12-2020

Utilizing Earth's Microbiology to Develop the Framework for a Manufactured Martian Nitrogen Cycle

Kyle Valgardson
Utah State University

UTILIZING EARTH'S MICROBIOLOGY TO DEVELOP THE FRAMEWORK FOR A MANUFACTURED MARTIAN NITROGEN CYCLE.

by

Kyle Valgardson

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Biochemistry

Approved:

Lance Seefeldt, Ph.D. Major Professor	Nicholas Dickenson, Ph.D. Committee Member
Ryan Jackson, Ph.D. Committee Member	D. Richard Cutler, Ph.D. Interim Vice Provost of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2020

ABSTRACT

Utilizing Earth's microbiology to develop the framework for a manufactured Martian nitrogen cycle.

by

Kyle Valgardson, Master of Science

Utah State University, 2020

Major Professor: Lance Seefeldt, Ph.D.
Department: Chemistry and Biochemistry

As humans aspire to explore the reaches of space, processes that seem insignificant on earth become more critical. One such process is the nitrogen cycle. Nitrogen is a significant element in biological molecules, making it essential for life as we know it. There are vast stores of dinitrogen gas on earth; this form of nitrogen, however, is unavailable to most organisms except for a few bacteria known as diazotrophs. These bacteria contain an enzyme known as nitrogenase, which reduces dinitrogen gas to ammonia, which can then be metabolized by other bacteria and plants. Consumption of plants and bacteria is how other organisms, higher in the food chain, obtain nitrogen for their protein and DNA. To our knowledge, there is not a native nitrogen cycle on Mars; as such, we must design a synthetic nitrogen cycle to support a manned mission to the planet. In this thesis we address this by looking at the functional system design for the central steps of a nitrogen cycle, including nitrogen fixation, transfer and recycling.

Nitrogen fixation is not exclusive to nitrogenase as there is a robust chemical

reaction, known as the Haber-Bosh process, which is used in the production of most conventional nitrogen fertilizers. The Haber-Bosch process is highly scalable but consumes large quantities of energy and requires extreme temperature and pressure, 500°C and 200 atm. These energetic requirements, in addition to the potential catastrophic consequences of a reactor failure, make the Haber-Bosch process unsuitable for space applications. Utilizing diazotrophic bacteria to reduce dinitrogen on Mars is a feasible alternative, as this biological process occurs near standard room temperature and pressure. The scalability, however, is not well understood as the application variables are so vast. We address this by comparing bacterial species growth with varying inputs to identify the most advantageous growth mode for a biological nitrogen fixation reactor. We found that when designing a nitrogen fixation system, balancing nitrogen fixation rates and input requirements is necessary. Factoring both of these metrics led us to photoheterotrophic growth of *Rhodopseudomonas palustris* CGA009 NifA* grown on acetate acetate as the optimal mission relevant system.

Over the last 30 years, Biofertilizer applications have increased as a means of reducing the environmental impacts of inorganic fertilizers. However, there are very few studies addressing a universal application of biomass, as a biofertilizer, to numerous plant cultivars and no studies relative to space travel. A functional Nitrogen transfer from *R. palustris* biomass to plants and other bacteria needs to be constructed as the second step in a synthetic nitrogen cycle. We have begun investigating biomass treatment methods and the compatibility of biomass with multiple plant cultivars. Treatment of biomass by acidification, at room temperature, with Sulfuric acid is capable of some level of degradation while maintaining nitrogen levels in solution. Even though acidified biomass has shown some minor inhibition on germination in very high concentrations (¿500mgN/L), evidence suggests that at functional concentrations (100mgN/L), acidified biomass is not inhibitory to germination and may be

a plausible means of nitrogen transfer for plant growth.

A cyclic nitrogen process is crucial for sustainability as it eliminates nitrogen accumulation and prevent depletion of dinitrogen gas. It is plausible that waste remediation by *R. palustris* NifA* would accomplish this by consuming organic compounds in nitrogen rich waste of a Mars-based research facility. *R. palustris* could grow using the organic carbon as a feed source while incorporating nitrogen sources such as ammonia a urea into its biomass, which could be used as a biofertilizer for further plant growth. We have only scratched the surface of this possibility as we have identified to some level the feasibility of growth in simulated waste effluents. Waste remediation combined with nitrogen reduction and transfer technologies complete the cycle as the foundation structure for a synthetic nitrogen cycle on Mars.

(100 pages)

PUBLIC ABSTRACT

Utilizing Earth's microbiology to develop the framework for a manufactured Martian nitrogen cycle.

Kyle Valgardson

History has shown us that space travel is a complicated activity not to be taken lightly. Extended missions such as those that would accompany a manned mission to Mars are guaranteed to have increased complexity and require creative solutions to problems we likely take for granted. One such issue is how to supply the necessary amount of nitrogen to the astronauts to keep them alive. Nitrogen is an essential component to life on Earth as most biological molecules, such as protein and DNA, contain a significant amount of it. Most organisms have to get it from what they eat, but some bacteria at the bottom of the food chain are capable of getting it from the air in our atmosphere. These bacteria take nitrogen gas from our atmosphere and convert it into ammonia, which is then taken by plants as they grow and incorporated into their proteins and DNA. As other organisms eat these plants this nitrogen is used to build their cells, furthering the transport through the food chain.

The natural process of converting nitrogen gas to ammonia by bacteria was not sufficient for supplying all of the plant growth as the world's population increases. To address this, an industrial process, known as the Haber-Bosch process, was developed to make artificial fertilizers that are now used from back yard gardens to mega-farms across the globe. Making this type of fertilizer on Mars is not plausible as it requires an extensive infrastructure to support it's production, and shipping the necessary amount of fertilizer would take too much payload. Because nitrogen gas is present on

Mars, sending a small supply of bacteria and using as much of the resources on Mars as possible, such as sunlight and water, we may be able to supply the nitrogen for the mission while keeping the payload requirement low. The foundational research necessary to check if this is possible is found in chapter 2 of this thesis. We compared different bacteria and reactor inputs and found that a bacteria, known as *Rhodopseudomonas palustris* NIfA*, that uses light as energy and acetate as carbon to produce the highest level of usable nitrogen. This fist step of nitrogen fixation is crucial as it allows us to convert the nitrogen gas on mars into biologically relevant nitrogen sources for plant and bacterial growth.

In most ecosystems, the bacteria that supply nitrogen to plants are specific depending on the plant species and the area the bacteria are living. This is problematic as we likely need to provide nitrogen to crop plants with various natural sources of nitrogen. In chapter 3, we show that by using acid to break down the bacteria, we may be able to use a single bacteria to supply nitrogen to various plants. One other aspect of nitrogen on Earth is that there is an extensive system of organisms and reactions that turn the unusable nitrogen in the food chain back into nitrogen gas, creating a cycle. This type of cyclic process is necessary to avoid depleting the nitrogen gas in the atmosphere and for supporting a long-lasting global ecosystem. There is even less nitrogen on Mars, so we want to create a cyclic process there as well. Using mission waste to support bacterial growth and nitrogen recycling would allow us to close the loop and complete an artificial nitrogen cycle.

To my wife and kids for your incredible love and support, there is no way I could have done it without you. And to my god and my faith for the perspective and gratitude this gives me.

ACKNOWLEDGMENTS

There is one major thing I have learned while working in this master's program, which is that surrounding yourself with great people, in and out of the lab, is more important than your own capacity and intelligence. I would not be giving credit where it is due without acknowledging those who have contributed to my life over these last two years.

My wife and kids - No one is more directly connected to my success as my wife and kids. Mikell, your example inspires me each and every day, you are an unwavering source of comfort and encouragement and I thank you with all my heart for the person that you have helped me to become. Mack, you are my accomplice in driving your mother crazy and I love spending that time with you. Twins to be named later I look forward to meeting you soon.

My family - You are the foundation of who I am and my work ethic. Mom, I thank you for driving me to accomplish great things and never letting me slack on a task. Dad, without your counsel and the opportunities you gave me I would not be as well rounded and driven so I thank you. Brothers, The shenanigans we participated in growing up is the primary reason for my personality and fun loving attitude. Thank you for the examples and friends you have been. Trichelle, your an inspiration to my life and I look forward to seeing you again someday.

My advisor - Dr. Seefeldt, thank you for giving me this opportunity as you have allowed me to find a way to use my abilities and strengths within the bounds of this grant. I thank you for considering my best interest as you have helped guide not only my research but my future career.

My labmates - We are only as strong as our weakest link and I thank you for

allowing me to be that link. Anna, as we worked together your intelligence and patience was appreciated as you waited for me to understand all these simple concepts. Mattie, you gave me the foundations understanding necessary to accomplish what I have. Hayden, thank you for listening as I tried to reason through concepts, our drink runs were appreciated. Spencer, thanks for letting me interrupt your studies as I thought out loud at you. Derek, Yang, and Artavazd, your experience has been invaluable in structuring world class experiments I couldn't have done it without you.

The rest of the department -Cindy, I thank you for your time and effort in helping me as my goals have shifted. Your an incredible person who goes above and beyond your job description for your students, I think you deserve a raise. My professors thank you for your personal touch as you have imparted your biochemical and life experience. Other graduate students thank you for helping me along the path with both biochemistry feedback and personal entertainment.

My funding agency - When I tell people I work for NASA they are always impressed and I now understand why. You are a world-class organization that allow labs to collaborate and work together to solve seemingly insurmountable tasks. The benefit of working within your organization will extend far beyond my resume.

Kyle Valgardson

CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER 1

INTRODUCTION

1.1 Biology as a solution to deep space exploration challenges

There are multiple things to consider when designing a novel process aimed at a specific goal, and in today's climate, sustainability is a major focus. Sustainability often is a focus as a means of limiting the environmental impact of a process; however, other benefits have been observed as sustainability measures are satisfied. Sustainable design is often most beneficial when done with a resource-conscious mindset, attempting to utilize highly prevalent and easily obtained local resources, known as *in situ* resources (Badiru, 2010). In addition to environmental impact reduction, this mindset often results in lower build costs as a result of shipping burden reduction through *in situ* resource utilization. The importance of local resource utilization increases as the distance between resource location and implementation increases. The benefits of *in situ* resource utilization in space exploration is magnified. As we venture further from Earth the shipment burden increases dramatically, as payload is often a bottleneck for mission design. Each unit mass of payload requires as much as 100 additional units of support mass for its transport (Menezes et al., 2015a; Nangle et al., 2020). This aspect of space travel requires us to rethink processes that seem insignificant to previous space exploration. For example, due to the travel distance and duration of a Mars research mission, total food supplies cannot be shipped as it would take a considerable portion of the allowable payload, estimated to be as high as 5 tons of shipped food (Menezes et al., 2015a; Nangle et al., 2020). A feasible solution to this would be to transport plant seeds and utilize Martian resources such as water,

N_2, and CO_2 to grow the food needed for the mission. food production processes alone would rely heavily on the established processes to supply plant relevant to feed stocks from *in situ* resources on Mars

In addition to food production, there may be other problems with a deep space mission that *in situ* resource utilization can address. Such as: fuel generation, drug manufacturing, radiation monitoring, and waste management. Biology has the potential to act as a catalyst, using many Martian resources to address some of these issues (Menezes et al., 2015a,b). Bacteria have been used as biocatalysts to address similar problems on Earth with benefits including, but not limited to: self-assembling, high input versatility, multi-process capability, integration, and self-healing (Sheldon and Woodley, 2018). If specific biological processes are feasible for space exploration missions, the scale of the benefits of using biocatalysts would only increase when compared to earth bound applications.

1.2 Nitrogen cycle

Nitrogen is one of the more abundant elements in biological material, such as DNA and proteins, making it pivotal to life as we know it. Nitrogen is found in relatively high abundance on Earth makeing up more than 70% of the atmosphere, in the form of dinitrogen gas. Even though dinitrogen gas is highly prevalent, this form of nitrogen is not readily biologically available as most organisms have no way of metabolizing it and incorporating it into their biomass. On Earth, evolution has addressed this by developing the enzyme nitrogenase, that is responsible for converting the abundant stores of dinitrogen gas to ammonia (Canfield et al., 2010). Following the generation of ammonia, plants and other microbes are capable of metabolizing ammonia through a process known as nitrogen assimilation. Nitrogen assimilation starts with uptake of free ammonium into a plant or bacterial cell through ammonium trans-

porters; ammonium is then assimilated into glutamine and glutamate through the glutamine synthetase/glutamine-2-oxoglutarate aminotransferase cycle (Miflin and Habash, 2002; Xu et al., 2012). Once ammonia has been assimilated into these amino acids, nitrogen can then be assimilated into proteins or transferred to other nitrogen-containing biological molecules. Other organisms that cant utilize nitrogen in the form of ammonium must consume plant tissue or bacterial mass proteins containing nitrogen, which they use to construct their nitrogen-rich organic structures. Nitrogen balance in most organisms is crucial, as high levels of ammonia are toxic. To address this toxicity, animals excrete excess nitrogen, mostly as ammonia, urea or uric acid (Wright, 1995). In order to prevent a nitrogen sink, nitrogen waste molecules are converted to nitrates through a bacterial process known as nitrification, then converted to nitrogen gas via denitrifying bacteria (Canfield et al., 2010). Nitrogen in biological molecules, such as protein and DNA, is very stable and must be broken down into ammonium through a bacterial process known as ammonification (Ladd and Jackson, 2015). The combination of all of these processes, from nitrogen reduction, assimilation, and recycling, is known as the nitrogen cycle (Ferguson, 1998). Like many well known or simple processes, when moved from our biosphere to other planetary bodies in our solar system, a fundamental aspects of life, such as the nitrogen cycle, become increasingly complex. In order to solve the complex problem of establishing an artificial nitrogen cycle on Mars, a reliable foundation for the bacterial processes needs to be developed. The comprehensive chapters of this thesis contain foundational principles for the development of an extraterrestrial artificial nitrogen cycle.

1.3 Nitrogen fixation

To design a nitrogen cycle on Mars, the first step is the reduction of dinitrogen

gas to ammonia. This process is achieved for many man-made fertilizers by a process known as the Haber-Bosh process, which consumes large quantities of energy and requires high temperature and pressure (Erisman et al., 2015). These aspects make this energetically costly and unsafe for utilization in a Martian research facility. Biological nitrogen reduction, on the other hand, typically occurs at room temperature and pressure, and is facilitated by nitrogenase, this process occurs in microorganisms known as diazotrophs. Within these diazotrophs, there are three nitrogenase isozymes that are characterized by the metal utilized in the catalytic site: Mo, V, and Fe nitrogenase (Eady, 1996). All of these isozymes perform the same basic nitrogen reduction chemistry, illustrated in the stoichiometric equation 1.1. These isozymes vary in their efficiencies as hydrogen ions are redueced to hydrogen gas without being coupled to ammonia generation at different ratios (Harris et al., 2018).

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i \tag{1.1}$$

Nitrogenase is a two-component enzyme with the catalytic site responsible for chemical reduction events and a dinitrogen reductase, which supplies energy through ATP hydrolysis (Hoffman et al., 2014; MacKay and Fryzuk, 2004). Equation 1.1 shows that for a perfectly efficient nitrogenase, 8 equivalent ATP are required for each ammonia produced. Because of enzymatic inefficiencies, this is not what occurs as nitrogenase evolves additional hydrogen during nitrogen reduction that is not coupled with ammonia generation (Harris et al., 2018). Consequently, more ATP is consumed increasing the energy demand for ammonia production. This enzymatic inefficiency is a significant factor to consider as we design a synthetic nitrogen reduction system. If we maximize the energetic flux through nitrogenase, we will in turn increase the ammonia production; this could be accomplished by increasing the ATP

levels or reducing competing metabolic processes. Additionally the effects of increasing ATP flux through nitrogenase needs to be considered, as changes in energetic flux could be detrimental to bacteria health, reducing overall ammonia production.

In diazotrophic bacteria, comparing growth modes such as phototrophic with chemotrophic and heterotrophic with autotrophic may indicate the optimal growth mode to maximize ATP flux through nitrogenase. Phototrophic bacteria are capable of converting light radiation to ATP through photosynthesis, while chemotrophic bacteria oxidize carbon compounds for ATP production. Even though ATP generated photosynthetically requires little to no chemical input, the rates of this growth mode are often slower when compared to chemotrophic (Schaub and van Gemerden, 1994). There is a similar trend when comparing autotrophic with heterotrophic, where autotrophic growth consumes ATP and electrons to reduce CO_2, supplying carbon for cell growth. In contrast, heterotrophic growth requires oxidation of high-value carbon compounds extracting energy and electrons. Autotrophic growth requires less chemical input, but heterotrophic growth rates are often faster (Bell et al., 2006; Ranaivoarisoa et al., 2019). One major issue in selecting a bacterial species and growth mode for chemical production is selecting a metric to use for comparison of each system. Due to the increased value of conserving volume with deep space missions, we use the metric of nitrogen fixation per reactor volume in our reactor design. In chapter 2 we use this metric to identify the optimal bacterial species and growth mode for our nitrogen reduction reactor.

1.4 Biomass for plant fertilization in space

Reducing nitrogen would be a futile effort unless the form of nitrogen produced by diazotrophs can be used for other mission processes. One of the major processes

would be plant growth as a food source for astronauts. Diazotrophs fall into two categories: symbiotic and free-living. Symbiotic diazotrophs can be found in plant root nodules where they supply bioavailable nitrogen to the host plant and in return receive nutrients from the plants. This process is species-specific, meaning a single bacteria cannot be universally applied to all plant species (Smercina et al., 2019). Free-living diazotrophs do not depend on plant nutrients for growth and reduce dinitrogen gas in more diverse environments, making them better candidates for the development of a broad application system. As awareness of the detrimental effects of artificial fertilizers on ecosystems increases, free-living diazotrophs are being studied as an alternative biofertilizer source. Artificial nitrogen fertilization has been necessary to support food production demand, as the global population increases. Artificial fertilizers allow for increased crop production in normally less fertile regions, as well as the ubiquitous application with numerous plant species. We are only now beginning to truly understand the detrimental aspects of artificial fertilizers as they have been shown to reduced natural plant diversity and have exhibit levels toxicity in aquatic environments where the excess fertilizer washes to (Crawley et al., 2005; Vitousek et al., 1997). Biofertilizers are a possible solution to this as they tend to be more environmentally friendly, and exhibit reduced toxicity and promote healthy soil bacteria growth, while still increasing the nitrogen levels in the soil (Rao, 1982).

Various diazotrophs have been used in a host of plant investigations, with many indicating competitive growth stimulation when compared to artificial nitrogen fertilizers (Jha and Prasad, 2006; Kantachote et al., 2016). Even though there is a substantial amount of research done applying diazotrophs as biofertilizers, they vary greatly not only in the plant and bacterial species but also in the mode of fertilization (Sakarika et al., 2019). Bacterial biofertilizer is commonly applied in two main modes: either as a direct fertilizer where dead biomass is added to the root substrate, or in-

directly where living bacteria are inoculated into the root zone where they actively supply nutrients to the plants (Sakarika et al., 2019). One major issue with indirect fertilization is the difficulty of controlling the amount of nitrogen added to the system, as it is difficult to control and monitor bacterial production following the inoculation event. Direct fertilization is not subject issues with nitrogen metering, because the biomass added can be accurately measured for the nitrogen content and dosage easily controlled. Purple non-sulfur bacteria (PNSB) are nitrogen-fixing phototrophs with a highly versatile metabolism. This ability to metabolize many different compounds has resulted in these bacteria populating environments across the globe; this pervasive presence has also lead to numerous investigations of PNSB as a biofertilizer, with the majority being applied indirectly (Sakarika et al., 2019). Few of these PNSB applications have been done with the same bacteria or plant strain in addition to a wide variance in inocula production, treatment, and performance indicators. This high level of variance highlights the need for a more systematic fertilizer production and compatibility screen. Even though environmental concerns for a Martian research center are small, the benefits of a versatile PNSB bio-fertilization system would ultimately arise from the moderate reactor conditions and self-assembling nature of this biocatalyst. In chapter 3 we use some basic treatment methods of bacteria biomass and plant compatibility screening in development of universal biofertilizer production.

1.5 Nitrogen waste remediation

One of the fundamental concepts of sustainable system design is the development of closed loop systems where the waste at the end of a product life cycle is converted into raw materials for the generation of new products (King et al., 2006). This type of system is inherent to biogeochemical processes such as the carbon and nitrogen

cycle, to prevent resource depletion. To prevent a similar depletion on Mars designing a synthetic nitrogen cycle would rely heavily on these engineering principles of remanufacturing and recycling (Garland, 1992; Mackowiak et al., 1996). A closed-loop system maintains the added value of products formed in the early stages of the synthetic nitrogen cycle, and remanufacturing biofertilizer from waste streams using PNSB may be a feasible strategy to accomplish this.

On Earth, PNSB are commonly utilized in remediation systems for a diverse set of waste streams including livestock, food production, agricultural, and domestic waste (Chitapornpan et al., 2013; Getha et al., 1998; Kim et al., 2004; Kornochalert et al., 2014; Nagadomi et al., 2000; Pintucci et al., 2015; Sepúlveda-Muñoz et al., 2020). This diversity is primarily due to the versatility of PNSB in metabolizing numerous compounds and their high tolerance to generally toxic materials. In addition to remanufacturing, biofertilizers using waste streams as a carbon and energy source have the ability for biofuel production, in the form of hydrogen gas (Basak and Das, 2007). Hydrogen is generated in PNSB via nitrogenase activity in both light and dark conditions, with light increasing the hydrogen production (Kim et al., 2011; Oh et al., 2004). Nitrogenase is typically regulated as a response to ammonia levels: expression is high with low ammonia levels and repression occurs as ammonia levels increase. Repression of nitrogenase in high ammonia is problematic with waste stream remediation as the typical mission waste would contain high ammonia levels (Verostko et al., 2004), which would significantly reduce nitrogenase activity limiting biofertilizer and biofuel production. *R. palustris* CGA009 NifA* mutant is a PNSB that exhibits constitutive nitrogenase expression and reduced ammonia sensitivity (Adessi et al., 2012), making it ideal for waste applications. Appendix A contains some initial characterization of waste remediation by *R. palustris* NifA*. The combination of this process with the nitrogen reduction system described in chapter 2, and

the nitrogen transfer found in chapter 3, gives a foundational outline of a synthetic nitrogen cycle for a Mars research center.

REFERENCES

Adessi, A., McKinlay, J. B., Harwood, C. S., and De Philippis, R. (2012). A Rhodopseudomonas palustris nifA* mutant produces H2 from NH4+-containing vegetable wastes. *International Journal of Hydrogen Energy* 37, 15893–15900. doi:10.1016/j.ijhydene.2012.08.009

Badiru, A. B. (2010). The many languages of sustainability: IE's should push for better resource utilization across all fields. *Industrial Engineer* 42

Basak, N. and Das, D. (2007). The Prospect of Purple Non-Sulfur (PNS) Photosynthetic Bacteria for Hydrogen Production: The Present State of the Art. *World Journal of Microbiology and Biotechnology* 23, 31–42. doi:10.1007/s11274-006-9190-9

Bell, S. G., Hoskins, N., Xu, F., Caprotti, D., Rao, Z., and Wong, L.-L. (2006). Cytochrome P450 enzymes from the metabolically diverse bacterium Rhodopseudomonas palustris. *Biochemical and Biophysical Research Communications* 342, 191–196. doi:10.1016/j.bbrc.2006.01.133

Canfield, D. E., Glazer, A. N., and Falkowski, P. G. (2010). The Evolution and Future of Earth's Nitrogen Cycle. *Science* 330. doi:10.1126/science.1186120

Chitapornpan, S., Chiemchaisri, C., Chiemchaisri, W., Honda, R., and Yamamoto, K. (2013). Organic carbon recovery and photosynthetic bacteria population in an anaerobic membrane photo-bioreactor treating food processing wastewater. *Bioresource Technology* 141, 65–74. doi:10.1016/j.biortech.2013.02.048

Crawley, M. J., Johnston, A. E., Silvertown, J., Dodd, M., Mazancourt, C., Heard, M. S., et al. (2005). Determinants of Species Richness in the Park Grass Experiment. *The American Naturalist* 165. doi:10.1086/427270

Eady, R. R. (1996). StructureFunction Relationships of Alternative Nitrogenases. *Chemical Reviews* 96. doi:10.1021/cr950057h

Erisman, J. W., Galloway, J. N., Dise, N. B., Sutton, M. A., Bleeker, A., Grizzetti, B., et al. (2015). *Nitrogen : too much of a vital resource : Science Brief*. Tech. rep., WWF Netherlands, Zeist, The Netherlands

Ferguson, S. J. (1998). Nitrogen cycle enzymology. *Current Opinion in Chemical Biology* 2, 182–193. doi:10.1016/S1367-5931(98)80059-8

Garland, J. L. (1992). *Coupling plant growth and waste recycling systems in a controlled life support system (CELSS)*. Tech. rep.

Getha, K., Vikineswary, S., and Chong, V. (1998). Isolation and growth of the phototrophic bacterium Rhodopseudomonas palustris strain B1 in sago-starch-processing wastewater. *World Journal of Microbiology and Biotechnology* 14, 505–511. doi:10.1023/A:1008855125634

Harris, D. F., Lukoyanov, D. A., Shaw, S., Compton, P., Tokmina-Lukaszewska, M., Bothner, B., et al. (2018). Mechanism of N2 Reduction Catalyzed by Fe-Nitrogenase Involves Reductive Elimination of H2. *Biochemistry* 57. doi:10.1021/acs.biochem.7b01142

Hoffman, B. M., Lukoyanov, D., Yang, Z.-Y., Dean, D. R., and Seefeldt, L. C. (2014). Mechanism of Nitrogen Fixation by Nitrogenase: The Next Stage. *Chemical Reviews* 114. doi:10.1021/cr400641x

Jha, M. N. and Prasad, A. N. (2006). Efficacy of New Inexpensive Cyanobacterial Biofertilizer Including its Shelf-life. *World Journal of Microbiology and Biotechnology* 22, 73–79. doi:10.1007/s11274-005-7024-9

Kantachote, D., Nunkaew, T., Kantha, T., and Chaiprapat, S. (2016). Biofertilizers from Rhodopseudomonas palustris strains to enhance rice yields and reduce methane emissions. *Applied Soil Ecology* 100, 154–161. doi:10.1016/j.apsoil.2015.12.015

Kim, M. K., Choi, K.-M., Yin, C.-R., Lee, K.-Y., Im, W.-T., Lim, J. H., et al. (2004). Odorous swine wastewater treatment by purple non-sulfur bacteria, Rhodopseudomonas palustris, isolated from eutrophicated ponds. *Biotechnology Letters* 26, 819–822. doi:10.1023/B:BILE.0000025884.50198.67

Kim, M.-S., Kim, D.-H., Son, H.-N., Ten, L. N., and Lee, J. K. (2011). Enhancing photo-fermentative hydrogen production by Rhodobacter sphaeroides KD131 and its PHB synthase deleted-mutant from acetate and butyrate. *International Journal of Hydrogen Energy* 36, 13964–13971. doi:10.1016/j.ijhydene.2011.03.099

King, A. M., Burgess, S. C., Ijomah, W., and McMahon, C. A. (2006). Reducing waste: repair, recondition, remanufacture or recycle? *Sustainable Development* 14. doi:10.1002/sd.271

Kornochalert, N., Kantachote, D., Chaiprapat, S., and Techkarnjanaruk, S. (2014). Use of Rhodopseudomonas palustris P1 stimulated growth by fermented pineapple extract to treat latex rubber sheet wastewater to obtain single cell protein. *Annals of Microbiology* 64, 1021–1032. doi:10.1007/s13213-013-0739-1

Ladd, J. N. and Jackson, R. B. (2015). Biochemistry of Ammonification. In *Nitrogen in Agricultural Soils* (John Wiley & Sons, Ltd). doi:10.2134/agronmonogr22.c5

MacKay, B. A. and Fryzuk, M. D. (2004). Dinitrogen Coordination Chemistry: On the Biomimetic Borderlands. *Chemical Reviews* 104. doi:10.1021/cr020610c

Mackowiak, C., Garland, J., and Sager, J. (1996). Recycling crop residues for use in recirculating hydroponic crop production. *Acta Horticulturae* , 19–24doi:10.17660/ActaHortic.1996.440.4

Menezes, A. A., Cumbers, J., Hogan, J. A., and Arkin, A. P. (2015a). Towards synthetic biological approaches to resource utilization on space missions. *Journal of The Royal Society Interface* 12. doi:10.1098/rsif.2014.0715

Menezes, A. A., Montague, M. G., Cumbers, J., Hogan, J. A., and Arkin, A. P. (2015b). Grand challenges in space synthetic biology. *Journal of The Royal Society Interface* 12. doi:10.1098/rsif.2015.0803

Miflin, B. J. and Habash, D. Z. (2002). The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. *Journal of Experimental Botany* 53. doi:10.1093/jexbot/53.370.979

Nagadomi, H., Kitamura, T., Watanabe, M., and Sasaki, K. (2000). Simultaneous removal of chemical oxygen demand (COD), phosphate, nitrate and H2S in the synthetic sewage wastewater using porous ceramic immobilized photosynthetic bacteria. *Biotechnology Letters* 22, 1369–1374. doi:10.1023/A:1005688229783

Nangle, S. N., Wolfson, M. Y., Hartsough, L., Ma, N. J., Mason, C. E., Merighi, M., et al. (2020). The case for biotech on Mars. *Nature Biotechnology* 38. doi:10.1038/s41587-020-0485-4

Oh, Y.-K., Seol, E.-H., Kim, M.-S., and Park, S. (2004). Photoproduction of hydrogen from acetate by a chemoheterotrophic bacterium Rhodopseudomonas palustris P4. *International Journal of Hydrogen Energy* 29, 1115–1121. doi:10.1016/j.ijhydene.2003.11.008

Pintucci, C., Padovani, G., Giovannelli, A., Traversi, M. L., Ena, A., Pushparaj, B., et al. (2015). Hydrogen photo-evolution by Rhodopseudomonas palustris 6A using pre-treated olive mill wastewater and a synthetic medium containing sugars. *Energy Conversion and Management* 90, 499–505. doi:10.1016/j.enconman.2014.11.045

Ranaivoarisoa, T. O., Singh, R., Rengasamy, K., Guzman, M. S., and Bose, A. (2019). Towards sustainable bioplastic production using the photoautotrophic bacterium Rhodopseudomonas palustris TIE-1. *Journal of Industrial Microbiology & Biotechnology* doi:10.1007/s10295-019-02165-7

Rao, N. S. S. (1982). Biofertilizers. *Interdisciplinary Science Reviews* 7. doi:10.1179/isr.1982.7.3.220

Sakarika, M., Spanoghe, J., Sui, Y., Wambacq, E., Grunert, O., Haesaert, G., et al. (2019). Purple non-sulphur bacteria and plant production: benefits for fertilization, stress resistance and the environment. *Microbial Biotechnology* n/a. doi:10.1111/1751-7915.13474

Schaub, B. E. M. and van Gemerden, H. (1994). Simultaneous phototrophic and chemotrophic growth in the purple sulfur bacterium Thiocapsa roseopersicina M1. *FEMS Microbiology Ecology* 13. doi:10.1111/j.1574-6941.1994.tb00065.x

Sepúlveda-Muñoz, C. A., de Godos, I., Puyol, D., and Muñoz, R. (2020). A systematic optimization of piggery wastewater treatment with purple phototrophic bacteria. *Chemosphere* doi:10.1016/j.chemosphere.2020.126621

Sheldon, R. A. and Woodley, J. M. (2018). Role of Biocatalysis in Sustainable Chemistry. *Chemical Reviews* 118. doi:10.1021/acs.chemrev.7b00203

Smercina, D. N., Evans, S. E., Friesen, M. L., and Tiemann, L. K. (2019). To Fix or Not To Fix: Controls on Free-Living Nitrogen Fixation in the Rhizosphere. *Applied and Environmental Microbiology* 85. doi:10.1128/AEM.02546-18

Verostko, C. E., Carrier, C., and Finger, B. W. (2004). Ersatz Wastewater Formulations for Testing Water Recovery Systems. 2004–01–2448. doi:10.4271/2004-01-2448

Vitousek, P. M., Aber, J. D., Howarth, R. W., Likens, G. E., Matson, P. A., Schindler, D. W., et al. (1997). Human Alteration of the Global Nitrogen Cycle: Sources and Consequences. *Ecological Applications* 7. doi:10.1890/1051-0761(1997)007[0737:HAOTGN]2.0.CO;2

Wright, P. A. (1995). Nitrogen excretion: three end products, many physiological roles. *Journal of Experimental Biology* 198

Xu, G., Fan, X., and Miller, A. J. (2012). Plant nitrogen assimilation and use efficiency. *Annual review of plant biology* doi:10.1146/annurev-arplant-042811-105532

CHAPTER 2
BACTERIAL STRAIN AND MEDIA COMPOSITION ANALYSIS TO DESIGN A BIOLOGICAL NITROGEN FIXATION SYSTEM ON MARS

2.1 Abstract

Every aspect of life support becomes increasingly more important as we send humans into deeper reaches of space. On Mars, we must address such problems with maximum infrastructure and maximal safety and stability. Biocatalysts housed in bacteria have distinct advantages as they are usually grown in moderate conditions and have the ability of self-assembly, and healing. Here we address the first steps in a synthetic nitrogen cycle using diazotrophs to reduce dinitrogen gas to bioavailable forms. In exploring some basic growth modes for diazotrophs it was not surprising that when focusing on growth rates, heterotrophic growth is more robust than autotrophic, and chemotrophic more so than phototrophic. However, upon measuring input requirements, the amount of nitrogen reduced per acetate consumed was significantly higher with chemotrophic bacteria. To understand which growth mode would be optimal as the foundation for a synthetic nitrogen cycle, it is imperative to consider both nitrogen fixation rates as well as carbon consumption rates. We identified that *Rhodopseudomonas palustris* CGA009 NifA* grown photoheterotrophically had the highest nitrogen fixation rates and efficiency relative to acetate consumption.

2.2 Introduction

A large portion of biological material, such as DNA and protein, contains significant levels of nitrogen making it essential for life. Nitrogen is found in relatively high abundance on Earth as it makes up more than 70% of our atmosphere. However, the nitrogen in our atmosphere is in the form of dintirogen gas (N_2) which is chemically inert and cannot be utilized by most organisms (Canfield et al., 2010). Evolution has addressed this by developing an enzyme known as nitrogenase that is responsible for converting N_2 into ammonia. Ammonia can be metabolized by plants and other bacteria, which in turn supply nitrogen to other organisms. Like many well known or simple processes, when moved from our biosphere to other planetary bodies, nitrogen supply becomes increasingly essential. To support life on Mars there needs to be a simple and efficient process for fertilizer production from *in situ* dinitrogen gas (Menezes et al., 2015). Nitrogen-fixing bioreactors are appealing alternatives to conventional inorganic fertilizer production, which require high pressure, temperature, and energy consumption (Erisman et al., 2015). Nitrogenase is capable of ammonia production at relatively low temperature and pressure, making it more conducive to applications in heavily controlled environments such as an early planetary research facility on Mars.

Nitrogenase may be capable of reducing dinitrogen in moderate conditions; however, this reduction comes at a substantial metabolic cost. N_2 is chemically inert, in order to surpass the energetic threshold required to reduce this stable molecule, nitrogenase couples electron transfer with MgATP hydrolysis (Hoffman et al., 2014; MacKay and Fryzuk, 2004). The minimum reaction stoichiometry (equation 2.1), shows that for every two molecules of ammonia produced 16 ATP and 8 electrons are

consumed (Hoffman et al., 2014).

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i \tag{2.1}$$

This energetic demand illustrates the energetic strain that diazotrophs are subject to while fixing nitrogen. As with organisms, other metabolic processes exist and are in competition with nitrogenase for the energetic resources available within the cell. Many diazotrophs are autotrophic, meaning they are capable of reducing carbon dioxide and assimilating it into biological molecules. Each carbon molecule that is assimilated requires 3-4 electrons and 2-6 ATP, depending on the anabolic process and carbon product formed (Mangiapia and Scott, 2016). Even though autotrophic bacteria have more resource competition between nitrogenase and carbon assimilation, it is not apparent that this should be avoided when designing a nitrogen fixing reactor, as autotrophic growth doesn't require the consumption of high-value carbon compounds. For heterotrophic growth, acetate is a good option as a carbon source as it is a simple molecule that can be produced inorganically through robust electrocatalytic processes that utilize CO as the chemical input (Li et al., 2014; Ripatti et al., 2019), or from CO_2 by a class of organisms known as acetogens (Adessi et al., 2012; Drake et al., 2008; Gildemyn et al., 2015). These bacteria have been utilized in numerous electrocatalytic systems which have increased the acetate production efficiency of the bacteria (Jourdin et al., 2016; Patil et al., 2015). Because CO_2 is readily available on Mars, and CO_2 conversion to CO and O_2 is plausible through a process known as MOXIE (Hecht et al., 2016), it is reasonable to assume that acetate will be our principle carbon source for heterotrophic growth in our nitrogen fixing bioreactor on Mars.

When considering efficiency in biosystem design for nitrogen reduction, it is valuable to note that there are diazotrophs that are also phototrophic (Adessi et al., 2012; Chen et al., 2006; Kannaiyan et al., 1997). Phototrophic bacteria are capable of converting light energy into ATP and reducing equivalents within the cell. However, one caveat with these bacteria is that they often take longer to grow when compared to heterotrophic bacteria (Kaufmann et al., 1982). This diversity of metabolic character found within diazotrophic bacteria creates an overwhelming number of options for bacteria selection and feedstock inputs for a nitrogen reducing biosystem. Identifying mission relavant metrics to consider in designing the optimal bioreactor system is not a trivial task. Here we will address how we developed our framework for a biological nitrogen reducing system that shows available applications in a synthetic Martian nitrogen cycle.

2.3 Materials and Methods

2.3.1 Bacterial species

All bacteria tested were diazotrophs. *Azotobacter vinelandii* is a chemoheterotrophic bacteria that was selected for its relatively fast growth rates, while consuming organic carbon. All other bacteria used were purple non-sulfur phototrophic bacteria strains capable of both autotrophic and heterotrophic growth, including *Rhodospirillum rubrum, Rhodobacter capsulatus, Rhodobacter spheroides, Rhodopseudomonas palustirs* TIE-1 wild type, and CGA009 nifA*. The nifA* mutation resulting in constitutive nitrogenase expression (McKinlay and Harwood, 2010).

2.3.2 Culture Media

A. vinelandii media containing 4.5mM K_2HPO_4 and 1.5mM KH_2PO_4 as the buffering components, 0.015mM ferric citrate, carbon source was either 50mM sucrose or 30mM acetate, concentrated salts solution to a final concentration of 0.1% (v/v) and 5M urea concentrate added to a final concentration of 0.2% (v/v). [salt solution concentrate (g/L concentration): $MgSO_4$-$7H_2O$ (2), $CaCl_2$-$2H_2O$ (0.9), Fe(II)SO_4-$7H_2O$ (0.05), Na_2MoO_4-$2H_2O$ (0.002)].

R. palustris minimal salts media containing 12.5mM K_2HPO_4 and 12.5mM KH_2PO_4 as the main buffering components, a concentrated base solution at a final concentration of 0.1% (v/v), Wolf's vitamins at a 0.5%(v/v) final concentration and depending on the modality of growth different carbon sources were implimented, 30mM acetate as the carbon source in heterotrophic growths or $NaHCO_3$ for autotrophic. [Concentrated base stock solution (mM concentrations): nitrilotriacetic acid (105), $MgSO_4$ (240), $CaCl_2$-$2H_2O$ (45), Na_2MoO_4-$2H_2O$ (0.78),$FeSO_4$-$7H_2O$ (2.5), EDTA (0.86), $ZnSO_4$-$7H_2O$ (3.81), $MnSO_4$-H_2O (0.91), $CuSO_4$-$5H_2O$ (0.15) $Co(NO_3)-2$-$6H_2O$ (0.085), $Na_2B_4O_7$-$10H_2O$ (0.046)]. [Wolfe's Vitamins stock solution [containing (g/L): p-aminobenzoid acid (0.005), folic acid (0.002), lipoic acid (0.005). Riboflavin (0.005), thiamine (0.005), nicotinic acid (0.005), pyridoxamine (0.01), pantothenic acid (0.005), cobalamin (0.0001), biotin (0.002)].

R. capsulatus minimal salts media consisting of 12.5mM K_2HPO_4 and 12.5mM KH_2PO_4 as the main buffering components, 30mM acetate carbon source, 0.054mM Na_2EDTA, 0.081mM $MgSO_4$-$7H_2O$, 0.051mM $CaCl_2$-$2H_2O$, a concentrated base solution at a final concentration of 0.1% (v/v), Wolf's vitamins at a 0.5%(v/v) final concentration, and biotin-thiamine 0.1% (v/v) final concentration. [Concentrated base stock solution (mM concentrations): EDTA (18), $FeSO_4$-$7H_2O$ (7.6), $CoCl_2$-$6H_2O$ (0.85), Na_2MoO_4-$2H_2O$ (0.78), $ZnSO_4$-$7H_2O$ (0.51), $MnCl_2$-$4H_2O$ (0.51), $CuCl_2$-$2H_2O$

(0.1), $NiCl_2$-$6H_2O$(0.11), $VOSO_4$-H_2O (0.15), H_3BO_3 (0.097), Na_2WO_4-H_2O (0.0061), Na_2SeO_3 (0.013)]. [Wolfe's Vitamins stock solution containing (g/L): p-aminobenzoid acid (0.005), folic acid (0.002), lipoic acid (0.005). Riboflavin (0.005), thiamine (0.005), nicotinic acid (0.005), pyridoxamine (0.01), pantothenic acid (0.005), cobalamin (0.0001), biotin (0.002)].

R. rubrum minimal salts media consisting of 12.5mM K_2HPO_4 and 12.5mM KH_2PO_4 as the main buffering components, 30mM acetate carbon source, 1.0mM $MgSO_4$-$7H_2O$, 1.0mM $CaCl_2$-$2H_2O$, a concentrated base solution at a final concentration of 0.1% (v/v), Wolf's vitamins at a 0.5%(v/v)final concentration, and biotin-thiamine 0.1% (v/v) final concentration. [Concentrated base stock solution (mM concentrations): EDTA (60), H_3BO_3 (45), Ferric citrate (15), $CoCl_2$-$6H_2O$ (0.85), Na_2MoO_4-$2H_2O$ (0.78), $ZnSO_4$-$7H_2O$ (0.51), $MnCl_2$-$4H_2O$ (0.51), $CuCl_2$-$2H_2O$ (0.1), $NiCl_2$-$6H_2O$(0.11), $VOSO_4$-H_2O (0.15), Na_2WO_4-H_2O (0.0061),]. [Wolfe's Vitamins stock solution containing (g/L): p-aminobenzoid acid (0.005), folic acid (0.002), lipoic acid (0.005). Riboflavin (0.005), thiamine (0.005), nicotinic acid (0.005), pyridoxamine (0.01), pantothenic acid (0.005), cobalamin (0.0001), biotin (0.002)]. [Biotin thiamine stock solution containing 0.014mM biotin and 3.7mM thiamine].

R. sphaeroides minimal salts media consisting of 12.5mM K_2HPO_4 and 12.5mM KH_2PO_4 as the main buffering components, 30mM acetate carbon source, 54uM Na_2EDTA, 81uM $MgSO_4$-$7H_2O$, 51uM $CaCl_2$-$2H_2O$, 0.04mM ferric citrate, a concentrated base solution at a final concentration of 0.1% (v/v), and Wolf's vitamins at a 0.75%(v/v) final concentration. [Concentrated base stock solution (mM concentrations): EDTA (18), $FeSO_4$-$7H_2O$ (7.6), $CoCl_2$-$6H_2O$ (0.85), Na_2MoO_4-$2H_2O$ (0.78), $ZnSO_4$-$7H_2O$ (0.51), $MnCl_2$-$4H_2O$ (0.51), $CuCl_2$-$2H_2O$ (0.1), $NiCl_2$-$6H_2O$(0.11), $VOSO_4$-H_2O (0.15), H_3BO_3 (0.097), Na_2WO_4-H_2O (0.0061), Na_2SeO_3 (0.013)]. [Wolfe's Vitamins stock solution containing (g/L): p-aminobenzoid acid (0.005), folic acid (0.002),

lipoic acid (0.005). Riboflavin (0.005), thiamine (0.005), nicotinic acid (0.005), pyridoxamine (0.01), pantothenic acid (0.005), cobalamin (0.0001), biotin (0.002)].

2.3.3 Growth conditions and monitoring

Azotobacter vinelandii growths were prepared using 2.6L Erlenmeyer flasks filled with 1L of the phosphate buffer as described previously (Harris et al., 2018). One flask was filled with 500mL to act as an overnight inoculum culture for the other flasks. Each flask was covered with aluminum foil, the salts concentrate, and urea stock were autoclaved to achieve sterility. Using aseptic microbiology practices to maintain sterility, salts concentrate was added to the 500mL media flask to reach a final concentration of 0.1% (v/v), urea stock to a final concentration of 10mM. The flask was inoculated from a starter plate, and placed in a shaker incubator overnight at 30°C and 300 rpm. Salts concentrate was added to the 1L buffer to achieve a 0.1% (v/v) final concentration and inoculated from the overnight culture to a starting OD_{600} between 0.05 and 0.1, and grown in the shaking incubator at the previously described conditions. Growths were monitored via optical density at 600nm using a spectrophotometer (Cary 50 UV-vis, Varian instruments, CA, united states), with measurements taken every 2-3 hrs through the entire growth period. After optical density was measured, samples were frozen in a -20C freezer for later analysis.

Photoautotrophic growths of *R. palustris* TIE-1 were prepared by adding the *R. palustris* media without Wolf's vitamines or $NaHCO_3$ to serum vials filled to a 50% volume. Anaerobicity was achieved by sparging the media with nitrogen for at least 20 min and headspace for 10 min. Vials were then sealed with blue butyl 20mm rubber stoppers and autoclaved. Upon cooling, Wolf's vitamines were added to the vials through a 0.2 μm syringe filter. Headspace was again exchanged with nitrogen gas

and normalized to ambient pressure, hydrogen gas equal to the headspace volume was added to each serum vial. Vials were again normalized to achieve a 1:1 ratio of N_2 to H_2, degassed $NaHCO_3$ was then added to make a 30mM final concentration. TIE-1 cells, grown heterotrophically in small screwcap vials, were used as an inoculum: this was accomplished by calculating the cell mass needed to achieve a starting OD_{660} between 0.05-0.1 and pelleting cells to separate cell mass. The pellets were washed with nitrogen-free media and re-pelleted, the pellets were resuspended in media taken from the photoautotrophic vials and added to vials via syringe. Samples were taken using sterile syringes and monitored by measuring OD at 660nm with the carry UV-vis spectrophotometer. Samples were taken every 1-2 days until the stationary phase. Cultures were grown at 25-30°C and illuminated with a halogen lamp; vials were placed at a constant distance from a light source to achieve a photon flux between 150-250 uM photon/m^2. Photon flux was measured with a 340-1040 quantum flux meter (Apogee Instruments, UT, United States). Storage procedures were identical to previously described.

Photoheterotrophic growths of purple non-sulfur bacteria were prepared in a similar fashion to the phototoautotrophic growths with some minor modificaitons. They were prepared with media containing acetate, hydrogen gas and bicarbonate additions were omitted. Samples were taken as previously described. Variable acetate concentration growths were prepared as described with the photoheterotrophic growth only with changes in the acetate concentration contained in the media. Acetate was added to the media to the appropriate concentration in each serum vial before autoclaving. The addition of Wolf's vitamines, sparging, inoculation, growth monitoring, and storage procedures were identical to previously described cultures.

2.3.4 Sample Analysis

Ammonia was quantified using a fluorescence-based assay previously described with minor modifications (Corbin, 1984). In 1cm cuvettes, 25μL of each sample was added to 1mL of reagent containing 3.5mM 2-mercaptoethanol, 20mM phthalaldehyde dicarboxaldehyde, 200mM potassium phosphate, and 5% (v/v) ethanol. The sample and reagent were allowed to react in the dark for 30 minutes, and fluorescence measurements were made with excitation wavelength 410nm and emission wavelength 472nm. A ammonium standard was created the same way only a series of ammonium chloride concentrations added to reagent.

Hydrogen gas quantification was conducted with a Shimadzu GC-8A gas chromatograph (Shimadzu Scientific Instruments Inc.) using a thermal conductivity detector. The injector/detector temperature was 100°C, column temperature 60°C, and argon carrier gas (135kPa). 200 μL samples from the serum vial headspace were injected into the column with a gas-tight syringe (Hamilton, NV, United States). A hydrogen standard curve was prepared by the addition of known concentrations of hydrogen into the headspace of vials filled 50% with H_2O. The peak areas from the generated chromatograms were then used to develop a linear curve to calculate the hydrogen in the headspace of the growth vials.

Due to small sample volumes collected, pH was measured using MColorpHast pH test strips, pH 6.5-10 (EMD Millipore Corporation, MA, United States). pH measurements were conducted immediately following sample collection from serum vials before OD_{660} measurements and freezing.

2.4 Results

To understand which general mode of growth would be most effective for a nitrogen-fixing system *R. palustris* TIE-1 was grown both autotrophically (with CO_2

and H_2) and heterotrophically (with acetate). Autotrophically, *R. palustris* was able to reach a maximal OD_{660} of 0.9 over the course of 30 days, heterotrophically the same strain was able to grow to an OD_{660} of 2.7 in 5 days (figure 2.1). Using the measured correlation of biomass to optical density (0.33g CDW/L/OD_{660}), we converted these values to dry cell mass. Bacterial dry cell mass typically contains 8-13% nitrogen (Rittmann and McCarty, 2001); this composition was used to determine the amount of nitrogen fixed per unit volume. Autotrophic growths were determined to produce 35mg N/L and the heterotrophic growth produces 90mgN/L. To calculate the production rate, we divide these values by the days. The autotrophic growth was found to be 1.16mg N/L/day and heterotrophic 18mg N/L/day. Heterotrophic growth with *R. palustris* shows a greater than 15-fold increase in nitrogen fixation rates relative to autotrophic.

To contrast phototrophic and chemotrophic growth we compared the growth rate of *A. vinelandii* to *R. palustris*. *A. vinelandii*, a fast-growing diazotroph, grew to an OD_{600} of 10 in 2 days (figure 2.2). This correlates to a nitrogen fixation rate of 165mgN/L/day, this rate exceeds *R. palustris* by a factor of 9. This lead us to believe that chemoheterotrophic growth, of *A. vinelandii*, may be a better candidate. However, the initial 30mM of acetate was insufficient to accommodate the entire growth requiring subsequent additions of acetate making the total consumed equal to over 180mM. Even when accounting for acetate consumption, the nitrogen fixation rate per unit of acetate with *A. vinelandii* was similar to those calculated from *R. palustris*. It was also previously demonstrated that *Azotobacter* grown on acetate has reduced levels of nitrogenase activity (Strandberg and Wilson, 1968). The literary evidence of limited nitrogenase activity and comparable fixed N per acetate consumed settled us on a photoheterotrophic growth mode as the base for the nitrogen fixation system

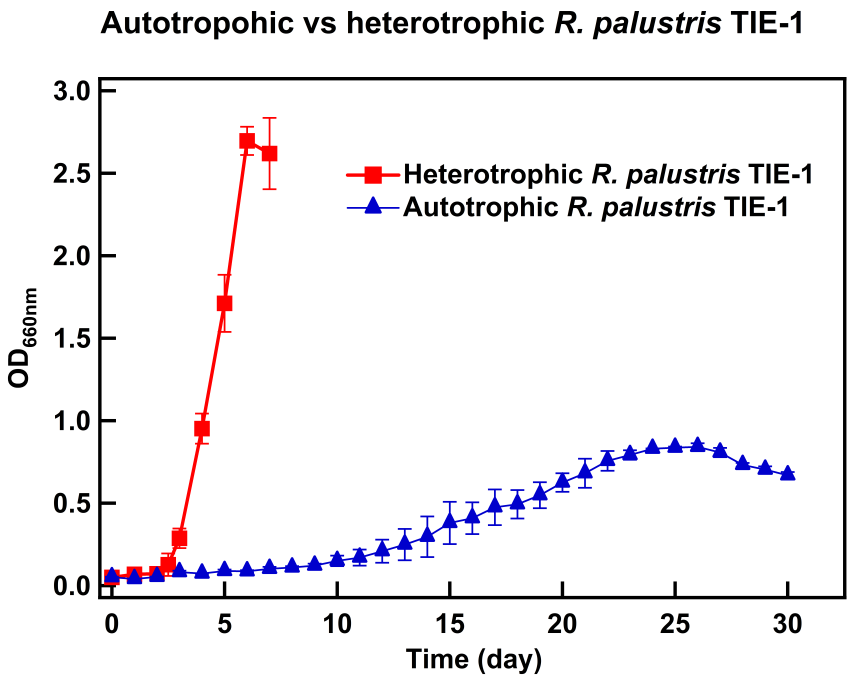

Fig. 2.1: Direct comparison of heterotrophic and autotrophic growth of *R. palustris* CGA009 NifA*.

with acetate.

Purple non sulfur species selection was conducted by growing each of the five strains simultaneously and comparing the growth rates directly (figure 2.3). *R. rubrum* grew to a maximal OD_{660} of 0.52 over the course of 4 days; *R. spheroides* grew to a similar OD_{660} of 0.59 over the 4 days; *R. capsulatus* had a faster initial growth rate and hit a maximal OD_{660} of 0.84 in 4 days; *R. palustris* NifA* reached a maximal OD_{660} of 1.8 in 4 days and TIE-1 reached an OD_{660} of 2.0 in 5 days. Converting these values into nitrogen fixation rates (table 2.1), we see that the two *R. palustris* strains nitrogen fixation rates are at least double the other purple non-sulfur strains.

The initial species selection experiments were all performed with 30mM acetate.

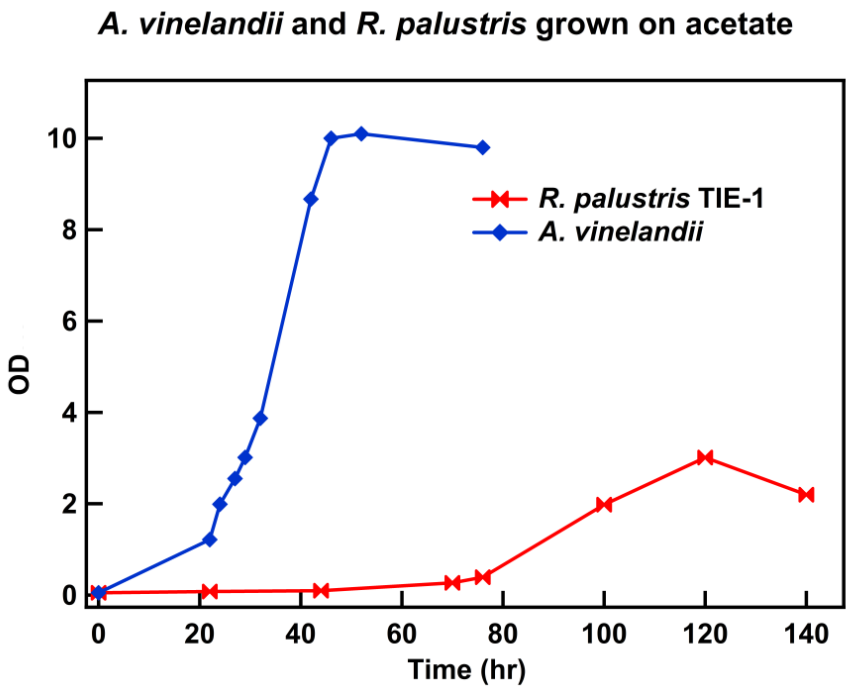

Fig. 2.2: Indirect optical density comparison growth of *R. palustris* CGA009 NifA* (measured at 660nm) and *A. vinelandii* (measured at 600nm).

This concentration was selected as a result of communications with a colleague informing us that their acetate reactor was producing acetate at this level, as well as comparison to ATCC media recipes for purple non-sulfur bacteria. Further optimization and characterization of the bacterial growth on acetate were necessary. Growths with extensive concentrations of acetate were conducted to identify the range of acetate where near-optimal growth occurred (figure 2.4). The 10, 25 and 50 mM acetate have similar growth with the 25 and 50 mM reaching the highest maximal OD within one day of each other, and a slight lag in the 50mM growth. The 100mM grew, but exhibited a 5-day lag phase as it acclimated to this high level of acetate.

The second series of variable acetate growths were performed with the acetate levels from 10-40mM. To better understand the activity of NifA* grown on acetate,

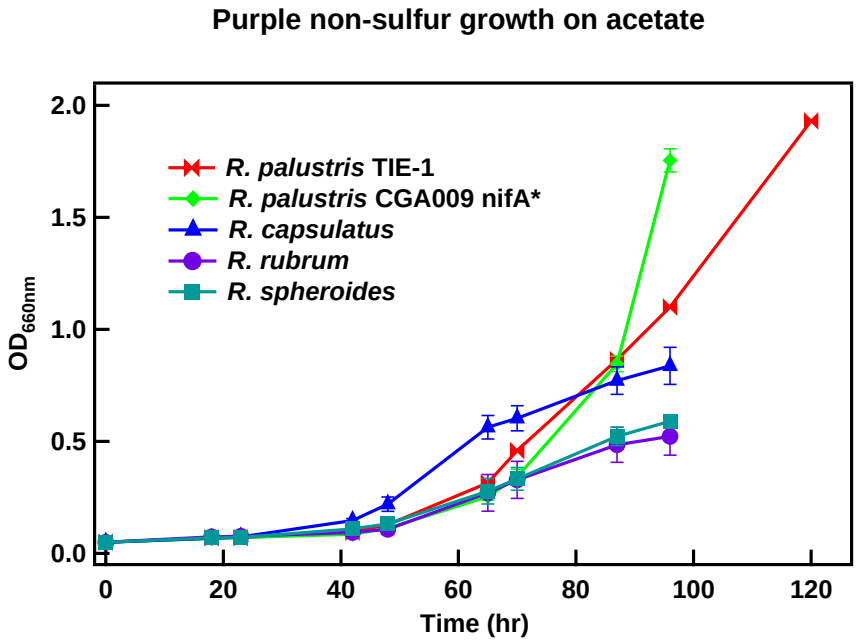

Fig. 2.3: Direct comparison of multiple PNSB species including*R. palustris* TIE-1 and CGA009 NifA*, *R. capsulatus*, *R. rubrum*, and *R. spheroides* (measured at 660nm).

in addition to optical density, we measured pH, hydrogen gas formation and extracellular ammonium. We can see from the OD_{660} measurements (figure 2.5A) that there is little to no difference between the 20-40mM acetate conditions with similar initial doubling rates, max OD, and lag times. The 40mM concentration did however reach a slightly lower maximal OD, around 75% of the maximal OD of the 20 and 30 mM growths. The 10mM has a similar initial growth rate but plateaus at a far lower cell density, around 30% of the maximal OD of the 20 and 30mM growths. These trends were also observed in the pH and hydrogen evolution measurements (Figure 2.5C and D). The combination of these results indicates that the growth kinetics for *R. palustris* are maximal with the 20 and 30mM conditions, indicating that they are saturated but not inhibited at these acetate concentrations. Acetate consumption rates were relatively comparable between initial acetate concentrations (figure2.5D). Extracellular ammonium levels were measured however none of the samples tested

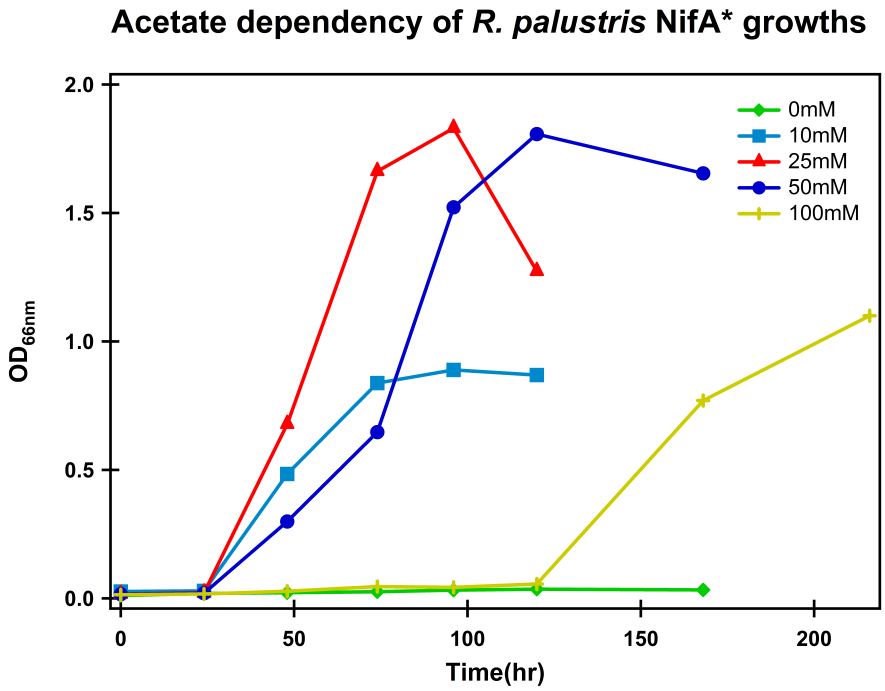

Fig. 2.4: Wide acetate concentration growth of *R. palustris* CGA009 NifA*, 0,10,25,50,100 mM starting concentrations

exhibit any detectable level of ammonia production (<100μM).

2.5 Discussion

Nitrogen reduction is essential for life support of a Martian research mission; however, for a feasible nitrogen-fixing reactor we would need to minimize our input requirements as well as our reactor size. In our first attempt to design the most efficient system, we focused on utilizing photoautotrophic bacteria. Photoautotrophic growth was appealing due to the energy capture capacity of the photosystem and the lack of high-value carbon requirements; however, underestimated the energetic competition that carbon fixation would have on the nitrogen reduction process, as the growth rates were 15 times larger heterotrophically when compared to autotrophic.

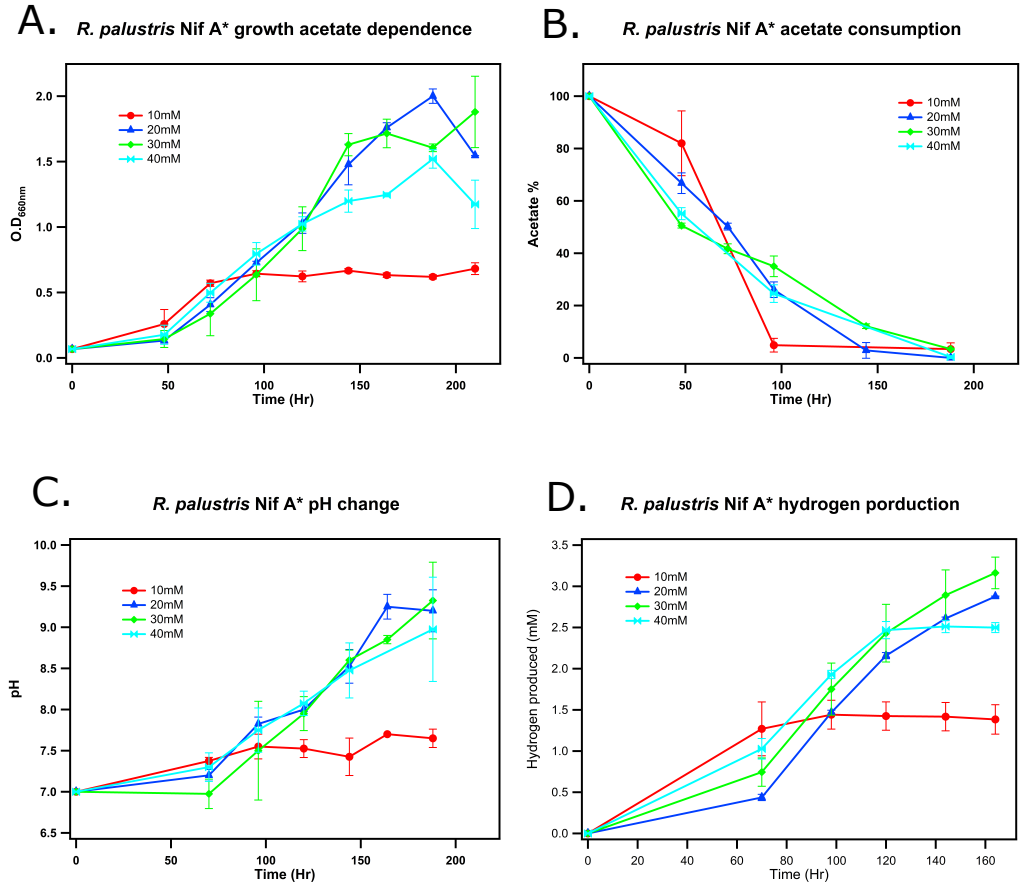

Fig. 2.5: In depth optimal acetate characteristics of *R. palustris* CGA009 NifA* growths, 10-40mM inintial acetate concentrations with multiple regular measurements of growth characteristics through the entire growth period. A: optical density measured at 660nm, B: percent extracellular acetate, C: pH changes, D: Hydrogen in headspace.

An autotrophic approach resulted in an unrealistic reactor volume required to satisfy the nitrogen demand increasing the mission burden, even when considering the consumption of readily available CO_2 compared to a higher-value acetate. Even though growth rates clearly indicate problems with an autotrophic reactor, when compared to heterotrophic growth, this measure alone is insufficient for selecting a microbial growth mode. Analysis of chemical input as well as growth rates indicate that there are benefits to phototrophic growth, even though it has a lower growth rates. This

is illustrated with the comparison of *A. vinelandii* to *R. palustris*. Fixation rates alone indicate a nine-fold advantage of reactor size with *Azotobacter*, but this is accompanied by a significant increase in acetate input, resulting in an increased mission burden. These calculations have led us to focus on PNSB as the bacteria for a mission critical nitrogen fixation reactor.

Of our PNSB, *R. palustris* is the optimal bacteria as it has higher growth rates while consuming the same amount of acetate per nitrogen reduced. As we narrow on the bacterial strain, it is helpful to consider the changes in the cellular nitrogen content through the growth phase. Nitrogenase expression and activity is typically regulated as a result of the ammonium levels within the cell (Masepohl et al., 2002). *NifA* is a transcriptional regulator of nitrogenase that is repressed as the ammonium levels increase, a mutation in this gene has been shown to reduce the sensitivity of a bacteria to ammonium levels (Adessi et al., 2012). Of the two *R. palustris* strains, the NifA* mutant has the largest upside in a nitrogen-fixing reactor due to the potential of fixing nitrogen in higher external ammonia environments. When we look at the hydrogen production in these two strains, we see higher levels of hydrogen generated with the CGA009 NifA* strain which is indicative of higher nitrogenase activity. It was also observed that *R. palustris* CGA009 NifA* has an increased nitrogen composition often exceeding 17%, where the wild type strain is typically 13% (data not shown).

Growth measurments of NifA* from experiments conducted with varying acetate concentrations indicate that optimal acetate concentration is 20-30mM. Even though cultures grew with acetate exceeding 30mM, they either exhibited increased lag phase, or reduced maximal OD, when compared to 30mM. Even though the growth was sub optimal, this shows the potential of NifA* to adapt to higher acetate levels if the more extensive system requires them to do so. Lower acetate concentrations bellow

20mM would support growth, if the acetate supply was limited and acetate generation was only capable of achieving low levels. However, this is unlikely as it is has been demonstrated that acetogens are capable of producing acetate levels exceeding 20mM (Batlle-Vilanova et al., 2016; Jourdin et al., 2016). Acetate generating bioreactors typically experience a decrease in pH from the high levels of acetic acid formation. This could potentially be mitigated by direct co-culture of acetogens with nitrogen-fixing bacteria. In a nitrogen-fixing reactor nitrogenase converts hydrogen ions to hydrogen gas increasing the pH; these opposing pH trends may balance the pH to a level optimal for both bacteria in a co-cultured reactor.

The central motivation for nitrogen fixation within natural and artificial nitrogen cycles is the use of fixed nitrogen as a biofertilizer for plant growth. A large portion of prior biofertilizer research has been done by isolating bacteria from specific plant species root zones (Gamal-Eldin and Elbanna, 2011; Y. Hafeez et al., 2006). Applications of these bacteria result in robust plant growth but are often not capable of acting as a biofertilizer with broad plant species application. Here we address the first step toward a ubiquitous biofertilizer by identifying the most effective growth modality for future bioreactor design. Future work will be necessary to facilitate the use of photoheterotrophically grown *R. palustris* NifA* biomass as a nitrogen source for plant/bacteria growth. However, this study shows promise in the capacity for a feasible biofertilizer reactor for life support of a Martian research base.

2.6 Acknowledgements

This material is based upon work supported by NASA under grant or cooperative agreement award number NNX17AJ31G/NNH16ZOA001N-16STRII_B3. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National

Aeronautics and Space Administration (NASA).

I would like to thank Mathangi Soundararajan and Anna Doloman for their thoughts and direction while designing experiments and communicating results.

REFERENCES

Adessi, A., McKinlay, J. B., Harwood, C. S., and De Philippis, R. (2012). A Rhodopseudomonas palustris nifA* mutant produces H2 from NH4+-containing vegetable wastes. *International Journal of Hydrogen Energy* 37, 15893–15900. doi:10.1016/j.ijhydene.2012.08.009

Batlle-Vilanova, P., Puig, S., Gonzalez-Olmos, R., Balaguer, M. D., and Colprim, J. (2016). Continuous acetate production through microbial electrosynthesis from CO2 with microbial mixed culture. *Journal of Chemical Technology & Biotechnology* 91. doi:10.1002/jctb.4657

Canfield, D. E., Glazer, A. N., and Falkowski, P. G. (2010). The Evolution and Future of Earth's Nitrogen Cycle. *Science* 330. doi:10.1126/science.1186120

Chen, C.-Y., Lee, C.-M., and Chang, J.-S. (2006). Feasibility study on bioreactor strategies for enhanced photohydrogen production from Rhodopseudomonas palustris WP3-5 using optical-fiber-assisted illumination systems. *International Journal of Hydrogen Energy* 31, 2345–2355. doi:10.1016/j.ijhydene.2006.03.007

Corbin, J. L. (1984). Liquid Chromatographic-Fluorescence Determination of Ammonia from Nitrogenase Reactions: A 2-Min Assay. *Applied and Environmental Microbiology* 47

Drake, H. L., Gößner, A. S., and Daniel, S. L. (2008). Old acetogens, new light. *Annals of the New York Academy of Sciences* doi:10.1196/annals.1419.016

Erisman, J. W., Galloway, J. N., Dise, N. B., Sutton, M. A., Bleeker, A., Grizzetti, B., et al. (2015). *Nitrogen : too much of a vital resource : Science Brief*. Tech. rep., WWF Netherlands, Zeist, The Netherlands

Gamal-Eldin, H. and Elbanna, K. (2011). Field Evidence for the Potential of Rhodobacter capsulatus as Biofertilizer for Flooded Rice. *Current Microbiology* 62, 391–395. doi:10.1007/s00284-010-9719-x

Gildemyn, S., Verbeeck, K., Slabbinck, R., Andersen, S. J., Prévoteau, A., and Rabaey, K. (2015). Integrated Production, Extraction, and Concentration of Acetic Acid from CO2 through Microbial Electrosynthesis. *Environmental Science & Technology Letters* 2. doi:10.1021/acs.estlett.5b00212

Harris, D. F., Lukoyanov, D. A., Shaw, S., Compton, P., Tokmina-Lukaszewska, M., Bothner, B., et al. (2018). Mechanism of N2 Reduction Catalyzed by Fe-Nitrogenase Involves Reductive Elimination of H2. *Biochemistry* 57. doi:10.1021/acs.biochem.7b01142

Hecht, M. H., Hoffman, J. A., and Moxie Team (2016). The Mars Oxygen ISRU Experiment (MOXIE) on the Mars 2020 Rover 1980

Hoffman, B. M., Lukoyanov, D., Yang, Z.-Y., Dean, D. R., and Seefeldt, L. C. (2014). Mechanism of Nitrogen Fixation by Nitrogenase: The Next Stage. *Chemical Reviews* 114. doi:10.1021/cr400641x

Jourdin, L., Freguia, S., Flexer, V., and Keller, J. (2016). Bringing High-Rate, CO2-Based Microbial Electrosynthesis Closer to Practical Implementation through Improved Electrode Design and Operating Conditions. *Environmental Science & Technology* 50. doi:10.1021/acs.est.5b04431

Kannaiyan, S., Aruna, S. J., Merina Prem Kumari, S., and Hall, D. O. (1997). Immobilized cyanobacteria as a biofertilizer for rice crops; Intl. Conference on Applied Algology, Knysna, South Africa, April 1996. *Journal of Applied Phycology* 9, 167–174. doi:10.1023/A:1007962025662

Kaufmann, N., Reidl, H.-H., Golecki, J. R., Garcia, A. F., and Drews, G. (1982). Differentiation of the membrane system in cells of Rhodopseudomonas capsulata after transition from chemotrophic to phototrophic growth conditions. *Archives of Microbiology* 131, 313–322. doi:10.1007/BF00411178

Li, C. W., Ciston, J., and Kanan, M. W. (2014). Electroreduction of carbon monoxide to liquid fuel on oxide-derived nanocrystalline copper. *Nature* 508. doi:10.1038/nature13249

MacKay, B. A. and Fryzuk, M. D. (2004). Dinitrogen Coordination Chemistry: On the Biomimetic Borderlands. *Chemical Reviews* 104. doi:10.1021/cr020610c

Mangiapia, M. and Scott, K. (2016). From CO2 to cell: energetic expense of creating biomass using the Calvin–Benson–Bassham and reductive citric acid cycles based on genome data. *FEMS microbiology letters* 363

Masepohl, B., Drepper, T., Paschen, A., Groß, S., Pawlowski, A., Raabe, K., et al. (2002). Regulation of nitrogen fixation in the phototrophic purple bacterium Rhodobacter capsulatus. *Journal of molecular microbiology and biotechnology* 4, 243–248

McKinlay, J. B. and Harwood, C. S. (2010). Carbon dioxide fixation as a central redox cofactor recycling mechanism in bacteria. *Proceedings of the National Academy of Sciences* 107. doi:10.1073/pnas.1006175107

Menezes, A. A., Cumbers, J., Hogan, J. A., and Arkin, A. P. (2015). Towards synthetic biological approaches to resource utilization on space missions. *Journal of The Royal Society Interface* 12. doi:10.1098/rsif.2014.0715

Patil, S. A., Arends, J. B. A., Vanwonterghem, I., van Meerbergen, J., Guo, K., Tyson, G. W., et al. (2015). Selective Enrichment Establishes a Stable Performing Community for Microbial Electrosynthesis of Acetate from CO2. *Environmental Science & Technology* 49. doi:10.1021/es506149d

Ripatti, D. S., Veltman, T. R., and Kanan, M. W. (2019). Carbon Monoxide Gas Diffusion Electrolysis that Produces Concentrated C2 Products with High Single-Pass Conversion. *Joule* 3. doi:10.1016/j.joule.2018.10.007

Rittmann, B. E. and McCarty, P. L. (2001). *Environmental Biotechnology: Principles and Applications* (McGraw-Hill Education: New York, Chicago, San Francisco, Athens, London, Madrid, Mexico City, Milan, New Delhi, Singapore, Sydney, Toronto)

Strandberg, G. W. and Wilson, P. W. (1968). Formation of the nitrogen-fixing enzyme system in Azotobacter vinelandii. *Canadian Journal of Microbiology* 14

Y. Hafeez, F., Yasmin, S., Ariani, D., Renseigné, N., Zafar, Y., and A. Malik, K. (2006). Plant growth-promoting bacteria as biofertilizer. *Agronomy for Sustainable Development* 26

CHAPTER 3
DEVELOPING A BROAD APPLICATION BIOFERTILIZER FROM *RHODOPSEUDOMONAS PALUSTRIS* BIOMASS

3.1 Abstract

As the world's population increases so does the demand for agricultural food production. To meet this demand more land is converted to agricultural land and aggresive fertilization strategies are conducted to increase crop production. inorganic nitrogen fertilizers are effective for plant growth, but they have severe environmental impacts as they contaminate surrounding areas, are highly toxic to aquatic environments, and reduce natural plant and microbe diversity. One approach to address these issues is to use organic matter rich in nitrogen as a biofertilizer to support food production as these typically have reduced ecological impacts. Here we identify the feasibility of *Rhodopseudomonas palustris* CGA009 NifA* as a ubiquitous biofertilizer supporting plant growth in multiple cultivars. The fertilization method was the first hurdle as adding the biomass directly to the substrata would result in at least 30% of the biomass being washed out of the system as well as uncontrolled nitrogen feed rates to the plant root zone. Acidification of the biomass stablized the nitrogen so it could be directly added to the nutrient solution allowing for a more measurable feed rate of nitrogen, and reducing nitrogen loss in the system. Germination screening was performed to identify plant species compatibility as many biofertilizers exhibit cultivar specific interactions. Seedling germination tests suggest that acidified biomass may stimulate plant growth relative to a nitrogen-free nutrient solution; however, there is still some level of growth inhibition with specific plant species when compared to

inorganic nitrogen fertilizers. Understand the full effects of *R. palustris* biomass on plant growth would require long term growth experiments to see if this inhibition is significant to adult plants.

3.2 Introduction

With an increasing population, the worldwide food demand has dramatically risen, resulting in increases in the amount of land converted into agricultural areas and the need for higher crop yield within established agriculture. Most current agricultural strategies require inorganic nutrient suplimentation to create and sustain soil capable of supporting global food demand. These strategies often result in reduced ecological diversity, as well as nutrients washing out into surrounding aquatic environments, where they exhibit high levels of toxicity to plant and animal life. (Crawley et al., 2005; Vitousek et al., 1997). Simulations show that using current practices and conventional fertilizers will have devastating environmental impacts in the coming decades (Tilman et al., 2011). Fertilization management strategies are being explored in an attempt to reduce fertilizer mobility (Malhi et al., 2001; Tagliavini et al., 1996), but there will need to be technological advances with less detrimental fertilizers to help limit the future impacts. Using biofertilizers as a replacement of, or supplement to, inorganic fertilizer, is a possible solution to these problems. In addition to essential nutrients such as nitrogen, potassium and phosphorus; biofertilizers are rich in plant growth-promoting compounds, such as carotenoid pigments, vitamines, and phytohormones (Sakarika et al., 2019). When compared to inorganic fertilizers, Biofertilizers tend to have reduced environmental impacts as they are less mobile and show lower levels of toxicity. (DaSilva et al., 1992; Rao, 1982).

Agricultural nitrogen run-off is one of the worst contributors to fertilizer toxicity

and contamination of surrounding ecosystems; there are three forms of nitrogen in conventional fertilizers; ammonia, nitrates, or urea. These compounds alone have been linked to significant decreases in biodiversity, as well as exhibiting persistence in environments, often detected for decades (Crawley et al., 2005; Heinsoo et al., 2020). These problems are not application site-specific; inorganic nitrogen has shown high levels of mobility as it unintentionally leaches into surrounding areas (Vitousek et al., 1997). Inorganic nitrogen fertilizer production consumes nearly 2% of the global energy as well as contributing significantly to atmospheric polution through emission of nitrogen oxides and CO_2 (Erisman et al., 2015). Biological fertilizers are capable of supplying nitrogen to plants while mitigating much of the environmental impact, as they are often sustainably produced, and the decomposition rate of these organic nutrients is slower when compared to soluble inorganic fertilizers, and depends on the soil microbial activity (DaSilva et al., 1992; Sakarika et al., 2019). Biofertilizer manufacturing, utilizing nitrogen-fixing bacteria, may be capable of reducing the use of traditional inorganic fertilizers, resulting in restored plant diversity and reduced contamination of surrounding ecosystems (Malhi et al., 2001). However, there are many unknown factors with the use of biofertilizers, such as general plant application, production efficiencies, or specific fertilization behaviors in different environments.

The development and application of biofertilizers are as diverse as the organisms that fix nitrogen themselves. Nitrogen-fixing bacteria occur as both plant symbiotic and free-living (Canfield et al., 2010). Symbiotic nitrogen-fixing bacteria are often found with plant and bacteria species-specific associations making them non-feasible for wide agricultural applications. Free-living bacteria exist in various environments and are not dependent on plant associations, increasing the possibility for biofertilizer production within this nitrogen-fixing class (Jha and Prasad, 2006; Tripathy and Ayyappan, 2005). Purple non-sulfur bacteria (PNSB) are phototrophic nitrogen-

fixing bacteria with a versatile metabolism, allowing these organisms to be distributed across the globe. There are many examples of PNSB being utilized as a biofertilizer, with the majority being applied as indirect (living cultures) to actively supply nutrients to the plants (Sakarika et al., 2019). Although indirect application is plausible, understanding the nutrient dosage is difficult due to the lack of growth control. Direct fertilization (dead cell mass) allows for metered control of the nitrogen content added because it can be directly measured before addition to plants. Many PNSB biofertilizer characterization has been done with rice as the cultivar, as rice paddy fields are one of the most common locations of PNSB isolation (Elbadry et al., 1999; Gamal-Eldin and Elbanna, 2011; Kantachote et al., 2016; Maudinas et al., 1981). In addition to rice studies, other cultivars have been tested; however, few studies have utilized the same bacterial strain, bio-fertilization mode (direct or indirect), or growth condition (Sakarika et al., 2019). Comparison of these trials to identify cultivar-specific compatibility difficult, due to this high level of variance between studies, illustrating a need for a more universal application process. Here we investigate the universal application of acidified *Rhodopseudomonas palustris* nifA* biomass as a sole nitrogen source for multiple plant species.

3.3 Materials and methods

3.3.1 Media composition

R. palustris CGA009 NifA* mutant strain was used in all our experiments. The bacteria was grown in a minimal salts media consisting of 12.5mM K_2HPO_4 and 12.5mM KH_2PO_4 buffering components, a concentrated base solution at a final concentration of 0.1% (v/v), Wolf's vitamins at a 0.5% (v/v), and 30mM acetate as

the carbon source. [Concentrated base stock solution (mM concentrations): nitrilotriacetic acid (105), $MgSO_4$ (240), $CaCl_2$-$2H_2O$ (45), Na_2MoO_4-$2H_2O$ (0.78),$FeSO_4$-$7H_2O$ (2.5), EDTA (0.86), $ZnSO_4$-$7H_2O$ (3.81), $MnSO_4$-H_2O (0.91), $CuSO_4$-$5H_2O$ (0.15) $Co(NO_3)$2-$6H_2O$ (0.085), Na_2B4O7-$10H_2O$ (0.046)]. [Wolfe's Vitamins stock solution [containing (g/L): p-aminobenzoid acid (0.005), folic acid (0.002), lipoic acid (0.005). Riboflavin (0.005), thiamine (0.005), nicotinic acid (0.005), pyridoxamine (0.01), pantothenic acid (0.005), cobalamin (0.0001), biotin (0.002)].

3.3.2 Photobioreactor operation

For bioreactor growth experiments, the R.palustris nifA* media was autoclaved first, aseptically pumped into the vessel, and flushed with N_2:CO_2 (80:20) mixture for 2 hours. Cultures were grown at 30±1°C with 3x6 fluorescent lamps, 17 W each, delivering a constant photon flux of 340 mol / m^2s (wavelength of 340 - 1040 nm). The 3L photobioreactor was inoculated with 5% (v/v) of late-exponential phase grown *R.palustris* nifA* culture and operated in a fed-batch mode for 3 weeks. The pressure inside the reactor was kept at 1.22 atm, with pressure CO_2= 0.24atm and pressureN_2= 0.98 atm. The gas outlet was kept open throughout the operation to eliminate pressure build-up due to the production of H_2 gas. Bacterial biomass was harvested in the mid-late exponential growth phase by centrifugation for 45 min in (10,500 rpm, Sorvall Lynx 400 centrifuge with Fiberlite F10 rotor, Thermo Scientific, MA, United States). Harvested biomass was stored at -80°C until further use as a biofertilizer in germination study.

3.3.3 Biomass testing and treatment

Ammonium measurements were made using a fluorescence-based assay previously described with few modifications (Corbin, 1984). Total nitrogen was measured using the HACH total nitrogen high range test kit based on the persulfate digestion method 10072 (HACH, CO, United States). Some dilutions were made so the anticipated nitrogen content of the biomass was between 2-150mgN/L.

Leachate columns were made with 2.5 inch diameter PVC pipe with a small mesh screen on the bottom to hold in the substrata. Each column was filled with 300 mL of dry washed substrata. Substratas tested were coarse sand, ultra fine sand, calcined clay, and diatomaceous earth. The biomass suspension had 0.3g of wet *R. palustris* cells suspended in 50mL water. The suspension was poured on the dry substrata, and each column was allowed to sit for 1 hour before water was poured through the columns until 50mL of flow-through was collected, and subsequent water washing was performed daily. Flowthrough was measured for optical density at 660nm (Cary 50 UV-vis, Varian instruments, CA, United States), electrical conductivity (DiST3 electrical conductivity tester, Hanna Instruments, RI, United States), and pH (FiveEasy pH meter, Mettler Toledo LLC, OH, United States).

Biomass acidification tests were conducted with 10mM solution of sulfuric acid which was used to resuspend biomass paste to a concentration of 2g/L of N. This concentrate was then diluted with sulfuric acid to 75mg/L and added to serum vials and sealed with butyl rubber stoppers and incubated for 2 weeks at room temperature. Half of the vials were shaken, and the others were stagnant throughout the two weeks. pH was monitored intermittently, and ammonium was measured as previously described at day 0 and day 14. Biomass preparation for plant treatments was created by resuspending cell mass in a 1mM sulfuric acid solution, the concentrate (6.9 gN/L) was then stored in the refrigerator until it was used.

3.3.4 Germination test

Acidified *R. palustris* nifA* biomass with a stock concentration of 6.9 g/L of N was used to prepare nutrient solutions for plant growth with varying concentrations of N: 650 mg/L, 130 mg/L, 13 mg/L, and 1.3 mg/L. The hydroponic nutrient solution for plants was prepared with the following formulation (in mM): $CaSO_4$*$2H_2O$ (1), K_2SO_4 (1), KH_2PO_4 (0.2), $MgSO_4$ (0.5), K_2SiO_3 (0.3), $FeCl_3$ (5), EDDHA (20), $MnCl_2$ (2), $ZnCl_2$ (3), H3BO_3 (40), $CuCl_2$ (2), Na_2MoO_4 (0.1). For nitrate-containing solution, positive control treatment, $Ca(NO_3)_2$, and KNO_3 were used instead of correspondent sulfates, to yield 84 mg/L of N in the final solution.

Nitrogen from acidified bacterial biomass was tested for the potential inhibitory effect on the seeds of five cultivars: Romaine lettuce (*Lactuca sativa*, var. Parris island), tomato (*Solanum lycopersicum*, var. Early girl), wheat (*Triticum aestivum* L, var. Apogee), barley (*Hordeum vulgare*), and cucumber (Cucumis sativus, var. Straight 8). Germination was conducted on cellulose germination paper in plastic germination boxes, with two-three seed types per box, depending on the seed size. Eight to ten seeds per plant cultivar were placed in each germination box for each of the six nitrogen treatments: NO_3-N (84 mg/L), 650 mg/L (biofertilizer), 130 mg/L (biofertilizer), 13 mg/L (biofertilizer), 1.3 mg/L (biofertilizer) and no N control. Seeds were watered with the correspondent nutrient solution every other day (20 ml per germination box) and kept in a stable temperature room at 20±2°C, under 12 hrs of illumination with 200μmol/m^2s of photons. Seedlings were harvested when shoots reached the top germination box cover (7-12 days).

Percent germination was calculated as a total number of seeds that developed shoots divided by the total number of seeds placed in the germination box of that

same cultivar. Wet weight of shoots (g) and shoot length (cm) were used as a qualitative means to assess the effect of using *R. palustris* nifA* biomass as a biofertilizer and a source of nitrogen. Statistical analysis of the differences in the resulting shoot lengths and shoot wet weights, compared to the no nitrogen control, were conducted with student T-test, assuming two-tailed distribution and two-sample unequal variance. The threshold for significant difference between two sets of data was set to 5% ($p<0.05$).

3.4 Results

3.4.1 Biomass preparation and soil addition

Substrata retention of biomass was tested in leachate columns with various substratas (coarse sand, fine sand, diatomaceous earth, and calcined clay). Biomass was added to the substratas as described in the Methods section, and optical density at 660nm was used to estimate the amount of biomass, that was washed from the columns, in the flow-through. Ratios of OD_{660} of flow-through were compared to the initial solution to calculate biomass loss percentages, which are provided in table 3.1 . We see that there is significantly more biomass retained in the diatomaceous earth and calcined clay relative to the sand substratas; however, there is still a considerable amount of biomass leaching out of the calcined clay and diatomaceous earth substratas. pH results showed that the highest retention substratas, diatomaceous earth and calcined clay, had the most extreme pH values 8.85±0.12 and 7.17±0.32, both sand substratas averaged near 7.9 which was similar to the pH of the washing solution (table 3.1). Electrical conductivity showed no conserved trends relative to retention. Upon performing a total nitrogen test of the initial solutions, it was observed that a

significant loss of nitrogen had occurred.

Substrate	Initial loss %	Subsequent daily loss%	Average pH of flow through
Diatomaceous Earth	38.7±4.8	11.1±2.0	8.8±0.1
Fine Sand	76.5±5.6	3.5±1.47	7.8±0.1
Coarse sand	64.23±3.3	5.25±1.5	7.9±0.05
Calcined Clay	17.6±2.1	3.26±0.6	7.2±0.3

Table 3.1: Biomass retention and pH measurements for solid substratas, diatomaceous earth, fine sand coarse sand and calcined clay, in leachate columns. % loss was calculated by measuring the OD of the flowthrough and dividing by the initial optical density of cells added to each column, converted to a percentage.

Acidification of biomass was done to address an alternative means of incorporating biomass to the growth system, and stabilizing nitrogen in solution. We wanted to see if we could develop a solution containing nitrogen derived from biomass that could be added to a watering solution and poured onto the plants to limit loss. We first acidified the biomass as indicated in the methods and let the solution mature. pH was monitored intermittently as a potential indicator of degradation and was stable in all vials throughout the maturation process. The most drastic change in the vials was the color, which gradually changed from a deep red to a pale brown in about a week. The only contrast between the shaking and stagnant vials was the settling of the degraded products, but the stagnant solutions resembled the shaken with gentle agitation. Ammonium levels were measured before and after experiment, and at no point were there any detectable levels of ammonia. Total nitrogen was also measured pre and post and showed no nitrogen loss as the pre and post-measurements were the same. These measurements and observations of the acidified biomass indicate, that there is some level of degradation, as observed in the solution color change, and the

nitrogen is stable in solution. However, as we did not see any detectable levels of ammonia, we are unclear of what form or nitrogen is in the acidified product.

3.4.2 Germination trials

Seeds of five different plant cultivars (tomato, wheat, cucumber, lettuce, and barley) were tested for their germination response in the presence of acidified bacterial biomass. Acidified biomass of *R. palustris* nifA*, grown in a 3L photobioreactor under fluorescent lights, was used as a biofertilizer for the germination trials and was applied to the plant seeds as described in the Methods section. The results of plant seedlings germination with varying concentrations of bacterial biomass applied, expressed in the mg/L of N, are available in table3.2. As can be noted from Table 3.2, there was no differential inhibition of plant germination rates with any of the applied biofertilizer N concentrations. The cucumber seeds demonstrated a varying degree of germination rate in all of the applied biofertilizer concentrations, but there is no indication that a specific biofertilizer amount is inhibitory to the seedling germination.

Treatments	Germination, %				
	Barley	Apogee wheat	Cucumber	Lettuce	Tomato
NO_3^--N, 84 mg/L	100	87.5	80	100	90
650 mg/L of N	87.5	100	90	100	90
130 mg/L of N	100	100	100	90	80
13 mg/L of N	100	100	90	100	90
1.3 mg/L of N	100	87.5	87.5	100	100
No N	100	100	70	100	90

Table 3.2: Percent germination of the tested plant cultivars with varying nitrogen source in the watering nutrient solution.

The effect of various biofertilizer concentrations on the resulting seedlings shoot wet weight and shoot length (Figure 3.1 and 3.2). The wet shoot weight for all cultivars was compared to the no nitrogen, NO_3-N, control (Figure 3.1). In all cases, the application of NO_3-N yielded increased shoot weight, but only for barley, cucumber, and lettuce was the difference statistically significant relative to the negative (no-N) control application. Despite the high variability of the collected shoot weight results among all cultivars, a prominent inhibitory effect can be noted from additions of biofertilizer at nitrogen concentrations of 650 mg/L. Wheat, cucumber, and lettuce were more negatively affected by higher concentrations of biofertilizer when compared to the other cultivars (tomato and barley). In general, biofertilizer concentrations of 13 mg/L and 1.3 mg/L of N were non-inhibitory and had an overall positive effect on seed germination.

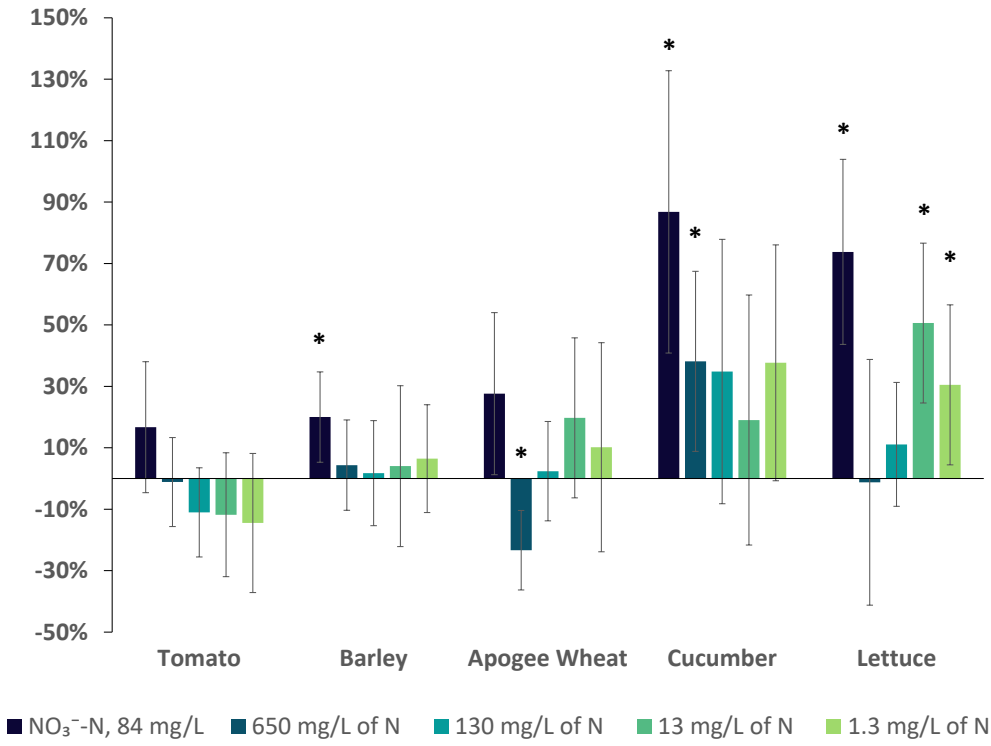

Fig. 3.1: Changes in the shoot wet weights of the plant seedlings compared to the negative, no nitrogen control treatment group. Plant seedlings were germinated with varying nitrogen source in the watering nutrient solution (NO3-N 84 mg/L, Organic (biomass) N at concentrations of 650 mg/L, 130 mg/L, 13 mg/L and 1.3 mg/L). Shoot wet weight was measured at the end of the germination study, after 7 days. (*) denotes statistical significance ($p<0.05$).

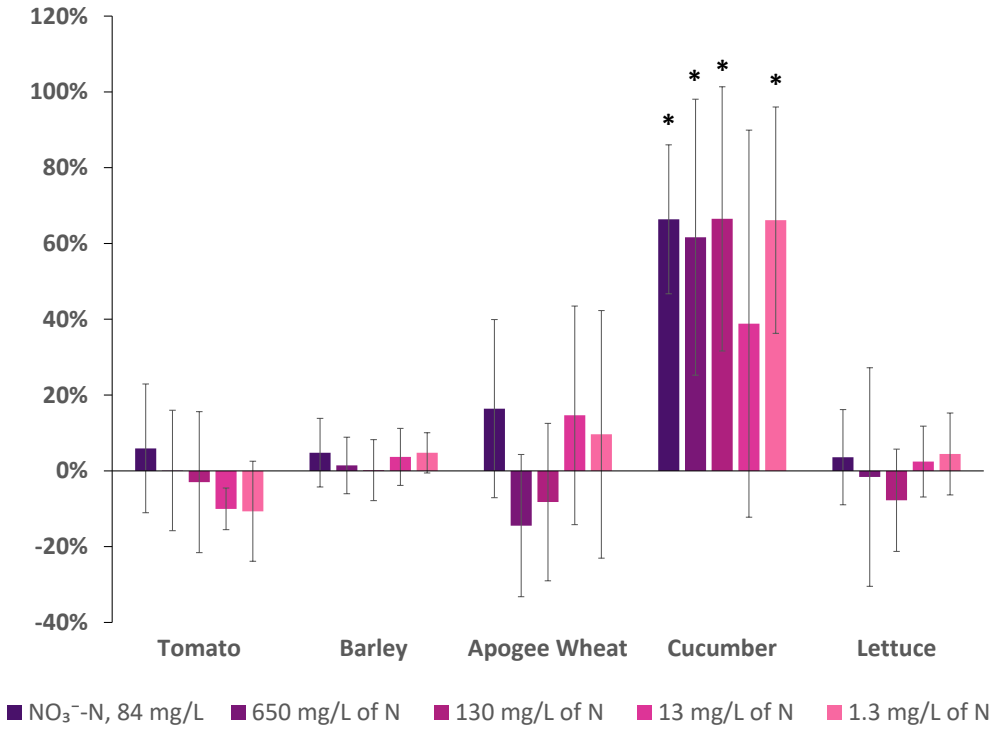

Fig. 3.2: Changes in the shoot lengths of the plant seedlings compared to the negative, no nitrogen control treatment group. Plant seedlings were germinated with varying nitrogen source in the watering nutrient solution (NO_3-N 84 mg/L, Organic (biomass) N at concentrations of 650 mg/L, 130 mg/L, 13 mg/L and 1.3 mg/L). Shoot length was measured at the end of the germination study, after 7 days. (*) denotes statistical significance ($p<0.05$).

Measuring shoot length, on the other hand, shows that this part of the plant is less indicative of the fertilizer effects (figure 3.2). Cucumber seeds were the only ones that demonstrated a statistically significant positive difference from any of the applied biofertilizer amounts. Unfortunately, we were not able to analyze the root characteristics of the germinated seedlings: all the roots were highly intertwined, and separation of individual plant roots was not possible. Full growth plant trials with varying concentrations of biofertilizer will yield more detailed comparison data, and both roots and shoots will be available for the length and weight analysis.

3.5 Discussion

The utilization of diazotrophs for the production of biofertilizers is not a new concept as blue-green algae have shown to act as a biofertilizer as early as 1955 (Allen and Arnon, 1955). Recently biofertilizer research has grown due to increased environmental awareness of the problems that arise with over-fertilization by inorganic ammonia (Schröder, 2014). Many diazotrophs have shown promise as possible biofertilizers, often being used to increase plant growth in controlled lab environments. Much of the existing research investigates bacterial nutrient supply to a limited number of plant species. To our knowledge, this study is the first demonstration of an extensive screening of various plant cultivars for the effect of using acidified PNSB biomass as a biofertilizer.

The results from the leachate column tests were the primary motivation for acidification as a pretreatment step, as biomass is difficult to incorporate directly into solid substratas. Nitrogen dosage with total biomass added at a single point would also be difficult to monitor, as biomass degradation rates are dependent on microbial soil composition which vary significantly across ecosystems (Alef and Kleiner, 1986; Fierer et al., 2009). If degradation is too slow, the plant would be limited in available nitrogen, and growth stunted. If degradation is too fast ammonia levels will rise quickly and may become toxic for plant growth (Vines and Wedding, 1960). Acidification shows promise to address this as it has some level of degradation while keeping all nitrogen in solution. This acidified biomass concentrate can then be added to nutrient solutions in a controllable fashion, reducing toxicity effects. One caveat with simple acidification is that there was no degradation of organic nitrogen to ammonium, meaning the nitrogen was still likely contained in proteins or amino

acids and will require microbial ammonification in the soil before plants can utilize the nitrogen (Ladd and Jackson, 2015). Plant response to this microbial action is yet to be investigated, but some research suggests that this may result in metabolic accumulation or increases in nitrogen use efficiency (Rouphael et al., 2020).

The germination results presented can be interpreted in a number of ways, based on the effective chemical form of nitrogen present. Ammonia or organic nitrogen forms will differentially affect plant growth, with high ammonium levels having a negative effect on plant germination (Barker et al., 1970; Bittsánszky et al., 2015; Esteban et al., 2016; Goyal and Huffaker, 2015). The nutrient solution used as a positive control in this study contained NO_3-N, which will in turn also have a different effect, likely stimulating plant germination (Fawcett and Slife, 1978; Hendricks and Taylorson, 1974). If plant growth trials indicate inhibition by biomass but not ammonium, further investigation of the form of nitrogen of acidified biomass may be necessary.

Careful examination of the obtained germination results does not seem to suggest ammonium toxicity through the germination stage of growth. None of the plants demonstrated any significant decreases in shoot length, with regards to the added acidified PNSB biomass, though this is common for plants under ammonium stress (Goyal and Huffaker, 2015). There were also no significantly different germination percentages among the cultivars with varying dosages of PNSB biomass. Significant inhibition of germination with the addition of NH_4OH at the concentrations of 1 g/L of N has previously been reported (Bremner and Krogmeier, 1989); however, this concentration was not tested in our study as this concentration is ten times higher than normal plant dosage. Ammonium toxicity, if present, might be more evident with full plant growth trials, as seedlings grow and deplete the seed-stored nitrogen.

Since there is no literary data describing the effects that mildly acidified or hy-

drolyzed bacterial biomass can have on plant growth or germination, the closest we can compare to is plant protein hydrolyzate (PH) (Calvo et al., 2014; du Jardin, 2015). Plant hydrolyzate is used as a biostimulant with beneficial effects in plant growth, and promotion of plant-associated microbiome activity (Colla et al., 2017). This hydrolyzate consists of peptides and free amino acids and is obtained through enzymatic proteolysis of plant biomass at temperatures lower than 60°C. Partially, we can anticipate some similar composition in our *R. palustris* hydrolysate, even though we used sulfuric acid at room temperature (22±2°C) for the hydrolysis process. PH has been used to stimulate root and shoot biomass gains in corn, tomato, pepper, and other horticulturally-relevant plants (Colla et al., 2017; Schiavon et al., 2008). Enhanced nitrogen uptake rates and assimilation in tomato plants were reported, when treated with PH (Colla et al., 2014). An apparent gain from adding PH to the plants was in it's stimulating effect on the beneficial plant-associated microbial activity (Colla et al., 2017; du Jardin, 2015; Rouphael et al., 2020), leading to increased plant resilience to environmental stress factors as well as an enhanced nutrient uptake in the root zone. All those factors may be present with the *R. palustris* hydrolysate used in this study, but further testing to analyze the amino acid profile of the bacterial hydrolysate is needed. Knowledge of the amino acid profile in the acidified *R. palustris* biomass will also help in evaluating whether there might be a feedback inhibition loop within genetic systems of plants that is characteristic to the decreased NO_3-N and NH_4^+-N uptake in roots for plants in the environment with multiple nitrogen sources, specifically, in the presence of amino acids (Kronzucker et al., 1999; Miller et al., 2008).

3.6 Acknowledgements

This material is based upon work supported by NASA under grant or cooperative agreement award number NNX17AJ31G/NNH16ZOA001N-16STRII_B3. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Aeronautics and Space Administration (NASA).

I would like to thank Anna Doloman for her joint work with this project as well Paul Kausuma and Bruce Bugbee for their direction with the germination trials.

3.7 Disclaimer

This chapter was co-authored by Kyle Valgardson and Anna Doloman. Work contributed by Kyle Valgardson included leachate column test and biomass acidificaiton. Work contributed by Dr. Anna Doloman included 3L photbioreactor operation and germination trails statistical analysis. Germination experiment was a joint effort between Kyle Valgardson and Anna Doloman. None of this chapter has been or will be used in any other thesis or dissertation.

REFERENCES

Alef, K. and Kleiner, D. (1986). Arginine ammonification, a simple method to estimate microbial activity potentials in soils. *Soil Biology and Biochemistry* 18, 233–235. doi:10.1016/0038-0717(86)90033-7

Allen, M. B. and Arnon, D. I. (1955). Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by Anabaena cylindrica Lemm. *Plant Physiology* 30

Barker, A. V., Maynard, D. N., Mioduchowska, B., and Buch, A. (1970). Ammonium and Salt Inhibition of Some Physiological Processes Associated with Seed

Germination. *Physiologia Plantarum* 23. doi:10.1111/j.1399-3054.1970.tb06487.x

Bittsánszky, A., Pilinszky, K., Gyulai, G., and Komives, T. (2015). Overcoming ammonium toxicity. *Plant Science* 231, 184–190. doi:10.1016/j.plantsci.2014.12.005

Bremner, J. M. and Krogmeier, M. J. (1989). Evidence that the adverse effect of urea fertilizer on seed germination in soil is due to ammonia formed through hydrolysis of urea by soil urease. *Proceedings of the National Academy of Sciences* 86. doi:10.1073/pnas.86.21.8185

Calvo, P., Nelson, L., and Kloepper, J. W. (2014). Agricultural uses of plant biostimulants. *Plant and Soil; Dordrecht* 383. doi:http://dx.doi.org/10.1007/s11104-014-2131-8

Canfield, D. E., Glazer, A. N., and Falkowski, P. G. (2010). The Evolution and Future of Earth's Nitrogen Cycle. *Science* 330. doi:10.1126/science.1186120

Colla, G., Hoagland, L., Ruzzi, M., Cardarelli, M., Bonini, P., Canaguier, R., et al. (2017). Biostimulant Action of Protein Hydrolysates: Unraveling Their Effects on Plant Physiology and Microbiome. *Frontiers in Plant Science* 8. doi:10.3389/fpls.2017.02202

Colla, G., Rouphael, Y., Canaguier, R., Svecova, E., and Cardarelli, M. (2014). Biostimulant action of a plant-derived protein hydrolysate produced through enzymatic hydrolysis. *Frontiers in Plant Science* 5. doi:10.3389/fpls.2014.00448

Corbin, J. L. (1984). Liquid Chromatographic-Fluorescence Determination of Ammonia from Nitrogenase Reactions: A 2-Min Assay. *Applied and Environmental Microbiology* 47

Crawley, M. J., Johnston, A. E., Silvertown, J., Dodd, M., Mazancourt, C., Heard, M. S., et al. (2005). Determinants of Species Richness in the Park Grass Experiment. *The American Naturalist* 165. doi:10.1086/427270

DaSilva, E. J., Ratledge, C., Ratledge, P. o. B. C., and Sasson, A. (1992). *Biotechnology: Economic and Social Aspects: Issues for Developing Countries* (Cambridge University Press)

du Jardin, P. (2015). Plant biostimulants: Definition, concept, main categories and regulation. *Scientia Horticulturae* 196, 3–14. doi:10.1016/j.scienta.2015.09.021

Elbadry, M., El-Bassel, A., and Elbanna, K. (1999). Occurrence and dynamics of phototrophic purple nonsulphur bacteria compared with other asymbiotic nitrogen fixers in ricefields of Egypt. *World Journal of Microbiology and Biotechnology* 15, 359–362. doi:10.1023/A:1008971515966

Erisman, J. W., Galloway, J. N., Dise, N. B., Sutton, M. A., Bleeker, A., Grizzetti, B., et al. (2015). *Nitrogen : too much of a vital resource : Science Brief*. Tech. rep., WWF Netherlands, Zeist, The Netherlands

Esteban, R., Ariz, I., Cruz, C., and Moran, J. F. (2016). Review: Mechanisms of ammonium toxicity and the quest for tolerance. *Plant Science* 248, 92–101. doi:10.1016/j.plantsci.2016.04.008

Fawcett, R. S. and Slife, F. W. (1978). Effects of Field Applications of Nitrate on Weed Seed Germination and Dormancy. *Weed Science* 26. doi:10.1017/S0043174500064626

Fierer, N., Carney, K. M., Horner-Devine, M. C., and Megonigal, J. P. (2009). The Biogeography of Ammonia-Oxidizing Bacterial Communities in Soil. *Microbial Ecology* 58, 435–445. doi:10.1007/s00248-009-9517-9

Gamal-Eldin, H. and Elbanna, K. (2011). Field Evidence for the Potential of Rhodobacter capsulatus as Biofertilizer for Flooded Rice. *Current Microbiology* 62, 391–395. doi:10.1007/s00284-010-9719-x

Goyal, S. S. and Huffaker, R. C. (2015). Nitrogen Toxicity in Plants. In *Nitrogen in Crop Production* (John Wiley & Sons, Ltd). doi:10.2134/1990.nitrogenincropproduction.c6

Heinsoo, K., Sammul, M., Kukk, T., Kull, T., and Melts, I. (2020). The long-term recovery of a moderately fertilised semi-natural grassland. *Agriculture, Ecosystems & Environment* 289, 106744. doi:10.1016/j.agee.2019.106744

Hendricks, S. B. and Taylorson, R. B. (1974). Promotion of Seed Germination by Nitrate, Nitrite, Hydroxylamine, and Ammonium Salts. *Plant Physiology* 54. doi:10.1104/pp.54.3.304

Jha, M. N. and Prasad, A. N. (2006). Efficacy of New Inexpensive Cyanobacterial Biofertilizer Including its Shelf-life. *World Journal of Microbiology and Biotechnology* 22, 73–79. doi:10.1007/s11274-005-7024-9

Kantachote, D., Nunkaew, T., Kantha, T., and Chaiprapat, S. (2016). Biofertilizers from Rhodopseudomonas palustris strains to enhance rice yields and reduce methane emissions. *Applied Soil Ecology* 100, 154–161. doi:10.1016/j.apsoil.2015.12.015

Kronzucker, H. J., Glass, A. D. M., and Siddiqi, M. Y. (1999). Inhibition of Nitrate Uptake by Ammonium in Barley. Analysis of Component Fluxes. *Plant Physiology* 120. doi:10.1104/pp.120.1.283

Ladd, J. N. and Jackson, R. B. (2015). Biochemistry of Ammonification. In *Nitrogen in Agricultural Soils* (John Wiley & Sons, Ltd). doi:10.2134/agronmonogr22.c5

Malhi, S. S., Grant, C. A., Johnston, A. M., and Gill, K. S. (2001). Nitrogen fertilization management for no-till cereal production in the Canadian Great Plains: a review. *Soil and Tillage Research* 60, 101–122. doi:10.1016/S0167-1987(01)00176-3

Maudinas, B., Chemardin, M., Yovanovitch, E., and Gadal, P. (1981). Gnotobiotic cultures of rice plants up to ear stage in the absence of combined nitrogen source but in the presence of free living nitrogen fixing bacteriaAzotobacter vinelandii andRhodopseudomonas capsulata. *Plant and Soil* 60, 85–97. doi:10.1007/BF02377114

Miller, A. J., Fan, X., Shen, Q., and Smith, S. J. (2008). Amino acids and nitrate as signals for the regulation of nitrogen acquisition. *Journal of Experimental Botany* 59. doi:10.1093/jxb/erm208

Rao, N. S. S. (1982). Biofertilizers. *Interdisciplinary Science Reviews* 7. doi:10.1179/isr.1982.7.3.220

Rouphael, Y., Lucini, L., Miras-Moreno, B., Colla, G., Bonini, P., and Cardarelli, M. (2020). Metabolomic Responses of Maize Shoots and Roots Elicited by Combinatorial Seed Treatments With Microbial and Non-microbial Biostimulants. *Frontiers in Microbiology* 11. doi:10.3389/fmicb.2020.00664

Sakarika, M., Spanoghe, J., Sui, Y., Wambacq, E., Grunert, O., Haesaert, G., et al. (2019). Purple non-sulphur bacteria and plant production: benefits for fertilization, stress resistance and the environment. *Microbial Biotechnology* n/a. doi:10.1111/1751-7915.13474

Schiavon, M., Ertani, A., and Nardi, S. (2008). Effects of an Alfalfa Protein Hydrolysate on the Gene Expression and Activity of Enzymes of the Tricarboxylic

Acid (TCA) Cycle and Nitrogen Metabolism in Zea mays L. *Journal of Agricultural and Food Chemistry* 56. doi:10.1021/jf802362g

Schröder, J. J. (2014). The Position of Mineral Nitrogen Fertilizer in Efficient Use of Nitrogen and Land: A Review. *Natural Resources* 05. doi:10.4236/nr.2014.515080

Tagliavini, M., Scudellazi, D., Marangoni, B., and Toselli, M. (1996). Nitrogen fertilization management in orchards to reconcile productivity and environmental aspects. In *Fertilizers and Environment: Proceedings of the International Symposium "Fertilizers and Environment", held in Salamanca, Spain, 26–29, September, 1994*, ed. C. Rodriguez-Barrueco (Dordrecht: Springer Netherlands), Developments in Plant and Soil Sciences. 161–170. doi:10.1007/978-94-009-1586-2_27

Tilman, D., Balzer, C., Hill, J., and Befort, B. L. (2011). Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences* 108, 20260–20264. doi:10.1073/pnas.1116437108

Tripathy, P. P. and Ayyappan, S. (2005). Evaluation of Azotobacter and Azospirillum as Biofertilizers in Aquaculture. *World Journal of Microbiology and Biotechnology* 21, 1339–1343. doi:10.1007/s11274-005-3787-2

Vines, H. M. and Wedding, R. T. (1960). Some Effects of Ammonia on Plant Metabolism and a Possible Mechanism for Ammonia Toxicity 1234. *Plant Physiology* 35, 820–825

Vitousek, P. M., Aber, J. D., Howarth, R. W., Likens, G. E., Matson, P. A., Schindler, D. W., et al. (1997). Human Alteration of the Global Nitrogen Cycle: Sources and Consequences. *Ecological Applications* 7. doi:10.1890/1051-0761(1997)007[0737:HAOTGN]2.0.CO;2

CHAPTER 4
SUMMARY AND FUTURE DIRECTIONS

4.1 Introduction

As we venture further from our planet to explore the reaches of space, sustainable design practices become drastically more important and one of the main principles of sustainability is *in situ* resource utilization. The benefits of *in situ* focused processes are magnified for space travel as the payload burden is the limiting factor for deep space exploration (Menezes et al., 2015; Nangle et al., 2020). When focusing on long term life support for a Martian research mission, nitrogen is a crucial element to consider as plants, grown for food, are dependent on external nitrogen sources. In addition to food production, other processes within a Martian research mission will require biologically available nitrogen. Bacteria capable of reducing nitrogen to ammonia via the enzyme nitrogenase have the capacity to utilize*in situ* dinitrogen gas to generate ammonia, in support these processes, while limiting the material payload burden. The chapters of this thesis present a foundation for a feasible synthetic extraterrestrial nitrogen cycle facilitated by nitrogen-fixing bacteria for biofertilizer production and utilization.

4.2 Growth mode analysis for bacterial nitrogen reduction

As we are unaware of any existing life on Mars, a manned research base would require the implementation of an artificial nitrogen cycle with bacteria capable of reliable and consistent nitrogen reduction. In Chapter 2, the first steps toward this

goal was addressed by finding the growth modality and activity of multiple diazotrophs. Following the analysis of some initial growth measures, it was evident that heterotrophic growth drastically increased energy flux through nitrogenase. Even though *Azotobacter* strains have higher growth rates, PNSB have significant systematic advantages as the amount of carbon input required to facilitate growth is drastically lower when compared to *Azotobacter*. When designing a Martian nitrogen-fixing reactor, the need for balance between reactor size and input requirements should both be considered to maximize efficiency. Here I use simple metrics to compare growth conditions to select the most mission compatible bacterial strain and media composition for the nitrogen fixation process of the synthetic nitrogen cycle.

4.2.1 Identification of additional high-value reactor outputs

Having a firm understanding of growth characteristics by measuring relevant inputs and outputs allows for the identification of costly process inputs, but also the realization of additional value that can be drawn from the system. On Earth, bioreactors are often utilized for multiple products to maximize process value; on Mars, this need is elevated due to less infrastructure being available. When considering other useful outputs of PNSB, hydrogen is an interesting product as it is a well studied high-value byproduct of nitrogenase, often the target product of bioreactors utilizing nitrogenase (Barbosa et al., 2001; Oh et al., 2004). The value of hydrogen on Mars would be immense as there are no *in situ* fuel reserves available, and hydrogen gas is a highly energetic, clean, and efficient fuel. These benefits are not exclusive to a Mars-based research facility as the interest in utilizing hydrogen gas as a systemic fuel source on Earth is increasing. Hydrogen interest is primarily because hydrogen gas can be burned, with oxygen, to generate energy. This process releases high

amounts of energy with water as the only byproduct, making it possible to reduce the global greenhouse gas emissions (Goltsov et al., 2006). Current hydrogen production is reliant on fossil fuels as hydrogen is generated by steam reforming of natural gas, which is currently the most economically favorable process for hydrogen generation. Progress is being made in biohydrogen generation directly or through refining other biologically produced molecules such as methanol (Ni et al., 2007). These processes are in the early stages of development and generally result in higher production costs for hydrogen generation, when compared to steam reforming of natural gas. One sizeable economic issue with these systems is the sole focus on hydrogen production; if there were a secondary output, the monetary value of these systems would inevitably increase, making them more feasible for real applications. A system such as ours would accomplish this goal, as the hydrogen generated would be considered a byproduct of our nitrogen fixing reactor.

Another avenue to consider is the benefit of specific products, not for direct extraction, but to be used in culturing different strains of bacteria directly. Co-culturing is a process that uses controlled microbial consortia to enhance a targeted biological process (Diender et al., 2019; Fan et al., 2019; Hu et al., 2011). These bacteria often supply molecules to one another in a reciprocal fashion or remediate toxic compounds in a reactor resulting in higher growth rates and product yields. In PNSB, activity of nitrogenase results in an increase of pH as hydrogen ions are consumed producing hydrogen gas. We have regularly seen that as a culture reaches the stationary phase the pH is often above 8, which is detrimental to cell culture health (Wang et al., 2010). Conversely, acetic acid production is often shown to reduce the pH of a culture to levels also inhibitory to cellular processes (Patil et al., 2015). It may be advantageous to co-culture a diazotroph and an acetogen to balance pH, resulting in a longer growth phase and increased cellular activity.

4.3 Viability of *R. palustris* as a ubiquitous biofertilizer

It is not a new concept to use diazotrophs for the production of biofertilizers, as blue-green algae were shown to promote plant growth more than 60 years ago (Allen and Arnon, 1955). However, due to global economic reasons, inorganic fertilizers are the norm in agricultural fertilization practices. Recently biofertilizers have received more attention as inorganic fertilizers have been identified as being detrimental to ecosystems, especially due to underlying ammonia toxicity (Schröder, 2014). PNSB have been used to supply nutrients for plant growth; however, much of the existing research investigates living bacteria inoculants with only a few plant species (Sakarika et al., 2019). In chapter 3, we explore the feasibility of using biomass, stabilized through acidificaiton, as a controlled nutrient supply for diverse plant species as the first step for a ubiquitous PNSB biofertilizer.

4.3.1 Biomass processing and application

Direct biomass addition to solid plant growth supporting substrata showed little promise, as the inert substrata were not capable of appreciable biomass retention. To address this issue, we used acid hydrolysis with sulfuric acid to kill the bacteria and disrupt the major cell structure. We also found that the high pH of late growth phase solution leads to the loss of nitrogen through volitalization of ammonia. Acidification lowers the pH to levels below the pKa of ammonia, resulting in the retention of the nitrogen in a solution. This solution can be precisely added to a nutrient solution, this gives the capacity of higher levels of control over the nutrients added while supporting plant growth. This is also mission-relevant as a Mars research base would use

hydroponics for plant growth, and the acidified biomass solution would be easier to incorporate into a hydroponic solution, when compared to bacteria paste.

This moderate acid hydrolysis was not capable of degrading organic nitrogen to ammonia, so further ammonification needeeds to take place in the nutrient solution before plants could utilize the nitrogen. The addition of a protein hydrolysate based biostimulant derived from plant matter, coupled with a bacterial inoculant, has demonstrated increased plant growth and metabolite accumulation in corn (Rouphael et al., 2020). Further studies are underway to monitor the degradation of biomass through ammonification in the nutrient solution, as well as the root zone of plant growth trials. A more detailed investigation of the metabolomic effect of acid hydrolyzed biomass on plant cultivars would have to be performed to understand the mechanism of promotion better.

4.3.2 Germination trials

Field trials with PNSB applied as a biofertilizer to rice and some other plant cultivars has previously demonstrated feasibility of this approach, however, most of this evidence is found with inoculant or from root zone microbiome analysis (Elbadry et al., 1999; Gamal-Eldin and Elbanna, 2011; Sakpirom et al., 2017). We demonstrated that acidified biomass has a limited effect on germination and early growth with many different cultivars; however, we have not yet demonstrated acidified biomass effects on full plant growth. Current experiments are underway comparing acidified biomass to numerous inorganic nitrogen sources through extended plant growth of wheat and lettuce cultivars. We hope that this will allow us to identify the fertilization mode or inhibition effects of acidified *R. palustris* biomass. If acidified biomass shows toxicity to full plant growth, it may be prudent to examine the form of nitrogen in the acid-

ified solution to identify if nitrogen is the toxic compound. However, if these plant growth trials indicate that PNSB biomass can be used as a biofertilizer for multiple plant species, it would be interesting to extend these experiments beyond the primary plant species tested. We are also limited in our understanding of what bacteria are responsible for the degradation of the biomass to ammonium in the nutrient solution, so investigation into the microbiome composition would be necessary, as we will need to construct the hydroponic microbiome when applied on Mars. In addition to the mechanism of nitrogen transfer, it would be important to explore the scalability of crop production in a Mars simulated system. Expanding these trials to larger levels of plant mass from germination through harvest will give us a clear idea of the feasibility of this process for a Mars-based application.

4.4 Nitrogen recycling

One unique aspect of PNSB is that they are capable of four main growth modes: photoheterotrophic, photoautotrophic, chemoheterotrophic, and chemoautotrophic (Larimer et al., 2004). Within the heterotrophic growth modes, they are also capable of metabolizing a vast array of carbon and nitrogen compounds. These characteristics result in PNSB being very resilient bacteria and have led to their colonization of diverse ecosystems across the globe (Bell et al., 2006; Harwood and Gibson, 1988). This diversity and stability have resulted in PNSB being used for a diverse set of waste remediation processes (Chitapornpan et al., 2013; Getha et al., 1998; Kornochalert et al., 2014; Pintucci et al., 2015; Sepúlveda-Muñoz et al., 2020). Waste recycling is crucial to the nitrogen cycle as it prevents accumulation of nitrogen by recycling it back to dinitrogen gas (Canfield et al., 2010; Ladd and Jackson, 2015).

A typical waste remediation system's primary purpose is to reduce toxic com-

pounds from water, but they also have the capacity to use the organics present in waste as a fuel for other biological processes (Chen et al., 2006; Gosse et al., 2007). A Martian waste remediation system would be necessary to complete the nitrogen cycle, and could also be used to fuel a nitrogen reduction system, through utilization of the organic carbon coumpounds present. Careful bacterial selection should be considered while investigating this possibility to target the most mission beneficial processes. Typical waste streams contain high levels of ammonia or urea, which are generally inhibitory to nitrogenase expression and activity, essentially turning nitrogen fixation off in these systems (Masepohl et al., 2002; Verostko et al., 2004). The *R. palustris* NifA* strain may be capable of fixing nitrogen in high external nitrogen environments as the ammonia sensitivity is eliminated (Adessi et al., 2012). In Appendix A we display data for *R. palustris* GCA009 NifA* mutant growth on a simulated early Martian planetary base wastewater. We are trying to determine the nitrogen recycling capacity and nitrogen fixation activity of this bacteria in a waste treatment role on Mars. Characterization of biofertilizer production through remediation of ammonia and urea, in addition to fixing nitrogen gas into biomass, is the main target for this project. Coupling this waste remediation with the foundational nitrogen fixation in chapter two, and nitrogen transfer in chapter 3, would close our nitrogen cycle and act as the fundational principles for the nitrogen component of the life support system on Mars.

4.5 Acknowledgements

This material is based upon work supported by NASA under grant or cooperative agreement award number NNX17AJ31G/NNH16ZOA001N-16STRII_B3. Any opinions, findings, and conclusions or recommendations expressed in this material

are those of the author(s) and do not necessarily reflect the views of the National Aeronautics and Space Administration (NASA).

I would like to thank Anna Doloman, Paul Kusuma, and Mathangi Soundararajan for their comments and thoughts.

REFERENCES

Adessi, A., McKinlay, J. B., Harwood, C. S., and De Philippis, R. (2012). A Rhodopseudomonas palustris nifA* mutant produces H2 from NH4+-containing vegetable wastes. *International Journal of Hydrogen Energy* 37, 15893–15900. doi:10.1016/j.ijhydene.2012.08.009

Allen, M. B. and Arnon, D. I. (1955). Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by Anabaena cylindrica Lemm. *Plant Physiology* 30

Barbosa, M. J., Rocha, J. M. S., Tramper, J., and Wijffels, R. H. (2001). Acetate as a carbon source for hydrogen production by photosynthetic bacteria. *Journal of Biotechnology* 85, 25–33. doi:10.1016/S0168-1656(00)00368-0

Bell, S. G., Hoskins, N., Xu, F., Caprotti, D., Rao, Z., and Wong, L.-L. (2006). Cytochrome P450 enzymes from the metabolically diverse bacterium Rhodopseudomonas palustris. *Biochemical and Biophysical Research Communications* 342, 191–196. doi:10.1016/j.bbrc.2006.01.133

Canfield, D. E., Glazer, A. N., and Falkowski, P. G. (2010). The Evolution and Future of Earth's Nitrogen Cycle. *Science* 330. doi:10.1126/science.1186120

Chen, C.-Y., Lee, C.-M., and Chang, J.-S. (2006). Feasibility study on bioreactor strategies for enhanced photohydrogen production from Rhodopseudomonas palustris WP3-5 using optical-fiber-assisted illumination systems. *International Journal of Hydrogen Energy* 31, 2345–2355. doi:10.1016/j.ijhydene.2006.03.007

Chitapornpan, S., Chiemchaisri, C., Chiemchaisri, W., Honda, R., and Yamamoto, K. (2013). Organic carbon recovery and photosynthetic bacteria population in an anaerobic membrane photo-bioreactor treating food processing wastewater. *Bioresource Technology* 141, 65–74. doi:10.1016/j.biortech.2013.02.048

Diender, M., Parera Olm, I., Gelderloos, M., Koehorst, J. J., Schaap, P. J., Stams, A. J. M., et al. (2019). Metabolic shift induced by synthetic co-cultivation promotes high yield of chain elongated acids from syngas. *Scientific Reports* 9, 1–11. doi:10.1038/s41598-019-54445-y

Elbadry, M., El-Bassel, A., and Elbanna, K. (1999). Occurrence and dynamics of phototrophic purple nonsulphur bacteria compared with other asymbiotic nitrogen fixers in ricefields of Egypt. *World Journal of Microbiology and Biotechnology* 15, 359–362. doi:10.1023/A:1008971515966

Fan, G., Teng, C., Xu, D., Fu, Z., Minhazul, K. A., Wu, Q., et al. (2019). Enhanced production of ethyl acetate using co-culture of Wickerhamomyces anomalus and Saccharomyces cerevisiae. *Journal of Bioscience and Bioengineering* doi:10.1016/j.jbiosc.2019.05.002

Gamal-Eldin, H. and Elbanna, K. (2011). Field Evidence for the Potential of Rhodobacter capsulatus as Biofertilizer for Flooded Rice. *Current Microbiology* 62, 391–395. doi:10.1007/s00284-010-9719-x

Getha, K., Vikineswary, S., and Chong, V. (1998). Isolation and growth of the phototrophic bacterium Rhodopseudomonas palustris strain B1 in sago-starch-processing wastewater. *World Journal of Microbiology and Biotechnology* 14, 505–511. doi:10.1023/A:1008855125634

Goltsov, V. A., Veziroglu, T. N., and Goltsova, L. F. (2006). Hydrogen civilization of the future—A new conception of the IAHE. *International Journal of Hydrogen Energy* 31, 153–159. doi:10.1016/j.ijhydene.2005.04.045

Gosse, J. L., Engel, B. J., Rey, F. E., Harwood, C. S., Scriven, L. E., and Flickinger, M. C. (2007). Hydrogen Production by Photoreactive Nanoporous Latex Coatings of Nongrowing Rhodopseudomonas palustris CGA009. *Biotechnology Progress* 23. doi:10.1021/bp060254

Harwood, C. S. and Gibson, J. (1988). Anaerobic and aerobic metabolism of diverse aromatic compounds by the photosynthetic bacterium Rhodopseudomonas palustris. *Applied and Environmental Microbiology* 54

Hu, H. L., van den Brink, J., Gruben, B. S., Wösten, H. A. B., Gu, J. D., and de Vries, R. P. (2011). Improved enzyme production by co-cultivation of Aspergillus niger and Aspergillus oryzae and with other fungi. *International Biodeterioration & Biodegradation* 65, 248–252. doi:10.1016/j.ibiod.2010.11.008

Kornochalert, N., Kantachote, D., Chaiprapat, S., and Techkarnjanaruk, S. (2014). Use of Rhodopseudomonas palustris P1 stimulated growth by fermented pineapple extract to treat latex rubber sheet wastewater to obtain single cell protein. *Annals of Microbiology* 64, 1021–1032. doi:10.1007/s13213-013-0739-1

Ladd, J. N. and Jackson, R. B. (2015). Biochemistry of Ammonification. In *Nitrogen in Agricultural Soils* (John Wiley & Sons, Ltd). doi:10.2134/agronmonogr22.c5

Larimer, F. W., Chain, P., Hauser, L., Lamerdin, J., Malfatti, S., Do, L., et al. (2004). Complete genome sequence of the metabolically versatile photosynthetic bacterium Rhodopseudomonas palustris. *Nature Biotechnology* 22, 55–61. doi:10.1038/nbt923

Masepohl, B., Drepper, T., Paschen, A., Groß, S., Pawlowski, A., Raabe, K., et al. (2002). Regulation of nitrogen fixation in the phototrophic purple bacterium Rhodobacter capsulatus. *Journal of molecular microbiology and biotechnology* 4, 243–248

Menezes, A. A., Cumbers, J., Hogan, J. A., and Arkin, A. P. (2015). Towards synthetic biological approaches to resource utilization on space missions. *Journal of The Royal Society Interface* 12. doi:10.1098/rsif.2014.0715

Nangle, S. N., Wolfson, M. Y., Hartsough, L., Ma, N. J., Mason, C. E., Merighi, M., et al. (2020). The case for biotech on Mars. *Nature Biotechnology* 38. doi:10.1038/s41587-020-0485-4

Ni, M., Leung, D. Y. C., and Leung, M. K. H. (2007). A review on reforming bio-ethanol for hydrogen production. *International Journal of Hydrogen Energy* 32, 3238–3247. doi:10.1016/j.ijhydene.2007.04.038

Oh, Y.-K., Seol, E.-H., Kim, M.-S., and Park, S. (2004). Photoproduction of hydrogen from acetate by a chemoheterotrophic bacterium Rhodopseudomonas palustris P4. *International Journal of Hydrogen Energy* 29, 1115–1121. doi:10.1016/j.ijhydene.2003.11.008

Patil, S. A., Arends, J. B. A., Vanwonterghem, I., van Meerbergen, J., Guo, K., Tyson, G. W., et al. (2015). Selective Enrichment Establishes a Stable Performing Community for Microbial Electrosynthesis of Acetate from CO2. *Environmental Science & Technology* 49. doi:10.1021/es506149d

Pintucci, C., Padovani, G., Giovannelli, A., Traversi, M. L., Ena, A., Pushparaj, B., et al. (2015). Hydrogen photo-evolution by Rhodopseudomonas palustris 6A using pre-treated olive mill wastewater and a synthetic medium containing sugars. *Energy Conversion and Management* 90, 499–505. doi:10.1016/j.enconman.2014.11.045

Rouphael, Y., Lucini, L., Miras-Moreno, B., Colla, G., Bonini, P., and Cardarelli, M. (2020). Metabolomic Responses of Maize Shoots and Roots Elicited by Combinatorial Seed Treatments With Microbial and Non-microbial Biostimulants. *Frontiers in Microbiology* 11. doi:10.3389/fmicb.2020.00664

Sakarika, M., Spanoghe, J., Sui, Y., Wambacq, E., Grunert, O., Haesaert, G., et al. (2019). Purple non-sulphur bacteria and plant production: benefits for fertilization, stress resistance and the environment. *Microbial Biotechnology* n/a. doi:10.1111/1751-7915.13474

Sakpirom, J., Kantachote, D., Nunkaew, T., and Khan, E. (2017). Characterizations of purple non-sulfur bacteria isolated from paddy fields, and identification of strains with potential for plant growth-promotion, greenhouse gas mitigation and heavy metal bioremediation. *Research in Microbiology* 168, 266–275. doi:10.1016/j.resmic.2016.12.001

Schröder, J. J. (2014). The Position of Mineral Nitrogen Fertilizer in Efficient Use of Nitrogen and Land: A Review. *Natural Resources* 05. doi:10.4236/nr.2014.515080

Sepúlveda-Muñoz, C. A., de Godos, I., Puyol, D., and Muñoz, R. (2020). A systematic optimization of piggery wastewater treatment with purple phototrophic bacteria. *Chemosphere* doi:10.1016/j.chemosphere.2020.126621

Verostko, C. E., Carrier, C., and Finger, B. W. (2004). Ersatz Wastewater Formulations for Testing Water Recovery Systems. 2004–01–2448. doi:10.4271/2004-01-2448

Wang, Y.-Z., Liao, Q., Zhu, X., Tian, X., and Zhang, C. (2010). Characteristics of hydrogen production and substrate consumption of Rhodopseudomonas palustris CQK 01 in an immobilized-cell photobioreactor. *Bioresource Technology* 101, 4034–4041. doi:10.1016/j.biortech.2010.01.045

APPENDICES

APPENDIX A
FUTURE DIRECTIONS: WASTEWATER REMEDIATION AS A MEANS OF NITROGEN RECYCLING

A.1 Introduction

Purple non-sulfur bacteria (PNSB) are a group of bacteria that are common to many natural and urban environments around the globe. Many PNSB, including *Rhodopseuodmonas palustris*, belong to the subclass alpha- and beta-proteobacteria. Like most PNSB, *R. palustris* is capable of 4 different metabolic growth modes: photoheterotrophic, photoautotrophic, chemoheterotrophic, and chemoautotrophic (Larimer et al., 2004). There exists even further metabolic diversity within these 4 growth modes as *R. palustris* is capable of growth on a wide range of carbon compounds from simple small alcohols to complex long-chain fatty acids (Bell et al., 2006; Harwood and Gibson, 1988). In addition to diverse carbon metabolism, *R. palustris* has the ability to grow diazotrophically (reducing dinitrogen gas to ammonia) or from other organic and inorganic nitrogen forms, as well as in the presence or absence of oxygen (Trüper and Pfennig, 1981). In addition to the diversity of metabolic growth modes, *R. palustris* contains three times as many transport system genes as most other bacteria, which may be a rational explanation for the diverse environments they grow in, and tolerance to pollution (Larimer et al., 2004).

This unique combination of growth exhibited by *R. palustris* has inevitably led to the utilization of this organism as a biocatalyst for production of both complex compounds, such as polyhydroxyalkanoates (Brandl et al., 1989; Kranz et al., 1997; Ranaivoarisoa et al., 2017), or simple biofuels, such as hydrogen (Oh et al., 2004).

The application of *R. palustris* may be most valuable within wastewater treatment, where the innate ability to metabolize a vast number of complex and simple organic compounds shines. This is illustrated by the diversity of remediation for various wastewater sources including but not limited to livestock, food production, agricultural, and domestic waste (Chitapornpan et al., 2013; Getha et al., 1998; Kim et al., 2012; Kornochalert et al., 2014; Nagadomi et al., 2000; Pintucci et al., 2015; Sepúlveda-Muñoz et al., 2020). *R. palustris* is not only effective at remediating a diversity of waste sources but is often utilized in biofuel production (Adessi et al., 2012a; Barbosa et al., 2001; Hu et al., 2018; Nath et al., 2005; Oh et al., 2004). Hydrogen is generated in *R. palustris* primarily as a byproduct of nitrogenase activity. Nitrogenase is active in both light and dark conditions, with light increasing the hydrogen production due to increased levels of ATP generated by the bacterial photosystem (Kim et al., 2011; Ormerod et al., 1961). Nitrogenase expression is highly sensitive to ammonium concentration, which is typically very high in wastewaters, and this often limits the hydrogen production of phototrophic bacteria in these environments. *R. palustris* NifA* mutants have been shown to have little to no ammonium sensitivity and maintain high levels of nitrogenase activity in high nitrogen environments (Adessi et al., 2012b; Heiniger et al., 2012; McKinlay and Harwood, 2010).

R. palustris has been grown in photobioreactors for waste remediation and hydrogen production with a wide range of light sources (Chen et al., 2006b; Gosse et al., 2007). Depending on light intensity *R. palustris* will regulate photosynthesis through changes in bacterial chlorophyll and carotenoid ratios in the bacterias light-harvesting complex (Brotosudarmo et al., 2015). It has also been demonstrated that light intensities will affect nitrogenase activity and, ultimately, hydrogen production (Carlozzi, 2009). Even though there are extensive applications of *R. palustris* in photobioreactors and the light intensity responses are fairly well understood there are few studies

investigating how different light sources will affect growth (Chen et al., 2006a; Kuo et al., 2012; Qi et al., 2017), and even fewer studies utilizing waste streams to study the effect of a light source (Zhao et al., 2019; Zhi et al., 2019). Here we address hydrogen production of *R. palustris* NifA * mutant grown under different light sources while remediating ERSATZ early planetary base synthetic wastewater (Verostko et al., 2004).

A.2 Methods

Nitrogen fixation (NF) media consisted of 12.5mM K_2HPO_4 and 12.5mM KH_2PO_4 buffering components, a concentrated base solution at a final concentration of 0.1% (v/v), Wolf's vitamins at a 0.5% (v/v), and 30mM acetate as the carbon source. [Concentrated base stock solution (mM concentrations): nitrilotriacetic acid (105), $MgSO_4$ (240), $CaCl_2$-$2H_2O$ (45), Na_2MoO_4-$2H_2O$ (0.78),$FeSO_4$-$7H_2O$ (2.5), EDTA (0.86), $ZnSO_4$-$7H_2O$ (3.81), $MnSO_4$-H_2O (0.91), $CuSO_4$-$5H_2O$ (0.15) $Co(NO_3)_2$-$6H_2O$ (0.085), Na_2B4O_7-$10H_2O$ (0.046)]. [Wolfe's Vitamins stock solution [containing (g/L): *p*-aminobenzoid acid (0.005), folic acid (0.002), lipoic acid (0.005), Riboflavin (0.005), thiamine (0.005), nicotinic acid (0.005), pyridoxamine (0.01), pantothenic acid (0.005), cobalamin (0.0001), biotin (0.002)].

ERSATZ early planetary base wastewater concentrates were made as previously described (Verostko et al., 2004). Full wastewater media consisted of each concentrate added in appropriate dilutions with the same phosphate, concentrated base, and wolfs vitamines as the NF media. Inhibition studies were conducted by adding individual concentrates to NF media growth vials in the proper dilutions.

All growths of purple non-sulfur bacteria were prepared by adding the appropriate media without Wolf's vitamines to serum vials filled to a 50% volume. Sterile ERSATZ concentrates 1, 4 and 5 were added after autoclaving, as sparging would

drive off carbonate, or foam to aggressively with the soap. Anerobicity was achieved by sparging the media with nitrogen for at least 20 min and headspace for 10 min. Vials were then sealed with blue butyl 20mm rubber stoppers and autoclaved. Wolf's vitamines were then added to a final concentration of through a 0.2μm syringe filter. Growths were inoculated with *R. palustris* CGA009 NifA* mutant strain to a starting OD660 between 0.1-0.05.samples were taken using sterile syringes and monitored by measuring OD at 660nm with the spectrophotometer (Cary 50 UV-vis, Varian instruments, CA, United States). Samples were taken daily into the stationary phase. Cultures were grown at 25-30°C and illuminated via warm white LED; vials were placed at a constant distance from a light source to achieve a photon flux between 150-250 μM photon/M2. Photon flux was measured with a 340-1040 quantum flux meeter (Apogee Instruments, UT, United States). Due to the small volume of samples, pH was measured using MColorpHast pH test strips, pH 6.5-10 (EMD Millipore Corporation, MA, United States).

A.3 Results and Discussion

R. palustris NifA* grown in full wastewater media, prepared as described (Verostko et al., 2004), grows to a maximal OD660 that is roughly half of what we see in NF media growths (Figure A.1). If this were a nitrogen-free media, this would be less alarming, but as the wastewater contains relatively high levels of inorganic nitrogen without inhibition, we would anticipate measurements in the 3-4 range. The next logical step led us to examine each individual waste concentrate on growth inhibition. To address this, we added corresponding waste concentrates to NF media and compared these growths to the NF control (figure A.2). We see from this experiment that there are three main growth patterns depending on the concentrate. Concentrate 14,

2, and 6 all contain inorganic nitrogen sources and exhibit higher initial growth rates, and maximal OD increases. Concentrate 3 has a similar growth rate and maximal OD660 as the nitrogen-fixing control; it also contains no inorganic nitrogen making it reliant on nitrogenase activity for growth. Concentrate 5 has a significantly long lag phase and slower growth rates, and it will eventually grow to an appreciable cell density; however, this is considered inhibitory to growth, due to the extensive lag phase.

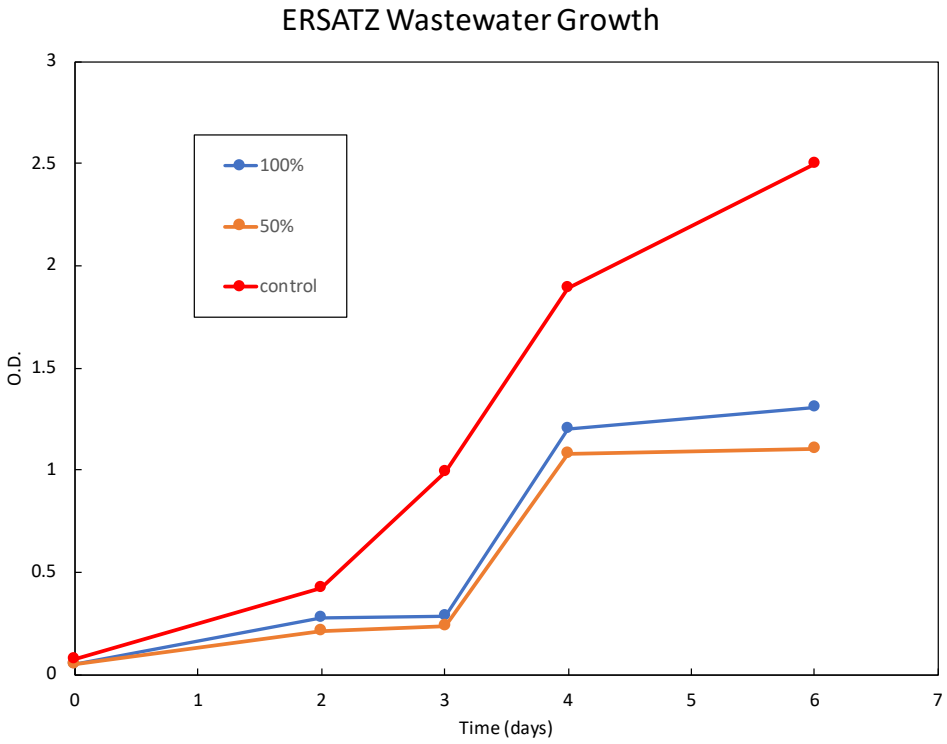

Fig. A.1: Growth monitoring of *R. palustris* CGA009 NifA* in full ERSATZ wastewater as measured by optical density at 660nm. 100% and 50% are dilutions of the full waste media and the control is nitrogen fixing minimal salts media with acetate.

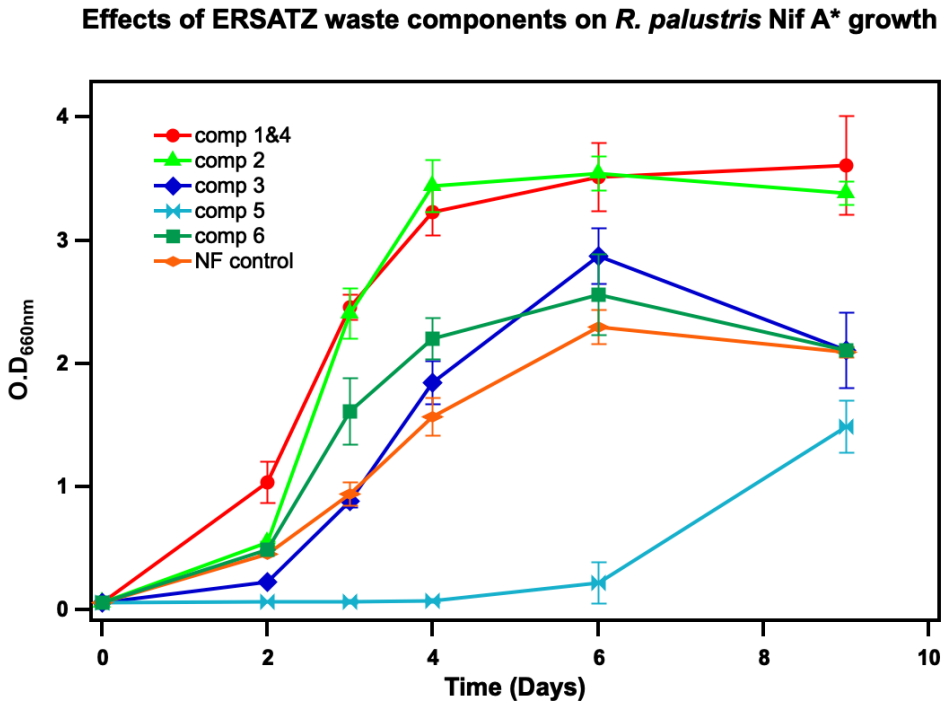

Fig. A.2: Toxicity screening of individual waste components on *R. palustris* CGA009 NifA* growth as measured by optical density at 660nm. Each concentrate from the ERSATZ waste media was added to NF minimal salts media prior to inoculation.

One interesting observation with these trials is that as each growth enters the stationary phase the pH measurements have consistently exceeded 8.5, which is high enough to inhibit growth. To address this we grew *R. palustris* NifA* on wastewater with phosphates added to increase the buffering capacity in the neutral region. Phosphates were added in different amounts near the same concentration as the NF media to find an optimal growth range (Figure A.3). The concentrations that exhibited the highest growth rates were 12.5 and 6.25mM. This was a full wastewater media, and there was some level of inhibition as the cell density never grew well, so we decided to use 12.5 not 6.25 going forward as it exhibited the same growth but has twice the buffering capacity. upon removal of concentrate 5, and addition of phosphates to the wastewater media, we still observed inhibition of growth.

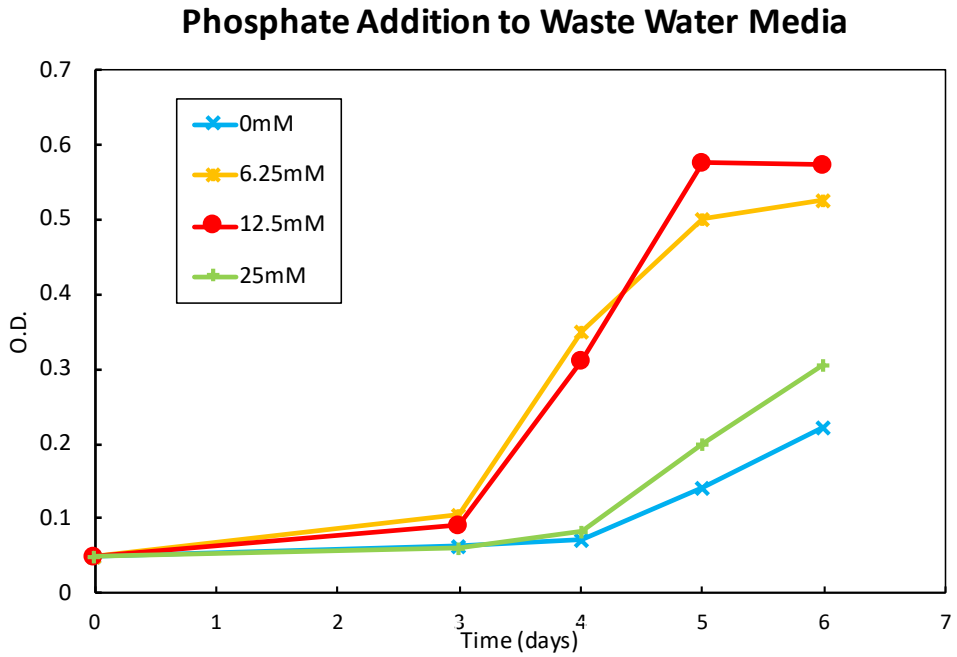

Fig. A.3: Growth measurements of *R. palustris* CGA009 NifA* to identify optimal phosphate concentration as a suppliment for ERSATZ wastewater to regulate pH. measured by optical density at 660nm.

A.4 Summary and future directions

Data collected in these early experiments suggest that early planetary based wastewater could be a potential feedstock for a nitrogen fixation reactor or at least a nitrogen recycling reactor. Going forward we will need to identify the best wastewater adjustments to support the growth of *R. palustris* NifA* however we want to limit modifications of individual waste concentrates as that would require pretreatment of specific effluents prior to implementation in the system. One typical downside to using wastewater to facilitate nitrogen fixation is that typically waste streams contain high levels of inorganic nitrogen, which down-regulates nitrogenase expression and activity. The NifA* mutant has the potential to overcome this boundary as the mutation has

lost the ability to regulate the expression of nitrogenase, limiting the ammonia sensitivity (Adessi et al., 2012a). We would verify if *R. palustris* NifA* could enrich the nitrogen content, through sustained nitrogenase activity in ammonium rich solution, by doing a total nitrogen assay of the system to see if there is an increase in reduced nitrogen levels. To confirm nitrogenase activity we would use hydrogen production as well as acetylene reduction to measure activity, as both hydrogen and acetylene can be reduced by nitrogenase.

Successful remediation of nitrogen rich waste streams for the growth of *R. palustris* NifA* are still early in their development, but hold valuable process application to complete the martian nitrogen cycle. In addition to applications on Mars this research would be very useful in remediation of Earth based waste water as it is possible to use waste as the main input for a biofertilizer production system.

A.5 Acknowledgements

I would like to thank Anna Doloman for her expertise and direction with this project

REFERENCES

Adessi, A., McKinlay, J. B., Harwood, C. S., and De Philippis, R. (2012a). A Rhodopseudomonas palustris nifA* mutant produces H2 from NH4+-containing vegetable wastes. *International Journal of Hydrogen Energy* 37, 15893–15900. doi:10.1016/j.ijhydene.2012.08.009

Adessi, A., Torzillo, G., Baccetti, E., and De Philippis, R. (2012b). Sustained outdoor H2 production with Rhodopseudomonas palustris cultures in a 50L tubular photobioreactor. *International Journal of Hydrogen Energy* 37, 8840–8849. doi:10.1016/j.ijhydene.2012.01.081

Barbosa, M. J., Rocha, J. M. S., Tramper, J., and Wijffels, R. H. (2001). Acetate as a carbon source for hydrogen production by photosynthetic bacteria. *Journal of Biotechnology* 85, 25–33. doi:10.1016/S0168-1656(00)00368-0

Bell, S. G., Hoskins, N., Xu, F., Caprotti, D., Rao, Z., and Wong, L.-L. (2006). Cytochrome P450 enzymes from the metabolically diverse bacterium Rhodopseudomonas palustris. *Biochemical and Biophysical Research Communications* 342, 191–196. doi:10.1016/j.bbrc.2006.01.133

Brandl, H., Knee, E. J., Fuller, R. C., Gross, R. A., and Lenz, R. W. (1989). Ability of the phototrophic bacterium Rhodospirillum rubrum to produce various poly (-hydroxyalkanoates): Potential sources for biodegradable polyesters. *International Journal of Biological Macromolecules* 11, 49–55. doi:10.1016/0141-8130(89)90040-8

Brotosudarmo, T. H. P., Limantara, L., Heriyanto, and Prihastyanti, M. N. U. (2015). Adaptation of the Photosynthetic Unit of Purple Bacteria to Changes of Light Illumination Intensities. *Procedia Chemistry* 14, 414–421. doi:10.1016/j.proche.2015.03.056

Carlozzi, P. (2009). The effect of irradiance growing on hydrogen photoevolution and on the kinetic growth in Rhodopseudomonas palustris, strain 42OL. *International Journal of Hydrogen Energy* 34, 7949–7958. doi:10.1016/j.ijhydene.2009.07.083

Chen, C.-Y., Lee, C.-M., and Chang, J.-S. (2006a). Feasibility study on bioreactor strategies for enhanced photohydrogen production from Rhodopseudomonas palustris WP3-5 using optical-fiber-assisted illumination systems. *International Journal of Hydrogen Energy* 31, 2345–2355. doi:10.1016/j.ijhydene.2006.03.007

Chen, C.-Y., Lee, C.-M., and Chang, J.-S. (2006b). Hydrogen production by indigenous photosynthetic bacterium Rhodopseudomonas palustris WP3–5 using optical fiber-illuminating photobioreactors. *Biochemical Engineering Journal* 32, 33–42. doi:10.1016/j.bej.2006.08.015

Chitapornpan, S., Chiemchaisri, C., Chiemchaisri, W., Honda, R., and Yamamoto, K. (2013). Organic carbon recovery and photosynthetic bacteria population in an anaerobic membrane photo-bioreactor treating food processing wastewater. *Bioresource Technology* 141, 65–74. doi:10.1016/j.biortech.2013.02.048

Getha, K., Vikineswary, S., and Chong, V. (1998). Isolation and growth of the phototrophic bacterium Rhodopseudomonas palustris strain B1 in sago-starch-processing wastewater. *World Journal of Microbiology and Biotechnology* 14, 505–511. doi:10.1023/A:1008855125634

Gosse, J. L., Engel, B. J., Rey, F. E., Harwood, C. S., Scriven, L. E., and Flickinger, M. C. (2007). Hydrogen Production by Photoreactive Nanoporous Latex Coatings of Nongrowing Rhodopseudomonas palustris CGA009. *Biotechnology Progress* 23. doi:10.1021/bp060254

Harwood, C. S. and Gibson, J. (1988). Anaerobic and aerobic metabolism of diverse aromatic compounds by the photosynthetic bacterium Rhodopseudomonas palustris. *Applied and Environmental Microbiology* 54

Heiniger, E. K., Oda, Y., Samanta, S. K., and Harwood, C. S. (2012). How Post-translational Modification of Nitrogenase Is Circumvented in Rhodopseudomonas palustris Strains That Produce Hydrogen Gas Constitutively. *Applied and Environmental Microbiology* 78. doi:10.1128/AEM.07254-11

Hu, B.-B., Li, M.-Y., Wang, Y.-T., and Zhu, M.-J. (2018). High-yield biohydrogen production from non-detoxified sugarcane bagasse: Fermentation strategy and mechanism. *Chemical Engineering Journal* 335, 979–987. doi:10.1016/j.cej.2017.10.157

Kim, M.-S., Kim, D.-H., Cha, J., and Lee, J. K. (2012). Effect of carbon and nitrogen sources on photo-fermentative H2 production associated with nitrogenase, uptake hydrogenase activity, and PHB accumulation in Rhodobacter sphaeroides KD131. *Bioresource Technology* 116, 179–183. doi:10.1016/j.biortech.2012.04.011

Kim, M.-S., Kim, D.-H., Son, H.-N., Ten, L. N., and Lee, J. K. (2011). Enhancing photo-fermentative hydrogen production by Rhodobacter sphaeroides KD131 and its PHB synthase deleted-mutant from acetate and butyrate. *International Journal of Hydrogen Energy* 36, 13964–13971. doi:10.1016/j.ijhydene.2011.03.099

Kornochalert, N., Kantachote, D., Chaiprapat, S., and Techkarnjanaruk, S. (2014). Use of Rhodopseudomonas palustris P1 stimulated growth by fermented pineapple extract to treat latex rubber sheet wastewater to obtain single cell protein. *Annals of Microbiology* 64, 1021–1032. doi:10.1007/s13213-013-0739-1

Kranz, R. G., Gabbert, K. K., Locke, T. A., and Madigan, M. T. (1997). Polyhydroxyalkanoate production in Rhodobacter capsulatus: genes, mutants, expression, and physiology. *Applied and Environmental Microbiology* 63, 3003–3009

Kuo, F.-S., Chien, Y.-H., and Chen, C.-J. (2012). Effects of light sources on growth and carotenoid content of photosynthetic bacteria Rhodopseudomonas palustris. *Bioresource Technology* 113, 315–318. doi:10.1016/j.biortech.2012.01.087

Larimer, F. W., Chain, P., Hauser, L., Lamerdin, J., Malfatti, S., Do, L., et al. (2004). Complete genome sequence of the metabolically versatile photosynthetic bacterium Rhodopseudomonas palustris. *Nature Biotechnology* 22, 55–61. doi:10.1038/nbt923

McKinlay, J. B. and Harwood, C. S. (2010). Carbon dioxide fixation as a central redox cofactor recycling mechanism in bacteria. *Proceedings of the National Academy of Sciences* 107. doi:10.1073/pnas.1006175107

Nagadomi, H., Kitamura, T., Watanabe, M., and Sasaki, K. (2000). Simultaneous removal of chemical oxygen demand (COD), phosphate, nitrate and H2S in the synthetic sewage wastewater using porous ceramic immobilized photosynthetic bacteria. *Biotechnology Letters* 22, 1369–1374. doi:10.1023/A:1005688229783

Nath, K., Kumar, A., and Das, D. (2005). Hydrogen production by Rhodobacter sphaeroides strain O.U.001 using spent media of Enterobacter cloacae strain DM11. *Applied Microbiology and Biotechnology* 68, 533–541. doi:10.1007/s00253-005-1887-4

Oh, Y.-K., Seol, E.-H., Kim, M.-S., and Park, S. (2004). Photoproduction of hydrogen from acetate by a chemoheterotrophic bacterium Rhodopseudomonas palustris P4. *International Journal of Hydrogen Energy* 29, 1115–1121. doi:10.1016/j.ijhydene.2003.11.008

Ormerod, J. G., Ormerod, K. S., and Gest, H. (1961). Light-dependent utilization of organic compounds and photoproduction of molecular hydrogen by photosynthetic bacteria; relationships with nitrogen metabolism. *Archives of Biochemistry and Biophysics* 94, 449–463. doi:10.1016/0003-9861(61)90073-X

Pintucci, C., Padovani, G., Giovannelli, A., Traversi, M. L., Ena, A., Pushparaj, B., et al. (2015). Hydrogen photo-evolution by Rhodopseudomonas palustris 6A using

pre-treated olive mill wastewater and a synthetic medium containing sugars. *Energy Conversion and Management* 90, 499–505. doi:10.1016/j.enconman.2014.11.045

Qi, X., Ren, Y., Tian, E., and Wang, X. (2017). The exploration of monochromatic near-infrared LED improved anoxygenic photosynthetic bacteria Rhodopseudomonas sp. for wastewater treatment. *Bioresource Technology* 241, 620–626. doi:10.1016/j.biortech.2017.05.202

Ranaivoarisoa, T. O., Rengasamy, K., Guzman, M. S., Singh, R., and Bose, A. (2017). Towards sustainable bioplastic production in resource limited environments using the photoferroautotrophic and photoelectroautotrophic bacterium Rhodopseudomonas palustris TIE-1. *bioRxiv* , 214551doi:10.1101/214551

Sepúlveda-Muñoz, C. A., de Godos, I., Puyol, D., and Muñoz, R. (2020). A systematic optimization of piggery wastewater treatment with purple phototrophic bacteria. *Chemosphere* doi:10.1016/j.chemosphere.2020.126621

Trüper, H. G. and Pfennig, N. (1981). Characterization and Identification of the Anoxygenic Phototrophic Bacteria. In *The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria*, eds. M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (Berlin, Heidelberg: Springer). 299–312. doi:10.1007/978-3-662-13187-9_18

Verostko, C. E., Carrier, C., and Finger, B. W. (2004). Ersatz Wastewater Formulations for Testing Water Recovery Systems. 2004–01–2448. doi:10.4271/2004-01-2448

Zhao, X., Zhao, C., Yang, S., and Luo, J. (2019). The Growth-promoting Mechanism of Unusual Spectroscopic Form of LH2 (LH4) from Rhodopseudomonas palustris CGA009 in Low Light. *Photochemistry and Photobiology* 95. doi:10.1111/php.13134

Zhi, R., Yang, A., Zhang, G., Zhu, Y., Meng, F., and Li, X. (2019). Effects of light-dark cycles on photosynthetic bacteria wastewater treatment and valuable substances production. *Bioresource Technology* 274, 496–501. doi:10.1016/j.biortech.2018.12.021