Analysis of Bacterial Abundance and Species Diversity in Various Soils

by McKenzie Roth

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Faculty Mentor: Dr. Andrew Greene, Associate Professor of Biology/Toxicology
Additional Reader: Dr. Andrew Trimble, Assistant Professor of Biology/Toxicology

Abstract

Different soil types display a wide range of nutrient content, microbial abundance and diversity. Many of the microbes viable in soil have practical functions in the agricultural and medical fields while others are pathogenic. Garden topsoil, potting soil, forest soil, compost and manure were the five soil types investigated in this study for bacterial abundance, species diversity, and nutrient content. We hypothesized that manure and compost would have the most nutrients and be highest in number and diversity of bacteria. Likewise, we hypothesized that potting soil would be low in all three areas. Each soil sample was diluted, plated on Plate Count Agar and incubated at 37^{o}C for 2 days in order to maximize the number of colonies able to grow on the media. The bacteria count was determined by plate count assay and the microbial abundance by using 16S rDNA to sequence isolated species and compare the number of genera present in each soil type. A qualitative nutrient analysis was conducted to evaluate the relative nutrient content of each soil type. It was determined that the compost was richest in nitrate, phosphorus and potassium, while the other soils were low in all three. The manure exhibited a significantly higher bacterial count than all the other samples. Potting soil, compost, garden topsoil and forest soil followed with less significant variance. Similarly, the manure represented the greatest number of genera from the 16S rDNA sequencing than the other samples.

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Introduction

Soil ecosystems depend largely upon the microbial abundance, species diversity and nutrient content of the soil (Condron et al. 2010). Microorganisms, such as nitrogen-fixing *Rhizobium*, antibiotic-secreting *Streptomyces*, and mycorrhizae, play pivotal roles in soil ecosystems; they cycle nutrients, convert atmospheric nitrogen to ammonia and nitrate, forms that are usable to other organisms, and promote plant growth. Additionally, they have practical applications in agriculture (Sharma et al. 2011) and medicine (Zarantonello 2001). In this study, we analyzed the species diversity and abundance of bacteria in five soil types; garden topsoil, potting soil, forest soil, compost and manure. These five soil samples were chosen due to anticipated variation in their nutrient content. The bacteria from the various soils were cultured on synthetic media and identified via 16S rDNA sequencing. Bacterial abundance was quantified by the plate count assay. We expected the more nutrient-rich soils, such as manure and compost, to harbor a greater amount and variety of bacteria because of increased nutrient supply, whereas, the samples with less nutrients, potting soil and forest soil, would support less microbial life.

Importance of Microbes in Soil

Soil is the most diverse microhabitat on earth (Willey et al. 2011). With rainfall, oxygen availability, and organic matter constantly changing, soil is a dynamic environment that is able to support a diverse abundance of life. Soil formation, plant growth, nitrogen fixation, control of plant pathogens and nutrient cycling are several significant roles bacteria, fungi, protists, and other microorganisms play (Condron et. al 2010). Likewise, they are also greatly advantageous in numerous fields including agriculture and medicine (Sharma et al. 2011, Schultz et. al 2012, Zarantonello 2001).

Microbial community structure and diversity can serve as indicators for evaluating soil quality (Sharma et al. 2011). High quality soil is an essential natural resource for maintaining productive crops and food security. Soil nutrients are exhausted by the rigorous practices of tillage, planting, harvesting and use of fertilizers and pesticides. Microbes respond quickly to natural stresses and are the driving forces behind many fundamental chemical processes in the soil, thus making them an influential parameter for monitoring soil quality. Shifts in microbial population and activity, with emphasis on *Rhizobium*, mycorrhizae and nitrifying bacteria, reflect the quality of the soil overall. These microorganisms in particular are good indicators for measuring soil quality because they are ubiquitous in the soil environment, are sensitive to agrochemicals, and their functions are most clearly understood.

More specifically, *Rhizobium* is a genus of nitrogen-fixing bacteria that symbiotically forms nodules on the roots of legumes (Rejili et al. 2012). They convert atmospheric nitrogen to ammonia and nitrate, forms that plants and animals are able to absorb and use. This legume-rhizobium symbiosis elevates the activity of the enzymes phosphatase, β-glucosidase, and hydrogenase, which promotes greater microbial activity and increased rate of decomposition (Rejili et al. 2012). It is generally agreed that high microbial diversity is indicative of good soil quality, because high diversity allows for microecosystems to re-equilibrate more quickly after an environmental stress. Various agricultural management practices have been adapted to maintain the soil microbial communities and thus sustain soil quality (Sharma et al. 2011).

Additionally, soil microbes have aided in advancements in the medical field. Living in close vicinity, soil microorganisms often compete with one another for space and energy. As a result, some species, such as those from the genus *Streptomyces*, have adapted by excreting antibiotics that inhibit the growth of neighboring microbes (Schultz et. al 2012). While some

species of *Streptomyces* are pathogenic, most produce helpful chemicals that promote plant growth and are used in human medicine to treat tumors, prevent infections, and serve as immunosuppressant drugs (Seipke et al. 2011). Isolated from the rhizosphere, *Streptomyces althioticus* has been found to produce the antibiotic silvalactam, a secondary metabolite that has powerful antiproliferative activity against cancer cells (Schultz et. al 2012). Its highly potent and selective qualities make it an antobiotic of particular interest to the medical field (Zarantonello 2001). Furthermore, the advantageous metabolites produced by *Streptomyces* are chiefly the result of interactions with other microbes, fungi, plants and animals (Seipke et al. 2011). With further study, scientists can isolate specific antibiotic-producing microbes to harvest their helpful metabolites, or determine a method of synthesizing the antibiotics in the laboratory.

Identifying Microbes in the Soil

There are numerous methods for identifying microbes in the soil. Historically the common approach has been to culture the microorganisms onto synthetic media, which unfortunately only supports less than 1% of bacteria and less than 17% of fungi (Balser et al. 2010). As a result, nondiscriminatory techniques have been developed, such as phospholipid fatty acid analysis, which identifies microorganisms by analyzing specific lipid content of the soil. After cell death, the lipid-based membrane degrades rapidly, thus analysis of the membranes in a soil sample give a more accurate estimation of the viable microbial community (Balser et al. 2010). Another newly developed method is the extraction of DNA directly from cells in the soil (Hirsch 2010). Although both techniques eliminate the requirement of culturing the species on media, they primarily identify organisms that are present rather than giving any insight into their functionality in the soil (Balser et al. 2010). Understanding the roles specific microorganisms have in their ecosystems remains an obstacle today in microbiology research.

Despite its evident shortcomings, culturing microorganisms on synthetic media has proven to be advantageous for establishing a base knowledge of microbiology. Culture media makes it possible to grow and maintain microorganisms in the laboratory (Willey et al. 2011). Furthermore, specialized media has practical applications such as testing for antibiotic resistance, food and water analysis, and studying the ability of microorganisms to metabolize specific compounds (Willey et al. 2011). For soil in particular, the ingredients in synthetic media can be manipulated to determine if an isolate can metabolize certain carbon sources (Vasileva-Tonkova and Gesheva 2004). For example Vasileva-Tonkova and Gesheva (2004) prepared media with common hydrocarbon pollutants the only carbon source. Any growth that occurred came from microbes that were able to catabolize the pollutants into less toxic products, a process known as biodegradation (Vasileva-Tonkova and Gesheva 2004). In addition to identifying the microbes in the soil, it is essential to understand the habitat itself. How soil is formed and what it is made of greatly influences what types of microbes can exist there.

Soil Formation

Understanding how soil is formed is essential for differentiating soil types as well as predicting nutrient content and the types of microbial species present. Parent material, climate, topography, biological factors and time are the five factors soil scientists use to explain how soils form ("Soil Formation and Classification" 2011, Balser et al. 2010). Soil tells a story of progress over time, starting from a parent rock being weathered away and carried over indefinite distances. Rock particles find a new resting place and are joined by other particles from different sources ("Soil Formation and Classification" 2011). If left on low-sloping or flat ground, horizontal layers of sediment will accumulate in fine strata, each particle retaining characteristic

chemical features of its parent rock. Together the mix of sediment is altered by the temperature and moisture of its new climate, as it develops into something more cohesive.

Soil formation is a long and continual process that depends chiefly on climate, landscape position and biological activity ("Soil Formation and Classification" 2011). The final structure of the soil, its nutrient content, and the climate of the environment influence the types and abundance of microbial growth (Balser et al. 2010). Every soil sample in this study varied in structure, source, and nutrient content allowing for a wide diversity of microbial abundance and species.

Characteristics of the five soil types

In this study, a nutrient-rich medium, Plate Count Agar (PCA) was used for plating in order to obtain the highest level of growth. Five soil samples, including topsoil, potting soil, manure, compost, and forest soil were diluted and pour plated with PCA. Bacterial colonies of distinct morphology were isolated, purified, and underwent DNA extraction. In order to quantify the amount of growth, the numbers of colonies that grew on the plates were counted. Lastly, a nutrient analysis was conducted to identify any correlation between bacterial diversity and abundance with nutrient content. Much effort has been invested in refining methodologies used to study microbes in the soil, because of the importance these microbes have in our daily lives and in the balance of entire ecosystems.

Topsoil, forest soil, potting soil, manure, and compost were the soil samples investigated in this study. A high variance in bacterial abundance and species diversity was expected from the five soil types due to their varied nutrient richness and location. One analysis of manure found that it contains 3.10-5.00 g of nitrogen per kilogram of sample, or 0.31-0.50% by weight (Dou et. al 2001). Furthermore, the manure samples had broad ranges of phosphorus and

potassium available (Dou et. al 2001). Another study looked into the nutrient content of manure from a sedentary horse and found it to typically have 0.36% nitrogen, 0.12% phosphorus, and 0.13% potassium by weight (Murphy). Because manure is made from the biological excretions of livestock, it is expected to have elevated levels of nutrient richness and microbial diversity. The manure sample analyzed in our study was taken from the field of a sedentary horse and was found to have low content of nitrate, phosphorus, and potassium and was slightly acidic. In spite of its low nutrient content, it still harbored a large abundance and diversity of microbial life.

Potting soil was expected to have the least diversity of microbes because it did not come from a natural environment. The nitrogen, phosphorus, and potassium composition by weight is measured for all potting soils. For example, Miracle-Gro is 0.21% nitrogen, 0.7% phosphorus, and 0.14% potassium by weight (*Scotts Miracle-Gro Co*. 2012). In comparison with the manure, the potting soil has fewer nutrients by weight percent. Because the potting soil has a consistent nutrient analysis, it was expected to have a consistently high abundance of microbes. However, because the nutrients found in potting soil are mainly synthetic and the soil is not formed naturally, it was hypothesized that there would be lower diversity.

Comprised mainly of food scraps, a home compost pile was the source of the third soil sample. This compost sample contained earthworm excreta known as casts that aid in the cycling of nutrients, maintaining soil structure, increasing enzyme levels, and supporting greater microbial populations (Chaoui et al. 2003). According to the study done by Chaoui et al. (2003), the soil that underwent the conventional composting method had a microbial biomass of 3980 mg per kilogram. Compared to other forms of composting analyzed within the same study, this is relatively high. In addition, the compost had 0.51% carbon, 0.031% nitrate, 0.24% phosphorus, and 1.14% potassium (Chaoui et al. 2003). With a constant input of decomposable

fare, the compost was expected to have one of the most active and diverse microbiological ecosystems of the soil types investigated.

The fourth soil type came from the topsoil of a deciduous temperate forest habitat. The fallen leaves and decomposing trees led to the prediction that the forest soil would be rich in nitrogen and organic carbon. However, the data from the nutrient analysis did not support this prediction. The forest soil sample tested low for nitrogen, potassium, and phosphorus. A study conducted by Sackett et al. (2012) investigated the effects of earthworm populations on soil nutrients in North American forest ecosystems. According to their soil nutrient analysis, the forest soil contained 0.0492% microbial biomass carbon, 0.00012% nitrate, 0.00641% ammonium, and 0.00069% phosphate (Sackett et al. 2012). These numbers are consistently lower than the other soil types, leading to the prediction that the forest soil would support a smaller amount and diversity of bacteria.

The nutrient content of topsoil largely depends on the location of its original sampling. In the case of this study, the topsoil was sampled from a cultivated garden before the planting season, thus no crops were present. Because plant roots provide nutrients for microbial growth, lack of plant life would negatively affect the species abundance and diversity (Condron et al. 2010). Consequently, the topsoil tested in the low range for potassium and phosphorus, but in the medium range for nitrate. Due to sufficient aeration in the garden, the topsoil sample had high oxygen availability which would support growth of aerobic microorganisms. As a result of the elevated quantities of oxygen and carbon, topsoil was also expected to have an increased abundance and diversity of bacteria.

In our study, we focused on how abundance and diversity of bacteria varied between garden topsoil, potting soil, forest soil, compost, and manure. We expected greater growth in the

soil types with greater nutrient content, specifically the manure and compost samples. In contrary, less growth was expected in the less nutritious soils such as potting soil and forest soil. After culturing microbes and sequencing DNA, we gathered data that suggested manure has the highest abundance of bacteria followed by potting soil, compost, top soil, then forest soil. In terms of species diversity, manure had the greatest number of genera represented followed by forest soil, compost, potting soil, and then topsoil.

Materials and Methods

Collection of Soil Samples

Bellville, Ohio was the site where manure, garden topsoil, compost, and forest soil were sampled. The samples were taken in early December 2011 before the ground had frozen. The garden topsoil sample came from a small garden before any plants had been planted. This garden had been regularly planted and maintained each year over a series of 20 years, thus it received a habitual amount of plants, nutrients and cultivation. The manure sample was taken from a farm containing one horse. A compost pile that regularly received food wastes was the site of the third sample. Likewise, a local deciduous forest area was the location of the forest soil sample.

The potting soil was sampled from the greenhouse at Ashland University. Rather than being taken directly from the bag in its dry form, the potting soil sample was taken from a pot where it was being presently used and watered often. Samples were stored in glass quart jars and covered with foil. Furthermore, they were regularly aerated and watered throughout the duration of the experiment.

Nutrient Analysis of the Soil Types

Approximately 5 grams of soil was dried completely overnight in an oven at 90^{o}C. Afterwards, the samples were filtered through a 2mm sieve to break up any large soil aggregates to smaller size. We tested each soil type for pH, nitrate, phosphorus, and potassium content using LusterLeaf Products Nutrient Analysis Kit (Woodstock, IL). This was a primarily qualitative analysis that yielded the relative nutrient content of each soil sample in study.

Determination of optimal growth conditions

Potting soil was used as a baseline to determine the optimal growth conditions to be used for the duration of the study. Nutrient content of media, temperature and length of incubation, magnitude of dilution, and consistency of dilution technique were tested. One gram of potting soil was diluted to 10^{-3}, 10^{-4}, and 10^{-5} mL in 0.9% saline solution in test tubes, and 0.1 mL was added to one of three molten media; therefore, the soil dilutions that were being plated were 10^{-4}, 10^{-5}, and 10^{-6} mL. Tryptic Soy Agar (TSA), Plate Count Agar (PCA) and Nutrient Agar (NA) were the three media used, all of which are nutrient rich (Table 1). A set of three plates of each medium from each dilution were allowed to incubate at room temperature, 30^{o}C and 37^{o}C. The growth was quantified after one day, 48 hours and 72 hours. It was concluded that the PCA medium incubated at 37 ^{o}C for 48 hours were the most optimal conditions to obtain the most microbial growth.

Table 1. The nutrient contents of the media that were compared to determine which media supported the most bacterial growth. TSA, tryptic soy agar, PCA, plate count agar, and NA, nutrient agar were the three media compared, each varying in amount and type of nutrients.

Medium	Nutrient (g/L)	
TSA	15.0	Peptone from casein
	5.0	Peptone from soymeal
	5.0	Sodium Chloride
	15.0	Agar
PCA	5.0	Tryptone, pancreatic digest of casein
	2.5	Yeast Extract
	1.0	Dextrose/glucose
	15.0	Agar
NA	3.0	Beef Extract
	5.0	Peptone
	15.0	Agar

Pour Plating and Quantification

Soil types analyzed in this study included topsoil, potting soil, forest soil, compost and manure, which were diluted in 0.9% saline solution to 10^{-4}, 10^{-5}, and 10^{-6}mL. Then 0.1 mL of each prepared dilution was added to approximately 20 mL of molten PCA and pour plated. The resulting dilutions that were plated were 10^{-4}, 10^{-5}, and 10^{-6} mL. After 2 days of incubation, the number of colonies that grew were enumerated and recorded. The plate exhibiting growth within the range of 25-250 colonies was utilized for quantification. Dilution and quantification was conducted at least three times with each soil type and the average abundance was calculated.

Isolation and purification of colonies

Each colony represents a single bacterial species capable of growing under the given conditions on the media, thus colonies were isolated and purified as preliminary steps for determining the actual species. Colonies of interest had distinct morphologies, sufficient growth, and lack of any other interfering growth, such as fungal. After a colony was selected, it was re-

streaked onto a solidified plate of PCA. The re-streaking was repeated at least three times, or until only one colonial morphology was apparent.

DNA extraction and purification

The purified colonies were inoculated into liquid PCA and incubated at 37°C in a shaking incubator for at least two days or until ample growth was evident. Once there was growth, the solution was centrifuged at 13,000 rpm for one minute. The supernatant was discarded, and the pellet containing the bacterial cells was retained and stored at -20°C.

DNA was extracted using a Promega kit by the following procedure (Promega, Madison, WI). The bacterial cells were thawed, re-suspended in 400µL of DNA Extraction Buffer (47mM EDTA, 25µg/mL lysozyme) and vortexed thoroughly. After incubation for 1.5 hours at 37°C, 7µL of Proteinase K was added and the samples were incubated in a 55°C bath for another hour. A half a milliliter of nuclei lysis solution and 7µL of RNase were added and set for a final incubation period of 10 minutes at 80°C. The remainder of the extraction was completed according to the manufacturer's instructions. Following extraction, samples were stored at -20°C.

PCR Amplification

PCR was prepared that targeted the 16S rDNA region of the genomic DNA. The DNA sample, PCR master mix containing Taq polymerase (Invitrogen, Carlsbad, CA) and the Bact 8f and Bact 1391R primers were added to centrifuge tubes and put in the thermocycler. The primers had specific sequences that matched a region upstream and downstream of the target 16S rDNA gene (Table 2). The thermocycler facilitated amplification of the target gene through a specific rotation of temperatures starting at 95°C melting step for 5 minutes, proceeded by 30

cycles of melting at 95 ^{o}C for 30 seconds, annealing at 56 ^{o}C for 30 seconds, and elongation at 72 ^{o}C for 90 seconds, with a final elongation step for 8 minutes.

Table 2. The sequences for the primers used for PCR. Note that the "R" nucleotide in the Bact-1391R primer indicates either adenine of guanine to compensate for variation observed at this location (Eckburg et al., 2005).

Upstream Primer	Bact-8F	5'-AGAGTTTGATCCTGGCTCAG-3'
Downstream Primer	Bact-1391R	5'-GACGGGCGGTGTGTRCA-3'

In order to test what samples were successfully amplified, agarose gel electrophoresis was conducted, and the distance the DNA fragments traveled was visualized using Kodak 1D 3.6 UV imaging. DNA fragments approximately 1500 base pairs in length were indicative that the 16S rDNA gene was amplified. The PCR products were purified using the GeneJet Gel Extraction Kit (Fermentas Inc, Burlington, ON). The purified PCR products were then quantified and the ones with greater than 20 ng/µL nucleic acid concentration were sent to an off-site facility (Functional Biosciences, Madison, WI) using the Bact-8F primer for sequencing.

16S rDNA Sequence Analysis

Specific nucleotide sequences resulted that allowed for each sample to be identified using the Seqmatch tool from the Ribosomal Database Project at Michigan State University (Cole et al., 2007; Cole et al., 2009). The following options were used: Strain: Type, Source: Isolates, Size: Both, Quality: Good, Taxonomy: Nomenclature. The sequences generated from this study were compared to sequences of known bacteria; the sequences that matched closest led to the identification of the most likely bacterial species from which the sample originated. The percent sequence identity was determined by comparing the sequences from our study to known sequences of the NCBI database using the BLAST algorithm (Altschul et al. 1997).

Results

Nutrient Analysis of Soil Types

In this study, we used a qualitative analysis of the soil types to illustrate their relative nutrient content. Variation of nutrient levels between the different soil types contributed to their diversity in bacterial species and abundance. The soils were tested for pH, nitrate, phosphorus, and potassium content (Table 3).

Table 3. Qualitative nutrient analysis of the 5 soil types sampled using LusterLeaf Products Nutrient Analysis Kit (Woodstock, IL). The values given are approximated amounts to give a relative idea of the nutrient content.

Soil Type	pH	Nitrate	Phosphorus	Potassium
Garden topsoil	Slightly acidic (6.5)	Medium to low (10mg/L)	Low (5mg/L)	Low (0-50mg/L)
Potting soil	Acidic (6.0)	Low (<10mg/L)	Low (5mg/L)	Low (0-50mg/L)
Forest soil	Neutral (7.0)	Low (<10mg/L)	Low (5mg/L)	Low (0-50mg/L)
Manure	Slightly acidic (6.5)	Low (<10mg/L)	Low (5mg/L)	Low (0-50mg/L)
Compost	Alkaline (7.5)	Medium to low (10mg/L)	Medium to High (50mg/L)	Medium (400mg/L)

Overall, the soils tested low in nitrate, potassium and phosphorus levels with the exception of the compost sample, which tested medium above in all nutrient areas. Additionally, the compost sample was characteristically alkaline in contrast to the other types that were acidic to neutral. An increased understanding of how the soil types differed from each other allowed us to predict which types would harbor greater microbial diversity and abundance.

Determination of Optimal Growth Conditions

The microbial content of the soils was investigated by using a nutrient-rich media under optimal growth conditions to yield the highest number of bacterial colonies. After comparing the number of colonies on different media, at different incubation temperatures, for varied length of duration, we concluded that Plate Count Agar media incubated at 37^{o}C for two days was optimal.

Quantification of Colonial Growth

Colonial growth on the PCA was enumerated after two days of incubation at 37^{o}C (Table 4). It was expected that the more nutrient-rich soils would harbor a higher number of microbes and thus would exhibit greater colonial growth on the PCA. In order to retrieve a representative sample of the whole microbial population from the given soil type, the soil was diluted and plated at least six times. The average bacterial count per gram of soil and standard deviation for each soil type were calculated and 95% confidence intervals were determined (Figure 1). We also conducted a one-way ANOVA with a Tukey HSD Post-hoc analysis to determine whether or not there were statistically significant differences between the groups. Statistical analyses were conducted using IBM SPSS Statistics 19 (Armonk, NY).

Table 4. P-values genereated from the one-way ANOVA with Tukey HSD Post-Hoc analysis. The mean difference between soil types is significant if $p \leq 0.05$. All the p values that are above 0.05 are bold and represent the samples that are not significantly different. PS = potting soil, CP = compost, TS = garden topsoil, FS= forest soil, and MN= manure.

Soil Type	Compared to	P value
PS	CP	**0.266**
	TS	0.001
	FS	0.000
	MN	0.000
CP	PS	**0.266**
	TS	**0.241**
	FS	0.002
	MN	0.000
TS	PS	0.001
	CP	**0.241**
	FS	**0.202**
	MN	0.000
FS	PS	0.000
	CP	0.002
	TS	**0.202**
	MN	0.000
MN	PS	0.000
	CP	0.000
	TS	0.000
	FS	0.000

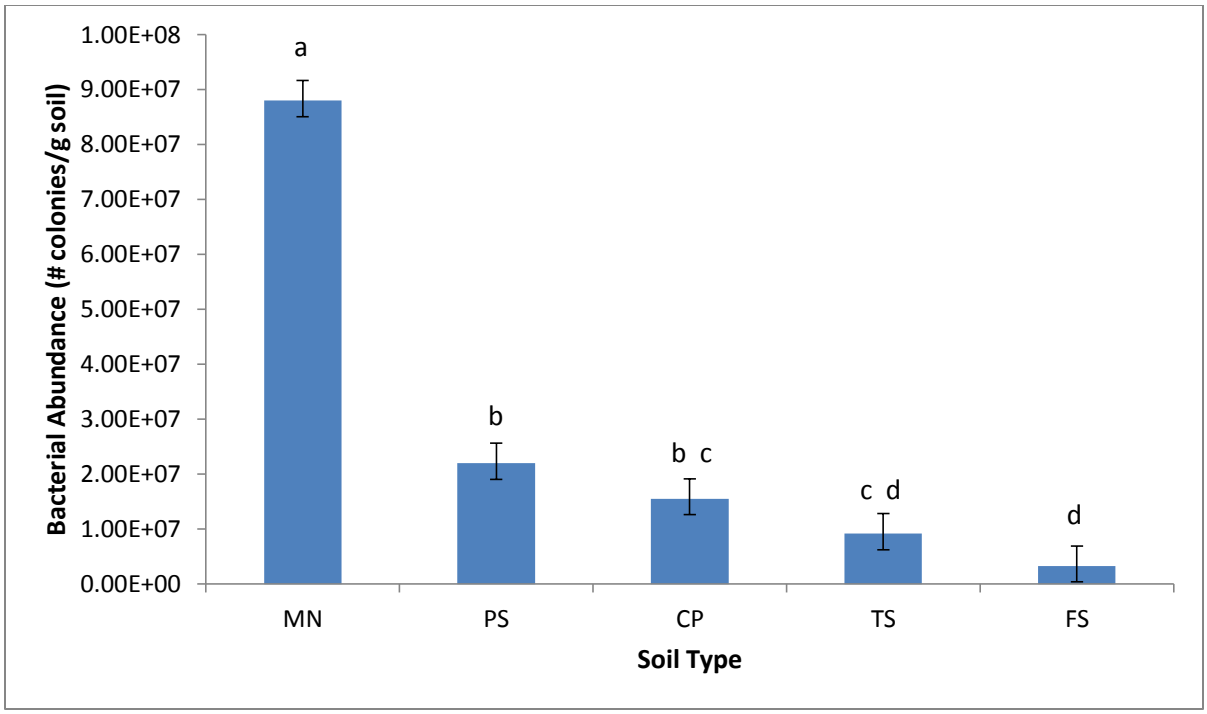

Figure 1. Average number of colonies per gram of soil that were quantified through plate count assay. Each soil type is had a different population size; manure (MN) n=6, potting soil (PS) n=8, compost (CP) n=7, garden topsoil (TS) n=10, and forest soil (FS) n=11. Error bars are given for the 95% upper and lower confidence intervals. Significant statistical difference was evaluated by One-Way ANOVA with a Tukey HSD Post-Hoc analysis. Matching letters above the data series represent that those soil types do not have statistically different bacterial counts.

As illustrated in Figure 1, the manure exhibited significantly higher bacterial growth than the other soil types (P= 0.00). Potting soil and compost followed with counts that were not significantly different (P= 0.266). Likewise, the bacterial count of the compost did not significantly differ from the garden topsoil (P = 0.241). The garden topsoil and forest soil were also statistically identical (P = 0.202). While plate count assay was used to determine the bacterial abundance, our next method allowed us to investigate which soil type supported the greatest diversity of bacterial life.

PCR Amplification and 16S rDNA Sequencing

DNA from 108 colonies was used for PCR. Those reactions were evaluated using gel electrophoresis. Fifty of them exhibited a 1500 bp DNA band indicative that the 16S rDNA gene

was successfully amplified (Figure 2). These 50 samples were sent to an office-site facility for sequencing (Functional Biosciences, Madison, WI).

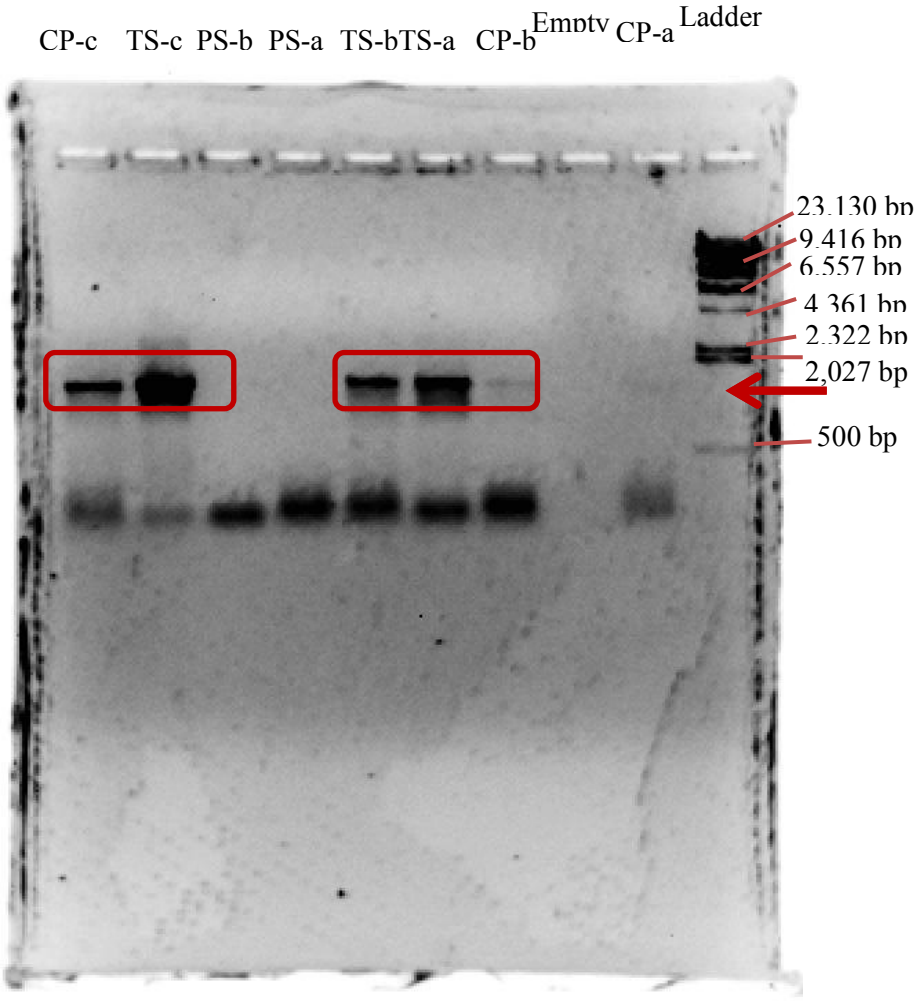

Figure 2. An example of a gel electrophoresis. The PCR products were evaluated by gel electrophoresis to determine if they contained the amplified 16S rDNA gene with a length of 1500 bp. Circled on this gel are five samples; compost sample b (CP-b), topsoil sample a (TS-a), topsoil sample b (TS-b), topsoil sample c (TS-c), and compost sample c (CP-c) that had a band at 1500 bp. The arrow is at 1500 bp.

The DNA fragments circled above are examples of successful PCR products because they had fragments at 1500 bp. Of the 50 samples that were sent in for analysis, 45 yielded sequences and were compared to a database of 16S rDNA sequences to identify the genus and species of the isolate, given by the Ribosomal Database Project. Thirty-five of these were

unique bacterial species. The percent identity was determined from the two sequence BLAST comparison. Additionally, the number of times isolated represents the species that were isolated multiple times independently from the same sample (Tables 5-9).

Table 5. The 16S rDNA results for the nine unique sequences obtained from the garden topsoil. The closest species matches are given as found from the Ribosomal Database Project, with the % identity obtained from two sequence BLAST comparison, and the number of times each species was isolated independently from the sample. A physical description of the bacteria colonies is also given.

Sample	*Closest Match(es)*	*% Identity*	*# Times Isolated*	*Colonial Morphology*
TS-1	*Streptomyces althioticus* (T); KCTC 9752	99% (599/602 bp)	1	Small circular white
TS-2	*Streptomyces griseochromogenes* (T); ISP 5499	100% (661/661 bp)	1	Small circular white
	Streptomyces resistomycificus (T); ISP 5133	100% (661/661 bp)		
	Streptomyces violascens (T); ISP 5183; CSSP183	100% (661/661 bp)		
	Streptomyces albidoflavus (T); NBRC 13010	100% (661/661 bp)		
	Streptomyces hydrogenans (T); NBRC 13475	100% (661/661 bp)		
TS-3	*Bacillus simplex* (T); DSM 1321T	100% (711/711 bp)	1	Small circular white
TS-4	*Bacillus subtilis* (T); DSM10	100% (769/769 bp)	1	Large white, antibiotic-secreting
TS-5	*Bacillus thuringiensis* (T); IAM 12077	100% (744/744 bp)	1	Smaller filamentous
TS-6	*Streptomyces bluensis* (T); NBRC 13460	98% (758/772 bp)	1	Faint white, smaller
TS-7	*Streptomyces humidus* (T); NRRL B-3172T	99% (764/768 bp)	1	Small circular white
TS-8	*Streptomyces griseoloalbus* (T); NBRC 13046	100% (637/637 bp)	1	Circular white
	Streptomyces lusitanus (T); NBRC 13464	100% (637/637 bp)		
TS-9	*Streptomyces viridochromogenes* (T); NRRL B-1511T	99% (691/694 bp)	1	Opaque white with brown toxin

Table 6. The 16S rDNA results for the six unique sequences obtained from the potting soil sample. The closest species matches are given as found from the Ribosomal Database Project, with the % identity obtained from two sequence BLAST comparison, and the number of times each species was isolated independently from the sample. A physical description of the bacteria colonies is also given.

Sample	*Closest Match(es)*	*% Identity*	*# Times Isolated*	*Colonial Morphology*
PS-1	*Streptomyces mexicanus* (T); NBRC 100915	99% (773/778 bp)	2	Opaque, pale yellow
PS-2	*Streptomyces phaeoluteichromatogenes* (T); NRRL B-5799	99% (744/746 bp)	2	White with harder outside
PS-3	*Bacillus thuringiensis* (T); IAM 12077	100% (706/706 bp)	2	Large yellow
PS-4	*Gordonia terrae* (T); X79286	99% (765/766 bp)	1	Peach-colored
PS-5	*Streptomyces avermitilis* (T); MA-4680	98% (744/758 bp)	1	Small circular white
PS-6	*Streptomyces thermocoprophilus* (T); B19	99% (615/618 bp)	1	Opaque white with brown toxin

Table 7. The 16S rDNA results for the six unique sequences obtained from the compost sample. The closest species matches are given as found from the Ribosomal Database Project, with the % identity obtained from two sequence BLAST comparison, and the number of times each species was isolated independently from the sample. A physical description of the bacteria colonies is also given.

Sample	*Closest Match(es)*	*% Identity*	*# Times Isolated*	*Colonial Morphology*
CP-1	*Bacillus anthracis* (T); ATCC 14578 *Bacillus cereus* (T); ATCC 14579	100% (668/668 bp) 100% (668/668 bp)	2	Off white with a brown secretion
CP-2	*Streptomyces antibioticus* (T); NRRL B-1701	100% (748/748 bp)	2	Off white with a brown secretion
CP-3	*Bacillus megaterium* (T); IAM 13418	99% (698/702 bp)	1	Disc-shaped white
CP-4	*Flavobacterium cucumis* (T); R2A45-3	97% (726/747 bp)	1	Small, yellow, jell-like
CP-5	*Bacillus drentensis* (T); LMG 21831	100% (588/588 bp)	1	Smaller yellow
CP-6	*Bacillus amyloliquefaciens* (T); NBRC 15535	99% (741/743 bp)	1	Large white, antibiotic-secreting

Table 8. The 16S rDNA results for the six unique sequences from the forest soil sample. The closest species matches are given as found from the Ribosomal Database Project, with the % identity obtained from two sequence BLAST comparison, and the number of times each species was isolated independently from the sample. A physical description of the bacteria colonies is also given.

Sample	*Closest Match(es)*	*% Identity*	*# Times Isolated*	*Colonial Morphology*
FS-1	*Viridibacillus arvi* (T); LMG 22165	100% (634/634 bp)	1	Small filamentous
FS-2	*Streptomyces olivochromogenes* (T); DSM 40451	99% (740/747 bp)	1	Small circular white
FS-3	*Streptomyces phaeoluteigriseus* (T);ISP 5182	99% (736/740 bp)	1	Small circular white
	Streptomyces pseudovenezuelae (T); NBRC 12904	99% (736/740 bp)		
	Streptomyces chartreusis (T); NBRC 12753	99% (732/741 bp)		
FS-4	*Bacillus weihenstephanensis* (T); DSM11821	99% (620/621 bp)	3	Large white
	Bacillus mycoides (T); ATCC6462	99% (620/621 bp)		
FS-5	*Bacillus thuringiensis* (T); IAM 12077	99% (666/667 bp)	2	Large white
FS-6	*Enterobacter nimipressuralis* (T); LMG 10245-T	99% (694/697 bp)	1	Opaque white with brown toxin

Table 9. The 16S rDNA results for the eight unique sequences from the manure sample. The closest species matches are given as found from the Ribosomal Database Project, with the % identity obtained from two sequence BLAST comparison, and the number of times each species was isolated independently from the sample. A physical description of the bacteria colonies is also given.

Sample	*Closest Match(es)*	*% Identity*	*# Times Isolated*	*Colonial Morphology*
MN-1	*Microbacterium kitamiense* (T); kitami C2	98% (733/747 bp)	1	Opaque, pale yellow
MN-2	*Bacillus megaterium* (T); IAM 13418	99% (647/651 bp)	1	Large white
MN-3	*Acinetobacter radioresistens* (T); DSM 6976	97% (714/734 bp)	1	Small circular white
	Acinetobacter venetianus (T); ATCC 31012	98% (715/732 bp)		
MN-4	*Microbacterium oleivorans* (T); DSM 16091	99% (772/778 bp)	2	Small, yellow, jell-like
	Microbacterium paraoxydans (T); CF36	98% (769/781 bp)		
MN-5	*Enterococcus faecalis* (T); JCM 5803	99% (673/674 bp)	1	Small circular white
MN-6	*Bacillus anthracis* (T); ATCC 14578	99% (651/654 bp)	1	Filamentous
	Bacillus cereus (T); ATCC 14579	99% (651/654 bp)		
MN-7	*Acinetobacter lwoffii* (T); DSM 2403	99% (753/754 bp)	1	Small, yellow, jell-like
MN-8	*Enterobacter asburiae* (T); JCM6051	99% (726/734 bp)	1	Tiny, clearish white
	Enterobacter ludwigii (T); 108491	98% (721/734 bp)		

Discussion

Bacterial Abundance

In this study we compared the bacterial abundance and species diversity of samples from potting soil, garden topsoil, forest soil, manure, and compost. Our predictions of abundance and diversity were largely based on a qualitative nutrient analysis of the soils. The nutrient analysis showed compost as the soil sample richest in nitrate, phosphorus, and potassium. The remaining samples tested low for all of the nutrients except for the garden topsoil testing medium to low in nitrate. We hypothesized that compost would have the greatest abundance and diversity of

bacteria because of its high nutrient content relative to the other samples. Also, manure was hypothesized to have high abundance and diversity because it came from the microbiologically-active intestine of a horse. Likewise, previous research suggested that horse manure is rich in nitrogen (Dou et. al 2001). Similarly, the garden topsoil was expected to harbor moderately high levels of bacterial diversity and abundance because of its moderate level of nutrients. Lastly, we hypothesized that potting soil (*Scotts Miracle-Gro Co*. 2012) and forest soil (Sackett et al. 2012) would have the least abundance and diversity of bacteria due to their low nutrient levels.

Our data indicate that manure supports the highest bacterial abundance of all the soil types investigated. According to the One-Way ANOVA, the average bacterial count for the manure sample was statistically different from all the other soil types (Figure 1). The high abundance of bacteria in the manure sample conflicted with its low nutrient content. This suggests that nutrient availability was not the most influential factor for determining the manure's ability to support bacteria. In contrast, the microbes isolated from the manure sample could have originated from the horse intestine rather than the soil. Likewise, *Enterococcus faecalis* is known to inhabit mammalian intestines and was one of the possible species isolated from the manure sample (Otawa et al. 2012).

According to our data, the potting soil and compost had the second highest bacterial abundances of the five soil types. The average bacterial count for potting soil and compost were not statistically different (Figure 1). Contrary to the results, we hypothesized that potting soil would be one of the lowest due to its lower nutrient content and synthetic formation (*Scotts Miracle-Gro Co*. 2012). The main ingredients in most potting soils include organic components such as peat moss, compost, and bark in addition to inorganic perlite and vermiculite (Pelczar 2009). Because these ingredients are processed and packaged, they are considered fairly sterile.

However, it is plausible that the potting soil was contaminated after production from the plant roots and water supply.

We hypothesized compost as having the greatest microbial diversity due to its high nutrient content. Although it did not have the greatest, the compost had the second highest abundance along with potting soil, which partially supports our hypothesis. The high nutrient analysis of the compost most likely contributed to its high bacterial count. The compost was also significantly similar to the garden top soil in terms of abundance (Figure 1). Supporting our hypothesis, the garden topsoil sample had moderate bacterial abundance compared to the other soil samples. This could be due to its moderate nutrient content, but would have to be investigated further. One factor that negatively affected the bacterial count of the garden top soil was the time in which the sample was taken; before any crops were planted. Therefore, there were no plant roots in the soil exuding carbon and nitrogen-rich compounds to attract microbes (Condron et al. 2010). If a sample were to be taken from the same garden location after the crops had begun to grow, we would expect a higher number of microbes.

According to the One-Way ANOVA with Tukey HSD Post-Hoc analysis, the forest soil and garden topsoil were not significantly different (Figure 1). Supporting our hypothesis, the data suggest that the forest soil was low in bacterial abundance. The forest soil exhibited both low number of bacteria and low nutrient content. Determining an accurate number for microbial abundance of forest soil is challenging due to spatial variability of the soil and different types of trees growing in the soil (Fernandez 2008). Accordingly, different tree types vary in amount of leaf litter which affects the rate of decomposition and nutrient release (Fernandez 2008). These factors could have also impacted the microbial population in our forest soil sample.

Bacterial Diversity

The data for microbial diversity was largely dependent upon the culturability of the microbes from the raw soil samples and on successful DNA extraction, PCR and 16S rDNA sequencing. 16S rDNA is a group of genes that codes for the small ribosomal subunit for bacteria. It is ideal for studying microbial evolution and relatedness because it has genes that are highly conserved over time and others that exhibit some variability. Stable sequences allow for the comparison of distantly related species; whereas, the variable sequences are used to compare similar species (Willey et al. 2011). 16S rDNA sequencing is a reliable method for identifying microorganisms at the genus level but not the species level; thus, our results do not show the exact species we isolated, but the closest known match. In some cases, several species of the same genus resulted as the possible closest match (see sample TS-2 in Table 5).

Further investigation would have to be conducted to confidently identify the species of the isolated microorganism. Discriminating between strains that belong to the same genus can be achieved by evaluating multiple genes through a process called multilocus sequence typing (MLST). If two isolates exhibit the same alleles for multiple genes, this strongly suggests they are closely related if not the same species (Willey et al. 2011). More specifically, one study proposed that three *Bacillus* species, *B. anthracis, B. cereus, and B. thuringiensis*, were all the same microorganism based on genetic evidence (Helgason et al. 2000). Although these three organisms demonstrated a wide range of pathogenic effects, they contained identical genotypes at 13 enzyme loci that were analyzed by multiloci enzyme electrophoresis (Helgason et al. 2000). Likewise, two strains we independently isolated from the compost and manure samples both had *B. cereus* and *B anthracis* as the closed possible matches. Thus it is reasonable that the two *Bacillus* species are either very closely related or indeed one bacterium.

Of the 50 samples sent in for 16S rDNA sequencing, 35 samples yielded sufficient results. We evaluated the species diversity of the soil types based on the number of genera present. There were nine different genera represented between all the soil types; 5 genera in manure, 4 in forest soil, 3 in potting soil and compost, and 2 in garden topsoil. Because monetary and temporal restrictions, not every bacterium present in the soils was isolated and sequenced. Saturation, or the point at which the same species are isolated repetitively, was not reached within our study. Most of the bacterial species identified from the soil types were only isolated once. Rather than only 50 isolates, thousands are required to reach saturation of a microbial rich environment. Therefore, no conclusion could be made as to which soil type was the most diverse, but several bacteria with practical applications were sequenced from the different soil types. Not every bacterium that was identified is discussed below, but a variety of unique and practical ones are showcased.

Garden topsoil exhibited the least number of genera (Table 5). Nine unique species from the garden topsoil were identified, 7 of which were from the genus *Streptomyces* and the other two were from the genus *Bacillus*. Species from *Streptomyces* are ubiquitous in soil and many produce commercially-used antibiotics (Kim et al. 2012**,** Jung et al. 2003). For example, we cultured *S. griseochromogenes* that produces tautomycetin, a T cell-specific immunosuppressive drug with anticancer activities (Kim et al. 2012). Additionally, *S. bluensis*, the bacterium responsible for producing bluensomycin, a member of the aminoglycoside family of antibiotics, was also isolated from the garden topsoil (Jung et al. 2003).

Members from the genus *Bacillus* are characterized by their ability to produce endospores that are highly resistant to extreme physical and chemical conditions. Two of the species we isolated from garden top soil, *B. subtilis* and *B. simplex*, have also been found in previous studies

to inhabit extreme environments such as Antarctic soils, deep sea floor sediments, spacecraft assembly facilities, and the interior of near subsurface granites. The endospores that are produced by members of the *Bacillus* genus are so resilient and long-lived that they contribute to the theory that viable microbes can be transferred through space (Fajardo-Cavazos and Nicholson 2006). In addition to producing endospores, some members of Bacillus produce metabolites that can be used as insecticides. Specifically, *B. thuringiensis* produces Cry toxins that are commonly used in bioinsecticides against the Colorado Potato Beetle. Furthermore, it is employed in the transgenic plants (Garcia-Robles 2012). Overall, the garden top soil harbored a wide variety of bacteria with practical functions ranging from insect control to antibiotics.

B. thuringiensis was also one of the closest matches for a bacterium we isolated from the potting soil sample. In addition, we cultured 4 different species of *Streptomyces* and one species of *Gordonia* (Table 6). Isolated from the potting soil, *Streptomyces avermitilis* is of industrial importance; it is known for the production of avermectins, antibiotics used for treating humans, animals and crops from parasitic infections (Zhang et al. 2012). We isolated another *Streptomyces* strain from the potting soil that was discovered and named in 2003; *S. mexicanus*, was isolated as a novel species with the ability of breaking down xylan, a polysaccharide found in the cell walls of some algae and plants (Petrosyan et al. 2003). In a previous study conducted by Chen et al. (2012), *Gordonia terrae* was isolated from a composting aquaculture waste mixture of molasses and rice bran. A thermophilic bacterium with high cellulase activity, *G. terrae* was studied for its ability to accelerate composting. Collectively, a diverse amount of microbes with a wide array of practical applications were isolated from the potting soil sample.

Similar to the potting soil, the compost exhibited 3 different genera, two of which were *Streptomyces* and *Bacillus* with the third as *Flavobacterium* (Table 7). The single species from

Streptomyces that was most closely matched our isolate was *S. antibioticus*. According to Oppegard et al. (2012), this bacterial strain produces the antibiotic Simocyclinone D8 that influences topoisomerases and has an antiproliferative effect against some cancer cells. Additionally, *Flavobacterium cucumis* was the closest match of another bacterium we isolated from the compost sample. Previous research shows that this same microbe was sampled from greenhouse soils in Korea. It received its name from its original discovery in the soil surrounding cucumber plants (Weon et. al 2007).

One of our compost samples resulted with two *Bacillus* species as the potential closest match; *B. anthracis* and *B. cereus*. This same species was also independently isolated from the manure sample. Research shows that both of these species are pathogenic. *B. anthracis* is known for its production of the potent toxin anthrax that is absorbed by breathing in endospores (Cote et al. 2012). Likewise, *B. cereus* can cause food-borne illnesses. Various isolates of *B. cereus* have developed resistance to the antibiotic tetracycline by horizontally transferring the gene for antibiotic resistance (Rather et al. 2012). One study analyzed these two *Bacillus* species in addition to *B. thuringiensis* and concluded that they were genetically indistinguishable from each other because of their identical genotypes at multiple locations (Helgason et al. 2000). From antibiotic-producing to disease-causing bacteria, compost harbored a unique variety of microorganisms as well.

Even more microbial diversity was exhibited by the forest soil. Of the six unique species that were sequenced, 4 different genera were represented (Table 8). Found in the top soil and potting soil, *Bacillus thuringiensis* was also cultured from the forest sample. A second *Bacillus* species was identified to be either *B. weihenstephanensis* or *B. mycoides*. Both of these microbes belong to the *B. cereus* group and can cause food-borne illnesses (Antolinos et al. 2012). From

the *Streptomyces* genus, the species *S. chartreusis* was isolated from the forest soil. Like many of the other members of *Streptomyces*, it also has antibiotic capabilities. Specifically, it produces tunicamycins that inhibit the biosynthesis of peptidoglycan in the bacterial cell wall, thus preventing cellular division and growth of certain pathogenic bacteria (Doraghazi et al 2011).

The third genus represented in the forest soil was *Viridibacillus*, whose name means "green rod" describing the pigment and shape of the individual cells. The species we isolated, *V. arvi*, was originally included with the *Bacillus* genus until research suggested it had sufficient difference in cell wall lipids and spore formation (Albert et al. 2007). *Enterobactor nimipressuralis* was the closest match from the final genus we found in the forest soil. *E. nimipressuralis* was previously identified as a disease-causing contaminant on saline cotton used in hospitals (Kim et al. 2010). Furthermore, E. nimipressuralis is an invasive species in forests causing bacterial wetwood (Forestry Images 2008). Forest soil exhibited the second highest microbial diversity, representing four genera that ranged from newly discovered species from *Viridibacillus* to ones that have been comprehensively investigated like those related to *Bacillus* and *Streptomyces*.

The soil sample that modeled the highest degree of bacterial diversity was manure. *Bacillus, Microbacterium, Acinetobacter, Enterobacter,* and *Enterococcus* were the five genera sequenced from the manure (Table 9). Like the other soil samples, manure harbored many unique microbes including *Enterococcus faecalis*, a pathogenic bacterium commonly found in bovine feces. We were not surprised to find this in the horse manure sample since it is a microbe that thrives in the intestinal tract of livestock (Otawa et al. 2012). In addition, we isolated carotenoid-producing *Microbacterium kitamiense*. One of the most diverse classes of natural pigments, carotenoids are used in food, pharmaceuticals, and cosmetics; thus, *M. kitamiense* can

be utilized by industries to synthesize specific carotenoids used in products (Zhang Yong et al. 2005). Another microorganism from the genus *Microbacterium* that was isolated from the manure sample most closely matched the species *M. paraoxydans*. This exact species of bacteria was also found at a contaminated soil site in Sanganer, India that regularly received effluent from local textile industries. The research showed that *M. paraoxydans* was highly resistant to the metal contaminants in that area, such as arsenic and nickel; furthermore, metal-resistant microbes like *M. paraoxydans* could potentially be used for bioremediation of contaminated sites (Kaushik et al. 2012).

Also cultured from the manure sample was a pathogenic bacterium, *Acinetobacter lwoffii* that is one of many *Acinetobacter* species to develop a resistance to carbapenem antibiotics. According to Figueiredo et al. (2012), *A. lwoffii* possesses genes that code for enzymes capable of hydrolyzing carbapenem and making it ineffective at fighting infection. The sequencing results for the one manure sample identified two possible *Enterobacter* species as its closest match; *E. asburiae* and *E. ludwigii*. *E. asburiae* is a phosphate-solubilizing bacterium that promotes the growth of certain plants such as mung beans (Zhao et al. 2011). Likewise, *E. ludwigii* is another helpful microbe that has the ability to degrade toxic compounds called tannins that would otherwise cause gastrointestinal distress in livestock. Zhao et al. (2011) found *E. ludwigii* living mutualistically in the rumen of goats where it could receive ample nutrients while metabolizing potentially harmful substances for the goat.

Overall, a vast diversity of bacteria from the genera *Streptomyces, Bacillus, Gordonia, Flavobacterium, Microbacterium, Acinetobacter, Enterococcus, Viridibacillus*, and *Enterobacter* were represented by the five soil types investigated. These genera were identified by 16S rDNA sequencing, which is most useful identifying isolates at the genus level but not the species level.

Further investigation by multilocus sequence typing (MLST) would have to be conducted in order to confidently identify the species of the isolated bacteria (Willey et al. 2011). A One-Way ANOVA with a Tukey HSD Post- Hoc statistical analysis of the bacterial abundance revealed manure as being significantly highest in abundance followed by potting soil and compost, compost and garden topsoil, and garden topsoil and forest soil. The soil types listed in pairs did not exhibit significantly different bacterial counts from each other. The qualitative nutrient analysis showed how the compost sample had the greatest nutrient content, garden topsoil with a moderate amount, and the potting soil, forest soil and manure with the lowest. There was no strong correlation between the results of the nutrient analysis and the microbial abundance and diversity.

Further research could be conducted on these soils by taking more random samples of each type to generate a larger sample size. Additionally, samples could be taken at different times of the year to see how the abundance of different bacteria fluctuates with changing temperature and precipitation. Different soil samples such as cow manure, soil near a river or pond, or soil from a farm that uses pesticides, could be investigated as well.

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Author Biography

McKenzie Roth was raised in the small town of Bellville, Ohio where she graduated from Clear Fork High School in 2009. Following her aspiration of becoming a high school science teacher, McKenzie is majoring in Integrated Science Education at Ashland University. While at Ashland, McKenzie has competed on the varsity track and cross country teams, earning Academic Excellence All GLIAC each semester for competing at the conference meet and having a GPA of greater than 3.5. More so, McKenzie earned Academic All American for finishing in the top third at the regional cross country meet and qualifying to nationals with her team in 2011. In addition to the Honors Program, she is also a College of Arts and Sciences scholar and has been on the Dean's List each semester. Beyond athletics and academics, McKenzie is involved in Religious Life where she serves as a small group leader for The Well. Furthermore, she is enjoying her second year of being a Resident Assistant.

Upon graduation, McKenzie plans to obtain a high school science teaching job in a low-income, high-need school in Ohio.