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Bench scale and microplate format assay of soil enzyme activities using spectroscopic and fluorometric approaches

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ABSTRACT

Method standardization and validation are essential for meaningful data comparison and interpretation. The objective of this study was to compare bench scale and microplate format assays of soil enzyme activities using spectroscopic (*p*-nitrophenyl or *p*NP) and fluorometric (4-methylumbelliferyl or MUF) based approaches. Three assay approaches, including *p*NP-bench, *p*NP-microplate and MUF-microplate, were compared. Data from microplate format assays in the presence of soil suspension suggested that MUF-based assays were about 14 times more sensitive than *p*NP-based assays. MUF detection provided measures as low as 50 pmol of MUF in a microplate well. However, the *p*NP bench scale assay was the most sensitive of the three assays in quantifying enzyme activities. The limit of quantification (LOQ) values expressed as corresponding enzyme activities using the protocols tested were 0.049, 0.242, and 0.0936 mmol kg^{-1} h^{-1} for *p*NP bench, *p*NP microplate, and MUF microplate assays, respectively. Of the microplate assays, the MUF-based assay was more precise than the procedure using *p*NP. The presence of soil suspension increased standard errors, which more than doubled for the detection of *p*NP in microplate assays, but showed little effect on the standard error for the detection of MUF. For meaningful data interpretation, we suggest that LOQ values be calculated for enzyme assays and caution be exercised when interpreting data below LOQ values. Based on evaluations of three different enzymes in 16 diverse soils, the activities measured by the three protocols were often significantly different, but were in the same order of magnitude and significantly correlated, suggesting that the same pool of isoenzymes were measured by the different protocols.

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1. Introduction

Quantification of soil enzyme activities is widely employed to evaluate functional diversity, environmental quality and anthropogenic impacts on ecosystem health (Marx et al., 2005; Ai et al., 2012). Assays of soil enzyme activities often employ one of two categories of substrate analogs at different assay conditions and scales of sample size. Conventional assays are conducted using *p*-nitrophenol (*p*NP)-labeled substrate analogs and assayed at bench scales (Tabatabai, 1994). In recent years, there is growing interest in microplate format assays that use fluorometric or spectroscopic approaches that employ a much smaller soil sample size than the conventional method (Wirth and Wolf, 1992; Popova and Deng, 2010).

One advantage of microplate format assays is the potential for simultaneous multiple enzyme assays of the same sample for enhanced data comparison (Wirth and Wolf, 1992). Comparability of data obtained by *p*NP-bench scale and 4-methylumbelliferyl (MUF)-microplate format assays has been evaluated (Marx et al., 2001; Drouillon and Merckx, 2005). Following comparison of β-glucosidase and acid phosphomonoesterase activities using these two enzyme assay approaches, Marx et al. (2001) found that both approaches generated similar V_{max} values, but the affinity was greater for the MUF-labeled substrates compared to the *p*NP-labeled substrates with their K_m values two orders of magnitude different. Moreover, the enzyme activities determined by these two approaches were considerably different. The observed differences between these two approaches are, to a certain degree, expected due to considerable differences in the assay conditions employed in the study by Marx et al. (2001). One distinct difference is the incubation temperature used for determining enzyme activities. In the microplate assays reported, enzyme activities were determined at 30 °C, while 37 °C was used in the bench scale assays as described by Tabatabai (1994).

Drouillon and Merckx (2005) evaluated phosphomonoesterase activity in soil using *p*NP-phosphate or MUF-phosphate as substrate analogs in seven unmanaged ecosystem soils and eight agricultural soils that have a range of soil properties. Their studies showed that

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phosphomonoesterase activities were significantly lower when *p*NP-phosphate was used as the substrate, especially when soil organic carbon (OC) was >130 mg C kg^{-1} soil. The activities of the two classes of substrate analogs were significantly correlated with contents of OC and total nitrogen (TN) in soils tested. However, activities from these two approaches were not correlated unless expressed on a soil OC unit basis. In their study, enzyme activities were determined by adjusting the incubation buffer to the pH value of the assayed soil, instead of the pH for optimal activity of the enzyme assayed. This approach may reflect potential activity in the assayed soils more accurately, but adds another variable (pH for the enzymatic reaction) that limits comparability of activities between soils. Based on studies reported by Tabatabai et al. (Eivazi and Tabatabai, 1977; Browman and Tabatabai, 1978; Juma and Tabatabai, 1977, 1978), the optimal pH in soil is 6.0 for acid phosphomonoesterase and 11.0 for alkaline phosphomonoesterase, respectively. Depending on the pH value of soils tested, the detected activity may be predominately due to activities of acid or alkaline phosphomonoesterase, or a combination of both phosphomonoesterases in various relative proportions. Moreover, the reported phosphomonoesterase activities detected by *p*NP- or MUF-based approaches were two orders of magnitude different, with activities detected using MUF-based assay being about 100-fold of those detected using *p*NP-based assays (Drouillon and Merckx, 2005). Sensitivity of the detection methods should not affect the activities. The observed differences between the two approaches suggest that two different pools of enzymes may have been measured.

Comparison and standardization of assay methods for soil enzyme activities are generating renewed interest (Marx et al., 2001; Drouillon and Merckx, 2005; Dick, 2011). This, to a certain degree, was driven by the increasing interest in the use of microplate format assays and the increasing popularity of MUF-based assay approaches. However, Drouillon and Merckx (2005) found 100-fold differences in enzyme activities between the MUF microplate assays and the conventional bench scale *p*NP assays, which raises serious questions about the interpretation and comparability of these approaches. There is relatively little information on sensitivity, reproducibility, and reliability of microplate enzyme assays in comparison to the conventional bench scale *p*NP assay.

Therefore, the objective of this study was to compare bench scale and microplate format assays of soil enzyme activities using MUF- and *p*NP-based approaches. We hypothesized that data obtained from these different approaches are not directly comparable because of different soil to buffer ratios and substrate analogs. Further, we hypothesized that the same pool or significant overlaps of enzyme pools are being detected by different assay approaches. Therefore, when assay conditions are optimized and controlled, activities determined using different approaches were expected to be within the same order of magnitude.

2. Materials and methods

2.1. Soils and analysis of basic properties

Sixteen surface soils (0–10 cm) with diverse properties were selected for evaluation (Table 1). Field moist soil samples were ground, sieved through a 2 mm screen, and then each was divided into two parts. One part was air-dried and stored at room temperature for determination of chemical properties. The other part was kept field-moist and stored sealed at 4 °C for enzyme assays. A portion of the air-dried samples was also passed through an 80-mesh screen for determination of OC and TN content. Soil pH values were determined using a combination glass electrode (soil: 0.01 M $CaCl_2$ (w/v) = 1:2.5). Contents of OC and TN were determined by dry combustion using a Carlo-Erba NA 1500 nitrogen/carbon/sulfur analyzer (Schepers et al., 1989).

2.2. Enzyme assay

Activities of β-glucosaminidase, β-glucosidase, and acid phosphomonoesterase in soils were evaluated using *p*NP bench, *p*NP microplate and MUF microplate assay approaches. MUF assays require developing a calibration curve for each batch of sample under identical assay conditions at the same time (Deng et al., 2011), making it challenging to conduct MUF-based bench scale assays. The detected enzyme activities by different approaches should be comparable when expressed on molar basis per unit of incubation time.

The bench scale procedure was described by Tabatabai (1994) and Parham and Deng (2000). Briefly, 1 g soil was incubated at 37 °C for 1 h with respective *p*NP substrates at the optimal pH for the specific enzymatic reaction, which was pH 5.5 for β-glucosaminidase, and pH 6.0 for β-glucosidase and acid phosphomonoesterase, respectively. The *p*-nitrophenol released during incubation was quantified by measuring its yellow color intensity at 415 nm with a spectrophotometer and calculated by referencing to a *p*NP standard curve. Duplicate analyses were conducted for each sample assay.

For microplate enzyme assays, two soil suspensions were prepared for each sample and the assay procedure is similar to that described by Deng et al. (2011). Briefly, each soil suspension was prepared by mixing 1 g of soil with 120 ml of deionized water in a 150 ml Pyrex beaker for 30 min using a 3.75 cm magnetic stir bar at 600 rpm (a speed that was sufficient for complete homogenization of the soil suspension). Aliquots (100 μl each) of the soil suspension were removed during continuous mixing using a 0–250 μl multi-channel pipette and placed into microplate wells that each contained 50 μl of modified universal buffer (MUB) at pH optimal for the enzyme assayed. During pipetting of the aliquots, the same stirring plate and speed were used for all suspensions and samples. Subsequently, 50 μl of respective substrate (*p*NP- or MUF-labeled, 5 mM) solutions was added to each microplate well. The well contents were mixed by pipetting and discharging several times before incubating the microplate at 37 °C for 16–24 h for *p*NP-based assays, or 1 h for MUF-based assays. The total volume of the reaction mixture during incubation was 200 μl. After incubation, 50 μl of NaOH (0.5 M) or THAM (0.1 M, pH 12) was added to each microplate well to terminate the enzymatic reaction. Autohydrolysis of substrates were tested and included in the calculations (Deng et al., 2011). MUB, MUF substrates, and MUF standards were prepared as described by Deng et al. (2011). *p*-Nitrophenyl substrates (5 mM, Sigma Chemicals, St. Louis, MO, USA) were prepared by dissolving 0.171 g of *p*-nitrophenyl-β-D-glucosaminide (N9376), 0.151 g of *p*-nitrophenyl β-D-glucopyranoside (N7006), or 0.186 g of *p*-nitrophenyl phosphate disodium salt hexahydrate (P4744) in about 70 ml of DI water, and adjusting the volume to 100 ml with DI water. These solutions were stored at 4 °C for up to 2 weeks or −20 °C for months. THAM (0.1 mM, pH 12) was prepared as described by Tabatabai (1994).

For the microplate assays using *p*-nitrophenol labeled substrates, absorbance of the soil suspension was measured at 415 nm with a Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The controls were performed using the same procedure but with the substrate added after the termination step to ensure the same matrix as the sample assay. Costar flat-well medium binding polystyrene 96-well microplates (well capacity 360 μl) were used throughout the study. Unless specified, four replicated analyses and four controls were conducted for each soil suspension and two suspensions were prepared for each sample. The average absorbance of the controls was subtracted from the absorbance readings for each well. *p*-Nitrophenol concentrations in