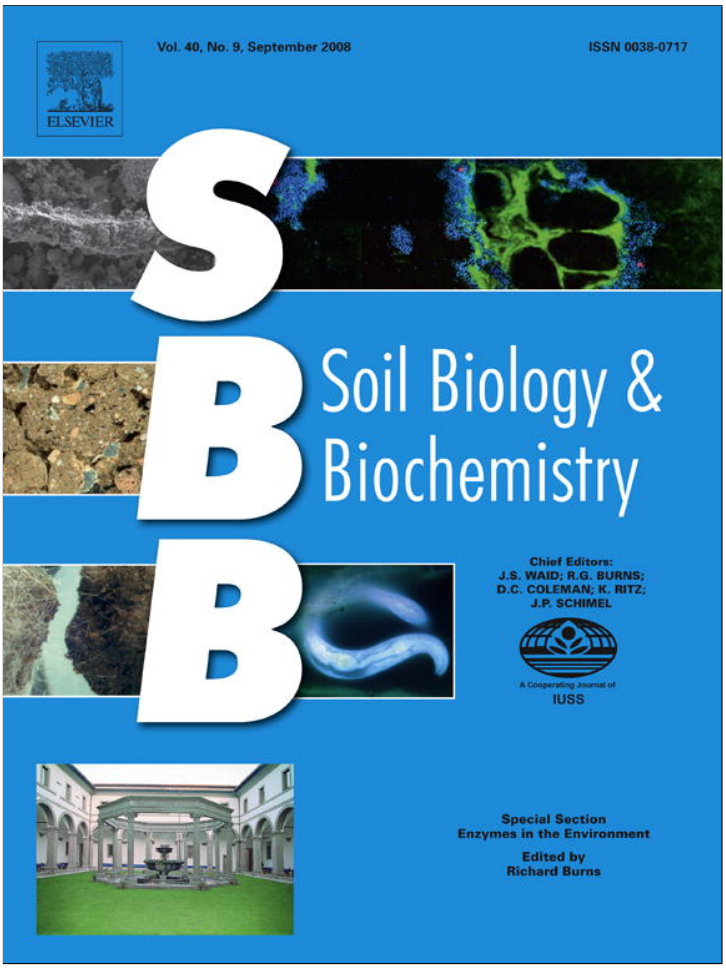

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Emerging tools for measuring and modeling the *in situ* activity of soil extracellular enzymes

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ABSTRACT

Current soil enzyme methods measure potential enzyme activities, which are indicative of overall enzyme concentrations. However, they do not provide insight in the actual rates of enzymatically catalyzed reactions under natural *in situ* conditions. The objectives of this review are to (1) clarify what is being measured by current standard soil enzymology methods; (2) present an overview of the factors that control *in situ* activities of soil enzymes; and (3) evaluate how emerging technologies and modeling approaches could enhance our understanding of *in situ* extracellular enzyme activity (EEA). Genomic studies targeting functional genes coding for extracellular enzymes can identify the genetic potential of microbial communities to produce enzymes. Microbial regulation of enzyme production can be assessed with transcriptomic studies of mRNA. Emerging proteomic tools could be used assess the pool sizes, diversity, and microbial source of soil enzymes. New mass-spectrometry approaches can be used to quantify the products of enzymatic degradation. The insights gathered from these approaches will foster improved models of decomposition that explicitly include enzymes and microbial species or functional groups. A comprehensive approach to measuring *in situ* activity and elucidating the regulation of enzyme production and stabilization is required to advance our understanding of the biochemistry of decomposition.

1. Introduction

Current soil enzyme methods (e.g. Saiya-Cork et al., 2002) measure potential enzyme activities, which are indicative of overall enzyme concentrations. However, they do not provide insight into the actual rates of enzymatically catalyzed reactions under natural *in situ* conditions. Actual *in situ* enzyme activities are controlled by complex interactions between substrate availability, enzyme concentration, soil physics, and thermodynamics. The concentration of active enzymes at any microsite within the soil matrix results from the balance of enzyme production, stabilization, and degradation rates. New approaches are required to improve our understanding of the drivers of *in situ* enzyme activities.

Extracellular enzymes are the primary means by which microbes degrade the insoluble macromolecules that comprise soil organic matter (SOM) and detritus into smaller, soluble molecules that can be assimilated (Fig. 1; Burns, 1982; Sinsabaugh, 1994; Burns and Dick, 2002). This depolymerization and solubilization is the initial, rate-limiting step of decomposition and nutrient mineralization, thus extracellular enzymes allow microbes to access the otherwise biologically unavailable carbon and nutrients in SOM. Some of the more abundant soil organic compounds that are degraded enzymatically include lignin, cellulose, starch, lipids, chitin, and proteins.

Extracellular enzymes may be associated with a cell's plasma membrane, periplasmic space, cell wall, or glycocalyx, or may be completely released into the cell's microenvironment (Sinsabaugh, 1994). Although extracellular enzymes that are released by cells into their environment may be stabilized, denatured, or degraded, some will survive in solution. Once exposed to the soil environment, extracellular enzymes complex with their target substrate molecules and either hydrolyze or oxidize the substrates into smaller molecules. These soluble, low molecular mass products can then be utilized as carbon and/or nutrient sources by the cell. A great diversity of enzymes exists in soil, and due to the diversity of compounds contained within SOM, the diversity of the soil community, and the diversity of the physical soil matrix, multiple soil

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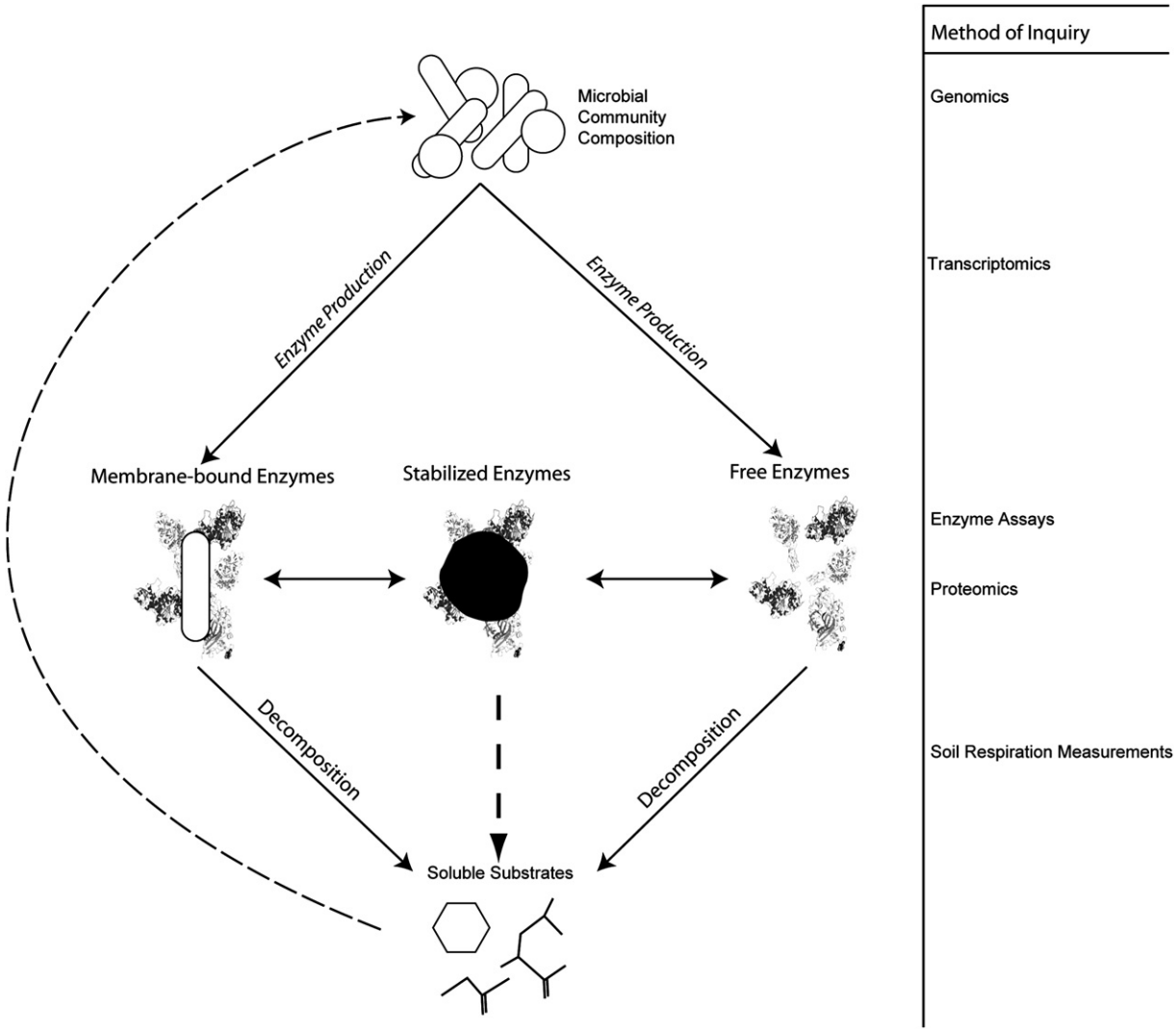

Fig. 1. Linkages between enzyme production by microbes, enzyme stabilization, enzyme activity, and decomposition. Microbial community composition determines the potential pool of enzymes, whose production (transcription and translation) is regulated by environmental conditions and substrate availability. The stabilization of enzymes and thus, potential activity, results from interactions of EE with soil particles. Enzyme activity is controlled by numerous factors including availability of substrates, temperature, and pH. The activity of EE results in the decomposition of organic matter, which releases soluble substrates that are metabolized by soil microbes, feeding back to microbial community composition. The text on the right side of the figure indicates the tools available to study each aspect of soil enzymology.

enzymes are required to efficiently degrade different chemical fractions of SOM.

While current methods continue to enhance our understanding of the roles of soil enzymes in ecosystem functioning, new emerging technologies in combination with modeling will result in a much more comprehensive understanding of controls on and consequences of *in situ* soil enzyme activity. The objectives of this review are to (1) clarify what is being measured by current standard soil enzymology methods, (2) present an overview of the factors that control *in situ* activities of soil enzymes, and (3) evaluate how emerging technologies and modeling approaches could enhance our understanding of *in situ* extracellular enzyme activity (EEA).

2. What are we *really* measuring with current enzyme techniques?

The most common method of measuring soil enzyme activity involves adding either a synthetic substrate linked to a fluorescent molecule (fluorophore) or a substrate that forms a colored compound (chromophore) to a dilute homogenized soil slurry and measuring the increase in fluorescence or absorbance over a fixed incubation time (Saiya-Cork et al., 2002). Soil slurries are used rather than filtered extracts because many of the active extracellular enzymes are bound to soil particles. In recent years, the throughput of these methods has increased through the use of 96-well microplates and microplate readers (Wirth and Wolf, 1992; Kremer, 1994; Marx et al., 2001; Saiya-Cork et al., 2002), and HPLC has been used to measure several enzyme activities simultaneously (Freeman and Nevison, 1999). While current soil extracellular enzyme methods have greatly enhanced our understanding of soil function, there are a number of limitations that provide opportunities for improvement. For example, current soil enzyme methodology is limited in several respects:

1. Only potential enzyme activities are measured under lab conditions with non-limiting amounts of substrates, which does not provide adequate information on *in situ* activities.
2. Only the sizes of enzyme pools (as potential activity) are measured, and there is no information provided on enzyme production or turnover rates.
3. Relatively simple, soluble substrates are used, which may not adequately represent the activities of enzymes that degrade

larger, complex insoluble polymers into soluble oligomers and monomers. Furthermore, only a limited number of fluorescently labeled complex substrates are commercially available.
4. Lab- based enzyme assays include the activity of stabilized (i.e. complexed with organic matter or clays) enzymes that may not be active under *in situ* conditions
5. No information is provided on which microbes produced the enzymes being detected.
6. Enzyme activity is typically measured at a single temperature, often not representative of typical soil conditions, and providing no information on reaction temperature sensitivities.
7. Measured enzyme activity rates can be affected by small variations in lab procedure, leading to measurement errors when all samples are not handled equivalently, and limiting comparison between data collected by different labs (Lee et al., 2007).
8. No positive controls are used to calibrate or validate activity rates.

The most important limitation of the standard soil enzyme lab assay is that it measures potential enzyme activity, which may not be a good indication of actual *in situ* activity. Potential enzyme activity is a measure of the maximum rate of an enzymatically catalyzed reaction at a particular temperature. In terms of Michaelis-Menten enzyme kinetics, potential enzyme activity is an empirical measurement of V_{max} (Michaelis and Menten, 1913). When potential enzyme activity is measured, substrate is added to ensure that the enzymes are not substrate limited, and the sample is homogenized with the substrate in a buffered slurry to make certain that diffusion does not limit the enzymes' access to substrate. Since the activity of the enzyme is limited neither by substrate concentration nor diffusion in the lab assay, the observed reaction rate is a function solely of enzyme concentration, and should be the maximum rate possible for that enzyme concentration at the given temperature and pH. In studies that focus on enzyme physiology, EEA is typically measured at the optimal pH and temperature of the enzyme to maximize reaction rates, even if the assay conditions differ markedly from *in situ* conditions. Studies that wish to make more realistic comparisons of EEA across systems, however, typically measure enzyme activity under conditions that mirror *in situ* pH and temperature. Measurements of potential EEA are likely to become more useful as our understanding of actual EEA under different conditions improves.

2.1. What factors control in situ extracellular enzyme activity?

2.1.1. Microbial production of extracellular enzymes

Because microbial production of extracellular enzymes is carbon, nitrogen, and energy intensive, microbes should only produce enzymes when nutrients and soluble C are scarce (Koch, 1985). When a nutrient is available in the soil solution, microbes reduce production of the enzymes that acquire that nutrient to avoid the costs of making them (Pelletier and Sygush, 1990; Chróst, 1991; Sinsabaugh and Moorhead, 1994). When particular nutrients are scarce, on the other hand, microbes can produce enzymes to liberate them from organic matter (Harder and Dijkhuizen, 1983). However, this strategy is only successful if the appropriate organic substrates are present. As a result, the production of some extracellular enzymes may be induced only in the presence of a suitable substrate (Allison and Vitousek, 2005).

In some cases, microbes may produce extracellular enzymes regardless of substrate availability as a sensing mechanism to detect substrate availability in the environment (Chróst, 1991; Koroljova-Skorobogatko et al., 1998; Klonowska et al., 2002). When substrate is available, these constitutive enzymes generate low concentrations of reaction products that induce additional enzyme synthesis. Once concentrations of products are sufficient to meet demand, enzyme production becomes suppressed and returns to constitutive levels (Chróst, 1991).

Because decomposition is largely extracellular, the products of organic matter breakdown may potentially be intercepted by "cheater" microbes, who may not be investing resources in enzyme production. This "cheating" can reduce the efficiency of microbial extracellular enzymes decomposition (Worm et al., 2000; Allison, 2005). However, some microbes may have evolved mechanisms to reduce cheating, such the use of antibiotics against "cheaters", and cell-bound enzymes which limit diffusion losses. The coordination of enzyme production by quorum sensing and the spatial aggregation of enzyme producers may also mitigate or reduce competitive interference from cheater microbes (Ekschmitt et al., 2005).

Our current understanding of microbial regulation of enzyme production comes from either pure culture studies, or from patterns of enzyme potential across C and nutrient gradients or in response to experimental additions (Carreiro et al., 2000; Saiya-Cork et al., 2002; Michel and Matzner, 2003; DeForest et al., 2004; Gallo et al., 2004; Allison and Vitousek, 2005). In the case of pure culture studies, microbial physiology in artificial media may not reflect *in situ* behavior, and the few cultures studied may not represent the diversity of microbes present in soils. On the other hand, observations of changes in enzyme potentials in response to C or nutrient additions are suggestive of increased enzyme production, but are confounded with changes in enzyme stabilization, degradation, and changes in microbial biomass or community structure (Waldrop et al., 2000). Our understanding of microbial regulation of enzyme production will be advanced by studies using genomic, transcriptomic, and proteomic tools, as discussed below.

2.1.2. Stabilization and turnover of extracellular enzymes

After extracellular enzymes are released by microbes, many enzymes become stabilized through interactions with clay minerals and SOM (Fig. 1; Burns, 1982; Nannipieri et al., 1996, 2002), or tannins (Joanisse et al., 2007). In fact, most of the potential enzyme activity in soils is adsorbed to particles rather than being free in solution (Kandeler, 1990). Stabilized enzymes often have lower or even no *in situ* activity, as complexation can make substrates unavailable, occlude enzyme active sites, and cause conformation changes in enzymes (Allison and Jastrow, 2006; Nannipieri, 2006). Enzymes stabilized with organomineral complexes may still retain some activity (Tate, 2002). However, this may vary between specific enzymes. For example, Gianfreda et al. (1994) found that when urease complexes with tannic acid are formed in the presence of ferric ions and aluminum hydroxide species, the enzyme retains its conformation and function (Tate, 2002). Pflug (1982) found that clay-bound cellulase enzymes maintained activity, whereas starch degrading amylase and amyloglucosidase activities were inhibited by clays. Enzyme stabilization may also protect against proteolysis and other denaturing agents (Skujins, 1976; Nannipieri et al., 1978; Nannipieri et al., 1988). It is possible that enzymatic function may be restored if enzymes are later detached from organomineral complexes. However, irreversible deactivation is common with enzymes adsorbed onto surfaces as a result of changes in protein conformation (Quiquampoix et al., 2002). If the enzyme is unfolded and the number of points of contact with the surface increase, more energy will be required to reverse the unfolding of the adsorbed enzyme, and may exceed the thermal energy available (Quiquampoix et al., 2002). Thus the turnover time of extracellular enzymes complexed with humic molecules or adsorbed by clay minerals is longer than that of free extracellular enzymes. The active proportion of stabilized soil enzymes represents a reservoir of potential enzyme activity that may be important under some conditions.

Determining the size of the stabilized enzyme pool is difficult without disturbing soils in lab assays. The standard lab assay releases many of these stabilized enzymes through physical disturbance of the soil (sieving and/or homogenizing), and by releasing electrostatically bound enzymes with the addition of buffer. Therefore, the activity of stabilized enzymes is likely over-represented in the standard lab assay relative to their *in situ* activity. Advancing our understanding of enzyme stabilization probably does not require technological advances so much as modifications of standard enzyme assays to distinguish stabilized from free enzymes (Gianfreda et al., 1994; Caldwell, 2005), and experiments that examine the effects of abiotic interactions on enzyme activities (Allison, 2006; Joanisse et al., 2007).

2.1.3. The underappreciated role of temperature in controlling in situ enzyme activities

Temperature is a strong control on enzyme activities, yet soil enzymes are typically only measured at a single temperature in lab assays, and too often that temperature is far from the median temperature of the field site. It is widely assumed that enzyme activity roughly doubles with a 10 °C increase in temperature ($Q_{10} = 2$), however the accumulated evidence of numerous studies suggests a wide range in temperature sensitivities for different enzymes, and measured Q_{10}s are often <2 (McClaugherty and Linkins, 1990; Frankenberger and Tabatabai, 1991a,b, Lai and Tabatabai, 1992; Wirth and Wolf, 1992; Criquet et al., 1999; Parham and Deng, 2000; Elsgaard and Vinther, 2004; Trasar-Cepeda et al., 2007).

Under lab assay conditions, with non-limiting amounts of substrate, temperature sensitivity is primarily a function of enzyme activation energy, and can be modeled with Arrhenius kinetics (Davidson and Janssens, 2006). However, under field conditions, where substrate availability is limited and fluctuates in space and time, temperature sensitivity is affected by Michaelis–Menten kinetics (Michaelis and Menten, 1913), where substrate availability affects the apparent temperature sensitivity (Davidson and Janssens, 2006). Therefore, lab assay measurements of enzyme temperature sensitivity should be interpreted with caution.

Several studies have demonstrated that the temperature sensitivity of extracellular enzymes changes seasonally (Fenner et al., 2005; Koch et al., 2007; Trasar-Cepeda et al., 2007; Wallenstein and Schimel, unpublished data). The most likely explanation is that the measured enzyme pool consists of different isoenzymes (enzymes with the same function, but different structure) through time, which may be produced by different organisms, or by a single species capable of producing multiple isoenzymes (Loveland et al., 1994). Consistent with this hypothesis, Di Nardo et al., 2004 found temporal changes in laccase and peroxidase isoenzymes during leaf litter decomposition. There is also some evidence for biogeographical patterns in enzyme temperature sensitivity. For example, many studies have observed that enzymes from microbes inhabiting cold environments have unusually low temperature optima (Huston et al., 2000; Coker et al., 2003; Feller, 2003). Temperature optima of extracellular enzymes are generally much higher than native soil temperatures, and therefore their ecological significance is debatable. Nonetheless, these observations suggest that microbes producing enzymes that maintain optimal activity under native soil conditions are favored. Thus, soil microbial community composition is likely controlled to some extent through feedbacks with enzyme efficacy.

Because different enzymes have different temperature sensitivities, changes in soil temperature may alter the relative rates of decomposition of different components of soil organic matter. Therefore, seasonal changes in temperature can alter the balance of SOM components contributing to soil respiration *without any changes in soil enzyme pools* (or measured enzyme potentials). Koch et al. (2007) found that at low temperatures, the relative temperature sensitivity of C-degrading enzymes was greater than aminopeptidases (which degrade N-rich proteins), suggesting that relative N availability could be decreased directly by temperature. Natural or human-driven changes in climate could also alter the relative rate of decomposition of SOM components, and ultimately, the quantity and composition of SOM.

There is insufficient data to elucidate general patterns in enzyme temperature sensitivity. Is the temperature sensitivity of specific soil enzymes consistent across ecosystems? How do seasonal changes in enzyme temperature sensitivity affect SOM decomposition? Studies of isoenzymes using proteomic tools will help elucidate the drivers of seasonal changes and inter-site differences in enzyme temperature sensitivity. Since current enzyme methods can be used to examine enzyme temperature sensitivities, our understanding of this important driver of *in situ* activities could be easily improved.

2.2. Emerging tools for studying soil EEA

2.2.1. Genomic studies of functional genes coding for extracellular enzymes

Genomic studies that target functional genes coding for extracellular enzymes can elucidate both the genetic potential for producing enzymes in soil microbial communities and the factors that regulate the transcription of those genes. By improving our understanding of enzyme production, we will be better able to model and predict enzyme pools under different conditions, and thus *in situ* enzyme activities. The primary limitation to studying functional genes coding for extracellular enzymes is that only a relatively small number of functional genes have been sequenced. This makes it difficult to design PCR primers that target functional genes for the large diversity of soil microbes that possess them. There have been have some pioneering investigations of genes coding for extracellular enzymes involved in soil organic matter decomposition, and these studies show great promise for linking genomic information to enzyme activities. The development of PCR primers for functional genes has led to several studies examining the composition of organisms that produce chitinases (Williamson et al., 2000; Metcalfe et al., 2002; LeCleir et al., 2004) or laccases (Luis et al., 2004; Luis et al., 2005b; Blackwood et al., 2007). Functional genes could also be quantified using quantitative PCR (qPCR), which may indicate the genetic potential for producing extracellular enzymes. As genomic technology continues to develop, massive amounts of data will be supplied by microarray (Yergeau et al., 2007) and high-throughput sequencing (Edwards et al., 2006; Roesch et al., 2007) efforts, greatly expanding the catalog of environmental functional genes in need of ecological interpretation.

While studies targeting functional genes in soil DNA provide information on the genetic potential for producing extracellular enzymes, they do not provide insights into gene regulation and transcription, which can be evaluated by studying mRNA transcripts using reverse transcriptase– PCR (RT–PCR) in soils (Luis et al., 2005a). Concentrations of mRNA transcripts may correlate more closely to enzyme production rates than functional gene concentrations in DNA.

While PCR-based studies require the design of primers to target known functional gene sequences, other techniques have been developed to examine the entire genome without *a priori* knowledge of a target gene. Metagenomic (or community genome) studies have already led to discoveries of new families of many proteins, including hydrolytic enzymes (Ferrer et al., 2005). Techniques are also being developed to screen metagenomes for specific enzymatic functions (Kuznetsova et al., 2005; Uchiyama et al., 2005; Yun and Ryu, 2005). While most metagenomic studies have

targeted DNA, some have examined the RNA of microbial communities (the meta-transcriptome) (Aneja et al., 2004; Sharma et al., 2006). Recently, RNA transcripts extracted from environmental samples were analyzed with a microarray containing probes for thousands of functional gene sequences (Gao et al., 2007). Studying the presence, abundance, and diversity of genes coding for soil enzymes or their transcripts will enable modeling of enzyme production rates under a range of conditions.

2.2.2. Metaproteomic tools for investigating enzyme diversity

Knowledge of alterations in gene expression is valuable, but cannot necessarily be interpreted to mean that an equivalent increase or decrease in enzyme production rates has occurred. Several studies (e.g. Lee et al., 2003) have demonstrated a poor correlation between the concentrations of a specific mRNA and the corresponding protein, caused by factors such as transcriptional control and protein stability. In addition, post-translational modifications, which cannot be detected through mRNA analysis, can be important determinants of function. Proteomic profiling can provide this missing information. Recent publications (Ram et al., 2005; Benndorf et al., 2007; Lacerda et al., 2007) have demonstrated the potential of the meta-proteomic approach. While there are several challenges in microbial community proteomics, including representative protein extraction, separation of proteins in complex samples, and the scarcity of genomic sequences for the microorganisms in these environmental communities, researchers have successfully used meta-proteomics to obtain insights into microbial community function that were otherwise unavailable. For example, Lacerda et al. (2007) used two-dimensional electrophoresis and MALDI-TOF/TOF mass spectrometry to follow the dynamics of nearly 200 identified proteins from a microbial community exposed to a cadmium shock. Ram et al. (2005) used shotgun proteomics to identify more than 2000 proteins from an acid mine drainage biofilm community. These efforts have been aided by the increasing availability of shotgun genomic sequencing of environmental samples and protein bioinformatics software that can find homology to proteins from the more than 950 bacterial genomes that have been sequenced to date.

Proteomics has great potential to elucidate the diversity and source of extracellular enzymes in soils, and can be used as discovery tool to characterize novel extracellular enzymes. For example, extracellular proteins can be separated on a native (non-denaturing) gel, and specific enzyme activities can be detected using fluorometric substrates (Kim et al., 2007). Individual isoenzymes that show activity could be excised from the gel and characterized using MALDI-TOF/TOF mass spectrometry and *de novo* sequencing (Kim et al., 2007). The protein can then be compared to public databases of known protein sequences to potentially identify the microbe that produced the enzyme. Because *de novo* sequencing provides amino acid sequences for peptides, it should be possible to develop degenerate primers to target previously unknown functional genes using PCR techniques. In this way, proteomic studies will help drive advances in genomics and transcriptomics.

2.2.3. Direct measurement of in situ enzyme activity

Recently, Dong et al. (2007) developed a technique for quantifying EEA *in situ* by placing a membrane saturated with a chromophore or a fluorophore, against a vertical soil surface. The surface was exposed by removing a pre-installed root window. By imaging the membranes, they were able to quantify enzyme activities, and to identify "hot-spots" of enzyme activity associated with roots or fungal mats. This method is particularly useful in identifying spatial patterns in soil enzyme activity, and could be linked to spatially explicit investigations of microbial community structure. However, it suffers some of the limitations of standard methodology, in that artificial substrates are supplied in non-limiting amounts, and soil moisture conditions are altered by the root windows. Thus, this method is more accurately interpreted as measuring *in situ* enzyme potential.

Direct measurement of *in situ* enzyme activity would be the ultimate tool to fully understand the complex interactions between microbes, enzymes, and SOM. There has been some progress in imaging enzymes in soils using scanning electron microscopy (Foster and Martin, 1981; Foster, 1985) and on fungal hyphae using confocal laser scanning microscopy (Alvarez et al., 2006), however, electron-dense soil components such as minerals and humic substances interfere with detection of enzymes. A potential advancement that could overcome many of these limitations involves the use of Quantum Dots (Qdots), which are nanoscale crystals that emit in the near-infared wavelengths and are more photostable than current fluorophores (Michalet et al., 2005). Qdots are already widely used in biomedical research to detect protease activity using Qdots that are quenched till they bind to their target enzyme (Blum et al., 2005, 2007), and this technology could be adapted to soils. In fact, any soil enzyme could be directly detected by covalently linking Qdots to antibodies. Qdots can be engineered to emit many different wavelengths, thus numerous enzymes could be detected simultaneously. Alternatively, enzyme production and turnover could be measured by sequential labeling of enzymes with different Qdots at specific time intervals. Like any other imaging technique, there are unique challenges in applying this approach to soils, including interferences from background fluorescence, non-specific binding of Qdots, and spatial heterogeneity.

2.2.4. Direct detection of the products of enzymatic reactions

Another potential approach to detect *in situ* enzyme activity is to directly quantify the products of enzymatic degradation. New mass-spectrometry technologies (e.g. LC-Q-TOF, Pyrolysis GC-MS, Orbitrap) allow the simultaneous detection of thousands of small molecules, which has been termed "metabolomics" (Dettmer et al., 2007). Prokushkin et al. (2007) used pyrolysis-GC-IRMS to characterize DOM in terrestrial sources and identify the source of dissolved organic carbon (DOC) in rivers. Gruber et al. (2006) used LC-MS to examine the effects of a predator on bacterial degradation of DOC. While these studies were not focused on enzymatic processes *per se*, they were characterizing the dissolved products of enzymatic degradation at the molecular level. The main challenge in extrapolating from concentrations of enzymatic products in DOC to enzyme activity rates is that enzymatic products are quickly incorporated into plant and microbial biomass. For example, Weintraub and Schimel (2005) found that tundra soil amino acid concentrations were at their lowest when protease activity was at its highest, indicating extremely rapid uptake of the protein breakdown products. They also observed that soluble protein concentrations increased following increases in protease activity. This suggests that while enzyme activities may be difficult to estimate from concentrations of the monomers ultimately released from the enzymatic degradation of biological polymers, soluble reaction intermediates may have a longer turnover time, and therefore may be more useful in estimating *in situ* activities. It may be possible to measure concentrations of some enzymatic reaction intermediates (e.g. soluble proteins, cellobiose) using new metabolomic approaches. Furthermore, enzyme production and turnover could be estimated by using isotopic labeling in combination with metabolomic approaches.

2.2.5. Incorporating enzymes into terrestrial biogeochemical models

It is well understood that decomposition is largely driven by extracellular enzymes that are produced by microorganisms.

However, traditional SOM decomposition models (e.g. CENTURY; Parton et al, 1987) have included neither microbes nor their enzymes as explicit drivers of decomposition. Rather these are incorporated implicitly into a fundamental decay constant, such that $dC/dt = KC$ where C is the carbon pool size, K is a first order rate constant (Parton et al, 1987; Schimel and Weintraub, 2003). Different K values are assigned to different pools of SOM; the lower the OM quality, the lower the K value. Such models assume that C is the limiting resource for decomposition and that it can therefore be modeled using simple first order decay of particular OM pools. Modeled microbial biomass grows until maintenance demand meets C supply, inducing C limitation (Schimel and Weintraub, 2003). These models work reasonably well at large spatial scales under relatively steady-state conditions with no rapid changes in climate, or C or N availability. However, there are major issues that such models are very poor at addressing. These include rapidly fluctuating or changing conditions and those where C is not the resource most limiting to microorganisms. Such conditions are relatively common in nature.

Enzyme dynamics can be added to a decomposition model by having microbes allocate C and N to producing enzymes and having the actual decomposition rates become a function of both C supply to microbes and enzyme concentration. For example, Schimel and Weintraub (2003) formulated a simple C only decomposition model that includes enzymes, such that $dC/dt = KCE$, where C is the carbon pool size, K is a first order rate constant, and E is size of the exoenzyme pool. While the decomposition rate of the C pool in this model is still ultimately controlled by OM quality (the K value), the extent of microbial C limitation is controlled by the total C return on microbes' C investment in enzymes (see Schimel and Weintraub (2003) for detailed descriptions of the equations they use to govern the production and loss of enzymes, microbial biomass C, and C fate). When enzymes are added to decomposition models in this manner, several phenomena emerge that qualitatively change the behavior of the models and the conclusions that can be drawn from them.

1. The microbial investment in enzymes can comprise a substantial amount of C or N that is diverted from microbial growth. That can alter the fate of C and N in the soil system.
2. A mechanism is required to produce a non-linear response of decomposition rates to enzyme concentrations. Schimel and Weintraub (2003) hypothesize a likely mechanism is that as microorganisms produce more enzymes, they must diffuse farther out from the cells, reaction products must diffuse farther back, and enzymes may compete with one another for binding sites. These dynamics result in non-linearities in enzyme kinetic responses that can produce a C-limited microbial biomass even in the presence of surplus C.
3. Under N-limited conditions, adding N can actually reduce respiration, as C is diverted from "overflow metabolism" to microbial growth.
4. The dynamics of pulse events (e.g. rewetting a dry soil) can be much better captured than traditional first- order decomposition models can achieve.

Building on previous efforts to refine decomposition models by including more ecological interactions between microorganisms and substrates, Moorhead and Sinsabaugh (2006) developed a decomposition model that resolves the decomposer community into three functional guilds, defined by different metabolic characteristics and enzyme activities, that are differentially associated with the decomposition of three classes of litter constituents: labile compounds, holocellulose and lignin. By combining general observations about the physiology of key groups of decomposers and the biochemistry of different litter constituents, this model explores the ramifications of microbial community behavior on decomposition.

Decomposition models often estimate the maximum rate of substrate decay (V_{max}) based on either substrate availability ($k \cdot C$) or microbial activity ($r \cdot B$), but in reality, decomposition is limited by both factors. Moorhead and Sinsabaugh (2006) calculated the realized rate of decomposition based on microbial activity in the substrate-limited case: $dC/dt = (k \cdot C) \cdot B/(K_b + B)$; and based on substrate availability in the microbial activity-limited case: $dC/dt = (r \cdot B) \cdot C/(K_c + C)$; where r and k are rate coefficients, K_c and K_b are half-saturation coefficients, B is microbial biomass and C is substrate. These two equations are equivalent when decay is optimal, suggesting that decay is limited by substrate availability if $C < r \cdot B/k$ or by microbial activity if $B < r/(k \cdot C)$. This conceptual approach can be applied to specific chemical constituents of litter, which have different intrinsic decay rate coefficients, and guilds of decomposer microorganisms, which have different metabolic and enzymic characteristics.

A critical feature of litter decay is the interaction between cellulose (C_2) and lignin (C_3), usually expressed as a function of lignocellulose index ($LCI = C_3/[C_2 + C_3]$) with no relationship to microbial activities (Meentemeyer, 1978; Melillo et al., 1982, 1989; Taylor et al., 1989; Aber et al., 1990). In contrast, Moorhead and Sinsabaugh (2006) calculate the rate of cellulose decay with Michaelis–Menten reaction kinetics: $dC_2/dt = V_{max} \cdot C_2/(K_2 + C_2)$ when $LCI = 0$; $dC_2/dt = 3/7 \cdot dC_3/dt$ when $LCI = 0.7$ (Melillo et al. (1982, 1989) found that LCI values generally increase during decomposition to a constant value of 0.7, regardless of initial chemistry), and using an exponential function of LCI (not shown) to scale K_2 when $0 < LCI \leq 0.7$, where V_{max} and K_2 are the maximum rate and half-saturation coefficient, respectively.

Microbial control of decomposition emerges from combinations of values for V_{max} and K assigned to different guilds for the degradation of different litter constituents. General knowledge of physiology, growth, and enzymology of different groups of microorganisms associated with different stages of litter decay roughly define different characteristics of the guilds. Guild 1: opportunists rapidly colonize newly available litter, consuming intermediate metabolites and soluble polymers. Guild 2: decomposers degrade the cellulose and lignocellulose that comprises most of the mass of plant litter by expressing several classes of hydrolytic and oxidative enzymes. They grow slower than opportunists but have access to a broader range of substrates. Guild 3: miners degrade recalcitrant organic matter with powerful oxidative enzymes that break the covalent bonds of aromatic rings and hydrocarbon chains, and thus gain access to protected glycosides, peptides and lipids. V_{max} is a function of growth rate and thus is greatest for opportunists and lowest for miners. The half-saturation coefficients for substrate degradation are specific to both substrate and guild, i.e., all guilds have high enzyme affinity (=low K values) for labile substrates, only decomposers and miners have relatively high affinity for cellulose, and only miners have relatively high affinity for lignin. Competition between guilds results from the relative activity of each guild, scaled to the degradative potential of each guild for each substrate.

The most significant insight from the Moorhead and Sinsabaugh Guild Decomposition Model (GDM) is the interaction between microbial behavior, litter chemistry, and nutrient availability (N) at the threshold between litter decay and stabilization. The GDM describes how the availability of labile C and N can inhibit lignin degradation by influencing the expression of extracellular enzymes responsible for microbial C acquisition (Setala and McLean, 2004), elucidating the mechanisms controlling C sequestration during decomposition.

Current and emerging techniques in proteomics and genomics can improve decomposition models such as the GDM by helping us to define microbial "guilds" or functional groups. However,

while the GDM has more explicit ecological interactions between microorganisms and substrates than previous decomposition models, it is not possible to predict *in situ* enzyme activity using this modeling approach. In the GDM, microbial control of decomposition emerges from combinations of values for V_{max} and K assigned to different guilds and litter constituents; it does not explicitly allocate C and N to a pool of enzymes. In order to model *in situ* enzyme activity, it is necessary for the model to allocate resources to a discrete extracellular enzyme pool, separate from microbial biomass, so that it is possible to relate enzyme activity to enzyme pool size (D. Moorhead, personal communication). The essential parameters required are enzyme production, stabilization, and turnover rates, as well as estimates of substrate availability. While empirical data on enzyme production, stabilization, and loss are not readily available, model estimates of *in situ* activity may be constrained by empirical determinations of substrate disappearance (e.g. using litter bags to quantify cellulose breakdown rates in leaf litter), metabolite production rates, microbial biomass, respiration rates, enzyme pool size and potential activity (V_{max}).

The model proposed by Schimel and Weintraub (2003) is a simple example of the type of model structure that could be used to estimate *in situ* enzyme activity, since it explicitly allocates resources to a pool of enzymes, and models the production and loss of enzymes, as well as microbial biomass, and the fate of substrate C. By modeling enzymes as a discrete resource pool, and constraining the model with available data, it is possible to estimate *in situ* extracellular enzyme activity. The accuracy of such estimates is limited, however, by the availability of data on enzyme production, stabilization, and loss, as well as reaction product formation and substrate availability.

Emerging technologies in genomics and proteomics have the potential to make a significant contribution toward the measurement of enzyme production, stabilization, and loss, while measurements of enzyme substrate availability and reaction product release are likely to improve with advances in metabolomics. Furthermore, isotopic labeling of enzyme substrates and reaction products, as well as enzymes themselves, in combination with these new approaches, holds great promise in elucidating the controls on *in situ* enzyme activity.

3. Conclusions

While current methods continue to enhance our understanding of soil enzymes and their role in ecosystem functioning, a comprehensive approach to measuring or modeling *in situ* activity is required to advance our understanding of the biochemistry of decomposition. At this point our knowledge of the role of microbial extracellular enzymes as the agents of decomposition is limited by our inability to (1) link potential EEA measured in the lab with *in situ* rates, (2) measure microbial enzyme production and turnover rates, and (3) determine the proportion of stabilized enzymes and their activity. In addition, more information on enzyme temperature sensitivities will be required even to estimate *potential in situ* enzyme activities.

In Fig. 1, we presented a conceptual model of the many factors that affect *in situ* enzyme activity, and emerging tools to evaluate them. While new imaging approaches will improve our ability to detect the presence and location of enzymes *in situ*, it will remain a challenge to detect enzyme-substrate interactions without the addition of artificial substrates. Thus, we believe that progress in understanding enzyme production, stabilization, turnover and activity can contribute to integrated models of soil enzymology. Specifically, genomic tools can be used to detect the genetic potential for producing extracellular enzymes, and studies of mRNA transcripts under experimental or natural conditions will advance our understanding of the regulation of enzyme production at the molecular level. Proteomic tools can be used to determine which microbes are producing extracellular enzymes, and to detect extracellular enzymes independently of substrate-based analyses. Metabolomic tools can be used to quantify the products of enzymatic degradation and potentially evaluate their production and uptake. The information gained from each of these techniques, in combination with potential enzyme activities measured with current techniques, will be required for the development of new, more microbially explicit biogeochemical models that will be better able to predict the impacts of changes in nutrient availability, precipitation, and temperature on enzymatically mediated soil processes.

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