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Short communication

A modified incubation method reduces analytical variation of soil hydrolase assays

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ABSTRACT

Given that analytical precision affects the number of biological replicates required to achieve adequate statistical power, and analytical variation is often high for soil assays, we investigated ways to reduce variation of soil hydrolase assays. For two mineral soils (sandy and loamy) and one organic soil (peat), we compared variation of fluorescence for acid phosphatase, β-glucosidase, and N-acetyl-glucosaminidase using incubation methods that differed in assay volume and the ability of reagents to mix. For fluorescence of β-glucosidase and N-acetyl-glucosaminidase from the mineral soils, which had coefficients of variation that exceeded 10% on average, well-mixed, larger volumes significantly reduced variation. This reduction was not observed for fluorescence of acid phosphatase from peat, which had consistently low variation. For all soils tested, thorough mixing of NaOH also reduced variation. Because the goal of enzyme assays is to estimate the total enzyme pool in a sample of soil, modifications to reduce analytical variation, such as those proposed here, should be considered. These modifications can potentially increase our ability to detect treatment differences within the heterogeneous soil matrix.

1. Introduction

As the proximate drivers of decomposition and nutrient cycling in soil (e.g. Ref. [1]), extracellular enzymes are important indicators of soil fertility [2,3] and are useful as parameters in biogeochemical models [4]. In order for estimates of extracellular enzymes to be informative, however, measurements must be accurate and precise. In terms of precision, large variation at any level of experimental design (e.g. biological replication or experimental plots, analytical replication of measurements) can reduce our ability to detect biologically meaningful treatment effects [5]. This variation may be especially problematic because subtle responses of decomposition (enzyme activity) can scale to influence carbon (C) and nutrient cycling between plants and microbes, and ultimately fluxes among soil, land and water. Because it is often difficult to increase biological replicates in large-scale field experiments, minimizing variation among analytical replicates is often essential for detecting differences among ecosystems, treatments or over time. Reconciling these methodological challenges, along with the effect of soil physiochemical interactions (e.g. adsorption to soil colloids, pseudo-enzymatic catalysis), is important for linking extracellular enzymes and nutrient cycling.

For measurements of hydrolytic enzymes, the 4-methylumbelliferone-(MUB)-based fluorescence method in microplate format is standard in ecological literature [6–9]. MUB-based detection is considered sensitive [8–10] and the high-throughput nature of the microplate format means that multiple enzymes can be analysed in parallel. Yet, analytical precision (i.e. well-to-well variation) using this method is usually relatively low [11]; therefore, reducing variation among analytical replicates is one way to increase precision of sample estimates [5].

While analytical variation of the soil assay is an important methodological factor to consider, causes of, and potential solutions to, are rarely explicitly investigated. Because soil slurries have floating particles that can settle during the incubation period, mixing during the enzyme assay is one factor that may affect variability. It is generally assumed that the small volumes associated with microplates (e.g. 250 μL) mix by diffusion. Surface tension, however, can restrict movement of solution within wells, even with shaking during the incubation period. Given the inherently heterogeneous nature of soil, larger volumes, either in deep well 96-well plates (<2000 μL) or 12-well plates or centrifuge vials (>300 mL), may also reduce analytical variation. For resolutions such as these, there is likely a trade-off between potential

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reductions in analytical variation and reagent use. The aim of this study was to test the effect of three methods that vary in assay volume and their degree of reagent mixing on the analytical precision of estimates of hydrolase activity by fluorescence. We hypothesized that thorough mixing in test tube incubations would lower coefficients of variation (CVs) compared to samples in 96 well plates, irrespective of maximizing fluorescence with the use of NaOH.

2. Materials and methods

We sampled two mineral soils (0–10 cm deep, sieved to 4-mm) and one organic soil (20–40 cm deep). The first mineral soil ("sandy soil"; >90% sand) was an Alfic Udipsamments (Zimmerman soil series, fine sand, pH 5.6), from a tall grass prairie in Anoka County, Minnesota. The second mineral soil ("loamy soil"; ~50% sand, 30% silt, 20% clay) was a Typic Hapludolls (Clarion soil series, fine loam, pH 6.5) from cultivated maize (*Zea mays* L.) field in Boone County, Iowa. The organic soil ("peat") was a Typic Haplohemist (Greenwood soil series, peat, pH 4.3) from a sphagnum bog in Itasca County, Minnesota. We froze all samples at −20 °C before analyses.

To test for the effect of assay volume and mixing during incubation, we developed a modification to the standard MUB-based method [6,7], hereafter called the "tube incubation method". Compared to the standard method of incubating assay mixtures in 96-well plates (microplate or deep well), the tube incubation method entailed incubating reagents in 5 mL tubes prior to transferring reaction products to a microplate, where we measured sample fluorescence. This modification does not change the number of biological replicates run (i.e. it does not pseudo-replicate). Rather, the method modifies how analytical replicates are prepared and, therefore, remains an estimate of the total enzyme pool in a sample of soil.

We compared the tube incubation method to the standard incubation in microplates and deep well plates for acid phosphatase (EC 3.1.3.2), β-glucosidase (EC 3.2.1.21), and N-acetyl-glucosaminidase (EC 3.2.1.52). We chose to restrict our tests to these three methods because they represent the most practical combination of factors that can be easily applied in the laboratory, as opposed to a method with large volume in multiple vessels with thorough mixing, for example. In addition, we used four treatments to test the effect of incubation method with the addition of 10 μL of 1 M NaOH: (i) microplate incubation followed by addition of NaOH, (ii) deep well plate incubation, centrifugation and transfer to microplate, followed by addition of NaOH, (iii) tube incubation and transfer to microplate, followed by addition of NaOH, and (iv) tube incubation followed by addition of NaOH and subsequent transfer to microplate.

For each soil type and replicate ($n = 5$), we homogenized 1 g soil with a Tissue Tearor (BioSpec Products, Inc, Bartlesville, OK) in 125 mL buffer adjusted to the pH of the soil for 30 s on high and then constantly mixed on a stir plate while pipetting. We then dispensed soil homogenate into microplates, deep well plates and tubes within 2 min of stirring. For all methods, we dispensed soil homogenate with wide orifice tips to permit the transfer of suspended soil particles. For assays incubated using the standard method in microplate, we transferred 200 μL of soil homogenate to 8 wells each containing 50 μL of substrate for a total of 8 analytical replicates per treatment and substrate combination. Similarly, for reactions incubated in 2 mL deep well plates, we transferred 1600 μL of soil homogenate to 8 wells, each containing 400 μL of substrate. For incubations performed in tubes, we transferred 1800 μL of soil homogenate to 5 mL tubes each containing 450 μL of substrate, for a total of 3 tubes (1 per substrate) per biological replicate ($n = 5$). We incubated 5 mL tubes on their sides and plates in the dark at 140 rpm and 23 °C for 2.5 h, which fell within the linear range of the reaction. At the end of the incubation, we inverted each tube to mix, and transferred 250 μL of assay mixture (soil homogenate + substrate) to 8 wells of a microplate using the multidispense function of an automatic pipette. Following previous protocols for deep well plates [12], we centrifuged at 2000 rpm for 2 min and then transferred 250 μL of supernatant from each well to a microplate using a multichannel pipette. In this way, the final plate set-up, including the number of analytical replicates and assay volumes, was the same for all methods. We read all samples using a fluorometer (360 nm excitation and 460 nm emission; BioTek Instruments, Inc., Winooski, VT). We stored all substrates at 4 °C and warmed them to room temperature prior to use. We ran all reactions at saturating substrate concentrations (in-well concentrations of 400 μM for β-glucosidase and N-acetyl-glucosaminidase and 800 μM acid phosphatase) and, for the purposes of demonstrating variation in raw fluorescence, we used units of fluorescence g^{-1} hr^{-1}.

To evaluate the effect on analytical variation of each incubation method with and without NaOH addition for each soil, we calculated CVs of the 8 analytical replicates (microplate wells) for a total of 5 CVs ($n = 5$ biological replicates) for each enzyme and method combination per soil. CV data were non-normally distributed. Data also did not meet the assumption of homogeneity of variance, which we expected since the goal of the experiment was to test for differences in variance. To avoid violating assumptions of ANOVAs, we used randomization tests with 9999 permutations to test for the effects of incubation method and/or NaOH addition on analytical variation. We performed all statistical analyses in R (V2.15.2).

3. Results

Across the soil types, enzymes and methods, CVs using the tube incubation method consistently ranged from 1 to 6%, which is comparable to or lower than CVs previously reported from bench-scale assays [13,14] and a tube incubation method [15] for absorbance-based methods using p-Nitrophenyl. For estimates of β-glucosidase and N-acetyl-glucosaminidase, the sandy soil exhibited lower CVs using the tube incubation method as compared to both the microplate method (β-glucosidase, $P < 0.01$; N-acetyl-glucosaminidase, $P < 0.01$) and deep well plate method (β-glucosidase, $P = 0.01$; N-acetyl-glucosaminidase, $P = 0.03$; Fig. 1). Similarly, the loamy soil exhibited lower CVs using the tube incubation method as compared to the microplate (β-glucosidase, $P < 0.01$; N-acetyl-glucosaminidase, $P = 0.04$) and the deep well plate methods (β-glucosidase, $P = 0.02$; N-acetyl-glucosaminidase, $P = 0.02$; Fig. 1). While incubating in a tube also reduced the CV of acid phosphatase, a reduction in CVs was only significant when compared to the microplate incubation method (sandy soil, $P = 0.02$; loamy soil, $P = 0.03$; Fig. 1). For the peat, the deep well incubation method produced lower CVs compared to the tube incubation method for acid phosphatase ($P = 0.02$) and N-acetyl-glucosaminidase ($P = 0.03$), and CVs among the three methods were not significantly different for β-glucosidase ($P > 0.09$ for all; Fig. 1). When NaOH was mixed with the assay reagents in tubes (method iv), average CVs for the sandy and loamy soil was over 4× lower when NaOH was added to the tubes (data not shown). This was observed for acid phosphatase (sandy soil, $P < 0.01$; loamy soil, $P < 0.02$), β-glucosidase (sandy soil, $P < 0.01$; loamy soil, $P < 0.03$) and N-acetyl-glucosaminidase (sandy soil, $P < 0.01$; loamy soil, $P < 0.01$). This is in contrast to the effect of NaOH addition on the peat, which was not as consistently pronounced when compared to the other three methods (data not shown).