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Microbiological characterization of the structures built by earthworms and ants in an agricultural field

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Abstract

The genesis and architecture of the structures built by ants and earthworms differ markedly, suggesting that—in addition to having different physical and chemical properties—the resident microbial community should also have unique properties. We characterized the inorganic N, biomass C, C mineralization rate, and functional diversity of the microbial communities of earthworm casts, earthworm burrow soil, ant mounds, and bulk soil from an agricultural field. Mound soil was most enriched in inorganic N and had the lowest pH, moisture content, and C mineralization rate. Functional diversity was evaluated by determining the ability of microorganisms to grow on 31 substrates using Biolog[®]EcoPlates in combination with a most probable number (MPN) approach. Casts had MPNs that were one to two orders of magnitude higher than burrow, mound and bulk soil for most substrates. Casts also had the highest MPNs for particular substrate guilds relative to bulk soil, followed by mound and burrow soil. Indices of substrate diversity and evenness were highest for casts, followed by burrow, mound, and bulk soil. Differences in the type of habitat provided by the structures built by ants and earthworms result in the differential distribution of nutrients, microbial activity, and metabolic diversity of soils within an agricultural field that affect soil fertility and quality.

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1. Introduction

The activities of ants and earthworms—both ecosystem engineers—have a significant impact on the physical, chemical, and biological properties of soil. These effects include changes in soil aggregation, preferential translocation of large soil particles, pore size distribution (Edwards and Bohlen, 1996; Lavelle and Spain, 2001; Eldridge, 1993), enhanced levels of inorganic nutrients (Dauber and Wolters, 2000; Görres et al., 1997; Wagner and Jones, 2006), and changes in the size and activity of microbial and microfaunal communities (Boulton and Amberman, 2006; Dauber et al., 2001; Savin et al., 2004; Wagner et al., 1997). The structures built by ants and earthworms differ in their genesis and architecture. Earthworms ingest soil and excrete it as casts, which they deposit in middens above ground or weld to the burrow walls (Edwards and Bohlen,

1996). Gut passage of soil reduces pores space (Görres et al., 2001) and changes the composition of the microbial community (Karsten and Drake, 1995). In contrast, material excavated by ants and subsequently deposited in mounds does not pass through the organism. The excretions of ants and earthworms may also select for different microorganisms (Amador and Görres, 2005).

There has been considerable interest in characterizing the genetic and functional diversity of the soil microflora over the past two decades (O'Donnell et al., 2005). While progress has been made in that direction, less is known about the factors that control microbial diversity at the field scale. Plants clearly play an important role as organizers of soil microbial communities (Wardle, 2005), but the role of the soil macrofauna remains poorly understood. The limited number of published studies suggests that environmental heterogeneities created by faunal disturbances in soil affect the distribution of nutrients and microorganisms, which may function to maintain diversity in ecosystems (Dihillion, 1999).

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The purpose of our study was to characterize the size, activity, and functional diversity of the microbial community of structures built by earthworms (casts and burrows) and ants (mounds), two ecosystem engineers commonly found in agricultural fields. Differences in the genesis and architecture of these structures may be expected to result in microbial communities that diverge in composition and activity.

2. Materials and methods

2.1. Site description and sampling

The study site was an agricultural field in Kingston, RI (USA) planted to tomatoes, cucumbers and pumpkins for five growing seasons prior to sampling. The soil was limed in the fall of 2002. Sampling took place on 27 May 2003, approximately 24 h after a high-intensity rain event (42.6 L m⁻² total precipitation, 7.7 L m⁻² h⁻¹ peak intensity; measured within a few kilometers of the field). This ensured that casts and mounds were deposited during the 24 h following the end of the rain event. A single sample each of ant mound, anecic earthworm cast and burrow soil, and bulk soil was taken from each of 10 randomly selected areas within the field. Burrow and bulk soil were taken from the top 2.5 cm of soil. Burrows were found by looking for casts. Only soil from burrows that were clearly inhabited (i.e. earthworms were found in burrow) was sampled. Burrow soil was removed by scraping the burrow to a distance of approximately 4 mm from the burrow wall with a small, narrow spatula. Bulk soil was sampled at least 10 cm away from the sampled burrow and other burrows. Ant mounds (generally < 10 cm in diameter) and casts were sampled with a small spatula. Samples were stored overnight in sealed plastic bags at 4 °C prior to analysis.

Equal amounts of unsieved soil were bulked by structure type for analysis of community-level physiological profiles (CLPP). All other soil properties were determined on subsamples removed prior to compositing. Roots and other debris were removed by hand and the composite samples mixed thoroughly before analysis. The use of composite samples is common in analyses of soil microbial community structure that involve molecular analyses (e.g. Smit et al., 2001), as well as those that employ CLPP (Preston-Mafham et al., 2002). The wide range of scales over which the spatial distribution of soil microorganisms is structured (Ettema and Wardle, 2002) makes bulking a practical approach to obtain an unbiased representation of the microbial community associated with different faunal structures at the field scale.

2.2. Physiological profiles

EcoPlates (Biolog, Inc., Hayward, CA, USA) were employed to determine microbial CLPPs using the method of Gamo and Shoji (1999). In conventional CLPP analyses, the ability of microorganisms to grow on multiple

substrates is tested using a single inoculum density. Differences in the growth kinetics of microorganisms using a particular substrate prevent interpretation of the data in terms of the population density of microorganisms growing on that substrate. The method developed by Gamo and Shoji (1999) involves varying inoculum density to determine the most probable number (MPN) of microorganisms capable of growing on 31 different substrates. The MPN approach involves determination of the presence or absence of microorganisms in individual portions of several consecutive dilutions of soil (Alexander, 1982). Combining determination of CLPPs with enumeration of microorganisms using MPNs addresses problems that arise from differences in inoculum density and kinetics of color development (Konopka et al., 1998; Preston-Mafham et al., 2002). A list of the substrates tested and the guilds (Zak et al., 1994) they were assigned to can be found in Table 1.

A major concern using the MPN approach to enumerate soil microorganisms is that they dwell on surfaces and removing them from these surfaces is difficult. For inoculation of the Biolog[®] EcoPlates the method identified by Balsler et al. (2002) to give the highest cell counts and inoculum densities was used. A known amount of soil (1.0 g f.w.) was diluted 1:10 with 50 μM phosphate buffer

Table 1
Growth substrates tested using Biolog[®] EcoPlates, corresponding well numbers, and associated guild groupings (Zak et al., 1994)

Guild (Abbrev.)	Substrate	Well no.
Amino acids (AA)	L-arginine	A4
	L-asparagine	B4
	L-phenylalanine	C4
	L-serine	D4
	L-threonine	E4
Amines and amides (AM)	Phenylethylamine	G4
	Putrescine	H4
Carboxylic acids (CA)	D-galactonic acid γ -lactone	A3
	D-galacturonic acid	B3
	2-hydroxybenzoic acid	C3
	4-hydroxybenzoic acid	D3
	γ -hydroxybutyric acid	E3
	D-glucosaminic acid	F2
	Itaconic acid	F3
	Glycyl-L-glutamic acid	F4
	α -ketobutyric acid	G3
	D-malic acid	H3
Carbohydrates (CH)	β -methyl-D-glucoside	A2
	D-xylose	B2
	i-erythritol	C2
	D-mannitol	D2
	N-acetyl-D-glucosamine	E2
	D-cellobiose	G1
	α -D-lactose	H1
Miscellaneous (M)	Pyruvic acid methyl ester	B1
	Glucose-1-phosphate	G2
	D,L- α -glycerol phosphate	H2
Polymers (P)	Tween 40	C1
	Tween 80	D1
	α -cyclodextrin	E1
	Glycogen	F1

(pH 7.2). The resulting suspension was shaken vigorously in a reciprocal shaker for 2 h. Six, ten-fold dilutions were used, with each dilution replicated three times to assess analytical error. Well inoculation volume was 150 μL . After inoculation, plates were incubated at 22 °C for 6 days. Absorbance at 595 nm was measured with a Model EL311 Automated Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). The minimum absorbance for positive wells was 0.30. MPNs were calculated as described by Woomer (1994).

2.3. Soil analysis

Soil pH was determined using a 1:10 (wt/vol) soil/water ratio and a pH meter. Moisture content was determined gravimetrically at 105 °C. The concentration of NO_3^- and NH_4^+ in soil samples was determined using a KCl extraction (Keeney and Nelson, 1982) and an automated nutrient analyzer. To determine C mineralization rate (C_{min}) a sample of field-moist soil (1 g) was placed in a 20-mL glass headspace vial and the vial sealed and incubated statically for 24 h in the dark at 22 °C. At the end of the incubation period, a sample of headspace gases was removed and the concentration of CO_2 determined by gas chromatography as described by Görres et al. (2001). Microbial biomass C (C_{mic}) was determined using the substrate-induced respiration method (West et al., 1986). A 1-g (dry weight) soil sample was incubated with 2 mL glucose solution (final concentration = 30 mg mL^{-1}) in a 20-mL glass vial. Vials were sealed, incubated at 22 °C and the CO_2 concentration in the headspace determined by gas chromatography (Görres et al., 2001) after incubation for 30 and 250 min.

2.4. Data analysis

Because we bulked 10 samples of each soil type for CLPP determinations, analysis of variance and means separation tests were not appropriate. Instead, we explored the data by comparing the analytical variation with the differences among the MPNs of soil types determined for each substrate. Analytical error was determined as the 95% confidence interval (Woomer, 1994) from the three replicates of each dilution. Specifically, we determined whether the mean MPN for a substrate, i , and soil type, j ,

fell within the 95% confidence interval of another soil type, k . If the mean MPN of soil j , $\langle \text{MPN}_j \rangle$, was an element (ε) of the 95% confidence interval of soil k , $[\text{MPN}_k]_{95\%}$, the test was assigned a score of 0, and a score of 1 if it did not. We then tested whether the mean MPN of soil k , $\langle \text{MPN}_k \rangle$, was an element of the 95% confidence interval of soil j , $[\text{MPN}_j]_{95\%}$. Scores of 1 and 0 were ascribed as before. Only if both tests resulted in a score of 1 did we assume that a difference between the soils was likely. This test can be described by the Boolean expression

$$B_{ijk} = \langle \text{MPN} \rangle_{ij} \varepsilon [\text{MPN}_k]_{95\%} \text{ AND } \langle \text{MPN} \rangle_{ik} \varepsilon [\text{MPN}_j]_{95\%}. \quad (1)$$

We further aggregated the sole carbon sources into guilds (Table 1). For each guild with N members an average score was calculated as

$$B_{\text{group}} = \frac{1}{N} \sum_{i=1}^N B_{ijk}. \quad (2)$$

B_{group} varies from 1 (soils j and k differ in all substrates) to 0 (soils j and k do not differ in any substrate). We computed the average for all soil comparisons within a guild.

We emphasize that this test is not a significance test, but a conservative measure of whether the MPNs for a carbon substrate, or for a guild, were sufficiently different between two soils to separate them by more than the analytical error of the method used.

Indices of substrate diversity (H), evenness (E), and richness (S) were calculated according to Zak et al. (1994).

Differences in soil properties among soil types were evaluated using a one-way analysis of variance ($P < 0.05$) and a pair-wise multiple comparison procedure (Student–Newmann–Keuls method).

3. Results

Cast, mound and burrow soil had a lower pH than bulk soil (Table 2). Cast and mound soil exhibited 10- and 3-fold NO_3^- enrichment, respectively, relative to bulk soil. Burrow soil was not enriched with NO_3^- . The concentration of NH_4^+ in mound soil was more than 25 times higher than in bulk soil, followed by casts (20 fold) and burrow soil (2 fold). Values of C_{mic} , C_{min} , and qCO_2 were similar for

Table 2

Mean values ($n = 10$) of pH, soil moisture, concentration of NO_3^- and NH_4^+ , microbial biomass (C_{mic}), C mineralization rate (C_{min}), and metabolic quotient (qCO_2) in soil from ant mounds, earthworm casts, earthworm burrows, and bulk soil in an agricultural field

Sample type	pH	Moisture content (%)	NO_3^- ($\mu\text{g N g}^{-1}$)	NH_4^+ ($\mu\text{g N g}^{-1}$)	C_{min} ($\mu\text{g CO}_2\text{-C g}^{-1} \text{d}^{-1}$)	C_{mic} ($\mu\text{g C g}^{-1}$)	qCO_2 ($\text{ng CO}_2\text{-C } \mu\text{g}^{-1} \text{C}_{\text{mic}} \text{d}^{-1}$)
Mounds	5.83	12.9a	3.4a	83.8a	14a	152a	94a
Casts	6.00	18.4b	10.5b	58.5b	28b	216b	130b
Burrows	6.34	15.0b	1.1c	6.3c	28b	193b	143b
Bulk soil	6.60	17.8b	1.0c	3.0d	21c	168ab	127b

Values followed by the same letter within a column were not significantly different ($P < 0.05$).

burrow soil and casts, and both were higher than bulk soil. In contrast, mound soil had lower values than bulk soil for all of these variables.

Casts had MPNs that were generally one to two orders of magnitude higher than for bulk, burrow, and mound soil

(Fig. 1). Microorganisms from bulk soil did not grow on 2-hydroxybenzoic acid, α -ketobutyric acid, or D,L- α -glycerol phosphate. In contrast, microorganisms from casts, burrow, and mound soil were capable of growth on all substrates except D,L- α -glycerol phosphate.

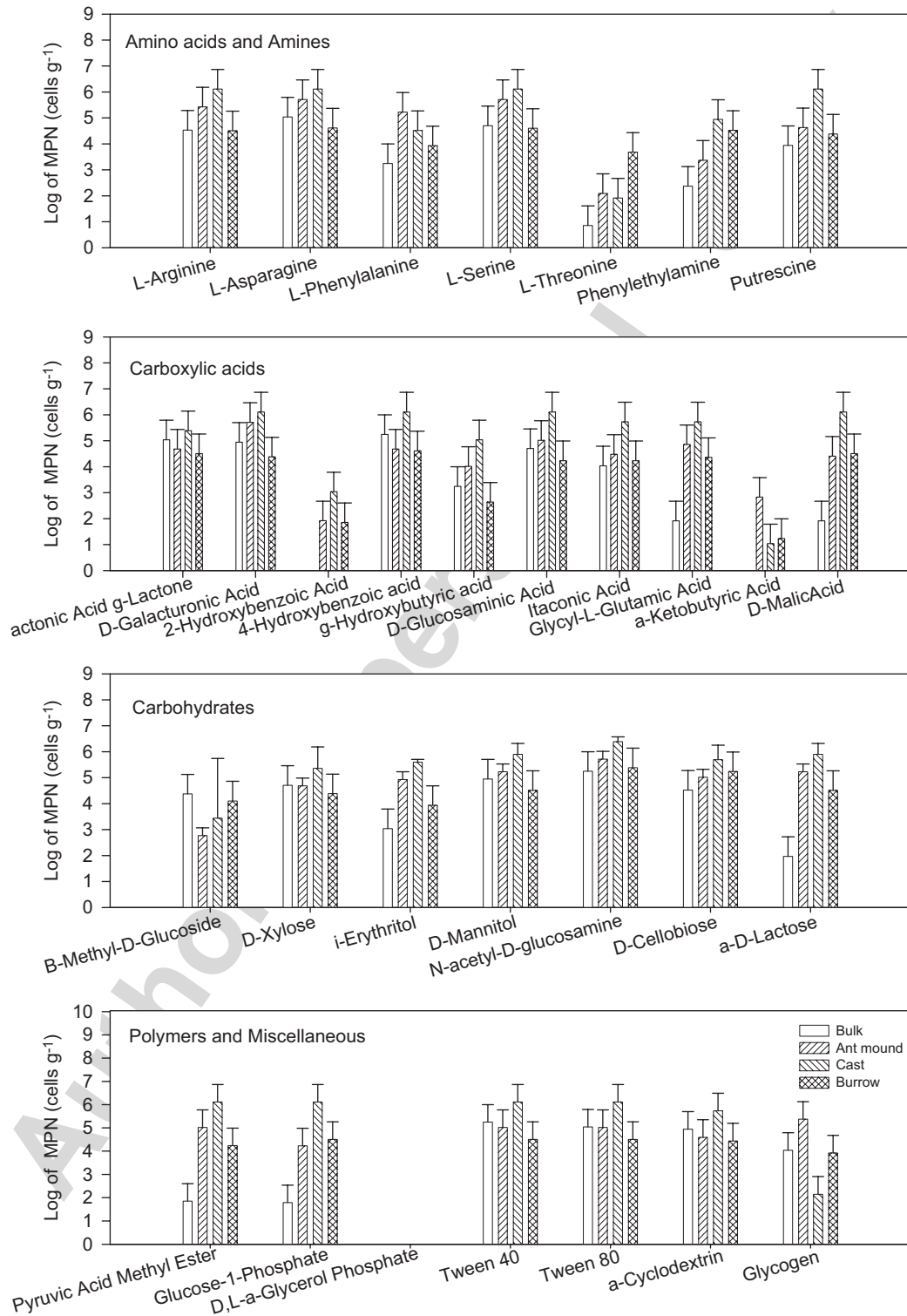


Fig. 1. Mean MPNs for bulk soil, earthworm burrow soil, earthworm casts, and ant mound soil for different substrate guilds. Three replicates drawn from a composited sample ($n = 10$) were analyzed per soil type; bars represent the 95% confidence level of the measurement. No growth was observed on D,L- α -glycerol phosphate.

Casts had MPNs that were higher than those in bulk soil for 29 of 31 substrates, with the remaining substrates being equal according to Eq. (2). All of the substrates in the carbohydrate, amino acid, and amide guilds differed in MPNs between casts and bulk soil (Table 3, Fig. 1). MPNs in mound soil were higher than in bulk soil for 19 of the 31 substrates tested, and substrates in the amide and amino acid guilds were different between mound and bulk soil (Table 3, Fig. 1). In contrast, MPNs differed little between burrow and bulk soil. Only 13 substrates differed by our criteria, with none of the guilds averaging a score of 1 (Table 3, Fig. 1).

Among engineered structures, the above-ground ones—ant mounds and earthworm casts—differed in MPNs for 78% of the substrates ($B_{\text{total}} = 0.78$, Table 3) with the greatest divergence observed for the amides, carboxylic acids, and polymers. Casts generally had greater MPNs than ant mounds.

Earthworm casts and burrows differed in 81% of the substrates ($B_{\text{total}} = 0.81$, Table 3) with the greatest divergence found in the carboxylic acid, carbohydrate and polymer guilds. Casts generally had greater MPNs than the burrow soils.

Values for substrate diversity (H) and evenness (E) followed the order: casts > burrows > mounds > bulk soil (Table 4). Average MPN per substrate was highest for casts, followed by mound, bulk and burrow soil.

4. Discussion

Enrichment of inorganic N at levels similar to those observed in the present study has been reported for structures built by ants (Dauber et al., 2001; Wagner and Jones, 2006) and anecic earthworms (Görres et al., 1997). C_{min} is also generally enhanced in earthworm burrow soil and casts (Edwards and Bohlen, 1996), as was the case in the present study. Microbial biomass may or may not be higher in these structures, depending on the timing of sampling relative to the inception of the burrow or excretion of casts and the availability of carbon and nutrients (Doube and Brown, 1998). Ant mounds had the lowest levels of C_{mic} and C_{min} , and the highest level of inorganic N of all the soils examined. The effects of ants on

these variables depend on species and age of mounds (Dauber et al., 2001). Soils with coarse textures have lower surface area and thus may have less habitat space for microorganisms, particularly bacteria (Postma and vanVeen, 1990). Likewise, moisture affects microbial activity because it controls both substrate and oxygen diffusion. If soils are too dry, substrates may not be sufficiently mobile to support microbial activity. The coarse texture and lower moisture content of ant mound soil may be responsible for the low microbial biomass and activity. Higher levels of inorganic N in ant mound soil can result from enhanced resource availability and improved conditions for microorganisms involved in N mineralization, as well as a low rate of N uptake by plants (Wagner and Jones, 2006). The low values of $q\text{CO}_2$, coupled with the high inorganic N levels observed in ant mound soil relative to other structures, may indicate a microbial community that processes C and N more effectively. Alternatively, the microbial community in the ant mounds may have been less active, with lower rates of N immobilization relative to mineralization.

The structures built by ants and earthworms differed in substrate richness, diversity, and evenness relative to bulk soil, with casts exhibiting the highest level of differentiation. Casts also had higher MPNs for most substrates relative to bulk soil. These results indicate that there is considerable divergence in substrate utilization patterns and in the relative contribution of different microbial populations in these structures. Because the above-ground structures we characterized can be presumed to have been built no more than 24 h prior to sampling, the data indicate that differentiation takes a relatively short time to develop. Differences in

Table 4
Substrate diversity (H), evenness (E), and richness (S), and average MPN per substrate for ant mounds, earthworm casts, earthworm burrows, and bulk soil

Type of structure	H	E	S	MPN per substrate (cells g^{-1})
Mounds	2.77	0.816	30	1.35×10^5
Casts	3.08	0.906	30	8.25×10^5
Burrows	2.83	0.831	30	3.50×10^4
Bulk soil	2.64	0.793	28	5.00×10^4

Table 3
Comparison of average Boolean scores, B , for substrate guilds among bulk, ant mound, earthworm burrow soil, and casts

Comparison		Value of B for substrate guild (No. of substrates in guild):						
		AA (5)	AM (2)	CA (10)	CH (7)	M (3)	P (4)	Total (31)
Bulk	Mounds	1.00	1.00	0.60	0.43	0.67	0.25	0.66
	Burrows	0.40	0.50	0.40	0.43	0.67	0.25	0.44
	Casts	1.00	1.00	0.90	1.00	0.67	1.00	0.93
Mounds	Burrows	1.00	0.50	0.40	0.57	0.33	0.25	0.51
Burrows	Casts	0.80	0.50	0.90	1.00	0.67	1.00	0.81
Casts	Mounds	0.40	1.00	0.90	0.71	0.67	1.00	0.78

Boolean scores can vary from 0 to 1, indicating the lowest and highest chance of a difference, respectively. Guild abbreviations as in Table 1.

microbial community composition have been observed between ant mounds and bulk soil based on substrate utilization patterns (Dauber and Wolters, 2000) and among earthworm casts, burrows and bulk soil using phospholipid fatty acid analysis (Enami et al., 2001) and nucleic acid and culture-based methods (Furlong et al., 2002).

A number of mechanisms may be responsible for differences in substrate utilization patterns. Utilization of a larger number of substrates in ant and earthworm structures than in bulk soil may be explained by soil translocation. Building of anecic earthworm burrows and ant mounds involves moving soil from deeper horizons to the soil surface, with consequent mixing of surface and deep soil. While there are fewer microorganisms in deeper soil horizons (Alexander, 2005), these may have different metabolic capacities from those at the soil surface. They can be brought to the surface on soil particles attached to body surfaces and, in the case of earthworms, in ingested soil. For casts and burrow soil—which may be lined with cast material—the source of microorganisms may also be the gut, which develops a stable microflora (Jolly et al., 1993; Karsten and Drake, 1995). In contrast, soil excavated to build ant mounds does not pass through the gut of the organism. Foraging by both earthworms and ants, and predation by ants, may also result in transport of microorganisms from food resources to faunal structures.

Changes in microbial habitat induced by the soil fauna may further select for some organisms over others. These changes can include closer contact with substrates and changes in pore and particle size distribution, and associated effects on moisture content and aeration. Ant mounds consist primarily of sand-sized particles, resulting in lower water holding capacity and increased drainage and aeration relative to bulk soil, conditions that can favor particular microbial populations. In the case of earthworms, repeated disruption of fungal hyphae in burrows may favor certain types of bacteria (Doube and Brown, 1998). Savin et al. (2004) found increased dominance of bacterial populations in earthworm burrow soil relative to bulk soil.

The chemical composition of gut and dermal exudates of earthworms are also likely to affect community composition. For example, Karsten and Drake (1995) observed that the intestinal environment of earthworms had equivalent aerobic and anaerobic microbial growth potential, and that the gut microflora consumed glucose, cellobiose, and ferrulate more readily than soil homogenates. In addition, various short-chain fatty acids were produced from the fermentation of glucose in the gut of these earthworms. Others have observed that gut transit in *Lumbricus terrestris* selects against bacterial populations in the α -, β - and γ -subdivisions of the Proteobacteria, whereas there is enhancement of bacteria in the δ -subdivision of the Proteobacteria and the *Cytophaga-Flavobacterium* cluster of the CFB phylum in cast soil (Schönholzer et al., 2002). For their part, ants produce antibacterial (Lavelle and Spain, 2001) and antifungal (Beattie et al., 1986) secretions

that may select against particular populations, promoting divergence of the microbial community from that of bulk soil.

Caution must be exercised in interpreting the data resulting from analysis of substrate utilization patterns of the microbial community from different soils (Konopka et al., 1998). Although using the MPN approach eliminates problems associated with differences in inoculation density, the method still detects only those organisms capable of growing under the assay conditions (e.g. high nutrient and substrate concentrations and oxygen availability). Thus, the divergence in community composition observed in the present study only represents the fraction of the microbial community capable of using the test substrates under the specific conditions of this assay. Furthermore, we did not include in our study the soil surrounding the tunnels and chambers of ant nests, which may make a unique contribution to the distribution of microorganisms in soil.

Our results show that the structures built by anecic earthworms and ants have microbial communities with characteristics that diverge from each other and from bulk soil. In addition to enhancing inorganic N levels and microbial activities, these animals affect growth substrate diversity, evenness, and richness in an agricultural field soil.

The number of earthworm burrows in soil generally ranges from 50 to 200 m⁻², and the mass of earthworm casts produced in arable soils in temperate areas can exceed 27 Mg ha⁻¹ yr⁻¹ (Edwards and Bohlen, 1996). In contrast, the mass of soil translocated by ants can range from 0.3 to 0.6 Mg ha⁻¹ yr⁻¹ (Lavelle and Spain, 2001), with fewer than 5 ant mounds m⁻² found in a temperate arable soil (Dauber and Wolters, 2004). These differences suggest that the structures built by earthworms may have a greater influence on microbial community structure. However, the relative contribution of the structures built by these two taxa to microbial diversity may be affected by ecological interactions and management practices. For example, wood ants do not affect earthworm abundance significantly (Laakso, 1999) or increase their abundance in particular in the nests of wood ants (Laakso and Setälä, 1997), but earthworms sometimes attract predatory ants (Yamaguchi and Hasegawa, 1996). In the present study we found ants and earthworms coexisting, but we did not examine how one affected the distribution of the other. Coexistence between the two taxa may increase soil microbial diversity at the field scale while spatially partitioning microbial taxa and their effects on soil properties and processes, with interaction between ants and earthworms possibly favoring specific microbial guilds. Within agricultural ecosystems, the spatial differences in microbial community composition associated with the structures built by these organisms likely translate into functional differences (e.g. residue decomposition, nutrient transformation, carbon sequestration, aggregate formation, disease suppression). This has implications for agricultural management approaches that rely on soil foodweb interactions for the maintenance of soil fertility and soil structure, such as conservation tillage,

that require a diversity of habitats to work effectively. Management practices that affect the distribution and activities of ants and anecic earthworms have the potential to affect the distribution and activities of soil microbial communities important for crop production and soil quality.

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