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The Effects of Varying Levels of Perchlorate on the Metabolism of Methanogens

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Abstract

The search for life on Mars has been one of the more intriguing pursuits of NASA and astrobiologists over the last fifteen years. With the help of NASA's Martian landers and orbiters, discoveries about the red planet have narrowed the range of possible known organisms that could survive on its surface or beneath its icy crust. The discoveries of water ice, methane, and perchlorate on Mars have excited astrobiologists and prompted experimentation on Earth to help determine whether life could exist on Mars. Methanogens seem an ideal candidate to be the organism responsible for biological production of methane on Mars, and many experiments have shown their potential to grow in different Mars-like conditions.

In this experiment, four different species of methanogens were grown under the effects of the oxidizing agent perchlorate. Perchlorate in Martian soil was a discovery made by the Phoenix Lander and hints at the possibility that aqueous brine could exist in the Martian subsurface. It was hypothesized that increasing concentrations of perchlorate would lead to decreasing levels of methane production in each species of methanogen.

In all four levels of perchlorate concentration (0, 1%, 5%, and 10%), all methanogens produced methane. However, the control and 1% groups showed significantly more metabolic activity than the higher concentration groups. This supported the initial hypothesis, but cannot conclusively determine the inability for methanogens to grow with perchlorate under the Martian crust.

Introduction

In late 2010, a team of researchers led by Felisa Wolfe-Simon published a controversial article in *Science* with an assertion that promised to change the way Astrobiologists look for extraterrestrial life. Wolfe-Simon isolated the bacterial microbe GFAJ-1 (which stands for Give Felisa A Job) from Mono Lake in California (Davies, 2010). Mono Lake is unique as "a hypersaline and alkaline water body" with high concentrations of arsenic, making it highly uninhabitable to most organisms (Wolfe-Simon et al., 2010). Funded by NASA and under the tutelage of the accomplished astrobiologist Ariel Anbar, Wolfe-Simon ambitiously hypothesized that GFAJ-1 had the capability of incorporating arsenic into its biomolecules including the backbone of its DNA (Davies, 2010). A chemical analog of phosphorus, arsenic lies directly below phosphorus on the periodic table and has a similar atomic radius and electronegativity (Fekry et al., 2011). Thus the substitution of arsenic for phosphorus in GFAJ-1 seemed possible at its root, and this possibility seemingly turned the fundamentals of organic life on its head. Previously, the dogma that every organism subsists on the six major nutrient elements (Carbon, Hydrogen, Nitrogen, Oxygen, Sulfer, and Phosphorus) restricted the search for organic life (David, 2011). This restriction dissolved in the aftermath of Wolfe-Simon's announcement, and the possibilities of what life could be and what it could look like seemingly changed completely. Using the analogs of the six major nutrient elements, organisms could exist with molecular scaffolding exponentially larger than any organism found on Earth. Most importantly, Wolfe-Simon's results redefined the environments in which life could exist and asserted that the typical restrictions and patterns of behavior for earth-bound organisms did not necessarily apply for

extraterrestrial life. Thus overnight the search for extraterrestrial life suddenly became far more difficult with the prospect that everything known about life on Earth did not necessarily apply to life elsewhere.

The aftermath of Wolfe-Simon's announcement has been filled with speculation and criticism. In May of 2011, *Science* published "eight 'technical comments' by 13 distinguished scientists" refuting Simon's research as flawed, suspecting contamination of her samples and errors in measurement (David, 2011). Fekry, Tipton, and Gates (2011) assert that the proposed arsenodiester linkages of GFAJ-1's DNA backbone would hydrolyze in 25° water in less than a second, making it kinetically unstable and not well suited to long term preservation of genetic material. Wolfe-Simon and her colleagues continue to stand by her initial hypothesis even though she noted the partial validity of her critics' comments (David, 2011). Regardless of the true nature of GFAJ-1 and its involvement with arsenic, Wolfe-Simon has sparked a great deal of speculation within the Astrobiology field and highlighted the ability of earthbound scientists to learn more about extraterrestrial life by studying organisms living in terrestrial environments. Determining the identity, behavior, and metabolic properties of organisms living in extreme environments on Earth could reveal the key to finding life on other planets.

Mars has been the destination of many NASA missions and had the attention of many Astrobiologists over the past fifteen years as our closest neighbor most likely to harbor life. Every 26 month period, the red planet is only six months to a year's trip away from Earth, making it relatively accessible for unmanned orbiters and landers (Zorpette, 1999). Also, the possibility that it could support or has supported life in the past has

tantalized scientists since the Odyssey orbiter discovered evidence of water ice just under the Martian crust in 2001 ("NASA Martian orbiter…", 2010).

In 2004, Formisano et al. reported the detection of methane in the Martian atmosphere using the Mars Express spacecraft while two separate teams led by Mumma and Krasnopolsky (2004; 2004) confirmed the findings. Krasnopolsky and Formisano both measured an average planetary abundance of 10 ppbv; however, the gas was not uniformly distributed across the red planet. This is a very small but significant amount of methane (Atreya, 2007). The lifetime of methane on Mars (300-600 years to drop by a factor of the mathematical constant *e* or ~2.72) and the presence of localized concentrations of methane on the planet despite strong winds that should mix it uniformly both lead to the assumption that methane is either being replenished into the atmosphere or is being sequestered into sinks periodically (Atreya, 2007). Volcanic activity can produce methane, but Martian volcanoes have been extinct for far too long (hundreds of millions of years) to be responsible for present concentrations of the gas (Krasnopolsky, 2006). Extraplanetary sources like comet impacts could have brought enough methane to result in a planetary average of 10 ppbv; however, the gas is not uniformly concentrated across Mars, and seasonal plumes of methane found by Mumma suggest there is some mechanism releasing and possibly trapping the gas over time (Atreya, 2007; Hand, 2008). Finally, methane on Mars could come from geochemical sources such as serpentinization, a process in which subterranean concentrations of iron or magnesium produce hydrogen which in turn reacts with a carbon source to form methane (Krasnopolsky, 2005). Methane produced by serpentinization could be trapped underground and gradually seep through fissures in the crust. It could also be stored as clathrates, or stable hydrates of

methane gas that is buried in subsurface reservoirs for long periods of time (Gainey & Elwood Madden, 2012).

The detection of methane on Mars has been one of the most encouraging discoveries over the past fifteen years because methane is usually associated with living things and could indicate the presence of microbial methane producers living on Mars. Of the methane on Earth (which has about 175 times more methane on average than Mars) around eighty percent is produced biologically (Atreya, 2007; Etiope, 2011). Biological methane production occurs most often in the digestive tract of ungulates due to methanogens, anaerobic archaea that reduce carbon dioxide to methane with hydrogen as the reducing agent (Jarrell & Kalmokoff, 1987). Methanogens have been shown to grow in very inhospitable environments on Earth, and as chemoautotrophs, they only require inorganic fuel (CO_2 and H_2) meaning they can grow without sunlight or liquid water (Abbasi, 2011; Jarrell & Kalmokoff, 1987). Thus methanogens could be living in the Martian subsurface and be the true source of methane production on the red planet either presently, or in the past having produced methane that was stored as clathrates only to be released later (Gainey & Elwood Madden, 2012).

In 1976, still early in NASA's exploration of Mars, the two Viking Mars Landers discovered the organic compounds, chloromethane and dichloromethane which researchers excused as "contamination from cleaning fluid" ("Viking revisited," 2010). However, the two organic molecules could have been formed from the reaction of another organic compound, perchlorate, with the extreme heat created by the Lander (Smith, 2011). Viking's discovery was not truly understood or believed by researchers until the Phoenix Lander confirmed the presence of perchlorate in Martian soil many

years later (Kerr, 2010). The Phoenix Lander mission of 2007 not only confirmed Viking's findings, but also provided confirmation of the Odyssey orbiter's discovery of water, as well as made its own discoveries of Mars' soil composition and geological history (Smith, 2011).

Using its Thermal and Evolved-Gas Analyzer (TEGA) and Wet Chemistry Lab (WCL), Phoenix discovered two important components of the Martian soil, perchlorate (conc. 0.5%) and calcium carbonate (conc. 5%) (Smith, 2011). The calcium carbonate suggests that Mars soil may have been wet recently, and alkaline soil was a unique find for the Phoenix Lander that previous missions missed (Smith, 2011). However, perchlorate was the biggest surprise from the Phoenix mission because it is rare on Earth and adds more possibility for life in the Martian soil (Marion et al., 2010). On Earth it is often synthesized for use as a jet propellant, but in nature it rarely accumulates except for in very dry places (like the Atacama Desert in Chile) due to the lack of water to wash it away (Ader et al., 2008). On Mars, where droughts are common, high concentrations of perchlorate could conceivably accumulate under the surface (Ader et al., 2008). Perchlorate salts such as $Mg(ClO_4)$, $Ca(ClO_4)$, and $Na(ClO_4)$ with low eutectic temperatures could lower the freezing point of water to around -70° C, and with warm enough weather, could form a supersaturated aqueous solution (Marion et al., 2009; Gough et al. 2011). This brine could support life, especially microbes that can anaerobically reduce perchlorate (Dudley et al., 2007).

Thus, methanogens and perchlorate-reducing organisms have the necessary food and possibly even an aqueous habitat under the Martian crust. They are unlikely to live together as perchlorate is a strong oxidizing agent that could interfere with the strict

anaerobic metabolism of methanogens (Jarrell & Kalmokoff, 1987). However, the potential aqueous environment created by perchlorate deposits could be the most suitable habitat for Martian methanogens, and thus it is worthwhile to observe the methanogen's ability to grow in the presence of perchlorate. Previous research has indicated methanogens have the ability to grow relatively normally in up to 1% solutions of perchlorate (Goodhart, 2011). This experiment aimed to observe the metabolism of methanogens in solutions of perchlorate up to 10% concentration with the hypothesis that the methane output would decrease with the increasing concentrations of perchlorate.

Materials and Methods

In order to get a broad understanding of perchlorate's effect on methanogen metabolic activity, four different methanogens (*Methanothermobacter wolfeii, Methanosarcina barkeri, Methanobacterium formicicum,* and *Methanococcus maripaludis)* were involved in this experiment. These methanogens were grown in the presence of differing levels of calcium perchlorate, $Ca(ClO_4)$.

Each methanogen was cultured in anaerobic test tubes with species specific standard methanogen growth media. Each species also had a specific temperature at which it was incubated in order to ensure optimal growth. The components of each different growth medium and the temperature at which each species was incubated can be found as Appendix A. As methanogens are obligate anaerobes, their media had to be made in an oxygen-free environment. This was accomplished using an anaerobic chamber. The media were prepared outside of the chamber, then placed inside the chamber, and divided into aliquots for each test tube. Each test tube was then sealed

using a rubber stopper, crimped with a metal cap, and finally sterilized in an autoclave in order to avoid contamination. The test tube and media are designed to provide the methanogens with an adequate environment to grow optimally.

Twenty-eight culture tubes were prepared for each of the four organisms being tested in this experiment. This number allowed for four groups of seven tubes within the twenty-eight for each organism. These four groups were differentiated by the concentrations of $Ca(ClO_4)$ injected into the tubes using a syringe. The first seven acted as a control for each organism, meaning no perchlorate was included. The latter three groups had 1%, 5%, and 10% perchlorate concentrations respectively. These three solutions were prepared and sterilized in the autoclave before injection.

Once the media and perchlorate concentrations had been prepared and separated into the 112 tubes, they were ready for inoculation. Just before inoculation, 0.125mL of 2.5% sodium sulfide (Na_2S) was injected into each tube to act as a reducing agent. Anaerobes cannot grow in the presence of even the smallest amount of oxygen, so it is necessary to use a reducing agent to rid the tubes of any residual oxygen. To inoculate the tubes, organisms were taken from stock and injected using a syringe into each tube. Each tube was then pressurized using 2 atm of hydrogen (H_2) they would later rely on for energy. At this point the methanogens were placed into their respective incubators in order to begin growing. As the methanogens grow, they take in hydrogen and produce methane; thus this methane is a suitable indicator of growth.

In order to measure this methane, 1mL samples of the gas in the headspace of each test tube were removed using a syringe and injected into a gas chromatograph. The

gas chromatograph then measures the methane concentration of the sample as a percentage. Measurements were taken beginning three days after inoculation and then every seven days afterwards for three weeks. The results were averaged and graphed in order to compare the average values over time.

Data and Results

The results of this experiment show a negative correlation between the average methane production as a percent of headspace volume and the increasing concentrations of calcium perchlorate. The biggest producers of methane in all the species tested were the controls and methanogens exposed to only 1% solutions of perchlorate as seen in Figures 1-4. In each of these figures, the performance of all four groups within each species is compared. In the *M. wolfeii* and *M. formicicum* populations, a significant difference was found between the control and 1% groups. However, the difference between the control and 1% groups was shown to be within the standard error bounds for the *M. barkeri* and *M. maripaludis* populations. All four populations showed a significant difference between the lower perchlorate concentrations (Control and 1% groups) and the higher concentrations (5% and 10%) which only produced over 1% methane composition in a *M. wolfeii* sample.

By comparing all four organisms' production together within each group of perchlorate concentration (as seen in Figures 5-8), species specific performance is exhibited. *M. wolfeii* outperformed the other three organisms in all concentrations of perchlorate. *M. barkeri* produced the lowest final methane compositions in all groups apart from the 5% group. In all perchlorate concentrations apart from 10%, *M.*

formicicum produced the most methane after three days, but in each concentration *M. wolfeii* eventually surpassed it.

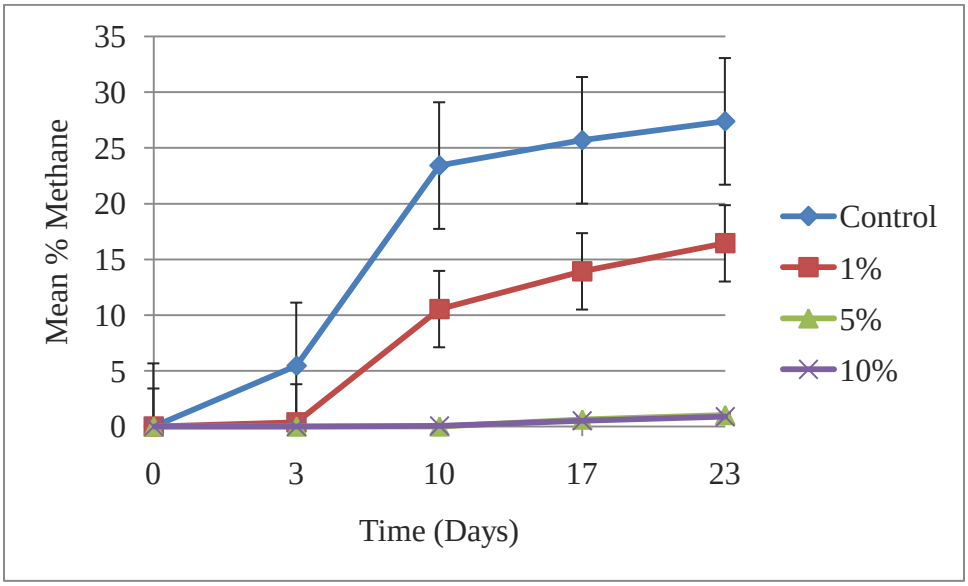

Figure 1. Mean methane composition as percentage of headspace volume in culture tubes of *Methanothermobacter wolfeii* in MM medium with four different perchlorate concentrations over the course of twenty-three days.

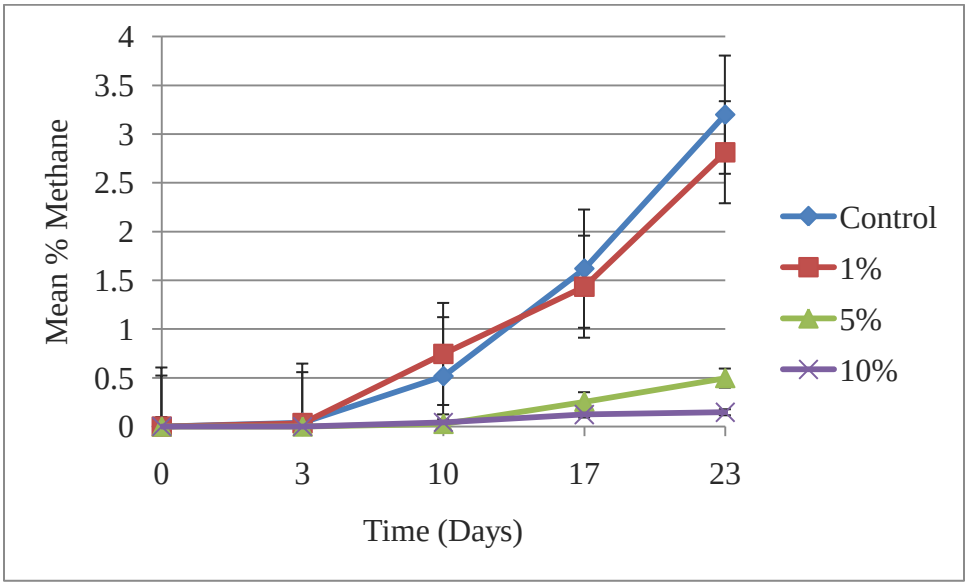

Figure 2. Mean methane composition as percentage of headspace volume in culture tubes of *Methanosarcina barkeri* in MS medium with four different perchlorate concentrations over the course of twenty-three days.

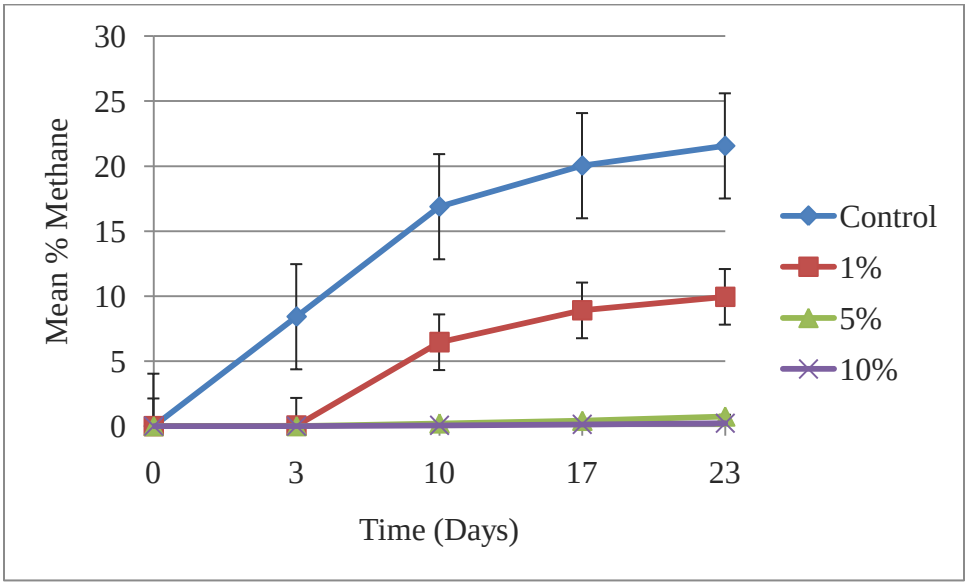

Figure 3. Mean methane composition as percentage of headspace volume in culture tubes of *Methanobacterium formicicum* in MSF medium with four different perchlorate concentrations over the course of twenty-three days.

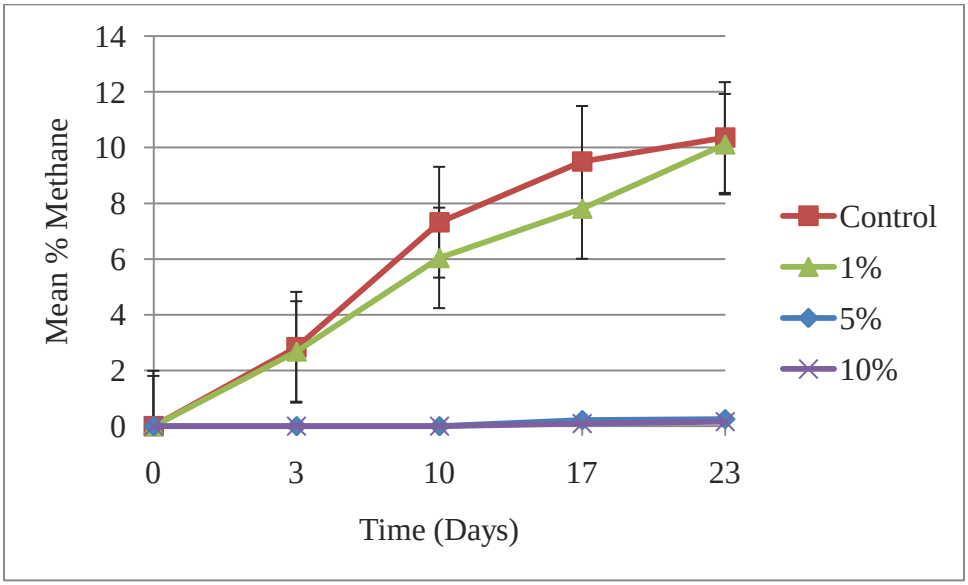

Figure 4. Mean methane composition as percentage of headspace volume in culture tubes of *Methanococcus maripaludis* in MSH media with four different perchlorate concentrations over the course of twenty-three days.

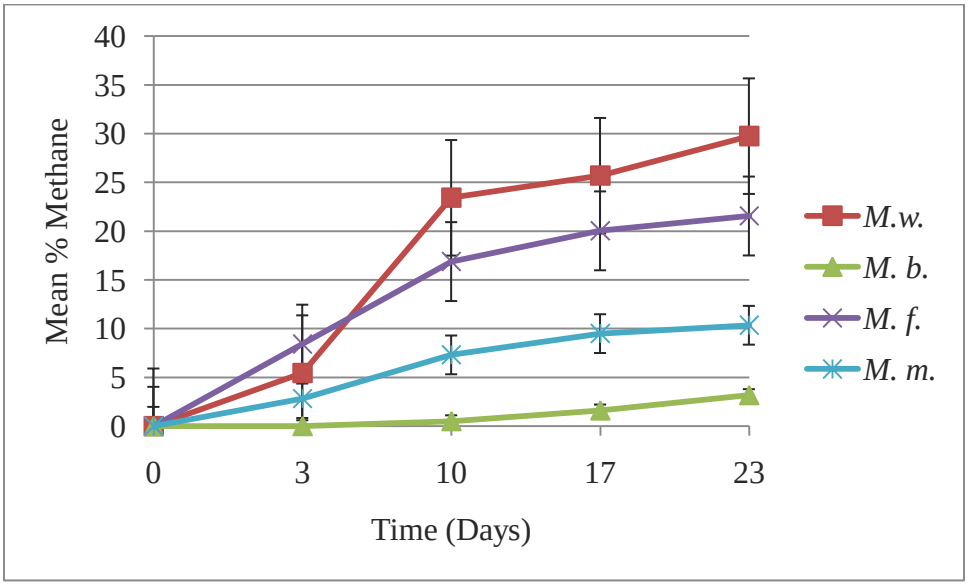

Figure 5. Mean methane composition as percentage of headspace volume in culture tubes of four different species of methanogens over the course of twenty-three days. *M. w.* - *Methanothermobacter wolfeii, M. b. – Methanosarcina barkeri, M. f.* - *Methanobacterium formicicum, M. m.* - *Methanococcus maripaludis*

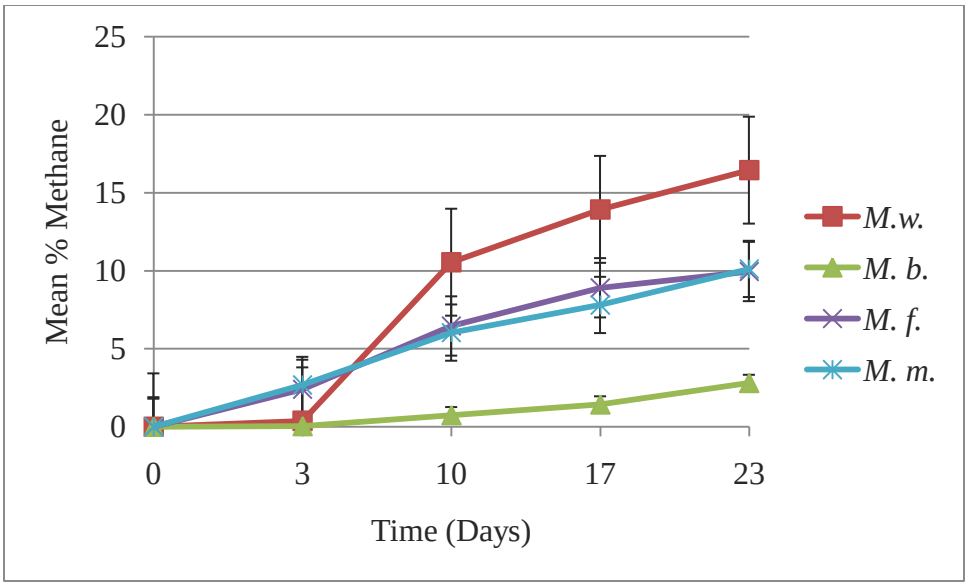

Figure 6. Mean methane composition as percentage of headspace volume in culture tubes of four different species of methanogens with a perchlorate concentration of 1% over the course of twenty-three days. *M. w.* - *Methanothermobacter wolfeii, M. b.* – *Methanosarcina barkeri, M. f.* - *Methanobacterium formicicum, M. m.* - *Methanococcus maripaludis*

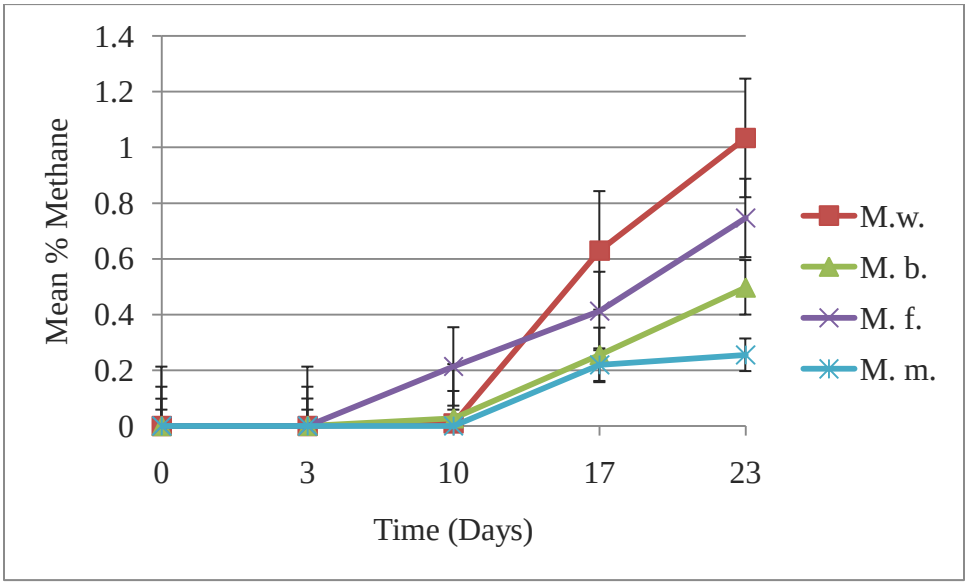

Figure 7. Mean methane composition as percentage of headspace volume in culture tubes of four different species of methanogens with a perchlorate concentration of 5% over the course of twenty-three days. *M. w. - Methanothermobacter wolfeii, M. b. – Methanosarcina barkeri, M. f. - Methanobacterium formicicum, M. m. - Methanococcus maripaludis*

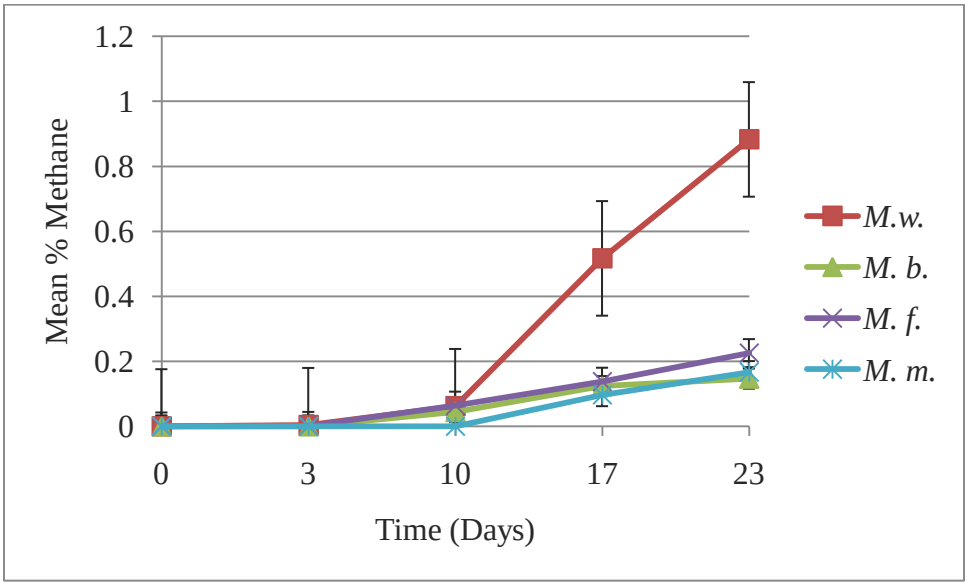

Figure 8. Mean methane composition as percentage of headspace volume in culture tubes of four different species of methanogens with a perchlorate concentration of 10% over the course of twenty-three days. *M. w. - Methanothermobacter wolfeii, M. b. – Methanosarcina barkeri, M. f. - Methanobacterium formicicum, M. m. - Methanococcus maripaludis*

Discussion

On November 26, 2011, Curiosity, the most recent Mars rover mission, launched from Cape Canaveral Air Force Station in Florida (Kruesi, 2012). The car-sized rover is en route for Mars with an expected landing date of August 6, 2012 (Kruesi, 2012). The

rover is packed with 10 instruments it will use during its year-long (Martian year) trek across the Gale Crater, an area expected to have been carved out of the landscape in an ancient "water-rich era" (Smith, 2011). As essentially a laboratory on wheels, the nuclear-powered Curiosity will retrieve samples and analyze them for evidence of organic materials and other remnants of past life. If it happens to find evidence of methane, it has the instruments to determine whether the methane is biological or geological in origin (Spotts, 2011). Thus the Curiosity rover could take much of the speculation surrounding life on Mars out of the equation even though it is still not equipped to find life itself yet (Spotts, 2011).

The results of this experiment support the previously asserted notion that methanogens could survive reasonably in environments containing 1% concentrations of perchlorate (Goodhart, 2011). However, the prospect that methanogens could function normally at concentrations of perchlorate greater than 1% was not supported by the data. In the 5% perchlorate concentration groups, all methanogens produced at most $1/30^{th}$ of their production in the control group. In the 10% group, all organisms showed a considerable decrease in production from the 5% group, and apart from *M. wolfeii*, all other organisms produced negligible amounts of methane at this high level of salt concentration.

Both *M. barkeri* and *M. maripaludis* produced relatively equivalent amounts of methane in the control and 1% groups, which could indicate their heightened ability to metabolize in the presence of perchlorate. However, both of these organisms produced considerably less overall methane production than the other two methanogens and also produced the least at the 5% and 10% concentrations.

Thus, the results of this experiment support the hypothesis that environments containing higher concentrations of perchlorate correspond to lower methanogen metabolic activity. This inverse relationship was shown to be more apparent in higher levels of perchlorate than lower concentrations which implies that methanogens could reasonably survive in the presence of small amounts of perchlorate. This relationship is most likely a result of the negative effect of strong oxidizing agents, such as perchlorate, on anaerobic organisms undergoing redox reactions (Jarrell & Kalmokoff, 1987).

These results do not, however, conclusively determine the inability for methanogens to exist in the possible aqueous brine reservoirs under the Martian surface. Even at high levels of perchlorate, all organisms were shown to produce some level of methane. This small amount of production would only be diminished at the incredibly cold temperatures of the Martian climate, but over long periods of time, it is possible that methanogens could produce a portion if not all of the methane present on Mars.

This experiment was run at the optimal temperature for each methanogen species, which does not attempt to emulate the Martian climate. Also, the reducing agent sodium sulfide could have had some effect on the oxidizing effects of perchlorate and skewed the results. In the future, experiments should be attempted at lower temperatures and in a soil composition made to replicate the Martian subsurface in order to deliver more conclusive results.

The results of this experiment and many other earthbound attempts to support the existence of life on Mars rely on the information gained through use of Mars landers and orbiters. Starting in August, the findings of the Curiosity Lander promise to give

perspective on the results of this experiment and others while also giving astrobiologists leads for future pursuits. Experiments such as this are necessary to narrow the possibilities of how or where life could exist on Mars. Conclusions gained from experimentation with methanogens or perchlorate-reducing bacteria can be used to decipher the findings of Mars landers and to specialize their equipment, destination, and behavior to better the odds of finally finding extraterrestrial life.

Appendix A

	Standard Methanogen Growth Medium for 50.00mL			
Ingredient	MM	MS	MSF	MSH
500µL Solution A	X	X	X	X
100µL Solution B	X	X	X	X
100µL Solution C	X	X	X	X
50µL Solution D	X	X	X	X
0.1g Yeast Extract		X	X	X
0.1g Trypticase Peptone		X	X	X
0.025g Mercaptoethane Sulfonic Acid		X	X	X
500µL Sodium Formate			X	X
1.475g NaCl				X
0.085g $MgCl_2$				X
0.025g KCl				X

Solution A:

100g/L NH_4Cl
100g/L $MgCl_2$-$6H_2O$
40g/L $CaCl_2$-$2H_2O$

Solution B:

200g/L K_2HPO_4-$3H_2O$

Solution C:

0.5g/L Resazurin

Solution D:

500mg/L Na_2-EDTA-$2H_2O$
150mg/L $CoCl_2$-$6H_2O$
100mg/L $MnCl_2$-$4H_2O$
100mg/L $FeSO_4$-$7H_2O$
100mg/L $ZnCl_2$
40mg/L $AlCl_3$-$6H_2O$
30mg/L Na_2WO_4-$2H_2O$
20mg/L $CuCl_2$-$2H_2O$
20mg/L $NiSO_4$-$6H_2O$
10mg/L H_2SeO_3
10mg/L H_3BO_3
10mg/LNa_2MoO_4-$2H_2O$

Medium	Organism	Incubation Temperature (C)
MM	*Methanothermobacter wolfeii*	55º
MS	*Methanosarcina barkeri*	37º
MSF	*Methanobacterium formicicum*	37º
MSH	*Methanococcus maripaludis*	Room temperature ~23º C

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