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Microbial community PLFA and PHB responses to ecosystem restoration in tallgrass prairie soils

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Abstract

Native North American prairie grasslands are renowned for the richness of their soils, having excellent soil structure and very high organic content and microbial biomass. In this study, surface soils from three prairie restorations of varying ages and plant community compositions were compared with a nearby undisturbed native prairie remnant and a cropped agricultural field in terms of soil physical, chemical and microbial properties. Soil moisture, organic matter, total carbon, total nitrogen, total sulfur, C:N, water-holding capacity and microbial biomass (total PLFA) were significantly greater ($p<0.05$) in the virgin prairie remnant as well as the two long-term (21 and 24 year) prairie restorations, compared with the agricultural field and the restoration that was begun more recently (7 years prior to sampling). Soil bulk density was significantly greater ($p<0.05$) in the agricultural and recently restored sites. In most cases, the soil quality indicators and microbial community structures in the restoration sites were intermediate between those of the virgin prairie and the agricultural sites. Levels of poly-β-hydroxybutyrate (PHB) and PLFA indicators of nutritional stress were significantly greater ($p<0.05$) in the agricultural and recent restoration sites than in the long-term restorations or the native prairie. Samples could be assigned to the correct site by discriminant analysis of the PLFA data, with the exception that the two long-term restoration sites overlapped. Redundancy analysis showed that prairie age ($p<0.005$) was the most important environmental factor in determining the PLFA microbial community composition, with C:N ($p<0.015$) also being significant. These findings demonstrate that prairie restorations can lead to improved quality of surface soils. We predict that the conversion of farmland into prairie will shift the soil quality, microbial community biomass and microbial community composition in the direction of native prairies, but with the restoration methods tested it may take many decades to approach the levels found in a virgin prairie throughout the soil profile.

Keywords: Microbial biomass; Community structure; Soil quality; PLFA; PHB; RDA; Prairie; Grassland; Ecosystem restoration

1. Introduction

As more than 99% of the pre-settlement tallgrass prairie in North America has been lost to agriculture and development (Samson and Knopf, 1994; Noss et al., 1999), interest in prairie reclamation and restoration has grown over the past 20 years in an effort to preserve this endangered ecosystem and its species (Samson and Knopf, 1996; Kindscher and Tiezen, 1998). Since European settlement, some 50–80% of the original soil organic matter has been lost (Tiessen et al., 1994; Knops and Tilman, 2000) at a rate of about 1% loss per year (Tiessen et al., 1982). Discontinuation of cultivation has a positive effect on soil quality, including increases in organic carbon, nitrogen and C:N ratios (Knops and Tilman, 2000), and land use changes from cropland to perennial grassland have led to increases in soil organic carbon, microbial biomass and activity in surface soils (Karlen et al., 1999; Potter et al., 1999). Grasslands with higher plant litter diversity have exhibited greater microbial biomass and decomposition rates (Bardgett and Shine, 1999). Thus, restoration with native plant species can potentially have positive effects on soil quality and microbial communities.

We had shown previously (McKinley, 2001) that some prairie restorations have surface soil organic matter and microbial biomass contents similar to those of adjacent virgin native prairies, but there were no cropped fields nearby to act as a control. The goal of this study was to use

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a representative set of physical, chemical and microbiological indicators to assess the quality of the soils in a variety of tallgrass prairie restorations by directly comparing them with nearby remnant prairie and agricultural land.

The chosen study site provided us with a virgin prairie remnant, an adjacent long-term cultivation field and three tallgrass prairie restorations of various ages. We sampled the top 5 cm of surface soil, since this is where any changes in soil characteristics are most likely to appear over the short term (Karlen et al., 1999; Potter et al., 1999; McKinley, 2001) and used PLFA profiles to compare the microbial community structures of the five sites. Community-level PLFA profiles have been found to be useful in detecting the responses of soil microbial communities to a variety of land uses or disturbances in other ecosystems (Hedrick et al., 2000; Yao et al., 2000; Fang et al., 2001; Harris, 2003), and certain marker PLFAs can indicate relative amounts of certain functional groups of organisms in soils (Zak et al., 1994; Ringelberg et al., 1997; White and Macnaughton, 1997; Zelles, 1997; Zogg et al., 1997).

We hypothesized that the surface soils in the prairie restorations would show improvement in soil quality (e.g. decreased bulk density and increased organic matter, WHC and C:N), microbial biomass and diversity of PLFAs compared with the agricultural site, and that the soils that had been in restoration the longest would show the most improvement. We predicted that, if this ecosystem was resilient, the microbial community structures of the restoration soils would approach that of the native prairie soil over time, and we wanted to determine which soil characteristics were most important in driving this succession. We also predicted that the native prairie soil community would exhibit fewer indicators of nutritional imbalances or stress (e.g. PHB, saturated:unsaturated PLFA, iso:anteiso PLFA ratios) than the other sites due to greater ecosystem stability (less disturbance) and better soil quality.

2. Materials and methods

2.1. Site description

Goose Lake Prairie State Park, in Grundy County near Morris, Illinois, is 1.6 km southwest of the confluence of the Kankakee and Des Plaines rivers, and about 80 km southwest of Chicago, Illinois. Totaling 1027 ha, it is the largest prairie in Illinois, and more than half of the area is a dedicated nature preserve. The landscape is one of low relief with clay-based Mollisol soils due to the Wisconsin Glaciation (Reinertsen et al., 1992). Later deposition of loess gave these topsoils a loamy texture overall (Table 1), and the sites sampled in this study have been characterized as loamy, mixed, mesic Argiudolls and Haplaquolls (Reineback, 1980).

Soil was sampled on August 21, 1999, from five different sites within the park (Table 1). At the time of sampling, the cultivated agricultural field had been in nearly continuous production since the 1860s through the summer of 1998. Row crops, primarily maize and soybeans, were grown using conventional tillage, and in the last year sunflowers were grown. Adjacent to the agricultural field was a small virgin tallgrass prairie remnant that had historically been fenced off from grazing or plowing and contained a diverse mix of warm season grasses and prairie forbs. Directly across the park access road from the agricultural field (less than 50 m away) was a recent prairie restoration that was seeded in 1992, primarily with big bluestem (*Andropogon gerardii*). Less than 0.8 km away was a relatively long-term prairie restoration that had been established as a diverse, high quality restoration in 1975 with a variety of prairie grasses and forbs. Both of these prairie sites had historically been farmed prior to restoration. About 1.5 km south of this site was an area that was established as a prairie restoration over soils that had been disturbed by a strip mine. In 1972, and again in 1978, a demonstration strip mine reclamation project was undertaken to reshape spoil piles and pits, spread 15 cm of topsoil over the exposed spoil, lime acidic soils and ponds, fertilize, and seed with several grasses and a few forbs. All of the prairie sites are maintained by burning every three to five years and, with the exception of the strip mine restoration, were topographically level.

2.2. Sampling

Soil samples were taken using a small corer (2.2 cm i.d.) to a depth of 5 cm. Samples were taken between, but often close to, plant stems. Four randomly-selected replicate areas at least 10 m apart were sampled at each of the five sites. In the field, 8–10 cores from each replicate area were pooled and sieved (2 mm^2 sieve openings) immediately after sampling, and the few visible roots and stones were removed. Subsamples for PLFA extractions were immediately placed on dry ice. Samples for the other assays were placed in a separate cooler (0 °C). Upon returning to the laboratory, subsamples for PLFA (phospholipid fatty acid) extractions were frozen at −80 °C, and subsamples for physical and chemical analyses were frozen at −20 °C.

2.3. Physical properties

Soil color of crushed, rough, oven-dried samples was determined by comparison with the Munsell Soil Color Charts (Munsell, 1994). Soil texture was determined by a modified hydrometer method (Cox, 1990; Brower et al., 1997; McKinley and Wolek, 2003). Bulk density (BD) was determined from intact cores removed from the ground (Culley, 1993). Water-holding capacity (WHC) was measured in soil cores on a sand bed (Öhlinger, 1996b).

2.4. Chemical characteristics

Moisture content (Öhlinger, 1996a) was determined by drying samples at 80 °C (±1 °C) overnight to constant

Table 1
Goose Lake Prairie sampling sites and physical characterization of the soils (mean ± standard deviation, $n=4$)

Code	Site sampled[a]	Texture	% Sand	% Silt	% Clay	Bulk density (g/cm^3)	WHC (g/g dry soil)	Temp. (°C)	Color (dry)
A	Agriculture since the 1860s	Loam	43.2 ± 1.5	38.4 ± 1.8	18.3 ± 1.2	1.60 ± 0.41	0.340 ± 0.039	19.6	10YR 4.5/2
RR	Recent prairie restoration (1992)	Loam	48.6 ± 4.9	36.4 ± 4.1	15.0 ± 1.5	1.22 ± 0.11	0.389 ± 0.019	19.0	10YR 2/1.5
LR	Long-term prairie restoration (1975)	Loam[b]	52.1 ± 2.5	30.2 ± 1.8	17.6 ± 1.6	0.97 ± 0.03	0.587 ± 0.050	19.4	10YR 3.5/1
SR	Strip mine prairie restoration (1978)	Loam	42.8 ± 2.5	35.3 ± 1.7	21.8 ± 1.1	0.84 ± 0.11	0.648 ± 0.010	17.6	ND
V	Virgin prairie remnant	Loam	40.5 ± 1.1	37.0 ± 3.0	22.6 ± 3.4	0.95 ± 0.08	0.669 ± 0.036	19.2	10YR 2/1

[a] 0–5 cm of surface soil.
[b] One of the four replicates at this site was a sandy loam.

weight (±0.01 g). The dried samples were then ashed in a muffle oven at 350 °C (±5 °C) overnight to determine organic matter (Brower et al., 1997). Total soil carbon (C), nitrogen (N) and sulfur (S) were measured by dry combustion (Matejovic, 1997) using a LECO CNS 2000 analyzer (LECO, St Joseph, MI).

2.5. Microbial communities: PLFA, PHA and sterols

Microbial biomass was estimated as the total extractable phospholipid fatty acids (PLFA), and PLFA profiles were analyzed to determine microbial community composition. To extract the lipids from the soils, the frozen soils were lyophilized and then 5.00 g was extracted using a single-phase chloroform–methanol–aqueous buffer system (Bligh and Dyer, 1959) as modified (Peacock et al., 2000). The total lipid extract was fractionated into neutral lipids, glycolipids, and polar lipids by silicic acid chromatography (Guckert et al., 1985) and the polar lipid fraction containing the phospholipids was isolated and transesterified into fatty acid methyl esters using a mild alkaline methanolysis reaction (Guckert et al., 1985). Fatty acid methyl esters (FAMEs) were analyzed by capillary gas chromatography with flame ionization detection on a Hewlett-Packard 5890 Series 2 chromatograph using a 50 m non-polar column (0.2 mm i.d., 0.11 μm film thickness) with the injector and detector maintained at 270 and 290 °C, respectively. The column temperature was programmed to start at 60 °C for 2 min and then ramp up at a rate of 10 °C min^{-1} to 150 °C, followed by a ramp of 3 °C min^{-1} to 312 °C. Methyl nonadecanoate was used as a quantitative internal standard and definitive peak identification was made for representative samples by gas chromatography/mass spectrometry using a Hewlett-Packard 5890 series 2 gas chromatograph interfaced with a Hewlett-Packard 5971 mass selective detector under the same column and temperature program described. Mass spectra were determined by electron impact at 70 eV.

Poly-β-hydroxyalkonates (PHAs) were quantified from the glycolipid fraction by GC and GC–MS following an ethanolysis derivatization, and using malic acid as an internal standard (Findlay and White, 1983). Sterols, including ergosterol, were quantified from the neutral lipid fraction following alkaline saponification and using cholestane as an internal standard (Nichols et al., 1983).

2.6. Statistics

Data were compiled and transformed in Microsoft Excel. Statistics and graphs were generated using Statistica for Windows, '99 Edition, release 5 (Statsoft, 1999), except for the redundancy analysis (RDA), which was calculated and graphed using CANOCO for Windows, version 4.02 (ter Braak, 1998).

Significant differences between groups (sites) were determined by comparing the means of each variable using a Kruskal–Wallis ANOVA and the Median Test. Significance of post hoc pairwise comparisons was determined using Tukey's HSD, or the Kolmogorov–Smirnov test for variables that were not normally distributed. Correlations between variables were made using a linear Pearson's r coefficient, and arcsine square root transformations were used for variables that were proportions of other variables. For all tests of significance $\alpha = 0.05$.

A principal components analysis (PCA) was used to select a subset of PLFAs important in explaining the variation in the data, and a forward stepwise discriminant analysis was used to determine whether these variables could be used to discriminate between the sites (Statsoft, 1999). The discriminant model was tested by omitting one case (replicate sample) from each site for the initial construction of the model. Then, the omitted replicates

were entered into the model to test whether the model could place them in the correct site.

Redundancy analysis (RDA), a linear canonical community ordination method, was used to visualize the relationships between the response variable values (PLFAs), the environmental variable gradients and the samples. RDA is similar to canonical correspondence analysis (CCA), except that RDA is a linear model while CCA is unimodal (the PLFA data in this study did not conform to a unimodal distribution). In RDA, the ordination axes are constrained to be linear combinations of the environmental variables, allowing the relationships between the environmental variables and the response variables to be directly compared. The sites are represented by points in the ordination space (a distance diagram), and the PLFA response variables (called 'species' in ordination models) and environmental variables are represented by arrows projecting from the origin (ter Braak and Prentice, 1988).

For the RDA in this study PLFAs were used as 'species'. The focus of the ordination was on the inter-species correlations, species scores were post-transformed (divided by the standard deviation) to reduce the effects of extreme values, and the ordination was centered by species and samples. Because the PLFA data was compositional in nature (mole percentages of the total sample), a log ratio transformation was used: $Y' = \log(100 \times Y + 1.00)$ (ter Braak, 1998, p. 294). The arrows in the resulting ordination diagrams point in the direction of maximum variation in the PLFAs, and the arrow length is proportional to the rate of change. PLFAs near the edge of the plot are most important in explaining site differences, while PLFAs near the center are of lesser importance. PLFA arrows pointing in the same general direction as environmental arrows can be interpreted as correlating well with that variable, and the longer the arrows, the more confidence can be had in that correlation (ter Braak, 1994). Environmental gradient arrows that are longest allow more confidence in the inferred correlations, roughly indicate a larger effect of that variable on the total species variation (ter Braak, 1998, p. 168), and point in the direction in which the site scores would move if the value of that environmental variable increased.

3. Results

3.1. Physical and chemical soil properties

All of the soils were loams, but the agriculture site (A) and the recent restoration site (RR) had significantly higher bulk density and lower water holding capacity (WHC) than the other sites (Table 1). The nutrients in the soils also varied (Table 2), with the agriculture and recent restoration sites having significantly lower amounts of organic matter, C, N, S and C:N than the other sites. In general, the values of the soil quality indicators for the prairie restoration sites were intermediate between those of the agriculture site and the virgin prairie site (V), with the long-term restorations (LR and SR) being closest to the virgin prairie values. An exception was the elevated amount of sulfur in the strip mine reclamation site (SR).

3.2. Community composition: PLFA, PHB and ergosterol

Total PLFA, the sum of the 76 fatty acids that were identified in the samples, was significantly higher in the virgin prairie and long-term restoration sites than in the agriculture and recent restoration sites (Fig. 1a). Total PLFA biomass correlated significantly with soil organic matter, N, C, moisture and WHC ($p < 0.001$), and negatively with bulk density ($p = 0.001$).

The proportion of terminally-branched saturated PLFAs, largely contributed by Gram (+) organisms, was significantly greater ($p < 0.05$) in the virgin prairie site compared with the other four sites, while the total for the polyunsaturates was significantly lower (Table 3). The proportion of monounsaturated PLFAs, mainly from Gram (−) organisms, was significantly lower ($p < 0.05$) in the agriculture and recent restoration sites than in the other three sites. The proportions of normal saturated PLFAs of all chain lengths were greater in the agriculture site than in the virgin prairie site, with the restoration sites being intermediate.

The variability in ergosterol levels in these soils was so high that no significant differences could be detected between the sites, and ergosterol did not correlate with fungal PLFA markers (18:2ω6 or a combination of the fungal markers 18:2ω6 and 18:1ω9c). The virgin prairie site had more bacterial PLFAs relative to total microbial

Table 2
Chemical characteristics of the soils sampled (mean ± standard deviation, $n = 4$)

Site[a]	% Moisture	% Carbon[b]	% Nitrogen[b]	% Sulfur[b]	C:N
A	15.6 ± 1.3	1.68 ± 0.06	0.139 ± 0.003	0.014 ± 0.002	12.1 ± 0.6
RR	18.4 ± 0.7	2.30 ± 0.25	0.160 ± 0.011	0.018 ± 0.002	14.3 ± 0.6
LR	19.8 ± 1.4	4.32 ± 0.24	0.275 ± 0.018	0.035 ± 0.002	15.7 ± 0.4
SR	26.0 ± 1.3	9.60 ± 0.87	0.415 ± 0.028	0.098 ± 0.021	23.1 ± 1.3
V	25.6 ± 1.4	7.69 ± 0.99	0.502 ± 0.064	0.049 ± 0.006	15.3 ± 0.3

[a] See Table 1 for site descriptions.
[b] Total organic carbon determined by LECO analyzer.

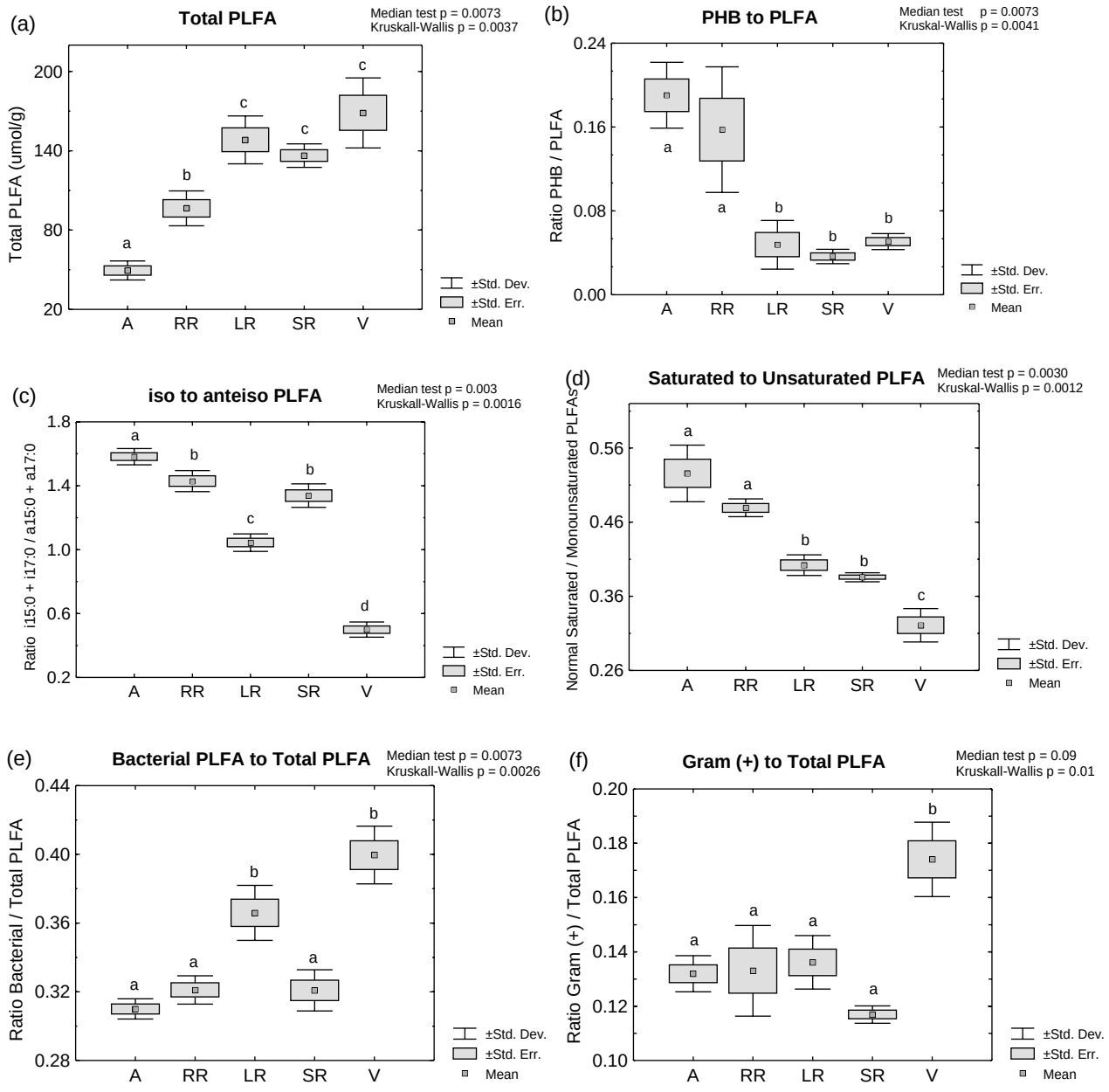

Fig. 1. Amount of (a) total phospholipid fatty acids (PLFA), (b) poly-β-hydroxybutyrate (PHB) relative to total PLFA, (c) iso to anteiso branched PLFA (i15:0+ i17:0/a15:0+ a17:0), (d) normal saturated to monounsaturated PLFA, (e) bacterial PLFA markers to total PLFA, and (f) Gram (+) PLFA markers to total PLFA found in the top 5 cm of the soils from the prairie sites sampled (mean ± one standard deviation and one standard error, $n=4$, lower case letters indicate significant differences of $p<0.05$ in Tukey's HSD and Kolmogorov–Smirnov tests). See Table 1 for site abbreviations and Table 3 for a list of saturated and monounsaturated PLFAs detected. Bacteria were estimated as the sum of i15:0, a15:0, 15:0, i16:0, 17:0, cy17:0, cy19:0, 18:1ω7c, and 18:1ω7t; PLFAs used to indicate Gram (+) bacteria were i10me16:0, a10me16:0, i15:0, a15:0, i16:0, and 17:0.

biomass (Fig. 1e), largely due to an increase in the proportion of PLFAs indicative of Gram positives at this site (Fig. 1f).

The amount of poly-β-hydroxybutyrate (PHB) relative to total PLFA biomass was significantly higher ($p<0.05$) and more variable in the agriculture and recent restoration sites than in the other sites (Fig. 1b). The PHB:PLFA ratio correlated significantly with bulk density, and correlated negatively with organic matter, C, N, S, C:N, moisture, WHC and clay ($p<0.01$). Other detectable poly-β-hydroxyalkonates (PHAs) were not significantly different between sites. PLFA indicators of nutrient stress, the ratio of iso to anteiso 15:0 and 17:0 PLFAs (Fig. 1c) and the ratio of normal saturated to monounsaturated PLFAs (Fig. 1d), correlated with PHB and exhibited a similar pattern, with the exception that in the long-term restoration soils (LR and SR) these PLFA ratios were significantly higher than those in the virgin prairie soil. Another PLFA ratio often

Table 3
Phospholipid fatty acid (PLFA) composition (mean mole%) of surface soils from prairie samples (site codes are listed in Table 1; $n=4$)

Fatty acid	A	RR	LR	SR	V	Marker
Normal saturated	20.5	19.7	17.6	17.0	13.8	
Mid-chain branched saturated	9.8	9.9	8.9	10.2	11.3	Actinomycetes[a]
10me16:0	4.1	3.6	3.6	5.0	5.4	Gram (+)[b,c]; actinomycetes[a]
10me18:0	1.3	1.7	1.2	1.3	1.2	Actinomycetes[a,d]
Terminally branched saturated	16.8	16.0	17.1	14.4	21.9	
i14:0	0.4	0.3	0.5	0.3	1.4	Gram (+)[e]
i15:0	6.2	5.9	5.4	5.1	4.1	Gram (+)[b,c,d,f,g]
a15:0	3.3	3.6	4.8	3.3	9.3	Gram (+)[b,d,f,g]
i16:0	2.3	2.3	2.1	1.8	2.1	Gram (+)[b,c,f,g]
i17:0	2.1	1.9	1.8	1.8	1.9	Gram (+)[b,d,f,g]
a17:0	1.9	1.8	2.0	1.9	2.8	Gram (+)[b,d,g]
Branched monounsaturated	7.0	7.7	7.4	8.7	7.0	
i17:1w7c	3.1	2.7	2.7	3.4	2.3	*Desulfovibrio*[a,d,h]
Monounsaturated	39.1	41.1	43.8	44.0	43.1	
16:1w9c	1.3	1.0	1.1	1.2	1.1	Gram (−)[b,g]
16:1w7c	5.8	5.1	4.6	5.4	4.0	Gram (−)[b,d]
16:1w7t	0.2	0.4	0.3	0.3	0.3	Gram (−)[b,g]
16:1w5c	5.0	5.5	4.1	4.8	3.5	Gram (−)[b]; fungi[i]
cy17:0	3.5	2.8	2.8	3.0	2.6	Gram (−)[b,c,d,f,g]
18:1w9c	6.5	6.9	7.8	8.6	6.9	Fungi[b,c,e,i]; plants[d,e]
18:1w7c	8.3	10.1	10.1	9.9	11.2	Gram (−)[b,c,d,f,g]
18:1w7t	0.3	0.3	0.5	0.3	0.4	Gram (−)[c]
18:1w5c	0.9	1.5	1.3	1.3	1.2	Gram (−)[b]
cy19:0	5.9	6.1	9.9	7.6	9.6	Gram (−)[b,c,d,f,g]
20:1w9c	0.6	0.4	0.3	0.4	0.3	Fungi[i]
Polyunsaturated	6.7	5.7	5.2	5.7	2.8	
18:2w6	3.0	2.6	3.4	3.6	1.8	Fungi[b,c,d,h,i], plants[b,e]
18:3w3	0.3	0.1	0.1	0.1	0.1	Fungi[h]; plants[h]
20:4w6	1.4	1.0	0.7	0.9	0.4	Protozoa[a,d,h]
20:5w3	1.0	0.7	0.5	0.5	0.2	Fungi[i]; plants[d]

[a] White et al. (1997).
[b] Zak et al. (1996).
[c] Zogg et al. (1997).
[d] Ringelberg et al. (1997).
[e] Zelles (1997).
[f] Bardgett et al. (1996).
[g] Frostegård and Bååth (1996).
[h] Pinkart et al. (2002).
[i] Madan et al. (2002).

indicative of stress, the ratio of cyclopropyl fatty acids to their monoenoic precursors, did not exhibit significant differences between sites.

3.3. Multivariate analyses

Discriminant analysis was performed to determine whether the PLFA profiles could be used to discriminate between the sites (Fig. 2). Because the PLFA data set had far more variables than sites, a principal components analysis (PCA) was first carried out to select a subset of PLFAs having high loading scores on the first two PCA axes. Eleven high-loading PLFAs from the PCA were entered into a forward stepwise discriminant analysis (br14:0, a15:0, 15:0, a17:0, 17:0, 19:1, 20:4ω6, 2me21:0, 22:6ω3, 23:0, 24:0), and six of these were retained by the model (20:4ω6 plus those shown as dominating the negative or positive ends of the axes in Fig. 3). One-fourth of the samples (one from each site) were excluded from the initial discriminant analysis so that they could be used to test the resulting model. All of the samples were correctly classified according to site in the initial analysis. When the excluded samples were added to the model, one of the five (LR) was incorrectly classified as another long-term restoration (SR). The samples from the agriculture and virgin prairie sites were well-separated from each other along axis 1 (influenced largely by 22:6ω3, 23:0, 19:1 and a17:0), while the samples from the three restoration sites all fell in the same general area, separating from the agriculture site along axis 2 (having higher amounts of a15:0 and a17:0).

Fig. 3a shows the site points and the environmental gradient arrows for the RDA ordination of the PLFA data. In Fig. 3b, the species (PLFAs) are shown with the sites. Prairie age, C, N, WHC and clay increased in the general

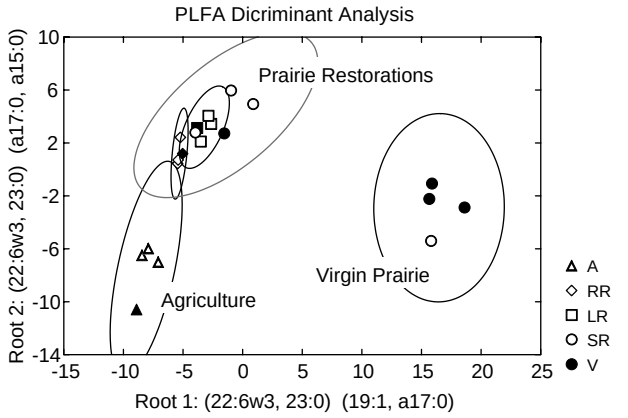

Fig. 2. Discriminant analysis of the PLFAs (Wilks' $\lambda = 0.0000099$, $p < 0.00001$ from the soils in the five prairie sites. Variables entered into the discriminant analysis were chosen by PCA. Of the 11 PLFAs entered, six were selected for the discriminant model by a forward stepwise method. Axis labels show variables that were strong negative or positive factors on each axis (PLFA 20:4ω6 was influential on the third and fourth axes of the model, not shown). Elipses represent 80% confidence levels. One of the four replicate samples from each site (solid symbols, open symbol for V) was left out of the initial model so that the model could be tested for correct classification of samples according to site (the LR sample was miss-classified as coming from the SR site in the PLFA model).

direction of the virgin prairie (V) sites, while bulk density increased toward the agricultural (A) sites (Fig. 3a), as would be expected from the site comparisons (Tables 1 and 2). Proportions of certain PLFAs (Fig. 3b) were highly correlated with prairie age, N, WHC and clay (br14:0, a15:0, a17:0, 19:1, 20:1, 22:1, i14:0, a10me16:0, i10me16:0, cy19:0), C:N and S (br16:1, 16:1ω7t, i16:1, i18:1), or bulk density (i15:0, i16:0, i17:0, cy17:0, 16:0, 18:0, 22:0, 23:0, 24:0, 20:5ω3, 22:6ω3).

The first axis of the RDA (Fig. 3) was significant in explaining the variation in the PLFAs ($p = 0.0050$), as were all four axes together ($p = 0.0450$). A total of 67.5% of the fitted PLFA data could be explained by the model (from the canonical sum of the eigenvalues), with the first two axes explaining 42.8% of this variation. The percentage of this variation that is explainable using the environmental variables that were measured was 41.5, 63.5, 73.0 and 79.9%, respectively, for the four axes (the cumulative species-environment variation explained). Axis 1 correlated most strongly with prairie age, N, and WHC, axis 2 correlated negatively with C:N, and axis 4 correlated with S and C:N.

In addition to being able to examine the relationships between samples, species and environmental gradients altogether on the same diagram, another advantage to this model is that, unlike discriminant analysis, there is no limit on the number of species that can be used relative to the number of samples. Although all 76 PLFAs were included in the RDA ordination, for the sake of clarity only the PLFAs with the highest species scores on each of the first two ordination axes, those that correlated well with environmental variables, and those that are important biological markers are displayed in Fig. 3b. Some general patterns emerge from this diagram. Among the mid-chain branched saturated PLFAs, the short-chained (14- and 16-carbon) fatty acids are on the positive end of axis 1, while the longer-chained (17-, 18- and 21-carbon) fatty acids are on the negative side. The opposite is true of the monounsaturates, with the 16- and 17-carbon fatty acids on the negative end of axis 1, the 18-carbon fatty acids in the middle, and the 19-, 20- and 22-carbon fatty acids on the positive side. For the terminally-branched saturated PLFAs, the anteiso-branched fatty acids (a15:0 and a19:0), are in the upper right quadrant while the iso-branched fatty acids (i16:0, i17:0 and i19:0, but not i14:0) are in the upper left. Axis 1 had several protozoal, fungal, and actinomycete markers toward the negative side (between sites A and RR). The PLFAs that loaded most heavily on axis 4 (correlated with S and C:N; not shown in Fig. 3) were 16:1ω9c, 18:1ω9c and 20:1ω9t.

4. Discussion

4.1. Physical and chemical soil properties

The sites sampled in this study have surface soils that differ in physical, chemical and biological composition, and many of these differences appear to be related to the amount of time that the site has been planted in tallgrass prairie (prairie age). With a few exceptions, the largest differences were almost always found between the agriculture site (A) and the virgin prairie remant site (V), with the prairie restoration sites generally intermediate between these two. This indicates that successional processes may be continuing in the microbial communities of the restoration site soils. No samples are available from these sites prior to their restoration, but it is known that the recent restoration (RR) and long-term restoration (LR) sites were farmed for many decades prior to restoration, and the SR site was strip-mined. Although we have no time zero control samples for these sites, nonetheless we believe that useful comparisons may be made since all of the sites are in close proximity and had the same type of native soil.

The results of the physical and chemical soil analyses (Tables 1 and 2) were comparable to those of similar studies. The agricultural soils (A) were clearly degraded in quality relative to the virgin prairie soils (V). The agricultural soils were lighter in color, had significantly higher bulk density, less soil organic matter and total C, N and S, lower C:N ratios, less moisture, and less microbial biomass (as PLFA) than the virgin prairie soil (Tables 1 and 2). All of these results are typical of long-term cultivation of North American prairie soils (Bradfield, 1937; Tiessen et al., 1994; Wander and Bollero, 1999) and other temperate grasslands (Low, 1972).

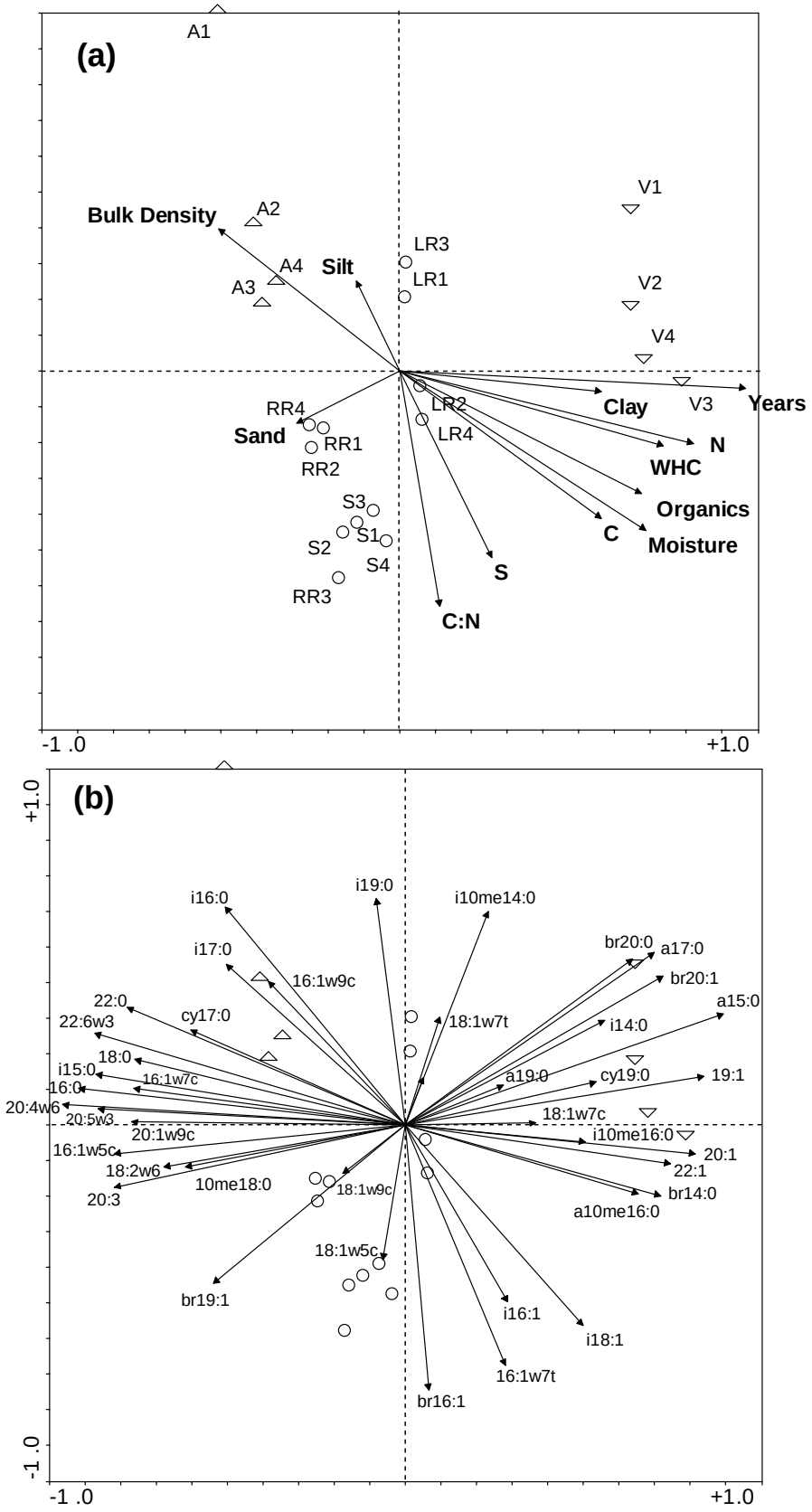

Fig. 3. Redundancy analysis (RDA) of the PLFA data set for the 20 soil samples, using 76 PLFAs as species and 12 environmental variables. Site codes for each sample in (a) are given in Table 1. (b) shows the PLFAs that had the highest absolute species scores on each of the first two axes, along with additional PLFA markers of biological interest (Table 3).

The establishment of native prairie plants on the restoration sites (RR, LR, SR) had a significant effect on these soil quality indicators, generally shifting them in the direction of the native prairie values. A detailed analysis of the soil properties from these samples was published previously (McKinley and Wolek, 2003). The extent of the changes appears to be partially determined by the amount of time under restoration. The strip mined restoration site (SR) site did not always follow this pattern exactly (e.g. total sulfur), probably due to its history of disturbance as a strip mine and the hilly topography resulting from the mining spoils. In addition to being the youngest restoration, the RR site had the least plant diversity (qualitative observation) of the prairie sites, which may also have affected the microbial community structure.

4.2. Community composition: PLFA, PHB, ergosterol

The soil microbial community composition also differed among the sites, as measured by phospholipid fatty acid profiles (PLFAs, Table 3). Community substrate utilization patterns (Biolog GN) were also analyzed for these samples, but were found to be ineffective at discriminating between sites (data not shown). As previously reported, plate counts of these same soil samples showed no significant differences between sites (McKinley and Wolek, 2003), and thus the PLFA method does appear to detect many organisms that are not culturable (White and Macnaughton, 1997; Pinkart et al., 2002). A total of 76 different PLFAs were detected in these soil samples. There were no significant differences in the number of PLFAs found in the five sites, with an average of 75.5 ± 0.6 PLFAs (range 75–76) in the virgin prairie samples and 73.5 ± 1.3 PLFAs (range 72–75) in the agriculture samples. However, the proportions of the PLFAs differed among the five sites (Table 3; Fig. 3b).

Prairie age correlated positively with total PLFA biomass, and negatively with protozoal and fungal markers. PLFAs that have been documented as markers for taxonomic groups or metabolisms in soil microbes are noted in Table 3. While these PLFA markers are not absolute in their specificity, generalizations can usually be made (Peterson and Klug, 1994; White et al., 1996; White and Macnaughton, 1997; Griffiths et al., 1999; Pinkart et al., 2002). For example, although some Gram (−) bacteria have been found to contain the branched PLFAs typically used as markers for Gram (+) bacteria (Kaneda, 1991), with the possible exception of *Desulfovibrio* none of these Gram (−) organisms (animal pathogens and GI inhabitants, aquatic organisms) would be found in significant numbers in most soils.

Although the total amounts of PLFAs typically attributable to Gram (−) and Gram (+) organisms both increased with prairie age, the proportion of the total community comprised of Gram (+) bacteria increased (Fig. 1f). In a study utilizing PCR-DGGE and culture techniques, a greater diversity of Gram (+), *Bacillus*-related species were found in permanent grassland compared with arable land in the Netherlands (Garbeva et al., 2003). However, in another study the proportion of Gram-negatives (indicated by the marker cy17:0) was found to increase with loadings of simulated root exudates, while the proportion of Gram-positives declined, although the biomass of each increased (Griffiths et al., 1999). There are more organic substrates (SOM, C and N), and presumably more root exudates, present in the virgin prairie soils than in the agricultural site, so this type of root exudate explanation does not appear to fully account for the increase in the proportion of cy17:0 in the agriculture site in the present study. Increased root decomposition does not appear to be a likely explanation either, since fungal and actinomycete markers were also in lower proportions in the virgin prairie site. The proportions of cy17:0 can, however, be affected by the nutrient status and physiological state of the community and may not always be a reliable indicator of taxonomic differences. Mono-unsaturated PLFAs, attributable largely to Gram (−) organisms, have also been found to increase in proportion with increasing amounts and diversity of carbon sources (Peacock et al., 2000) and decrease in soils subjected to disturbance and compaction from vehicular traffic (Peacock et al., 2001). When we used a group of PLFAs (Fierer et al., 2003) as a Gram (−) marker (16:1ω7c, 16:1ω7t, 18:1ω7c, 18:1ω9c, cy17:0, cy19:0) rather than cy17:0 alone, however, there were no significant differences in the proportion of Gram (−) organisms between sites. It is apparent that the changes in the microbial community structures in these soils following restoration are more complex than simply a broad taxonomic shift from Gram (+) to Gram (−), or vice versa. Therefore, when PLFAs are used to monitor ecosystem restoration it is important to have a nearby undisturbed reference site for comparison.

A clear trend of decreasing nutritional stress of the bacterial community with age of the prairie could be seen in three different measures: the ratios of PHB/PLFA, iso/anteiso PLFA, and saturated/monounsaturated PLFA ratios (Fig. 1b–d). PHB (poly-β-hydroxybutyrate) is an endogenous bacterial carbon storage product that usually accumulates under conditions of unbalanced growth when carbon is available but nitrogen or another essential nutrient is limiting (Tunlid et al., 1985; Tunlid and White, 1992). Bacteria associated directly with roots in the rhizosphere have been shown to have very low PHB/PLFA, while those in the surrounding soil contained much higher ratios (Tunlid et al., 1985). It is possible that the high levels of organic matter and mature development of the extensive root systems of the prairie grasses and plants provide enough nutrients, root exudates and growth factors that the bacterial communities in these ecosystems are undergoing balanced growth, even if they are not attached directly to a root. Shifts in the iso/anteiso and saturated/unsaturated PLFA

ratios have been associated with nutrient stress, physical or chemical disturbance, or communities undergoing changes in composition (Zelles et al., 1992; White et al., 1996; Kieft et al., 1997; Peacock et al., 2001; Pinkart et al., 2002; Fierer et al., 2003).

In the present study, bacterial and fungal PLFA biomass both increased over time in the restoration sites, but the bacterial:total PLFA ratio (Fig. 1e) was higher in the virgin prairies site, indicating that the bacterial biomass had increased more than the fungal biomass. Bacterial and fungal biomass was reported to increase with substrate loading in the root exudate study, but the bacterial:fungal ratio decreased (Griffiths et al., 1999). Although 18:2ω6 and the other polyunsaturated PLFAs found in the soils in this study can occur in both fungi and plants (Madan et al., 2002), the majority of these PLFAs were probably of fungal origin because most prairie plants are deep-rooted, the few visible root fragments were removed prior to extraction, plant roots have a much smaller surface area-to-volume ratio than fungal hyphae (Klamer and Bååth, 2004), and no PLFAs exclusive to higher plants (e.g. 16:2, 18:1ω11c, 26:0) were found in these samples. The PLFA marker 18:2ω6 has previously been found to correlate well with fungal respiration rates in soils when bacteria are suppressed by selective antibiotics (Bardgett et al., 1996), and has also been found to correlate with ergosterol (Frostegård and Bååth, 1996; Bååth and Anderson, 2003).

No significant differences in ergosterol or ergosterol/PLFA were seen between the sites in this study, and ergosterol did not correlate with 18:2ω6. The proportion of polyunsaturated PLFAs, indicative of total eukaryotes, also decreased with prairie age, and others have found this proportion to be lower in grasslands than in forests or salt marshes (Zelles and Bai, 1994). It is unusual, however, for grasslands or native prairies to have lower proportions of fungi (West and Grant, 1987; Djajakirana et al., 1996; Stahl and Parkin, 1996; Stahl et al., 1999; Bailey et al., 2002) or fungal activity (Kennedy and Gewin, 1997) than cultivated sites, but a study of a short-grass, semi-arid prairie in Colorado did find lower fungal hyphal lengths than typical for grasslands or agricultural sites (Ingham et al., 1986), and others have found no significant difference between the 18:2ω6/total PLFA ratios in a grassland and several different cropped fields (Zelles et al., 1995). Although fungi have been found to increase along moisture gradients in prairies and fens (Turner et al., 2001), it may be that fungi were more prevalent in the soils with the lower water-holding capacities (A, RR, LR) because they are often better able to withstand high levels of osmotic stress than bacteria (Griffin, 1972). However, both the amount and composition of fungal PLFAs and sterols in cells have been found to vary with environmental conditions, fungal developmental stages (Lösel, 1988; Madan et al., 2002) and taxonomy, and these factors may partially obscure fungal biomass changes as reflected by PLFA markers and ergosterol levels. It may also be that by sampling only the top 5 cm of soil in our prairies we missed some key fungal habitats, or the populations may be more patchy than the bacterial populations, requiring more sample replication to detect meaningful patterns of distribution. The large variability in the ergosterol measurements support this idea, but the fact that the fungal PLFA markers were not as variable as ergosterol indicate possible inefficiencies in the ergosterol extraction or processing.

The use of discriminant analysis (Fig. 2) showed that PLFA profiles were distinctly different in sites undergoing ecosystem restoration compared with both nearby cropped soils and native prairie soils, and that the microbial community composition of the restoration soils appear to be intermediate with respect to these two end points in many ways. A relatively small subset of PLFAs can be used to discriminate between these sites. This appears to be both a sensitive and robust assay, correctly assigning four out of five samples to the correct sites. Most of the PLFAs selected as important discriminators in the stepwise iteration are markers for nutritional status (a15:0, a17:0) or are typical of certain taxonomic groups (a15:0 for Gram positives, 19:1 for Gram negatives, and 22:6ω3 for eukaryotes).

The RDA analysis allowed us to examine the patterns in the PLFA data in terms of both the sites and the measured environmental gradients (Fig. 3). The environmental variables measured in this study explained 80% of the variability in the PLFAs. It was not surprising that C:N gradients were important environmental factors in the PLFA ordination, increasing in the direction of the native prairie site and away from the agriculture site. Native prairies are known for having high levels of organic matter but low available nitrogen, and the plants growing there are adapted to this condition (Seastedt et al., 1991; Tilman et al., 1996). We also expected soil organic matter to be a strong influence on the microbial communities in the prairie restorations and native prairie because grasslands are carbon sinks (Samson and Knopf, 1994), microbial biomass is commonly correlated with SOM (Dick, 1992; Baldock and Nelson, 2000) and SOM is often cited as one of the most important indicators of soil quality (Kennedy and Papendick, 1995; Romig et al., 1995; Singer and Ewing, 1997).

The prairie restoration soils appear to be responding to their new plant communities and land management regimes, and progressing toward the state of the native virgin prairie. This result agrees with the trends seen in soil characteristics (Tables 1 and 2) and total PLFA biomass (Fig. 1a) across the sites (noting that the SR site has a different pre-restoration history than the other sites, and therefore does not always fit the trend line). We had expected the virgin prairie to exhibit not only greater microbial biomass, but also perhaps a wider range of PLFA markers. However, just as many fatty acids exhibited a strong negative correlations (21 PLFAs) with prairie age as showed a strong positive correlation (17 PLFAs).

Although the proportion of Gram (+) markers was higher in the virgin prairie site than in the other sites (Fig. 1f), there was no clear segregation of Gram (+)

or Gram (−) markers along the ordination axes (Fig. 3b). However, some interesting patterns of PLFAs were found in relation to the ordination axes were found in terms of chain length and iso vs. anteiso branching among certain groups of PLFAs. The PCA/discriminant analysis also pointed to the Gram (+) markers a15:0 and a17:0 as being important discriminators of these communities. These patterns may be related to nutritional status (iso/anteiso) or other environmental factors, rather than taxonomy, indicating that measures of microbial activity may also be important for following the progress of ecological restorations (Harris, 2003).

In this study only the top 5 cm of soil was sampled, which is most likely to exhibit short-term changes in response to plant communities (Woods, 1989; Gebhart et al., 1994; Karlen et al., 1999; Potter et al., 1999; McKinley, 2001). Based on the levels of SOM in the soils in this study, we estimated that it would take at least 45 years for the restorations to reach the level of SOM found in the virgin prairie site, in the top 5 cm alone (McKinley and Wolek, 2003). How long it will take for the entire root zone of the soil to respond in terms of physical, chemical and biological characteristics is an open question requiring more detailed sampling by depth. A study of old fields in Minnesota concluded that it would take 230 years to reach a steady state of carbon (Knops and Tilman, 2000), and others have estimated time periods on the order of more than 1000 years to renew degraded topsoils (Willis and Evans, 1977). Whether or not the microbial communities will take that long to develop a similar composition to the virgin soils is difficult to predict.

In conclusion, PLFA profiles provided a sensitive and meaningful measure of microbial community compositions in prairie restorations compared with nearby agricultural fields and native prairie. The longer a formerly cropped or disturbed site had been planted and maintained as a prairie restoration, the more similar the soil physical, chemical and biological characteristics were to the native prairie, but none of the restorations had yet developed PLFA profiles identical to the native site. Prairie restoration does appear to enhance the quality of surface soils compared with cropped lands, and may propel a secondary succession in the microbial communities. Whether or not these communities will ever match those found in the virgin prairie soils is yet to be seen.

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