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**The Effects of Salt and Temperature on Three Methanogen Species:
Implications for Mars**

An Honors Thesis submitted in partial fulfillment of the requirements for Honors Studies in
Biological Sciences

By

Katy Dunlap

Spring 2023

Biological Sciences

Fulbright College of Arts and Sciences

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Acknowledgements

I would first like to thank Dr. Timothy Kral for giving me the opportunity to do research in his lab for the last two years. I appreciate all the guidance, patience, and encouragement he has given me throughout this process. I would also like to thank Dr. Brenda Monica Magnetti, Dr. Daniel Lessner, and Dr. Matt Clay for their time and support reviewing my thesis. Finally, I would like to thank the University of Arkansas Honors College for providing me a grant to pursue my research interests and continue my academic goals.

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Abstract

The question of whether life has ever existed on Mars – either in the past or currently – has been pursued for decades. This debate has been prompted by a variety of discoveries regarding similarities between Mars and Earth and more recently concerns over human extinction. Mars, like Earth, once had large amounts of water, a key ingredient for life. H₂O exists on Mars in various forms now, and it is theorized that there is liquid water beneath the surface as well. Mars also contains salt and is very cold at its surface, pointing researchers towards the idea of subsurface life. Methanogens – methane-producing archaea – are candidate/model organisms for the *Life on Mars* debate since they are anaerobic, non-photosynthetic, and often found in extreme environments on Earth. They have also been shown to survive and grow in various Martian conditions. The discovery of methane on Mars further entrenched methanogens in this debate.

This experiment analyzed the effect of 1% NaCl, 1% MgSO₄, 2% NaCl, and 2% MgSO₄ on the metabolism of *Methanothermobacter wolfeii*, *Methanosarcina barkeri*, and *Methanobacterium formicicum* when incubated at their ideal growth temperatures and sub-ideal temperatures. All of the methanogen species were able to grow in 1% and 2% salt concentrations at their ideal temperature, however growth was inhibited when incubated at a cooler temperature. *M. formicicum* displayed the most tolerance to higher salt conditions coupled with low temperatures. The presence of NaCl and MgSO₄ therefore does not eliminate the possibility of methanogens on Mars, and the inhibition of growth linked with changes in temperature further reinforces the argument for subsurface Martian life. Though the surface is subfreezing, subsurface temperatures would be more hospitable for life, including for methanogens.

Introduction

Discussion over life on Mars has existed for decades in both the scientific and public realms. For example, Catling (2004) describes jokes between Soviet Russians, comparing lifeless Mars to a lifeless Earth. Aside from jokes and popular films, many scientists have also contributed to this discussion, debating questions like “Has life ever existed on Mars?” and “Could life exist on Mars in the future?” These questions are not far-fetched considering the vast similarities between Earth and Mars. Current social and ecological issues like the exploitation of Earth’s resources have bolstered *Life on Mars* research as well (Buchanan, 2017).

Mars

Leovy (2001) notes several structural similarities between Mars and Earth. For example, Mars and Earth are both similar in size and axial tilt, with 25.19° for Mars and 23° for Earth (Leovy, 2001; Joseph et al., 2022). Joseph et al. (2022) note that Mars does undergo large changes in obliquity, with an extreme tilt at more than 80° , but that is not necessarily disastrous for *Life on Mars* research prospects. Research suggests that between periods of extreme obliquity, planetary changes such as global warming and increases in pressure and humidity would provide water to the planet (Joseph et al., 2022). Large bodies of water would form and stay for possibly millions of years, creating conditions for life.

As explored by Joseph et al. (2022), water is of utmost importance to the *Life on Mars* debates because of its significance to the evolution of life on Earth. Some of the earliest evidence of life on Earth can be traced back to fossil microbial mats dating back to 3.5 billion years ago, with other evidence possibly dating life back to 3.8 billion years ago (McKay, 2010). This would be around the time of the Late Heavy Bombardment, suggesting its importance to the evolution

of life (McKay, 2010; Schulze-Makuch et al., 2005). Brack (1996) also confirms its importance, describing how it was probably required for processing organic molecules. Not only was water present on early Earth, it could also be found on early Mars. However, water on early Mars would take form in wet and dry spells billions of years ago (Joseph et al., 2022; Schulze-Makuch et al., 2005). The geomorphic characteristics of the planet – channels, valleys, etc. – provide further evidence of ancient water sources (Nazari-Sharabian et al., 2020). Though modern-day Mars is generally acknowledged to be cold and dry, water still exists on the planet through ice and brines in parts of its soil, and it can be found as vapor in the atmosphere as well as (Joseph et al., 2022; Martínez and Renno, 2013). Mars also houses other materials rich in water that contain clay minerals and sulfates (Nazari-Sharabian et al., 2020). The presence of water deepens the *Life on Mars* discussion as it is tied with the evolution of life on Earth. Mars likely had the conditions necessary for life during its previous wet periods – periods that coincided with the evolution of terrestrial life – and current-day water presence invigorates the discussion over whether life could potentially exist on Mars now or in the future.

Aside from the water-rich materials and brines mentioned, Mars' soil contains a variety of salts. For example, perchlorates have been found in the soil by the Mars Phoenix Lander using ion-selective electrodes (Hecht et al., 2009). Parent molecules to these anions were found to be magnesium perchlorate and, to a slightly larger degree, calcium perchlorate. Similar results were found by Curiosity (Kounaves et al., 2014b; Glavin et al., 2013). However, other salts have been found in Mars' soil as well. Sulfates like gypsum and $MgSO_4$ are prominent on Mars' surface, and carbonates are predicted to be present as well (Chevrier and Mathé, 2007). Similar to perchlorates, chloride ions also take form in salts like NaCl and KCl (Möhlmann and Thomsen, 2011).

Other conditions significant to this experiment include Mars' climatic and atmospheric features. Mars is colder than Earth, with CO₂, H₂O, and dust cycles – as well as axial tilt and orbit eccentricity – influencing its climate. In its northern hemisphere, Mars has longer, colder winters and shorter, warmer summers because of these characteristics (Martínez et al., 2017). Martínez et al. (2017) also analyze the ground temperatures on Mars, finding a daytime maximum of ~259 K (-14.15 °C) during periods of high solar insolation. During periods of low solar insolation, the daytime maximum was only ~230K (-43.15 °C) while the nighttime minimum was ~180 K (-93.15 °C). Other research corroborates these low temperatures, citing an average surface temperature of -60 °C on Mars (McKay, 2010). Clearly, Mars is much colder than Earth, pointing many to the subsurface as a possible abode for life. Aside from its climate, Mars has a unique atmosphere. It is thin, comprising an average pressure only 0.6% that of Earth, and it is dry. Dust, as opposed to moisture, is predominant in its atmosphere (Banfield et al., 2020). It also differs in its chemical composition, containing gases like CO₂, H₂, N₂, CH₄, and other noble gases (Banfield et al., 2020; Krasnopolsky and Feldman, 2001; Webster et al., 2014). Because much of Earth's methane has biological origins, the discovery of CH₄ in Mars' atmosphere has greatly excited the field of research investigating the possibility of *Life on Mars* (Webster et al., 2014). Morozova et al. (2006) describe how methane's short half-life indicates origination from either volcanic activity or biological sources, both of which have been unobserved so far.

Methanogens

Methanogens are a group of methane-producing archaea that are known to occupy a wide variety of environments. Examples of environments where methanogens have been found

include volcanic vents, the human gastrointestinal tract, oil deposits, polluted water, and tundra wetland soil (Jabłoński et al., 2015; Simankova et al., 2003). Methanogens have also been found in hypersaline environments, including some having salt concentrations higher than seawater (McGenity and Sorokin, 2010). Consequently, methanogens can also grow in an array of temperatures – the optimal temperature for growth changing between species – with research suggesting a range between 0 °C to 122 °C (Jabłoński et al., 2015).

All organisms require a carbon source and an energy source for metabolism.

Methanogens are chemoautotrophic organisms, meaning they do not utilize organic compounds like glucose to make energy. Instead, inorganic compounds are utilized. Jabłoński et al. (2015) cite that the majority of methanogenic species prefer H₂ as their energy source and CO₂ as their carbon source, where the molecular hydrogen is used to reduce carbon dioxide to methane (CH₄). Because they produce methane – a greenhouse gas more potent than carbon dioxide – methanogens have also been important to research regarding climate change and the future of biofuel, not just to the *Life on Mars* discussion (Jabłoński et al., 2015).

This experiment utilized three different methanogen species: *Methanothermobacter wolfeii*, *Methanosarcina barkeri*, and *Methanobacterium formicicum*. *M. wolfeii* has an optimal growth temperature of 55 °C, while both *M. barkeri* and *M. formicicum* have an optimal growth temperature of 37 °C (Kral et al., 2011). Because methane is produced during their metabolism, it can be used as a marker of growth in methanogens.

As mentioned before, *Life on Mars* has been a point of discussion for decades even though there have been no confirmed sightings or record of life to date. Many have dismissed the surface of Mars because it is inhospitable given its thin atmosphere, lack of protection from radiation, and subfreezing temperatures (Schulze-Makuch et al., 2005). However, this does not

dismiss the possibility of life – either in the past or the future – at the subsurface level (Nazari-Sharabian et al., 2020). Earth’s early history reveals a similar story, with life only coming to the surface after the oxygenation of its atmosphere and the development of the ozone layer (Schulze-Makuch et al., 2005).

Mars’ cold and dry history would restrict what kinds of life could evolve, however, setting microbial life as the most likely form to evolve. This is not an all-together unfortunate conclusion given Mars’ history of water. Based on the materials found in the soil, the vapor in the atmosphere, the presence of ice, and the land’s geomorphic features, research suggest that Mars had warmer, wetter environments in its earlier history, environments that would be conducive to the evolution of microbial life forms (Schulze-Makuch et al., 2005; Nazari-Sharabian et al., 2020; McKay, 2010). Subsurface liquid water sources would also provide habitats and protection for microbes on Mars now, further fueling the discussion of *Present or Future Life on Mars* (Krasnopolsky et al., 2004; Kral et al., 2011).

Methanogens are described as good candidate species for Martian life research. As Kral et al. (2016) describe, methanogens are anaerobic – oxygen exposure is fatal to these organisms – and most do not require organics. This means methanogens would not be limited by Mars’ general lack of organic compounds and limited oxygen (Krasnopolsky et al., 2004; Clark et al., 2021). They are also not photosynthetic, meaning their growth would not need to take place above the surface in Mars’ subfreezing temperatures (Kral et al., 2016).

Purpose

Research has shown that methanogen species can survive in many extreme environments, making them good candidates for Mars research. These conditions include extreme hot and cold

temperatures and high salt concentrations (Morozova et al., 2006; McGenity and Sorokin, 2010). Methanogens have also been shown to grow in a variety of Martian conditions, including in low temperatures, on Mars soil simulants, and in low pressure (Morozova et al., 2006; Kral et al., 2004; Kral et al., 2011). However, the literature regarding methanogens' response to salts found on Mars has been focused on different perchlorates as opposed to others found in the soil.

This research therefore analyzed the growth of *M. wolfeii*, *M. barkeri*, and *M. formicicum* when exposed to NaCl and MgSO₄, two salts found on Mars. In addition to investigating the effect of salts on growth, the methanogens were also subjected to two temperatures. All three species were incubated at their optimal growth temperature as well as a colder temperature. Adding two temperature conditions would provide insight into how the response to salt would change with increased environmental stress. A colder temperature would also be more akin to Mars' conditions (though the test temperatures were warmer than what would be found on Mars' surface).

This research will therefore contribute to the *Life on Mars* debate by analyzing how three model/candidate species react when exposed to salts found on Mars at ideal and cooler temperatures.

Methods

Media Preparation

The three methanogen species used in this study were grown in their own specific media. *M. wolfeii* was grown in MM medium, *M. barkeri* was grown in MS medium, and *M. formicicum* was grown in MSF medium, which were all prepared as described by Kral (2016). Most notably, MM constitutes a minimal medium, while MS and MSF are MM medium with additional

ingredients (Kral, 2016). The contents of the media can be found in the Appendix. Media, made on the countertop in the lab, were transferred to the Coy anaerobic chamber to deoxygenate overnight. These media were brought up to proper volume with bicarbonate buffer. Buffer was prepared by dissolving 4 g of sodium hydroxide in 1 liter of deionized water. This solution was saturated with carbon dioxide gas using a gassing manifold (Kral, 2016). The carbon in the buffer served as the carbon source for the methanogens. Each trial utilized 300 ml of MM, MS, and MSF.

Pre-Inoculation, Inoculation, and Post-Inoculation

NaCl and MgSO₄ salts were used in this experiment. Trial 1 utilized 1% wt/vol salt concentrations, while trials 2 and 3 utilized 2% wt/vol salt conditions. In all cases, the media – MM, MS, and MSF (Appendix) – were added to the appropriate amount of each salt. The control group consisted of 0% salt concentrations in all three trials. It should be noted that the standard growth medium for each organism contains a small amount of salt, less than 0.25% total salt. None of the media contain NaCl or MgSO₄, so the 1% or 2% added in these experiments is in addition to the salts in the standard media. Following deoxygenation in the anaerobic chamber as mentioned in the previous section, 9.5 ml of each salt medium was dispersed into tubes, with three tubes per salt condition (for a total of 54 tubes). Inside the chamber, the tubes were sealed with rubber stoppers and removed for crimping. All tubes were then autoclaved for 30 minutes at 121 °C and 15 psi. Tubes were left to cool overnight.

Fifteen drops (approximately 0.125 mL) of 2.5% sodium sulfide solution were injected into each tube to remove residual oxygen. After approximately 15 minutes had passed, each tube was inoculated with 0.5 ml of its corresponding culture.

After inoculation, each tube was pressurized with H₂ gas and incubated at its appropriate temperature. For *M. wolfeii*, incubation occurred at 55 °C (optimal growth temperature) and 37 °C, while incubation for *M. barkeri* and *M. formicicum* occurred at 37 °C (optimal growth temperature) and 22 °C. Higher than optimal growth temperatures were not investigated because they would most likely be fatal.

Data Collection and Analysis

To gauge the growth of the methanogens, 1 ml of headspace gas was removed from each tube and analyzed by gas chromatography (GC; Varian Micro-GC, model CP-4900). The gas chromatograph measured the percent of methane. Consistent readings of 0.5% or lower methane of the same cultures with time were not considered to be growing. This was due to the inaccuracy of the GC at very low purported methane concentrations.

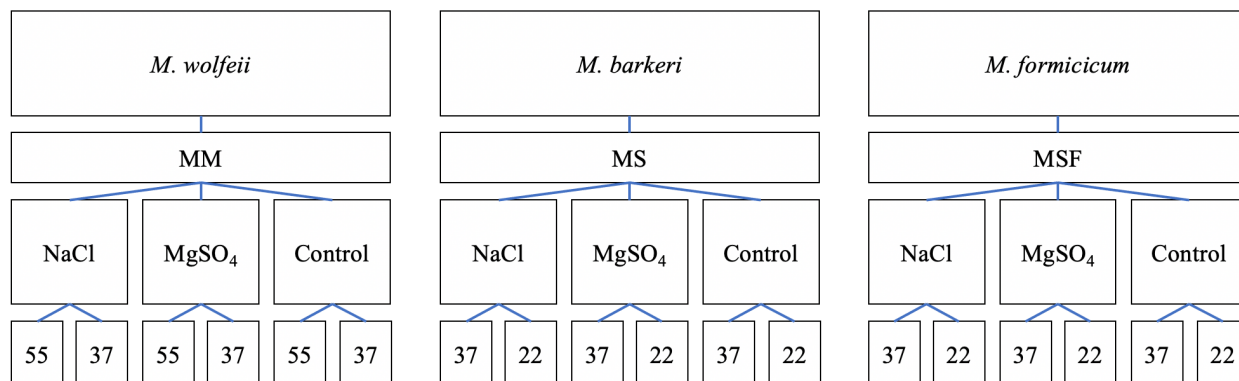
Data collection occurred every 7 days for 6 weeks (with an exception for hazardous weather in Trial 3). Each reading was subsequently recorded. Figure 1 provides a schematic demonstrating the experimental design, with each condition shown having three replicates per trial.

After recording the reading for each tube, the percent methane produced by the replicates of each condition was averaged. If a culture had turned pink, indicating contamination with oxygen, the tube was discarded and not measured further.

The standard deviation of each condition was calculated and used for the error bars in each Figure.

Figure 1

The Experimental Design Demonstrated via Flow-Chart



The first row is the organism, the second is the medium, the third is the salt [control is no salt], and the fourth is the incubation temperature measured in °C. Each condition (i.e. species, salt concentration, and temperature) has three replicates.

Results and Discussion

Figures 2 – 7 show the methane production for the three methanogens incubated in the presence or absence (Controls) of 1% NaCl or 1% MgSO₄ at two different temperatures, the organisms' optimal growth temperatures and lower temperatures. The 1% salt concentration was Trial 1. Figures 8 – 13 (Trial 2) show methane production with 2% salts with the other conditions being the same as in Trial 1. Figures 14 – 19 (Trial 3) are a repeat of Trial 2. Trials 2 and 3 were not averaged together since they were separate experiments. Table 1 is a summary of the highest methane measured for each organism under each condition in each Trial.

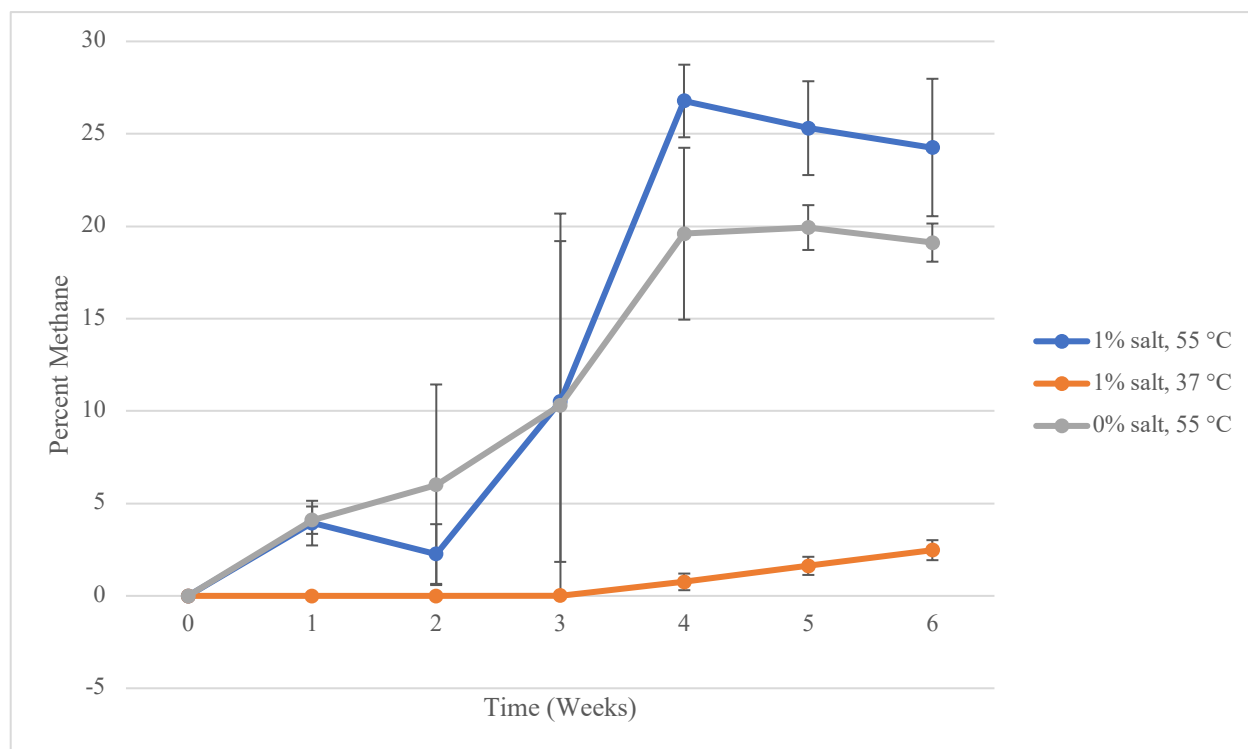


Figure 2. Methane production by *Methanothermobacter wolfeii* when grown in medium containing 1% NaCl wt/vol at 55 °C and 37 °C compared to the control (no salt) These data were from Trial 1.

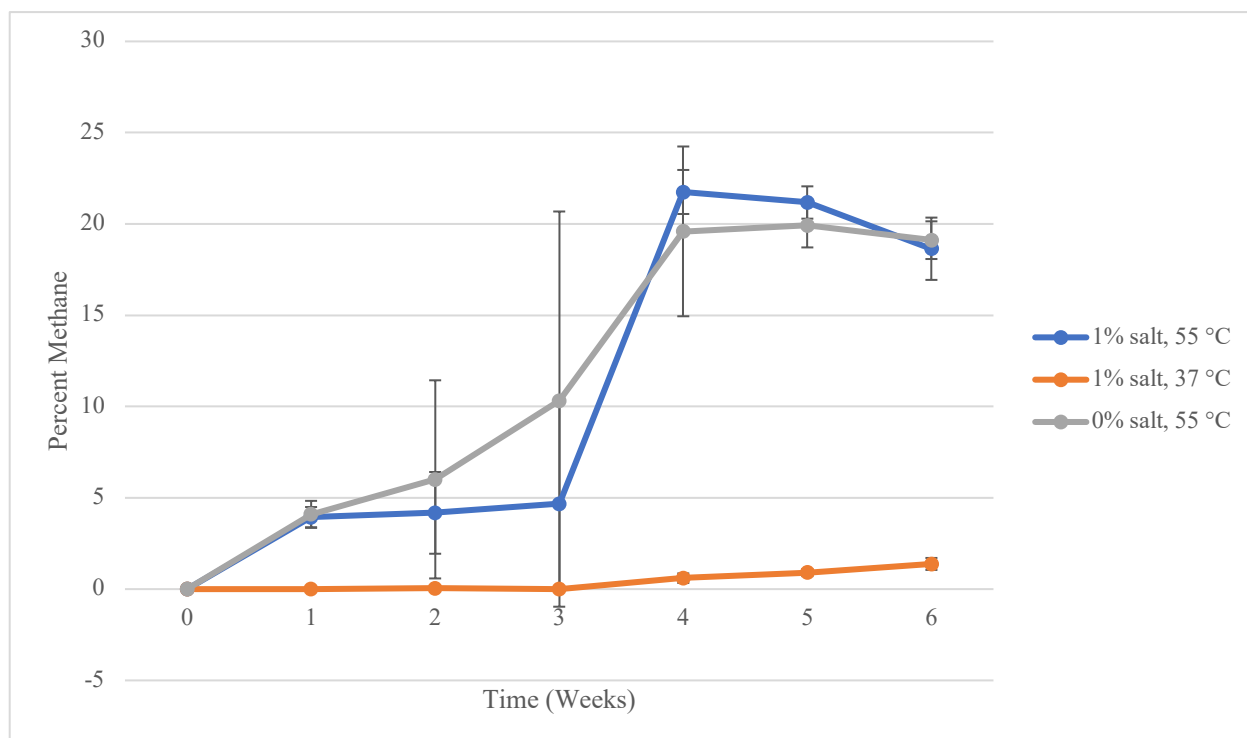


Figure 3. Methane production by *Methanothermobacter wolfeii* when grown in medium containing 1% MgSO₄ wt/vol at 55 °C and 37 °C compared to the control (no salt). These data were from Trial 1.

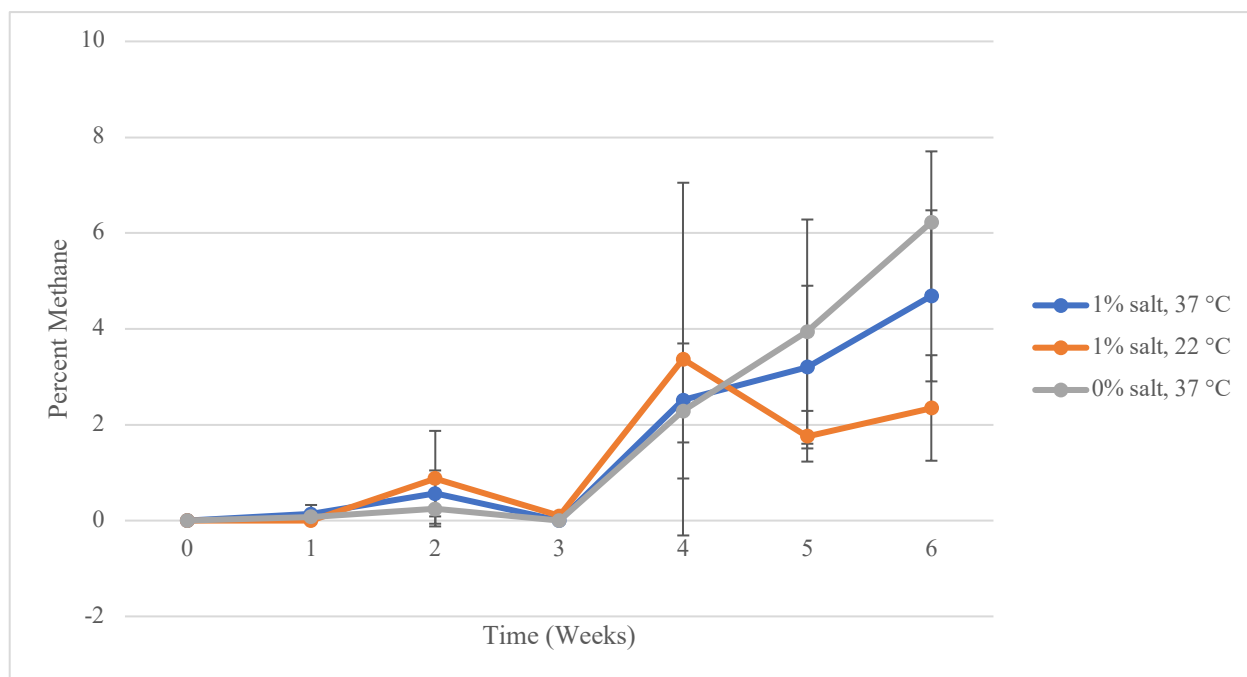


Figure 4. Methane production by *Methanosarcina barkeri* when grown in medium containing 1% NaCl wt/vol at 37 °C and 22 °C compared to the control (no salt). These data were from Trial 1.

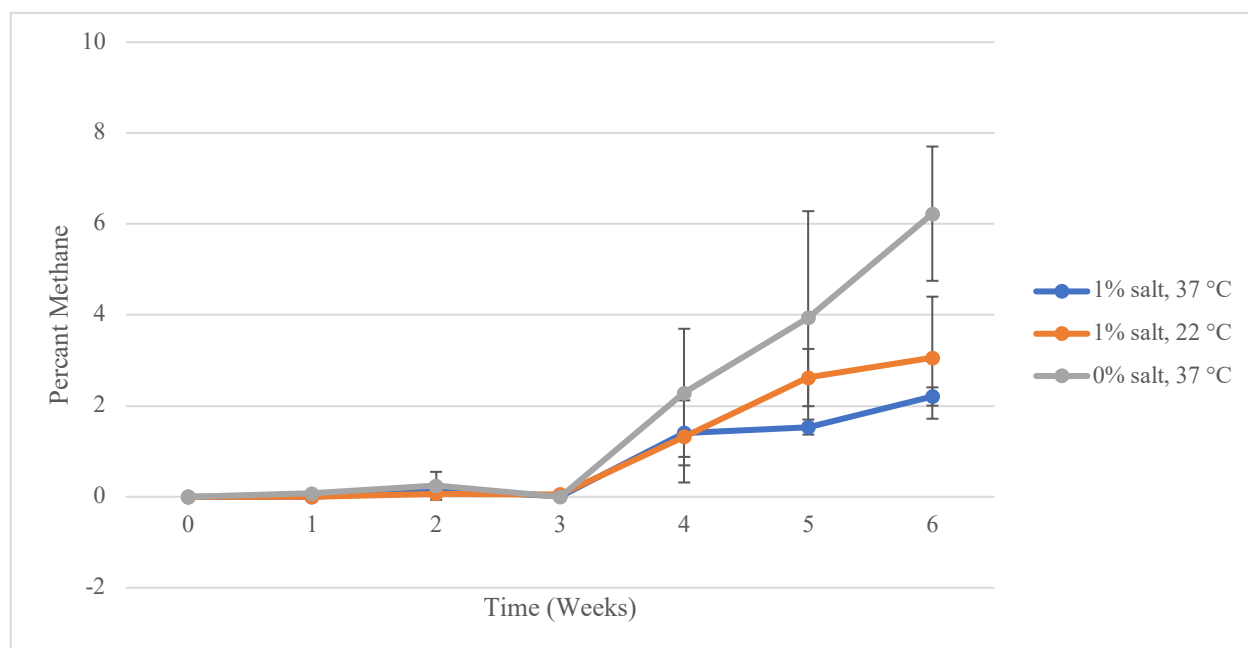


Figure 5. Methane production by *Methanosarcina barkeri* when grown in medium containing 1% MgSO₄ wt/vol at 37 °C and 22 °C compared to the control (no salt). These data were from Trial 1.

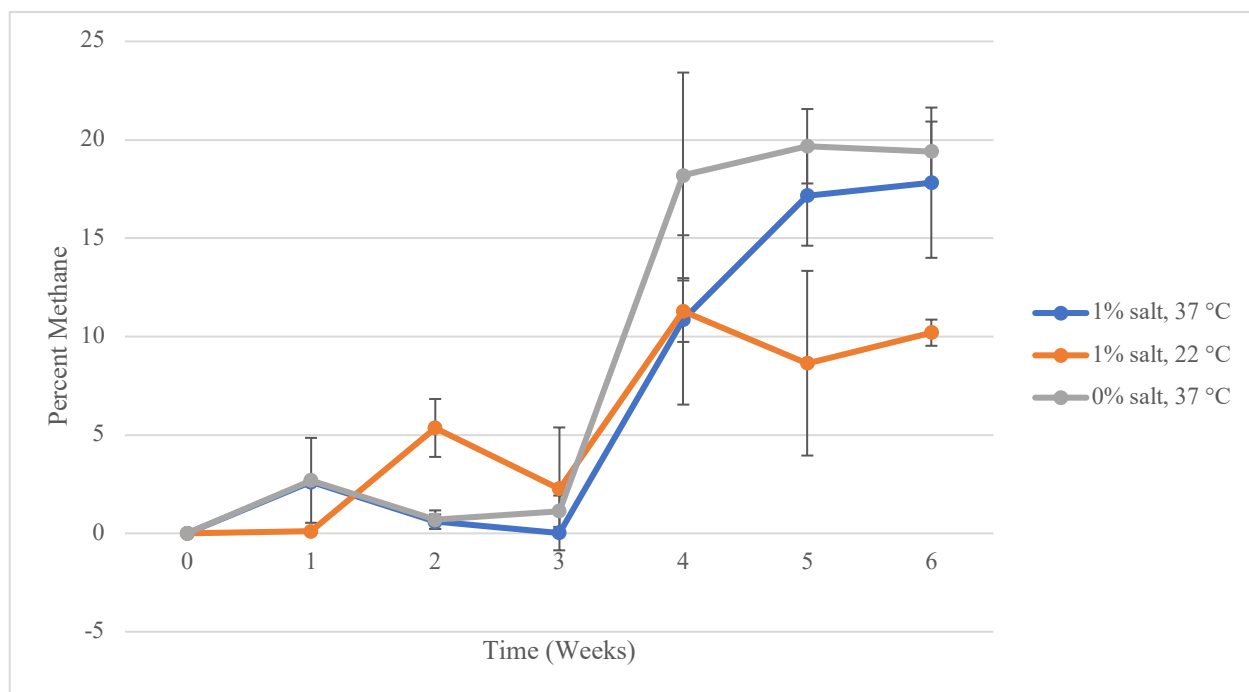


Figure 6. Methane production by *Methanobacterium formicum* when grown in medium containing 1% NaCl wt/vol at 37 °C and 22 °C compared to the control (no salt). These data were from Trial 1.

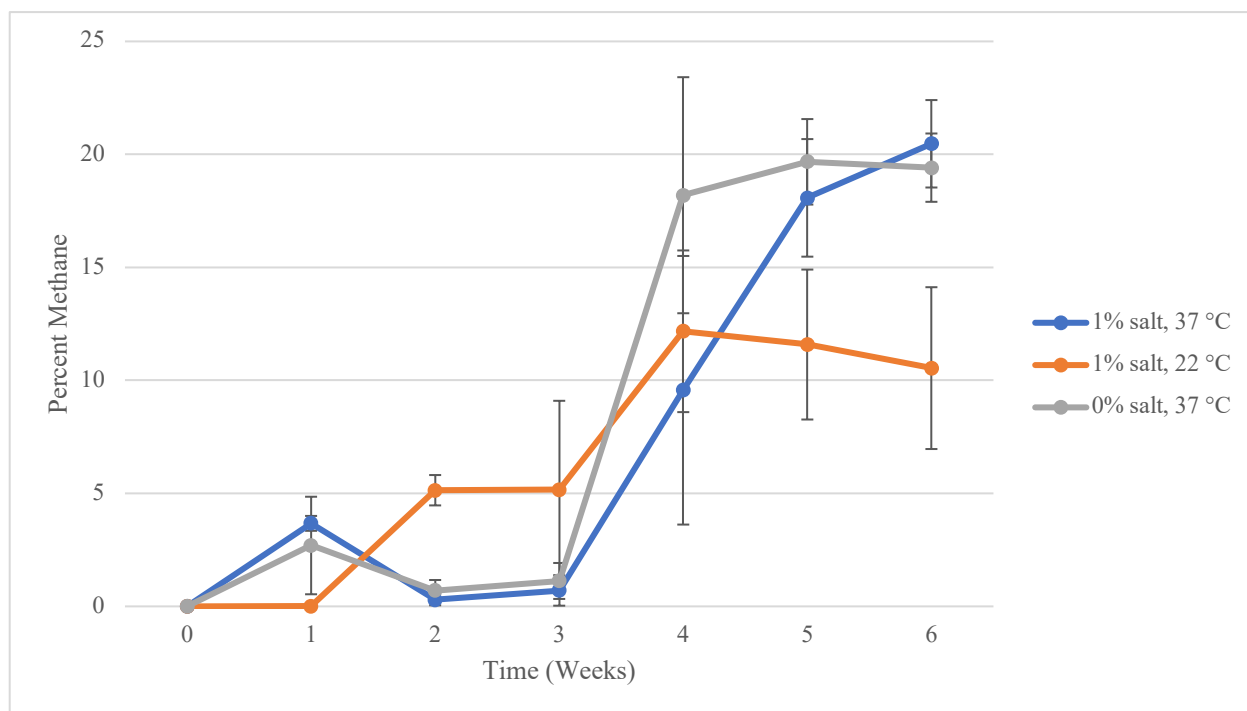


Figure 7. Methane production by *Methanobacterium formicicum* when grown in medium containing 1% MgSO₄ wt/vol at 37 °C and 22 °C compared to the control (no salt). These data were from Trial 1.

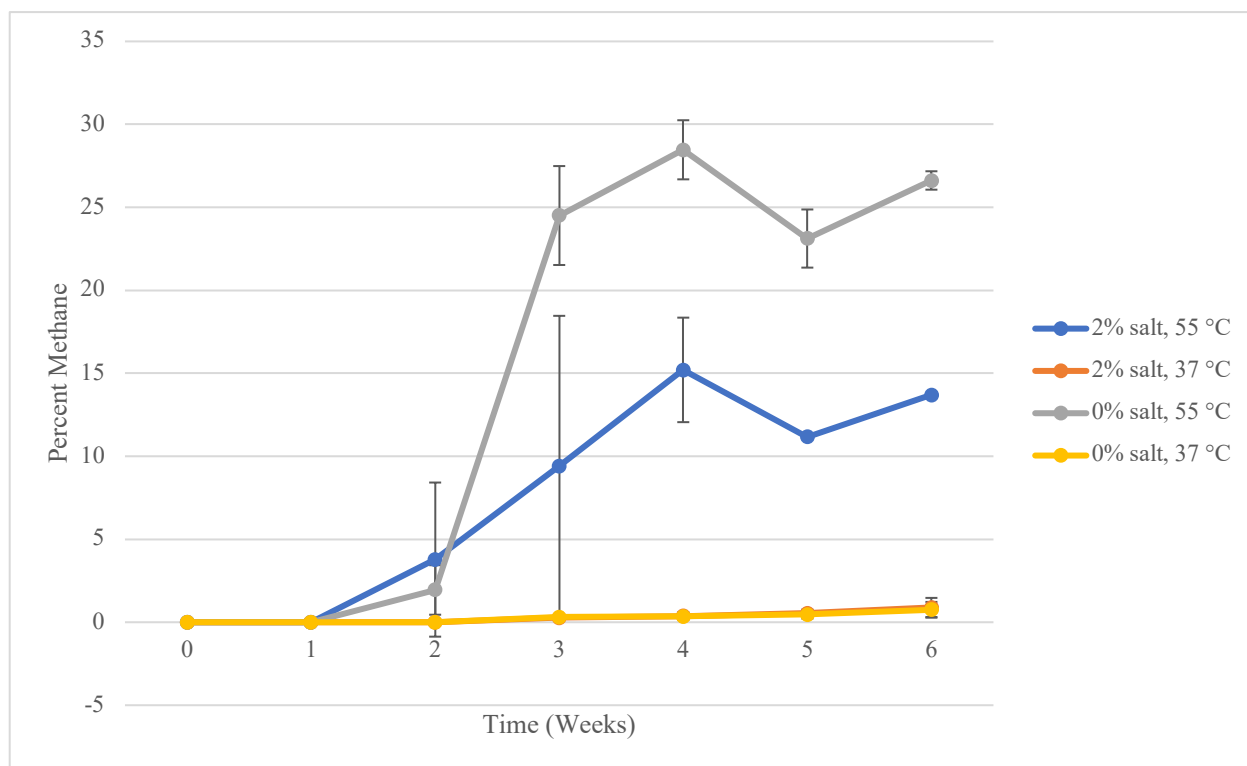


Figure 8. Methane production by *Methanothermobacter wolfeii* when grown in medium containing 2% NaCl wt/vol at 55 °C and 37 °C compared to the control (no salt). These data were from Trial 2.

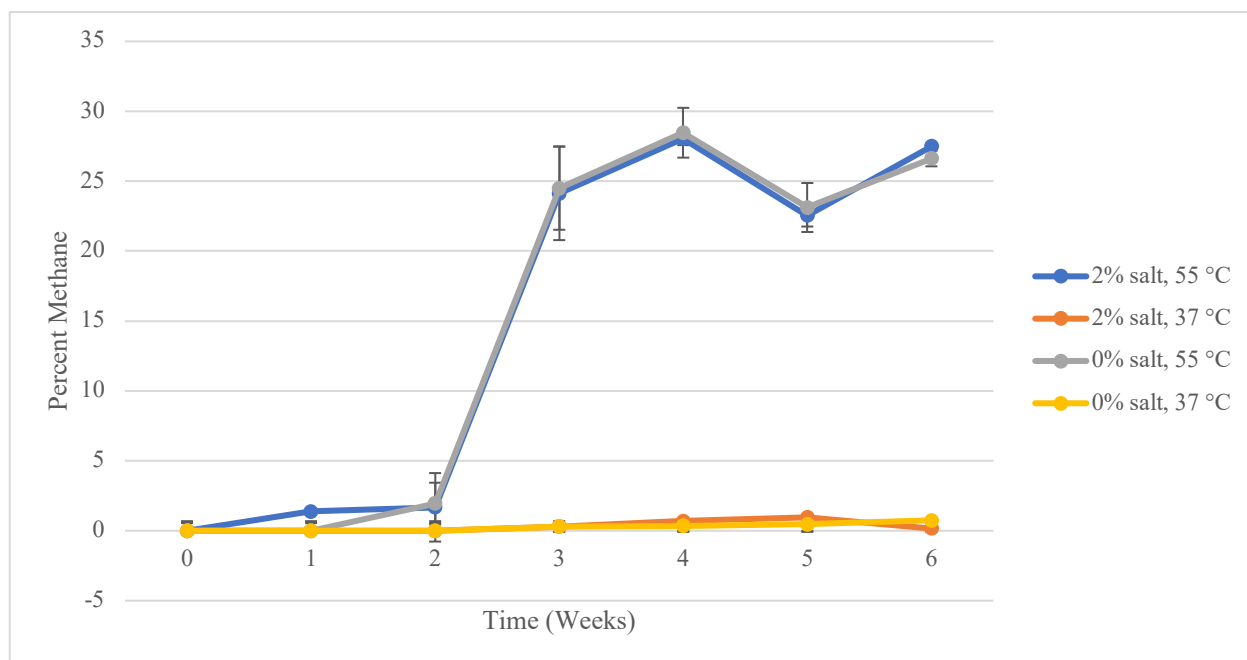


Figure 9. Methane production by *Methanothermobacter wolfeii* when grown in medium containing 2% MgSO₄ wt/vol at 55 °C and 37 °C compared to the control (no salt). These data were from Trial 2.

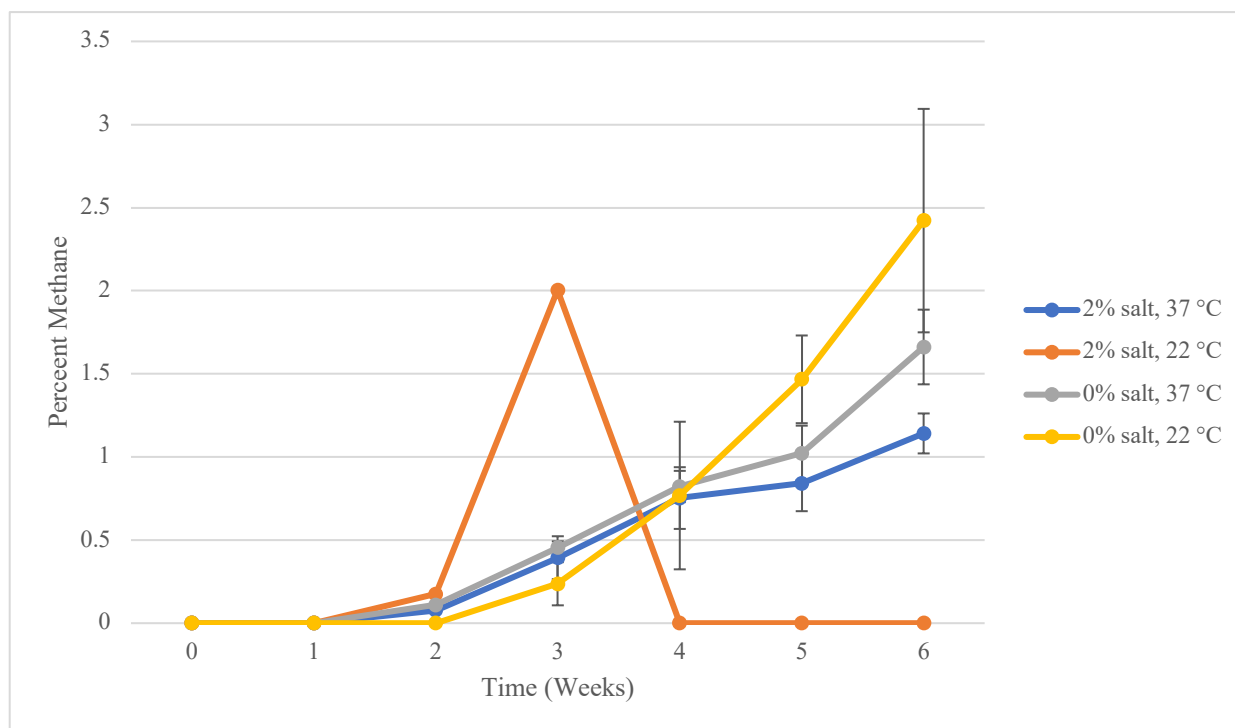


Figure 10. Methane production by *Methanosarcina barkeri* when grown in medium containing 2% NaCl wt/vol at 37 °C and 22 °C compared to the control (no salt). These data were from Trial 2.

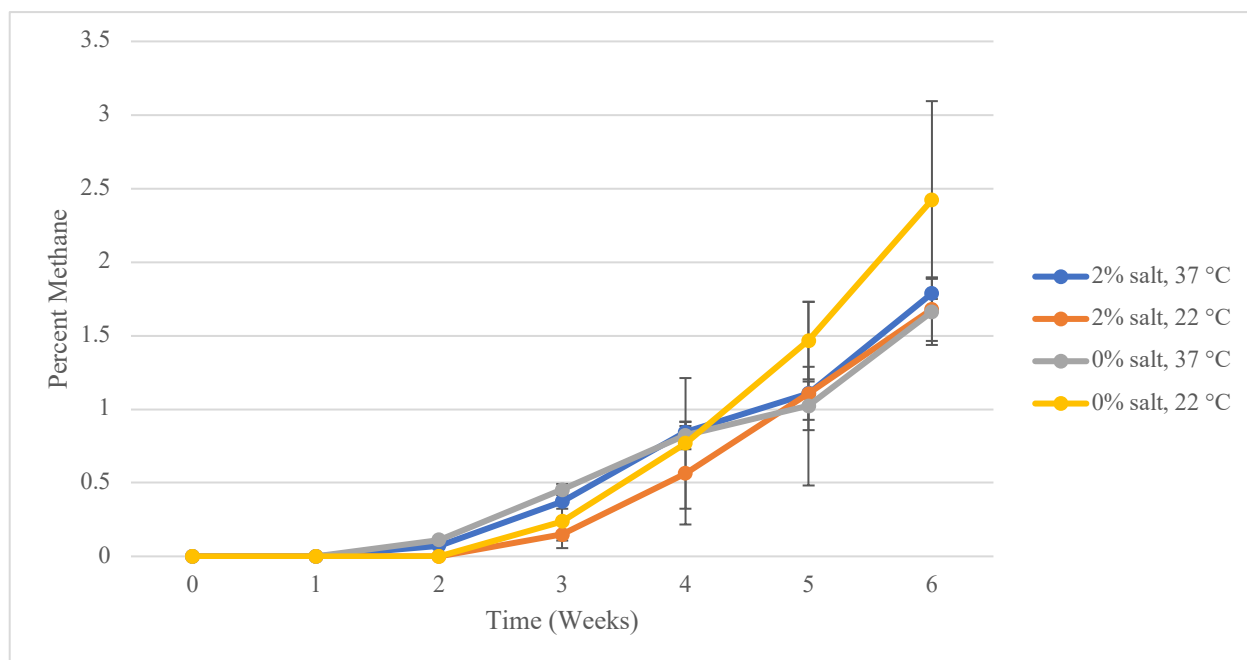


Figure 11. Methane production by *Methanosarcina barkeri* when grown in medium containing 2% MgSO₄ wt/vol at 37 °C and 22 °C compared to the control (no salt). These data were from Trial 2.

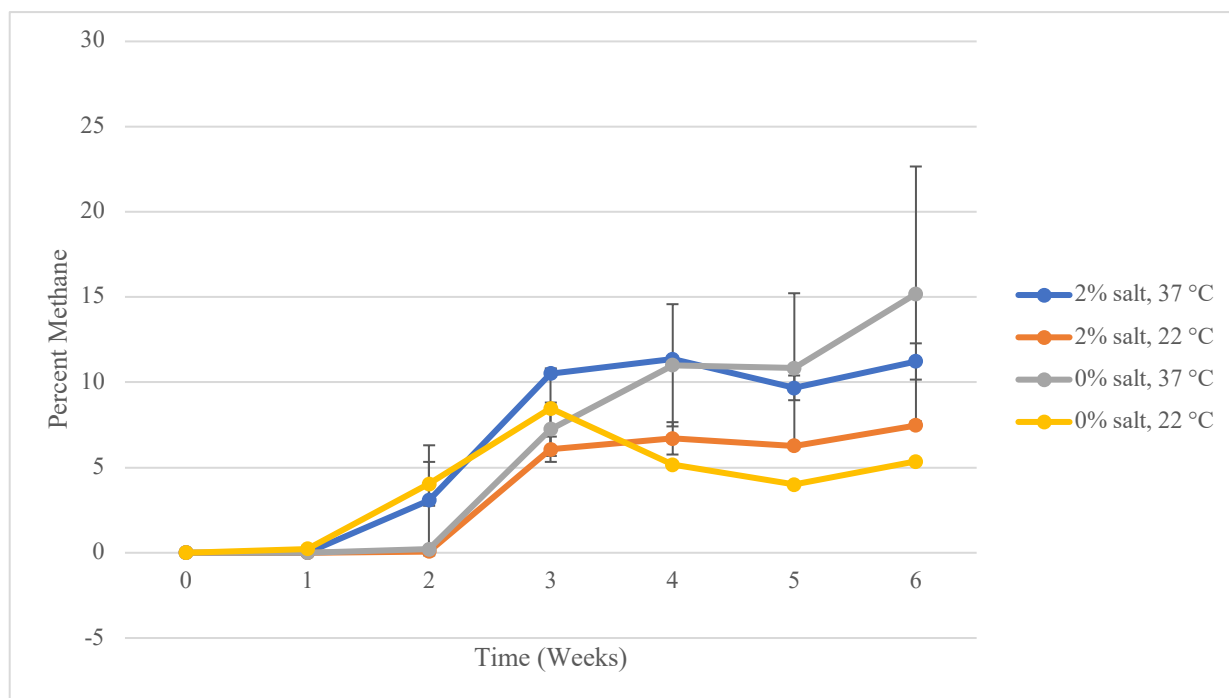


Figure 12. Methane production by *Methanobacterium formicicum* when grown in medium containing 2% NaCl wt/vol at 37 °C and 22 °C compared to the control (no salt). These data were from Trial 2.

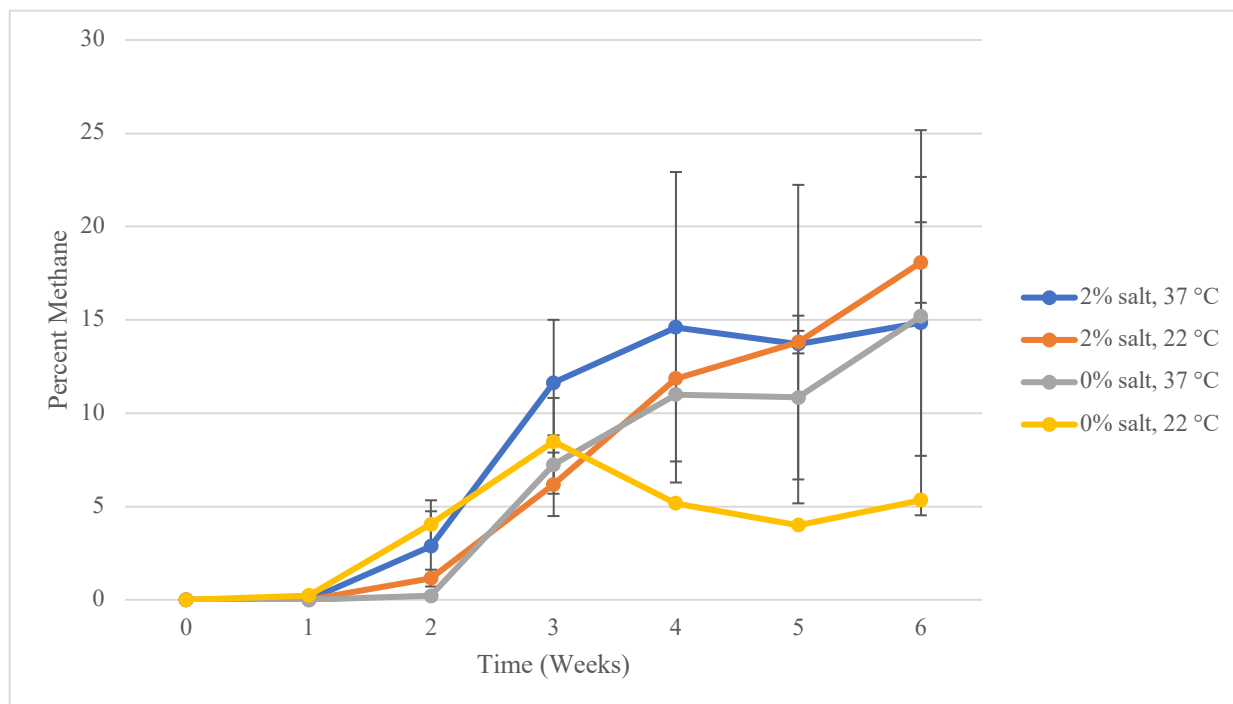


Figure 13. Methane production by *Methanobacterium formicicum* when grown in medium containing 2% MgSO₄ wt/vol at 37 °C and 22 °C compared to the control (no salt). These data were from Trial 2.

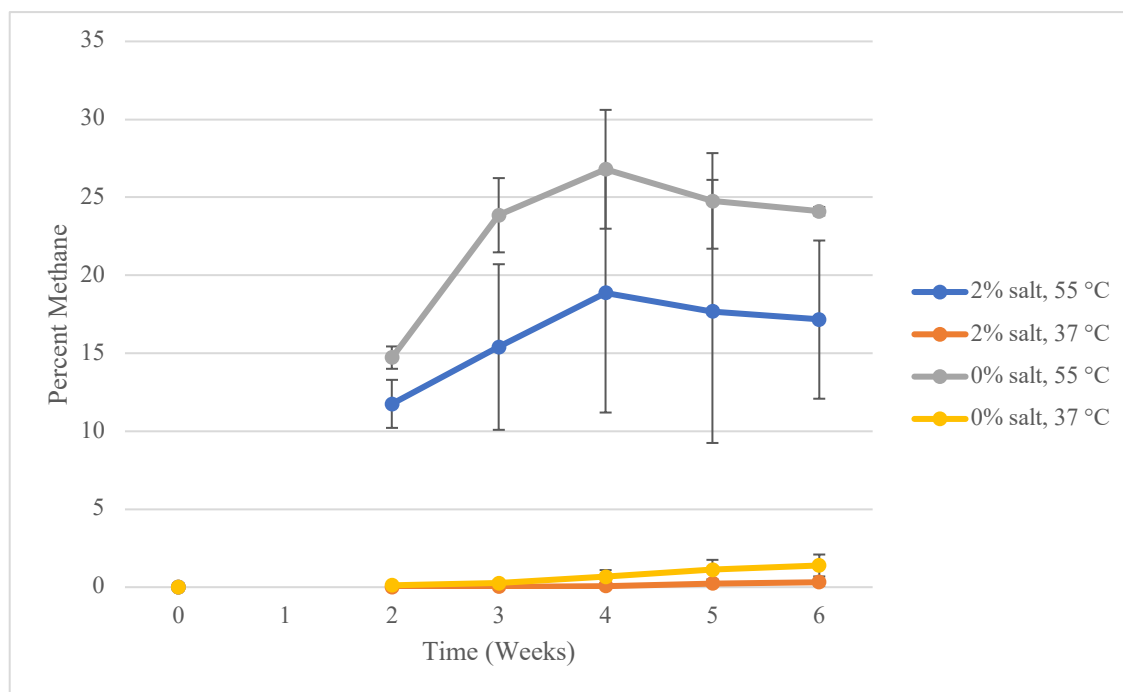


Figure 14. Methane production by *Methanothermobacter wolfeii* when grown in medium containing 2% NaCl wt/vol at 55 °C and 37 °C compared to the control (no salt). These data were from Trial 3.

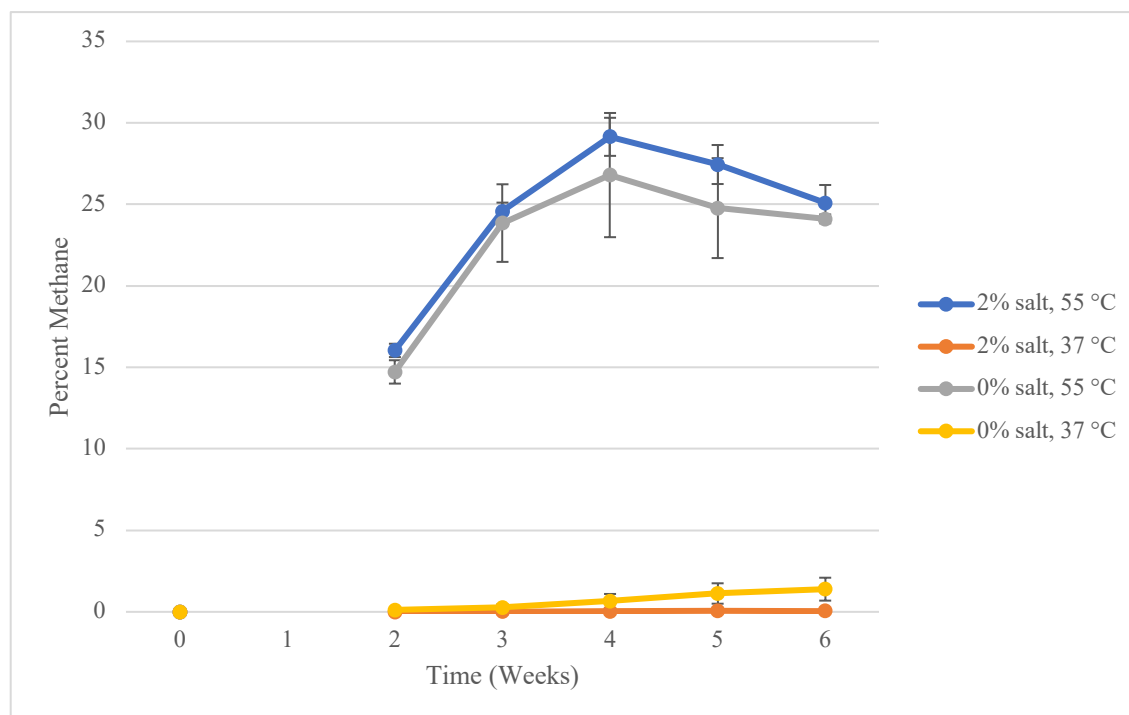


Figure 15. Methane production by *Methanothermobacter wolfeii* when grown in medium containing 2% MgSO₄ wt/vol at 55 °C and 37 °C compared to the control (no salt). These data were from Trial 3.

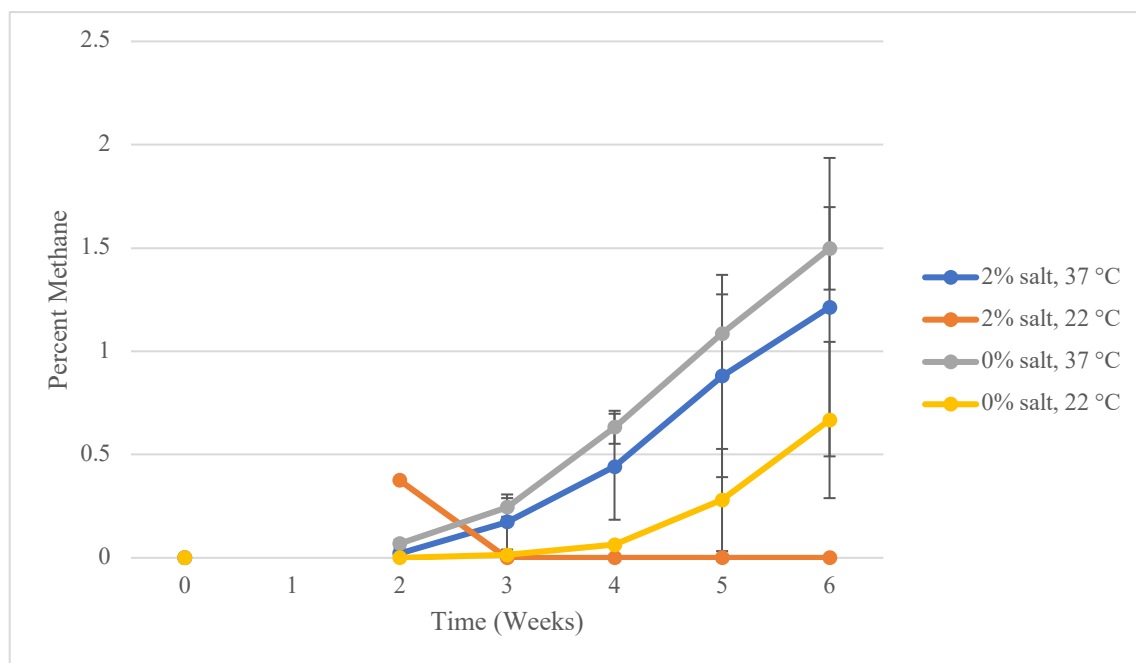


Figure 16. Methane production by *Methanosarcina barkeri* when grown in medium containing 2% NaCl wt/vol at 37 °C and 22 °C compared to the control (no salt). These data were from Trial 3.

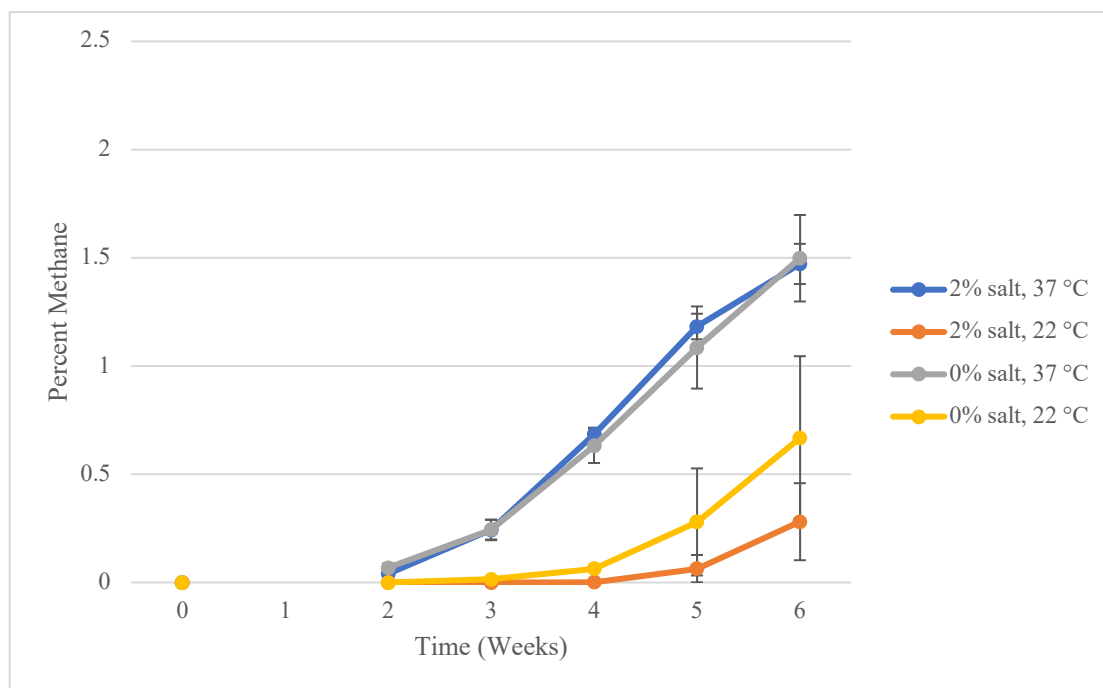


Figure 17. Methane production by *Methanosarcina barkeri* when grown in medium containing 2% MgSO₄ wt/vol at 37 °C and 22 °C compared to the control (no salt). These data were from Trial 3.

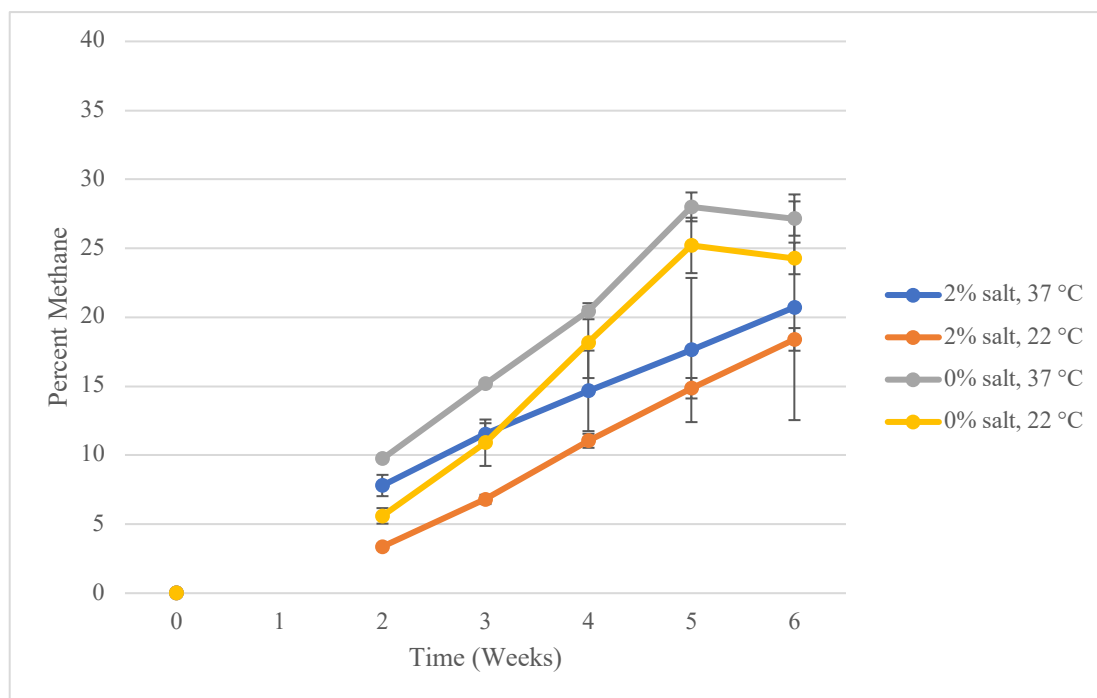


Figure 18. Methane production by *Methanobacterium formicicum* when grown in medium containing 2% NaCl wt/vol at 37 °C and 22 °C compared to the control (no salt). These data were from Trial 3.

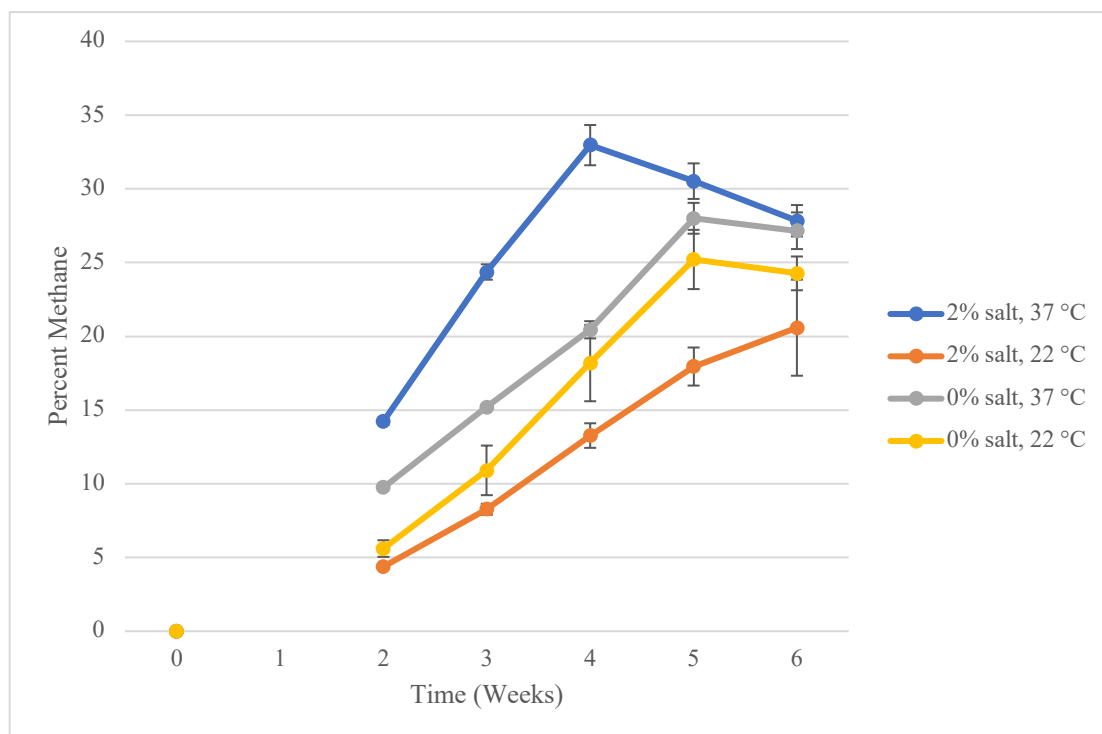


Figure 19. Methane production by *Methanobacterium formicicum* when grown in medium containing 2% MgSO_4 wt/vol at 37 °C and 22 °C compared to the control (no salt). These data were from Trial 3.

Table 1. Highest amounts of methane produced by each methanogen under each condition.

Species and Condition	Maximum Percent Methane	Week
<i>M. wolfeii</i> , 1% NaCl, 55 °C	26.78%	4
<i>M. wolfeii</i> , 1% NaCl, 37 °C	2.47%	6
<i>M. wolfeii</i> , 1% MgSO ₄ , 55 °C	21.75%	4
<i>M. wolfeii</i> , 1% MgSO ₄ , 37 °C	1.38%	6
<i>M. wolfeii</i> , 0%, 55 °C	19.93%	5
<i>M. barkeri</i> , 1% NaCl, 37 °C	4.69%	6
<i>M. barkeri</i> , 1% NaCl, 22 °C	3.37%	4
<i>M. barkeri</i> , 1% MgSO ₄ , 37 °C	2.21%	6
<i>M. barkeri</i> , 1% MgSO ₄ , 22 °C	3.06%	6
<i>M. barkeri</i> , 0%, 37 °C	6.23%	6
<i>M. formicicum</i> , 1% NaCl, 37 °C	17.82%	6
<i>M. formicicum</i> , 1% NaCl, 22 °C	11.29%	4
<i>M. formicicum</i> , 1% MgSO ₄ , 37 °C	20.47%	6
<i>M. formicicum</i> , 1% MgSO ₄ , 22 °C	12.17%	4
<i>M. formicicum</i> , 0%, 37 °C	19.67%	5

Trial 1

Species and Condition	Maximum Percent Methane	Week
<i>M. wolfeii</i> , 2% NaCl, 55 °C	15.21%	4
<i>M. wolfeii</i> , 2% NaCl, 37 °C	0.89%	6
<i>M. wolfeii</i> , 2% MgSO ₄ , 55 °C	28.04%	4
<i>M. wolfeii</i> , 2% MgSO ₄ , 37 °C	0.96%	5
<i>M. wolfeii</i> , 0%, 55 °C	28.47%	4
<i>M. wolfeii</i> , 0%, 37 °C	0.75%	6
<i>M. barkeri</i> , 2% NaCl, 37 °C	1.14%	6
<i>M. barkeri</i> , 2% NaCl, 22 °C	2.00%	3
<i>M. barkeri</i> , 2% MgSO ₄ , 37 °C	1.79%	6
<i>M. barkeri</i> , 2% MgSO ₄ , 22 °C	1.68%	6
<i>M. barkeri</i> , 0%, 37 °C	1.66%	6
<i>M. barkeri</i> , 0%, 22 °C	2.42%	6
<i>M. formicicum</i> , 2% NaCl, 37 °C	11.37%	4
<i>M. formicicum</i> , 2% NaCl, 22 °C	7.47%	6
<i>M. formicicum</i> , 2% MgSO ₄ , 37 °C	14.85%	6
<i>M. formicicum</i> , 2% MgSO ₄ , 22 °C	18.07%	6
<i>M. formicicum</i> , 0%, 37 °C	15.19%	6
<i>M. formicicum</i> , 0%, 22 °C	5.34%	6

Trial 2

Species and Condition	Maximum Percent Methane	Week
<i>M. wolfeii</i> , 2% NaCl, 55 °C	18.87%	4
<i>M. wolfeii</i> , 2% NaCl, 37 °C	0.32%	6
<i>M. wolfeii</i> i, 2% MgSO ₄ , 55 °C	29.15%	4
<i>M. wolfeii</i> , 2% MgSO ₄ , 37 °C	0.06%	5
<i>M. wolfeii</i> , 0%, 55 °C	26.80%	4
<i>M. wolfeii</i> , 0%, 37 °C	1.39%	6
<i>M. barkeri</i> , 2% NaCl, 37 °C	1.21%	6
<i>M. barkeri</i> , 2% NaCl, 22 °C	0.38%	2
<i>M. barkeri</i> , 2% MgSO ₄ , 37 °C	1.47%	6
<i>M. barkeri</i> , 2% MgSO ₄ , 22 °C	0.28%	6
<i>M. barkeri</i> , 0%, 37 °C	1.50%	6
<i>M. barkeri</i> , 0%, 22 °C	0.67%	6
<i>M. formicicum</i> , 2% NaCl, 37 °C	20.73%	6
<i>M. formicicum</i> , 2% NaCl, 22 °C	18.40%	6
<i>M. formicicum</i> , 2% MgSO ₄ , 37 °C	32.96%	4
<i>M. formicicum</i> , 2% MGSO ₄ , 22 °C	20.58%	6
<i>M. formicicum</i> , 0%, 37 °C	28.00%	5
<i>M. formicicum</i> , 0%, 22 °C	24.27%	6

Trial 3

M. wolfeii is clearly affected by lowered temperature considering the methane production is drastically different when comparing the 55 °C groups and the 37 °C groups. For example, in all trials the maximum percent methane produced by the methanogens – whether exposed to 1% NaCl, 1% MgSO₄, 2% NaCl, 2% MgSO₄, or no salt – was reduced by more than half when incubated at a lower temperature.

At their ideal temperature of 55 °C, *M. wolfeii* seemed to be affected by increasing salinity, especially NaCl. The 1% MgSO₄, 2% MgSO₄, and 1% NaCl groups produced methane comparably to their control (no salt) groups, but methane production decreased when the NaCl concentration increased to 2%. This decrease was seen in both Trial 2 and Trial 3. Increased salt concentrations may also affect growth at the cooler temperature. In Trial 1 and Trial 2, the 1% and 2% NaCl and MgSO₄ groups produced methane comparably to the control groups, however in Trial 3, *M. wolfeii* exhibited reduced growth. Neither the 2% NaCl group nor the 2% MgSO₄ group surpassed 0.5% methane in their headspace gas, which is considered as no growth. Trial 2 saw very minimal methane production as well (<1% methane in headspace gas), so it is possible that increased salt can reduce growth, especially when combined with lower temperature conditions. The effect of this combination can be variable – as is shown by the small discrepancies between Trial 2 and Trial 3 – but in general it is inhibitory. *M. wolfeii* is thus inhibited by lower temperatures and increasing salt concentrations. When incubated at its ideal temperature of 55 °C, it seems *M. wolfeii* is most affected by NaCl, but at a lower temperature both 2% NaCl and 2% MgSO₄ can be inhibitory.

With some variation, temperature does seem to influence the methane production/metabolism/growth of *M. barkeri*, though this influence was not as staunch as what was seen with *M. wolfeii*. For example, methane production did not seem to be altered by

lowered temperatures when exposed to 1% salt concentrations, which is supported by the overlapping standard deviation error bars (Figures 4 and 5). In trial 2, the temperature also did not seem to largely influence methane production, with overlapping error bars for the 2% MgSO₄ and control group (Figure 11). In trial 3, however, there seemed to be a greater reduction in methane production within the 22 °C or cooler temperature group. The error bars did not overlap, and the maximum percent values were more distinct. Distinct growth patterns between two temperature groups were also seen when *M. barkeri* was exposed to 2% NaCl. For example, the 2% NaCl/22 °C group achieved a maximum of 2.00% methane in its headspace gas at week 3, but after this point no methane was measured by the gas chromatograph. A similar pattern was seen in Trial 3, where the 2% NaCl/22 °C group achieved a maximum of 0.38% at week 2, after which no methane was detected. Their 2% NaCl/37 °C counterparts did produce methane for the entire period. For these reasons, temperature seems to influence the metabolism of *M. barkeri* but not to a large extent. The 2% NaCl concentrations/higher salinity could exacerbate the negative influence of colder temperatures.

Both 1% and 2% MgSO₄ did not seem to influence the methane production of *M. barkeri* when incubated at 37 °C. The percent methane recorded was fairly consistent, with no obvious or large deviations from the control group. In Trial 1, the 1% MgSO₄ group did have a smaller maximum percentage compared to the control group (2.21% and 6.26% respectively), but the controls from other trials had similar maximums as the 1% MgSO₄ group (1.66% for Trial 2 and 1.50% for Trial 3). The 1% NaCl concentration also did not greatly influence methane production when *M. barkeri* was incubated at 37 °C and 22 °C, as the NaCl groups performed similarly to the MgSO₄ and control groups. However, *M. barkeri* experienced reduced methane production when exposed to 2% NaCl. When incubated at 37 °C, the methane production of the

NaCl group was slightly lower than the control group in both Trial 2 and Trial 3. When incubated at 22 °C, there were more noticeable differences in methane production. In Trial 2, the 2% NaCl group had a maximum of 2.00% methane at week 3, but methane was not detected after that point. A similar phenomenon happened in Trial 3, where the 2% NaCl group had a maximum of 0.38% methane at week 2, after which no methane was detected. These 2% NaCl/22 °C groups were therefore not able to grow under these conditions. This means that while 1% and 2% MgSO₄ did not have a largely inhibitory effect on *M. barkeri* methane production at either temperature, NaCl did have an inhibitory effect. This effect was amplified with increasing salinity and lower temperatures. *M. barkeri* thus was able to grow in the presence of increasing MgSO₄ concentrations and at a lower temperature, but exposure to NaCl would inhibit growth, especially at a lower temperature.

The methane production of *M. formicicum* was also reduced when incubated at 22 °C compared to 37 °C. In Trial 1, for example, the methane production of the 1% NaCl and 1% MgSO₄ groups was reduced at 22 °C. As mentioned, a control for 22 °C was not measured during Trial 1, but the maximum methane production of the control for 37 °C was roughly similar to that of the 22 °C control of Trial 3 (19.67% and 25.21% respectively). The controls for Trial 3 had similar methane production too, with a 28.00% maximum for the 37 °C control and a 25.21% maximum for the 22 °C control. This pattern was not seen in Trial 2. The 55 °C control had a maximum of 15.19% while the 22 °C control had a maximum of 8.48%. However, this 8.48% may not be reliable due to oxygen exposure. Because the control groups experienced a decrease in methane production when temperature was reduced, it seems the growth of *M. formicicum* can be influenced by changes in temperature. It should be noted that *M. barkeri* normally produces far less methane than *M. wolfeii* and *M. formicicum* under all conditions.

Similar to *M. barkeri*, MgSO₄ concentrations seemed to play a less inhibitory role compared to NaCl concentrations. At 1% concentration and 37 °C, MgSO₄ maximum methane production was within 1% of the control, but maximum methane production in the presence of 1% NaCl was slightly lower. Still, these maximum percentages were relatively similar to one another. When incubated at 22 °C, the methane production of *M. formicicum* was also similar between the 1% NaCl and 1% MgSO₄ groups. At 2% salt concentrations and 37 °C, methane production was still similar to the control group. As in Trial 1, the 2% MgSO₄ group performed comparably to the control group in both Trial 2 and Trial 3. Methane production in the presence of 2% NaCl was also reduced compared to the control group in both Trial 2 and Trial 3. However, a different pattern emerged when *M. formicicum* was incubated at 22 °C and exposed to 2% salt concentrations. Both 2% NaCl and 2% MgSO₄ experimental groups experienced reduced methane production compared to the control. Their maximums in Trial 3 would be 18.40%, 20.58%, and 25.21% respectively. A different pattern was seen in Trial 2 – with a 7.47% maximum for the 2% NaCl group, 18.07% maximum for the 2% MgSO₄ group, and 8.48% maximum for the control group – but both the NaCl and control groups experienced oxygen exposure that affected more than one replicate. In general, these findings and patterns suggest that *M. formicicum* is influenced by increasing salinity, but not to a large degree. When the environment is both high in salt and low in temperature – when there is more stress – growth is more greatly reduced.

It should be noted that all readings in Trial 1 (Figures 2-7) experienced either a dip in percent methane or a large standard deviation at week 3. This might be explained by an unknown malfunction in the gas chromatograph possibly involving the injection port. Unfortunately, due to an oversight, there were no controls at the lower temperature condition during Trial 1 (i.e. 37

°C for *M. wolfeii* and 22 °C for *M. barkeri* and *M. formicicum*), but comparisons can be made to those controls in Trial 2 and Trial 3. No readings were taken at week 1 of Trial 3 (Figures 14 - 19) due to inclement weather.

All three methanogen species experienced inhibited methane production when incubated at a cooler temperature and exposed to higher concentrations of salts. NaCl seemed to influence growth more than MgSO₄, especially for *M. barkeri*, which did not continue to grow in the presence of 2% NaCl and incubated at 22 °C. It should be noted that 1% NaCl and 1% MgSO₄ are only the same in wt/vol. The molecular weight of NaCl is 58.5 g/mol while that of MgSO₄ is 120.366 g/mol (Tro, 2015). A 1% solution of NaCl is 0.17 M while a 1% solution of MgSO₄ is 0.083 M. Thus, the number of molecules of NaCl in a 1% solution is approximately twice the number of molecules in a 1% solution of MgSO₄. So, the greater effect of NaCl may be due to the greater number of molecules. *M. formicicum* seemed most resilient to the changes in both temperature and salt, as it continued to grow in all conditions and produce the most amount of methane.

Many of the methanogen groups achieved their maximum methane production before Week 6, after which the percent methane detected would dwindle ever so slightly until the end of the trial period. This phenomenon can be seen in Figures 14 and 15. The major reason that methane production slows down is that the energy source, H₂, is being depleted. Also, if enough samples are removed for GC analysis, the methane concentration will appear to decrease. This decrease can be explained by the fact that methane is far less soluble in the medium, 0.025 g per liter while carbon dioxide's solubility is 1.69 g per liter, both at 25 °C and 1 atm (Kaye and Laby, 1986). Since there is far more carbon dioxide in the media than methane, every time that a sample of headspace gas is removed for GC analysis, more carbon dioxide comes out of solution

than methane, thus diluting the remaining methane in the headspace, causing a reduction in the percent methane detected.

As mentioned in previous sections, salts – including the NaCl and MgSO₄ tested in this experiment – have been found in Mars' soil (Möhlmann and Thomsen, 2011; Chevrier and Mathé, 2007). Liquid brines are also largely theorized to exist on Mars, with some evidence noting their temporary formation above surface (Martínez and Renno, 2013). They are largely theorized to exist below the surface of Mars as well. Recent radar evidence found reflective surfaces below the SPLD, for example, and attributed these reflective surfaces to bodies of liquid water (Orosei et al., 2018). Liquid brines were emphasized in this situation due to the prevalence of salts in the Martian soil and the cool temperature of the polar deposits (Orosei et al., 2018; Diez, 2018). The presence of salt in the soil as well as its association with subsurface water – which is required for all known lifeforms – demonstrates its importance to the *Life on Mars* debate. *M. wolfeii*, *M. barkeri*, and *M. formicicum* are not halophilic methanogen species, yet they were able to metabolize and grow when exposed to increasing NaCl and MgSO₄ salt conditions. Growth was more inhibited when the methanogens were also subjected to sub-ideal temperatures.

Conclusion

Both salt and temperature are vital to the *Life on Mars* debates. The Martian soil contains salts, and many of the places on the planet theorized to be hospitable to life – such as subglacial liquid water – contain salt as well (Diez, 2018; Nazari-Sharabian et al., 2020; Schulze-Makuch et al., 2005). However, Mars is colder than Earth, with an average surface temperature of -60 °C (McKay, 2010). For this reason, the methane production of *M. wolfeii*, *M. barkeri*, and *M.*

formicicum were tested when exposed to increasing NaCl and MgSO₄ concentrations as well as when incubated at ideal and sub-ideal temperatures.

Kral et al. (2016) found that perchlorates would not be a limiting factor to methanogens on Mars, as *M. wofeii*, *M. barkeri*, *M. formicicum*, and *M. maripaludis* were able to produce methane in the presence of various perchlorate concentrations. A similar response occurred in this experiment, with *M. wolfeii*, *M. barkeri*, and *M. formicicum* producing methane at 1% NaCl and MgSO₄ concentrations when incubated at their ideal growth temperatures (although temperature would decrease methane production, especially for *M. wolfeii*). The three methanogen species tested were also able to metabolize in the presence of 2% NaCl and MgSO₄ concentrations when incubated at their ideal temperatures. However, at a reduced temperature metabolism was challenged, especially for *M. wolfeii* and *M. barkeri*. *M. barkeri*, for example, was unable to metabolize in 2% NaCl/22 °C conditions. *M. formicicum* seemed to withstand both changes in salinity and changes in temperature the best as it produced the most methane (based on the percent detected via gas chromatography) with arguably less variability between the different conditions.

Based on these data, the presence of NaCl and MgSO₄ does not rule out the existence of methanogens on Mars. However, the colder temperatures on Mars would be inhibitory, strengthening the case for subsurface life where temperatures are supposedly warmer.

Future research could adapt this experimental design, opting to replenish the molecular hydrogen and observe the methane production over a longer exposure time/trial period. Similarly, the response to more elevated concentrations of NaCl and MgSO₄ could be analyzed as well, given the importance of brines to the *Life on Mars* debate. However, other salts are present on Mars too, and future research could investigate these as well.

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Appendix

Components for 100 mL Media

MM Medium:

1000 uL Solution A

200 uL Solution B

200 uL Solution C

100 uL Solution D

MS Medium:

1000 uL Solution A

200 uL Solution B

200 uL Solution C

100 uL Solution D

0.2 g Trypticase Peptone

0.2 g Yeast Extract

0.05 g Mercaptoethane Sulfonic Acid

MSF Medium

1000 uL Solution A

200 uL Solution B

200 uL Solution C

100 uL Solution D

0.2 g Trypticase Peptone

0.2 g Yeast Extract

0.05 g Mercaptoethane Sulfonic Acid

1000 uL Sodium Formate

Solution A

100 g l⁻¹ NH₄Cl

100 g l⁻¹ MgCl₂·6H₂O

40 g l⁻¹ CaCl₂·2H₂O

Solution B

200 g l⁻¹ K₂HPO₄·3H₂O

Solution C

0.5 g l⁻¹ Resazurin

Solution D

500 mg l⁻¹ Na₂-EDTA·2H₂O

150 mg l⁻¹ CoCl₂·6H₂O

100 mg l⁻¹ MnCl₂·4H₂O

100 mg l⁻¹ FeSO₄·7H₂O

100 mg l⁻¹ ZnCl₂

40 mg l⁻¹ AlCl₃·6H₂O

30 mg l⁻¹ Na₂WO₄·2H₂O

30 mg l⁻¹ CuCl₂·2H₂O

20 mg l⁻¹ NiSO₄·6H₂O

10 mg l⁻¹ H₂SeO₃

10 mg l⁻¹ H₃BO₃

10 mg l⁻¹ Na₂MoO₄·2H₂O