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Postmortem Interval Estimation Based on Eukaryotic Community Associated with Soil Under Decomposing Porcine Remains

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Forensic Science at Virginia Commonwealth University.

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Abstract

POSTMORTEM INTERVAL ESTIMATION BASED ON EUKARYOTIC COMMUNITY ASSOCIATED WITH SOIL UNDER DECOMPOSING PORCINE REMAINS

By Amanda Haase, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Recent studies have shown that bacteria associated with soils under a carcass change significantly both temporally and spatially. These studies were done in both laboratory and field conditions, but with limited replications. In addition, limited information exists on temporal and spatial changes in eukaryotic community structure associated with soil under decomposing remains. This study was designed to fill in these gaps and expand on previous studies to improve postmortem interval (PMI) estimation techniques as well as to identify cadaver decomposition islands (CDI) based on eukaryotic community structure associated with soil under vertebrate remains. To accomplish this goal, soil samples were collected at 0 m (beneath the carcass) and 3 m away (control) from the porcine carcasses (N=6) on day 1 (T1/27 accumulated degree day (ADD)), day 2 (T2/57 ADD), day 26 (T26/734 ADD), day 33 (T33/930 ADD), day 40 (T40/1130 ADD), day 47 (T47/ 1326 ADD), day 54 (T54/ 1516 ADD), and Day 61 (T61/1703 ADD). The eukaryotic community for each sample was determined using 18S rDNA dual-index MiSeq sequencing on MiSeq FGx sequencing platform. Sequence data were analyzed using Mothur v1.39.5 and in RStudio. Results showed significant differences in eukaryotic community structure between soil collected under the carcass (0 m) and at 3 m away (control), but the same was not true for 0 m soil samples collected at different time points/ADD. The spatial difference in eukaryotic community composition was mainly due to decreased relative abundance of

Nematoda (e.g., Ascaridida and Araeolaimida) and increased relative abundances of Basidiomycota (e.g., Trichosporonaceae) and Ascomycota (e.g., Dipodascaceae). During initial period of decomposition (0-57 ADD), eukaryotic phylogenetic diversity did not differ much between soil under the carcass (i.e., 0 m) and the control site (i.e., 3 m away) but after 57 ADD, eukaryotic α-diversity declined sharply in soil under the carcass but remained unchanged in control soil. In conclusion, soil eukaryotes associated with decomposing porcine remains significantly differ spatially but not temporally. These results highlight the importance of the microbial eukaryote community during the process of decomposition and in the identification of CDI.

Keywords: forensic science, 18S rDNA, high throughput sequencing (HTS), CDI

Introduction

Soil ecology has many applications, and recent interest has been focused on the necrobiomic microbial communities in soils under decomposing carcasses (Hyde et al., 2013; Metcalf et al., 2013; Pechal et al., 2014, 2013). The effects of vertebrate carrion decomposition on soil and microbial ecology are not fully understood, and hence additional research in this area is needed (Singh et al., 2018). Benbow et al. (2013) defines the necrobiome as the community of both prokaryotic and eukaryotic species that are present on decomposing animals and human remains (Benbow et al., 2013). The surrounding soils of a carcass undergo chemical, biological, and geochemical changes due to the necrobiome, as microorganism communities change rapidly and measurably (Finley et al., 2015a). Microscopic postmortem evidence from the carcass, as well as the soil underneath it, may have potential importance in criminal investigations, such as providing postmortem interval (PMI) estimation and potentially identifying the location of decomposition sites (Finley et al., 2015b).

The postmortem interval (PMI), or time since death, can be estimated based on an analysis of a carcass' necrobiome. Environmental changes lead to a predictable response from microbial communities (Metcalf et al., 2017) and, therefore, decomposition processes may be correlated to PMI. Decomposition is a continuous process, but is often described as following five rough, non-rigid stages identified by the physical appearance of the carcass when exposed to insects (Galloway et al., 1989). Length of each stage varies among cadavers and environmental conditions. The fresh stage is the initial stage that follows the moment of death and proceeds until the body begins to bloat. The body is not discolored, and insect activity has not begun. The following stage is early decomposition, which is initiated by bacteria that create gas as a by-

product. This stage displays discoloration of the flesh as well as inflation of the body, and eventually, the bodily fluids are purged. The next phase is advanced decomposition where decomposition and insect activity increases. The later phase is skeletonization characterized by decreasing insect activity up until very little tissue is present. Over half the body had exposed bones. The final extreme decomposition stage is when bones begin to bleach and fragment into small pieces (Galloway et al., 1989).

Because the rate of decomposition depends on many biotic (e.g., insect activities, microbial activities, etc.) and abiotic (e.g., temperature, precipitation, etc.) factors, the stages cannot be strictly defined and relied upon (Galloway et al., 1989). To provide PMI estimates, a total body score (TBS) system is used to quantify the stages of decomposition (Megyesi et al., 2005). It involves the scoring of three regions, which include the head and neck, trunk, and limbs; lower values represent less decay, while higher values represent more advanced decay. The value for each body region is added to provide a score of the total body that equates to a rough stage of decomposition (Megyesi et al., 2005). Accumulated degree days (ADD), which combines thermal energy with time, is often used with TBS for PMI estimation. Specifically, ADD is the addition of thermal energy that is necessary for chemical and biological reactions during decomposition to occur (Simmons et al., 2010). Using ADD instead of, e.g. days, allows a more reliable comparison of decomposition studies in various geographical regions that may experience greatly different temperatures.

The decomposition process also affects the associated environment including soil. One study demonstrated that microbial communities within the soil underneath decomposing remains influence the rate of decomposition (Lauber et al., 2014). As leakage of fluids from the body occurs during decomposition, the necrobiome and nutrient-rich fluids are introduced to the

underlying soil, in turn affecting the soil's microbial community. The effects observed in the soil decrease as distance from the remains increases (Singh et al., 2018), and these effects include increased soil microbial biomass, microbial activity, and nematode abundance (Carter et al., 2009). Interestingly, Damann et al. (2012) conducted a study at the University of Tennessee Anthropology Research Facility to observe the long-term effects on continuous human decomposition on soil. It was found that the soil within the facility was similar regarding moisture content, pH, organic content, total carbon and nitrogen content, and biomass by lipid-bound phosphorus, and total extracted DNA, while it was unlike the soil outside of the facility that had not experienced human decomposition (Damann et al., 2012).

Whilst study of the necrobiome associated with decomposition is of interest, traditional culture-dependent techniques are unable to document the total community structure (Vass, 2001). When technology became more advanced, the exploration of molecular techniques to analyze the entirety of microbes present was initiated (Hyde et al., 2015). Microbial community diversity may be assessed by PCR amplification of the 16S rRNA gene (for bacteria), 18S rRNA gene (for eukaryotes), and the nuclear ribosomal internal transcribed spacer (ITS) for fungi (Schoch et al., 2012). Initially, genetic fingerprinting-based approaches (e.g., T-RFLP, RFLP etc.) were used for estimation of microbial diversity associated with an environment, but these approaches had several limitations (e.g., difficulty in microbial identification, inconsistent results in repeated studies etc.). The development of high throughput sequencing technologies (e.g., Roche 454 pyrosequencer, the Illumina HiSeq/MiSeq sequencer, the Ion PGM™/Ion Proton™ System, and the PacBio® RS II sequencing system) in the last decade has completely revolutionized the non-culture based microbial ecological studies. These high-throughput sequencing platforms have made it possible to analyze the changes that occur on and below

carrion during the decomposition process, in much greater detail and at much lower cost (Hyde et al., 2013a).

In recent years, the eukaryotic tree of life underwent several revisions, mainly because several taxonomic groups previously thought to be monophyletic are now clearly a non-monophyletic group (Adl et al., 2018, 2007, 2005; Burki, 2014; Tedersoo, 2017). Eukaryote names remain the same, but taxonomic relationships have been altered. The most current and commonly used reference file for classification of eukaryotes is SILVA NR v123. This utilizes a six-tiered system (Levels 1 to 6) that resembles the previous Domain to Genus taxonomic levels. UniEuk taxonomic framework for eukaryote sequence classification is utilized, which is made universal by combining and updating new research (Berney et al., 2017).

Studies assessing necrobiomes throughout the processes of decomposition to estimate PMI should ideally incorporate an adequate number of replications using a human model, or a model that is very similar to humans. It is important to also use or mimic natural field conditions and carry out the study for long-term decomposition. Limitations of previous studies include few replications (Pechal et al., 2014), the use of a model organism that didn't mimic human remains (Metcalf et al., 2013), carrying out decomposition in a lab setting (Metcalf et al., 2013), and studying a fraction of the decomposition process (Hyde et al., 2013b; Pechal et al., 2014). Specifically, mice are much smaller than humans, have more hair, and do not put the same amount of pressure on the soil as they weigh less. For soil studies, it is particularly important to consider all variables, including pressure, as it directly affects the soil. A porcine model has frequently been used as a substitute for human cadavers, as pigs have similar weight and hair distribution and, importantly, they are much more accessible than humans for research. Previous studies have used very few human cadavers (Metcalf et al., 2016), so the results cannot be used

to make general inferences about decomposition. Mice and rat models allow for a larger sample size, but are not very representative of the human model. Controlled field conditions are also ideal to incorporate naturally occurring temperature fluctuation, precipitation, insect access, etc., and long-term studies are preferred to allow PMI estimation over an extended period of decomposition.

Recent studies have applied next-generation sequencing of necrobiome communities to PMI estimation. For example, Pechal et al. (2014) studied bacterial communities associated with decomposition using a porcine model. Three replicates in anti-scavenging cages were examined over the course of five days. They found that the bacterial community structure changes over time/ADD, and bacterial community succession could be utilized for the estimation of PMI (Pechal et al., 2014). This study was relatively short term with little replication, and only bacterial communities from porcine remains were studied.

Pechal et al. (2013) published another study estimating PMI based on decomposition across seasons. A porcine model with six replicate carcasses was again used during spring and summer, while three replicate carcasses were used for the fall and winter. The buccal cavity, skin, and interior anal cavity were sampled every three days until the dry stage was reached. They found that microbial activity during decomposition increased during all seasons except autumn, and the community structure could be predicted per season (Pechal et al., 2013). Again, a small number of replicates were used, but both Pechal et al. studies initiated interest in the predictability of necrobiomes throughout decomposition. It is also important to note that both studies used anti-scavenging conditions, which would occur in natural field conditions.

Metcalf et al. (2013) used a mouse model in a laboratory setting to show that the necrobiome associated with decomposition can help assess PMI due to noticeable and repeated

changes within the community. This study examined a span of 48 days, used 40 replicates, and utilized next generation sequencing to obtain their data. Samples included the abdominal cavity, skin, and the soil beneath the remains. In this study, estimated PMI based on both bacterial and eukaryotic community succession, was within 3 days of actual PMI (Metcalf et al., 2013). Although there were many replicates, the major downfall was the study was performed in a laboratory setting (i.e. carrion didn't face field conditions such as insects and varying environmental conditions) and mouse carrion used in this study did not mimic humans very well as a model organism.

Hyde et al. (2013) performed a study on bacteria associated with the bloat stage of decomposition using two human cadavers in natural field conditions. Samples were taken at the beginning and end of the bloat stage from the mouth and anal cavities. They found that bacterial communities were different between the two cadavers, the two sample sites, and the time points of sample collection. Also, the communities became more similar between sample sites as decomposition progressed (Hyde et al., 2013a). Different body sites have their own native bacterial flora, and the increasing similarities through decomposition are likely due to the breakdown of tissue barriers in the body. For the results to have more impact, the sample number and cohort replicates need to be increased over the entire process of decomposition.

In another study, Hyde et al. (2015) again used two human cadavers to determine the change in bacterial community over time. They were placed in field conditions and samples were collected from the mouth, skin, and anal cavities over the course of decomposition. The cadavers were found to experience a change in the bacterial community throughout decomposition. They also decomposed at differing rates, which could be due to previous body conditions (physical, medical, cause of death, postmortem conditions). This resulted in inconsistent sampling events

for each corpse because the sample time points were the stages of decomposition identified by the researchers rather than, e.g. set ADD intervals (Hyde et al., 2015). Again, this study would have benefited from additional replicates so there would be a better representation of the normal population.

In 2016, Metcalf et al. studied microbial communities during decomposition using a human model while incorporating different soil substrates. Two replicate human cadavers were used in the winter and two more in the spring. The human study was performed outdoors in natural conditions and samples were taken from the skin and the soil associated with decomposition. High throughput sequencing (HTS) was used to characterize bacterial, eukaryotic, and fungal communities. They found that certain microbes appear at predictable times and that the communities mostly derive from the soil (Metcalf et al., 2016). Although human cadavers were utilized in this study, they were not well replicated. Also, the remains were placed too close to each other where contamination between cadavers is likely (Metcalf et al., 2016), so the impact of the study is not clear.

To begin to combat the limitations of previous studies, Woolf et al. (2016) performed a replicated field study with porcine remains over the course of 1703 ADD (60 days). Skin samples were collected from six porcine remains throughout decompositions and were assessed for bacterial community structure. Changes in the structure were seen throughout decomposition and the study provided additional evidence that bacterial succession can be used to estimate PMI. Forger et al. (2018) also used porcine skin samples collected from Woolf et al. (2016), but instead of bacterial succession, studied eukaryotic community succession and developed a statistical model that allowed PMI estimation within two days of true PMI.

This project builds on research performed by Woolf et al. (2016) and Forger et al. (2019) that utilized a porcine model in field conditions over an extended period (60 days) to fill in the gaps from previous studies. Site design, sample collection, and bacterial succession study on porcine skins for PMI estimation were performed previously by Woolf et al. (2016), whereas a study of eukaryotic succession associated porcine skins for PMI estimation were performed previously by Forger et al. (2019).

The main aim of this study is to determine eukaryotic community structure associated with soil under (0 m) and at 3 m away (control) from decomposing porcine remains for the prediction of PMI and the cadaver decomposition sites (CDS). The objectives of the study are:

1.) To determine eukaryotic community structure associated with soil under porcine remains and at control sites, using 18S rDNA high-throughput sequencing technology.

2.) To determine indicator eukaryotic taxa for postmortem interval based solely on eukaryotic structure associated with soil samples.

3.) To determine indicator eukaryotic taxa for carrion decomposition sites based solely on eukaryotic structure associated with soil samples.

Materials & Methods

The field experiment for carrion decomposition was conducted at Virginia Public Safety Training Center (VPSTC) and the Department of Corrections' AgriBusiness operation known as Pamunkey Farm, in Hanover, Virginia (approximately 37.70806°, -77.43444°). Twelve swine were euthanized by a gunshot wound to the head, weighed, and then immediately transported to the experimental site in individual 2 mm thick, 55-gallon heavy duty bags. An experimental

group with free insect access (ACC) was composed of six randomly selected carcasses (20.5-32.74 kg; 5 female and 1 male) of twelve total carcasses, whereas the other six were designated as insect exclusion remains. The six ACC carcasses were utilized in this study. The carcasses were laid on their left sides with snouts pointing eastward inside a specialized vertebrate scavenger exclusion cage (5' x 3.5'x 3'). The cages were made of hex mesh poultry netting around a 14-gauge welded wire fencing frame. The tops of all enclosures were covered with green shade cloth to prevent direct sunlight from shining on the remains. The enclosed remains were placed 10-15 m apart within an open field site as shown in Figure 1 (Woolf et al., 2016).

Total body score (TBS) and accumulated degree days calculation

The total body score (TBS) of each carcass was recorded using a modified Megyesi (Megyesi et al., 2005) total body scoring system (Moffatt et al., 2016). Each porcine carcass was regularly photographed to visually document the stages of decomposition. Ambient temperature (°C) was recorded every hour using a single Onset® HOBO® Water Temp Pro v2 U22-001 data logger and Onset® Waterproof Shuttle (Onset Computer Corporation, Bourne, MA) throughout the study. Accumulated degree days (ADD) for each timepoint were calculated using 0 °C as a base temperature. TBS data allow the correlation of ADD and the stages of decomposition.

Sample Collection

Soil samples were collected using 3-inch sections of sterilized iron pipe (electrical conduit) at 0 m (beneath or immediately adjacent to the carcass) and 3 m from each insect access porcine remains (n=6). At each distance for each time point, samples from three sites were collected and then combined. The sample collections started at T0 and were collected daily through T3. They were then collected every other day through T15 and once weekly through

T61. Because not all these sample collections were sequenced, this study focuses on only eight collection points: T1 (27 ADD), T2 (57 ADD), T26 (734 ADD), T33 (930 ADD), T40 (1130 ADD), T47 (1326 ADD), T54 (1516 ADD), and T61 (1703 ADD). Total 95 (n=47 for 0 m and n=48 for 3 m) soil samples were collected and utilized for this study. All soil samples used in this study were collected by Woolf et al. (2016).

DNA extraction

DNA was extracted from 0.25 g of soil sample using DNeasy PowerSoil Kit (Qiagen Inc., USA) following manufacturer's protocol. The final elution volume was 100 μL. The DNA extracts were stored in -80 °C freezer.

18S rDNA amplification & MiSeq® sequencing

The hypervariable region V9 of the 18S rRNA gene was amplified on an Applied Biosystems VeritiTM 96-Well Thermal Cycler (Thermo Fisher Scientific, Waltham, MA) utilizing the primers and protocol referred to by the Earth Microbiome Project (http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/18s/). The amplified product was sequenced using dual-index strategy referred to by Kozich et al. (2013) and using primers and protocol as described in Forger et al. (2019). Briefly, each primer included an Illumina® adapter sequence (5'-AATGATACGGCGACCACCGAGATCTACAC-3' for forward primer and 5'-CAAGCAGAAGACGGCATACGAGAT-3' for reverse primer), an 8 nucleotide index sequence, a 10 nucleotide pad sequence (5- TATCGCCGTT-3' for forward primer and 5'-AGTCAGTCAG-3' for reverse primer), a 2 nucleotide non-complimentary linker sequence (CG for forward primer and CA for reverse primer), and a 18S rRNA gene specific forward (V8_1391f: 5'- GTACACACCGCCCGTC-3' (Amaral-Zettler et al., 2009) and reverse

(V9_EukBr: 5'- TGATCCTTCTGCAGGTTCACCTAC -3' (Amaral-Zettler et al., 2009) primer. Each well of the 96-well PCR plate included 12.5 μL of Promega 2X PCR master mix (Promega, Madison, WI), 1 μL (5 μM) each of forward (Euk_1391f) and reverse (EukBr) primers, 4 μL (10 μM) of mammalian blocking primer (http://press.igsb.anl.gov/earthmicrobiome/protocols-and-stand- ards/18s/), 0.69 ng to 5 ng of template DNA (total DNA was quantified on an Invitrogen™ Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA)) and remaining volume of water for a final total volume of 25 μL. The 96-well PCR plate included a negative (nuclease free water, Promega Corporation, USA) control sample. Following amplification, all PCR products were visualized on a 1.6% agarose gel to ensure successful PCR amplification and that the PCR products were of expected size. Because majority of the samples had two bands, gel extractions were performed on all samples using the QIAquick Gel Extraction Kit (Qiagen, MD, USA) following the manufacturer's protocol. All PCR products were cleaned using Agencourt AMPure post-PCR purification system (Beckman Coulter, Brea, CA) using the manufacturer's protocol. Purified amplicons from each sample were pooled in equimolar concentrations (1 ng/μL). The pooled 18S rDNA library was sequenced for 2X250 paired-end sequencing-by-synthesis using Illumina MiSeq v2 Reagent Kit (MiSeq, Illumina, San Diego, CA) on Illumina's MiSeq FGx (MiSeq, Illumina, San Diego, CA) sequencing system using the manufacturer's protocol.

Data analysis

The raw sequence data were analyzed using MiSeq SOP (Kozich et al., 2013) (http://www.mothur.org/wiki/MiSeq_SOP) on Mothur v 1.39.5 (Schloss et al., 2009). All sets of reads (forward and reverse) were combined by creating contigs using command *make.contig* and the default options in Mothur v 1.39.5 (Schloss et al., 2009) except *trimoverlap* set to "true" and

insert set to “30”. Contigs that had an ambiguous base or unusually long/short read lengths were removed from future analysis. Overlapping paired-end sequences were aligned using SILVA v132 NR reference alignment with default settings in Mothur v 1.39.5 (Schloss et al., 2009). Any sequence that did not align with the targeted region of the SILVA alignment was eliminated from analysis. The sequences that remained were checked for chimera formation with program UCHIME (Edgar et al., 2011) in Mothur v 1.39.5 (Schloss et al., 2009). The most abundant sequence was used as a reference data for detection of suspected chimera, and singletons were eliminated. Hierarchical classification of good quality 18S rDNA sequences was carried out using Naïve Bayesian rRNA classifier version 2.2 (Wang et al., 2007) in Mothur v 1.39.5 (Schloss et al., 2009). The SILVA NR v132 alignment file (https://www.arb-silva.de/download/arb-files/) was used as the reference file for eukaryotic hierarchical classification. Only the sequences with ≥ 70% bootstrap support were considered classified at any hierarchical level. Some sequences remained unclassified especially those at lower taxonomic levels (e.g., Level 6), since no reference data are perfectly complete.

Bar graphs of relative sequence abundances at different taxonomic levels (ranks) were constructed in Microsoft Excel (Microsoft Corporation, WA, USA). Distance between sequences were calculated using command *dist.seq* with *cutoff* set to 0.20 and *countends* set to F. This distance file was used for generation of neighbor-joining tree using Clearcut program as implemented in Mothur v 1.39.5. Neighbor-joining (NJ) tree was used as an input file for the determination of α- diversity (phylogenetic diversity) and β-diversity (weighted and unweighted UniFrac) associated with each sample. Box plots of α-diversity indices were created using the ggplot2 package in RStudio Version 1.2.5033 (RStudio Team, 2019). Weighted UniFrac distances were also used for visualization of sample clustering in principal coordinate analysis

(PCoA) and Analysis of Molecular Variance (AMOVA). Principal coordinate analysis (PCoA) data from first two axes for all treatments were plotted using the ggplot2 package in R version 3.5.2 (2019).

Results

The results of the study are presented under general sequence characteristics, taxonomic distribution, and eukaryotic diversity subheadings.

General sequence characteristics

Before processing, there were 2,475,638 raw reads, ranging from 20 reads to 64,152 reads per sample. After processing, 616,207 good quality sequences remained, ranging from 4 to 20,992 sequences per sample. The quality control steps during data analysis are attributed to the loss of sequences generated. An average of 6,418 reads could be attributed to each sample, with final read lengths ranging from 83 to 149 bases (x = 108.15 bases). At Level 2, vertebrates made up 0.17% (1,021 reads) of all reads. This was also true for lower levels of classification; at Level 4, Mammalia comprised 0.17% (1,006 reads) of all reads. These were removed from further calculations as they belonged to the porcine host DNA. Additionally, two samples that had less than 184 reads were removed from calculations as they were not taxonomically informative.

Taxonomic distribution

A total of 616,207 sequences reads were classified into 38 phyla (Level 2), 82 classes (Level 3), 190 orders (Level 4), 161 families (Level 5), and 389 genera (Level 6). Because reference data are not complete, a great percentage of reads on average (e.g., 3.4% (at phylum level) (Figure 2) to 15% (at Genus level)) were unable to be classified.

Overall, over 63% of all sequences at the phylum level (Level 2) belonged to Apicomplexa (25%), Nematoda (15%), Basidiomycota (13%), and Ascomycota (11%) (Figure 2). At the family level (Level 5), over 47% of all sequences belonged to Neogregarinorida (25%), Trichosporonaceae (11%), Ascaridida (6%), and Araeolaimida (5%) (Figure 3). During the initial period of decomposition (0-57 ADD), eukaryotic structure did not differ much between soil under the remains (0 m away) and the soil at 3 m distance (control) but, after that interval, eukaryotic community structure associated with soil under porcine carcasses (0 m) differed significantly from the control soil samples (3 m away) (Figures 2, 3; Table 1). This difference was mainly because of decreased relative abundance of Nematoda (e.g., Ascaridida and Araeolaimida) and increased abundances of Basidiomycota (e.g., Trichosporonaceae) and Ascomycota (e.g., Dipodascaceae). Temporally, eukaryotic community structure did not differ significantly between ADD in both soil under the carcass (0 m) and at the control site (3 m away) (Table 2).

Eukaryotic diversity

During initial period of decomposition (0-57 ADD), eukaryotic phylogenetic diversity exhibited few differences in the soil samples between 0 m and the 3 m distance, but after ADD 57 eukaryotic α-diversity declined sharply in the 0 m soil samples but remained unchanged in the control soil (3 m away) samples (Figure 4). Distinct clustering in weighted UniFrac distances was observed between soil samples collected at 0 m and at 3 m (Figure 5). Except for samples collected during the initial period of decomposition (i.e. 27 ADD and 57 ADD), soil samples collected under the porcine remains (0 m) did not cluster according to ADD (Figure 6).

Discussion

An 18S rDNA MiSeq sequencing approach was used in this study to determine changes in eukaryotic community structure spatially and temporally in soil associated with porcine remains. Because most currently available, up-to-date eukaryotic reference databases (e.g., SILVA NR v132) are incomplete (Porter et al., 2012), a high percentage of unclassified sequences were observed at different taxonomic levels. Additionally, the conflict between morphology-based and DNA-based phylogenies has resulted in an increase of recently proposed taxonomies for eukaryotes (Adl et al., 2012, 2018; Tedersoo, 2017). Distribution and relationships to other taxa have changed while the organisms' names have not. For fungal classification at lower taxonomic levels, 18S rDNA generally does not perform as well as other commonly used markers (e.g., ITS) because the ITS region is more variable than 18S rDNA (Schoch et al., 2012).

The relative abundances of eukaryotic phyla in soil under porcine remains (0 m distance) did not change significantly with time, and was not similar to what was observed on porcine skin samples by Forger et al. (2019). For example, Forger et al. (2019) observed a nematode bloom on porcine skin at ADD 267-448 whereas this study observed a sharp decline in nematode abundance after ADD 57 (Figure 2). This study did not include soil samples that were collected between ADD 57 and ADD 734, and hence it is not clear exactly when the nematode population started disappearing from the soil under the carcass (0 m). It is hypothesized that nematodes move out of soil towards a carcass throughout decomposition in order to feed on its associated bacteria and flies, so the porcine skin samples were expected to show a nematode bloom (Griffiths, 1994). Also unlike Forger et al. (2019), Rhabditida (Nematoda) did not have a strong presence in this study. Other nematodes such as Ascaridida and Araeolaimida were the main families associated with the soil samples. Trichosporonaceae was observed in earlier and later

ADDs in Forger et al. (2019) whereas in this study it was observed only during the latter part of decomposition (i.e., at greater ADD). This study, and Forger et al. (2019), reported a high relative abundance of Dipodascaceae during later part of decomposition. Although there are some similarities between eukaryotic communities on porcine skin and in soil samples, these samples cannot be combined statistically, as they do not have the same indicator taxa.

Fungi present at later ADD are expected, because they are known saprotrophs that break down organic matter and assist in nutrient cycling (Burke et al., 2011). Some fungi can even produce collagenases that break down bone matrix (Michelle et al., 2016). Both Ascomycota and Basidiomycota observed in this study are normal fauna of the human gut (Hallen-Adams et al., 2015; Sam et al., n.d.) and they are known to play an active role in leaf litter decomposition (Kuramae et al., 2013).

Eukaryotic α-diversity was observed to decrease with time, which has been previously observed for bacterial α-diversity on porcine (Pechal et al., 2014), murine (Metcalf et al., 2013), and human (Metcalf et al., n.d.) remains. Conversely, this study did not find significantly higher eukaryotic phylogenetic diversity during later collection points compared with earlier collection points, as was observed by Metcalf et al. (2013) on the skin of murine remains. The opposite effect was observed in the soil beneath porcine remains, which is consistent with previous literature; Metcalf et al. (2013) seems to be the only exception to the trend of decreasing diversity as decomposition progresses. Eukaryotic phylogenetic diversity during the first two collection points was higher than the eukaryotic phylogenetic diversity observed during the later collection points (Figure 4). The differences observed between Metcalf et al. (2013) and this study could be due to differences in the model organisms (porcine vs. murine), sample (skin vs. soil), or the experimental design (field vs. laboratory conditions). Additionally, a PCoA plot

(Figure 5), showed that 0 m samples had greater dispersion from the centroid than soil samples that were collected at 3 m away (control). This pattern is similar to what was observed by Singh et al. (2018), and indicate either 0 m soil samples have greater β-diversity than 3m (control) soil samples or the multivariate datasets have too much variability.

Because the eukaryotic structure between the soil under the remains and at the control site were significantly different, a cadaver decomposition island (CDI) model may be possible. Taxa indicative of the soil under the remains are also indicative of carrion decomposition sites. Even though the control soil was significantly different and experienced little change throughout decomposition, minor changes were observed.

Conclusion

Overall, the eukaryotic community associated with soils under a vertebrate carcass changes significantly with space but not with time (or at least not within two months after placement of the carcass); this indicates that eukaryote community structure associated with vertebrate carcasses can be utilized for development of a statistical model to predict characteristics of the CDI. This study moves towards improved methodologies by potentially extending the capabilities of CDI identification and filling the gaps of previous studies. Despite this, there is still a way go before necrobiome prediction modeling for PMI estimation and CDI identification is a normal practice in forensic science and in DNA laboratories. Future research in this area should involve collecting soil samples at more frequent intervals and for a longer period of time. This would also include expanding the field conditions, such as different geographic locations, different times of the year, allowing scavenging, burring or submersion of the remains, and using humans instead of pigs.

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Figures and Tables

(a)

(b)

(c)

Figure 1. Photographs of Virginia Public Safety Training Center field experiment site (Woolf et al., 2016).
(a) NE to SW view of test site with all 12 swine seen from the vehicle entry point with subject A6 closest to the camera. (b) Subject A1 within wire scavenger exclusion cage without shade cloth. (c) Closer view of test site showing shade cloth over the test subjects.

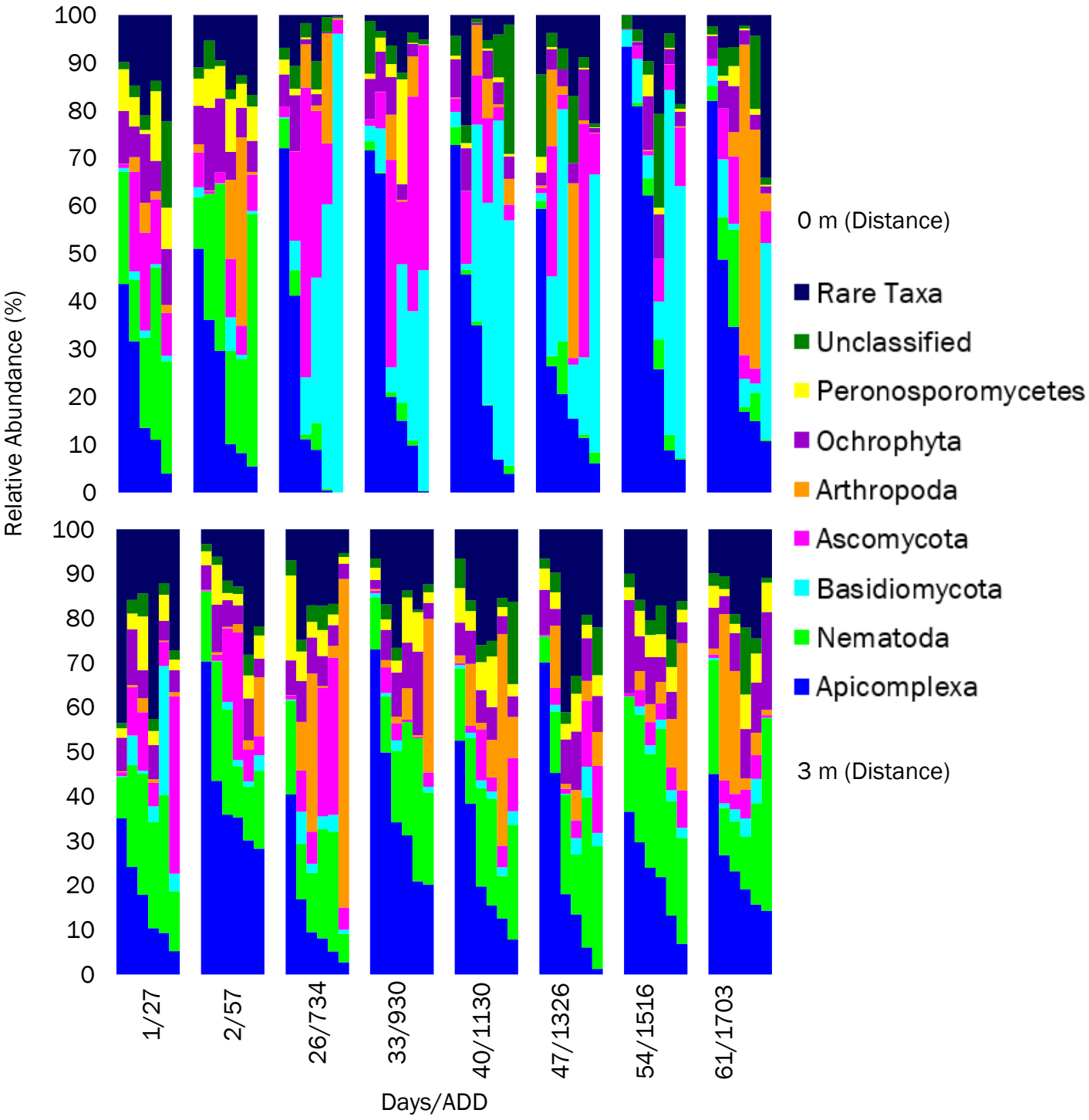

Figure 2. Eukaryotic community profile (at phylum level i.e., level 2) of soil under (0 m) and at 3 m away (control) from porcine remains. "Unclassified" include all unclassified taxa at the phyla level. "Rare taxa" include all taxa present at the phyla level that are not listed in the

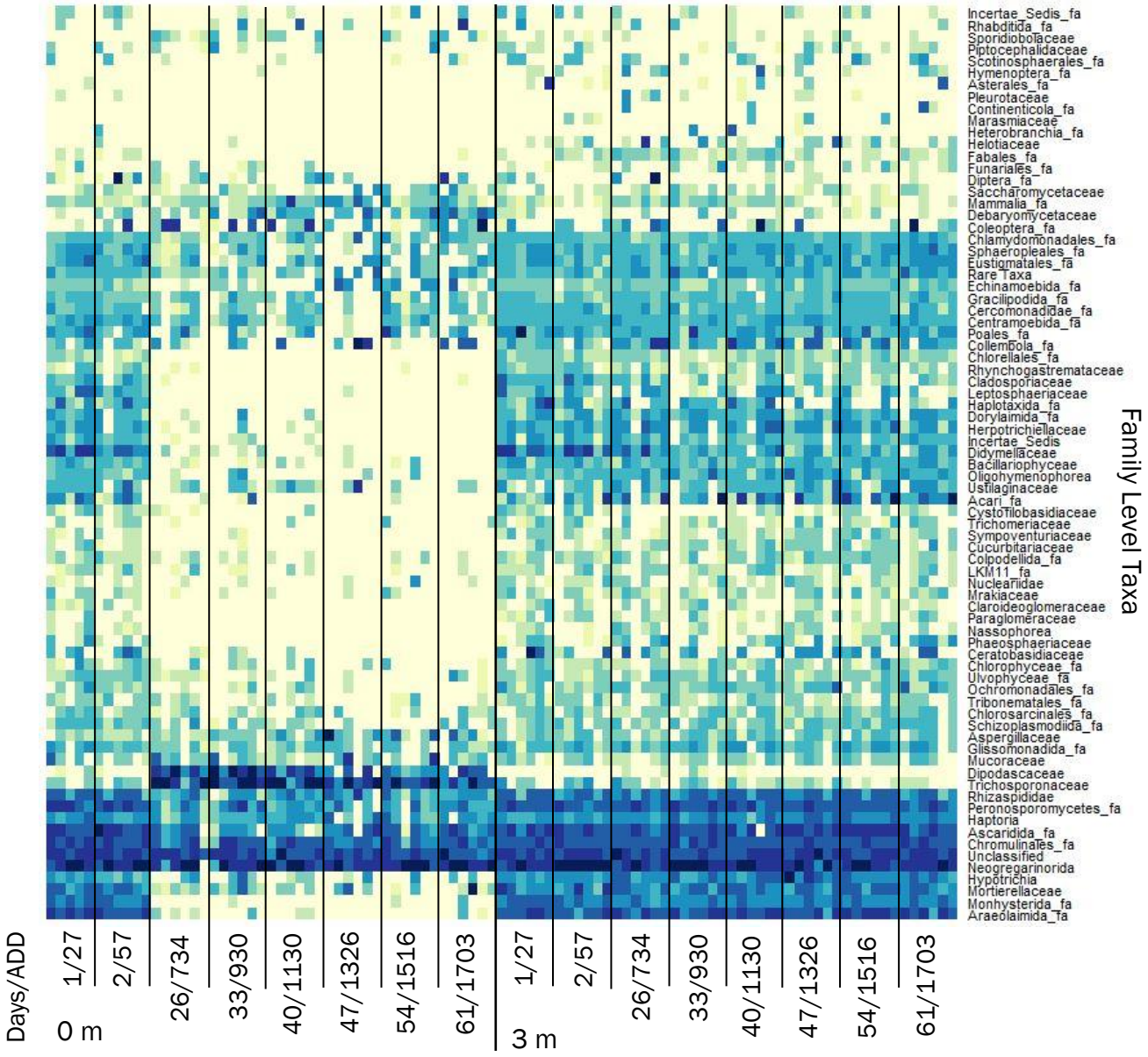

Figure 3. Heat map of family (level 5) taxonomic changes with increased Days/ADD at 0 m and 3 m (control). Deep blue color indicates 100% relative abundance while no color (white) indicates 0% relative abundance. As the blue color value increases from white to deep blue (darkens), relative abundance also increases.

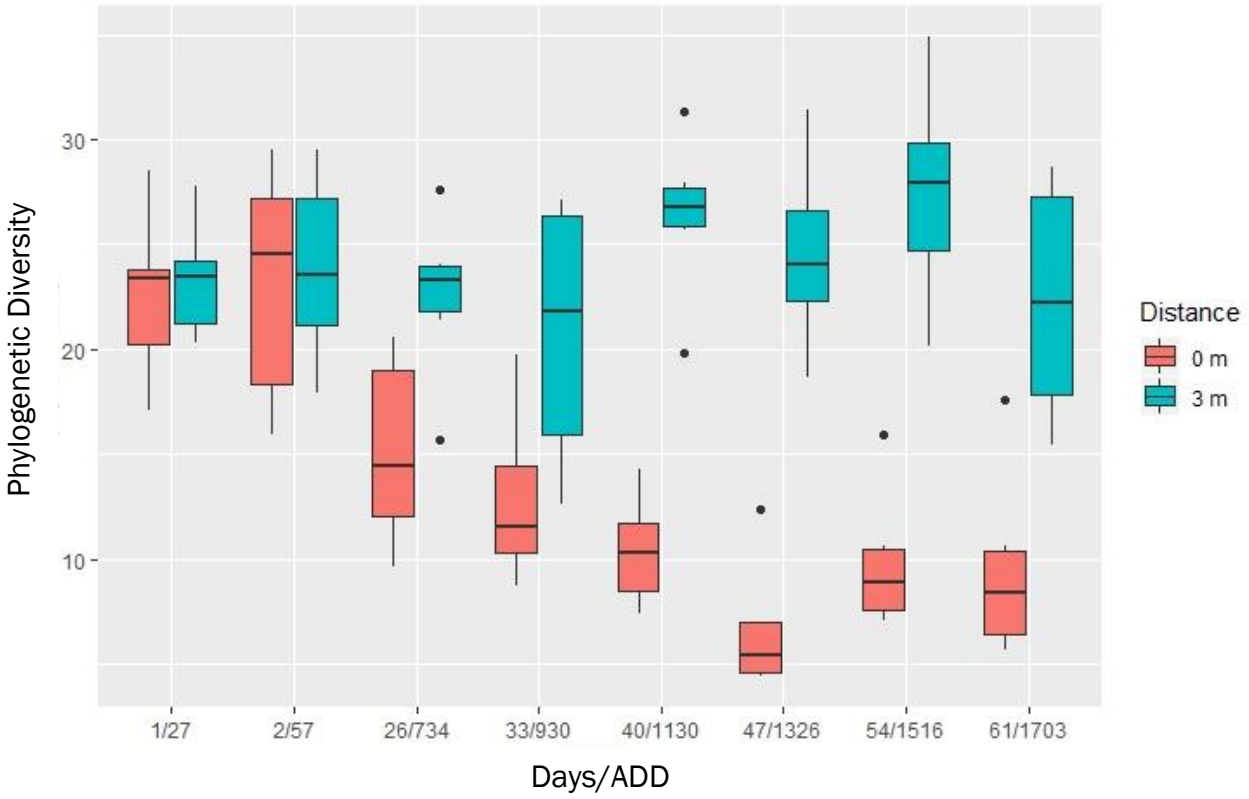

Figure 4. Temporal changes in eukaryotic α-diversity of soil under (0 m) and 3 m away (control) from porcine remains.

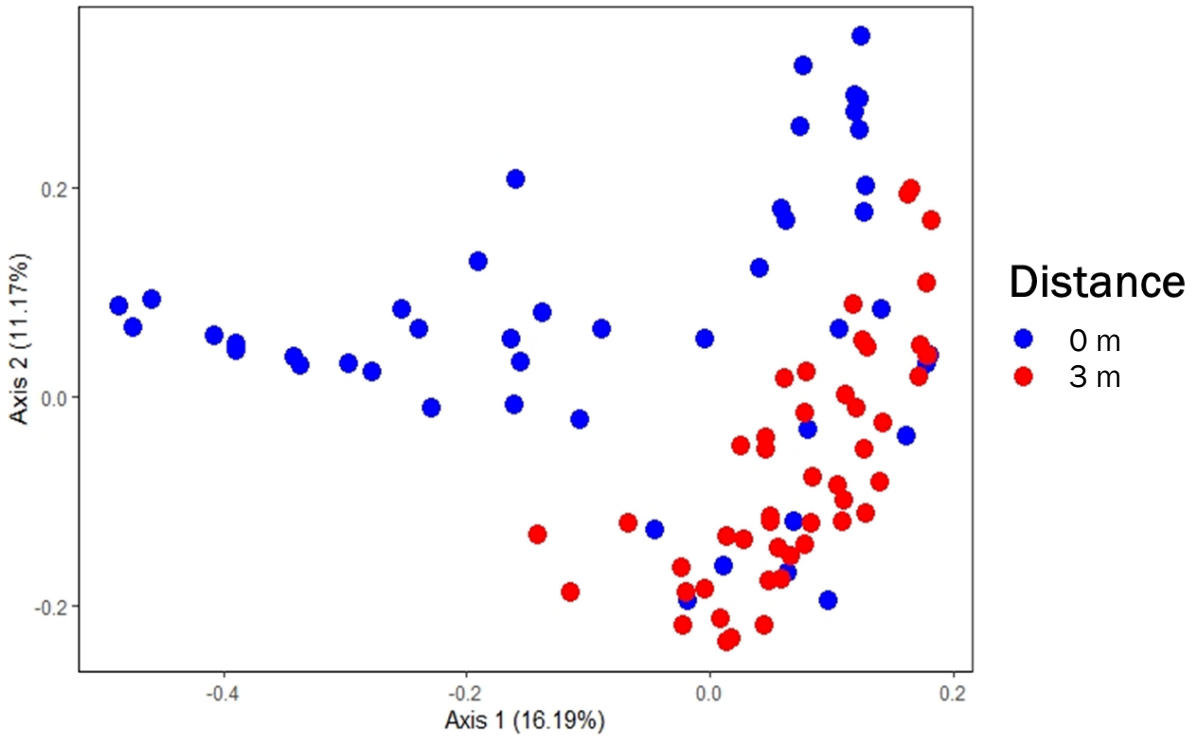

Figure 5. Two-dimensional principle coordinate analysis (PCoA) plot of eukaryotic β-diversity (based on weighted UniFrac distances) associated with soil under (0 m) and 3 m away (control) from porcine remains. R^2 value for this plot was 0.8207.

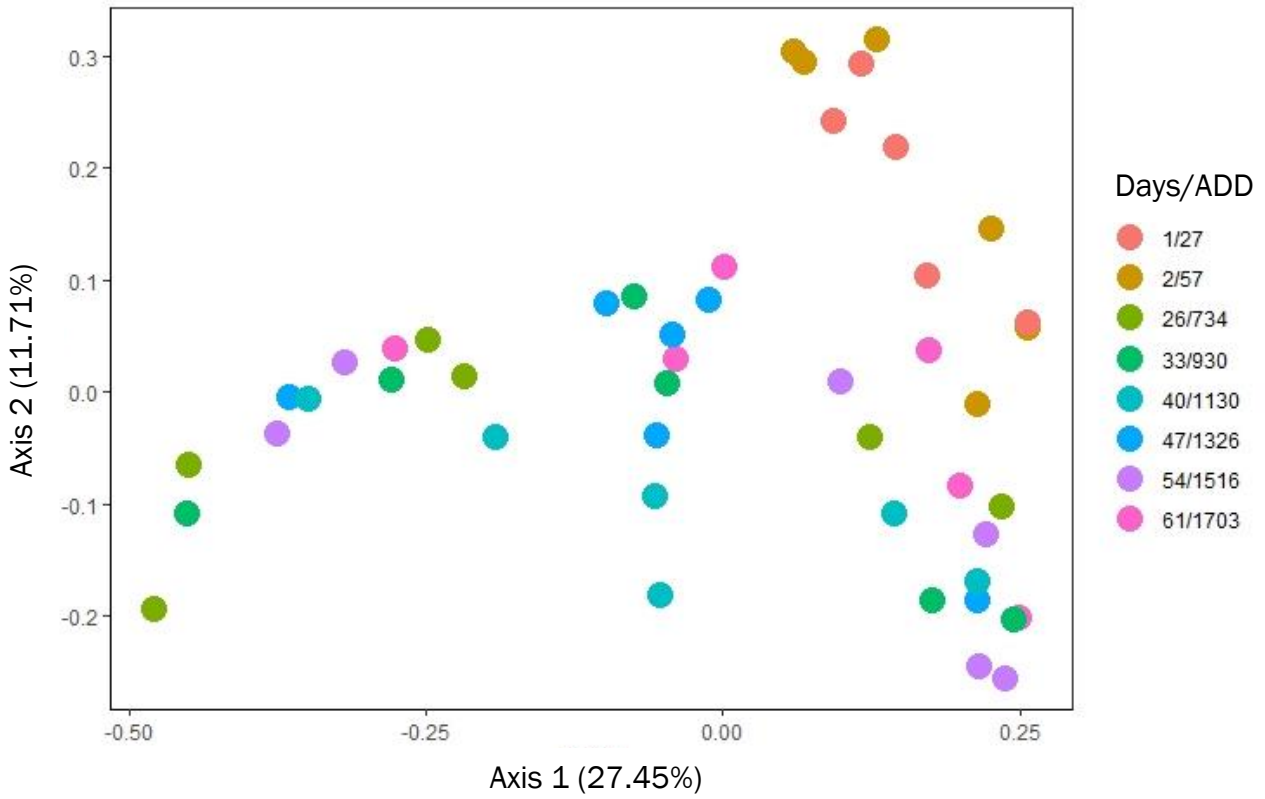

Figure 6. Two-dimensional principle coordinate analysis (PCoA) plot showing temporal changes in eukaryotic β-diversity (based on weighted UniFrac distances) of soil under the porcine remains (0 m). R^2 value for this plot was 0.695607.

Table 1. P value of pairwise AMOVA comparisons of each distance based on weighted UniFrac distances. Experiment-wise error rate: 0.05.

Distances	**0 m**
3 m	<2e-05*

*Significant difference

Table 2. P values of pairwise AMOVA comparisons of each ADD based on weighted UniFrac distances of 0 m soil samples. Experiment-wise error rate: 0.05. Pair-wise error rate (Bonferroni): 0.000416667

Days/ADD	**T1**	**T2**	**T26**	**T33**	**T40**	**T47**	**T54**
T2	0.66992						
T26	0.01522	0.01022					
T33	0.006	0.0042	0.65842				
T40	0.00072	0.00156	0.49516	0.47544			
T47	0.00532	0.00772	0.65662	0.47338	0.87248		
T54	0.01106	0.01024	0.28414	0.38108	0.60318	0.61366	
T61	0.00928	0.02116	0.2194	0.279	0.39212	0.55504	0.63328

Vita

Amanda Marie Haase was born on October 26, 1995, in Fairfax, Virginia, and is an American citizen. She graduated from Eastern View High School, Culpeper, Virginia in 2014 as an Honors Graduate. She received her Bachelor of Science from Wilson College, Chambersburg, Pennsylvania in 2018 and subsequently attended Virginia Commonwealth University in Richmond for the Master of Science in Forensic Science program.