USING ENZYMES TO LINK SOIL STRUCTURE AND MICROBIAL COMMUNITY FUNCTION IN A PRAIRIE CHRONOSEQUENCE

By

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USING ENZYMES TO LINK SOIL STRUCTURE AND MICROBIAL COMMUNITY FUNCTION IN A PRAIRIE CHRONOSEQUENCE

Abstract

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Recently attention has focused on the potential of using soil as a sink for atmospheric CO_2. The objective of this study was to use soil enzymes and classical methods of soil aggregate fractionation to explore the relationship between microbial community function and soil structure of a tallgrass prairie chronosequence. The soils within the chronosequence were: (1) remnant native prairie, (2) agricultural soil, and (3, 4) tallgrass prairies restored from agriculture in 1979 and 1993. β-glucosidase (E.C. 3.2.1.21) and N-acetyl-β-glucosaminidase (NAGase, EC 3.2.1.30) assays were conducted on four different aggregate size fractions (>2 mm, 1 -2 mm, 250µm-1 mm, and 2 - 250 µm) from each soil. Specific activities for both enzymes (µg PNP g^{-1} soil h^{-1}) were greatest in the microaggregate (2 µm -250 µm) fractions across the chronosequence; however, this size fraction makes up only a small proportion of the whole soil. Therefore, it is the larger macroaggregate-derived enzyme activities that have the greatest impact on the activity of

the whole soil. Analyzing both enzymes and the physical structure, a reversion from an agricultural soil through the restored to more like the prairie soil, was not detected. It appears that the function of these microbial community systems in the native tallgrass prairie and agricultural soils of the chronosequence are in equilibria while the lands restored to tallgrass prairie are in an ongoing state of recovery.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

Introduction

Investigations of carbon sequestration are necessary to discover potential ways to reduce the rising levels of greenhouse gases. Soils are a source and sink of greenhouse gases including N_2O, CH_4 and CO_2. However, recent attention has focused on soils as a potential sink for atmospheric CO_2 until new sources of CO_2-free power are developed or CO_2 released to the atmosphere is reduced. It is hypothesized that through management of soils, such as no-tillage in agricultural systems, an increase in soil C can occur until a carrying capacity is reached (Smith 1994). An important consequence of increasing soil C is the concurrent increase in soil aggregation, which affects many physical and chemical parameters that relate to soil quality (Doran and Parkin 1994). Thus the ability to develop management systems that increase soil C will offset greenhouse gas emissions through C storage and also increase soil quality.

Management of arable lands will affect soil aggregation by changing the microbial community structure and function. In turn, the microbial community composition affects the carbon (C) dynamics in a soil system. Thus, it is this complex link between aggregation and microbial composition that will affect soil C storage in soils. In native systems, plant debris decomposes slowly on the surface, allowing for the accumulation of soil organic matter (SOM) and the development of fungi that result in the formation of soil aggregates (Gupta and Germida 1988). In contrast, agricultural systems often involve tillage of the plant debris into the surface soil, which results in little fungal development due to the shearing action from tillage that which can result in less new

aggregate formation. In addition, rapid microbial oxidation of the plant residue decreases the C that could have been stored as soil organic matter (SOM).

Soil aggregates consist of a hierarchy of decaying SOM and soil mineral particles made of clay, sand, and silt. The most simple soil aggregates, called microaggregates, contain small pieces of recalcitrant SOM, which are physically protected from decomposition by mineral soil particles that accumulate on and around the surface of the SOM (Cambardella and Elliott 1994). Particulate organic matter (POM) C may account for about 40% of the total soil organic C, most of which occurs in the macroaggregate size fractions (Cambardella and Elliott 1994). Microbial exudates, fungal hyphae, and POM combine to form a glue that holds microaggregates into larger macroaggregate structures (Jastrow and Miller 1998; Tisdall and Oades 1982).

Macroaggregates are beneficial in C storage due to the physical protection of the SOM within the aggregate. The rate of C turnover in macroaggregates is faster than microaggregates as indicated by the greater amount of freshly added C incorporated into macroaggregates than into microaggregates (Jastrow 1996; Six et al. 2001). In addition, C accumulation can be the result of slow decomposition of SOM, no priming of native C with continuous OM addition, or a slow turnover rate of macroaggregates (Six et al. 2001). A slow turnover rate in the macroaggregate size fraction is a physical process that results in a subsequent decrease in the rate of biological SOM decomposition due to the physical protection of the SOM.

Enzymes produced by microorganisms or microbial communities are potentially sensitive indicators of change in the biochemical component of the SOM, or of C content in the soil (Bandick and Dick 1999; Deng and Tabatabai 1996; Dick 1997; Ekenler and Tabatabai 2003). Changes in SOM, particularly microbial biomass C and potentially mineralizable substrate, should be evident in enzyme activity before changes in the SOM pool are measurable (Deng and Tabatabai 1996). β-glucosidase (EC 3.2.1.21) is a well studied enzyme from the Glycosidase enzyme family and is important in C cycling . The C content of a soil is significantly correlated to glycosidase enzyme activity, since the enzyme is instrumental in the breakdown of cellulose and is produced by both bacteria and fungi (Deng and Tabatabai 1996). Fungi are a major source of chitin as it is the main constituent of fungal cell walls. Chitin, upon hydrolysis by N-acetyl glucosaminidase (NAGase, EC 3.2.1.52) yields glucosamine as a product, making NAGase an important enzyme in C cycling (Guggenberger et al. 1999). Miller et al. (Miller et al. 1998) compared the activity of a diverse group of fungi to that of chitinolytic bacteria and actinomycetes and determined that only the fungi displayed constitutive NAGase activity.

Enzyme activities in different aggregate size fractions may provide insights to the mechanisms of SOM dynamics in whole soils and possibly, how C is sequestered in soil systems. The objectives of this study were to investigate the relationship between microbial community function and soil aggregate structure by assessing the enzyme activity for the aggregate fractions across a chronosequence of similar soil types and vegetation at different stages of ecosystem development. Based on these objectives three hypotheses were formulated:

1) Soil macroaggregates will be a greater component of the soil fabric as the soil chronosequence ages because the largest size fraction will not be physically disrupted by tillage. Instead, the determining factors of aggregate size will be natural processes.

2) There will be more enzyme activity in the largest size fraction because the labile SOM dominates this fraction. The presence of labile SOM binds microaggregates into macroaggregates and encourages the growth of the microbial biomass within aggregate structures. As the microbial community gets larger, the amount of enzymes secreted from the biomass also increases.

3) Enzyme activity within each aggregate fraction will increase with the increasing age of the soil chronosequence due to the increased physical protection of the SOM provided by the aggregate structures.

By identifying physical locations within the soil structure that display the greatest amount of biochemical activity across a carbon aggrading chronosequence we can understand how aggregate protection and C sequestration are related. This relationship can be used to enhance C sequestration.

Materials and Methods

Soil sampling

The tallgrass prairie restoration chronosequence at Fermi National Laboratory (N41.8º and W 88.3º, Batavia, IL, USA) was sampled during the spring of 2003. The soils (located within a 1.5 mile radius) collected were from remnant native prairie (Prairie), agricultural land that was restored to prairie grassland in 1979 and 1993 (R79 and R93

respectively), and agricultural land (Ag) that continues to be in a long-term rotation of row crops (*Zea mays* and *Phaseolus vulgaris*). The soil in the Drummer series classified as a fine-silty, mixed, superactive, mesic Typic Endoaquoll (Table 1). The mean precipitation was 92 cm and the mean temperature was 11ºC (Jastrow 1987; Soil Survey Staff 2004). Soil from each plot was sampled to a depth of 0-5 cm, passed through a 4-mm sieve, visible plant debris was removed by hand, and the soil was stored at 4ºC until analysis.

Wet sieving

The wet sieving method for aggregate distribution was used in our experiment(s) and adapted from those of Jastrow (2003) and Angers and Mehuys (1993). The sieve sizes were 2 mm, 1 mm, 250 μm, and 53 μm, therefore the size fractions collected were: >2 mm, 1-2 mm, 250 μm-1 mm, and 2-250 μm The sieves were nested with the largest mesh on top and placed into a large basin. Distilled water was added to the basin until the water level reached 1 cm below the wire mesh of the 2-mm sieve. The soil (100g dry wt) was spread evenly over the surface of the 2-mm mesh and the water level was raised just until the soil could be wet by capillarity. The soil was allowed to moisten for ten minutes, and then the water level was raised to 1.5 cm above the mesh of the top sieve. Sieving consisted of raising and lowering the nest of sieves 4 cm at a rate of 50 times per min for 2 min.

After sieving, the soil remained in the basin undisturbed for 5 minutes to allow fine particles to settle. Floating organic matter was aspirated into a vacuum flask and rinsed

onto a 20-µm nylon filter. The nest of sieves was slowly removed from the basin and placed onto a catch pan to collect any remaining water. The sieves were separated and placed into a 27°C forced air oven and air dried until the aggregates could easily be removed from the sieve. The aggregates were air dried to -0.33 MPa water content (pressure plate method) (Klute 1986).

Water from the basin and catch pan were combined and centrifuged in a Beckman J2-21 centrifuge at 14500×g (9500 rpm) for 30 min to collect particles greater than 2 µm that were not retained on the 250-µm sieve (the 2 mm-250 µm fraction). Stoke's law was used to determine the force and time required for the particles to be removed from the solution. After centrifuging, the soil particles from this fraction were combined in a preweighed container and allowed to dry to -0.33 MPa water content in a forced air oven at 27°C. Three macroaggregate size fractions were collected (>2 mm, 2-1 mm, 250 µm - 2 mm) and one microaggregate fraction was collected (250-2 µm).

All of the soil fractions were incubated at the -0.33 MPa water content for three days before further analyses to minimize skewing of metabolic activity caused by handling the soil.

Enzyme assays

β-glucosidase (EC 3.2.1.21) and N-acetyl-β-glucosaminidase (NAGase, EC 3.2.1.30) assays were performed on whole soil and all aggregate size fractions from the four soils of the chronosequence. The protocol for the N-acetyl-β-glucosaminidase assay was

derived from (Parham and Deng 2000), and β-Glucosidase was assayed as described by (Eivazi and Tabatabai 1988).

For the β-Glucosidase activity assay, soil (1 g OD at -0.33 MPa water content) was placed into a plastic tube and treated with 4 mL of modified universal buffer (MUB, pH 6) and 1 mL of 0.5 M p-nitrophenyl-β-D-glucopyranoside substrate solution. The solution was mixed thoroughly and allowed to incubate in the dark for 1 hour at 37°C. After incubation, the reaction product was stopped and the yellow color from the *p*-nitrophenol was developed by the addition of 1 mL 0.5 M calcium chloride and 4 mL of Tris buffer (pH 10). The solution was mixed and filtered through a 0.45 µm syringe tip filter. Controls were performed for both field moist and all wet aggregate size fractions by adding the substrate after the reaction was stopped. The *p*-nitrophenol was measured by absorption on a spectrophotometer at a wavelength of 405 nm and quantified by comparison with a standard curve.

For the N-acetyl-β-glucosaminidase assay 1 g (OD equivalent) soil was weighed into a plastic tube and 4 mL of 100 mM acetate buffer (pH 5.5) and 1 mL of 10 mM *p*-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma, St. Louis, MO) substrate solution were added. The solution was thoroughly mixed and allowed to incubate for 1 hour in the dark at 37°C. After 1 h the reaction was stopped by adding 1 mL of 0.5 M calcium chloride and 4 mL of 0.5 M sodium hydroxide. This also promotes the development of the yellow *p*-nitrophenol color that is the basis of the enzyme product colorimetric assay of activity (Ekenler and Tabatabai 2002). The solution was mixed and filtered through a 0.45 µm

syringe tip filter. Controls were performed for both field moist and all wet aggregate size fractions by adding the substrate after the reaction was stopped. The *p*-nitrophenol intensity was measured by absorption on a spectrophotometer at a wavelength of 405 nm and the absorption quantified by comparison to a standard curve.

Standard Curve

Solutions of 0-5 µg *p*-nitrophenol (15 mM *p*-nitrophenol in MUB, pH 6) were made to develop a standard curve for β-glucosidase and NAGase assays (Tabatabai and Bremner 1969). The solutions were incubated for 1 hour at 37ºC. After incubation, the yellow *p*-nitrophenol color was developed by adding 1 mL of 0.5 M $CaCl_2$ and 4 mL of Tris buffer (pH 10). The standard solutions were measured by absorption on a spectrophotometer at a wavelength of 405 nm and a standard curve developed by plotting absorption vs concentration.

Statistical Analysis

The data were analyzed using Systat 10 (SPSS Inc, Chicago Illinois). For all multiple comparisons, Bonferroni's pairwise comparison test was used following a one-way ANOVA to detect significant differences at $P<0.05$. The enzyme activity data were grouped and analyzed by 1) comparing the activity of an enzyme in the different size fractions of each soil and by 2) comparing the activity of an enzyme in one size fraction across all four soils.

Results

Aggregates

The Prairie and Ag soils had a similar aggregate size distribution (Fig. 1). However, in the Ag soil there were about twice the amount of microaggregates 2μm-250μm in size and 25% fewer macroaggregates (>2 mm and 1-2 mm) than the Prairie soil. The R93 plot had about 66% more mass in large macroaggregates (>2 mm) than both the Prairie and Ag plots, accompanied by a small proportion of 1-2 mm, 250μm-1 mm, and 2-250 μm macroaggregates and microaggregates. In the R79 plot more of the mass is distributed into the 1-2 mm and 250 μm-1 mm macroaggregate and >2 mm large macroaggregate size fractions compared to the R93 plot. The R79 plot had the smallest microaggregate size fraction of the plots in the soil chronosequence with the microaggregate size fraction making up less than 4% of the total mass distribution for that plot.

β-Glucosidase

Enzyme activities across a soil chronosequence of native prairie, restored prairie, and continuous row crop agriculture were compared in this study. The age of each of the members of the chronosequence affected the amount of β-glucosidase activity that was present in each of the aggregate size fractions. β-Glucosidase specific activity (Eq. 1) was significantly greater in the smallest size fraction (2μm-250μm) for all the chronosequence soils, except in the Ag soil (Fig. 2a).

$$\text{Specific enzyme activity} = \frac{\text{activity } (\mu\text{g PNP})}{\text{mass of aggregates in the size fraction (g)}} \qquad \text{[Eq. 1]}$$

The trend exhibited by the Ag soil showed decreased specific activity across all fractions compared to the activities detected in the other soils and aggregate fractions in the chronosequence (Fig. 2b). After restoration back to grassland prairie (R79 and R93), β-glucosidase specific activity generally increased over that of the Ag fraction.

In the >2 mm size fraction, the β-glucosidase specific activity of the Ag plot was lower and significantly different from the R93 plot, but not the R79 and Prairie plots (Fig. 2b). In the 250 μm -2 μm microaggregate size fraction the Prairie and R79 plots were significantly different from the Ag and R93 plots of the chronosequence.

β-glucosidase enzyme activity in the smallest size fraction of all the chronosequence soils was sensitive to the C and N content of each soil aggregate size fraction, in that the enzyme activity increased as the amount of C and N associated with that size fraction increased (Table 2 and Fig. 3a,b).

When the distribution of β-glucosidase activity was weighted to the size of the aggregate fractions in each soil (Aggregate-derived activity; Eq. 2), the Prairie (macroaggregate size fractions dominate) and the Ag soil had approximately similar activity distributions within each aggregate size fraction, though the Ag activities were consistently lower (Fig. 4).

$$\text{Aggregate - derived activity} = \frac{\text{specific enzyme activity } (\mu\text{g PNP g}^{-1}\text{ soil h}^{-1})}{\text{proportion of aggregates in fraction (g/100g soil)}} \qquad \text{[Eq. 2]}$$

Both restored plots exhibited larger amounts of β-glucosidase aggregate-derived activity in the >2 mm macroaggregate size fraction than that of the Prairie or Ag plots.

N-acetyl-β-glucosaminidase

In the Ag soil N-acetyl-β-glucosaminidase (NAGase) activity among all of the size fractions within the soil is smaller than the aggregate size fractions in the remaining soils of the chronosequence (Fig. 5a). The smallest size fraction in each soil had the highest specific activity of all the aggregate size fractions in that soil, except in the Ag soil which had no significant differences between the aggregate size fractions within that soil. NAGase specific activities were greatest in the smallest (2 μm -250 μm) microaggregate size fractions across the chronosequence (Fig. 5b).

The NAGase enzyme aggregate-derived activity values in the small microaggregate size fraction increased as the amount of C and N associated with those fractions increased (Table 2 and Fig. 6a, b).

Most of the NAGase aggregate-derived activity in the Prairie soil was located in the 1-2 mm and 250 μm-1 mm aggregate size fractions (Fig. 7). The Ag soil had a uniform distribution of aggregate-derived NAGase activity, such that all four aggregate size fractions contributed approximately equally to the soil activity (Fig. 7). In the R93 soil, the >2 mm size fraction had the greatest aggregate-derived activity. In the R79 soil, the specific activity was mainly derived from the 2-250 μm size fraction. However, the aggregate-derived enzyme activity of the R79 soil was found to be divided approximately

equally among the three macroaggregate size fractions (>2 mm, 1-2 mm, and 250μm-1 mm).

Discussion

Analysis of the aggregate size distribution within each soil of the chronosequence revealed an increase in the amount of large macroaggregates that contribute to the structure of the whole soil compared to the contribution the aggregates make in the Ag soil. This supports the hypothesis that soil macroaggregates will be a greater component of the soil fabric as the soil chronosequence ages, which also agrees with the previously proposed aggregate hierarchy models (Jastrow and Miller 1998; Six et al. 1999; Tisdall and Oades 1982). These hierarchy models propose that surface litter deposition contributes to the formation of more stable soil aggregates by binding together microaggregates to make macroaggregates. The binding agents are organic matter and microbial byproducts of litter decomposition (hyphae, other biomass, and exudates).

The specific activity of the enzymes in each of the four aggregate size fractions of the chronosequence did not provide evidence to support the hypothesis that the enzyme activity would be the greatest in the largest aggregate size fraction. Instead, the specific activity of both enzymes revealed that the largest amount of activity was associated with the microaggregate size fraction in all of the chronosequence soils (Figs. 2, 5). The specific enzyme activity did, however, support the hypothesis that enzyme activity within each aggregate size fraction will increase with the increasing age of the soil chronosequence (Figs. 2, 5). This could be due to the observation that enzyme activity in

a particular size fraction tended to increase as the chronosequence ages. However, when the proportion that each aggregate fraction contributes to the whole soil is considered, *i.e.,* aggregate-derived activity, the microaggregate size fraction actually contributes a small amount to the activity of the whole soil.

For both β-Glucosidase and NAGase, the enzyme activity derived from each aggregate size fraction suggests a change in microbial functions across the soil chronosequence (Fig. 2). The Prairie and Ag soils had a similar distribution of aggregate-derived enzyme activity among the aggregate size fractions however, the magnitude of the activities differed. The Ag soil had less aggregate-derived activity in each fraction than the corresponding fraction in the Prairie soil; for both enzymes, the enzyme activity in the whole Ag soil was only about a quarter of that in the Prairie whole soil (data not shown). Both the Prairie and Ag soils have been under the influence of their respective management regimes for an extended amount of time, allowing each soil to reach some type of steady state. It is likely that enzyme activity exhibited in the Ag soil would have been different from what we measured if measured following the early cultivation of the native prairie sod, however, the enzyme activity measurements were made on the Ag soil after approximately 150 years of row crop cultivation. This long history of consistent management may have fostered a new, steady state of biochemical dynamics. The similar distribution of aggregate-derived activity in the Prairie and Ag soils may also occur due to the complex nature of the trophic levels in the Prairie soil that may not be present in the Ag soil, *i.e.*, arthropod variability and plant species diversity (Berkelmans

et al. 2003). In that case, the similar distributions would be mere coincidences, artifacts of the different soil conditions.

The distribution of aggregate-derived activities across the aggregate size fractions of the restored prairie soils differed from the Ag and Prairie soils (Fig. 4, 7). In the R93 soil, the β-glucosidase aggregate-derived activity was higher in the large macroaggregate (>2 mm) size fraction than in the smaller fractions (Fig. 4). Similar changes in the β-glucosidase activity distribution of the R93 soil were seen in the R79 soil but lower in magnitude (Fig. 7). An increase in the aggregate-derived β-glucosidase activity of the >2 mm, 1-2 mm, and 250 μm-1 mm macroaggregate size fractions of the R93 and R79 soils compared to the Ag and Prairie soils, showed that the microbial community was adapting to the changes caused by the restoration. It is possible that the microbial community was responding to an increase in organic matter deposition on the soil surface due to the reduction in tillage and reversion back to a natural grassland system (Beare et al. 1992). This agrees with other researchers (Gupta and Germida 1988; Miller and Dick 1995), who likewise concluded that macroaggregate structures provide habitat for microbial biomass, the enzyme activity of which is probably stabilized by the physical protection offered by the macroaggregate structure. Alternatively, the function of the microbial community as seen via enzyme activity, could be responding to a change in the fungal portion of the microbial community due to proliferation of fungi after cessation of tillage.

Macroaggregate (>2 mm, 1-2 mm, 250 μm-1 mm)-derived NAGase activity (Fig .7) was also suggestive of microbial function shifts across the soil chronosequence. In the R93

soil, the activity was highest in the large macroaggregate size fraction (> 2 mm) (Fig. 7). The increase in enzyme activity relative to the Ag soil indicates that the function *i.e.,* NAGase activity, that the microbial community was expressing had changed, perhaps in response to the addition of organic matter to the surface of the soil (Beare et al. 1992). The R79 plot suggests other community shifts, seen as the reduction of aggregate-derived activity in the large (> 2 mm) macroaggregate size fraction and the subsequent increase in activity of the 1-2 mm and 250 μm -1 mm macroaggregate size fractions. This may be due to aggregate turnover as the microbial decomposition of the organic matter binding the macroaggregates breaks the large >2 mm aggregates into new microaggregates, which then re-form into macroaggregates > 250 μm in size (Six et al. 1999).

The microaggregate fraction did not contribute much to the enzyme activities of the whole soils, in spite of its large specific activity for both enzymes, because this fraction is a very small component (4-11%) of the whole soil from each plot (Fig. 1). It is possible that residual enzyme activity was protected in these microaggregates by having been once incorporated into a larger macroaggregate structure. Evidence for this conclusion is supported by $\delta^{13}C$ data collected by Jastrow et al. (Jastrow et al. 1996; Jastrow and Miller 1998) in which the age of C3 and C4 derived C in aggregate size fractions of pasture and corn soils was compared. The large macroaggregates were found to have a greater proportion of recently deposited C and thus a more rapid apparent turnover rate (74 years) than that of the microaggregate structures. The C associated with microaggregate structures had a turnover time of approximately 412 years (Jastrow et al. 1996). This reflects the age and degree of protection of the C and possibly the associated microbial

enzymes within each aggregate size fraction. Aggregate turnover may keep the newly formed and newly released microaggregates supplied with fresh secretions of enzyme from microorganisms residing within macroaggregates; these enzymes may be sorbed to and protected by clay minerals in the soil (Burns 1986).

It is important to consider the possible source of the enzyme activity in the aggregate size fractions of the soil chronosequence. The β-glucosidase enzyme activity is associated with the both bacterial and fungal processes; as a result, the contribution that each individual microbial group makes to the activity of the aggregate size fractions of the soils in the chronosequence is unclear. Miller et al. (Miller et al. 1998) found NAGase activity to be highly correlated with fungally-dominated systems. The NAGase data presented here reflects the activity of the microbial biomass as the biomass population changes from a bacterially-dominated active community in the Ag soil (as indicated by a F:B ratios of whole soils in Table 1) to a fungally dominated active community in the Prairie and Restored Prairie (R93 and R79) soils (Bailey et al. 2002).

Conclusions

The microbial systems in the native tallgrass prairie and agricultural soils of the chronosequence are in equilibrium while the lands restored to tallgrass prairie are in an ongoing state of recovery. The native prairie system developed over thousands of years under conditions that are different from today. Similarly, the agricultural plots have been so managed for about 150 years. A new steady state may eventually be reached in the restored systems; this new equilibrium will reflect human influences and changes in

climate under which the systems re-stabilize. We are still unable to predict the time to a new equilibrium, nor if will it be similar to the native prairie.

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Table 1. Soil characteristics.

Plot	pH	C (mg g^{-1} soil)	N (mg g^{-1} soil)	F:B	Texture
Currently farmed (Ag)	5.6	41	3.1	0.85	Clay Loam
Restored prairie 1993 (R93)	6.6	59	5.6	10.7	Clay Loam
Restored prairie 1979 (R79)	7.3	60	5.1	13.5	Silt Loam
Native prairie (Prairie)	7.5	118	8.9	ND	Silt Loam

ND = not determined

Table 2. Carbon and Nitrogen content of each aggregate size fraction across the soils of the chronosequence.

Soil	Aggregate size fraction	mg C g^{-1} soil in each aggregate size fraction	mg C g^{-1} whole soil	mg N g^{-1} soil in each aggregate size fraction	mg N g^{-1} whole soil
Ag	>2 mm	10.36		0.77	
	1-2 mm	12.14		0.91	
	250µm-1 mm	14.44		1.07	
	2-250 µm	4.14		0.30	
	total	41.08	41.16	3.06	3.06
Restored prairie 1993 (R93)	>2 mm	40.38		2.85	
	1-2 mm	7.71		0.56	
	250µm-1 mm	4.59		0.32	
	2-250 µm	2.90		0.21	
	total	55.57	58.82	3.94	5.55
Restored prairie 1979 (R79)	>2 mm	30.83		2.45	
	1-2 mm	16.40		1.28	
	250µm-1 mm	11.16		0.85	
	2-250 µm	1.85		0.14	
	total	60.24	60.43	4.72	5.09
Native Prairie	>2 mm	24.49		1.86	
	1-2 mm	39.40		3.14	
	250µm-1 mm	43.53		3.48	
	2-250 µm	5.87		0.48	
	total	113.29	118.40	8.97	8.90

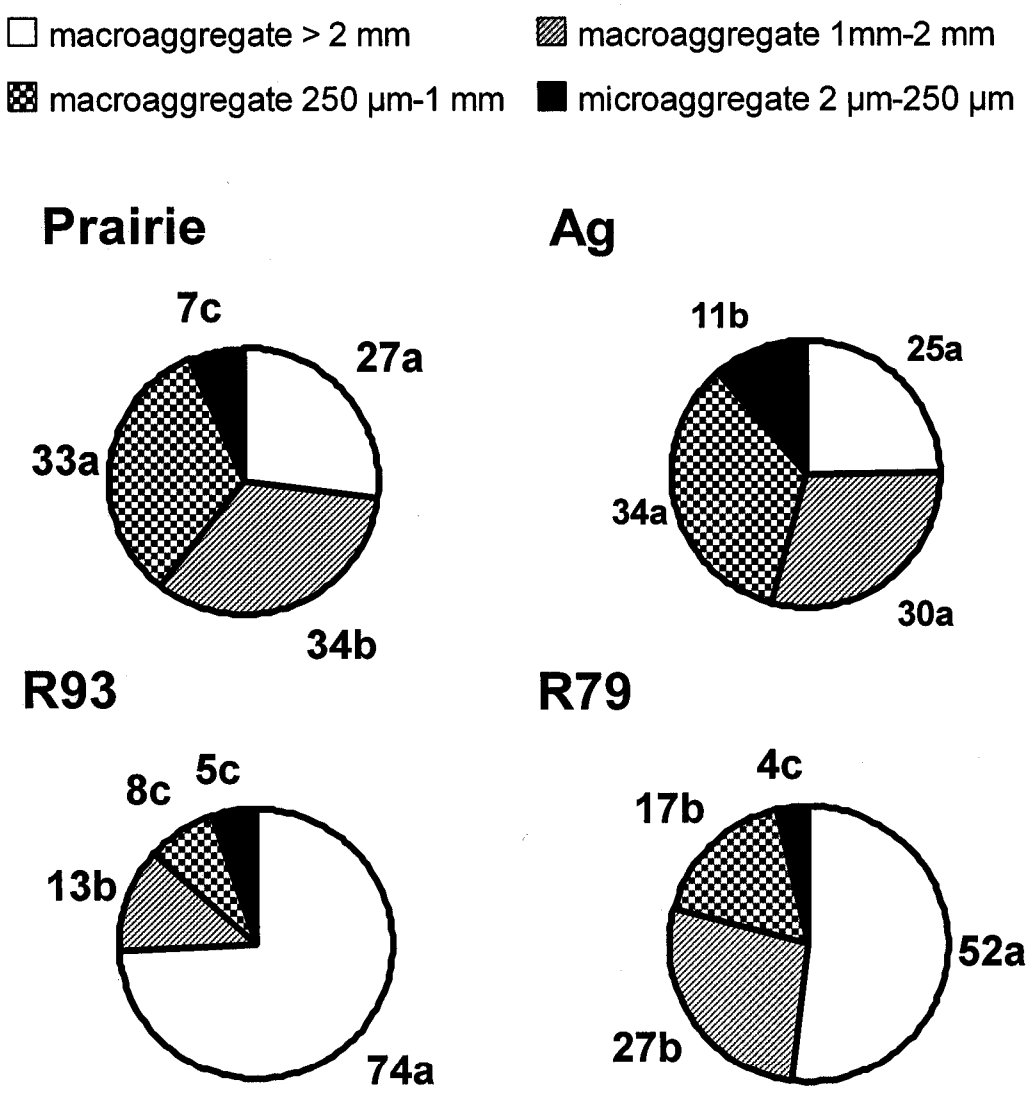


Figure 1. Aggregate size distribution of each plot in the soil chronosequence. Wedges within soil each accompanied by the same letter are not significantly different (Bonferroni, $P < 0.05$).

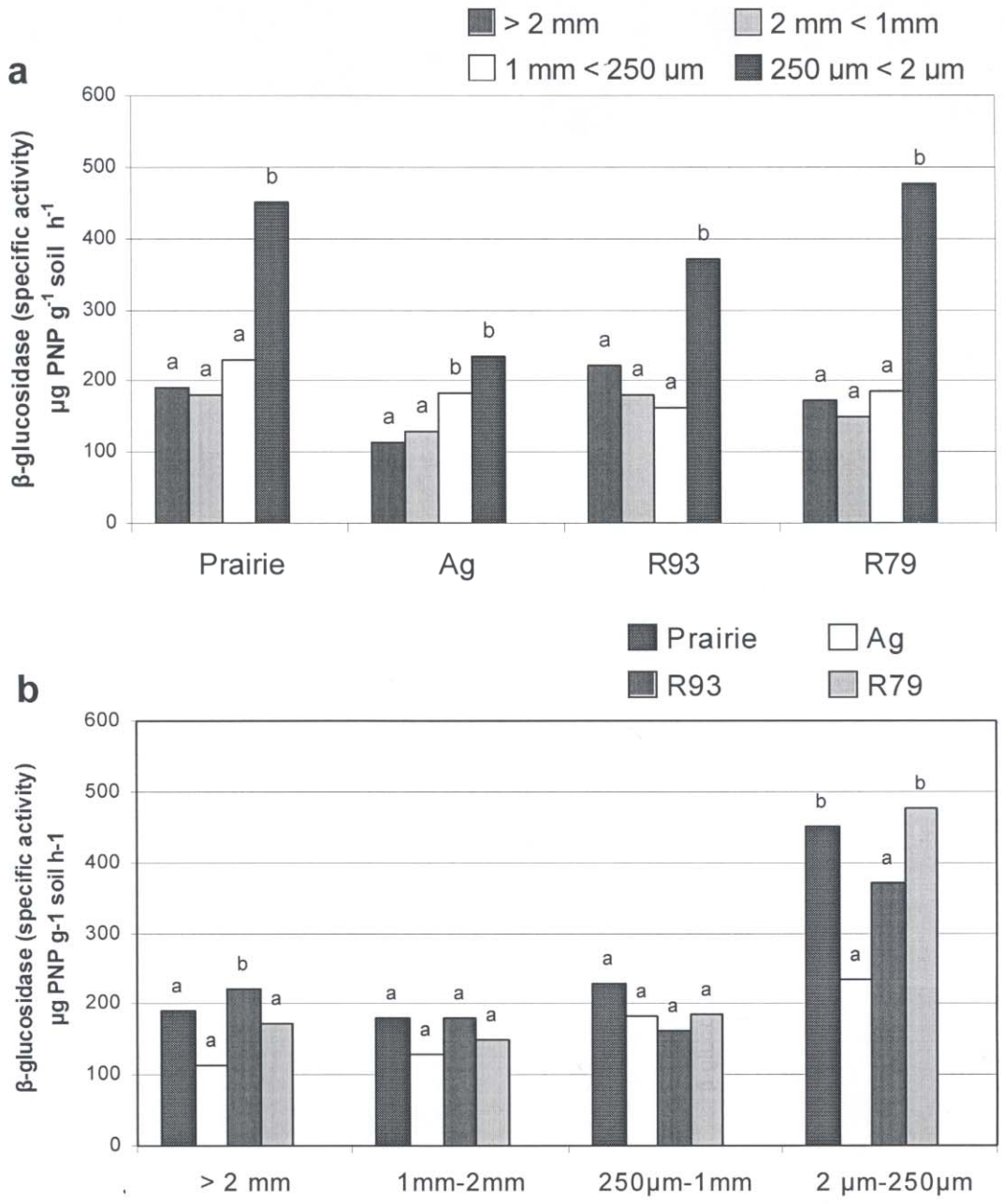


Figure 2. a) β-Glucosidase specific activity of each aggregate size fraction within each soil of the chronosequence. **b)** The same data is rearranged to show β-glucosidase specific activity (g^{-1} soil in that fraction) of each aggregate size fraction in each chronosequence plot. Bars topped by the same letter are not significantly different, Bonferroni ($P<0.05$). Comparisons only valid within soils (a) and size fraction (b).

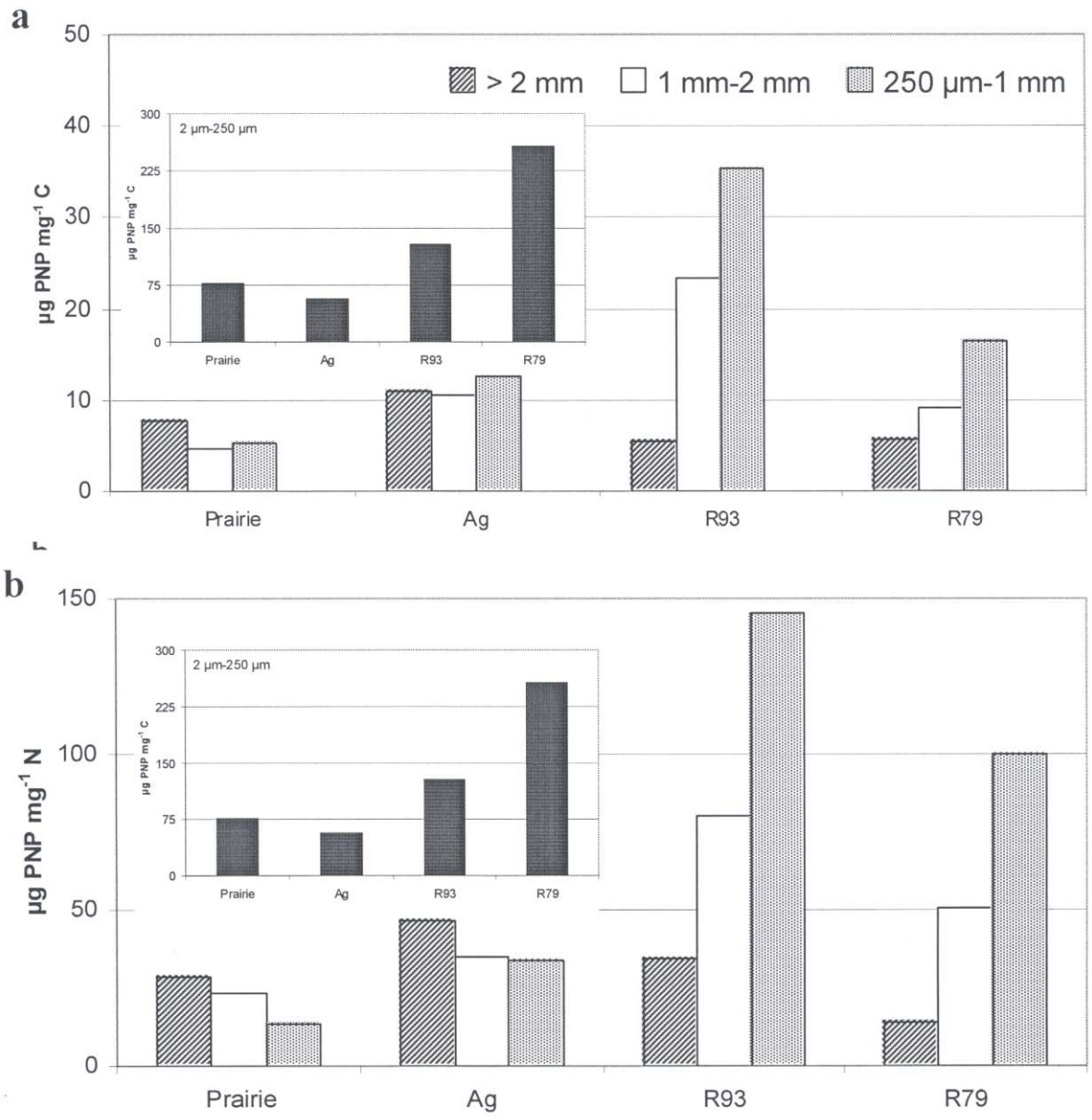


Figure 3. β-glucosidase enzyme activity (µg PNP) of each aggregate size fraction expressed per unit of C and N content within each aggregate size fraction in each plot of the chronosequence. Relationship of C (a) and N (b) to β-glucosidase enzyme activity.

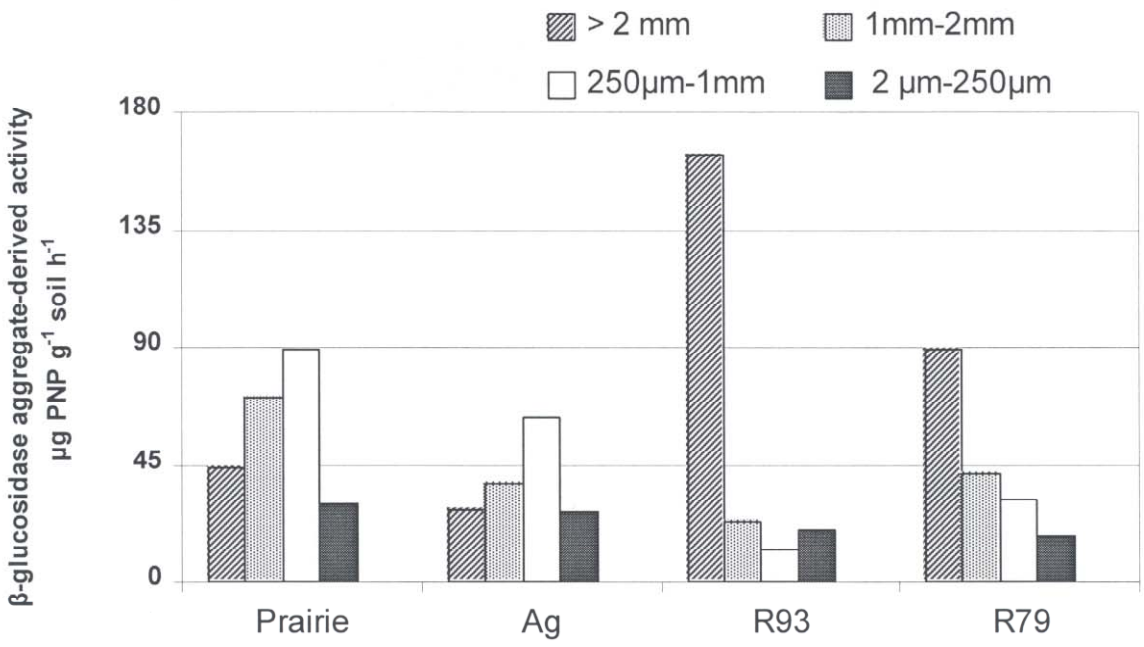


Figure 4. β-glucosidase aggregate-derived enzyme activity (enzyme activity (µg PNP g^{-1} soil h^{-1})/proportion of aggregate in fraction (g/100 g soil)).

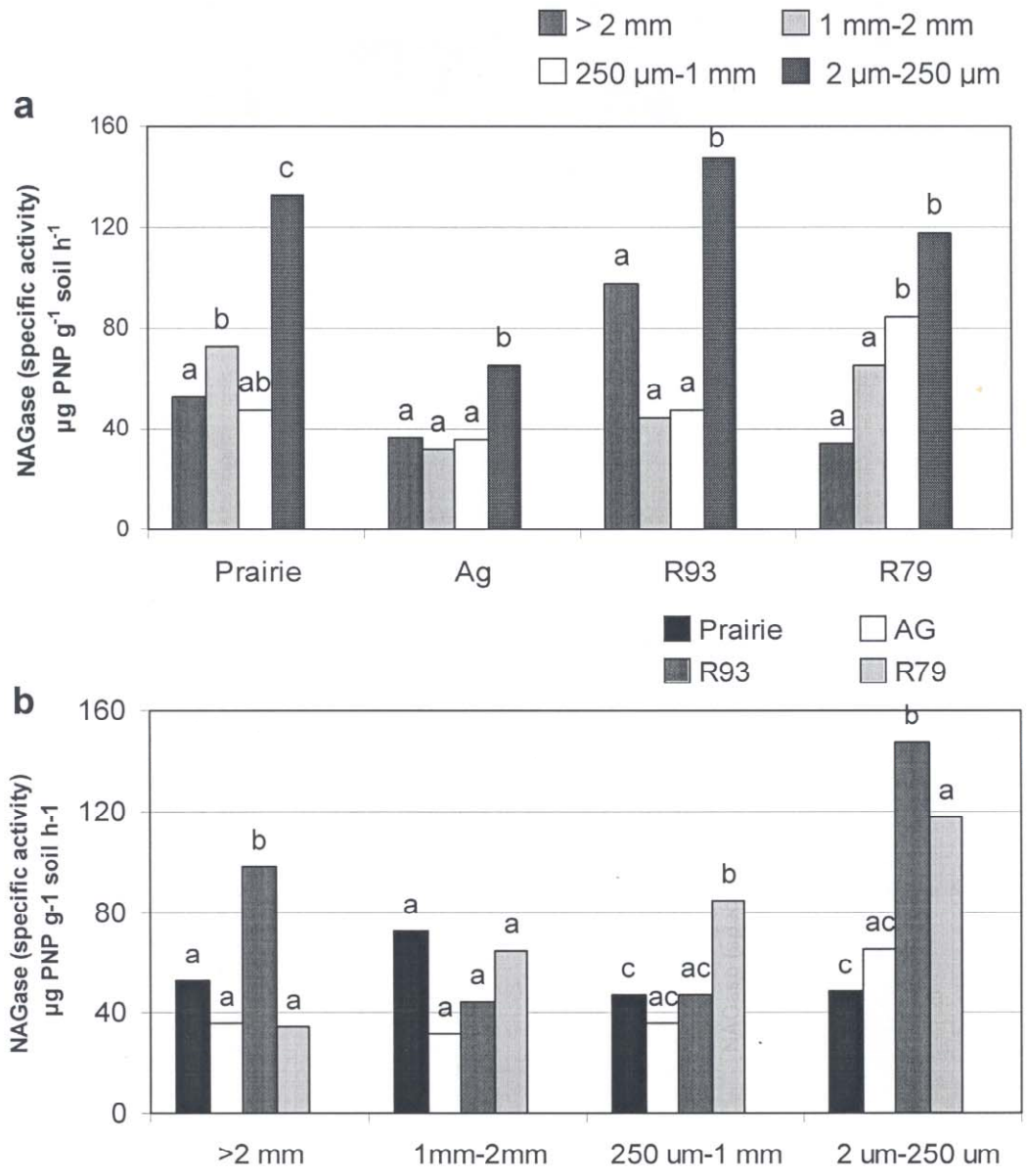


Figure 5. a) N-acetyl-β-glucosaminidase (NAGase) specific activity of each aggregate size fraction within each soil of the chronosequence. **b)** The same data is rearranged to show NAGase specific activity (g^{-1} soil in that fraction) of each aggregate size fraction in each chronosequence plot. Bars topped by the same letter are not significantly different, Bonferroni ($P<0.05$). Comparisons only valid within soils (a) and size fraction (b).

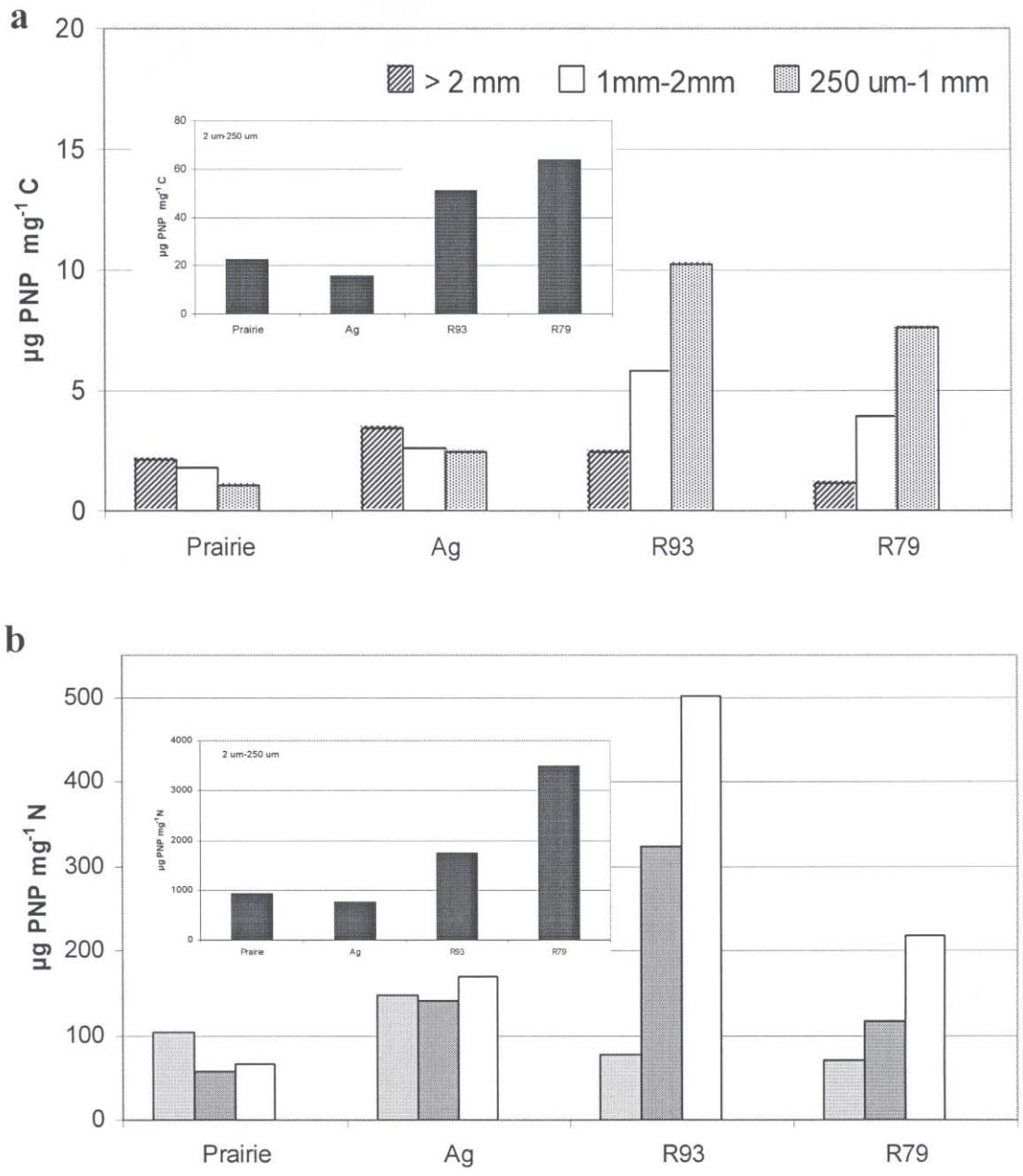


Figure 6. Enzyme activity of each aggregate size fraction expressed per unit of C and N content within each aggregate size fraction in each plot of the chronosequence. Relationship of C (a) and N (b) to NAGase enzyme activity.

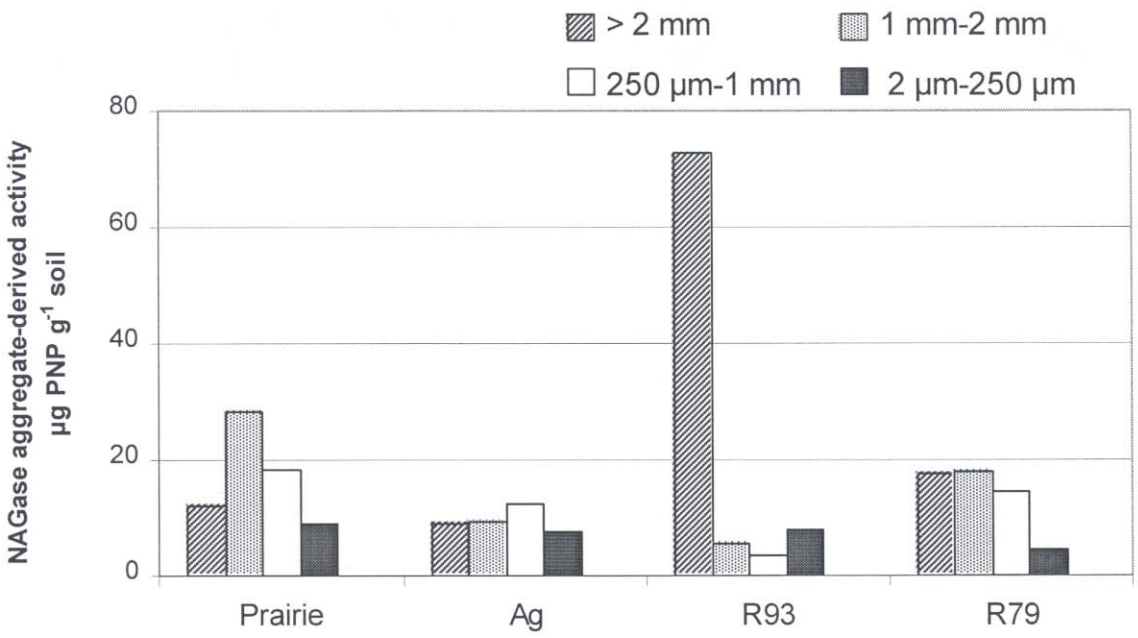


Figure 7. N-acetyl-β-glucosaminidase aggregate-derived enzyme activity. Aggregate-derived activity= enzyme activity (µg PNP g^{-1} soil h^{-1})/proportion of aggregate in fraction (g/100 g soil).

APPENDIX A: SELECTION OF ENZYMES FOR STUDY

Six enzymes assays were surveyed to select those that met the following criteria:

1. Is the enzyme activity robust to water saturation resulting from the wet sieving procedure?
2. Does the enzyme activity vary across the chronosequence?
3. Are published gene sequences available for the enzyme?

These criteria were queried on the 6 enzymes that are presented in this appendix using the following basic procedure. To address question 1, soils were saturated on the nest of sieves by the method described in chapter one, without sieving the soil. These enzyme activities were compared to those in soils that had been analyzed in their field moist state following conditioning that consisted of bringing the water content of each soil to -0.33 Mpa. Only the R93 soil was used for this test due to limited quantities of the other three soils from the prairie chronosequence. A t-test was used to compare samples and a *P*-value less than 0.05 was considered a significant difference.

To address question 2, all six enzyme assays were surveyed on all four field moist soils of the prairie chronosequence (except the cellulose assay in which only R79, R93, and Ag soils were used) at -0.33 Mpa water content. For all multiple comparisons, Bonferroni's pairwise comparison test was used following a one-way ANOVA to detect significant differences at $P<0.05$.

Arylsulphatase

Function

Sulphatases catalyze the hydrolysis of organic sulphate esters. Arylsulphatase catalyses the irreversible reaction:

$$ROSO_3^- + H_2O = ROH + H^+ + (SO_4)^{2-}$$

which has been detected in soil microorganisms. The activity of arylsulphatase is also correlated with soil organic carbon, nitrogen, and cation exchange capacity. In this assay system, *p*-nitrophenyl sulphate is hydrolyzed to produce *p*-nitrophenol, which can be measured spectrophotometrically at 405 nm (Alef and Nannipieri 1995a).

Method

Soils were conditioned with a water content of -0.33 MPa prior to assay.

In a test tube, 1 g (dry weight) of soil and add 0.25 mL of toluene, 4 mL acetate buffer (0.5M, pH 5.8), and 1 mL of *p*-nitrophenyl sulphate (25 mM in acetate buffer, store at 4ºC) were combined. The tube was capped, the contents mixed thoroughly, and incubated for one hour at 37ºC. After the incubation 1 mL of $CaCl_2$ (0.5 M) and 4 mL of NaOH (0.5 M) were added to each tube, (for the controls *p*-nitrophenyl sulphate substrate was added before adding the $CaCl_2$ and NaOH) and the tube was then gently mixed. The suspension was filtered through a 0.45 µm filter and the abundance of *p*-nitrophenol was measured colorimetrically on a spectrophotometer at 405 nm.

Calibration curve

One milliliter of standard (PNP) was diluted in 100 mL H_20 to create the PNP stock solution. The calibration standards were made by pipetting stock and water in the following ratios into Erlenmeyer flasks:

standard µg PNP	PNP (mL)	H_2O (mL)
0	0	5
2	1	4
4	2	3
6	3	2
8	4	1
10	5	0

Calibration standards were incubated and analyzed with the rest of the samples according to the procedure above.

Calculation

$$\rho - Nitrophenol\ (\mu g\ g^{-1}\ dwt\ h^{-1}) = \frac{C \times V}{s \times t}$$

C = measured concentration of *p*-nitrophenol (µg/mL)

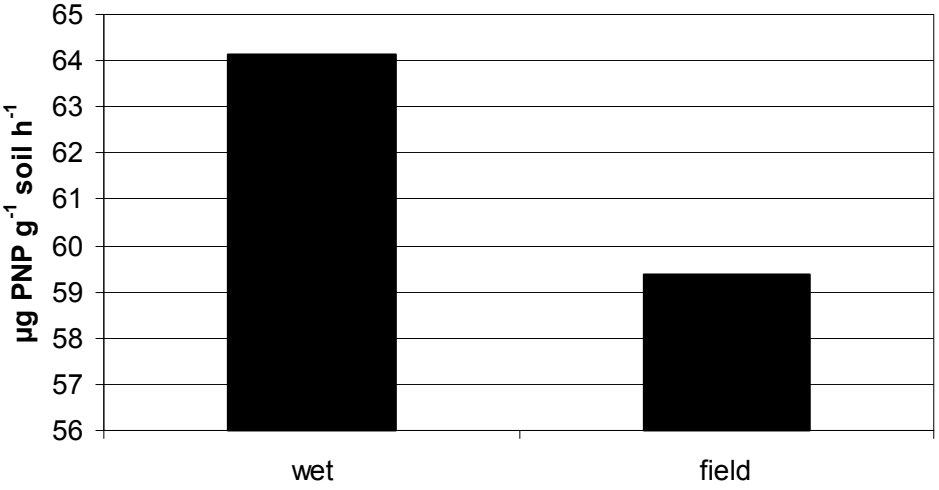
s = dry weight of soil used (g)

V = total volume of soil suspension (mL)

t = incubation time (h)

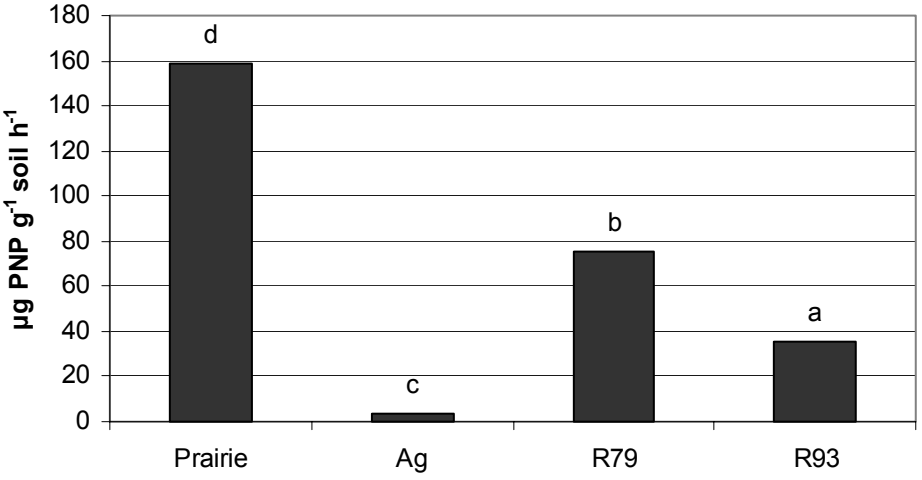
Results

1. Is the activity of arylsulphatase robust to the water saturation that will result from wet sieving?



The t-test was used and a *P* value of 0.004 was generated, therefore wetting the soil significantly increased the activity of arylsulphatase.

2. Does arylsulphatase activity vary across the chronosequence?



The arylsulphatase assay was a sensitive indicator of change across the prairie chronosequence. Bars topped by the same letter are not significantly different (Bonferroni, $P<0.05$).

3. Are published gene sequences available for the enzyme?

Few gene sequences for microorganisms were available for this enzyme.

Conclusion

Arylsulphatase was rejected as a study enzyme because wet sieving increased the amount of enzyme activity measured in the soil sample, and the enzyme is not closely related to the carbon cycle.

β-glucosidase and N-acetyl-glucosaminidase

Function

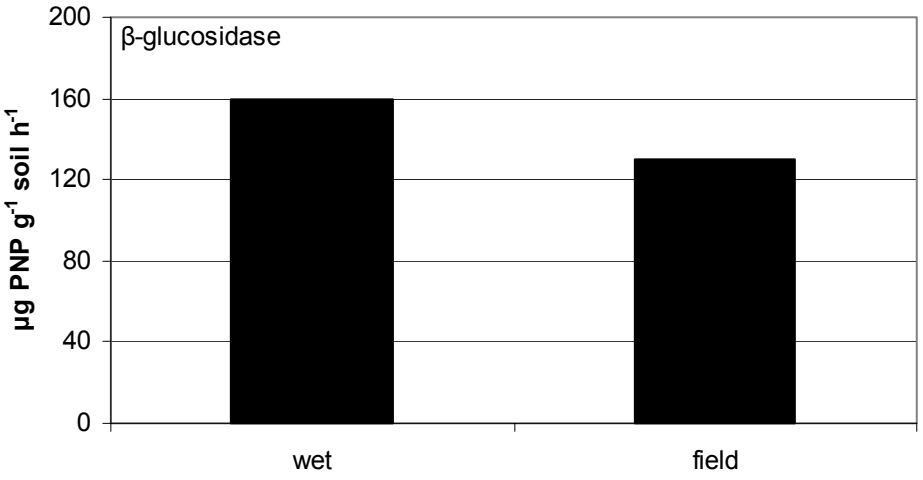
The functions of these enzymes are detailed in chapter one.

Method

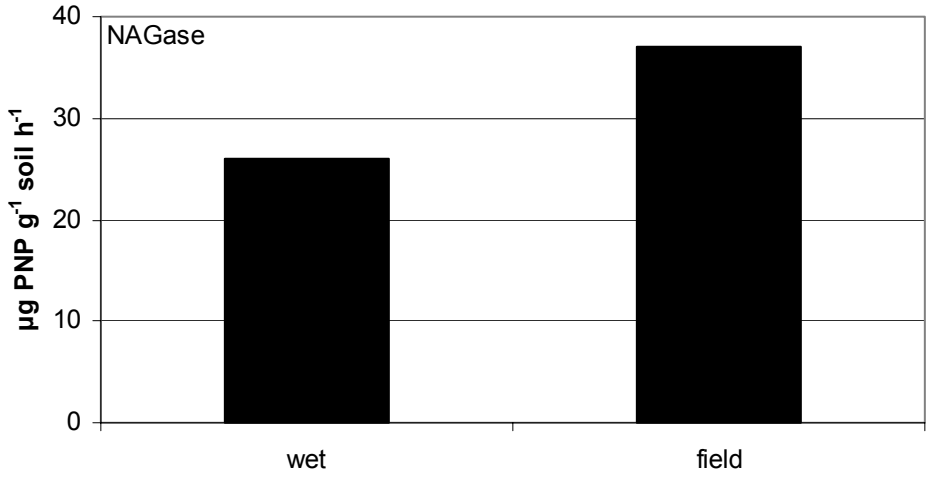
The methods for assaying these enzymes are detailed in chapter one.

Results

1. Is the activity of β-glucosidase robust to the water saturation that will result from wet sieving?

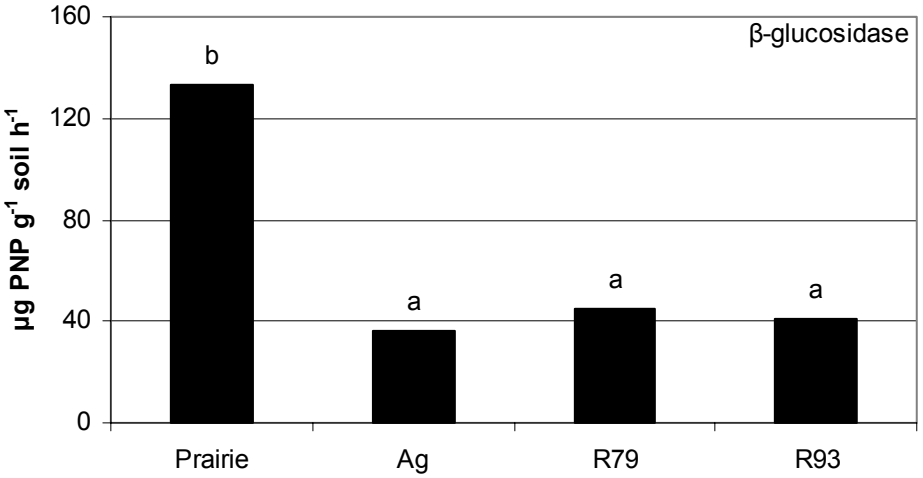


The t-test was used and a p value of 0.031 was generated, therefore wetting the soil significantly increased the activity of β-glucosidase.

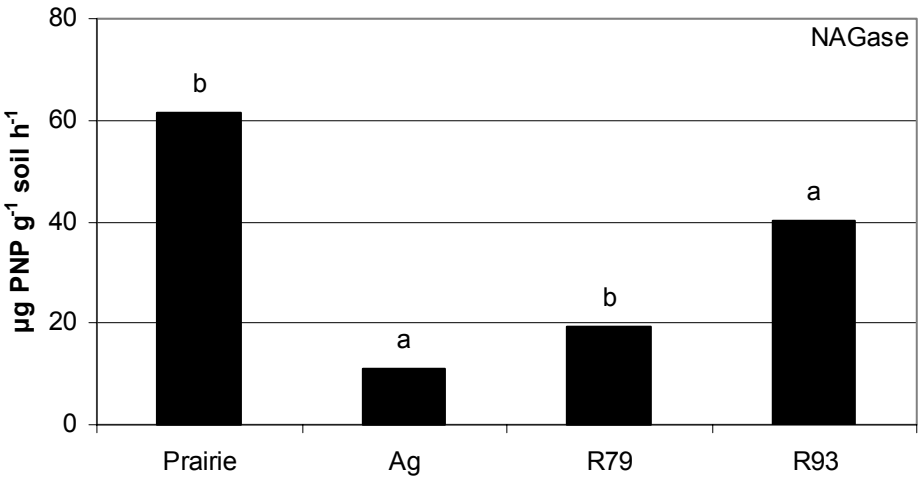


The t-test was used and a p value of 0.015 was generated, therefore wetting the soil significantly decreased the activity of β-glucosidase.

2. Does arylsulphatase activity vary across the chronosequence?



The β-glucosidase activity was a sensitive indicator of change across the prairie chronosequence. Bars topped by the same letter are not significantly different (Bonferroni, $P<0.05$).



The NAGase activity was a sensitive indicator of change across the prairie chronosequence. Bars topped by the same letter are not significantly different (Bonferroni, $P<0.05$).

3. Are published gene sequences available for the enzyme?

Yes, abundant gene sequences for microorganisms are available for these enzymes.

Conclusion

The β-glucosidase and NAGase assays were studied further because the assays were sensitive indicators of change across the prairie chronosequence soils.

Cellulase

Function

The cellulase assay measures the hydrolysis of cellulose to b-glucose by measuring the release of reducing sugars such as the glucose monohydrate that is used to calibrate the assay (Alef and Nannipieri 1995b).

Method

The following reagents are needed for the assay:

Acetate buffer:

2 M, pH 5.5

CMC solution:

0.7% (w/v) in acetate buffer.

Reagent A:

16 g of anhydrous sodium carbonate and 0.9 g of potassium cyanide in 1 L water.

Reagent B:

0.5 g of potassium ferric hexacyanide in H_2O, in 1 L water. Store in a brown (light shielded) bottle.

Reagent C:

1.5 g of ferric ammonium sulphate , 1 g sodium dodecyl sulphate, and 4.2 mL of concentreated sulphuric acid in 1 L H_2O (at 50°C).

Day one:

Five grams (OD) of field-moist (-0.33 MPa water content) soil were placed in a test tube and 7.5 mL of acetate buffer and 7.5 mL CMC solution were added. The tube was capped tightly and incubated at 50ºC for 24 hours.

Day two:

The CMC solution was added to the control samples (without the CMC substrate on day one). All soil suspension samples were filtered through a 0.45 µm filter. A 0.5 mL aliquot of filtrate from each sample was placed into a fresh test tube and 10 mL of H_2O added. A 1 mL aliquot of this diluted solution was removed into a fresh glass tube. To the diluted solution, 1 mL of reagent A and 1 mL of reagent B were added. The tubes were closed tightly and the solutions were mixed well. The samples were then placed into a boiling in water bath at 100ºC for 15 minutes. After cooling for 5 minutes at 20 ºC, 5 mL of reagent C were added to each sample. The solutions were mixed and allowed to stand for one hour at 20ºC. The reduced sugar content of the samples was measured on a spectrophotometer at 690 nm.

Calibration curve

Prepare a stock glucose solution of 28 mg glucose in 1 L water.

Glucose (µg)	Glucose solution (mL)	H_2O (mL)
2.8	0.1	0.9
5.6	0.2	0.8
8.4	0.3	0.7
11.2	0.4	0.6
14	0.5	0.5
16.8	0.6	0.4
19.6	0.7	0.3
22.4	0.8	0.2
25.2	0.9	0.1
28	1.0	0

Measure the absorption generated by the calibration standards with the rest of the samples in the above procedure.

Calculation

Glucose equivalent (ug/g dry weight soil/24 hours) = C*v*f/s

$$\text{Glucose equivalent } (\mu g\ g^{-1}\ \text{dry weight soil day}^{-1}) = \frac{C \times V \times F}{s \times t}$$

C = measured glucose concentration (μg ml^{-1})

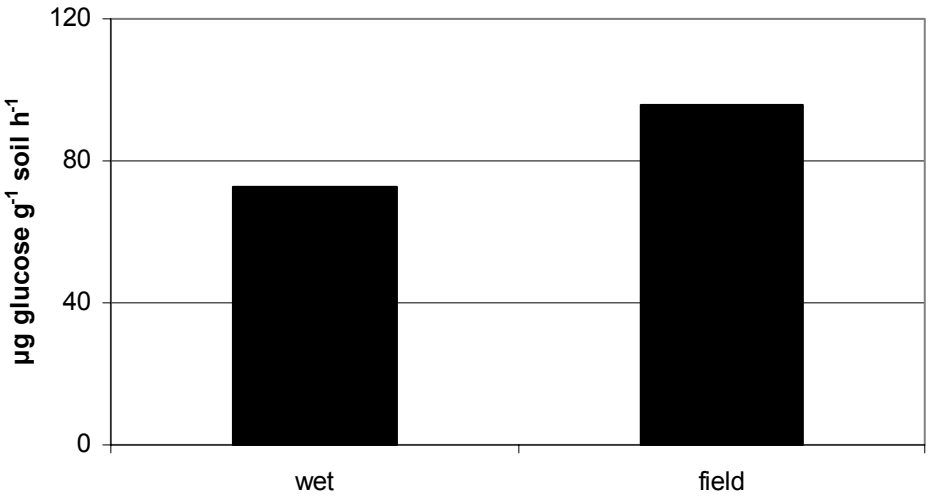
V = volume of suspension (30 mL)

F = dilution factor (20 for ag)

s = dry weight of soil used (g)

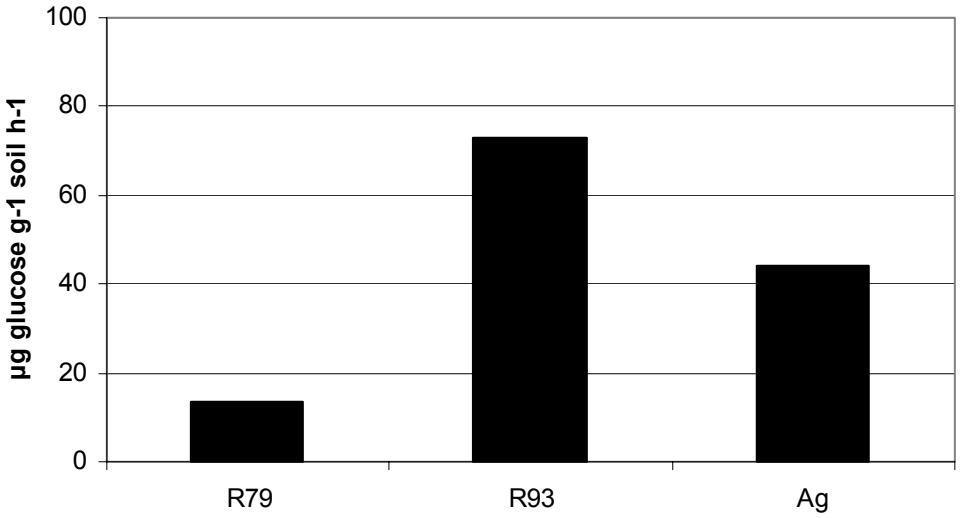
Results

1. Is the activity of cellulase robust to the water saturation that will result from wet sieving?



The t-test was used and a p value of 0.02 was generated, therefore wetting the soil significantly decreased the activity of cellulase.

2. Does cellulase activity vary across the chronosequence?



There was no significant differences in cellulase activity among the chronosequence soils (P=0.078). The cellulase assay was not sensitive indicator of change across the prairie chronosequence.

3. Are published gene sequences available for the enzyme?

Yes, many gene sequences are available for microbial sources of this enzyme.

Conclusion

The cellulase assay was removed from further analysis due to its lack of discrimination among the soils in the prairie chronosequence.

Chitinase activity

Function

(Alef and Nannipieri 1995c)

Method

Stock solution of 4-(dimethyl amino)benzo aldehyde (DMBA)

10 g DMBA dissolved in 87.5 mL concentrated acetic acid and 12.5 mL concentrated hydrochloric acid, store at 4C.

DMBA working solution

Mix one volume stock with four volumes of concentrated acetic acid. This solution must be prepared fresh daily.

Day one:

One gram of soil, 5 mL of phosphate buffer (0.12 M, pH 6.0) and 5 mL chitin suspension (5% w/v chitin in 0.2g/L sodium azide) were added to a test tube. The tube was capped tightly, mixed, and incubated at 37ºC for 16 hours.

Day two:

The chitin suspension was added to the controls, after which 10 mL of potassium chloride (2 M) were added to each sample. The samples were placed on a shaker at room temperature for 30 min. The soil suspensions were filtered using 0.45 µm filter. Next, 0.5 mL of the filtrate of each sample was placed into a fresh tube and 1.5 mL of H_2O and 0.4 mL borate buffer (0.8 M, pH 9.1) were added. The samples were boiled for three minutes and cooled to room temperature. Once the samples were cool, 5 mL of DMBA

working solution was added. The samples were mixed and incubated at 35ºC for 30 minutes. Color development was measured on a spectrophotometer at 405 nm.

Calibration Curve

Calibration Concentration ($\mu g\ mL^{-1}$)	N-Acetyl glucosamine (45 mM) (mL)	Phosphate buffer (mL)	KCL (mL)	H_2O (mL)
0	0	12.5	25	12.5
50	0.5	12.5	25	12
100	1.0	12.5	25	11
150	1.5	12.5	25	10.5
200	2.0	12.5	25	10
250	2.5	12.5	25	9.5

Calculation.

$$\text{N} - \text{Acetyl glucosamine}\ (\mu\text{g g}^{-1}\text{dwt hour}^{-1}) = \frac{C \times V}{dwt * t}$$

C= measured N-Acetyl glucosamine concentration (µg/mL)

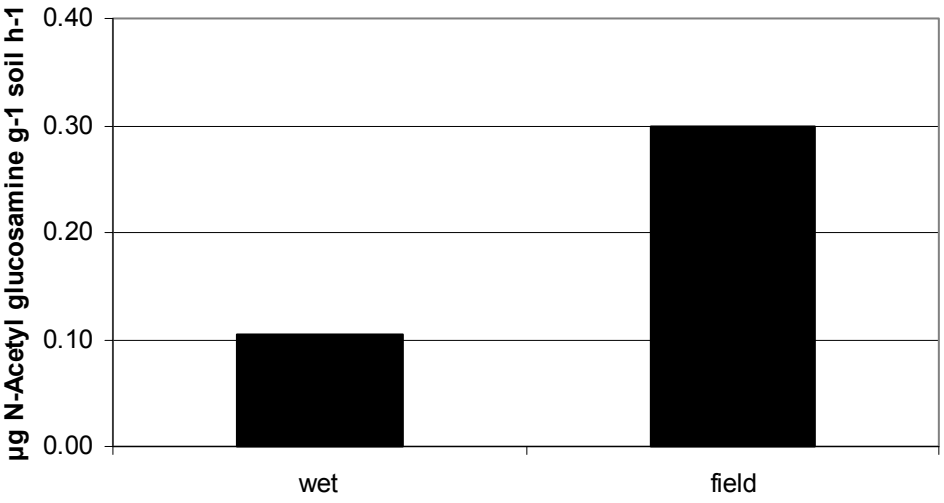
V = final volume of assay (20 mL)

dwt = dry weight of 1 g moist soil (g)

t = incubation time (h)

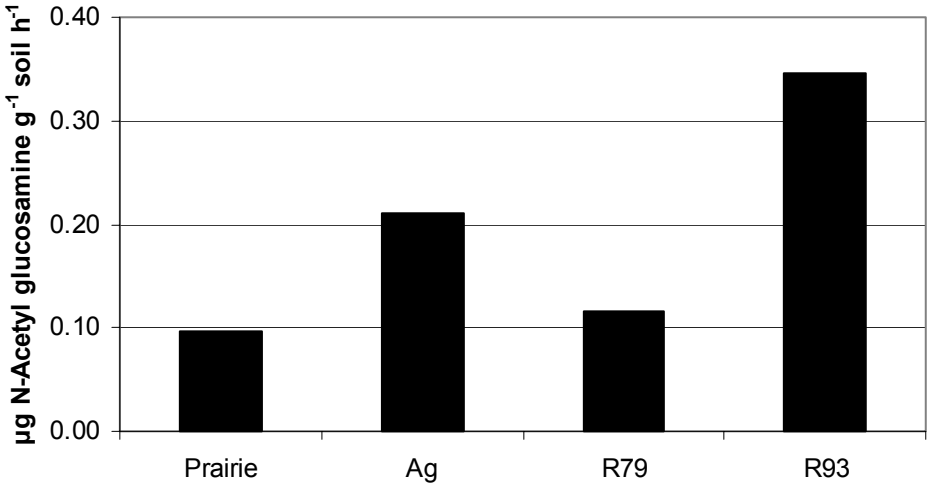
Results

1. Is the activity of chitinase robust to the water saturation that will result from wet sieving?



The t-test was used and a *P* value of 0.09 was generated, therefore wetting the soil did not significantly change activity of chitinase.

2. Does chitinase activity vary across the chronosequence?



The chitinase assay had a *P* value of 0.768, therefore there were no differences in chitinase activity among the soils of the chronosequence.

3. Are gene sequences published for the enzyme?

Yes, abundant gene sequences are available for this enzyme in microorganisms.

Conclusion

The chitinase assay was not pursued further because of the assay's inability to differentiate between the different soils of the prairie chronosequence.

Dehydrogenase activity

Function

The oxidative activity of dehydrogenase in a cell is used as a measure of microbial activity in soil. Dehydrogenase is a respiratory enzyme that removes electrons from its respective substrate and moves the electrons along through NAD+ and ubiquinone. Eventually the electrons will be dumped into the cytochrome system, in which the final electron acceptor is O_2. This method utilizes triphenyltetrazolium chloride (TTC) as an electron acceptor. TTC is reduced by the addition of electrons to triphenyl formazan (TPF). The reduction rate of TTC to TPF is measured colorimetrically at a wavelength of 546 nm (Alef).

Method

The following solutions were prepared ahead of time.

TTC solution

0.8 g of TTC dissolved in 100 mL Tris buffer (100 mM).

TPF calibration solution

Dissolve 50 mg of TPF in 100 mL acetone.

Day one:

In a test tube combine and mix 5 g of soil and 5 mL of TTC solution. The control samples contained only soil and Tris buffer (10 mM). The samples were incubated for 24 hours at 30ºC.

Day two:

Each tube received 40 mL of acetone. The samples were mixed thoroughly and incubated for 2 hours at room temperature with shaking every 30 minutes. The soil suspensions were filtered, and the TPF concentration was measured at 546 nm.

Calibration Curve

Prepare the following solutions in flasks.

Standard concentration (µg TPF/mL)	TPF (mL)	Tris (mL)	Acetone (mL)
0	0	8.3	41.7
5	0.5	8.3	41.2
10	1.0	8.3	40.7
20	2.0	8.3	39.7
30	3.0	8.3	38.7
40	4.0	8.3	37.7

Calculation

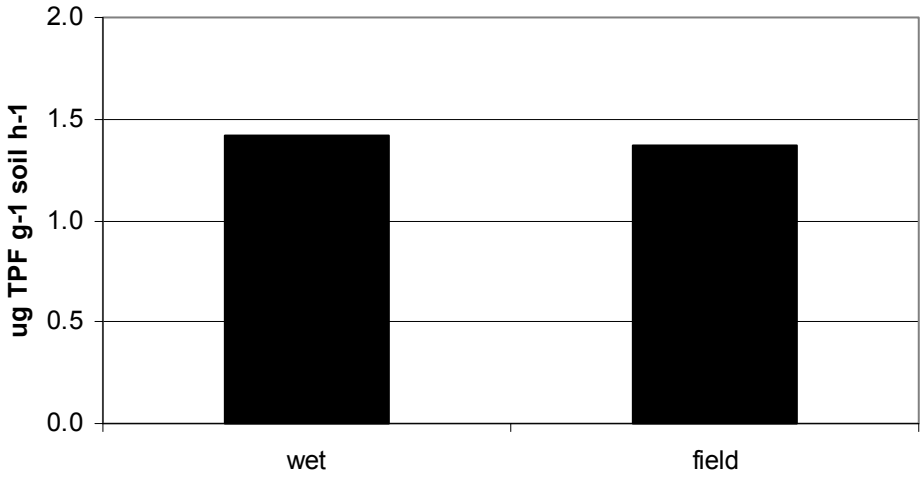
$$\text{Dehydrogenase activity with TPF } \mu\text{g g}^{-1} \textit{ dry } \text{weight soil} = \frac{\text{TPF } (\mu\text{g mL}^{-1}) \times sv}{s}$$

s = dry weight of soil used (g)

sv = volume of solution added to the sample (ml)

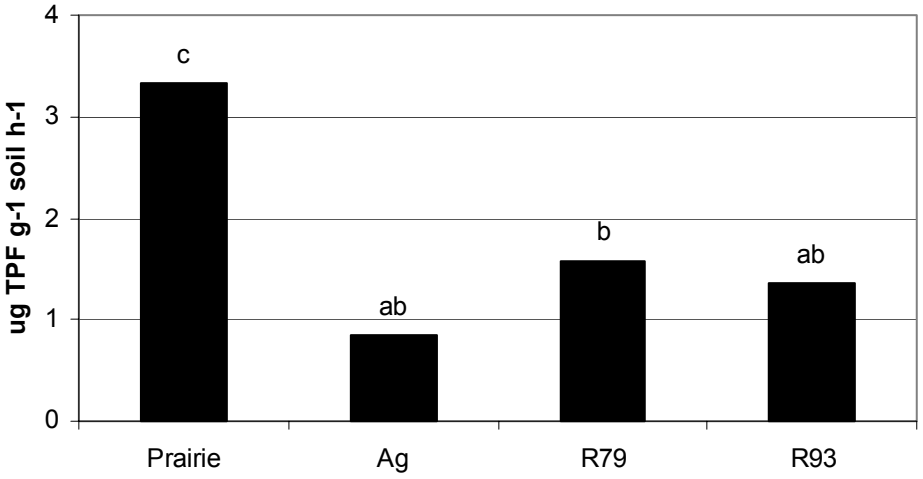
Results

1. Is the activity of dehydrogenase robust to the water saturation that will result from wet sieving?



The t-test was used and a *P* value of 0.435 was generated, therefore wetting the soil did not significantly change the activity of dehydrogenase.

2. Does dehydrogenase activity vary across the chronosequence?



The activity does vary among the soils of the chronosequence; bars topped by the same letter are not significantly different (Bonferroni, $P<0.05$).

3. Are gene sequences published for the enzyme?

Gene sequences are available for this enzyme; however, the sequences available for this enzyme are very generic.

Conclusion

Dehydrogenase activity was rejected as a study enzyme because it is a very generic, ubiquitous soil process that is not exclusively related to the carbon cycle. Identifying individual gene sequences specific to the relevant dehydrogenases for use in future microarray work would be a challenge beyond the scope of this project.

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APPENDIX B: THE INFLUENCE OF MICROBIAL ACTIVITY IN PED DEVELOPMENT

Introduction

Some of the processes of pedogenesis are additions, subtractions, transformations, and translocations of soil mineral and organic material. The factors that drive these processes are time, parent material, climate, topography, and organisms. The pedogenic structure that evolves over time is a result of the combination and intensity of the pedogenic processes. Pedogenic soil structure is important for:

1. Vertical and lateral water movement through a soil profile
2. Crop nutrient uptake/root penetrability
3. Nutrient cycling
4. Erosion control.

In order to contribute to our understanding of how a soil develops, my objective was to focus on a single part of the pedogenic system, specifically, the biochemical transformations mediated by soil microorganisms.

Microbial enzyme activity can by an indicator of carbon and nitrogen transformations in soil (Ekenler and Tabatabai 2002). The enzyme activity of a soils' microbial population is sensitive to soil perturbations (Dick 1997). I have selected two extracellular enzymes β-glucosidase and N-acetyl-β-glucosaminidase as indicators of soil microbe populations. N-acetyl-β-glucosaminidase (E.C. 3.2.1.52) breaks down chitobiose (chitin dimers) molecules to produce N-acetyl-D-glucosamine units. The enzyme is predominately produced by fungi (Waldrop et al. 2000). In this study, it is assumed that the glucosaminidase activity detected is fungal in origin.

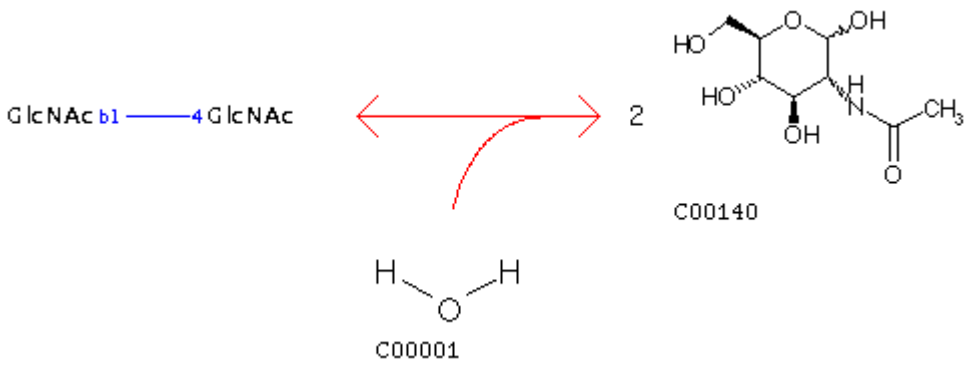

Figure 1. N-acetyl-D-glucosaminidase hydrolyzing chitobiose units (GlcNAc), yielding water and two N-acetyl-D-glucosamine (coo140) molecules.

β-glucosidase (E.C. 3.2.1.21) hydrolyzes the non-reducing β-D-glucose residues releasing β-D-glucose units. This enzyme activity is predominately bacterial in orgin (Waldrop et al. 2000). In this study all β-glucosidase activity is assumed to be from bacteria.

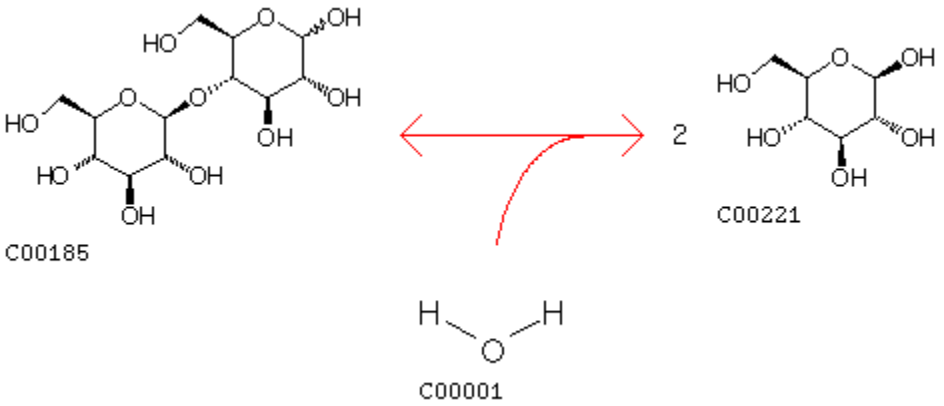

Figure 2. β-D-Glucopyranosyl-4-D-glucopyranose (c00185) units are hydrolyzed by β-glucosidase yielding water and two β-D-glucose monomers.

N-acetyl-β-glucosaminidase activity is more strongly correlated to fungal expression and less linked to bacterial expression (Tisdall and Oades 1982). Fungi have been linked to

aggregate stability (Miller et al. 1998). Microbial activity is favored by the aerobic conditions at ped surfaces (exterior 5 mm). I hypothesize that greater activities of β-glucosidase and N-acetyl-β-glucosaminidase will be measured in the soil samples from the outer 5 mm of a ped than its interior.

Materials and Methods

Soil Sampling

Soil was sampled from a pre-dug pit during the fall of 2003. The soil is a Palouse series, fine-silty, mixed, superactive, mesic Pachic Ultic Haploxerolls. Currently the land is farmed with a conservation tillage and direct seed program. Samples were taken from the northeast corner of the pit using a soil knife. Intact peds were removed from the pit from the Ap, Bt1, Bt2, and Bt3 horizons. The horizons span a vertical distance of 0-150 cm. Once sampled the soil peds were kept at 4ºC until use.

The exterior and interior of each ped was separated in a sterile laminar flow hood using a sterile metal scalpel. The exterior 5 mm of each ped surface was removed, passed through a 2 mm sieve, and homogenized. The interior of each ped was also passed through a 2 mm sieve and homogenized.

The water content of each both the interior and exterior of each ped was determined by drying in a forced air oven at 105ºC for 48 hours. The samples were conditioned at approximately -0.03 Mpa water content (~37%) for five days before further analysis.

Enzyme assays

N-acetyl-β-glucosaminidase (NAGase, EC 3.2.1.30) and β-glucosidase (EC 3.2.1.21) assays were completed on all samples. The protocol for the glucosaminidase assay was derived from (Parham and Deng 2000). β-Glucosidase method was derived from (Eivazi and Tabatabai 1988).

Briefly, for Glucosaminidase; 1 g OD equivalent soil was weighed into a plastic tube. Then 4 mL of 100 mM acetate buffer (pH 5.5) and 1 mL of 10 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma) substrate solution were added. The assay was thoroughly mixed and allowed to incubate for 1 hour in the dark at 37°C. Post incubation 1 mL of 0.5 M calcium chloride and 4 mL of 0.5 M sodium hydroxide were added to the assay to stop the reaction and promote the development of the yellow p-Nitrophenol color (Ekenler and Tabatabai 2002). Controls were assayed by adding substrate after the reaction was stopped with the addition of NaOH. The assay was mixed and filtered through a 0.45 μm syringe tip filter. Controls were carried out for both the exterior and interior of the peds. *p*-Nitrophenol was measured on a spectrophotometer at a wavelength of 405 nm.

β-Glucosidase activity was measured based on the method developed by (Eivazi and Tabatabai 1988). Soil (1 g OD) was placed into a plastic tube and treated with 4 mL of modified universal buffer (MUB) and 1 mL of 0.5 M p-nitrophenyl-β-D-glucopyranoside substrate (Sigma). The solution was mixed thoroughly and allowed to incubate in the dark for 1 hour at 37°C. For controls substrate was added after the

reaction was terminated. After incubation the reaction was stopped and the yellow color from the p-Nitrophenol developed from the addition of 1 mL 0.5 M calcium chloride and 4 mL of Tris buffer (pH 10). The assay solution was mixed and filtered through a 0.45 µm syringe tip filter. P-Nitrophenol was measured on a spectrophotometer at a wavelength of 405 nm.

All enzyme assays were conducted in replicates of three. Significant differences in enzyme activities among soil samples were detected using a one-way ANOVA, and the means were compared using the Bonferroni test at $P<0.05$. Statistical analyses were conducted in SYSTAT v.10 (SPSS Inc, Chicago IL)."

Results

N-acetyl-β-glucosaminidase activity was greater in the exterior ped faces of the A and Bt 1 horizons. The variability of N-acetyl-β-glucosaminidase activity between the exterior ped face and the interior of the ped equilibrates as depth increases.

β-glucosidase activity was greater in the exterior ped faces of the lower horizons Bt 2 and Bt3. There was no variability β-glucosidase enzyme activity between exterior ped face and the interior ped in the upper most A and Bt1 horizons.

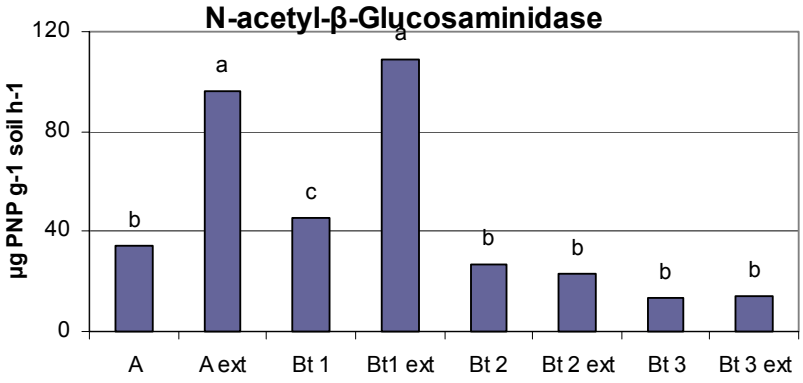

Fig. 3. Bars topped by the same letter are not significantly different (Bonferroni, $P<0.05$).

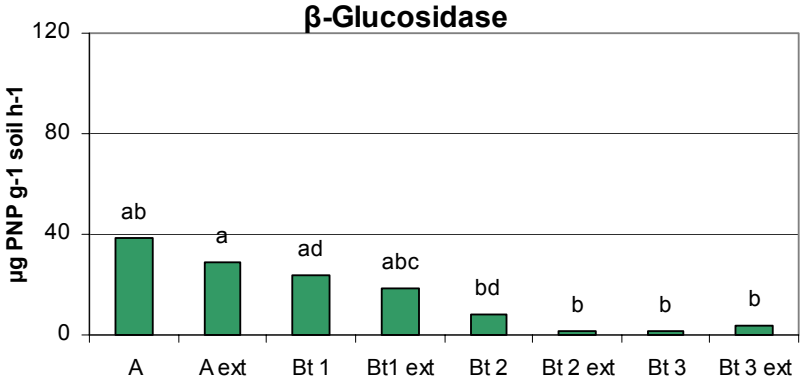

Fig. 4. Bars topped by the same letter are not significantly different (Bonferroni, $P<0.05$).

Discussion

The data provides evidence suggesting the possibility that N-acetyl-β-glucosaminidase activity may play a part in the development of soil structural characteristics. Aggregate stability is linked to pedogenic structure. A small amount of residual enzyme activity remains in the profile that could possibly serve to sustain aggregate and thus pedogenic structure.

Due to the amount of clay accumulated on ped surfaces, future research regarding extracellular enzymes and their attachment to clays will be an important link in understanding what effect microbial activity has on ped structure. Variability in clay content in the different horizons could affect the availability of the enzymes for substrate degradation. Extracellular enzymes can sorb to the edges of clay minerals, preventing the enzymes' active site from binding substrate. As clay content increases the amount of enzyme bound to the edges could increase. This could lead to a reduction in the efficiency of the microorganisms to break down available substrate while protecting the enzyme from degradation itself. The existence of microbial enzyme activity deep with in a soil profile may thus be greater then initially observed.

Conclusion and Interpretation

N-acetyl-β-glucosaminidase activity was the only enzyme of the two studied that was differently expressed between the exterior and interior of the ped. As this enzyme is dominantly associated with fungal activity, the formation of the mollic epipedon of the soil may therefore be partly attributable to fungal biomass and the persistent residues of fungal activity. Stable aggregate formation is a precursor to the structural development of a soil and is the building block of a dynamic soil system.

Acknowledgements

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APPENDIX C: DEVELOPMENT OF A DNA MICROARRAY TO DETECT β-GLUCOSIDASE AND N-ACETYL-GLUCOSAMINIDASE

A microarray was designed to analyze the possible microbes in the microbial community that could be contributing to the enzyme (β-glucosidase and N-acetyl-β-D-glucosaminidase) activity measured in the prairie soil chronosequence. The optimization of microarray conditions is currently ongoing and is the focus of the follow-up research to this thesis.

1. Probe Design

DNA probes for genes sequences for these enzymes were obtained by searching the NCBI database with the E.C. number for each enzyme (β-glucosidase E.C. 3.2.1.21; NAGase E.C. 3.2.1.30). The sequences selected for further study were of both bacterial and fungal origin. Pathogenic microorganism gene sequences were excluded.

Selected sequences were aligned using Clustal X software (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html). After alignment, the sequences were imported into BoxShade (http://www.ch.embnet.org/software/BOX_form.html) to identify areas of homology. Sequence areas (70 nucleotides long) of interest for probe design were located in non-homologous regions of the sequences. After 70-base (70-mer) non-homologous regions were identified in each sequence, the possible probe sequences were submitted to the Blast function of the NCBI alignment software (http://www.ncbi.nlm.nih.gov/BLAST/) to confirm the uniqueness of these sequences.

Result

Table 1. Organisms and DNA sequences for a microarray designed to interrogate the possible microbial origins of β-glucosidase and NAGase activities.

Enzyme	Description	Kingdom, Genus or Class	Organism and Probe name	sequence for probe
B-Glu	Eukaryote	Saccharomycetes	P.capsulata 235-303	ATGCTGGTAACGGTGTCAGGGGCACTGATATGGTCAACGCTTATGCTTCGGGTCTGCACGTTGGTGCTTC
	Eukaryote	Ascomycota	S.lycopersici 1100-1168	CTAGAACATACAACTACCCGGATGAATACTTGACACTTGGAGGCTTGGATCCTTACAACCCCCCGCCTG
	Eukaryote	Ascomycota	T.reesei 1449-1617	TCATTCTGGAGCAGATTCTTGCTCTTCCGCAGGTCAAGGCCGTTGTCTGGGCGGGTCTTCCTTCTCAGG
	Eukaryote	Ascomycota	B.fuckeliana 3147-3216	ATAGGATGGAGAGAAGAAATGTGGGGGGAATTGGGGGGCGGGGGCTTGACGGAGAGAATGGAGAAATGAA
	Bacteria	Actino	M.bispora 361-430	ACGCGGGGCCGACCTCGTGGCCGCGGTCGTCCAGGGACACGCGGCGGCGAGCGACGCCGCCGGGGACCTG
	Bacteria	Thermotogae	T.maritima 696-765	AGAAGAAGACATCAGAGCGGTGAGATTCATGCATCAGTTCAACAACTATCCTCTCTTTCTCAATCCGATC
	Bacteria	Actino	S.reticuli 15-84	TTCGTCCCTGACCTTTCCTCCCGCCTTCCTCTGGGGCGCCGCGACCTCGGCGTACCAGATCGAGGGTGC
	Bacteria	Bacilli	B.circulans 601-669	CGGGATCGCGCCGAACACGTCATGGGCCGTTCCTTACCGACGCACCAAGGAGGATATGGAGGCTTGCCT
	Bacteria	Actino	Streptomyces 802-870	CTCCAGGGCGCCTACCCGGAGGACCTCGTCAAGGACACCGCCGGGCTGACCGACTGGTCCTTCGTCCGC
	Bacteria	Neisseriaceae	C.violaceum 825-894	AATTACTATTACACCTTGAGCTACGCCCACAATCCGCCGGACGGCGTCGGCATGCAGCGAATCAACACCA
	Bacteria	Bacilli	B.subtilis 440-511	AAGTCATAGAGTTTTATGAACGCTATGCCAAAACGGTTTTCAAACGCTATCAACACAAAGTGAAGTATTGG
	Bacteria	Proteobacteria	E.herbicola 574-643	CCGAGAAACGACTGAAAGGTGAGGAGCTTACACTCCCTCCTATTGTTCCTGCTGGTTCCTATCTCGAAAC
	Eukaryote	Ascomycota	T.emersonii 1537-1606	TGGACATCCTGATGGAGGACAAGAACGGCAACCCCATCGGCCCCGAGACCCAATGCGAGTGGCTGCGGCC
	Eukaryote	Ascomycota	C.wickerhamii 798-867	GCTGTTGAAGGTGGTGATGAGGCTATCTTAACTGTAGTTAATGGTGAAACTTACTATCAATGGGAAGGTA
	Bacteria	Clostridia	C.stercorarium 146-215	AGACAACGGGATCCCACTGATCCCGGTAAGAAAACCACTGTCCCGGCCACATGTTTCCCC
	Bacteria	Proteobacteria	R.leguminosarum 168-240	TCCGAATGGCTCTTCATTCGCCTGTTCGTGGGACCTCGATCTGGCTTTCCAGCTCGGAACAGCATTGGCAGCC
	Bacteria	Proteobacteria	A.tumefaciens 2245-2311	GCCGTTCAAGGAGCTGCGTGCCTTTGCGAAGCTCAAGCTGGCCCCGGGCGCGACCGGTACGGCGGTGC
NAGase	Bacteria	Proteobacteria	N.europaea 33-92	GGGCACAGAACTGACTGAAACGGATCGTGTCCGACTAAGTCATCCCTTGGTGGGAGGCGT
	Bacteria	Proteobacteria	R.solanacearum 371-438	ACATCGACCTGAGCTTCACGCCGGTGCTGGACCTGGACTATGGCACCAGCGGCGTGATCGGTGACCGC
	Bacteria	Actino	C.fimi 1874-1946	GGCCCGACAGCACCGCGCCGGCGCGCCAGTCCCGGAACCGCAGCAGCCACACGACGGCGACGATGACCGCGGC
	Bacteria	Actino	S.olivaceoviridis 440-509	CCCCGGCCGC CGCCGAACCCGGCGGCTCCCCGTACCGCATCACCGGCGACACGCGGATCCGCGCCGCATC
	Bacteria	Actino	Arthrobacter 2676-2751	CTTGCCGGTGGCAGTCTCCCCCGGCAAGAGCCTGAAGCTTGACGTTGGCCAGCTGGCAGCCCCCGGCACCAAGGC
	Bacteria	Proteobacteria	C.violaceum 186-255	CACAGCATCCGCCGCATCCTGAAGCTGGACACGCCGCAGTTCACCCTCCGCCACATCACCGGCGACCTGT
	Bacteria	Bacilli	L.lactis 244-332	TAGTCCAGGTCACATGGGCTCCTTATTAGAGCAAAATCCTGAATTTGCACTACCTGACAGCAATCAACAAGCGGTA
	Eukaryote	Fungi	C.albicans 1888-1959	GACTACAAAGATATGGCCACGTACTGCAGCACTTGCTGAATTGACTTGGAGCGGGAACAAGGATTCCAACGG
	Eukaryote	Fungi	P. chrysogenum 303-373	CCGGAATCCATAACGTCGATGTTCATGTGGACAACGATGCCGATCTCCAATACGGTGTGGATGAATC

2. Microarray Hybridization and Signal Detection

Genomic DNA from soil samples was isolated using a method that was modified from Zhou et al. and Gabor et al. (Gabor et al. 2003; Zhou et al. 1996). Briefly, 1 g soil and 1 mL of lysis buffer were added to a 2.0 mL capacity conical tube (BioPlas, Inc. San Rafael, CA). The lysis buffer consisted of 100 mM Tris pH 8, 100 mM sodium EDTA pH 8, 100 mM NaH_2PO_4 pH 8, 1.5 M NaCl, 1% CTAB (CAS#57-09-0), and 5% polyvinylpolyprilidone. After the sample was vortexed for one minute at maximum speed, 10 µL proteinase K (10 mg/mL), 40 µL lysozyme (10 mg/mL), and 0.5 g silica-zirconia beads (0.1 mm, BioSpec, Bartlesville, OK) were added. The sample was mixed and incubated at 37ºC for 30 min. After the incubation 200 µL 20% SDS was added, the sample mixed and allowed to incubate for 2 hours at 65ºC. The samples were then placed in a bead-beater and homogenized for two minutes. Following bead beating, the samples were centrifuged at 16000× g for 10 minutes; the supernatant was removed and placed into a clean DNAase-free tube. The pellet was extracted once more with lysis buffer, vortexed for 1 minute, incubated for 10 minutes at 65ºC, centrifuged and the supernatant removed and pooled with the previous sample.

The DNA in the supernatant was purified by the addition of an equal volume of chloroform-isoamyl alcohol. The samples were centrifuged and the aqueous phase was removed. The DNA was precipitated from the aqueous phase, the pellet washed, and the DNA resuspended in water.

A biotin-labeled nucleotide (dATP) was incorporated into genomic DNA per manufacturers' instructions (BioNick DNA Labeling System, Invitrogen, Carlsbad, CA). Target hybridization occurred for 16 hours at room temperature. Trymide signal amplification (TSA) was used to maximize the detection of hybridized target to probe sequences (TSA Biotin System, Perkin-Elmer, Boston, MA). Slides were washed by rinsing with fresh TNT buffer, followed by a four-minute agitation in TNT buffer. After completion of the TSA procedure an Alexa Fluor 546 streptavidin conjugate (2 $\mu g\ ml^{-1}$ 1x SSC 5x Denhardt's) was added to the slide (Call et al. 2003). Fluor attachment was followed by a final wash and rinse in 0.6x SSC. The slides were dried and imaged on a ScanArray Express HT (Perkin-Elmer, Boston, MA) imager.

Results

TSA experiment

a

4	3C	3C	3C	3C	B	B	B	B	CY3	CY3	CY3	CY3	B	B	B	B
3	3A	3A	3A	3A	B	B	B	B	3B	3B	3B	3B	B	B	B	B
2	2B	2B	2B	2B	B	B	B	B	2C	2C	2C	2C	B	B	B	B
1	1	1	1	1	B	B	B	B	2A	2A	2A	2A	B	B	B	B
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

1	Biotin labeled primer	3A	nicked B. subtilis 10 ng/uL
2A	nicked PUC19 10 ng/uL	3B	nicked B. subtilis 100 ng/uL
2B	nicked PUC19 100 ng/uL	3C	nicked B. subtilis 250 ng/uL
2C	nicked PUC19 250 ng/uL	CY3	

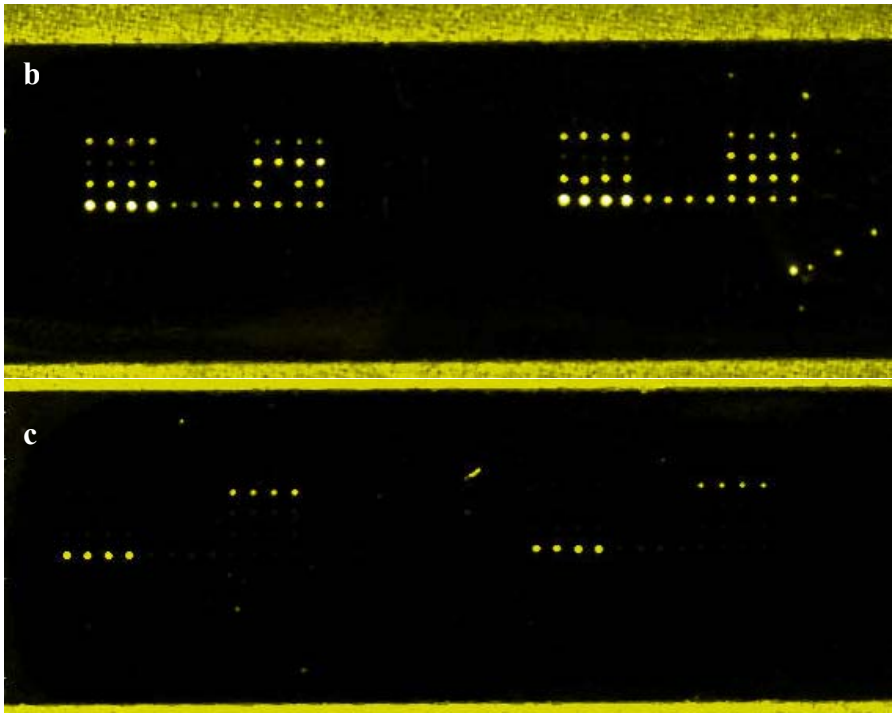
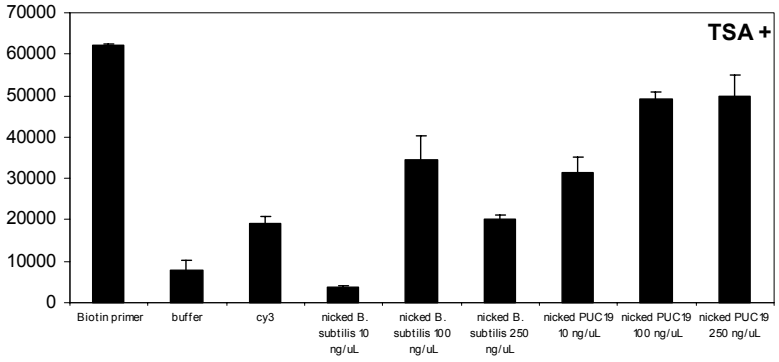
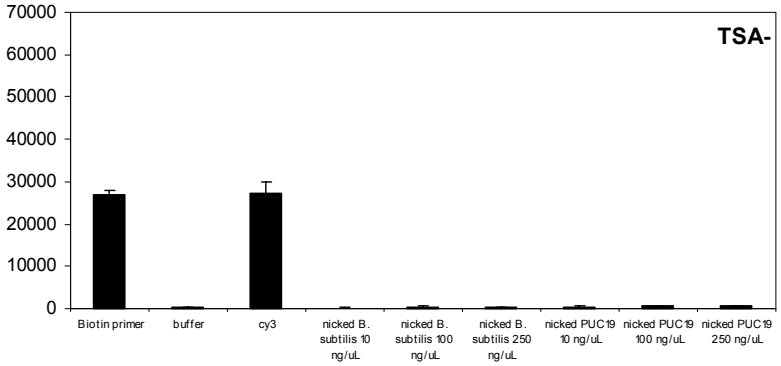


Figure 1. The TSA +/- experiment. a) Array map. b) Microarray images of the TSA + and c) TSA- experiment. Slide "a" followed the procedure above. Slide "b" did not undergo TSA amplification, only Alexa Fluor 546-streptavidin attachment.

a



b



c

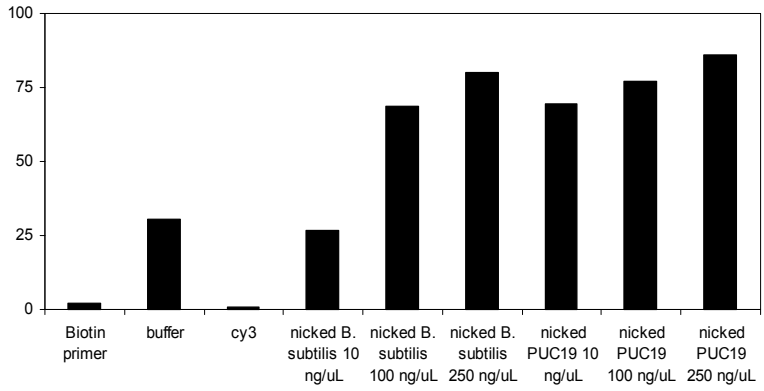
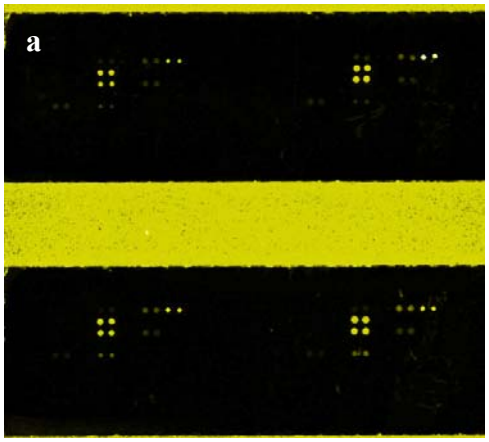


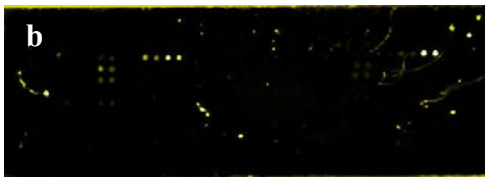
Figure 2. a) TSA+ slide signal pixel intensity. b) TSA- slide signal pixel intensity. c) The TSA+/TSA- pixel intensity ratio. TSA amplification increased pixel signal intensity >75 times.

Formamide concentration experiment

9	C.albicans 1888-1959	Buffer	P. shrysogenum 303-373	Buffer	PUC19	CY3						
8	Arthrobacter 2676-2751	Buffer	C.violaceum 186-255	Buffer	L.lactis 244-332	Buffer						
7	R.solanacearum 371-438	Buffer	C.fimi 1874-1946	Buffer	S.olivaceoviridis 440-509	Buffer						
6	R.leguminosarum 168-240	Buffer	A.tumefaciens 2245-2311	Buffer	N.europaea 33-92	Buffer						
5	T.emersonii 1537-1606	Buffer	C.wickerhamii1 798-867	Buffer	C.stercorarium 146-215	Buffer						
4	C.violaceum 825-894	Buffer	B.subtilis 440-511	Buffer	E.herbicola 574-643	Buffer						
3	S.reticuli 15-84	Buffer	B.circulans 601-669	Buffer	Streptomyces 802-870	Buffer						
2	B.fuckeliana 3147-3216	Buffer	M.bispora 361-430	Buffer	T.maritima 696-765	Buffer						
1	P.capsulata 235-303	Buffer	S.lycopersici 1100-1168	Buffer	T.reesei 1449-1617	Buffer						
	1	2	3	4	5	6	7	8	9	10	11	12

Figure 3. Array map for the formamide experiment.





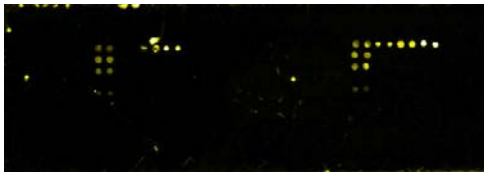


Figure 4. Array images from the formamide hybridization experiment. Top wells contained soil 500 ng DNA extract, 10 ng PUC19 plasmid DNA and 100 ng *B. subtilis* DNA in the hybridization.

Bottom wells contained only 100 ng *B. subtilis* DNA and 10 ng PUC19 DNA in the hybridization;

a) Hybridization buffer contained 0% formamide

b) Hybridization buffer contained 25 % formamide

c) Hybridization buffer contained 50 % formamide

Summary

Including formamide in the hybridization step of the microarray improves hybridization specificity of the target DNA to the probe. Using 25% formamide in the hybridization buffer allowed for less inhibition of target to probe binding as exhibited by the amount of PUC19 plasmid DNA that bound to the appropriate probe. When 0% and 50% formamide was used during hybridization, PUC19 DNA and *B. subtilis* binding to its respective probe was inhibited in the non-soil DNA extract slides.

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