STRATIFICATION IN TOPSOIL: A PRELIMINARY ANALYSIS OF BACTERIAL BIODIVERSITY AT TWO SITES IN SAN ANTONIO, TEXAS

K. N. Treviño and D. E. Starkey*
School of Natural Sciences and Mathematics, Department of Biology, University of the Incarnate Word, San Antonio, TX 78209
**Corresponding author; Email: dstarkey@uiwtx.edu*

Abstract.–The purpose of this study was to determine whether the A horizon (topsoil) exhibits stratification across two depths, 1 cm and 4 cm. Two sites in San Antonio, TX, were chosen to address this question. The first was on the campus of the University of the Incarnate Word. The second was adjacent to the university in the Headwaters Sanctuary. At each locality, soil samples were analyzed in order to examine the morphological diversity of bacteria across site and depth. Preliminary results suggest that the bacterial biodiversity is different when depths are considered as a single entity versus individually. These finding have implications for investigations into soil biodiversity and suggest that in order to gain an accurate estimate of bacterial biodiversity in topsoil multiple depths within this layer of soil should be examined.

Keywords: 16S rRNA, biodiversity, bacteria, topsoil, stratification, A horizon

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In any ecosystem, terrestrial or aquatic, microorganisms, such as bacteria or fungi, are the most prevalent and diverse organisms present (Whitman et al. 1998; Torsvik et al. 2002; Kemp & Aller 2004; Venter et al. 2004). In terrestrial environments in particular, bacteria are incredibly abundant as they play critical roles in nutrient cycling, plant health and nutrition, as well as, soil structure, and fertility (e.g., Mishra 1996; Madsen 2005; Ferris & Tuomisto 2015; Kaiser et al. 2016). In turn, bacterial diversity is impacted by abiotic factors such as pH, soil moisture, carbon and nitrogen content, and the concentration of key nutrients (e.g., Buckley & Schmidt 2002). Of these factors, numerous studies suggest pH is the key component driving microbial diversity with low pH soils containing decreased biodiversity compared to neutral pH soils (Fierer & Jackson 2006; Rousk et al. 2010). In addition, bacterial biodiversity can be impacted by biotic factors such as plant diversity and abundance and the

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presence of competing microorganisms (Barthlott et al. 1999; ter Steege et al. 2003; Goberna et al. 2016).

Soils may contain multiple horizons. Each horizon has distinct characteristics and may, or may not, be found in all soil types. For example, the A horizon (topsoil) often contains a higher percentage of organic matter and minerals, whereas the B horizon (subsoil) contains a higher percentage of iron and other minerals that have leached into this layer. As a result of the differences in composition between horizons, microhabitats are often present within, and between, horizons (Kirk et al. 2004). This can result in a tremendous amount of genetic and morphological diversity in the soil microbes present within and across different horizons (e.g., Ovreas et al. 1998). Unfortunately, a large percentage of soil microbes cannot be examined using traditional techniques. In fact, estimates suggest only 1-10% of bacterial isolates can be cultured using standard microbiological practices (e.g., Borneman et al. 1996; Pham & Kim 2012). This often leads to the isolation of bacteria that thrive under the chosen conditions and, as a result, an inaccurate estimate of total biodiversity.

The complexity of soil is driven by the interaction of factors such as water and organic content, biotic activity, and pH (e.g., Robe et al. 2003). These factors interact to provide a diverse array of habitats for microorganisms. Unfortunately, the complex nature of soil also increases the level of difficulty when isolating microorganisms as various means of binding to soil are utilized by bacteria (e.g., Bakken & Lindahl 1995). For example, bacteria may possess pili or fimbriae that aid in their attachment to soil particles (Weiss 1973; Rosenberg et al. 1982). In addition, many bacterial species excrete extracellular polysaccharides that are used to bind the bacteria to soil, or to clay particles in soil, resulting the in formation of stable soil aggregates which may interfere with bacterial extraction (Martens & Frankenberger 1992; Chenu 1993; Lindahl & Bakken 1995). As a result, a variety of methods, indirect and direct, have been developed to extract bacteria from soil (e.g., Torsvik 1980; Ogram et al. 1987).

There are two primary steps involved in direct extraction methods: 1) disruption/lysis of the bacterial cell wall and 2) the separation of nucleic acids and soil. The disruption of the bacterial cell wall can be achieved using physical (freeze/thaw or beads), chemical (SDS), or enzymatic (lysozyme) methods individually or in combination. After lysis, nucleic acids are purified using an organic solvent (phenol or chloroform) followed by precipitation using ethanol or isopropanol.

In contrast, indirect methods do not lyse the bacterial cell wall. Instead, the goal is to separate intact bacteria from soil. A variety of methods utilizing sonication, shaking, and centrifugation alone, or in combination, have been developed to achieve this goal. In addition, chemicals such as Chelex and SDS have been utilized to aid in the separation of bacteria from soil. After separation, intact bacteria can be plated, isolated, and examined for morphological differences. DNA can then be isolated from each colony followed by sequencing.

In a comparison of methods, direct methods are generally considered to result in a higher yield of nucleic acids and to be less biased (in terms of recovered bacterial biodiversity) than indirect methods. However, the nucleic acids that are recovered are often contaminated, contain eukaryotic nucleic acids, or are sheared, which can limit their utility. In comparison, although more time consuming, indirect methods result in the recovery of higher quality and a larger volume of nucleic acids (see Robe et al. 2003 for a more in-depth review of these methods).

After isolation of DNA, individual isolates require identification to family and genus. This is routinely accomplished utilizing Barcoding genes such as 16S. In many cases, isolates can be accurately identified utilizing a single gene but in some cases the results can be ambiguous and multiple genes need to be analyzed to conclusively identify unknown isolates (e.g., Janda & Abbott 2007; Rossi-Tamisier et al. 2015). With the focus of the present study on the potential stratification of the A horizon (topsoil), the decision was made that intact bacteria would be required in order to reanalyze isolates if they

proved inconclusive utilizing a single gene. Therefore, the indirect method of bacterial/soil separation was chosen to allow intact bacteria to be isolated in the event there was a need for reanalysis. The choice to use a mechanical, rather than chemical, method to separate bacteria from soil was made to reduce the chance that bacterial cells would be lysed. In a comparison of mechanical methods, a blender or rotating shaker method has proven to be the most efficient means of separating bacteria from soil (Lindahl & Bakken 1995). As our sample volume was small, we chose to use a variation of the rotating shaker method with an intermediate speed and time of centrifugation (e.g., Faegri et al. 1977). This procedure was followed with the realization that, most likely, the results would be biased. However, the goal of this study was not to discover all bacteria present in the A horizon, but instead to determine if the bacteria present at each site, and depth, in this horizon were similar or different in terms of percent abundance and species composition.

The Headwaters Sanctuary (HWS) is a unique 53-acre urban site located in San Antonio, Texas. The site is incredibly important to south central Texas as the Blue Hole, the source spring of the San Antonio River, is located in the Sanctuary (Peterson 2006). The HWS is classified as a riparian forest. However, the floral distribution is not that of a typical riparian forest. The primary reason is that the HWS was cleared and utilized as farmland until the mid-1960's. As a result, the floral diversity is only decades old and the current distribution of plant species resembles a woodland, rather than riparian, community (Peterson 2006). The UIW garden (UIWG) serves as a campus community garden. This site was chosen because it is managed i.e., new plants (e.g., tomatoes, *Solanum lycopersicum*, and cabbage, *Brassica oleracea*) are normally grown in the garden each semester although fertilizers are not routinely applied. Therefore, a comparison could be made between a managed site (UIWG) and an unmanaged site (HWS) to determine if the pattern of usage could play a role in bacterial biodiversity.

The A horizon is normally found at depths between 5-20 cm, depending upon soil type, and is generally considered to contain the highest biodiversity of soil microbes. In San Antonio, Texas, the soils are normally described as vertisols. This soil type has a high clay content and is prone to swelling and shrinkage based upon moisture level. In addition, this feature of vertisols often results in a lack of distinct soil horizons. In previous studies, the A horizon has been treated as a single, uniform layer (e.g., Goberna et al. 2016; Zhou et al. 2017). In order to test whether or not the A horizon should be treated as uniform layer, bacterial colonies, with unique morphologies, were isolated from two sites and depths generally considered to lie within the A horizon. These depths were chosen because they represent the upper and lower limits of the most conservative definition of the A horizon. After unique colonies were isolated, DNA was extracted and a segment of the 16S rRNA gene corresponding to hypervariable regions 3-4 was sequenced to identify the isolate to family and genus. In order to determine if there was stratification at these sites, the total number of colonies representing each taxon was determined for each depth at each site. Then, the individual depths were compared to determine if the Relative Abundance of each taxon (number of identified colonies of a single taxon divided by the total number of all colonies) was the same across, or if there is evidence of stratification between, depths.

MATERIALS & METHODS

Site Characteristics.–The organic layer (O horizon) was removed prior to soil collection and samples were collected at 1 cm and 4 cm depths below the soil surface from each site (UIWG 29.27953^{o}N, 98.27957^{o}W and (HWS 29.28284^{o}N, 98.28314^{o}W). Temperatures at both sites were monitored for approximately six weeks before and after collection. Overall, surface temperatures at both sites ranged from a high of 28^{o}C to a low of 12^{o}C. Temperatures at 1 cm at each site ranged from a high of 27^{o}C to a low of 13^{o}C while temperatures at

4 cm at each site ranged from a high of 26°C to a low of 13°C. The week prior to collection, the temperature variation between depths was no more than 1-1.5°C. On the day of sample collection, 3 March 2017, the temperatures at 1 cm and 4 cm, at both sites, were 23°C and 22°C respectively. For 2 weeks prior to collection, there was no rainfall so the soil at each site was drained, and dry and moisture levels should have been homogeneous between soil depths. *Cynodon dactylon* (bermudagrass) was the sole plant species identified at the HWS collection site. At the time of collection, there had been no plants growing in the UIWG for a period of ~6 mo.

Bacterial Extraction and Culture.–0.25 g of soil from each depth, and site, were mixed and combined with 1 mL of sterile distilled water in a 1.5 mL test tube. Samples were vortexed for 5 min to homogenize the soil/water mixture. Tubes were centrifuged for 4-5 min at 6,000xG to separate the soil particulate. Serial dilutions of the isolated supernatant were made at 1:100, 1:200, and 1:500. 250 µL of each dilution was plated, in triplicate, on nutrient agar (NA) plates containing 0.5% peptone, 0.3%, beef extract/yeast extract, 0.5% NaCl, and 1.5% agar. Plates were incubated for 12-16 hr at 37°C. Samples were then examined at 100x and 400x magnification under a dissecting microscope. Morphological data were recorded for each unique colony using standard morphological descriptions. Distinct colonies were isolated and grown overnight at 37°C in nutrient broth (NB) containing 0.5% peptone, 0.3%, beef extract/yeast extract, and 0.5% NaCl. Cultures were plated and grown a second time at 37°C on NA. Single colonies were isolated and grown in 2 mL of NB. Two 500 µL samples were preserved in glycerol and stored -80°C for future studies. The second mL was used for DNA isolation.

DNA Extraction, PCR, and PCR Clean-up.–Isolates were centrifuged at 10,000xG for 1 min to pellet bacteria. 200 µL of Quick Extract DNA Extraction buffer (Epicentre) was added to the bacterial pellet and the sample was vortexed until pellets dissolved. The resulting homogenate was incubated for 20 min at 65°C followed by an additional 20 min at 100°C.

For PCR, samples contained 1 µL of DNA (~250 ng/µL), 25 µL MangoMix™ (1.5 mM $MgCl_2$, MangoTaq™ DNA Polymerase, and 100 mM dideoxy Nucleotides), 1 µL of Forward primer (10 mM), 1 µL Reverse primer (10 mM), and 24 µL of water for a final reaction volume of 50 µL. Standard PCR conditions (35 cycles: 95° for 30 sec, 53° for 90 sec, 72° for 90 sec with a final extension at 72° for 5 min) were utilized to amplify a fragment of the 16S gene. Positive samples were purified using the Illustra ExoProStar PCR Purification Kit (GE Healthcare Life Sciences) containing shrimp Alkaline Phosphatase and Exonuclease I. Sequences were generated using both primers and standard protocols at the UT-Health DNA Core Facility, San Antonio, TX.

Family/Genus Identification.–Initially, all sequence reads were edited using Finch TV (Geospiza, Inc.) to ensure nucleotide positions were identified correctly. After verification, unknown sequences were compared to GenBank reference sequences to identify each isolate to family and genus. After identification, isolates identified as the same genus were aligned using Clustal X and reexamined using Finch TV to validate sequences (Thompson et al. 2003). Once a final sequence had been determined, unique isolates were reanalyzed in GenBank to validate preliminary identifications. To determine percent divergence between unique isolates, sequences were compared in MEGA7 using 3 models of DNA evolution: Jukes and Cantor, Kimura 2p, and Maximum Likelihood (Kumar et al. 2016).

Soil Analysis.–1 cm and 4 cm samples for soil testing were collected from the HWS and UIWG sites. Samples were sent to the Texas A&M AgriLife Extension Service Soil, Water and Forage Testing Laboratory, College Station, Texas. All samples were analyzed for the following nutrients: phosphorus, potassium, calcium, magnesium, sulfur, and sodium. In addition, pH, conductivity, and nitrate concentration were determined. Samples were also analyzed for percent sand, silt, and clay.

RESULTS

Seventy-eight unique colony morphologies were identified from our study sites. Examples of representative colony morphologies are described in Table 1. Of the 78 colonies, sequences were generated for 73 isolates (~93.6%). All unique isolates were resolved as members of the family Bacillaceae and tentatively identified as members of the genus *Bacillus*. Overall, isolates tentatively identified as *B. subtilis* were the most common representing 67 of 73 isolates. The other putative species recovered, *B. megaterium* and *B. cereus*, each comprised 3 of 73 isolates.

Differences in taxon composition between depths at each site are summarized in Table 2. When both depths are considered as one site, the average number of unique colonies is 12.5 in the UIWG and 26.5 in the HWS. At UIWG, the 1 cm depth resolved the largest number of morphological isolates (10) and total number of colonies (152) tentatively as *B. subtilis.* A similar result was recovered from the 4 cm depth. There was a second putative species identified at each depth, *B. megaterium* from 1 cm and *B. cereus* from 4 cm. As in the UIWG, both HWS depths resolve the largest number of isolates and total colonies putatively as *B. subtilis*. An additional species, *B. cereus*, was recovered from the 1 cm depth whereas two additional species, *B. cereus* and *B. megaterium*, were recovered from the 4 cm depth.

Table 3 summarizes the results as a percentage of the total number of isolates if the depths are considered as a single site or if they are separated into 1 cm and 4 cm depths. If both depths at a single site are considered, Species Richness is 3. However, Relative Abundance varied across site and depth (Table 3). For example, isolates tentatively identified as *B. subtilis* ranged from 62.16-96.97%. The only other putative species identified to comprise more than 25% of the total diversity at any site or depth was *B. cereus* at the HWS 1 cm depth. The only other putative species identified to comprise more than 25% of the total diversity at any site or depth was *B. cereus* at the HWS 1 cm depth.

Table 1. Morphological descriptions for 6 isolates from the UIWG 1 cm site illustrating the diverse colony morphologies that were recovered as the same putative species of bacteria.

Colony #	Size	Shape	Edge	Color	Species ID
KT-1	Small	Circular	Uniform	Gray	*B. subtilis*
KT-2	Medium	Circular	Filamentous	Gray	*B. subtilis*
KT-3	Small	Irregular	Filamentous	Light Gray	*B. subtilis*
KT-4	Punctiform	Circular	Filamentous	Cream	*B. subtilis*
KT-8	Small	Circular	Filamentous	Dark Gray	*B. subtilis*
KT-9	Punctiform	Irregular	Filamentous	Tan	*B. subtilis*

Table 2. The putative species identified from each site and depth at the UIWG and HWS sites. Total Isolates were calculated based upon the number of unique colonies putatively identified as each taxon multiplied by the dilution factor used to generate each serial dilution. DNS reflects colonies that could not be sequenced. DNS isolates were not utilized in the Total Isolates calculation. Abbreviations and species names follow those in text.

Site	Depth (cm)	Species	Total Isolates
UIWG	1	*B. subtilis*	6080
		B. megaterium	1560
		DNS	880
Total			7640
	4	*B. subtilis*	2560
		B. cereus	80
Total			2640
HWS	1	*B. subtilis*	3680
		B. cereus	2240
		DNS	480
Total			5920
	4	*B. subtilis*	11600
		B. megaterium	1520
		B. cereus	480
Total			13600

Table 3. The percentage of each putatively identified species as a percentage of the total if depths are considered as a single data entity (UIWGtot or HWStot) versus as separate data points (e.g., UIWG1 and UIWG4). Abbreviations and species names follow those in text.

Taxon	UIWG 1	UIWG 4	UIWGtot	HWS 1	HWS 4	HWStot
B. subtilis	79.58	96.97	84.05	62.16	85.29	78.28
B. megaterium	20.42	0	15.16	0	11.18	7.79
B. cereus	0	3.03	0.78	37.84	3.53	13.93

In order to determine if sites show a different pattern of Relative Abundance, data from each depth were combined and the sites compared. Results suggest that although Species Richness is the same, Relative Abundance differs between sites ($P_{0.05}$=4.9x10^{-5}, 2 *df*). When Relative Abundance at a single depth is compared to Relative Abundance at that site, differences are also evident. For example, the 1 cm and 4 cm HWS depths differ from what would be expected if depths were considered as a single entity ($P_{0.05}$=4.74x10^{-12}, 2 *df* and $P_{0.05}$=7.2x10^{-3}, 2 *df*, respectively). If depths at UIWG are compared to Relative Abundance across this site, the 4 cm depth is different ($P_{0.05}$=7.28x10^{-6}, 2 *df*); the 1 cm depth is not ($P_{0.05}$=0.243, 2 *df*).

The total number of colonies/gram of soil also differed between site and depth (Table 2). At the UIWG, there was a 65.4% decrease in total colonies/gram of soil between the 1 cm and 4 cm depths. In comparison, the 1 cm and 4 cm depths at the HWS show a 129.7% increase in total colonies/gram of soil. When the 2 sites are compared, the HWS showed an 89.9% increase in total colonies/gram of soil.

The three putative species *Bacillus subtilis*, *B. cereus*, and *B. megaterium* identified in this study each contained multiple haplotypes. *B. subtilis* resolved two haplotypes that differ by a single nucleotide. These haplotypes were found at both locations and depths. In total, 19 unique isolates resolved with the first haplotype and 48 unique isolates resolved with the second. The three isolates identified as *B. cereus* also contained two haplotypes. The isolates found at the UIWG 4 cm and HWS 1 cm depths were identical. The isolate found

Table 4. The change in nutrient concentration across measured depths at HWS (a) and UIWG (b) sites. The values are reported as mg/kg of dry weight soil. A negative sign in the % change column indicates a reduction between the 1 cm and 4 cm depths; a positive sign indicates an increase.

(a)	HWS1	HWS4	% Change
phosphorus	176	181	+2.841
nitrate	3	4	+33.333
potassium	703	684	-2.703
calcium	9292	8610	-7.340
magnesium	410	373	-9.024
sulfur	22	17	-22.727
sodium	8	8	n/a
pH	7.9	7.9	n/a

(b)	UIWG1	UIWG4	% Change
phosphorus	205	60	-70.732
nitrate	5	2	-60.000
potassium	415	519	+25.060
calcium	12274	14739	+20.083
magnesium	577	540	-6.412
sulfur	43	110	+155.814
sodium	15	15	n/a
pH	7.9	8.2	n/a

at HWS 4 cm depth differed from these by a single nucleotide. Two haplotypes were also found amongst the three isolates putatively identified as *B. megaterium*. The isolate recovered from UIWG 1 cm depth differed by a single nucleotide from two isolates recovered from the HWS 4 cm depth.

In the HWS, the pH at both depths was 7.9 indicative of a slightly alkaline soil. Phosphorus and nitrate were the only nutrients tested that increased in concentration between the 1 cm and 4 cm depths – all other nutrients decreased in concentration between depths (Table 4). The soil profile for the 1 cm depth was indicative of a sandy loam soil. The soil profile for the 4 cm depth was indicative of a loam soil. In the UIWG, three nutrients, potassium, calcium, and sulfur, increased in concentration between the 1 cm and 4 cm depths – all

other nutrients decreased in concentration between depths (Table 4). The pH at the 1 cm depth was 7.9, whereas the pH at the 4 cm depth was 8.2 indicative of a slightly alkaline soil. The 1 cm and 4 cm depths were both characterized as sandy loam soils.

DISCUSSION

Morphology is one of the primary methods used to identify microorganisms. Morphological characteristics such as shape, size, elevation, color, and border are routinely used to identify bacterial unknowns to family and genus (e.g., Souza et al. 2015). However, studies have demonstrated that environmental changes such as a nutrient stress, a decrease in O_2, or the introduction of antibiotics can alter colony morphology (Goerke et al. 2007; Souza et al. 2013). As a result, the identification of a bacterial unknown to family and genus can be dramatically impacted. In the present study, we recovered a diverse array of colony morphologies that were putatively identified as the same family and genus (Table 1). This suggests that growth conditions may not have been optimal and the morphological characteristics evident reflect environmental rather than genetic differences between isolates. Furthermore, these data reinforce the fact that morphology alone is not sufficient to identify bacterial unknowns and additional methods are required for accurate identification.

Perhaps, the most common method used to identify bacterial unknowns is the examination of overall sequence similarity (Vos 2011). A variety of protein coding and structural genes are commonly utilized including 16S, *rpoB*, *Gyra*, *GyrB*, and *SodA* (e.g., Reller et al. 2007). Of these genes, 16S has become the standard for several reasons including its occurrence in all Prokaryotes and the existence of multiple hypervariable regions (e.g., Choi et al. 1996; Van de Peer et al. 1996; Baker et al. 2003; Munson et al. 2004; Petti et al. 2005; Srinivasan et al. 2015). Of the 9-hypervariable regions contained in the gene, numerous studies suggest that regions 3, 4, & 5 are the most

informative for bacterial identification (e.g., Takahashi et al. 2014; Parada et al. 2015; Yang et al. 2016).

Based on the widespread usage of this gene, standardized criteria based on percent similarity have been proposed to define unknowns as unique taxa. For example if 2 isolates share <97% sequence similarity, they may represent unique taxa (Stackebrandt & Goebel 1994). As additional data has been collected this criterion has been altered and now limits of <98.7% sequence similarity have been proposed to delineate taxa (Stackebrandt & Ebers 2006). However in numerous instances, the 16S gene has been unable to resolve closely related species, or the percent divergence does not follow the proposed guidelines, but the unknowns represent valid species (e.g., Alexander et al. 2002; Janda & Abbott 2007; Rossi-Tamisier et al. 2015). Additionally, the percent similarity recovered when examining the 16S gene does not always correlate to genome diversity (e.g., Welch et al. 2002; Spencer et al. 2003; Wolfgang et al. 2003). For example, 16s diversity is often negligible between *E. coli* isolates, but genome similarity can be as low as 40% (Welch et al. 2002).

The unknowns identified in this study exceed the proposed criteria utilizing all 3 models of evolution suggesting they represent unique taxa. For example, isolates putatively identified as *B. subtilis* exhibit between 5.7-6.4% percent sequence divergence from the isolates identified as *B. cereus*. In addition, these isolates exhibit between 6.4-6.8% percent sequence divergence from the isolates identified as *B. megaterium*. Isolates putatively identified as *B. cereus* exhibit between 4.5-4.9% percent sequence divergence from isolates putatively identified as *B. megaterium*. If full-length 16S sequences are analyzed, the percent divergence is only 20-30% larger than what is identified with partial 16S sequences. This suggests that the partial sequences utilized in this study can provide the resolution required to distinguish between these isolates. Overall, the large percent divergence recovered between isolates suggests they likely represent distinct taxa, but additional data will be required to conclusively identify these isolates.

Of the species identified, *B*. *subtilis* is the most widely studied (Sonenshein et al. 2002). In an examination of *B*. *subtilis* isolates collected from desert soils, all isolates exhibited greater than 99.8% sequence similarity in the 16S gene. However after isolates were examined using RFLP's from 3 genes (*rpoB*, *polC*, and *gyrA*) distinct differences were observed suggesting 2 distinct groups, possibly subspecies, were contained among this group of isolates (Roberts & Cohan 1995; Nakamura et al. 1999). In this study, multiple, closely related, haplotypes were recovered. Based on present criteria, the level of diversity is not sufficient to propose additional species but could suggest that a higher level of biodiversity exists than can be recognized using the 16S gene alone. Moreover, the number of isolates that were identified with each unique haplotype suggests that this is not a sequencing error but instead reflects true genetic diversity at these sites.

The goal of this study was to analyze the bacterial diversity recovered at 2 depths within the A horizon. Therefore, the choice was made to use an indirect method of bacterial isolation given that these methods have a reduced risk of lysing, or destroying, bacterial cell walls. This is a critical factor in that the primary goal of this study was to identify unique isolates that could be identified to family and genus. If direct methods were utilized, the isolates could have been lysed and this would prevent the identification of isolates that were ambiguous utilizing only the 16S gene. As a result of this choice, bacterial cells may have remained coagulated to soil particles, as the means of separation utilized were not stringent enough to dislodge them. Furthermore, the speed of centrifugation and/or vortex times may not have been optimal to separate bacteria from soil resulting in inadequate separation. In addition, the contents of vertisol soils, e.g., clay, could have hampered the separation of bacteria from soil and resulted in the low level of biodiversity recovered in this study. The choice was made to utilize 37^{o}C as the incubation temperature given that the majority of morphological descriptions of bacteria utilize this as the standard temperature. The choice of incubation temperature could also have favored specific bacterial genera and resulted in decreased Species Richness. In total, this suggests that the methods

utilized may have been insufficient to separate and identify all bacterial isolates present in the A horizon. However, the goal of this study was not to isolate and identify all bacterial isolates, but instead to determine if there was a difference in bacterial biodiversity between soil depths i.e., stratification. To this extent, the procedures utilized did allow us to investigate the differences in Relative Abundance across depths and suggests there is a difference between the depths examined supporting our hypothesis that stratification exists within the A horizon.

With the exception of nitrate and sodium, all nutrients examined in the present study were recovered at, or above, concentrations (mg/kg of dry weight soil) that would require the addition of supplemental nutrients to support plant growth. As a result, the concentrations of the additional nutrients examined may exceed concentrations required for growth by many bacterial genera thereby inhibiting their ability to thrive. Overall, the nutrient concentrations at each site and depth depict very different soil chemistries (Table 4). In fact at these sites, with the exception of magnesium, all nutrients examined exhibit different trends e.g., calcium decreases in concentration between 1 cm and 4 cm at the HWS but increases in concentration between 1 cm and 4 cm at the UIWG (Table 4).

B. subtilis was the only taxa recovered from both sites and depths. At both sites, the overall percentage of this taxon increased between 1 cm and 4 cm (Table 3). This suggests that in spite of the fact that these sites exhibit different chemical compositions, the concentrations of the nutrients examined were not inhibitory to this taxon (Tables 3 and 4). However, the other recovered taxa suggest that the chemical composition could, in fact, have an impact on the ability of certain bacteria to thrive. For example at the HWS, the overall percentage of *B. megaterium* increased between depths whereas the overall percentage of *B. cereus* decreased between depths. The opposite trend is evident at the UIWG (Table 3). Overall, this suggests that the chemical composition of these sites could have an impact on bacterial growth and the lack of biodiversity evident in this study could have

resulted from nutrient concentrations that prevented, or inhibited, the growth of specific bacterial genera.

The lack of plant diversity at the UIWG suggests that the nutrients in the soil most likely had not been depleted by plants. However, the same cannot be said for the HWS. This difference in usage could also have played a role in the differences in species composition identified at these sites. In fact, the UIWG has a reduced microbial biomass suggesting that usage could have an impact on biodiversity. In addition, the alkaline pH identified at these localities could be selective and also prohibit the growth of specific bacterial genera . For example, if *Bacillus* species thrive, or outcompete other genera, in soils with an alkaline pH, the lack of bacterial diversity recovered in the present study may not be surprising. In total, these results suggest that additional factors, biotic and abiotic, must be playing a role in shaping the bacterial diversity present at these sites. Studies are currently underway utilizing alternative methods of bacterial extraction and growth conditions to determine if these results accurately reflect biodiversity at these sites or if these results are biased based upon the method of isolation and culture conditions utilized.

Overall, these data suggest Relative Abundance differs when depths are treated individually versus as a single entity (Table 3). For example, the HWS 1 cm depth shows an absence of *B. megaterium* whereas if the HWS is considered as a single entity *B. megaterium* comprises nearly 8% of the total biodiversity. A similar pattern regarding *B. megaterium* is evident in the UIWG. However, this pattern was not evident in all instances. A comparison between the UIWG 1 cm depth and the UIWG total did not exhibit a statistically significant deviation. Overall, these data suggest that stratification may exist within the A horizon (topsoil) but given the small sample size of this study more data from additional sites will be required to confirm these preliminary findings.

CONCLUSIONS

Soil provides a diverse array of habitat for microorganisms. In turn, microorganisms provide key nutrients and breakdown organic matter improving soil health. Due to the close association between soil and microbes, there have been a wide variety of studies directed towards understanding soil biodiversity. One of the fundamental issues in these studies is the method of separation of bacteria from soil. The conditions utilized, the growth media, and growth temperature can all be selective. As a result, the recovered biodiversity can be skewed towards bacteria that thrive under the chosen conditions and, as a result, not be a true reflection of biodiversity.

In the present study, we utilized a mechanical method of separation and standard bacterial growth conditions. These choices could have skewed the results towards bacterial genera that are favored under these conditions e.g., *Bacillus*. However, the present study was not designed to determine the overall distribution of bacteria instead the focus of this study was to determine if bacterial biodiversity is similar across soil depths or if stratification exists. Overall, our preliminary results suggest bacterial taxa due occur with different frequencies at the depths and sites examined indicating there is the potential for stratification within the A horizon. In addition, our results suggest that the chemical composition of the soil being examined could impact the biodiversity recovered. As a result, analyses focused on understanding the structure of any soil horizon should target multiple layers within that horizon, methods of extraction, and growth conditions in order to obtain a clearer picture of overall soil biodiversity.

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