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# Methanogen Metabolism in the Presence of Iron Compounds: A Martian Environment Simulation

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# Methanogen Metabolism in the Presence of Iron Compounds:

# A Martian Environment Simulation

An Honors Thesis submitted in partial fulfillment

of the requirements for Honors Studies in

Biochemistry

By

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**<u>Table of Contents</u>**

**<u>Abstract</u>**

The climate on the face of Mars is hostile towards life. Thus, any organisms potentially living on Mars would likely inhabit a subsurface environment, in which conditions are wetter and warmer. However, organisms living beneath the surface would be significantly deprived of light and some organic compounds necessary for the sustenance of most life.

The discovery of methane gas in Mars' atmosphere has led to speculation that methanogens, obligate anaerobes which produce methane as waste, are the organisms most likely to survive in a subsurface environment. These members of domain Archaea subsist on water, hydrogen, and carbon dioxide. In addition, like all organisms, methanogens depend on micronutrients such as vitamins and minerals for optimum growth.

Iron is synonymous to Mars, and research by NASA has confirmed the existence of the compound ferric sulfate on the planet. It has been suggested that ferric sulfate brines could account for liquid water in the subsurface of Mars, in addition to the formation of the gullies via episodic transition from solid to viscous liquid flows.

The goal of this project was to examine a potential link between methanogens and the iron compounds of the red planet. To this end, experiments were conducted in which the growth of methanogens in solutions of ferric sulfate and ferric nitrate was monitored. In order to replicate the droughts and floods of Mars, methanogens were desiccated and exposed to those same chemicals. The results of this study show that certain methanogens can survive in the presence of iron compounds. However, no results indicated that the organisms were utilizing these compounds as fuel.

**<u>Introduction</u>**

The premier question in astrobiology has always been whether or not life exists outside of planet Earth, and if it does, whether it exists in forms recognizable to terrestrial science. Both manned and unmanned technological expeditions, as well as radio broadcasts, have been sent to the far reaches of our solar system in an effort to contact alien life forms, though we do not yet have the ability to travel to other planets and search them in person.

As Earth's closest planetary neighbor, Mars has often been the object of wonder, fantasy, and scientific inquiry, including the possibility of extraterrestrial life. Several debates have sprung up concerning this topic: Can life exist in such a barren landscape? After all, liquid water is the solvent of life on Earth, and none has been seen on the surface of Mars. If organisms do exist on Mars, do they follow the biological standards we take for granted, such as Darwinian evolution, carbon basis, and genetics dependent on nucleic acids (Poole 2007)?

On Earth, the presence of oxygen in our atmosphere is responsible for the prolific amount of higher life. On Mars, however, carbon dioxide ($CO_2$) is the most prevalent gas, accounting for 95.3% of the atmosphere. Trace amounts of other gases make up the remainder. In descending order of concentration, nitrogen ( $N_2$), argon ($Ar$), oxygen ($O_2$), carbon monoxide ($CO$), and water vapor ($H_2O$) are all present (Mumma 2009). In 2003, Mumma and colleagues noted the presence of relatively large amounts of methane ($CH_4$) in the atmosphere of Mars. Implementing high-dispersion infrared spectrometers at three ground-based telescopes, they were able observe around 90% of the surface of Mars (Mumma 2009). In three specific areas, Terra Sabae, Nili Fossae,

and Syrtis Major, Mumma and team observed 19,000 metric tons of methane. For scale, a hydrocarbon vent at Coal Oil Point offshore of Santa Barbara, California, emitted about the same amount (Mumma 2009).

This gas is not a permanent fixture in the atmosphere. It is destroyed gradually by UV radiation, resulting in a lifespan of 300 to 600 years (Stoker & Bullock, 1997). With methane levels remaining nearly constant over time, it must be assumed that methane is somehow being restored.

On Earth, 90% of atmospheric methane is created by biological systems. The remaining 10% is rooted in geochemical phenomena (Mumma 2009). The geochemical trends of Mars are far from well known. Several theories as to the origin of this gas have sprung up, including meteor strikes and volcanic eruptions (Atreya 2007).

Given the fact that the vast majority of methane on Earth is produced by organisms, as well as the localized discovery of methane on Mars, it would be remiss to not test the possibility that a portion of methane on Mars is produced biologically. Since there are evidently no macroorganisms on Mars, we must consider microorganisms.

Methanogens are microorganisms which utilize hydrogen gas ($H_2$) and simple inorganic carbon compounds ($CO_2$), and produce methane ($CH_4$) as waste (Yu 1994). This process is known as methanogenesis. Methanogenesis is a form of anaerobic respiration, in which the terminal electron acceptor is carbon, not oxygen. Methanogenesis is summarized by *Equation 1.*

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$

*Equation 1*

Because their energy source is inorganic, methanogens need not rely on energy from the sun.

The surface of Mars is cold, dry, and highly oxidizing, making it unlikely that any microorganism could survive there. However, it is possible that certain hardy microorganisms, methanogens for example, could survive below the surface given the right conditions, receiving their hydrogen from volcanoes or other geothermal sources (Kral 2004).

Methanogens thrive in extreme conditions on our planet. For example, one methanogen community has been found miles below the surface within the Witwatersrand Basin in South Africa (Mumma 2009). These remarkable organisms are able to chemically reduce hydrogen sulfate ($HSO_4^-$), using hydrogen or methane as an electron donor, and producing hydrogen sulfide ($H_2S$) and methane.

If this level of versatility is possible on Earth, it may also be possible that, if methanogens exist on planet Mars, they exist below the surface. There, they could oxidize hydrogen to create methane, which could then escape through the numerous cracks, craters, and ravines of the planet. Four examples of methanogens are *Methanococcus maripaludis*, *Methanosarcina barkeri*, *Methanothermobacter wolfeii*, and *Methanobacterium formicicum*.

*M. maripaludis* has been shown to grow well in media rich in hydrogen and carbon dioxide (Jones 1983). *M. maripaludis* is weakly motile, gram negative, strictly anaerobic, and grows in circular colonies whose surfaces are smooth (Jones 1983). These organisms are classified as mesophiles, meaning that they prefer intermediate temperatures. *M. maripaludis* can grow between 18 and 47 degrees centigrade, with optimal growth occurring at 38 degrees (Jones 1983). *M. maripaludis* grows best at a nearly neutral pH, in a relatively high concentration of salt (Jones 1983).

*M. barkeri*, like *M. maripalus*, is mesophilic. It grows best between 37 and 42 degrees centigrade. Optimum growth occurs in media rich in $H_2$ and methanol (Maestrojuan 1991). *M. barkeri* is gram positive and immotile. While most members of the genus *Methanosarcina* prefer a neutral pH, *M. barkeri* have been found in much more acidic environments, with a pH as low as 4.3. (Maestrojuan 1991). Unlike *M. maripaludis*, *M. barkeri* does not grow well in high salt concentrations (Maestrojuan 1991).

*M. wolfeii* is classed as a thermophile, an organism which prefers elevated temperatures. *M. wolfeii* grows best at 55 degrees centigrade. *M. wolfeii* is gram positive and immotile (Winter 1984).

*M. formicicum* is another mesophile. This organism prefers temperatures ranging from 37 to 48 degrees centigrade (Bryant 1987). *M. formicicum* is rod-shaped. They occur most often as single cells, but sometimes in chains or filaments. *M. formicicum* grow well in media containing hydrogen and carbon dioxide (Bryant 1987).

The overall composition of Mars' soil and atmosphere is important when considering whether or not Mars can sustain life. After all, we do not search for life on gas giants like Jupiter hoping to find some miniscule alcove where life could thrive; the probability is just too small! Many attempts have been made by rover missions in order to determine the composition of soil on Mars. For the most part, however, it is still unclear. The Mars Global Surveyor missions, the Mars Exploration Rover (MER), and the Mars Express missions have determined that the most ubiquitous compounds on the planet are composed of iron and sulfur. The Viking missions, Mars Pathfinder (MPF), and MER missions measured large amounts of sulfur at their landing sites. This,

in combination with the tendency of compounds made of lead to oxidize in the Martian atmosphere, have lead some to believe that the surface contains a large amount of sulfate compounds (Lane 2004).

The core of Mars is thought to be composed of iron sulfide ($Fe_2S$, or pyrite, in its most stable form), and the surface is thought to consist of an iron (III) oxide ($Fe_2O_3$) layer (Marion 2008). Surveys have found large amounts of ferric sulfate, and its salt derivatives, in the soil of the planet (Marion 2008). Infrared spectroscopy tests made by MER and other missions found IR results in the 1200 to 1700 $cm^{-1}$ range, closely matching hydrous iron (III) sulfate (Lane 2004).

Ferric sulfate, molecular formula $Fe_2(SO_4)_3$ is much more reactive than its close relatives. Compared to the more common iron (II) sulfate, which has a one-to-one ratio of iron to sulfate ions, iron (III) sulfate has a two-to-three iron to sulfate ratio (Bishop 1995). The synthesis of ferric sulfate involves oxidation of iron (II) sulfate using sulfuric acid, according to Equation 2.

$$2FeSO_4 + H_2SO_4 + H_2O_2 \rightarrow Fe_2(SO_4)_3 + 2H_2O \qquad \textit{Equation 2}$$

Ferric sulfate has a melting point of 480°C, and thus exists in solid form on both Earth and Mars (Bishop 1995). Jarosite, a salt derivative of ferric sulfate, chemical formula $KFe^{3+}_3(OH)_6(SO_4)_2$, has been discovered in ore deposits on the surface of Mars via mass spectroscopy by MER in 2007 (Wills 2000).

Dr. Kral and his students have shown that, provided with all essential components of metabolism, *M. maripaludis*, *M. wolfeii*, *M. formicicum*, and *M. barkeri* are able to grow in various Martian soil stimulants. The research reported in this paper tested the ability of these same organisms to grow on media containing varying

concentrations of ferric sulfate and a similar compound, ferric nitrate. In addition, select methanogens were desiccated and subsequently exposed to these same compounds in order to test their durability.

Monitoring the growth patterns and hardiness of methanogens in various iron compounds can tell us about the metabolism of these organisms. Testing them in simulated Martian conditions can help scientists to understand what life is possible on Mars, in addition to the origin of life on Earth and beyond. Domain Archaea constitutes the oldest and simplest life on planet Earth. Their nontraditional energy sources and ability to survive under extreme conditions can help us to understand the beginnings of life and how it has developed over billions of years.

**Materials and Methods**

***Organisms and Media.*** Four species of methanogens are typically studied in the Kral lab: *M. wolfeii, Methanosarcina M. barkeri, M. maripaludis* and *M. formicicum.* These were grown in specialized media which Dr. Kral and colleagues have developed to facilitate growth of each of the methanogens, and incubated at growth temperatures of the organisms (55°C, 37°C, 25°C and 37°C, respectively). Four different types of media were used, MM, MS, MSH and MSF. Please refer to Appendix A for an in depth list of dry chemicals and solutions used to make these.

For Experiment One, only *M. wolfeii* (MM), *M. barkeri* (MS), and *M. formicicum* (MSF) were tested. Each medium type was prepared using 0.5% iron compound by mass. Part one of the first experiment was conducted using ferric nitrate, part two using ferric sulfate

For Experiment Two, *M. maripaludis* (MSH) and *M. formicicum* (MSF) were tested. These methanogens were centrifuged, washed, desiccated, exposed to concentrations of iron compounds at 10%, 30%, and 50% by mass, and inoculated into normal media. Part one of the second experiment was conducted using ferric nitrate, part two using ferric sulfate.

***Media preparation.*** The various media were prepared using an electronic scale for dry chemical ingredients and micropipettes for solution ingredients. All ingredients for each medium were mixed in Erlenmeyer flasks. During Experiment One, iron compounds would be mixed in with normal media ingredients at this point. During Experiment Two, this step served only to breed methanogens to use in the actual experiment.

Test tubes into which the medium would be divided were labeled. The newly mixed solution and all test tubes were placed in a Coy anaerobic chamber. In the chamber, bicarbonate buffer was poured into each flask up to the 50-mL line, if 50 milliliters of media were being prepared for example. The media solutions, still in flasks, would sit in the anaerobic chamber for 24 hours in order to thoroughly deoxygenate. Afterwards, all solutions were funneled into their respective tubes, with about 10 mL allotted for each tube. Each tube was then closed with a rubber stopper in order to prevent oxygen contamination. Once they were removed from the anaerobic chamber, the test tubes were sealed with metal caps. The tubes were then sterilized using an autoclave.

***Inoculation and Gassing.*** The sterilized test tubes were then injected with twelve drops of sodium sulfide ($Na_2S$) solution. Then, after a short waiting period, stock culture methanogens were injected into the tubes. Afterwards, each tube was pressured with 200 kPa $H_2$ using a gassing manifold. All tubes were then placed in incubators set to the organisms' respective growth temperatures.

After a week of incubation, the methanogens are ready for experimentation. During Experiment One, the tubes were simply measured for methane every week. For Experiment Two, the methanogens would proceed to the next step.

***Washing.*** Each 10 mL incubated test tube was poured into a large size centrifuge tube, each of which was centrifuged at 5,000 rpm for 20 minutes. It was at this point that the research subjects were narrowed down; MSF formed great pellets, MSH formed decent pellets, MS and MM formed no lasting pellets whatsoever. Therefore, *M. formicicum* became the backbone of Experiment Two, and *M. maripaludis* was tested only as a

supplement from this point onward. Only two tubes of *M. maripaludis* were used, pellet and no pellet.

After the first centrifuge cycle, the supernatant was poured off. Afterwards, 10 mL of sterile buffer with sodium sulfide were added to each centrifuge tube and centrifuging was repeated at 5,000 rpm for 20 minutes.

***Desiccation.*** After washing, the large centrifuge tubes were decanted again and filled with 1 mL of sterile buffer containing sodium sulfide. The pellet was gently dislodged into the buffer using a syringe, and then transferred via the same syringe into a 1 mL sterile microcentrifuge tube. These smaller tubes would then be put into a microcentrifuge for 10 minutes at 15,000 rpm. The supernatant was poured off and more sterile buffer with sodium sulfide was added to the microcentrifuge tubes, which were subsequently centrifuged again for 10 minutes at 15,000 rpm. These tubes were placed into desiccation jars containing Drierite ($CaSO_4$) inside the anaerobic chamber, and left to desiccate over night.

***Iron Compound Exposure.*** Iron compound solutions as previously described were prepared in deionized water. One milliliter of compound solution was injected per microcentrifuge tube, which at this point only contained dry pellet and very little moisture. Pellets were exposed to iron solution for one week. Afterwards, the micro centrifuge tubes were centrifuged to reform the pellet. The supernatant was decanted, and sterile buffer would be used again to wash the pellets. Next, a syringe was used to suspend the pellet in buffer and inject the methanogens into fresh media. The organisms were then incubated at their growth temperatures.

***Methane Measurements.*** In order to measure growth of methanogens, a gas chromatograph was utilized. One milliliter of headspace gas samples were taken from each test tube and processed using a Varian micro gas chromatograph (model CP-4900). Methane was measured as an indicator of growth. Measurements were taken once a week. Three tubes were measured for each concentration of iron.

## Results

***Experiment One.*** Methane production from the first experiment, using *M. wolfeii*, *M. barkeri*, and *M. formicicum* in media tubes containing 0.5% ferric nitrate, and then 0.5% ferric sulfate, can be seen in Figures 1-3. Any and all control tubes, which contained no methanogens, produced no methane and were thus not seen in the figures. Experiment One occurred over six weeks.

Ferric nitrate tubes showed greater peaks of methane production and a more gradual decline in methane levels for *M. wolfeii*, *M. barkeri*, and *M. formicicum*. In all organisms, methane increased over time, peaking around week three, and declined until the end of the experiment at the end of week six.

***Experiment Two.*** Methane production from the second experiment, using desiccated *M. maripaludis* and *M. formicicum* in 10%, 30%, and 50% iron compound, can be seen in Figures 4-7. Once again, no control tubes, which contained no methanogens, produced any methane, and thus were not seen in the figures. Experiment Two occurred over six weeks with a different round of methanogens being inoculated every week, and because of this, not every methanogen/iron compound combination was allowed the full six weeks to progress.

*M. maripaludis* displayed methane production after week two. *M. formicicum* displayed methane for both iron compounds at the 10% level, with ferric nitrate showing great levels of production. *M. formicicum* displayed methane only for ferric nitrate at the 30% level. At the 50% level, *M. formicicum* displayed methane for both iron compounds, with ferric sulfate tubes showing greater production.
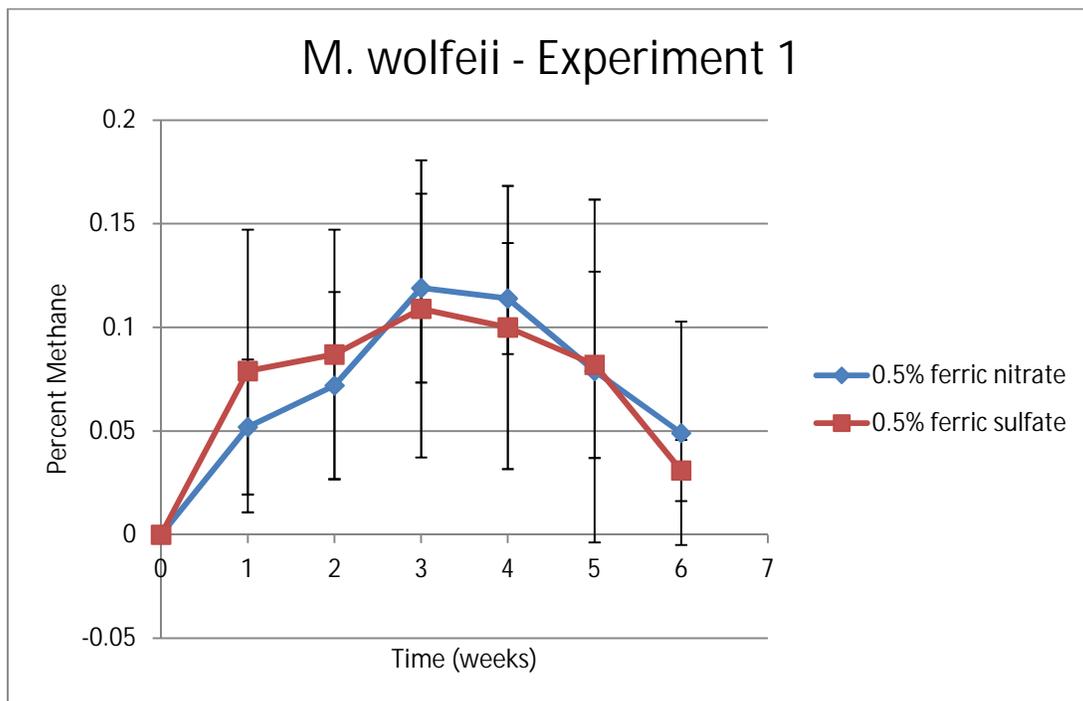
*Figure 1*: Methane production by *Methanothermobacter wolfeii* in MM medium
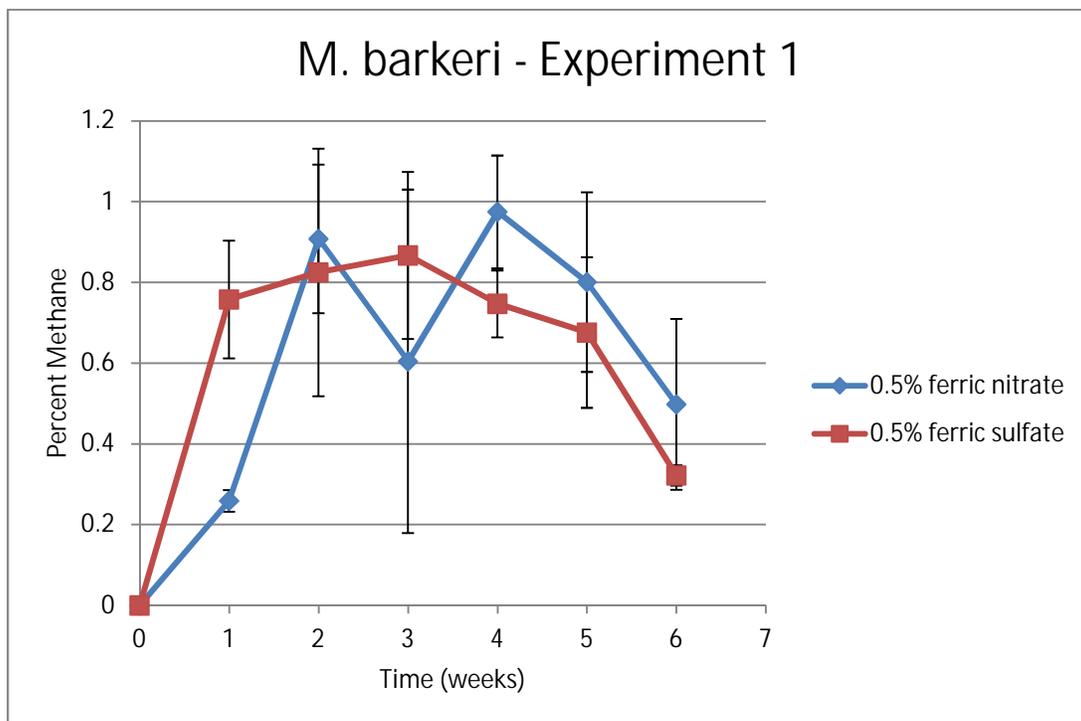
containing 0.5% iron compound.

*Figure 2*: Methane production by *Methanosarcina barkeri* in MS medium containing
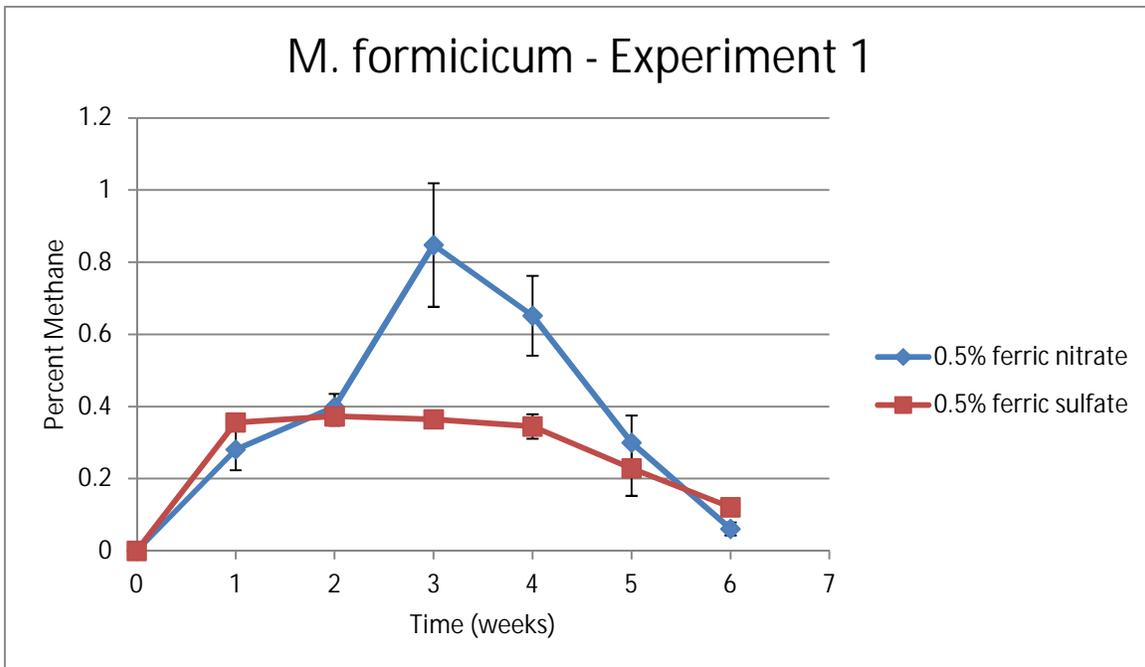
0.5% iron compound.

*Figure 3*: Methane production by *Methanobacterium formicicum* in MSF medium
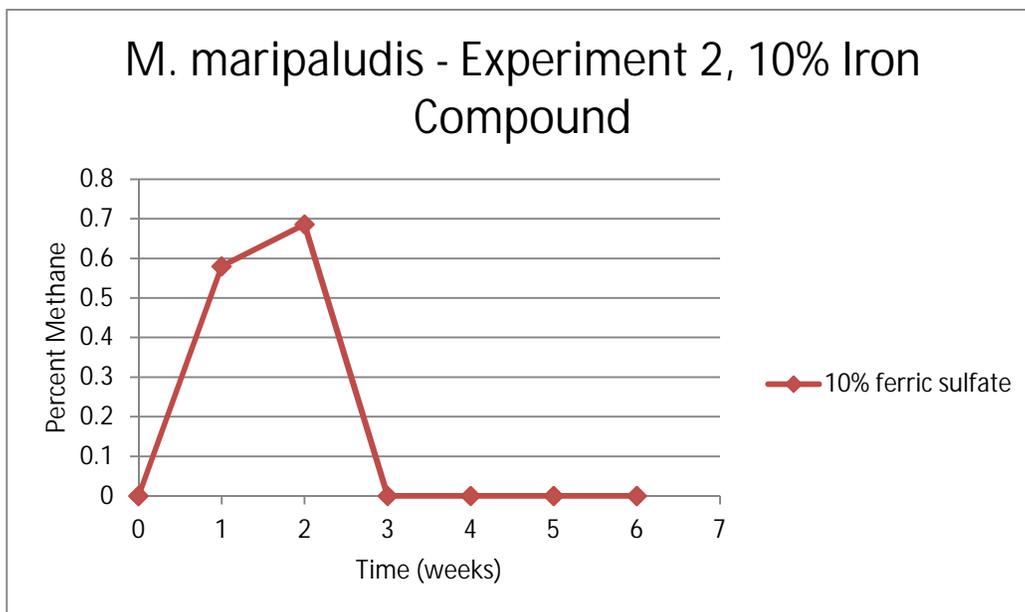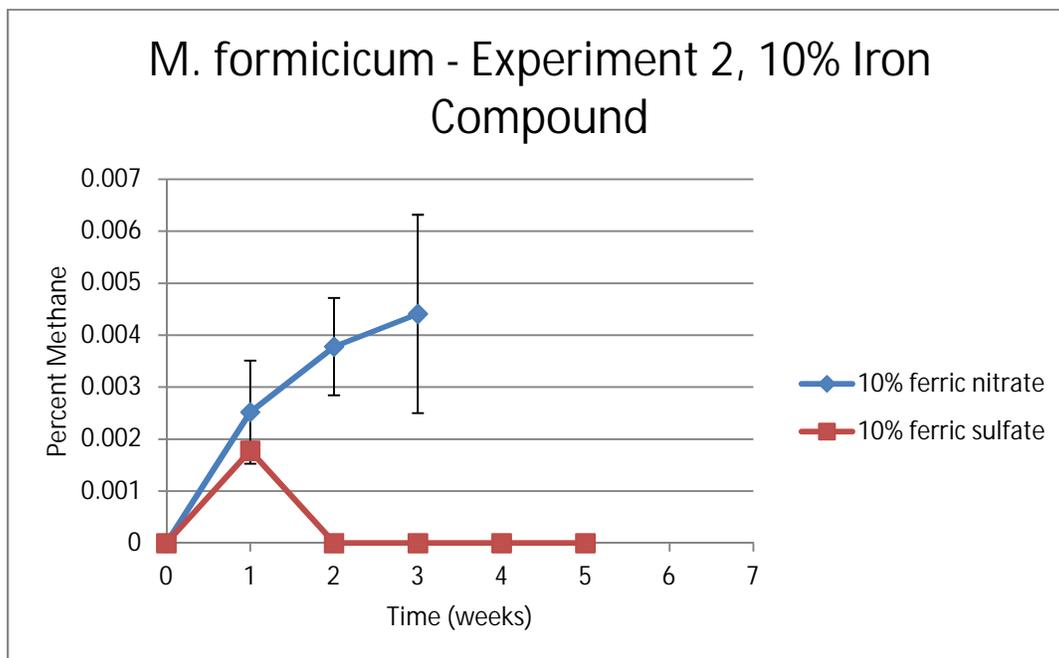
containing 0.5% iron compound.

*Figure 4*: Methane production by *Methanococcus maripaludis* in MSH medium following desiccation and exposure to 10% ferric sulfate solution for one week.

*Figure 5*: Methane production by *Methanobacterium formicicum* in MSF medium

following desiccation and exposure to 10% ferric sulfate solution or 10% ferric nitrate

solution for one week.

*Figure 6*: Methane production by *Methanobacterium formicicum* in MSF medium

following desiccation and exposure to 30% ferric sulfate solution or 30% ferric nitrate

solution for one week.
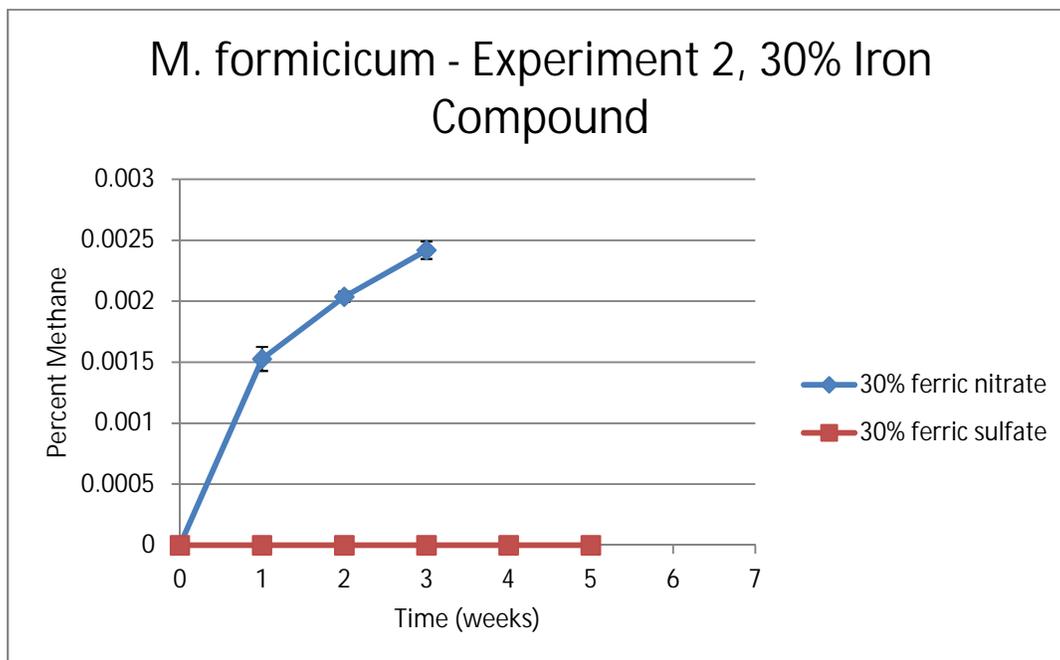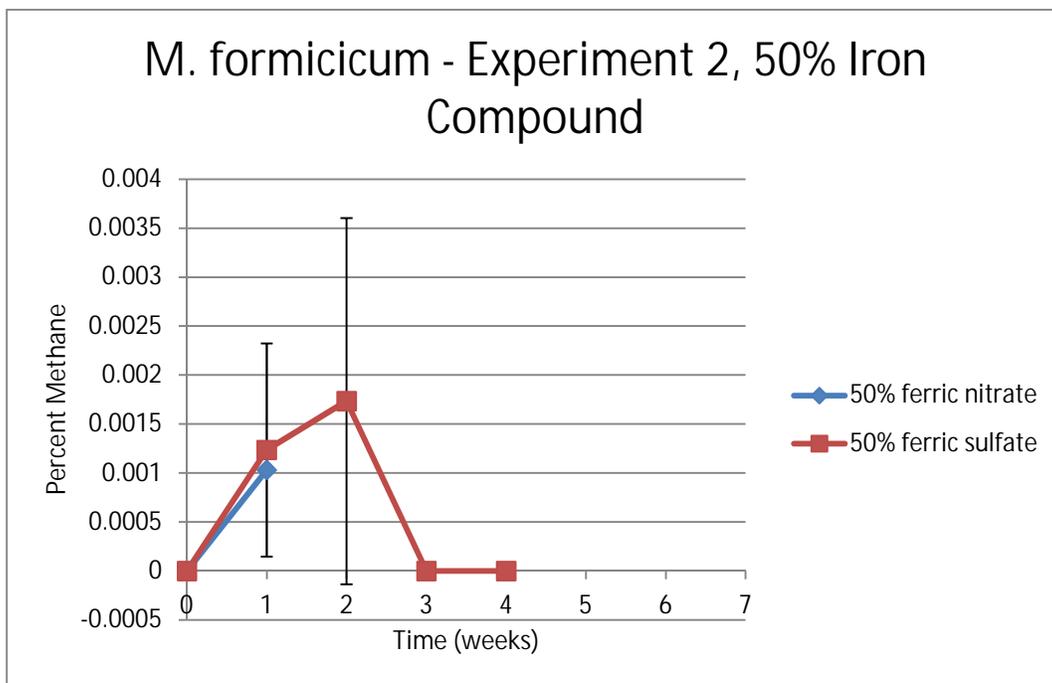
*Figure 7*: Methane production by *Methanobacterium formicicum* in MSF medium

following desiccation and exposure to 50% ferric sulfate solution or 50% ferric nitrate

solution for one week.

**Discussion**

In the first experiment, in which the organisms were grown in either 0.5% ferric nitrate or 0.5% ferric sulfate, the three species of methanogens tested were able to grow. While the percentages of methane created seem small, it is very encouraging that there was increasing methane with time seen in Figures 1, 2, and 3.

*M. barkeri*, grown in MS media, was by far the organism which most successfully adapted to the presence of both ferric nitrate and ferric sulfate. The percent methane for this organism peaked at 0.985%, measured after the third week of growth in 0.5% ferric nitrate solution.

*M. wolfeii*, grown in MM media, experienced the least growth when inoculated into ferric nitrate and ferric sulfate solutions. The percent methane for this organism peaked at 0.119% after three weeks of growth, a much lower figure even when compared to the other methanogens tested in the same experiment.

One outcome of Experiment One is that ferric nitrate appears to allow for more methane production than ferric sulfate. While ferric sulfate solution resulted in higher methane production initially, methane percentages for organisms in ferric nitrate solution peaked higher and stayed higher throughout the course of the study. This conclusion is interesting, if somewhat disappointing; ferric sulfate has been found on Mars but so far, there have been no reports of ferric nitrate.

In the second experiment, let us first address the data presented for desiccated *M. maripaludis* grown in 10% ferric sulfate MSH media. After the second week of growth, methane percentages for this organism peaked at 0.686%; during every other

week, however, no methane was measured. There are two possibilities. First, the methanogens may have been able to suddenly take advantage of the hydrogen and carbon sources available, only to be killed early by an unusual environment. Second, the week two measurement was a result of instrumental error. I believe that the second option is most likely, as methanogens typically grow more gradually that what the data present.

Of the three figures in Experiment Two which compare ferric nitrate to ferric sulfate, two indicate that ferric nitrate is superior at allowing methane production.

Methane percentages were so low, and growth was so rare, that I believe it is fair to say that concentrations of ferric nitrate or ferric sulfate at 10% or above do not very well facilitate the continued growth of desiccated methanogens. No trend appeared to be present relating growth and percentage of iron compound. It is possible for desiccated methanogens to grow in high concentrations of iron compound. However, the methane production resulting from this was sporadic and low in volume.

## **Appendix**

### *Components for 50 mL of Methanogen Growth Media*

| Ingredient | MM | MS | MSF | MSH |
|---|---|---|---|---|
| 500µL Soln. A | X | X | X | X |
| 100µL Soln. B | X | X | X | X |
| 100µL Soln. C | X | X | X | X |
| 50µL Soln. D | X | X | X | X |
| 0.1g Yeast Extract | | X | X | X |
| 0.1g Trypticase Peptone | | X | X | X |
| 0.025g Mercaptoethanosulfonic Acid | | X | X | X |
| 500µL Sodium Formate (2.5%) | | | X | |
| 1.5g NaCl | | | | X |
| 0.09g MgCl$_2$ | | | | X |
| 0.025g KCl | | | | X |

### *Solution A:*

100 g/L NH$_4$Cl

100 g/L MgCl$_2$ (6H$_2$O)

40 g/L CaCl$_2$ (2H$_2$O)

*Solution B:*

200 g/L $K_2HPO_4$ ($3H_2O$)

**Solution C:**

0.5 g/L Resazurin

**Solution D:**

500 mg/L $Na_2EDTA$ ($2H_2O$)

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 $Na_2MoO_4$ ($2H_2O$)

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