Developing Reliable Methods for Microbial Fingerprinting of Soil Evidence: Collection, Contamination, Storage, and Analysis

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Draft Summary Overview

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Soil is a ubiquitous substance that can readily act as trace evidence when it is transferred from a crime scene onto an individual or object. Historically, soil has generally been treated as class evidence, based on factors such as pH, organic content, particle size, etc. However, the recent advent of advanced molecular techniques has allowed scientists to generate massive amounts of data from small samples of soil, so much so that it may be possible to individualize soil evidence, making it far more valuable for forensic investigations.

In our previous research on soil (NIJ 2013-R2-CX-K010) we showed that the bacterial makeup of soil samples, based on DNA sequence difference in the variable regions of the 16S rRNA gene, could be used to differentiate soils originating from different habitat types, as well as soils from similar habitats (deciduous woodlots) within close proximity of one another. The influence of time (soil collection over a one year period) was examined, as were small spatial scales within habitats. Finally, soils were placed on mock evidence items (clothing, tires, shovels), and samples were collected over time to look for *ex-situ* temporal changes in bacterial profiles.

Based on promising and informative results from those experiments, multiple subsequent studies were proposed, the results of which are summarized here. Several of these findings have been presented at scientific conferences[1], and in one peer-reviewed publication to date[2] with more are forthcoming. The new lines of research examined how the storage of evidentiary and

[1]Badgley and Foran Storage Conditions and Time Alter the Association of Known and Questioned Soil Evidence Derived via Next-Generation Bacterial DNA Profiles. The American Academy of Forensic Sciences Annual Meeting, Feb. 2017, New Orleans, LA;

Foran Developing Reliable Methods for Microbial Fingerprinting of Soil Evidence: Collection, Contamination, Storage, and Analysis. NIJ Research Symposium, Feb. 2018, Seattle, WA.

Heinz and Foran The Influence of Depth and Mixtures on the Bacterial Profiling of Soil Using Next Generation Sequencing. The American Academy of Forensic Sciences Annual Meeting, Feb. 2018, Seattle, WA

Heinz and Foran Exogenous Factors Affecting Bacterial Profiling of Soil on Clothing Via Next Generation Sequencing. The American Academy of Forensic Sciences Annual Meeting, Feb. 2018, Seattle, WA

[2]Badgley, Jesmok and Foran 2018 Time radically alters *ex-situ* evidentiary soil 16S bacterial profiles produced via next-generation sequencing. Journal of Forensic Sciences 63, 1356–1365 doi:10.1111/1556-4029.13753

known soils influence subsequent bacterial DNA profiles, how limited quantities of soil affect bacterial DNA profiles, how different depths of forensic soil samples—as might result from a burial—affect bacterial DNA profiles, how the human microbiome (i.e. skin) affects bacterial profiles obtained from soils on worn clothing, and how the presence of blood mixed with soil affects bacterial DNA profiles. The abridged methods, results, and conclusions from these experiments are given below.

General Methods

Soil samples were collected at different times of the year from a variety of habitats, including lawns, deciduous woods, coniferous woods, a dirt road, and agricultural fields. With the exception of the depth samples (described below) soils were collected from the surface to approximately 1 cm deep using a clean trowel, from 3 – 4 spots within an ca. 0.5 m^2 area at each habitat location (for greater detail on all general methods, see Badgley et al, 2018). DNA was extracted from soils using PowerSoil DNA Isolation Kits, and hypervariable regions 3 and 4 of the bacterial 16S rRNA gene were amplified using barcoded primers. Amplicons were sequenced on an Illumina MiSeq, and sequences were processed using the software mothur. Operational taxonomic units were binned at 97% sequence similarity, and the data were analyzed using visual (taxonomic abundance charts[3] and non-metric multidimensional scaling *(*NMDS)) and statistical (supervised classification) measures.

Soil Storage Conditions

We have previously shown that *ex-situ* soil bacterial DNA profiles can change over time (Badgley et al. 2018), meaning that profiles obtained from aged evidence could differ from

[3] Herein these charts are shown at the taxonomic class level, as displaying phyla loses too much information, while orders produce excessive noise. Note that these charts reflect the *relative* differences in bacterial makeup (i.e., the percent of the total profile each class represents), not necessarily increases/decreases in the constituents of any one class, although for simplicity those terms are used.

recently collected known soils even though they shared a common origin, and likewise that profiles from known soil samples could change depending on their length and method of storage prior to processing. Obviously the first of these is largely beyond the control of law enforcement/forensic examiners, at least until the evidence is identified and collected. In contrast, once in police/scientist's hands, the evidence or known soils could be stored in a variety of ways. To examine how this might influence subsequent bacterial profiles, fresh soil samples were collected in plastic bags and stored at room temperature (ca. 24°C), 4°C, -20°C, and -80°C, as well as removed from the plastic bag and stored at room temperature in weigh boats. Soils were then sub-sampled after one day, one week, one month, and two months of storage. As exemplified in Figure 1 (agricultural field), soils of all habitats stored in bags did not change appreciably from Day 0 through two months of sub-sampling. In contrast, when soils from the same habitat were stored in open weigh boats at room temperature, substantial temporal changes in bacterial profile were apparent (Figure 2), consistent with those described in Badgley et al.

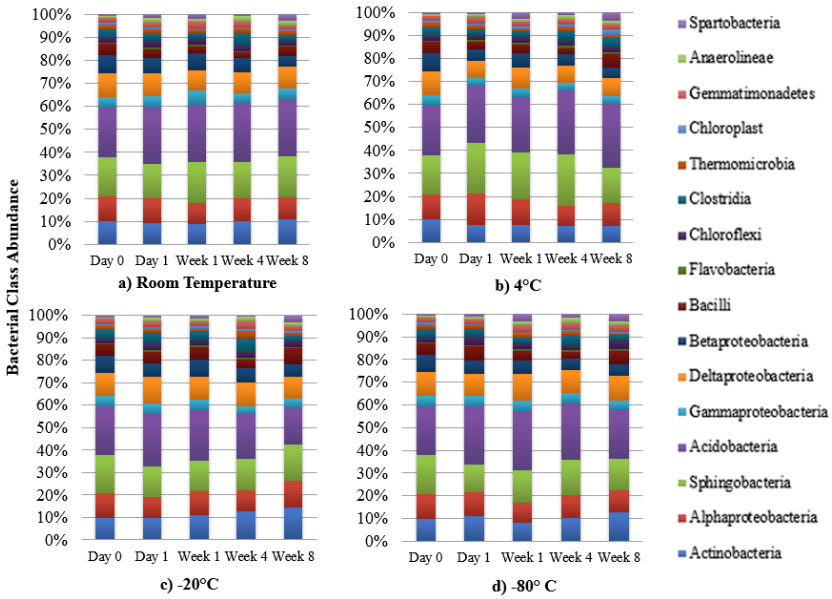

Figure 1. Bacterial class abundance from soils stored in plastic bags at different temperatures. Each color represents a different bacterial phylogenetic class, noted on the right, which also apply to Figure 2. The bacterial composition of soils did not change appreciably over time.[4]

[4] Figure from the Masters research of Alyssa Badgley. Available at: https://d.lib.msu.edu/etd/4185

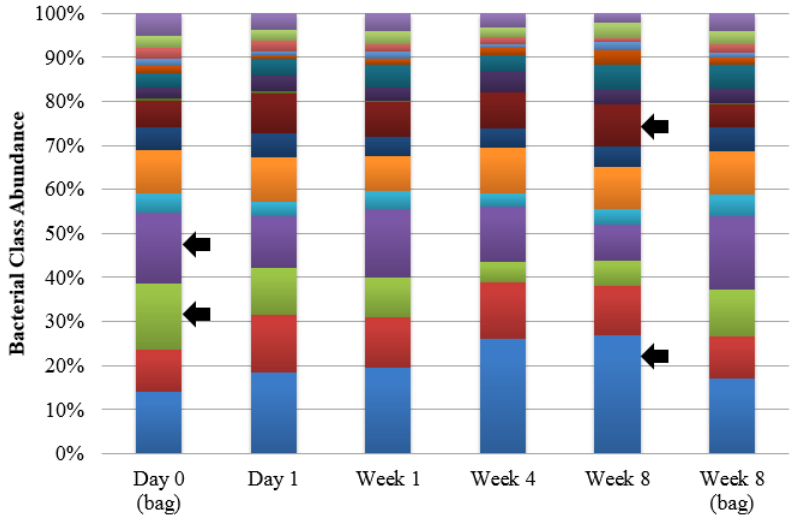

Figure 2. Bacterial class abundance changes from soils stored at room temperature. Soils were placed in open weigh boats on day zero. Similar to the changes detailed in Badgley et al. 2018, Actinobacteria and Bacilli (arrows in ascending order on the right) increased, and Sphingobacteria and Acidobacteria (arrows in ascending order on the left) decreased over eight weeks. The bacterial composition of Day 0 and Week 8 soils kept in the bag were similar.[5]

The results were confirmed using NMDS and statistical measures (data not shown). The upshot of this is that there must be careful consideration of how to store both retrieved evidentiary soils and known soil samples, such that the known soils are exposed to similar conditions for similar amounts of time as the evidentiary soils; simply freezing soils or storing them at room temperature upon collection could result in bacterial profiles that are artificially different from soils from the same location that were stored differently.

Soil Quantity

For most of our research on bacterial DNA profiling of soil, we collected excess soil from each habitat, and processed the 250 mg of soil that the manufacturer of the soil DNA isolation kit recommended. However, in a forensic setting it is quite possible that much less soil exists on an evidentiary item; for instance, as might be wiped on a piece of clothing. Given this, it was of interest to determine if the quantity of soil tested affected bacterial DNA profiling, and what level of soil is required to obtain consistent results.

[5] Figure from the Masters research of Alyssa Badgley. Available at: https://d.lib.msu.edu/etd/4185

Samples of 250, 100, 50, 25, 10, 5, and 1 mg of soil from different habitats were tested. The abundance charts for all habitats showed little internal difference based on the amount of input soil (data not shown), however NMDS plots indicated that at very small amounts of input soil (10 mg or less) the resultant profiles did not cluster as well with the known soils or higher amounts of input soils (Figure 3 for three habitats). When larger amounts of input DNA were used for PCR, the very small amounts of soil samples grouped closer to, or with the other samples (Figure 3), thus this problem was easily remedied by using slightly more input DNA.

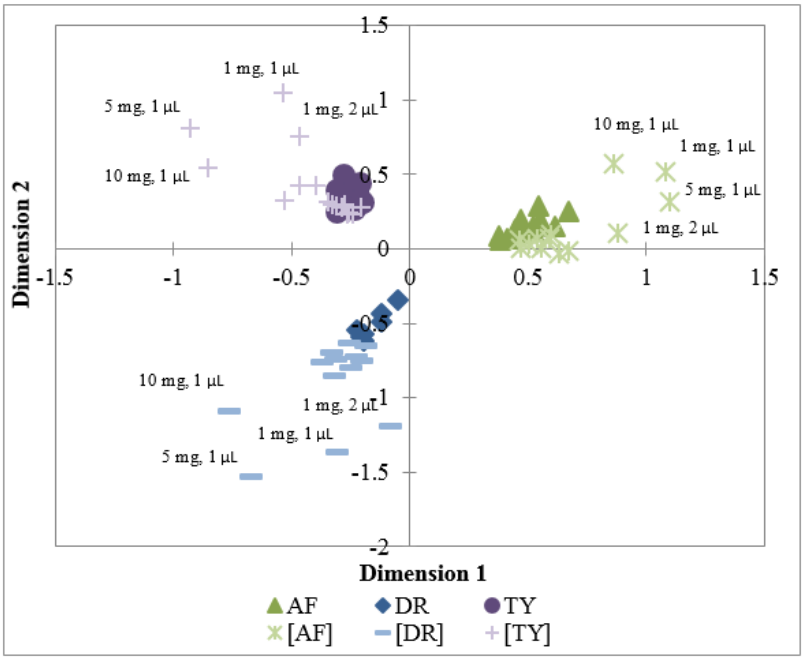

Figure 3. Habitat of origin ordination via NMDS of soils at different masses (line symbols) and known soils (geometric symbols). Profiles generated from the soils of different masses are denoted by brackets in the legend. The 10, 5, and 1 mg soils amplified with 1 µL DNA were less similar to the habitat of origin. Profiles from the same masses but amplified using 2 or 5 µL of DNA clustered more closely or with the knowns. AF = agricultural field, DR = dirt road, TY = (herbicide) treated yard.[6]

The Influence of Soil Depth

While most of our experiments utilized surface soils as they are the most likely to come into contact with an individual or evidence item, it is possible that under some circumstances, such as a burial, soil from below the surface comes into play. To examine this, corings were

[6] Figure from the Masters research of Alyssa Badgley. Available at: https://d.lib.msu.edu/etd/4185

taken at each habitat and samples collected at the surface and at depths of 5, 10, 20, 40 and 60 inches (earlier work showed little or no differences in bacterial profiles from 0 – 5 inches; data not shown), as well as mixtures of equal masses of soil from each depth, as a burial might include. Results for two of the habitats are displayed in Figure 4, both of which show differences with depth, most noticeably at 60 inches for the yard, where clay was struck. However, the mixed soils appear substantially like the surface material and not the deeper samples (confirmed via NMDS and statistical analyses; data not shown), indicating that surface soils contain far more bacteria than do deeper soils, thus bacteria from the former 'swamp out' those from the latter. Most importantly, these results indicate it is not necessary to collect deep samples in order to best represent a soil mixture from shallow and deeper depths.

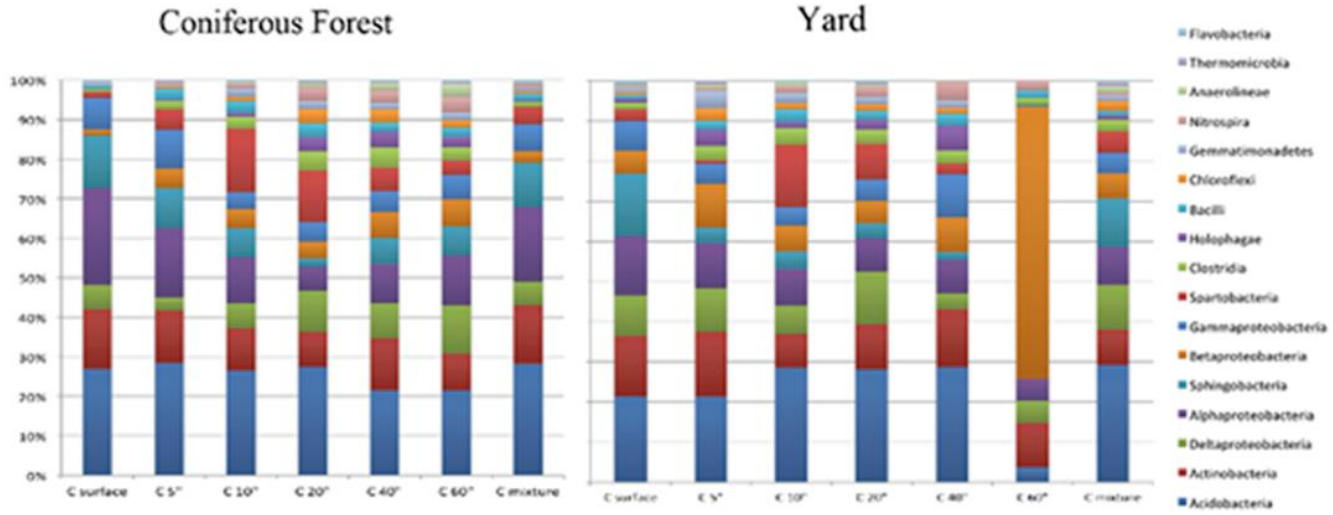

Figure 4. The influence of soil depth on bacterial profiles from two habitats, resulting from corings (C) from the surface to 60 inches (left to right). Note the changes in profiles with depth, particularly in the yard sample at 60 inches, which was clay. Markedly, mixtures of equal amounts of soil from each depth tested (far right for each habitat) most closely resembled the shallow samples (verified via NMDS and statistically).[7]

The Influence of the Human Skin Microbiome

A major focus of our research has been generation of bacterial DNA profiles from soil on evidence materials, including clothing. However, this clothing has been 'clean' (either new or

[7] Figure from the Masters research of Emily Heinz (unpublished).

laundered) prior to soil addition, thus there was little or no bacterial contribution from a wearer, as would be the case in a forensic setting. Based on this, 12 volunteers[8] wore new, lightly laundered t-shirts for 24 hours. Unworn and worn shirt samples were collected, and soil was then rubbed into the shirts, they were aged from 0 – 6 months, and ca. 1 cm^2 cuttings were taken monthly. Results in the form of an NMDS plot are displayed in Figure 5, where soils from the worn shirts always group with the known soils from the same habitat, and well away from the unworn and worn shirts without soils. These data show that the human skin microbiome has little or no influence on bacterial profiles obtained from soil on worn clothing, presumably because soil bacteria are so abundant that they 'swamp out' any results obtained from human bacteria.

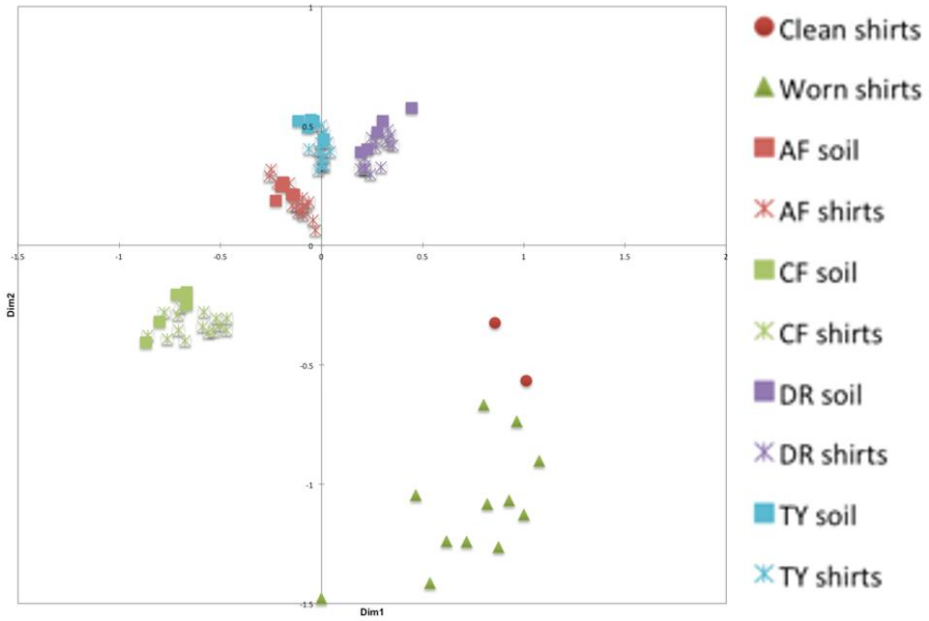

Figure 5. The influence of the human skin microbiome on soil samples collected from worn clothing based on an NMDS plot. Note that the unworn shirts (red circles) and worn shirts prior to soil addition (green triangles) cluster well away from the known soil samples and the soil samples placed on the worn shirts, and that the soils on worn shirts cluster tightly with the known soils from the same habitat. AF = agricultural field, CR = coniferous forest, DR = dirt road, TY = (herbicide) treated yard.[9]

[8] IRB approval was obtained for experiments involving human subjects.
[9] Figure from the Masters research of Emily Heinz (unpublished).

The Influence of Blood on Bacterial Profiles

It is not uncommon for blood to be associated with violent crimes. Given that blood has long been used as a growth medium for bacteria (e.g. 'blood agar'), we tested different mixture ratios of soils and fresh pig blood (10 to 1, 1 to 1, 1 to 10 by volume) placed on evidence items (shirts and trowels) and sampled over two months, in order to determine what effect the blood might have on bacterial profiles. The evidence items were allowed to dry in the open or stored in plastic bags. In the bags the 10 to 1 soil to blood samples dried quickly, the 1 to 1 samples were dry after a few days, while the 1 to 10 samples remained moist throughout the testing.

Exemplary bacterial profiles from these experiments are shown in Figure 6. As displayed on the left, the bacterial profiles from items that were stored in the open (dried) showed the same general changes in taxonomic class changes as did soils themselves (e.g., Figure 2). In stark contrast, the 1 to 1 and 1 to 10 soil to blood ratios stored in plastic bags (Figure 6 right) showed tremendous increases in Bacilli and Gammaproteobacteria (both of which are well represented in human disease, including blood-borne), with the former increasing almost immediately (see day 0 results), and the latter by the first week. Relative increases and decreases in other classes, quite different from *ex-situ* soils without blood, occurred as well.

Conclusions

The experiments and results described here show that different factors will need to be considered when utilizing bacterial 16S rRNA gene profiling for identification of soil, while other factors have little or no influence on bacterial profiles. Included in the latter is the human skin microbiome, which has no measurable effect on soil profiles, even though volunteers wore the shirts against their skin for 24 straight hours. It seems likely that even the small amounts of soil rubbed into the shirts contains such an abundance of bacteria that it fully overshadows

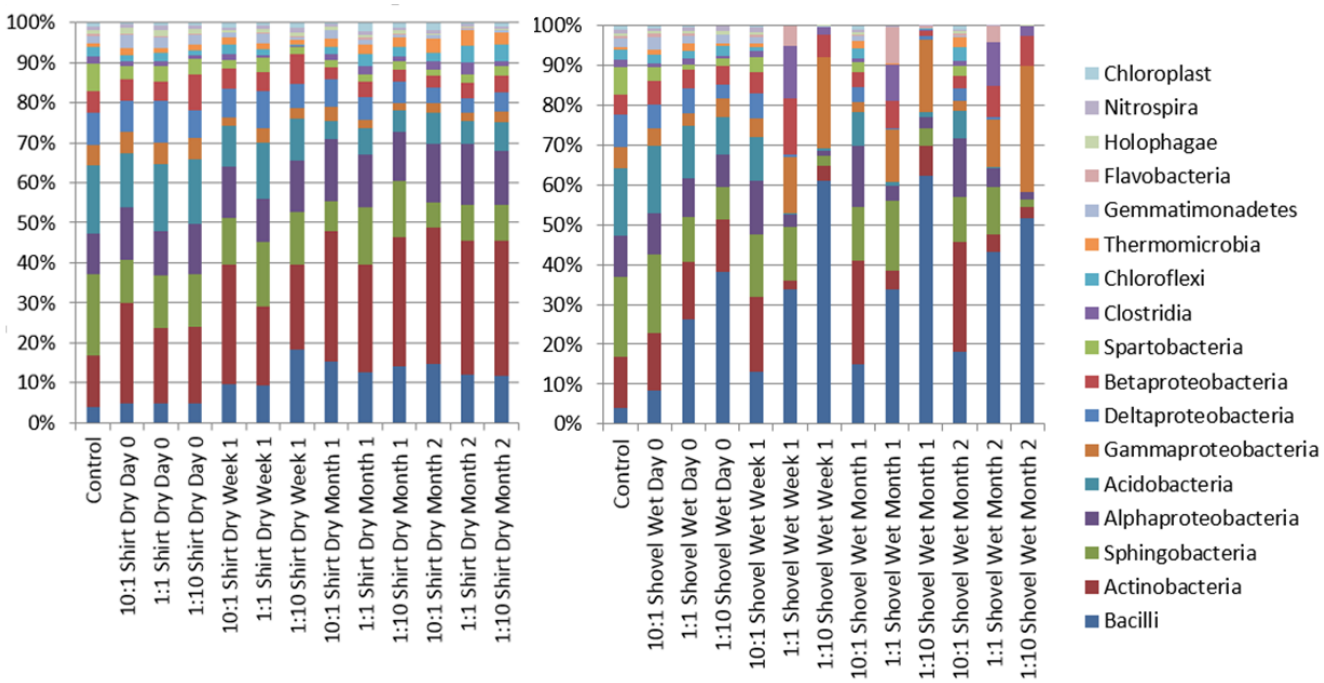

Figure 6. The influence of blood on soil DNA profiles. On the left are various ratios of soil:blood placed on shirts, and allowed to dry. The changes in bacterial classes are similar to those from other dried materials, as exemplified in Figure 2. In contrast, evidence stored in plastic bags ('wet', on the right) showed tremendous relative increases in Bacilli and Gammaproteobacteria. ('Shovel' = trowel)[10]

the skin contribution, which is clearly beneficial when attempting to profile soil from worn clothing. Similarly, while depth does influence the bacterial makeup of soil samples from different habitats, the bacterial profile from a mixture of soils from differing depth is so dominated by the (assumedly) much more abundant bacteria from the shallow soils, the mixed depth soils produce a profile most similar to the surface, meaning that in the event of a burial where depth soils are mixed, only surface knowns will come into play. Finally, when storing known soils or soils collected from evidence, the storage temperature, from room temperature to -80°C, has little or no influence on subsequent DNA profiles, as long as the *ex-situ* soil is stored such that it does not dry out.

[10] Figure from the Masters research of Emily Heinz (unpublished).

In contrast, soil that does dry out, which would likely be the case when existing on evidentiary material such as clothing, shoes, and the like, produces bacterial profiles that change temporally. This will need to be considered during an investigation involving forensic soil analysis, particularly for known samples, which will need to be treated in a way similar to how the *ex-situ* evidentiary soil existed. If that soil evidence was collected a day after the crime occurred and then stored properly, it will resemble the correct known soil collected subsequently. However, if the soil has existed on the evidence for a week or month or longer before it is collected, then known samples will need to be equivalently aged.[11]

Similarly, there is the chance that some tertiary factor, such as the blood tested here, could influence soil bacterial profiles. However, much as exogenous factors (e.g., mold, cleaning agents, UV) that can affect a standard forensic DNA profile do not somehow make that person's profile look like a different person's profile, exogenous factors that might affect a soil bacterial profile, such as the blood tested here, will either make a bacterial profile very dissimilar to any known soil profiles under consideration, or simply destroy it completely. Still, one must be cognizant of such artifacts, as they could lead to erroneous exclusions.

Taken together, the results from these studies help confirm the value of soil bacteria profiling for forensic investigations. As with any evidence, potential influences from exogenous factors that might affect findings or conclusions must be considered, examination of which was the exact goal of this research. The data produced here, in combination with our earlier research, indicate that with appropriate care and consideration, bacterial profiling of soil is a viable, rigorous, and worthwhile tool for forensic investigation.

[11] The manuscript 'Bacterial Profiling of Soil For Forensic Investigations: Consideration of *Ex Situ* Changes in Questioned and Known Soil Samples' was submitted to the Journal of Forensic Sciences' on 6-24-19.