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Activities of extracellular enzymes in physically isolated fractions of restored grassland soils

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Abstract

Extracellular enzymes degrade complex organic compounds and contribute to carbon turnover in soils. We used physical fractionation procedures to investigate whether soil carbon is spatially isolated from degradative enzymes across a prairie restoration chronosequence in Illinois, USA. We found that carbon-degrading enzymes were abundant in all soil fractions, including macroaggregates, microaggregates, and the clay-sized fraction. The activities of two cellulose-degrading enzymes and a chitin-degrading enzyme were 2–10 times greater in particulate organic matter (POM) fractions than in bulk soil, consistent with the rapid turnover of POM fractions. Polyphenol oxidase activity in the clay-sized fraction was 3 times that in the bulk soil, despite a higher mean residence time for carbon in the clay-sized fraction. For most enzymes, differences in activity among fractions and across the restoration chronosequence diminished when adjusted for differences in carbon concentrations. However, glycine aminopeptidase activity per unit carbon increased four-fold across the chronosequence in the clay fraction, while polyphenol oxidase activity declined by 40%. These results suggest that enzyme production and carbon turnover occur rapidly in POM fractions, but slowly in mineral-dominated fractions where enzymes and their carbon substrates are immobilized on mineral surfaces. Soil carbon accumulation in mineral fractions and across the prairie restoration chronosequence probably reflects increasing physical isolation of enzymes and substrates on the molecular to micron scale, rather than exclusion of enzymes from entire soil fractions. Based on these mechanisms, land managers could increase soil C stocks by reducing the physical disruption of soil structure associated with cultivation.
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1. Introduction

Soils contain up to 2300 Pg of carbon (C) globally (Jobbagy and Jackson, 2000), and changes in soil C cycling due to human activities could have major impacts on concentrations of atmospheric CO_2 and the global climate system (Houghton et al., 2001). Soil cultivation for agriculture frequently converts soil C stocks into CO_2, with negative consequences for soil quality, agricultural productivity, and global climate (Hendrix et al., 1998; Tilman et al., 2002). While losses of soil C under agriculture are well documented and often problematic, development of strategies to stabilize or increase C stocks requires better knowledge of the mechanisms regulating C turnover in soils (Paustian et al., 2000; Six et al., 2000, 2002).

In both natural and agricultural soils, mineral sorption and soil aggregation are important mechanisms leading to C stabilization (Elliott, 1986; Sollins et al., 1996). Sorption by soil minerals can protect organic material from microbial attack, thereby increasing the mean residence time of mineral-stabilized C (Christensen, 1995; Torn et al., 1997; Kaiser and Guggenberger, 2003). Aggregation also physically protects soil C, with smaller aggregates generally containing relatively more old C (Six and Jastrow, 2002). Roots and mycorrhizal fungal hyphae are important drivers of the relationship between aggregate stability and C storage because they serve as binding agents and a source of organic material to soil aggregates (Tisdall and Oades, 1982; Guggenberger et al., 1999) and create conditions

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favoring the formation of microaggregates within macroaggregates (Miller and Jastrow, 1992; Six et al., 2000). When soils are converted for agriculture, C inputs from plants and fungi decline, and cultivation breaks up existing soil aggregates, leaving C within aggregates more vulnerable to decomposition (Elliott, 1986; Cambardella and Elliott, 1993; Six et al., 1998). Therefore, loss of soil aggregation is probably both a result and a cause of C loss (Juma, 1994; Beare et al., 1997). As soils recover from agricultural disturbance, restoration of perennial root systems and aggregate structure lead to increases in C stocks, which then feed back to further enhance aggregate stability (Jastrow, 1996; Jastrow and Miller, 1998).

Given that most organic inputs to soil are polymeric, the decomposition of soil C depends on the microbial production of extracellular enzymes that convert complex compounds into smaller products (Ratledge, 1994; Kögel-Knabner, 2002; Nannipieri et al., 2002). For example, root and leaf litter contain large amounts of cellulose and hemicellulose, and microbes must produce cellulose-degrading enzymes to access these substrates (Ljungdahl and Eriksson, 1985; Beguin, 1990). Plant material is also rich in lignin, which is degraded by polyphenol oxidase and peroxidase enzymes that are produced primarily by fungi (Blanchette, 1991). Other microbes may produce extracellular enzymes to degrade chitin, an insoluble polymer of amino sugars (Ratledge, 1994; Keyhani and Roseman, 1999) that is a major component of the cell walls of fungal mycelia. Carbon-degrading microbes must also produce nutrient-acquiring enzymes, such as phosphatases and proteases, to obtain nutrients for growth and enzyme synthesis (Sinsabaugh et al., 1993; Asmar et al., 1994). Over time, soil microbes and abiotic reactions reprocess plant inputs into even more complex (and often insoluble) materials, such as humic compounds (Tate, 1987; Spaccini et al., 2000) that require oxidative enzymes for their degradation (Mangler and Tate, 1982; Freeman et al., 2001).

How do these enzymes interact with soil structure to control C balance? We hypothesized that minerals and aggregates could enhance C stability by physically impeding the enzymatic degradation of C compounds. Soil aggregation could interfere with enzymatic decomposition by preventing microbes and enzymes from making contact with substrates inside aggregates (Sollins et al., 1996). Such restriction might cause C to build up within aggregates. To test whether this mechanism is important for C storage in grassland soils, we measured extracellular enzyme activities in macro- and micro-aggregates from a prairie restoration chronosequence in northern Illinois, USA. We also predicted that increases in soil aggregation and C concentration across the chronosequence would be accompanied by a reduction in enzyme activities within soil aggregates.

There is good evidence from studies using radiocarbon and ^{13}C isotopes that soil fractions differ widely in their C turnover rates, with larger size fractions having higher turnover rates (Balesdent and Mariotti, 1996; Bird et al., 2002). In a Kansas grassland soil similar to our Illinois grassland soils, coarse and fine particulate organic matter (POM) fractions had mean residence times of 9 and 31 years, respectively, while silt- and clay-sized fractions showed much higher values of 73 and 196 years, based on ^{13}C measurements following a C4–C3 vegetation transition that occurred 62 years ago (Jastrow, unpublished data). Since extracellular enzymes catalyze the decomposition of organic C, we hypothesized that enzyme activities would be greater in soil fractions with rapid C turnover, such as POM, but lower in fractions with slow turnover, such as clay-sized particles. Alternatively, enzymes could accumulate on clay surfaces via the same stabilization mechanisms that decrease C turnover. If both enzymes and C are immobilized on or within soil minerals, enzymes could coexist with C substrates without degrading them (Zimmerman et al., 2004).

2. Methods

2.1. Sample collection

We collected soil samples in late August 2003 from 4 sites in a tallgrass prairie restoration chronosequence within the National Environmental Research Park at the Fermi National Accelerator Laboratory (Fermilab) in Batavia, IL, USA (Jastrow, 1987, 1996). These sites were (1) a conventionally cultivated row-crop field in soybeans [*Glycine max* (L.) Merr.], (2) an 11-year-old restored prairie, (3) a 25-year-old restored prairie, and (4) a native prairie remnant (never cultivated). The first site has been cultivated, hayed, or grazed for over 100 years, including a corn–soybean rotation since 1988 or 1989 and continuous corn between 1975 and 1988. The site was tilled by moldboard plow and disking until the early 1980s, and by some form of conservation tillage thereafter (including chisel plowing, shallow tillage, and no-till). Plant species composition in the prairie sites was a mixture of C4 grasses, C3 grasses and sedges, and C3 forbs (Jastrow, 1987). All sites were located on Drummer silty clay loam (fine-silty, mixed, superactive, mesic Typic Endoaquoll) with soil pH values of 6.1–7.0 (1:1 soil:deionized water slurry). Mean temperature in 2003 was ~1 °C above the 30-y mean of 8.8 °C, and precipitation was near the 30-y mean of 999 mm (National Climatic Data Center, 2003). Climate during the 3 months preceding the study reflected the 2003 averages.

At each site, we removed surface litter and collected 3 soil cores from each of 5 evenly spaced locations along a randomly located 50-m transect. The 3 cores (4.8 cm diameter × 15 cm depth) at each sampling location were taken 30 cm apart in a line perpendicular to the transect and composited to give 5 samples for each site. Soils were transported to the laboratory on ice and stored at 4 °C.

2.2. Soil processing

Field-moist soils were processed within 2 days of collection by gently breaking apart cores along natural break points, then passing the soil through an 8-mm sieve. Root pieces and organic debris (not incorporated into aggregates and longer than 8 mm) that passed through the sieve were removed. After thorough mixing, a subsample was dried at 105 °C for gravimetric determination of the water content of each sample. Another subsample of the bulk soil was frozen at −20 °C for enzyme analysis. The remaining soil was stored at 4 °C until it was needed in one of three separate fractionation procedures (below) within another 10 days. Soil bulk density was calculated by dividing the total dry weight of each soil core by the core volume.

2.3. POM, silt, and clay fractionation

We fractionated soils into POM, silt-sized, and clay-sized fractions by using a modified version of the Cambardella and Elliott (1992) procedure. Ten grams of field-moist processed soil, 40 ml of water, and 20 glass beads (4-mm diameter) were combined in 60-ml polyethylene bottles and shaken for 60 min on a wrist action shaker at ~250 strokes min^{-1}. We optimized the time and number of beads in preliminary tests to ensure the most complete break-up of aggregates with the least disruption of POM. We dispersed soil particles physically by using the bead method rather than by chemical dispersion with sodium hexametaphosphate to avoid ionic solutions that can denature enzymes. The resulting soil homogenate was poured through a 250-μm sieve stacked on top of a 53-μm sieve to collect coarse and fine POM fractions, respectively. After rinsing both sieves sequentially, material that passed both sieves was transferred to 1-l centrifuge bottles for separation of silt-sized and clay-sized particles. Bottles were centrifuged at 1000 rev min^{-1} (270*g*) for 3 min to pelletize silt-sized particles. The suspended clay-sized particles were aspirated into another 1-L bottle and centrifuged at 4500 rev min^{-1} (5550*g*) for 30 min. Both silt-sized and clay-sized pellets were resuspended in 50 ml of deionized water, then centrifuged at 3400 rev min^{-1} (2440*g*) in 50-ml centrifuge tubes for 30 min. We removed excess water from the silt-sized and clay-sized fractions by re-centrifuging in a smaller volume and then discarding the supernatant.

2.4. Macroaggregate fractionation

To separate water-stable macroaggregates from bulk soil, we placed another 10 g of processed field-moist soil on a 250-μm sieve and submerged the sieve in deionized water. After 5 min, the sample was wet-sieved by hand for 50 up–down strokes over a 2-min period (Elliott, 1986). Floating litter was aspirated away, and the sieve was removed from the water and allowed to drain. Aggregates were transferred to a 50-ml centrifuge tube with 35 ml of Ludox AS-40 (Aldrich; density $\leqslant 1.3\ g\ cm^{-3}$, adjusted to pH 7 with HCl) to remove the free and released inter-aggregate POM that was caught on the sieve but not incorporated into macroaggregates. We used Ludox rather than sodium polytungstate (SPT) solution because the ionic strength of the SPT could denature soil enzymes. After the tube containing Ludox and macroaggregates was gently inverted 4 times, we allowed the mixture to settle for 1 min, then removed the light fraction and Ludox by aspiration. Macroaggregates were washed 3 times with deionized water to remove residual Ludox, then transferred to preweighed containers.

2.5. Microaggregate fractionation

Stable microaggregates were separated from bulk soil by using a microaggregate isolator in a third fractionation procedure modified from Six et al. (2000). Field-moist, processed soil (10 g) was immersed in deionized water for 10 min and then transferred to the microaggregate isolator. In this device, the soil was shaken with 50 stainless steel beads (4-mm diameter) over a 250-μm sieve at 180 strokes min^{-1} on a reciprocating shaker. Continuous, steady water flow (0.5 ml min^{-1}) flushed microaggregates and other soil components smaller than 250 μm through the device and carried them to a 53-μm sieve below. Shaking was stopped when water below the 250-μm sieve ran clear. With some samples, a few macroaggregates remained on the 250-μm sieve; these were gently pushed through the sieve with a rubber spatula. Material retained on the 53-μm sieve was wet-sieved (50 up–down strokes in 2 min). The microaggregates were gently transferred from the sieve into preweighed containers. We did not distinguish free microaggregates from those inside macroaggregates.

We attempted to separate inter-microaggregate fine POM caught on the 53 μm sieve from stable microaggregates by using Ludox, but this method was ineffective. The Ludox could not be removed from the microaggregates without centrifugation, which caused sedimentation of silica particles from the Ludox. Therefore, the microaggregate fraction probably contains a substantial amount of inter-microaggregate fine POM. To address this issue, we assumed that all fine POM in the soil (determined from the first fractionation procedure) was captured on the 53-μm sieve and mathematically removed this fine POM contribution from the microaggregate results. These corrected data are presented along with the original data. Because the effect of the correction was generally small, we used the original data in statistical analyses.

Dry weights of all soil fractions were calculated from the total wet weight of each fraction and the dry:wet ratio of a subsample dried at 65 °C. The remaining wet soil fractions were frozen at −20 °C for enzyme analyses immediately after separation procedures were completed.

2.6. Enzyme and carbon analyses

We measured the activities of extracellular enzymes (Table 1) with assay techniques modified from Sinsabaugh et al. (1993) for hydrolytic enzymes or Sinsabaugh et al. (1992) for polyphenol oxidase (PPO). Soil fractions and bulk soil samples were combined with 60 ml of 50 mM Tris buffer, pH 7, and homogenized in a blender for 1–2 min. We used pH 7 buffer instead of the pH 5 buffer used by Sinsabaugh et al. (1993) to obtain a more realistic estimate of potential enzyme activity in our neutral pH soils. In a 2 ml centrifuge tube, 0.75 ml of homogenate was combined with 0.75 ml of substrate (Table 1) in 50 mM Tris buffer and shaken vigorously at 21 °C. Shaking proceeded until spectrophotometric measurements of the accumulated reaction products were reliable (see Table 1 for assay lengths). For all enzymes except PPO, we centrifuged the tubes after shaking and measured the absorbance of *p*-nitrophenol (*p*NP) in the supernatant at 410 nm on a Hitachi U-2000 spectrophotometer (Hitachi Instruments, Inc.). To assay PPO activity, homogenates were diluted 10-fold to reduce interference from native soil polyphenols, and absorbance of the reaction product was measured at 460 nm. Instead of using L-DOPA as a PPO substrate (Sinsabaugh et al., 1992), we chose pyrogallol (PG) because preliminary experiments indicated that PG was less susceptible to non-enzymatic reactions with soil minerals. For all enzymes, we included appropriate controls to account for the background absorbances of the substrate and homogenate solutions. Enzyme activities were expressed as micromoles of product formed per gram of fraction dry weight per hour (μmol product $[\text{g fraction}]^{-1}\,\text{h}^{-1}$) by using a standard curve of absorbance versus *p*NP (Sigma 104-1) concentration for all enzymes except GAP and PPO. For GAP, we decreased the slope of the *p*NP standard curve by a factor of 1.68 to account for the lower extinction coefficient of *p*-nitroaniline (Sroga and Dorick, 2001). We standardized PPO activities by completely oxidizing a known amount of PG substrate with commercially prepared PPO (Sigma T7755) and measuring the absorbance of the reaction products; therefore PPO activity is expressed in terms of substrate consumed. Sample homogenization, shaking, and the use of soluble substrates ensured that nearly 100% of the potential activity in each sample was assayed.

Dried subsamples of soil fractions and bulk soil were ground and homogenized in a Spex mill and analyzed for C and N contents by dry combustion gas chromatography with a Carlo Erba NA1500 elemental analyzer. Total C was equivalent to organic C because no carbonates were present in these soils.

2.7. Statistical analyses

The effects of chronosequence site and soil fraction on C and N concentrations and enzyme activities were evaluated by two-way analysis of variance (ANOVA). Results from this analysis should be interpreted with caution since soil fractions are not independent. Bulk density data were subjected to a one-way ANOVA with site as the main effect. Data were log- or square root-transformed where necessary to improve normality. Mean separations were accomplished by using Bonferroni *t* tests for post hoc multiple comparisons. We used SAS version 9 for all statistical tests (SAS Institute, 2004).

3. Results

Across all sites, more than 75% of the soil mass was composed of silt-sized particles, and the remaining soil mass was evenly split between clay-sized particles and POM (Table 2). Most (87.7%) of these particles were bound into water-stable macroaggregates, whereas only 32.9% of the soil mass existed as stable microaggregates. Most of the microaggregates were isolated from within water-stable macroaggregates, but some portion (especially in the cultivated soil) was not bound into stable macroaggregate structures. Recovery from cultivation increased the fraction of soil in macroaggregates from 76.0% to 93.9% and decreased the microaggregate fraction from 37.7% to 30.7% of the soil mass (Fig. 1).

Overall, bulk soil C concentrations averaged $46\,\text{g kg}^{-1}$ (Table 3). The average C concentration in the silt-sized fraction was significantly lower ($39\,\text{g kg}^{-1}$), but C

Table 1
Extracellular enzymes and their functions, substrates, and assay time

Enzyme	Function	Substrate	Time (h)
β-Glucosidase	Releases glucose from cellulose	5 mM *p*NP-*β*-*D*-glucopyranoside[a]	2–5
Cellobiohydrolase	Releases disaccharides from cellulose	2 mM *p*NP-cellobioside	3–8
Polyphenol oxidase	Oxidizes lignin; degrades and synthesizes humic compounds	50 mM pyrogallol, 50 mM EDTA	1
N-acetyl-glucosaminidase	Degrades chitin	2 mM *p*NP-acetyl-*β*-*D*-glucosaminide	3–5
Glycine aminopeptidase	Degrades proteins	2 mM glycine *p*-nitroanilide (predissolved in a small volume of acetone)	2–3
Phosphatase	Releases inorganic phosphate from organic matter	5 mM *p*NP-phosphate	1–2

[a] *p*NP = *p*-nitrophenyl.

Table 2
Mean distribution of soil mass in particle size fractions and aggregates across all sites ($n = 20$)

Fraction	Soil mass(%)	(SE)
Coarse POM[a]	2.3	(0.3)
Fine POM	9.2	(0.7)
Silt-sized	77.3	(0.8)
Clay-sized	9.9	(0.2)
Macroaggregates	87.7	(1.8)
Microaggregates	32.9	(1.1)

Percentages are derived from independent procedures and are not intended to sum to 100.
[a]Particulate organic matter.

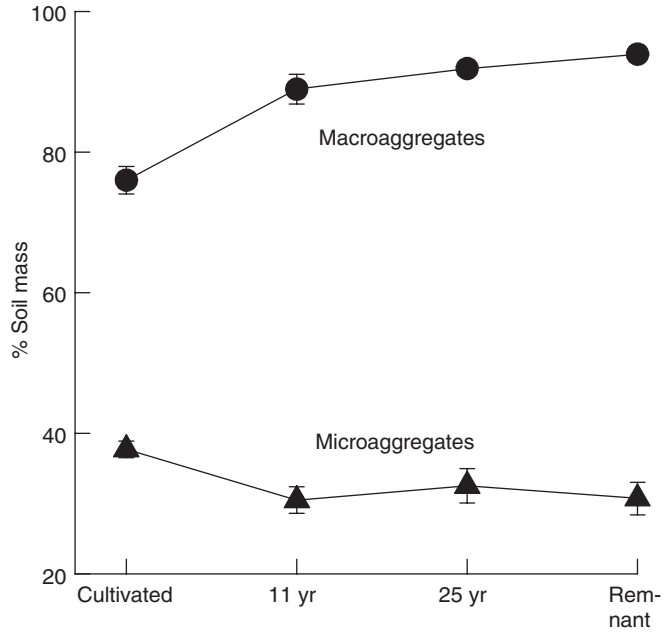

Fig. 1. Changes in percent soil mass (mean and SE) of macroaggregates and microaggregates across the prairie restoration chronosequence ($n = 5$).

concentrations in all other fractions were greater than or equal to the bulk value. Although coarse POM had the highest C concentration (118 g kg^{-1}), the clay-sized fraction was also relatively high in C, with concentrations exceeding those of fine POM. As the largest soil fraction, macroaggregate C concentrations generally reflected bulk soils, whereas microaggregates had significantly higher C concentrations, indicating that they were enriched in fine POM and/or clay-sized particles relative to bulk soil. Across the chronosequence, C concentrations in bulk soils doubled from 33 to 65 g kg^{-1} (Table 3). A similar doubling occurred in each of the soil fractions, except that within the coarse POM fraction, the C concentration remained at 10–14 g kg^{-1} across the chronosequence. On a whole-soil basis, C accumulated in roughly similar proportions in each fraction across the chronosequence, although the increase was smaller in the clay-sized and microaggregate fractions (Fig. 2). Despite a significant site × fraction interaction (Table 4), all fractions showed a similar pattern of low C content in the cultivated soil, intermediate levels in the restoration sites, and the highest C content in the prairie remnant (Supplementary Data, Table S1).

Nitrogen concentrations increased from 2.9 to 5.7 g kg^{-1} in bulk soils across the chronosequence (Table 3). This trend caused a decline in C:N starting after 11 y of restoration; however, C:N in the cultivated soil was low because of relatively low C concentrations (Table 3). Nitrogen concentrations were highest in the clay-sized fraction (7.7 g kg^{-1}) and lowest in the silt-sized fraction (3.5 g kg^{-1}). Carbon:nitrogen ratios were lowest in the clay-sized fraction, greatest in the POM fractions, and intermediate in the bulk soil, silt-sized, and aggregate fractions.

Measurable activities of all six assayed enzymes were found in nearly every soil fraction at every site. Only cellobiohydrolase (CBH) and *N*-acetyl-glucosaminidase (*N*AG) activities were below detection limits in some clay-sized fractions. Although we were concerned that extracellular enzymes could be leached by the hour-long particle size dispersion, enzyme recoveries were >80% (Table 5). Recoveries could not be determined for the aggregate fractions, but we assume they also exceed 80% because the aggregate fractionation procedures were less disruptive than the dispersion procedure used for the other fractions.

Activities of cellulose-degrading enzymes were greatest in POM fractions, reaching 1.46 μmol *p*NP (g fraction)$^{-1}$ h^{-1} for β-glucosidase (BG) and 0.109 μmol *p*NP (g fraction)$^{-1}$ h^{-1} for CBH in coarse POM, averaged across all sites (Fig. 3A, 3B). BG and CBH activities were lowest in the clay-sized fraction, with values of 0.26 and 0.032 μmol *p*NP (g fraction)$^{-1}$ h^{-1}, respectively. In the cultivated site, BG and CBH activity g^{-1} fraction was particularly high in the POM fractions relative to the other fractions (Table S1; significant site × fraction interaction, Table 4). In all fractions, these enzyme activities declined initially, and then recovered across the chronosequence. Macroaggregates contained less cellulose-degrading enzyme activity than the bulk soil, whereas activities were higher in microaggregates. BG and CBH activities in the silt-sized fraction were slightly lower than bulk soil values and were similar to activities in macroaggregates.

In contrast to cellulose-degrading enzymes, PPO was highest in the clay-sized fraction, with average activities reaching 188 μmol PG (g fraction)$^{-1}$ h^{-1} (Fig. 3C). PPO activities were lowest in the POM fractions (~45 μmol PG [g fraction]$^{-1}$ h^{-1}), similar in the bulk soil, silt-sized, and macroaggregate fractions (62–72 μmol PG [g fraction]$^{-1}$ h^{-1}), and slightly elevated in microaggregates. Across the chronosequence, PPO activities g^{-1} fraction remained constant or declined, with a pronounced reduction at the 25 yr-old site in most fractions (Table S1).

*N*AG activity was strongly associated with the coarse POM fraction, where values reached 1.18 μmol *p*NP (g fraction)$^{-1}$ h^{-1} (Fig. 3D). Activity in the fine POM

Table 3
Mean C and N concentrations and C:N for bulk soils by chronosequence site and soil fraction across sites

	Bulk density ($g\,cm^{-3}$)		Carbon concentration ($g\,kg^{-1}$)		Nitrogen concentration ($g\,kg^{-1}$)		C:N	
	Mean	(SE)	Mean	(SE)	Mean	(SE)	Mean	(SE)
Chronosequence site (n = 5)								
Cultivated	1.046[a]	(0.024)	33[c]	(4)	2.9[b]	(0.1)	11.4[a]	(0.2)
11-yr restoration	1.011[ab]	(0.028)	45[b]	(3)	3.7[b]	(0.3)	12.2[a]	(0.4)
25-yr restoration	0.923[b]	(0.015)	46[b]	(1)	3.9[b]	(0.1)	12.0[a]	(0.7)
Prairie remnant	0.824[c]	(0.011)	65[a]	(4)	5.7[a]	(0.4)	11.4[a]	(0.1)
Fraction[1] *(n = 20)*								
Bulk soil			46[d]	(3)	4.1[cd]	(0.3)	11.7[c]	(0.2)
Coarse POM[2]			118[a]	(7)	4.3[cd]	(0.4)	30.3[a]	(2.5)
Fine POM			63[c]	(5)	4.7[bc]	(0.4)	14.2[b]	(0.3)
Silt-sized			39[e]	(3)	3.5[d]	(0.3)	11.7[c]	(0.2)
Clay-sized			77[b]	(4)	7.7[a]	(0.4)	10.2[d]	(0.2)
Macroaggregates			46[d]	(3)	4.1[cd]	(0.2)	11.7[c]	(0.1)
Microaggregates			58[c]	(4)	5.2[b]	(0.4)	11.8[c]	(0.1)
Microaggregates (no fine POM)[3]			57	(4)	5.5	(0.4)	11.0	(0.1)

Within sites or fractions, means followed by the same letter are not significantly different ($P > 0.05$, Bonferroni post-hoc multiple comparisons).
[1]Average value for 5 samples in each of 4 sites.
[2]Particulate organic matter.
[3]Contribution of fine POM to microaggregate C and N removed.

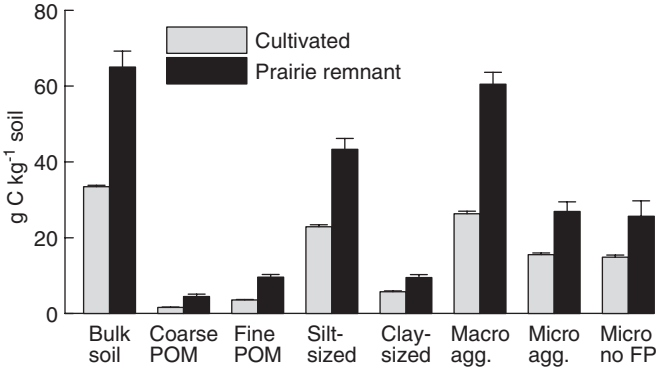

Fig. 2. Mean (SE) amount of C in soil fractions on a whole-soil basis for cultivated and prairie remnant soils ($n = 5$). POM = particulate organic matter. "Micro no FP" is the amount of C in microaggregates, excluding the contribution of C from fine POM.

fraction exceeded the bulk soil value of 0.127 μmol *p*NP $(g\,fraction)^{-1}\,h^{-1}$ by a factor of 3, while activity in macroaggregates was similar to that in the bulk soil. Activity in microaggregates also exceeded the bulk soil value, but this difference disappeared when the contribution from fine POM was removed. The clay- and silt-sized fractions contained the least *N*AG activity (0.055–0.062 μmol *p*NP $[g\,fraction]^{-1}\,h^{-1}$). *N*AG activity g^{-1} fraction showed a general increase across the chronosequence, except in the coarse POM fraction where it declined (Table S1; significant site × fraction interaction, Table 4).

The activities of glycine aminopeptidase (GAP; Fig. 3E) and especially phosphatase (PA; Fig. 3F) were distributed across different soil fractions largely in proportion to C concentration. Average PA activity ranged from a low of 1.66 μmol *p*NP $(g\,fraction)^{-1}\,h^{-1}$ in the silt-sized fraction to a high of 6.28 μmol *p*NP $(g\,fraction)^{-1}\,h^{-1}$ in the coarse POM fraction. Across the chronosequence, PA activity in all fractions showed a consistent pattern of increase in the 11 yr-old site, followed by a decline the 25 yr-old site, and another increase in the prairie remnant (Table S1). Average GAP activity in the microaggregate, clay-sized, and POM fractions all exceeded the bulk soil value of 0.523 μmol pNA $(g\,fraction)^{-1}\,h^{-1}$ by at least 40%, although the magnitude of the differences varied by site along the chronosequence (significant site × fraction interaction, Table 4). This interaction arose because GAP activity increased more dramatically in the mineral soil fractions than in the POM fractions across the chronosequence (Table S1). In the silt-sized and macroaggregate fractions, GAP activities were similar to the bulk soil values.

Since C concentrations varied dramatically among soil fractions and across the restoration chronosequence, we also expressed enzyme activities per unit C (Table S2). Cellulase activities were more similar across fractions when expressed per unit C than when expressed per gram soil (Table S2). By contrast, PPO activity g^{-1} C was lower in the POM fractions. *N*AG activity g^{-1} C was greatest in the POM fractions, despite their high C concentrations, while GAP and PA activities g^{-1} C were similar across all fractions (Table S2).

Because soil C concentrations generally increased across the chronosequence, most enzyme activities per unit C

Table 4
Analysis of variance results for effects of chronosequence site and soil fraction on carbon contents and enzyme activities

Effect	d.f.	*F*-value						
		Carbon content	β-Glucosidase	Cellobio-hydrolase	Polyphenol oxidase	*N*-acetyl-glucosaminidase	Glycine aminopeptidase	Phosphatase
			Data per gram fraction					
Site	3, 112	148.6***	49.2***	44.3***	29.1***	38.9***	62.6***	66.0***
Fraction	6, 112	908.3***	139.9***	26.5***	96.1***	185.8***	19.4***	105.8***
Site × Fraction	18, 112	5.4***	5.7***	4.0***	2.3**	2.9***	5.1***	2.6**
			Data per gram fraction carbon					
Site	3, 112		51.6***	43.8***	150.4***	9.2***	15.8***	76.8***
Fraction	6, 112		139.2***	28.3***	249.9***	91.3***	6.6***	5.9***
Site × Fraction	18, 112		6.7***	2.5**	2.7***	1.9*	3.5***	2.7***

*$P<0.05$; ** $P<0.01$; *** $P<0.001$.

Table 5
Mean recoveries for assayed extracellular enzymes, calculated as the weighted sum of enzyme activity in the POM, silt-sized, and clay-sized fractions, divided by the bulk soil activity

Enzyme	Recovered (%)	(SD)
β-Glucosidase	85.7	(5.8)
Cellobiohydrolase	82.4	(7.9)
Polyphenol oxidase	111.4	(5.7)
N-acetyl-glucosaminidase	80.8	(7.1)
Glycine aminopeptidase	96.0	(3.5)
Phosphatase	85.4	(4.4)

declined or remained the same as restoration proceeded. This decline was particularly dramatic for PPO activity g^{-1} C, which decreased by ~50% in all soil fractions across the chronosequence, including the clay-sized fraction where it was most concentrated (Fig. 4B). By contrast, GAP activity per unit C increased across the chronosequence despite rising C concentrations (Table S2), and this increase was most pronounced in the clay-sized fraction (Fig. 4A). Although there were significant site × fraction interactions for all enzyme activities g^{-1} C (Table 4), the pattern of similar or declining activity with restoration was generally consistent across the different fractions for the cellulases, PPO, and PA, though the magnitude of the response differed (Table S2). In contrast, *N*AG and GAP activities g^{-1} C showed a pattern of increase across the chronosequence in the mineral fractions, but a slight decline in the POM fractions (Table S2).

4. Discussion

Biotic and abiotic mechanisms that reduce C degradation can allow C to accumulate in soil after cultivation ceases (Paustian et al., 2000). Specifically, we predicted that aggregation might physically impede access of soil enzymes to C within the interiors of soil aggregates. Contrary to this prediction, we found that enzyme activities in aggregates generally equaled or exceeded those in the bulk soil, and may have been even greater if there were enzyme losses during the fractionation procedure. Only BG and CBH activities were lower in macroaggregates than in bulk soil, and all enzymes (except possibly *N*AG) were elevated in microaggregates. Thus, we reject the hypothesis that C accumulation associated with soil aggregation is driven by a lack of enzyme activity.

High enzyme activities could reduce C concentrations in soil fractions, but in contrast, we observed that degradative enzyme activities were greatest in C-rich fractions. Marx et al. (2005) also found positive relationships between hydrolase activities and C concentrations in grassland soil particles separated by size. This relationship is reasonable for fractions with abundant labile C, such as the >200 μm particle size class of Marx et al. and our POM fractions. Here, active microbes may be using plant-derived C for growth and enzyme production, thereby increasing enzyme activities in these fractions (Schulten et al., 1993; Gregorich et al., 1996; Kandeler et al., 1999b). In the coarse POM fraction, high *N*AG activity may stem from enzyme production by microbes that degrade the chitin-rich cell walls of dead fungi, especially mycorrhizal fungi (Gooday, 1990; Guggenberger et al., 1999). Elevated cellulase activities in the POM fractions are probably produced by a microbial community that exploits the cellulose from plant material (Ratledge, 1994). Enzymes such as *N*AG, BG, and CBH may drive rapid C turnover in POM fractions, which retain high C concentrations only because they are derived from inputs of recent plant material with high C:N and low mineral content. This rapid decomposition occurs in the POM fraction despite relatively low activities of PPO (Fig. 3C), an enzyme required to degrade the lignin component of plant-derived POM (Kirk and Farrell, 1987).

Carbon concentrations and some enzyme activities were also high (relative to bulk soil) in the microaggregate and clay-sized fractions. Clearly, some mechanism must prevent enzymes from mineralizing C efficiently in these mineral-associated fractions, which contain C pools with very slow turnover rates (Torn et al., 1997; Six and

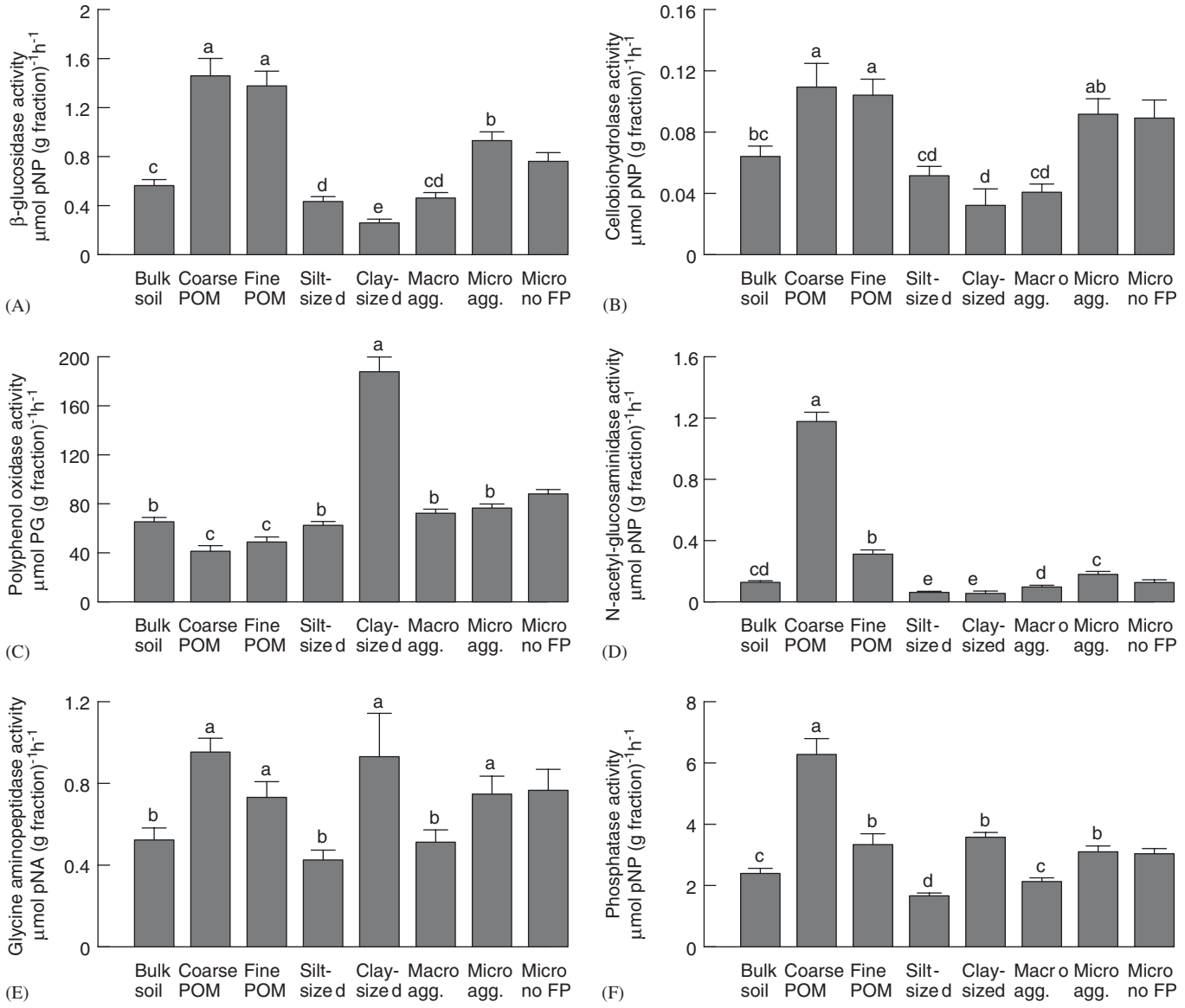

Fig. 3. Mean (SE) enzyme activities per unit fraction mass for measured soil fractions pooled across all sites ($n = 20$). (A) β-glucosidase, (B) cellobiohydrolase, (C) polyphenol oxidase, (D) *N*-acetyl-glucosaminidase, (E) glycine aminopeptidase, (F) phosphatase. "Micro no FP" is the amount of C in microaggregates, excluding the contribution of C from fine POM. For each enzyme, means with the same letter are not significantly different ($P > 0.05$, Bonferroni post hoc multiple comparisons).

Jastrow, 2002). One possible explanation is that appropriate enzymes are not abundant enough in mineral-associated fractions to degrade the C substrates found there. In the clay-sized fraction, we did find low activities of BG, CBH, and *N*AG, but these enzymes are not critical for decomposition in this fraction because it contains little cellulose and chitin (Cornejo and Hermosín, 1996). However, PPO activity in the clay-sized fraction was three times that in bulk soil. The substrates for PPO include humic materials, which—along with aliphatic compounds—make up a large fraction of clay-associated C (Christensen, 1995). In addition, C concentrations in the clay-sized fraction nearly doubled across the prairie restoration chronosequence (Fig. 2) in the presence of this elevated PPO activity. Therefore, we need another mechanism to explain why elevated PPO activity does not increase C turnover in the clay-sized fraction, and similarly, why elevated hydrolase activities do not degrade more C in the microaggregate fraction.

One likely mechanism is the physical isolation of enzymes and their C substrates at the molecular scale. Sorption on mineral surfaces probably reduces actual enzyme activities in the soil well below the potential activities we measured under assay conditions (Burns, 1982; Sinsabaugh and Moorhead, 1994). The process of soil organic matter degradation may contribute to this potential activity as microbes generate C compounds and enzymes that ultimately become sorbed to adjacent mineral surfaces (Golchin et al., 1994). Over time, this process may generate stable C and enzyme pools within

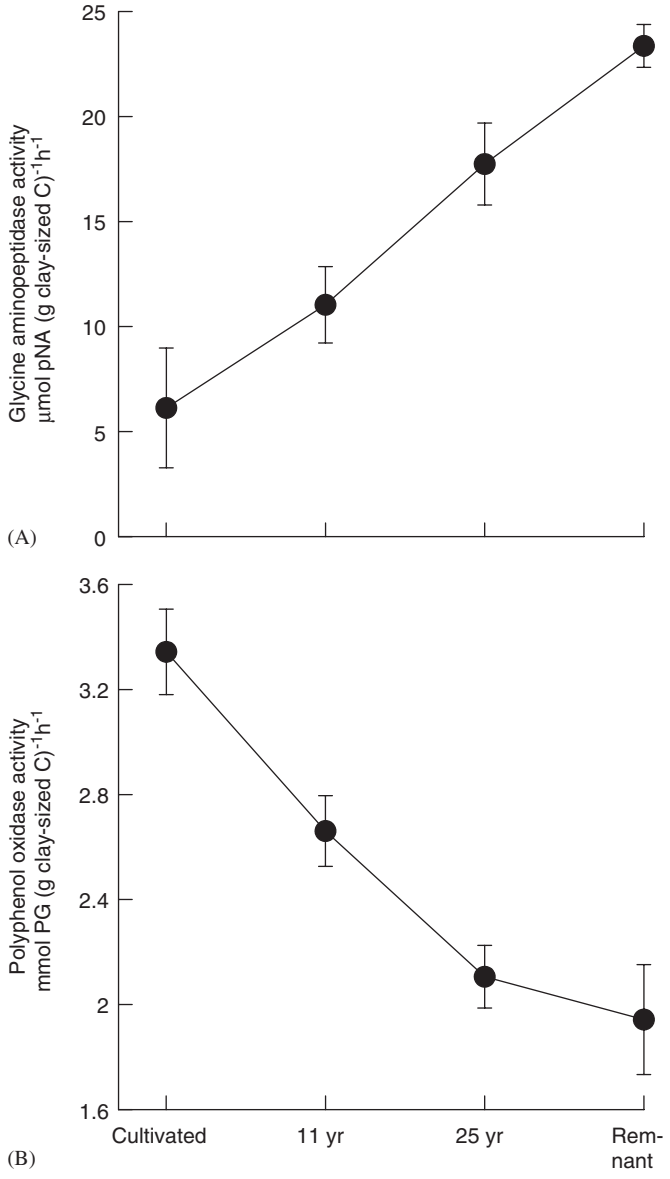

Fig. 4. Change in mean (SE) enzyme activity per unit C in the clay-sized fraction across the prairie restoration chronosequence ($n = 5$): (A) Glycine aminopeptidase; (B) Polyphenol oxidase.

mineral-associated fractions, particularly microaggregates. Thus, at least two "pools" of enzyme may coexist in our grassland soils: a mineral-stabilized pool with high potential activity but low actual activity against soil C substrates, and a POM-associated enzyme pool that rapidly degrades recent inputs of plant C.

This multi-pool model suggests that process rates do not always relate to enzyme concentrations, especially in mineral-rich soils. Our data show that old C pools in the clay and microaggregate fractions coexist with high potential enzyme activities, and presumably high enzyme concentrations. Within a soil fraction, the concentration of active enzyme increases with enzyme production or stabilization and decreases when enzymes are degraded by proteases or chemical breakdown. In the POM fractions, potential enzyme activities (and concentrations) of BG, CBH, and *N*AG are probably high because active microbes are producing large quantities of hydrolytic enzymes to degrade plant compounds. In the mineral-dominated fractions, enzyme production and microbial activity are probably lower because labile C is scarce, yet enzymes persist because they are stabilized on mineral surfaces and protected from degradation (Lähdesmäki and Piispanen, 1992; Sollins et al., 1996; Quiquampoix, 2000). We found that GAP and PPO in particular accumulated in the clay-sized fraction, suggesting that these enzymes are preferentially stabilized on clay surfaces and/or produced near clay particles. However, the stability of C in mineral fractions indicates that these enzymes are decoupled from substrate degradation and decomposition. Additional studies should be done to confirm that mineral-stabilized enzymes cannot efficiently degrade their substrates under field conditions.

Changes in potential activities of PPO and GAP per unit C across the prairie restoration chronosequence also suggest important interactions among enzymes and mineral surfaces. The most dramatic changes occurred in the clay-sized fraction, where PPO activity decreased by over 40% and GAP increased more than three-fold in the prairie remnant relative to cultivated soil (Fig 4A, B). PPO activity declined because the clay-sized fraction accumulated more C across the chronosequence, while PPO per unit soil mass remained constant. This finding suggests that the potential activity of PPO in the clay-sized fraction depends on mineral sorption processes rather than C substrate availability.

Alternatively, increasing GAP (a protease) within the clay-sized fraction may be sufficient to degrade PPO (a protein) before it becomes stabilized on the clay surface. If the two-pool model is correct, PPO sorbed on mineral surfaces should be derived from enzyme production by active, lignin-degrading microbes in the POM fraction. This production should increase across the chronosequence as litter inputs increase, and thereby raise the concentration of PPO in mineral fractions. However, PPO concentrations will not increase if higher protease activities enhance enzyme degradation before PPO becomes stabilized on mineral surfaces.

Increasing GAP activity across the chronosequence could reflect higher GAP production as microbes become more dependent on organic sources of N. In the cultivated soil where N-fixing crops and fertilizer application increase mineral N availability, microbes benefit little by producing GAP to degrade proteins (Chróst, 1991; Allison and Vitousek, 2005). As restoration proceeds, increasing sorption of amino compounds on clay surfaces likely contributes to the high N concentrations in the clay-sized fraction (Table 3) and could provide a source of N for GAP-producing microbes if the sorbed compounds are accessible to microbes (Christensen, 1995; Chevallier et al., 2003).

Aside from mineral stabilization, other explanations for slow C turnover in the face of high enzyme activity are also possible. For the PPO results, the net degradation rate of humic compounds may have been low because of competing synthesis reactions also catalyzed by PPO during the humification process (Katase and Bollag, 1991; Zavarzina et al., 2004). Enzymes other than the ones we assayed might also have limited decomposition in some of the fractions, though this explanation is unlikely given that we studied six different, functionally important enzymes. Finally, the mineral fractions may contain small, labile pools of C that elevate C concentrations and support high enzyme activities, despite a predominance of C pools with slow turnover rates. If true, this explanation suggests that C and enzyme pools in mineral fractions are heterogeneous and poorly characterized by a single mean residence time (Trumbore, 2000).

Some of the mechanisms that affect enzyme activity within soil fractions might also help explain C accumulation across the restoration chronosequence. Here, enzyme activities remained constant or increased per unit soil mass, even as soil C concentrations increased. As with the soil fractions, this paradoxical relationship between C and enzyme activity might result from increased enzyme production with greater C availability or from stabilization mechanisms that act on both C and enzymes (Kandeler et al., 1999a). Early in the chronosequence, cultivation reduces litter inputs, breaks up soil aggregates, and rearranges the spatial structure of the soil. Under these disturbed conditions, C-limited microbes may produce fewer enzymes, but these enzymes interact more efficiently with their substrates. As soils recover from cultivation, C availability and enzyme production may increase, but a large fraction of the enzyme activity may become isolated from C substrates because of aggregate formation and mineral stabilization.

5. Conclusions

Within soil fractions, the amount of enzyme activity and the rate of C turnover depend on enzyme production by decomposers as well as stabilization by physical processes. Enzymes within POM fractions are produced by active microbial populations, which rapidly degrade C from recent plant inputs. Within mineral fractions, microbial activity and enzyme production is lower, but potential enzyme activities may be high due to stabilization on mineral surfaces. These fractions contain high concentrations of old C, suggesting that mineral-stabilized enzymes are poor catalysts for decomposition. Thus, multiple pools of enzymes are present in the soil that differ in their stability and capacity to degrade C compounds.

As grassland soils recover from plowing disturbance, C stocks increase because enzymes and their C substrates sorb to mineral surfaces and become trapped in aggregates. Although enzyme production stays constant or increases as grassland vegetation re-establishes, changes in C inputs and increases in soil macroaggregates (Fig. 1) contribute to enzyme stabilization and allow C stocks to increase. Therefore, land managers trying to promote C sequestration in agricultural soils or restored prairies should employ techniques that reduce the physical disruption of soil aggregates.

Supplementary data

The file ''Allison Jastrow Tables S1 and S2.xls'' contains a spreadsheet with supplementary data Tables S1 and S2, which show carbon contents and enzyme activities per unit mass and per unit C in each soil fraction across the prairie restoration chronosequence.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.soilbio.2006.04.011

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