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**SOIL MICROBIAL COMMUNITIES FROM THE ALIMENTARY CANAL OF
THE EARTHWORM *LUMBRICUS TERRESTRIS* (OLIGOCHAETA:
LUMBRICIDAE)**

by

Joshua A. Chapman

Thesis submitted to the Davis College of Agriculture, Forestry and Consumer Science
at West Virginia University
in partial fulfillment of the requirements
for the degree of

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in
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manure, CLPP, DGGE

ABSTRACT

SOIL MICROBIAL COMMUNITIES FROM THE ALIMENTARY CANAL OF THE EARTHWORM *LUMBRICUS TERRESTRIS* (OLIGOCHAETA: LUMBRICIDAE)

by Joshua A. Chapman

The effect of earthworm (*Lumbricus terrestris*) activity on microbial community composition was investigated in a silt loam soil. Fingerprints were constructed using PCR amplicons of bacterial 16S *rDNA* separated by denaturing gradient gel electrophoresis. Analysis of similarity established that significant differences existed between community fingerprints. Differences were explored using non-metric multidimensional scaling (MDS) and unweighted pair-group method with arithmetic mean (UPGMA). Microbial communities of earthworm casts separated from soil treatments for both CD DGGE and CI DGGE profiles. Cast communities were determined by the worm's food source. Soils with earthworms and manure separated from all other soil treatments. Major trends observed above also were evident in separate analyses employing PCR amplification of *nirK* for DGGE, and BIOLOG ECO MicroPlates™ for community level physiological profiles (CLPP). Data suggest bacterial communities are modified after ingestion, however resultant changes in the soil bacterial community only occur when earthworms consume a soil manure mixture.

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GENERAL INTRODUCTION

Sustainable production of grass-fed pastured beef cattle is dependent on soil fertility and the ability to produce high quality forage. Since 1998, experimental grasslands at the West Virginia University Reedsville Experiment Farm located in Preston County, WV have been managed to promote sustainable grass-fed beef production. These pastures contain mixtures of orchardgrass (*Dactylis glomerata* L.), Kentucky bluegrass (*Poa pratensis* L.), and meadow fescue (*Festuca elatior* L.) in combination with red clover (*Trifolium pretense* L.) and white clover (*Trifolium repens* L.). Periodically, experimental plots are over-seeded with legumes (circa 5 kg ha⁻¹), amended according to two fertility regimes (P, K and lime additions), and the same stocking rate. Nitrogen is supplied by legumes in the pastures and by inputs from animal excreta (urine and manure) deposited by grazing cattle. Distribution of manure in the pasture is uneven resulting in patches where an enhanced microbial activity may lead to changes in soil nutrient cycling and fertility.

Soil quality generally is assessed using a combination of physical, chemical, and microbiological measurements and is operationally defined as the capacity of a soil to function within ecosystem boundaries to sustain biological productivity, maintain environmental quality and promote plant and animal health (Doran and Parkin, 1996). Since microorganisms are responsible for decomposition and recirculation of organic matter in soil, prokaryotic diversity potentially is a useful indicator of soil quality obtained by measuring changes in the composition and function of the soil bacterial community. In a previous study, Sutanto (2005) established experimental subplots at the

Reedsville farm on high and low fertility pasture sites and amended them with a manure treatment to examine the potential effects on microbial indices of soil quality. She observed shifts in the soil microbial community in these upland pasture soils as a function of the long-term levels of fertilizer input (high vs. low). However, data on the short-term impact of manure pats on soil bacterial communities were more variable, with greater differences generally observed in the upper 5 cm of soil compared with a 15 cm profile, suggesting that proximity to the decomposing manure pat was an important determinant. Field observations revealed large numbers of earthworms always were present under decomposing manure pats. The potential impact of earthworms on the redistribution of organic matter and microorganisms present in the manure was not systematically assessed. The working hypothesis of the present thesis was that earthworm feeding behavior affects bacterial community composition in pasture soil.

Earthworms play a major role in the degradation and stabilization of soil organic matter and strongly influence soil quality. Earthworms often are present in higher numbers around organic matter compared with bulk soil (Hendrix *et al.*, 1992). Earthworms consume soil and organic materials which enter the alimentary canal, pass through the digestive tract and are deposited back on the soil as fecal material, commonly referred to as earthworm casts. Earthworms excavate burrows that can extend from surface to subsurface soil horizons. Earthworms affect soil porosity by burrowing and depositing their casts as loosely packed aggregates on the soil surface. The diameter of burrows ranges from 1 to 10 mm (Lee, 1985). Pore spaces of this size are among the largest found in soil. This increase in soil porosity results in better soil aeration and increased water infiltration rates resulting in reduced erosion from heavy rain events

(Tomlin *et al.*, 1995). Earthworms can ingest and turn over large amounts of soil, thus affecting soil structure through the production of high organic matter containing casts that become soil aggregates. In addition, earthworms secrete copious amounts of epidermal mucus that is rich in mucopolysaccharides and proteins which can bind soil particles together (Lee, 1985).

Earthworms have an enormous influence on the dynamics and decomposition of organic matter. Their diet consists mainly of organic detritus in various stages of decay and includes bacteria, fungi, microfauna and mesofauna (Lee, 1985). The distribution of organic residues through the soil is related to the feeding behavior of different species.

Anecic species such as *Lumbricus terrestris* feed mainly on surface organic matter which they incorporate vertically into the soil profile or deposit as residue around the mouth of their burrows (Doubé and Brown, 1998). Other earthworm species burrow continuously through the upper soil horizons, mixing organic matter with mineral particles.

Earthworms affect nutrient cycling and availability of nutrients in soil. Plant available N, P and K often are higher in fresh earthworm casts than in bulk soil (Doubé and Brown, 1998).

Interactions between earthworms and soil bacteria still are not well understood. During cast formation in the earthworm gut, bacteria colonize actively decomposing organic residues. Several studies have shown higher microbial numbers in the earthworm gut and in the drilosphere compared to bulk soil (Daniel and Anderson, 1992; Devliegher and Verstraete, 1997; Edwards and Fletcher, 1988; Kristufek *et al.*, 1992; Parle, 1963; Schonholzer *et al.*, 1999; Tomlin *et al.*, 1995; Wolter and Scheu, 1999; Pedersen and Hendriksen, 1993). However, increases in microbial biomass within the earthworm gut

were not always observed (Daniel and Anderson, 1992; Scheu, 1987, Devliegher and Verstraete, 1995). Recent studies exploring this relationship have found that earthworm gut/cast microbial populations differ from that of soil. Specific phylogenetic groups have been found in higher numbers in earthworm guts/casts such as *Proteobacteria* (Schonholzer *et al.*, 2002; Devliegher and Verstraete, 1997; Fischer *et al.*, 1995; Furlong *et al.*, 2002) and *Actinobacteria* (Kristufek *et al.*, 1993). Microbial communities of earthworm guts/casts seem to be mostly derived from their food source but are modified within the earthworm due to different physiological conditions (Furlong *et al.*, 2002; Egert *et al.*, 2004). The microaerophilic intestines of earthworms have been found to activate ingested denitrifiers and other nitrate-dissimilating bacteria, which may impact nitrogen transformations within soils (Ihssen *et al.*, 2003). Earthworms may transport and disseminate specific microbial groups through the soil by their burrowing behavior (Thorpe *et al.*, 1996).

The present study was designed to investigate the influence of earthworms on bacterial populations in pasture soil impacted by cattle manure. Laboratory incubations of pasture soil, manure, and earthworms were performed using replicated soil mesocosms. The objective was to determine if earthworm feeding behavior triggered shifts in soil microbial communities. Molecular approaches (denaturing gradient gel electrophoresis; DGGE), microbial community physiological profiles (CLPP's), and microbial enumerations were employed to address this question. DGGE is a genetic fingerprinting method that allows comparison among samples and detection of shifts in populations over time or under different environmental conditions (McCaig *et al.*, 2001). CLPP's are used to assess the functional diversity of microorganisms based on sole-

source carbon utilization patterns (Zak *et al.*, 1994). Genetic diversity measured by DGGE and functional diversity measured by CLPP provide complementary experimental approaches to investigate the potential impact of cattle manure redistribution by earthworms on bacterial community composition and soil quality in upland pasture soil of northern West Virginia.

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CHAPTER I: MODIFICATION OF BACTERIAL COMMUNITIES IN A PASTURE SOIL BY THE FEEDING ACTIVITY OF EARTHWORMS

INTRODUCTION

Agricultural practices affect the diversity of soil microbial populations both temporally and spatially (McCaig *et al.*, 2001). Deposition of cattle manure onto pasture adds nutrients, organic carbon, and large number of microorganisms from the bovine gut microflora (Frostegard *et al.*, 1997). Microbiological hot spots occur in soil as the indigenous soil microbial community responds to the addition of readily decomposable organic matter. Earthworms play a major role in redistribution and recycling of manure through pasture soils and thus may be important determinants of soil microbial community structure (Edwards and Fletcher, 1988). Fertilized temperate pastures are among the most favorable habitats for earthworms, with densities of up to 2000 individuals per m⁻² (Edwards and Bohlen, 1996). This translates into a belowground earthworm biomass that can exceed the weight of the surface herbivores supported by pasture grazing (Blakemore, 2002). Earthworms ingest mineral soil and particulate organic matter, mix them with organic secretions in the gut, and then excrete them as fecal pellets called casts. Casts contain microorganisms present in the original food source and also are colonized by gut microorganisms all of which are subsequently deposited into soil. *Lumbricus terrestris*, the earthworm used in the present study, is an anecic species that forms extensive vertical burrows, pulls organic matter down into them, then excretes casts within the soil matrix or on the soil surface. Deposition onto the soil surface can constitute 90% of cast production, but casts also persist as stable

microaggregates in subsurface soils for long periods of time (Whalen *et al.*, 2004; Ge *et al.*, 2001). In temperate grasslands it was estimated that up to 70 t ha⁻¹ yr⁻¹ of soil was turned over into earthworm casts (Bouche, 1983). The daily rate of earthworm cast production was measured at 60 g m⁻² day⁻¹ in a grazed New Zealand pasture (Chevallier *et al.*, 2006).

The overall purpose of this study was to explore changes in composition of microbial communities in a silt loam pasture soil as earthworms (*Lumbricus terrestris*) fed on different food sources. We tested the following hypotheses: *i*) fingerprints of the microbial community in earthworm casts differ according to food source *ii*) microbial communities in earthworm casts differ from those in soil *iii*) deposition of earthworm casts alter the soil microbial community. Fingerprints of bacterial community structure were constructed using PCR amplicons of bacterial 16S *rDNA* separated by denaturing gradient gel electrophoresis (DGGE). Complementary analyses also were performed employing PCR amplification of *nirK* for DGGE and BIOLOG[®] ECO MicroPlates[™] for community level physiological profiles (CLPP).

MATERIALS AND METHODS

Sample Site and Mesocosms

All soils used in this study were collected from pastures on the West Virginia University Research Farm, Reedsville, West Virginia. The pastures are part of a beef cow/calf production system. Soils were a Gilpin silt loam (fine-loamy, mixed, semiactive, mesic Typic Hapludults) and a Wharton silt loam (fine-loamy, mixed, mesic Aquic Hapludults) (Patton, 1959). The predominant grasses in the area were orchard grass (*Dactylis glomerata* L.), Kentucky bluegrass (*Poa pratensis* L.), and meadow fescue (*Festuca elatior* L.) with red clover (*Trifolium pretense* L.) and white clover (*Trifolium repens* L.). Since 1998 these experimental pastures have been fertilized with two level of fertilizer inputs; i.e. high fertility (maintain soil available P at 134 kg ha⁻¹ and available K at 390 kg ha⁻¹) or low fertility (maintain soil available P at 67 kg ha⁻¹ and available K at 195 kg ha⁻¹). Soil was collected on June 1, 2005 from an area that had not received any experimental treatment, but had been set aside as reserve (area adjacent to plots 35 and 36, Appendices II). Prior to the current experiment the area was a hayfield and received minimum inputs of lime, phosphorus and potassium. The sod layer was removed and soil was collected with a spade at a depth of approximately 15 cm and put into large plastic bags then transported on ice back to the lab and stored at 4°C until the following day. Four mesocosms were constructed from approximately 19 L (height = 35 cm, base = 26 cm) cylindrical plastic containers that had ventilation holes (1 cm) placed in the bottom and side to prevent anaerobic zones. Plastic fine mesh screen (2 mm) was placed over ventilation holes to prevent earthworms from escaping. Prior to addition of

soil, mesocosms were surface sterilized with 95% ethanol and allowed to dry. Soil was sieved through a 4-mm-diameter screen and manually homogenized. Mesocosms were filled with soil to a level 10 cm below the top. The volume of soil was approximately 13.3 L in each mesocosm. Mesocosms were assigned to one of four treatments: *i*) control *ii*) earthworms *iii*) manure *iv*) earthworm and manure (Table 1).

Cattle manure samples also were collected from the Reedsville Research Farm the same day as soil samples. Manure pats were sampled two weeks following removal of cattle from grazed pasture plots (plots 15 and 16, Appendix II). This area (approx. 1 ha) was grazed as one paddock by eight cows and eight calves for seven days. Pats were sampled with a spade and placed in large plastic bags and transported back to the lab on ice where samples were stored at 4°C until the following day. Manure was sieved through a 4-mm-diameter screen and manually homogenized. To standardize manure addition to mesocosms, manure was packed into 100-mm-diameter Petri dishes and brought to approximately 100 g wet weight. Plates were inverted and manure placed on the soil surface of two mesocosm systems. Three simulated manure pats were added to each mesocosm that received manure.

Lumbricus terrestris earthworms were obtained from a commercial distributor (DMF Baits, Waterford, MI). Three earthworms picked randomly from each batch of 24 were confirmed to be *L. terrestris* by following an identification key (Dindal, 1990). Only adult earthworms were used as confirmed by the presence of a clitellum. Prior to addition to mesocosms the earthworms were washed three times in sterile distilled water and placed in sterilized 100-mm Petri dishes with sterile filter paper lining the bottom. Earthworms were starved and depurated for a period of 48 hr to remove previous gut

contents. Following the starvation period, earthworms again were washed three times as before and placed in designated treatments. Forty-five earthworms were added to each of the two mesocosms that were to receive an earthworm treatment. After addition of earthworms and manure mesocosms were kept in the dark at 25°C for a period of 120 hr (5 days).

Sampling: Soil, Manure and Casts

Following the incubation period soil, manure and earthworms were removed from the mesocosms. Each simulated manure pat was first removed using sterile forceps and placed in individual sterile bags and stored at 4°C until further analysis. Soil from each individual mesocosm was transferred into large sterile plastic bags. Forty-five earthworms were recovered from each treatment and divided into three groups of fifteen. Individual earthworms were washed three times in sterile distilled water and placed in sterile 100-mm Petri dishes with sterile filter paper. Earthworm casts were collected from Petri dishes every few hours and stored at 4°C until further analysis. Cast material was pooled from each of the three groups of each treatment. Soil was then manually homogenized, three separate samples (200 g sample⁻¹) were taken from each treatment and placed in sterile bags and stored at 4°C until further analysis.

Heterotrophic Plate Counts

Heterotrophic bacteria from soil, cast and manure samples were quantified with a standard plate count method (Zuberer, 1994). Heterotrophic plate count data obtained for each of the three samples of each material / treatment combination. Samples (1 g) were

diluted (1/100 dilution) in 1X Winogradsky Salt solution (WSS) (99 ml) and shaken vigorously (100 reps) to disperse bacterial cells. The solution was then serially diluted (1/10) to obtain a 10^{-5} , 10^{-6} , 10^{-7} dilution for soil, cast and manure samples, respectively. A 0.1 ml sample from dilutions 10^{-3} to 10^{-5} was spread on the surface of R2A medium (Ingredients L⁻¹: 0.5 g bacto yeast extract; 0.5 g bacto proteose peptone no. 3; 0.5 g bacto casamino acids; 0.5 g bacto dextrose; 0.5 g soluble starch; 0.3 g sodium pyruvate ($\text{CH}_3\text{COCO}_2\text{Na}$); 0.3 g potassium phosphate, dibasic (K_2HPO_4); 0.05 g magnesium sulfate (MgSO_4); 15 g bacto agar) (Difco, Detroit, MI) (100 mm) (3 reps) (n = 9). Dilutions of 10^{-4} to 10^{-6} were used for cast samples while 10^{-5} to 10^{-7} dilutions were used for manure samples. After incubating for 14 days at 25°C the number of colony forming units (CFU's g⁻¹ material⁻¹ (dry weight)) in each replicate of a sample was determined. Plates were only counted that had between 30 and 300 CFU's and data was log-transformed to obtain geometric means.

Community Level Physiological Profiles - BIOLOG[®]

The capability of soil microbial communities to utilize a variety of carbon sources was assessed using BIOLOG[®] ECO MicroPlates[™] (Biolog Inc., Hayward, CA) which contain triplicate wells of 31 carbon sources useful for soil community analysis. Three replicate samples (1 g dry wt.) were taken from each of the three samples per material / treatment combination (n = 27). Replicate samples were diluted (1/100) in WSS and vigorously shaken (100 reps) to homogenize the suspension. This suspension was then further diluted (1/10) to obtain a 10^{-3} suspension. The 10^{-3} dilution was used to inoculate (100 μL well⁻¹) BIOLOG[®] ECO MicroPlates[™]. Plates were incubated in the

dark at 25°C for 156 hr. The rate utilization of the carbon source was monitored by measuring absorbance at 590 nm. Readings were taken every 12 hr. using an automated plate reader (SpectraMAX 340 pc, Molecular Devices, Sunnyvale, CA). Average Well Color Development (AWCD) was calculated as the average optical density across all wells per plate. AWCD was used as an indicator of general microbial activity.

DNA Extraction

Culture-dependent

Aliquots (100 μ L) of the 10^{-2} dilution of each material / treatment combination ($n = 9$), described previously for CLPP, were spread onto four replicate R2A agar plates to obtain a 10^{-3} dilution (Difco, Detroit, MI). Plates were incubated aerobically for two weeks at 25°C. Cultured cells were washed and pooled from all four replicate plates using a total of 4 ml of sterile WSS and a sterile inoculating loop. The resulting cell suspensions were vortexed and frozen at -20°C until DNA was extracted. Samples were thawed, vortexed and DNA was extracted from aliquots of each cell suspension (1.8 ml) using the MoBio Microbial DNA extraction kit (MoBio Labs, Carlsbad, CA) following the manufacturer's protocol.

Culture-independent

Three replicate samples (0.5 g dry weight) were taken from each of the three samples of each material / treatment combination ($n = 9$). Total DNA was extracted using the MoBio Power Soil DNA Extraction Kit (MoBio Labs, Carlsbad, CA) following manufacturer's protocol. A Fastprep (FP 120-Bio101, Savant Instruments, NY, USA)

machine was used for the bead-beating step of the procedure. DNA was stored at -20°C until further analysis.

Polymerase Chain Reaction

16S rRNA gene

The variable V3 region of the 16S *rRNA* gene from the domain Eubacteria was amplified using the PRBA338F primer (5'-ACTCCTACGGGAGGCAGCAG-3') with a GC clamp and the PRUN518R primer (5'-ATTACCGCGGCTGCTGG). The PCR protocol included a 5-minute initial denaturation at 94°C, 30 cycles of 92°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, followed by 7 minutes at 72°C. The final 50-μL reaction mixture contained 1× PCR buffer (Promega, Madison, WI.), 3.2 μmol of MgCl₂, 0.8 μM of deoxynucleoside triphosphates (Promega, Madison, WI), 2% bovine serum albumin (Fischer Biotech, Pittsburgh, PA), 0.5 μM (each) of forward and reverse primers (IDT DNA, San Diego, CA), 0.5 units of Taq polymerase (Promega, Madison, WI), and ~50-200 ng of template DNA. PCR products were pooled, cleaned and concentrated using QIAquick PCR Purification Kit (Qiagen, Valencia, CA), and the amount of PCR product in the samples was determined by running gel electrophoresis on a 1.5% agarose gel with a mass ladder stained with ethidium bromide. Quantification of DNA in the PCR product was done using volume analysis in Quantity One Software (BioRad, Hercules, CA).

nirK gene

An approximately 450 b.p. region of the gene that encodes nitrite reductase (*nirK*) in denitrifying bacteria was amplified using the F1aCu primer (5'- ATC ATG GTS CTG CCG CG-3') and the R3Cu primer (5'- GCC TCG ATC AGR TTG TGG TT-3') with a GC clamp attached to the 5' end (Hallin and Lindgren, 1999). The PCR protocol included a 2-minute initial denaturation at 94°C, 35 cycles of 94°C for 30 seconds, 67°C for 60 seconds, and 72°C for 60 seconds, followed by 10 minutes at 72°C. The final 50- μ L reaction mixture contained 1 $\times$ PCR buffer (Promega, Madison, WI.), 1.5 mM of MgCl₂, 200 μ M of deoxynucleoside triphosphates (Promega, Madison, WI), 400 ng ml⁻¹ bovine serum albumin (Fischer Biotech, Pittsburgh, PA), 1 μ M (each) of forward and reverse primers (IDT DNA, San Diego, CA), 1.25 units of Taq polymerase (Promega, Madison, WI), and ~50-200 ng of template DNA. PCR products were pooled, cleaned and concentrated using QIAquick PCR Purification Kit (Qiagen, Valencia, CA), and the amount of PCR product in the samples was determined by running gel electrophoresis on a 1.5% agarose gel with a mass ladder stained with ethidium bromide. Quantification of DNA in the PCR product was done using volume analysis in Quantity One Software (BioRad, Hercules, CA).

Denaturing Gradient Gel Electrophoresis

16S rRNA genes

Approximately 150 ng for CD gels and 100 ng for CI gels of pooled PCR product was loaded into a polyacrylamide gel (8%) with a 40% (16.8 g urea; 16 ml formamide/100 ml) to 60% (25.2 g urea; 24 ml formamide/100 ml) denaturing gradient.

Gels were electrophoresed using the DCode™ Universal Mutation Detection System (BioRad, Hercules, CA) initially for 4 min at 200 V to drive samples into the gel and then voltage was reduced to 50 V at 60°C for 16 hrs. Gels were subsequently visualized by silver staining using the protocol of Caetano-Anolles and Gresshoff (1994). The developed gels were then scanned using Hewlett Packard ScanJet 7400c (Hewlett-Packard Co., Palo Alto, CA) in 600 pixel per inch resolution and saved in uncompressed Tiff format for further analysis.

nirK genes

Approximately 125 ng for CD gels and 100 ng for CI gels of pooled PCR product was loaded into a polyacrylamide gel (6%) with a 50% (21 g urea; 20 ml formamide/100 ml) to 70% (29.4 g urea; 28 ml formamide/100 ml) denaturing gradient. Gels were electrophoresed using the DCode™ Universal Mutation Detection System (BioRad, Hercules, CA) initially for 4 min at 200 V to drive samples into the gel and then voltage was reduced to 50 V at 60°C for 18 hr. Gels were subsequently visualized by silver staining using the protocol of Caetano-Anolles and Gresshoff (1994). The developed gels were then scanned using Hewlett Packard ScanJet 7400c (Hewlett-Packard Co., Palo Alto, CA) in 600 pixel per inch resolution and saved in uncompressed Tiff format for further analysis.

Data Analysis

Community Level Physiological Profiles - BIOLOG[®]

Statistical analyses were performed using SAS Statistical Software (SAS Institute Inc., Cary, NC). BIOLOG[®] ECO MicroPlates[™] plates were evaluated using Proc GLM with LSMeans and the tdiff option using a double nested analysis of variance (Nested ANOVA), where the values for each of the three replicate per plate were nested within the appropriate sample. Samples were nested within the appropriate treatments. The type III error term was used to test significance based on a P value of less than or equal to 0.05. A few plates inoculated with soil exhibited anomalous color development and were removed from further analyses because they appeared defective. These plates had very low AWCD and did not follow the color development exhibited by other plates. For BIOLOG[®] ECO MicroPlates[™] total well color development (TWCD) was calculated as the sum of absorbance values. Average well color development (AWCD) took the mean for those 31 absorbance values. Substrate diversity (H) was calculated as $H = -\sum p_i \ln p_i$, where p_i is the proportion (substrate absorbance divided by TWCD) of total microbial activity on a particular carbon source. Substrate richness (S) was calculated from the total number of wells with absorbance values greater than 0.25. Substrate richness per guild was calculated from the number of wells with absorbance values greater than 0.25 within each guild. Substrate evenness (E) was calculated as $E = H/\ln(S)$. Principal component analysis (PCA) was done on normalized data obtained by dividing each substrate absorbance value by the AWCD (Garland and Mills, 1991). Pearson's correlation was used to assess correlations between principal components and AWCD of each substrate.

Denaturing Gradient Gel Electrophoresis

DGGE gels were analyzed using Quantity One gel analysis software (BioRad, Hercules, CA). Analysis of the profiles was done by setting the background subtraction at 15 using a rolling disk method. Bands were manually picked based on the intensity profile for each lane. Bands that produced intensity peaks that were sharp and defined and greater than the base intensity value (0.05) were considered to be bands. Unweighted pair-group method with arithmetic mean (UPGMA) dendrograms were generated in Quantity One using the Dice similarity index. Bootstrap analysis (1,000 replications) was done using DGGestat (Van Hanne, 2005). Sorenson similarity index based on the presence/absence of bands was used for analysis of similarity (ANOSIM) test and ordination of data via multidimensional scaling (MDS). The Sorenson similarity index is analogous to the Bray-Curtis index when simple binary (presence/absence) data are used.

The result of MDS ordination is a plot where the position of each sample is determined by its distance from all other points in the analysis. An analysis of similarity ANOSIM routine was used to examine statistical significance between samples. ANOSIM tests the null hypothesis that the average rank similarity between objects within a group is the same as the average rank similarity between objects between groups. ANOSIM is based on the rank similarities between samples in the similarity matrix and produces a test statistic R which can range from -1 to 1. Objects that are more dissimilar between groups than within groups will be indicated by an R statistic greater than 0. An R value of 0 indicates the null hypothesis is true. A level of significance P value also is produced for the analysis. ANOSIM involves a test based on the permutations of all

samples and replicate samples and is free from any assumptions of normality, but not from the assumption of equal within-group dispersion (Rees *et al.*, 2004).

Heterotrophic Plate Counts

Statistical analyses were performed using SAS Statistical Software (SAS Institute Inc., Cary, NC). Log-transformed plate count data were evaluated using Proc GLM with LSMeans and the tdiff option using a nested analysis of variance (Nested ANOVA), where the geometric mean CFU values of 3 replicate plates for each samples were nested with the appropriate treatment. The type III error term was used to test significance based on a P value of less than or equal to 0.05.

RESULTS

Preliminary Observations and Experimental Design

A preliminary feasibility study was performed using mesocosms constructed to contain various combinations of pasture soil, cattle manure, and earthworms (Appendix I A, B). Earthworms rapidly established an extensive burrow system within the mesocosms and by visual estimate consumed greater than 50% of the added manure within a five day incubation period. Samples were conveniently recovered from the mesocosms for laboratory measurements such as enumeration of heterotrophic bacteria, CLPP, and DGGE analyses performed on soils and earthworm casts. Preliminary results from all analyses suggested that bacterial numbers, functional diversity, and genetic fingerprints changed after exposure of soils to cattle manure in combination with earthworms (Appendix I C, D, E, F, G, H). Based on these preliminary observations a replicated experiment was performed employing larger mesocosms (Figure 1) to investigate the impact of earthworm feeding behavior on soil bacterial communities. A summary flow sheet schematic of the experiment is presented in Figure 2.

Heterotrophic Plate Counts

Soil, earthworm casts, and manure were recovered from the various treatments (Table 1) and heterotrophic bacterial enumerations performed by spread plating onto R2A medium (Table 2). Counts were analyzed using a nested ANOVA and geometric treatment means separated using Tukey's HSD procedure ($P < 0.05$). Plate counts ranged from 10.28 to 6.97 and were significantly greatest in manure, decreased in earthworm casts, followed by bulk soil. Numbers of bacteria in casts recovered from worms feeding

on manure were significantly greater than those feeding on soil alone. In the former case, numbers in casts were lower than those obtained directly from manure; in the latter case, numbers were significantly higher than those obtained directly from soil.

Community Level Physiological Profiles

Functional diversity indices are presented in Table 3. Diversity indices were analyzed using a double-nested ANOVA and treatment means separated using Tukey's HSD procedure ($P < 0.05$). Functional diversity indices were significantly lower in unamended control soil compared with all other samples (Table 3). Intermediate values generally were observed in soil containing worms and in the resultant cast material obtained from worms feeding only on soil. The highest values generally were observed from soil containing worms feeding on manure, cast materials from these worms, and samples obtained directly from manure. Richness values, calculated as the mean number of positively utilized substrates, ranged from 21 to 28 and follow the above trend.

Substrates within BIOLOG[®] ECO MicroPlates[™] can be divided into six substrate guilds (carbohydrates, carboxylic acids, polymers, amino acids, amines/amides, and a miscellaneous category). Table 4 presents mean richness values separated by substrate guild. Richness was generally lowest in control soil and greatest in manure samples for the carbohydrate, polymer, amines/amides, and miscellaneous guilds. No significant differences between treatments were observed for the carboxylic acid and amino acid guilds.

Principal component analysis (PCA) used to explore multivariate BIOLOG[®] ECO MicroPlates[™] data is an ordination technique used to arrange multidimensional space into a low-dimensional space such that similar samples are close together and dissimilar samples are far apart. PCA based on adjusted average well color development is presented in Figure 3. Ordination using PCA resulted in distinct separations between treatments. Unamended control soil was well separated from all other treatments along the first principal component axis (PC1), followed by soil treated only with manure and soil treated only with worms. The remaining treatments were separated along the second principal component axis (PC 2). Soil with earthworms fed manure grouped closely with casts from earthworms fed only soil. Casts from earthworms fed manure grouped closely with both remaining manure treatments. Forty four percent of the total observed variation in data was accounted for by the first two principal components (PC 1= 26%, PC 2= 18%). PC 1 was most positively correlated with pyruvic acid methyl ester ($r = 0.907$), D-mannitol ($r = 0.875$) and, D-galactonic acid- γ -lactone ($r = 0.770$); and most negatively correlated with D-malic acid ($r = -0.602$), D-xylose ($r = -0.534$) and, x-D-lactose ($r = -0.525$). PC 2 was most positively correlated with 4-hydroxy benzoic acid ($r = 0.827$), L-phenylalanine ($r = 0.824$) and, Tween 40 ($r = 0.813$); and most negatively correlated with x-D-lactose ($r = -0.656$), D,L-x-glycerol phosphate ($r = -0.554$) and, D-galactonic acid γ -lactone ($r = -0.530$).

Denaturing Gradient Gel Electrophoresis

16S rRNA gene: Comparison of CD DGGE and CI DGGE bacterial fingerprints

Fingerprints of bacterial community structure were constructed using PCR amplicons of bacterial 16S *rRNA* genes separated by denaturing gradient gel electrophoresis. Both a culture-dependent and a culture-independent DGGE (CD DGGE and CI DGGE) method were used to examine untreated pasture soil; soil treated with manure; pasture soil that contained *L. terrestris* feeding on soil or a soil manure mixture; and *L. terrestris* casts obtained from earthworms feeding on soil or a soil manure mixture. Limited space on the DGGE gels precluded the inclusion of manure-alone samples. CD and CI derived PCR amplicons of bacterial 16S *rRNA* were separated on a single DGGE gel to directly compare the methods (Figure 4a). In order to fit all samples onto a single DGGE gel this experiment utilized a single replicate of each treatment composed of nine pooled PCR reactions.

Dice similarity coefficients were calculated and the unweighted pair-group method with arithmetic mean (UPGMA) was used to generate dendrograms for examination of clustering within community fingerprints (Figure 4b). CD and CI community fingerprints were highly dissimilar (20% similarity) resulting in two well-separated clusters. These data suggest that the two methods characterize a distinctly different segment of the soil bacterial community. Within each cluster separated by method, *L. terrestris* casts were distinctly dissimilar from all soil treatments (58% and 41% similarity for CD and CI, respectively). Bacterial communities in cast materials varied according to the worm's food source (67% and 66% similarity, for CD and CI respectively). Differences in community fingerprints between soil treatments were less

distinct, regardless of methodology. Control soils and those receiving only worms tended to cluster distinctly from soils receiving manure treatments.

Analysis of similarity (ANOSIM) derived from Sorenson's similarity values established that highly significant differences existed between community fingerprints. The ANOSIM R value was $R = 0.95$ ($P < 0.001$) for the combined CD and CI DGGE fingerprint. Differences in similarity data also were explored using non-metric Multidimensional Scaling (MDS). The large separation between CD and CI data (Figure 5) supported the clustering previously observed with UPGMA and indicates that different bacterial communities were detected by the two methods. Earthworm casts separated from all soil treatments for CI samples but not CD samples. No clear separation between any material or treatment combination was observed when CD profiles were analyzed via MDS.

The number of bands observed in the combined CI and CD DGGE gel are presented in Table 5, as are the number of distinct bands unique to each treatment. The total number of bands resolved using both the CD and CI approach were similar. CD gel analysis resulted in 74 total bands, with 20 unique to soil treatments and 18 unique to earthworm cast samples; CI analysis resulted in 61 total bands, with 16 unique to soil treatments and 26 unique to earthworm casts. CD and CI analysis each resulted in 9 bands unique to earthworm cast with manure; the greatest number of unique bands compared to all other treatments.

16S rRNA gene: Individual CD DGGE and CI DGGE bacterial fingerprints

In separate analyses, CD and CI 16S amplicons were separated on individual DGGE gels (Figure 6a and 8a). In these analyses, there were two replicate lanes per treatment for each material (soil and cast). These lanes were composed of three pooled PCR reactions that were amplified from three sub-samples of each of the three main samples. The general trends previously observed in the combined CD and CI analysis (Figure 4a) were comparable to results obtained on these separate DGGE gels employing increased replication. The greatest dissimilarity again was observed between microbial communities in earthworm casts and those in soil (Figure 6b and 8b). The similarities between these clusters were 46% and 27% in CD DGGE and CI DGGE, respectively. Bacterial communities in cast materials again varied according to the worm's food source. Soil-fed and manure-fed earthworm casts were 64% (CD) and 74% (CI) similar. Not previously observed, UPGMA cluster analysis separated soil with earthworms feeding on manure from other soil treatments (CD DGGE, 68% similarity; CI DGGE, 70% similarity). ANOSIM R values were as follows: $R = 0.93$ ($P < 0.001$) for CD-DGGE fingerprint; $R = 0.89$ ($P < 0.001$) for CI DGGE fingerprint. Ordination plots clearly separated earthworm casts from soil treatments in both CD DGGE and CI DGGE profiles (Figure 7 and 9). MDS analysis of the CD DGGE profile resulted in a significant separation between earthworm casts with manure and earthworm casts without manure. A different pattern to that from previous data was observed in the soil treatments. A small separation was observed between soils containing both earthworms and manure from other CD DGGE treatments. No separation of soil treatments was observed in the CI DGGE analysis.

DGGE: nirK gene

A parallel set of DGGE analyses to those described above for the 16S *rRNA* gene were performed using PCR amplicons derived from the *nirK* gene. Unlike the 16S *rRNA* gene which is universally present in all bacteria, *nirK* is a functional gene coding for a copper-containing nitrite reductase present in a subset of the denitrifying bacteria. Denitrification activity is known to be stimulated by the presence of earthworms (Horn *et al.*, 2003) therefore fingerprinting populations of these bacteria might provide a more sensitive indicator of bacterial population shifts due to earthworm feeding behavior. A combined CD/CI as well individual CD and CI DGGE gels were constructed for *nirK* using similar treatments and the same replication schemes reported for the 16S gels (Figure 10a, 12a, 14a). One *nirK* DGGE gel (Figure 10) did not include a control soil or soil plus worms treatment due to the inability to PCR amplify *nirK* from bacterial community DNA extracted from these samples.

Banding patterns of CD and CI derived *nirK* amplicons were distinctly dissimilar (17% similarity; Figure 10b). Within these major clusters earthworm casts clearly separated from all soil treatments (42% and 66% similarity, CD and CI analysis, respectively). ANOSIM derived from Sorenson's similarity values confirmed that significant differences existed between *nirK* community fingerprints. ANOSIM R value was $R = 1$ ($P < 0.001$) for the combined CI and CD DGGE fingerprint. MDS analysis (Figure 11) separated earthworm casts from all soil treatments for CD samples but not CI samples. Little separation occurred between CI treatments or materials. No separations in CD soil treatments were evident. Fewer total bands were produced using the *nirK*

gene than with the 16S *rRNA*. No substantial differences in total bands or unique bands were evident within a given treatment for the *nirK* combined gel (Table 6).

With both the individual *nirK* CD DGGE and CI DGGE gels the greatest dissimilarity was observed between microbial communities in earthworm casts and those in soil samples. The similarities between these clusters were 43% and 59% in CD DGGE and CI DGGE, respectively (Figure 12b and 14b). ANOSIM R values were: 0.64 ($P < 0.001$) for CD DGGE fingerprint; 0.70 ($P < 0.002$) for CI DGGE fingerprint. Ordination plots separated microbial communities from earthworm casts versus soil treatments for both CD DGGE and CI DGGE profiles (Figure 13 and 15). A large separation was observed between earthworm casts treatments in the CD analysis. In the CI DGGE experiment a small separation was observed between soil with earthworms feeding on manure versus other treatments.

DISCUSSION

Ingestion, digestion, and excretion of soil or manure by *L. terrestris* changed the number of heterotrophic bacteria detected in the resultant cast material compared with the original food source. Numbers of bacteria detected in casts obtained from manure-feeding worms were significantly lower than in the original manure. Numbers of bacteria in casts obtained from soil-feeding worms were significantly higher than in the original soil.

As a food source passes through the earthworm alimentary tract, numbers of bacteria present in the original material can be increased or decreased. This suggests that deposition of earthworm casts within the soil profile should modify numbers of bacteria present. However, in the present study no significant differences in bacterial numbers were found among the various soil treatments. This might be due to the relatively short experimental incubation period in the presence of worms resulting in a relatively small volume of cast material, or could be due to a dilution artifact from recovery and mixing of the all soil present in the mesocosm prior to further analyses. Similar data were reported by Pedersen and Hendriksen (1993) who also observed differential survival and growth of various gram negative bacteria within the earthworm alimentary canal. Several other studies have reported increased microbial numbers in the earthworm gut compared to bulk soil (Daniel and Anderson, 1992; Edwards and Fletcher, 1988; Kristufek *et al.*, 1992; Parle, 1963; Schonholzer *et al.*, 1999; Tomlin *et al.*, 1995; Wolter and Scheu, 1999). Devliegher and Verstraete (1997) found greater numbers of fluorescent pseudomonads associated with casts of *L. terrestris* compared to bulk soil. High

concentrations of organic residues and easily-degradable mucopolysaccharides can provide energy rich substrates supporting microbial growth within the earthworm digestive tract (Fischer *et al.*, 1997). Ingested microorganisms must also endure digestive enzymes and microaerophilic and/or anaerobic conditions, which could inhibit microbial growth or lead to cell death. In theory, the alimentary canal of the earthworm may inhibit the growth of some bacterial populations, favor the growth of others, while allowing others to pass unaffected. As a result, earthworms may selectively transport and disseminate specific microbial groups through the soil by their burrowing behavior (Thorpe *et al.*, 1996), thereby modifying soil bacterial community structure. Image analyses of stained thin soil sections recently have been used to directly demonstrate the presence of enhanced microbial numbers associated with earthworm casts in upper horizons of upland grassland soils (Bruneau *et al.*, 2005)

Garland and Mills (1991) first used BIOLOG[®] microtitre plates to measure community level physiological profiles (CLPPs) from the number and types of carbon substrates used by the heterotrophic bacterial community. BIOLOG[®] ECO MicroPlates[™] were developed for environmental studies and contain three replicate sets of 31 carbon substrates relevant to the growth of soil microorganisms (Campbell *et al.*, 1997). Inoculation of samples into BIOLOG[®] ECO MicroPlates[™] produces community-level physiological profiles (CLPP) of potential microbial C-source utilization. Limitations to CLPP measurements have been examined extensively (Garland, 1997, Konopka *et al.*, 1998, Preston-Mafham *et al.*, 2002). The assay primarily characterizes metabolically active copiotrophic bacteria, therefore correlations with actual *in situ* carbon utilization are limited. However, the assay allows rapid characterization of

relative changes in microbial functional diversity and is complementary to molecular techniques for community characterizations (Singh *et al.*, 2006). Previous studies have been able to ascertain shifts in the physiological processes of soil microbial communities due to soil type (Widmer *et al.* 2001; Girvan *et al.* 2003), amendment with crop residues and urine (Sharma *et al.* 1998; Williams *et al.* 2000; Bending *et al.* 2002; Malosso *et al.* 2005), and crop species and crop rotation (Bending *et al.* 2000; Grayston *et al.*, 2004, Meithling *et al.* 2000, Singh *et al.*, 2006).

Only one previous study has examined CLPP patterns in earthworm casts. Aira *et al.* (2006), reported that after gut transit through the earthworm *Eudrilus eugeniae*, the microbial CLPP profiles obtained from a food source comprised of pig manure were clearly differentiated from resultant earthworm casts. Functional diversity indices were higher in cast material, despite reduced overall respiration, biochemical enzyme activities, and C and N concentrations compared with the pig slurry.

In the present study, statistically significant differences were detected between treatments using functional diversity indices and clear separation of treatments by PCA were observed. In contrast to observations by Aira *et al.* (2006), diversity indices generally were lower in casts compared with cattle manure, but were higher than values observed in soil samples. Casts were separated from all soil samples except soil with earthworms fed manure. Casts from earthworms fed manure clustered most closely with manure samples.

As demonstrated by PCA, digestion of unamended soil by earthworms changed the microbial physiological profile. In contrast, digestion of manure-amended soil resulted in CLPP's more similar to the original manure food source. This difference is

likely attributed to the available organic carbon in the two materials. Manure bacteria in the earthworm gut probably experienced little change in carbon availability. Soil bacteria probably experience a more carbon and nutrient rich environment in the earthworm gut perhaps leading to activation of dormant and/or endospore-forming bacteria or enrichment of selected microbial groups. Resuscitation of bacteria would prime them for rapid growth within BIOLOG[®] ECO MicroPlates[™] (Horn *et al.*, 2003).

There was a large separation in PCA ordination between the soil control and all other soil treatments. The largest differences observed in soil samples occurred when earthworms feeding on manure were present. Earthworms in soil, especially when a microbe rich food source such as manure is present, appear to affect the dominant microbial physiological types enriched and detected in the BIOLOG[®] ECO MicroPlates[™] assay. This does not automatically imply long-term changes in soil physiological processes leading to changes in soil fertility or quality. Broad functional redundancy of soil microorganisms results in the ability of multiple taxa to carry out the same physiological function (Langenheder *et al.*, 2006). Therefore large-scale changes in microbial community composition may be necessary to induce shifts in the physiological activities that dominate soil microbial processes.

Muyzer *et al.* (1993) used denaturing gradient gel electrophoresis (DGGE) as a method to fingerprint the genetic diversity of environmental bacterial communities (Ovreas and Torsvik, 1998; Bruns *et al.*, 1999; Smalla *et al.*, 2001; McCaig *et al.*, 2001; Girvan *et al.*, 2003; Nicol *et al.*, 2003; Salles *et al.*, 2004). DGGE separates PCR amplicons of a target gene based on their percent GC content in a polyacrylamide gel containing a gradient of urea and formamide. DGGE fingerprints of soil microbial

communities often result in complex banding patterns, which are hard to resolve and compare, particularly when CI DGGE of the 16S *rRNA* gene is employed (Ovreas and Torsvik, 1998; Nakatsu *et al.*, 2000). CD DGGE is an alternative method that may provide simpler, more distinct fingerprints. Ellis *et al.* (2003) suggested that profiles of culturable bacteria may provide a sensitive ecological indicator due to their rapid response to environmental change. Duineveld *et al.* (1998) previously demonstrated that CI DGGE and CD DGGE profiles of soil microbial community 16S *rRNA* genes produced dissimilar community fingerprints. Gram-positive bacteria and *Proteobacteria* are the predominant bacteria reported using culture-dependent methods (Dunbar *et al.*, 1999). Members of the *Acidobacteria* and *Verrucomicrobia* are commonly detected using culture-independent methods (Hugenholtz *et al.*, 1998; Barns *et al.*, 1999).

In the present study, comparison on the same gel of CD DGGE and CI DGGE 16S *rRNA* fingerprints resulted in broad separation by methodology. Similar patterns of treatment separation were observed within each method. Cast microbial communities always were distinctly dissimilar from soil. Presence of manure in a cast or soil treatment generally resulted in distinct fingerprints compared with unamended samples. Microbial communities of earthworm guts/casts are derived from their food source but are modified within the earthworm due to different physiological conditions. Using terminal restriction length polymorphisms (T-RFLPs) of 16S *rRNA* genes, Egert *et al.* (2004) found significant differences in community profiles between soil and casts of *L. terrestris*. In contrast, work by Furlong *et al.* (2002) suggested similar community composition but increased abundance of phyla such as γ -*Proteobacteria* in casts compared to soils inhabited by *L. rubellus*.

Other soil-feeding invertebrates such as humivorous beetle larvae (Egert *et al.*, 2003) and soil feeding termites (Schmitt-Wagner *et al.*, 2003) possess characteristic indigenous microbial communities. Previous studies have suggested that earthworms also possess an indigenous bacterial population (Jolly *et al.*, 1993; Toyota and Kimura, 2000), however more recent studies refute these findings. Analysis of 16S *rRNA* genes in earthworm guts and casts suggest that the microbial communities in the earthworm gut is largely food derived (Furlong *et al.*, 2002; Egert *et al.*, 2004). Unique bands (bands not found in any other treatment) in DGGE profiles generated from earthworm casts could represent bacteria specific to the earthworm's alimentary canal. Earthworms used in this study were obtained from a commercial distributor and were kept in an artificial bedding material which could be a source of non-soil bacteria. Earthworms were depurated prior to use, however some of the original non-soil bacteria may have remained in earthworms digestive tract. If these bacteria persisted during soil consumption and passed through the digestive tract then perhaps they would be detectable by DGGE in cast materials. Enrichment and growth of soil bacteria within the earthworm gut to levels detectable by DGGE could provide an alternative explanation for the appearance of unique bands in cast material.

Earthworms fed both manure produced casts distinct from those fed soil alone. Disappearance of bands after passage through the earthworm digestive system suggests that some bacteria were consumed and digested rather than passing through unharmed. Bands present in soil but not in earthworm casts could result from selective grazing (Wolter and Scheu, 1999). It has been suggested that fungi, and protozoa, are the primary source of nutrients for earthworms, while bacteria and algae are less important as

nutrient resources and are not digested by earthworms (Brown, 1995; Edwards and Bohlen, 1996; Edwards and Fletcher, 1988). The greatest numbers of unique DGGE bands were associated with casts from manure fed worms, most likely due to the preferential feeding of the earthworms on the supplemented manure and associated microorganisms. Egert *et al.* (2004) found that cast microbial communities that were distinct from soil became divergent from soil communities when earthworms consumed a mixture of soil and leaf litter.

The capacity to denitrify is widely distributed among members of the *Proteobacteria* which are numerically prevalent in *L. terrestris* cast material (Fischer *et al.*, 1997). The earthworm gut is an ideal habitat for denitrification: anoxic conditions, high water content, sugars, organic acids, amino acids, nitrate, nitrite, and ammonium are all present in nonlimiting amounts (Horn *et al.*, 2003). The intestines of earthworms have been found to activate ingested denitrifiers and other nitrate-dissimilating bacteria, which may impact nitrogen transformations within soils (Ihssen *et al.*, 2003). Earthworms have a greater capacity to denitrify than bulk soil, producing large amounts of both N₂O and N₂ (Ihssen *et al.*, 2003; Karsten and Drake, 1997; Elliott and Anderson, 1990; Matthies *et al.*, 1999; Horn *et al.*, 2006). Various mechanisms have been proposed to explain differences in denitrification between earthworms, their casts, and bulk soil: *i*) the denitrifying community found in casts resulted from a pre-existing community attached to the gut wall (Singleton *et al.*, 2001), *ii*) digestive processes differentially lysed or stimulated ingested bacteria (Oliver, 1993), *iii*) selective feeding by earthworms (Wolter and Scheu, 1999), *iv*) culture-dependent resuscitation of viable but dormant cells (Egert *et al.*, 2003), and *v*) activation of dormant endospore-forming denitrifying bacteria

stimulated by availability of sugars and amino acids (Fischer *et al.*, 1995). Fischer *et al.* (1997) found passage through the gut of *L. terrestris* initiated spore germination of *Bacillus megaterium*, a significant member of the earthworm cast microbial community (Furlong *et al.*, 2002).

Functional genes encoding enzymes involved in the denitrification pathway can be targeted for community fingerprint analyses (Wolsing and Prieme, 2004). PCR primers producing amplicons suitable for DGGE recently have been developed for two genes; *nirK* and *nosZ* (Throback *et al.*, 2004). In the present study, denitrifying communities of pasture soil and *L. terrestris* casts were compared based on nitrite reductase (*nirK*) genes separated via DGGE. Similarities values from both CD and CI DGGE profiles resulted in significant differences between denitrifying communities of casts and soil. This dissimilarity became more evident when earthworms were fed on manure as an additional food source. These data contrast with the results of Horn *et al.* (2003) who cloned and sequenced nitrous oxide reductase (*nosZ*) genes from the gut of the earthworm *Aporrectodea caliginosa* to characterize denitrifying microbial populations. Results of this phylogenetic study revealed no significant differences between soil and earthworm gut communities, however higher numbers of *Pseudomonas*-related denitrifiers occurred in soil compared with the earthworm gut.

Nitrite reductase is a key enzyme in the process of denitrification. Two distinct structural types of dissimilatory nitrite reductases are known: i) a copper-containing enzyme (Cu-Nir) encoded by the *nirK* gene ii) and a heme c and heme d₁-containing enzyme (cd₁-Nir) encoded by the *nirS* gene. Suitable DGGE primers exist only for *nirK*, therefore the community fingerprints reported in the present study do not universally

cover the expected diversity of denitrifying bacteria. Numerically important soil denitrifiers such as *Pseudomonas fluorescens* contain the *nirS* gene and therefore would not be detected in the current analyses. (Gamble *et al.*, 1977; Coyne *et al.*, 1989). Casts of *L. terrestris* are reported to contain 100 fold more fluorescent pseudomonads that corresponding untreated soil (Devliegher and Verstraete, 1997).

Treatment differences observed with *nirK* DGGE were consistent with differences seen with 16S *rRNA* gene and with CLPP analyses. Previous reports found that physiological (CLPP) and molecular results were qualitatively similar suggesting the two approaches may be reporting on similar members of the microbial community (Crecchio *et al.*, 2004, Grayston *et al.*, 2004, Singh *et al.*, 2006). However, others report a lack of correlation. For example, Perez-Piqueres *et al.* (2006) saw changes in bacterial T-RFLP fingerprints caused by compost amendments that were not related to changes in CLPP. In the present study similarities between CLPP and DGGE were most apparent in respect to CD DGGE. These two methods may produce qualitatively similar results due to the fact that both methods employ the heterotrophic cultivable fraction of the soil community. The observation that CLPP and DGGE produce analogous results adds strength to the concept that the multiple method approach can be used to better characterize changes in soil microbial communities.

CONCLUSIONS

Data suggest that bacterial communities contained in the earthworms food source are modified after ingestion; however resultant changes in the soil bacterial community primarily occurred when earthworms consumed a soil manure mixture. Bacterial densities and substrate utilization changed upon digestion and excretion by earthworms, especially when manure was a food source, compared to unamended pasture soil. Molecular fingerprints of two genes (16S *rRNA* and *nirK*) also suggested changes in total and denitrifying communities upon ingestion and excretion of soil or manure. The greatest alterations in soil microbial communities generally were observed when earthworms consumed manure as a food source. Grazing beef cattle introduce exogenous bacteria to the soil environment. As earthworms move through the soil depositing cast material they translocate microorganisms that otherwise would be spatially limited to the area in which the manure was deposited. Whether these bacteria are able to persist and overcome the indigenous soil population or influence inherent nutrient cycles will require longer-term study.

In future studies it would be worthwhile to further characterize the microbial communities of earthworm casts through sequencing of unique and common DGGE bands to better understand how the digestive process of earthworms specifically changes microbial populations. Identifying specific organisms that are positively or negatively regulated by earthworms could provide insight into the properties of the earthworm alimentary canal that change the soil microbial community. Knowledge of the changing members of the soil community coupled with measurements of microbial processes (i.e., enzyme activity, biomass, denitrification and respiration) could provide a better

understanding of the interactions between soil bacteria and earthworms and their effect on soil quality and fertility. Parallel utilization of the functional genes *nosZ* and *nirS* for community fingerprinting should provide additional insights.

Table 1. Description of the experimental treatments.

Material	Treatment	Description
Soil	Control	Pasture soil that received no treatment
	Worm	Pasture soil incubated with <i>L. terrestris</i>
	Manure	Pasture soil incubated with manure pats on the soil surface
	Worms + Manure	Pasture soil incubated with <i>L. terrestris</i> and manure on the soil surface
Cast	Soil	<i>L. terrestris</i> casts collected from worms feeding only on pasture soil (from soil with worms treatment)
	Manure	<i>L. terrestris</i> casts collected from worms feeding on pasture soil and manure (from soil with worms and manure treatment)
Manure	w/out Worms	Manure collected from soil with manure treatment
	w/ Worms	Manure collected from soil with worms and manure treatment

Table 2. Heterotrophic plate counts obtained using R2A medium incubated aerobically at 25°C for 48 h.

Material	Treatment	CFU g⁻¹ material ^a	
Soil	Control	7.04	a
	Earthworms	6.97	a
	Manure	7.04	a
	Earthworms + Manure	7.06	a
Cast	Soil	8.09	b
	Manure	8.76	c
Manure	w/out Earthworms	10.28	d
	w/ Earthworms	10.18	d

^a Geometric mean values (n = 9) followed by the same letter are not significantly different (p < 0.05) as determined by Tukey's Studentized Range (HSD) Test).

Table 3. Bacterial functional diversity indices determined using BIOLOG[®] ECO MicroPlates[™].

Material	Treatment	n ^g	TWCD ^a	AWCD ^b	Richness ^c	Diversity ^d	Evenness ^e
Soil	Control	21	31.2 a	1.00 a	21 a	2.78 a	0.93 a
	Worm	21	34.7 b	1.12 b	23 b	3.00 b	0.95 b
	Manure	12	42.1 d	1.35 d	24 b	3.01 bc	0.95 b
	Worms + Manure	21	45.0 e	1.45 e	27 c	3.16 d	0.96 c
Cast	Soil	27	33.3 b	1.07 b	24 b	3.05 c	0.96 c
	Manure	27	39.0 c	1.25 c	27 c	3.17 d	0.97 c
Manure	w/out Worms	27	38.4 c	1.24 c	28 c	3.20 d	0.96 c
	w/ Worms	27	37.8 c	1.22 c	28 c	3.20 d	0.96 c
ANOVA ^f	P value		P<0.01	P<0.01	P<0.002	P<0.005	P<0.04

Mean values in each column within a particular treatment followed by the same letter are not significantly different (P<0.05) as determined by Tukey's Studentized Range (HSD) Test.

^a Total well color development (TWCD) = mean sum of blanked absorbance values.

^b Average well color development (AWCD) = TWCD/31.

^c Richness = mean number of wells greater than 0.25.

^d Diversity = $H = -\sum(\pi_i \ln \pi_i)$, where π_i = mean of each absorbance value / TWCD.

^e Evenness = $E = H/\ln(S)$, where H is diversity and S is richness.

^f P values for overall double-nested ANOVA test for substrate guild richness

^g Uneven treatment replication is due to unusual well color development in some BIOLOG[®] Eco plates. These plates were removed from the analysis.

Table 4. Richness within substrate guilds as determined by utilized substrates within BIOLOG[®] ECO MicroPlates[™].

Material	Treatment	n ^d	Carbohydrates (7) ^a		Carboxylic Acids (9)		Polymers (4)		Amino Acids (6)		Amines/Amides (2)		Misc. ^b (3)	
Soil	Control	21	4.1	a	6.4	-	3.1	a	3.9	-	1.4	ab	1.9	a
	Earthworms	21	4.4	a	7.5	-	3.5	c	4.3	-	1.2	ab	2.3	b
	Manure	12	5.0	b	7.1	-	3.7	cd	4.3	-	1.5	b	2.3	b
	Worms + Manure	21	5.4	bc	8.0	-	3.9	d	5.0	-	1.9	c	2.6	b
Cast	Soil	27	5.6	cd	6.4	-	3.3	ab	4.1	-	1.9	c	2.6	bc
	Manure	27	6.0	d	7.3	-	3.9	d	4.4	-	2.0	c	3.0	d
Manure	w/out Worms	27	7.0	e	7.2	-	3.9	d	4.6	-	2.0	c	3.0	d
	w/ Worms	27	6.9	e	6.9	-	4.0	d	4.9	-	2.0	c	3.0	cd
ANOVA ^c		P value	P < 0.0001		P < 0.1		P < 0.0025		P < 0.2		P < 0.0028		P < 0.0001	

Mean values in each column within a particular treatment followed by the same letter are not significantly different ($P < 0.05$) as determined by Tukey's Studentized Range (HSD) Test.

^a Values in parenthesis are number of substrates in each substrate guild.

^b Misc. = Miscellaneous

^c P values for overall double-nested ANOVA test for substrate guild richness

^d Uneven treatment replication is due to unusual well color development in some BIOLOG[®] Eco plates. These plates were removed from the analysis.

Table 5. Total and unique band counts for combined culture dependent and culture independent 16S *rRNA* DGGE profile (Figure 4a.).

Material	Treatment	Total Bands*		Unique Bands**	
		CD	CI	CD	CI
Soil	Control	33	26	1	3
	Worm	38	19	0	0
	Manure	41	17	3	0
	Worm + Manure	43	21	3	5
Cast	Soil	38	33	4	6
	Manure	43	34	9	9
Total	Soil	ND	ND	20	16
	Cast	ND	ND	18	26
	Soil & Cast	74	61	61	48

*Total values refer to the sum of all treatments for each material (soil or cast) or the sum of all treatments for both materials (soil and casts)

**Unique bands are defined as bands that are found in only one material/treatment and are not shared with any other material/treatment

ND = not determined

Table 6. Total and unique band counts for combined culture dependent and culture independent *nirK* DGGE profile (Figure 10 a.).

Material	Treatment	Total Bands*		Unique Bands**	
		CD	CI	CD	CI
Soil	Control	22	ND	0	ND
	Worm	24	ND	1	ND
	Manure	21	21	0	2
	Worm + Manure	23	24	0	3
Cast	Soil	16	20	1	1
	Manure	21	18	7	0
Total	Soil	ND	ND	15	10
	Cast	ND	ND	12	5
	Soil & Cast	38	31	33	26

*Total values refer to the sum of all treatments for each material (soil or cast) or the sum of all treatments for both materials (soil and casts)

**Unique bands are defined as bands that are found in only one material/treatment and are not shared with any other material/treatment

ND = not determined

Pasture Soil

Sample Soil

Remove contents of
intestines

5 Day Incubation

Collect worm casts

Cattle Manure

Sample manure pats

Figure 1. Depiction of the experimental process.

Figure 2. Experiment 2 flow chart.

Figure 3. Principal component analysis based on normalized well color development (average absorbance value for each substrate divided by AWCD) from BIOLOG[®] ECO MicroPlates[™] for pasture soil, *L. terrestris* casts and manure. Values in parenthesis on the axis indicate the proportion of the total variation accounted for by each principal component. See Table 3 for number of replicates per treatment (n).

(a)

(b)

Figure 4. (a) DGGE gel of PCR-amplified 16S *rRNA* genes amplified from culture-dependent and culture-independent bacterial communities of pasture soil and *L. terrestris* casts. Each lane is a pooled sample composed of nine PCR reactions (b) UPGMA dendrogram constructed using the Dice similarity matrix generated from culture-dependent and culture independent DGGE profiles of PCR-amplified 16S *rRNA* genes from pasture soil and casts from *L. terrestris* under different feeding conditions. The scale located above the tree indicates percent similarity. Numbers at nodes represent bootstrap values (1,000 resamplings). Bootstrap values below 40 were not reported.

Figure 5. Multidimensional scaling (MDS) ordination plot based on mean Sorenson similarity values of combined culture-dependent and culture-independent 16S *rRNA* DGGE profile of pasture soil and *L. terrestris* casts (Figure 4a.). Each material / treatment combination is a pooled sample composed of nine PCR reactions. ANOSIM $R = 0.95$ ($P < 0.001$) indicates a significant difference exists between treatment groups. CD (culture-dependent); CI (culture-independent).

(a)

(b)

Figure 6. (a) DGGE gel of PCR-amplified 16S *rRNA* genes amplified from culture-dependent bacterial communities of pasture soil and *L. terrestris* casts. Each treatment has two replicate lanes each composed of three pooled PCR reactions (b) UPGMA dendrogram constructed using the Dice similarity matrix generated from culture-dependent DGGE profiles of PCR-amplified 16S *rRNA* genes from pasture soil and casts from *L. terrestris* under different feeding conditions. The scale located above the tree indicates percent similarity. Numbers at nodes represent bootstrap values (1,000 resamplings). Bootstrap values below 40 were not reported.

Figure 7. Multidimensional scaling (MDS) ordination plot based on mean Sorenson similarity values of culture-dependent 16S *rRNA* DGGE profile of pasture soil and *L. terrestris* casts (Figure 6a). Each material / treatment combination has two replicate samples composed of three pooled PCR reactions. ANOSIM $R = 0.93$ ($P < 0.001$) indicates a significant difference exists between treatment groups.

(a)

(b)

Figure 8. (a) DGGE gel of PCR-amplified 16S *rRNA* genes amplified from culture-independent bacterial communities of pasture soil and *L. terrestris* casts. Each treatment has two replicate lanes each composed of three pooled PCR reactions (b) UPGMA dendrogram constructed using the Dice similarity matrix generated from culture-independent DGGE profiles of PCR-amplified 16S *rRNA* genes from pasture soil and casts from *L. terrestris* under different feeding conditions. The scale located above the tree indicates percent similarity. Numbers at nodes represent bootstrap values (1,000 resamplings). Bootstrap values below 40 were not reported.

Figure 9. Multidimensional scaling (MDS) ordination plot based on mean Sorenson similarity values of culture-independent 16S *rRNA* DGGE profile of pasture soil and *L. terrestris* casts (Figure 8a). Each material / treatment combination has two replicate samples composed of three pooled PCR reactions. ANOSIM $R = 0.89$ ($P < 0.001$) indicates a significant difference exists between treatment groups.

(a)

(b)

Figure 10. (a) DGGE gel of PCR-amplified *nirK* genes amplified from culture-dependent and culture-independent bacterial communities of pasture soil and *L. terrestris* casts. Each lane is a pooled sample composed of nine PCR reactions (b) UPGMA dendrogram constructed using the Dice similarity matrix generated from culture-dependent and culture-independent DGGE profiles of PCR-amplified *nirK* genes from pasture soil and casts from *L. terrestris* under different feeding conditions. The scale located above the tree indicates percent similarity. Numbers at nodes represent bootstrap values (1,000 resamplings). Bootstrap values below 40 were not reported. Soil control and soil with worm treatments were removed from analysis due to insufficient PCR amplification.

Figure 11. Multidimensional scaling (MDS) ordination plot based on mean Sorenson similarity values of combined culture-dependent and culture-independent *nirK* DGGE profile of pasture soil and *L. terrestris* casts (Figure 10a.). Each material / treatment combination is a pooled sample composed of nine PCR reactions. ANOSIM $R = 1$ ($P < 0.001$) indicates a significant difference exists between treatment groups. Soil control and soil with worm treatments were removed from analysis due to insufficient PCR amplification. CD (culture-dependent); CI (culture-independent).

(a)

(b)

Figure 12. DGGE gel of PCR-amplified *nirK* genes amplified from culture-dependent bacterial communities of pasture soil and *L. terrestris* casts. Each treatment has two replicate lanes each composed of three pooled PCR reactions (b) UPGMA dendrograms constructed using the Dice similarity matrix generated from culture-dependent DGGE profiles of PCR-amplified *nirK* genes from pasture soil and casts from *L. terrestris* under different feeding conditions. The scale located above the tree indicates percent similarity. Numbers at nodes represent bootstrap values (1,000 resamplings). Bootstrap values below 40 were not reported. Soil control and soil with worm treatments were removed from analysis due to insufficient PCR amplification.

Figure 13. Multidimensional scaling (MDS) ordination plot based on mean Sorenson similarity values of culture-dependent *nirK* DGGE profile of pasture soil and *L. terrestris* casts (Figure 12a). Each material / treatment combination has two replicate samples composed of three pooled PCR reactions. ANOSIM $R = 0.64$ ($P < 0.001$) indicates a significant difference exists between treatment groups.

(a)

(b)

Figure 14. (a) DGGE gel of PCR-amplified *nirK* genes amplified from culture-independent bacterial communities of pasture soil and *L. terrestris* casts. Each treatment has two replicate lanes each composed of three pooled PCR reactions (b) UPGMA dendrogram constructed using the Dice similarity matrix generated from culture-independent DGGE profiles of PCR-amplified *nirK* genes from pasture soil and casts from *L. terrestris* under different feeding conditions. The scale located above the tree indicates percent similarity. Numbers at nodes represent bootstrap values (1,000 resamplings). Bootstrap values below 40 were not reported.

Figure 15. Multidimensional scaling (MDS) ordination plot based on mean Sorenson similarity values of culture-independent *nirK* DGGE profile of pasture soil and *L. terrestris* casts (Figure 14a). Each material / treatment combination has two replicate samples composed of three pooled PCR reactions. ANOSIM $R = 0.70$ ($P < 0.001$) indicates a significant difference exists between treatment groups.

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APPENDIX I

Appendix I A. Summary flow chart describing a preliminary feasibility study used to determine the final experimental design employed in this thesis

Appendix I B. Feasibility study diagram

(C)

(D)

Appendix I. (C) DGGE gel of PCR-amplified 16S *rRNA* genes amplified from culture-dependent bacterial communities of pasture soil and *L. terrestris* casts. Pre treatment samples are before addition of manure and incubation in soil mesocosms. Post treatment samples are after addition of manure and incubation in soil mesocosms (D) UPGMA dendrograms constructed using the Dice similarity matrix generated from culture-dependent DGGE profiles of PCR-amplified 16S *rRNA* genes from pasture soil and casts from *L. terrestris* before and after treatment with cattle manure. The scale located above the tree indicates percent similarity.

(E)

(F)

Appendix I. (E) DGGE gel of PCR-amplified 16S *rRNA* genes amplified from culture-independent bacterial communities of pasture soil and *L. terrestris* casts. Pre treatment samples are before addition of manure and incubation in soil mesocosms. Post treatment samples are after addition of manure and incubation in soil mesocosms (F) UPGMA dendrograms constructed using the Dice similarity matrix generated from culture-independent DGGE profiles of PCR-amplified 16S *rRNA* genes from pasture soil and casts from *L. terrestris* before and after treatment with cattle manure. The scale located above the tree indicates percent similarity.

Appendix I G. Heterotrophic plate count enumerations of soil, *L. terrestris* casts and manure obtained from R2A media after 48 hr. incubation at 25⁰C*

Material	Treatment	CFU g ⁻¹ material ^{-1**}	
		Pre-Treatment	Post Treatment
Soil	Soil	7.10	7.03
	Manure	7.16	6.97
Cast	Soil	8.84	8.19
	Manure	8.88	8.87
Manure	-		9.39

* Pre treatment samples are before addition of manure and incubation in soil mesocosms. Post treatment samples are after addition of manure and incubation in soil mesocosms

** Geometric mean CFU values (n = 8)

ND = not determined

Appendix I H. Principal component analysis based on normalized well color development (average absorbance value for each substrate divided by AWCD) from BIOLOG® GN2 MicroPlates™ inoculated with soil, earthworm casts or manure. Soil 1 and 2 are soil samples prior to manure treatment. All other samples are post manure treatment. (n = 6)

APPENDIX II

Appendix II. WVU Agricultural Experiment Farm, Reedsville, WV