Report Title: Characterization of bacterial and microbial eukaryotic communities (including fungal) associated with corpse decomposition using next generation sequencing

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Abstract

Our research focused on two major aims of forensic science: to estimate the postmortem interval (PMI) of corpses and to locate clandestine gravesites. Establishing the time since death is critical in every death investigation, yet existing techniques are susceptible to a range of errors and biases. For example, forensic entomology is widely used to assess PMI, but errors in estimates can range from days to months. Microbes may provide a novel method for estimating PMI that circumvents these limitations. Furthermore, if distinctive microbial communities form in the soil, these could potentially be used to identify clandestine gravesites. Our purpose was to determine whether the succession of bacterial and microbial eukaryotic communities associated with corpses and their gravesoil are sufficiently predictable to be useful for forensic science and criminal justice. Our goals were to determine: 1) whether bacterial and microbial eukaryotic, in particular fungal, decomposer communities change in a predictable manner as corpse decomposition proceeds, 2) whether decomposer communities change the endogenous soil community in detectable ways, and 3) whether decomposer communities are

universal or source-specific by characterizing variation in these communities across soil types and mammalian corpse species. To accomplish these goals, we proposed a multi-phase project to characterize the basic successional dynamics of bacterial and fungal communities associated with corpses and their gravesoils. We discovered that microbial community change provided an accurate estimate of the postmortem interval in a mouse model system. We also demonstrated that if an endogenous soil community is not present beneath a mouse corpse (e.g. sterilized soil treatment), bacterial decomposers become as abundant on the skin of the corpse as they would in the presence of a soil microbial community, but microbial eukaryotic communities were highly different. Further to this, we determined that if a soil substrate is present, bacterial and microbial eukaryotic decomposer communities on the skin and in the gravesoil exhibit some universality in their composition regardless of soil type. For example, in an experiment in which mice were decomposed on three contrasting soil types (shortgrass prairie, desert, and pine forest), including soil type as a feature in training our regression model for estimating PMI did not improve model performance. Instead, important features for training the model included microbial operational taxonomic units (OTUs) that were abundant in all decomposition data sets, regardless of soil type. This universality of microbial decomposer taxa has enabled us to identify signature organisms that may be useful for locating clandestine graves. Finally, we present results from both swine and human outdoor decomposition experiments and discuss how these microbial diversity trends translate outside of the laboratory and are consistent across different ecoregions and host taxa. Swine and human cadaver experiments confirmed that a succession of microbes during decomposition occurs. However, both experiments also suggest that seasonal

variation affects the decomposer community, at least to some extent. We conclude that tracking of microbial community change using next-generation sequencing approaches has potential applications for forensic science and medicolegal death investigation.

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Executive Summary

Two major aims in forensic science are to estimate the postmortem interval of corpses and to locate clandestine gravesites. Research focused on these aims often leverages information contained in biotic products associated with decomposition. For example, the succession of insects that visit the corpse and identification of chemicals produced during decomposition have been established as important tools by forensic practitioners to estimate the post-mortem interval, e.g.(Horenstein et al. 2010), and to identify clandestine graves (Vass et al. 1992, Carter et al. 2008b). Decomposition is accomplished by communities of organisms from across the tree of life, with microbes (e.g. bacteria, nematodes, and fungi) representing a major component of these communities. Although anecdotes about the role and source of microbes in corpse decomposition permeate both peer-reviewed and popular scientific literature, rigorous experimental research on microbial community ecology associated with decomposition has only recently begun to emerge (Carter and Tibbett 2006, Carter et al. 2008a). Tracking microbial communities associated with decomposition may be a useful tool in forensic science given the ubiquity and abundance of microbes coupled with emerging tools that enable community composition to be rapidly determined across many samples and time points. In particular, a predictable succession of bacterial and fungal communities associated with corpse decomposition may be a useful tool for estimating the postmortem interval. Likewise if distinctive communities form in the soil, these could potentially be used to identify clandestine gravesites. However, basic research characterizing the dynamics of bacterial and fungal communities associated with corpse decomposition is a critical first step

towards establishing whether these dynamics are sufficiently predictable to be useful in forensic science.

Our purpose was to determine whether the succession of bacterial and microbial eukaryotic (including fungal) communities associated with corpses and their gravesoil are sufficiently predictable to be useful for forensic science and criminal justice. Our goals were to determine: 1) whether bacterial and microbial eukaryotic, in particular fungal, decomposer communities change in a predictable manner as corpse decomposition proceeds, 2) whether decomposer communities change the endogenous soil community in detectable ways, and 3) whether decomposer communities are universal or source-specific by characterizing variation in these communities across soil types and mammalian corpse species. To accomplish these goals, we completed a multi-phase project to characterize the basic successional dynamics of bacterial and fungal communities associated with cadavers of three mammalian taxa and their gravesoils in both controlled and outdoor settings.

For our Phase 1 project, we characterized the succession of bacteria and microbial eukaryotes associated with mouse corpses and gravesoil using next-generation sequencing methods that allow for deep sequencing of microbial communities through the stages of decomposition. We sampled the microbial communities from the mouse corpse abdominal cavity and skin (head and torso), as well as from the associated gravesoil from 5 replicate corpses over 8 time points that spanned 48 days. By using a mouse model, we were able to perform a highly replicated experiment with destructive

sampling that enabled us to sample both the surface and interior of each corpse at each sampling time point. The Phase 1 experiment included two treatments – forty mice were allowed to decompose on an untreated soil, which had an endogenous microbial community (Metcalf *et al*. 2013) and a second set of forty mice were allowed to decompose on soil that had been subjected to three rounds of sterilization in an autoclave (Lauber *et al*. *in review*). Therefore, the sterilized soil treatment did not have an endogenous microbial community contributing to decomposition.

First, we analyzed data for the untreated soils group to characterize fundamental information about the ecology of decomposition and the potential for using microbes to estimate the postmortem interval (PMI). We provided the first 16S bacterial high-throughput sequence time-series data set for the abdominal cavity and gravesoil as well as the first 18S microbial eukaryotic high-throughput sequence time-series data set for the abdominal cavity, gravesoil, and skin sites for decomposing mammal. We discovered such a strong pattern of succession in both bacterial and microbial eukaryotic communities that we were able to estimate postmortem interval within ± 3 days. Our regression approach also allowed us to identify the most important features (i.e. OTUs) influencing our model accuracy. These results were described in a publication in the open-access, peer-reviewed journal *eLIFE* (Metcalf *et al*. 2013).

Second, we compared two treatment groups,untreated versus sterilized soils, to understand the effects of soil microbial communities on the dynamics of decomposition. We discovered that decomposition of mice proceeded two to three times faster in the

presence of an endogenous soil community. We also discovered differences in the overall composition of the decomposer communities associated with the skin of mice depending on whether an endogenous microbial community was present or not. For bacterial communities, many of the most abundant taxa were similar (e.g. Enterobacteriaceae were abundant on the skin regardless of treatment, although the untreated group had a high abundance of the genus *Morganella* specifically). Microbial eukaryotic communities, however, were highly different between treatments. Mouse skin microbial communities in the sterilized soil treatment group were dominated by fungi and in the untreated soil group dominated by the nematode *Oscheius tipulae*. These results suggest that some constituents of bacterial communities may be universal, but further research is required. It appears that the microbial eukaryotic community may be highly influenced by soil.

For Phase 2, we performed a laboratory experiment in which mouse corpses decomposed on three different soil types to determine the specificity of decomposer communities to soil source communities. We generated 16S (bacterial), 18S (microbial eukaryotic) and ITS (fungal specific) next-generation sequence data sets for samples from Phase 2 and analyses for these data are underway. Similar to Phase 1, we discovered that microbial communities changed consistently across replicates over time at each sample site. For both abdominal and skin sites, bacterial community change was significantly associated with time, regardless of soil type. Additionally, bacterial communities were initially highly different among soil types, but became progressively more similar as decomposition progressed. For example, Gammaproteobacteria in the family Pseudomonadaceae, Betaproteobacteria of the family Alcaligenaceae, and Firmicutes of

the family Bacillaceae increased in abundance in each sample site during decomposition. These trends suggest that a proportion of the soil bacterial decomposer communities are common across contrasting soil types, which makes bacterial communities associated with decomposition very attractive as a forensic tool.

Microbial eukaryotic communities also showed consistent shifts during decomposition with late stage communities and were dominated by nematodes, amoebae, cercozoa, and fungi. Sequencing of 18S amplicons revealed that the most abundant organisms in each sample type were nematodes of the Class Chromadorea that feed on bacteria or fungi. This bloom of nematodes, which occurred after rupture, was likely in response to the increase in bacterial and fungal biomass during early Active Decay. Samples associated with each soil type were dominated by a different family of Chromadorea (desert soil: Aphelenchidae, forest soil: Cephalobidae, and grassland: Pangrolaimidae and Aphelenchidae). The amoeba *Heterolobosea tetramitia* was also prevalent in late stage decomposer communities regardless of soil type, but at highly variable levels. Sequencing of fungal specific ITS amplicons revealed a shifts over time that appear soil type specific.

Finally, for Phase 2 we tested our ability to predict PMI with bacterial and microbial eukaryotic data. We discovered that we our regression models were most accurate during the Active Decay stage (root mean square error of model ~2-3 days) of decomposition, and less so during Advanced Decay (rmse ~ 6 days). We also discovered that our models in similar predictive accuracy regardless of whether we informed the regression model

with soil type. This surprising finding is very encouraging for the potential use of the microbes for estimating PMI because it suggests that the same OTUs are changing during decomposition regardless of starting soil community.

As part of Phase 3, we generated 16S data for gravesoil associated with *Sus scrofa* decomposition experiments (samples originally collected by Co-PI Carter as part of previous experiments). Our research on swine-associated gravesoils demonstrated that carcass mass did influence the structure of gravesoil microbial communities; the decomposition of 1 kg carcasses did not result in significant changes of dominant microorganisms although larger carcasses were associated with significant changes in the abundance of several dominant bacterial communities. Furthermore, we show that microbial community shifts in gravesoils are different across seasons. We conclude that the structure of postmortem gravesoil microbial communities is affected significantly by season and initial carcass mass. We also conclude that significant changes in bacterial communities are observed before significant changes in eukaryote communities.

Finally, we teamed up with Dr. Sibyl Bucheli and Dr. Aaron Lynne at Sam Houston State University to collect soil and skin samples associated with decomposition of *Homo sapiens*. We discovered that significant and predictable microbial community change associated with decomposition exists with human cadavers in outdoor scenarios. We also confirmed that seasonal variation might affect microbial community composition, a result also discovered in swine carcasses. Understanding the extent to which seasons affect our ability to predict PMI will be an important focus of future research.

Our results demonstrate that an accurate estimate of PMI can be obtained by tracking microbial community change with next-generation sequencing. This suggests a potentially expanded role for microbiological forensic indicators within the criminal justice system. The methods described in this report have several advantages for use in criminal investigations, including a continued rapid decline in the cost of DNA sequencing and the ease and familiarity of swab-based sampling at potential crime scenes to law enforcement officers.

Results from our research have been communicated to peers and the public in a number of ways. Results from Phase 1 and preliminary results from Phase 2 were presented at a well-attended oral presentation at the 2013 Annual Meeting of the American Academy of Forensic Sciences (J. Metcalf, Friday, February 22, 2pm Physical Anthropology Section). Additionally, R. Knight presented the results and progress of our research program at the NIJ R&D Grantee's meeting Tuesday, February 19th (followed by 3 additional live-streamed NIJ presentations in April), which provided us with the opportunity to disseminate our results to a broader forensic community and receive feedback from a variety of people in the field. Results of Phase 1 are described in two manuscripts, the first of which was published in the open-access, peer-reviewed journal *eLIFE*. Our second manuscript, which describes the effects of decomposition on a sterilized substrate, is currently under review (Lauber et al. 2014). Results from Phase 2 research were recently shared with peers at the 2014 Annual Meeting of American Academy of Forensic Science during the Pathology/Biology, Session: "Microbes Continued". Results

from research of Phases 2 and 3 are described in two additional manuscripts that are currently in prep (Carter et al. 2014), which are in the final stages of completion and will be submitted for publication in a peer-reviewed journal in the spring of 2014. Co-PI Carter shared results from Phase 3 swine experiments in the 2014 AAFS Pathology/Biology, Session: "Microbes Continued" session as well. Furthermore, the Phase 3 sampling effort with collaborators Dr. S. Bucheli and Dr. A Lynne at Sam Houston State University and our overall forensics research was recently highlighted by NPR's science correspondent Rob Stein (http://www.npr.org/blogs/health/2013/09/23/219375086/could-detectives-use-microbes-to-solve-murders). Finally, Co-PI's Carter and Metcalf organized (Carter) and participated (Carter and Metcalf) in a day-long workshop entitled "Forensic Microbiology: Where Do We Begin?".

Main Body of the Final Technical Report

I. Introduction

We live in a world dominated by microbes. This ubiquity coupled with recent knowledge gained from high-throughput sequencing studies about their diversity makes microbial ecology a promising field to search for new forensic tools. Microorganisms dominate the diversity of most—perhaps all—environments, from soils to oceans to animal intestines. The vastness of this diversity can now be explored with the advent of culture-independent methods and high-throughput DNA sequencing. Due to intensive research on specific environments such as soils (Lauber et al. 2009), the mammalian gut (Ley et al. 2008a), and the human microbiome (Costello et al. 2009), we know that the diversity of microbes

both within and between habitats (including the human body) is high, 99% of species cannot be cultured, distinct environments often host distinct microbial communities, and microbial communities may assemble in new environments in a predictable manner (Fierer et al. 2010b, Ramirez et al. 2010, Koenig et al. 2011). Importantly, bacterial communities that are associated with vertebrates are radically different from communities in free-living habitats (Ley et al. 2008b). Most culture-independent microbiome research has focused on bacterial communities, but similar research is now being done on fungal communities (Rousk et al. 2010).

Two characteristics of microbial diversity make microbial community analysis appealing as a potential forensic tool: 1) as in other environments, the succession of microbial communities associated with decomposing corpses is likely predictable over time, and 2) microbial communities in different environments (including both animal-associated and soil-associated environments) are quantifiably distinct. Just as we have demonstrated 'proof of concept' that we can use hand-associated bacteria for forensic identification of objects touched by specific individuals (Fierer et al. 2010a, Hsu et al. 2012), we may be able to use the signature of those communities associated with corpses to determine time since death and to locate possible grave sites.

Microbes play an important role in decomposition (Vass 2001, Hopkins 2008, Mondor et al. 2012). During decomposition, the corpse progresses through several recognized stages of decomposition, including Fresh, Active Decay, which includes Bloating and Rupture, and Advanced Decay (Carter et al. 2007, Parkinson et al. 2009). For example, from the

Fresh stage to the Bloat stage, enteric microbes likely contribute to putrefaction by digesting the corpse macromolecules, which in turn generates metabolic byproducts that cause the corpse to bloat (Mondor et al. 2012). Evans (1963) proposed that a major shift in microbial communities occurs at the end of bloat when the body cavity ruptures, as this key event likely shifts the abdominal cavity from anaerobic to aerobic. Additionally, at the Rupture stage, nutrient rich body fluids are released into the environment often increasing pH (Carter et al. 2010) likely altering endogenous microbial communities. The microbiology of corpse decomposition can now be investigated in detail by utilizing sequencing advances that enable entire communities to be characterized across the timeline of decomposition.

One of the most challenging forms of evidence to establish is the amount of time that has lapsed since death, also known as the postmortem interval (PMI). Establishing PMI is critical because it facilitates many aspects of a death investigation, such as the identification of victims and suspects and the acceptance or rejection of suspect alibis. However, PMI is difficult to establish because we have a relatively poor understanding of the ecology of corpse decomposition. When a mammal dies it becomes a large nutrient resource that can support a complex and phylogenetically diverse community of organisms (Mondor et al. 2012). Biotic signatures associated with these stages of decomposition, such as the development rate of blow fly larvae (Amendt et al. 2007), succession of insects (Horenstein et al. 2010), and changes in the biochemistry of corpse-associated "gravesoil" (Tullis and Goff 1987, Vass et al. 1992, Benninger et al. 2008, Carter et al. 2008a, Horenstein et al. 2010), can be used to estimate PMI, but no method

is successful in every scenario (Tibbett and Carter 2008). For example, limitations of forensic entomology include uncertainty in the interval between death and egg deposition (Tomberlin et al. 2011), lack of insects during particular weather events or seasons (Archer and Elgar 2003), and region-specific blowfly larval growth curves and insect communities (Gallagher et al. 2010). Using microbial community change to track the progression of decomposition may circumvent many of these limitations because microbes are ubiquitous in the environment, located on humans before death, and can be reliably quantified using high-throughput DNA sequencing. We need to determine whether microbial community change is sufficiently measurable and directional during decomposition to allow accurate estimates of past events such as the PMI.

Another very important problem in criminal investigations is locating buried bodies in an unknown location, which are often referred to as clandestine graves. It is possible that clandestine graves could be located by testing soils near or in recent contact with decomposing corpses. The fundamental ecological question here is whether cadaver decomposition modifies the soil microbial community sufficiently to be useful for gravesoil identification.

Our research consisted of three phases. The objectives of each phase are listed below as outlined in the original proposal.

Phase 1 - Lab-based assessment of bacterial and fungal communities associated with the decomposition of a model organism, mouse (*Mus musculus*).

We aimed to characterize the basic structure and source of bacterial and fungal communities associated with mouse corpses in the presence and absence of endogenous soil organisms under controlled environmental conditions.

Objective 1: Determine whether the changes in decomposer communities over time are predictable.

Objective 2: Identify those bacterial and fungal taxa derived from the corpse itself by measuring community change in the presence of sterile soil.

Objective 3: Determine whether taxa associated with corpse decomposition communities are detectable in the gravesoils.

Phase 2 – Lab-based assessment of bacterial and fungal communities associated with the decomposition of mice on three contrasting soil types.

Next, we assessed whether corpse-associated microbial communities exhibit predictable shifts in composition over time regardless of soil type and whether signatures of decomposition persist in soil after corpses are removed.

Objective 1: Determine whether decomposer communities are similar given different source soil communities and if they exhibit similar changes over time.

Objective 2: Determine whether signatures of decomposer communities persist in soil after a corpse is removed.

Phase 3 –A survey of gravesoils associated with pig cadavers (*Sus scrofa*), and human cadavers (*Homo sapiens*).

Finally, we assessed whether bacterial and fungal decomposer communities in soil are corpse-specific, and whether successional patterns are similar across mammalian taxa.

Objective 1: Determine which features of studies involving model organisms can be used to study microbial decomposer community patterns associated with humans.

II. Methods

Experimental set-up

Phases 1 and 2 – Characterization of bacterial and microbial eukaryotic (including fungal) communities associated with the decomposition of a model organism, mouse (*Mus musculus*).

We characterized bacterial and microbial eukaryotic decomposer community succession by studying the decomposition of a model mammal organism, *Mus musculus*, in a controlled laboratory setting. By using a mouse model, we were able to perform a highly replicated experiment with destructive sampling that enabled us to sample both the surface and interior of each corpse at each sampling time point. Importantly, this approach allowed us to sample the abdominal cavity prior to natural corpse rupture, and thus, our data addresses forensic hypotheses that abdominal microbes play a key role in corpse decomposition (Evans 1963). This approach contrasts with typical forensic studies of decomposition where one to three donated corpses of human or swine (used as a

human model) are used for experiments where only externally accessible body sites are sampled, e.g. (Pechal et al. 2013).

We performed two separate experiments. For Phase 1, we allowed mice to decompose on two substrates: untreated soil and sterile soil. For Phase 2, we allowed mice to decompose on three contrasting soil types (pine forest soil, desert soil, and shortgrass steppe). Soils were collected from the field (top 10 cm) and sifted with a 4mm sieve (Figure 1). Water holding capacity was calibrated to ~50%. Soils were homogenized with a large shovel. For both experiments, mice were placed on their right side on top of soil in a clean Tupperware-type container with a small hole drilled into each side above soil level to prevent anaerobic conditions (Figure 1). Six individual mouse graves were placed in an autoclavable polycarbonate Nalgene rat cage with a microfilter top to exclude insects (Figure 1). For each experiment, individual mouse graves were grouped into the secondary containers by treatment group (i.e. Phase 1 – untreated versus sterile; Phase 2 – shortgrass, desert, and pine forest soils). Both experiments were conducted at the CU Transgenic Mouse Facility in a room dedicated to the experiment with little foot traffic. Temperature was regulated between 20–21°C (68 – 70°F). To avoid loss of water from the soils in the dry Colorado climate, humidity in the polycarbonate cages was kept near 100% by lining the cages with soaked lab bench paper. We sampled skin on the head, skin on the belly, and abdominal cavity of each mouse as well as the soil underneath the mouse while still in an aboveground position on the gravesoil using sterile swabs (BD BBL™ CultureSwab™ , Becton Dickinson, USA).

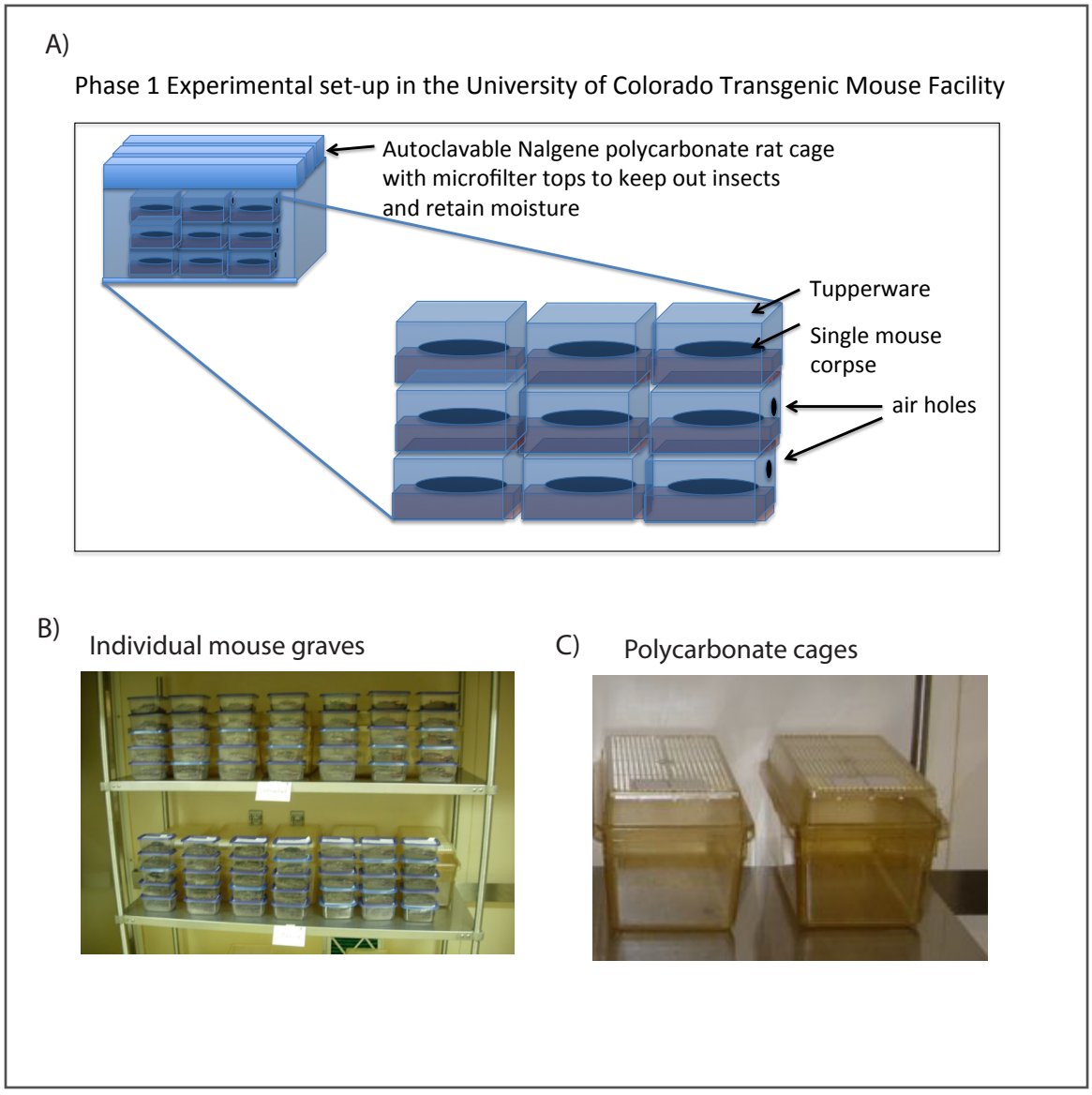

Figure 1. Experimental set-up for Phase 1 shown in A) schematic of the set-up is illustrated B) photo of individual "Tupperware" mouse graves and C) photo of polycarbonate rat cages that housed multiple individual graves. Each mouse corpse will be placed on either sterile or untreated soil in a Tupperware container "grave" with air holes to prevent anaerobic conditions. Tupperware containers were grouped by treatment (sterilized soil vs. untreated soil) in the secondary filter-top polycarbonate cages.

Experimental set-up

Phase 1 – mouse decomposition on untreated soil versus sterile soil

We sampled the microbial communities from the mouse corpse abdominal cavity and skin (head and torso), as well as from the associated gravesoil from 5 replicate corpses in each treatment over 8 time points that spanned 48 days. At each time point before sampling the five mouse corpses, we recorded a visual body score estimate for the head and torso following Megyesi *et al*. (Megyesi et al. 2005). For the sterilization treatment, soil was sterilized by subjecting it to heat and pressure (121 °C, 100 kPa for 30 minutes) in an autoclave 3 times over 4 days to destroy microbes, fungi and their spores.

Experimental set-up

Phase 2 – mouse decomposition on three contrasting soils

We allowed mice to decompose on three contrasting soil types from grassland, hardwood forest, and desert soils, which vary in dominant plant species, pH, carbon:nitrogen ratio, total carbon and total nitrogen. The experimental set-up was very similar to Phase 1 (Figure 1) with a few modifications (Figure 2). We used mice raised in a conventional facility and not a sterile transgenic facility as in the first experiment. This allowed for higher microbial biomass for skin samples at early time points. Additionally, we sampled the abdominal cavity in two ways. After an incision was made to access the abdomen, the cavity was swabbed. Next, the cecum was removed and placed in a 1.5 ml Eppendorf tube. The cecum was later swabbed for DNA extraction. We did not use a syringe to flush the abdominal cavity with saline solution to sample microbial DNA. In the first

experiment, this did not work well, particularly at late stages when the saline flush would simply flow into the soil beneath the mouse, which required us to take liquid samples as well as abdominal swabs. Furthermore, we hypothesize that using a syringe may bias sampling of microbial eukaryotic communities. Mice decomposed for 71 days and 5 mice were sampled from each soil type 8 times over the experiment with sampling events occurring more frequently (~every 3 days) over the first two weeks. We generated 16S, 18S, and ITS (targeting fungi specifically) sequences for over 790 samples collected during the experiment.

Figure 2. Phase 2 experiment with mice decomposing on three contrasting soil types (from left to right - shortgrass prairie, desert, and pine forest). The experimental set-up is shown in Figure 1.

Experimental set-up

Phase 3 - Survey of pig and human gravesoil samples

Swine gravesoils

We took advantage of soils collected as part of previous research projects by Co-PI Carter to perform a broad survey of bacterial and fungal decomposer communities in gravesoils associated with swine (*Sus scrofa domesticus*) (Anderson et al. 2013, Meyer et al. 2013). For the soil samples from Meyer et al. (2013), in which swine were allowed to decompose in two different seasons, we hypothesized that soils associated with pig carcasses decomposing in the summer season exposed to 1412 accumulated degree days (ADD) would undergo more substantial microbial community change than soils associated with pig carcasses decomposing in the winter exposed to only 325 ADD. These experiments allowed us to assess whether decomposer communities associated with mouse corpses are host-specific or similar across mammal species.

Human decomposition

Finally, we explored microbial communities associated with human decomposition through a collaboration with Dr. Sibyl Bucheli and Dr. Aaron Lynne at Sam Houston State University (SHSU) Southeast Texas Applied Forensic Science (STAFS) Facility, which is a willed body donation facility designed for decomposition studies and training. The STAFS facility is approximately 5 km north of Huntsville, Texas (Walker County) and is an outdoor, two-acre area fenced off within the larger 247 acres of The Center for Biological Field Studies (CBFS) located in the Pineywoods ecoregion characterized by a humid and subtropical climate and a sparse forest covering of pine trees and a ground covering of herbaceous plants. We sampled soil and skin sites associated with human (*Homo sapiens*) cadavers. Originally, we planned to only sample gravesoil associated

with human cadavers, but after the results from our mouse experiments demonstrated that both soil and skin microbial communities were informative for estimating PMI, we decided to sample both soil and skin samples from the experiments at the SHSU STAFS facility. We collected samples during two experiments over two seasons (February = winter, April = spring). Skin sites were sampled by non-abrasively swabbing with a sterile cotton tipped applicator (Puritan, 25-806 1WC) or (BD BBL™ CultureSwab™ , Becton Dickinson, USA). The cotton tip was removed and placed in a collection tube (VWR Sterile screw-cap cryo tube, 89004-310) containing 0.5 ml standard Phosphate buffered saline (pH 7.4) and stored at -80°C until processing for bacterial genomic DNA.

Soil samples were collected from the top 10cm of the soil horizon using a sterile metal spatula and stored in a 15 ml sterile polypropylene centrifuge tube (VWR, 89039-666). All samples were immediately frozen after collection at -20°C. Soil was later swabbed for DNA work and the remainder of the soil was utilized for pH measurements. For the winter experiment two bodies were placed in the field on February 26, 2013, and samples were collected from three skin sites (left hip, right hip, and left knee) and four soil sites (next to the left hip, right hip, left knee, and head) in addition to three non-corpse associated control soil sites. In the winter experiment, samples were collected daily for the first month, and then every three to four days for an additional month until April 29th, 2013. Additionally, samples were collected once a month in May, June, and July, at which point sampling was terminated. For the spring experiment, bodies were placed on April 17th, 2013, and samples were collected from eight skin sites (left hip, right hip, left bicep, right bicep, left lateral, right lateral, groin, and head) and six soil

sites (next to the left hip, right hip, left armpit, right armpit, left knee, groin, and head) as well as three control soil sites that were not associated with the corpse.

Soil chemistry

For both mouse and human decomposition experiments, pH was measured. For each soil sample, we suspended 1g of soil in 5ml of deionized water and measured pH using an Orion 3 Star benchtop pH meter (Thermo Scientific, USA). Triplicate pH measurements were averaged for a final pH estimate for each sample.

For the Phase 2 mouse decomposition experiment, we also measured total carbon, nitrogen, ammonia, and nitrate for soil samples. Based on previous soil chemistry research by Co-PI Carter, we suspected that soils would experience substantial changes in chemistry (Carter et al. 2010). For nitrate and ammonia measurements, soils were extracted using 2M KCl immediately following harvest by weighing 5 grams of wet soil, adding 2M KCl, shaking for 1 hour at 200 rpm, allowing the samples to settle for 1 hour after shaking, and vacuum filtering using Whatman No. 1 filter papers. Soil extracts were analyzed for inorganic nitrogen (NH_4^+ and NO_3^-) at the Colorado State University Soil, Water, and Plant Testing Laboratory (http://www.soiltestinglab.colostate.edu/). Another soil subsample was collected, dried at 60°C for 48hrs, sieved through 2mm sieves, removing all rocks and plant material, ground, and analyzed for total carbon and nitrogen content using an elemental analyzer (ECS 4010; Costech Analytical, Valencia, CA, USA) at the University of Colorado's Arid Land's Ecology Laboratory.

We followed the soil chemistry protocols available at http://www.colorado.edu/eeb/facultysites/barger/protocols.html.

DNA extraction and next-generation sequencing

DNA extraction, amplicon generation, and 16S and 18S amplicon preparation for sequencing followed the protocols described in Caporaso *et al*. (2011) and Metcalf *et al*. 2013 and can also be found on the Earth Microbiome Project (EMP) webpage (http://www.earthmicrobiome.org/emp-standard-protocols/). Briefly, all DNA extractions were performed using the 96-well high throughput PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) at the University of Colorado, Boulder.

The most efficient and cost-effective way to characterize microscopic communities is through culture-independent sequencing. Deep sequencing of amplicons generated from environmental DNA samples is becoming a widely used approach to profile communities not just for bacteria and archaea, but for many different branches of the tree of life. For example, Bates *et al*. (2013b) surveyed the diversity of protists (unicellular eukaryotes) in a global set of soils representing many of the major biomes on earth. This approach utilized barcoded primers for 18S rRNA amplicons in a similar fashion as the more widely used, high-throughput 16S rRNA amplicon surveys for bacteria and archaea. Similarly, McGuire *et al*. (2013) utilized the fungal marker (Schoch et al. 2012), nuclear ribosomal internal transcribed spacer (ITS) to characterize fungal communities in New York City's parks and urban green roof soil communities. Amplicon-based approaches

are not limited to ribosomal RNA, but can be used for any marker that provides taxonomic resolution for the group of interest and has an available reference database. A combination of these amplicon-based, community-profiling approaches could be applied to studies of carrion decomposition to better understand taxonomic and diversity changes across multiple trophic levels over time. Although, amplicon-based approaches will have inherent limitations (biases in pcr), surveys have shown similar results using different primer sets and 16S gene regions, confirming that it is often not a significant factor. We amplified the 16S rRNA variable region 4 using the bacterial primer set 515F/806R. 18S primers were based on Amaral-Zetter *et al*. (Amaral-Zettler et al. 2009), Euk1391f (GTACACACCGCCCGTC) and EukBr (TGATCCTTCTGCAGGTTCACCTAC). A blocking primer specific to mammals was used to minimize amplification of host DNA (GCCCGTCGCTACTACCGATTGGIIIIITTAGTGAGGCCCT C3 Spacer), with the design based on (Vestheim and Jarman 2008) as described in the EMP 18S protocol. For Phases 2 and 3, we also generated ITS amplicon data following (Bates et al. 2013a, McGuire et al. 2013).

To accurately characterize these highly diverse microbial communities, we sequenced amplicons at a depth of millions of sequences using the Illumina HiSeq platform. Sequencing technology has become immensely more accurate and simultaneously less expensive over the last few years. Since writing our 2011 proposal, CU has launched a high-throughput sequencing core facility (http://biofrontiers.colorado.edu/core-facilities/next-gen-sequencing) that currently hosts an Illumina HiSeq 2000 and a benchtop Illumina MiSeq. Instead of using Roche's 454 technology as originally

proposed, we have utilized the Illumina HiSeq 2000 platform for generating sequence data for our forensics research because the accuracy and number of sequence reads is higher. Both the core facility and the Knight lab are located in CU's new BioFrontiers Institute, which is a new multidisciplinary biotechnology center focused on research in critical areas of unknown biology and translating that knowledge into applications, such as forensic technology. A general overview of the sample processing and data analysis pipeline may be seen in Figure 3.

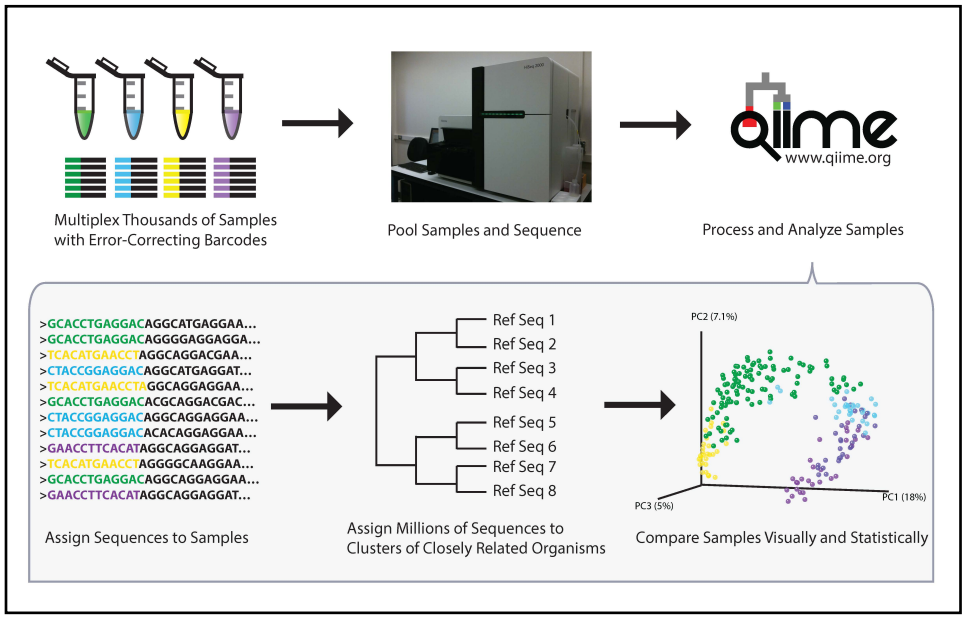

Figure 3. Overview of data generation, processing and analysis workflow using QIIME. After DNA is extracted, a marker gene (e.g. the small subunit ribosomal RNA) or genomic region is amplified using barcoded PCR primers. Next, the barcoded sequences are pooled and sequenced on a high-throughput DNA sequencing instrument. Finally, the open source software package QIIME is used to assign sequences to samples based on their barcodes, perform quality filtering, assign sequences to operational taxonomic units (optionally using a reference database such as Greengenes or SILVA), and generate

publication-quality visualizations and statistics (e.g. ANOSIM and PCoA plots). This schematic is based on a figure originally published in Hamady & Knight (2009).

Sequence data processing and analysis

16S rRNA sequence processing

Using the default settings in QIIME) (Caporaso et al. 2010b), barcoded Illumina 16S rRNA sequences were quality filtered and demultiplexed using error-correcting Golay codes that reduce the possibility of sample mis-assignment. All sequences were 100 bp in length. We classified sequence reads into Operational Taxonomic Units (OTUs) on the basis of sequence similarity. As our sample set included soil and highly decomposed material, we suspected that a substantial number of sequences would not be represented in the reference Greengenes alignment. Therefore, we utilized the QIIME software open-reference OTU picking protocol. Briefly, sequence reads were initially clustered against the Greengenes 97% OTU reference dataset (http://greengenes.secondgenome.com) (DeSantis et al. 2006, McDonald et al. 2012). The 16S data analyzed for Metcalf *et al*. (2013) utilized the February 2011 release of Greengenes. The Greengenes reference data set is updated and expanded approximately every six months to one yearwith the most recent version available at (http://greengenes.secondgenome.com). Future papers will use more recent releases of Greengenes (e.g. 2013 release). Sequences from our study that did not have significant similarities to sequences in the Greengenes dataset were subsequently clustered into *de novo* OTUs at 97% similarity using UCLUST. Taxonomy was assigned using the RDP classifier (Cole et al. 2009) within QIIME retrained on the

Greengenes 2011 reference dataset. The representative sequences of all OTUs were then aligned to the Greengenes reference alignment using PyNAST (Caporaso et al. 2010a) and this alignment was used to construct a phylogenetic tree using FastTree (Price et al. 2010) within QIIME. Sequences that did not align to Greengenes with a 70% similarity threshold were assumed to be non-16S artifacts and removed from further analysis. The resulting tree topology with associated branch lengths was used for subsequent diversity analyses. We removed low abundance OTU's making up <0.0005% of reads in the total data set as recommended for Illumina generated data (Bokulich et al. 2013).

18S rRNA sequence processing

Raw 18S rRNA sequence data were subjected to similar processing and demultiplexing protocols within QIIME as described above, except that a curated version of the Silva 108 database was used as the reference database (original: http://www.arb-silva.de/documentation/release-108/; Pruesse *et al*. (2007), curated version available at qiime.org/home_static/dataFiles.html). Sequences not corresponding to eukaryotic 18S were removed from the dataset prior to analysis by excluding reads that failed to align to the eukaryotic portion of Silva 108 at a low similarity threshold (70% sequence similarity) with PyNAST (Caporaso *et al*. 2010) within QIIME. The dataset was further filtered to exclude all sequences assigned to vertebrate animals, as these likely correspond to the mouse host. Similar to 16S data, we removed low abundance OTU's making up <0.0005% of reads in the total data set as recommended for Illumina generated data (Bokulich *et al*. 2013) and samples were rarified to 2500 sequences per sample. A phylogenetic tree was constructed for subsequent diversity analyses by placing

representative sequences into a tree of the Silva 108 eukaryotic representative set using the maximum likelihood EPA algorithm within RAxML (Berger et al. 2011). Taxonomy was assigned to *de novo* reads using RDP (Cole *et al*. 2009) retrained on the Silva 108 eukaryotic reference database at the genus level within QIIME.

ITS sequence processing

We processed the ITS fungal amplicon data following methods outlined in (McGuire et al. 2013) using the QIIME pipeline is a similar way as for 16S rRNA and 18rRNA amplicon data.

Statistical analysis of microbiome data

We performed the majority of our statistical analyses using the QIIME software package (Caporaso et al. 2010). We explored beta diversity patterns by performing principle coordinate analyses (PCoA) with phylogeny-based (UniFrac) unweighted distances. Alpha diversity was estimated using the phylogenetic diversity metric as well as Shannon diversity and tested for significance using the nonparametric option in the compare_alpha_diversity.py command while differences in taxon abundances were performed using the bootstrapped Mann-Whitney U test in QIIME. We utilized Bayesian source-tracking software (Knights et al. 2011), which is wrapped in QIIME, to better understand which sample sites may have hosted bacterial communities that contributed to the late stage decomposer communities. To assess the correlation between microbial community change and time, we performed Mantel tests (with recommended option for

single factor tests: unrestricted permutation of raw data) using the software package PRIMER v6 (Clarke and Gorley 2006). Additionally, we tested for significant changes in microbial communities between the Fresh and Advanced Decay stages for each site and data type with a PERMANOVA test either in QIIME (Caporaso et al 2010) or in PRIMER v6 (Clarke and Gorley 2006). For statistical analysis, duplicates of samples (e.g. abdominal swab and abdominal liquid/syringe) were removed.

We regressed PMI directly on the taxon relative abundances using the Random Forests model (Breiman 2001) with version 4.6-7 of the *randomForest* package in *R (Liaw and Wiener 2002)* with default settings. We directly assessed the predictive performance of the regression using leave-one-out (LOO) error estimation, predicting the PMI of each sample using a model trained on the remaining samples. From these LOO errors we calculated the mean error, and standard deviation of the errors with units of days. We performed these regressions within each sample site separately and for bacteria and eukaryotes separately with and without the first and last time points (0 and 48 days) to determine the best body site and timeframe for predicting PMI. Since skin and soil demonstrated the best predictive power (see results), we also merged OTU tables from skin and soil sites as well as marker type to potentially further improve our PMI estimates.

For Phases 2 and Phase 3 (human decomposition experiments only) data, we compared regression methods, namely, random forest, boosting tree and cubist (R package caret) to investigate whether a particular method performs best. For each method, the input data was partitioned into 10 subsets and models were built on 9 subsets and validated using

the rest subset. This cross-validation process was then repeated 10 times, with each of the 10 subsets used exactly once as the validation data. This so-called 10-fold cross validation (CV) is an effective way to measure the accuracy of models as root mean squared error (RMSE) in unit of days, and to choose the most parsimonious model (in order to avoid overfitting to the input training data) within one standard deviation of the optimal accuracy.

We estimated PMI as a function of sample sites and soil types to determine which location(s) of the body host microbial communities that are most informative for accurate PMI prediction, and to measure whether and how various soil types, such as dessert, forest, and grassland, bias our prediction. Additionally, we also performed regression analyses using microbial samples collected within various time points to assess the time window in which microbial signatures provided the most accurate PMI estimate, and how large the errors are for this and other time windows. We performed PMI estimates primarily on 16S data, and we are currently exploring the utility of 18S and ITS for estimating PMI as well (18S results shown for Phase 2).

III. Results

Phase I – Mouse decomposition on soil with an endogenous microbial community (untreated soil)

We found evidence for a microbial clock to estimate the postmortem interval (PMI) in a mouse model system. We assessed the temporal dynamics of the microbial communities in the untreated soil data set. We collected 223 abdominal cavity, skin, corpse-associated

soils samples (gravesoil), and no-corpse soil controls. After sequence quality filtering and removal of failed samples and samples with low numbers of sequences, the HiSeq Illumina sequence data set included 167 samples successfully sequenced for 16S and 142 samples successfully sequenced for 18S rRNA (Metcalf *et al*. 2013). Most samples with too few sequences to be included in the final datasets (i.e. failures) were collected at early time points (e.g. days 0 and 3) when microbial biomass was likely low. This was especially true for eukaryotic skin samples and abdominal cavity swab or liquid samples. In the second experiment, we improved our success with skin samples at early time points by using conventional mice that were raised in a non-sterile facility and fed non-sterile food (see results of Phase 2).

It has long been assumed that endogenous gut-associated bacteria dominate cadaver decomposition prior to rupture, following which non-enteric and aerobic (soil-borne, dermal) microbes bloom and dominate the community (Evans 1963). Rupture is a crucial stage during decomposition, in which bloating due to putrefaction breaks open the abdominal cavity, and is expected to result in shifts of the microbial community because the cavity becomes aerobic. Early culture-based investigations conducted without soil lent support to this assumption by showing that many bacteria exploiting a carcass are members of the gut microbiota (Ingram and Dainty 1971, Corry 1978). Our results also lend support to these long-held hypotheses. During the Bloating Stage (approximately days 6-9), endogenous anaerobes and facultative anaerobes that are known to be common members of the gut community such as Firmicutes in the families Lactobacillaceae and Bacteroidetes in the family Bacteroidaceae increase in the abdominal cavity (Figure 4).

However, after rupture occurs (~9 days after the start of the experiment), these taxa decrease dramatically, and exposure of the abdominal cavity to oxygen allows aerobes such as members of the Rhizobiales in the families Phyllobacteriaceae, Hyphomicrobiaceae, and Brucellaceae to dominate (Figure 4). Additionally, facultative anaerobes in the Gammaproteobacteria family Enterobacteriaceae, which are widely recognized as opportunistic pathogens and are associated with sewage and animal matter (Leclerc et al. 2001), become abundant after rupture.

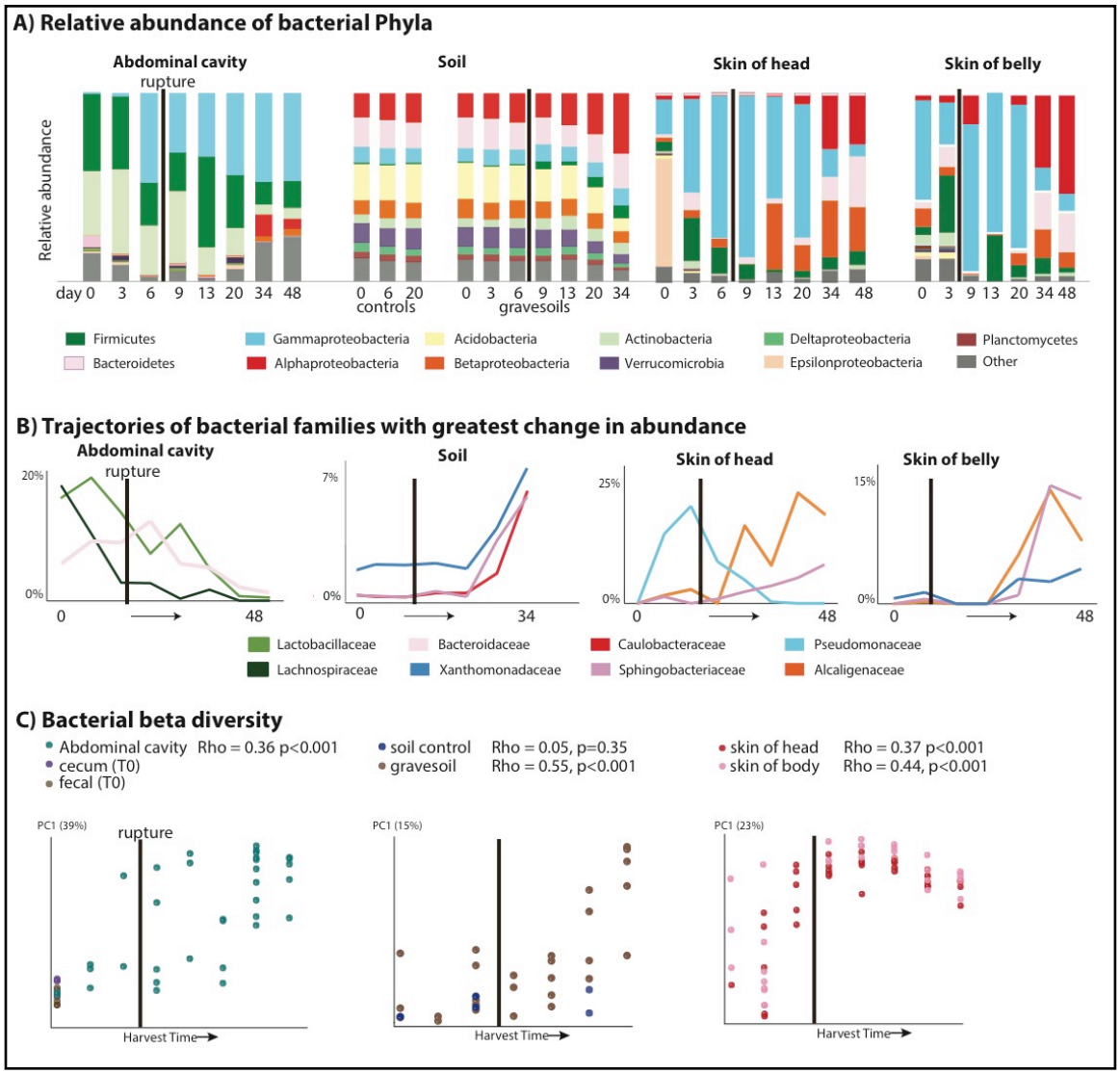

Figure 4. Bacterial community composition changes significantly and consistently over the course of decomposition. A) Relative abundance of phyla of bacteria over time for all body sites. Control soils were averaged across time points. For the abdominal site, Day 0 includes cecum, fecal, and abdominal swab and liquid samples. For the soil site, control soils collected on days 0, 6 and 20 are shown on the left of the plot B) The three bacterial families that show the greatest change in abundance over time are plotted for each site. C) PCoA plot based on unweighted UniFrac distances displaying bacterial community change at all sites during decomposition. Results from Mantel tests (Rho and p-values) show that bacterial community change correlated significantly with time. The point of rupture is marked with a thick vertical black line on each plot. This figure was originally published in Metcalf *et al*. (2013) and is open access under the Creative Commons-Attribution License (CC-BY).

When corpses rupture they release an ammonia-rich, high nutrient fluid that alters both the pH and nutrient content of the soil (Meyer et al. 2013). Accordingly, we saw a predictable spike in gravesoil pH from ~6.0 to ~8.5 and a decline in Acidobacteria (Metcalf et al. 2013), the abundance of which is known to be inversely related to soil pH (Lauber et al. 2009). Acidobacteria prefer oligotrophic conditions (Fierer et al. 2007) and grow much more slowly than most other taxa (Ward et al. 2009, Castro et al. 2010), thus the decline of Acidobacteria may be related to the huge influx of nutrients into the soil rather than shifts in soil pH. In this study, Alphaproteobacteria abundance (mainly the Rhizobiales) increased across the sampling period and became most abundant post-Rupture stage soil samples, suggesting this group prefers a relatively nutrient rich

environment and is able to outcompete the Acidobacteria (Marilley and Aragno 1999, Klappenbach et al. 2000, Smit et al. 2001) for the corpse derived resources. Such shifts in taxonomic composition were typical in these Advanced Decay associated soil communities (after ~20 days), which were significantly different from the time-zero soil communities as well as the no-corpse soil controls (Figure 4, Table 1, PERMANOVA p-value 0.007). No-corpse control soils, which were collected throughout the experiment, did not change significantly over time (Table 2, Mantel Rho 0.05, p-value 0.35), linking soil community change to the presence of a corpse. Though preliminary, our work suggests that it may be possible to identify gravesoil by an increase in the abundance of copiotrophic taxa relative to oligotrophic taxa.

Table 1. For each sample site and each marker type, PERMANOVA results of UniFrac distance (unweighted) for Fresh (day 0 -3 days) versus Advanced Decay (days 20-48) decomposition microbial communities. For soil sites, we also include comparisons of control soils versus Advanced Decay gravesoils. For the 18S skin of head, there were not sufficient samples for statistical analysis.

	PERMANOVA Pseudo F	p-value (999 permutations)
16S soil with corpse	2.38	0.007
16S ctrl soil vs Advanced Decay soil	2.54	0.001
16S abdominal	6.31	0.001
16S skin on head	8.19	0.001

16S skin on body	5.81	0.001
18S soil with corpse	10.17	0.001
18S ctrl soil vs Advanced Decay soil	5.23	0.001
18S abdominal	5.34	0.001
18S skin on head	--	--
18S skin on body	5.96	0.001

Table 2. For each sample site and each marker type, Mantel test results using Spearman's rank correlation coefficient to assess the correlation between microbial community UniFrac distance (unweighted) and time. Importantly, control soil microbial communities did not change significantly over time.

	Spearman Rho	**Spearman p-value**
16S soil	0.548	0.001
16S ctrl soil	0.051	0.352
16S abdominal	0.364	0.001
16S skin of head	0.368	0.001
16S skin of body	0.437	0.001
18S soil	0.772	0.001
18S ctrl soil	0.127	0.154
18S abdominal	0.209	0.029
18S skin of head	0.279	0.004

18S skin of body	0.079	0.143

Bacterial communities associated with the decomposing corpses became increasingly differentiated from starting communities over time in the abdominal cavity, gravesoil, and skin sites (Figure 4, Table 2, Mantel test Rho values 0.36 – 0.55, $p < 0.001$ for all sites). Although they did not converge completely, similar taxa became abundant at each sample site in the later stages of decomposition (Metcalf *et al*. 2013). For example, in both soil and skin, several bacterial families within the Bacteroidetes (Sphingobacteriaceae), Alphaproteobacteria (Brucellaceae, Phyllobacteriaceae, and Hyphomicrobiaceae), and Betaproteobacteria (Alcaligenaceae) increase in abundance during the Advanced Decay stage of decomposition (Figure 4). This trend is consistent with previous findings that bacterial skin communities are often a reflection of the surrounding environment with which they are in contact (Costello et al. 2009, Song et al. 2013), and this convergence may also arise because the low biomass initially found on the skin is easily overwhelmed by soil taxa.

The community of microbial eukaryotes also changed significantly and consistently over the time course of decomposition at all sampled sites except the skin of the torso (Figure 5). Beginning at approximately 20 days, the microbial eukaryotic community at all sites became dominated by a nematode, *Oscheius tipulae,* in the family Rhabditidae (Figure 5). Microbial eukaryotic community composition in the no-corpse control soils did not change significantly over time (correlation with time: Mantel Rho 0.15, $p = 0.13$, Table

2). Furthermore, the nematode *Oscheius tipulae* was not detected at a level of > 1% in any control soil sample.

Oscheius tipulae, a bacterivorous representative of the family Rhabditidae, is considered a common nematode species of terrestrial habitats such as soil, leaf litter, and compost all over the world (Baille et al. 2008). As a consequence of the nematode bloom, Shannon diversity (community evenness) declines at all sample sites for eukaryotic communities (t-test Fresh Stage versus Advanced Decay stage decomposition: soil $p<0.001$, abdominal $p= 0.002$, skin $p =0.03$). Phylogenetic distance diversity estimates were variable across sample sites with a significant decrease only detected in soil (Metcalf et al. 2013). The nematode bloom is decoupled from rupture, and it appears that this generalist consumer of bacteria responds to the increase in bacterial biomass that is associated with decomposition (Benninger et al. 2008, Carter et al. 2008a, Parkinson et al. 2009, Carter et al. 2010, Damann et al. 2012) and outcompetes other community members. One potential contributing factor to the nematode bloom may have been the fact that the mouse graves were relatively closed systems that would have prevented entry of organisms preying on nematodes. Future experiments with open systems will be necessary to determine the impact of higher trophic levels on community dynamics.

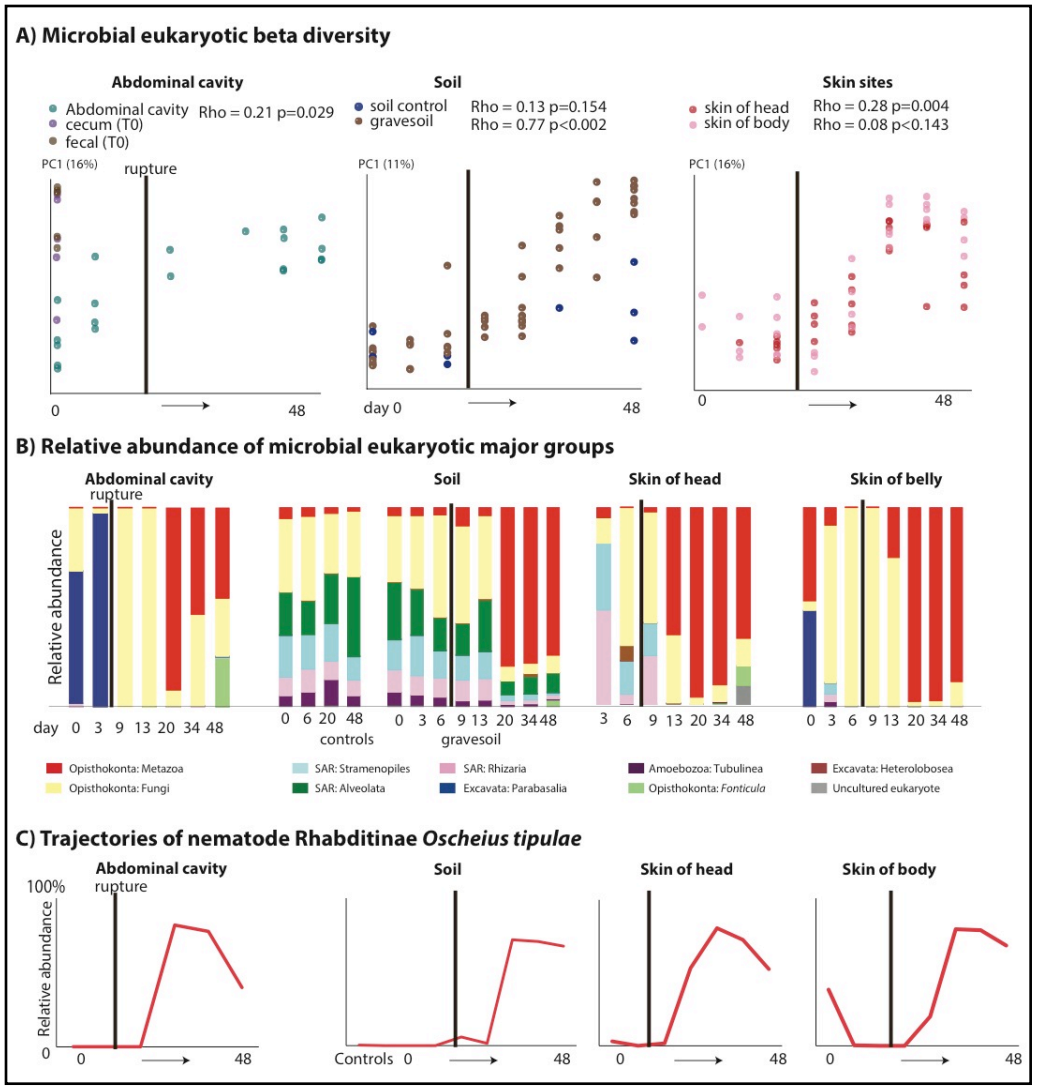

Figure 5. Eukaryotic community composition changes directionally and becomes dominated by the nematode *Oscheius tipulae*. A) PCoA plot based on unweighted UniFrac distances displaying microbial eukaryotic community change during decomposition. Results from Mantel tests (Rho and p-values) show that microbial eukaryotic community change correlated significantly with time except for the skin of the belly. B) Relative abundance of microbial eukaryote taxa at the class level over time. Microbial eukaryotic community composition changes significantly and predictably over the course of decomposition. C) The eukaryotic nematode *Oscheius tipulae* became

highly abundant at each sample site at late stages of decomposition. The point of rupture is marked with a thick vertical black line on each plot. This figure was originally published in Metcalf et al. 2013 and is open access under the Creative Commons-Attribution License (CC-BY).

Because consistent shifts in the presence and abundance of specific bacterial and eukaryotic taxa occurred during known stages of decomposition, these data suggested that the succession of bacterial and microbial eukaryotic communities may be used to estimate PMI. By regressing known postmortem interval directly on the taxon relative abundances using a Random Forests model. Random Forests is a machine learning technique that creates random decision trees based on subsets of the features (e.g. taxa) in the data, then chooses the subsets of features that are best able to classify samples into predefined groups using only part of the data. This technique has been useful in other microbial ecology studies, providing high classifier accuracy (Knights et al. 2011). We discovered that the temporal change in microbial communities of the skin of the head allowed us to estimate PMI within as little as 3.30 +/- 2.52 days (mean absolute error +/- standard deviation) (Figure 6). Regressions performed for the timeframe of 0-34 days resulted in the smallest mean absolute error, which suggests that microbes may be more highly informative for estimating PMI during the earlier stages of decomposition, at least for the sites we sampled. However, this trend may also be due to less frequent sampling events between days 13-48. It may be possible to further improve error estimates with more frequent sampling.

For both 16S and 18S datasets, skin and soil sites were more informative for estimating PMI than data from the abdominal cavity (Figure 6). This observation was initially surprising because the marked shifts from anaerobic to aerobic communities that occurred in the abdomen would appear to be a good marker of timing. However, these major microbial changes, which correspond to the time of Rupture, occurred at different times for the replicate corpses in the experiment (Figures 4 & 5), and this variability likely reduced the usefulness of the abdominal data for estimating PMI.

Overall, bacteria did not perform significantly better in our PMI estimates than microbial eukaryotes and combining both 16S and 18S data sets resulted in the best estimates of PMI, although the improvement was not significant (Metcalf et al. 2013). For each sample site, we estimated the "importance" of each taxon, a measure of its contribution to the PMI estimate, by removing each taxon from the predictive model and calculating the mean percent increase in mean squared error. For bacteria, taxa in the Order Rhizobiales were among the most important predictive taxa at each sample site. For microbial eukaryotes, *Oscheius* was the most important taxon for skin and abdominal sites and the second most important for gravesoil. Fungal taxa of the family Boletales were the most important contributors to PMI regressions for gravesoil. An analysis of models built using small numbers of highly predictive taxa indicates that 5-10 taxa provide approximately the same prediction accuracy as models built with all taxa, as assessed by leave-one-out analyses (Metcalf et al. 2013). This holds across domains and sample sites, and identifying diagnostic taxa may be a fruitful avenue for forensic investigation. Future

studies involving varied habitat conditions, soil types, etc. are needed to identify the most general subset of predictive taxa.

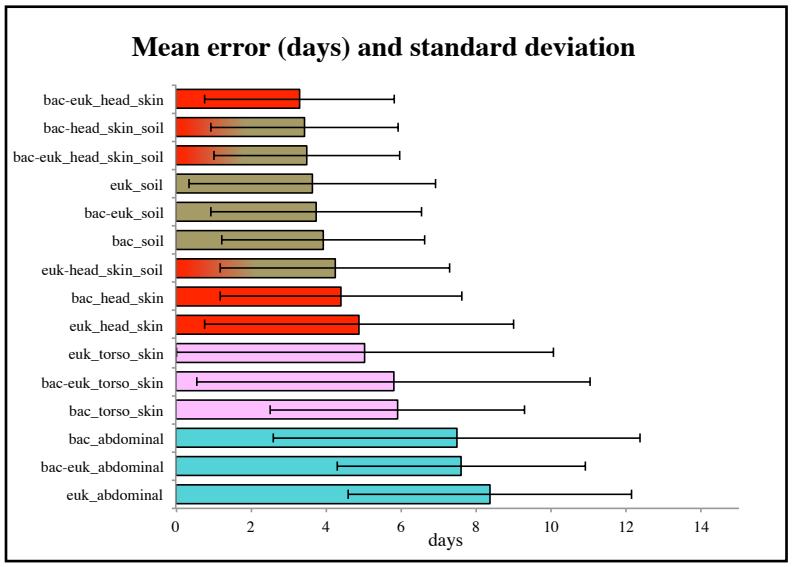

Figure 6. Estimates of the mean absolute error (MAE) and standard deviations for PMI regressed directly on the taxon relative abundances (in days) based on bacterial ("bac") and eukaryotic ("euk") microbiome composition for each sampled site - soil (brown), abdominal cavity (teal), skin of the head (red), and skin of the body (pink). Results are show for the timeframe of 0-34 days, which produced smallest errors. Results are displayed with the smallest MAE shown at the top (skin of head with combined 16S and 18S data) and largest error at the bottom (18S abdominal data). This figure was originally published in Metcalf et al. (2013) and is open access under the Creative Commons-Attribution License (CC-BY).

Phase I – Mouse decomposition on untreated versus sterile soils substrates (untreated vs. sterilized treatment groups)

By comparing mouse decomposition on untreated soil to mouse decomposition on soil subjected to three rounds of sterilization, we were able to draw conclusions about the influence of endogenous soil community on decomposition rates and decomposer microbial community composition. We have found evidence for increased rates of decomposition when mice decompose on soils with a normal microbial community. The time point that rupture occurred was estimated by measuring the pH of the soil because ammonia-rich, high nutrient fluids that are released during rupture increase the pH of the soil (Meyer et al. 2013). For the untreated soil, rupture occurred between days 6 and 9, while for the sterile soil treatment samples, rupture occurred between days 13 and 20 (Figure 7).

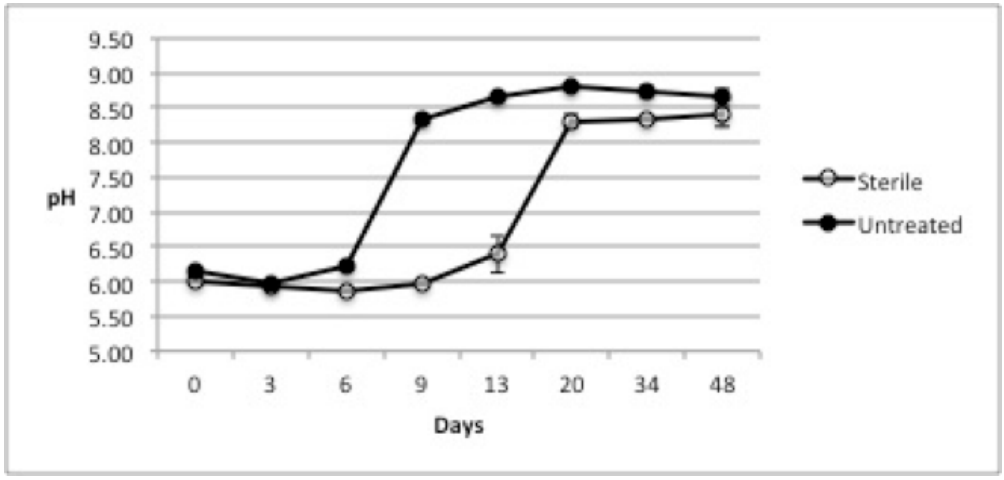

Figure 7. Average pH of soil over time with standard error. For the untreated soil group, a dramatic increase in pH occurred between day 6 and day 9, which is when rupture of body fluids and subsequent leakage into the soil likely occurred. For the sterilized soil, a dramatic increase in pH was detected between days 13 and 20. Therefore, rupture

occurred much later in the sterile treatment group. Figure generated for Lauber *et al*. *in review*.

Furthermore, visual key estimates of decomposition state (Figure 8) also supported the conclusion that decomposition progressed more slowly for mice placed on a sterilized treated soil.

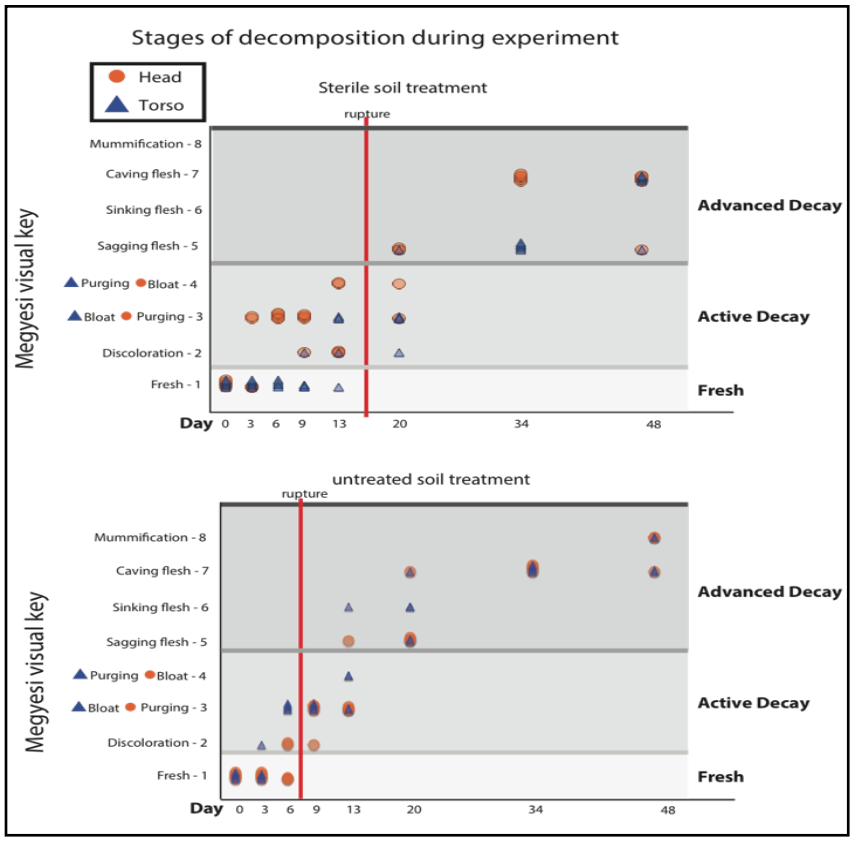

Figure 8. We used a visual body score estimate of decomposition following the Megyesi Key (Megyesi *et al*. 2005). Visual key estimates for the head (orange circle): Fresh - no discoloration (1 point); Active Decay - Discoloration (2 points), Purging of decomposition fluids out of eyes, nose, or mouth (3 points), Bloating of neck and/or face (4 points); Advanced Decay - Sagging of flesh (5 points), Sinking of flesh (6 points),

Caving in of flesh (7 points), Mummification (8 points). The key for the torso (blue triangle) is the same as above except that Bloating of abdominal cavity (3 points) precedes Rupture and/or purging of fluids (4 points). Gray boxes around points indicate generally with which stage of decay each time point is associated. Figure generated for Lauber *et al. in review*.

Our sterilization treatment resulted in a significant decrease in the alpha diversity of the treated soil relative to the untreated soil in both bacteria and microbial eukaryotic communities (Figure 9). Sterilization nearly or totally exterminated the soil microbial eukaryotic community, which could not be successfully amplified until was after corpse rupture at Day 20 of the experiment. During Advanced Decay, microbial gravesoil communities also differed dramatically. For bacterial communities, Firmicutes (mostly of the endospore-forming genus *Bacillus*) dominated after sterilization (0.7-99%) while the unsterilized soils contained similar abundances of major taxa in all three samples (Figure 9). In gravesoils subjected to sterilization treatments, four fungi (*Eurotiomycetes* 51%, *Leotiomycetes* 14%, *Dothidiomycetes* 12% and the *Sordariomycetes* 8.5%) dominated during Advanced Decay Figure 9). Although still dominated by fungal taxa, untreated soils hosted abundant taxa (>10% of total community in most samples) from *Metamonada*, *Metazoa*, *Apicomplexia*, and *Stramenopiles* (Figure 9).

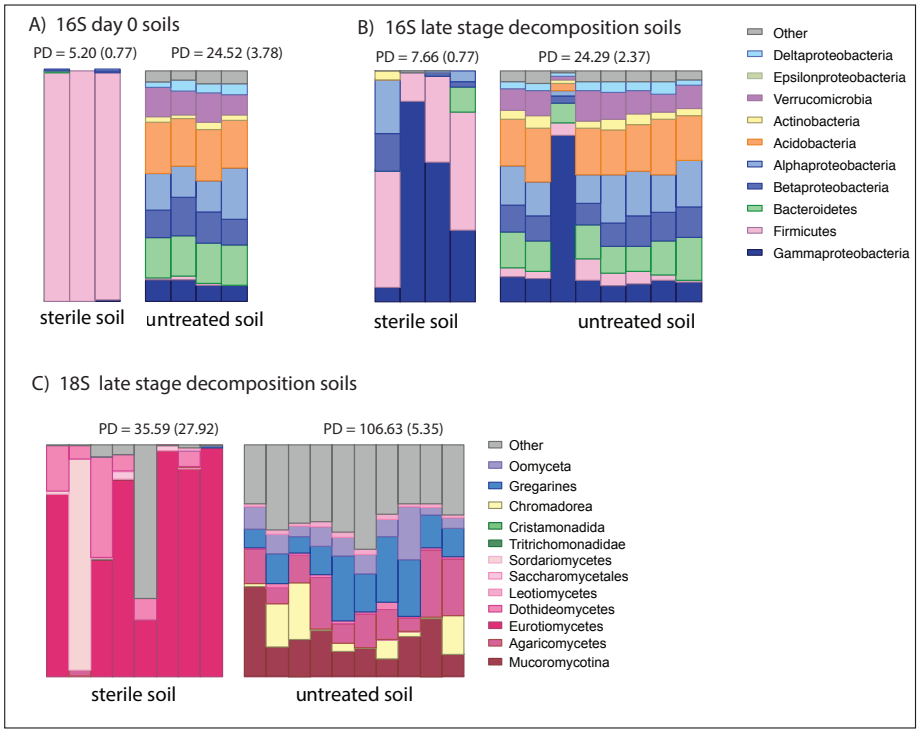

Figure 9. Effects of sterilization treatment on soils. Relative abundance of A) bacterial (16S) taxa in day 0 soils, and B) bacterial taxa in late stage decomposition soils and C) microbial eukaryotic (18S) late stage decomposition soils. Microbial eukaryotic DNA was not detected in day 0 soils after sterilization treatment. For 18S, taxa are shaded by Phylum (Fungi - red/pink, Excavata - green, Nematoda - yellow, Alveolata - blue, Stremenopiles - purple, and other low abundance taxa are represented as gray). Figure generated for Lauber *et al*. *in review*.

The effect of sterilized soils had a significant effect on the post-rupture host microbial communities. We compared the microbial communities at the two time points following

rupture (sterile group – days 20 and 34, untreated group -- days 13 and 20). We discovered that bacterial communities of skin and soils were different between the treatments (PERMANOVA p<= 0.024 in all cases) while those in the abdomen and the skin of the belly were not significantly affected by the presence of an endogenous soil bacterial community (Figure 10).

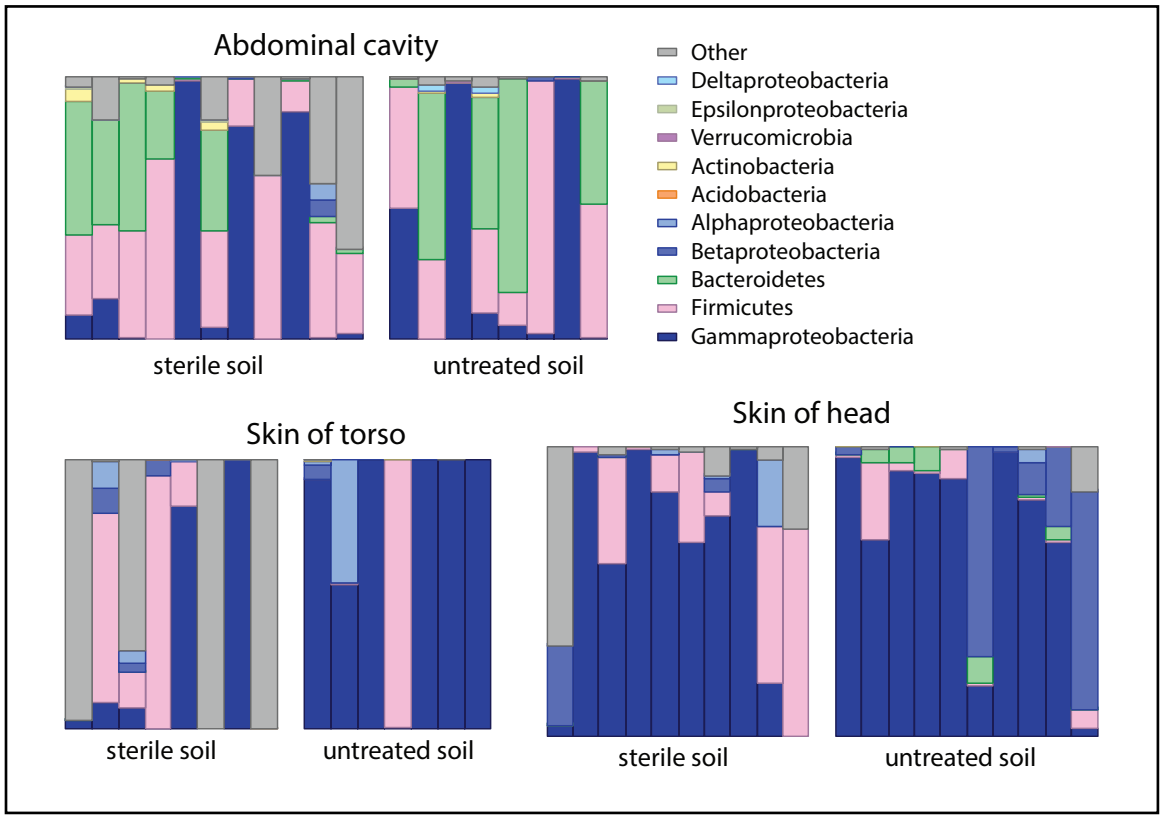

Figure 10. Relative abundance of bacterial Phyla for each treatment (sterile or untreated soils) in each sample site during late stage decomposition. Figure generated for Lauber *et al. in review*.

A comparison of the microbial eukaryotic community (Figure 11) unweighted UniFrac

distance showed that the skin of the torso (PERMANOVA p=0.017) and soil communities (PERMANOVA p=0.001) were significantly different between the treatments while the skin on the head community was only marginally affected (PERMANOVA p=0.059).

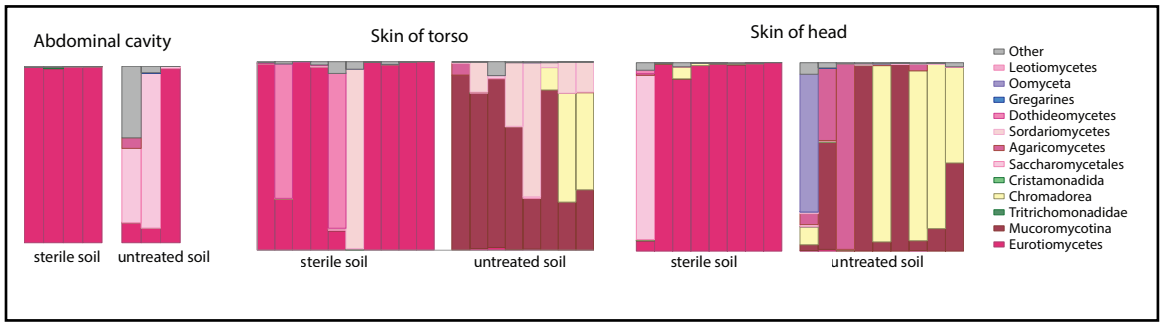

Figure 11. Relative abundance of microbial eukaryotic taxa are shown at the highest level of taxonomic resolution for each sample site and each treatment (sterile vs. untreated soil substrate). Taxa are shaded by Phylum (Fungi - red/pink, Excavata - green, Nematoda - yellow, Alveolata - blue, Stremenopiles - purple, and other low abundance taxa are represented as gray). Figure generated for Lauber *et al. in review*.

To estimate the proportion of each starting, endogenous bacterial community (i.e. soil, abdominal, skin) in each late stage decomposer community, we used the Bayesian source-tracking software, SourceTracker (Knights et al. 2011). Late stage decomposition communities were similar to day 0 skin communities and a community not well represented by any source (an "unknown" community) in both skin sites as well as the abdominal site. The late stage decomposition untreated soil community was very similar

to the starting soil community (Figure 12). In contrast, the late stage decomposition sterilized soil community was highly influenced by skin. Interestingly, the abdominal cavity did not appear to contribute substantially to the late stage bacterial decomposer community.

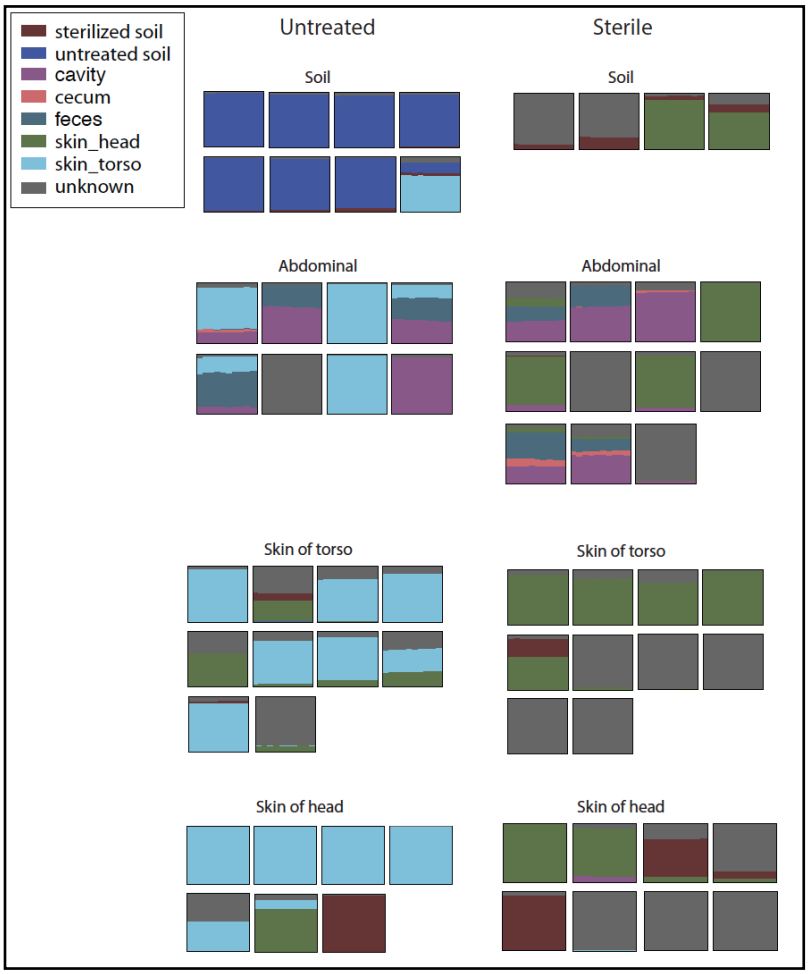

Figure 12. Bayesian source tracking analysis of late stage decomposition bacterial communities. Source communities included day 0 abdominal swab/liquid, skin, soils, and an unknown community not represented by the sources.

Phase 2 – decomposition of mice on three contrasting soils

We generated 16S, 18S, and ITS (targeting fungi) for 792 samples collected during the experiment. We had a much higher success rate for samples in the Phase 2 experiment than in Phase 1. For 16S amplicon sequencing rarified at 14,000 sequences per sample, 765 samples successfully sequenced, a >95% success rate (Phase 1 success rate was ~74%). For 18S rarified at 3000 sequences per sample, 637 samples sequenced successfully, a >75% success rate (Phase 1 had a 64% success rate). Unfortunately for ITS, we had a substantially lower success rate (~60%), which is likely an issue with the primer set and not the samples. The Knight Laboratory is currently optimizing and trouble-shooting fungal-specific primers that work better for a range of samples.

On the skin of the mice, bacterial communities changed very consistently across replicates during decomposition, resulting in a very striking PCoA plot of unweighted UniFrac distances (Figure 13).

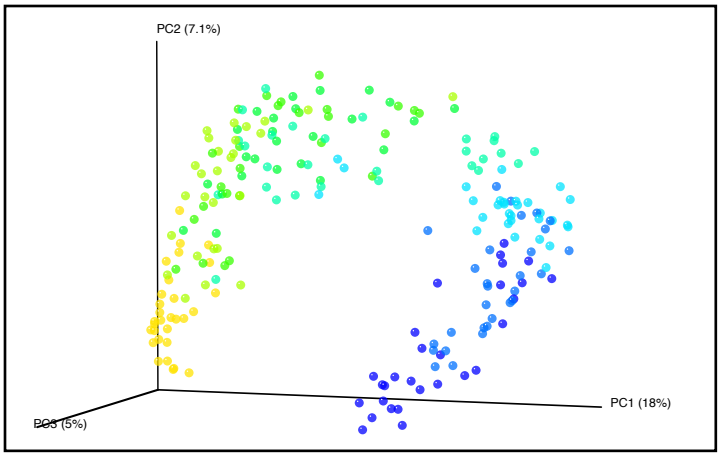

Figure 13. PCoA plot is colored by time over a gradient from yellow (day 1) to blue (day 71). Mantel test of Unifrac distance matrix and time (mantel Rho= 0.553 p<0.001) Significance: microbial communities sampled from skin (head and body) of mice corpses become differentiated from the starting community over the course of decomposition.

Skin on the head of the mouse and the torso of the mouse experienced similar bacterial community changes during decomposition (Figure 14).

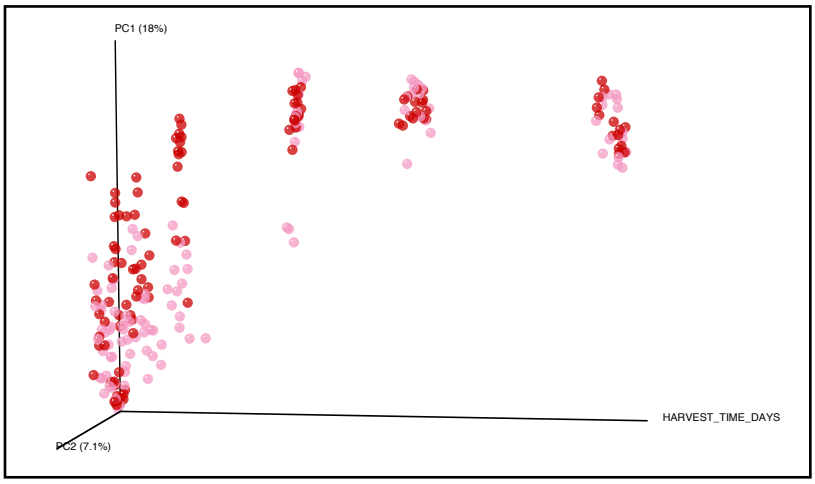

Figure 14. PCoA plot of skin bacterial communities (unweighted UniFrac) with time of decomposition as an explicit axis (red = skin of head, pink = skin of body). This is a different view of the data shown in the figure above (time is forced as an axis). Skin bacterial communities sampled from different body sites are highly similar at late stages of decomposition and fairly similar at early stages of decomposition.

Skin bacterial communities on the head and the torso of the mouse were not greatly influenced by soil type, although mice on desert soils show higher variability across replicates (Figure 15-16). Furthermore, late stage decomposer communities of mice on the desert soils were somewhat distinct (Figure 15-16).

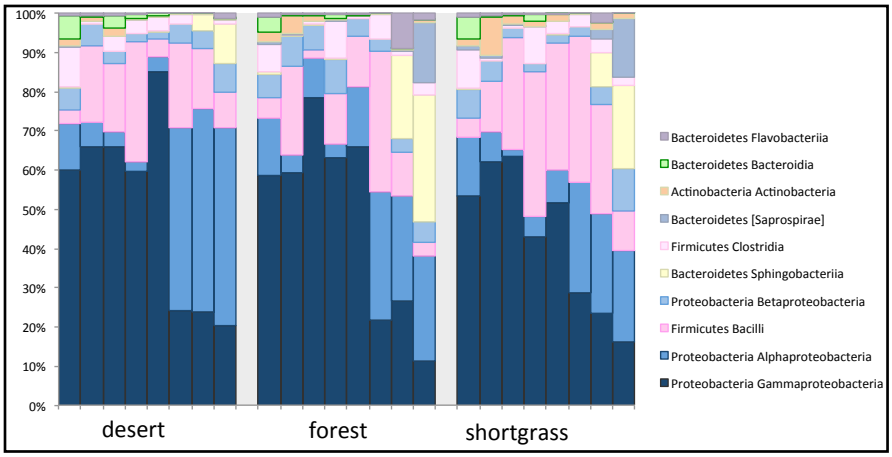

Figure 15. Relative abundance of skin bacterial Phyla averaged for each time point across the five skin of head and five skin of torso swabs. Results are shown by soil treatment. A similar change in relative abundance of skin taxa is detected during decomposition regardless of soil type. Figure prepared for Metcalf *et al. in prep*.

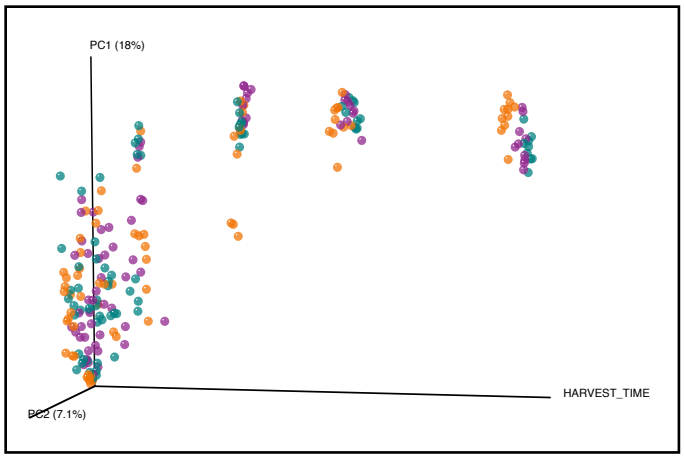

Figure 16. PCoA plot of skin UniFrac distances with time of decomposition on the x-axis. Skin communities are colored by soil type. Soil type influences the skin community, particularly at late stages of decomposition (when the corpse is starting to integrate into the soil). However, the skin bacterial communities are much more influenced by time than soil type.

During decomposition, bacterial communities sampled from soils with a corpse become highly differentiated from no-corpse soils quickly. This trend can be seen for each soil type (Figures 17-19).

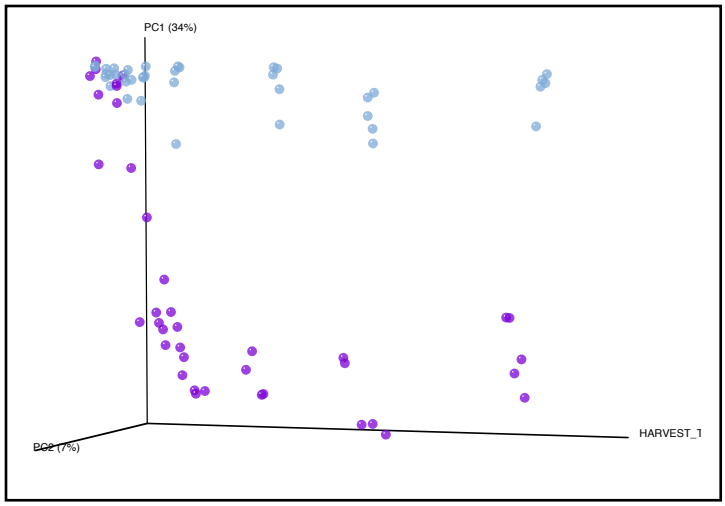

Figure 17. PCoA plot of shortgrass prairie soil bacterial (16S) community UniFrac distance (unweighted). Light blue dots indicate the no-corpse control soils samples. Darker purple indicates soil samples taken from underneath mouse corpses. Change in bacterial community is significantly correlated with time (mantel Rho= 0.248 p-value < 0.003). Control soils are significantly different than soils with corpses (permanova control vs. treatment soil, Pseudo-F 20.8, p< 0.001).

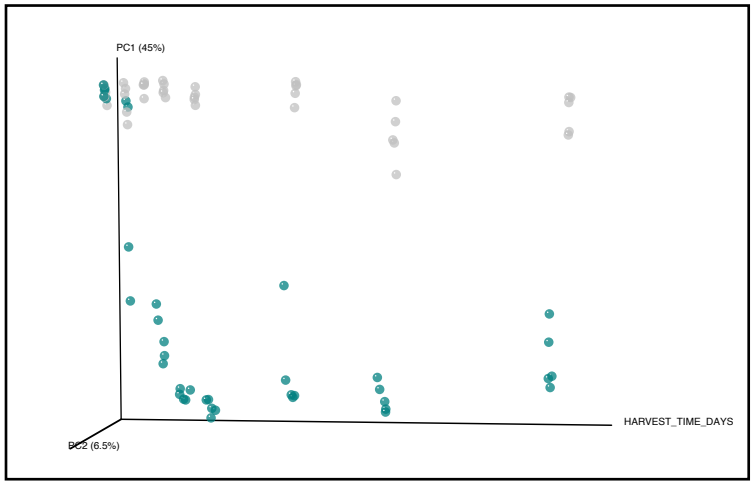

Figure 18. PCoA plot of forest soil bacterial (16S) community UniFrac distance (unweighted). Light gray dots indicate the no-corpse control soils samples. Darker teal dots indicate soil samples taken from underneath mouse corpses. Change in bacterial community is significantly correlated with time (mantel Rho= 0.333 p-value < 0.001).). Control soils are significantly different than soils with corpses (permanova control vs. treatment soil, Pseudo-F 32.5, p< 0.001).

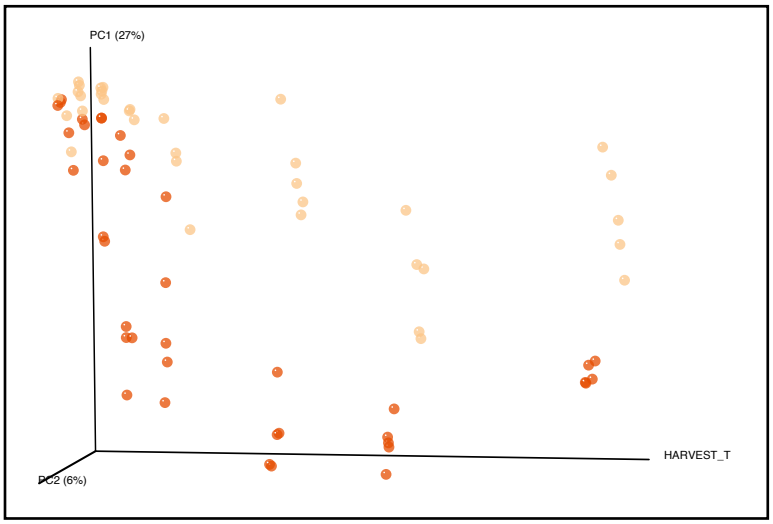

Figure 19. PCoA plot of desert soil bacterial (16S) community UniFrac distance (unweighted). Light tans dots represent the no-corpse control soils bacterial communities. Dark orange dots represent communities from soil samples taken from underneath mouse corpses. Change in bacterial community is significantly correlated with time (mantel Rho= 0.383 p-value < 0.001). Control soils are significantly different than soils with corpses (permanova control vs. treatment soil, Pseudo-F 7.2, p< 0.001).

Overall soil bacterial communities show similar patterns across contrasting soil types with a significant difference between corpse-associated and no-corpse late stage bacterial communities for each soil type. Furthermore, day 0 starting bacterial communities are quite different, but become more similar to each other during decomposition (Figure 20).

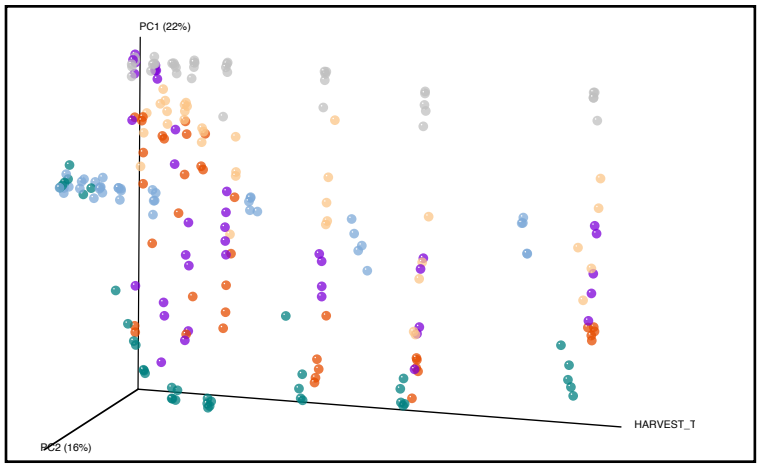

Figure 20. PCoA plot showing all soil samples with colors consistent with figures 14-16 with time of decomposition as an explicit x-axis. Light gray – forest no-corpse control, teal– forest soils with corpses, light blue – grassland no-corpse control, purple – grassland soils with corpses, light tan - Desert no-corpse control, orange – Desert soils with corpses. Soil bacterial communities start off highly different, but samples associated with decomposing corpses (bright colors) become more similar during decomposition.

The bacterial community of the mouse abdominal cavity was highly variable across replicates during decomposition (Figure 21). However, community change was still highly correlated with time (mantel Rho= 0.581 p-value < 0.001). These trends are consistent with results from the Phase 1 experiment.

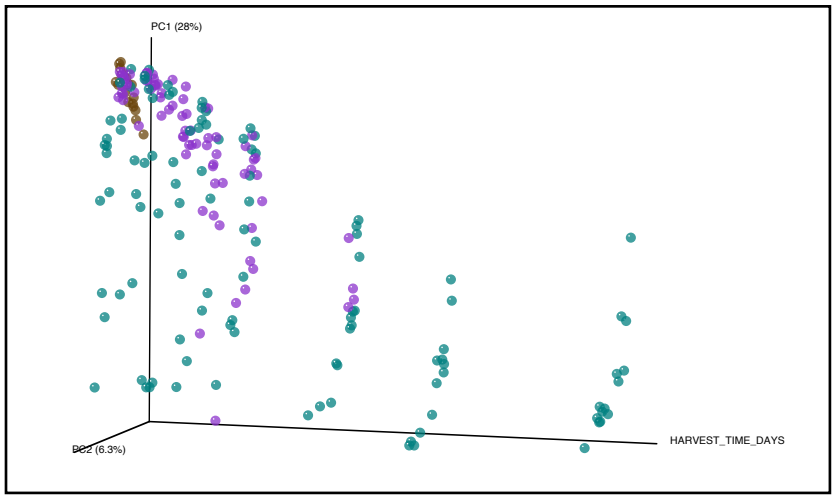

Figure 21. PCoA plots of abdominal bacterial communities (unweighted UniFrac) with time of decomposition as an explicit axis colored by specific abdominal sample type: brown – fecal (day 0 only), Purple – cecum (day 0 – day 30), Teal – abdominal cavity swab (day 0 – day 71). At early time points, cecum samples appear less variable than abdominal cavity samples.

Abdominal bacterial communities were not significantly influenced by soil type (permanova soil type, Pseudo-F 1.2, p=0.249), although there is some differentiation at the last two collection points (Figure 22).

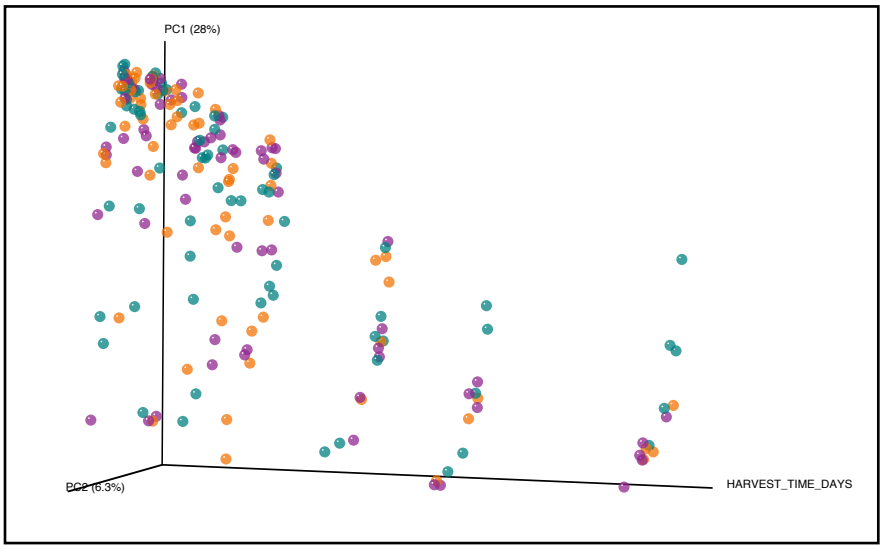

Figure 22. PCoA plots of abdominal bacterial communities (unweighted UniFrac) with time of decomposition as an explicit axis colored by soil type (orange = desert, purple = grassland, teal = forest).

18S

Microbial eukaryotic data revealed shifts from fungal dominated communities to nematode dominated communities during decomposition (Figure 23). After corpse rupture, when a nutrient pulse entered the soil, a bloom of nematodes appeared at every

sample site. The dominant nematode varied by soil type, but were all of the class Chromadorea and feeders of bacteria or fungi. For example, a fungal feeding nematode of the family Aphelenchidae dominated post-rupture grassland and desert samples. PCoA plot of soils (not shown) exhibited a similar trend, which is also evident in the taxa summary plots (Figure 25).

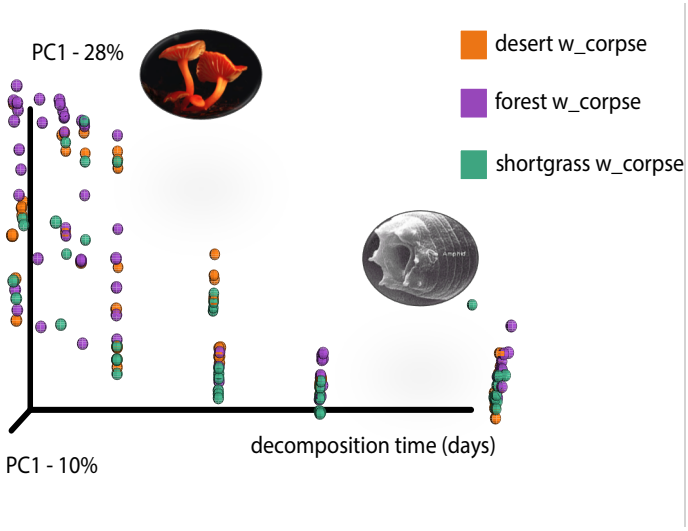

Figure 24. PCoA plot of microbial eukaryotic unweighted UniFrac distances. Spheres representing microbial eukaryotic communities of the skin are colored by soil treatment. A shift from a fungal to a nematode dominated community is driving the trend over time. A nematode of the class Chromadorea blooms during Active decay in each soil treatment.

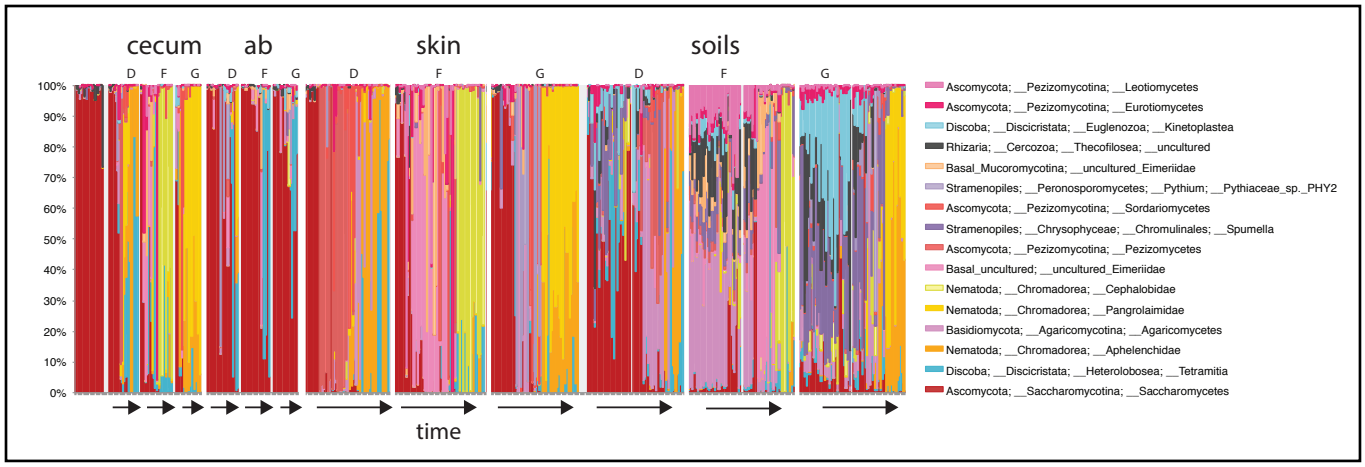

Figure 25. Relative abundance of microbial eukaryotes associated with each sample site during decomposition. Fungal taxa are represented by shades of red or pink and nematodes are represented with shades of yellow or orange. A shift from red to yellow is evident in each sample site.

ITS

From our ITS data, we detected a shift from fungi during decomposition, but each soil type was dominated by a different decomposer fungal community (Figure 26). Decomposer fungal communities in desert and shortgrass prairie soils and corpse skin were dominated by multiple unidentified taxa in the group Ascomycota, including Sordariomycetes. Forest soils, however, were dominated during late stage decomposition by Mortierella, a taxon in the group Zygomycota.

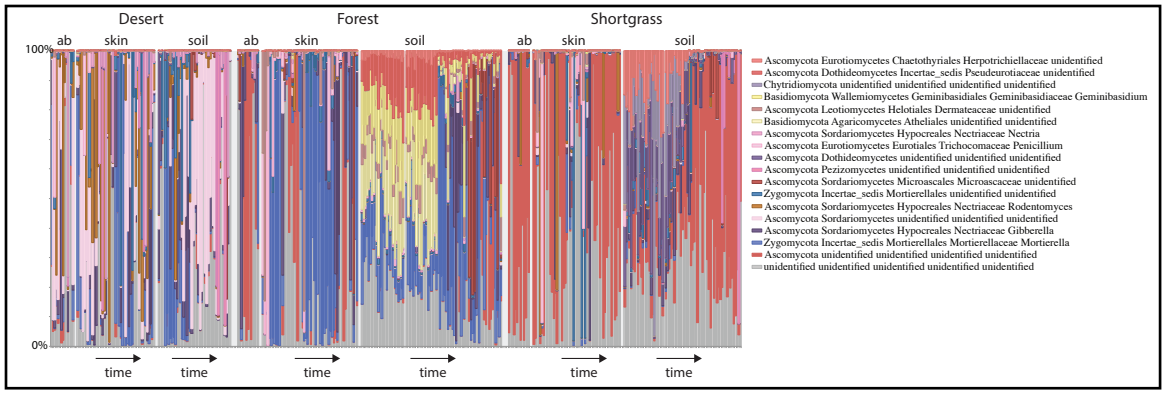

Figure 26. Relative abundance of fungal taxa (y-axis) at all sites in order from day 0 to day 71 of decomposition. Taxa in the group Ascomycota are shaded red/pink, Basidiomycota are shaded yellow, and Zygomycota are shaded blue.

Resampling of gravesoils after corpse removal over 30 days

For one pre-rupture and one post-rupture time point (day 4 and day 30), we resampled soils three additional times after the mouse cadaver was removed – at 10, 20, and 30 days. We discovered that for both the day 4 pre-rupture sample and day 30 post-rupture sample, gravesoil bacterial communities samples 10, 20, and 30 days after the mouse cadaver was removed remained significantly different than controls soils, but were also significantly different between resampling days (Table 3).

Table 3. Pseudo-F and p-values of PERMANOVA tests for significant differences between 1) resampled bacterial communities (within each soil type) and 2) between resample communities and control soils (within each soil type).

		Day 4 resample		Day 30 resample	
PERMANOVA (999 permutations)	**soil_type**	**Pseudo F**	**p-value**	**Pseudo F2**	**p-value 3**
are resample communities different from each other ?	desert	2.3691	0.001	2.7789	0.001
	forest		0.001	2.1215	0.001
	shortgrass	1.7451	0.001	2.5138	0.001
are resample communitiesdifferent from control soils?	desert	2.5157	0.005	14.8459	0.001
	forest	1.7166	0.028	47.9501	0.001
	shortgrass	1.8235	0.008	29.5946	0.001

Although bacterial communities differed significantly between resample days, the difference was much less than the difference between resamples and control soils (Table 3 – compare Pseudo F values, and Figure 27)

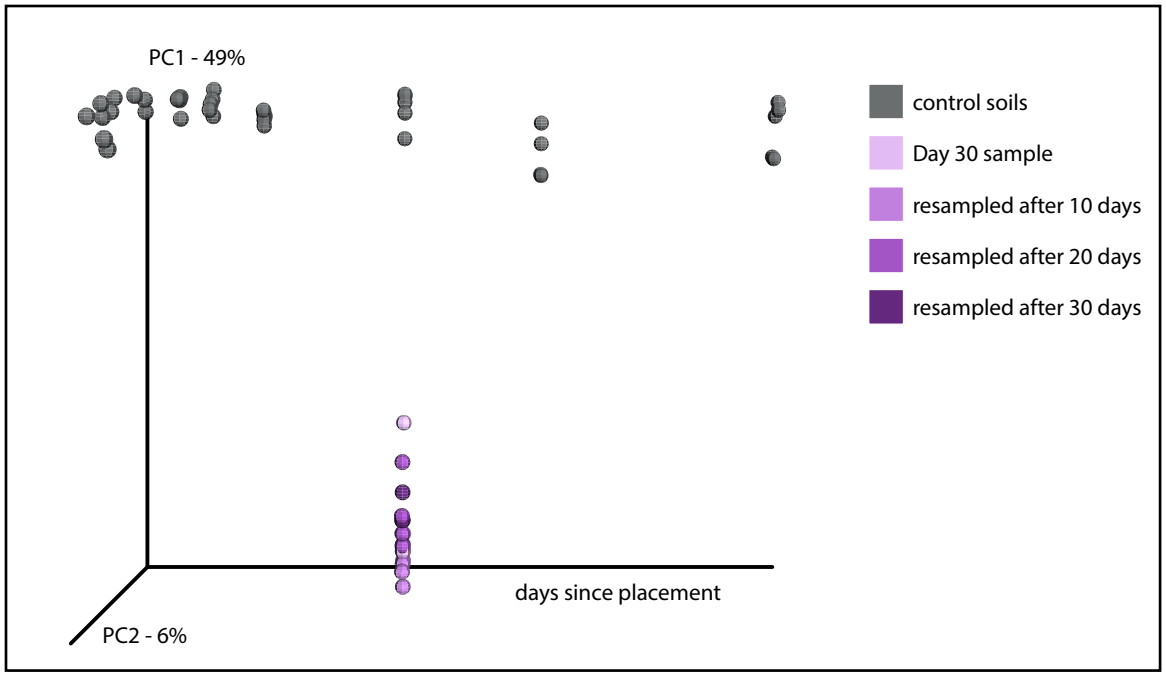

Figure 27. PCoA plot based on unweighted UniFrac displaying forest control soils, Day 30 of decomposition soils, and resampling soils after 10, 20, and 30 days of mouse cadaver removal. All resample soils cluster with the original Day 30 gravesoil and are remain highly differentiated from control soils.

Soil chemistry

We also characterized changes in biochemical properties of gravesoils during decomposition. We discovered a striking increase in nitrate soil concentrations during decomposition (Figure 28).

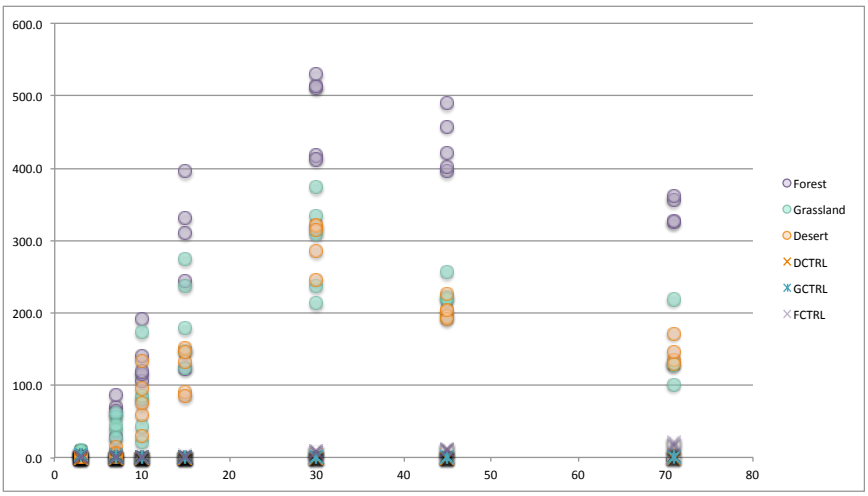

Figure 28. Ammonia levels for gravesoils (circles shaded by soil type) and control soils (crosses). Ammonia levels increase during decomposition and peak at the fifth samples point, which was day

Estimating PMI

We estimated the root mean square error (rmse) for predicting PMI for each sample site. We tested random forest, generalized boosting model, and cubist regression modeling approaches and discovered that each method resulted in similar rmse estimates. For 16S, we discovered that our error was between one and four days. We had the smallest rmse for the cecum, soil, and skin of head sites, and the largest rmse for the skin of torso and abdominal cavity sites (Figure X). This trend is fairly consistent with results from Phase 1, in which skin of head and soil provided the most accurate estimates of PMI. Because of the large rmse for estimating PMI with the abdominal cavity microbial communities in Phase 1, we sampled the cecum separately from the abdominal cavity (which is simply a

swab of the cavity) for the Phase 2 experiment. It appears that a much more predictable succession of bacteria is occurring in the cecum, which is connected to the intestinal bacterial community, than in the abdominal cavity, which is likely fairly sterile in living animals.

We repeated our PMI regression models with and without soil type as an importance feature. If a microbial decomposer community is uniquely defined by its environment, we expect that including soil type as an importance feature would improve (decrease) rmse. If microbial decomposer communities are universal, however, we expect that including soil type as a feature would not change the rmse. As shown in Figure 29, rmse estimates were almost identical with (left) and without (right) including soil type as an importance feature. This result implies that the bacterial decomposer community is universal, at least in the presence of a soil substrate.

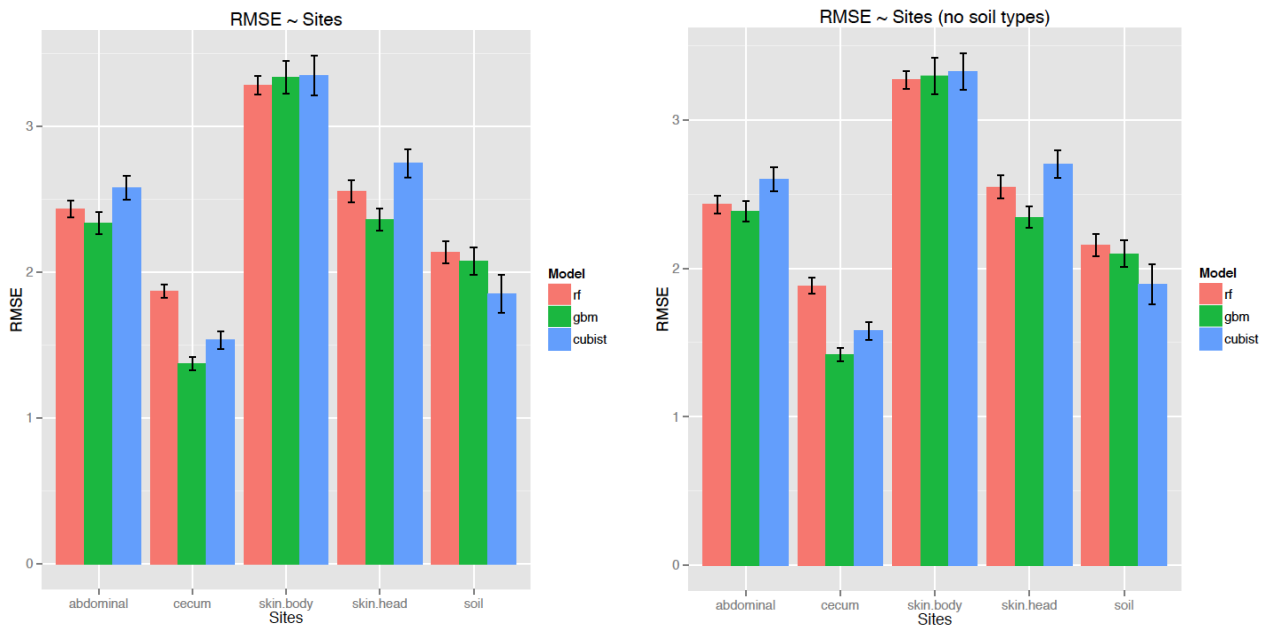

Figure 29. Root mean squared error for three regression models (rf – random forest, gbm – generalized boosting model, and cubist) for each site sampled in Phase 2. The graph on the left includes soil type as a feature in the model, whereas the graph on the right does not. The errors are not improved (decreased) when soil is included as a feature in the regression models, which suggests that starting soil community does not have a major influence on the bacterial decomposer community.

We also assessed how rmse varies during different stages of decomposition by estimating rsme for different windows of time. We find that errors are smallest during the Active Decay (earlier timepoints) stage of decomposition **(Figure 30).**

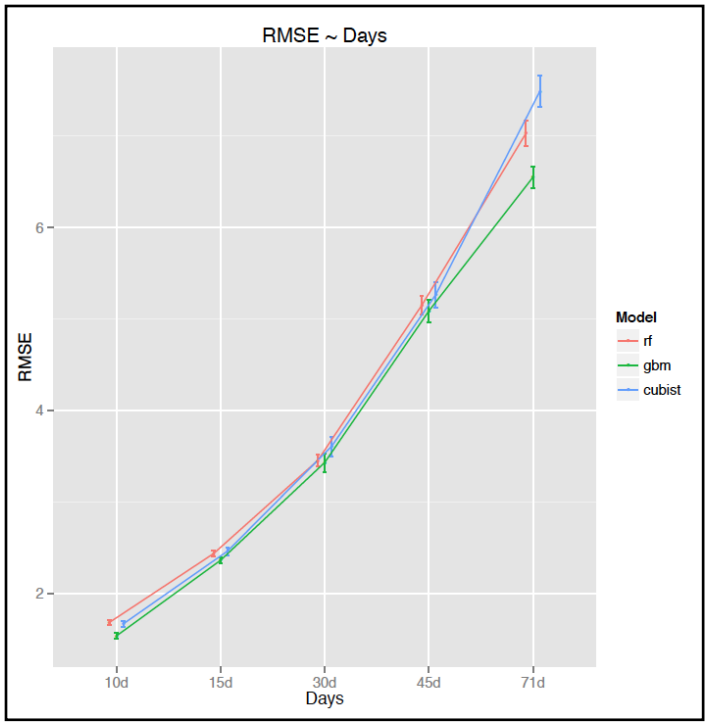

Figure 30. Root mean square error is assessed across decomposition, as increasingly more days of the experiment are included in the analysis. Errors are smallest when only the data during Active Decay is included, which suggest that the bacterial clock is most accurate during this stage of decomposition. Although errors become substantially larger during Advanced Decay, it is still notable that the error is a maximum of ~6 days over a 71 day experiment.

We estimated rmse of PMI predictions using the 18S microbial eukaryotic data as well (Figure 31). We discovered that we had similar prediction accuracy as with 16S data (rsme of ~2-3 days). In contrast to the 16S data, the skin of the torso provided the most accurate estimates of PMI (rsme ~2 days).

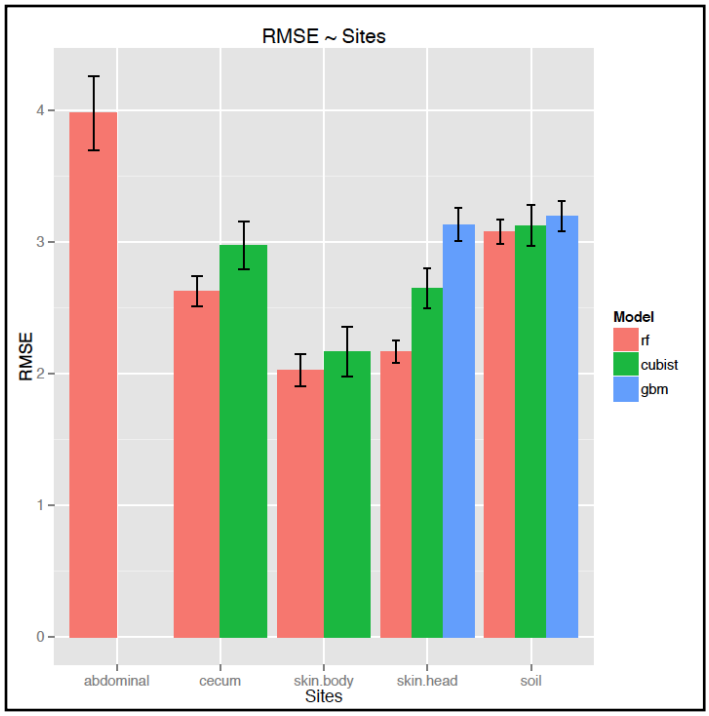

Figure 31. Root mean square error for PMI estimates using microbial eukaryote taxa as features. For several sample sites (abdominal cavity), we did not have sufficient numbers of successfully sequenced sampled to generate a robust model.

Future research will include combining 16S and 18S data sets to assess whether our prediction power is improved. Furthermore, we will explore the potential to estimate PMI with ITS fungal data, however, the lower sequencing success rate for this gene marker may prohibit robust modeling.

Phase 3 – decomposition microbiomes of different host species

Gravesoil associated with decomposing swine

We generated 16S bacterial and 18S microbial eukaryotic sequence data for soils collected from swine decomposition experiments that were originally performed by Co-PI Carter and colleagues in Lincoln, Nebraska (Meyer *et al*. 2013; Anderson *et al*. 2013). We investigated the effect of carcass mass on gravesoil microbial communities by decomposing carcasses of varying masses - 1 kg, 20 kg, 40 kg, and 50 kg. To understand the seasonal dynamics of the gravesoil microbiome we investigated the structure of microbial communities associated with gravesoil and control soils between summer and winter.

We compared bacterial gravesoil communities to control soils and discovered that they were significantly different ($P < 0.001$). The abundance of several dominant taxa in gravesoils was significantly different to control soils, but none of these differences were observed between control soils and 1 kg carcasses. Rather these differences were typically associated with 20 kg, 40 kg, and 50 kg carcasses. The decomposition of these larger carcasses significantly ($P < 0.05$) lessened the abundance of "*Candidatus* Chthoniobacteraceae", Gaiellaceae, "*Candidatus* Nitrososphaera", Acidobacteria, and Rhodoplanes relative to control soils. Increases in the abundance of Planococcaceae, Bacillaceae, *Sporosarcina* sp., *Ignatzschineria* sp., *Acinetobacter* sp., and Chitinophagaceae (Figures 32-33).

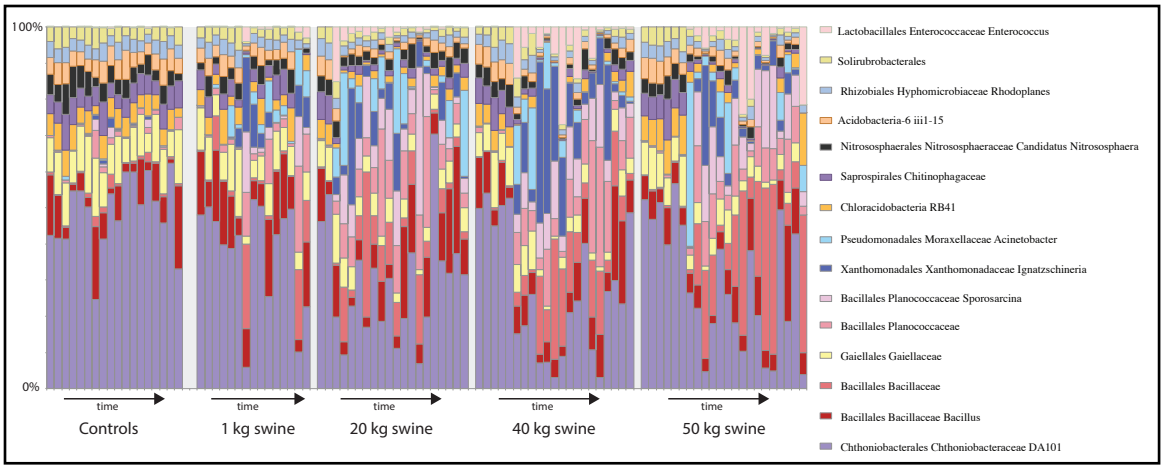

Figure 32. Relative abundance of bacterial genera during decomposition for control soils and for each mass treatment group (1, 20, 40, and 50 kg swine). This figure was prepared for Carter *et al*. *in prep*.

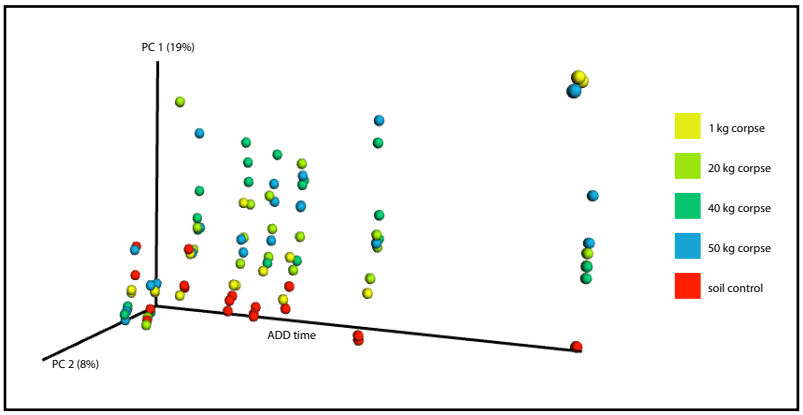

Figure 33. PCoA plot of bacterial community unweighted UniFrac distance with spheres representing soil samples, which are colored by mass treatment (1, 20, 40, and 50kg) or control (no cacaver). This figure was prepared for Carter *et al*. *in prep*.

Gravesoil microbial eukaryote communities were also significantly ($P < 0.001$) different from control communities (Figure 34). However, the abundances of individual dominant

taxa were not significantly greater than in control soils, even though a conspicuous increase in the abundance of nematode Rhabditidae was observed (Figure 35). Also there was no significant difference between carcasses of contrasting mass.

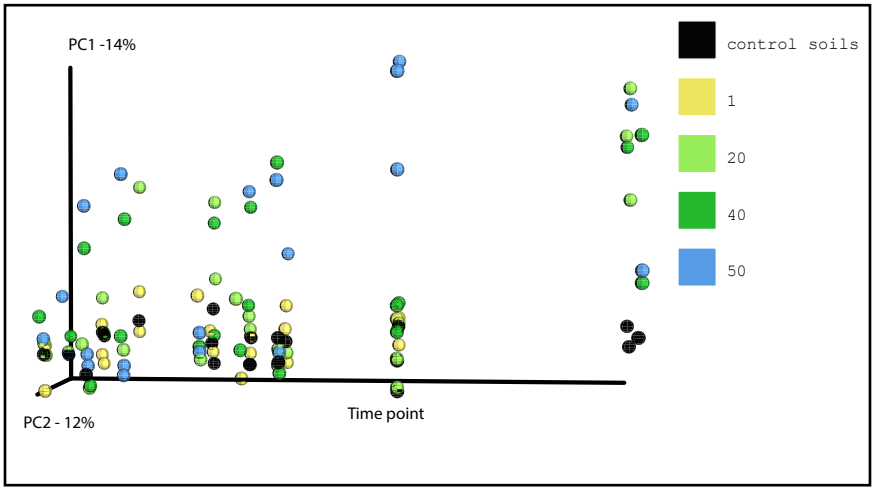

Figure 34. PCoA plot of microbial eukaryotic unweighted UniFrac distance with spheres representing graveoil or control soil samples.

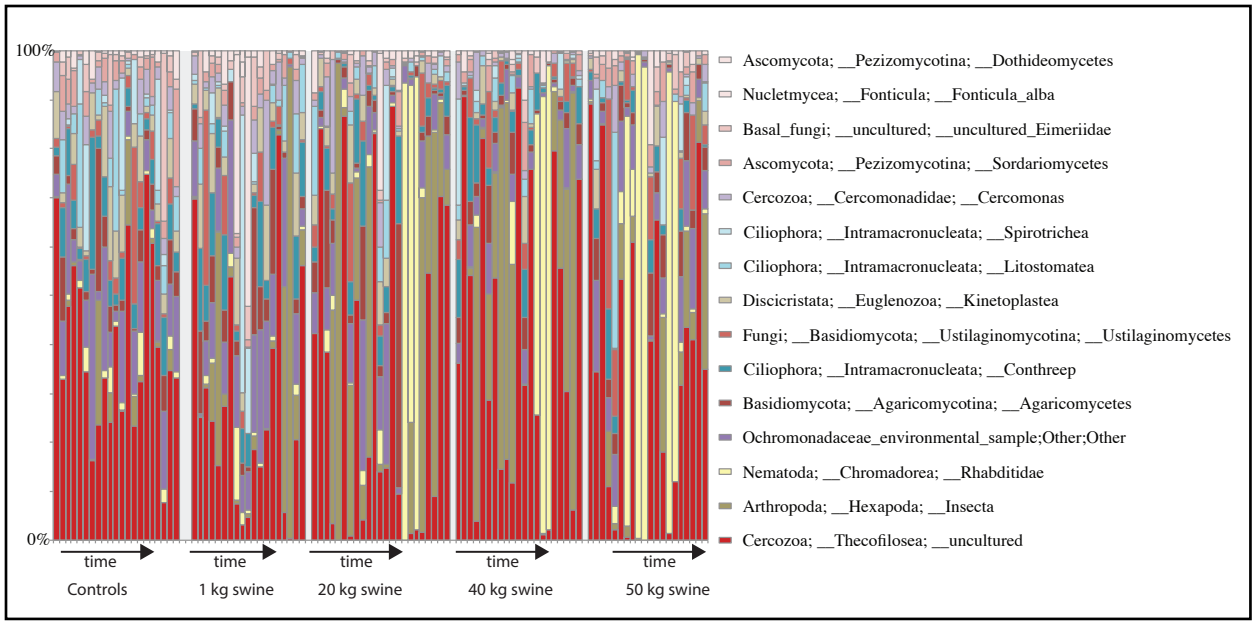

Figure 35. Relative abundances of microbial eukaryotic taxa in control soils and grave soils associated with decomposing swine of different masses. Taxa are shaded according to major groups - Fungi (red/pink), Excavata (green), Nematoda (yellow), Alveolata (blue), Stramenopiles (purple), Rhizaria (gray/black), Amoebozoa (tan), Insecta (green), Discoba (teal), Basal (orange).

We also generated and analyzed data for swine gravesoil during different seasons, an experiment originally described in (2013). We hypothesized that soils associated with pig carcasses decomposing in the summer season exposed to 1412 accumulated degree days (ADD) would undergo more substantial microbial community change than soils associated with pig carcasses decomposing in the winter exposed to only 325 ADD. For comparisons between control soils between seasons and gravesoils and control soils within season, we report p-values resulting from PERMANOVA analyses of unweighted UniFrac distances below and Two-Way ANOVA.

First, we investigated the differences in control (no corpse) soil communities between the winter and summer seasons. The structure of the bacterial communities in control soils was significantly ($P < 0.05$) different between summer and winter. Among the dominant ($\geq$ 1% of total data set) taxa this was observed only as a significantly ($P < 0.001$) greater abundance of the bacterial order Solirubrobacterales (phylum Actinobacteria) (Figure 36) in the winter; seasonal variation was primarily due to shifts in rare bacterial lineages.

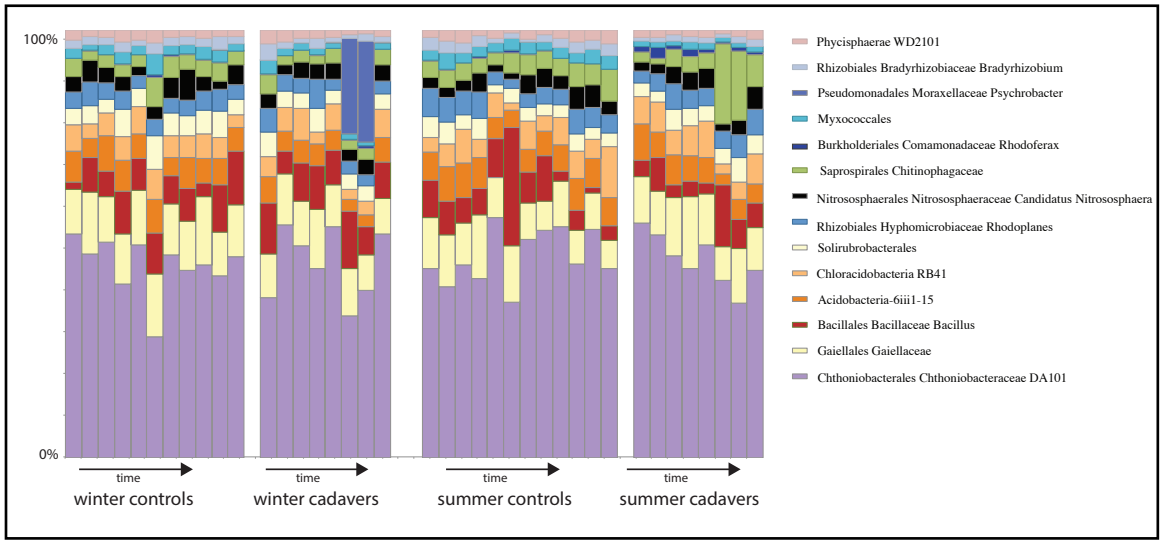

Figure 36. Relative abundance of bacterial taxa in control soils and gravesoils during decomposition in winter (left) and summer (right). Different taxa become abundant during decomposition in the different seasons.

In contrast, the structure of the eukaryotic microbial communities in control soils was not significantly ($P = 0.375$) different between seasons (Figure 37). Among the dominant eukaryotic taxa, however, one significant ($P < 0.05$) difference was observed: the

abundance of the protozoan Kinetoplastia (phylum Euglenozoa) was greater in the summer.

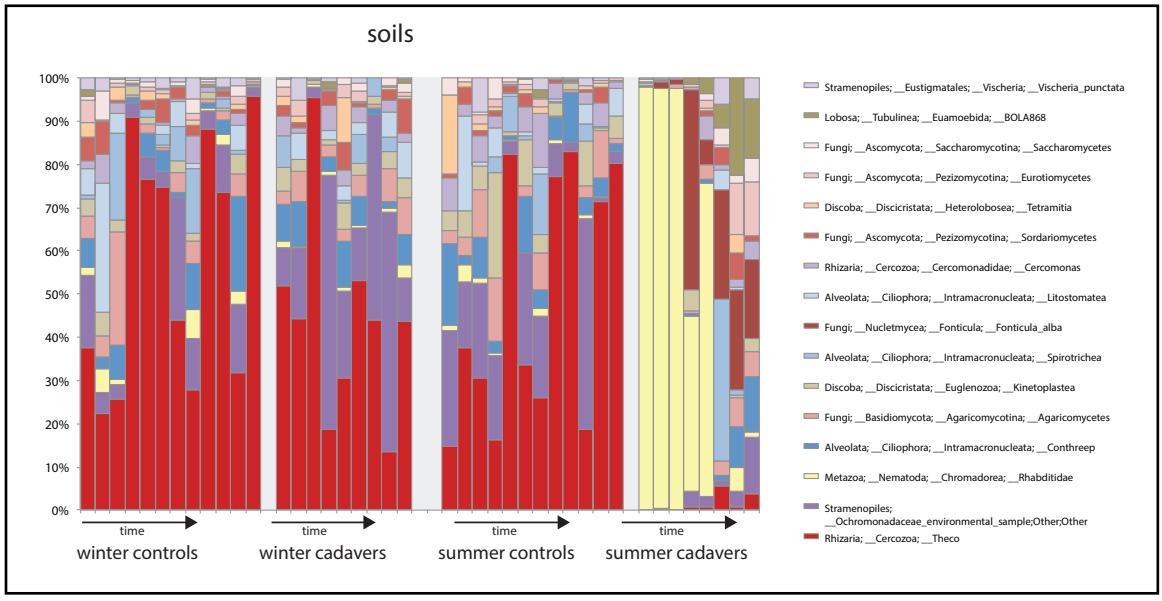

Figure 37. Relative abundance of microbial eukaryotic taxa during decomposition in control and gravesoils in winter (left) and summer (right). A nematode bloom was only detected in the gravesoils collected during the summer season.

Next, we compared bacterial communities in gravesoils to control soils in each season. Summer gravesoils associated with post-rupture time points were significantly ($P < 0.001$) different than summer control soils (Figure 38). Summer gravesoils contained a significantly ($P < 0.001$) greater abundance of bacteria *Sphingobacterium* sp. but carcass decomposition lessened the abundance of several dominant bacterial taxa. The abundances of Phycisphaerae (Planctomycetes; $P < 0.05$), *Bradyrhizobium* sp. (Proteobacteria: Alphaproteobacteria; $P < 0.01$), Myxococcales (Proteobacteria:

Deltaproteobacteria), and "*Candidatus* Chthoniobacteraceae" (Verrucomicrobia; $P < 0.01$) were significantly lower in gravesoils. Winter gravesoil communities were also significantly ($P < 0.01$) different than control soils. Winter gravesoil contained a significantly ($P < 0.05$) greater abundance of *Psychrobacter* sp. (Proteobacteria: Gammaproteobacteria) than control soils. These gravesoils also contained significantly lesser abundances of Solirubrobacterales ($P < 0.05$) and Myxococcales ($P < 0.05$) than control soils.

Bacterial gravesoil communities were significantly ($P < 0.001$) different between seasons. The summer gravesoils were associated with a significantly ($P < 0.01$) greater abundance of bacterial family Chitinophagaceae (Bacteroidetes: Sphingobacteriales) relative to winter gravesoils. Winter gravesoils contained a significantly greater abundance of bacteria *Sphingobacterium* sp. (Bacteroidetes: Sphingobacteriales; $P < 0.001$), *Psychrobacter* sp. ($P < 0.05$), and "*Candidatus* Chthoniobacteraceae" ($P < 0.05$) relative to summer gravesoils.

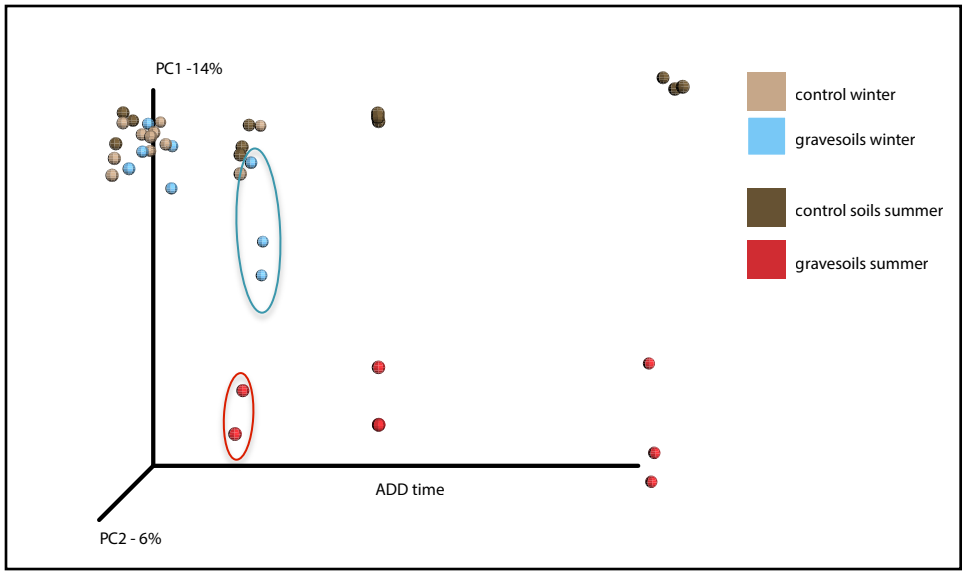

Figure 38. PCoA plot of unweighted UniFrac distances of control and gravesoil samples. Additive degree day (ADD) is shown as an explicit x-axis. Both winter and summer gravesoil bacterial communities become different than control soils during decomposition. At ADD 325, winter samples (blue ellipse) and summer samples (red ellipse) do not host similar bacterial communities.

Gravesoil microbial eukaryote communities were significantly ($P < 0.001$) different to control soils in the summer but not ($P = 0.134$) in the winter. Summer gravesoil microbial eukatyotic communities were associated with a significantly greater of several taxa relative to control soil including nematode Rhabditidae ($P < 0.001$), slime mold *Fonticula alba* ($P < 0.001$), amoeba Euamoebida ($P < 0.001$), fungus Eurotiomycetes ($P < 0.01$), and fungus Tremellomycetes ($P < 0.01$). One of the dominant eukaryotic taxa was significantly ($P < 0.01$) greater in control soils relative to gravesoil: protozoa Thecofilosea.

Gravesoil eukaryote communities differed significantly between seasons. Several differences were observed between the eukaryotic communities in summer and winter gravesoils. Summer gravesoils contained a significantly greater abundance of nematode (Rhabditidae; $P < 0.001$), protozoa (Thecofilosea; $P < 0.05$), slime mold (*Fonticula alba*; $P < 0.001$), amoeba (Euamoebida; $P < 0.001$) and fungus (Tremellomycetes; $P < 0.01$) (Figure 39). Winter gravesoil were associated with a greater abundance of the fungus from class Dothideomycetes ($P < 0.05$) (Figure 39).

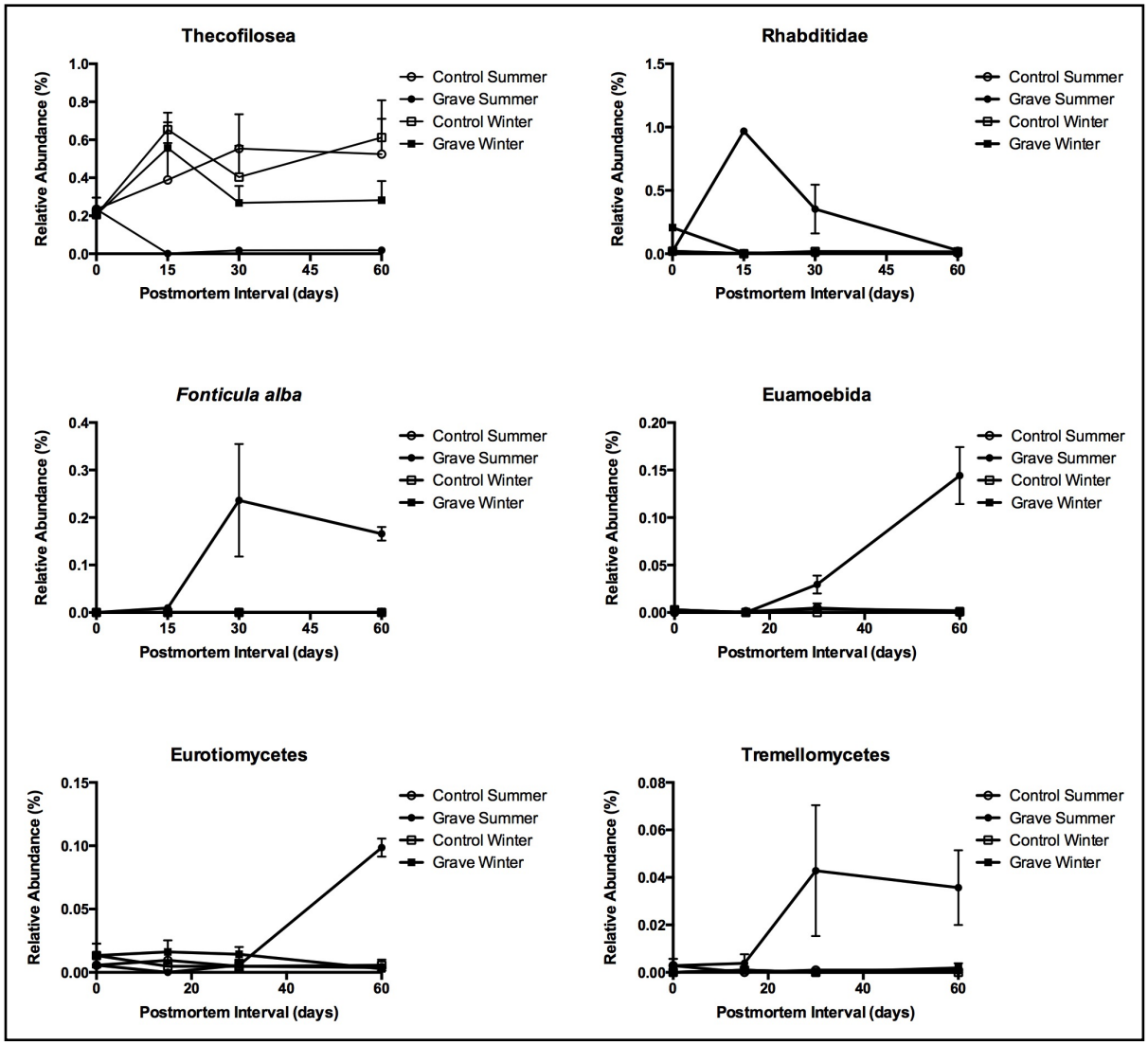

Figure 39. Eukaryotes associated with significant differences between seasons and/or carcass treatment in soils underneath swine (*Sus scrofa domesticus*) carcasses placed on he soil surface of a pasture near Mead, Nebraska, USA in the summer (June) or winter (February). Bars represent standard errors.

Gravesoil and skin samples associated with decomposing humans

We sampled skin, gravesoils, and control soils from decomposing human cadavers in Huntsville, Texas during two seasons, winter and spring. Samples associated with two cadavers were collected during each season. For comparisons of gravesoils to control soils, a PERMANOVA test was utilized and we report p-values below.

Winter (February) experiment

Bacterial communities associated with skin and gravesoils changed substantially during decomposition (Figure 40), and post-rupture gravesoil bacterial communities were significantly different from control soils ($p < 0.001$).

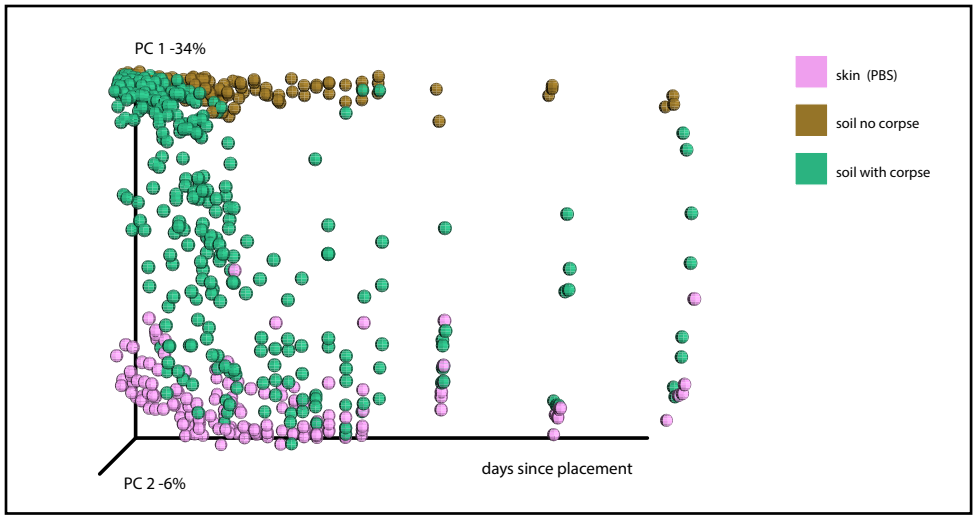

Figure 40. PCoA plot based on unweighted UniFrac distances displaying skin, gravesoil, and control soil samples with days since cadaver placement (time of decomposition) on the x-axis.

Gravesoils were collected from four areas near each decomposing cadaver – left of the hip, knee and head as well as the right hip (Figure 41). All gravesoil sample sites become differentiated from control soils during decomposition.

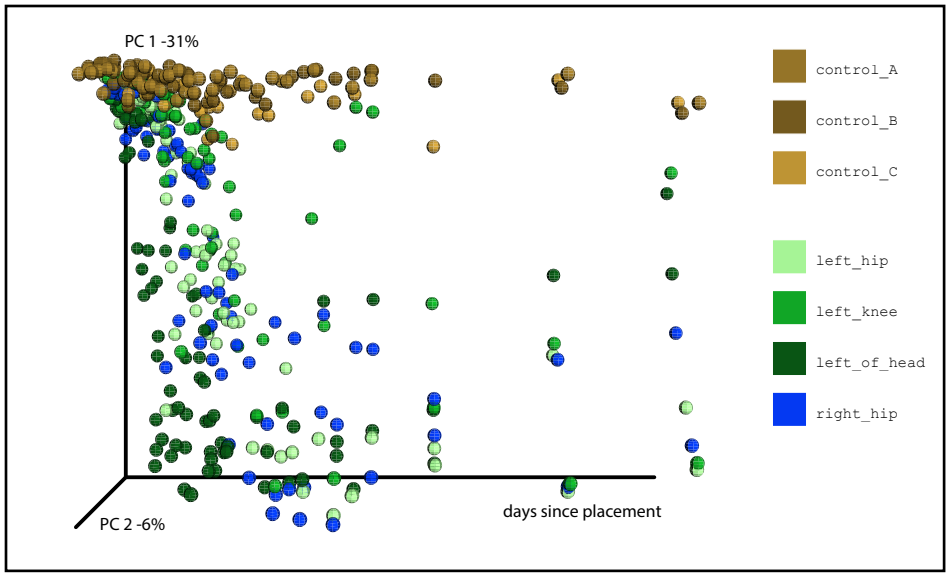

Figure 41. PCoA plot based on unweighted UniFrac distances showing control soils and graveoil soils colored by collection site.

During decomposition, the relative abundance of taxa in the Phylum Firmicutes increased in both gravesoils and skin sites (Figure 42). In gravesoils, the relative abundance of Bacteroidetes taxa also increased. In skin, taxa in the Phylum Actinobacteria increased in relative abundance.

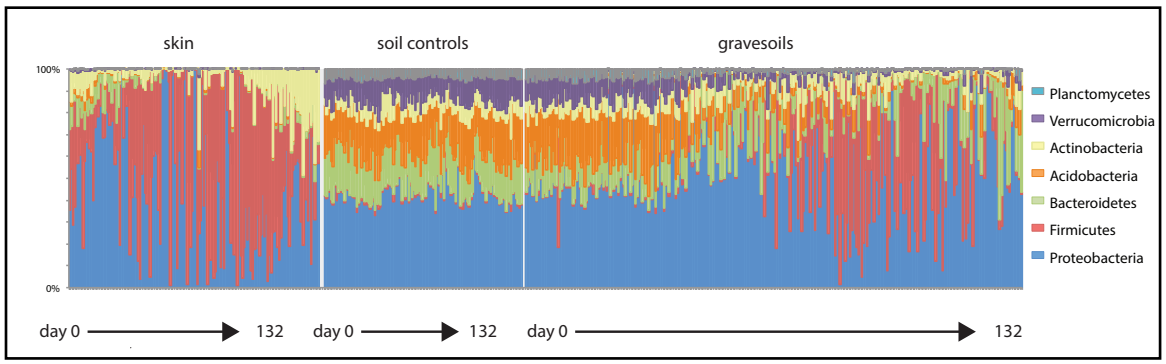

Figure 42. Relative abundance of bacterial Phyla during decomposition in skin, soil controls, and gravesoils. An increase in relative abundance of taxa in the Phylum Firmicutes was detected in both skin and soils. Only Phyla comprising >1% of the community are shown.

18S

Skin microbial eukaryotic communities were dominated by fungal taxa (Figure 43). Control soils and early decomposition gravesoils hosted a diverse microbial eukaryotic community that included numerous taxa in the groups stramenopiles, alveolates, discobes, nematodes, and fungi (Figure 43). Late stage decomposition gravesoils, however, are dominated by fungi and nematodes (Figure 43).

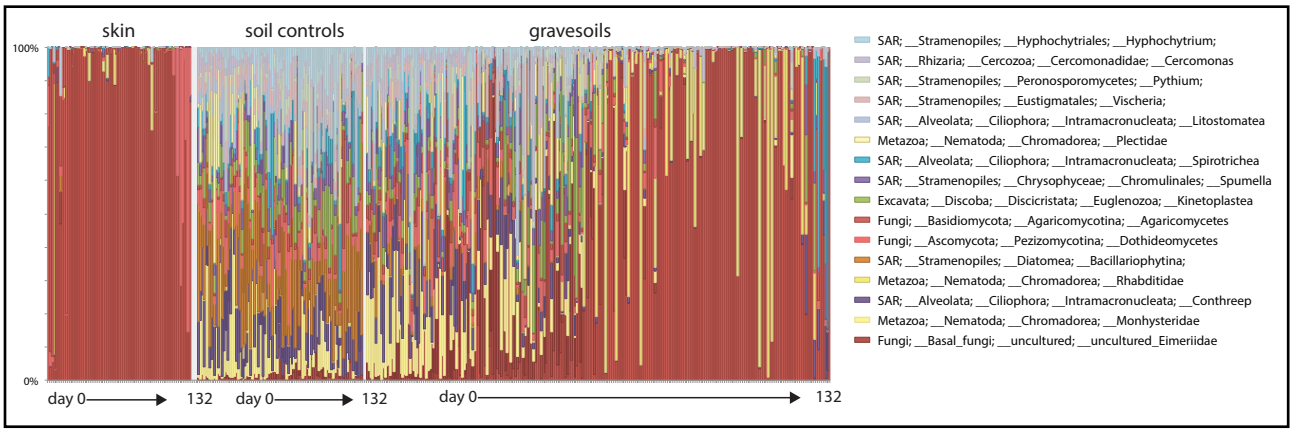

Figure 43. Relative abundance of microbial eukatyotic taxa during decomposition in skin, soil controls, and gravesoils. Fungal taxa are shaded in red/pink and nematode taxa are shaded in yellow. Only phyla comprising >1% of the community are shown.

Spring (April) experiment

Bacterial communities of skin and gravesoils associated with decomposing cadavers changed during decomposition (Figure 44). Gravesoils were significantly different than control soils ($p < 0.001$).

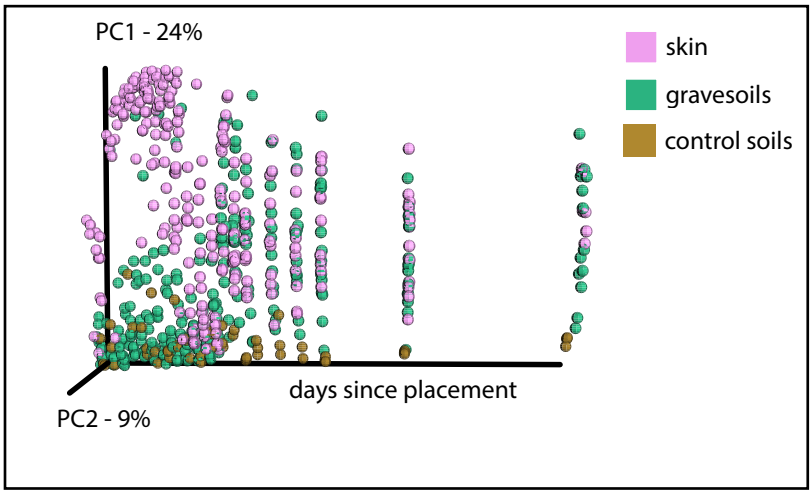

Figure 44. PCoA plot based on unweighted UniFrac distances showing control soils and graveoil soils colored by collection site.

Each location from which gravesoil was collected changed in a similar manner during decomposition (Figure 45).

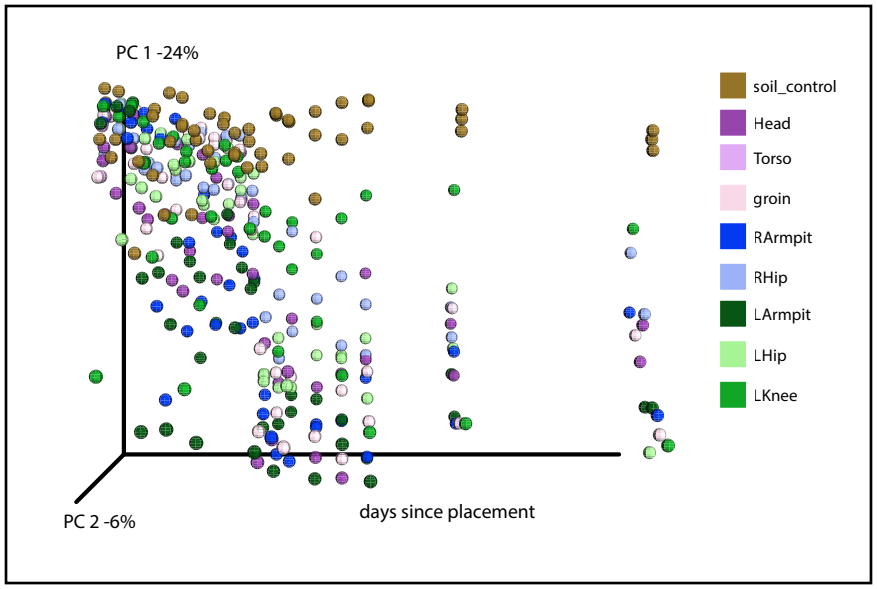

Figure 45. PCoA plot based on unweighted UniFrac distances showing control soils and graveoil soils colored by collection site.

As in the winter (February) experiment, taxa in the Phylum Firmicutes and Actinobacteria increased in relative abundance during decomposition at all skin sites (Figure 46). We did not see an increase in the relative abundance of Firmicutes in the gravesoils, however. We detected an increase in taxa of the Phyla Bacteroidetes (as detected in the winter experiment), Proteobacteria and Actinobacteria (Figure 46).

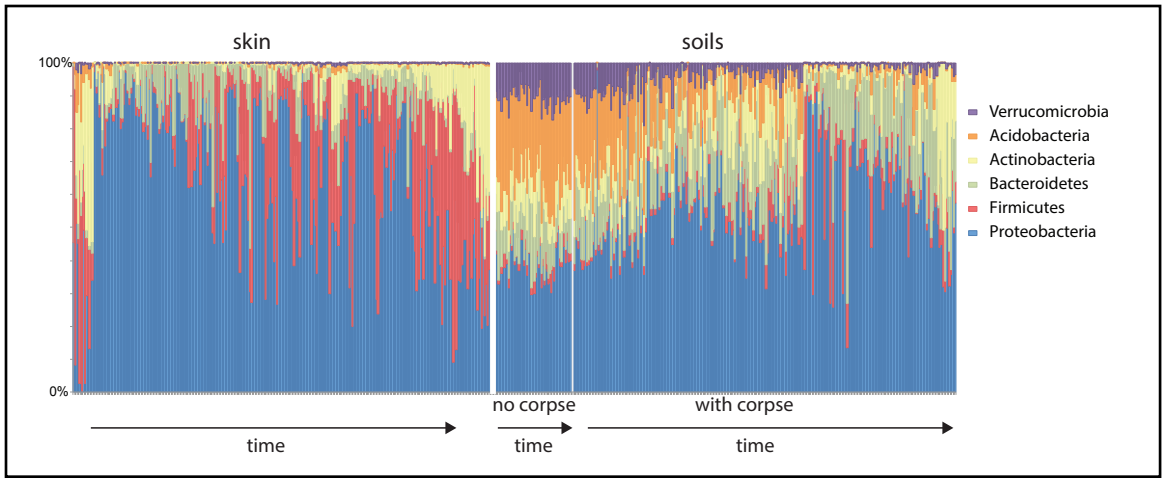

Figure 46. Relative abundance of bacterial Phyla in skin, control soils, and gravesoils.

18S

Skin microbial eukaryotic communities are dominated by nematodes and fungi during Active Decay (Figure 47). During Advanced Decay, fungi almost completely dominate all skin sites (Figure 47). Gravesoils also experience an increase in relative abundance of nematodes and fungi, and become almost completely dominated by fungi in very late Advanced Decay (Figure 47).

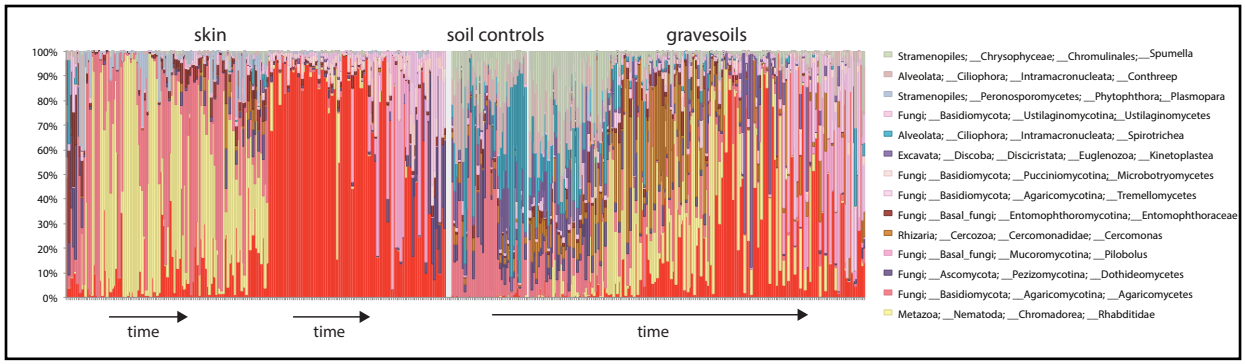

Figure 47. Relative abundance of microbial eukatyotic taxa in skin, soil controls, and gravesoils during decomposition. Fungal taxa are shaded in red/pink and nematode taxa are shaded in yellow. Only Phyla comprising >1% of the community are shown.

PMI estimates

We discovered that we had excellent predictive power for estimating PMI for both human cadaver experiments. Similar to Phase 2 experiment results, we discovered that our ability to predict PMI was more accurate during decomposition. Hence, we used the first three weeks of sample collections to generate 16S based regression models with errors of less than 4 days (Figure 48, Figure 49).

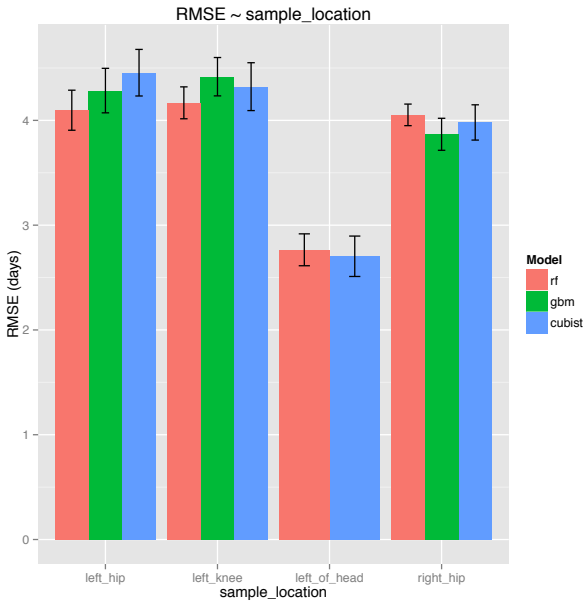

Figure 48. Root mean square error (rmse) for each sample location from which skin and soil were collected. Sample type (skin or soil) was included as a feature in the regression model. The soil collected from the left of the head provided the most accurate prediction.

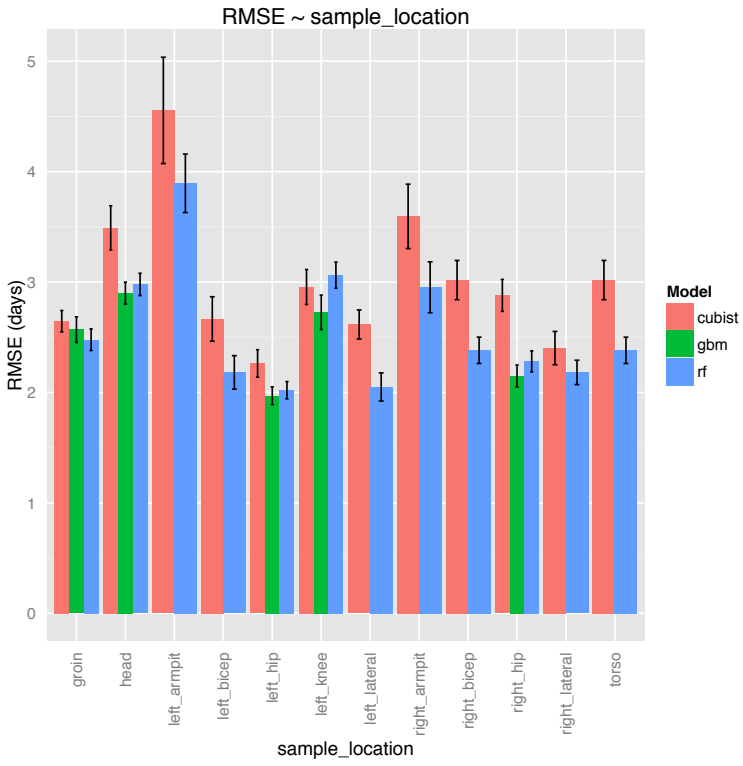

Figure 49. Root mean square error (rmse) for each sample location from which skin and soil were collected. Sample type (skin or soil) was included as a feature in the regression model. The soil collected from the left of the armpit was the least accurate for predicting PMI.

IV. Conclusions

In three phases of research we determined that 1) bacterial and microbial eukaryotic decomposer communities change in a predictable manner as corpse decomposition proceeds, 2) decomposer communities modify the endogenous soil community in significantly detectable ways, and 3) microbial decomposer communities are universal, at least to some extent (e.g. soil type).

With our Phase 1 experiment, we provided a proof-of-principle demonstration that microbial community ecology of decomposing corpses has potential to be developed into a complementary forensic tool for estimating the postmortem interval (PMI). The approach described in Metcalf *et al*. (2013) has several advantages that make widespread adoption of these techniques likely in the future, including continued rapid decline in the cost of DNA sequencing and the ease and familiarity of swab-based sampling at potential crime scenes to law enforcement officers. Given the accuracy of our estimates of PMI, our work suggests a potentially expanded role for microbiological forensic indicators within the criminal justice system.

As part of Phase 1, we also discovered that when a carcass in the absence of a soil substrate, the decomposer community is far less diverse and is dominated by fungal taxa and bacterial taxa from the Phyla Firmicutes (primarily Bacillaceae) and Proteobacteria (primarily Enterobacteriaceae). The less diverse decomposer community associated with the sterile treatment was associated with a slower rate of carrion decomposition. The

mice placed on an endogenous soil community reached late stage decomposition 2-3 times more quickly than those placed on sterilized soil. Soil invertebrates play a critical role in breaking down much of the plant detritus deposited on the soil surface (Smith 1986, Dadour and Harvey 2008) and this study suggests that they could also have an impact on the rate at which mammalian carcasses decompose as they were, for the most part, non-existent in the sterilized soils in this study.

Our Phase 2 experiment allowed us to understand that microbial decomposer communities are not soil specific. These results have important implications for using microbial communities as potential gravesoil indicators. We were able to predict PMI with a root mean square error of ~ 3days, even when the soil type was not included as a feature of the regression models. Sequencing of the 18S amplicons revealed that the highest abundance organism in each sample type was nematodes of the Class Chromadorea. Samples associated with each soil type were dominated by a different family of Chromadorea (desert soil: Aphelenchidae, forest soil: Cephalobidae, and grassland: Pangrolaimidae and Aphelenchidae). These trends suggest that a proportion of the soil microbial decomposer communities are common across contrasting soil types, which makes bacterial communities associated with decomposition very attractive as a forensic tool.

Additionally, we discovered that the microbial community change that occurs in the soil due to decomposition remains for at least 30 days after the cadaver was removed. These results suggest that the presence of corpse may be detectable even after it is moved.

The conclusions for Phase 3 experiments (swine and human outdoor experiments) are critical for understanding how our controlled laboratory experiments translate into potential real-world forensic applications. Our research on swine-associated gravesoils show that carcass mass did influence the structure of gravesoil microbial communities; the decomposition of 1 kg carcasses did not result in significant changes of dominant microorganisms although larger carcasses were associated with significant changes in the abundance of several dominant bacterial communities. Furthermore, we show that soils can undergo seasonal shifts in their microbial communities and these shifts are modified by carcass decomposition. These shifts are due to increases and decreases in the abundance of soil-dwelling microorganisms; microorganisms typically associated with mammals or putrefaction were not dominant in gravesoils. We conclude that the structure of postmortem gravesoil microbial communities is affected significantly by season and initial carcass mass. We also conclude that significant changes in bacterial communities are observed before significant changes in eukaryote communities.

Finally, we confirmed that significant and predictable microbial community change associated with decomposition exists with human cadavers in outdoor scenarios. We also confirmed that seasonal variation (originally detected in swine carcasses) also occurs with human cadavers. Understanding the extent to which seasons affect our ability to predict PMI will be an important focus of future research. It is possible that although seasonal decomposer communities differ, they may have similar features (OTUs) that allow for accurate predictions across seasons.

Our results show that postmortem soil microbial communities have great potential as physical evidence because they follow predictable trends. Future studies are crucial because previous forensic investigations using traditional indicators has shown that environmental parameters such as temperature, moisture, soil type, and soil texture can affect the rate at which decomposition occurs and the ability of decomposer organisms to function (Carter et al. 2008a, 2010). It is likely that these parameters will affect postmortem microbial communities. Consequently, the relationship between these parameters, decomposition, and postmortem microbial communities must be understood to develop a method to estimate PMI that is admissible as physical evidence within the criminal justice system. However, the present work strongly indicates that decomposition is sufficiently reproducible as an indicator of PMI to motivate these additional studies.

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VI. Dissemination of Research Findings

Results from our research have been communicated to peers and the public in a number of ways. Results from Phase 1 and preliminary results from Phase 2 were presented at a well-attended oral presentation at the 2013 Annual Meeting of the American Academy of Forensic Sciences (J. Metcalf, Friday, February 22, 2pm Physical Anthropology Section). Additionally, Rob Knight presented the results and progress of our research program at the NIJ R&D Grantee's meeting Tuesday, February 19th (followed by 3 additional live-streamed NIJ presentations in April), which provided us with the opportunity to disseminate our results to a broader forensic community and receive feedback from a variety of people in the field. Finally, a manuscript describing the results is in the open-access, peer-reviewed journal *eLIFE* (Metcalf et al. 2013). Results from research of Phases 2 and 3 are described in two additional manuscripts that are currently in prep (Carter et al. 2014), which are in the final stages of completion and will be submitted for publication in a peer-reviewed journal in the spring of 2014. Co-PI Carter shared results

from Phase 3 swine experiments in the 2014 AAFS Pathology/Biology, Session: "Microbes Continued" session as well. The Phase 3 sampling effort with Sibyl Bucheli and Aaron Lynne at Sam Houston State University and our overall forensics research was recently highlighted by NPR's science correspondent Rob Stein (http://www.npr.org/blogs/health/2013/09/23/219375086/could-detectives-use-microbes-to-solve-murders). We have a number of manuscripts in preparation that summarize the results from our research. Finally, Co-PI's Carter and Metcalf organized (Carter) and participated (Carter and Metcalf) in a day-long workshop entitled "Forensic Microbiology: Where Do We Begin?".