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Biowaste to Biofuel: Using Methane-Producing Microorganisms Found in Soil Samples from Local Wetlands

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Summary

In this study, we investigated whether methane-producing microorganisms are present in the soil samples obtained from local bogs and wetlands in the Puget Sound region of Washington state. Furthermore, we determined the temperature at which these methane-producing microorganisms can be used to produce methane, a biofuel, from kitchen and yard waste. Producing methane for making biofuel as renewable energy is better than sending kitchen and yard waste to landfills, which do not utilize or collect these wastes for energy production. We show that methane-producing microorganisms are indeed present in the soil samples we collected and that they remain active at both indoor (68 °F) and outdoor (36 to 70 °F) temperatures. A significant amount of methane was detected after 45–75 days of soil incubation with fed biowaste. Importantly, our work establishes the potential of using these methane-producing microorganisms for the production of biogas in colder climates of the Pacific NW and paves the way for the future research and design of a kitchen-scale biodigester for use in colder climates.

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Introduction

The inspiration for our project comes from the Millennium Project [1], which is a global foresight network on behalf of humanity for building a better future that informs the world of the emerging energy crisis. The Millennium Project was initially founded in 1996 after a three-year feasibility study with the United Nations University, Smithsonian Institution, Futures Group International, and the American Council. The Millennium Project is participated and sponsored by various companies, organizations, and individuals of over 3,500 scholars, business planners, policy makers and futurists. Fifteen global challenges were set to make a better world for all of us; from climate change, clean water, energy, population and resources, to global ethics. One of the global challenges facing humanity is energy, where it is defined as "How can growing energy demands be met safely and efficiently?" by the Millennium Project [1]. This is where harvesting the benefits of clean, local, and renewable energy comes in. One source of such energy is biogas energy. Currently, efforts are being made to advance biofuel technologies around the world, and biogas is an important source of biofuel [2,3]. Biogas can be used for heating and cooking purposes, or to power motor vehicles [4].

Biogas is landfill gas, swamp gas, or digester gas produced when microorganisms decompose wet organic waste present in a landfill, swamp or digester. Biogas contains methane, the product of anaerobic digestion of organic waste by anaerobic organisms [5]. Anaerobic digestion consists of four key steps or processes in which different bacteria break down biodegradable materials in the absence of oxygen: 1) hydrolysis 2) acidogenesis 3) acetogenesis and 4) methanogenesis (**Figure 1**, ref. 6). Methane in biogas is 20 times more potent as a greenhouse gas than carbon dioxide [7]. Therefore, uncontained landfill gas, which escapes into the atmosphere, may significantly contribute to the effects of global warming. We therefore argue that it is very important to use biogas as a source of energy rather than allow it to move to the atmosphere [8].

A biodigester is a technology that can be designed

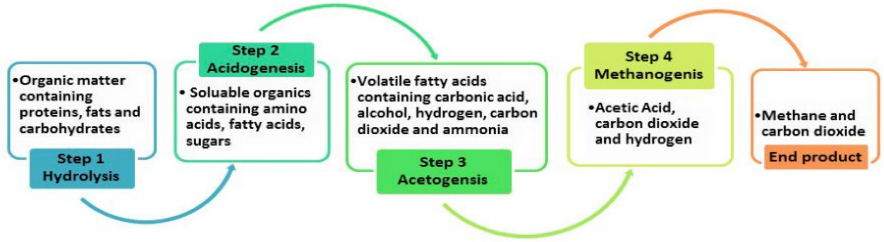

Figure 1: Four Step Process of Methanogenesis. Each step is carried out by different bacteria. [6]

and used to harvest biogas energy. Its advantage over other approaches is that the organic wastes from local communities, schools and households can be collected and sent to the biodigester. Microorganisms use organic wastes to produce biogas or methane, contained, and can be used for local energy generation applications. There are three types of methanogenic anaerobic bacteria that can be found and cultivated for the purpose of designing a biodigester: thermophilic, mesophilic, and psychrophilic bacteria. Thermophilic bacteria thrive in extremely hot temperatures (113 to 252 °F), mesophilic bacteria in tropical climate temperatures (68 to 113 °F), and psychrophilic bacteria in moderate cold to extreme cold temperatures (5 to 68 °F) [9]. Most bio-digesters use mesophilic bacteria found in animal manure and are engineered to provide suitable conditions to allow the bacteria to produce methane [10]. However, methane-producing bacteria can be found naturally in the Puget Sound region.

In this project, we wanted to know whether methane-producing microorganisms are present in the soil samples obtained from local bogs and wetlands and whether we can use these methane-producing microorganisms to produce methane from kitchen and yard waste for potential use as a biofuel. We also wanted to know how temperature affects methane production by local methane-producing microorganisms, such as in fluctuating outdoor conditions of our neighborhood, as well as a constant indoor environment. Our hypothesis was that there are many methane-producing microorganisms present in the soil samples from local bogs and wetlands, and that we would detect methane production in both indoor and outdoor temperature tests.

Results

We first collected soil samples from Shadow Lake Bog in Renton, WA to obtain the methane-producing microorganisms. The location was chosen because it consists of waterlogged peat bogs. Peat bogs are known to have low oxygen levels, suggesting that anaerobic methane-producing microorganisms might live in the lake sediment [11,12]. Three experimental vials at room temperature and three at outside temperature were set up. Two measurements on methane and carbon dioxide (CO_2) were done at 15-day intervals. In the measurement, headspace gas samples from the vials were harvested through a needle in a gastight locking valve syringe, and injected into a gas chromatograph. The results of Trial-1 are reported as the average methane and carbon dioxide concentrations (**Table 1**).

Conditions	1st Incubation, 15 days		2nd Incubation, 15 days	
	Methane (% v/v)	CO_2 (% v/v)	Methane (% v/v)	CO_2 (% v/v)
Room Temp	0.00±0.00	10.17±2.36	0.00±0.00	10.90±5.28
Outside	0.00±0.00	23.33±7.57	0.00±0.00	12.67±2.08

Table 1: Average for all vials for soil samples from Shadow Lake bog. Indoor room temperature: 68 °F for first and second incubation. Outdoor temperature: 36 to 47 °F for first incubation and 37 to 52 °F for second incubation. Each vial has 30.0 g feedstock + 3 g soil + 10 mL water (trial discontinued as methane was undetectable). Errors indicate standard deviation.

The results in **Table 1** show that no methane but a high level of CO_2 was detected in the vials when the first measurement was taken at 15 days. After the first measurement, it was found that pH was very low (pH 3.5–4.0). We hypothesized that the environment could be too acidic and unsuitable for the methanogenesis step to happen. Therefore, pH was adjusted with a sodium hydroxide buffer to a neutral pH of 7.0. The second measurement was done after another 15-day interval, but methane was not detected even after a month of incubation. High levels of CO_2 were detected in all the vials, indicating that some soil microorganisms were able to digest the biowaste and make it to the acetogenesis stage, as determined by the low pH and CO_2 formation [6,13,14]. We believe that the first three steps of the biodegradation of organic wastes (**Figure 1**) occurred in our samples; however, the last step of methanogenesis did not occur, since no methane was detected. Furthermore, we were not sure that the methane-producing microorganisms were present in the soil sample that we obtained from Shadow Lake. The color of soil sediment at Shadow Lake was reddish, which also indicated acidic conditions that may not be suitable for methanogenesis [6,13,14].

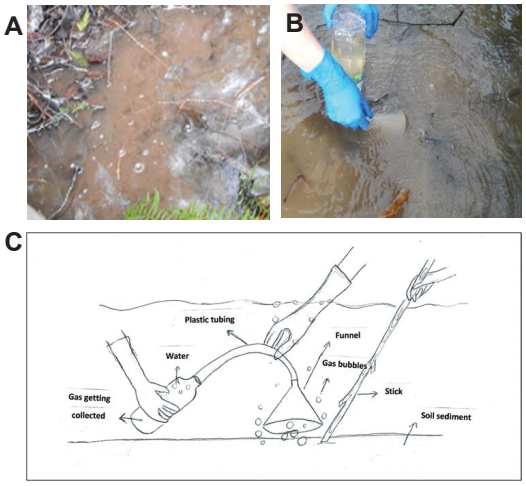

Figure 2: Methane Collection from West Hylebos. A) Methane bubbles appearing in the sediment after it was prodded with a stick. B) Using a bottle, tubing and funnel to collect methane gas from deep sediment. C) Schematic of gas collection from soil sediment.

To deal with the absence of methanogenesis in the Shadow Lake samples, we decided to obtain soil samples containing methane-producing microorganisms

from a different location. We picked West Hylebos wetland in Federal Way, WA. The color of the sediment was a dark gray, and many bubbles emerged from the deep soil when probed with a stick, consistent with methanogenesis. (**Figure 2A**) We decided to test for the presence of methane at this location. With a gas collection set up (**Figure 2**), we collected these gas bubbles in bottles from 3 different areas in the wetland to be analyzed for methane in a gas chromatograph. The results revealed that the deep pit area shows maximum methane production (**Table 2**). A flaming test also

No.	Soil Bubble Sample	Methane (%)
Methane standard		3.99
1	Marlake	2.83
2	Deep pit	10.81
3	Fallen tree	1.91

Table 2: Soil samples from three sites at West Hylebos wetland.

indicated that the collected gas sample showed a quick blue flame, confirming the presence of methane in those areas.

Sediment (soil sample) was collected from water-logged areas in the deep pit at the wetland, known to have very low oxygen levels. Instead of bringing the soil sample to the lab and setting up the test vials, in Trial-2, the soil was filled directly into the vials, which were sealed underwater to avoid any exposure to air (**Figure 3A**). The vials therefore did not have exactly identical amounts of soil sediment. Once the vials were brought back to the laboratory, the top layer of water sample was removed to allow headspace for methane accumulation. The headspace was purged with an anaerobic inert gas to keep an anaerobic condition in each vial and remove any headspace methane from each vial before vial incubation. All vials were divided into two sets, each containing three vials. One set was stored at indoor temperature (**Figure 3B**), and the other was stored outdoors (**Figure 3C**). No biowaste substrate was added to any of these vials.

The objective of this initial experiment was to see whether there was any methane production from the solid sediment and organic residue without biowaste substrate addition. We first incubated these vials for 9 days to check whether the methane-producing microorganisms remained active after sample harvesting. After the first 9-day incubation, we confirmed by gas chromatography the presence of methane, indicating that the methane-producing microorganisms were in the soil sample vials. After sampling, we purged the headspace of all the vials with anaerobic gas to strip off any methane in the vial headspace. To ensure that the microorganisms were still active after the first 9-day incubation, the vials were incubated for 6 more days (second incubation). All the vials were sampled again for methane analysis. It

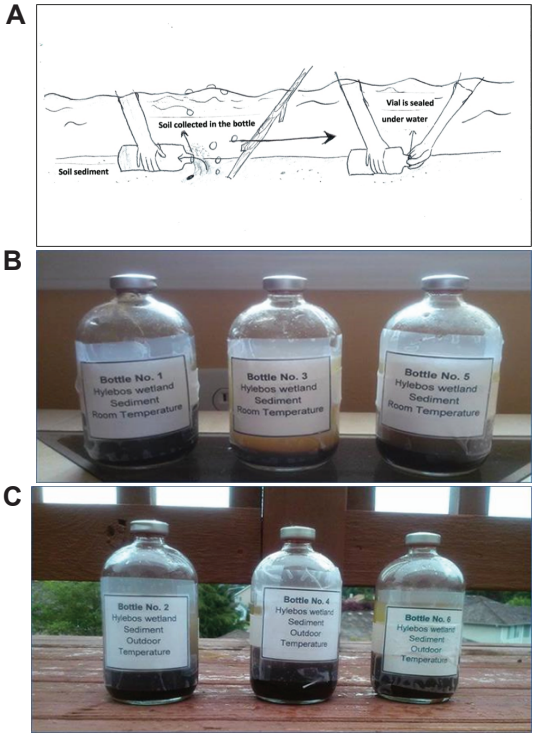

Figure 3: Soil Sediment Collection and Incubation. A) Schematic of soil collection into vials. B) Vials 1, 3, 5 were kept at room temperature. C) Vials 2, 4, 6 were kept outdoors.

was again confirmed that methane was produced in each vial, indicating that 1) hydrolysis 2) acidogenesis 3) acetogenesis 4) methanogenesis were present in all vials (**Figure 1**). The results averaged from three vials at room (indoor) temperature and three at outdoor temperature, are shown in the **Table 3**. From these two initial tests of methane formation, it can be seen that the level of methane and carbon dioxide were low without any biowaste substrate addition. Further, it should be noted that the soil sample amount is very small as compared to the entire bottle test volume. Thus, the amount of methane produced from these initial tests

Conditions	1st Incubation, 9 days		2nd Incubation, 6 days	
	Methane (% v/v)	CO_2 (% v/v)	Methane (% v/v)	CO_2 (% v/v)
Room Temp	2.23±1.39	1.92±0.85	1.86±0.71	1.47±1.02
Outside	0.81±1.06	1.31±0.69	1.86±1.28	1.43±0.77

Table 3: Methane (average for all vials) analysis after multiple days of incubation. Indoor or room temperature: 68 °F for first and second incubation. Outdoor temperature: 38 to 55 °F for first incubation and second incubation. Errors indicate standard deviation.

without additional feedstock is very small or low in concentration, as shown in **Table 3**.

After the second incubation and sampling, we sought to increase methane production by adding 10 mL of partially digested feedstock from Trial-1 to the vials. It should be noted that soil microorganisms from both

Shadow Lake bog and West Hylebos wetland are present in our test vials due to the addition of digested feedstock from Trial-1 vials to Trail-2 vials. Three measurements for methane and CO_2 were done at about 15-day intervals and the final fourth measurement was done after roughly a 30-day interval. After each sampling and methane analysis, the sample bottle headspace was purged with anaerobic gas to remove any methane in the headspace and thus set the baseline for calculating the methane production rate in each test interval. The results of these four methane measurements are plotted in **Figure 4**.

After the addition of feedstock, we found that CO_2 levels started rising and only a very small amount of methane was produced in each vial, indicating that the soil methanogens were still active in each vial (**Figures 4 and 5**). In the first and second incubation periods, high CO_2 concentration indicated a strong acetogenesis, and low methane production indicated that the initial methanogenesis is weaker. At the third incubation, methane production was considerably higher for all the indoor and outdoor vials. In particular, the indoor vials in the third incubation period had a higher average methane concentration of 27.18% than the outdoor vials at the average of 2.12%. The indoor vial production rate was 1.81% per day, while the outdoor rate was 0.14% per day. In the fourth incubation period with 30 days, the outdoor vials increased methane production, probably due to the warmer temperature present in May. The bottle headspace methane was generated at very high concentrations, with levels at 60.53% for the indoor temperature and 46.98% for the outdoor temperature. These methane levels are comparable to those produced with good biodigesters in warmer climates [15]. These tests showed that the added biowaste substrate improves methane production. One caveat is that the warmer temperatures in May could be a stimulating factor for the outdoor vials to produce more methane, while the outdoor colder temperature in April (late winter)

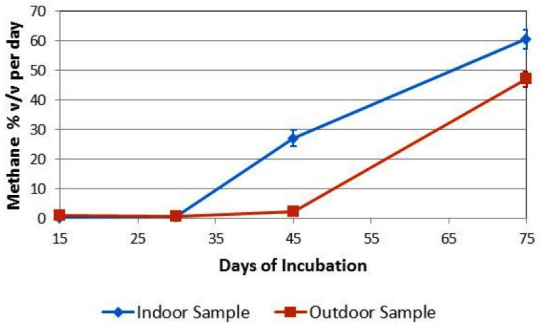

Figure 4: Vial methane content. Both indoor and outdoor test vials show a spike of methane production around the same time, indicating methanogenesis. Indoor temperature: 68 °F for all tests; Outdoor temperatures: 42-59 °F for first and second incubation, 47-65 °F for third incubation, and 58-70 °F for fourth incubation. Error bars indicate standard deviation.

still suppressed methane production. Thus, it would be more efficient to build a smaller indoor biogas digester than a bigger outdoors one based on our results.

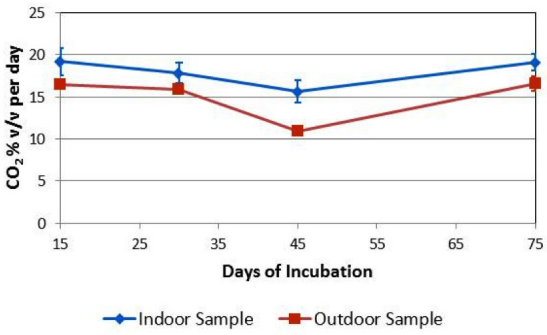

Figure 5: Vial carbon dioxide content. CO_2 levels dip around the same time that methane levels spike up (see **Figure 4**). Indoor temperature: 68 °F for all tests. Outdoor temperatures: 42-59 °F for first and second incubation, 47-65 °F for third incubation, and 58-70 °F for fourth incubation. Error bars indicate standard deviation.

Discussion

Our findings support our hypothesis and establish the potential of using these methane-producing microorganisms for the production of biogas in colder climates of the Pacific Northwest. Our hypothesis that methane-producing microbes exist in local wetlands is supported by this study. We were able to enrich the soil methane-producing microorganisms with blended kitchen and yard biowaste to produce methane in the indoor temperature and in the colder outdoor temperature conditions. Although more methane formation was detected at room temperature, the vials kept at the colder, winter outdoor temperatures also showed significant methane production. The methane-producing microbes were able to digest the feedstock simulating the kitchen and yard waste common in any household and produced biogas.

We deduced that the reason why the Shadow Lake bog samples did not work well was that the bog had acidic conditions, which were not suitable for methane-producing microorganisms. Hydrolytic, acidogenic, and acetogenic bacteria were likely present and biologically active; however, methane-producing bacteria were not active in the Shadow Lake sediment at that particular location. It has been established [16] that acidogenic bacteria lower the pH prior to the stage when methanogenic bacteria start working, a stage which needs a higher pH level. The process of methanogenesis is pH sensitive; the pH must be between 6.5 and 8.0 [6,16] for better methane production. In the biogas digester starting up, literature indicates that during the biodigester startup, the acid producing and hydrolyzing microbes might outgrow the methanogens, causing a drop in pH. If the pH ever drops below 6.8, methanogen

growth will be inhibited. At or below a pH of 6.0, cleaning out and restarting the bio digester is recommended [13,14]. We infer that this explains our results in Trial-1 using the Shadow Lake samples.

The West Hylebos wetlands had a significant amount of methane production in the sediment. The bacteria remained active at both indoor and outdoor temperatures during the trial period. In the test setup, it should be noted that soil microorganisms from both Shadow Lake bog and West Hylebos wetland were present in our test vials due to the addition of digested feedstock from Trial-1 vials to Trail-2 vials, Although Trial-1 with Shadow Lake bog soil samples did not produce methane, they were able to digest feedstock well. Thus the microorganisms from Trial-1 might have helped, in part, Trial-2's hydrolysis, acidogenesis, and acetogenesis. (**Figure 1**)

The production of methane was higher at room temperature than in colder temperatures. In the months of May and June, as the outdoor temperatures rose, the methane generation in the vials that were kept outdoors also rose. This observation is congruent with the idea that methanogens outgrow acid producing organisms at higher temperatures, and that bio digesters should be started at higher temperatures rather than lower temperatures [13]. Similar to our test results, researchers involved in the project done by Cordova Electric Co-op, University of Alaska Fairbanks and Cordova High School also report that temperature range is a major restricting factor for most existing biogas digesters and microbes found naturally at colder temperatures can be used to produce biogas although at only 28-56% of rates typical of warmer temperature regimes [18].

It can be concluded that the methane-producing microorganisms in our samples were using the biowaste in the feedstock provided. That is why we saw immediate changes in the levels of CO_2 and methane. The bacteria involved in the first three steps of methanogenesis became active, and when the raw material for methanogenesis was created, the methanogenic bacteria became active (**Figure 1**). After that time, the levels of CO_2 remained consistent and levels of methane continued to elevate, indicating that all four steps of methanogenesis were occurring in the vials. The time period of approximately 45 days for methanogenesis has been noted in other references also [13].

Thus, the West Hylebos wetlands' anaerobic microbial consortium was able to digest the feedstock simulating the kitchen and yard waste common in any household and produce biogas. A significant amount of methane (60%) was detected in two and a half months' time [17,19], which is longer compared to warmer climates where biogas is generated in a matter of a few days or weeks [10]. Our results show that although methane generation is slow to start, it accelerates much faster later as compared to its initial rate. The results establish the potential of using these methane-producing microorganisms for the production of biogas in colder climates of the Pacific Northwest. This paves the way for the future research on designing parameters of a bio-digester that can be used in schools or at home in colder climates.

In spite of the encouraging results we obtained during our experiments, we acknowledge that our experiments had some time limitations. This was one of the reasons for using partially digested feedstock from Trial-1 in the following Trial-2. If we have a chance to repeat the experiment, we would make several improvements. First, we would incubate the samples in several constant, experimentally induced temperatures (for example 39 °F, 68 °F, and 99 °F). This would enable us to clearly determine how the microbes perform under these temperatures for biogas or methane production. Secondly, we would also have a negative control for each temperature group without feedstock, which would be incubated for the same amount of time. This would help clearly establish that we have developed a feedstock that is degraded by the microorganisms in the soil samples to produce methane. Finally, we would find a method to keep an equal amount of soil samples in each test vial. Equal volumes of soil in each vial would make the data cleaner and easier to interpret.

Methods

In the experiments, we collected soil samples that contained methane-producing microorganisms. We tested the output of methane gas in six vials containing the methane-producing microorganisms from the soil sample and the feedstock made up of kitchen and yard waste. Three vials were kept at constant indoor temperature (68 °F), and three vials were kept at the fluctuating outdoor temperatures (between 36 and 70 °F) of the local Puget Sound area to test the possibility of methane production under these conditions.

Obtaining Bacterial Cultures

Soil samples containing a consortium of microorganisms were obtained from local wetlands. We found two local wetlands with potential for obtaining good testing cultures: 1) Shadow Lake and 2) West Hylebos. In the wetland area we looked for mud which has a gray undertone and is part of a body of water where bubbles can be seen emerging when sediment under the water is poked. The gas bubble collection method is shown in **Figure 2**. This area of water is likely to contain methane-producing microorganisms [12]. The collected gas bubbles inside the bottle are used for methane confirmation in a gas chromatograph.

Soil samples containing the microorganisms were

collected in the following manner: First, the collection bottle is completely filled up with water to displace any air inside the bottle. The bottle is kept submerged in the water. The sediment is then poked with a stick to check for gas bubbles. Then soil is collected from the sediment deep under water, as shown in **Figure 3A**. Finally, the bottle is sealed when it is still under water to avoid any air from getting in because oxygen is toxic to anaerobic microorganisms.

Preparing the Feedstock

The feedstock should have a carbon to nitrogen ratio of 25 to 30:1 for microbes to effectively decompose organic materials [20], while this ratio can be as low as 20:1 or as high as 40:1 [21]. Feedstock was prepared to match this ratio using materials that can be commonly found in any household kitchen waste and yard waste as follows: chicken, potatoes, lettuce, pasta (just plain cooked noodles), spinach, coffee ground, apples, dead leaves, grass clippings and beans [22]. These were combined in equal proportions. Then all the ingredients were blended together in a blender. The feedstock needs to be consistent in all the trials where feedstocks were added as shown in **Table 4**.

Ingredients (and amount-1 part each)	Carbon to Nitrogen Ratio
1. Chicken/fish	5:1
2. Potatoes	25:1
3. Lettuce	18:1
4. Pasta noodles/rice	22:1
5. Spinach	18:1
6. Grass	19:1
7. Coffee grounds	25:1
8. Apple/fruit	40:1
9. Leaves (yard waste)	60:1
10. Beans	5:1
Average Ratio: (rounded up)	24:1

Table 4: Carbon:nitrogen content in various ingredients of the feedstock [20,21].

Calculating Feedstock Moisture Content

The feedstock was reported with a moisture content of 80.99% and a solid content of 19.01% [5]. On a digital scale, an aluminum pan was weighed and tared. A small amount of feedstock was placed on the aluminum pan and weighed to find the wet weight. Then the pan was placed in an oven at 220 °F for five hours until the feedstock is completely dry. Then the tray was weighed to find the dry weight of the feedstock. Moisture content is calculated by 100% - (dry weight / wet weight) the moisture content in our feedstock was 80% as detected by Ohaus MB45 moisture analyzer. The desirable ratio of feedstock to culture is 9:1 OR 90% feedstock and 10% culture. Therefore, 900 grams of feedstock would need 100 grams of culture [23]. Good pH should be between 6.5 and 8.5 for biogas production [13].

Setting Up the Experiment

For Trial-1, 6 vials were obtained and numbered 1-6. In each vial, about 30 grams of the feedstock was deposited. Then 13 mL of soil sample + water from the wetland was deposited into each vial. The vials were then fitted with an airtight cap and purged using the following method.

An anaerobic inert gas (argon) or nitrogen without oxygen is used for vial headspace purging to remove any headspace oxygen during trial set up. Anaerobic gas is turned on at about 3 psi pressure. Tubing and needle coming from the anaerobic gas cylinder is first purged for approximately two minutes by flushing the anaerobic gas through the gas line. One spare needle is first inserted into the vial to act as an outlet needle for the anaerobic gas flow. The incoming anaerobic gas needle (inlet needle) is inserted into septum on the test vial to develop positive flow of anaerobic gas into vial headspace. The vial headspace is flushed with anaerobic gas for 5 minutes. The vial is agitated gently throughout to release any air bubbles that might be trapped in the liquid sample. Outlet needle is then removed from the septum at 5 minute purging. Inlet needle is then removed from the septum. Since anaerobic gas is heavier than air, it easily displaces the air in the test vial creating an O_2 free atmosphere. Then vials 1, 3, and 5 were placed outdoors at least 10 feet away from the house for the duration of the trial period; vials 2, 4, and 6 were placed indoors for the duration of the trial period. The indoor temperature was maintained around 68 °F. Outdoor temperatures varied according to the season.

Results were collected at approximately 15-day intervals and methane and CO_2 were measured using (Hewlett-Packard) HP 5890 Gas Chromatograph (GC) equipped with thermal conductivity detection and a Altech CTR dual pack column, specifically designed for methane and other gas analysis. The carrier gas is helium. The GC was first calibrated with a gas standard – Scotty Analyzed Gases No. 14. Methane standard is 3.99% and carbon dioxide standard is 5.04%. After the gas chromatography was calibrated, from a test vial headspace, a gas sample of 0.5 mL volume equilibrated to room atmosphere was harvested through a needle in a gas tight syringe with a locking value and injected into the gas chromatography. If there is any methane, the thermal conductivity detection can detect the methane after the methane is separated using the Altech CTR dual pack column.

For Trial-2, the soil was filled directly in the vials, which were sealed under water to avoid any exposure to air. The vials therefore did not have identical amounts of culture. In each 150 mL bottle, 100 mL lake sediment and lake water were retained while 50 mL lake water was removed from the top with a plastic pipette. The vial was resealed and subsequently the headspace was purged with anaerobic

gas without oxygen. Initially no feedstock was added in the bottles because we wanted to confirm the presence of methane-producing microorganisms in the soil samples with minimum organic matters in the sediment.

After the existence of the organisms in the soil sample was detected and established by methane formation, a small amount of partially digested feedstock from Trial-1 was added to the vials following the method below:

1) 10 mL top lake water was first taken out from each second trial bottle. (The second trial bottle has a total volume 100 mL liquid and sediment, and 50 mL headspace).

2) From the first trial bottles, 10 mL partially digested feedstocks were taken out and added to each second trial bottle.

3) Each second trial bottle was then sealed with a rubber stopper and an aluminum cap with a crimper and purged with anaerobic gas for 8 minutes.

Three measurements for methane and CO_2 were performed at 15-day intervals, and the final fourth measurement was completed after a 30-day interval. (Approximately + or – 2 days depending on the availability of the equipment). After each gas analysis, the vial headspace CO_2 was purged off with the anaerobic gas.

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