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Eubacterial community structure and population size within the soil light fraction, rhizosphere, and heavy fraction of several agricultural systems

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Abstract

The hypothesis that soil light fraction and heavy fraction harbor distinct eubacterial communities and have differing numbers and sizes of bacterial cells was tested in three agronomic cropping systems. This hypothesis would imply that these soil fractions are distinct microbial habitats. Shoot residue and rhizosphere soil were also included in the analysis. Terminal restriction fragment length polymorphism (T-RFLP) of 16S ribosomal DNA was used to assay eubacterial community structure. T-RFLP profiles were affected by both soil fraction and cropping system, accounting for 35–50% of the variance in the profiles. T-RFLP profiles separated samples into two distinct eubacterial habitats: soil heavy fraction, which includes the mineral particles and associated humified organic matter, and soil light fraction/shoot residue and rhizosphere, which includes particulate soil organic matter. Differences were not based on organic C content of fractions alone; T-RFLP profiles were also differentiated by cropping system and by rhizosphere versus light fraction/shoot residue. Heavy fraction communities had the least amount of random variability in T-RFLP profiles, resulting in the clearest cropping system effects, while rhizosphere and shoot residue communities were the most variable. Profiles from organically managed corn soil were more variable than for either conventionally managed corn or alfalfa. The log number of bacterial cells per gram fraction was affected by soil fraction but not cropping system, being highest in the light fraction. The percentage of cells $>0.18\ \mu m^3$ was also greater in the light fraction than in other fractions. While bacterial cell density was generally correlated with C content of the soil fraction, heavy fraction did have a significantly greater number of cells per μg C than other soil fractions. The results show that habitat diversity in soil, related both to the amounts and types of organic matter, as well as other potential factors, are important in maintaining the high soil bacterial species diversity and evenness that is found in soil.

Keywords: Light fraction; Rhizosphere; Heavy fraction; Shoot residue; Soil microbes; Microbial community analysis; Terminal restriction fragment length polymorphism; Agricultural management

1. Introduction

Particles of soil organic matter isolated by flotation are known as light fraction (LF). These have been shown to be useful predictors of soil respiration and N mineralization (Janzen et al., 1992; Biederbeck et al., 1994; Hassink, 1995). The LF is sensitive to soil management (Cambardella and Elliott, 1993; Bremer et al., 1994) and has been proposed as an important component of soil quality (Gregorich et al., 1994; Yakovchenko et al., 1998). It is also important in the formation and stabilization of soil aggregates (Golchin et al., 1994a). Compared to other soil fractions, the LF is composed of more recently deposited organic matter particles (plant residues), with greater rates of turnover (Buyanovsky et al., 1994; Gregorich et al., 1995), higher carbohydrate contents, and higher C:N ratios (Christensen, 1996). The LF can be further divided into a more labile 'free' LF isolated from outside of soil aggregates, and LF 'occluded' within soil aggregates (Golchin et al., 1994b, 1995).

These characteristics of LF raise the question of whether LF constitutes a unique soil microbial habitat, which can be physically isolated for direct characterization. Such habitat diversity in soil could explain the high microbial species richness and evenness (or non-dominance by any species)

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detected by ribosomal sequencing (Borneman and Triplett, 1997; Nüsslein and Tiedje, 1998; Nakatsu et al., 2000) and DNA rehybridization rate studies (Torsvik et al., 1990; Sandaa et al., 1999). An analogous soil fraction that is well-recognized as a distinct microbial habitat is the rhizosphere, or the region of soil adjacent to and under direct influence of plant roots (Bolton et al., 1993). Root exudates and sloughed cells support both enhanced microbial growth (e.g. Söderberg and Bååth, 1998) and a microbial community different from that in the 'bulk soil' (e.g. Ringelberg et al., 1997).

The fundamental test of whether a soil fraction forms a distinct microbial habitat is to determine whether the composition of the microbial community residing within it is distinct from those within other soil fractions. This has not been performed for LF. Microbial biomass in the LF has been investigated, but reports of whether microbial biomass is more concentrated in LF than in heavy fraction (HF) are conflicting (Gregorich and Janzen, 1996). On the other hand, soil microsites containing decomposing plant residues, which may be similar to LF, have been recognized to harbor increased numbers of bacterial cells for a long time (Thom, 1935). Other studies have examined the effects of experimental additions of plant residue to soil microcosms, noting increases in dehydrogenase activity (Rønn et al., 1996; Gaillard et al., 1999), microbial biomass (Chotte et al., 1998), soil microfauna (Gaillard et al., 1999), and bacterial conjugation (Sengeløv et al., 2000).

We tested the hypothesis that the LF, HF, rhizosphere, and shoot residue $>$ 2 mm are distinct soil habitats containing differing eubacterial communities. Community analysis was conducted using terminal restriction fragment length polymorphism (T-RFLP) of the 16S ribosomal gene after PCR-amplification from eubacteria. Numbers of bacteria within soil fractions were assessed to examine the relationship between the enrichment of C and bacterial biomass in soil microhabitats. The responses of the communities to differing cropping systems was also examined to determine whether communities in different microhabitats respond differently to environmental conditions varying at a larger scale.

2. Materials and methods

2.1. Field sites and sample collection

Soils at the W.K. Kellogg Biological Station (KBS) in SW Michigan are Typic Hapludalfs and approximately 43% sand and 40% silt (Robertson et al., 1997). Conventionally managed continuous corn and organically managed corn were sampled at the Living Field Laboratory at KBS. The conventionally managed continuous corn receives synthetic fertilizer and pesticides on a regular basis. The organically managed corn is in a corn–corn–soybean–wheat rotation, with cover crops planted after corn and wheat. Organically managed corn receives compost (dairy manure and deciduous tree leaves) at the beginning of each growing season (Jones et al., 1998). Samples were also collected from alfalfa in the adjacent KBS LTER site. Alfalfa fields were established in 1989, and plants were killed every five years with herbicide and replanted to maintain stand vigor. This occurred in the spring of 1999, between sampling dates in this study. Alfalfa plants receive occasional lime amendments and pesticide application (http://lter.kbs.msu.edu/Agronomics).

Soil samples (0–10 cm depth) were collected in September of 1998 and August of 1999 from four replicate field plots of each treatment. Samples were from between corn plants in the Living Field Lab and nearby permanent sampling locations in the LTER alfalfa. In 1998, nine soil cores were pooled from each plot. In 1999, three 350 g soil blocks were excavated and pooled per plot. Samples were stored at 4 °C until fractionation was complete, which was within three weeks in 1998 and eight weeks in 1999.

2.2. Soil fractionation

Soil cores from 1998 were mixed by hand and a subsample was removed for moisture determination. Samples were forced through a 2 mm sieve by breaking aggregates along planes of weakness. Roots and shoot residue $>$ 2 mm were removed with forceps. Layers of soil $<$1 mm thick clinging to the roots and shoot residue were included with those fractions. Density separation was performed using a procedure modified from Golchin et al. (1994b) to isolate free or inter-aggregate LF. Preliminary experiments showed that isolation of LF using sodium polytungstate at a density of 1.7 g cm^{-3} damaged bacterial cells. Therefore further isolations of LF for microbiological assays were performed using water. However, to facilitate comparison of this study with past reports, LF was also isolated using sodium polytungstate in 1998. To distinguish between the fractions isolated using different densities, the density at which the fraction was isolated will follow its abbreviation; hence LF isolated using water will be designated LF-1. Bulk soil samples (with roots and shoot residue removed) weighing 50–75 g were placed in sterile centrifuge bottles. Sterile solution was added to a total volume of 180 ml. The bottles were inverted by hand 10 times and material clinging to sides of tubes and caps was washed into the suspension with sterile solution. The total volume within the bottle was brought to 200 ml, and particles were allowed to settle 30 min. Bottles were then centrifuged in a swinging-bucket centrifuge at 3000 rpm for 30 min. The liquid and floating particles were isolated by aspiration into a filtration flask. This suspension was rinsed on a 20 μm sieve and the LF-1 particles were transferred to filter paper and collected with forceps. All fractions (roots/rhizosphere, shoot residue, LF-1 and HF-1) were divided into three subsamples. These were: (1) frozen for DNA extraction 2) stored in

4.9% formaldehyde and (3) weighed before and after drying at 65 °C.

Samples collected in 1999 were fractionated in essentially the same way except the soil was divided into separate aggregate size-classes prior to density separation as part of another study (Blackwood et al. in preparation). Soil clods were broken down along planes of weakness until they fit through a 6.3 mm sieve. A nest of sieves with the soil sample on top was shaken by hand resulting in isolation of aggregates of sizes 4–6.3, 2–4, and 0–2 mm. Density separation was performed as described above after all macroaggregates had been forced through a 2 mm sieve. This resulted in a LF-1 and HF-1 for each aggregate size class. Isolation of roots/rhizosphere and shoot residue was performed during sieving steps.

2.3. Carbon and nitrogen analyses

Carbon and nitrogen contents of dried, ground 1998 samples were obtained using a Carlo-Erba NA1500 series 2 Nitrogen–Carbon–Sulfur Analyzer.

2.4. Direct microscopy

Samples preserved with formaldehyde were dispersed by blending in a Waring blender (HF and whole soil), or by vortexing a sample diluted with 5 ml of water for 5 min with approximately 1 ml of 1 mm diameter glass beads (LF, rhizosphere, shoot residue). Bacterial cell numbers were determined microscopically in samples from 1998 (Paul et al., 1999). Calculations of cell biovolume were based on formulas in Paul and Clark (1996). Cells were divided into size classes using the categories of Christensen et al. (1995): $>0.18\ \mu m^3$, large; $<0.065\ \mu m^3$, small (or dwarf).

2.5. T-RFLP analysis

T-RFLP was performed as described by Blackwood et al. (2003). Community DNA was extracted from 0.25 to 1 g samples using the Ultraclean Soil DNA extraction kit (Mo Bio Laboratories, Solana Beach, CA). The 16S ribosomal gene was amplified directly from community DNA using general eubacterial PCR primers: eubacterial primer 8-27F (AGAGTTTGATCCTGGCTCAG, *E. coli* numbering; Amann et al., 1995; Integrated DNA Technologies, Coralville, IA) and the universal primer 1392-1406R (ACGGGCGGTGTGTACA). Optimized PCR reactions were performed in triplicate for each sample using hexachlorofluoroscein (hex)-labeled 8-27F (Integrated DNA Technologies). PCR replicates were pooled and purified using the Promega PCR Preps Wizard Kit as directed by the supplier, except that elution was performed with 19 μl of sterile water heated to 55–65 °C.

Five microliters of purified PCR product was mixed with 5 μl of restriction enzyme master mix containing 1.5 U/μl of restriction enzyme and 1 μl of the accompanying reaction buffer (Gibco). Restriction reactions were incubated for 3 h at 37 °C, followed by 16 min at 65 °C to denature the restriction enzyme. Three microliters of the restricted PCR product was mixed with 1 μl of 2500 TAMRA size standard (Applied Biosystems Instruments, Foster City, CA). DNA fragments were separated by size by electrophoresis at 1800 V for 14 h on an ABI 373 automated DNA sequencer at Michigan State University's DNA Sequencing Facility. The 5′ terminal fragments (T-RFs) were visualized by excitation of the hex molecule attached to the forward primer. The gel image was captured and analyzed using Genescan Analysis Software 3.1. A peak height threshold of 50 fluorescence units was used in the initial analysis of the electropherogram. Negative controls (extraction blanks) were conducted with every PCR and run on several Genescan gels. Small peaks occasionally appeared in negative control lanes on Genescan gels, but the cumulative peak height was always below 1000 units. Samples were re-run if the cumulative peak height was below 9500 fluorescence units.

T-RFLP profiles for all samples were generated using the restriction enzyme *Rsa* I. Additional profiles were generated using the restriction enzyme *Msp* I in order to confirm results obtained with *Rsa* I, and to assist in the effort to assign tentative phylogenetic affiliations to T-RFs which differ between treatments. To reduce costs associated with these confirmatory analyses, a representative subset of the replicates was randomly chosen to be analyzed by *Msp* I (replicates 1–3 of 1998 rhizosphere, LF-1, and HF-1 samples; replicates 3 and 4 of LF-1 and HF-1 from 0–2 and 4–6.3 mm aggregates, and all rhizosphere samples, in 1999). T-RFs of sizes between 50 and 500 bp were aligned against a previously defined database with identities of samples concealed (Blackwood et al., 2003).

2.6. Statistical analysis

Most statistical analyses were performed using SAS Version 8 Stat and IML components, and Microsoft Excel. Redundancy analysis was performed using Canoco (Microcomputer Power, Ithaca NY). Percent C and N were analyzed using one-way analysis of variance (ANOVA) to test for a significant effect of cropping system. Total numbers of cells and percent of cells in the largest and smallest size classes were analyzed by two-way ANOVA with cropping system and soil fraction as fixed factors. Soil fractions were treated as split-plots by specifying field replicate as a random factor in Proc Mixed in SAS. The relationship between cell counts and C and N contents of samples was assessed by regression, and the significance of the increased fit by more complex ANOVA models compared to regression models was determined with a partial F-test. Cell counts were converted to a per square meter basis using bulk density calculated from the weight and volume of the sample cores collected in 1998.

The hypothesis that T-RFLP profiles from different soil fractions and cropping systems were significantly different was tested using redundancy analysis (Legendre and Anderson, 1999; Blackwood et al., 2003). This procedure finds that portion of the variability in community profiles that can be explained by experimental treatments. The significance of treatments can be tested by random permutation of the identities of the profiles. Separate analyses were based on Hellinger distance, which takes into account relative peak heights, and Jaccard distance (or one minus Jaccard's coefficient of community), which takes into account the presence of T-RFs only (Blackwood et al., 2003). Distributions of partial pseudo-F statistics were generated with 9999 random permutations. The significance of soil fraction was also tested by permutation of identities within field replicates in order to nest soil fraction within the cropping system experimental unit.

Tests performed using the 4–6.3 mm aggregate fractions from 1999 were randomly chosen to be presented since the results from different-sized aggregate fractions are essentially identical. In order to ensure that all treatments had the same number of replicates and to simplify permutation testing, one missing profile was replaced in the 1999 *Rsa* I and *Msp* I datasets by the mean vector generated from the other three replicates of the respective treatment. This procedure has no effect on the results of the hypothesis test since the mean and variance of the treatment involved is unaffected, and the pseudo-F statistic is being compared to a distribution generated by random permutation rather than a multivariate normal distribution (Legendre and Anderson, 1999). Relationships between profiles were examined using canonical principal components plots obtained from the redundancy analysis and Ward's method of hierarchical cluster analysis. Percent C and N were tested for significant effects on T-RFLP profiles using redundancy analysis.

T-RFs that were associated with particular treatments were identified by examination of the percent variability explained by the different factors in redundancy analysis. Indicator values (IndVals) were calculated for these T-RFs for each treatment, with and without interaction between treatments, from Hellinger-transformed data (Dufrêne and Legendre, 1997). IndVal is maximum when all occurrences of a T-RF are within the treatment being examined. T-RFs that were consistently affected by treatment in 1998 and 1999 were compared to sequences in the Ribosomal Database Project version 8.1 (http://rdp.cme.msu.edu) using the online TAP-TRFLP software (Marsh et al., 2000).

3. Results

3.1. Fraction mass and C and N content

Weights and percent C and N of soil fractions are shown in Table 1. C and N were more concentrated in roots/rhizosphere, shoot residue and LF than in HF. A somewhat lower percent C and N in LF-1.7 compared to LF-1 indicates

Table 1
Biogeochemical characteristics of soil fractions

Fraction	Cropping system	% of total soil weight	%C	%N	% of total soil C	% of total soil N	C:N
Roots/rhizosphere	Alfalfa	0.22 (0.06)	10.2 (1.4)	0.6 (0.06)	1.7 (0.4)	1.1 (0.3)	17 (1)
	Conv. corn	0.45 (0.12)	6.4 (1.2)	0.4 (0.06)	2.8 (0.7)	1.7 (0.5)	18 (1)
	Org. corn	0.42 (0.07)	5.6 (0.9)	0.3 (0.05)	1.7 (0.4)	1.2 (0.3)	16 (1)
	p	0.0078	0.0008	0.0002	0.0260	NS	NS
Shoot residue >2 mm	Alfalfa	0.13 (0.07)	21.7 (6.1)	1.2 (0.3)	2.1 (0.9)	1.3 (0.8)	18 (3)
	Conv. corn	0.37 (0.12)	14.4 (0.7)	0.7 (0.07)	5.2 (1.7)	2.6 (0.8)	22 (2)
	Org. corn	0.33 (0.04)	17.0 (2.2)	0.9 (0.1)	4.0 (1.0)	2.5 (0.5)	18 (3)
	p	0.0069	NS	0.0086	0.0217	NS	NS
LF-1	Alfalfa	0.05 (0.01)	27.3 (3.1)	1.6 (0.09)	1.0 (0.3)	0.6 (0.1)	18 (1)
	Conv. corn	0.13 (0.03)	21.5 (2.0)	1.2 (0.06)	2.7 (0.5)	1.7 (0.4)	18 (2)
	Org. corn	0.10 (0.01)	23.8 (0.9)	1.4 (0.1)	1.7 (0.2)	1.1 (0.2)	18 (1)
	p	0.0005	0.0145	0.0021	0.0003	0.0008	NS
HF-1	Alfalfa	99.6 (0.1)	1.2 (0.1)	0.1 (0.01)	95 (1.4)	97 (1.1)	10 (1)
	Conv. corn	99.0 (0.1)	0.9 (0.03)	0.09 (0.004)	89 (1.6)	94 (1.0)	11 (1)
	Org. corn	99.1 (0.06)	1.3 (0.1)	0.1 (0.004)	93 (0.9)	95 (0.1)	11 (1)
	p	< 0.0001	0.0003	0.0015	0.0007	0.0037	NS
LF-1.7	Alfalfa	0.40 (0.07)	21.4 (1.8)	1.3 (0.2)	7.1 (1.2)	3.4 (2.4)	18 (1)
	Conv. corn	0.69 (0.08)	17.6 (3.0)	1.1 (0.2)	11.3 (3.4)	5.9 (4.2)	18 (2)
	Org. corn	1.02 (0.2)	20.2 (1.7)	1.3 (0.2)	14.4 (2.4)	10.6 (3.0)	18 (1)
	p	0.0031	NS	NS	0.0142	0.0341	NS

Values in parentheses are standard deviations; $N = 4$. p-values indicate the significance of the effect of cropping system within the fraction in a one-way ANOVA. NS implies cropping system not significant at $p = 0.05$ level. LF-1.7 includes LF-1 and additional material.

that part of the additional material of LF-1.7 is more decomposed. LF-1 weighed 10–20% as much as LF-1.7. In general, cropping system had a significant impact on weights and C and N contents of soil fractions (Table 1). Proportion of total soil weight in the different soil fractions was similar for 1999 samples (data not shown).

3.2. Microscopic bacterial cell counts

Soil fraction significantly affected log bacterial cells/g fraction ($p < 0.0001$), while cropping system did not ($p > 0.1$). A log transformation was used in statistical testing where necessary to reduce the effects of an increase in variance with increasing mean value of treatments. Residual (split-plot) error was not different from whole-plot error. The lowest number of bacterial cells/g fraction was present in HF-1, while LF-1 had the highest (Fig. 1). The population size in whole soil samples was not significantly different from HF-1 samples, indicating that fractionation did not result in loss of bacteria. LF-1 contained 0.5–1.2% of bacterial cells detected in soil, which is similar to the proportion of the total amount of C in the LF-1 (1.2–1.6%). HF-1 contained 94–98% of all bacteria detected.

A significant linear relationship was found between the log number of cells/g fraction and percent C of the fraction ($R^2 = 0.72$), although percent C did not explain log number of cells/g as well as soil fraction categories (tested by partial F-test, $F = 23.6$, d.o.f. $= 2,44$, $p = 1.1 \times 10^{-7}$). Results were similar to those described regardless of whether cell counts or bacterial biomass per gram of fraction was analyzed. The majority of bacterial C per square meter was contained in HF-1 since it constituted most of the soil volume (Fig. 2). Shoot residue >2 mm and rhizosphere contained equal amounts of bacterial biomass per square meter, with LF-1 containing slightly less (Fig. 2).

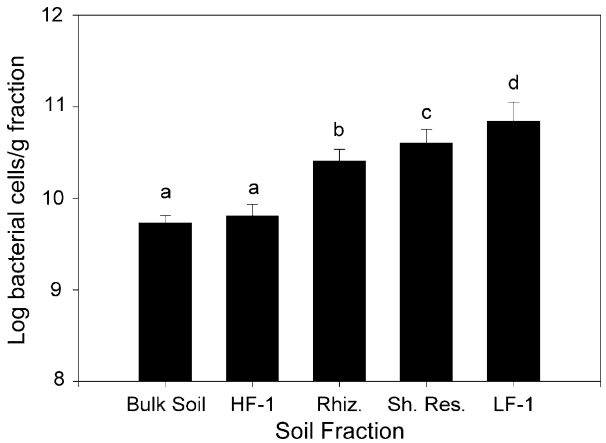

Fig. 1. Number of DTAF-stained cells counted per gram of soil fraction. Bars with different letters are significantly different via the Bonferroni-corrected least significant difference test ($p < 0.005$). $N = 12$ per soil fraction, error bars represent standard error.

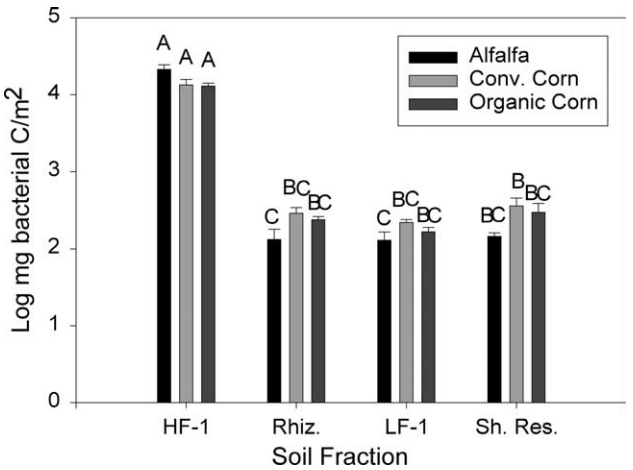

Fig. 2. Log mg bacterial C per square meter (10 cm depth). Soil fractions with different letters have significantly different means via the Bonferroni-corrected least significant difference test ($p < 0.0008$). $N = 4$ per soil fraction-cropping system combination, error bars represent standard error.

The number of cells/μg fraction-C was significantly affected by fraction ($p < 0.0001$) and fraction-cropping system interaction ($p = 0.0246$) in two-way ANOVA. The number of cells/μg fraction-C was significantly greater in HF-1 than in other fractions (Fig. 3). Results were similar when μg bacterial C/μg fraction-C was analyzed (data not shown).

There was a significantly greater percentage of large cells in the LF-1 and shoot residue than in the rhizosphere or HF-1 (Fig. 4). Whole-plot error was lower than residual error for the percentage of cells in the largest size class (>0.18 μm^3), so analysis was performed with whole-plot (or field treatment replicate) as a random factor in Proc Mixed in SAS. The percentage of large cells also increased with C content of fraction (data not shown). The percentage of cells in the smallest size class (< 0.065 μm^3) followed

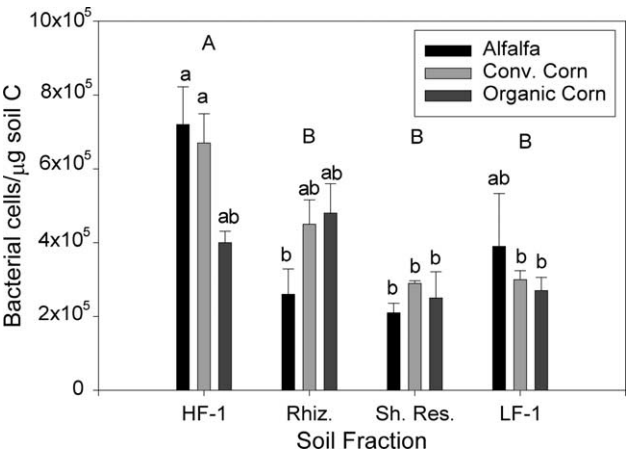

Fig. 3. Number of DTAF-stained cells counted per μg fraction C. Soil fractions with different capital letters have significantly different means via the Bonferroni-corrected least significant difference test ($p < 0.008$), soil fraction-cropping system bars with different uncapitalized letters are also significantly different ($p < 0.0008$). $N = 4$ per soil fraction-cropping system combination, error bars represent standard error.

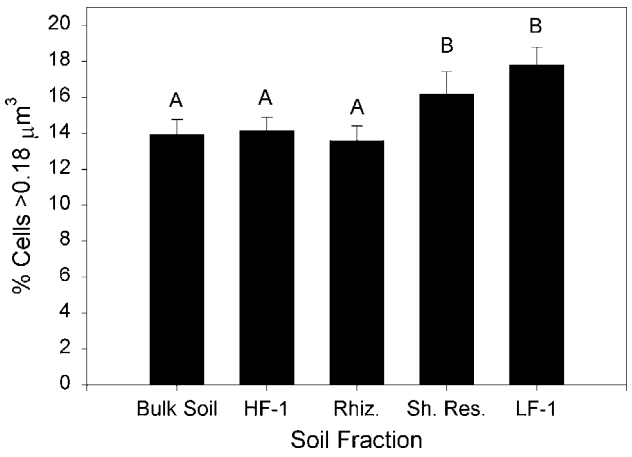

Fig. 4. Percentage of DTAF-stained cells with biovolume $> 0.18\ \mu m^3$. Bars with different letters are significantly different via the Bonferroni-corrected least significant difference test ($p < 0.005$). $N = 12$ per soil fraction, error bars represent standard error.

the inverse of the trends in the largest size class (data not shown). While cells in the smallest size class constituted the majority of cells in all samples (50–70%), they made up only 10–15% of the soil bacterial biomass.

3.3. Analysis of treatment effects on T-RFLP profiles

There were 120 T-RFs detected in the 48 profiles generated by the restriction enzyme *Rsa* I in 1998, and 104 in the 95 profiles from 1999. There were 124 *Msp* I T-RFs detected in the 27 profiles in 1998, and 114 in the 35 profiles from 1999. Approximately 25 to 50 T-RFs were detected in each profile. There were significant effects of cropping system and soil fraction on both *Rsa* I and *Msp* I T-RFLP profiles in both 1998 and 1999 (Table 2). Interaction effects were also significant, indicating that the effects of soil fraction were not uniform for different cropping systems, and/or the effects of cropping systems were not uniform for different soil fractions. Treatment effects accounted for 40 to 55% of the variance in Hellinger-transformed profiles, and 35–38% of the variance in Jaccard distances between profiles (Table 2). Limiting permutation of profiles to within field replicates when testing soil fraction had no effect on the *p*-value. Percent C and N, and C to N ratio of samples could also account for a significant amount of variation in the Hellinger-transformed T-RFLP profiles (15.5%, $p = 0.0001$, data not shown).

Table 2
Proportion of total variance in T-RFLP profiles accounted for by experimental factors according to redundancy analysis

	Soil fraction[a]	Cropping system[b]	Interaction[c]	Sum
Hellinger distance				
1998 *Rsa* I	16.6*	16.2*	14.8*	47.6*
1998 *Msp* I	22.3*	18.5*	14.0**	54.8*
1999 *Rsa* I	16.4*	15.6*	14.2*	46.2*
1999 *Msp* I	17.2*	10.3*	13.7*	41.2*
Jaccard distance				
1998 *Rsa* I	10.9*	11.6*	15.3*	37.8*
1998 *Msp* I	16.8*	14.6*	15.2**	36.6*
1999 *Rsa* I	10.5*	11.5*	13.6*	35.6*
1999 *Msp* I	12.5*	9.5*	13.9*	35.9*

$N = 4$ except 1998 *Msp* I where $N = 3$. *$p = 0.0001$, generated by 9999 random permutations of data. **$p < 0.01$, generated by 9999 random permutations of data.

[a] Soil fractions compared included HF-1, LF-1, shoot residue, and rhizosphere.

[b] Cropping systems compared included continuous alfalfa, continuous conventional corn, and organic corn in rotation.

[c] Interaction refers to soil fraction *X* cropping systems interaction terms.

Fig. 5 shows partial canonical principal components plots derived from the redundancy analysis of 1998 *Rsa* I profiles, which are similar to those derived from 1999 and *Msp* I profiles. These plots are useful because the effects of each experimental factor can be displayed one at a time, after effects of other experimental factors have been

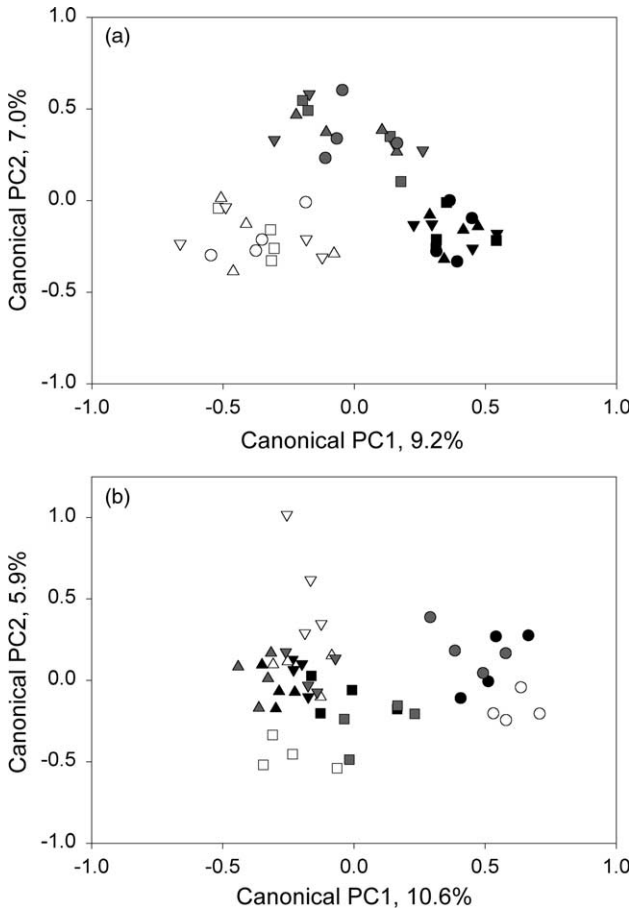

Fig. 5. Partial canonical principal components plots derived from redundancy analysis of 1998 *Rsa* I T-RFLP profiles. Percentages on axes indicate proportion of the variance explained by each axis. See Table 2 for total variance explained and significance of treatments and Results for interpretation of plots. (○) HF-1, (△) LF-1, (▽) shoot residue, (□) rhizosphere; white: alfalfa, black: conventional corn, grey: organic corn. (A) Cropping system axes (2 of 2) with effects of soil fraction and interactions partialled out. (B) Two of the nine soil fraction and interaction axes with cropping systems effects partialled out.

'partialled out' (e.g. separation of samples will be due to cropping system only in the cropping system plot). This makes them an efficient way to compare treatments within one experimental factor (e.g. did one treatment within the cropping system factor diverge from the other two, or were all three different?). However, with multiple experimental factors this may also make the percent of the total variability explained by individual axes seem low. The variability explained within the entire experiment, and its significance, is shown in Table 2. Fig. 5a shows the effects of cropping system, with soil fraction and interaction effects partialled out. The samples are clearly separated by cropping system. One hundred percent of the variability that can be explained by cropping system in this dataset is shown in this plot since only two canonical principal components can be derived from an experimental factor with three treatments. The inclusion of interaction effects in the cropping systems plot had little effect on the arrangement of samples.

The effects of soil fraction and interactions between soil fraction and cropping system are shown in Fig. 5b, with cropping system effects partialled out. The first two canonical axes shown in Fig. 5b account for 16.5% of the total variance in the Hellinger-transformed profiles, or approximately half of the variance accounted for by soil fraction and soil fraction-cropping system interactions. The remaining effects of these treatments were captured in the remaining seven higher-order canonical principal components, which are not shown because of the relatively small amount of variability associated with each. The major effect of soil fraction was a division between HF-1 and other fractions, although some corn rhizosphere samples fell between these two major groups. Rhizosphere and LF-1/shoot residue samples were also separated from each other, although not as completely as they were from HF-1. The primary effect of the interaction terms was the separation of the alfalfa samples from corn samples (organic or conventional) within each soil fraction group (Fig. 5b).

Relationships between T-RFLP profiles were also examined through cluster analysis using Ward's method on both Hellinger and Jaccard distances. Conclusions from examination of clustering dendrograms were similar to those described above. It was observed that clustering of samples by cropping system occurred for rhizosphere in 3 of 8 dendrograms, while it occurred in seven dendrograms for LF-1/shoot residue and in six for HF-1. This led to the hypothesis that soil fractions had differences in the variability of T-RFLP profiles. To test this hypothesis, Hellinger variance (or 1/2 the mean Hellinger distance between samples) was calculated for each treatment. The different cropping systems were used as replicates, resulting in three replicate values for mean Hellinger variance for each soil fraction. One-way ANOVA was used to test the null hypothesis of no differences in mean variance of T-RFLP profiles between soil fractions. This analysis was performed separately for *Rsa* I and *Msp* I in 1998 and 1999. Differences in variability were found to be significant for *Rsa* I in 1999 ($p = 0.02$) but not in the other analyses ($p > 0.05$). In 1999 the mean Hellinger variance of profiles followed the trend HF-1 < LF-1 < Rhizosphere = Shoot Residue, and in 1998 the trend was HF-1 = LF-1 < Rhizosphere < Shoot Residue.

Table 3
Number of T-RFs that were associated with particular soil fractions or cropping systems

Experimental factor	*Rsa* I			*Msp* I		
	1998	1999	Common	1998	1999	Common
Soil fraction	20	13	6	22	27	3
Cropping system	16	18	6	35	11	4
Interaction	24	23	0	28	27	7

'Common' refers to the number of T-RFs that were associated with the same treatments in 1998 and 1999.

3.4. Analysis of individual T-RFs

T-RFs were considered to be affected by an experimental treatment if greater than 20% of the variability in Hellinger-transformed peak heights was explained by a factor (i.e. soil fraction, cropping system, or interaction effects) after accounting for variability due to other factors in redundancy analysis. Eleven to 35 T-RFs were affected by each factor each year (Table 3). Twenty-six of these T-RFs were found to be associated with the same treatments in 1998 and 1999, as indicated by IndVal scores (Table 3). Each of these T-RFs corresponded with multiple sequences in the RDP. Sequences associated with these *Rsa* I and *Msp* I fragments were compared to identify a sequence which could have generated a fragment of each type, but no sequence was found that would produce both an *Rsa* I and an *Msp* I T-RF associated with the same soil fraction or cropping system.

One *Msp* I fragment of size 405–408 bp that was found to be associated predominantly with roots/rhizosphere is close in size to the *Msp* I fragment that would be generated from the garden pea chloroplast sequence in the database (404 bp). The 405–408 bp *Msp* I fragment detected in the rhizosphere may be due to the presence of plant DNA in the genomic DNA extract from those samples. The overall importance of any particular T-RF to the statistical tests and community ordination described above is small, however, due to the large number of fragments that were found to be important in separating different fractions (>20 *Msp* I fragments in each year). *Rsa* I fragments generated from the garden pea chloroplast sequences are outside the range analyzed (i.e. >500 bp).

4. Discussion

Results of this study show that LF and HF form distinct microbial habitats in soil, which are also different from

the rhizosphere. The LF-1 and rhizosphere communities were more similar to each other than either was to the HF-1 community. This is not surprising: LF and rhizosphere are both characterized by high amounts of labile C, which supports increased bacterial growth rates and predator populations. LF and rhizosphere differ in that the rhizosphere supports symbiotic associations between roots and other organisms (e.g. mycorrhizal fungi, root pathogens). Also, the availability of complex carbohydrates contained within plant cell walls is probably much greater in LF, presenting different catabolic enzyme requirements to the microbial community. LF-1, shoot residue, and the rhizosphere had C to N ratios of approximately 18, lower than that found in plant tissue (typically 60–80), and higher than that in bulk soil or HF-1 (10-13). For LF-1 the intermediate C to N ratio is evidence of the microbial biomass present and the partially decomposed state of the organic particles, since mineral soil particles are washed away in its isolation. For the rhizosphere the increased C to N ratio represents the balance between the C to N ratios of the soil particles clinging to the roots and of the roots themselves, which were included in rhizosphere in this study.

The LF-1 and shoot residue >2 mm can be considered a single homogeneous microbial habitat since they had statistically indistinguishable C and N contents, community compositions, and bacterial cell numbers and size distributions. Shoot residue and LF-1 differ primarily by particle size and artifacts associated with their isolation. Artifacts include time of isolation and the washing away of mineral soil particles clinging to the organic particles of LF-1 but not shoot residue. The LF-1/shoot residue effect cannot be attributed simply to the steps involved in fractionation since the communities of these fractions are so similar. In the systems we studied, LF-1 and shoot residue >2 mm together contained approximately twice as much C, N, and bacterial biomass as did the rhizosphere, per unit of fraction or per unit of whole soil. The eubacterial community of LF-1/shoot residue was significantly different from either rhizosphere or HF-1, and cells were slightly larger. LF-1.7 made up approximately 1% of soil weight and 10% of soil C. LF-1.7 and shoot residue > 2 mm together represent a pool of active organic matter greater than 5 times as large as the rhizosphere (including root mass). If we assume that the microbiology of LF-1.7 is similar to that of LF-1, then it is apparent that this fraction may be the true seat of activity for soil microorganisms dependent on freshly deposited labile organic matter.

The majority of soil microorganisms and C and N reside in the HF, which is dominated by soil mineral particles. This can be considered the habitat matrix within which patches of LF/shoot residue and rhizosphere are deposited. Organic matter within this fraction is humified and recalcitrant, but a unique HF community exhibited both lower random variability and greater sensitivity to agronomic management. Therefore selection for species well adapted to their environment may be greatest in this habitat. This was the opposite of the expected trend, since the rhizosphere is frequently cited as exerting a selective pressure on microbial communities. The temporal scale of the cropping system treatments may be important in determining the stability of changes within fractions. In this study the treatments had been in place 6–10 years, whereas studies finding larger effects of plant species on the rhizosphere compared to bulk soil are often in place one year (e.g. Latour et al., 1996; Maloney et al., 1997). The rhizosphere community is different from other habitats, but may be assembled more randomly, perhaps due to proliferation of randomly encountered dormant bacteria in HF-1 that have the life history traits enabling them to respond to high amounts of labile C. A similar situation may be true for shoot residue >2 mm, while more specialized communities may have developed on LF-1 since this habitat is older than shoot residue and more complex substrate is left.

Cropping system had a large effect on T-RFLP profiles. The cropping systems examined differed in many ways, including amounts and types of plant tissue, rhizodeposition, synthetic fertilizers, compost, pesticides, and cultivation. The alfalfa produced the least amount of plant biomass, while organic first-year corn produced more plant biomass than continuous conventional corn over the course of the growing season, but not averaged over the entire organic crop rotation (which included a year of soybeans and a year of wheat). These differences between systems led to changes in T-RFLP profiles that were consistent across all soil habitats (i.e. LF, HF, rhizosphere), as well as changes in profiles unique to certain soil habitat-cropping system combinations. The increased variability of the organically managed corn system was expected since it involved three crop species and cover crops, and also received composted manure and leaves, which has been shown to contribute to soil particulate organic matter (Wilson, 2001). These findings support previous reports of an effect of cropping system or other agricultural soil management on microbial community structure at other sites (Zelles et al., 1995; Bossio et al., 1998; Lukow et al., 2000). However, in a study performed on other treatments at the KBS LTER site, Buckley and Schmidt (2001) used 16S ribosomal RNA oligonucleotide probing and found no differences in the proportion of bacteria in major phylogenetic groups due to different cropping systems. Also, no changes in nitrifier community composition were detected due to agronomic treatments at the KBS LTER using DGGE of the nitrifier 16S ribosomal genes (Phillips et al., 2000). Reasons for the different conclusions of this study may be the focus on different cropping systems compared to previous studies, or use of different techniques.

The oligonucleotide probes used by Buckley and Schmidt (2001) each targeted a very broad group of organisms, lumping the dynamics of many potentially

independent populations together. Phillips et al. (2000) focused on nitrifiers, which are known to have small populations and little diversity, and their results cannot be extrapolated to other groups. The T-RFLP method used here was applied broadly to most eubacteria in the soil due to the PCR primers used. The phylogenetic resolution of the method was intermediate, providing a finer division of groups than obtained by probing, but not limited to a small number of organisms.

Attempts to identify the T-RFs that were consistently important in defining differences between treatments were limited by our use of very broad PCR primers. The number of sequences that can produce any individual T-RF is potentially large when using domain-specific eubacterial primers. We attempted to work around this problem by performing digests with multiple restriction enzymes, but no sequences could be found in the database that would match similarly acting T-RFs from the different digests. This could be due to the importance of organisms with no sequences currently represented in the database (i.e. unculturable organisms). It may be necessary to use more narrow primers and/or more laborious methods such as DNA sequencing if the goal is to obtain phylogenetic information about very complex communities, as has also been pointed out by Dunbar et al. (2001).

The size of the bacterial populations present in LF and rhizosphere in this study may seem low; however they are very close to the proportion of the total amount of C found in the fractions. This proportional relationship between bacterial cells and organic C is similar to what was found by Kanazawa and Filip (1986). The relationship was further generalized in this study because it was found that percent C could explain the majority of the variation in cell numbers across cropping systems and fractions. However, the partial F-test results indicate that effects of other differences between soil fractions on populations were also important. One such factor may be the accumulation of less active cells in HF-1. The lower activity of HF-1 cells is indicated by the larger percentage of cells with biovolume $<0.065\ \mu m^3$ in this fraction (Christensen et al., 1995; Baath, 1994). This resulted in an increased ratio of cells to organic C in HF-1. The ratio of bacterial C to total organic C was also increased in HF-1 because the change in cell size was not large enough to offset the increased cell density per unit C in this fraction. The relationship between C and cell numbers contrasts with the results of Ahmed and Oades (1984), who found that 0.2–0.4% of the total soil ATP and 11–12% of the total C was contained in the LF. This difference may be due to the use of $ZnBr_2$ at a density of $1.6\ g\ cm^{-3}$ for density separation and more vigorous soil dispersion by Ahmed and Oades (1984). Our preliminary experiments with the fluorescent redox indicator 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) indicated that sodium polytungstate solution with density of $1.7\ g\ cm^{-3}$ inactivated cells, probably due to osmotic pressure; we therefore recommend the use of water for isolation of LF for microbiological work until a more complete analysis of the effects of dense liquids on microorganisms is carried out. Increased growth rate of bacteria in LF and decomposing shoot residue is supported by the finding in this study that there was an increase in the percentage of cells with biovolume $>0.18\ \mu m^3$ in those fractions (Baath, 1994; Christensen et al., 1995), which was also explained in part by increased C concentration.

The hypothesis that habitat diversity enhances soil microbial diversity is supported by the detection of unique communities in different soil fractions. Future studies should examine the individual mechanisms by which habitat diversity can enhance microbial diversity, as well as possible changes in the variability of communities associated with different soil fractions or cropping systems. It is necessary to learn how microbial communities organize themselves to be able to manage them to the degree that has been called for (e.g. Smith and Paul, 1990; Beare, 1997; Kennedy, 1999). If communities are organized into separate habitats, these should be taken into consideration when looking for management effects, because effects may be present in some habitats and not others. In this study HF-1 made up over 90% of whole soil samples, and the whole-soil community composition was indistinguishable from HF-1 despite containing several significantly different communities in other habitats. Different habitats may also represent opportunities for management, such as refuges from competition or locations of enhanced nutrient availability to be exploited by beneficial organisms.

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