5-2020

Characterization of Rhodopseudomonas Palustris Strains for the Production of Fixed Nitrogen Fertilizer for Mars

Andrew James Walters
Utah State University

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

by

Andrew James Walters

Capstone submitted in partial fulfillment of the requirements for graduation with

UNIVERSITY HONORS

with a major in

Biological Engineering
in the Department of Biological Engineering

Capstone Mentor Dr. Ronald Sims	**Departmental Honors Advisor** Dr. Elizabeth Vargis

University Honors Program Director
Dr. Kristine Miller

UTAH STATE UNIVERSITY
Logan, UT

Spring 2020

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). Fourteen liters of both NifA* and PB23 were then grown at scale. 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 study.

Acknowledgements

First, I would like to thank my team members Kade Derrick, Caleb Walker, and Drew Porter for their efforts in this research project. Additionally, I would like to thank Dr. Lance Seefeldt and Dr. Bruce Bugbee for supporting this project. This project would not have been possible without the assistance of their graduate students: Mathangi "Mattie" Soundararajan, Kyle Valgardson, and Paul Kusuma, and Dr. Anna Doloman, a post-doctorate researcher. I would like to thank the NASA Microbial Media and Feedstock Division of the Center for the Utilization of Biological Engineering in Space (CUBES) for funding this project and providing the opportunity to present this project to domain experts.

A special thanks to Dr. Charles Miller, Dr. Ron Sims, and Dr. Ryan Jackson for support on this project and for their encouragement and mentorship over the many years. Also, my gratitude to Dr. Elizabeth Vargis for providing Honors Advising and helping me to overcome many personal barriers in order to be successful.

I am indebted to the Honors Department for years of support and believing in me.

Finally, I would like to thank my wife, Sierra Walters, and my parents, Glen and Lori Walters, for their unwavering support. Mom, thanks for buying me that microscope.

A Note to the Reader

This capstone report is the culmination of several years of work, including the efforts of Kade Derrick, Caleb Walker, and Drew Porter, my Biological Engineering Senior Design Team. For the purposes of this Honors Capstone, blocks of text in italics designate my contributions that separates this capstone from my Senior Design Project. See "Reflective Writing" in Appendix B for a retrospective analysis of the impact of this experience.

Table of Contents

Final Written Product **Word count = 9,817 words**

Introduction

Honors Note on Introduction

Throughout this project, I was responsible for literature review and curating the foundation of knowledge for R. palustris because of my background with the microbe. I have been working with R. palustris since I helped the Miller Lab discover a novel strain in 2016.

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

Honors Note on Aims and Objectives

Early in this project, Kade and Caleb made a connection with Dr. Lance Seefeldt. I then led the early discussions that determined our ultimate objectives and deliverables.

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 was identified due to its performance under nitrogen fixing conditions.

Fourth, the best strains of *R. palustris* were then confirmed based on the results from the baseline quantification of NH_4^+ production and the growth curve.

Fifth, the strains were grown at scale to generate biomass.

Sixth, 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

Honors Note on Background and Literature Review

As previously mentioned, literature reviews and introductions were my responsibility. This literature review is a compilation of years of independent work and hours of reading papers to better understand R. palustris. This section constitutes the largest fraction of Honors work.

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] These changes combined through genetic engineering might represent a solution for the production of excreted ammonium in the growth media. However, due to a change in design focus, genetic engineering was not attempted.

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.

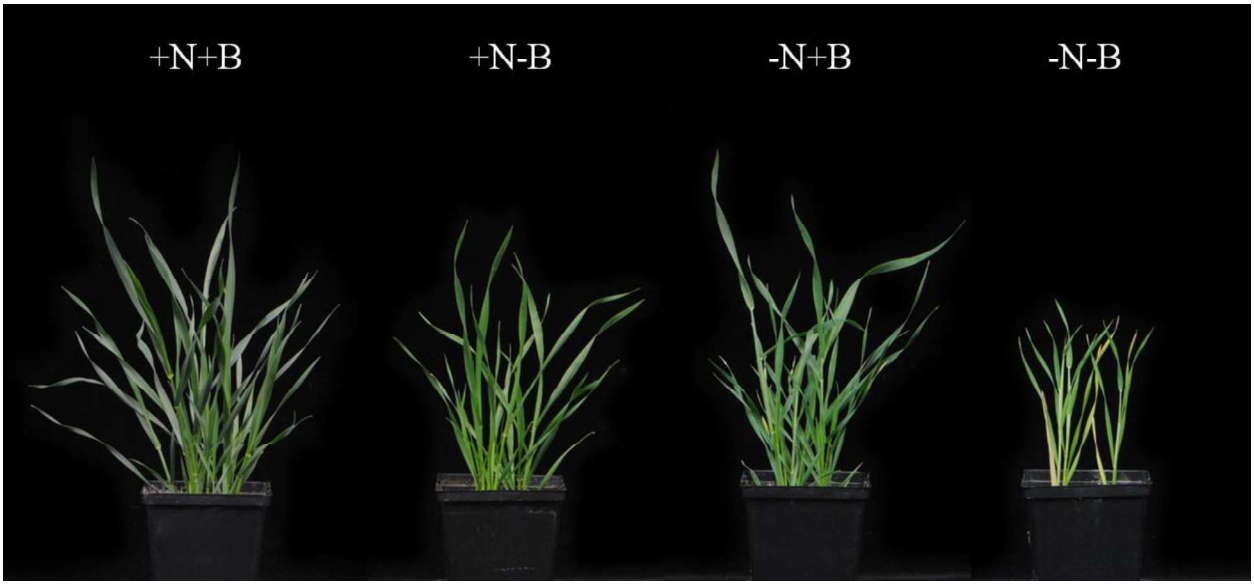


Figure 1. *Plant trial demonstrating the use of bacteria as fertilizer. N: traditional slow release nitrogen fertilizer. B: Azotobacter added as nitrogen source. Image credit: Paul Kusuma, Dr. Bruce Bugbee's Plant Physiology Lab, Utah State University.*

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 strain 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

Honors Note on the Growth Trials

This section constitutes the second largest fraction of my Honors work. I was responsible for maintaining the cultures of R. palustris. My background with the microbe made me particularly well suited for this role, but it demanded a significant amount of time and effort beyond that required of my peers. I primarily made the growth media, inoculated starter cultures, curated isolated colonies, and attempted to maintain pure cultures. It was a challenge to maintain pure cultures as so many team members were working on the project, and we were frequently shuttling between two labs.

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 µL 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 µL 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 µg/mL) were also created and measured in duplicate so that a standard curve could be created.

The 25 μL 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 100C and the column temperature was 60C. 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, Wolf's 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 2. 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 3-4. 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.*

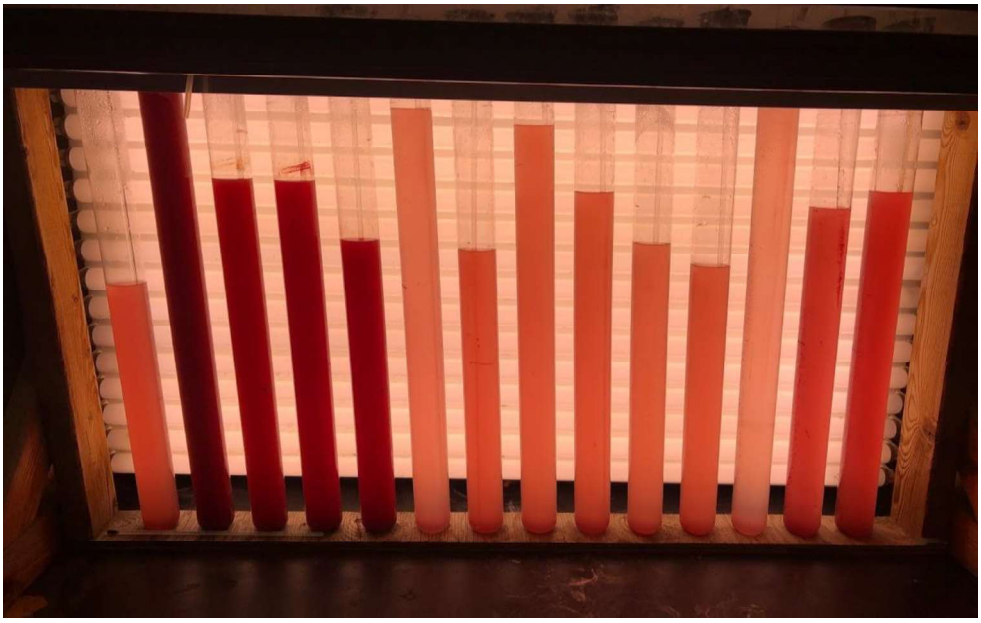


Figure 4. *Growth of the NifA* strain just before the cell mass was harvested.*

Figure 5. *Harvested R. palustris.*

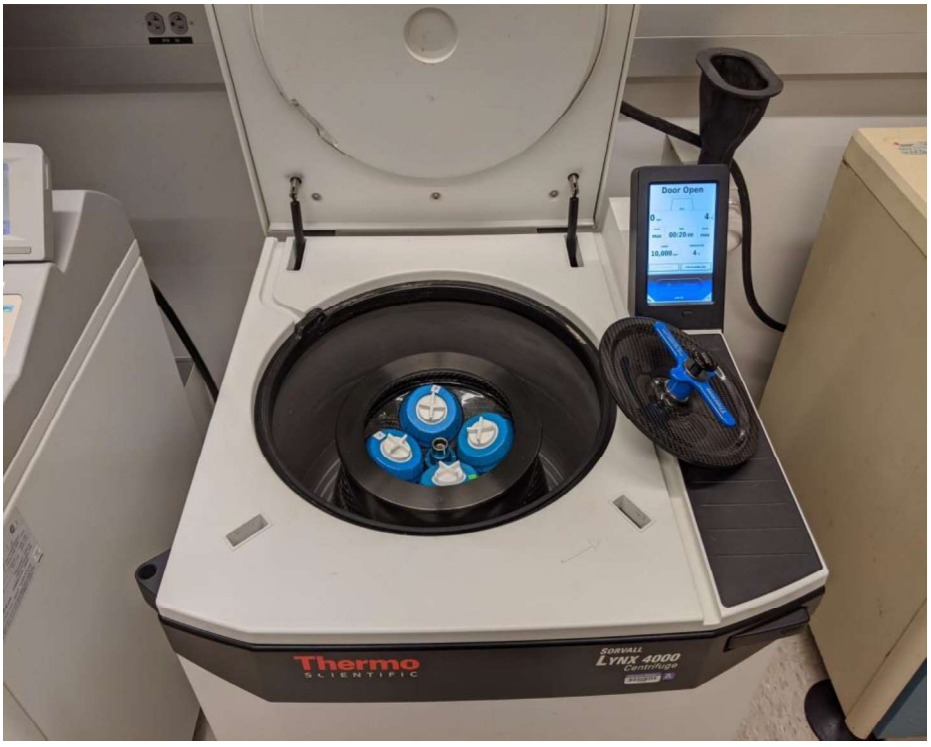


Figure 6. *Harvesting of R. palustris via centrifugation.*

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.

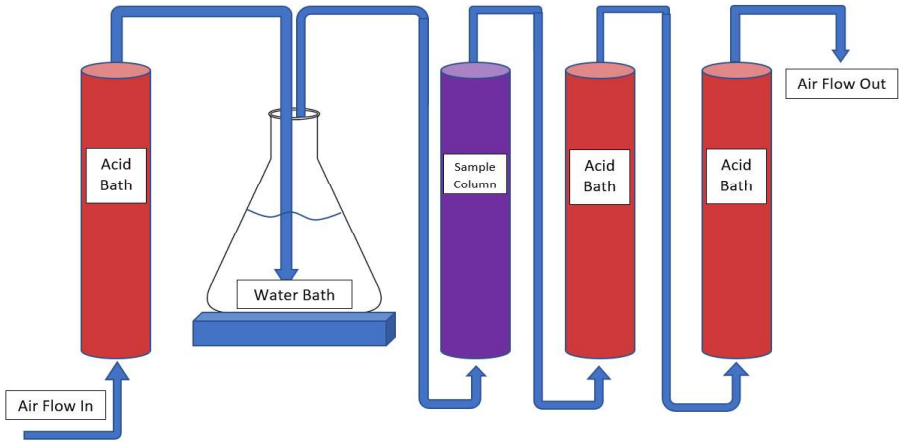


Figure 8. *A schematic illustrating the design of the volatile ammonia degradation system.*

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.*

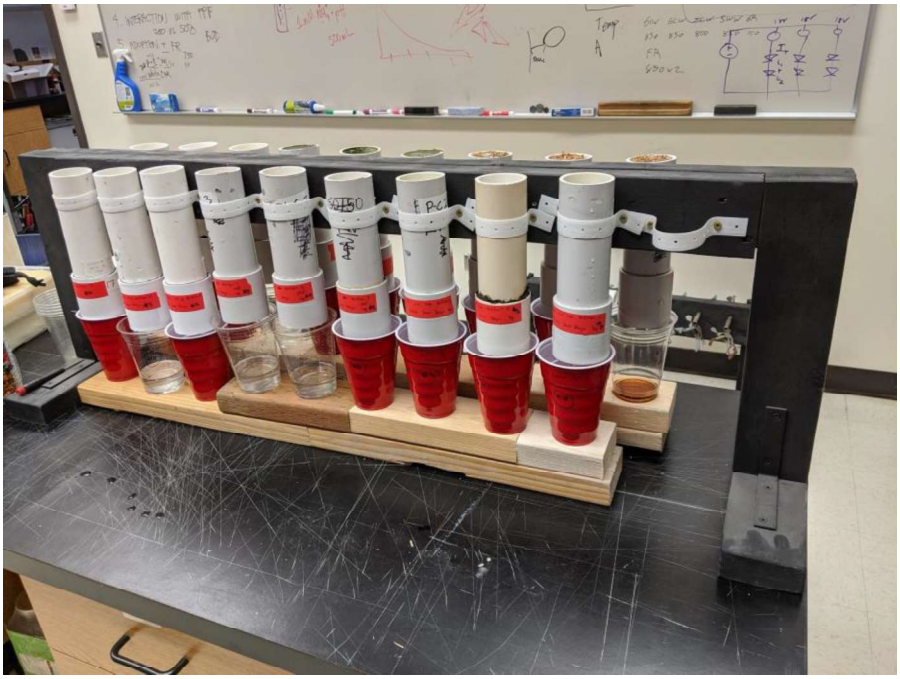


Figure 12. *The columns used in the soluble nitrogen degradation study.*

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 lab of Kathryn Fixen of the University of Minnesota, 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

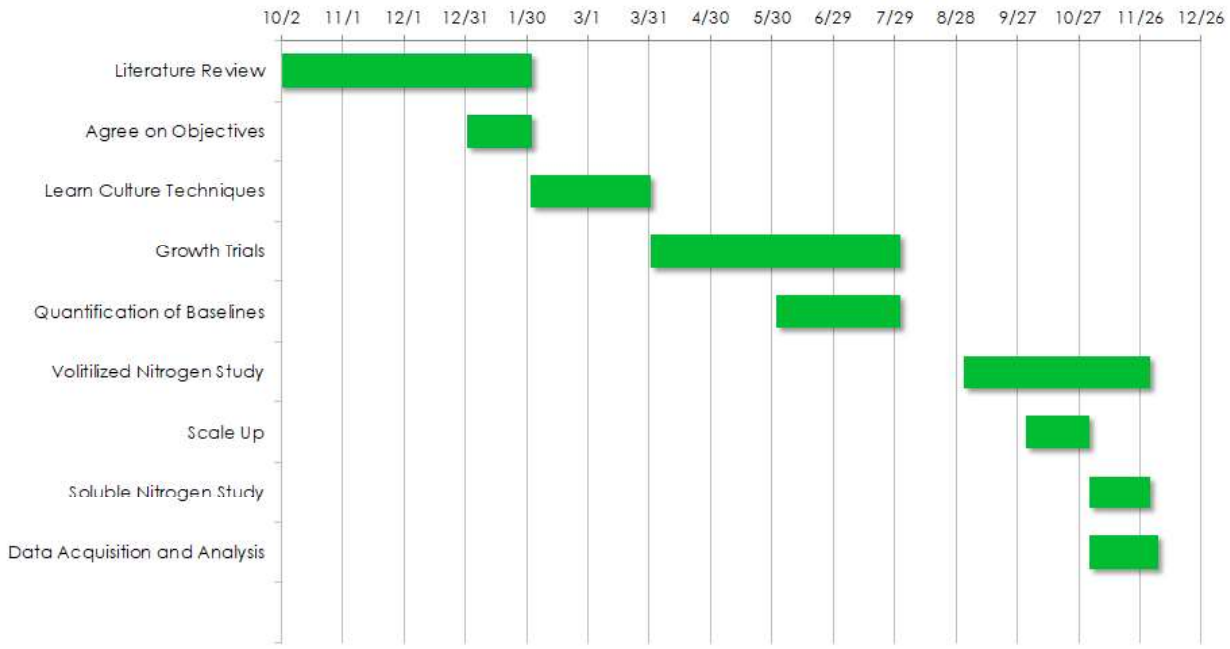


Figure 13. *A Gantt chart showing the timeline for the project and the individual tasks to be completed.*

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.

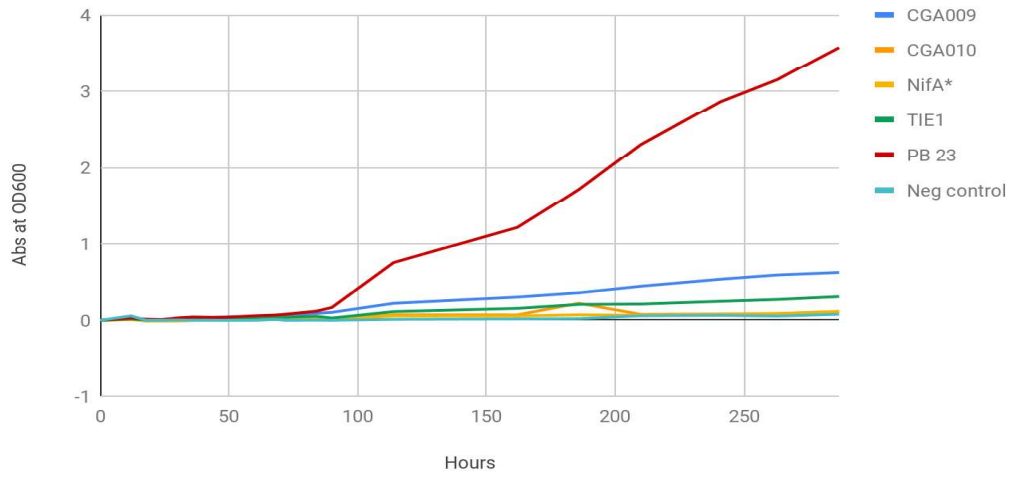


Figure 14. *Growth trial 1 of several R. palustris strains under nitrogen fixing conditions. PB23 was initially observed to be the fastest growing. Unfortunately, this trial became contaminated and data was unreliable.*

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.

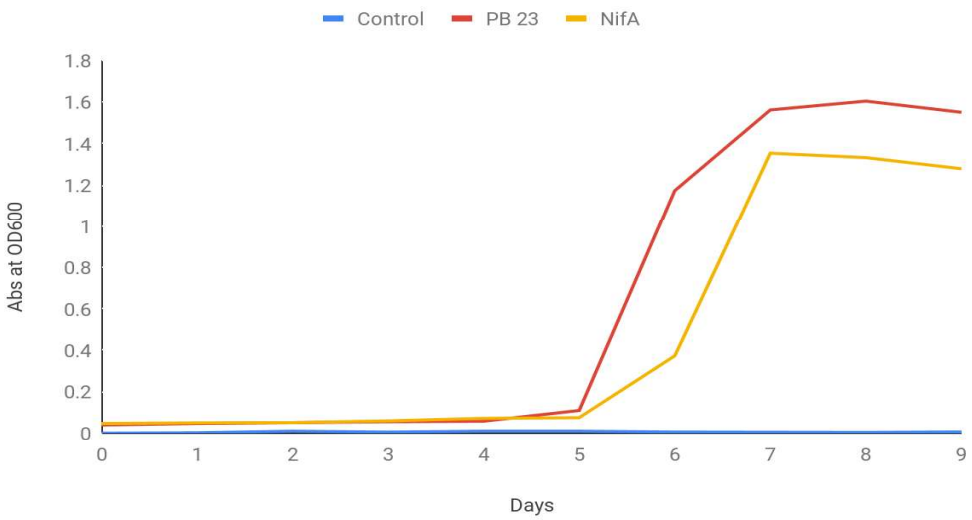


Figure 15. *Averaged OD600 Values for the Second Growth Trial*.

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: °C
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.

Honors Note on Results of the Growth Trials

This was one of the most disappointing results of my Senior Design project, and quite frankly, the biggest failure of my Honors Capstone. As a team, we were really struggling to get R. palustris to grow consistently. Our growth curves lacked the characteristic lag, exponential, and stationary growth phases that can be seen in Figure 15. I spent hours meticulously measuring the optical density of R. palustris. My measurements had low standard deviations, and everything was growing as it should: or so I thought. After measuring the ammonium concentrations, it was apparent that ammonium was present even at day zero when there was no way that R. palustris had fixed any nitrogen. The assay turns yellow depending on the concentration of ammonium. Within seconds, the first samples from day zero were banana yellow. My heart sank, and I realized what I had done: I had used the seed media with a high concentration of ammonium instead of the nitrogen free media. In fact, the concentration was so high it couldn't be measured using our assay. In the end, I generated the best growth curve for R. palustris I had ever done during 4 years of work with the microbe. However, that curve was meaningless in the context of this project. With a "fail forward" mentality, the technique did improve future iterations.

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. Another error made was that optical density had previously been sampled at a wavelength of 600 nm. In Trial 3 the strains were successfully grown to the previously observed maximum optical density of between 2.5 and 3. For Trial 3, samples were measured at 660 nm. See Figure 16.

Honors Note on the Discussion of the Growth Trials

As previously mentioned, the meticulous technique I practiced generating the textbook growth curve in Figure 15 improved future iterations of the growth trial. We finally got the curve we expected, with a reduced lag time due to seeding the cultures correctly. The exponential growth phase was extended, as expected. As the microbe needed to synthesize its own nitrogen from N_2 gas, its doubling time was significantly reduced, giving the extended exponential phase. It was reassuring to finally get the result we were looking for.

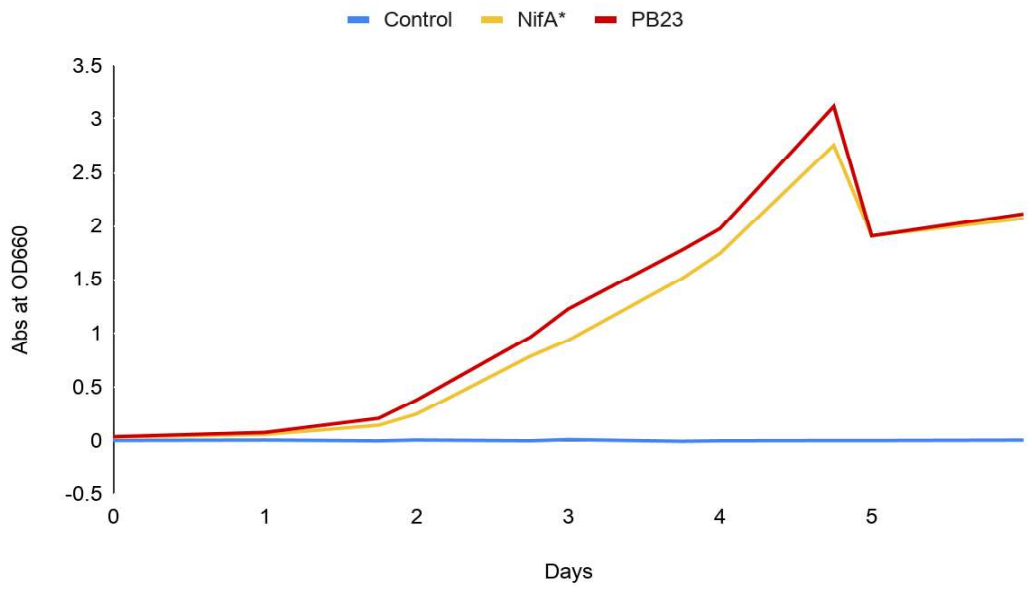


Figure 16. *OD660 of the third growth trial.*

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.

Honors Note on the Results of Ammonium Assays

The data from the assay trial that was largely dismissed was due to contamination of cultures due to cross over between samples. In the end, R. palustris was growing in every sample, including the negative controls. This resulted from multiple people working on it, after which I ended up taking responsibility for growth trials on my own to reduce variability.

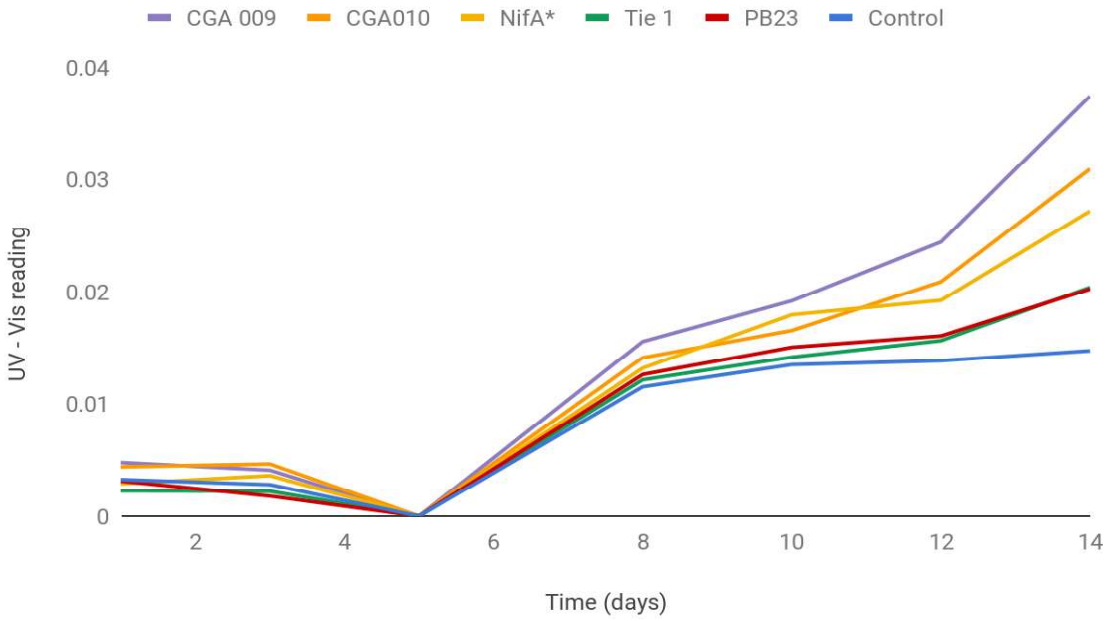


Figure 17. *Averaged ammonium assay data for growth trial #1.*

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.

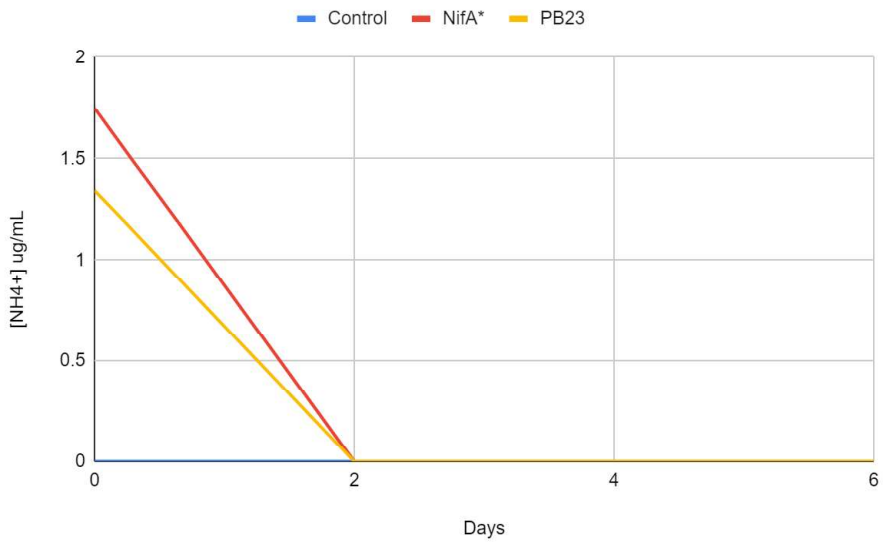


Figure 18: *Results of the ammonium assay from growth trial #3.*

Honors Note on the Discussion of the Results of Ammonium Assays

This is where we realized that I had messed up yet again. We didn't include the results of the ammonium assay from growth trial #2 because everything was banana yellow. In this trial, we realized that I was growing seed cultures on media containing ammonium, then transferring the bacteria to the nitrogen free medium. I forgot to wash the cells, and the residual ammonium in the seed cultures was more than enough to encourage robust growth. We see in the ammonia assay that the ammonia concentration dropped as the bacteria completed log phase, then was flatlined during the exponential and stationary phases. It is possible that they assimilated enough nitrogen to complete the growth curve without it, it is more likely that the nitrogen was consumed during lag phase as the microbe became accustomed to the new media, which reduced the lag phase, and then only made enough for growth and maintenance metabolism during the exponential and stationary phases. Our hypothesis was that nifA would generate more ammonium than was needed and the concentration of ammonium would increase over time. Unfortunately, we didn't see this result, and we ran out of time on the project to try other things. Our advisors encouraged us to treat the cells themselves as slow release fertilizers instead of trying to collect the extracellular media as a fertilizer. This decision pivoted and informed the rest of our project and enabled us to move forward despite a negative finding.*

My role moving forward in this for the Honors Capstone was to inform the microbiology of scale up. We had to grow these microbes in volumes nearly 15 times as large as we had ever done. I made the media and made informed decisions on growth conditions.

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. palustris* 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

Honors Note on Scale Up

As previously mentioned, my microbiology experience became partner to Drew Porter's scale up expertise. Together, we completed this objective.

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 Dr. Doloman's 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

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.

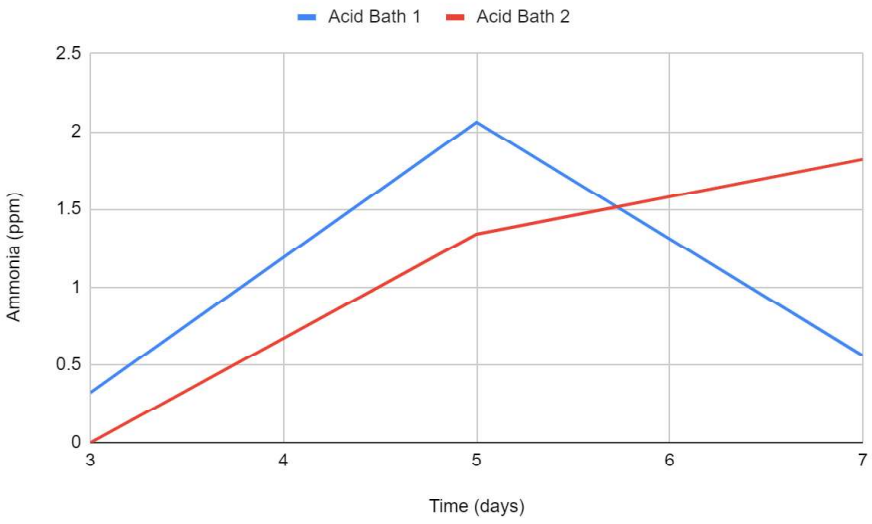


Figure 19. *Concentration of volatilized ammonia during the third test trial of the volatilized ammonia system with NifA* at OD 2.*

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.

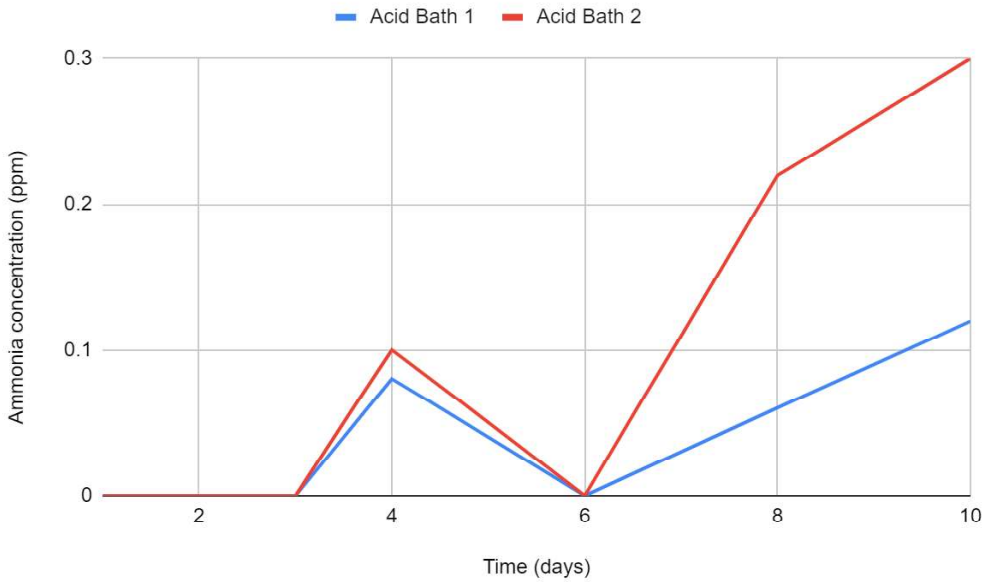


Figure 20. *The curve of the final degradation trial for NifA* bacteria.*

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.

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.

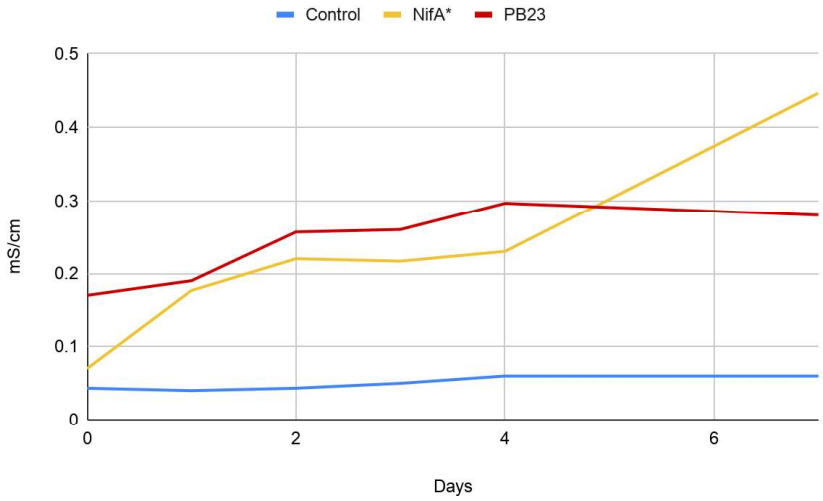


Figure 23. *Electrical conductivity of the NifA* and PB23 strains.*

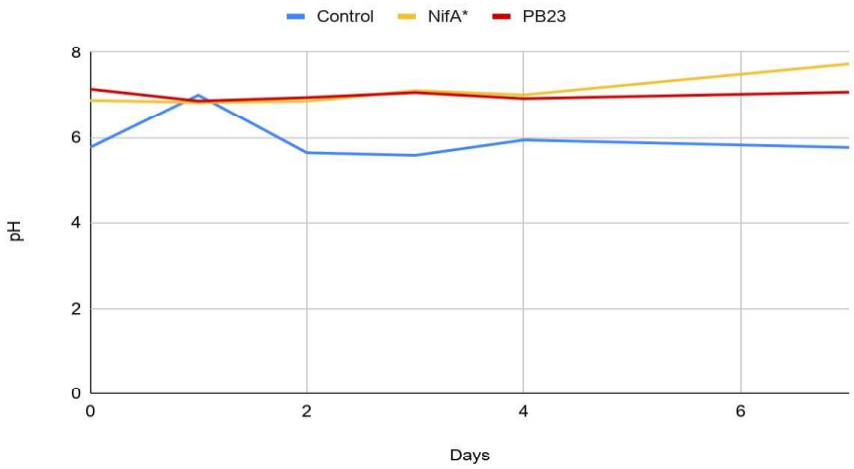


Figure 24. *The pH of the NifA* and PB23 strains.*

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 will be given to both Dr. Seefeldt and Dr. Sims on December 11, 2019. The report will be relayed to any of Dr. Seefeldt's colleagues at NASA if he desires. The project will be presented during the spring semester of the 2020 calendar year at the Utah State University Capstone Design Summit to the general public, biological engineering department, and any of the faculty involved in the project.

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°C
- 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.

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.

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 three research aims. First, he has worked for the past three years with Dr. Charles Miller isolating, characterizing, and sequencing the genome of a novel isolate of *R. palustris*. Second, he is assisting Drew Porter with the development of the educational kits. He also works with Dr. Ryan Jackson characterizing a Type IV CRISPR system and developing an educational CRISPR kit. 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 will be 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 will also come 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

Reflective Writing (1,197 words)

As I reflect on this project, I pondered why this was called a capstone and not a thesis, and thought of a funny story from my past. I grew up working on my grandma's farm and learned how to drive at about 12 years old. For context, I was less than 5 feet tall when I started

my junior year of high school and was 6’0” when I graduated. It was really difficult for me to reach the clutch of that old Ford stick shift diesel. To circumvent this, I’d drop the truck into neutral, start the truck, and then pull myself forward in the seat to push the clutch and only shift when I was on a straightaway. That wasn’t hard to do on a farm!

Well, when I was about 14 or 15 my dad and I built a cinderblock mailbox at our house. He pulled up in his green, stick shift Chevy S10, with the fender nearly rubbing the tires when he brought home the massive capstone for the mailbox. I was too short to lift it, so he got his friends to help. He backed the truck up carefully to the mailbox, then as a team, they lifted it on the mailbox. My dad asked me to move the truck quickly so he could cement on the capstone. I’d driven the truck a hundred times by that time: no problem. Well, in my haste, my nasty habit of leaving the truck in neutral caught up to me. I thought it was in neutral, pressed in the clutch, and started the truck. Then I popped back out the clutch to get situated to drive (like I always did) the truck (which was actually in reverse, not neutral) FLEW back the 6 or so inches to the mailbox at full speed and SLAMMED into the mailbox. Everyone shouted and jumped out of the way. I quickly moved the truck forward and stepped out of the truck. We all watched as the mailbox rocked back and forth in what felt like slow motion. I must have been pale as a ghost: I’ll never forget the look on my dad’s face. Anger, disbelief, shock, then a slump of the shoulders as he realized that what was done was done. Luckily, the capstone didn’t fall off, it didn’t crack, and the mailbox didn’t fall over. But it has always been a little crooked since then, and the dent in the tailgate of my dad’s truck was hard to miss.

This capstone project is a lot like that experience. Throughout my education (and quite frankly throughout my life) I’ve always struggled to finish things. There are a lot of half-finished projects laying around: a scale model of T-rex that fell apart last year that I haven’t glued back

together, and an open hole in my room back in Tooele that I said I'd cover up 10 years ago, to name a few. This project is a lot like that mailbox. I had developed some nasty habits like procrastination, miscommunication, and project jumping. The capstone came lumbering in to crown my undergraduate education, but the truck I brought it on didn't have the right suspension, and it took a lot of friction to get it done. I'm a lot like that green pickup truck: a work in progress, trying to take on more than I can handle, but mostly dependable (except for that time the transmission slipped and we found it parked on the neighbor's lawn after rolling off our driveway). My mentors are like my dad's friends: they helped me get to the finish line and then helped me lift the heavy capstone onto the mailbox. Those habits caught up to me a few times during the project, leaving my final project slightly askew like the mailbox, and probably a dent in the relationships with some mentors. I hope they can forgive me for that and can see how much I've grown over these past few years. I can see it: just the act of me actually finishing a project is a huge milestone in my life. I'm so grateful for their time and support.

This project (and the associated years of research that led up to it) changed the course of my life and career. Even though the project didn't work and was extremely frustrating at times, the experiences that came along the way mean so much to me (in retrospect of course). I helped discover the PB23 strain of *R. palustris* that we used in this project, and it has been with me (for better or for worse) for nearly 4 years. It was part of my research proposal for which I was awarded an Honorable Mention for the Goldwater Scholarship. I was awarded the American Society for Microbiology Undergraduate Research Fellowship to work on this microbe and had the privilege to present at a massive and prestigious conference, ASM Microbe. This work also afforded me the opportunity to present at Posters on the Hill in Washington, D.C. and meet many of our elected representatives.

One of the best privileges of working on this project was getting to interact with the Center for the Utilization of Biological Engineering in Space. It was a pleasure to work with Dr. Lance Seefeldt and Dr. Bruce Bugbee, the local leaders of this group at USU. We got to present at the annual CUBES meeting that was held at USU this year (serendipitously), where I got to meet government leaders like Dr. John Hogan from NASA, academic leaders from MIT, Berkeley, and Stanford, and industry professionals from Ginkgo Bioworks. My work on this microbe and with NASA was specifically mentioned as why I was offered a place in Boston University's prestigious Biomedical Engineering PhD program. I'm sure it was instrumental in my acceptance to Rice University's Bioengineering PhD program, where I decided to go.

This project taught me how to fail forward in research. A negative result is an opportunity to learn, and an invitation to "Charge on!" as Dr. Ron Sims would say. It helped me think critically and apply some of the scale up theory I had learned in my Biological Engineering coursework. This capstone also helped broaden my experiences: in a single project we worked in a Biological Engineering laboratory, a Biochemistry laboratory, and a Plant Physiology Greenhouse. The interdisciplinary nature of this project made it all the more impactful.

Even though this project was frustrating at times, it wasn't without merit or benefit. We laid a foundation for further work on developing this microbe as a fertilizer for the Martian Missions. If it is successful, it could represent another method for generating fertilizer here on Earth, which farmers could implement. Locally, we were able to answer questions for the Seefeldt lab (what are the ideal growing conditions for *R. palustris*?) and the Bubgee lab (how long does the nitrogen in the cells take to degrade and become bioavailable?). Finally, I was also able to serve my team with my experience with *R. palustris* and its microbiology. I am grateful for this time to reflect on how much this microbe and this project did to help me grow.

References

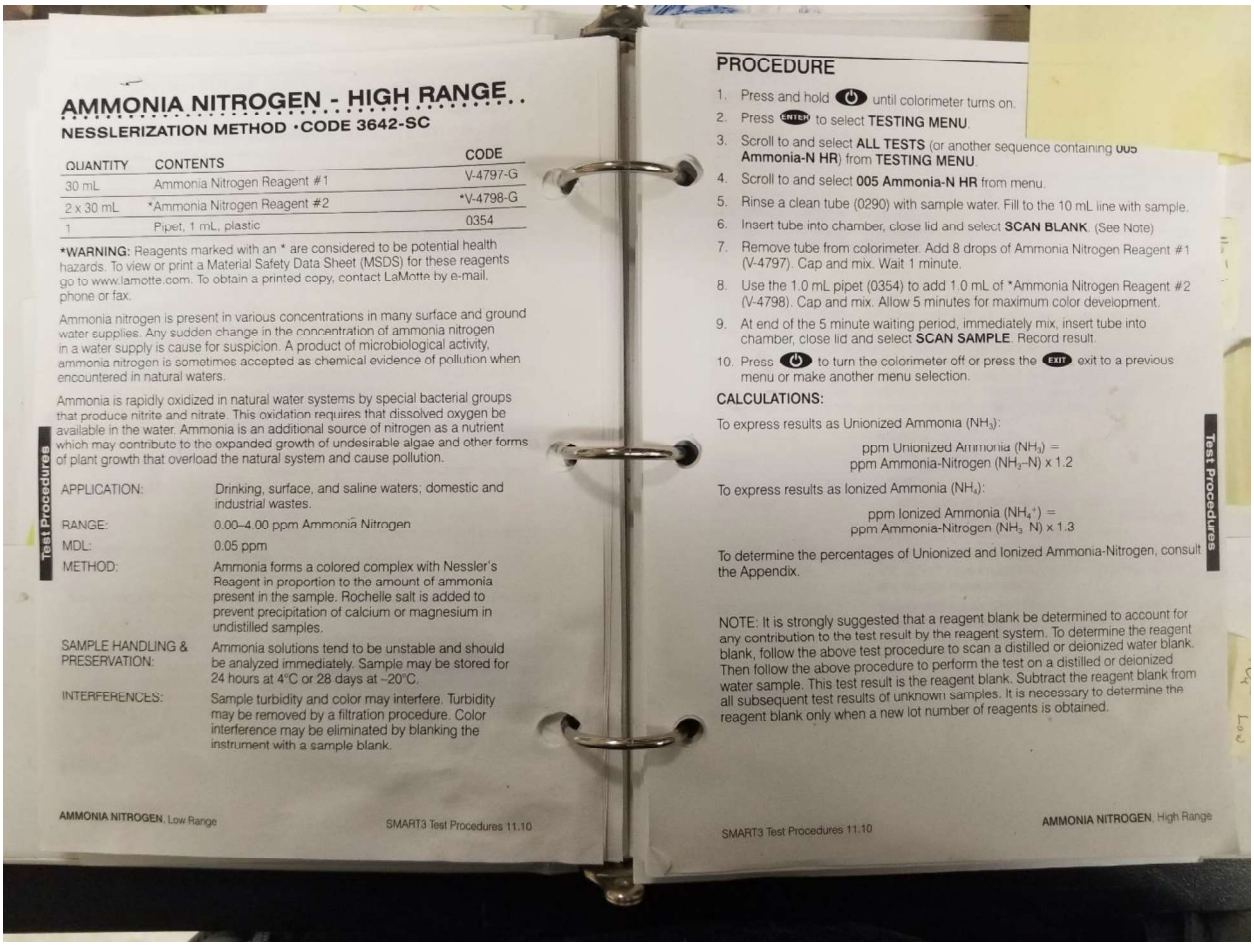
1. Menezes AA, Cumbers J, Hogan JA, Arkin AP. Towards synthetic biological approaches to resource utilization on space missions. 2015;
2. Larimer FW, Chain P, Hauser L, Lamerdin J, Malfatti S, Do L, et al. Complete genome sequence of the metabolically versatile photosynthetic bacterium Rhodopseudomonas palustris. Nat Biotechnol. 2004;22(1):55–61.
3. Sasikala C, Ramana C, Rao PR. Nitrogen fixation by Rhodopseudomonas palustris OU 11 with aromatic compounds as carbon source / electron donors. 1994;122:75–8.
4. Ripatti, D. S., Veltman, T. R., & Kanan, M. W. (2019). Carbon monoxide gas diffusion electrolysis that produces concentrated C2 products with high single-pass conversion. *Joule*, *3*(1), 240-256.
5. Wong W-T, Tseng C-H, Hsu S-H, Lur H-S, Mo C-W, Huang C-N, et al. Promoting Effects of a Single Rhodopseudomonas palustris Inoculant on Plant Growth by Brassica rapa chinensis under Low Fertilizer Input. Microbes Environ. 2014;29(3).
6. Larimer FW, Chain P, Hauser L, Lamerdin J, Malfatti S, Do L, et al. Complete genome sequence of the metabolically versatile photosynthetic bacterium Rhodopseudomonas palustris. Nat Biotechnol [Internet]. 2004;22(1):55–61. Available from: http://www.nature.com/doifinder/10.1038/nbt923
7. Adessi A, McKinlay JB, Harwood CS, De Philippis R. A Rhodopseudomonas palustris nifA* mutant produces H2 from NH 4 + -containing vegetable wastes. Int J Hydrogen Energy [Internet]. 2012;37(21):15893–900. Available from: http://dx.doi.org/10.1016/j.ijhydene.2012.08.009
8. Doud DFR, Holmes EC, Richter H, Molitor B, Jander G, Angenent LT. Metabolic

engineering of Rhodopseudomonas palustris for the obligate reduction of n-butyrate to n-butanol. Biotechnol Biofuels. 2017;10(1):1–11.

9. Jiao Y, Kappler A, Croal LR, Newman K, Newman DK. Isolation and Characterization of a Genetically Tractable Photoautotrophic Isolation and Characterization of a Genetically Tractable Photoautotrophic Fe (II) -Oxidizing Bacterium , Rhodopseudomonas palustris Strain TIE-1. Appl Environ Microbiol. 2005;71(8):4487–96.
10. Xu W, Chai C, Shao L, Yao J, Wang Y. Metabolic engineering of Rhodopseudomonas palustris for squalene production. J Ind Microbiol Biotechnol. 2016;43(5):719–25.
11. Williams DR, NASA. Earth Fact Sheet [Internet]. [cited 2018 Dec 4]. Available from: https://nssdc.gsfc.nasa.gov/planetary/factsheet/earthfact.html
12. Williams DR, NASA. Mars Fact Sheet [Internet]. [cited 2018 Dec 4]. Available from: https://nssdc.gsfc.nasa.gov/planetary/factsheet/marsfact.html
13. Fixen KR, Zheng Y, Harris DF, Shaw S, Yang Z-Y, Dean DR, et al. Light-driven carbon dioxide reduction to methane by nitrogenase in a photosynthetic bacterium. Proc Natl Acad Sci. 2016;113(36).
14. Yakunin AF, Hallenbeck PC. AmtB Is Necessary for NH4+ -Induced Nitrogenase Switch-Off and ADP-Ribosylation in Rhodobacter capsulatus. J Bacteriol. 2002;184(15):4081–8.
15. Adams, C., Frantz, J., & Bugbee, B. (2013). Macro-and micronutrient-release characteristics of three polymer-coated fertilizers: Theory and measurements. *Journal of plant nutrition and soil science*, *176*(1), 76-88.

Appendix – Procedures

Ammonia Assay: Bugbee Lab



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.

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.

Test Procedures

AMMONIA NITROGEN, Low Range SMART3 Test Procedures 11.10

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

SMART3 Test Procedures 11.10 AMMONIA NITROGEN, High Range

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

Bacterial Growth Media Recipes

PM medium (with thiosulfate as electron donor)

PM component	Volume (ml) per 1 L	Final concentration
0.5 M Na_2HPO_4	25	12.5 mM
0.5 M KH_2PO_4	25	12.5 mM
10% $(NH_4)_2SO_4$	10	0.1%
Concentrated base	1	---

0.1 M $Na_2S_2O_3$	100 (autotrophic) 1 (heterotrophic)	10 mM (autotrophic) 0.1 mM (heterotrophic)
PABA (2 mg/ml)	1	0.002 mg/ml

- pH should be around 6.8
- Aliquot medium into wanted volumes for growths
    - Anaerobic bottles should be flushed with argon or nitrogen gas
    - For photoheterotrophic growths, screw-cap vials can be used, but just filled to the top with medium; the cells will use any residual oxygen
- Autoclave
- Add carbon sources (i.e. acetate or succinate) from stock solutions if growing heterotrophically

PM medium (no thiosulfate- H_2 as electron donor)

PM component	**Volume (ml) per 1 L**	**Final concentration**
0.5 M Na_2HPO_4	25	12.5 mM
0.5 M KH_2PO_4	25	12.5 mM
10% $(NH_4)_2SO_4$	10	0.1%
Concentrated base	1	---
PABA (2 mg/ml)	1	0.002 mg/ml

- pH should be around 6.8
- Aliquot into wanted volumes for growths and autoclave
- 80% hydrogen:20% CO_2 at 200 kPa
- 20 mM bicarbonate
- Add 1 μM NiCl2 if growing the cells with hydrogen as the electron donor (hydrogenase has Ni in its cofactor)

NF (nitrogen-fixing) medium (with thiosulfate as electron donor)

NF component	**Volume (ml) per 1 L**	**Final concentration**
0.5 M Na_2HPO_4	25	12.5 mM
0.5 M KH_2PO_4	25	12.5 mM
Concentrated base	1	---
0.1 M $Na_2S_2O_3$	100	10 mM
PABA (2 mg/ml)	1	0.002 mg/ml

- pH should be around 6.8
- Aliquot medium into wanted volumes for growths
    - Anaerobic bottles should be flushed with argon or nitrogen gas
    - For photoheterotrophic growths, screw-cap vials can be used, but just filled to the top with medium; the cells will use any residual oxygen

- Autoclave
- Add carbon sources (i.e. acetate or succinate) from stock solutions if growing heterotrophically

NF (nitrogen-fixing) medium (without thiosulfate- H_2 as electron donor)

NF component	Volume (ml) per 1 L	Final concentration
0.5 M Na_2HPO_4	25	12.5 mM
0.5 M KH_2PO_4	25	12.5 mM
Concentrated base	1	---
PABA (2 mg/ml)	1	0.002 mg/ml

- pH should be around 6.8
- Aliquot into wanted volumes for growths and autoclave
- 80% hydrogen:20% CO_2 at 200 kPa
- 20 mM bicarbonate

Add 1 μM NiCl2 if growing the cells with hydrogen as the electron donor (hydrogenase has Ni in its cofactor)

Component for Rhodopseudomonas media

Concentrated base

Concentrated base component	For 1000 mL concentrated base
Nitrilotriacetic acid (NTA-free acid)	20 g
$MgSO_4$ anhydrous	28.9 g
$CaCl_2.2H_2O$	6.67 g
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0185 g
$FeSO_4.7H_2O$	0.198 g
Metal 44	100 mL

Dissolve NTA separately in 600 mL of water and neutralize with KOH (14.6 g KOH), add other components, and dissolve in order given. Adjust to pH 6.8 with KOH before making to final volume of 1000 mL. A precipitate forms when adjusting the pH from the acid side of 6.8 with KOH (need about 100 mL of 1M KOH), but eventually will redissolve with stirring. When the pH is near 6.8, the colour of the solution changes from a deep yellow to straw colour. Then, filter sterilize and store in glass bottle wrapped with aluminium foil. Store at 4°C for at least one year.

Metal 44

Metal 44 component	For 1000 mL metal 44
EDTA (free acid, not sodium salt)	2.5 g
$ZnSO_4.7SO_4$	10.95 g
$FeSO_4.7H_2O$	5 g
$MnSO_4.H_2O$	1.54 g
$CuSO_4.5H_2O$	0.392 g
$Co(NO_3)_2.6H_2O$	0.25 g
$Na_2B_4O_7.10H2O$	0.177 g

Add EDTA to 800 mL distilled water stirring and adjust pH about 5.0 with 10 M NaOH to get EDTA dissolved. Add other metals in order given (Do not add components until the previous one has dissolved), and then make the final a volume of 1000 mL (final plus pH is around 2.4, a clear and lime green solution). Then, filter sterilize and store in glass bottle wrapped with aluminium foil. Store at 4°C indefinitely.

Phosphate solutions

To make	For 1000 mL	For 500 mL
0.5M Na_2HPO_4 (FW 141.96)	70.98 g	35.49 g
0.5M KH_2PO_4 (FW 136.09)	68.045 g	34.0225 g

Filter sterilize.

Others

To make	For 500 mL	For 250 mL
10% $(NH_4)2SO_4$	50 g	25 g
0.1M $Na_2S_2O_3.5H_2O$ (FW 248.2)	12.41 g	6.205 g
2 mg/mL p-aminobenzoic acid (PABA)	1g	0.5 g

Filter sterilize (PABA bottle should be wrapped with aluminium foil).

Carbon solutions

To make	For 200 mL	For 100 mL
2M Sodium acetate, trihydrate (FW 136.08)	54.432 g	27.216 g
1M Sodium succinate (FW 270.15)	54.03 g	27.015 g

Filter sterilize 2 times. For anaerobic stocks, filtrate solution (once filter sterilized) into autoclaved bottle and flush with N_2 gas for 30 min.

Yeast extract solutions

To make	For 200 mL	For 100 mL
10% Yeast extract	20 g	10 g
5% Yeast extract	10 g	5 g
1% yeast extract	2 g	1 g

Pour solution into bottle, flush with N_2 gas for 30 min and autoclave for 30 min. After autoclave, flush with N_2 gas for 5 min.

Vanadium solution

To make	For 200 mL	For 100 mL
1 mM VCl_3 (FW 157.3)	0.03146 g	0.01573 g

Pour solution into bottle, flush with N_2 gas for 30 min (wrapped with aluminum foil), and autoclave for 30 min. After autoclave, flush with N_2 gas for 5 min (bottle should be wrapped with aluminum foil).

Wolfe's vitamin solution (WV): add 0.05 mL/10 mL medium

Vitamin	g/L	Final mg/L
Para-aminobenzoic acid	0.005	0.025
Folic acid	0.002	0.01
Lipoic acid (Thioctic acid)	0.005	0.025
Riboflavin	0.005	0.025
Thiamine	0.005	0.025
Nicotinic acid amide (Nicotinic acid)	0.005	0.025
Pyridoxamine (Pyridoxine HCl)	0.01	0.05
Pantothenic acid	0.005	0.025
Cobalamin (B-12)	0.0001	0.0005
Biotin	0.002	0.01
Adjust pH 7.0 with NaOH solution, and keep in -20°C		

Filter sterilize 2 times. For anaerobic stocks, filtrate solution (once filter sterilized) into autoclaved bottle and flush with N_2 gas for 30 min (wrapped with aluminum foil) (bottle should be wrapped with aluminum foil)

Ammonia Assay: Seefeldt Lab

Ammonia assay buffer

o-Phthalaldehyde(Phthalic dicarboxaldehyde (Aldrich P39400)	270 mg
Ethanol (190 proof, nondenatured)	5 mL
2-mercaptoethanol	25 mL
0.2 M Phosphate buffer pH 7.3	100 mL

The o-Phthalaldehyde and ethanol mix should be exposed as little to light as possible. Add it and the beta mercaptoethanol quickly to the phosphate buffer, bubble the solution for a few minutes with Argon gas, and then seal in an amber bottle in the dark. The buffer is good for no more than 1 month.

A stock solution of ammonium chloride (FW 53.49) is prepared with 50 mg in 500 mL. Then standards are prepared by adding different amounts of the stock (0.1 mg/mL or 1.869 mM) to make the following;

5 ug/mL	add 50 uL to 950 uL DW	2.336 nmol in 25 uL
10 ug/mL	add 100 uL to 900 uL DW	4.674 nmol in 25 uL
15 ug/mL	add 150 uL to 850 uL DW	7.011 nmol in 25 uL

20 ug/mL	add 200 uL to 800 uL DW	9.347 nmol in 25 uL
25 ug/mL	add 250 uL to 750 uL DW	11.68 nmol in 25 uL
30 ug/mL	add 300 uL to 700 uL DW	14.02 nmol in 25 uL

For the analysis, 1.0 mL of the assay buffer and 25 mL of the standards are added to each disposable cuvette, and allowed to sit in the dark for 30 minutes. For the samples, the same is done but with 25 uL of the sample.

The excitation wavelength is 410nm, and the emission wavelength is 472nm.

Author Biography

Andrew James (AJ) Walters will graduate in May 2020 with his Bachelor of Science in Biological Engineering from Utah State University, with minors in Entrepreneurship, Computer Science, Chemistry, Biology, and Spanish. He is an accredited Engineer in Training after passing the "Other Disciplines" Fundamentals of Engineering Exam. He specialized in Synthetic Biology through 5 years undergraduate research, including two summer internships as a Genome Engineering Intern at Synthetic Genomics, Inc. in San Diego, California. He presented his research at prestigious conferences such as the Synthetic Biology: Engineering, Evolution, and Design (SEED) conference, the American Society for Microbiology (ASM) Microbe meeting, and the Council for Undergraduate Research's Posters on the Hill. He was awarded the Utah State University Undergraduate Research Fellowship, the ASM Undergraduate Research Fellowship, and an Honorable Mention for the Goldwater scholarship.

He will join Rice University's Bioengineering Ph.D. program in Fall 2020 to further his career in synthetic biology. After graduation, he plans to work in the biotechnology industry or start his own biotechnology company. Eventually he hopes to be the Chief Scientific Officer of one of these companies.