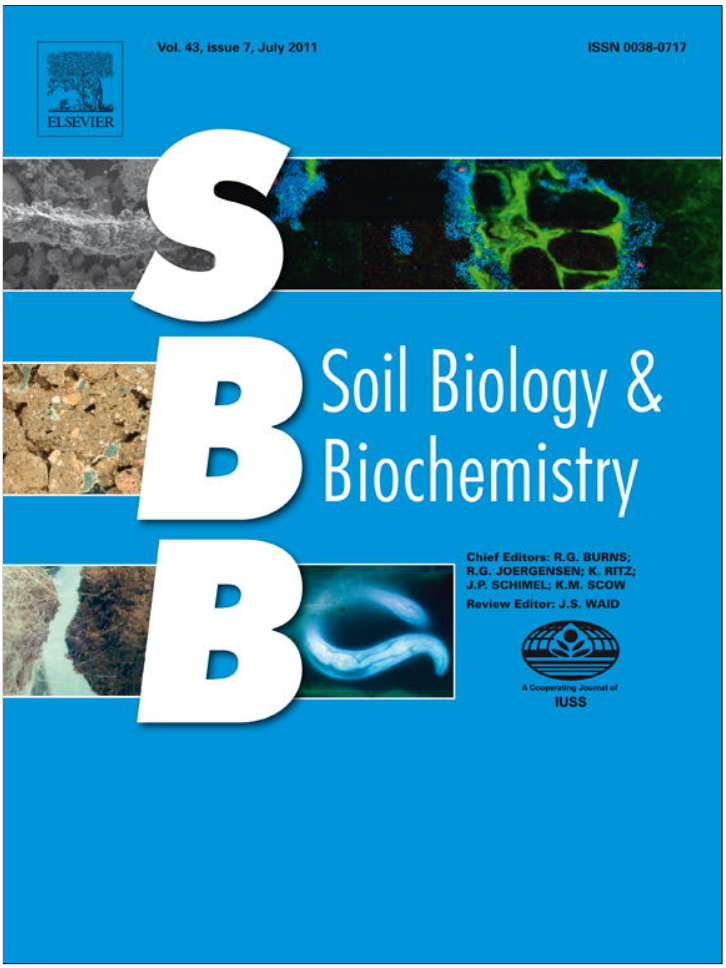

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Review

Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies

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ABSTRACT

Microbial digestive enzymes in soil and litter have been studied for over a half century, yet the understanding of microbial enzymes as drivers of ecosystem processes remains hindered by methodological differences among researchers and laboratories. Modern techniques enable the comparison of enzyme activities from different sites and experiments, but most researchers do not optimize enzyme assay methods for their study sites, and thus may not properly assay potential enzyme activity. In this review, we characterize important procedural details of enzyme assays, and define the steps necessary to properly assay potential enzyme activities in environmental samples. We make the following recommendations to investigators measuring soil enzyme activities: 1) run enzyme assays at the environmental pH and temperature; 2) run proper standards, and if using fluorescent substrates with NaOH addition, use a standard time of 1 min between the addition of NaOH and reading in a fluorometer; 3) run enzyme assays under saturating substrate concentrations to ensure V_{max} is being measured; 4) confirm that product is produced linearly over the duration of the assay; 5) examine whether mixing during the reaction is necessary to properly measure enzyme activity; 6) find the balance between dilution of soil homogenate and assay variation; and 7) ensure that enzyme activity values are properly calculated. These steps should help develop a unified understanding of enzyme activities in ecosystem ecology.

1. Introduction

Decomposition of organic matter in the environment is a microbial process (Swift et al., 1979). Microorganisms use secreted or membrane-bound digestive enzymes (DEs) to degrade polymeric substances (e.g., cellulose, chitin), and rely on diffusion to access the degradation products (Burns, 1982; Sinsabaugh et al., 1991; Sinsabaugh, 1994). The products of enzymatic degradation (e.g., glucose, amino acids, phosphate) are then used by microorganisms for metabolism and growth. Interest in DEs in environmental samples can be traced to the early 20th century (and likely earlier; Skujins, 1978), when Selman Waksman studied the proteolytic capabilities of bacteria and fungi isolated from soils (Waksman, 1918). Work on soil microbial DEs increased throughout the 20th century (Briggs and Segal, 1963; Galstian, 1959; Skujins, 1978), and today there are dozens (if not more) of articles published every year on the topic (e.g., Sinsabaugh et al., 2008). The broad interest in DEs reflects the fact that DEs represent the initial, rate limiting step of decomposition, and therefore, understanding variation in DE activity levels can have broad implications in ecosystem studies (Burns, 1978b, 1982; Wallenstein and Weintraub, 2008).

Research on microbial DEs traditionally focused on the isolation and characterization of novel proteins for potential industrial uses. Studies on the role of DEs in soil processes only began in earnest in the late 1950's (e.g., Galstian, 1959; Voets and Dedeken, 1964), but the throughput rate of enzyme assays was slow enough to limit the understanding of soil enzyme diversity and substrate specificity (Galstian, 1974). Since the 1990's, however, there have been major advances in soil enzyme methodology that have increased the diversity of ecosystems and enzymes under investigation. In particular, assays using fluorescent dye-conjugated substrates [e.g., 4-methylumbelliferone (MUB), 7-amino-4-methylcoumarin (AMC); Marx et al., 2001; Saiya-Cork et al., 2002] have been developed that allow rapid and highly sensitive determination of multiple DEs that control C, N, and P cycling, and thus, provide the

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opportunity to answer questions about DEs on broader scales. Because of these developments, we can now test a larger array of hypotheses related to DEs and their role in biogeochemical cycling (Sinsabaugh et al., 2009). Furthermore, we can answer some of these questions at larger scales by combining data from different studies in meta-analyses (e.g., Sinsabaugh et al., 2009, 2008).

Despite the widespread adoption of high-throughput assays for soil enzymes (e.g., Marx et al., 2001), an updated, comprehensive discussion of the details and pitfalls of modern enzyme methodology is not available in the literature. In fact, a major motivation for writing this article is that the authors (and our colleagues) receive dozens of inquiries each year on the execution and interpretation of soil enzyme assays. We recognize that several excellent reviews of enzyme methodology have been written over the years (e.g., Alef and Nannipieri, 1995; Burns, 1978a; Gianfreda and Ruggiero, 2006; Nannipieri et al., 2002; Roberge, 1978; Tabatabai, 1982, 1994; Tabatabai and Dick, 2002), but the prevalent use of high-throughput microplate methods has created the need for an updated review on the current state of enzyme methodology in ecosystem studies. The current knowledge gap affects the quality and utility of contemporary soil enzyme data, often resulting in DE activity levels that are incomparable among different studies, even though the same DEs are assayed. For example, the same enzyme can be assayed in soils from different habitats, but because of differences in substrate used, substrate concentration, soil mass, and calculation of enzymatic activity, the activities may have different meanings from different studies. Such methodological differences have impeded efforts to make broad generalizations about DEs across environmental samples and different conditions (DeForest, 2009).

Therefore, the objective of this review is to address some of the methodological concerns that have developed as more researchers delve into the enzyme ecology of microbial decomposers, but may be unfamiliar with past considerations of enzyme methodology (e.g., Burns, 1978a). In an effort to offer a broad perspective on enzyme methodology in bulk soil and litter samples, we will discuss the major issues surrounding biochemical assays of DE activities and make suggestions on how to optimize the methods used. Methodological optimization will better enable the ecological community to perform larger scale meta-analyses and improve understanding of how microbial DEs drive ecosystem processes. For specific methodologies involving enzymes in different fractions of soils (i.e., bound vs. free enzymes), we refer readers to several excellent reviews that discuss this topic in more detail (Burns, 1978a, 1982; Gianfreda and Ruggiero, 2006; Nannipieri, 2006; Nannipieri et al., 2002; Quiquampoix et al., 2002; Tabatabai, 1994; Tabatabai and Dick, 2002).

2. Conducting enzyme assays

One of the most important points to consider when running enzyme assays on environmental samples is that these assays measure “maximum potential” enzymatic activity, not the actual rates of enzymatically catalyzed reactions (“realized activity”) in soil or litter (Burns, 1978a; Tabatabai and Dick, 2002; Wallenstein and Weintraub, 2008). Thus, like any *in situ* biochemical technique, soil DE assays require optimization. Commonly cited assay methods (e.g., Saiya-Cork et al., 2002) were developed to address specific ecological questions, and application of the method to new sites or questions will usually require verification and adjustment of the assay protocol. Several key variables must be determined before running enzyme assays on environmental samples to ensure that potential enzyme activity is properly estimated: sample storage, assay pH, assay substrate concentration, assay temperature, assay duration and mixing, and assay homogenate preparation and dilution. We will address each of these in turn.

2.1. Sample storage

Soil and litter samples must be collected, transported, and stored in a consistent manner to prevent alteration and degradation of enzyme activity. So important are collection and storage conditions that analyses have been performed (e.g., Lee et al., 2007) and reviews written on this topic (e.g., Forster, 1995). For this review, however, we simply want to make the point that consideration must be taken of the enzymes under study and the differential effects that different storage methods can have on different enzymes (Gianfreda and Ruggiero, 2006). For instance, it may be appropriate to freeze samples for certain enzyme assays (e.g., β-glucosidase), but not others (e.g., *N*-acetyl-β-D-glucosaminidase; DeForest, 2009). Moreover, the storage method and/or time may also affect the activities of different enzymes in different ways, causing increases in some, and decreases in others (Lee et al., 2007). Thus, if one knows precisely how samples will be transported and stored prior to enzymatic assays, enzymes that can withstand a particular treatment regime should preferentially be studied over those that might be sensitive to that treatment regime. Alternatively, we suggest conducting a small pilot study to test if there will be substantial effects due to storage. We recommend conducting assays on fresh samples whenever possible.

2.2. Assay pH

Enzymes are sensitive to pH and display specific pH optima (Tabatabai, 1994; Turner, 2010). However, enzymes in soil and/or litter may not operate at their pH optimum (Burns, 1978a). Unlike animal digestive tracts, for example, most microbes cannot control the environmental pH for their DEs. Thus, in order to estimate potential enzyme activities in environmental samples, enzyme assays should be run at a pH appropriate for that sample, as opposed to relying on a pH used in a published method (Turner, 2010). For instance, an article by Saiya-Cork et al. (2002) has been cited over 50 times specifically for its soil enzyme method (ISI Web of Science, June 2010). Only twelve of these >50 studies reported that enzyme assays were conducted at a pH appropriate for the environmental samples, if the pH was mentioned at all. Of course, the chosen assay pH can be influenced by the question being asked. For instance, a researcher may be interested in the optimal pH for a specific enzyme (Gallo et al., 2004; Turner, 2010), or the enzyme activities specific to the rhizosphere, the pH of which may differ from the bulk soil pH (Burns, 1982). But, the point of this review is to call for optimization of enzyme methodology for each study site. Thus, bulk soil or litter pH is the desirable pH to use for the estimation of potential digestive enzyme activities in bulk soil or litter samples.

2.2.1. Buffers

DE assay protocols for soil and litter samples usually call for the use of an aqueous buffer to control assay pH and dilute the sample. The pH range of the chosen buffer system should match the environmental sample. For example, sodium acetate/acetic acid buffer has a pH range of 3.6–5.6 and would be inappropriate for samples with pH >5.6. Buffer choice can also directly affect the activity level of an enzyme. For instance, phosphate buffer may interfere with the measurement of phosphatase activities, tris is in an inhibitor of α-glucosidase (Dahlqvist, 1968), and citrate can chelate iron (Essington et al., 2005), thereby inhibiting enzymes with iron-heme prosthetic groups (e.g, lignin peroxidase; Sinsabaugh, 2010). Universal buffer systems (e.g., Skujins et al., 1962; Tabatabai, 1994) contain many of these compounds, and hence, may be inappropriate for some enzymes. Because a wide range of buffers can be used depending on the question being asked or the conditions of a specific habitat (e.g., Turner, 2010), we cannot make specific buffer

recommendations here. Instead, we wish to make the point that buffer should be chosen carefully when beginning enzyme assays on environmental samples (Tabatabai and Dick, 2002).

Although nearly all soil/litter DE assays use buffers, some researchers have called for the elimination of buffers in certain enzyme assays (e.g., urease; Zantua and Bremner, 1975). Buffers have traditionally been used by biochemists to control the assay pH for isolated enzymes, which have been removed from the compartmentalized cellular environment, or the controlled environment of a digestive tract. Soils contain an array of compounds (e.g., clays, ions, solutes) that effectively control the pH within the soil environment. Thus, a potential alternative to using a buffer is to simply use water as the diluent for sample homogenates, which would allow the sample itself to control the pH of the assay (Zantua and Bremner, 1975). A potential advantage of this approach is that the assay would run at the environmental pH without interference from buffer components. However, pH fluctuations have been observed in some assays performed in the absence of buffer (Burns, 1978a). Moreover, all studies of isolated enzymes are done in the presence of buffer, so if any comparisons are to be made among enzyme activities in soils and those of isolated enzymes, a buffering system must be used. Hence, we suggest using an appropriate buffer for enzyme assays, but call for more work on the potential of using water as a diluent, particularly on whether pH fluctuations during assays with water as the diluent are high enough to influence measured enzyme activities.

2.2.2. NaOH addition for fluorescent substrates

A key consideration for high-throughput assays using MUB- or AMC-conjugated substrates is that the fluorescent dye released during the assay fluoresces best at alkaline pH values (>9; Mead et al., 1955). Since assays are typically conducted at a pH lower than 9, NaOH is often added to raise the pH immediately before reading the samples in a fluorometer (e.g., DeForest, 2009; Saiya-Cork et al., 2002; Wallenstein et al., 2009). However, not all buffers respond to NaOH addition in the same manner – that is, assays conducted in 50 mM sodium acetate buffer at pH 5 may require one volume/concentration of NaOH to raise the pH to the appropriate range for fluorescence readings, whereas a different buffer (e.g., 25 mM maleate buffer, pH 5.8) might require more NaOH. Assays run under alkaline conditions may not require the addition of any NaOH. These details must be resolved prior to running assays to ensure consistent fluorescence of the dyes.

Another issue regarding NaOH addition is that the fluorescence of MUB and AMC vary with time following the addition of NaOH to the assay wells (DeForest, 2009). MUB fluorescence increases until ~20 min after NaOH addition, at which time the fluorescence begins to decrease, whereas AMC shows a decrease in fluorescence with time following the addition of NaOH (Fig. 1). Likewise, we and other workers (De Cesare et al., 2000) have observed that the color intensity of p-nitrophenol changes with time after NaOH addition in colorimetric assays. Thus, the amount of time between the addition of NaOH and the fluorescence/absorbance reading should be standardized to eliminate this source of variation in enzyme activity calculations. Consistency is also important because MUB standard and MUB-conjugated substrate fluorescence diverge with time following NaOH addition (DeForest, 2009). Hence, in congruence with DeForest (2009), we recommend a time frame of 1 min between NaOH addition and the reading of plates in a fluorometer to reduce analytical variation. Care should be taken to ensure that the same time interval is used for all samples in a given study.

The issues associated with NaOH addition led us to ask the question of whether MUB and AMC based enzyme assays would yield satisfactory results without adding NaOH altogether. To test this possibility we conducted enzyme assays in two Udipsamments soils with different pH values (pH 4.5 and 6.5) and determined the fluorescence produced in the assays with and without NaOH addition. β-glucosidase, β-1,4-*N*-acetyl-glucosaminidase, acid phosphatase, and leucine aminopeptidase activities were measured as described by Saiya-Cork et al. (2002), using 50 mM sodium acetate buffer at pH 4.5, and 50 mM sodium bicarbonate buffer at pH 6.5. At both pH levels, β-1,4-N-acetyl glucosaminidase activities were significantly higher with NaOH addition, whereas β-glucosidase activities were marginally, although still significantly, higher with NaOH addition at pH 4.5, but not at pH 6.5 (Table 1). Acid phosphatase activity also responded differently depending on soil pH: at pH 6.5, activity was significantly lower with added NaOH, whereas no significant effect of NaOH addition was detected at pH 4.5. Leucine aminopeptidase activity was unchanged by NaOH addition at pH 6.5 and not detectable at pH 4.5. Examination of the raw data indicated that the variation in activity levels in response to NaOH addition was caused by varying fluorescence in the control wells rather than assay wells. Because the control values are part of the activity calculations (see below), this still affects the final activity values.

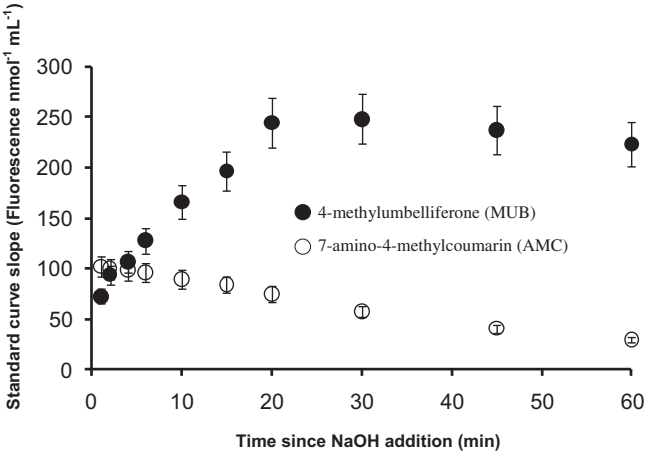

Fig. 1. Slopes of MUB and AMC standard curves as a function of time since NaOH addition. Values are mean ± standard deviation.

Table 1
Activity levels of four hydrolytic enzymes as determined with fluorescent dye-conjugated substrates with or without the addition of NaOH in Udipsamments soils (from northwest Ohio) of pH 4.5 and 6.5.

Condition	β-glucosidase	β-1,4-*N*-acetyl-glucosaminidase	Acid phosphatase	Leucine aminopeptidase
Soil pH: 4.5				
NaOH added	461.072 ± 92.3	464.819 ± 46.3	182.859 ± 51.2	N/A
NaOH not added	363.367 ± 71.8	378.793 ± 42.4	149.357 ± 30.9	N/A
$F_{1,11}$	4.184	11.274	1.887	N/A
P	*0.068*	**0.007**	0.199	N/A
Soil pH: 6.5				
NaOH added	45.881 ± 10.1	25.979 ± 6.9	93.220 ± 11.8	11.854 ± 3.4
NaOH not added	38.973 ± 10.1	18.007 ± 3.7	233.800 ± 106.8	11.818 ± 2.5
$F_{1,11}$	1.421	6.382	5.146	0.004
P	0.261	**0.030**	**0.045**	0.984

Activity levels presented as nmol product produced hour^{-1}g dry soil^{-1}. Values are mean ± standard deviation (N=6 for each enzyme and each soil). Activity levels for each enzyme were compared among assays with and without NaOH addition in each of the soils with ANOVA. Activity levels were considered significantly different at $P \leq 0.05$, as indicated in **bold**.

Despite the alkaline pH optima of MUB and AMC, we had no difficulty detecting the accumulation of the dyes released by enzyme activity at pH 4.5, even without increasing the sensitivity setting of the fluorometer. The variability (standard deviation) of the calculated enzyme activities was also similar regardless of NaOH addition for most enzymes (Table 1). These results indicate that it is possible to conduct enzyme assays using MUB- or AMC-conjugated substrates without adding NaOH prior to fluorescence measurements. Given the variability introduced by inconsistencies in the time between NaOH addition and fluorescence measurements (DeForest, 2009), and the differential effects of NaOH addition on the different buffers, substrates, controls, and samples used in these assays, conducting the assays without NaOH addition is an option worth considering in future methodological studies. However, we do not recommend amending this practice in the middle of a study due to potential changes in the calculated enzyme activities.

2.3. Substrate concentration

With the exception of phenol oxidase and peroxidase, the majority of DEs measured in ecosystem studies are hydrolytic enzymes, which generally follow Michaelis–Menten kinetics (e.g., β-glucosidase; Fig. 2). Two important parameters can be inferred from Michaelis–Menten kinetics: the maximal velocity (V_{max}) and the Michaelis–Menten constant (K_m; the substrate concentration at ½ the maximal velocity). The K_m provides information on enzyme affinity for substrate and enzyme efficiency (Davidson et al., 2006; Marx et al., 2005; Nannipieri and Gianfreda, 1998; Tabatabai, 1994). Because most researchers investigating DEs in environmental samples strive to measure potential enzyme activity – a measure of V_{max} – it is important to confirm that each hydrolytic enzyme is assayed under saturating conditions, as activities measured at lower substrate concentrations will underestimate potential DE activity (Nannipieri and Gianfreda, 1998).

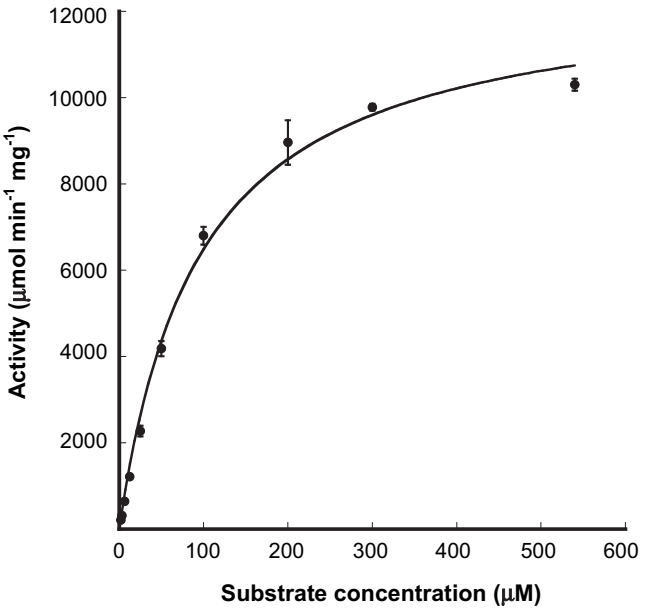

Fig. 2. Biochemical activity of purified β-glucosidase (from *Aspergillus niger*) as a function of substrate (4-methylumbelliferyl β-D-glucopyranoside) concentration. Values are Mean ± standard deviation. Line was fit with non-linear regression using the Michaelis–Menten equation: $\{(V_{max}\,[substrate])/(K_m + [substrate])\}$. K_m was estimated at 87.92 μM substrate, and Vmax as 12,046 μmol min^{-1} mg^{-1}. See text for definitions.

Underestimating activity has the consequence of reducing the power to detect differences in enzyme activity (Fig. 3). For example, β-glucosidase activities measured at saturating substrate concentrations (200 μM; Fig. 3) reveal differences that could be missed at sub-saturating substrate concentrations (50 μM; Fig. 3). Statistical comparisons of β-glucosidase activity at the lowest enzyme concentrations in Fig. 3 (concentrations reflective of enzyme concentrations in environmental samples; Fig. 4) reveal that the activity at 0.5 μg of enzyme is lower than the 0.8 μg enzyme concentration for both the 200 μM and 50 μM substrate concentrations. However, the statistical significance of the difference is an order of magnitude greater in the activities measured at saturating substrate concentrations compared to sub-saturating substrate concentrations (Fig. 3). An important point here is that this assay was conducted with purified enzyme with low analytical error. Thus, given the high variability commonly encountered in soil and litter samples, differences among sites or treatments are more likely to be detected if the enzyme assays are run at saturating substrate concentrations than at sub-saturating conditions (Marx et al., 2001). Of the >50 articles citing Saiya-Cork et al. (2002) for the methodology, only five used final assay substrate concentrations greater than 40 μM (the concentration used by Saiya-Cork and colleagues) or optimized the substrate concentrations for their specific samples. Our own investigations in California grassland soils revealed saturating substrate concentrations >100 μM for β-glucosidase (Fig. 4), and saturating concentrations that vary by site and enzyme. Therefore, underestimation of potential enzyme activity in samples may be a widespread problem and may have been a source of type II error in previous investigations. See Nannipieri and Gianfreda (1998) for an excellent discussion of enzyme kinetics in soils.

The exceptions to the premise of saturating conditions are the oxidases – phenol oxidase and peroxidase which are involved in lignin degradation. Because oxidases may not follow Michaelis–Menten kinetics, the assays for these enzymes are more complex and difficult to optimize (Sinsabaugh, 2010). It is likely, however, that swamping the sample with an overabundance of substrate (e.g., 25 mM substrate concentration) will allow the enzymatic reaction to proceed at some maximal rate, whatever that might be. See below ("Current assay techniques for phenol oxidase and peroxidase") for a discussion of assay methodology for phenol oxidase and peroxidase.

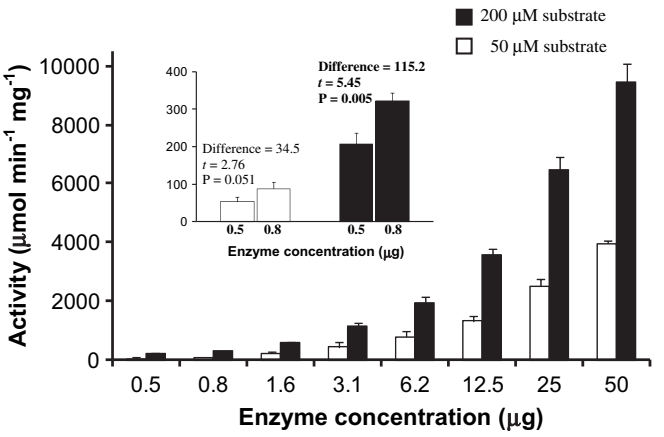

Fig. 3. Biochemical activity as a function of enzyme concentration of purified β-glucosidase (from *Aspergillus niger*) at two substrate concentrations (substrate: 4-methylumbelliferone-β-D-glucoside). Values are mean ± standard deviation. The inset shows the enzyme activities for the 0.5 and 0.8 μg enzyme concentrations at the two substrate concentrations. Activity levels were compared among the two enzyme concentrations with *t*-test for each substrate concentration individually.

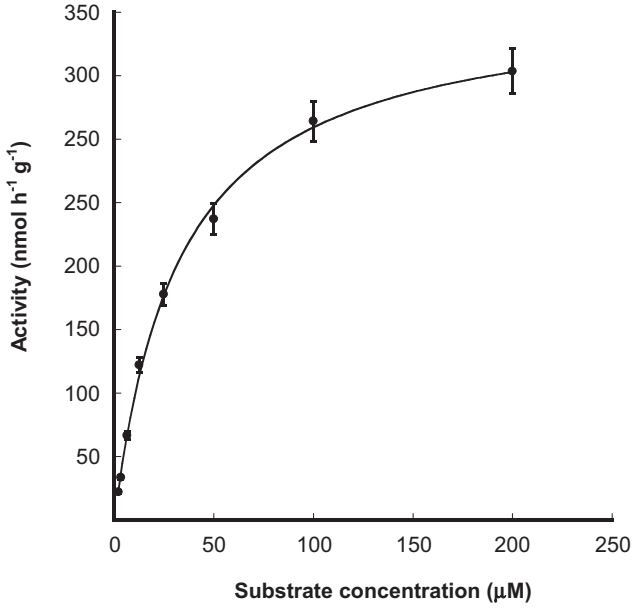

Fig. 4. β-glucosidase activity (at 22 °C) as a function of substrate concentration in grassland soils from Irvine, California, USA. Values are Mean ± standard error. See Fig. 2 for line calculation. K_m was estimated at 32.77 μM substrate, and V_{max} as 410 nmol min^{-1} g^{-1}.

2.4. Assay temperature

Similar to pH sensitivity, enzymes are sensitive to temperature and show temperature optima. Temperature sensitivity of enzymes in environmental samples can vary with latitude (Kang and Freeman, 2009) and even season at a single site (Fenner et al., 2005; Koch et al., 2007; Wallenstein et al., 2009). Therefore, it is important to choose assay temperatures that are relevant to the sampling time and location (Koch et al., 2007). If temperature sensitivity of enzymes is a central question, then it will be necessary to run assays at multiple temperatures (e.g., Trasar-Cepeda et al., 2007; Wallenstein et al., 2009).

In the context of global change, many studies have examined the effects of temperature on DE activities in environmental samples. Although V_{max} sensitivity to temperature is well documented and expected (e.g., Davidson et al., 2006; Fenner et al., 2005; Koch et al., 2007; Trasar-Cepeda et al., 2007), the sensitivity of K_m to temperature in microbial DEs is less well-studied (Davidson et al., 2006). Modeling efforts have indicated that the temperature sensitivity of K_m can have dramatic effects on decomposition rate in response to increasing temperature (Allison et al., 2010; Davidson et al., 2006), especially under changing substrate conditions. In Californian soils, we have observed that the K_m of β-glucosidase increases with increasing temperature (German and Allison, unpublished data), which suggests that the efficiency of an enzyme may be compromised at higher temperatures, and especially in areas where substrate concentrations are close to or below K_m concentrations. Substrate concentrations close to K_m are probably more common than concentrations approaching saturating conditions in soils, thus making K_m a worthwhile parameter to measure, not only for optimizing enzyme assays, but for understanding enzyme function in the environment.

2.5. Assay duration and mixing

Because enzyme activity is calculated as a rate (under the assumption of zero order kinetics), it is important to ensure that the amount of fluorescence or absorbance increases linearly with time. Thus, for hydrolytic enzymes, measuring fluorescence or absorbance over time (e.g., every 10 min) should produce a linear relationship. As most reported enzyme methods are end point assays (e.g., Saiya-Cork et al., 2002), care should be taken to use an assay duration that produces a linear response with time. The exact method used for assay duration optimization will vary by site and depend upon assay pH: assays run at acidic pH arrested by the addition of NaOH must be measured in different wells at each time interval, whereas assays run at alkaline pH conditions can be read periodically over time. Although, assays run under acidic conditions can also be read repeatedly without the addition of NaOH (see "Assay pH" above).

Because soil and/or litter particles can settle during the assay, it is important to consider whether assay reagents need to be mixed during the reaction period. For larger assay volumes (e.g., >300 μL) run in 12-well plates or centrifuge vials, we recommend mixing the assay reagents on a shaker to ensure constant contact of enzymes and substrate. However, smaller volumes (i.e., ≤250 μL) run in microplate wells may mix sufficiently by diffusion, although this should be investigated before running assays on a large scale. Since phenol oxidase requires molecular oxygen to operate, it may be helpful to run this assay in larger volumes that are constantly mixed (see below).

2.6. Assay homogenate preparation and dilution

Homogenization technique can affect the outcome of DE assays. The most consistent results come from the use of a homogenizer or blender (e.g., Polytron homogenizer; Grandy et al., 2007), which breaks up particles and disperses enzymes in buffer with minimal cell lysis and associated release of intracellular enzymes (e.g., compared to bead beating, a homogenization technique intended to lyse cells; Weintraub unpublished data). Over-homogenization should be avoided to eliminate alterations of enzymes in the sample or the release of intracellular enzymes. To confirm that microbial cell lysis has not occurred, a comparison could made of the enzyme activity levels in samples dispersed with a homogenizer and those measured in samples in which microbial cells were purposefully lysed (e.g., using bead beating or tip sonication; German and Bittong, 2009). To ensure that assays are being performed on consistent soil or litter fractions, soil samples should be passed through a 2 mm screen, and litter samples must be chopped into smaller pieces before homogenization. The particles in a homogenate will settle once blending has ceased. Therefore, the homogenate must be constantly stirred (e.g., on a magnetic stir plate) while pipetting to ensure that a homogenous mixture is being pipetted into each well or vial. An examination of the variation among enzyme replicates will provide information on how well the sample is homogenized.

Another variable to consider is the ratio of buffer-to-sample used to make the homogenate, which affects the turbidity of the homogenate. Increasing amounts of particulate and/or organic material in the homogenate (i.e., less dilution) will cause a "quenching" of fluorescence or interference with absorbance readings for colorimetric assays. However, homogenates that are too dilute may lead to undetectable absorbance/fluorescence or increase analytical variability due to fine scale heterogeneity of the homogenate. Therefore, we recommend that researchers strike a balance between interference and variability and not assume a buffer-to-sample ratio (or dilution factor) used in a previous investigation is appropriate for all samples. As a guideline, we recommend that samples be diluted further if quench values are less than 0.5.

The storage of homogenate may also introduce variability into enzyme assays. DeForest (2009) found that homogenate stored for 2 h at 4 °C produced variable assay results for some enzymes (i.e., β-1,4-*N*-acetyl-glucosaminidase, acid phosphatase, and phenol oxidase) and not for others, and the direction and amount of change varied by enzyme and sample location. This finding leads us to suggest that homogenate be made just before starting the assay, a consistent practice in the animal and plant literature.

3. Standards

The use of a single concentration standard in DE assays is common in the literature, whereas enzyme activity calculations in plants or animal studies ubiquitously use standard curves (e.g., German and Bittong, 2009). Why, then, are environmental samples so different that we would use a single concentration standard, especially when the fluorescence values produced in our assays vary so widely from the fluorescence produced by the actual standard? The answer may lie in convenience – it is simpler to pipette a single concentration of the standard solution than it is to pipette eight different concentrations into a microplate. Pipetting multiple concentrations of standards in a single microplate takes up valuable well space and takes significantly more time than pipetting a single concentration in each plate. But are there any drawbacks to using a single concentration standard as opposed to a standard curve? A comparison of standard curves in the presence of buffer and in the presence of homogenate shows that neither the slope (fluorescence $nmol^{-1}$ mL^{-1}) nor the quenching is different between a standard curve and three separate single concentration standards (Table 2). Therefore, single concentration standards may be suitable for some samples, but this assumption should be verified before beginning a new study. For example, Wallenstein et al. (2009) reported that quenching varied with standard concentration, meaning that a standard curve might be necessary under such circumstances.

4. Activity calculations

After measuring fluorescence or absorbance over time, data must be converted into units of enzyme activity. Several controls and standards need to be run simultaneously with the enzyme assay to account for background noise in the assay reagents and to convert fluorescence or absorbance values into the correct units (DeForest, 2009). In this section we will discuss calculations used for fluorescent and colorimetric assays.

Table 2
Slopes (fluorescence $nmol^{-1}$ mL^{-1}) of standard curves and single concentration standards of 4-methylumbelliferone (MUB) in the presence of buffer or homogenate prepared with boreal forest soil (Delta Junction, Alaska, USA).

Standard type	Buffer fluorescence ($nmol^{-1}$ mL^{-1})	Homogenate fluorescence ($nmol^{-1}$ mL^{-1})	Quench
Standard curve	212.14 ± 8.44	158.36 ± 8.44	0.75 ± 0.04
Single conc.			
0.63 nmol mL^{-1}	211.73 ± 12.25	165.07 ± 21.65	0.78 ± 0.10
2.50 nmol mL^{-1}	207.47 ± 5.85	152.20 ± 22.87	0.73 ± 0.11
5.00 nmol mL^{-1}	213.47 ± 8.70	160.37 ± 7.56	0.75 ± 0.04
$F_{3,11}$	0.30	0.25	0.18
P	0.82	0.86	0.90
	NS	NS	NS

Values are mean ± standard deviation ($n = 3$). Quench is the ratio of the slope in the presence of homogenate over the slope in the presence of buffer. Fluorescence and quench values were compared among the standard curve and each of the single concentrations with ANOVA. NS = not significant.

4.1. Calculations for fluorescent assays

These calculations are appropriate for assays using MUB- or AMC- conjugated substrates, which are used to detect many hydrolytic enzymes in environmental samples (Table 3). The necessary controls are: fluorescence from the substrate (substrate control), fluorescence from the homogenate (homogenate control), MUB (or AMC) standards in the presence of buffer (standard), and MUB (or AMC) in the presence of homogenate (quench control). Activity is calculated as follows:

$$\text{Activity}\left(\text{nmol g}^{-1}\text{ h}^{-1}\right) = \frac{\text{Net Fluorescence} \times \text{Buffer volume(mL)}}{\text{Emission coefficient} \times \text{Homogenate Volume(mL)} \times \text{Time(h)} \times \text{Soil mass(g)}} \quad (1)$$

Where,

$$\text{Net Fluorescence} = \left(\frac{\text{Assay} - \text{Homogenate Control}}{\text{Quench Coefficient}}\right) - \text{Substrate Control} \quad (2)$$

$$\text{Emission Coeff.}\left(\text{fluorescence nmol}^{-1}\right) = \frac{\text{Standard Fluorescence}}{\left[\frac{\text{Standard Concentration(nmol)} \times \text{Assay Volume(mL)}}{\text{Volume of Standard(mL)}}\right]} \quad (3)$$

$$\text{Quench Coeff.} = \frac{\text{Quench Control} - \text{Homogenate Control}}{\text{Standard Fluorescence}} \quad (4)$$

Buffer volume is the volume of buffer used for homogenate preparation, and soil mass is the mass of soil added to the buffer.

The above equations work for assays in which a single concentration standard and quench standard are used to determine the emission coefficient. If a standard curve is used to examine the emission coefficient across a gradient of standard concentrations, then the equations can be modified as follows:

$$\text{Emission Coefficient}\left(\text{fluorescence nmol}^{-1}\right) = \frac{\text{Standard Curve Slope}\left[\frac{\text{Fluorescence}}{\frac{\text{nmol}}{\text{mL}}}\right]}{\text{Assay Volume(mL)}} \quad (5)$$

$$\text{Quench Coefficient} = \frac{\text{Slope of Standard Curve(in presence of homogenate)}}{\text{Slope of Standard Curve(in presence of buffer)}} \quad (6)$$

The standard curve used for the emission coefficient is in the presence of homogenate. All other equations are the same.

4.2. Calculations for colorimetric assays

The calculations for colorimetric assays are similar to those for fluorimetric assays, except there is no “quench” control for chromogenic dyes. Standard curves must be constructed with the

Table 3
Enzymes commonly measured in environmental samples, and their functions, substrates, and classification.

Enzyme	Enzyme function	Substrate	EC
β-D-1,4-cellobiosidase	Catalyzes the hydrolysis of 1,4-β-D-glucosidic linkages in cellulose and cellotetraose, releasing cellobiose. Enzyme is also called cellobiohydrolase.	4-MUB-β-D-cellobioside	3.2.1.91
β-1,4-glucosidase	Catalyzes the hydrolysis of terminal 1,4-linked β-D-glucose residues from β-D-glucosides, including short chain cellulose oligomers.	4-MUB-β-D-glucoside	3.2.1.21
β-1,4-xylosidase	Degrades xylooligomers (short xylan chains) into xylose.	4-MUB-β-D-xyloside	3.2.1.37
α-1,4-glucosidase	Principally a starch degrading enzyme that catalyzes the hydrolysis of terminal, non-reducing 1,4-linked α-D-glucose residues, releasing α-D-glucose	4-MUB-α-D-glucoside	3.2.1.20
β-1,4-*N*-acetyl-glucosaminidase	Catalyzes the hydrolysis of terminal 1,4 linked *N*-acetyl-beta-D-glucosaminide residues in chitooligosaccharides (chitin derived oligomers).	4-MUB-*N*-acetyl-β-D-glucosaminide	3.1.6.1
Leucine aminopeptidase	Catalyzes the hydrolysis of leucine and other amino acid residues from the N-terminus of peptides. Amino acid amides and methyl esters are also readily hydrolyzed by this enzyme.	L-Leucine-7-amino-4-methylcoumarin	3.4.11.1
Urease	Catalyzes the hydrolysis of urea into ammonia and carbon dioxide.	Urea	3.5.1.5
Acid Phosphatase	Mineralizes organic P into phosphate by hydrolyzing phosphoric (mono) ester bonds under acidic conditions.	4-MUB-phosphate	3.1.3.2
Polyphenol oxidase	Also known as polyphenol oxidase or laccase. Oxidizes benzenediols to semiquinones with O_2.	L-DOPA, pyrogallol, ABTS	1.10.3.2
Peroxidase	Catalyzes oxidation reactions via the reduction of H_2O_2. It is considered to be used by soil microorganisms as a lignolytic enzyme because it can degrade molecules without a precisely repeated structure	L-DOPA, ABTS	1.11.1.7

Modified from Grandy et al. (2007).

chromogenic dye (e.g., p-nitrophenol or p-nitroaniline) to generate the extinction coefficient (slope of the absorbance vs. concentration relationship for the chromophore). As with fluorimetric assays, substrate and homogenate controls are necessary to calculate the enzyme activity. Activity is calculated as follows:

$$\text{Activity}\left(\mu\text{mol g}^{-1}\text{h}^{-1}\right) = \frac{\text{Net Absorbance} \times \text{Buffer volume(mL)}}{\text{Extinction coeff.} \times \text{Homogenate Volume(mL)} \times \text{Time(h)} \times \text{Soil mass(g)}} \quad (7)$$

$$\text{Net Absorbance} = \text{Assay} - \text{Homogenate Control} - \text{Subsrate Control} \quad (8)$$

5. Enzymes

Of the many enzymes operating in soil and litter, ten are commonly studied in environmental samples (Table 3; although other enzymes are assayed, depending on the study; Gianfreda and Ruggiero, 2006). The 10 common enzymes are chosen because their activity levels represent the potential capacity of microbes to acquire C (for energy), N, and P, and are relevant for C, N, and P cycling (Allison et al., 2007; Saiya-Cork et al., 2002). Moreover, many of the enzymes degrade the major C-containing compounds in soil and litter: cellulose, hemicellulose, starch, chitin, protein, and lignin. The exact enzymes chosen for assay will depend on the question being asked. For example, if one is interested in the effects of N-addition (e.g., via fertilizer amendment) on C and N cycling specifically, one would likely assay the hydrolytic enzymes involved in carbohydrate and protein degradation, as well as the oxidative enzymes directed at lignin substrates. On the other hand, if a researcher is interested in phosphorus cycling, the focus of the assays may be directed solely at enzymes that produce phosphates from the available substrates. However, because most DE assays are high-throughput, we recommend that researchers assay as many enzymes as possible in order to obtain the most complete picture of microbial DE activity within a given sample, especially because the activity levels of a single enzyme can be misleading (Gianfreda and Ruggiero, 2006). Additional enzymes for ecosystem studies can be found in Tabatabai (1994) and Alef and Nannipieri (1995).

6. Normalization of activity levels – dry mass, organic matter, or microbial biomass C?

The calculations shown in section four above express enzyme activities on a mass basis. Four different variables are commonly used to represent the mass of the sample: 1) dry soil or litter mass; 2) organic matter content; 3) C content; or 4) microbial biomass C. Enzyme activity varies as a function of microbial decomposer biomass, which in turn depends on organic matter and C availability (Gianfreda and Ruggiero, 2006). Hence, the currency used to normalize the data will depend on the question being asked.

Of the terms in which to express DE activity, sample dry mass is the simplest and most common. Many researchers dry soil or litter to a constant mass at 60 °C, but true dry mass is determined by drying samples at 105 °C, above the boiling point of water. Drying at 60 °C can leave up to 10% of original moisture content in the sample (Van Soest, 1994), which can be substantial in saturated soils. Because soil dry mass inherently includes organic matter and mineralogical differences, it may be the best normalized activity with which to make comparisons of DE activities among different sites. Thus, even if DE activities are published as a function of organic matter or microbial biomass C, we recommend that soil dry mass data be made available so that other researchers can convert activities for direct comparison.

Total organic matter in a sample is determined by combusting dried sample at high temperatures (400–550 °C) for at least 3 h. The mass difference between the dry mass of a sample prior to combustion and the mass following combustion represents the organic matter. The amount of C present in a sample can be determined using an elemental analyzer. Normalizing activity levels with total organic matter or C content can help reveal differences in substrate quality or quantity (Jordan et al., 1995).

Microbial biomass C in a sample can be determined by several methods, with chloroform fumigation as the most common (Brookes et al., 1985; Vance et al., 1987). Other microbial biomass metrics against which to normalize environmental DE activity

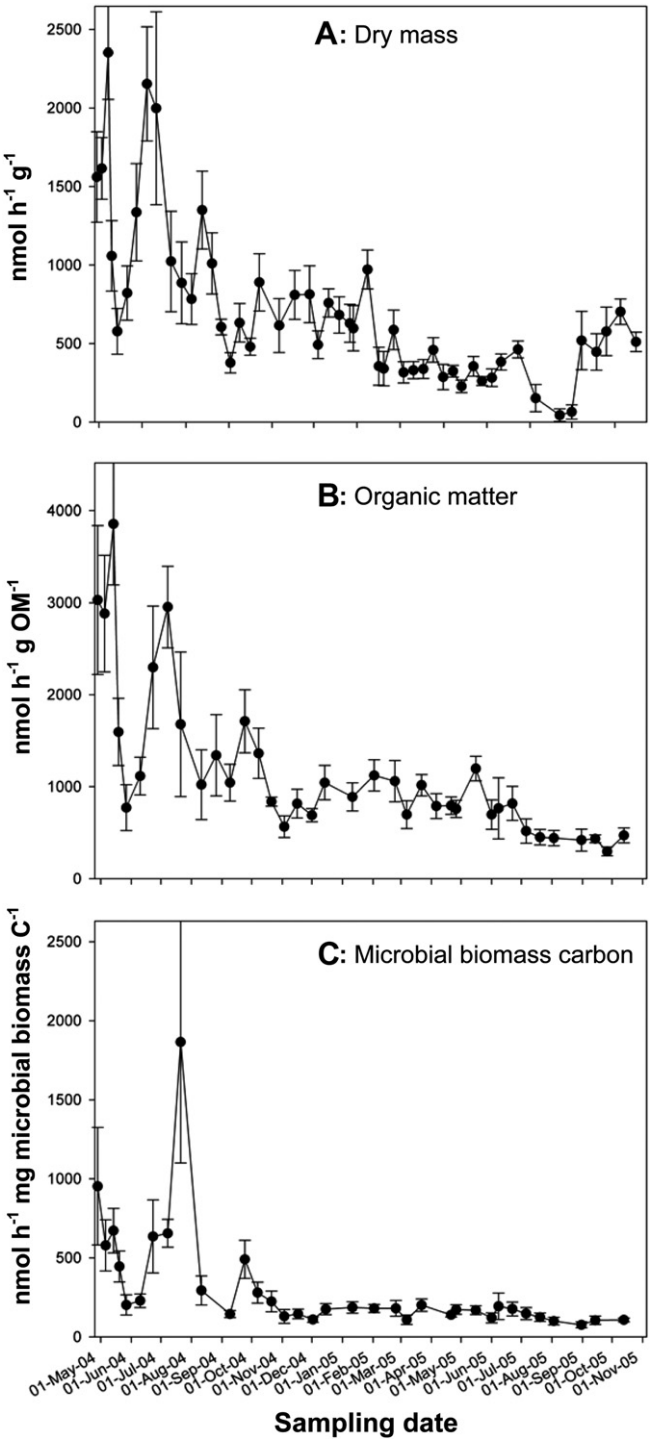

Fig. 5. β-glucosidase activities measured across time in coniferous forest soils (Colorado, USA), as a function of A, soil dry mass; B, soil organic matter; and C, microbial biomass carbon. Values are mean ± SEM.

include phospholipid fatty acid (PLFA) analysis (Hassett and Zak, 2005), adenosine triphosphate (ATP) concentrations (Nannipieri et al., 1996), and total soil DNA concentrations (Brankatschk et al., in press). Normalizing activity levels to microbial biomass allows for the observation of changes in enzymatic production per unit biomass, which may be a qualitative metric of microbial community function in response to specific treatments or conditions (Brankatschk et al., in press; Hassett and Zak, 2005; Kandeler and Eder, 1993; Landi et al., 2000; Nannipieri et al., 1996).

We determined β-glucosidase activities in coniferous forest soil across an 18-month period and calculated the activities on each sampling date as a function of soil dry mass, soil organic matter, and microbial biomass C. Activities normalized to each currency captured similar patterns of activity over time, with a spike in the first four months of the study, followed by dwindling activities thereafter (Fig. 5). However, there was progressively less variability among sampling dates when the data were normalized by soil organic matter content and microbial biomass C. In this case, normalization helps to eliminate the impacts of spatial variability in soil organic matter or microbial biomass C content between samples collected on different dates. In fact, most differences in DE activity among sites or treatments are likely the result of differences in soil organic matter content or microbial biomass C (Sinsabaugh et al., 2008). Thus, differences in DE activity among sites or treatments normalized to organic matter or microbial biomass C can indicate that some other environmental factor is affecting DE activity. Researchers should evaluate the specific question to be answered prior to beginning an investigation and normalize the activities accordingly. For example, normalization by soil dry mass may be most appropriate when investigating the absolute variation in enzyme activities among sites or treatments (Jordan et al., 1995), whereas normalization by organic matter accounts for differences in organic matter content among the samples (e.g., Sinsabaugh and Findlay, 1995). Normalization to microbial biomass C allows for the quantification of how much enzyme activity there is per unit microbial biomass. Either way, the more variables collected during a study and made available to the scientific community (e.g., online), the more broadly applicable the dataset.

7. Current assay techniques for phenol oxidase and peroxidase

Compared to the hydrolytic enzymes assayed with fluorimetric or colorimetric substrates (see above; Table 3), oxidase assay protocols are more complicated and less well-resolved. Many oxidases mediate non-specific free–radical reactions under variable conditions and require different cofactors. For more detailed discussions of oxidases, we defer to recent reviews by Sinsabaugh (2010) and Theuerl and Buscot (2010). Here, we will focus specifically on the most commonly used techniques to assay oxidase activities in soil and litter.

Oxidative enzyme activity (phenol oxidase and peroxidase) is most often quantified by the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) because it is affordable, relatively soluble, and produces oxidation products that can be detected with a spectrophotometer (Mason, 1948; Pind et al., 1994; Sinsabaugh et al., 2008; Sinsabaugh and Linkins, 1988). However, there are several issues with the assay that must be resolved. For instance, the product of the assay (2-carboxy-2,3-dihydroindole-5,6-quinone; Mason, 1948) is not commercially available for standard curve construction. Therefore, a common method of generating the product is to allow a commercially available oxidase (e.g., tyrosinase or horseradish peroxidase) to react with L-DOPA until the substrate is completely reacted (Allison and Vitousek, 2004). The product can then be diluted and used to construct a standard curve. This method assumes, without verification, that all of the substrate has been converted to product, and therefore, that the product concentration is equal to the starting substrate concentration. Extinction coefficients will be incorrect if this assumption does not hold, but it is encouraging that they are within a factor of 2–3 across several studies performed in different laboratories (e.g., Allison and Vitousek, 2004; DeForest, 2009; Saiya-Cork et al., 2002; Shi et al., 2006; Yao et al., 2009). However, the chromophore

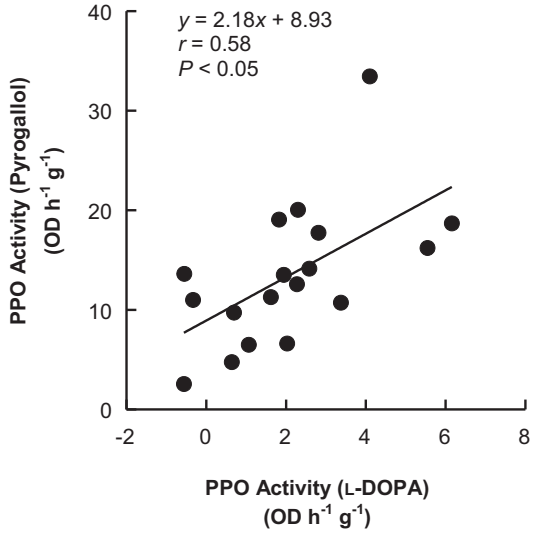

Fig. 6. Phenol oxidase (PPO) activities in Alaskan boreal forest soils from L-DOPA and pyrogallol assays. Both assays were conducted in 50 mM sodium acetate buffer, pH 5.0 with 25 mM EDTA. OD = optical density units.

produced by the oxidation of L-DOPA is not stable over time, and the calculated extinction coefficient is dependent upon the rate at which the L-DOPA is oxidized (Weintraub, unpublished data). Moreover, the oxidation reaction with L-DOPA has a pH optimum near 9 (Pind et al., 1994), perhaps affecting its feasibility for use in oxidase assays of acidic soils and litter, such as those of the boreal forest (Allison et al., 2008).

Another issue is that L-DOPA is vulnerable to chemical oxidation by several metal species, and oxidative products can be consumed by secondary reactions in the soil homogenate, thereby limiting color development during the assay (Sinsabaugh, 2010). Thus, the increase in color intensity may not proceed linearly with time, calling into question its feasibility as a substrate for the calculation of enzymatic activity, which is assumed to follow zero order kinetics with time.

Potential side reactions in the L-DOPA assay make it difficult to design appropriate controls. For instance, autoclaved soils could be used as negative controls that maintain organic matter content of soil, and combusted soils could be used to specifically examine side reactions solely due to mineral species. However, autoclaving can cause significant changes to the stability of soil organic matter (Conrad, 1996; Serrasolses et al., 2008), and enzymes stabilized in organic matter or on mineral surfaces may not be destroyed by autoclaving (Carter et al., 2007; Stursova and Sinsabaugh, 2008). Combusted soils contain no organic matter that may interact with minerals to drive oxidation reactions in natural soils (Sinsabaugh, 2010). Thus, there may not be a realistic way to construct negative controls that account for side reactions that occur in soils (note that most of these problems are absent in litter).

Peroxidase activity is calculated by running the phenol oxidase assay concurrently with assay wells containing H_2O_2 (DeForest, 2009; Saiya-Cork et al., 2002). The difference in activity between the phenol oxidase assay and assay wells containing H_2O_2 represents the net peroxidase activity. The peroxidase protocol inherently has all of the shortcomings of the phenol oxidase assay, plus any additional side reactions caused by H_2O_2.

Oxidase substrates must result in product formation that changes linearly over time, yet L-DOPA does not appear to meet this basic requirement in soil assays. Different protocols have been devised to address some of the problems with the L-DOPA assay. For instance, pyrogallol (1,2,3-trihydroxybenzene) has been used successfully as a substrate for phenol oxidase under acidic conditions (pH 5; Allison et al., 2008, 2006; Allison and Jastrow, 2006; Allison and Treseder, 2008). Moreover, the addition of ethylenediamine tetraacetic acid (EDTA) as a chelating agent may reduce metal oxidation of L-DOPA and pyrogallol in assays of phenol oxidase activity in soils (Allison, 2006). However, phenol oxidases and peroxidases can require metal species as cofactors (Bourbonnais et al., 1998; Camarero et al., 2005), and thus, EDTA could interfere with the enzyme reaction.

Another potential substrate for phenol oxidase and peroxidase assays in acidic soils is 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) or ABTS (Floch et al. 2007). ABTS has the advantage that that it is not oxidized by abiotic constituents of soil, and thus, potential controls (e.g., autoclaved soil) can be used for phenol oxidase and peroxidase assays. Comparisons of phenol oxidase activity measured with L-DOPA and pyrogallol in the same samples shows that the activities produced by the two substrates are comparable (Fig. 6). ABTS can be more sensitive than L-DOPA as a phenol oxidase and peroxidase substrate in acidic samples, producing activity levels that are 2–3× higher than those observed with L-DOPA. The greater absorbance values observed in the ABTS assays is due to the higher extinction coefficient of this compound, resulting in a greater color change per μmol oxidized (Weintraub and Rinkes, unpublished data). Sinsabaugh (2010) provides several other possibilities for the assay of phenol oxidase and peroxidase activities. These assays all use different substrates under different conditions, so consensus among the scientific community for these assays will be crucial if we are to develop a method using a single, appropriate substrate. Overall, phenol oxidase and peroxidase methodologies are less resolved than they are for hydrolases, and this will remain a fertile area for methodological research in the years to come. The biggest challenge will be finding a substrate that is stable enough across a wide range of pH values, so as to allow assays to be conducted under ambient pH conditions.

8. Moving forward: optimization of assay protocols and future directions

Clearly there are many challenges associated with assaying DEs in soil and litter samples. Therefore, we would like to conclude with a set of recommendations to improve assay data quality and facilitate the sharing of optimization procedures across laboratories:

1) Run enzyme assays at the pH and temperature of the collection site so that the measured activities will be relevant to the study site.
2) Include proper standards, and if using fluorescent substrates with NaOH addition, use a standard time of 1 min between the addition of NaOH and reading in a fluorometer.
3) Ensure that V_{max} is being measured in soil or litter, if the study objective is to determine maximal potential activity. In order to do this, researchers must ensure that saturating substrate concentrations are used.
4) Measure enzyme activities over time to confirm that product is produced linearly over the duration of the assay.
5) Examine whether mixing of the reagents during the reaction is sufficient to properly measure DE activity.
6) Find a balance between dilution of soil homogenate and assay variation, and make homogenates just before beginning an assay, not ahead of time.
7) Ensure that enzyme activity values are properly calculated.

If all of these steps are followed, then researchers can be more certain that measured enzyme activities are indeed reflective of enzymatic potential in their environmental samples. Furthermore,

if enzyme activities are normalized to different currencies (i.e., soil dry mass, soil organic matter, microbial biomass) for a particular study, making the other currencies available online will allow for better use of the data in meta-analyses. All of these steps will encourage better collaboration among researchers investigating the links between DE activities and decomposition. Furthermore, properly estimated enzyme activity levels may have more meaning when used in conjunction with emerging proteomic and/or genomic tools that are expanding our ability to understand microbial decomposers and the significant roles they play in ecosystems (Nannipieri, 2006; Wallenstein and Weintraub, 2008).

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References

Alef, K., Nannipieri, P., 1995. Enzyme activities. In: Alef, K., Nannipieri, P. (Eds.), Methods in Applied Soil Microbiology and Biochemistry. Academic Press, London, pp. 311–373.

Allison, S.D., 2006. Soil minerals and humic acids alter enzyme stability: implications for ecosystem processes. Biogeochemistry 81, 361–373.

Allison, S.D., Czimczik, C.I., Treseder, K.K., 2008. Microbial activity and soil respiration under nitrogen addition in Alaskan boreal forest. Global Change Biology 14, 1–13.

Allison, S.D., Gartner, T.B., Holland, K., Weintraub, M., Sinsabaugh, R.L., 2007. Soil enzymes: linking proteomics and ecological process. In: Hurst, C.J., Crawford, R.L., Garland, J.L., Lipson, D.A., Mills, A.L., Stetzenbach, L.D. (Eds.), Manual of Environmental Microbiology, third ed. ASM Press, Washington D.C., pp. 704–711.

Allison, S.D., Jastrow, J.D., 2006. Activities of extracellular enzymes in physically isolated fractions of restored grassland soils. Soil Biology and Biochemistry 38, 3245–3256.

Allison, S.D., Nielsen, C., Hughes, R.F., 2006. Elevated enzyme activities in soils under the invasive nitrogen-fixing tree *Falcataria moluccana*. Soil Biology and Biochemistry 38, 1537–1544.

Allison, S.D., Treseder, K.K., 2008. Warming and drying suppress microbial activity and carbon cycling in boreal forest soils. Global Change Biology 14, 2898–2909.

Allison, S.D., Vitousek, P.M., 2004. Extracellular enzyme activities and carbon chemistry as drivers of tropical plant litter decomposition. Biotropica 36, 285–296.

Allison, S.D., Wallenstein, M.D., Bradford, M.A., 2010. Soil-carbon response to warming dependent on microbial physiology. Nature Geoscience 3, 336–340.

Bourbonnais, R., Leech, D., Palce, M.G., 1998. Electrochemical analysis of the interactions of laccase mediators with lignin model compounds. Biochimica et Biophysica Acta 1379, 381–390.

Brankatschk, R., Töwe, S., Kleineidam, K., Schloter, M., Zeyer, J. Abundances and potential activities of nitrogen cycling microbial communities along a chronosequence of a glacier forefield. The ISME Journal doi:10.1038/ismej.2010.184. in press.

Briggs, M.H., Segal, L., 1963. Preparation and properties of a free soil enzyme. Life Sciences 1, 69–72.

Brookes, P.C., Landman, A., Pruden, G., Jenkinson, D.S., 1985. Chloroform fumigation and the release of soil nitrogen: a rapid direct extraction method to measure microbial biomass nitrogen in soil. Soil Biology and Biochemistry 17, 837–842.

Burns, R.G., 1978a. Enzyme activity in soil: some theoretical and practical considerations. In: Burns, R.G. (Ed.), Soil Enzymes. Academic Press, London, pp. 295–340.

Burns, R.G., 1978b. Soil Enzymes. Academic Press, London.

Burns, R.G., 1982. Enzyme activity in soil: location and a possible role in microbial ecology. Soil Biology and Biochemistry 14, 423–427.

Camarero, S., Ibarra, D., Martinez, M.J., Martınez, A.T., 2005. Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. Applied and Environmental Microbiology 71, 1775–1784.

Carter, D.O., Yellowlees, D., Tibbett, M., 2007. Autoclaving kills soil microbes yet soil enzymes remain active. Pedobiologia 51, 295–299.

Conrad, R., 1996. Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O, and NO. Microbiological Reviews 60, 609–640.

Dahlqvist, A., 1968. Assay of intestinal disaccharidases. Analytical Biochemistry 22, 99–107.

Davidson, E.A., Janssens, I.A., Luo, Y., 2006. On the variability of respiration in terrestrial ecosystems: moving beyond Q_{10}. Global Change Biology 12, 154–164.

De Cesare, F., Garzillo, A.M.V., Buonocore, V., Badalucco, L., 2000. Use of sonication for measuring acid phosphatase activity in soil. Soil Biology and Biochemistry 32, 825–832.

DeForest, J.L., 2009. The influence of time, storage temperature, and substrate age on potential soil enzyme activity in acidic forest soils using MUB-linked substrates and L-DOPA. Soil Biology and Biochemistry 41, 1180–1186.

Essington, M.E., Nelson, J.B., Holden, W.L., 2005. Gibbsite and goethite solubility: the influence of 2-ketogluconate and citrate. Soil Science Society of America Journal 69, 996–1008.

Fenner, N., Freeman, C., Reynolds, B., 2005. Observations of a seasonally shifting thermal optimum in peatland carbon-cycling processes; implications for the global carbon cycle and soil enzyme methodologies. Soil Biology and Biochemistry 37, 1814–1821.

Floch, C., Alarcon-Gutiérrez, E., Criquet, S., 2007. ABTS assay of phenol oxidase activity in soil. Journal of Microbiological Methods 71, 319–324.

Forster, J.C., 1995. Soil sampling, handling, storage, and analysis. In: Alef, K., Nannipieri, P. (Eds.), Methods in Applied Soil Microbiology and Biochemistry. Academic Press, London, pp. 49–121.

Gallo, M., Amonette, R., Lauber, C., Sinsabaugh, R.L., Zak, D.R., 2004. Microbial community structure and oxidative enzyme activity in nitrogen-amended north temperate forest soils. Microbial Ecology 48, 218–229.

Galstian, A.S., 1959. The activity of soil enzymes and the rate of soil respiration. Proceedings of the USSR Academy of Sciences 127, 1099–1102.

Galstian, A.S., 1974. Enzymatic activity of soils. Geoderma 12, 43–48.

German, D.P., Bittong, R.A., 2009. Digestive enzyme activities and gastrointestinal fermentation in wood-eating catfishes. Journal of Comparative Physiology B 179, 1025–1042.

Gianfreda, L., Ruggiero, P., 2006. Enzyme activities in soil. In: Nannipieri, P., Smalla, K. (Eds.), Nucleic Acids and Proteins in Soil. Soil Biology, vol. 8. Springer-Verlag, Berlin Heidelberg, pp. 257–311.

Grandy, A.S., Neff, J.C., Weintraub, M.N., 2007. Carbon structure and enzyme activities in alpine and forest ecosystems. Soil Biology and Biochemistry 39, 2701–2711.

Hassett, J.E., Zak, D.R., 2005. Aspen harvest intensity decreases microbial biomass, extracellular enzyme activity, and soil nitrogen cycling. Soil Science Society of America Journal 69, 227–235.

Jordan, D., Kremer, R.J., Bergfield, W.A., Kim, K.Y., Cacnio, V.N., 1995. Evaluation of microbial methods as potential indicators of soil quality in historical agricultural fields. Biology and Fertility of Soils 19, 297–302.

Kandeler, E., Eder, G., 1993. Effect of cattle slurry in grassland on microbial biomass and on activities of various enzymes. Biology and Fertility of Soils 16, 249–254.

Kang, H., Freeman, C., 2009. Soil enzyme analysis for leaf litter decomposition in global wetlands. Communications in Soil Science and Plant Analysis 40, 3323–3334.

Koch, O., Tscherko, D., Kandeler, E., 2007. Temperature sensitivity of microbial respiration, nitrogen mineralization, and potential soil enzyme activities in organic alpine soils. Global Biogeochemical Cycles 21, GB4017.

Landi, L., Renella, G., Moreno, J.L., Falchini, L., Nannipieri, P., 2000. Influence of cadmium on the metabolic quotient, L-:D-glutamic acid respiration ratio and enzyme activity: microbial biomass ratio under laboratory conditions. Biology and Fertility of Soils 32, 8–16.

Lee, Y.B., Lorenz, N., Dick, L.K., Dick, R.P., 2007. Cold storage and pretreatment incubation effects on soil microbial properties. Soil Science Society of America Journal 71, 1299–1305.

Marx, M.C., Kandeler, E., Wood, M., Wermbter, N., Jarvis, S.C., 2005. Exploring the enzymatic landscape: distribution and kinetics of hydrolytic enzymes in soil particle-size fractions. Soil Biology and Biochemistry 37, 35–48.

Marx, M.C., Wood, M., Jarvis, S.C., 2001. A microplate fluorimetric assay for the study of enzyme diversity in soils. Soil Biology and Biochemistry 33, 1633–1640.

Mason, H.S., 1948. The chemistry of melanin III. Mechanism of the oxidation of dihydroxyphenylalanine by tyrosinase. Journal of Biological Chemistry 172, 83–99.

Mead, J.A.R., Smith, J.N., Williams, R.T., 1955. Studies in detoxification. 67. Biosynthesis of the glucuronides of umbelliferone and 4-methylumbelliferone and their use in fluorimetric determination of b-glucuronidase. Biochemical Journal 61, 569–574.

Nannipieri, P., 2006. Role of stabilised enzymes in microbial ecology and enzyme extraction from soil with potential applications in soil proteomics. In: Nannipieri, P., Smalla, K. (Eds.), Nucleic Acids and Proteins in Soil. Soil Biology, vol. 8. Springer-Verlag, Berlin Heidelberg, pp. 75–94.

Nannipieri, P., Gianfreda, L., 1998. Kinetics of enzyme reactions in soil environments. In: Huang, P.M., Senesi, N., Buffle, J. (Eds.), Structure and Surface Reactions of Soil Particles. John Wiley & Sons, New York, pp. 449–479.

Nannipieri, P., Kandeler, E., Ruggiero, P., 2002. Enzyme activities and microbiological and biochemical processes in soil. In: Burns, R.G., Dick, R.P. (Eds.), Enzymes in the Environment. Marcel Dekker, New York, pp. 1–33.

Nannipieri, P., Sastre, I., Landi, L., Lobo, M.C., Pietramellara, G., 1996. Determination of extracellular neutral phosphomonoesterase activity in soil. Soil Biology and Biochemistry 28, 107–112.

Pind, A., Freeman, C., Lock, M.A., 1994. Enzymatic degradation of phenolic materials in peatlands - measurement of phenol oxidase activity. Plant and Soil 159, 227–231.

Quiquampoix, H., Servagent-Noinville, S., Baron, M.H., 2002. Enzyme adsorption on soil mineral surfaces and consequences for the catalytic activity. In: Burns, R.G.,

Dick, R.P. (Eds.), Enzymes in the Environment: Activity, Ecology and Applications. Marcel Dekker, New York, pp. 285–306.
Roberge, M.R., 1978. Methodology of soil enzyme measurement and extraction. In: Burns, R.G. (Ed.), Soil Enzymes. Academic Press, London, pp. 341–370.
Saiya-Cork, K.R., Sinsabaugh, R.L., Zak, D.R., 2002. The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. Soil Biology and Biochemistry 34, 1309–1315.
Serrasolses, I., Romanyà, J., Khanna, P., 2008. Effects of heating and autoclaving on sorption and desorption of phosphorus in some forest soils. Biology and Fertility of Soils 44, 1063–1072.
Shi, W., Dell, E., Bowman, D., Iyyemperumal, K., 2006. Soil enzyme activities and organic matter composition in a turfgrass chronosequence. Plant and Soil 288, 285–296.
Sinsabaugh, R.L., 2010. Phenol oxidase, peroxidase and organic matter dynamics of soil. Soil Biology and Biochemistry 42, 391–404.
Sinsabaugh, R.L., Antibus, R.K., Linkins, A.E., 1991. An enzymic approach to the analysis of microbial activity during plant litter decomposition. Agriculture, Ecosystems & Environment 34, 43–54.
Sinsabaugh, R.L., Findlay, S., 1995. Microbial production, enzyme activity, and carbon turnover in surface sediments of the Hudson River estuary. Microbial Ecology 30, 127–141.
Sinsabaugh, R.L., Hill, B.H., Follstad Shah, J.J., 2009. Ecoenzymatic stoichiometry of microbial organic nutrient acquisition in soil and sediment. Nature 462, 795–798.
Sinsabaugh, R.L., Lauber, C.L., Weintraub, M.N., Ahmed, B., Allison, S.D., Crenshaw, C., Contosta, A.R., Cusack, D., Frey, S., Gallo, M.E., Gartner, T.B., Hobbie, S.E., Holland, K., Keeler, B.L., Powers, J.S., Stursova, M., Takacs-Vesbach, C., Waldrop, M.P., Wallenstein, M.D., Zak, D.R., Zeglin, L.H., 2008. Stoichiometry of soil enzyme activity at global scale. Ecology Letters 11, 1252–1264.
Sinsabaugh, R.L., Linkins, A.E., 1988. Exoenzyme activity associated with lotic epilithon. Freshwater Biology 20, 249–261.
Sinsabaugh, R.S., 1994. Enzymic analysis of microbial pattern and process. Biology and Fertility of Soils 17, 69–74.
Skujins, J.J., 1978. History of abiontic soil enzyme research. In: Burns, R.G. (Ed.), Soil Enzymes. Academic Press, London, pp. 1–49.
Skujins, J.J., Braal, L., McLaren, A.D., 1962. Characterization of phosphatase in a terrestrial soil sterilized with an electron beam. Enzymologia 25, 125–133.
Stursova, M., Sinsabaugh, R.L., 2008. Stabilization of oxidative enzymes in desert soil may limit organic matter accumulation. Soil Biology and Biochemistry 40, 550–553.
Swift, M.J., Heal, O.W., Anderson, J.M., 1979. Decomposition in Terrestrial Ecosystems. University of California Press, Berkeley, CA, USA.
Tabatabai, M., 1982. Soil enzymes. In: Page, A.L., Miller, R.H., Keeney, D.R. (Eds.), Methods of Soil Analysis, Part 2. American Society of Agronomy, Madison, pp. 903–947.
Tabatabai, M., 1994. Soil enzymes. In: Weaver, R.W., Angle, S., Bottomley, P. (Eds.), Methods of Soil Analysis Part 2: Microbiological and Biochemical Properties. Soil Science Society of America, Madison, pp. 775–833.
Tabatabai, M., Dick, W., 2002. Enzymes in soil: research and developments in measuring activities. In: Burns, R.G., Dick, R.P. (Eds.), Enzymes in the Environment. Activity, Ecology and Applications. Marcel Dekker, New York, pp. 567–596.
Theuerl, S., Buscot, F., 2010. Laccases: toward disentangling their diversity and functions in relation to soil organic matter cycling. Biology and Fertility of Soils 46, 215–225.
Trasar-Cepeda, C., Gil-Sotres, F., Leirós, M.C., 2007. Thermodynamic parameters of enzymes in grassland soils from Galicia, NW Spain. Soil Biology and Biochemistry 39, 311–319.
Turner, B.L., 2010. Variation in pH optima of hydrolytic enzyme activities in tropical rain forest soils. Applied and Environmental Microbiology 76, 6485–6493.
Van Soest, P., 1994. Nutrional Ecology of the Ruminant. Cornell University Press, Ithica, NY. 476 p.
Vance, E.D., Brookes, P.C., Jenkinson, D.S., 1987. An extraction method for measuring soil microbial biomass C. Soil Biology and Biochemistry 19, 703–707.
Voets, J.P., Dedeken, M., 1964. A new method for measuring the activity of proteolytic soil enzymes. Die Naturwissenschaften 51, 267–268.
Waksman, S.A., 1918. Studies on the proteolytic enzymes of soil fungi and actinomycetes. Journal of Bacteriology 3, 509–530.
Wallenstein, M.D., McMahon, S.K., Schimel, J.P., 2009. Seasonal variation in enzyme activities and temperature sensitivities in Arctic tundra soils. Global Change Biology 15, 1631–1639.
Wallenstein, M.D., Weintraub, M.N., 2008. Emerging tools for measuring and modeling the in situ activity of soil extracellular enzymes. Soil Biology and Biochemistry 40, 2098–2106.
Yao, H., Bowman, D., Rufty, T., Shi, W., 2009. Interactions between N fertilization, grass clipping addition and pH in turf ecosystems: implications for soil enzyme activities and organic matter decomposition. Soil Biology and Biochemistry 41, 1425–1432.
Zantua, M.I., Bremner, J.M., 1975. Comparison of methods of assaying urease activity in soils. Soil Biology and Biochemistry 7, 291–295.