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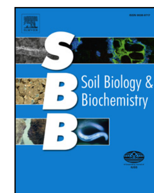

Methodological recommendations for optimizing assays of enzyme activities in soil samples

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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

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Table 1
Description of forest soils (0–5 cm depth of A horizon) from the Sierra Nevada, CA, USA used to evaluate *para*-nitrophenol (*p*NP) substrate assays of soil enzyme activities.

Site ID	Parent material	Dominant Vegetation[†]	Elevation (masl)	MAP (cm)	MAT (°C)	Soil classification
An-BO[a]	Andesite	Blue oak	160	46	17.0	loamy-skeletal, mixed, superactive thermic Lithic Ultic Haploxeroll
An-PP[a]	Andesite	Ponderosa pine	1150	125	11.5	fine, parasesquic, mesic Andic Palehumult
An-WF[a]	Andesite	White fir	1700	140	8.5	medial-skeletal, amorphic, mesic Humic Haploxerand
An-RF[a]	Andesite	Red fir	2150	135	6.0	medial-skeletal, amorphic, frigid Humic Vitrixerand
Ba-BO[b]	Basalt	Blue oak	280	78	16.7	fine-loamy, mixed, superactive thermic Ultic Haploxeralf
Ba-PP[b]	Basalt	Ponderosa pine	920	99	14.2	fine, parasesquic, mesic Xeric Palehumult
Ba-WF[b]	Basalt	White fir	1600	115	8.3	medial-skeletal, amorphic, mesic Typic Haploxerand
Ba-RF[b]	Basalt	Red fir	2300	134	6.5	sandy-skeletal, mixed, frigid Vitrandic Xerorthent
Gr-BO[c]	Granite	Blue oak	198	33	16.7	coarse-loamy, mixed, thermic Typic Xerochrept
Gr-PP[c,d]	Granite	Ponderosa pine	1390	91	11.1	fine-loamy, mixed, semiactive, mesic Ultic Haploxeralf
Gr-WF[c,d]	Granite	White fir	1800	101	9.1	coarse-loamy, mixed, superactive, mesic Humic Dystroxerept
Gr-RF[d,e]	Granite	Red fir	2200	102	5.0	mixed, superactive, frigid Dystric Xeropsamment

[†] Blue oak, Quercus douglasii; Ponderosa pine, Pinus ponderosa; White fir, Abies concolor; Red fir, Abies magnifica.
[a] Rasmussen et al., 2007.
[b] Rasmussen et al., 2010.
[c] Dahlgren et al., 1997.
[d] Rasmussen et al., 2006.
[e] Bales et al., 2011.

Table 2
General soil properties of twelve forest soils (0–5 cm of A horizon, n = 4) used to optimize *para*-nitrophenol (*p*NP)-based assays of soil phosphomonoesterase activity. Soils represent combinations of three parent materials (andesite, An; basalt, Ba; granite, Gr) and four elevations defined by dominant tree species (blue oak; BO; ponderosa pine; PP; white fir; WF; red fir, RF) from the California Sierra Nevada (USA).

Soil	Texture			pH		Total C		Total N		Total P		Available P[a]	
	clay	silt	sand			mg g^{-1}		mg g^{-1}		μg g^{-1}		μg g^{-1}	
	g kg^{-1}			mean	se	mean	se	mean	se	mean	se	mean	se
An-BO	122	552	326	5.81	0.01	34	1	2.4	0.0	526	34	2.1	0.1
An-PP	65	460	476	6.02	0.03	72	3	2.6	0.1	837	28	4.3	0.2
An-WF	32	397	571	6.16	0.01	127	3	5.3	0.2	1503	51	1.3	0.2
An-RF	29	366	606	5.84	0.01	97	2	4.2	0.1	1411	71	3.8	0.4
Ba-BO	94	516	390	6.70	0.00	35	0	2.9	0.0	578	26	0.7	0.0
Ba-PP	77	475	448	6.18	0.01	80	2	3.2	0.1	435	21	0.4	0.0
Ba-WF	26	307	667	6.01	0.01	68	5	3.2	0.2	720	49	0.8	0.1
Ba-RF	19	180	801	6.97	0.01	37	1	1.9	0.1	393	66	5.9	0.5
Gr-BO	22	347	631	6.43	0.01	31	5	2.6	0.3	364	49	19.9	1.0
Gr-PP	27	508	464	6.09	0.01	62	4	2.6	0.1	723	120	20.1	0.2
Gr-WF	29	338	633	6.03	0.01	52	4	1.9	0.1	539	54	2.8	0.6
Gr-RF	26	301	673	5.79	0.01	61	6	2.1	0.2	567	97	9.8	0.1

[a] Anion-exchange membrane extractable P.

differences in PHO activity as well as artifacts in *p*NP-based enzyme assays (Table 2). Soil pH ranged from acidic to neutral (pH 5.8–7.0). Soils represented a 4-fold gradient in SOC (31–127 mg g^{-1}), and varied by an order of magnitude in clay content (19–122 mg g^{-1}) and by two orders of magnitude in available P (0.4–20.1 μg g^{-1}).

2.3. Alkaline termination

Four methods of assay termination representing combinations of base and $CaCl_2$ were evaluated:

(1) 0.5 M NaOH + 0.5 M $CaCl_2$ (Tabatabai, 1994)
(2) 0.2 M NaOH + 2.0 M $CaCl_2$ (Schneider et al., 2000)
(3) 0.1 M Tris buffer (pH 12) + 0.5 M $CaCl_2$ (Klose et al., 2003)
(4) 0.5 M NaOH + 2.0 M $CaCl_2$ (this study)

Assays were performed using the conditions described by Tabatabai (1994) for acid phosphomonoesterase (PHO, Enzyme Commission 3.1.3.2) because the buffer, incubation time and temperature, and substrate concentration of this method is widespread in soil science and our objective was to quantify the effects of potential artifacts in soil enzyme activity assays as commonly practiced. Four mL of modified universal buffer (MUB, pH 6.5) and 1 mL of 50 mM *para*-nitrophenyl phosphate (*p*NPP) in MUB (pH 6.5) was added to 1 g of soil. This provided a final substrate concentration of 10 mM g^{-1} soil. The slurry was briefly mixed by vortexing and incubated for 1 h at 37 °C.

Reactions were terminated immediately at the conclusion of the 1 h incubation by adding 4 mL of base and 1 mL of $CaCl_2$ solutions. Assays were centrifuged to remove sediment from the supernatant, from which *p*NP was quantified spectrophotometrically using absorbance at 410 nm. Centrifugation yields similar *p*NP values as filtration (Elsgaard et al., 2002), which was confirmed for a subset of our samples. Mean absorbance from triplicate negative controls (i.e., substrate but no soil) specific to each termination method was subtracted from absorbance of soil assays. The activity calculated for PHO in this manner, without further correction for DOM or *p*NP recovery, is referred to hereafter as uncorrected PHO activity.

2.4. Evaluation of DOM interference and recovery of *p*NP

The effect of DOM on absorbance at 410 nm was evaluated by performing assays as described above, but without substrate (i.e., 5 mL

Table 3
Uncorrected activity of phosphomonoesterase (PHO) in 12 forest soils (0–5 cm of A horizon) for 4 methods of alkaline termination of *para*-nitrophenol (*p*NP) based enzyme assays. Soils represent combinations of three parent materials (andesite, An; basalt, Ba; granite, Gr) and four elevations defined by dominant tree species (blue oak; BO; ponderosa pine; PP; white fir; WF; red fir, RF) from the Sierra Nevada, USA. For a given soil, letters indicate significant differences in PHO activity by termination method as determined by Tukey's test ($p < 0.05$), and the F-statistic indicates the magnitude of the termination effect.

Soil	Uncorrected PHO activity (μmol *p*NP g^{-1} h^{-1})				F-statistic
	0.5 M NaOH + 0.5 M $CaCl_2$	0.2 M NaOH + 2.0 M $CaCl_2$	0.1 M Tris (pH 12) + 0.5 M $CaCl_2$	0.5 M NaOH + 2.0 M $CaCl_2$	
	(Tabatabai, 1994)	(Schneider et al., 2000)	(Klose et al., 2003)	(this study)	
An-BO	2.95 ± 0.09 a	2.81 ± 0.17 a	2.27 ± 0.05 b	1.69 ± 0.04 c	24.4
An-PP	3.77 ± 0.25 a	2.91 ± 0.04 b	1.91 ± 0.11 c	1.34 ± 0.03 c	43.6
An-WF	0.62 ± 0.05 c	2.86 ± 0.39 a	3.17 ± 0.12 a	1.82 ± 0.07 b	23.5
An-RF	0.81 ± 0.07 c	2.14 ± 0.14 a	1.78 ± 0.04 a	1.28 ± 0.03 b	36.8
Ba-BO	3.00 ± 0.06 a	3.50 ± 0.02 a	3.56 ± 0.15 a	1.69 ± 0.20 b	33.5
Ba-PP	2.86 ± 0.09 a	2.28 ± 0.08 b	1.91 ± 0.10 b	1.49 ± 0.02 c	40.8
Ba-WF	4.93 ± 0.27 a	4.34 ± 0.30 a	3.16 ± 0.06 b	1.77 ± 0.15 c	30.1
Ba-RF	1.15 ± 0.02 b	0.96 ± 0.04 c	2.78 ± 0.05 a	0.49 ± 0.02 d	602.8
Gr-BO	4.09 ± 0.08 a	3.37 ± 0.11 b	0.51 ± 0.01 d	1.35 ± 0.04 c	418.5
Gr-PP	3.17 ± 0.10 a	2.42 ± 0.18 b	2.57 ± 0.11 b	1.13 ± 0.07 c	38.2
Gr-WF	2.02 ± 0.04 a	1.56 ± 0.03 b	2.08 ± 0.04 a	0.84 ± 0.01 c	281.5
Gr-RF	2.12 ± 0.10 a	1.73 ± 0.10 b	2.06 ± 0.06 ab	1.00 ± 0.01 c	35.2

of buffer), for all four termination treatments. Absorbance of substrate-free assays was used to express DOM interference on a *p*NP basis (μmol *p*NP equivalent g^{-1} h^{-1}). From each uncorrected PHO activity (4 analytical replicates per termination per soil), the corresponding mean DOM *p*NP equivalent was subtracted to yield the DOM-corrected PHO activity. The DOM-corrected PHO activity was then used to calculate the degree of mis-estimation (%) of PHO activity that resulted from not accounting for DOM absorbance (Equation (1)).

$$\frac{(\text{uncorr. PHO activity}) - (\text{DOM corr. PHO activity})}{\text{DOM corr. PHO activity}} \times 100\% \quad (1)$$

Differences in DOM interference and DOM-corrected activities among the 4 termination methods were tested for each soil with analysis of variance (ANOVA) and mean differences were determined by Tukey's test ($p < 0.05$) using PROC GLM in SAS v9.4 (SAS Institute, Cary, NC).

Recovery of *p*NP was evaluated by performing assay incubations in which substrate was replaced with *p*NP to obtain the same final concentration as in assays described above (i.e., 10 mM *p*NP per g soil). This was performed for all four termination methods. To evaluate the additive effects of DOM and *p*NP recovery, mis-estimation of PHO activity with *p*NP recovery correction was separately calculated without correction for DOM (Equation (2)), and with correction for DOM by subtracting mean DOM absorbance from absorbance of *p*NP recovery assays (4 analytical replicates per termination per soil) (Equation (3)).

$$\frac{(\text{uncorr. PHO activity}) - (p\text{NP recovery corr. PHO activity})}{p\text{NP recovery corr. PHO activity}} \times 100\% \quad (2)$$

$$\frac{(\text{DOM corr. PHO activity}) - (\text{DOM corr. } p\text{NP recovery corr. PHO activity})}{\text{DOM corr. } p\text{NP recovery corr. PHO activity}} \times 100\% \quad (3)$$

Differences in *p*NP recovery, *p*NP-corrected activities and DOM + *p*NP-corrected activities among the 4 termination methods were tested for each soil with ANOVA and mean differences were determined by Tukey's test ($p < 0.05$).

2.5. Effect of substrate concentration

To evaluate the effect of substrate concentrations on activity assays across a range of SOM concentrations expected to entail variability in PHO activities, soils were assayed in quadruplicate at final substrate concentrations of 0, 2, 5, 10, 15, 20, 30, and 50 mM per g soil, using 1 g soil. Assays were terminated by the method of Schneider et al. (2000) because this method was found to minimize potential errors in measurement of PHO activity relative to other termination methods. For each soil, PHO activity was plotted as a function of substrate concentration (Michaelis and Menten, 1913; Johnson and Goody, 2011). Apparent K_m and V_{max} were calculated based on the Lineweaver-Burk transformation, in which inverse enzyme activity [1/V] is plotted as a linear function of inverse substrate concentration [1/S] (Lineweaver and Burk, 1934). The correlation coefficient (R) for the linearized fit was calculated using PROC CORR in SAS v9.4 (Cary, NC). The recommended substrate concentration for determing PHO activity in each soil was calculated as $5 \times K_m$. To test the effect of substrate concentration on comparison of enzyme activities among soils, ANOVA was performed for PHO activity at each substrate concentration (2, 5, 10, 15, 20, 30, 50 mM). Sensitivity of comparisons of PHO activity among soils were evaluated using the F-statistic, and significant differences in activity among soils at each substrate concentration were determined by Tukey's test ($p < 0.05$).

3. Results

3.1. Soil PHO activity

Potential activities of soil PHO as commonly reported in the literature—without corrections for DOM interference or *p*NP recovery—differed significantly by termination method (Table 3). PHO activity was more influenced by termination method (F = 222) than by soil type (F = 79). Highest PHO activities were measured when assays were terminated with 0.5 M NaOH + 0.5 M $CaCl_2$ (mean 2.62 μmol *p*NP g^{-1} h^{-1}) and 0.2 M NaOH + 0.5 M $CaCl_2$ (mean 2.58 μmol *p*NP g^{-1} h^{-1}), followed by 0.1 M Tris + 0.5 M $CaCl_2$ (mean 2.31 μmol *p*NP g^{-1} h^{-1}). Measured PHO activities were markedly lower for the alternative termination tested in this study, 0.5 M NaOH + 2.0 M $CaCl_2$ (mean 1.32 μmol *p*NP g^{-1} h^{-1}). However, the effects of termination method on measured PHO activity varied by soil (termination × soil F = 32, $p < 0.0001$), and did not necessarily reflect SOC (Table 3). For example, the relative effect of termination method on uncorrected PHO activity was greatest in Ba-RF (F = 603, 37 mg C g^{-1}), and least in An-BO (F = 24, 34 mg C g^{-1}) and An-WF (F = 24, 127 mg C g^{-1}).

Moreover, differences in uncorrected PHO activity by termination method did not show the same trend among soils. For example, uncorrected PHO activity was greatest for An-PP and least for An-RF following termination with 0.5 M NaOH + 0.5 M $CaCl_2$. Due to multiple two- and three-way interactions, there were no simple effects

Table 4
Enzyme activity equivalents of dissolved organic matter (DOM) and contribution to mis-estimation of soil phosphomonoesterase activity in *para*-nitrophenol (*p*NP)-based assays of 12 forest soils (0–5 cm of A horizon). Soils represent combinations of three parent materials (andesite, An; basalt, Ba; granite, Gr) and four elevations defined by dominant tree species (blue oak; BO; ponderosa pine; PP; white fir; WF; red fir, RF) from the Sierra Nevada, USA. For a given soil, letters indicate significant differences by termination method on DOM interference (expressed as *p*NP equivalents) determined by Tukey's test ($p < 0.05$), and the F-statistic indicates the magnitude of this effect.

Soil	DOM *p*NP equivalent (μmol *p*NP g^{-1} h^{-1})				F-statistic
	0.5 M NaOH + 0.5 M $CaCl_2$	0.2 M NaOH + 2.0 M $CaCl_2$	0.1 M Tris (pH 12) + 0.5 M $CaCl_2$	0.5 M NaOH + 2.0 M $CaCl_2$	
	(Tabatabai, 1994)	(Schneider et al., 2000)	(Klose et al., 2003)	(this study)	
An-BO	0.11 ± 0.00 a	0.01 ± 0.00 c	0.12 ± 0.00 a	0.02 ± 0.00 b	426.2
An-PP	0.91 ± 0.26 a	0.08 ± 0.00 b	0.22 ± 0.00 b	0.04 ± 0.00 b	7.3
An-WF	0.27 ± 0.03 b	0.27 ± 0.01 b	0.40 ± 0.00 a	0.11 ± 0.00 c	36.4
An-RF	0.63 ± 0.09 a	0.32 ± 0.01 bc	0.35 ± 0.02 b	0.10 ± 0.00 c	17.3
Ba-BO	0.09 ± 0.01 b	0.01 ± 0.00 c	0.16 ± 0.00 a	0.02 ± 0.00 c	204.1
Ba-PP	0.60 ± 0.06 a	0.05 ± 0.00 c	0.21 ± 0.01 b	0.05 ± 0.00 c	53.2
Ba-WF	3.51 ± 0.17 a	0.37 ± 0.03 b	0.38 ± 0.02 b	0.07 ± 0.00 b	270.2
Ba-RF	0.36 ± 0.01 a	0.09 ± 0.00 c	0.27 ± 0.01 b	0.04 ± 0.00 d	646.6
Gr-BO	0.16 ± 0.01 a	0.02 ± 0.00 c	0.11 ± 0.00 b	0.02 ± 0.00 c	172.2
Gr-PP	0.28 ± 0.01 a	0.06 ± 0.00 b	0.28 ± 0.00 a	0.02 ± 0.00 c	710.9
Gr-WF	0.36 ± 0.01 a	0.07 ± 0.00 c	0.30 ± 0.01 b	0.02 ± 0.00 d	592.7
Gr-RF	0.78 ± 0.06 a	0.13 ± 0.00 c	0.39 ± 0.02 b	0.04 ± 0.00 c	96.7

explaining uncorrected PHO activity by parent material, climate, or termination method. When considered separately by termination method, the interaction of parent material and climate in influencing uncorrected PHO activity was greatest for 0.5 M NaOH + 0.5 M $CaCl_2$ (F = 76), similar to 0.1 M Tris + 0.5 M $CaCl_2$ (F = 70), and least for 0.2 M NaOH + 2.0 M $CaCl_2$ (F = 19) and 0.5 M NaOH + 2.0 M $CaCl_2$ (F = 12).

3.2. DOM interference

The four methods of alkaline termination produced soil- and termination-specific DOM interference, resulting in varying miscalculation of enzyme activity (Table 4, Fig. 1). For all tested terminations and soils, the contribution of DOM to absorbance at 410 nm consistently resulted in overestimation of PHO activity. Though the magnitude of overestimation due to DOM was strongly influenced by the termination method (F = 295), followed by soil (F = 94), DOM interference for a given termination method depended on the particular soil (termination × soil, F = 63, $p < 0.0001$).

DOM interference was lowest for terminations using 2.0 M $CaCl_2$ in conjunction with 0.5 M NaOH (0.02–0.11 μmol *p*NP equivalents g^{-1} h^{-1}) or 0.2 M NaOH (0.01–0.37 μmol *p*NP equivalents g^{-1} h^{-1}). The termination proposed in this study (0.5 M NaOH + 2.0 M $CaCl_2$) consistently yielded the lowest magnitude of DOM interference across soils. The greatest magnitude and soil-specificity of DOM interference occurred for 0.5 M NaOH + 0.5 M $CaCl_2$ (0.09–3.51 μmol *p*NP equivalents g^{-1} h^{-1}).

Overestimation of PHO activity followed trends in DOM absorbance at 410 nm (Fig. 1). The degree of overestimation (%) of enzyme activity was least for terminations with 2.0 M $CaCl_2$ (+0.2 M NaOH, 0.2–17.3%; +0.5 M NaOH, 1.2–8.6%). Termination with 0.1 M Tris + 0.5 M $CaCl_2$ yielded greater variation among soils in activity overestimation from DOM (4.6–29.3%). Greatest and most soil-specific overestimation of activity occurred for the widely employed method of termination of 0.5 M NaOH + 0.5 M $CaCl_2$ (3.2–346.6%). Greatest DOM interference occurred for the three Andisols (77% for An-WF, 347% for An-RF, 246% for Ba-WF), two of which (An-WF, An-RF) also had the highest SOC of the twelve soils (Table 2).

Overestimation of enzyme activity due to DOM was positively correlated with SOC and negatively correlated with clay content (Supplementary Table 1). The correlation of SOC and activity overestimation (%) was positive under 0.5 M NaOH + 0.5 M $CaCl_2$ termination ($p = 0.016$), and under 0.1 M Tris + 0.5 M $CaCl_2$ termination there was a stronger and negative correlation between clay content and DOM mis-estimation ($p = 0.002$). For only the termination using 0.2 M NaOH + 2.0 M $CaCl_2$, the *p*NP equivalent of DOM was positively correlated with SOC ($p = 0.029$) and negatively correlated with clay content ($p = 0.012$).

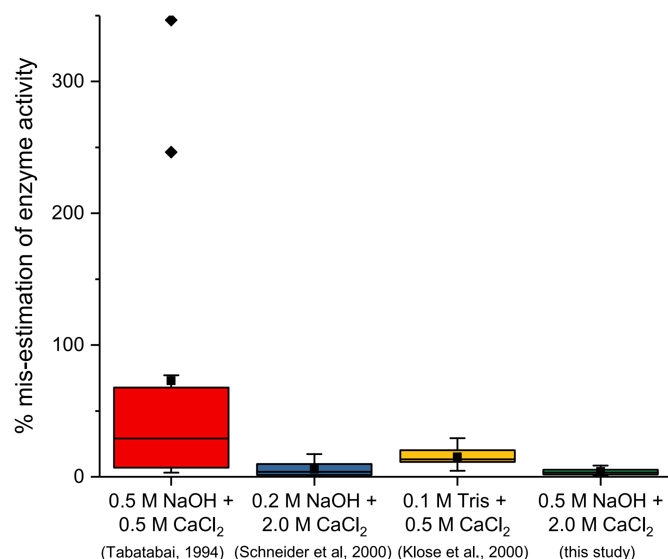

Fig. 1. Mis-estimation (%) of soil phosphomonoesterase (PHO) activity resulting from not accounting for dissolved organic matter (DOM) interference, for four different methods of enzyme assay termination. PHO activity was determined using the *para*-nitrophenol (*p*NP) enzyme assay. Each termination method was tested using the same set of diverse forest soils (n = 12) from the Sierra Nevada, USA. For a given termination method, the mis-estimation of PHO activity was calculated as the difference in uncorrected PHO activity relative to PHO activity corrected for DOM interference (*p*NP equivalents calculated from absorbance at 410 nm).

3.3. *p*NP recovery

Recovery of a *p*NP spike (10 mM g^{-1} soil) varied less strongly among terminations than DOM interference. Recoveries of *p*NP (accounting for DOM interference) were highest for terminations with 0.5 M NaOH + 0.5 M $CaCl_2$ (86–105%) and 0.2 M NaOH + 2.0 M $CaCl_2$ (85–98%), with lower recoveries with 0.1 M Tris + 0.5 M $CaCl_2$

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Table 5
Recovery of *para*-nitrophenol (*p*NP) and corresponding mis-estimation of phosphomonoesterase (PHO) activity from not accounting for *p*NP recovery for 12 forest soils. *p*NP recoveries were corrected for DOM interference. Soils (0–5 cm of A horizon) represent combinations of three parent materials (andesite, An; basalt, Ba; granite, Gr) and four elevations defined by dominant tree species (blue oak; BO; ponderosa pine; PP; white fir; WF; red fir, RF) from the Sierra Nevada, USA. For a given soil, letters indicate significant differences by termination method on recovery (%) of a 10 mM g^{-1} soil spike of *p*NP determined by Tukey's test ($p < 0.05$), and the F-statistic indicates the magnitude of this effect.

Soil	*p*NP recovery (%)				F-statistic
	0.5 M NaOH + 0.5 M $CaCl_2$	0.2 M NaOH + 2.0 M $CaCl_2$	0.1 M Tris (pH 12) + 0.5 M $CaCl_2$	0.5 M NaOH + 2.0 M $CaCl_2$	
	(Tabatabai, 1994)	(Schneider et al., 2000)	(Klose et al., 2003)	(this study)	
An-BO	96.7 ± 1.8 a	95.9 ± 2.5 a	86.0 ± 0.7 b	79.1 ± 1.2 b	19.3
An-PP	93.6 ± 0.3 a	90.4 ± 1.8 a	80.5 ± 0.7 a	79.7 ± 1.1 a	30.0
An-WF	101.5 ± 1.1 a	85.2 ± 3.4 b	64.4 ± 1.5 c	80.4 ± 1.4 b	41.4
An-RF	105.1 ± 2.1 a	89.9 ± 1.0 b	72.1 ± 1.8 c	78.5 ± 1.5 c	55.8
Ba-BO	97.4 ± 1.6 a	91.8 ± 1.3 a	83.6 ± 0.9 b	85.7 ± 0.8 b	21.1
Ba-PP	96.7 ± 2.4 a	88.8 ± 2.5 ab	73.4 ± 1.6 c	79.7 ± 0.6 bc	21.5
Ba-WF	102.9 ± 1.0 a	93.4 ± 3.7 b	82.0 ± 0.8 c	84.3 ± 0.3 bc	17.7
Ba-RF	100.4 ± 1.0 a	91.4 ± 0.7 ab	90.8 ± 3.3 b	82.8 ± 1.2 b	11.2
Gr-BO	98.2 ± 0.6 a	95.5 ± 1.3 a	87.9 ± 0.4 b	84.4 ± 0.0 b	54.5
Gr-PP	98.9 ± 2.7 a	98.4 ± 0.5 ab	82.3 ± 6.2 b	82.6 ± 0.7 ab	5.7
Gr-WF	102.8 ± 1.3 a	91.3 ± 1.4 b	97.9 ± 0.8 a	85.4 ± 0.5 b	22.0
Gr-RF	86.0 ± 0.7 a	96.0 ± 1.5 a	85.4 ± 3.3 b	82.6 ± 0.9 b	16.6

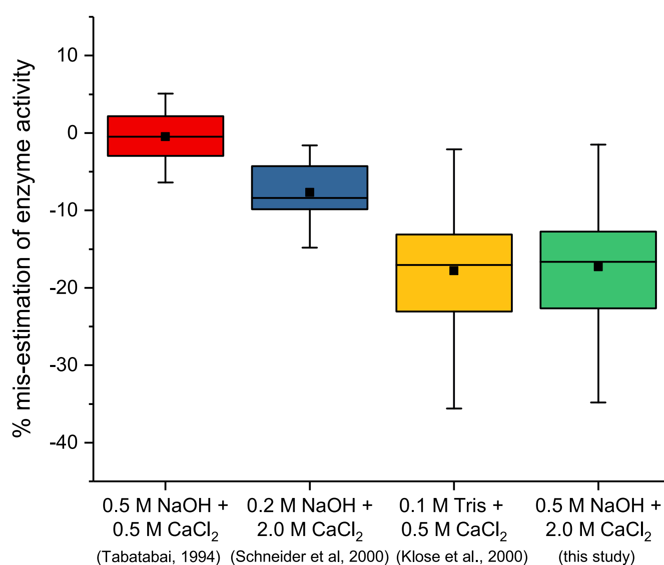

Fig. 2. Mis-estimation (%) of soil phosphomonoesterase (PHO) activity resulting from not accounting for incomplete *para*-nitrophenol (*p*NP) recovery, for four different methods of enzyme assay termination. Each termination method was tested using the same set of diverse forest soils (n = 12) from the Sierra Nevada, USA. For a given termination method, the mis-estimation of PHO activity was calculated as the difference in uncorrected PHO activity relative to PHO activity corrected for *p*NP recovery.

(64–98%) and 0.5 M NaOH + 2.0 M $CaCl_2$ (79–86%) (Table 5, Fig. 2). As a result, not correcting for incomplete recovery of *p*NP generally resulted in underestimation of enzyme activity, the degree of which varied substantially by termination method (F = 189) compared to soil type (F = 10). A weaker albeit significant difference in *p*NP recovery by termination depending on soil type (termination × soil F = 5, $p < 0.001$) indicated that differences in *p*NP recovery among termination depended on soil type.

Recoveries of *p*NP without correcting for DOM interference (Supplementary Table 2) were highly similar to *p*NP recovery calculated to account for *p*NP equivalents of DOM (Table 5). For example, *p*NP recovery was still more strongly influenced by termination method (F = 195) than by soil type (F = 9), and the interaction of these persisted (termination × soil F = 5, $p < 0.001$) (Supplementary Table 2).

Underestimation of PHO activity by not correcting for *p*NP recovery was consequently greatest in soils under terminations with lowest *p*NP recovery (Fig. 2). The degree of misestimating PHO activity was least for 0.5 M NaOH + 0.5 M $CaCl_2$ (+5.1 to −6.4%), followed by 0.2 M NaOH + 2.0 M $CaCl_2$ (−1.6 to −14.8%) and was greatest for 0.1 M Tris + 0.5 M $CaCl_2$ (−2.1 to −35.6%) and 0.5 M NaOH + 2.0 M $CaCl_2$ (−1.5 to −34.8%).

3.4. Substrate concentration

PHO activities increased with substrate concentration, and more than doubled from the low substrate concentrations commonly employed in soil studies (< 10 mM g^{-1} soil) to the highest substrate concentration (50 mM g^{-1} soil) (Fig. 3). Comparison of PHO activities among soils were strongly influenced by substrate concentration, with statistically weaker differences in PHO activity when measured at low compared to high substrate concentrations. For example, PHO activity did not significantly differ between two soils (An-BO, Ba-PP) when assayed at typical substrate concentrations (≤10 mM g^{-1} soil), but were statistically different at substrate concentrations that approached or exceeded 5 × K_m (Supplementary Table 3). Differences in PHO activity among soils were also enhanced at higher (e.g., F = 102 at 30 mM per g) versus lower substrate concentrations (e.g., F = 16 at 10 mM g^{-1}). PHO K_m across the 12 soils varied from 4.2 to 13.3 mM g^{-1} soil, meaning that recommended minimums of 5 × K_m were at least 20.9 mM g^{-1} soil and as much as 66.3 mM g^{-1} soil (Supplementary Table 3).

4. Discussion

4.1. Effect of alkaline termination on DOM

Though the choice of termination method significantly impacted PHO activity with and without correction for DOM, our results identify the importance of accounting for DOM absorbance for accurate measurement of enzyme activities. Although uncorrected PHO activities were most similar and tended to be highest for terminations using 0.5 M NaOH + 0.5 M $CaCl_2$ (Tabatabai, 1994) and 0.2 M NaOH + 2.0 M $CaCl_2$ (Schneider et al., 2000), neglecting to account for DOM interference resulted in gross overestimation of enzyme activity when the former termination method was used. This indicates (1) greater DOM co-extraction when terminated with high NaOH and low flocculent concentrations (Tabatabai, 1994), and thus (2) less inaccuracy of enzyme activities calculated without correction for DOM using the 0.2 M

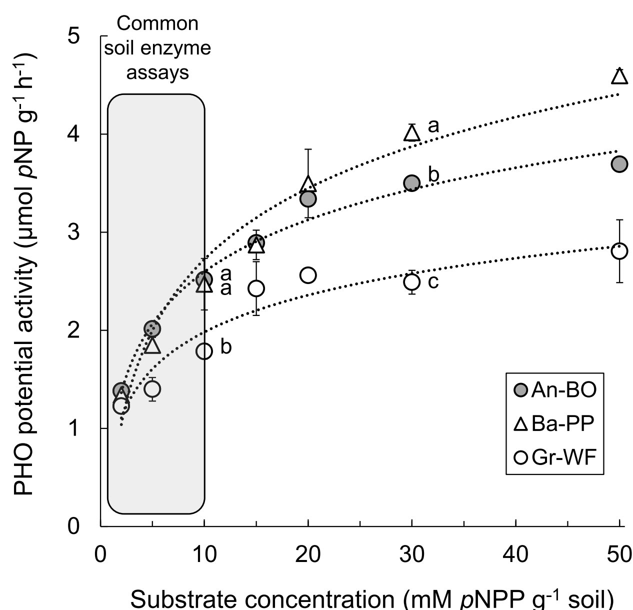

Fig. 3. Phosphomonoesterase (PHO) activity of select soils across a range of *para*-nitrophenyl phosphate (*p*NPP) substrate concentrations. Different letters indicate significant differences ($p < 0.05$) of PHO activity among soils measured at a specific substrate concentration. The grey box corresponds to the substrate concentrations commonly employed in studies on soil PHO activity. Soils (0–5 cm of A horizon) are from the California Sierra Nevada and developed on varying parent materials (An, andesite; Ba, basalt; Gr, granite) and under distinct ecotones (BO, blue oak; PP, ponderosa pine; WF, white fir).

NaOH + 2.0 M $CaCl_2$ termination proposed by Schneider et al. (2000). In low SOM samples, it may be possible that termination with 0.2 M NaOH + 2.0 M $CaCl_2$ (Schneider et al., 2000) could yield sufficiently negligible DOM interference so as to enable enzyme activities to be accurately measured without the additional step of DOM correction.

4.2. Effect of alkaline termination on pNP recovery

Though recovery of *p*NP varied less among termination methods for the diverse soils in this study than for DOM interference, calculated enzyme activities were more influenced by omitting the correction for *p*NP recovery than by omitting the correction for DOM interference. This likely reflects the relatively low magnitude of *p*NP DOM equivalents compared to the amount of *p*NP not recovered. The notable exception to this were two Andisols when terminated by the widespread method of Tabatabai (1994) using 0.5 M NaOH + 0.5 M $CaCl_2$.

Incomplete recovery of *p*NP, as may be the case for much of soil enzyme activity data in the literature, can lead to underestimated activities and thus incorrect comparisons of enzyme activities among soils and/or treatments. Though omitting correction for *p*NP sorption may be defensible for comparing relative differences in enzyme activities among soils of the same mineralogy and similar SOC content, or among soils with similar *p*NP sorption (Margesin et al., 2002), comparisons with other studies can be compromised. Though not tested in this study, soils containing pyrolyzed C (e.g., biochar, charcoal) may be especially sensitive to this source of error because of high sorption capacity of *p*NP to these components (Cornelissen et al., 2005; Jindo et al., 2014). The specific surface area of soil samples is likely a key driver of incomplete *p*NP recovery; non-specific binding of *p*NP (e.g., monolayer formation) (Boyd, 1982; Ko et al., 2007) enables the use of *p*NP to calculate specific surface area of soils and minerals (Ristori et al., 1989; Saggar et al., 1996; Theng et al., 1999; Hedley et al., 2000).

In addition to ensuring accuracy of soil enzyme activities, accounting for incomplete recovery of *p*NP can improve the accuracy of kinetic characterizations. Correcting for *p*NP sorption tends to result in lower apparent K_m values (i.e., higher affinity of enzyme for substrate) (Cervelli et al., 1973; Trasar-Cepeda and Gil-Sotres, 1988). In contrast, correction for *p*NP recovery may not necessarily affect apparent V_{max}. For example, in temperate forest soils (northwestern Spain), accounting for incomplete *p*NP recovery decreased the apparent K_m of PHO, from a range of 3.7–16.8 mM to 1.7–7.0 mM, whereas V_{max} (2.8–73.5 µmol *p*NP g^{-1} h^{-1}) was largely unchanged (Trasar-Cepeda and Gil-Sotres, 1988). These potential artifacts in apparent kinetic parameters are in contrast to 'real' shifts in K_m and V_{max} of mineral-bound enzymes as a result of conformational and steric changes induced by binding (Makboul and Ottow, 1979).

It should be noted that *p*NP recovery was estimated by measuring single-point sorption (one *p*NP concentration) that matched the concentration of *para*-nitrophenyl-linked substrate. Construction of *p*NP calibration curves for each soil (similar to conducting sorption isotherms) has been recommended (Vuorinen, 1993). However, the primary objective of this study was to illustrate how *p*NP recovery in soil samples can be incomplete, depend on termination method and potentially compromise the accuracy of calculated enzyme activities. The use of 10 mM *p*NP g^{-1} soil in this study is meant to provide a measure of differences in recovery of *p*NP among diverse soils.

4.3. Effect of substrate concentration

This study demonstrates that the widespread use of low substrate concentrations relative to saturation target ($5 \times K_m$) can compromise the accuracy of enzyme activities measured in soil samples. Under non-saturating substrate conditions, enzyme activity will increase with substrate concentration, challenging valid comparisons of activities among samples and studies using different concentrations (Acker and Auld, 2014). In addition to enabling comparisons among soils, achieving conditions of substrate saturation tends to maximize the signal-to-noise ratio in enzyme assays (Acker and Auld, 2014). There is also a risk of false negatives in the use of low substrate concentrations in soil enzyme assays. Though this has been noted by others (e.g., Malcolm, 1983), our study explicitly addresses and illustrates the potential magnitude of these artifacts.

In the nearly 50 years since *p*NP substrates were first applied for measuring enzyme activities in soils, the recommended or commonly used substrate concentration has fluctuated over 3 orders of magnitude

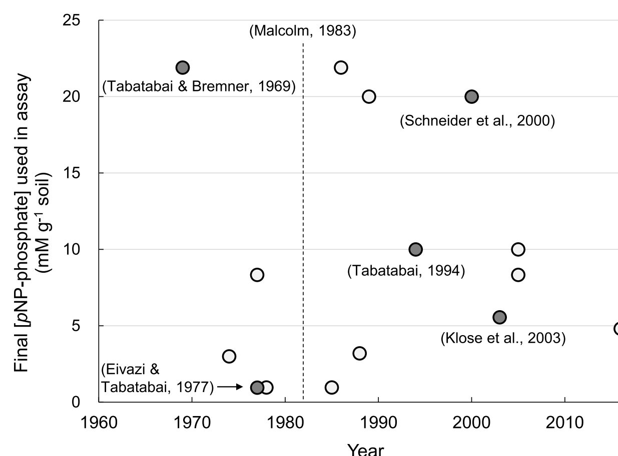

Fig. 4. Illustration of historical variability in the amount of substrate used in *para*-nitrophenyl-based enzyme activity assays in soil samples, using the example of *para*-nitrophenyl phosphate (*p*NPP) for phosphomonoesterase. Substrate concentrations are shown for the final concentrations used in the assay on a soil mass basis (mM g^{-1}). Darkened and labeled circles represent studies of extremes of substrate concentrations, or indicate the first reported use of assay termination methods evaluated in this study.

(Fig. 4). The first application of *p*NP substrates for measuring enzyme (PHO) activities in soils by Tabatabai and Bremner (1969) employed a 1 mL addition of 115 mM *p*NP substrate to 4 mL of buffer. With the addition of toluene (0.25 mL) to suppress microbial activity, this resulted in a final substrate concentration of 21.9 mM g^{-1} soil. The increasing abandonment of toluene in enzyme assays given weak or no effects in short-duration (1 h) assays (Tabatabai, 1994; Verchot and Borelli, 2005) and methodological complications (Kaplan and Hartenstein, 1979; Frankenberger and Johanson, 1986) means that studies using this recommended substrate concentration employed a final substrate concentration of 23 mM g^{-1} soil. However, substrate concentrations as low as 0.95 mM g^{-1} soil (e.g., Eivazi and Tabatabai, 1977; Juma and Tabatabai, 1978) have been used. Though the widespread method of soil enzyme activity assays outlined by Tabatabai (1994) used 10 mM final concentration per g soil (adding 1 mL of 50 mM to a final volume of 5 mL), many studies since then use lower concentrations and in the majority of cases do not provide a rationale for this (e.g., calculated K_m).

This study also indicates that blanket recommendations of substrate concentrations for a given enzyme across soil as well as enzyme types are problematic. As evidenced by the literature and supported by our findings, activity of a particular enzyme in soils can vary significantly. Hui et al. (2013) found that reported values of PHO activity measured in soils and sediments varied by as much as 4 orders of magnitude. Additionally, blanket recommendations of a substrate concentration for C-, N-, P-, and S-cycling enzymes that can be assayed with *p*NP substrates are in conflict with knowledge that the activities can vary substantially for different enzymes. For example, PHO activity is generally greater than phosphodiesterase activity (Turner and Haygarth, 2005), and the activity of both these phosphatases appears to be 1–2 orders of magnitude greater than that of cellobiohydrolase (e.g., Verchot and Borelli, 2005; Turner, 2010). If substrate recommendations are to be made, they should be specific to enzyme type. Only a handful of studies perform preliminary, empirical determination of substrate concentrations necessary for saturation of a target enzyme, largely in fluorescence-based assays using 4-methylumbelliferone (MUF)-linked substrates, by varying substrate concentration by up to 3 orders of magnitude (Kandeler and Gerber, 1988; Kang and Freeman, 1999; Marx et al., 2001; Stemmer, 2004; German et al., 2011).

4.4. Enzyme saturation and kinetics in soil samples

In order to achieve conditions approaching substrate saturation of an enzyme, the use of $5 \times K_m$ is recommended and common practice in 'traditional' biochemistry. This guideline was suggested for soils by Burns (1978) and reiterated by Malcolm (1983). It does not appear that this guideline has been observed in the majority of soil studies, because it requires calculation of K_m for each soil sample, and these continue to be absent in most studies since this issue was raised by Malcolm (1983). Among early studies that determined soil enzyme K_m values, many did not use the determined K_m value to adjust substrate concentrations in activity assays (e.g., Eivazi and Tabatabai, 1977; Perucci and Scarponi, 1985; Trasar-Cepeda and Gil-Sotres, 1988). The issue of substrate concentration in soil enzyme assays extends beyond *p*NP-based assays to those employing MUF-linked substrates (German et al., 2011).

The recommended method for determining K_m in biochemistry is an iterative processes, in which an initial K_m is estimated using 4 to 6 substrate concentrations followed by a more careful determination with up to 8 substrate concentrations ranging 0.2 to $5 \times$ initial K_m (Brooks et al., 2012). Though early work in soil enzymes employed linear transformations, these are no longer used in biochemistry due to the sensitivity of linear transformations to extreme values and the advent of nonlinear regression software (Brooks et al., 2012). Achieving substrate saturation of an enzyme is theoretically impossible because true saturation would require an infinite amount of substrate given the hyperbolic nature of Michaelis-Menten kinetics (Michaelis and Menten, 1913; Bisswanger, 2014). For example, Bisswanger (2014) points out that though enzyme activity is 50% of V_{max} at K_m, adding $5 \times K_m$ substrate to occupy the remaining 50% of binding sites only results in an additional 33% of sites being bound (total 83% at $5 \times K_m$), because the ratio of substrate-filled sites determines the degree of saturation. Since certain enzymes may be inhibited at high substrate concentrations even well below concentrations that approach substrate saturation for other enzymes (i.e., non-Michaelis-Menton kinetics) (Leskovac, 2004) it is possible that using high substrate concentrations may inhibit enzyme activities in environmental samples such as soils (German et al., 2012a; Steen and Ziervogel, 2012). However, this further supports the need for *a priori* determination of K_m because potential inhibition of enzyme activity as a function of substrate concentration can be detected in the initial parameterization of assay conditions (e.g., German et al., 2012b).

Kinetic parameters of PHO in A horizons of Sierra Nevada forests in our study are consistent with previous studies. For example, temperate agricultural soils (Midwestern USA) exhibited apparent K_m values of 1.2–3.4 mM g^{-1} soil and apparent V_{max} of 1.4–4.5 µmol *p*NP g^{-1} h^{-1} for PHO (17.7–54.5 g kg^{-1} organic C, pH 5.8–7.8). In surface soils of temperate forests (northwestern Spain), K_m ranged 1.7–7.0 mM g^{-1} soil, and V_{max} ranged 2.8–73.5 µmol *p*NP g^{-1} h^{-1} (Trasar-Cepeda and Gil-Sotres, 1988). Though there have been few empirical determinations of the ability of recommended substrate concentrations to achieve $5 \times K_m$, our results agree with the range of values reported by others. For example, Schneider et al. (2000) calculated a substrate concentration of 20 mM g^{-1} soil for PHO in forested Humic Cambisols with high SOC (13.1–96.0 mg g^{-1}), similar to the original concentration of 21.9 mM g^{-1} soil first used in soil PHO assays (Tabatabai and Bremner, 1969).

4.5. Methodological recommendations for pNP-based enzyme assays of soil samples

Based on the twelve soils representing diverse mineralogy and gradients of SOC content and PHO activity, we suggest three modifications of enzyme assays to improve the accuracy of activity data, *viz.*

Substrate concentrations that approach conditions of saturation should be used to increase assay sensitivity. Soil enzymology should use the rule-of-thumb that is standard for enzyme characterizations: $5 \times K_m$. This requires *a priori* determination of K_m, which would provide additional data (K_m, V_{max}) on enzymes in soils.

Incomplete recovery of pNP must be accounted for, because sorption of *p*NP to inorganic and organic components of soil samples is expected to consistently result in underestimation of enzyme activities. As we demonstrate, this artifact may be aggravated depending on termination as well as the soil type. Soil-specific sorption of *p*NP precludes assumption of a universal 'correction factor' or recovery constant, in contrast to other methods such as microbial biomass carbon (e.g., Joergensen, 1996), and as noted previously (Vuorinen, 1993) necessitates empirical determination for each soil sample. Accounting for incomplete recovery is standard in other soil biochemistry methods, notably fixation of inorganic P released by fumigation in determinations of microbial biomass P (e.g., Brookes et al., 1982; Kouno et al., 1995; Oberson et al., 1997).

Correction for DOM interference is necessary when terminating with 0.5 M NaOH, as in the widely employed method of Tabatabai (1994). Though SOC tends to increase overestimation of enzyme activity via DOM absorbance at 410 nm, the magnitude of DOM interference in enzyme assays is generally less than incomplete *p*NP recovery, despite greater soil and termination variability in DOM interference. The exception to this may be Andisols, which in our study exhibited orders of magnitude greater DOM interference than other soils. This could reflect the ability of short-range order minerals common in Andisols (e.g., allophane) to bind large amounts of soluble OM (Buurman et al., 2007; Takahashi and Dahlgren, 2016).

We therefore recommend assay termination with 0.2 M NaOH + 2.0 M $CaCl_2$ (Schneider et al., 2000) as it minimizes DOM interference and maximizes *p*NP recovery among the four termination methods evaluated. We note that future studies should evaluate additional termination methods of varying concentration of Tris and/or $CaCl_2$ (e.g., > 0.1 M Tris, 0.1 M Tris + 2.0 M $CaCl_2$), combinations of NaOH and Tris, and/or additional bases or base mixtures (e.g., 0.1 M glycine-NaOH; Kwapiszewski et al., 2014). There may be additional considerations beyond DOM interference and *p*NP recovery not assessed in this study that could be helpful to determine termination method suitability. For example, Deng et al. (2013) reported decreased variability in fluorescence quantification for MUF-based assays terminated with 0.1 M Tris (pH 12) compared to 0.5 M NaOH.

4.6. Additional considerations for assays of enzyme activities in soil samples

Inconsistencies among previous studies in reporting the substrate concentrations used in *p*NP-based enzyme assays challenge comparisons of activities and kinetic parameters. We therefore propose the following to improve reporting of activity data from soil enzyme assays:

Reporting the concentration of substrate for the final volume used in enzyme assay. Studies should report the final concentration of substrate (mM) used in the assay because this is the intensity of substrate to which enzyme in the soil sample is exposed. In contrast, many studies report the initial concentration of the substrate stock solution added to the buffer (e.g., in the 1 mL of substrate added to 4 mL of buffer). This consideration extends to reporting the range of substrate concentrations used in kinetic characterizations. Consistency in reporting these values is crucial for allowing the direct comparison between the substrate concentration used in the actual assay versus the guideline of 5 × K_m substrate concentration. For example, Pang and Kolenko (1986) reported the use of 5–115 mM g^{-1} to assay PHO activity, but it is not clear if this was the final or initial (i.e., the 1 mL addition) substrate concentration. It appears that initial substrate concentration was used to calculate K_m because values as high as 91 mM were reported. Recalculating these K_m values (i.e., dilution factor of 5.25) provides an apparent K_m value of 17.3 mM g^{-1} soil, consistent with the range of soil PHO K_m reported in this and other studies (Trasar-Cepeda and Gil-Sotres, 1988; Schneider et al., 2000).

Reporting K_m values on a soil mass basis. Unlike purified enzymes in traditional biochemistry, the measurement of enzymes that are a minute mass component of soils and the variation in the amount of soil used means that the amount of substrate to achieve ½ V_{max} is specific to the mass of soil used in the assay. We therefore recommend that K_m for enzymes in soil samples be reported on a soil mass basis (i.e., mM g^{-1} soil).

Reporting enzyme activities on a molar, not mass, p*NP basis*. The mass of *p*NP is arbitrary with respect to enzyme activity because it is an artificial substrate that serves as a proxy for mineralization of 'natural' or non-artificial substrates in soils. Reporting enzyme activities on a molar basis is more conceptually relevant to understand potential rates of enzymatic transformation of organic C, N, P, and S in soils. This also enables comparison of enzyme activities determined using other natural and artificial substrates (e.g., MUF-linked substrates), and can be used to calculate stoichiometric ratios of enzyme activities (e.g., C- to *N*-cycling enzymes) (Sinsabaugh et al., 2008, 2009). Consistency in reporting enzyme activities would also improve comparability among studies, which have mixed reporting of enzyme activities on a µg and µmol *p*NP basis, and potentially avoid errors in reporting. For example, soil PHO activities reported by Doelman and Haanstra (1989) and Radersma and Grierson (2004a) of 538 and 299 µmol *p*NP g^{-1} h^{-1}, respectively, are likely misreported on a *p*NP mass basis and correspond to 3.9 and 2.2 µmol *p*NP g^{-1} h^{-1} (*p*NP = 139.11 g mol^{-1}). Furthermore, the use of 5 mL of 10 mM *p*NPP per 0.5 g soil (Doelman and Haanstra, 1989) or 5 mM *p*NPP g^{-1} soil (Radersma and Grierson, 2004b) means that the maximum enzyme activity possible was 100 µmol *p*NP g^{-1} h^{-1} and 5 µmol *p*NP g^{-1} h^{-1}, respectively—approximately 5- and 60-fold lower than reported.

Qualifying enzyme activities, K_m, and V_{max} as "apparent" if corrections for interferences are not performed.

These recommendations are relevant to other methods of assaying enzyme activities in soil samples, including MUF-linked substrates.

Finally, though the present study examined the effect of termination methods on enzyme activities, other methodological aspects of soil enzyme assays can influence measured enzyme activities and compromise the comparison among studies. Additional aspects of soil enzyme assays include assay pH, incubation temperature, shaking during the assay, and choice of buffer, as reviewed by others (Tabatabai and Bremner, 1971; Malcolm, 1983; Burns et al., 2013). These parameters could have greater effects on enzyme activities, and thus comparison among studies, than termination methods reported here. For example, shaking assays during incubation produced apparent PHO K_m values that were 3.2-fold less than without shaking (Tabatabai and Bremner, 1971). Standardizing these additional components of soil enzyme assays to enable comparability among studies and laboratories (German et al., 2011; Deng et al., 2017) would necessitate additional methodological comparisons.

5. Conclusion

The application of *p*NP-based enzyme assays to soil samples was a key development in soil biochemistry, and enzyme assays continue to be an essential tool for understanding transformations of soil organic C, N, P, and S. However, the accuracy of *p*NP-based enzyme assays can be compromised by DOM interference and/or low *p*NP recovery, which depend on the method of assay termination (base + $CaCl_2$). Furthermore, the variability in the substrate concentrations reported in the literature and the general absence of verifying substrate concentrations that achieve the recommended 5 × K_m means there may be limited comparability of soil enzyme activity data derived from such studies. For the first time, this study quantified errors associated with these potential interferences and practices, and proposes procedures to improve the accuracy and comparability of enzyme activities measured with *para*-nitrophenyl-linked substrates. Our results identify a general overestimation of activity from DOM interference, which is more variable by soil and termination type but is generally less in magnitude than the underestimation of activity that results from incomplete recovery of *p*NP released. To minimize these artifacts and ensure accuracy of activity measurements, we recommend assay termination using 0.2 M NaOH + 2.0 M $CaCl_2$ proposed by Schneider et al. (2000). Additionally, we demonstrate the importance of employing substrate concentrations 5 × K_m, because enzyme activities, and thus statistically detectable differences between soils, increase with substrate concentration. The range of substrate concentrations used in the majority of studies to measure PHO activity (0.9–21.3 mM g^{-1} soil) are well below 5 × K_m for several soils in this and other studies. The methodological and conceptual recommendations for soil enzyme assays supported by this study can improve the reliability of data on enzyme in soils to better support insights to soil C-, N-, P-, and -S cycling uniquely afforded by this biochemical tool.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.11.006.

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