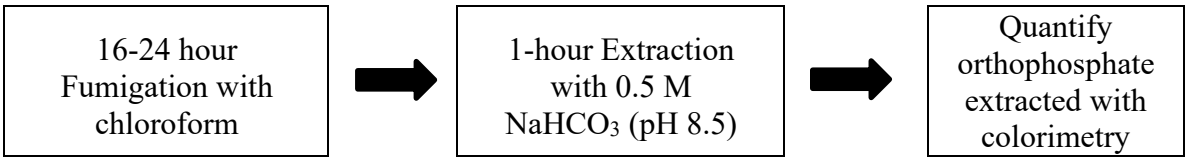
SOP: **MBP (Chloroform)**

Overview:

This standard operating procedure (SOP) describes a protocol for measuring microbial biomass phosphorus using gas fumigation with chloroform. The method was originally reported by Brookes et al. (1982). Key instruments are desiccators, shaker, and spectrophotometer. A key safety consideration is the use of a fume hood during chloroform fumigation. Soils that are field-moist are always used.
Note: Estimation of MBP in this SOP is based on quantification of inorganic P



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

- Always handle chloroform in well-ventilated fume hood
- 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

Reagent preparation

- $NaHCO_3$
- Murphy-Riley:
  - Ammonium molybdate
  - Antimony potassium tartrate
  - Ascorbic acid
- Magnetic stir bar and heated stir plate

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
  - 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)
- 50 mL dispensette
- 0.5 M $NaHCO_3$ (40 mL per sample)
- Shaker
- Centrifuge

Colorimetry (Murphy Riley)

- Analytical balance capable of weighing to two decimal places
- Commercial P standard (1000 mg P/L)
- Murphy-Riley solution
- 96 well microplates
- Microplate spectrophotometer capable of reading at 882 nm
- Pipette and tips (60-1000 uL)

Detailed Procedure:

I. Sample Preparation

1. Measure 5 ± .05 g oven-dry equivalent of field-moist soil into 50 mL Falcon tubes. Hand crumbling can help homogenize field-moist soil samples before weighing. Each soil sample requires at least 1 replicate for fumigation and 1 replicate for the non-fumigation control. If you plan to do correction with P spike, another tube with same amount of soil should be weighed for each sample
    i. The mass of the soil is based on the 1:8 soil (g) to $NaHCO_3$ (40 mL) ratio
2. Include a blank (empty Falcon tube) that will be treated the same as samples to account for background P throughout fumigation, extraction, and colorimetry

II. Reagent Preparation

1. 0.5 M $NaHCO_3$
    i. Dissolve in water
    ii. Use heated stir plate (50ºC) and stir bar to dissolve
2. Murphy-Riley
    i. Murphy-Riley Solution A
        1. Dissolve 4.3 g ammonium molybdate in 400 mL of deionized water in a 1-litre beaker.
        2. Dissolve 0.40 g antimony potassium tartrate in 400 mL deionized water, then add to the ammonium molybdate solution in the beaker.
        3. Slowly and carefully, with stirring and while cooling in an ice bath, add 54 mL conc. H_2SO_4.
        4. Allow to cool and make to 1000 mL with deionized water. Mix well and store in a dark bottle in a refrigerator. *The reagent is stable for 4 weeks at 4°C
    ii. Murphy-Riley Solution B
        1. Ascorbic acid, 1%: Dissolve 1.00 g of ascorbic acid in 100 mL deionized water. Make a fresh solution daily as needed.
    iii. Final Murphy-Riley (MR) reagent: combine 56 mL of solution B + 44 mL of solution A, and mix (should turn to light yellow color)

III. Fumigation (only for the set of samples to be fumigated)

1. Place the desiccators under the fume hood. With the ventilation system on, pour 30-40 mL of chloroform (depending on the volume of the desiccator) 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. Place rubber-banded, uncapped tubes into the desiccators. Pack them tightly to prevent spills.
    i. Include 1-2 true 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 5-6 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 16-24 hours (standardized by project). Cover fume hood sash to prevent chloroform degradation by light.
    i. Non-fumigated controls can be stored at 4ºC during this time.
5. After 16-24 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 closed waste container. Do not dispose of boiling chips, as these can be re-used.

IV. Extraction

1. Prepare 0.5 M $NaHCO_3$ with pH at 8.5 (40 mL per centrifuge tube)
    i. If made in batches, combine before use
    ii. Store in a closed container at room temperature
2. Using a dispensette, add 40 mL of 0.5 M $NaHCO_3$ (pH 8.5) to the fumigated replicates, non-fumigated replicates, and true blanks.
3. Recap tubes and place on the shaker (120 rpm; "low" for Eberbach E6010.00) for 1 hour
4. After 1 hour, remove tubes from the shaker and centrifuge (4000 rpm for 10 min). Pipette the supernatant out while avoiding plant residues and particles and use extract for colorimetry (described in the next section). Filtration may be necessary if the supernatant does not clear after centrifugation

V. P spike (if needed)

1. For the tube weighed for P spike, add 38 mL of 0.5 M NaHCO3 pH 8.5 and 2 mL of 125 ppm inorganic P (125 mg P L^{-1} = 50 µg P g^{-1} soil) made separately in the same bicarbonate extraction solution, cap, and shake for

1hr. Obtain supernatant the same way described above as other samples and proceed to colorimetry step

VI. P Colorimetry (prepare reagents before extraction)

1. Calibration standards (typically ranging from 0 – 20 mg P/L) need to be made in extracting solution (same bicarbonate solution used for extraction). Dilute commercial standard (1000 mg P/L) in each extracting solution and sequentially dilute them to make calibration standards that are within the linear range and cover the concentration you are expecting for your soil samples.
    i. It is essential to use the same extracting solution because molybdate colorimetry of P is sensitive to pH (color development and intensity, precipitation).
2. React extract and final MR solution at the ratio of 3:7 (e.g., 60 µL extract to 140 µL MR solution) in well plate and read the absorbance at 882 nm.
    i. The samples should be ready to be read when absorbance of standards stabilizes and constructed standard curve is linear (typically $R^2 > 0.99$; approx. 40 min)

VII. Clean up

1. Dispensette 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. Rinsing with dilute acid before water can help further cleaning the dispensette.
2. If tubes leak in the centrifuge, remove tube holders and wipe away any liquid at the bottom. Shaker should also be cleaned if leaks occur during shaking.
3. Pour residual chloroform into closed waste container
4. Any remaining 0.5 M $NaHCO_3$ solution may be drained in the sink after diluted 20x and flushing the sink with 1-2 L of tap water after
5. Any solution containing MR solution should be collected in a waste bottle clearly labelled with contents and their concentrations
6. Falcon tubes may be thrown away in regular trash bins

VIII. Calculation

Measurement of MBP is usually expressed in units of µg P g^{-1} soil. P content of fumigated and non-fumigated samples are converted to the final unit separately and then MBP is calculated by difference at the end.

1. Convert raw absorbance to concentration (mg P L^{-1}) using calibration curve constructed (as noted previously, calibration standards should be treated exactly the same way as samples, so the absorbance can be directly converted to concentrations in the extract, before dilution if samples were separately diluted). Multiply the concentration by dilution factor if diluted.
2. Multiply the concentration by the extract volume (i.e., 0.04 L), divide by soil mass (≈ 5 g), and multiply by 1000 (to convert from mg to µg) to yield concentration in µg P g^{-1} soil
3. If you have done P spike, you can correct the concentration (fumigated) by dividing it by the recovery rate

i. MBP = Fumigated P conc. /(recovery) – Non-fumigated P conc.
where recovery = (SPIKE P conc. – Non-fumigated P conc.)/50
e.g., a recovery of 0.8 means that 80% of 50 ppm is recovered

Example calculation:

Given
Fumigated
Absorbance = 0.383
Soil weight = 5.00 g
Non-fumigated
Absorbance = 0.199
Soil weight = 5.00 g
Standard curve: y = 8.0894x - 0.5096
Extraction volume = 0.04 L

Concentration in μg P g^{-1} soil (fumigated) = (8.0894*0.383 - 0.5096) mg P/L * 0.04 L / 5g * 1000 μg/1mg = 20.7 μg P g^{-1} soil
Concentration in μg P g^{-1} soil (non-fumigated) = (8.0894*0.199 - 0.5096) mg P/L * 0.04 L / 5g * 1000 μg/1mg = 8.80 μg P g^{-1} soil
MBP = 20.7 - 8.80 = 11.9 μg P g^{-1} soil

References:

Brookes, P.C., Powlson, D.S., Jenkinson, D.S., 1982. Measurement of microbial biomass phosphorus in soil. Soil Biology and Biochemistry 14, 319-329.

Suggested reading:

Brookes, P.C., Powlson, D.S., Jenkinson, D.S., 1984. Phosphorus in the soil microbial biomass. Soil Biology and Biochemistry 16, 169-175.

Citation:

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

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