Food and Agriculture Organization of the United Nations

Standard operating procedure for soil enzyme activities

β-glucosidases, arylsulfatase, N-acetyl-β-glucosaminidase, dehydrogenase, phosphomonoesterases

GLOBAL SOIL PARTNERSHIP

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Abbreviations

CEC	cation exchange capacity
CL	critical limit
DI	deionized
EA	enzyme activity
EC	Enzyme Commission
EPA	United States Environmental Protection Agency
GlcNAc	N-acetyl-β-D-glucosamine
GLOSOLAN	Global Soil Laboratory Network
GSP	Global Soil Partnership
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride
JWG	joint working group
MUB	modified universal buffer
NAG	N-acetyl-β-D-glucosaminidase
NETSOB	International Network on Soil Biodiversity
NRCA	National Resource Conservation Service
PME	phosphomonoesterase
PT	proficiency test
PNAG	*p* nitrophenyl N acetyl β-D-glucosaminide
PNG	*p*-nitrophenyl-β-D-glucopyranoside
PNP	*p*-nitrophenol
PNPP	*p*-nitrophenyl phosphate
PNS	*p*-nitrophenyl sulphate
RCY	relative cumulative yield
RPD	relative percent difference
SDS	safety data sheet
SH	soil health
SMAF	Soil Management Assessment Framework
SOC	soil organic carbon
SOM	soil organic matter
SOP	standard operating procedure
THAM	tris(hydroxymethyl)aminomethane
TPF	triphenylformazan
TTC	2,3,5-triphenyltetrazolium chloride
USDA	United States Department of Agriculture

Chemical elements

Al	aluminium
C	carbon
Fe	iron
N	nitrogen
P	phosphorus
S	sulphur

1. Introduction to the determination of soil enzymes

Healthy soils are biologically active, productive and resilient (Lehman *et al.,* 2015; Karlen and Rice, 2015; Mbuthia *et al.*, 2015; Hatfield, Sauer and Cruse, 2017; Mendes *et al.,* 2024; Porto Muniz *et al.,* 2021; Passinato *et al.*, 2021; Chaer *et al.*, 2023; Serafim *et al.*, 2023), with a better capacity for carbon sequestration (Stott *et al.,* 2010; Dick and Burns, 2012; Lal, 2015; Hok *et al.*, 2018, Acosta-Martinez *et al.*, 2021, Chaer *et al.*, 2023), nutrient use efficiency (Drinkwater and Snapp, 2007; Anghinoni and Vezzani, 2021; Chaer *et al.*, 2023), soil pathogen control (Larkin, 2015) and pesticide bioremediation (Portilho *et al.*, 2015). All these functions result in greater sustainability from both an economic profitability and an improved environmental quality point of view (Prado *et al.*, 2016; Rinot *et al.*, 2019).

Therefore, assessment tools are needed to evaluate the impact of agricultural management systems on critical soil functions related to soil health (SH). Due to the limitations of traditional soil chemical analyses in their ability to fully express the benefits of implementing sustainable soil management practices (Drinkwater and Snapp, 2007; Anghinoni and Vezzani, 2021), research was conducted into other methods more able to fully assess soil health (Stott *et al*., 2010; Raiesi and Kabiri, 2016; Karlen, Stott and Mikha, eds, 2021). Several studies have shown that because soil microbial indicators are associated with the living portion of soil, they are more sensitive to changes in management than chemical and physical properties (Miller and Dick, 1995; Bandick and Dick, 1999; Kandeler, Tscherko and Spiegel,1999; Bending *et al.*, 2004; Geisseler and Horwath, 2009; Peixoto *et al.*, 2010; Singh *et al.*, 2018; Lorenz *et al.*, 2020; Santos *et al.*, 2022). For this reason, several soil microbial indicators were included in the Food and Agriculture Organization of the United Nations (FAO)'s "Protocol for the assessment of Sustainable Soil Management" (FAO-ITPS, 2020). In this regard, soil enzymes stand out, since they are more sensitive to soil management changes than the overall quantification of soil organic matter (SOM) (Bandick and Dick, 1999; Ndiaye *et al.*, 2000; Balota *et al.*, 2004; Peixoto *et al.*, 2010; Stott *et al.*, 2010; Dick and Burns, 2011; Hok *et al.*, 2018; Singh *et al.*, 2018; Acosta-Martínez *et al.*, 2021; Santos *et al.*, 2022; Chaer *et al.*, 2023). Some advantages in using soil enzymes as SH indicators are that their assays are relatively inexpensive, rapid, and have high throughput analytical procedures that have been highly correlated to other soil biological analyses (Dick and Burns, 2011; Nannipieri, Trasar-Cepeda, and Dick, 2018; Acosta-Martinez *et al.*, 2021; Passinato *et al*. 2021).

Soil's capacity to stabilize and protect enzymes is an early-warning indication of conditions favouring SOM stabilization and storage. Given all these advantages, in Brazil, for example, arylsulphate sulphohydrolase (arylsulphatase) and β-D-glucoside-glucohydrolase (β-glucosidase) determinations are performed in routine commercial soil analyses (Soil Bioanalysis, SoilBio, technology) and have been used successfully on large-scale SH assessments since 2020 (Mendes *et al.*, 2021, 2024). Enzyme assays are also becoming part of commercial laboratories in the United States of America.

The intensity of tillage in conjunction with the quality and quantity of plant residues returned to soil are strong influences on the soil biological functioning of agricultural systems (Acosta-Martinez, Klose and Zobeck, 2003; Acosta-Martinez *et al*., 2010; Bandick and Dick, 1999; Peixoto *et al.*, 2010). Thus, different management systems leave their "fingerprint", or "biological signature" in the soil environment. Soil´s ability to hold a "memory" of the type of management to which it has been submitted is closely associated with its biological component (Bandick and Dick, 1999; Balota *et al.,*

2004; Dick and Burns, 2011; Peixoto *et al.*, 2010; Mankolo *et al.*, 2012; Singh *et al.*, 2018). In particular, extracellular enzymes enable the persistence of past management effects in soil nutrient cycling and SOM dynamics. The measurement of potential enzyme activities (EAs) represents the sum of activities associated with living organisms (microorganisms, plants and animals) and enzymes secreted by past generations of organisms (an abiontic component). The enzyme abiontic component is associated with the non-living fraction which accumulates in soil and is protected from proteases through their adsorption onto clay particles and organic matter (Wallenstein and Burns, 2011). Since the stabilized fraction (abiontic component) is not part of the living cell, it can be more protected from seasonal or environmental factors (Knight and Dick, 2004), and persist longer on soil surfaces than other measurements of the living component, which are important advantages of enzyme activities as SH indicators.

There is no doubt that a rigorous standardization of methods and protocols, along with appropriate proficiency testing, will facilitate the major goal of the harmonization process to collect high quality data on soil enzymes all over the world (Karlen, Stott and Mikha, eds, 2021; Nannipieri, Trasar-Cepeda, and Dick, 2018). The universal adoption of standardized protocols across laboratories and regions will also make regional and national compilations of SH data easier to interpret.

During the fifth Global Soil Laboratory Network (GLOSOLAN) meeting (held virtually 23–25 November 2021), as part of the decisions made about the harmonization of the standard operating procedures (SOPs) for soil biological analysis, a joint working group (JWG) on soil microbes and soil fauna was established from GLOSOLAN and the International Network on Soil Biodiversity (NETSOB) (two technical networks of the Global Soil Partnership [GSP]).

At the first GLOSOLAN/NETSOB JWG meeting on soil biological analysis on 15 March 2022, it was decided that soil enzymes would be the first SOPs to be harmonized, with five soil enzymes being identified: β-glucosidase (Chemical Abstracts Service [CAS] 9001-22-3), arylsulphatase (CAS 552858-79-4), acid phosphomonoesterase (PME) (CAS 9001-77-8), alkaline PME (CAS 9001-78-9), N-acetyl-β-D-glucosaminidase (NAG) (CAS 3459-18-5) and dehydrogenase (CAS 9028-88-0). With the exception of dehydrogenase, the four soil enzymes selected by the group are among the most typically evaluated in agricultural soils, having been previously selected by national SH initiatives, such as the United States Department of Agriculture (USDA)'s Natural Resource Conservation Service (NRCA), based on their importance to carbon (C) (β-glucosidase), C and nitrogen (N) (NAG), phosphorus (P) (acid and alkaline PMEs) and sulphur (S) (arylsulphatase) cycling (Acosta-Martinez *et al.*, 2021).

Traditionally, Tabatabai's procedures for β-glucosidase, arylsulphatase, NAG, and acid and alkaline phosphatase (performed under optimal conditions, and as described in this document), have provided the foundation for the current understanding of enzymatic responses to soil and crop management, as well as their fate and persistence mechanisms (Acosta-Martinez *et al.*, 2021). Since the early 1970s, thousands of papers have been published on using these procedures and they have been shown to be sensitive in detecting changes due to land management, physical disturbance and selected contaminants worldwide (Nannipieri, Trasar-Cepeda, and Dick, 2018; Acosta-Martinez *et al.*, 2021).

As an assay methodology is determined by time, labour, and reagent costs, each methodological decision should be weighed against the relative improvement in accuracy (Nakayama *et al.,* 2023). A thorough investigation on pH optima, controls for non-enzymatic contributions to apparent activity, matrix type (buffer or water), substrate concentration, and on how the accuracy and feasibility of soil

enzyme activity assays can be maximized is provided in Daughtridge, Nakayama and Margenot (2021), Li, Wade and Margenot (2021), Wade *et al.* (2021) and Nakayama *et al.* (2023).

Before the presentation of each enzyme SOP, aspects common to all will be discussed jointly in Section 1.1, Section 1.2 and Section 1.3. These sections build on Dick *et al.* (1996), Lorenz and Dick, (2011) and Acosta-Martinez *et al.* (2021).

1.1. Soil sampling

To measure soil microbial properties, sampling times should be carefully selected to overcome problems related to seasonal variability. Overall, the most important action for valid, multiyear comparisons is to collect samples at the same time every year and to report the sampling date to make possible comparisons across studies.

In temperate regions, either the mid-to-late spring period or late autumn period (when there have been no fresh inputs of organic matter or fertilizers), have been suggested as the best times to collect soil samples for microbial analyses (Lorenz and Dick, 2011). Spring sampling coincides with soil fertility testing (Acosta-Martinez *et al.*, 2021).

In contrast to temperate soils, there is little information regarding the advantages and disadvantages of using different sampling times in the assessment of microbial indicators in tropical soils. However, Brazil is a tropical country in which soil enzymes have been used for large scale on-farm SH assessments since July 2020 (SoilBio technology), and can be used as an example. The country's sampling of soil enzymes (arylsulphatase and β-glucosidase), is based on the adoption of the FERTBIO soil sample concept, requiring soil samples to have been collected at the end of the rainy season, at the post-harvest stage and air-dried prior to laboratory assays (Mendes *et al.*, 2019). The FERTBIO soil sample concept considers the advantages (primarily reductions in operational costs) offered to farmers and commercial laboratories from standardizing the sampling time and pretreatment procedures for soil chemical fertility (FERT) and biological (BIO) analyses.

Regarding the soil sampling protocol, unless there is strong evidence for a lack of uniformity at the sampling site, (such as obvious changes in soil type, topography, or land use) a simple random sampling is appropriate for a SH assessment. If the site is not uniform and subareas can be identified, then the stratified random sampling approach should be used. This requires a random sample to be collected within each subarea (Lorenz and Dick, 2011).

Judgment sampling (where an area is selected at a site that is seen as "typical" while other areas thought to be nonrepresentative of the larger area are avoided [Dick *et al.*, 1996; Wollum II, 1994]) is a highly-biased approach that is dependent on the expertise of the investigator and is therefore not recommended. Composite sampling is recommended instead, by taking several field samples to adequately represent the area of consideration, ensuring that they are then thoroughly mixed to form one composite or bulk sample (Dick, Breakwell and Turco, 1996; Wollum II, 1994).

Typically for sampling, the topsoil is the most sensitive zone for measuring the effects of soil use, disturbance, or management on soil enzyme activities. Studies have shown that hot spots for biological properties tend to be within the top 0–15 cm of soil, with some studies taking shallower soil depth increments, and finding significant differences between 0–5 cm and 5-10 cm or 5–15 cm (Lorenz and Dick, 2011; Bajouco *et al.,* 2020; Acosta-Martinez *et al*., 2021). In the United States of America,

the standard depth for sampling agricultural soils is 0–15 cm because that is the typical, historical depth of tillage (Acosta-Martinez *et al.,* 2021). However, for large scale on-farm SH assessments in Brazil, the diagnostic soil layer chosen is 0–10 cm depth, using the same sampling procedures for soil chemical fertility analyses (Mendes *et al.,* 2021, 2024; Chaer *et al.,* 2023). Considering the lack of a global consensus on a depth value, and as the more superficial the soil layer, the greater the chances of detecting the impacts of different management systems, we recommend using 0–10 cm as the sampling depth in this protocol, to be adopted worldwide. Using the 0–10 cm layer as a diagnostic layer, especially in areas under no-till systems and/or with minimal soil preparation, maximizes the probability of detecting differences between treatments, and preventing potential effects from being diluted when sampling at a wider depth range of 0–15 cm or 0–20 cm.

1.2. Sample preparation and storage

The objective for measuring enzyme activities will dictate the method of soil sample handling and storage (Lorenz and Dick, 2011).

If the goal is to have enzyme activities that reflect the true state of the ability of the soil to perform a given enzymatic reaction under *in situ* conditions, field-moist soil samples and cold storage at 4 °C are the best approach (Lorenz and Dick, 2011). In this case, field-moist soil samples should be analysed as soon as possible (within days), to obtain results that are most characteristic of the sampling day. Soil samples should be kept fresh in a sealed plastic bag, transported to the laboratory in an insulated cooler box and stored at 4 °C until they can be processed (Acosta-Martinez *et al.,* 2021)

If the goal is to use enzyme activities to assess SH, then air-dried soil samples are preferred (drying in a forced air oven at 35 °C [± 5 °C] is also acceptable).

The effects of the pretreatment of soils (air-drying or field-moist samples) and sample storage methods (freezing at −20 °C or −80 °C, or air-drying and kept cold at 4 °C) prior to enzyme analysis are complex and depend on the combination of soil enzyme, soil type, sampling location and season. The effect of sample storage has been widely discussed in the literature (such as Speir and Ross, 1975, 1981; Ladd 1985; Bandick and Dick, 1999; Rao *et al.*, 2003; Nannipieri, Kandeler and Ruggiero, 2002; Hinojosa *et al.*, 2004; Zornoza *et al.*, 2006; Lee *et al.*, 2007; Wallenius *et al.*, 2010; Turner and Romero, 2010; Abellan *et al.*, 2011; Lopes *et al.*, 2013; Mendes *et al.*, 2019; Acosta-Martinez *et al.,* 2021). These effects are related to changes occurring in the different components that contribute to the overall activity of a soil enzyme (Nannipieri, Kandeler and Ruggiero, 2002; Rao *et al.*, 2003; Abellan *et al.*, 2011). As pointed out by Peoples and Koide (2012), storage effects may be tolerable if relationships among “treatments” are unaltered.

Air-drying has the largest impact on enzymes associated with a viable microbial biomass (such as intracellular enzymes), with less effect on the extracellular enzymes (in particular the extracellular fraction stabilized in the soil matrix). After air-drying, enzyme activities are generally reduced (Pancholy and Rice, 1972; Speir and Ross, 1981; Bandick and Dick, 1999; Lee *et al.*, 2007; Wallenius *et al.*, 2010; Abellan *et al.*, 2011; Lopes *et al.*, 2015; Mendes *et al.*, 2019; Reardon *et al.*, 2022). However, some reports have shown that specific enzyme activities can increase (El-Shinnawi, Shehata and El-Shimi, 1982; Eivazi and Tabatabai, 1977; Eivazi and Tabatabai, 1990; Gianfreda and Bollag, 1996; Bandick and Dick, 1999), remain unchanged (Zornoza *et al.*, 2006), or become altered in unpredictable ways (Rao *et al.*, 2003).

Air-drying is an advantage for SH evaluations as it reduces the impact of conditions that affect the highly-variable living microbial component relative to enzyme activity (Acosta-Martinez *et al.*, 2021). Furthermore, enzymes activities after air-drying are likely to better reflect the true long-term trajectory of a given management practice on SH (Lorenz and Dick, 2011). Air-drying soil samples causes a rapid cessation of microbial activity and is a useful means of storing soil samples and standardizing experiments as moisture levels become minimal. Therefore, it is an advantage that the SOPs for the four enzymes considered here – β-glucosidase, NAG, arylsulphatase, and the acid and alkaline PMEs – can be performed on air-dried soil, overcoming cold storage requirements (Acosta-Martinez *et al.*, 2021). Since dehydrogenases are intracellular enzymes that transfer electrons from the substrate to an electron carrier, they need to be determined in field-moist soil samples.

Although some authors consider that air-drying should be avoided due to it reducing soil enzyme activities (see Turner and Romero, 2010; Abellan *et al.*, 2011), the ability to air-dry soil samples facilitates the universal adoption of standardized protocols across laboratories and regions, by unifying pretreatment operations and reducing operational costs (Lorenz and Dick, 2011; Lopes *et al.*, 2013; Mendes *et al.*, 2019; Acosta-Martinez *et al.*, 2021; Reardon *et al.*, 2022). Therefore, even though air-drying reduces enzyme activities, as long as its effect is well-established and preliminary tests provide evidence that the air-drying process preserves the ranking of the treatments, it should be preferred. Additionally, many soils experience the equivalent of "air-drying" naturally, as part of the climate in which they are found. For example, the dry season in tropical climates (Mendes *et al.*, 2019), Mediterranean climates, or soils in arid regions in the irrigation off-season are often in an "air-dried" status for long periods of time.

After air-drying, soil samples must be sieved with a 2 mm sieve. It is important that the sample is completely mixed before sieving. Dry soils that do not readily pass through the sieve can be crushed carefully before sieving. According to Dick *et al.* (1996), it is essential that the whole sample, or a representative aliquot, passes through the sieve. If aggregates are discarded because they are difficult to force through the sieve, the sample will be biased, as the entire soil sample will not be represented after sieving (Lorenz and Dick, 2011).

1.3. Interpretation of results

The absence of reference values for microbial indicators in SH assessments requires a comparison of different treatments. The use of reference criteria (comparative assessments) has been suggested, as the ideal values for the bioindicators can vary with climate, soil type, mineralogy, management and land use (Lopes *et al*., 2013). Two different approaches to establishing reference criteria for SH assessments have been proposed: (i) the comparative use of native, undisturbed soils under climax vegetation and with minimal anthropogenic impacts; and (ii) the comparative use of reference soils capable of maintaining a high level of productivity and environmental performance (Doran and Parkin, 1994; Gil-Sotres *et al.*, 2005). Another alternative is a dynamic assessment, that assesses trends over time (Kandeler, Tscherko and Spiegel, 1999). In fact, comparative and dynamic assessments are complementary, and allow different rating scales, with each approach holding advantages and disadvantages (Gil-Sotres *et al.*, 2005).

The main obstacle for the inclusion of these parameters in routine commercial soil analyses was the interpretation of individual values of microbial indicators. To be helpful for agricultural management decisions, target values of microbial indicators are necessary and represent a major challenge to SH

assessments (Dick, 1992; Trasar-Cepeda *et al.*, 1998; Gil-Sotres *et al.*, 2005; Gonzalez-Quiñones *et al.*, 2011; Lopes *et al.*, 2013; Lupwayi *et al.*, 2015; Biswas *et al.*, 2017; Mei, Yang and Tian, 2019; Mendes *et al.*, 2019; Tian *et al.*, 2020; Hemmati, Yaghmaeian and Farhangi, 2022). Therefore, an essential part of a SH monitoring programme is the development of an interpretative framework with the establishment of reference values, to allow ameliorative actions to be taken at an appropriate time (Gonzalez-Quiñones *et al.*, 2011).

In the United States of America, the Soil Management Assessment Framework (SMAF) provides site-specific interpretations for SH indicators, including β-glucosidase, based on the development of non-linear scoring curves and their relationships with soil functions which can be of three types: (i) more is better (upper asymptotic sigmoid curve); (ii) less is better (lower asymptotic sigmoid curve); and (iii) having a midpoint optimum (Karlen and Stott, 1994; Andrews, Karlen and Cambardella, 2004; Stott *et al.*, 2010). Indicators must be scored to interpret how each measure relates to the soil function of interest and to allow the indicators to be integrated by eliminating unit differences.

In Brazil, Lopes *et al.* (2013), described an interpretative framework that applies the principles of soil nutrient calibration tests to several microbial indicators, including the activity of soil enzymes, β-glucosidase, cellulase, arylsulphatase and acid phosphatase. In that study, microbial indicators were interpreted as a function of long-term relative cumulative yields (RCYs) of maize, soybean and soil organic carbon (SOC) in linear regression models. Adequacy classes for each microbial indicator as a function of the RCY and SOC were established based on the following criteria:

- ≤40 percent = low;
- 41 to 80 percent = moderate; and
- >80 percent = adequate.

Using this strategy, Biswas *et al.* (2017) suggested critical limits (CLs) for the soil enzymes β-glucosidase, urease and dehydrogenase in rice cropping systems in different soil types in India. The interpretative framework based on crop yield and SOM has also been used to generate proposed CLs for β-glucosidase in Canada (Lupwayi *et al.*, 2015).

Accounting for soil texture is crucial for a meaningful SH assessment, since it is the controlling factor in numerous soil physical, chemical, and biological attributes, such as cation exchange capacity (CEC), soil aggregation, and the retention of organic matter (Karlen *et al.*, 2017). The interpretation strategy based on SOC and RCYs was replicated in soils representing the most important textural classes found in Brazil (from sandy to clayey) and interpretative algorithms for ARYL and GLU were developed as a function of the percentage of clay content (Mendes *et al.* 2019 and 2024; Lopes *et al.*, 2021). Appropriate interpretation algorithms are also needed to show how well a specific soil is performing a production or environmental function and can be used to support on-farm management decision-making.

In summary, local research is of paramount importance to establish reference values, since they will vary according to soil type and climate conditions. This is valid not only for soil enzymes, but for all biological indicators. The key to robust interpretations is to use consistent and standard methods. Without applying the rigorous standardization of the SOPs for soil enzymes, variation among laboratories will hinder the development of interpretations for various soil types and climate scenarios, which in turn will make regional and national compilations of soil enzyme data challenging to interpret.

2. β-glucosidases

2.1. A brief introduction to β-glucosidases

Carbohydrates are the most abundant of the four main classes of biomolecules, exceeding proteins, lipids, and nucleic acids. In biological systems, carbohydrates function as energy storage (starch) and structural components (cellulose and hemicellulose in plants, and chitin in fungi and insects) (Deng and Popova, 2011). In nature, carbohydrates include mono-, di-, oligo- and polysaccharides. Most carbohydrates in the soil environment originate from plant biomass, which is composed of 15 to 60 percent cellulose, from 10 to 30 percent hemicelluloses, from 5 to 30 percent lignin, and from 2 to 15 percent protein (Sylvia *et al.*, 2005).

Glycosidases are carboxyhydrolases, which hydrolyse degradation products of cellulase. β-glucosidase catalyses the hydrolysis of water-soluble cellobiose releasing β-D-glucose. Activity of this enzyme is important in providing labile C and energy sources to support microbial life in soil.

β-glucosidase is one of the most investigated enzyme activities for SH assessments, and the first enzyme activity to be included in SH indexes (such as the SMAF) (Stott *et al.*, 2009). In Brazil, along with arylsulphatase, β-glucosidase has been used as part of routine commercial soil analyses, for on-farm SH assessments (Mendes *et al.*, 2021, 2024) and to evaluate C trends in clayey Oxisols (Chaer *et al.*, 2023).

2.2. Scope and field of application

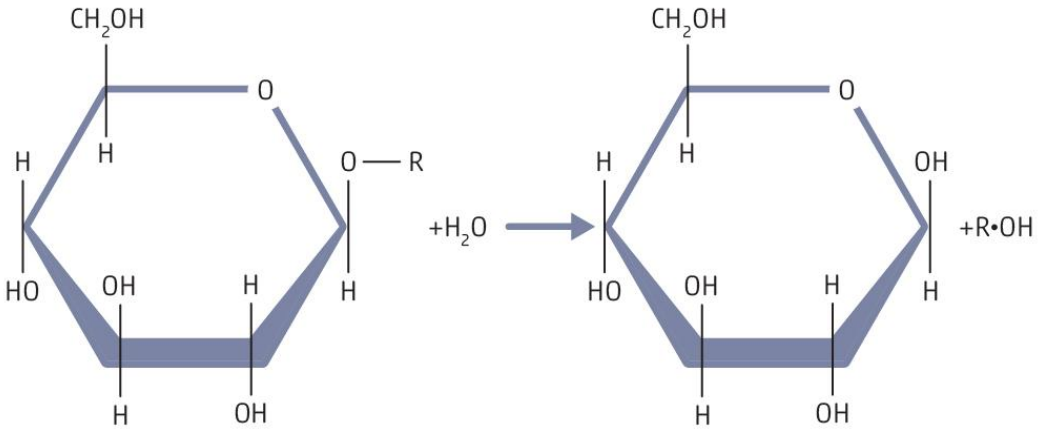
This protocol applies to the determination of potential β-glucosidase activity in the soil, based on the colorimetric determination of *p*-nitrophenol (PNP) (also known as 4-Nitrophenol) that is released when soil is incubated with the respective *p*-nitrophenyl substrate. This method is described in Tabatabai (1994), Dick *et al.* (1996), Deng and Popova (2011) and Acosta-Martinez *et al.* (2021).

The test method described here does not use toluene. Multiple studies have demonstrated that it can be omitted from the 1-hour incubation (Eivazi and Tabatabai 1977; Tabatabai, 1994). Eliminating toluene also reduces safety concerns and environmental risks associated with the waste that is generated (Acosta-Martinez *et al.*, 2021).

2.3. Principle

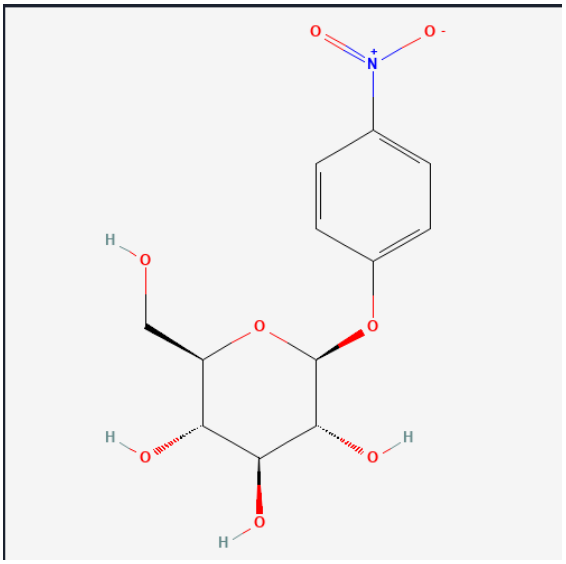
The assay of β-glucosidase is based on the colorimetric determination of PNP released when soil is incubated with the respective *p*-nitrophenyl substrate ([PNG]) at the pH optimal for the specific enzymatic reaction (Figure 1 and Figure 2). The incubation is performed at 37 °C for 1 hour. The PNP released is quantified after the addition of 0.5 molarity (M) $CaCl_2$ and 0.1 M tris(hydroxymethyl)aminomethane (THAM) (CAS 77-86-1) buffer pH 12.

Figure 1:The reaction involving β-glucosidase hydrolysing p-nitrophenyl β D glucopyranoside



Note: R represents the *p*-nitrophenyl radical.

Figure 2. Chemical structure of p-nitrophenyl β D glucopyranoside



2.3.1. Aspects to be noted

Because enzymes are difficult to extract from soils and usually lose their integrity, soil enzymes are characterized by measuring their activity under optimal conditions (pH buffer, temperature, and substrate concentration). Therefore, since this measurement is performed under optimal conditions, it provides a measure of potential activity and not *in situ* activity.

2.4. Apparatus

The following apparatus will be needed:

- 50 ml Erlenmeyer flasks, fitted with No. 2 rubber stoppers or best option in the laboratory;

- an incubator (37 °C);
- Whatman No. 2V filter paper (folded) or similar; and
- a spectrophotometer or colorimeter that can be adjusted to measure absorbance from 400 to 420 nm.

2.5. Materials

The following materials will be needed:

- A stock solution of modified universal buffer (MUB): Dissolve 12.1 g of THAM, 11.6 g of maleic acid, 14.0 g of citric acid, and 6.3 g of boric acid (H_3BO_3) in about 800 M sodium hydroxide (NaOH), adjust to 1 L with 0.5 M NaOH, and store at 4 °C.
- A MUB (pH 6.0): Place 200 ml of MUB stock solution in a 1 L beaker containing a magnetic stirring bar, which is placed on a magnetic stirrer. Titrate the pH of the solution to 6.0 with HCl (0.1–0.5 M) and adjust the volume to 1 L with deionized (DI) water.
- *p*-nitrophenyl-β-D-glucoyranoside (50 mM): Prepare by dissolving 0.753 g of PNG in about 40 ml of MUB pH 6.0 and adjusting to 50 ml with the same buffer. Prepare daily or the solution can be stored at 4 °C for days and −20 °C for weeks.
- Calcium chloride ($CaCl_2$) (0.5 M): Dissolve 73.5 g of $CaCl_2 \cdot 2H_2O$ in DI water with the final volume adjusted to 1 L.
- Tris(hydroxymethyl)aminomethane buffer (100 mM, pH 12): Dissolve 12.1 g of THAM in about 800 ml of DI water. Adjust the pH of the solution to 12 with 0.5 M NaOH and adjust the volume to 1 L with DI water.
- Standard PNP solution (10 mM): Dissolve 1.0 g of PNP in about 800 ml of DI water in a 1 L volumetric flask and adjust to 1 L with DI water. Store the solution in the dark at 4 °C.

2.6. Health and safety

This procedure involves the use of hazardous chemicals. Refer to the laboratory safety guidelines or the safety data sheet (SDS) before proceeding.

2.6.1. Personnel safety

Wear proper personal protective equipment. Use laboratory coat, closed shoes, appropriate gloves and safety glasses when performing the chemical analysis to mitigate the harmful effects of chemical exposure. Wash hands and clean other exposed areas with mild soap and water after using all chemical reagents. Use safety showers and eyewash to dilute spilled acids and bases. Use sodium bicarbonate and water to neutralize and dilute spilled acids.

Always work in a fume hood when weighing and handling PNP and *p*-nitrophenyl sulphate (PNS) solutions.

2.6.2. Chemical hazards

p-nitrophenol is a common environmental pollutant owing to its wide application in pharmaceuticals, explosives, dyes and agrochemicals. Inhalation or ingestion causes headaches, drowsiness, nausea, and a blue colour in lips, ears, and fingernails (cyanosis). Contact with eyes or skin causes irritation

and can also be absorbed through the skin to give the same symptoms as for inhalation (USCG, 1999). No information is available on the chronic (long-term) effects of PNP in humans or animals from inhalation or oral exposure. No information is available on the reproductive, developmental, or carcinogenic effects of PNP in humans. The United States Environmental Protection Agency (EPA) has not classified PNP for potential carcinogenicity. All PNP waste is considered a hazardous waste and must be discarded accordingly (NCBI, 2024a).

Boric acid may be harmful if swallowed or inhaled and may cause moderate eye irritation. Avoid breathing dust, and wash hands with soap and water after handling (NCBI, 2024b).

2.7. Soil sample preparation and storage

The objective for measuring enzyme activities will dictate the method of soil sample preparation and storage.

If the goal is to have enzyme activities reflect the true state of the ability of the soil to perform a given enzymatic reaction under *in situ* conditions, field-moist soil samples and cold storage at 4 °C present the best approach (Lorenz and Dick, 2011).

If the goal is to use enzyme activities to assess SH, air-dried soil samples are preferred (drying in a forced air oven at 35 °C [± 5 °C] is also acceptable). Air-drying reduces the impact of conditions that affect the highly variable microbial component relative to EA. Furthermore, EAs after air-drying are likely to better reflect the true long-term trajectory of a given management practice on SH (Lorenz and Dick, 2011; Mendes *et al.*, 2019; Acosta-Martinez *et al.*, 2021, Chaer *et al.*, 2023).

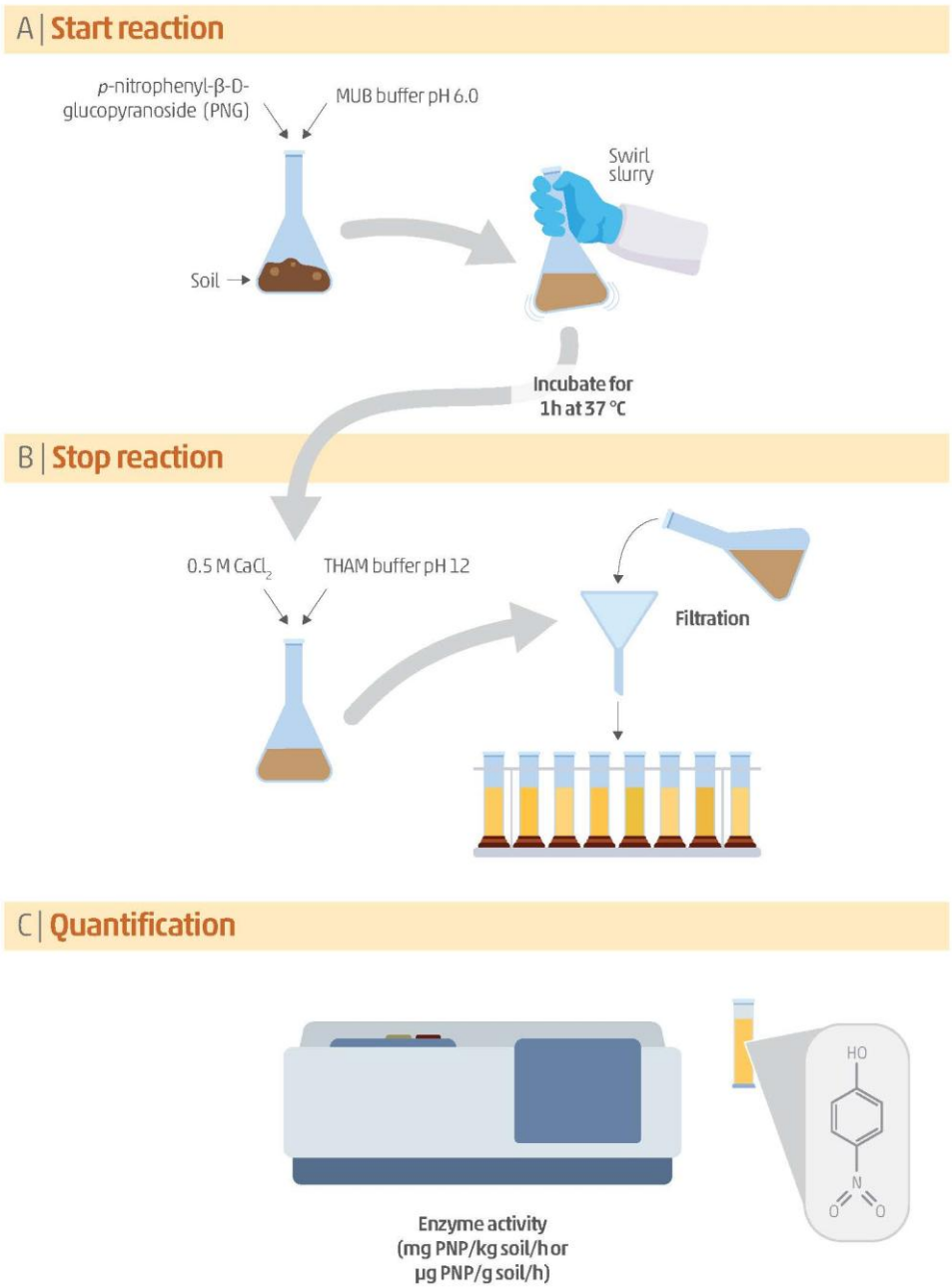
In both cases, soil samples must be sieved to ≤2.0 mm size.

2.8. Procedure

Label three 50 ml Erlenmeyer flasks with A and B as replicates, and the control sample as C. In each flask, place 1 g of soil (<2 mm), then add 4 ml of MUB pH 6.0 and 1 ml of PNG solution, stopper the flask, mix thoroughly, and incubate the soil suspension at 37 °C. After 1 hour, remove the stopper, add 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.1 M THAM buffer (pH 12), mix the contents, and filter the soil suspension through a Whatman No. 2V folded filter paper. Measure the yellow colour intensity of the filtrate with a spectrophotometer at 400 nm and calculate the amount of *PNP* released by reference to a calibration curve (see Table 1). The generalized pipeline to measure β-glucosidase activity in soil samples is shown in Figure 3.

Enzyme assays are performed in duplicates (Erlenmeyer flasks A and B) plus a control (Erlenmeyer flask C). A soil control should be performed with each soil analysed to allow for colour not derived from PNP released by β-glucosidase activity. To perform controls, follow the same procedure as for a sample, but with the addition of 1 ml of the substrate PNG solution after the additions of 0.5 M $CaCl_2$ and 4 ml of THAM buffer (pH 12) (this should be done immediately before filtration of the soil suspension).

Figure 3. Generalized pipeline to measure β-glucosidase activity in soil samples



2.8.1. Preparation of the calibration curve

The calibration curve is developed with standards containing 0 µg, 10 µg, 20 µg, 30 µg, 40 µg, and 50 µg of PNP in each flask (Table 1).

To prepare the calibration curve, dilute 1 ml of the standard solution (10 mM) to 100 ml in a volumetric flask with water and mix the solution thoroughly. Then pipette 0 ml, 1 ml, 2 ml, 3 ml, 4 ml,

and 5 ml of this diluted standard solution (0.1 mM) into 50 ml Erlenmeyer flasks, adjust to 5 ml by adding water (5 ml, 4 ml, 3 ml, 2 ml, 1 ml and 0 ml, respectively). Follow with the addition of 0.5 M $CaCl_2$ and 4 ml of 0.1 M THAM, mix and filter, as described for soil samples (Table 1). Measure the yellow colour intensity of the filtrate with a spectrophotometer adjusted to a wavelength of 400 nm.

When filtrates from soil samples have a colour intensity exceeding that of the highest PNP standard solution, dilute the filtrate with a 1:1 mixture of MUB pH 6.0 and 0.1 M THAM pH 12 until the absorbance readings fall within the limits of the calibration curve.

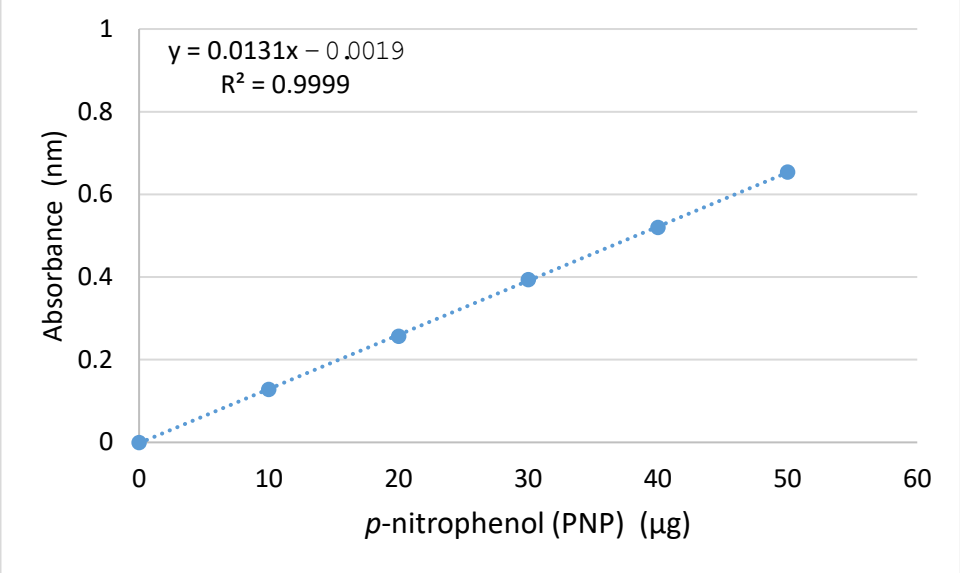
Table 1. Preparation of the calibration curve

µg *p*-nitrophenol	Distilled water (ml)	Diluted standard solution (ml)	$CaCl_2$ (ml)	THAM (ml)
0	5.0	0	1	4
10	4.0	1.0	1	4
20	3.0	2.0	1	4
30	2.0	3.0	1	4
40	1.0	4.0	1	4
50	0	5.0	1	4

2.8.2. Calculation

The PNP released by the soil samples in the filtrate is calculated by reference to a calibration graph (absorbance reading versus PNP content). A regression equation is used to convert the absorbance readings into PNP concentrations (Figure 4). All the results must be expressed in a dry weight basis (Table 2). The concentration value of the controls must be subtracted from the concentration values obtained in the duplicates (Table 2).

Figure 4. Calibration graph and regression equation



Based on the regression equation shown in Figure 4 (y = 0.0131x − 0.0019), the PNP content of the filtrates is calculated and expressed in a dry weight basis (Table 2). The control values must be subtracted from the replicates.

Table 2. Example of calculation to obtain final results of β-glucosidase activity in soil

Sample 1	**Absorbance (nm)**	**µg PNP**	**Dry weight (g)**	**PNP/g**	**Replicate A (minus control)**	**Replicate B (minus control)**	**Average**
Control	0.049	3.88	1.0	3.88	31.303	33.745	32 524
Replicate A	0.459	35.183	1.0	35.183			
Replicate B	0.491	37.625	1.0	37.625			

2.8.3. Results reporting

Enzyme activity can be expressed as either µg PNP/g soil/h or mg PNP/kg soil/h.

2.9. Quality assurance and quality control

2.9.1. Accuracy test

There should be participation in an interlaboratory proficiency test (PT) at least once a year. The PT z-score should be less than 3. If not, the root cause should be identified, and corrective and preventive actions developed.

2.9.2. Precision test

A replicate analysis should be performed every 20 to 30 samples in each batch test. The relative percent difference (RPD) should not be greater than 15 percent between results, as follows:

$$RPD = \frac{M_1 - M_2}{\left(\frac{M_1 + M_2}{2}\right)} \times 100\%$$

where: M_1 is the result of the sample and M_2 is the result of the sample's duplicate.

If the precision test fails, the cause of the failure must be identified, and corrective or preventive actions must be developed.

2.9.3. Laboratory control sample

The measurement of a sufficiently available sample of known enzyme activity value can be analysed per batch, to ensure that normal conditions have been maintained for the materials and throughout the process. This laboratory control sample can be labelled as the internal reference sample or master sample (see the GLOSOLAN basic guidelines on how to prepare a sample for internal quality control [Gowing and Hayr, 2020]).

2.10. Remarks

The original protocol for a β-glucosidase assay used toluene as a bacteriostatic (Tabatabai, 1994). It is a colourless, water-insoluble liquid with the smell associated with paint thinners and has the potential of causing severe neurological harm. Multiple studies have demonstrated that it can be omitted from the 1-hour incubation (Eivazi and Tabatabai 1977; Tabatabai, 1994). Eliminating toluene also reduces safety concerns when performing the assay (by avoiding the need to perform assays under the hood) and any environmental risks associated with the waste generated (Acosta-Martinez and Tabatabai, 2011; Acosta-Martinez *et al.*, 2021).

Calcium chloride ($CaCl_2$) is added to prevent the dispersion of clay and any extraction of SOM. Tabatabai (1994) suggested that the substrates are hydrolysed with time in the presence of excess NaOH. Therefore, treating the incubated soil samples with THAM buffer pH 12 is recommended. The rate of such chemical hydrolysis of substrates is highly variable, depending on the substrates. Daughtridge, Nakayama and Margenot (2021) and Nakayama *et al.* (2023) proposed additional controls (blanks) to account for dissolved organic matter interference and the abiotic hydrolysis of substrate.

The absorbance of PNP can be measured at wavelengths from 400 to 420 nm with minimal difference in enzyme activity, as long as the same wavelength is used for standards and samples (Acosta-Martinez *et al.*, 2021).

Laboratories should only consider centrifugation after comparing the results of filtering versus centrifugation for the soils of the region or country to be evaluated, and if it is considered more convenient.

For sandy soils with low soil organic matter (1 percent or less) a longer incubation time (at least 2 hours, with swirling after 1 hour) is recommended (Acosta-Martinez *et al.*, 2021). For soils with high SOM (>3 percent) or low pH (such as <5), the molarity of $CaCl_2$ can be increased without interfering with the reactions related to PNP release (Acosta-Martínez, Cano and Johnson, 2018).

Acosta-Martinez *et al.* (2021) reported that a modification in the β-glucosidase protocol is possible by using half of all solutions and amount of soil, without changing the proportion of the original assay. The same absorbance is obtained per sample and the time to perform the assays remains the same. However, it significantly reduces the amount of soil, resources and waste generated in the long term.

A thorough investigation on pH optima and controls for non-enzymatic contributions to apparent activity of β-glucosidase, is provided in Daughtridge, Nakayama and Margenot (2021), Li *et al.* (2021), Wade *et al.* (2021), and Nakayama *et al.* (2023).

3. Arylsulphatase

3.1. A brief introduction to arylsulphatase activity

Sulphur occurs in soil in inorganic and organic forms, with the organic fraction accounting for 90 to 98 percent of the total S present in most topsoils of humid and semihumid regions (Klose *et al*., 2011). Soil S is continuously cycled between organic and inorganic S forms. Since organic S compounds are unavailable to plants, they must be converted by biochemical or microbial mineralization to inorganic S for plant uptake (Castellano and Dick, 1991). Ester sulphates – where the linkage with sulphate is in the form of R–O–S (R representing a diverse group of organic moieties) – represent an important fraction of total organic S in soil (30 to 75 percent) and are considered to be the most labile form of organic S in soil (Scherer, 2001; Tabatabai, 2005). Additionally, there is evidence that ester sulphates are of a more transitory nature than is C-bonded S and serve as a temporary sink of SO_4^{2-} in soil, and thus are an important source of plant-available S (Klose *et al.*, 2011).

Sulphohydrolases (EC 3.1.6), including arylsulphatase, are enzymes of the esterase class that catalyse the hydrolysis of ester sulphates (Klose *et al.*, 2011). Considering the predominance of ester sulphates in topsoils, arylsulphatase play an important role in the mineralization of organic S. Although several types of sulphatases occur in nature, including alkylsulphatases, steroid sulphatases, glucosulphatases, chondrosulphatases, and mycosulphatases (Germida, Wainwright and Gupta, 1992; Tabatatabai, 2005; Haneklaus, Bloem and Schnug, 2007), most of the studies have been focused on arylsulphatase, which was the first sulphatase to be detected in nature (Fitzgerald, 1978; Klose *et al.*, 2011). Arylsulphatase catalyses the hydrolysis of ester-bonded S (arylsulphate anion) by fission of the O–S linkage, in the following pathway: $R\text{-}OSO_3^- + H_2O \rightarrow R\text{-}OH + H^+ + SO_4^{2-}$ (arylsulphate + water → phenol + hydrogen (H) ion + sulphate).

Although both bacteria and fungi produce sulphatases, this enzyme has been specifically associated with fungal biomass because fungi, unlike bacteria, accumulates ester sulphate, which is the substrate for arylsulphatase (Saggar, Battany and Stewart, 1981). Thus, this enzyme provides a reflection of the amount of recent fungal biomass production and turnover: the more fungal biomass produced, the more the microbial community is induced to produce sulphatases (Acosta-Martinez *et al.*, 2021). In Brazil, arylsulphatase has been used along with β-glucosidase, as part of routine commercial soil analyses for on-farm SH assessments (Mendes *et al.*, 2021, 2024) and to evaluate C trends in clayey Oxisols (Chaer *et al.*, 2023).

3.2. Scope and field of application

This protocol applies to the determination of potential arylsulphatase activity in soil, based on the colorimetric determination of PNP released when soil is incubated with the respective *p*-nitrophenyl substrate.

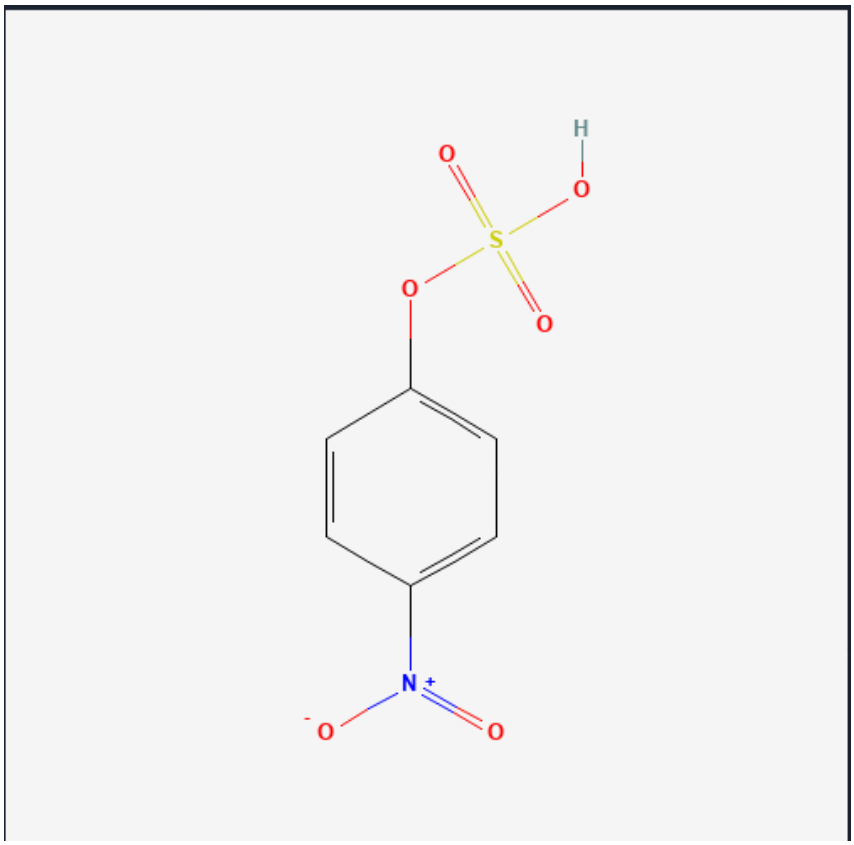
This method is described in Tabatabai (1994), Dick *et al.* (1996); Klose *et al.* (2011), and Acosta-Martinez *et al.* (2021).

The test method described here does not use toluene. Multiple studies have demonstrated that it can be omitted from the 1-hour incubation (Eivazi and Tabatabai 1977; Tabatabai, 1994). Eliminating toluene also reduces safety concerns and environmental risks associated with the waste generated (Acosta-Martinez *et al.*, 2021).

3.3. Principle

The method is based on the spectrophotometric determination of PNP released by arylsulphatase activity when soil is incubated with a buffered (pH 5.8) potassium *p* nitrophenyl sulphate solution (colourless). The soil–buffer–substrate mixture is incubated at 37 °C for 1 hour. The PNP released is extracted by filtration after the addition of $CaCl_2$ and NaOH reagents. The spectrophotometric method used for the determination of PNP depends on the fact that alkaline solutions (such as achieved by the addition of 0.5 M NaOH) of this phenol have a yellow colour (whereas, acid solutions of PNP and acid and alkaline solutions of PNS are colourless) (Figure 5). The $CaCl_2$ is added to prevent the dispersion of clay and any extraction of SOM during the treatment with NaOH. Clay dispersion complicates filtration, and any dark yellow-brown-coloured organic matter extracted with NaOH interferes with the colorimetric determination of PNP.

Figure 5. Chemical structure of p-nitrophenyl sulphate (PNS)



3.4. Apparatus

The following apparatus will be needed:

- Erlenmeyer flasks (50 ml), fitted with No. 2 stoppers or best option in the laboratory;
- Whatman No. 2 filter paper (folded) or similar;
- an incubator;
- funnels; and
- a spectrophotometer or colorimeter that can be adjusted to measure absorbance from 400 to 420 nm.

3.5. Material

The following materials will be needed:

- An acetate buffer (0.5 M, pH 5.8): Dissolve 68 g of sodium acetate trihydrate in about 700 ml of DI water, add 1.70 ml of glacial acetic acid (99 percent), and dilute the volume to 1 L with DI water.
- *p*-nitrophenyl sulphate solution (0.05 M)*:* Dissolve 0.614 g of potassium *p*-nitrophenyl sulphate in about 40 ml of acetate buffer and dilute the solution to 50 ml with buffer. Store this solution in a refrigerator at 4 °C.

- Calcium chloride ($CaCl_2$) (0.5 M): Dissolve 73.5 g $CaCl_2 \cdot 2H_2O$ in about 700 ml DI water and dilute the volume to 1 L with DI water.
- Sodium hydroxide (NaOH) (0.5 M): Dissolve 20 g NaOH in about 700 ml DI water, and dilute the volume to 1 L with DI water.
- Standard PNP solution (10mM): Dissolve 1.0 g PNP in about 700 ml DI water, and dilute the solution to 1 L with water. Store the solution in a refrigerator at 4 °C.

3.6. Health and safety

This procedure involves the use of hazardous chemicals. Refer to the laboratory safety guidelines or the SDS before proceeding.

3.6.1. Personnel safety

Wear proper personal protective equipment. Use laboratory coat, closed shoes, appropriate gloves and safety glasses when performing the chemical analysis to mitigate the harmful effects of chemical exposure. Wash hands and clean other exposed areas with mild soap and water after using all chemical reagents. Use safety showers and eyewash to dilute spilled acids and bases. Use sodium bicarbonate and water to neutralize and dilute spilled acids.

Always work in a fume hood when weighing and handling PNP and PNS solutions.

3.6.2. Chemical hazards

p-nitrophenol is a common environmental pollutant owing to its wide application in pharmaceuticals, explosives, dyes and agrochemicals. Inhalation or ingestion causes headaches, drowsiness, nausea, and a blue colour in lips, ears, and fingernails (cyanosis). Contact with eyes or skin causes irritation and can also be absorbed through the skin to give the same symptoms as for inhalation. (USCG, 1999). No information is available on the chronic (long-term) effects of PNP in humans or animals from inhalation or oral exposure. No information is available on the reproductive, developmental, or carcinogenic effects of PNP in humans. The EPA has not classified PNP for potential carcinogenicity. All PNP waste is considered a hazardous waste and must be discarded accordingly (NCBI, 2024a).

Glacial acetic acid is a flammable liquid, and harmful in contact with skin. It causes severe skin burns and eye damage and is toxic if inhaled (NCBI, 2024c).

Sodium hydroxide is also known as anhydrous caustic soda. Sodium hydroxide solution appears as a colourless liquid, denser than water, toxic by ingestion, and causes severe skin burns and eye damage. **Inhalation** of the dust may cause damage to the upper respiratory tract and lungs, producing mild nose irritation to pneumonitis. **Ingestion** causes severe damage to mucous membranes and severe scar formation or perforation may occur. **Eye contact** produces severe damage (NCBI, 2024d).

3.7. Sample preparation

The objective for measuring enzyme activities will dictate the method of soil sample preparation and storage.

If the goal is to have enzyme activities reflect the true state of the ability of the soil to perform a given enzymatic reaction under *in situ* conditions, field-moist soil samples and cold storage at 4 °C are the best approach (Lorenz and Dick, 2011).

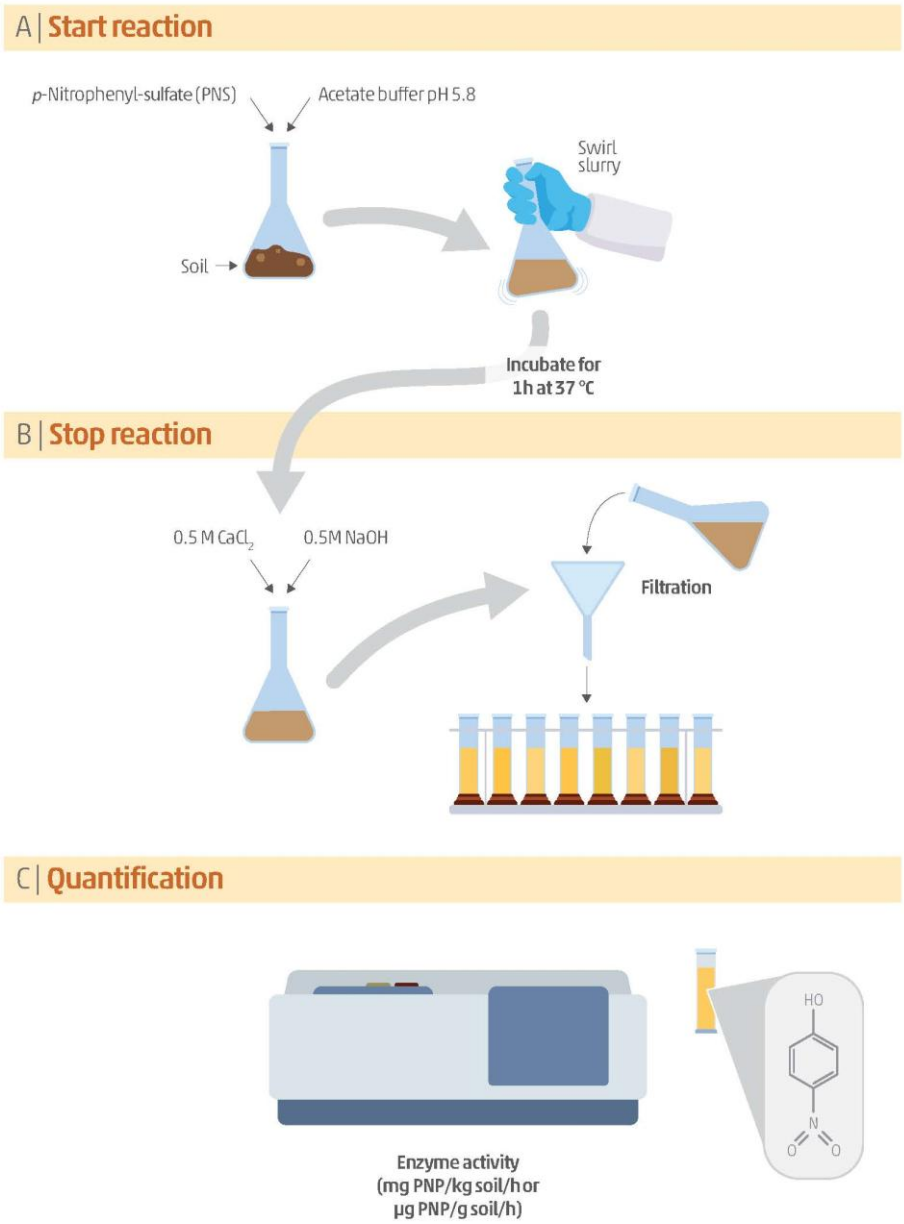
If the goal is to use enzyme activities to assess SH, air-dried soil samples are preferred (drying in a forced air oven at 35 °C [± 5 °C] is also acceptable). Air-drying reduces the impact of conditions that affect the highly variable microbial component relative to EA. Furthermore, EAs after air-drying are likely to better reflect the true long-term trajectory of a given management practice on SH (Lorenz and Dick, 2011; Mendes *et al.*, 2019; Acosta-Martinez *et al.*, 2021; Chaer *et al.*, 2023).

In both cases, soil samples must be sieved to <2.0 mm size.

3.8. Procedure

Label three 50 ml Erlenmeyer flasks with A and B as replicates, and the control sample as C. In each flask, place 1 g of air-dried soil (<2 mm), add 0.25 ml 4 ml of acetate buffer, and 1 ml of PNS solution (0.05 M) and swirl the flask for a few seconds to mix the contents. Stopper the flask and place it in an incubator at 37 °C. After 1 hour, remove the stopper, add 1 ml $CaCl_2$ (0.5 M) and 4 ml NaOH (0.5 M), swirl the flask for a few seconds, and filter the soil suspension through a Whatman No. 2 filter paper. Measure the yellow colour intensity of the filtrate with a spectrophotometer adjusted to a wavelength of 400 nm (see Figure 6).

Figure 6. Generalized pipeline to measure arylsulphatase activity in soil samples



Enzyme assays are performed in duplicates plus a control. Controls should be included for each assay by following the procedure described above, but adding the substrate PNS solution after termination of the reaction using NaOH (0.5 M).

3.8.1. Preparation of the calibration curve

The calibration curve is developed with standards containing 0 µg, 10 µg, 20 µg, 30 µg, 40 µg, and 50 µg of PNP in each flask (Table 3). To prepare this curve, dilute 1 ml of the standard PNP solution to 100 ml in a volumetric flask and mix the solution thoroughly. Then pipette 0 ml, 1 ml, 2 ml, 3 ml, 4 ml, and 5 ml aliquots of this diluted standard solution into 50 ml Erlenmeyer flasks, adjust the volume to 5 ml by the addition of DI water, add 1 ml $CaCl_2$ (0.5 M) and 4 ml NaOH (0.5 M), mix, filter, and measure the yellow colour intensity of the filtrate with a spectrophotometer adjusted to a wavelength of 400 nm.

Controls should be performed with each soil analysed to account for colour not derived from PNP released by arylsulphatase activity. Controls are conducted by adding 1 ml of *p*-nitrophenyl sulphate solution (0.05 M) after addition of the 1 ml $CaCl_2$ (0.5 M) and 4 ml NaOH (0.5 M) and immediately before filtration of the soil suspension. The PNP yellow colour formed is stable for at least several hours if stored in the dark, but direct sunlight causes rapid fading.

If the colour intensity of the filtrates from soil samples exceeds the highest PNP standard solution, dilute the filtrate with a 1:1 mixture of acetate buffer (pH 5.8) and 100 mM THAM (pH 12) until the absorbance readings are within the limits of the calibration curve.

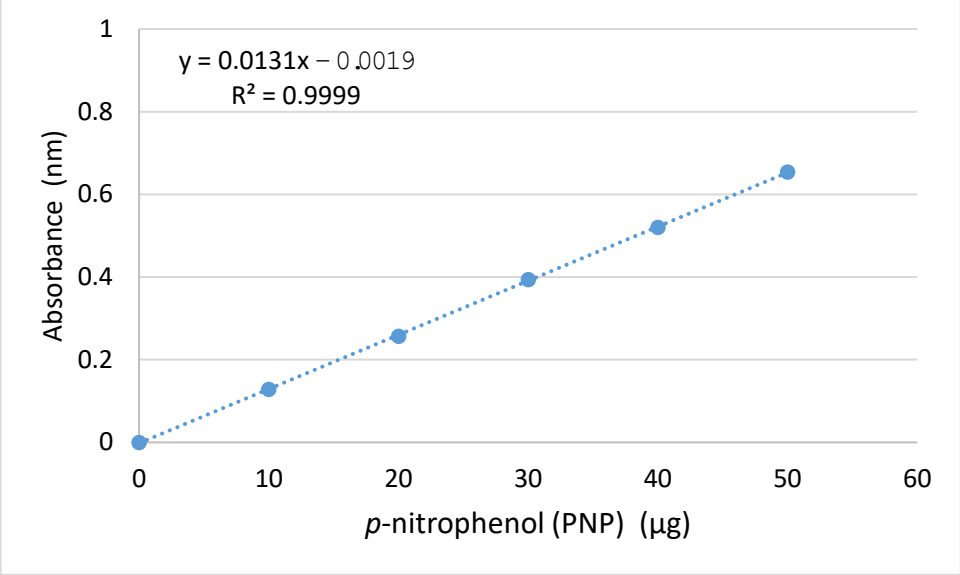
Table 3. Preparation of the calibration curve

µg *p*-nitrophenol (PNP)	Distilled water (ml)	Diluted standard solution (ml)	$CaCl_2$ (ml)	NaOH (ml)
0	5.0	0	1	4
10	4.0	1.0	1	4
20	3.0	2.0	1	4
30	2.0	3.0	1	4
40	1.0	4.0	1	4
50	0	5.0	1	4

3.9. Calculations

The PNP released by the soil samples in the filtrate is calculated by reference to a calibration graph (absorbance reading versus PNP content) (Figure 7). A regression equation is used to convert the absorbance readings into PNP concentrations (Figure 7). All the results must be expressed in a dry weight basis. The concentration value of the controls must be subtracted from the concentration values obtained in the duplicates.

Figure 7:Calibration graph and regression curve



Based on the regression equation, shown in Figure 7 (y = 0.0131x − 0.0019), the PNP content of the filtrates is calculated and expressed in a dry weight basis (Table 4). The control values must be subtracted from the replicates.

Table 4. Example of calculation to obtain final results of arylsulphatase activity in soil

Sample 1	**Absorbance (nm)**	**µg PNP**	**Dry weight (g)**	**PNP/g**	**Replicate A (minus control)**	**Replicate B (minus control)**	**Average**
Control	0.049	3.88	1.0	3.88	31.303	33.745	32.524
Replicate A	0.459	35.183	1.0	35.183			
Replicate B	0.491	37.625	1.0	37.625			

3.9.1. Results reporting

Enzyme activity can be expressed as either µg PNP/g soil/h or mg PNP/kg soil/h.

3.10. Quality assurance and quality control

3.10.1. Accuracy test

There should be participation in an interlaboratory proficiency test (PT) at least once a year. The PT z-score should be less than 3. If not, the root cause should be identified, and corrective and preventive actions developed.

3.10.2. Precision test

A replicate analysis should be performed every 20 to 30 samples in each batch test. The relative percent difference (RPD) should not be greater than 15 percent between results, as follows:

$$RPD = \frac{M_1 - M_2}{\left(\frac{M_1 + M_2}{2}\right)} \times 100\%$$

where: M_1 is the result of the sample and M_2 is the result of the sample's duplicate.

If the precision test fails, the cause of the failure must be identified, and corrective or preventive actions must be developed.

3.10.3. Laboratory control sample

The measurement of a sufficiently available sample of known enzyme activity value can be analysed per batch of analysis, to ensure that normal conditions have been maintained for the materials and throughout the process. This laboratory control sample can be labelled as the internal reference sample or master sample (see the GLOSOLAN basic guidelines on how to prepare a sample for internal quality control [Gowing and Hayr, 2020]).

3.11. Remarks

The original protocol for an arylsulphatase assay used toluene as a bacteriostatic (Tabatabai, 1994). It is a colourless, water-insoluble liquid with the smell associated with paint thinners and has the potential of causing severe neurological harm. Multiple studies have demonstrated that it can be omitted from the 1-hour incubation (Eivazi and Tabatabai 1977; Tabatabai, 1994; Acosta-Martinez *et al.*, 2021). Eliminating toluene also reduces safety concerns when performing the assay (such as avoiding the need to perform assays under the hood) and any environmental risks associated with the waste generated (Acosta-Martinez and Tabatabai, 2011; Acosta-Martinez *et al.*, 2021). Therefore, to simplify the arylsulphatase assay and make the universal adoption of standardized protocols across laboratories easier, the recommendation is to omit toluene.

The addition of $CaCl_2$ (0.5 M) is necessary to prevent the dispersion of clay and any extraction of soil organic matter following the NaOH treatment of the soil suspension. Controls account for the presence of trace amounts of PNP in some commercial samples of *p*-nitrophenyl sulphate and for the extraction of trace amounts of coloured soil material by the $CaCl_2$–NaOH treatment. Daughtridge, Nakayama and Margenot (2021) proposed additional controls (blanks) to account for dissolved organic matter interference and abiotic hydrolysis of substrate.

In organic soils or organic layers of forest soils, NaOH should be replaced with THAM buffer (pH 12) to prevent any extraction of organic matter and humic substances, which would interfere with the yellow colour measurement of the PNP. For information about how to conduct enzyme assays based on the determination of the PNP yellow colour in organic or forest soils, see Klose *et al.* (2021).

Absorbance of PNP can be measured at wavelengths from 400 to 420 nm with minimal difference in enzyme activity, as long as the same wavelength is used for standards and samples (Acosta-Martinez *et al.* 2021).

Laboratories should only consider centrifugation after comparing the results of filtering versus centrifugation for the soils of the region or country to be evaluated, and if it is considered more convenient.

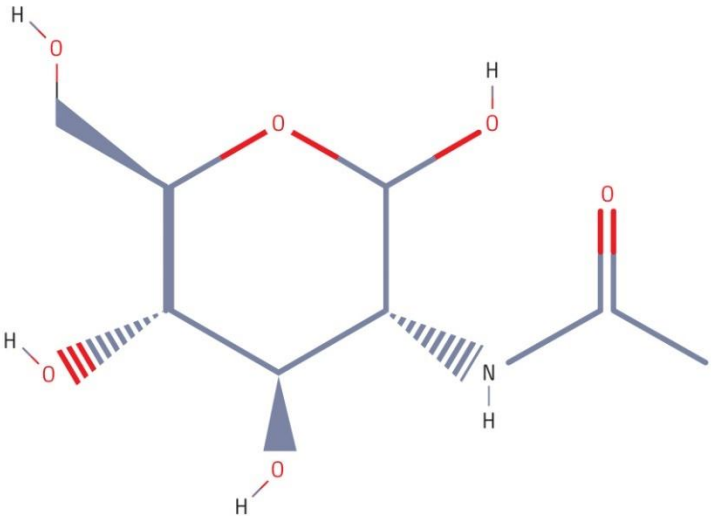
Acosta-Martinez *et al.* (2021) reported that a modification in the arylsulphatse protocol is possible by using half of all solutions and amount of soil, without changing the proportion of the original assay. The same absorbance is obtained per sample and the time to perform the assays remains the same. However, it significantly reduces the amount of soil, resources and waste generated in the long term.

4. N-acetyl-β-glucosaminidase

4.1. A brief introduction to N-acetyl-β-glucosaminidase

The assays of carboxyhydrolases are frequently based on the determination of end products (monosaccharides) of their hydrolytic activities. Monosaccharides are the simplest forms and basic units of carbohydrates. The predominant form of glucose, a six-carbon carbohydrate, is β-D-glucopyranose. When the hydroxyl group on the C-2 of β-D-glucopyranose is substituted with acetylated amino group, the resulting sugar is N-acetyl-β-D-glucosamine (GlcNAc) (Figure 8).

Figure 8. Chemical structure of N-acetyl-β-D-glucosamine



N-acetyl-β-D-glucosamine residues, joined by a β-1,4-glycosidic bond, form a long, straight chains of chitin. N-acetyl-β-D-glucosamine is the major component of cell walls of bacteria, fungi and the exoskeletons of arthropods. Chitin is the second most abundant polysaccharide in nature after cellulose. The degradation of this complex compound (chitin) leads to the release of amino sugars, thereby playing a crucial role in supporting microbial life in soil, global C and nitrogen (N) cycling (Deng and Popova, 2011; Acosta-Martinez *et al.*, 2021).

N-acetyl-β-D-glucosaminidase (NAG) is an enzyme that hydrolyses chitin chains from the terminal non-reducing end, releasing simple N-acetyl-β-D-glucosamine units (Tronsmo and Harman, 1993).

4.2. Scope and field of application

This protocol applies to the determination of potential NAG activity in soil based on the colorimetric determination of PNP released when soil is incubated with the respective *p*-nitrophenyl-N-acetyl-glucosaminide (PNAG) (CAS 3459-18-5 [N9376, Sigma-Aldrich]) substrate. This hydrolysis is important for C and N cycling in soils with the release of amino sugars (a vital source of mineralizable N in soils) from chitin (Ekenler and Tabatabai, 2004). N-acetyl-β-D-glucosaminidase has been reported to correlate significantly with fungal biomass (Miller *et al.*, 1998).

The application of toluene has been eliminated from this method as an antiseptic. Several studies have reported benefits of not using toluene during the 1-hour incubation (Eivazi and Tabatabai 1977; Tabatabai, 1994; Deng and Popova, 2011), allowing a reduction in safety concerns when performing an assay to less hazardous waste (that poses an environmental risk) (Acosta-Martinez *et al.*, 2021).

4.3. Principle

The assay of NAG activities in soil is based on the colorimetric determination of PNP released when air-dried soil is incubated with an acetate buffer (100 mM, pH 5.5) and the respective PNAG substrate

for 1 hour at 37 °C. After incubation, 0.5 M $CaCl_2$ is added followed by 0.1 M THAM buffer (pH 12) and the released PNP is then quantified.

4.4. Apparatus

The following apparatus will be needed:

- 50 ml Erlenmeyer flasks, fitted with No. 2 rubber stoppers;
- volumetric flasks, acid washed (100 ml and 1000 ml);
- long stem funnels;
- test tubes and rack;
- pipettes and tips;
- cuvettes;
- an electronic weighing scale (± 1.0 mg sensitivity);
- an incubator (37 °C);
- Whatman No. 2V folded filter papers; and
- a spectrophotometer or colorimeter that can be adjusted to measure absorbance from 400 to 420 nm.

4.5. Materials

The following materials will be needed:

- Stock solution of modified universal buffer (MUB): Dissolve 12.1 g of THAM, 11.6 g of maleic acid, 14.0 g of citric acid, and 6.3 g of boric acid (H_3BO_3) in about 800 ml of 0.5 M sodium hydroxide (NaOH), adjust to 1 L with 0.5 M NaOH, and store at under 4 °C.
- Acetate buffer (100 mM, pH 5.5): Dissolve 13.6 g of sodium acetate trihydrate (CAS 6131-90-4) in about 800 ml of DI water. Titrate to pH 5.5 with 99 percent glacial acetic acid (CAS 64-19-7) and adjust the volume to 1 L with DI water.
- *p*-nitrophenyl-N-acetyl-β-D-glucosaminide (10 mM): Dissolve 0.342 g of PNAG in about 80 ml of acetate buffer pH 5.5 and adjusting to 100 ml with the same buffer. Prepare daily or the solution can be stored at 4 °C for days and −20 °C for weeks.
- Calcium chloride (0.5 M): Dissolve 73.5 g of $CaCl_2 \cdot 2H_2O$ (CAS 10035-04-8) in 700 ml DI water with the final volume adjusted to 1 L.
- Tris(hydroxymethyl)aminomethane buffer (100 mM, pH 12): Dissolve 12.2 g of THAM in about 800 ml of DI water. Adjust the pH of the solution to 12 by titration with 0.5 M NaOH, stirring constantly, and adjust the volume to 1 L with DI water.
- Standard PNP solution (10 mM): Dissolve 1.0 g of PNP (CAS 100-02-7, spectrophotometric grade) in about 800 ml of DI water in a 1 L volumetric flask and adjust to 1 L with DI water. Store the solution in the dark at 4 °C.

4.6. Health and safety

This protocol requires the use of hazardous chemicals, so take caution and refer to the safety guidelines provided in the appropriate safety data sheet (SDS).

4.6.1. Personnel safety

Wear proper personal protective equipment. Use laboratory coat, closed shoes, appropriate disposable gloves for chemical resistance, and safety glasses when performing the chemical analysis to mitigate the harmful effects of chemical exposure. Adequately wash hands and clean other exposed areas with mild soap and water after using all chemical reagents. Use safety showers and eyewash to dilute spilled acids and bases. Use sodium bicarbonate and water to neutralize and dilute spilled acids.

Always work in a fume hood when weighing and handling PNP and PNAG solutions.

4.6.2. Chemical hazards

p-nitrophenol is a common environmental pollutant owing to its wide application in pharmaceuticals, explosives, dyes and agrochemicals. Inhalation or ingestion causes headaches, drowsiness, nausea, and a blue colour in lips, ears, and fingernails (cyanosis). Contact with eyes or skin causes irritation and it can be absorbed through the skin to give the same symptoms as for inhalation (USCG, 1999). No information is available on the chronic (long-term) effects of PNP inhalation or oral exposure in humans or animals. No information is available on the reproductive, developmental, or carcinogenic effects of PNP in humans. The EPA has not classified PNP for potential carcinogenicity. All PNP waste is considered a hazardous waste and must be discarded accordingly (NCBI, 2024a).

4.7. Sample preparation

The objective for measuring enzyme activities will dictate the method of soil sample preparation and storage.

If the goal is to have enzyme activities reflect the true state of the ability of the soil to perform a given enzymatic reaction under *in situ* conditions, field-moist soil samples and cold storage at 4 °C present the best approach (Lorenz and Dick, 2011).

If the goal is to use enzyme activities to assess SH, air-dried soil samples are preferred (drying in a forced air oven at 35 °C (± 5 °C) is also acceptable). Air-drying reduces the impact of conditions that affect the highly variable microbial component relative to EA. Furthermore, EAs after air-drying are likely to better reflect the true long-term trajectory of a given management practice on SH (Lorenz and Dick, 2011; Mendes *et al.*, 2019; Acosta-Martinez *et al.*, 2021; Chaer *et al.*, 2023).

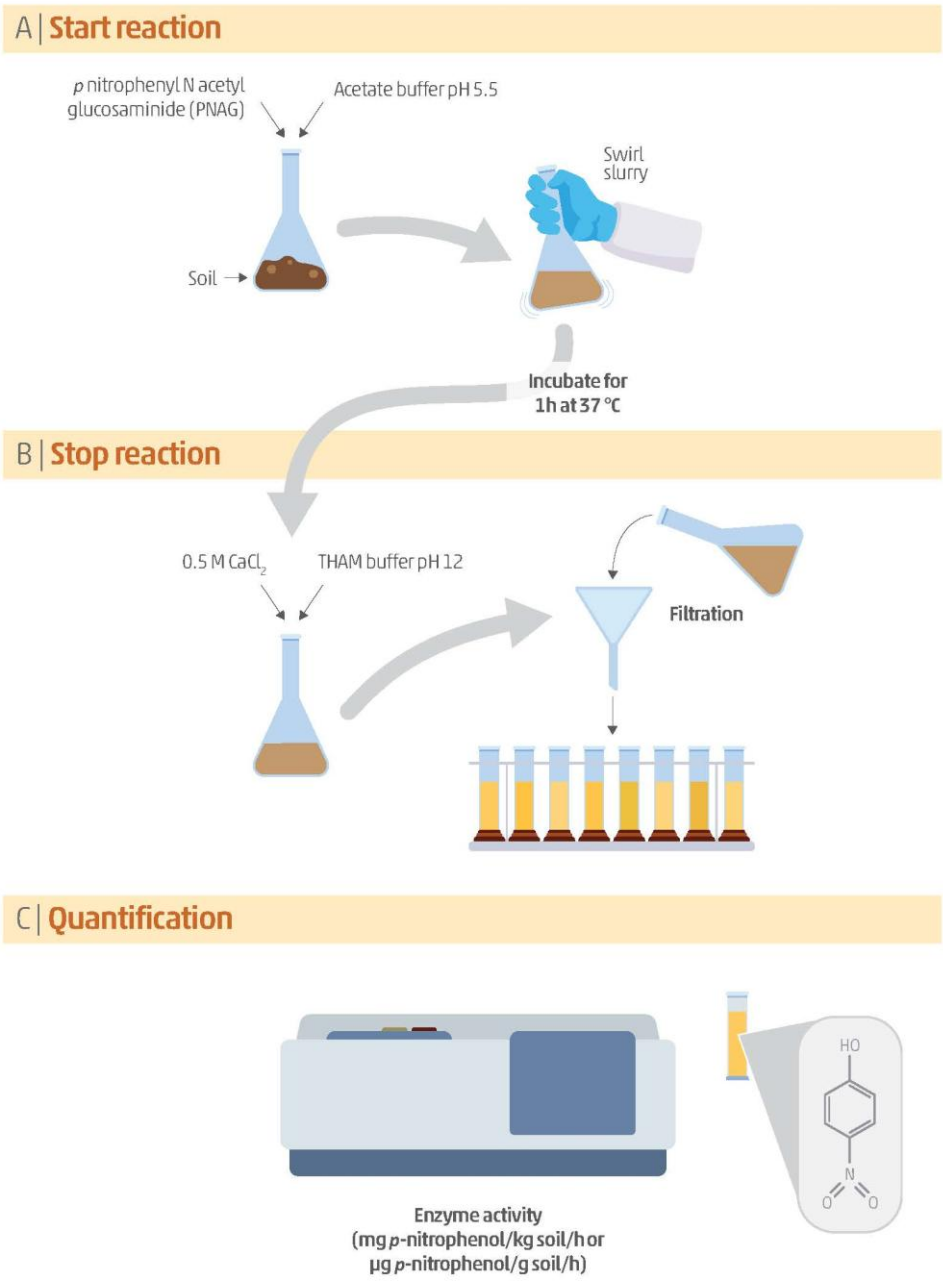
In both cases, soil samples must be sieved to ≤2.0 mm size.

4.8. Procedure

Label three 50 ml Erlenmeyer flasks with A and B as replicates, and the control sample as C. Add 1 g (± 0.02 g) of air-dried soil (<2 mm) to each Erlenmeyer flask (A, B and C), then add 4 ml of acetate

buffer to each Erlenmeyer flask (A, B and C). Add 1 ml of PNAG solution (substrate) to replicates (A and B) only. Place a stopper in each flask and swirl each flask gently to thoroughly homogenize the admixture. Then place each flask in an incubator for 1 hour at 37 ^{o}C. After 1 hour, remove the flasks from the incubator, and uncap the flasks. Add 1 ml of 0.5 M $CaCl_2$ to the soil in each flask and then add 4 ml of 0.1 M THAM buffer (pH 12) to the replicate flasks (A and B) first, before the control (C). Swirl gently to mix the soil solution in each flask. Then add 1 ml of PNAG solution (substrate) to the flask C (control). Then swirl to mix content. Pour the soil solution into a funnel lined with folded filter paper (Whatman 2V) capturing the filtrate into test tubes or clean flasks. Let filtration continue for up to 30 minutes or until fully filtered. Remove funnel from test tubes or flasks and discard the filter paper appropriately. Using a pipette, transfer an aliquot of the filtrate into a cuvette and measure the colour intensity in a spectrophotometer at 400 nm (Figure 9). Since PNP is light sensitive, read the samples as soon as possible after filtration.

Figure 9. Generalized pipeline to measure N-acetyl-β-D-glucosaminidase activity in soil samples



If the colour intensity of the filtrate exceeds the highest PNP standard solution, dilute the filtrate with a 1:1 mixture of acetate buffer (pH 5.5) and 100 mM THAM (pH 12) until the absorbance readings are within the limits of the calibration curve.

4.8.1. Preparation of the calibration curve

The calibration curve is developed with standards containing 0 µg, 10 µg, 20 µg, 30 µg, 40 µg, and 50 µg of PNP in each flask (Table 5). Transfer 1 ml of the PNP standard solution (10 mM) into a volumetric flask and fill to the volume mark with water. After mixing the solution thoroughly, pipette 0 ml, 1 ml,

2 ml, 3 ml, 4 ml, and 5 ml of diluted PNP (working standard) solution into 50 ml flasks, adjust to 5 ml by adding the corresponding amount of water (5 ml, 4 ml, 3 ml, 2 ml, 1 ml, and 0 ml respectively). For an alkaline termination of each reaction, add the 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.1 M THAM as described for enzyme assays of soil samples. Filter the suspension and measure with the spectrometer at 400 nm.

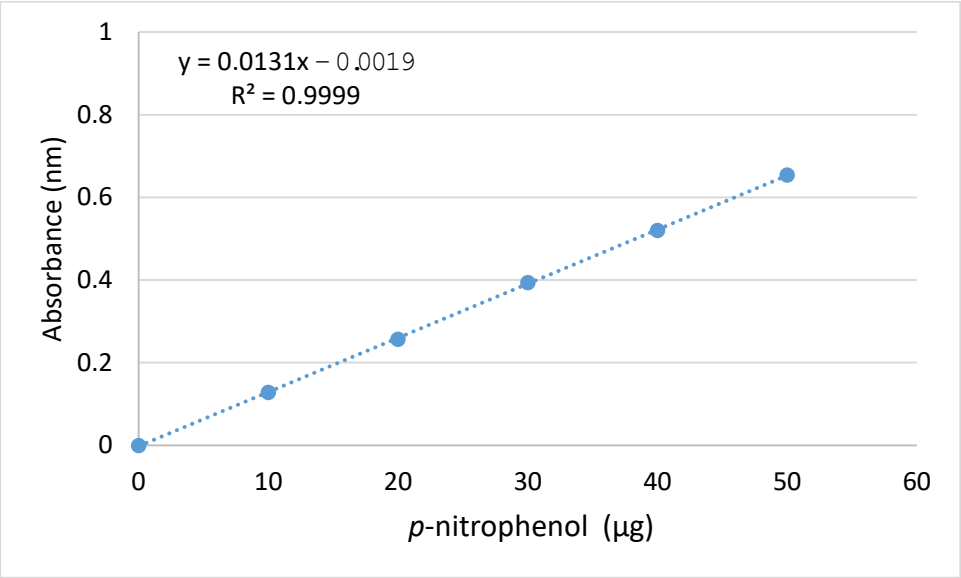
Table 5. Preparation of the calibration curve

µg *p*-nitrophenol	Distilled water (ml)	Diluted standard solution (ml)	$CaCl_2$ (ml)	THAM (ml)
0	5.0	0	1	4
10	4.0	1.0	1	4
20	3.0	2.0	1	4
30	2.0	3.0	1	4
40	1.0	4.0	1	4
50	0	5.0	1	4

4.9. Calculation

The PNP released by the soil samples in the filtrate is calculated by reference to a calibration graph (absorbance reading versus PNP content) (Figure 10). A regression equation is used to convert the absorbance readings into PNP concentrations (Figure 10). All the results must be expressed in a dry weight base. The concentration value of the controls must be subtracted from the concentration values obtained in the duplicates.

Figure 10. Calibration graph and regression equation



Based on the regression equation shown in Figure 10 (y = 0.0131x – 0.0019), the PNP content of the filtrates is calculated and expressed in a dry weight basis (Table 6). The control values must be subtracted from the replicates.

Table 6. Example of calculation to obtain final results of N-acetyl-β-D-glucosaminidase activity in soil

Sample 1	**Absorbance (nm)**	**µg PNP**	**Dry weight (g)**	**PNP/g**	**Replicate A (minus control)**	**Replicate B (minus control)**	**Average**
Control	0.049	3.88	1.0	3.88	31.303	33.745	32.524
Replicate A	0.459	35.183	1.0	35.183			
Replicate B	0.491	37.625	1.0	37.625			

4.9.1. Results reporting

Enzyme activity can be expressed as either µg PNP/g soil/h or mg PNP/kg soil/h.

4.10. Quality assurance and quality control

4.10.1. Accuracy test

There should be participation in an interlaboratory proficiency test (PT) at least once a year. The PT z-score should be less than 3. If not, the root cause should be identified, and corrective and preventive actions developed.

4.10.2. Precision test

A replicate analysis should be performed every 20 to 30 samples in each batch test. The relative percent difference (RPD) should not be greater than 15 percent between results, as follows:

$$RPD = \frac{M_1 - M_2}{\left(\frac{M_1 + M_2}{2}\right)} \times 100\%$$

where: M_1 = the result of the sample and M_2 = the result of the sample's duplicate.

If the precision test fails, the cause of the failure must be identified, and corrective or preventive actions must be developed.

4.10.3. Laboratory control sample

The measurement of a sufficiently available sample of known enzyme activity value can be analysed per batch of analysis, to ensure that normal conditions have been maintained for the materials and throughout the process. This laboratory control sample can be labelled as the internal reference sample or master sample (see the GLOSOLAN basic guidelines on how to prepare a sample for internal quality control [Gowing and Hayr, 2020]).

4.11. Remarks

Calcium chloride ($CaCl_2$) is added to prevent the dispersion of clay and any extraction of soil organic matter. Tabatabai (1994) suggested that the substrates are hydrolysed with time in the presence of excess NaOH. Therefore, treating the incubated soil samples with THAM buffer pH 12 was recommended. The rate of such chemical hydrolysis of substrates is highly variable, depending on the substrates. Daughtridge, Nakayama and Margenot (2021) proposed additional controls (blanks) to account for dissolved organic matter interference and the abiotic hydrolysis of substrate.

Acosta-Martinez *et al.* (2021) reported a modification to the NAG protocol by using half of all reagents and soil used in this original protocol without changing the proportions of the original assays.

The absorbance of PNP can be measured at wavelengths from 400 to 420 nm with a minimal difference in enzyme activity, as long as the same wavelength is used for standards and samples (Acosta-Martinez *et al.*, 2021).

5. Dehydrogenase activity

5.1. A brief introduction to the assay to measure dehydrogenase activity

The oxidoreductases (EC 1) comprise the largest enzyme group and consist of enzymes that catalyse reactions in which one substrate is oxidized (the donor) while another is reduced (the acceptor) (Dixon and Webb, 1979). In common with all redox reactions, the reaction mechanism involves electron transfer, expressed in a simplistic representation as:

$A^- + B \rightarrow A + B^-$

However, the observed reaction usually involves the transfer of two H atoms from the donor to the acceptor (dehydrogenation) and, consequently, most of the enzymes are called dehydrogenases. The entire dehydrogenase-catalysed reaction system is an enzyme donor–acceptor complex, located inside the cell, and does not involve ions or electrons reacting in solution (Dixon and Webb, 1979).

5.2. Scope and field of application

This protocol applies to the determination of potential dehydrogenase activity in soil, based on the colorimetric determination of red-coloured 1,3,5-triphenylformazan (TPF) released when the soil is incubated with the respective 2,3,5-triphenyl-tetrazolium chloride (TTC) substrate (TTC is also known as triphenyl tetrazolium chloride or tetrazolium chloride).

This protocol builds on the work done by Casida, Klein Jr and Santoro (1964), Moeskops *et al.* (2010), and Małachowska-Jutsz and Matyja (2019).

5.3. Principle

For the assay of soil dehydrogenase activity, two principal electron acceptors have been used: TTC and 2-(4-iodophenyl)-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT). This protocol focuses on TTC as an electron acceptor (Malachowska-Jutsz and Matyja, 2019).

5.3.1. 2,3,5-triphenyltetrazolium chloride as an electron acceptor

2,3,5-triphenyltetrazolium chloride is an artificial terminal H acceptor in the electron transport chain and is reduced to a red-coloured TPF. Triphenylformazan is extracted using organic solvents (such as methanol), and the colour intensity of the extract is determined by spectroscopic methods. The intensity of the colour is directly proportional to the concentration of the produced TPF.

5.4. Apparatus

The following apparatus will be needed:

- glass vials;

- a glass funnel;
- volumetric flasks;
- pipette and tips;
- cuvettes;
- an electronic weighing scale;
- a linear shaker (125 rpm);
- a shaking incubator set at 37 °C;
- a vortex mixer; and
- a spectrophotometer or colorimeter.

5.5. Materials

The following materials will be needed:

- Tris(hydroxymethyl)aminomethane buffer (0.1 M): Dissolve 12.1 g of THAM in 700 ml distilled water, adjust with HCl (1 M) to pH 7.8 for acid soils with pH values less than 6.0, to pH 7.6 for neutral soils with pH values ranging from 6.0 to 7.5, and to pH 7.4 for alkaline soils with pH values higher than 7.5. Bring up to 1 000 ml with distilled water.
- 2,3,5-triphenyltetrazolium chloride solution (3 percent): Dissolve 3 g of TTC in 80 ml THAM buffer, dilute to 100 ml with THAM buffer. Store in the dark.
- Triphenylformazan standard solution (500 µg TPF/ml): Dissolve 50 mg TPF in 80 ml methanol and bring up with methanol to 100 ml. Store in the dark.
- Methanol.

5.6. Health and safety

This procedure involves the use of hazardous chemicals. Refer to the laboratory safety guidelines or the safety data sheet (SDS) before proceeding.

5.6.1. Personnel safety

Wear proper personal protective equipment. Use laboratory coat, closed shoes, appropriate gloves and safety glasses when performing the chemical analysis to mitigate the harmful effects of chemical exposure. Perform the chemical analysis inside the safety hood. Wash hands and clean other exposed areas with mild soap and water after using all chemical reagents.

5.6.2. Chemical hazards

Tris(hydroxymethyl)aminomethane is used in chemical manufacturing (surface-active agents, vulcanization accelerators, and pharmaceuticals), as an emulsifying agent (cosmetics, mineral oil and paraffin wax emulsions, leather dressings, textile specialties, polishes, cleaning compounds, and soluble oils), and as an absorbent for acidic gases, a biological buffer, an acidimetric standard, and a therapeutic alkalinizing agent. It is used in buffer systems for the freezer storage of sperm, used to regulate pH in the transport of live aquatic species, in cell culture media, and is anti-inflammatory. It

can cause skin irritation, serious eye irritation or eye damage, and may cause respiratory irritation (NCBI, 2024e).

2,3,5-triphenyl-tetrazolium chloride is used in analytical chemistry for staining plant and animal tissues, and to test the ability of seeds to germinate, and is a redox indicator commonly used in biochemical experiments, especially to indicate cellular respiration. It can cause skin irritation, serious eye irritation and may cause respiratory irritation (NCBI, 2024f).

1,3,5-triphenylformazan is acutely toxic and harmful if swallowed. It is also very toxic to aquatic life (NCBI, 2024g).

Methanol is a clear colourless liquid, used in hydraulic fracturing mixtures. It is also used as dehydrator of natural gas, fuel for utility plants (methyl fuel), feedstock for the manufacture of synthetic proteins by continuous fermentation, a source of H for fuel cells, and a home heating oil extender. Exposure to excessive methanol vapour causes eye irritation, headache, fatigue and drowsiness. High concentrations can produce central nervous system depression and optic nerve damage. 50 000 ppm will probably cause death in 1 to 2 hours, and can be absorbed through the skin. Swallowing may cause death or eye damage (USCG, 1999).

Human studies: Humans (and non-human primates) are uniquely sensitive to methanol poisoning. Nearly all of the available information on methanol toxicity in humans relates to the consequences of acute rather than chronic exposures. A vast majority of poisonings involving methanol have occurred from drinking adulterated beverages and from methanol-containing products. The minimum lethal dose of methanol in the absence of medical treatment is between 0.3 and 1 g/kg. Wide interindividual variability of the toxic dose is a prominent feature in acute methanol poisoning. Two important determinants of human susceptibility to methanol toxicity appear to be: (i) concurrent ingestion of ethanol, which slows the entrance of methanol into the metabolic pathway; and (ii) hepatic folate status, which governs the rate of formate detoxification. The symptoms and signs of methanol poisoning, which may not appear until after an asymptomatic period, include visual disturbances, nausea, abdominal and muscle pain, dizziness, weakness and disturbances of consciousness ranging from coma to clonic seizures. Visual disturbances range from mild photophobia and misty or blurred vision to markedly-reduced visual acuity and complete blindness. In extreme cases, it results in death. The principal clinical feature is severe metabolic acidosis of the anion-gap type.

Ecotoxicity studies: Methanol is of low toxicity to aquatic organisms, and effects due to environmental exposure to methanol are unlikely to be observed, except in the case of a spill (NCBI, 2024h).

5.7. Sample preparation

Fresh soil should be homogenized by thoroughly mixing and sieving to ≤2 mm size. Soil should be analysed fresh and as soon after collection as possible (less than 1 month), as results have shown that dehydrogenase activity is adversely affected by air-drying or storage, even at 4 °C (Ross, 1970).

5.8. Procedure

Because of the light sensitivity of TTC and TPF, all procedures should be performed under diffused light. All measurements are carried out in duplicate with one blank.

Label three glass vials with A and B as replicates, and the control sample as C. Place 5 g of field-moist soil in 50 ml glass vial. Add 2 ml TTC solution and 2 ml THAM buffer. Add 4 ml of THAM buffer to the blanks (no TTC). Stopper the vials, mix the contents thoroughly and incubate for 24 hours at 37 °C in the dark. After incubation, add 20 ml of methanol to each vial, and shake the vials for 2 hours in the dark in a reciprocal shaker (125 rpm). The soil suspensions are then filtered on Whatman filter papers No. 5 that are pre-wetted with methanol. Filtrates are received in volumetric flasks of 25 ml or 50 ml, depending on the expected activity. To extract all produced TPF, the remaining soil in the vials is washed twice with methanol and finally the filter papers are flushed twice with methanol. The filtrates in the volumetric flasks are diluted until the mark with methanol. The optical density of the filtrates is measured at 485 nm.

5.8.1. Preparation of the calibration curve

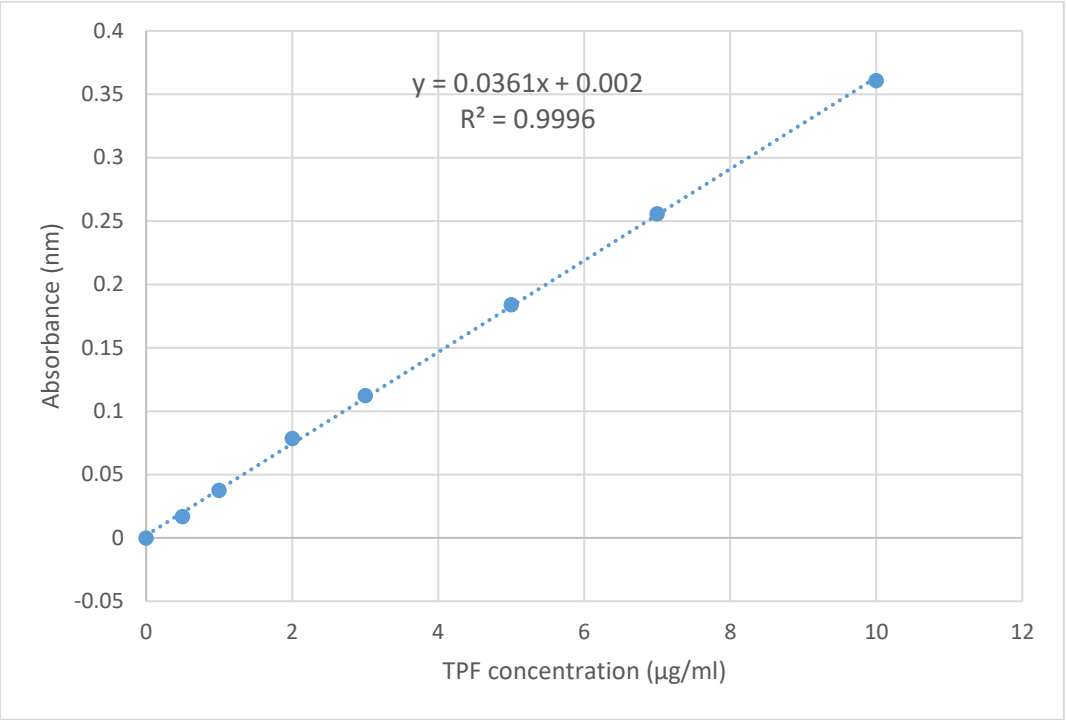
Pipette 0.5 ml TPF standard solution in a volumetric flask (100 ml) and bring up with methanol to 100 ml to obtain a concentration of 2.5 µg TPF/ml.

Pipette 0.5 ml, 1 ml, 1.5 ml, 2 ml, 2.5 ml and 3 ml of TPF standard solution in a volumetric flask (50 ml) and bring up with methanol to 50 ml to obtain the following concentrations: 5 TPF/ml, 10 TPF/ml, 15 TPF/ml, 20 TPF/ml, 25 TPF/ml and 30 µg TPF/ml.

Prepare the standards like samples. Do not directly pipette the TPF into the volumetric flasks but onto pre-wetted filter paper. After pipetting TPF, add 20 ml methanol and flush the filter papers twice.

Adapt the calibration according to the expected concentrations (Figure 11). For example, because of the low activity of the dehydrogenase, a standard of 2.5 µg TPF/ml is used. Alternatively, a standard of 30 µg TPF/ml is used for high dehydrogenase activity in forest soil.

Figure 11:Calibration graph and regression equation



5.9. Calculations

Dehydrogenase activity is expressed as µg of TPF/g dry soil/h Using the calibration curve, TPF concentrations can be determined from the corresponding absorbance value at 485 nm (Table 7, Table 8 and Table 9). The regression equation is used to convert absorbance readings into TPF concentrations.

Table 7. Example of results obtained in an enzyme activity assay

Sample 1	**Absorbance**	**Concentrate** (µg/ml**)**
Control	0.003	0.147216782
Replicate A	0.2559	7.799438
Replicate B	0.2545	7.757528305

Table 8. Calculation formula

Calculation	Value or formula	unit
Weight of fresh soil used for extraction (mass [m])	*5*	g
Soil water content (moisture content [mc])	*gravimetric*	g/g
Extractant volume (V)	*50*	ml
Triphenylformazan (TPF) concentration in the extract from zero (a)	*Spectrophotometer measurement*	µg/ml
TPF concentration in the extract from blank (b)	*Spectrophotometer measurement*	µg/ml
TPF concentration in the extract from soil (replicate 1) [c1]	*Spectrophotometer measurement*	µg/ml
TPF concentration in the extract from soil (replicate 2) (c2)	*Spectrophotometer measurement*	µg/ml
Adjusted TPF concentration in the extract from blank (d)	$\mathrm{d} = (\mathrm{b} - \mathrm{a})$	µg/ml
Adjusted TPF concentration in the extract from soil (e1)	$\mathrm{e1} = (\mathrm{c1} - \mathrm{a})$	µg/ml
Adjusted TPF concentration in the extract from soil (e2)	$\mathrm{e2} = (\mathrm{c2} - \mathrm{a})$	µg/ml
TPF produced by soil (x)	$x = \left(\frac{(\mathrm{e1} + \mathrm{e2})}{2 - d}\right)$	µg/ml
TPF produced in 1 g of dry soil (Y)	$\mathbf{Y = x \times \left(\frac{V}{\left(\frac{m}{(1 + mc)}\right)} \right)}$	**µg/g**

Table 9. Example of calculation to obtain final results of dehydrogenase activity in soil

Sample ID	1
Weight of fresh soil used for extraction (g)	5
Gravimetric soil water content (g/g)	0.1403
Weight of dry soil used (g)	4.38
Volume after extraction (ml)	50
TPF concentration in the extract from zero (µg/ml)	0.0445
TPF concentration in the extract from blank (µg/ml)	0.1472
TPF concentration in the extract from soil (replicate A) (µg/ml)	7.7994
TPF concentration in the extract from soil (replicate B) (µg/ml)	7.7575
Adjusted TPF concentration in the extract from blank (µg/ml)	0.1027
Adjusted TPF concentration in the extract from soil (replicate A) (µg/ml)	7.7549
Adjusted TPF concentration in the extract from soil (replicate B) (µg/ml)	7.6103
TPF produced by soil (µg/ml)	7.58
TPF produced dry soil (µg/g dry soil/h)	86.44

5.9.1. Results reporting

Express enzyme activity as µg TPF/g soil/h.

5.10. Quality assurance and quality control

5.10.1. Accuracy test

There should be participation in an interlaboratory proficiency test (PT) at least once a year. The PT z-score should be less than 3. If not, the root cause should be identified, and corrective and preventive actions developed.

5.10.2. Precision test

A replicate analysis should be performed every 20 to 30 samples in each batch test. The relative percent difference (RPD) should not be greater than 15 percent between results, as follows:

$$RPD = \frac{M_1 - M_2}{\left(\frac{M_1 + M_2}{2}\right)} \times 100\%$$

where: M_1 is the result of the sample, and M_2 is the result of the sample's duplicate.

If the precision test fails, the cause of the failure must be identified, and corrective or preventive actions must be developed.

5.10.3. Laboratory control sample

The measurement of a sufficiently available sample of known enzyme activity value can be analysed per batch of analysis to ensure that normal conditions have been maintained for the materials and throughout the process. This laboratory control sample can be labelled as the internal reference

sample or master sample (see the GLOSOLAN basic guidelines on how to prepare a sample for internal quality control [Gowing and Hayr, 2020]).

5.11. Remarks

Soil should be analysed fresh and as soon after collection as possible (<1 month), as results have shown that dehydrogenase activity is adversely affected by air-drying or storage, even at 4 °C (Ross, 1970). 2,3,5-triphenyl-tetrazolium chloride and TPF are very sensitive to light and should be kept in the dark at all times. This method varies strongly with the change of the incubation time (1–40 hours), TTC concentration, soil sample weight (1–10 g), incubation temperature (20–37 °C). The dehydrogenase activity strongly depends on the method used to measure it, even when activity is measured in the same soil sample. Therefore, it is difficult to compare results from different sources (Małachowska-Jutsz and Matyja, 2019).

In addition to TTC, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) also has been used as principal electron acceptor. Regarding the limitations, none of the methods (TTC and INT) can estimate the exact dehydrogenase activity, but rather give an indication of the intensity of DHG activity. The TTC methodology is the most commonly used for the dehydrogenase activity. Prosser, Speir and Stott (2011) listed some of the advantages of INT as follows: (i) it is a better competitor with O_2 than other electron acceptors; (ii) it is less toxic to microorganisms; (iii) it is more rapidly reduced than TTC; (iv) it performs in both aerobic and anaerobic conditions; and (v) it is sensitive over a wide range of temperatures (Benefield, Howard and Howard, 1977; Trevors, Mayfield and Inniss, 1982; Trevors,1984; Friedel, Mölter and Fischer, 1994; Mosher, Levison and Johnston, 2003). It has been noted that approximately 10 percent of the evolved H is transferred to INT (Benefield *et al.*, 1977), compared with only 2 to 3 percent with TTC (Öhlinger, 1996).

6. Phosphomonoesterases

6.1. Brief introduction to phosphomonoesterases activities

Organic phosphorus constitutes a significant proportion of soil total P (Dalal, 1977). In soils, organic P largely occurs in two major forms defined by bond type: phosphomonoester and phosphodiester P. These two forms are hydrolysed by hydrolytic enzymes known as phosphatases. Phosphatases play a major role in the transformation of soil organic P, mediating the transformation and recycling of P forms in soil (including from inputs such as crop residues and manure), into phosphate anions ($H_2PO_4^-$/HPO_4^{2-}) that can be taken up by plants and soil microorganisms, or adsorbed by clays and metal (iron [Fe] and aluminium [Al]) oxides/hydroxides.

Phosphatases participate in the hydrolysis of phosphate monoesters, which represent up to one-third of the soil organic P, including inositol phosphates, sugar phosphates, and mononucleotides (Grindel and Zyrin, 1965; McKercher and Anderson, 1968; Veinot and Thomas, 1972; Turner and Newman, 2005). These enzymes are also responsible for the transformation of other organic P (phosphate esters) from plants and microbial biomass, including phospholipids (Kowalenko and McKercher, 1970), glycerol phosphates, phosphatidyl choline (Hance and Anderson, 1963), and nucleic acids (Anderson, 1970). Microbial biomass P represents a relatively minor proportion (<1 to 5 percent) of the total soil P (Torsvik and Goksoyr, 1978; Turner *et al.*, 2013), but it is the most active labile P pool in soil P cycling.

Phosphatases are more specifically categorized as phosphomonoesterases (PMEs) and phosphodiesterases. In classic enzymology in which purified, single enzymes are studied, the terms acid PME (EC 3.1.3.2) and alkaline PME (EC 3.1.3.1) are used to refer to two distinct intracellular PMEs that differ in their pH-constrained catalytic mechanisms for hydrolysing the phosphomonoester bond. In soils, these pH optima sometimes manifest in distinct acid and alkaline pH optima, but often, soil enzyme activities do not exhibit clear pH optima (Wade *et al.*, 2021). Thus, the term "acid phosphatase" technically refers to PME activity assayed under acidic conditions (operationally speaking), and for purified enzymes (not soil samples), a PME with an acidic pH activity optimum. The opposite applies to alkaline phosphatase.

Currently, there is substantial evidence that not all soil PME activity with acid or alkaline pH is necessarily due to discrete enzymes (specifically acid [EC 3.1.3.2] and alkaline [EC 3.1.3.1] PMEs). Instead, PME activity in soils is derived from the contributions of multiple isozymes, which in aggregate generally results in acid and alkaline optima (Wade *et al.*, 2021).

Phosphatase activity is a valuable enzyme activity for the evaluation of the P-cycle in native ecosystems where fertilization is not used. However, it may not be a good SH indicator when certain management practices are in use, such as inorganic phosphate fertilizers and limestone (Dick *et al.*, 2000; Acosta-Martinez *et al.*, 2021). Inorganic phosphate fertilizers naturally suppress this enzyme by a feedback mechanism, as the PO_4 product of the reaction is the same form of P found in fertilizers (Mathur and Rayment, 1977; Clarholm, 1993; Janes-Bassett *et al.*, 2022).

6.2. Scope and field of application

This protocol applies to the determination of potential PME (EC 3.1.3) activity in soil, based on colorimetric determination of PNP released when soil is incubated with *p*-nitrophenyl phosphate (PNPP) substrate.

Two protocols are described here for the estimation of the activities of PMEs in soils. The method developed by Tabatabai and Bremner (1969) has been used for more than 50 years. It is rapid, simple, reproducible and has been shown to be sensitive for detecting changes due to land management, physical disturbance and selected contaminants worldwide (Acosta-Martinez *et al.*, 2021). It involves the colorimetric estimation of the PNP released when soil is incubated with toluene and buffered disodium PNPP solution pH 6.5 or pH 11, for acid phosphatase or alkaline phosphate activity, respectively.

The second protocol aims for a broader measure of PMEs. This assay does not use a buffer but instead employs water as the matrix for the assay, to avoid assumptions on the pH optima of "acid" or "alkaline" phosphatase (such as PME) (Turner, 2010; Wade *et al*., 2021) which entails an assay pH that aligns with soil pH and thus *in situ* conditions (Burns *et al.*, 2013). Recent studies have shown that the pH optima of soil enzymes may co-vary with some basic soil properties, including PME (Wade *et al.,* 2021). Water can hold the assay pH constant as well as buffers (Lessard *et al.*, 2013; Li, Wade and Margenot, 2021), thus avoiding the potential suppression of enzyme activity.

Neither of the tests described here apply toluene. Multiple studies have demonstrated that it can be omitted from the 1-hour incubation (Eivazi and Tabatabai, 1977; Tabatabai, 1994). Eliminating toluene also reduces safety concerns and environmental risks associated with the waste generated (Acosta-Martinez *et al.*, 2021).

6.3. Principle

The method developed by Tabatabai and Bremner (1969) involves the colorimetric estimation of PNP released when soil is incubated with toluene and buffered disodium PNPP solution pH 6.5 or pH 11, for acid phosphatase or alkaline phosphate activity, respectively. Treatment of $CaCl_2$–NaOH (pH 10) after incubation is used to extract the PNP released by phosphatase activity, which develops a stable yellow colour used to estimate this phenol and gives quantitative recovery of PNP added to soils.

The assay for the broader measure of PMEs is also based on the use of PNPP substrate with the spectrophotometric determination of PNP (yellow colour) released by enzyme activity when soil is incubated with a solution (no buffer, only water) of PNPP. The soil–substrate mixture is incubated at 37 °C for 1 hour, and the PNP released is extracted by filtration after the addition of $CaCl_2$ and THAM reagents.

Both spectrophotometric methods used for determination of PNP depend on the fact that alkalinized solutions (as achieved by the addition of 0.5 M NaOH or 0.1 M THAM at pH 12) of this phenol have a yellow colour, due to the deprotonation of PNP. Solutions of PNPP are generally colourless or have a slight yellow tinge due to background PNP impurities from the synthesis procedure or from abiotic hydrolysis during storage.

6.4. Apparatus

The following apparatus will be needed:

- 50 ml Erlenmeyer flasks, fitted with No. 2 stoppers or best option in the lab;
- long stem funnels;
- pipettes and tips;
- cuvettes;
- electronic weighing scale (± 1.0mg sensitivity);
- an incubator (37 °C);
- Whatman No. 2V folded filter paper; and
- a spectrophotometer or colorimeter that can be adjusted to measure absorbance from 400 to 420 nm.

6.5. Materials

The following materials will be needed:

- Deionized water.
- Modified universal buffer (MUB) stock solution: Dissolve 12.1 g of tris(hydroxymethyl)aminomethane (THAM), 11.6 g of maleic acid, 14.0 g of citric acid, and 6.3 g of boric acid (H_3BO_3) in 488 ml of 1 M sodium hydroxide (NaOH) and dilute the solution to 1 L with DI water. Store it in a refrigerator.
- Modified universal buffer (pH 6.5 and 11): Place 200 ml of MUB stock solution in a 500 ml beaker containing a magnetic stirring bar, and place the beaker on a magnetic stirrer. Adjust the solution to pH 6.5 with 0.1 M hydrochloric acid (HCl), and bring the volume to 1 L with DI water. Adjust another 200 ml of the MUB stock solution to pH 11 by using 0.1 M NaOH, and bring the volume to 1 L with water.
- *p*-nitrophenyl phosphate solution (0.05 M): Dissolve 0.840 g of disodium *p*-nitrophenyl phosphate tetrahydrate (PNPP) (CAS 4264-83-9 [Sigma 104, Sigma-Aldrich]) in about 40 ml of MUB pH 6.5 (for assay of acid phosphatase) or pH 11 (for the assay of alkaline phosphatase) and adjust to 50 ml with MUB of the same pH. Store the solution in a refrigerator.
- Calcium chloride ($CaCl_2$) (0.5 M): Dissolve 73.5 g of $CaC1_2 \bullet 2H_2O$ in about 700 ml of water, and adjust the volume to 1 L with DI water.
- Sodium hydroxide (NaOH) (0.5 M): Dissolve 20 g of NaOH in about 700 ml of water, and adjust the volume to 1 L with DI water.
- A standard PNP solution: Dissolve 1.0 g of PNP in about 700 ml of water and adjust the solution to 1 L with DI water. Store the solution in a refrigerator.

6.6. Health and safety

This procedure involves the use of hazardous chemicals. Refer to the laboratory safety guidelines or the safety data sheet (SDS) before proceeding.

6.6.1. Personnel safety

Wear proper personal protective equipment. Use laboratory coat, closed shoes, appropriate gloves and safety glasses when performing a chemical analysis to mitigate the harmful effects of chemical exposure. Wash hands and clean other exposed areas with mild soap and water after using all chemical reagents.

6.6.2. Chemical hazards

p-nitrophenol is a common environmental pollutant owing to its wide application in pharmaceuticals, explosives, dyes and agrochemicals. Inhalation or ingestion causes headaches, drowsiness, nausea, and a blue colour in lips, ears, and fingernails (cyanosis). Contact with eyes or skin causes irritation and can also be absorbed through skin giving the same symptoms as for inhalation. (USCG, 1999). No information is available on the chronic (long-term) effects of PNP from inhalation or oral exposure in humans or animals. No information is available on the reproductive, developmental, or carcinogenic effects of PNP in humans. The EPA has not classified PNP for potential carcinogenicity. All PNP waste is considered a hazardous waste and must be discarded accordingly (NCBI, 2024a).

Boric acid may be harmful if swallowed or inhaled, and may cause moderate eye irritation. Avoid breathing dust and wash hands with soap and water after handling (NCBI, 2024b).

6.7. Sample preparation

The objective for measuring enzyme activities will dictate the method of soil sample preparation and storage.

If the goal is to have enzyme activities reflect the true state of the ability of the soil to perform a given enzymatic reaction under *in situ* conditions, field-moist soil samples and cold storage at 4 °C present the best approach (Lorenz and Dick, 2011).

If the goal is to use enzyme activities to assess SH, air-dried soil samples are preferred (drying in a forced air oven at 35 °C (± 5 °C) is also acceptable). For a detailed description of alkaline and acid phosphates activities as affected by soil conditions (fresh versus air-dried) and several common cold storage temperatures (such as 4 °C, −20 °C, and −80 °C), Lee *et al.* (2007) is recommended. According to several studies, including Lee *et al.* (2007), the effects of storage treatments on the PMEs are complex and can vary as a function of the soil type. A decrease in the activity of these phosphatases is generally observed in the air-dried soil compared with the field-moist soil (such as Lopes *et al.*, 2015; Mendes *et al.*, 2019). However, the soil enzyme activity levels were similar among most of the storing conditions (air-dried at 4 °C or −20 °C) (Lee *et al.*, 2007).

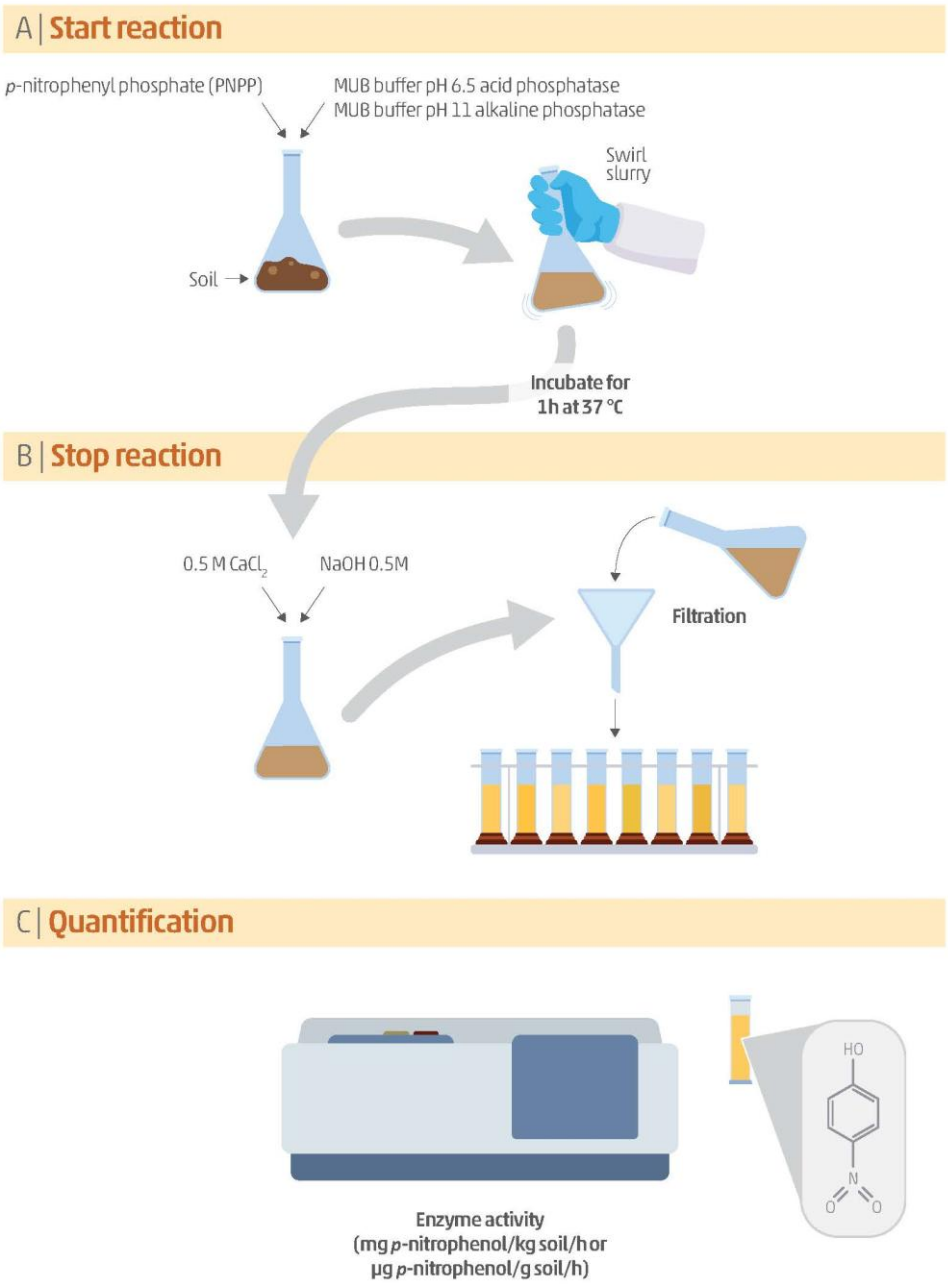
Air-drying reduces the impact of conditions that affect the highly variable microbial component relative to EA. Furthermore, EAs after air-drying are likely to better reflect the true long-term trajectory of a given management practice on SH (Lorenz and Dick, 2011; Mendes *et al.*, 2019; Acosta-Martinez *et al.*, 2021).

In both cases, soil samples must be sieved to ≤2.0 mm size.

6.8. Procedure

Label three 50 ml Erlenmeyer flasks with A and B as replicates, and the control sample as C. In each flask, place 1 g of soil (<2 mm), 4 ml of MUB (pH 6.5 for an assay of acid phosphatase or pH 11 for an assay of alkaline phosphatase), 1 ml of PNPP solution made in the appropriate buffer (pH 6.5 for an assay of acid phosphatase or pH 11 for an assay of alkaline phosphatase), and swirl the flask for a few seconds to mix the contents. Stopper the flask, and place it in an incubator at 37 °C. After 1 hour, remove the stopper, add 1 M $CaC1_2$ and then 4 ml of 0.5 M NaOH, swirl the flask for a few seconds, and filter the soil suspension through a Whatman No. 2 folded filter paper. Measure the yellow colour intensity of the filtrate with a spectrophotometer at 400 nm (Figure 12).

Figure 12. Generalized pipeline to measure acid and alkaline phosphomonoesterase activity in soil samples



Enzyme assays are performed in duplicates (Erlenmeyer flasks A and B) plus a control (Erlenmeyer flask C). A soil control should be performed with each soil analysed to allow for colour not derived from PNP released by phosphatase activity. To perform controls, follow the same procedure as for a sample, but with the addition of 1 ml of PNPP solution after the additions of 0.5 M $CaCl_2$ and 4 ml of 0.5 M NaOH (immediately before filtration of the soil suspension).

6.8.1. Calibration curve

The calibration curve is developed with standards containing 0 µg, 10 µg, 20 µg, 30 µg, 40 µg, and 50 µg of PNP in each flask (Table 10). To prepare this curve, dilute 1 ml of the standard PNP solution to 100 ml in a volumetric flask and mix the solution thoroughly.

Pipette 0 ml, 1 ml, 2 ml, 3 ml, 4 ml, and 5 ml aliquots of this diluted standard solution into Erlenmeyer flasks (50 ml), adjust the volume to 5 ml by addition of water (5 ml, 4 ml, 3 ml, 2 ml, 1 ml, and 0 ml, respectively), and proceed as described in the enzyme assay protocol after incubation of the soil sample (add 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.5 M NaOH, mix, and filter the resultant suspension) (Table 10). Measure the yellow colour intensity of the filtrate with a spectrophotometer at the same wavelength of 400 nm as for the soil enzyme assay, and prepare a calibration curve (PNP concentration versus absorbance).

When filtrates have a colour intensity exceeding that of the highest PNP standard solution, dilute the filtrate with a 1:1 mixture of MUB pH 6.0 and 0.1 M THAM, pH 12 until the absorbance readings fall within the limits of the calibration curve.

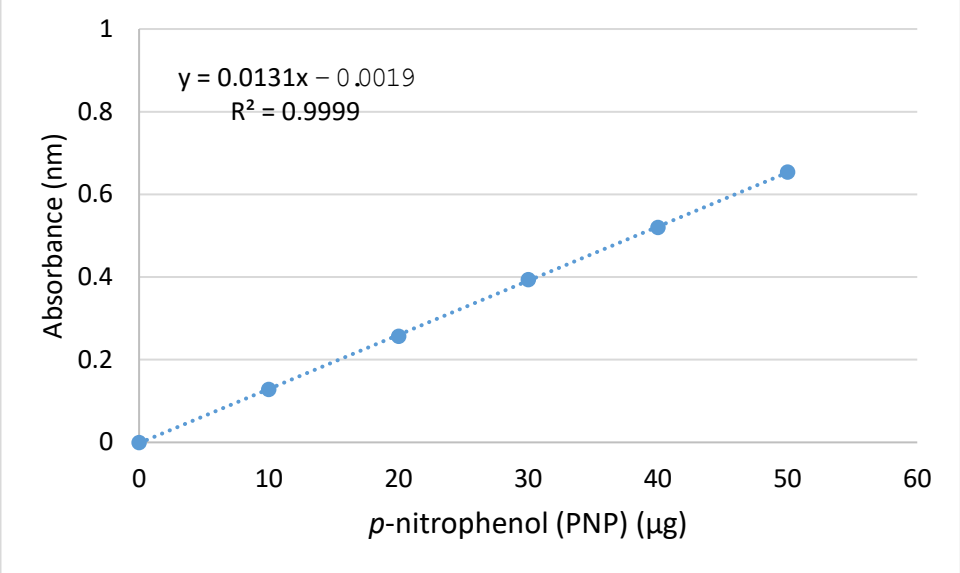
Table 10. Preparation of the calibration curve

µg *p*-nitrophenol	Distilled water (ml)	Diluted standard solution (ml)	$CaCl_2$ (ml)	NaOH (ml)
0	5.0	0	1	4
10	4.0	1.0	1	4
20	3.0	2.0	1	4
30	2.0	3.0	1	4
40	1.0	4.0	1	4
50	0	5.0	1	4

6.9. Calculation

The PNP released by the soil samples in the filtrate is calculated by reference to a calibration graph (absorbance reading versus PNP content) (Figure 13). A regression equation is used to convert the absorbance readings into PNP concentrations (Figure 13), with all the results being expressed in a dry weight basis. The concentration value of the controls must be subtracted from the concentration values obtained in the duplicates.

Figure 13:Calibration curve and regression equation



Based on the regression equation shown in Figure 13 (y = 0.0131x − 0.0019), the PNP content of the filtrates is calculated and expressed in a dry weight basis (Table 11). The control values must be subtracted from the replicate.

Table 11. Example of calculation to obtain final results of phosphomonoesterase activity in soil

Sample 1	**Absorbance (nm)**	**µg PNP**	**Dry weight (g)**	**PNP/g**	**Replicate A (control)**	**Replicate B (control)**	**Average**
Control	0.049	3.88	1.0	3.88	31.303	33.745	32.524
Replicate A	0.459	35.183	1.0	35.183			
Replicate B	0.491	37.625	1.0	37.625			

6.9.1. Results reporting

Enzyme activity can be expressed as either µg PNP/g soil/h or mg PNP/kg soil/h.

6.10. Quality assurance and quality control

6.10.1. Accuracy test

There should be participation in an interlaboratory proficiency test (PT) at least once a year. The PT z-score should be less than 3. If not, the root cause should be identified, and corrective and preventive actions developed.

6.10.2. Precision test

A replicate analysis should be performed every 20 to 30 samples in each batch test. The relative percent difference (RPD) should not be greater than 15 percent between results, as follows:

$$RPD = \frac{M_1 - M_2}{\left(\frac{M_1 + M_2}{2}\right)} \times 100\%$$

where: M_1 is the result of the sample and M_2 is the result of the sample's duplicate.

If the precision test fails, the cause of the failure must be identified, and corrective or preventive actions must be developed.

6.10.3. Laboratory control sample

The measurement of a sufficiently-available sample of known enzyme activity value can be analysed per batch of analysis, to ensure that normal conditions have been maintained for the materials and throughout the process. This laboratory control sample can be labelled as the internal reference sample or master sample (see the GLOSOLAN basic guidelines on how to prepare a sample for internal quality control [Gowing and Hayr, 2020]).

6.11. Remarks

The original protocol for phosphatase assays used toluene as a bacteriostatic (Tabatabai, 1994). Toluene is a colourless, water-insoluble liquid with a smell associated with paint thinners and has the potential of causing severe neurological harm. Multiple studies have demonstrated that it can be omitted from the 1-hour incubation (Eivazi and Tabatabai 1977; Tabatabai, 1994). Eliminating toluene also reduces safety concerns when performing the assay (avoiding the need to perform assays under the hood) and reduces environmental risks associated with the waste generated (Acosta-Martinez and Tabatabai, 2011; Acosta-Martinez *et al.*, 2021).

It is necessary to add $CaCl_2$ before the addition of NaOH to prevent the dispersion of clay and any extraction of SOM. The use of 0.5 M NaOH stops the reaction catalysed by PMEs. Clay dispersion complicates filtration, and if any SOM is extracted, the filtrate shows a dark colour which interferes with the colorimetric analysis for PNP. The procedures described give a quantitative recovery of PNP added to soils.

Absorbance of PNP can be measured at wavelengths from 400 to 420 nm with minimal difference in the enzyme activity, as long as the same wavelength is used for standards and samples (Acosta-Martinez *et al.*, 2021).

The solutions of the substrates used for the assay of PMEs are stable for several days if stored in a refrigerator. The compounds used for the assay of these enzymes are artificial substrates and are not expected to be found in soils.

The dry substrates should be stored in a freezer. The standard PNP solution is stable for a few weeks if stored in a refrigerator. The substrate concentrations in the incubation mixtures during the assay of the activities of PMEs are about 5 to 10 times greater than the Michaelis constants (K_m values) determined for these soil enzymes. K_m is a key parameter in enzyme kinetics that represents the

substrate concentration at which the reaction rate is half of its maximum value (V_{max}). It is an indicator of the affinity of an enzyme for its substrate. If necessary, these substrate concentrations can be changed to meet the objectives of the assay (Acosta-Martínez and Tabatabai, 2011).

At least two laboratory replicates are necessary to be analysed per sample.

The use of a soil control is so designed that it allows for the detection of trace amounts of PNP in some commercial samples of PNPP and for extraction of trace amounts of coloured soil material by the $CaCl_2$–NaOH treatment used for extraction of PNP in the assay of PME.

Acosta-Martinez *et al.* (2021) reported that a modification in the phosphatase protocol is possible by using half the amount of all solutions and half the amount of soil, without changing the proportion of the original assay. The same absorbance is obtained per sample and the time to perform the assays remains the same. However, it significantly reduces the amount of soil, resources and waste generated in the long term.

7. Assay method for broader activity of phosphomonoesterases

7.1. Apparatus

The following apparatus will be needed:

- 50 ml Erlenmeyer flasks, fitted with No. 2 stoppers or best option in the lab;
- long stem funnels;
- pipettes and tips;
- cuvettes;
- an electronic weighing scale (± 1.0 mg sensitivity);
- an incubator (37 °C);
- Whatman No. 2V folded filter paper; and
- a spectrophotometer or colorimeter that can be adjusted to measure absorbance from 400 to 420 nm.

7.2. Materials

The following materials will be needed:

- Deionized water or similar purity (such as 18.2 MΩ•cm water).
- *p*-nitrophenyl phosphate solution (0.05 to 0.200 M) (the exact amount depends on the research question): dissolve 0.840 g of disodium *p*-nitrophenyl phosphate tetrahydrate in approximately 40 ml of DI or distilled water, and adjust to 50 ml with DI or distilled water. Store the solution in a refrigerator for up to 2 weeks.

Note: sufficient substrate should be tested, by first determining K_m of soil PME activity, to ensure activities are measured in conditions that approach V_{max}.

- Calcium chloride (0.5 M): Dissolve 73.5 g of $CaC1_2•2H_2O$ in about 700 ml of water, and adjust the volume to 1 L with water.
- Tris(hydroxymethyl)aminomethane (0.1 M): Dissolve 12.1 g of THAM in about 900 ml of water, adjust the pH using dilute HCl or NaOH solutions, and adjust the volume to 1 L with water.
- Standard PNP solution: Dissolve 1.0 g of PNP in about 700 ml of water and adjust the solution to 1 L with water. Store the solution in a refrigerator.

7.3. Sample preparation

The objective for measuring enzyme activities will dictate the method of soil sample preparation and storage.

If the goal is to have enzyme activities reflect the true state of the ability of the soil to perform a given enzymatic reaction under *in situ* conditions, field-moist soil samples and cold storage at 4 °C present the best approach (Lorenz and Dick, 2011).

If the goal is to use enzyme activities to assess SH, air-dried soil samples are preferred (drying in a forced air oven at 35 °C (± 5 °C) is also acceptable). For a detailed description of alkaline and acid phosphates activities as affected by soil conditions (fresh versus air-dried) and several common cold

storage temperatures (4 °C, −20 °C, and −80 °C), it is recommended to refer to Lee *et al.* (2007). According to several studies, including Lee *et al.* (2007), the effects of storage treatments on the PMEs are complex and can vary as a function of the soil type. A decrease in the activity of these phosphatases is generally observed in the air-dried soil compared with the field-moist soil. However, the soil enzyme activity levels were similar among most of the storing conditions (air-dried, 4 °C or −20 °C) (Lee *et al.*, 2007).

Air-drying reduces the impact of conditions that affect the highly variable microbial component relative to EA. Furthermore, EAs after air-drying are likely to better reflect the true long-term trajectory of a given management practice on SH (Lorenz and Dick, 2011; Mendes *et al.*, 2019; Acosta-Martinez *et al.*, 2021).

In both cases, soil samples must be sieved to ≤2.0 mm size.

7.4. Procedure

Place 1 g of soil (<2 mm) in a 50 ml Erlenmeyer flask. Add 4 ml of water, 1 ml of PNPP solution and swirl the flask for a few seconds to mix the contents. Alternatively, 5 ml of substrate solution at the final assay concentration can be added directly. Stopper the flask, and place it in an incubator at 37 °C. After 1 hour, remove the stopper, add 1 ml of 0.5 M $CaCl_2$ and then 4 ml of 0.1 M THAM, swirl the flask for a few seconds, and filter the soil suspension through a Whatman No. 2V folded filter paper. Measure the yellow colour intensity of the filtrate with a spectrophotometer at 405 nm.

A soil control should be performed with each soil analysed to allow for colour not derived from PNP released by PME activity. To perform controls, follow the same procedure as for a sample, but with the addition of 1 ml of PNPP solution after the additions of 0.5 M $CaCl_2$ and 4 ml of 0.5 M THAM (immediately before filtration of the soil suspension).

7.4.1. Calibration curve for *p*-nitrophenol standard

The calibration curve is developed with standards containing 0 µg, 10 µg, 20 µg, 30 µg, 40 µg, and 50 µg of PNP in each flask (Table 12). To prepare this curve, dilute 1 ml of the standard PNP solution to 100 ml in a volumetric flask and mix the solution thoroughly. Then pipette 0 ml, 1 ml, 2 ml, 3 ml, 4 ml, and 5 ml aliquots of this diluted standard solution into 50 ml Erlenmeyer flasks, adjust the volume to 5 ml by the addition of distilled water (5 ml, 4 ml, 3 ml, 2 ml, 1 ml, and 0 ml, respectively), and proceed as described in the enzyme assay protocol after incubation of the soil sample (adding 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.1 M THAM, mix, and filter the resultant suspension) (Table 12). Measure the yellow colour intensity of the filtrate with a spectrophotometer at 400 nm (at the same wavelength as for the soil enzyme assay) and prepare a calibration curve (PNP concentration versus absorbance).

When the filtrates have a colour intensity exceeding that of the highest PNP standard solution, dilute the filtrate with a 1:1 mixture of distilled water and 0.1 M THAM until the absorbance readings fall within the limits of the calibration curve.

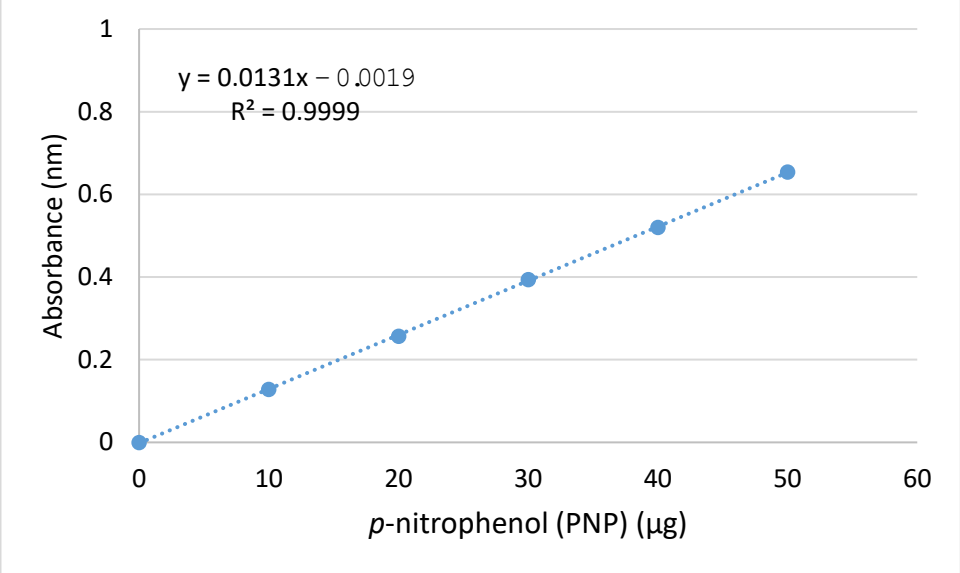
Table 12. Preparation of the calibration curve

µg *p*-nitrophenol	Distilled water (ml)	Diluted standard solution (ml)	$CaCl_2$ (ml)	NaOH (ml)
0	5.0	0	1	4
10	4.0	1.0	1	4
20	3.0	2.0	1	4
30	2.0	3.0	1	4
40	1.0	4.0	1	4
50	0	5.0	1	4

7.5. Calculation and reporting

The PNP released by the soil samples in the filtrate is calculated by reference to a calibration graph (absorbance reading versus PNP content) (Figure 14). A regression equation is used to convert the absorbance readings into PNP concentrations (Figure 14), with all the results being expressed in a dry weight basis. The concentration value of the controls must be subtracted from the concentration values obtained in the duplicates.

Figure 14. Calibration graph and regression equation



Based on the regression equation shown in Figure 14, the PNP content of the filtrates is calculated and expressed in a dry weight basis (Table 13). The control values must be subtracted from the replicate.

Table 13. Example of calculation to obtain final results of phosphomonoesterase activity in soil

Sample 1	**Absorbance (nm)**	**µg PNP**	**Dry weight (g)**	**PNP/g**	**Replicate A (minus control)**	**Replicate B (minus control)**	**Average**
Control	0.049	3.88	1.0	3.88	31.303	33.745	32.524
Replicate A	0.459	35.183	1.0	35.183			
Replicate B	0.491	37.625	1.0	37.625			

7.5.1. Results reporting

Enzyme activity can be expressed as either µg PNP/g soil/h or mg PNP/kg soil/h.

7.6. Remarks

The original protocol for phosphatase assays used toluene as a bacteriostatic (Tabatabai, 1994). It is a colourless, water-insoluble liquid with a smell associated with paint thinners and has the potential of causing severe neurological harm. Multiple studies have demonstrated that it can be omitted for the 1-hour incubation (Eivazi and Tabatabai 1977; Tabatabai, 1994). Eliminating toluene also reduces safety concerns when performing the assay (such as avoiding the need to perform assays under the

hood) and reducing environmental risks associated with the waste generated (Acosta-Martinez and Tabatabai, 2011; Acosta-Martinez *et al.*, 2021).

It is necessary to add $CaCl_2$ before the addition of THAM to prevent the dispersion of clay and any extraction of soil organic matter. The use of 0.1 M THAM is proposed to attenuate or stop the reaction catalysed by PMEs, under the assumption that alkalization denatures extracellular enzymes in the soil sample. However, this assumption has yet to be tested. Dispersion of clay complicates filtration, and if organic matter is extracted, the filtrate shows a dark colour, which interferes with the colorimetric analysis for PNP. The procedures described give a quantitative recovery of PNP added to soils.

The solutions of the substrates used for the assay of PMEs are stable for several days if stored in a refrigerator. The compounds used for the assay of these enzymes are artificial substrates and are not expected to be found in soils.

The dry substrates should be stored in a freezer. The standard PNP solution is stable for at least 3 weeks if stored in a refrigerator (Daughtridge, Nakayama and Margenot, 2021). The substrate concentrations in the incubation mixtures during the assay of the activities of PMEs are about five to ten times greater than the K_m values determined for these soil enzymes. If necessary, these substrate concentrations can be changed to meet the objectives of the assay (Acosta-Martínez and Tabatabai, 2011; Margenot, Nakayama and Parikh, 2018).

At least two laboratory replicates are necessary to be analysed per sample.

The use of a soil control is so designed that it allows for the PNP in some commercial samples of PNPP and for the extraction of trace amounts of coloured soil material by the $CaCl_2$–THAM treatment used for the extraction of PNP in the PME assay.

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The Global Soil Partnership (GSP) is a globally recognized mechanism established in 2012. Our mission is to position soils in the Global Agenda through collective action. Our key objectives are to promote Sustainable Soil Management (SSM) and improve soil governance to guarantee healthy and productive soils, and support the provision of essential ecosystem services towards food security and improved nutrition, climate change adaptation and mitigation, and sustainable development.

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INTERNATIONAL NETWORK ON SOIL BIODIVERSITY

The International Network on Soil Biodiversity (NETSOB) was established in December 2021 to promote the sustainable use and conservation of soil biodiversity and to bring experts in this field and existing initiatives together to form the human talent that contributes to the implementation of the Global Soil Biodiversity Observatory (GLOSOB).

GLOSOLAN
GLOBAL SOIL LABORATORY NETWORK

GLOSOLAN is a Global Soil Laboratory Network which aims to harmonize soil analysis methods and data so that soil information is comparable and interpretable across laboratories, countries and regions. Established in 2017, it facilitates networking and capacity development through cooperation and information sharing between soil laboratories with different levels of experience. Joining GLOSOLAN is a unique opportunity to invest in quality soil laboratory data for a sustainable and food secure world.

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