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Microbial Biomass and Activity in Soils from Virgin Prairies Compared with Prairie Restoration, Forest and Agricultural Sites in Illinois

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Little is known about the communities of free-living (asymbiotic) microbial communities inhabiting prairie soils. In this study, total viable microbial biomass (from phospholipids), culturable asymbiotic nitrogen-fixers, and Biolog metabolic profiles were compared in soils from a prairie restoration, a soybean field, a forest and three virgin prairie remnants. The virgin prairies consistently had much greater total viable biomass than the prairie restoration or an agricultural field planted in soybeans, but the soybean field had a higher count of free-living nitrogen-fixing bacteria. Viable microbial biomass correlated positively with soil organic content. Except for the agricultural field, the top 1 cm of soil had more biomass per gram of soil than the 5–7 cm depth. Rates of carbon substrate metabolism also differed significantly in the different treatments, as shown by a discriminant analysis. The prairie restoration soil also showed significantly less diversity in the substrates metabolized by the microbiota, and for those substrates in which metabolic rates differed between sites, the virgin prairies always had higher rates than the corresponding restoration or agricultural site. We conclude that the conversion of farmland into prairie may significantly improve the biomass and activity of the soil microbial communities, but it may take decades or longer to approach the levels found in a virgin prairie. Eventually, we hope to be able to predict this progress and to determine the effects of these changes on the rates of soil processes.

INDEX DESCRIPTORS: soil, bacteria, microbes, microbial biomass, microbial metabolism, asymbiotic nitrogen-fixers, organic matter, Biolog, community-level physiological profiles (CLPP), CLPP, substrate utilization, prairie restorations, agriculture, flatwood forest, virgin prairie, mesic prairie.

Prior to European settlement, 8,900,000 ha of Illinois were tallgrass prairie (Samson and Knopf 1994). More than 99.9% of the original tallgrass prairie of Illinois has been lost to the plow (Noss et al. 1999), with only about 930 ha of prairie remaining. Although the impact of farming on plant species composition, biomass and diversity is obvious, the impact on the soil communities is less apparent and more difficult to study directly.

Soil microorganisms (bacteria, fungi, protozoa and algae) play vital roles in maintaining the health and integrity of the soil itself as well as helping to maintain the health of the plant and animal communities. Although some symbiotic microorganisms (e.g., mycorrhizae, *Rhizobium,* etc.) interact specifically with particular species of plants or animals, most microorganisms in the soil are free-living or can associate in a less specific and obligate manner with macroorganisms. These free-living microbes are essential to terrestrial ecosystems, carrying out many metabolic activities key to decomposition processes and biogeochemical cycles that return nutrients and organic matter to the soil. Nitrogen fixation, for example, has been called the second most important biological process on Earth after photosynthesis (Graham 2000), and asymbiotic nitrogen fixation has been found to be the principle source of biologically-fixed nitrogen in tallgrass prairies (Kapusta 1980). Nitrogen has been proposed as the nutrient having the most importance in limiting and structuring tallgrass prairie ecosystems (Blair et al. 1998). Carbon cycling and sequestration are also mediated by microorganisms. It has been shown that cultivation has significant effects on these rates and ultimately results in loss of soil organic matter to the atmosphere as carbon dioxide (Tiessen et al. 1982, Elliot 1986, Dick 1992, Wander and Bollero 1999).

Soil microorganisms also modify the soil structure, affecting such properties as aggregation, water holding capacity, wetability and porosity. Microbial biomass, diversity and activity are often listed as important indicators of soil health and quality (Pankhurst 1997, Roper and Ophel-Keller 1997, Singer and Ewing 1997, Sparling 1997, White and Macnaughton 1997), in addition to such characteristics as soil organic matter, texture, pH, bulk density and other physical-chemical properties. However, it has been estimated that less than 10% of all microbes in nature (including soils) are culturable (Winding et al. 1994, Palojärvi et al. 1997), and, therefore, microbial communities cannot be characterized solely by methods that rely upon growing the organisms in the laboratory.

The goals of this study were to (1) assess a set of microbial methods for use in monitoring soil microbial communities during prairie restorations and (2) determine the effects of prairie restoration on microbial soil communities and other soil parameters. We hypothesized that the soils from the prairie restorations would exhibit some signs of recovery from disturbance by having soil and microbial characteristics intermediate between those in the agricultural site and the virgin prairie sites. The years of lack of tillage and increased plant diversity should allow the soil microbial communities to increase in biomass and functional diversity.

We compared the microbial communities in the surface layers of soils from a virgin prairie, an adjacent prairie restoration, and an adjacent forest in Cook County, IL, as well as another nearby high-quality virgin prairie site. Also, a virgin prairie was compared with an adjacent agricultural field at a railroad remnant site in McHenry County, IL. In each case, the microbial communities were characterized by measuring the total viable microbial biomass (as phospholipid phosphate), numbers of culturable asymbiotic N_2-fixing bacteria (heterotrophic diazotrophs), and community substrate utiliza-

Table 1. Soil texture data for the sites sampled (top 5 cm of soil, means of five replicate samples at each site), site codes and dates of sampling.

Site Sampled	Code	Date	Texture	% Sand	% Silt	% Clay
James Woodworth Virgin Prairie	JWV	24 June	Loamy sand	82	10.5	7.5
Bunker Hill Virgin Prairie	BHV	8 July	Sandy loam	77	16	7
Bunker Hill Prairie Restoration	BHR	15 July	Sandy loam	56	30	14
Bunker Hill Forest	BHF	15 July	Loam	51	39	10
Huntley Virgin Prairie	HV	5 August	Sandy loam	65	27	8
Huntley Agricultural Field	HA	5 August	Sandy loam	65	22	13

Table 2. Soil environmental characterization for each of the sites sampled (see Table 1 for site codes). Samples of surface soils (top = 0–1 cm depth) and slightly deeper soils (bottom = 5–7 cm depth) were taken from the same cores (nd = no data). Values are means ± standard deviations (n = 5).

Site	Depth	Temp. (°C)	Organics (%)	Moisture (%)	pH
JWV	top	31.2 ± 2.8	18.63 ± 0.57	10.98 ± 1.75	7.16 ± 0.20
	bottom	26.2 ± 2.4	17.36 ± 0.37	21.98 ± 1.54	6.96 ± 0.19
BHV	top	20.6 ± 1.2	7.72 ± 0.55	19.28 ± 2.00	6.64 ± 0.08
	bottom	19.7 ± 0.5	5.81 ± 0.39	17.17 ± 0.93	6.62 ± 0.11
BHR	top	26.6 ± 0.4	7.94 ± 0.80	6.34 ± 1.07	6.38 ± 0.24
	bottom	26.0 ± 0.3	5.27 ± 0.79	10.10 ± 1.03	6.24 ± 0.27
BHF	top	25.3 ± 0.3	8.21 ± 1.16	15.70 ± 1.88	6.63 ± 0.26
	bottom	24.5 ± 0.2	6.04 ± 0.36	13.87 ± 1.56	6.22 ± 0.32
HV	top	21.7 ± 0.3	9.52 ± 1.82	25.13 ± 3.95	7.98 ± 0.23
	bottom	21.4 ± 0.2	6.25 ± 1.39	17.36 ± 5.68	8.12 ± 0.20
HA	top	22.2 ± 0.2	3.78 ± 0.23	21.10 ± 1.01	5.88 ± 0.88
	bottom	22.0 ± 0.1	nd	26.32 ± 4.75	5.98 ± 0.99

tion patterns (community-level physiological profiles, or CLPPs) using the Biolog GN assay. This information will help us to determine whether prairie restorations improve soil microbial diversity and function and soil quality.

METHODS

Site Descriptions

Soils were sampled from three separate locations in northeastern Illinois, and in two of these cases, more than one ecosystem (site) was sampled in a given location. Table 1 lists the sites in the order that they were sampled in the summer of 1995, along with soil texture data. Table 2 details the general soil environmental conditions (temperature, moisture, organic matter and pH) at each of the soil sampling sites and depths.

The James Woodworth Prairie Preserve (also known as Peacock Prairie, after the original homesteader) is a 2.1 ha tallgrass prairie remnant which is now surrounded by urban commercial and residential development. The Preserve is owned and managed by the University of Illinois at Chicago (UIC) and is located northwest of Chicago on the edge of the city of Niles, Maine Township, Cook County, IL, on the east side of Milwaukee Avenue 0.8 km (0.5 mi.) north of the intersection with Golf Road. Numerous studies have been conducted in the preserve on the insect communities (Paintin 1929, Auerbach 1951, Park et al. 1953, Hamilton 1981) and plant communities (Betz and Cole 1969, Apfelbaum and Rouffa 1981, Apfelbaum and Rouffa 1982). The area sampled during this study (site JWV) is mesic "virgin" tallgrass prairie (Albert S. Rouffa, pers. comm.) with little or no soil disturbance. Over 200 plant species have been identified in this area, showing a high degree of native plant species richness (Apfelbaum and Rouffa 1982). The area sampled is just east of the path leading through the prairie from the Interpretation Center in approximately the center of the eastern half of the property. The soils here are typical Mollisols of black-soil prairie, containing a thick horizon of organic-rich, dark soil in the root zone and having a hue of 5YR, value of 2.5, and chroma of 1 for dry soil using the Munsell Soil Color Chart for comparison (Munsell Color 1994).

Bunker Hill Prairie (3.2 ha) and Edgebrook Flatwoods (31.6 ha) are adjacent areas which are listed in the Illinois Natural Areas Inventory, owned by the Forest Preserve District of Cook County, and managed together by the North Branch Prairie Project and the Volunteer Stewardship Network of The Nature Conservancy (North Branch Prairie Project 1990, 1994). Bunker Hill Prairie is on the northwestern edge of the city of Chicago, near Niles, IL, and about 6 km (3.75 miles) south and 5 km (3.1 miles) east of the James Woodworth Prairie Preserve. The prairie and forest areas sampled are located in a triangle bordered by Devon Avenue on the south, Caldwell Avenue on the northeast, and the North Branch of the Chicago River on the west. The prairie area is a wet to wet mesic prairie/oak opening that may have standing water in spring but is very dry by midsummer (North Branch Prairie Project 1990). Three different sites were sampled at Bunker Hill. One prairie site (BHV) is believed to be undisturbed in terms of the soil profile, so it is considered "virgin prairie" for the purposes of this study. It may have been grazed, and it was mowed as a play area in the past (stewards Jane and John Balaban, personal communication). An adjacent prairie site (BHR) has soils that were disturbed in the past by farming but is currently being managed as a prairie restoration. Restoration began at Bunker Hill (BHR) in 1983, including burning sections of the prairie each year from 1984 until 1994. The adjacent flatwoods site (BHF) is second growth forest and lies just east of the north branch

of a creek that drains into the river. As with most Cook County Forest Preserves, these woods are heavily foraged by deer. The topography at Bunker Hill is flat, with clayey, hardpan soil that is slowly permeable. None of the Bunker Hill soils were as dark in color as the soil from the James Woodworth Prairie. The forest soil (BHF) was the darkest in color (Munsell color 10YR, 3.5/1), with the virgin prairie (BHV) having a Munsell color of 5 to 7.5YR, 5/1 and the restoration soil (BHR) having a Munsell color of 10YR 4.5/2 for dry soil. The restoration soil seemed to be more compacted and was much harder to core than the other two sites.

The third location sampled is a railroad-associated prairie remnant (site HV) near Huntley in McHenry County, IL, approximately 72 km (45 miles) west-northwest of Chicago on the border between Grafton and Coral townships. The site is located on the west side of the main tracks of the Chicago and Northwestern R.R. (formerly the Huntley-Union-Marengo R.R.) between Huntley and Union, IL. This high-quality wet mesic virgin tallgrass prairie remnant (HV) is an Illinois Nature Preserve and is managed by the McHenry County Conservation District. A soybean field (site HA) west of the prairie, and separated from it by a set of siding tracks, was also sampled for comparison. The prairie remnant had rich, dark soil (Munsell color 10YR 4/1 for dry soil) while the adjacent farmland soil had a lighter, reddish color, was more cloddy and very clayey in feel. Because it had rained recently, the farm soil was so dense and sticky that it could not be sieved.

Soil Sampling

Soils at each location were sampled in the morning and immediately returned to the laboratory for analysis. Five randomly chosen replicate areas were sampled within each of the six sites (30 total soil samples), but areas in unrepresentative small topographic depressions or mounds were avoided. Samples were taken between plants, avoiding as many roots as possible. Soil was extracted from the ground using small corers (2.2 cm diameter, 11 cm long cork borers), with enough cores taken from each replicate area to fill a small, sterile Whirl-Pak (Nasco, Fort Atkinson, WI.) bag ¾ full (to the 4 oz fill line). The corers were marked at measured intervals, and the soil from a depth of 5–7 cm (the bottom of the core) was analyzed separately from the soil from a depth of 0–1 cm (the top of the core).

Samples were immediately placed into a cooler containing ice packs, but the soil bags were not allowed to come in direct contact with the ice in an attempt to keep the microorganisms cool but not frozen. When all five of the replicates had been taken from a given site, the soil samples were sieved though a wire mesh with square openings that were 2 mm on each side, and stones, insects and roots were removed. Five grams of sieved soil were removed for lipid extraction, and the remainder of the sample was placed in sterile plastic Whirl-Pak bags and returned to the laboratory in the cooler. All sampling and sieving gear was cleaned, but not sterilized, between samples. Sampling and sieving were carried out as quickly as possible in order to minimize changes in the soil microbial communities prior to analysis. In general, it took about one hour to take and sieve the five replicate samples from each site, and the samples arrived in the laboratory no more than four hours after the start of sampling.

Soil Analysis

As soon as the soil samples arrived in the laboratory, microbiological analyses were begun on subsamples of each replicate, and the remainder was stored at 4°C until further analysis. Within the week, moisture content was determined by drying weighed samples at 80°C (± 1°C) overnight to constant weight and reweighing (± 0.01 g). The dried samples were then ashed in a muffle oven at 350°C (± 5°C) overnight and reweighed to determine the percentage organic matter (ash-free dry weight; Brower et al. 1997).

Soil texture was determined by a modified hydrometer method (Cox 1990, Brower et al. 1997). Fifty grams of sieved, oven-dried soil were stirred together with 100 ml of 5% sodium hexametaphosphate for two hours. The suspension was then combined with 400 ml of distilled water in a Waring blender and mixed for two 1-minute cycles at low speed. The suspension was then rinsed into a 1000 ml graduate and brought up to 1000 ml with distilled water. The cylinder was covered, mixed gently by inverting 30 times, and placed on a flat surface. Hydrometer and temperature readings were taken at 40 sec, 60 sec, 2 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 or 8 h, and 24 h after mixing. A blank cylinder was made up with no soil to obtain temperature correction factors. For each time, the maximum particle diameter in solution was plotted against the % soil particles less than that diameter remaining in solution (Brower et al.1997), and from the smoothed curve, the percent sand (≥ 50 μm), silt (< 50 μm but > 2 μm) and clay (≤ 2 μm) were determined.

Microbial Biomass

Microbial biomass was determined as the amount of extractable phospholipid phosphate per gram of dry soil, which gives a good estimate of the total viable microbial biomass because phospholipids have been shown to break down rapidly after cell death (White et al. 1979). Total lipids were quantitatively extracted from sieved soil samples in the field immediately after sampling (Bligh and Dyer 1959, White et al. 1979). In the field, 5 grams of soil were mixed with 20 ml of methanol and 10 ml of chloroform in a glass jar having a Teflon-lined lid, and the jars were returned to the laboratory. Up to one week later, the soil extracts were quantitatively transferred to 50 ml Teflon Oak Ridge tubes and centrifuged at 3000 × g for five minutes. The supernatants were transferred to 60 ml separatory funnels, and the soil dry weight was determined from the dried pellet. To each separatory funnel was added an additional five ml of chloroform and 15 ml of distilled water, giving a ratio of water/methanol/chloroform of 0.9:1:1 (V/V/V). The funnels were shaken, the mixtures allowed to separate overnight, and the organic phase was collected from the bottom layer. The solvent was removed in a rotary evaporator at 35°C, the concentrated samples were resuspended in 2–4 ml of chloroform, transferred to glass test tubes having Teflon-lined caps, dried under a stream of nitrogen gas at 35°C, and stored at −20°C until analysis. Prior to analysis, the samples were resuspended in 5.00 ml of chloroform, and 500 μL aliquots were placed into 16 mm × 15 cm acid-washed test tubes (HCl : distilled water (1:1, vol/vol)). All of the chloroform was removed under a stream of nitrogen prior to the addition of 1.5 ml of 23% perchloric acid. In order to oxidize the lipids and leave only inorganic phosphate, samples were refluxed with the acid in a 180°C heating block at a 45° angle in a perchloric acid fume hood until colorless (about 2 hours) but were not allowed to become dry. After cooling, 2.4 ml of molybdate reagent (4.4 g ammonium molybdate, 14 ml concentrated H_2SO_4 per L distilled water) and 2.4 ml of Fiske & Subbarow reducer (Sigma) were added. After 10 min in a boiling water bath, the blue color was measured at the absorption maximum of 830 nm on a Spectronic 20. A standard curve (r^2 = 0.997) was constructed using known phosphate concentrations.

Asymbiotic nitrogen fixing bacteria

Within two to four days of sampling, soils were plated onto a nitrogen-free medium to get plate counts of nitrogen-fixing bacteria. One gram of sieved soil was shaken vigorously in 99 ml of sterile distilled water for two min, followed by serial dilution. Then 0.1 ml

aliquots of the 10^{-3}, 10^{-4}, and 10^{-5} dilutions were plated onto Winogradsky agar plates (Bergey's Manual 1984) containing 1% glucose, giving final dilutions of 10^{-4}, 10^{-5}, and 10^{-6}, respectively. Plates were incubated at 25°C for three days prior to counting.

Substrate Utilization Profiles

Immediately after the samples arrived in the laboratory, portions of each sample were prepared for inoculation into Biolog GN® microtiter plates (Biolog, Inc., Hayward, CA). These commercially-prepared plates consist of 95 wells, each containing a different sole carbon source, nutrients, and the redox indicator dye tetrazolium violet which detects respiration as the formation of NADH. The 96th well is a blank containing no carbon source. The range of substrates includes 28 carbohydrates, 24 carboxylic acids, 20 amino acids, 4 additional aromatic compounds, 5 polymers, 3 amides, 3 amines, 3 phosphorylated compounds, 2 esters, 2 alcohols and a brominated compound, all of which have been described previously (Garland and Mills 1991).

Ten grams (± 0.01 g) of sieved soil were added to 50 ml of sterile 54 mM phosphate buffer (pH 7.0) in a square dilution bottle and shaken vigorously for 30 sec. To promote settling of soil particles, 5 mg each of magnesium carbonate and calcium chloride were added, the bottles shaken for 15 sec, and then allowed to settle for 10 minutes. After settling, 20 ml of the suspension was pipetted into an empty sterile bottle. Using a Spec 20 spectrophotometer at 590 nm, the OD of the suspension was adjusted to 0.45 (± 0.01) using sterile phosphate buffer.

After all of the replicate samples were adjusted to the same OD, 150 µL was pipetted into each well of the microtiter plates using a multi-channel pipettor. Two replicate plates were inoculated for each of the five replicate soil samples (except for JWV, for which three replicate plates were inoculated for each soil sample). Incubation times were kept as short as possible in order to minimize the effects of growth and population shifts in the wells. After 24 and 48 hours of incubation at 27°C, plates were read by eye from below against fluorescent lights using a scale of 0–4 to record the density of blue dye formation in each well. A standard template for comparison with each plate was made using blue markers on clear plastic. A score of 0 indicated no visible blue color, 1 indicated detectable but very pale blue, and 4 indicated very dark blue to black. When totaling the number of substrates utilized by each sample, only readings greater than one were counted as "positive". A modified Shannon Diversity Index, H′, was calculated based upon the utilization of substrates by each sample as follows: $H' = -\Sigma\, p_i(\ln p_i)$, where p_i is the ratio of the intensity of utilization of a particular substrate (absorbance or relative intensity of dye formation) to the sum of the intensities of all of the 95 substrates (Zak et al. 1994).

Statistics

Data were entered and transformed in Microsoft Excel, and statistical analyses were conducted in Statistica '99 (StatSoft, Inc., Tulsa, OK). Bacterial colony counts were log transformed. Pairwise comparisons of soil organics, biomass and colony counts were made using Tukey's HSD test following an ANOVA, correlations were calculated using Pearson's r, and multi-way comparisons were made using the non-parametric Kruskal-Wallis test. A principal components analysis (PCA) with a biquartimax normalized rotation was used to select the Biolog substrates that most explained the variance between samples. Substrates selected in the PCA were entered into a stepwise discriminant analysis (DA) to test the hypothesis that microbial substrate utilization could allow one to discriminate between sites (ecosystems).

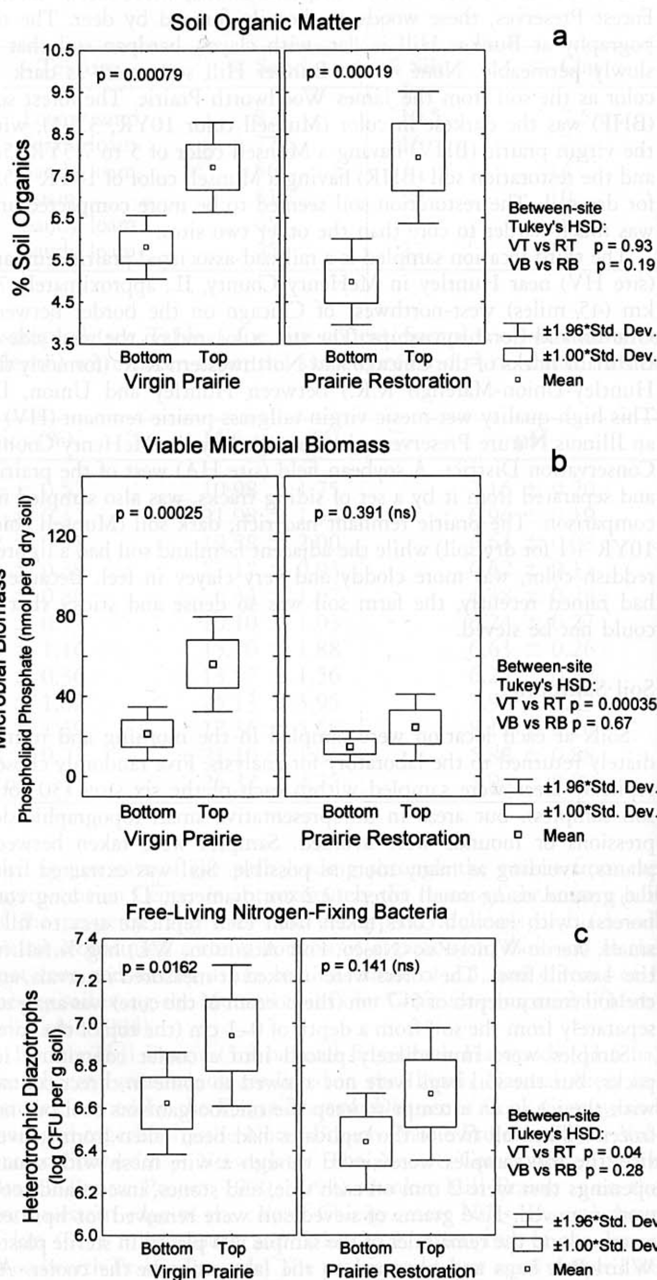

Fig. 1 Bunker Hill Prairie soil (a) organic matter, (b) viable microbial biomass, and (c) free-living nitrogen-fixing bacteria in a virgin prairie (site BHV) and an adjacent 12-year prairie restoration (site BHR). The "Bottom" of the core was 5–7 cm below the soil surface, and the "Top" of the core was 0 to 1 cm from the surface. Values shown are the means ± one and 1.96 Std. Dev. for five replicate samples at each site; P values are for post hoc tests (Tukey's HSD) following an ANOVA.

RESULTS

Organic Matter, Microbial Biomass and Nitrogen-fixers

There were significant differences between these ecosystems in the soil characteristics and in the soil microbial communities (Figs. 1 and 2). The virgin prairie soils at JWV contained more organic matter than any of the other sites (Table 2). The differences in or-

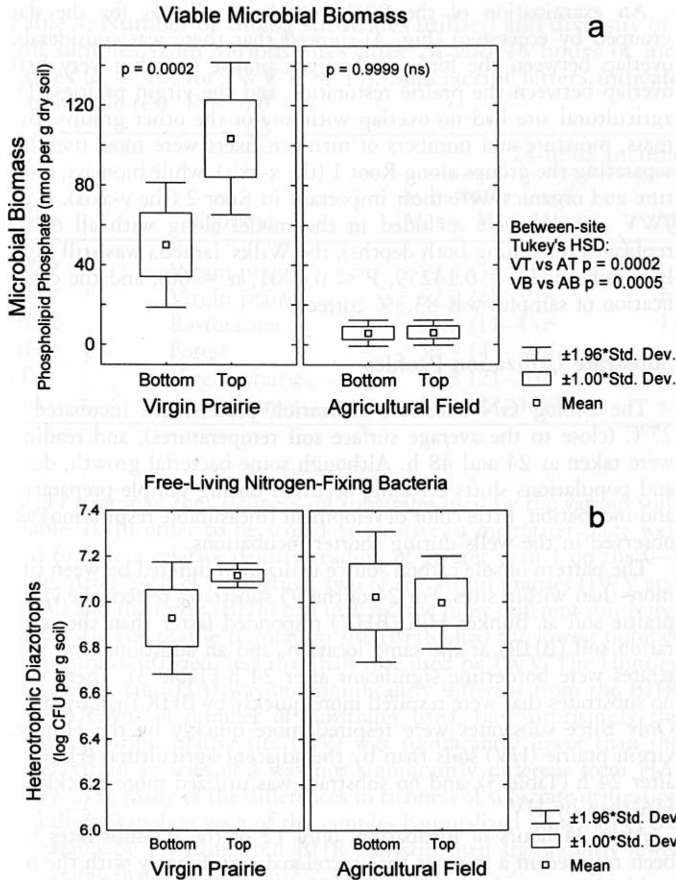

Fig. 2 Huntley railroad remnant prairie soil (a) viable microbial biomass, and (b) free-living nitrogen-fixing bacteria in a virgin prairie (site HV) and an adjacent soybean field (site HA). The "Bottom" of the core was 5–7 cm below the soil surface, and the "Top" of the core was 0 to 1 cm from the surface. Values shown are the means ± one and 1.96 Std. Dev. for five replicate samples at each site; p values are for post hoc tests (Tukey's HSD) following an ANOVA.

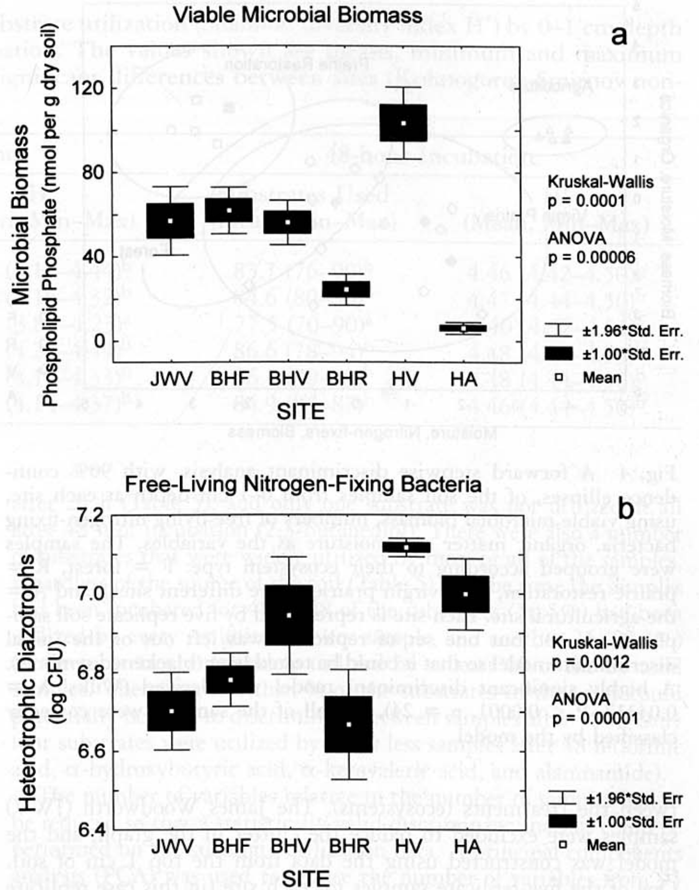

Fig. 3 Soil (a) viable microbial biomass and (b) numbers of free-living nitrogen-fixing bacteria in each of six sites sampled (see Tables 1 and 2 for site descriptions). Soil was sampled from a depth of 0–1 cm. Values shown are the means ± one Std. Error and one Std. Dev. for five replicate samples at each site; p values are for an ANOVA and a non-parametric ANOVA (Kruskal-Wallis test).

ganic matter were not significant between sites at the Bunker Hill location (Fig. 1a), but the Huntley virgin prairie (HV) had almost twice as much organic matter ($P < 0.01$, data from 1998) than the adjacent agricultural field (HA). In all sites except HA (no data available), the top 1 cm of soil contained significantly more organic matter ($P < 0.05$) than the soil sampled from between 5 and 7 cm.

Viable microbial biomass (phospholipid phosphate) was significantly different between sites at each location (BH and H; Figs. 1b, 2a) with the exception that the forest site (BHF) at Bunker Hill was not significantly different from the virgin prairie site (BHV; Fig. 3a). The differences in microbial biomass between the virgin (BHV) and restored (BHR) prairies at Bunker Hill were only significant in the top 1 cm of soil, but not at the 5–7 cm depth (Fig. 1b), possibly due to the greater tendency for the BHR soil to dry out at the surface (mean ± SD % moisture for BHR = 6.3 ± 1.1; BHV = 19.3 ± 2.0). However, the differences in microbial biomass between the two Huntley sites were highly significant in both depths sampled (Fig. 2a), with the agricultural site containing very little viable biomass compared to any of the other sites sampled (Fig. 3a). Although virgin prairie soil sampled at the James Woodworth Prairie (JWV) site had significantly more organic matter than any of the other sites regardless of depth (Table 2), it had about the same amount of microbial biomass as BHV and less than HV (Fig. 3a). So, although viable microbial biomass was significantly positively correlated with % organic matter overall (Pearson's $r = 0.529$, $P < 0.01$, $n = 50$), there were some exceptions to this rule. These differences might also be attributable to moisture because the Huntley site was sampled after a heavy rain and was wetter at the surface than the other sites, whereas the Bunker Hill and James Woodworth soils had roughly equivalent moisture contents when sampled (Table 2).

At the Bunker Hill location, the same pattern held for nitrogen-fixers as for total microbial biomass (Figs. 1b, 1c). In contrast, although the agricultural field (HA) had significantly less total microbial biomass than the virgin prairie (HV), the counts of nitrogen-fixers were not significantly different (Figs. 2a, 2b) and were among the highest of any of the sites sampled (Fig. 3b). Subsequent soil analyses performed in 1998 did not find significant differences in nitrate concentrations between these soils. It may be that a lack of organic nitrogen in the HA soil was selecting for an enrichment of nitrogen fixers relative to total microbial biomass, although only total soil organic matter (not organic nitrogen) was measured in this study.

When the biomass, nitrogen-fixer, organic matter and moisture data were entered as variables into a forward stepwise discriminant analysis, the model (Fig. 4) was able to accurately discriminate be-

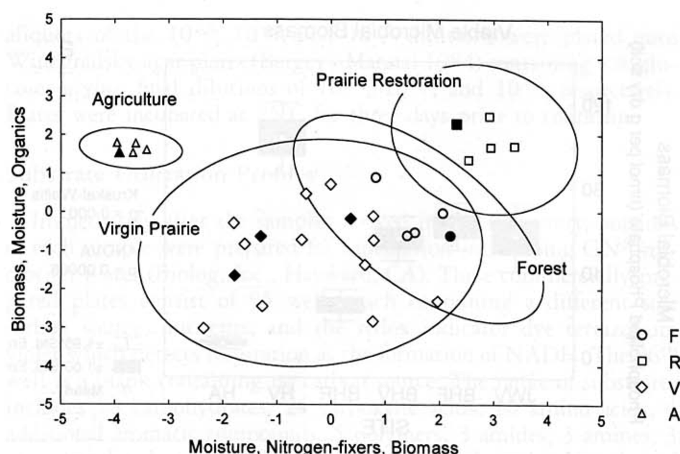

Fig. 4 A forward stepwise discriminant analysis, with 90% confidence ellipses, of the soil samples from 0–1 cm depth at each site, using viable microbial biomass, numbers of free-living nitrogen-fixing bacteria, organic matter and moisture as the variables. The samples were grouped according to their ecosystem type: F = forest, R = prairie restoration, V = virgin prairie (three different sites), and A = the agricultural site. Each site is represented by five replicate soil samples (n = 30), but one set of replicates was left out of the initial discriminant model so that it could be tested later (blackened symbols). A highly significant discriminant model was derived (Wilks' λ = 0.04317, $P < 0.0001$, n = 24), and all of the samples were correctly classified by the model.

tween the treatments (ecosystems). The James Woodworth (JWV) samples were excluded to reduce the clutter in the graph, and the model was constructed using the data from the top 1 cm of soil. One of the five replicate samples for each site (in this case replicate #3 for each site) was excluded to later test the model for classification accuracy. The Wilks' Lambda was highly significant (λ = 0.04317, $P < 0.0001$, n = 24), and only one of the 24 samples entered into the original model was misidentified (BHV5 was identified as a BHF forest sample), giving a total of 95.8% correct identifications. When the six excluded replicates were then added into the model to test its validity, they were classified with 100% accuracy. The significance of the results was not dependant upon which set of replicates was initially excluded.

An examination of the 90% confidence ellipses for the data grouped by ecosystem (Fig. 4) showed that there was considerable overlap between the forest and virgin prairie sites but very little overlap between the prairie restoration and the virgin prairies. The agricultural site had no overlap with any of the other groups. Biomass, moisture and numbers of nitrogen fixers were most useful in separating the groups along Root 1 (the x-axis), while biomass, moisture and organics were most important in Root 2 (the y-axis). If the JWV samples were included in the model along with all of the replicates (including both depths), the Wilks' lambda was still highly significant (λ = 0.14259, $P < 0.0001$, n = 60), and the classification of samples was 83.3% correct.

Substrate Utilization Profiles

The Biolog GN substrate-utilization plates were incubated at 27°C (close to the average surface soil temperatures), and readings were taken at 24 and 48 h. Although some bacterial growth, death and populations shifts certainly occurred during sample preparation and incubation, little color development (measurable respiration) was observed in the wells during shorter incubations.

The pattern of sole carbon source utilization differed between sites more than within sites. For 24 of the 95 substrates tested, the virgin prairie soil at Bunker Hill (BHV) responded faster than the restoration soil (BHR) at the same location, and an additional five substrates were borderline significant after 24 h (Table 3). There were no substrates that were respired more quickly by BHR than by BHV. Only three substrates were respired more quickly by the Huntley virgin prairie (HV) soils than by the adjacent agricultural (HA) soil after 24 h (Table 3), and no substrate was utilized more quickly by HA than by HV.

After 24 hours of incubation, only 12 of the 95 substrates had been respired in a manner that correlated significantly with the microbial biomass of the soil sample. The utilization rates of cellobiose and D-trehalose correlated best with the organic content of the samples (Pearson's r = 0.72, n = 25), with D-gluconic acid close behind (r = 0.70); respiration of 17 other substrates also correlated significantly with organic content. None of the substrates' utilization rates correlated negatively with biomass or organic matter, but the use of four substrates correlated negatively with the number of nitrogen fixers (xylitol, D-mannose, gentibiose and D-trehalose). Nine other substrates correlated positively with the number of nitrogen-fixers.

The total number of substrates utilized by each sample was tallied

Table 3. Substrates metabolized at significantly different rates after 24 hours of incubation in the Biolog assay by virgin prairie (BHV) soils and by prairie restoration (BHR) soils at the Bunker Hill site (0–1 cm depth). In each case, the BHV soil microbial communities metabolized the substrates at a significantly faster rate than did the BHR soil communities (P values are given below for a Kolmogorov-Smirnov two-sample nonparametric test). Substrates that were also metabolized faster by the Huntley virgin prairie (HV) communities than by the Huntley agricultural site (HA) communities are marked with **($P < 0.025$) or with *($P < 0.100$).

$P < 0.001$	$P < 0.005$	$P < 0.025$	$P < 0.100$
D, L-lactic acid	acetic acid	L-arabinose	γ-hydroxybutyric acid*
quininc acid	citric acid	tween 40	α-ketobutyric acid
L-histidine	D-saccharic acid	tween 80	succinic acid
hydroxy L-proline	sebacic acid	cis-aconitic acid	L-asparagine
L-serine*	p-hydroxyphenylacetic acid	α-ketoglutaric acid	putrescine
urocanic acid**	L-aspartic acid	D-galactonic acid lactone	
	γ-aminobutyric acid	succinamic acid**	
	inosine	L-pyroglutamic acid	
		thymidine	
		2-aminoethanol	

Table 4. Number of Biolog substrates utilized and diversity of substrate utilization (Shannon diversity index H′) by 0–1 cm depth soil samples from various sites after 24 and 48 hours of incubation. The values shown are means, minimum and maximum values (n = 10; for JWV n = 15). Superscript letters indicate significant differences between sites (Kolmogorov-Smirnov nonparametric test. $P < 0.05$).

		24-hour Incubation		48-hour Incubation	
Site	Ecotype	Substrates Used (Mean, Min–Max)	H′ (Mean, Min–Max)	Substrates Used (Mean, Min–Max)	H′ (Mean, Min–Max)
JWV	Virgin prairie	59.4 (50–71)[c]	4.26 (4.14–4.44)[b]	83.7 (76–90)[b]	4.46 (4.42–4.50)[b]
BHV	Virgin prairie	52.9 (47–59)[c]	4.24 (4.16–4.32)[b]	84.6 (80–90)[b]	4.47 (4.44–4.50)[b]
BHR	Restoration	26.6 (11–43)[a]	4.07 (3.80–4.23)[a]	77.5 (70–90)[a]	4.40 (4.32–4.51)[a]
BHF	Forest	50.9 (43–56)[c]	4.28 (4.20–4.44)[b]	86.6 (78–94)[b]	4.48 (4.41–4.53)[b]
HV	Virgin prairie	39.3 (21–50)[b]	4.25 (4.16–4.33)[b]	86.1 (79–89)[b]	4.48 (4.44–4.50)[b]
HA	Agriculture	37.4 (27–47)[ab]	4.21 (4.14–4.37)[b]	84.9 (83–87)[b]	4.46 (4.44–4.50)[b]

to get a sense of the "richness" of substrates used by the various soils (Table 4). In order to be scored as positive, a microtiter plate well had to have a relative density reading of greater than 1 on the 0–4 scale. After 24 h of incubation, two of the virgin prairies (JWV and BHV) and the forest (BHF) had utilized the most different substrates (Table 4). The prairie restoration site (BHR) had the lowest number of substrates utilized, less than half that used by JWV. The Huntley agriculture site (HA) was not significantly different from the BHR site in terms of number of substrates used, but surprisingly the Huntley virgin prairie site (HV) was significantly lower than the other virgin prairies and was not significantly different from HA. After 48 h, many of the differences in richness of substrate utilization had disappeared, as each of the samples had utilized nearly all of the substrates by this time, but BHR had still used significantly fewer substrates than any other site.

In order to get a sense of the relative "diversity" of substrates utilized, a modified Shannon Diversity Index, H′, was also calculated for each sample (Table 4). The diversity indices showed fewer differences between sites than richness, with only BHR being significantly ($P < 0.05$) lower than all of the other samples at both 24 and 48 h incubation periods (Table 4).

There were also some differences in which substrates were utilized (reading > 1 on the relative scale) by various samples according to ecosystem or location. The two Cook County virgin prairies, JWV and BHV, had several substrates in common which were not used as frequently by any of the other samples after 24 h: Tween 40, Tween 80, mono-methylsuccinate, acetic acid, propionic acid, sebacic acid, L-proline, thymidine and D-galactonic acid lactone (which was also used by BHF). Each of these sites had only one substrate unique to that site and not used by any other sites after 24 h (D-arabitol for JWV, and succinamic acid for BHV), indicating that these two sites had more metabolic potentials in common than they had differences. Sites JWV and HV (virgin prairies from different counties) had only two substrates in common after 24 h that were not used by the other samples: γ-hydroxybutyric acid and 2-amino ethanol. Also emphasizing the importance of location, there were several substrates that were utilized less frequently or not at all by the two McHenry County sites (HV and HA) but were used by soils from the other sites after 24 h (D-galactose, gentibiose, D-raffinose, L-rhamnose, glycerol, D,L-α-glycerol phosphate and glycogen) or not used at all after 48 h (acetic acid and formic acid). Only one substrate was utilized at a greater frequency by both the HV and HA sites than by the other sites (α-cyclodextrin was used by every Huntley replicate within 48 h).

As might be expected from rich, mixed-species communities, there were only nine substrates not utilized by any of the samples after 24 h (Table 5), and only one substrate was not utilized at all after 48 h of incubation (2,3-butanediol). There were also a number of substrates that were widely utilized by most or all of the samples, regardless of the source of the soil (Table 5). By the time the samples had been incubated for 48 h, 48 of the substrates (50.5%) had been utilized by every replicate of every sample, and another 21 (22.1%) of the substrates were widely utilized by at least 60 of the 65 total samples. Therefore, less than ⅓ of the substrates in the assay could potentially be used to discriminate between samples after 48 h. Only four substrates were utilized by 15 or less samples after 48 h (formic acid, α-hydroxybutyric acid, α-ketovaleric acid, and alaninamide).

The number of variables relative to the number of samples had to be reduced so that a statistically valid discriminant analysis could be performed on the substrate utilization data. A principal components analysis (PCA) was used to reduce the number of variables from 95 to 20 by selecting those substrates that best explained the differences between the samples in terms of the constructed factors (axes). Only the Bunker Hill samples were used in the PCA so as not to bias the discriminant analysis results. Only those substrates with significant factor loadings on the first two factors (axes) of the PCA were included in the discriminant analysis.

Of the 20 substrates entered into the forward stepwise discriminant analysis of the samples from the 24 h incubation of the soil from the top 1 cm depth, 13 were included in the discriminant model (Table 6). Nine of these substrates were ones that had shown significant differences between treatments (Table 3). The other four substrates included in the model either exhibited significance only in a multi-way comparison (D-mannitol and D,L-α-glycerol phosphate) or were metabolized significantly faster by the forest sample (BHF) than by all of the other sites (D-galactose and D-melibiose).

Replicate #3 from each site was initially excluded from the discriminant model for later testing. The discrimination was significant (Wilks' lambda = 0.02568, $P < 0.0001$, $n = 52$), and only one sample was misclassified (BHF 4b was classified as virgin prairie; Fig. 5). The factor structure included quinic acid, inosine, urocanic acid, D-saccharic acid, L-serine and hydroxy L-proline on Root 1 and D-galactose, quinic acid, cic-aconitic acid, D,L-α-glycerol-phosphate, D-melibiose and γ-hydroxybutyrate on Root 2. When the 13 replicates initially omitted were added as a test of the discriminant model, only two were misclassified (BHF 3b was classified as virgin prairie, and JWV 3c was classified as forest), giving an overall accuracy of 84.6%.

Samples from the 5–7 cm depth that were incubated for 24 h also provided a significant discrimination between treatments. Samples incubated for 48 h did not provide a clear separation between treatments, and the discrimination was not significant.

Table 5. After 24 hours of incubation, substrates not utilized by any soil sample replicates, substrates rarely utilized (no more than 3 positive samples), or widely utilized (at least 50 positive samples), regardless of the site sampled. For the six sites sampled (BHF, BHR, BHV, HV, HA, JWV), 10 replicate Biolog plates were incubated for each site (except JWV, which had 15 replicate plates); total n = 65. For these substrates, there were no significant between-site differences.

Not Utilized	Rarely Utilized	Widely Utilized
i-erythritol	α-cyclodextrin	N-acetyl-D-glucosamine
α-D-lactose lactulose	adonitol	D-fructose
xylitol	L-fucose	α-D-glucose
α-hydroxybutyric acid	formic acid	D-mannitol
glycyl-L-aspartic acid	D-glucosaminic acid	D-mannose
glycyl-L-glutamic acid	α-ketobutyric acid	β-methyl-D-glucoside
L-leucine	α-ketovaleric acid	D-sorbitol
L-threonine	alaninamide	sucrose
2,3-butanediol	L-ornithine	D-trehalose
	L-phenylalanine	D-galacturonic acid
	D, L-carnitine	D-gluconic acid
		D-glucuronic acid
		D, L-lactic acid
		glucose-1-phosphate
		glucose-6-phosphate

Table 6. Biolog substrates included in the discriminant analysis of the 24-hour incubation of soils sampled from the top 1 cm. A principal components analysis (PCA) was used to determine which substrates explained the most variance between samples. These 20 substrates were then entered into a stepwise discriminant analysis, which resulted in the inclusion of the 13 substrates listed below in the discriminant model.

Carboxylic Acids	Carbohydrates	Amino Acids	Aromatics	Phosphorylated Compound
quinic acid	D-galactose	L-serine	urocanic acid	D, L-α-glycerol phosphate
cis-aconitic acid	D-mannitol	hydroxy L-proline	inosine	
γ-hydroxybutyric acid	D-melibiose			
succinamic acid				
D-saccharic acid				

In a further test to determine whether the discrimination in the 24 h samples was specific to the ecosystem treatment, discriminant analyses were also performed using soil sample replicate number or Biolog plate replicate number as the grouping variables. As would be expected if the discrimination of the sites depended upon ecosystem treatment and was not random, the discriminant models using replicate number or plate number were not significant and could not correctly classify samples by these categories. When site location (Bunker Hill, James Woodworth or Huntley) was used as the grouping variable, the program could separate the samples by location, indicating that some of the differences between samples could be accounted for by landscape heterogeneity rather than management treatment.

DISCUSSION

It is clear from these results that both the ecosystem (or ecosystem management) and the location of the sites had significant effects on the soil microbial communities. Total viable microbial biomass appeared to be limited primarily by soil moisture and organic matter, both of which were related and declined as a result of soil disturbance in this study (sites BHR and HA, Table 2 and Fig. 3a). Bacteria, in particular, depend upon films of water in soil for their activity (substrate uptake and metabolism) and motility (Gregorich and Janzen 2000, Rice et al. 1998). Soil organic matter increases the water-holding capacity of the soil (Baldock and Nelson 2000), among other characteristics favorable to microbes (increased cation exchange capacity, buffering capacity, and sources of slow-release nutrients).

The biomass of the agricultural site (HA) may also have been depressed somewhat due to increased acidity (mean pH 5.9) because most soil bacteria have pH optima between 6.0 and 7.5 (Gregorich and Janzen 2000). Fungi easily withstand lower pH values, but they often decline in tilled soils due to physical disruption of their hyphae (Wardle 1995) or lack of moisture (Frey et al. 1999). Similar reductions in the overall microbial biomass of grassland soils due to agricultural activities have been reported in many countries (Patra et al. 1990, Zelles et al. 1995, Lavahun et al. 1996, Alvarez et al. 1998).

The variability in microbial biomass within sites was greatest in the undisturbed virgin prairie and forest soils. As would be expected, the more recently a site had been disturbed (plowed), the more uniform the distribution of microbial biomass was with both depth and area (Figs. 1b, 2a, 3a). Similar depth results have been found by others (Lavahun et al. 1996, Alvarez et al. 1998). Our results also agreed with those of others who have found that microbial C and N in tallgrass prairie soils are concentrated at the surface (Garcia and Rice 1994) where the concentration of organic matter is also highest (Ransom et al. 1998).

In general, the viable biomass results agreed with those previously obtained by direct counting of live bacterial cells in a tallgrass prairie soil in Missouri (Herman and Kucera 1976). Those authors found

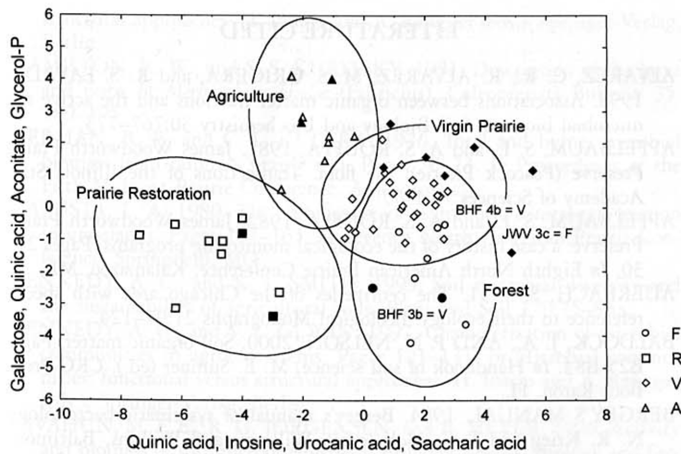

Fig. 5 A discriminant analysis, with 90% confidence ellipses, of the soil samples from 0 to 1 cm depth incubated in the Biolog assay for 24 hours. Twenty substrates chosen by PCA were entered into the forward stepwise analysis, which used 13 of those substrates in the discriminant model (Table 6). The samples were grouped according to their ecosystem type: F = forest, R = prairie restoration, V = virgin prairie (three different sites), and A = agricultural site. Each site is represented by five replicate soil samples, and two replicate Biolog plates were inoculated for each sample (except for JWV, which had three replicate plates; total n = 65). One set of replicates for each site was left out of the initial discriminant model for later testing (blackened symbols), and a highly significant model was derived (Wilks' lambda = 0.02568, $P < 0.0001$, n = 52). In the initial construction of the model, one sample was misclassified (BHF replicate 4b); when the replicates initially omitted were later entered into the model, two were misclassified (JWV 3c and BHF 3b), giving an overall accuracy of discrimination of 84.6%

1.6×10^8 live bacterial cells/g dry soil in a control area and 2.3×10^8 cells/g in an annually burned area. The virgin prairies sampled in the present study ranged from 3.8×10^8 to 1.2×10^9 live cells/g dry soil, if one uses a conversion factor of 1×10^7 cells per nmol phosphate from lipids as a rough estimate of cell numbers, or 7.6×10^{10} to 2.4×10^{11} if a conversion factor of 2×10^9 cells per nmol P is used (Dobbs and Findlay 1993). In contrast, the agricultural site had more than an order of magnitude fewer cells/g of soil. One might expect higher estimates of biomass from the phospholipid phosphate assay than from direct counting, because the lipid assay will include bacteria obscured by debris as well as live fungi, protozoa, algae and possibly microinvertebrates.

Surprisingly, in the agricultural site (HA) the asymbiotic nitrogen-fixing bacteria were significantly enriched relative to the total microbial biomass (Figs. 2b, 3b). Possible explanations include natural selection based upon nitrogen limitations at periods between fertilizer applications, possibly as a result of lower levels of stable soil organic nitrogen and a reduced ability to hold nutrients via cation exchange and adsorption due to the relative lack of organic matter in this soil (Baldock and Nelson 2000). One would think, however, that the lack of soil organics would also restrict the growth of heterotrophic diazotrophs, because it has been proposed that these organisms are most limited by the availability of a suitable carbon source (Kapusta 1980). It is possible that the presence of a legume crop (soybeans) in the field at the time of sampling may have increased the numbers of nitrogen fixing bacteria in the soil, because *Rhizobium* can survive saprophytically in the soil for extended periods (Graham 2000). However, a wide variety of colony morphologies were observed on the plates containing nitrogen-free media, suggesting that increased numbers of *Rhizobium* alone were unlikely to account for the increase in nitrogen fixers seen at this site. The presence of colonies on these plates does not reflect actual nitrogen-fixation activity in the soils, and we have no way of telling from these data whether or not the organisms were active *in situ*. Nitrogen-fixing bacteria generally perform best when nitrogen is limiting, the pH is near neutral (Brady 1974), and clays are present to protect them from oxygen and predators (Pankhurst 1997).

Substrate utilization profiles like those provided by the Biolog assay have been proposed as useful indicators of functional phenotypic diversity in microbial communities (Garland and Mills 1991, Griffiths et al. 1997, Palojärvi et al. 1997). In the current study, clear differences were seen between sites in the short-term (24 h) metabolic responses of the microbial communities to certain substrates (Tables 3, 4, Fig. 5). However, by the time the samples had been incubated for 48 h, most of them were metabolizing nearly all of the substrates, and we were unable to discriminate between sites based upon substrate utilization patterns.

At least half of the substrates utilized within 24 h by nearly all of the samples (≥ 50 out of 65 samples) were carbohydrates, particularly simple sugars, sugar phosphates, or disaccharides commonly found in plant litter or plant root exudates (Table 5). However, the substrates that were metabolized at significantly different rates within 24 h by virgin prairies (BHV, HV) compared with disturbed sites (BHR, HA) included (Table 3) 13 carboxylic acids, six polar amino acids, four aromatics, and an unusual sugar (L-arabinose), indicating that the differences between treatments may rest largely in the ability to respond quickly to a wider variety of molecular arrangements. The rapid response of the virgin prairie soils in particular in terms of the number of substrates utilized (Table 4) was not surprising, because microbial activity in prairies is often found to be much higher than in soils from other native ecosystems in the same climate (Rice et al. 1998). It may be that the wealth and diversity of organic matter and plant exudates in prairie soils stimulates the development of a population of microbes capable of utilizing a wide range of substrates. The long-term stability of these systems may allow them to develop species-rich "climax" microbial communities exhibiting the highest possible diversity and biomass under the given environmental conditions (Fliebbach and Mäder 1997), while the constant disturbance of cultivated sites may leave them in a constant state of early succession with lower species diversity.

It was somewhat surprising, however, that the agricultural soil (HA) exhibited slightly greater richness and diversity of substrate utilization than the site that had been under prairie restoration management for 12 years (BHR; Table 4), even though BHR had significantly more viable microbial biomass (Fig. 3a) and organic matter. The difference in initial substrate utilization rates may have been due in part to the lower soil moisture levels of the BHR site on the day that it was sampled, possibly leading to increased cell dormancy and lag times for the bacteria.

No other studies reported thus far have evaluated tallgrass prairie soils using the Biolog system. In a comparison of farming practices in Switzerland, the organically farmed soils had higher microbial biomass and utilized more carbohydrate substrates than the conventional and NPK-fertilized soils, but there were no significant differences in the use of the other Biolog substrates (Fliebbach and Mäder 1997). Unlike the present study, these investigators did not find correlations between the microbial parameters and the organic carbon or organic nitrogen content of the soils (Fliebbach and Mäder 1997). Another European study found that the Biolog assay could discriminate between agricultural soils from different countries that had been under different management practices for many years but could not discriminate between different short-term treatments like mulching or straw addition (Palojärvi et al. 1997), echoing our findings of

long-term, landscape-level differences in substrate utilization patterns.

Although they did not use the Biolog assay, Kennedy and Smith (1995) compared substrate utilization rates of pure cultures isolated from "natural prairie" and cultivated sites near Pullman in eastern Washington. They found that the prairie soil had greater microbial biomass carbon and enzyme activity rates for phosphatase, dehydrogenase, denitrification and nitrification but that both ecosystems utilized about the same range of substrates tested. However, they found that the cultivated soil microbes were often more resilient in their response to stressors such as heavy metals, antibiotics and osmotic pressure. Our results may differ because they reported fungi to be dominating the decomposition activity at the prairie site (Kennedy and Gewin 1997), while our inoculation and assay methods favored the growth and detection of bacteria.

Inoculation of Biolog plates with diluted soil suspensions always carries with it the possibility that some carbon sources and nutrients may be carried over from the soil to the inoculated wells (White et al. 1997), confounding the carbon substrate utilization results. However, because the HA sample had the lowest soil organic content and yet responded more quickly to certain substrates than BHR and almost as quickly as HV (Table 4), we doubt that this was a significant problem in this study. The Biolog assay conditions also typically stimulate the growth of bacteria within the microtiter plate wells whether or not the organisms utilize the carbon source being tested. This opens up the possibility of microbial selection and competition occurring within the wells over time (Winding and Hendriksen 1997), which is why we chose to use the shortest incubation times possible in this study. However, the microbial communities in the wells cannot be assumed to be identical to those *in situ,* considering the possible effects of sample processing and incubation. It is possible that the mixing, dilution and settling techniques used to equalize the OD of the soil solutions may have been biased toward certain cell shapes (cocci vs. bacilli, for example) or certain cell sizes (starved microcells vs. viable cells).

Overall, these results reinforce the notion that there are negative, long-term effects of conventional agriculture on soil and microbial community health. With the loss of soil organic matter, microbial biomass and some microbial functions, these highly disturbed soils may have lost a good deal of their overall function and resilience. Even though the agricultural site (HA) was sampled at a time that soil moisture should not have been limiting (after a recent rain), it still had significantly less viable microbial biomass than the somewhat dryer prairie restoration site (BHR). Further studies need to be done to examine seasonal trends and spatial variability in greater detail as well as to better determine the physical and chemical factors structuring the microbial communities in these soil ecosystems. It appears that prairie restoration projects may improve the microbial biomass, and ultimately the soil organic matter, of disturbed sites, but it may take many years or decades for these sites to reach the microbial biomass, activity and functional diversity levels found in virgin prairies.

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