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Soil Biology & Biochemistry 38 (2006) 1608–1614

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Endogeic earthworms differentially influence bacterial communities associated with different soil aggregate size fractions

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Received 13 June 2005; received in revised form 14 October 2005; accepted 6 November 2005
Available online 2 February 2006

Abstract

Endogeic earthworm activities can strongly influence soil structure. Although soil microorganisms are thought to be central to earthworm-facilitated aggregate formation, how and where within the soil matrix earthworm-facilitated influences on soil microbial communities are manifested is poorly defined. In this study we used 16S rRNA gene-based terminal restriction fragment polymorphism (T-RFLP) analyses to examine bacterial communities associated with different aggregate size fractions (macroaggregates, microaggregates-within-macroaggregates and inner-microaggregates-within-macroaggregates) of soils incubated for 28 d with and without earthworms. We hypothesized that bacterial communities in different soil aggregate size fractions are differentially influenced by earthworm activities. Our results indicate significantly enhanced aggregate formation (both macroaggregates and microaggregates within macroaggregates) in earthworm-worked soils relative to soils receiving only plant litter. Although significant differences were found between bacterial communities of earthworm and litter-only treatments for all soil fractions, communities associated with earthworm-worked macroaggregate fractions exhibited the least similarity to all other soil fractions regardless of treatment. In addition to differences in terminal restriction fragment (T-RF) size distributions, T-RFLP profiles of earthworm-worked soil macroaggregates had significantly fewer T-RF sizes, further suggesting less species evenness and more extensive alteration of bacterial communities within this fraction. These findings suggest that, due to rapid occlusion of organic materials, microbial communities associated with microaggregates-within-macroaggregates formed during or shortly after passage through the earthworm gut are relatively inactive, and therefore change relatively little over time compared to macroaggregate populations as a whole.

Keywords: Earthworms; Soil aggregates; Soil structure; T-RFLP

1. Introduction

The activities of earthworms have been the subject of ongoing research due to their profound influence on soil physical and chemical properties (Edwards and Bohlen, 1996). Earthworms contribute to soil aggregation mainly through cast production and burrowing activities (Edwards and Bohlen, 1996). Intimate mixing of organic and mineral particles in the earthworm gut creates conditions conducive to aggregate formation (Shipitalo and Protz, 1989). Burrowing produces aggregates through axial and radial pressures, which can orient clay and organic materials, and through deposition of external mucus, which acts as both a microbial substrate and a soil binding agent (Brown et al., 2000).

The activities of microorganisms are thought to be integral to aggregate formation and turnover (Oades, 1993; Degens, 1997). In turn, it is well known that earthworm activities can strongly influence microbial activity (Barois, 1992; Daniel and Anderson, 1992; Edwards and Fletcher, 1988; Pedersen and Hendriksen, 1993; Wolter and Scheu, 1999). Concurrent with changes in microbial activity, a number of studies have demonstrated microbial community alteration in cast and/or drilosphere environments relative to soil as a whole (Brown et al., 2000; Furlong et al., 2002). However, the extent to which microbial communities associated with different soil microhabitats are affected is virtually unknown. Such knowledge is central to in-depth understanding of the

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0038-0717/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2005.11.014

mechanisms by which earthworms drive microbial community compositional changes and soil aggregation.

One way to view or conceptualize soil microbial habitats is in the context of the hierarchical structure of soil aggregates. The aggregate hierarchy model, as proposed by Tisdall and Oades (1982), implicates different binding agents acting at different hierarchical stages of aggregation. Small primary particles and organo-mineral complexes are bound together into microaggregates (<250 μm), which are in turn bound within macroaggregates (>250 μm). Mirroring the aggregate hierarchy is a hierarchical pore size distribution (Oades, 1984), with smaller aggregate size fractions typically containing smaller sized pores. Pore structure, including connectivity between pores, largely determines habitable space, water and substrate distributions, interactions between organismal groups (Young and Crawford, 2004) and, therefore, microhabitat characteristics.

In addition to potentially altering microbial populations of different aggregate size fractions by altering soil organic matter release and occlusion dynamics (Brown et al., 2000), earthworms may strongly influence microbe redistribution within the soil matrix through dispersal of microorganisms by ingestion at one location and egestion elsewhere (Edwards and Bohlen, 1996), and through smaller-scale redistribution. A number of researchers have shown that microbial community composition typically exhibits pronounced stratification within and between aggregate size fractions (Tisdall and Oades, 1982; Sessitsch et al., 2001; Mummey and Stahl, 2004; Mummey et al., 2006). Since soil structure is largely destroyed during passage through the earthworm gut (Shipitalo and Protz, 1988; Barois et al., 1993), these small scale spatial relationships are likely to be largely disrupted or destroyed as well.

The aim of the current study was to determine the extent to which earthworm activities influence the composition of bacterial communities in macroaggregate and inner- and total-microaggregate soil fractions using culture-independent, molecular methods. We hypothesized that, due to spatiotemporal constraints on substrate diffusion within different aggregate size classes and rapid occlusion of organic materials during microaggregate reformation in cast materials, earthworm facilitated changes in microbial community composition are most pronounced at the scale of the macroaggregate.

2. Materials and methods

2.1. Site description and soils

Surface soils (0–10 cm) were collected from a gently sloping undisturbed grassland site. The site is located 10 km north of Missoula, MT (46°45′00N, 114°07′30W) and receives 34–42 cm average annual precipitation. Soil at the site is classified as a cobbly loam Argixeroll. Soil textural analysis and nutrient concentrations are described in Lutgen and Rillig (2002) and Lutgen et al. (2003). Soils were air-dried after collection (moisture content after drying was 2–3%) and sequentially sieved to pass 2 mm and 500 μm sieves. In order to produce media having bulk density conducive to earthworm growth, coarse material greater than 2 mm was discarded and soil particles greater than 500 μm and less than 500 μm were remixed in a 1:4 ratio.

2.2. Incubation

Earthworms (*Aporrectodea caliginosa*) were collected by hand-sorting from pasture sod (cobbly loam Argixeroll) in the vicinity of Missoula, MT. Prior to incubation, worms were kept for a week in a moist soil subsample (14 °C). Soil-mixture subsamples (100 g) were subjected to three separate treatments: (i) plant litter and earthworms; (ii) plant litter, no earthworms; (iii) control (no additions). For treatments receiving plant litter, finely ground grass-leaf (*Festuca idahoensis*) litter (1 g) and soil mixtures were thoroughly mixed. Soil mixtures were brought up to 30% water content. For earthworm treatments, two adult earthworms were added to each sample. All samples were incubated in the dark for 28 d (14 °C). Every 3 d containers were weighed to determine moisture losses and de-ionized water was added when losses approached 7%.

2.3. Soil physical analyses

For all treatments, soil aggregate size distributions were determined after transfer of soil to large weight boats, removal of earthworms, and subsequent air-drying for 3 d, which allows for stabilization of earthworm casts (Marinissen and Dexter, 1990). After slow remoistening, macroaggregates were isolated by wet sieving, which entailed gentle movement of 5 g soil on 250 μm sieves up and down 4 cm for 5 min using a sieving machine (Five Star Scientific, Twin Falls, Id.) (Kemper and Rosenau, 1986). Microaggregates-within-macroaggregates (miM) were determined using the method of Six et al. (2000). Briefly, macroaggregates were immersed in deionized water on top of a 250 μm sieve with 50 glass beads (4 mm diameter) and shaken on a reciprocal shaker for 3 min. Continuous water flow through the device allowed microaggregates to pass through the 250 μm sieve as they were released from macroaggregates, minimizing microaggregate exposure to disruptive forces. The miM fraction was subsequently captured on a 53 μm sieve. Samples were retained for molecular and physical analyses. Samples of macroaggregate and miM fractions of earthworm and litter-only treatments were retained after isolation for nucleic acid extraction and subsequent molecular analyses. Additional samples were retained for weight determination after being oven dried (104 °C) for 24 h.

2.4. Biological analyses

Isolation of inner-miM fractions was achieved using high-energy UV radiation. This procedure is based on the ability of high-energy UV radiation to oxidize organic

materials including nucleic acids. Since UV radiation does not penetrate soil mineral components to a great extent (Skjemstad et al., 1993), organic matter, including microbes and their nucleic acids, within miM are largely protected. Our photo-oxidation reactor system and procedure are as described in Mummey and Stahl (2004) and modified as in Mummey et al. (2006). Briefly, quartz tubes (50 ml) containing aqueous miM suspensions were fixed to a vertically aligned rotating wheel centered on a horizontally aligned 450 W mercury vapor UV bulb encased in a cooling water-jacket. Tube rotation ensured that the miM remained suspended, without excessive abrasion, and that they were irradiated evenly from all angles. After 24 h photo-oxidation treatment, the inner-miM fractions were collected and analyzed as below.

DNA was extracted from each soil fraction (0.25 g, wet weight) using PowerSoil DNA isolation kits (MoBio Laboratories, Carlsbad, CA). Polymerase chain reaction (PCR) amplification of genomic templates utilized the fluorescently labeled forward primer 27f, with 927r as the unlabeled reverse primer (Lane 1991). The 50 μl reaction mixtures contained 1X PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer, 1.25 U HotMaster^{tm} Taq DNA polymerase (Brinkmann Instruments, Westbury, NY), and 1 μl soil DNA extract. PCR amplification began with a denaturing step of 95 °C for 3 min, followed by 32 cycles of 95 °C for 30 s, 56 °C for 45 s, and 72 °C for 2 min; followed by a final extension at 72 °C for 3 min. PCR was performed using a Eppendorf Mastercycler (Brinkmann Instruments).

PCR product quantities were estimated after gel electrophoresis by comparing band intensities after staining with ethidium bromide. Approximately 25 ng of PCR product was digested with 6 U of *HhaI* (New England Biolabs, Beverly, MA) for 4 h in the manufacturer's recommended reaction buffer. Digests were purified by passage through gel filtration cartridges (Edge Biosystems, Gaithersburg, MD) and T-RF sizes subsequently determined by capillary electrophoresis using an ABI 3100 automated DNA sequencer. T-RF sizes between 50 and 500 bp were determined using GeneMapper analytical software (Applied Biosystems Inc., Fremont, CA) with Genescan 500 ROX (Applied Biosystems) as the size standard.

Comparison of T-RFLP profiles from different samples requires standardization of relative fluorescence between samples (Dunbar et al., 2001). Briefly, relative fluorescence was standardized to the smallest quantity by proportionally reducing each peak area in profiles having greater total relative fluorescence. After proportional reduction of larger profiles, peaks having fluorescence values less than the threshold value (50 relative fluorescent units) were eliminated from subsequent analyses.

2.5. Statistical analyses

Relationships between T-RFLP peak height data of all samples, and earthworm and litter-only treatments, were examined using ordination techniques. Initial detrended correspondence analysis indicated that the data exhibited a linear, rather than a unimodal, response to sample origin, justifying the use of linear ordination methods (Lepš and Šmilauer, 2003). Therefore, relationships between T-RFLP profiles of samples were evaluated by redundancy analysis (RDA), using Canoco software (Microcomputer Power, Ithaca, NY). This technique identifies the proportion of the variability that can be explained by experimental treatments. A Monte Carlo permutation test based on 199 random permutations was used to test the null hypothesis that bacterial community profiles were unrelated to sample treatment.

A modification of the Mantel test (Mantel, 1967), employing the computer program zt (Bonnet and Van de Peer, 2002), was utilized to test for differences between soil fractions of earthworm and litter-only treatments. In this analysis, two matrices were constructed, one consisting of Pearson correlation coefficients for all sample pairs, and the second consisting of 1 or 0, depending upon whether two samples belonged to the same group or not. Test statistics were calculated based on Monte Carlo sampling (999 permutations).

Differences in the percentage of macro- and microaggregates between treatments were examined by one-way ANOVA using the statistical program SPSS (v9.0).

3. Results

3.1. Aggregate size distributions

While no significant differences were found for stable macroaggregate quantities between plant litter without earthworm and no litter treatments (Fig. 1), addition of earthworms resulted in significant macroaggregate formation ($F = 134$, $P < 0.001$). The miM fraction also increased significantly in earthworm treatments compared to no-addition and litter-only treatments (Fig. 1) ($P < 0.0001$).

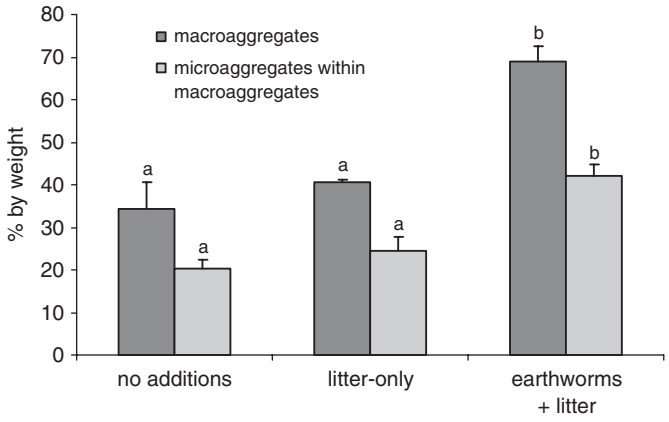

Fig. 1. Percent macroaggregates and microaggregates within macroaggregates (miM) for soil receiving no additions, plant litter only, and plant litter plus earthworms. *Different letters indicate significant differences in either macroaggregates or miM fractions.

3.2. T-RFLP analyses

Ordination analysis of T-RFLP profiles derived from macroaggregate, miM and inner-miM fractions (Figs. 2A, B and C, respectively) indicate clusters corresponding to sample identity. Analysis using the Mantel test found T-RFLP profiles of earthworm-treated soil fractions to be significantly different from corresponding fractions of the litter-only treatment (Fig. 2).

Ordination analysis of T-RFLP profiles derived from all earthworm treatment fractions indicated that the macroaggregate fraction formed a distinct cluster in relation to miM and inner-miM fractions (Fig. 3A), while inner-miM samples formed a group within the miM cluster. Subsequent

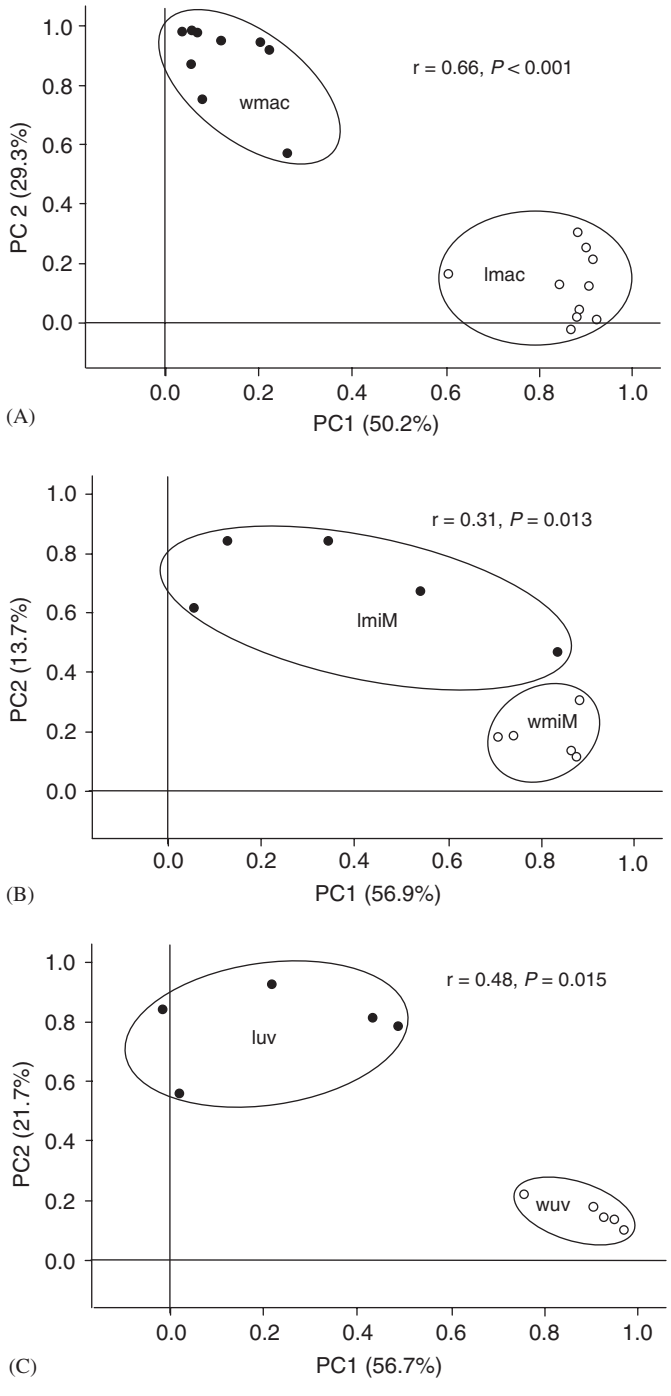

Fig. 2. Ordination plots comparing bacterial communities of (A) macroagggregates (wmac and lmac indicate earthworm-treated and litter-only treatments, respectively), (B) microaggregate (wmiM and lmiM indicate earthworm-treated and litter-only treatments, respectively), and (C) inner-microaggregate fractions (wuv and luv indicate earthworm-treated and litter-only treatments, respectively). Numbers in the upper, right-hand corner of each plot indicate the results of Mantel tests.

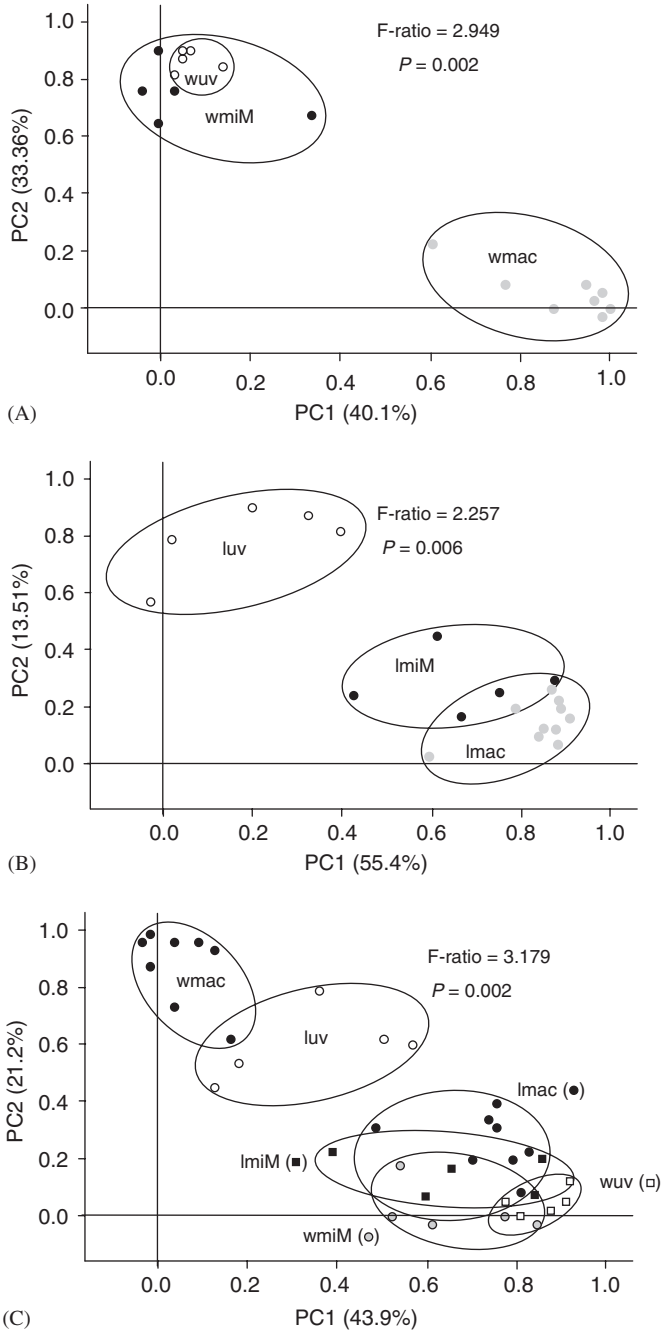

Fig. 3. Ordination plots comparing bacterial communities in fractions of (A) earthworm-treated soils, (B) litter-only soils, and (C) all soil fractions. Sample identifiers are the same as in Fig. 2. Numbers in the upper, right-hand corner of each plot indicate the results of redundancy analyses testing the hypothesis that sample identity accounts for a significant amount of the total variance.

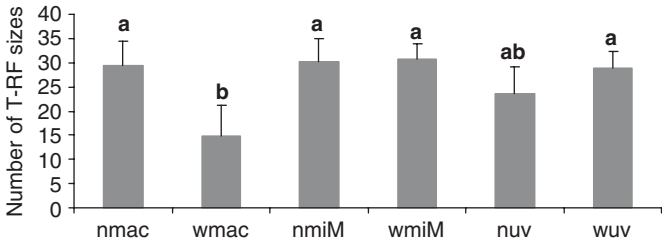

Fig. 4. Comparison of terminal restriction fragment numbers obtained from treatments and soil fractions. Different letters indicate significant difference at the alpha = 0.05 level (ANOVA, Bonferoni adjustment).

redundancy analysis indicates that sample identity accounted for a significant amount of the total variance ($F = 2.95$, $P = 0.002$).

Three distinct clusters, corresponding to macroaggregate, miM, inner-miM samples, were apparent in ordination plots of T-RFLP profiles derived from litter-only soil fractions (Fig. 3B). However, unlike earthworm treatment soil fractions, inner-miM samples formed a distinct group distant from both macroaggregate and miM samples. Redundancy analysis also indicated that sample identity accounted for a significant amount of the variance in this dataset ($F = 2.26$, $P = 0.006$).

Ordination of all sample T-RFLP data showed that clusters corresponding to sample identity were formed. Although substantial overlap between some of the sample groups was apparent, a significant amount of the variance could be accounted for by sample identity ($F = 3.18$, $P = 0.002$). Separation was most pronounced for macroaggregates of earthworm soil followed by inner-miM fractions of the litter-only treatment.

Oneway ANOVA of average ribotype numbers (Fig. 4) indicated that there were significant differences among groups ($F = 12$, $P < 0.0001$). Multiple comparisons (Tukey-Kramer HSD) indicated that the number of ribotypes detected in the earthworm treatment macroaggregate fraction was significantly smaller than all other earthworm or litter-only soil fractions, with the exception of inner-miM of the litter-only treatment.

4. Discussion

Most soil bacteria are inactive at a given time due to low nutrient availability (Morita, 1997; Coleman, 2001). Earthworm foraging activities resulting in the release of soil organic matter can temporally activate segments of these dormant soil bacterial populations (e.g. Brown et al., 2000; Furlong et al., 2002; Bruneau et al., 2005). Release of microbial substrates and alteration of soil physical structure facilitated by earthworm activities would be expected to differentially influence microbial community composition in different soil compartments, potentially in the long term. Unlike all studies to date examining overall soil, cast or drilosphere environments, our analyses were directed toward bacterial communities associated with different aggregate fractions, thus allowing for discussion of our findings within the framework of a hierarchical aggregate structure.

Similar to numerous studies (e.g. Edwards and Bohlen, 1996; Lee and Foster, 1991; Bossuyt et al., 2004), our results indicate that the presence of earthworms significantly enhanced macroaggregate formation (Fig. 1). Our results also correspond with a few recent studies (Bossuyt et al., 2004; Pulleman and Marinissen, 2004; Bossuyt et al., 2005) showing significant new miM formation of earthworm-worked soils (Fig. 1). These results highlight the pervasive influence of earthworm activities on soil structure.

Our results also indicate significant differences between bacterial populations of soil fractions associated with earthworm and litter-only treatments, indicating that earthworms influence bacterial populations across hierarchical aggregate structures. Although earthworms facilitated alteration of bacterial community composition in all soil fractions analyzed, changes were greatest in macroaggregate communities. In addition to differences in T-RF size distributions, which are indicative of altered species composition, T-RFLP profiles derived from earthworm-treated macroaggregate fractions contained significantly fewer total T-RF sizes than all other soil fractions with the exception of the inner-miM fraction of the litter-only treatment (Fig. 4). The relatively few numbers of T-RF sizes in the litter-only inner-miM fraction is consistent with previous analyses of total inner-miM fractions of two different Wyoming soils (Mummey et al., 2006). This result is not surprising in that inner-miM fractions of undisturbed soils would be expected to be relatively limited in microhabitats and would therefore be expected to exhibit less diversity than analyses of more complex structures such as whole-microaggregates or macroaggregates.

Since a given target sequence must represent approximately 1% of sequences homologous to a specific primer pair to be detected using PCR-based methods (Moeseneder et al., 1999), T-RFLP-based methods detect only relatively dominant ribotypes present within a sample. Thus, fewer T-RF numbers in T-RFLP profiles of earthworm-worked macroaggregate fractions suggest less species evenness in this fraction, further suggesting that earthworm activities select for bacterial groups that dominate macroaggregates as a whole.

Additionally, ordination analyses indicated that alteration of bacterial communities due to earthworm activities was most pronounced at the macroaggregate scale (Fig. 3). These results are consistent with observations suggesting that after earthworm ingestion microbial substrates may rapidly become limited in microaggregates, and to a greater extent than in macroaggregates as a whole. For example, Bossuyt et al. (2005) found that *Aporrectodea caliginosa* activities resulted in rapid physical protection of newly added plant residue C in the miM fraction, but almost no protection of newly added C at the macroaggregate scale. These results imply pronounced differences in

spatiotemporal constraints on substrate resources between different aggregate size classes during aggregate formation and that residues not protected within miM remained largely available for microbial growth. A few studies suggest that microaggregates, specifically, begin to form before or rapidly after cast excretion (Shipitalo and Protz, 1989; Barois et al., 1993; Bossuyt et al., 2004; Bossuyt et al., 2005). Moreover, clays (Shipitalo and Protz, 1988) and organic matter dispersed within the earthworm gut may directly form microaggregates; thus, unlike current models for particulate organic matter or active root facilitated microaggregate formation in temperate soil (as outlined in Six et al., 2002) microbial activities (decomposition) may not necessarily be required for initial microaggregate formation in earthworm guts or casts (Shipitalo and Protz, 1989; Bossuyt et al., 2005; Pulleman et al., 2005).

The results of the ordination analysis of miM fractions are consistent with a model for homogenization of microbial populations during soil disruption in the earthworm gut and relative inactivity of microbes captured within newly formed miM. In treatments receiving only plant litter, macroaggregates and miM clustered distantly from inner-miM (Fig. 3B), suggesting distinctly different inner-miM and whole-miM populations. In contrast, earthworm-treated soil miM and inner-miM clustered distantly from macroaggregates (Fig. 3A), suggesting similar populations in inner- and whole-miM fractions.

The above discussion focused primarily on events occurring within cast materials. However, earthworm activities would be expected to differentially influence microbial populations in other regions within the soil matrix as well. Based upon aggregate hierarchical size and positional relationships, each aggregate size fraction, including interior and exterior regions of aggregates, would be subject to different constraints on substrate and gaseous diffusion. Hattori (1988) presented a conceptual framework for microbial habitats in soils consisting of two distinct environments: the inner and outer fractions of aggregates. Inner aggregate habitats would be expected to offer relatively stable water availability and protection against predation compared to outer-aggregate habitats. Due to differences in diffusional constraints, and competition with organisms residing in outer-aggregate regions, inner-aggregate habitats would be expected to be relatively limited in terms of substrates available for microbial growth. For example, Chenu et al. (2001) demonstrated that slow addition of glucose to soil macroaggregate fractions increased numbers of microorganisms on aggregate surfaces, but not in aggregate interiors. Earthworm-derived mucus, which contains water-soluble, readily decomposable organic compounds (Edwards and Bohlen, 1996), would also be expected to be subject to similar diffusional constraints. Thus, substrates released by earthworm activities may influence microbial populations inhabiting established macroaggregates to a greater extent than populations inhabiting microaggregates residing inside macroaggregates, especially those in proximity to burrows.

In this study we show that short-term earthworm-facilitated alteration of soil bacterial community composition occurs predominantly at the macroaggregate scale. This result is consistent with observations that new residue C is rapidly protected from decomposition in cast materials at the microaggregate scale but largely available for microbial decomposition at the scale of the macroaggregate. Further research is clearly needed to determine both earthworm-facilitated long-term spatially defined changes in microbial populations throughout the soil matrix, and temporal changes in bacterial populations associated with initial microaggregate formation at different stages during passage through the earthworm gut. The relative activity, functionality and population structure of microorganisms in different aggregate fractions as they age will potentially provide insights into long-term aggregate stabilization and C-sequestration; additionally, this will provide insights into how multi-organismal relationships influence the development and maintenance of soil microbial communities.

Acknowledgments

This work was supported by a grant from the United States Department of Agriculture-National Research Initiative.

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