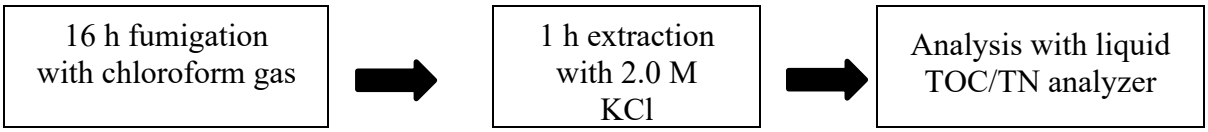
SOP: **Microbial Biomass C and N: sequential fumigation-extraction (chloroform gas)**

Overview:

This standard operating procedure (SOP) describes a protocol for determining the C and N within soil microbial biomass via chloroform fumigation. The method was originally reported by Vance, Brookes, and Jenkinson (1987). Key adaptations made to the original method include the use of 2.0 M KCl for extraction instead of 0.5 M K_2SO_4. In addition, correction factors for estimated proportion of microbial C/N in CO_2 are not used. Key instruments are desiccators, shaker table, and liquid C and N analyzer. A key safety consideration is the use of a fume hood during chloroform fumigation. Soils that are field-moist are always used.



Safety:

All standard safety protocols and online safety training via UIUC Division of Research Safety (DRS) are required.
Personal protection (PPE) for this procedure include:
Eye Protection: Laboratory glasses or goggles
Body Protection: Laboratory coat
Hand Protection: Nitrile gloves
Particularly hazardous substances: Chloroform

- Do not inhale, swallow, or allow contact with skin.
- IF SWALLOWED: Call a POISON CENTER/doctor if you feel unwell.
- ON SKIN: Wash with plenty of water.
- IF INHALED: Remove person to fresh air and keep comfortable for breathing. Call a POISON CENTER/doctor.
- IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
- IF exposed or concerned: Get medical advice/ attention.

Specific details on this substance are incorporated in the **Detailed Procedure** below.

Instrumentation & Consumables:

Sample preparation

- Analytical balance capable of weighing to two decimal places
- 50 mL disposable polypropylene centrifuge tubes (Falcon tubes)

- Centrifuge tube racks

Fumigation

- Fume hood with vacuum line and hose
- Empty desiccator(s)
  - Size and quantity dependent on number of samples
  - Remove wire plate and desiccant
  - The rim and cover should be lightly greased with vacuum grease
- 100 mL beaker(s) (one per desiccator)
- Boiling chips
- Chloroform (liquid)
  - Chloroform should be handled only under the fume hood, while wearing gloves
  - Refer to safety section above
- Rubber bands
- Oil-based pencil
  - Although optional, this is recommended for labeling Falcon tubes. Regular sharpie labels may become hard to read after fumigation
- Parafilm sealing film

Extraction

- 50 mL disposable polypropylene centrifuge tubes (Falcon tubes)
  - A second set of tubes are needed for the filtered extractions. It is recommended to have these organized and labeled before the time of extraction.
- 50 mL dispensette
- 2.0 M KCl (***NOTE***: 0.5 M K_2SO4 can also be used)
  - If made in batches, combine before use
  - Store in a closed container indefinitely at room temperature
- Shaker table
- Whatman 70 mm filter paper, grade 42
- Centrifuge

Analysis

- Liquid TOC/TN analyzer

Detailed Procedure:

I. Sample Preparation

1. Measure 6.00 ± 0.05 g oven-dry equivalent of field-moist soil into Falcon tubes. Each soil sample must include at least 1 replicate for fumigation and 1 replicate for the non-fumigation control. If possible, include 2-3 replicates per sample.

i. The mass of the soil is based on the 1:5 soil (g) to KCl (mL) ratio; 5 g oven-dry equivalent may be used with 25 mL KCl

II. Fumigation

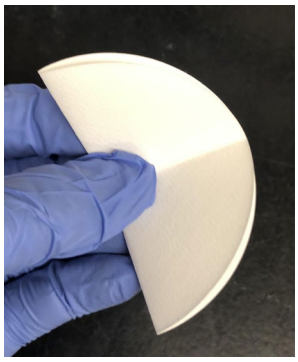
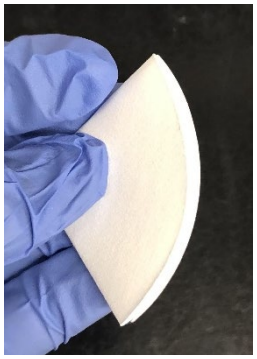
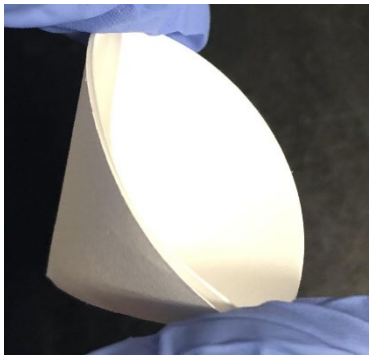
1. Place the desiccators under the fume hood. With the ventilation system on, pour 30-40 mL of chloroform (note: you may need to increase volume for larger desiccators) into 100 mL beaker with a thin layer of boiling chips, and place one beaker in each of the desiccators.
2. Wrap rubber bands around 7 Falcon tubes in a honeycomb pattern (see pictures below). Place rubber-banded, uncapped tubes into the desiccators. Pack them tightly to prevent spills.
    i. Include 1-2 soil-free blanks (empty tubes) in each desiccator to account for any contamination during fumigation, extraction, and filtration steps.
    ii. **Do not throw away the caps**; these will be needed for the extraction.

3. Place the desiccator cover on with a tight seal by sliding it horizontally along the rim. Depressurize the desiccator.
    i. With the desiccator's vacuum nozzle open, connect the hosing and turn on the vacuum flow. Let run for 1-2 minutes; bubbles should begin forming in the chloroform.
    ii. With the vacuum still running, close the vacuum nozzle tightly. A vacuum should be pulled within the desiccator. Turn off vacuum flow and remove hose.
        1. Bubbles should continue to form after sealing the desiccator
    iii. Allow to rest for 5-25 minutes and release pressure to listen for a hissing sound, to ensure a proper seal. Pull the vacuum again.
        1. Optional: wrap parafilm around any potential areas of leakage, including the rim of the desiccator, rim of mobile head of desiccator, and vacuum hose connection point.
4. Fumigate for 16 hours. Cover fume hood sash to prevent chloroform degradation by light.
    i. Non-fumigated controls can be stored at 4ºC during this time.
5. After 16 hours, repressurize desiccators by opening the nozzle.

i. Listen for a hissing noise when breaking the seal. If there is no sound, the vacuum was likely broken early. The fumigation will need to be repeated. Also, make sure there is some chloroform remaining in the beakers, as this confirms that there was enough chloroform for fumigation overnight.
ii. Open the desiccator and remove the chloroform beaker, then vacuum at least 8 times to ensure no chloroform is left in the samples. Then allow desiccators to vent with the cover off for ~20 min before removing samples.
iii. Dispose of remaining chloroform into a capped waste jug. Do not dispose of boiling chips, as these can be re-used.

III. Extraction

1. Using a dispensette, add 30 mL of 2.0 M KCl to the fumigated replicates, non-fumigated replicates, and true blanks.
    i. Remember to use 1:5 ratio of soil (g) to KCl (mL); 25 mL KCl can be used for 5 g oven-dry soil
2. Recap tubes and place on the shaker table (low setting – 120 rpm) for 1 hour
3. While extractants are shaking, fold filter paper (must be grade 42) into funnels on each of the labeled filtration tubes. This can be done by folding the circular filter paper into quarter-circles, then opening from the top and pressing into tube (see pictures).

4. After 1 hour, remove tubes from the table and pour the extractants through the filters until 15 mL of clear solution has accumulated. If extractants are not clear after filtration, repeat the process with a new tube and filter paper. Centrifugation prior to filtration is not necessary but highly recommended for time efficiency.
5. Extractants may be stored at -20 ºC for 2-4 weeks prior to analysis

IV. Analysis

Measurements of microbial biomass C and N are usually expressed in units of µg C or N g^{-1} soil. For a description of MBC/N analysis using a liquid TOC/TN analyzer, please see the **SOP for the AnalytikJena multi N/C 2100S**. See below for preparation needed before analysis:

1. Extractants must be diluted 1:5 using deionized water (18.2MΩ-cm water if possible) due to the high salt concentration.
2. Pipette 5 mL of your diluted extractants into clean, labeled vials.

Following analysis on the AnalytikJena, use this equation to express results in µg C or N g^{-1} soil:

$$\mu g\ C\ or\ N\ g^{-1} = \frac{A \times V}{W} \times \frac{1000\ \mu g}{mg}$$

A = C or N concentration in KCl solution (mg C or N / L)
V = Extraction volume (L)
W = Soil mass (g)

V. Clean up

1. Dispensettes should be cleaned **immediately** after use to prevent crystal formation. Return to maximum volume, then pump deionized water 5-10x. Empty and allow to dry before storing.
2. If the centrifuge was used, remove Falcon tube holders and wipe away any liquid at the bottom. If KCl crystalizes around the tube holders, they can be extremely difficult to take apart and change tube sizes.
3. Dispose of remaining chloroform in a capped waste bottle.
4. Any remaining 2.0 M KCl solution may be drained in the sink after diluted 20x and flushing the sink with 1-2 L of tap water after
5. Falcon tubes and filters may be thrown away in regular trash bins

References:

Vance, E. D., Brookes, P. C., & Jenkinson, D. S. (1987). An extraction method for measuring soil microbial biomass C. *Soil Biology and Biochemistry*, *19*(6), 703-707.

Citation:

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https://margenot.cropsciences.illinois.edu/methods-sops/

Questions can be directed to Andrew Margenot at margenot@illinois.edu