# Procedure for Soil Respiration (CO_2 Evolution from Rewetting a Dried Soil)

Procedure Overview:

This document describes a procedure for the determination of CO_2 respired from air-dried soil that has been rewetted. The method was originally reported by Franzluebbers et al. (1996) and expanded by Franzluebbers et al. (2000). Several methods are available for quantifying evolved CO_2. This procedure facilitates a high-throughput framework, using 50 mL centrifuges tubes and an infrared gas analyzer (IRGA). Soils that are ground to <2 mm are typically used.

10 g of soil + pre-determined volume of H_2O	→	Incubate at 25.0^oC for 24 hours	→	Read on IRGA
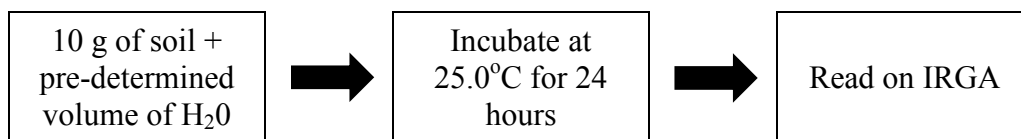
Instrumentation and Materials:

Sample Preparation and Reaction
- Analytical balance capable of weighing to two decimal places
- 50 mL disposable polypropylene centrifuge tubes (Falcon tubes)
- 10 mL pipettor and tips
- Falcon tube caps with septa installed and sealed with silicone caulk
- Parafilm

Reading Samples on Spectrophotometer
- Carrier gas tank (100% N_2 gas + regulator)
- Air Control Valve (Dwyer RMA-150-VSSV)
- LI-840a infrared gas analyzer (IRGA)
- LI-840a software
- 0.5 mL and 1 mL syringe with needle
- CO_2 standard gas tank (1% CO_2, 99% N_2 + regulator)

Detailed Procedure:

I. Sample Preparation:

1. Label 50 mL tubes with unknown sample ID's.
2. Weigh out 10 g (± 0.05) of the unknown soil sample in corresponding tube (may be done in advance).
3. Leave tubes uncapped until start of sample reaction.
4. Soil checks should be prepared in the same manner as the unknown soils and serve as laboratory reference samples. It is recommended to pulverize and homogenize a large batch of air-dried soil for long-term use. The soil checks allow for a quality control check across respiration runs performed on different batches and over multiple days.
5. Label 3 empty tubes that contain no soil to use as blanks. These will be used later in calculations.

II. Sample Rewetting and Incubating:

1. Place samples in a fume hood to make sure the initial level of carbon dioxide in each tube is uniform.
2. While keeping the tube under the hood, use the 10 mL pipette to dispense pre-determined volume of deionized water (typically 3 mL, see Appendix A1) into each tube. The water should be dispensed in a circular motion to prevent any splashing or disturbance of soil. Keep a timesheet of when the water is added to each sample. This should be done in 1 minute intervals. Be careful not to breathe into sample tube as this will increase the carbon dioxide levels and give false values.
3. Cap the samples while the tube is still under the hood.
4. Wrap parafilm over the cap onto the sides of the tube to seal.
5. The 3 blanks should be treated in the same way without adding soil or water.
6. Place sealed samples in an incubator set to $25^{\circ}C$. Incubate for 24 hours. Record incubation temperature to be used later in calculations.

III. Preparing the IRGA:

1. Turn on gas tank containing IRGA carrier gas (N_2) 20-30 minutes prior to sample start time. Set carrier gas flow to 70 mL/min (50-100 mL/min is an acceptable range). Measuring standards typically takes 10 minutes.
2. On the computer connected to the IRGA, open LI-COR software to run LI-840A.
3. Click File > Connect and connect to appropriate port.
4. When flow rate of carrier gas remains constant (typically takes 10 minutes), click:
    View > Calibration and calibrate CO_2.
5. To start logging measurements, click: Logging > Start. Enter a file name for the data file and save. The CO_2 values will be logged at 1 second intervals.

IV. Measuring CO_2 Standards:

1. There will be 3 volumes of standards taken (1 mL, 0.5 mL, and 0.25 mL).
2. Turn on standard tank (1% CO_2, 99% N_2).
3. Insert the 1 mL syringe into standard tank septa and draw 1 mL of the CO_2 standard.
4. Inject 1 mL quickly into the injection port. Record time of injection. The CO_2 concentration will be digitally logged.
5. Repeat steps 3-4 until there are 3 readings with 1 mL. Wait 1 minute between standards, or until the baseline of CO_2 returns to zero.
6. Using the 0.5 mL syringe, repeat steps 3-5 with 0.5 mL and with 0.25 mL of the CO_2 standard. Note we are manipulating CO_2 standard volumes as a proxy for different CO_2 concentrations here. This negates the need for 3 different tanks with 3 different CO_2 concentrations.
7. The standards should be completed before and after reading unknown sample.

V. Reading Samples on IRGA

1. Remove samples from the incubator and record the temperature.
2. These should be read as close to the 24hr mark as possible, hence the recording of time when

water was added.

3. At exactly 24 hours since rewetting began, insert 1 mL syringe into septa in lid of unknown soil sample.
4. Pump syringe 5 times to thoroughly mix air inside the tube.
5. Draw 1 mL of air from the sample tube and insert into the IRGA septa at the minute mark, keeping in time with the time recorded 24hrs earlier.
6. Inject 1 mL quickly into the injection port. Do this in the same manner as the standards (record time and wait for the CO_2 level to return to 0).
7. Repeat steps with remaining samples, soil checks and blanks.
8. At the end, repeat the CO_2 standard measurements again as described above in section IV.

VI. Equipment Clean Up

1. After taking CO_2 readings, stop logging to save as a .txt file.
2. Turn off the carrier gas tank and standard tank, making sure to release the pressure.
3. LI-COR recommends leaving the IRGA on all the time.

VII. Calculations

Respiration measurements are most often reported as a rate of C mineralization, or the amount of C respired per kilogram of dry soil per day (mg C kg soil^{-1} day^{-1}). There are several calculations that are required to take raw IRGA CO_2 ppm measurements to a rate, as outlined below:

1) **Identify peaks of standards, blanks and unknown samples:**
   a. The LiCor software logs CO_2 concentrations every second. Samples injected will produce CO_2 concentrations that increase quickly over time, peak, then decrease back to baseline. The peak, or highest concentration of CO_2 is the value used for each corresponding sample. Peaks that correspond to each standard, blank and unknown need to be identified and all remaining CO_2 concentrations should be eliminated.
   b. This can be done manually in excel or automatically using the *turnpoints*() function in the package *pastecs* in R.

2) **Calculate Adjusted IRGA ppm:**
   a. Average the 3 blanks to get a baseline IRGA ppm.
   b. Subtract the averaged baseline IRGA ppm from each unknown soil sample IRGA ppm value. This step accounts for CO_2 concentration at start of incubation.

3) **Calculate a Standard Curve:**
   a. Average the 3 replicates for each of the 3 standards at the beginning of the readings. Repeat this step with the standards read after the unknown samples.
   b. Average the 2 corresponding standards before and after readings in step 3a. Note any systematic drifting of values (increase/decrease) over the course of the readings.
   c. Calculate a regression line equation between the known CO_2 gas standards and the empirically-derived gas standard.
      i. Y-axis: the known CO_2 gas standards: i) 1 mL: 1% or 10,000 ppm, ii) 0.5 mL: 0.5% or 5,000 ppm and iii) 0.25 mL: 0.25% or 2,500 ppm.
      ii. X-axis: the calculated averaged IRGA ppm measurements.
      iii. Extract slope and intercept terms from regression line.

4) **Calibrate the Adjusted IRGA ppm results to the Standard Curve:**

a. Multiply the slope of the regression line by the adjusted IRGA ppm and then add intercept to calibrate to the known ppm.

5) **Convert the calibrated and adjusted ppm CO_2 (which is on a volume basis) to µg CO_2-C per L headspace with the below equation (ideal gas law):**
   a. Cm (µg CO_2-C L headspace^{-1}) = (Cv x M x P) / (R x T), where
      i. Cv = ppm (volume) CO_2
      ii. M = molecular weight of C (12 µg /µumol)
      iii. P = Barometric pressure (1 atm)
      iv. R = universal gas constant (0.0820575 L· atm / K · mole)
      v. T = incubation temp in °K (273.15 + °C)

6) **Convert µg CO_2-C L headspace^{-1} to µg CO_2-C gram soil^{-1}:**
   a. Multiply µg CO_2-C L headspace^{-1} by the volume of the incubation chamber (in L) and divide by the weight of the soil used in the incubation. This is equivalent to the more commonly reported mg CO_2-C kg soil^{-1}.

7) **Finally, to convert mg CO_2-C kg soil^{-1} to a rate, divide by the number of days incubated.**

Example Calculation:

Suppose we have 10g of an unknown soil sample that we incubate for 1 day at 25 °C (298.15 K) after rewetting. The adjusted volume of the incubation chamber (50 mL), after accounting for average soil volume is 0.0451 L. The average blanks ppm = 77.06 and the unknown soil sample ppm = 950.10. The standard IRGA readings were 249.23, 551.38, 1209.77 ppm for the standards 2,500, 5,000, 10,000 ppm, respectively. The following calculations correspond to the steps above.

Steps:
1) Extract all peaks from exported LiCor data file.
2) Adjusted IRGA = 950.10 – 77.06 = 873.04 ppm
3) Y-axis: 2,500, 5,000, 10,000; X-axis: 249.23, 551.38, 1209.77) => y = 7.77x + 724.13
4) Calibrated and adjusted IRGA = (7.77 x 873.04) + 724.13 = 7510.71 ppm
5) Cm (µg CO_2-C L headspace^{-1}) = (Cv x M x P) / (R x T) =
   (7510.71 ppm x 12 µg /µumol x 1 atm)/ (0.0820575 x (273.15 + 25)) =
   3698.80 µg CO_2-C L headspace^{-1}
6) µg CO_2-C gram soil^{-1} = (3698.80 µg CO_2-C L headspace^{-1} x 0.0451 L)/ 10 g soil =
   16.68 µg CO_2-C gram soil^{-1} or 16.68 mg CO_2-C kg soil^{-1}
7) Rate: 16.68 mg CO_2-C kg soil^{-1} / 1 day = 16.68 mg CO_2-C kg soil^{-1} day^{-1}

References:

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Citation: Procedure for Soil Respiration, Ohio State University Soil Fertility Lab, 2019.
https://soilfertility.osu.edu/protocols

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Appendix:

A1. Issues with Determining Volume of H_2O with Unknown Soil Samples

- A major limitation of this method is the challenge of accurately determining the amount of water need to rewet each soil sample. Some procedures use a set volume of water regardless of the soil sample, others use capillary action to rewet soils, while others require determining volume of water for each individual soil sample based on either 50% water-filled pore space or 50% water hold capacity (WHC). Wade et al. (2018) reported large differences in final respiration values depending on the procedure, with rewetting via capillary action returning markedly lower values than surface wetting based on 50% WHC.
- Determining the amount of water to add for each individual soil sample takes time and is likely outside the scope of most commercial soil testing labs concerned with processing large numbers of samples. Until alternative methods are developed to rapidly estimate the appropriate amount of water to add to rewet a sample, there will always be tradeoffs with optimizing the respiration potential of a soil and speed and efficiency of obtaining data.
- Franzluebbers (2016) uses 50% water-filled pore space using the weight and volume of a soil to calculate the amount of water needed for each sample/ set of samples. This method also requires hand sieving samples to 4.75 mm.
- The OSU Soil Fertility lab uses one of two approaches: Either adding 3 mL of water for speed and efficiency, or to empirically determine the 50% WHC for each sample. The 3 mL of water was empirically derived as the volume that returned highest respiration values across a large and diverse sample set of Ohio soils. The 2nd approach, determining 50% WHC for each sample is often done with research samples using the procedure outlined below.

Empirically Determining 50% Water Holding Capacity (WHC) of a Soil (rapid method)
1. Place a funnel into a beaker and place on a balance.
2. Fold filter paper in quarters and place into funnel. Tare balance to zero.
3. Add ~20 g of soil that has been oven-dried at 105°C into the funnel.
4. Record the weight to the nearest 2 decimal places. This is W_1.
5. Add DI water until the soil is saturated, and let it to completely drain from the funnel (~30 min).
6. Transfer filter paper with saturated soil to an aluminum tin and record wet weight (W_2 = tin + filter paper + moist soil).
7. Put the tin containing soil in the oven @105°C overnight.
8. Record dry weight (W_3 = tin + filter paper + oven-dry soil)
9. Calculate how much water the soil per gram of soil: 100% WHC = (W_2 – W_3) / W_1
10. Calculate 50% WHC for 10 g of soil: 50% WHC = (100% WHC ÷ 2) * 10 g
11. Note this procedure assumes that any water absorbed by the filter paper is negligible.

A2. Septa Installation into Falcon Tube Caps

1. Use a 7/16 drill bit to drill a hole in the middle of the tube cap (50 mL falcon tube).
2. Insert the septa (Sigma-Aldrich: Supelco Butyl Septa, 20mm Cat# 27232) into the cap so that the lip of the septa fits securely around the hole in the cap.
3. Use 100% clear silicon caulk to seal around the septa on the top and underneath.
4. Allow to dry overnight before use.

A3. IRGA Instrumentation Setup

Materials used to set up a continuous flow IRGA with N_2 as a carrier gas. See pictures below for reference.

- Dwyer RMA-150-VSSV
- LI-840a (or LI-820a) infrared gas analyzer (IRGA)
- LI-840a software
- Harris Specialty Gas: Gas regulator Cat# HP722C-050-580-D
- 9.5mm Injection port septa- Chromatography Research Supplies: Cat# 230675
- Licor: Gelman 1 Micron Filter Assembly Cat# 9967-008
- Licor: Bev-A-Line Tubing, 1/4" OD, 1/8" ID Cat# 222-01824
- Quick connects for tubing

Micron filter

Carrier gas

Carrier gas tank:
100% N_2 gas

Standard tank:
1% CO_2, 99% N_2

Air inlet

Air outlet

Micron filter

Carrier gas

Injection port

Dwyer RMA-150-VSSV