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Using landscape and depth gradients to decouple the impact of correlated environmental variables on soil microbial community composition

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Abstract

Simultaneously assessing shifts in microbial community composition along landscape and depth gradients allows us to decouple correlations among environmental variables, thus revealing underlying controls on microbial community composition. We examined how soil microbial community composition changed with depth and along a successional gradient of native prairie restoration. We predicted that carbon would be the primary control on both microbial biomass and community composition, and that deeper, low-carbon soils would be more similar to low-carbon agricultural soils than to high carbon remnant prairie soils. Soil microbial community composition was characterized using phospholipid fatty acid (PLFA) analysis, and explicitly linked to environmental data using structural equations modeling (SEM). We found that total microbial biomass declined strongly with depth, and increased with restoration age, and that changes in microbial biomass were largely attributable to changes in soil C and/or N concentrations, together with both direct and indirect impacts of root biomass and magnesium. Community composition also shifted with depth and age: the relative abundance of sulfate-reducing bacteria increased with both depth and restoration age, while gram-negative bacteria declined with depth and age. In contrast to prediction, deeper, low-C soils were more similar to high-C remnant prairie soils than to low-C agricultural soils, suggesting that carbon is not the primary control on soil microbial community composition. Instead, the effects of depth and restoration age on microbial community composition were mediated via changes in available phosphorus, exchangeable calcium, and soil water, together with a large undetermined effect of depth. Only by examining soil microbial community composition shifts across sites and down the soil column simultaneously were we able to tease apart the impact of these correlates environmental variables.
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1. Introduction

Soil microbes control many belowground processes critical to ecosystem functioning, through their influence on decomposition of organic matter, and creation of soil structure. Shifts in soil microbial community composition and abundance, in turn can significantly influence the dynamics of these essential processes. The composition of the microbial community can be influenced by environmental perturbations, including soil management practices (Moore and de Ruiter, 1991; Bardgett et al., 1993; Cambardella and Elliot, 1994; Lovell et al., 1995; Beare, 1997; Bardgett and McAlister, 1999; Stahl et al., 1999; Zeller et al., 2001; Bailey et al., 2002; Grayston et al. 2004; Allison et al., 2005; McKinley et al., 2005). The mechanisms responsible for changes in microbial community composition have been difficult to establish, because soil variables are highly correlated. We suggest that by examining microbial community composition shifts in two dimensions simultaneously, we can decouple correlations among soil variables to reveal underlying controls.

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For example, the strong environmental gradients that occur across the landscape can parallel those that occur with depth in the soil column. However, variables that are strongly correlated in one dimension are not necessarily correlated in another. While C is highly correlated with water in surface soils, this correlation breaks down deeper in the soil column (pers. obs.). Thus, it will be possible to decouple the impact of carbon, water, and other correlated variables by simultaneously examining shifts that occur across the landscape and down the soil column.

We test this idea by examining changes in microbial community composition and environmental variables with depth at seven sites along a tallgrass prairie restoration chronosequence. Transformation of this agroecosystem soil, which had been under continuous tillage-based cultivation for the last century, to a prairie soil dominated by rhizospheric processes, results in a dramatic increase in above- and belowground plant production (Jastrow, 1987, 1996; Cook et al. 1988), litter accumulation on the soil surface, and an aggregated soil structure (Jastrow, 1987; Miller and Jastrow, 1990; Jastrow et al., 1998), together with an increase in soil carbon (Jastrow, 1996). These changes can have profound effects on soil microbial community composition (Allison et al. 2005).

Soil microbial community structure also shifts with depth (Ahl et al., 1998; Ekelund et al., 2001; Blume et al., 2002; Griffiths et al., 2003; Agnelli et al., 2004) and the strong environmental gradients that exists within a depth profile can parallel those generated across the landscape by restoration age. Depth gradients exist because resource inputs are highly stratified, entering the system either at the soil surface (in the case of litter) or strongly declining with depth (in the case of root inputs) (Feng et al., 2003; LaMontagne et al., 2003). Conversely, mineral nutrients sourced from parent material, such as inorganic phosphorus, calcium, magnesium, iron and aluminum may increase with soil depth because of protection from weathering. In addition, surface soils are more exposed to frequent wetting and drying (Van Gestel et al., 1992; Ekelund et al., 2001), freeze/thaw cycles, and have higher levels of oxygen (Agnelli et al., 2004). These factors have been found to influence microbial community composition, with declining abundances of fungi relative to bacteria (Zelles and Bai, 1994; Blume et al., 2002; Jörgensen et al., 2002; Feng et al., 2003), and an increase in actinomycetes and gram-positive bacteria relative to gram-negative bacteria (Zelles and Bai, 1994; Feng et al., 2003; Fierer et al., 2003) with depth.

We assessed microbial community composition and biomass by using phospholipid fatty acid (PLFA) analysis, and used structural equations modeling (SEM) to determine the relative impact of environmental factors on those variables. We predicted that changes in soil carbon would have the strongest direct impact on both total microbial biomass and community composition (Ahl et al., 1998; Blume et al., 2002; Feng et al., 2003; Fierer et al., 2003), and thus that deeper, low-carbon soils will be more similar to low-carbon agricultural soils than to high-carbon remnant prairie soils (see Fig. 1). We also expected water to be important in structuring the microbial community (Ekelund et al., 2001), with water determined both by soil carbon, and by depth due to proximity to the water table. Soil pH was also expected to be an important regulator of microbial community composition, with pH in turn regulated by soil calcium (Grayston et al., 2004; Reich et al., 2005), and calcium regulated by soil texture and depth. Roots were expected to have both a direct impact on the microbial community by providing both a habitat and carbon resources, and also an indirect effect mediated through changes in soil water availability and soil carbon. In addition, phosphorus was expected to directly alter community composition by increasing the abundance of bacteria relative to fungi (Grayston et al., 2004) and by inhibiting arbuscular mycorrhizal fungi (AMF) (Marschner and Dell, 1994), and also indirectly influence community composition by increasing plant biomass. No direct effects

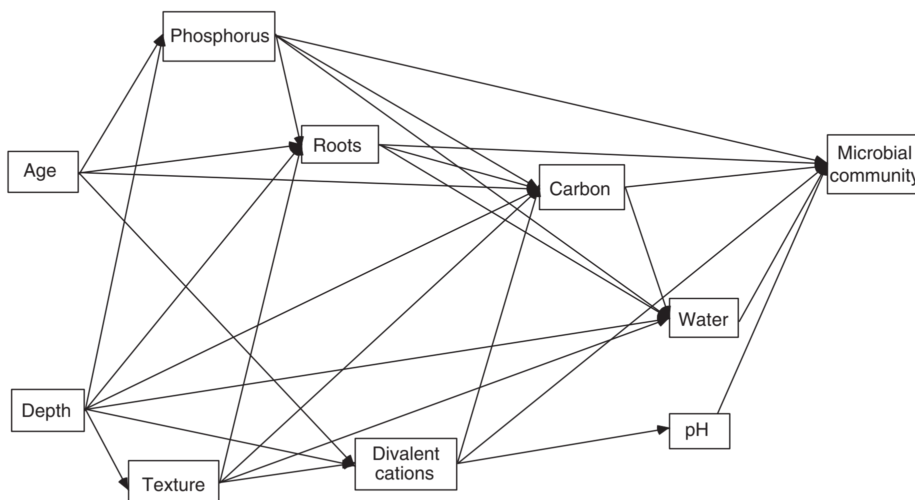

Fig. 1. Hypothetical model by which age and depth indirectly affect microbial community composition and biomass.

of site age or depth were predicted to occur. Any direct effects of age and depth suggest that our model does not incorporate important physical mechanisms by which the soil environment influences the microbial community.

2. Materials and methods

2.1. Site description

Samples were collected within the National Environmental Research Park at the Fermi National Accelerator Laboratory (Fermilab) in Batavia, Illinois, USA (N 41°51′22.5″, W 88°14′19.0″). The 30-yr mean air temperature and precipitation are 8.8 °C and 999 mm, respectively (National Climatic Data Center, 2003). All sampling sites were located on Drummer series soil (fine-silty, mixed, mesic Typic Haplaquoll); a deep, poorly drained soil that is very typical of the soils of the Prairie Peninsula of Illinois and neighboring states. Fermilab was prairie before European settlement, but has been in cultivation since the 1830s. The site was under continuous corn since at least 1969 (when Fermilab was established). Beginning in 1992, the agricultural fields have been rotated between corn and soybean. The fields are chisel-plowed most years, and fertilizer is applied when required. Since 1975, between 2 and 25 ha have been restored to tallgrass prairie annually (Betz, 1986; Jastrow, 1987). These restorations represent a chronosequence, which allows us to examine successional processes by substituting space for time, i.e. substituting similar soils (space) to obtain a chronosequence of different restoration ages (time). Space for time substitution can be problematic because of the potential for site differences to obscure trends due to time, or even generate unrelated patterns (Pickett, 1989). In this system, the past history of sites is very similar, time at which succession began is accurately documented, restoration procedures were similar, and we ensured that sampling occurred on the same soil type, thus keeping site variation to a minimum.

2.2. Sampling

Five restored prairie plots, one agricultural field, and one remnant prairie were sampled. The restored prairie plots were planted in spring 1977 (23 growing seasons (gs), plot 3D), fall 1981 (18 gs, plot 8D), spring 1992 (8 gs, plot 18D), summer 1993 (7 gs, plot N3D), and spring 1997 (3 gs, plot PLD). The agricultural field used in the study was planted to soybean (plot BD) during the year of sampling. The agricultural field and restored prairie samples were collected over an 11-day period from late August to early September 1999. The remnant prairie was sampled in early October 2001. Although the time delay may be of some concern, we assume that conditions at this native, never cultivated prairie site are at equilibrium, and thus there is little change in microbial community composition and soil physical variables with time.

In the restored and remnant prairies, three quadrats were randomly distributed perpendicular to a 50 m transect. In the agricultural fields, two quadrats along the transect were located within rows, and one quadrat was placed between rows to capture the variation of a cultivated field. In each quadrat, three soil cores (diameter 4.8 cm) were taken to a depth of 25 cm, and three smaller-diameter cores (diameter 3 cm) were taken from the bottom of the hole made by the first core to a depth of 100 cm. The cores were cut into depth increments of: 0–5 cm, 5–15 cm, 15–25 cm, 25–50 cm, 50–75 cm and 75–100 cm, and composited by quadrat. Soil cores were frozen at the end of each day.

2.3. Laboratory analyses

Frozen soil cores were thawed overnight in a refrigerator, weighed, and passed through an 8-mm sieve. During processing, roots were collected from the sieve by hand, washed in water, and dried at 65 °C to constant weight. To determine bulk density (Db) and soil moisture content, a subsample of soil was weighed fresh, then dried at 105 °C to constant weight. Bulk density was calculated by multiplying the fresh weight of cores of known volume by the ratio of fresh:dry weights of the subsample, then dividing by the total soil core volume.

A second subsample of soil was dried at 65 °C for 48 h, finely ground with a Spex mill (Spex-Certiprep Inc., Metucher, NJ), and analyzed for soil organic carbon (SOC) and total nitrogen (TN) contents by using a LECO CN-2000 analyzer (LECO Corporation, St Joseph, MI, USA). Shallow soil samples (0–50 cm) were run at 1350 °C, while deeper samples were run at 1350 °C for N, and 1040 °C for C, to avoid combustion of carbonates in deeper soils (Wright and Bailey, 2001).

A third subsample was air dried and analyzed for pH, available phosphorus, exchangeable cations, and soil texture at the Kansas State University soil testing Laboratory (Manhattan, KS). Soil pH was analyzed using a 1:1 slurry method, as described by Watson and Brown (1998), but without the addition of calcium chloride. For Mehlich-3 phosphorus, soil was extracted in acetic acid, ammonium nitrate, ammonium fluoride, nitric acid, and EDTA, according to the extraction and colorimetric assay procedures described in Frank et al. (1998). Exchangeable cations (Ca, K, and Mg) were determined by the ammonium acetate (1 M, pH 7.0) method described by Warncke and Brown (1998), and analyzed by an Inductively Coupled Plasma (ICP) Accuris Spectrometer (ARL/Fisons, Eclublens, Switzerland), or a Model 3110 Flame Atomic Absorption (AA) Spectrometer (Perkin Elmer Corp., Norwalk, CT). Particle size (texture) was estimated by a modification of the Bouyoucos hydrometer method (Bouyoucos, 1962).

A fourth soil subsample was processed to assess microbial community composition and biomass using PLFA analysis, by passing it through a 2-mm sieve, and then freeze-drying (−50 °C, 80×10^{-3} Mbar) for 48 h in a

Labconco Freezone 4.5 freeze-drier (Labconco, Kansas City, MO). Lipids were extracted from freeze-dried soil in a single-phase mixture of chloroform, methanol, and phosphate buffer (pH 7.4) in a ratio of 1:2:0.8, by an adaptation of the method described by Bligh and Dyer (1959). After 3 h, water and chloroform were added to separate the mixture into polar and nonpolar fractions, and total lipids were extracted from the nonpolar chloroform phase. The PLFAs were separated from other lipid classes by using silicic acid column chromatography (Vestal and White, 1989; Zak et al., 1996), methylated by using a mild-alkaline solution, and the samples frozen until analysis.

Prior to analysis, PLFAs were thawed and dissolved in hexane, as an internal standard. PLFA separation was by high-resolution fused-silica capillary gas chromatography (GC), using an HP 6890 GC, with an HP7683 autosampler (Agilent Technologies, Palo Alto, CA). A 25 m HP-5 column was used, with hydrogen as the carrier gas at a constant flow rate of 4.9 ml min^{-1}. A 1 μl splitless injection was made for each sample, with the inlet temperature set at 230 °C, and the inlet purged at 47.0 ml min^{-1}, 0.75 min after injection. The oven temperature was held at 80 °C for 1 min, increased at a rate of 20 °C min^{-1}–155 °C, and then increased at 5 °C min^{-1} to a final temperature of 270 °C and held for 5 min. Detection of PLFAs was by flame ionization at 350 °C. PLFAs were identified by retention time in comparison to known standards, and quantified using FAME 19:0 (Matreya Inc, PA) as an internal standard.

Fatty acid nomenclature is in the form of A:$B\omega C$, where 'A' is the number of carbon atoms in the chain, 'B' is the number of double bonds, and 'C' is the position of the double bond from the methyl end of the molecule; *cis* geometry is indicated by the suffix '*c*'. The prefixes 'i', 'a', and 'me' refer to iso, anteiso, and midchain methyl branching, respectively, with 'cy' indicating a cyclopropyl ring structure.

2.4. Data analysis

Individual PLFAs were summed to give a measure of total microbial biomass in each sample. The composition of the soil microbial community was assessed on relative molar abundances of signature PLFAs, by using a principle components analysis (PCA) to summarize the PLFA composition of each sample. In PCA, samples representative of multispecies communities are sorted so that the distance between samples is related to their similarity. The axes along which samples are positioned are not necessarily representative of actual environmental variables but are artificially created variables that explain the maximum amount of variation (Lepš and Smilauer, 1999). The data met the assumptions necessary for using PCA as an ordination technique.

We used SEM to test a hypothetical mechanistic model by which environmental variables influence microbial biomass and community composition (Fig. 1). SEM is a flexible multivariate statistical technique that is ideally suited to testing the importance of pathways in hypothesized models, and to compare models to experimental data. In SEM, a path coefficient (analogous to a standardized correlation coefficient) is calculated for each connecting path between variables, and these path coefficients are used to determine the direct and indirect effects of environmental variables on the dependent variable. These path coefficients are equivalent to the standardized partial regression coefficients of multiple regression, and represent the effect of a one-standard deviation change in an independent variable on a dependent variable, with all other variables statistically held constant (Mitchell, 1992). These path coefficients can then be used to determine the direct and indirect impacts of environmental variables on the dependent variable. In addition, there are numerous indices by which SEM tests how well a theoretical model fits the data. We present two indices: the χ^2 and Hoelter N. The χ^2 statistic tests the null hypothesis that there is no significant difference between the data and the proposed model (Mitchell, 1992). A significant χ^2 ($P \leqslant 0.05$) indicates that the null model should be rejected, because the model and data are significantly different. Thus, a non-significant P value indicates that the data support the hypothesized model. However, the χ^2 statistic is vulnerable to low sample size, with models more readily accepted when N is small. As a result, we also present the Hoelter N index: the number of sampling units required to reject a model. A high (>100) Hoelter N provides further evidence that the data fit the model well.

We developed a single SEM model to represent all sites: although a multi-group analysis with separate model for each site would be more appropriate (Shipley, 2000), the number of samples required is prohibitive. After rejecting our hypothetical model on the grounds of poor fit, we used SEM as a heuristic device and evaluated additional models. In an iterative process, we rejected models on the grounds of poor fit, or the more subjective grounds of lacking a mechanistic basis, until we arrived at a model giving the best fit while still maintaining a plausible mechanistic basis. However, as is always the case with correlative models, we cannot exclude the possibility that equally well-fitted models exist.

The SEM was performed in Amos 5 (Arbuckle, 2003), while PCAs were performed in PC-Ord (McCune and Mefford, 1999). Correlations were performed in Systat 10 (SPSS, 2000). Data were transformed as necessary (age, depth, water, root biomass, soil C and N, phosphorus, and total PLFA) to meet assumptions of normality and homogeneity of variance, and tests considered significant at $P \leqslant 0.05$.

3. Results

The ratio of total PLFA per soil C (an indirect measure of carbon quality) declined with depth, and increased with age (Fig. 2A). Water availability was greater in the surface

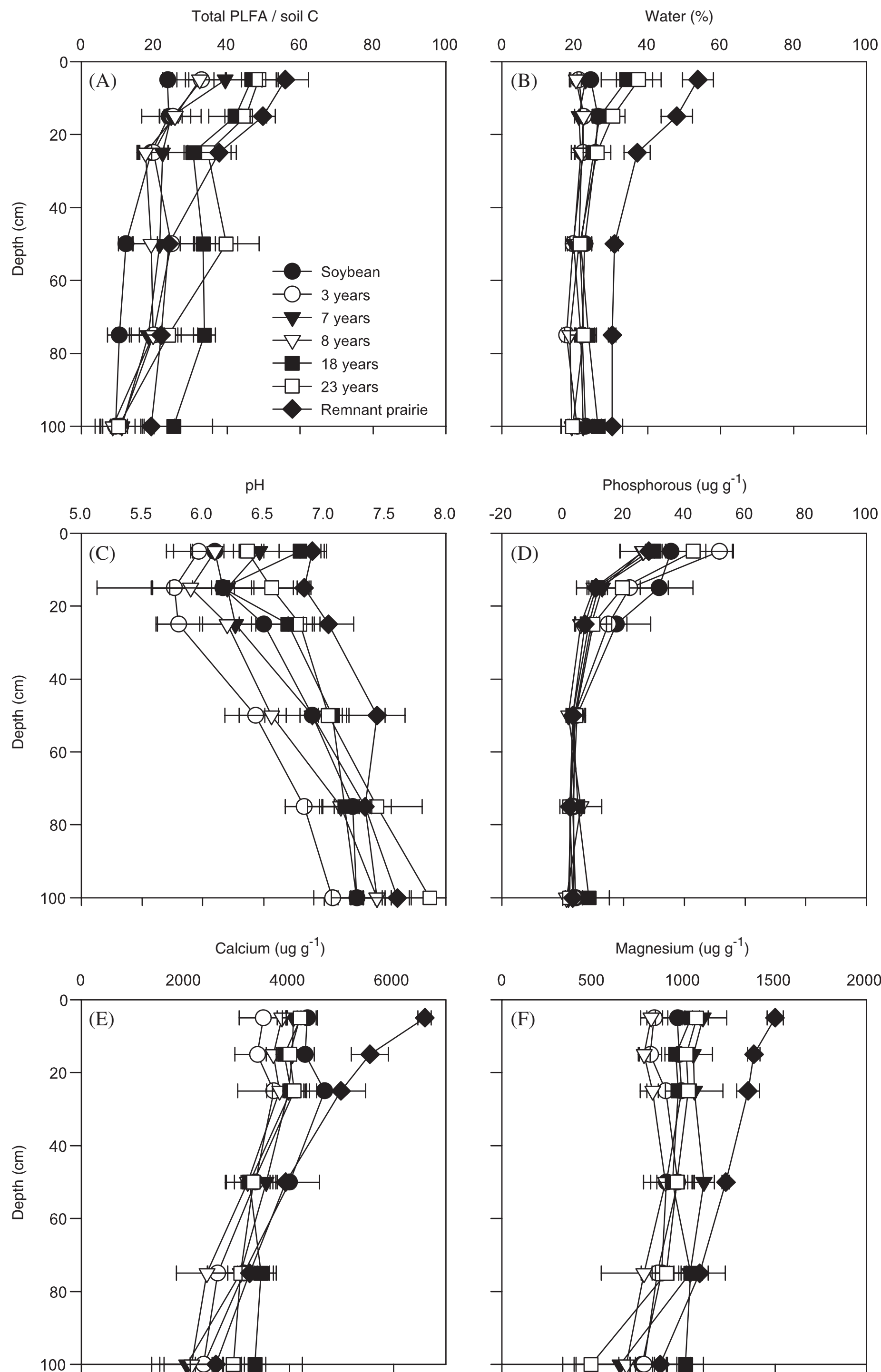

Fig. 2. Changes in selected environmental variables with site age (yr) and depth (cm) (mean$\pm$1 SD, $n = 3$).

soil of older restored plots and the remnant prairie than in deeper soil, or in shallow soil of the agricultural or younger restored plots (Fig. 2B). The pH increased with soil depth and restoration age (Fig. 2C). Phosphorus declined with depth (Fig. 2D). Calcium declined somewhat with depth, and was higher in the remnant than restored or agricultural plots (Fig. 2E). Magnesium declined somewhat in the deepest layers, and was higher in the remnant than agricultural or restored plots (Fig. 2F). Data on soil C, root biomass, and bulk density will be presented separately (Matamala et al., in preparation).

Total PLFA decreased strongly with depth in all except the soybean field (Fig. 3), where total PLFA was relatively constant over the first 25 cm, then declined abruptly. Total PLFA increased with age of restoration, with a maximum concentration in surface soils in the remnant prairie (Fig. 3).

The hypothetical model did not provide a good fit to the data for total PLFA (results not shown). However, a component of the proposed model does adequately fit the data ($\chi^2 = 10.468$, df $= 5$, $P \leqslant 0.063$, Hoelter $N = 133$), and we were able to explain 94% of the variation in total PLFA (Fig. 4). As previously explained, P value >0.05, and high Hoelter N value indicates that the data support the hypothesized model. Total PLFA increased most strongly with soil C (or N) (Table 1), although we were unable to distinguish between the impact of soil C and N because they were very highly correlated across all depths and restoration ages ($R^2 = 0.99$). The effect of depth was stronger than restoration age (Table 1). The impacts of depth and age on total PLFA were indirect, and mediated through weak, direct changes in magnesium, root biomass, and soil C (Fig. 4, Table 1).

Plotting relative abundance of microbial functional groups by site and depth, we find that bacteria dominate the microbial community through all ages and depths (Fig. 5). There is a weak pattern of increasing relative abundances of actinomycetes and sulfate-reducing bacteria (Sulfobacter) with depth, and of decreasing gram-negative bacteria (Fig. 5). In addition, the relative abundance of sulfate-reducing bacteria is higher in the remnant prairie than either the agricultural or restored sites.

PCA axis 1 explained 56% of the variation in PLFA composition, while axis 2 explained a further 17% (Fig. 6). Deeper soils fell to the left of PCA axis 1, as did older restored soils (Fig. 6). Deeper, and older, samples had higher relative abundances of 10me16:0, a signature for sulfate-reducing bacteria and actinomycetes, and somewhat higher relative abundances of the actinomycetes signature 10Me18:0, while relative abundance of gram-negative bacterial signature 18:1ω7c declined with depth and restoration age (Table 2). Although both depth and age had a weak influence on soil microbial community composition, the longer length of the depth regression line (Fig. 6) indicates than depth had a stronger impact than restoration age. Because PCA axis 2 is not strongly related

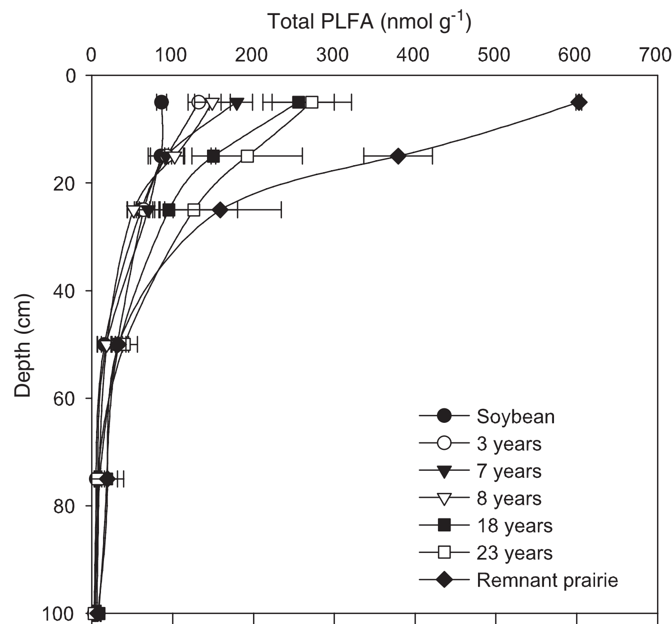

Fig. 3. Impact of site age (yr) and depth (cm) on total PFLA (nmol g^{-1} of soil) (mean ± 1 SD, $n = 3$).

Table 1
Standardized direct and indirect effects of age and depth on total PLFA

	Direct effects	Indirect effects	Total effects
Age (yr)[a]	0.000	0.243	0.243
Depth (cm)[a]	0.000	−0.823	−0.823
Root biomass (g m^{-3})[a]	0.170	0.104	0.274
Magnesium (μg g^{-1})	0.153	0.110	0.263
Soil C (%)[a]	0.760	0.000	0.760

[a]Indicates that ln values were used.

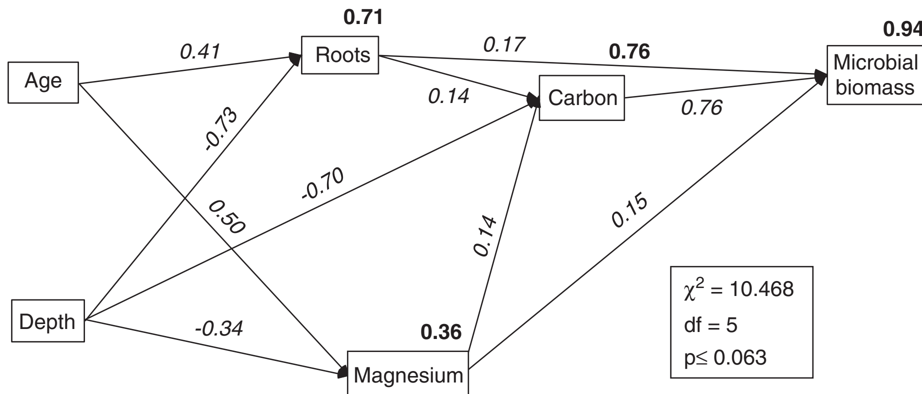

Fig. 4. Pathways by which age (yr) and depth (cm) directly and indirectly influence total PLFA (nmol g^{-1} soil). Arrows connecting environmental variables to the independent variable (total PLFA) indicate direct effects, while environmental variables linked to the independent variable via other environmental variables constitute indirect effects. Numbers are standardized path coefficients (*italics*), and the proportion of total variance explained (**bold**) for each endogenous variable ($n = 126$). A P value >0.05, and high Hoelter N value indicate that the model and data are not significantly different: the model fits.

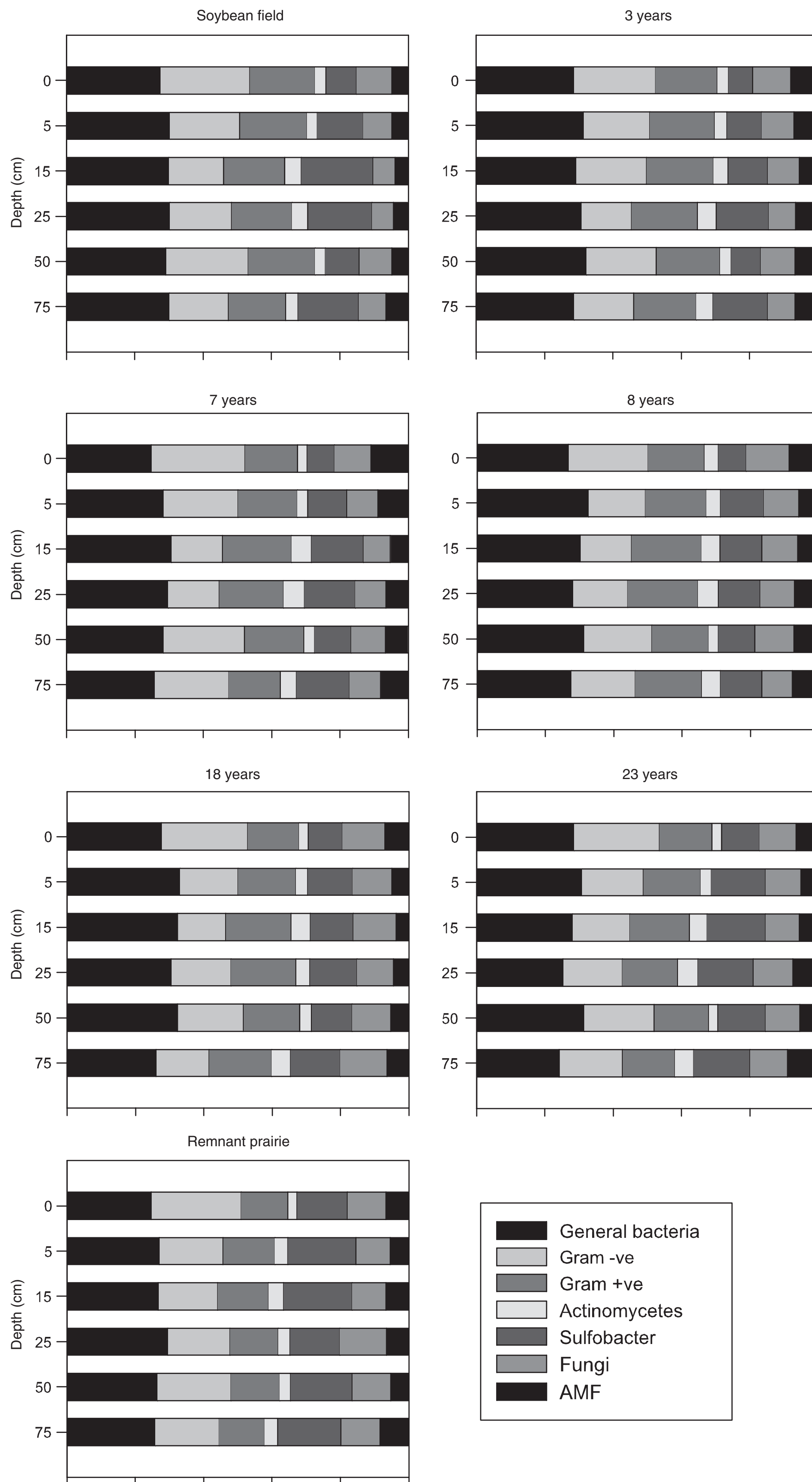

Fig. 5. Impact of site age (yr) and depth (cm) on the relative abundance of microbial functional groups.

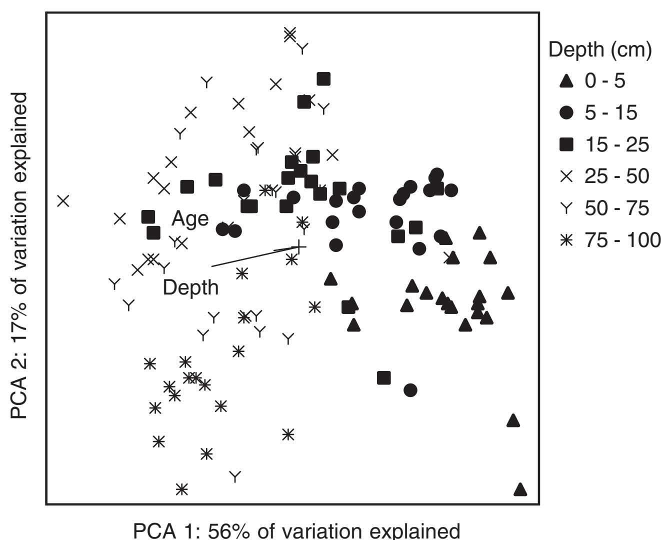

Fig. 6. Soil microbial community composition assessed as relative abundance of signature PLFAs, with each community summarized to a single point by using a principle components analysis (PCA). Length of regression lines indicates impact of that variable.

Table 2
Impact of signature PLFAs on PCA axis 1 position

PLFA	Functional group	Eigenvalue (PCA axis 1)
14:0	Bacteria[a]	−0.0823
a15:0	Bacteria[b,c]	0.0613
i17:0	Bacteria[b,c]	−0.0026
a17:0	Bacteria[b,c]	−0.0217
cy19:0a	Bacteria[b,c]	0.0846
i15:0	Gram-positive[b,c]	0.0610
i16:0	Gram-positive[b,c]	−0.0048
16:1ω7c	Gram-positive[b,c]	0.1914
cy17	Gram-negative[b,c]	0.0156
18:1ω7c	Gram-negative[b,c]	0.4425
10Me16:0	Actinomycetes [c]/Sulfobacter[d]	−0.8330
10Me18:0	Actinomycetes[c]	−0.1485
16:1ω5c	AMF[e]	0.1079
18:2ω6,9c	Saprophytic fungi[b,c]	−0.0047
18:1ω9c	Saprophytic fungi[a]	0.1333

[a]Zak et al. (1996);
[b]Frostegard et al. (1993);
[c]Zelles (1999);
[d]Coleman et al. (1993);
[e]Olsson (1999).

to age of depth, or to environmental gradients, this axis is not discussed further.

We tested whether our hypothetical model explained environmental impacts on microbial community composition using position on PCA axis 1 as our dependent variable. The hypothetical model provided an extremely poor fit to the data (results not shown). However, a subset of the proposed model did fit the data ($\chi^2 = 10.977$, df $= 8$, $p \leqslant 0.203$, Hoelter $N = 177$), and explained 75% of the variation in sample position on PCA axis one (Fig. 7). Again, in SEM, a P value >0.05 and high Hoelter N value indicate that the data support the hypothesized model. Depth had a strong, negative direct effect on position on PCA axis 1, while the effect of age was indirect and mediated though changes in soil C, water, calcium and phosphorus (Fig. 7). The impact of carbon is also indirect, and mediated via positive impacts on calcium and phosphorus (Fig. 7). Phosphorus has a positive impact on sample position on PCA axis 1, while calcium and water have a negative impact (Fig. 7). The direct effect of depth has the strongest impact on microbial community, with calcium and phosphorus having stronger impacts than water or carbon (Table 3).

We cannot rule out the possibility that sampling the remnant prairie later in the growing season compared to the rest of the sites affected total PLFA. Surface soils would be most affected because they respond more rapidly to fluctuations in air temperature. Since total PLFAs in the top 20 cm were nearly twice as high in the remnant prairie than any other site (Fig. 3), the cooler temperature would only underestimate these values. Soil microbial community composition would likely be more sensitive to soil moisture fluctuations. Since total precipitation was 0.6 and 0.7 inches 2 weeks prior to sampling in 1999 and 2001, respectively, and 0 inches 5 days prior to sampling in both years, the effect on the soil microbial community composition is likely to be insignificant.

4. Discussion

Our prediction that soil C would have the greatest direct influence on microbial biomass was supported by the model. In contrast, soil C only indirectly affected soil microbial community composition and in fact, the large direct influence of depth suggests that the model did not incorporate a mechanistic control on composition that varied with depth.

As expected, microbial biomass in the prairie sites declined strongly with depth, and increased with successional age (Fig. 3), patterns consistent with higher organic matter inputs in surface soils and in older prairie sites (Feng et al., 2003; LaMontagne et al., 2003). The relatively constant microbial biomass values we measured in the top 25 cm in the soybean field is commonly found in surface layers of cropped soils (Kaiser and Heinimeyer, 1993; Dodds et al., 1996; Martens et al., 2003), followed by a sharp decline in the deeper depths. This pattern is generated by tilling, which incorporates organic matter homogeneously through the tilling layer.

The effects of restoration age and depth on microbial biomass were indirect (Fig. 4), suggesting that we have a good mechanistic understanding of controls on microbial biomass along gradients of depth and successional age. Direct effects of age and depth in the model would mean that the model did not include mechanistic variables that control microbial biomass since depth and age themselves cannot directly influence microbial biomass. The model revealed that the primary direct controls on microbial biomass are soil C and/or soil N, together with root

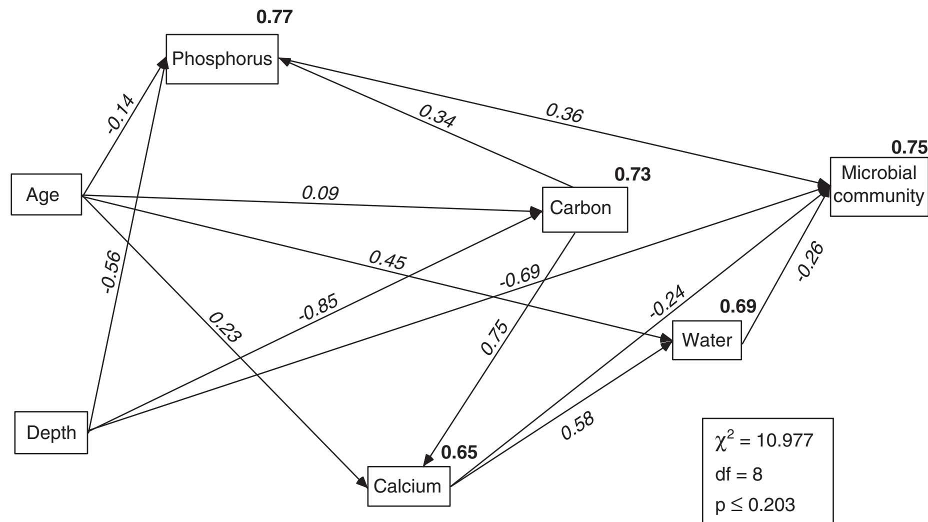

Fig. 7. Pathways by which age (yr) and depth (cm) directly and indirectly influence microbial community composition, summarized by PCA axis 1. Arrows connecting environmental variables to the independent variable (total PLFA) indicate direct effects, while environmental variables linked to the independent variable via other environmental variables constitute indirect effects. Note that in some cases, the direction of arrows is reversed relative to our proposed model. Numbers are standardized path coefficients (*italics*), and the proportion of total variance explained (**bold**) for each endogenous variable ($n = 126$). A P value >0.05 and high Hoelter N value indicate that the model and data are not significantly different: the model fits.

Table 3
Standardized direct and indirect effects of age and depth on microbial community composition, summarized by PCA axis one

	Direct effects	Indirect effects	Total effects
Age (y)[a]	0.000	−0.275	−0.275
Depth (cm)[a]	−0.695	−0.054	−0.748
Soil C (%)[a]	0.000	−0.175	−0.175
Calcium ($\mu g\, g^{-1}$)	−0.244	−0.150	−0.394
Water (%)[a]	−0.259	0.000	−0.259
Phosphorous ($\mu g\, g^{-1}$)[a]	0.359	0.000	0.359

[a]ln values used.

biomass and magnesium (Fig. 4). Although there is considerable evidence from correlative studies that carbon controls microbial biomass (Wardle 1992; Yao et al. 2000; Steenworth et al. 2002), this study provides stronger causative evidence, by decoupling soil carbon from a multitude of other correlated variables. We are still unable to distinguish between soil C and N, as there is little variation in the C:N ratio with either depth or restoration age. Relatively constant C:N ratios presumably occur because the inputs of both are plant derived, and they are mineralized at similar rates. Carbon quality may also influence microbial biomass: although not assessed directly, the ratio of total PLFA to total carbon is higher in surface soils and in remnant prairie, than in deeper or younger soils (Fig. 2A), suggesting higher carbon quality in these high microbial biomass soils. However, the very strong impact of total carbon (Fig. 4) suggests changes in quality are of secondary importance. Total soil carbon provides both an energy source for microbial growth, and a high surface area substrate for colonization. The relative importance of these two aspects is unknown.

Root biomass also has a positive direct impact on total PLFA (Fig. 4), suggesting a rhizosphere effect, with a large microbial community supported by root exudates and recently sloughed material. Although the hypothesized positive effect of root biomass on soil carbon does occur, the correlation is surprisingly weak (Fig. 4). This may be due to differences in rates of root decomposition and carbon turnover with depth (Gill et al., 1999). In addition, magnesium has a weak positive effect on microbial biomass (Fig. 4). Although this may be due in part to the promotion of soil aggregation through cation bridging (Muneer and Oades, 1987), magnesium cannot be replaced by calcium in this model, suggesting this effect is not restricted to promotion of aggregation. An alternative explanation is that magnesium limits plant growth (Tilman, 1984), and thus indirectly limits soil C availability.

Relative abundance of bacteria is much higher than that of fungi in this tallgrass prairie restoration chronosequence, and is highest in the older restored sites and remnant prairie (Fig. 5). Higher relative abundances of bacteria were also found by McKinley et al. (2005) in restored Illinois prairie, and by McCulley and Burke (2004) at the Konza tallgrass prairie. These findings contrast with previous studies which report increasing fungal dominance in restored systems relative to agricultural (e.g. Bardgett et al., 1993; Cambardella and Elliot, 1994; Beare, 1997; Bardgett and McAlister, 1999; Stahl et al., 1999; Zeller et al., 2001; Bailey et al., 2002). We suggest that high relative abundances of bacteria in prairie systems results from the high surface area offered for colonization in these high root-biomass, high-carbon, soils.

As previously demonstrated (Joergensen and Scheu, 1999; Feng et al., 2003; Fierer et al., 2003), depth has a stronger impact on microbial community composition than

does age (Table 3, Fig. 6). Deeper, and older restored soils have higher relative abundances of actinomycetes and sulfate-reducing bacteria (Fig. 6, Table 2), a pattern consistent with other studies (Zelles and Bai, 1994; Blume et al., 2002; Jörgensen et al., 2002; Feng et al., 2003; Fierer et al., 2003). Feng et al. (2003) suggest that high relative abundances of actinomycetes may reflect the higher proportion of carbon in recalcitrant forms deeper in the soil column, while an increase in relative abundances of sulfate-reducing bacteria indicate developing anaerobic conditions deeper in the soil column. Similarly, Fierer et al. (2003) suggest that water is an important variable structuring microbial community composition with depth at their semi-arid research site.

Both depth and restoration age indirectly affect microbial community composition via changes in phosphorus, calcium and water, with calcium and phosphorus regulated in part by soil carbon (Fig. 7). An impact of calcium on the microbial community (Grayston et al., 2004), and also on earthworms (Reich et al., 2005), has been suggested to occur via changes in pH. However, we find only weak relationships between calcium and pH in this system ($R^2 = 0.13$). Alternatively, calcium influences soil aggregation by promoting bridging between charged clay particles and humic acids (Muneer and Oades, 1989), and consequently, aggregation may influences microbial community composition by supplying habitable microsites. There is some support for this supposition: Väisänen et al. (2005) found that the bacterial community differed among different aggregate size classes, while the fungal community was relatively unresponsive. Phosphorus has also been demonstrated to influence the bacterial community in comparisons of improved and unimproved grasslands (Grayston et al., 2004), while Bünemann et al. (2004) found that as phosphorus increased, relative abundance of 10me16:0, 10me17:0, and 10me18:0 declined. Although the mechanism is not known, this finding does corroborate our observation of higher relative abundances of 10me16:0 and 10me18:0 in deeper soils with lower levels of available phosphorus.

There was a strong direct effect of depth on microbial community composition (Fig. 7), suggesting that we have not assessed an important, depth-dependent environmental variable controlling microbial community composition. This variable may be anoxia: high relative abundance of the PLFA signature for sulfate-reducing bacteria occurs in deeper soils (Table 2), indicating reducing conditions. Low oxygen may occur in deeper samples, with air-filled pore space strongly related to depth (Barber et al., 2004). We suggest that changes in carbon quality are not responsible for this depth effect on microbial community composition: deeper and older soils fall to the left on axis 1 (Table 1), and while deeper soils have lower carbon quality (as assessed by the ratio of total PLFA to soil C), older soils have higher carbon quality (Fig. 2A).

Several components of our hypothesized model (Fig. 1) were found to be of limited value in explaining variation in microbial community composition at this site. Most notably, texture had no significant effect (results not shown), although predicted to be important in determining soil carbon, calcium, and water availability. A probable explanation is that when establishing this chronosequence, plots were intentionally restricted to a single soil type in an attempt to isolate changes due to successional time. As a result, differences in soil texture are minimized. This variable may prove to be of great importance when broader gradients are considered.

A potential weakness of this two dimensional gradient analysis is that it can obscure relationships, particularly in a non-equilibrium system such as a chronosequence. Most notably, the relationship between age and soil C is weak in the whole soil column model (Fig. 7), whereas in the surface soil there is a strong pattern of carbon accumulation with successional time (results not shown). Changes in soil carbon early in succession are restricted to the surface soils, an area where the majority of microbial biomass is concentrated (Fig. 3). As such, while two-dimensional gradient analysis is useful in developing mechanistic models, they should be interpreted cautiously when drawing conclusions about compartments within the model.

We had previously suggested soil carbon was the primary control on microbial community composition (Allison et al., 2005), and predicted that low-carbon, deep soils would be more similar to low-carbon agricultural soils than to high-carbon remnant prairie soils. Instead, we find the opposite, increasing site age and depth have similar affects on microbial community composition. Although in surface layers soil C, bulk density and water are highly correlated, these correlations break down as depth increases. By simultaneously using environmental gradients generated by restoration age and depth, we have decoupled these correlations, revealing that the role of carbon in structuring community composition is indirect. Carbon may instead influence microbial community composition by providing binding sites for charged particles such as calcium, as well as promoting water retention in surface soils. The similarity between deep soils and older restored soils is due to different factors: the depth effect is largely direct, while the effect of age is indirect and mediated via changes in calcium and water. Overall, we demonstrate that simultaneously examining composition shifts in two dimensions enabled us to tease apart the impact of otherwise correlated environmental variables, revealing underlying controls on the composition of the microbial community.

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References

Agnelli, A., Ascher, J., Corti, G., Ceccherini, M.T., Nannipieri, P., Pietramellara, G., 2004. Distribution of microbial communities in a forest soil profile investigated by microbial biomass, soil respiration and DGGE of total and extracellular DNA. Soil Biology & Biochemistry 36, 859–868.

Ahl, C., Joergensen, R.G., Kandeler, E., Meyer, B., Woehler, V., 1998. Microbial biomass and activity in silt and sand loams after long-term shallow tillage in central Germany. Soil and Tillage Research 49, 93–104.

Allison, V.J., Miller, R.M., Jastrow, J.D., Matamala, R., Zak, D.R., 2005. Changes in soil microbial community structure in a tallgrass prairie chronosequence. Soil Science Society of America Journal 69, 1412–1421.

Arbuckle, J.L., 2003. Amos 5. SmallWaters Corp., Chicago, IL.

Bailey, V.L., Smith, J.L., Bolton, H., 2002. Fungal-to-bacterial ratios in soils investigated for enhanced C sequestration. Soil Biology & Biochemistry 34, 997–1007.

Barber, K.R., Leeds-Harrison, P.B., Lawson, C.S., Gowing, D.J.G., 2004. Soil aeration status in a lowland wet grassland. Hydrological Processes 18, 329–341.

Bardgett, R.D., McAlister, E., 1999. The measurement of soil fungal:-bacterial biomass ratios as an indicator of ecosystem self-regulation in temperate grasslands. Biology and Fertility of Soils 19, 282–290.

Bardgett, R.D., Frankland, J.C., Whittaker, J.B., 1993. The effects of agricultural practices on the soil biota of some upland grasslands. Agriculture, Ecosystems and the Environment 45, 25–45.

Beare, M.H., 1997. Fungal and bacterial pathways of organic matter decomposition and nitrogen mineralization in arable soils. In: L. Brussaard, L., Ferrera-Cerrato, R. (Eds.), Soil Ecology in Sustainable Agricultural Systems. CRC Press, Boca Raton, FL, pp. 37–70.

Betz, R.F., 1986. One decade of research in prairie restoration at the Fermi National Accelerator Laboratory (Fermilab), Batavia, Illinois. In: Clambey, G.K., Pemble, R.H. (Eds.), The Prairie: Past, Present and Future. In: Proceedings of the Ninth North American Prairie Conference. Moorhead State University, Moorhead, MN, pp. 179–185.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37, 911–917.

Blume, E., Bischoff, M., Reichert, J.M., Moorman, T, Konopka, A., Turco, R.F., 2002. Surface and subsurface microbial biomass, community structure and metabolic activity as a function of soil depth and season. Applied Soil Ecology 20, 171–181.

Bouyoucos, G.J., 1962. Hydrometer method improved for making particle size analysis of soils. Agronomy Journal 54, 464–465.

Bünemann, E.K., Bossio, D.A., Smithson, P.C., Frossard, E., Oberson, A., 2004. Microbial community composition and substrate use in a highly weathered soil as affected by crop rotation and P fertilization. Soil Biology & Biochemistry 36, 889–901.

Cambardella, C.A., Elliot, E.T., 1994. Carbon and nitrogen dynamics of soil organic matter fractions from cultivated grassland soils. Soil Science Society of America Journal 58, 123–130.

Coleman, M.L., Hedrick, D.B., Lovley, D.R., White, D.C., Pye, K., 1993. Reduction of Fe(III) in sediments by sulphate-reducing bacteria. Nature 361, 436–438.

Cook, B.D., Jastrow, J.D., Miller, R.M., 1988. Root and mycorrhizal endophyte development in a chronosequence of restored tallgrass prairie. New Phytologist 110, 355–362.

Dodds, W.K., Banks, M.K., Clenan, C.S., Rice, C.W., Sotomayor, D., Strauss, E.A., Yu, W., 1996. Biological properties of soil and subsurface sediments under abandoned pasture and cropland. Soil Biology & Biochemistry 28, 837–846.

Ekelund, F., Rønn, R., Christensen, S., 2001. Distribution with depth of protozoa, bacteria and fungi in soil profiles from three Danish forest sites. Soil Biology & Biochemistry 33, 475–481.

Feng, Y., Motta, A.C., Reeves, D.W., Burmester, C.H., van Santen, E., Osborne, J.A., 2003. Soil microbial communities under conventional-till and no-till continuous cotton systems. Soil Biology & Biochemistry 35, 1693–1703.

Fierer, N., Schimel, J.P., Holden, P.Q., 2003. Variations in microbial community composition through two soil depth profiles. Soil Biology & Biochemistry 35, 167–176.

Frank, K., Beegle, D., Denning, J., 1998. Phosphorous. In: Brown, J.R. (Ed.), Recommended Chemical Soil Test Procedures for the North Central Region. North Central Regional Publication No. 221 (Revised). University of Missouri Agricultural Experiment Station, Columbia, MO, pp. 21–30.

Frostegård, A., Bååth, E., Tunlid, A., 1993. Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. Soil Biology & Biochemistry 25, 723–730.

Gill, R., Burke, I.C., Milchunas, D.G., Lauenroth, W.K., 1999. Relationship between root biomass and soil organic matter pools in the shortgrass steppe of Eastern Colorado. Ecosystems 2, 226–236.

Grayston, S.J., Campbell, C.D., Bardgett, R.D., Mawdsley, J.L., Clegg, C.D., Ritz, K., et al., 2004. Assessing shifts in microbial community structure across a range of grasslands of differing management intensity using CLPP, PLFA and community DNA techniques. Applied Soil Ecology 25, 63–84.

Griffiths, R.I., Whiteley, A.S., O'Donnell, A.G., Bailey, M.J., 2003. Influence of depth and sampling time on bacterial community structure in an upland grassland soil. FEMS Microbiology and Ecology 43, 35–43.

Jastrow, J.D., 1987. Changes in soil aggregation associated with tallgrass prairie restoration. American Journal of Botany 74, 1656–1664.

Jastrow, J.D., 1996. Soil aggregate formation and the accrual of particulate and mineral-associated organic matter. Soil Biology & Biochemistry 28, 665–676.

Jastrow, J.D., Miller, R.M., Lussenhop, J., 1998. Interactions of biological mechanisms contributing to soil aggregate stabilization in restored prairie. Soil Biology & Biochemistry 30, 905–916.

Joergensen, R.G., Scheu, S., 1999. Depth gradients of microbial and chemical properties in moder soils under beech and spruce. Pedobiologia 43, 134–144.

Jörgensen, R.G., Raubuch, M., Brandt, M., 2002. Soil microbial properties down the profile of a black earth buried by colluvium. Journal of Plant Nutrition and Soil Science 165, 274–280.

Kaiser, E.-A., Heinemeyer, O., 1993. Seasonal variations of soil microbial biomass carbon within the plough layer. Soil Biology & Biochemistry 25, 1649–1655.

LaMontagne, M.G., Schimel, J.P., Holden, P.A., 2003. Comparison of subsurface and surface soil bacterial communities in California grassland as assessed by terminal restriction fragment length polymorphisms of PCR-amplified 16S rRNA genes. Microbial Ecology 46, 216–227.

Lepš, J., Smilauer, P., 1999. Multivariate analysis of ecological data (course notes). Faculty of Biological Sciences. University of South Bohemia.

Lovell, R.D., Jarvis, S.C., Bardgett, R.D., 1995. Soil microbial biomass and activity in long-term grassland: effects of management changes. Soil Biology & Biochemistry 27, 969–975.

Marschner, H., Dell, B., 1994. Nutrient uptake in mycorrhizal symbiosis. Plant and Soil 159, 89–102.

Martens, D.A., Reedy, T.E., Lewis, D.T., 2003. Soil organic carbon content and composition of 130-year crop, pasture and forest land-use managements. Global Change Biology 10, 65–78.

McCulley, R.L., Burke, I.C., 2004. Microbial community composition across the Great Plains: Landscape versus regional variability. Soil Science Society of America Journal 68, 106–115.

McCune, B., Mefford, M.J., 1999. Multivariate analysis of ecological data 4.20. MjM Software, Gleneden Beach, OR.

McKinley, V.L., Peacock, A.D., White, D.C., 2005. Microbial community PLFA and PHB responses to ecosystem restoration in tallgrass prairie soils. Soil Biology & Biochemistry 37, 1946–1958.

Miller, R.M., Jastrow, J.D., 1990. Hierarchy of root and mycorrhizal fungal interactions with soil aggregation. Soil Biology & Biochemistry 22, 579–584.

Mitchell, R.J., 1992. Testing evolutionary and ecological hypotheses using path analysis and structural equation modelling. Functional Ecology 6, 123–129.

Moore, J., de Ruiter, P.C., 1991. Temporal and spatial heterogeneity of trophic interactions within belowground food webs. Agriculture, Ecosystems and the Environment 34, 371–397.

Muneer, M., Oades, J.M., 1989. The role of Ca-organic interactions in soil aggregate stability. III. Mechanisms and models. Australian Journal of soil Research 27, 411–423.

Olsson, P.A., 1999. Signature fatty acids provide tools for determination of the distribution and interactions of mycorrhizal fungi in soil. FEMS Microbiology Ecology 29, 303–310.

Pickett, S.T.A., 1989. Space-for-time substitution as an alternative to long-term studies. In: Likens, G.E. (Ed.), Long-term Studies in Ecology. Springer-Verlag, New York, NY, pp. 110–135.

Reich, P.B., Oleksyn, J., Modrzynski, J., Mrozinski, P., Hobbie, S.E., Eissenstat, D.M., et al., 2005. Linking litter calcium, earthworms and soil properties: a common garden test with 14 tree species. Ecology Letters 8, 811–818.

Shipley, B., 2000. Cause and Correlation in Biology. Cambridge University Press, Cambridge, UK.

SPSS, 2000. Systat 10. SPSS Science, Chicago, IL.

Stahl, P.D., Parkin, T.B., Christensen, M., 1999. Fungal presence in paired cultivated and uncultivated soils in central Iowa, USA. Biology and Fertility of Soils 29, 92–97.

Steenworth, K.L., Jackson, L.E., Calderón, F.J., Stromberg, M.R., Scow, K.M., 2002. Soil microbial community composition and land use history in cultivated and grassland ecosystems of coastal California. Soil Biology & Biochemistry 34, 1599–1611.

Tilman, G.D., 1984. Plant dominance along an experimental nutrient gradient. Ecology 65, 1445–1453.

Väisänen, R.K., Roberts, M.S., Garland, J.L., Frey, S.D., Dawson, L.A., 2005. Physiological and molecular characterization of microbial communities associated with different water-stable aggregate size classes. Soil Biology & Biochemistry 37, 2007–2016.

Van Gestel, M., Ladd, J.N., Amato, M., 1992. Microbial biomass responses to seasonal change and imposed drying regimes at increasing depths of undisturbed topsoil profiles. Soil Biology & Biochemistry 24, 103–111.

Vestal, J.R., White, D.C., 1989. Lipid analysis in microbial ecology: quantitative approaches to the study of microbial communities. BioScience 39, 535–541.

Wardle, D.A., 1992. A comparative assessment of factors that influence microbial biomass carbon and nitrogen levels in soil. Biological Reviews 67, 321–358.

Warncke, D., Brown, J.R., 1998. Potassium and other basic cations. In: Brown, J.R. (Ed.), Recommended Chemical Soil Test Procedures for the North Central Region. North Central Regional Publication No. 221 (Revised). University of Missouri Agricultural Experiment Station, Columbia, MO, pp. 31–34.

Watson, M.E., Brown, J.R., 1998. pH and lime requirements. In: Brown, J.R. (Ed.), Recommended Chemical Soil Test Procedures for the North Central Region. North Central Regional Publication No. 221 (Revised). University of Missouri Agricultural Experiment Station, Columbia, MO, pp. 13–16.

Wright, A.F., Bailey, J.S., 2001. Organic carbon, total carbon, and total nitrogen determinations in soils of variable calcium carbonate contents using a LECO CN_2000 dry combustion analyzer. Communications in Soil Science and Plant Analyses 32, 3243–3258.

Yao, H., He, Z., Wilson, M.J., Campbell, C.D., 2000. Microbial biomass and community structure in a sequence of soils with increasing fertility and changing land use. Microbial Ecology 40, 223–237.

Zak, D.R., Ringelberg, D.B., Pregitzer, K.S., Randlett, D.L., White, D.C., Curtis, P.S., 1996. Soil microbial communities beneath *Populus grandidentata* grown under elevated atmospheric CO_2. Ecological Applications 6, 257–262.

Zeller, V., Bardgett, R.D., Tappeiner, U., 2001. Site and management effects on soil microbial properties of subalpine meadows: a study of land abandonment along a north-south gradient in the European Alps. Soil Biology & Biochemistry 33, 639–649.

Zelles, L., 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterization of microbial communities in soil: a review. Biology and Fertility of Soils 29, 111–129.

Zelles, L., Bai, Q.Y., 1994. Fatty acid patterns of phospholipids and lipopolysaccharides in environmental samples. Chemosphere 28, 391–411.