

The Effect of Microbe Invasions on the Methanotroph Metabolism and Community Structure in Microcosms

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Abstract

. The theory of maximum entropy production (MEP) states that steady state systems with sufficient degrees of freedom will organize to maximize the rate of entropy production (Vallino, Proposal). There have been values for entropy calculated in abiotic systems that have been very useful for predictive models and it would be a great advancement if we were able to apply these predictions to biological systems. My experiment incorporated invasive species into this theory, hypothesizing that a species will be more likely to exhibit a successful invasion they are able to maximize the rate at which the system dissipates energy. I conducted this study using microcosms that contained microbial communities that had been functioning for over two years. By introducing new microbial species to these preexisting microbial I found that a change of species composition within one of the microcosms did in fact increase the rate of methane respiration (or the rate of entropy production).

Introduction

The theory of maximum entropy production (MEP) states that steady state systems with sufficient degrees of freedom will organize to maximize the rate of entropy production (Vallino, Proposal). In other words, a system will strive to maximize the rate at which it dissipates energy, converting its energy source from a form with high energy potential to a form with low energy potential (Vallino, *Per Comm*). An example of this is methane oxidation, which was the focus of my experiment. When methane is oxidized it transitions from having a high energy potential in the form of CH_4 to having a low energy potential in the form of CO_2 . The MEP theory indicates that a community that oxidizes methane faster should be more stable (or more probable) than one that oxidizes methane at a lower rate.

There have been other speculations about the MEP theory, (Ulanowicz and Hannon 1987, and Swenson 1989), but the lack of support behind the theory has prevented it from acquiring much attention (Vallino, Proposal). In the field of non-equilibrium thermodynamics Dewar (2003, 2005) has recently presented a provisional proof of this theory. Thermodynamics have not typically been applied to biological systems, but it is necessary that they are in order to understand more about the fundamental principles of how these systems function (Vallino, Proposal). This theory

allows for more predictive capabilities in regards to biological systems (Vallino, *Per Comm*).

In my experiment, I incorporated invasive species into this theory. In regards to the principles of the MEP theory, my hypothesis was that there is a higher probability that an introduced species will undergo a successful invasion if it increases the rate of entropy production (or energy dissipation) of a system. In order to test this idea I investigated the effect of microbe invasions on the biogeochemistry of microcosms. Under steady state conditions (that is, there is not a lot of carbon accumulating inside the microcosms), the methane oxidation rate is proportional to entropy production (Vallino, *per comm*). Microbial systems are excellent to use in order to study how biological systems function because they operate on a much shorter timescale than other systems. For the experiment I used four microbial microcosms that Joe Vallino has been working with for over two years. I introduced new species of microbes to two of the four microcosms by inoculating them with pond water containing a great diversity of microbial species.

The microcosms are closed systems with inputs of only 2.9% methane in oxygen and outputs from the system that are monitored by ongoing gas line data. By monitoring the rate of methane oxidation within the microcosms (through the gas line data) I was able to assess the rate of entropy production and how it shifted post inoculation.

Methods

Inoculation

In order to introduce new species of microbes to two of the four microcosms I inoculated them each with a 1 liter mixed sample of pond water from four different ponds in Falmouth, MA. The ponds I chose were Crooked Pond, Deep Pond, Coonamessett Pond, and Jenkins Pond. I chose these because they were all fresh water ponds with a pH around 7 which is similar the conditions within the microcosms. Since the addition of this pond water incorporated the introduction of nutrients as well, I inoculated the second two microcosms with an autoclaved sample (by heating it to a temperature that killed any living organisms within the sample) so that they would receive the same nutrients yet

none of the new microbial species. These two microcosms served as the controls for the experiment.

Sampling

Throughout the experiment I sampled the microcosms five times for bacterial and protist counts, and for the microbial DNA in order to characterize the community on the molecular level. I sampled them each once before they were inoculated, and four times afterwards. I also took a sample of the inoculum itself. The bacterial/protist samples were preserved using 1 ml of glutaraldehyde, and the DNA samples were kept frozen in a Sterivex 0.22 µm filter unit with 2 ml of a cell lysis buffer.

Bacterial and protist counts were conducted to analyze the communities, to assess which species were present, and to examine the densities of which they existed. The method used for these counts incorporated the use of 6-diamidino-2-phenylindole (DAPI) to stain cells for direct epifluorescence counting (Limnol, Oceanogr 1980). The stock solution was 1000 µg/ml which was diluted to a working solution of 200 µg/ml by adding 5 ml of the stock to 4 ml of deionized water.

In order to count the bacteria for each microcosm, 0.2 ml of the microcosm sample was diluted by the addition of 1.8 ml Phosphate buffered saline (PBS). The cells in the sample were then stained for 5 minutes using 100 µl of the DAPI working solution. After 5 minutes, the stained sample was filtered through a 0.22 µm pore size black polycarbonate filter which was placed on top of a glass fiber (Whatman GF/F) backing filter. The sample was poured into a Millipore tower and was then pulled through the filters using a hand pump (which always kept at low pressure to avoid breaking the cells). The sides of the filter tower were rinsed with about 1 ml of the PBS solution. Once everything was filtered through, the 0.22 µm pore size filter was placed on a slide with a cover slip and a drop of oil and then observed using the 100x oil-immersion objective. The average bacteria concentration in cells/ml was calculated as shown below:

$$\ell_{bacteria} = \frac{\langle n \rangle \frac{A_w}{A_g}}{V_f}$$

Where $\langle n \rangle$ is equal to the average number of bacteria in the grid, A_w equals the area of the filter, A_g equals the area of the grid, and V_f equals the volume of the sample filtered.

The protist counts were conducted in a similar way, but since protists were at a much lower concentration in the sample 8 ml of each sample were filtered through a 0.8 μm membrane filter, and the PBS was only used at the end of the filtering process to rinse the sides of the Millipore tower. I calculated the average number of cells per milliliter using the equation shown below:

$$\ell_{protist} = \frac{\langle n \rangle \frac{A_w}{A_{sweep}}}{V_f}$$

The only difference from the bacterial concentration calculation was that for the protist calculation the area of the filter was divided by the area of the section of the filter that was in the field of view as I panned across the diameter of the filter.

The DNA was extracted from the Sterivex filter unit using a Puregene Kit (Zettler). Each extraction then underwent a Polymerase Chain Reaction (PCR) in order to amplify the DNA (Head, Saunders, Pickup 1998). I analyzed the DNA in each sample using a Denaturing gradient gel electrophoresis (DGGE, 2004). The DGGE is a molecular fingerprinting method that separates the PCR generated DNA products. The DGGE gels were able to separate the different size PCR products in each sample. The gels were created by combining two solutions containing acrylamide and differing amounts of denaturants (one 60% and one 25%). This forms a gradient of denaturant which will denature the different PCR products with different sequences during the electrophoresis. By staining the gel, the different banding patterns can be observed which shows how many different sequences (and therefore species) were in each sample. Each band theoretically represents a different microbial species and by comparing banding patterns from sample to sample, the changes in species composition can be analyzed.

Results

Gas Line Data:

Immediately after the inoculation of microcosms 1 and 2 (or Treatments 1 and 2), Treatment 2 exhibited an increase in methane oxidation. This was observed through the

gas line data which showed an increase in the percentage of carbon dioxide (Fig. 1) leaving the microcosm, and a decrease in both the oxygen and methane percentages leaving the microcosm (Fig. 2, 3). Treatment 1 and Controls 1 and 2 did not show any significant changes in methane oxidation from the gas line data (Fig. 1, 2, 3).

Protist and Bacteria results:

There was one type of ciliate that was observed in all of the microcosms both before and after the inoculum (Fig. 4). It was around 175 microns long and 75 microns wide. It was also observed in the inoculum along with a few other unidentified protists (Fig. 5). One protist was observed in the inoculum and appeared in Treatment 2 only after the inoculation (Fig. 5). This protist was around 125 microns long and 100 microns wide.

Ciliate counts showed that there was no significant oscillation in ciliate numbers in any of the microcosms (Fig. 6). Both Treatment 1 and 2 appeared to decrease slightly in their ciliate concentrations, and Control 1 and 2 appeared to increase slightly.

The bacterial concentrations for the two treatments each followed a similar pattern, decreasing initially and then increasing around 5 days after the inoculation (Fig. 7). The bacterial concentrations did not seem to follow much of a pattern in the two controls, however (Fig. 7). Control 1 increased very gradually over the sampling period, while Control 2 increased from around 3×10^6 cells/ml to around 6×10^6 cells/ml within the first two weeks, but then began to decline again in the next week.

DGGE results:

There was no change in the banding pattern from the DGGE for Control 2 (Fig. 8) which was expected because there were no new species introduced to this microcosm. There was a change, however, in the banding pattern observed in the DGGE for Treatment 2 (Fig. 9). The gel showed that there was a band (or species) present at the time of the first sampling (the day before the inoculation) that was not present ever again, not even half an hour after the inoculation.

Discussion

The gas line data reacted to the inoculum very quickly in Treatment 2 (Fig. 1, 2, 3). The methane oxidation increased, as shown by the increase in carbon dioxide leaving the microcosm, and the increase in both oxygen and methane that was leaving. This increase in methane oxidation was not observed in Treatment 1, even though they both

experienced the same inoculation. The immediate change in Treatment 2 implies that the microcosm may have already been in the middle of a transition. The system appeared to be very easily altered by the addition of the inoculum in a way that Treatment 1 did not. Although all four microcosms were initially replicates of each other (when they were first constructed two years ago), they have all evolved in their own way and can no longer be considered identical, even though they all appeared to be functioning on the same level. This might explain why Treatments 1 and 2 did not react in the same way to the inoculum.

The two controls did not appear to experience much of a change in methane oxidation after the addition of the nutrients, showing that it is highly probable that the great increase in methane oxidation observed in Treatment 2 was due to a species shift as opposed to the addition of nutrients.

The ciliate counts showed that none of the microcosms experienced a big change in the number of ciliates present (Fig. 6). It was shown that the two controls increased in their ciliate numbers slightly which could be due to the addition of nutrients without the addition of new predators. Both Treatment 1 and 2 showed a slight decrease in ciliate numbers which could be explained by the fact that the nutrient addition in the two treatments was accompanied by the addition of new organisms that may have preyed upon the ciliates. These were all very slight changes, however.

The bacterial concentrations exhibited an interesting change in the two treatments (Fig. 7). The fluctuation in the bacterial concentration of Treatment 2 could be explained by the addition of the new protist to that microcosm. If that protist preyed upon a specific non-methanotrophic bacteria that was in the microcosm, that could explain the initial decrease in bacterial numbers that was observed. By consuming these bacteria, the protist may have recycled some of the nutrients that were locked up in their biomass, making them available for the methanotrophs to take up, accelerating their growth rate (this cycling within the microcosm is diagramed in Fig. 10). This could be what caused the increase in methane oxidation for Treatment 2. There was a protist that was observed in the inoculum that only appeared in Treatment 2 after the inoculation (Fig. 5). This could have been the mark of a successful invasion in Treatment 2.

In the DGGE for Control 2 the banding pattern was the same for each sample, showing that there was no species change during the time that I sampled. This is exactly what was expected because there were no new species added to the controls. The banding pattern in the DGGE for Treatment 2 was a bit more surprising. There was a band present in the sample taken before the inoculation that was not seen in any of the samples taken after the inoculation (not even the sample taken only half an hour after). This implies that Treatment 2 might have already been undergoing some sort of shift when I inoculated it. It is surprising that it would be able to react as quickly as it did otherwise. If this was the case, it would explain why Treatment 2 responded to the inoculum while Treatment 1 remained unaffected.

Conclusion

Analyzing how these closed microbial systems altered their rate of methane oxidation in response to the addition of new species was a valuable way of looking at how the MEP theory might apply to biological systems and the introduction of a new species. It was shown in the results that there was an increase in methane oxidation that accompanied a change in species composition in Treatment 2. It was also clear that there was a new protist introduced to Treatment 2. This supports the MEP theory in the sense that a change in species composition for Treatment 2 did in fact result in an increase in methane oxidation. The theory predicts that it is more probable that the system will undergo a change that increases the entropy production, which is exactly what happened in Treatment 2.

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Figure 1. Change in the percentage of methane leaving the microcosms over time.

Black= Treatment 1
Blue= Treatment 2
Red= Control 1
Green= Control 2

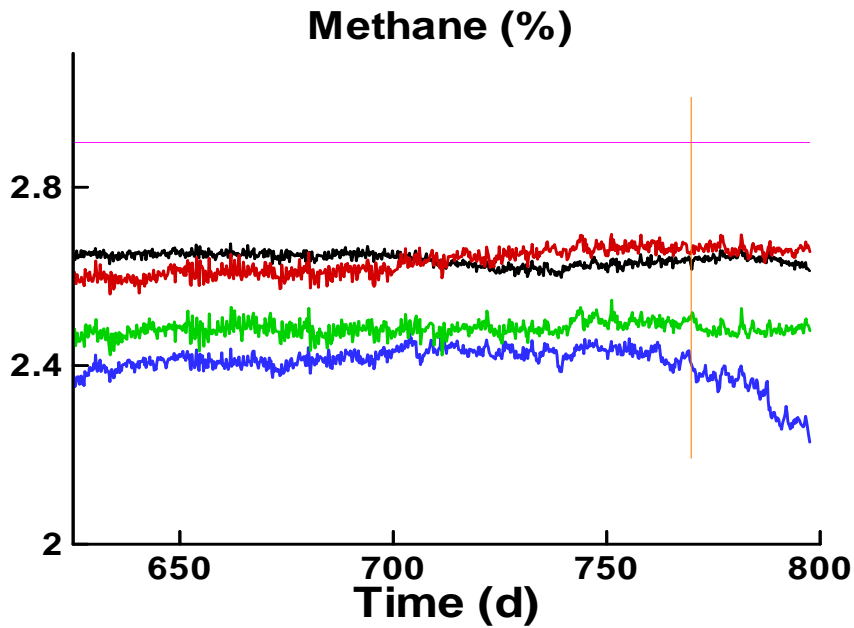


Figure 2. Change in the percentage of oxygen leaving the microcosms over time.

Black= Treatment 1
Blue= Treatment 2
Red= Control 1
Green= Control 2

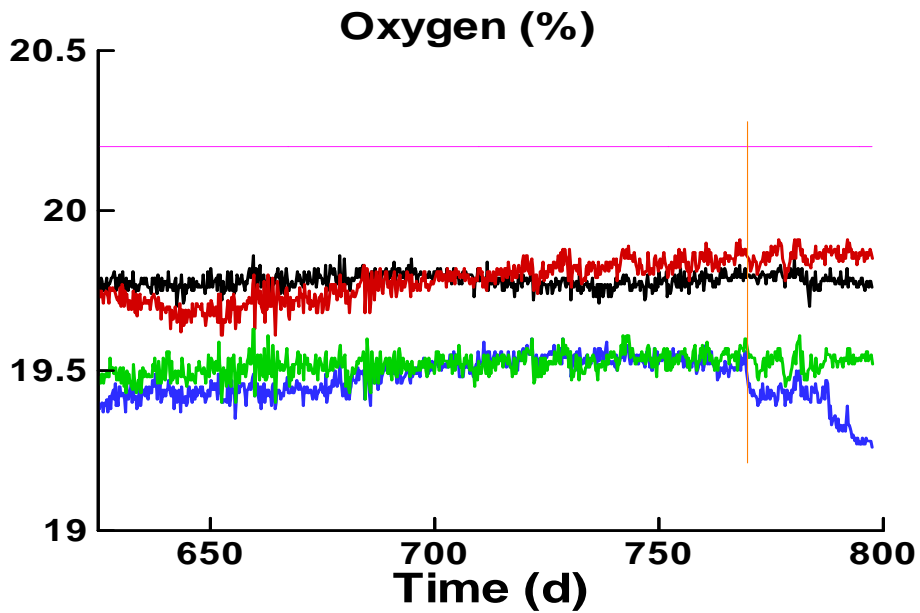


Figure 3. Change in percentage of carbon dioxide leaving the microcosms over time.

Black= Treatment 1

Blue= Treatment 2

Red= Control 1

Green= Control 2

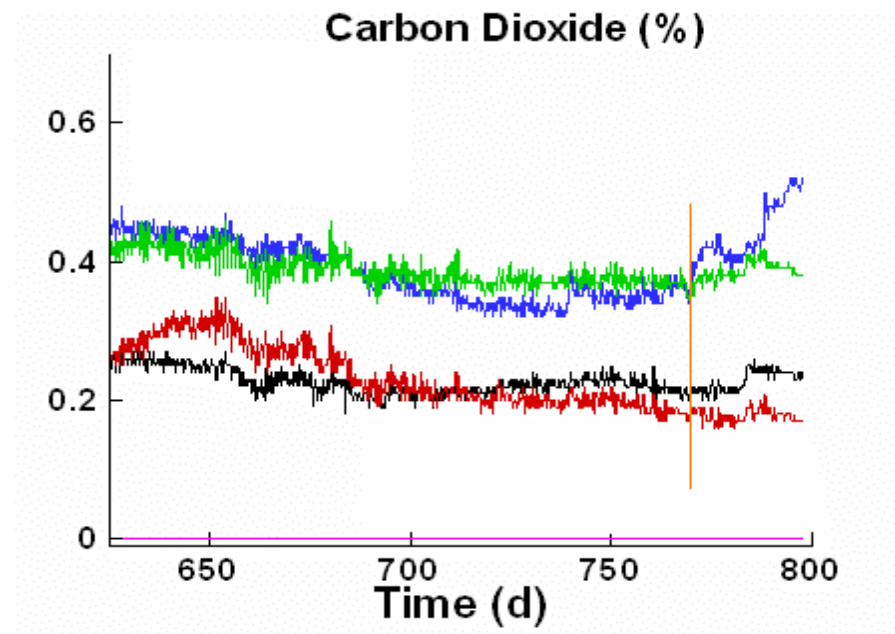


Figure 4. Common ciliate seen in each microcosm and in the inoculum.



Figure 5. Protists seen in the inoculum vs. protists seen in the four different microcosms.



Figure 6. Ciliate concentrations in the four microcosms over time.

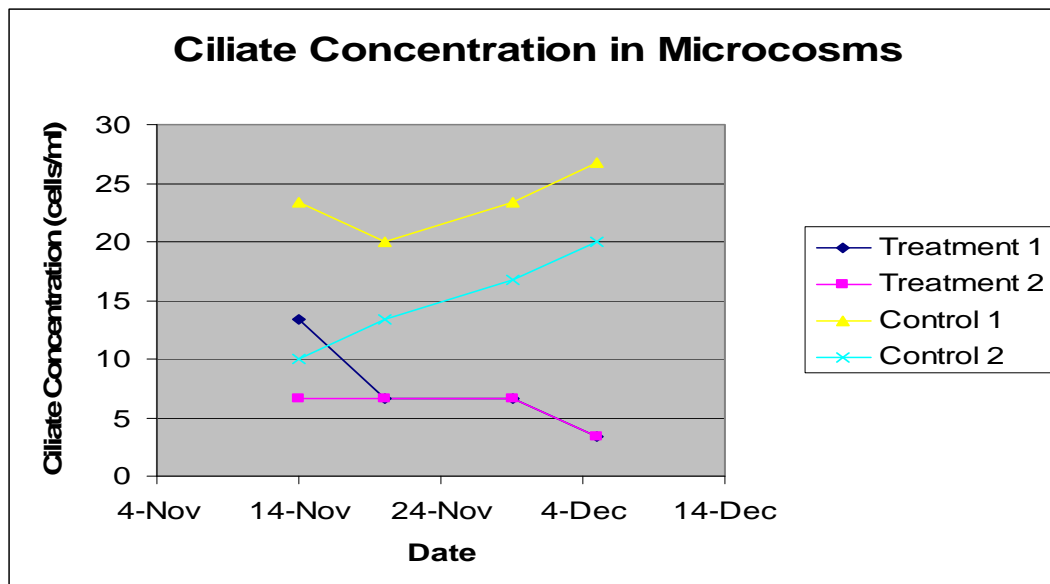


Figure 7. Bacterial concentrations in the four microcosms over time.

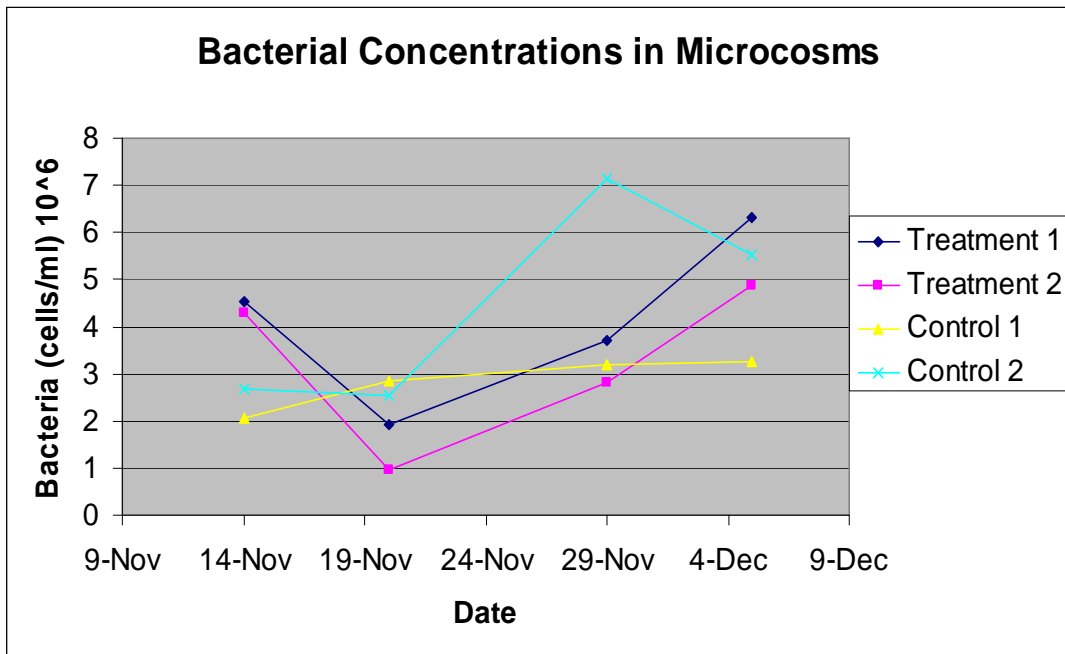


Figure 8. DGGE for Control 2.

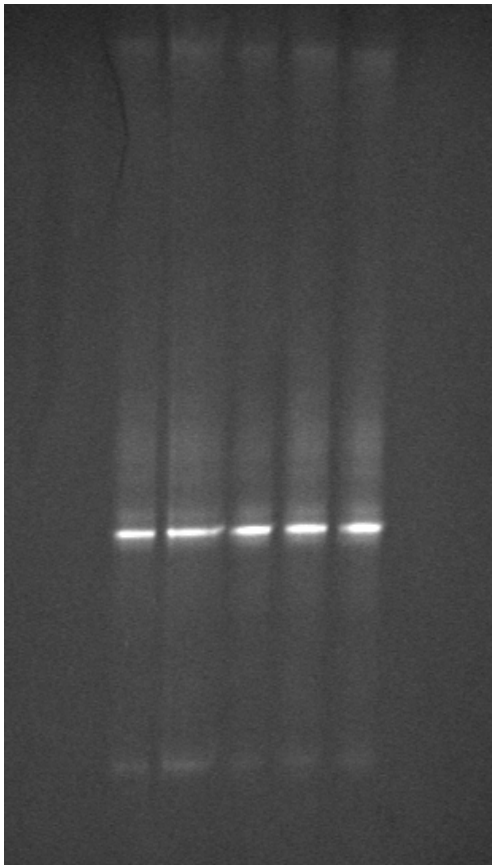


Figure 9. DGGE for Treatment 2.

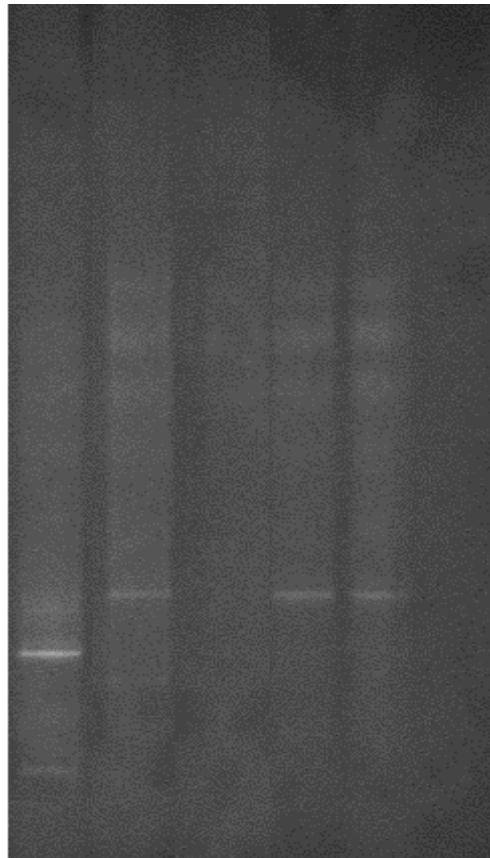


Figure 10. Diagram of the methanotrophic microbial system within the four microcosms.

