

Protocol for Moisture Temperature Response Surface Incubations of Great Plains Soils

Updated 5 September 2012

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1 Overview of Moisture Temperature Response Surface (MTRS)

1.1 Significance of Methane and Soil

1.1.1 Methane is a potent greenhouse gas that plays an important role in global climate change. Soils can affect large scale methane flux in the environment due to their ability to act as methane sinks or sources (depending on the microbial activity in the soil). Methane flux is determined by the change in methane concentration over time and is affected by methane production of methanogenic bacteria and by methane consumption of methanotrophic bacteria. It is possible to quantify methane flux of a given soil by measuring the change in methane concentration over time, however this does not account for the individual components of flux: simultaneous production and consumption. The technique of Isotope Pool Dilution allows for both production and consumption of methane to be determined based on additions of isotopically labeled $^{13}\text{CH}_4$. In ambient air methane exists at approximately 1.9 ppm, and approximately 1% of methane in natural abundance exists as $^{13}\text{CH}_4$, leaving $^{12}\text{CH}_4$ as the remaining 99% of ambient methane. The rate of methane consumption can be determined by increasing the concentration of $^{13}\text{CH}_4$ and observing the rate of $^{13}\text{CH}_4$ disappearance in the headspace of a sealed container of soil. If total methane concentration over time is also measured, then the rate of methane production can be determined by difference using the total methane concentration minus the methane consumption.

1.2 Significance of Moisture Temperature Response Surface (MTRS)

1.2.1 The Moisture Temperature Response Surface seeks to determine methane production and consumption in combinations of environmental conditions for each field site by combining five (5) moisture levels with five (5) temperature levels in a matrix design, making 25 combinations in total. See Table 1 of Appendix A. The full MTRS for a field site requires two incubations in total due to available resources and space. The incubations are set up as displayed in Table 2. A suggested schedule for a MTRS incubation can be found in Table 3 of Appendix A.

2 Preparing Soil Samples

2.1 Reasoning

2.1.1 Soil samples are kept in the von Fischer lab refrigerators to keep microbial activity and chemical processes to a minimum. Microbes require a 24 hour period of time for “pre-incubation” to reach their optimal activity prior to incubation. It is important to plan when the Methane Carbon Isotope Analyzer (MCIA) is available for an extended period of time (between 24 and 72 hours of continuous availability, depending on the field site incubation time) and to work backward to schedule when the soil pre-incubation period should begin. The purpose of this section is to describe

the steps necessary to calculate the moisture and temperature levels and explain the pre-incubation process for soil samples in a MTRS incubation.

2.2 Materials and Methods

2.2.1 Materials

- Large soil sample bags from Great Plains Methane field sites
- GPM Temperature Moisture Response Surface Soil Prep Sheet 2012.docx
- 2 mm sieve
- g-sensitive scale
- Gravimetric tins
- Drying oven
- Spray bottle of deionized (DI) water
- Gravimetric Water Content (GWC) and Volumetric Water Content (VWC) regression for the desired field site
- SurfaceAverageVWC data sheet
- SurfaceAverageTemp data sheet
- Excel template for incubation
- Clean and dry 10" x 15" jelly roll pans
- Clean and dry metal fork
- Calibrated incubators in Antolin Lab (Anat/Zool Room E212)
- Plastic bag
- Clips

2.2.2 Determining Gravimetric Water Content (GWC) of soil

1. Sieve and homogenize large bags of soil.
2. It is important to start the GWC of fresh soil approximately 48 hours before the desired start of pre-incubation so that soils can sit out overnight before pre-incubation if water evaporation is necessary.
3. On the von Fischer lab network drive (the "J drive") go to J drive>Great Plains Methane>MCIA testing>2012 Temperature Moisture Response>MTRS Templates and print the Word document "GPM Temperature Moisture Response Surface Soil Prep Sheet 2012.docx." Fill in the field site information.
4. Weigh 50.00 g within 0.1g for the gross weight of soil + gravimetric tin and record value in "Gross wet weight soil" column on the top half of the Soil Prep Sheet under the heading "Soil Gravimetrics Before Incubation."
5. Place gravimetric tin with soil in drying oven at 105°C for 24 hours.
6. After 24 hours of oven drying, record the gross weight (tin+soil) in "Gross dry weight" column of Soil Prep Sheet.
7. On the von Fischer lab network drive go to J drive>Great Plains Methane>MCIA testing>2012 Temperature Moisture Response and create a folder with the field site name and date the soil was sampled (e.g. SVB 6.25.12 for Sevilleta B soil that was sampled on June 25, 2012).

8. Within the folder, make a folder for the T1M1-Control1 incubation and one for the T4M3-Control3 incubation for that field site with the date soil was sampled.
9. On the von Fischer lab drive go to J drive>Great Plains Methane>MCIA testing>2012 Temperature Moisture Response>MTRS Templates open the proper Excel template for MTRS incubation (either T1M1-Control1 or T4M3-Control3) and Save As the field site name and date soil was sampled in front of the incubation ID; save the newly named template in the new folder for that field site and for that incubation. Use this newly named template to enter all data from the incubation.
10. In the “Gravimetrics” tab of the Excel template enter data from the Soil Prep Sheet.

2.2.3 Determining 100% soil saturation

1. Sieve and homogenize soil old soil cores to be saturated. Determine the gravimetric water content with same process as section 2.2.2 instructions #4-6, then record data on the bottom half of the Soil Prep Sheet under the heading “Soil Gravimetrics Before Saturation.”
2. Tare a g-sensitive scale to jelly roll tray.
3. Weigh 400 g fresh soil onto jelly roll tray. Record soil weight in table under “Soil Saturation Data” heading of Soil Prep Sheet.
4. Tare scale with tray and soil.
5. Use DI spray bottle to spray DI water onto soil. At first the soil will readily accept water, but as soil approaches 100% saturation, sprays should be administered more slowly. This ensures the water will fully saturate soil, but not pool. See Figure 1.
6. When soil begins to glisten (NO pooling water), 100% saturation is achieved.
7. A good check that the soil is not over-saturated is to hold the tray of soil vertically and ensure there is no water pooling in the corners of the tray and that no water runs from the soil.
8. Record weight of water in “Soil Saturation Data” table of the Soil Prep Sheet. These data will aid in avoiding over-wetting of soils and give a reasonable number for water holding capacity of sieved soil from the field site.
9. Enter data into “100% Saturation” tab in Excel template.
10. Saturation data need only be quantified once for each plot.



Figure 1. Soil to be 100% saturated with DI water.

2.2.4 Calculating Moisture Levels

1. Locate the black 1.5" MCIA binder that contains the "SurfaceAverageVWC" data, "SurfaceAverageTemp" data, and Gravimetric Water Content (GWC) & Volumetric Water Content (VWC) Regressions for each field site.
2. On the SurfaceAverageVWC sheet under "Quantiles" use the 97.5% VWC value for the Level 5 moisture level, and use the 2.5% VWC value for the Level 1 moisture level. It is desired to have three (3) moisture levels between the high (level 5) and low (level 1). See Table 4 of Appendix A.
3. Volumetric water levels will be used to calculate the desired Gravimetric Water Content (GWC) levels for incubation. Use the regression equation specific to each field site in the MCIA notebook to relate VWC to GWC. In the regressions, GWC is on the y-axis and VWC is on the x-axis. See Table 5 of Appendix A for example.
4. The Excel template can be used to enter the high and low VWC levels in the corresponding yellow cells of the "VWC and Grav" tab of the Excel template. The regression formula can be entered in the orange cells on the "VWC and Grav" tab of the Excel template. Check several of the calculated values manually to ensure the formulas have been entered correctly.
5. The Excel template should also calculate the change in g of water from the GWC of fresh soil to the desired level for incubation in the blue cells on the "VWC and Grav" tab of the Excel template. **If the desired GWC for the lower moisture levels lies below the GWC of the fresh soil, then the proper mass of fresh soil must be air-dried.**
6. To mass out soil samples in order to air-dry overnight on lab counter, refer to section 2.2.6 and use instructions #4-9.

2.2.5 Determining Temperature Levels

1. Temperature levels will be determined using the same calculation method as the VWC levels by using the "AverageSurfaceTemp" data sheet for the desired field site.
2. Under the Quantile heading, use the 97.5% temperature value as the Level 5 temperature and use the 2.5% temperature value as the Level 1 temperature. Divide the difference in Level 1 and Level 5 temperatures by 4 to determine the increase in degrees per temperature level. Determine temperature Levels 2, 3 & 4 (same mathematical process at determining VWC levels).
3. Set the Percival incubators in the Antolin lab (Anatomy/Zoology room E212- the key to the Antolin lab lives on a yellow star-shaped key chain in the drawer under the molecular bench in the von Fischer lab) to the proper temperatures. The incubators are pictured in Figure 2.



Figure 2. Incubators in the Antolin lab.

4. It is a good idea to calibrate the incubators before each incubation by permanently placing a beaker of water with a glass thermometer in each incubator to verify the digital reading on the outside of the incubator matches the reading on the thermometer. Figure 3 shows the beaker of water inside the incubator.
5. To calibrate the incubator, first adjust the digital number on outside of the incubator with a Philip's head screwdriver to match the temperature of the glass thermometer.
6. Loosen the temperature setting knob with a flat-head screwdriver and turn the knob to point at the correct inside temperature, then tighten the knob to that position.
7. Record temperature levels in the Excel template on the "Pre-Incubation data" tab.



Figure 3. Glass thermometers are used to verify the inside temperature of each incubator.

2.2.6 Starting Pre-Incubation

1. Print the tab "Pre-Incubation Data" from the Excel template.
2. Use the role of plastic in the von Fischer lab to cut a ~22" section of plastic for soil sample and use the impulse sealer to heat seal one end of the section of plastic (set the knob to ~3) to create a bag for pre-incubation.
3. Take out the large bags of soil from the JvF fridges.
4. The jelly roll trays are numbered and on the "Pre-Incubation Data" tab of the Excel template the tray number that belongs to each moisture-temperature combination is listed.
5. Tare a g-sensitive scale to the jelly roll tray and record the tray number on the Soil Prep Sheet.
6. Wearing gloves, weigh out the desired g of fresh soil. Write the actual weight of the soil next to its corresponding tray number in the "Fresh soil weight (g)" column of the Pre-Incubation data sheet.
7. Refer to the column "Desired gross weight for pre-incubation" column to determine the target weight of each tray of soil for pre-incubation.
8. Tare the scale with nothing on it.
9. Place the tray with soil on the scale and slowly spray DI water onto the soil. Mix in the water additions with a clean metal fork. Add sprays of water until the weight reaches the value in the "Desired gross mass for pre-incubation" column.
10. Record the actual gross weight (tray+soil+water) in the column "Gross mass before pre-incubation."
11. Set a gray aerated micropipette tip box in the center of the soil sample and insert the tray of soil into the plastic bag.
12. Clip the open end of the bag closed so water will not evaporate from the soil, but do not seal the bag. It is desired that methane can diffuse into the bag during pre-incubation.
13. Set the bagged soil tray into the proper incubator to begin pre-incubation (the Incubation ID will tell you which temperature level it belongs to- e.g. T4M3 should go in the T4 incubator).

14. Record the time that pre-incubation starts.
15. Return to the soils at least once (ideally halfway through the pre-incubation period) during pre-incubation to refresh the air in the bags so that the methanotrophs do not become starved of methane. Refresh the air by removing the clips and wafting new air in the bag, then clipping the bag shut once again.

3 Creating Gas Dilutions and Gas Bags

3.1 Reasoning

3.1.1 The Moisture Temperature Response Surface requires different gas concentrations of CH₄ for the standard and soil samples. To make specific dilute CH₄ concentrations from high concentrations of both ¹²CH₄ and ¹³CH₄ it is necessary to make step-wise dilutions in gas-tight bags. The purpose of this section is to outline the various gas dilutions used in the MTRS incubation and explain how to make gas-tight bags in which dilutions may be held.

3.2 Materials and Methods:

3.2.1 Materials

- 99 atom% ¹³CH₄
- Compressed Breathing Air gas cylinder
- 1% CH₄ gas cylinder
- SF₆ tank
- Wrenches
- Syringes
- 5 gallon gas tank
- LDPE tubing
- Snoop
- Large mylar bag for Enriched Breathing Standard
- Small mylar bags for Controls
- Metal 5/16 hole punch
- Cardboard backing
- O-rings
- ¼" brass Swagelok bulkhead fittings
- Impulse heat sealer
- Conventional iron
- Orange insulated gloves
- Evacuator
- Flow meter
- Timer

3.2.2 Preparing the 5 gallon gas tank

1. Ensure the pressure has been released from the Yellow 5 gallon gas tank. Wait a few minutes after there is no hissing sound to be sure there is no remaining positive pressure in tank.
2. To ensure tank fittings do not leak, do a pressure check on the tank fittings by pressurizing the tank to ~10psi, then pouring a small amount of Snoop (located in 2nd blue cupboard in GC room) on each fitting to check for leaks at every junction. Leakage will be apparent by Snoop forming continuous bubbles at the fittings. If continuous bubbles appear, tighten that fitting.
3. Release all tank pressure as described in step 1.
4. Evacuate the 5 gallon tank down to ~500mTorr (or lower). This will take 45-60 minutes to do. Before removing the tank from the evacuation manifold, make sure all valves are CLOSED to ensure tank remains sealed and evacuated as it is removed from the evacuator.
5. Prepare the needed amount of labeled $^{13}\text{CH}_4$ (see Table 6 of Appendix A in Excel), connect syringe to the evacuated tank, and let the negative pressure of the tank pull it in. Close the tank fitting after the label injection.
6. Prepare the needed amount of methane from the 1% CH_4 gas cylinder (see Table 6 of Appendix A in Excel).
7. Connect the yellow 5 gallon tank to the pressurized large gas cylinder that will fill the Yellow 5 gallon tank to the correct pressure with LDPE tubing in the GC room. Make sure the connections are tightened with wrenches. DO NOT USE TYGON TUBING.
8. Pressurize the 5 gallon tank with the desired gas (from one of the grey standard cylinders in the GC room). Increase the pressure in increments of 5psi and wait until you cannot hear the gas entering the 5 gallon tank before increasing the pressure (when gas flow is no longer audible the 5 gallon tank is at pressure equilibrium with the pressure of the regulator of the large gas cylinder). Using Snoop at the connections between the 5 gallon tank and the gas cylinder would be a good idea to make sure no more leaks become apparent at higher pressures.
9. Once you have reached your target pressure (usually between 80 - 100 psi) let the tank fully equilibrate for a couple of minutes before closing ALL the valves of the 5 gallon tank.
10. Disconnect the LDPE tubing from the regulator of the large gas cylinder. There will probably be a popping sound. Decrease the pressure on the regulator and then release the gas still in the system. This is both out of courtesy and to prevent accidental de-pressurization events.

3.2.3 Making a small gas-tight mylar bag with a fitting

1. In order to make a gas-tight mylar bag with a fitting, first locate the desired size of mylar bag.
2. Open the mylar bag and insert a piece of cardboard as backing when making a hole for the fitting. See Figure 4A.
3. Take the 5/16 metal punch and create a hole in the mylar material against the cardboard backing. See Figure 4B.
4. Gently remove the mylar circle cut by the hole punch if necessary. Be sure not to rip the mylar around the hole when detaching the circle cut by the punch. See Figure 4C.

5. Adhere several 4" pieces of masking tape on the insides of the non-punched wall of the mylar bag (it is not necessary to put tape on the inside of mylar bags intended to incubate soil samples). This will protect the mylar bag from being damaged by the fitting on the other wall. See Figure 4C.
6. Take a ¼" brass Swagelok bulkhead fitting and slide one O-ring against the fixed hexagonal nut. See Figure 4D.
7. Insert the bulkhead fitting into the mylar bag so that the O-ring sits snugly to the outside of the mylar bag.
8. Slide a second O-ring onto the bulkhead fitting against the inside of the mylar bag. This will create a seal with the mylar material between two O-rings. It is best to use O-rings that are the same thickness.

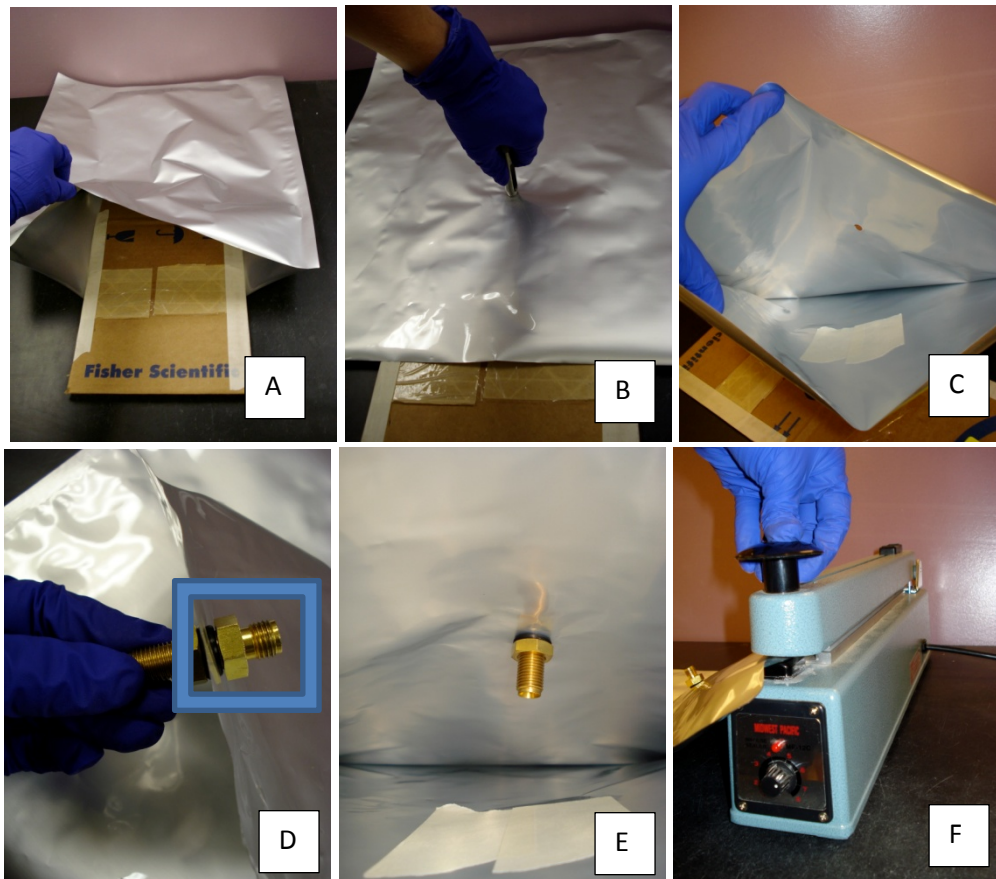


Figure 4. Making a mylar gas bag.

9. To tighten the seal of the fitting, screw a flat brass nut onto the bulkhead fitting on the inside of the bag. Ensure that the nut is tightened just past finger-tight. Do not over tighten the flat nut because this can cause the O-rings to shift and may damage the seal. See Figure 4E.
10. For small mylar bags use the impulse heat sealer to seal the open side of the bag (step 13). For the Enriched Breathing Standard, use the iron to seal the open end of the mylar bag (step 11).
11. For large mylar bags: Lay a metal ruler on the black von Fischer lab bench and lay the mylar bag over the ruler, making sure the metal ruler is under the area intended to create a heat seal and acts as a backing to the seal.

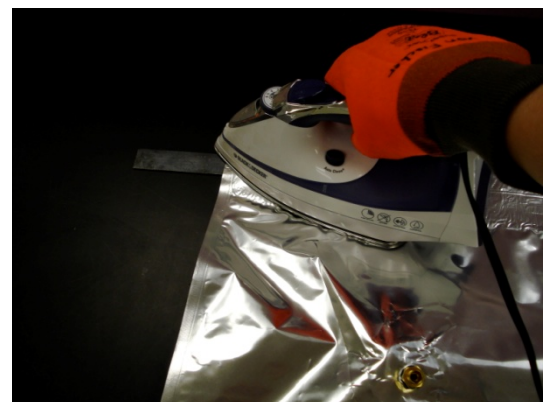


Figure 5. Ironing a seal for a large mylar bag.

12. Wear the orange insulated gloves and turn on the iron to the cotton setting (hottest available). Once the iron is hot, gently glide the iron on the mylar bag where the metal ruler is placed for backing. Iron at least two seals across the large mylar bag. See Figure 5.
13. For small mylar bags: Make sure the impulse heat sealer is plugged into an outlet. Check that the heat setting knob is at ~6 for sealing mylar bags. See Figure 4F.
14. Open the impulse heat sealer by lifting the handle (Note: the impulse heat sealer is only hot for the first few seconds that the handle is depressed and the red light on the side of the sealer will light up briefly during the time that the metal strip is hot).
15. Place the mylar bag across the heat strip and depress the handle for 10 seconds.
16. Create several seals across the mylar bag. Inspect each seal to ensure it lines up across the full width of the mylar bag.
17. Make a gas port Luer slip fitting for the gas bag by taking 1/4" OD Tygon tubing and ferruling a 1/4" Swagelok capped nut to one side of the Tygon tubing. Attach a Luer slip to the other side of the Tygon tubing. See Figure 6.
18. Connect the Swage nut of the Luer slip fitting to the bulkhead fitting of the mylar bag by wrenching 1/4 turn past finger-tight. This completes making a gas-tight mylar bag.
19. If ever a patch is needed for a small hole or to reinforce a mylar bag, use Tough Tags to patch the mylar material.

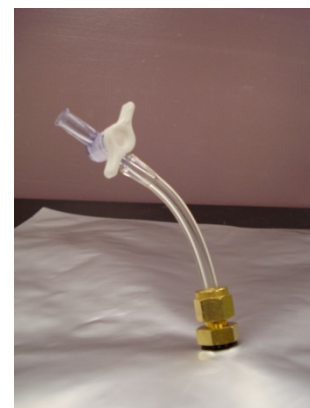


Figure 6. A picture of a Luer slip fitting on a mylar gas bag.

3.2.4 Rinsing Mylar Bags

1. To rinse a mylar bag attach the Luer slip to the nitrogen tank (or fill with the gas from a standard tank that you plan to use in the dilution- e.g. use the 5.03 ppm CH₄ tank to rinse the mylar bag if the dilution will be made with the 5.03 ppm CH₄ standard) and fill bag approximately 1/4 full.
2. Remove mylar bag from the air tank and attach it to the evacuator.
3. Evacuate the mylar bag completely, being careful not to let the bulkhead fitting suction the mylar material into the fitting, which can create a hole in the bag. Rinse and evacuate 2-3 times.
4. The Enriched Breathing Standard mylar bag must be rinsed and evacuated prior to incubation.

3.2.5 Filling the Enriched Breathing Standard

1. Once the large mylar bag for the Enriched Breathing Standard has been rinsed and evacuated it can be filled with the proper dilution for incubation. See Table 6 of Appendix A for recipe.

2. Inject the proper volume of labeled gas from the Yellow Tank dilution into the evacuated mylar bag (see Table 6 of Appendix A in Excel).
3. To use the flow meter, locate the “Flow Data” sheet (J drive>Great Plains Methane>MCIA testing “Flow Meter Rates.pdf”) or use the “Flow Meter Rate” tab of the Excel template to identify corresponding flow meter levels with flow rates. Connect the flow meter with flow controller to the Compressed Breathing Air gas cylinder in the GC room and open the regulator to the desired flow rate on the flow meter.
4. Connect the mylar bag to the flow meter and fill bag to desired volume. See Table 7 of Appendix A.

3.2.6 Filling the SF₆ dilution

1. Use a rinsed gas bag with a Luer slip fitting to fill the SF₆ dilution.
2. Turn on the regulator to the small pure SF₆ tank outside and clear the tubing by letting fresh gas flow out of the tubing to the outside air.
3. Rinse the 10mL syringe 2-3 times with the pure SF₆ gas, then take 5.0mL in the syringe and inject it into the evacuated bag.
4. Rinse the Hamilton syringe and fill the remainder of the SF₆ dilution according to the recipe in Table 6 of Appendix A in Excel.

4 Incubation procedures

4.1 Reasoning

4.1.1 MTRS incubations are tailored to each Great Plains Methane field site by using the specific moisture and temperature levels, and the duration of incubation follows suite. in that the total incubation time differs among field sites. The process leading up to incubation is held constant, however, and the goal of this section is to describe the procedures required to start an incubation.

4.2 Materials and Methods

4.2.1 Materials

- MCIA
- mylar bags with bulkhead fittings (1 per soil sample)
- 1 O-ring per soil sample
- g-sensitive scale
- data sheet
- SF₆ dilution
- 10 mL syringe for SF₆ injection

- 14 evacuated vials
- 30 L syringe for evacuated vial filling
- 30 mL syringe for $^{13}\text{CH}_4$ injection
- Medical Grade Compressed Breathing Air gas tank (Antolin tank)
- Flow meter
- Timer
- 1 mylar bag/soil sample
- Tape and sharpie to write soil IDs on outside of mylar bags
- Data sheet
- 14 lunch trays
- 1 Luer slip per soil sample

4.2.2 Determine the incubation run time

1. Determine how long the incubation should run (how many hours in total?).
2. Each incubation consists of 5 cycles of the MCIA taking one measurement from soil samples and controls per cycle. Determine the amount of time per cycle by dividing the total incubation time by the number of cycles (5). See Table 8 of Appendix A. This calculation is also on the Stall Time tab of the Excel template.
3. It is useful to space out the time between MCIA gas measurements of soil samples by connecting a Medical Grade Compressed Breathing Air gas tank to one port of the Multi-Inlet Unit (MIU) by an open split (serves the function of stalling at the end of each cycle to increase the amount of time between MCIA measurements of soil samples and controls).
4. Calculate the duration of time the open split will occupy per cycle by subtracting the amount of time taken by the MCIA to measure 29 gas samples (400 sec each) from the cycle time.
5. The MCIA pulls approximately 0.109 L/min of gas. Using the flow rate of the MCIA, check by calculation that the volume of the Enriched Standard bag is sufficient for all cycles of the incubation so it will not run out of gas.

4.2.3 Preparing the MCIA

1. The MCIA must be powered on 12 hours prior to incubation in order to warm up the analyzer. To turn on the MCIA, flip the switch on the back of the MCIA to the ON position.
2. Make sure the external pump and Multi-port Inlet Unit (MIU) are also powered on.
3. In the main panel on the MCIA screen use the mouse to click on the Rate button until 0.5 Hz is displayed.
4. In the main panel on the MCIA screen use the mouse to click on the Setup button. The screen will prompt for confirmation that it is desired to pause measurements to enter the Setup Menu. Click Okay.
5. Click on the Dilution button and disable dynamic dilution. See Figure 7.

6. Return to the Setup Menu and click the Manifold button.
7. If the manifold screen is inactive because the manifold is disabled, enable the gas manifold by clicking on the “Enable gas manifold box.” See Figure 8 for an example of the Manifold screen.
8. Ensure that the reference cycle is programmed to be measured first by making sure the “Start with reference cycle” box is highlighted. In the drop-down menu on the right side ensure there is only one (1) Unknown Gas cycle per Reference cycle. See Figure 8.
9. It is necessary that the manifold alternates the Enriched Breathing Standard between soil samples and controls. Make sure the valve order proceeds as 1, 2, 1, 3, 1, 4... etc. and that each sample (except the open split) will be measured for 400 seconds. See Figure 8.



Figure 7. MCIA screenshot showing how to disable dynamic dilution of gas.

Unknown Gas			Reference Gas			Gas Manifold Control
Valve	Seconds	Description	Valve	Seconds	Description	
1	400	--ENRICHED_STDN	1	400	--ENRICHED_STDN	<input type="checkbox"/> Enable Gas Manifold Number of unknown cycles per reference cycle: <input type="text" value="1"/> <input type="checkbox"/> Start with reference gas cycle.
10	400	-sample	2	400	-sample	
1	400	-ENRICHED_STDN	1	400	--ENRICHED_STDN	
11	400	-sample	3	400	-sample	
1	400	-ENRICHED_STDN	1	400	--ENRICHED_STDN	
12	400	-sample	4	400	-sample	
1	400	--ENRICHED_STDN	1	400	--ENRICHED_STDN	
13	400	-sample	5	400	-sample	
1	400	--ENRICHED_STDN	1	400	--ENRICHED_STDN	
14	400	-sample	6	400	-sample	
1	400	-ENRICHED_STDN	1	400	--ENRICHED_STDN	
15	400	-sample	7	400	-sample	
1	400	-ENRICHED_STDN	1	400	--ENRICHED_STDN	
16	5680	OPEN_SPLIT	8	400	-sample	
0	0	-	1	400	--ENRICHED_STDN	
0	0	-	9	400	-sample	

Figure 8. MCIA screenshot of the Gas Manifold Control that programs the MIU.

10. If necessary, change the number of seconds in the entry for the open split to the open split stall time seconds that was previously calculated. See Figure 8.

4.2.4 Preparing incubation materials

1. Evacuate 14 vials for each incubation. These will be filled with gas samples and analyzed for the [SF6] to determine volume of the samples by dilution.
2. Make one mylar bag with a bulkhead fitting for each soil sample. Do not seal the open end of the mylar bag until prompted to do so in the next section.
3. Ensure the “Incubation data” tab of Excel template is printed.
4. Use a cart to take all materials to Antolin lab.
5. Connect flow meter to Medical Grade Compressed Breathing Air tank in the Antolin lab (referred to as “Antolin Breathing tank”).
6. Ensure the middle of the glass float in the flow meter is at 70 (or whatever flow rate has been previously calculated and desired) when gas cylinder regulator is open.

4.2.5 Sealing soil sample bags for incubation

1. Remove one soil at a time from the incubator.
2. Take off the plastic bag and set aside (use the same plastic pre-incubation bags for Incubation 1 and Incubation 2 for each field site).
3. Remove the aerated micropipette tip box from the soil and brush off any soil on box back onto the tray.
4. Open the mylar bag intended to hold the soil sample and connect the aerated micropipette tip box to inside of the mylar bag for the soil sample by sliding the long end of the bulkhead fitting into the hole in the top of the box. See Figure 9A.
5. Secure the box to the bulkhead fitting by sliding an O-ring onto the end of the bulkhead fitting (this is not meant to create a seal, rather it is to stop the box from falling off the fitting inside the bag). See Figure 9B.
6. Use tape to make a label with the proper incubation ID (e.g. T1M1) and stick tape to outside of mylar bag.
7. Tare the scale with nothing on it.
8. Weigh and record the gross weight (tray + soil) of the sample after the pre-incubation period in the “Gross weight after pre-incubation” column on the data sheet.
9. Insert the soil into the mylar bag and ensure the aerated micropipette tip box sits in the middle of the tray of soil.
10. Set the mylar bag that holds the soil tray on a lunch tray. The lunch tray provides support and protection for the mylar bag and will travel with the soil sample mylar bag for the rest of the incubation.

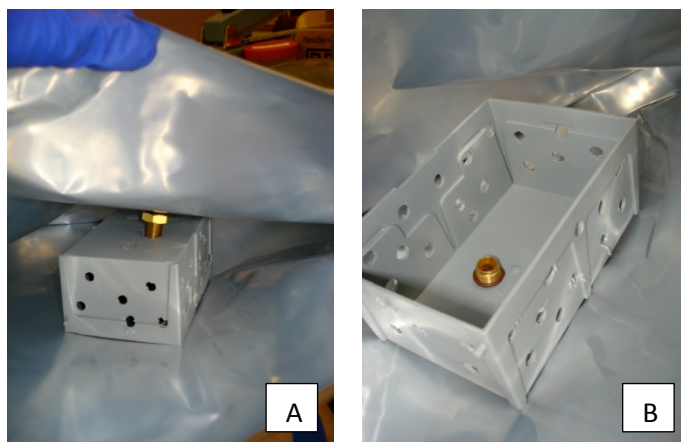


Figure 9. Insert the bulkhead fitting into the aerated micropipette tip box, then use an O-ring to hold it in place.

11. Fasten a Luer slip to the outside of the mylar bag with wrenches (1/4 turn past finger tight).
12. Use the impulse heat sealer (heat setting ~6) to seal the mylar bag with the soil sample and aerated micropipette tip box inside.
13. Rinse 10mL syringe with SF₆ dilution 2-3 times. Pump gas several times in SF₆ dilution bag with the rinsed syringe, then fill syringe to 5.0 mL.
14. Inject 5.0 mL of the SF₆ stock dilution into the sealed soil sample mylar bag.
15. Rinse ¹³CH₄ label syringe 2-3 times with the Yellow tank dilution. Allow pressure of the Yellow tank to fill rinsed syringe with 17.0 mL of Yellow tank dilution.
16. Inject 17.0 mL of Yellow tank dilution into sealed soil sample bag.
17. Open regulator of Antolin Breathing cylinder to clear out old gas from the tubing connected to the flow meter, then close regulator valve.
18. Set timer to desired fill time (25 seconds for soil sample bags if flow meter is set to 70; 41 seconds for control bags if flow meter is set to 70) and connect soil sample mylar bag to flow meter of the Breathing Tank.
19. Start timer and open regulator of Antolin breathing tank simultaneously to fill soil sample bag for the proper duration of time (this will ensure gases are well-mixed).
20. Record the evacuated vial number in the "SF₆ Vial #" column" on the data sheet.
21. Rinse evacuated vial syringe with room air 2-3 times.
22. Use evacuated vial filling syringe to take a 30.0 mL sample from soil sample bag and inject sample into evacuated vial.
23. Place soil sample back into the proper incubator. Complete this for each soil sample.

4.2.6 Filling Control bags for incubation

1. Control bags should be evacuated prior to incubation.
2. Use the rinsed 10mL syringe to inject 5.0 mL SF₆ dilution into control bag.
3. Use the rinsed 30mL syringe to inject 17.0 mL Yellow tank dilution into control bag.
4. Fill control bag with 7.0 L of Antolin Breathing tank (fill for 41 seconds if flow meter is set to 70).

4.2.7 Connecting the open split and Samples to MCIA and starting the incubation

1. Turn down the flow meter to ~ 700 mL/min (level 10-15 on the flow meter) and connect the flow meter to the open split.
2. Ensure the open split is connected to port #16 of the MIU and ensure all Swagelok connections are wrenched down ¼ turn past finger tight.

3. Ensure the Antolin Breathing tank is on and the regulator is open so gas is constantly flowing out of the tank through the flow meter into the open split to the MCIA tubing.
4. It is important to pause the MCIA while connecting samples to the MIU ports so that gas is not drawn from the samples before the intended start of the incubation. Click the Setup button on the main screen of the MCIA and pause the MCIA by navigating to the Setup Menu screen. Stay on the Setup Menu screen while connecting the MIU ports to the mylar bags.
5. Connect the Enriched Breathing Standard to port #1 of the MIU.
6. It is necessary to remove the plugs on the lower left side of each incubator so the MCIA's MIU tubing can be threaded through the hole to reach the mylar bags inside the incubators.
7. Refer to the printed data sheet to see which numbered MIU port should be connected to each soil sample and control. Thread the MIU tubing lines into the proper incubators.
8. Use wrenches to remove the Luer slip and quickly tighten the MIU tubing to the bulkhead fitting of the mylar bag. Wrench the MIU tubing to the mylar bag $\frac{1}{4}$ " past finger tight.
9. Once the Enriched Breathing Standard and the mylar bags in the T3 incubator (MIU ports 2-5) are connected to the proper MCIA MIU ports and wrenched down, it is time to start the MCIA sampling.
10. It is necessary to have the Enriched Standard and the first few mylar sample bags connected before allowing the MCIA to start sampling in order to have a head start in connecting the other bags (e.g. the MCIA will sample port 1 for 400 sec, port 2 for 400 sec, port 1 for 400 sec, port 3 for 400 sec, port 1 for 400 sec etc while the rest of the bags are being connected to the MIU tubing.- the goal here is to stay well ahead of the MCIA sampling while connecting the other samples; this also saves time for the person working the incubation and makes the sampling start earlier than waiting for all MIU ports to be connected before starting the MCIA).
11. Click the Return button on the Setup Menu screen to start the first cycle of the incubation. Wait until "Slow Reference Flow" appears on the screen. It is a good idea to watch the MCIA measure the first two samples to ensure the Manifold is set to sample the MIU valves in the correct order. See Figure 10.
12. On the data sheet record the incubation file number (displayed at the bottom of the screen).
13. After the MCIA begins sampling, continue connecting all of the other samples to the MCIA's MIU tubing in the incubators.

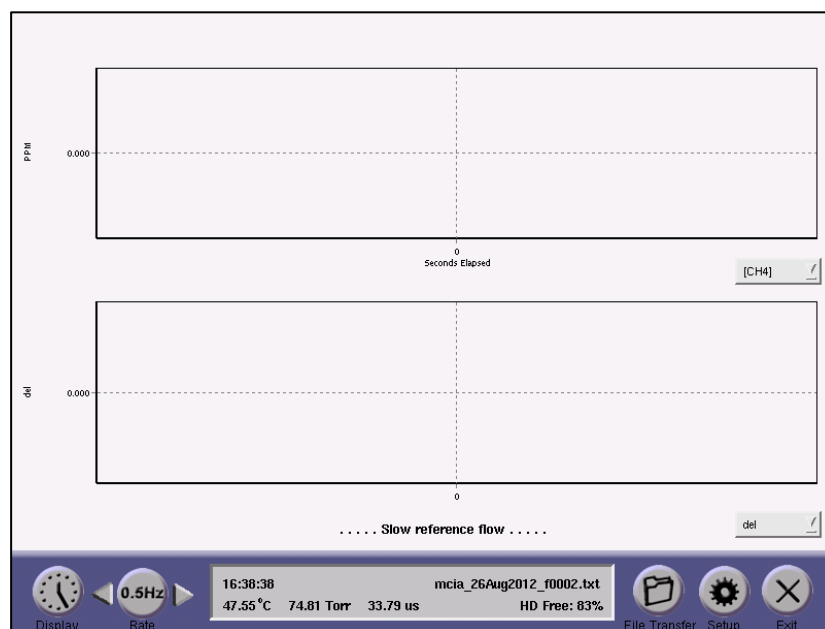


Figure 10. MCIA screenshot showing when the MCIA begins to sample the first cycle of an incubation.

14. The MCIA will continue to cycle until it is manually stopped. Determine the end time of the incubation and plan to return to the MCIA at that time to stop the incubation and download the MCIA files.
15. Before leaving the incubation to run, check that the flow meter is still set between 10-15.

5 Ending the incubation

5.1 Reasoning

5.1.1 The MCIA will cycle until it is manually stopped, thus it is important to know when the incubation should end.

5.2 Materials and Methods

5.2.1 Materials

- Wrenches
- Cart
- Soil dumping bucket
- Non-nitrogen soap
- DI water
- Drying rack
- Ethernet cord or USB drive
- Excel template

5.2.2 Ending and incubation and cleaning up

1. Measurements of the MCIA should be stopped at the end of the last cycle. This can be accomplished by clicking the Setup button and navigating to the Setup Menu screen (halts data acquisition on the current file and starts a new data file) or by clicking the File Transfer button and manually transferring files to a USB drive.
2. If manual file transfer from the MCIA to a USB drive is desired, refer to pages 9-13 of the Los Gatos MCIA Instructions Manual.
3. Turn off the Antolin Breathing tank.
4. Disconnect the open split, Enriched Standard and all mylar bags from the MIU tubing and remove tubing from inside the incubators.
5. Coil MIU tubing and fasten tubing with orange Velcro ties, then stack coiled tubing on the MCIA cart.
6. Use a cart to take mylar soil sample bags back to von Fischer lab.
7. Cut open mylar bags and dump soil from trays into the designated soil dumping bucket.

8. Remove O-rings, aerated micropipette tip boxes and bulkhead fittings from mylar bags and wash with non-nitrogen soap, then rinse three (3) times with DI water. Set Swagelok pieces and O-rings on paper towel to dry. Hang aerated micropipette tip boxes on lab drying rack above sink.
9. Wash jelly roll trays with non-nitrogen soap and rinse three (3) times with DI water. Set trays on lab drying rack above sink.
10. Lunch trays do not require washing.

5.2.3 Downloading data using the Local Area Network (LAN)

1. In order to download data using the LAN, the Ethernet cord must be plugged into the MCIA when the MCIA is powered on, or the MCIA must be restarted after the Ethernet is plugged in.
2. Make sure to use a computer in the von Fischer lab that is connected to the Local Area Network.
3. On the PC go to Start>Run and in the drop down menu that says "open:" enter \\LGR-11-0016 then click "okay."
4. A window will appear for the LGR Analyzer with two icons. Click on the "lgrdata" icon to navigate to the desired files.
5. A listing of files should appear in the LGR Analyzer window and will be organized by date. Find the folders of the files from the incubation. Data files on the MCIA are set to last for 12 hours, so each incubation is comprised of multiple files.
6. Open the "Flow" folder and open the zipped folder with the desired .txt data file.
7. Do NOT modify or delete any files on the MCIA!! Rather, copy the file and paste it into the proper folder for that incubation on the J drive.
8. To open a text file in Excel copy and paste the text file in the incubation folder on the J drive change the file type of the copied text file to a comma separated value file (e.g. Copy of aaaaaaa.txt => Copy of aaaaaaa.csv).
9. Open the .csv and save as an Excel workbook.
10. It is necessary to paste the files together in chronological order in an Excel workbook.
11. After the files have been compiled into one Excel workbook, review the Excel incubation template in the "MCIA raw data" tab to see which columns of data should be copied and reordered.
12. After the columns have been organized, copy and paste the proper data columns from the entire incubation into the Excel template for that incubation.
13. Create a folder that will be accessed by Evan for database entry by going to J drive>Great Plains Methane>Moisture-Temperature Response. Create a folder with the field site name and date the soil was sampled. Within that folder create a folder with the proper incubation title (either T1M1-Control1 or T4M3-Control3).
14. Create a copy of the entire Excel template and paste it in the proper folder for database entry.