

**AN INVESTIGATION OF INTRINSIC AND EXTRINSIC FACTORS
THAT INFLUENCE SOIL MICROBIAL SUCCESSION DURING HUMAN
DECOMPOSITION**

**A Dissertation Presented for the
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Degree
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ABSTRACT

Decomposer organisms play a vital role in terrestrial nutrient cycling, breaking down complex organic compounds and providing nutrients for primary producers. Microbial communities and other decomposers associated with a vertebrate carcass, or the “necrobiome”, are critical for degradation and recycling of soft tissues following vertebrate mortality. The impact of microbial decomposers in vertebrate decomposition has been shown with regard to soil microbial communities, where the presence of soil microbes impact decomposition rate and undergo succession. However, current assessments of microbial ecology within decomposition-impacted soils have primarily focused on one aspect of microbial succession: succession of bacterial taxa for forensic applications. While this has provided a foundation of knowledge to build upon, it is still unclear how this succession is affected by extrinsic (*e.g.*, environmental) and intrinsic (*e.g.*, cadaver-related) factors and how microbial functions are impacted. These knowledge gaps are important for understanding how ecosystem processes are impacted by decomposition. The ultimate aim of this dissertation was to improve our understanding of soil microbial succession during decomposition by assessing these knowledge gaps. Thus, this dissertation consisted of three research objectives: (1) evaluation of the effects of intrinsic factors on soil microbial succession, (2) evaluation of the effects of extrinsic factors on soil microbial succession, and (3) evaluation of soil microbial functional succession during decomposition. Two separate field studies were conducted. The first observed the early stages of decomposition of 19 human subjects, while the second observed a longer term (one year) decomposition of three subjects. All subjects decomposed on the soil surface at the University of Tennessee Anthropology Research Facility. Overall, we show that soil microbial succession during decomposition is impacted by both intrinsic and extrinsic factors. This included body mass index, which affected soil chemical response and microbial succession, making it a factor of interest for future investigations. We also used metatranscriptomics to show that microbial gene expression shifted during decomposition and was still impacted following one year. Together, our investigation of factors that influence multiple aspects of microbial

succession lays an important framework for understanding the ecology of human and vertebrate decomposition in terrestrial ecosystems.

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INTRODUCTION

Vertebrate decomposition is an important process in terrestrial ecosystems (**Figure 0.1**), impacting nutrient availability via carbon and nitrogen cycling and landscape heterogeneity. This process is mediated, in part, by microbes within multiple microhabitats such as within the body (internal), on the body (external), and in the surrounding environment (especially the soil below), as microbial decomposers contain enzymes that target various macromolecules (*i.e.*, protein, lipids, carbohydrates, and nucleic acids). Decomposition results in a flush of liquified decomposition products to the soil, altering substrate availability and physicochemical conditions experienced by soil microbial communities. Additionally, internal and external microbes from the body are deposited into the soil, leading to a coalescence of host-associated and soil microbial communities. Together, vertebrate mortality events cause a disturbance to native soil microbial communities, resulting in microbial succession, *i.e.*, changes in microbial community composition and function over time.

Human taphonomy facilities in the United States and around the world allow us to study human decomposition processes in terrestrial ecosystems to address both ecological impacts of vertebrate decomposition on microbial communities and their applications for forensic science. However, most microbial human decomposition studies focus on communities from a forensic science perspective, with the goal of predicting time since death from taxonomic succession or identifying microbial biomarkers. This forensic perspective has led to identification of general successional patterns in decomposition-impacted soil; however, it has also shifted our scientific questions away from decomposition ecology, limiting our holistic understanding of microbial responses to and roles during human decomposition.

Specifically, questions pertaining to the effects of drivers, including extrinsic (*i.e.*, environmental) and intrinsic (*i.e.*, cadaver-related) factors, on microbial succession and microbial functional succession in response to decomposition require further investigation. For example, soil physicochemical parameters are a subset of extrinsic variables that can influence microbial community dynamics. Although soil physiochemistry has been described during decomposition, and patterns between chemical and microbial changes have been suggested, few studies have linked chemical changes to microbial succession in a comprehensive investigation. Intrinsic

factors (*e.g.*, age, sex, or body mass index[BMI]) may also impact microbial decomposers, either through variation in host-associated microbial communities and/or differences in resources for decomposition. Some associations have been suggested between various intrinsic factors (*e.g.*, heart disease, sex, BMI) and internal microbial communities during decomposition, although less is known about their effects on soil microbial communities. To our knowledge, the only intrinsic factors to be investigated within decomposition soils have been the intrinsic factors body mass and lead dosing. Additionally, a majority of human decomposition studies have investigated microbial structural succession (*i.e.*, changes in taxonomic composition over time); however, decomposition, as an environmental disturbance, can impact microbial community functions as well. This emphasis on composition has left our understanding of the specific role(s) of soil microbial decomposers in decomposition processes and soil microbial functional succession unelucidated.

Together, three knowledge gaps in our understanding of microbial ecology and succession during terrestrial vertebrate decomposition events have been identified: (1) effects of intrinsic factors on microbial succession, (2) effects of extrinsic factors on microbial succession, and (3) characterizing microbial functional succession during decomposition. The overall goal of this dissertation research was to advance our understanding of soil microbial community succession during human decomposition by investigating the effects intrinsic and extrinsic factors on soil microbes and characterizing changes in microbial functionality.

RESEARCH QUESTIONS

Three questions were assessed in this dissertation to address knowledge gaps that hinder our understanding of soil microbial succession during terrestrial vertebrate decomposition:

- 1) How do intrinsic factors affect soil microbial structural succession during human decomposition?
- 2) How do extrinsic factors impact the predictability of microbial structural succession in decomposition-impacted soils?
- 3) How does soil microbial gene expression change in response to human decomposition over a one-year period?

DISSERTATION ORGANIZATION

This dissertation contains a literature review and three research chapters. The first chapter is a review that focuses on microbial succession during terrestrial vertebrate decomposition. In this chapter, we propose that decomposition should be viewed from an ecological lens to better understand microbial community dynamics and ecosystem impacts that result from mortality events. The second through fourth chapters focus on our research questions and have been prepared as manuscripts for journal submissions. In Chapter 2, we focus on our first research question and evaluate the impact of intrinsic factors (*e.g.*, age, sex, body mass index, and disease status at time of death) on soil microbial structural succession during decomposition of nineteen human individuals. Chapter 3 leverages data collected in Chapter 2 to evaluate the predictability of soil microbial structural succession from the context of estimating of postmortem interval (PMI) and assess the impact of extrinsic factors on this predictability. Collectively, these first two studies evaluated microbial community composition, with the goal of identifying factors that alter the presence and succession of soil microbial decomposers. Chapter 4 focuses on functional succession of soil microbial communities, an understudied aspect of vertebrate decomposition. In this chapter we assess gene expression in soils below three decomposing individuals (human) over one year. The final chapter of this dissertation (Chapter 5) summarizes our conclusions and discusses avenues for future research.

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APPENDIX

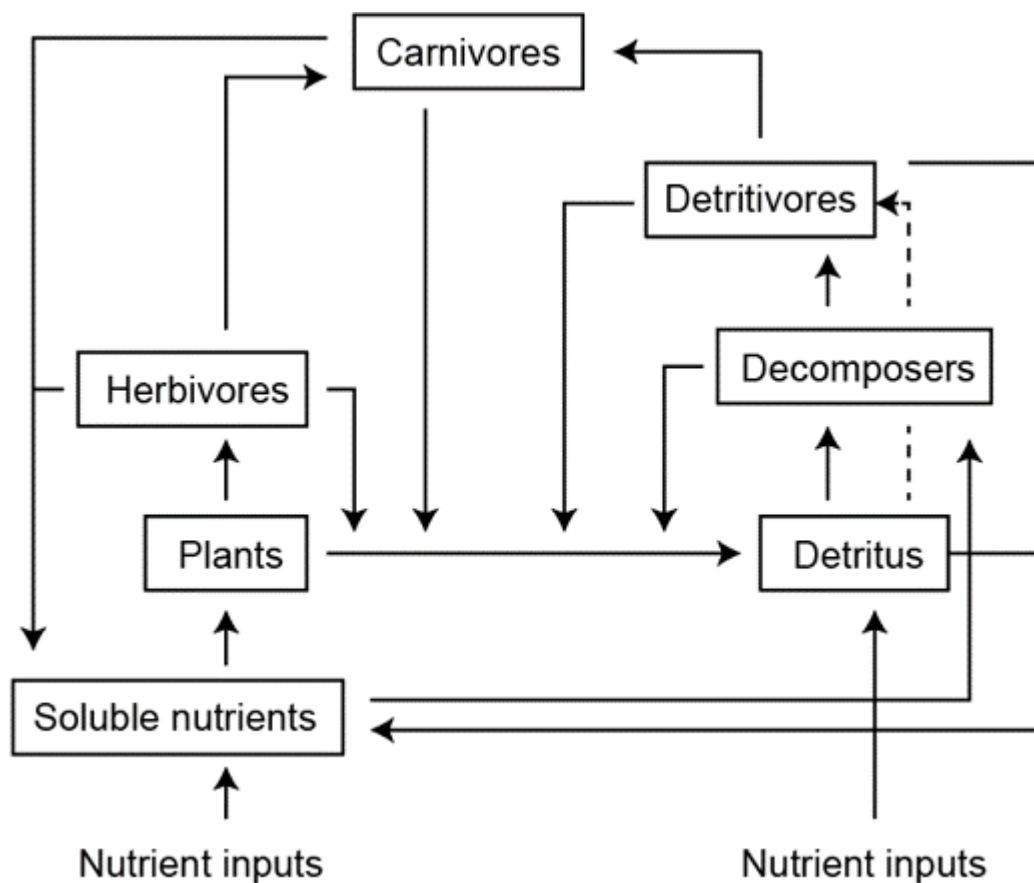


Figure 0.1 Flow of nutrients through a terrestrial food web. In this diagram, decomposition of detritus, or non-living organic matter (i.e., dead plants and animal carcasses and waste) by decomposer organisms, including soil microbes, is central to nutrient flow in terrestrial ecosystems. Figure adapted from Moore et al. (2004).

CHAPTER 1 MICROBIAL ECOLOGY OF VERTEBRATE DECOMPOSITION IN TERRESTRIAL ECOSYSTEM

PREFACE

This first chapter is a systematic review discussing microbial community succession during vertebrate decomposition in terrestrial ecosystems. We summarize microbial successional patterns occurring in different microhabitats of a decomposition system, with the goal of contextualizing current literature within an ecological framework. With the advent of next generation sequencing, investigation of microbial communities associated with decomposing bodies (*i.e.*, necrobiome) vertebrate decomposition has increased in the past ten years. Thus, I synthesized these studies to help realize the current status of the field, while also identifying knowledge gaps for future investigation. For this chapter, I obtained literature of human and animal decomposition studies that focused on microbial communities from Web of Science following preferred reporting items for systematic reviews and meta-analyses (PRISMA) guidelines.

A version of this chapter has been submitted to mBio as a minireview and is currently under review: Allison R. Mason, Lois S. Taylor, and Jennifer M. DeBruyn (2022). Microbial ecology of vertebrate decomposition in terrestrial ecosystems.

Contributions: This project was conceived by ARM. Systematic literature review, including obtaining and evaluation of pertinent literature, was completed by ARM. The manuscript was written by ARM, with contributions from LST and JMD.

ABSTRACT

Vertebrate decomposition results in an ephemeral disturbance of the surrounding environment. Microbial decomposers are recognized as key players in the breakdown of complex organic compounds, controlling carbon and nutrient fate in the ecosystem and potentially serving as indicators of time since death for forensic applications. As a result, there has been increasing attention on documenting the microbial communities associated with vertebrate decomposition, or the “necrobiome”. These necrobiome studies differ in the vertebrate species, microhabitats (*e.g.*, skin vs. soil), and geographic locations studied, but many are narrowly focused on the forensic application of microbial data, missing the larger opportunity to understand the ecology of these communities. To further our understanding of microbial dynamics during vertebrate decomposition and identify knowledge gaps, there is a need to assess the current works from an ecological systems perspective. In this review, we examine recent work pertaining to microbial community dynamics and succession during vertebrate (human and other mammals) decomposition in terrestrial ecosystems, through the lens of a microbial succession ecological framework. From this perspective, we describe three major microbial microhabitats (internal, external, and soil) in terms of their unique successional trajectories and identify three major knowledge gaps that remain to be addressed.

INTRODUCTION

Microbes act as decomposers in many ecosystems, mediating processes that break down macromolecules and impacting nutrient cycling (*e.g.*, carbon, nitrogen, etc.) (1). In terrestrial ecosystems, detritus (non-living organic matter) represents an important nutrient pool for microbial decomposers. Detritus is often considered one resource pool within ecosystems (2), without considering its origin or composition. For example, detritus is comprised of dead plant matter (*e.g.*, litter), as well as dead animal carcasses. Microbial degradation of plant litter has received extensive research over the past four decades. However, the composition of nutrients and decomposition patterns differ between plants and animals (3-5). The role(s) of microbes during animal carcass decomposition has received less attention, limiting our understanding of nutrient cycling through this key detritus pool.

Animals are important sources of carbon (C) and nitrogen (N) in terrestrial ecosystems as their tissues represent large pools of organic C and N (6). Upon death, carbon and nutrients stored in an animal carcass are redistributed throughout the ecosystem via scavengers and decomposers, with microbial decomposition playing a key role (4, 7). The localized flush of decomposition products from the decomposing carcass causes an ephemeral disturbance to soil microbial communities by altering the soil chemical (2, 8) and physical environment (1). These disturbed areas are referred to as hotspots, exhibiting increased biogeochemical reaction rates compared to their surroundings (4, 9), and contribute to landscape heterogeneity (9). Nutrient inputs from animal decay increases resource availability in the soil, thereby stimulating microbial primary production and nutrient cycling (4, 10). This influx also alters chemical transformations and increases microbial biomass, leading to enhanced carbon and nutrient losses via emission of greenhouse gasses (CO₂, N₂O, and CH₄) (8, 11, 12).

Research investigating microbes during vertebrate decomposition in terrestrial ecosystems has increased in the past decade. This is largely attributable to the general increase in microbiome research due to advancements in sequencing and bioinformatic technologies, as well as the recognition that microbes are central to the decomposition

process. Microbial communities during vertebrate decomposition have been assessed in different microhabitats (*e.g.*, skin vs. soil), species (*e.g.*, humans, pigs, mice), and geographic locations. As we start to amass more data from these studies, there is a need to distill trends in microbial data to understand what knowledge gaps remain and where the field should focus for future investigations.

While decomposition is an important ecosystem process, the majority of microbial-focused vertebrate decomposition studies center around the potential forensic applications of microbes. Specifically, many studies aim to assess if the changes observed in microbial taxa can be predictive of time since death, or postmortem interval (PMI). This perspective, while important to forensic science, shifts the focus away from the ecological mechanisms occurring within vertebrate decomposition systems. This leaves gaps in our understanding of ecosystem impacts due to vertebrate decomposition, and the roles of microbes in these processes remain unclear. Recent review papers of postmortem microbial communities have focused on internal microbial communities only (13), forensic applications (14, 15), or methodologies (16). Therefore, the goal of this review is to discuss microbial community dynamics during vertebrate decomposition from an ecological lens, while highlighting knowledge gaps to be addressed in future work. This review will focus on microbial community dynamics during surface vertebrate (human and animal) decomposition in terrestrial ecosystems. As a result, literature pertaining to burial and aquatic decomposition systems, and the role of insects and scavengers in decomposition, as well as their likely interactions with microbial decomposers will not be explicitly addressed. By understanding the ecology of terrestrial vertebrate decomposition, we can gain more information about the mechanisms impacting microbes during this process and move towards a better understanding of nutrient flow through terrestrial detritus pools.

CONCEPTS FROM ECOLOGY: MICROBIAL SUCCESSION

Carcass decomposition alters the surrounding ecosystem, creating a disturbance that impacts microbial communities. A carcass represents a source of nutrients for macro- and

microorganisms. Animal carcasses have high water content (2, 16) and are rich in nitrogen (11), which, compared to plant decomposition, contributes to rapid decomposition rates as detritivores and decomposer microbes use those nutrients. Additionally, plant organic matter is composed of large, recalcitrant molecules (*e.g.*, lignin, cellulose, etc.) that decompose slowly via specified microbial metabolic pathways (17). Breakdown of carcass tissues results in a concentrated input of carbon and nitrogen compounds into the surrounding environment, altering the chemical and physical environment for microorganisms. For example, changes in pH, salinity, gas release, and presence of nutrients such as NH_4^+ , NO_3^- , PO_4^{3-} , and Ca^{2+} have been documented in soils below decomposing carcasses (8, 10, 18, 19). These changes in nutrient availability and physical conditions leads to a succession of microorganisms over time.

Succession describes the series of semi-predictable changes in biological populations over time, sometimes in response to altered environmental conditions or disturbances. Ecosystem successional theory originated from observations of changes to plant populations: In the late 19th century, Henry Cowles described how plant populations change along an ecological gradient, developing the idea of a chronosequence of species over time (20). Frederick Clements expanded upon this idea, proposing that vegetation succession is predictable and ends when a climax community is reached. Clements suggested that a climax community, which he termed a super-organism, contains members that interact in a way that serves the purpose of the community as a whole (21). Henry Gleason later challenged the idea of climax communities as a super-organism, instead proposing that members of the climax community act in an independent manner. Gleason recognized the random component to population dynamics, suggesting that succession may not be as predictable as thought by Clements (22). From these original theories, two types of succession were defined: primary and secondary succession. Primary succession describes the series of species that colonize a previously sterile environment, while secondary succession describes the re-colonization of a disturbed environment that previously contained life (23). Over time ecologists have built upon these fundamental theories, applying them to new systems and different organisms.

As microbial ecology has boomed in the past decade due to advancements in next-generation sequencing, microbial ecologists have started to adapt these successional theories to microbial communities. Microbes are more physiologically diverse compared to plants; therefore, microbial succession ecology is rooted in, but differs from, plant succession ecology. For example, plants use CO₂ and light as carbon and energy sources, respectively; while microbes can use a diverse range of compounds to satisfy these requirements. Microbial ecologists have proposed multiple categories of succession that differ from traditional primary and secondary succession recognized in plant ecology. Fierer et al. (2010) described autotrophic, endogenous heterotrophic, and exogenous heterotrophic microbial succession, as forms of primary succession, based on the diversity of microbial carbon requirements (heterotrophs vs. autotrophs) and resource inputs. Autotrophic succession is expected to occur in environments that have little to no organic carbon present, where autotrophs would be the first colonizers (23). Endogenous and exogenous heterotrophic succession can be characterized by the source of organic carbon and the distribution of copiotrophs (*i.e.*, prefer high nutrient conditions) and oligotrophs (*i.e.*, prefer low nutrient conditions) over time. In endogenous heterotrophic succession, there is a single event that results in organic carbon C input into the system, impacting local nutrient concentrations/availability. In this scenario, initial colonizers are copiotrophs that metabolize local nutrients, altering the substrate and thus resulting in a shift towards oligotrophic biomass at later timepoints (23). In contrast, exogenous succession is driven by nutrients that are added continuously to the system via external organic inputs. In this environment both copiotrophs and oligotrophs respond, however nutrient quality is variable over time leading to fluctuations in both copio- and oligotrophic biomass (24).

Secondary succession in microbial communities would include temporal changes in microbial communities as well as community responses to environmental stress or disturbance. Microbial community structure (*i.e.*, taxa present and their relative abundances in a community) and/or function may be altered in response to environmental disturbance, leading to a semi-predictable succession (25, 26). An ecosystem disturbance

can be defined as an event that directly or indirectly alters the community (24, 25). In the case of indirect disturbance, altered environmental conditions impact communities, while a direct disturbance directly influences the types and abundance of taxa present in a community (25). The type, magnitude, and duration of a disturbance all impact how microbial communities are affected and how they respond to disturbance (24, 25). In their review, Shade et al. (2012) apply disturbance ecology to microbial communities and proposes that community outcomes depend on the balance between disturbance parameters (*e.g.*, timing and magnitude) and community resistance/resilience. For example, microbial communities may resist changes following disturbance, or they may shift to a different steady state. Some communities are resilient and ultimately return to their starting composition after a disturbance, or resilience is low and/or the disturbance is too great and the community is pushed into a new alternative steady state (23, 24). These concepts of microbial succession and disturbance ecology provide a lens through which vertebrate decomposition can be assessed.

MICROBIAL SUCCESSION IN VERTEBRATE DECOMPOSITION: WHERE ARE WE?

Microbial succession during vertebrate decomposition has been investigated over a wide range of timescales and in a variety of species and microhabitats within a decomposition system. There are two distinct microbial communities involved in vertebrate decomposition within terrestrial ecosystems (**Figure 1.1**). The first is the host-associated microbiota, which can be divided into internal microbial communities (*e.g.*, oral, gastrointestinal tract, vaginal, etc.) and external skin microbial communities. The second is the soil microbiota present in soil below and surrounding the decomposing carcass. Each of these microhabitats undergoes different types of microbial succession that occur on different timescales (**Figure 1.1**). This section of the review will present what is known about microbial communities during vertebrate decomposition in each microhabitat, while tying in microbial successional theory and discussing knowledge gaps specific to each microhabitat.

Methods

Papers pertaining to vertebrate decomposition were obtained from Web of Science using the search terms in **Figure 1.2**. This yielded a total of 309 publications dating from 1960 to 2021. After manually removing duplicate research articles, articles that did not fit the scope of this review, and review articles, 56 primary literature sources remained (**Figure 1.2**). Of the 56 papers included in this review, 33 pertain to microbial succession during vertebrate decomposition, meaning they: 1) included more than three timepoints; 2) were longitudinal timeseries, or chronosequences; and 3) directly reported community structure (composition or function).

Internal carcass microbial succession

Internal host-associated microbes undergo a succession of taxa as host tissues are broken down and internal organs are colonized. In antemortem conditions vertebrates have organs that are colonized by microorganisms (*e.g.*, gastrointestinal tract, vagina, mouth) and those that are uncolonized, or normally sterile sites (*e.g.*, blood, heart, brain, liver, etc.). Ecologically, the type(s) of microbial succession that occur in sterile and unsterile organs during decomposition are expected to differ. Organs that were previously sterile are expected to exhibit a succession that may be described as primary succession, since microbes had not previously colonized these microhabitats. Of the primary heterotrophic succession models that have been described for microbial communities, endogenous heterotrophic succession (27) best describes the type of succession that would be expected to occur in sterile organs. In this model, primary colonizers would be copiotrophic heterotrophs that use organic carbon from the degradation of tissues. In contrast, decomposition represents a disturbance to microbial communities in non-sterile tissues. Changes in their chemical and physical environment, caused by cell and tissue degradation, are expected to drive microbial response resulting in secondary succession.

Following death, host microbes are involved in the breakdown of tissues and both microbial diversity and composition changes as decomposition progresses (28). Microbial communities, mainly bacteria, have been surveyed in different organs and internal body habitats including the eyes (28), nose (28, 29), ears (29, 30), heart (31-35), liver (32, 33,

36), spleen (30, 32, 33, 36), brain (30, 33, 34, 36), intestinal tract (28, 34, 37-43), and mouth (28, 30, 33, 42, 44) and over time scales ranging from one hour to 30 days (**Table 1.1**).

The taxa present and their succession in body regions such as the mouth and rectum appear to differ from those that occur in previously sterile internal organs, especially at the family and genus levels, suggesting that antemortem colonization status (*i.e.*, sterile, or non-sterile) impacts the successional model postmortem. These studies suggest that bacterial community composition between organs might be expected to become more similar as decomposition progresses; however, there is no consensus on timing of the convergence as experiments differ in the timeframe observed, species studied, and decomposition environment (*i.e.*, laboratory vs. terrestrial). All of these factors can impact the decomposition environment, leading to differences in decomposition patterns. Even with this variation, there are some common observations; for example, internal bacterial communities appear to shift toward a community dominated by opportunistic facultative anaerobes, indicated by increased abundance of *Clostridiales* (30, 32, 35, 38, 40) and decreased relative abundance of obligate anaerobes, such as *Bacteroides* (37, 38, 40, 45). This is likely because as organs and skin break down and carcass integrity is compromised, we expect that internal conditions become more like external conditions and oxygen-tolerant organisms become more competitive.

Determining general trends in internal microbial succession during decomposition is difficult due to sampling limitations and variability between individuals. While the mouth and rectum can be swabbed with minimal impact, sample collection from other tissues is invasive and can alter the decomposition environment, changing physical conditions and thus the microbial communities. As a result, many tissue succession studies employ destructive sampling of carcasses at different times (*i.e.*, a chronosequence) as opposed to repeated longitudinal sampling of the same individual(s). This single sample per individual only provides a snapshot of decomposition, making it difficult to attribute changes to, or control for, potential sources of variation (*e.g.*, age, disease, etc.). This is

particularly important as recent studies suggest microbial communities during decomposition differ due to some cadaver-related intrinsic factor(s) (31, 40).

Another challenge for this field is that most of what is known about internal postmortem microbial communities comes from laboratory studies or samples taken at time of autopsy, which do not include factors such as insect and scavenger access, presence of soil, and variable environmental conditions that occur during outdoor terrestrial decomposition. These factors could potentially alter microbial communities, either directly (via introduction of new microbes from insects and scavengers or the environment) or indirectly (via altering microbial habitats). As a result, greater variability in succession is expected between individuals during outdoor decomposition relative to those conducted under laboratory settings. To date, only six of the 33 successional studies we identified have observed internal flora in an outdoor setting (37, 39, 41, 42, 46), and all but one of these studies were limited to the mouth and rectum. While these studies have shown similar trends to indoor studies, particularly at the phylum level, variability has been observed between individuals (40, 42, 47). Fu et al. (2019) show differences in fungal decomposer taxa present and clearer compositional shifts during indoor swine decomposition compared to outdoor, suggesting that decomposition setting does impact microbial succession.

External carcass microbial succession

Skin microbial communities are expected to undergo some form of secondary succession during decomposition. Skin is the largest organ and is made up of multiple microhabitats, each with unique microbial composition (42). As skin contains its own microbiota prior to death, decomposition would represent a disturbance to this established community as they respond to new conditions created by tissue decomposition and release of internal microbes as structures breakdown. Microbial succession in different skin habitats has been observed during decomposition (42, 44, 48, 49). These studies range in the time frame of observation (five to 143 days) and species used, with the most common species studied being mouse (*Mus musculus*) and swine (*Sus scrofa*) (**Table 1.1**). Similar to internal postmortem studies, postmortem skin microbiome studies are divided between

those conducted in the laboratory (42, 49) and those in outdoor terrestrial settings (42, 44, 49).

Skin microbial communities change in response to vertebrate decomposition. While the skin contains different antemortem microbial habitats, terrestrial decomposition studies suggest that the succession between different skin habitats is similar in both humans and swine at the phylum level (44, 49). Of the external microbial succession studies that apply 16S rRNA gene sequencing, only three have taken place in an outdoor setting (42, 44). All of these outdoor studies observed that skin microbial communities exhibit increased relative abundances of *Firmicutes* and *Actinobacteria* and decreased *Proteobacteria* as decomposition progresses (42, 48, 49). This is likely the result of decomposition fluids being released from the body, exposing the skin to opportunistic *Firmicutes* from internal communities. This is in contrast to skin microbial succession during controlled mice decomposition experiments conducted in the laboratory (48-50), where the increase in *Firmicutes* on the skin is less pronounced than in outdoor vertebrate (human and pig) studies. These differences suggest that smaller vertebrates may exhibit different succession (either different taxa or different timeframes) than larger vertebrates and/or setting (*i.e.*, laboratory vs. outdoor) influences microbial succession as decomposition progresses. In outdoor settings, the activity and movement of other decomposers (*e.g.*, insects and scavengers) during terrestrial decomposition may lead to bioturbation and mixing, resulting in additional disturbance to the microbial environment and movement of opportunistic microbes. No work to date has directly compared skin succession between different vertebrate species within the same study (*i.e.*, same environmental conditions); this comparison would be useful for expanding findings to other carrion in terrestrial environments.

Soil microbial succession

Soil microbial communities directly below and surrounding a decomposing carcass are altered in response to decomposition products. These communities contain numerous bacterial and fungal decomposers that are involved in the breakdown and cycling of organic matter in the soil environment, including organic matter from the carcass. Indeed,

previous work has shown that soil microbes are involved in and help accelerate vertebrate decomposition (49) and decomposer organisms found on the carcass have been suggested to originate from soil (51). Not only do native soil microbes respond to highly concentrated carcass-derived products, but also to the influx of host-associated microbes that are flushed into the soil with decomposition products. This coalescence of microbial communities results in a unique succession of taxa. The roles of host vs. soil microbes within these coalesced communities remains unclear. Research centered around soil microbial succession during vertebrate decomposition has primarily focused on bacterial community composition (49, 52-54); however, microbial succession using fatty acid profiles (55) and soil fungal succession (49, 52) has also been assessed.

Changes in the dominant bacterial phyla have been described for soil microbial communities during vertebrate decomposition. In all outdoor studies assessing bacterial succession, phyla containing anaerobic taxa, such as *Firmicutes* and *Bacteroidetes*, have been shown to increase following release of decomposition products, as aerobic heterotrophs consume oxygen during metabolism of decomposition products, creating hypoxic or anoxic conditions in the soil (49, 51-53, 56, 57). Soil decomposers from the phylum *Actinobacteria* also increase during soft tissue decomposition, likely because *Actinobacteria* are known to have enzymes and pathways for the breakdown of complex and varied organic compounds (48, 49, 51, 53, 56). Changes in these taxa, while still observable, are not as pronounced during laboratory mouse decomposition (55) compared to human decomposition in the field, suggesting that differences between species (*e.g.*, size, microbial composition, etc.) or decomposition setting (*e.g.*, variable environment or presence of insects and scavengers) may impact the magnitude of disturbance created by carcass decomposition leading to variable microbial succession.

Recognizing that fungi are also important decomposers of organic matter in terrestrial ecosystems, a few studies have focused on fungal succession during vertebrate decomposition (48, 49, 57, 58) (**Table 1.1**). These studies differ in species used (human, pig, or mice), decomposition setting (indoor or outdoor), and the method used to characterize the fungal communities (culturing, 18S rRNA gene, or ITS). Only two

studies have assessed soil fungal changes during decomposition via fungal marker gene sequencing in an outdoor setting (55, 59). Both of these studies observed a shift in soil fungal composition, whereby communities became dominated by *Saccharomycetes*. Interestingly, the taxa within this fungal class responsible for these shifts differed slightly. In Fu et al. (2019), the *Saccharomycetes* genera *Yarrowia* and *Candida* dominated soil communities by day 14, while dominant taxa in Metcalf et al. (2016) included *Saccharomycetes* genera *Yarrowia* and *Dipodascaceae*. Members of the fungal class *Saccharomycetes* are fermentative yeasts that are often found living in vertebrate microbiomes (e.g., skin, oral, gut) (49). In the context of decomposition, their increased abundance in soil is likely due to the release of these species from the carcass via decomposition fluid and their subsequent proliferation in nutrient-rich conditions. As the labile carcass-derived nutrients are consumed by copiotrophic bacteria and fungi in the soil, the relative abundance of *Saccharomycetes* is expected to decrease as conditions shift to favor oligotrophic species; however, this has not been observed in the timeframe of current studies (143 days) (60), thus long-term studies are needed to test this hypothesis.

Shifts in microbial taxa during vertebrate decomposition are likely driven by individual species' nutritional requirements (C:N:P) and growth characteristics combined with environmental filtering due to altered soil chemical and physical conditions. Borrowing from plant and animal ecology, previous work has shown that some soil bacterial taxa can be described as copiotrophs or oligotrophs (61), where copiotrophs thrive under high nutrient conditions and oligotrophs dominate microbial communities under low nutrient conditions. This relationship has also been expanded to compare general bacterial and fungal life strategies. Fungi generally require less nitrogen (relative to carbon) and exhibit more oligotrophic features than bacteria (62, 63). As a result, fungi:bacteria biomass ratios shift toward greater proportions of fungi when high C:N resources are input into a system and vice versa (52, 64). In the context of vertebrate decomposition, nutrient-rich decomposition fluids are expected to stimulate copiotrophic microbes and shift toward a more bacteria-dominated community. As decomposition progresses, we would expect low C:N labile compounds to be depleted leaving more recalcitrant

compounds. This shift in nutrient pools would favor oligotrophic bacteria and fungi that prefer low nutrient conditions. While some bacterial taxon shifts in decomposition-impacted soils have been described in this context (49), only one study (25) has compared both bacterial and fungal (via ITS sequencing) communities from the same individuals. Metcalf et al. (2016) observed that fungal community composition shifted towards saprobes of the phyla *Ascomycota* and *Zygomycota*, while *Basidiomycota*, made up of many oligotrophic taxa, decreased in relative abundance.

Unlike internal and external microbial communities, soil microbial communities remain present after soft tissue decomposition is complete. This is important when considering microbial community response to disturbance and whether they ultimately return to pre-disturbance conditions or stay in a new state (56). The majority of soil decomposition studies focus on the period of soft tissue decomposition, up to 200 days into decomposition (56). During this timeframe, soil microbial communities, both bacteria and fungi, do not appear to return to their native composition (49, 52); for example, in one study, relative abundance of *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* were all still elevated while *Acidobacteria* was still reduced after 200 days (65). Moving forward, longer term studies are needed to test the resiliency of soil microbial communities following vertebrate decomposition.

MICROBES IN VERTEBRATE DECOMPOSITION: KNOWLEDGE GAPS AND FUTURE DIRECTIONS

While the investigation of microbial communities involved in vertebrate decomposition has increased in the past decade, many questions still remain unanswered. The body of work to date has helped to identify general trends in decomposer community composition, but the factors impacting microbial decomposer response during vertebrate decomposition have yet to be disentangled. Characterizing these responses and their variability will allow us to better understand the ecological impacts created by carrion and the role microbes play in recycling these nutrients. We see three main areas of opportunity for understanding microbial ecology of vertebrate decomposition systems: the roles of extrinsic variation, intrinsic variation, and a focus on functional succession.

Extrinsic variation

Extrinsic (*e.g.*, environmental) and intrinsic (*e.g.*, carcass-related) factors impact decomposers during carcass decomposition and ultimately, the timing and magnitude of the disturbance. Previous work has revealed extrinsic factors impacting the rate of decomposition (66); specifically, temperature and moisture are shown to be primary influences on microbial activity during decomposition (57, 67, 68), governing the rate of microbial metabolism and nutrient availability (8) in the soil environment. Beyond temperature and moisture, little work has been done to relate other physical or chemical factors of the decomposition environment to changes in microbial communities. Specifically, the soil chemical environment changes during vertebrate decomposition due to the release of saline and nutrient-rich fluids (45, 69, 70). This chemical environment is altered further as abiotic and biotic breakdown of organic matter occurs (71). This creates phases of unique soil biochemical profiles over time, as described in Keenan et al. (2018). While microbial succession and changes in soil biogeochemistry have been assessed independently of each other, little work has been done to disentangle the relationship between soil microbial community structure and/or function and the chemical and biogeochemical changes that occur during vertebrate decomposition. These major physicochemical changes in the soil environment likely result in filtering of microbial taxa, partly explaining changes in community structures. Future studies should integrate microbial sequence data with physicochemical environment and biogeochemical process data, to better understand microbial successional patterns within the soil, and how microbial communities constrain impacts of vertebrate decomposition on important ecological processes, such as nutrient cycling.

Intrinsic variation

Intrinsic factors, that is, factors related to the carcass itself, can also impact microbial decomposers, leading to variation between individuals. This could be, but is not limited to, body composition, sex, ancestry, diet, or even illness status and/or drug loading at time of death. Intrinsic factors can alter the internal microbiome and/or the alter the carcass as a nutrient source antemortem (28, 72-75), decreasing the predictability of

microbial succession by increasing variability between individuals. Effects of intrinsic factors on microbial succession during vertebrate decomposition are not well studied. In fact, of all the microbial-related literature captured by our search terms, only eight papers investigated effects of intrinsic factors (30, 31, 33, 35, 37, 58, 76), and only four of these met our criteria as a microbial succession study (longitudinal or chronosequence studies with at least three timepoints) (**Table 1.1**).

Preliminary studies suggest intrinsic factors affect vertebrate decomposition dynamics; however, results are conflicting. It is challenging to control for these variables as interactions between multiple intrinsic factors creates a problem of co-variables that can be difficult to disentangle (*e.g.*, age at time of death often correlates to higher prevalence of illnesses and/or medications). As a result, large study sizes are needed. Three studies to date have investigated the effects of intrinsic factors on internal microbial community composition (28, 30, 33). Pechal et al. (2018) found that of the intrinsic factors assessed (*e.g.*, sex, body mass index [BMI], manner of death), only heart disease was correlated with different mouth microbial diversity, and only for samples taken within 24 hours of death. However, it was unclear if this was an effect of heart disease or age, as heart disease was more prevalent in older populations compared to those dying of other causes (58). Javan et al. (2016) reported no effect of age, but sex did explain some of the variation in bacterial communities. Interestingly, Lutz et al. (2021) reported that BMI and sex explained some of the variation in bacterial composition within their study, but no effect of age or manner of death. While these results are conflicting, it is important to note that all three studies employed a single timepoint sample collection at the time of autopsy or investigation, and so only provide a snapshot of a dynamically changing postmortem community. Aneyo et al. (2020) looked at the impact of lead dosing in swine (*Sus scrofa*) on soil microbes, finding no significant impact on soil microbes; however, only the aerobic, cultivatable community was assessed and no replicates were included within treatment groups (28, 30). Additionally, all of these studies investigate different microhabitats (internal (28, 58) and soil (58)) within a decomposition system, and only one of them took place in an outdoor setting (77). Moving forward more investigation

into the role of intrinsic variables during vertebrate decomposition will be crucial to understand variable decomposition patterns between individuals and species.

Functional succession

Microbes regulate nutrient cycling in ecosystems via the expression of enzymes that break down macromolecules and transform compounds, which influence nutrient availability. Observing succession of microbial functions tells us how microbes are contributing to decomposition processes, as well as provide insight about how broad ecosystem processes, such as nutrient cycling, may be impacted by a decomposing carcass. The majority of studies investigating microbial responses to vertebrate decomposition have been limited to community membership (*i.e.*, taxa present) via DNA sequencing approaches, leaving a considerable gap in understanding functional changes (*i.e.*, changes to decomposition activities of those microbial members).

Functional assessments are extremely important when considering the fact that taxon identity or abundance does not always equate to microbial activity (78-80). Several studies have inferred general microbial activity (8, 45, 52, 70), but no one has yet shown how different taxa contribute to decomposition processes and how the functionality of different microbial communities change over time. Additionally, there is a disconnect between the number and types of functional studies in the three different microhabitats shown in **Figure 1.1**. Most of the microbial activity work in decomposition settings has been conducted in soil (45, 52, 80), however these are largely based on detection of metabolic by-products, such as release of CO₂ or metabolites. In soils, studies have reported a general increase in heterotrophic respiration following the release of decomposition fluids (45, 70), as well as altered lipase gene abundance (45) and enzyme activity (8, 45). Additionally, DeBruyn et al. (2021) employed untargeted metabolomics to reveal changes in microbial metabolite profiles in response to both pig and human decomposition (45). Specifically, increased metabolites related to amino acid metabolism and citric acid (TCA) cycle were observed, suggesting that organic nitrogen metabolism pathways are important. In addition, the metabolite profiles differed between humans and pigs lending credence to the idea that species and/or other intrinsic factors (*e.g.*, drugs,

body composition, etc.) influence decomposition timing and trajectory (81). As a result, we not only need to begin to focus efforts on understanding microbial functional succession, but also identifying factors that may lead to variation in functional changes.

In contrast to the research in soils, functional assessment of internal and external microbial communities during vertebrate decomposition is lacking but see (46, 82). Burcham et al. (2019) investigated gene expression of postmortem internal microbes by applying metatranscriptomics, observing up-regulation of pathways involved in amino acid and carbohydrate metabolism within 72 hours in the heart. The authors suggested that functional redundancy of communities at timepoints greater than 48 hours led to similar expression patterns; however, it is unclear if these results are due to functional redundancy or due to delays in expression changes. This novel study provides a foundation for the investigation of microbial gene expression in other microhabitats during vertebrate decomposition. Moving forward, we need to evaluate the functional activity of microbial communities in all three microhabitats over time to accurately evaluate the role(s) of different microbial taxa during the course of vertebrate decomposition.

CONCLUSIONS

Animal carcasses are important carbon and nutrient inputs in terrestrial ecosystems, driving landscape heterogeneity and nutrient cycling. Considering animal decomposition from an ecological perspective will provide new insights into decomposition ecology. Specifically, this lens will allow us to answer questions about community assembly, succession, and turnover, which in turn will improve our understanding of the ecological impact(s) of vertebrate decomposition. In order to move this field of study forward, we should address ecologically driven questions that help disentangle microbial community dynamics across microhabitats during decomposition. This will ultimately provide a foundation for understanding the impacts of animal decomposition on ecosystems.

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APPENDIX

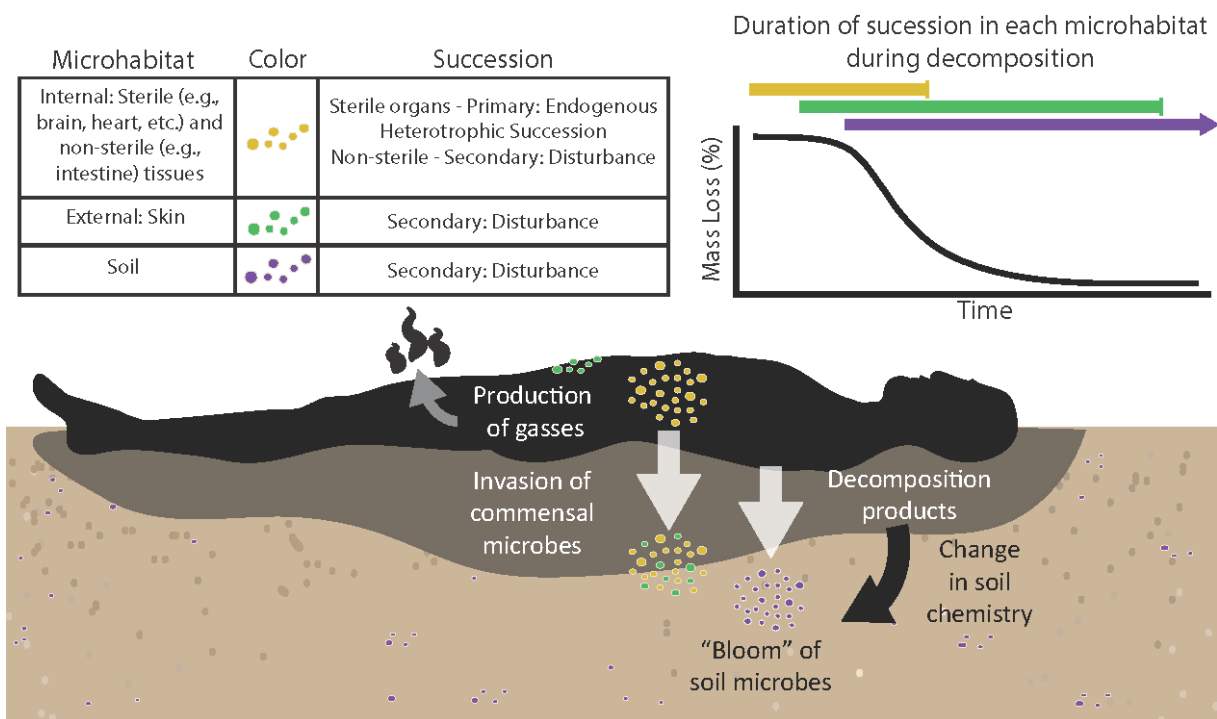


Figure 1.1 Microbial communities in different microhabitats in and on a decomposing animal or human undergo a succession that occur on different timescales (top, right panel). Commensal microbial communities, internal (yellow) and external (green), are the first to respond to decomposition and have a defined endpoint corresponding to the complete disintegration of the carcass tissue (represented by blunt-end arrows). Soil microbial communities (purple) undergo succession as decomposition products enter the soil and this succession continues after soft tissue decomposition is complete (represented by pointed arrow).

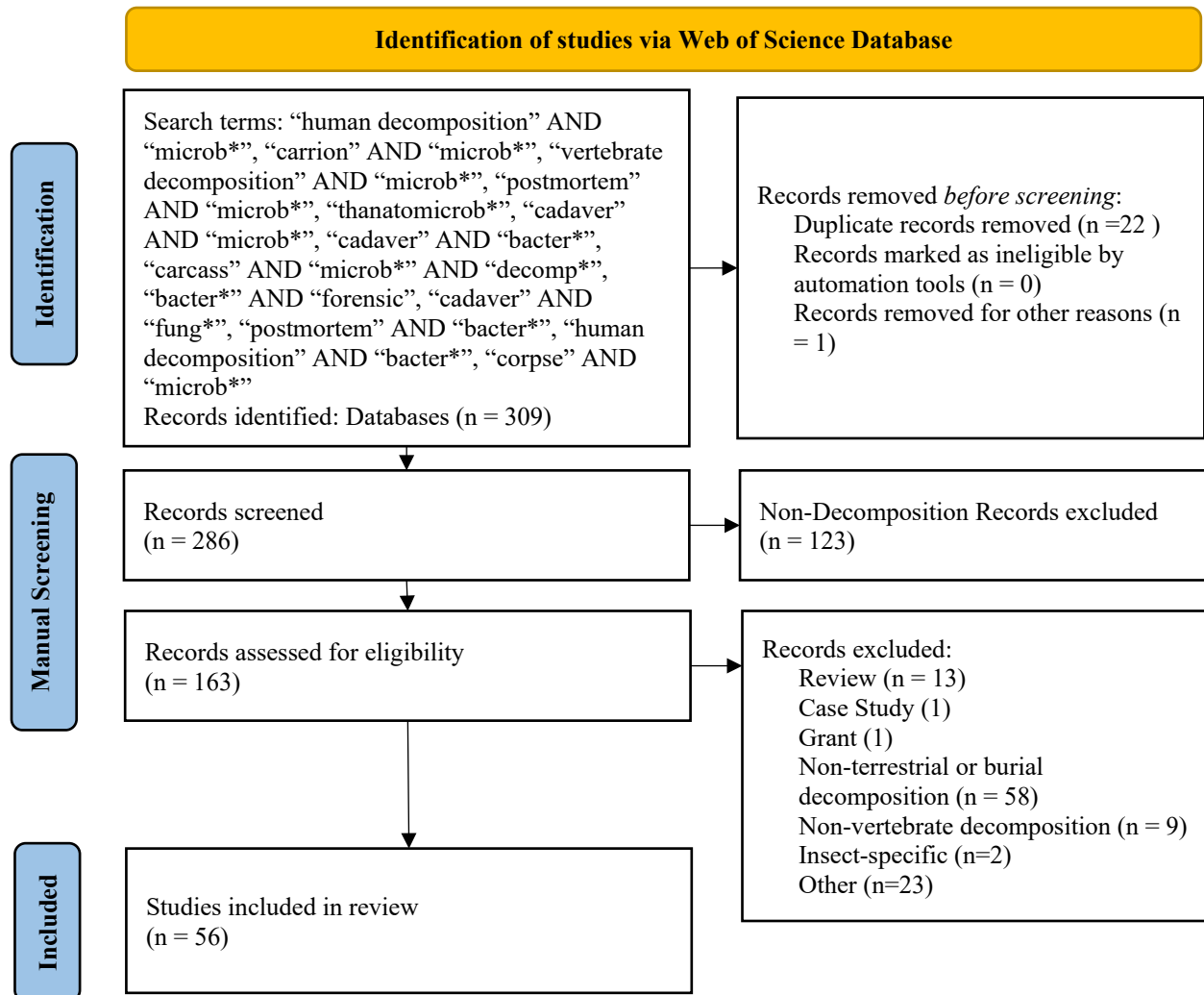


Figure 1.2 Flow chart showing criteria and screening processes for decomposition literature included in this review. Flow chart and guidelines for reported were followed according to PRISMA guidelines. Within the manual screening guidelines, insect-specific referred to articles that probes insect microbiomes. As insect were not within the scope of this review, these references were removed.

Table 1.1 List of the 33 microbial succession studies captured in our search. Papers pertaining to vertebrate decomposition were obtained from Web of Science using the following search terms: “human decomposition” AND “microb*”, “carrion” AND “microb*”, “vertebrate decomposition” AND “microb*”, “postmortem” AND “microb*”, “thanatomicrob*”, “cadaver” AND “microb*”, “cadaver” AND “bacter*”, “carcass” AND “microb*” AND “decomp*”, “bacter*” AND “forensic”, “cadaver” AND “fung*”, “postmortem” AND “bacter*”, “human decomposition” AND “bacter*”, “corpse” AND “microb*”. Duplicate research articles, articles that did not fit the scope of this review, review articles, and those that did not describe succession were excluded (Figure 1.2).

Reference	Microhabitat	Vertebrate Species	Number of Individuals	Geographical Location	Indoor or Outdoor study	Decomposition Timeframe	Study Design	Dependent Variables considered	Microbiology Methodology
Hu et al. (2021)	Internal: appendix, colon	Human	63	China	Indoor	8 days	Destructive		DNA sequencing (16S)
Can et al. (2014)	Internal: blood, brain, heart, liver, spleen	Human	11	Alabama, USA	Indoor	20-240 h	Destructive		DNA sequencing (pyro: 16S)
Javan et al. (2016)	Internal: blood, brain, mouth, heart, liver, spleen	Human	27	Alabama, USA	Indoor	3.5-240 hours	Destructive	Intrinsic: Age, Sex	DNA sequencing (16S)
Bell et al. (2018)	Internal: heart	Human	10	Alabama and Florida, USA	Indoor	6-58 hours	Destructive	Intrinsic: Sex	DNA sequencing (16S)

Table 1.1 continued

Reference	Microhabitat	Vertebrate Species	Number of Individuals	Geographical Location	Indoor or Outdoor study	Decomposition Timeframe	Study Design	Dependent Variables considered	Microbiology Methodology
Lutz et al. (2020)	Internal: brain, heart, liver, spleen, prostate, uterus	Human	40	Italy	Indoor	1-18 days	Destructive	Intrinsic: Sex, Age, Cause of death, BMI	DNA sequencing (16S)
Heimesaat et al. (2012)	Internal: mesenteric lymph nodes, spleen, liver, kidney, blood	Mouse	not reported	Germany	Indoor	72 hours	Destructive		DNA qPCR (9 intestinal bacteria taxa)
Liu et al. (2020)	Internal: brain, heart, cecum	Mouse	240	China	Indoor	15 days	Destructive		DNA sequencing (16S)
Ashe et al. (2021)	Internal: oral	Human	3	North Carolina, USA (FOREST)	Outdoor	253-392 accumulated degree days	Longitudinal		DNA sequencing (16S), metagenome, metatranscriptome
Dong et al. (2019)	Internal: oral	Mouse	24	China	Indoor	10 days	Longitudinal		DNA sequencing (16S)
Iancu et al. (2016)	Internal: oral, rectum	Swine	3	Romania	Outdoor	14 weeks	Longitudinal		DNA sequencing (16S)

Table 1.1 continued

Reference	Microhabitat	Vertebrate Species	Number of Individuals	Geographical Location	Indoor or Outdoor study	Decomposition Timeframe	Study Design	Dependent Variables considered	Microbiology Methodology
Hauther et al. (2015)	Internal: rectum	Human	12	Tennessee, USA (ARF)	Outdoor	9-20 days	Longitudinal	Intrinsic: Cause of death, Sex, Weight	DNA qPCR (3 intestinal bacteria taxa)
DeBruyn et al. (2017)	Internal: rectum	Human	4	Tennessee, USA (ARF)	Outdoor	14-30 days (~800 accumulated degree days)	Longitudinal		DNA sequencing (16S)
Li et al. (2021)	Internal: rectum	Rat	8	China	Outdoor	15 days	Longitudinal		DNA sequencing (16S)
Pechal et al. (2018)	External: ears, nose, mouth, eyes, umbilicus, rectum	Human	188	Michigan, USA	Indoor	1-73+ h	Destructive	Extrinsic: Season, Location of death; Intrinsic: Sex, Ethnicity, BMI, Heart disease, and manner of death	DNA sequencing (16S)
Chun et al. (2015)	External: head, limbs, and maggot masses	Swine	3	Hawaii, USA	Outdoor	144.5 hours	Longitudinal		Culturing: bacteria (aerobic); MALDI-TOF
Hyde et al. (2015)	External: mouth, cheeks, bicep, torso, rectum	Human	2	Texas, USA (STAFS)	Outdoor	24 days	Longitudinal		DNA sequencing (pyro: 16S)

Table 1.1 continued

Reference	Microhabitat	Vertebrate Species	Number of Individuals	Geographical Location	Indoor or Outdoor study	Decomposition Timeframe	Study Design	Dependent Variables considered	Microbiology Methodology
Pechal et al. (2014)	External: oral, skin	Swine	3	Ohio, USA	Outdoor	5 days	Longitudinal		DNA sequencing (pyro: 16S)
Metcalf et al. (2013)	External: head, torso; Soil	Mouse	40	Colorado, USA	Indoor	48 days	Destructive		DNA sequencing (16S, 18S)
Metcalf et al. (2016)	External: skin, abdomen; Soil	Mouse, Human	120 (Mouse); 4 (Human)	Colorado, USA (Mouse); Texas, USA (STAFS: Human)	Indoor and outdoor	71 days (Mouse); 82-143 days (Human)	Destructive (mice); Longitudinal (humans)	Extrinsic: Season (humans only)	DNA sequencing (16S, ITS, 18S)
Fu et al. (2019)	External: torso; Soil	Swine	6	China	Indoor and outdoor	indoor: 8 weeks; outdoor: 2 weeks	Longitudinal		DNA sequencing (ITS)
Adserias-Garriga et al. (2017)	Soil	Human	3	Tennessee, USA (ARF)	Outdoor	12 days	Longitudinal		DNA sequencing (16S)
Moerno et al. (2011)	Soil	Human	9	Tennessee, USA (ARF)	Outdoor	16 weeks	Longitudinal		DNA sequencing (16S, <i>nifH</i>)
Cobaugh et al. (2015)	Soil	Human	4	Tennessee, USA (ARF)	Outdoor	83-198 days	Longitudinal		DNA sequencing (16S)
Finley et al. (2016)	Soil	Human	17	Texas, USA (FARF)	Outdoor	up to 240 days	Destructive		DNA sequencing (16S)
Parkinson et al. (2009)	Soil	Human	3	Tennessee, USA (ARF)	Outdoor	up to 1 year (5370 ADD)	Longitudinal		DNA (T-RFLP); PLFA

Table 1.1 continued

Reference	Microhabitat	Vertebrate Species	Number of Individuals	Geographical Location	Indoor or Outdoor study	Decomposition Timeframe	Study Design	Dependent Variables considered	Microbiology Methodology
Aneyo et al. (2020)	Soil	Swine	3	Nigeria	Outdoor	53 days	Longitudinal	Intrinsic: Lead contamination	Culturing: bacteria and fungi (aerobic)
Carter et al. (2015)	Soil	Swine	3	Nebraska, USA	Outdoor	60 days	Longitudinal	Extrinsic: Season	DNA sequencing (16S, 18S)
Brenton et al. (2016)b	Soil	Swine	12	Canada (UOITDF)	Outdoor	90 days	Longitudinal	Extrinsic: Season	FAME
Howard et al. (2010)	Soil	Swine	1	Louisiana, USA	Outdoor	71 days	Longitudinal		DNA qPCR (Group 1 lipase genes); Culturing: bacteria (aerobic)
DeBruyn et al. (2021)	Soil	Human, Swine	9 Human, 8 Swine	Tennessee, USA (ARF)	Outdoor	5-21 weeks	Longitudinal	Extrinsic: Season	Heterotrophic respiration, Leucine amino peptidase assay
Pechal et al. (2013)b	External: oral, skin, rectal	Swine	21	Ohio, USA	Outdoor	1,862-12,504 accumulated degree hours	Longitudinal		DNA sequencing (16S); Biolog EcoPlates™
Brenton et al. (2016)a	Soil	Swine	12	Canada (UOITDF)	Outdoor	90 days	Longitudinal	Extrinsic: Season	Fluorescein diacetate assay
Damann et al. (2015)	Bone	Human	12	Tennessee, USA (ARF)	Outdoor	571-18,918 accumulated degree days	Destructive		DNA sequencing (16S)

CHAPTER 2 BODY MASS INDEX (BMI) IMPACTS ON SOIL CHEMICAL AND MICROBIAL RESPONSE DURING HUMAN DECOMPOSITION

PREFACE

This chapter focuses on assessing the potential impacts on intrinsic factors (*e.g.*, age, sex, disease, and body mass index [BMI]) on soil chemistry and microbial community structure (*i.e.*, taxonomic succession) during human decomposition. For this project, I was able to collaborate with Dr. Dawnie Steadman and their team from the Anthropology department at the University of Tennessee, Knoxville (UTK), Dr. Shawn Campagna and their team from the Chemistry department at UTK, and the Forensic Anthropology Center (ARF) at UTK to conduct a large-scale multidisciplinary human decomposition study. For this project, I led soil and decomposition fluid collection from 19 human individuals and used a combination of soil chemical analyses and amplicon sequencing (16S rRNA, ITS) to observe environmental and microbial responses to decomposition. This project led to the identification of BMI as an intrinsic factor that can impact chemistry and microbial succession in human decomposition-impacted soils.

A version of this manuscript is currently under review at mSphere and has been edited by co-authors: Allison R. Mason, Hayden S. McKee-Zech, Katharina M. Hoeland, Mary C. Davis, Shawn R. Campagna, Dawnie W. Steadman, and Jennifer M. DeBruyn.

Contributions: Funding for this study was awarded to DWS, SRC, and JMD (National Institute of Justice Grant # 2018-DU-BX-0180). This study was conceived by DWS, SRC, and JMD. Donor enrollment and placements were conducted by MCD, HSMZ, and ARM. Collection of daily photos and field observations were collected by HSMZ, with acknowledgments to Sarah Schwing, Thomas Delgado, and Erin Patrick. Soil and decomposition fluid sampling protocols were developed by ARM and JMD. Field collections of soil and fluid samples were completed by ARM, HSMZ, and KMH, with acknowledgements to Tori Beard, Morgan White, Sarah Schwing, Thomas Delgado, and Erin Patrick. Laboratory soil physicochemical analyses were completed by ARM, with acknowledgements to Tori Beard, Morgan White, and Mallari Starrett. Next generation sequencing and microbial community analyses were conducted by ARM. Data analysis and visualization were completed by ARM. The manuscript was written by ARM and JMD, with input from all authors.

ABSTRACT

Abstract

Microorganisms are key decomposers of vertebrate mortalities, breaking down body tissues and impacting decomposition progress. During human decomposition, both extrinsic environmental factors and intrinsic cadaver-related factors have potential to impact microbial decomposers either directly, or indirectly via altered physical or chemical conditions. While extrinsic factors (e.g., temperature, humidity) explain some variation in microbial response during human decomposition in terrestrial settings, recent work has noted that even under the same environmental conditions, individuals can have different decomposition patterns, highlighting the potential for intrinsic factors to impact microbial decomposers. The goal of this study was to investigate the effects of several intrinsic factors (age, sex, diseases at time of death, and body mass index [BMI]) on chemical and microbial changes in decomposition-impacted soils. In a field study conducted at the University of Tennessee Anthropology Research Facility, soils were collected from the decomposition-impacted area surrounding 19 deceased human individuals through the end of active decomposition. Soil physicochemical parameters were measured, and microbial (bacterial and fungal) communities were assessed via amplicon sequencing. BMI was shown to explain some variation in soil pH and microbial response to human decomposition. Hierarchical linear mixed effects models revealed that BMI category significantly explained variation in pH response within decomposition-impacted soils over time (HLM $F = 9.647$; $p < 0.001$). Additionally, the relative abundance of soil *Saccharomycetes* in decomposition soils under underweight donors displayed little to no changes (mean maximum change in relative abundance: +6.6 %), while all other BMI categories displayed increased relative abundance of these organisms over time (normal: +50.6 %, overweight: +64.4 %, obese: +64.6%) (HLM $F = 3.441$; $p = 0.11$). Together, these results reveal intrinsic factors influencing decomposition patterns, especially within the soil environment, and suggest BMI is an important factor for controlling decomposition processes.

Importance

This work begins to address questions about inter-individual variation in vertebrate decomposition attributed to intrinsic factors, that is, properties of the carcass or cadaver itself.

Most research on factors affecting decomposition have focused on the extrinsic environment, such as temperature or humidity. While these extrinsic factors do explain some variation in decomposition patterns, inter-individual variability is still observed. Understanding how intrinsic factors influence microbial decomposers will help reveal the ecological impacts of decomposition. This work also has forensic applications, as soil chemical and biological changes have been suggested as indicators of postmortem interval. We reveal factors that explain variation in the decomposition environment that should be considered in these estimates. This is particularly important as we consider the implications of variations in human populations due to diet, age, BMI, disease, toxicological loading, etc. on forensic investigations dealing with decomposing remains.

INTRODUCTION

Carcass decomposition is an important ecosystem process, stimulating biological activity and nutrient cycling in the local environment. In terrestrial settings, nutrient rich fluids from decomposing animal carcasses are flushed into the surrounding soil during decomposition, altering soil chemistry and increasing soil microbial activity. Changes in soil chemistry include increased electrical conductivity, ammonium concentrations, and dissolved organic carbon and nitrogen content (1-3). Soil microbes also respond to decomposition products, resulting in altered community composition and activity. This includes decreased alpha diversity and increased relative abundance of Firmicutes and Bacteroidetes has also been observed during human decomposition (4, 5).

While general patterns in soil responses to human decomposition have been identified, variability in the direction and magnitude of soil responses have been noted. For example, change in soil pH is highly variable between studies. While Perrault and Forbes (2016) and Aitkenhead-Peterson et al. (2012) reported decreased pH in decomposition-impacted soils, other studies have reported increased pH (3, 8-10). Further, Fancher et al. (2017) observed both increases and decreases in pH between individuals within the same study. Variability in pH response has also been observed between species: for example, DeBruyn et al. (2021) observed decreased pH under humans but increased pH under pigs concurrently decomposing at the same site under the same environmental conditions. It is unknown why differences in soil pH response are observed between sites and studies. However, given that pH is a key abiotic control on microbial communities, this variation in soil chemical response likely impacts the activity and succession of soil microbial decomposers during decomposition (11), and may constrain the fate of carcass-derived carbon and nutrients in the ecosystem.

Both extrinsic environmental factors (*e.g.*, temperature, season, moisture, etc.) and intrinsic cadaver-related, factors (*e.g.*, sex, diseases, mass, etc.) have potential to impact decomposition rates and patterns. Specifically, warmer temperatures increase insect and microbial activity, and more rapid decomposition rates are observed compared to cooler temperatures (12). Moisture also affects decomposition by mediating microbial activity (13). However, even when extrinsic factors are controlled for, such as when multiple carcasses start decomposition at the same time

and experience the same local environmental conditions, differential decomposition patterns have been observed among individuals (1, 2, 14), suggesting that intrinsic factors may also impact decomposition patterns. Genetics, age, sex, diet, body mass index (BMI) and diseases (including therapeutic interventions) can lead to differences in body physiology or chemistry and microbiome between individuals (15-20) that may ultimately impact decomposer activity during decomposition. This could be a direct effect on soil physiochemistry and microbes because of changes in tissue decomposition product chemistry, or an indirect effect, for example by influencing insect or scavenger behavior which then alters decomposition progression (**Figure 2.1**). Spicka et al. (2011) observed that a greater body mass (20-50 kg pigs) resulted in slower decomposition rate but overall greater release of ninhydrin reactive nitrogen in decomposition soils compared to individuals with lower body mass (1 kg pigs), highlighting variability in decomposition patterns due to body mass.

Changes in soil chemistry and microbial succession in response to human decomposition have been suggested as potential markers to estimate the postmortem interval (PMI) as evidence in death investigations (22-24), therefore understanding sources of variation in human decomposition is critical for forensic applications. Both extrinsic and intrinsic factors are sources of variability that can impact PMI; however, a majority of decomposition studies have focused on extrinsic factors (1, 10, 25-28). A few terrestrial decomposition studies have shown that inclusion of BMI in soil chemistry-based PMI models improves model prediction (2, 29). In contrast, microbial abundance-based PMI models (24, 30) have not incorporated any extrinsic or intrinsic variables in model construction and rely solely on sequencing data. Additionally, the few studies addressing the effects of intrinsic factors on microbial succession concentrate on internal microbial communities (31-33), leaving questions about the influence of intrinsic factors on soil microbes during decomposition. Identifying intrinsic factors which influence human decomposition will lead to a better understanding of soil and microbial responses, ultimately improving PMI estimations from these markers.

The purpose of this study was to investigate the effects of several intrinsic factors (age, sex, diseases at time of death, and BMI) on chemical and microbial changes in decomposition fluids and soils impacted by the fluids during human decomposition. Our overarching hypothesis was

that differences in these intrinsic factors would lead to changes in the magnitude of soil chemical response and altered microbial successional patterns. Based on the demographics of our study population (age ≥ 40) BMI is a reasonable proxy for body composition, where a higher BMI is associated with a greater proportions of fat tissue relative to muscle tissue (34, 35). We hypothesized that differences in fat:muscle composition of a body changes the nutrient resources available to the environment during decomposition, and therefore may alter microbial communities (*e.g.*, a higher fat content might result in selection for lipid metabolizers). Additionally, we examined the effects of diseases on decomposition. Diseases are known to alter microbiome composition and tissue chemistry antemortem (18); thus, we hypothesized this may also affect postmortem diversity and activity of microbial decomposers. To address our questions, 19 deceased human individuals were studied in an outdoor surface decomposition experiment at the University of Tennessee Anthropology Research Facility (ARF) from February 2019 – March 2020. Soil chemical analyses were combined with characterization of soil bacterial and fungal communities using amplicon sequencing to link these differences to intrinsic factors, ultimately gaining a better understanding of soil dynamics during human decomposition.

METHODS

Study design

A series of human decomposition studies were conducted at the University of Tennessee Anthropology Research Facility (ARF), located in Knoxville, TN (35° 56' 28" N, 83° 56' 25" W). The ARF is a roughly 2-acre area of temperate mixed deciduous forest dedicated to studying human decomposition (36). The soil type at ARF consists of clay loam and channery clay loam overlaying a bedrock of limestone, shale, and sandstone. These soils are classified as Coghill-Corryton complex (CcE), containing 25 – 65% slopes and described as rocky and well drained (<https://websoilsurvey.sc.egov.usda.gov/>).

Nineteen deceased human individuals (hereafter, “donors”) were selected from those donated through the ARF body donation program. Donors were selected independent of age, weight, ancestry, or sex. Donors without open wounds were selected in order to avoid altering insect behavior and/or microbial activity during decomposition. Medical histories and known prescribed medications lists were obtained for all individuals enrolled in the study. Donors

ranged in age from 40-91 years and were near evenly distributed by sex (**Table 2.1**). All donors identified as White. BMI (kg/m^2) was calculated using cadaver height (m) and weight (kg) recorded upon arrival at the Forensic Anthropology Center (FAC). From this, BMI of donors ranged from 14.2 to 55.1 and was used to group donors into BMI groups using Centers for Disease Control (CDC) categories (Underweight < 18.5 ; Normal 18.5 - 24.9; Overweight 25 – 29.9; Obese ≥ 30). Donors were also categorized by the presence or absence of six broad disease categories, determined by conditions reported as contributing to cause of death. We recognize that categorizing by disease encompasses altered physiology or microbiome due to disease, as well as medications and other therapeutic interventions used to treat the disease. Disease categories included: diabetes, cancer, cardiovascular diseases, respiratory diseases, neurological diseases, and pneumonia (**Table 2.1**). In most cases, more than one condition was reported as contributing to cause of death, therefore some donors were attributed multiple conditions.

Donors were placed within the facility to decompose as they were received between February 2019 and March 2020 (**Table 2.1**). Prior to placement all individuals were kept at 4 °C for no longer than one week following death. Each individual was placed supine on the soil surface without clothing. For every donor site, a control site was identified at least 1 m away from the donor and uphill if possible. Hourly temperature was monitored using TinyTag temperature and humidity loggers (Gemini Data Loggers, UK) placed within 0.5 m of the donor and 1 m above the ground. Temperature readings were taken every hour from time of placement until the donor was unenrolled from the study at the end of active decomposition. Active decomposition is the decomposition stage characterized by the release of gasses and fluid from the remains, the cessation of fluid purge, and complete collapse of the abdomen (37), all of which were used as indicators of the end of active decomposition here. In this study, 0 ADH was defined as time of placement within the ARF, and a baseline temperature of 10 °C was used for ADH calculations to keep our results consistent with additional insect data collected from these donors.

Soil sampling

Soil samples were collected at 0, 100, 250, 500, 750, 1000, and thereafter at 500 accumulated degree hour (ADH) intervals until the end of active decomposition when donors were unenrolled from the study. For each soil sample, a sterile 10 ml syringe with the tip end cut off (Norm-Ject®

Henke-Ject™ syringe) was used to collect five to eight soil cores of 5 cm depth. At each timepoint, soils were collected from within the area of visibly saturated soils around decomposing donors and at the control site. Soils were then homogenized by hand and debris larger than 2 mm was removed (*i.e.*, rocks, roots, insects, etc.). A 20 g subsample was weighed into a 4 oz. Whirl-Pak™ bag (Nasco), flash frozen in liquid nitrogen and stored at -80 °C prior to DNA extraction. Remaining soil was stored at 4 °C prior to soil physicochemical analyses.

Decomposition fluid sampling

Decomposition fluids emitted from the donors were collected as they pooled on the surface of the soil surrounding the donor. Fluid was collected using a sterile 30 ml syringe (BD 30 ml Luer-Lok™ tip syringe). The tip of the syringe was carefully inserted into the surface of pooling fluid and slowly drawn up. This process was repeated until ~10 ml of fluid was collected. The syringe was then carefully inverted two to three times to homogenize, and fluid was evenly dispensed into four 2 ml cryovials. Cryovials were flash frozen in liquid nitrogen and stored at -80 °C prior to DNA extraction.

Soil physicochemical analysis

A subset of soil physicochemical analyses were conducted within 4 days of collection using soils stored at 4 °C. Soils were acclimated to room temperature for at least 30 minutes prior to all measurements. Soil electrical conductivity (EC) and pH were measured on a 1:2 soil to deionized water mixture by weight using an Orion Star™ A329 pH/ISE/Conductivity/Dissolved Oxygen portable multiparameter meter (ThermoFisher). Soil gravimetric moisture was calculated after determining the water weight of soil by oven drying duplicate 2 to 3 g soil aliquots at 105 °C for 72 hours.

Soil biological activity

After soil samples acclimated to room temperature, respiration rates were measured via the accumulation of CO₂ over 24 hours as described previously (36). Briefly, 6 g of soil was sealed in 60 ml serum bottles and CO₂ was measured with duplicate injections, immediately after capping and after 24 hours, into a LI-820 CO₂ analyzer (LI-COR) with manual injection. Incubations were conducted in the dark at room temperature (~20 °C).

Extracellular enzyme activities and 16S rRNA gene amplicon sequencing was performed for all donors on a subset of soil samples. For most donors, five soil samples corresponding to 0, 1500, 3000, and 4500 ADH plus the final (T_f) sample was used for enzyme assays and sequencing, as these ADH values were the median 25, 50, and 75 percentiles across all donors. Some donors completed active decomposition at/or before 4500 ADH or extended longer (*i.e.*, TOX010 T_f = 17500 ADH), therefore fewer or additional timepoints were assessed.

The activity of four common soil extracellular enzymes were evaluated: β -glucosidase (BG; sugar degradation), phosphatase (PHOS; phosphorous mineralization), *N*-acetyl- β -D-glucosaminidase (NAG; chitin degradation), and leucine amino peptidase (LAP; protein degradation). Enzyme assays were conducted in triplicate for all donors as previously described in Bell et al. (2013) (38), with some modifications. Briefly, 2.75 g subsamples were weighed from soils stored at -80 °C and held at -20 °C prior to assays. Soil samples were then thawed at room temperature and slurried in 50 mM TRIS buffer at pH 6.7 (average pH of our soil samples) in a blender (Waring commercial blender, model WF2212114). Assays were conducted using 800 μ l of slurry and 200 μ l of enzyme substrate (1500 μ M). Optimum substrate concentrations were determined prior to conducting assays on soil samples and was found to be 1500 μ M for all enzymes. Additionally, standard curves were conducted using MUB and MUC concentrations ranging from 0 μ M to 200 μ M. All standard curves and soil samples were assessed in triplicate and included blanks for each sample to evaluate background concentrations.

DNA sequencing

DNA was extracted from all soil and decomposition fluid samples using the DNeasy Powerlyzer PowerSoil kit (QIAGEN Inc.). Extractions were conducted according to manufacturer's instructions with the following modifications. Briefly, 0.25 g of soil/fluid was used for all extractions and homogenized (MO BIO PowerLyzer™ Bench Top Bead-Based Homogenizer) using settings suggested for high organic soils (2,500 RPM for 45 s). DNA was eluted in 100 μ l of 10 mM Tris buffer and stored at -20 °C prior to sequencing. Total DNA concentrations were determined using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen) using an assay volume of 200 μ l and 1 μ l of DNA. Control and decomposition fluid extracts for each donor were pooled, ensuring equal amounts of DNA (determined by concentration) were added. DNA extracts

were sent to the University of Tennessee Knoxville Sequencing Core Facility (Knoxville, TN) for library preparation and sequencing. Both bacterial and fungal communities were sequenced; the primer set 515F (39)/806R (40) was used to amplify the V4 region of the 16S rRNA gene, the ITS2 region in fungi was amplified using primers described previously(41). Libraries were sequenced on the Illumina MiSeq platform to generate paired-end reads.

Reads were processed using Mothur (42) (v.1.43.0). Briefly, low-quality (16S: $Q > 20$, $bp \leq 50$; ITS $Q > 20$, $bp < 200$), sequences containing ambiguous bases (≥ 1), and non-biological (primers and adapters) sequences were removed. 16S reads were aligned to the SILVA non-redundant database (v132), while ITS were not aligned. VSEARCH was used to remove chimera sequences. Bacterial and fungal sequences were classified using the SILVA non-redundant database (43) (v132) and UNITE RefS database (44) (version 02.02.2020), respectively. Bacterial sequences were then clustered into operational taxonomic units (OTUs) based on $\geq 97\%$ sequence similarity and the default optclust method, while fungal sequences were clustered using abundance-based greedy clustering.

Statistics

To account for natural variation in soil parameters over time and space and allow for comparison between donors, we normalized measured soil parameter values by calculating the log response ratio ($LRR = \ln[\text{treatment value}/\text{control value}]$) (45). LRR values greater than 0 were higher in decomposition-impacted soils compared to control soils, while values less than 0 were lower in decomposition-impacted soils compared to control soils.

Effects of time (as ADH), season, BMI, sex, and diseases on soil parameter's response during decomposition were assessed with hierarchical linear mixed effects models, allowing for random slopes and/or intercepts by donor. Random effects terms were chosen for each response variable based on best fit (determined by Akaike information criterion, AIC). Due to small sample sizes, the effects of diabetes ($n = 2$) and pneumonia ($n = 2$) were not evaluated. Statistical differences were then assessed using a type III analysis of variance (ANOVA) with Satterthwaite's method. All models were run in R using the *lmer()* function (R package lme4 version 1.1.25) and statistically analyzed using the *anova()* function from lmerTest (version 3.1.3) package. Due to differences in ADH to complete active decomposition, patterns of soil chemical responses differed

between donors with some exhibiting linear patterns and others displaying saturating trends. Thus, only data points ≤ 5000 ADH were used in hierarchical linear mixed effects models to capture the linear response period (436 samples across all donors).

Alpha (Chao1 richness and Inverse Simpson) and beta (Bray-Curtis dissimilarity) diversity of soil and fluid communities, respectively, were calculated in R and beta diversity was visualized using principal coordinate analysis (PCoA) within the R package phyloseq (version 1.32.0). Changes in alpha diversity within soil communities were assessed using hierarchical linear mixed effects models and statistical differences assessed using type III analysis of variance (ANOVA) with Satterthwaite's method. Differences in soil community structure were evaluated with permutational analysis of variance (PERMANOVA), via the *adonis()* function in the R package vegan (version 2.5.6). The functions *betadispr()* and *permutest()*, both from vegan (version 2.5.6), were used to evaluate multivariate homogeneity of dispersions between specified groups (*i.e.*, Donor, ADH) from Bray-Curtis dissimilarity values.

RESULTS

BMI and decomposition time

BMI of the nineteen individuals used in this study ranged from 14.2 to 55.1, with a median BMI of 24.6. In general, as BMI of the individual increased, the time (in accumulated degree hours, ADH) to complete active decomposition increased (linear mixed effects model $F = 39.58$, $p < 0.001$) (**Figure 2.2**). ADH to complete active decomposition ranged from 1500 for the lowest BMI donor to 18,500 for the highest BMI donor, where the completion of active decomposition was determined as the stage when the abdomen was completely collapsed, and body was no longer actively producing visible decomposition fluids. While not significant, males in this study had slightly higher BMIs than females (Wilcoxon $W = 34$, $p = 0.4002$) and BMI decreased with age at death (ANOVA $F = 3.121$, $p = 0.095$) (**Figure 2.3A,B**). Differences in BMI by end-of-life diseases were only observed for cardiovascular diseases ($W = 10$, $p = 0.019$), where donors with cardiovascular diseases were associated with higher BMI (**Figure 2.4**).

Decomposition effects on soil chemistry and microbial activity

Decomposition significantly increased soil electrical conductivity (EC) compared to control soils but had no consistent or significant effect on soil pH (hierarchical linear mixed effects model, HLM pH $p = 0.149$; EC $p = 0.002$) (**Figure 2.5**). When normalized to the control soils (to account for background variability), the log response ratio of soil EC significantly increased over time ($F = 27.93$; $p < 0.001$). In contrast, the log response ratio of soil pH was not significantly different over time ($F = 0.242$; $p = 0.623$) due to high variability between donors. In particular, while a majority of donors ($n = 14$) displayed decreases in soil pH, some donors ($n = 5$) resulted in increased pH (**Figure 2.5**). Similar to EC, decomposition resulted in increased heterotrophic respiration during decomposition ($F = 11.39$; $p = 0.029$). The log response ratio of soil parameters did not significantly differ between seasons or due to sex of the individual (**Table 2.2**). It was also noted that at the end of active decay, all the soil parameters measured were still altered compared to initial conditions.

To assess soil microbial activity in soils, we measured the activity of four common soil extracellular enzymes: β -glucosidase (BG), *N*-acetyl- β -D-glucosaminidase (NAG), phosphatase (PHOS), and leucine aminopeptidase (LAP). Decomposition significantly altered the activity of NAG ($F = 6.523$; $p = 0.012$); however, no significant differences were detected between decomposition and control soils over time for BG ($F = 0.626$; $p = 0.431$), PHOS ($F = 2.300$; $p = 0.133$), and LAP ($F = 0.054$; $p = 0.817$) in decomposition-impacted soils within our dataset. Generally, NAG and PHOS activity increased (**Figure 2.5**), while the protein degrading enzyme, LAP, was variable between individuals ($n = 13$ decreased) in soils during decomposition (**Figure 2.5**).

Principal component analysis (PCA) of normalized (LRR) soil parameters (pH, EC, BG, NAG, PHOS, and LAP) revealed relationships between soil parameter responses during decomposition (**Figure 2.6**). Principal component (PC) 1 was associated with time and related to an increase in soil parameters EC, PHOS, NAG, and BG. Soil response varied along PC2, which was most related to differences in pH and LAP activity between individuals.

BMI and soil responses

We observed a relationship between variability in soil pH response and donor BMI. Hierarchical linear mixed effects models revealed that BMI category significantly explained variation in pH response within decomposition-impacted soils over time (HLM $F = 9.647$; $p < 0.001$) (**Table 2.2**). Specifically, soil pH increased in decomposition-impacted soils of underweight individuals, but decreased under normal, overweight, and obese donors (**Figure 2.7**). Soil pH and LAP activity were positively correlated (Spearman $r = 0.73$; $p < 0.001$) during the study period, with LAP activity increasing with pH ($F = 4.781$; $p = 0.032$) (**Figure 2.8**). However, change in LAP activity over time ($F = 2.444$; $p = 0.131$) was not significantly different between BMI categories.

Diseases and soil responses

We sought to determine if donors' diseases explained some of the variability in the measures soil parameters. The primary effects of presence and absence of four disease categories (cancer, respiratory diseases, cardiovascular diseases, and neurological diseases) and their influence on soil parameters over time were evaluated with HLM and results are reported in **Table 2.3**. A few trends were observed between donor disease state and soil responses during decomposition. First, pH decreased to a greater extent in decomposition soil of those with neurological diseases compared to those without (HLM $F = 30.79$; $p < 0.001$). Microbial respiration increased to a greater extent for those with cardiovascular diseases over time ($F = 5.077$; $p = 0.026$). Individuals with cancer displayed a greater decrease in soil LAP activity ($F = 4.201$; $p = 0.045$) and less of an increase in microbial respiration ($F = 8.776$; $p = 0.004$) with progress of decomposition.

Microbial communities

To assess changes in soil microbial community composition during decomposition, selected soil and pooled decomposition fluid samples were sent for 16S rRNA gene (V4 region) and internal transcribed spacer (ITS) amplicon sequencing. 16S rRNA gene sequencing yielded a total of 17,372,327 raw reads. Removal of primers, erroneous reads (≥ 1 ambiguous bases, $50 > \text{bp} > 275$), sequences that aligned poorly, and chimera and non-bacterial sequences resulted in 10,844,074 remaining reads. In R, control samples (*e.g.*, extraction blanks) and singletons were removed leaving 10,579,899 reads across 134 samples with a mean library size of 78,954 reads.

Reads were clustered into 33,363 operational taxonomic units (OTUs) at 97% similarity, with a mean of 3,958 OTUs per sample. Good's coverage for bacterial libraries were greater than 0.994 for all samples, therefore libraries were randomly subsampled to the smallest 16S library size ($n = 26,780$) prior to alpha and beta diversity analyses.

Gene amplification of the ITS2 region yielded a total of 13,044,056 raw reads. Removal of primers, erroneous reads (≥ 1 ambiguous bases, $bp < 200$), and chimera and non-fungal sequences resulted in 9,052,099 remaining reads. After removal of control samples and singletons in R, 9,037,408 reads across 134 samples remained, with a mean library size of 67,443. Reads were clustered into 13,536 operational taxonomic units (OTUs) at 97% similarity, with a mean of 852 OTUs per sample. For all fungal libraries, Good's coverage was greater than 0.991. Libraries were randomly subsampled to the smallest ITS library size ($n = 13,193$) prior to alpha and beta diversity analyses.

Decomposition fluid microbial communities

Chao1 richness estimates of the decomposition fluid bacterial and fungal communities ranged from 228.93 to 739.95 and 53.49 to 381.38, respectively, while Inverse Simpson diversity values ranged from 4.31 to 24.87 and 1.08 to 47.07, respectively. Generally, alpha diversity (Chao1 richness and inverse Simpson diversity estimate) of decomposition fluid bacterial communities increased with greater total ADH to complete active decomposition. Mean richness was 318.3 for donors that completed decomposition quickly ($ADH \leq 5000$) and 517.1 for donors that took longer to decompose ($ADH \geq 10,000$). Other than total ADH, no variables tested explained the variation in Chao1 or Inverse Simpson estimates of decomposition fluid bacterial communities between donors, while none of the variables tested significantly explained fluid fungal community diversity changes (**Table 2.4**). Further, no extrinsic or intrinsic factors tested, such as season, age, sex, BMI, and respective diseases significantly explained (PERMANOVA $p > 0.05$) variation in bacterial or fungal community composition of decomposition fluid communities as assessed by permutational multivariate analysis of variance (PERMANOVA) (**Table 2.4**).

Microbial communities found in the decomposition fluid emitting from the donors were comprised of two to five different bacterial phyla (**Figure 2.9**). A majority of the bacterial communities were comprised of Firmicutes and Proteobacteria with a mean relative abundance

across individuals of 63% and 28%, respectively. At the class level, decomposition fluid communities were dominated by Clostridia (39%), Gammaproteobacteria (29%), and Bacilli (28%). High inter-individual variability was observed between fluid bacterial communities at the genus level, however *Oblitimonas* (25%), *Lactobacillus* (22%), and *Ignatzschineria* (20%) were commonly observed. Fungal communities in the decomposition fluid were comprised of one to four dominant phyla (**Figure 2.9**) depending on the individual. Across all individuals, the fungal phylum Ascomycota dominated the decomposition fluid community, with an average relative abundance of 91% across individuals. A majority of these Ascomycota belonged to the fungal class *Saccharomycetes* (78%) (**Figure 2.9**), however a subset of individuals (n = 7) also contained *Sordariomycetes* in their decomposition fluids. Aside from one individual (TOX002), a majority of fungal organisms in decomposition fluid (mean 65%) were of the genus *Yarrowia*, a *Saccharomycete*. The genus *Dipodascus* was also present in fluid samples from a subset of individuals (n = 5). Interestingly, the common human fungal commensal, *Candida* (class *Saccharomycetes*), was only detected in the decomposition fluid of one individual (TOX017) at relative abundances higher than 5%.

Soil microbial communities

Alpha diversity

Soil bacterial communities had greater diversity than decomposition fluid communities (6 times higher Chao1 and 6.5 times higher Inverse Simpson index). In general, species richness and diversity of soil bacterial and fungal communities decreased as decomposition progressed (**Figure 2.10**). The contribution of intrinsic and extrinsic factors to inter-individual variability in diversity was investigated using HLM. Factors of interest included season, body mass index (BMI), sex, and the presence/absence of different diseases at time of death (**Table 2.2**, **Table 2.3**). We found that neither season nor sex had a significant effect ($p > 0.05$, **Table 2.2**) on Chao1 richness or Inverse Simpson estimates. BMI category was not related to bacterial richness, however there were differences in diversity (Inverse Simpson estimates) over time by BMI category (HLM $F = 3.705$; $p = 0.017$). Specifically, diversity in soils below underweight and normal individuals decreased, while diversity in soils below overweight and obese

individuals remained constant through 5000 ADH. BMI category did not explain differences soil fungal richness or alpha diversity (**Table 2.2**).

Changes in soil chemistry were related to some of the differences in microbial communities over time. Within bacterial communities, richness ($F = 13.09$; $p < 0.001$) and diversity ($F = 6.331$; $p = 0.014$) estimates decreased as soil EC increased. For fungal communities, richness ($F = 5.424$; $p = 0.023$) and diversity ($F = 17.19$; $p < 0.001$) increased with increasing LAP activity in decomposition soils. Fungal community diversity was also shown to increase with increasing BG activity ($F = 12.41$; $p < 0.001$) and soil moisture ($F = 30.06$; $p < 0.001$).

Some trends were observed with microbial richness (Chao1 estimates) and disease (**Table 2.3**). Specifically, change in bacterial Chao1 estimates over time differed between donors with and without respiratory ($F = 4.640$; $p = 0.035$) or neurological ($F = 5.237$; $p = 0.026$) diseases. While no overall relationship between Chao1 estimates and the presence of cancer was observed using HLMs, we did observe lower richness in decomposition soils surrounding individuals with cancer compared to those without cancer at one time point: 4500 ADH (Wilcoxon $p = 0.01$). Presence or absence of disease at time of death did not explain differences in fungal richness or diversity. (**Table 2.3**). We did not observe any relationship between the presence of cancer, cardiovascular, respiratory, or neurological diseases and bacterial diversity in decomposition soils.

Community structure

Microbial community structure

Microbial community composition was altered in response to human decomposition (**Figure 2.11A,B**). Both bacterial (PERMANOVA $F = 2.25$, $r^2 = 0.025$, $p = 0.004$) and fungal ($F = 2.97$, $r^2 = 0.023$, $p = 0.002$) community composition was significantly different in decomposition-impacted soils compared to controls. Constrained Analysis of Principal coordinates (CAP) of Bray-Curtis distances was used to relate changing microbial community composition to the measured soil environmental parameters. The first two CAP axes explained 14.6% and 16% of bacterial and fungal community variation, respectively (**Figure 2.11A,B**). In both bacterial (**Figure 2.11A**) and fungal (**Figure 2.11B**) CAP analyses, gravimetric moisture and temperature

was related to CAP2, while pH, EC, extracellular enzyme activities, and time (as percent ADH) were related to CAP1. Interestingly, the relationships between measured soil chemical variables and enzyme activities and microbial compositional shifts follow those identified in the soil chemical responses during decomposition (**Figure 2.6**). Microbial community composition shifted along CAP1, which was strongly correlated to increased EC in decomposition soils. Similar to soil chemistry shifts, soil pH and LAP activity were positively correlated in constrained ordinations. Composition also varied along CAP2, where soil samples with increased temperature had lower gravimetric moisture.

Some differences in bacterial and fungal compositional shifts in response to decomposition were observed. Specifically, while soil bacterial communities changed as decomposition progressed, they did not become more similar with time. Statistical beta dispersion, a measure of variability between groups, shows that the statistical beta dispersion of bacterial communities increases slightly over time (**Figure 2.12A**). In contrast, dispersion of fungal communities decreased with time (Beta dispersion $F = 11.17$, $p = 0.001$) (**Figure 2.12C**), showing that fungal communities become more similar during active decomposition. Additionally, inter-individual variability was greater within bacterial communities than within fungal communities (**Figure 2.12B,D**). Beta dispersion of bacterial communities was significantly different between individuals ($F = 4.239$; $p = 0.001$), while the beta dispersion of fungal communities was not ($F = 0.796$; $p = 0.711$).

Two-way PERMANOVAs assessing the effects of time and respective factors on bacterial and fungal community composition were performed, while beta dispersion results for all variables are reported in **Table 2.5**. Within bacterial communities, all variables were significant (PERMANOVA $p < 0.05$), while all but cancer were significant for fungal communities. This was likely due to repeated measures within each donor causing lower p-values. As a result, we also evaluated potential effects on community composition within respective timepoints (0, 1500, 3000, 4500, and 6000 ADH) using one-way PERMANOVA with each factor. An individual's age explained some of the variation in bacterial (PERMANOVA $F = 1.611$; $p = 0.017$) and fungal ($F = 1.318$; $p = 0.0489$) communities at 1500 ADH (**Table 2.6**). No other variables were significant for bacterial communities, but season did significantly impact soil fungal communities. Specifically, variation in fungal community composition in decomposition-

impacted soils was partially explained by season at 3000 and 4500 ADH. Additionally, fungal communities differed by season ($F = 1.271$; $p = 0.033$) in control soils, while bacterial communities did not ($F = 1.124$; $p = 0.2707$). We also noted that soils collected from decomposition plots prior to placement were not significantly different from controls in regard to bacterial ($F = 1.217$; $p = 0.2328$) and fungal ($F = 1.148$; $p = 0.1958$) community composition.

Bacterial community composition

Relative abundance of bacterial taxa in soil changed throughout decomposition (**Figure 2.13A, 2.14A**). At the phylum level, increases in relative abundance of *Proteobacteria* (median 13.8% increase across all donors) and *Firmicutes* (20.4%) were observed over time, while median relative abundance of *Verrucomicrobia* (median 7.7% decrease across all donors) and *Acidobacteria* (12%) decreased. Changes in *Bacteroidetes* and *Actinobacteria* were variable among individuals: *Bacteroidetes* increased in 14 individuals and decreased in five, while *Actinobacteria* increased in 11 and decreased in 8. At the class level, *Gammaproteobacteria* (median 15.7% increase across all donors), *Clostridia* (9.5%), *Actinobacteria* (7.2%), and *Bacilli* (6.9%) increased in decomposition soils; notably, these were taxa also detected in fluid samples (**Figure 2.9**). Relative abundance of taxa in class *Verrucomicrobiae* (median 7.7% decrease across all donors) and *Thermoleophilia* (6.1%) decreased in decomposition-impacted soils, while *Bacteroidia* response was variable ($n = 13$ individuals increased, $n = 5$ decreased, and $n = 1$ no change). At the genus level, the soil organism Subgroup_6_ge within the phylum *Acidobacteria* was found prior to cadaver placement and decreased (median 6.6% decrease across all donors) during active decomposition, while *Acinetobacter* increased (median 8.6% increase across all donors).

Fungal community composition

Fungal community structure in decomposition-impacted soils also changed over time. Prior to placement, soil fungal communities included the phyla *Ascomycota*, *Basidiomycota*, *Mortierellomycota*, and *Rozellomycota*, among others. As decomposition progressed, members of *Ascomycota* (median 29% increase across all donors) and *Mortierellomycota* (+4%) increased in relative abundance, while members of *Basidiomycota* (median 11% decrease across all donors), *Rozellomycota* (-1%), and *Chytridiomycota* (-1%) decreased (**Fig. 2.13B**). At the class

level, the most notable change in fungal community structure was an increase in *Saccharomycetes* between 3000 to 4500 ADH (median 57% increase across donors), corresponding with the timing of fluid introduction to soil. Within *Saccharomycetes*, we observed increases in the genera *Yarrowia* (median 22% increase across all donors) and *Dipodascus* (23%) associated with the same time period.

Saccharomycetes and BMI

Increases in *Saccharomycetes*, previously observed during outdoor juvenile pig decomposition (46), in decomposition-impacted soils was notably related to donor BMI. In particular, decomposition soils below underweight donors displayed little to no changes in relative *Saccharomycetes* abundance over time (**Figure 2.15**) (HLM $F = 3.441$; $p = 0.11$). This included individuals 002, 015, and 018, whose BMI ranged from 14.2 to 20.2 and had visibly little fat tissue. We also found that as donor BMI increased, the maximum relative abundance of *Saccharomycetes* observed in decomposition soils during active decomposition increased (ANOVA $F = 5.582$; $p = 0.03$). None of the other factors tested, including disease categories, season, sex, or age, were found to be related to relative *Saccharomycetes* abundance in decomposition soils (**Table 2.3**). Variation in relative *Saccharomycetes* abundance in decomposition soils was also positively related to heterotrophic respiration (HLM $F = 3.972$; $p = 0.052$), but not to any other soil parameter, including pH.

DISCUSSION

The main goal of this study was to assess the impact(s) of intrinsic, or cadaver-related, factors on soil chemical and microbial patterns during human decomposition. Specifically, we evaluated the relationships between cadaver body mass index (BMI), sex, and end of diseases contributing to death to soil chemical responses and microbial community structure and activity in decomposition-impacted soils. Data were collected from nineteen individuals, and we were able to identify intrinsic factors, including BMI, related to variable pH responses and microbial activity in decomposition-impacted soil.

BMI and soil chemistry

In addition to noting general decomposition trends in soil pH and EC and microbial community response, our study observed inter-individual variation in soil chemical and microbial responses. Some of this variation was explained by donor BMI, the relationship between an individual's weight and height. BMI is often used as an indirect measure of body fat in humans, since BMI and percent body fat are typically positively correlated (47). However, BMI is an indirect measure, and does not account for variability in body composition (48). For example, an individual with high lean muscle mass and low body fat may have a high BMI. While we did not measure body fat percent in our study, we visually observed that the individuals with high BMI had a greater amount of fat tissue as opposed to lean muscle tissue, so we can assume that BMI generally reflects body fat content for our study sample. Additionally, BMI was calculated from cadaver measurements of height and weight as opposed to values reported in medical history or on a driver's license. While cadaver height may be problematic due to inaccuracy when positioning the cadaver for measurement, reported antemortem values may not be up to date (49). However, results by Ferorelli et al. (2017) suggest differences between antemortem and cadaver height are minimal.

We found that soil pH response varied between individuals, with soil pH increasing for individuals with BMI < 18.5 and decreasing for those with BMI > 18.5. Variable pH response is in accordance with previous vertebrate decomposition studies in terrestrial ecosystems, where some studies report increased soil pH (1, 3, 9), while others report decreased pH (6, 8, 10). Fancher et al. (2017) observed both increased and decreased soil pH within the same study assessing human decomposition. Further, we observed that direction of soil pH change (increase vs. decrease) was partially explained by differences in BMI, suggesting that BMI influences pH response during human decomposition. While BMI has not been linked to any specific soil response during human decomposition previously, Fancher et al. (2017) and Aitkenhead-Peterson et al. (2021) noted that the inclusion of BMI in models improved the estimation of PMI from multiple soil responses, including pH. Soil pH response may have varied due to relative ratios of fat and muscle decomposition products associated with individuals of different BMIs. Fat tissue contains 60 to 85% lipids (50), which are hydrolyzed into stearic, oleic, and palmitic

acid; while decomposition of muscle tissue releases ammonium (NH_4^+). Therefore, a greater proportion of acidic products would be expected in individuals with more fat tissue, resulting in decreased soil pH. Alternatively, muscle tissue contains more nitrogen than fat tissue (51) and ammonification of proteins, peptides and amino acids present in muscle tissue release NH_4^+ . In individuals with less fat tissue and more muscle, a greater relative proportion of NH_4^+ is expected, resulting in increased pH.

pH is arguably one of the most important environmental factors impacting soil microbial communities and activities directly and indirectly (11). In this study, we observed a positive relationship between soil pH and leucine aminopeptidase (LAP) activity during decomposition, suggesting that pH regulates LAP response during human decomposition and/or LAP activity generates additional NH_4^+ thereby increasing soil pH. LAP, a general aminopeptidase, has been shown to exhibit optimum activity under alkaline conditions (pH 6 to 8) (52), potentially explaining greater LAP activity in soils of individuals whose pH increased during decomposition. Microbes produce LAP for nitrogen acquisition (53), increasing nitrogen availability for the community. As a result, variable LAP activity may impact the rate of microbial-mediated peptide degradation and nutrient availability for soil microbes, leading to increased variability in microbial community composition and function between individuals. Our results are consistent with previous decomposition studies that recorded soil LAP activity (3, 36). For example, Keenan et al. (2018a) reported increased pH and LAP activity during terrestrial beaver decomposition, while Keenan et al. (2018b) observed decreased pH and LAP activity in a multi-individual human grave. DeBruyn et al. (2021) conducted a surface decomposition experiment comparing pigs and humans and observed that pH and LAP activity decreased in human decomposition-impacted soil but increased under pigs (1). Thus, the pH-LAP relationship appears robust, and further work will be needed to determine the mechanism behind this relationship and implications for the fate of decomposition products.

Saccharomycetes and BMI/pH

We also observed that the relative abundance of *Saccharomycetes* in decomposition soils was related to donor BMI. While *Saccharomycetes* relative abundance increased in decomposition soils below normal, overweight, and obese donors, underweight individuals displayed no such

trend. Taylor et al. (2021) had also identified the fungal class *Saccharomycetes* as one of the main taxa responsible for fungal community compositional shifts in decomposition soils below donors ranging in BMI from 24.6 to 29.6 (BMI categories: normal and overweight). *Yarrowia*, a *Saccharomycete*, has also been shown to increase in soils during juvenile pig decomposition (46). The relationship between *Saccharomycetes* relative abundance and BMI may be due to differences in proportions of fat and muscle tissue between individuals. The dominant *Saccharomycetes* genera we observed (*Yarrowia* and *Dipodascus*) are found in a variety of environments, including insects and humans, and display diverse metabolic capabilities (55). Specifically, *Yarrowia* species are known for their ability to metabolize lipids (55), suggesting that high BMI individuals with more fat provide a favorable substrate, giving *Yarrowia* a competitive advantage over other fungi.

As BMI was related to soil pH changes and pH can impact microbial communities, we thought that the variable soil *Saccharomycetes* response may be related to pH response during decomposition. However, we did not observe a linear relationship between soil pH and *Saccharomycetes* relative abundance in our data set, suggesting the response of *Saccharomycetes* was not due to an altered pH. While many members of *Saccharomycetes*, including *Saccharomyces cerevisiae*, prefer low pH, *Yarrowia lipolitica* has been suggested to adapt to a wide range of pH conditions (55, 56). As *Yarrowia* and *Dipodascus* were the dominant *Saccharomycetes* genera present in our communities, this adaptability to variable pH conditions may explain why soil pH was not significantly related to *Saccharomycetes* relative abundances in decomposition soils.

Relative ratios of fat and muscle tissue impacts soil chemical and microbial responses

Observed relationships between BMI and microbial community composition may be partially explained using ecological stoichiometry theory, which posits that microbial community composition and activity is driven by the balance of the organism's nutritional requirements and nutrient content of available resources (i.e., C:N) (57, 58). Within this framework, individual taxon responses to disturbance may be predictable when the difference between organismal C:N:P requirement and C:N:P of resources is considered. Release of nutrient-rich fluids from the cadaver into the surrounding soil alters carbon and nitrogen pools during decomposition (9, 59,

60). Yet, C:N of various body tissues differs. Specifically, muscle tissue contains more nitrogen and thus a lower C:N than fat. For example, Keenen et al. (2019) recorded a C:N of 49 and 3.3 for fat and muscle tissue, respectively, in beavers (*Castor canadensis*). This difference in C:N between tissue types may have important implications when considering nutrient composition of different individuals. For example, we can consider two individuals with the same weight and muscle mass, but different body fat percentage (15% vs. 30%). Assuming similar elemental composition of fat and muscle across vertebrates, we can use values from Keenan et al. (2019) to calculate that the C:N ratio of the fat and muscle fraction of these individuals would be 4.07 and 4.83, respectively (**Figure 2.16**). For a 93kg individual, this roughly equates to 4.62 kg more carbon for a 30% body fat individual, compared to the 15% body fat individual. While these are clearly rough estimates based on several assumptions, it illustrates that differences in body composition (fat, muscle, and bone) between individuals may alter resource pools and proportions of breakdown products, both of which may impact microbial decomposer presence and activity.

Trends in disease status and soil responses

In addition to a relationship with BMI, we also identified some relationships between reported diseases contributing to the individual's cause of death and soil chemical and microbial responses. Donors with cancer at time of death had altered microbial decomposer communities with lower diversity and respiration rates compared to donors without cancer. This could be due to differences in body tissues due to disease or the presence of chemotherapeutics and other drugs (*e.g.*, morphine) in the system. It could also be an indirect effect of altered microbiome due to the disease and/or therapeutic interventions (*e.g.*, radiotherapy). For example, cancer has been associated with altered gut microbiome composition (18), with some cancer patients having a greater proportion of Firmicutes relative to Bacteroides (61). Wang et al. (2015) observed lowered diversity and decreased Firmicutes:Bacteroides ratio after radiotherapy, suggesting cancer treatments and therapeutics alter gut communities further. Thus, cancer may lead to different microbiome composition at time of death, altering decomposer communities and impacting decomposition patterns. Notably, we did not observe differences in bacterial or fungal community composition of fluids released from the body during decomposition between disease

categories. This could be because we pooled the collected decomposition fluids throughout decomposition to make a composite sample for each individual, which may have masked any initial variability in microbiomes between individuals. To see if decomposer communities do indeed differ due to disease, an investigation into the initial microbiome at time of death, length of time to initial fluid release, and time-series analysis of fluid communities would be informative.

Variation in bacterial and fungal response during active decomposition

One interesting observation we noted was the variation in soil bacterial and fungal community structures during human decomposition. During active decomposition soil fungal community composition shifted, becoming more similar in composition between individuals. In contrast, soil bacterial community composition did not become more similar, suggesting that soil bacterial community response is more variable than soil fungal response during active decomposition. Additionally, we generally observed greater inter-individual variability in bacterial communities compared to fungal communities, and seasonal differences in bacterial communities that were not seen in fungal communities. These differences may result from distinct responses to environmental stressors caused by the release of decomposition products due to overall diversity differences between bacterial and fungal groups. This study and others have shown that the soil chemical environment is heavily impacted by vertebrate decomposition (2, 3, 7, 59, 62). In our study, soil EC (correlating to salinity) increased up to 48 times higher than background conditions. This level of disturbance impacts soil microbial communities, altering their structure and activity. Both bacterial and fungal communities make up soil microbial communities, however differences in their diversity can impact their resistance (*i.e.*, ability to withstand change) and/or resilience (*i.e.*, rate of community recovery) to disturbance events (63). In this study, bacterial diversity was much higher than fungal diversity in our pre-decomposition soils. As higher diversity can be associated with greater resistance to disturbance events (63), this may explain why bacterial communities did not become more similar during active decomposition. In contrast, fungal communities, with lower diversity, were less resistant to disturbance, resulting in the filtering of those species in unfavorable conditions.

Limitations/future directions

There are several aspects of this study that limit our interpretation and provide avenues for future research. First, while our results show a relationship between BMI and soil pH response, it is important to note that BMI is an indirect measure based solely on an individual's height and weight, and does not account for differences in fat proportion or distribution due to sex and age (48). This may explain some of the variability we observed in moderate BMI individuals. A more direct measure of body composition (percentages of fat, muscle, and bone), such as Dual-energy x-ray absorptiometry (47), would be needed to evaluate relationships between elemental stoichiometry and microbial responses in future decomposition studies. Second, while we were able to investigate relationships with some individual intrinsic factors, there are other intrinsic factors (*e.g.*, age, diet, ancestry, and drugs in the system at time of death) that were not considered that may impact decomposition patterns. Additionally, our observations were likely limited by the demography of our sample population: all donors had a mean age of 71. Lastly, while we investigated general effects of disease states, as they contributed to cause of death, we were not able to look at interactions between diseases or address seasonal effects due to the size of our dataset and distribution of donors within disease categories across seasons. As a result, these trends should be followed up with controlled, targeted study to identify the effects of diseases and their therapeutics on decomposition patterns.

CONCLUSIONS

The goal of this research was to assess the impact(s) of sex, BMI, and disease status at time of death on the soil decomposition environment and microbiome. Our results suggest an individual's BMI can influence decomposition patterns, leading to inter-individual variability in soil chemical and microbial responses during human decomposition. We also observed preliminary effects of diseases on decomposition, notably that cancer (and/or its therapeutic interventions) may have a potential inhibitory effect on microbial decomposers. Together these results indicate that intrinsic factors, such as BMI, likely play a role in driving decomposition rates and patterns and should be considered in taphonomy studies. Further, our results may have implications for postmortem interval estimation methods that rely on bacterial community data. In particular, even in our demographically narrow study, the decomposer microbiome (or

necrobiome) varied between individuals suggesting successional patterns may not be universal when larger sample sizes and more diverse populations are considered. Intrinsic factors helped explain some of this variability, so inclusion of intrinsic and environmental data may help improve postmortem interval models.

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APPENDIX

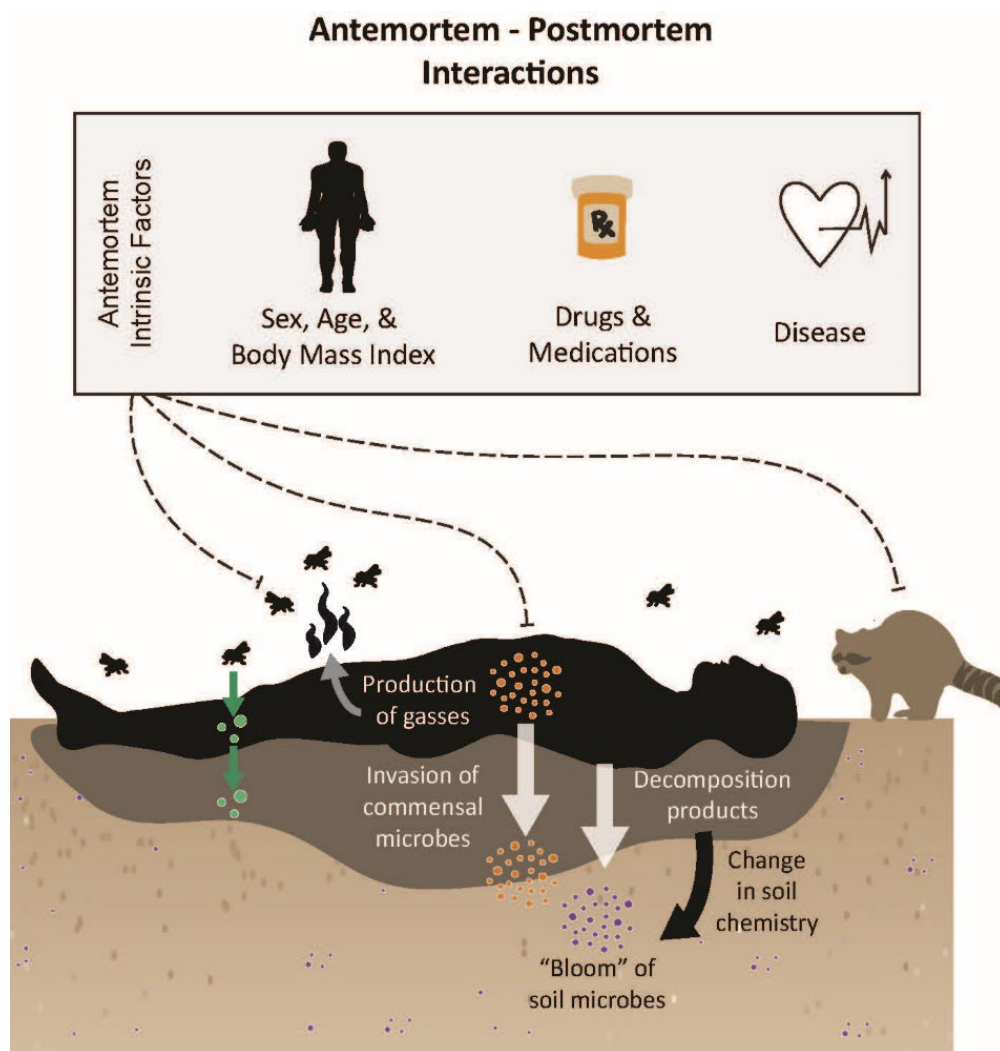


Figure 2.1 Diagram of interactions between antemortem intrinsic factors and decomposer organisms. During human decomposition, host-associated microbes, environmental microbes, insects, and scavengers work together to break down body tissues. Liquified decomposition products are flushed into soil where microbes respond to the influx and changes in soil chemistry. Antemortem conditions, such as body mass, age, diet, diseases, or drugs and other treatments, can influence decomposer (i.e., scavengers, insects, and microbes) activity leading to variability in decomposition rate and progression.

Table 2.1 Demographics for all donors enrolled in study. Total ADH is the ADH at the end of active decomposition. Disease presence was based on those reported in medical histories as contributing to cause of death.

Donor ID	Sex	Age	BMI	BMI Category	Season of Placement	Total ADH	Total Days	Reported Diseases					
								Diabetes	Cancer	Respiratory Diseases	Cardiovascular Diseases	Neurological Diseases	Pneumonia
TOX001	Female	63	33.4	Obese	Winter	15,500	93	Y	N	N	Y	N	N
TOX002	Female	78	20.2	Normal	Spring	3,750	37	N	Y	N	N	N	N
TOX003	Male	71	19.9	Normal	Spring	4,500	27	N	N	Y	N	N	N
TOX004	Female	84	19.3	Normal	Spring	3,500	15	N	Y	N	N	N	N
TOX005	Female	71	23.2	Normal	Spring	8,500	32	N	N	Y	N	N	Y
TOX006	Male	64	24.9	Normal	Spring	4,500	15	N	Y	N	Y	Y	N
TOX007	Male	89	26.2	Over	Spring	6,000	18	N	N	N	Y	N	N
TOX008	Male	40	24.3	Normal	Summer	6,500	21	N	Y	N	N	N	N
TOX009	Female	72	23.8	Normal	Summer	4,500	13	N	Y	N	N	N	N
TOX010	Male	65	54.1	Obese	Summer	17,500	50	N	N	Y	Y	N	Y
TOX011	Male	81	22	Normal	Summer	7,000	19	N	N	N	N	Y	N
TOX012	Female	77	29.4	Over	Summer	6,000	17	Y	N	N	N	Y	N
TOX013	Male	54	41.6	Obese	Summer	7,500	25	N	N	N	N	N	N
TOX015	Male	78	14.2	Under	Fall	1,500	27	N	N	Y	N	N	N
TOX016	Male	62	37.5	Obese	Fall	1,500	57	N	N	Y	N	N	N
TOX017	Female	62	55.1	Obese	Winter	18,500	191	N	Y	N	Y	N	N
TOX018	Female	85	16	Under	Winter	4,000	74	N	N	N	N	Y	N
TOX019	Female	67	19.6	Normal	Winter	5,500	72	N	N	N	N	N	N
TOX020	Male	91	31.2	Obese	Spring	6,000	48	N	N	Y	N	N	N

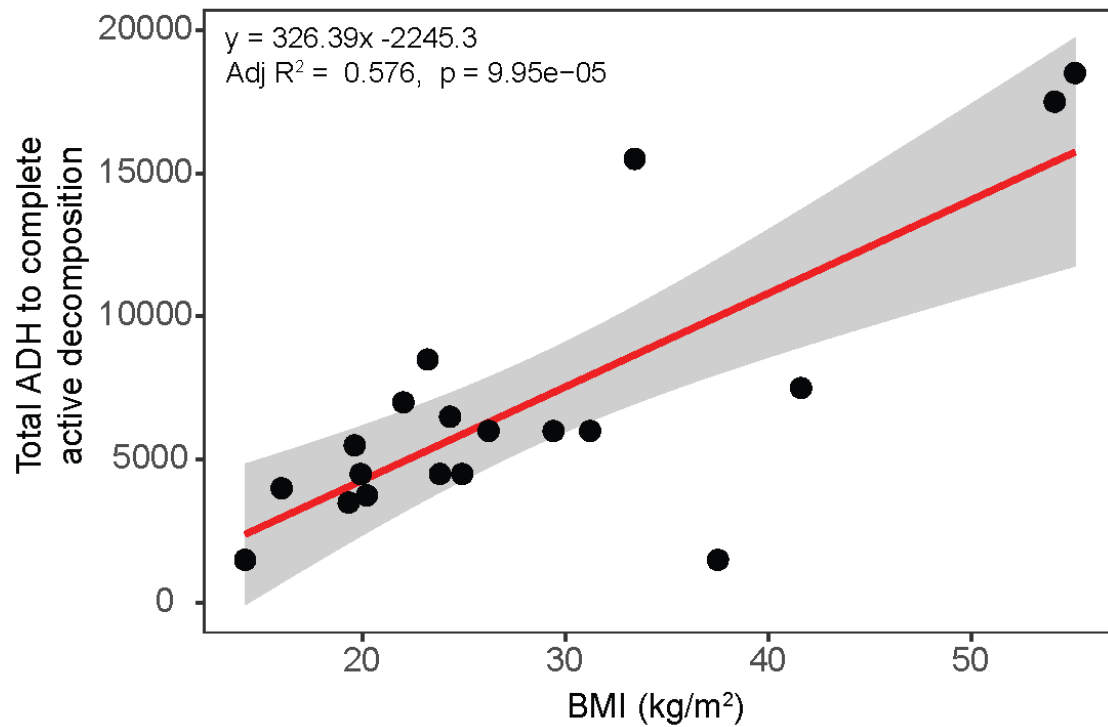


Figure 2.2 Linear relationship between total ADH to complete active decomposition and BMI. Donor body mass index (BMI) impacts total time to complete active decomposition. Specifically, total ADH is greater for high BMI individuals. The red line represents the linear trend between variables and the gray shaded area is the 95% confidence interval. Time is presented in accumulated degree hours (ADH).

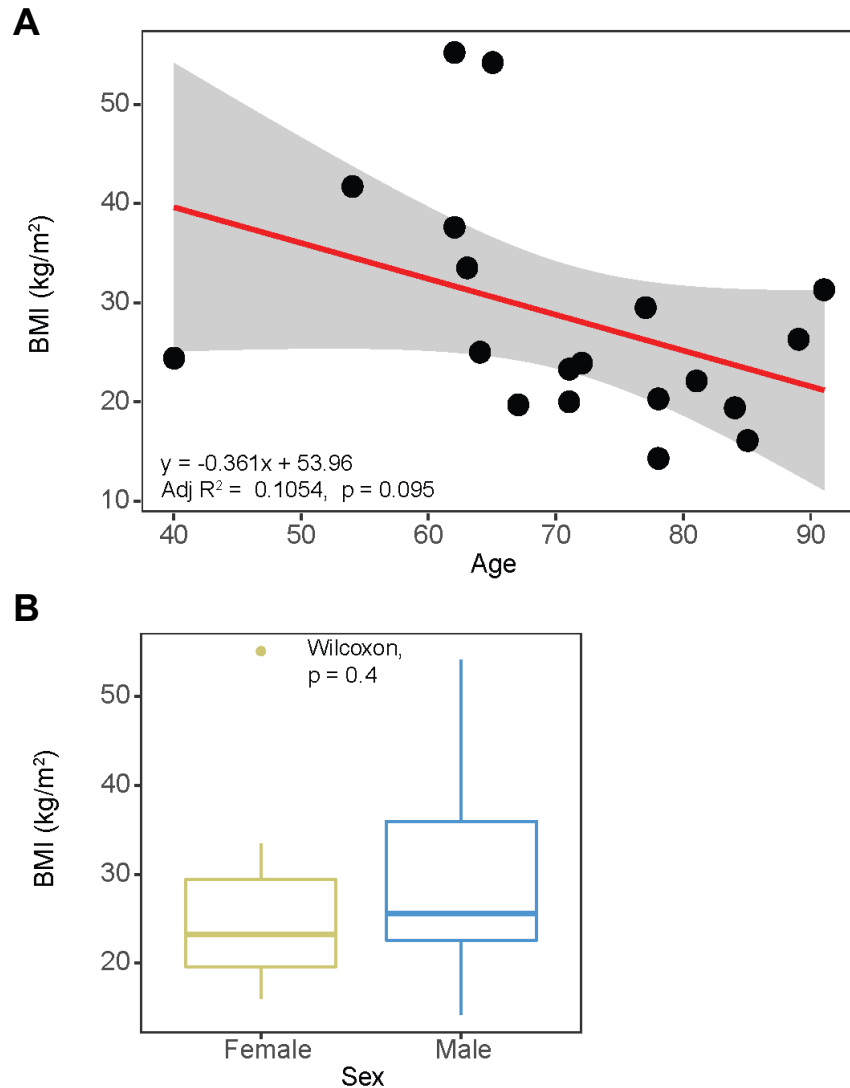


Figure 2.3 Relationship between donor age (A) and sex (B) and BMI for the study population. In panel A, BMI decreases with age, while in panel B, males (blue) have slightly higher mean BMI compared to females (green), but the difference is not significant. In panel B, the red line represents the linear trend between variables and the gray shaded area is the 95% confidence interval.

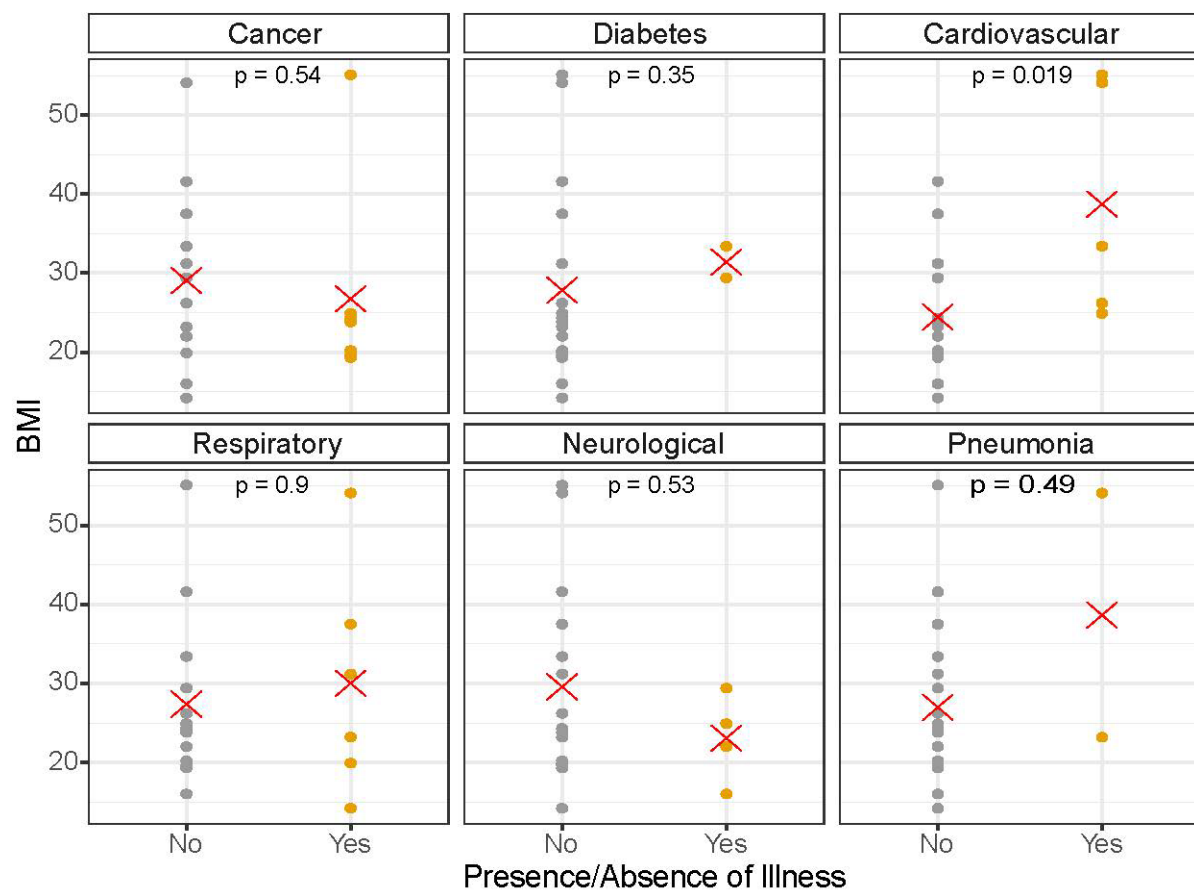


Figure 2.4 Comparing the BMI between donors with (yellow) and without (grey) different illnesses (panel: A-cancer, B-diabetes, C-cardiovascular, D-respiratory, E-neurological, F-Pneumonia). Red X indicates group means and p-values derived via Wilcoxon non-parametric tests.

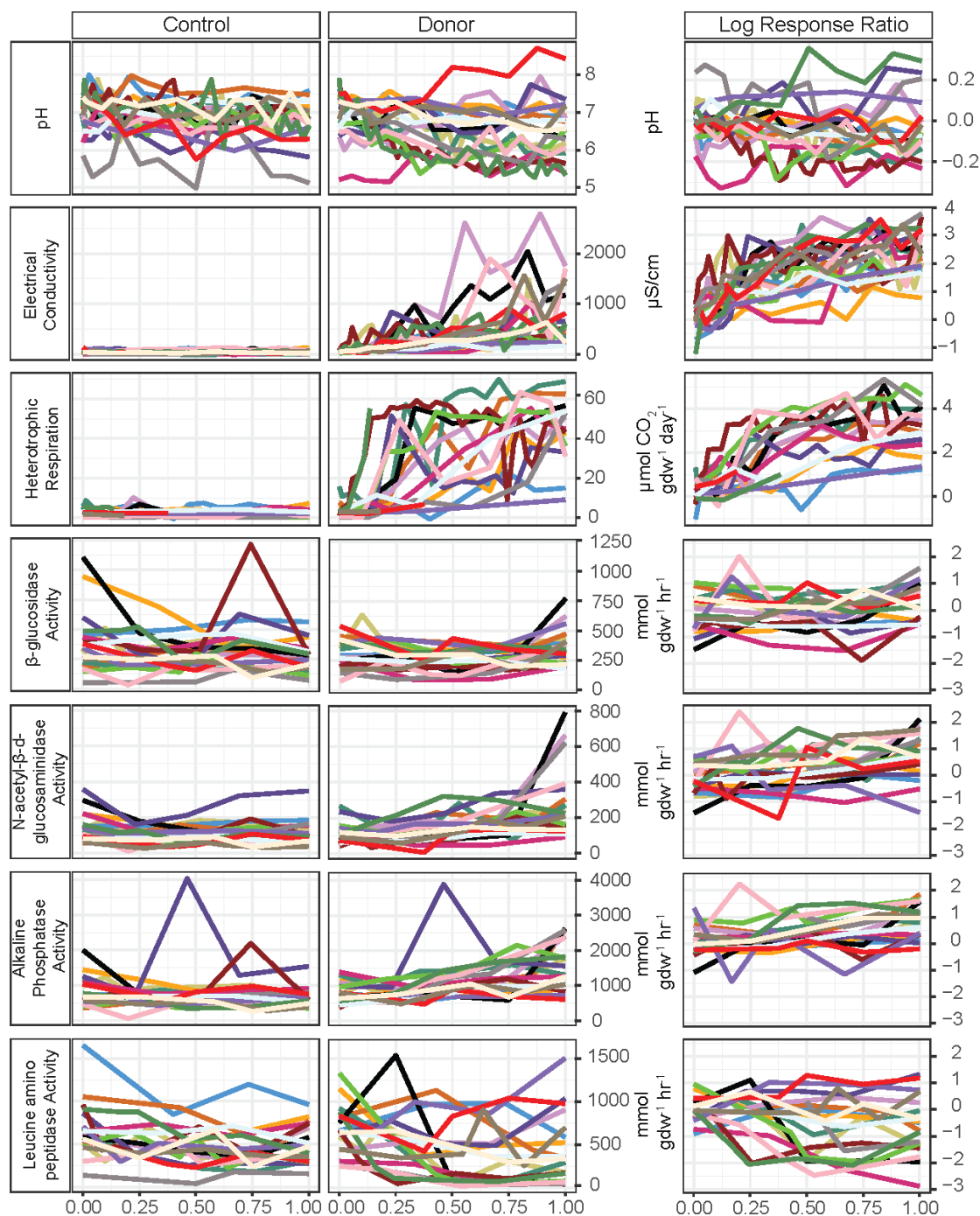


Figure 2.5 Raw and normalized soil physicochemical and extracellular enzyme responses. Comparing pH (row 1), electrical conductivity (row 2), heterotrophic respiration (row 3), and extracellular enzyme activities (rows 4-7) between control (column 1) and donor (column 2) soils and normalized log response ratio values (column 3) over time. Trends for each donor (names “TOX###”) are delineated by line color. Time, in ADH, has been normalized across donors as percent ADH. Percent ADH for a single sample is defined as the sample ADH divided by total ADH required to complete active decomposition, specific to the donor.

Table 2.2 Analysis of variance (ANOVA) results from hierarchical linear mixed effects models for the log response ratio (LRR) of each soil parameter and bacterial (16S) and fungal (ITS) community Chao1 richness and Inverse Simpson. Only samples ≤ 5000 ADH were used to capture the linear section of parameter responses. Significant ($p < 0.05$) models indicated in bold.

		ADH	BMI category	Sex	Season	ADH: BMI cat	ADH: Sex	BMI cat : Sex	ADH : BMI cat : Sex
pH LRR	F	0.242	5.392	0.001	1.880	9.647	0.017	3.833	3.066
	p	0.623	0.017	9.81	0.208	< 0.001	0.897	0.045	0.029
Electrical Conductivity LRR	F	27.93	2.659	0.145	0.195	1.709	0.645	0.604	0.247
	p	< 0.001	0.050	0.704	0.899	0.235	0.439	0.613	0.861
Heterotrophic Respiration LRR	F	11.39	0.640	0.173	0.550	0.353	0.010	0.211	0.016
	p	0.029	0.595	0.680	0.652	0.796	0.922	0.888	0.904
β -glucosidase LRR	F	1.117	0.250	0.844	0.583	0.578	3.655	1.923	1.364
	p	0.296	0.860	0.379	0.643	0.632	0.062	0.179	0.265
N-acetyl- β -D-glucosaminidase LRR	F	0.364	0.519	1.487	1.173	3.096	6.157	2.500	4.945
	p	0.549	0.677	0.249	0.379	0.035	0.016	0.107	0.004
Alkaline phosphatase LRR	F	1.44	0.583	0.005	0.031	0.875	0.188	0.236	0.476
	p	0.235	0.636	0.945	0.992	0.460	0.670	0.870	0.701
leucine amino peptidase LRR	F	1.844	1.803	0.179	0.595	2.444	0.056	0.509	0.298
	p	0.201	0.158	0.674	0.621	0.131	0.817	0.678	0.826
16S Chao1	F	5.944	0.1612	0.121	0.108	1.334	0.665	0.041	0.932
	p	0.018	0.920	0.725	0.953	0.274	0.419	0.988	0.432
16S Inverse Simpson	F	5.335	1.400	0.432	0.015	3.705	2.239	0.478	0.009
	p	0.025	0.287	0.525	0.997	0.017	0.141	0.703	0.999
ITS Chao1	F	15.00	0.081	0.136	0.662	0.899	0.056	0.056	0.524
	p	< 0.001	0.970	0.718	0.598	0.448	0.799	0.982	0.667
ITS Inverse Simpson	F	5.700	1.100	0.005	0.815	0.957	0.006	0.923	1.075
	p	0.021	0.370	0.943	0.522	0.420	0.939	0.447	0.368

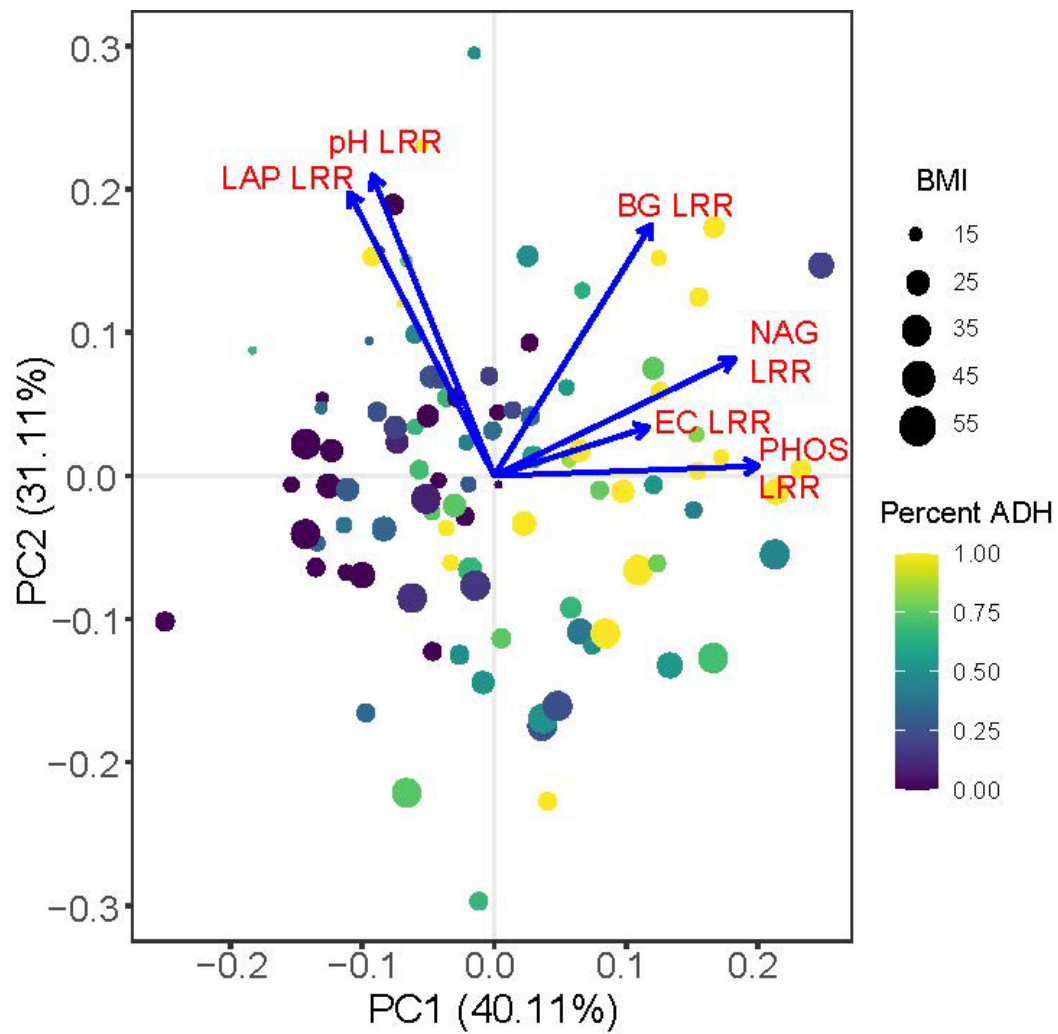


Figure 2.6 Principal Component Analysis (PCA) of soil chemical profiles during decomposition. Blue arrows represent principal component (PC) loadings for each variable included in the analysis. Point size corresponds to donor BMI while color denotes sample time as a percent of the total time required for that donor to complete active decomposition, in ADH.

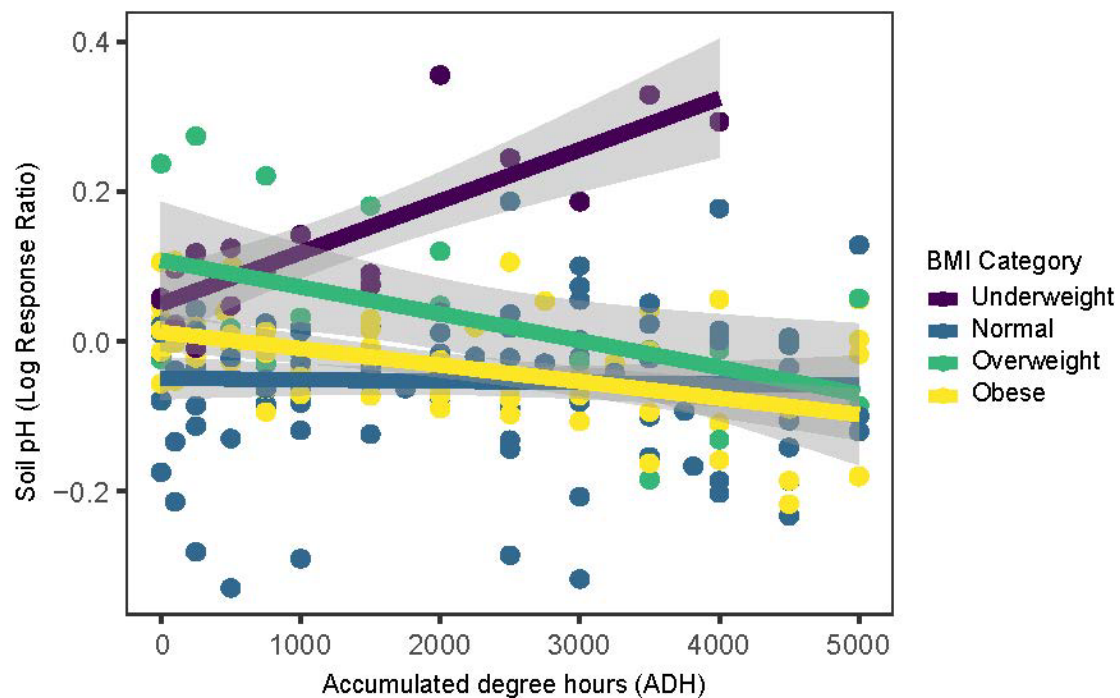


Figure 2.7 Donor BMI impacts soil pH response during human decomposition. Decomposition of underweight (purple) donors resulted in increased soil pH, while pH increased in decomposition of normal (blue), overweight (green), and obese (yellow) donors. Lines represent the linear relationship between soil pH and time (in ADH) for each BMI category (line color) and grey shading shows the 95% confidence interval for each linear relationship.

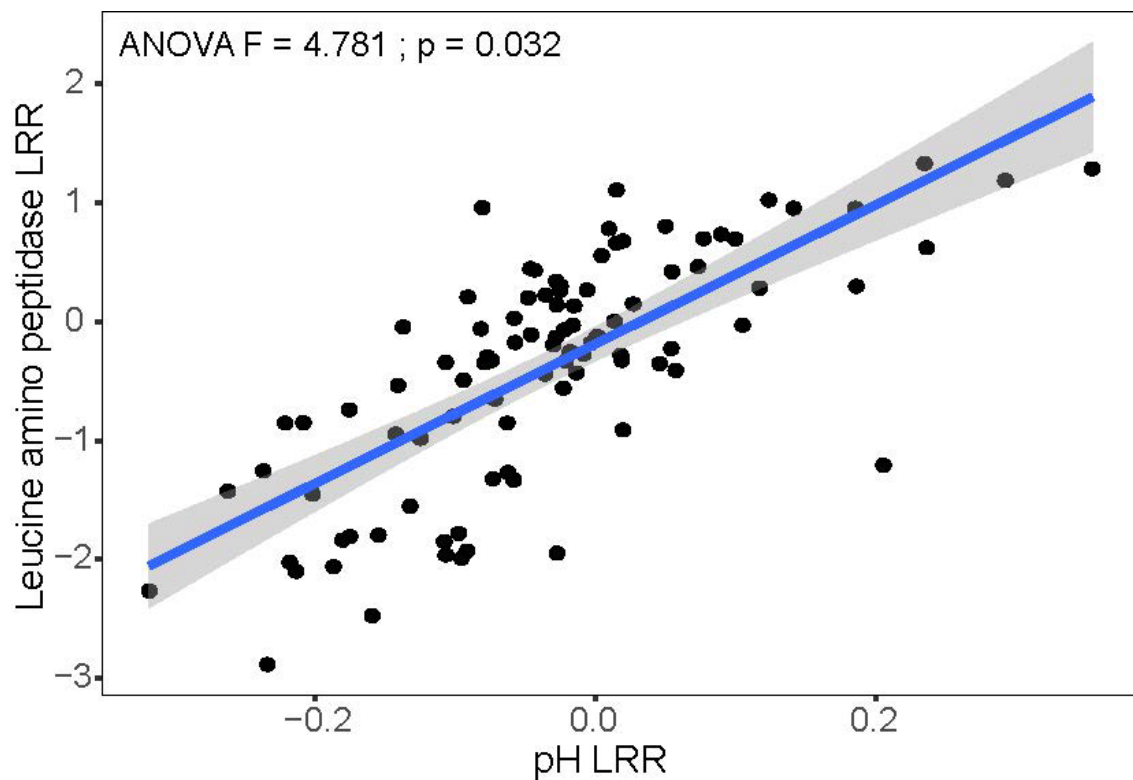


Figure 2.8 Leucine amino peptidase activity is impacted by changes in pH within decomposition soils. Specifically, leucine amino peptidase activity is greater in soils with higher pH. The blue line represents the linear trend between variables and the gray shaded area is the 95% confidence interval. Both soil leucine amino peptidase activity and pH are presented in normalized log response ratio values.

Table 2.3 ANOVA results from linear mixed effects models testing for the effects of time and the presence/absence of illness categories on respective soil responses. Only samples ≤ 5000 ADH were used to capture the linear section of parameter responses. Normalized log response ratio values for each parameter were used in models. All pH models included BMI as a co-variate as it was found to impact pH via HLM.

		ADH	Cancer	ADH: Cancer	Cardio- vascular	ADH: Cardio	Resp- iratory	ADH: Resp	Neuro- logical	ADH: Neuro
pH LRR*	F	12.89	3.765	1.156	0.619	3.333	0.031	3.845	0.898	30.79
	p	< 0.001	0.068	0.284	0.442	0.069	0.862	0.051	0.357	< 0.001
Electrical	F	35.12	0.887	0.129	11.65	0.508	0.376	0.005	1.210	0.225
Conductivity LRR	p	0.237	0.348	0.811	0.001	0.751	0.540	0.973	0.273	0.810
Heterotrophic	F	330.1	0.047	8.776	0.274	5.077	0.070	0.732	0.011	6.093
Respiration LRR	p	< 0.001	0.830	0.004	0.606	0.026	0.794	0.394	0.917	0.015
β -glucosidase	F	0.455	1.736	0.129	1.860	0.367	0.148	0.482	6.311	1.662
LRR	p	0.503	0.196	0.721	0.181	0.547	0.703	0.490	0.016	0.203
N-acetyl- β -D- glucosaminidase	F	11.60	0.066	0.504	2.161	0.206	0.308	0.294	0.427	0.090
LRR	p	0.001	0.799	0.481	0.150	0.652	0.583	0.590	0.518	0.766
Alkaline	F	12.26	0.311	0.069	2.136	0.025	0.881	0.320	0.918	0.153
phosphatase LRR	p	0.001	0.581	0.794	0.151	0.875	0.355	0.574	0.344	0.697
leucine amino	F	16.14	2.513	4.201	< 0.001	0.726	0.101	< 0.001	0.606	0.329
peptidase LRR	p	< 0.001	0.122	0.045	0.983	0.398	0.752	0.995	0.441	0.569
16S Chao1	F	6.914	0.368	3.121	0.793	0.956	0.003	4.640	0.036	5.237
	p	0.012	0.548	0.082	0.381	0.332	0.954	0.035	0.851	0.026
16S Inverse Simpson	F	8.172	0.908	2.567	0.053	0.248	0.010	0.022	0.448	0.861
	p	0.006	0.346	0.114	0.819	0.619	0.754	0.882	0.506	0.357
ITS Chao1	F	13.47	1.144	0.518	0.396	0.002	< 0.001	0.583	0.017	0.274
	p	< 0.001	0.291	0.474	0.532	0.966	0.986	0.448	0.897	0.603
ITS Inverse Simpson	F	6.613	2.148	1.163	0.924	1.101	0.020	0.335	1.652	1.303
	p	0.013	0.285	0.285	0.340	0.298	0.889	0.565	0.204	0.258
Saccharomycetes	F	23.93	1.698	0.159	0.420	0.092	1.282	1.545	0.380	0.039
(Relative Abundance)	p	< 0.001	0.198	0.697	0.519	0.766	0.262	0.233	0.540	0.846

Table 2.4 ANOVA results from linear models testing for the individual effects various intrinsic and extrinsic factors on bacterial and fungal Chao1 richness and Inverse Simpson estimates of decomposition fluid samples (top) and PERMANOVA results from Bray-Curtis distances for bacterial and fungal decomposition fluid communities. PERMANOVA were only assessed for categorical variables, and as a result numeric continuous variables were not assessed (reported as NA).

			Total ADH	Season	Sex	Age	BMI (numeric)	BMI category	# of drugs (serum)	Cancer	Cardio	Resp	Neuro
Decomposition Fluid ANOVA results	16S Chao1	F p	9.271 0.009	1.163 0.364	3.516 0.082	0.585 0.457	2.223 0.158	0.542 0.594	0.044 0.837	0.799 0.386	0.319 0.581	0.122 0.732	1.354 0.264
	16S Inverse Simpson	F p	6.960 0.019	1.493 0.266	3.959 0.066	0.024 0.878	3.653 0.077	0.477 0.631	0.563 0.469	0.621 0.444	1.321 0.270	0.068 0.798	0.001 0.974
	ITS Chao1	F p	0.092 0.765	1.129 0.376	0.244 0.629	0.291 0.598	0.303 0.591	0.422 0.664	1.25 0.297	1.052 0.322	0.247 0.627	0.083 0.777	0.006 0.941
	ITS Inverse Simpson	F p	0.567 0.464	0.389 0.763	1.477 0.244	0.500 0.491	1.075 0.317	0.652 0.537	0.57 0.466	2.139 0.166	0.618 0.445	0.536 0.48	0.171 0.685
Decomposition Fluid PERMANOVA results	16S Bray- Curtis	F <i>r</i> ² p	1.075	1.500	0.889	NA	NA	0.871	NA	0.722	0.395	1.812	0.898
			0.071	0.273	0.060	NA	NA	0.118	NA	0.049	0.027	0.115	0.060
			0.376	0.067	0.505	NA	NA	0.616	NA	0.697	0.973	0.076	0.588
	ITS Bray- Curtis	F <i>r</i> ² p	0.928	1.173	1.010	NA	NA	0.764	NA	1.265	0.552	0.902	0.677
			0.062	0.227	0.067	NA	NA	0.105	NA	0.083	0.038	0.060	0.046
			0.419	0.301	0.394	NA	NA	0.603	NA	0.229	0.794	0.431	0.528

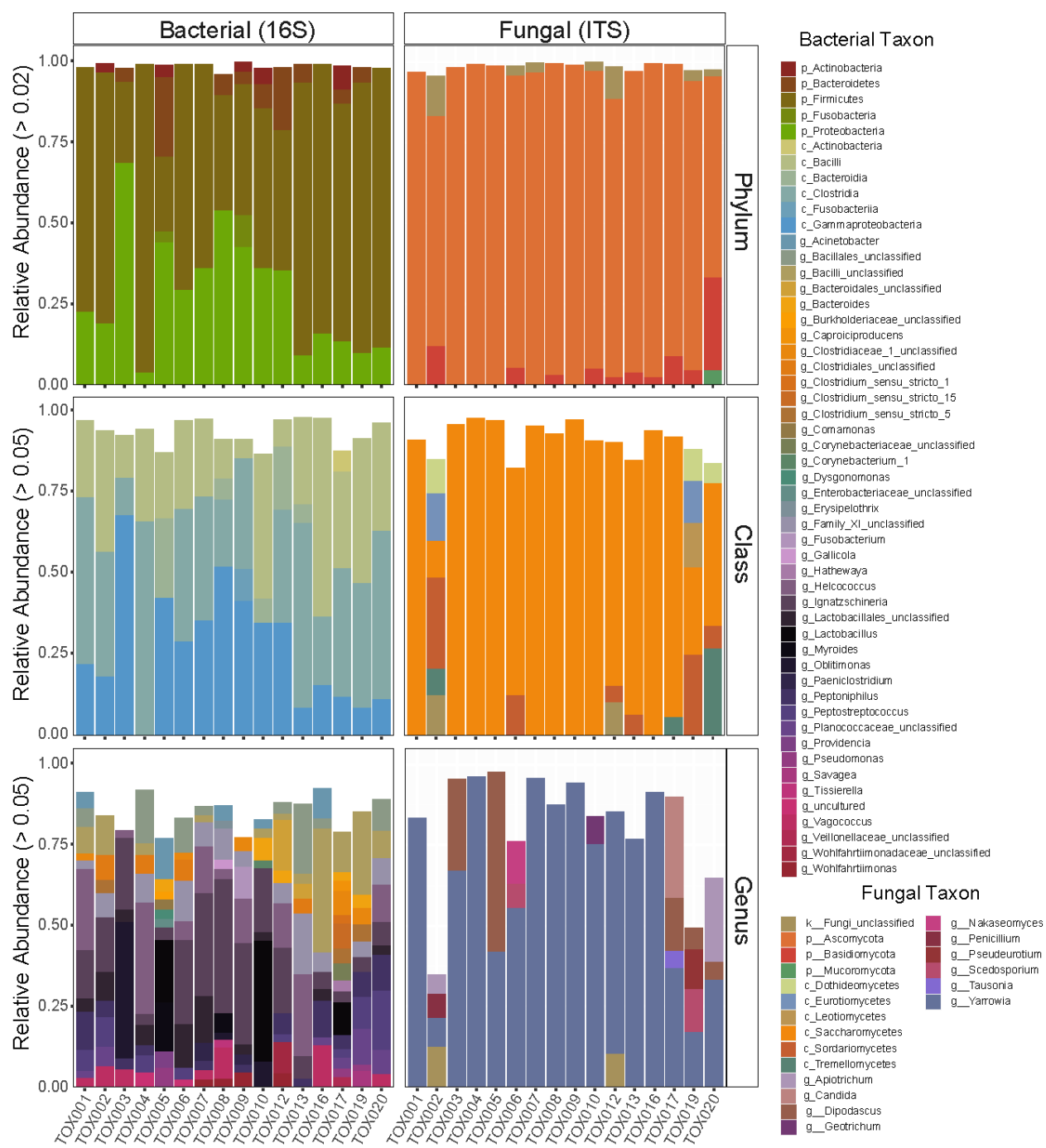


Figure 2.9 Relative abundance of bacterial (column 1) and fungal (column 2) taxa in decomposition fluid samples at the phylum (row 1), class (row 2), and genus (row 3) levels.

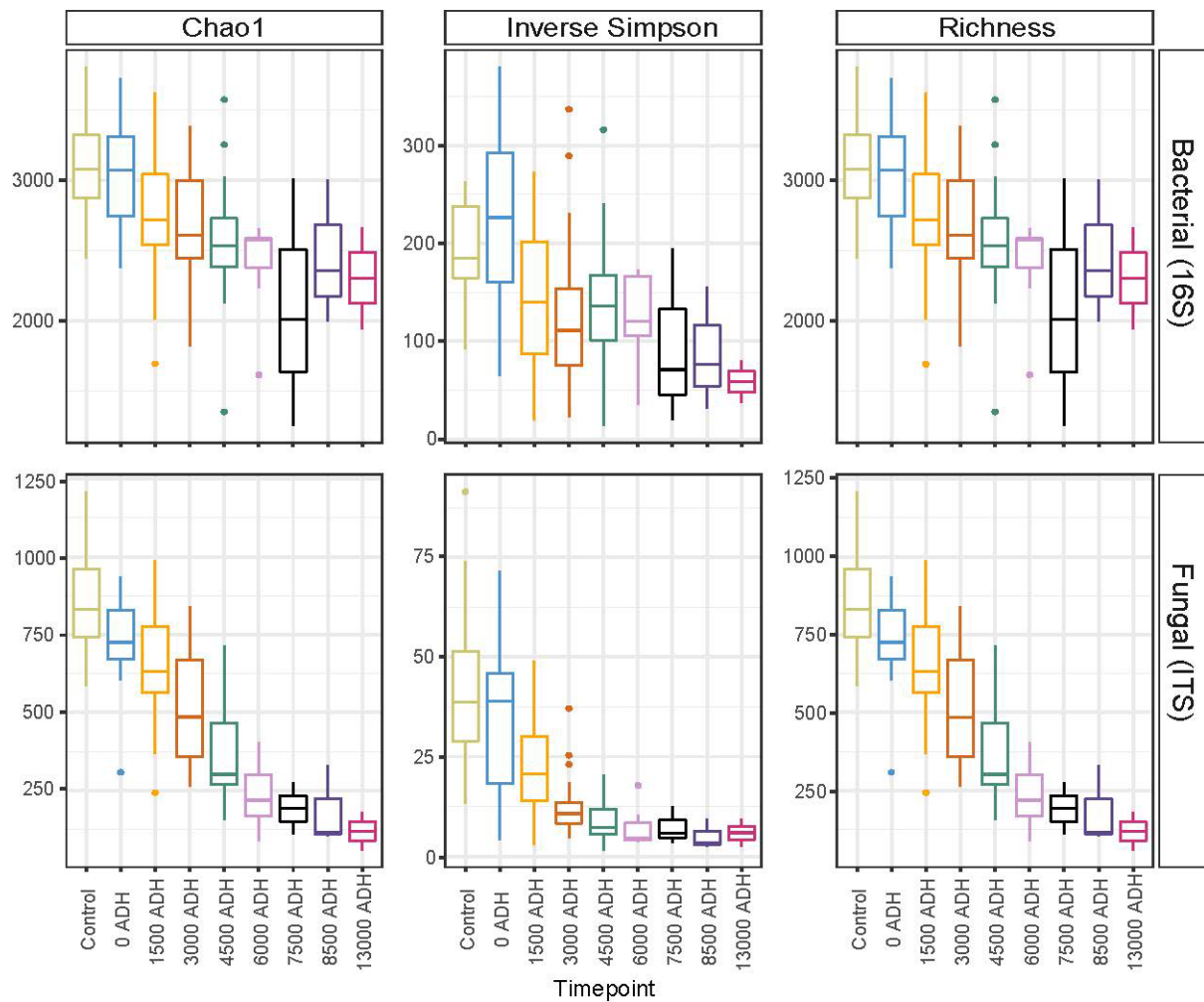


Figure 2.10 Chao 1 (column 1), Inverse Simpson (column 2), and richness (column 3) estimates for bacterial (row 1) and fungal (row 2) communities in soil samples over time (ADH). Boxplot color denotes sample timepoint.

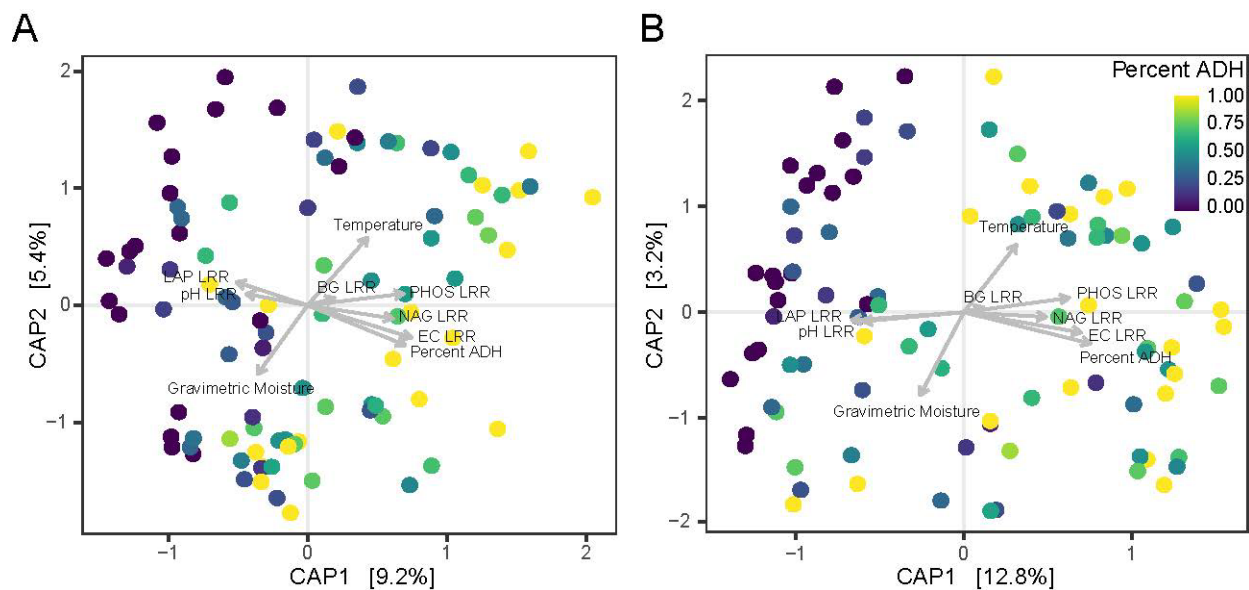


Figure 2.11 Constrained Analysis of Principal coordinates (CAP) of Bray-Curtis distances in bacterial (A) and fungal (B) community structure within decomposition soil samples. Grey arrows represent the influence of each environmental parameter included in the analysis. Data point color denotes decomposition time as percent ADH (sample ADH divided by total ADH required to complete active decomposition).

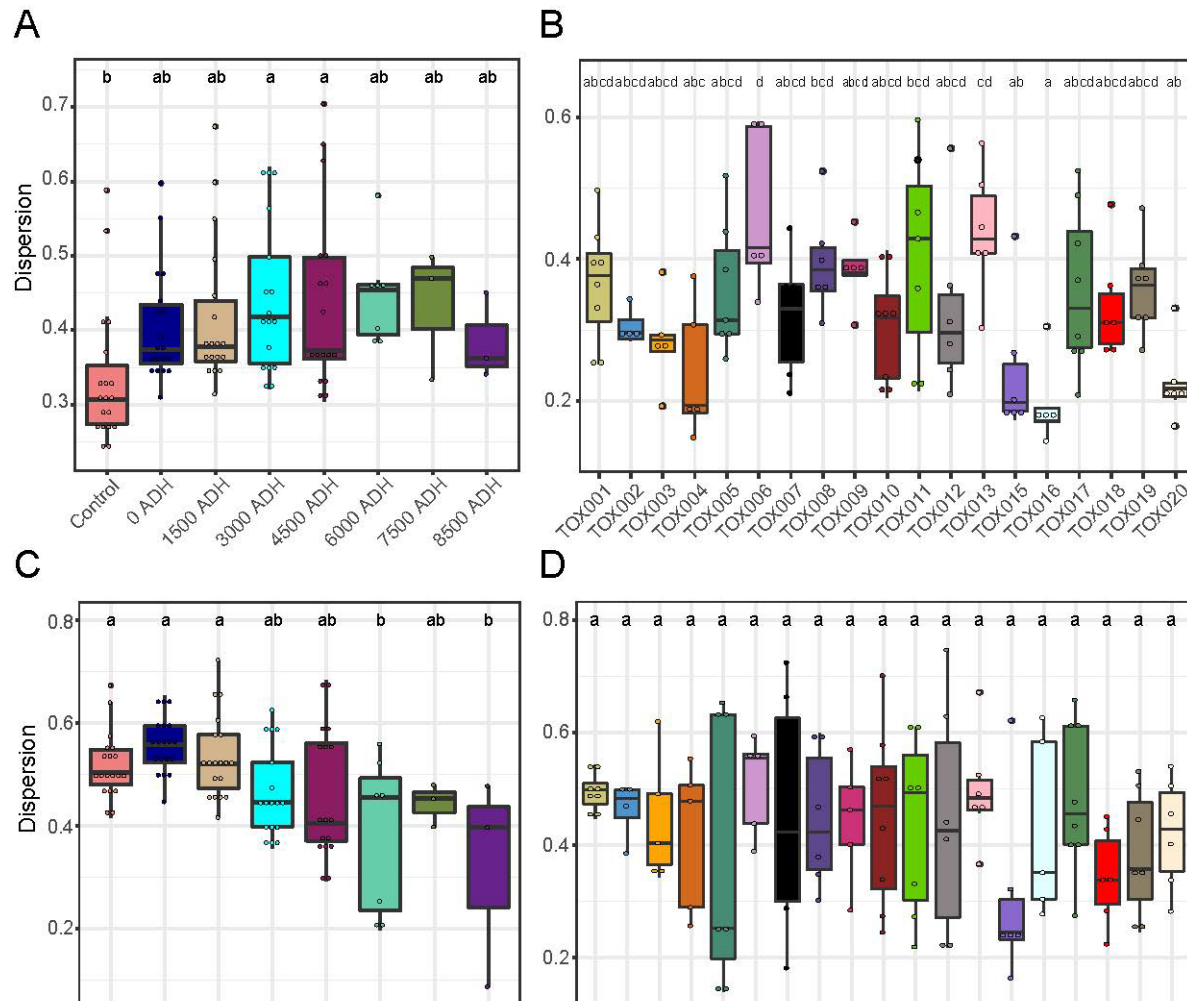


Figure 2.12 Bacterial and fungal community composition shifts differ between soil bacterial and fungal communities during active decomposition. Beta dispersion increases with time in bacterial communities (A) but decreases in soil fungal communities (C). Additionally, dispersion between donors differs for bacterial communities (B), while remaining similar across donors for fungal communities (D). In panel A and C, color denotes sample timepoint, while in B and D color represents donor. Letters above boxplots are the result of post-hoc Tukey tests. ADH = Accumulated Degree Hours

Table 2.5 Beta dispersion results for various intrinsic and extrinsic factors for bacterial and fungal communities.

	Bacterial (16S)			Fungal (ITS)		
	F	N Perm	Pr(>F)	F	N Perm	Pr(>F)
Donor	3.528	999	0.001	0.796	999	0.711
Season	6.662	999	0.002	2.408	999	0.063
Sector	5.969	999	0.002	6.004	999	0.002
Treatment	22.36	999	0.001	1.184	999	0.270
Sex	0.168	999	0.668	0	999	0.992
Cancer	3.675	999	0.063	1.188	999	0.284
Cardiovascular	0.991	999	0.315	0.880	999	0.341
Respiratory	16.89	999	0.001	6.434	999	0.009
Neurological	4.002	999	0.048	1.342	999	0.212
Timepoint	7.318	999	0.001	11.174	999	0.001
BMI category	5.116	999	0.004	8.782	999	0.001

Table 2.6 PERMANOVA results assessing the effects of and various respective intrinsic and extrinsic factors on soil bacterial and fungal community composition within the selected timepoints (0, 1500, 3000, 4500, and 6000 ADH). Pre-placement samples included controls and 0 ADH samples and were compared to see if control soils differed from decomposition sites prior to donor placement.

		Pre-placement		Control		0 ADH		1500 ADH		3000 ADH		4500 ADH		6000 ADH	
		16S	ITS	16S	ITS	16S	ITS	16S	ITS	16S	ITS	16S	ITS	16S	ITS
Treatment	F	1.27	1.15												
	r ²	0.032	0.031												
	p	0.233	0.196												
Season	F			1.124	1.271	1.447	1.029	1.236	0.978	1.422	2.297	1.316	2.106	0.958	0.760
	r ²			0.184	0.203	0.224	0.171	0.209	0.173	0.169	0.247	0.158	0.231	0.324	0.275
	p			0.271	0.033	0.046	0.374	0.139	0.516	0.084	0.001	0.107	0.006	0.489	0.778
Sex	F			1.018	0.886	0.726	0.857	1.238	1.002	1.033	1.276	1.056	1.626	0.856	0.511
	r ²			0.056	0.049	0.041	0.048	0.072	0.059	0.064	0.078	0.066	0.098	0.146	0.093
	p			0.381	0.695	0.772	0.726	0.202	0.43	0.377	0.178	0.337	0.071	0.794	0.962
Age Category	F			0.983	1.051	1.158	1.064	1.611	1.318	1.19	0.887	0.875	0.934	0.877	0.698
	r ²			0.164	0.174	0.188	0.175	0.257	0.220	0.215	0.17	0.168	0.177	0.467	0.411
	p			0.483	0.34	0.211	0.312	0.017	0.049	0.187	0.685	0.722	0.560	0.725	0.782
BMI category	F			1.272	1.118	1.349	1.193	0.915	0.954	1.114	0.923	1.251	1.121	1.203	1.333
	r ²			0.202	0.183	0.212	0.193	0.164	0.17	0.205	0.176	0.224	0.206	0.375	0.4
	p			0.142	0.167	0.083	0.096	0.604	0.580	0.299	0.591	0.193	0.316	0.188	0.218
Cancer	F			0.804	0.922	0.72	0.725	0.793	0.844	0.626	0.601	1.083	0.987	0.963	1.012
	r ²			0.045	0.051	0.041	0.041	0.047	0.050	0.04	0.038	0.067	0.062	0.161	0.168
	p			0.640	0.594	0.783	0.954	0.723	0.706	0.923	0.913	0.315	0.451	0.580	0.45
Cardio	F			0.959	0.1	1.045	1.204	1.136	0.993	0.806	0.817	0.766	0.82	0.768	0.456
	r ²			0.053	0.055	0.058	0.066	0.066	0.058	0.051	0.052	0.049	0.052	0.133	0.083
	p			0.414	0.481	0.367	0.172	0.261	0.44	0.657	0.658	0.790	0.604	0.860	0.859
Resp	F			0.916	0.804	0.833	0.853	0.774	0.799	0.884	1.074	0.830	1.491	0.830	0.830
	r ²			0.511	0.045	0.047	0.048	0.046	0.048	0.056	0.067	0.052	0.090	0.052	0.142
	p			0.479	0.873	0.601	0.707	0.733	0.784	0.554	0.328	0.637	0.117	0.637	0.616
Neuro	F			1.673	1.036	0.792	0.695	0.922	0.922	0.956	0.672	0.962	0.796	1.062	2.181
	r ²			0.090	0.057	0.044	0.039	0.054	0.054	0.06	0.043	0.060	0.050	0.175	0.304
	p			0.074	0.374	0.682	0.973	0.518	0.562	0.443	0.840	0.464	0.659	0.445	0.095

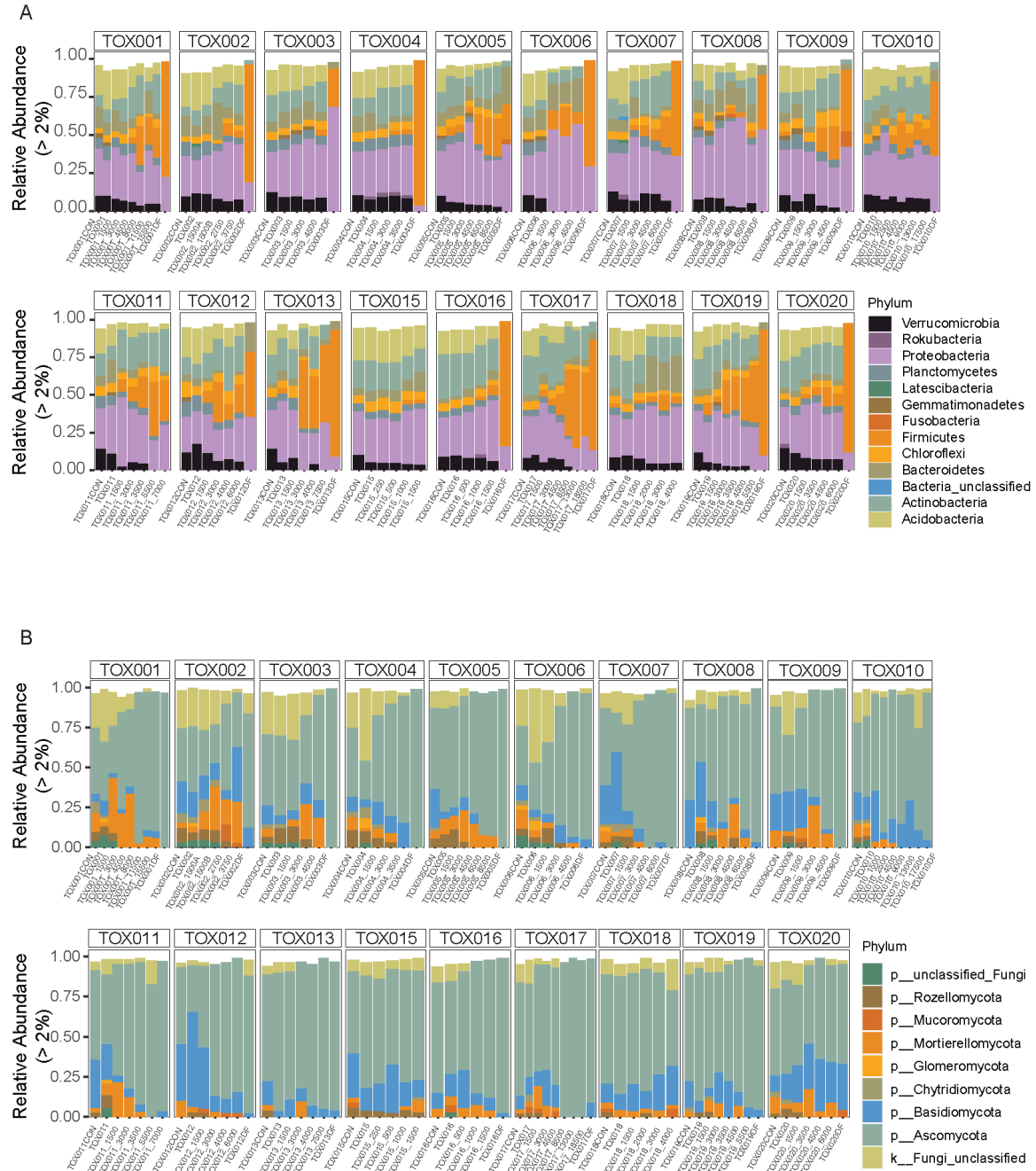
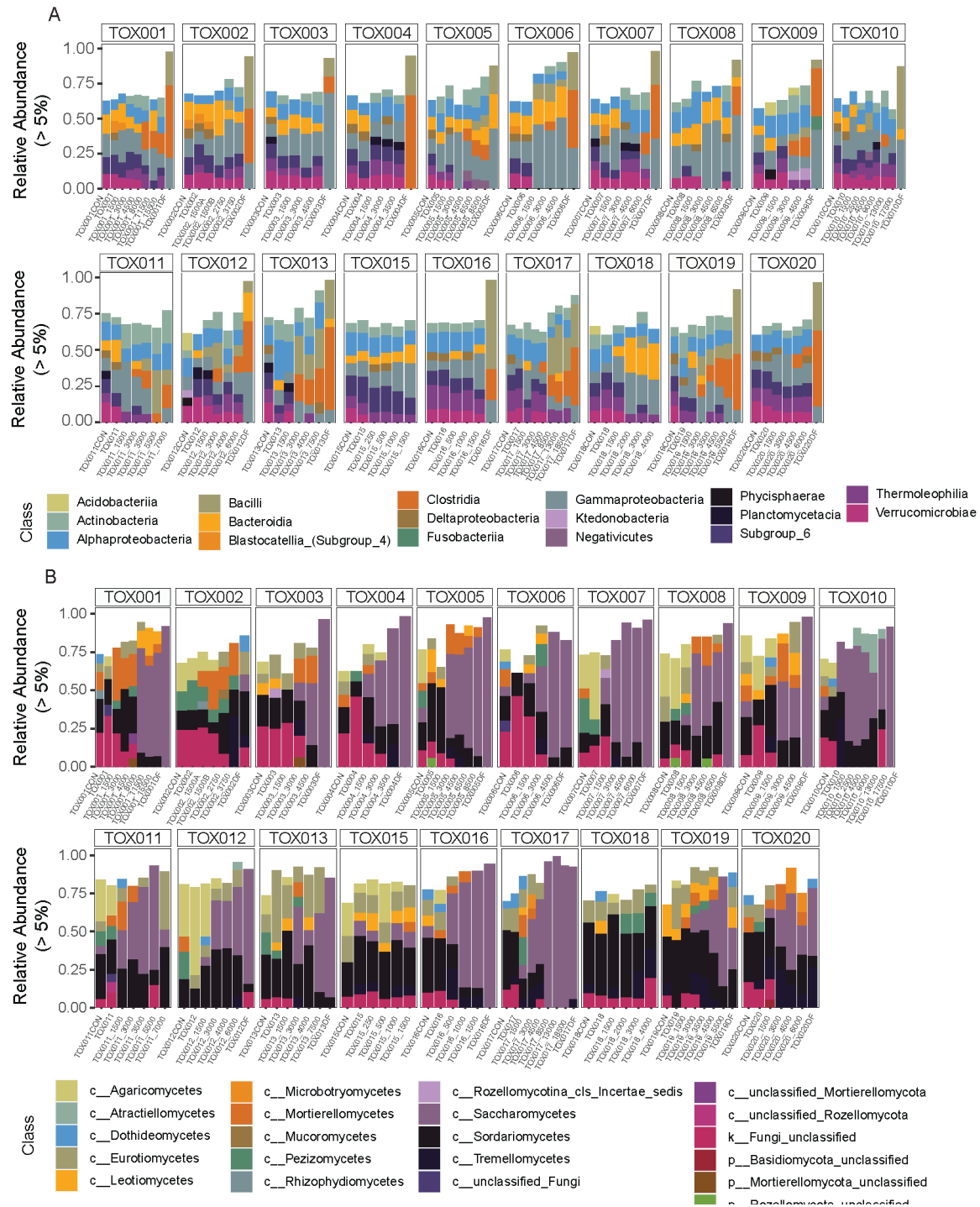


Figure 2.13 Relative abundance of bacterial (A) and fungal (B) phyla over time (x-axis) for each of the nineteen individuals in the study, labeled TOX###.



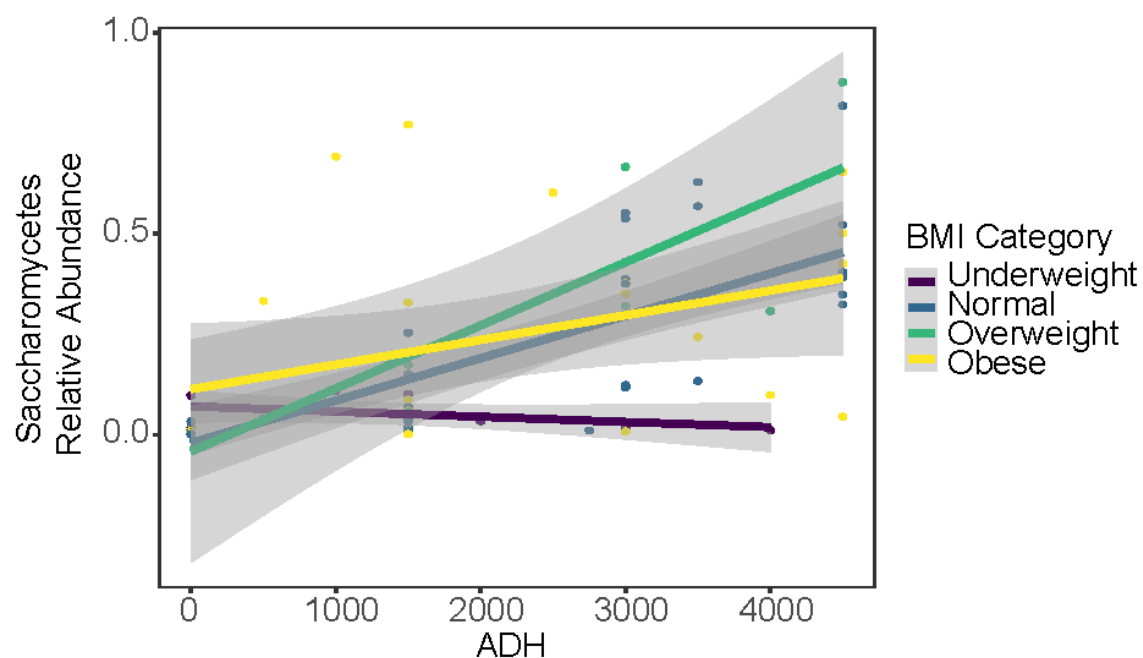


Figure 2.15 Relative abundance of the fungal class *Saccharomycetes* over time (ADH) in decomposition-impacted soils. Relative *Saccharomycetes* abundance increased as decomposition progresses in soils below normal (blue), overweight (green), and obese (yellow) donors, but does not change in soils below underweight (purple) donors. Lines represent the linear relationship between relative *Saccharomycetes* abundance and time (in ADH) for each BMI category (line color), while gray shading is the 95% confidence interval for each linear relationship.

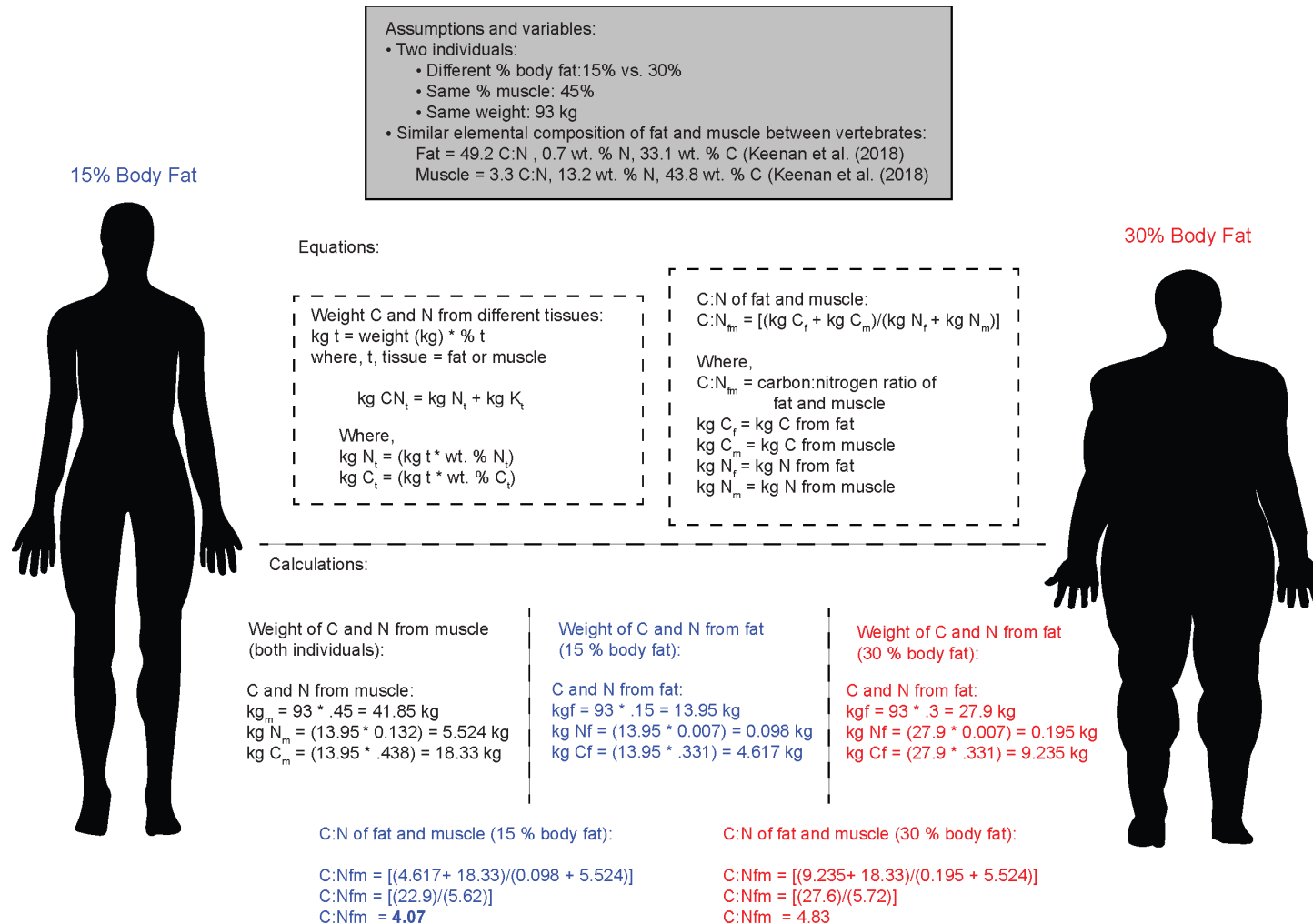


Figure 2.16 Calculation of C:N of fat and muscle between individuals with different body fat percentage, here 15 % and 30 %. Assumptions are outlined in the top gray box, while equations are presented in dashed boxes. Calculations pertaining to the 15% body fat individual are in blue, while calculations for the 30% body fat individual are in red text.

**CHAPTER 3 ENVIRONMENTAL PREDICTORS IMPACT MICROBIAL-
BASED POSTMORTEM INTERVAL (PMI) ESTIMATION WITHIN
HUMAN DECOMPOSITION SOILS**

PREFACE

While Chapter 2 focused on intrinsic factors, this chapter focuses on the effects of extrinsic (*e.g.*, environmental) factors on microbial community structural succession (*i.e.*, changes in the relative abundance of taxa) during human decomposition from the context of estimating postmortem interval (PMI). This project leverages data collected in Chapter 2, specifically amplicon (16S rRNA and ITS) sequencing and soil biogeochemical data; however, we address a different aspect of decomposition: estimation of PMI and the effects of extrinsic factors on that estimation. For this project, I applied supervised machine learning (random forest regression) to assess how different factors (biological marker, taxonomic level, and environmental features) impacted estimation of PMI from soil microbial taxonomic succession. This combination of supervised machine learning on a large dataset of human individuals ($n = 19$) showed that the measured environmental features sometimes improve model prediction, depending on the biological marker and taxonomic level considered for model generation. It also led to the identification of taxa whose changes in relative abundance correlated with decomposition time.

A version of this manuscript is currently being prepared for journal submission.

Contributions: This study was conceived by ARM. Laboratory soil physicochemical, and enzyme assays analyses were completed by ARM, with acknowledgements to Tori Beard, Morgan White, and Mallari Starrett. Next generation sequencing, microbial community analyses, random forest model construction, statistical analyses, and data visualization were conducted by ARM. The manuscript was written by ARM.

ABSTRACT

Microbial community data has been suggested to supplement established postmortem interval (PMI) estimation methods from insect succession and development on human remains. This is due to limitations of entomological methods, including reduced accuracy for longer PMIs and variation in insect colonization and/or access. In contrast, microbes are present at all stages of decomposition, making them a favorable target for forensic applications. A few studies have assessed the predictability of microbiome succession in decomposition soils, producing error rate ranges from two and a half to seven days; however, these models are built from small datasets ($n = 4$ individuals) that do not have the statistical power to capture inter-individual variation. Additionally, microbial-based models are built solely from amplicon sequencing of biomarkers (*e.g.*, 16S rRNA and ITS) and do not consider extrinsic factors that influence the presence and abundance of microbial decomposers. Therefore, this study aimed to build upon current research by evaluating the effects of environmental data on microbial-based PMI estimates from decomposition soil samples. We used relative taxon abundances from two biomarkers (16S rRNA and ITS2) via amplicon sequencing along with soil biogeochemical datasets of 19 deceased human individuals that decomposed on the soil surface at the University of Tennessee's Anthropology Research Facility (ARF) in Tennessee, USA. Random forest regressions were applied to datasets with different combinations of biological marker (16S only, ITS only, 16S and ITS combined) and taxonomic level (phylum, class, order, OTU), both with and without measured environmental predictors (*e.g.*, pH, conductivity, moisture, enzyme activities). Model performance was then compared by calculating the mean absolute error (MAE). MAE ranged from 796 to 997 ADH across all models. 16S only models outperformed ITS only models, while combining 16S and ITS did not improve 16S models alone. R^2 of the top models for each biological marker ranged from 0.9159 to 0.95 when predicting PMI within the training dataset. Inclusion of measured environmental data in PMI prediction models had varied effects depending on the biological marker and taxonomic level conserved. Specifically, inclusion of the measured environmental features improved (reduced MAE) all ITS models, but only improved 16S models at higher taxonomic levels (phylum and class), suggesting differences between bacterial and fungal community responses during decomposition. Overall, we show there is some

predictability in soil microbial succession in response to human decomposition, however error rates were high when considering a large population of donors.

INTRODUCTION

Microbes undergo a succession of taxa and/or function in response to disturbance events (1). Vertebrate death, and subsequent decomposition, in terrestrial ecosystems represent one such event, where microbial community composition is altered in response to nutrient deposition and altered environmental conditions (2-6)(Chapter 1). Microbial succession has been studied in various decomposition microhabitats, including internal organs (7-13), skin (3, 4, 14-17), and soil (5, 17, 18). These studies suggest that microbial taxonomic succession is consistent, thus work has begun to assess the ability to predict postmortem interval (PMI) from microbial communities for forensic applications. This work was inspired by the need to improve upon current forensic entomology methods, which link insect succession or development to PMI. Entomological methods are widely used in forensic cases but are limited to cases where insects are present and have access to the remains. Unlike insects, microbes are present during all stages of decomposition, making them potentially useful for forensic application. PMI, or the time elapsed since death (and/or beginning of decomposition), can be important evidence for death investigations, allowing law enforcement to establish a timeline of events (19).

Thus far microbial abundance-based PMI prediction models have been assessed in all major microhabitats and three different mammalian species. This includes internal (20, 21), external/skin (3, 16, 17, 22), and soil (22) microbial communities during pig (*Sus scrofa*) (3), mouse (*Mus musculus*) (17, 20, 22), and human (*Homo sapiens*) (16, 17, 21, 22) decomposition. These models suggest some level of predictability in microbial succession; however, results differ based on the microhabitat and taxonomic level considered and algorithm used for model construction. So far, the most common approach is to apply supervised machine learning algorithms to taxonomic abundance data of a few taxa (3) or whole communities (11, 16, 17, 21, 22). Within these studies, random forest regression is the most frequently used supervised machine learning algorithm. These models report error ranging from ~15 hours in mouse intestine samples to ~58 hours in mouse brain samples (20), up to 138 accumulated degree days in human skin samples (16), and two to five days in soil samples (22). Predictability within all three decomposition microhabitats (*i.e.*, internal, external, and soil) have been investigated by at least one study, with the soil microhabitat receiving the least attention. To date only one in-depth

study has sought to predict PMI from soil microbial community succession from amplicon sequencing (22); however, this study only included $n = 4$ humans, leaving our understanding of the predictability of soil microbial succession across a larger population of humans unclear.

Within the soil environment, effects of sample size and factors impacting model performance when predicting PMI have not been investigated. To date, the only study assessing predictability of microbial succession within decomposition-impacted soils have trained models with mixed human and non-human datasets (22) or with human datasets up to $n = 4$ individuals (22). While this previous study provides a foundation to build upon, it is unclear how inter-individual variation and potential species differences may impact model performance. Recent work suggests differences in decomposition patterns may exist between carcass species (23, 24) and even within species, due to intrinsic carcass properties (*e.g.*, body composition) (Chapter 2). Thus, there is a need to assess the predictability of soil microbial succession within a large human dataset in order to assess applicability of these models for forensic science. Additionally, current microbial-based PMI estimation models are trained using relative abundance of microbial taxa as model features. However, changes in soil environmental parameters are also observed over time. For example, soil electrical conductivity (correlates to salinity) and ammonium and nitrate concentrations have been shown to undergo shifts within human decomposition soils (25-27). It is possible that the inclusion of other model predictors (rather than only taxa relative abundance) may help to improve model estimation by providing additional resolution over time.

To build upon previous studies and address questions surrounding predictability of soil microbial succession during decomposition, the goals of this study were to 1) determine the ability of soil microbial communities to predict decomposition time during human decomposition 2) determine which biological marker (16S rRNA, ITS, or both) and taxonomic level (phylum, order, class, or OTU) results in the most accurate model predictions in these soils, and 3) assess how inclusion of soil environmental parameters as model features affects model accuracy. Our first aim was to investigate model performance with different biological markers, *i.e.*, genes most commonly used as sequencing targets. Previous work reported that 16S-based models performed better than 18S or ITS models in soil (22). However, we hypothesized that ITS-based models would be more accurate than 16S-based models: In previous work, we observed that fungal community

composition between individuals became more similar over decomposition time, while bacterial communities did not (Chapter 2). This may lead to less noise and better predictability for fungal models, compared to 16S models when considering a large number of subjects. Our second aim was to assess which taxonomic level(s) resulted in the greatest model accuracy. Based on results reported by Belk et al. (2018) and patterns in our data showing increased inter-individual variability at lower taxonomic levels (Chapter 2), we hypothesized that higher taxonomic levels, such as phylum and class would provide better PMI prediction. Our third goal was to probe the impact of environmental features on model prediction. While no previous studies have addressed this question, we hypothesized that inclusion of environmental predictors would help to improve PMI model predictions. Multiple soil parameters (*e.g.*, electrical conductivity, pH, ammonium concentrations etc.) have been observed to consistently change with decomposition time (5, 24-28), thus models may be improved by overlying soil physicochemical changes with taxa succession. We addressed our study aims using sequencing (16S rRNA and ITS2 amplicon) and soil physicochemical datasets of 19 deceased human individuals (Chapter 2) decomposed on the soil surface at the University of Tennessee's Anthropology Research Facility (ARF) in Tennessee, USA. Random forest regressions were applied to datasets with different combinations of biological marker (16S only, ITS only, 16S and ITS) and taxonomic level (phylum, class, order, OTU), both with and without environmental predictors. Model performance was then compared by calculating the mean absolute error (MAE).

METHODS

Study design

This work stems from our previous study, which aimed to assess the influence of intrinsic, or cadaver-related factors, on soil microbial ecology during human decomposition. While our previous work focused on variation in soil microbial successional patterns, this study aims to assess the effects of extrinsic factors on predictability of this succession to estimate PMI. Full experimental details are reported in Chapter 2. Briefly, decomposition of 19 deceased human individuals was observed through the end of active decomposition, here denoted by collapse of the abdomen and cessation of fluid leaking from the trunk (29). Decomposition took place at the Anthropology Research Facility (ARF), located at the University of Tennessee in Knoxville, TN,

USA (35° 56' 28" N, 83° 56' 25" W). The ARF is a forested outdoor facility consisting of clay loam and channery clay loam soils of the Coghill-Corryton complex (CcE) (Chapter 2)(30).

As described in Chapter 2, individuals with no open wounds or had not been autopsied were chosen for this study, as this could alter microbial decomposers prior to and during our study. Individuals were selected independent of demographic, however all individuals identified as White and ranged in age from 40-91 years (Chapter 2). Individuals were placed supine on the soil surface as they were received between February 2019 and March 2020. Hourly temperatures were recorded using TinyTag temperature and humidity loggers (Gemini Data Loggers, UK) until unenrollment at the end of active decomposition, characterized by (29) (Chapter 2). For this study accumulated degree hours (ADH) was calculated using hourly temperature readings and represented one measure of decomposition time. In this study, 0 ADH was defined as time of placement within ARF, and a baseline temperature of 10 °C was used for ADH calculations to keep our results comparable with entomology-based methods (31).

Soil sampling

As previously described in Chapter 2, five cm soil cores were collected from the decomposition-impacted area surrounding each individual at predetermined accumulated degree hour (ADH) intervals up until the end of active decomposition. ADH intervals included prior to placement (0 ADH), 100, 250, 500, 750, and 1000 ADH, and thereafter at 500 ADH intervals until unenrollment. For each respective sample, cores were homogenized, hand-picked to removed debris (*e.g.*, roots, insect larvae, rocks, etc.), and flash frozen in liquid nitrogen. Soil samples were then stored at -80°C prior to DNA extraction. Soil pH and electrical conductivity (EC) were measured from soil slurries using an Orion Star™ A329 pH/ISE/Conductivity/Dissolved Oxygen portable multiparameter meter (ThermoFisher), as described in Chapter 2 and gravimetric was measured in duplicate by oven drying 2 to 3 g soil aliquots at 105 °C for 72 hours. Enzyme activities of β -glucosidase (BG), *N*-acetyl- β -D-glucosaminidase (NAG), leucine amino peptidase (LAP), and alkaline phosphatase (PHOS) were measured according to a modified procedure by Bell et al. (2013) (Chapter 2).

DNA extraction

DNA extracts were obtained as described in Chapter 2. Briefly, 0.25 g of soil was extracted with the DNeasy Powerlyzer PowerSoil kit (QIAGEN Inc.) following manufacturer's instructions with modifications for our soil texture (clay loam) and condition (high organic content). Specifically, soils were homogenized under parameters suggested for high organic soils (2,500 RPM for 45 s). DNA concentration was determined using a fluorometric assay (Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen)) with a total volume of 200 µl and 1 µl of DNA. All DNA extracts were sent to the University of Tennessee Knoxville Sequencing Core Facility (Knoxville, TN) for 16S rRNA and ITS2 region amplicon sequencing on the Illumina MiSeq platform (2 x 150 bp). The primer set 515F (33)/806R (34) was used to amplify the V4 region of the 16S rRNA gene, while the ITS2 region in fungi was amplified using primers described previously (35)(Chapter 2).

Amplicon sequencing analysis

Raw sequences were processed in Mothur (36) to generate operational taxonomic unit (OTU) read count tables for both 16S and ITS datasets as described in Chapter 2, however in this study all OTUs were retained, as singletons and doubletons were not removed. Additionally, control samples and samples greater than 5000 ADH were removed in R (37). Samples were cut off at 5000 ADH to capture the linear response of soil parameters and account for variation in decomposition timeframes between individuals observed in Chapter 2. This resulted in 113 sequence libraries for model construction.

For remaining samples, read counts were total sum scaled (TSS) by determining the relative abundance of each OTU and normalizing to a standard library size (10,000 for all samples). This allowed for comparison of reads across samples and between biomarkers. We also removed OTUs with less than 10 reads across all samples in TSS normalized count tables to reduce noise in the datasets. 16S and ITS TSS read count tables were generated at the phylum, order, and class levels by summing the corresponding OTU table at each respective taxonomic level and then applying the TSS normalization as described above. One of our goals was to compare predictability of bacterial (16S only), fungal (ITS only), or both (16S-ITS) communities; therefore, after TSS normalization, 16S-ITS combined datasets for each taxonomic level were

generated by merging respective 16S and ITS TSS count tables. As a result, 12 datasets were created and used for random forest models.

Machine learning

We chose to apply random forest regression to datasets to predict PMI in ADH. This kept our study similar to those previously conducted in decomposition soil (22), while also providing a first assessment of the predictability of soil microbial succession during human decomposition in our geographical region (Knoxville, TN). Model construction was completed in R using the Ranger (38) package. First, samples were assigned to testing or training datasets. This was completed by randomly assigning 6 donors (~1/3) to the test set, while the remaining 13 were grouped into the training set. This approach was conducted following Belk et al. (2018), to ensure that all samples from a single individual were in either the testing or training set, respectively.

Next, random forest regressions were applied to microbial taxa TSS count tables in Ranger. First, random forest model parameters node size (3, 5, 7, 9) and sample size (0.55, 0.632, 0.70, 0.80) were hyper-tuned, by comparing models with different combinations of the parameters listed. The optimal model was chosen by assessing out of the bag mean square error (OOB MSE) of each model and choosing the set of parameters with the lowest OOB MSE. The optimum model for each biomarker and taxonomic level was assessed by calculating the OOB MSE of the model and the root mean square error (RMSE) and mean absolute error (MAE) for predictions of the testing set in 100 runs of the optimum model. This process was repeated for models including measured environmental parameters, however values for ambient temperature (°C), pH, electrical conductivity (EC), moisture, β -glucosidase (BG) activity, *N*-acetyl- β -D-glucosaminidase (NAG) activity, leucine amino peptidase (LAP) activity, and alkaline phosphatase (PHOS) activity were included as model features (**Table 3.1**). For all environmental parameters, aside from temperature, log response ratio normalized values (39)(Chapter 2), were used to account for natural seasonal differences in these parameters. The top 25 most influential model features were extracted from each optimum model to assess taxa/environmental factors influencing model predictions. To evaluate the potential differences in model predication between biomarkers and taxonomic levels, linear regression was applied to the average MAE

values (mean of 100 runs per model). Variation in MAE due to treatment variables was assessed with ANOVA, while differences between treatment groups were determined with *post-hoc* t-tests in R.

RESULTS

Soil environmental parameters

The measured soil biogeochemical parameters were altered in response to human decomposition (Chapter 2). Of the original parameters reported in Chapter 2, soil pH, EC, and four enzyme activities were assessed in this study. Soil pH and EC are known drivers of microbial community dynamics (40, 41), while enzyme activities provide insight into functionality of the microbial community. Soil EC generally increased with progression of decomposition. Soil pH was variable between individuals, with pH increasing ($n = 5$), decreasing ($n = 12$), or displaying minimal change relative to the controls ($n = 2$) (Chapter 2). Extracellular enzyme activities were also variable between individuals, however general trends included increased NAG and PHOS over time. BG and LAP were more variable, with LAP activity correlated to soil pH (Chapter 2).

General model statistics

In total, 24 models were built in R. Twelve of the models contained environmental features and the other twelve did not. The number of taxa included as features in models without environmental data are reported in **Table 3.2**. Bacterial (16S) and fungal (ITS) features ranged from 35 to 5195 and 16 to 2219, respectively, increasing with taxonomic level. For all models, MAE ranged from 796 to 997 ADH (**Figure 3.1**). Across all variables considered, the best performing model was the 16S phylum level model with environmental predictors (MAE 796). In contrast, the worst performing model was the ITS phylum level without environmental data (MAE 997). Overall, ITS models made up seven out of 10 worst performing models. In fact, no ITS-only model was in the top 10 best performing models, measured by MAE values. The best performing ITS model was the ITS order level model with environmental data (MAE 874). Combined 16S-ITS models were dispersed among the best and worst models for predicting ADH. For example, the 16S-ITS order level model without environmental data had the second lowest MAE (MAE 801) overall, but also the 16S-ITS OTU level model with environmental data had the sixth worst MAE (MAE 893). Predictability, assessed by the linear relationship between

predicted and observed values, for the training (**Figure 3.2A-C**) and testing (**Figure 3.2D-F**) datasets for the top 16S only, ITS only, and 16S-ITS models are shown in **Figure 3.2**. R^2 for these models ranged from 0.9159 to 0.95, however these values were reduced when making predictions for the testing dataset ($r^2 = 0.7051 - 0.5884$).

Model comparison: biological marker

Ability of random forest regressions to predict ADH varied depending on the biological marker used to build models (ANOVA $F = 9.455$, $p = 0.001$). ITS models were generally less accurate in predicting ADH compared to 16S or 16S-ITS models independent of taxonomic level and environmental data (**Figure 3.3**). ITS models ranged in MAE from 874 to 997 ADH, with a mean MAE of 910 ADH. Post-hoc t-tests show that ITS models, in general, had higher MAE than both 16S (t-test $p = 0.001$) and 16S-ITS ($p = 0.007$) models. In comparison, 16S and 16S-ITS models performed similarly ($p = 0.37$). 16S models ranged in MAE from 796 to 889 ADH, with an average MAE of 837 ADH, while 16S-ITS models ranged in MAE from 801 to 893 ADH (average MAE = 852 ADH).

Model comparison: taxonomic level

Some variation was observed in MAE due to taxonomic level considered for model development, however these differences were not significantly different (ANOVA $F = 1.538$; $p = 0.24$). When considering the potential influence of taxonomic level within biomarkers, no significant difference in MAE by taxonomic level was observed for 16S ($p = 0.2$), 16S-ITS ($p = 0.15$), or ITS ($p = 0.63$) models (**Figure 3.4**). While these results were not significant, some trends were observed. First, order level models had the lowest MAE for all three biological markers assessed. This was also observed in **Figure 3.1**, where order level models had the lowest MAE for all models without environmental data. Trends for the other taxonomic levels varied depending on the biological marker in consideration. Phylum and class level models performed similarly within 16S models, with OTU models generating the highest MAE. Within 16S-ITS models, class and OTU level models performed similarly, displaying the first and second highest MAE, respectively. For ITS models, phylum level models had the highest MAE, followed by OTU and class.

Model comparison: environmental parameters

Overall, inclusion of environmental parameters in random forest models to predict ADH from soil microbial taxa impacted model accuracy. However, if model MAE increased or decreased was dependent on biological marker and taxonomic level considered (**Figure 3.1**). For ITS models, inclusion of environmental factors reduced MAE irrespective of the taxonomic level. This reduction was most pronounced for the phylum level model, in which MAE was reduced by 59 ADH. In models containing 16S sequencing data (16S and 16S-ITS), effect of environmental features differed by taxonomic level. Specifically, phylum, class, and order level models performed better and OTU level models performed worse within 16S models. This was similar for the combined 16S-ITS datasets at the phylum and OTU levels; however, class and order level models performed worse (*i.e.*, increased MAE) when environmental factors were included.

Model features: top models

In addition to assessing the predictability of different random forest models, we also looked at important model features to observe which taxa and/or environmental parameters impacted model performance. Here we highlight the top 25 features of best performing 16S (phylum + environmental), 16S-ITS (order), and ITS (order + environmental) models, determined by lowest MAE within the test set. Both 16S and ITS top models included environmental predictors, while the combined 16S-ITS model did not (**Figure 3.5A-C**). For the 16S phylum model with environmental data, the most important model feature, as assessed by decrease in MSE, was soil electrical conductivity (EC). The remaining top features included *Firmicutes*, *Acidobacteria*, *Epsilonbacteraeota*, and *Proteobacteria*, respectively. Other features of interest for this model included leucine aminopeptidase activity (#6) and *Nitrospirae* (#8) (**Figure 3.5A**). For the ITS order model with environmental predictors, the most important model feature was *Pleosporales* with EC, *Rhizophydiales*, Unclassified *Basidiomycota*, and *Auriculariales* rounding out the top five (**Figure 3.5C**). In this model, no other environmental parameters were among the top 25 important features. Other top taxonomic features of interest included *Saccharomycetales*' (#10) and Unclassified *Sordariomycetes* (#25), as their members are present in the human mycobiome and feces, respectively (42, 43). The top performing 16S-ITS model was the order level model without environmental features. Top features for this model were the bacterial order

Lactobacillales and the fungal order *Pleosporales*. Bacterial orders *Clostridiales*, *Bacteroidales*, and *Cardiobacteriales* were third, fourth, and fifth most important features, respectively (**Figure 3.5B**). Relative abundance of anaerobic bacterial taxa identified in random forest models, including *Firmicutes* (**Figure 3.6A**) and *Clostridiales* (**Figure 3.6C**), increased as decomposition progressed. In contrast, relative abundance of the aerobic nitrifying organisms of the phylum *Nitrospirae* decreased (**Figure 3.6B**). Relative abundance of the bacterial order *Cardiobacteriales* (**Figure 3.6D**) and fungal order *Pleosporales* (**Figure 3.7**), identified in the mixed 16S-ITS order model, increased and decreased, respectively, over time.

DISCUSSION

The goal of this work was to assess the effect of extrinsic factors on the predictability of soil microbial communities during human decomposition. Within this context, we sought to address the influence of biological marker, taxonomic level, and inclusion of non-taxa abundance data, here a few environmental parameters, on model prediction of PMI. To address these questions, we applied random forest regressions to various microbial abundance-based datasets collected from decomposition soils below 19 deceased human individuals in Knoxville, TN. Model analysis revealed differences between model accuracy due to the biological marker, taxonomic level, and environmental parameters considered for model construction. Overall, models generally performed poorly, ranging in MAE from 796 to 997 ADH. In East Tennessee, these error rates correspond to roughly 2.5 to 3.5 days in July and greater than 28 days in February. Further, considering our total decomposition time of 5000 ADH, errors of 796 to 997 ADH equates to 15.9 % to 19.9 % of the total decomposition time (ADH). This wide error range when including a larger number of subjects across multiple seasons suggests microbiome-based models may have low accuracy, particularly when considering a diverse population of individuals and seasonal differences. Thus, assessment of singular (*i.e.*, data across multiple seasons) and season-specific models is needed.

One important source of variation in our study was the different rates of decomposition between individuals. While we attempted to correct for differences due to thermal energy input by using accumulated degree hours (ADH), there was still variability. 5000 ADH represented different morphological stages of decomposition for different individuals: For example, 5000 ADH

represented the end of active decomposition for individual 009, but only about 25 % of active decomposition for individual 010. Both of these individuals were placed within the facility in the summer, experiencing the same local environmental conditions and insect and scavenger communities, suggesting there are additional factors leading to variation in microbial communities within decomposition-impacted soils. These may include additional extrinsic, or environmental parameters, not considered in our models, as well as intrinsic differences between the individuals themselves (*e.g.*, age, weight, medications, medical conditions etc.). Moving forward, we will need to employ a strategy to combine antemortem and environmental data in order to investigate which factors help improve model predictions.

Influence of diversity and taxa succession on PMI estimations

The trends we observed in model MAE between different biological markers, taxonomic levels, and inclusion of environmental data may all be linked to differences in diversity between bacterial and fungal (16S vs. ITS) communities and the number of taxa (*i.e.*, features), ultimately impacting resolution for predicting PMI. Overall, Chao1 richness and Inverse Simpson diversity were 10 and 15 times lower, respectively, in fungal communities compared to bacterial communities (Chapter 2). This translated to differences in the total number of model features for 16S and ITS models, where 16S models had roughly 1.7 to 2.3 times more features, depending on the taxonomic level considered.

In our previous work, we observed that fungal community composition became more similar as decomposition progressed, with only a few taxa driving fungal successional patterns (Chapter 2). This was also observed in Fu *et al.* (2019), in which only a few taxa (*e.g.*, *Ascomycota* sp., *Yarrowia lipolitica*, etc.) displayed relationships with PMI. While we hypothesized that ITS-based models would be more accurate than 16S-based models because of these studies, our results actually revealed that 16S models generally outperformed ITS-based models and combining 16S and ITS did not improve 16S models alone. This result coincides to those reported by Belk *et al.* (2018), in which 16S models had lower error than ITS or 18S models. Reduced diversity, in combination with the number of taxa linked to PMI, may explain why ITS models had higher MAE than 16S models. Specifically, reduced number of taxa observed in fungal communities with compositional shifts driven by relatively few taxa may provide lower

resolution than shifts in bacterial community composition. This may also explain why combining 16S and ITS datasets, which would increase overall diversity, did not outperform either marker alone. With only a few fungal taxa displaying changes over time, their inclusion may not have added additional resolution to the model.

Diversity differences between bacterial and fungal communities may also drive some of the trends observed between taxonomic levels and with or without environmental factors. In this study, order level models performed best for all biological markers when not considering environmental features. This is in contrast to findings by Belk et al. (2018), where lower error was reported for phylum and class level models. This may be linked to a balance between taxonomic resolution and noise for this time period of decomposition. Previous decomposition studies have observed an increase in inter-individual variation at lower taxonomic levels (17, 27)(Chapter 2). This may explain why OTU level models displayed the highest MAE for all biological markers when not considering environmental data. Within the field, microbial taxonomic succession has mostly been characterized at higher taxonomic levels, at which general patterns are more similar between individuals. However, aggregating microbial abundances at higher taxonomic levels, such as phyla and class, inherently reduces data dimensionality. It is possible that this decrease in features, in conjunction with trends in taxon abundance over time reduces the ability of the random forest regression to resolve timepoints at the highest taxonomic levels.

This balance between diversity and features with resolution over time may also explain environmental features effect on model MAE. Here, we hypothesized that inclusion of environmental parameters would improve all model predictions, by combining soil chemical and microbial successional patterns. While inclusion of environmental predictors did reduce MAE for some models, MAE was increased for others. This effect appears to be linked to biological marker and taxonomic level considered for model creation. Specifically, inclusion of environmental parameters into the lower diversity fungal models added features that helped to improve overall resolution to predict PMI. In contrast, inclusion of environmental data may have added additional noise to high diversity bacterial datasets at lower taxonomic levels, overall leading to decreased model performance.

Model features

In addition to our main goals, we also assessed model features for each top performing 16S, 16S-ITS, and ITS model as determined by lowest MAE. This included the 16S phylum model with environmental data, the 16S-ITS order level model, and the ITS order level model with environmental data. Top model features for 16S phylum level models included taxa observed in previous human and animal decomposition studies, such as the bacterial phyla *Firmicutes*, *Acidobacteria*, and *Proteobacteria* (5, 17). In our study, *Firmicutes* and *Nitrospirae* were shown to decrease as decomposition progressed, while *Acidobacteria* decreased and *Proteobacteria* remained consistent. These changes seem to be linked to differences in metabolism and environmental changes that occur when decomposition products are released into the surrounding soil. For example, it has been suggested heterotrophic microbial activity responding to nutrient availability results in depletion of soil oxygen (27, 28). This would impact the presence of anaerobic gut and soil taxa. While we did not measure soil oxygen in this study, soil respiration was increased in these soils, and so oxygen depletion is to be expected (Chapter 2). The increased presence of taxa containing facultative and obligate anaerobic members, *Firmicutes* and *Clostridiales* in phylum and class models, respectively, and decrease in *Nitrospirae*, containing nitrifying bacteria that oxidize nitrogen under aerobic conditions, support this hypothesis. Increases in *Firmicutes* and *Clostridiales* follow successional trends observed in internal (*e.g.*, organs) microbial communities (45-47). Specifically, increased relative abundance of *Clostridium* has been termed the “Clostridium Effect” by Javan et al. (2017) and observed in various organs (6, 45) and rectum (47) postmortem. We also observed this effect within decomposition-impacted soils following deposition of decomposition fluid in this study, suggesting some of these organisms may be host-derived. Decreased presence of *Acidobacteria* in decomposition-impacted soils is likely linked to their oligotrophic characteristics in response to high nutrient deposition (5, 48). Succession of these taxa and other taxa past 5000 ADH and the potential implications for PMI models is unclear.

Order level models also revealed some information about soil microbial succession during human decomposition. The 16S-ITS order level model had the lowest MAE among all 16S-ITS models. Within this model, important taxa were a combination of 16S and ITS features present in

respective models. Among the top bacterial features, *Lactobacillales*, *Bacteroidales*, and *Clostridiales* were all shown to display general increases as decomposition progressed. This is consistent with previous literature (5). One interesting find was *Cardiobacteriales* as the fifth most important model feature. *Cardiobacteriales* is a bacterial order of gram-negative rods, whose members are generally capable of fermentation of various sugars (49). Within this order, only the genus *Ignatzschineria* was identified based on SILVA non-redundant database (v132) (50). This genus has been identified in previous outdoor decomposition studies focusing on gut (47), skin (14), and soil (5) microbial communities. *Ignatzschineria* are associated with insect species (51, 52) and first appear in the soil during release of fluid. We previously observed this taxon in bacterial decomposition fluid communities (Chapter 2), further suggesting these organisms come from the body. Their association with insects highlights the potential for macro-decomposer and scavenger activity to impact microbial succession during decomposition and suggests that PMI estimation models specific to decomposition setting (indoor or outdoor) may be required.

Within the ITS order level models, the fungal order *Pleosporales* was among the most influential taxon for PMI estimation. *Pleosporales*, a member of Ascomycota fungi, decreased as decomposition progressed. This was similar to observations by Fu *et al.* (2019), where *Pleosporales* sp. was shown to be associated with non-decomposition soils by LEfSe (linear discriminant analysis effect size). *Pleosporales* are often associated with plants, found as endophytes, epiphytes, and the rhizosphere (53). Reduced relative abundance of these fungi in decomposition soils is interesting considering *Pleosporales* have been shown to positively respond to nitrogen amendments (54, 55). Their response to decomposition products may suggest sensitivity to highly concentrated nitrogen amendments and/or other soil chemical parameters, such as osmotic stress in response to high EC, observed in decomposition soils.

In regard to environmental predictors, electrical conductivity (EC) appeared to be most influential of those assessed. EC was recorded as the top and second most important feature for the 16S phylum and ITS order models with environmental data, respectively. This is likely due to patterns in soil EC being more consistent between individuals over time. Specifically, EC was shown to increase within decomposition soil over time for all nineteen individuals. This increase

in EC has been shown to positively correlate with changes in soil ammonium concentrations (28, 56), suggesting ammonium also correlates to microbial community dynamics. Previous studies have observed initial increases in ammonium concentrations following deposition of decomposition fluids (26-28, 56, 57). Other parameters, such as pH and LAP activity were more variable both over time as well as between individuals, displaying increases and decreases in response to human decomposition (Chapter 2). While we did not consider all possible environmental parameters in this study, these results suggest that feature selection may help to identify relevant environmental parameters for model construction.

Limitations/considerations

Little is known about the complete trajectory of soil microbial succession in response to decomposition, and if the complete successional pattern can be modeled for forensic science applications. This is due to variation in decomposition studies, including vertebrate species observed, study design, and overall small sample sizes (Chapter 1,2). Additionally, a majority of decomposition studies focus on the early stages of decomposition, when decomposers are most active in degrading soft tissues (58, 59). While informative for initial compositional shifts, this timeframe does not allow us to assess for how long these communities may be impacted or if they return to pre-decomposition conditions (1). While this study starts to address factors that influence PMI estimations from soil microbial succession using a large dataset with human individuals, many foundational questions still remain. Below we discuss multiple areas to be expanded upon with future investigation.

First, we did not include all possible environmental and soil data as model predictors. Other factors, such as respiration rates, oxygen concentration, N species, dissolved organic carbon and nitrogen, among others may be relevant features for models predicting PMI within the soil environment. In addition, it is possible that changes in soil parameters during human decomposition differ based on the soil type/region due to climatic differences impacting decomposition progression or presence of microbial taxa (60). Therefore, further work is needed to determine relevant taxonomic and environmental features for these PMI estimation models.

Second, we chose to implement the random forest regression algorithm as it is not as sensitive to non-linear data and has high interpretability compared to other forms of supervised and

unsupervised machine learning algorithms (61). This allowed us to assess prediction of PMI and identify taxa and environmental features that correlate with PMI, as well as kept our results similar to previous decomposition studies within the soil environment (22). However, recent studies have compared multiple machine learning algorithms in other decomposition microhabitats (*i.e.*, skin, organs), showing variation both between internal organs (20) and within the same habitat (16). Both Liu et al. (2020) and Johnson et al. (2016) observed other machine learning algorithms performed better than random forest in higher diversity microhabitats such as the skin and cecum. As the soil environment is among the most diverse on the planet, it is necessary to assess different machine learning approaches when predicting PMI within this microhabitat (62).

Third, total PMI considered for model construction will likely impact the performance and applicability of these models (62). Here we showed that order level models had the lowest MAE in models that do not include environmental features. This is in contrast to findings by Belk et al. (2018) and our hypothesis, in which we expected lower error for phylum and class level models, suggesting differences between studies, such as region, sampling strategy, number of individuals, species, study timeframe or intrinsic differences between donor populations may impact model performance. For example, our study went through 5000 ADH, while Belk et al. (2018) presented data up to 25 days. In our study, 5000 ADH corresponded to 13 to 115 days depending on the time of year. Additionally, Belk *et al.* (2018) observed decreased model error when only using datapoints from the first 25 days of decomposition compared to the first 50 days. Therefore, future work is needed to determine the optimum PMI at which random forest regression is useful in decomposition soils and if this optimum can be improved with other machine learning algorithms.

Finally, we chose to utilize operational taxonomic units (OTUs) and ADH calculated with a baseline of 10°C, as opposed to amplicon sequence variants (ASVs) and ADH with a baseline of 0°C or 4°C. The recent application of denoising methods to generate ASVs has become popular in microbial studies using amplicon sequencing. However, we chose to cluster sequences into OTUs to reduce our raw dataset and the probability of splitting single genomes across multiple ASVs (63). While Glassman and Martiny (2021) observed similar results for alpha and beta

diversity from OTUs and ASVs in leaf litter communities, other studies have shown differences in diversity when comparing the two methods (65). Thus, it is unclear if using OTUs or ASVs will impact machine learning algorithms such as random forest to predict PMI. Future work should investigate differences in PMI estimations from models constructed with OTUs as well as ASVs. Additionally, we chose to use a baseline of 10°C, which is commonly used for entomological methods due to the developmental threshold of regional insects (31). However, other decomposition studies within the soil environment have also used 0°C or 4°C as thresholds for ADH or accumulated degree hour (ADD) calculations. These differences may impact PMI estimates; however, no one has addressed effects of different thresholds for ADH/ADD calculation on PMI estimates from microbial successional patterns within the soil. Therefore, a comprehensive comparison between different ADH calculations is necessary.

CONCLUSIONS

This study aimed to assess microbial abundance-based prediction of PMI from soil microbial communities. Within this, we compared models with different biological markers, taxonomic levels, and presence/absence of environmental variables to expand upon previous estimations of PMI from machine learning algorithms. From this large dataset, including nineteen individuals across multiple seasons, we observed decreased model precision compared to previously published models based on small datasets. However, our results show that 16S and 16S-ITS models performed similarly and outperformed ITS models. Further, order level models have the lowest MAE when not considering environmental parameters. We also show that the addition of other factors, such as environmental parameters, have the potential to impact PMI estimations. These results show there is some level of predictability in soil microbial succession, however high error rates were seen across nineteen individuals and across seasons. For example, our study size did not account for all intrinsic factors that are related to an individual's agency (*i.e.*, choices based on the resources available to an individual) that can alter antemortem conditions; therefore, when we expand the study sample size beyond nineteen, the observed variation and error in PMI estimations may increase. Together this means microbial-based PMI models would need considerable validation and refinement across a diverse study sample prior to implementing in a forensic context.

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APPENDIX

Table 3.1 Overview of variables used to construct models.

Type of data	Predictor variables tested
Bacterial and fungal taxa (OTUs) relative abundances	16S OTUs, ITS OTUs, both 16S and ITS OTUs
Phylogenetic level	Phylum, Order, Class, OTU
Environmental parameters	Ambient Temperature, EC, pH, moisture, β -glucosidase, <i>N</i> -acetyl- β -D-glucosaminidase, leucine amino peptidase, and alkaline phosphatase

Table 3.2 Number of microbial taxonomic features provided as input for random forest regression for various biomarkers and all taxonomic levels investigated in this study. 16S-ITS microbial features are the sum of 16S and ITS features, alone.

	16S features	16S-ITS features	ITS features
Phylum	5195	7414	2219
Class	264	411	147
Order	111	177	66
OTU	35	51	16

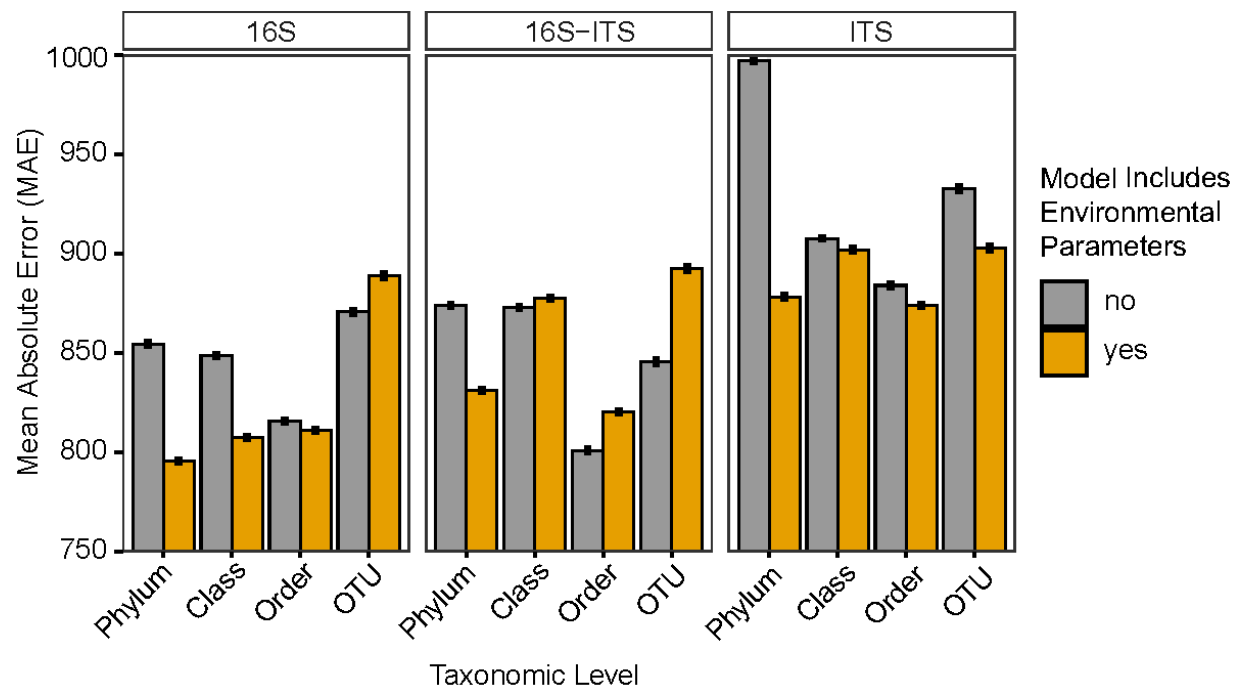


Figure 3.1 Mean absolute error (MAE) from 100 runs of each respective model against the testing dataset. Data are reported by biological marker (column), while color compares models with (gold) and without (gray) environmental predictors.

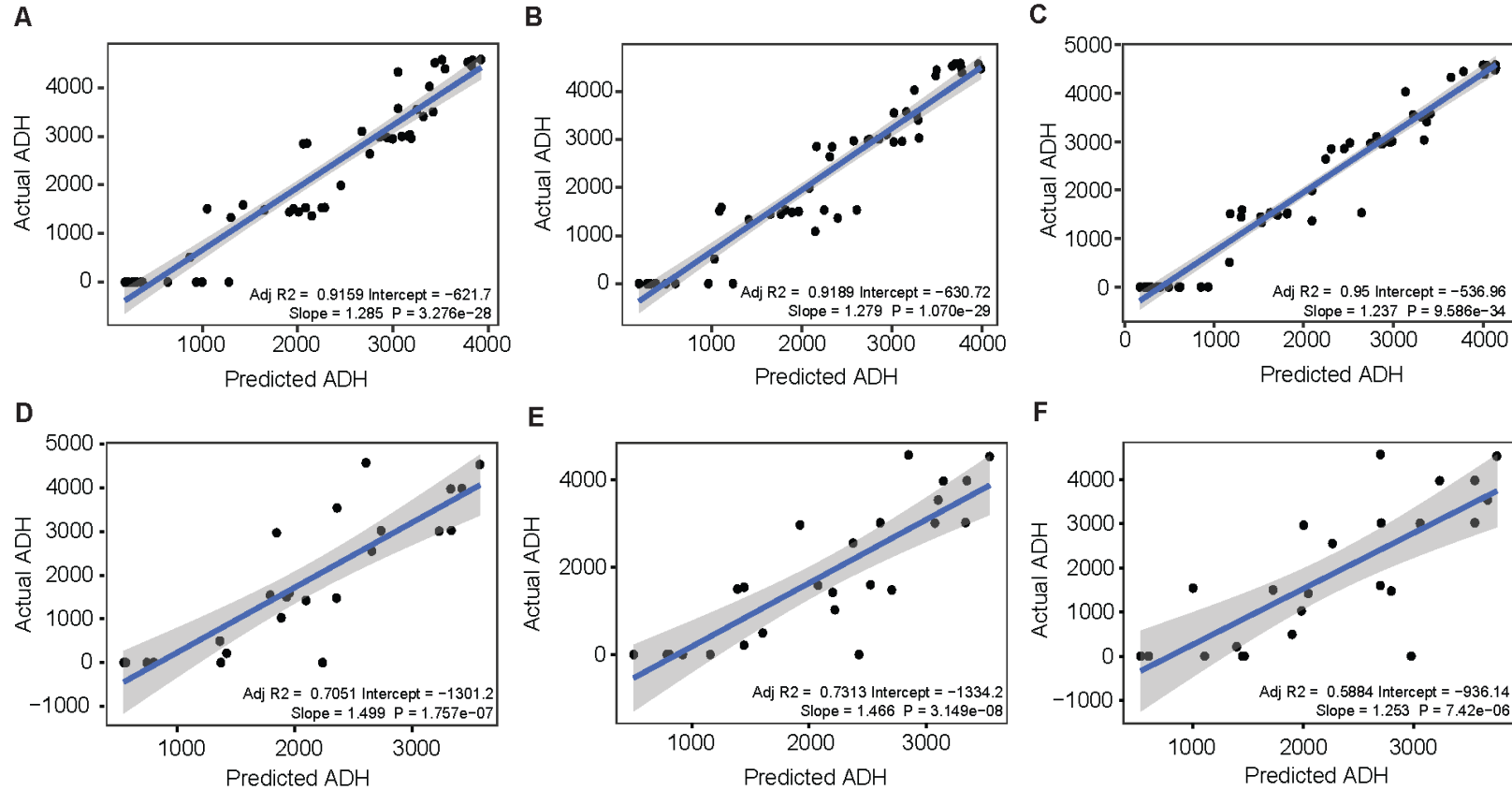


Figure 3.2 Model predictions for the training set (A-C) and testing set (D-F) for the top performing model for each biological marker as determined by lowest MAE. For each biological marker, top models included 16S phylum + environmental (A,D), 16S-ITS order (B,E), and ITS order + environmental (C,F). Predictability of each model is greater for the training set (A-C) compared to the testing set (D-F). A-F displays the linear relationship (blue lines) and 95 % confidence interval (gray shading) between actual PMI, in ADH, and predicted ADH within each respective dataset (training vs. testing).

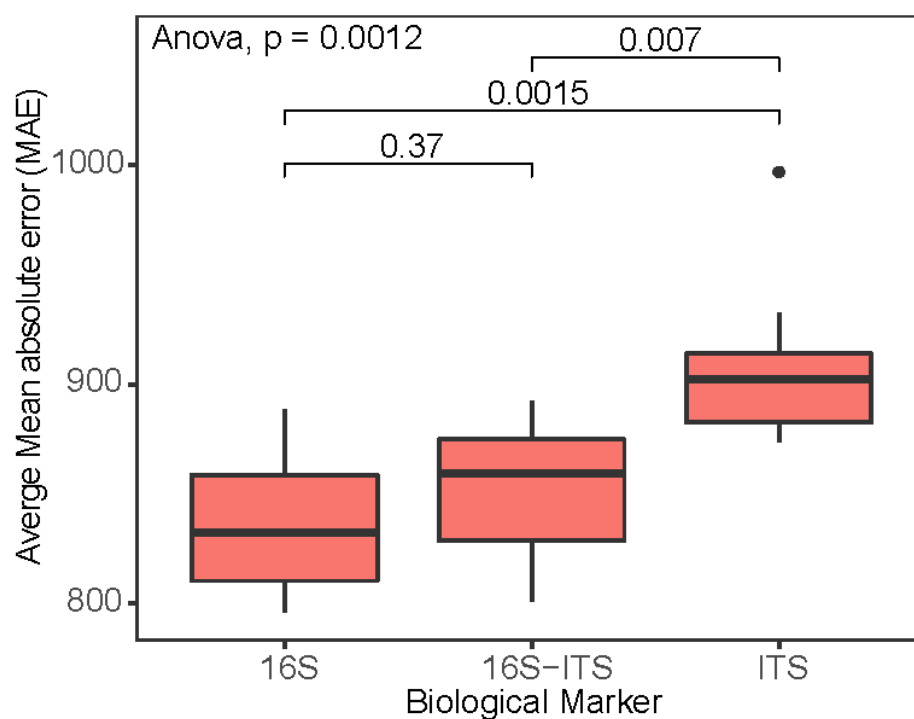


Figure 3.3 Mean absolute error (MAE) varies as a result of biological marker (16S, 16S-ITS, or ITS) used for model construction. Average MAE is the result of 100 runs of the 24 respective models. Reported p-values are the result of *post-hoc* t-tests.

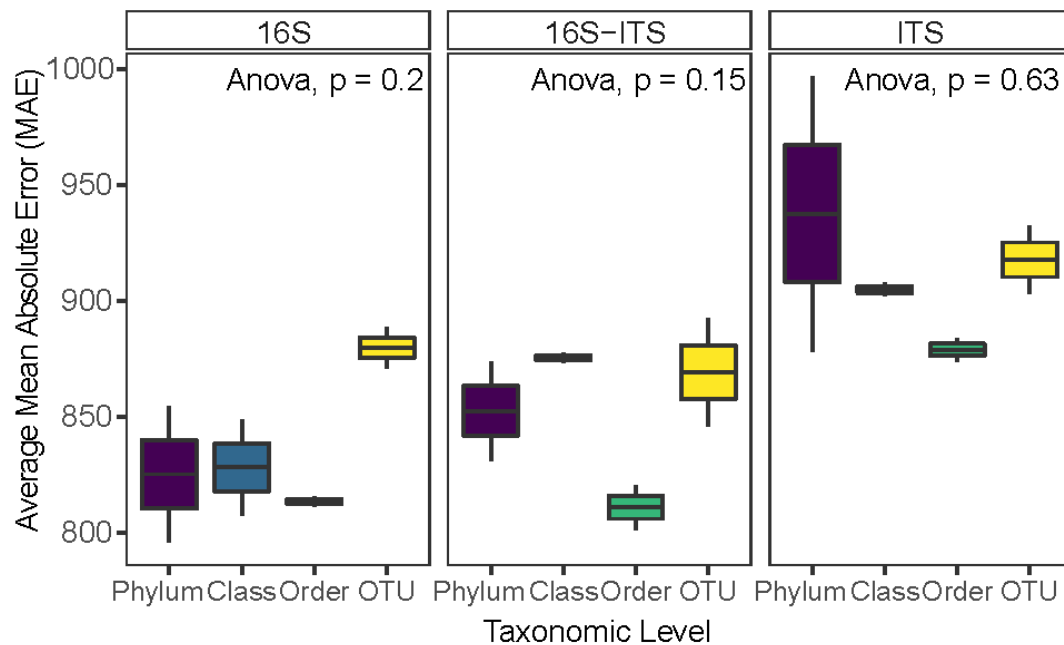


Figure 3.4 Mean absolute error (MAE) does not vary as a result of taxonomic level (color) used for model construction for any of the biological markers assessed (column). Average MAE is the result of 100 runs of the 24 respective models. Order level models (green) generally had the lowest MAE, compared to phylum (purple), class (blue), and OTU (yellow) models.

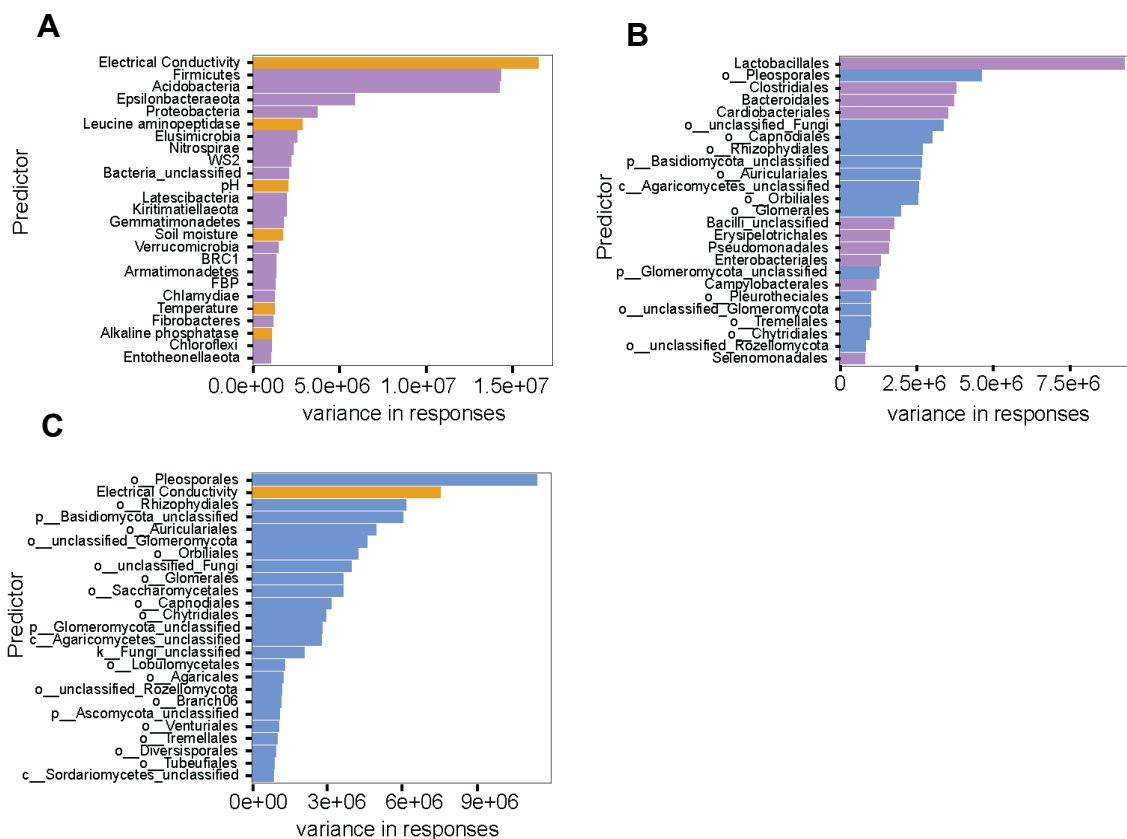


Figure 3.5 Top 25 model features as determined by variance of responses in Ranger. For each biological marker, top models included 16S phylum + environmental (A), 16S-ITS order (B), and ITS order + environmental (C). Bar color denotes whether the feature is a 16S taxon (purple), ITS taxon (blue), or environmental feature (gold).

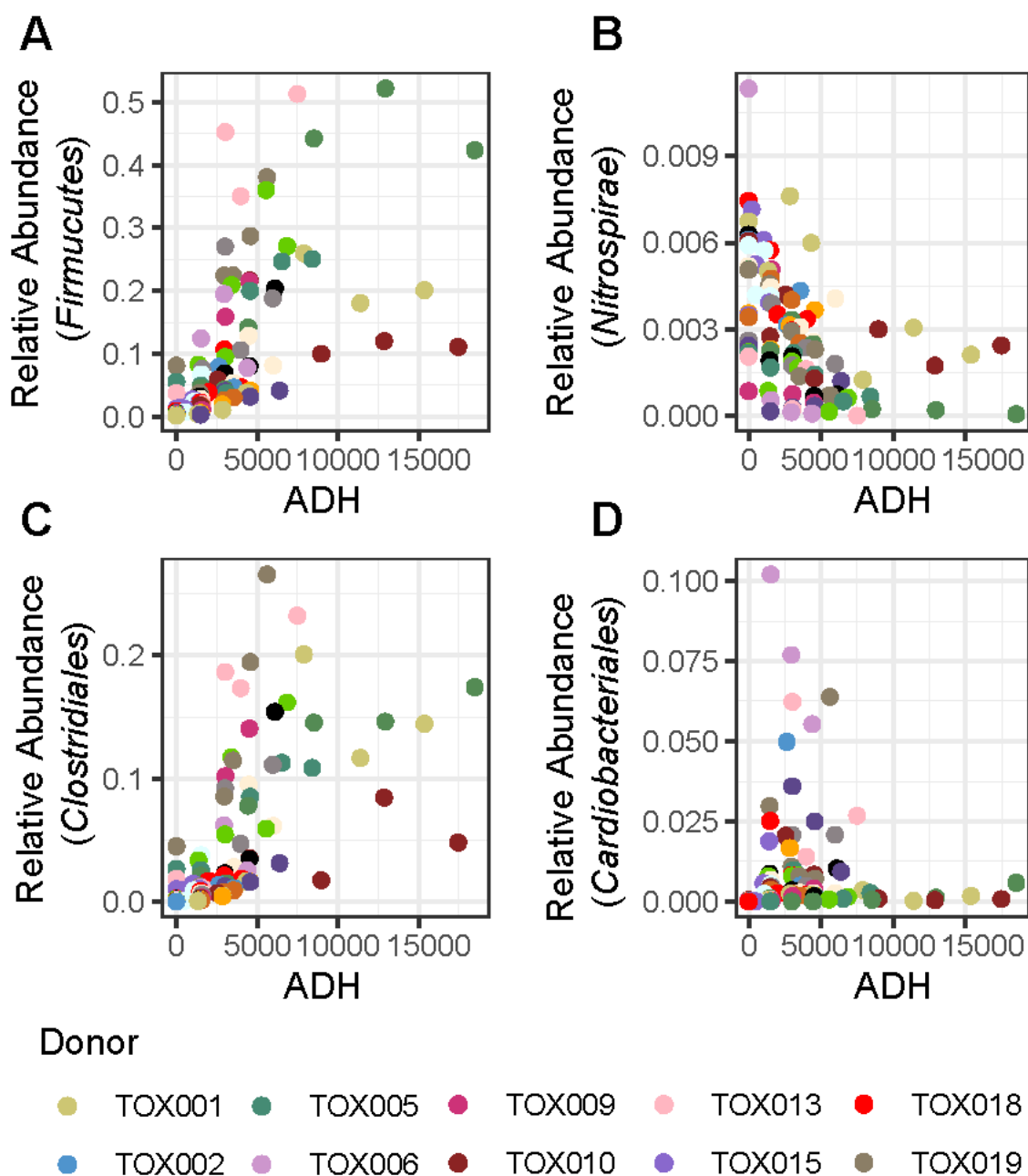


Figure 3.6 Relative abundance of select bacterial taxa identified as important features in random forest regressions. Relative abundance of the phyla *Firmicutes* (A) and *Nitrospirae* (B) and orders *Clostridiales* (C) and *Cardiobacteriales* (D) change over time, here accumulated degree hours (ADH), within decomposition-impacted soils. Trends for each donor (names “TOX####”) are delineated by line color.

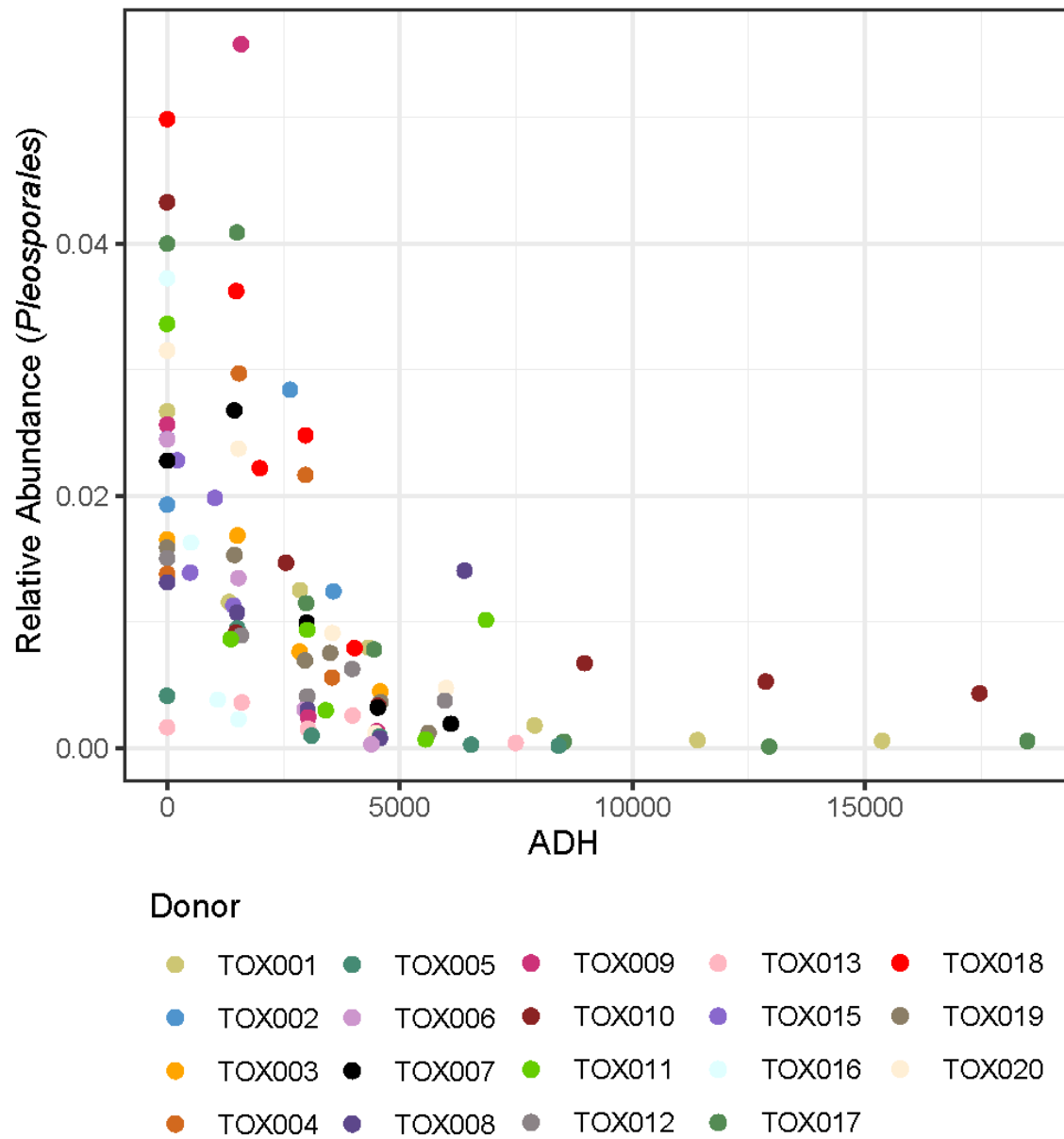


Figure 3.7 Relative abundance of the fungal order *Pleosporales* over time, in accumulated degree hours (ADH). Trends for each donor (names “TOX####”) are delineated by line color.

CHAPTER 4 SOIL MICROBIAL FUNCTIONAL SUCCESSION OVER ONE YEAR OF HUMAN DECOMPOSITION

PREFACE

Where Chapter 2 and 3 investigated microbial structural succession, Chapter 4 focuses on another aspect of microbial community response to disturbance: functionality. Specifically, we investigated soil microbial functional succession during one year of human decomposition via application of metatranscriptomics. For this project, I collaborated with Dr. Lois Taylor to leverage soil samples collected as a part of her dissertation (1). In the original study, Dr. Taylor observed decomposition of three human individuals for one year. For my project, we chose a subset of timepoints, based on soil physicochemical data, for RNA extraction and subsequent sequencing. This combination of metatranscriptomics with soil physicochemical analyses allowed us to characterize gene expression of the soil community over the course of decomposition while linking some of these changes to extrinsic soil physicochemical factors. Specifically, we show that gene expression profiles within decomposition soils are still altered after one year and soil oxygen may be a key extrinsic factor, impacting microbial gene expression (*e.g.*, nitrogen cycling genes).

Contributions: Original field study was designed by Dr. Lois Taylor and Dr. Jennifer DeBruyn. Soil collections were conducted by LST and ARM, with acknowledgements to Tori Beard, Morgan White, and Mallari Starrett. Soil physicochemical data were collected by LST, while RNA extraction, next generation sequencing, microbial gene expression analyses were conducted by ARM. All data analysis and visualizations were completed by ARM. A version of this manuscript is currently being prepared for journal submission.

ABSTRACT

Taxonomic succession of soil microbial communities in response to vertebrate decomposition has been observed in various settings over the past decade, documenting the presence of microorganisms as decomposition progresses. While this describes community composition and structure, this does not inform about which members or genes are active. This has left gaps in our understanding of what functional roles microorganisms play in vertebrate decomposition, which ultimately impacts ecosystem functioning. Here we present the first study investigating gene expression in human decomposition soils over one year. Total RNA was extracted and metatranscriptomes obtained from soil samples collected prior to decomposition (day 0) and at days 12, 58, 86, 168, and 376 from below three decomposing human bodies. Analyses show that microbial gene expression profiles shift over time, clustering by timepoint, with profiles most different from controls at day 86. Shifts in gene expression were partially explained by environmental and soil physicochemical variables, including internal body accumulated degree hours (ADH) ($F = 5.41$, $r^2 = 0.152$, $p = 0.001$), as well as soil temperature ($F = 2.60$, $r^2 = 0.073$, $p = 0.045$), pH ($F = 2.60$, $r^2 = 0.073$, $p = 0.042$) and electrical conductivity ($F = 2.97$, $r^2 = 0.084$, $p = 0.037$). Differential expression analysis revealed that decomposition soils are associated with increased expression of stress response genes (mean fold change 3.48), particularly heat shock proteins ($p < 0.001$), whose expression increased between days 0 and 58 and remained elevated through day 376. Further, we identified genes whose expression was altered at certain timepoints. This included increased expression of nitrogen cycling genes HAO (85x), *norB* (83x), and *nosZ* (19x) at day 86 when dissolved oxygen was reduced to approximately 85%, suggesting that hydroxylamine may be converted to nitric oxide during reduced oxygen conditions. We additionally show that soil microbial gene expression profiles remained altered compared to starting profiles after one year of decomposition. Together, these results provide evidence of altered microbial metabolisms (e.g., taurine metabolism, nitrogen cycling, and lipid metabolism) and reveal the potential of vertebrate decomposition to have both ephemeral and lasting effects on ecosystem processing in response to mortality events.

INTRODUCTION

Soil microbial communities are important drivers of ecosystem processes in terrestrial environments. Many soil microbes are decomposers that are involved in degradation of complex organic matter and nutrient cycling in terrestrial ecosystems. Environmental disturbance can impact the presence and/or activity of soil microorganisms that are involved in these cycles, ultimately affecting nutrient availability and the release of greenhouse gas emissions, such as CO₂ and N₂O (2, 3). Vertebrate death, and subsequent carcass deposition in terrestrial ecosystems, is one disturbance event that impacts soil biogeochemical cycling and microbial community structure. Specifically, altered carbon and nitrogen transformations have been observed in response to release of concentrated decomposition products into the surrounding soil (4-10).

While carbon (C) and nitrogen (N) transformations have been documented during decomposition, the functional response of microbes and their roles in nutrient cycles remains unclear. Previous work from our lab (11, 12) and others (13-16) have conducted surveys of decomposition-impacted soil microbial communities through amplicon sequencing of marker genes (*i.e.*, 16S rRNA, ITS). This has allowed us to investigate how microbial biodiversity and composition change in response to vertebrate decomposition, revealing patterns such as increases in the anaerobic taxa *Firmicutes* and *Bacteroidetes*. However, few studies have investigated soil biogeochemistry and microbial communities within the same study. Taylor (2020) suggested that observed fungal community compositional shifts were linked to soil dissolved oxygen.

Additionally, Metcalf et al. (2016) started to connect chemical and microbial shifts, applying dynamic Bayesian networks to identify interactions between specific taxa and physicochemical data (*e.g.*, nitrate, pH) during controlled mouse decomposition experiments. While insightful for making potential connections between taxa and physiochemistry, these analyses to date have not been able to inform which taxa are active members of the community and responsible for chemical transformations, which functional pathways/genes are present, and how these pathways are altered in response to decomposition.

Methods such as RNA sequencing (*i.e.*, metatranscriptomics), and metabolomics can be used to investigate microbial community functional succession in response to decomposition by

measuring gene expression and metabolites, respectively. Where DNA methods are limited to explaining structural succession, analysis of microbial community functional succession is necessary to determine which functional pathways are altered in response to decomposition. This can help inform how ecological functioning, including C and N cycling, is impacted by decomposition events in terrestrial ecosystems. To date, only two studies have applied metatranscriptomic approaches to assess mRNA in vertebrate decomposition samples (17, 18): Burcham et al. (2019) examined gene expression of internal organ microbial communities during mouse decomposition, while Ashe et al. (2021) examined gene expression of oral microbial communities during human decomposition. Both studies suggest that the host microbial community functionality is altered during decomposition, including altered expression of amino acid and carbohydrate metabolism in the heart (17) and shifts in gene transcripts across different taxa (18). We expect that soil microbial community gene expression profiles are also altered; however, this has never been examined. The decomposition-impacted soil metabolome was assessed by DeBruyn et al. (2020), showing changes in soil metabolites over time, however it is unclear which microbes are responsible for these shifts. Additionally, DeBruyn et al. (2020) showed the soil metabolome was still altered compared to starting conditions at the end of the 21-week study, suggesting long-term impacts of decomposition on soil microbial functioning.

The purpose of this study was to investigate microbial gene expression during a one-year period of human decomposition and address the following questions: (1) how does gene expression differ between control and decomposition soils? (2) how does gene expression change over time in decomposition-impacted soils? (3) do microbial gene expression profiles return to pre-decomposition conditions after one year? We hypothesized that decomposition would lead to altered gene expression in soils, as decomposition alters soil physical and chemical conditions (8, 9, 19). We also hypothesized that gene expression would change over time as resources are used and transformed and soil chemical and physical conditions change as a result of tissue degradation. For example, we expected to observe changes in the expression of nitrogen cycling genes over time, as increased nitrogen transformations have been previously observed in decomposition soils (8). Additionally, we expected to observe increased expression of lipid metabolizing genes during active and advanced decomposition as microbes degrade lipids

deposited in the soil (20). Finally, multiple studies have shown that soil chemistry (5, 8) and microbial community composition (11, 13) (via 16S rRNA gene amplicon sequencing) are still impacted after one year, therefore we expected to observed altered soil functional profiles at the end of the study.

To answer these questions, metatranscriptomes of soil samples collected at six timepoints over one year of human decomposition were used to determine the active populations and expression of genes and pathways relevant to the enhanced biogeochemical cycling observed in decomposition hotspots. Further, we assessed differentially expressed genes between decomposition and control soils and analyzed genes whose expression changed over time, identifying functions or functional pathways of interest. Differentially expressed genes were used to generate hypotheses about ecosystem processes and provide insight on the functional response of microbes occurring during decomposition. This assessment of functional profiles within decomposition-impacted soils allows us to better understand microbial response to vertebrate decomposition in terrestrial settings and provides vital information about the biogeochemical activity within these hotspots.

METHODS

Study design

In February 2018, three deceased male human subjects (hereafter, “donors”) were placed supine on the soil surface at the University of Tennessee Anthropology Research Facility (ARF) and allowed to decompose. Located in Knoxville, TN (35° 56' 28" N, 83° 56' 25" W) the ARF is a roughly 2-acre outdoor facility dedicated to studying human decomposition (12). The soils at the ARF are comprised of the Loyston-Talbott-Rock outcrop (LtD) and Coghill-Corryton (CcD) complexes. LtD soils are a silty clay loam and channery clay overlaying lithic bedrock, while CcD soils are comprised of clay from weathered quartz limestone (1, 12). A site that had not been previously exposed to decomposition was used for this study.

The decomposition field experiment is fully described in Taylor (2020). Briefly, experiments were conducted in a block design, where each block consisted of one decomposition site and one control site (1). In total three blocks, *i.e.*, three donors paired with three respective control sites,

were included in the study. Each control site was chosen in a manner to ensure their location was uphill and roughly 2 m away from decomposition sites (1). Donor internal temperatures were recorded by probes located in the abdomen, while ambient air temperatures were monitored via sensors located roughly 50 cm above the soil surface. Soil temperature and salinity were measured with sensors placed directly underneath each individual (Decagon Devices, GS3) (1). Decomposition of all subjects was observed for one year. Donor ages ranged from 65 to 86 and were within 1 kg of each other with regard to weight (90.7 to 91.6 kg); donor BMI varied between 27.7 to 29.6 (1).

Sampling and physiochemistry

During the one-year study period, soils were sampled at 20 timepoints chosen to correspond with morphological stages of decomposition as described by (21). Once advanced decay was reached, soils were collected at intervals of 350 accumulated degree days (ADD), calculated using ambient air temperatures, up to one year. Soil samples were collected from all six sites at two depths: 0-1 cm (interface) and 1-16 cm (core), for a total of 12 samples at each timepoint. All soil cores were taken using a 1.9 cm (3/4 inch) diameter soil auger. Decomposition soils were taken from directly beneath the cadavers, taking care to not re-sample the same location more than once. At the time of sampling, soil dissolved oxygen was measured in triplicate using an Orion Star™ A329 pH/ISE/Conductivity/Dissolved Oxygen portable multiparameter meter (ThermoFisher) (1).

Soil samples were transported back to the lab at the University of Tennessee (Knoxville, TN) and processed within 24 hours of collection. Soils were homogenized by hand to remove insect larvae, roots, rocks, and other debris (> 2 mm). A subset of soils were used to measure pH, electrical conductivity (EC), and evolved CO₂ as described in Taylor (2020). Soil nitrogen species (NH₄⁺, NO₃⁻) and total carbon (TC) and nitrogen (TN)) were measured in all soil samples as described in (1). Roughly 10 g of soil was reserved for nucleic acid extraction, placed in a 4 oz. Whirl-Pak™ bag (Nasco), and flash frozen in liquid nitrogen. All samples were stored at -80 °C until further analysis. Bacterial and fungal community composition was assessed via amplicon sequencing of the 16S rRNA gene and ITS2 region as described in Taylor (2020).

RNA extraction and sequencing

A subset of 24 soil samples representing 6 study timepoints were chosen for metatranscriptomic analysis. Study days 0, 12, 58, 86, 168, and 378 were chosen as they represent distinct morphological and soil biogeochemical stages during decomposition. Study day 0 was chosen as a baseline sample prior to cadaver placement. Study day 12 was chosen because this was the start of active decomposition and corresponded to maximum soil ammonium concentrations and minimum soil oxygen (approximately 39%). Study day 58 was chosen as this sample represented the pH minimum, and respiration and soil temperature were at a maximum (1). Additionally, ammonium concentrations began to decrease around day 58. Study day 86 was chosen as the time period when soil oxygen had started to return and nitrate levels began to increase. Study day 168 was chosen as nitrate was at its maximum and soil dissolved oxygen had returned to 99%. Finally, day 376 was chosen to represent the end of the study, approximately 1 year since cadaver placement.

Each study day was represented by four soil samples: one pooled control sample and one sample from each donor. In the original study by Taylor (2020), soil cores were sectioned into 0-1 cm (interface) and 1-16 cm (core) samples. We recreated the full 16 cm core by combining interface and core soils from each respective donor/site 1:16 and 15:16, respectively, prior to RNA extraction. For control samples, each recombined soil sample (control 1, control 2, and control 3) was pooled prior to RNA extraction.

RNA was extracted from 2 g of recombined soil sample using Qiagen's RNeasy® PowerSoil® Total RNA kit (catalog no. 12866-25). Manufacturer's instructions were followed with a few modifications. Soils become saline during decomposition; therefore, we followed manufacturer's suggestion and incubated all extracts at -20 °C following addition of solution SR4 (step 9) to decrease salt precipitation. All RNA samples were resuspended in 40 µl of Solution SR7. RNA concentrations were assessed fluorometrically using Qubit® RNA HS assay (catalog no. Q32852) using 1 µl of RNA. DNA contamination was removed by DNase treating samples twice using Qiagen's DNase Max® kit in 50 µl reactions. RNA concentrations were remeasured after DNase treatment. PCR with general V4 16S rRNA gene primers (22, 23) was conducted using RNA extracts as the template to confirm removal of all DNA prior to sequencing. RNA aliquots

were shipped to HudsonAlpha Discovery (Huntsville, AL) for library preparation and RNA sequencing. Dual-indexed libraries were prepared using the Illumina® Stranded Total RNA prep with ribosomal RNA depletion via ligation with Ribo-Zero Plus. Libraries were then pooled and sequenced on Illumina's NovaSeq 6000 v4 platform, resulting in demultiplexed fastq files for each sample.

Bioinformatics

Read quality control (QC) was conducted in KBase (24) using Trimmomatic (25). Paired fastq files were imported to KBase through Globus. Poor quality reads were removed and adapters trimmed via Trimmomatic (v0.36) using default settings and the TruSeq3-PE-2 adapter file. After QC check with FastQC, trimmed libraries were exported as fastq files from KBase through Globus. Remaining ribosomal RNA was filtered using bbmap (maxindel = 20, minid = 0.93) from the Joint Genome Institute's (JGI) bbtools suite (26). After this step, all non-ribosomal reads from all 24 samples were merged into one file. This file was then used to co-assemble reads into contigs using the *de novo* assembler MEGAHIT (v1.2.9) (27) (--12 --k-min 23, --k-max 123, --k-step 10).

Gene identification and annotation from assembled contigs was performed using Prodigal (28) and eggNOG mapper (29), respectively. Briefly, the fastq containing all contigs was submitted to Prodigal (v2.6.3) for protein coding gene predication for a meta-sample (-p meta -f gff). Next, predicated genes were functionally and taxonomically annotated using eggNOG mapper (v2.1.6) using basic settings to perform a diamond blastp search (30). Only genes that were both functionally and taxonomically annotated by one of the databases used by eggNOG mapper and identified as bacterial, archaeal, or fungal were chosen as genes of interest. Transcript counts for all genes of interest were obtained by mapping reads from each respective sample to genes of interest obtained from co-assembly using QIAGEN CLC Genomics Workbench 20.0 (<https://digitalinsights.qiagen.com/>).

Differential expression

Transcript counts from all samples were combined in a single workable data file and imported into R for differential expression analysis using the R packages edgeR (31) and limma (32)

following a modified pipeline by Phipson et al. (2020) (33). Briefly, the transcript count table was imported into R and converted to a DGElist object. Genes without sufficient counts for statistical analysis were removed to increase power using the edgeR function *filterByExpr()*. Raw counts were then log2 normalized and gene expression profiles compared via multidimensional scaling (MDS) and hierarchical clustering. For differential expression analysis, raw filtered reads were normalized using edgeR's trimmed mean of M values (TMM) normalization using the function *calcNormFactors()*. TMM normalized reads were then log2 transformed using limma's *voom()* and differential expression assessed. Empirical Bayes shrinkage was used correct to p-values for false discovery rates. The topmost up and down regulated genes for each comparison, determined by log2 fold change and adjusted p-values, were then reported. Expression of certain genes were assessed after performing transcripts per million (TPM) normalization and statistical analyses with a combination of analysis of variance (ANOVA) and *post-hoc* Tukey tests. ANOVA across all timepoints were applied to hierarchical linear mixed effects models to account for repeated sampling within each donor block.

RESULTS

Sequencing

Illumina sequencing yielded a total of 5,073,476,730 reads, or 2,536,738,365 paired reads, with a mean of 105,697,432 paired reads per sample. Removal of adapters and low-quality reads removed 4.7 % of all reads, leaving 4,834,123,062 total reads. Filtering of ribosomal RNA further removed 7.3 % of reads, leaving 4,479,804,360 reads for assembly. After co-assembly, a total of 6,257,674 proteins were identified by Prodigal. From this, 1,048,573 proteins were annotated by eggNOG-mapper (16.7 %). A majority of the annotated proteins were taxonomically annotated as bacteria (91.3 %), followed by eukaryotes (7.6 %), and archaea (0.81 %). Of the 7.6 % of eukaryotic proteins, 64.4 % (4.9 % of all proteins) were annotated as fungi. For this work, genes of interest included all bacterial, archaeal, and fungal proteins, therefore all non-fungal eukaryotic proteins (32,004) were removed for downstream analysis. This reference file of genes was then used to determine gene transcript counts in all samples using CLC genomic workbench. The percent of reads mapped to genes of interest ranged from 21 to 38 %,

with an average of 31 % reads mapped. Gene counts were then combined in a single file and used for downstream analyses in R.

General characteristics in soil gene expression profiles

Soil gene expression profiles differed between decomposition timepoints. Multidimensional scaling (MDS) showed functional profile shifts over time, where gene expression in decomposition soils clustered by timepoint and all control and day zero samples clustered together (**Figure 4.1**). Gene expression profiles shifted away from controls as decomposition progressed. Expression was most different from controls on study days 58 and 86 (**Figure 4.2**), before shifting back toward control conditions on study days 168 and 376. After one year of decomposition, soil gene expression profiles had not returned to pre-decomposition conditions, as evidenced by their clustering away from controls in the MDS plot (**Figure 4.1**).

Soil gene expression and physiochemistry

As shown in the original study, soil chemistry was altered in response to human decomposition, with multiple parameters still impacted after one-year (1). Generally, soil pH decreased and remained low in decomposition soils of all but one individual. Soil EC increased in response to decomposition, remaining elevated through approximately day 58 before gradually decreasing throughout the remainder of the study (**Figure 4.3**). Respiration (evolved CO₂) increased, beginning at day 12, which corresponded to a reduction in soil dissolved oxygen (DO). Specifically, minimum soil DO was observed at day 12, while respiration remained elevated between days 12 and 86. Ammonium concentrations reached a maximum between days 12 and 58. This was followed by decreased ammonium and increased nitrate concentrations at day 86, with nitrate concentrations reaching a maximum at day 168. (**Figure 4.3**)

Some correlations were observed between gene expression shifts and soil physicochemical data at decomposition timepoints. Canonical correspondence analysis was used to correlate gene expression data and soil physicochemical data (**Figure 4.4**). Samples along CCA1 corresponded to soil C:N, while samples along CCA2 corresponded to changes in soil temperature, pH, EC, gravimetric water content, and evolved CO₂. CCA1 and CCA2 explained 36.2 and 20.9 percent of the variance in gene expression, respectively. Transcript profiles at day 12 were associated

with an increase in soil C:N. Gene expression profiles at days 58 to 86 were correlated with increased soil temperature, EC, and evolved CO₂, while study day 168 was associated with elevated levels of soil NO₃. Further, Permutational Analysis of Variance (PERMANOVA) revealed that internal ADH, soil temperature, pH, and EC significantly explained some of the variation in gene expression profiles ($p < 0.05$). No other variables were significant at $\alpha = 0.05$ (**Table 4.1**).

Differential expression in decomposition soils

Overall, decomposition changed soil gene expression profiles over the one-year study relative to control soils. Differential expression analysis identified 7,047 and 38,425 down and up-regulated genes in decomposition soils when compared to control soils. Gene transcripts that were associated with control soils were from a wide variety of clusters of orthologous genes (COG) functional categories. Specifically, the top 20 genes whose expression was higher in control soils belonged to ten unique COG categories, including signal transduction mechanisms, transcription, and those of unknown function. In contrast, the top 20 genes whose expression was higher in decomposition soils only fell into four COG categories (**Figure 4.5A**). The most commonly found COG category in decomposition soils was post-translational modification, protein turnover, and chaperones. Within this category, several heat shock stress response genes were identified, including SSA2, HSP82, and *clpB* (**Table 4.2**). Further investigation into these genes shows their expression increased in response to decomposition, typically reaching maximum expression around study days 58 and 86 (**Figure 4.6**). This corresponded to elevated soil temperature below decomposing bodies between study days 12-80, with soil temperatures up to approximately 43 °C (1), and maximum soil electrical conductivity measurements between days 12 and 58 (**Figure 4.3**).

Taxonomy associated with top differentially expressed gene transcripts also differed between control and decomposition soils. The top 30 significantly differentially expressed gene transcripts in decomposition soils were associated with Fungi, *Actinobacteria*, and *Xanthomonadales*, while gene transcripts in controls were associated with *Acidobacteria*, *Cyanobacteria*, *Proteobacteria* (α , δ , and γ), and *Planctomycetes* (**Figure 4.5B**). Comparison of control and decomposition soils at each study day showed differences: Specifically, gene

expression profiles in decomposition soils were most different from controls at day 86, as shown by relative distance between study days using MDS (**Figure 4.1**). Also, the greatest number of differentially expressed genes relative to control samples was observed at day 86, where we saw 145,460 and 124,883 up- and down-regulated genes, respectively (**Figure 4.7**).

Differential expression between decomposition timepoints

Differential expression analysis between respective sequential study days further revealed which genes were altered between decomposition timepoints. The top ten significantly up- and down-regulated genes, determined by lowest p-values from differential expression analysis, are reported in **Table 4.3**. In general, the topmost up and down regulated transcripts corresponded to a variety of COG functional categories (**Figure 4.8A**), with patterns specific to each timepoint described below. Additionally, a majority of the transcripts were taxonomically associated with bacteria, however transcripts associated with fungi and archaea were also identified (**Figure 4.8B**).

Expression of genes annotated as COG categories cell wall/membrane/envelope biogenesis, inorganic ion transport and metabolism, and carbohydrate transport and metabolism increased at day 12. In contrast, expression of secondary metabolite biosynthesis, transport, and catabolism genes decreased during this period (**Figure 4.8A**). Transcripts from *Bacilli* and *Clostridia* increased, while transcripts from *Actinobacteria* decreased between study days zero and 12 (**Figure 4.8B**). Four of the ten genes that decreased at day 12 were related to taurine metabolism, with their annotations associated with *tauD*, encoding taurine dioxygenase. (**Table 4.3**). Further investigation into *tauD* showed that mean expression of these genes decreased steadily over one year, beginning at day 12 (**Figure 4.9B**); however, *tauD* expression in response to human decomposition was variable across taxonomic associations. Most *tauD* transcripts were associated with *Gammaproteobacteria*, *Actinobacteria*, *Betaproteobacteria*, *Alphaproteobacteria*, and fungi. While a majority of the *tauD* gene queries displayed reduced expression over time, expression of fungal-associated and a few *Betaproteobacteria*-associated *tauD* genes increased at day 58 (**Figure 4.10**). Sources of taurine in the human body include taurine absorbed from the diet and taurine produced from anaerobic microbial deconjugation of bile salts via bile salt hydrolase (BSH) enzymes (34). Therefore, we also looked at expression of

genes encoding BSH enzymes in decomposition soils. Expression of these genes was elevated at days 12, 58, and 86 before converging toward pre-decomposition levels at days 168 and 376 (**Figure 4.9A**). Hierarchical linear mixed effects (HLM) models showed that both *tauD* (HLM $F = 13.77$, $p < 0.001$) and BSH ($F = 7.38$, $p = 0.002$) gene expression was significantly different over time (**Figure 4.9A,B**).

Between days 12 and 58, 90 % of the topmost upregulated genes were associated with the COG translation, ribosomal structure and biogenesis and all were taxonomically associated with *Betaproteobacteria* (**Figure 4.8A,B**). Many of these genes are associated with the *rpl* protein family, involved in ribosomal binding. Genes across multiple COG categories and annotated as *Bacilli* and *Clostridia* decreased between study days 12 and 58, six of which were transcripts that increased between days zero and 12 (**Figure 4.8B, Table 4.3**).

Multiple transcripts associated with the energy production and conversion, as well as transcripts annotated as inorganic transport and metabolism, and translation, ribosomal structure and biogenesis, increased between days 58 and 86 (**Figure 4.8A**). Two of the upregulated energy and production and conservation transcripts were associated with cytochrome c oxidase subunits in *Betaproteobacteria*; another was annotated as *hao*, encoding the enzyme hydroxylamine dehydrogenase which is involved in conversion of hydroxylamine to nitrite during nitrification (**Table 4.3**). Further investigation into hydroxylamine dehydrogenase showed a significant increase in *hao* transcripts at day 86 followed by subsequent decreases at days 168 and 376 ($F = 4.183$; $p = 0.0196$) (**Figure 4.11**). This increase corresponded to decreased soil ammonium levels and subsequent accumulation of nitrate (**Figure 4.3**). Half of the topmost downregulated genes between days 58 and 86 were not assigned to a COG (*i.e.*, unclassified) or were of unknown function. During this period *Bacteroidetes* transcripts decreased, however three genes associated with this taxon were also among the topmost upregulated genes.

Differential expression comparing study days 86 and 168 and 168 and 376 identified genes across a variety of functional categories, with many unclassified in the COG database or with unknown function (**Figure 4.8A**). Expression of carbohydrate transport and metabolism genes associated with *Bacilli* decreased between day 168 and 376. Additionally, *Acidobacteria*

transcripts increased in decomposition-impacted soils between study day 168 and 376 (**Figure 4.8B**). These transcripts were not associated with any single COG category, however.

Nitrogen cycling genes

Expression of nitrogen cycling genes was impacted in response to human decomposition. Due to the detection of *hao* in our differential expression analysis, and our hypotheses predicting changes to nitrogen transformation processes, the expression of genes encoding common enzymes involved in nitrogen cycling (*nifH*, *nirB*, *nirK*, *norB*, *nosZ*, *nrfA*, *nxrA*, and *amoA*) were assessed using their enzyme commission numbers (**Figure 4.12A,B**). *nifH*, encoding a subunit of nitrogenase which is involved in nitrogen fixation, displayed little to no changes in gene expression between control and decomposition soils. Transcripts for two genes encoding enzymes contributing to the last two steps of denitrification, *norB* (encodes nitric oxide reductase) and *nosZ* (encodes nitrous oxide reductase), increased between study days 12 and 86, and decreased at study day 168 before increasing again at day 376. In contrast, expression of genes encoding nitrate reductase, *narG*, and NO-forming nitrite reductase, *nirK*, remained low until day 376 when transcripts for both genes increased. As noted above, expression of *hao*, encoding hydroxylamine dehydrogenase, increased at study day 86 before decreasing at remaining timepoints (**Figure 4.8A**, **Figure 4.12B**). Expression of *amoA*, encoding a subunit of ammonia monooxygenase, and *nxrA*, encoding a subunit of nitrite oxidoreductase, which are involved in nitrification, changed in response to decomposition. *amoA* transcripts initially decreased at day 12, remaining reduced until study day 376 when transcripts increased. Similarly, abundance of genes that encode for enzymes involved in dissimilatory nitrate reduction, *nirB*, and *nrfA*, was low for the first 168 days, and then *nrfA* expression increased at day 376 (**Figure 4.12B**).

Lipase gene transcripts

We also investigated changes in triacylglycerol lipase (enzyme commission number: 3.1.1.3) gene transcription in decomposition-impacted soils, as we hypothesized that lipid degrading enzymes would be impacted during decomposition. Generally, lipase transcripts decreased as decomposition progressed (HLM $F = 6.564$, $p < 0.001$), however we also observed a significant interaction between study day and taxonomic annotation ($F = 8.786$; $p < 0.001$). Specifically,

lipase gene transcripts annotated as bacterial decreased with decomposition time ($F = 10.39$; $p = 0.001$), while fungal lipase transcripts increased, reaching a maximum at study day 58 ($F = 4.509$; $p = 0.015$) (**Figure 4.13**).

DISCUSSION

The goal of this study was to assess soil microbial gene expression in response to human decomposition. Metatranscriptomics were applied to soil samples collected over one-year from below three decomposing human bodies. This was the first study (to our knowledge) to investigate gene expression profiles in vertebrate decomposition-impacted soils. From this, we showed that microbial gene expression shifted over time, with samples reproducible between individuals. Additionally, we showed that gene expression profiles had not recovered to pre-decomposition conditions after one year. Comparison of control and decomposition expression profiles showed that heat-shock proteins were elevated in response to decomposition. We also described expression patterns between decomposition timepoints, noting changes in functional gene categories at certain timepoints. Further, expression of common nitrogen cycle marker genes suggest nitrogen transformation pathways are altered over time.

Gene expression profiles may be less sensitive to inter-individual variability compared to taxonomic profiles derived from rRNA gene amplicon sequencing. Specifically, gene expression profiles clustered by timepoint with relatively good reproducibility between individuals. For example, we observed bacterial community composition based on 16S amplicons changed with time but did not become more similar in Chapter 2. However, Chapter 2 was focused on the early stages of decomposition, while sampling in this study was spread over one year. To see if gene expression displays similar or less sensitivity to inter-individual variation observed by compositional studies, a high-resolution investigation of gene expression during the early stages of decomposition is needed.

Gene expression is impacted after one year of decomposition

We noted that gene expression profiles were still altered compared to the starting communities and controls after one year of study. It is unclear if the microbial communities, in terms of gene expression profiles, have reached a new steady state as a result of decomposition, or if they

would eventually return to pre-decomposition conditions. Barton et al. (2020) also showed long lasting effects in soil chemistry in response to human and pig decomposition. Specifically, they reported that total phosphorus and nitrogen, ammonium, nitrate, and electrical conductivity were still elevated above background levels even after 500 days of human decomposition. These results suggest that microbial community structure and function may be affected well past one year and potentially longer than one and a half years. This has implications for terrestrial ecosystem processing, as decomposition alters functionality soil microbial communities and therefore nutrient cycling and release of greenhouse gasses. Further work with extended sample collections beyond one year are needed to address how long microbial gene expression is impacted.

Bacteria and fungi both contribute to decomposition processes

Bacteria, fungi, and archaea were all active during decomposition, suggesting that members of all three domains have the potential to contribute to decomposition processes and nutrient cycling. While a majority of annotated transcripts were identified as bacteria, fungal transcripts were the second most abundant group. Within differential analyses, fungal transcripts made up 46% of the top 15 significantly differentially expressed genes associated with decomposition-impacted soils. Additionally, with respect to expression shifts between decomposition timepoints, fungal transcripts were among the topmost upregulated genes at study day 86. The presence of fungal transcripts is not surprising as fungi are key decomposers, involved in the degradation of organic matter in terrestrial ecosystems (36). It was interesting to see an increase in certain fungal transcripts, such as lipase, at study days 58 and 86 when soil oxygen began to recover, suggesting that fungal activity in decomposition soils may be constrained by environmental factors such altered oxygen levels. Prior work with these soils showed fungal community compositional shifts related to oxygen (1), indicating that fungal gene expression may correlate to changes in microbial community dynamics observed in amplicon DNA sequencing.

Heat-shock/stress response

Soil microbial communities expressed stress response genes in response to human decomposition. Differential expression analysis identified increased expression of multiple heat

shock proteins associated with the taxa *Xanthomonadales*, *Actinobacteria* and fungi. Upon further investigation, expression of these genes increased through day 58 and remained high for the remainder of the year. Soil temperature was elevated up to 10°C relative to controls between study days 12 and 100. Soil electrical conductivity, which is correlated with ionic strength (37) and can indicate soil salinity, is also elevated in decomposition soils (1, 8-10). In this study, soil EC increased up to 663 $\mu\text{S}/\text{cm}$ (16 times higher than background) through day 58 before slowly decreasing through the remainder of the study. As a result, we would expect these microbes to be experiencing both heat and osmotic stress during this period. Prior work has observed increased heat shock gene expression during salt stress in paddy soils (38) and the presence of both heat and osmotic stress genes in desert soils along a salt gradient (39). In our study we showed that soil microbial communities elicit a stress response during human decomposition, however, at this time, it is unclear if expression of these genes is in response to heat stress alone, or in combination with osmotic stress.

Taurine metabolism

Taurine, an amino acid involved in bile acid formation (34), is present in the human body, with taurine being absorbed from the diet or synthesized in the liver (40). However, taurine is also produced as a byproduct of deconjugation of bile salts via bile salt hydrolases (BSH) present in the anaerobic gut taxa *Lactobacillus* and *Clostridium* (34). In our study, we observed increased expression of genes encoding BSH enzymes between days 12 and 86. Given that increased expression of BSH genes followed release of decomposition products and occurred during the period of reduced dissolved oxygen in our study, it is likely that taurine accumulation is the result of BSH enzyme activity by anaerobic microorganisms. While we did not measure taurine in this study, our results correspond to previous decomposition studies that report accumulation of taurine in various organs and body regions (41-43) and decomposition soils (19, 44) via metabolomics and increased relative abundance of *Clostridium* and *Lactobacillus* within the body (45-47) and in decomposition soils (11)(Chapter 3)(Taylor et al., *unpublished*) via DNA sequencing methods.

Trends in BSH expression in our study are similar to changes in taurine observed by DeBruyn et al. (2021) during pig and human decomposition. In their study, taurine was elevated at week 8

and reduced at week 14. Eight weeks roughly corresponds to day 58 of our study, where BSH expression was elevated. This is also consistent with Hoeland (2021), which observed taurine accumulation in soils during the early stages of human decomposition. We did not capture a sample corresponding to week 14, when DeBruyn et al. (2021) observed reduced taurine relative to controls; however, we did observe a decrease in BSH between days 86 and 168, suggesting that taurine may be reduced at some point during this period due to microbial taurine metabolism.

One pathway of taurine metabolism is through desulfurization via the α -ketoglutarate-dependent enzyme taurine dioxygenase (TauD). Specifically, this enzyme, encoded by the gene *tauD*, converts 2-oxoglutarate and taurine to produce aminoacetaldehyde, succinate, sulfite, and CO₂ (48). Succinate and sulfite from this reaction can then be used for the citric acid cycle and sulfur metabolism, respectively. Given increased BSH expression in our study and reported taurine accumulation in others, we would expect taurine to be present for microbial metabolism by TauD. However, we observed a general decrease in *tauD* expression between days 12 through 376. This trend was driven by reduced expression of *tauD* genes associated with *Proteobacteria*, *Gammaproteobacteria*, and *Actinobacteria*; although, expression of *tauD* genes associated with fungi and a few *Betaproteobacteria* displayed increased expression at day 58. This corresponded to increased expression of bile salt hydrolases (BSH) between days 12 and 86.

Trends in *tauD* expression may be driven by sulfur dynamics in decomposition soils. While we did observe increased expression of some *tauD* genes at day 58, a majority of *tauD* gene expression was reduced in decomposition soils. These genes were associated with taxa (*e.g.*, *Proteobacteria*, *Gammaproteobacteria*, and *Actinobacteria*) whose relative abundance have been shown to remain consistent or increase during human decomposition (11), suggesting that *tauD* expression is regulated under decomposition conditions. It is known that taurine desulfurization by TauD is used by microbes for sulfur acquisition during periods of sulfur limitation (49). We did not measure sulfur species in this experiment, although a few human decomposition studies have observed elevated sulfate (50) and elemental sulfur (51) in decomposition-impacted soils. Therefore, an explanation may be that *tauD* expression is reduced in response to deposition of sulfur during decomposition. Additionally, while we did not measure taurine levels in this study,

taurine may be processed through other pathways. For example, taurine can be deaminated by taurine dehydrogenase to produce sulfite and acetyl-CoA for carbon metabolism (48, 52). Therefore, future investigation into other taurine metabolism pathways will help elucidate taurine patterns in decomposition-impacted soils.

Overall, these results suggest that human decomposition has potential impacts on sulfur metabolism through deposition of inorganic (sulfate) and organic (sulfur-containing amino acids) sulfur compounds. However, changes in these metabolites have yet to be linked to sulfur transformations and/or sulfur-cycling microbes during human decomposition. Thus, further investigation with a target study combining soil physiochemistry, including measurements of sulfur species, and quantification of sulfur metabolism is needed to elucidate these patterns.

Nitrogen cycling

The human body is a concentrated source of nitrogen that is released into the surrounding soil during decomposition, therefore we also evaluated expression of genes involved in nitrogen cycling. Expression of common nitrogen cycling marker genes was altered in decomposition soil and suggested nitrogen transformations during human decomposition were driven by soil oxygen concentrations with hydroxylamine an important intermediate. In our study, soil dissolved oxygen levels dropped below 40% at day 12 and did not fully reoxygenate until day 168. During this period of depleted oxygen levels, ammonium concentrations reached a maximum at day 12 and slowly recovered over remaining timepoints. In contrast, soil nitrate concentrations remained low during the early stages of decomposition, and then increased by day 168 when soil oxygen levels recovered. These nitrogen dynamics are consistent with observations by Keenan et al. (2018), who proposed three phases of biogeochemical cycling corresponding to oxygen availability. Specifically, the first phase precedes release of decomposition products when nitrogen species are in equilibrium. The second phase follows the release of decomposition products, which stimulates microbial activity thereby diminishing oxygen levels. This phase is also characterized by elevated ammonium concentrations. The third phase occurs following recovery of soil oxygen, where nitrification and denitrification are stimulated (8). There are some parallels between Keenans' phases of biogeochemical cycling and expression of nitrogen cycling genes in our study. For example, we observed low or reduced expression of nitrification genes

nxrA and *amoA* between days 12 and 86, during reduced oxygen conditions. This would be consistent with accumulation of ammonium while nitrate remained low, as described in Keenan et al. (2018)'s second biogeochemical phase.

We observed increased expression of *hao*, which encodes the enzyme hydroxylamine dehydrogenase (HAO) at day 86 while oxygen was reduced. This corresponded to simultaneous increases in expression of genes encoding nitric oxide reductase (*norB*) and nitrous oxide reductase (*nosZ*). Traditionally HAO has been thought to process hydroxylamine to nitrite during nitrification, while NorB and NosZ are enzymes involved in the last two steps of denitrification converting nitric oxide (NO) to dinitrogen gas (N₂). However, recent work has suggested hydroxylamine can be converted to nitric oxide (NO), as well as can interact with multiple phases of the nitrogen cycle (53). Even though *amoA* expression was shown to decrease during reduced oxygen conditions, *amoA* transcripts were still present and likely able to convert ammonium to hydroxylamine as soil oxygen was not completely depleted during decomposition. Additionally, a previous study showed that the growth of the ammonia oxidizing bacteria *Nitrosomonas europaea* under anoxic conditions lead to accumulation of hydroxylamine in a chemostat bioreactor (54), suggesting anaerobic ammonium oxidation (anammox) may also be occurring in decomposition soils. However, we did not observe increases in *nirK* expression, which might suggest conversion of nitrite to NO for use in the anammox pathway. NO produced via HAO activity may be used for anammox in these soils; however, the role of hydroxylamine as an intermediate in anammox is still debated (53). Therefore, our current hypothesis is that hydroxylamine accumulates under anaerobic conditions during decomposition, which can then be converted to NO by HAO. This NO would then be present for anaerobic denitrifying bacteria to convert to nitrous oxide (N₂O) by NorB and finally to N₂ by NosZ. This may also explain the brief increase in N₂O observed by Keenan et al. (2018) during biogeochemical phase 2.

As soils fully reoxygenated, we observed increased expression of genes encoding enzymes involved in aerobic nitrification, *amoA* and *nxrR*. Activity of these genes could then continue processing ammonium to nitrate following reoxygenation, supplying nitrate for traditional denitrification pathways. We observed increased expression of marker genes encoding all four enzymes in the complete denitrification pathway (*narG*, *nirK*, *norB*, and *nosZ*) at day 376.

Increased expression of nitrification and denitrification marker genes is consistent with accumulation of nitrite, nitrate, and N₂O described by Keenan et al. (2018)'s third biogeochemical phase. Our results provide further insight into nitrogen transformations during vertebrate decomposition, highlighting an important role of hydroxylamine, and future work could include monitoring of nitrogen intermediates hydroxylamine and hydrazine (anammox) concentrations over time.

Lipase genes

Humans are also composed of fats, containing lipids that are broken down during decomposition; Therefore, we also assessed expression of triacylglycerol lipase genes in decomposition soils. Our results show that expression of triacylglycerol lipase genes was altered in response to decomposition, and these shifts differed between bacteria and fungi. Bacterial triacylglycerol lipase expression decreased in response to decomposition. This is in contrast to previous work by Howard et al. (2010), who observed increased gene copy number of Group 1 lipase genes via qPCR during swine decomposition. This may be due to differences in human compared to swine decomposition. Studies have noted differences in pH response between humans and pig decomposition, whereby pigs exhibit increased pH and humans generally exhibit decreased pH (19) and differences in fatty acid composition in human compared to pig tissue (55). Therefore, lipase expression patterns may differ between species due to compositional differences and/or pH response. For example, in this study pH decreased; With many triacylglycerol lipases having a pH optimum that is neutral to basic (56-58), this may explain why we observed decreased lipase gene expression in our study. These results may also reflect a difference between functional potential and gene expression in decomposition-impacted soils, especially as it relates to lipase genes.

In contrast to bacterial lipases, fungal triacylglycerol lipase expression increased as decomposition progressed. These results suggest that fungi are important triacylglycerol degraders in vertebrate decomposition-impacted soils. Additionally, expression of these genes was correlated to the relative abundance of the fungal classes *Saccharomycetes*, *Sordariomycetes*, and *Eurotiomycetes* (59). These fungi have been previously associated with decomposition soils (14, 16) and are known to contain triacylglycerol lipase genes in their

genomes (60, 61), suggesting that these organisms are important for lipid degradation in decomposition soils. Further work would be needed to confirm the taxonomy of fungal lipases in decomposition soils. It is important to note that while we focused on triacylglycerol lipase (EC 3.1.1.3), there are other phospholipases involved in metabolism and degradation of other lipid compounds that may display different expression over time and between bacterial and fungal populations.

CONCLUSIONS

This study represents the first investigation of soil microbial gene expression during human decomposition, where we collected decomposition soils from three human individuals over one year. Metatranscriptomic analysis of these samples show that decomposition impacted microbial community gene expression profiles, exhibiting functional shifts over time. This included altered expression of nitrogen cycling and lipase genes as microbes process the nutrient-rich tissues of the human body. Additionally, we noted that functionality within decomposition-impacted soils was still affected after one year and had not returned to starting conditions. Together, these results show that vertebrate decomposition has lasting impacts within the soil environment. These results have important implications for understanding the larger ecosystem impacts due to vertebrate mortality events in terrestrial ecosystems and the roles soil microbial communities play during these events.

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APPENDIX

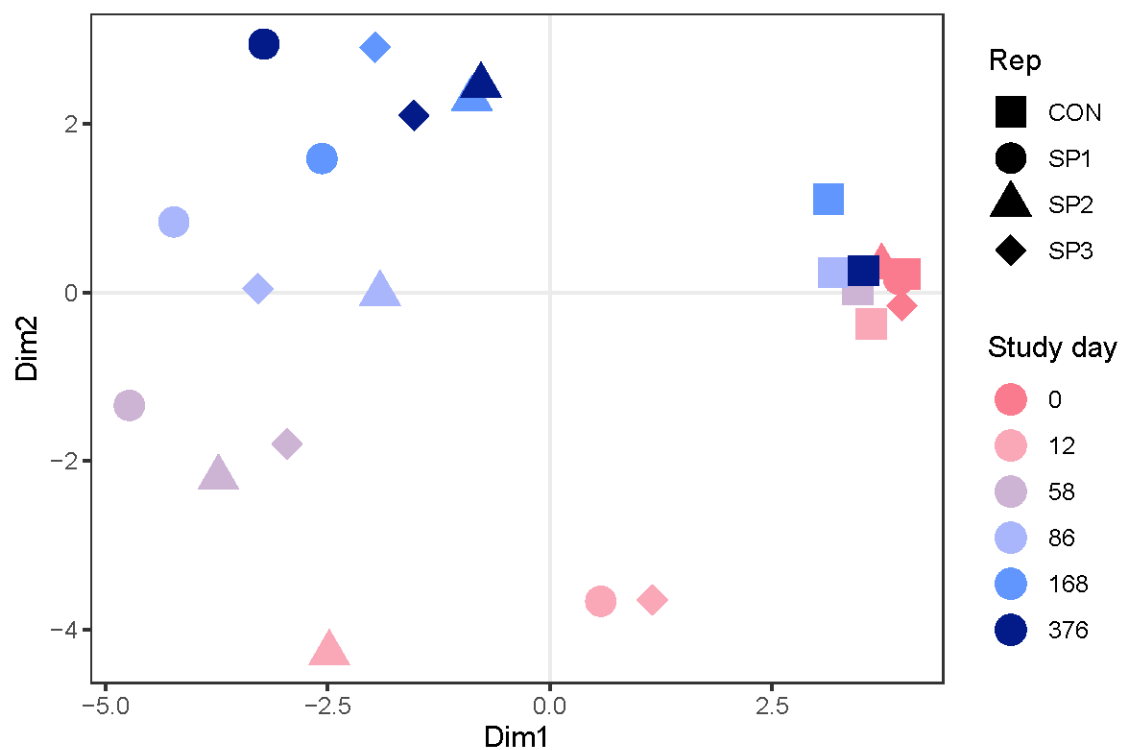


Figure 4.1 Multidimensional scaling (MDS) shows shifts in microbial gene expression profiles in response to human decomposition. Soils from controls (CON) and each individual (SP1, SP2, SP3), are denoted by symbol shape. Progression of study day is represented by a gradient in symbol color.

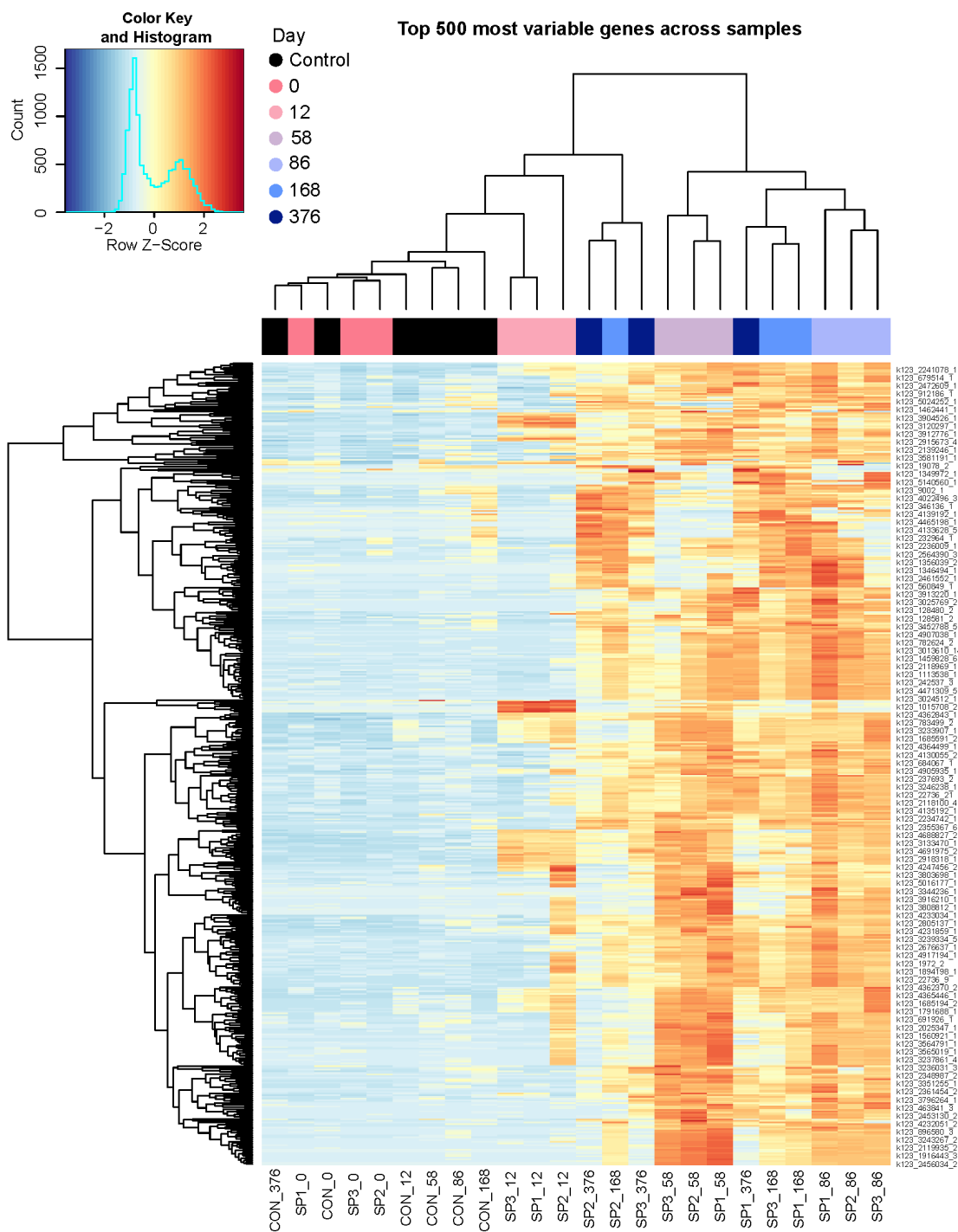


Figure 4.2 Hierarchical clustering heatmap showing the log counts per million (CPM) of the top 500 most variable genes across samples. Variable genes were determined by selecting genes with the highest variance in gene expression as calculated in R. Samples are clustered along the x-axis using Euclidean distances between samples calculated from the log counts per million (CPM) and colored by study day.

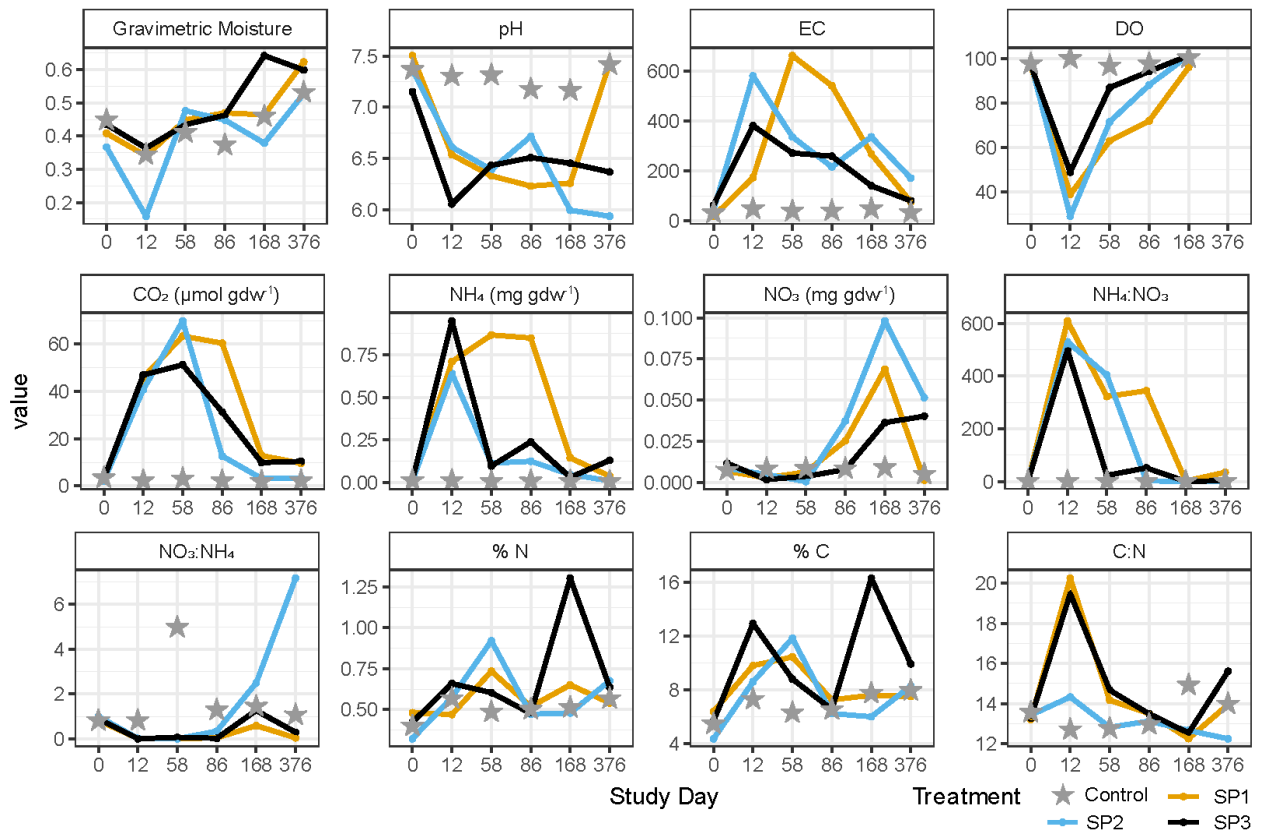


Figure 4.3 Soil physicochemical parameters in decomposition soils during the one-year study. Data is shown for each individual donor: SP1 (gold), SP2 (blue), and SP3 (black). Values for the full 16cm core samples were estimated by summing values interface (0-1 cm) and core (0-16 cm) reported by Taylor et al, (2020) in 1:16 and 15:16 ratios, respectively. Controls reported here are averages of estimates from all three experimental controls and are represented by stars.

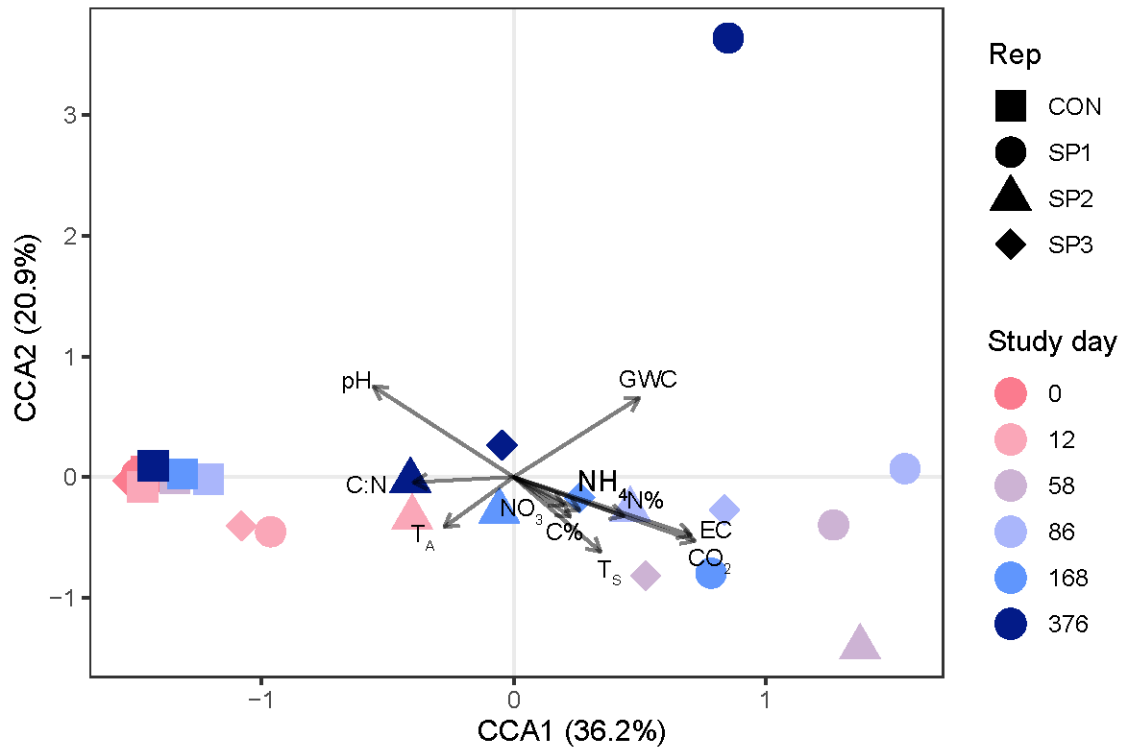


Figure 4.4 Canonical correspondence analysis (CCA) shows the relationship between gene expression profiles at each study day to soil physicochemical data from Taylor et al. (2020). Soils from controls (CON) and the three donors (SP1, SP2, SP3) are denoted by symbol shape. Soil physicochemical variable loadings are represented by arrows: Gravimetric water content (GWC), electrical conductivity (EC), pH (pH), evolved CO₂ (CO₂, $\mu\text{mol gdw}^{-1}$), ammonium (NH₄) and nitrate (NO₃) concentrations (mg gdw⁻¹), percent carbon (%C), percent nitrogen (%N), and carbon:nitrogen ratio (C:N), ambient temperature (T_A), and soil temperature (T_S). Environmental variables explained 64.3% of the variation in gene expression profiles across all ordinates.

Table 4.1 Permutational analysis of variance (PERMANOVA) results identifying significant environmental parameters which explain some of the variation in soil gene expression profiles. Environmental parameter data is from Taylor et al. (2020).

	Df	SumOfSqs	MeanSqs	F.Model	R2	Pr(>F)
ADH (ambient)	1	0.2873	0.28725	2.8986	0.08167	0.053
ADH (internal)	1	0.5359	0.53595	5.4082	0.15239	0.001
ADH (soil)	1	0.1540	0.15400	1.5539	0.04379	0.170
Temperature (Ambient; °C)	1	0.1849	0.18487	1.8655	0.05256	0.138
Temperature (Soil; °C)	1	0.2579	0.25791	2.6025	0.07333	0.045
Gravimetric moisture	1	0.1168	0.11682	1.1788	0.03322	0.302
pH	1	0.2581	0.25814	2.6048	0.07340	0.042
EC	1	0.2945	0.29450	2.9718	0.08374	0.037
CO ₂ (μmol gdw ⁻¹)	1	0.1105	0.11049	1.1150	0.03142	0.333
NH ₄ (mg gdw ⁻¹)	1	0.1296	0.12957	1.3074	0.03684	0.217
NO ₃ (mg gdw ⁻¹)	1	0.0768	0.07678	0.7747	0.02183	0.550
N%	1	0.0793	0.07935	0.8007	0.02256	0.509
C%	1	0.0785	0.07846	0.7917	0.02231	0.520
C:N	1	0.0611	0.06107	0.6162	0.01736	0.699
Residuals	9	0.8919	0.09910		0.25359	
Total	23	3.5171			1.0000	

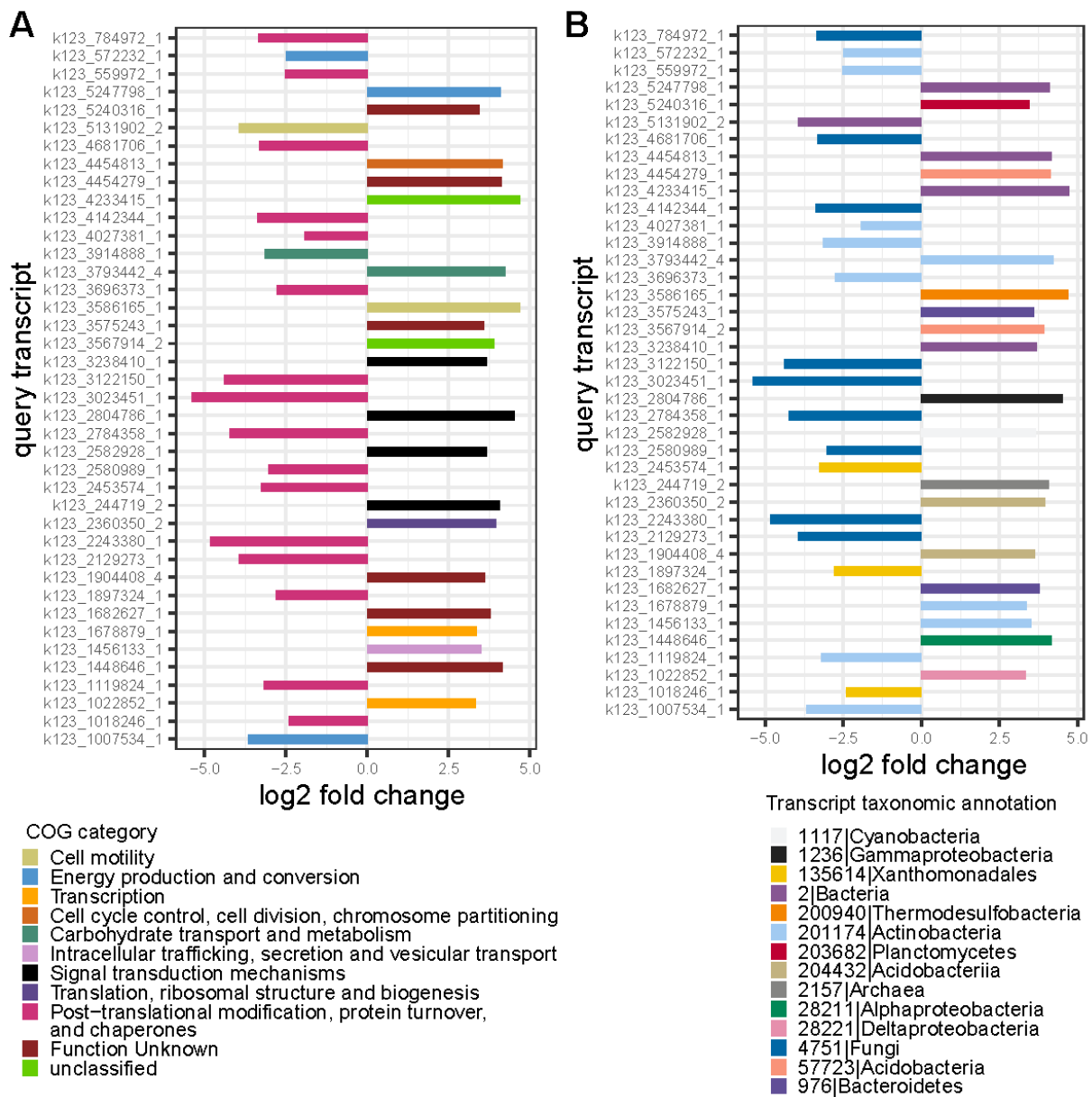


Figure 4.5 Top 30 up- and down-regulated genes in controls relative to decomposition soils across all study days, colored by COG functional category (A) and taxonomic annotation (B). Positive values denote higher expression in controls, while negative values are higher in decomposition soils.

Table 4.2 Top 15 most up- and down-regulated gene queries, determined by log2 fold change and adjusted p-values, in decomposition, compared to control soils. Positive log2 fold change values represent genes whose expression was higher in control soils, while negative log2 fold change values are higher in decomposition soils. Taxonomic annotation, COG categories, gene description, gene names, and EC were assigned via eggNOG-mapper.

Query	log2 fold change	p-value	Taxonomic annotation	COG category	COG annotation	Gene Description	Gene name	EC
k123_3586165_1	4.6990	9.73 E-10	Thermodesulfobacteria	NU	Cell motility	Type II secretion system (T2SS), protein E, N-terminal domain	-	-
k123_5240316_1	3.4439	2.41 E-08	Planctomycetes	S	Function Unknown	TIGRFAM type VI secretion protein, VC_A0111 family	-	-
k123_4233415_1	4.7085	2.67 E-08	Bacteria	-	-	-	-	-
k123_5247798_1	4.1013	2.81 E-08	Bacteria	CO	Energy production and conversion	amine dehydrogenase activity	-	-
k123_1022852_1	3.3399	3.38 E-08	Deltaproteobacteria	K	Transcription	ROS/MUCR transcriptional regulator protein	-	-
k123_4454813_1	4.1586	3.58 E-08	Bacteria	D	Cell cycle control, cell division, chromosome partitioning	protein conserved in cyanobacteria	-	-
k123_3793442_4	4.2293	6.16 E-08	Actinobacteria	G	Carbohydrate transport and metabolism	pfkB family carbohydrate kinase	kdgK	2.7.1.45
k123_3567914_2	3.9102	7.15 E-08	Acidobacteria	-	-	-	-	-
k123_3575243_1	3.5862	7.5E-08	Bacteroidetes	S	Function Unknown	Protein of unknown function (DUF1501)	-	-
k123_1456133_1	3.4962	7.77 E-08	Actinobacteria	U	Intracellular trafficking, secretion and vesicular transport	TadE-like protein	-	-

Table 4.2 continued

Query	log2 fold change	p-value	Taxonomic annotation	COG category	COG annotation	Gene Description	Gene name	EC
k123_1678879_1	3.3644	8.25 E-08	Actinobacteria	K	Transcription	DNA binding	-	-
k123_1448646_1	4.1544	8.46 E-08	Alphaproteobacteria	S	Function Unknown	Type VI secretion system effector, Hcp	-	-
k123_2582928_1	3.6654	1.02 E-07	Cyanobacteria	T	Signal transduction mechanisms	Signal Transduction Histidine Kinase	-	-
k123_4454279_1	4.1149	1.07 E-07	Acidobacteria	S	Function Unknown	Type VI secretion system effector, Hcp	-	-
k123_2804786_1	4.5185	1.11 E-07	Gammaproteobacteria	T	Signal transduction mechanisms	Domain present in phytochromes and cGMP-specific phosphodiesterases.	-	-
k123_4681706_1	-3.3329	1.54 E-05	Fungi	O	Post-translational modification, protein turnover, and chaperones	Belongs to the heat shock protein 70 family	SSA2	-
k123_2129273_1	-3.9390	1.82 E-05	Fungi	O	Post-translational modification, protein turnover, and chaperones	Belongs to the heat shock protein 70 family	SSA2	-
k123_2784358_1	-4.2260	2.08 E-05	Fungi	O	Post-translational modification, protein turnover, and chaperones	Heat shock protein	HSP82	-
k123_2453574_1	-3.2513	2.36 E-05	Xanthomonadales	O	Post-translational modification, protein turnover, and chaperones	Part of a stress-induced multi-chaperone system, it is involved in the recovery of the cell from heat-induced damage, in cooperation with DnaK, DnaJ and GrpE	clpB	-

Table 4.2 continued

Query	log2 fold change	p-value	Taxonomic annotation	COG category	COG annotation	Gene Description	Gene name	EC
k123_2580989_1	-3.0343	2.43 E-05	Fungi	O	Post-translational modification, protein turnover, and chaperones	MreB/Mbl protein	SSA2	-
k123_784972_1	-3.3612	2.98 E-05	Fungi	O	Post-translational modification, protein turnover, and chaperones	Belongs to the heat shock protein 70 family	SSA2	-
k123_5131902_2	-3.9446	3.14 E-05	Bacteria	N	Cell motility	domain, Protein	-	3.2.1.81, 3.2.1.97
k123_1897324_1	-2.8043	3.53 E-05	Xanthomonadales	O	Post-translational modification, protein turnover, and chaperones	Part of a stress-induced multi-chaperone system, it is involved in the recovery of the cell from heat-induced damage, in cooperation with DnaK, DnaJ and GrpE	clpB	-
k123_1007534_1	-3.6688	4.03 E-05	Actinobacteria	C	Energy production and conversion	Ferredoxin	fdxA	-
k123_1119824_1	-3.1908	4.12 E-05	Actinobacteria	O	Post-translational modification, protein turnover, and chaperones	Heat shock 70 kDa protein	dnaK	-
k123_3696373_1	-2.7683	4.18 E-05	Actinobacteria	O	Post-translational modification, protein turnover, and chaperones	Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions	groL2	-

Table 4.2 continued

Query	log2 fold change	p-value	Taxonomic annotation	COG category	COG annotation	Gene Description	Gene name	EC
k123_3023451_1	-5.3879	5.67 E-05	Fungi	O	Post-translational modification, protein turnover, and chaperones	Hsp90 protein	HSP82	-
k123_1018246_1	-2.4002	6.58 E-05	Xanthomonadales	O	Post-translational modification, protein turnover, and chaperones	Part of a stress-induced multi-chaperone system, it is involved in the recovery of the cell from heat-induced damage, in cooperation with DnaK, DnaJ and GrpE	clpB	-
k123_4142344_1	-3.3757	6.975 E-05	Fungi	O	Post-translational modification, protein turnover, and chaperones	Hsp90 protein	HSP82	-
k123_3914888_1	-3.1463	7.28 E-05	Actinobacteria	G	Carbohydrate transport and metabolism	Phosphoketolase	-	4.1.2.2, 4.1.2.9

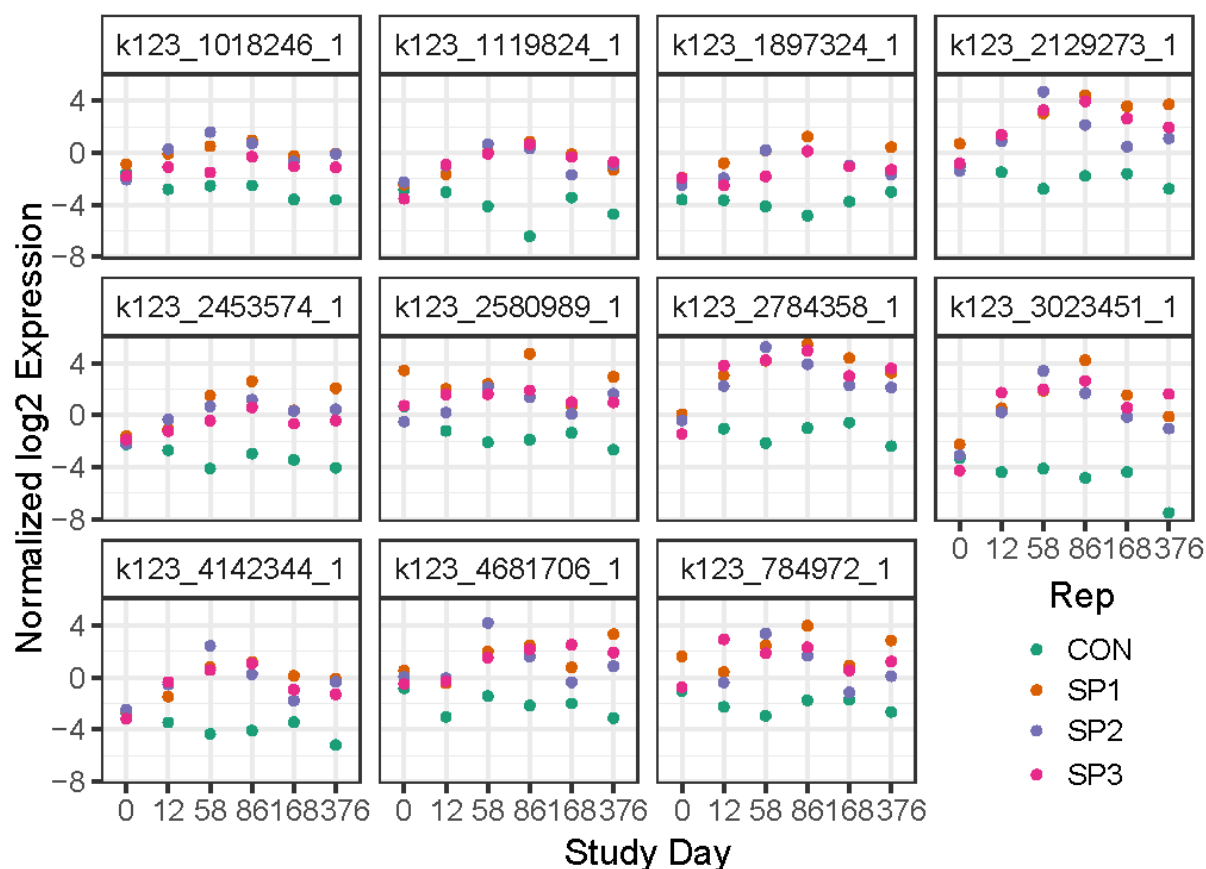


Figure 4.6 Normalized log₂ expression of all heat shock proteins identified by differential expression analysis comparing decomposition and control soils. Symbol color denotes if the sample is a control (CON, green), or one of three individuals: SP1 (orange), SP2 (purple), or SP3 (pink).

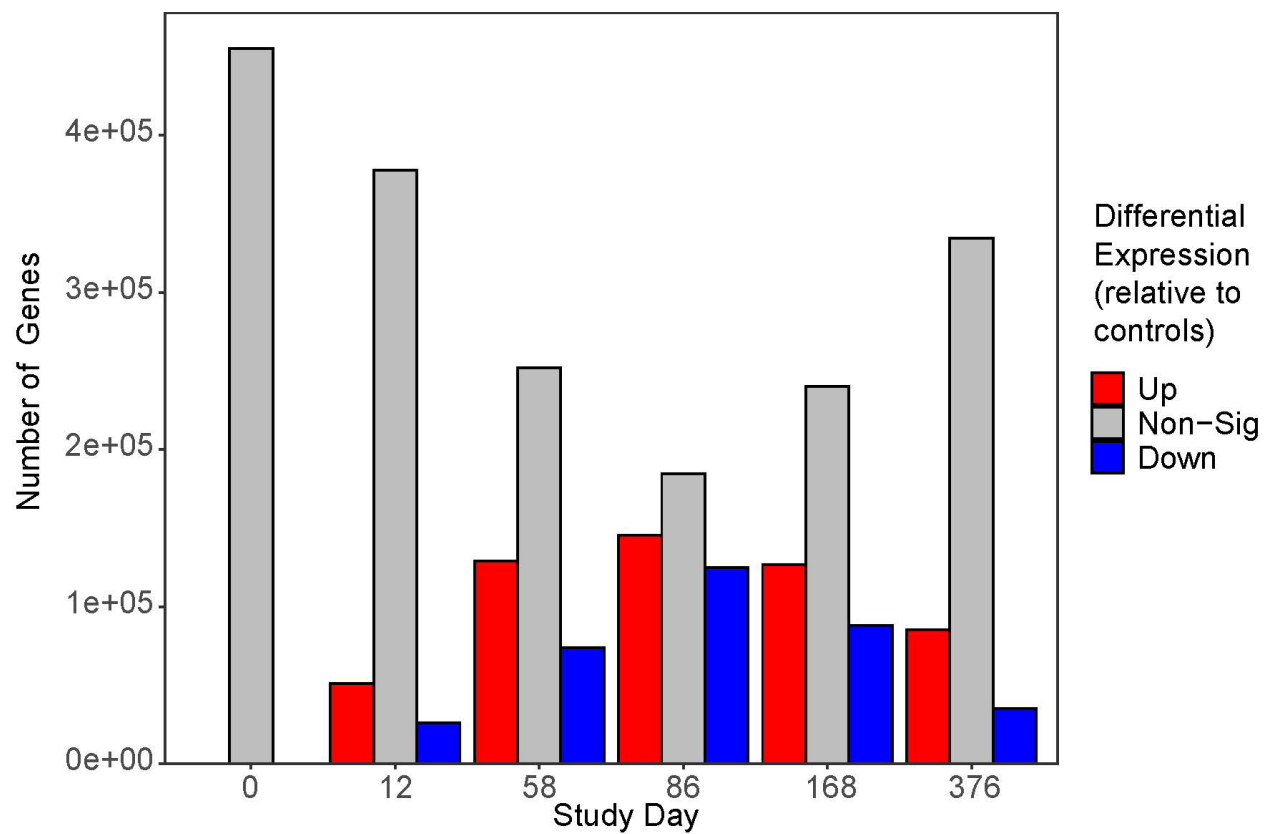


Figure 4.7 Number of up- (red) and down- (blue) regulated genes at each study day, relative to control soils. Number of genes that were not significantly differentially expressed between decomposition and control soils are denoted in gray.

Table 4.3 Top 10 most up- and down-regulated genes, determined by log2 fold change and adjusted p-values, for each sequential timepoint comparison. Positive log2 fold change values represent genes whose expression was higher in the later decomposition timepoint soils, while negative log2 fold change values are higher in earlier decomposition timepoint soils. Taxonomic annotation, COG categories, gene names, and EC were assigned via eggNOG-mapper. The comparison column distinguishes each timepoint comparison.

Query	Comparison	log2 fold change	p-value	Taxonomic annotation	COG category	COG annotation	Gene name	EC
k123_3351426_1	12v0	11.41	1.04E-15	Clostridia	S	Function Unknown	-	-
k123_3689959_6	12v0	12.86	2.09E-15	Bacilli	GM	Carbohydrate transport and metabolism	-	-
k123_1682409_1	12v0	12.51	3.31E-15	Bacilli	C	Energy production and conversion	nifJ	1.2.7.1
k123_4131385_2	12v0	11.77	7.08E-15	Bacilli	M	Cell wall/membrane/envelope biogenesis	-	-
k123_3474748_6	12v0	11.25	8.26E-15	Bacilli	E	Amino acid transport and metabolism	oppA	-
k123_2018420_1	12v0	11.13	1.46E-14	Bacilli	G	Carbohydrate transport and metabolism	fruA	2.7.1.202
k123_4134106_1	12v0	10.77	1.5E-14	Bacilli	C	Energy production and conversion	pdhC	2.3.1.12
k123_2451258_1	12v0	12.57	1.58E-14	Clostridia	M	Cell wall/membrane/envelope biogenesis	-	-
k123_3683359_3	12v0	12.86	1.61E-14	Bacilli	P	Inorganic ion transport and metabolism	copA	3.6.3.54
k123_2018420_3	12v0	9.998	1.61E-14	Bacilli	GKT	Carbohydrate transport and metabolism	manR	-
k123_4030449_2	12v0	-7.20	1.28E-08	Gammaproteobacteria	C	Energy production and conversion	-	-

Table 4.3 continued

Query	Comparison	log2 fold change	p-value	Taxonomic annotation	COG category	COG annotation	Gene name	EC
k123_1903136_2	12v0	-6.77	3.62E-08	Actinobacteria	Q	Secondary metabolites biosynthesis, transport, and catabolism	-	1.14.11.17
k123_4017556_1	12v0	-6.74	7.12E-08	Betaproteobacteria	Q	Secondary metabolites biosynthesis, transport, and catabolism	-	1.14.11.17
k123_2470867_1	12v0	-6.35	1.06E-07	Actinobacteria	Q	Secondary metabolites biosynthesis, transport, and catabolism	tauD	1.14.11.17
k123_2231885_1	12v0	-5.43	1.13E-07	Actinobacteria	E	Amino acid transport and metabolism	-	-
k123_1903136_1	12v0	-5.51	1.31E-07	Actinobacteria	K	Transcription	-	-
k123_3697194_1	12v0	-5.45	1.54E-07	Actinobacteria	Q	Secondary metabolites biosynthesis, transport, and catabolism	tauD	1.14.11.17
k123_4238273_1	12v0	-6.11	1.81E-07	Deltaproteobacteria	S	Function Unknown	-	-
k123_2680211_1	12v0	-5.15	3.09E-07	Proteobacteria	Q	Secondary metabolites biosynthesis, transport, and catabolism	-	1.16.3.3
k123_4688925_2	12v0	-6.01	3.68E-07	Betaproteobacteria	C	Energy production and conversion	-	-
k123_785677_1	58v12	12.11	2.79E-15	Betaproteobacteria	J	Translation, ribosomal structure and biogenesis	rplC	-

Table 4.3 continued

Query	Comparison	log2 fold change	p-value	Taxonomic annotation	COG category	COG annotation	Gene name	EC
k123_2456034_2	58v12	11.23	1.88E-14	Betaproteo bacteria	J	Translation, ribosomal structure and biogenesis	rplD	-
k123_3024550_1	58v12	10.82	2.62E-14	Betaproteo bacteria	J	Translation, ribosomal structure and biogenesis	ctc	-
k123_1916443_2	58v12	11.32	4.43E-14	Betaproteo bacteria	J	Translation, ribosomal structure and biogenesis	rplT	-
k123_794397_5	58v12	12.05	4.57E-14	Betaproteo bacteria	J	Translation, ribosomal structure and biogenesis	rplQ	-
k123_1010408_1	58v12	11.83	4.68E-14	Betaproteo bacteria	J	Translation, ribosomal structure and biogenesis	rplE	-
k123_1115623_7	58v12	10.79	5.29E-14	Betaproteo bacteria	JKL	Translation, ribosomal structure and biogenesis	rhIE1	-
k123_3802697_1	58v12	10.71	6.49E-14	Betaproteo bacteria	JKL	Translation, ribosomal structure and biogenesis	rhIE1	-
k123_5130820_1	58v12	11.59	6.93E-14	Betaproteo bacteria	JKL	Translation, ribosomal structure and biogenesis	rhIE1	-
k123_2572977_1	58v12	11.37	6.94E-14	Betaproteo bacteria	U	Intracellular trafficking, secretion and vesicular transport	secY	-
k123_351426_1	58v12	-10.85	3.76E-15	Clostridia	S	Function Unknown	-	-

Table 4.3 continued

Query	Comparison	log2 fold change	p-value	Taxonomic annotation	COG category	COG annotation	Gene name	EC
k123_3689959_6	58v12	-12.30	6.47E-15	Bacilli	GM	Carbohydrate transport and metabolism	-	-
k123_4131385_2	58v12	-11.20	2.41E-14	Bacilli	M	Cell wall/membrane/envelope biogenesis	-	-
k123_1682409_1	58v12	-11.43	2.73E-14	Bacilli	C	Energy production and conversion	nifJ	1.2.7.1
k123_3028891_1	58v12	-10.74	6.30E-14	Clostridia	K	Transcription	-	-
k123_2018420_3	58v12	-9.43	6.75E-14	Bacilli	GKT	Carbohydrate transport and metabolism	manR	-
k123_3469192_2	58v12	-9.63	1.12E-13	Clostridia	T	Signal transduction mechanisms	-	-
k123_3683359_3	58v12	-11.78	1.21E-13	Bacilli	P	Inorganic ion transport and metabolism	copA	3.6.3.54
k123_335030_2	58v12	-11.42	1.38E-13	Bacilli	C	Energy production and conversion	adhB	1.1.1.1, 1.1.1.202
k123_3012292_1	58v12	-9.30	1.72E-13	Bacilli	S	Function Unknown	yugP	-
k123_3581297_1	86v58	8.05	8.56E-11	Fungi	-		-	-
k123_133381_1	86v58	8.36	3.25E-10	Betaproteobacteria	C	Energy production and conversion	-	1.7.2.6
k123_3681927_1	86v58	8.47	3.28E-10	Betaproteobacteria	C	Energy production and conversion	-	1.9.3.1
k123_4461993_6	86v58	10.38	5.74E-10	Bacteroidetes	S	Function Unknown	-	-
k123_1691746_1	86v58	8.75	7.32E-10	Rhodospirillales	C	Energy production and conversion	nuoH	1.6.5.3

Table 4.3 continued

Query	Comparison	log2 fold change	p-value	Taxonomic annotation	COG category	COG annotation	Gene name	EC
k123_1674520_1	86v58	8.66	7.36E-10	Alphaproteobacteria	C	Energy production and conversion	atpA	3.6.3.14
k123_4907595_1	86v58	8.47	7.64E-10	Oceanospirillales	J	Translation, ribosomal structure and biogenesis	rpsB	-
k123_788629_1	86v58	8.02	7.71E-10	Bacteroidetes	PT	Inorganic ion transport and metabolism	-	-
k123_784141_1	86v58	6.60	9.60E-10	Bacteroidetes	-		-	-
k123_2678388_2	86v58	7.97	1.34E-09	Betaproteobacteria	C	Energy production and conversion	coxB	1.9.3.1
k123_903075_1	86v58	-7.91	8.60E-12	Bacteroidetes	-		-	-
k123_2460567_2	86v58	-7.06	4.81E-11	Bacteroidetes	S	Function Unknown	mltG	-
k123_680086_2	86v58	-6.94	6.93E-11	Bacteria	-		-	-
k123_3353720_2	86v58	-7.04	8.18E-11	Bacteroidetes	U	Intracellular trafficking, secretion and vesicular transport	secG	-
k123_4342821_1	86v58	-6.57	2.41E-10	Bacteroidetes	CO	Energy production and conversion	-	-
k123_680086_6	86v58	-6.70	4.86E-10	Bacteroidetes	G	Carbohydrate transport and metabolism	-	-
k123_3687116_4	86v58	-6.68	5.70E-10	Bacteroidetes	S	Function Unknown	yhhW	-
k123_349053_1	86v58	-6.99	5.997E-10	Bacteroidetes	F	Nucleotide transport and metabolism	guaB	1.1.1.205
k123_2348950_2	86v58	-6.36	6.10E-10	Bacteroidetes	O	Post-translational modification, protein turnover, and chaperones	-	-

Table 4.3 continued

Query	Comparison	log2 fold change	p-value	Taxonomic annotation	COG category	COG annotation	Gene name	EC
k123_1004837_2	86v58	-7.16	6.80E-10	Bacteroidetes	S	Function Unknown	-	-
k123_465331_1	168v86	6.83	2.46E-10	Bacteroidetes	M	Cell wall/membrane/envelope biogenesis	-	-
k123_1247591_2	168v86	6.66	1.53E-09	Xanthomonadales	-		-	-
k123_356907_3	168v86	7.01	1.95E-09	Actinobacteria	I	Lipid transport and metabolism	desA3_2	-
k123_1802620_3	168v86	6.78	3.42E-09	Actinobacteria	S	Function Unknown	-	-
k123_4137171_1	168v86	7.25	3.66E-09	Actinobacteria	U	Intracellular trafficking, secretion and vesicular transport	-	-
k123_1449660_1	168v86	7.11	5.70E-09	Actinobacteria	S	Function Unknown	-	-
k123_3015796_1	168v86	6.13	6.52E-09	Actinobacteria	S	Function Unknown	-	-
k123_4469568_1	168v86	8.52	6.95E-09	Gammaproteobacteria	NU	Cell motility	pilA	-
k123_4013561_1	168v86	6.72	7.47E-09	Xanthomonadales	N	Cell motility	fliD	-
k123_4136401_2	168v86	6.10	7.50E-09	Actinobacteria	O	Post-translational modification, protein turnover, and chaperones	fkfB	5.2.1.8
k123_2576445_1	168v86	-7.81	3.50E-10	Xanthomonadales	M	Cell wall/membrane/envelope biogenesis	mopB	-
k123_2013370_2	168v86	-7.41	3.71E-10	Gammaproteobacteria	S	Function Unknown	-	-

Table 4.3 continued

Query	Comparison	log2 fold change	p-value	Taxonomic annotation	COG category	COG annotation	Gene name	EC
k123_1784040_1	168v86	-7.54	4.48E-10	Clostridia	L	Replication, recombination and repair	-	-
k123_2784492_8	168v86	-7.10	6.19E-10	Actinobacteria	Q	Secondary metabolites biosynthesis, transport, and catabolism	-	-
k123_1671881_1	168v86	-7.71	1.20E-09	Bacteroidetes	P	Inorganic ion transport and metabolism	-	-
k123_115662_2	168v86	-8.93	1.21E-09	Bacilli	C	Energy production and conversion	sdhA	1.3.5.1, 1.3.5.4
k123_2013542_1	168v86	-7.29	1.57E-09	Betaproteobacteria	S	Function Unknown	-	-
k123_784141_1	168v86	-6.40	1.83E-09	Bacteroidetes	-		-	-
k123_126934_1	168v86	-8.88	2.72E-09	Bacteroidetes	M	Cell wall/membrane/envelope biogenesis	-	-
k123_3912958_2	168v86	-7.10	4.80E-09	Bacteroidetes	C	Energy production and conversion	ccoG	-
k123_4347668_1	376v168	7.99	4.14E-10	Archaea	-		-	-
k123_2010423_2	376v168	8.46	5.97E-10	Acidobacteria	J	Translation, ribosomal structure and biogenesis	rplJ	-
k123_4021403_1	376v168	6.60	7.84E-10	Acidobacteria	O	Post-translational modification, protein turnover, and chaperones	groL	-
k123_2899693_1	376v168	6.55	1.28E-09	Acidobacteria	O	Post-translational modification, protein turnover, and chaperones	-	-

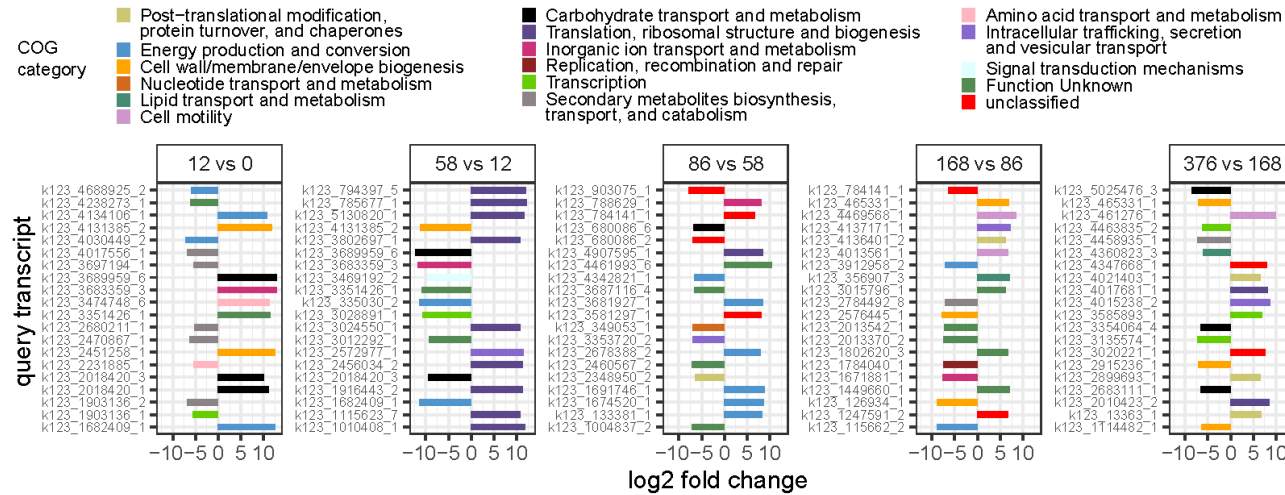
Table 4.3 continued

Query	Comparison	log2 fold change	p-value	Taxonomic annotation	COG category	COG annotation	Gene name	EC
k123_461276_1	376v168	9.98	2.42E-09	Bacteria	N	Cell motility	-	-
k123_13363_1	376v168	6.77	2.66E-09	Acidobacteria	O	Post-translational modification, protein turnover, and chaperones	groL	-
k123_3585893_1	376v168	6.94	2.91E-09	Acidobacteria	K	Transcription	rpoC	2.7.7.6
k123_3020221_1	376v168	7.64	3.43E-09	Xanthomonadales	-		-	-
k123_4015238_2	376v168	8.72	4.32E-09	Acidobacteria	U	Intracellular trafficking, secretion and vesicular transport	-	-
k123_4017681_1	376v168	8.22	4.97E-09	Acidobacteria	J	Translation, ribosomal structure and biogenesis	rpsC	-
k123_465331_1	376v168	-7.07	1.08E-10	Bacteroidetes	M	Cell wall/membrane/envelope biogenesis	-	-
k123_4458935_1	376v168	-7.20	1.65E-09	Epsilonproteobacteria	Q	Secondary metabolites biosynthesis, transport, and catabolism	-	-
k123_5025476_3	376v168	-8.47	2.40E-09	Actinobacteria	G	Carbohydrate transport and metabolism	ptsH	-
k123_1114482_1	376v168	-6.40	2.84E-09	Bacteroidetes	MU	Cell wall/membrane/envelope biogenesis	-	-
k123_4360823_3	376v168	-6.01	3.23E-09	Gammaproteobacteria	IQ	Lipid transport and metabolism	-	1.1.1.159
k123_3135574_1	376v168	-7.25	4.14E-09	Actinobacteria	K	Transcription	fruR	-

Table 4.3 continued

k123_ 3354064_4	376v168	-6.63	8.46E-09	Actinobact eria	G	Carbohydrate transport and metabolism	-	-
k123_ 2683111_1	376v168	-6.49	9.34E-09	Actinobact eria	G	Carbohydrate transport and metabolism	-	5.3.1.4
k123_ 4463835_2	376v168	-6.19	1.02E-08	Actinobact eria	K	Transcription	-	-
k123_ 2915236_1	376v168	-7.06	1.57E-08	Actinobact eria	M	Cell wall/membrane/env elope biogenesis	-	-

A



B

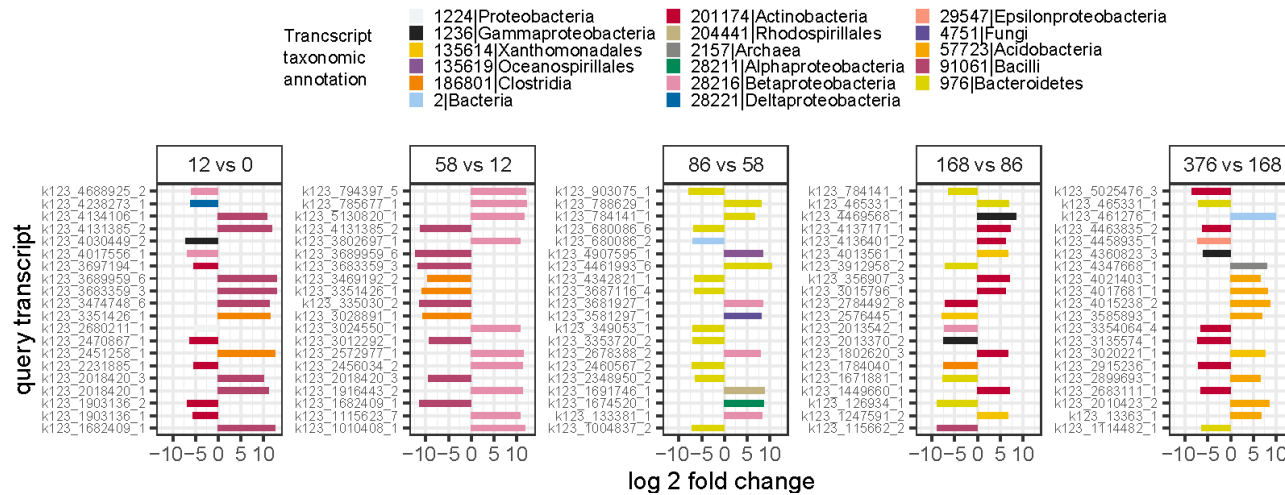


Figure 4.8 Top twenty up- and down-regulated genes in decomposition soils comparing sequential study days (0, 12, 58, 86, 168, 376) colored by COG functional category (A) and taxonomic annotation (B). Positive values denote increased expression compared to the preceding timepoint, while negative values denote a decrease.

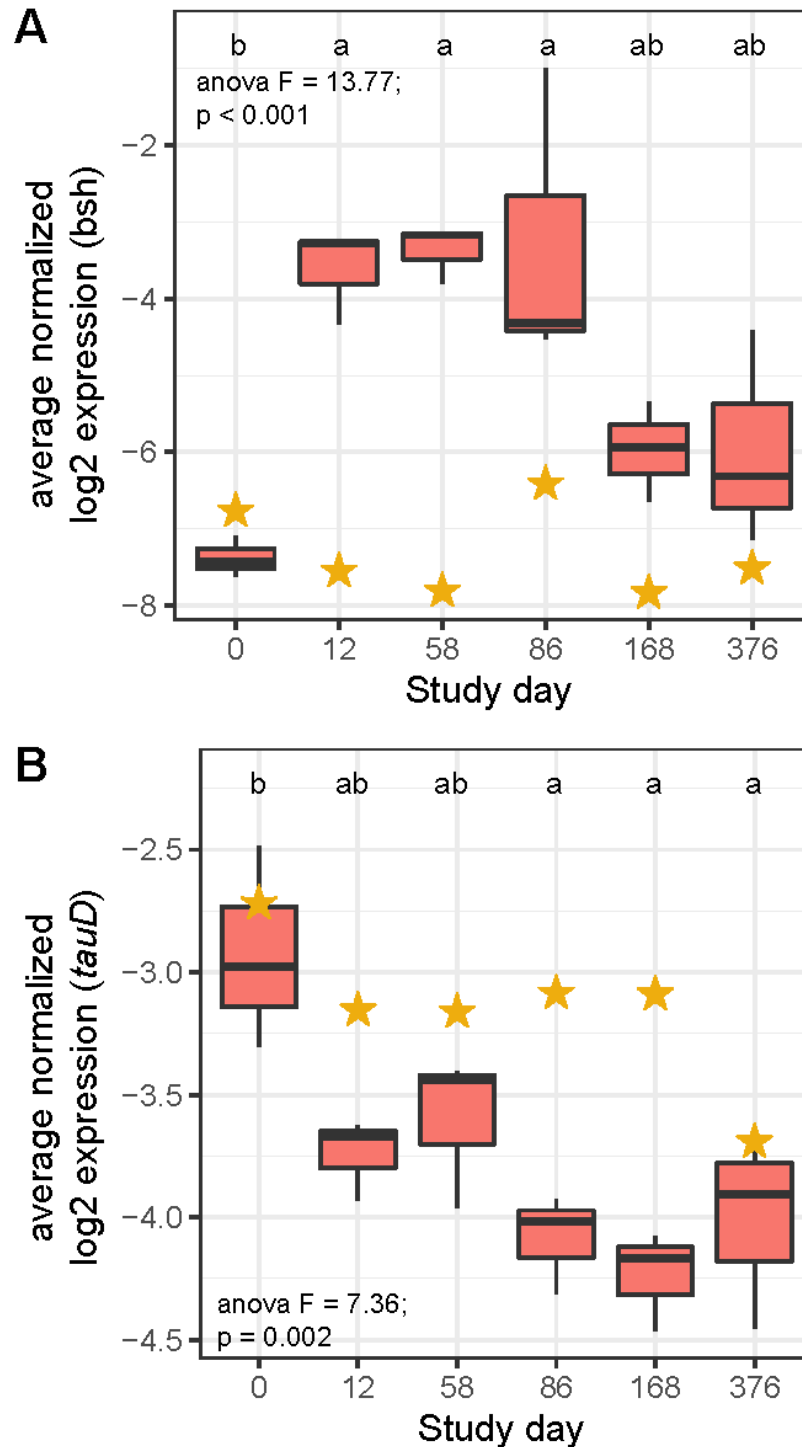


Figure 4.9 Mean bile salt hydrolase, BSH, (A) and *tauD*, taurine dioxygenase, (B) log2 normalized expression in controls (gold stars) and study days (boxplots). Boxplots display the 25th and 75th quartiles and median log2 normalized values between all three individuals at each timepoint. ANOVA p-value is the result of a hierarchical linear mixed effects model accounting for repeated measures of each donor block, while letters denote the results of *post-hoc* Tukey test.

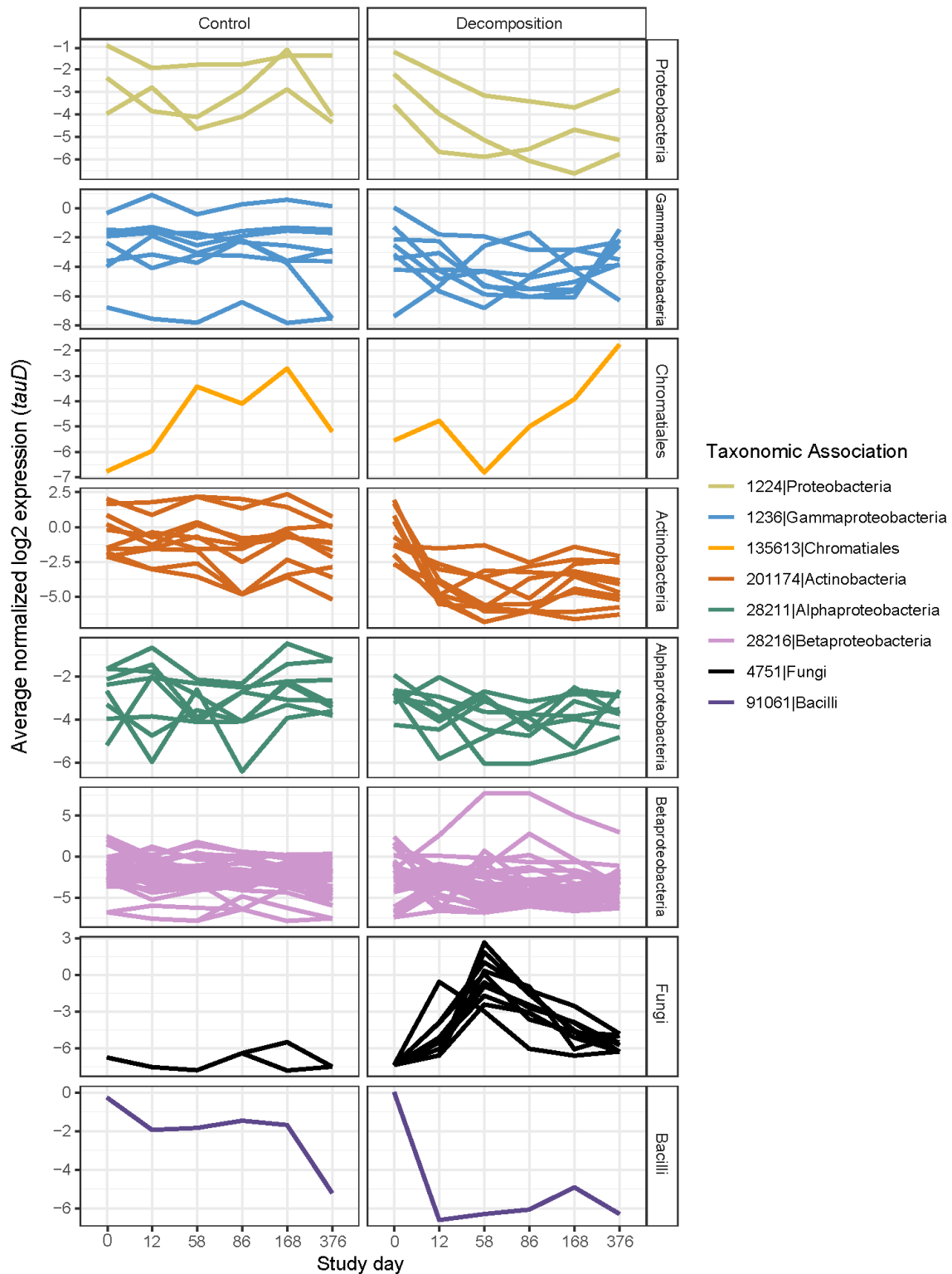


Figure 4.10 Mean normalized log₂ expression of *tauD* gene queries by taxonomic association (color) in control and decomposition soils at each study day. Each line represents one gene query, while color denotes taxonomic association as determined by eggNOG-mapper.

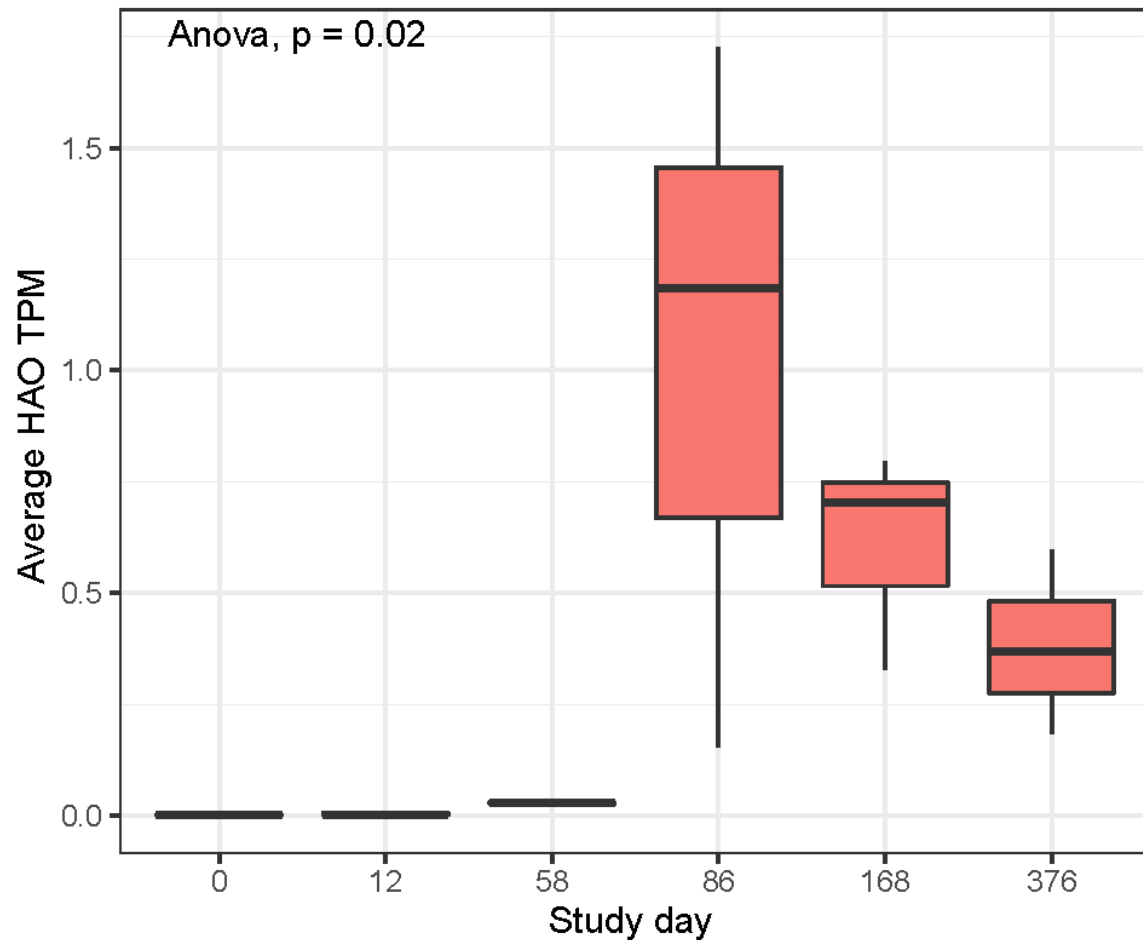


Figure 4.11 Mean *hao*, encoding hydroxylamine dehydrogenase, transcripts per million (TPM) in decomposition soils at each study day. Boxplots display the 25th and 75th quartiles and median TPM values between all three individuals at each timepoint. ANOVA p-value is the result of a hierarchical linear mixed effects model accounting for repeated measures of each donor block.

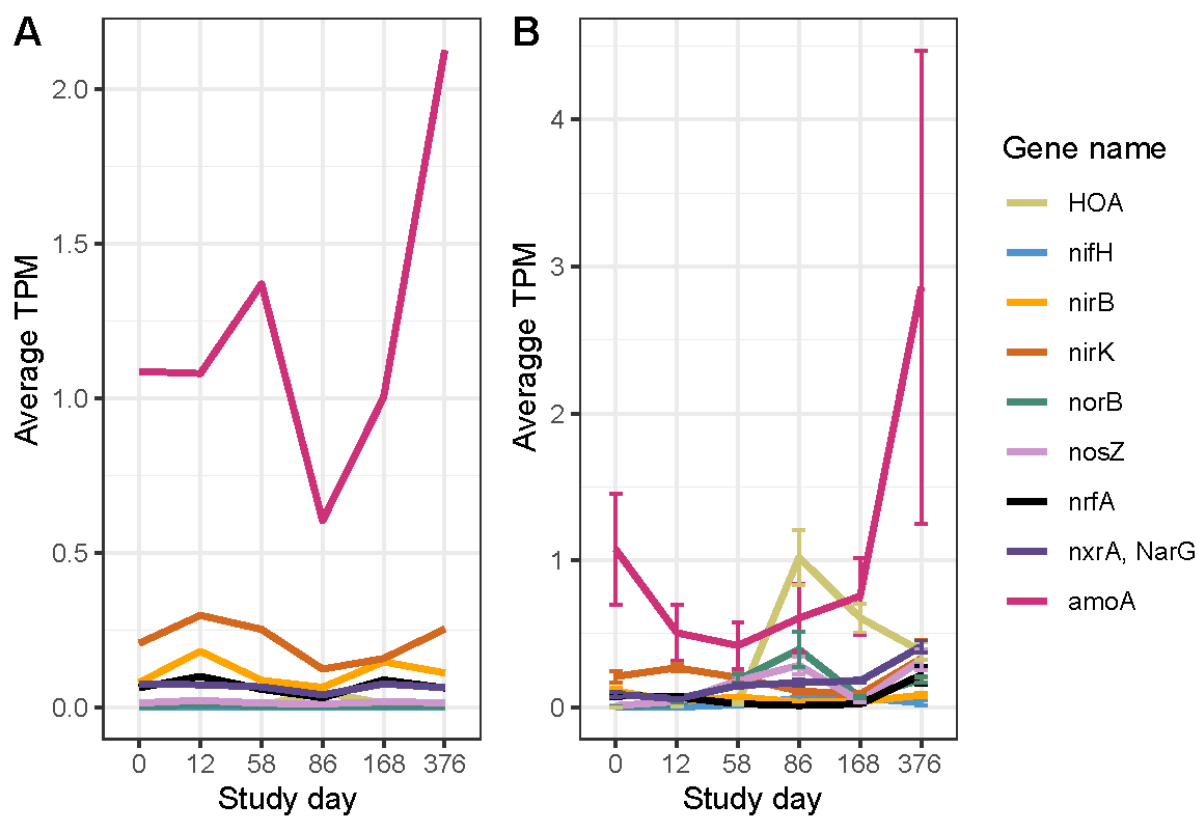


Figure 4.12 Average gene expression, in transcripts per million (TPM), of commonly used marker genes for enzymes involved in nitrogen cycling over time in controls (A) and decomposition (B) soils. Error bars in B represent mean of three individuals.

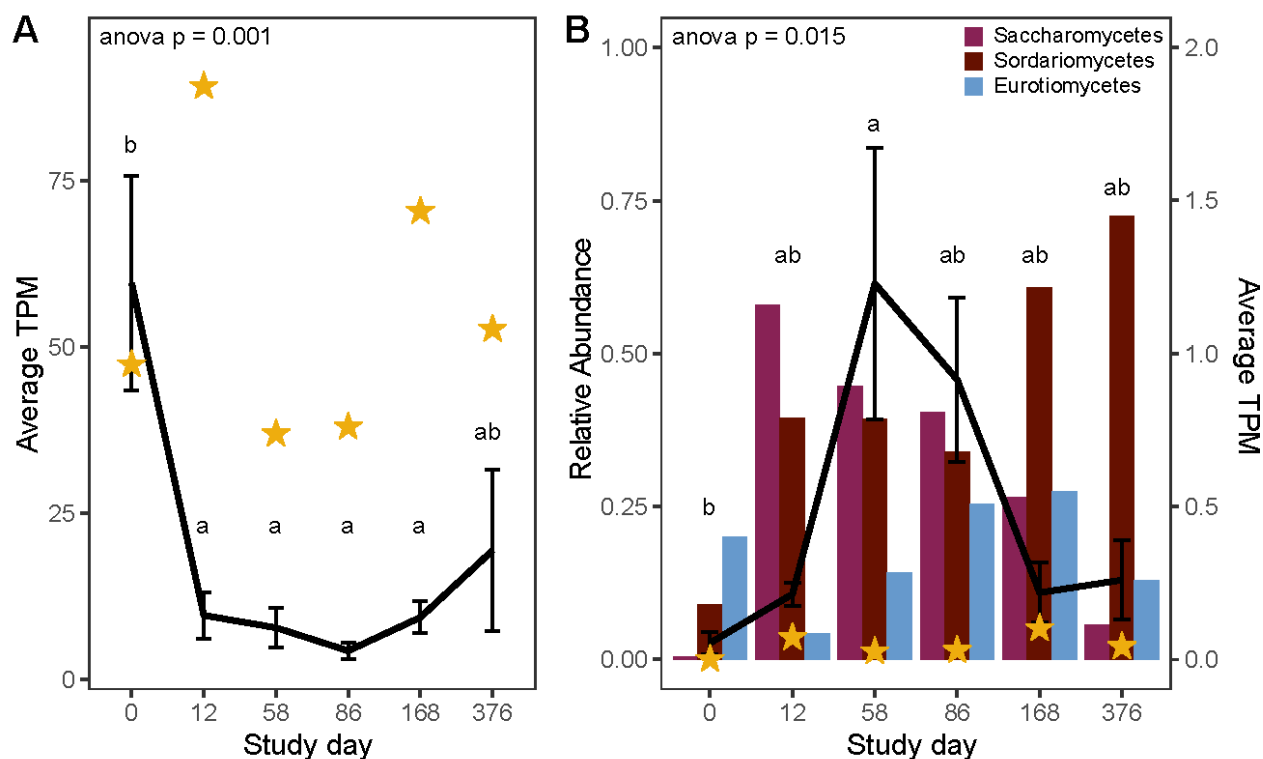


Figure 4.13 Mean transcript abundance, in transcripts per million (TPM), of all bacterial (A) and fungal (B) associated triacylglycerol lipase (EC 3.1.1.3) gene queries over time. Black lines (A,B) report mean TPM from all three individuals (black line) with error bars, while gold stars denote mean TPM in control soils. P-values are the result of ANOVAs where average TPM and study day are the dependent and independent variables, respectively, while letters are the result of *post-hoc* Tukey tests between decomposition timepoints. In B, bars show the relative abundance of the fungal classes *Saccharomycetes*, *Sordariomycetes*, and *Eurotiomycetes*, reported by Taylor et al, (2020).

CONCLUSION

Microbes are important decomposers involved in the degradation of animal tissues following mortality events. In terrestrial ecosystems, vertebrate decomposition causes a disturbance to the surrounding soil, resulting in microbial community succession. Most studies of microbial succession during human decomposition have been inspired by forensic entomology and aim to predict postmortem interval (PMI) for forensic applications. While important, this focus has left considerable knowledge gaps in our understanding of microbial ecology of decomposition systems, including factors that influence successional patterns. Further, little is known about microbial functional succession as these studies have emphasized community composition. Therefore, the goal of this dissertation was to address these knowledge gaps to better understand the microbial ecology within decomposition-impacted soils. In this work, three research chapters show that intrinsic factors effect soil microbial communities during decomposition (Chapter 2), extrinsic factors impact PMI prediction from structural succession (Chapter 3), and gene expression profiles of soil microbial communities change as decomposition progresses (Chapter 4).

EXTRINSIC VARIATION

The relationship between microbial communities and extrinsic drivers was investigated throughout all chapters, including relationships between environmental factors and microbial community structure (Chapters 2,3) and function (Chapter 4). Results across all three chapters show that microbial communities change over time, and these shifts are linked to extrinsic variables. As decomposition progresses, both the soil physicochemical environment, particularly soil pH and EC, and microbial communities are impacted. In Chapter 2, we observed that microbial community structure shifted during the early stages of human decomposition. These shifts correlated to changes in soil electrical conductivity (EC), which was also observed to be an important environmental feature in random forest models to predict PMI (Chapter 3) and was related to variation in gene expression (Chapter 4).

Another important constraint on soil microbial composition and function appeared to be soil oxygen. We know from previous work that excess microbial respiration results in a depletion of

oxygen in decomposition soils. While oxygen was not directly measured in the study reported in Chapters 2 and 3, we did observe increases in facultative and anaerobic taxa following release of decomposition fluid. Similarly, prior study of fungal community structure in soils from Chapter 4 (1) showed a potential relationship between fungal community structure and dissolved oxygen measurements over time. In our investigation of microbial gene expression, day 58 represented a period when dissolved oxygen had partially returned and we saw a respiration maxima.

Interestingly, this is when we observed increased expression of multiple fungal genes, including lipase and *tauD*, suggesting that fungal gene expression may also be regulated by soil oxygen.

Additionally, results from Chapter 4 showed that nitrogen cycling in decomposition-impacted soils may be occurring via an alternative pathway linked to shifts in soil oxygen. Expression of *hao* (encoding hydroxylamine oxidoreductase) corresponded to increased expression of *norB* (encoding nitric oxide reductase) and *nosZ* (encoding nitrous oxide reductase) during periods of reduced soil oxygen, implying that hydroxylamine is an important intermediate for nitrogen cycling during human decomposition. In particular, HAO may be converting hydroxylamine to nitric oxide, diverting nitrogen from downstream nitrification processes and into denitrification. To test this hypothesis, a cumulative examination of hydroxylamine concentrations within decomposition soils and release of nitric oxide and nitrous oxide as decomposition progresses is needed. Additionally, future work should also assess anaerobic ammonium oxidation (anammox) to see if these processes are contributing to the observed expression and biogeochemical patterns observed in decomposition soils.

INTRINSIC VARIATION

We also found that intrinsic factors can impact microbial succession in human decomposition soils. In Chapter 2, we showed that body mass index (BMI) was related to changes in soil pH and relative abundance of the fungal taxa *Saccharomycetes*. These results suggest that the presence of certain decomposer microbes and microbial community succession may be affected by the types and abundance of substrates present for decomposition in a vertebrate body. Specifically, we hypothesized that the effects of BMI on soil responses is likely due to different ratios of tissues, particularly fat and muscle. However, more work is needed to test this hypothesis and identify mechanisms by which BMI influences soil communities. Additionally, BMI is only a

proxy for estimating body fat across a diverse population, as it does not account for distribution of fat due to age or sex. Thus, testing effects due to body composition (*i.e.*, proportion of fat and lean tissue) would be more appropriate. We recommend future studies test microbial and chemical responses during decomposition of different body compositions.

Additionally, results from Chapter 2 suggest that expression of fungal lipase genes may be also impacted by an individual's BMI. Chapter 4 revealed a correlation between changes in fungal lipase expression and abundance of *Saccharomycetes* and *Sordariomycetes*, while Chapter 2 showed that the abundance of these taxa are impacted by intrinsic factors, specifically BMI. This may lead to variation in fungal lipase activity between individuals with different body compositions. BMIs of the three donors used in Chapter 4 were within two kg/m² of each other, thus large differences in lipase expression were not observed. However, there could be differences between underweight and obese groups, similar to trends observed in Chapter 2. Therefore, future work should investigate expression of fungal lipases between individuals with differing body composition, as well as between vertebrate species with different body compositions.

In regard to applications for forensic science, the influence of intrinsic factors, particularly BMI, has implications for PMI estimations as this variation can alter the predictability of microbial successional patterns. This may explain the high error rates of PMI estimation models we reported in Chapter 3. Specifically, inter-individual variation due to intrinsic factors may increase model error when predicting PMI from a diverse population of donors. We did not include any intrinsic factors in creation of PMI estimation models in Chapter 3, as we focused on extrinsic factors. However, future studies should assess the incorporation of intrinsic factors, such as BMI, into soil microbial abundance-based PMI estimation models. This will require integration of categorical (*e.g.*, sex, disease, cause of death) and continuous (*e.g.*, age, BMI) intrinsic variables, specific to each individual, with microbial abundance and environmental data.

Previous studies have observed differences in soil chemical responses during decomposition of different vertebrate species. Specifically, changes in soil pH are highly variable between studies. Decomposition of non-human vertebrate subjects typically display increased soil pH. In contrast studies with humans generally display decreased soil pH, however, have increases and decreases

have both been observed. This variation has also been observed for pigs and humans decomposing under the same environmental conditions: where DeBruyn et al. (2021) observed decreased pH under humans but increased pH under pigs. From an ecological perspective, our results relating variation in soil pH to BMI may explain differences in soil pH response observed between humans (3) and between humans and other vertebrate species (2) as microbes respond to changing substrates. This is likely due to differences in body composition between species that may impact our ability to fully relate carrion ecology to forensically motivated human decomposition studies, as we may not be accounting for differences in host-associated decomposer communities or soil responses that ultimately influence the presence and succession of microbial communities. Therefore, we recommend that the research focus be taken into consideration when choosing appropriate model organisms for studying microbial communities during decomposition.

Chapter 2 focused on effects of BMI specific to soil chemistry and microbial succession, however we also observed a correlation between total decomposition time and BMI, suggesting that intrinsic factors can have broad impacts on multiple aspects of decomposition, including other decomposers (*e.g.*, insects and scavengers). Therefore, future studies should investigate how these intrinsic factors relate to other facets of decomposition, such as decomposition rate or other presence/succession of decomposer communities. This will help us better understand variation in soil microbial response to decomposition as activity of one decomposer group (*e.g.*, insects) can impact soil communities.

Chapter 2 also revealed some difficulties in identifying intrinsic factors that impact decomposition processes. Humans are complex individuals with multiple underlying conditions and characteristics. As a result, it was difficult to identify relationships related to a single intrinsic factor. This was particularly difficult when considering potential effects due to disease, as no individual had a single disease present. To assess these and other relationships a large sample of humans is needed, which may not be realistically attainable. Alternatively, care should be taken to select individuals with similar intrinsic factors and compare decomposition processes and microbial responses between groups.

We also showed that intrinsic factors had the potential to influence soil extrinsic parameters. Specifically, variable soil pH response was explained by an individual's BMI in Chapter 2. This has important implications for understanding microbial response during vertebrate decomposition and variation in successional patterns. This was apparent in Chapter 3, with less variable soil parameters, such as EC, consistently among the top environmental features in random forest models, while variable parameters (*i.e.*, pH) exhibited less influence on model prediction. It is important to note, however, that we were only able to investigate a few soil parameters in these studies, thus future investigations should include a wider range of variables. Respiration rates, oxygen concentration, nitrogen species, dissolved organic carbon and nitrogen, among others, are all of interest as they include variables that constrain and/or describe microbial activity and provide a snapshot of carbon and nitrogen dynamics in decomposition-impacted soils.

FUNCTIONAL SUCCESSION

Chapter 4 addressed our knowledge gap of microbial functional succession. In this chapter we showed that soil microbial gene expression shifted in response to vertebrate decomposition and do not return to pre-decomposition conditions after one year. While this confirmed that decomposition has strong and long-lasting effects on soil microbial functionality, we were unable to determine for how long soil communities were impacted following decomposition. Therefore, we still need to capture decomposition processes that occur past one year. Additionally, this study only captured six timepoints over one year. While informative for general patterns in gene expression, this does not capture fluctuations that might occur within decomposition stages. For example, we observed high inter-individual variability in microbial community structure during the initial stages of decomposition in Chapter 2. Although this study was based on 16S rRNA gene and ITS amplicon sequencing, it may suggest inter-individual variability in gene expression shifts as well.

Alternatively, inter-individual variability in gene expression profiles may be reduced due to functional redundancy between taxa. Thus, we recommend a high-resolution investigation of gene expression to fully capture succession in functional gene expression. These studies should also investigate functional redundancy by combining metagenomic and metatranscriptomic

approaches and assess PMI estimations from functional succession. This would have major implications for forensic science, as stability in gene expression shifts may result in more accurate PMI estimations compared to taxa-abundance based models tested in Chapter 3. Combining metagenomic approaches with metatranscriptomes will also help to identify differences between community functional potential and gene expression by active microbes and begin to link compositional and functional shifts.

Further supporting the presence of functional redundancy in this system, we observed changes in gene expression that correspond to trends captured by metabolomic approaches, while differing from changes in relative abundance captured by DNA sequencing approaches. One example of this was our observation of reduced *tauD* expression corresponding to recent metabolomics studies that report elevated levels of taurine in decomposition soils, the primary substrate for *tauD*. While we observed decreased *tauD* expression over time, relative abundance of taxa associated with these genes remained consistent or increased in soils. These taxa (*e.g.*, *Gammaproteobacteria*, *Actinobacteria*, etc.) displayed similar shifts in Chapters 2 and 3. These results highlight differences between genomic potential and active functional pathways, where here expression of *tauD* within these organisms is likely regulated in response to lowered sulfur limitation. However, our study covered a one-year period, while soil metabolomics studies have mostly focused on changes up to six months, and none have linked metabolomic changes to microbial genes or taxa. Future studies should also investigate soil transcriptomes and metabolomes within the same study to identify other relationships between gene expression and metabolites and further link microbial gene expression to metabolic processes.

The role of archaea has not been addressed during human decomposition. These organisms are present in soils and are known to contribute to biogeochemical cycles, particularly nitrogen cycling via ammonia oxidizing archaea. We observed some transcripts associated with archaea in our differential expression analysis (Chapter 4) but did not look into general expression patterns or functional associations of these organisms. Therefore, future investigation should assess if there are functions specific to archaea within decomposition soils, in comparison to bacteria and fungi, to further determine functional roles of different microbial decomposers.

BACTERIA VS FUNGAL COMMUNITY RESPONSES TO HUMAN DECOMPOSITION

One theme revealed by all three chapters was a difference between bacterial and fungal successional patterns during human decomposition. In Chapter 2, soil bacterial community structure was more sensitive to inter-individual variation than fungal community structure. This led to the hypothesis in chapter 3 that fungal community shifts would provide more accurate estimates of PMI. Ultimately, we show this was not the case, as bacterial models outperformed fungal PMI estimation models. We also observed differences in fungal and bacterial gene expression during decomposition in Chapter 4. Specifically, expression of lipases differed between bacteria and fungi. Together, these studies suggest an interesting contrast between these two communities, likely driven by differences in diversity and growth characteristics. Specifically, diversity was lower within soil fungal communities compared to bacterial communities. This may have reduced resistance of fungal communities to disturbance, leading to a greater response to altered environmental conditions during decomposition, including oxygen and EC. However, the first two chapters only assessed bacterial and fungal community composition dynamics during the early stages of decomposition. We might expect fungal biomass to increase at later timepoints as tissues are decomposed and labile nutrients as processed. Only a few studies have assessed both bacterial and fungal communities simultaneously within decomposition-impacted soils. However, these have all been in relation to predicting PMI from successional patterns. Thus, we recommend that future work includes long-term investigation focused on soil ecology to better understand bacterial and fungal community dynamics during vertebrate decomposition.

Together, these three studies begin to address knowledge gaps that hinder our full understanding of microbial succession within decomposition-impacted soils. We have used a variety of molecular techniques to assess both compositional and functional succession in combination with soil physicochemical analyses to contextualize these shifts. We show that soil microbial succession during decomposition is a complex process that is affected by both intrinsic and extrinsic factors. Further, evaluation of functional succession has resulted in a more holistic view of the role of soil microbes in decomposition processes. It is our recommendation to expand

microbial studies past taxonomic evaluation to fully understand microbial ecology in decomposition-impacted soils.

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VITA

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