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The Essential Nutrients for Methanococcus maripaludis: Growth on Montmorillonite

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COEHP: An Honors Thesis

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Acknowledgements

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Abstract

When the topic of life on Mars gets brought up, our minds automatically jump to thinking of the definition of life as any advanced creature. But I am looking at life on a much smaller scale. A group of Archaea called methanogens live on Earth in some very normal as well as extreme conditions, and in the last few decades scientists have been questioning if they could even be found on the planet Mars. These microbes use hydrogen gas as their energy source and produce methane as a byproduct, which has been found in relatively significant amounts on Mars. It has been known for quite some time that methanogens grow and/or survive under some Mars-like conditions. This is because there is carbon dioxide, hydrogen gas, potential liquid water, and anaerobic environments (no oxygen) on Mars. All of these things are conducive to the survival of methanogens. Some of the soil on Mars has been shown to resemble that of the silicate montmorillonite. My research dealt with the specific methanogenic strain called *Methanococcus maripaludis*. It has been documented that *M. maripaludis* did not grow on montmorillonite, but would if growth medium was present. My research sought to determine which essential compound or compounds in the medium allowed for growth of *M. maripaludis* on montmorillonite. This was done by creating eight conditions (including the control) in which different compounds or solutions were taken out of the growth medium in order to see what the effect of its absence was. This allowed me to draw certain conclusions about the different situations and see which was essential for the growth of *M. maripaludis*. The findings of my research were that there was indeed an essential compound, and it was that of the salt solution. The *M. maripaludis* could not grow at all without it, and it grew minimally with only the salt solution present on the soil.

Introduction

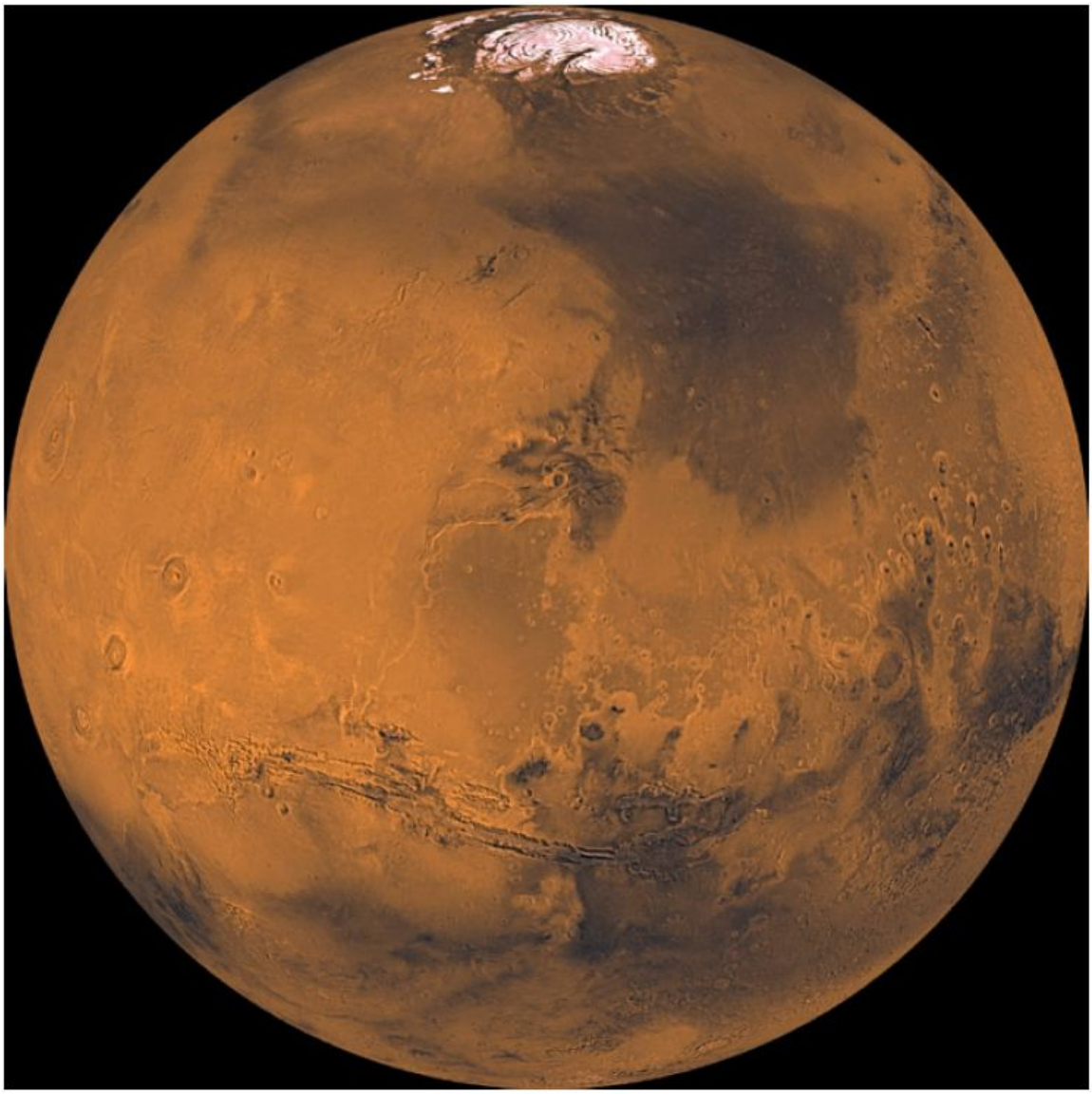

Figure 1. Photograph of the planet Mars as seen by the Hubble space telescope (Wild, 2014).

I was a sophomore sitting in Dr. Kral's Microbiology course when it struck me that I enjoyed the subject matter that this class was covering. I had been telling myself to start looking for research positions around this time so I did a quick search on Dr. Kral's research and found out that the main theme of his research was life on Mars. This immediately intrigued me. It was not until I went to his office to start on this research journey that I fully understood what type of

life we were talking about. Like many people when you hear life on Mars, our minds often drift to Marvin the Martian or some equivalent. But that was not what this lab's focus was.

Before I go any further I would like to give a brief overview of this place we call the Red Planet. Mars is about one-sixth the size of Earth and it is approximately 142 million miles from the sun (Wild & Dunbar, 2008). The environment of Mars is fairly hostile; its temperature varies across its entirety, it is covered in rocky craters and volcanoes, and the planet is susceptible to severe dust storms due to the red particles that sit atop its surface (Wild & Dunbar, 2008). Liquid water is essential to life as we know it, and scientists are searching for any evidence of water on the planet currently. But there is some evidence showing that water was once on the surface. There have been images of the terrain of the planet in which carved out gullies are shown, which was most likely created by the flow of rivers that emptied into larger bodies of water (Carr, 1996).

You may be asking why would we look to Mars for life out of all of the other planets and moons throughout the galaxies and even nebula? The answer is that it is the easiest to explore. It takes extensive resources, time, and money to be able to explore a region of space. There are a few options for life in space that have been considered. There is Titan and Enceladus, which are both moons of Saturn. Titan is a reasonable option because it has a very dense atmosphere and there is evidence of liquid water on its surface (Stofan et al., 2006). Enceladus is also an option because there is thought to be liquid water beneath its southern polar cap, as well as having notable amounts of methane and water vapor present (McKay et al., 2008). These options may sound relatively persuasive, except when faced with the fact that they are very far from Earth, which makes exploration and study quite difficult. It is much more economical to pour funds into

the exploration of Mars because of its distance as well as the environmental aspects that make it conducive for life.

Before I get into the types of life that we expect are living on Mars, I think it is important to mention some other key aspects of Mars that scientists have taken note of in the previous years. Carbon dioxide and methane have been found in relatively high amounts on Mars. In fact, there was a very large plume of methane identified using IR (infrared) spectrometers on Earth that found an estimated 19,000 metric tons of this gas (Mumma et al., 2009). Also over this last year, there was also a significant amount of methane located at the Gale crater of Mars discovered over a 20-month period of observation using a tunable laser spectrometer (Webster et al., 2014). This gas has been found in many regions of Mars, and it is of note to mention that the gas has a half-life between 300 and 600 years; it can be destroyed by large amounts of UV radiation (Stroker & Bullock, 1997). In the beginning of this introduction I mentioned that dust storms were common on this planet, these storms are capable of moving the gas around to different areas of the planet. Yet we still see significant amounts of methane in certain areas, telling us that there must be a source continuously supplying the gas (Wild & Dunbar, 2008). There could be a number of culprits, if you will, for this concentration of methane on the Red Planet. The first that comes to mind would have to be volcanoes, because as I stated earlier there is quite a large number of volcanoes found on Mars. According to the United States Geological Survey, volcanoes release large amounts of methane and sulfur dioxide (2010). This fact alone eliminates volcanoes as the source because the main gasses on Mars include methane, argon, carbon dioxide, nitrogen, and trace amounts of oxygen and carbon monoxide (Greicius & Dunbar, 2012). It could also come from meteorites, but they strike the surface of Mars too rarely for there to be significant amounts of methane (Atreya, Mahaffy, & Wong, 2007; Krasnopolsky,

Maillard, & Owen, 2004). The third and most likely source of this methane is microorganisms living in the subsurface of Mars.

There are three domains of life as we know it: Eukaryotes, Bacteria, and Archaea (Smith, 2005). The domain that I am going to be honing in on is that of Archaea, and specifically the types of cells we call methanogens. Methanogens, as you can probably guess from their name, produce the gas methane (CH_4). Methanogens play a vital role in the global carbon cycle and they are responsible for the vast majority of methane found in the Earth's atmosphere (Pavlov et al., 2000). These microorganisms are obligate anaerobes (survive only in the absence of oxygen) that carry out the reduction of carbon dioxide to methane by using molecular hydrogen as their source of energy (Leon & Larson, 2011). They have been found in very extreme environments such as hydrothermal vents, hot springs, permafrost, and many others (Leon & Larson, 2011; Morozova et al., 2005). This shows us that it would not be shocking for the organism to be able to thrive in Martian conditions. Most methanogens need water, a carbon source, and hydrogen as their energy in order to thrive (Leon & Larson, 2011) As stated earlier, the byproduct of methanogen metabolism is methane, and the amount of this gas present may point to how well they are thriving.

Now that we have covered some of the main bits of background information, we can delve into the main topics that I have been tasked with in my research. Spectroscopic analyses have shown that there is a significant similarity between the energies and band strengths in reflectance spectra between montmorillonite and the soil on Mars (Bishop, Pieters, Burns, Edwards, Mancinelli, & Froschl, 1995). Montmorillonite is a silicate or bentonite that contains sodium, calcium, aluminum, silicon, hydrogen, and oxygen. The high sulfate content of the soil on Mars provides some evidence that ferric sulfate minerals may be present, and allow it to be a

good analog to use in experimentation (Bishop et al., 1995). There is a special character of ferrihydrite in the visible to near infrared section that is similar to that of bright regions on Mars surface, but ferrihydrite contains too much iron to be the main component of the surface. Since significant amounts of sulfur have been found in the soil on Mars, mixtures of sulfates have been added to synthetic montmorillonites, which has proven to exhibit very similar spectroscopic readings to those on Mars (Bishop et al., 1995). Montmorillonite is a smectite group phyllosilicate, and its presence on Mars could have aided in some of the past climate conditions on the planet (Clark et al., 2007). Orbiting instrumentation has been used to assess that there are indeed phyllosilicates present in certain areas of the planet; some areas have more present than others (Clark et al., 2007). These studies add to the well known stipulation that montmorillonite is indeed a main component of the soil on Mars surface. This is key to my research because it gives me the conditions that I can use to replicate Mars- like environments. My project dealt with a specific strain of methanogen called *Methanococcus maripaludis*. It has been shown that *M. maripaludis* cannot grow on the Mars analog montmorillonite. Other methanogens have been shown to be able to grow on montmorillonite in the Kral lab, but why not *M. maripaludis*? However, it will grow on montmorillonite with growth media present, so what specific compound or compounds in the media allow for this growth?

My research showed that the essential nutrient for *M. maripaludis* was salt solution. This is not surprising since *M. maripaludis* is a halophile (salt-loving). This is certainly relevant to the search for life on Mars. Researchers at Arizona State University have discovered the first evidence for chloride deposits, otherwise known as salts, on the surface of Mars (Burnham & Johnson, 2008). These salt deposits were found in over 200 locations across the surface of Mars, and this discovery also adds to the evidence of liquid water that was previously discussed. These

salt deposits that were discovered imply that Mars was much wetter long ago, because nearly all of them are located in depressed areas of land indicating the possibility of water erosion (Burnham & Johnson, 2008; Stroker & Bullock, 1997). This discovery also has huge implications for the water debate, a team of astrobiologists with NASA have stated that there is reason to believe that these salts could cause liquid water to form when it comes in contact with water in its solid form, otherwise known as ice (Fischer, Martinez, Elliott, & Renno, 2014). These new discoveries are exciting because it is working to link all of the previous research that has been done. My research has shown that the essential nutrient for *M. maripaludis* survival is indeed salt solution, which has been shown to exist in quite large amounts in these deposits along the planet's surface. My results and their implications will be elaborated on in the following sections.

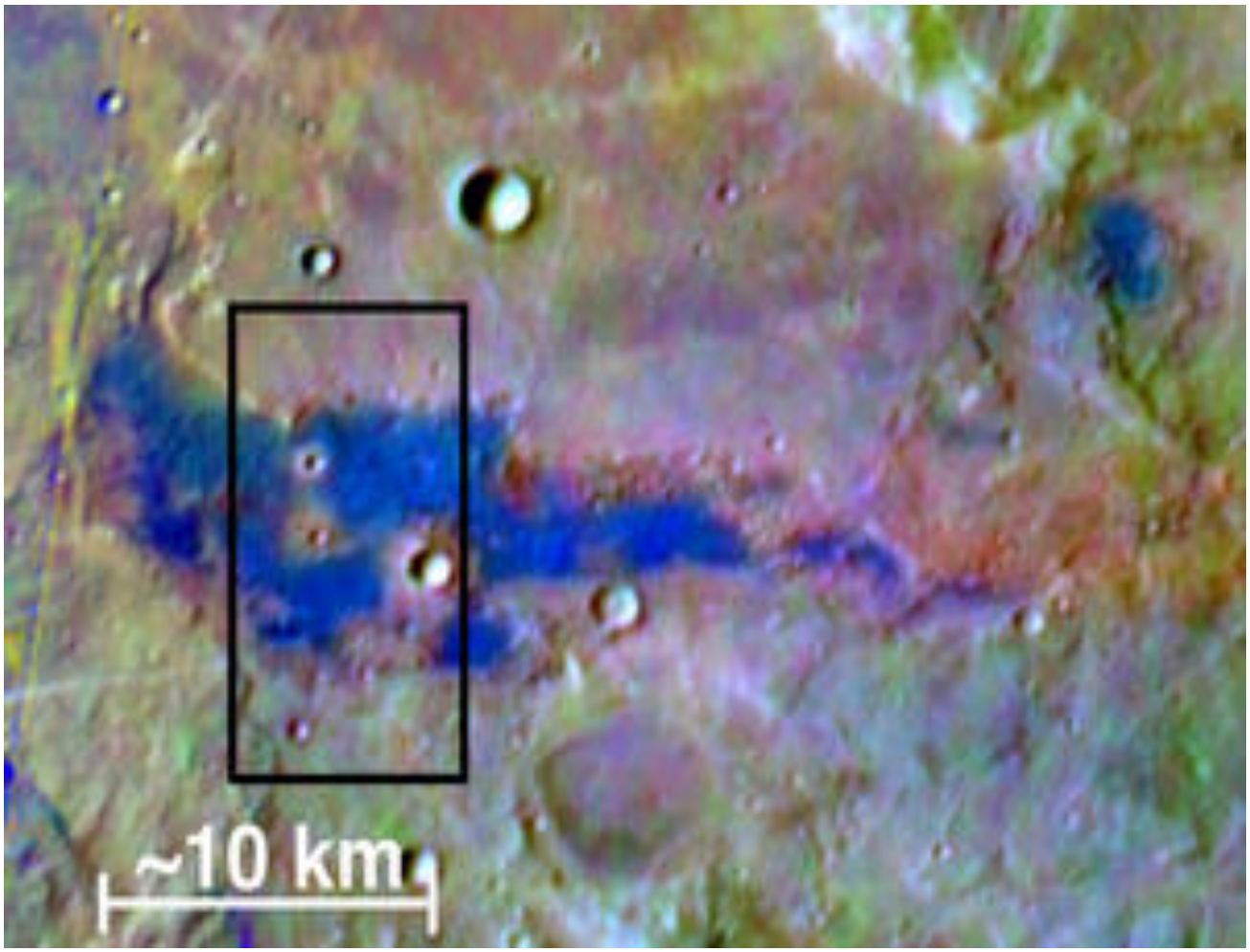

Figure 2. An image of the salt deposits recently found in the southern highlands of Mars ("Salt Deposits Found in Martian Highlands", 2008).

Materials and Methods

Organisms and Media. There was only one strain of methanogen used in my experiments and that was *Methanococcus maripaludis*. I split up my experiment into eight conditions, which will be described in detail. *M. maripaludis* was grown in the standard methanogen growth (SMG) medium called MSH medium. For a list of the ingredients found in MSH medium, please refer to Appendix A.

Growing M. maripaludis in MSH Media. Using an electronic scale and micropipettes, ingredients and solutions for the MSH medium were measured into separate Erlenmeyer flasks that represented the eight different conditions I had assigned for my experimentation. Here are the eight conditions:

1. Control: MSH medium with no montmorillonite present.
2. Montmorillonite + Salt Solution: Only salt solution present.
3. Montmorillonite - Solution A: Everything except Solution A.
4. Montmorillonite - Solution B: Everything except Solution B.
5. Montmorillonite – Solution D: Everything except Solution D.
6. Montmorillonite – Salt Solution: Everything except salt solution.
7. Montmorillonite + Buffer: Only buffer present, no other solutions.
8. Montmorillonite + MSH medium: Everything present.

The flasks were transferred from the work area where I measured everything out to the Coy anaerobic chamber. This chamber is an oxygen free bubble like contraption that has glove ports so that you can work with the contents that are inside the chamber. Once inside the chamber, a sterile buffer solution saturated with carbon dioxide was poured into each flask so that they were up to the 50-mL line. I then swirled the flasks in the chamber so that the contents would dissolve

and become an even solution. The flasks were then left overnight in the chamber so that they could become fully saturated with carbon dioxide and deoxygenated. The next day, the media in the flasks were carefully poured into culture tubes (test tubes). I had three test tubes for each condition, making for a total of 24 tubes. Every test tube except for the control contained 0.5 grams of montmorillonite, then they were put into the anaerobic chamber so that the eight Erlenmeyer flasks could be poured into their respective test tubes. Each flask had 50 mL of medium in it; I poured 10 mL of medium into each test tube. The tubes were labeled according to their specific condition, along with my initials and the date that they were to be inoculated with methanogen. Once the tubes were filled with their respective media, I sealed them with blue rubber stoppers while still in the chamber. At this point I could take them out of the chamber so that they could be crimped. Crimping was done by putting an aluminum crimp on top of the rubber stopper and using an instrument to fully tighten it around the edges, prohibiting the rubber stopper from coming off as well as further sealing the test tube from the outside world to make it as close to Martian conditions as possible. The test tubes were then sterilized in the autoclave.

After sterilization in the autoclave, I used a syringe to add 0.5 mL of 2.5% sodium sulfide (Na_2S) into each of the 24 tubes in order to eliminate any remaining oxygen, about an hour prior to inoculation. An aliquot of 10 mL of *M. maripaludis* culture was placed into each of two sterile centrifugation tubes. I turned on the centrifuge for 15 minutes at 5000 rpm. With the methanogen cells pelleted at the bottom, the supernatant was carefully poured off. Each tube was then re-suspended with 6 mL of the sterile bicarbonate buffer. The process was then repeated once more. The two centrifugation tubes full of culture were then combined so that there was 12 mL of culture in one container. Syringes were then filled with the culture and injected into each of the 24 test tubes. Since there were 24 tubes and 12 mL of culture available, 0.5 mL of methanogen

culture was injected into each individual test tube. Once all of the test tubes were inoculated with *M*. *maripaludis*, 200 kPa of hydrogen gas was added to each test tube using the gassing manifold. The 24 test tubes were then allowed to incubate at room temperature (roughly 20-26 degrees Celsius), and the methanogens were allowed to grow for one week. Once the week had come to an end I measured the methane content in each test tube. This was done through the use of a micro gas chromatograph; 1 mL of gas from each test tube was drawn by way of syringe and run through the gas chromatograph in order to see how much methane there was. For my records I labeled this first day of measurement as 'day one'. I then continued to measure the methane values every three to four days for a total of five measurements or data collections. This was done for all three experimental trials, so that there were data collections for all 24 test tubes for days 1, 4, 8, 12, and 15. It is important to note that this is where my data collection stopped but I did check all tubes again after roughly 20 days and the values had not changed significantly, so it indicated that there had been a plateau. Therefore the 15th day was chosen as a stopping point for the data collection.

Results

The results for *M. maripaludis* methane production in the eight conditions of MSH medium components and montmorillonite clay can be seen in the following figures. The values reported in the figures were examined as averages across the three experiments. In other words, for one condition I would have nine data readings (three for each trial) in which I took the average and standard deviation. This is the value that can be found in these figures. Montmorillonite was present in every situation except the first one, which was the control. The highest average methane concentration for the control was 36.98% (Figure. 3). The highest average for MSH medium and montmorillonite was 31.20% (Figure. 4). There was no methane measured for buffer plus montmorillonite (Figure. 5) or MSH medium without salt solution plus montmorillonite (Fig. 6). The methane concentration for MSH medium without solution D plus montmorillonite was 27.58% (Figure. 7), for MSH medium without solution B plus montmorillonite was 33.38% (Figure. 8) and MSH medium without solution A plus montmorillonite was 6.08% (Figure. 9). The methane concentration for montmorillonite and only salt solution was 5.23% (Fig. 10. There is a summary figure (Figure. 11) showing all eight conditions to make comparisons easier to see.

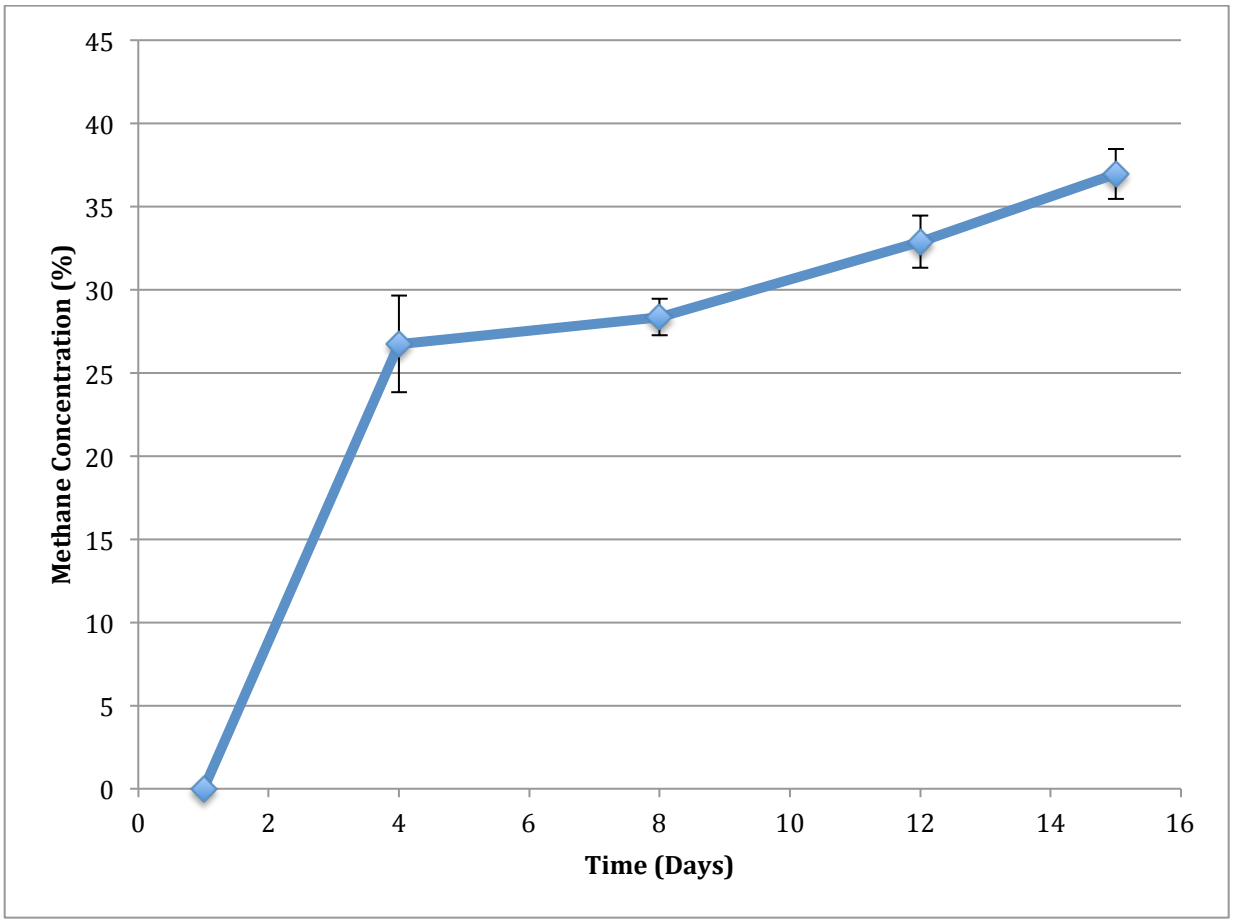

Figure 3. Methane production by *Methanococcus maripaludis* growing in MSH medium (Control).

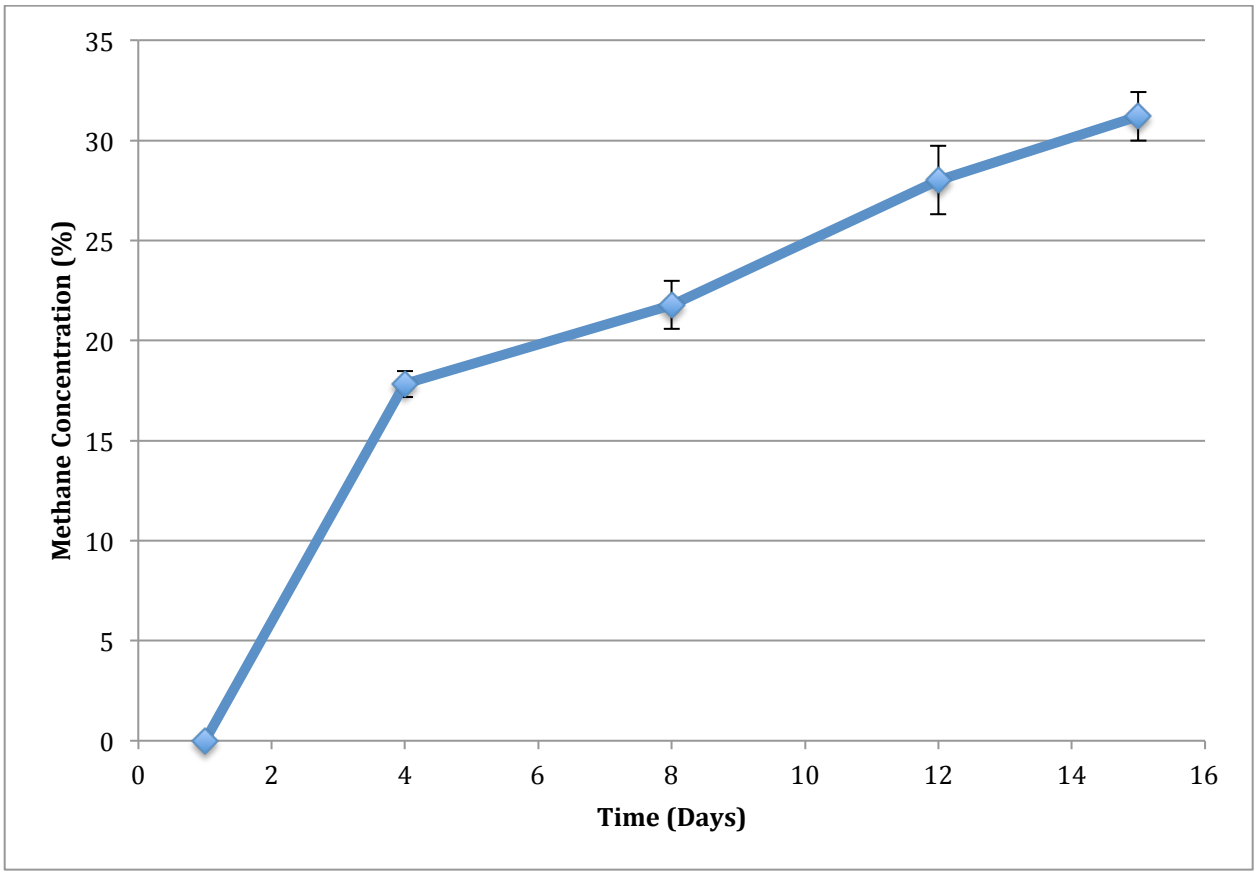

Figure 4. Methane production by *Methanococcus maripaludis* growing in MSH medium while montmorillonite was present.

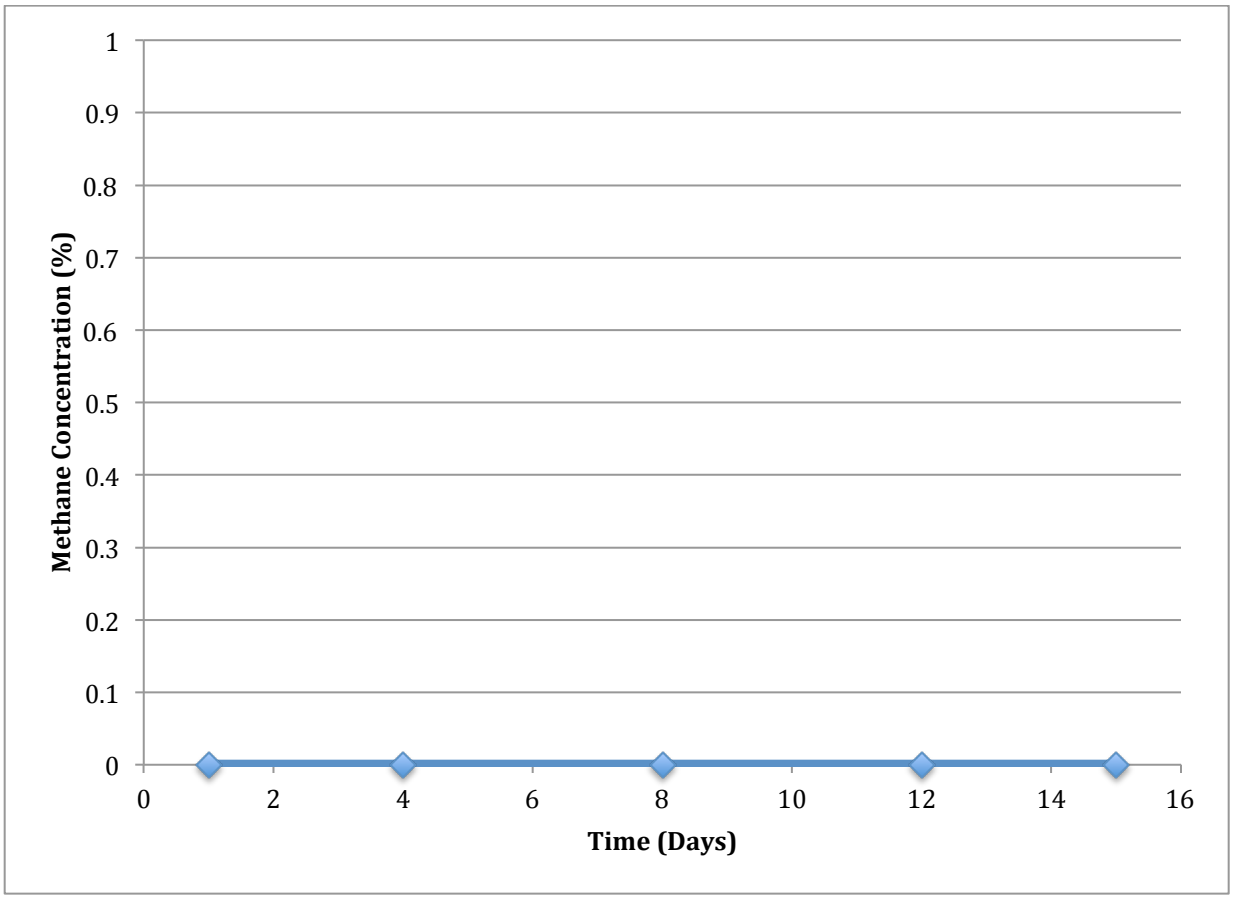

Figure 5. Methane production by *Methanococcus maripaludis* growing in buffer solution while montmorillonite was present.

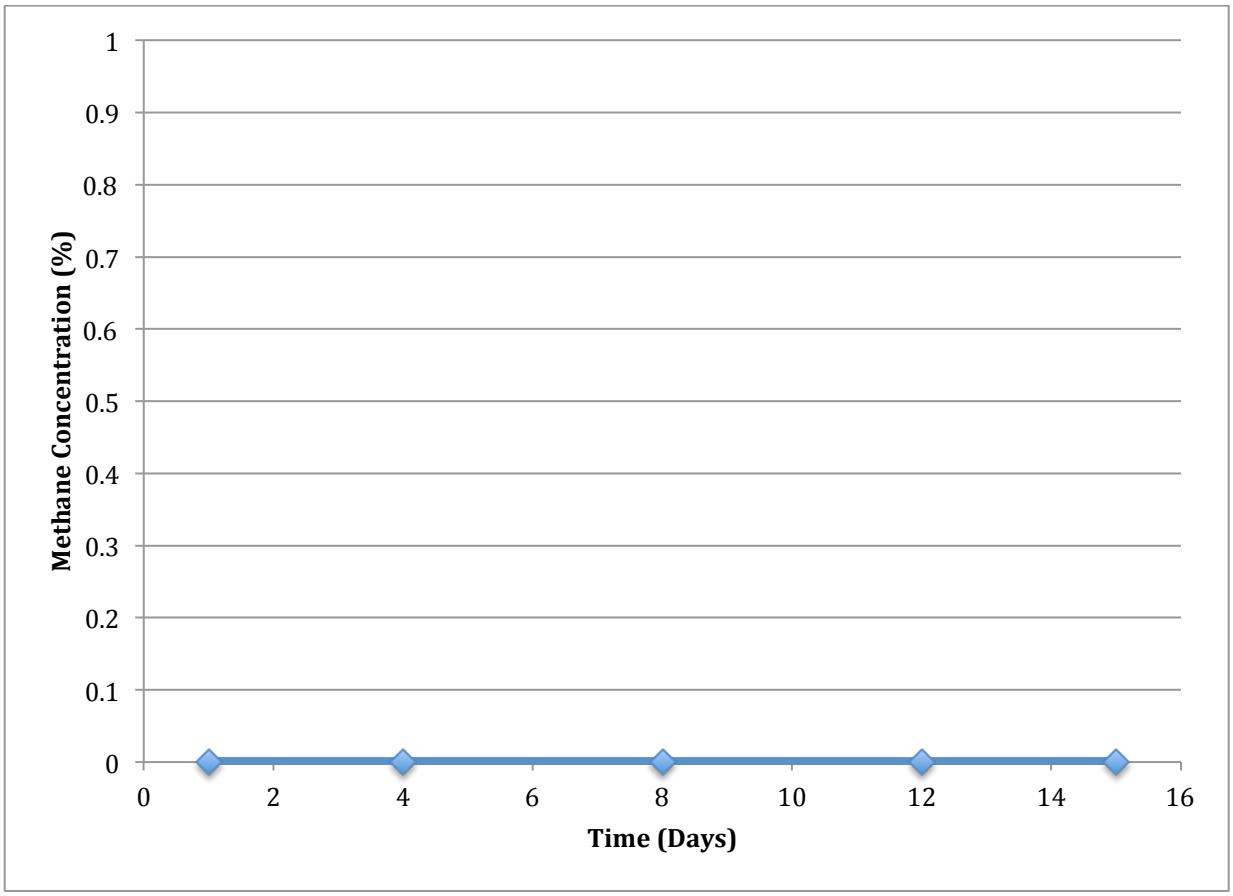

Figure 6. Methane production by *Methanococcus maripaludis* growing in MSH medium made without salt solution while montmorillonite was present.

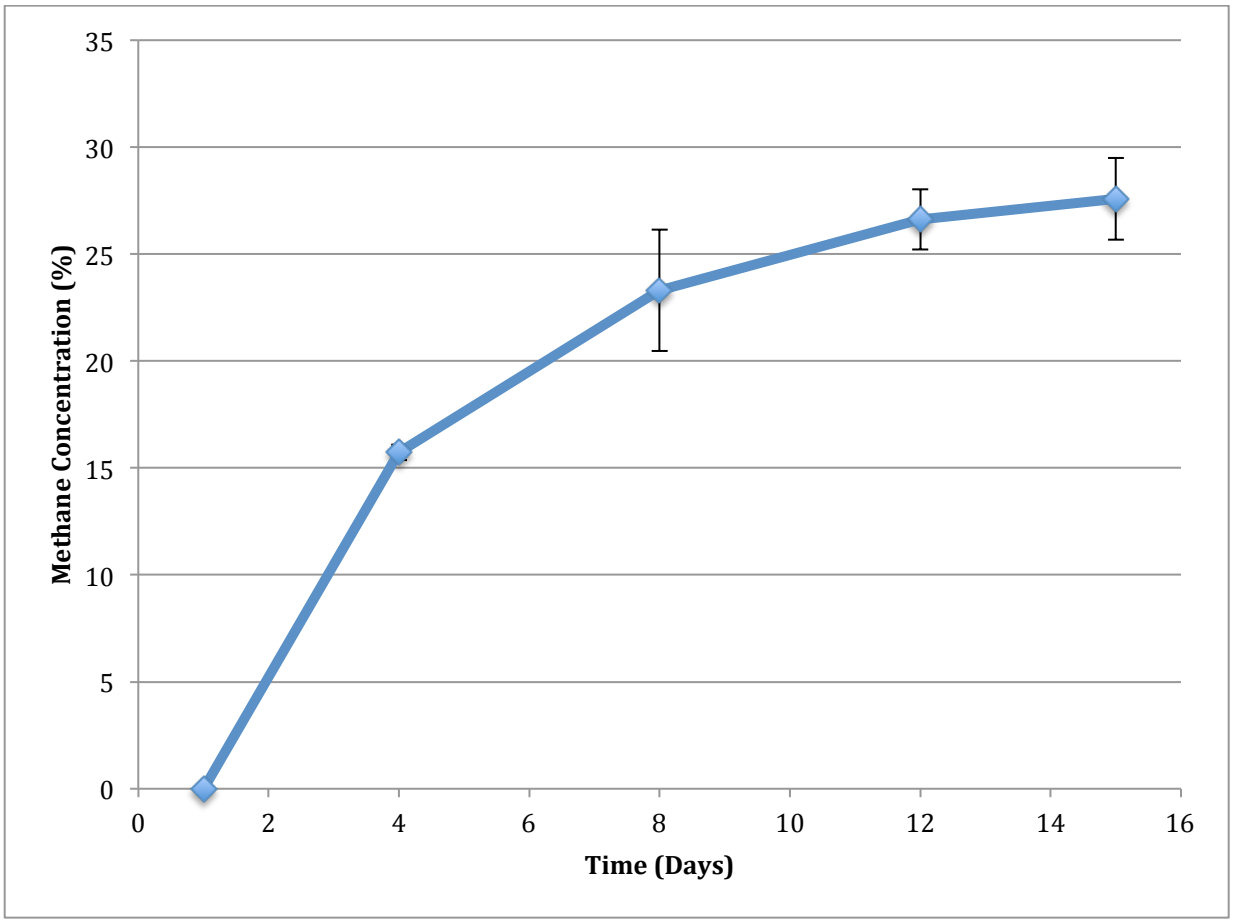

Figure 7. Methane production by *Methanococcus maripaludis* growing on MSH medium made without Solution D while montmorillonite was present.

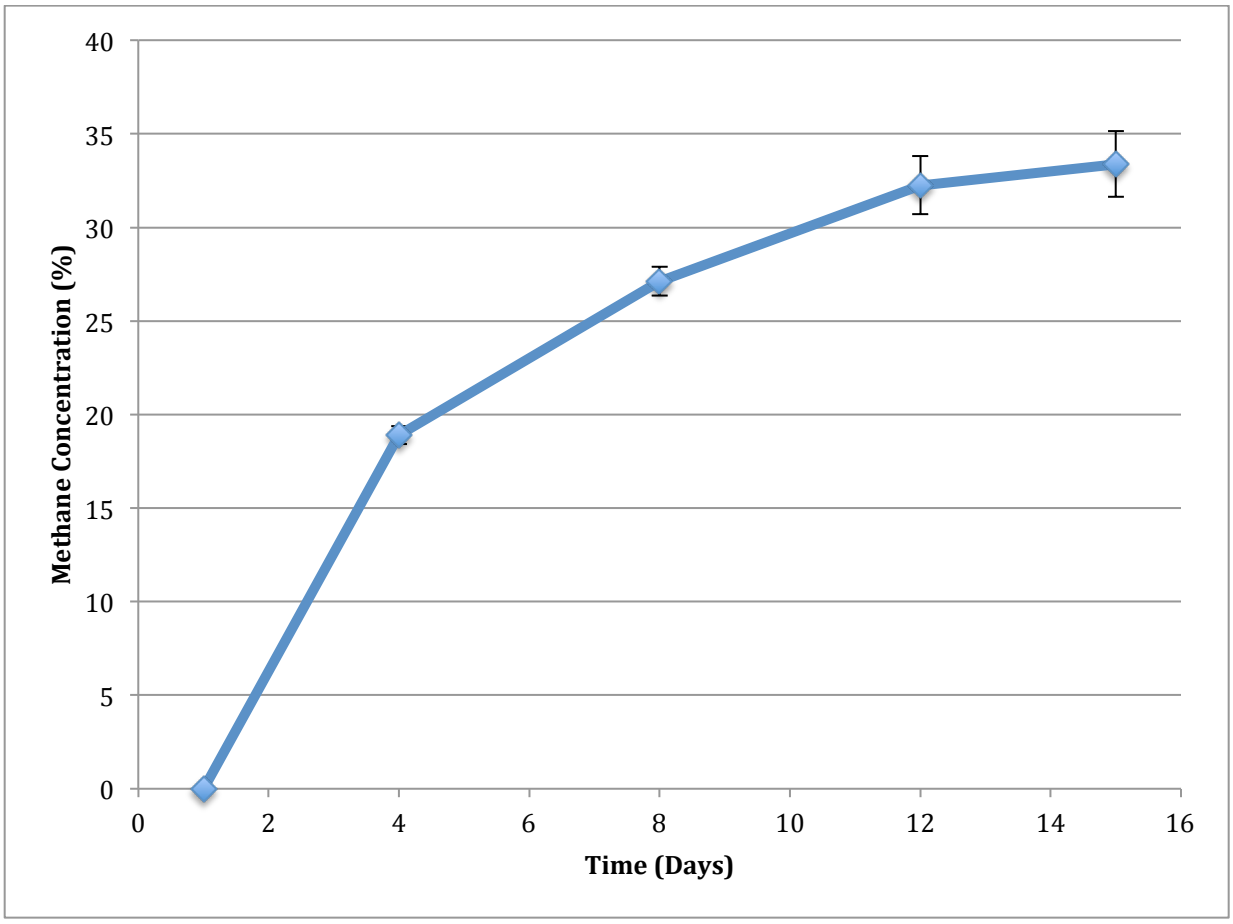

Figure 8. Methane production by *Methanococcus maripaludis* growing on MSH medium made without Solution B while montmorillonite was present.

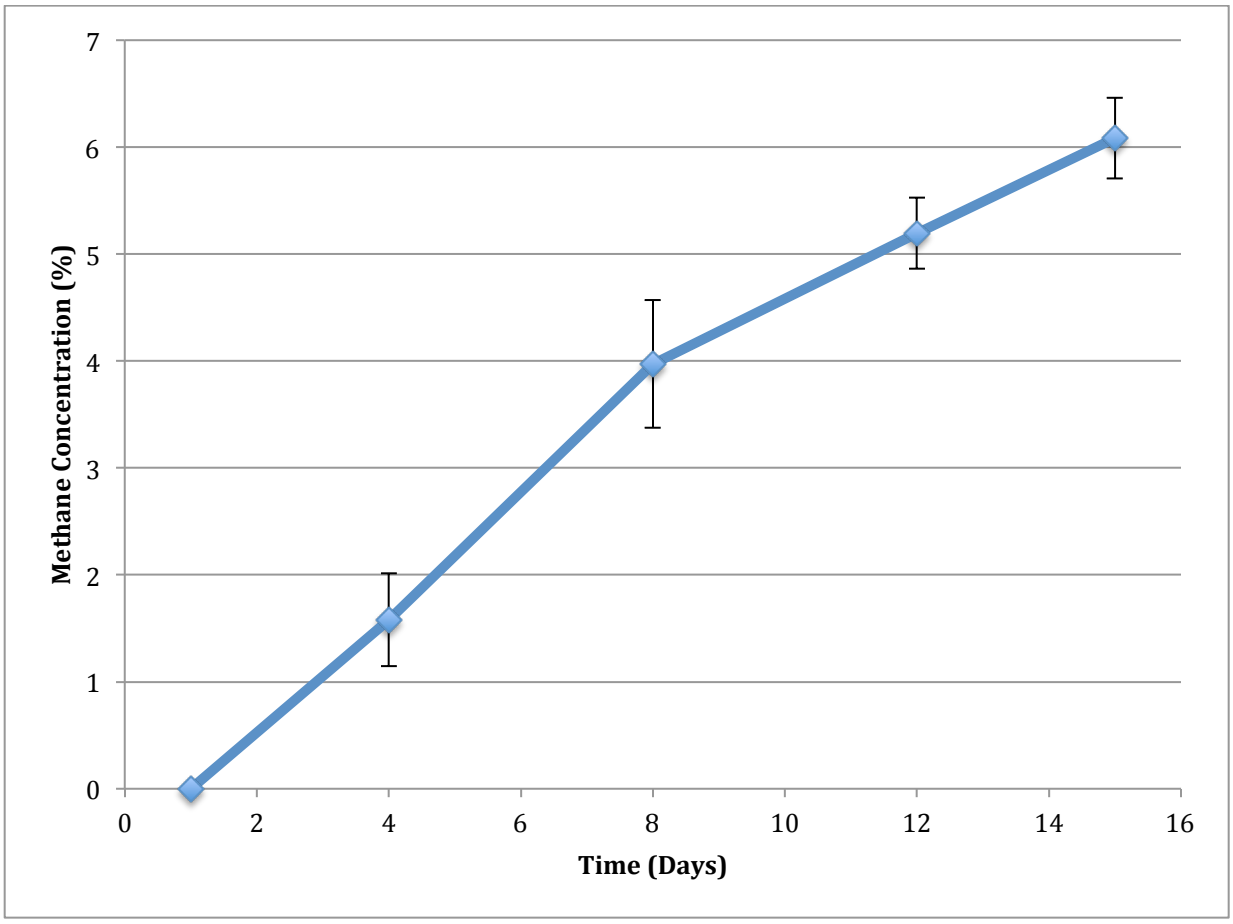

Figure 9. Methane production by *Methanococcus maripaludis* growing on MSH medium made without Solution A while montmorillonite was present.

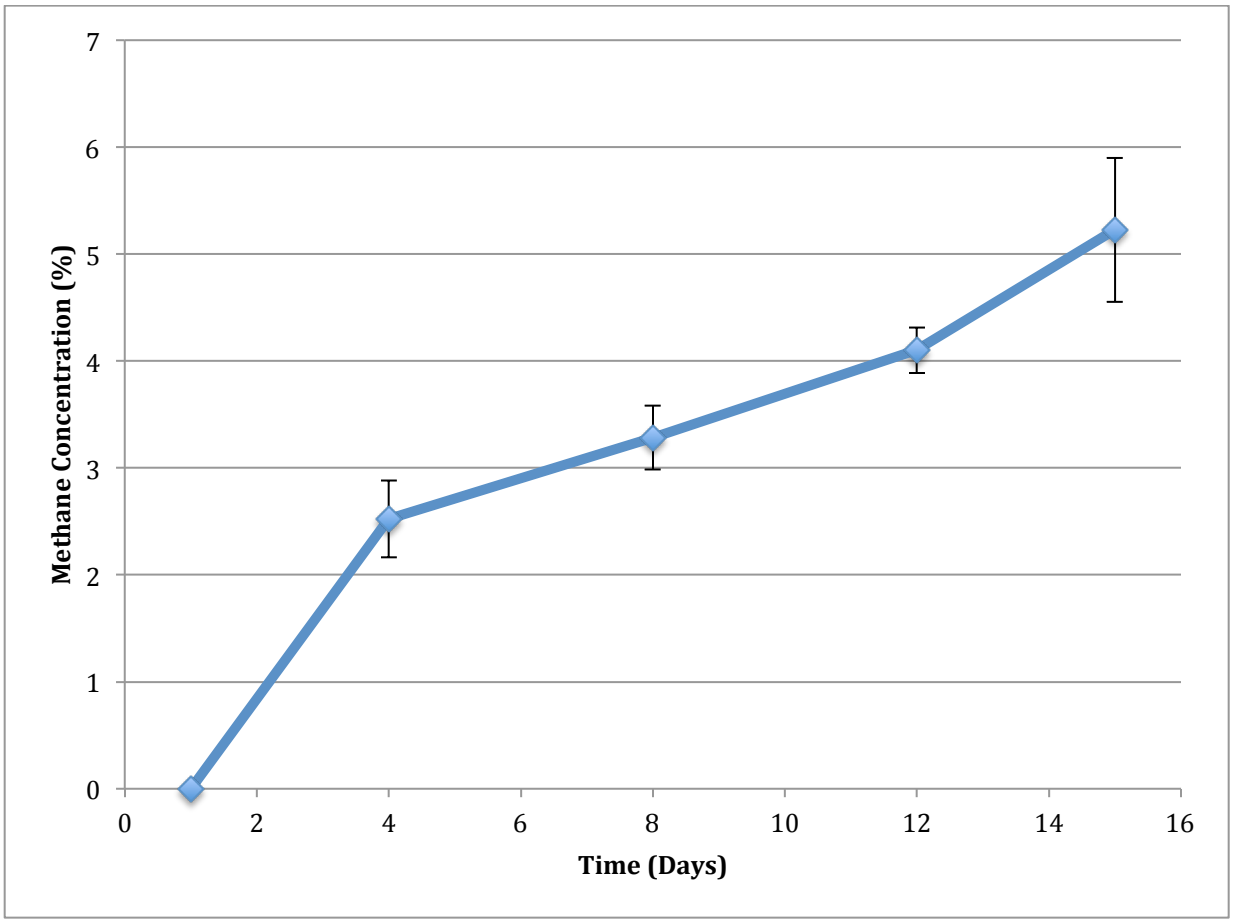

Figure 10. Methane production by *Methanococcus maripaludis* growing on salt solution while montmorillonite was present.

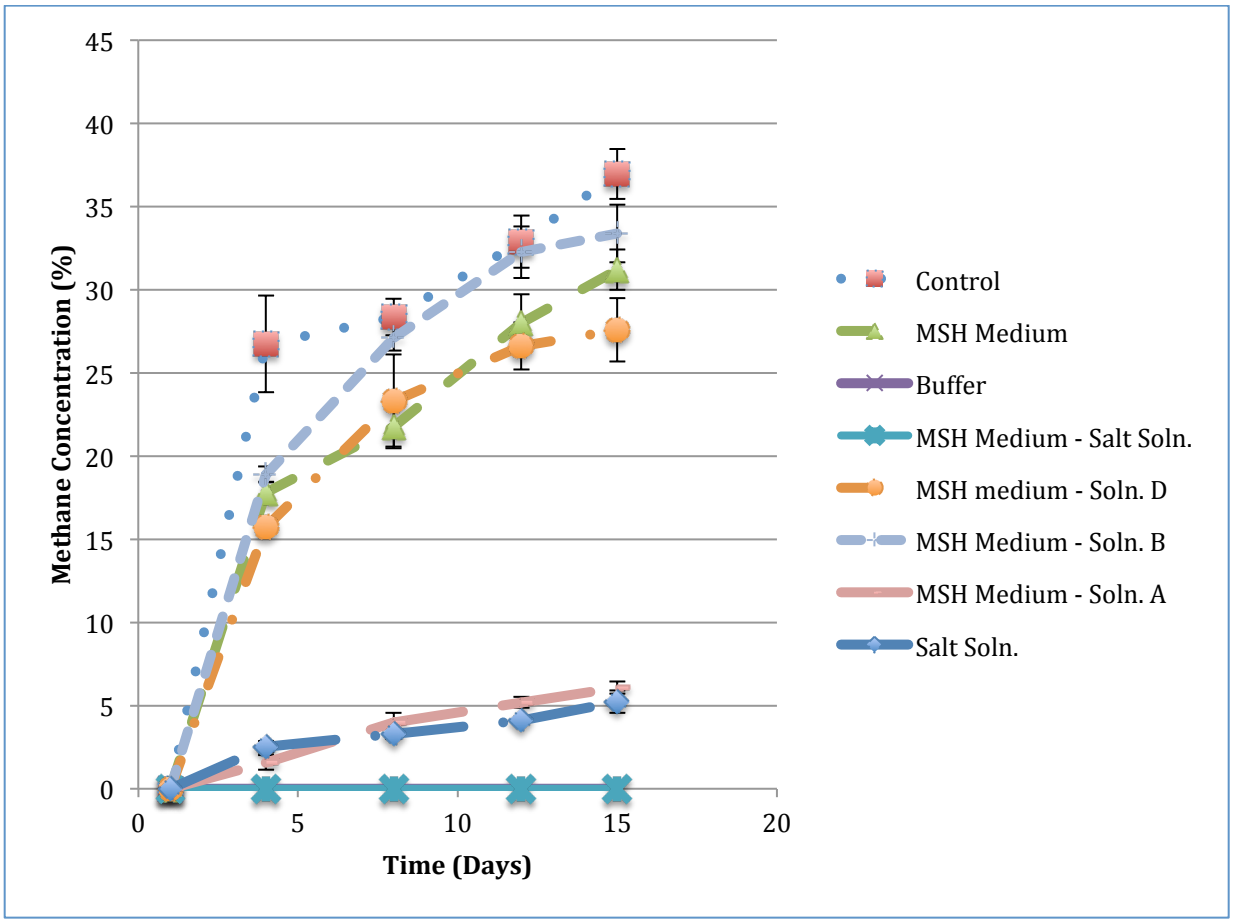

Figure 11. Methane production by *Methanococcus maripaludis* while growing on montmorillonite (except control) with seven different nutrient combinations.

Discussion

The results for all three of the trials of this study were fairly consistent. The control had the highest levels of growth on average for all of the trials. The condition with buffer only had no growth as expected. Most of the conditions decreased the amount of methane produced slightly as compared to the control, but there are three conditions that are the most evident upon analysis of the data. The first is that of the montmorillonite with MSH medium made without solution A added. This condition had everything in it except for solution A, and it is clear upon looking at the results that it made a significant difference in methane production. Even though it seems as if solution A is of importance for the growth of *M. maripaludis* on montmorillonite clay, further examination of the data shows that there is an even more essential component for the production of methane by the microorganism. The most essential component for the growth of *M. maripaludis* was observed to be salt solution. I have come to this conclusion based on two key observations from the data. The condition when the methanogen was grown on montmorillonite with MSH medium made without salt solution resulted with no methane production over the course of the measurement period. This was interesting to me, and it did not make complete sense until I had found a second condition that worked as corroborative evidence for salt solution being the essential component. This condition was that of the growth of *M. maripaludis* on montmorillonite with only salt solution added and no other medium. It may not seem apparent that it is significant upon first glance simply because its methane concentration was so low. But the fact that it grew even minimally on the montmorillonite clay with only salt solution present is definitely of note. And when the condition previously mentioned with the MSH medium with no salt solution present was compared to this one, it became apparent that salt solution was a very essential component to the growth of *M. maripaludis*. This is an acceptable conclusion, because

as I stated in my introduction there have been many large salt deposits found along the surface of Mars. Just as life on earth has preferred locations, I think it is safe to assume the same for Mars. And the locations of these salt deposits would conceivably provide *M. maripaludis* with the means it needs to grow.

In conclusion, though solution A seems to be of importance, the data from this research suggest that salt solution is the most essential component for the growth of *M. maripaludis*. This is significant because it shows that these microorganisms may be able to thrive quite well in Mars like conditions.

Appendix A

	Standard Methanogen Growth Medium (100 mL)			
Ingredient	MM	MS	MSF	MSH
500 µL Solution A	X	X	X	X
100 µL Solution B	X	X	X	X
100 µL Solution C	X	X	X	X
50 µL Solution D	X	X	X	X
0.1 g Yeast Extract		X	X	X
0.1 g Trypticase Peptone		X	X	X
0.025 g Mercaptoethane Sulfonic Acid		X	X	X
500 µL Sodium Formate			X	X
1.475 g NaCl				X
0.085 g $MgCl_2$				X
0.025 g KCL				X

Solution A:

100 g/L NH_4CL

100 g/L $MgCl_2$-$6H_2O$

40 g/L $CaCl_2$-$2H_2O$

Solution B:

200 g/L K_2PO_4-$3H_2O$

Solution C:

0.5 g/L Resazurin

Solution D:

500 mg/L Na_2-EDTA-$2H_2O$

150 mg/L $CoCl_2$-$6H_2O$

100 mg/L $MnCl_2$-$4H_2O$

100 mg/L $FeSO_4$-$7H_2O$

100 mg/L $ZnCl_2$

40 mg/L $AlCl_3$-$6H_2O$

30 mg/L Na_2WO_4-$2H_2O$

20 mg/L $CuCl_2$-$2H_2O$

20 mg/L $NiSO_4$-$6H_2O$

10 mg/L H_2SeO_3

10 mg/L H_3BO_3

10 mg/L Na_2MoO_4-$2H_2O$

Buffer:

4 g NaOH/L deionized H_20, saturated with CO_2

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