

Examining the Growth of Methanotrophic Bacteria Immersed in Extremely Low-Frequency Electromagnetic Fields

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SUMMARY

The demand for natural resources, and consequently greenhouse gas (GHG) emissions, are only set to increase in the next several decades. Methane gas, having 80 times more warming power than carbon dioxide over a 20 year period, accounts for about 10% of all U.S. GHG emissions primarily due to the agriculture and waste management industry. GHGs trap heat in Earth's atmosphere causing rising surface temperatures and sea levels, ocean acidification, and extreme weather patterns. *Methylomicrobium alcaliphilum* 20Z^R is a methanotrophic bacterium currently being explored as a means for mitigating these emissions and/or production of value-added compounds from wasted sources of methane. Based on related studies, we predicted that exposure to low-frequency electromagnetic fields for 5 and 15 minutes would be a feasible method to increase the growth rates of *M. alcaliphilum* 20Z^R, while 30 minutes would decrease the growth rates. We hypothesized that this increase in growth could make this method of catabolizing methane more practical given the slow growing nature of methanotrophs in standard conditions. However, our data showed that exposure to an electromagnetic field density of 0.1 milliTesla (mT) at a frequency of 50 hertz (Hz) for 5, 15, and 30 minutes had no statistically significant effects on the growth of *M. alcaliphilum* 20Z^R when compared to the control. The results of this study therefore do not fully support our hypothesis that an electromagnetic field can positively impact microbial growth, but future research should be done to further solidify and expand upon the data collected.

INTRODUCTION

The methane concentration in Earth's atmosphere has doubled since the Industrial Revolution, far exceeding natural levels, and accounts for about 20% of the warming in our planet (1). Given that methane is 80 times more potent in terms of heat capture than carbon dioxide, the effects that methane has on our environment are only magnified (1). In order to lessen this effect, researchers and environmentalists have recently taken particular interest in the use of methanotrophs, bacteria that consume methane as their source of energy for metabolism. Some byproducts of this unique metabolism

can even be used for commercial and industrial production to produce biofuels and various chemicals like 2,3-butanediol for use as a precursor to plastics and pesticides (2).

However, numerous studies have addressed the limitations associated with methanotrophic oxidation, including most commonly, the issue of slow bacterial growth rate, and thus slow methane uptake (3-5). Extremely low-frequency (ELF) electromagnetic fields (EMFs) are a form of non-ionizing, low-energy, non-thermal radiation resulting from the combination of electric fields generated by differences in a circuit's voltage and the magnetic fields generated by current flowing through a conductive medium (6-7). Several peer-reviewed studies quantifying the impacts of ELF EMFs on biological systems have shown that specific parameters like the frequency, time duration, and magnetic flux density (the number of magnetic field vectors passing through a given area) can accelerate normal cell potentials, increase the rate of healing, reduce inflammation, ameliorate bruising, and promote cell proliferation (8-11). Furthermore, these same positive impacts have also been observed on the growth of various types of bacteria (12-13). Optical density, used as a tool to quantify such growth, is a logarithmic intensity ratio of light absorption by a particular substance. If more light is absorbed, there exists a greater density of material as opposed to if less light was absorbed, thereby serving as a direct representation of growth over time.

One of the most common exposure mechanisms in these studies is the use of the Helmholtz coil, which consists of two identical magnetic coils facing parallel to each other with the radius of each matching the distance separating them. As current passes through both coils, a uniform magnetic field is generated within the center of the structure, creating an ideal area for samples to be placed into. The positive characteristics of exposure, along with the well-documented methods for the construction and utilization of EMF exposure mechanisms like the Helmholtz coil configuration used in our study, show the vast potential for its implementation in a variety of industries (14-16).

Here, we examine the effects of ELF EMFs on *Methylomicrobium alcaliphilum* 20Z^R, a halotolerant, alkaliphilic, obligate methanotrophic, Gram-negative bacterium isolated from moderately saline soda lakes in Tuva (Central Asia), with the aim of catalyzing species growth and consequently, methane consumption (17). We chose this strain specifically because of its tractable metabolic network

and its versatility to a wide range of pHs and temperatures (2). The microbe's ability to grow at higher salinities also helps to decrease contamination. After being exposed to 0.1mT 50Hz EMFs for 5, 15, and 30 minutes, we found that ELF EMFs do not have statistically significant effects on the growth of *M. alcaliphilum* 20Z^R. Despite the results not supporting our hypothesis, the premise of our study is still important as increasing the growth of such bacteria could have drastic impacts on reducing greenhouse gasses in our atmosphere.

RESULTS

Our experiment aimed to test the feasibility of 0.1mT 50 Hz ELF EMFs on increasing the growth of *M. alcaliphilum* 20Z^R. We divided the samples into four groups, each containing three bottles (biological replicates). The control group was not exposed to the EMF, while the three experimental groups were exposed for 5 minutes, 15 minutes, or 30 minutes, with the exposure occurring at hour 20. We quantified the concentration (and therefore growth) of the bacteria by measuring the optical density (OD) of the samples periodically over 67 hours.

The growth curve, which shows the OD reading as a function of time, suggests that at the end of exponential phase (hour 23.5), samples exposed to the EMF for 5

Table 1: The percent increase or decrease of growth, maximum OD, and growth rate for each of the three exposure times when compared to the controls. Calculated from values at hour 20 immediately after exposure to hour 23.5 when the exponential phase ended.

Exposure Time	Growth Curve	Max OD	Growth Rate
0 mins	N/A	N/A	N/A
5 mins	11.99%	16.79%	64.59%
15 mins	10.98%	8.94%	51.23%
30 mins	1.73%	-5.45%	41.80%

minutes had an approximate 11.99% increase in growth when compared to the control group (Figure 1). Similarly, samples exposed for 15 minutes had an approximate 10.98% increase in growth, and those exposed for 30 minutes experienced around a 1.73% increase in growth over the control (Table 1). OD readings were taken well into the bacteria's stationary phase in order to examine the longer lasting effects of this treatment. We observed that in each sample's highest OD reading at hour 45.5, those exposed for 5 minutes had around a 16.79% higher maximum OD as compared to the control group. Additionally, those exposed for 15 minutes had an

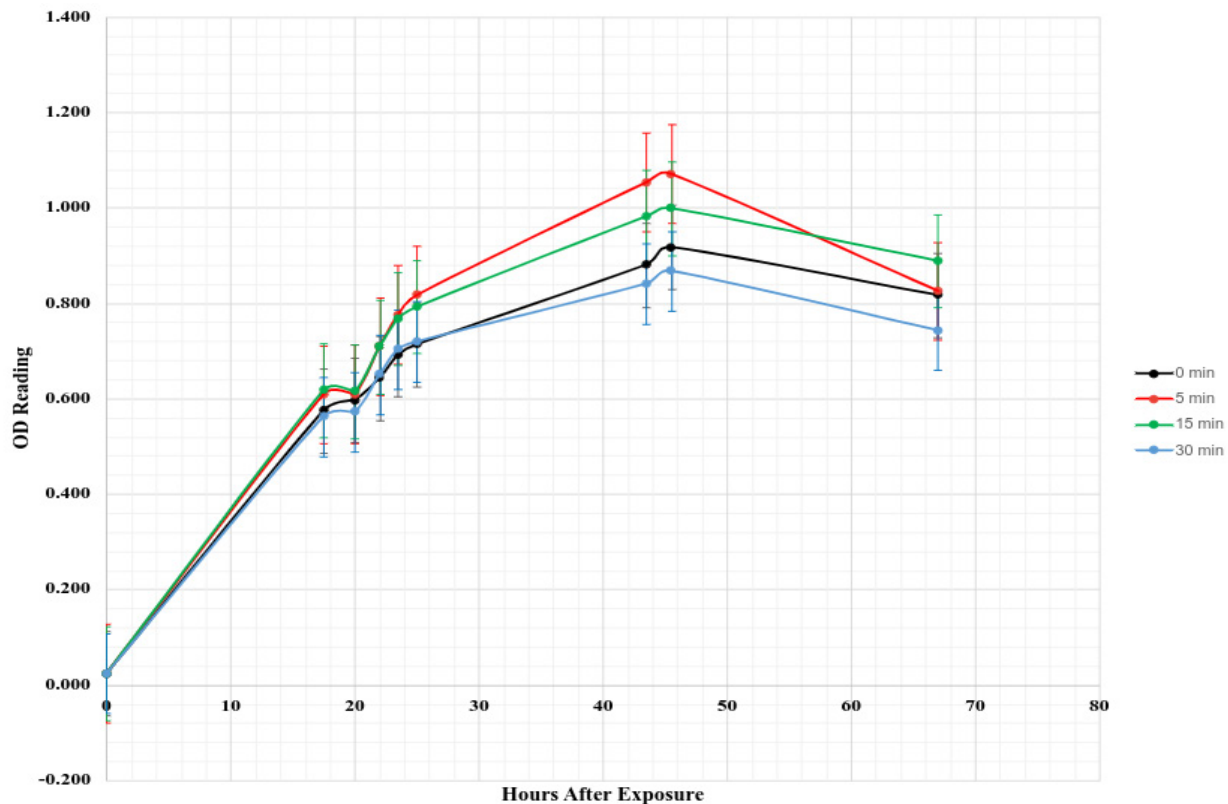


Figure 1: Growth curve after exposure to 0.1mT 50Hz EMF. *M. alcaliphilum* 20ZR growth was impacted by the length of exposure to ELF EMF. *M. alcaliphilum* 20Z^R was exposed to 0, 5, 15, and 30 min of 0.1mT 50Hz electromagnetic fields. OD readings were recorded at hour 0, 17.5, 20, 22, 23.5, 25, 43.5, 45.5, and 67. Growth curve taken from average of 3 biological replicates. Error bars represent standard error

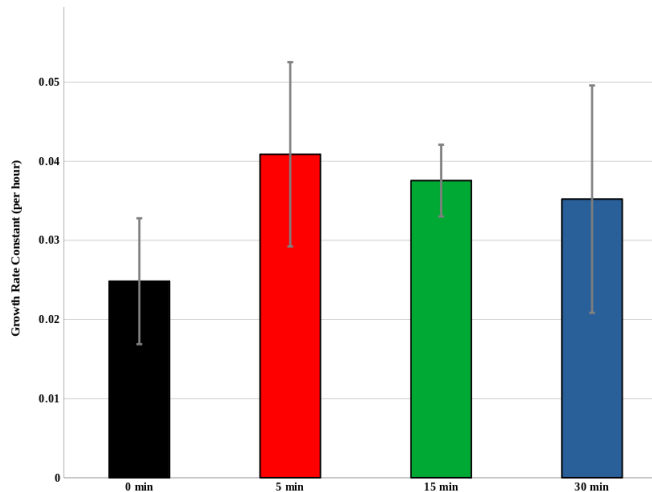


Figure 2: Growth rate after exposure to 0.1mT 50Hz EMF. *M. alcaliphilum* 20Z^R growth rate was impacted by length of exposure to ELF EMF. *M. alcaliphilum* 20Z^R was exposed to 0, 5, 15, and 30 min of 0.1mT 50Hz electromagnetic fields. OD readings were recorded at hour 0, 17.5, 20, 22, 23.5, 25, 43.5, 45.5, and 67 and compiled using the bacterial growth rate equation $N_t = N_0(1 + r)t$ using data from hour 20 to hour 23.5. Error bars represent standard error.

approximate 8.94% higher maximum density. On the other hand, the group exposed for a consecutive 30-minute time period experienced a 5.45% decrease in the maximum OD reading. It is important to note that the slight decrease in OD of the bacteria between hour 18 and 20 depicts the period in which samples were taken off the shaker whilst being exposed to the generated field.

The growth rate, defined as the rate of change in the number of bacteria per unit of time, was calculated from the moment of exposure (hour 20) to the end of exponential phase (hour 23.5). Our results show that samples exposed to the EMF for 5 minutes had a 64.59% increase in bacterial growth rate, while those for exposed 15 minutes had a 51.23% increase, and finally those exposed for 30 minutes

had a 41.80% increase when compared to the control group (Figure 2).

Using the average OD values from the moment of exposure (hour 20) to the end of exponential phase (hour 23.5), we determined that, when analyzed against the control group, the growth of the experimental samples exposed for 5 minutes (t -test, $p = 0.20$), 15 minutes ($p = 0.19$), and 30 minutes ($p = 0.49$) was not statistically significantly different because the p -values are higher than our significance level ($\alpha = 0.016$). Therefore, the null hypothesis, being that there is no correlation between exposure time and general growth, cannot be rejected and our data can only provide support for correlation between the variables being studied.

DISCUSSION

Methane is considered to be one of the most effective heat-trapping greenhouse gasses (1). With the rise in emissions from open-pit landfills, farms, and factories, environmental scientists are scrambling for new methods to combat the release of this chemical. This study examines the effects of ELF EMFs on the growth of the methanotrophic bacterium *M. alcaliphilum* 20Z^R in hopes of stimulating bacterial proliferation but ultimately found that there were no statistically significant impacts.

To this day, there have been mixed results on the effects of ELF EMF exposure on biological systems. Some studies have claimed these fields accelerate cell potentials, promote healing, and reduce inflammation, while others have noticed its detrimental effects on both bacterial colony forming unit count (the number of bacterial cells in a sample viable for binary fission) and OD readings (18-19). Perhaps the effects are dependent solely on the type of biological organism being exposed, or possibly there exists a complex relationship between the duration of time and EMF intensity a bacterial sample can be exposed to before causing negative/harmful effects (20). The wide range of positive and negative results

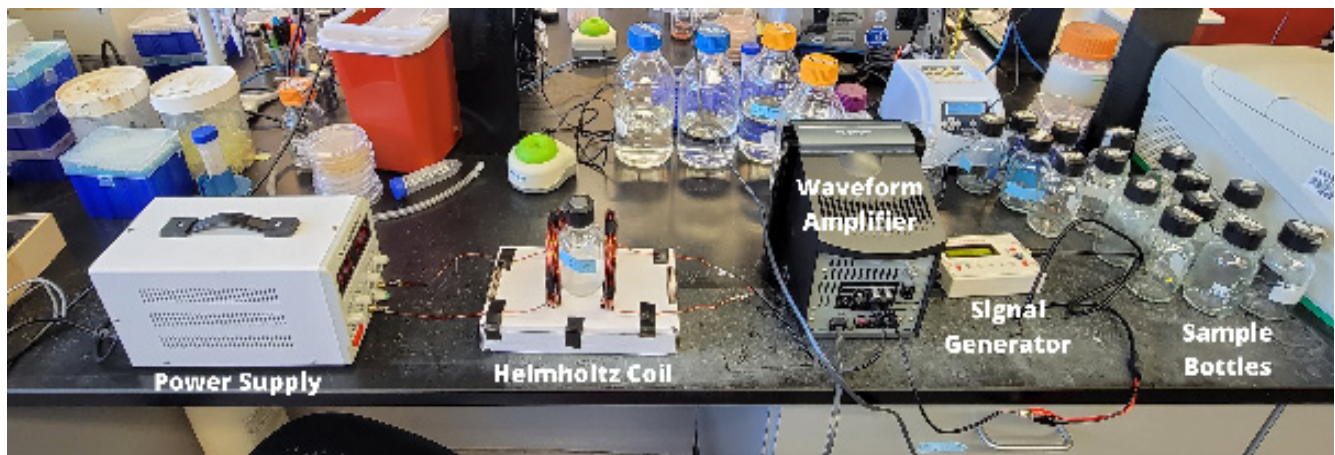


Figure 3: The extremely low-frequency electromagnetic field generator with each component labeled.

generated by the experiments cited in this manuscript make it difficult to identify an exact reference point. Additionally, it is not known how large a change in growth could theoretically be, especially for each strain of bacteria, given the fact that research in this area on a molecular level is still novel.

As our results indicate, exposure to a 0.1mT 50Hz ELF EMF for 5, 15, and 30 minutes does not appear to have a significant effect on the growth rates or growth curves of methanotrophic bacteria. However, this could very well be due to the low statistical power of this experiment, therefore making it important to continue experimentation under identical conditions. Some factors and limitations that could have influenced the results of this study was the fact that the ELF EMF exposure system was designed and developed in-house, meaning that small imperfections in the wounding of the wire may have had miniscule impacts on the perfect uniformity of the field created.

The reason for choosing our specific parameters as outlined in our hypothesis was because of prior research done in the field with similar frequencies and intensities that demonstrated that exposure for 10 minutes could lead to positive effects on bacterial growth, enzyme kinetics, and gene expression, while exposure for longer periods of time greater than 30 minutes showed negative effects on these attributes (21-23). Future studies to expand on this one could test the effects of a larger range of field densities (0.1mT–5mT) and a larger range of exposure times (5–60 minutes). In short, similar and altered conditions with a larger sample size will help paint a better, more solid picture of the effects of ELF EMF exposure on methanotrophs and gain a stronger understanding of which variables could lead to the most positive effects.

Although the results generated by this experiment supported the null hypothesis, the basis for this study is still important because successfully increasing the growth rates of methane-consuming bacteria can lead to an increase in methane oxidation rates and thus has the potential to reduce the detrimental methane concentration in Earth's atmosphere.

MATERIALS AND METHODS

Construction of the Helmholtz Coil

A Helmholtz coil consists of two identical magnetic coils facing parallel to each other. The radius of each coil matches the distance separating the two coils. When current passes through both coils, it generates a uniform magnetic field. In this particular experiment, each coil had a radius of 50mm, each composed of 25 wounds of 14 American Wire Gauge insulated copper magnetic wire. One individual coil was attached to a power supply (Lavolta BPS-305) running at ~535mA and 0.05V, while the other to a waveform amplifier (Sony CMX-E22) and a signal generator (FG-100 DDS) (Figure 3). The intersection of the magnetic field produced by each coil summates to form a sinusoidal wave with a direct current offset.

Preparation of Liquid Medium

The bacterium *M. alcaliphilum* 20Z^R was grown in a nitrate mineral salts (NMS) medium which contained 1M potassium nitrate (KNO₃), 0.2M magnesium sulfate (MgSO₄) x 7•H₂O, 0.02M calcium dichloride (CaCl₂) x 2•H₂O, and distilled, deionized water (24). The medium was supplemented with 1mL/L trace solution, 20mL/L phosphate solution, and 40mL/L carbonate solution. The trace solution contained 5M ethylenediaminetetraacetic acid disodium salt (Na₂EDTA), 2M ferrous sulfate (FeSO₄) x 7•H₂O, 0.3M zinc sulfate (ZnSO₄) x 7•H₂O, 0.03M manganese(II) chloride (MnCl₂) x 4•H₂O, 0.2M cobalt chloride (CoCl₂) x 6•H₂O, 0.6M copper sulfate (CuSO₄) x 5•H₂O, 0.05M nickel chloride (NiCl₂) x 6•H₂O, 0.05M sodium molybdate (Na₂MoO₄) x 2•H₂O, and 0.03M boric acid (H₃BO₃). The phosphate solution contained 5.44M potassium dihydrogen phosphate (KH₂PO₄) and 5.68M disodium hydrogen phosphate (Na₂HPO₄) while the carbonate solution contained 75.6M sodium bicarbonate (NaHCO₃), and 10.5M sodium carbonate (Na₂CO₃).

Preparation of Liquid Culture

Twelve 100mL bottles were autoclaved at 121°C for 45 minutes. In order to increase the pH and use it as a buffer, 3mL of phosphate and 6mL of carbonate solutions were then added into an already prepped bottle containing 150mL of NMS medium. Once mixed, 25mL of this solution was then inserted into three bottles for three biological replicate samples. Cultures were inoculated with bacteria grown from a frozen stock on a petri dish containing NMS agar. 50cm³ of gaseous methane via needle and syringe was introduced into each bottle. The prepared containers were then placed in the 30°C shaker overnight at 200rpm. Once again, 3mL of phosphate and 6mL of carbonate were then each added into two new 150mL bottles of NMS medium and then divided evenly (25mL each) into the 12 previously autoclaved 100mL bottles. They were then labeled based on their associated biological replicates and time of exposure (control or 0 minutes, 5 minutes, 15 minutes, or 30 minutes).

OD readings were taken of the three initial biological replicate bottles at 600nm. All 12 cultures were then split to OD = 0.024. It was determined that 0.67mL of biological replicate 1, 0.66mL of biological replicate 2, and 0.75mL of biological replicate 3 was needed to obtain these concentrations. After inoculation of the bacteria into the bottles, 50cm³ of methane gas was then added to each of the 12 bottles via needle and syringe and then placed in the 30°C shaker overnight until it reached an OD of 0.50 the next morning.

ELF EMF Exposure

Each sample was then exposed to the ELF EMF for the specified duration of time just before reaching the exponential phase (hour 20). In order to maintain a controlled experiment, all bottles were taken off the shaker for the same amount of time, meaning those not facing exposure at the moment were placed on the side away from the generating source.

Optical Density Readings

OD readings were taken every 1–2 hours apart until hour 67. A needle was inserted into the lid of each bottle and 1mL of solution was extracted. The liquid was then placed in a spectrophotometer (Jenway 6320D) at 600nm and values were recorded on a table.

Data Analysis

The average values for the three biological replicates were taken and used to calculate growth, maximum optical density, and growth rate along with the respective percent change. Growth is simply the average OD values recorded at each period in time. By subtracting the OD value of the control at the end of exponential phase (hour 23.5) from each of the exposed samples, dividing it by the control, and then multiplying by 100, the percent difference can be seen. A similar process was done for maximum OD except at hour 45.5. Growth rate for each condition was calculated from the moment of exposure at hour 20 to the end of exponential phase at hour 23.5 using the exponential growth equation $N_t = N_o (1 + r)^t$, where N_t is the final OD value, N_o is the initial OD value, r as the unknown rate constant, and t as time. The percent difference equation was applied to the newfound r values in an identical fashion to the growth and optical density calculations.

The built-in Microsoft Excel one-tailed t-test was used to calculate significance between the control and exposed samples. To account for multiple comparisons a Bonferroni corrected α -level of 0.016 was used.

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