SOP: **Enzyme assays (*p*NP)**

Overview:

This standard operating procedure (SOP) describes the measurement of potential soil enzyme activities using *para*-nitrophenyl (*p*NP) linked substrates that releases *para*-nitrophenol (*p*NP) when hydrolyzed by soil enzymes. The method was originally reported by Tabatabai and Bremner (1969) and further modified in Tabatabai (1994), Margenot et al. (2018), and Daughtridge et al. (2021). Field moist soil samples (stored at ≈ 4 °C and hand-crumbled) or air-dried and ground to pass a < 2 mm sieve are used.

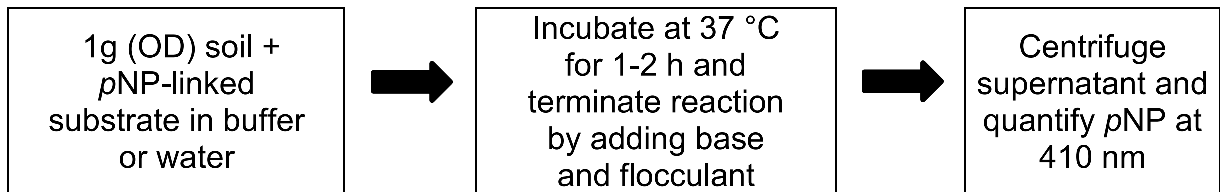
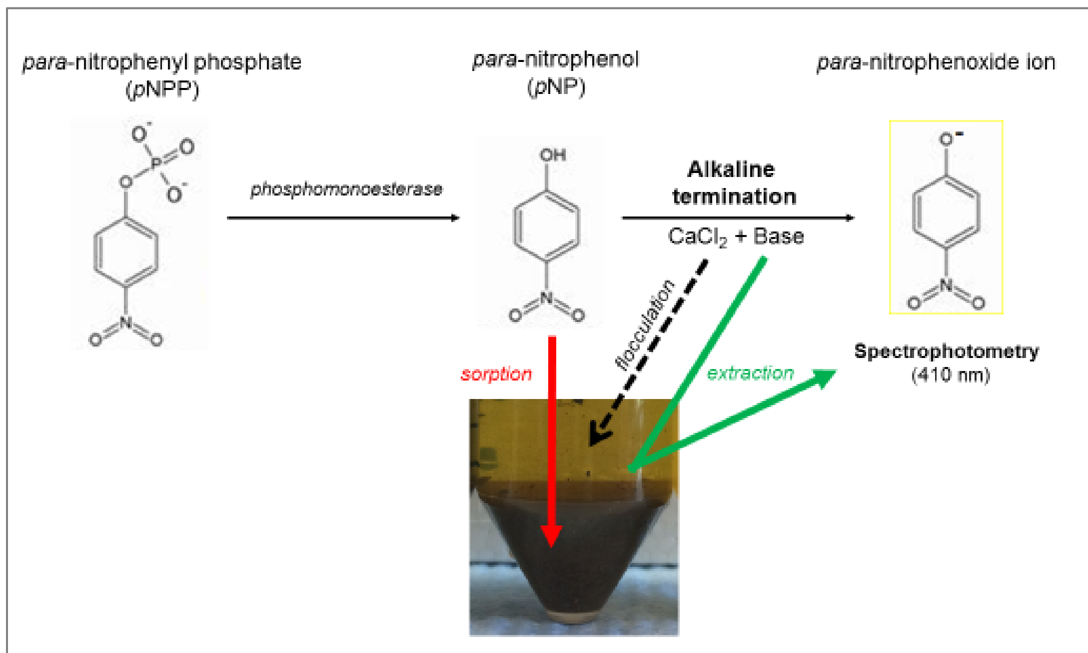


Figure shows how a *p*NP-linked substrate yields *p*NP during enzyme assay and how *p*NP interacts with the soil matrix.



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: Lab coat

Hand Protection: Gloves
Particularly hazardous substances: *para*-nitrophenol (also called 4-nitrophenol) and hydrochloric acid
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

Reagent preparation

- Analytical balance (at least three decimal places sensitivity)
- pH meter
- Pipettes & tips (100 µL – 4 mL)
- 500 mL volumetric flask
- Boric acid
- Citric acid (monohydrate)
- Maleic acid
- NaOH
- HCl
- *p*NP-substrate
- THAM (also called Tris, Trizma base)
- $CaCl_2 \cdot 2H_2O$
- *p*NP (4-nitrophenol)

Enzyme assay

- Pipettes & tips capable of dispensing 100 µL – 4 mL
- Water bath capable of maintaining 37 °C

Colorimetry (quantification of *p*NP)

- microcentrifuge and microcentrifuge tubes (1-2 mL)
- microplate spectrophotometer (absorbance measured at 410 nm)
- 96-well microplates

Detailed Procedure:

I. **Sample and Blank Preparation**
   1. Measure 1.00 ± 0.02 g of oven-dried equivalent of soil into 50 mL centrifuge tube. Record exact weight of soil to at least 1/100th of one gram (1.XX g)

2. In addition to the soil samples, prepare blanks (first two are required) that are going to be treated the same way as samples (section II).
    i. True blank with no soil and no substrate: accounts for background absorbance of matrix solution and also helps detect any contamination throughout assay. This can be used as 0 mM *p*NP standard for the calibration curve.
    ii. Blank with same *p*NP-substrate solution as samples, but with no soil: accounts for abiotic hydrolysis of substrate during storage, incubation, and termination.
    iii. Blank with *p*NP solution (not *p*NP-substrate) and soil: accounts for sorption/recovery of *p*NP as *p*NP can be bound to soil particle surface. Soils can vary in sorption capacity of pNP, and thus a priori testing of representative samples from the study site is recommended to check if recovery correction is necessary. The concentration of *p*NP to be spiked should be close to the amount your soil hydrolyzes.
    iv. Blank with soil but without substrate: account for dissolved organic matter (DOM) interference. This is critical when you see dark brown-black color in your terminated assay solutions even after centrifugation because this can contribute to absorbance at 410 nm that is not from *p*NP.
    * ii and iv accounts for overestimation of activity due to absorbance that is not from soil enzyme activity, whereas iii accounts for underestimation of activity. Detailed illustration of these blanks can be found in Daughtridge et al. (2021).

II. Reagent preparation

1. Modified universal buffer (MUB)

   * This is not necessary if you use water as matrix instead of buffer. Buffer has been traditionally used to maintain reaction solution at assumed pH optimum (for each enzyme) during assay. However, evidence suggests that buffer does not maintain the pH at target pH during assay (Li et al., 2021), and assumed pH optimum does not necessarily correspond to highest activity (Wade et al., 2021). Therefore, the use of water is suggested to avoid false assumption of pH optimum and conduct assay in more natural condition (i.e., soil pH).
    i. Stock, as per Turner method (Turner, 2010)
        - Make solution of the following for a final volume of 500 mL using a volumetric flask
            a. 6.3 g boric acid
            b. 14.0 g citric acid (monohydrate)
            c. 11.6 g maleic acid
            d. 12.1 g Trizma base
            e. 19.5 g NaOH
        - Stock keeps in fridge for ~2 weeks without risk
    ii. Dilute this stock 4x (1:3) with distilled water to make a working MUB solution

- Use NaOH and HCl solutions (5-10 w/v or v/v%) to adjust the working MUB solution pH to target. See table below for target pH values (based on enzyme optima)

Soil enzyme activities that can be assayed with *p*NP-linked substrate, and commonly used assay conditions. Adapted from Table 2 of Daughtridge et al. (2021). EC number is Enzyme Commission number.

Enzyme		Substrate	pH	Incubation Time	[S]* (mM)	EC number
P-cycling	Acid phosphomonoesterase	*p*NP-phosphate	6.5	1	10	3.1.3.2
	Alkaline phosphomonoesterase	*p*NP-phosphate	11	1	10	3.1.3.1
	Phosphodiesterase	*bis-p*NP-phosphate	8	1	10	3.1.4
S-cycling	Arylsulfatase	*p*NP-sulfate	5.8	1	10	3.1.6.1
C-cycling	β-Glucosidase	*p*NP-β-glucopyranoside	6	1	10	3.2.1.21
	β-Galactosidase	*p*NP-β-galactopyranoside	6	1	10	3.2.1.23
	Cellobiohydrolase	*p*NP-β-D-cellobioside	6	2	2	3.2.1.4
C- & N-cycling	Chitinase	*p*NP-N-acetyl- β -D-glucosaminide	5.5	1	10	3.2.1.14

** Substrate concentration ([S]) may be well below saturation for many enzymes. It is recommended that Km be determined for each soil sample or that a high [S] be used. See Margenot et al. 2018 for discussion.*

2. *p*NP-substrate solution, made in buffer at final assay pH or in water
    i. For traditional, operational assessments of enzyme activities, *p*NP-substrate solution of 0.010 M = 10 mM per g soil is used
        - Use 20 mM for P-cycling enzymes
        - However, high concentrations of substrate achieve substrate saturation of an enzyme → better reflect activity differences at V_{max}
            a. Lower risk of false negatives
            b. See Margenot et al. (2018) for details
        - Most of the substrate solutions should be stable at 4 °C for ~ 1 week (Daughtridge et al., 2021)

    ii. Note that this SOP describes single-point enzyme assay involving single substrate concentration. For kinetic characterizations, you need to vary substrate concentrations. See Margenot et al. 2018 SBB for example for phosphomonoesterase.
3. 0.1 M THAM at pH 12
    i. Prepare 0.1 M THAM and adjust the pH using NaOH and HCl solutions (5-10 w/v or v/v%) to yield final pH of 12.
        - Base is used to halt reaction and activate *p*NP chromophore via alkalinization (see Fig. 1)
        - The base also helps extract *p*NP sorbed to soil particles
        - 0.5 M NaOH was originally used until evidence of its hydrolysis of substrates and thus potential overestimation of

potential activities appeared. Although this can be corrected with your soil-free blank, abiotic hydrolysis may be higher than enzyme activity in some cases (Daughtridge et al., 2021). THAM is also preferred because NaOH can cause precipitation of Ca due to high pH after termination of reaction

    ii. THAM is also called Tris, Trizma base

4. 2.0 M $CaCl_2$
    i. Prepare 2.0 M $CaCl_2$ solution
        - It is used to flocculate soil after termination with base
        - 2.0 M is higher than traditional 0.5 M concentration. This is to avoid DOM co-extraction with alkaline termination, especially for high OM soils. See Margenot et al. (2018) for rationale.
5. *p*NP (4-nitrophenol) standards made in same matrix (buffer or water) as enzyme assays and terminated the same way as samples, for calibration curve for colorimetry.

    Example Approach:

    i. Make 5 mM (or lower) *p*NP solution using the same solution you used for making *p*NP-substrate solution. 5 mM *p*NP solution should be stable for ~ 1 week (at least for water matrix).
    ii. Dilute the *p*NP solution with same solution you just used to make working standards with *p*NP concentration ranging from 0-0.8 mM.
    iii. Terminate the working *p*NP standards the same way as you do with your samples (refer to Detailed Procedure); for example, 5 ml of 0.8 mM *p*NP solution terminated with 4 ml of 0.1M THAM + 1 mL of 2 M $CaCl_2$ to get 0.4 mM *p*NP solution in same solution matrix as samples after termination. The terminated *p*NP standards would be the calibration standards that will be used to construct standard curve.

III. **Reaction**

1. Turn on the water bath in advance so that the temperature stabilizes at 37 °C. Add water if the water level is too low.
2. Uncap the 50 mL centrifuge tube (with weighed soils) and add 5 mL of *p*NP-linked substrate solution to the 50 mL centrifuge tube
3. Swirl the centrifuge tubes for 1 min. Place plastic wrap on top of the tubes to avoid evaporation during incubation. Then incubate the tubes at 37 °C for 1 or 2 hr (depends on which enzyme being evaluated) using the water bath. To keep the tubes inside the water bath, place weight (e.g., tray + flasks filled with water) on top of the tubes.

IV. Alkalization and centrifugation

1. At the end of incubation, take centrifuge tubes out of the water bath and terminate reaction by adding 4 mL of 0.1 M THAM (reactions of soils and working standards should turn yellow in the presence of *p*NP)
2. Add 1 mL 2.0 M $CaCl_2$ to flocculate; let sit ~5 min or until clear aliquot is evident

3. Transfer 1 mL of supernatant of resting reaction solution into a microcentrifuge tube and spin down (14,000 rpm for 1 min 45 s) or until a clear supernatant is obtained.
4. Colorimetry
5. Making sure to not disturb sedimented pellet at bottom of microcentrifuge tube, transfer 200 µL to the 96-well microplate. For some enzymes (in particular phosphomonoesterase) and/or some soils, you may need to dilute supernatant using nano-pure water to achieve an absorbance value in the linear range of the calibration curve. You can dilute the sample directly in well plate (e.g., adding 100 µL of sample + 100 µL of water)
6. Turn on the microplate spectrophotometer to warm up, set absorbance reading at 410 nm (literature uses absorbance at 405-420 nm to quantify *p*NP).
7. Measure absorbance at 410 nm of the calibration standards, soil reaction extracts, and blanks. Make sure that the standard curve is linear (typically $R^2 > 0.99$), and absorbance of your samples and blanks are within the linear range of constructed standard curve.

V. Clean up

1. Collect all solutions containing *p*NP or *p*NP-linked substrate (and buffer if used) 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.

VI. Calculations

Potential enzyme activities are usually expressed in units of µmol *p*NP g^{-1} soil hr^{-1}. You may also see these reported as mg *p*NP g^{-1} soil hr^{-1}, but this is inaccurate because the mass of *p*NP is arbitrary when considering the capacity of an enzyme to hydrolyze real substrates. Note that enzyme activities are rates of substrate transformation but is “potential” because substrate concentrations in assays are likely much higher than in situ conditions (actual activities). However, this method has been widely applied and “potential” activity has been shown to detect differences in factors such as land use and management.

1. Convert absorbance reading to concentration (mM) based on the calibration curve you obtained from *p*NP standards
2. If you have diluted the samples, multiply the concentration by dilution factor
3. If you have done the *p*NP recovery blank, divide the concentration from step 2 by the recovery ratio to correct for the sorption. Recovery calculated as ratio of mole of *p*NP recovered (concentration of recovery blank from step 2 minus concentration of DOM blank, then multiplied by final volume) to mole of *p*NP spiked (concentration of spike solution multiplied by volume spiked).
4. Convert the hydrolysis blank absorbance to concentration (mM) using the same standard curve as step 1 and subtract the resulting concentration from corresponding sample concentrations calculated in step 3 (make sure that initial substrate concentration of blanks and samples correspond to each other)

5. If you have done the DOM blank, convert the DOM blank absorbance to concentration (mM) using the same standard curve as step 1 and subtract the resulting concentration from sample concentration with same soil used
6. Regardless of whether you have done the above correction (you should) or not, convert the concentration (mM) to the amount (mmol) of *p*NP hydrolyzed in the final solution by multiplying the concentration with the volume of your final solution in L after termination (10 mL = 0.01L). Then, multiply the value by 1000 to convert to μmol.
7. Divide the μmol *p*NP hydrolyzed by the amount of oven-dry soil (g) and duration of incubation (hr) to obtain μmol *p*NP g^{-1} soil hr^{-1}

Example calculation:

Given

Sample Absorbance = 1.021

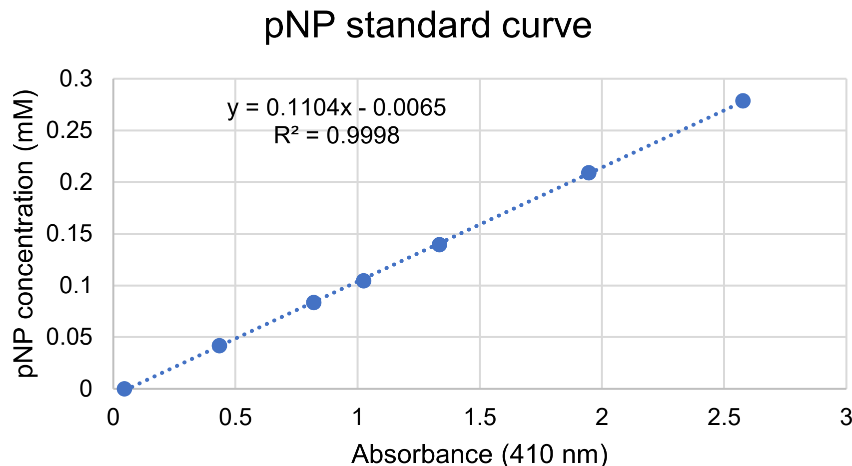
Dilution = 2 (for both sample and blank)

Standard curve y = 0.1104x - 0.0065

*p*NP recovery = 0.973

Hydrolysis blank absorbance = 0.115

DOM blank absorbance = 0.09



Convert absorbance to concentration

Sample conc. = 0.1104*1.021 - 0.0065 = 0.106 mM

Hydrolysis blank conc. = 0.1104*0.115 - 0.0065 = 0.00484 mM

DOM blank conc. = 0.1104*0.09 - 0.0065 = 0.00203 mM

Convert sample concentration to activity (with correction for DOM and sorption)

$$Potential\ activity = \frac{\left(\frac{0.106mM * 2}{0.973} - 0.00484mM * 2 - 0.00203mM * 2\right) * 0.01L * \frac{1000\mu mol}{mmol}}{1g\ soil * 1hr}$$

$$= 2.05 \ \mu\text{mol} \ p\text{NP g}^{-1} \text{ soil h}^{-1}$$

Convert sample concentration to activity (without correction for DOM and sorption)

$$Potential\ activity = \frac{(0.106 * 2 - 0.00484 * 2) * 0.01 * 1000}{1 * 1}$$
$$= 2.02 \ \mu\text{mol} \ p\text{NP g}^{-1} \text{ soil h}^{-1}$$

References:

Daughtridge, R.C., Nakayama, Y., Margenot, A.J., 2021. Sources of abiotic hydrolysis of chromogenic substrates in soil enzyme assays: Storage, termination, and incubation. Soil Biology and Biochemistry 158, 108245.

Li, C., Wade, J., Margenot, A.J., 2021. Modified universal buffer does not necessarily maintain soil enzyme assay pH. Biology and fertility of soils 57, 869-872.

Margenot, A.J., Nakayama, Y., Parikh, S.J., 2018. Methodological recommendations for optimizing assays of enzyme activities in soil samples. Soil Biology and Biochemistry 125, 350-360.

Tabatabai, M., 1994. Soil enzymes. Methods of soil analysis: part 2—microbiological and biochemical properties, 775-833.

Tabatabai, M., Bremner, J., 1969. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biology and Biochemistry 1, 301-307.

Wade, J., Li, C., Vollbracht, K., Hooper, D.G., Wills, S.A., Margenot, A.J., 2021. Prescribed pH for soil β-glucosidase and phosphomonoesterase do not reflect pH optima. Geoderma 401, 115161.

Suggested reading:

1. Burns et al 2013. Soil enzymes in a changing environment: Current knowledge and future directions. *Soil Biology and Biochemistry* 58:216. https://doi.org/10.1016/j.soilbio.2012.11.009

2. Nannipieri et al. 2018. Soil enzyme activity: a brief history and biochemistry as a basis for appropriate interpretations and meta-analysis. *Biology and Fertility of Soils* 54:11. https://doi.org/10.1007/s00374-017-1245-6

Citation:

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