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Methodological recommendations for optimizing assays of enzyme activities in soil samples

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ABSTRACT

Assays of enzyme activities in soil samples based on *para*-nitrophenol (*p*NP) spectrophotometry are a powerful tool in soil biochemistry. We evaluated potential sources of error and optimization strategies for soil enzyme assays across 12 diverse soils (6 USDA orders, 31–127 mg g^{-1} soil organic carbon [SOC]), using the activity of soil phosphomonoesterase (PHO) as an example. We hypothesized that dissolved organic matter (DOM) interference, *p*NP recovery, and substrate concentration would affect calculated enzyme activities, and that this would reflect the method of assay termination: 0.5 M NaOH + 0.5 M $CaCl_2$ (Tabatabai, 1994), 0.2 M NaOH + 2.0 M $CaCl_2$ (Schneider et al., 2000), 0.5 M NaOH + 2.0 M $CaCl_2$ (this study), and 0.1 M Tris (pH 12) + 0.5 M $CaCl_2$ (Klose et al., 2003). Terminations using 0.5 M NaOH increased *p*NP recovery compared to termination with 0.1 M Tris, but resulted in greater DOM interference (absorbance at 410 nm), which for terminations using NaOH but not Tris was positively correlated with total SOC (R^2 = 0.45–0.38). Greatest DOM interference occurred for Andisols for termination with 0.5 M NaOH + 0.5 M $CaCl_2$, which for two Andisols of intermediate SOC (97 and 68 mg g^{-1}) was 1–2 orders of magnitude greater than other soils (346 and 246% overestimation of PHO activity). Increasing $CaCl_2$ concentration (0.5 M–2.0 M) decreased DOM interference, but this effect was less pronounced than the effects of base type or concentration. Enzyme activity tended to be overestimated in assays terminated with NaOH due to DOM interference, and was more greatly underestimated in assays terminated with Tris buffer due to low recovery of *p*NP, which was soil-specific. Soil PHO K_m values, which were not correlated with SOC, varied by soil (4.2–13.3 mM g^{-1} soil) demonstrating that substrate concentrations routinely employed (typically ≤ 10 mM g^{-1} soil) are likely insufficient to achieve recommended substrate conditions (5 × K_m) for accurate measurement of PHO activity. This study illustrates the importance of *a priori* determination of soil enzyme K_m to achieve conditions nearing substrate saturation, and recommends termination with 0.2 M NaOH + 2.0 M $CaCl_2$, correction for *p*NP recovery, and correction for DOM absorbance at 410 nm to increase the accuracy of *p*NP-based enzyme assays in soils. Finally, to improve communication and thus comparison of measured enzyme activities among studies and assay methods (*p*NP vs 4-methylumbelliferone [MUF]), it is suggested that studies report the concentration of substrate for the final volume used in enzyme assays, report K_m values on a soil mass basis, express enzyme activities on a molar *p*NP basis, and qualify enzyme activities, K_m, and V_{max} as 'apparent' if corrections for interferences are not performed.

1. Introduction

Para-nitrophenol (*p*NP)-based enzyme assays are widely employed to measure the activities of enzymes that drive soil nutrient cycling. The basis of this approach is that hydrolysis of the *para*-nitrophenyl-linked substrate by enzymes present in a soil sample releases *p*NP. The concentration of *p*NP released, and thus the activity of the substrate-specific enzyme in the soil sample, can be inexpensively and rapidly quantified by spectrophotometry (410 nm) under alkaline conditions. Since its first application to soils in 1969 to assay phosphomonoesterase (PHO) activity (Tabatabai and Bremner, 1969), a suite of additional *para*-nitrophenyl substrates have been developed to assay enzymes that mediate organic carbon (C), nitrogen (N), phosphorus (P), and sulfur (S) mineralization in soils.

Less than two decades after the first application of *para*-nitrophenyl assays to soils, the importance of adhering to best practices of enzyme assays while also accounting for potential artifacts unique to soil samples was raised (Malcolm, 1983). Malcolm (1983) argued that "[i]n

Abbreviations: *p*NP, *para*-nitrophenol; *p*NPP, *para*-nitrophenyl phosphate; PHO, phosphomonoesterase; DOM, dissolved organic matter; SOC, soil organic carbon

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many of the publications concerning the measurement of phosphatase activities in soils, the basic rules governing enzyme assays have, at best, been only partially obeyed." Methodological aspects of enzyme assays in their application to soil samples may compromise the accuracy and comparability of measured activities. Potential sources of error include (1) dissolved organic matter (DOM) interference with spectrophotometry, (2) incomplete recovery of released *p*NP, and (3) substrate concentrations insufficient to achieve saturation of the enzyme (Malcolm, 1983). Furthermore, the method of assay termination has been suggested to influence DOM interference and *p*NP recovery, the extent of which are likely soil-specific, but only limited comparisons of soil types and termination methods have been made (Schneider et al., 2000). The objective of this study was to quantify the effects of these potential sources of error in *p*NP-based enzyme assays using soils of diverse pedogenic states and soil organic matter (SOM) content, with PHO as an example enzyme.

The method of alkaline termination affects DOM interference with *p*NP spectrophotometry and thus the accuracy of soil enzyme assays (Schneider et al., 2000). *p*NP-based assays are terminated with a solution of base and flocculating agent, commonly sodium hydroxide (NaOH) and calcium chloride ($CaCl_2$), respectively (Tabatabai and Bremner, 1969). The role of NaOH is to both extract *p*NP released by enzyme activity from the soil matrix and to develop the color for spectrophotometry via conversion of *para*-nitrophenol to *para*-nitrophenoxide (400–415 nm); $CaCl_2$ serves as a flocculent to increase supernatant clarity for spectrophotometry (Tabatabai, 1994). However, NaOH can co-extract DOM, which can contribute to absorbance at 400–415 nm. To address these artifacts, two alternative terminations have been proposed for high SOM samples. Schneider et al. (2000) proposed decreasing the concentration of NaOH from 0.5 M to 0.2 M while increasing the concentration of $CaCl_2$ from 0.5 M to 2.0 M to reduce co-extraction of DOM, which interfered with spectrophotometry of PHO activity assays in forest soils. For the same reason, Klose et al. (2003, 2011) proposed replacing 0.5 M NaOH with 0.1 M tris(hydroxymethyl)aminomethane (Tris, also referred to as THAM) buffer (pH 12) in activity assays of PHO as well as glucosidase and sulfatase in peat samples.

Malcolm (1983) emphasized the need to account for soil-specific sorption of the released *p*NP, which can sorb to mineral and organic components of soil samples (Boyd, 1982). Soil-specific recovery of *p*NP among a variety of soils was first quantified in assays of PHO activities in forest soils (Harrison, 1979), and it has been demonstrated that adjusting for soil-specific *p*NP sorption is necessary to accurately measure enzyme activities as well as kinetic characterization parameters (e.g., Michaelis constant [K_m], maximal velocity of catalysis [V_{max}]) (Cervelli et al., 1973; Skujiņš and Burns, 1976; Trasar-Cepeda and Gil-Sotres, 1988; Vuorinen, 1993; Margesin et al., 2002). However, less understood is the extent to which incomplete *p*NP recovery may compromise soil enzyme activity measurements. Nor is it known how different termination methods influence *p*NP recovery.

A final challenge to the reliability of soil enzyme activity measurements is the high variability in substrate concentrations among studies. Substrate concentrations in assays should be sufficiently high as to approach or achieve V_{max}, because as measured activities near V_{max} they are more reliably comparable among soils within and across studies (Malcolm, 1983; Schneider et al., 2000; German et al., 2011). Employing a substrate concentration 5-fold greater than the empirically determined K_m in assays of enzyme activities (Brooks et al., 2012) has been suggested for soils (Burns, 1978, 1982), yet the majority of soil studies do not assess whether the substrate concentration employed achieves this. For example, assays of soil PHO activity reportedly encompass as much as four orders of magnitude of substrate concentration (e.g., 0.05–20 mM) (see reviews by Malcolm, 1983; Nannipieri et al., 2011) and differences in the amount of soil (e.g., 0.2–2.0 g) used means that substrate concentration per unit soil (mM g^{-1}) may vary even more. While proposed in the first descriptions of enzyme assays (Tabatabai and Bremner, 1969) and emphasized several decades after (Burns, 1978; Malcolm, 1983), these normative prescriptions on substrate concentration have not been explicitly assessed for potential to compromise soil enzyme activity data obtained by the *p*NP assay method.

This study sought to evaluate three parameters known to affect the accuracy and comparability of enzyme activities determined by *p*NP-based assays. Four termination methods were evaluated: the widespread modification (i.e., no toluene) (Tabatabai, 1994) of the original method for soil enzyme assays of PHO (Tabatabai and Bremner, 1969), two alternatives proposed for high SOM samples (Schneider et al., 2000; Klose et al., 2003), and an alternative termination reported for the first time in this study. The effects of not correcting for DOM interference and *p*NP recovery were investigated for these four termination methods across 12 soils (6 USDA orders) with diverse properties, including SOM content. Additionally, we illustrate the importance of employing substrate concentrations that approximate V_{max} based on the recommended use of $5 \times K_m$. Finally, we review historic trends in substrate concentrations used for PHO activity assays and draw upon traditional biochemistry literature to highlight theoretical considerations to improve the accuracy and, as importantly, the communication of soil enzyme assay conditions and activities to ensure the comparability of enzyme data within soil science and across diverse disciplines.

2. Methods

2.1. Sites and soil sampling

Soils were sampled at a total of twelve locations in the California Sierra Nevada to furnish a diversity of soil properties relevant to enzyme activities (e.g., SOC). These twelve sites represent combinations of three parent materials (basalt, granite, andesite) and four climate zones. Climate zones are defined by elevation ranges with characteristic forests dominated by blue oak (*Quercus douglasii*; BO), ponderosa pine (*Pinus ponderosa*; PP), white fir (*Abies concolor*; WF), and red fir (*Abies magnifica*; RF) (Table 1). Precipitation occurs primarily as rain at lower elevations (BO, PP) and as snow at higher elevations (WF, RF), and is concentrated in November–March. Soil orders include Alfisols (2), Andisols (3), Entisols (2), Inceptisols (2), Mollisols (1), and Ultisols (2). Pedogenesis and soil C cycling at these sites have been extensively investigated (Dahlgren et al., 1997; Rasmussen et al., 2006, 2007; Graham and O'Geen, 2010; Rasmussen et al., 2010).

Soils at each of the twelve sites were sampled at four locations. Similar to the sampling procedure described by Rasmussen et al. (2008), for consistency soil sampling locations within each site were separated by at least 10 m, on a similar landform (midslope) and at least 3 m away from the nearest tree. Overlying litter and/or O horizons were removed by gentle raking or excavating with a hand trowel, respectively. Mineral soil (A horizon) was sampled at 0–5 cm depth. The four soil samples were combined into a composite for this study.

2.2. Soil properties

Soil pH was measured in deionized water (1:5) after 30 min of equilibration. Soil texture was determined by laser diffraction (Eshel et al., 2004). Total soil C and N were determined with an ECS 4010 CHNSO Analyzer (Valencia, CA). Available P was determined as anion-exchange membrane (AEM) extractable inorganic P (P_i). AEM strips (1×4 cm, VWR International, West Chester, PA) were loaded with carbonate as the counterion. Soils were extracted with AEM in distilled water (1:20 soil:water) by shaking for 18 h. Inorganic P was desorbed from the membranes by shaking for 1 h in 0.25 M H_2SO_4 and quantified by absorbance at 880 nm (Murphy and Riley, 1962). Total soil P was estimated as P_i following ashing (550 °C, 1 h) and acid extraction (1 M H_2SO_4, 1:50 soil:extractant, 18 h) (Dieter et al., 2010).

Soils encompassed a diversity of properties expected to entail