Methanotrophy and the Environment

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Abstract

Methane is a powerful greenhouse gas that is essential to understanding climate change and its feedbacks. Soil microbial methane oxidation is a significant methane sink, yet many questions about this process remain unanswered, especially those that address environmental effects on methanotrophy. For my short project I attempted to use molecular methods to analyze methanotrophy in the Trunk River, an environment very different from others studied with these techniques. In addition, I attempted to test inhibition and stimulation of methane oxidation due to carbon dioxide levels, and acetate and ammonium additions. Unfortunately, due to time constraints, unsuccessful DNA extraction, and the slow growth rate of methanotrophs, I was unable to obtain any conclusive data during my time at Woods Hole.

Introduction

Understanding changes in methane oxidation is critical to understanding climate change and its feedbacks. Methane (CH$_4$) is a powerful greenhouse gas, 20 to 30 times more effective than CO$_2$ on a per molecule basis (le Mer and Roger 2001), that has increased by a factor of 2.5 since the industrial revolution (Whalen 2005). Soil microbial methane oxidation is a significant methane sink with removal of methane by hydroxyl radicals being the largest methane sink. Despite concerns about global change and the clear implications of soil microbial methane oxidation, how this process functions in the environment and how it is both influenced by and responds to feedbacks from global and land use changes are still poorly understood.

Although methane oxidation has been studied for 50 years now, modern molecular techniques have only been used extensively in few ecosystems, mainly rice paddies, freshwater systems with standing water, forests, and grasslands. These techniques take advantage of the pmoA gene which encodes particulate methane monooxygenase (MMO), a functional gene present in all known methanotrophs except Methylocella spp. (Dumont and Murrell, in press). As more of these studies are performed, novel gene sequences and novel methanotrophs are being discovered.

Upon visiting the Trunk River, I noticed that this was a very different ecosystem, oscillating between a freshwater and saltwater environment with changes in tide and inputs from rivers. In addition, large amounts of organic matter were present and methanogenesis appeared to be occurring at high rates. Bubbles which appeared to allow methane gas to escape were evident. Knowing that in rice paddies and other wetland ecosystems (which have high rates of methanogenesis), most of the methane produced is consumed by methanotrophs while often more than 80% of the emitted methane escapes through plants, aerenchyma and otherwise (King 1996), I determined the Trunk River would be an excellent environment for methanotrophs yet unlike any others described in the literature I had read.

I decided to enrich for methanotrophs using two types of media--freshwater and saltwater, and to make a clone library of the pmoA gene from the DNA I would extract. Having just learned to use the software program Arb, I planned to align the sequences and compare the pmoA sequences from my clone library with those available from GenBank (over 1000).

In addition to my queries about how the environment of the Trunk River affects methanotrophy, I am interested in how the environmental changes associated with global
change affect rates of methane oxidation land use and climate changes. A recent study by Horz et al. (2005) examined the diversity of methane oxidizers and the pmoA genes in a field study with independent variables related to climate change, (elevated CO₂, temperature, precipitation, nitrogen deposition), but rates of methane oxidation were never quantified.

Due to the time constraints of this project, I decided to test methane consumption using pure cultures of methanotrophs with only a few variables, varying CO₂ concentrations, acetate additions, and ammonium additions. Because methanotrophs fall into two broad categories, Type I and Type II, with different carbon assimilation pathways, the ribulose monophosphate (RuMP) pathway and the serine pathway, I tested a pure culture of each. The serine pathway does require carbon dioxide for carbon assimilation in addition to methane, but the RuMP pathway does not. Because carbon dioxide is also an end product for methanotrophs, thermodynamics may cause high concentrations of CO₂ to inhibit methanotrophy. Research has shown that elevated CO₂ can increase rates of methanotrophy, but I wanted to see at what point that stopped and if, at high concentrations, carbon dioxide would inhibit methanotrophy. Based on the differences in carbon assimilation pathway, I think testing both Type I and Type II methanotrophs separately for the effect of high concentration CO₂ is essential.

I chose to look at the effects of acetate additions as a proxy for increased carbon availability. With rises in atmospheric levels of CO₂, plants allocate resources differently, increasing root exudates and carbon availability in the soil. Although methanotrophs are autotrophs, no studies have shown that they are not mixotrophs, and if they can use acetate, they should be able to grow faster in its presence as it should cost them less energy than fixing their own carbon.

The rationale for performing an ammonium additions experiment was not to perform novel research on the effects of fertilization, but rather as a control because in previous studies ammonium has been shown to inhibit methane oxidation. The inhibitory effect is easily explained by the similarity between MMO and ammonia monooxygenase (AMO), which is essential to nitrification. Laboratory studies show that MMO can perform the function of AMO in high NH₄⁺ environments.

Methods

*Trunk River Methanotroph Community Analysis*

Sample Collection

The soil used for the enrichments was collected into 50 ml sterile plastic tubes from the Trunk River in Falmouth, MA. I collected soil from methane bubbles on the sand bar during low tide by inverting the tube and pressing while twisting the tube down into the sand with the methane bubbles in the center. Most of these bubbles were evident in the beige sand, but I did also collect from a methane bubble coming through sand with very high amounts of organic material (black but still sandy).

Enrichments

Because the Trunk River oscillates between salt water and freshwater depending on tides and river inputs, I decided to make enrichments using two types of media, salt water and freshwater, although a medium in between the two would also have been interesting.
Saltwater Medium (500 ml)
1 L Saltwater Base
2.5 ml 1M MOPS Buffer (pH 6.8)
2.5 ml .2M K$_3$PO$_4$
2.5 ml 1M NaNO$_3$
125 ul 1M NaSO$_4$
125 uL of 12 vitamin solution
125 ul vitamin B$_{12}$ solution
500 ul 1M H$_2$CO$_3$
500 ul EDTA-Chelated Trace Elements Solution

Freshwater Medium (500 ml)
5 ml Freshwater Base
2.5 ml 1M MOPS Buffer (pH 6.8)
2.5 ml .2M K$_3$PO$_4$
2.5 ml 1M NaNO$_3$
125 ul 1M NaSO$_4$
125 uL of 12 vitamin solution
125 ul vitamin B$_{12}$ solution
500 ul 1M H$_2$CO$_3$
500 ul EDTA-Chelated Trace Elements Solution
485 ml Sterile Deionized Distilled water

To set up the enrichments, I used serum vials with stoppers in order to manipulate the gas concentrations of the headspace. I added 30 ml of each medium to 7 120 ml serum vials, stopped with a blue mushroom cap and crimp top, and autoclaved for 35 minutes. When cool, I removed the crimp and stopper to add approximately 5 g of soil to 5 saltwater medium and 5 freshwater medium serum vials. After stopping and crimping once again, I vacuumed and gassed the vials (using a sterile filter) with gas containing 5% CO$_2$, 21% O$_2$, and 74% N$_2$ several times, ending with a final pressure of about 1.7 atm. Then, I removed 60 ml with a syringe and added 30 ml of methane (99.97%) using the syringe and a sterile filter.

For incubating the enrichments, I put some bottles on a shaker table at 27.5°C; however, due to space availability, others incubated on my bench top. Growth was apparent after 5 days of incubation. Biofilms were especially visible on the vials that had been left on the bench top but the solutions were less turbid than those of the vials that had been shaking. Turbidity in the shaking vials became evident 1 week after incubation (2 days after the biofilms appeared).

Due to time constraints for clone libraries, I extracted DNA from the vials 5 days after initial incubation, when I first noticed biofilms. I used the shaking cultures and scraped any biofilms I could see off of the sides of the vials and into solution. Using the Ultraclean Soil DNA kit from MoBio with the instructions from the course (bead beating instead of using the vortex adapter), I extracted DNA from 1 ml of 4 different enrichments, 2 saltwater and 2 freshwater.

My next step was performing a polymerase chain reaction (PCR) using primers specific to the gene for particulate methane monooxygenase. I used the most inclusive
primer set for pmoA to find the highest diversity of methanotrophs possible. Unavoidably, these primers are not inclusive of every known methanotroph; *Methylocella* does not have the pmoA gene. In addition, in the trade off between specificity and inclusiveness, the primers I chose will also amplify amoA, the gene encoding ammonia monooxygenase in nitrifying bacteria.

Primers: A189 5’ –GGNGACTGGGACTTCTGG—3’
A682 3’ –GAASGCNGAGAAGAASG—3’

To run the PCR, my initial denaturing step was 94°C for 5 minutes. Then I ran 30 cycles of 94°C for 1 minute, 56°C for 1 minute, and 74°C for 1 minute. For the final extension, I set the program to 74°C for 5 minutes (Dumont and Murrell, in press).

With successful PCR product, I wanted to clone the pmoA genes, sequence them, and analyze the communities in the different enrichments with each other and with those found in previous research. I was going to adjust the sequences in ARB and compare them to the large number of pmoA sequences previously submitted to GenBank. I wanted to compare clone libraries of pmoA from the different enrichments with previous research and with each other, saltwater with saltwater, freshwater with freshwater, and saltwater with freshwater using LibShuff. This never happened (see Results).

**Environmental Effects on Methanotrophy**

Using pure cultures of Type I (*Methylomonas methanica* S1) and Type II (*Methylocystis parvus* OBBP) methanotrophs given to me by Colleen Cavanaugh, I tested inhibition and stimulation of methanotrophy due to environmental differences by measuring methane consumption. I added 10 ml of the freshwater medium described earlier to 27 ml Balch tubes, stopped and crimped them, and autoclaved the tubes. I vacuumed and gassed each tube using a sterile filter with gas containing 5% CO₂, 21% O₂, and 74% N₂ 11 times, ending with a final pressure of about 1 atm. Then, I added 2 ml of methane (99.97%) each tube using a sterile filter and a Hamilton syringe. Each treatment then received different additions (using a sterile filter) as shown in the table below:

<table>
<thead>
<tr>
<th>Treatment – 2 tubes of each</th>
<th>Additions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Highest carbon dioxide</td>
<td>20 ml of 80% N₂ / 20% CO₂</td>
</tr>
<tr>
<td>High carbon dioxide</td>
<td>10 ml of 80% N₂ / 20% CO₂, 10 ml N₂</td>
</tr>
<tr>
<td>Elevated carbon dioxide (5% of air mix)</td>
<td>20 ml N₂</td>
</tr>
<tr>
<td>Acetate addition</td>
<td>NaCH₃COOH to total 10mM</td>
</tr>
<tr>
<td>Ammonium addition</td>
<td>NH₄Cl to total 10mM</td>
</tr>
<tr>
<td>Control for both acetate and ammonium additions</td>
<td>NaCl to total 10mM</td>
</tr>
</tbody>
</table>

For each treatment, one tube was inoculated with 1 ml of a pure culture cell suspension of *Methylomonas methanica* S1 and the other with 1ml of *Methylocystis parvus* OBBP. The tubes were put in a wire basket lying flat and the basket placed on a shaker table in an incubator at 28°C.

Methane measurements of the headspace gas were taken at the start of the experiment, after 9, 32, and 64 hours using a locking Hamilton syringe and run on a
Shimadzu GC-14A gas chromatograph. I developed a standard curve in advance and used it to determine the number of moles of methane that correlated to the units of the GC. I was then able to calculate the number of moles of methane in the tube.

**Results**

*Trunk River Methanotroph Community Analysis*

Following two unsuccessful PCR reactions, one that was contaminated (source turned out to be the tubes) and another that showed no product, I was unable to clone in time for sequencing, concluding this project for the time being.

*Environmental Effects on Methanotrophy*

Unfortunately, in the time the experiment ran, the variability in measurement error exceeded any changes in methane concentration measured. Graphs 1 and 2 display data collected adjusted to moles CH$_4$ / L over time.

**Discussion**

*Trunk River Methanotroph Community Analysis*

The likely reason why my second PCR was unsuccessful was that the DNA extraction was either unsuccessful or too dilute. At the time of DNA extraction, my enrichments were still not very turbid; methanotrophs were just beginning to grow. I did run my DNA on the first gel, but it seemed to float away. Even if the ethanol (floating DNA) were not the problem, the DNA could have been too dilute to see a band.

In the future, I encourage students to repeat this experiment; the methane is already here (rather than having to wait until half way through the independent projects to start the enrichments). I definitely feel making a clone library from the Trunk River using enrichments is feasible and intriguing. Using soil from the river is also interesting; I attempted to do this as well, but I had the same problems with my DNA extractions and PCR and found out too late to make a clone library while here.

*Environmental Effects on Methanotrophy*

The variability due to measurement error when measuring the methane in the headspace of the tubes on the gas chromatograph (GC) necessitated significant methane consumption for a rate to be calculated. Because of the slow growth rate of the organisms and the delay in obtaining methane, I started the experiment with cultures that were not very dense and hoped the ones I expected to be stimulated by the experiment would grow fast enough for me to see methane consumption in those tubes. This did not happen. In the future, I would give the methanotrophs more time to grow before starting the experiment, or run the experiment much longer (2-3 weeks) to be able to confidently measure methane consumption.

**Conclusions**

Methanotrophs grow slowly. They also consume methane slowly. Few methanotrophs consume methane very slowly.

Future work on the methanotrophs in Trunk River and on how methanotrophy will change with global change is essential to understanding how our environment functions.
Future Work

I plan to take my enrichments back to my laboratory in Berkeley, CA, and extract the DNA again. I want to make a clone library of the pmoA genes recovered and compare the sequences to what has been found before.

Acknowledgements

I want to give special thanks to Tom Schmidt for patiently discussing my project with me many times as it transformed. Also, Bill Metcalf and Jared Leadbetter have been great about answering my many questions while providing me with different perspectives and approaches (as well as vast knowledge). In addition, Colleen Cavanaugh gave me pure cultures of methanotrophs and was very generous with her time and knowledge, talking to me for hours about methanotrophy. Adam Guss has helped me numerous times with what I am actually doing and how in addition to assisting me a dozen times with the GC, discussing ideas for future improvement of a project like this, and explaining biochemistry to me. Jeanne Poindexter is an amazing and inspiring woman with an immense amount of knowledge who has encouraged and guided me. I want to thank Colin Murrell for discussions about methane oxidation, and all of our speakers for inspiring conversation. Also, all of the teaching assistants, our amazing course coordinator, and enthusiastic course assistant have all been very helpful and giving of themselves. And, of course, all of the other students in the course have been helpful with bouncing ideas around, encouraging, and great fun.

Many people have helped me with this project, but I would never even be here if it were not for financial support from the Planetary Biology Internship Scholarship, the Marine Biological Laboratory, the College of Natural Resources and the Department of Environmental Science, Policy and Management at the University of California, Berkeley, and Mary Firestone. In addition, I would not be here without the encouragement I received from Mary Firestone, Don Herman, Kristen DeAngelis, Eric Dubinsky, Christine Hawkes, Damon Bradbury, Jennifer Pett-Ridge, and my family to pursue my interests and to apply to the Microbial Diversity program. Also, I want to thank whoever decided to accept me into the program, giving me this wonderful opportunity to be immersed in microbiology. This has been an amazing growth experience for me, scientifically and otherwise, expanding my horizons and showing me a new lens through which to look at my research and the world.

References


Figures

**Methane Calibration Curve**

![Methane Calibration Curve](image)

Figure 1. Calibration curve used to correlate peak area output given by the GC with actual methane concentration.
Figure 2. Methane concentration under headspace with varying amounts of carbon dioxide. The initial values should be $4.7 \times 10^{-5}$ mol/L. The change in methane concentration is consistently less than measurement error.
Figure 3. Methane concentration over time for ammonium and acetate additions and control. The initial values should be $4.7 \times 10^{-5}$ mol/L. The change in methane concentration is consistently less than measurement error.
Enrichments

Figure 4. Freshwater enrichments (A) and a microscope image taken from contents (B).

Figure 5. Saltwater enrichments (A) and microscope photographs taken from contents (B and C). Biofilms were present along the sides. At one point I scraped the biofilm into the solution, removed a sample and examined. Image 5C is likely from the biofilm.
Pure Cultures

Figure 6. Microscope images of *Methylomonas methanica* S1, the Type I methanotroph used in the second experiment. Figure 6A is the *M. methanica* growing on a Petri dish while 6B is from inoculated tubes.

Figure 7. Microscope images of *Methylocystis parvus* OBBP, the Type II methanotroph used in the second experiment. Figure 7A is the *M. parvus* growing on a Petri dish while 7B is from inoculated tubes.