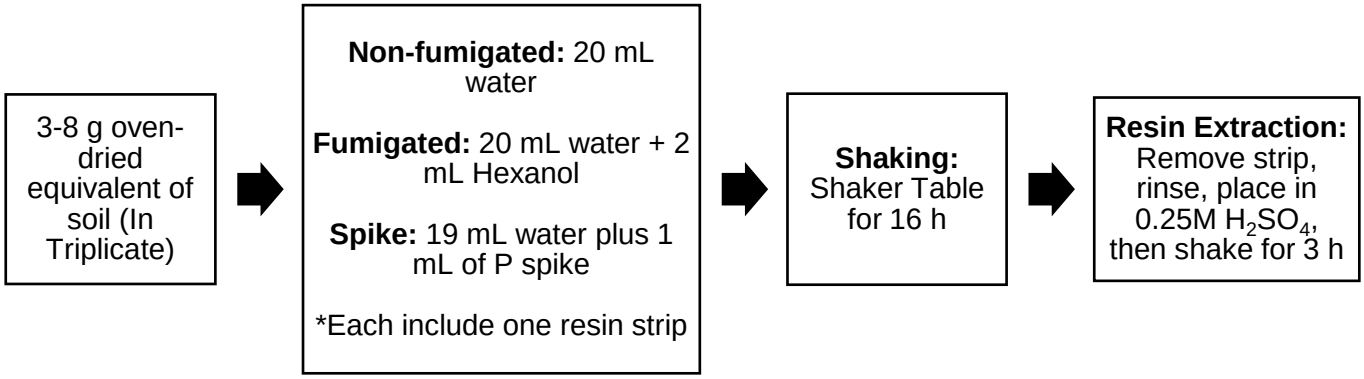
SOP: **Microbial Biomass Phosphorus (hexanol)**

Overview:

This standard operating procedure (SOP) describes a commonly used protocol for estimating pools of soil microbial biomass phosphorus (MBP). The method was originally reported by Kouno et al. (1995) for highly P-fixing soils, using liquid chloroform with resin to trap P released by lysed cells before their capture by colloid binding sites. The method was then modified by others to avoid dissolution of VWR/BDH sourced resins by chloroform, by switching to hexanol as a liquid fumigant. Key consumables are anion-exchange (AEM) or resin strips, hexanol, 0.25 M H_2SO_4, Murphy-Riley reagent, 50 mL and 15 mL centrifuge tubes. Key instrumentation are pipettes and tips, dispensette, horizontal shaker, and UV-spectrophotometer. Key safety considerations are the use of PPE at all times and a fume-hood when manipulating particularly hazardous substances (see below).



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: Safety goggles
Body Protection: Laboratory coat
Hand Protection: Nitrile gloves
Particularly hazardous substances: Concentrated sulfuric acid and hexanol should be handled in the fume-hood. Make sure to check Safety Data Sheet if unsure about how to handle these chemicals.
Specific details on these substances are incorporated in the **Detailed Procedure** below.

Instrumentation & Consumables:

Sample preparation

- Analytical balance (two decimal places sensitivity)
- 50 mL centrifuge tube
- Resin strip (1×4 cm), VWR anion-exchange BDH

Reagent preparation

- Analytical balance (at least two decimal places sensitivity)
- Sulfuric acid (H_2SO_4)
- Ascorbic acid
- Sodium bicarbonate ($NaHCO_3$)
- Ammonium molybdate
- Antimony potassium tartrate
- 5% sodium hydroxide (NaOH) solution
- Phosphate-P standard

Fumigation

- Horizontal shaker
- Dispensette
- Pipette and tips (100-1000 µL and 1-5 mL)
- Hexanol (2- or 3-hexanol)
- Phosphate-P standard

Extraction

- 15 mL centrifuge tubes
- Tweezers
- Squirt-bottle for water (18.2 MΩ cm^{-1})

Colorimetry

- 96-well microplate
- Spectrophotometer (microplate) capable of reading at 882 nm
- Pipette and tips (100-1000 uL)

Detailed Procedure:

I. **Sample Preparation**

1. Measure 3.00 – 8.00 g (± 0.1 g) of oven-dried equivalent of soil into 50 mL centrifuge tubes. Performing duplicates is recommended if possible.
   Note: 3.00 g is the minimum; 8.00 g is recommended for low P soils.

   i. Falcon brand tubes are recommended for avoiding leaks during fumigation.

ii. Record exact weight of soil to at least two decimal places (e.g. 5.00 g).

2. Prepare empty 50 mL centrifuge tube for blanks with no soil, but treated the same way as samples to account for background P.

II. **Reagent preparation**

1. Extractants and digestant
   i. AEM strip
      a. Shake AEM strips in 0.5 M $NaHCO_3$ for at least 3 h.
      b. Decant the $NaHCO_3$ careful not to drop any strip, and then shake the AEM strips in water (18.2 MΩ cm^{-1}).
      c. Repeat this procedure three times and this will ensure that AEM is pre-loaded with bicarbonate.

      Note: AEM strip can be reused until they deform, but make sure to pre-load them before every use.
   ii. 1.25 M (50 g/L = **5 % m/v**) NaOH
   iii. 0.25 M H_2SO_4

2. Standards
   i. Calibration standards (ranging from 0 – 10 mg phosphate-P/L) need to be made in H_2SO_4. Dilute commercial standard (1000 mg P/L) in H_2SO_4 to make calibration standards that are within the linear range and cover the concentration you are expecting for your soil samples.

3. Colorimetry reagents
   i. Murphy-Riley Solution A
      a. Dissolve 4.3 g ammonium molybdate in 400 mL of deionized water in a 1-litre beaker.
      b. Dissolve 0.40 g antimony potassium tartrate in 400 mL deionized water, then add to the ammonium molybdate solution in the beaker.
      c. Slowly and carefully, with stirring and while cooling in an ice bath, add 54 mL of H_2SO_4 [95-98%].
      d. 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
      a. 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). This must only be prepared on the day of use.
   iv. 5% NaOH (1.25 M or 50 g/L) (for adjusting pH for colorimetry)

III. Fumigation

1. To each 50 mL centrifuge tube with soil samples, add:
    i. Non-fumigated: 20 mL H_2O via dispensette + resin strip (using clean tweezers)
    ii. Fumigated: 20 mL H_2O via dispensette + resin strip + 2 mL hexanol via pipette
    iii. Spike: 19 mL H_2O via dispensette + resin strip + 1 mL of P spike **equivalent to 50 µg P per g soil*** (50 mg/kg) in sample.
        *e.g., for 2.00 g soil, this would entail adding 1 mL of 100 mg P/L.
2. Cap tightly and place on horizontal shaker for 16 hours on the low setting (120 rpm).

IV. Resin Extraction

1. After removing the samples from the horizontal shaker, remove resin strips from tubes (using clean tweezers). Rinse strips with deionized H_2O and place in 12 mL of 0.25 M H_2SO_4, in 15 mL tubes.
2. Place 15 mL tubes on horizontal shaker for 3 hours.
3. After removing from horizontal shaker, these extracts are now stable. Resin strips should be removed within 3 days to prevent degradation.
4. Colorimetry is performed directly in the well plate.
5. In each microplate well, add 120 µL of extract with 140 µL Murphey-Riley and 20 µL of 5% (1.25 M or 50 g/L) NaOH. The same ratio should be used for calibration standards at this time.
6. Let stand 45 minutes–1 hour (or until the highest standard stabilizes).
7. Read absorbance at 882 nm using a spectrophotometer.

V. Clean up

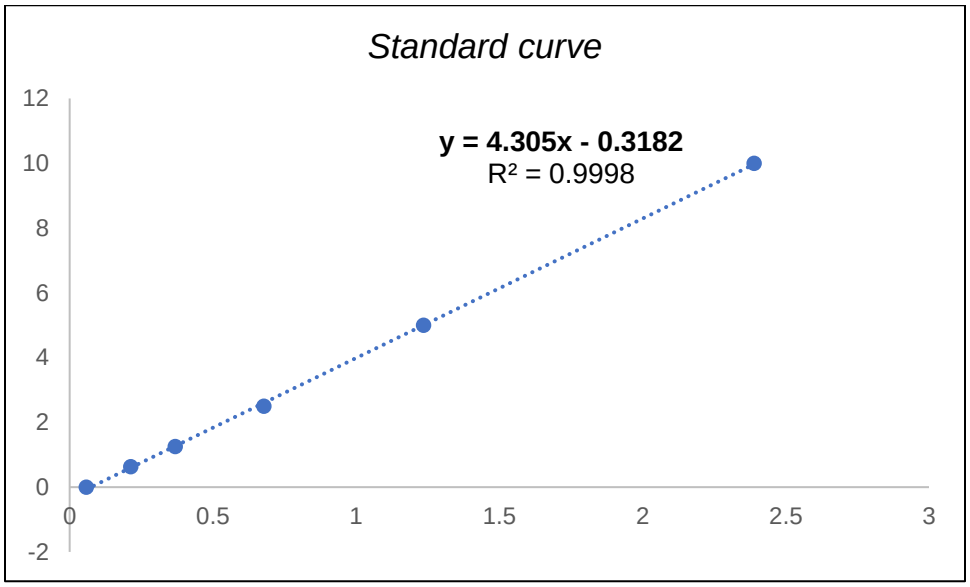
1. Make sure to clean up dispensette (rinse with water) and shaker (especially if tubes leak).
2. Collect solutions (except those that can go into drain with copious amount of water; check DRS for further information) into chemical waste bottle, clearly labelled with contents and their concentrations. Request pick up when the bottle is almost full. Clean up any spills with absorptive tissues and soap water immediately as needed. For spilled dilute acids, immediately neutralize with sodium bicarbonate and then clean up.

VI. Calculations

Measurement of microbial biomass P is expressed in units of mg P kg^{-1} soil.

1. Convert raw absorbance to concentration (mg P L^{-1}) using calibration curve.
2. Multiply the concentration by the extract volume and divide by soil mass to yield concentration in mg P kg^{-1} soil.
3. Determine spike recovery (equations below) and correct values, then subtract Non-fumigated from Fumigated value for MBP.

Example calculation:



i. Convert raw absorbance to concentration (mg P L^{-1}) using equation from standard curve.

ii. Correct for dilution by multiplying the measured concentration × its dilution factor.

		(i)		**(ii)**
Sample	**Absorbance (882 nm)**	**Concentration (mg P/L)**	**Dilution factor**	**Concentration corrected for dilution (mg P/L)**
Fumigated	1.364	5.55	1	5.55
Non-fumigated	0.916	3.63	1	3.63
Spike	1.286	5.22	4	20.87

iii. Multiply the concentration corrected for dilution by the volume of H_2SO_4 in extraction (12 mL), to obtain the total P in soil sample (µg).

iv. Divide the total P in soil sample (µg) by the mass of soil (g), to convert to µg P/g soil (also equivalent to mg P/g soil).

		(iii)		**(iv)**
Sample	**Volume of H_2SO_4 (mL)**	**Total P in sample (µg)**	**Soil mass (g)**	**Convert to (µg P / g soil)**
Fumigated	12	66.65	5.01	13.30
Non-fumigated	12	43.50	5.02	8.67
Spike	12	250.47	5.00	50.09

Then,

Spike Recovery is calculated as:

$$Spike\ Recovery = \frac{S - NF}{50\ mg/kg}$$

Example:

$$Spike\ Recovery = \frac{50.09\frac{mg}{kg} - 8.67\frac{mg}{kg}}{50\frac{mg}{kg}} = 0.829$$

Note: Recoveries of 50-90% are common except in highly P-fixing soils (e.g., Oxisols).

MBP Spike Corrected is calculated as

$$MBP\ \left(\frac{mg}{kg}\right) = \frac{(F - NF)}{Spike\ Recovery}$$

Example:

$$MBP\ \left(\frac{mg}{kg}\right) = \frac{(13.30\frac{mg}{kg} - 8.67\frac{mg}{kg})}{0.829} = 5.60\ mg\ P\ kg^{-1}\ soil$$

Abbreviations: S, spike; F, fumigated; NF, non-fumigated.

Note: Be cautious with soils that have high resin P background, i.e., NF samples (> 50 mg P/L) because microbial P may not be detectable.

References:

Hedley, M.J., Stewart, J.W.B., Chauhan, B.S., 1982. Changes in Inorganic and Organic Soil Phosphorus Fractions Induced by Cultivation Practices and by Laboratory Incubations. Soil Science Society of America Journal 46, 970-976.

Kouno, K., Tuchiya, Y., & Ando, T. 1995. Measurement of soil microbial biomass phosphorus by an anion exchange membrane method. Soil Biology and Biochemistry, 27(10), 1353-1357.

Suggested reading:

Hedley, M.J., White, R.E. & Nye, P.H. (1982). Plant-Induced Changes In The Rhizosphere Of Rape (*Brassica napus* Var. Emerald) Seedlings. New Phytologist, 91: 45-56. https://doi.org/10.1111/j.1469-8137.1982.tb03291.x

McLaughlin, M. J., Alston, A. M., & Martin, J. K. (1986). Measurement of phosphorus in the soil microbial biomass: a modified procedure for field soils. Soil Biology and Biochemistry, 18(4), 437-443.

Citation:

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