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IMPROVED MICROPLATE FLUOROMETRIC SOIL ENZYME ASSAY FOR β-GLUCOSIDASE DETECTION

Emily C. Hoehn
University of Nebraska-Lincoln, emily.hoehn@huskers.unl.edu

IMPROVED MICROPLATE FLUOROMETRIC SOIL ENZYME ASSAY FOR β-GLUCOSIDASE DETECTION

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Emily C. Hoehn

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Emily C. Hoehn, M.S.

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Adviser: Daniel D Snow

Soil microbes produce extracellular enzymes responsible for degrading complex organic compounds to release energy and nutrients. Measurement of soil enzymes can be considered an indicator of soil health and microbial community composition because of its sensitivity to agricultural and management practices. Fluorescence enzyme assays tend to be more sensitive than spectrophotometric (ie. colorimetric) assays and a 96-well plate has the potential capacity for high-throughput use. Development of a newly modified enzyme assay using fluorometric (4-methylumbelliferone), automated pipetting system and sonication, as well as a reduction in replicates allows for a higher sample throughput rate suitable for service laboratory use. The newly modified enzyme assay was applied to a long-term cropping system and varying fertilizer nitrogen management to determine the use of air-dried versus fresh soil enzyme activity. No significant difference was found in air-dried and fresh soils, but fertilizer N differences were only apparent under air-dried conditions. The positive linear relationship between β-glucosidase and grain yield support the use of enzymes as a soil quality indicator.

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Soil Enzymes: A brief introduction to common soil enzymes and analysis methods

ABSTRACT

The critical role of soil enzymes as catalysts in soil nutrient cycling and soil organic matter decomposition are consider expressions of soil microbial community diversity, metabolic requirements and nutrients available in the soil environment. Soil enzymes are sensitive to a variety of environmental factors such as temperature, pH, nutrients and water content. Understanding the presence and activity of soil enzymes in soil nutrient cycles may have important implications the microbial community structure and reactions to ecosystem disturbances. This review provides a brief introduction to seven soil enzymes involved in important nutrient cycle in the soil: β-glucosidase, β-glucosaminidase, arylsulfatase, amidase, urease, alkaline phosphatase and acid phosphatase. In addition, an overview of two common assay enzyme analysis, spectrophotometric and fluorometric, is discussed with an emphasis on what the measurements mean and the supportive role of soil enzyme activity in research. The importance of enzyme research as an indicator of soil health is pivot for future understanding of microbial community composition and diversity in disturbed and natural ecosystems to maintain and support a sustainable, healthy soil ecosystem.

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INTRODUCTION

The diversity and abundance of soil microorganisms play a critical role in global nutrient cycling. Measuring small changes in organic matter is difficult due to high soil heterogeneity (Carter, 2002). As a result, there is increasing interest in soil health indicators that are more sensitive to small changes in nutrients may provide a gauge for soil quality and microbial communities responses (Arias et al., 2005). Healthy soils have a diverse community of microbes able to provide some natural control of plant disease and pests (Singh et al., 2004; Mendes et al., 2011), foster symbiotic relationships with plants (Marschner et al., 2001; Berg and Smalla, 2009; Li et al., 2014), promote nutrient cycles, and improve soil structure (Arias et al., 2005). Microbial decomposition of large molecules in soil organic matter is performed by extracellular enzymes responsible for catalyzing the breakdown of macromolecules into simple monomers for easier microbial consumption (Wallenstein and Weintraub, 2008). The abundance of enzymes and correlation to microbial communities (Tabatabai, 1994b) make enzymes an ideal indicator of soil health.

Soil enzymes are sensitive to changes in soil organic matter, soil physical properties, and microbial activity (Dick et al., 1996; Alves de Castro Lopes et al., 2013) and thus act as an early indicator of soil quality (Pankhurst et al., 1995; Sinsabaugh et al., 2008; Deng et al., 2013). Disruption of soil microbial activities seen through changing levels of metabolic enzyme activity can help estimate ecosystem disturbance caused by human disturbance and cultivation (Pankhurst et al., 1995; Deng et al., 2013) and are more sensitive than the chemical and physical properties of the soil (Alves de Castro Lopes et al., 2013). Enzyme activities selected for soil health indicators should be easily

quantified and have the ability to reflect differences in ecosystems and anthropogenic activity (Tate III, 2002).

Enzymes can exist within in the microbial cell (intracellular) or in the environment (extracellular). Intracellular enzymes are created by heterotrophic soil microbes (Knight and Dick, 2004) which can later be transported out of the cell and become extracellular. Extracellular enzymes can be attached to the exterior cell wall in the periplasmic space or be completely released into the environment (Sinsabaugh, 1994; Wallenstein and Weintraub, 2008). Extracellular enzymes can be quickly degraded, denature (Yan et al., 2010) or immobilized on clay minerals, humic acids and particulate organic matter (Burns et al., 2013).

Due to carbon(C):nitrogen(N):phosphorus(P) ratio constraints on microbial biomass (Cleveland and Liptzin, 2007), specific enzymes are generated depending upon the environmentally limiting nutrient needed to meet microbial demands (Sinsabaugh et al., 2008; Allison et al., 2011). Understanding enzyme characteristics (ie origin, mode of action, reaction substrates, and products) can link microbial community function, organic matter composition and environmental conditions that are involved in organic matter breakdown. The objective of the first section of this review is to describe seven common soil enzymes, their role within the soil system, and optimal laboratory conditions required to measure the enzyme activity. Soil enzymes discussed are involved in the C cycle (β-glucosidase, β-glucosaminidase), the sulfur (S) cycle (arylsulfatase), the N cycle (amidase, urease) and the P cycle (alkaline phosphatase, acid phosphatase). In the second section, a short review on methods used to measure soil enzyme activity is discussed with an emphasis on fluorometric and spectrophotometric assay and their interferences.

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β-Glucosidase

The β–glucosidase enzyme (β-G, EC 3.2.1.21) is a constitutive and well described enzyme (Chrøbst, 1989; Marx et al., 2001; Caldwell, 2005) pivotal in the degradation of cellulose, an important process in the cycling of soil organic matter (Turner et al., 2002). Changes in β-G activity can be easily detected in managed ecosystems within a relatively short period (e.g. 1 to 3 years) (Bandick and Dick, 1999; Knight and Dick, 2004) and is considered stable with low seasonal variability (Knight and Dick, 2004; Moscatelli et al., 2012a). The large scale role in C cycling and sensitivity to soil management makes β-G an ideal bioindicator of soil health (Ndiaye et al., 2000; Moscatelli et al., 2012b).

β-glucosidase catalyzes the hydrolysis of cellobiose, the smallest repetitive unit of cellulose, and in some cases cellooligosaccharides, releasing glucose (See Figure 1) for consumption by microorganisms (Deobald, 1997). β-glucosidase abundance is a rate-limiting factor in cellulose degradation (Saha et al., 1994). Endoglucanases and cellobiohydrolases, other cellulose degrading enzymes, are responsible for cleaving cellulose into random low crystalline cellulosic fibers and removing cellulobiose from the non-reducing end of the cellulose molecule, respectively (Saha et al., 1994). These enzymes are inhibited by the presence of cellobiose thus, the reduction of cellobiose to glucose by β-G prevents cellobiose inhibition (Bisaria and Ghose, 1981; Kumar et al., 2008). β-glucosidase is also subject to end product inhibition and thermal inactivation. Thus, β-G does not accumulate in the soil which slows the degradation of organic matter and nutrient release to the environment (Kumar et al., 2008).

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Fungi are estimated to be the largest producers (~87%) of β-G although it has been detected in various plants, animals and bacteria (Knight and Dick, 2004). Commercially, β-G is produced by the fungal species in the *Trichoderma* and *Aspergillus* genera (Esterbauer et al., 1991; Kirk et al., 2002; Cherry and Fidantsef, 2003) and is used in a wide range of industries including textiles, fermentation, pulp and paper (Percival Zhang et al., 2006) and potential use in biofuels production (Kumar et al., 2008).

β-Glucosaminidase

In tropical environments, chitin degradation plays a key role in C and N cycling. Chitin, a biopolymer with β-1,4 linkages, is mineralized to C and N in humid soils to create amino sugars (Ekenler and Tabatabai, 2002; Acosta-Martínez et al., 2007) by β-glucosaminidase. β-glucosaminidase (EC 3.2.1.30) hydrolyzes the N-acetyl-β-D-glucosamine (NAG) residues from chitooligosaccharide non-reducing terminal ends (See Figure 2) (Parham and Deng, 2000; Acosta-Martínez et al., 2007). β-glucosaminidase activity has been positively correlated to soil N mineralization (Acosta-Martínez et al., 2007), C and N microbial biomass (Parham and Deng, 2000) and are impacted by land management and soil order (Acosta-Martínez et al., 2007).

β-glucosaminidase is widely distributed in nature and can be found in fungi such as *Trichoderma harzianum*, bacteria, plants, and insects (Parham and Deng, 2000; Acosta-Martínez et al., 2007). β-glucosaminidase activities have been proposed as an indicator of soil fungal biomass (Parham and Deng, 2000).

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Arylsulfatase

The abundance of organic S (~95%) in the top layer of soil (Piutti et al., 2015) highlights the importance of enzymatic organic S mineralization (Tabatabai, 1994a; Acosta-Martínez et al., 2007). Organic S compounds are classified as carbon-bound sulfur (e.g. amino acids, sulfonates, and heterocyclic S) and non-carbon bound sulfur (e.g. sulfates esters and sulfamates) (Freney et al., 1975; Scherer, 2001; Piutti et al., 2015). Non-carbon bound S is the most labile for S mineralization from organic matter (Piutti et al., 2015) and ester sulfates represent 70% of organic S in aerobic soils (Knauff et al., 2003). Soil organic S in ester sulfate form is hydrolyzed by arylsulfatase (ARS, EC 3.1.6.1) activity to mineralize organics to SO_4^{2-} (Kellogg et al., 1972; Gupta et al., 1993). The release of plant available sulfate (SO_4^{2-}) by ARS (Bandick and Dick, 1999) is vital to plant growth and metabolism (Droux, 2004).

The ARS enzyme catalyzes the hydrolysis of sulfate esters by cleaving the O-S bonds to release sulfates (See Figure 3) (Tabatabai, 1994a). Arylsulfatase activity increases with soil organic matter content (Tabatabai, 1994b; Klose et al., 1999; Knights et al., 2001) and decreases with soil depth (Tabatabai, 1994b). The importance of C substrates for microbial growth could govern S cycling (Knights et al., 2001). Conflicting research correlating ARS activity with other nutrient additions accentuates the complexity of the soil S cycle. Increased ARS activity has been correlated to organic C in the rhizosphere (Knauff et al., 2003) and organic fertilization under maize and winter wheat rotation (Giacometti et al., 2014), but ARS was unresponsive to organic and inorganic fertilization amendment under the same crop rotation (Liang et al., 2014) and on a clay loam soil in northern Spain (Mijangos et al., 2006). Inhibition of ARS activity

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by anionic trace elements such as molybdate (MoO_4^{2-}), tungstate (WO_4^{2-}), arsenate (AsO_4^{3-}) and phosphate (PO_4^{3-}) (Tabatabai, 1994a) could account for variations in results. Arylsulfatase enzymes are primary produced by bacteria in *Actinobacteria* and *Pseudomonas* (Cregut et al., 2009) and by fungal species in *Ascomycotina* (Piutti et al., 2015) have been identified (Tabatabai, 1994a).

Amidase

The importance of N in plant growth and production is normally the most limiting nutrient for plants. Amidase (acylamide amidohydrolase, EC 3.5.1.4) is responsible for the production of ammonia (NH_3) and carboxylic acid through the hydrolysis of amides (See Figure 4) (Tabatabai, 1994a; Fraser et al., 2013). The production of ammonia (NH_3) is an important transformation in the N cycle. Amidase can be increased in the presence of C, but excess C can lead to both N and P limitation (Allison et al., 2011). Positive amidase activity has been correlated with high molecular weight amide substrates (propionamide) but not for low weight substrates (formamide) (Fraser et al., 2013).

Amidase is produced by a wide variety of microorganisms, plants, and animals including bacteria in the *Rhodococcus* (Nawaz et al., 1994) and *Bacillus* (Thalenfeld et al., 1977) genera. Fungal production of amidase has been noted in *Aspergillus* (Benke, 1979) and *Fusarium* (Reichel et al., 1991) genera. The wide variety of substrates available to amidase is indicative of the diverse nature and presence of amidase. Irreversible inhibition of amidase activity by organophosphate insecticides such as fenitrothion and trichlorphon have been described (Rasool et al., 2014).

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Urease

Urease is another enzyme active in soil N cycling. Urease (urea amidohydolase, EC 3.5.1.5) is responsible for the hydrolysis of urea to form ammonia (NH_3) (See Figure 5) (Tabatabai, 1994a). Over application of fertilizer alongside urease activity results in N leaching and N pollution worldwide (Rasool et al., 2014).The application of urea as a N fertilizer has been used worldwide in agriculture (Singh et al., 2013). Urease is monitored as an indicator of eutrophication in agricultural and grassland ecosystems (Kandeler et al., 1999) and heavy metal pollution (Kim et al., 2008). The volatilization of ammonia by urease have lead to extensive studies of inhibitors for agricultural application (Tabatabai, 1994a). The wide variety of both natural and synthetic urease inhibitors are categorized by: reaction with sulfhydryl (mercapto) groups of sulfase (e.g. hydroquinone, p-benzoquinone), metal chelating compounds (e.g. acetohydroxamic acid) and similarly structured inhibitors (e.g. hydroxyurea, phosphoroamides) that bind to urease, inhibiting activity (Singh et al., 2013).

Decreasing urease is associated with a decrease in microbial biomass which is controlled by soil C (Singh et al., 2013). Higher urease activity in uncultivated soil in comparison to cultivated soil is attributed to differences in soil organic C and microbial activity (Singh et al., 2013). Urease is universally distributed by plants, fungi and bacteria (Singh et al., 2013). Between 17%-70% of bacteria and 78%-98% of fungi produce extracellular urease (Lloyde and Sheaffe 1973). Two of the best commercial sources of urease are Jack beans (*Canavlia ensiformis)* and *Bacillus pasteurii* (Varner, 1960).

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Alkaline Phosphatase

Phosphatase measurements are an important indicator of soil organic P mineralization (Tabatabai, 1994a). Phosphate is the second most important nutrient for plant growth but is largely unavailable for root uptake because it is rapidly immobilized by complexing with cations or adsorption to organic matter (Lambers et al., 2008; Tran et al., 2010). Enhancing P release through high expression of phosphatase has led to a greater interest in phosphatases from an agricultural standpoint (Maseko and Dakora, 2013). Phosphatases are classified according to the enzyme predominantly expressed at different pH vales: acid phosphatase and alkaline phosphatase. These two classifications of enzymes have the ability to hydrolyze a large variety of phosphomonoesters including β-glycerophosphate, phenylphosphate, β-naphthyl phosphate and p-nitrophenyl phosphate (Tabatabai, 1994b).

As the name suggests, alkaline phosphatase is predominantly found in alkaline soils (Tabatabai, 1994b). Alkaline phosphatase (orthophosphoric monoester phosphohydrolase EC 3.1.3.1) hydrolyzes phosphate groups ("dephosphorylation") from a wide variety of molecules including nucleotides, proteins and alkaloids (See Figure 6)(Tabatabai, 1994a). The ratio of enzyme activities of acid phosphatase to alkaline phosphatase has been suggested as a more sensitive and accurate reflection of soil pH (Dick et al., 2000). The inability of many plants to survive highly alkaline conditions suggests that alkaline phosphatases are largely derived from microorganisms (Dick et al., 1996), including bacteria, fungi but is also found in earthworms (Herbien and Neal, 1990).

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Acid Phosphatase

Acid phosphatase functions like alkaline phosphate but at a pH of less than 7 (Tabatabai, 1994a). Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) hydrolyzes organic phosphates to produce inorganic phosphate, a form that is easily accessible to plants (See Figure 6) (Maseko and Dakora, 2013). As a catalyst of a variety of organic and inorganic phosphomonoesters (Bowles et al., 2014), it is often studied due to its pivotal role in P mineralization for plant nutrient requirements.

Considerable research into phosphatases has been dominated by acid phosphatase due to its relationship to plants and P mineralization in agricultural systems (Maseko and Dakora, 2013). Acid phosphatase is a root exudate of many plant species when P is limiting (Ström et al., 2005). For example, fynbos legumes *Cyclopia genistoides, Aspalathus caledonensis,* and *Aspalathus aspalathoides* all express acid phosphatase in the rhizosphere (Maseko and Dakora, 2013).

ANALYTICAL METHODS

The methodologies adopted for soil enzyme measurement are not universal and create difficulty in comparing soil enzyme research. Differences between substrates, assay conditions, incubation times and detection methods (Nannipieri et al., 1980; Marx et al., 2001; Burns et al., 2013) contribute to differences in enzyme readings.

As with any assay, functionality and sensitivity are important, especially due to variability of enzyme activity in response to environmental factors. The final measurement of activity is the potential enzyme activity within the soil sample and may not reflect the actual activity in the field. Substrate concentration, pH, temperature and

time of incubation must be optimized to measure potential enzyme activity, and assays must include a terminal enzyme inhibitor to prevent further enzyme activity (Kandeler et al., 1999). An outline of laboratory conditions used in previous studies is presented in Table 1. Soil enzyme assays have been used to measure differences between soil managements, soil types, and plant community types and are intended to reflect microbial community functions. Here, two common enzyme assays, colorimetric and fluorometric, will be briefly discussed.

Colorimetric

Colorimetric analysis is dependent on the release of *p*-nitrophenol (*p*NP), a commonly used substrate, when soil is incubated with a *p*NP at optimal enzyme pH (Deng et al., 2013). The distinct yellow color is indicative of enzymatic reaction and can be measured with a spectrophotometer and a standardized calibration curve (Tabatabai, 1994b; Deng et al., 2013). Colorimetric enzyme analysis is a bench assay that is generally labor intensive, time consuming and constrained to one enzyme per sample run (Deng et al., 2011). As a result, soil sample storage is unavoidable which can impact enzyme activity (DeForest, 2009; Deng et al., 2011; Dick et al., 2013). Aside from issues associated with sample storage and soil processing conditions (i.e. fresh, frozen or air-dried) (Dick et al., 2013), other detection interferences are associated with soil pH, temperature, sample volume, soil homogenization, and reaction time between termination and data reading (Turner et al., 2002; Sinsabaugh et al., 2008; DeForest, 2009; German et al., 2011; Dick et al., 2013).

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Fluorometric

Fluorometric assays measure potential enzyme commonly using fluorogenic 4-methylumbelliferyl (MUF) labeled substrates. Fluorescence readings are less sensitive to the effects of turbidity compared to adsorption-based detection used in colorimetry (Deng et al., 2011). Microplate readers can be used in both colorimetric and fluorometric assays, and are rapid, sensitive, selective and generally cost effective for large sample loads and high throughput data analysis (Piletsky et al., 2001; Dick et al., 2013). A typical concern for fluorescence-based methods is the uniformity of diluted soil suspensions. Dick et al. (2013), however, found no significant effect on the variability of enzyme activity measured from different layers of suspension while being stirred, and sonication before sampling was not found to improve reproducibility (Deng et al., 2013). To account for different soil extract matrices without introducing other methodological issues, a calibration curve for each soil type is required.

Fluorescence based assays must account for quenching, a process that decreases the fluorescence intensity and results in underestimating the actual reaction rate being measured. Quenching can be caused by energy transfer, excited state reactions, and complex formation and is heavily dependent on pressure and temperature (Deng et al., 2011). In soil extracts, turbidity and suspended soil particles can also contribute to quenching. Quenching has been shown to reduce fluorescence by 27-61% and is highly soil-dependent (Deng et al., 2011). Although quenching could be compensated for by using longer incubation periods, longer periods can lead to potential microbial growth and consumption of substrates or reagents, resulting in other analytical artifacts (Deng et

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al., 2011; Dick et al., 2013).To compensate for quenching, fluorescence assays use multiple controls, blanks, and soil specific calibration curves.

CONCLUSIONS

Understanding the role of enzymes in the microbial community and soil nutrient cycling can increase our understanding of soil microbial relationships, community structure and ecosystem processes. The enzymes presented in this review are only a small representation of the catalysts involved in soil nutrient cycling. Further enzyme research and refined methods could help determine anthropogenic impacts, such as agricultural practices and soil management programs or nutrient cycling. Monitoring anthropogenic impacts can provide valuable information for maintaining healthy, sustainable ecosystems.

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Table 1: Soil enzymes included in this chapter with their classifications, functions, substrates, and assay conditions.

General Enzyme EC number	Name	Soil Function	Assay conditions: Substrate	pH	Source(s)
Cellulases					
3.2.1.21	β-glucosidase	Cellulose degradation; produces glucose for plant and microorganism consumption	*p*-Nitrophenyl-β-D-glucopyranoside (10mM)	6.0[a]	(Tabatabai, 1994b; Acosta-Martínez et al., 2007)
			4-MUF-β-D-glucoside (100μM)	5.5, 6.0[b]	(Marx et al., 2001; Giacometti et al., 2014)
3.2.1.30	β-glucosaminidase	chitin degradation; provides amino sugars	*p*-Nitrophenyl-*N*-acetyl-β-D-glucosaminidemine (10mM)	5.5[a]	(Parham and Deng, 2000)
			4-MUF-N-acetyl-β-D-glucosamide (200μM)	5.5, 6.0[b]	(Marx et al., 2001; DeForest, 2009; Giacometti et al., 2014)
Sulfatases					
3.1.6.1	Arylsulfatase	Produces plant available sulfates	*p*-Nitrophenyl sulfate (10mM)	5.5-6.02[a]	(Tabatabai, 1994b; Acosta-Martínez et al., 2007)
			4-MUF-sulfate (100μM)	5.5[b]	(Giacometti et al., 2014)
Amidohydrolases					
3.5.2.4	Amidase	Produces ammonia and carboxylic acid; releases organic N	Nα-Benzoyl-DL-arginine 4-nitroanilide hydrochloride	8.5[b]	(Bandick and Dick, 1999)(Tabatabai 1994)
3.5.1.5	Urease	Volatilizes ammonia from urea: releases organic N	Urea (20mM)	5.0[a]	(Saiya-Cork et al., 2002)
			Urea	10.0[b]	(Kandeler and Gerber, 1988)
Phosphomonoesterases					
3.1.3.1	Alkaline Phosphatase	Produces plant available phosphates in alkaline soil conditions	disodium p-nitrophenyl phosphate tetrahydrate (20mM)	10.0[a]	(Kandeler et al., 1999)
3.1.3.2	Acid Phosphatase	Produces plant available phosphates in acid soil conditions	*p*-Nitrophenyl sulfate (10mM)	5.8[a]	(Tabatabai, 1994b; Acosta-Martínez et al., 2007)
			4-MUF-phosphate (200μM)	5.5-6.0[b]	(Marx et al., 2001; DeForest, 2009; Giacometti et al., 2014)

EC: enzyme commission classification

MUF: 4-methylumbelliferone
[a]:indicates colorimetric analysis
[b]: indicates fluorescence analysis

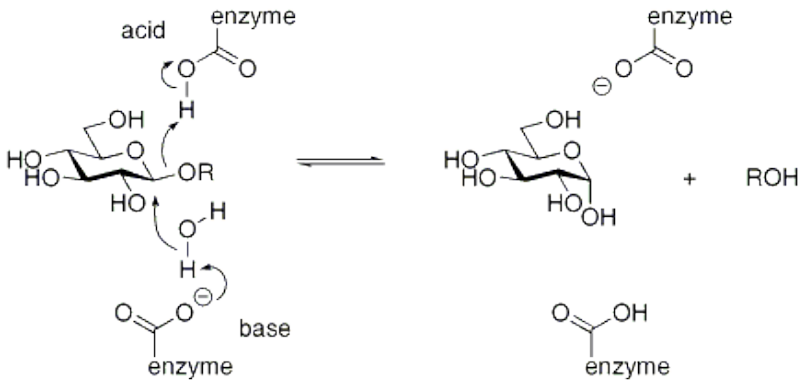

Figure 1: β-Glucosidase Enzymatic Reaction from Kumar et al. (2008). β-Glucosidase catalyzes the hydrolysis of cellobiose to release glucose.

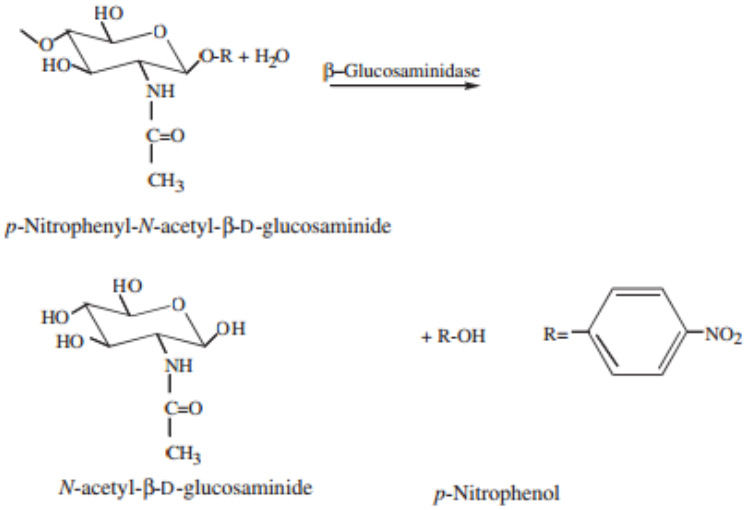

Figure 2: β-Glucosaminidase Enzymatic Reaction from Ekenler and Tabatabai (2004). β-glucosaminidase hydrolyzes the NAG residues from chitooligosaccharide non-terminal end.

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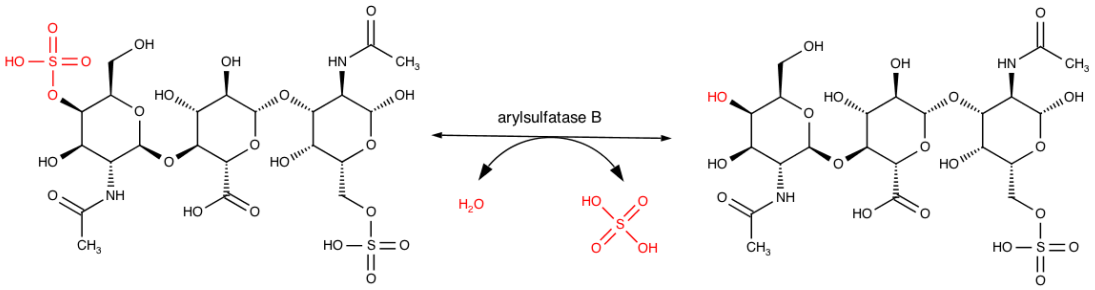

Figure 3: Arylsulfatase Enzymatic Reaction from Klose et al. (2011). Arylsulfatase catalyzes the hydrolysis of sulfate esters by cleaving the O-S bond to release sulfates.

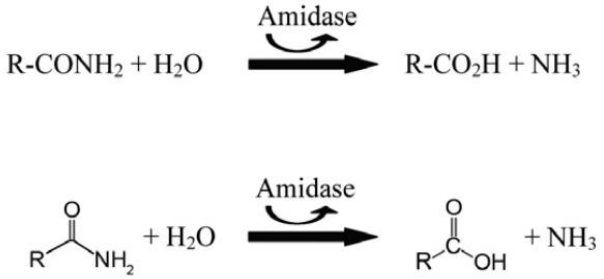

Figure 4: Amidase Enzymatic Reaction from Kandeler et al. (2011). Amidase hydrolyzes amides to produce ammonia (NH_3) and carboxylic acid.

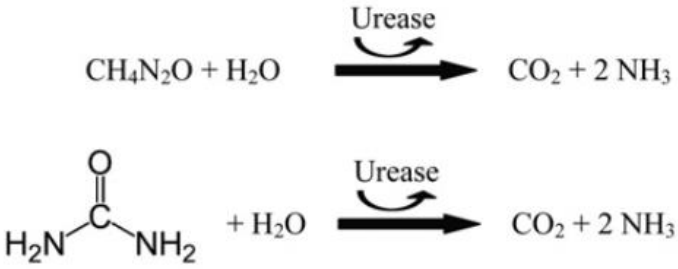

Figure 5: Urease Enzymatic Reaction from Kandeler et al. (2011). Urease hydrolyzes urea to release ammonia (NH_3).

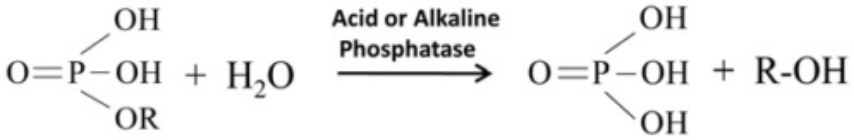

Figure 6: Alkaline and Acid Phosphatase Enzymatic Reaction from Acosta-Martinez et al. (2011). Alkaline and Acid Phosphatase hydrolyze phosphate groups from a wide variety of molecules including nucleotides, proteins, and alkaloids.

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CHAPTER 2

A modified microplate method for high throughput determination of β-glucosidase activity

ABSTRACT

Soil microbes produce extracellular enzymes which degrade complex organic compounds to release energy and nutrients. Soil enzymes are sensitive to ecosystem disturbances and are suggested as indicators of soil health, microbial community composition and microbial function. Fluorescence enzyme assays tend to be more sensitive than spectrophotometric (ie. colorimetric) assays, and use of a 96-well plate supports high-throughput use. The objective of this study was to develop and compare a new, faster microplate assay to a common microplate assay, and a traditional bench scale assay used to measure an important soil health enzyme, β-glucosidase, using fluorometric (4-methylumbelliferone) and spectroscopic (p-nitrophenyl or *p*NP) detection. Evaluation includes the use of an automated pipetting system and sonication, as well as a reduction in analytical replicates, allowing higher sample throughput suitable for service laboratory use.

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INTRODUCTION

Bacteria, fungi and archaea communities actively contribute and control multiple biogeochemical cycles that produce, stabilize, and destabilize soil organic matter and cycle nutrients throughout the soil system (Burns et al., 2013). The inability of microorganisms to metabolize high molecular weight molecules found in organic matter constituents requires the production of extracellular enzymes to cleave bonds, creating monomeric products for microbial consumption (Sala et al., 2001; Hoppe et al., 2002; Arrieta et al., 2004) and liberating micro- and macronutrients to the soil environment (Bell et al., 2013).

Enzyme synthesis is governed by microbial response to environmental conditions, termed "quorum sensing" (Shank and Kolter, 2009). This phenomenon may control the distance that enzymes diffuse from the cell, as a response to substrate availability in the environment (Allison et al., 2011). Once released from the cell, enzymes will attach to cell walls, diffuse from the parent cell and degrade, or become immobilized on clay minerals, humic acids and particulate organic matter (Burns et al., 1994). Sorption of soil microbe enzymes to extracellular environments and strong carbohydrate-enzyme complexes cause extraction to be difficult (Tabatabai, 1994a), and extreme pH's necessary to release bonds could lead to cell lysing and inaccurate enzyme activity measurements (Dick et al., 2013)**.** Instead of direct extraction of enzymes, potential enzyme activity is correlated to microbial activity by detecting the degradation rate of target substrates and production of products.

Methodologies for measuring extracellular enzymes are limited by contrasting philosophies concerning *'in vivo'* and *'in situ'* approaches. The classical '*in vivo*'

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approach uses controlled conditions (ie. temperature, pH) and maximum substrate availability to provide an optimal enzyme activity measurement (Tabatabai, 1994b). The '*in situ*' approach mimics sample site conditions to provide the natural potential enzyme activity (Saiya-Cork et al., 2002), but pH fluctuations have been observed in samples lacking buffers (German et al., 2011) and may not properly represent '*in situ*' conditions. These approaches produce markedly differing results and appropriate use will depend on the research question to be answered (Burns et al., 2013). Reliance on '*in vivo*' methods for universal laboratory analysis allows comparison of optimal enzyme activities. Effective soil enzyme activity is controlled by concentration of enzyme and substrate availability (Bell et al., 2013). Optimal conditions for enzyme detection are extensively outlined in other studies and in the previous chapter (Tabatabai and Bremner, 1969; Klose et al., 1999; Parham and Deng, 2000; Marx et al., 2001; Deng et al., 2013).

The use of microplate-scale fluorometric assays based on 4-methylumbelliferone (MUF) substrates have increased in research as a fast, higher throughput method compared to the traditional colorimetric bench top assay (Marx et al., 2001; Vepsäläinen et al., 2001; Deng et al., 2011). Though a microplate reader can also measure absorbance, fluorescence readings are less sensitive to the effects of turbidity compared to adsorption-based detection (Deng et al., 2011). Microplate readers are rapid, sensitive, selective and generally cost effective for large sample loads and high throughput data analysis (Piletsky et al., 2001; Dick et al., 2013). Interferences are generally due to variation in fluorescence intensity due to quenching (Deng et al., 2011). Turbidity and soil composition also contribute to quenching and can decrease fluorescence by 27-61% (Deng et al., 2011)

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requiring compensation by creating a calibration curve for each soil type. A comparison of the three enzyme assays is presented in Table 1.

In a study conducted by Dick et al. (2013), bench scale assays using pNP had higher detection limits (8.55 nmol kg^{-1} h^{-1}) in comparison to the pNP and MUF microplate methods (1.700 nmol kg^{-1} h^{-1} and 0.026 nmol kg^{-1} h^{-1} respectively). Deng et al. (2012) found MUF-based assays to be 14 times more sensitive and precise than pNP based assays. Improved sensitivity of MUF microplate assays is balanced by the higher cost for equipment. The purpose of this research was to further optimize and automate a microplate method outlined by Deng et al., (2011) by employing an automated pipetting system, improving sample preparation, and reducing calibration points. In addition, solution storage stability and expiration of substrate was tested. Twenty-five representative soils with a range of colorimetric β-glucosidase values, soil types, pH values and organic carbon (C) contents were selected for this study. For evaluations of the automated pipetting system and sonication, only one representative soil was selected. These improvements will permit processing and measurement of a larger number of samples over shorter time frames and ultimately increase the application of soil enzymes in monitoring soil health.

MATERIALS AND METHODS

Soil collection and characterization

Soils were selected from Kellogg Soil Survey Laboratory (KSSL) and collected over the last two years by Natural Resource Conservation Service (NRCS) field office employees in Nebraska, North Dakota, South Dakota, and Montana (See Table 1). These

soils are representative of a wide variety of research projects including: agricultural, greenhouse and grazing projects. The soils samples were selected based on β-glucosidase values from previous bench scale colorimetric readings run within the year. These samples were all treated to the same collection and storage methods. Upon collection, the twenty-five soils (0-150cm) were sieved through a 2mm screen and air-dried at room temperature. Soil pH values were determined using a standard glass body electrode connected to a digital pH/Ion Meter (soil: 0.01 M $CaCl_2$ (w/v) = 1:2.0). Soil organic carbon (SOC) and total nitrogen (TN) were determined on soil air dried, finely ground soil aliquots on an elemental analyzer (Elementar Americas, Inc., Mt. Laurel, NJ). Air-dried, 2 mm sieved samples were archived at room temperature until enzyme analysis.

Precision pipette, homogenization and reagent stability

One sample was randomly selected among the 25 test soils to evaluate precision and accuracy of the Precision Pipetting System (BioTek, Winooski, VT) for automated dispensing of reagents and preparation standards in a 96-well plate. A 5-point calibration using low standard 10 μM and high standard 50 μM was replicated and diluted to 5 μM, 20 μM, and 30 μM. Eight replicate standard curves were compared between automated and manually prepared standards.

Enzyme extractions using a sonication bath or magnetic stir bar were compared for another soil sample. Samples were either stirred with a magnetic stir plate for 30 min or sonicated at 10 watts (W), 15 W, and 20 W for 120 seconds (s) as described in Marx et al. (2001) and Dick et al. (2013) prior to enzyme analysis.

The effect of storage method on solution stability was tested on MUF-G and standards by comparing newly made solutions kept in the refrigerator overnight to frozen

solutions. Solutions were mixed and aliquots taken and placed in the freezer (4°C) until analysis. Five day stability of MUF-G was tested on 8 extracts of a single soil after being warmed in the water bath for 30 mins at 37°C. Single day solution stability was tested on solutions made the previous day and tested in 30 min increment for 6 hours.

Soil amount, replication amount and MUF amount

Two contrasting soils with low and high β-glucosidase activities were used to optimize conditions for sample size, extraction ratio and MUF amounts for precision and accuracy. Three different soil sample sizes (0.5 g, 1.0 g, and 2.0 g), two different MUF concentrations (2 μM, 4 μM) and 3 extraction replications and 4 analytical replications per extraction were tested. Enzyme activity was calculated as an average of four treatments minus the average of four controls to account for variances often encountered in biological soil samples (Parham and Deng, 2000; Deng et al., 2011).

Enzyme Assays

Twenty-five soils were testes for β-glucosidase were measured using a bench scale *p*-nitrophenol (*p*NP) method, a microplate-based MUF assay by Deng et al., (2011) and a modified microplate MUF assay (See Table 2). The traditional colorimetric bench scale reference methods were performed as described in the Kellogg Soil Survey Laboratory Methods Manuel (Burt, 2014). Briefly, a modified universal buffer (pH 6.0), substrate and p-nitrophenyl-β-glucosidase was added to a 1 g sample and incubated at 37°C for 1 hr. After incubation, 2-amino-2(hydroxymethyl)-1-3-propanedio (THAM, pH 12) was added to quench the enzyme reaction. Each sample was pipetted, filtered and diluted. Sample absorbance was measured at 410 nm with a spectrophotometer (Cary 50

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Conc, Varian Australia Pty Ltd.) and concentrations calculated using a standard calibration curve created with each batch. Duplicates and controls were analyzed for every soil sample.

The original microplate enzyme assay was performed as described by Dick et al (2014), which is a modified version of Deng et al. (2011). Briefly, each soil suspension was prepared by adding 120 mL de-ionized water to 1 g dry soil in a 150 mL beaker. The soil suspension was mixed for 30 mins using a 3.75 cm magnetic stir bar at 600 rpm. Four-channel pipettes were used to measure 100 µL aliquots of soil slurry taken during mixing and added to a 96-well microplate containing 50 µL modified universal buffer (MUB, pH 6.0). Methylumbelliferone (MUF-G)-labeled substrate (50 µL) or MUF standard (50 µL) was added to each respective well and thoroughly mixed by aspirating and dispensing well contents. Plates were incubated for 1 hr. After incubation, 50 µL tris-hydroxymethyl aminomethane (THAM, 0.1 M, 12 pH) was added to terminate enzymatic reaction. Autohydrolysis controls for substrates were tested and included in calculations (Deng et al., 2011). Four replicates of six point calibration curves were used for each soil sample. Solutions and standards were prepared as described by Deng et al. (2011).

The second microplate enzyme assay was modified from Dick et al. (2014) by optimizing a soil slurry homogenization method, adding an automated pipetting system and using a 3-point calibration curve. Reagents, including MUB, soil standards, and dilutions were distributed to wells by a Precision Microplate Pipetting System (BioTek). Homogenization of soil slurry was performed by sonication samples at 15 W for 120 s before using a 3.75 cm magnetic stir bar at 600 rpm to prevent the soil slurry from

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settling. Incubation and final calculations were performed as described in Deng et al. (2011).

Data Analysis

Precision Pipette System was analyzed with a one-way analysis of variance (ANOVA) using PROC (SAS v. 9.2, Cary,NC). Methylumbelliferone labeled standard concentrations were fixed factors with β-glucosidase activity treated as a variable. There were eight replicates for each standard. Pretreatment of soils were analyzed using a one-way ANOVA using PROC with homogenization methods as a fixed factor and β-glucosidase activity as a variable. Methylumbelliferone stability was tested over 5 days using a one-way ANOVA using PROC with days as treatments and β-glucosidase activity as a variable. Soil amount, soil replications, and MUF concentrations were tested using a two-way GLIMMIX ANOVA procedure. Soil amount, replicates, and MUF concentrations were fixed factors with β-glucosidase activity used as a random factor. Tukey-Kramer HSD post hoc test were used on all ANOVA procedures to determine potential statistically differences between treatments. Comparison of soil enzyme assays and comparison of soil properties and soil enzyme activity were analyzed with a linear regression model using SigmaPlot (Systat v. 11.0, San Jose, California). All statistical tests were considered significant at $p \leq 0.05$.

RESULTS

Calibration curves developed using the Precision Pipetting System were reproducible for both 5-point and 3-point calibration curves (Figure 1)**.** Plates are typically prepared in less than seven minutes using the Precision Pipetting System. There

was no statistical difference on the impact of pretreatment on enzyme activity ($p=0.8172$). Three sonication levels evaluated as a pretreatment for soil all resulted in higher enzyme activities (Table 3). Stirring soils for 30 mins resulted in the lowest enzyme activity level while sonication at 15 W ml^{-1} produced the highest enzyme activity levels and had a comparable variability to stirring (SD=0.255). Sonication at 20 W ml^{-1} produced the second lowest enzyme activity level but was the least variable (SD=0.221).

Refrigerated MUF-G substrate signal efficiency over 5 days was found to have no statistical difference ($p=0.2610$) but decreased with prolonged use (Figure 2). The highest activity was found on the second day after MUF-G was prepared but also had the greatest standard deviation (0.266), variation (0.071) and standard error (0.0940). Fluorescence intensity was more than doubled in refrigerated reagents though variability was greater than frozen reagents (See Figure 3). Initial increase in fluorescence intensity of frozen solutions attributed to sample aliquot not reaching assay temperature until after 60mins in water bath (data not shown) which significantly impacted analysis results and was thus, removed from presented results.

One-way ANOVA performed on sample sizes MUF amount and replications needed revealed no significant difference in replications measurement ($p=0.6241$) or MUF concentration amount ($p=0.2015$) within both soils. Sample mass was significant for both soils ($p=0.0131$; $p\leq0.0001$) (Figure 4). Differences between means were found in 0.5 g-2.0 g and 1 g-2 g in soil 2643 (0.6090, 0.0641) and 0.5 g-1.0 g and 1 g-2 g in soil 9467 (0.5138, 0.3773). A breakdown of means indicated a higher activity and suitable standard deviations in soil 2643 (0.4506, 0.0677) and soil 9467 (2.96311, 0.81872).

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Select soil characteristics and their relationship to soil enzyme activity were analyzed by the bench reference assay and the modified microplate assay indicated no clear trends between soil sand percentage (Figure 5), soil silt percentage (Figure 6), soil clay percentage (Figure 7) or soil pH (Figure 8). A positive linear relationship was evident for β-G activities analyzed by both assays (Figure 9). A slightly stronger correlation was found under the modified microplate assay (R^2=0.7630) than the bench reference assay (R^2=0.7328).

The comparison of bench, microplate Deng et al. (2011) and the modified microplate are shown in Figure 10. Using the colorimetric analysis as the reference assay, the modified microplate method results were more strongly correlated to bench *p*NP results (R^2=0.936) compared to the original Deng et al. (2011) microplate assay (R^2=0.656).

DISCUSSION

Precision Pipette, Homogenization and Reagent Stability

Use of the automated Precision Pipetting System reduced errors, was faster than hand pipetting and freed the technician to prepare additional plates. The instrument was able to consistently create calibration curves (R^2 >0.99), supporting the use of a three point calibration curve.

Ultrasonic energy has been widely used to disperse soil aggregates and expose immobilized enzymes to substrates, maximizing potential enzyme activity. Sonication pretreatment of soil has been previously evaluated with mixed results. Dick et al. (2014) performed a sonication pretreatment with MUF microplate assay and found similar

results in enzyme activity to the results presented here, where 15W mL^{-1} had the highest enzyme activity. Unlike Dick et al. (2014), results indicated similar variability as using a stir bar. Difference in assay conditions such as an ice bath using for sonication in Dick et al. (2014) may have contributed to differences in enzyme activity. Dick et al. (2014) reporting internal soil suspension temperatures of 17 to 25°C before collecting sample aliquots. Variable sample temperatures can reduce consistency in measured enzyme activities since reaction and extraction temperatures play a critical role in measuring enzyme activity. For example, measured activity roughly doubles for each 10°C change (Allison et al., 2011) and may also contribute to a "lag" stage before optimal enzyme activity occurs. Dick et al. (2013) reported using shorter incubation times, smaller sample slurry aliquots and differing reagents that may also contribute to the variation in results. Increased incubation times have been suggested as a means to compensate for quenching, but also may increase the potential for microbial growth and consumption of substrates or reagents, resulting in other artifacts (Deng et al., 2011), but shorter incubation times could result in higher interferences due to quenching. Larger slurry aliquots obtain in this assay (100 μL) differed from Dick 2014 (50 μL) and is thought to be a more effective approach to capture within-sample soil variation in enzyme levels (Bell et al., 2013). The use of NaOH quenching buffer can also result in fluorescence variations. MUB fluoresce has been noted to increase until ~20 mins after addition, then begins to decrease (DeForest, 2009; German et al., 2011; Bell et al., 2013) unlike THAM, which is stable for up to an hour.

Reagent storage stability is an important characteristic determining reproducibility of enzyme assays. Storage time and temperature of substrate has been shown to affect

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results (Pesaro et al., 2003; Lee et al., 2007; DeForest, 2009; Wallenius et al., 2010). Though it may advantageous to make larger quantities of reagents, storage of reagents at 4°C resulted in markedly drastic loss of fluorescence (Figure 3). Fresh, refrigerated reagents, which are reportedly good for up to two weeks (Deng et al., 2013), have a much higher fluorescence. It is important to note that standards and substrate containing fluorescent compounds tend to photodegrade (Bell et al., 2013) so reagents must be protected from light by using amber glass or wrapping containers with aluminum foil.

Soil amount, replication amount and MUF amount

Bench-scale assays for measuring soil enzymes are sample limited, labor intensive and time consuming with storage impacting enzymatic activity, creating errors in data analysis and interpretation (Deng 2011). No significant differences in replications or MUF amount indicates the need for only one extraction replicate and a concentration of 2 μM MUF is acceptable when performing this assay. Variations in results with sample mass may be attributed to soil heterogeneity (Figure 4). The 1 g samples had the highest enzyme activity, and variability in results were slightly higher than 0.5 g and 2.0 g samples. Variations in results due to sample mass between the two soils were insignificant at 0.5 g and 1.0 g for low enzyme activity soils and insignificant at 0.5 and 2.0 g.

Comparison of methods for measuring soil enzymes

The statistically similar activity measurements reported here can be attributed to differences in temperature, pH and time to read fluorescence after termination which has been shown to alter fluorescence readings (Deng et al., 2011). A positive linear

relationship was detected in both assays for percent organic carbon (%OC) in the samples (See Figure 9). The modified assay had a slightly better correlation than the reference assay to %OC in the sample (R^2=0.7630, R^2=0.7328, respectively). The relationship between organic C and β-G activity can be attributed to role of the enzyme in C cycling.

CONCLUSION

The modified microplate-scale fluorometric assay has the potential for processing large volumes of samples through automation but is limited by the necessity for individual calibration curves caused by fluorescence quenching. The use of a 3-point calibration curve, automated pipetting system and sonication can enable a faster and correlates closely with traditional bench-scale spectrophotometric enzyme assay. A lack of a standardized soil enzyme assay creates difficulties for comparison of data between studies. Adding to the difficulties associated with soil enzyme assays is the heterogeneous nature of soils and sediment that vary with time, space and depth, even at microsites within the soil (Burns et al., 2013) requiring further research into sampling techniques and storage. This modified method can process 36 soil samples in ~2 hrs.

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ID	Year	Sample Location	Sampling Depth	pH[1]	OC[2] (%)	Sand (%)	Silt (%)	Clay (%)	β-G activity[3]
9465	2014	N. Dakota	0.0-5.0	7.5	3.3	31.8	53.3	14.9	482
9467	2014	N. Dakota	0.0-5.0	7.4	4.1	33.3	52.1	14.6	453
9469	2014	N. Dakota	0.0-5.0	7.4	3.9	31.0	53.8	15.2	446
9519	2014	N. Dakota	0.0-5.0	7.1	4.2	32.3	53.9	13.8	425
9503	2014	N. Dakota	0.0-5.0	7.1	4.3	33.1	52.1	14.8	407
9513	2014	N. Dakota	0.0-5.0	7.3	4.7	30.1	56.2	13.7	380
9508	2014	N. Dakota	5.0-15.0	7.3	5.0	30.4	56.1	13.5	360
9511	2014	N. Dakota	0.0-5.0	7.4	4.1	30.1	56.3	13.6	340
1160	2013	N. Dakota	0.0-5.0	7.2	3.1	31.7	53.6	14.7	320
9522	2014	N. Dakota	5.0-15.0	7.2	3.7	31.8	54.8	13.4	300
9480	2014	N. Dakota	5.0-15.0	7.5	3.1	33.9	52.3	13.8	282
1155	2013	N. Dakota	5.0-15.0	7.3	4.4	35.4	49.7	14.9	262
1179	2013	N. Dakota	5.0-15.0	7.3	4.6	31.8	53.6	14.6	240
5616	2014	Nebraska	0.0-5.0	5.4	2.4	5.7	68.7	25.6	223
8552	2013	N. Dakota	5.0-10.0	6.9	3.6	28.8	55.8	15.4	201
5420	2014	Nebraska	0.0-5.0	6.8	2.1	6.6	60.3	33.1	177
5371	2014	Nebraska	0.0-5.0	5.6	1.7	14.9	54.3	30.8	160
5351	2014	Nebraska	5.0-10.0	6.2	1.3	6.7	56.1	37.2	138
5231	2014	Nebraska	0.0-5.0	6.2	1.7	6.6	57.6	35.8	120
5365	2014	Nebraska	5.0-10.0	6.0	1.1	13.5	53	33.5	100
2661	2013	Montana	5.0-15.0	6.2	1.3	7.5	76.3	16.2	80
2643	2013	Montana	5.0-15.0	6.0	1.3	5	80.3	14.7	60
5330	2014	Nebraska	5.0-10.0	4.6	1.0	11	51.5	37.5	39
1500	2013	S. Dakota	26.0-37.0	7.5	0.3	52.5	26.9	20.6	21
1504	2013	S Dakota	127.0-150.0	7.9	0.4	37.5	36.4	26.1	0

[1]pH determined by glass electrode

[2]Estimated Organic Carbon
[3]β-G; β-glucosidase activity (μmols g^{-1} soil hr^{-1}) determined by colorimetric analysis

Table 1: Twenty-five soil sample characterization including sample depth, pH, OC, soil characteristics and β-glucosidase activity calculated as outlined in Kellogg Soil Survey Laboratory Methods Manual (Burnt, 2014).

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Detection	Instrument	Mass (g)	Homogenization	Extraction	Time (hrs)	Samples	Source(s)
Colorimetric	Spectrophotometric	1.0	vortex	4mL MUB 1 ml PNG	2.5	24	Tabatabai, (1994)
Fluorometric	Microplate Reader	1.0	Stir plate (30m)	160 mL RODI 50µL MUB 50 µL substrate	2	16	Deng et al. (2011), Dick et al. (2014)
Fluorometric	Microplate Reader	1.0	Sonication (2m)	160 mL RODI 50µL MUB 50 µL substrate	2	32	Deng et al. (2011), Dick et al. (2014), This study

Table 2: Comparison of mass, homogenization, extraction and assay time among the three soil enzyme assays

Homogenization method	Replications	Time (mins)	Mean (µmols g^{-1} h^{-1})	SD	CV (%)
Stir (700 rpm)	8	30	1.81 A	0.255	14
Sonication (10 W ml^{-1})	8	2	1.93 A	0.321	17
Sonication (15 W ml^{-1})	8	2	1.94 A	0.255	13
Sonication (20 W ml^{-1})	8	2	1.90 A	0.221	12

Table 3: Effect of homogenization method on measured β-glucosidase activity. Capital letter indicates no statistical difference between pretreatments. Enzyme activities were calculated using Deng et al. (2011) procedure.

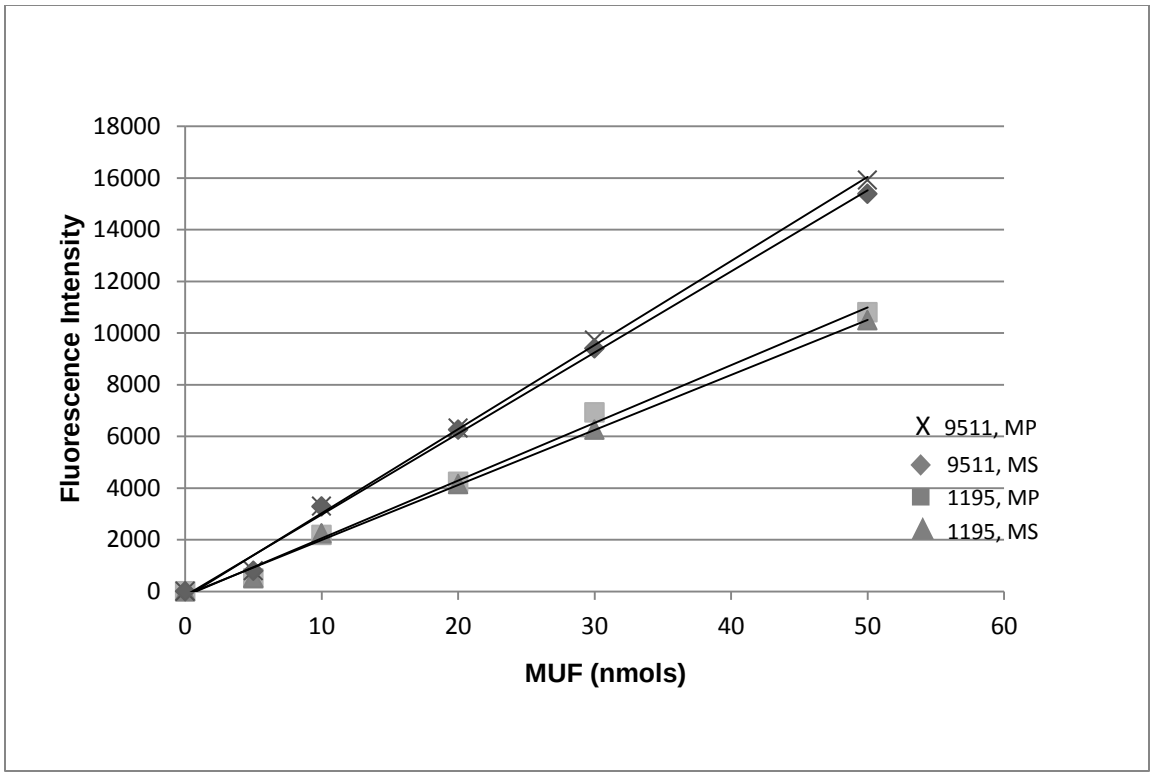

Figure 1: Automated dispensing of reagents and standards by the Precision Pipette System (MP) was compared to manually prepared standards (MS). No significant differences were found between MS and MP supporting the use of the automated process within the presented assay.

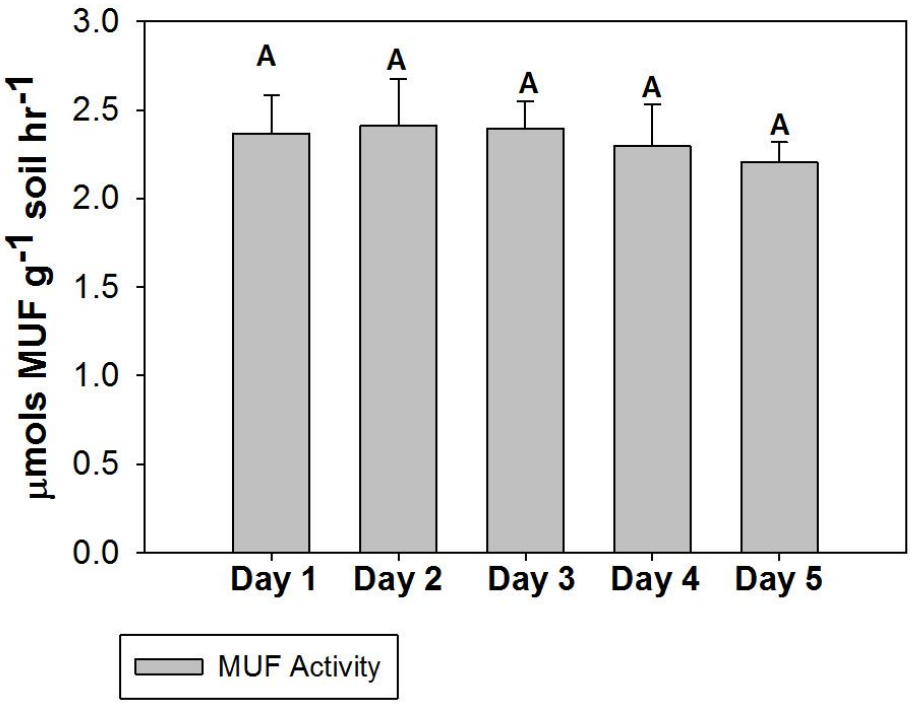

Figure 2: Methylumbelliferone substrate stability over five days. There was no statistical difference (p=0.2610) in measured enzyme activity but a decreasing trend in enzyme activity is noted after day 3.

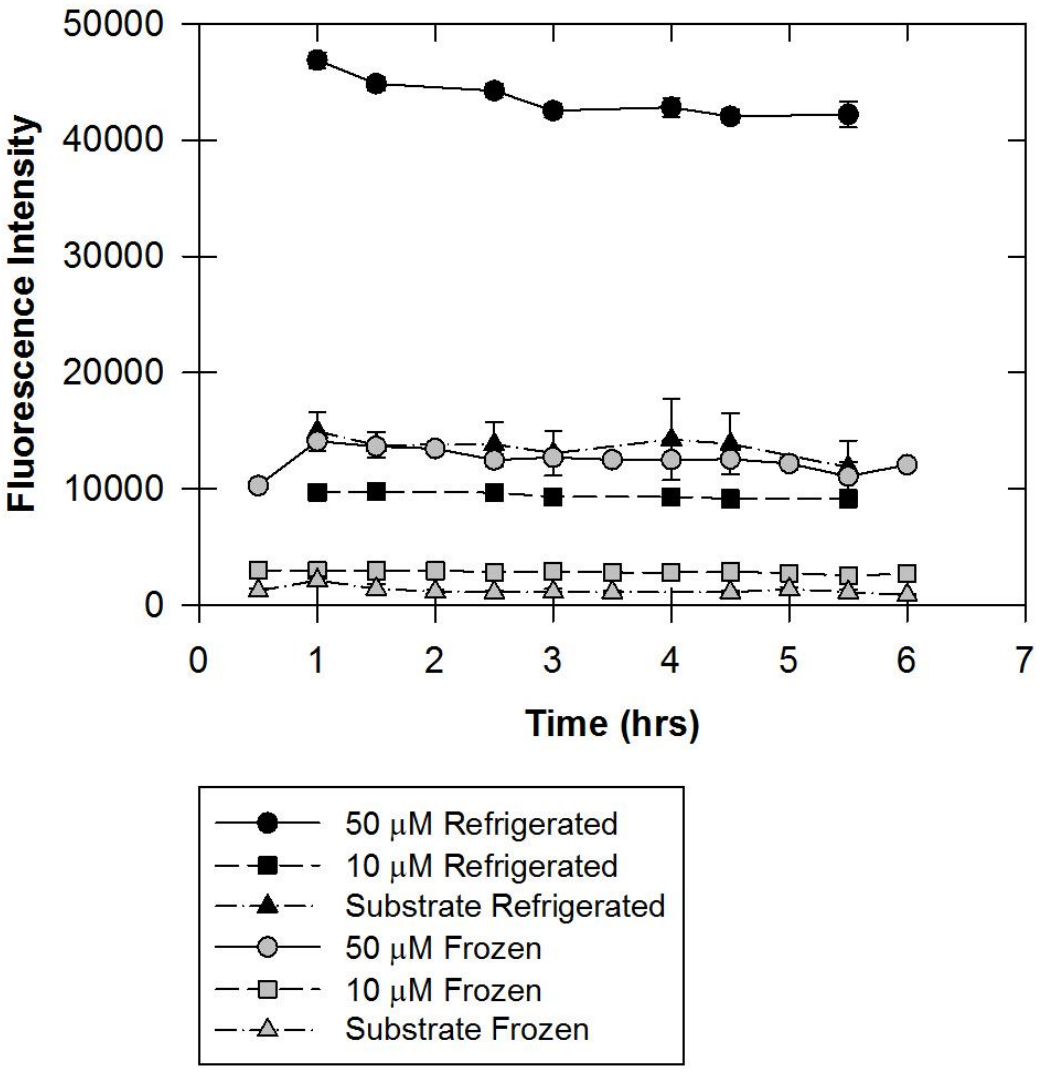

Figure 3: Fluorescence intensities measured over a six hour period for stability of refrigerated and frozen reagents. Refrigerated reagents produced a much higher fluorescence signal than frozen reagents supporting the use of refrigerated reagents this soil enzyme assays.

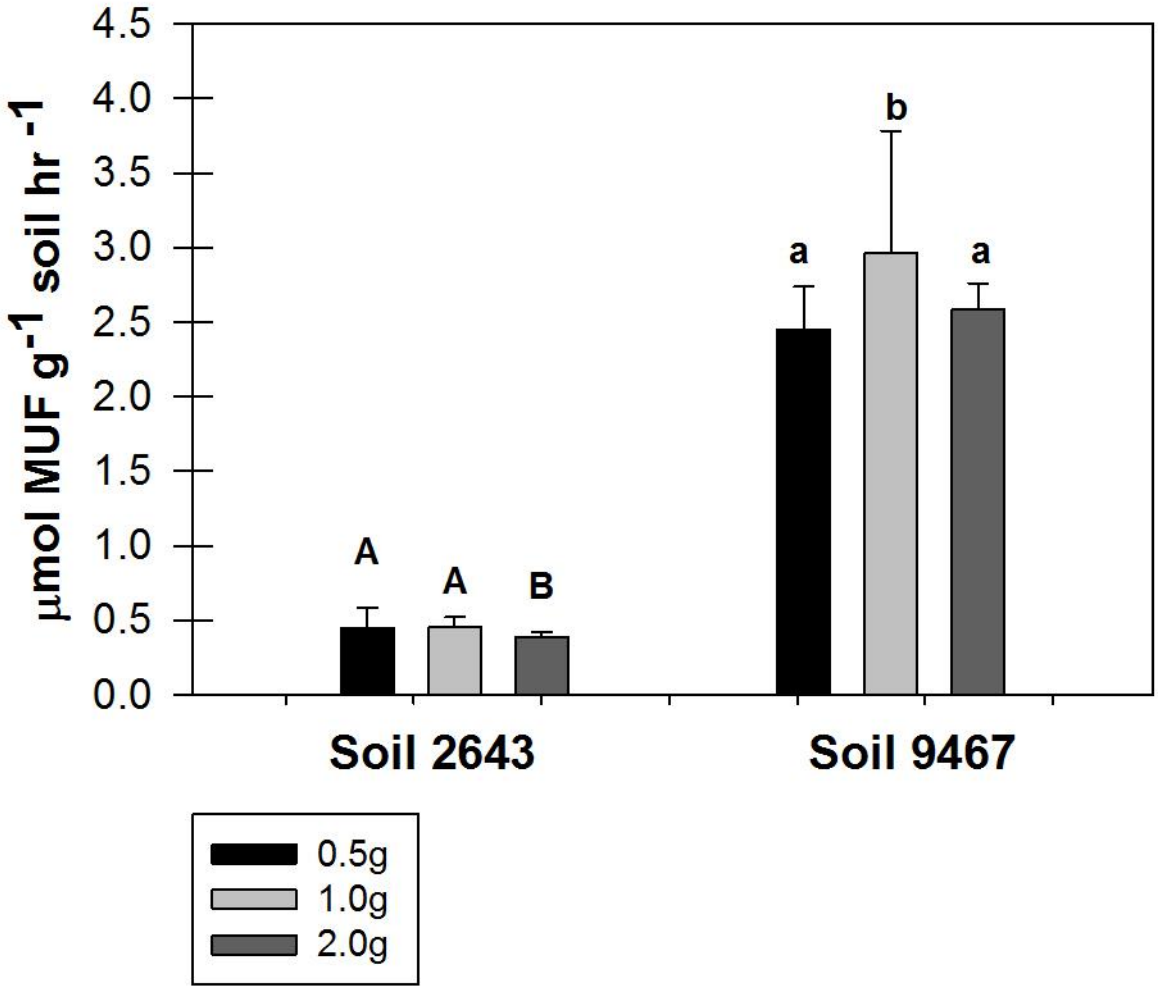

Figure 4: ANOVA results for soil sample sizes were analyzed on soils with high β-glucosidase activity (Soil 9467) and a soil with low β-glucosidase activity (Soil 2643). Significant differences were found in 2 g samples for Soil 2643 and 1.0 g samples for Soil 9467. The

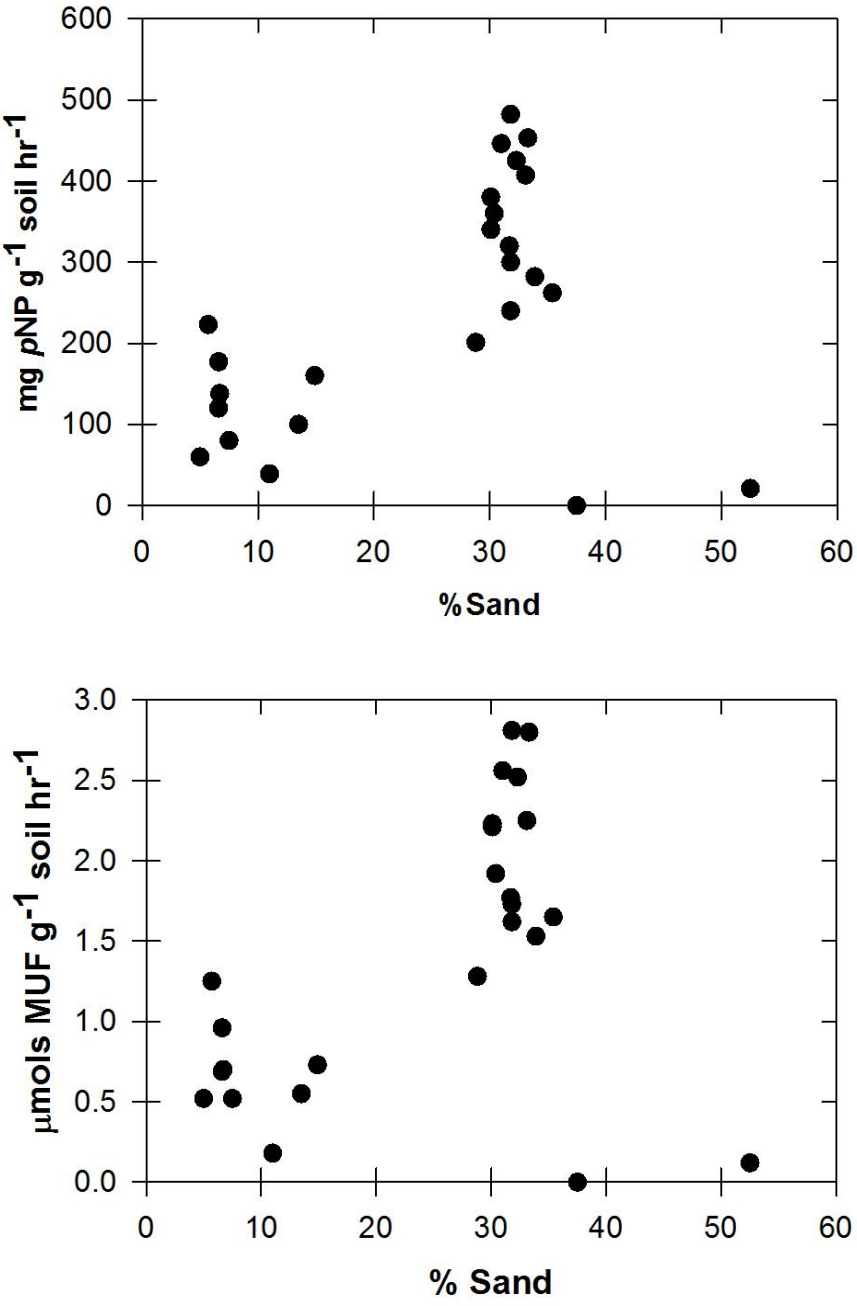

Figure 5: Relationship between percent sand defined by the reference assay results (above) and modified enzyme assay results (below). Both enzyme assays produce similar results in response to sand characteristics in the 25 soil samples tested in this paper.

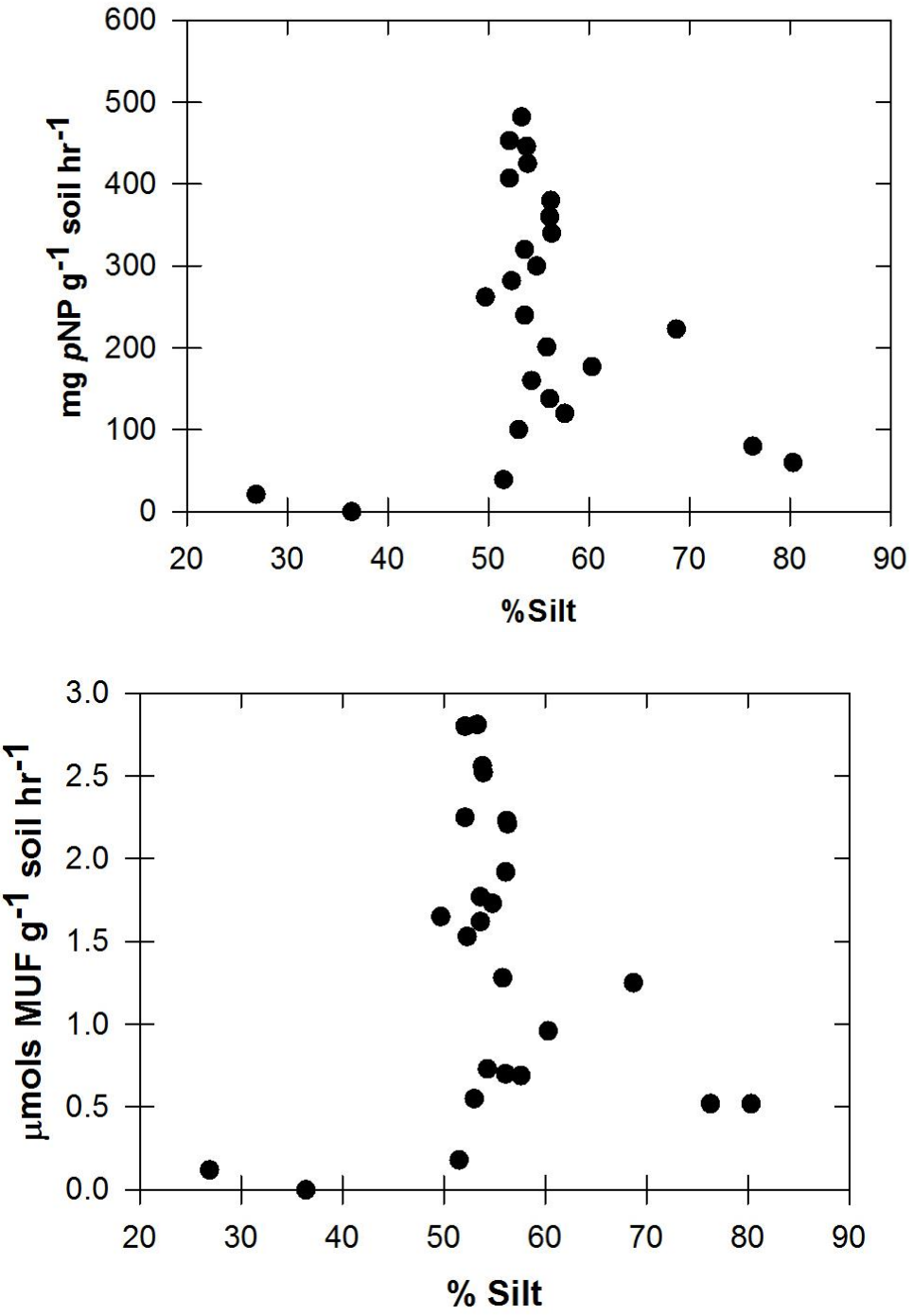

Figure 6: Relationship between percent silt defined by the reference assay results (above) and modified enzyme assay results (below). Both enzyme assays show similar trends in response to percent silt found in the 25 soil samples tested in this paper.

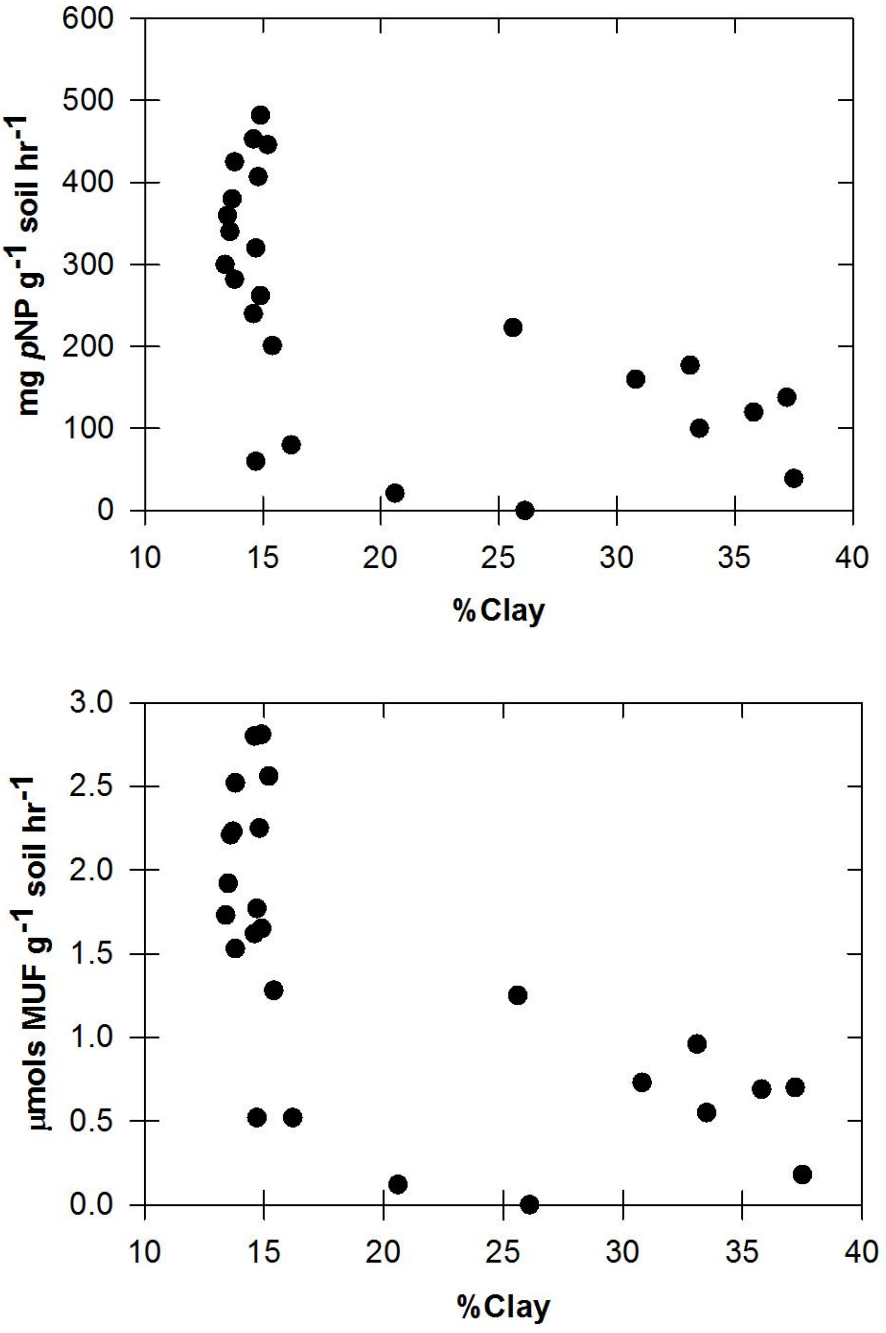

Figure 7: Relationship between percent clay defined by the reference assay results (above) and modified enzyme assay results (below). Both enzyme assays show similar trends around 15% clay content within the 25 soil samples tested in this paper.

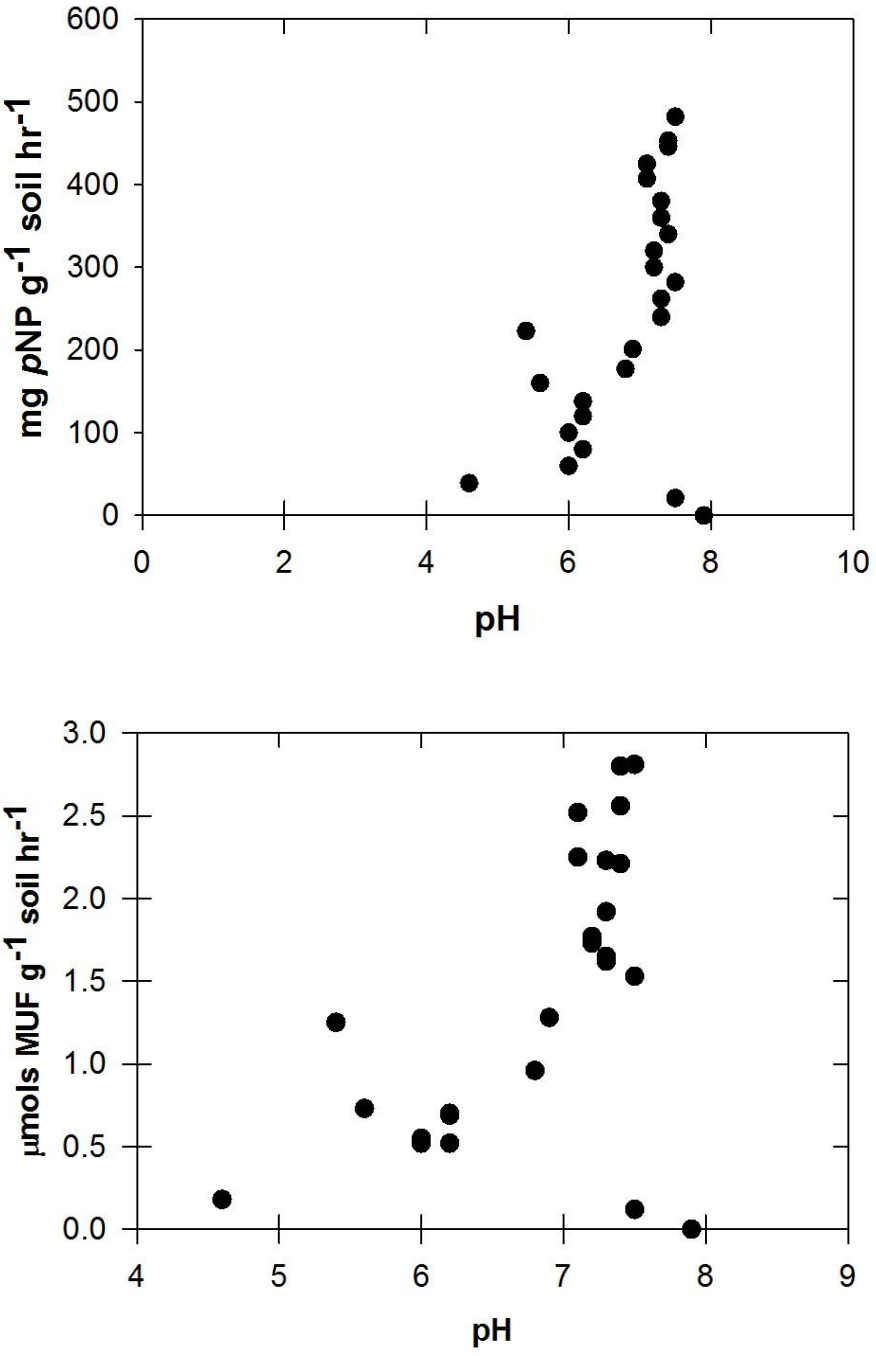

Figure 8: Relationship between soil pH and soil enzyme activity analyzed by the reference assay (above) and modified enzyme assay (below). The wide response of activities detected between a pH 6-8 indicate pH does not play a large role in enzyme activity.

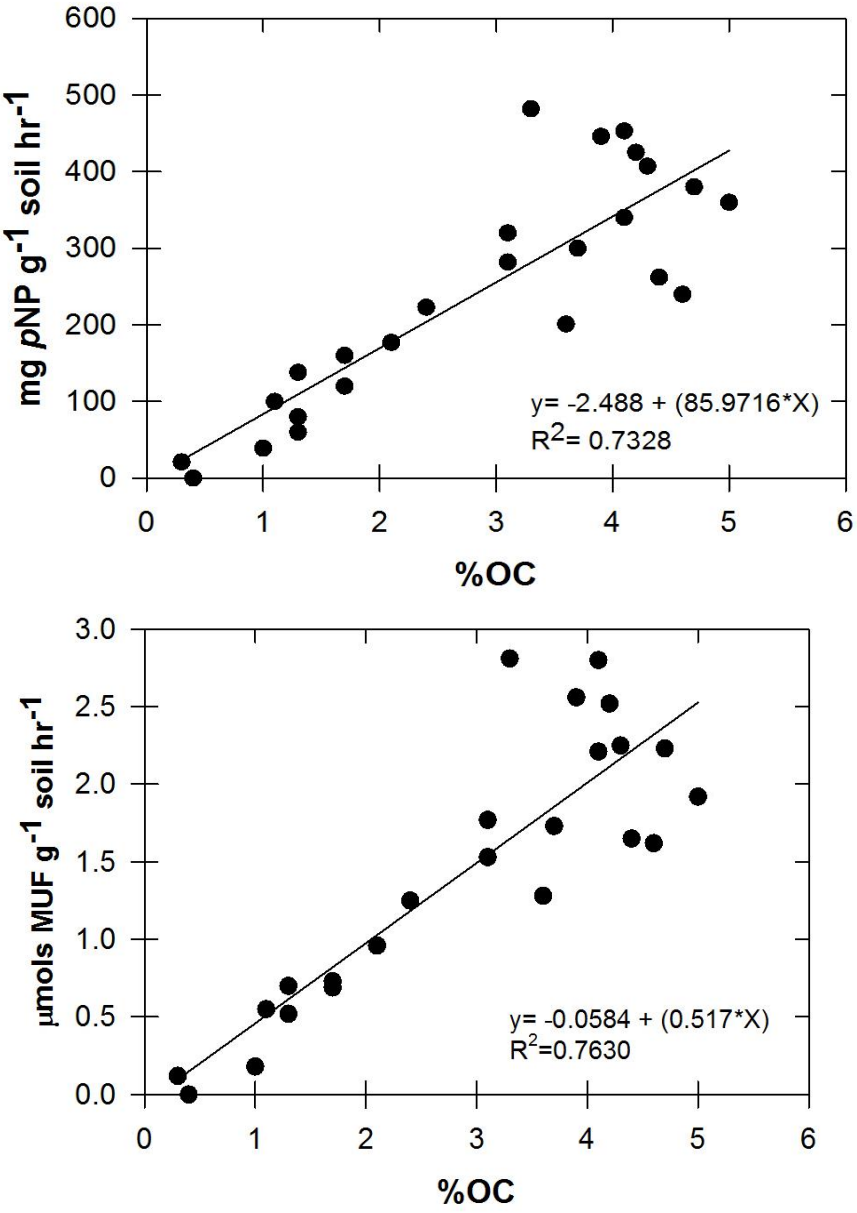

Figure 9: Linear relationship between percent organic carbon (%OC) and β-glucosidase activity analyzed by the reference assay (above) and modified enzyme assay (below). Both enzyme assays show similar linear trend with the modified enzyme assay having a lightly tighter correlation (R^2=0.7630) than the reference assay (R^2=0.7328).

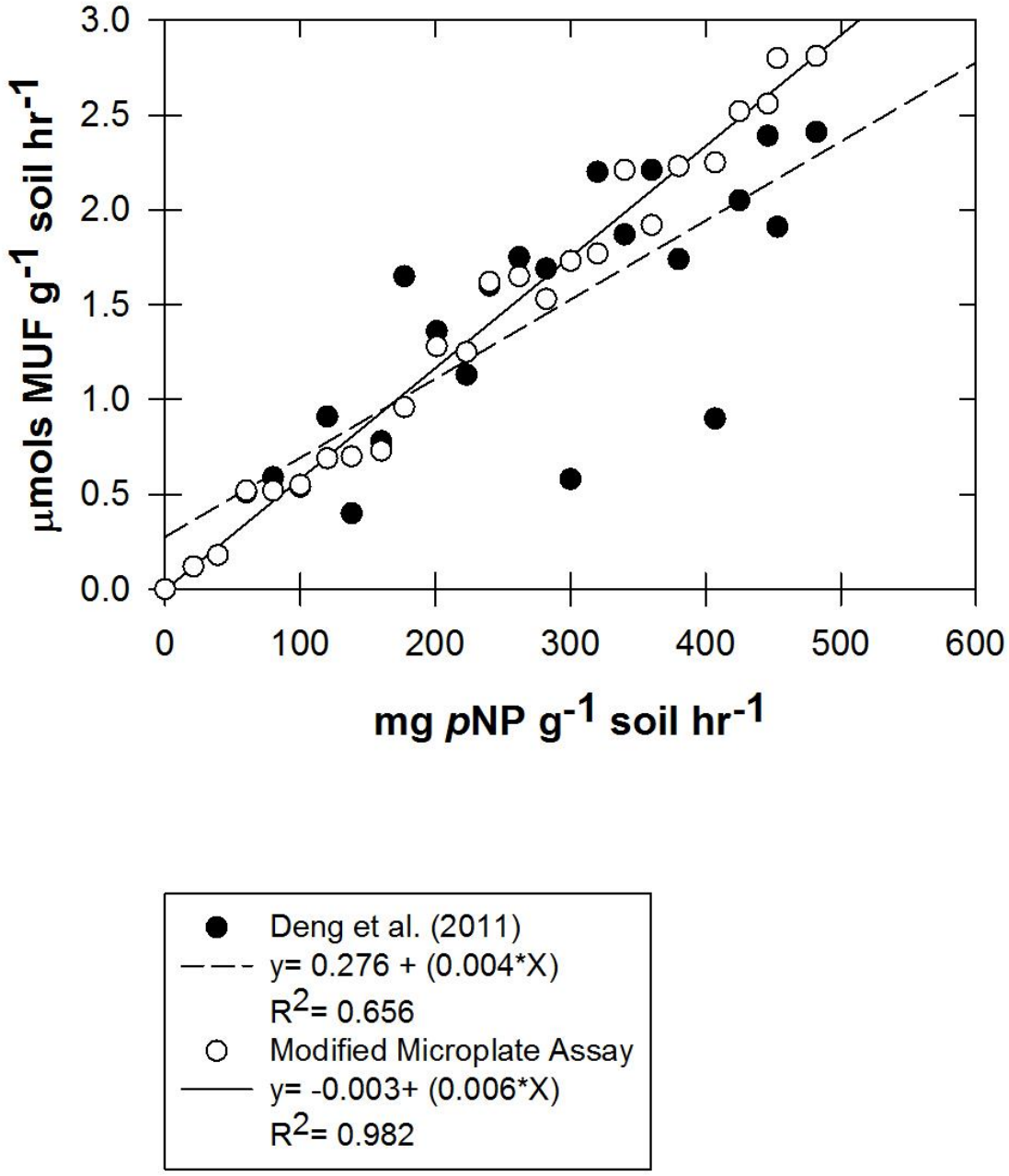

Figure 10: Linear regression comparison between the Deng et al. (2011) microplate assay and the modified microplate assay presented in this paper. The modified enzyme assay has a tighter correlation (R^2=0.982) to the reference assay than the Deng et al. (2011) (R^2= 0.656) indicating the compatibility of the modified assay to the reference assay.

CHAPTER 3

Microplate-scale fluorometric soil enzyme assay for impacts of nitrogen fertilizer and crop rotation

ABSTRACT

Soil extracellular enzyme activities reflect soil microbial functioning and can be used as indicators of soil quality. The impacts of crop rotation complexity and nitrogen (N) fertilizer addition was assessed using a modified fluorometric assay for β-glucosidase activity in soils under corn (*Zea mays* L.) in a long-term rain-fed, no-till system in eastern Nebraska. The effects of soil processing/storage and crop inputs (e.g. grain yield, stover yield, total aboveground biomass) were also evaluated relative to soil β-glucosidase activity. Crop rotation treatments included continuous corn monoculture (CC), a two-year corn-soybean [*Glycine max* (L.) Merr.] rotation (CS), and two four-year rotations consisting of corn-soybean-grain sorghum [*Sorghum bicolor* (L.) Moench]-oat [*Avena sativa* (L.)]/clover mixture [80% *Melilotus officinalis* Lam. +20% *Trifolium pretense* L.] (CSGO), or corn-oat/clover-grain sorghum-soybean (COGS). Nitrogen treatments were 0 kg N ha^{-1} yr^{-1} and 90 kg N ha^{-1} yr^{-1}. Four-year crop rotations increased grain yield, stover yield and total aboveground biomass, and fertilizer N addition increased yields and biomass across all cropping systems. Soil β-glucosidase activity was not different between thawed fresh and air-dried soils, but fertilizer N differences were only significant in air-dried soils. The positive linear relationship between β-glucosidase and grain yield support the use of enzymes as a soil quality indicator.

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INTRODUCTION

Soil enzyme activities reflect changes in soil organic matter, soil physical properties, and microbial activity, thus are indicative of soil productivity (Dick et al., 1996; Alves de Castro Lopes et al., 2013). As soil biological indicators, soil enzymes are more sensitive than the chemical and physical properties of the soil (Alves de Castro Lopes et al., 2013). The sensitivity to soil biotic and abiotic characteristics allow enzymes to be considered an early indicator of soil quality (Pankhurst et al., 1995; Sinsabaugh et al., 2008; Deng et al., 2013). By monitoring changing levels of metabolic enzymes, ecosystem disturbances from human interference and cultivation can be measured (Pankhurst et al., 1995; Deng et al., 2013). Enzymes selected for soil quality indicators should be easily quantified and have the ability to reflect differences in ecosystems and anthropogenic activity (Tate III, 2002). Because carbon (C) is an important energy source for microbes, β-glucosidase (β-G), an important cellulose enzyme, is an ideal enzyme to monitor.

β-glucosidase is a constitutive, well documented enzyme that hydrolyzes cellobiose to glucose, a simple sugar that acts as an important secondary energy source (Chrøbst, 1989; Marx et al., 2001; Caldwell, 2005). β-glucosidase is pivotal in the degradation of cellulose, an important process in the cycling of soil organic matter (Turner et al., 2002). β-Glucosidase controls the rate of cellulose decomposition, making it among the best indicators of C cycling (Bisaria and Ghose, 1981; Saha et al., 1994; Kumar et al., 2008). Changes in β-G activity can be easily detected in managed ecosystems within a relatively short period (e.g. 1 to 3 years) (Bandick and Dick, 1999; Knight and Dick, 2004) and is considered stable with low seasonal variability (Knight

and Dick, 2004; Moscatelli et al., 2012) unlike other biological methods that may fluctuate within a short period of time (Knight and Dick, 2004). The stability and low variability of β –G makes it an excellent early indicator of organic matter changes and turnover rates (Moscatelli et al., 2012). The effects of storage treatments on soil enzyme activity is a concern (Lee et al., 2007; DeForest, 2009; Burns et al., 2013) and several options of storage conditions have been suggested (Turner, 2010; Wallenius et al., 2010). Ultimately, enzyme response to storage conditions is highly dependent on enzyme and soil type (Bandick and Dick, 1999), but β-G has indicated little activity fluctuations, regardless of storage condition (Lee et al., 2007).

The reliance of microbial communities on plant C stimulates microbial enzyme synthesis, which also results in the release of valuable plant needed minerals such as nitrogen (N), phosphorous (P) and other minerals as organic matter decomposes (Singh et al., 2004). Cover crops been noted to affect the activity and composition of microbial communities (Alvey et al., 2003; Marriott and Wander, 2006; Smukler et al., 2008; Kallenbach and Grandy, 2011; Bowles et al., 2014a) and can shift in response to substrate availability provided by diverse crop systems (Alvey et al., 2003; Singh et al., 2004; Cenini et al., 2016). Due to C:N:P ratio constraints on microbial biomass (Cleveland and Liptzin, 2007), specific enzymes are generated dependent on the most limiting nutrient needed to meet microbial demands (Sinsabaugh et al., 2008; Allison et al., 2011). In the presence of inorganic N, β-glucosidase activity increases in grassland and forest soils (Ajwa et al., 1999; Saiya-Cork et al., 2002; Tiemann and Billings, 2011; Bowles et al., 2014a) and conventionally managed agricultural systems (Bandick and Dick, 1999). Long-term N additions have been correlated to higher soil C content, which then drives

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positive β-G activity responses to increased C substrate availability (Cenini et al., 2016). Nitrogen fertilization stimulated the production of C-containing compounds in the soil, derived from plant root decomposition and root exudates, which enhance the decomposition of stable forms of organic matter, stimulating mineralization of C-containing compounds, and contributing to the incorporation of soil forms into stable soil fractions (Mergel et al., 1996; Cenini et al., 2016).

Monitoring soil enzyme activity as an added measure of crop rotation and N management productivity and efficiency can provide valuable information on microbial community activity. The unique long-term, four crop rotation can provide valuable information on soil management practices. The objective of this paper was to evaluate the effect of monoculture, two-year and four-year cropping rotations under different N management on β-glucosidase activity during the R3 stage of corn production. The response of β-glucosidase to different storage effects was then assessed to determine soil storage requirements and relationship responses to air-dried and fresh frozen soil samples.

MATERIALS AND METHODS

Site Experimental Design and Soil Sampling

Soil experimental design and sampling were performed as outlined in Sindelar et al. (2015). Briefly, soils were collected from an experiment farm in Saunders County County (Ithaca, NE, USA; lat: 41.167667, long:-96.419457). This long-term, no-till site has two dominant soil types: Yutan silty clay loam and Tomek silt loam. Site elevation is 366 m with a mean temperature of 9.82 °C and a mean precipitation of 83.56 mm at time of sampling.

For this study, a randomized complete block design was used with a split plot arrangement for five replicates. Crops included corn [*Zea mays* L.], soybean [*Glycine max* (L.) Merr.], sorghum [*Sorghum bicolor* (L.) Moench] and an oat/clover mix [80% *Melilotus officinalis* Lam. +20% *Trifolium pretense* L.]. Crop rotations included continuous corn (CC), a two year rotation of corn-soybean (CS), and two four year roations: corn-soybean-oat/clover-sorghum (CSGO) and corn-sorghum-soybean-oat/clover (COGS). Each block contained on cropping rotation and was split into plots with different N managements (0, 90, 180 kg N ha^{-1} yr^{-1} for corn and grain sorghum; 0, 34, 69 kg N ha^{-1} yr^{-1} for soybean and oat/clover).

Details concerning planting times, seed inoculations, fertilizer N (urea CH_4N_2O), crop hybrids, and herbicides applications are extensively outlined in Sindelar et al. (2015). Once crops had matured, dry matter was collected for corn, soybean and grain sorghum. Corn ears and sorghum heads were removed then plant matter was stripped, homogenized and dried at 60 °C to a constant mass then weighed. Corn ears and sorghum heads were then dried, threshed and weighed for total aboveground biomass calculations (Sindelar et al 2015).

Surface soil samples (0-20 cm) were collected in August 2014 from all crop rotation treatments in only plots under corn in the 0 and 90 kg N ha^{-1} yr^{-1} treatments (0N, 90N, respectively) (n=40). Soil sampling time corresponded with the R3 developmental stage of corn. Step-down soil probes (2.2 cm diameter) were used to collect 10 soil cores in inter-row microsites and 5 soil cores in-row between plants to represent a 2:1 ratio of inter-row:row microsite types within the plot. All 15 cores were composited and homogenized per plot, stored in zip-lock bags, and transported on ice to the laboratory. In

the laboratory, soil samples were sieved through a 2 mm screen. Approximately 100g of fresh soil was frozen immediately at 4°C until prior to analysis. Soil water content was determined in a subsample by drying to constant mass at 105 °C.

Soil β-glucosidase activity assay

The activity of β-glucosidase was determined with a modified microplate assay using 4-methylumbelliferone (MUF) substrate (Deng et al., 2011; Dick et al., 2013). The assay was conducted on thawed fresh frozen soil samples (4°C) and on thawed samples that were allowed to air-dry. Frozen samples were removed from the freezer, weighed and allowed to acclimate to room temperature (~30 mins) before analysis. Solutions and standards were created as described in Deng et al. (2011) except only the 10 μM and 50 μM β-glucosidase standards were created. Solutions were made the previous day and stored in the refrigerator until analysis. Prior to analysis, solutions were warmed in a water bath at 37°C. An automated Precision Microplate Pipette System™ (BioTek Instruments, VT, USA) was used to fill all microplate wells with 50 μL modified universal buffer (MUB) and calibration wells with standards. This method is a modified version of Deng et al. (2011) by optimizing soil slurry, adding an automated pipetting system and using a 3-point calibration curve.

Briefly, a 1-g sample was placed in a 150 mL beaker and 120 mL deionized water was added to the sample. The samples were sonicated for 120 s at 15 watts (W) to homogenize samples and improve enzyme extraction. The soil solution was mixed continuously until sample aliquots were taken for analysis (~2 mins). A four-channel pipette was used to measure 100 μL aliquot of soil slurry and added to a 96-well

microplate containing 50 µL modified universal buffer (MUB, pH 6.0). Methylumbelliferone-labeled substrate (4-methylumbelliferone) (MUF-G) or methylumbelliferone standard (methylumbelliferone sodium salt) (MUF) were added to appropriate wells and thoroughly mixed by aspirating and dispensing well contents. Plates were covered and placed in a water bath set at 37°C. MUF-G was added to control columns and enzyme activity was quenched after ~3 minutes using tris(hydroxymethyl)aminomethane (THAM). The relative fluorescence was measured using a fluorescence microplate reader (365 nm excitation and 450 nm emission). Enzyme activity was reported as µmol MUF g^{-1} soil hr^{-1} and was calculated as outlined in Deng et al. (2011).

Data Analysis

Grain yield, stover yield, and total aboveground biomass were analyzed with a two-way analysis of variance (ANOVA) using the GLIMMIX procedure (SAS v. 9.2, Cary, NC). Rotation and N were fixed factors with treatment blocks used as a random factor. There were five replicates for each treatment combination (CC/0N; CC/90N; CS/0N; CS/90N; COGS/0N; COGS/90N; CSGO/0N; CSGO/90N). Sample handling and storage effects on β-G activities were tested using a three-way GLIMMIX procedure, where soil condition (thawed fresh, air-dried), rotation, and N were fixed effects and block used as the random factor. To evaluate the effect of soil handling and storage on the sensitivity of β-G activity to agronomic management practices, individual ANOVAs were run using GLIMMIX on each thawed fresh and air-dried soils. Normality was tested using the Shapiro-Wilks statistic, and significant treatment effects were further evaluated using Tukey multiple comparisons tests. Linear correlations were evaluated between β-G

activities and crop growth parameters (grain yield, stover yield, total aboveground biomass). All statistical tests were considered significant at $p \leq 0.05$.

RESULTS

Grain yield, stover yield, and total aboveground biomass results were affected by crop rotation ($p < 0.0005$) and fertilizer-N addition ($p < 0.0001$) (Table 1), but with no interactive treatment effects. Significantly higher grain yield, stover yield, and total aboveground biomass was consistently found under COGS rotation when compared to CC (Table 2), and crop responses in CS and CSGO did not differ. All yields increased under 90N fertilizer applications for all rotations.

There were no significant differences in enzyme activity between thawed fresh or air-dried soils ($p = 0.1511$) (Table 3). Fresh soil enzyme activity was significantly affected by rotation ($p < 0.0001$) only, with soils under the four-year cropping rotations having significantly higher activity than the monoculture system (Figure 1). Air-dried soil enzyme activity indicated an effect on rotation ($p < 0.05$) and nitrogen management ($p = 0.0089$). Nitrogen fertilizer was only significantly different in AD soils (Figure 2). Four-year crop rotations also had the highest enzyme activity levels but CSGO was only significantly different than CC.

Thawed fresh soils were positively correlated to dry matter grain yields (Figure 6) though the relationship was stronger in air-dried soils (Figure 3). Stover yield had no correlation with either soil condition (Figure 4 and 7) which influenced total aboveground

biomass relationships (Figure 5 and 8) as total aboveground biomass was the sum of dry matter grain yields and stover yields.

DISCUSSION

Analysis of both air-dried and fresh soils indicated no significant differences between β-G activities and the relationship between the two soil conditions revealed the same relationships between the cropping rotations. Both conditions highlighted differences between diverse cropping systems and monoculture cropping system as observed by other research (Yin et al., 2010; Vargas Gil et al., 2011; Alves de Castro Lopes et al., 2013) and responded to N-fertilizer additions (CITES?), supporting use of β-glucosidase as a bioindicator of soil health.

Both crop yield and enzyme analysis resulted in the higher activity under diverse cropping rotations in comparison to monoculture systems, similar to other studies (Bandick and Dick, 1999; Knight and Dick, 2004; Moscatelli et al., 2012) . In this study, COGS crop rotation resulted in the highest yields and CC was significantly lower, consistent with other research comparing diverse cropping systems and monoculture cropping systems (Alvey et al., 2003; Xuan et al., 2012). In some studies, crop rotation had no effect on bacterial communities (Navarro-Noya et al., 2013) while others have identified an influence on bacteria (Alvey et al., 2003; Salles et al., 2004; Xuan et al., 2012) as well as soil fungal communities (Zhang et al., 2014), but none have investigated a four crop rotation system.

The impacts of a diverse cropping system may be due to intricate symbiotic relationships that occur between microbial communities and plant rhizosphere. Microbial

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community shifts in the rhizosphere have been associated with plant species, soil types and root structure (Marschner et al., 2001; Berg and Smalla, 2009). Plant-associated shifts in microbial community structure, deemed the "legacy effect", have been identified in various studies (Alvey et al., 2003; Vargas Gil et al., 2011) and may contribute to higher yields in diverse cropping systems (Vargas Gil et al., 2009) alongside suppression of disease (Singh et al., 2004; Mendes et al., 2011) and weed control (Narwal and Haouala, 2006; Mhlanga et al., 2015). Diverse crop rotations allow different plants to release specific compounds in the rhizosphere (Vargas Gil et al., 2009) contributing to a diverse microbial community. The linear relationship established in both dry and thaw-fresh soil reflected a positive linear relationship indicating a positive trend associated with higher yields and higher enzyme activity. In corn, community structure has been strongly linked to corn growth stages (Li et al., 2014), most likely driven by labile substrate availability (Fierer et al., 2007; Goldfarb et al., 2011). Corn yields were significantly impacted by diverse yields (Figure 1) and impacted the activity of β-G under both soil conditions.

In addition to microbial shifts as a response to substrate availability, enzyme activity and crop yields positively responded under fertilized conditions, similar to other N management studies (Ajwa et al., 1999; Henriksen and Breland, 1999; Carreiro et al., 2000; Saiya-Cork et al., 2002; Geisseler and Horwath, 2009; Tiemann and Billings, 2011; Fraser et al., 2013; Bowles et al., 2014b; Cenini et al., 2016), but significant differences between fertilizer-N amendments were only identified under air-dried soil conditions. The response of enzyme activity to N additions correlate to higher soil C content which cause a positive β-G activity response due to new substrate availability (Cenini et al.,

2016). Though this same effect of substrate availability on β-G was expected under fresh conditions, sample condition or microbial response to moisture added within the assay may have caused early enzyme activity and lead to a lack of enzyme activity. Fresh soil samples did not have a high soil water content, but small ice crystal did form. Samples were allowed to reach room temperature (~30 mins) prior to analysis and liberation of water from the ice crystals may have provided contact between soils enzymes and available C substrates in the soil resulting in enzyme activity or predation prior to analysis.

Soil storage for enzyme analysis has been greatly debated with emphasis on intended use of the data collect and the enzyme in question (Burns et al., 2013). The effects of storage treatments on soil enzymes has been heavily studied (Lee et al., 2007; DeForest, 2009; Burns et al., 2013) and is often dependent on enzyme and soil type (Bandick and Dick, 1999). β-G enzyme activities were generally lower in AD than FR soils, consistent with other results (Bandick and Dick, 1999; Lee et al., 2007). Microbial C has been shown to be significantly affected by drying and storing soil at room and 4°C, respectively (Lee et al., 2007) which could impact enzyme activity levels.

Drying soil often decreases soil water potential and increases all osmotic potential that may further affect enzyme activities (Lee et al., 2007). Microbial pulse responses associated with re-wetting of soil has been noted in many studies postulated to be a result of substrate exposure due to lysed cells (Pesaro et al., 2003) or soil disruption of stabilized enzymes to substrates (Tabatabai, 1994; Xiang et al., 2008). Similarly, freeze-thaw storage of soil samples can also cause cell lysis, though β-G has reported no effect (DeForest, 2009). Fresh soil samples were allowed to reach room temperature (~30 min)

prior to enzyme analysis which may have stimulated enzyme activity due new substrate availability and microbial response. Bacteria have be shown to respond to moisture changes in less than 15 mins (Halverson et al., 2000) while fungi and spore germination can activate within 10-30 mins of water contact (Metz et al., 2011). Air-dried soil was only exposed to water directly prior to analysis (~7-10 mins), resulting in minimal microbial community growth prior to analysis, and potentially greater microbial community growth during incubation.

CONCLUSION

Enzyme synthesis, as a response to substrate available, adds an important view of microbial community activity and is an important component of soil quality. In this study, we showed air-dried soil samples are able to identify the same crop rotation relationships as fresh soil samples, providing easier storage and time requirements for an industrial laboratory setting. The ability to detect fertilizer-N amendments can be attributed to microbial stimulation when fresh soils were allowed to acclimate to room temperature. Microbial stimulation in air-dried soils may have occurred during incubation and high substrate availability enabling the fertilizer-N amendment to be detected. Ultimately, diverse crop rotations were shown to have a higher enzyme activity and provide a higher yield that monoculture systems, supporting the theory of diverse microbial systems occur under diverse cropping systems. Though enzyme activities alone cannot describe soil decomposition processes within the soil, it can provide valuable information about specific organic compounds within the soil under different management systems.

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Effect	Air-dried β-G Activity (μmol g^{-1}soil hr^{-1})	Fresh β-G Activity (μmol g^{-1} soil hr^{-1})	Grain Yield (kg ha^{-1})	Stover Yield (kg ha^{-1})	Total Biomass (kg ha^{-1})
Rot	0.0072	<.0001	0.0007	0.0307	0.0022
N	0.0089	0.1553	<.0001	<.0001	<.0001
Rot*N	0.2058	0.6368	0.5879	0.2479	0.6331

Table 1: Significance of P values for BG activities, and dry matter (0% moisture) grain yields, stover yields and total aboveground biomass.

	Dry Matter Grain (kg ha^{-1})		Stover yield (kg ha^{-1})		Total Dry Matter (kg ha^{-1})	
Fertilizer N **Crop Rotation†**	0N	90N	0N	90N	0N	90N
CC	1958	5971	2632	5277	4590	11248
CS	4738	7925	4511	5797	9249	13722
COGS	5509	8188	4471	6578	9980	14766
CSGO	4753	7687	4106	5243	8859	12931

†CC, continuous corn; CS, corn-soybean; COGS, corn-oat/clover-grain sorghum-soybean; CSGO, corn-soybean-grain sorghum-oat/clover

Table 2: Dry matter (0% moisture) yields for grain, stover and total dry matter response to crop rotation and fertilizer-N.

Cropping Rotation	Fertilizer-N (kg N ha^{-1})	Fresh β-G Activity (μmol g^{-1} soil hr^{-1})	Air-dried β-G Activity (μmol g^{-1}soil hr^{-1})
CC	0	0.64 ± 0.04	0.56 ± 0.03
	90	0.74 ± 0.04	0.73 ± 0.05
CS	0	0.66 ± 0.06	0.61 ± 0.02
	90	0.84 ± 0.02	0.93 ± 0.07
CSGO	0	1.17 ± 0.04	1.00 ± 0.08
	90	1.13 ± 0.06	1.09 ± 0.06
COGS	0	0.93 ± 0.08	0.89 ± 0.04
	90	1.04 ± 0.06	0.90 ± 0.05

Table 3: β-G enzyme activities (±SD) for air-dried and fresh soils under different crop rotations and fertilizer-N managements.

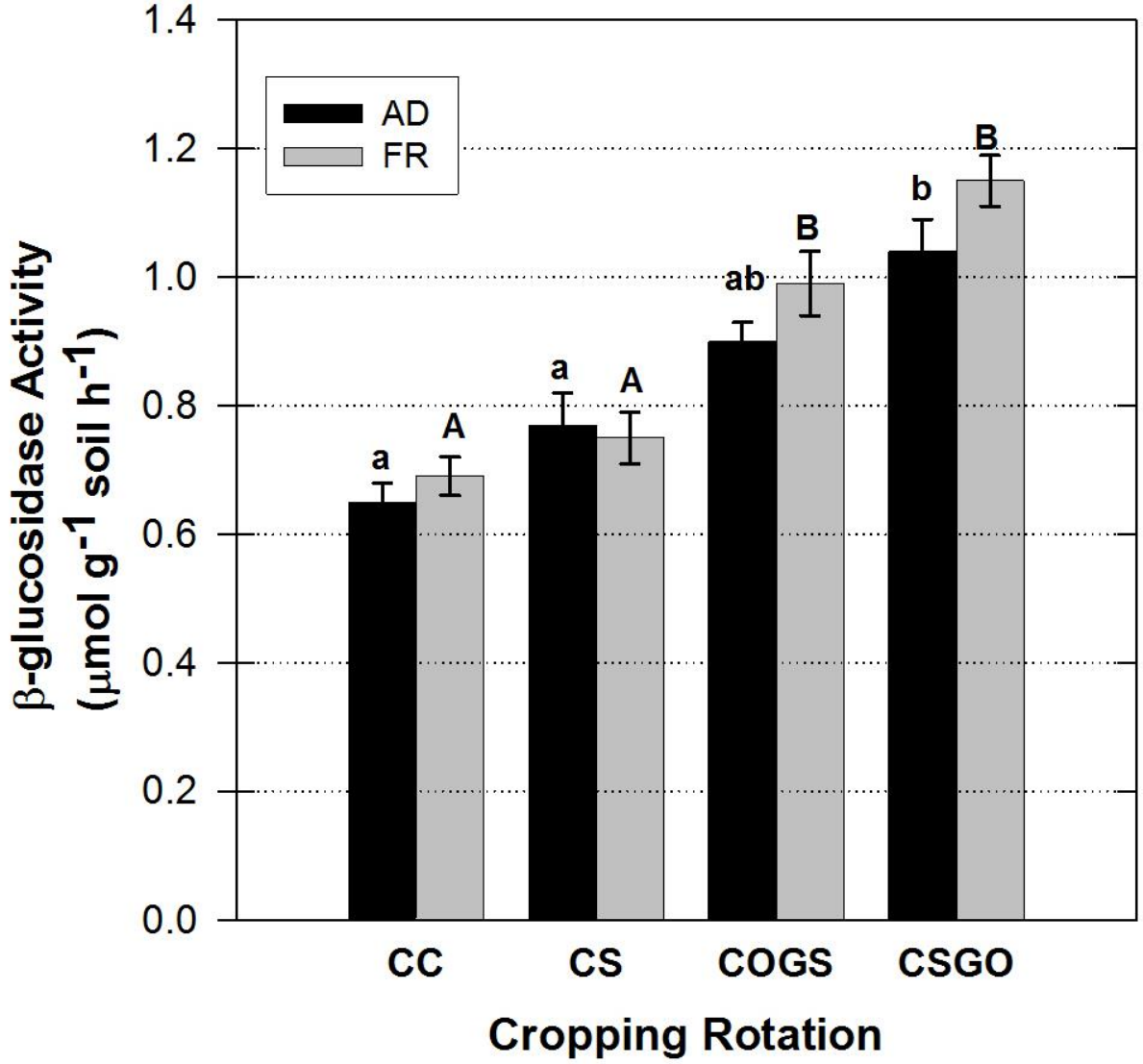

Figure 1: Air-dried (AD) and fresh (FR) soil enzyme activities for all four cropping rotations. No statistical differences were found between AD and FR soils (p = 0.1511) although different relationships were apparent under different soil conditions. In both conditions, diverse cropping systems had higher enzyme activity then monoculture systems.

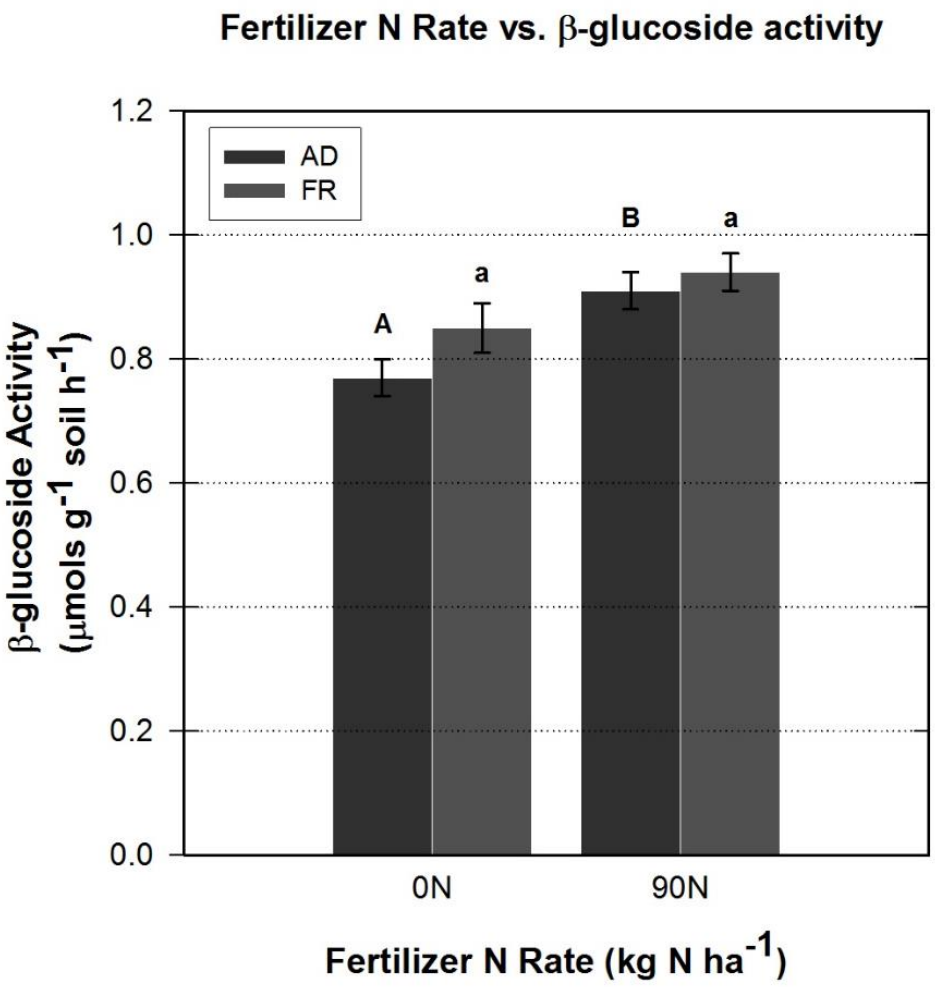

Figure 2: β-glucosidase enzyme activity detected for air-dried (AD) and fresh (FR) soils under different fertilizer-N treatments. Statistical differences were only noted under AD conditions (p = 0.0089) and is attributed microbial stimulation.

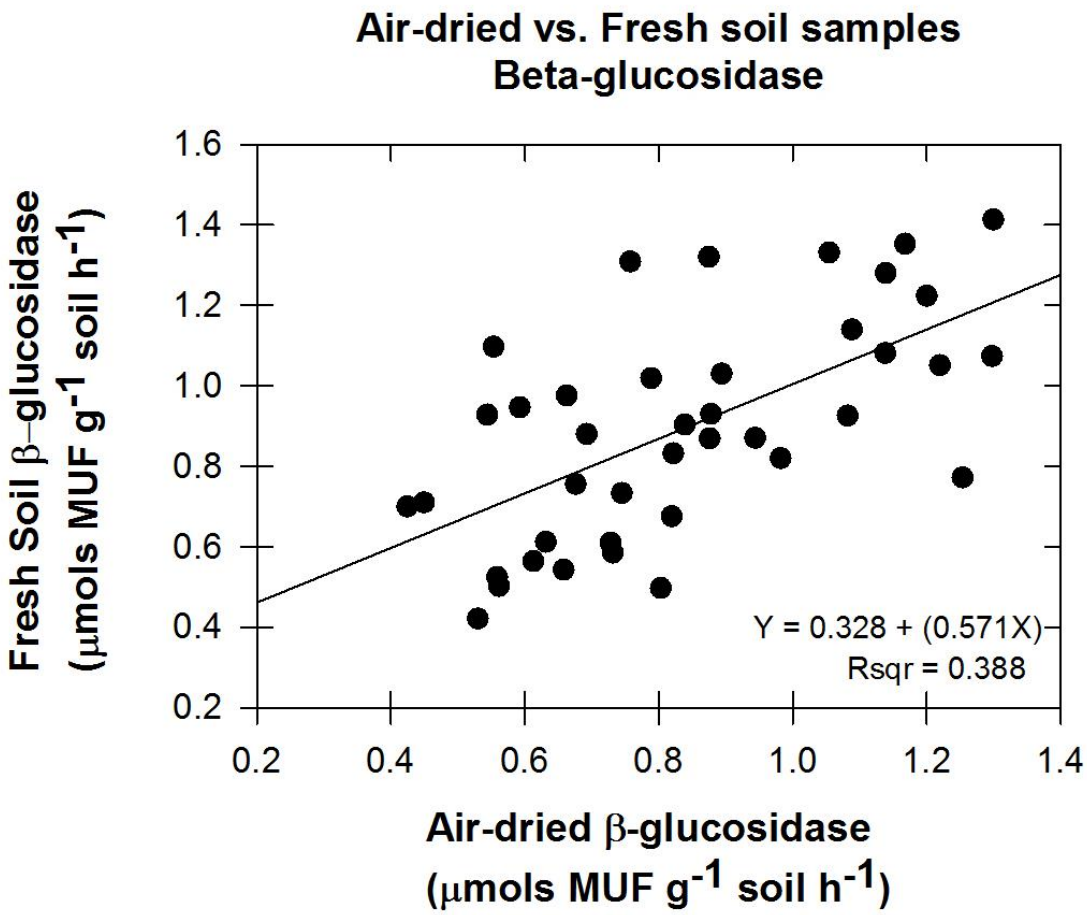

Figure 3: Linear regression analysis of fresh and air-dried on β-glucosidase activities indicating air-dried soils showed only 57% of activity compared to fresh soil samples.

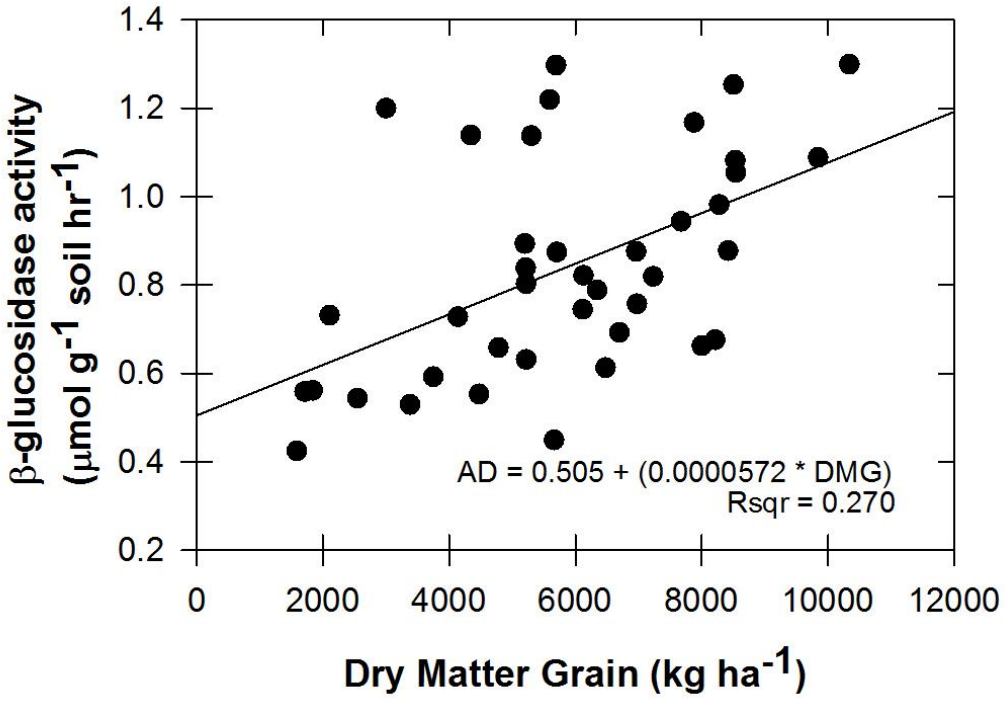

Figure 4: Air-dried β-glucosidase enzyme activity associated with dry matter grain.

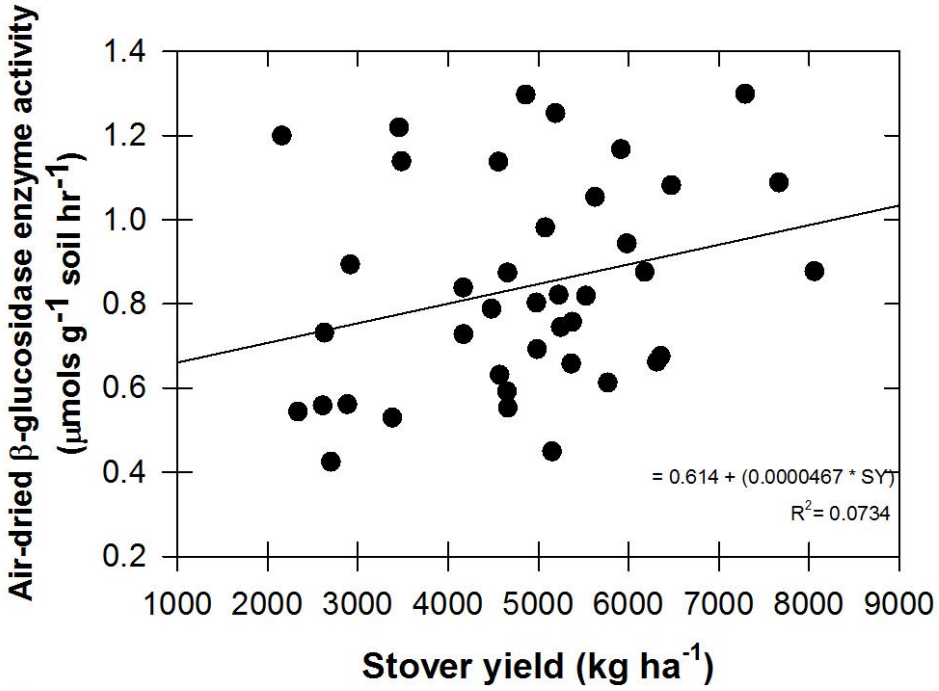

Figure 5: Air-dried β-glucosidase enzyme activity associated with stover yield.

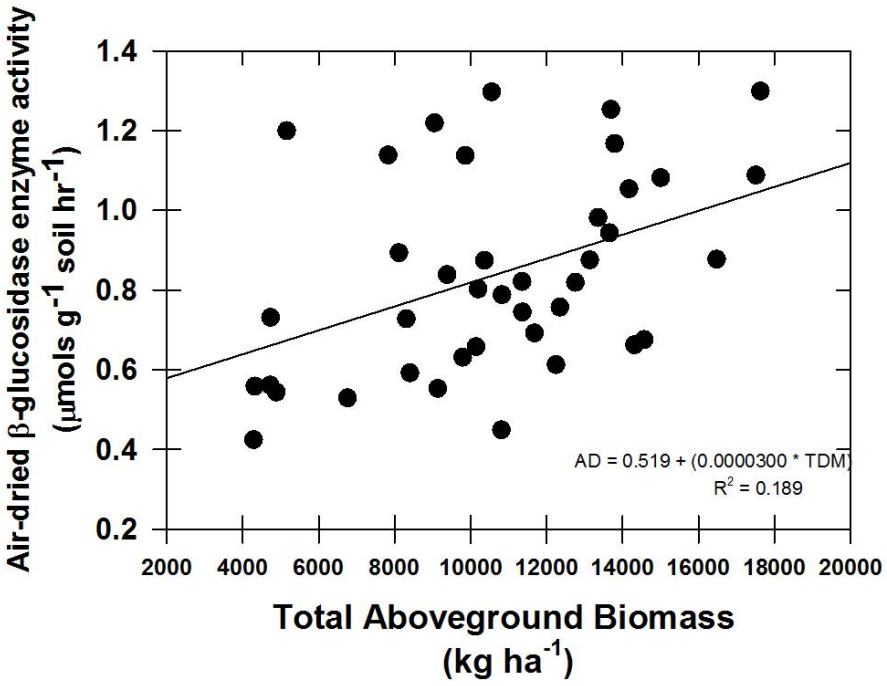

Figure 6: Air-dried β-glucosidase enzyme activity associated with aboveground biomass.

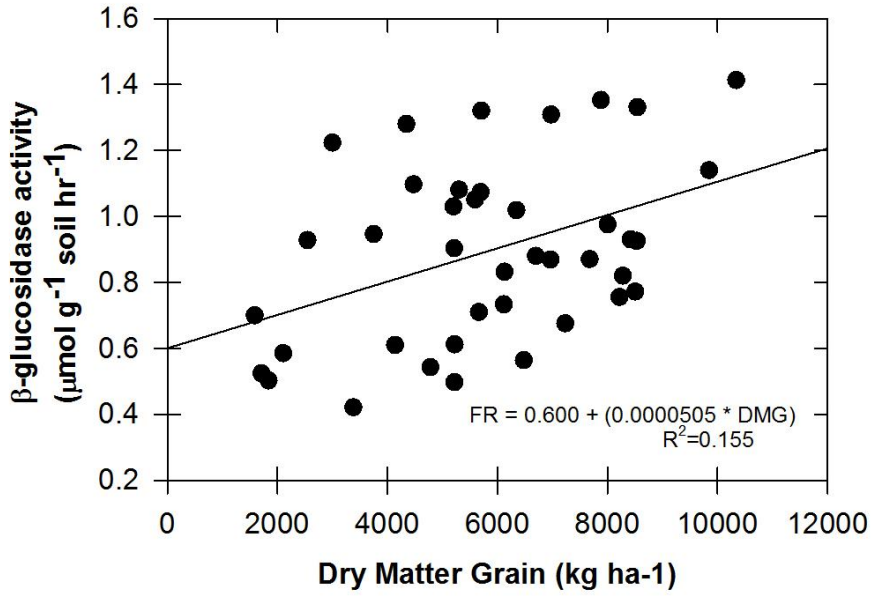

Figure 7: Thaw-fresh β-glucosidase enzyme activity associated with dry matter grain.

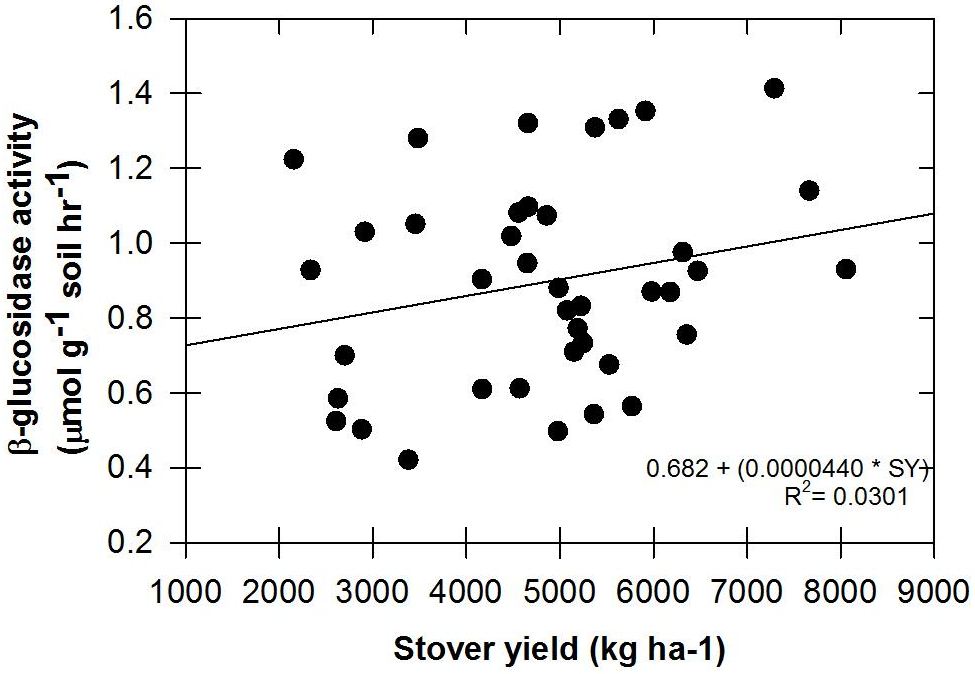

Figure 8: Thaw-fresh β-glucosidase enzyme activity associated with stover yield.

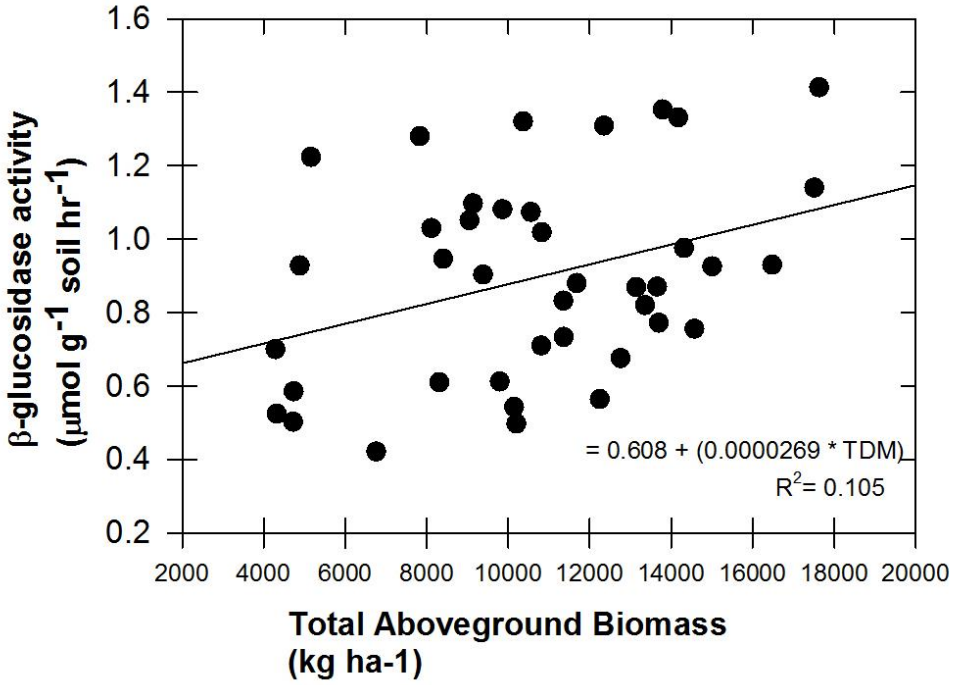

Figure 9: Thaw-fresh β-glucosidase enzyme activity associated with total aboveground biomass.

APPENDIX I

SOIL ENZYME ASSAY PROTOCOL FOR KELLOG SOIL SURVEY LABORATORY

Soil Analysis

Soil Enzymes

methylumbelliferone (MUF)

β-glucosidase

Air-Dry, <2mm

Field-Moist, <2mm

1. Application

Soil enzymes are sensitive to changes in soil organic matter, soil physical properties, microbial activity and are indicative of soil productivity (Dick et al., 1996; Alves de Castro Lopes et al., 2013) and thus act as an early indicator of soil quality (Pankhurst et al., 1995; Sinsabaugh et al., 2008; Deng et al., 2013). Disruption of soil microbial activities seen through changing levels of metabolic enzymes can help estimate ecosystem disturbance caused by human interference and cultivation (Pankhurst et al., 1995; Deng et al., 2013) and are more sensitive than the chemical and physical properties of the soil (Alves de Castro Lopes et al., 2013). Enzyme activities selected for soil health indicators should be easily quantified and have the ability to reflect differences in ecosystems and anthropogenic activity (Tate III, 2002).

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The β –glucosidase enzyme (BG, EC 3.2.1.21) is a constitutive and well document enzyme (Chrøbst, 1989; Marx et al., 2001; Caldwell, 2005) pivotal in the degradation of cellulose, an important process in the cycling of soil organic matter. Changes in β-glucosidase activity can be easily detected in managed ecosystems within a relatively short period (1-3 years)(Bandick and Dick, 1999; Knight and Dick, 2004). Enzyme activity is stable and reported low seasonal variability (Knight and Dick, 2004; Moscatelli et al., 2012a) unlike other biological methods that may fluctuate within a short period of time(Knight and Dick, 2004). The stability and low variability of β – glucosidase alongside it's large-scale role in C cycling and sensitivity to soil management makes BG an ideal bioindicator of soil health (Ndiaye et al., 2000; Moscatelli et al., 2012b).

2. Summary of Method

Reagents are warmed to assay temperature (37°C) in a water bath prior to analysis. A 1.0g soil sample with 8.35mL DI water is placed a sonication bath for 120s at 15W. DI water is added to 120mL. Due to quenching and interferences encountered with soil, each soil requires a separate calibration. Calibration standards (0µM, 10µM, 50µM) use methylumbelliferone sodium salt while sample standards use methylumbelliferone substrate. The calibration microplate, herein referred to as "Plate 1", is filled with 50µL Modified Universal Buffer (MUB), 100µL sample aliquot and 50µL calibration standard. Plate 1 is covered with sealing tape and water bathed for 1 hr. The sample plate, herein referred to as "Plate 2", is filled with 50µL MUB and 100µL sample aliquot. Methylumbelliferone substrate is added to odd rows in the sample microplate, covered and water bathed for 1hr. At the conclusion of the hour for Plate 1, 50µL Tris

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(hydroxymethyl) aminomethane (THAM, pH 10) is added to terminate reaction. Plate 1 is promptly read using a microplate spectrophotometer at 360nm excitation and 460nm emission. The gain setting for the assay is determined using the autogain setting on Plate 1. At the conclusion of the hour for Plate 2, 50µL methylumbelliferone substrate is added to even rows, and then 50µL THAM is added to terminate reaction. Plate 2 is read promptly using the same settings from Plate 1. Data are reported as µmol MUF g^{-1} dry soil h^{-1}.

3. Interferences

The β-glucosidase assay can be determined on both air-dry and field-moist samples though air-drying makes handling easier. Enzyme activities are quantified through fluorometric methods using methylumbelliferone (MUF) labeled substrates that are easily cleaved by soil enzymes. All soil enzymes have an optimal temperature, specific substrates and specific pH ranges. The sensitivity of enzymes require controlled temperature and pH to produce maximum enzyme activity (Tabatabai, 1994). Fluorometric analysis requires the use of a soil specific calibration curve due to soil interferences with activity readings. Proper pipetting technique and understanding of the basic principles of enzyme assays is required for proper execution of soil enzyme assays by technicians. The ability to automate a large proportion of soil enzyme assays limits potential technician error and aids in ease of analysis.

4. Safety

Wear protective clothing (coats, aprons, sleeve guards, and gloves) and eye protection (face shields, goggles, or safety glasses) when preparing reagents, especially concentrated acids and bases. Dispense concentrated acids and bases in a fume hood.

Thoroughly wash hands after handling reagents. Use safety showers and eyewash stations to dilute spilled acids and bases. Use sodium bicarbonate and water to neutralize and dilute spilled acids. Standard laboratory equipment includes fire blankets and extinguishers for use if necessary. Follow the manufacturer's safety precautions when using the spectrophotometer.

5. Equipment

5.1 Electronic balance, ±0.01g, ±0.0001g sensitivity

5.2 Volumetric flasks, 50mL, 100mL, 1000mL

5.3 Plastic bottle, amber, 1000mL

5.4 Eight-channel pipette, electronic digital, 250µL

5.5 Pipette, electronic digital, 50mL

5.6 Sonication bath

5.7 Nine-position magnetic mixing plate, 600 rpm

5.8 Precision Microplate Pipetting System, BioTek

5.9 Water bath, 37°C

5.10 Synergy H1 Multi-Mode Reader, BioTek

5.11 Disposable pipettes

5.12 Magnetic stir bars, 3.75cm

5.13 Disposable Tips, 200µL

5.14 Beakers, 150mL

5.15 96-well Microplates

5.16 Corning Microplate Sealing Tape

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6. Reagents

6.1 Reverse osmosis deionized (RODI) water, ASTM Type I grade of reagent water

6.2 Hydrochloric acid (HCl), concentrated, 12 N, trace pure grade

6.3 HCL, 1M. Carefully add 16.7mL HCl in 200mL RODI

6.4 0.5 M NaOH, 20.0g NaOH dissolved in 1L RODI

6.5 Modified universal buffer (MUB). Dissolve 12.1g tris (hydroxymethyl) aminomethane (THAM), 11.6g maleic acid, 14.0g citric acid, and 6.3g boric acid in 800mL 0.5 M NaOH. Adjust to 1 L with 0.5 M NaOH and store at 4°C.

6.6 MUB working solutions (pH 6.0). Place 200mL MUB stock solution in 1-L beaker containing a magnetic stir bar. Place on stir plate and titrate to pH with 1M HCl while stirring. Adjust the volume to 1L with DI water.

6.7 Methylumbelliferyl substrates (2mM). Weigh 0.068g methylumbelliferyl-β-D-glucoside (MUF-G; Sigma Aldrich M3633) into a 100mL volumetric flask and adjust the volume to 100mL with DI water. Store in the dark at 4°C. Prepare new solutions after 3 days.

6.8 THAM (0.1 M, pH 10). Dissolve 12.1 g Tris (hydroxymethyl) aminomethane (THAM) (MW 121.14) in 700mL DI water. Adjust pH to 10.0 with 1M HCl (a few drops required, typically), then adjust volume to 1 L. Store in a plastic container at room temperature.

6.9 Methylumbelliferone (MUF) stock solution (100µM). Dissolve 0.0202g 4-methylumbelliferone sodium salt (98%; M1508; Sigma Aldrich in 700mL DI water, then adjust volume to 1L. Sonicate for 30s for full dissolution. Store in the dark at 4°C for no more than 2 weeks.

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6.10 MUF working standards (10µM, 50µM). MUF standards are prepared by diluting 50mL and 10mL of MUF stock solution (100µM) in 100mL DI.

7. Procedure

7.1 Blank microplates are tested for impurities by measuring fluorescence at 360nm excitation and 460nm emission, gain set at 65. Readings greater than 20 are discarded.

7.2 Warm reagents in water bath at assay temperature (37°C) for 30mins prior to initiating assay.

7.3 Weigh 1.0g <2mm air-dry soil to nearest mg on an electronic balance and place in 150mL glass beaker

7.4 Place two microplates on Dock C and D of Precision Pipetting System to dispense 50µL buffer, 50µL 10µM Standard, and 50µL 50µM standards (See Figure 1, Program 1)

7.5 Place 8.65mL RODI water to soils samples and sonicate samples for 120s.

7.6 Add 111.35mL RODI water each sample to reach 120mL.

7.7 Using a magnetic stir bar, allow samples to homogenize while aliquots are taken.

7.8 A multichannel pipette with 4 tips is used to dispense 100µl samples into Plate 1. Ensure the wells are properly mixed by pipetting up and down several times (See Figure 2).

7.9 Cover Plate 1 with sealing tape, label with plate and time and water bath at 37°C for 1 hour.

7.10 Using a multichannel pipette with 4 tips, dispense 100µl samples into Plate 2 (See Figure 3).

7.11 Add 50µL MUF substrate to odd rows of sample plate. Ensure the wells are properly mixed by pipetting up and down several times

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7.12 Cover Plate 2 with sealing tape, label with plate and time and water bath at 37°C for 1 hour.

7.13 Following Plate 1 incubation, add 50µL of 0.1 M THAM (pH 10) to each well to terminate reaction (Program 2).

7.14 Read Plate 1 fluorescence at 360nm excitation and 460nm emission, setting the gain to AutoGain the highest calibration (Row 3).

7.15 Following Plate 2 incubation, add 50µL substrate to even rows of Plate 2.

7.16 Add 50µL of 0.1 M THAM (pH 10) to each well to terminate reaction (Program 2).

7.17 Read Plate 2 fluorescence at 360nm excitation and 460nm emission, setting the gain to the setting determined in Plate 1.

8. Calculations

-The intercept of the calibration curve should be forced through zero to prevent negative values

-Average autohydrolysis control is calculated by subtracting the average fluorescence intensity of the wells incubated with substrate added after the reaction is terminated from the average of the wells with substrate added before incubation.

$$\boldsymbol{\mu mol\ g^{-1}h^{-1}\ MUF} = \frac{\frac{\frac{(A-B)-C}{D}}{0.1}\ \mathbf{x\ 120\ x}\ \left(\frac{1}{10^6}\right)}{E/_F\ \mathbf{x\ 1}}$$

where:

A= Average raw sample florescence intensity read from Plate 2 for sample

B= Average raw control florescence intensity read from Plate 2 for sample

C= Autohydrolysis control, calculated by taking the average difference between

substrate administered before and after incubation

D= slope of the calibration curve created in Plate 1 (pmols/fluorescent intensity)

0.1 Constant= Sample aliquot amount (0.1mL)

120 Constant= Amount of RODI water used to homogenize sample (120mL)

$1/10^6$ Constant= Conversion of 1pmol to 10^6 μmols

1 Constant= Water bath time (1hr)

E= Sample weight (g)

F= ADOD (g)

9. Report

Report data to the nearest 0.01 μmol MUF g^{-1} dry soil h^{-1}.

10. Precision and Accuracy

Precision and accuracy data are available from the KSSL upon request.

11. References

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12. Supplementary

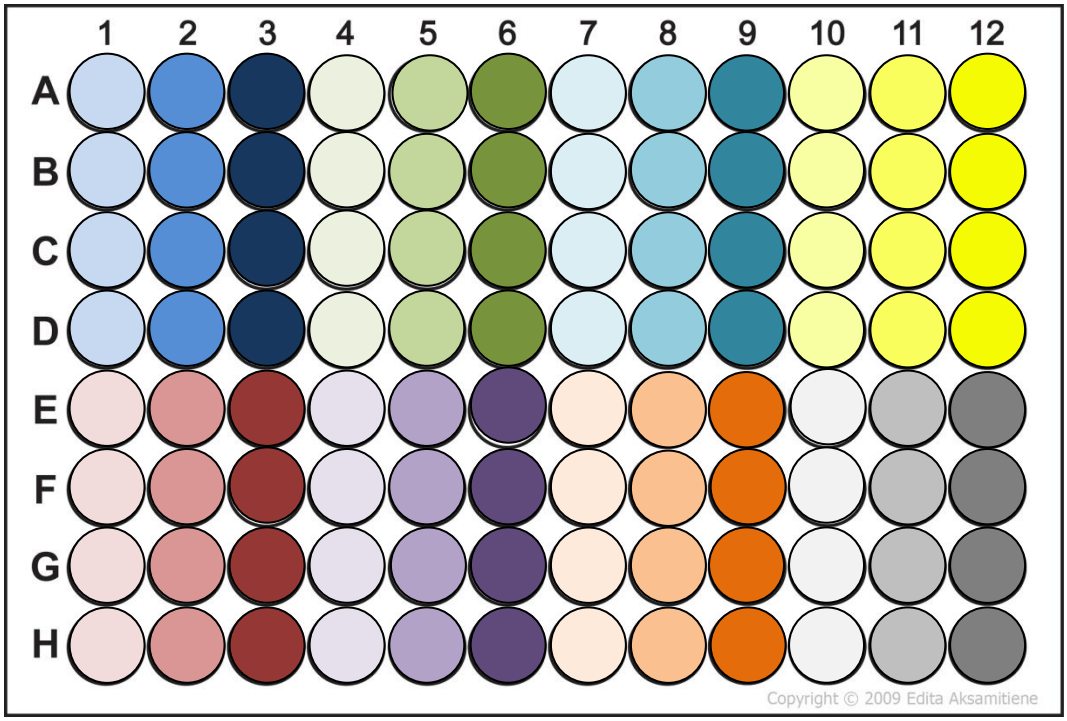

Figure 1: Plate 1 Layout (Calibration plate)

-Colors indicate different soil sample

-Light color: 50µL DI added

-Medium color: 50µL 10µM added

-Dark color: 50µL 50µM added

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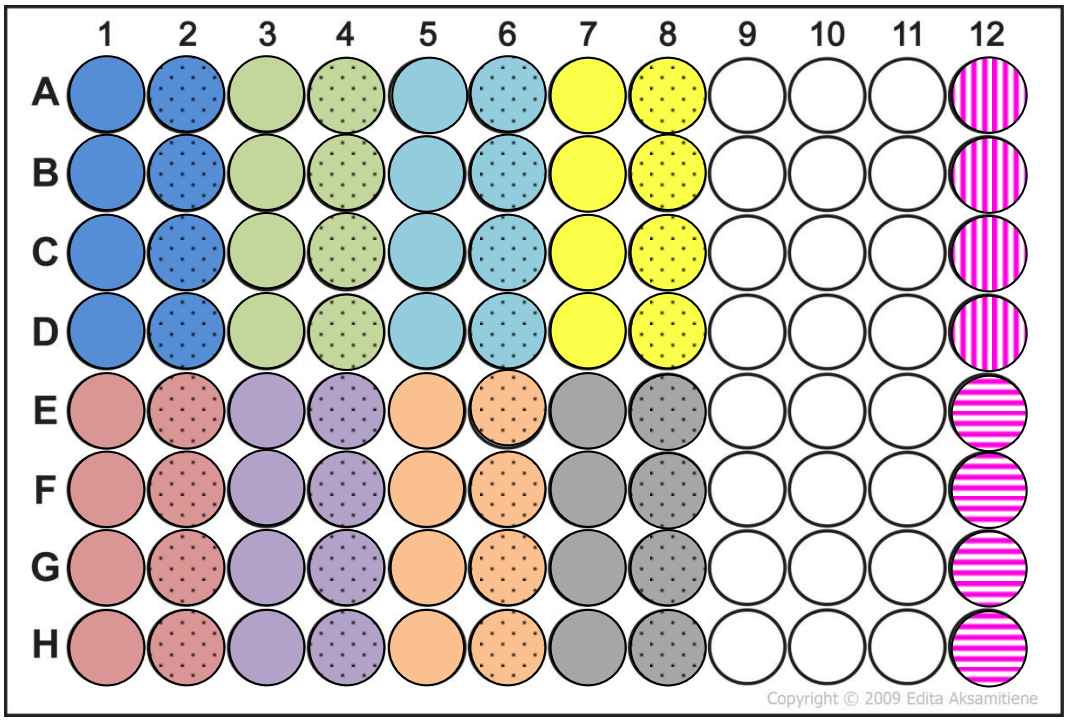

Figure 2: Plate 2 Layout (Sample plate)

-Colors indicate different soil sample

-Solid color indicate sample

-Dotted color indicate control

-Vertical lines indicate autohydrolysis with substrate before incubation

-Horizontal lines indicate autohydrolysis with substrate after incubation

Program 1: Preparation of Standard and Sample Plates (Dispense of MUB and Standards)

Instrument: Precision Power Automated Pipette System

Program: 3 Cal

Supply List:

-50mL MUB placed in Sta. B, column 1

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-50mL 10µM Standard placed in Sta. B, column 2

-50mL 50µM Standard placed in Sta. B, column 3

Sta.	Cols.	Supply	Vol
A	1-12	LABCON200ROBOTIC	-
B	4-4	R_1X4	20mL
C	1-12	CORNING96FLAT	200µL
E	1-12	CORNING96FLAT	200µL
F	1-12	CORNING96FLAT	200µL

Program Commands:

Loop Max times (Level 1)

Tips from Sta. A Colum1 (+0) using Rack for tips, waste for residual

Aspirate 50µL from Sta. B column 1 (+0) using Pre5

Dispense 60µL into Sta. C column 1 (auto-incr) using DP BG

Loop Off

Loop Max times (Level 1)

Tips from Sta. A Colum1 (+0) using Rack for tips, waste for residual

Aspirate 50µL from Sta. B column 1 (+0) using Pre5

Dispense 60µL into Sta. F column 1 (auto-incr) using DP BG

Loop Off

Loop Max times (Level 1)

Tips from Sta. A Colum1 (+0) using Rack for tips, waste for

`

residual

Aspirate 50µL from Sta. B column 1 (+0) using Pre5

Dispense 60µL into Sta. E column 1 (auto-incr) using DP BG

Loop Off

Loop 1 times (Level 1)

Tips from Sta. A column 2 (auto-incr) using Rack for tips and residual

Aspirate 50µL from Sta. B column 2 (+0) using Pre5

Dispense 60 µL into Sta. C column 2 (+0) using DP BG

Tips from Sta. A column 2 (auto-incr) using Rack for tips and residual

Aspirate 50µL from Sta. B column 2 (+0) using Pre5

DIspense 60 µL into Sta. C column 5 (+0) using DP BG

Tips from Sta. A column 2 (auto-incr) using Rack for tips and residual

Aspirate 50µL from Sta. B column 2 (+0) using Pre5

DIspense 60 µL into Sta. C column 8 (+0) using DP BG

Tips from Sta. A column 2 (auto-incr) using Rack for tips and residual

Aspirate 50µL from Sta. B column 2 (+0) using Pre5

DIspense 60 µL into Sta. C column 11 (+0) using DP BG

Loop Off

Loop 1 times (Level 1)

`

Tips from Sta. A column 3 (auto-incr) using Rack for tips and residual

Aspirate 50µL from Sta. B column 3 (+0) using Pre5

DIspense 60 µL into Sta. C column 3 (+0) using DP BG

Tips from Sta. A column 3 (auto-incr) using Rack for tips and residual

Aspirate 50µL from Sta. B column 3 (+0) using Pre5

DIspense 60 µL into Sta. C column 6 (+0) using DP BG

Tips from Sta. A column 3 (auto-incr) using Rack for tips and residual

Aspirate 50µL from Sta. B column 3 (+0) using Pre5

DIspense 60 µL into Sta. C column 9 (+0) using DP BG

Tips from Sta. A column 3 (auto-incr) using Rack for tips and residual

Aspirate 50µL from Sta. B column 3 (+0) using Pre5

DIspense 60 µL into Sta. C column 12 (+0) using DP BG

Loop Off

DIspense 50µL into Sta. C column 1 (+0) using D1D01002

DIspense 50µL into Sta. C column 4 (+0) using D1D01002

DIspense 50µL into Sta. C column 7 (+0) using D1D01002

DIspense 50µL into Sta. C column 10 (+0) using D1D01002

`

Program 2: THAM DIspense

Instrument: Precision Power Automated Pipette System

Supply List: 50mL THAM placed in Sta. B, 4th column

Sta.	Cols.	Supply	Vol
A	1-12	LABCON200ROBOTIC	-
B	4-4	R_1X4	25mL
C	1-12	CORNING96FLAT	200µL
F	1-12	CORNING96FLAT	200µL

Program Commands:

Loop Max times (Level 1)

Tips from Sta. A Colum1 (+0) using Rack for tips, waste for residual

Aspirate 50µL from Sta. B column 4 (+0) using Pre5

Dispense 60µL into Sta. column 1 (auto-incr) using DP BG

Loop Off

Program 3: Microplate Settings

Program: GB_Blank Plate Read

Instrument: Synergy H1 Microplate reader

Procedure

Detection Method: Fluorescence intensity

Read Type: Endpoint/ Kinetic

`

Optics Type: Monochromators

Read Step:

Wavelengths: 1

Excitation: 360

Emission: 460

Optics Position: Top

Gain: 65

Read Speed: Normal

Read Height: 7.00mm

Program: GB_Fluor_Test

Instrument: Synergy H1 Microplate reader

Calibration Plate

Temperature:

Incubator: On

Temperature: 37°C

Preheat before continuing with next step: selected

Shake:

Shake Mode: Linear

Duration: 0:05

Linear Frequency: 567 cpm (3mm)

Read:

Detection Method: Fluorescence intensity

Read Type: Endpoint/ Kinetic

`

Optics Type: Monochromators

Read Step:

Wavelengths: 1

Excitation: 360

Emission: 460

Optics Position: Top

Gain: AutoGain (Autoscale)

Read Speed: Normal

Read Height: 7.00mm

Sample Plate

Temperature:

Incubator: On

Temperature: 37°C

Preheat before continuing with next step: selected

Shake:

Shake Mode: Linear

Duration: 0:05

Linear Frequency: 567 cpm (3mm)

Read:

Detection Method: Fluorescence intensity

Read Type: Endpoint/ Kinetic

Optics Type: Monochromators

Read Step:

`

Wavelengths: 1

Excitation: 360

Emission: 460

Optics Position: Top

Gain: Set to match Calibration Plate

Read Speed: Normal

Read Height: 7.00mm

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