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Isolating and Manipulating Microorganisms using Ureolysis for Creating Extraterrestrial Microbial Biotechnology Systems

Honors Thesis

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Department: Chemistry

Advisor: Justin Biffinger, Ph.D.

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Abstract

The conversion of CO_2 into valuable feedstocks, such as high energy sugars would create paradigm shifting technologies for applications on earth and for interplanetary exploration. Microbes and microbe consortia may be one way to accomplish this conversion. Approximately 70% of the Earth's microorganisms live in the dark marine biosphere (DMB). The DMB, which covers more than two-thirds of the Earth, is known as the most isolated region of the Earth's largest CO_2 sink. Despite its role in reducing CO_2 and its vast majority of microorganisms, only about 5% of the sea floor has been explored. Due to the limited knowledge of the DMB and its microorganisms it, it is one of the best resources in discovering new dark carbon fixation pathways and carbon fixing microorganisms. We will explore how some DMB microorganisms may use urea as a nitrogen source for fixing carbonate. To start, microbes found in sediment extracted from the sea floor of the Gulf of Mexico were grown, in 96-wellplates, under varying concentrations of HCO_3^-, urea, and acetate. The growth of the microbes was monitored using OD600 readings with a plate reader. Consortia which appeared to show growth were transferred to 10mL of the successful media and continued to be monitored. Growth was confirmed by using IR spectroscopy and successfully isolating DNA from the consortia. Following confirmed growth, one successful consortia, grown with a media containing 10mM HCO_3^-, 10mM urea, and 1mM acetate under anaerobic conditions with a pH of 7.6 and a temperature of 4˚C was followed, using iron chromatography, in a 72-day experiment to determine how the levels of HCO_3^-, urea, and acetate changed with time and the success of carbonate fixation within the consortia. Individual microbes from the consortia and their DNA are also to be isolated.

Acknowledgements

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Introduction

In order to sustain extraterrestrial life in outer space or on another planet, specifically Mars, food, medicine, fuel, and more will be essential. Many of these necessary products, also including plastics and adhesives, are composed primarily of carbon, hydrogen, oxygen, and nitrogen atoms [8]. These essential molecules can be found in the form of CO_2 and N_2, in the Martian atmosphere, and H_2O, in the surface water on the planet, and could ideally be manipulated to help produce the desired and necessary products [5]. Some products may be produced using physicochemical methods or photosynthetic organisms, such as plants and algae, but heterotrophic microbial production systems may prove to be the most effective approach to producing a varied of desired products [2]. On Earth, bio-products are generated rapidly by using heterotrophic microbes with high concentrations of readily metabolized organic substrates [3]. The rate and efficiency of a microbial system can be based on the type of organic substrate used. Typically, sugars, such as D-glucose, which are plentiful in energy and carbons, are the chosen substrate for commercial terrestrial microbial production systems and experimentation [4]. Less complex sugars may also prove to be effective at supporting rapid rates of growths in these systems.

If microbial bio-manufacturing platforms are to be used on Mars, or other planetary bodies, local materials must be used to produce the carbon substrates while on site, but this has its problems. While on Earth, sugar-based substrates can be made efficiently and inexpensively from plant biomass [7]. In space and on Mars these methods will not be accessible, and another approach will be necessary. There are known processes, such as photo/electrochemical and thermal catalytic systems, which have proven to make smaller organic compounds and alcohols from CO_2, which could be found in the Martian atmosphere and, as shown in Figure 1, is one of the major wastes not recycled during space travel. However, these processes have not been developed enough to produce the more complex molecules, such as sugars, due to the expense compared to already successful terrestrial methods of producing sugars and a few technical challenges [6]. A new method, or perhaps a new microbe, may need to be discovered or researched further

to help produce these sugars efficiently enough to produce necessary products when exploring, or possibly inhabiting, new planetary bodies, such as Mars.

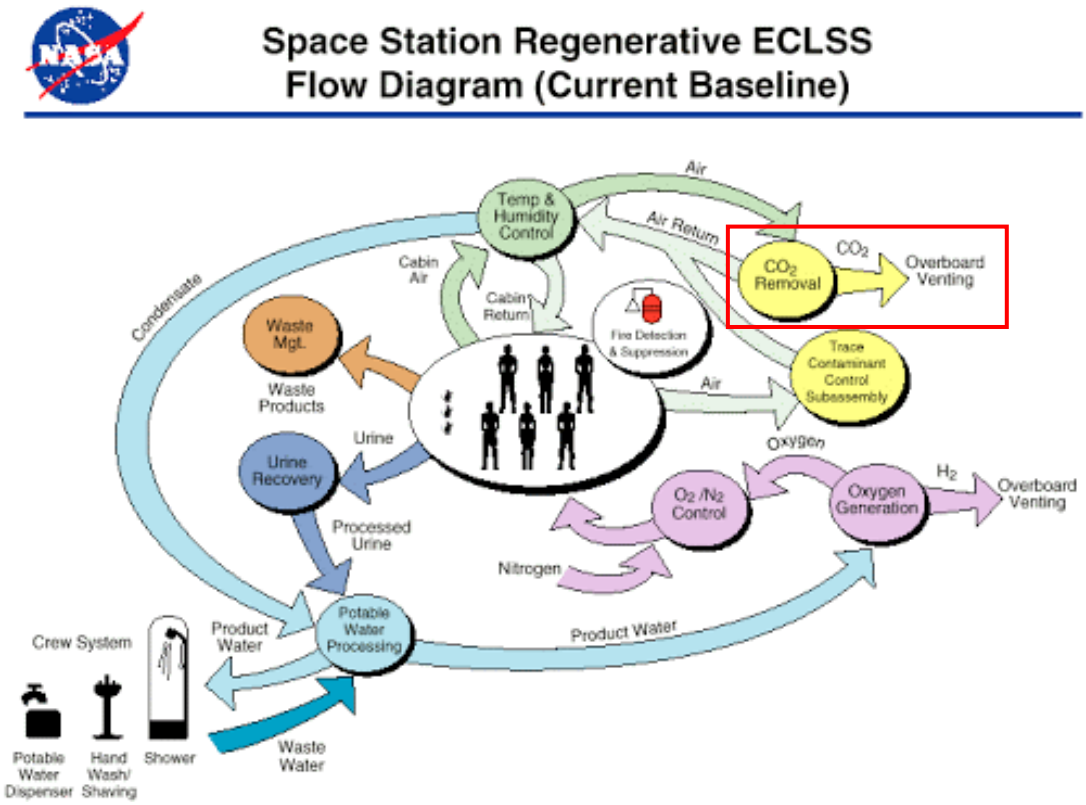

Figure 1 Functioning of the environmental control and life support system on the International Space Station, where a major nonrecycled waste is Carbon Dioxide

The dark marine biosphere (DMB) is an excellent place to begin searching for a microorganism, which can be used in these microbial biotechnology systems. The DMB covers over two-thirds of the surface of the Earth and is home to approximately 70% of the Earth's microorganisms. It contributes immensely to Earth's largest CO_2 sink and is known as the most isolated region of it. Despite the role it plays in reducing CO_2 and the incredible numbers of microorganisms that are known to be there and likely even more living there unknown, very little of the DMB has been researched and discovered. In fact, only about 5% of the sea floor has been explored [1]. Due to the limited knowledge of the DMB and the microorganisms it houses, it may prove to be one of the best resources in discovering new dark carbon fixation pathways and carbon fixing

microorganisms. If discovered, these microorganisms may be researched and utilized to fix carbon from the Martian atmosphere to help produce useful carbon substrates for an effective microbial biotechnology system. The goal of this project is to discover and identify a microbe consortium, from the DMB, which can effectively participate in carbon fixation under anaerobic conditions, using Urea as a nitrogen source. Urea was chosen as the nitrogen source for this experiment because it would be easy to access in space, either in the form of slow-release formate urea fertilizers, or if necessary, through urine, which is how humans naturally expel excess nitrogen in the form of urea. A proposed mechanism for how carbon fixation and ureolysis, the breakdown of urea, may be connected is shown in Figure 2.

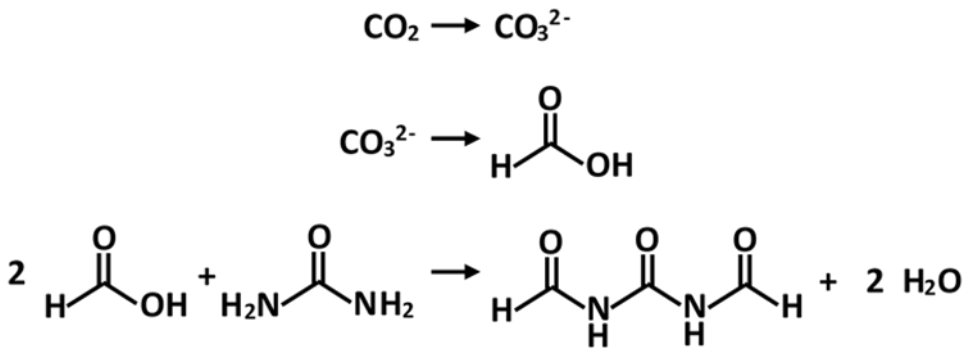

Figure 2 Possible mechanism to combine carbon fixation and ureolysis

Methodology

This project has had three separate phases. In Phase I, different salt medias were made to determine the best environment to grow a microbe consortium with DMB sediment. Phase II included selecting successful medias to grow on a larger scale and to monitor further. In Phase III, the most successful consortium was monitored even closer on a 72-day experiment to monitor the fluctuating concentration of the salt media.

For Phase I, the first step was to make well plates with solutions containing varying concentrations of urea, HCO_3^-, and acetate. The base for each solution was a modified ONR7A salt media, shown in Table 1. Each well contained a version of the salt solution containing either 1 mM or 10 mM urea, 1mM or 10 mM HCO_3^-, and 0 mM or 1 mM

acetate. The well plate set-up and media formulations are shown in Table 2. 2 µL of sediment sample was loaded into each well, as well as 1 µL vitamin mix and 1 µL of an Fe^{2+} solution to help encourage growth. Four well plates in total were created, two using a Modified ONR7A Salt Medium at a pH of 7.6 as a base, one to be held at 4˚C and one to be held at 27˚C, and two using a Modified ONR7A Salt Medium at a pH of 8.5 as a base, again being held at either 4˚C or 27˚C. All well plates were held under anaerobic conditions, which was accomplished using an anaerobic chamber. A plate reader was used to take OD600 readings every few days to monitor the growth of each sample.

Modified ONR7A Medium**			
	Quantity Added	Formula Weight	Concentration (mM)
Solution 1:			
NaCl	22.79 g	58.44	410.50
KCl	0.72 g	74.55	10.17
NaBr	83.00 mg	102.89	0.85
$NaHCO_3$	31.00 mg	84.01	0.39
H_3BO_3	27.00 mg	61.83	0.46
NaF	2.60 mg	41.99	0.07
Na_2HPO_4 x 7 H_2O	89.00 mg	268.07	0.35
TAPSO	1.30 g	259.28	5.28
H_2O	500.00 mL		
Adjust to pH 7.6 or 8.5 with NaOH			
Solution 2:			
$MgCl_2$ x 6 H_2O	11.18 g	203.3	57.89
$CaCl_2$ x 2 H_2O	1.46 g	147.01	10.45
$SrCl_2$ x 6 H_2O	24.00 mg	266.62	0.09
H_2O	450.00 mL		
Autoclave the solutions separately. Solutions should be mixed after autoclaving when they have cooled to at least 50°C			

Table 1 Formulation of Modified ONR7A Salt Medium

	1	2	3	4	5	6	7	8	9	10	11	12
A	1 mM HCO3- 10 mM Urea	10 mM HCO3- 10 mM Urea	10 mM HCO3- 10 mM Urea 1 mm Acetate	1 mM HCO3- 1 mM Urea	10 mM HCO3- 1 mM Urea	10 mM HCO3- 1 mM Urea 1 mm Acetate	1 mM HCO3-	10 mM HCO3-	10 mM HCO3- 1 mm Acetate	1 mM HCO3-	1 mM HCO3- 10 mM Urea	10 mM HCO3- 1 mM Urea
B	1 mM HCO3- 10 mM Urea	10 mM HCO3- 10 mM Urea	10 mM HCO3- 10 mM Urea 1 mm Acetate	1 mM HCO3- 1 mM Urea	10 mM HCO3- 1 mM Urea	10 mM HCO3- 1 mM Urea 1 mm Acetate	1 mM HCO3-	10 mM HCO3-	10 mM HCO3- 1 mm Acetate	1 mM HCO3-	1 mM HCO3- 10 mM Urea	10 mM HCO3- 1 mM Urea
C	1 mM HCO3- 10 mM Urea	10 mM HCO3- 10 mM Urea	10 mM HCO3- 10 mM Urea 1 mm Acetate	1 mM HCO3- 1 mM Urea	10 mM HCO3- 1 mM Urea	10 mM HCO3- 1 mM Urea 1 mm Acetate	1 mM HCO3-	10 mM HCO3-	10 mM HCO3- 1 mm Acetate	1 mM HCO3-	10 mM HCO3- 10 mM Urea	10 mM HCO3- 1 mM Urea 1 mm Acetate
D	1 mM HCO3- 10 mM Urea	10 mM HCO3- 10 mM Urea	10 mM HCO3- 10 mM Urea 1 mm Acetate	1 mM HCO3- 1 mM Urea	10 mM HCO3- 1 mM Urea	10 mM HCO3- 1 mM Urea 1 mm Acetate	1 mM HCO3-	10 mM HCO3-	10 mM HCO3- 1 mm Acetate	1 mM HCO3-	10 mM HCO3- 10 mM Urea	10 mM HCO3- 1 mM Urea 1 mm Acetate
E	1 mM HCO3- 10 mM Urea	10 mM HCO3- 10 mM Urea	10 mM HCO3- 10 mM Urea 1 mm Acetate	1 mM HCO3- 1 mM Urea	10 mM HCO3- 1 mM Urea	10 mM HCO3- 1 mM Urea 1 mm Acetate	1 mM HCO3-	10 mM HCO3-	10 mM HCO3- 1 mm Acetate	1 mM HCO3-	10 mM HCO3- 10 mM Urea 1 mm Acetate	
F	1 mM HCO3- 10 mM Urea	10 mM HCO3- 10 mM Urea	10 mM HCO3- 10 mM Urea 1 mm Acetate	1 mM HCO3- 1 mM Urea	10 mM HCO3- 1 mM Urea	10 mM HCO3- 1 mM Urea 1 mm Acetate	1 mM HCO3-	10 mM HCO3-	10 mM HCO3- 1 mm Acetate	1 mM HCO3-	10 mM HCO3- 10 mM Urea 1 mm Acetate	
G	1 mM HCO3- 10 mM Urea	10 mM HCO3- 10 mM Urea	10 mM HCO3- 10 mM Urea 1 mm Acetate	1 mM HCO3- 1 mM Urea	10 mM HCO3- 1 mM Urea	10 mM HCO3- 1 mM Urea 1 mm Acetate	1 mM HCO3-	10 mM HCO3-	10 mM HCO3- 1 mm Acetate 1 mm Acetate	1 mM HCO3-	1 mM HCO3- 1 mM Urea	
H	1 mM HCO3- 10 mM Urea	10 mM HCO3- 10 mM Urea	10 mM HCO3- 10 mM Urea 1 mm Acetate	1 mM HCO3- 1 mM Urea	10 mM HCO3- 1 mM Urea	10 mM HCO3- 1 mM Urea 1 mm Acetate	1 mM HCO3-	10 mM HCO3-	10 mM HCO3- 1 mm Acetate	1 mM HCO3-	1 mM HCO3- 1 mM Urea	

Table 2 Formulation of each well media. Wells of the same color contain the same formulation of media. Media in columns 11 and 12 represent controls of medias in columns 1-6. Medias in control wells were not given any sediment samples.

Once growth was thought to occur, based on the OD600 readings of the well plates, Phase II was started. 100 µL of samples of apparent growing consortia were removed from their wells and transferred to vials containing 10mL of the successful media from their well plates. The growth of each consortia, then continued to be monitored using OD600 readings, every few days. Once a sample showed growth once again, a small amount of the sample was frozen, and some was used in DNA isolation to be sent in and analyzed. One sample, growing with 10 mM urea, 10 mM HCO_3^-, and 1 mM acetate, appeared to be thriving more so than the others. This sample was held at a pH of 7.6 and a temperature of 4˚C.

The successful sample was then used in Phase III. This phase consisted of a 72-day experiment to monitor the concentrations of urea, HCO_3^-, and acetate, and how they changed with time in hopes of observing carbonate fixation. To conduct this, five serum bottles, each with 80 mL of the successful pH 7.6 media, were used. Two of the bottles contained glass beads (to observe if any of the microbes in the consortium made a biofilm and if it affected carbonate fixation), two had no glass beads, and one served as a control. Two additional serum bottles, one with glass beads and one without, were also created to

be untouched throughout the experiment and to be used for DNA isolation. These bottles were all stored under anaerobic condition at a temperature of 4˚C. Every three days or so, 3 samples were taken from each serum bottle: 1 to be frozen, 1 to track the pH, and 1 used in an IC reading to track concentrations. The urea concentrations were also attempted to be tracked using a HPLC with a Urea column and a Urea Nitrogen Colorimetric Detection Kit. At the end of the 72 days a sample from each of the undisturbed vials was used for DNA isolation to be sent in and further analyzed.

Results and Data

After Phase I, a total of 22 samples were removed from their well plates to be grown on a larger scale. Thirteen were from a pH of 7.6, nine being held at 4˚ and four being held at 27˚. Nine were from a pH of 8.5, five being held at 4˚ and four being held at 27˚. The media formulations resulted in apparent growth are shown in Table 3.

Media	[HCO3-]	[Urea]	[Fe2+]	[Acetate]	pH	Temperature
1	1 mM	10 mM	.0016 mM		7.6	4
2	1 mM	10 mM	.0016 mM		7.6	27
3	10 mM	10mM	.0016 mM	1 mM	7.6	4
4	10 mM	10mM	.0016 mM	1 mM	7.6	27
5	1 mM	1 mM	.0016 mM		7.6	4
6	10 mM	1 mM	.0016 mM	1 mM	7.6	4
7	10 mM	1 mM	.0016 mM	1 mM	7.6	27
8	10 mM	1 mM	.0016 mM		7.6	27
9	1 mM	10 mM	.0016 mM		8.5	4
10	10 mM	10 mM	.0016 mM	1 mM	8.5	4
11	10 mM	10 mM	.0016 mM	1 mM	8.5	27
12	1 mM	1 mM	.0016 mM		8.5	4
13	10 mM	10 mM	.0016 mM		8.5	4
14	10 mM	10 mM	.0016 mM		8.5	27
15	10 mM	1 mM	.0016 mM		8.5	27

Table 3 Successful media formulations from well plates

Of the 22 samples that were attempted to be grown on a larger scale, in Phase II, 9 appeared to continue to grow. These successful media were Medias 3 (three samples), 7 (one sample), 8 (one sample), 9 (one sample), 10 (one sample), and 12 (two samples), as shown in Table 3. DNA isolation was attempt on all 9 of these samples. DNA was successfully isolated for 5 of them, including from two samples of Media 3, one from Media 9, one from Media 10, and one from Media 12.

The most successful consortium was grown from Media 3 and moved on to Phase III. This sample was tracked in a 72-day experiment. The IC results from the media throughout the experiment are shown in Figure 3. The IC readings indicated that the acetate in the solution was diminished to 0 by approximately day 15 and the level of carbonate lowered throughout the 72 days. The vials with and without beads seemed to show no significant difference. Unfortunately, the levels of urea could not be shown with the IC data. An HPLC ran with a urea column also failed to show the change in the urea levels of our salt solution. A final attempt to monitor the Urea concentrations was attempted using a Urea Nitrogen Colorimetric Detection Kit. The results of this test indicated that there was no significant change in the Urea concentrations throughout the 72-day experiment. The results of the Urea Nitrogen Colorimetric Detection Kit are shown in Figure 4.

After the 72-day experiment, the five isolated DNA samples from Phase II, as well as DNA isolated from the end of the 72-day experiment, were sent in to be identified. All of the isolated DNA came from consortia of bacteria, so many different bacteria were identified. Most notably, there were five species in the 72-day experiment DNA that were not able to identified on a species level, indicating that some bacteria that has yet to be discovered may be present. All bacteria, in all of the consortia, were determined to not be harmful.

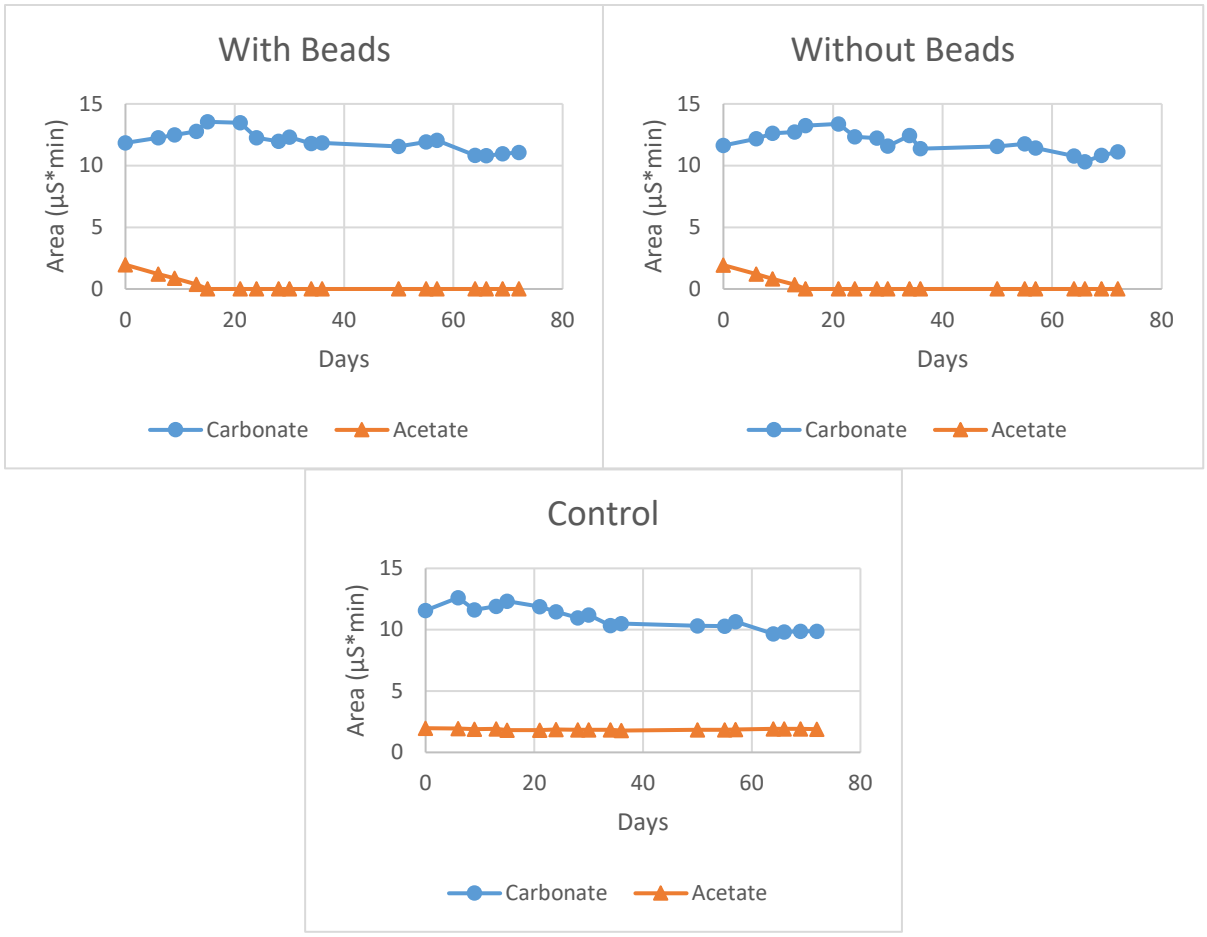

Figure 3 Results of Ion Chromatography for 72-day Experiment

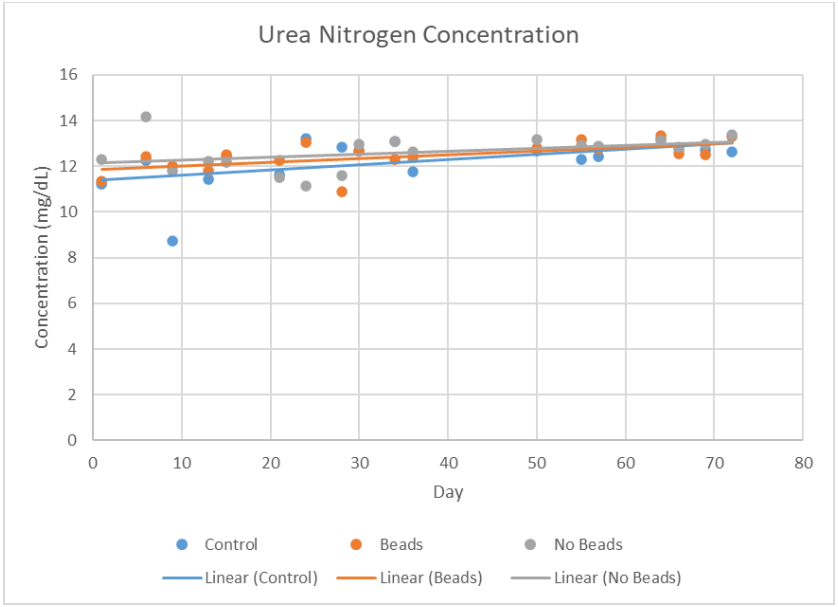

Figure 4 Urea concentration throughout the 72-day experiment, as determined using a Urea Nitrogen Colorimetric Detection Kit.

Discussion and Conclusion

This is the very early stages of this area of research, so there are few definitive conclusions to draw. Of the different medias that were successful there were no clear concentrations of HCO_3^-, Urea, or Acetate that microbes from the sediment sample favored the most. However, there was consistently more diversity and growth in microbes that were cultured at 4˚C instead of 27˚C, as well as at a pH of 7.6 instead of a pH of 8.5. The 72-day experiment did not indicate enough change to determine if carbonate was being used as a sole carbon source or if/how the microbes were interacting with the urea present in the solution. This could be due to the cells not growing at a high enough density, or for a long enough time, to consume a detectable amount of carbonate and urea. In the future a longer, more in depth look at the microbe growth may be necessary. Since there were many unidentified microbes, there is also future work that needs to be done to isolate and identify individual bacterial species. Throughout each phase of this experiment, samples of successfully growing consortia were frozen in order to continue on with this experiment in many different ways in the future. Ultimately, this research was successful in creating a solid base to add to in further investigations, when considering what will be necessary for planetary colonization.

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