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**Methanogens, Plausible Extraterrestrial Life Forms on Mars, and their Tolerance to
Increasing Concentrations of Illite Clay**

An Honors College Thesis Proposal submitted in accordance to the J. William Fulbright Honors
College of Arts & Science's requirements for demonstration of competency in the Biological
Sciences

By

Chandler Kern

2018

Biological Sciences

J. William Fulbright College of Arts and Sciences

The University of Arkansas

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Abstract

Methanogens, some of Earth's most primitive prokaryotic organisms, are candidates for possible life forms capable of inhabiting Mars. Specifically, four different species (*Methanobacterium formicicum*, *Methanococcus maripaludis*, *Methanosarcina barkeri*, *Methanothermobacter wolfeii*) were analyzed for their tolerance to the presence of illite clay. Illite is a crystalline mineral that has been identified from regions of Mars's surface. Results indicated that all four species grew with some success in the illite at different concentrations. This experimentation with methanogens' abilities to survive and reproduce in the presence of illite allows for a more accurate understanding of the potential capability of microbial growth in Martian conditions.

Methanogens, a Plausible Extraterrestrial Life Form on Mars, and their Tolerance to Increasing Concentrations of Illite Clay

1. Introduction

Since NASA's *2001 Mars Odyssey* detected ice deposits under the surface of Mars (Mars Odyssey, 2017), the planet has been considered an environment that could possibly be suitable to sustain extraterrestrial life, and a plausible candidate capable of bearing Mars's extreme environmental conditions are methanogens. These anaerobic prokaryotes are theorized to have been one of the first living organisms to have evolve and survive in Earth's pre-oxygenated atmosphere (Gribaldo and Brochier-Armanet, 2006), and now with the discovery of water, the elixir of life, on Mars, extensive research is being conducted to see if methanogens are capable of potentially inhabiting Mars.

Mars, nicknamed the "Red Planet", is the fourth planet from the sun and the second smallest to Mercury in our solar system. Mars appears to be red in color to the naked eye due to the iron oxide on the planet's surface. Mars, also the name of the god of war in Roman mythology, is a terrestrial planet with a very thin atmosphere. Having bearable seasonal temperatures, ranging from lows of -143°C to highs of 35°C , Mars can further be considered a possible planet capable of supporting life ("Mars, 2018.").

With that being said, the conditions of Mars are certainly not favorable for life as we know it on Earth. As mentioned before, the relatively extreme temperatures of Mars are not suitable for a large portion of Earth's known species; however, methanogens are known for their

ability to survive in extreme environmental conditions. For example, they have been collected and studied from Earth's wetlands, hot springs, submarine hydrothermal vents, and even the digestive tracts of animals such as humans. Methanogens have even been shown to be capable of surviving in "solid" rock on the Earth's crust. This ability, along with their tolerance of temperatures varying from about -40°C to 145°C , makes them a reasonable candidate for possibly being able to survive on Mars's surface ("Methanogen, 2018.").

One of the leading researchers on methanogens as a possible life form on Mars is Dr. Timothy Kral at the University of Arkansas (Chastain and Kral, 2010a; Chastain and Kral, 2010b; Kendrick and Kral, 2006; McAlister and Kral, 2006; Kozup and Kral, 2009; Kral et al., 1998; Kral et al., 2004; Kral et al., 2011; Kral and Altheide, 2013; Kral et al., 2014; Kral et al., 2016; Mickol and Kral, 2016; Sinha, 2016). In his laboratory, Kral has replicated various characteristics of Mars's surface as well as subsurface, and he has performed investigations to determine whether or not certain Martian environmental conditions inhibit methanogen growth. Members of Dr. Kral's lab have investigated whether some clays found on Mars are inhibitory to methanogens, and if not, whether they contain nutrients that can support growth and reproduction. Similarly, in my project, I intended to study methanogens' ability to grow in the presence illite.

Illite is a crystalline mineral that has been identified in clay from regions of Mars such as the Oxia Palus quadrangle (Wray et al., 2010). The purpose of my research was to identify whether or not illite inhibits methanogen growth, and then, given that it does not completely

prevent growth, determine if illite can support the growth of our methanogens. All four species of methanogens in my project are hydrogenotrophic; this specific variation of methanogenesis uses carbon dioxide as a carbon source and hydrogen as an energy source, and methane and water are produced and expelled into the surrounding environment. The balanced equation for this particular metabolic pathway is as follows:



Furthermore, the Martian atmosphere is composed of a gas mixture that is 96% carbon dioxide. Pair that with the presence of atmospheric hydrogen gas from “vigorous volcanic outgassing from a highly reduced early Martian mantle (Ramirez et al., 2013),” and the results are the correct reactants for hydrogenotrophy to occur. To further this hypothesis, studies have shown that some areas of Mars’s atmosphere were historically concentrated with methane gas (“Atmosphere of Mars, 2017.”). Now, the question becomes: where does this methane gas in the Martian atmosphere originate? Conservative views credit volcanic outgassing as the source of these high concentrated areas of atmospheric methane, but until an inhibiting abiotic Martian growth factor is identified, methanogens exist as a possible explanation for the presence of methane on Mars. My experiment is intended to further the research on methanogens as a possible form of extraterrestrial life on Mars.

2. Experimental Methods

As mentioned previously, in this experiment, four different species of methanogens were exposed to concentrations of illite in order to determine if illite is inhibitory to their growth.

Concentrations started at 1% wt/vol and increase to 2% wt/vol. The four methanogens selected to be used in this experiment and their respective growth media were as follows:

- *Methanobacterium formicicum* (MSF Media)
- *Methanococcus maripaludis* (MSH Media)
- *Methanosarcina barkeri* (MS Media)
- *Methanothermobacter wolfeii* (MM Media)

These particular methanogens were chosen for this research because each demonstrates variable characteristics and are the type strain for their respective species.

To assess how methanogens grow in the presence of illite, many solutions were prepared with varying concentrations of illite. Each species was grown in these solutions in order to determine if methanogens can tolerate, grow, and reproduce in the presence of illite. For each methanogen used in this experiment, a specific medium was used to promote their growth (Appendix). However, illite was added in equal concentrations to every batch of medium prepared in order to be certain that each methanogen was exposed to the exact same amount of illite. Each species of methanogen was grown in 1% wt/vol illite solutions. Each variation of growth medium contained carbon dioxide to serve as the methanogen's carbon source. Then, all test tubes were pressurized with hydrogen to serve as the methanogen's energy source. It is important to note that methanogens are obligate anaerobes and exposure to even trace amounts of atmospheric oxygen could be lethally toxic. If oxygen ever contaminated the test tubes in which

methanogens were being grown, the methanogens no longer produced methane as a metabolic byproduct. Because of this, after the growth media preparation, the test tubes, flasks containing the four types of growth media, and rubber test tube stoppers were transferred into a Coy anaerobic chamber containing approximately 90% carbon dioxide and 10% hydrogen (necessary for the palladium catalysts to properly deoxygenate the chamber) where they remained for a period of at least 36 hours in order to completely deoxygenate. After the deoxygenation process occurred, the different growth media were distributed into the test tubes, capped with rubber stoppers, and properly labeled with their respective contents. Following this step, all of the newly created test tubes and their growth media contents were autoclaved at 121°C in preparation for the introduction of methanogens. Next, prior to the addition of methanogens, sodium sulfide was added to each test tube in order to remove whatever residual oxygen still remained. Finally, the methanogens were inoculated and allowed to grow in their previously specified growth media. Methanogen growth was quantified through the use of gas chromatography. Using a syringe, a sample of the gas mixture within a test tube was removed and injected into the gas chromatograph. The results produced a numerical value for the percentage of methane in the gas sample. The amount of methane in a gas sample of a test tube was directly proportional to the number of surviving methanogens for that species, and using this information was how it was intended to assess the methanogens' tolerances to increasing concentrations of illite. It was likely that methanogen growth would decrease as the concentrations of illite increased because each growth medium already serves as an ideal environment for methanogen growth and reproduction. Thus, addition of any other compound would likely decrease the growth rate and survivorship of the methanogens. However, the underlying purpose of my research was to explore whether or not methanogens were inhibited by

illite. The growth of each methanogen in their varying concentrations of illite was then compared to a control group where no illite was added to the methanogens' media.

3. Results

An overall analysis of the methane production by the methanogens at both the 1% and 2% concentrations of illite, suggested that illite did not appear to exhibit significant inhibitory effects on the growth or reproduction of the methanogens. The mean values of methane percent concentrations for each methanogen in the presence of varying levels of illite was compared to the average methane percent concentrations of the methanogens in their respective controlled environment.

When examining the methanogens growth and reproduction in the presence of 1% illite, the overlapping standard error of mean (SEM) bars at every recorded point suggested that there appeared to be no significant difference in growth between *M. wolfeii* in the presence of 1% illite in comparison to *M. wolfeii* in their controlled environment (Figure 1), *M. maripaludis* in the presence of 1% illite in comparison to *M. maripaludis* in their controlled environment (Figure 2 and Figure 7), *M. formicicum* in the presence of 1% illite in comparison to *M. formicicum* in their controlled environment (Figure 3), and *M. barkeri* in the presence of 1% illite in comparison to *M. barkeri* in their controlled environment (Figure 11). The potentially remarkable exceptions to this trend are that in one trial a significantly greater methane percent concentration existed at day 16 between *M. wolfeii* in the presence of 1% illite in comparison to *M. wolfeii* in their controlled environment suggesting that illite potentially has a stimulating effect on *M. wolfeii*'s growth (Figure 5). Additionally, in another trial *M. formicicum* in the presence of 1% illite appeared to

produce significantly more methane than *M. formicicum* in their controlled environment from day 16 and beyond suggesting that illite could possibly exhibit stimulating effects on *M. formicicum*'s growth and reproduction (Figure 9). Similarly, in another trial *M. barkeri* in the presence of 1% illite appeared to produce significantly more methane than *M. barkeri* in their controlled environment from day 13 and beyond suggesting that illite could possibly exhibit stimulating effects on *M. barkeri*'s growth and reproduction (Figure 4). However, for all of the exceptional cases mentioned, due to the inconsistency of methanogen growth rates in laboratory, further experimentation must be completed in order to definitively make the conclusion that illite could possibly possess stimulating growth properties for *M. wolfeii*, *M. formicicum*, and *M. barkeri*.

When examining the methanogens growth and reproduction in the presence of 2% illite, a significantly greater methane percent concentration existed at day 8 between *M. wolfeii* in the presence of 2% illite in comparison to *M. wolfeii* in their controlled environment further suggesting that illite potentially has a stimulating effect on *M. wolfeii*'s growth (Figure 6). Similarly, a significantly greater methane percent concentration existed at day 31 between *M. barkeri* in the presence of 2% illite in comparison to *M. barkeri* in their controlled environment further suggesting that illite potentially has a stimulating effect on *M. barkeri*'s growth (Figure 12). Likewise, in another trial *M. formicicum* in the presence of 2% illite appeared to have produced significantly more methane than *M. formicicum* in their controlled environment from day 16 and beyond further suggesting that illite could possibly exhibit stimulating effects on *M. formicicum*'s growth and reproduction (Figure 10). Again though, for all of the remarkable cases mentioned, due to the inconsistency of methanogen growth rates in laboratory, further

experimentation must be completed in order to definitively make the conclusion that illite could possibly possess stimulating growth properties for *M. wolfeii*, *M. formicicum*, and *M. barkeri*.

On the other hand, *M. maripaludis* in the presence of 2% illite appeared to have produced significantly less methane than *M. maripaludis* in their controlled environment from day 24 and beyond suggesting that illite at greater concentrations could possibly exhibit inhibitory effects on *M. maripaludis*'s growth and reproduction (Figure 8). However, further experimentation must be completed in order to verify this phenomenon.

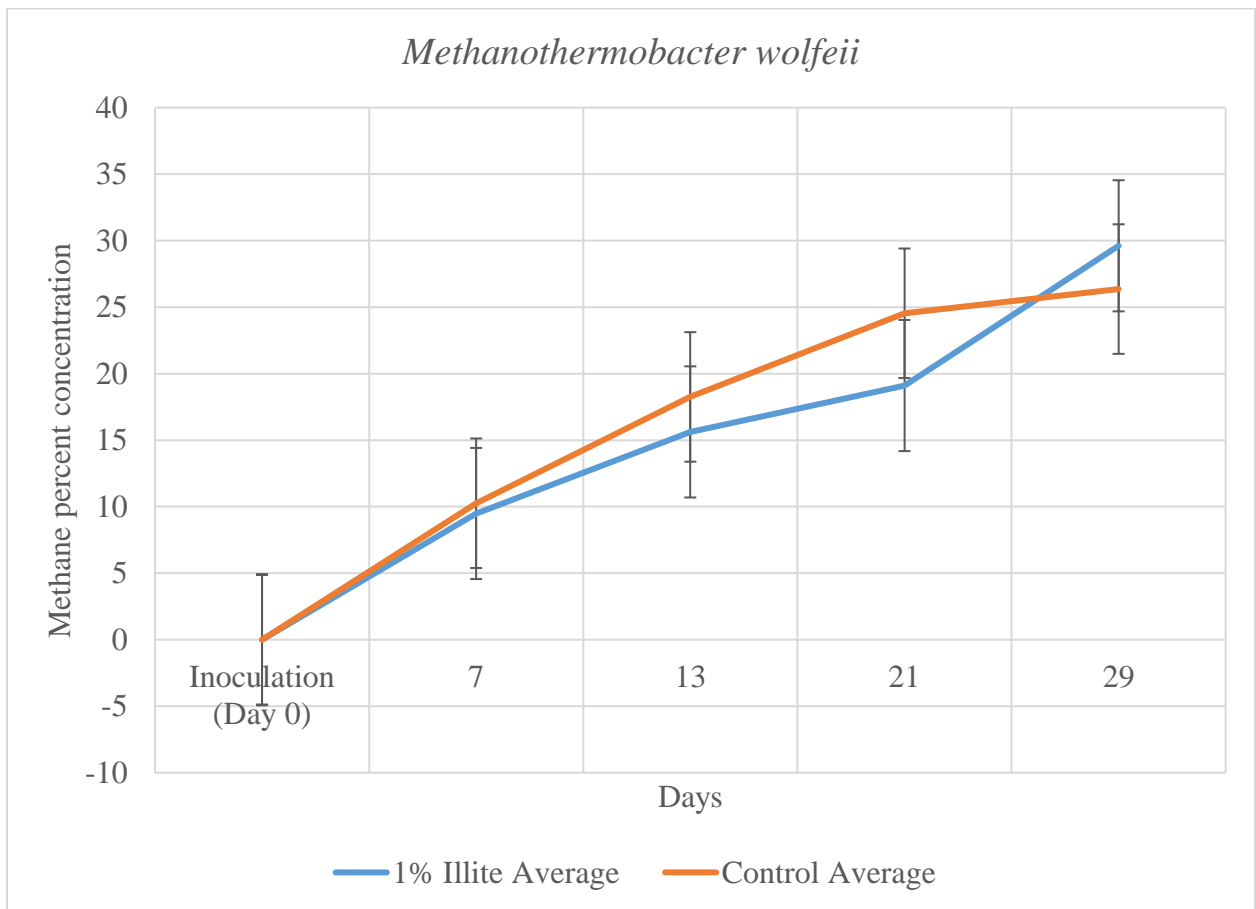


Figure 1: Methane production by *Methanothermobacter wolfeii* in the presence of varying concentrations of the illite clay in MM medium. The error bars depict plus or minus one standard error of the mean (SEM).

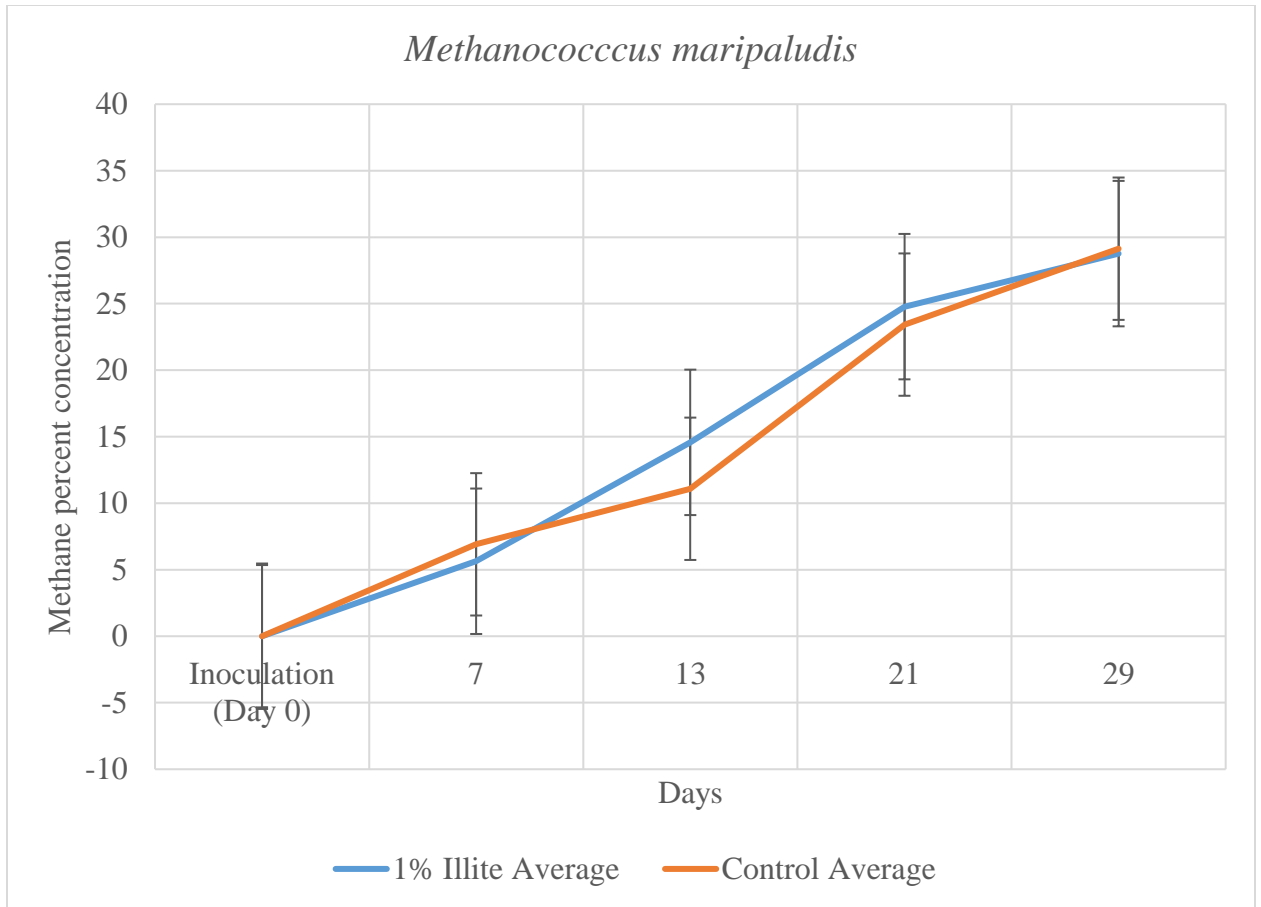


Figure 2: Methane production by *Methanococcus maripaludis* in the presence of varying concentrations of the illite clay in MSH medium. The error bars depict plus or minus one standard error of the mean (SEM).

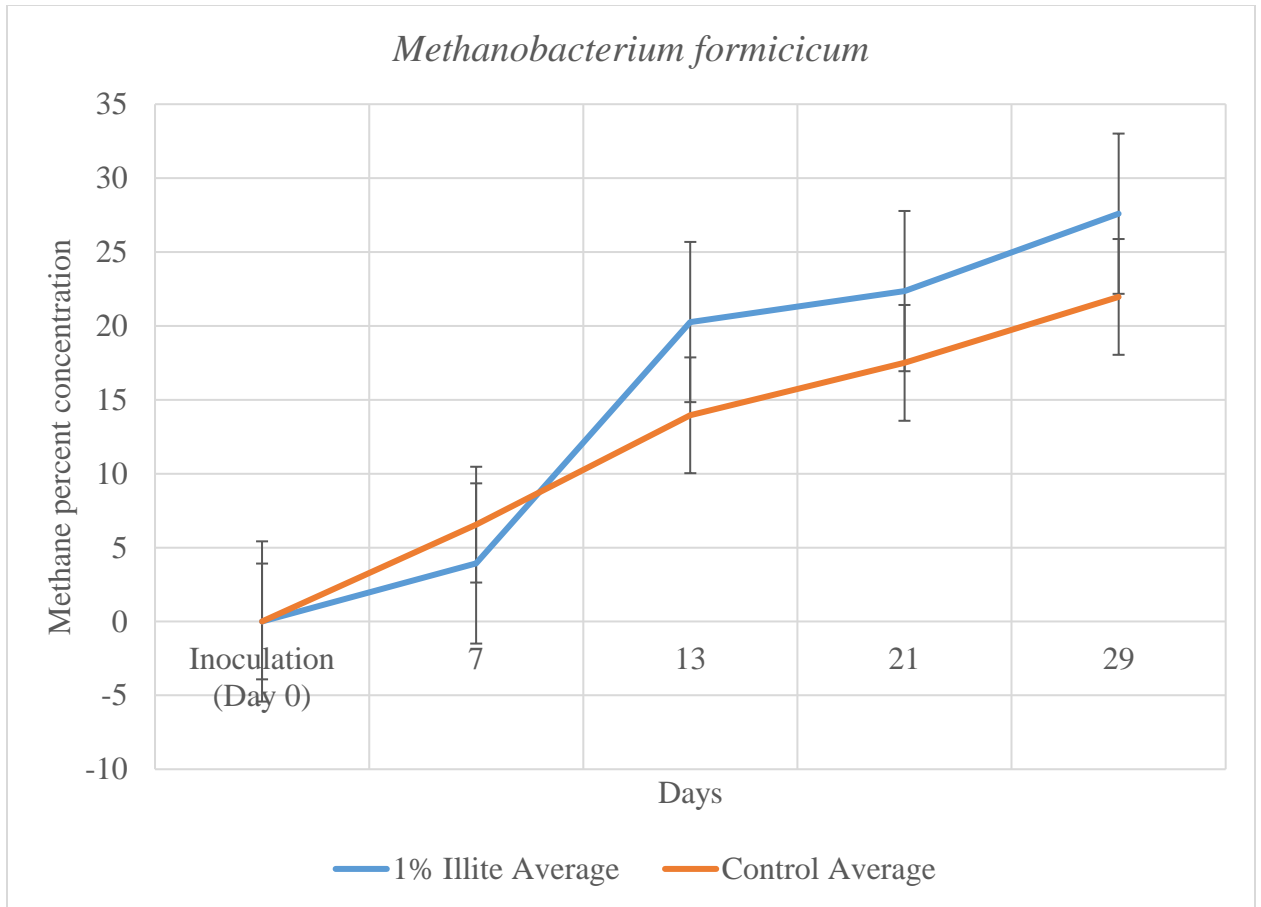


Figure 3: Methane production by *Methanococcus formicicum* in the presence of varying concentrations of the illite clay in MSF medium. The error bars depict plus or minus one standard error of the mean (SEM).

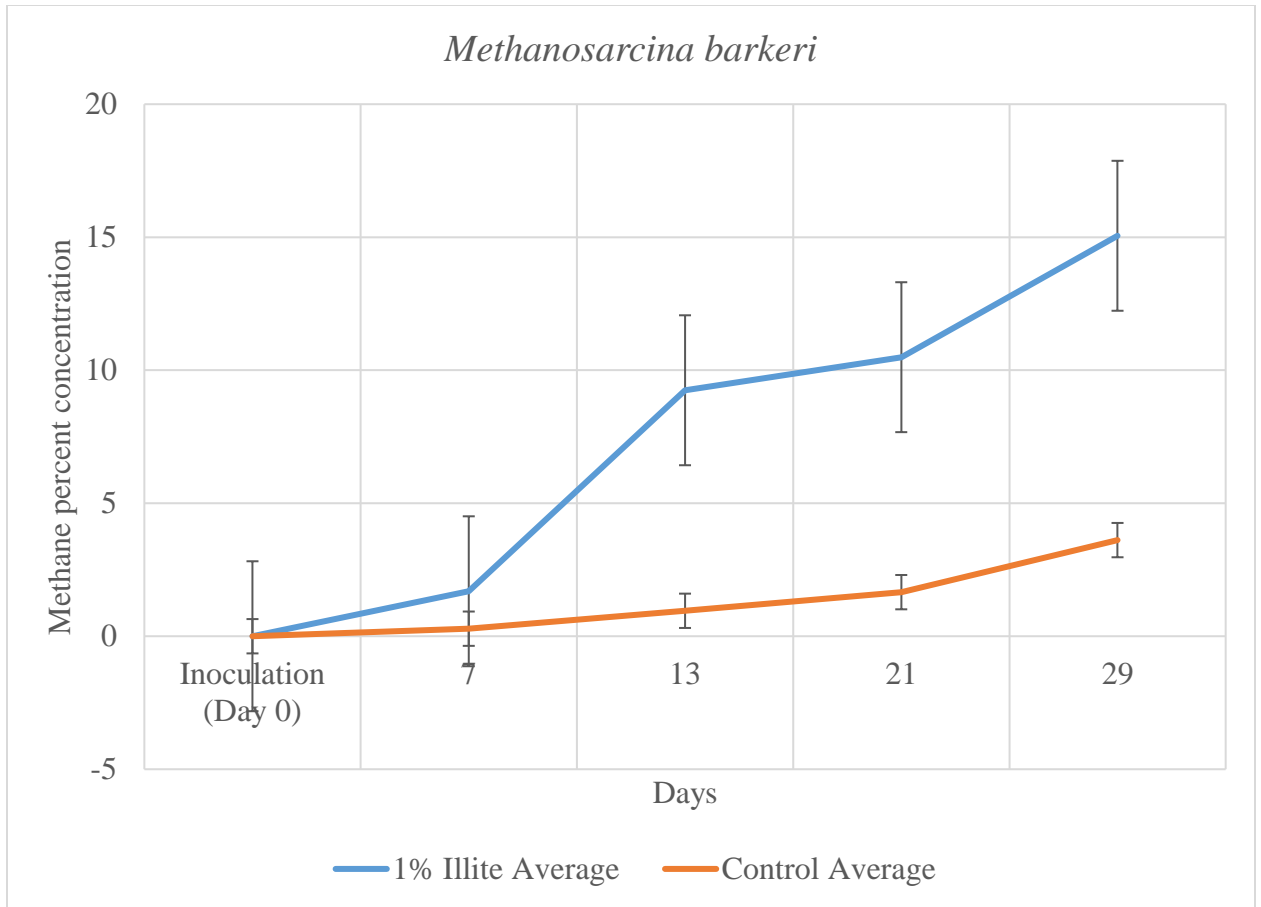


Figure 4: Methane production by *Methanosarcina barkeri* in the presence of varying concentrations of the illite clay in MS medium. The error bars depict plus or minus one standard error of the mean (SEM).

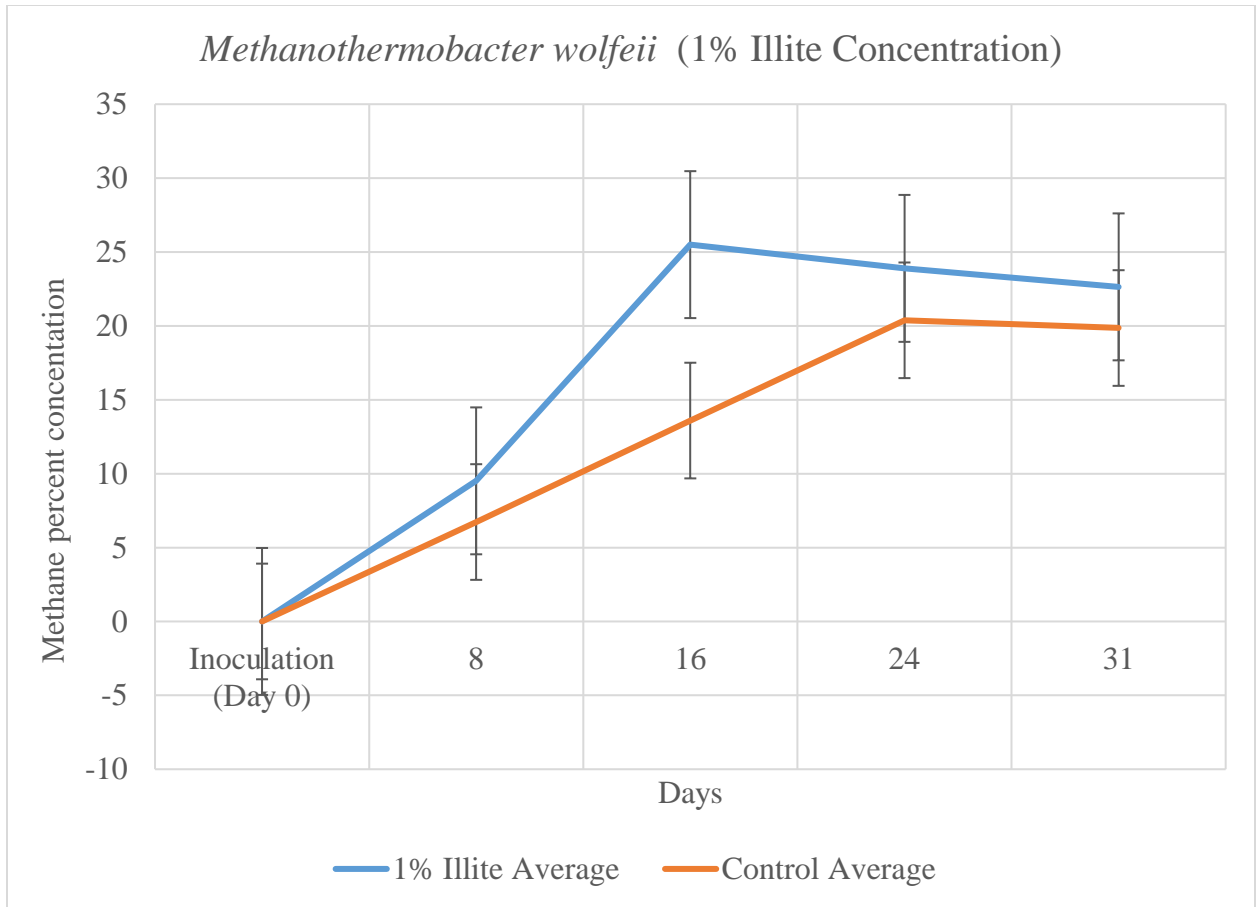


Figure 5: Methane production by *Methanothermobacter wolfeii* in the presence of varying concentrations of the illite clay in MM medium. The error bars depict plus or minus one standard error of the mean (SEM).

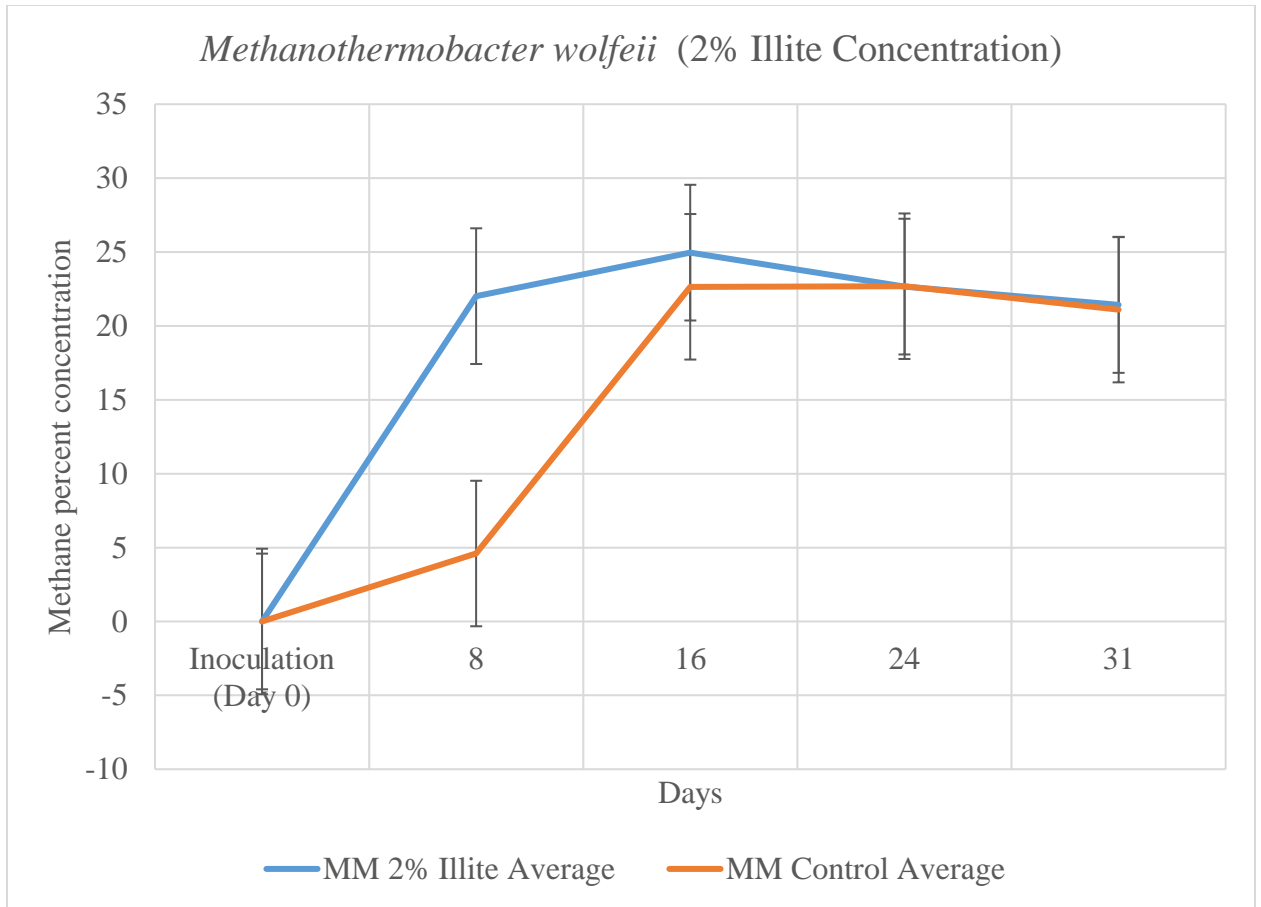


Figure 6: Methane production by *Methanothermobacter wolfeii* in the presence of varying concentrations of the illite clay in MM medium. The error bars depict plus or minus one standard error of the mean (SEM).

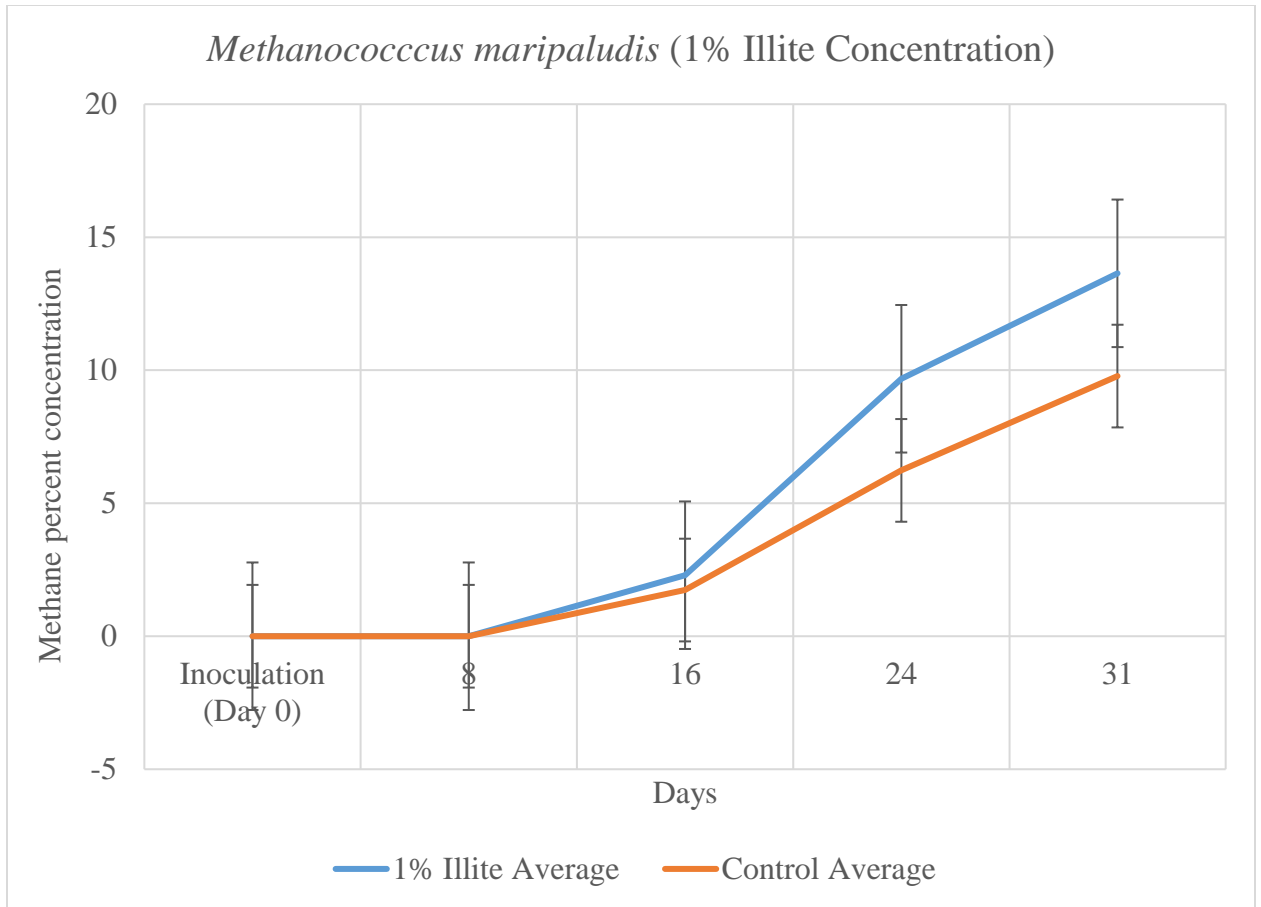


Figure 7: Methane production by *Methanococcus maripaludis* in the presence of varying concentrations of the illite clay in MSH medium. The error bars depict plus or minus one standard error of the mean (SEM).

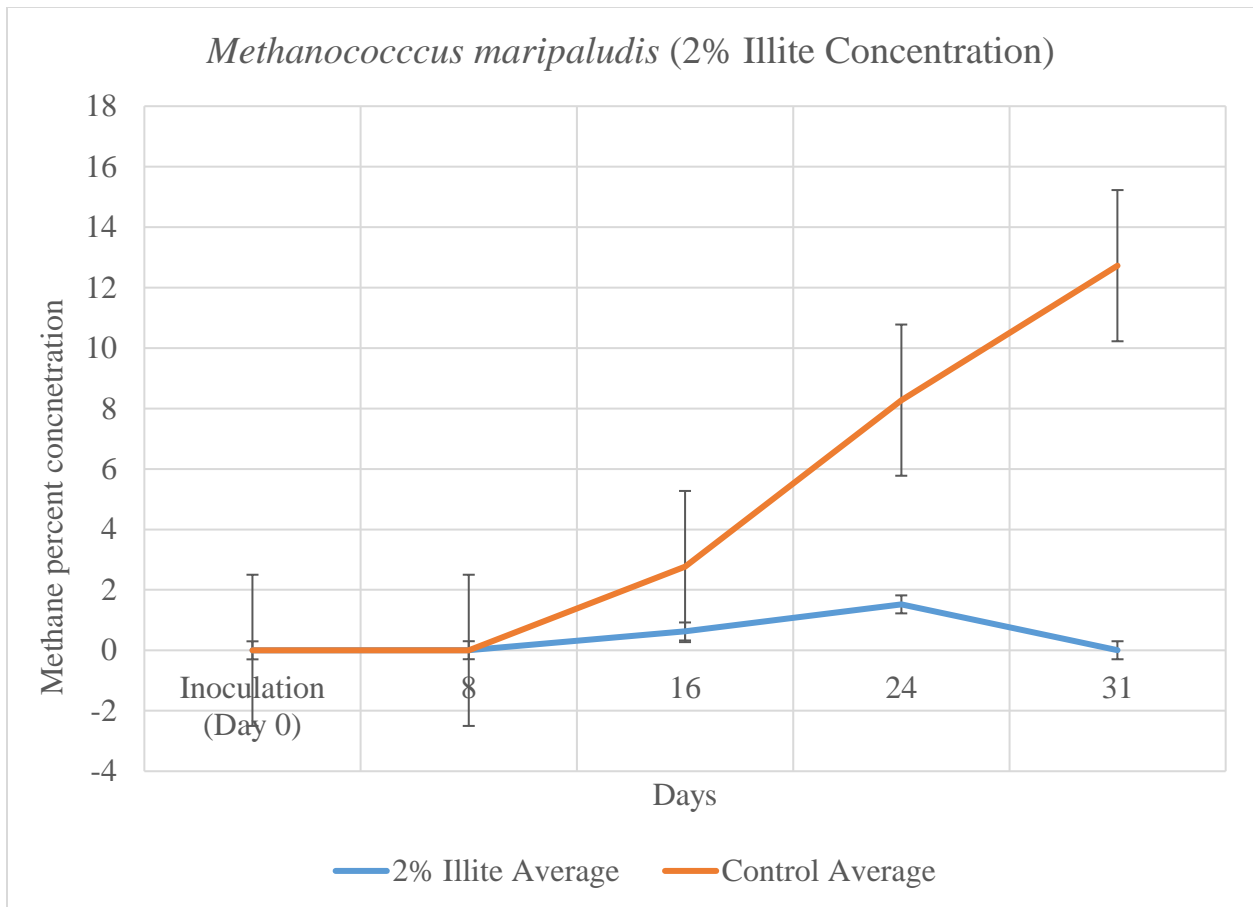


Figure 8: Methane production by *Methanococcus maripaludis* in the presence of varying concentrations of the illite clay in MSH medium. The error bars depict plus or minus one standard error of the mean (SEM).

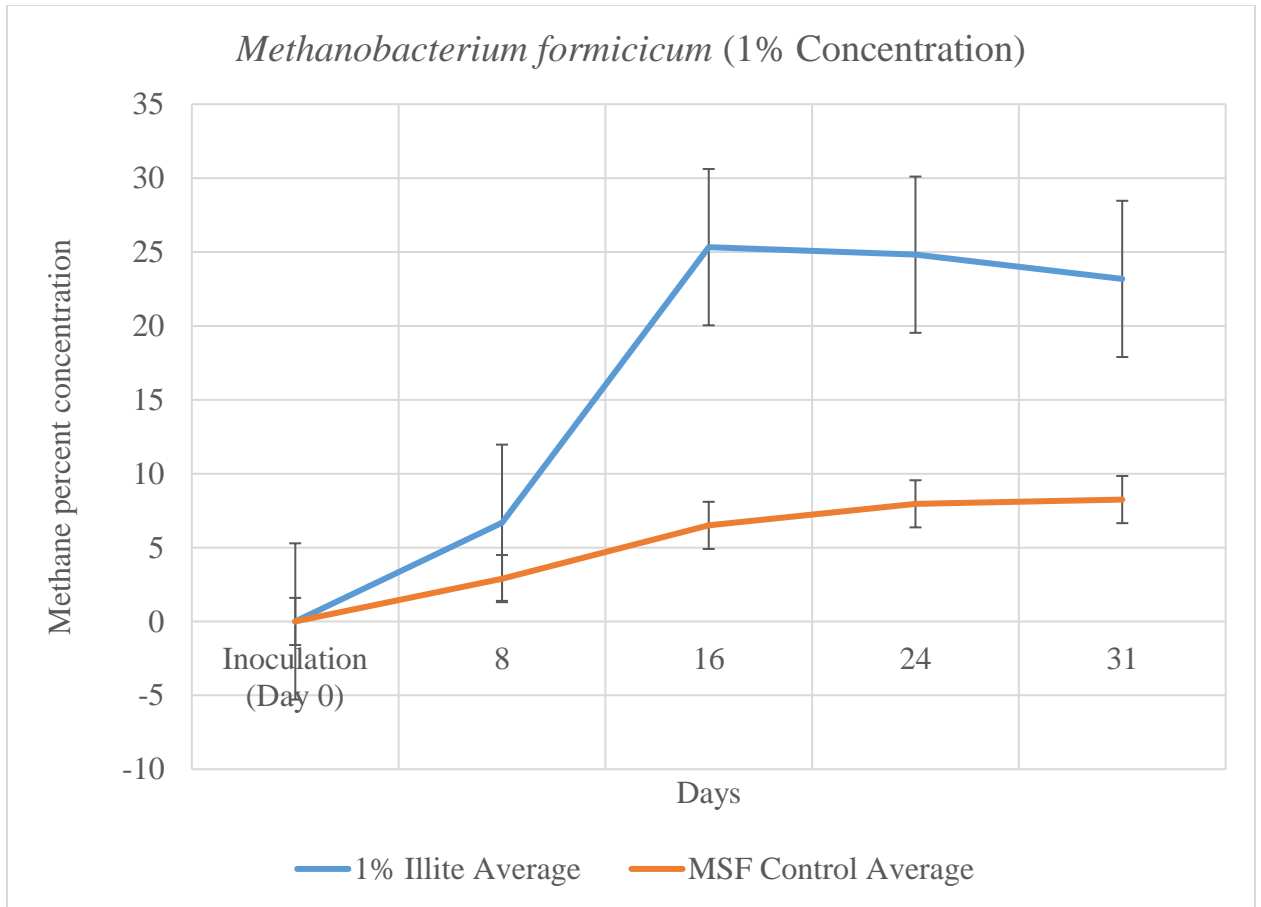


Figure 9: Methane production by *Methanococcus formicicum* in the presence of varying concentrations of the illite clay in MSF medium. The error bars depict plus or minus one standard error of the mean (SEM).

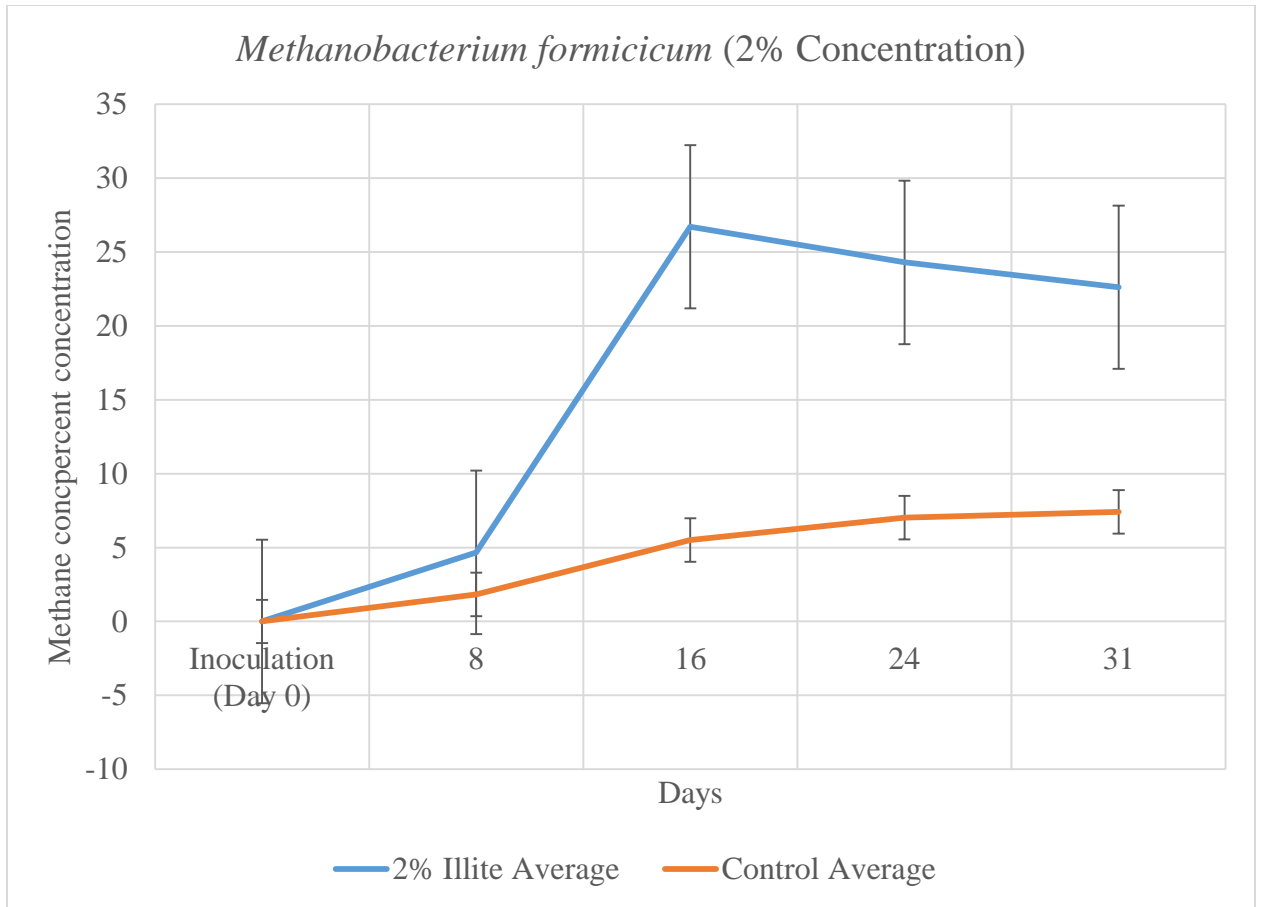


Figure 10: Methane production by *Methanococcus formicicum* in the presence of varying concentrations of the illite clay in MSF medium. The error bars depict plus or minus one standard error of the mean (SEM).

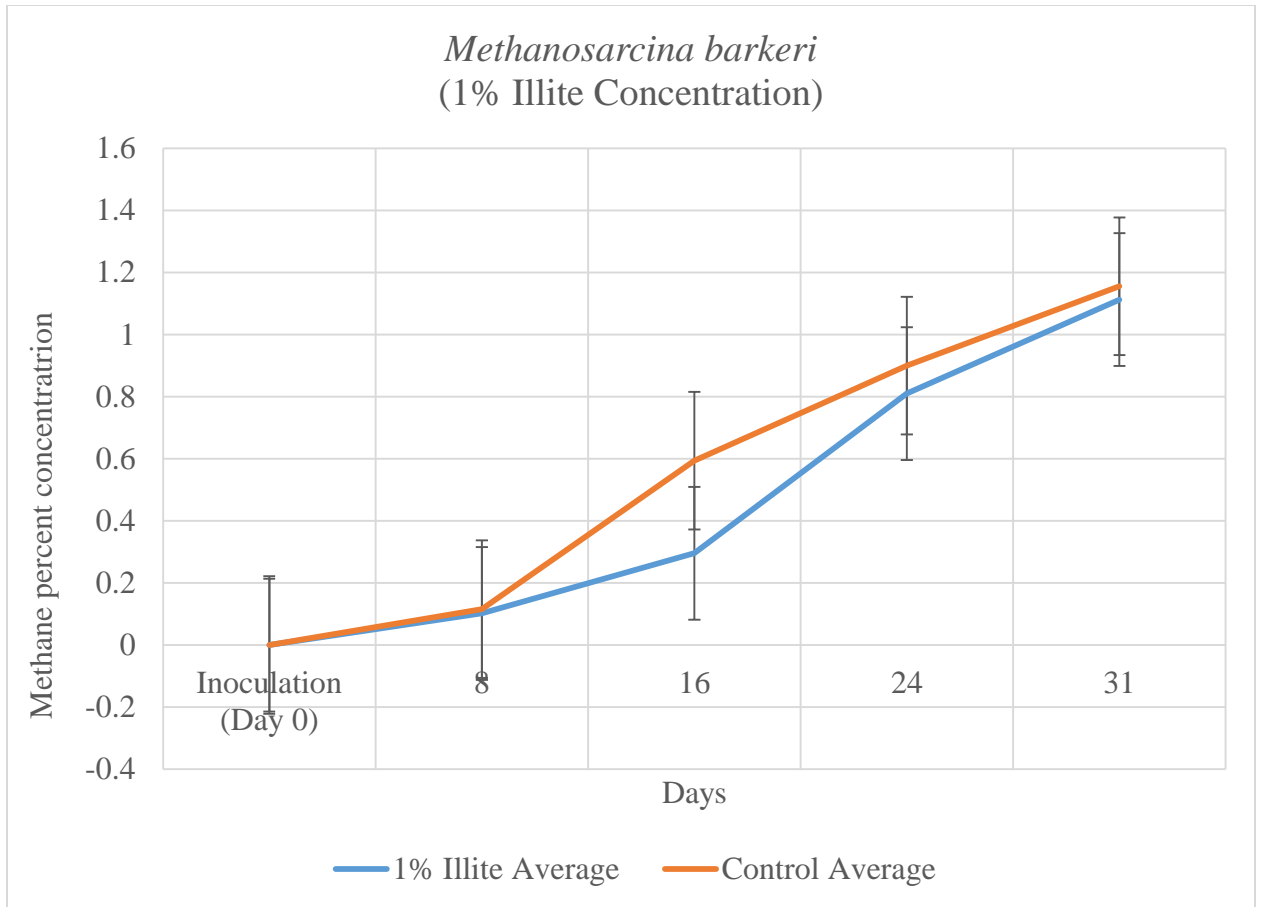


Figure 11: Methane production by *Methanosarcina barkeri* in the presence of varying concentrations of the illite clay in MS medium. The error bars depict plus or minus one standard error of the mean (SEM).

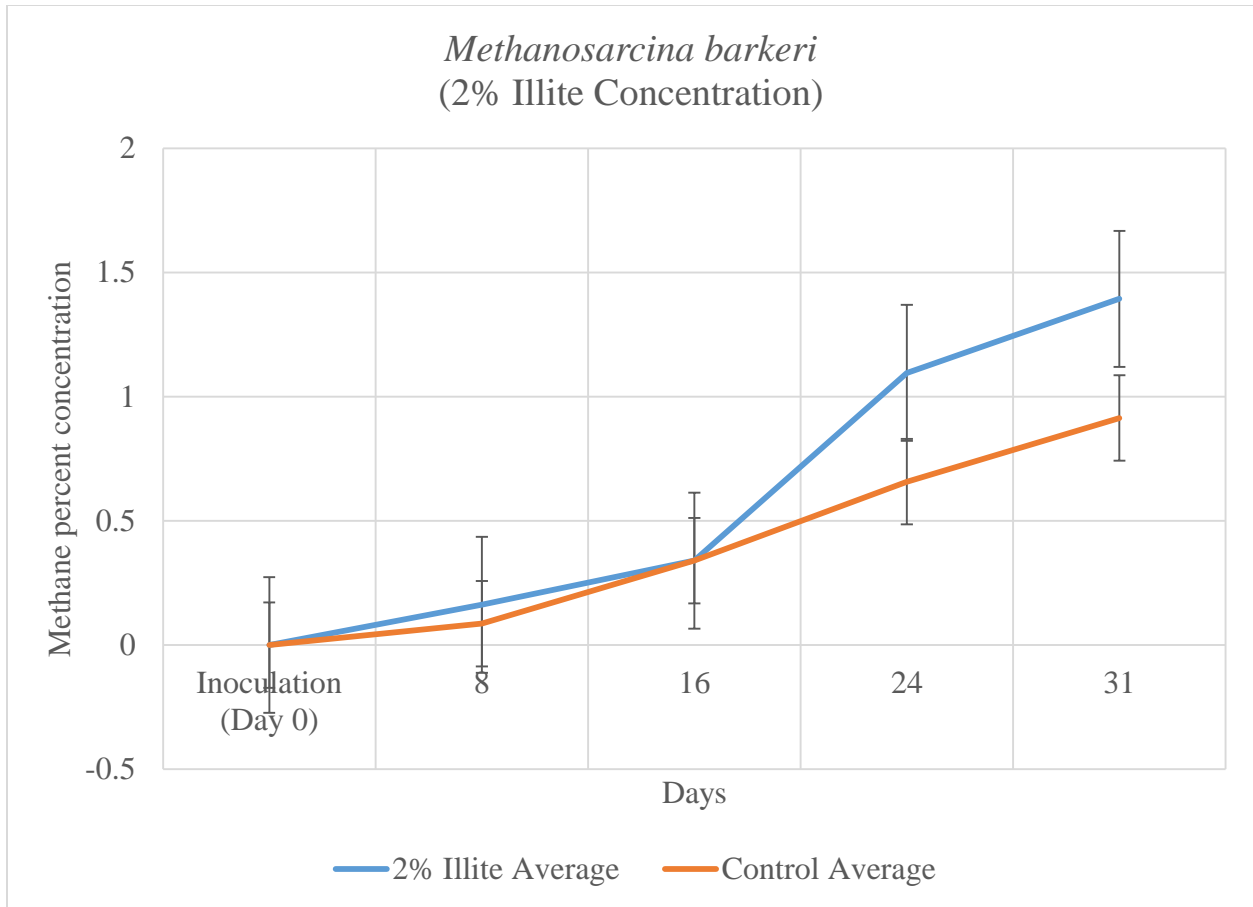


Figure 12: Methane production by *Methanosarcina barkeri* in the presence of varying concentrations of the illite clay in MS medium. The error bars depict plus or minus one standard error of the mean (SEM).

4. Discussion

The methanogens' abilities to grow and reproduce with high success in the presence of varying concentrations of illite clay provides further evidence for their potential existence on or beneath the surface of Mars. With that being said, the results can only suggest the possibility of life being capable of enduring Martian surface conditions. Firsthand evidence of life on Mars can only be obtained from probes sent there to collect samples. Nevertheless, the results indicated that Martian life remains possible, and the endeavors to continue to search for life on Mars are still very much worthwhile.

Since the results of the experiment suggested that methanogens were indeed able to produce methane in the presence of illite, it should further be tested to see if illite possesses nutrients that can support their growth. Methanogenic cells should be washed free of any media components by centrifugation, then added to sterile tubes containing illite in buffer. Illite concentration should go from 10% wt/vol to 50% wt/vol. The buffer should be the same used to make methanogenic growth media, and it should be saturated with CO₂, the carbon source for the methanogens. Sodium sulfide should be added to remove residual O₂, and H₂ should be added as the energy source. If methane production (growth) occurs over time, it would indicate that the methanogens are getting required nutrients (other than H₂, CO₂, H₂O and sulfur) from the illite. This has been demonstrated with other Martian clays such as montmorillonite (Chastain and Kral, 2010a).

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Appendix

Media Preparation

MM medium (per liter) Preparation:

4.0 g NaOH
0.25 g $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$
1.0 g NH_4Cl
0.4 g $\text{K}_2\text{HPO}_4\cdot 3\text{H}_2\text{O}$
1.0 $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$
0.4 $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$
1.0 mg Resazurin
5.0 mg $\text{Na}_2\text{-EDTA}\cdot 2\text{H}_2\text{O}$
1.5 mg $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$
1.0 mg $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$
1.0 mg $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$
1.0 mg ZnCl_2
0.4 mg $\text{AlCl}_3\cdot 6\text{H}_2\text{O}$
0.3 mg $\text{Na}_2\text{WO}_4\cdot 2\text{H}_2\text{O}$
0.2 mg $\text{CuCl}_2\cdot 2\text{H}_2\text{O}$
0.2 mg $\text{NiSO}_4\cdot 6\text{H}_2\text{O}$
0.1 mg H_2SeO_3
0.1 mg H_3BO_3
0.1 mg $\text{NaMoO}_4\cdot 2\text{H}_2\text{O}$

MS medium (per liter) Preparation:

MM medium composition, plus:
2.0 g Yeast Extract
2.0 g Trypticase Peptone
0.5 g Mercaptoethane sulfonic acid

MSF medium (per liter) Preparation:

MS medium composition, plus:

10,000 uL Sodium Formate

MSH medium (per liter) Preparation:

MSF medium composition, plus:

29.5 g NaCl

1.7 g MgCl₂

0.5 g KCl

Preparation of Buffer:

The standard bicarbonate buffer solution was prepared without boiling by dissolving NaOH in water free of O₂, and then, the solution was equilibrated with N₂-CO₂.

(Source: Boone *et al.* 1989)