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Preliminary Analyses of the Diversity of Soil Microbes on the Campus of the University of the Incarnate Word

Viridiana Wheeler
University of the Incarnate Word, vwheeler@student.uiwtx.edu

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PRELIMINARY ANALYSES OF THE DIVERSITY OF SOIL MICROBES ON THE CAMPUS OF THE UNIVERSITY OF THE INCARNATE WORD

by

VIRIDIANA WHEELER

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PRELIMINARY ANALYSES OF THE DIVERSITY OF SOIL MICROBES ON THE CAMPUS OF THE UNIVERSITY OF THE INCARNATE WORD

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University of the Incarnate Word, 2017

Soil samples were examined outside Bonilla Science Hall on the campus of the University of the Incarnate Word in San Antonio, Texas at three time points between the calendar years 2016 and 2017. These time points correspond to Fall, Winter, and Spring. Samples were taken at two topsoil depths, 1 cm and 4 cm, to determine if there is a difference in bacterial load or distribution across the timeframe examined.

Soil samples were diluted and plated on nutrient agar plates in order to identify unique colony morphologies. A total of 132 distinct morphological isolates were identified and sequenced. Of the 132 distinct colonies identified, sequences were generated for 95. These 95 colonies were found to represent 2 genera, *Bacillus* and *Arthrobacter*. The six unique *Bacillus* species identified among the isolates were: *subtilis*, *cereus*, *megaterium*, *niancini*, *pumilis*. A single unidentified species of *Arthrobacter* was also discovered.

B. subtilis and *B. cereus* were present for all time points and depths. *B. megaterium* was present at all time points and depths with the exception of time point 3.4. Upon comparison of 1cm and 4 cm depths at each time point, bacterial load was found to decrease by as much as 91%. If similar depths are compared across time points, bacterial load was also found to decrease with the exception of time point 1.4 vs. 2.4, or 3.4, which show an increase in bacterial load. In terms of bacterial diversity, the only comparison that demonstrated a similar distribution of

bacteria was a comparison between time points 1.1 and 1.4. Macronutrient concentration and soil temperature were sampled at time point 3 and found to differ across depths at this time point. This suggests that these factors play a role in the bacterial diversity present at this time point. Overall, the results of this study suggest that the topsoil is a dynamic environment and the distribution and load of bacteria can differ significantly across both depth and time.

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Insights into Bacterial Diversity

There are three domains used to classify living cells: Archaea, Bacteria, and Eukarya [6]. Each domain possesses unique features differentiating it from the others, however there are shared features among the domains [6]. For example in the Archaea, cells walls are composed of glycoproteins and lack peptidoglycan [6, 8]. This is in contrast to the cell walls of Bacteria that are composed of polysaccharides and peptidoglycan [6, 8]. Furthermore, the Archaea can be subdivided on their ability to thrive in certain, usually extreme, environments – normally conditions that would prevent growth of bacteria or eukaryotes [6, 8]. The Kingdoms within Eukarya are classified according a variety of other characteristics. For example, eukaryotes are classified as to how they obtain nutrients and whether, or not, they can use CO_2 as a source of carbon. [6, 18]. However, there are similarities between the domains. For example, both the archaea and the eukarya contain histone proteins and introns. In addition, antibiotics do not inhibit growth in either of these domains [6].

Bacteria are among the most diverse organisms on Earth. Bacteria are often classified using five distinct morphological features [6]. First, they can be classified depending on their mode of nutrition or how they metabolize resources [6]. There are four major nutritional modes found in bacteria: Autotroph, Heterotroph, Phototroph, and Chemotroph [12, 17]. Bacteria are classified as Autotrophs if they can produce their own food - normally by using inorganic compounds such as CO_2 [2]. Heterotrophic bacteria typically need organic sources of Carbon [2]. Phototrophic bacteria utilize the energy from sunlight by performing photosynthesis to create cellular energy [2]. Lastly, Chemotrophic bacteria require chemical energy to make ATP [2]. Bacteria can also be classified according to their ability to produce endospores, which are

resistant structures containing their genetic material i.e., chromosome [6]. Another feature widely used to classify bacteria is the source of motility, e.g., whether, or not, they contain flagella [6].

The most widely used features that are used to classify bacteria are shape and cell wall structure [6]. Bacteria exhibit a variety of morphologies and these alternate shapes are often used to classify them into Genera and/or species [11]. Bacterial shapes vary between cocci, spirilla, and bacilli [6]. Cocci are described as spherical shaped bacteria, bacilli as rod-shaped bacteria, and spirilla as elongated spiral-shaped bacteria [6, 19]. Having these different shapes allows bacteria to thrive in different environments due to their ability to cope with, and adapt to, specific environments via their shape [19]. These shapes also allow bacteria to cope with, and thrive in, specific environments due to issues related to nutrient access, cell division, attachment to surfaces, and the need to evade predators [20]. Therefore, shape not only allows bacteria to adapt to different environments, but plays a role is how they acquire nutrients, how they divide, and how they deal with predators [19].

Rod-shaped bacteria are among the most commonly found bacteria, in fact they are the first type of bacteria that is hypothesized to have evolved [19]. Examples of rod-shaped bacteria include common bacteria such as *Escherichia coli* (*E. coli*) and *Bacillus subtilis* (*B. subtilus*) [11]. The Rod-shape results from mutations that have been linked to envelope synthesis, which helps determine overall bacterial shape [11]. Although bacteria cannot be identified to genus or species by morphology alone, you can identify distinct colonies shapes under a microscope which can help determine whether, or not, you are examining different genera or species. As a result, it is often difficult to classify bacteria by morphology alone due to the limited number of morphologies and the fact that many of the morphological characteristics used to distinguish different genera are similar when examined visually [6].

As previously mentioned, bacteria can also be identified by their cell wall structure. These structural differences can be identified using gram-staining procedures. There are 2 basic cell wall structures in bacteria: gram positive (g+) and gram negative (g-). The difference between the 2 structures is that g+ bacteria contain a single lipid membrane surrounded by a cell wall that is composed of a thick layer of peptidoglycan. On the other hand, g- bacteria contain a thin layer of peptidoglycan between an inner and outer layer of the lipid membranes [4]. Therefore, it is easy to distinguish between g- and g+ bacteria when utilizing gram-staining techniques making it an effective technique for preliminary identification. However, as was evident with morphological analyses, cell wall structure is not an effective means to conclusively identify genera/species of bacteria.

Soil Bacteria

Beneficial, as well as harmful, bacteria are found in a wide variety of ecosystems. Bacteria are ubiquitous and humans encounter them on a daily basis. Bacteria are found in everything from playground equipment and cell phones, to the air we breathe. Bacteria are also present in the soil where children play, on their toys, as well as in basically every aspect of their daily lives. Therefore, it is necessary for children to have exposure to bacteria, as it will help in the development of the immune system [13].

How much is really known about the amount of bacteria children are exposed to when playing outside? For decades, scientists have been conducting research on soil microbes. However, most of these studies have focused on the surface layer, or topsoil, without investigating what lies beneath the surface layer of the soil. Topsoil is generally considered to be the upper 2 inches (5.1 cm) to 8 inches (20 cm) of soil [3]. To determine if the type of bacteria present is different at different soil depths, one must determine the bacterial load - the amount of

bacteria that is present in a certain location or on a specific object. The bacterial load in soil is a subject that is not very well understood and a relatively small amount of research has been conducted on this subject. Therefore, it is essential that research be done in this area in order to understand which microbes are present in the lower topsoil layers, as opposed to the entire topsoil layer, as differences in the number and type of bacteria present could have a significant impact on the soil and the organisms that thrive in this environment [15].

As mentioned previously, topsoil (the upper 2 to 8 inches of soil) has been shown to have high densities of microorganisms [3]. However, studies have not examined different depths within the topsoil, so very little is known about the stratification of this layer of soil. However, subsurface microbes are believed to play an important role in the formation of the topsoil, the health of the ecosystem, biogeochemistry, decomposition, as well as the maintenance of groundwater quality [10]. Subsurface microbes are believed to maintain soil structure as well as alleviate the degradation of organic material as well as carbon sequestration and nutrient cycling [4]. In general Soil microbes are thought to be directly linked to the maintenance of soil temperature and moisture, however there has not been adequate research conducted to support this hypothesis [4]. Soil microbes also trigger CO_2 production, or respiration, and significant changes in temperature or soil moisture could alter CO_2 production [16]. A study focused on determining the impact of soil respiration on global warming showed that initially respiration would decrease, but overall the effect would not be dramatic [16].

Climatological Factors that Influence Bacteria

Bacterial growth is influenced by a wide variety of climatological factors. For example, bacterial growth can be affected by soil and air temperature as well as precipitation, due to the fact that many bacteria are specific regarding the environmental conditions under which they will

thrive and reproduce [10]. Previous studies have shown that the daily temperature range of soil has a negative effect on microbial biomass as well as growth [10]. Apart from this, seasonal temperature changes in microbial dynamics along with nutrient transformation have a negative impact on water availability [10]. This is because water availability tends to promote microbial turnover along with soil organic matter decomposition and mineralization [10]. As mentioned previously very little research has been conducted to determine the relationship between temporal changes and soil microbes, thus it remains unclear as to how these abiotic factors affect microbial growth [4]. Therefore, it is important to determine the soil conditions at a specific location that is being targeted for the analysis of bacterial load [16]. For example, the sites one might examine could contain different levels or moisture, different extremes of temperature or different concentrations of macro/micronutrients all of which could inhibit, or stimulate, the growth of certain genera, or species, of bacteria.

Rationale for this Project

In this study, the differences in bacterial load at 2 soil depths were investigated in order to determine if there are any difference in the bacterial load and diversity between these depths. This investigation targeted a minimal depth of 1 cm and lower depth of 4 cm, the lower and upper limits of topsoil, in order to determine the presence and Load of bacterial genera and species in the upper soil layers. The present study focused on comparing 3 different time points fall (November), winter (January), and spring (March) to determine which genera were present and what effect soil depth had on the growth of microbes.

Morphological analysis was utilized to identify isolates with distinct morphologies. All isolates were found to exhibit unique morphologies were sequenced and identified to genus and species (if possible). Ultimately, we compared the 2 soil depths in order to gain a better

understanding of the microbes present at each soil depth. While conducting the experiment, we were able to identify the bacterial load present at each time point in order to determine if the bacterial composition is changing over time. The results of this study were then compared to a past study conducted at the same site, and at several of the same time points, utilizing pill bugs. This comparison allowed us to determine if the diversity of soil bacteria is changing, the diversity of bacteria on pill bugs is changing, or whether changes in both occur.

Expected Findings

In this study, we expect to identify differences in bacterial diversity across the time points examined. If the present study corroborates previous analyses, we would expect to identify a wider variety of bacteria in fall (November) vs. winter (January). We also expect to identify a wider variety of bacteria in the 1 cm layer of soil. We are expecting this result due to the possibility that 1 cm layer will be affected more by abiotic factors such as temperature and moisture. In addition, the 1 cm layer will be subject to increased anthropogenic disturbance when compared to the 4 cm level. Furthermore, we are expecting the bacterial load to be similar across the depths examined, although we do not expect the bacterial composition to be the same.

MATERIALS AND METHODS

Sample Collection

Soil samples were collected on the campus of the University of the Incarnate Word outside of Bonilla Science Hall (29° 27' 58.1256" N, 98° 27' 56.4084" W). Soil samples were taken at 2 distinct depths: 1 cm and 4 cm. Samples were taken at 3 distinct time points, the first in November, the second in January, and the third in March. Soil and surface temperatures were taken during February and April, during sampling time point 3, to determine the stochasticity of the site.

Determination of Bacterial Load

Immediately after soil samples were collected, bacteria were extracted from the sample. For extraction, soil samples were weighed to ~ 0.25g. Next, 1 mL of water was added to the sample. The samples were vortexed for approximately 5 minutes, followed by centrifugation for 5 minutes at a speed of 6,000 rpm in order to separate the soil from the bacterial sample. Next, the samples were serially diluted. The 1 cm samples were serially diluted using a 1:1 ratio utilizing 500 uL of H_2O and 500 uL of sample. The 4 cm samples were not diluted since we had an adequate number of colonies present when samples were plated. The samples were then transferred to a Nutrient agar plates. 200 uL of sample was pipetted on to each plate. Samples were plated in triplicate for each soil depth to ensure the validity of the results. All plates were incubated for 12-16 hours at 37°C. The total number of colonies growing on each plate was counted. Only plates containing between 30-300 colonies were used to determine overall bacterial load for that time point and soil depth. The bacterial load was calculated using the following formula: Colony forming units x Plating Factor x Dilution Factor x 4 = bacteria load. For example, the number of colonies on a plate is 78. Therefore, the formula used to determine

the bacterial load would be 78 x 5 (assuming a 200ul sample size) x 2 (assuming a 1:1 dilution) x 4 (to standardize to bacteria/gram of soil) = 3,120 bacteria/gram of soil. For each soil depth, the average bacterial load for the 3 replicates was determined and if the bacterial load was not statistically different, 1 sample was chosen to determine the overall bacterial load for that depth. This sample was then used in comparisons to other time points and depths.

After counting and identification of colony morphology, each distinct colony was isolated and grown in Nutrient broth for 12-16 hours at 37ºC. Following isolation in Nutrient broth, each individual colony was regrown on an individual Nutrient agar plate. Agar plates were incubated for 12-16 hours at 37°C. After successful growth, individual colonies were isolated from agar plates and grown in Nutrient broth for a second time. After incubation at 37ºC, samples were regrown on Nutrient agar plates for 12-16 hours at 37°C to ensure the isolation of individual colonies.

Polymerase Chain Reaction and Gene Sequencing

Overnight cultures for each unique colony were grown at 37ºC in 2 ml of Nutrient broth. After 16-18 hours, broth was transferred to a sterile 1.5 ml tube. Each tube was centrifuged at 9,000 RPM to pellet bacteria. This process was continued until the Nutrient broth was exhausted. Cell pellets were re-suspended in 175 ul of Quick Extract DNA Extraction Solution (Epicentre). The suspensions were incubated for 20 minutes at 65ºC. This incubation was followed by an additional incubation at 100ºC for 20 minutes. After incubation, samples were subjected to gel electrophoresis and DNA quality was assessed. All DNA samples were stored at -20ºC until needed.

Samples underwent PCR utilizing MangoMix™, containing 1.5 mM $MgCl_2$, DNA Polymerase, and dideoxy Nucleotides. Reverse primer 805 and Forward primer 337 were used to

amplify a 440bp segment of the 16s rRNA gene. For those samples that gave no results with this primer set, Forward primer 27 and Reverse primer 1492 were utilized. For each reaction, 5 ul of 805R primer, 5 ul of 337F primer, 14 ul of H2O, 1 ul of DNA and 25 ul of mango mix were combined. Standard PCR conditions (95ºC for 30 seconds, 53ºC for 1 minute and 72ºC for 1 minute and 30 seconds) were used to amplify the 16s rRNA gene. Samples underwent gel electrophoresis using the eGel system. Samples that demonstrated positive results were then purified using a standard shrimp Alkaline Phosphatase and Exonuclease I protocol. Samples were taken to UT-Health to generate a DNA sequence for each sample.

Sequence Analysis

DNA sequences obtained from UT-Health were initially analyzed using FinchTV to ensure the fidelity of all sequencing data. Once verified, the sequences were put into GenBank in order to identify the genus of the isolate - and species if possible. After the identification of each isolate, the isolates that were identified as belonging to the same genus and species of bacteria were compared using the following software: Clustal X [21] and MEGA [22]. Clustal X allowed the comparison of individual sequence reads to ensure the nucleotide differences that were previously identified were valid. After analysis of all sequences, those sequence found to be unique were analyzed in MEGA to determine the level of sequence divergence between all unique isolates.

Soil Chemistry

Approximately 500 grams of soil was sampled from each depth in order to determine whether the macronutrient concentration was different between our sample sites. After collection, samples were sent to the Texas A&M Agricultural Extension Service laboratory in College Station, Texas where they were assayed for the concentration of the following macronutrients:

Nitrate, Phosphorus, Potassium, Calcium, Magnesium, Sulfur, and Sodium. In addition, pH and Soil Conductivity were determined for each sample.

RESULTS

A total of 3 time points were sampled at soil depths of 1 cm and 4 cm. Samples were obtained outside of Bonilla Science Hall on the campus of the University of the Incarnate Word. Sampling time points correspond to fall (November 2016), winter (January 2107), and spring (March 2017). The area sampled is regularly maintained and transited and is therefore subject to a wide variety of anthropogenic disturbance.

Determination of Bacterial Load

At each time point, 2 distinct depths were examined to determine bacterial load. These examinations involved serial dilutions of 1:1 for the 1 cm samples, whereas 4 cm samples did not require dilution. All sites were examined in triplicate to ensure validity of the bacterial load calculation. All morphologies that were found to represent the same genus and species of bacteria, after DNA sequence analyses, were add together to obtain a final estimate for the bacterial load represented by each taxa at each depth. Analyses of replicates from each time point and sample did not reveal statistically significant differences. Therefore, a single replicate was chosen and used to determine differences in bacterial load and distribution. The results of these calculations are presented in Tables 1-6. The bacterial load was determined to be 3,680 bacteria/gram of soil (b/g) at time point 1 at 1 cm and 4,760 b/g at 4 cm. At time point 2, the bacterial load was determined to be 1,720 b/g at 1 cm and 1,740 b/g at 4 cm. Finally at time point 3, the bacterial load was determined to be 2,040 b/g at 1cm and 4,640 b/g at 4 cm. These data suggest that the total number of bacteria found at each time point and at each depth is different. Overall, these finding suggest that the number of bacteria is not similar across the time points examined and results in different distributions of bacteria at both time point and depth.

Morphological Differences

Tables 1-6 present the different colony morphologies present at each site during the 3 time points as well as for each depth. Overall, there were a wide variety of distinct morphologies identified. In fact, several colonies were identified as the same species despite obvious differences in morphology. For example, *B. cereus* presented as large, flat, and circular or punctiform, flat, and circular at time point 1.1 (time point 1, 1 cm depth). In contrast, *B*. *cereus* presents as large, flat, and filamentous, or moderate, flat, and filamentous, or punctiform, flat, and irregular at time point 1.4 (Tables 1 and 2). Tables 3-6 illustrate additional examples of species that present with different, and distinct, morphologies. Overall, no genus and species of bacteria was identified with a consistent morphology in terms of size, elevation, or form.

16S rRNA Sequencing

132 distinct bacterial isolates were examined during the course of this study. Primers 337 Forward and 805 Reverse were used to amplify a 440bp region of 16s rRNA gene in each isolate. While those were the primary primers used, primers 27 Forward and 1492 Reverse were used to amplify a 1460bp region – if the primary set of primers did not work. This primer set was chosen because it has been shown to be phylogenetically informative in previous bacterial studies. These primers amplify region II-IV of the 16s rRNA gene. Even with the utilization of primers covering a wider range of basepairs in the 16s gene, there were some isolates that had to be removed from the study due to a lack of useable sequence data. Overall, of the 132 samples analyzed in the present study, DNA sequences were generated for 97 (~ 74%) of these samples.

Six time points were sampled in this study with an average of 22 samples analyzed per time point. The most common genus of bacteria recovered was *Bacillus*. This genus was found at all time points and depths examined. Two species of *Bacillus*, *subtilis* and *cereus*, were present at all timepoints and depths (Table 7). A third species of *Bacillus*, *megaterium*, was found at 5 of 6

time points (Table 7). Two additional species of *Bacillus* were found at a single time point (Table 7). Finally, bacteria identified as *Arthrobacter* was found at both depths at a single time point (Table 7).

Of the samples where DNA sequences were generated, 93.7% were identified as member of the genus *Bacillus*. The most common species identified was *subtilis* representing 46, of 95, samples. The second most common identified species was *cereus* representing 27, of 95, samples. The third most common species was *megaterium* representing 14, of 95, samples. There were 2 additional *Bacillus* species, *niancini* and *pumilis*, identified from a single time point. All remaining samples (a total of 6) were identified as *Arthrobacter*.

After identification of samples to genus and species, all samples identified as the same species were examined for diversity between isolates. There were 3 isolates found to be unique amongst samples identified as *B. subtilis*. The first group corresponded to samples isolated during time points 1.1, 1.4, and 2.1. The second group of isolates was identified from time points 2.4, 3.1, and 3.4. There were no instances where an isolate from the first group was found at the same time as isolates identified from the second time point. Overall, the sequences from these groups differed in a single nucleotide, ~ a 0.3% sequence difference (Table 8). The third group was found only at time point number 3 at the 4 cm depth. These samples showed 3, ~ 0.8%, and 4 , ~1.1%, sequence differences from group 1 and 2, respectively (Table 8). The remaining species of *Bacillus* showed no nucleotide diversity between isolates based upon depth or time point. A similar result was found upon examination of the *Arthrobacter* isolates. Finally, the species recognized as *Bacillus* demonstrated significant levels of sequence diversity ranging between 3.5 and 7.5% (Table 8). Isolates recognized as *Arthrobacter* demonstrated even higher levels of sequence divergence ranging from 20.2 to 21% (Table 8). Figure 1 illustrates a

Maximum Parsimony analysis of the relationships amongst the unique isolates identified in this study.

Depth and Time Point Analyses

After all isolates were identified, the soil depth and time points were compared in order to determine if the distribution and bacterial load differed. The results of these analyses are presented in Tables 1-6 & 9. These results suggest that there is a reduction in bacterial load between time point 1 compared to time points 2 and 3 at both depths. At time points 2 and 3, there was a 112% and 166% increase in bacterial load between the 1 cm and 4 cm depths. If the depths are examined across time points, e.g., 1.1 vs. 2.1, no similar distribution can be found. Overall, the data suggest there is an increase in load at time point 3 but a decrease in load at time point 2. If the distribution of taxa is examined across time points or depth, the first time point is the sole comparison that indicated a similar distribution of taxa (Tables 10 and 11). The remaining comparisons suggest that no time point had a similar distribution of taxa at the depths or time points examined in this study (Tables 10 and 11).

Soil Chemistry

Analysis of the soil chemistry in the study area revealed that there were differences in macronutrients levels, as measured in parts per million (ppm), between the soil depths examined. Of the *seven* macronutrients examined, only Sodium, showed no change in concentration between depths. Three of the macronutrients examined showed an increase in concentration ranging from 20-156% (Table 12). In contrast, 3 macronutrients showed a decrease in concentration ranging from 6.5-71% (Table 12). Conductivity also differed between the depths with a 22.5% decrease in conductivity between the 1 and 4-cm depths indicating the 1 cm depth

contained higher levels of moisture. pH was similar between depths, 7.9 for 1c m and 8.2 for 4 cm, indicating an alkaline soil at both soil depths.

Soil Temperature

Figure 2, illustrates the changes in temperatures over the time frame examined. These time points correspond to time point 3. This data suggests there is variation in temperature between the depths examined and the soil surface. These data could be used to suggest that temperature does play a role in bacterial load and distribution and could in part explain the differences in these parameters evident at time point 3 (Figure 2 and Tables 5, 6, 10, and 11).

DISCUSSION

Bacterial survival is dependent on the correct nutrients being present in the environment. In addition, environmental conditions will have a dramatic impact on bacterial growth. The abundance of nutrients found in the environment will be affected by various abiotic factors including temperature, rainfall, and humidity and as a result the abundance of bacteria can change as these resources are altered. This suggests that there could be seasonal variation in the environment and in turn this could cause variation in the abundance and distribution of bacteria. In the present study, the primary focus was to investigate whether or not the number and distribution of bacteria change as depth or climatological factors change. In order to assess these factors and their importance in determining the abundance of bacteria, we chose to examine 3 time points representing Fall, Winter, and Spring.

Bacterial Load

The bacterial load differed significantly between time points and soil depths. In all comparisons, the distribution of taxa and the bacterial load differed between time point and depth. There was a decrease in bacterial load between time points 1 and 2 in both depths and an increase in bacterial load between time points 1 and 3 in both depths. In total, these results suggest that there is significant turnover in the distribution of bacteria in topsoil suggesting this environment is dynamic and in a constant state of flux. Furthermore, these data suggest that the topsoil is not a homogenous layer in terms of bacterial diversity across the time points examined. As a result, the diversity of bacteria found in a given environment could differ greatly across soil depth and time of year and depths that are considered topsoil could, and do, differ greatly in bacterial load and distribution.

Bacterial Morphology

The results of the morphological analyses conducted in the present study suggest that the bacterial species recovered do not have a consistent morphology. For example, *B. cereus* colonies were identified that present with the following morphologies: punctiform/flat/irregular or large/flat/filamentous. Moreover, *B. subtilis* colonies were identified that presented with the following morphologies: large/flat/filamentous or large/irregular/circular. This suggests that there is a significant amount of plasticity in this character and morphology may not be a reliable criterion for bacterial genus/species identification. Overall, this could lead to a large number of isolates being examined that were thought to be different, but were actually the same genus/species. This could also suggest that the conditions under which the bacteria were grown, 37ºC, were not optimal or the isolation techniques utilized need to be refined in order to distinguish between different soil bacteria isolates.

Overall, these results suggest that certain species of bacteria do not grow with a consistent morphology. These results suggest that using morphology alone is not the most reliable method to identify soil bacteria and other techniques should be utilized to identify soil bacteria. Lastly, there could also be bacteria that are in fact different species that are showing similar morphologies and would be missed if morphology were the sole method of identification.

Sequencing Analyses

In total, 132 isolates were examined in the present study. Of these, sequences were generated for 95 isolates using a primer set that amplified 440 bp of the 16s rRNA gene. The 95 isolates examined were found to represent 2 genera, *Bacillus* and *Arthrobacter*. There were at least 6 unique species identified, *B. subtilis*, *B. cereus*, *B. megaterium*, *B. pumilis*, *B. niancini*, and *Arthrobacter* ssp. Upon examination of the sequence diversity among isolates recognized as

the same species only *B*. *subtilis* showed any level of sequence divergence among its isolates. The level of sequence divergence was low, 1-4 bp, but could be used to suggest that we were recovering 3 different strains of *B*. *subtilis*. Interestingly, the first unique isolate was found only at time points 1.1, 1.4, and 2.1. The second unique isolate was found only at time points 2.4, 3.1, and 3.4. The third isolate was found at a single time point, 3.4. The isolates from the first 2 time points differed by a single nucleotide – 0.3%. This could be a sequencing error, but this is unlikely given the fact that 17 (1.1, 1.4, and 2.1) and 24 (2.4, 3.1, and 3.4) isolates were recovered from these time points. The third isolate was 4 nucleotides (1.1%) different from the first isolate and 3 nucleotides (0.8%) different from the second. In total while we do not advocate using percent sequence divergence to identify novel bacterial species, these results suggest that there may be as many as 3 different strains of *B*. *subtilis* present at this locality. The single nucleotide difference found between the first 2 *B*. *subtilis* isolates could result from a novel mutation occurring at the 4-cm depth that ultimately replaced the first isolate from the 1-cm depth at the third time point. This would strengthen the argument that this environment is dynamic and the bacteria found are subject to selective forces that require novel adaptations to ensure their survival. Studies are being planned to investigate this question further.

These results suggest that there is a low level of species diversity at the depths and time points examined in this study. The most widespread species found in the present study was *B*. *subtilis* (includes all 3 isolates). This species was found at all time points and represented 46 of 95 (48.4%) isolates. This species was also the most common at both depths representing 50% of the isolates identified from the 1 cm samples and 48% from the 4 cm samples. The next most common taxa identified in the present study was *B*. *cereus*. This species was also found at all time points and depths and represented 27 of 95 (28.4%) isolates. This species represented 21%

of the 1cm and 33% of the 4cm isolates. The third most common species identified was *B. megaterium*. This species was identified at all time points except 3.4. This species represented 18% of the 1-cm and 13% of the 4-cm isolates. Isolates recognized as *Arthrobacter* were the next most common isolate and represented 6/95 (6.3%) isolates. This species was found only at time points 1.1 and 1.4 and represented 6% of the 1-cm and 7% of the 4-cm isolates. The 2 remaining species of *Bacillus*, *pumilis* and *niancini*, were found only at time point 3.1 and represented 3% each of the 1-cm isolates. Overall, these results suggest that the environment examined is fairly homogeneous and contains only 1 to 2 dominant species at any given time point.

X^2 Analyses

In order to investigate these results further, the samples were examined using X^2 analyses to determine if there was a significant difference between bacterial distribution at each time point and depth. These results suggest that the bacterial load and distribution is not different between the 1 cm and 4 cm samples taken at time point 1. However, the remaining comparisons suggest that the bacterial load and distribution are statistically different between depths and time point. In total, these results suggest that the bacterial load and distribution can change in a very short time frame and that they can change quite dramatically. This suggests that although the environment examined contained a limited number of species, the distribution did change significantly. For example, at time point 1.1 *B. subtilis* represents 25% of the total bacterial load and is present with 4 other species. At time point 3.4, there is something dramatically different. *B. subtilis* now comprises 78% of the total bacterial load, but is present with only 1 other species.

Soil Chemistry and Temperature

The soil analysis was only conducted at the third time point, so comparisons could not be made across all time points examined in the present study. The relationships between soil

characteristics are likely to remain similar through time, however. These data suggest that there is a difference in soil chemistry between the depths examined. There was only a single macronutrient, Sodium, which did not show a difference in concentration. All other macronutrients exhibited differences in concentration. The most significant differences were evident in Nitrates, Phosphorus, and Sulfur. If these macronutrients are important to growth and survival of the bacteria recovered in the present study, this could help explain the difference in bacterial load and distribution evident between the 1 and 4 cm samples evident at the third time point. In addition, temperature readings were found to fluctuate between soil depths. This could also contribute to the change in bacterial load and distribution evident at time point 3.

CONCLUSIONS

The present study was designed to investigate the distribution of bacteria at 2 soil depths and 3 distinct time points. In addition to the isolation of bacteria, soil chemistry and temperature were examined to investigate whether these abiotic factors play a role in bacterial load and distribution. The results of this study suggest that there is a limited distribution of bacteria in the study area – the number of different species was similar across all depths and time points. However, there is a significant difference in bacterial load across depths and time points. *B. subtilis* was the most common bacteria recovered in the present study. This species represented 48.4% of the total isolates examined. However, this species was not always the most abundant taxa – at 2 time points, 1.1 and 2.4, *B. cereus* was the most abundant species. If all time points are examined, *B. subtilis* represents between 24 and 78% of the total bacterial diversity. A similar result is found in all taxa present at more than a single time point. In addition, all time point comparisons, other than 1.1 vs. 1.4, show a significant difference in species distribution. The present study also recovered a wide variety of morphologies that were identified as the same

genus, and species, of bacteria. This result suggests that morphology alone is not sufficient to determine the identity of isolates of soil bacteria. In addition, the chemistry of the soil was found to be significantly different between the depths examined. These differences, along with differences in temperature, could be a driving force for the differences that are evident in bacterial diversity at time point 3 in this study.

In total, the results of the present study suggest that both soil chemistry and temperature could play a critical role in determining the bacterial load and distribution for a given environment. Moreover, these results suggest that the environment examined is constantly changing and the distribution of bacteria can change significantly in short timeframe ~ 6-8 weeks. Upon comparison of these data with data collected from soil insects (pill bugs), there is a broader distribution of genera (9 vs. 2) and species (18 vs. 6) recovered from pill bugs compared to topsoil. In addition, all genera and species of bacteria present in topsoil were also isolated from pill bugs. In total, these results suggest that compared to the soil in which they live insects may harbor a unique bacterial fauna. Given the results of the present study, topsoil cannot be considered a uniform soil layer and if the bacterial load and distribution are to be determined multiple layers within topsoil should be examined to accurately access the number and distribution of bacteria at that site.

REFERENCES

1. Alhajouj, S. (2016). *Preliminary Examination of the Bacterial Diversity Harbored by the Pill Bug.* Master's thesis, University of the Incarnate Word, 2016. San Antonio, Texas.

2. Altidor, M. (2017). Nutritional Types of Bacteria. Retrieved from https://sciencing.com/nutritional-types-bacteria-2515.html

3. Bell, C., Mcintyre, N., Cox, S., Tissue, D., & Zak, J. (2008). Soil Microbial Responses to Temporal Variations of Moisture and Temperature in a Chihuahuan Desert Grassland. Microbial Ecology,56(1), 153-167. doi:10.1007/s00248-007-9333-z

4. Bell, C. W., Acosta-Martinez, V., Mcintyre, N. E., Cox, S., Tissue, D. T., & Zak, J. C. (2009). Linking Microbial Community Structure and Function to Seasonal Differences in Soil Moisture and Temperature in a Chihuahuan Desert Grassland. Microbial Ecology,58(4), 827-842. doi:10.1007/s00248-009-9529-5

5. Brown, L., Wolf, J. M., Prados-Rosales, R., & Casadevall, A. (2015). Through the wall: Extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. Nature Reviews Microbiology Nat Rev Micro, 13(10), 620-630. doi:10.1038/nrmicro3480

6. Edwards, G. I., Cimmino, M., & Edwards, G. I. (1997). Barrons AP biology: advanced placement test in biology. Hauppauge, NY: Barrons Educational Series.

7. Eilers, K. G., Debenport, S., Anderson, S., & Fierer, N. (2012). Digging deeper to find unique microbial communities: The strong effect of depth on the structure of bacterial and archaeal communities in soil.Soil Biology and Biochemistry, 50, 58-65. doi:10.1016/j.soilbio.2012.03.011

8. Eme, L., & Ford Doolittle, W. (2015). Primers: Archaea. *Current Biology*, *25*, R845-R875.

9. Fierer, N., Schimel, J. P., & Holden, P. A. (2003). Variations in microbial community composition through two soil depth profiles. Soil Biology and Biochemistry, 35(1), 167-176. doi:10.1016/s0038-0717(02)00251-1

10. Gestel, N. C., Dhungana, N., Tissue, D. T., & Zak, J. C. (2015). Seasonal microbial and nutrient responses during a 5-year reduction in the daily temperature range of soil in a Chihuahuan Desert ecosystem. Oecologia, 180(1), 265-277. doi:10.1007/s00442-015-3452-x

11. Jones, L. J., Carballido-López, R., & Errington, J. (2001). Control of Cell Shape in Bacteria. Cell, 104(6), 913-922. doi:10.1016/s0092-8674(01)00287-2

12. Madigan, Michael & Martinko, John. (1997). Brock Biology of Micro-Organisms. Prenticehall, Inc.

13. Mazmanian, S. K., Liu, C. H., Tzianabos, A. O., & Kasper, D. L. (2005). An Immunomodulatory Molecule of Symbiotic Bacteria Directs Maturation of the Host Immune System. *Cell, 122*(1), 107-118. doi:10.1016/j.cell.2005.05.007

14. Michel, H. M., & Williams, M. A. (2011). Soil Habitat and Horizon Properties Impact Bacterial Diversity and Composition. Soil Science Society of America Journal, 75(4), 1440. doi:10.2136/sssaj2010.0171

15. Probandt, D., Knittel, K., Tegetmeyer, H., Ahmerkamp, S., Holtappels, M., & Amann, R. (2017). Permeability shapes bacterial communities in sublittoral surface sediments. *Environmental Microbiology, 19*(4), 1584-1599.

16. Romero-Olivares, A. L., Allison, S. D., & Treseder, K. K. (2017). Soil microbes and their response to experimental warming over time: A meta-analysis of field studies. *Soil Biology and Biochemistry, 107*, 32-40.

17. Whittaker, R. H. (1969). New Concepts of Kingdoms of Organisms. *Science, 163*, 150-160. Retrieved August 13, 2017.

18. Woese, C. R., Kandler, O., & Wheelis, M. L. (1990). Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria, and Eucarya. *Proc. Nati. Acad. Sci. USA, 87*, 4576-4579. Retrieved August 08, 2017.

19. Young, K. D. (2007). Bacterial morphology: why have different shapes? *Current Opinion in Microbiology, 10*(6), 596-600. doi:https://doi.org/10.1016/j.mib.2007.09.009

20. Young, K. D. (2006). The Selective Value of Bacterial Shape. *Microbiology and Molecular Biology Reviews,70*(3), 660-703. doi:10.1128/mmbr.00001-06

21. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, 23, 2947-2948.

22. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets Kumar S, Stecher G, and Tamura K (2016). Molecular Biology and Evolution 33:1870-1874.

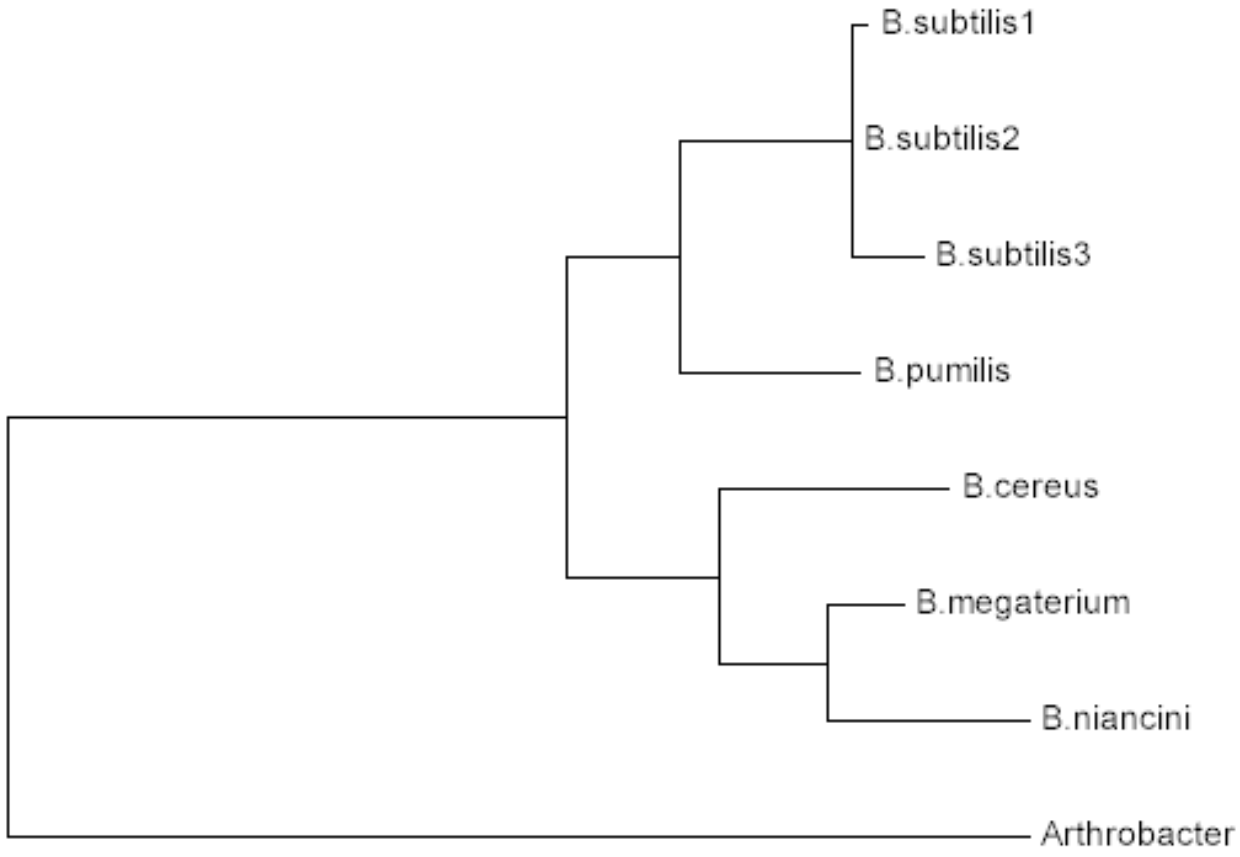

Figure 1. Maximum Parsimony analysis of the unique isolates of recovered in the present study. The phylogeny consists of 140 steps with a Consistency Index of 0.857.

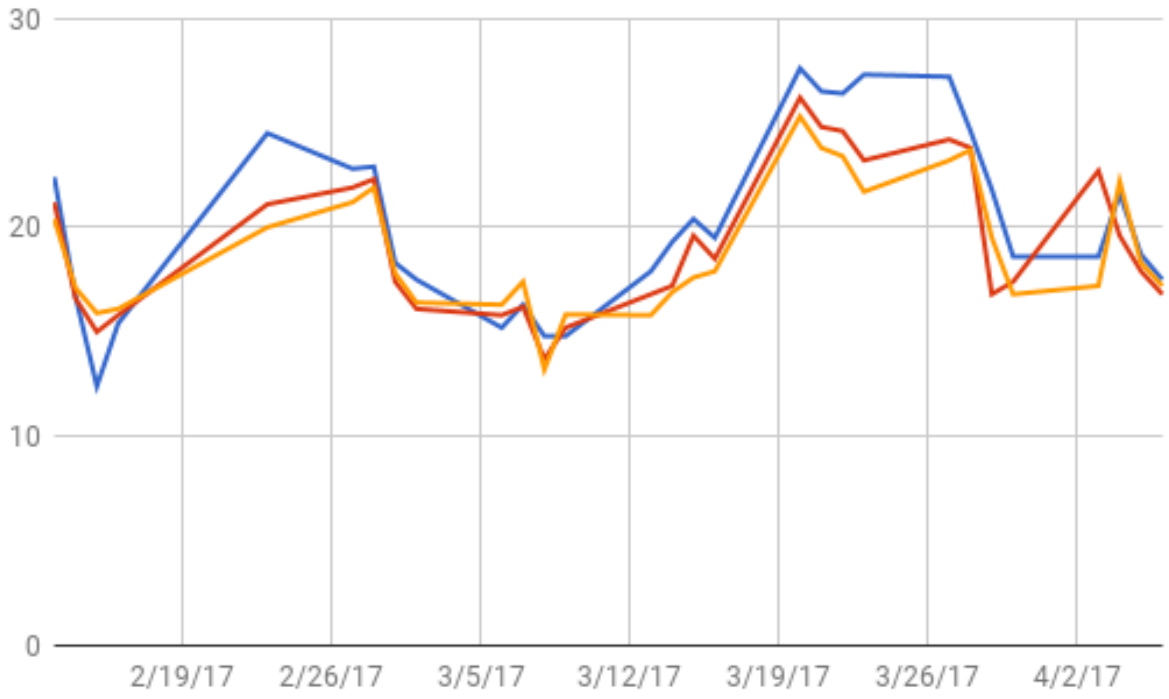

Figure 2. Temperature variation recorded from February through April of 2017. This timeframe corresponds to time point 3. All temperatures are reported in Celsius. The blue line represents the surface layer of the topsoil. The red line represents the 1-cm depth. The gold line represents the 4-cm depth.

Table 1. Morphology and colony count from bacteria collected at Bonilla Science Hall located on the University of the Incarnate Word Campus of San Antonio, Texas during the 1st time point examined during Fall 2016 at a depth of 1 cm. Bacterial load was determined using 1:1 dilution. Bacterial load was found to be 3,680 b/g. *B. cereus* was found with 2 distinct morphologies at this time point.

Colony	Size	Elevation	Form	Count	Total	Genus
1-1A	large	flat	circular	10	400	*B. cereus*
1-1B	moderate	flat	circular	11	440	*B. megaterium*
1-1C	punctiform	flat	circular	32	1280	*B. cereus*
1-1D	punctiform	flat	filamentous	15	600	*B. subtilis*
1-1E	small	flat	filamentous	14	560	*Anthrobacter*
1-1F	small	flat	filamentous	3	120	DNS
1-1G	moderate	flat	filamentous	5	200	DNS
1-1H	large	flat	filamentous	2	80	DNG
Total				92	3680	

Table 2. Morphology and colony count from bacteria collected at Bonilla Science Hall located on the University of the Incarnate Word Campus of San Antonio, Texas during the 1st time point examined during Fall 2016 at a depth of 4 cm. Bacterial load was determined using an undiluted sample. Bacterial load was found to be 4,760 b/g. *B. cereus* was found having 3 distinct morphologies whereas *B. subtilis* had 2 distinct morphologies at this time point.

Colony	Size	Elevation	Form	Count	Total	Genus
1-4A	large	flat	filamentous	1	20	*B. cereus*
1-4B	moderate	flat	filamentous	2	40	*B. cereus*
1-4C	punctiform	flat	irregular	70	1400	*B. cereus*
1-4D	small	flat	filamentous	4	80	*Anthrobacter*
1-4E	small	flat	filamentous	1	20	*B. subtilis*
1-4F	small	elevated	irregular	1	20	*B. megaterium*
1-4G	small	flat	irregular	5	100	*B. subtilis*
1-4H	large	flat	irregular	8	160	DNS
1-4I	moderate	flat	filamentous	41	820	DNG
1-4J	small	flat	filamentous	105	2100	DNG
Total				238	4760	

Table 3. Morphology and colony count from bacteria collected at Bonilla Science Hall located on the University of the Incarnate Word Campus of San Antonio, Texas during the 2nd time point during Winter 2016 at a depth of 1 cm. Bacterial load was determined using 1:1 dilution. Bacterial Load was determined to be 1,720 b/g. *B. cereus* and *B. subtilis* were found with 2 and 4 distinct morphologies, respectively, at this time point.

Colony	Size	Elevation	Form	Count	Total	Genus
2-1A	large	flat	filamentous	3	120	*B. subtilis*
2-1B	large	flat	filamentous	1	40	*B. cereus*
2-1C	moderate	flat	filamentous	11	440	*B. subtilis*
2-1D	moderate	flat	undulate	3	120	*B. cereus*
2-1E	small	flat	filamentous	8	320	*B. megaterium*
2-1F	small	flat	circular	3	120	*B. subtilis*
2-1G	punctiform	flat	irregular	5	200	*B. subtilis*
2-1H	punctiform	flat	filamentous	3	120	DNS
2-1I	large	flat	filamentous	2	80	DNS
2-1J	small	flat	filamentous	4	160	DNS
Total				34	1720	

Table 4. Morphology and colony count from bacteria collected at Bonilla Science Hall located on the University of the Incarnate Word Campus of San Antonio, Texas during the 2nd Time Point during Winter 2016 at a depth of 4 cm. Bacterial load was determined using undiluted samples. Bacterial load was found to be 1,740 b/g. *B. cereus, B. subtilis* and *B. megaterium* were found with 4, 2, and 2 distinct morphologies, respectively, at this time point.

Colony	Size	Elevation	Form	Count	Total	Genus
2-4A	large	flat	filamentous	1	20	*B. subtilis*
2-4B	large	flat	filamentous	1	20	*B. megaterium*
2-4C	large	flat	filamentous	1	20	*B. cereus*
2-4D	large	irregular	circular	3	60	*B. subtilis*
2-4E	moderate	flat	filamentous	8	160	*B. cereus*
2-4F	moderate	irregular	filamentous	3	60	*B. cereus*
2-4G	small	flat	filamentous	16	320	*B. megaterium*
2-4H	punctiform	flat	filamentous	51	1020	*B. cereus*
2-4I	large	flat	filamentous	3	60	*DNS*
Total				84	1740	

Table 5. Morphology and colony count from bacteria collected at Bonilla Science Hall located on the University of the Incarnate Word Campus of San Antonio, Texas during the 3rd Time Point during Spring 2017 at a depth of 1 cm. Bacterial load was determined using 1:1 dilution. Bacterial load was found to be 2,040 b/g. *B. cereus* and *B. subtilis* were found with 2 and 5 distinct morphologies, respectively, at this time point.

Colony	Size	Elevation	Form	Count	Total	Genus
3-1A	large	flat	filamentous	2	80	*B. subtilis*
3-1B	moderate	flat	filamentous	2	80	*B. cereus*
3-1C	moderate	flat	filamentous	1	40	*B. subtilis*
3-1D	moderate	flat	filamentous	2	80	*B. megaterium*
3-1E	moderate	flat	filamentous	1	40	*B. niancini*
3-1F	small	flat	irregular	3	120	*B. subtilis*
3-1G	small	flat	filamentous	3	120	*B. subtilis*
3-1H	small	flat	filamentous	1	40	B. cereus
3-1I	puncti	flat	filamentous	32	1280	B. subtilis
3-1J	small	flat	irregular	2	80	DNS
3-1K	large	flat	filamentous	2	80	DNS
3-1L	small	flat	filamentous	3	120	DNG
Total				47	2040	

Table 6. Morphology and colony count from bacteria collected at Bonilla Science Hall located on the University of the Incarnate Word Campus of San Antonio, Texas during the 3rd Time Point during Spring 2017 at a depth of 4 cm. Bacterial load was determined using undiluted samples. Bacterial load was found to be 4,620 b/g. *B. cereus* and *B. subtilis* were found with 3 and 6 distinct morphologies, respectively, at this time point.

Colony	Size	Elevation	Form	Count	Total	Genus
3-4A	large	flat	irreg	1	20	*B. subtilis*
3-4B	large	flat	filamentous	1	20	*B. subtilis*
3-4C	large	flat	filamentous	1	20	*B. cereus*
3-4D	moderate	flat	curled	10	200	*B. subtilis*
3-4E	moderate	flat	filamentous	2	40	*B. cereus*
3-4F	moderate	flat	filamentous	1	20	*B. subtilis*
3-4G	moderate	flat	irreg	1	20	*B. subtilis*
3-4H	small	flat	filamentous	2	40	*B. cereus*
3-4I	small	flat	filamentous	1	20	*B. subtilis*
3-4J	moderate	flat	filamentous	4	80	DNS
3-4K	puncti	flat	circular	140	2800	DNG
3-4L	small	flat	filamentous	34	680	DNG
3-4M	moderate	flat	filamentous	33	660	DNG
Total				231	4620	

Table 7. Presence, or absence, of each genera and species of bacteria identified in the present study. "B." refers to the genus *Bacillus*. "ssp" refers to an unidentified species. *B. subtilis* refers to each of the 3 isolates. X^1 refers to the first, X^2 the second, and X^3 the third isolate. 1.1-3.4 refers to individual time points and depth of that sample.

	1.1	1.4	2.1	2.4	3.1	3.4
B. subtilis	X^1	X^1	X^1	X^2	X^2	$X^{2, 3}$
B. cereus	X	X	X	X	X	X
B. megaterium	X	X	X	X	X	
B. niancini					X	
B. pumilis					X	
Arthrobacter ssp.	X	X				

Table 8. Percent sequence divergence found between unique isolates of bacteria identified in the present study. Subtilis 1-3 represent the 3 unique *B*. *subtilis* isolates identified. All calculations represent uncorrected-p distances.

	subtilis 1	*subtilis* 2	*subtilis* 3	*megaterium*	*niancini*	*pumilis*	*cereus*
subtilis 2	0.003						
subtilis 3	0.011	0.008					
megaterium	0.057	0.054	0.046				
niancini	0.054	0.057	0.049	0.030			
pumilis	0.038	0.035	0.043	0.065	0.075		
cereus	0.062	0.059	0.051	0.038	0.049	0.065	
Arthrobacter	0.208	0.205	0.210	0.205	0.208	0.202	0.202

Table 9. Comparison of Bacterial Load between depths at a single site and between the same depths at multiple time points. Comparisons in Bold represent an Increase in Bacterial Load between the samples.

Comparison	% Change in Bacterial Load
1.1 vs. 1.4	91% Reduction
2.1 vs. 2.4	50% Reduction
3.1 vs. 3.4	37.5% Reduction
1.1 vs. 2.1	71% Reduction
1.1 vs. 3.1	81% Reduction
2.1 vs. 3.1	33% Reduction
1.4 vs. 2.4	**60% Increase**
1.4 vs. 3.4	**33% Increase**
2.4 vs. 3.4	17% Reduction

Table 10. The percentage of the Bacterial Load found for each species at each time point. All values are standardized to 100% and do not represent actual counts for that species. “B” refers to the genus *Bacillus*. 1.1-3.4 refers to individual time points and depth of each sample.

	1.1	1.4	2.1	2.4	3.1	3.4
B. subtilis	25	35	60	24	55	78
B. cereus	38	29	13	48	18	22
B. megaterium	13	12	27	29	9	
B. niancini					9	
B. pumilis					9	
Arthrobacter ssp.	25	24				

Table 11. X^2 Analysis of the standardized data presented in Table 10. 1.1-3.4 refers to individual time points and depth of each sample. DF is the degrees of freedom for that analysis. NO indicates that the P-value was not significant at $P_{0.05}$. YES indicates a significant difference at $P_{0.05}$.

	DF	X^2 Value	$P_{0.05}$	Significance
1.1 vs 1.4	3	6.249	0.1001	*NO*
2.1 vs 2.4	2	79.659	5.038×10^{-18}	**YES**
3.1 vs 3.4	4	37.507	1.410×10^{-7}	**YES**
1.1 vs 2.1	3	105.524	1.008×10^{-27}	**YES**
1.1 vs 3.1	5	72.575	1×10^{-135}	**YES**
2.1 vs 3.1	4	55.843	2.200×10^{-11}	**YES**
1.4 vs 2.4	3	63.989	8.253×10^{-14}	**YES**
1.4 vs 3.4	3	90.518	1.695×10^{-19}	**YES**
2.4 vs 3.4	2	164.583	1.825×10^{-36}	**YES**

Table 12. Soil was analyzed at both the 1cm and 4cm depths to determine the macronutrient concentration. Concentration of macronutrients was measured in ppm. Results are shown as % difference – increase or decrease. NC = no change in concentration. Values in Bold represent an increase in concentration. Values with a "^" represent no change. Values with a "*" represent a decrease in concentration.

Nutrients	**1 cm**	**4 cm**	**% Difference**
Nitrogen	5	2	60*
Phosphorus	205	60	70*
Potassium	415	519	**25**
Calcium	12,274	14,739	**20**
Magnesium	577	540	6*
Sulfur	43	110	**156**
Sodium	15	15	0^
Conductivity	338	262	22.5*