

Soil Microbial Functional Succession Over One Year of Human Decomposition

Allison R. Mason¹, Lois S. Taylor², Naomi Gilbert¹,
Steven W. Wilhelm¹, Jennifer M. DeBruyn^{1,2*}

¹Department of Microbiology, University of Tennessee-Knoxville, 1311
Cumberland Avenue, Knoxville, 37996.

²Department of Biosystems Engineering and Soil Science, University of
Tennessee-Knoxville, 2506 E.J. Chapman Drive, Knoxville, 37996.

*Corresponding author(s). E-mail(s): jdebruyn@utk.edu;

Abstract

Vertebrate decomposition is a key ecosystem process in terrestrial environments, altering the soil environment and nutrient pools (C, N, S, etc.). Soil microbial communities are hypothesized to drive biogeochemical cycling in these ephemeral hotspots, however it is unclear which members are active or metabolic pathways they might be expressing. This has left a foundational knowledge gap regarding the functional roles of microorganisms in vertebrate decomposition. We use metatranscriptomes obtained from soil samples collected over the course of one year from below three decomposing human bodies to address this knowledge gap. Computational analyses reveal shifts in gene expression as decomposition progressed, remaining altered after one year. Microbes in decomposition soils displayed increased expression of heat shock proteins, whose expression increased between days 0 and 58 and remained elevated through day 376. Further, expression nitrogen cycling genes coincides soil dissolved oxygen and suggests that microbial communities may be converting hydroxylamine to nitric oxide and reducing nitrous oxide to nitrogen gas during reduced oxygen conditions. Together, we show that human decomposition alters soil microbial

gene expression, providing 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.

Keywords: Human Decomposition, Microbial Succession, Metatranscriptomics, Soil Microbial Ecology

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 drive nutrient cycling in terrestrial ecosystems. Environmental disturbances 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 [1, 2]. Vertebrate death and subsequent carcass deposition in terrestrial ecosystems is one disturbance resulting in the deposition of large quantities of organic C and N [3–10], along with other elements (P, K, S, *etc*) [11], which collectively contribute to microbially-mediated biogeochemical cycling. In addition to this, changes in pH, temperature, and fluctuations in soil oxygen provide abiotic filtering further impacting microbial metabolic strategies [7–9, 11, 12].

While C and N transformations have been documented during decomposition, the functional response of microbes and their roles in nutrient cycles remain unclear. The composition and structure of decomposition-impacted soil microbial communities have been investigated using amplicon sequencing of marker genes (*i.e.*, 16S rRNA, 18S rRNA, ITS), revealing successional dynamics [13]. This has allowed us to investigate

changes in microbial biodiversity and composition 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, which can further help to describe microbial ecology in human and animal decomposition systems. Taylor et al. (2024) [14] suggested that fungal community shifts were linked to changes in soil dissolved oxygen, highlighting interactions between soil microbes and the surrounding environment. While insightful for making potential connections between taxa and physiochemistry, these analyses cannot inform which taxa are active members of the community responsible for chemical transformations, which functional pathways/genes are expressed, 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. This can inform how ecological functions, including C and N cycling, are impacted by decomposition events in terrestrial ecosystems. To date, only two studies have applied metatranscriptomic approaches to assess mRNA in vertebrate decomposition samples [15, 16]: Burcham et al. (2019) [15] examined gene expression of internal organ microbial communities during mouse decomposition, while Ashe et al. (2021) [16] examined gene expression of oral microbial communities during human decomposition. Both studies suggest that the host microbial community functionality is altered during decomposition, including differential expression of amino acid and carbohydrate metabolism in the heart [15] and shifts in gene transcripts across different taxa [16]. We expect that soil microbial community gene expression profiles are also altered; however, this has never been examined to our knowledge. The decomposition-impacted soil metabolome was assessed by DeBruyn et al. (2021) [17], showing changes in soil metabolites over time including increased prevalence of amino acids, however it is unclear which

139 microbes are responsible for these shifts. Additionally, DeBruyn et al. (2021) [17]
 140 showed the soil metabolome was still altered compared to starting conditions at the
 141 end of the 21-week study, suggesting long-term impacts of decomposition on soil
 142 microbial functioning.
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 144 Here, we investigate soil microbial gene expression during a one-year period of human
 145 decomposition. The overarching goal of this work was to assess the impacts of verte-
 146 brate decomposition on ecosystem functioning by characterizing community-level shifts
 147 in soil microbial function. We hypothesize that: (i) gene expression would change over
 148 time as resources are used and transformed and soil chemical and physical conditions
 149 change due to the influx of decomposition products during soft tissue degradation
 150 [8, 9, 17]; (ii) expression of genes encoding enzymes involved in nitrogen cycling will
 151 be altered, as changes in nitrogen pools have been previously described in decompo-
 152 sition soils [8]; (iii) lipid metabolism will increase, as we expect lipids from the body
 153 to enter the soil during decomposition and previous studies showed an increase of
 154 lipolytic organisms in decomposition soils [12, 18]; (iv) soil expression profiles will not
 155 return to pre-decomposition conditions as multiple studies have shown soil chemistry
 156 [5, 8] and microbial community composition [19, 20] (via 16S rRNA gene amplicon
 157 sequencing) are still impacted after one year. We analyzed metatranscriptomes of soil
 158 samples collected at six key timepoints over one year of human decomposition to
 159 determine the active populations and expression of genes and pathways relevant to
 160 the enhanced biogeochemical cycling observed in decomposition hotspots. We com-
 161 pared gene expression between decomposition timepoints and control soils that were
 162 unexposed to decomposition products to identify functions or functional pathways
 163 of interest. We show: (i) that decomposition impacts soil microbial community gene
 164 expression after one year; (ii) expression of genes for stress response are elevated
 165 in decomposition soils; (iii) expression of genes encoding triacylglycerol lipase dif-
 166 fered between fungal (increased) and bacterial (decreased) organisms; (iv) evidence
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for phased nitrification and denitrification, driven by changes in soil dissolved oxygen; (v) evidence for organic sulfur processing (taurine) via bile salt hydrolases. This assessment of functional profiles within decomposition-impacted soils provides insight into the microbial response(s) to vertebrate decomposition in terrestrial settings and biogeochemical cycling within these hotspots.

Results

Soil Physiochemistry

Soil chemistry was altered in response to human decomposition, with multiple parameters still impacted after one year [14]. Generally, soil pH decreased and remained low in decomposition soils of all but one individual. Soil electrical conductivity (EC) increased in response to decomposition, remaining elevated through approximately day 58 before gradually decreasing throughout the remainder of the study (Supplementary Material 1). Respiration (evolved CO₂) increased by an order of magnitude beginning at day 12, which corresponded to a reduction in soil dissolved oxygen (DO) to 29% - 48.9%. Ammonium concentrations increased 78-fold, reaching maximum concentrations 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 (Supplementary Material 1).

Microbial gene expression in response to human decomposition

Gene expression profiles in decomposition-impacted soils shifted away from controls and day zero samples as decomposition progressed (Fig 1A). Expression was most different from controls on study days 58, 86, 168 (Supplementary Material 2), before shifting back toward control conditions on study day 376. After one year of decomposition, soil gene expression profiles had not returned to pre-decomposition conditions,

231 as evidenced by their clustering away from controls and day zero samples in the MDS
232 plot (Fig 1A).
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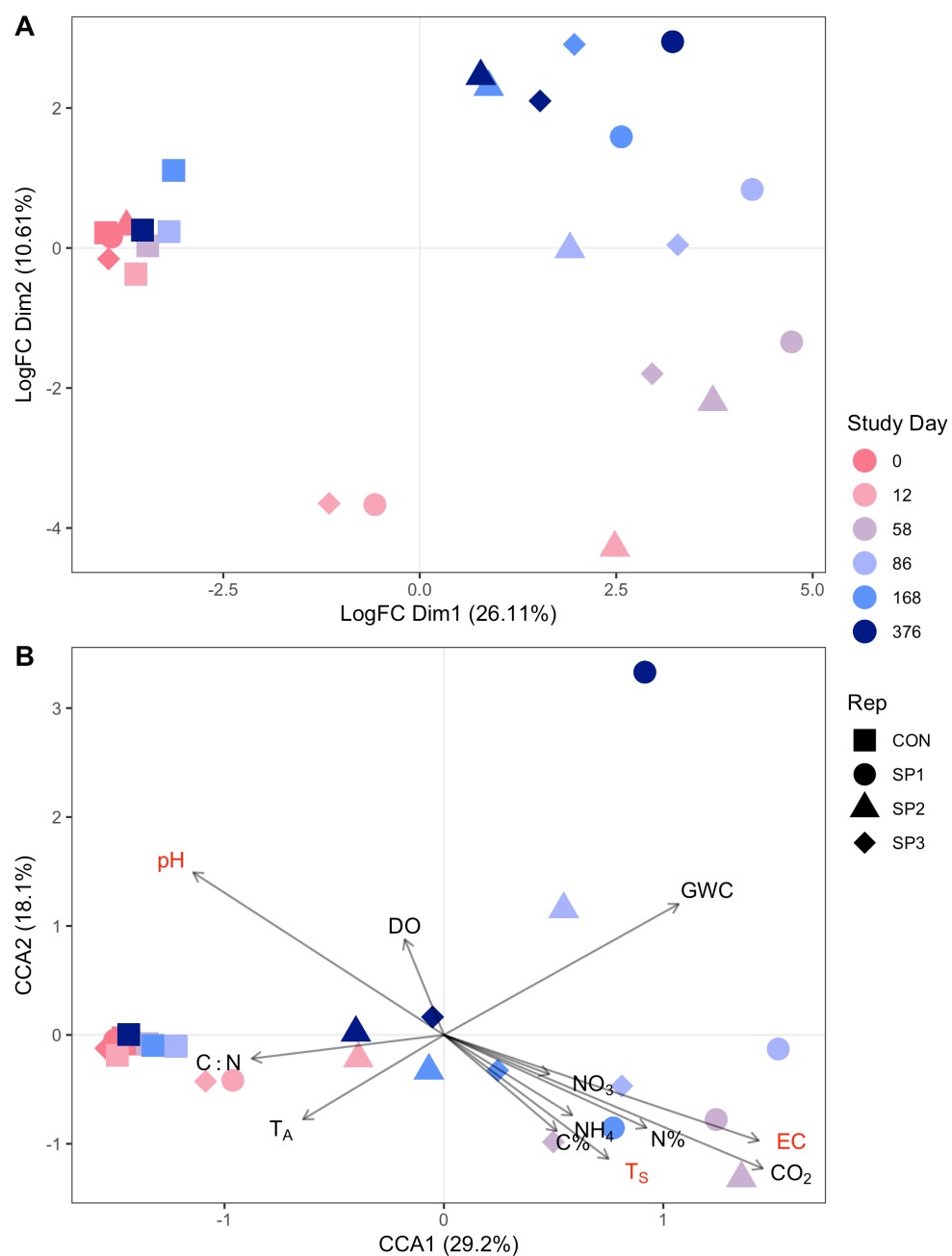
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Figure 1: Microbial gene expression profiles are altered during human decomposition. Multidimensional scaling (MDS) shows gene expression within soils changed as decomposition progressed (A). Additionally, canonical correspondence analysis (CCA) shows that environmental variables explained 47.3% of the variation in gene expression profiles (B). Variables in bold red type significantly ($p < 0.05$) explained some of the variation in gene expression profiles as assessed by Permutational Analysis of Variance (PERMANOVA). In both panels soils from controls (CON) and the three donors (SP1, SP2, SP3) are denoted by symbol shape, while color represents study day. In B, soil physiochemical variable loadings are represented by arrows: Gravimetric water content (GWC), electrical conductivity (EC), pH (pH), dissolved oxygen (DO), respiration (evolved CO_2 $\mu\text{mol gdw}^{-1}$), ammonium (NH_4), and nitrate (NO_3) concentrations (mg gdw^{-1}), percent carbon (%C), percent nitrogen (%N), carbon:nitrogen ratio (C:N), ambient temperature (T_A), and soil temperature (T_S).



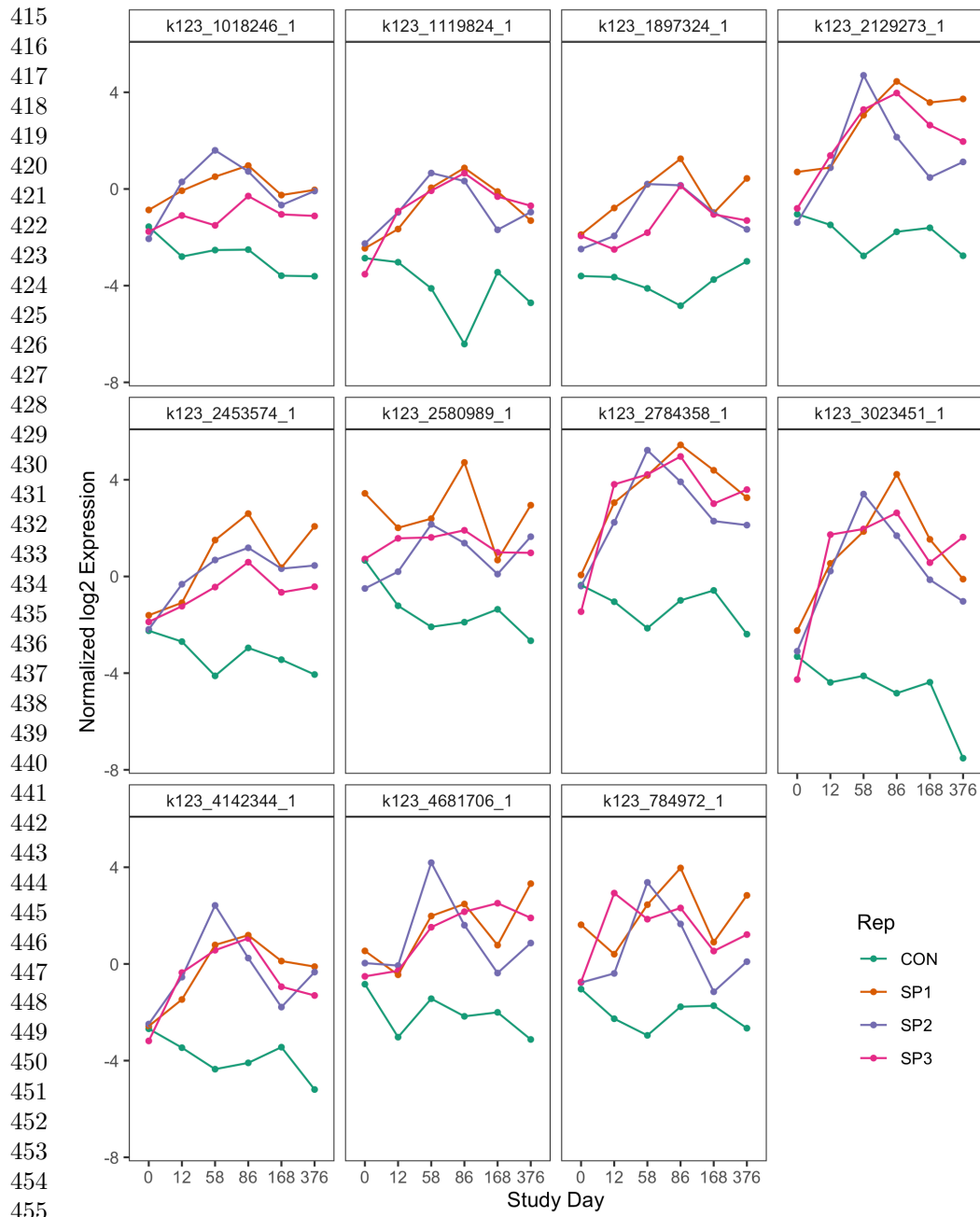
Some correlations were observed between gene expression shifts and soil physiochemical data at decomposition timepoints. Canonical correspondence analysis (CCA) was

323 used to constrain gene expression data with soil physiochemical data (Fig 1B). CCA1
 324 and CCA2 explained 29.2% and 18.1% of the variance in gene expression, respec-
 325 tively. Transcript profiles at day 12 were associated with an increase in soil carbon to
 326 nitrogen ratio (C:N). Gene expression profiles at days 58 to 86 were positively corre-
 327 lated with increased soil temperature, EC, and evolved CO₂, while study day 168 was
 328 associated with elevated levels of soil NO₃. Further, Permutational Analysis of Vari-
 329 ance (PERMANOVA) revealed that internal accumulated degree hours (ADH), soil
 330 temperature, pH, and EC significantly explained some of the variation in gene expres-
 331 sion profiles ($p < 0.05$). No other soil chemical variables were significant at $\alpha = 0.05$
 332 (Supplementary Material 3).

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 340 Overall, decomposition changed soil gene expression profiles over the one-year study
 341 relative to control soils. Differential expression analysis between decomposition and
 342 control soils identified 7,047 down-regulated and 38,425 up-regulated genes. Gene tran-
 343 scripts that were associated with control soils belonged to a wide variety of clusters of
 344 orthologous genes (COG) functional categories. Specifically, the top 20 genes whose
 345 expression was higher in control soils belonged to ten unique COG categories, includ-
 346 ing signal transduction mechanisms, transcription, and those of unknown function. In
 347 contrast, the top 20 genes whose expression was higher in decomposition soils only
 348 fell into four COG categories (Supplementary Material 4 A): 1) post-translational
 349 modification, protein turnover, and chaperones; 2) energy production and conversion;
 350 3) cell motility; and 4) carbohydrate transport and metabolism. The most common
 351 COG category represented in decomposition soils (80% of the top 20 genes) was post-
 352 translational modification, protein turnover, and chaperones. Within this category,
 353 several heat shock stress response genes were identified, including SSA2, HSP82, and
 354 clpB (Supplementary Material 5). Further investigation into these genes shows their
 355 expression increased in response to decomposition, typically reaching maximum tran-
 356 script levels around study days 58 and 86 (Fig 2). This corresponded to elevated soil
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temperatures below decomposing bodies between study days 12-80, with soil temperatures increasing to approximately 43°C [14], and maximum soil EC and minimum dissolved oxygen measurements between days 12 and 58 (Supplementary Material 1).

Figure 2: Normalized log2 expression of heat shock proteins identified by differential expression analysis comparing decomposition and control soils. Each panel represents a single heat shock transcript, labeled with query ID. Symbol color denotes if the sample is a control (CON, green), or one of three individuals: SP1 (orange), SP2 (purple), or SP3 (pink).



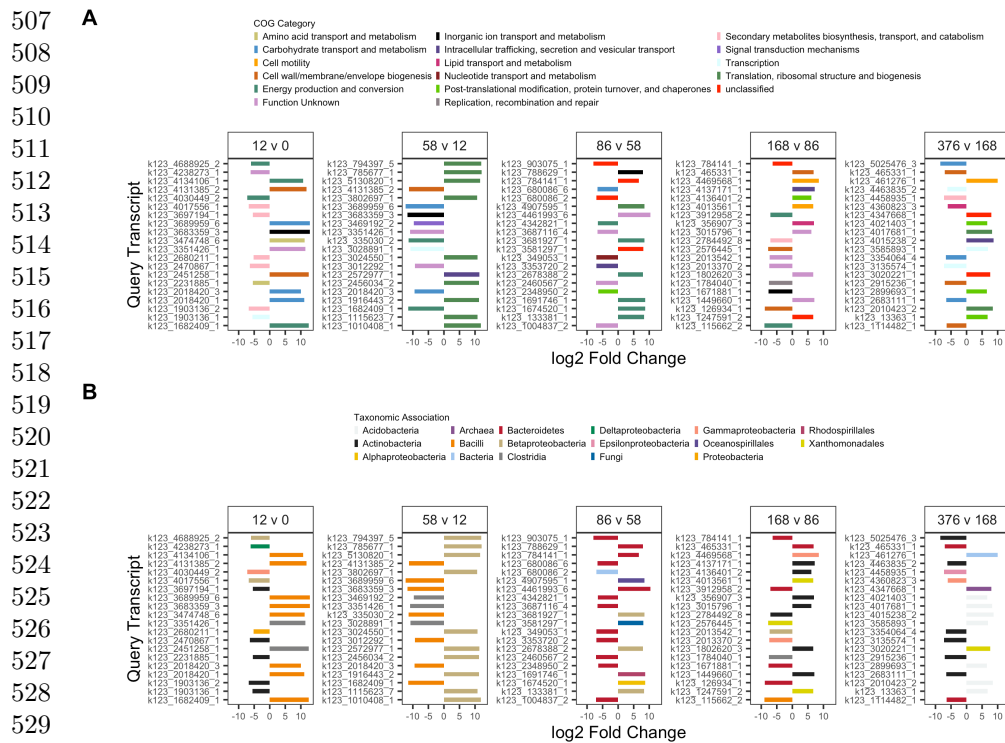
457 Taxonomy associated with top differentially expressed gene transcripts also dif-
 458 ferred between control and decomposition soils. The top 40 significantly differentially
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expressed gene transcripts in decomposition soils were associated with Fungi, *Actinobacteria*, and *Xanthomonadales*, while gene transcripts in controls were associated with *Acidobacteria*, *Cyanobacteria*, *Proteobacteria* (α , δ , γ), and *Planctomycetes* (Supplementary Material 4 B). 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.

Fate of decomposition products as evidenced in gene expression profiles over time

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 the lowest p-values from differential expression analysis (< 0.05), are reported in Supplementary Material 6 and Fig 3.

Figure 3: 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.



Expression of genes annotated with the COG categories cell wall/membrane/envelope biogenesis, inorganic ion transport and metabolism, and carbohydrate transport and metabolism increased from day 0 to 12. In contrast, expression of secondary metabolite biosynthesis, transport, and catabolism genes decreased during this period (Fig 3A). Transcripts from *Bacilli* and *Clostridia* increased, while transcripts from *Actinobacteria* decreased between study days zero and 12 (Fig 3).

Between days 12 and 58, 90% of the topmost upregulated genes were associated with the translation, ribosomal structure and biogenesis COG and all were taxonomically associated with *Betaproteobacteria* (Fig 3A,B). Many of these genes were annotated as ribosomal protein large (RPL), involved in ribosomal binding. Genes across multiple COG categories with taxonomic associations to *Bacilli* and *Clostridia* decreased

between study days 12 and 58, six of which were transcripts that previously increased
between days zero and 12 (Fig 3B, Supplementary Material 6).

Multiple transcripts associated with the energy production and conversion COG, as
well as transcripts annotated with the COGs inorganic transport and metabolism,
and translation, ribosomal structure and biogenesis, increased between days 58 and
86 (Fig 3A). Two of the upregulated energy and production and conservation tran-
scripts were associated with cytochrome c oxidase subunits in *Betaproteobacteria*,
while another was annotated as *hao*, encoding the enzyme hydroxylamine dehydroge-
nase which is involved in conversion of hydroxylamine to nitrite during nitrification
(Supplementary Material 6). Further investigation into hydroxylamine dehydrogenase
showed a significant increase in *hao* transcripts at day 86 followed by subsequent
decreases at days 168 and 376 ($F = 4.183$; $p = 0.02$). This increase corresponded to
decreased soil ammonium levels and subsequent accumulation of nitrate (Supplemen-
tary Material 1). 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.

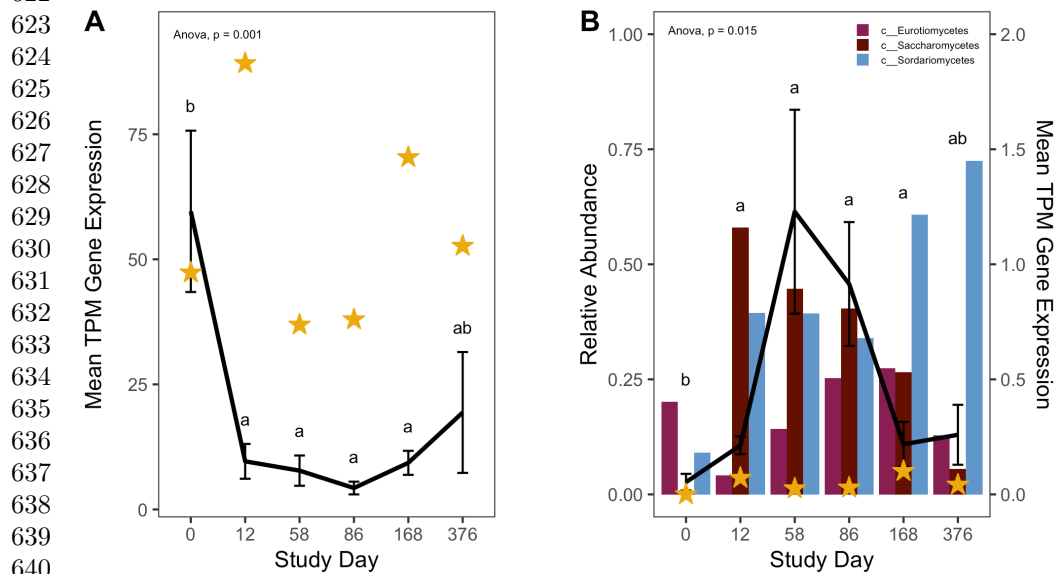
Differential expression comparing study days 86 with 168 and 168 with 376 identified
genes across a variety of functional categories, with many unclassified in the COG
database or with unknown function (Fig 3A). Expression of carbohydrate transport
and metabolism genes associated with *Bacilli* decreased between day 168 and 376.
Acidobacteria transcripts increased in decomposition-impacted soils between study
day 168 and 376, but were not associated with any single COG category (Fig 3B).

Carbon compound metabolism

We expected to observe increased expression of lipid metabolizing genes during active
and advanced decomposition as microbes degraded lipids deposited in the soil [18].

Therefore, we investigated changes in triacylglycerol lipase (enzyme commission number: 3.1.1.3) gene transcription in our soils. 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 bacteria decreased with decomposition time ($F = 10.392$; $p = 0.001$), while fungal lipase transcripts increased, reaching a maximum at study day 58 ($F = 4.509$; $p = 0.015$) (Fig 4).

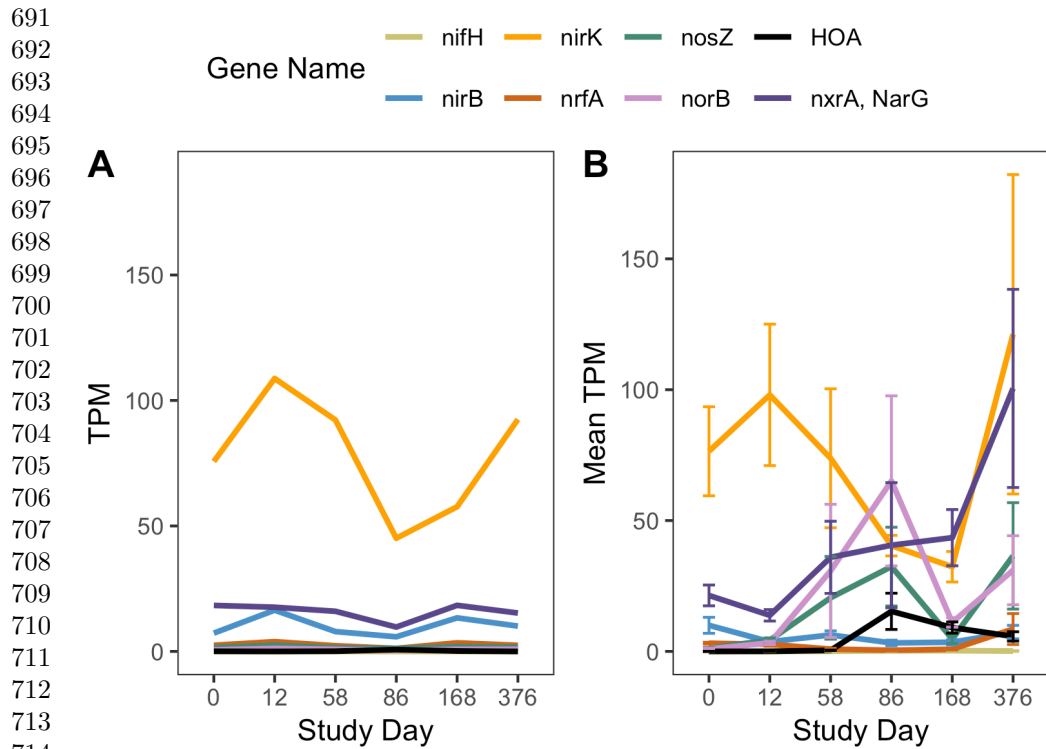
Figure 4: Mean transcript abundance, in transcripts per million (TPM), of all bacterial (A) and fungal (B) triacylglycerol lipase (EC 3.1.1.3) genes over time. Black lines (A, B) report mean and standard deviation of TPM from three individuals (black line), while gold stars denote mean TPM in control 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 in Taylor et al. (2024).



Nitrogen- and sulfur compound transformations

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*, *nrrA*, and *amoA*) were assessed using their enzyme commission numbers (Fig 5A,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 (Fig 3A, Fig 5B). Expression of *amoA*, encoding a subunit of ammonia monooxygenase, and *nrrA*, 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. Similarly, abundance of genes that encode for enzymes involved in dissimilatory nitrate reduction, *nirB*, and *nrfA*, was low for the first 168 days, with *nrfA* expression increasing at day 376 (Fig 5B).

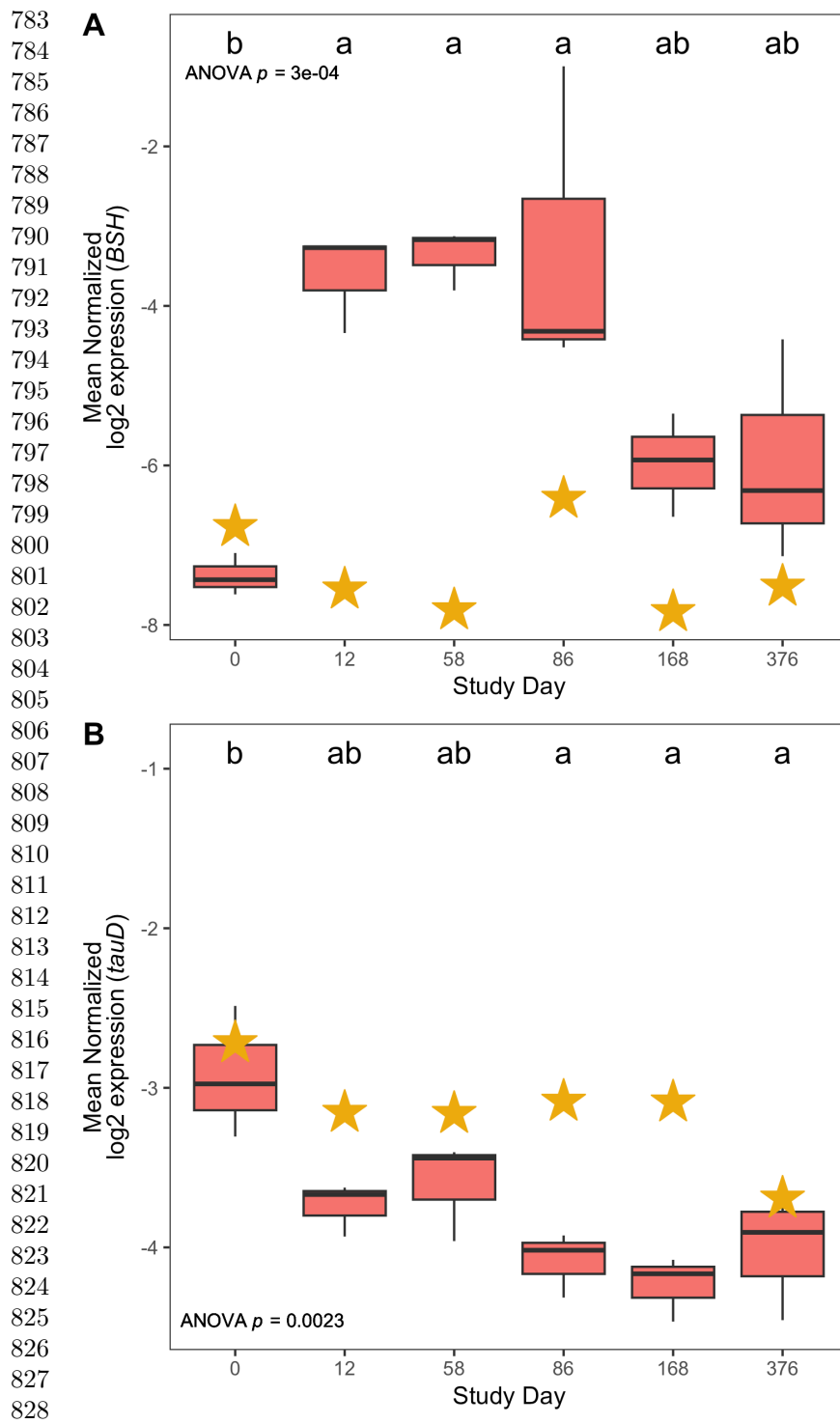
Figure 5: Mean gene expression, in transcripts per million (TPM), of commonly used marker genes for enzymes involved in nitrogen cycling over time in controls (A) and decomposition (B) soils. Data in B represent mean and standard deviation of TPM from three individuals.



Expression of genes involved in metabolism of nitrogen and sulfur-containing compounds were also impacted by human decomposition. Specifically, four of the top ten genes whose expression decreased at day 12 were related to taurine metabolism, with their annotations associated with *tauD*, encoding taurine dioxygenase. (Supplementary Material 6). Further investigation into *tauD* showed that mean expression of these genes decreased steadily over one year, beginning at day 12 (Fig 6B); 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 (Supplementary Material 7). 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 [21]. 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 (Fig 6A). Hierarchical liner mixed effects (HLM) models showed that both *tauD* (HLM $F = 7.356$, $p = 0.002$) and BSH ($F = 13.768$, $p < 0.001$) gene expression was significantly different over time (Fig 6A,B).

Figure 6: Mean bile salt hydrolase, BSH, (A) and *tauD*, taurine dioxygenase, (B) log2 normalized expression in controls (gold stars) and decomposition (boxplots) soils. 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.



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. From this, we found 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 revealed 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, in particular with respect to lipid, nitrogen and sulfur metabolism.

Decomposition impacted soil community gene expression, even after a year

Gene expression profiles remained altered after one year of decomposition. It is unclear if soil 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. The soil pH, EC, NH_4^+ , NO_3^- , and total nitrogen (TN) exhibited differences (although not statistical) in these soils following a year of decomposition, however bacterial and fungal community structures, as assessed by rRNA amplicon libraries, were still altered [14]. This indicates that decomposition can continue to structure microbial communities and impact their function for extended periods of time. While nutrient pools and communities both demonstrate less rapid change at later time points in the study, there is not evidence suggesting an arrival at a steady-state post-disturbance microbial community. In some studies, human decomposition can result in elevated carbon and nutrients (organic nitrogen, ammonium, nitrate, and phosphate) for longer than a year [3], suggesting decomposition events

875 have long lasting effects on the local ecosystem. Together, this has implications for
876 terrestrial ecosystem processing (*e.g.*, nutrient cycling, emission of greenhouse gasses,
877 etc.), as we show that decomposition alters functional metabolism pathways within
878 soil microbial communities. Further work with extended sample collections beyond
880 one year are needed to address how long microbial communities and their functions
881 are impacted.
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885 Bacteria, fungi, and archaea were all represented in expressed genes throughout
886 decomposition, suggesting that members of all three domains have the potential to
887 contribute to decomposition processes and nutrient cycling. While a majority of anno-
888 tated transcripts were identified as bacteria, fungal transcripts were the second most
889 abundant group. Fungal transcripts made up almost half (seven of the top 15) of the
890 significantly differentially expressed genes associated with decomposition-impacted
891 soils. Additionally, with respect to expression shifts between decomposition time-
892 points, fungal transcripts were among the topmost upregulated genes at study day
893 86. The presence of fungal transcripts is not surprising as fungi are key decomposers,
894 involved in the degradation of organic matter in terrestrial ecosystems [22]. It was
895 interesting to see an increase in certain fungal transcripts, such as lipase, at study
896 days 58 and 86 when soil oxygen began to recover. We would expect lipids to enter
897 the soil as tissues are broken down during decomposition, so we were surprised to
898 see bacterial lipase genes decrease during decomposition. This suggests that microbial
899 activity in decomposition soils may be constrained by the changing chemical environ-
900 ment, potentially altered oxygen levels in the case of bacterial lipase gene expression.
901 Prior work with these soils showed that soil oxygen concentration was a key driver of
902 changes in both bacterial and fungal community composition [14].
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Increased stress responses during decomposition	921
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Soil microbial communities expressed stress response genes in response to human	923
decomposition. Differential expression analysis identified increased expression of mul-	924
tipl heat shock proteins associated with the taxa <i>Xanthomonadales</i> , <i>Actinobacteria</i> ,	925
and fungi. Upon further investigation, expression of these genes increased through day	926
58 and remained high for the remainder of the year. Soil temperature was elevated rel-	927
ative to controls between study days 8 and 80, with maximum temperatures $>40^{\circ}\text{C}$,	928
while soil electrical conductivity increased up to 663 $\mu\text{S}/\text{cm}$ (16X higher than back-	929
ground) through day 58 before slowly decreasing through the remainder of the study.	930
Soil electrical conductivity (correlates with ionic strength [23] and can indicate soil	931
salinity) has previously been shown to increase in decomposition soils [8–10, 14]. As	932
a result, we would expect these microbes to be experiencing both heat and osmotic	933
stress during this period. Prior work has observed increased heat shock gene expres-	934
sion during salt stress in paddy soils [24] and the presence of both heat and osmotic	935
stress genes in desert soils along a salt gradient [25], suggesting saline conditions can	936
alter the expression of heat and/or osmotic stress genes. In our study we observed	937
that stress response within soil microbial communities is stimulated during human	938
decomposition, however, at this time, it is unclear if expression of these genes is in	939
response to heat stress alone, or in combination with osmotic stress.	940
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Increased expression of fungal lipase genes during	954
decomposition	955
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Human fat tissue contains lipids that are broken down during decomposition. There-	958
fore, we assessed expression of triacylglycerol lipase genes in decomposition soils. Our	959
results show that expression of triacylglycerol lipase genes was altered in response	960
to decomposition, and these shifts differed between bacterial and fungal transcripts,	961
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specifically bacterial triacylglycerol lipase transcripts decreased in response to decomposition, while fungal triacylglycerol lipase transcripts increased. Further, expression of these genes corresponded to changes in relative abundance of the fungal classes *Saccharomycetes*, *Sordariomycetes*, and *Eurotiomycetes* [14]. These fungi have been previously associated with decomposition soils [26, 27] and are known to contain triacylglycerol lipase genes in their genomes [28, 29], suggesting that they play a role in lipid degradation in decomposition soils.

Our observation of an overall decrease in triacylglycerol lipase transcripts contrasts with previous work by Howard et al. (2010) [18], who observed increased gene copy number of Group 1 lipase genes via qPCR during swine decomposition. Fatty acid composition differs in human compared to pig tissue [30], potentially altering the lipid profile available for microbes, leading to differences in decomposition products within the soil [17]. These products can then directly or indirectly alter community composition and/or activity of functional proteins via substrate availability or the chemical environment. Further, decomposition of humans and pigs resulted in increased pH in soils below pigs, and decreased pH below humans [17]. Altered pH and soil chemistry could result in a different functional potential and/or gene expression in decomposition-impacted soils. Many triacylglycerol lipases have a pH optimum that is neutral to basic [31–33], so cells may be decreasing expression under acidic conditions in human decomposition soils. Availability of lipid species and changes to pH may select for taxa that favor these substrates/pH conditions; for example, Mason et al. (2022) [12] suggested the abundance of the fungal taxa *Saccharomycetes* was related to antemortem BMI due to relative proportions of fat and muscle tissue.

Evidence for phased denitrification and nitrification

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 marker genes for nitrogen cycling was altered in decomposition soil and suggested nitrogen transformations during human decomposition are driven by soil oxygen concentrations with hydroxylamine as an important intermediate. We observed low or reduced expression of nitrification genes *nrrA* and *amoA* between days 12 and 86, during a period when oxygen was reduced to 39% - 85%. This was concomitant with accumulation of ammonium, which reached a maximum on day 12, and low nitrate conditions indicating that nitrification was inhibited. This period of reduced soil oxygen constraining nitrification was also described in a decomposition experiment with beaver carcasses Keenan et al. (2018) [8].

We observed increased expression of *hao*, which encodes the enzyme hydroxylamine dehydrogenase (HAO) at day 86 while oxygen was reduced (~85%). 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 [34]. 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 reported that the growth of the ammonia oxidizing bacteria *Nitrosomonas europaea* under anoxic conditions lead to accumulation of hydroxylamine in a chemostat bioreactor [35], 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 [34]. 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. Keenan et al. (2018) [8] also noted a brief increase in N₂O emissions, which suggests denitrification was occurring during this phase of reduced soil oxygen concentrations. As soils fully reoxygenated by day 168, we observed increased expression of genes encoding enzymes involved in aerobic nitrification, *amoA* and *nxrR*. Nitrification is an oxygen-dependent process which would be converting the accumulated ammonium to nitrate; the increase in nitrate concentrations may then serve as a substrate for denitrification. We observed increased expression of marker genes encoding all four enzymes in the complete dissimilatory 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 after oxygen is reintroduced to soils described in Keenan et al. (2018) [3, 8]. Together, gene expression patterns in our study provide further insight into nitrogen transformations in during vertebrate decomposition, suggesting an important role of hydroxylamine.

Increased expression of bile salt hydrolases

Sulfur is present in various organic molecules, including taurine, a sulfur- and nitrogen-containing acid involved in bile acid formation [21]. Taurine is present in the human body, where it can be absorbed from the diet or synthesized in the liver [36]. However, taurine is also produced as a byproduct of the deconjugation of bile salts via bile salt hydrolases (BSH) present in the anaerobic gut taxa *Lactobacillus* and *Clostridium* [21]. In our study, we observed increased expression of genes encoding BSH enzymes between days 12 and 86. Given that increased expression of BSH genes corresponded to the beginning of active decomposition, when decomposition products were observed

to enter the soil, and 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 concentrations in this study, our results correspond to previous decomposition studies that report accumulation of taurine in various organs and body regions [37–39] and soils [17, 40] during decomposition via metabolomics, and increased relative abundance of *Clostridium* and *Lactobacillus* within the body [41–43] and in decomposition soils [19] via DNA sequencing methods, including in these soils [14].

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 [44]. 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* transcripts associated with *Proteobacteria*, *Gammaproteobacteria*, and *Actinobacteria* whose relative abundance have been shown to remain consistent or increase during human decomposition [19], suggesting that *tauD* expression is downregulated under decomposition conditions. However, we noted that expression of *tauD* genes associated with fungi and a few *Betaproteobacteria* displayed increased expression at day 58, corresponding to increased expression of bile salt hydrolases (BSH) between days 12 and 86. The reduction in *tauD* expression may be due to increased sulfur availability. We did not measure sulfur species in this experiment; however, others have observed increased sulfur concentrations in decomposition-impacted soils [3, 7, 11]. Thus, sulfur scavenging pathways such as taurine desulfurization by TauD [45], whose genes are expressed under sulfur-limiting

1151 conditions, likely display reduced expression under sulfur replete conditions. Addition-
1152 ally, taurine may be processed through other pathways. For example, taurine can be
1153 deaminated by taurine dehydrogenase to produce sulfite and acetyl-CoA for carbon
1154 metabolism [44, 46]. Overall, our results suggest that human decomposition has poten-
1155 tial impacts on soil sulfur biogeochemistry through deposition of inorganic (sulfate)
1156 and organic (sulfur-containing amino acids) sulfur compounds.
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1161 1162 **Conclusion** 1163

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1165 This study represents a novel investigation of soil microbial gene expression dur-
1166 ing human decomposition. Metatranscriptomic analysis of soils from three human
1167 individuals over one year shows that decomposition impacted microbial community
1168 gene expression profiles, exhibiting functional shifts over time. This included altered
1169 expression of genes involved in lipid, N and S metabolism as microbes processed
1170 the nutrient-rich tissues of the human body. Additionally, we noted that function-
1171 ality within decomposition-impacted soils was still affected after one year and had
1172 not returned to starting or background conditions. Together, these results show that
1173 vertebrate decomposition has lasting impacts on local soil ecosystems, including soil
1174 microbial communities. These results have important implications for understanding
1175 biogeochemical changes due to vertebrate mortality events in terrestrial ecosystems.
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1184 **Materials and Methods** 1185

1186 1187 **Study design** 1188

1189 In February 2018, three deceased male human subjects (hereafter, “donors”) were
1190 placed supine on the soil surface at the University of Tennessee Anthropology Research
1191 Facility (ARF) and allowed to decompose. Located in Knoxville, TN (35° 56’ 28” N,
1192 83° 56’ 25” W) the ARF is a roughly 2-acre outdoor facility dedicated to studying
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human decomposition [47]. 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 [14, 47]. A site that had not been previously
exposed to decomposition was used for this study.

The decomposition field experiment is fully described in Taylor et al. (2024) [14].
Briefly, experiments were conducted in a block design, where each block consisted of
one decomposition site and one control site [14]. 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 [14]. 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) [14].
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 [14].

Sampling and physiochemistry

Decomposition of all subjects was observed for one year. During the one-year study
period, soils were sampled at 20 timepoints chosen to correspond with morphological
stages of decomposition as described by [48]. 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. All soil cores were taken using a 1.9 cm
(3/4 inch) diameter soil auger to a depth of 16 cm. Soils were divided into two depth
fractions: 0-1 cm (interface) and 1-16 cm (core) for the analyses reported in Taylor et
al. (2024) [14]; the entire 0 to 16 cm core was used for this current study. Decomposi-
tion soils were taken from directly beneath the cadavers, taking care to not re-sample

1243 the same location more than once. At the time of sampling, soil dissolved oxygen was
 1244 measured in triplicate using an Orion Star™ A329 pH/ISE/Conductivity/Dissolved
 1245 Oxygen portable multiparameter meter (ThermoFisher) [14].
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 1248 A subset of 6 study timepoints were chosen for metatranscriptomics analysis. Study
 1249 days 0, 12, 58, 86, 168, and 376 were chosen as they represented distinct morphologi-
 1250 cal and soil biogeochemical stages during decomposition. Study day 0 was chosen as
 1251 a baseline sample prior to cadaver placement. Study day 12 was the start of active
 1252 decomposition and corresponded to maximum soil ammonium concentrations and
 1253 minimum soil oxygen (approximately 39%). Study day 58 was chosen as this sample
 1254 represented the pH minimum, and respiration and soil temperature were at a maxi-
 1255 mum [14]. Additionally, ammonium concentrations began to decrease around day 58.
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 1257 Study day 86 was when soil oxygen started to recover and nitrate levels began to
 1258 increase. Study day 168 was chosen as nitrate was at its maximum and soil dissolved
 1259 oxygen had returned to 99%. Finally, day 376 was chosen to represent the end of the
 1260 study, 1 year since cadaver placement. Each study day was represented by four soil
 1261 samples for RNA extraction: one pooled control sample which was a mix of the three
 1262 control locations, plus one sample from each of the three donors, yielding a total of
 1263 24 samples for this study.
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 1266 Soil samples were transported back to the University of Tennessee (Knoxville, TN)
 1267 and processed within 24 hours of collection. Soils were homogenized by hand to remove
 1268 insect larvae, roots, rocks, and other debris (> 2 mm). A subset of soils were used
 1269 to measure pH, electrical conductivity (EC), and evolved CO_2 as described in Taylor
 1270 (2024). Soil nitrogen species (NH_4^+ , NO_3^-) and total carbon (TC) and nitrogen (TN)
 1271 were measured in all soil samples as described in [14]. Reported values for soil phys-
 1272 iochemistry represent the full 16 cm core; estimated by summing interface and core
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values reported by Taylor et. al, (2024) [14] in 1:16 and 15:16 ratios, respectively. Control reported here are means of the three experimental controls that were unimpacted by decomposition.

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 et al. (2024).

RNA Extraction and Sequencing

RNA was extracted from 2 g of soil using Qiagen's RNeasy® PowerSoil® Total RNA kit. Manufacturer's instructions were followed with a few modifications. Soils became saline during decomposition; therefore, we followed the 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 the Qubit® RNA HS assay (catalog no. Q32852) with 1 µl of RNA. DNA contamination was removed by DNase treating RNA extracts twice using Qiagen's DNase Max® kit in 50 µl reactions. RNA concentrations were remeasured after DNase treatment. PCR with V4 16S rRNA gene primers [49, 50] 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.

1335 Bioinformatics

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1337 Illumina sequencing of the 24 libraries yielded a total of 5,073,476,730 reads, or
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1339 2,536,738,365 paired reads, with a mean of 105,697,432 paired reads per sample. Read
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1341 quality control (QC) was conducted in KBase [51] using Trimmomatic [52]. Paired
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1343 fastq files were imported to KBase through Globus. Poor quality reads were removed
1344 (4.7% of all reads), and adapters trimmed via Trimmomatic (v0.36) using default set-
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1346 tings and the TruSeq3-PE-2 adapter file, resulting in 4,834,123,062 total reads. After
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1348 QC check with FastQC, trimmed libraries were exported as fastq files from KBase
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1350 through Globus. Remaining ribosomal RNA was filtered using bbmap (maxindel =
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1352 20, minid = 0.93) from the Joint Genome Institute's (JGI) bbttools suite [53]. Fil-
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1354 tering of ribosomal RNA further removed 7.3% of reads, leaving 4,479,804,360 reads
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1356 for assembly. Following this step, all non-ribosomal reads from all 24 samples were
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1358 merged into one file. Reads were then co-assembled into contigs using the de novo
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1360 assembler MEGAHIT (v1.2.9) [54] (-12 -k-min 23, -k-max 123, -k-step 10).

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1360 Gene identification and annotation from co-assembled contigs was performed using
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1362 Prodigal [55] and eggNOG mapper [56], respectively. Briefly, the fastq containing all
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1364 contigs was submitted to Prodigal (v2.6.3) for protein coding gene predication for a
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1366 meta-sample (-p meta -f gff). After co-assembly, a total of 6,257,674 proteins were
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1368 identified by Prodigal. Next, predicated genes were functionally and taxonomically
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1370 annotated using eggNOG mapper (v2.1.6) using basic settings to perform a diamond
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1372 blastp search [57]. From this, 1,048,573 proteins were annotated by eggNOG-mapper
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1374 (16.7%). Most of the annotated proteins were taxonomically annotated as bacteria
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1376 (91.3%), followed by eukaryotes (7.6 %), and archaea (0.81 %). Of the 7.6% of eukary-
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1378 otic proteins, 64.4% (4.9% of all proteins) were annotated as fungi. For this study,
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1380 genes of interest included all bacterial, archaeal, and fungal proteins, therefore all
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1382 non-fungal eukaryotic proteins (32,004) were removed prior to downstream analysis.
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1384 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/>). The percent of reads mapped to genes of interest ranged from 21% to 38% between samples, with an average of 31% reads mapped. Gene counts were then combined in a single file and used for downstream analyses in R.

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 [58] and limma [59] following a modified pipeline by Phipson et al. (2020) [60]. 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(), using study day as the comparison group.

Raw counts were then log2 normalized and gene expression profiles compared via multidimensional scaling (MDS) and hierarchical clustering. Multidimensional scaling (MDS) was conducted using plotMDS() from the limma package to assess differences between samples. MDS values were extracted from the MDS object, and the first two dimensions plotted using ggplot2 [61]. We also assessed the relationship between gene expression profiles and changes in the soil environment using canonical correspondence analysis (CCA). Environmental variables of interest included decomposition time in accumulated degree hours (ADH) based on ambient temperatures, ADH based on internal gut temperatures, ADH based on soil temperatures, gravimetric moisture, pH, electrical conductivity (EC), dissolved oxygen (DO), CO₂ (mol gdw⁻¹), NH₄ (mg gdw⁻¹), NO₃ (mg gdw⁻¹), N %, C %, and CN ratio. First, permutational multivariate analysis of variance (PERMANOVA) with adonis() (vegan v2.6.7) [62] was used to identify significant soil parameters. Then the vegan functions cca() and scores() were

1427 applied to run the CCA and extract scores, respectively. Scores for the first two
1428 dimension were plotted using ggplot2, with loadings extracted from the CCA biplot.
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1431 For differential expression analysis, raw filtered reads were normalized using edgeR's
1432 trimmed mean of M values (TMM) normalization using the function calcNormFac-
1433 tors(). TMM normalized reads were then log2 transformed using limma's voom() and
1434 differential expression assessed. Empirical Bayes shrinkage was used correct to p-
1435 values for false discovery rates. The topmost up and down regulated genes for each
1436 comparison, determined by log2 fold change and adjusted p-values, were then reported.
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1440 Expression of certain genes were assessed after performing transcripts per million
1441 (TPM) normalization and statistical analyses with a combination of analysis of vari-
1442 ance (ANOVA) and post-hoc Tukey tests. ANOVA across all timepoints were applied
1443 to hierarchical linear mixed effects models to account for repeated sampling within
1444 each donor block.
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1451 **Data availability**

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1453 Raw RNA sequence files from the Illumina Novaseq are available at the National Cen-
1454 ter for Biotechnology Information's (NCBI) Sequence Read Archive (SRA) as a part
1455 of [BioProject PRJNA1066312](#) under BioSample accession numbers SAMN45195141-
1456 SAMN45195164. Additional datasets supporting the conclusions of this article are
1457 available on [GitHub](#).
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1463 **Code availability**

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1465 The code used for analysis and to generate figures are available on [GitHub](#).
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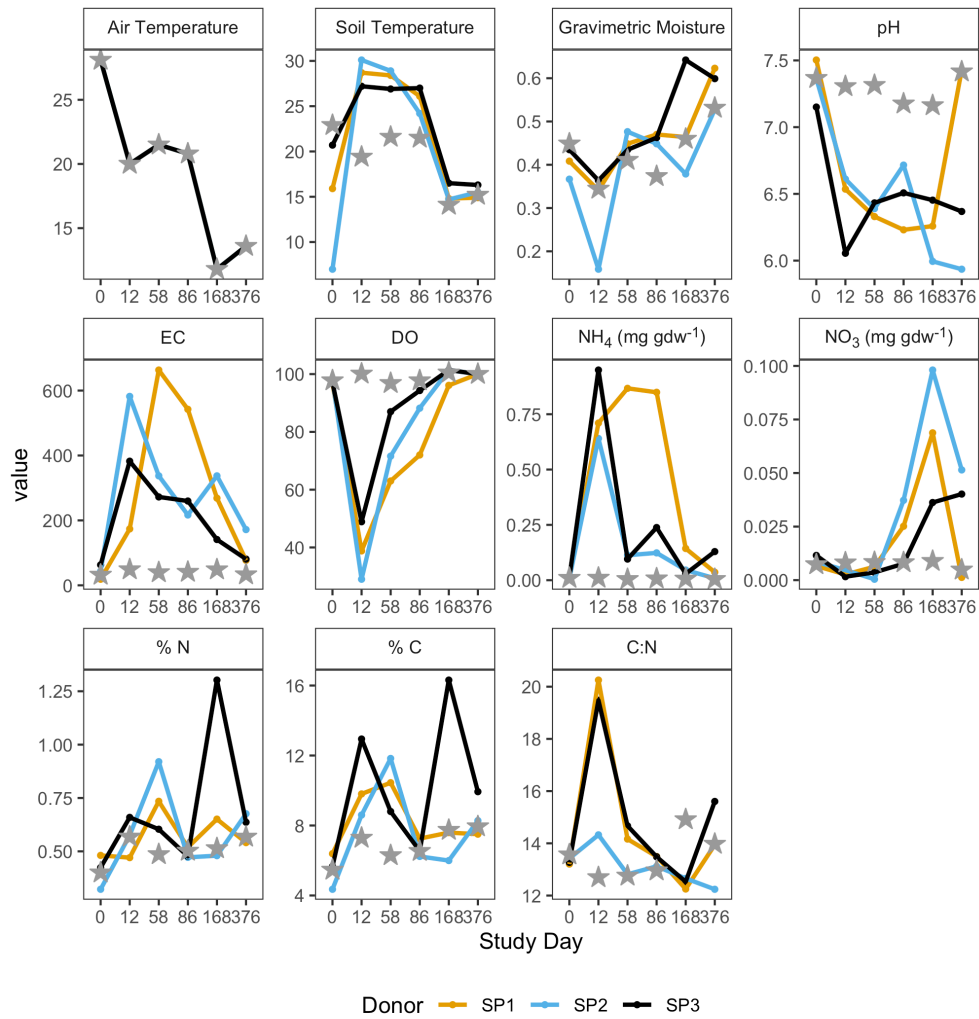
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1808 Acknowledgements

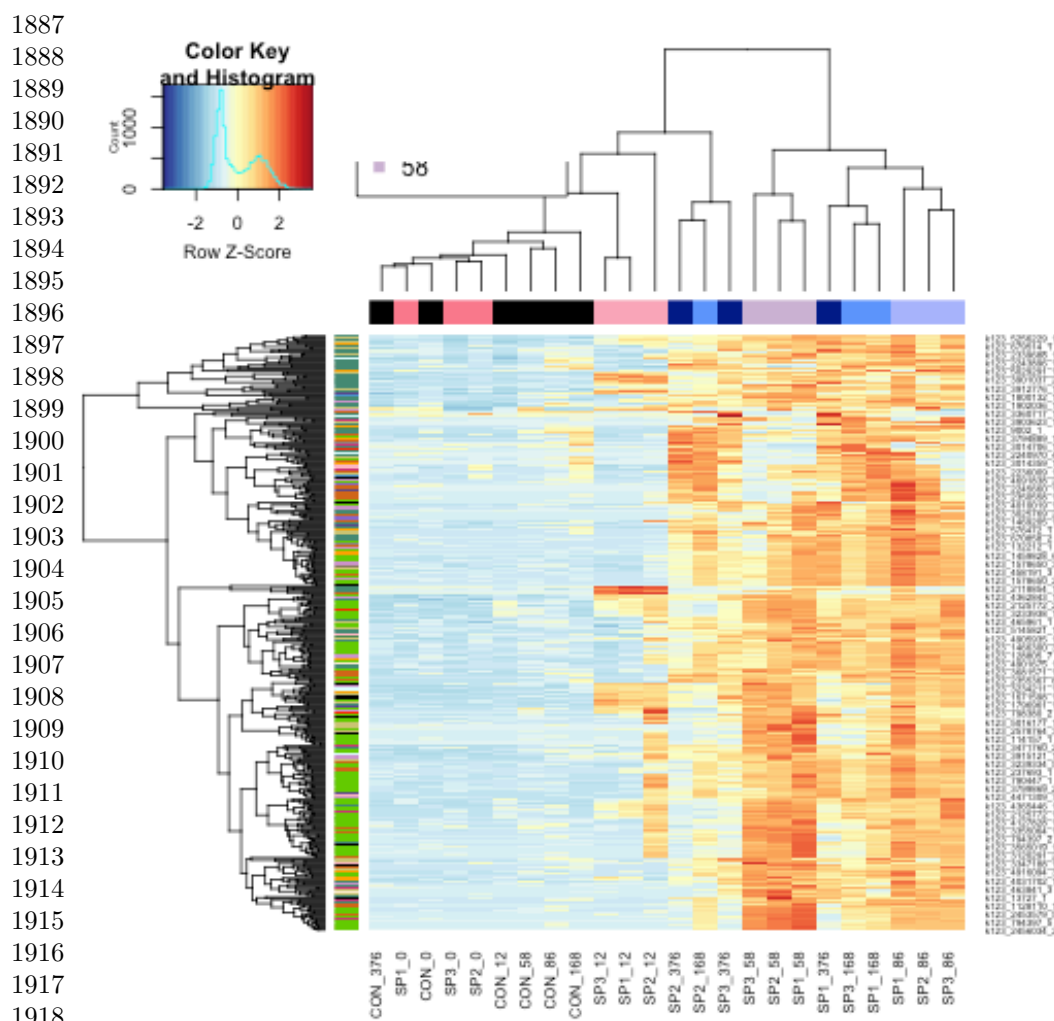
1809
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1820 Supplementary Information

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Supplementary Material 1: Figure S1. Soil physiochemical parameters in decomposition soils during the one-year study. Data is shown for each individual donor: SP1 (gold), SP2 (blue), and SP2 (black). Values for the full 16 cm core samples were estimated by summing values interface (0-1 cm) and core (0-16 cm) reported by Taylor et al, (2024) in 1:16 and 15:16 ratios, respectively. Controls reported here are means of three experimental controls that were unimpacted by decomposition and are represented by stars.



1919 Supplementary Material 2: Figure S2. Hierarchical clustering heatmap showing the log
1920 counts per million (CPM) of the top 500 most variable genes across samples. Variable
1921 genes were determined by selecting genes with the highest variance in gene expression.
1922 Samples are clustered along the x-axis using Euclidean distances between samples and
1923 colored by study day.

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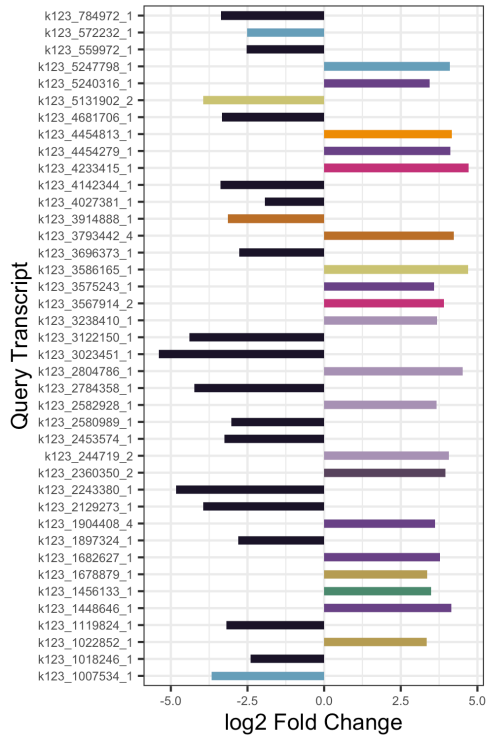
Table S1. Permutational analysis of variance (PERMANOVA) results identifying significant environmental parameters which explain some of the variation in soil gene expression profiles. Environmental parameter data is from Taylor et al. (2024).

Variables with $p < 0.05$ are indicated in bold.

Supplementary Material 3

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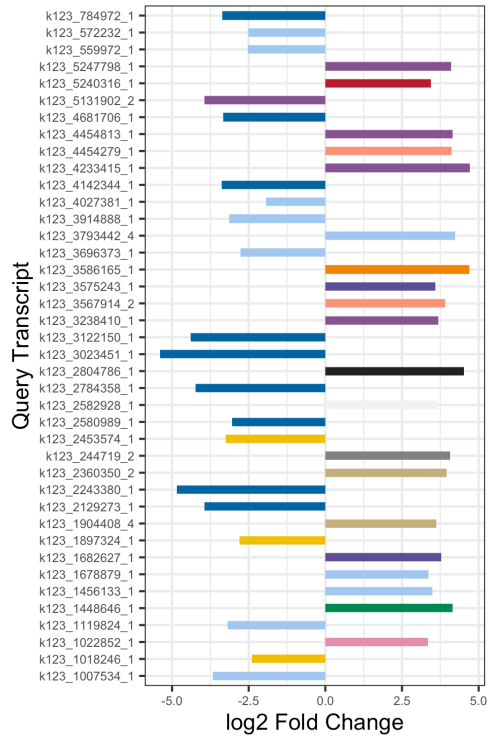
A



COG Category

- Cell motility
- Energy production and conversion
- Transcription
- Cell cycle control, cell division, chromosome partitioning
- Carbohydrate transport and metabolism
- Intracellular trafficking, secretion and vesicular transport
- Signal transduction mechanisms
- Translation, ribosomal structure and biogenesis
- Post-translational modification, protein turnover, and ch:
- Function Unknown
- unclassified

B



Taxonomy

- 1117|Cyanobacteria
- 1236|Gammaproteobacteria
- 135614|Xanthomonadales
- 2|Bacteria
- 200940|Thermodesulfobacteria
- 201174|Actinobacteria
- 203682|Planctomycetes
- 204432|Acidobacteriia
- 2157|Archaea
- 28211|Alphaproteobacteria
- 28221|Deltaproteobacteria
- 4751|Fungi
- 57723|Acidobacteria
- 976|Bacteroidetes

Supplementary Material 4: Figure S3. Top 40 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.

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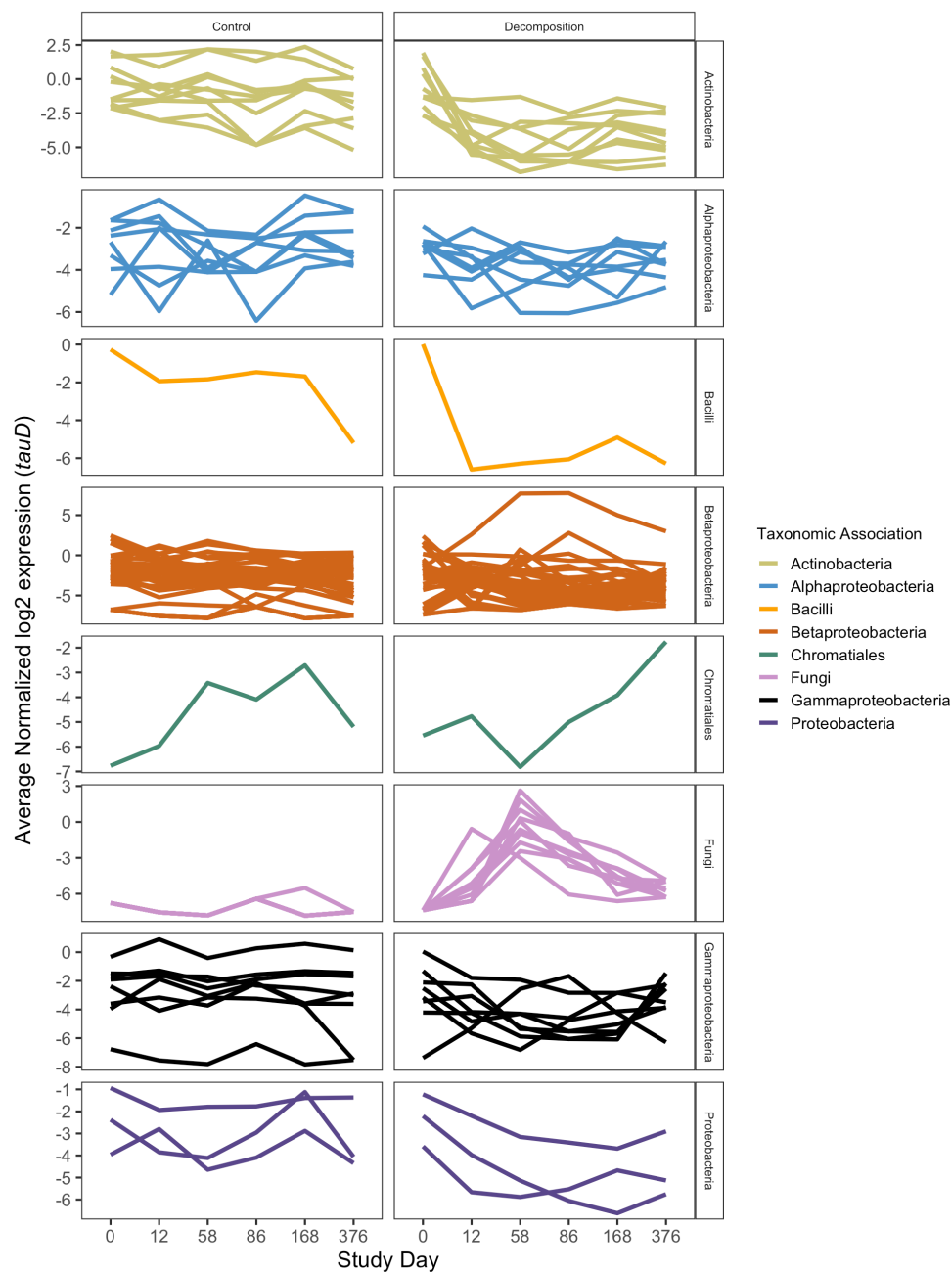
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Table S2. Top 20 most up- and down-regulated gene queries, determined by log2 fold change and adjusted p-values, in control relative to decomposition soils. Positive log2 fold change values represent genes whose expression was higher in control soils, while negative log2 fold change values were higher in decomposition soils. Taxonomic annotation, COG categories, gene description, gene names, and EC were assigned via eggNOG-mapper.

Supplementary Material 5

Table S3. 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.

Supplementary Material 6



Supplementary Material 7: Figure S4. Mean normalized log₂ expression of *tauD* genes by taxonomic association (color) in control and decomposition soils at each study day. Each line represents one *tauD* gene query, while color denotes taxonomic association as determined by eggNOG-mapper.