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Molecular and functional characterization of bacterial communities in organic farm and pasture soils

Sherie L. Edenborn
West Virginia University

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Molecular and Functional Characterization of Bacterial Communities in Organic Farm
and Pasture Soils

By

Sherie L. Edenborn

Dissertation submitted to the Davis College of Agriculture,
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at West Virginia University
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for the degree of

Doctor of Philosophy
in
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Approved by

Alan J. Sexstone, Ph.D., Chair
Gary K. Bissonnette, Ph.D.
Kenneth P. Blemings, Ph.D.
James B. Kotcon, Ph.D.
Daniel G. Panaccione, Ph.D.

Division of Plant and Soil Sciences

Morgantown, West Virginia
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bacteria, DGGE, CLPP, Small-celled bacteria, Spatial analysis

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ABSTRACT

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Sherie L. Edenborn

Culture-dependent denaturing gradient gel electrophoresis (CD DGGE) fingerprinting of the 16S rRNA gene was developed to characterize mixed soil bacterial communities recovered on agar plates. CD DGGE analyses resulted in clear banding patterns of sufficient complexity (16–32 major bands) and reproducibility to investigate differences in bacterial communities in silt loam soils. Replicate CD DGGE profiles from plates inoculated with less-dilute samples (10^{-3}) had a higher band count and were more similar (72–77%) than profiles from more-dilute samples (51–61%). When CD DGGE fingerprints were compared to profiles constructed from 16S rRNA genes obtained from culture-independent clone libraries (CB DGGE profiles) 34% of the bands were unique to the culture-dependent profiles, 32% were unique to the culture-independent profiles and 34% were found in both communities. CD DGGE fingerprints of the 16S rRNA gene were used to compare culturable microbial communities in soils from an organic farm and pastures that were subjected to differing agronomic treatments. Multiple and simple correlation analyses revealed significant relationships between microbial biomass carbon (MBC), potentially mineralizable nitrogen (PMN), and variables derived from principal component analysis (PCA) of community-level physiological profiling (CLPP), as well as non-metric multidimensional scaling (NMDS) of CD DGGE data. All measures clearly separated soil types, but varied in their ability to distinguish among treatments within a soil type. Overall, MBC, PMN, and CLPP were most responsive to additions of compost (organic farm) and manure (pasture); while CD DGGE resolved differences in legume cropping (organic farm) and inorganic fertilization (pasture). Bacteria from filtered ($< 0.45 \mu\text{m}$) and non-filtered slurries of soils from an organic farm were cultivated on R2A agar for up to one month. According to CD DGGE analyses, filtered and unfiltered communities were highly dissimilar and distinct small-celled bacteria were isolated from soils collected one year apart. Filterable bacteria were classified by phylogenetic analyses as α -, β -, γ -, or δ -*Proteobacteria* or *Bacteroidetes* with the genera *Polaromonas* and *Ramlibacter* representing 23% of the non-chimeric sequences in clone libraries. Other sequences were associated with known copiotrophic ultramicrobacteria, endophytic nitrogen-fixing bacteria in the genera *Herbospirillum* and *Azospirillum*, and bacteria adapted to inter- or intracellular growth. Sequences obtained from slow-growing, pinpoint-sized colonies were highly similar to an amoeba-resistant bacterium affiliated with the genus *Rhodoplanes*, a putative diazotroph that may play a potentially important role in soil nitrogen dynamics. Finally, the spatial variability and heterogeneity of microbial substrate utilization in tilled and untilled organic farm soils were explored using CLPP coupled with multivariate and geostatistical analyses. Robust semivariograms based on the kinetics of substrate utilization in BIOLOG GN2 plates were constructed. Microbial substrate utilization was spatially structured in some untilled

soils and subsurface tilled soils over ranges > 4 m. Rotary tillage completely disrupted the spatial structure of substrate utilization in surface soils. Amines and amides were the only substrate classes that exhibited a tillage-induced loss of spatial structure at lower soil depths. Spatial structure at lower soil depths in untilled soils was most pronounced for utilization of amino acids, amines and amides. Collectively, data presented in this dissertation support arguments that culturable soil microbial communities provide non-redundant and useful information for characterization of selected agronomic perturbations.

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INTRODUCTION

The productivity of agricultural soils is impacted by poor management and exploitive farming practices (Kaiser and Proffitt, 2004). As a result there has been an increased interest in the effects of agricultural stress on the sustainability and overall quality of soil (Parr et al., 1992). Because healthy ecosystems are characterized by high species diversity (Mader et al., 2002), and bacteria are known to play important roles in many biogeochemical cycles in soil, it has been suggested that the structural and functional diversity of bacterial communities can be used as indicators of changing soil quality (Parr et al., 1992).

Bacterial communities can be characterized using both culture-independent and culture-dependent methods. Over the past decade, culture-independent analyses of bacterial genes have become the preferred methods to profile the diversity of bacterial communities in environmental samples (Tiedje et al., 1999; Amann, 2000). Culture-independent methods are thought by some investigators to be superior to culture-dependent methods because they provide a more complete profile of total species richness (Amann, 1995; Ovreas and Torsvik, 1998). Others have argued that culturable bacteria are ecologically relevant because these are the members of the community that contribute most to biomass and activity (Ellis et al., 2003).

A current paradigm in microbial ecology states that less than 0.1–5% of bacterial cells observed directly in soil are able to be cultivated on bacteriological media (Ritz, 2007). It is also widely accepted that culture-dependent methods fail to detect novel groups, such as the *Acidobacteria* and *Verrucomicrobia*, which have been found to be

numerically abundant and widespread in soil when analyzed using culture-independent methods (Hugenholtz et al., 1998; Barns et al., 1999). Recently, these ideas have been challenged. Studies have demonstrated that novel bacteria, once thought to be unculturable, can be grown in culture when conditions include longer incubation times and diverse media (Janssen et al., 2002; Joseph et al., 2003; Schoenborn et al., 2004; Stevenson et al., 2004; Sangwan et al., 2005). Joseph et al. (2003) reported that 60 different family-level groups were represented by a total of 350 isolates grown on dilute nutrient agar. Thirty-three percent of these isolates were not validly named or described species, and 27% belonged to novel family-level groups. Approximately 4% were classified into four family-level groups that have not been formally described but may contain validly-named species. Twenty-nine of the isolates (8%) were characterized as *Acidobacteria* and one was from the division *Verrucomicrobia*. In a separate study, Janssen et al. (2002) were able to recover members *Acidobacteria* and *Verrucomicrobia* on dilute nutrient agar plates that were incubated for 12 weeks. Based on the actual viable counts (6.03×10^7 – 1.49×10^8 CFU/g soil), they estimated that approximately 20% of the viable cells could be cultivated on dilute media.

Culture-independent profiles of bacterial communities are constructed by extracting DNA or cells directly from soil. In the late 1970s and early 1980s, microbial DNA was extracted from soils using a cell extraction method that removed soil microorganisms from soil particles prior to lysis (Faegri et al., 1977; Torsvik and Goksoyr, 1978; Holben et al., 1988). One limitation of this procedure is the low recovery of bacterial cells bound to soil particles (Steffan et al., 1988). Holben et al. (1988) and Steffan et al. (1988) reported recoveries of 33% and 30% of the bacterial cells in soil,

respectively. Techniques such as mechanical blending have been used to increase the release of bacterial cells from soil. Lindahl and Bakken (1995) found that 30% of cells could be released from soil after six minutes of dispersion in a Waring blender, while higher rates (50–87%) were observed by Faegri et al. (1977) and Prieme et al. (1996). The ability to recover cells from soil is dependent on soil type. Hopkins et al. (1991) examined the recovery of cells from a clay loam (Hallsworth series—Typic Ochroaqualf), a sandy loam (Rivington series—a brown earth, Dystrochrept) and the peaty horizon from an upland stagnohumic-gley. They found that 56–61% of bacterial cells were released from all soils. In contrast, Frischer et al. (2000) reported that 96% of cells could be recovered from salt marsh sediments without phylogenetic bias.

Although cell extraction methods provided cleaner samples for extraction of DNA, the low recovery of cells from certain soils has limited the broad use of this technique. Ogram et al. (1987) developed a technique that involved direct lysis of cells attached to the soil matrix followed by the extraction of DNA. Various modifications of this technique have been developed to increase extraction efficiency (Zhou et al., 1996; Miller et al., 1999) and allow co-extraction of RNA with DNA (Griffiths et al., 2000; Hurt et al., 2001). Overall, the direct lysis method has been shown to result in much higher recoveries of DNA from soil samples (Steffan et al., 1988). However, substances such as humic acids interfere with the extraction, detection, and quantification of DNA (Zhou et al., 1996) and the amplification of genes using PCR. Theoretical calculations of extraction efficiency can be made based on the number of bacterial cells in soil before and after extraction and the average amount of DNA per cell. Zhou et al. (1996) used this approach to examine the efficiency of extraction from eight different types of soil.

Extraction efficiency ranged from 26–92%, with one soil showing higher cell counts in the soil post-extraction compared to pre-extraction. Based on their results, it was concluded that extraction efficiency was influenced by the clay content of the soil and the type of bacteria (gram-positive bacteria being more difficult to lyse than gram-negative bacteria). To study the potential loss of DNA via binding to the soil matrix, Frostegard et al. (1999) added varying amounts of naked lambda DNA to different soil types and then tried to recover it using different extraction methods. They found that grinding the soil increased the recovery of DNA from dry soil; however, no more than 6% of the added lambda DNA was ever recovered for any of the soil types.

A major criticism of culture-independent analyses is that they do not differentiate active cells from inactive cells. Stable isotope probing (SIP) is an emerging technology that links microbial activity to the organisms that are responsible for these activities (Boschker et al., 1998). Using this technique, a substrate labeled with a stable-isotope such as ^{13}C is added to an environmental sample, the labeled substrate is assimilated by certain cells in the population, and biomolecules containing the stable isotope are extracted and isolated from the total community and analyzed using standard methods such as polar lipid-derived fatty acid analysis, DGGE, or gene sequencing and cloning. Direct extraction of 16S rRNA from soil or co-extraction of DNA and RNA (Felske et al., 1998; Griffiths et al., 2000; Hurt et al., 2001) and selective removal of DNA from dead cells using ethidium monoazide (Nocker and Camper, 2006) are other approaches that have been used to characterize soil bacteria that may play a more active role in soil processes.

Neither culture-independent nor culture-dependent techniques can provide an absolute picture of soil bacterial diversity, suggesting that a combined approach may be more robust. Lipson and Schmidt (2004) used culture-independent and culture-dependent methods to study seasonal changes in bacterial communities in alpine soil of the Colorado Rocky Mountains. They concluded that seasonal shifts could be consistently demonstrated using both methods, although different groups of organisms were detected. Similar conclusions were drawn by Kisand and Wikner (2003), who compared the richness of estuarine bacterioplankton by using DNA-DNA hybridization of cultured isolates versus cloning and DGGE of 16S rRNA genes from culture-independent samples. They demonstrated that similar taxa were detected by all three methods at the genus level and higher; however, there was very little overlap at the species level.

The importance of combining culture-dependent methods with culture-independent analysis is being realized. Teske et al. (1996) stressed the ability of parallel analyses to provide a framework in which the strengths and biases of different methods can be evaluated. Joseph et al. (2003) and Ward (2006) pointed out that laboratory cultivation is needed to fully understand the biochemical and ecological potentials of bacteria that are currently only known from metagenomic and phylogenetic analysis of gene sequences. A recent debate in the journal FEMS Microbiology Ecology (volume 60(3): 2007) highlights the following question; “are studies of culturable communities still useful in the field of environmental microbial ecology?”. Ritz (2007) contends that culturable organisms are of limited use because they represent a minor fraction of the soil community and their growth in culture does not reflect conditions that exist *in situ*. In

contrast, Nichols (2007) points out that cultured organisms provide an essential complement to culture-independent studies by corroborating findings and providing a foundation for culture-independent analyses. In this dissertation, I provide information that is relevant to this debate by: i) developing an appropriate culture-dependent fingerprinting methodology to facilitate comparisons of culturable bacterial communities, ii) assessing culturable bacterial communities as a function of agronomic treatment, and iii) examining culturable small-celled bacteria, a numerically dominant, potentially important, and seldom-cultured fraction of the soil bacterial community. Specific objectives addressed by my research are listed below.

Objectives

1. To compare and contrast the culture-independent and culture-dependent methods to characterize soil bacterial communities in soils from an organic farm that have received different agronomic treatments. To develop and evaluate a DNA fingerprinting technique to characterize culturable bacterial communities in soil **(Chapter 1)**.
2. To culture and characterize small-celled soil bacteria, a numerically dominant fraction of the soil bacterial community that remains largely uncultivated **(Chapter 2)**.
3. To evaluate the effects of fertility regimes used on an organic farm and upland pasture on the structure and function of culturable soil bacterial communities, microbial biomass carbon and potentially mineralizable nitrogen **(Chapter 3)**.

4. To contrast the effects of tillage and no tillage on the variability and spatial heterogeneity of microbial substrate utilization in organic farm soils using multivariate and geostatistical techniques (**Chapter 4**).

The results of each chapter stand on their own to address questions that must be answered in order to better understand the diversity and ecology of soil bacteria and assess how agronomic practices impact the structure and function of soil microbial communities.

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Chapter 1: DGGE Fingerprinting of Culturable Soil Bacterial Communities Complements Culture-independent Analysis

Abstract

Culture-dependent DGGE (CD DGGE) fingerprinting of the 16S rRNA gene was used to characterize mixed soil bacterial communities recovered on agar plates. Using R2A agar as a growth medium, CD DGGE analysis resulted in clear banding patterns of sufficient complexity (16–32 major bands) and reproducibility to investigate differences in bacterial communities in a silt loam soil. Replicate CD DGGE profiles from plates inoculated with less-dilute samples (10^{-3}) had a higher band count and were more similar (72–77%) than profiles from more-dilute samples (51–61%). Different culture media and incubation conditions resulted in distinct community fingerprints and increased the cumulative number of unique bands detected. When CD DGGE fingerprints were compared to profiles constructed from 16S rRNA genes obtained from culture-independent clone libraries (CB DGGE profiles) 34% of the bands were unique to the culture-dependent profiles, 32% were unique to the culture-independent profiles and 34% were found in both communities. These data demonstrate that culture-independent DGGE profiles were supplemented by the distinct bands detected in culture-dependent profiles. CD DGGE can be a useful technique to follow the dynamics of distinct culturable fractions of the soil bacterial community.

Keywords: Culturable bacteria, DGGE, Plate wash, Bacterial community structure, Culture-dependent, Culture-independent.

Introduction

A current paradigm in soil microbiology states that bacterial communities are highly diverse and only a small percentage of environmental bacteria can be isolated in pure culture using standard laboratory media (Janssen et al., 2002). As a result, various non-culturing methodologies that depend on nucleic acid extraction and gene amplification have superceded the isolation and identification of bacteria growing on agar plates and have become the preferred approach to the study of population dynamics and genetic diversity of soil microbial communities (Hugenholtz et al., 1998; Ellis et al., 2003). However, recent studies suggest that culture-dependent and culture-independent methods each recover and resolve distinct soil bacteria, which suggests that a suitable combination of these approaches would be useful to obtain a more comprehensive overview of the soil community. In general, culture-based methods better represent copiotrophs including high GC gram-positive bacteria, whereas culture-independent methods provide better profiles of difficult-to-culture but widespread genera such as *Acidobacteria* and *Verrucomicrobia* (Barns et al., 1999; Smit et al., 2001; Lipson and Schmidt, 2004). Since many culturable bacteria exhibit fast growth rates and larger cell sizes (Bakken and Olsen, 1987; Bakken 1997), it is probable they also exhibit more short-term variation in community structure than slow-growing, difficult-to-culture bacteria. Ellis et al. (2003) have argued that culturable bacteria are important to soil ecosystem functions because of their higher total biomass and metabolic activity. Therefore, culturable bacteria may provide an ecologically-relevant complement to culture-

independent community characterizations and serve as responsive indicators of physical, chemical, and biological changes in the soil environment.

Significant impediments to routine characterization of culturable bacterial communities include isolation and identification of representative pure culture collections from many individual soil samples. I have approached this problem by pooling the bacterial cells growing on individual agar plates. Total DNA was extracted from this culture-dependent "community", followed by polymerase chain reaction (PCR) amplification of the 16S rDNA gene, and development of a community fingerprint using denaturing gradient gel electrophoresis. Culture-dependent DGGE (CD DGGE) is complementary to the culture-independent DGGE methodology first proposed by Muyzer et al. (1993) and since widely employed to investigate the dynamics of soil microbial communities in relation to changing environmental factors (Muyzer and Smalla, 1998; Øvreås 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).

The utility of CD DGGE for soil bacterial community characterization has not been systematically explored. Duineveld et al. (1998) and Ellis et al. (2003) previously used both culture-independent and CD DGGE to compare bacterial communities recovered from the rhizosphere of potted chrysanthemums and metal-contaminated soils, respectively. The two methods produced dissimilar community fingerprints; however, variable results obtained in the CD DGGE profiles suggested the need for better characterization and standardization of the technique.

The objectives of this study were to: 1) compare and contrast CD DGGE profiles with DGGE fingerprints constructed from 16S rRNA genes obtained from culture-

independent clone libraries, and 2) evaluate the complexity and reproducibility of DGGE fingerprints of the 16S rRNA genes from bacterial communities grown on agar plates. The results indicate acceptable reproducibility with the CD DGGE method and the ability to discern statistical differences between bacterial communities in different soil samples. I also demonstrate that by combining soil pre-treatments, incubation conditions, and various culture media, it was possible to resolve unique DGGE bands that were not resolved in culture-independent profiles.

Materials and Methods

Soil sampling and experimental design

Soil samples were collected three times in June 2004, February 2005 and August 2005 from market garden plots at the West Virginia University (WVU) Organic Research Farm located in Morgantown, West Virginia, USA (39° 39' 0.18" N; 79° 56' 11.6" W). Four studies were conducted to address various technical questions about the CD DGGE procedure. Details of the studies conducted, the experimental design, and sampling strategy are presented in **Table 1.1**. Soils on the farm are Dormont and Guernsy silt loams: fine loamy, mixed, superactive, mesic, Oxyaquic Hapludalfs (Wright et al., 1982).

Culture-dependent bacterial communities

Twenty grams (dry weight) of composite soil samples from each plot were placed in sterile Waring blenders and agitated for one minute at high speed (3x with intermittent

cooling on ice) in 180 ml of sterile Winogradsky's salts solution (WSS; Zuberer, 1994). Blended suspensions were serially diluted in sterile WSS prior to inoculation (100 µl) by spread plating onto 4 replicate agar plates. The media used included R2A agar, Tryptic Soy agar, Pseudomonas isolation agar (Difco, Detroit, MI), and Oil agar prepared as described by Sexstone and Atlas (1977). Unless otherwise noted, 1,000-fold (10^{-3}) diluted soil was routinely used to obtain heterotrophic soil bacterial communities grown on agar plates and incubated aerobically for two weeks at 25°C (**Figure 1.1**). In study 4, (**Table 1.1, Figure 1.4**), plates were incubated anaerobically and/or the diluted soil was heat shocked (80°C for 15 min) prior to inoculation of the agar plates.

Culture-dependent DNA extraction and PCR amplification of the V3 region of the 16S rRNA gene

Cultured cells were washed sequentially from four replicate plates using WSS (4 ml) and a sterile disposable inoculating loop. The resulting cell suspensions were vortexed and frozen (-20°C). DNA was extracted from aliquots of each thawed cell suspension (1.8 ml) using the MoBio Microbial DNA extraction kit (MoBio Labs, Carlsbad, CA). The variable V3 region of the 16S rRNA gene from the domain *Eubacteria* was amplified using the PRBA338F primer with a GC clamp (5'-ACTCCTACGGGAGGCAGCAG-3') and the PRUN518R primer (5'-ATTACCGCGGCTGCTGG). The final 50-µl reaction mixture contained 1× PCR buffer (Promega, Madison, WI), 3.2 mM of MgCl₂, 0.8 mM of a mixture of deoxynucleotide triphosphates (Promega), 0.2% bovine serum albumin, 0.5 µM (each) of the forward and reverse primers (Integrated DNA Technologies, San Diego, CA), 2.5 units of *Taq* DNA

polymerase (Promega), and ~50–200 ng of template DNA. The PCR protocol included a five-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. PCR products were cleaned and concentrated using QIAquick PCR Purification Kit (Qiagen, Valencia, CA). The amount of PCR product in the samples was determined by agarose gel electrophoresis of samples and compared with a mass ladder (Fermentas Inc., Hanover, MD). Quantification of DNA in the PCR product was done by volume analysis using Quantity One Software (Biorad, Hercules, CA).

Culture-independent DGGE fingerprints derived from pooled 16S rDNA clone libraries

In a preliminary study (unpublished data) I constructed conventional culture-independent DGGE profiles by direct amplification of 16S rRNA genes from soil DNA (Muyzer et al., 1993). As observed by others, DGGE fingerprints constructed using this method had complex profiles and smeared regions that complicated the identification and scoring of bands (Øvreås and Torsvik, 1998; Nakatsu et al., 2000). Therefore, I employed an alternative culture-independent approach that allowed me to partition 16S rDNA from soil by cloning PCR fragments into *E. coli* and then washing clones from plates in sufficient numbers to produce clear, interpretable DGGE profiles. This technique will be referred to as cloning-based DGGE (CB DGGE). An overview of the CB DGGE procedure and of the CD DGGE method are outlined in **Figure 1.1**.

Total soil DNA was extracted from sieved soil (~0.5 g wet weight) using the MoBio Soil DNA extraction kit following the manufacturer's protocol. A Fastprep (FP120-Bio101, Savant Instruments, NY) machine was used for the bead-beating step of

the experiment. The extracted DNA was used as a template for amplification of the 16S rRNA gene using primers 27f (5' AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTCTTACGACTT-3'). The final 100- μ l PCR mixture contained 1 $\times$ PCR buffer (Promega), 1.8 mM of MgCl₂, 200 μ M of a mix of deoxynucleotide triphosphates (Promega), 0.4% bovine serum albumin, 0.1 μ M (each) of forward and reverse primers (Integrated DNA technologies), 5 units of *Taq* polymerase (Promega), and ~300 ng of template DNA. The PCR protocol included 5-min initial denaturation at 94°C, 7 cycles touchdown with annealing temperatures decreasing in one-degree increments from 61°C to 56°C (92°C for 30 s (denaturation); 30 s (annealing); and 72°C for 90 s (elongation) for 30 s), 22 cycles as follows (92°C for 30 s (denaturation); 55°C for 30 s (annealing), and 72°C for 90 s), and 7 min at 72°C. PCR products were pooled, cleaned and concentrated using QIAquick PCR Purification Kit (Qiagen). Four microliters of the cleaned PCR product (~15–20 ng/ μ l) was then cloned using the Topo TA cloning kit for sequencing (Invitrogen, Carlsbad, CA). Cell suspensions were plated onto five or six Luria Broth (LB) plates with kanamycin (50 μ g/ml). Transformed clones were washed from each plate using a total of 4 ml of sterile WSS and a sterile disposable inoculating loop. Only positive recombinants are able to grow due to the disruption of the lethal *E. coli* gene *ccdB*; therefore, clones containing plasmids with no inserts are eliminated using this method (Bernard et al., 1994). The resulting cell suspensions were vortexed and frozen. Plasmid DNA was extracted from thawed aliquots of each cell suspension (1.8 ml) using the QIAprep Spin Miniprep Kit (Qiagen). The DNA was used as a template for PCR amplification of the V3 region of the 16S rRNA gene as described above.

Denaturing gradient gel electrophoresis

Composite PCR products (~ 150 ng) were 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) or a 40%–70% (29.4 g urea; 28 ml formamide/100 ml) denaturing gradient and electrophoresed for 14–16 hours at 50 V and 60°C using the DCode™ Universal Mutation Detection System (BioRad). The gel was visualized by silver staining using the protocol of Caetano-Anolles and Gresshoff (1994). The developed gel was scanned using Hewlett Packard ScanJet 7400c (Hewlett-Packard Co., Palo Alto, CA) at a resolution of 600 dpi and saved in uncompressed TIFF format for further analysis.

Statistical Analysis

Quantity One gel analysis software (BioRad) was used to analyze all DGGE gels. Band analysis was performed by setting background subtraction at 15 using the rolling disk method. Bands with intensity < 0.05 were excluded from the analysis. Unweighted pair-group method with arithmetic mean (UPGMA) trees were generated in Quantity One based on Sorensen's similarity index. Bootstrap analysis (1,000 replications) was done with the DGGESat program (Erik Van Hannen; <http://ddgehelp.blogspot.com>). Similarity matrices were calculated using Sorensen's index, and analysis of similarity (ANOSIM) was used to determine significant differences between DGGE banding profiles (R package Vegan; Oksanen, 2005). In ANOSIM, an R value greater than 0 indicates that the DGGE profiles between treatment groups are more dissimilar than profiles within groups. P values indicate the level of significance (Rees et al., 2004). Finally, the numbers of bands in each DGGE profile were compared using a

one-way Analysis of Variance (ANOVA) and students t-test. Statistical analyses were done in R (R Core Development Team, 2004).

Results

CD DGGE analysis of bacterial communities from R2A plates inoculated with different dilutions of soil

CD DGGE of the 16S rRNA gene was used to compare the bacterial communities that grew on R2A plates inoculated with different soil dilutions from high- and low-input soils (**Figure 1.2a**). More bands generally were observed in DGGE profiles of communities from plates inoculated with more concentrated soil suspensions (**Table 1.2**). The 10^{-3} profiles had an average of eight more bands than the 10^{-6} profiles, although the differences were not statistically significant (ANOVA, $P < 0.27$). A greater similarity was observed between replicate DGGE profiles of bacterial communities from the 10^{-3} dilution (72–77%) compared to those from the 10^{-6} dilution (51–61%). There were significant differences in the DGGE profiles between the 10^{-3} and 10^{-6} dilutions ($R=0.41$, $P < 0.05^*$), but no significant differences between the 10^{-4} and 10^{-5} dilutions ($R=0.25$, $P < 0.07$) as determined by ANOSIM, a non-parametric multivariate test. UPGMA analysis separated bacterial communities into two major clusters (**Figure 1.2b**), which suggested that the soil dilution used to inoculated the plates influenced the communities growing on the agar plates more than source of the soil (high- or low-input soil). No distinct clusters differentiated the 10^{-4} and 10^{-5} communities (data not shown). There were significantly more bands in the lower region (relative front > 0.60) of the DGGE gel (**Figure 1.2a**) in the 10^{-3} samples as compared to the 10^{-6} samples (t-test, $P < 0.002^{**}$).

Impact of plot variability on CD DGGE profiles of soil bacterial communities

Composite soils samples (10 cores per plot) were collected from the six treatment plots at the West Virginia University Organic Research Farm (n=3 for low-and high-input plots). CD DGGE profiles were constructed for four replicate sub-samples from each composite soil sample using 10^{-3} soil dilutions and R2A media (DGGE gels not shown). Cluster analysis (UPGMA) revealed that sub-samples were more similar to each other than to samples from replicate plots (**Figure 1.3**). Average percent similarity between replicate sub-samples was $84 \pm 6\%$ for the high-input soils and $85 \pm 3\%$ for the low-input soils. Among plots, the average percent similarity was $62 \pm 6\%$ for high-input soils and $73 \pm 5\%$ for low-input soils. ANOSIM indicated that the differences in DGGE profiles from different plots were highly significant for both the high-input samples ($R=0.99$, $P < 0.001^{***}$) and low-input samples ($R=0.96$, $P < 0.001^{***}$).

Comparison of CD DGGE profiles of soil bacterial communities subjected to different culture conditions and pretreatments

A single composite soil sample obtained from one low-input plot was subjected to different pretreatments and incubation conditions. UPGMA dendrograms (**Figure 1.4b**) revealed two distinct clusters of CD DGGE profiles of bacterial communities grown on different media (**Figure 1.4a**). One cluster was composed of communities capable of growing at low-nutrient concentrations (R2A) or on complex carbon sources (Oil agar). In contrast, communities in the second cluster were defined by media selective for copiotrophic bacteria (TSA and Pseudomonas isolation agar). Overall, the average

percent similarity between replicates was $80 \pm 8\%$ and between media $44 \pm 13\%$. Either five and six more bands were observed from communities grown on R2A (27 ± 2) and Oil agar (26 ± 3) than on Tryptic soy agar (21 ± 4) and Pseudomonas isolation agar (21 ± 2). ANOSIM demonstrated significant differences in the DGGE profiles as a function of media type ($R=0.98$, $P < 0.001^{***}$). Incubating plates under anaerobic conditions and/or heat shocking the soil resulted in distinctly different communities (ANOSIM, $R=1.00$, $P < 0.001^{***}$; **Figure 1.4c,d**). The percent similarity among replicates exhibited an overall average of $80 \pm 6\%$ and clear and distinct clusters were observed based on soil pretreatment. The percent similarity among treatments was the lowest observed in this study ($29 \pm 9\%$) indicating distinctly different communities. More bands were observed for communities that had been incubated aerobically (19–27) than anaerobically (12–19).

Comparison of CB and CD DGGE fingerprints

Culture-independent DGGE profiles constructed using the cloning-based approach are presented in **Figure 1.5**. Clone libraries of 16S rRNA genes conventionally are used for phylogenetic analysis of individual isolates; however, my results suggest that cloning is also an effective way to produce culture-independent DGGE fingerprints. I refer to this approach as cloning-based DGGE (CB DGGE) to distinguish this approach from conventional culture-independent analyses based on direct extraction and amplification of bacterial DNA in soil. Methods similar to CB DGGE previously have been employed to screen clone libraries (Muyzer and Smalla, 1998) and fractionate bacterial communities in biofilms into manageable subsets (Burr, 2006) but, to my knowledge, has not been used for comparison of soil bacterial communities.

When approximately 1000 clones were pooled for CB DGGE from high-input soils, complex profiles were observed (61 bands; **Figure 1.5, Lane 1**). In contrast, 77 clones from the low-input library produced 31 distinct DGGE bands (**Figure 1.5, Lane 2**). CB DGGE profiles were compared with composite CD DGGE profiles obtained using pooled PCR samples from separate studies previously reported in **Figure 1.2** through **Figure 1.4** (**Table 1.1, Figure 1.5**). In contrast to CB DGGE profiles, very few bands were resolved in the lower region of the CD DGGE profiles. I found that the number of bands unique to the CB DGGE decreased when communities cultured using different media and incubation conditions were included in the comparison (**Table 1.1, Figure 1.6**). When all the culturable communities were included in the analysis, the number of bands detected only in the CD analysis (40) was comparable to the number found only in the CB profile (37). Including CD DGGE profiles from all studies increased the number of unique resolvable bands to 116 and the number shared in both CB and CD profiles to 39.

Discussion

DGGE fingerprinting does not provide complete coverage of soil bacterial diversity since only a limited number of bands can be resolved in any fingerprint (Muyzer and Smalla, 1998; Nikolausz et al. 2005). Culture-independent analysis theoretically should provide improved coverage of community richness due to recovery of difficult-to-culture bacteria; however, factors such as DNA extraction bias and relative abundance of 16S rRNA genes in the environmental samples will affect the fingerprints obtained. The relative contribution of culturable compared with difficult-to-culture bacteria to DGGE community profiles is not commonly reported. In this study, I compared culture-

dependent and culture-independent DGGE fingerprints of 16S rRNA genes amplified from an organic farm soil. Communities recovered from culture plates were expected to represent a minor subset of the soil bacterial population due to physical, nutritional, and competitive selection on the growth medium employed. As summarized in **Figures 1.5** and **1.6**, this is not entirely what I observed. Culture-independent DGGE profiles contained some unique bands that resisted denaturation, migrated to the lower region of the gel, and may represent a difficult-to-culture fraction of the soil community. However, in upper regions of the gel there always were bands unique to culture-dependent DGGE analyses, even when a single growth medium (R2A) and soil dilution (10^{-3}) were employed (**Analysis 1, Figure 1.6**). When additional soil dilutions, soil pretreatments, culture media and culture incubation conditions were included in the analyses, the cumulative number of bands detected increased as did the number of bands unique to the CD DGGE profiles (**Analyses 2 and 3, Figure 1.6**). In the final analysis 34% of the bands were unique to the CD profiles, 32% were unique to the CI profiles and 34% were found in both communities. This demonstrates that culture-dependent and culture-independent profiles each can resolve unique, diverse, and equally complex fractions of the soil bacterial community. Because culture-dependent and culture-independent methods likely profile distinct fractions of the soil bacterial community with unique ecological roles (Garland, 1999), CD DGGE can provide a useful complement to culture-independent fingerprinting.

Other studies have reported improved assessment of soil bacterial diversity when both culture-independent and culture-dependent methods are used in combination (Teske et al. 1996; Kisand and Wikner, 2003; Lipson and Schmidt, 2004; Aislabie et al., 2006).

In these studies, community diversity was determined from identification of cultured bacterial isolates and sequencing of cloned 16S rRNA genes amplified from soil community DNA (Dunbar et al. 1999; Lipson and Schmidt, 2004; Kisand and Wikner, 2003). This approach is informative, but not always efficient for routine studies that involve the processing of large numbers of soil samples. Molecular fingerprinting techniques such as DGGE remain one of the best ways to rapidly explore the diversity and dynamics of soil bacterial communities. If more detailed phylogenetic information is required, DGGE offers the advantage that individual bands can be excised, cloned and sequenced (Sekiguchi et al., 2002), although methodological difficulties such as co-migration of bands with similar sequences (Gafan and Spratt, 2005), multiple bands from identical sequences (Nikolausz et al., 2005), formation of chimeric and heteroduplex molecules (Muyzer and Smalla, 1998) and limited phylogenetic information from sequenced bands (Muyzer and Smalla, 1998) must be taken in to account.

Previous studies suggested that CD DGGE profiles of bacterial communities from culture plates are too variable for rigorous analyses of differences among soil treatments (Duineveld et al. 1998; Ellis et al. 2003). In the present study, I evaluated the effects of dilution, replication, and culture conditions on the complexity and reproducibility of CD DGGE fingerprints. Overall, the CD DGGE gels showed clear banding patterns of sufficient complexity to investigate differences in soil microbial communities. ANOSIMS demonstrated that DGGE profiles from replicate sub-samples of soils from different plots were significantly more similar than profiles between different plots, a prerequisite if the method is to be used for meaningful comparisons of bacterial communities from different soil samples.

CD DGGE profiles from different soil dilutions demonstrated that the number of bands decreased and the structure of the bacterial communities shifted with dilution. Similar results have been observed using liquid cultures (Santegoeds et al., 1996; Jackson et al., 1998; Franklin et al., 2001), but are not commonly reported for growth on solid media. Replicate CD DGGE profiles from the least dilute plates were more similar (72–77%) than those from the most dilute plates (51–61%). Based on these results and those reported above, I recommend using a less-dilute sample (10^{-3}) to achieve a higher band count and less variation between replicate samples if only a single dilution and medium are employed. If more comprehensive profiles are desired, it is advantageous to characterize communities from different dilution plates simultaneously.

Recent advances in cultivation methods promise improved isolation of difficult-to-culture soil bacteria and a narrowing of the gap between taxa recovered using culture-dependent and culture-independent approaches (Janssen et al., 2002; Joseph et al., 2003; Schoenborn et al., 2004; Stevenson et al., 2004; Davis et al., 2005; Tamaki et al., 2005). Previous studies reporting the diversity of culturable bacteria on solid media generally have relied on the use of highly dilute soils as inocula using the classical spread-plate method (Zuberer, 1994). The usual objective is to obtain a “countable plate” containing between 30 and 300 well-separated colonies in order to facilitate picking individual isolates in pure culture. Therefore, less-diluted samples with colonies too numerous to enumerate accurately are normally discarded without further evaluation. This practice excludes examination of culturable bacteria that are lost through dilution (Button, 1993) or bacteria that exploit unique niches created by the growth of other bacteria on crowded plates (Kassen and Rainey, 2004). For example, Dedysh et al. (2006) reported enhanced

detection of *Acidobacteria* and *Planctomycetes* in co-cultures compared with pure cultures. They suggested that a method that includes mixed cultures may be superior to the “single-colony pick-up” approach for isolation of difficult-to-culture bacteria. In this study, I observed a higher band count on plates inoculated with less-dilute soils. Specifically notable was the collection of bands in the bottom region of the 10^{-3} profiles. Although the phylogenetic affiliations of individual bands were not assigned by sequence analysis, the absence of these bands on the countable (10^{-6}) plate suggests that unique bacteria are lost under less crowded growth conditions.

Conclusions

Although bacterial growth under different metabolic conditions or on different types of solid media is inherently selective, the ability to partition complex soil bacterial communities into definable physiological subsets using a variety of isolation media and incubation conditions should be viewed as a potential strength of the CD DGGE technique. My results suggest that CD DGGE detected unique 16S rRNA genes that were not represented by CB DGGE profiles of a large clone library (~ 1000 clones). Garland (1999) suggested the need to better understand the ecological interactions that exist between culturable and non-culturable types of bacteria because it is likely that changes in one fraction of a community lead to changes in others. Historically, culture-dependent methods such as community-level physiological profiling (CLPP) and plate counts have proven useful models for screening relative shifts in the functional diversity and size of microbial communities, conducting exploratory investigations, and generating hypothesis. My results suggest CD DGGE is sufficiently reproducible to investigate the

dynamics of culturable bacterial communities. Compared with culture-independent methods, CD DGGE requires more time for sample preparation. However, the ability of CD DGGE to partition soil bacteria into distinct subsets based on their growth characteristics provides a potential means to identify biological and environmental factors that influence bacterial community structure (Aislabie et al., 2006; Langenheder et al., 2006).

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Table 1.1. Summary of studies, experimental design^a, and sampling strategy.

	Sampling Date	Crops Planted in Spring Prior to Sampling	Type of Community Characterization ^b	Plots Sampled ^c (No. of plots)	No. of Replicate Soil Sub Samples Per Plot ^d	Lane No. in Composite DGGE Gel ⁱ (Figure 1.5)
1 ^e	Jun. 2004	Tomatoes and Peppers	CB DGGE	High-input (4)	1	1
				Low-input (4)	1	2
			CD DGGE	High-input (4)	1	3
				Low-input (4)	1	4
2 ^f	Feb. 2005	Peas and Beans	CD DGGE (plot variability)	High-input (3)	4	5, 6
				Low-input (3)	4	
3 ^g	Feb. 2005	Peas and Beans	CD DGGE (dilutions)	High-input (3)	1	7, 8
				Low-input (3)	1	
4 ^h	Aug. 2005	Tomatoes and Peppers	CD DGGE (media/pre-treatment)	Low-input (1)	3 per media or pre-treatment	9–14

- ^a The field experiment, which was established in 2000, is a completely randomized design with four plots (4.9 x 7.6 m) per treatment. Treatments included fertility input (compost and green manure) and annual crop rotation among four plant families (Fabaceae, Solanaceae, Cucurbitaceae, and Asteraceae). High-input plots received composted dairy manure (22.4 Mg ha⁻¹ based on wet weight) applied 1–3 days prior to planting and green manure (rye and vetch) tilled into the soil 1–3 prior to planting. Low-input plots received only green manure applied as described above. Fertility treatments were applied annual since 2000.
- ^b Culture-dependent (CD) and cloning-based (CB) denaturing gradient gel electrophoresis (DGGE). Sub-samples of bulk soils collected from each plot were processed for CB DGGE and/or CD DGGE as outlined in **Figure 1.1**.
- ^c Ten soil cores (16 cm depth x 2 cm diameter) were collected from each plot at ~1.5 meter intervals along two transects at midpoint between crop rows. Soil samples were bulked, placed into plastic bags, and immediately transported back to the laboratory, where they were sieved (< 2 mm) and stored at 4°C overnight.
- ^d For CB DGGE analysis soil sub-samples were composed of ~0.5 g of bulked soil from each plot. For CD DGGE analysis sub-samples were composed of 20 g of the bulked soil from each plot that was processed as outlined in **Figure 1.1**. CD DGGE profiles presented in each lane in **Figure 1.2** through **Figure 1.4** were constructed from one sub-sample each.
- ^e Initial comparison of CB DGGE and CD DGGE profiles. Pooled DGGE profiles from study 1 are presented in **Figure 1.5**.
- ^f Effects of plot variability on CD DGGE profiles. Replicate DGGE profiles were constructed from 50 g sub-samples of soil from composite samples collected from each treatment plot. UPGMA dendrograms for study 3 are presented in **Figure 1.3**.
- ^g Effects of soil inoculum size on CD DGGE profiles. Each sub-sample was diluted (10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁶), and the individual dilutions were plated onto R2A agar as outlined in **Figure 1.1**. DGGE profiles and UPGMA dendrograms for the 10⁻³ and 10⁻⁶ dilutions are presented in **Figure 1.2**.
- ^h Effects of media and pre-treatment on CD DGGE profiles. DGGE profiles and UPGMA dendrograms for study 4 are presented in **Figure 1.4**.
- ⁱ Composite gel with CB DGGE profiles and pooled CD DGGE profiles presented in **Figure 1.5** and analyzed in **Figure 1.6**.

Table 1.2. Average number of CD DGGE bands and average percent similarity in dilution study.

Sample	Average no.		Average percent similarity	
	of bands ^a	($\pm$ SD)	between replicates ^a	($\pm$ SD)
High-input 10^{-3}	26	(1.5)	77.2	(2.41)
High-input 10^{-4}	26	(3.8)	72.0	(11.41)
High-input 10^{-5}	25	(5.3)	67.8	(16.13)
High-input 10^{-6}	17	(2.6)	51.0	(4.98)
Low-input 10^{-3}	26	(0.6)	72.1	(3.17)
Low-input 10^{-4}	18	(2.9)	70.4	(9.47)
Low-input 10^{-5}	26	(3.0)	72.7	(9.64)
Low-input 10^{-6}	19	(5.7)	61.3	(8.78)

^a n=3

Figure 1.1. Flowchart of protocols used to construct culture-dependent (CD DGGE) and cloning-based (CB DGGE) fingerprints of bacterial communities from organic farm soils. In study 3, additional dilutions were used (10^{-4} , 10^{-5} and 10^{-6}) to construct CD DGGE profiles.

Figure 1.2. (a) CD DGGE profiles of 16S rRNA genes amplified from soil bacterial communities grown on R2A media inoculated with 10^{-3} or 10^{-6} dilutions of soil from high (H) and low (L) input plots from the WVU organic research farm and incubated aerobically for 2 weeks at 25°C. Samples were analyzed on a 40–60% DGGE gradient for 14 hours at 50 V. Box and arrow highlight fewer bands with relative fronts > 0.6 in the 10^{-6} compared with the 10^{-3} dilution. (b) UPGMA dendrograms constructed using Sorensen's similarity index generated from the above DGGE profiles. Bootstrap values are given at nodes (1,000 resamplings).

Figure 1.3. UPGMA dendrograms constructed using Sorensen's similarity index generated from DGGE profiles of bacterial communities from replicate soil sub-samples collected from (a) high and (b) low input plots at the West Virginia University organic research farm. Bootstrap values are given at nodes (1,000 resamplings).

Figure 1.4. CD DGGE profiles and UPGMA dendrograms constructed using Sorensen's similarity index generated from DGGE profile from soil bacterial communities (a, b) grown on four different media and (c, d) subjected to different pretreatments (heat shock vs. no heat shock) and incubation conditions (aerobic vs. anaerobic) prior to plating on R2A media. Media included: R2A Agar (R2A), Tryptic Soy Agar (TSA), Oil Agar (OA) and Pseudomonas Isolation Agar (PIA). Heat-shocked samples were incubated at 80°C for 15 minutes. Samples were run on a 40–70% DGGE gradient for 16 hours at 50 V. Bootstrap values are given at nodes (1,000 resamplings).

Figure 1.5. DGGE profiles of culture-independent (CB DGGE) and culture-dependent (CD DGGE) 16S rRNA genes of bacterial communities from organic farm soils. Profiles were constructed using pooled 16S rRNA genes from the studies outlined in Table 1.1. Lanes 1 and 2 represent CB DGGE profiles of clones (~ 1000) from high-input soil collected in study 1 and clones (~ 77) from low-input soil collected in study 1, respectively. The pooled CD DGGE profiles are from: high-input plots (4) in study 1 (Lane 3); low-input plots (4) in study 1 (Lane 4); high-input plots (12) in study 2 (Lane 5); low-input plots (12) in study 2 (Lane 6); high- and low-input dilution plates 10^{-3} and 10^{-4} (12) in study 3 (Lane 7); high- and low-input dilution plates 10^{-5} and 10^{-6} (12) in study 3 (Lane 8); R2A agar (3) in study 4 (Lane 9); Tryptic Soy Agar (3) in study 4 (Lane 10); Oil Agar (3) in study 4 (Lane 11); Pseudomonas Isolation Agar (3) in study 4 (Lane 12); anaerobically incubated plates with and without heat shock (6) in study 4 (Lane 13); and aerobically incubated plates with and without heat shock (6) in study 4 (Lane 14). Numbers in parentheses indicate the number of sub-samples included in each pooled profile.

Figure 1.6. Number of bands found in the composite DGGE gel (Figure 1.5) when different combinations of culture-dependent profiles were included in the analysis.

Analysis 1, lanes 1–4; Analysis 2, lanes 1–8; Analysis 3, profiles from all lanes. Samples included in each lane are defined in **Table 1**.

Chapter 2: Small-Celled Bacteria from Organic Farm Soils Show Phylogenetic Similarities to Diazotrophs and Bacteria that Favor Inter- and Intracellular Habitats

Abstract

The numerically dominant bacteria in soil have cell diameters $< 0.5 \mu\text{m}$; however, the phylogenetic identities of these organisms remain largely unknown, in part because small-celled bacteria are rarely grown in culture. In the present study, bacteria from filtered ($< 0.45 \mu\text{m}$) and nonfiltered slurries of organic farm soils were cultivated on R2A agar. The majority of colonies from the filtered samples were recovered from the 10^{-3} and 10^{-4} dilutions of compost-amended soils after incubation at 25°C for up to one month. To avoid a loss of diversity associated with sub-culturing, the colonies from primary isolation plates were pooled and 16S rRNA genes were PCR-amplified from DNA extracted from these “communities” of culturable bacteria. DGGE analysis of the 16S rRNA genes from the filtered and unfiltered communities indicated that the filterable soil bacteria were not similar to the dominant organisms cultured from unfiltered samples and that distinct small-celled bacteria were isolated from soils collected one year apart. Phylogenetic analysis of 16S rRNA genes from the filtered bacteria were classified as α -, β -, γ -, or δ -*Proteobacteria* or *Bacteroidetes*. The 16S rRNA genes affiliated with the genera *Polaromonas* and *Ramlibacter* represent 23% of the non-chimeric sequences in the clone libraries. Other sequences were associated with known copiotrophic ultramicrobacteria from soil, endophytic nitrogen-fixing bacteria in the genera *Herbospirillum* and *Azospirillum*, and bacteria that are adapted to inter- or intracellular growth. Almost half of the sequences from the slow-growing, pinpoint-sized colonies

were highly similar to an amoeba-resistant bacterium that is affiliated with the genus *Rhodoplanes*, a putative diazotroph that may play a potentially important role in soil nitrogen dynamics.

Keywords: Soil, Ultramicrobacteria, Small cells, 16S rRNA, DGGE, N-fixation, Endophytes, Intracellular bacteria.

Introduction

Bacteria residing naturally in soil are typically much smaller than bacteria grown in laboratory cultures. Cells in culture tend to have cell diameters $> 0.5 \mu\text{m}$ (Bae et al., 1972), while direct microscopic observations indicate that 65 to 78% of the bacteria in soil have diameters $< 0.5 \mu\text{m}$ (Bakken, 1985; Christensen et al., 1999). Although small-celled bacteria (diameter $< 0.5 \mu\text{m}$) are known to represent a numerically-dominant class of soil bacteria, there is limited information regarding the phylogenetic and physiological diversity of this community because they do not grow readily in culture (Bakken and Olsen, 1987).

Soil bacteria with small volumes or cell diameters are often referred to as “dwarf” bacteria (Bae and Casida, 1973; Bakken and Olsen, 1987; Rutz and Kieft, 2004). Small-celled bacteria can be further classified as ultramicrocells (UMCs), which are dormant, stress-resistant cells that are smaller versions of otherwise larger-sized cells; and ultramicrobacteria (UMB), which are cells with consistent volumes less than $0.1 \mu\text{m}^3$ (Schut et al., 1997; Cavicchioli and Ostrowski, 2003). Studies suggest that small-celled soil bacteria have the same amount of DNA as larger bacteria (Bakken and Olsen, 1989) but can be distinguished from larger cells based on their growth rates, metabolic profiles and community fingerprints (Bååth, 1994; De Fede et al., 2001; De Fede and Sexstone, 2001). Based on phospholipid fatty acid analyses, Lindahl et al. (1997) suggested that small-celled soil bacteria were primarily gram-positive organisms. However, both Iizuka et al. (1998) and Rutz and Kieft (2004) have isolated 16S rRNA sequences from small soil bacteria that are related to *Proteobacteria* as well as gram-positive bacteria. Small-celled bacteria affiliated with the *Verrucomicrobiales* (Janssen and Schuhmann, 1997),

Actinobacteria (Hahn et al., 2003) and archaea (Huber et al., 2002; Rutz and Kieft, 2004) also have been isolated or detected.

To recover small-celled bacteria from soil, it is necessary to exclude faster-growing species from the inoculum. Extinction dilution techniques have previously been used to isolate ultramicrobacteria from marine waters (Schut et al., 1993) and rice paddy soil (Janssen and Schuhmann, 1997). De Fede and Sexstone (2001) also used extinction dilution to recover small-celled bacterial communities from agricultural and forest soils. Although this technique has proven useful, it requires that the target bacteria be present in high number (Button et al., 1993) and amenable to growth in liquid culture. Bacteria that are lost through dilution may represent novel organisms (Edenborn and Sexstone, 2007).

Evidence suggests that liquid serial dilution is inferior to solid media for the recovery of diverse bacteria in culture (Schoenborn et al., 2004). Bacteria from undiluted soils can be recovered by inoculating filtrates of soil slurries directly onto agar plates. Direct filtration was not useful for the recovery of marine ultramicrobacteria (Cavicchioli and Ostrowski, 2003), but was used successfully by Iizuka et al. (1998) to recover fast-growing copiotrophic UMB from soil. Recent studies have demonstrated that incubation periods from 10 weeks to three months may be necessary to grow difficult-to-culture bacteria such as the *Acidobacteria* and *Verrucomicrobia* (Janssen et al., 2002; Joseph et al., 2003). Therefore, improved recovery of small-celled bacteria may be achieved by using direct filtration in combination with longer incubation periods and low-nutrient media (Janssen et al., 2002). To assess the feasibility of this approach, I used direct filtration to isolate small-celled bacteria from organic farm soils in June of consecutive years. Because my primary objective was to explore the potential to

cultivate small-celled bacteria rather than to isolate pure cultures, I pooled colonies that had been washed or picked from the primary isolation plates and constructed 16S rRNA clone libraries from community DNA. This method was used to minimize the significant loss of diversity that can occur when co-cultures containing one or more different types of bacteria are separated during subculturing (Dedysh et al., 2006). Further, this approach allowed me to compare bacterial communities using a culture-dependent DGGE fingerprinting technique (Edenborn and Sexstone, 2007).

Materials and Methods

Study site and soil sampling

Soil samples were collected in June of 2003 and 2004 from high- and low-input plots at the market garden at the West Virginia University (WVU) Organic Research Farm located in Morgantown, West Virginia, USA (39° 39' 0.18" N; 79° 56' 11.6" W). High-input plots received composted dairy manure (22.4 Mg ha⁻¹ based on wet weight) applied 1–3 days prior to planting and green manure (rye and vetch) tilled into the soil 1–3 days prior to planting. Low-input plots received only green manure applied as described above. Soils on the farm are Dormont and Guernsy silt loams: fine loamy, mixed, superactive, mesic, Oxyaquic Hapludalfs (Wright et al., 1982). Ten soil cores (16 cm depth x 2 cm diameter) were aseptically collected from each plot at ~1.5 meter intervals along two transects located midpoint between crop rows. Soil samples were bulked, sieved (< 2 mm), placed into plastic bags, and immediately transported back to the laboratory, where they were stored at 4°C overnight. In 2003, soils were collected from

one low-input and one high-input plot. In 2004, soils were collected from replicate (n=4) high- and low-input plots.

Enumeration and cultivation of filterable and nonfilterable soil bacteria

Composite soil samples from each plot were placed in sterile Waring blenders and agitated for one minute at high speed (3x with intermittent cooling on ice) in sterile Winogradsky's salts solution (WSS) (Zuberer, 1994). The primary soil dilutions were 50 g/200 mL WSS (1/5) in 2003 and 20 g/180 mL WSS (1/10) in 2004. The blended slurries from 2003 were serially diluted in sterile 0.1% (w/v) Bacto peptone (Difco, Detroit, MI), and aliquots from each dilution were inoculated by spreading either 50 μ l (2003) or 100 μ l (2004) onto replicate R2A agar (Difco, Detroit, MI). The final dilutions of soil on the plates ranged from 10^{-2} to 10^{-7} . To isolate small-celled bacteria, 5–10 mL of each soil dilution from 2003 (**Figure 2.1**) were filtered immediately through a series of 0.45 μ m polyethersulfone syringe filters (Whatman International, Clifton, NJ). In 2004, 25 mL of soil suspension from the blender was placed into a sterile centrifuge tube and allowed to settle overnight at 4°C. Five mL were then removed from the top of the suspension and filtered through a 0.45 μ m disposable polypropylene filter unit (Nalgene Nunc International, Rochester, NY). The resulting filtrates were plated directly onto replicate plates of R2A agar (n=3 in 2003; n=27 in 2004). All plates were incubated aerobically at 25°C for up to one month along with control plates that received no inoculum or 100 μ L of the sterile peptone (0.1%) diluent that had been filtered as described for the soil samples. The colony forming units (CFUs) on each plate were counted at 24 hours, one or two weeks and one month using a Quebec colony counter and

the colony morphologies were visually characterized based on pigmentation and colony size. Two colony classes were operationally defined based on colony diameter: small colonies (< 1 mm) and large colonies (> 1 mm).

DNA extraction from bacterial communities on agar plates

Pooled samples of culturable bacteria were obtained by: 1) sequentially washing cells from replicate plates using WSS (4 ml) and a sterile disposable inoculating loop (Edenborn and Sexstone, 2007); or, 2) picking and pooling individual colonies. In 2003, plate washes of both nonfiltered and filtered bacteria were obtained from replicate (n=3) dilution plates (10^{-3} , 10^{-4} , 10^{-5} and 10^{-6}) from one high- and one low-input soil sample. In 2004, nonfiltered bacterial communities from the 10^{-2} dilution from replicate (n=4) high- and low-input plots were collected using the plate washing technique, and filtered bacteria were picked and pooled based on colony size. All of the resulting cell suspensions were vortexed and frozen (-20°C). DNA was extracted from aliquots of each thawed cell suspension (1.8 ml) using the MoBio Microbial DNA extraction kit (MoBio Laboratories, Carlsbad, CA).

PCR-DGGE analysis of bacterial communities and 16S rRNA clones

The variable V3 region of the 16S rRNA gene from the domain *Eubacteria* was amplified using the PRBA338F primer with a GC clamp and the PRUN518R primer as previously described (Edenborn and Sexstone, 2007). 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 estimated by agarose gel

electrophoresis of samples and a mass ladder (Fermentas Inc., Hanover, MD). PCR samples from bacterial communities or individual clones were 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) or a 45% (18.9 g urea; 18 ml formamide/100 ml) to 75% (31.5 g urea; 30 ml formamide/100 ml) denaturing gradient and electrophoresed for 14–16 hours at 50 V and 60°C in the DCode™ Universal Mutation Detection System (Biorad, Hercules, CA). The gels were stained with silver according to the protocol of Caetano-Anolles and Gresshoff (1994). The stained gel was scanned with a Hewlett Packard ScanJet 7400c (Hewlett-Packard Co., Palo Alto, CA) at a resolution of 600 dpi and saved in uncompressed TIFF format for further analysis. DGGE gels were analyzed using Quantity One gel analysis software (BioRad). Band analysis was performed by setting background subtraction at 15 using the rolling disk method. Bands with intensity < 10 % of the maximum intensity observed in the profile were excluded from the analysis. Similarity matrices and unweighted pair-group method with arithmetic mean (UPGMA) trees were generated in Quantity One using Sorensen's index.

PCR amplification, cloning and sequencing of 16S rRNA genes from filtered bacteria

The 16S rRNA genes in pooled DNA samples of filtered bacteria were amplified using primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTCTTACGACTT-3'). The final 100-μl PCR mixture contained 1× PCR buffer (Promega, Madison, WI), 1.8 mM of MgCl₂, 200 μM of a equal mix of deoxynucleotide triphosphates (Promega), 0.4 % bovine serum albumin, 0.1 μM (each) of forward and reverse primers (Integrated DNA Technologies) 5 U of *Taq* DNA

polymerase (Promega), and ~300 ng of template DNA. The PCR protocol included 5-min initial denaturation at 94°C, six touchdown cycles of annealing temperatures decreasing in 1°C increments from 61°C to 56°C (92°C for 30 s (denaturation); 30 s (annealing); and 72°C for 90 s (elongation) for 30 s), 22 cycles as follows (92°C for 30 s (denaturation); 55°C for 30 s (annealing), and 72°C for 90 s), and 7 min at 72°C. Four microliters of the cleaned PCR (~15–20 ng/μl) product was then cloned using the Topo TA cloning kit for sequencing (Invitrogen, Carlsbad, CA), and transformed cell suspensions were plated onto 5 or 6 Luria broth agar plates (5 g NaCl, 5 g Yeast Extract (Difco), 10 g Bactopeptone (Difco), and 15 g agar per L) containing kanamycin (50 μg/ml). Clones with full-sized (~1400 bp) inserts were identified using colony PCR with the M13 forward and reverse primers and subjected to amplified ribosomal DNA restriction analysis (ARDRA) analysis using *Rsa*I and *Hha*I (New England Biolabs, Ipswich, MA). Restriction digests were done separately and then combined prior to electrophoresis. Plasmid DNA was extracted from selected clones using the QIAprep Spin Miniprep Kit (Qiagen).

Prior to sequencing, the V3 region of 16S rRNA from each clone was amplified and associated with bands in the community using the DGGE protocol described above. Partial 16S rRNA genes associated with specific DGGE and ARDRA patterns were sequenced by a commercial sequencing facility (Davis Sequencing, Davis, CA) using the T7 sequencing primers. Putative chimeric sequences were identified using the program Bellerophon (Huber et al., 2004) and a multiple alignment with closely-related bacterial sequences was performed using the NAST alignment tool on the Greengenes website (DeSantis et al., 2006a; DeSantis et al., 2006b). Further, the classification tool in

Greengenes was used to associate each sequence with a bacterial division. Aligned sequences were manually refined and imported into the Mega software package version 3.1 (Kumar et al., 2004). A neighbor-joining tree was constructed based on 950 bp of the 16S rRNA gene using the Kimura two-parameter substitution model with complete deletion of gapped sequences. Bootstrap analysis (1,000 resamplings) was done to estimate the statistical reliability of the groupings. Bootstrap values greater than 70% are indicated at tree nodes. The percent similarities between the known and unknown sequences in the final tree were determined using the gapped BLAST protocol (BLAST 2 sequences) on the NCBI website (Tatusova and Madden, 1999).

Results

Cultivation of filterable bacteria from organic farm soils

In 2003, filterable bacteria were recovered from 10-fold dilutions of high- and low-input soils that had been directly plated onto R2A medium and incubated aerobically for two weeks at 25°C (**Figure 2.1**). A total of 216 CFUs were counted among all plates; 67% were from high-input soils and 33% from low-input soils. Eighty-seven percent of the colonies observed on the plates had been inoculated with filtrate from the 10^{-3} and 10^{-4} soil dilutions. Only five CFUs were present on plates inoculated with the 10^{-6} filtrate. Forty-one percent of the CFUs classified as small colonies (< 1 mm at 14 days) were pinpoint-sized and 13% had yellow or orange pigments. In 2004, filterable cells from a 10-fold soil dilution were directly plated on R2A agar plates and incubated aerobically at 25°C. In contrast to the results from 2003, very few colonies were observed after a one-week incubation period (**Figure 2.2.**); however, at one month, a total of 360 CFUs were

counted on all plates (82% from the high-input soil and 18% from the low-input soil). As previously observed, a large percentage (53%) of the colonies were pinpoint-sized. No growth was observed on any of the control plates.

CD DGGE analysis of filtered and nonfiltered soil bacterial communities from R2A plates

In this study, composite CD DGGE fingerprints of filtered and nonfiltered bacterial communities were constructed by pooling partial 16S rRNA genes recovered from bacteria growing on R2A plates that had been inoculated with different soil dilutions (2003 samples) or soils from replicate treatment plots (2004 samples) (**Figure 2.3**). A 40–60% DGGE gradient was used for the 2003 samples. However, a 45–75% gradient was needed to resolve bands in the 2004 fingerprints. The number of bands observed in the 2003 and 2004 fingerprints ranged from 27 to 37 for the nonfiltered community and from 20 to 31 for the filtered community. In both 2003 and 2004, the most intensely-stained bands in the nonfiltered community were present in the upper region of the gel (as indicated by the black arrows in **Figure 2.3**) and were not detected in profiles of filterable bacteria. In contrast, many of the bands in fingerprints of the filterable bacteria were detected in the lower regions of the gel (**Figure 2.3**) and were present at different levels of intensity in the profiles from the nonfiltered community. Based on Sorensen's index, the percent similarities between filterable and nonfilterable profiles ranged from 14–19% in 2003 and 10–15% in 2004. The similarity between the community fingerprints from the high- and low-input soils (**Figure 2.3**) were: 44% (nonfilterable; 2003), 40% (filterable; 2003), 69% (nonfilterable; 2004) and 33%

(filterable; 2004). Finally, a DGGE analysis of all samples using the same gradient (45–75%; gel not shown) was done to evaluate temporal shifts in community structure. An UPGMA dendrogram (**Figure 2.4**) suggested that both the filtered and unfiltered communities from 2003 were only slightly similar (22 and 29%, respectively) to those from 2004. Comparison of filtered communities on the same gradient (45–75%) revealed considerably more bands in the lower region of the gel in the 2004 fingerprint than in the 2003 profile (**Figures 2.6a and 2.7a**). This resulted in a very low similarity (7%) between fingerprints. Intensity profiles of aligned DGGE fingerprints were generated using Quantity One software, and peaks related to each band were visually inspected. Peaks that clearly were present in the filtered community but not present in the nonfiltered community included 1, 11, and 13 (2003) and 6, 10, 14, 21 and 22 (2004) (**Figure 2.3**).

Phylogenetic diversity of filterable soil bacterial communities

Pooled 16S rRNA genes from the filterable bacterial communities from 2003 and 2004 were used to construct four clone libraries that were affiliated with isolation source (high-input and low-input soil) and colony size (large colony, > 1 mm and small colony, < 1 mm). From these libraries, 105 clones with inserts of the expected size (~ 1400 bp) were isolated and classified using DGGE and ARDRA. DGGE analysis of the V3 region was used to associate the 16S rRNA genes from clones with bands in the original DGGE profiles of the filterable bacterial communities (**Figures 2.3, 2.6a, and 2.7a**). ARDRA was done to identify potentially different 16S rRNA genes associated with each band. A numerical identification code was given to clones with unique RFLP types that specified

the DGGE band number and a unique ARDRA type associated with that band (eg., 5.1 refers to ARDRA pattern 1 associated with band 5). If bands were detected in both years, a third number was used to designate year 1 or year 2 (eg. 8.1.1, band 8, ARDRA pattern 1, year 1). The distribution of clones from 2003 and 2004 is presented in **Figure 2.5**. In general, multiple ARDRA patterns were associated with most bands. There also was more redundancy in the clones from 2003 compared to 2004. Most notable was clone 3.2, which dominated the 2003 library and was associated with both the high-and low-input soil.

The 16S rRNA genes from 78 clones with unique ARDRA patterns and three clones associated with pattern 3.2 were partially sequenced. Fourteen of the sequences were identified as putative chimeras based on analysis using the program Bellerophon, and were removed from the analyses. The remaining 67 sequences were assigned to major phyla using the classification tool in Greengenes, and phylogenetic clusters were inferred from a neighbor-joining tree. Sequences were assigned to the *α -Proteobacteria* (36%), *β -Proteobacteria* (37%), *γ -Proteobacteria* (16%), *δ -Proteobacteria* (4%) and *Bacteroidetes* (6%). Sub-clusters were identified in each group using the letters A through J (**Figures 2.6b and 2.7b**). In general, sequences associated with the same DGGE bands clustered together, and there was often very little difference in the sequences associated with distinct intra-band ARDRA patterns. As suggested by the DGGE fingerprints, there was little overlap between the sequences obtained from the 2003 and 2004 samples.

Thirty-seven percent of the ARDRA clones from 2003 were associated with the genera *Polaromonas*, *Ramlibacter* and *Variovorax* in the *β -Proteobacteria* family

(Figure 2.6; cluster D). All of the operational taxonomic units (OTUs) in cluster D except clone 1.1 were 98 to 99% similar to *Polaromonas ginsengisoli* str. Gsoil 115 (AB245355) based on local alignment using the gapped BLAST algorithm. Clone 1.1 was more similar (95%) to the *Variovorax* and *Ramlibacter* sequences. Bacteria associated with sequences in this cluster were isolated from high- and low-input soils from 2003.

OTUs represented in clusters B, C and G **(Figure 2.6)** were associated with filterable bacteria characterized in other studies. Clones 10.1 and 12.1 formed a tight phylogenetic cluster (97% similarity) to an ultramicrobacterium (AB008503) isolated by Iizuka et al. (1998) **(Figure 2.6; cluster B)**. Phylogenetic analysis and pair-wise alignment indicated that these sequences were similar to *Herbaspirillum* sp. (96 to 97%) and *Oxalicibacterium flavum* (94 to 98%). Both of these sequences were obtained from 2004 clones with a larger-colony size (> 1 mm). Four sequences from the 2003 high-input soils **(Figure 2.6; cluster G)** also were highly similar (99%) to a second ultramicrobacterium (AB008508). The phylogenetic affiliation of these clones with *Xanthomonas campestris* was supported by high sequence similarity (94 to 97%) and bootstrap values. Finally, the six sequences in cluster C showed 92 to 93% similarity with a 16S rRNA sequence from a deep-groundwater bacterium that was able to pass through a 0.2 µm filter (Miyoshi et al., 2005) and a 92 to 97% similarity with a sequence from a clone associated with a deep-sea coral (DQ395940). The cultured representative *Denitratisoma oestradiolicum* str, AcBE2-1 (AY879297) had a 92 to 93% similarity with the sequences in cluster C.

The last cluster in the β -*Proteobacteria* (**Figure 2.6; cluster A**) was composed of sequences associated with clones from 2004 with small-sized colonies (< 1 mm). Pair-wise alignments in BLAST indicated that clone 22.2 was 97% similar to *Methylovorus mays*. Clones 2.2, 2.3 and 2.4 were 92 to 94% similar to *Methylophilus sp.* and 91 to 97% similar to *Siderooxidans paludicola* (DQ386858). Other sequences classified in the *Gammaproteobacteria* were associated (92 to 97% similarity) with *Lysobacter sp.* (**Figure 2.6; cluster E**) and *Thermomonas dokdonensis* (**Figure 2.6; cluster F**).

The 16S rRNA sequences classified as α -*Proteobacteria* were broadly divided into two groups; those associated with bacteria with a large-colony size from predominantly high-input soils (**Figure 2.7; clusters H and I**) and bacteria with very small colony size (**Figure 2.7; cluster J**). Cluster I was highly similar (97 to 98%) to the sequence for the purple nonsulfur bacterium *Phaeospirillum fulvum* and cluster H was loosely affiliated (91 to 92%) with the nitrogen-fixing genera *Azospirillum*. The small-colony isolates (**Figure 2.7; cluster J**) were associated with known intracellular bacteria and ultramicrobacteria. Sequences 6.1 and 6.2 were 88% similar to *Rickettsia conorii* and *Rickettsia sibirica* and formed a cluster in the α -*Proteobacteria* that was supported by bootstrap analysis. Clone 15.2.1 had a 98% similarity to *Sphingomonas sp.* and clones 22.5, 21.1 and 8.22 had some relatedness (87 to 93%) to *Paravibaculum lavamentivorans*, *Bradyrhizobium japonicum*, *Beijerincki indica*, *Rhizobium* and *Rhodoplanes elegans*. Specifically notable was the number of sequences in cluster J (15.5.2, 22.6, 22.8, 22.7, 22.1, and 22, 4) that were found to be similar (95 to 96%) to a bacterium isolated using amoebal co-culture (DQ123621) and to *Rhodoplanes elegans*, D25311 (94 to 96%). The remaining sequences were classified as δ -*Proteobacteria* and

Bacteroidetes. Four clones in *Bacteroidetes* cluster were related to the known isolates in the genus *Flexibacter* (91 to 96%). However, the three clones associated with DGGE band number 14 and classified as δ -*Proteobacteria* had 16S rRNA genes significantly different from the closest validated species. Only 84% similarity was observed between δ -*Proteobacteria* clones and *Geobacter pickeringii* str. G13 and *Desulfobacterium catecholicum* str. DSM388.

Discussion

The majority of active bacteria in soil have cell diameters that are less than 0.5 μm (Christensen et al., 1999); however, the identities of these organisms are largely unknown because small-celled bacteria resist cultivation (Bakken and Olsen, 1987). Although it is possible to characterize 16S rRNA genes in filtered samples using molecular cloning technology (Rutz and Kieft, 2004), this approach is complicated by the probable presence of naked DNA from larger-sized cells in filtrates. Further, cloned genes do not provide a platform to study the physiology and ecology of small-celled bacteria. Therefore, efforts must be made to isolate cultured representatives of bacteria with small cell sizes. In this study, I characterized communities of filterable bacterial cells ($< 0.45 \mu\text{m}$) that grew on primary isolation plates in order to assess the diversity of bacteria that may serve as model organisms for future studies.

A significant number of small-celled bacteria were observed on R2A agar following direct filtration of soil through 0.45 μm filters. In 2003, most CFUs were found on plates inoculated with the least dilute soils, and only five CFUs were present on the 10^{-6} plate, which was one dilution above (10^{-7}) the “countable” plate (30–300

colonies). In 2004, colonies from the filtered samples appeared on growth plates after an incubation period of 1 month. However, there was no increase in the number of colonies on plates that had been inoculated with the most dilute (10^{-7}) unfiltered samples after two weeks. These data suggest that direction filtration may improve the recovery of culturable small-celled bacteria from soil.

Often a high percentage of bacterial isolates that appear on primary agar plates are lost during subsequent efforts to obtain representative isolates in pure cultures (Ellis et al., 2003). Therefore, I opted to use a plate wash technique (Edenborn and Sexstone, 2007) to isolate total DNA from bacterial communities on primary isolation plates, followed by PCR amplification of the 16S rDNA gene and CD DGGE analysis to screen communities from filtered and unfiltered soil samples for similarities and differences. Fingerprint analysis indicated that the filterable bacteria were not similar to the dominant organisms growing from the unfiltered soil samples. Distinct filterable bacteria were isolated in 2003 and 2004, perhaps due to the varied approaches used to isolate small cells or inherent variations in the soil bacterial communities. The parallel changes in both the filtered and unfiltered communities suggest real spatial and temporal shifts in these communities. However, it also is probable that filtration of less dilute soil slurries in 2004 increased our recovery of rare taxa that may not have been cultivated in 2003.

Although I did not attempt to classify the filtered bacteria as ultramicrobacteria (UMB) or ultramicrocells (UMC), several of the 16S rRNA genes in the 2003 samples were highly similar to the two aerobic copiotrophic UMB isolated by Iizuka et al. (1998). The 16S rRNA genes from clones 12.1 and 10.2 were 97% similar to the UMB isolate MY14, which has a cell volume of $0.07 \pm 0.03 \mu\text{m}^3$, a doubling time of 7.4 hours in

tryptic soy broth, and a 16S rRNA gene sequence with 95% similarity to *Paucimonas lemoignei* X92555 (previously classified as *Pseudomonas lemoignei*). I included the 16S rRNA sequences for *P. lemoignei* in the phylogenetic analysis to demonstrate the relationship of clones 10.1 and 12.1 and the known UMB. However, the 16S rRNA sequences of UMB MY14 and clones 10.1 and 12.1 were more closely related to those of *Herbaspirillum sp.* and *Oxalicibacterium flavum* than *P. lemoignei*. UMB related to MY14 has been previously designated as the nearest neighbor to endophytic nitrogen-fixing isolates classified as *Herbaspirillum frisingense* (Kirchhof et al., 2001). Bacteria in this genus typically have cell widths between 0.5–0.7 μm (Elbeltagy et al., 2001); therefore, they could have moved through the filter via cell flexure (Haller et al., 2000). However, it has been shown that populations of *Herbaspirillum* rapidly fall below detection limits following inoculation into soil but recolonize and multiply when plants are introduced (Olivares et al., 1996). Therefore, smaller forms of *Herbaspirillum* cells may represent dormant or viable-but-non-culturable cells that persist in soil. Interestingly, we also found sequences associated with the endophytic nitrogen-fixing bacterium *Azospirillum* in the 2004 clone library.

A second group of clones (**Figure 2.6; cluster G**) had 16S rRNA gene sequences that were 99% similar to a second fast-growing, copiotrophic UMB strain DYO1 (Iizuka et al., 1998). The sequences from our study and that of Iizuka et al. (1998) showed 94 to 97% similarity to the 16S rRNA genes of the plant pathogen *Xanthomonas campestris*. The cells in the genus *Xanthomonas* have diameters ranging from 0.2–0.6 μm and lengths from 0.8–2.9 μm (Swings and Civerolo, 1993), which suggests that these cells could easily pass through a 0.45 μm filter. UMB are thought to be organisms that thrive

primarily under nutrient-limited conditions (Roszak and Colwell, 1987). However, Fegatella et al. (1998) have pointed out that the obligate intracellular parasite *Rickettsia prowazekii* represents an UMB that lives in nutrient-rich cytoplasm. As a plant pathogen, *X. campestris* gains entry into the intracellular spaces in xylem tissue through hydathodes, stomates or wounds, and then kills plant cells by activating genes in the plant via bacterial effector proteins across membranes (Büttner and Bonas, 2002). Although *X. campestris* is not an obligate resident inside plant cells, the characteristics of this bacterium appear to be adapted for the niche that it occupies *in situ*. A small cell size can facilitate entry and survival in plant tissue and the ability to respond rapidly to nutrients can prove useful when cytoplasmic contents are released from dying cells. The other sequences classified in the γ -*Proteobacteria* were similar to the 16S rRNA genes from the genera *Lysobacter* and *Thermonomas*, which are also members of the family Xanthomonadaceae.

In contrast to the filtered bacteria from 2003, most of the colonies from the 2004 samples did not appear on the culture plates until one month following inoculation. A large percentage of these colonies were pinpoint-sized. Torrella and Morita (1981) originally described autochthonous ultramicrobacteria as very small bacteria that form colonies on agar very slowly and are unable to be detected with the naked eye after 24 hours of growth. Our colonies could be seen without magnification; however, they were not detected until plates had been incubated for one month. Therefore, these pinpoint colonies could represent oligotrophic UMB. Analysis of the 16S rRNA sequences from the filterable bacteria indicated that most of the bacteria from 2004 with a very small colony size were related to bacteria in the genera *Rickettsia*, *Sphingomonas*,

Bradyrhizobium, *Rhizobium*, *Parvibaculum*, *Beijerinckia*, *Methylophilus*, *Methylovorus*, and *Rhodoplanes*. Several of these genera included bacteria that are noted for growing inside animal or plant cells (*Rickettsia*, *Rhizobium* and *Bradyrhizobium*). Others, related to the bacteria *Sphingomonas* and *Methylophilus*, were among the ultrasmall bacteria ($< 0.1 \mu\text{m}^3$) isolated from a 120,000-year-old glacial ice core in Greenland (Miteva and Brenchley, 2005). Rutz and Kieft (Rutz and Kieft, 2004) also reported isolating clones of dwarf bacteria that were related to *Sphingomonas* from desert soils.

Interestingly, the majority of the small-colony sequences from our study were highly similar (95–96%) to an isolate “*Rhodoplanes* sp. strain laus-2,” that was isolated using an amoebal co-culture technique from a hospital water network (Thomas et al., 2006). This strain was characterized as a very slow-growing gram-negative bacterium that formed loose colorless colonies on CYE agar incubated at 32° C for 3 weeks. Buckley et al. (2007) recently used stable isotope probing with $^{15}\text{N}_2$ to demonstrate that bacteria in the genus *Rhodoplanes* may represent a new and important group of free-living nitrogen-fixing bacteria. It is known that protozoa living in the rhizosphere increase the nitrogen available to plants by releasing nutrients from consumed bacterial biomass (Bonkowski, 2004); however, the role of protozoa as hosts for symbiotic nitrogen-fixing bacteria has only recently been demonstrated (Kneip et al., 2007).

Significantly more filterable bacteria were isolated from soils amended with compost (high-input) compared to those that were not (low-input). The added nutrients in compost may have promoted the resuscitation of starved cells or selected for copiotrophic organisms. The clones that affiliated with the ultramicrobacteria characterized as *X. campestris* were isolated only from high-input plots and appeared on

the primary isolation plate within 2 weeks, which supports the characterization of this UMB as fast-growing and copiotrophic. Other sequences related to *Phaeospirillum fulvum* also were found only in high-input soils. However, the dominant clones in the 2003 library were found in both high- and low-input soils. Sequences related to *Polaromonas*, *Ramlibacter* and *Variovorax* (cluster D) comprised 37% of the clones in the 2003 library. Rutz and Kieft (2004) reported cloning a 16S rRNA gene (AF443568) from dwarf bacteria from shrubland soils that was related to *Ramlibacter* and *Acidovorax*. This sequence, which was 92 to 94% similar to our sequences in cluster D, made up 35% of the clones isolated from soils collected from beneath creosote bush canopies, but was not detected in soils collected from open areas between bushes. These data suggest that the bacterium associated with this sequence persists in soils with higher levels of carbon and nitrogen. However, we obtained clones associated with cluster D that were isolated from soils both with and without compost. A common near neighbor in both of these studies, *Ramlibacter tataouinensis*, is a pleomorphic bacterium that was isolated from a meteorite found in desert soils, produces yellow-orange pigments, and forms cysts and ‘nanometric’ rods (diameter of 0.2 μm) (Heulin et al., 2003). The ecological role of this bacterium in temperate soil is unclear; however, evidence suggests that it may be involved in the transformation of inorganic elements in soil. In culture, this bacterium has been shown to preferentially colonize orthopyroxene crystals (Benzerara et al., 2004b) and control precipitation of calcium phosphate (Benzerara et al., 2004a). The other near neighbors in cluster D belonged to the genera *Polaromonas* and *Variovorax*. Like *Ramlibacter tataouinensis*, several species of *Polaromonas* are adapted to live in extreme environments such as the Antarctic seas, Alaskan soil and oligotrophic caves and

karsts (Irgens et al., 1996; Barton, 2006; Sizova and Panikov, 2007). Other species, however, have been isolated from naphthalene-contaminated sediments (Jeon et al., 2004) and tap water (Kaempfer et al., 2006), which suggests that bacteria in this genus are cosmopolitan organisms with broad niches. Although the reported cell diameters of most *Polaromonas* are $> 0.5 \mu\text{m}$, the possibility that UMC form in response to environmental stress or starvation cannot be excluded.

The remaining 16S rRNA sequences for the clones libraries were affiliated with the δ -*Proteobacteria* and *Flexibacter-Cytophaga-Bacteroidetes* (CFB) group. The *Flexibacter* are known to be highly pleomorphic, forming long threads that fragment into very short rods as cultures age (Reichenbach and Kleinig, 1974). Members of the CFB division have been found in associations with many organisms including: the arbuscular mycorrhizal fungi *Glomus geosporum* and *Glomus constrictum* (Roesti et al., 2005), Charophycean green algae (Fisher et al., 1998), and the nematodes *Cephalobus brevicauda* (Salinas et al., 2007) and *Eubostrichus diana* (Polz et al., 1999). Barbieri et al. (2000) also identified an uncultured CFB-affiliated bacterium in the subgroup *Sphingobacter* that was found within the mycelium of the ectomycorrhizal fungus *Tuber borchii*. In this study, the nearest neighbor (91–93% similarity) to three of the clones from 2003 was a *Flexibacter* clone (DQ279370) associated with *Tuber magnatum*.

Conclusions

A small cell size is a phenotypic trait that helps bacteria adapt to and survive in different environments. Some bacteria exhibit plasticity in cell size and may only become small under stressful conditions, while others maintain a fixed cell volume.

Selective forces that favor a small cell size include nutrient availability, passive dispersal, sieving through geological strata and predation by protozoa and nematodes (Young, 2006; Salinas et al., 2007). Small cell sizes are also associated with bacteria that have host-dependent life strategies (Iizuka et al., 1998). In contrast to other studies (Rutz and Kieft, 2004; Miteva and Brenchley, 2005), we did not find any clones related to small-celled gram-positive bacteria. However, we isolated 16S rRNA genes from culturable small-celled soil bacteria that are highly similar to known soil ultramicrobacteria, filterable bacteria recovered in groundwater and desert soils, and bacteria that favor intracellular, endophytic or epibiotic growth. There also were many 16S rRNA sequences that were affiliated with bacteria that are well-established or newly discovered nitrogen-fixing bacteria. Although these bacteria were not isolated in pure culture and their dimensions were not confirmed, their growth on agar plates and unique community fingerprints are evidence that they represent viable soil bacteria that are not routinely recovered using the classical spread-plate method (Zuberer, 1994). By expanding efforts to cultivate filterable soil bacteria, we can target the numerically-dominant bacteria in soil and improve our ability to “culture the unculturable.”

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Figure 2.1. Number of small cell colonies on R2A plates inoculated with four dilutions of soils from the high-input (black bars) and low-input (white bars) plots at the market garden at the West Virginia University organic research farm on June 26, 2003. Plates were incubated for 2 weeks at 25°C.

(a)

(b)

Figure 2.2. Appearance of bacterial colonies on R2A agar 24 hrs (white bars) , 1 week (gray bars) and 1 month (black bars) for (a) nonfiltered communities (10^{-7} dilution) and (b) filtered (10^{-2} dilution). Bacteria were isolated from soil collected from high- and low-input plots located at the West Virginia University organic market garden on June 8, 2004.

Figure 2.3. PCR-DGGE fingerprint of partial 16S rRNA genes from filtered and nonfiltered bacterial communities isolated from high- and low-input soils in (a) 2003 and (b) 2004. Band numbers are associated with sequenced 16S clones presented in **Figures 2.6 and 2.7**. Black arrows designate bands referenced in text.

Figure 2.4. UPGMA dendrogram of filtered and unfiltered bacterial communities in high- and low-input soils from the organic farm in June 2003 and 2004.

Figure 2.5. Distribution of ARDRA patterns for 16S rDNA clones from small-celled bacteria from (a) 2003 (b) and 2004. Clones from 2003 were classified into two groups based on the source (filterable bacteria from high- or low-input soil) of the 16S rRNA sequences. Clones from 2004 were classified into two groups based the colony size of the filterable bacteria that the 16S rRNA sequences were isolated from. Asterisks indicate putative chimeric sequences identified using Bellerophon.

Figure 2.6. (a) PCR-DGGE of partial 16S rRNA genes and (b) neighbor-joining phylogenetic tree of β and γ *Proteobacteria* 16S rDNA sequences of clones from filterable bacteria from 2003 and 2004. Clone identifications refer to the soil source (high- or low-input) or the bacterial colony type (large or small colony) associated with the bacterial sources of the 16S rRNA genes and the DGGE band and ARDRA pattern associated with each clone. Asterisks indicate bands with 16S rDNA sequences detected in both 2003 and 2004 and gray boxes highlight important reference sequences discussed in the text. Sub-clusters are identified in each group using the letters A through G. Bootstrap values greater than 70% are presented at nodes. Scale bar represents 0.02 substitutions per nucleotide position.

Figure 2.7. (a) PCR-DGGE of partial 16S rRNA genes and (b) neighbor-joining phylogenetic tree of α and δ *Proteobacteria* and *Bacteroidetes* 16S rDNA sequences of clones from small-celled bacteria from 2003 and 2004. Clone identifications refer to the soil source (high- or low-input) or the bacterial colony type (large or small colony) associated with the bacterial sources of the 16S rRNA genes and the DGGE band and ARDRA pattern associated with each clone. Asterisks indicate bands with 16S rDNA sequences detected in both 2003 and 2004 and gray boxes highlight important reference sequences discussed in the text. Sub-clusters are identified in each group using the letters H through J. Bootstrap values greater than 70% are presented at nodes. Scale bar represents 0.02 substitutions per nucleotide position.

Chapter 3: Response of Culturable Bacterial Communities in Upland Pasture and Organic Farm Soils to Agronomic Treatment

Abstract

Culturable soil bacteria may exhibit significant short-term variation in community structure, providing a responsive indication of anthropogenic disturbances. In the present study, microbial biomass carbon (MBC), potentially mineralizable nitrogen (PMN), community level physiological profiling (CLPP), and culture-dependent DGGE (CD DGGE) fingerprinting of the 16S rRNA gene were used to compare culturable microbial communities in organic farm and pasture soils subjected to differing agronomic treatments. Multiple and simple correlation analyses revealed significant relationships between MBC, PMN, and variables derived from principal component analysis (PCA) of CLPP data as well as non-metric multidimensional scaling (NMDS) of CD DGGE data. All measures clearly separated soil types, but varied in their ability to distinguish among treatments within a soil type. Overall, MBC, PMN, and CLPP were most responsive to additions of compost (organic farm) and manure (pasture), while CD DGGE resolved differences in legume cropping (organic farm) and inorganic fertilization (pasture). Assessment of culturable soil bacteria using CLPP and CD DGGE provided non-redundant and useful indications of selected agronomic perturbations.

Keywords: Culturable bacteria, Cultivable, DGGE, CLPP, Community structure, Functional diversity, Compost, Manure, Organic Farm, Pasture.

Introduction

Well-functioning agricultural soils sustain biological productivity, promote environmental quality, and maintain plant and animal health (Doran and Parkin, 1994). Measurements of soil functionality encompass a variety of physical, chemical, and microbiological parameters. Microorganisms serve as dynamic indicators of a soil's quality due their essential roles in organic matter decomposition, nutrient cycling, and plant productivity (Kennedy and Papendick, 1995; Sun et al., 2004). Parameters that integrate diverse microbial populations into a single measure, such as microbial biomass carbon (MBC) and potentially mineralizable nitrogen (PMN), historically have proven to be useful and are widely employed measures of soil quality (Stenberg, 1999; Ibekwe et al., 2002). Microbial biomass C encompasses a small labile fraction of total soil organic carbon that responds actively to changes in soil fertility, supports soil aggregation, and can be related to environmental factors such as climate, soil moisture, texture, and organic matter quality (Templer et al., 2003). Potentially mineralizable nitrogen provides an index of a soil's nitrogen supplying capacity and has been positively correlated with other chemical and physical indicators of soil quality (Ibekwe et al., 2002).

More recently, workers have attempted to open the "black box" of soil microbial dynamics, seeking to assess and compare detailed changes in microbial populations resulting from implementation of differing agronomic practices (Tiedje et al., 1999). Various measures of microbial community structure have been proposed as appropriate indicators of changing soil quality (Stenberg, 1999; Schlöter et al., 2003). Functional diversity assessed via community-level physiological profiling (CLPP) employs a culture-based technique that characterizes the relative growth of the soil microbial

community on a variety of carbon substrates. CLPP has been used successfully to discriminate soil microbial communities based on 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; Miethling et al., 2000). A variety of non-culturing methodologies have been developed that rely on nucleic acid extraction followed by gene amplification to characterize the soil microbial community (Nakatsu, 2007; Thies, 2007). Culture-independent molecular methods theoretically provide a better representation of total species richness, since they include novel, numerically-abundant, but previously unculturable groups such as the *Acidobacteria* and *Verrucomicrobia* (Amann, 1995; Hugenholtz et al., 1998; Øvreås and Torsvik, 1998; Barns et al., 1999; Girvan et al., 2003). The study of soil microbial communities is challenging, and currently no single methodology is without limitation nor provides a complete picture of total diversity (Farrelly et al., 1995; Konopka et al., 1998; Polz and Cavanaugh, 1998; Garland, 1999; Schmalenberger et al., 2001; Preston-Mafham et al., 2002; Thies, 2007). In order to provide more comprehensive profiles and a framework to evaluate the strengths and biases of each method, it is beneficial to examine multiple techniques in parallel to study bacterial communities (Girvan et al., 2003).

Despite the current prevalence of the culture-independent approach, evidence is emerging that culture-dependent profiles may be more responsive to anthropogenic perturbations (Ellis et al., 2003). In a recent study, I investigated a plate wash method to construct culture-dependent DGGE (CD DGGE) profiles of the 16S rRNA gene (Edenborn and Sexstone, 2007). The CD DGGE gels showed clear banding patterns of

sufficient complexity and variability to investigate differences in microbial communities. In this paper CD DGGE, CLPP, MBC and PMN were used to compare and contrast bacteria in soils from a pasture and organic farm that have been subjected to different fertility regimes. The objectives of this study were to: to 1) evaluate the utility of CD DGGE to characterize the response of culturable bacterial communities to different soil fertility amendments and 2) compare and contrast the responsiveness of CD DGGE, CLPP, MBC and PMN microbial indicators to these agronomic treatments.

Materials and Methods

Description of field sites and soil sampling

The West Virginia University (WVU) Organic Research Farm is located in Monongalia County, WV (39°38'34"N, 79°56'04"W). Soils on the farm are Dormont and Guernsey silt loams (fine loamy, mixed, superactive, mesic, Oxyaquic Hapludalfs). The market garden, established in 2000, is a completely randomized design with four plots (4.9 x 7.6 m) per treatment. Soil pH averaged 6.7 and was not significantly different between treatments (Childers, 2005). Treatments included fertility input (compost and green manure) and annual crop rotation among four plant families (Fabaceae, Solanaceae, Cucurbitaceae, and Asteraceae). High-input plots received composted dairy manure (22.4 Mg ha⁻¹ based on wet weight) and green manure (rye and vetch) applied and tilled into the soil 1–3 days prior to planting. Low-input plots received only green manure applied as described above. Details of compost analyses, soil fertility, and crop yield in the market garden have been reported by Childers (2005). In 2004, compost was applied to plots on April 19 (peas), May 11 (beans), and May 25

(tomatoes/peppers). Composite (12 cores) surface soil samples (15 cm depth x 2.5 cm diameter) were collected on June 8 and August 2 of 2004 from high- and low-input plots that had been planted with beans and peas (Legumes) and tomatoes and peppers (Solanaceae). Ten soil cores (16 cm depth x 2 cm diameter) were collected from each plot at ~1.5 meter intervals along two transects at midpoint between crop rows. Soil samples were bulked, placed in polyethylene bags, transported on ice to the laboratory, sieved (< 2 mm) and refrigerated at 4°C.

The West Virginia University Reedsville Experiment Farm is located in Preston County, WV (39°30'55"N, 79°48'70"W). Soils on the farm are mapped in the Gilpin (fine-loamy, mixed, semiactive, mesic Typic Hapludults) and Wharton (fine-loamy, mixed, mesic Aquic Hapludults) soil series. Since 1998, this experimental pasture has been limed to maintain a pH circa 6.5 and fertilized with two levels of inorganic fertilizer inputs; i.e., high fertility (P 134 kg ha⁻¹ and K 390 kg ha⁻¹) or low fertility (P 67 kg ha⁻¹ and K 195 kg ha⁻¹). The pasture was subjected to the same annual grazing pressure (Prigge et al., 1999). The predominant pasture grasses were orchard grass (*Dactylis glomerata* L.), Kentucky bluegrass (*Poa pratensis* L.), and meadow fescue (*Festuca elatior* L.) interspersed with white clover (*Trifolium repens*) and red clover (*Trifolium pratense*).

In summer 2004, fenced experimental subplots were established on adjacent high- and low-fertility pasture sites and amended with a manure treatment. The percentage of legumes averaged 36% and 10% in high- and low-fertility plots, respectively (Sutanto, 2005). Each plot was subdivided into quadrants (1 m x 1 m), which were randomly assigned to receive a manure patch or remain as an untreated control. Manure patches

(25 cm diameter, 5 cm height) were fabricated from fresh manure collected from the same fields. Four replicate quadrants from each treatment were sampled at day 0, 21, 40, 63, 123 after site establishment. On day 0 only the non-manured (control) soils were sampled. Twelve soil core samples (15 cm depth x 2.5 cm diameter) were collected from each quadrant. Core samples were bulked, placed in polyethylene bags, transported on ice to the laboratory, sieved (< 2 mm) and refrigerated at 4°C.

Microbial biomass carbon and potentially mineralizable nitrogen

MBC was extracted from moist soil (80% water-filled porosity) according to the method of Islam and Weil (1998). Total carbon in soil extracts was measured using a Dohrmann® DC-190 TOC analyzer (Rosemount® Analytical Inc., Santa Clara, CA). PMN was determined according to the methods described in Keeney (1982). Duplicate field moist soil samples (5 gram) were placed in 20 x 150 mm test tubes with 12.5 mL of sterile water. One replicate was extracted at time 0 with 12.5 mL of 4 M KCl. The headspaces of the other replicates were filled with N₂ to ensure an anaerobic incubation and then were incubated in the dark at 20°C for 7 days. At the end of 7 days, the incubated samples were shaken (60 minutes, 30 rpm) with an additional 12.5 mL 4 M KCl. Supernatant was filtered through 0.45 µm Millipore filter and frozen until further analysis. Ammonia in each sample was measured using a Lachat flow injection analyzer (Hach Company, Loveland, CO) using the phenolate method.

Community level physiological profiling

The ability of soil microbial communities to utilize a variety of carbon sources was assessed using community-level physiological profiling originally developed by Garland and Mills (1991). Sterile Waring blenders were used to homogenize (1 min, max speed, 3x with intermittent cooling on ice) 20 g of soil in 180 mL Winogradsky Salts Solution (WSS; Zuberer, 1994). Homogenate was diluted (10^{-3}) and used to inoculate ($100\ \mu\text{L well}^{-1}$) BIOLOG[®] ECO Plate MicroPlates[™] (Biolog Inc., Hayward, CA). The microplates were incubated at 25°C for five days. Utilization of the carbon source was monitored by measuring absorbance at 590 nm. Readings were taken every 24 hours using an automated plate reader (SpectraMAX 340 pc, Molecular Devices, Sunnyvale, CA).

Culture-dependent denaturing gradient gel electrophoresis

Twenty grams (dry weight) of composite soil samples from each plot were placed in sterile Waring blenders and agitated for one minute at high speed (3x with intermittent cooling on ice) in 180 mL of sterile WSS. Blended suspensions were diluted (10^{-2}) in sterile WSS and 100 μL was plated onto each of four replicate agar plates (final dilution = 10^{-3}) and incubated aerobically for two weeks at 25°C. Cultured cells were washed sequentially from four replicate R2A plates using WSS (4 mL) and a sterile disposable inoculating loop. The resulting cell suspensions were vortexed and frozen (-20°C). DNA was extracted from aliquots of each thawed cell suspension (1.8 mL) using the MoBio Microbial DNA extraction kit (MoBio Labs, Carlsbad, CA). The variable V3 region of the 16S rRNA gene from the domain *Eubacteria* was amplified using the PRBA338F

primer with PRUN518R primer as previously described (Edenborn and Sexstone, 2007). Composite PCR products (~ 150 ng) were 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 and electrophoresed for 14–16 hours at 50 V and 60°C using the DCode™ Universal Mutation Detection System (BioRad). The gel was visualized by silver staining according to the protocol of Caetano-Anolles and Gresshoff (1994). The developed gel was scanned using Hewlett Packard ScanJet 7400c (Hewlett-Packard Co., Palo Alto, CA) at a resolution of 600 dpi and saved in uncompressed TIFF format for further analysis. A detailed description of this procedure is presented in Edenborn and Sexstone (2007).

Data analysis

Differences between MBC organic farm (OF) and pastures (PAS) soils were tested using an analysis of variance (ANOVA) in a 2 X 3 factorial design (factor A: high- and low-input (OF) or high- and low-fertility (PAS); factor B: time-crop combination (OF) or time-manure amendment combination (PAS)). PMN in organic farm soils was compared using a 2 X 2 Factorial (Factor A: high- and low-input; Factor B: time-crop combination). For pastures soils, the same factorial design used for MBC was used for PMN. Tukey's HSD test was used for post-ANOVA pair-wise comparisons.

Total well color development (TWCD) for BIOLOG® ECO plates was calculated as the sum of absorbance values determined after 63 hours of incubation. Average well color development (AWCD) was calculated from 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 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 evenness (E) was calculated as $E = H/\ln(S)$. Data were normalized by dividing each substrate absorbance value by the AWCD (Garland and Mills, 1991). Robust principal component analysis (PCA) was done on a covariance matrix of normalized data using $\alpha=0$.

DGGE gels were analyzed using Quantity One gel analysis software (BioRad, Hercules, CA). Analysis of the bands was done by setting background subtraction at 15 using a rolling disk method and generating intensity profiles to evaluate and compare peak size, shape and location. Bands were picked manually for a limit of intensity > 0.05 . Similarity matrices were calculated with Sorensen's index based on the presence/absence of peaks. Analysis of similarity (ANOSIM) was used to determine significant differences between DGGE banding profiles. In ANOSIM, an R value greater than 0 indicates that objects (DGGE profiles) are more dissimilar between groups than within groups and a P value indicates the level of significance (Rees et al., 2004). Enhanced non-metric multi-dimensional scaling (NMDS) was used to ordinate DGGE data (Fromin et al., 2002; Rees et al., 2004). The ability of NMDS to provide a good representation of similarity data in two-dimensional space was evaluated based on Kruskal's stress values. Stress values < 0.15 indicate that the ordination plots presented were useful representations of the CD DGGE data (Rees et al., 2004).

Multiple and simple correlations analysis was used to assess associations between principal component, NMDS axis scores, MBC and PMN data. Statistical analyses were

done in R (R Development Core Team 2004) or SAS (SAS Institute Inc., Cary, NC). ANOSIM and NMDS were done using the R package Vegan (Oksanen, 2005).

Results

Microbial biomass carbon and potentially mineralizable nitrogen

In the present study, levels of MBC were found to be an order of magnitude higher in pasture soils compared with the organic farm (**Table 3.1**). In both systems, amendment with manure or compost and season resulted in significant differences in MBC. MBC levels in organic farm soils were significantly affected by input ($F=17.57$, $P < 0.001^{***}$) and the time-crop combination ($F=7.51$, $P < 0.004^{**}$). The interaction between these two factors was not statistically significant ($F=2.19$, $P < 0.134$). Multiple comparison using Tukey's HSD test indicated that the MBC in high-input plots (June) was significantly different from the other soils except the high-input plots planted with legumes (August) (**Table 3.1**). In pastures, MBC was significantly affected by manure amendment and time ($F=17.78$, $P < 0.001^{***}$), but not fertilizer amendment ($F=1.20$, $P < 0.287$). The interaction was not significant ($F=2.58$, $P < 0.103$). MBC was significantly higher in September than in May, and the greatest amount of MBC was found in plots that had been amended with manure (**Table 3.1**). Clear statistical differences between inorganic fertilizer applications were not demonstrated; however, lower levels of MBC generally were observed in low- compared with high-fertility plots, both in May and September.

Pasture soils on average contained four times more PMN than organic farm soils (**Table 3.1**). In organic farm soils, a significant difference in PMN was found due to input ($F=4.87$, $P < 0.048^{**}$) but neither time-crop combination ($F=0.99$, $P < 0.339$) nor the

interaction of these two factors was significant ($F=0.757$, $P < 0.402$). When individual means were compared using Tukey's HSD, no differences were found in PMN between different soils (**Table 3.1**). In pastures, both fertilizer input ($F=4.93$, $P < 0.040^{**}$) and time-manure amendment ($F=7.43$, $P < 0.004^{**}$) were found to have a significant effect on PMN without any interaction ($F=0.203$, $P < 0.979$). Plots with manure contained significantly higher levels of PMN than plots that had not been amended with manure (**Table 3.1**).

Community level physiological profiling

Shannon diversity indices ranged between 3.26–3.35 and 3.27–3.29; richness values ranged from 28–30 and 28–29; and evenness from 0.975–0.982, and 0.975–0.977 for the organic farm and pasture soils, respectively. Using Tukey's HSD test to separate means, I observed no significant differences ($P > 0.05$) in functional diversity indices based on soil type or agronomic treatment (data not shown).

Despite the lack of treatment discrimination by diversity parameters, ordination of the CLPP data using principal component analysis clearly distinguished low-input organic farm soils from other soils. Bryant and Yarnold (2005) suggest that the minimum number of samples should be at least five times the number of variables in order to achieve a reliable PCA analysis. Thirty-one substrate vectors (substrates) also confound the interpretation of biplots. Therefore, I first performed PCA on all 31 substrates to identify the most strongly-loaded, non-colinear substrates. Ten of these substrates were then used for a subsequent PCA. These substrates were: L-arginine, D-cellobiose, cyclodextrin, D-erythritol, 2-hydroxybenzoic acid, D-lactose, L-phenylalanine,

phenylethylamine, L-threonine, and D-xylose. Ordination of these data is presented in **Figure 3.1** where the distinct cluster formed by low-input organic farm soils can be visualized. In this analysis, 75% of the variance was accounted for by the first three principal components. The first principal component was most strongly correlated with the utilization of L-phenylalanine (-0.94), D-lactose (-0.68) and L-threonine (-0.61). Principal component 2 was most strongly correlated with 2-hydroxybenzoic acid (0.87), and principal component 3 was most strongly correlated with phenylethylamine (0.95) and L-arginine (0.69).

Culture-dependent denaturing gradient gel electrophoresis

To facilitate comparison of all soils and treatments on a single gel, replicate PCR products from samples obtained in August or September were first pooled. The resultant CD DGGE profiles of 16S rDNA showed distinct differences between culturable bacterial communities in organic farm and pasture soils (**Figure 3.2a**). Culturable bacterial communities in the two soil types were highly distinct (ANOSIMS; $R=1.00$, $P < 0.023^{**}$). Ordination of the data using NMDS demonstrated that legume cropping (OF) and fertilizer (PAS) had more impact on the structure of culturable soil bacterial communities than organic matter amendments (compost or manure) (**Figure 3.2b**).

Temporal changes in CD DGGE fingerprints were determined following application of manure pats to pasture soil treatment plots. Prior to application of manure (day 0), five replicate soil samples were collected from high- and low-fertility plots and the culturable bacterial communities characterized. Significant differences were found between the high- and low-fertility plots on day 0 (ANOSIMS; $R=0.36$, $P < 0.018^{**}$).

NMDS analysis of these data is presented in **Figure 3.3a**. As the manure pats decomposed, additional samples were collected on days 21, 40, and 63. At each sampling time, PCR products from three replicate plots per treatment were combined to create a composite profile. There were significant differences in bacterial communities due to time (ANOSIMS; $R=0.36$, $P < 0.013^{**}$); however, no significant differences were observed due to treatment ($R= -0.162$, $P < 0.835$). Ordination of the data also revealed distinct clustering based on sampling time (**Figure 3.3b**).

In June and August, soils collected from three replicate plots per treatment at the organic farm were used for CD DGGE analysis. There were no significant differences in CD DGGE profiles from different treatments in June (ANOSIMS; $R=0.44$, $P < 0.087$); however, significant differences were observed in August ($R=0.454$, $P < 0.006^{**}$). Bacterial communities from tomato and pepper plots (high and low fertility) collected in both June and August clustered together (**Figure 3.4a**). In contrast, the communities from soils that had been planted with legumes clustered separately from those from soils planted with tomatoes and peppers and from each other (**Figure 3.4b**).

In this study, Kruskal's stress values for all analyses were < 0.15 , indicating that the ordination plots presented were useful representations of the CD DGGE data (Rees et al., 2004).

Correlations between MBC, PMN, CLPP, and CD DGGE

Multiple correlation analysis was used to explore potential relationships between the principal component scores from the PCA analysis of CLPP data (**Figure 3.1**) and other measured variables. Significant correlations were observed between the CLPP

principal components and MBC ($R=0.71$, $P < 0.001^{***}$) and PMN ($R=0.47$, $P < 0.007^{**}$). When organic farm and pasture sample were analyzed separately the correlations improved ($R > 0.85$, $P < 0.001^{***}$) for MBC and PMN from both systems. Multiple correlations between the CLPP principal components (**Figure 3.1**) and the axis scores from NMDS of CD DGGE data from the organic farm samples in August 2004 (**Figure 3.4b**) also were highly significant (dimension 1: $R=0.99$, $P < 0.001^{***}$; dimension 2: $R=0.98$, $P < 0.001^{***}$). Multiple correlation analysis was not performed on pasture data because samples for CD DGGE and CLPP were collected on different sampling dates. Simple correlations were performed for both agronomic systems using axis scores from NMDS of CD DGGE data and the MBC and PMN data. In both soil systems, significant correlations were found between the DGGE axis scores and MBC (**Figure 3.5**) but not between DGGE axis scores and PMN (data not shown).

Discussion

Microbial biomass carbon and potentially mineralizable nitrogen

Microbial biomass carbon and potentially mineralizable nitrogen are broad indicators of soil quality that assess microorganisms at the level of the whole microbial community (Stenberg, 1999). In the present study, both MBC and PMN were responsive to compost and manure input to organic farm and pasture soils, respectively. The effects of compost amendment in organic farm soils generally were transitory. For example, MBC levels in the high-input plots planted with tomatoes and peppers were significantly higher than in low-input soils two weeks after application of compost, but decreased to similar levels by ten weeks. This decreasing trend in MBC following compost

amendment may be due to increased carbon mineralization rates following regular soil tillage employed to control weeds in this organic production system (Childers, 2005). High-input legume plots at the organic farm sustained the highest levels of MBC throughout the sampling period, perhaps due to higher N availability in these plots. Both MBC and PMN in manure-treated high-fertility pastures remained significantly higher than other treatments 17 weeks following amendment. In the absence of manure, clear differences were not apparent among MBC and PMN as a function of inorganic fertilizer applications in pasture soils.

Changes in MBC and/or PMN due to crop rotation, legume cropping, manure amendment, and compost application (Moore et al., 2000; Andrews et al., 2002; Parham et al., 2002; Ginting et al., 2003; Girvan et al., 2003; Canali et al., 2004; Kang et al., 2005) have been observed previously. Reported effects of nitrogen additions on MBC and PMN have been contradictory. Some studies have shown increased MBC following N fertilizer application (Fraser et al., 1994; Omay et al., 1997), while others have found no effect (Moore et al., 2000; Cookson et al., 2005). Ginting et al. (2003) reported that soils receiving nitrogen fertilizer had PMN values lower than those receiving manure and compost but higher than those of control soils. In contrast, Kang et al. (2005) observed slightly higher levels of PMN in fertilized soils than in control soils, and Cookson et al. (2005) reported no differences in PMN in fertilized and unfertilized soils. Fisk and Fehey (2001) have suggested that biomass pools in fertilized soils may be smaller but more physiologically active than those found in unfertilized soil.

Community-level physiological profiling

CLPP is a widely-used technique appropriate for screening relative shifts in microbial communities, conducting exploratory investigations, and corroborating other methods (Garland, 1999). Principal component analysis was first employed by Garland and Mills (1991) to reduce complex multivariate CLPP data into a smaller number of interpretable variables. In the present study I observed no statistically significant differences in calculated functional diversity indices (diversity, richness, evenness) resulting from different agronomic treatments within each system; however, ordination of CLPP data by PCA clearly distinguished low-input organic farm soils from other soils and identified substrates that best discriminated between the different samples. Utilization of strongly-loaded aromatic substrates such as L-phenylalanine and 2-hydroxybenzoic acid determined much of the observed variation among soil treatments revealed by PCA. Positive substrate loadings discerned by PCA probably do not represent microbial physiologies that predominate *in situ* since the assay enriches and selects only a few bacterial types on each individual carbon source (Konopka et al., 1998; Preston-Mafham et al., 2002). However, it is reasonable that culturable bacteria capable of catabolizing aromatic substrates could serve as responsive indicator organisms that are capable of discriminating among agronomic treatments.

Evidence suggests that CLPP is a culture-based enrichment method that primarily characterizes fast-growing, copiotrophic bacteria that can be distinct from the dominant bacteria in soil inocula (Smalla et al., 1998; Garland, 1999). Therefore, the ecological significance of CLPP data often is questioned. In this study I used multiple correlation analysis to explore potential associations between CLPP data and MBC and PMN. My

results indicated that CLPP principal component scores were significantly correlated with MBC and PMN in both systems. Although correlation does not imply causation, correlative relationships suggest that these variables respond in a similar manner to environmental perturbation (Cookson et al., 2007). Other workers also have found significant correlations between CLPP data, gross N flux, and soil respiration (Cookson et al., 2007). These results suggest a link between the functional diversity of culturable bacteria and soil ecosystem functions.

Culture-dependent denaturing gradient gel electrophoresis

Ellis et al. (2003) compared metal-contaminated and uncontaminated soils, and provided strong evidence supporting the use of culturable bacteria to discriminate between soil microbial communities. Culture-independent DGGE profiles of the 16S rRNA gene were of limited value since they produced similar community fingerprints, regardless of the level of soil contaminants. When soil bacteria were first cultivated on agar plates the total number of organisms decreased with increasing metal content, and the resulting “communities” growing on the plates produced distinct DGGE fingerprints of 16S rRNA. Ellis et al. (2003) hypothesized that culturable bacteria were an ecologically-relevant fraction of the soil community and provided unique information when compared with culture-independent methodologies, since they were responsive to environmental change. The clear difference in culturable communities observed by these workers was somewhat expected due to strong selection by high soil levels of toxic metals. Further work with CD DGGE is warranted to determine whether this technique

can discriminate subtle community changes that might occur in agronomic systems that are characterized by high species diversity and minimal selective pressures.

Recently, I demonstrated that CD DGGE fingerprints resolved a unique and diverse fraction of the soil bacterial community that complemented fingerprints produced using a culture-independent approach (Edenborn and Sexstone, 2007). In that study, CD DGGE analyses resulted in clear banding patterns of sufficient complexity (16–32 major bands) and reproducibility to investigate differences in bacterial communities in a silt loam soil. In the present study, we employed CD DGGE to examine whether culturable bacterial communities varied with soil amendment. I found no statistically-significant differences in CD DGGE fingerprints of 16S rDNA as a function of manure (PAS) or compost amendment (OF); however, significant differences were observed as a result of inorganic fertilizer amendment in pasture soils and legume cropping in organic farm soils. Chu et al. (2007) have observed previously that legumes and nitrogen availability impact bacterial community structure. Girvan et al. (2003) also hypothesized that soil type and legume cropping were the overriding factors controlling the structure of bacterial communities in agricultural soils. It is likely that the fertilizer effect I observed in pasture soils also involved a legume effect. For the past decade, experimental pastures at Reedsville, WV, have been subjected to equal grazing pressure and animal waste deposition but have received two different levels of phosphorus, potassium and lime amendments. The higher fertility treatment has been shown to favor the persistence of pasture legumes, such as red and white clover, in these soils (Bryan et al., 1987).

Natural selection acts on phenotype, and physiological characteristics are often linked to more than one gene. Therefore, it is insufficient merely to catalog 16S rRNA

genes to understand how environmental factors govern shifts in community structure. The ability to partition soil bacteria into distinct physiological subsets according to their growth characteristics on laboratory media provides a potential means to relate 16S rRNA fingerprints to phenotype. Previously, I demonstrated that utilization of multiple media and incubation conditions increased the number of unique bands resolved by CD DGGE relative to culture-independent analyses (Edenborn and Sexstone, 2007). In the present study, multivariate analyses of CLPP profiles suggested that culturable bacteria catabolizing aromatic substrates were highly discriminatory. Langenheder et al. (2006) have suggested that bacterial community structure may be related more to narrow functions such as degradation of semilabile carbon compounds than to broad functions such as respiration and biomass production. If this is true, characterizing culturable bacterial communities with specific physiological capabilities or narrow niche preferences may improve our ability to distinguish between soils that have been impacted by anthropogenic perturbations. Culture-based studies can never duplicate the complex environment that exists in soil or completely characterize the vast diversity of bacteria revealed by culture-independent analyses. However, results from culture-based studies provide a framework for more sophisticated modes of analysis (Ward, 2006). Recently, some investigators have argued that culturable communities have no utility in contemporary microbial ecology and cannot be related to soil fertility (Ritz, 2007). I disagree. Rather, I support arguments presented by Nichols (2007) and agree with the contention that microbial cultivation is a re-emerging frontier that provides information that is not directly observable from culture-independent and sequencing efforts alone.

Conclusions

The organic farm and upland pasture represent systems with contrasting soil types, land use, and fertility regimes. In this study, I used MBC, PMN, CLPP and CD DGGE of the 16S rRNA gene to compare microbial communities as a function of agronomic amendments. There were significant correlations between MBC and PMN and variables derived from analysis of the functional and genetic diversity of soil communities; however, each measurement technique exhibited a different response to the fertility amendments. Overall, MBC, PMN, and CLPP were most responsive to additions of manure and compost to soils; however, only CD DGGE revealed clear differences in soils that were amended with inorganic fertilizers and/or cropped with legumes. Because multivariate models are often used to evaluate the effects of agronomic perturbations on soil, it is necessary to identify variables that increase the ability to discriminate accurately between different systems. My study suggests that measures of microbial diversity do not provide redundant information in soil quality analysis, and that culturable bacterial communities provide useful indicators of select agronomic perturbations.

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Table 3.1. Microbial biomass carbon (MBC) and potentially mineralizable nitrogen (PMN) in organic farm and pasture soils^a.

Average Organic Farm						235 ± 77	23 ± 6
ID	Symbol ^b	System	Fertility Regime	Crop/Amendment	Sampling Date	MBC	PMN
						(mg C/kg)	(mg N/kg)
C1	◆	Organic Farm	High Input ^c	Tomatoes/Peppers	June 2004	369 ± 48 a	ND
NC1	◇	Organic Farm	Low Input ^d	Tomatoes/Peppers	June 2004	207 ± 36 b	ND
C2	◆	Organic Farm	High Input	Tomatoes/Peppers	Aug 2004	199 ± 13 b	24 ± 6 a
CL2	▲	Organic Farm	High Input	Legumes	Aug 2004	282 ± 29 ab	31 ± 4 a
NC2	◇	Organic Farm	Low Input	Tomatoes/Peppers	Aug 2004	156 ± 13 b	18 ± 4 a
NCL2	△	Organic Farm	Low Input	Legumes	Aug 2004	195 ± 12 b	19 ± 2 a
H1	■	Pasture	High Fertility ^e	None	May 2004	1659 ± 133 c	110 ± 18 abc
L1	□	Pasture	Low Fertility ^f	None	May 2004	1931 ± 79 bc	121 ± 11 ab
H2	●	Pasture	High Fertility	None	Sept 2004	1915 ± 126 bc	59 ± 8 c
HM2	■	Pasture	High Fertility	Manure	Sept 2004	2666 ± 126 a	125 ± 10 ab
L2	○	Pasture	Low Fertility	None	Sept 2004	2214 ± 79 abc	85 ± 11 bc
LM2	□	Pasture	Low Fertility	Manure	Sept 2004	2443 ± 196 ab	142 ± 13 a
Average Organic Farm						235 ± 77	23 ± 6
Average Pasture						2138 ± 374	107 ± 30

^a Mean values $n=4$ ($\pm$ SEM) followed by the same letter are not significantly different ($p < 0.05$) as determined by Tukey's Studentized Range (HSD) Test.

^b Symbols used in Figures throughout paper.

^c High Input = 22.4 Mg ha⁻¹ of composted dairy manure + green manure (rye + vetch) applied and tilled into soil 7–10 days prior to planting.

^d Low Input = Green manure (rye + vetch) tilled into soil 7–10 days prior to planting.

^e High Fertility = Inorganic fertilizer, P, 134 kg ha⁻¹ and K, 390 kg ha⁻¹

^f Low Fertility = Inorganic fertilizer, P, 67 kg ha⁻¹ and K, 195 kg ha⁻¹

Figure 3.1. Ordination biplots of principal components (PC1 and PC2) from CLPP analysis of organic farm and pasture soils subjected to varying fertility regimes and crop rotations from August and September 2004. Symbol designations are as described in **Table 1.**

Figure 3.2. (a) DGGE gel of pooled PCR-amplified 16S rRNA genes from culture-dependent bacterial communities of organic farm and pasture from August–September 2004 and (b) ordination plots of dimensions 1 and 2 from NMDS analysis of CD DGGE data. Stress of the plot = 0.000053. Symbol designations are as described in **Table 1**.

Figure 3.3. Ordination plots of DGGE data from pasture soils in: (a) May and (b) May–September, 2004. Stress of the plot = (a) 0.15 and (b) 0.15. Symbol designations are as described in **Table 1**.

Figure 3.4. Ordination plots DGGE data from organic farm market garden soils in: (a) June and (b) August, 2004. Stress of the plot = (a) 0.013 and (b) 0.13. Symbol designations are as described in **Table 1**.

Figure 3.5. Scatterplots, trendlines and Pearson correlation coefficients between MBC and dimensions 1 and 2 from NMDS analysis of CD DDGE profiles from organic farm in August, 2004 (a, b) and pasture days 0, 21, 40 and 63 between May and September, 2004 (c, d). Significant correlations are indicated by: $^{**} P < 0.01$ and $^* P < 0.05$.

Chapter 4: Spatial Variability and Heterogeneity of Community-level Physiological Profiles in Tilled and Untilled Soils from an Organic Farm

Abstract

Spatial variability and heterogeneity of microbial substrate utilization in tilled and untilled plots was explored using community-level physiological profiling (CLLP) and multivariate and geostatistical analysis. Robust semivariograms were constructed based on the area under the kinetic curve for substrate utilization in BIOLOG GN2 plates. Microbial substrate utilization was spatially structured in untilled soils and some tilled soils. Rotary tillage disrupted the spatial structure of substrate utilization in the upper few centimeters of soil; however, bacterial utilization of amines and amides exhibited a tillage-induced loss of spatial structure at 13–14 cm. Variability of microbial substrate utilization increased or decreased depending on the degree of perturbation. Spatial structure was more evident at lower soil depths in untilled soils, especially for amino acids, amines and amides. Flux of CO₂ was not spatially dependent and was significantly correlated with utilization of the amines and amides.

Keywords: Tillage, Spatial heterogeneity, Spatial variability, Geostatistics, Community-level physiological profiling (CLPP), Microbial substrate utilization.

Introduction

High spatial variability and heterogeneity of soil microenvironments hinders the development of accurate predictive models capable of relating biotic and abiotic factors to microbial community structure and activity at the field scale (Parkin, 1993). Spatial variability and heterogeneity of available carbon is thought to be an important factor regulating the structure and function of soil communities; however, details of *in situ* spatial distribution of soil microbial populations are just beginning to emerge (Ettema and Wardle, 2002).

Variability describes general changes in the value of a property over time and space. In contrast, heterogeneity refers to variability that exhibits spatial structure (Ettema and Wardle, 2002). Conventional parametric statistics such as the coefficient of variation can be used to quantify variability, while autocorrelation is used to determine heterogeneity in spatial data by quantifying the resemblance between neighbors as a function of spatial separation distances (Rossi et al., 1992; Ettema and Wardle, 2002; Legendre et al., 2002). Data that are spatially autocorrelated violate the assumption of independence required for parametric statistical procedures such as Analysis of Variance (ANOVA) and can inflate type I errors associated with significance testing of regression or correlation coefficients (Rossi, 1992; Legendre et al., 2002). Therefore, *a priori* assessment of spatial dependence is essential for optimal experimental design and accurate statistical inference (Ettema and Wardle, 2002).

Although a complete understanding of microbial processes cannot be derived by studying the structure and function of microbial communities at a single spatial scale (Franklin and Mills, 2003), knowledge of plot-level heterogeneity is necessary when

evaluating treatment effects (Parkin, 1993). Microbial dynamics may be controlled at the field scale by manipulation of soil structure through practices such as tillage (Elliott and Coleman, 1988). Spatial analyses are needed to accurately quantify the impact of agronomic practices such as tillage on microbial variables in soil.

Certified organic practices preclude the use of herbicides; therefore, rotary-tillage and manual cultivation routinely are employed to control weeds in the market garden of the West Virginia University Organic Research Farm (Childers, 2005). In a previous study, the effects of fertility and cropping on culturable microbial communities in market garden soils were evaluated using multivariate analyses (see Chapter 3). Since tillage was ubiquitous, potential impacts of this practice on market garden soil microbial communities remain unknown. Soil tillage has been shown to disrupt soil aggregation, accelerate the microbial decomposition of soil organic matter (Tisdall and Oades, 1982) and alter microbial community structure (Lupwayi et al., 2001; Feng et al., 2003). Mechanical perturbation is thought to affect bacterial diversity and activity by altering habitat space, water and substrate distribution, and the spatial arrangement of pores (Young and Ritz, 2000). Further, it induces changes in the distribution of soil organic matter as well as the macro- and micro-organisms that facilitate decomposition (Kladivko, 2001). In particular, tillage generally decreases soil organic carbon and nitrogen by exposing pools of previously protected organic matter to microbial degradation, thus negatively impacting soil aggregation and fertility (Kristensen et al., 2003). Such impacts are of considerable importance in organic farming systems, which cannot rely on inputs of inorganic fertilizers to sustain soil fertility and plant growth (Kuepper, 2001).

Geostatistical analyses have been used previously to quantify the spatial heterogeneity of microbial cells (Nunan et al., 2002), microbial communities (Grundmann and Debouzie, 2000; Franklin et al., 2002; Franklin and Mills, 2003; Ritz et al., 2004), and microbial activities such as flux of trace gases from soil (Robertson et al., 2000). However, geostatistical analyses rarely have been used to evaluate data from community-level physiological profiling (CLPP), even though this technique is widely applied to assess the impact of agricultural practices on soil bacterial communities (Sharma et al., 1998; Bending et al., 2000; Miethling et al., 2000; Williams et al., 2000; Bending et al., 2002; Malosso et al., 2005). Therefore, in the present study, I performed an exploratory investigation of spatial variability and heterogeneity of bacterial substrate utilization patterns in tilled and untilled organic farm soils at the inter- and intra-plot scale using multivariate and geostatistical techniques. The primary objective of this study was to assess the feasibility of using geostatistical analysis with CLPP data.

Materials and Methods

Site description, experimental design and soil sampling

West Virginia University (WVU) Organic Research Farm is located in Monongalia County, WV (39°38'34"N, 79°56'04"W). Soils on the farm are Dormont and Guernsy silt loams (fine loamy, mixed, superactive, mesic, Oxyaquic Hapludalfs). Surface layers extend to ~ 25 cm and subsoil to ~ 90 cm. Tillage plots were established on July 30, 2003, in a grassland area that had been uncultivated for ten years. Six plots (4.7 m x 4.7 m per plot) were established for two treatments (3 till and 3 no-till) (**Figure 4.2a**). Vegetation on all plots was cut back, raked, and mowed to 5 cm. Three plots were

tilled to 15 cm by running a rotary tiller through the soil in a single pass and three plots were left untilled. All plots were covered with black landscaping fabric (DuPont, Wilmington, DE) to prevent erosion of the soil. On days 0, 15 and 139 (following the first tillage event), twelve 15-cm deep cores were randomly collected from each plot. On day 347 after the primary tillage event, twelve soil cores were taken from each plot, and divided into three vertical sections (0–5 cm; 5–10 cm; 10–15 cm) (**Figure 4.2a**). All samples were placed into plastic bags and immediately transported back to the lab, where they were stored at 4°C overnight. Prior to analysis, soils were sieved (< 2 mm), and dry weight equivalents were determined.

After soil samples were collected on day 347, soils in the till plots were tilled again using a rotary tiller. Plots were left uncovered following the second tillage event. On day 456 after the second tillage event, soil samples were collected for geospatial analysis. Sampling points were identified, marked with nails and Licor collars (80 cm²) made of PVC pipe were centered around each nail. A single 15-cm soil core was removed from the center of the Licor collar after CO₂ flux measurements were completed. Two 1-cm sections (2–3 cm and 13–14 cm) were excised from each core in the field, crumbled, placed in sterile whirl-pak bags, and stored on ice until transport back to the lab.

Heterotrophic plate counts

Twenty grams of each composite soil sample from day 347 were blended for three 1-minute intervals (with intermittent cooling on ice) in 200 ml of Winogradsky's salt solution (WSS; Zuberer, 1994). Blended soils were then diluted (10^{-2} to 10^{-6}) in 0.1%

Bactopeptone (Difco, Detroit, MI) and spread on R2A media with a sterile glass rod. Colony forming units (CFUs) were counted at two weeks using a Quebec colony counter.

Microbial biomass carbon and *in situ* CO₂ flux.

Microbial biomass carbon (MBC) was extracted from moist soil (80% water-filled porosity) according to the method of Islam and Weil (1998). Total carbon in soil extracts was measured using a Dohrmann[®] DC-190 TOC analyzer (Rosemount[®] Analytical Inc., Santa Clara, CA). *In situ* CO₂ flux was measured using a LICOR 6400 Portable Photosynthesis System (Li-Cor Inc., Lincoln, NE) according to the manufactures instructions.

Community level physiological profiling

The capability of soil microbial communities to utilize a variety of carbon sources was assessed using community-level physiological profiling (CLPP) originally developed by Garland and Mills (1991). Sterile Waring blenders were used to homogenize (1 min, max speed, 3x with intermittent cooling on ice) 20 g of soil collected on day 347 in 180 mL Winogradsky Salts Solution (WSS). Soil samples collected for the geostatistical analysis were disrupted by placing 1 gram of sieved soil in 9 ml of WSS and agitating samples on a rotary shaker for 60 minutes at 30 rpm. Homogenate was diluted (10^{-3}) and used to inoculate (100 μ L well⁻¹) BIOLOG[®] Ecoplates or GN2 MicroPlates[™] (Biolog Inc., Hayward, CA). The microplates were incubated at 25°C for five days. The utilization rate of each carbon source was monitored by measuring absorbance at 590 nm. Readings were taken every 24 hours using an automated plate reader (SpectraMAX 340

pc, Molecular Devices, Sunnyvale, CA) and values less than 0.25 were set to zero. The Average Well Color Development (AWCD) was used as an indicator of general microbial activity and was calculated as the average optical density across all wells in a given plate or all wells associated with substrate guilds (carbohydrates, polymers, carboxylic acids, amines/amides, and amino acids) (Zak et al., 1994). Substrate diversity (H) was calculated as $H = -\sum p_i \ln p_i$, where p_i is the proportion 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 AWCD within each guild. Substrate evenness (E) was calculated as $E = H/\ln(S)$. The area under the kinetic curve (AUC) was calculated using an Excel-based trapezoidal approximation (Guckert et al., 1996).

Statistical analysis

Prior to statistical analysis, data were checked for normality using a normal quantile plot and a Shapiro-Wilk goodness of fit test. Data were normalized by transformation when necessary. The mean, standard deviation and coefficient of variation (% CV) were calculated for each treatment class and differences in MBC and heterotrophic plate counts were tested using an analysis of variance (ANOVA) in a 2 X 3 factorial design (factor A: tillage, factor B: time of sampling or sampling depth). Tukey's HSD test was used for post-ANOVA pair-wise comparisons. BIOLOG data from days 1, 15, 139 and 347 were normalized by dividing each substrate absorbance value by the AWCD (Garland and Mills, 1991), and principal component analysis (PCA) was done on

a covariance matrix using JMP software (SAS Institute, Cary, NC). For PCA analysis of CLPP data used for geostatistical analysis, the AUC for each substrate was normalized by dividing by the average AUC for the plate. Pearson product moment correlation coefficients were used to evaluate associations between the AUC data and CO₂ flux. Correlation analysis was done using the Hmisc package in R (R Development Core Team, 2004).

Geostatistical analysis

Stein and Ettema (2003) previously developed a sampling schemes to estimate experimental variogram parameters in roto-tilled plots. The locations of the 28 sampling points in these designs were determined using spatial simulated annealing (SSA) and the Warrick and Meyers criterion. The Warrick and Meyers criterion evaluates the quality of sampling points by assessing the distribution of sampling points over the distances classes and SSA is an iterative algorithm used to optimize the design based on the selected criterion. An optimal design is achieved when a sufficient number of samples (> 30) are evenly distributed over all the distance classes. In this study, a rescaled (4.3 x 4.3 m (14 ft x 14 ft) version of Stein and Ettema's sampling scheme for bare plots (**Figure 4.2 b**).

Spatial dependence (autocorrelation) was evaluated by analyzing the semivariance ($\gamma(h)$) as a function of distance Eq. (1).

$$(1) \gamma(h) = \frac{1}{2N(h)} (\sum (z(s_i) - z(s_i + h)))$$

Where:

$N(h)$ is the number of observation pairs separated by distance h

$z(s_i)$ is the value of interest at location s_i

$z(s_i + h)$ is the value at a location at distance h from s_i

Graphing semivariance versus the distance results in a semivariogram. Modeling the semivariogram reveals information about the spatial structure of a soil parameter such as the scale of heterogeneity and the spatially-dependent predictability of the property (Ettema and Wardle, 2002). Many soil variables exhibit spherical semivariograms (**Figure 4.2**). Information that can be obtained from the semivariogram includes the sill ($C + C_0$, estimate of total population variance), the range or patch size (distance beyond which the data are independent), the nugget (C_0 , variance due to sampling error or spatial dependence at scales not studied), and C (the spatially-dependent variance) (Rossi et al., 1992; Ettema and Wardle, 2002).

In this analysis, isotropic semivariograms were constructed using an active lag distance of 3 meters and lag class intervals ranging from 0.30 to 0.40. The active lag distance is the range over which semivariance will be calculated, and the lag class interval defines how pairs of points will be grouped into lag classes. Gaussian, exponential or linear models with the best fit (highest r^2) and a sufficient number of distance pairs in each lag class are presented in this paper. The ratio of the nugget to the sill (N/S) was used to define three groups based on the level of spatial dependence, where data with $N/S < 0.25$ = strong spatial dependence; N/S between 0.25 and 0.75 = moderate spatial dependence and $N/S > 0.75$ = no spatial correlation (Cambardella et al., 1994).

Variography and punctual kriging were done using GS+ software v 7.0 (Gamma Design Software, St. Plainwell, MI).

Results and Discussion

Exploratory data were obtained prior to implementing the geostatistical study. Replicate (n=3) tilled and untilled plots were established immediately adjacent to the market garden on soils previously undisturbed for a decade (**Figure 4.1a**). Based on samples collected to a 15-cm depth, CLPP parameters (diversity, richness, evenness) obtained from 31 substrate BIOLOG ECO plates did not show obvious changes between treatments at day 1, 15, and 139 following tillage (**Appendix 1.1**). Variability of the CLPP parameters, indicated by the % CV, was higher at day 1 in tilled compared with untilled plots. Principal component analysis (PCA) failed to separate tillage treatments (**Appendix 1.2**). At day 347 following tillage, samples again were collected, this time in 5-cm increments to a depth of 15 cm. PCA analyses of CLPP data clearly separated samples with depth within each tillage treatment (**Figure 4.3**) but not among tillage treatments (data not shown). Again, principal components were considerably more variable in tillage plots (**Figure 4.3**). These data were supported by determinations of microbial biomass carbon (MBC) and heterotrophic plate counts (**Table 4.1**), values of which significantly decreased with depth, but at a given depth were not significantly different due to tillage. The only apparent differences in microbial biomass involved the higher coefficient of variation (% CV) observed with depth in tilled compared with untilled plots. Collectively, the exploratory data did not reveal clear separation of tillage

effects by parametric and multivariate statistics, but did suggest differences in both variability and heterogeneity among treatment plots.

The impact of tillage on soil structure depends on the degree of soil perturbation and the type of tillage practice that is used (Young and Ritz, 2000). The higher variability initially observed in the tilled plots could have been due to incomplete disruption of soil structure by the minimal rotary-tillage employed. Prior to the second year, plots were tilled vigorously twice to achieve greater soil homogenization. At 475 days following this second tillage event (day 822), soil samples were collected from two different depths (2–3 cm and 13–14 cm) to investigate intra-plot variability and heterogeneity of CLPP data obtained using 95 substrate BIOLOG GN2 plates. A modified design (**Figure 4.1b**) based on 28 samples per treatment was employed (Stein and Ettema, 2003). Since the cost per test for CLPP analysis with BIOLOG GN2 plates is high (\$10.00 per sample), sampling designs must consider pragmatic factors such as affordability. This sampling design using only 26 BIOLOG plates per treatment was employed, which allowed construction of exploratory isotropic semivariograms with the necessary number of samples per lag class (Stein and Ettema, 2003).

Using parametric analyses, the calculated CV for the diversity, richness, and evenness demonstrated greater variability in untilled compared with tilled plots (**Appendix 1.2**). Specifically notable were the much larger CVs associated with the amines/amides, particularly at the 13–14 cm depth. PCA analysis of the area under the curve (AUC) data also suggested slightly more variability in the untilled versus tilled plots (**Figure 4.4**). PCA clearly separated soils by depth, but not by tillage treatment.

To assess the effects of tillage on the spatial heterogeneity of CLPP, semivariograms were constructed using values calculated from all 95 BIOLOG substrates or from each of five separate substrate guilds (polymers, carbohydrates, carboxylic acids, amines/amides, and amino acids). The AUC was used as a response variable for the geospatial analysis because kinetic parameters are thought to be superior to other summary statistics for CLPP analysis (Preston-Mafham et al., 2002). All AUC data were normally distributed except for the polymers and amine guilds at a depth of 13–14 cm. These data were normalized using a Box Cox transformation prior to analyses. The semivariogram is a geostatistical tool that models the similarity between data as a function of their separation distance (Rossi et al., 1992). To produce reliable semivariograms, it is recommended that a minimum of 30–50 distance pairs are represented in each lag class (Rossi et al., 1992). When a bin size between 0.3 to 0.4 m and an active lag distance of 3 m were used, the mean number of samples in each lag class ranged from 33 to 43 (**Table 4.2, 4.3**). Several lag classes contained fewer than 30 distance pairs due to the exclusion of outliers; however, the minimum never fell below 27.

Gaussian, exponential and linear models were fit to the data for the combined substrates and individual substrate guilds (**Table 4.2, 4.3, Figure 4.5 a,b,c,d**). Goodness of fits to these models (r^2 ranging from 0.55 to 0.97) reveal that microbial substrate utilization is spatially structured in no-till and some tilled organic farm soils. Robust models could not be constructed for tilled soils collected from the 3–4 cm depth, indicating no spatial dependence in these data (**Table 4.2, 4.3**). Structural variance (C) accounted for 56% of the variance in the Gaussian and exponential models ($r^2 > 0.50$)

(Table 4.2, 4.3). The degree of spatial dependence, characterized by the nugget/sill ratio, ranged from strong to moderate. Strong spatial dependencies were found for the combined substrates and the amino acids in the no-till plots at a depth of 13–14 cm (Table 4.2, 4.3). When substrate guilds were examined separately, the ranges of spatial structure tended to be greater in untilled soils compared to tilled soils. In the absence of tillage, the carbohydrates were spatially structured at both depth classes, but the amines/amides and amino acids only exhibited spatial structure at depths of 13–14 cm. The data from the carbohydrates had the best fit of all the spatial models (Table 4.3; Figures 4.5c,d).

Ritz et al., (2004) characterized spatial structure in grassland soils using CLPP and other microbial parameters. They reported that CLPP principal components as well as AWCD for amino acids were spatially autocorrelated over ranges from 2.1 to 5.9 m and accounted for 60–85% of the total variance. Although I observed similar values for the structural variance (56 to 87%), the analyses suggested that some of the ranges (> 4.5 m) exceeded the dimensions of the plot, particularly in untilled soils. This may be due to different response variables (AWCD vs. AUC), the experimental design (depths of the soil samples) or it may reflect real differences in the soils, topography or management strategies. The ranges also were larger than others observed at this scale using DNA fingerprint analysis (1.3 to 3 m; Franklin and Mills, 2003) and PFLA analysis (0.73 to 5.7 m; Ritz et al., 2004).

In the present study, spatial structure of microbial substrate utilization was characterized at two different depths. Data indicated large differences in the variability and heterogeneity of microbial substrate utilization over very small vertical distances.

Spatial structure was more explicit at lower soil depths probably because intensive rotary tillage disrupts microorganisms primarily in the upper few centimeters of soil. Doran (1987) reported that MBC and PMN were much higher in no-tilled soils than in the surface layers of tilled soils; however, there were very little differences below 7.5 cm. De Fede and Sexstone (2001) observed large differences in AWCD and PCA using GN2 plates between A and B horizon soils. Nunan et al. (2002) studied the spatial distribution of bacteria in an arable field by quantifying the number and location of bacteria in soil via fluorescence microscopy of ultra-thin sections of soil cores. They found that bacteria in the topsoil were only spatially structured at the microscale level (μm to cm), while communities in the subsoil were structured at multiple spatial scales. Finally, Franklin et al. (2002) reported that the patch size for bacterial abundance and microbial community structure was much smaller in the vertical versus the horizontal direction. These results support the premise that complex spatial patterns are present at different soil depths and the effects of tillage are pronounced over very small vertical distances.

The respiratory activities of plant roots, mesofauna and microorganisms are related to microbial substrate utilization; therefore, we measured CO_2 flux as part of the geostatistical study. Previous studies also have demonstrated that soil CO_2 flux often exhibits high temporal and spatial variability (Parkin and Kaspar, 2004) and can be spatially dependent (Stoyan et al., 2000). In the present study, we were unable to fit CO_2 flux data to any of the spatial models and a t-test ($P < 0.09$) indicated that there were no significant differences in the average CO_2 flux in tilled ($2.48 \pm 0.66 \text{ g CO}_2 \text{ m}^{-2} \text{ d}^{-1}$) and untilled ($2.83 \pm 0.85 \text{ g CO}_2 \text{ m}^{-2} \text{ d}^{-1}$) plots. The % CVs calculated from gas flux data were 27% and 30% for the till and no-till plots, respectively. Correlation analysis revealed that

there was a significant association between CO₂ flux and the AUC data from the amines/amides (**Table 4.4**). The microbial processes that regulate the relationship between CO₂ and soil nitrogen dynamics are ecosystem-specific and fluctuate with nitrogen and carbon status and time (Hu et al., 2006). Therefore, a more comprehensive study would be needed to better determine if the utilization of amines and amides is associated with CO₂ flux over time.

It is useful to investigate spatial patterns at multiple scales, because the factors that affect change are scale-dependent (Ettema and Wardle, 2002). Previous studies have established that microorganisms can be spatially structured at macro, meso, and micro-scales (Grundmann and Debouzie, 2000; Franklin et al., 2002; Nunan et al., 2002; Franklin and Mills, 2003). We focused on assessing the spatial variability and heterogeneity of substrate utilization at the plot scale because a plot-level scale is commonly used to evaluate the effects of agronomic treatments (Parkin, 2002). Our data support observations by Ritz et al. (2004) that CLPP data were associated with geostatistical ranges measured over meters at a given sampling depth, but also demonstrated that vertical spatial dependency varied over a scale of 0.1 meters. Subsurface soils exhibited the greatest spatial dependency, particularly in untilled soils. The lack of spatial structure in tilled surface soils (or the relatively small geostatistical ranges of < 2 m observed in subsurface tilled soil) suggests that application of classical parametric and multivariate statistics are appropriate to discern treatment differences in culturable microbial populations at the plot scale as was done in Chapter 3 of this dissertation. The large geostatistical range of spatial dependence (~20 m) observed in

untilled soils would necessitate a different plot size and design to effectively study this agronomic treatment in a rigorous and larger experiment.

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Table 4.1. Means and coefficients of variation (% CV) for biomass and heterotrophic plate count data for vertical subsections of soil collected on day 347.

Treatment	Depth (cm)	Biomass (mg C/kg)		Plate Count (Log CFU/ml)	
		Mean ^a	% CV	Mean ^a	% CV
No Till	0–5	481.81	a	16.67	9.04
	5–10	110.84	b	31.75	8.57
	10–15	85.29	b	22.37	8.02
Till	0–5	765.34	a	11.69	9.04
	5–10	188.42	b	40.28	8.58
	10–15	100.98	b	85.66	7.93

^a Mean values followed by the same letter are not significantly different ($P < 0.05$) as determined by Tukey's Studentized Range (HSD) test.

Table 4.2. Summary of results from geostatistical analysis of AUC data for all BIOLOG GN2 substrates from day 822.

Treatment	Depth	Model Type	r^2	Mean (SD) distance pairs per lag class	Nugget (N)	Sill (S)	Range (R)	Spatial Dependence (N/S)	% Structural Variance
Till	3–4 cm	Exponential	0.04	37 (4.7)	-	-	-	-	-
No Till	3–4 cm	Exponential	0.61	36 (4.6)	248	807	> 4.3	Moderate	69
Till	13–14 cm	Gaussian	0.97	40 (6.9)	141	321	4.9	Moderate	56
No Till	13–14 cm	Gaussian	0.83	33 (6.4)	76	592	2.7	Strong	87

Table 4.3. Summary of results from geostatistical analysis of AUC data from substrate classes from BIOLOG GN2 plates from day 822.

Substrate Class	Treatment	Depth	Model Type	r^2	Mean (SD) distance pairs per lag class	Nugget (N)	Sill (S)	Range (R)	Spatial Dependence (N/S)	% Structural Variance
Polymers	No Till	3–4 cm	Exponential	0.60	43 (7.2)	398	1207	> 4.3	Moderate	67
	Till	13–14 cm	Gaussian	0.73	39 (7.2)	149	496	1.3	Moderate	70
Carbohydrates	No Till	3–4 cm	Gaussian	0.94	43 (7.2)	479	1185	4.7	Moderate	60
	No Till	13–14 cm	Gaussian	0.55	36 (6.1)	392	1494	> 4.3	Moderate	74
	Till	13–14 cm	Exponential	0.91	35 (4.1)	204	501	2.9	Moderate	59
Carboxylic acids	No Till	13–14 cm	Exponential	0.52	37 (4.7)	153	617	> 4.3	Moderate	75
Amines/Amides	No Till	13–14 cm	Gaussian	0.74	33 (6.2)	203	823	2.3	Moderate	75
Amino Acids	No Till	13–14 cm	Gaussian	0.80	36 (6.1)	148	1106	4.6	Strong	87
	Till	13–14 cm	Linear	0.58	37 (4.7)	286	410	3.0	Moderate	30

Table 4.4. Pearson correlation coefficients between CO₂ flux and AUC data for substrate classes from BIOLOG GN2 plates.

	Top		Bottom	
	Till	No Till	Till	No Till
All substrates	0.17	0.24	0.18	-0.02
Polymers	-0.01	0.16	0.12	-0.04
Carbohydrates	0.15	0.20	0.30	-0.12
Carboxylic acids	0.19	0.28	0.12	0.04
Amines/Amides	0.38*	0.04	-0.04	0.01
Amino Acids	0.11	0.33	0.03	0.13

* Significant correlations ($P < 0.05$).

Figure 4.1. Dimensions and layout of plots for analyses done on day 347 following the primary tillage event (a) and sampling scheme and analyses for geospatial study done in A plots on day 456 following the second tillage event (b).

Figure 4.2. Generalized spherical semivariogram (From: Ettema and Wardle, 2002).

Figure 4.3. Ordination biplots of principal components (PC1 and PC2) from CLPP analysis of vertical subsections (a) no till and (b) till soils collected from plots A, B and C on day 347 following the initial tillage event in July 2003. PCA analysis was done on AWCD readings from BIOLOG Ecoplates. Error bars represent the standard deviations for PC1 and PC2.

Figure 4.4. Ordination biplots of principal components (PC1 and PC2) from CLPP analysis of vertical subsections of till and no-till soils collected on day 822 following the primary and secondary tillage events. PCA analysis was done on standardized values for the area under the kinetic curve from BIOLOG GN2 plates. Error bars represent the standard deviations for PC1 and PC2.

Figure 4.5. Semivariograms of AUC at 13–14 cm for (a) all substrates in the no-till plot, (b) all substrates in the till plot, (c) carbohydrates in the no-till plot (d), and carbohydrates in the till plot.

APPENDIX 1.1

Means and coefficients of variation (% CV) for CLPP parameters for BIOLOG Ecoplates for vertical subsections of soil collected on days 0, 13, 139 and 347.

Treatment	Day	Depth (cm)	AWCD		Diversity (H)		Richness (S)		Evenness (E)	
			Mean	% CV	Mean	% CV	Mean	% CV	Mean	% CV
No Till	1	0–15	1.63	4.6	3.30	0.4	29.78	2.2	0.97	0.5
	15	0–15	1.50	6.1	3.31	1.1	29.22	2.9	0.98	0.4
	139	0–15	1.50	5.7	3.27	0.8	29.33	4.2	0.97	0.7
Till	1	0–15	1.44	13.3	3.25	1.9	28.11	6.8	0.97	0.5
	15	0–15	1.49	7.9	3.31	0.5	30.00	2.4	0.97	0.6
	139	0–15	1.46	7.0	3.27	1.3	30.11	2.6	0.96	1.1
No Till	347	0–5	1.86	6.1	3.39	0.5	30.72	1.5	0.99	0.3
	347	5–10	1.61	6.4	3.34	0.7	30.11	2.2	0.98	0.5
	347	10–15	1.51	9.4	3.27	1.7	29.22	4.5	0.97	0.9
Till	347	0–5	1.82	9.7	3.38	0.6	30.78	1.4	0.99	0.4
	347	5–10	1.43	6.9	3.28	1.6	29.00	4.1	0.98	0.8
	347	10–15	1.27	14.0	3.17	2.9	26.56	8.7	0.97	1.2

APPENDIX 1.2

Means and coefficients of variation (% CV) for CLPP parameters from BIOLOG GN2 plates for vertical subsections of soil collected on day 822.

Substrate Group	Depth	Treatment	AWCD		Diversity (H)		Richness (S)		Evenness (E)	
			Mean	% CV	Mean	% CV	Mean	% CV	Mean	% CV
Polymer	2-3 cm	Till	1.67	17.0	1.55	5.8	5	6.4	0.98	2.8
		No Till	1.66	26.0	1.53	7.5	5	9.9	0.98	3.0
	13-14	Till	1.01	22.0	0.96	23.2	3	27.8	0.93	6.6
		No Till	1.12	25.1	1.02	25.5	3	29.7	0.93	7.3
Carbohydrates	2-3 cm	Till	1.81	11.7	3.21	2.0	27	4.0	0.97	1.1
		No Till	1.66	22.0	3.18	4.1	26	9.6	0.98	1.5
	13-14	Till	1.45	14.4	2.87	4.7	20	13.3	0.96	1.8
		No Till	1.45	18.8	2.88	6.5	20	16.9	0.96	2.0
Carboxylic Acids	2-3 cm	Till	1.15	13.4	3.01	2.6	23	8.2	0.96	1.0
		No Till	1.02	25.1	2.92	5.0	21	16.5	0.96	1.1
	13-14	Till	1.05	10.3	2.93	2.3	22	7.5	0.95	1.1
		No Till	1.12	11.5	2.96	3.0	23	10.1	0.95	1.3
Amines/Amides	2-3 cm	Till	1.16	20.0	1.31	10.0	4	17.0	0.94	4.9
		No Till	0.96	29.9	1.18	14.6	4	20.8	0.92	20.1
	13-14	Till	1.05	12.2	1.17	9.4	4	17.8	0.93	6.3
		No Till	1.06	25.2	1.18	23.0	4	23.1	0.88	20.4
Amino Acids	2-3 cm	Till	1.65	10.9	2.83	2.2	19	5.8	0.97	1.1
		No Till	1.50	20.2	2.79	3.4	18	8.1	0.97	1.2
	13-14	Till	1.59	9.6	2.76	1.8	18	6.0	0.96	0.9
		No Till	1.62	14.2	2.76	3.2	18	8.1	0.96	1.0

CURRICULUM VITAE

EDUCATION

Ph.D. Agricultural Sciences
with a concentration in environmental microbiology
(2007)

West Virginia University
Division of Plant and Soil Sciences, Morgantown, WV

Dissertation: Molecular and functional characterization
of bacterial communities in soils from an organic farm and pasture.

Doctoral Studies in Aquatic Ecology (1991-1992) – Kent State University, Kent, OH

M.S. Biology (1990) – Clarion University of Pennsylvania, Clarion, PA;
Thesis: Manganese toxicity and bioconcentration in the green algae *Mougeotia* sp.
(Chlorophyceae)

B.S. Medical Technology (1997) – Clarion University of Pennsylvania, Clarion PA
Certification: American Society of Clinical Pathologist (ASCP) - (MT-204770)

B.S. Biology, *summa cum laude* (1987) – Clarion University of Pennsylvania

EMPLOYMENT

Teaching Positions

- **Assistant Professor of Biology** (8/07) – West Virginia Wesleyan College, Buckhannon, WV.
- **Biology Instructor** (12/99–4/06) – Clarion University of Pennsylvania, Department of Biology, Pittsburgh Site, Venango and Main Campuses, PA.
Courses: Microbiology, Anatomy and Physiology, and Biology
- **Adjunct Faculty** (8/99–5/00) – Jefferson Community College, Steubenville, OH.
Course: Anatomy and Physiology
- **Graduate Teaching Fellow** (8/92–8/93) – Kent State University, Kent, OH.
Courses: General Biology and Microbiology
- **Graduate Teaching Assistant** (1/88–5/90) – Clarion University of Pennsylvania, Department of Biology, Clarion, PA. *Courses: Botany and Zoology*

Research and Clinical Positions

- **Doctoral Research Assistant** (8/02–Present) – West Virginia University, Division of Plant and Soil Science, Morgantown, WV
- **Research Associate** (7/98–1/99) – Department of Infectious Diseases, Children's Hospital of Pittsburgh, Pittsburgh, PA
- **Medical Technologist** (10/97–7/98) – Jefferson Hospital, South Hills Health Care System, Pittsburgh, PA
- **Environmental Microbiologist** (6/90–6/92) – U.S. Bureau of Mines, Pittsburgh, PA Research Center, Pittsburgh, PA
- **Biological Technician** (Summers 87, 88) – U.S. Department of the Interior, Office of Surface Mining and Reclamation Enforcement, Pittsburgh, PA

PUBLICATIONS

Edenborn, S.L. and A.J. Sexstone. (2007). DGGE fingerprinting of culturable soil bacterial communities complements culture-independent analyses. *Soil Biology and Biochemistry*. 39 (7):1570–1579.

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Edenborn, H.M., L.A. Brickett, D.H. Dvorak, and S.L. Edenborn. (1993). Monitoring iron and manganese diagenesis in constructed wetlands with continuous gradient gels. *Biohydrometallurgical Technologies* 1:631–639.

Dvorak, D.H., R.S. Hedin, S.L. Gustafson (Edenborn), and H.M. Edenborn (1991). Treatment of metal-contaminated water using bacterial sulfate reduction: results from pilot-scale reactors. International Conference on the Abatement of Acidic Drainage. Montreal, Canada, pp.301–314.

R.S. Hedin, D.H. Dvorak, S.L. Gustafson (Edenborn), D.M. Hyman, P.E. McIntire,

R.W. Nairn, R.C., Neupert, A.C. Woods and H.M Edenborn. (1991). Use of a constructed wetland for the treatment of acid mine drainage at the Friendship Hill National Historic Site, Fayette County, PA. Final Report Presented to the National Park Service.

PRESENTATIONS/ABSTRACTS

Edenborn, S.L. and A.J. Sexstone. (2006). Characterization of soil bacterial communities on culture plates using denaturing gradient gel electrophoresis of the 16S rRNA gene. Annual Meeting of the American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America, Indianapolis, Indiana.

Chapman, J.A., S.L. Edenborn and A.J. Sexstone. (2006). Modification of bacterial communities in a pasture soil by the feeding activity of earthworms. Annual Meeting of the American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America, Indianapolis, Indiana.

Edenborn, H.M., S.L. Edenborn, B. Ji, J.H. Doherty, and B. B. Casper. (2005). Observations on the relationships between bacteria and grasses at Eastern serpentine barrens and Midwestern prairie sites. Annual meeting of the Allegheny Branch of American Society for Microbiologists, Clearfield, PA.

Friedland, J.M., S.L. Edenborn, and A.J. Sexstone. (2004). Diversity of microbial populations within a constructed wetland treating wastewater. Annual Meeting of the American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America, Seattle, Washington.

Edenborn, S.L., Y. Sutanto, J. Chapman, and A.J. Sexstone. (2004). Culture-dependent assessment of bacterial diversity in northern West Virginia soils. Annual Meeting of the American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America, Seattle, Washington.

Salinas, K.A., J.B. Kotcon, and S. L. Edenborn. (2003). The effects of a bacterial biological control agent on taxis of *Clarkus papillatus* in a laboratory bioassay. Annual Meeting of the Society of Nematologist, Cornell University, Ithaca, New York.

Edenborn, H.M., L.A. Brickett, D.H. Dvorak, and S.L. Edenborn. (1993). Monitoring manganese diagenesis in constructed wetlands with continuous gradient gels. International Biohydrometallurgy Symposium, Jackson Hole, WY.

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Geological Society of America Annual Meeting. Cincinnati, OH. (Received best poster award).

Gustafson (Edenborn), S.L., R.S. Hedin and H.M. Edenborn. (1991). Rates of iron and manganese removal in waters from wetlands receiving acid mine drainage: The role of indigenous microorganisms. Gordon Research Conference in Applied and Environmental Microbiology, Colby-Sawyer College, New London, NH.

Gustafson (Edenborn), S.L. and S.A. Jatkari. (1991). Algal taxonomy of coal mine drainage sites and correlation with drainage chemistry. Abstract # 141. The Fourth International Phycological Congress, Duke University, Durham, North Carolina.

Gustafson (Edenborn), S.L., and H.M. Edenborn. (1991). Biological removal of manganese in acid mine drainage by a mixed algal-bacterial community. Abstract # 54, 91st Annual Meeting of the American Society for Microbiology, Dallas, Texas.

Gustafson (Edenborn), S.L., R.S. Hedin, R.W. Nairn and H.M. Edenborn. (1991). The importance of bacterial-iron oxidation and sulfate reduction in wetlands constructed to treat acid mine drainage. Symposium on the Biogeochemistry of Wetlands, Center for Wetland Resources, Louisiana State University, Baton Rouge, LA.

Edenborn, H.M., D.H. Dvorak, R.S. Hedin, and S.L. Gustafson (Edenborn). (1990). Treatment of metal-contaminated water via bacterial sulfate reduction in wetlands and pilot-scale reactors. National Association of Abandoned Mine Land Program, Breckenridge, CO.

HONORS AND AWARDS

Gamma Sigma Delta, Honor Society of Agriculture (2007); Provost Fellowship, Kent State University (1992); Commonwealth of Pennsylvania University Biologists; Outstanding Student Award (1987); Penn State University Student Travel Award to Symposium in Plant Physiology (1987); Beta Beta Beta National Biological Honor Society (1986); Phi Eta Sigma Freshman Honor Society (1984).

PROFESSIONAL SOCIETIES

American Society for Microbiology (ASM)