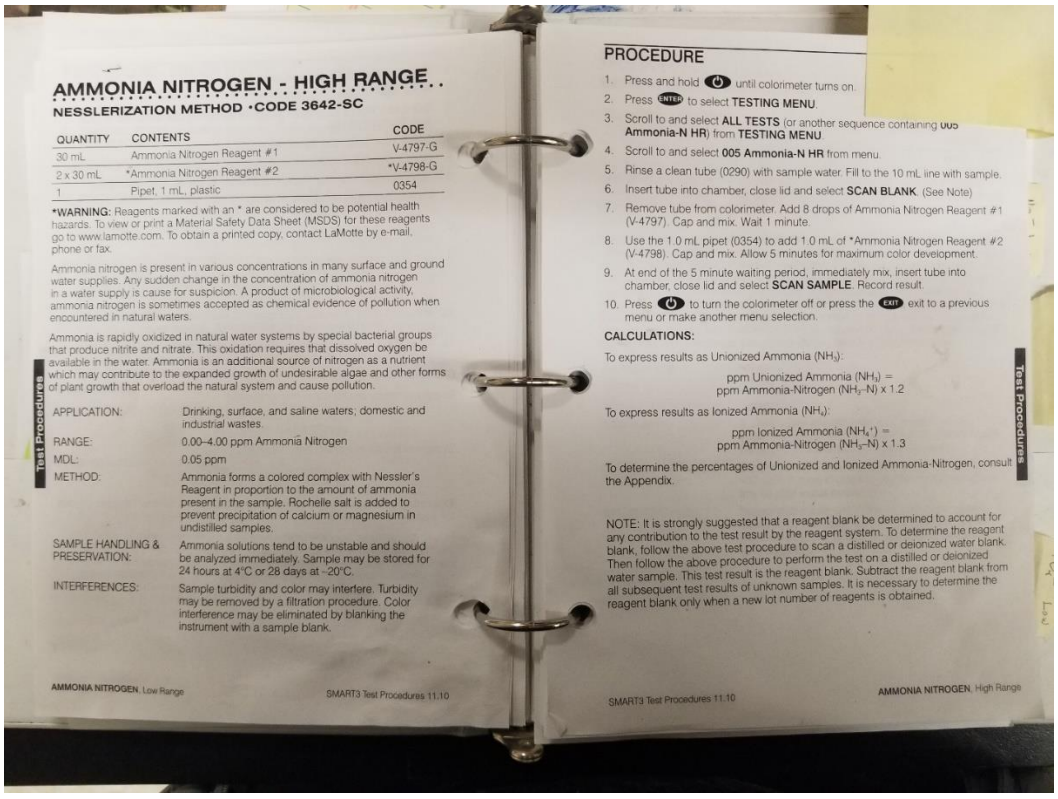
Appendix A – Procedures:



AMMONIA NITROGEN - HIGH RANGE

NESSLERIZATION METHOD •CODE 3642-SC

QUANTITY	CONTENTS	CODE
30 mL	Ammonia Nitrogen Reagent #1	V-4797-G
2 x 30 mL	*Ammonia Nitrogen Reagent #2	*V-4798-G
1	Pipet, 1 mL, plastic	0354

***WARNING:** Reagents marked with an * are considered to be potential health hazards. To view or print a Material Safety Data Sheet (MSDS) for these reagents go to www.lamotte.com. To obtain a printed copy, contact LaMotte by e-mail, phone or fax.

Ammonia nitrogen is present in various concentrations in many surface and ground water supplies. Any sudden change in the concentration of ammonia nitrogen in a water supply is cause for suspicion. A product of microbiological activity, ammonia nitrogen is sometimes accepted as chemical evidence of pollution when encountered in natural waters.

Ammonia is rapidly oxidized in natural water systems by special bacterial groups that produce nitrite and nitrate. This oxidation requires that dissolved oxygen be available in the water. Ammonia is an additional source of nitrogen as a nutrient which may contribute to the expanded growth of undesirable algae and other forms of plant growth that overload the natural system and cause pollution.

Test Procedures

APPLICATION:	Drinking, surface, and saline waters; domestic and industrial wastes.
RANGE:	0.00–4.00 ppm Ammonia Nitrogen
MDL:	0.05 ppm
METHOD:	Ammonia forms a colored complex with Nessler's Reagent in proportion to the amount of ammonia present in the sample. Rochelle salt is added to prevent precipitation of calcium or magnesium in undistilled samples.
SAMPLE HANDLING & PRESERVATION:	Ammonia solutions tend to be unstable and should be analyzed immediately. Sample may be stored for 24 hours at 4°C or 28 days at –20°C.
INTERFERENCES:	Sample turbidity and color may interfere. Turbidity may be removed by a filtration procedure. Color interference may be eliminated by blanking the instrument with a sample blank.

PROCEDURE

1. Press and hold ⏻ until colorimeter turns on.
2. Press ENTER to select **TESTING MENU**.
3. Scroll to and select **ALL TESTS** (or another sequence containing **005 Ammonia-N HR**) from **TESTING MENU**.
4. Scroll to and select **005 Ammonia-N HR** from menu.
5. Rinse a clean tube (0290) with sample water. Fill to the 10 mL line with sample.
6. Insert tube into chamber, close lid and select **SCAN BLANK**. (See Note)
7. Remove tube from colorimeter. Add 8 drops of Ammonia Nitrogen Reagent #1 (V-4797). Cap and mix. Wait 1 minute.
8. Use the 1.0 mL pipet (0354) to add 1.0 mL of *Ammonia Nitrogen Reagent #2 (V-4798). Cap and mix. Allow 5 minutes for maximum color development.
9. At end of the 5 minute waiting period, immediately mix, insert tube into chamber, close lid and select **SCAN SAMPLE**. Record result.
10. Press ⏻ to turn the colorimeter off or press the EXIT exit to a previous menu or make another menu selection.

CALCULATIONS:

To express results as Unionized Ammonia (NH_3):

ppm Unionized Ammonia (NH_3) =
ppm Ammonia-Nitrogen (NH_3–N) x 1.2

To express results as Ionized Ammonia (NH_4):

ppm Ionized Ammonia (NH_4^+) =
ppm Ammonia-Nitrogen (NH_3–N) x 1.3

To determine the percentages of Unionized and Ionized Ammonia-Nitrogen, consult the Appendix.

NOTE: It is strongly suggested that a reagent blank be determined to account for any contribution to the test result by the reagent system. To determine the reagent blank, follow the above test procedure to scan a distilled or deionized water blank. Then follow the above procedure to perform the test on a distilled or deionized water sample. This test result is the reagent blank. Subtract the reagent blank from all subsequent test results of unknown samples. It is necessary to determine the reagent blank only when a new lot number of reagents is obtained.

Test Procedures

Procedure followed for the colorimetric ammonia assay used to analyze the volatilized ammonia degradation trials.

Appendix B – Reflection:

Word Count: 1013

Participating in my Senior Capstone Design Project over the course of 18 months taught me a great deal about teamwork, communication, design, and research. While these senior design projects are typically proposed by an industry sponsor and presented to a group of junior students for the student's consideration, my colleagues and I sought out a project of our own design and were able to create a collaboration between two colleges at the university. This method of development presented our group of four students with a unique set of challenges and opportunities. Instead of an industry mentor and "expert" to guide us and provide us with a set of goals, we were able to meet with Dr. Lance Seefeldt and his graduate students working on similar projects and determine our own set of realistic goals. Instead of regular meetings with representatives from a local company, we instead reported to the group of graduate students in Dr. Seefeldt's lab. These circumstances forced our group to be proactive in our reporting and work ethic. When compared to other groups in my senior class, our group seemed to have less accountability and more freedom when dealing with our mentors. The responsibility to pass along information and reach out for help, as well as to drive the project forward, lied squarely on our shoulders. This dynamic allowed for an increased rate of personal growth, as we were never "babied" during our work. We learned to hold regular meetings among ourselves and developed a team dynamic that worked for us and we set up our own schedule for communication with our mentors and for group work.

Completing such an immense project with an interdisciplinary team did not come without several challenges, however. During our initial meetings, the majority of the group wanted to set unrealistic goals, including publishing several peer-reviewed publications based on our future work. We had to collaborate with our mentors to find a happy middle ground and set goals that were reasonable for the project while maintaining a good work-life balance. Our team dynamic also took some time to develop. Each of the group members, including myself, had worked as a lead on research projects, and each of us had the capability and intelligence to direct the project. Our mentors had little to no background in engineering and did not fully understand the Capstone Design process. These issues led to some deliberation, but we were able to find a system that worked for us, with each student often handling a portion of the project, allowing for autonomy and leadership for each of us.

I personally took the lead for a degradation trial that was run as part of the project. Coordinating with my mentors and team leaders as designing the experiment proved to be perhaps the most valuable experience of the capstone project. Although I had to commit more time to that portion of the project, it was rewarding to design, construct, troubleshoot, and run an experiment. Along with a pair of mentors and another group member, I designed an apparatus to test the degradation of our bacterium in soil and we developed a way to measure that degradation. I was able to plan meetings, schedule workdays, delegate tasks, and communicate

with our mentors over this portion of the project. This experience helped me to develop organization and leadership skills as I was stretched by our deadlines and need for data.

The project also had a complex set of ethical concerns and exploring and discussing them with our group also provided great learning experiences. As we were working with a group collaborating with NASA, our project was working towards manned Martian missions. Many ethical concerns can be raised about sending humans to a desolate planet to alter the environment in a way that makes it suitable for life. Those sent to Mars would experience intense hardship and uncertainty and would be forced to live a strict lifestyle with their schedule and diet largely decided for them. Aside from the daunting tasks and intense physical requirements, the astronauts will also certainly face issues revolving around mental health, loneliness, and discouragement. Discussing this situation and working on the project, which aimed to control one of the thousands of variables the astronauts would face, helped expose us to the many ethical dilemmas present in research. As a future researcher I believe that this experience will help me to consider the ethical implications of my research.

As part of the project we also had the opportunity to create and present a poster at the Intermountain Biological Engineering Conference. The assignment to present at the conference was given only a few days before the conference was to be held, not allowing us much time to prepare. While we put the poster together as a team, two of our team members were unable to make it to the presentation, so I was able to present along with one team member. The conference attracted industry members and students from all of Utah and many neighboring states. Preparing the poster and presenting at this professional conference was a great opportunity to hone my communication skills and was also a great platform for networking with business leaders and many working in engineering companies.

My experience in the Biological Engineering Department Senior Capstone Design Program has given me valuable research experience and helped me to further understand my own interests. In part due to this project, I have a desire to continue my involvement in research and hope to do while in medical school this fall. The technical skills and laboratory techniques I acquired while completing this project will certainly become an asset as I continue my research career. I am also grateful for the relationships I developed with my group members and mentors during the project and for all that I learned. I feel better prepared for the future engineering and research problems I will encounter, and the completion of this project has given me the confidence to tackle any task. This project was truly the capstone of my education here at Utah State University.

Personal Biography:

Daniel Kade Derrick was born and raised in St. George, Utah. Growing up, Kade was always fascinated by the world around him and spent time exploring nature, playing sports, and being involved in his community. Kade loves the outdoors, hunting, fishing, hiking, camping, and spending time with his wife and daughter.

During his Biological Engineering degree at Utah State University, Kade developed a passion for research and innovation and has worked with several university professors as he explored his interests. Kade has spent his time at USU working toward matriculation into a medical scientist training program. This highly competitive dual degree program allows students to attend medical school and complete a Ph.D. in 7-8 years. Kade scored in the 99th percentile on the MCAT and has already received multiple acceptances to prestigious universities. He hopes to receive a Ph.D. in Cancer Biology and pursue a career as an oncologist and physician-scientist.