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Eubacterial communities in different soil macroaggregate environments and cropping systems

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Abstract

Different positions within soil macroaggregates, and macroaggregates of different sizes, have different chemical and physical properties which could affect microbial growth and interactions among taxa. The hypothesis that these soil aggregate fractions contain different eubacterial communities was tested using terminal restriction fragment length polymorphism (T-RFLP) of the 16S ribosomal gene. Communities were characterized from two field experiments, located at the Kellogg Biological Station (KBS), MI, USA and the Ohio Agricultural Research and Development Center (OARDC), Wooster, OH, USA. Three soil management regimes at each site were sampled and management was found to significantly affect T-RFLP profiles. The soil aggregate erosion (SAE) method was used to isolate aggregate regions (external and internal regions). Differences between eubacterial T-RFLP profiles of aggregate exteriors and interiors were marginally significant at KBS (accounting for 12.5% of total profile variance), and not significant at OARDC. There were no significant differences among macroaggregate size classes at either site. These results are in general agreement with previous studies using molecular methods to examine microbial communities among different soil macroaggregate size fractions, although further study of communities within different aggregate regions is warranted. Analysis of individual macroaggregates revealed large inter-aggregate variability in community structure. Hence the tertiary components of soil structure, e.g. arrangement of aggregates in relation to shoot residue, roots, macropores, etc., may be more important than aggregate size or intra-aggregate regions in the determination of the types of microbial communities present in aggregates. Direct microscopic counts were also used to examine the bacterial population size in aggregate regions at KBS. The proportion of bacterial cells with biovolumes $>0.18\ \mu m^3$ was higher in aggregate interiors than in exteriors, indicating potentially higher activity in that environment. This proportion was significantly related to percent C of the samples, while total bacterial cell counts were not.

Keywords: Soil macroaggregates; Aggregate regions; Microbial community analysis; Terminal restriction fragment length polymorphism; Agricultural management; Soil structure

1. Introduction

The most common model of the spatial structure of soil particles at millimeter and smaller scales is a hierarchical arrangement of soil aggregates (Edwards and Bremner, 1967; Tisdall and Oades, 1982; Christensen, 1996). The process of aggregation is viewed as dynamic, with microaggregates (<250 μm in diameter) being bound together by plant roots, fungi, organic polymers, and plant residues to form macroaggregates (>250 μm in diameter). As organic particles encrypted within macroaggregates decompose, some macroaggregates disintegrate forming new microaggregates (Beare et al., 1994b; Golchin et al., 1997). Larger macroaggregates generally contain more labile organic matter (Beare et al., 1994a; Elliott, 1986; Gupta and Germida, 1988; Six et al., 2000; Denef et al., 2001) with a more rapid turnover time (Buyanovsky et al., 1994; Monreal et al., 1997). The significance of soil aggregates in structuring microbial communities, however, continues to be debated.

Another approach to studying aggregate structure is to divide aggregates into their internal and external regions.

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Macroaggregates can develop anaerobic cores due to microbial respiration (Lefelaar, 1993; Priesack and Kisser-Priesack, 1993; Zausig et al., 1993), allowing denitrification under unsaturated conditions (Hojberg et al., 1994; Sexstone et al., 1985). External and internal regions of macroaggregates can also differ in age of organic matter, and in contents of organic C and N, phosphorus, cations, and inorganic N (Santos et al., 1997; Smucker, 2003). It is also thought that there may be protection of soil microbes from predators such as nematodes and protozoans in the centers of aggregates (Elliott et al., 1980; Elliott and Coleman, 1988).

The studies described above suggest that the environments in different sized aggregates, or in different regions within an aggregate, may select for different microbial communities. Previous studies on the effects of size of soil aggregates on resident microorganisms have had contradictory results. Different studies have found microbial populations to be greatest in <0.05 mm aggregates (Kanazawa and Filip, 1986), 0.05–0.25 mm aggregates (Monreal and Kodama, 1997), and >0.25 mm aggregates (Gupta and Germida, 1988). Mendes et al. (1999) and Mendes and Bottomley (1998) found the trends in microbial biomass and number of *Rhizobium leguminosarum* across macroaggregate size classes were not consistent between sampling dates, although number of bacterial cells was consistent.

Hattori's (1988) washing-sonication method involves suspension of soil aggregates in water to separate bacteria located on the outer portions of aggregates, followed by sonication to isolate those bacteria in the inner portion. Hattori (1988) found that bacteria in the outer portions of soil aggregates isolated by this method tended to be different in several physiological aspects compared to bacteria in the inner portions. Drazkiewicz (1994) found most types of cultivable bacteria had increased populations in the inner portion of aggregates in a loam, but only a few had significantly increased populations in a silt loam. Location within the aggregate had no consistent effect on the response of cultivable bacteria to keratin-carbamide fertilization (Dabek-Szreniawska, 1993). The drawback of the washing-sonication method is that variability in adhesion of bacteria to soil particles, and the number and length of washes, will confound the physical location of the cells that are washed out of the aggregate. In this study, a method is used to separate external from internal regions of soil aggregates based on removal by mechanical erosion of the aggregate exteriors. This fractionation is dependent on physical characteristics of the aggregate only, and has been useful in studies of the location of soil organic matter, nutrients and pH (Santos et al., 1997) and intra-aggregate porosity and hydraulic conductivity (Park and Smucker, 2005).

The chemical, biological, and physical differences between aggregate fractions described above result in different environments for bacterial growth, which may affect bacterial community composition. In this study, we test the hypothesis that eubacterial communities differ based on the macroaggregate size class they reside in, and on their position within macroaggregates. A molecular community-profiling method, terminal restriction fragment length polymorphism (T-RFLP), is used to test these hypotheses. T-RFLP is a culture-independent community analysis method which has been shown to be sensitive to changes in microbial community composition due to management and soil habitat (Blackwood and Paul, 2003; Kennedy et al., 2004). The finding that eubacterial communities differ between physical soil fractions would imply that these fractions comprise spatially-distinct soil habitats.

2. Materials and methods

2.1. Field sites and sample collection

Samples were collected at two locations: Kellogg Biological Station (KBS), Hickory Corners, MI, USA, and the Ohio Agricultural Research and Development Center (OARDC), Wooster, OH, USA. Details of the field sites are described elsewhere (Blackwood and Paul, 2003; Collins et al., 2000). Soils at KBS are a combination of Kalamazoo fine loam and Oshtemo coarse loam (Typic Hapludalfs). Field treatments at KBS included conventionally-managed continuous corn, organically-managed first-year corn (from the Living Field Laboratory, established in 1993), and continuous alfalfa (from the Long Term Ecological Research site, established in 1988). The organically-managed first-year corn is in a corn–corn–soybean–wheat rotation, with cover crops planted after corn and wheat. Alfalfa plots are killed with herbicide and reseeded every 5 years to maintain stand vigor. Samples were collected between corn plants within the rows, or nearby permanent sampling stations in alfalfa fields. Four replicate field plots per treatment were sampled. Three 350 g soil blocks 10 cm deep were excavated per plot, and were transferred intact to glass jars for transportation. Samples were stored at 4 °C until fractionation was complete.

Soils from OARDC are classified as Wooster silt loam (Typic Fragiudalf). Treatments sampled at OARDC included no-till continuous corn, conventionally-tilled continuous corn, and native successional deciduous forest. Corn treatments were started in 1962. Corn plots have been managed using standard agronomic practices in the region. Three replicate plots per treatment were sampled by excavating a soil block 10 cm deep and storing in a plastic container at 4 °C until fractionation was complete.

2.2. Soil fractionation

Whole soil blocks from KBS were gently separated along planes of weakness over a nest of sieves with mesh sizes of 6.3, 4, and 2 mm, and roots and shoot residue >2 mm were

removed. Soil aggregates were separated until they passed through the 6.3 mm sieve, following recommendations by Jastrow and Miller (1991). The nest of sieves was then shaken by hand until only stable aggregates remained on the 4 and 2 mm sieves. This resulted in isolation of aggregates of sizes 4–6.3, 2–4, and 0–2 mm. The 0–2 mm size class included unconsolidated material as well as aggregates < 2 mm. Density separation was performed on approximately 50 g subsamples of KBS aggregates with water, resulting in the isolation of a heavy fraction (HF-1) and light fraction (LF-1) for each aggregate size class separately (Blackwood and Paul, 2003).

Soil samples from OARDC were allowed to air dry completely before fractionation. Soil blocks were then separated along planes of weakness over a nest of sieves with mesh sizes of 9.5, 6.3, 4, and 2 mm, and roots and shoot residue were removed. The sieves were shaken by hand, and aggregates 4–6.3 and 2–4 mm across were isolated for this study.

Separation of aggregates into external and internal regions was accomplished using stainless steel, soil aggregate erosion chambers (SAE). To peel concentric surface layers from whole aggregates, individual aggregates were weighed and placed in the upper compartments of the SAE. The SAE chambers were then rotated on an orbital shaker at 150–250 rpm as described by Park and Smucker (2005), causing the aggregate to roll across the knurled inner wall of the SAE. As material was eroded from the aggregate surface, it passed through a 352 μm screen into a lower collection chamber. The upper compartments with aggregates were weighed periodically to determine the proportion of the aggregate that had been removed by mechanical erosion. When the weight of the unpeeled portion of the aggregate had decreased by 33%, the material trapped in the bottom of the SAE chamber, referred to as the external region of the aggregate, was removed. Erosion was continued until the weight of the aggregate was reduced by another 33%, and the uneroded portion of the aggregate, referred to as the internal region, was isolated. The orbital shaker speed was gradually increased in order to minimize crumbling of aggregates, but those that broke into multiple fragments (<20%) during erosion were replaced.

Four 4–6.3 mm aggregates (not subjected to density separation) from one plot of each of the KBS cropping systems were randomly chosen for separation into external and internal regions. Individual aggregate regions from KBS samples were not pooled. Four 4–6.3 mm aggregates and eight 2–4 mm aggregates were chosen from each OARDC plot for separation into aggregate regions. The OARDC aggregate regions were pooled by field plot for all subsequent analyses. Fractionation of soil and aggregates was completed 8 weeks after sampling for KBS samples and 3 weeks after sampling for OARDC samples.

2.3. T-RFLP analysis

T-RFLP was performed as described previously (Blackwood et al., 2003). Briefly, community DNA was extracted from samples using the Ultraclean Soil DNA extraction kit (Mo Bio Laboratories, Solana Beach, CA, USA). The 16S ribosomal gene was amplified directly from community DNA using the general eubacterial PCR primer 8-27F (AGAGTTTGATCCTGGCTCAG, *E. coli* numbering, Integrated DNA Technologies, Coralville, IA) and the universal primer 1392-1406R (ACGGGCGGTGTGTACA). Optimized PCR reactions were performed in triplicate for each sample using hexachlorofluorscein (hex)-labeled 8-27F (Integrated DNA Technologies). PCR replicates were pooled and purified using PCR Preps Wizard Kits (Promega, Madison, WI), with elution in 19 μL of sterile water. Five microliter of purified PCR product was digested using 15 U of the restriction enzyme *Rsa*I (Gibco BRL, Gaithersburg, MD) in a 10 μL reaction, incubating 3 h at 37 °C, followed by 16 min at 65 °C. DNA fragments from 3 μL of the restricted PCR product were separated by size by electrophoresis on an ABI 373 automated DNA sequencer (Applied Biosystems Instruments, Foster City, CA) at Michigan State University's DNA Sequencing Facility. The 5′ terminal restriction 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.

T-RFLP profiles were generated for samples of HF-1 and LF-1 from different aggregate size classes of KBS soil, from external and internal regions of individual KBS 4–6.3 mm aggregates, and from pooled external and internal regions of 2–4 and 4–6.3 mm OARDC aggregates. Negative controls 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.

2.4. Direct microscopy

Cells were dispersed in formaldehyde-fixed individual KBS aggregate regions by diluting to 2 mL with water and vortexing for 5 min with 0.5 mL of 1 mm glass beads. Bacterial cell numbers were quantified in the individual aggregate samples following the procedure of Paul et al. (1999). Briefly, 4 μL of fixed sample was dried overnight in each of five 6 mm diameter wells of an analytical microscope slide (Cel-Line Associates, Newfield, NJ). Dried sample smears were then stained with 5-(4,6-dichlorotriazin-2-yl) aminofluoroscein (DTAF) for 40 min, followed by rinsing in phosphate buffer and water. Digital images of bacteria were obtained under oil immersion at

100× magnification using a Leitz Orthoplan 2 microscope and a Princeton Instruments microscope camera. Cells were counted and measured by a script written in the image analysis program IPLab (Princeton Instruments, Trenton, NJ). Calculations of cell biovolume were based on formulas in Paul and Clark (1996) assuming a prolate three-dimensional geometry. 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. Carbon and nitrogen analyses

Carbon and nitrogen contents of dried, ground KBS aggregate regions were determined by combustion in a Carlo-Erba NA1500 series 2 Nitrogen–Carbon–Sulfur Analyzer.

2.6. Statistical analysis

Statistical analyses were performed using SAS version 8 Stat and IML components (SAS Institute, Cary, NC) and Canoco (Microcomputer Power, Ithaca, NY). All analyses were performed taking into account blocking of replicates in the field and lab, which was not found to be significant.

Percent C and N and microscopic cell counts in individual aggregate regions were analyzed by ANOVA as a split-plot experimental design using Proc Mixed in SAS. Each individual aggregate was treated as a whole plot, with aggregate regions as the split plots. Linear regression analysis was also used to test for an effect of percent C and N on bacterial cell counts. This was also performed in Proc Mixed to take random inter-aggregate variability into account.

To match DNA fragments in different samples, T-RFs 50–500 bp in length were aligned against a previously-defined database of T-RFs with sample identities concealed. Cumulative peak height was standardized to 10,000 fluorescence units when possible since this was previously shown to reduce noise in T-RFLP data analysis (Blackwood et al., 2003). Differentiation of T-RFLP profiles due to cropping system, aggregate size class, or aggregate region was tested using redundancy analysis of Hellinger distance and Jaccard distance (Legendre and Anderson, 1999). Previously, Jaccard distance was found to be more sensitive if profiles are of adequate strength, but Hellinger distance is more robust and preserves information about which peaks are important in the analysis (Blackwood et al., 2003). The null hypothesis of no difference between profiles due to treatment was tested using 9999 random permutations of profile identities in the software Canoco. To test for aggregate region effects, profiles were permuted within individual aggregates as in a split-plot experimental design. Percent C and N were also tested as covariables for significant effects on T-RFLP profiles using redundancy analysis. Where exploratory data analysis was most appropriate, relationships between profiles were examined using hierarchical cluster analysis by Ward's method, and ordination by principal components analysis or principal coordinates analysis (Blackwood et al., 2003).

3. Results

3.1. KBS aggregate size classes and density fractions

Each aggregate size class (0–2, 2–4, and 4–6.3 mm) made up an approximately equal amount of the total soil weight, varying from 29 to 38% each in different samples. LF-1 made up less than 0.5% of total weight of each macroaggregate size class, but approximately 5% of the carbon present in macroaggregates.

Cropping system significantly affected T-RFLP profiles from aggregate size classes separated by density ($p=0.0001$). Cropping system accounted for 24% of the overall variance in HF-1 profiles, and 35% of the overall variance in LF-1 profiles. Aggregate size class did not have a significant effect on T-RFLP profiles of either LF-1 or HF-1 ($p>0.1$). Cropping system differences in both HF-1 and LF-1 are shown in Fig. 1, which also shows clear separation between density fraction communities and no differences between macroaggregate size classes. HF-1 profiles from different aggregate size-classes did not differ from whole (unfractionated) soil T-RFLP profiles ($p>0.1$).

3.2. Regions within 4–6.3 mm KBS aggregates

Fifteen to sixty milligrams of external or internal aggregate soil was obtained from each individual aggregate, and this material was divided up for DNA extraction, direct microscopy, and C and N analysis. Four aggregates per cropping system were analyzed by all assays. T-RFLP profiles were obtained from all samples, and there was a

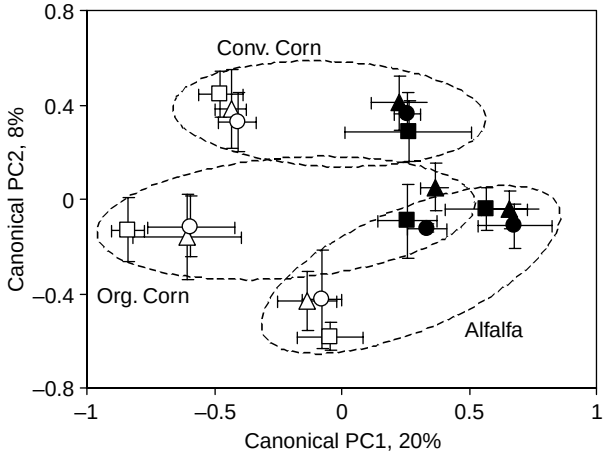

Fig. 1. T-RFLP profiles from KBS macroaggregate fractions separated into LF-1 (light fraction) and HF-1 (heavy fraction). Error bars show one standard deviation from treatment mean; $N=4$ field replicates. (▲) 0–2 mm, (■) 2–4 mm, (●) 4–6.3 mm; black: HF-1, white: LF-1.

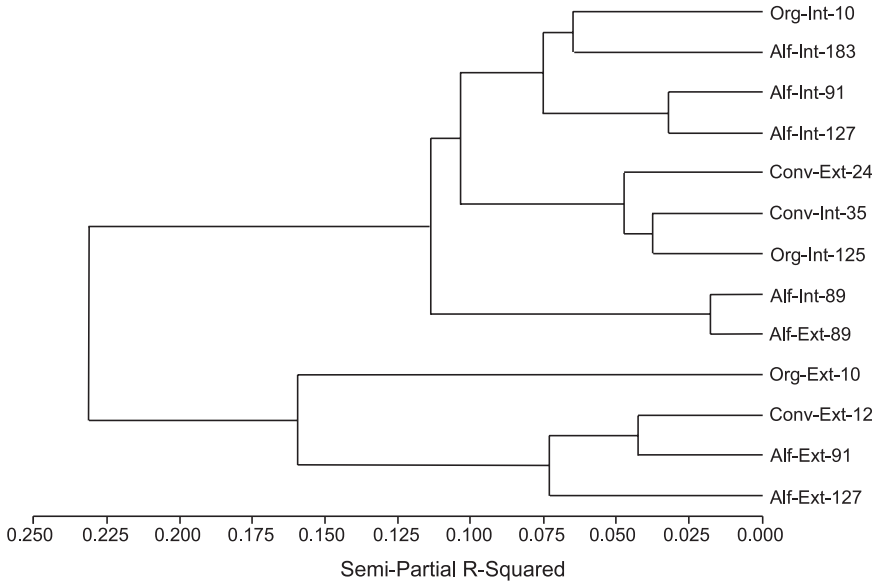

Fig. 2. Dendrogram of eubacterial community T-RFLP profiles from aggregate regions of individual 4–6.3 mm KBS aggregates. Each leaf or endpoint of the dendrogram represents a region from an individual aggregate. Sample names are coded as 'cropping system–aggregate region–aggregate identification number'. Alf = alfalfa, Conv = conventional corn, Org = organic corn, Ext = external region, Int = internal region.

large amount of variability in the cumulative peak height of T-RFLP profiles. It has been previously shown that standardizing cumulative peak height of T-RFLP profiles is an important step in generating robust results (Blackwood et al., 2003). We present both a hypothesis-testing analysis (redundancy analysis, Hellinger distance) and a conservative exploratory analysis (cluster analysis, Jaccard distance) using profiles with cumulative peak heights previously shown to be optimal for these methods.

Limiting samples to those with a cumulative T-RF peak height greater than 7500 fluorescence units, and where profiles for both the internal and external regions were obtained, resulted in a dataset including three aggregates from alfalfa, three from organic corn, and one from conventional corn. 48% of the variance in the profiles could be explained by differences between individual aggregates. Redundancy analysis indicated that the effect of aggregate region was marginally significant ($p = 0.077$) and accounted for 12.5% of the variation in the dataset. Differences between the alfalfa and organic corn cropping systems, as well as the interaction between aggregate region and cropping system, were not found to be statistically significant ($p > 0.1$). This is in contrast to results from the LF-1 and HF-1 samples derived from 50 g of aggregates (described above), where differences between cropping systems are evident because variability between individual aggregates is masked by using a large pool of aggregates.

Limiting samples to those with cumulative peak height greater than 10,000 fluorescence units for analysis by Jaccard distance resulted in 13 acceptable profiles. Exploratory data analysis methods were used on this dataset because it was heavily weighted toward alfalfa samples and only four aggregates were represented by both regions. While clustering of communities is not based on cropping system in Fig. 2, four of six external-layer samples are separated from the other samples, accounting for 23% of the variance in the dataset. This trend was verified in principal coordinates plots derived from the Jaccard distance matrix as well.

Aggregate region had a significant effect ($p = 0.047$) on percentage of cells in the largest cell size class ($> 0.18\ \mu m^3$), being 15.4% in aggregate exteriors and 16.7% in interiors. In 10 out of 12 aggregates examined, percent cells $> 0.18\ \mu m^3$ was higher in aggregate interiors than exteriors. There was significant inter-aggregate variability which was accounted for by designating aggregates as whole plots using Proc Mixed in SAS. One outlier aggregate reversed the trend for percent cells $> 0.18\ \mu m^3$ in conventional corn (Table 1). There were no significant effects of aggregate region or cropping system on percent C or N, bacterial cells per g fraction, or percentage of cells in the smallest ($< 0.065\ \mu m^3$) bacterial cell size class (Table 1).

Regression analysis showed that the relationship between percent of cells in the largest size class and percent organic C in the sample was significant (Fig. 3). Proc Mixed was again used to account for inter-aggregate variability. The regression of total numbers of bacterial cells on percent C was not significant ($p = 0.23$). Percent C and N did not explain a significant amount of the variation in the T-RFLP profiles ($p = 0.35$).

3.3. OARDC 2–4 and 4–6.3 mm aggregate regions

Two OARDC datasets were created to analyze aggregate region and management effects. This was done to avoid

Table 1
Bacterial population size and organic nutrient content for 4–6.3 mm KBS intra-aggregate external and internal regions

Cropping system	Aggregate regions	% C	% N	Log cells/g	% cells $>0.18\ \mu m^3$[a]	% cells $<0.065\ \mu m^3$
Alfalfa	External	1.4 (0.7)	0.14 (0.04)	9.7 (0.1)	15.6 (2.9)	58.7 (3.5)
	Internal	1.9 (1.2)	0.21 (0.08)	9.6 (0.5)	18.2 (2.9)	56.1 (9.3)
Conv. corn	External	1.1 (0.2)	0.12 (0.01)	9.6 (0.1)	14.2 (0.9)	61.3 (1.8)
	Internal	1.0 (0.2)	0.11 (0.07)	9.5 (0.2)	13.9 (2.2)	63.1 (1.8)
Org. corn	External	1.5 (0.3)	0.11 (0.08)	9.6 (0.2)	16.5 (2.5)	58.3 (6.1)
	Internal	1.4 (0.4)	0.13 (0.11)	9.7 (0.2)	17.9 (1.9)	53.6 (2.0)

Values are means of four individual aggregates, with standard deviation indicated in parentheses.
[a] Internal aggregate regions contained a significantly higher proportion of cells $>0.18\ \mu m^3$ compared to external layers, $p<0.05$.

the size class-treatment combination of 2–4 mm conventional tillage aggregates because only one replicate T-RFLP profile of adequate strength was obtained for internal aggregate regions of this sample type. The first dataset included 4–6.3 mm aggregates of all management treatments, while the second included 2–4 and 4–6.3 mm aggregates of no-till and successional vegetation only. Soil management significantly affected T-RFLP profiles in both datasets, although the effects were stronger in the first dataset (Table 2). This is likely because conventional-tillage corn communities were the most divergent of the three management treatments (Fig. 4), and this treatment was not included in the second dataset. Neither aggregate region nor aggregate size caused significant differences in OARDC T-RFLP profiles (Table 2).

4. Discussion

Blackwood and Paul (2003) had previously used T-RFLP to show that plant-tissue dominated soil fractions, such as light fraction, harbored a different microbial community than the mineral soil matrix at KBS. T-RFLP has previously been shown to be a sensitive method in a wide variety of environments (Blackwood et al., 2003; Kim and Marsh, 2004). In this study, whole soil was fractionated into different macroaggregate size classes, and into different regions within macroaggregates. The regional fractions eroded from whole aggregates are primarily mineral particles, and differ chemically, biologically, and physically (Santos et al., 1997; Smucker et al., 1998; Park and Smucker, 2005). While differences between T-RFLP profiles of aggregate external and internal regions were observed at one site (KBS), the overall variance explained was low due to high inter-aggregate variability. This is illustrated in Fig. 2, where there are two aggregate exterior profiles that do not cluster with the remaining four, and by the fact that 48% of the variance in individual aggregate region profiles was explained by similarities between regions of the same aggregate. No differences between aggregate regions were found at OARDC. Pooling regions from 4 or 8 aggregates likely did not overcome inter-aggregate variability for the OARDC soils, although variability was reduced enough to detect differences among soil management treatments.

If there are weak but consistent differences between communities of different aggregate regions, it may be possible to detect these differences by using a larger number of aggregates to average over the strong inter-aggregate variability. This is accomplished by the soil washing

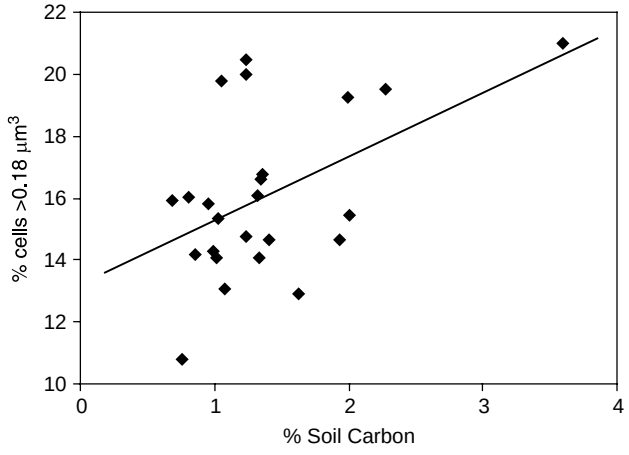

Fig. 3. Percent of total cells with biovolume $>0.18\ \mu m^3$ vs. percent soil carbon in individual soil aggregate interiors and exteriors. Linear regression shown is significant ($p=0.035$, $R^2=24\%$). Other cell size classes did not show significant relationships to soil C.

Table 2
Percent of variability explained by each experimental factor for OARDC soil aggregate T-RFLP profiles

	Region	Size	Management	Interaction	Sum
4–6.3 mm aggregates[a]					
Hellinger distance	NS[b]	NA[c]	23**	NS	23
Jaccard distance	NS	NA	19*	NS	19
2–4 and 4–6.3 mm aggregates[d]					
Hellinger distance	NS	NS	14**	NS	14
Jaccard distance	NS	NS	12*	NS	12

p-values generated by 9999 random permutations of sample identity. $*p<0.01$, $**p=0.0001$.
[a] Conventional tillage corn, no-till corn, successional vegetation.
[b] NS = not significant, $p>0.05$.
[c] NA = not applicable.
[d] No-till corn and successional vegetation.

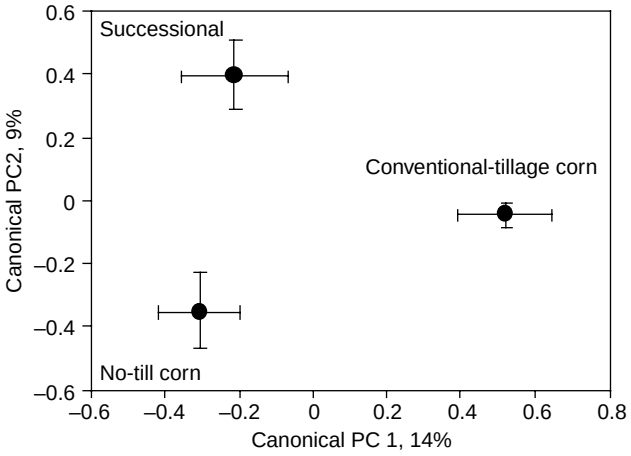

Fig. 4. T-RFLP profiles from 4 to 6.3 mm OARDC aggregates. Canonical axes are optimized to show significant effects of cropping system as detected by redundancy analysis. Error bars show one standard deviation from treatment mean; $N=6$ for each cropping system (three field replicates × two aggregate regions).

method of Hattori (1988). Soil washing has the disadvantage that the strength of adherence of cells confounds their spatial position. The aggregate peeling method does select against aggregates that are unstable or with very irregular shapes, although all methods of soil fractionation disrupt some aggregates. Ranjard et al. (2000b) used a soil washing method to separate 'outer' and 'inner' bacteria and detected minor differences between community profiles of these cell fractions using ribosomal intergenic spacer analysis. But in another study, community profiles of 'outer' and 'inner' fractions of bacteria were similar, as were their responses to mercury exposure (Ranjard et al., 2000a).

Total number of bacterial cells, and the proportion of cells $>0.18\ \mu m^3$, had previously been shown to correlate with the amount of C present in a soil fraction (Blackwood and Paul, 2003). In individual aggregate regions, both percent C and total number of cells varied much less compared to the soil fractions previously studied, and showed no relationship to each other, being typical of values for HF-1. However, there was a significant relationship between the proportion of cells $>0.18\ \mu m^3$ and percent C. Cell size is an indicator of activity (growth and metabolism), and cells with biovolumes $>0.18\ \mu m^3$ have been shown to represent the growing fraction of cells in incubations (Christensen et al., 1995). Cell size may therefore be a more sensitive indicator of average metabolic status than total numbers of cells for bacteria residing in the mineral soil matrix because of the accumulation of small, dormant cells in this environment (Blackwood and Paul, 2003). The values in Table 1 have not been adjusted for sand content because it would have been impossible to determine this without sacrificing one of our other analyses. We also have no evidence that sand is differentially sequestered in internal or external regions, or that management regime at KBS shifts soil texture.

At KBS, the test for differences between communities of different macroaggregate size classes was performed separately on the HF-1 and LF-1 for each sample, while at OARDC the same test was performed on pooled samples of external and internal regions from several aggregates for each sample. In neither case were differences detected between macroaggregate size classes. These results agree with other culture-independent studies which have found that macroaggregate size class has minimal effects on microbial phospholipid fatty acid profiles (Petersen et al., 1997), or archaeal 16S ribosomal T-RFLP profiles (Ramakrishnan et al., 2000).

Soil management had previously been found to cause shifts in the composition of the eubacterial communities in several soil fractions at KBS (Blackwood and Paul, 2003). The impact of soil management at KBS was further verified in this study, and soil management was found to alter eubacterial community composition at OARDC as well. However, when communities from individual KBS aggregates were analyzed, the impacts of soil management were masked by very large inter-aggregate variability.

What could be the cause of the large inter-aggregate variability in community composition, which results in little effect of size or position within macroaggregates? Tertiary soil structure, such as proximity of sites to macropores or decomposing shoot residue, may be the most important factor in determining the composition and activity of microbial communities in different aggregates and in different sites within aggregates (Parkin, 1993; Young and Ritz, 2000). While some consistent environmental differences may exist among macroaggregate size classes, and among regions within aggregates, the turnover time of macroaggregates in agronomic soil has been reported to be 2–10 years (Buyanovsky et al., 1994; Monreal et al., 1997). This may be too rapid relative to the low rates of microbial growth in soil to allow much differentiation of communities to occur. Bacteria in agronomic bulk soil at KBS only divide once every ~470 days (calculated from μ in Harris and Paul (1994), mean temperature = 8.8 °C [1996–2004], and assuming $Q_{10}=2$). More rapid growth in the rhizosphere, LF, and shoot residue, on the other hand, may allow for extensive changes in the makeup of the community (Blackwood and Paul, 2003) due to differential success of species under different conditions. If the structure of soil aggregates is truly hierarchical, differences between microaggregate size classes may also be important in determining community composition. Microaggregates turn over much more slowly than macroaggregates (Buyanovsky et al., 1994; Monreal et al., 1997), and various sizes are present in the external and internal regions of macroaggregates. In studies of one soil, differences between communities in different micro- and macroaggregate size classes have been found by Ranjard et al. (2000b) and Poly et al. (2001), although it is difficult to assess the statistical significance of their results. In addition to habitat turnover coupled with low growth rates, high rates of colonization by

new species could also contribute to variability in community composition and mask differentiation between environments (e.g. Hraber and Milne, 1997). Microbial dispersal in soil is generally viewed as passive and dependent on the soil water status, although more study of this phenomenon is needed (Murphy and Tate, 1996; Grundmann, 2004).

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