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Title: Decaying Pig Body's Impact on Forest Soil Microbiota
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Purpose
The original purpose of our study was to determine the effects of different soil treatments on microbial diversity in crop fields; however, we did not obtain enough DNA to perform the analysis. Our revised purpose was to determine the long-term effects of a decaying pig body on forest soil microbiota using culture-independent analysis.

Methods
Each student extracted genomic DNA from a different soil sample. We used polymerase chain reaction (PCR) to amplify bacterial small subunit (SSU or 16S) ribosomal RNA (rRNA) genes from bacteria in the crop field soil. We purified the mixed amplicon, measured its quantity, and analyzed it using agarose gel electrophoresis. The purified mixed amplicon was shipped for sequence analysis using Illumina MiSeq technology. Nonmetric multidimensional scaling (MDS) was used to compare bacterial diversity relative to time.

We followed the procedures on pp. 91-110 of the laboratory manual with the following exceptions (1): Step B-1 on p. 94: We added 5uL of community DNA to one PCR reaction but added 1uL of community DNA into the other PCR reaction. Step E-1-2 on p. 96: Nanodrop was not used to determine concentration of the mixed amplicon. Step G on p. 98-107: We skipped these step and instead followed the directions in the document "unit 2 instructions to process MiSeq data.doc."

Results
We amplified the 16S rRNA genes from the bacteria present in the soil samples using PCR. We purified the mixed amplicons and shipped them for Illumina MiSeq sequence analysis. We then analyzed the biodiversity in the soil samples using the Bray-Curtis dissimilarity index and the Shannon Diversity index.

We used gel electrophoresis to determine the size of the 16S rRNA genes. Lane 1 contained 500ng of 100-bp DNA ladder. Lanes 2 and 3 each contained 5ug of 16S rRNA gene DNA; the band in lane 3 was more intense than the band in lane 2. Lanes 4 through 7 served as no-template control and had no observable bands (Figure 1).

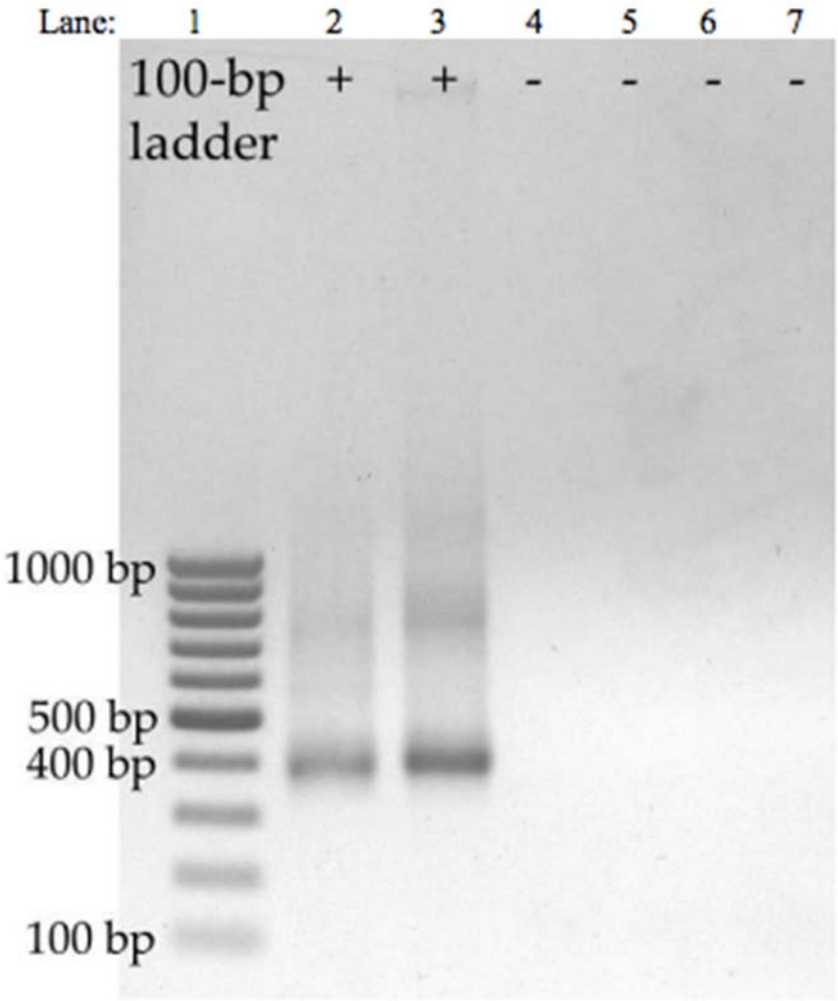

Figure 1: 2% Agarose gel of 16S rRNA genes from bacterial DNA isolated from soil microbial communities. Lane 1, 100-bp DNA ladder (500 ng); Lanes 2-3, contain purified 16S rRNA genes amplicons (5uL). Lanes 4-7, no-template controls (5ul).

We constructed a standard curve of the 100-bp DNA ladder to approximate the size of the 16S rRNA gene DNA. We compared the migration distances of the base-pair fragments of the DNA ladder against the migration distance of the 16S rRNA gene. The migration distance of the 16S rRNA gene was 10.6cm. Based on the standard curve plot the 16S rRNA genes are approximately 370bp in size.

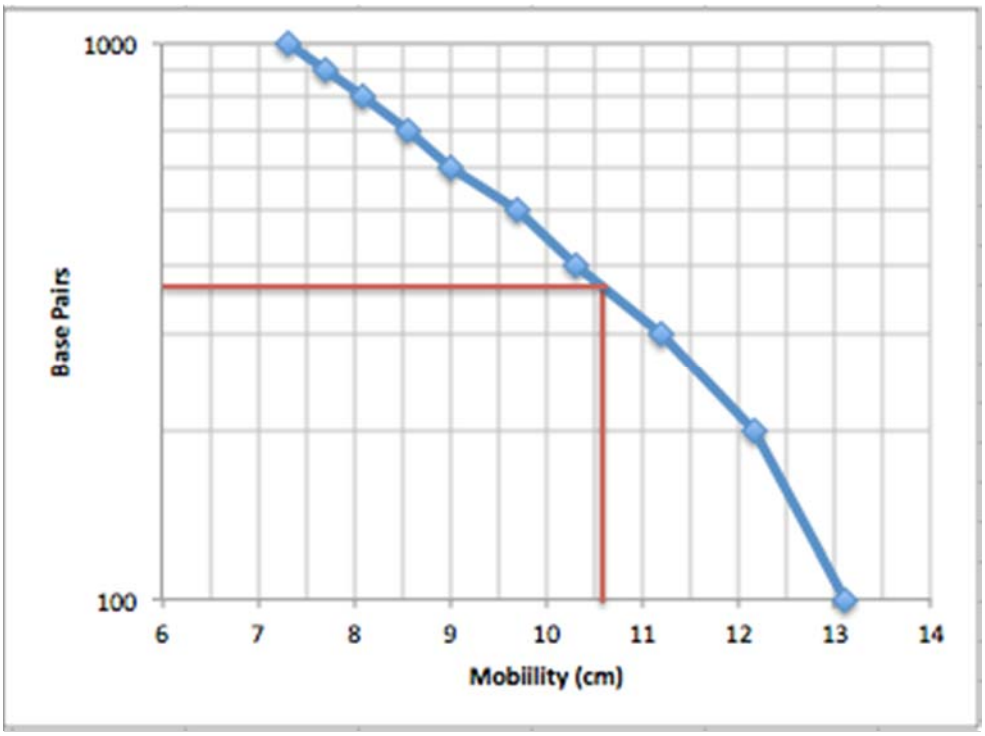

Figure 2. Standard curve of 100-bp DNA ladder molecular weight standard. The vertical red line indicates distance migrated by the 16S rRNA genes. The horizontal red line indicates the approximate base pair size of the 16S rRNA genes.

Central Services Lab required a qubit reading of 65 (2.6 ng/uL) to perform Illumina MiSeq analysis on the sample. The purified PCR product containing 5uL of community DNA had a qubit reading of 8.33 (0.333 ng/uL). The purified PCR product containing 1uL of community DNA had a qubit reading of 7.95 (0.318 ng/uL). Both purified amplicons mixtures did not contain enough DNA for sequencing. Instead we performed sequence analysis on data collected by Dr. Misty Weitzel and Larry Hodges, who were interested in analyzing the long-term effects of a decaying pig body on forest soil microbiota. Mothur computer program was used to align the sequences; the program removed 827 ambiguous sequences and 31,112 chimeras.

We determined microbial diversity by generating a Shannon diversity index for our samples. The Shannon diversity index measures species abundance and evenness. Higher values indicate greater microbial diversity. Microbial diversity was measured using samples collected 0, 0.5, 1, 6, 12, and 84 months after the pig body was left in the forest (Table 1). Microbial diversity was plotted and compared using a Bray-Curtis dissimilarity plot (Figures 3 and 4). The quantity of operational taxonomic units (OTUs) observed varied with time (Figure 5, 6, and 7).

Table 1. Shannon Diversity Index

Sample	Month	S	N	H'(loge)
3	0	6493	101652	5.90518180
6	0.5	5688	90600	5.75068356
11	1	4484	95969	5.29290920
14	6	6120	94510	5.87481691
19	12	1450	98518	4.36312397
23	84	3652	103161	5.25237408

S = total species, N = total individuals, H’ = Shannon diversity

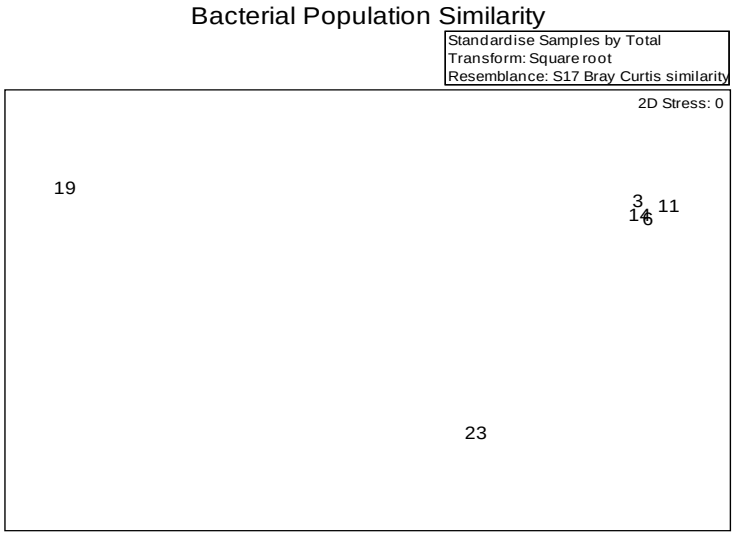

Figure 3. Bray-Curtis dissimilarity plot of microbial population resemblance.

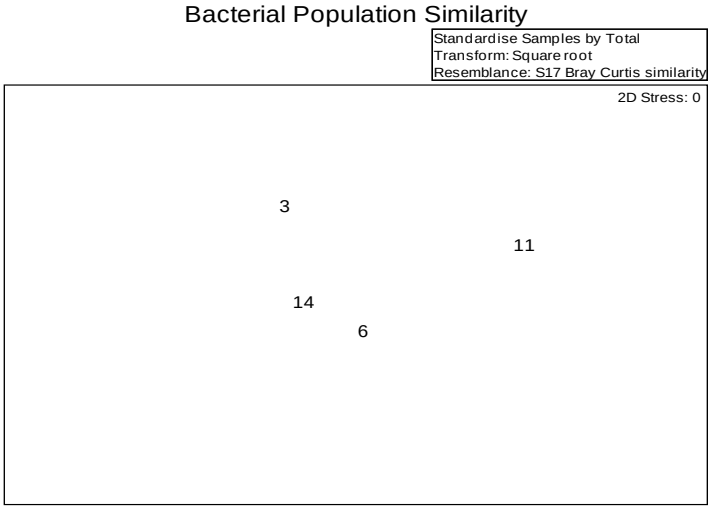

Figure 4. Cluster from Bray-Curtis dissimilarity plot enhanced.

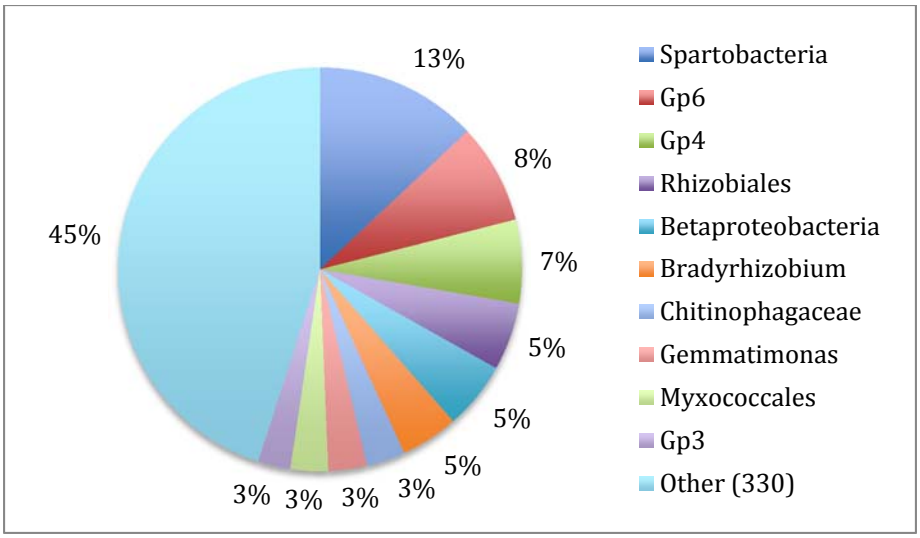

Figure 5. OTUs average from samples collected during the first six months.

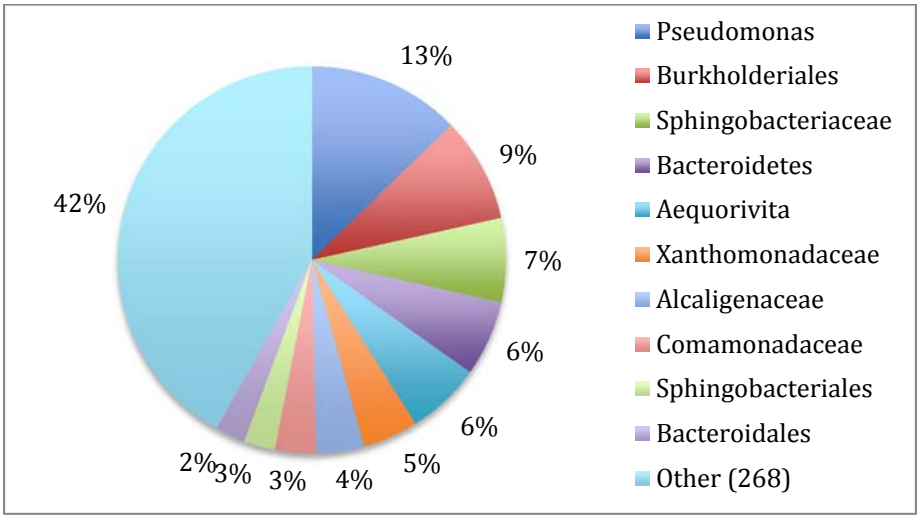

Figure 6. OTUs from sample collected after 12 months.

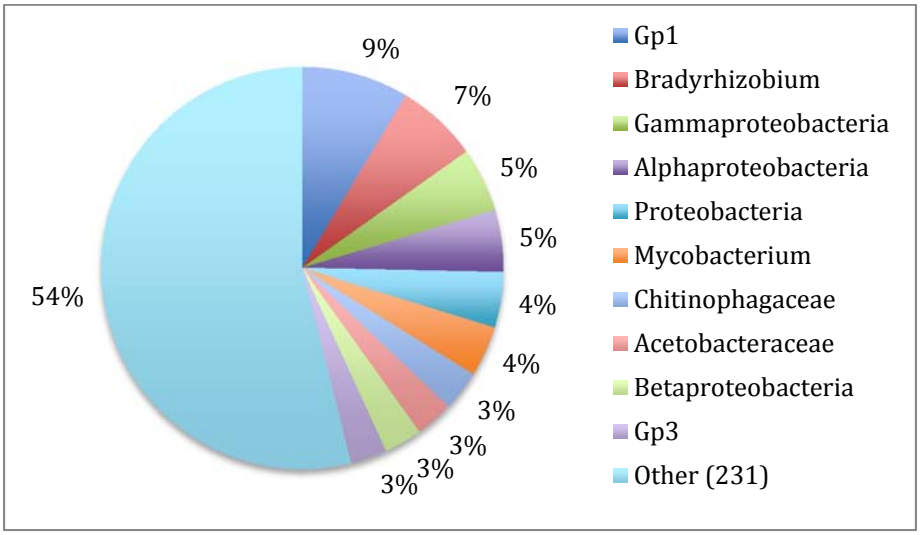

Figure 7. OTUs from sample collected after 84 months.

Discussion

The original purpose of our experiment was to analyze the effects of different soil treatments on microbial communities in crop fields; however, we changed the focus of our project after failing to produce enough DNA for sequencing. Our revised purpose was to determine the long-term effects of a decaying pig body on forest soil microbiota using a culture-independent analysis of the bacterial population. We amplified the DNA, purified the mixed amplicons, and shipped them for Illumina MiSeq sequence analysis. We then analyzed the soil samples using the Bray-Curtis dissimilarity index and the Shannon Diversity index.

We encountered an issue that prevented us from analyzing our original soil samples. The PCR primer we used to amplify the DNA did not work as expected. This primer was ineffective because it was either expired or it was not a good match for our sample. Our qubit readings were poor indicating low DNA concentration. Inadequate DNA impacted our ability to run a proper agarose gel and to sequence the purified amplicons from the soil samples.

We analyzed the agarose gel provided in the lab manual because our gels did not contain enough DNA. The amplicon has a base pair size of 370 based on the migration distance on the gel. This is within the expected size range for 16S rRNA genes.

Forest soil microbial diversity was measured by collecting samples within one meter of the pig body. Microbial diversity near the pig body changed over time according to the Shannon Biodiversity index and Bray-Curtis dissimilarity plot. The biodiversity readings from samples collected during the first six month period were clustered on the Bray-Curtis plot (Figure 4); they had similar Shannon diversity readings ranging from 5.29 to 5.91 (Figure 3). After 12 months the Shannon diversity reading dropped to 4.36, indicating a loss in microbial diversity. After 84 months the Shannon diversity reading increased to 5.25, indicating a partial recovery in soil microbial diversity; however, the soil sample from month 84 had less diversity compared to the original soil sample (Table 1).

The forest soil lost over 75% of its species diversity within one year of introducing the pig body. Initially there were 6,493 species present in the soil but after 12 months only 1,450 species were present. After 84 months 3,652 species were identified representing a 250% increase in total species compared to month 12 (Table 1). Analysis of unique species count supports the readings obtained from the Shannon diversity index.

Species dominance and presence in the forest soil changed during the study. Initially the dominant species was *Spartobacteria,* which composed 13% of the population. At month

12 *Pseudomonas* was the dominant species comprising 13% of the population. Finally, at 84 months *Gp1* was the dominant species comprising 9% of the population. In each sample the 10 dominant OTUs combined together were detected about as often as the remaining 200+ OTUs. The total number of OTUs consistently decreased during the study. Initially there were 340 OTUs detected on average during the first 6 months (Figure 5). After 12 months only 278 OTUs were detected (Figures 6). After 84 months only 241 OTUs were detected (Figure 7). The continuous decrease in OTUs appears to contradict the conclusions from the Shannon biodiversity index and Bray-Curtis plot. A possible explanation is that the Shannon diversity index does not group similar strains while OTUs group similar strains.

Future analysis of this study should explore the effect that the pig body has on the microbial biodiversity 10 meters away. Additionally, samples obtained at 24 and 72 months should be included in the analysis to accurately determine when the microbial diversity started to recover. Finally, additional samples should be collected at 96 and 108 months to determine if the recovery of microbial biodiversity continues. For similar studies in the future, researchers should collect samples before introducing the pig body because this will give a better picture of how soil microbiota changes over time. Furthermore, in future studies samples should be collected yearly to better understand the recovery process of microbial diversity.

Conclusion

The microbial diversity in the forest soil changed around the pig body. The biodiversity decreased after 12 months but signs of biodiversity recovery are present in samples taken at 84 months.

References

1. Muller, R., Ream, W. 2016. Molecular Microbiology Laboratory: A Writing-Intensive Course, 3rd edition. pp. 91-110.

Acknowledgements

I would also like to thank Xavier Gonzalez and Amanu Haile for helping edit this paper. Additionally, thank you to Ariel Liberda for demonstrating how to group OTUs. I would also like to thank the teaching assistants and Dr. Ream for providing us with the opportunity to conduct this experiment. Finally, I would like to thank Dr. Weitzel and Larry Hodges for providing the pig dirt data.

Questions

1. Can the approach used in experiment 2 detect dead bacteria?

Culture-independent analysis can detect dead bacteria, as long as the DNA is fairly intact. The analysis only requires DNA to be 250 base pairs in length. While we are able to detect more species than a culture-dependent approach, we also detected dead species that may no longer be present in the area. This impacts our understanding of the type of bacteria that are alive at different time periods.

2. Do the sequence counts for each species accurately reflect the bacterial population present in a sample? Explain.

The sequence counts do not provide a 100% accurate representation of the bacterial population in a sample. Some bacterial species amplify their DNA at higher than average rates during PCR, while other species amplify their DNA at lower than average rates. Additionally, species that are no longer alive in the area are included in our analysis because genomes from dead bacteria are sequenced. The major benefit of using culture-independent analysis is that 99% of the bacterial species present in a typical soil sample do not grow on laboratory media (culture-dependent analysis). Thus, culture-independent analysis provides a more accurate representation of the sequence counts than culture-dependent analysis.