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Whole Orchard Recycling Effects on Soil Organic Matter and Microbial Community Dynamics Across Space, Time, and Genomic Scales

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Whole Orchard Recycling Effects on Soil Organic Matter and Microbial Community Dynamics
Across Space, Time, and Genomic Scales

By

HELIX ROBLES
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Soils & Biogeochemistry

in the

OFFICE OF GRADUATE STUDIES
of the
UNIVERSITY OF CALIFORNIA
DAVIS

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Abstract

Whole orchard recycling (WOR) is an increasingly adopted management practice in perennial agroecosystems that incorporates wood chip biomass into soils during orchard re-establishment. This one-time organic amendment repurposes trees at the end of their productive lifecycle and increases soil organic matter (SOM) contents with lignocellulosic carbon (C). Here, temporal responses of soil microbial community, particulate organic matter (POM), and mineral associated organic matter (MAOM) compositions to WOR were elucidated in fine sandy loam soils. This dissertation integrates (Ch. 1) amplicon sequencing, (Ch. 2) POM and MAOM fractionation, spectroscopy, stable isotope analysis, and (Ch. 3) genome-resolved metagenomics to characterize how WOR influences soil microbial communities and SOM composition across 4- and 15-year time scales in almond orchards within the Central Valley, California, USA.

Across a four-year time series, following WOR application to a commercial almond orchard, the soil fungal community composition exhibited immediate but transient shifts, while bacterial and archaeal communities were comparatively stable. WOR initially slowed microbial community turnover and enriched specific microbial taxa, particularly within *Proteobacteria* and *Ascomycota*, with these effects diminishing over time as communities converged toward control conditions. Over this four-year soil chronosequence, WOR significantly increased the average SOM content of 0 – 15 cm soil from 2.70% to 4.38% ($p = 0.002$). Experimental orchards, 4 and 6 years post-WOR, revealed increases in total carbon (TC) and total nitrogen (TN) within both POM and MAOM pools, suggesting enrichment of POM with plant-derived organic functional groups and enrichment of MAOM with microbially-derived organic functional groups. However, these effects appear to attenuate by 15 years in fine sandy loam soil, with distinct C:N ratios and isotopic signatures between POM and MAOM for all soils analyzed here.

Genome-resolved metagenomic analyses further revealed that WOR effects on bacterial and archaeal communities were most pronounced at intermediate time points (4- and 6-year-old orchards), which received the highest wood chip application rates (61 and 85 metric tons per acre, respectively). WOR-enriched metagenome-assembled genomes (MAGs) were dominated by heterotrophic taxa, including *Alphaproteobacteria*, *Gammaproteobacteria*, and *Thermoleophilia*, with putative functions in metabolizing lignocellulosic decomposition products and reductive N transformations. Although sparse, several WOR-enriched MAGs were positively associated with TC, TN, and aromatic organic functional group contents of SOM.

In synthesis, these findings support a successional decomposition framework, in which the soil fungal community is initially responsive to WOR, within 1 year, while the most abundant bacteria and archaea opportunistically await inputs of wood chip degradation products into the surrounding <2 mm soil. The presence of chitin degradation pathways in many WOR-enriched MAGs, found in 4- and 6-year-old orchards, further expands on the potential for soil bacteria to degrade fungal biomass, which may partially explain the attenuation of SOM content observed in the 15-year-old orchard. Together, this suggests that most microbial biomass produced during wood decomposition is likely subject to re-metabolization during the first two decades of a typical orchard lifecycle, with limited potential for wood chip derived-C to stabilize onto silt and clay particles within fine sandy loam orchard soils.

Chapter 1

Soil Microbiome Shifts Associated with Organic Amendment by Whole Orchard Recycling

Key Points:

- Soil fungal community composition responded most within the first year after whole orchard recycling (WOR), although resembled the control soil community after 4 years.
- Soil bacterial and archaeal community composition was not significantly shifted by WOR across the four-year soil chronosequence, however changes over time were significant.
- For fungi, WOR enriched *Ascomycota* and many unidentified fungal taxa in <2 mm soil, with significant reductions in Simpson evenness.
- For bacteria and archaea, WOR enriched *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacteroidia*, *Vicinamibacteria*, *Verrucomicrobiae*, and other heterotrophic taxa in <2 mm soil without significant changes in Simpson evenness.
- WOR slowed the temporal turnover rates of soil bacteria, archaea, and fungi during the first year following orchard establishment, although an increase in the time-decay slope was observed in the second year for the bacterial and archaeal community.

Abstract

Organic amendments offer opportunities to maintain agricultural sustainability by restoring soil organic matter (SOM). Whole orchard recycling (WOR) is an organic management practice that repurposes agricultural waste trees at the end of their productive life cycle by grinding and tilling wood chips into the soil surface before planting the next orchard. WOR provides a carbon (C) rich substrate, which has been observed to increase topsoil SOM content and stability of large macroaggregates, however the response of the soil microbial community to

WOR is unknown. Here, the microbial community composition of wood chip amended and unamended control soils were characterized over a 4 years chronosequence, following WOR in January 2018, by 16S rRNA gene (16S) region V4 and fungal nuclear ribosome internally transcribed spacer region 1 (ITS1) amplicon sequencing following WOR in January 2018. The WOR treatment had a significant effect on the fungal community composition in 2018 and 2020. This treatment effect was not observed for bacterial communities or in 2019 and 2021 fungal communities. Temporal turnover was strongest in 2018 for both bacterial/archaeal and fungal communities, with significantly steeper Bray-Curtis time-decay slopes in control soils than WOR soils (bacterial/archaeal: control slope = 0.000962 vs. WOR = 0.000729, $p = 0.00299$; fungal: control = 0.000782 vs. WOR = 0.000463, $p = 0.0379$). Turnover weakened over time, stabilizing by 2021 with similar slopes between treatments. MetaLonDA identified 130 differentially abundant ASVs ($>0.01\%$ mean relative abundance) between WOR and control soils, including 82 bacterial/archaeal ASVs (51 WOR-enriched, primarily *Alphaproteobacteria* and *Gammaproteobacteria*) and 48 fungal ASVs (24 WOR-enriched, primarily Ascomycota and unidentified fungi). These findings suggest that 1) WOR has the greatest impact on the topsoil microbial community within the first year following application, 2) initially slowing microbial community turnover while driving targeted ASV enrichment, and 3) the community composition shifts back towards control conditions during wood chip decomposition and subsequent land management events.

Frequent used abbreviations: whole orchard recycling (WOR), soil organic matter (SOM), soil organic carbon (SOC), carbon (C), nitrogen (N), 16S rRNA gene (16S), fungal nuclear ribosome internally transcribed spacer region 1 (ITS1), amplicon sequence variant (ASV)

Introduction

Soil health is defined as the continued capacity of soil to function as a vital living ecosystem that sustains plants, animals, and humans^{1,2}. Its assessment requires is a holistic manifestation of physical, chemical, and biological properties in relation to the ability of soil to perform ecosystem functions¹ such as regulating water, sustaining biodiversity, filtering pollutants, cycling nutrients, and providing structural support. Although soil health cannot be determined by a single metric; the soil organic matter (SOM) content is one key indicator³ that serves critical functions in nutrient storage, moisture retention, aggregate stability⁴, and diversification of microbial niche spaces through heterogenization of the soil matrix⁵. Cultivation of SOM within agroecosystems can also contribute to the mitigation of climate change-induced stresses, which include elevated temperatures⁶, variations in precipitation⁷, and exacerbated drought conditions⁸. As soil organic carbon (SOC) is the main component of SOM⁹, practices that encourage maintenance of SOC are essential for sustaining soil properties relevant to ecosystem functioning and crop growth. For instance, wood mulches in orchards have been observed to regulate soil temperature¹⁰, increase soil water holding capacity¹¹, and SOC stabilization within the mineral-associated fraction¹².

Whole orchard recycling (WOR) is an organic management practice in which trees at the end of their productive life cycle are ground into wood chips and tilled into the surface soil before planting the next orchard. Like other organic amendment practices, WOR offers solutions to restore soil health while repurposing agricultural waste materials. Incorporation of woody biomass provides the opportunity for tree crop systems to restore soil health by increasing carbon (C) contents and serves as a sustainable solution for tree disposal. WOR has become the only economically viable strategy available for orchard farmers in the San Joaquin Valley, CA, where

the California Air Resources Board has voted to end agricultural burning by 2025, due to worsening air quality^{13–15}. Studies on WOR have reported increased irrigation water use efficiency, increased formation and stability of SOC in large macroaggregates¹⁶, and long term (10 years) reduction in nitrate leaching potential¹⁷ without reductions in almond tree growth and yield¹⁸. However, WOR can temporarily reduce plant available nitrogen (N) in the first year because the high C:N ratio of the wood chip amendment (~160¹⁶) stimulates microbial activity¹⁹ and has been observed to increase gross N immobilization rates by 10-fold¹⁷. Thus, the effects of WOR are temporally dynamic because the huge one-time amendment of wood chips, which provide ~32 - 106 metric tons of C per hectare²⁰, progressively decompose over multiple agricultural growing years. Double-decay modeling projected that 49% of WOR biomass decomposes after 4 years, with 6.6% remaining after 20 years²⁰.

Amendments of woody biomass provide soil microbes with complex lignocellulose polymers (i.e., cellulose, lignin, and hemicellulose polymers), which may diminish microbial diversity and consolidate C flow through specific adapted taxa. For fungi, a strong preference of *Thelephorales*, *Atheliales*, and *Sebacinales* for coarse woody debris has been found, whereas *Ascomycetes* and *Agaricales* appeared more in mineral soil²¹. Recently shed plant litter has been found to cultivate saprotrophic fungi until the C:N ratio of the substrate decreases sufficiently with decomposition²². Decreases in organic matter content and quality (i.e., material having a lower quantity of nutrients and/or energy yield relative to metabolic input) with soil depth has been associated with decreases in microbial biomass, diversity, and activity^{22–24}. Bacterial communities also show successional stages along the decay process, which has been described by an initial high abundance of cellulolytic taxa shifting towards degraders of fungal mycelia²⁴. For bacteria, a preference for niches with certain nutrient contents and organic matter quality is

indicative of their ecological strategy²⁴. Abundant copiotrophic bacteria in litter and deadwood include phyla from *Proteobacteria* and *Bacteroidetes*²⁴. *Acidobacteria* show a high level of metabolic versatility that allows them to decompose complex C substrates²⁴. Members of *Bacteroidetes* have also been shown to be potent decomposers of cellulose and other biopolymers²⁴. However, microorganisms that degrade cellulose utilize extracellular reactions that yield free by-products which can promote interactions with noncellulolytic organisms²⁵, so it is unclear how WOR integration of woody biomass into the mineral soil will compare to the existing studies described here. These studies exemplify specific microbial taxa that are known to respond to woody C substrates, however limited research has specifically addressed the effects of WOR on microbial community composition and diversity in agricultural soils.

Soil microbial communities are responsive to agricultural management, in which genomic techniques have emerged as the current gold standard approach to unravelling spatial and temporal patterns in community composition²⁶. Spatially, soil microbial communities are often correlated with environmental conditions, such as soil pH²⁷, depth²⁸, and C availability²⁹. Temporally, microbial communities tend to turnover on time scales of days to months³⁰. The temporal turnover of specific microbial guilds also varies within microbial communities. At a coarse taxonomic level, soil fungi have been found to have a temporal turnover rate 2.5 times faster than soil bacteria within temperate forest soils³¹. It has also been suggested that soil fungal communities have greater intra-annual than inter-annual temporal differences, such that compositional differences among seasons is equivalent to differences observed over thousands of kilometers in space³². At a finer taxonomic level, ectomycorrhizal fungi have displayed slightly faster temporal turnover than soil saprotrophs, while yeasts behaved differently than other fungal guilds³¹. Bacterial oligotrophs, such as *Planctomycetes* and *Verrucomicrobia*, have displayed

lower temporal turnover than copiotrophs³¹, as copiotrophs are more sensitive to rapid fluctuations in soil C^{27,33}. These patterns, which are largely genomically based, portray a framework for conceptualizing soil microorganisms spatially and temporally, with examples of nuances that are often conglomerated when analyzing bulk soil samples.

The main goal of this work is to identify temporal trends in microbial community composition that are associated with WOR. In this study, a four-year chronosequence of soil microbial community composition was constructed by repeated sampling of a commercial almond orchard containing WOR amended blocks and unamended control blocks. Taxonomic and functional profiles of microbial communities were assessed by amplicon sequencing (V4 region of the 16S rRNA gene and fungal ITS region 1) to address the following research questions and hypotheses.

Research Questions and Hypotheses

Question 1) How does the soil microbial community structure change following WOR?

- **Hypothesis 1a)** WOR, which is both a mechanical disturbance and massive one-time C application, will cause changes in microbial community composition.
- **Hypothesis 1b)** Subsequently, the microbial community will continue to shift over time following decomposition of applied C.

Question 2) Which microbial taxa are differentially abundant between WOR and control treatments? How do their abundances change over time within treatments?

- **Hypothesis 2a)** Secondly, wood chips, which are composed of complex polymeric C, will favor increases in relative abundances of saprotrophic taxa.
- **Hypothesis 2b)** Thereby, WOR will reduce microbial diversity and evenness.

Question 3) Are there differences in the temporal turnover rates of WOR and control soil microbial communities?

- **Hypothesis 3a)** Thirdly, changes in relative abundances of saprotrophic microbial taxa, which are adapted to degrading complex organic polymers, will increase temporal turnover rates of the soil microbial community.
- **Hypothesis 3b)** As applied C is metabolized, a decline in abundances of soil saprotrophs and temporal turnover rate will be observed.

Methods

Site description:

The study is set in an operational commercial almond orchard spanning 13 hectares, situated in Parlier, CA, covering 4 consecutive growing seasons (March to November 2018 to 2021). Originally planted with Westerner Plums in 1998, plum trees underwent excavation in late 2017. Subsequently, they were ground using a horizontal grinder and spread across the field using modified manure spreaders. Following this, stubble was disked into the ground in early 2018. In January 2018, a mixture comprising 50% Nonpareil and 50% Monterey almond varieties on Brights-H5 rootstock was planted. The soil texture of the root zone primarily consists of fine sandy loam. The soil is classified as coarse-loamy, mixed nonacid thermic Typic Xerorthents of the Hanford series. The National Cooperative Soil Survey characterized the Hanford series to typically contain less than 1% SOM content, decreasing with depth³⁴. The orchard experiences a Mediterranean climate, with precipitation levels generally falling below almond evapotranspiration requirements for a significant portion of the growing season. On average, the annual rainfall and temperature stand at 285 mm and 17°C, respectively.

Overview of the WOR treatment and control:

The experimental design follows a randomized complete block design, as shown in the appendix (Figure A1.1), wherein WOR (i.e., wood chip amended soil) and control (i.e., unamended ‘standard practice’) soils represent the treatments. The treatments were sampled in three replicate blocks, each containing about six rows of 13 trees. In 2018, before orchard replanting, the WOR plots received a one-time application of recycled wood chips, which was estimated at 40 metric tons of wood chips acre⁻¹ application. During the study period, all trees in the field were fertigated with soluble 32% urea ammonium nitrate (UAN32, 32-0-0) and calcium ammonium nitrate (CAN-17) by a double line drip irrigation system. The cumulative annual synthetic N fertigation rates were estimated as 76.5 kg N ha⁻¹ in 2018, 32.4 kg N ha⁻¹ in 2019, 70.8 kg N ha⁻¹ in 2020, and 109.8 kg N ha⁻¹ in 2021. Additionally, the entire field received winter manure applications, which were estimated as 63.3 kg N ha⁻¹ in January 2018, 63.3 kg N ha⁻¹ in November 2018, 63.3 kg N ha⁻¹ in December 2019, and 84.5 kg N ha⁻¹ in December 2020. The specific N application times and rates are shown in Figure 1.1 below. The combined annual manure and synthetic N application rates total to 139.8 kg N ha⁻¹ for 2018, 95.7 kg N ha⁻¹ for 2019, 155.3 kg N ha⁻¹ for 2020, and 194.5 kg N ha⁻¹ for 2021.

Cumulatively, the C and N amendments for this specific trial begin with a land use history of semi-annual manure application in the winter of each year, the WOR treatment in 2018, and semi-monthly N-fertilizer application during the growing season. Assuming that wood is 50% C by dry weight³⁵, the 40 metric tons of wood per acre applied to the WOR treatment soils is approximately 49,421 kg C ha⁻¹. In comparison, assuming a C:N ratio of 20:1 for fresh cow manure³⁶, the entire field received approximately 1,266 kg C ha⁻¹ in Jan. 2018, 1,266 kg C ha⁻¹ in Nov. 2018, 1,266 kg C ha⁻¹ in Dec. 2019, and 1,690 kg C ha⁻¹ in 2020. These calculations

were estimated from the annual manure N fertigation rates, which were 63.3 kg N ha⁻¹ for 2018, 63.3 kg N ha⁻¹ for 2019, 63.3 kg N ha⁻¹ for 2020, and 84.5 kg N ha⁻¹ for 2021 growing seasons. Across the entire 4-year sampling period, both treatments received approximately 333.3 kg synthetic N ha⁻¹, 274.1 kg manure N ha⁻¹, and 5,488 kg manure C ha⁻¹, whereas the WOR treatment soils received an additional 49,421.03 kg wood C ha⁻¹. The applications of manure relative to the soil sampling time points are conceptualized in Figure 1.1 below.

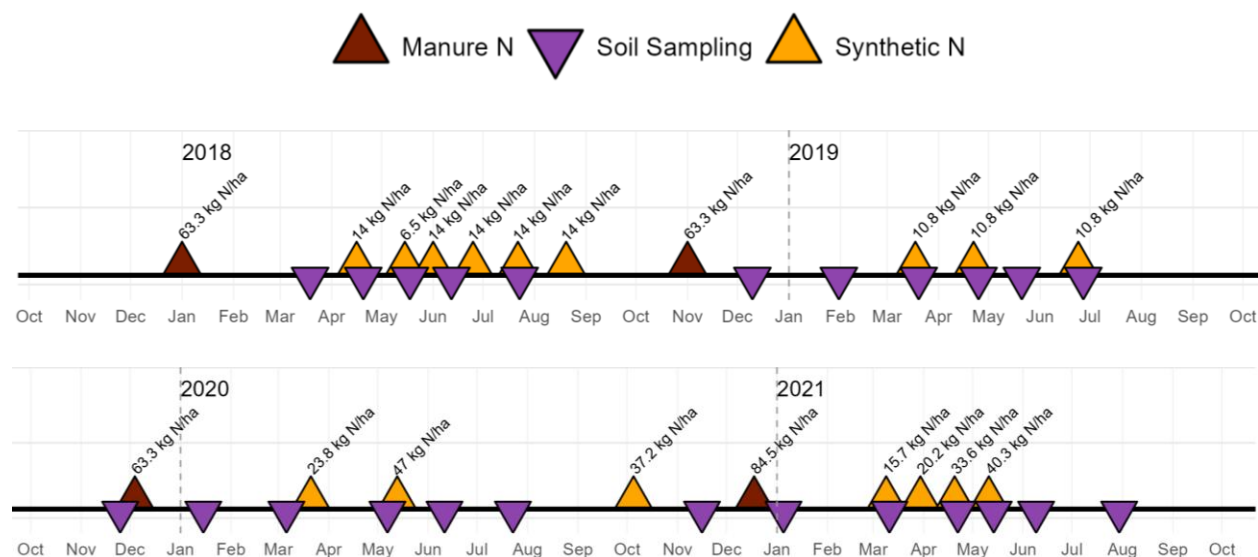


Figure 1.1: Timeline of N fertilization events and soil sampling time points. The N fertilization events were applied to the entire almond orchard, while soil samples were collected from the replicate treatment blocks shown in the appendix (Figure A1.1). The winter manure N application events (brown triangles), soil sampling events (purple triangles), and synthetic N application events (orange triangles) are organized to scale chronologically, with annotations of associated N application rates.

Soil sampling scheme:

Composited soil samples (0-15 cm) were collected monthly during the 2018 - 2021 agricultural growing seasons, along with an additional off-season winter sample. The specific months sampled for each year are shown in Figure 1.1 and the exact sampling dates are displayed along the x-axis of Figure 1.4. For each replicate, three subsamples within a 1-meter radius from the center of randomly selected trees formed a composite soil sample. A portion of

the composite sample was frozen in the field, by dry ice and isopropanol slurry, for DNA extraction. Soil to be used for physical and chemical characterizations was stored in coolers and sieved to <2 mm after transport.

Data Collection and Analysis:

Soil organic matter (SOM) content (g SOM / g dry soil) was measured by loss on ignition. Briefly, 10 g of dried and >2 mm sieved soil was combusted within a muffle furnace at 550C. The mass loss was measured using a three decimal point scale. DOC content (mg C g⁻¹ dry soil) was measured on 0.5 M K₂SO₄ soil extracts using a Shimadzu TOC-L analyzer. Briefly, 40 mL of 0.5 M K₂SO₄ solution was added to 8.00 g of dry soil. This was shaken on a lateral shaker at 250 rpm for 1 hour, centrifuged at 5000 rpm for 5 minutes, and filtered using an ashless Whatman 41 filter. DON content (mg N g⁻¹ dry soil) was measured on the same 0.5 M K₂SO₄ soil extract collected for DOC and using the Shimadzu TOC-L analyzer. To measure both C and N, the TOC-L was run using autosampler (ASI-L), high salt combustion tube, and total nitrogen unit (TNM) attachments. Soil pH was measured using a 1:1 soil and DIW extract.

Statistical analysis of SOM, DOC, and DON were conducted in R version 4.5.3 using the package lme4. Here, three SOM observations were identified as outliers and removed by the thresholds 10% > SOM > 0.001%, encompassing WOR.4 Day 0 (24% SOM), WOR.2 Day 402 (15% SOM), and Control.1 Day 0 (0.000897% SOM). Linear mixed-effects models were used to evaluate the treatment, time, and interaction on response variables. Treatment and sampling Day were included as specific fixed effects, while Block and the Block x Treatment interaction were included as random effects to account for the experimental design. Response variables were log₁₀-transformed when necessary to meet the assumptions of normality and homoscedasticity, as assessed by visual inspection of residuals versus fitted plots, Q-Q plots, and residual

histograms. Significance of fixed effects was determined using likelihood ratio tests based on chi-squared (χ^2) statistics, with $\alpha = 0.05$. All reported values correspond to models that satisfied assumptions and achieved appropriate fit.

DNA extractions were performed on 0.5 g of soil for 144 total samples using an MP Biomedical FastPrep Soil DNA extraction kit for Soil (MP Biomedicals, Irvine, CA, USA) and then purified via the OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, USA). DNA quality was assessed via gel electrophoresis and quantity measured using the Qubit™ dsDNA High Sensitivity assay (Invitrogen, Carlsbad, CA, USA). The microbial community composition was assessed by 16S rRNA gene and ITS amplicon sequencing. PCR amplification of the V4 region of the 16S rRNA gene was used to assess the bacterial and archaeal community composition³⁷. PCR amplification of ITS region 1 was used to assess the fungal community composition³⁸. PCR was performed in triplicate 25 μ L reactions using DreamTaq™ Hot Start PCR Master Mix (ThermoFisher Scientific, Waltham, MA, USA) with 400 ng μ L⁻¹ BSA and 5 ng of template DNA. No template controls were also amplified. Fungal ITS-1 amplicons were generated with a two-step PCR protocol using dual barcoded primers with heterogeneity spacers and Illumina adapters³⁹⁻⁴¹. In the first step PCR, the fungal ITS-1 region was amplified in 25 cycles using 800 nM each of tagged ITS1f⁴² and ITS2⁴³ primers using the following thermal cycling parameters: initial denaturation at 95°C for 2 minutes, 30 cycles of denaturation at 95°C for 30 seconds, primer annealing at 59°C for 30 seconds, and extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. The second step reaction consisted of 10 additional cycles using 1 μ L of the first PCR reaction as template and 800 nM of tagged barcoded primers with Illumina adapters; thermal cycling parameters were the same as above, with the exception of a 55°C annealing temperature. Forward-barcoded 515F and 806R primers⁴⁴ at a final

concentration of 500 nM each were used to amplify the 16S rRNA gene v4 region using the thermal cycling parameters described above and a primer annealing temperature of 55°C. For both primer sets, the triplicate PCR reactions from each sample were combined, checked via gel electrophoresis, and quantified via Qubit™ assay. PCR products from each sample were pooled in equimolar ratios into a single library, which was purified using AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA). Bacteria/archaeal and fungal libraries were sequenced (2 x 250 bp paired end reads) on an Illumina MiSeq (Illumina, Inc., San Diego, CA, USA) at the University of California Davis DNA Technologies Core facility.

Sequencing and Processing:

Samples were submitted to the UC Davis DNA Technologies Core Facility for sequencing on an Illumina MiSeq platform having 2 x 250 bp paired end reads. Raw sequence data was processed into amplicon sequence variants (ASVs) using dada2⁴⁵ and R⁴⁶ on the USDA high performance computing cluster Ceres. Fungal ASVs were classified via BLAST search against the NCBI nucleotide database⁴⁷. Bacterial and archaeal ASVs were classified against the SILVA rRNA database version 138⁴⁸ using the naïve Bayesian classifier with a cutoff of 0.80⁴⁹. Finally, the libraries were filtered to remove ASVs that were abundant in the no template control PCRs, classified as chloroplast or mitochondria, and present at sequencing depths of less than 10 reads. This filtering resulted in library sizes of 4,865,047 and 6,603,623 sequences for bacterial/archaeal and fungal community profiles, respectively.

Processing and analysis of microbial composition and structure was performed separately the 16S rRNA gene and ITS1 amplicon libraries. The package phyloseq version 1.48 was used to import ASV and taxonomy tables into R, normalize libraries, and for other data manipulations⁵⁰.

Bacterial/archaeal and fungal libraries were normalized to 4,730 and 12,104 sequences, respectively for alpha and beta diversity analyses.

Community Analysis and Statistics:

To determine if soil microbial beta-diversity changed significantly between treatments and over time in each experiment, the software Primer-e v7⁵¹ was used to perform a permutational analysis of variance (PERMANOVA) on Bray-Curtis dissimilarity matrices with treatment and sampling date as fixed factors and the Treatment:Block interaction as a random factor that was nested within treatment. For significant time or interaction effects, post hoc PERMANOVAs were performed within Primer-e v7 by permutation of residuals under a reduced model and a type III (partial) sum of squares. Principal coordinates analysis (PCoA) performed with vegan and plotted with ggplot2⁵² was used to visualize the Bray-Curtis distance matrices of fungal and bacterial/archaeal communities from each treatment. For each pair-wise comparisons of samples within the Bray-Curtis dissimilarity matrix, “days apart” was calculated as the absolute value of the difference between each sample pair. The temporal turnover figures were generated by plotting the pair-wise Bray-Curtis dissimilarity values on the y-axis by days apart on the x-axis⁵³. Differentially abundant bacterial/archaeal and fungal ASVs between treatments (WOR and control) and over all 24 time points (4 years * 6 months) were detected using the R package MetaLonDA⁵⁴.

Alpha diversity was calculated as Hill numbers q0 (richness), q1 (Hill-Shannon), and q2 (Hill-Simpson) using the R package HillR⁵⁵. To test significant differences in alpha diversity measures, a linear mixed model (lme4; degrees of freedom method by Kenward-Roger) was implemented with main effects of ‘Treatment’ and ‘Date’, an interaction of ‘Treatment × Block’, and ‘Block’ as random effects ($\alpha = 0.05$)⁵⁶. Models were compared using chi-squared (χ^2)

statistics to portray differences in deviance between models and determine p- values ($\alpha = 0.05$) based on likelihood ratio test comparisons⁵⁶. Multiple comparisons were determined using contrasts and Tukey's test for mean separation (see supplemental data for post-hocs), and significant differences are presented in the results. The bacterial/archaeal and fungal species richness measures, q_0 , was not transformed, however the Shannon diversity measure, q_1 , was logit transformed to attain normality. The figures and text shown display the untransformed values.

Results

Whole orchard recycling (WOR) treatment significantly increased soil organic matter (SOM) ($X^2 = 9.81$, $p = 0.0017$), dissolved organic carbon (DOC) ($X^2 = 12.084$, $p = 0.0005$), and dissolved organic nitrogen (DON) contents ($X^2 = 4.1382$, $p = 0.042$) over the 4-year chronosequence, as compared to the unamended control. Across all sampling years, the average SOM content was 2.70% in control soils and 4.38% under WOR treatment. Overall, SOM and DON contents varied more by WOR treatment, while DOC varied more by time ($X^2 = 23.91$, $p = 1.01e-06$). Time did not significantly influence variance of SOM ($X^2 = 0.567$, $p = 0.4514$) and DON ($X^2 = 0.1761$, $p = 0.6748$). Significant interactions between treatment and time were not observed for SOM ($X^2 = 0.0054$, $p = 0.9$), DOC ($X^2 = 0.0793$, $p = 0.7782$), and DON contents ($X^2 = 0.8669$, $p = 0.3518$).

SOM contents in the 0 – 15 cm layer ranged from 1.5 – 5.0 % in the control and 2.2 – 8.0 % in WOR treated soil (Figure 1.2A). The highest levels of SOM were observed in soils treated by WOR 1-year ago (2019). Dissolved organic C (DOC) concentrations ranged from 0.05 – 0.45 mg C g⁻¹ dry soil in the control and from 0.06 – 0.55 mg C g⁻¹ dry soil under the WOR treatment (Figure 1.2B). Average DOC concentrations were 0.14 mg C g⁻¹ in control soils and 0.23 mg C

g^{-1} under WOR, with the highest concentrations observed in 2018 and 2019 samples. DON concentrations ranged from 0.01 – 0.15 mg N g^{-1} dry soil in the control and 0.01 – 0.17 under the WOR treatment (Figure 1.2C). Average DON concentrations were 0.04 mg N g^{-1} in control soils and 0.05 mg N g^{-1} under WOR, with the highest concentrations observed in 2018 and 2021 samples.

At the beginning of the study period, in March 2018, the average soil pH was 7.13 in control soils and 7.29 under WOR treatment. Reductions in soil pH were observed in March 2019, June 2019, July 2020, and March 2021. In June 2019, the average soil pH was 6.99 in control soils and 6.80 under WOR treatment. In July 2020, the average soil pH was 6.75 in the control soils and 6.80 under WOR treatment. The lowest soil pH values were observed in March 2021, with an average of 6.49 in control soils and 6.30 under WOR treatment. At the end of the chronosequence, in July 2021, the average soil pH was 6.56 in control soils and 6.41 under WOR treatment.

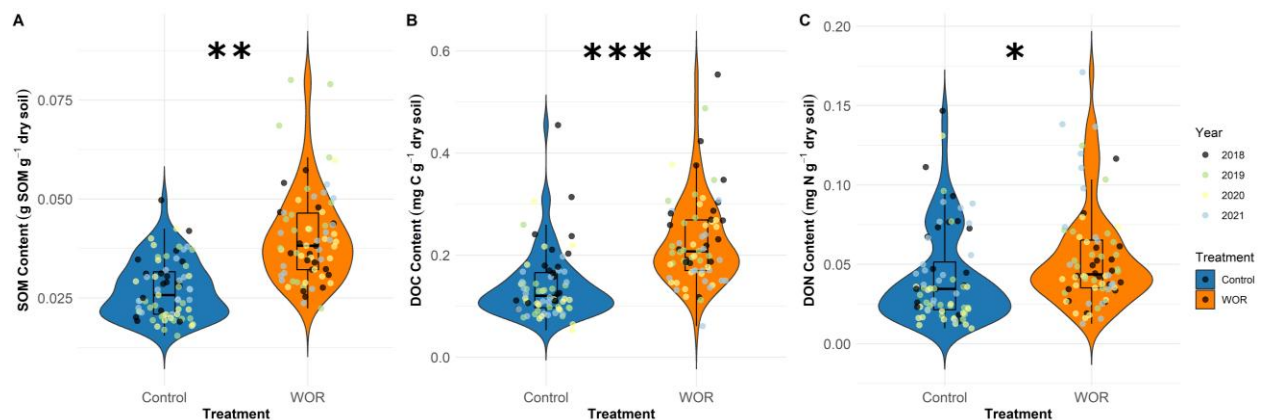
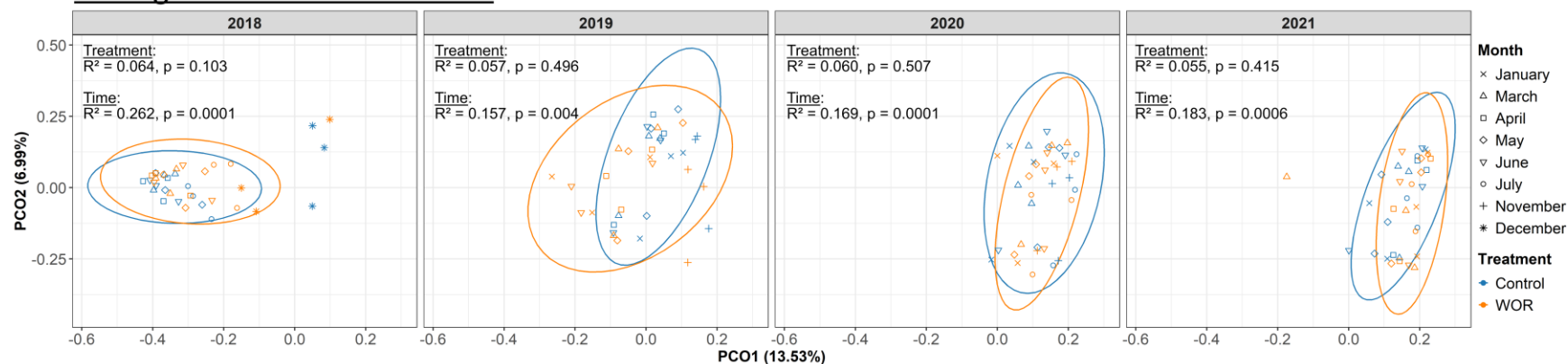


Figure 1.2: Violin plots showing the distribution of (A) soil organic matter (SOM), (B) dissolved organic carbon (DOC), and (C) dissolved organic nitrogen (DON) between treatments and the four-year chronosequence. Violin width reflects the density of observations at each value, and the embedded box indicates the interquartile range with the median shown as a line. Points are colored by sampling year within each treatment group. Three SOM outliers were removed prior to plotting: WC.4 Day 0 (24% SOM), WC.2 Day 402 (15% SOM), and C.1 Day 0 (0.000897% SOM).

Amplicon sequencing of the 16S rRNA gene V4 region and ITS1 yielded 11,151 bacterial ASVs, 183 archaeal ASVs, and 2,195 fungal ASVs. Principal coordinates analysis (PCoA) of Bray Curtis dissimilarities revealed temporal shifts of bacterial/archaeal and fungal communities, as shown in Figure 1.3. Though a small portion of the total variation is explained, PCO1 captured 13.53 and 11.57%, while PCO2 captured 6.99 and 5.16% of variation in community composition, for bacteria/archaea and fungi, respectively. PCO1 appears to be associated with changes over time and PCO2 appears to be associated with WOR treatment differences. Repeated measures PERMANOVAs performed on all years together (2018-2021) reinforce observations that the microbial community composition varied more across time (bacteria/archaea: $R^2 = 0.312$, $p = 0.0001$; fungi: $R^2 = 0.239$, $p = 0.0001$) than by treatment (bacteria/archaea: $R^2 = 0.025$, $p = 0.426$; fungi: $R^2 = 0.017$, $p = 0.0199$). A significant interaction between time and treatment was also observed for both bacterial/archaeal ($R^2 = 0.124$, $p = 0.0064$) and fungal ($R^2 = 0.171$, $p = 0.0026$) communities, prompting separate PERMANOVAs for each year.

Year-specific analyses indicated that bacterial/archaeal communities differed significantly among monthly timepoints within each year but did not respond significantly to the WOR treatment. Conversely, fungal communities exhibited significant treatment effects in 2018 ($R^2 = 0.114$, $p = 0.033$) and 2020 ($R^2 = 0.042$, $p = 0.0001$). For fungi, significant effects of time (i.e., differences between sampling months) were also observed in 2018 ($R^2 = 0.153$, $p = 0.0096$) and 2019 ($R^2 = 0.207$, $p = 0.0002$), but not 2020 and 2021. A significant interaction between treatment and time was not observed for any individual year, for both bacterial/archaeal and fungal communities.

V4 region of 16S rRNA Gene:



Fungal ITS region 1:

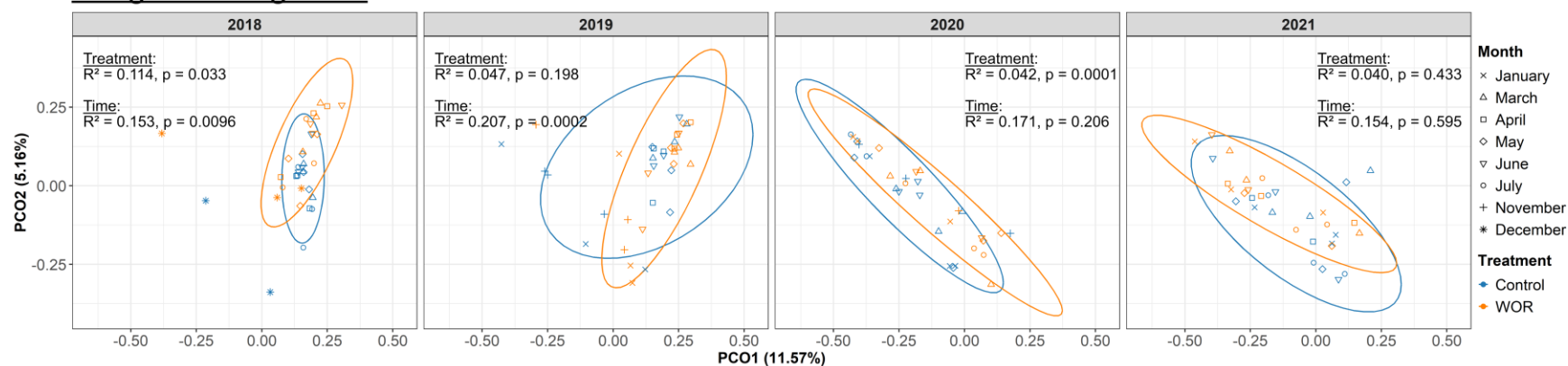


Figure 1.3: Principal coordinate analysis plot (faceted by year) based on Bray-Curtis dissimilarities of bacterial/archaeal (16S rRNA gene V4 region) and fungal (ITS1) communities in control and whole orchard recycling (WOR) soils across all years (2018-2021). This is PCoA is faceted by sampling year, where each point represents an individual soil sample, colored by treatment and shaped by sampling month, with 95% confidence interval ellipses and repeated measures PERMANOVA results overlaid.

Bacterial and archaeal (16S rRNA) communities showed moderate variation in alpha diversity across the chronosequence, with Shannon diversity (q_1) differing significantly over time ($X^2 = 119.92$, $df = 23$, $p = 4.307e-15$) and by treatment ($X^2 = 4.9026$, $df = 1$, $p = 0.02682$). Cumulatively, across treatments and time, measurements of bacterial and archaeal Shannon diversity were highest in 2018 and 2019 (Figure 1.4A). Simpson evenness was near 1 across all samples, with the lowest bacterial and archaeal evenness observed in 2021 (Figure 1.4A). Simpson evenness did not differ significantly between treatment groups and a significant difference was observed across time ($X^2 = 119.92$, $df = 23$, $p = 5.074e-15$). A significant interaction between treatment and time was not observed for either Shannon diversity or Simpson evenness indices.

Fungal (ITS1) communities in WOR soils had lower Shannon diversity (q_1) than the control in 2018, but differences between treatment groups were not observed in subsequent years (Figure 1.4B). Significant main effects of both treatment ($X^2 = 4.5586$, $df = 1$, $p = 0.03275$) and time ($X^2 = 37.512$, $df = 23$, $p = 0.02872$) were observed for fungal q_1 , but the interaction was insignificant. The Simpson evenness was lowest in the WOR amended 2018 soils (Figure 1.4B), with a significant difference between treatments ($X^2 = 4.6236$, $X^2 df = 1$, $p = 0.03154$), although evenness was near 1 across all samples from 2019, 2020, and 2021. No significant time or interaction effects were detected for fungal evenness.

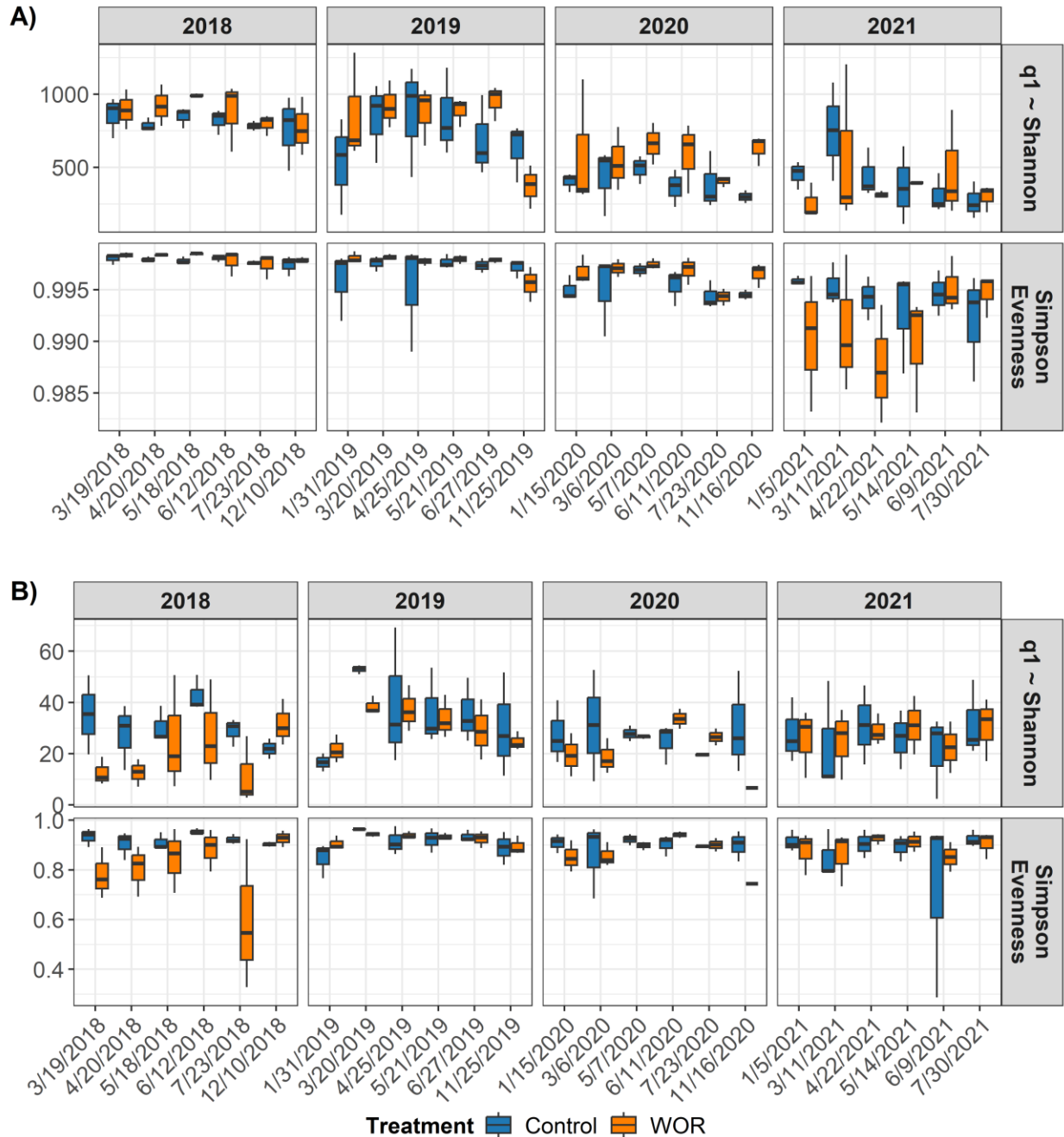


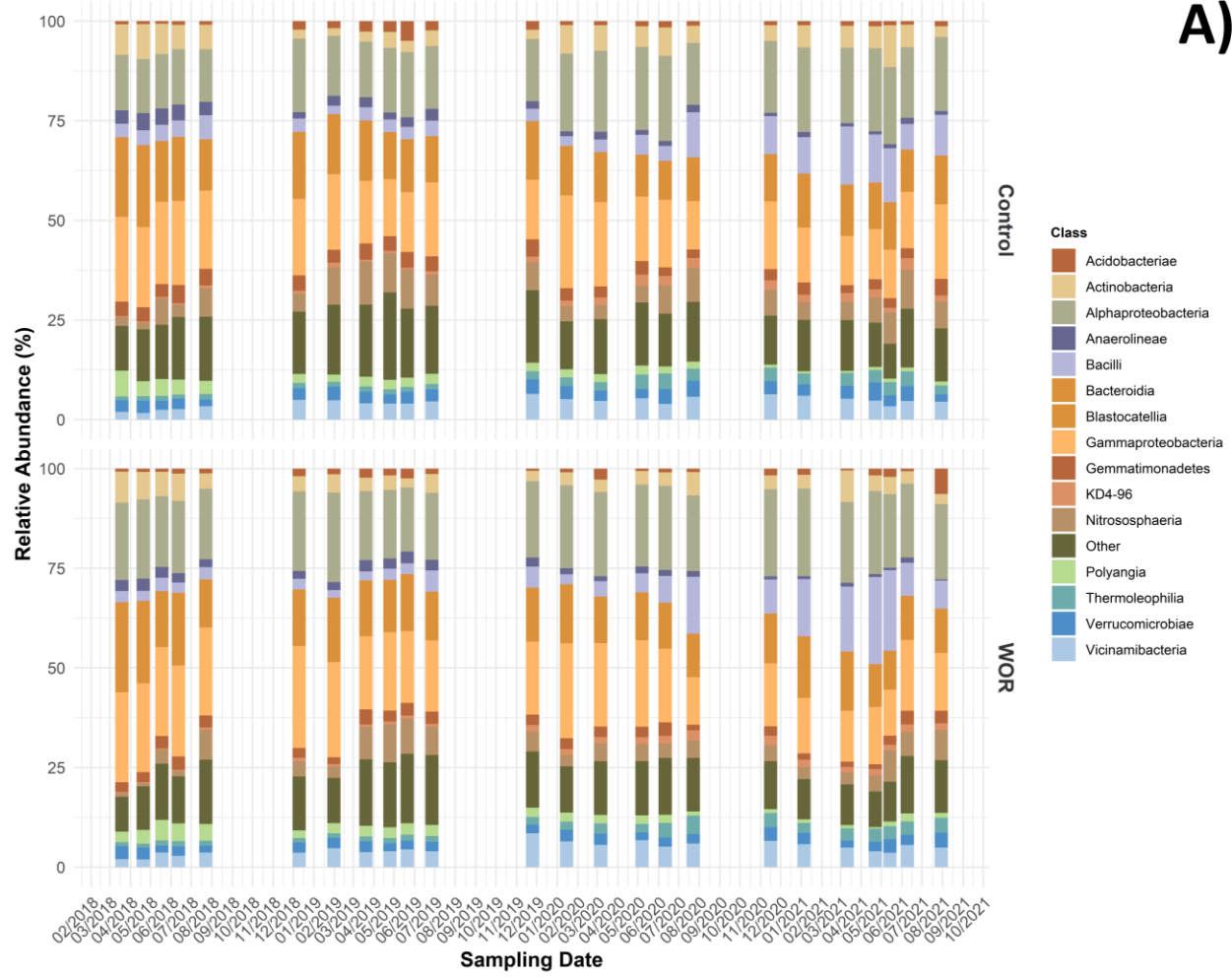
Figure 1.4: Alpha diversity metrics for bacterial/archaeal 16S rRNA gene communities (A) and fungal ITS1 communities (B) from 0–15 cm composite soil samples collected monthly from 2018 to 2021 in a commercial almond orchard in Parlier, California, USA. Treatments included control soils (blue) and whole orchard recycling soils (orange). Boxplots show Shannon diversity (q1) and Simpson evenness for each sampling date, grouped by year and treatment.

Among the top 15 most abundant bacterial and archaeal classes, changes in relative abundance over time were greater than differences between treatment groups. Bacterial and archaeal classes display both intra-annual and inter-annual changes. As observed in Figure 1.5A, *Gammaproteobacteria* and *Bacteroidia* relative abundances were highest in 2018 and lowest in 2021 for both treatment groups. Conversely, *Bacilli*, *Blastocatellia*, and *Vicinamibacteria* display the lowest relative abundances in 2018 and 2019, and the highest relative abundances in 2020 and 2021. *Verrucomicrobiae* displayed the most stable relative abundances over time, whereas *Nitrososphaeria* and *Actinobacteria* displayed greater intra-annual than inter-annual variability. Among all 16S rRNA gene counts, *Alphaproteobacteria* and *Gammaproteobacteria* were the most abundant classes detected, together composing 44.0% of the WOR amended soils and 40.2% for control soils. That is, if the treatment groups are compared by merging sample counts across all time points, the WOR amended soils consisted of 22.4% *Alphaproteobacteria*, 21.6% *Gammaproteobacteria*, 10.4% *Bacteroidia*, 7.7% *Bacilli*, 5.6% *Blastocatellia*, 5.4% *Nitrososphaeria*, 5.3% *Vicinamibacteria*, and 5.1% *Actinobacteria*. Similarly, the unamended control soils consist of 20.3% *Alphaproteobacteria*, 19.9% *Gammaproteobacteria*, 9.4% *Bacteroidia*, 7.1% *Bacilli*, 6.3% *Blastocatellia*, 7.0% *Nitrososphaeria*, 6.0% *Actinobacteria*, and 5.1% *Vicinamibacteria*. The highest abundance of *Gammaproteobacteria*, *Bacilli*, and *Alphaproteobacteria* was observed within WOR treated soils, in December 2018, May 2021, and March 2021 respectively.

Among the top 15 most abundant fungal classes, the greatest differences were observed between the WOR and control soils sampled in 2018. Most fungal classes display greater intra-annual than inter-annual variability. As observed in Figure 1.5B, relative abundance of unidentified fungi is nearly twice as high among WOR 2018 soil samples (91.9 – 50.2% RA),

relative to the 2018 unamended controls (56.5 - 27.1% RA). Conversely, relative abundances of *Tremellomycetes*, *Dothideomycetes*, *Sordariomycetes*, and *Pezizomycetes* were much lower in WOR 2018 soil samples than the 2018 control. Thereafter, relative abundances of these WOR 2018 fungal classes appear to converge with the control in 2019, 2020, and 2021. In WOR and control soils, the relative abundance of *Mortierellomycetes* is lowest in 2018 and appears to increase over 2019, 2020, and 2021. *Agaricomycetes* display greater intra-annual than inter-annual variability. When the sample counts were merged across all time points and ordered in descending order for each treatment group, WOR amended soils consist of 55.2% unidentified fungi, 17.1% *Tremellomycetes*, 7.3% *Dothideomycetes*, 6.9% *Sordariomycetes*, 5.6% *Agaricomycetes*, 2.6% *Mortierellomycetes*, 2.2% unidentified *Ascomycota*, and 0.7% *Pezizomycetes*. The unamended control soils consist of 47.0% unidentified Fungi, 17.1% *Tremellomycetes*, 10.3% *Dothideomycetes*, 9.5% *Sordariomycetes*, 5.1% *Agaricomycetes*, 4.3% *Mortierellomycetes*, 2.1% *Pezizomycetes*, and 1.4% unidentified *Ascomycota*. The highest abundance of *Tremellomycetes* and *Dothideomycetes* was observed within control soils, in January 2019 and December 2018 respectively.

A)



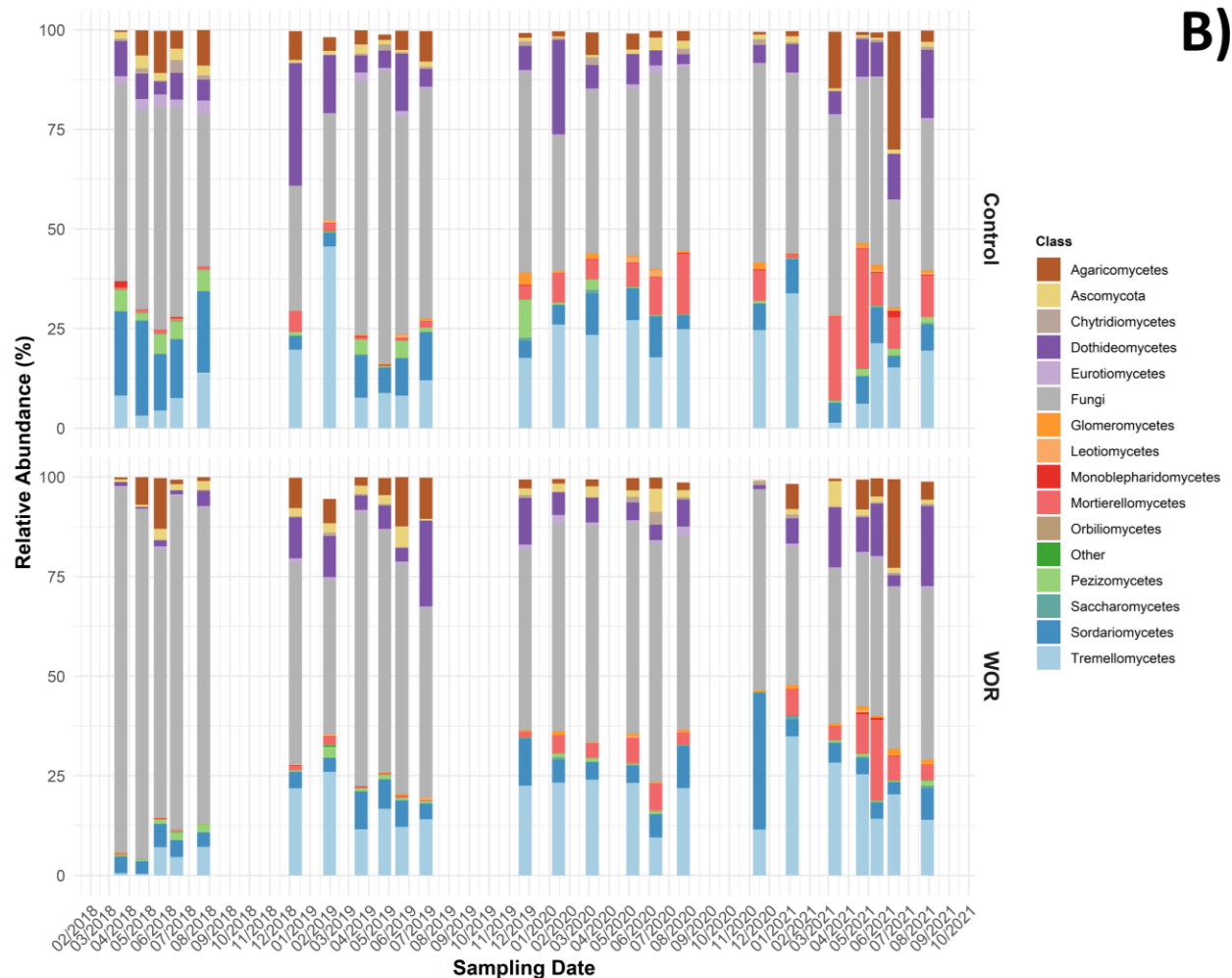


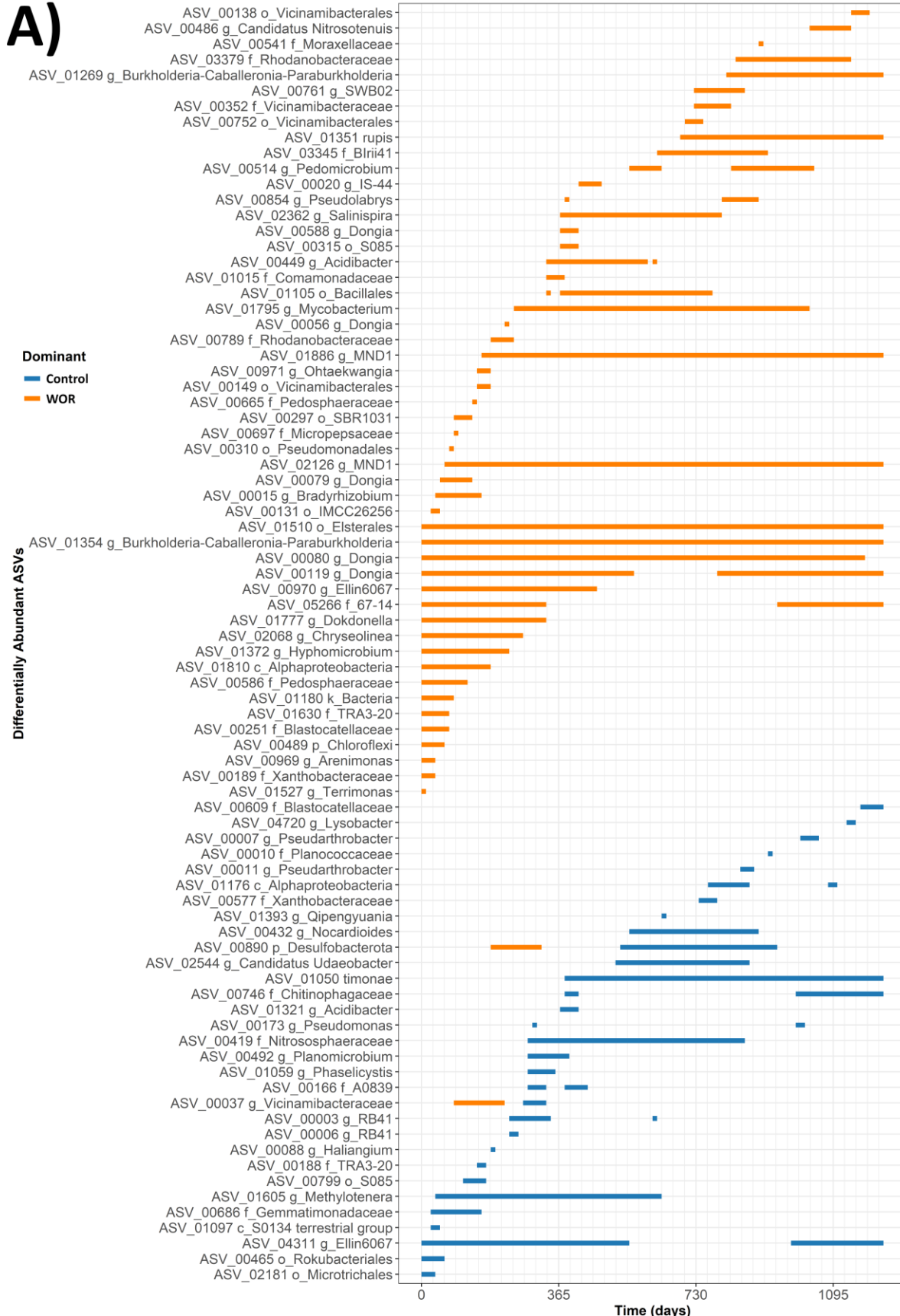
Figure 1.5: Top 15 most abundant 16S rRNA gene (A) and fungal ITS1 (B) classes. The amplicon sequence variant (ASV) counts were merged across the 3 blocks sampled for each treatment group, agglomerated to the class level, converted to relative abundances, and plotted over time. The stacked bar plots are organized chronologically along the x-axis, demonstrating monthly changes in relative abundance during the agricultural growing season, with two “off season” time points in-between each season. The plot is vertically faceted by treatment. ASVs that do not fall into the top 15 most abundant classes were grouped into the “Other” category.

Differential abundance tests, performed by MetaLonDA across the 2018 – 2021 growing seasons, identified 130 differentially abundant bacterial/archaeal and fungal ASVs, present at >0.01 % mean relative abundance, between the WOR and unamended control treatments. For the V4 16S rRNA amplicons, MetaLonDA detected 82 differentially abundant ASVs, of which 51 were WOR enriched, 29 were control enriched, and 2 were enriched in both WOR amended and

unamended soil (Figure 1.6A). At the class level, WOR enriched bacterial and archaeal ASVs were primarily *Alphaproteobacteria* (14 ASVs), *Gammaproteobacteria* (15 ASVs), *Bacteroidia* (4 ASVs), *Vicinamibacteria* (4 ASVs), and *Verrucomicrobiae* (2 ASVs). *Actinobacteriota*, and *Verrucomicrobiota* (Figure 1.6A). Less abundant but unique WOR enriched classes included *Acidimicrobiia*, *Actinobacteria*, *Anaerolineae*, *Bacilli*, *Dehalococcoidia*, *Nitrososphaeria*, *Polyangia*, *Spirochaetia*, and *Thermoleophilia*. Notably, only two archaeal ASVs were detected as differentially abundant by MetaLonDA, ASV_00419 (f_ *Nitrososphaeraceae*) and ASV_00486 (g_ *Candidatus Nitrosotenuis*). Differentially abundant ASVs that exhibited long-term WOR enrichment (≥ 730 days) include *Betaproteobacteria* (ASV_00970, 01886, 02126, and 01354), *Alphaproteobacteria* (ASV_01510, 00080, and 00119), and *Actinobacteria* (ASV_01795). In contrast, control enriched ASVs encompassed *Gammaproteobacteria* (7 ASVs), *Alphaproteobacteria* (4 ASVs), *Actinobacteria* (3 ASVs), *Blastocatellia* (3 ASVs), and *Bacilli* (2 ASVs), with individual representatives from *Acidimicrobiia*, *Bacteroidia*, *Dehalococcoidia*, *Methylomirabilia*, *Nitrososphaeria*, and *Verrucomicrobiae*.

For the ITS1 amplicons, MetaLonDA detected 48 differentially abundant ASVs, of which 24 were WOR enriched, 23 were control enriched, and 1 was enriched in both treatments (Figure 1.6B). Fungal WOR enriched ASVs were predominantly unidentified fungi (17 ASVs), *Ascomycota* (2 ASVs), and *Alternaria* species (2 ASVs), with individual representatives from *Mortierella alpina*, *Lipomyces tetrasporus*, and *Vishniacozyma globispora*. Among the two *Alternaria* ASVs identified here, ASV_00901 exhibited long-term enrichment (over 500 days) after orchard establishment and WOR treatment. In contrast, control enriched ASVs were predominantly unidentified fungi (11 ASVs), *Dothideomycetes* (5 ASVs), *Agaricomycetes* (2 ASVs), *Sordariomycetes* (2 ASVs), *Tremellomycetes* (2 ASVs), and *Pezizomycetes* (1 ASV).

A)



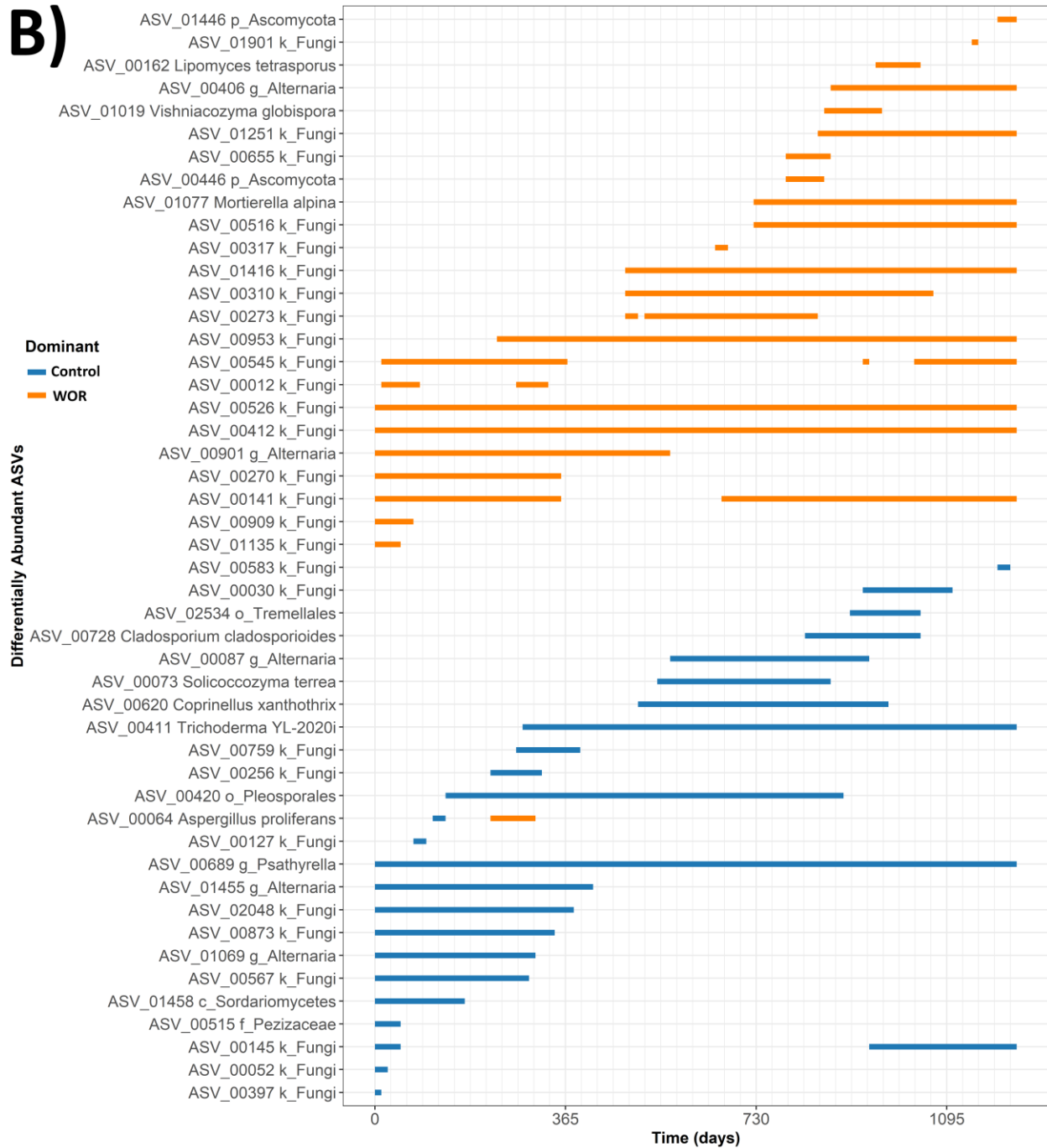


Figure 1.6: Differentially abundant V4 16S rRNA (A) and ITS1 (B) ASVs were detected using MetaLonDA, which identifies significant (p.value threshold = 0.05) time intervals when ASVs are enriched in either wood chip (orange) amended and unamended control (blue) soils. All ASVs were filtered to > 0.01 % mean RA and analyzed to the family level. Major gridlines occur annually relative to the first sampling time (day 0) on March 19, 2018. Minor gridlines occur monthly, at 30.417-day increments.

Temporal turnover in the bacterial/archaeal communities was evident across the chronosequence. Over all four years, the largest temporal shifts in community composition were observed in 2018, the first year following orchard replanting, while temporal turnover was weaker and more similar between treatments in 2020 and 2021 (Figure 1.7A). The Bray-Curtis dissimilarity increased significantly with time in the 2018 WOR, 2019 WOR, and 2018 unamended control soils. In 2018, the slope of the Bray-Curtis time-decay relationship was significantly steeper in control soils than in WOR soils (control slope = 0.000962, WOR slope = 0.000729; $p = 0.00299$). In 2019, the control slope was not significantly different from zero, whereas the WOR slope was positive and significant; furthermore, the slopes differed significantly from one another and the WOR slope was steeper overall (control slope = -1.16×10^{-5} , WOR slope = 0.000241; $p = 0.00487$). In 2020 and 2021, the WOR and control Bray-Curtis time-decay slopes were not significantly different from zero, nor did the time-decay slopes differ significantly between treatments in 2020 ($p = 0.744$) or 2021 ($p = 0.415$).

For the fungal communities, the largest temporal shifts were observed in 2018 and 2019, while temporal turnover was weaker in 2020 and 2021 (Figure 1.7B). In 2018, both WOR and control soils showed significant positive time-decay relationships, and the control slope was significantly steeper than the WOR slope (control slope = 0.000782, WOR slope = 0.000463; $p = 0.0379$). In 2019, both treatments again showed significant positive slopes, but slopes did not differ significantly from each other (control slope = 0.000549, WOR slope = 0.000581; $p = 0.813$). In 2020, the control slope was not significantly different from zero, whereas the WOR slope was positive and significant; however, slopes did not differ significantly from one another (control slope = 0.000172, WOR slope = 0.000398; $p = 0.175$). In 2021, the WOR and control Bray-Curtis time-decay slopes were not significantly different from zero and did not differ

significantly between treatments ($p = 0.402$). Overall, fungal temporal turnover under WOR was positive in 2018, 2019, and 2020, but was not significantly different from the control time-decay relationship in 2019, 2020 and 2021 (Figure 1.7B).

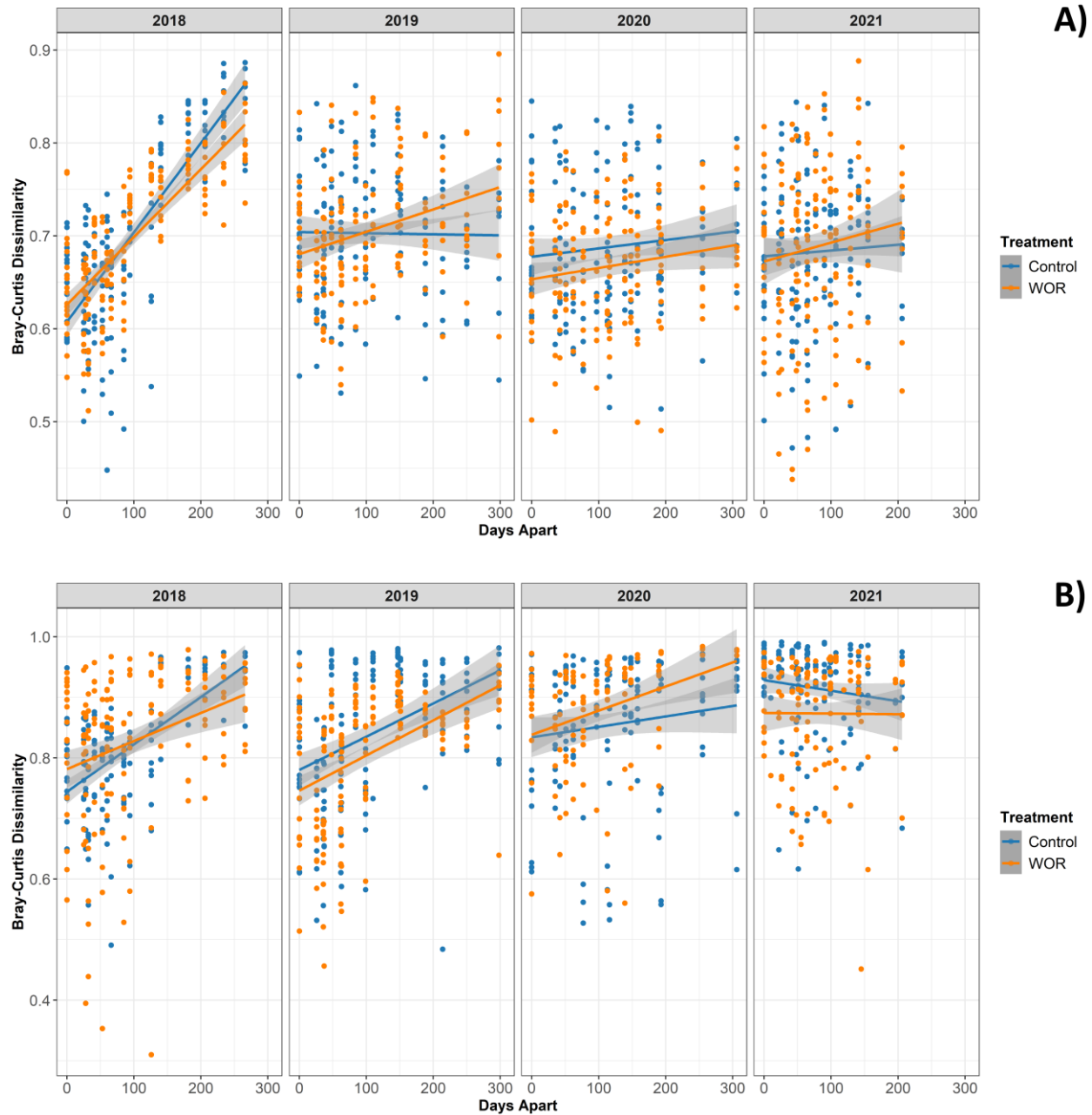


Figure 1.7: Temporal turnover of bacterial/archaeal (A) and fungal (B) communities, expressed as Bray-Curtis dissimilarities distances plotted over time. Data are separated by treatment and faceted by Year, including all pairwise comparisons between blocks. Day 0 is represented by the first soil sampling date (March 19, 2018). “Days Apart” is calculated as the number of days between the sampling dates for each of the pairwise comparisons of Bray-Curtis dissimilarity.

Discussion

WOR enhanced soil physiochemical properties

Averaging across the four-year soil chronosequence, whole orchard recycling (WOR) significantly increased soil organic matter (SOM), dissolved organic carbon (DOC), and dissolved organic nitrogen (DON) contents of sieved (< 2 mm) fine sandy loam soil (Figure 1.2), reinforcing existing literature related to soil health improvement by organic amendment. The SOM trends observed here are alignment with a 9-year WOR trial, that demonstrated an increase in the proportion of stable large macroaggregates, which had a higher relative proportion and total weight of occluded SOC¹⁶. Additionally, a larger proportion of total nitrogen (TN) was bound to minerals in the <53 μm silt and clay fraction¹⁶, which has been supported by enhanced incorporation of N into microbial biomass¹⁷. Here, the highest levels of SOM were observed among WOR treated soils in 2019, 1 year after wood chip incorporation, however it is likely that the unsieved orchard soil matrix experienced the highest levels of total carbon (TC) in 2018, immediately following wood chip incorporation.

Notably measurements in this study were conducted on sieved (< 2 mm) bulk soil samples taken from the top 15 cm of the berm, about a foot away from each tree, and may not capture fine localized changes occurring within soil aggregates, the rhizosphere, or at the surface of large woody debris. This is important to acknowledge because soil microbes are known for cycling nutrients and the distribution of nutrients within the soil matrix has been found to reflect the composition and activity of the soil microbiome^{57–59}. Microbial hotspots (i.e., small soil volumes with much faster process rates than the average soil conditions) have been observed in the rhizosphere, detritosphere, and aggregate surfaces³³ – in which soil microaggregates (<250 μm) have been found to have more diverse and dynamic microbial communities than large

macroaggregates (>2 mm)⁶⁰. Thus, granted that soils are highly heterogeneous⁶¹, bulk soil measurements represent an average of many microenvironments.

WOR effects on soil microbial community composition diminish over time

WOR presents a novel system to study the temporal shifts in the soil microbial community following a huge one-time amendment of woody organic material. Within this 4-year study, temporal change had a more prominent effect on the soil microbial community composition than the WOR treatment. The PCoAs (Figure 1.3) indicate that WOR did not significantly shift the community composition of bacteria and archaea (V4 16S rRNA), while time (i.e., sampling month) had a significant effect within and across all four sampling seasons. In contrast, WOR significantly shifted the fungal (ITS1) community composition (Figure 1.3) and decreased Simpson evenness (Figure 1.4B) in 2018 (i.e., the first year following WOR application and orchard replanting). This suggests a stronger immediate and inter-annually variable response of soil fungi to WOR, with WOR-induced shift dissipating in subsequent years (2019, 2020, 2021) as the fungal community composition converges with temporal trends observed in the control. However, in this commercial almond orchard, convergence of WOR-related changes in microbial community composition with the control may have been accelerated by winter manure applications. Studies on organic farming systems have reported shifts in both bacterial and fungal community composition following organic amendment with manure, with fresh manure having a stronger impact on the microbial communities than compost manure⁶². Similarly, an amplicon-based study reported dose-dependent changes in soil microbial community structure, with the strongest effect observed within the first few weeks of amendment at the highest green-manure compost amendment level ($60 \text{ m}^3 \text{ ha}^{-1}$)⁶³. Thus, in real commercial agricultural systems, it is important to consider how temporal change may be confounded with

land management events. Future research should characterize the microbes present within the organic material before incorporation into the soil matrix and then evaluate the persistence of identified non-native soil microbial community members across time.

Temporal turnover in WOR soils differed from the control

The temporal turnover of soil microbes is understudied within agricultural soil systems; however, the temporal change of the microbial community can be more important than the direct effect of organic amendment. Furthermore, microbial composition shifts induced by organic amendment may diminish over time, and it may be possible to predict a point when the soil microbial community returns to its original state. In this study, the strongest temporal shifts in both bacterial/archaeal and fungal communities occurred in 2018 and 2019, the first two years after orchard replanting and WOR implementation, whereas turnover was slower in 2020 and 2021 (Figure 1.7 A and B). The WOR amendment was expected to perturb temporal turnover rates of soil fungi more greatly than the bacterial rate, as soil fungi have been found to turnover 2.5 - 3 times faster than soil bacteria^{31,64}. More broadly, woodchip-C was anticipated to increase microbial activity and shift the relative abundances of ASVs based on their response to conditions imposed by the treatment. Amplicon-based metrics of microbial beta-diversity suggest that microbial temporal turnover rates are guild-specific with faster turnover rates in plant-litter than in soil³¹. Thus, the greatest differences in Bray-Curtis dissimilarity for both bacterial and fungal communities was expected in the months immediately following WOR, when the most wood material was present. Surprisingly, the control soils showed a significantly steeper time-decay slope than WOR soils in 2018, for both bacterial/archaeal and fungal communities. Specifically, temporal turnover of the control communities was roughly 1.3 times faster for bacteria/archaea and 1.7 times faster for fungi. This result supports the observation that

time exerts a more pronounced effect on the microbial community composition than WOR. However, it is important to consider all the components that are included within the time variable; for instance, the orchard re-establishment event in 2018 may have had an overarching temporal influence on the soil microbial community. Agricultural studies have indicated that strawberry, grape, and soybean rhizosphere soil communities recruit microbes to the rhizosphere from the bulk soil communities⁶⁵⁻⁶⁷, suggesting that freshly planted almond trees likely exert a temporal influence on the surrounding bulk soil community. Then in 2019, the WOR slope was significantly steeper than the control slope for bacteria/archaea. Assuming that critical sampling time points were not missed in January or February 2018, these patterns suggest that WOR interacted with the early post-replant period when microbial communities were likely responding to the mechanical disturbance and appearance of root exudates. Moreover, the temporal variable includes the variability of land management (e.g., N-fertilization, manure application, irrigation events), temperature, and climate. Another possibility is that the temporal change driven by initial N fertilization events was buffered by the initially high soil C:N ratio created by WOR amendment. Repeated application of urea-ammonium-nitrate (UAN) fertilizer, over six growing seasons, was found to increase the relative abundance of copiotrophic groups such as *Bacteroidetes*, *Firmicutes*, *Gammaproteobacteria*, *Alphaproteobacteria*, and decrease the relative abundance of oligotrophic groups such as *Verrucomicrobia* and *Betaproteobacteria*⁶⁸. Similarly, a global meta-analysis showed mineral fertilizer application increase microbial biomass C by 15% due to increases on crop productivity⁶⁹.

Differentially abundant ASVs associated with WOR change over time

Amendments of woody biomass provide soil microbes with complex plant cell wall polymers (i.e., cellulose, lignin, and hemicellulose polymers), which may diminish microbial

diversity and consolidate carbon flow through specific adapted taxa. SIP incubations of forest soils found few (168 out of 2,233) bacterial taxa to increase in relative abundance in response to chemically complex C substrates, such as cellulose, lignin, and tannin-protein⁷⁰. Further, Goldfarb et al., 2011 found that many cellulose-responsive taxa are capable of cellulose hydrolysis, such as bacteria from the families *Clostridiaceae* and *Lachnospiraceae*, *Sphingomonas* spp., and *Spirochetes*⁷⁰. However, microorganisms that degrade cellulose utilize extracellular reactions that yield free by-products which can promote interactions with noncellulolytic organisms²⁵. Holistically, a shotgun metagenomic-SIP experiment using ¹³C-cellulose delineated a framework for the competitive exclusion and metabolic dependency of cellulose economy in agricultural soil; wherein, Wilhelm et al., 2021 found cellulolytic *Cellvibrio* (*Gammaproteobacteria*) and *Chaetomium* (*Ascomycota*) to be most ¹³C-enriched, which exhibited rapid growth and antibiotic production²⁵. Noncellulolytic dependent taxa that accessed ¹³C from cellulose included members of *Planctomycetales*, *Verrucomicrobia*, and *Vampirovibrionales*²⁵.

It has been suggested that one of the main factors controlling soil microbial diversity is diversity of C substrates²⁹. As suggested by the decreased fungal evenness in 2018 (Figure 1.4B), the huge one-time wood chip amendment likely created a C-rich but relatively narrow niche, selecting for a subset of fungi capable of degrading lignin and cellulose and thereby reducing overall fungal diversity. As shown in Figure 1.6B, 10 fungal ASVs were detected as enriched in the 2018 WOR treated soils. These ASVs were predominantly unidentified fungi, indicating WOR initially favored increases in abundance of unique fungal species. This observation is supported by the class-level relative abundance data shown in Figure 1.5B, whereby all 2018 WOR treated soil communities are dominated by unidentified fungi. Even though WOR

treatment did not significantly shift the bacterial/archaeal community composition (Figure 1.3) and their evenness resembled the control (Figure 1.4A), 37 bacterial ASVs were detected as enriched in the 2018 WOR treated soils (Figure 1.6A). The bacterial/archaeal ASVs that responded positively to WOR predominantly belonged to the classes *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacteroidia*, *Vicinamibacteria*, and *Verrucomicrobiae* (Figure 1.6A).

Several differentially abundant taxa, especially those detected shortly after WOR in 2018, have putative functions associated with wood chip decomposition and broader nutrient cycling in soil. For example, bacterial ASV_01527 and ASV_001351 belong to the family *Chitinophagaceae*, which contains species that are capable of chitin and cellulose degradation⁷¹. Furthermore, these specific ASVs belong to the genus *Terrimonas*, which contains species that have been found to degrade complex carbohydrates⁷². In terms of N cycling WOR-enriched ASVs, ASV_00970 (g_ellin6067), ASV_01886 and ASV_02126 (g_MNDI), and ASV_00020 (g_IS-44) are ammonia oxidizing bacteria from the family *Nitrosomonadaceae*⁷³. Collectively, these nitrifying taxa were detected as differentially abundant within WOR soils from Spring 2018 through the end of the chronosequence in Summer 2021. This outcome is supported by a previous study that reported a heightened, although insignificant, abundance of the bacterial ammonia monooxygenase gene (*amoA*) in WOR treated soil¹⁷. Jahanzad et al., 2021 also reported that WOR, when combined with (NH₄)₂SO₄ fertilizer applications, resulted in a short-term (1 year) decrease of nitrite reducing functional gene (*nirK*) abundance¹⁷. Together, these results suggest that aerobic metabolism dominates the top 15 cm of WOR amended fine sandy loam soils.

For fungi, *Alternaria* ASV_00901 exhibited particularly long-term enrichment, from the beginning of the study in Spring 2018 to Summer 2019, consistent with its role as a common

mold of decay⁷⁴. Then in 2020, the increased relative abundance of ASV_00162 *Lipomyces tetrasporus* may provide evidence for fungal succession, as they have been found growing on lignocellulosic biomass hydrolysates⁷⁵. More broadly, MetaLonDA detected 24 WOR-enriched fungal ASVs, of which 17 are unidentified fungi. The 7 WOR-enriched ASVs, that were identified beyond the kingdom-level, consist of 5 *Ascomycota*, 1 *Basidiomycota*, and 1 *Mucoromycota*. Assuming that these proportions are representative of all WOR-associated fungi, the enrichment of *Ascomycota* is supported by observations in forest litter and soil. Stursová et al., 2012 found most fungal cellulose decomposers belonged to *Ascomycota*, with cellulose decomposition being 10 times faster in the fungi-dominated litter than in the bacteria-dominated soil⁷⁶. Future work on temporal patterns of microbial ecology in WOR soils may benefit from quantifying the relative contributions of fungi and bacteria to wood decomposition.

Limitations

Soil communities contain a large proportion of dormant organisms⁷⁷, limiting the ability of amplicon based techniques to detect shifts in the soil microbial community. High spatial heterogeneity of soils may also confound temporal patterns⁷⁸, despite efforts to sample and process the soils consistently. Within this specific study, sieving the soil to <2 mm removed larger wood chip fragments from the WOR treated soils, which may represent a genomic blind-spot or missed opportunity to capture microbes that colonize wood surfaces. Similarly, the low detection of archaeal ASVs may be an artifact of a persisting universal primer bias toward ligating to bacterial sequences (e.g., universal primer development is tailored to the sequences available within databases, which historically have been dominated by bacterial sequences) and use of short amplicon regions (e.g., amplification of multiple regions or full length 16S rRNA

gene may be more robust). Finally, differential abundance reflects relative, not absolute, changes; ASVs reduced under WOR may appear enriched in control soils, and vice versa.

Conclusion

Overall, this study demonstrates a time-dependent response of soil bacterial/archaeal and fungal community compositions to whole orchard recycling (WOR), while reinforcing existing knowledge on improvements to soil physiochemical properties by organic amendment. Here, the fungal community response was most pronounced in the first year following orchard replanting and WOR application (2018), displaying an enrichment of *Ascomycota* and many unidentified fungi, with significant reductions in Simpson evenness. For bacteria, WOR predominantly enriched *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacteroidia*, *Vicinamibacteria*, and *Verrucomicrobiae*, without significant changes in Simpson evenness. Temporal turnover rates were significantly higher in 2018 control soils relative to the WOR treated communities, for both bacteria/archaea and fungi (bacterial/archaeal: control slope = 0.000962 vs. WOR = 0.000729, $p = 0.00299$; fungal: control = 0.000782 vs. WOR = 0.000463, $p = 0.0379$). However, disentangling the effect of time from potential WOR interactions with confounding land management events (e.g., N-fertilization forms, irrigation intensity, subsequent organic C amendments, etc.) remains a major knowledge gap that limits further temporal inference from this data. Although, it is possible that co-amendment of WOR with organic N sources can enhance the magnitude of associated soil health benefits or reduce negative consequences. Tangentially, this work provides a basis for exploring how WOR-induced changes in soil microbial community composition can be mitigated or refined by the timing and chemical form of subsequent organic amendments.

Chapter 1 Appendix

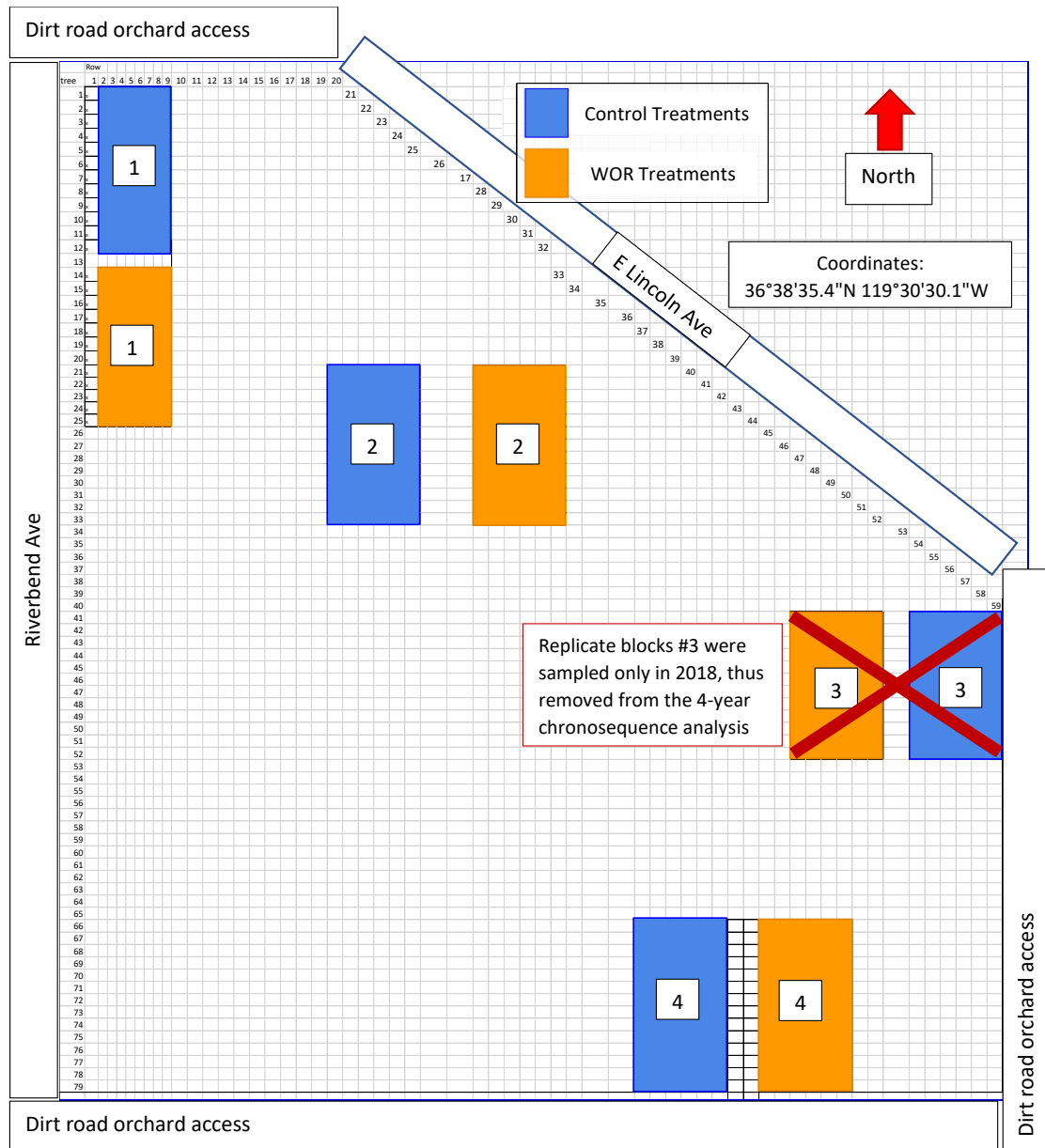


Figure A1.1: Site map of the commercial almond orchard, located in Parlier CA, USA, E Lincoln Ave & S Riverbend Ave, wherein WOR treated (orange) and control (blue) plots were sampled monthly during the agricultural growing seasons of 2018, 2019, 2020, and 2021.

Chapter 2

Characterization of Particulate and Mineral-Associated Organic Matter in Wood Chip Amended Sandy Loam Soil: Composition and Distribution Across Space-Time

Key Points:

- Whole orchard recycling (WOR) increased average soil organic matter (SOM) contents of fine sandy loam soil by 30–50% in 3-, 4-, and 6-yr orchards; attenuation of the WOR treatment effect was observed in the 15-year-old orchard.
- WOR increased total carbon (TC) and total nitrogen (TN) contents of both particulate and mineral associated (POM and MAOM) fractions of SOM in 3-, 4-, and 6-yr orchards.
- The C:N ratios and stable isotope signatures ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) suggest that POM is predominantly plant derived, while MAOM is predominantly microbially derived.
- Diffuse reflectance Fourier transform infrared spectroscopy (DRIFTS) of POM and MAOM revealed significant WOR-induced shifts in the ratios of aromatic $\nu(\text{C}=\text{C})$, aliphatic $\nu(\text{C}-\text{H})$, and carboxylic $\nu(\text{C}-\text{O})$ organic functional groups.

Abstract

Understanding the composition and persistence of soil organic matter (SOM) over time is fundamental to optimization of organic amendment practices for enhanced soil fertility and long-term agricultural sustainability. Whole orchard recycling (WOR) is an organic management practice in which trees at the end of their productive life cycle are ground into wood chips and tilled into the surface soil before planting the next orchard. This process provides a carbon (C) dense substrate that is predominantly composed of lignocellulose. Here we characterize the composition of the particulate organic matter (POM) and mineral-associated organic matter (MAOM) fractions of WOR treated fine sandy loam soils within a space-for-time substitution experimental design that spans 3-, 4-, 6-, and 15-year time points. Samples were collected in

summer 2023 from four experimental almond orchards established in 2008, 2017, 2019, and 2020 at the University of California Kearney Agricultural Research and Extension Center (USA). Organic functional groups composing soil POM and MAOM were analyzed using diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS). Evidence for inputs and transformations of POM into MAOM were evaluated using C:N ratios and stable isotope C ($\delta^{13}\text{C}$) and N ($\delta^{15}\text{N}$) contents. WOR increased SOM and TC contents by 30 – 50% in 3-, 4-, and 6-year-old orchards and enriched the relative absorbance of aromatic $\nu(\text{C}=\text{C})$ and aliphatic $\nu(\text{C}-\text{H})$ functional groups in POM. Differences were not observed in the 15-year-old orchard, however. WOR also increased the TC contents of MAOM in 3-, 4-, and 6-year-old orchards, without significant changes in organic functional group composition. Across treatments and orchard ages, our analyses suggest that MAOM is predominantly microbially derived in fine sandy loam soils, indicated by lower C:N ratios, enrichment of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, and organic functional group profiles divergent from POM. These findings articulate that WOR largely provides plant derived lignocellulose-C to POM, with microbial transformations into MAOM; although MAOM associations with silt and clay particles are likely limited in fine sandy loam soils. WOR effects on POM and MAOM were detected after 6 years but not after 15 years.

Frequently used acronyms: whole orchard recycling (WOR), diffuse reflectance infrared Fourier-transform spectroscopy (DRIFTS), Fourier-transform infrared spectroscopy (FTIR), mid-infrared (MIR), carbon (C), nitrogen (N), soil organic matter (SOM), particulate organic matter (POM), mineral associated organic matter (MAOM), soil organic matter (SOM), coefficient of determination (R^2),

Introduction

Maintenance of soil organic carbon (SOC) is essential for agricultural sustainability because SOC is the main elemental component of soil organic matter (SOM)⁹. SOM serves critical functions in nutrient storage, moisture retention, and binding soil mineral particles into aggregates⁴. Thus, restoring SOC may serve as a dual solution for mitigating C emissions to the atmosphere and improving soil fertility. Less stable pools of SOM can be easily enhanced from management practices like reducing tillage, applying compost or growing cover crops⁹. However, these approaches do not always produce long-term SOC gains, and the magnitude of soil C accumulation is not always equal to the amount of inputs^{79–81}. Thus, the ability of land management techniques to accumulate and maintain long-term SOC storage is limited by knowledge on the formation and composition of stable SOM pools⁸².

In perennial cropping systems, whole orchard recycling (WOR) is an organic management practice in which trees at the end of their productive life cycle are ground into wood chips and tilled into the surface soil before planting the next orchard. Incorporation of woody biomass provides the opportunity for tree crop systems to restore soil health by increasing soil C stocks and serves as a sustainable solution for tree disposal. In the San Joaquin Valley (California, USA), WOR has emerged as the primary strategy available to orchard farmers following the passage of the California Senate Bill 705 (2003), which restricts agricultural burning to improve air quality^{13–15}. Compared to the convention of burning waste tree biomass in piles on site or at cogeneration facilities, current literature promotes WOR as a climate-smart practice⁸³ that improves soil health through increased soil carbon (C) storage²⁰, microbial biomass nitrogen (N)¹⁷, water retention⁸⁴, and formation of large macroaggregates¹⁶. Although,

double-decay modeling in this region has projected that 49% of WOR biomass decomposes after 4 years, with 6.6% remaining after 20 years²⁰.

Soil amended with orchard wood provides soil microbes with a complex lignocellulose substrate that has a high C:N ratio of approximately 160¹⁶. WOR has already demonstrated increased formation and stability of large macroaggregates, which preserve a larger quantity of intra-aggregate SOC relative to other aggregate fractions¹⁶. Mechanistically, the production of microbial biomass has been identified as a major driver of SOM formation and soil aggregation⁹, specifically due to the stability of occluded cell wall components following microbial death^{80,85,86}. Microbial necromass stabilization in soil is often attributed to interactions with clay-sized mineral particles⁸⁷, which have higher surface area and reactivity than sand-sized particles⁸⁸. The effect of soil texture on decomposition is also indirectly mediated by soil water content⁸⁹. For silty loam soils, orchard floor mulching practices have been observed to stimulate microbial activity⁹⁰ and increase both particulate organic matter (POM) and mineral associated organic matter (MAOM) contents even after six years⁹¹, but it is uncertain if the same temporal dynamics apply to sandy loam soils. To date there are no studies that have examined temporal change in the organic functional groups that compose the POM and MAOM fractions of SOM within fine sandy loam soils amended by WOR.

Holistically, SOM represents a continuum of organic molecules differing in size, degrees of decomposition, and reactivity with the environment⁹. SOM can be operationally partitioned into POM and MAOM fractions, which differ in size, persistence time, and stabilization mechanisms^{92–95}. POM is composed of relatively undecomposed organic particles >53 μm in size, generally considered to be more susceptible to decomposition, with faster average turnover rates (e.g., years – decades) than MAOM^{93,96}. In contrast, MAOM consists of organic matter <53

μm is assumed to be protected from decomposition by physical and chemical associations with silt and clay particles, contributing to slower average turnovers (e.g., decades – centuries). Size fractionation remains a useful method to separate SOM into these pools, which assume differing stabilization mechanisms and ecological roles^{93,97,98}. However, the compositional boundaries between POM and MAOM are often blurred in heterogeneous soils. While POM and MAOM are widely assumed to represent distinct biogeochemical pools, their composition and turnover dynamics are not strictly dichotomous⁹⁹. Both fractions contain mixtures of rapidly and slowly cycling carbon, and both are influenced by plant- and microbially-derived compounds^{100–102}.

In the ongoing efforts to understand the relative contributions of plant- and microbially-derived compounds to stable SOM⁹, a framework for “decoupled” and “coupled” hypothesis testing has been proposed for POM and MAOM⁹⁹. Under the decoupled hypothesis, POM is dominated by partially decomposed plant residues that tend to have higher and more variable C:N ratios, greater abundance of aromatic functional groups, and lower enrichment of stable isotope C ($\delta^{13}\text{C}$) and N ($\delta^{15}\text{N}$) than MAOM, which is often enriched microbial necromass and metabolites^{95,99,103}. In contrast, the coupled hypothesis implies greater connectivity between pools, such that plant-derived compounds can sorb directly to soil minerals and become incorporated into aggregates, producing POM and MAOM pools that are more homogenous⁹⁹. Under this framework, coupling implies that POM and MAOM share sources and stabilization pathways, whereas decoupling implies that they represent more distinct pools with distinct input and protection mechanisms. To test the degree of connectivity between POM and MAOM pools, stable isotope measurements of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ provide powerful diagnostics because they integrate both composition and microbial processing history. Under the decoupled hypothesis, MAOM is expected to have consistently higher $\delta^{13}\text{C}$ enrichment than POM because soil

microbial biomass has been found to be more enriched in $\delta^{13}\text{C}$ than plant biomass^{99,103,104}. In terms of the molecular composition, POM is expected to contain higher and more variable aromatic (C=C) bonds relative to carbonyl (C=O) bonds than MAOM, as aromatic (C=C) is predominantly plant-derived whereas carbonyl (C=O) bonds are often microbially derived^{86,99,105–107}. Conversely, coupled conditions are expected to yield POM and MAOM pools that have similar isotopic and organic functional group profiles.

Fourier-transform infrared (FTIR) spectroscopy is a non-destructive, rapid, and cost-effective method for analyzing the chemical composition of SOM by probing vibrational modes of polar covalent bonds in organic molecules^{108–110}. In this study, we used diffuse reflectance infrared Fourier-transform spectroscopy (DRIFTS), which measures diffusely scattered mid-infrared radiation from the surface of powdered soil and is well suited for heterogeneous samples. In this method, soil is exposed to mid-infrared radiation ($4,000\text{--}400\text{ cm}^{-1}$), and molecular bonds with changing dipole moments absorb energy at characteristic frequencies. The diffusely reflected radiation contains absorption features corresponding to these vibrational modes, producing a spectrum that can be used to identify functional groups such as carboxylic acids, aromatics, and polysaccharides and to characterize the molecular composition of soil constituents. For example, wood is composed primarily of cellulose, hemicelluloses and lignin, each of which exhibits characteristic FTIR absorption bands that have been identified in two main regions¹¹¹; the $3800\text{--}3400\text{ cm}^{-1}$ region encompasses hydroxyl functional group stretching in hemicellulose and lignin molecules and the $1800\text{--}1500\text{ cm}^{-1}$ region encompasses aromatic double bonds of lignin¹¹¹. Additionally, plant lipids and microbial biomass both give rise to two sharp bands in the spectral region $3000\text{--}2800\text{ cm}^{-1}$, which have been found to increase during decomposition¹¹². Targeted spectral regions and peaks have been widely used to derive spectral

indices to evaluate SOM decomposition, transformation, and persistence¹¹³. Notably, The ratio of aliphatic to aromatic C is hypothesized to be a metric for decomposition^{114,115}, where a wood chip induced spike in aromatic C is expected to be initially high and decrease with decomposition. The ratio of C-rich aromatic C to oxygen-rich functional groups has been found to decline with increased biological activity^{113,116}. Similarly, the ratio of aliphatic C to oxygen-rich functional groups has been found to decrease during decomposition, with increased microbial transformation of plant litter^{107,117,118}.

Here, a space-for-time substitution design was utilized to evaluate the long-term influence of WOR on the molecular composition, origin, and associated stability of SOM fractions. Soil samples were collected from four experimental almond orchards representing 3, 4, 6, and 15 years since WOR implementation, allowing us to infer temporal changes in SOM on a timescale relevant to the average orchard. For this space-for-time series, C:N ratios and $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ contents were used to test the “decoupling” and “coupling” framework proposed for POM and MAOM pools, while DRIFTS was applied to characterize the organic functional groups that compose POM and MAOM in this system. Together, these datasets provide a chemical basis for assessing the transformation and persistence of wood chip C within a fine sandy loam agricultural soil matrix. Accordingly, the specific objectives of this study were to 1) characterize the influence of WOR on SOM composition, specifically for POM and MAOM fractions; 2) evaluate the potential for contributions of wood chip POM into MAOM by assessing signatures of plant and microbial organic functional groups, specifically for a 3, 4, 6, and 15 year space-for-time series. By integrating stable isotopic enrichment patterns with organic functional group composition, this work allows us to understand the relative contributions of WOR to POM and MAOM pools and infer the stability of SOC in woody perennial orchard systems.

Research Questions and Hypotheses

Question 1) How does WOR influence the distribution of organic functional groups that compose POM and MAOM? How do the compositions of POM and MAOM change over time?

- **Hypothesis 1a)** Wood chips incorporated into the soil by WOR will contribute lignocellulose-C to soil almost exclusively to POM, with minor contributions to MAOM.
- **Hypothesis 1b)** The composition of POM will fluctuate over time, according to soil TC contents, while the composition of MAOM will remain relatively stable.

Question 2) Is MAOM plant or microbially derived in fine sandy loam orchard soil?

Is there evidence for WOR contributions of wood chip POM directly into MAOM?

- **Hypothesis 2a)** Wood chips incorporated into fine sandy loam soil will initially and predominantly contribute lignocellulose-C to POM, with delayed and minimal contribution to MAOM.
- **Hypothesis 2b)** MAOM will be predominantly microbially derived in fine sandy loam orchard soils, which are characterized by low clay contents and frequent agricultural disturbance.

Methods

Field Site Description:

Soil was collected in July and August 2023 from four experimental almond orchards that received wood chip amendments by whole orchard recycling (WOR) in 2008 (15 years old), 2017 (6 years old), 2019 (4 years old), and 2020 (3 years old). The orchards are located at the University of California Kearney Agricultural Research and Extension Center (36.5966 °N, -119.5176 °W) in Parlier, California (USA), each having 4 replicated WOR and control blocks.

WOR treatments received 33 - 85 metric tons of wood chips per acre. Chronologically, the 3-year-old orchard received 60 tons of wood per acre, the 4-year-old orchard received 61 tons of wood per acre, the 6-year-old orchard received 85 tons of wood per acre, and the 15-year-old orchard received 33 tons of wood per acre. Control treatments followed conventional management practices during orchard establishment. For the orchards recycled in 2017, 2019, and 2020, the control soils were unamended and burning served as the control treatment in the 2008 orchard. This yields a chronosequence of soil samples at 15-, 6-, 4-, and 3-years post WOR, as a space for time substitution design. The soil texture primarily consists of fine sandy loam, characterized as coarse-loamy, mixed nonacid thermic Typic Xerorthents of the Hanford series. The National Cooperative Soil Survey characterized the Hanford soil series to typically contain less than 1 % soil organic matter (SOM) content, decreasing with depth³⁴. The orchard experiences a Mediterranean climate, with dry, hot summers and cool, wet winters and precipitation levels generally falling below almond evapotranspiration requirements for a significant portion of the growing season. On average, the annual rainfall and temperature stand at 285 mm and 17°C, respectively. Thus, all crop production in the region is irrigated.

SOM Fractionation:

Sampling yielded 32 soil samples from four agricultural fields, each containing two treatments (Control and WOR) with four replicate blocks. Immediately following collection, all soil samples were sieved to <2 mm and stored at 4 °C prior to fractionation. The samples were fractionated into particulate organic matter (POM) and mineral-associated organic matter (MAOM) pools following the method described by Cotrufo et al., 2019⁹⁸. Briefly, 25 g of soil was dispersed in sodium hexametaphosphate solution, shaken, and wet-sieved to 53 µm to separate the coarse POM fraction from the finer MAOM. Fractions were oven-dried at 58 °C. A

subsample of each dried POM and MAOM fraction was further oxidized with sodium hypochlorite (NaOCl) to remove organic matter, resulting in a “mineral-enriched” residue for use in subsequent FTIR spectral subtraction, as described by Margenot et al., 2019¹¹⁹.

DRIFTS Analysis:

Diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) was used to characterize POM and MAOM fractions of the <2 mm sieved soil samples. To isolate organic signals and minimize interference from mineral background absorbance, spectral subtraction techniques were applied^{120,119}. Specifically, spectra from mineral-enriched fractions were subtracted from those of SOM-containing fractions to reduce mineral contributions and enhance organic functional group signals. Thus, POM and MAOM fractions were analyzed in both untreated and NaOCl-treated forms. All samples were air-dried, diluted to 10% (w/w) with pre-ground potassium bromide (KBr), finely homogenized in an agate mortar, and oven-dried again at 58 °C to minimize water vapor interference. The composite diluted samples were analyzed using a PIKE Technologies EasiDiff DRIFT accessory (PIKE Technologies, Madison, WI) attached to a Thermo Nicolet 6700 FTIR spectrometer (Thermo Scientific, Waltham, MA) equipped with a deuterated triglycine sulfate (DTGS) detector and KBr beamsplitter. The instrument chamber was continuously purged with CO₂- and H₂O-free air for a minimum of 15 minutes before data acquisition. A KBr background spectrum was recorded every four hours and automatically subtracted from all sample spectra to correct for baseline and environmental variation.

Spectral subtractions (NaOCl-treated from untreated) and Kubelka–Munk transformations were carried out in OMNIC software (Thermo Scientific) to isolate organic functional group contributions from mineral absorption features. The subtracted spectra were

subset to 3700 – 1200 cm⁻¹ for clarity, as the region <1200 cm⁻¹ overlaps with non-target metal oxide and Si-O bands¹²¹. Functional group assignments were based on Baes & Bloom (1989)¹²², Calderón (2011)¹²³, Li (2017)¹²⁴, Margenot (2015)¹²⁵, and Parikh (2014)¹⁰⁸ with principal absorption peaks described in the appendix, Table A2.1 below.

To assess wood chip C decomposition, two peak height indices were calculated, as adaptations of Margenot et al., 2015¹²⁵ and Veum et al., 2014¹¹³. The wood chip decomposition indices below represent aliphatic-to-aromatic and aromatic-to-carboxylic functional group contents respectively.

$$\text{Aliphatic : Aromatic} = \frac{2931 \text{ cm}^{-1} + 2871 \text{ cm}^{-1}}{1660 \text{ cm}^{-1} + 1600 \text{ cm}^{-1} + 1540 \text{ cm}^{-1} + 1510 \text{ cm}^{-1}}$$

Equation 1

$$\text{Aromatic : Carboxylic} = \frac{1660 \text{ cm}^{-1} + 1600 \text{ cm}^{-1} + 1540 \text{ cm}^{-1} + 1510 \text{ cm}^{-1}}{1724 \text{ cm}^{-1} + 1320 \text{ cm}^{-1} + 1275 \text{ cm}^{-1}}$$

Equation 2

Analysis of Soil Physicochemical Properties:

Soil physicochemical properties were measured on bulk (<2 mm), POM, and MAOM fractions of soils collected from plots that were either unamended (control) or amended with wood chips via the WOR treatment in 2008, 2017, 2019, and 2020. Soil organic matter (SOM) content (g SOM g⁻¹ dry soil) was determined via loss-on-ignition. Ten grams of air-dried, <2 mm sieved soil were combusted at 550°C in a muffle furnace, and mass loss was recorded on a precision balance (±0.001 g). Total carbon (TC) and total nitrogen (TN) concentrations were analyzed by the UC Davis Analytical Laboratory using a combustion method (AOAC Official Method 972.43). Approximately 5 g of ground samples (<0.25 mm, 60-mesh) were combusted in an induction furnace equipped with a thermal conductivity detector for nitrogen and an infrared

(IR) detector for carbon. This flash combustion procedure oxidizes all organic and inorganic matter into combustion gases (CO_2 , N_2 , NO_x , H_2O), providing sensitive quantification of TC and TN with detection limits of $\sim 0.02\%$. TC and TN data were obtained for bulk soil, POM, and MAOM fractions. For each soil fraction, C:N ratios were subsequently calculated by dividing TC by TN (both expressed as percent of dry weight), providing an estimate of organic matter quality and decomposition potential across treatments and soil fractions.

Dissolved organic carbon (DOC) and dissolved organic nitrogen (DON) contents (mg C or N g^{-1} dry soil) were measured from $0.5 \text{ M K}_2\text{SO}_4$ soil extracts using a Shimadzu TOC-L analyzer equipped with a total nitrogen unit (TNM) and autosampler (ASI-L). For each extraction, 8.00 g of dry soil was shaken in 40 mL of $0.5 \text{ M K}_2\text{SO}_4$ for 1 h at 250 rpm , centrifuged ($3075 \times g$, 5 min), and filtered through ashless Whatman 41 filter paper. The TOC-L system used a high-salt combustion tube to simultaneously analyze DOC and DON. Soil pH was measured using a 1:1 (soil:deionized water) slurry and a calibrated glass electrode.

Water holding capacity (WHC) of field-moist, $<2 \text{ mm}$ sieved soils was determined gravimetrically using a standard saturation–drainage procedure¹²⁶. Approximately 30 g of soil was placed onto a pre-weighed filter paper fitted in a funnel above a beaker. The filter paper was saturated with deionized water, allowed to drain until non-dripping, and weighed. Soil was then saturated by repeatedly adding DI water until a thin layer of water remained at the surface. Funnels were covered with parafilm and allowed to drain for 24 h . After drainage, the saturated soil and filter paper were transferred to pre-weighed aluminum tins, dried at 105°C for 24 h , and reweighed. WHC (%) was calculated as the mass of water retained after drainage divided by oven-dry soil mass $\times 100$.

To assess short term microbial CO₂ respiration, 50 g of field-moist <2 mm sieved soil was brought to 50 % WHC, placed in sterile containers, covered with parafilm, and pre-incubated overnight at 25 °C to allow equilibration. Following acclimation, subsamples were weighed directly into 118 mL glass mason jars (Ball Quilted Crystal, Ace Hardware) fitted with gas-tight rubber septa. For each soil, three technical replicates of 2.00 g were prepared for the 24-h respiration assay. Jars were sealed and incubated at 25 °C for 24 h. Headspace CO₂ concentrations immediately before and after incubation were measured using an SRI 8610C gas chromatograph equipped with a flame ionization detector and methanizer. CO₂ respiration rates were calculated as the change in headspace CO₂ concentration over 24 h, corrected for jar volume and soil dry mass.

Stable Isotope Analysis:

Stable carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) isotope ratios of soil samples were determined at the UC Davis Stable Isotope Facility (SIF). For each sample, approximately 74 – 102 mg (mean = 85 mg) of finely ground, oven-dried soil from the POM and MAOM fractions was weighed into tin capsules for analysis. Samples were analyzed using Elementar vario MICRO cube elemental analyzer interfaced to an Elementar VisION IRMS (Elementar Analysensysteme GmbH, Langenselbold, Germany). Samples were combusted at 1080 °C in a reactor containing chromium oxide and silvered copper oxide. Following combustion, oxides are removed in a reduction copper reactor at 650°C. The helium carrier then flows through a water trap containing magnesium perchlorate and phosphorous pentoxide desiccants. Following removal of water, N₂ and CO₂ were separated chromatographically and introduced sequentially to the IRMS for isotope ratio determination^{127,128}.

Data Analysis and Statistics:

The spectral data was analyzed in R (R Core Team, 2025)⁴⁶, using packages ChemoSpec¹²⁹ to import the spectral object, then perform hierarchical clustering analysis (HCA) and principal component analysis (PCA). Then ggplot2¹³⁰ was utilized to refine the resulting figures and plot soil metadata.

Model selection, data transformation, and ANOVA tests were conducted in R using emmeans¹³¹, rcompanion¹³², and car¹³³ packages. Each soil physicochemical and SOM variable was analyzed with an analysis of variance (ANOVA) model that included year, which is confounded with year of orchard establishment, and treatment (i.e., WOR versus control) as fixed factors. Specifically, the model was written as `lm(variable ~ Year + Treatment, data = SFT_meta)`. The data were tested for normality and homogeneity of variances using inspection of quantile-quantile plots, histograms of residuals, and Shapiro-Wilk tests, and were transformed when necessary to meet model assumptions prior to inference.

Results

The total carbon (TC), total nitrogen (TN), and soil organic matter (SOM) concentrations in the <2 mm soil fraction (Figures 2.1a, 2.1b, and 2.2a respectively) were significantly elevated by whole orchard recycling (WOR) treatment relative to the control, with more pronounced differences in the younger orchards (i.e., 3-, 4-, and 6-years post-recycling). WOR plots showed 30–50% higher SOM and TC compared to controls in the 3-, 4-, and 6-year-old orchards ($p < 0.001$), but differences were diminished in the 15-year-old orchard. Chronologically, the unamended control SOM content means were 2.33% after 3 years, 2.09% after 4 years, 2.23% after 6 years, and 2.76% after 15 years relative to orchard establishment (Table 2.1). The WOR treated SOM content means were 2.82% after 3 years, 3.44% after 4 years, 3.48% after 6 years,

and 2.68% after 15 years relative to wood chip integration and orchard establishment (Table 2.1). Collectively, the treatment effect was strongest for SOM ($F = 41.94$, $p < 0.001$) and TC ($F = 37.08$, $p < 0.001$), with significant treatment $\times$ orchard age interactions for both variables ($F = 8$, $p < 0.001$), suggesting temporal variability in treatment response.

Similar patterns to the bulk (<2 mm) soil were observed in the corresponding POM and MAOM fractions (Figures 2.1a, b, and c), with TC nearly doubling in the 4- and 6-year-old orchards. Notably, significant variation of TC between orchard ages was observed in POM, however the temporal variation was insignificant in MAOM across the 3-, 4-, 6-, and 15-year space-for-time series. Although, MAOM TC and TN contents were highest in WOR soils for orchards 4 and 6 years post recycling. For this space-for-time orchard series, WOR increased soil POM TC means from 0.22 to 0.36 mg C kg⁻¹ after 3 years, 0.26 to 0.98 mg C kg⁻¹ after 4 years, and 0.43 to 0.96 mg C kg⁻¹ after 6 years (Table 2.2). POM TC showed little difference between treatments in the 15-year-old orchard, control and WOR means of 0.50 and 0.46 mg C kg⁻¹ respectively (Table 2.2). Across this space-for-time orchard series, WOR treatment also increased MAOM TC from a mean of 0.84 to 1.14 mg C kg⁻¹ after 3 years, 0.73 to 1.16 mg C kg⁻¹ after 4 years, 0.73 to 1.30 mg C kg⁻¹ after 6 years, with a small and insignificant increase from 0.97 to 0.99 mg C kg⁻¹ after 15 years (Table 2.2). A similar pattern was observed for TN means, with WOR increasing POM TN from 0.02 to 0.03 mg N kg⁻¹ after 3 years, 0.02 to 0.07 mg N kg⁻¹ after 4 years, and 0.03 to 0.06 mg N kg⁻¹ after 6 years, before converging with the control at 0.04 mg N kg⁻¹ in the 15 year old orchard (Table 2.2). Likewise, WOR increased MAOM TN means from 0.12 to 0.14 mg N kg⁻¹ after 3 years, 0.10 to 0.14 mg N kg⁻¹ after 4 years, and 0.09 to 0.14 mg N kg⁻¹ after 6 years, without significant treatment differences after 15 years (Table 2.2).

Table 2.1 Fine Sandy Loam Soil Physiochemical Means for 4 Orchards with WOR Treatments, as a Space-for-Time Substitution

Treatment	Orchard Age	TC (%)	SD	TN (%)	SD	C:N	SD	SOM (%)	SD	pH (1:1 DIW)	SD	DOC (mg C / g soil)	SD	DON (mg N / g soil)	SD	WHC (%)	SD	mg CO ₂ / 24 hrs	SD
Control	3 Years	0.62	0.16	0.07	0.02	8.63	0.75	2.328	0.260	7.66	0.26	0.78	0.17	0.10	0.04	25.19	5.32	25.28	9.53
WOR	3 Years	0.82	0.24	0.08	0.03	9.88	0.76	2.818	0.455	7.82	0.30	0.94	0.32	0.06	0.04	24.56	2.36	21.37	11.06
Control	4 Years	0.61	0.13	0.07	0.03	8.53	1.35	2.085	0.213	6.49	0.38	0.77	0.07	0.68	0.92	32.57	4.50	46.40	22.89
WOR	4 Years	1.38	0.37	0.12	0.02	11.03	1.30	3.435	0.796	6.50	0.27	1.46	0.23	0.50	0.51	42.60	3.95	39.41	19.52
Control	6 Years	0.74	0.06	0.06	0.00	12.17	0.31	2.228	0.112	7.82	0.09	0.84	0.12	0.04	0.02	30.63	1.47	102.93	32.19
WOR	6 Years	1.42	0.07	0.11	0.01	12.68	1.14	3.480	0.145	7.71	0.10	1.68	0.29	0.28	0.09	38.51	0.99	83.69	4.19
Control	15 Years	0.92	0.09	0.09	0.03	10.26	1.63	2.758	0.171	7.70	0.13	0.66	0.08	0.04	0.05	41.10	2.84	75.76	15.00
WOR	15 Years	0.87	0.11	0.08	0.01	10.80	0.34	2.683	0.269	7.72	0.21	0.82	0.09	0.05	0.05	39.45	0.74	52.91	28.76

Table 2.1: The means and standard deviations of the <2 mm soil physiochemical data presented here were calculated using four replicate blocks per combination of treatment and orchard age. Chronologically, the 3-year-old orchard received 60 tons of wood per acre, the 4-year-old orchard received 61 tons of wood per acre, the 6-year-old orchard received 85 tons of wood per acre, and the 15-year-old orchard received 33 tons of wood per acre.

Table 2.2 POM and MAOM Physiochemical Means for 4 Orchards with WOR Treatments, as a Space-for-Time Substitution

Treatment	Orchard Age	POM						MAOM					
		TC (%)	SD	TN (%)	SD	C:N	SD	TC (%)	SD	TN (%)	SD	C:N	SD
Control	3 Years	0.22	0.06	0.02	0.00	9.25	0.96	0.84	0.13	0.12	0.01	7.24	0.33
WOR	3 Years	0.36	0.13	0.03	0.01	11.97	2.31	1.14	0.22	0.14	0.02	8.16	0.47
Control	4 Years	0.26	0.06	0.02	0.01	9.86	0.71	0.73	0.18	0.10	0.02	7.04	0.45
WOR	4 Years	0.98	0.38	0.07	0.02	14.24	1.47	1.16	0.23	0.14	0.02	8.26	0.60
Control	6 Years	0.43	0.03	0.03	0.00	13.70	0.69	0.73	0.17	0.09	0.02	7.76	0.43
WOR	6 Years	0.96	0.14	0.06	0.01	15.26	0.64	1.30	0.18	0.14	0.02	9.50	0.28
Control	15 Years	0.50	0.06	0.04	0.00	13.85	0.51	0.97	0.08	0.12	0.01	8.32	0.24
WOR	15 Years	0.46	0.13	0.04	0.01	12.56	1.01	0.99	0.13	0.12	0.01	8.25	0.24

Table 2.2: The means and standard deviations of the POM and MAOM physiochemical data presented here were calculated using four replicate blocks per combination of treatment and orchard age. Chronologically, the 3-year-old orchard received 60 tons of wood per acre, the 4-year-old orchard received 61 tons of wood per acre, the 6-year-old orchard received 85 tons of wood per acre, and the 15-year-old orchard received 33 tons of wood per acre. These fractions were separated directly from the < 2 mm soil characterized in table 1.

Soil C:N ratio also exhibited clear treatment effects in the <2 mm, POM, and MAOM fractions (Figure 2.1c). The WOR-treated POM and MAOM fractions consistently had higher C:N ratios, particularly in orchards 3, 4, and 6 years old (POM C:N: $F = 16.55$, $p < 0.001$; MAOM C:N: $F = 45.87$, $p < 0.001$), indicating increased carbon content without a proportionate increase in nitrogen. Over this space-for-time series, WOR changed the POM C:N ratio from a mean of 9.3 to 12.0 after 3 years, 9.9 to 14.2 after 4 years, 13.7 to 15.3 after 6 years, and 13.8 to 12.6 after 15 years (Table 2.2). Concurrently, WOR increased the MAOM C:N ratio from a mean of 7.2 to 8.2 after 3 years, 7.0 to 8.3 after 4 years, and 7.8 to 9.5 after 6 years (Table 2.2). A treatment mean difference was not observed in the 15-year-old orchard, having C:N ratios of 8.3 and 8.2 in WOR and control soil respectively. A significant treatment effect was also detected in the <2 mm soil C:N ratio ($F = 10.47$, $p < 0.004$), following a very similar temporal trend as the POM fraction.

Dissolved organic carbon (DOC), extractable by 0.5 M K_2SO_4 from the <2 mm soil, (Figure 2.2e) was significantly higher in WOR plots ($F = 27.25$, $p < 0.001$), especially in the 4- and 6-year-old orchards, while DON did not vary significantly by treatment (Figure 2.2f). Chronologically, WOR increased the soil DOC means from 0.78 to 0.94 mg C g⁻¹ dry soil in the 3-year-old orchard, 0.77 to 1.46 mg C g⁻¹ in the 4-year-old orchard, 0.84 to 1.68 mg C g⁻¹ in the 6-year-old orchard, and 0.66 to 0.82 mg C g⁻¹ in the 15-year-old orchard (Table 2.1). Water holding capacity (WHC) of the <2 mm soil (Figure 2.2b) was also significantly increased by WOR ($F = 10.65$, $p = 0.003$), with a significant treatment $\times$ orchard age interaction ($F = 5.79$, $p = 0.004$). For these fine sandy loam soils, the WHC ranged from about 25–45 %, with wood chip-amended soils exhibiting higher WHC relative to unamended controls. Specifically, WOR increased soil WHC means from 32.57 to 42.60% in the 4-year-old orchard and from 30.63 to

38.51% in the 6-year-old orchard (Table 2.1). This increase was not observed for the soil in the orchards amended by WOR 3 and 15 years in age.

The soil pH values (Figure 2.2c) did not vary by treatment and were between 7.5 and 8.0 in all soils except for the 4-year-old orchard. Chronologically, the control soil pH means were 7.7 in the 3-year-old orchard, 6.5 in the 4-year-old orchard, 7.8 in the 6-year-old orchard, and 7.7 in the 15-year-old orchard (Table 2.1). The WOR soil pH means were 7.8 in the 3-year-old orchard, 6.5 in the 4-year-old orchard, 7.7 in the 6-year-old orchard, and 7.7 in the 15-year-old orchard (Table 2.1).

Soil CO₂ respiration rates (Figure 2.2d), measured by 24-hour jar incubations, did not differ between WOR and control treatments ($p > 0.05$), but varied strongly across orchards ($F = 18.50$, $p < 0.001$), suggesting site-level heterogeneity in microbial activity and mineralizable soil C. Across the chronosequence, bulk soil CO₂ respiration rates showed substantial differences among fields ($F = 18.50$, $p < 0.001$), with the highest fluxes measured in the 6-year-old orchard and the lowest fluxes measured in the 3-year-old orchard. Within each orchard, however, WOR and control treatments did not differ significantly in respiration (all pairwise treatment contrasts $p > 0.11$), despite clear treatment effects on SOM, TC, and TN.

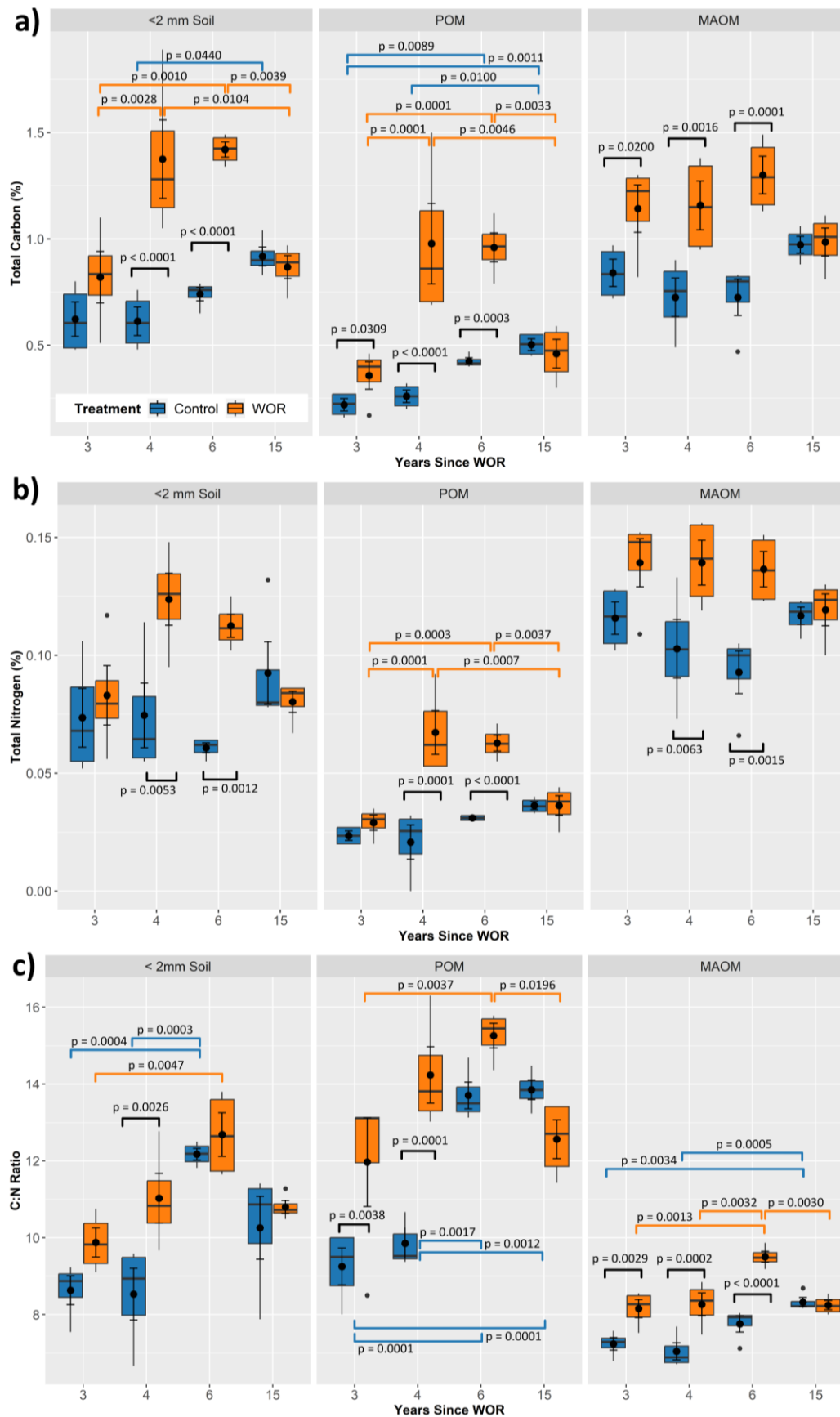


Figure 2.1: Box plots of total carbon (TC), total nitrogen (TN), and C:N ratio for the bulk (<2 mm), particulate organic matter (POM) and mineral associated organic matter (MAOM) fractions across four sampling years (3, 4, 6, and 15 years since WOR; a, b, c respectively). WOR-treated soils are shown in orange, and control soils in blue. Boxes represent the interquartile range with medians, whiskers extend to $1.5 \times$ IQR, and points indicate means $\pm$ SE. Significant ($p > 0.05$) post hoc comparisons between treatments are shown in black brackets, while comparisons between time points are shown in orange and blue brackets.

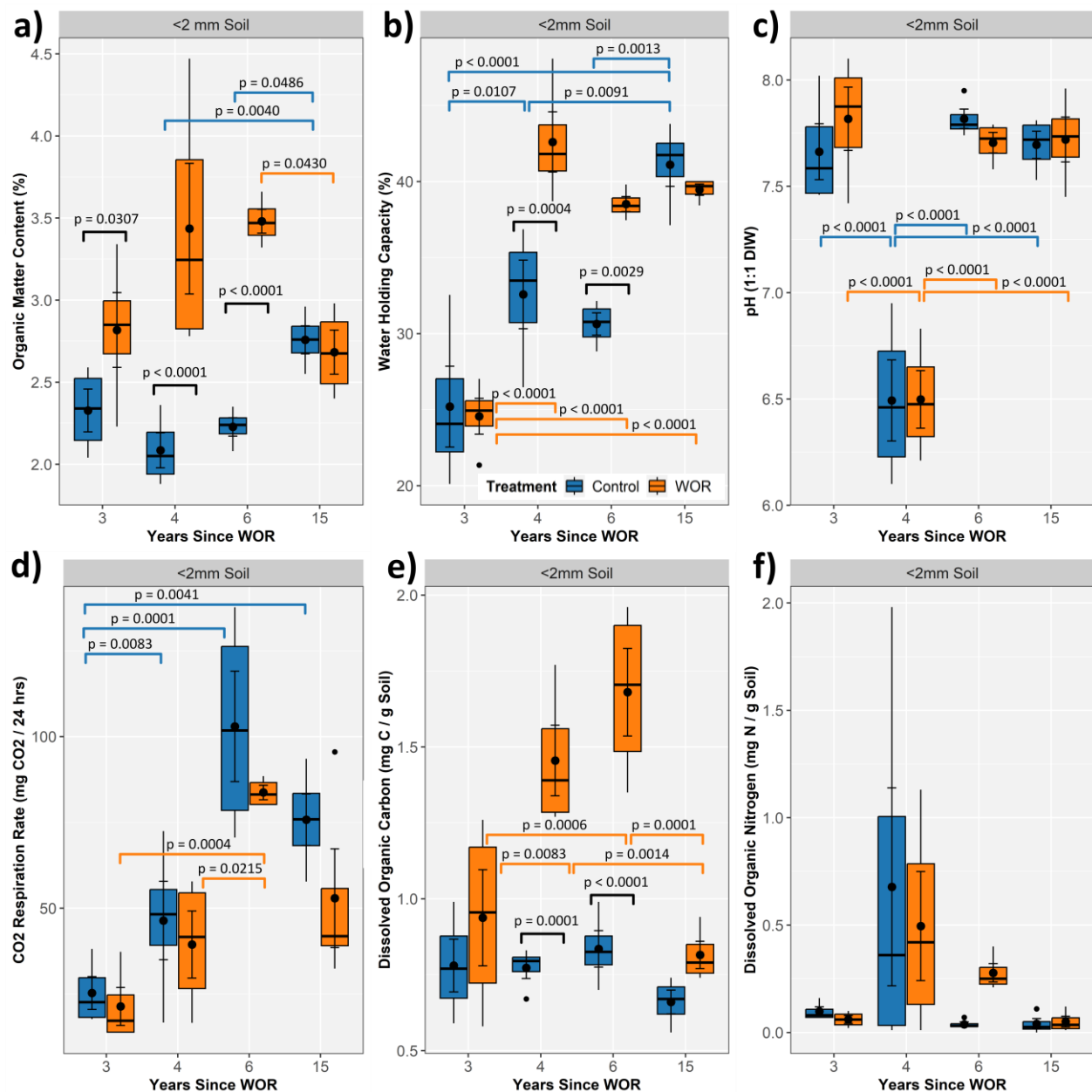


Figure 2.2: Box plots of soil organic matter (SOM), water-holding capacity (WHC), pH (equal parts soil and deionized water), CO₂ respiration, dissolved organic carbon (DOC), and dissolved organic nitrogen (DON) for the bulk (<2 mm) soils sampled across four sampling years (3, 4, 6, and 15 years since WOR; a, b, c, d, e, f respectively). WOR-treated soils are shown in orange,

and control soils in blue. Boxes represent the interquartile range with medians, whiskers extend to $1.5 \times$ IQR, and points indicate means $\pm$ SE. Significant ($p > 0.05$) post hoc comparisons between treatments are shown in black brackets, while comparisons between time points are shown in orange and blue brackets.

Comparing POM with each corresponding MAOM fraction, the C:N ratios were always higher for POM (Figure 2.3a). This pattern was also observed when comparing WOR (POM C:N mean: 13.5 ± 1.9 , MAOM C:N mean: 8.5 ± 0.7) and control treatments (POM C:N mean: 11.8 ± 2.3 , MAOM C:N mean: 7.6 ± 0.6). Despite these average differences, POM and MAOM C:N ratios were positively correlated (WOR: $r = 0.74$; Control: $r = 0.79$; both $p < 0.001$), with many samples aligning below the 1:1 line, shown in Figure 2.3a. This pattern is in alignment with the decoupling conceptual framework, which suggests that POM has major plant derived components and that MAOM has major microbially derived components in this system.

The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ stable isotope contents of POM and MAOM (Figure 2.3b and c) also display the patterns described in the decoupling framework, again for both treatment groups. Here, the $\delta^{13}\text{C}$ values were higher in MAOM (WOR $\delta^{13}\text{C}$: $-26.0 \pm 0.7\text{‰}$; Control $\delta^{13}\text{C}$: $-25.8 \pm 1.0\text{‰}$) than in POM (WOR $\delta^{13}\text{C}$: $-27.4 \pm 0.8\text{‰}$; Control $\delta^{13}\text{C}$: $-27.3 \pm 1.0\text{‰}$), indicating that MAOM is more enriched with ^{13}C . Similarly, the $\delta^{15}\text{N}$ values were consistently higher in MAOM (WOR mean: $5.2 \pm 1.3\text{‰}$; Control mean: $5.1 \pm 1.1\text{‰}$) relative to POM (WOR mean: $2.0 \pm 1.0\text{‰}$; Control mean: $2.0 \pm 0.9\text{‰}$), indicating that MAOM is more enriched with ^{15}N . Linear regressions of POM vs MAOM ^{13}C -enrichment (WOR: $r > 0.93$, $p < 0.001$; Control: $r > 0.92$, $p < 0.001$) and ^{15}N -enrichment (WOR: $r > 0.92$, $p < 0.001$; Control: $r > 0.93$, $p < 0.001$) were strongly correlated in both treatment groups, with points falling above the 1:1 line. This again suggests that POM has major plant derived components, which likely undergo microbial transformation into MAOM within this system, regardless of WOR treatment.

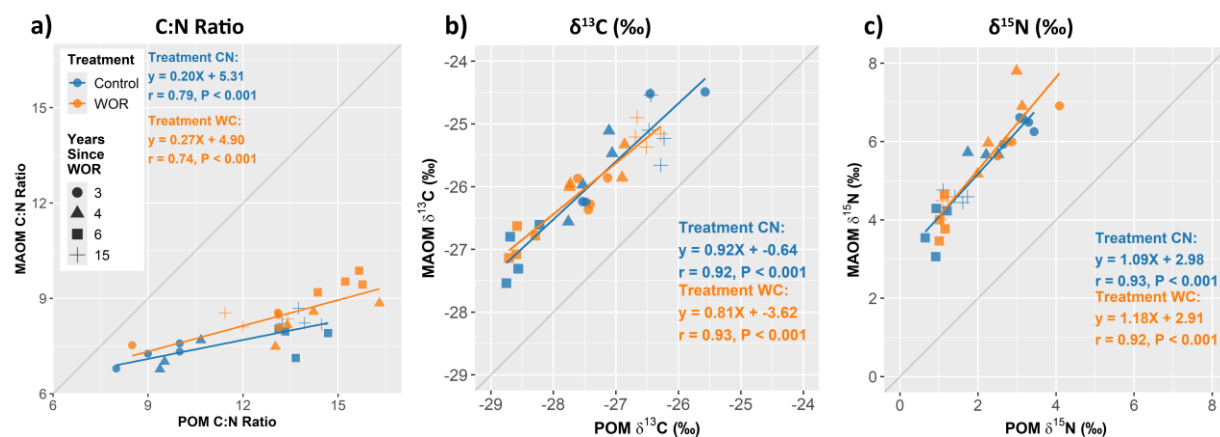


Figure 2.3: Evaluation of particulate organic matter (POM) and mineral associated organic matter (MAOM) coupling using linear regressions of C:N ratio, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$ contents (a, b, c respectively). Each point represents an individual sample of POM plotted against the corresponding MAOM fraction value. The whole orchard recycling (WOR) amended soils are plotted in orange and unamended controls are plotted in blue. Years since WOR, which is confounded with the field sampled, is expressed by shape. A faint grey 1:1 line is displayed in the background for comparison.

Diffuse reflectance infrared Fourier Transform spectroscopy (DRIFTS) and organic functional group assignment, based on reported SOM spectral reference regions (collated in the appendix, Table A2.1), identified 14 of the most prominent peaks within the $3700 - 1200 \text{ cm}^{-1}$ region. Here the POM and MAOM fractions predominantly displayed $\nu(\text{N-H})$ and $\nu(\text{O-H})$ stretching, asymmetric and symmetric aliphatic $\nu(\text{C-H})$ stretching, CO_2 $\nu(\text{C=O})$, carboxylic acid and ester $\nu(\text{C=O})$ stretching, aromatic $\nu(\text{C=C})$ stretching, amide $\delta(\text{N-H})$ bending, $\nu(\text{C=N})$ stretching, aliphatic $\delta(\text{C-H})$ bending, hydroxyl $\nu(\text{O-H})$ stretching, carboxylate $\nu(\text{C-O})$ and carboxylic acid $\nu(\text{C=O})$ stretching. Among the 14 prominent spectral features identified in POM and MAOM, three aromatic peaks and five aliphatic hydrocarbon peaks were detected.

Treatment-related differences in infrared absorbance were most pronounced in the intermediate-aged orchards (4 and 6 years since WOR amendment; Figure 2.4a and b). Aromatic $\nu(\text{C=C})$ bands were dominant across all DRIFT spectra; however consistently more intense in WOR-treated soils (both POM and MAOM fractions) compared to controls.

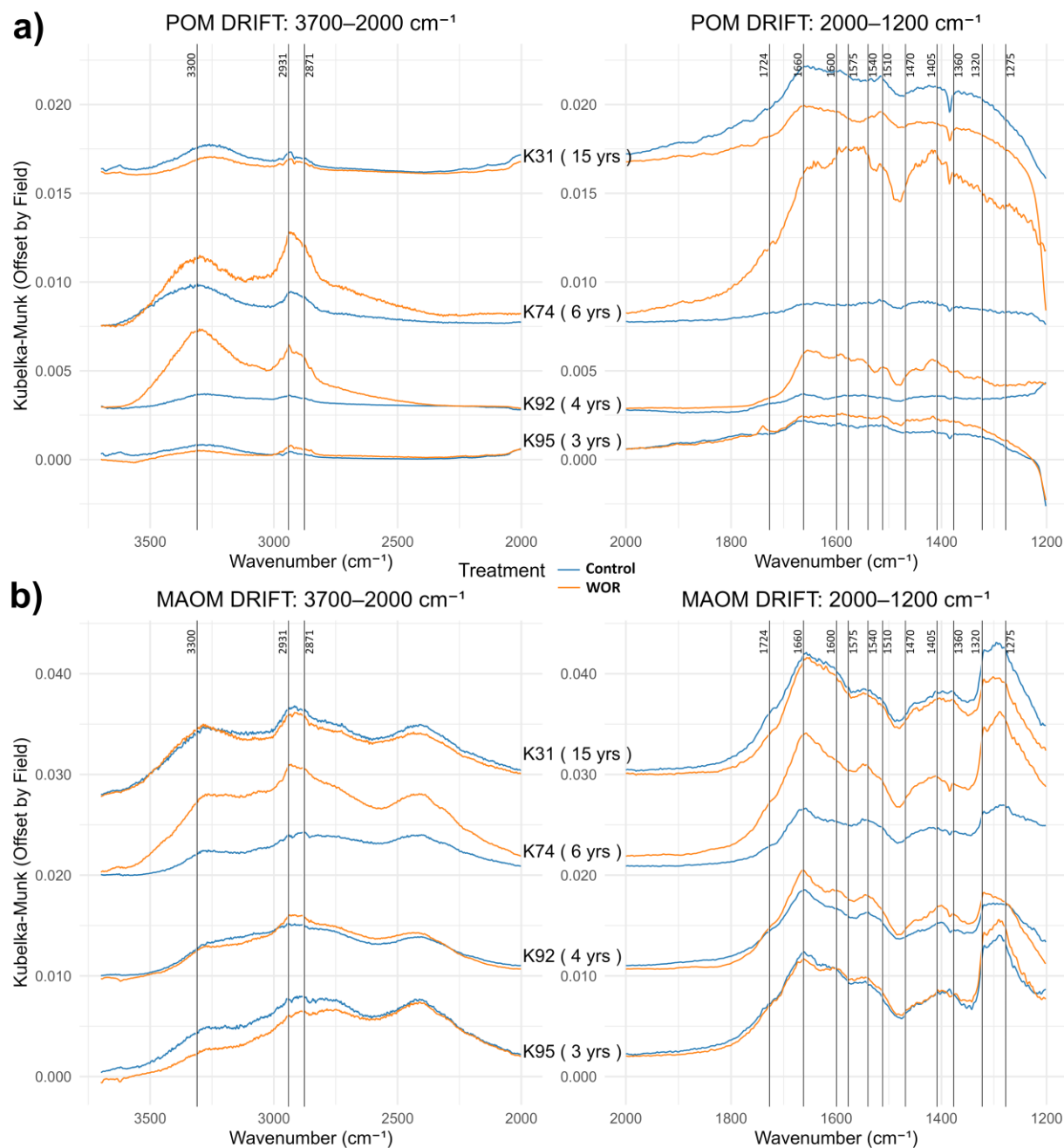


Figure 2.4: Diffuse reflectance infrared Fourier transform (DRIFT) spectra of particulate organic matter (POM) and mineral associated organic matter (MAOM) fractions (a and b respectively), shown as Kubelka-Munk transformed absorbance following spectral subtraction (i.e., NaOCl treated soil spectra subtracted from the untreated soil spectra), displayed are spectral averages of four replicate blocks for each treatment and year combination. Orchard blocks are arranged by establishment year from oldest (est. 2008, 15 yrs post-WOR; top) to youngest (est. 2020, 3 yrs post-WOR; bottom). WOR amended soils are shown in orange, while the controls are in blue. Spectra were collected using OMNIC software, and biological replicates (blocks 1–4) were averaged post-subtraction. The subtracted spectra were subset to 3700 – 1200 cm^{-1} for clarity, as

the region $<1200\text{ cm}^{-1}$ overlaps with non-target metal oxide and Si-O bands. The unaveraged spectra are shown in the appendix for POM (Figure A2.3) and MAOM (Figure A2.4).

Indices of Aliphatic C : Aromatic C and Aromatic C : Carboxylic C (Figure 2.5) were highest in the POM fractions from WOR treated soils collected from the 4-year-old orchard amendment, with significant reductions in both indices for 6- and 15-year-old orchards, relative to the WOR amendment. Increased Aliphatic C : Aromatic C index values indicate greater aliphatic contents [2931 cm^{-1} $\nu(\text{C-H})$ asymmetric and 2871 cm^{-1} $\nu(\text{C-H})$ symmetric stretches] relative to aromatic contents [1660 , 1600 , 1540 , and 1510 cm^{-1} , $\nu(\text{C=C})$ and $\nu(\text{C-H})$ stretches] in the POM fraction of WOR-treated soils. A higher Aromatic C : Carboxylic C index corresponds to a greater proportion of aromatic functional groups compared to carboxylic functional groups, with contributions from other carbonyl-containing groups [1724 cm^{-1} , 1320 cm^{-1} , 1275 cm^{-1} $\nu(\text{C=O})$ stretches].

For POM analyzed from the 4-year-old orchard, the WOR treatment significantly increased the contributions of aliphatic and aromatic functional groups relative to the carboxylic functional groups. Although insignificant, POM analyzed from the 6-year-old orchard display lower ratios of aliphatic to aromatic functional groups after WOR amendment. For MAOM from the 3-year-old orchard, WOR treatment resulted in significantly reduced ratios of both aliphatic to aromatic and aromatic to carboxylic functional groups. Significant differences in the ratios of aliphatic, aromatic, and carboxylic functional groups were not detected for MAOM from the 4-year-old orchard. For MAOM from the 6-year-old orchard, WOR treatment significantly increased the ratios of aliphatic to aromatic functional groups; although insignificant, the ratios of aromatic to carboxylic functional groups were reduced by WOR in the 6-year-old orchard. No treatment differences were detected after 15 years in any index ratio.

Across indices, significant WOR treatment effects were confined to sparse space-time points, while differences between orchard ages were more pronounced, often with a significant shift of WOR treated POM and MAOM ratios down to control conditions in the 15-year-old orchard. For the POM Aliphatic C : Aromatic C index, a marginally significant WOR treatment $\times$ orchard age interaction was observed ($F = 2.71$, $p = 0.071$). The MAOM Aliphatic C : Aromatic C index exhibited a significant WOR treatment $\times$ orchard age interaction ($F = 11.21$, $p < 0.001$). The WOR treatment $\times$ orchard age interaction was insignificant for both POM and MAOM Aromatic C : Carboxylic indices.

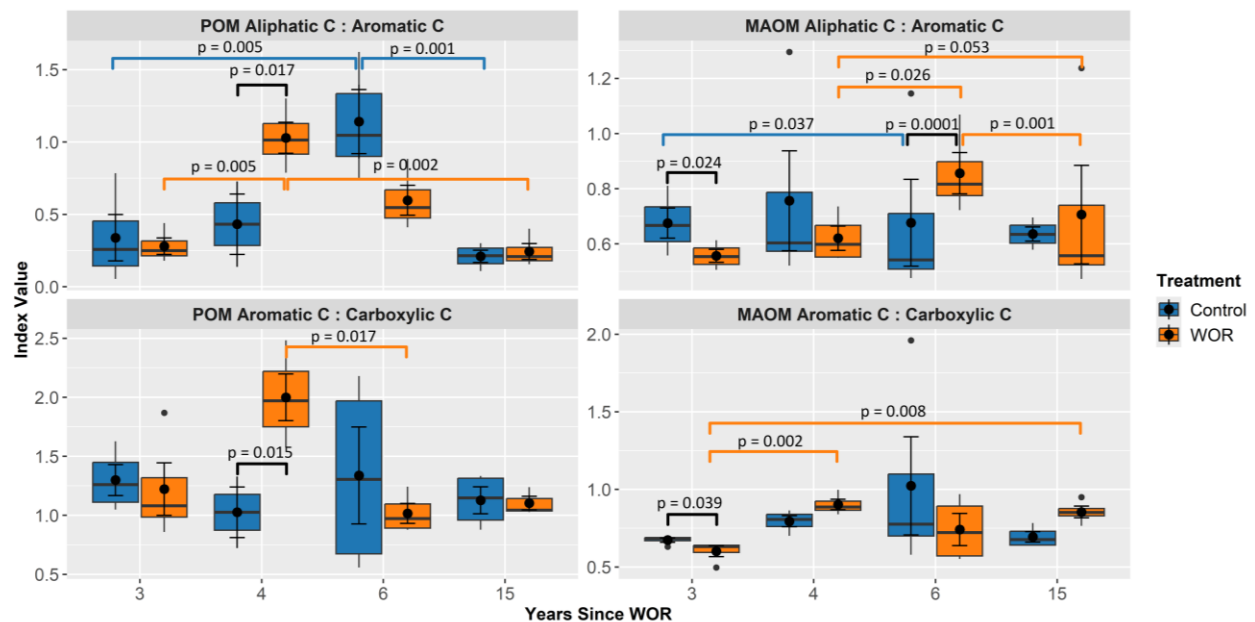


Figure 2.5: Box plots of Aliphatic C : Aromatic C and Aromatic C : Carboxylic C indices for particulate organic matter (POM) and mineral associated organic matter (MAOM) soil fractions across a space-for-time chronosequence of Central Valley, CA orchards at 3, 4, 6, and 15 years since Whole Orchard Recycling (WOR). Indices were derived from ratios of characteristic diffuse reflectance Fourier transform spectroscopy (DRIFTS) absorbance peaks, with values calculated for four replicate blocks per year and treatment combination. Treatments include WOR-amended soils (orange) and untreated control soils (blue). Boxes represent the interquartile range with medians, whiskers extend to $1.5 \times$ IQR, and points indicate means $\pm$ SE. Black points indicate the mean $\pm$ standard error (SE) for each group; error bars at means reflect SE across blocks. Significant ($p > 0.05$) post hoc comparisons between treatments are shown in black brackets, while comparisons between time points are shown in orange and blue brackets.

Discussion

Hanford fine sandy loam orchard soils treated by WOR showed 30–50 % higher soil organic matter (SOM) contents compared to controls in 3-, 4- and 6-year-old orchards (Figure 2.2a), with evidence for direct contributions of wood chips to the composition of particulate organic matter (POM) and microbial transformation into mineral associated organic matter (MAOM). However, these effects were not observed in the 15-year-old orchard samples, suggesting exhaustion of applied wood chip C within this timeframe. These results portray a temporal progression of wood chip decomposition into orchard fine sandy loam orchard soils, where coarse wood residues gradually fragment during catabolic and anabolic C transformations by soil organisms until they are fully mineralized. Within this space-for-time substitution, WOR increased SOM content means in a skewed bell curve pattern, where the difference in SOM content means was 0.49% in the 3-year-old orchard, 1.35 % in the 4-year-old orchard, 1.25% in the 6-year-old orchard, and 0.08% in the 15-year-old orchard (Table 2.1). This pulse of SOM was expected to follow the trend in cumulative CO₂-C, reported by Culumber et al., 2025, where soil CO₂ respiration was highest in the first year following WOR and attenuated to control conditions in subsequent years²⁰. Comparatively, the 3-year-old orchard might have been expected to show the greatest increase SOM content following WOR, however this may be a case where most wood chip fragments were still large (i.e., > 2 mm) and thus sieved from analysis. Jar incubations of Hanford fine sandy loam soils, over 8 months at 20 – 23°C and 65% of field capacity, have shown that larger woodchip sizes (e.g., 12.7 and 6.4 mm) exhibit delayed C mineralization while the smallest woodchip size (0.2 and 1.6 mm) gave highest initial CO₂ emissions, but lowest total loss of SOC¹³⁴. Thus, changes in SOM contents following WOR reflect a balance of wood-C decomposition into smaller molecules and mineralization to CO₂.

The study presented here extends existing literature on WOR by examining chemical changes in the composition of POM and MAOM fractions of SOM within 3-, 4-, and 6-year-old orchards. For fine sandy loam soils, WOR-derived C largely accumulated in the coarse and fast-cycling POM fraction, with comparatively smaller contributions to the finer and slower-cycling MAOM fraction. Here, WOR significantly increased MAOM TC contents in the 3-, 4-, and 6-year-old orchards, but this effect was not observed in the 15-year-old orchard. The observed trend in MAOM TC may arise more commonly in sandy loam soils, of which the Handford series is characterized by clay contents of 10% or less³⁴, due to physical limitations of organic compounds to associate with silt and clay particles⁸⁷. For temperate top soils, von Lützow et al., 2007 found ~50–75% of total organic C to be associated with clay-sized particles (<2 μm), ~20–40% with silt-sized particles (2–63 μm), and <10% with sand-sized particles (>63 μm)¹⁰⁰. Especially for agriculturally managed sandy loam soils that are frequently disturbed, transfer of POM (> 53 μm particles) into long-term stabilized MAOM (< 53 μm particles) was not expected because organic compounds are less easily associated with sand particles.

Conceptually, WOR directly contributes plant-derived C to soil as POM, but it was unclear if MAOM is predominantly plant or microbially derived in this orchard system. The results of study provide evidence that MAOM is predominantly microbially derived in fine sandy loam soils, regardless of WOR treatment. As shown in Figure 2.3a, the POM C:N ratios (~8 - 16) are higher than corresponding MAOM C:N ratios (~7 - 9), wherein MAOM is more consistent with the stoichiometric ratios of microbial necromass¹³⁵. The increased enrichment of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ within the MAOM, shown in Figures 2.3b and c, further supports the notion that plant-derived compounds were not directly transferred into MAOM because this would have resulted in more homogenous isotopic profiles. Directionally, greater enrichment of MAOM with stable

isotopes also support this notion because microbial biomass has been found to be more enriched in ^{13}C than plant biomass^{99,103,104} and it can be protected from degradation by sorption to soil colloids. These results are in alignment with projected ^{13}C turnover times in temperate topsoil, which range from 0.5 to 374 years in the sand fraction, 115 to 676 years in the silt fraction, and 76 to 190 years in the clay fraction¹⁰⁰. Lastly, ^{13}C natural abundance techniques suggest that POM fundamentally has a short mean residence time relative to C associated with clay- and silt-sized organomineral complexes⁷⁹. For the WOR treatment, this conceptually manifests in direct losses of woodchip-derived C as CO_2 from POM, with limited transformation into MAOM.

To elucidate the form and stability of C compounds within the WOR system, diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) was utilized to characterize the molecular composition of POM and MAOM fractions of SOM. Wood, which is composed of cellulose, hemicelluloses and lignin, has specific FTIR absorption bands that have been identified in two main regions; although, small shifts can occur depending on the wood species studied¹¹¹. Broadly, the $3800\text{--}3400\text{ cm}^{-1}$ region encompasses hemicellulose and lignin hydroxyl groups and the $1700\text{--}1500\text{ cm}^{-1}$ region encompasses aromatic $\text{C}=\text{C}$ bonds of the lignin¹¹¹. As shown in Figure 2.4, these regions are prominently expressed in POM that underwent WOR treatment 4 and 6 years ago. Notably, the 3- and 15-year-old POM display very minor differences between treatments and the resulting spectra are flatter. Aromatic $\nu(\text{C}=\text{C})$ bands were dominant across all DRIFT spectra, however consistently more intense for the WOR treated soils sampled from the 4- and 6-year-old. This enhancement likely reflects the high aromatic C content of lignin-rich wood residues because most aromatic $\text{C}=\text{C}$ in soils is plant-derived, while $\text{C}=\text{O}$ is often contributed by microbes^{86,99,105–107}. Likewise, aliphatic $\nu(\text{C}\text{--}\text{H})$ absorbance features were more pronounced in WOR-amended POM fractions, indicating the presence of lipids,

which can be detected in weakly decomposed plant matter^{99,136}. The aliphatic $\nu(\text{C-H})$ absorbance features can also increase with growth of microbial biomass, which has a lipid content of 10 – 15 % dry weight¹³⁷. Collectively, the spectral patterns observed here follow the TC and SOM contents displayed in Figure 2.1a and 2.2a, where TC contents rise and fall over the 3-, 4-, 6-, and 15-year space-for-time series.

Spectral indices have been largely utilized for assessment of SOM properties¹⁰⁸, such as decomposability¹³⁸, stability¹³⁹, hydrophobicity¹⁴⁰, and oxygen content¹¹⁶. Oxygen containing functional groups may be used as indicators of decomposition because oxygen is preferentially utilized in microbial metabolism¹¹³, where it may be incorporated into essential biomolecules such as amino acids, nucleic acids, and membrane components. In the context of studying wood decomposition, indices were developed by incorporating the prominent aromatic (1510, 1660 cm^{-1}), aliphatic (2931, 2871 cm^{-1}), and carboxylic (1760 cm^{-1}) functional group peaks observed in Figure 2.4. In alignment with Figures 2.1, 2.3, and 2.4, the POM index values (Figure 2.5) display a sloping temporal trend and a consistent significant difference between WOR treatments in the 4-year-old orchard. Here, the ratio of aromatic C to carboxylic C is 100% greater than the unamended control, which is expected for soil that is dominated by woodchip derived C. Then, comparing the 4- and 6-year-old orchards, the decline in relative aromatic C band intensity indicates lignin has decomposed, which likely enables microbial growth, as supported by persistently high TC, (Figure 2.1a), balancing of the POM aromatic C to carboxylic C ratio, and corresponding increases in MAOM aliphatic C to aromatic C ratios. Finally, comparisons with the 15-year-old orchard again suggest that these WOR-induced changes attenuate to control conditions for both POM and MAOM. Similar temporal trends have been described for organic amendments of plant residues, where primary decomposition of plant-C is followed by

secondary decomposition of microbial biomass-C¹⁴¹. In contrast, POM and MAOM fractions with identical ratios of aliphatic C-H, aromatic C=C, and carbonyl C=O bonds would suggest that MAOM has major plant-derived contributions⁹⁹, indicating that the POM and MAOM fractions were compositionally similar. For the fine sandy loam soils studied here, the ratio of aromatic C to carboxylic C is consistently elevated above 1.0 for all POM samples, regardless of WOR treatment, while this ratio is consistently below 1.0 for almost all MAOM samples (Figure 2.5). This result indicates that MAOM represents a pool of C that is more oxidized, likely due to microbial metabolic processing of POM within the topsoil of this orchard system.

Conclusion

Integrating the structural, isotopic, and spectroscopic evidence, our results suggest that the transient gains in soil organic matter (SOM) contents of sandy loam soils following whole orchard recycling (WOR) are concentrated within the particulate organic matter (POM) fraction, while corresponding mineral associated organic matter (MAOM) fractions remain predominantly microbially derived regardless of WOR treatment. In this system, gains in overall SOM contents appear to peak after about 4 and 6 years, then dissipate after 15 years during the decomposition of lignocellulose, which is gradually mineralized into CO₂. This implies that repeated organic amendments may be necessary for maintenance of healthy soil conditions in the sandy loam soils that are commonly found in California's Central Valley, USA. Collectively, these findings emphasize that WOR acts as a significant short-term pulse of C that fuels microbial processing of POM into MAOM. Here, the potential for top soils to accumulate and maintain stable SOM in the long-term appears to be physically limited by soil texture, with soil C stabilization capacity increasing with clay content.

Chapter 2 Appendix

Spectral Region Reference (cm ⁻¹)	Spectral Peak Maxima (cm ⁻¹)	Organic Functional Group Assignment (Collation and Comparison of SOM Literature)
3300 - 3400 (broad band)	3300	○ O-H stretching in alcohols, water, carboxylic acids and phenols (Calderón et al. 2011)¹²³, ○ $\nu(\text{N-H})$ and $\nu(\text{O-H})$ (Margenot et al. 2015)¹²⁵, O–H and N–H stretch of water and 2° amines (Parikh et al. 2014)¹⁰⁸, ○ Phenolic OH stretch (Baes & Bloom, 1989)¹²²
2840 - 2890 and 2930 - 2970	2871 and 2931	○ C-H stretching of aliphatic hydrocarbons (Calderón et al. 2011)¹²³, ○ 2850 asymmetric $\nu(\text{C-H})$, 2924 symmetric $\nu(\text{C-H})$ (Margenot et al. 2015)¹²⁵, ○ CH₂ asymmetric stretch and CH₃ symmetric stretch (Parikh et al. 2014)¹⁰⁸,
1700 - 1730	1724	○ C=O carbonyl C stretch of esters and carboxylic acid (Calderón et al. 2011)¹²³, ○ asymmetric $\nu(\text{COOH})$ (Parikh et al. 2014)¹⁰⁸, ○ C=O stretch of -COOH (Baes & Bloom, 1989)
1650 - 1660	1660	○ Aromatic $\nu(\text{C=C})$ stretching (Margenot et al. 2015)¹²⁵,

		○ Carbonyl C (Calderón et al. 2011)¹²³
1580 - 1620	1600	○ Aromatic C=C stretch and/or asymmetric — COO⁻ stretch (Baes & Bloom, 1989)¹²², ○ Aromatic C=C stretch (Parikh et al. 2014)¹⁰⁸
1575	1575	○ Amide d(N-H) bend and v(C = N) stretch (Margenot et al. 2015)¹²⁵
1540 - 1550	1540	○ Amide II: N-H, C-N (Parikh et al. 2014)¹⁰⁸
1500 - 1525	1510	○ Aromatic C-H and C=C (Calderón et al. 2011)¹²³, ○ Aromatic C=C stretch (Baes & Bloom, 1989)¹²²
1405 and 1470	1405 and 1470	○ Aliphatic bending d(C-H) (Margenot et al. 2015)¹²⁵, ○ 1454 – 1460 δ(CH₂) (Parikh et al. 2014), ○ CH deformation of -CH₃ and -CH bending of -CH₂ (Baes & Bloom, 1989)
1390 - 1400	1360	○ aliphatic d(C-H) bend, potential contributions from symmetric carboxylate v(C-O) stretch (Margenot et al. 2015)¹²⁵, ○ symmetric v(COO-) (Parikh et al. 2014)¹⁰⁸
1320	1320	○ hydroxylic C-O-H (Calderón et al. 2011)¹²³

1250 - 1270	1275	○ Phenol $\nu(\text{C-O})$ asymmetric stretch and carboxylic acid $\nu(\text{C-O})$ stretch (Margenot et al. 2015)¹²⁵, ○ Carboxylic COOH (Calderón et al. 2011)¹²³, ○ $-\text{OH}$ stretch of phenolic OH (Baes & Bloom, 1989)¹²²
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Table A2.1: Organic functional group assignments for particulate organic matter (POM) and mineral associated organic matter (MAOM) by diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) in the mid-infrared range (subset to $3600 - 1200 \text{ cm}^{-1}$ for clarity). Spectral peak maxima correspond to absorbances measured after spectral subtraction of soil mineral interference for the particulate organic matter (POM) and mineral associated organic matter (MAOM) fractions of fine sandy loam orchard soils amended with wood chips by whole orchard recycling (WOR) in 3-, 4-, 6-, and 15-year-old orchards. Corresponding reference articles for each spectral region are provided for each organic functional group assignment.

WOR & Control DRIFT Spectra (Untreated Samples)

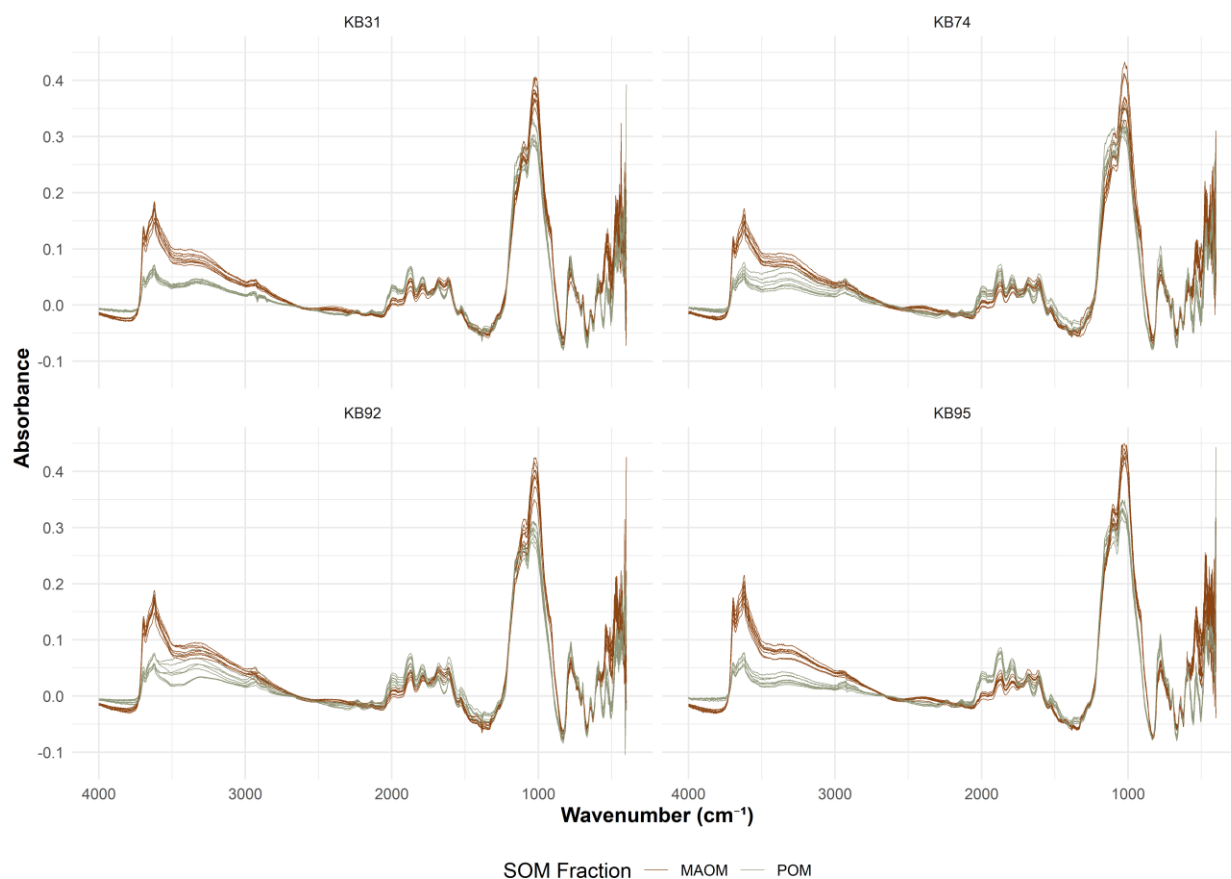


Figure A2.1: Spectra of particulate organic matter (POM; green hues) and mineral-associated organic matter (MAOM; brown hues) fractions of soil organic matter (SOM) collected from 15-year-old (field K31), 6-year-old (field K74), 4-year-old (field K92), and 3-year-old (field K95) orchards. Samples were taken from the top 15 cm of the berm near each tree in summer of 2023. Here, the MAOM fractions appear compositionally distinct from the POM fractions, most notably near $\sim 3600\text{ cm}^{-1}$ where MAOM samples show greater absorbance of mineral-surface $\nu(\text{OH})$ bonds. Raw spectra were baseline-corrected using iteratively reweighted least squares (IRLS) correction; peak assignments follow the reference regions compiled in Table A2.1.

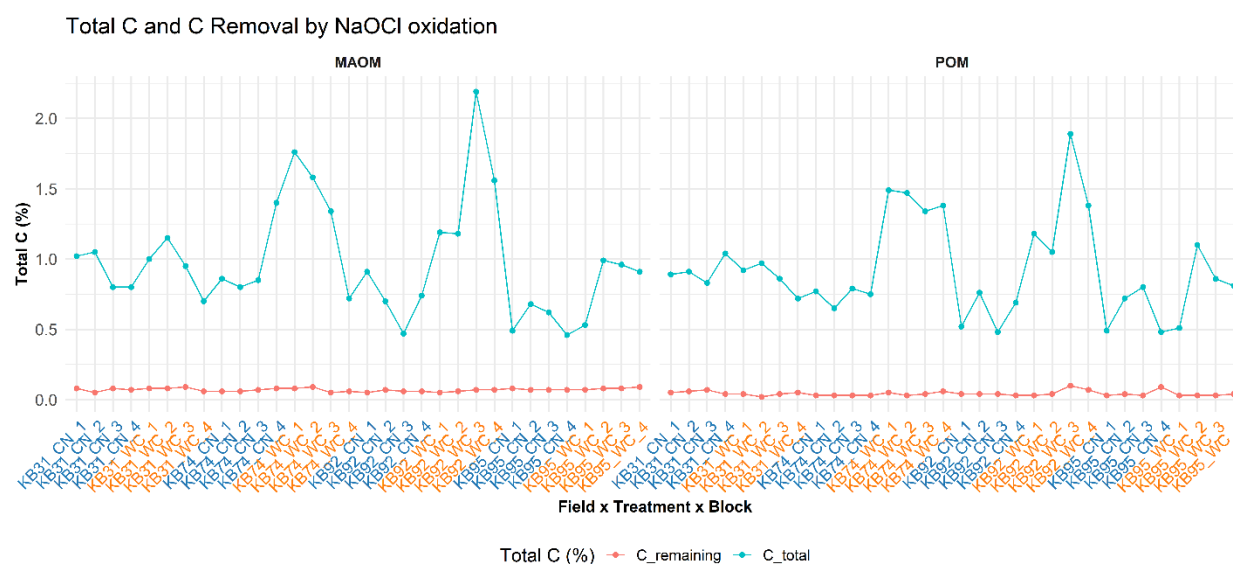


Figure A2.2: Total carbon (C) contents of particulate organic matter (POM) and mineral associated organic matter (MAOM) are shown in blue for all soil samples collected within the 3-, 4-, 6-, and 15-year-old orchards. C removal by bleach (NaOCl) oxidation, shown for each sample in red, supports the near complete removal of C within the corresponding oxidized fractions.

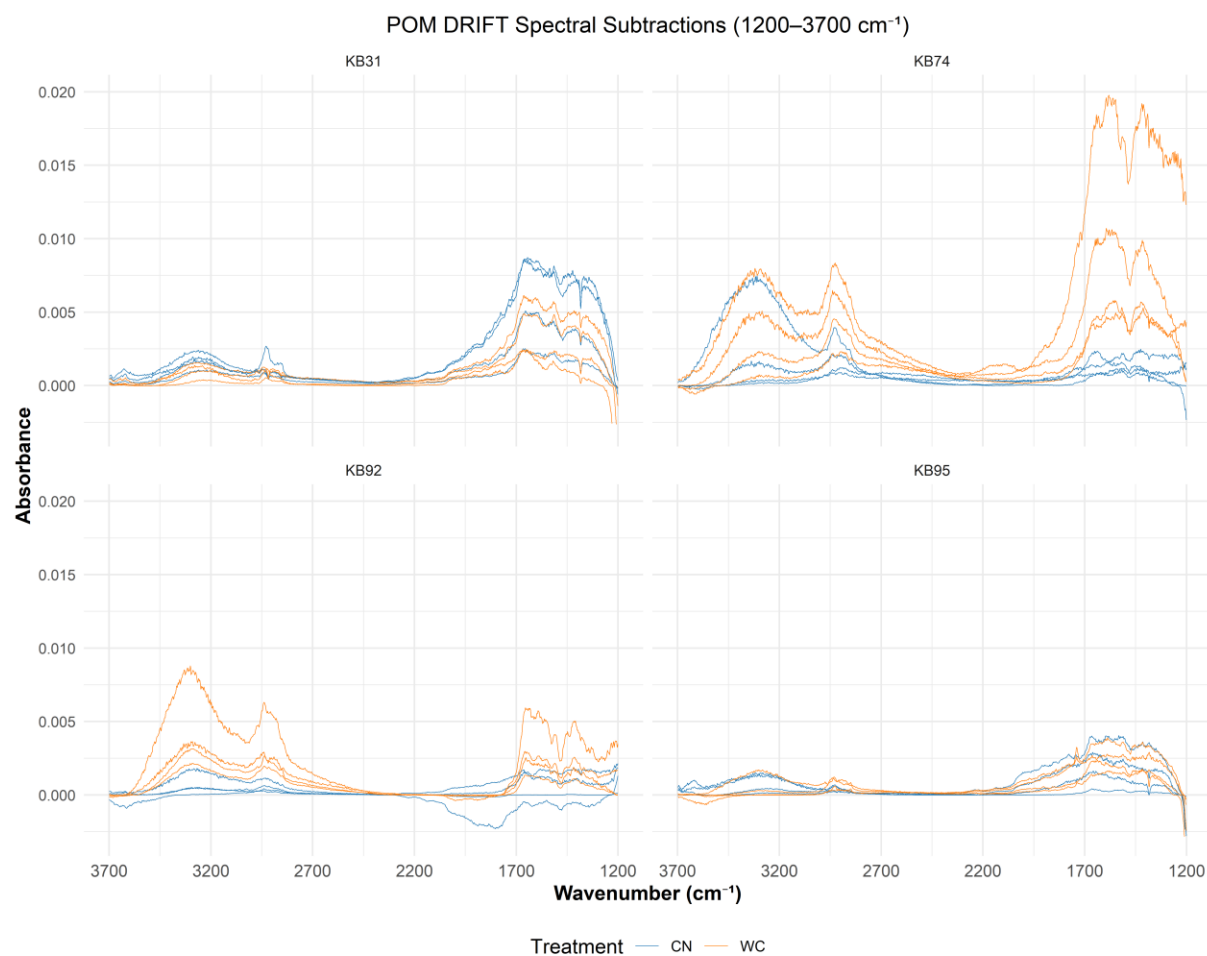


Figure A2.3: Unaveraged spectral subtractions of particulate organic matter (POM) samples, faceted by field to present a space-for-time substitution. Here 15-year-old (field K31), 6-year-old (field K74), 4-year-old (field K92), and 3-year-old (field K95) orchards were sampled in summer 2023.

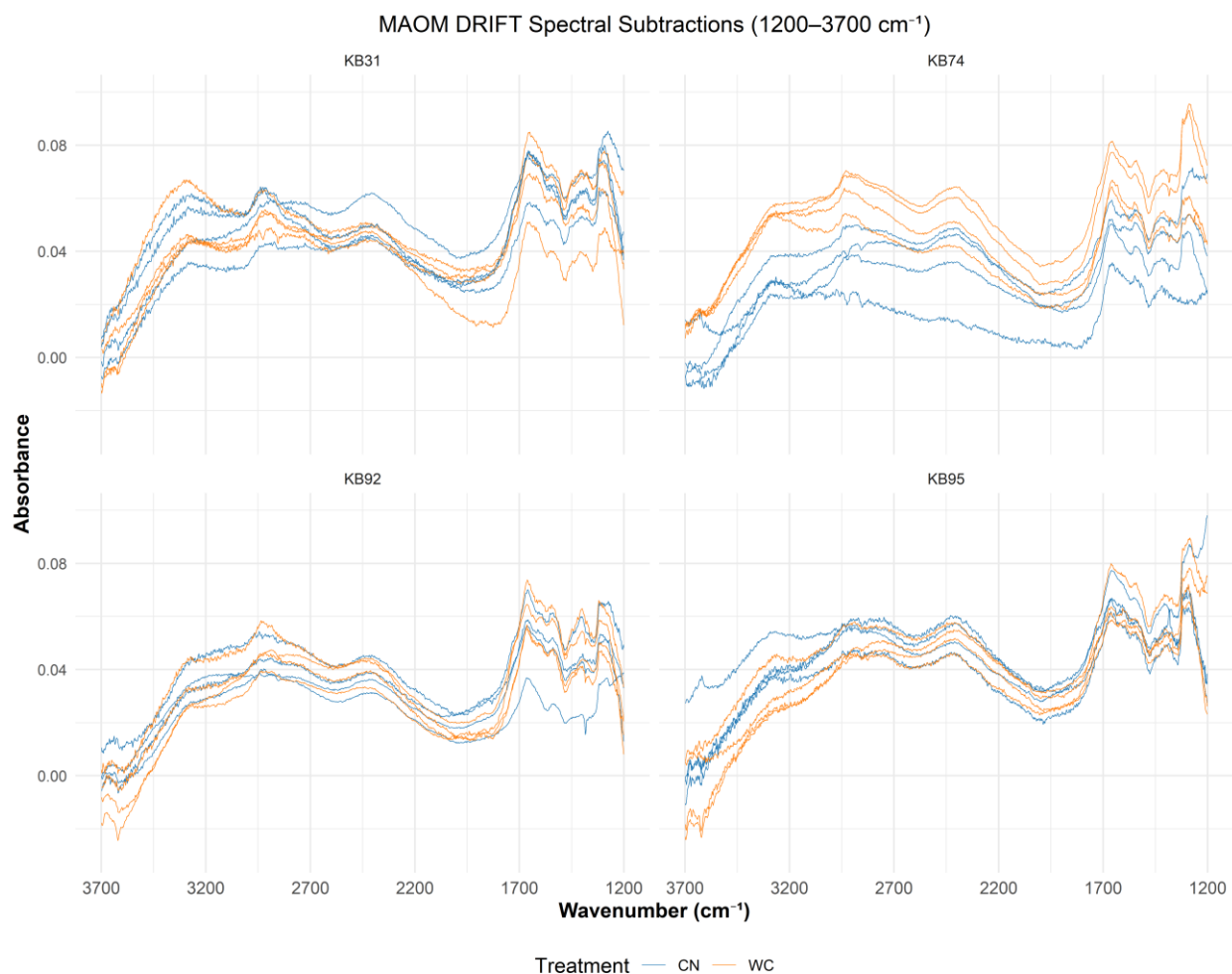


Figure A2.4: Unaveraged spectral subtractions of mineral associated organic matter (MAOM) samples, faceted by field to present a space-for-time substitution. Here, 15-year-old (field K31), 6-year-old (field K74), 4-year-old (field K92), and 3-year-old (field K95) orchards were sampled in summer 2023.

Chapter 3

Impact of Whole Orchard Recycling on Soil Bacteria and Archaea:

A Metagenomic Space-for-Time Substitution in Fine Sandy Loam Soils

Key Points:

- Topsoil Bacterial and archaeal communities were significantly affected by whole orchard recycling (WOR) in 4- and 6-year-old orchards, coinciding with the highest measured soil organic matter (SOM) and total carbon (TC) contents of <2 mm fine sandy loam soil.
- WOR did not significantly affect bacterial and archaeal metagenome-assembled genomes (MAGs) in 3- and 15-year-old orchards.
- Functional pathway profiles of WOR-enriched bacterial and archaeal MAGs suggest a preference for lignocellulose decomposition products, without the ability to initiate depolymerization of intact lignocellulose, and N reduction, rather than N oxidation.
- WOR-enriched MAGs appear to operate as a microbial consortium, with major contributions from *Alphaproteobacteria*, *Gammaproteobacteria*, *Thermoleophilia*, and other heterotrophic lineages that are common in soil.

Abstract

Understanding the response of soil microorganisms to carbon (C) amendments is imperative for the optimization of organic management practices to enhance soil fertility and agricultural sustainability. Whole orchard recycling (WOR) is an organic management practice in which trees at the end of their productive life cycle are ground into wood chips and tilled into the surface soil before planting the next orchard. This process provides a C-rich, primarily lignocellulose substrate. Among 3-, 4-, 6-, and 15-year-old almond orchards, sampled as a space-

for-time substitution design, WOR effects on bacterial and archaeal metagenome-assembled genomes (MAGs) were strongest in the 4- and 6-year-old orchards and were diminished in the 3- and 15-year-old orchards. The strongest separation between WOR and control bacterial/archaeal community composition occurred in the 6-year-old orchard, which received 85 metric tons of wood chips per acre, while the treatment effect was not evident in the 15-year-old orchard, which received 33 metric tons of wood chips per acre. The WOR-enriched MAGs were taxonomically dominated by *Alphaproteobacteria*, *Gammaproteobacteria*, *Thermoleophilia*, and other heterotrophic lineages, and their encoded pathways suggest preferential use of partially degraded lignocellulose polymers, rather than direct decomposition of intact wood chips, and reductive N transformations, rather than oxidative N transformations. Although these MAGs were only weakly aligned with SOM composition at the ordination level, several WOR-enriched taxa showed positive associations with TC, TN, and aromatic functional group contents of soil particulate organic matter (POM). In synthesis, these findings propose a successional decomposition framework, in which the response of topsoil bacteria and archaea to WOR is elicited by the availability of wood chip inputs into the surrounding <2 mm soil.

Frequently used acronyms: carbon (C), nitrogen (N), total carbon (TC), total nitrogen (TN), soil organic matter (SOM), whole orchard recycling (WOR), metagenome-assembled genomes (MAGs), Kyoto Encyclopedia of Genes and Genomes (KEGG), KEGG orthologs (KOs), particulate organic matter (POM), mineral associated organic matter (MAOM), diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS), principal coordinates analysis (PCoA), principal coordinate (PCO)

Introduction

California's Central Valley produces more than 75% of the worldwide almond supply^{142,143}, which covered approximately 1.5 million acres in 2025¹⁴⁴, and is valued at over \$5 billions of dollars annually¹⁴⁵. Almond orchards have a productive lifecycle for 15 – 30 years, with an average of 21.7 years, before they are replanted^{20,143}. Orchard reestablishment generates a large amount of woody waste biomass, which was burned in piles or in co-generation facilities¹⁴³. Open agricultural burning was banned in 2025 in the San Joaquin Valley, which comprises two-thirds of California's Central Valley, USA, when the California Air Resources Board passed Senate Bill 705 to improve air quality¹⁴⁶. Whole Orchard Recycling (WOR) is an emerging waste biomass management strategy in which old orchard trees are ground, chipped, and incorporated into the soil prior to replanting. WOR has been promoted as a climate-smart practice⁸³ that improves soil health through increased soil carbon (C) storage²⁰, microbial biomass nitrogen (N)¹⁷, water retention⁸⁴, and formation of large macroaggregates¹⁶. Microbial biomass has been identified as a major driver of soil organic matter (SOM) formation and soil aggregation, specifically due to the stability of occluded cell wall components following microbial death^{80,85,86}.

Soil microorganisms mediate the breakdown, transformation, and stabilization of plant-derived C and the cycling of N in agricultural ecosystems¹⁴⁷. While saprotrophic fungi are commonly considered the key wood decomposers^{148,149}, bacteria dominate soil microbial communities quantitatively¹⁵⁰ and contribute to substrate decomposition through secretion of extracellular enzymes¹⁴⁸, while driving key N transformations that influence plant nutrient availability¹⁵¹. For a soil system receiving an enormous one-time input of deciduous hardwood, which is composed of lignin (23-30%), cellulose (38-49%), and hemicellulose polymers (19-

26%)¹⁵², microbial decomposition is expected to follow a succession of microbial decomposers based on their ability to use particular substrates^{148,153}. Conceptually, soil microbial activity is more rapidly stimulated by compounds that are more readily available (e.g., soluble, simple, small)¹⁵⁴ and nutritionally dense¹⁵⁵, while complex aromatic carbohydrate polymers are expected to decompose more slowly. For fine sandy loam soil, double-decay modeling projected that 49 % of WOR biomass C remains in the soil after 4 years and 6.6 % will remain after 20 years²⁰. Within this system, most microbial biomass produced during wood decomposition is likely subject to predation and re-metabolization during the first two decades of a typical orchard lifecycle. Although, decomposition rates and microbial succession are also influenced by agricultural management intensity, such as irrigation and fertilizer application rates¹⁵⁶.

In parallel with C metabolism, oxidative and reductive N transformations (i.e., nitrification and denitrification) are influenced by soil depth^{157–159}, aggregate oxygen availability¹⁶⁰, and C substrate identity¹⁶¹. In agricultural soils, applications of mineral fertilizer N have been found to increase microbial biomass¹⁶². Hence, microbial N transformations are essential for N acquisition, retention, and detoxification strategies¹⁶³. The microbial N cycle can be conceptualized in six interconnected redox processes: N fixation, which converts atmospheric dinitrogen to ammonium; ammonification, which releases ammonium from organic N; nitrification, an oxidative pathway that converts ammonium to nitrite and then nitrate; denitrification, a reductive pathway that sequentially converts nitrate to nitrite, nitric oxide, nitrous oxide, and ultimately dinitrogen; anaerobic ammonium oxidation, which couples ammonium and nitrite to produce dinitrogen; and dissimilatory nitrate reduction to ammonium (DNRA)¹⁴⁷. A previous WOR trial found that wood chip amendment to fine sandy loam soil increased microbial N immobilization into biomass by 95% after one year, with a 71% increase

in the abundance of nitrite reductase (*nirK*) genes and decreased nitrate leaching potential after 10 years¹⁷. Notably, the abundances of bacterial ammonia monooxygenase (*amoA*) and nitrite reductase (*nirS*) did not significantly differ by WOR treatment after 1 and 10 years¹⁷.

Metagenome-assembled genomes (MAGs) provide a powerful tool for examining the functional potential of microbial communities in complex soil environments¹⁶⁴. MAGs have been widely used in soil systems for ecological inference via genome annotation for enzymes and metabolic pathways^{165,166}. To date there are no studies that have examined the functional potential of bacterial MAGs (e.g., C and N metabolism pathways) and their associations with soil chemistry within WOR amended soil. Chapter 2 of this dissertation showed that WOR increased SOM and TC contents by 30–50%, for orchards 3 – 6 years old, by enriching aromatic and aliphatic functional groups composing particulate organic matter (POM), with attenuation in 15 years. Building on these results, this chapter reconstructed MAGs from soil metagenomes, to identify WOR-enriched bacteria and archaea, characterize their encoded C and N metabolism pathways, and relate MAG community composition to the POM and MAOM functional group composition.

Here, we used shotgun metagenomics to reconstruct MAGs from soils in four experimental almond orchards that have replicated WOR and control treatments, as described in Chapter 2 Methods, to address the following:

Research Questions and Hypotheses

Question 1) How long do WOR-induced changes in microbial community composition persist?

- **Hypothesis 1a)** WOR-induced changes in microbial community composition will manifest most prominently within first 4 years, while wood chip-C is most present.

- **Hypothesis 1b)** WOR-induced changes in microbial community composition will dissipate in about 10 years with the exhaustion wood chip-C.

Question 2) What is the functional potential of WOR-enriched MAGs for C and N cycling?

- **Hypothesis 2a)** WOR-enriched MAGs will encode lignocellulose-related decomposition pathways, such as cellulases and hemicellulases.
- **Hypothesis 2b)** WOR-enriched MAGs will be capable of both oxidative and reductive N transformation pathways in topsoil.

Question 3) Are WOR-enriched MAGs positively correlated with POM and MAOM organic functional groups, such as the abundance of aromatic C bonds?

- **Hypothesis 3)** Yes, WOR-enriched MAGs that are capable of lignocellulose decomposition will be positively correlated with aromatic C contents of POM.

To address these questions, I used a space-for-time design across 3-, 4-, 6-, and 15-year-old orchards, comparing WOR and control treatments within each field. The metagenomic results are quantitatively related to soil chemistry and DRIFTS-based measurements presented in Chapter 2 using multivariate and correlation analyses. In comparison with the amplicon-based analyses presented in Chapter 1, which characterized WOR effects on bacterial/archaeal and fungal community composition, this chapter provides a complementary functional perspective by resolving WOR-enriched MAGs and inferring ecological responses of soil microbial communities to WOR over the timespan of a typical orchard lifecycle.

Methods

Soil Collection:

Soils were collected in summer 2023 from four experimental almond orchards that received WOR in different years (2008, 2017, 2019, 2020), representing 15-, 6-, 4-, and 3-year-old systems respectively. Here orchard age is confounded with field location, as a space-for-time substitution, wherein each field received a different wood chip application rate (33, 85, 61, 60 metric tons wood chips per acre respectively). Each orchard included four replicate blocks per treatment group, and soils were sampled from 0 to 15 cm, as three composite cores per treatment, in WOR and control plots (2 treatments * 4 fields * 4 replicate blocks = 32 total soil samples). See Chapter 2 Methods for more details.

DNA Extraction and Metagenomic Sequencing:

Genomic DNA was extracted from soil using Qiagen DNeasy PowerLyzer PowerSoil kit (Qiagen Inc., Hilden, Germany), purified using OneStep PCR Inhibitor Removal kit (Zymo Research, Irvine, CA, USA), and quality checked for concentration and integrity via fluorescence and gel electrophoresis, respectively. The resulting genomic DNA extracts (~25 μ L per sample at 24 to 124 ng DNA μ L⁻¹) were submitted to UC Davis DNA technologies core for shotgun metagenomic using 2 x 150 bp paired-end sequencing on an Illumina Novoseq (Nova1159), generating a total of 267 Gb of raw reads.

Metagenomic Assembly and Binning:

Raw reads from each sample were filtered for quality ($\geq Q15$) and length (≥ 50) using fastp version 1.0.1¹⁶⁷ and quality checked using FastQC version 0.11.9¹⁶⁸, resulting in 133.6 Gb forward and 133.6 Gb reverse sequence data. Individual forward and reverse fastq files of each sample were concatenated into single files (R1 and R2). These concatenated fastq files were

normalized to k-mer coverage and depth of 100X and 5, respectively using bbnorm.sh followed by read error correction using tadpole.sh (<https://sourceforge.net/projects/bbmap/>), BBTools version 39.01. The resulting normalized and error corrected reads were assembled into contigs using MEGAHIT version 1.2.9 with the meta-large preset¹⁶⁹. Then, the resulting co-assembly was filtered to retain contigs $\geq 1,500$ bp. The filtered co-assembly consisted of 1,762,218 contigs with a total assembly length of 4,980,850,988 bp.

Taxonomic screening of contigs was performed using Kraken2 version 2.1.3 to remove non-fungal eukaryotic sequences and retain bacterial, archaeal, fungal, and viral contigs for downstream analyses. Taxonomic screening resulted in a filtered co-assembly consisting of 1,745,819 contigs, with a total assembly length of 4,937,480,704 bp and an N50 of 2,768 bp. Quality-controlled reads from each sample were mapped back to the filtered assembly using bwa-mem2 version 2.2.1¹⁷⁰, and resulting alignments were processed into sorted and indexed BAM files using Samtools version 1.17¹⁷¹.

Metagenomic binning was performed on an assembly filtered to contain only bacterial, archaeal, and viral contigs using a combination of MetaBAT2 version 2.15¹⁷², BinSanity version 0.5.4¹⁷³, and VAMB version 3.0.3¹⁷⁴ to maximize genome recovery across diverse community members. Resulting bins from all methods were consolidated and refined using DASTool version 1.1.6¹⁷⁵. Genome quality was assessed using CheckM2 version 1.1.2¹⁷⁶, and bins with $\geq 50\%$ completeness and $\leq 10\%$ contamination were retained as metagenome-assembled genomes (MAGs). Relative abundances of MAGs across samples were calculated by mapping reads to a concatenated MAG reference using CoverM (<https://github.com/wwood/CoverM>) version 0.6.1¹⁷⁷ with parameters `--min-read-aligned=--percent 0.75`, `--min-read-percent-identity 0.95`, and `--min-covered-fraction 0`.

Genomic Bin Taxonomic Assignment and Annotation:

Taxonomic classification of MAGs was performed using Genome to Taxonomy Database toolkit (GTDB-Tk) version 2.4.0 with the GTDB reference database release 220^{178,179}.

Functional annotation of MAGs was conducted using Prokka version 1.14.6¹⁸⁰ to generate gene predictions and corresponding amino acid fasta and GFF annotation files. Protein sequences were annotated (assigned to KO groups) for metabolic potential using KEGG-based pathway inference implemented through eggNOG mapper version 2.1.12^{181,182}, enabling identification of pathways involved in plant-derived carbon degradation and nitrogen cycling. Pathway presence and completeness were assessed across MAGs to characterize functional differences among samples using KEGG-decoder version 0.6¹⁸³. All bioinformatic processing was performed on the USDA SCINet Ceres high performance computing (HPC) infrastructure.

Data Analysis and Statistics:

All statistical analyses and graphing were performed in R version 4.5.3 on MAGs with $\geq 50\%$ completeness and $\leq 10\%$ contamination. Principal coordinates analysis (PCoA) was performed on Hellinger-transformed relative abundance data using the package vegan version 2.7.3¹⁸⁴. Differential abundance analyses were performed on MAGs with relative abundances greater than 0.01% across samples using MaAsLin3 version 1.2.0¹⁸⁵, with log transformation and Benjamini-Hochberg false discovery rate correction. MAGs identified as significantly differentially abundant ($p < 0.05$) were further examined for enrichment of metabolic pathways related to soil C turnover and N transformations. The R package ggplot2 version 4.0.2¹⁸⁶ was used to generate heat maps of KEGG pathways completeness.

To assess concordance between MAG community composition and SOM composition, Procrustes analyses and PROTEST were conducted for 4-year-old (field K92) and 6-year old

(field K74) orchards using vegan. For each field, Bray–Curtis dissimilarities were calculated from MAG relative abundances and used to generate PCoA ordinations, and Euclidean distances were calculated from DRIFTS peak intensity matrices of POM and MAOM. DRIFTS peak matrices consisted of Kubelka–Munk transformed peak heights at 14 organic functional group bands (3300, 2931, 2871, 1724, 1660, 1601, 1576, 1540, 1510, 1469, 1406, 1320, 1275, and 1238 cm^{-1}) derived from spectral subtraction of soil mineral contribution. Symmetric Procrustes rotations were used to compare MAG ordinations with POM or MAOM ordinations, and the significance of the Procrustes correlation was evaluated using PROTEST with 999 permutations. Mantel tests based on Spearman’s rank correlation (999 permutations) were used in parallel to quantify correlations between MAG Bray–Curtis distance matrices and POM or MAOM Euclidean distance matrices. Spearman rank-order correlations were also calculated between Hellinger-transformed MAG relative abundances and z-score standardized soil chemistry and DRIFTS peak intensities of POM. Statistical significance was assessed using the Benjamini–Hochberg procedure to adjust for multiple testing ($p\text{-adj} < 0.05$). For visualization, results were filtered to include only significant correlations ($p\text{-adj} < 0.05$) within differentially abundant MAGs found in the 6-year-old orchard (field K74) and relevant soil properties, including SOM, TC, TN, C:N ratio, and pH.

SOM Fractionation:

As described in Chapter 2 Methods, the 32 soil samples were fractionated into particulate organic matter (POM) and mineral-associated organic matter (MAOM) pools following the method described by Cotrufo et al., 2019⁹⁸. A subsample of each POM and MAOM fraction was further oxidized with sodium hypochlorite (NaOCl) to remove organic matter, resulting in a

“mineral-enriched” residue for use in subsequent FTIR spectral subtraction, as described by Margenot et al., 2019¹¹⁹.

Soil Physiochemistry:

As described in Chapter 2 Methods, bulk (<2 mm), POM, and MAOM soil fractions were analyzed for SOM content, total carbon (TC), total nitrogen (TN), soil pH (1:1 ratio of soil to deionized water), dissolved organic carbon (DOC), dissolved organic nitrogen (DON), water holding capacity (WHC), and microbial CO₂ respiration.

Results

Co-assembly and metagenomic binning of 32 soil metagenomes yielded 228 metagenome-assembled genomes (MAGs) that were greater than 50% complete with less than 10% contamination. Out of the 228 MAGs, 14 were high quality (>90% complete, <5% contamination). Taxonomically, the 228 MAGs were classified by GTDB-tk into the archaeal phylum *Thermoproteota* (8) and 13 bacterial phyla – *Actinomycetota* (100), *Pseudomonadota* (36), *Acidobacteriota* (28), *Gemmatimonadota* (21), *Chloroflexota* (21), *Nitrospirota* (4), *Verrucomicrobiota* (3), *Desulfobacterota* (3), *Bacillota* (2), and *Methylomirabilota* (2). The relative abundance of the 228 MAGs within each of the 32 soil metagenomes were composed of 3 to 6 % of the microbial community sequenced (Figure 3.1). Differential abundance tests comparing MAG relative abundances between WOR and control treatment groups are shown in Figure 3.3 with corresponding class and family level annotations.

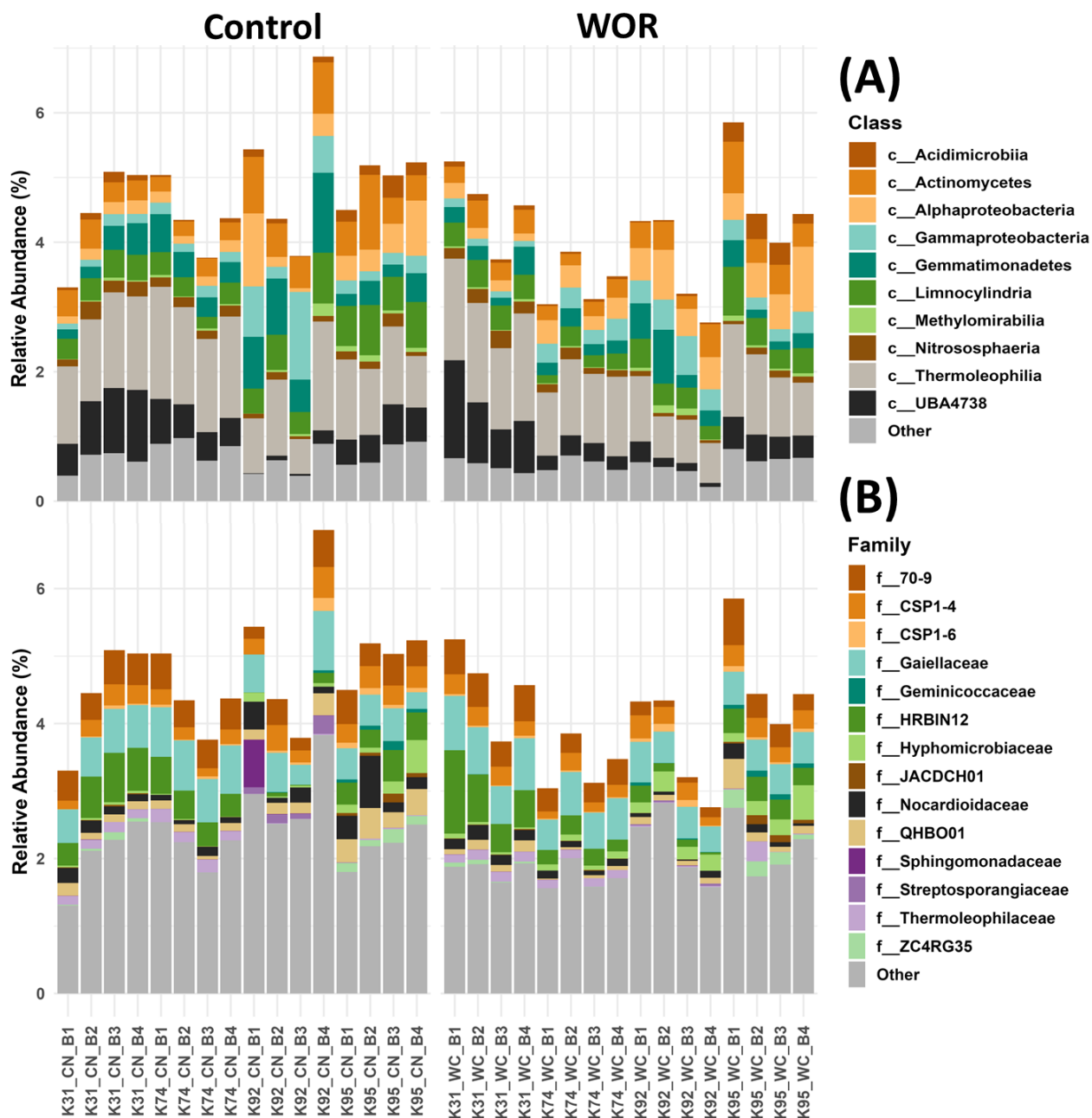


Figure 3.1: Relative abundance (%) of the top 10 mapped classes (A) and top 15 mapped families (B) represented for the 228 medium-quality MAGs (>50% complete, with <10% contamination) recovered from the 32 soil metagenomes. Bars are grouped by orchard, shown each of the 4 replicates, and faceted by WOR treatment. Here, 15-year-old (field K31), 6-year-old (field K74), 4-year-old (field K92), and 3-year-old (field K95) orchard soil samples are arranged from left to right.

Principal coordinates analysis (PCoA) of Bray Curtis dissimilarities calculated using the relative abundance of MAGs across samples revealed spatiotemporal shifts of bacterial and

archaeal MAGs (Figure 3.2). Here, 55% of the total variation across MAGs was explained by the first two principal coordinates; PCO1 captured 35.2% and PCO2 captured 20.09% of variation. Shifts along PCO1 appear to be associated with changes over time, wherein temporal differences encompass management related differences between experimental orchards, and separation along PCO2 appears to be associated with differences between treatment groups. PERMANOVA of Bray-Curtis dissimilarity distances across all samples detected a significant treatment-by-year interaction ($R^2 = 0.078$, $p = 0.0017$), suggesting that the effect of WOR treatment on bacterial/archaeal community composition differed among orchard fields that were amended by WOR in different years. PERMANOVAs performed on individual fields demonstrate significant effects on MAG community composition in the 6-year old (field K74; $R^2 = 0.657$, $p = 0.0251$) and 4-year-old (field K92; $R^2 = 0.368$, $p = 0.0251$) orchards, but not in the 15-year-old (field K31; $R^2 = 0.070$, $p = 1.00$) or (field K95; $R^2 = 0.183$, $p = 0.258$). Following Benjamini-Hochberg correction across the four field-level tests, the treatment effect was marginally significant for the 6- and 4-year-old orchards (fields K74 and K92; $p\text{-adj} = 0.0502$ in both cases).

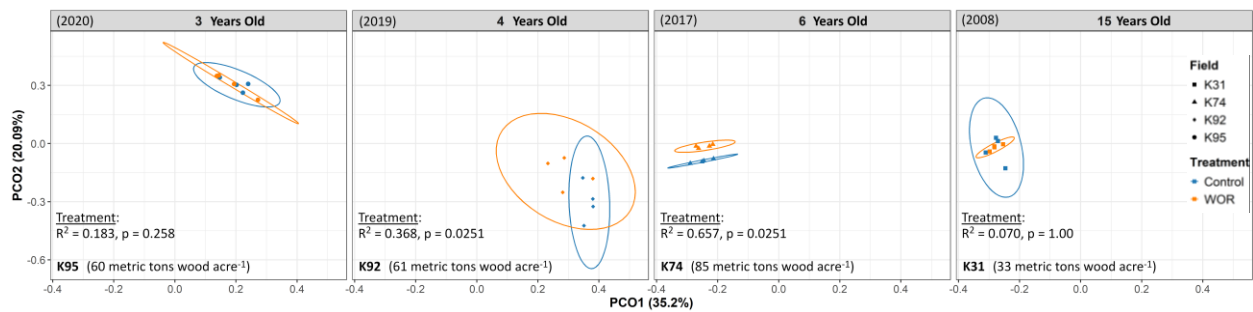


Figure 3.2: Principal coordinates analysis (PCoA) based on Bray-Curtis distances of the 228 medium-quality (>50% complete, with <10% contamination) MAGs recovered from the 32 soil metagenomes. This is PCoA is faceted by orchard age, where each point represents an individual soil sample, colored by treatment and shaped by sampling month, with 95% confidence interval ellipses and PERMANOVA results overlayed. Shifts in MAG community composition are shown over space-time (PCO1), highlighting separation between WOR and control soils (PCO2).

To identify MAGs that differed significantly between treatment groups, MaAsLin3 was performed separately for each of the four fields. No MAG abundances were significantly different in fields K95 (3 years post-WOR) or K31 (15 years post-WOR). Therefore, only the differential abundance results for fields K92 (4 years post-WOR) and K74 (6 years post-WOR) are shown below (Figures 3.3 and 3.4). In the 4-year-old orchard (field K92), 10 WOR-enriched and 15 control-enriched MAGs were significantly differentially abundant (Figure 3.3A). At the class level, WOR-enriched bacterial MAGs in the 4-year-old orchard (field K92) belonged to *Alphaproteobacteria* (4), *Thermoleophilia* (3), *Gammaproteobacteria* (1), *Blastocatellia* (1), and *Acidimicrobiia* (1). In contrast, control-enriched archaea and bacteria in the 4-year-old orchard (field K92) belonged to the classes *Nitrososphaeria* (1), *Thermoleophilia* (3), *Gemmatimonadetes* (3), *Actinomycetes* (2), *Bacilli* (1), *Blastocatellia* (1), *Limnocyndria* (1), *Gammaproteobacteria* (1), and *Chloroflexia* (1).

The C decomposition KEGG pathway heatmap for the 4-year-old orchard (field K92) demonstrates the functional potential of 10 WOR-enriched MAGs and 15 control-enriched MAGs (Figure 3.3B). Two WOR-enriched MAGs, SFT_217_g__CADEED01 and SFT013_g__JAJVID01, contained a complete cellulase pathway. More prominently, 7 WOR-enriched MAGs and 9 control-enriched MAGs encoded beta-glucosidase, which cleave cellobiose into glucose units. Pectin decomposition-related enzymes detected in WOR-enriched MAGs included D-galacturonate epimerase (4), D-galacturonate isomerase (2), and oligogalacturonide lyase (1). Starch-related enzymes were more common in the 4-year-old orchard (field K92) control-enriched MAGs, including alpha-amylase (4), glucoamylase (4), and pullulanase (1). Complete pullulanase pathways were detected in 2 WOR-enriched MAGs, whereas alpha-amylase and glucoamylase pathways were incomplete. For chitin-related

pathways, 5 WOR-enriched MAGs and 4 control-enriched MAGs contained complete diacetylchitobiose deacetylase enzymes. Notably, beta-N-acetylhexosaminidase was found in 16 out of the 25 significantly differentially abundant MAGs shown in Figure 3.3.

The N cycling pathway completeness heatmap based on KEGG assignments of protein-coding genes for the 4-year-old orchard (field K92) can be broadly conceptualized into N acquisition, oxidative N transformations, and reductive N transformations (Figures 3.3C). For N acquisition, the urea transporter appeared in 2 out of 15 control-enriched MAGs, although it was largely incomplete, and 3 out of 10 WOR-enriched MAGs. Nitrogen fixation was detected in only one MAG, SFT202_g__Bradyrhizobium, which was WOR-enriched. Oxidative N pathways detected here include hydroxylamine oxidation and nitrite oxidation; however, hydroxylamine oxidation was detected in only one WOR-enriched MAG, SFT013_g__JAUSIF01. Nitrite oxidation was detected in one WOR-enriched MAG, SFT202_g__Bradyrhizobium, and in two control-enriched MAGs, SFT047_g__JADGHA01 and SFT067_g__CALAED01. Most differentially abundant MAGs encoded reductive N pathways, including dissimilatory nitrate reduction (2 control-enriched and 1 WOR-enriched MAG), DNRA (2 control-enriched and 1 WOR-enriched MAG), nitrite reduction (9 control-enriched and 5 WOR-enriched MAGs), nitric oxide reduction (2 control-enriched and 1 WOR-enriched MAG), and nitrous oxide reduction (1 control-enriched and 1 WOR-enriched MAG).

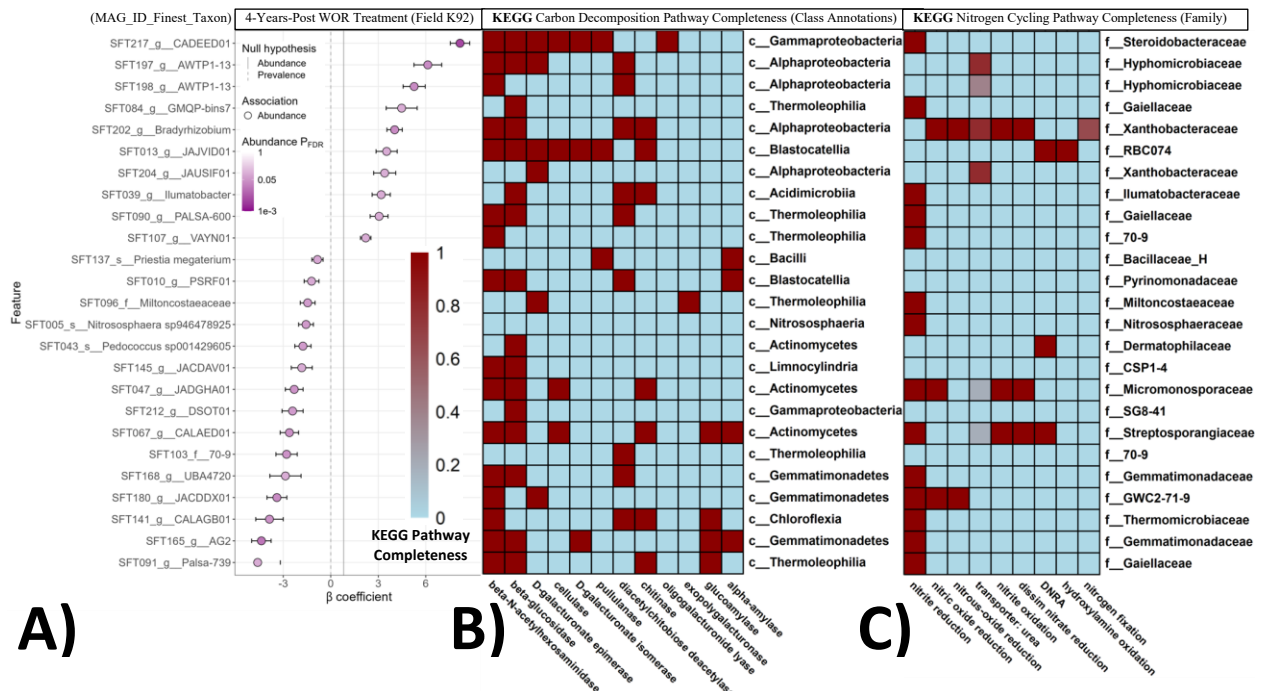


Figure 3.3: Differentially abundant MAGs from field K92 (A), sampled 4 years post-WOR, and corresponding C- and N-cycle KEGG pathway completeness heatmaps (B and C respectively). MaAsLin3 β coefficients indicate MAG enrichment in WOR soils (+ β) or control soils ($-\beta$). KEGG-Decoder C and N metabolic pathways were filtered to retain pathways relevant to carbon decomposition and nitrogen cycling. Pathways with zero completeness across all MAGs were removed. Class- and family-level taxonomic annotations for each MAG are aligned to the right.

We detected 23 WOR-enriched and 2 control-enriched MAGs that were significantly differentially abundant in field K74 (6 years post-WOR, Figure 3.4A). WOR-enriched bacterial MAGs primarily belonged to *Alphaproteobacteria* (6), *Gammaproteobacteria* (5), *Thermoleophila* (4), *Actinomycetes* (3), *UBA4738* (3), *Blastocatellia* (1), and *Acidimicrobiia* (1) classes, whereas the two control-enriched MAGs belonged to *Gemmatimonadetes*. WOR-enriched MAGs that were identified in both 4-year-old (field K92) and 6-year-old (field K74) orchards include SFT217_g__CADEED01, SFT197_g__AWTP1-13, SFT198_g__AWTP1-13, SFT202_g__Bradyrhizobium, SFT204_g__JAUSIF01, SFT039_g__Ilumatobacter, and SFT_g__PALSA-600. Control-enriched MAGs that were identified in the 6-year-old orchard (field K74) include SFT175_g__JACDDX01 and SFT167_g__UBA4720, which are

taxonomically similar to the 4-year-old orchard (field K92) control-enriched MAGs SFT180_g__JACDDX01 and SFT168_g__UBA4720.

The C decomposition KEGG pathway profiles detected demonstrate the functional potential of WOR-enriched MAGs to decompose plant cell wall components, such as cellulose, pectin, chitin, starches and other polysaccharides. Only one WOR-enriched MAG, SFT_217_g__CADEED01, contained a complete cellulase pathway, while 12 WOR-enriched MAGs encoded beta-glucosidase. For pectin decomposition, WOR-enriched MAGs contained complete D-galacturonate epimerase (7), D-galacturonate isomerase (4), and oligogalacturonide lyase (1) enzymes. Starch-related enzymes detected in WOR-enriched MAGs include pullulanase (3) and glucoamylase (3). Four WOR-enriched MAGs contained chitin-related pathways, 5 WOR-enriched MAGs contained diacetylchitobiose deacetylase enzymes, and 2 of these MAGs contained both pathways. Notably, beta-N-acetylhexosaminidase was present in 19 of the 25 significantly differentially abundant MAGs (Figure 3.4A).

The N-cycle KEGG pathway heatmap shows that differentially abundant MAGS found 6-year-old orchard (field K74) encode pathways associated with N acquisition, oxidative N transformations, and reductive N transformations (Figure 3.4C). In terms of N acquisition, 8 WOR-enriched MAGs demonstrate potential for urea transport, although some of these pathways were incomplete, and 1 WOR-enriched MAG (SFT202_g__Bradyrhizobium) displays potential for nitrogen fixation. Nitrite oxidation is the only other oxidative N pathway, detected within 4 WOR-enriched MAGS within the 6-year-old orchard (field K74). Most WOR-enriched MAGs encoded reductive N pathways, including dissimilatory nitrate reduction (4), DNRA (4), nitrite reduction (12), nitric oxide reduction (4), and nitrous-oxide reduction (1).

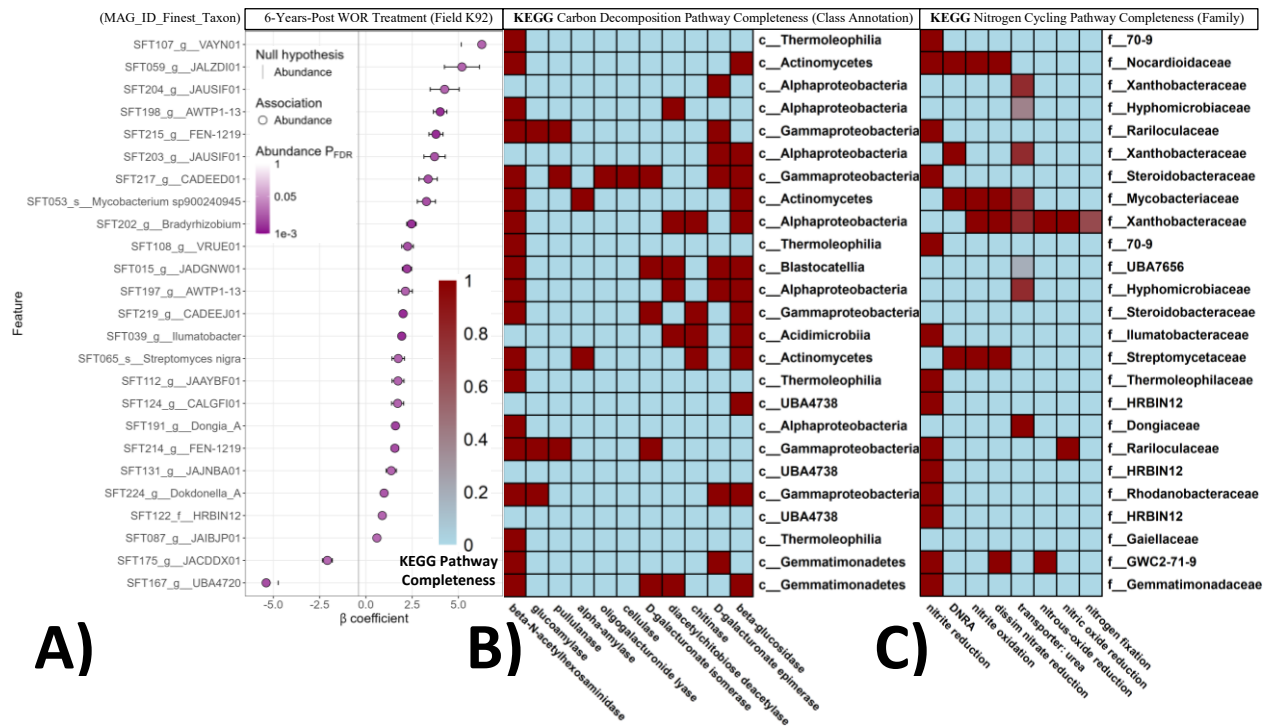


Figure 3.4: Differentially abundant MAGs from field K74 (A), sampled 6 years post-WOR, and corresponding C- and N-cycle KEGG pathway completeness heatmaps (B and C respectively). MaAsLin3 β coefficients indicate MAG enrichment in WOR soils ($+\beta$) or control soils ($-\beta$). KEGG-Decoder C and N metabolic pathways were filtered to retain pathways relevant to carbon decomposition and nitrogen cycling. Pathways with zero completeness across all MAGs were removed. Class- and family-level taxonomic annotations for each MAG are aligned on the right.

Bacterial and archaeal MAG community composition was marginally correlated with POM composition in both 6-year-old and 4-year-old (field K74 and K92) orchard soils, whereas relationships with MAOM composition were weak and not significant (Table 2.1). In the 6-year-old orchard (field K74), the Procrustes analysis indicated a moderate correlation between MAG and POM ordinations (Procrustes correlation = 0.61, $P = 0.07$), which was consistent with a marginal Mantel correlation between Bray–Curtis MAG dissimilarities and POM Euclidean distances ($r = 0.25$, $P = 0.07$). In the 4-year-old orchard (field K92), MAG and POM ordinations were also marginally correlated (Mantel $r = 0.41$, $P = 0.06$), but Procrustes correlations were weaker and non-significant. Thus, Spearman rank order correlation analyses were not performed for the 4-year-old orchard (field K92) but were focused on the 6-year-old orchard (field K74).

Field	Orchard_Age	Wood_Chip_application_rate	Fraction	Proc_corr	Proc_p	Mantel_r	Mantel_p
K74	6 years	85 metric tons per acre	POM	0.608	0.068	0.251	0.066
K74	6 years	85 metric tons per acre	MAOM	0.408	0.52	-0.066	0.61
K92	4 years	61 metric tons per acre	POM	0.431	0.49	0.408	0.059
K92	4 years	61 metric tons per acre	MAOM	0.459	0.35	0.215	0.21

Table 2.1: Procrustes and Mantel statistics relating bacterial/archaeal MAG community composition to soil organic matter composition in 4- and 6-year-old WOR-amended orchards. MAG communities were compared using Bray–Curtis dissimilarities of MAG relative abundances, and SOM composition was compared using Euclidean distances of DRIFTS peak intensities for particulate (POM) and mineral-associated (MAOM) organic matter fractions. DRIFTS peak intensities represent 14 organic functional group bands, with maxima at 3300, 2931, 2871, 1724, 1660, 1601, 1576, 1540, 1510, 1469, 1406, 1320, 1275, and 1238 cm⁻¹, quantified from Kubelka–Munk transformed subtraction spectra described in Chapter 2.

Spearman rank order correlation analysis revealed positive associations between the relative abundances of the 6-year-old orchard (field K74) differentially abundant MAGs and soil C and N pools (Figure 3.5). Among the 23 WOR-enriched MAGs, 8 showed positive correlations with SOM (%), TC, POM TC, or MAOM TC. SFT215_g__FEN-1219, SFT202_g__Bradyrhizobium, and SFT087_g__JAIBJP01 exhibited strong positive correlations ($p > 0.50$) across nearly all C and N pools. These 3 MAGs, together with SFT107_g__VAYN01, were the only MAGs significantly correlated with POM TC or POM TN. Most WOR-enriched MAGs were positively correlated with MAOM TC (10 MAGs) or MAOM TN (9 MAGs), and 7 were positively correlated with both. SOM (%) was positively correlated with 8 of the 23 WOR-enriched MAGs. By contrast, the two control-enriched MAGs, SFT175_g__JACDDX01 and SFT167_g__UBA4720, were negatively correlated with soil pH or MAOM TN. Seven MAGs showed no significant correlation with any soil chemistry variable: SFT059_g__JALZDI01, SFT108_g__VRUE01, SFT219_g__CADEED01, SFT191_g__Dongia_A, SFT131_g__JAJNBA01, SFT224_g__Dokdonella_A, SFT122_f__HRBIN12.

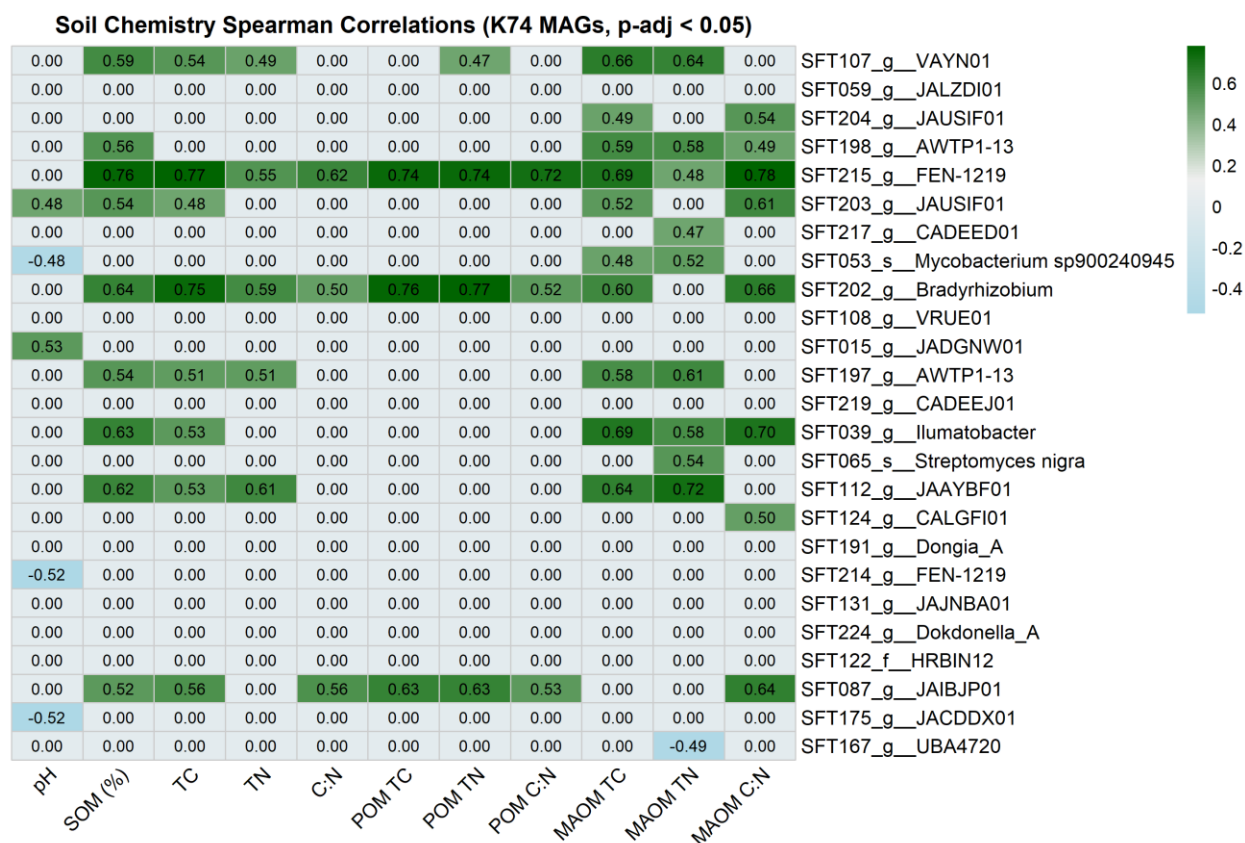


Figure 3.5: Heatmap of significant Spearman rank order correlations (p-adj. < 0.05) for 6-year-old (field K74) differentially abundant bacterial MAGs (Hellinger-transformed relative abundances) and soil variables (z-score standardized), including POM and MAOM fractions. As shown in Figure 3.4, MAGs aligned at the bottom were identified as control-enriched (SFT175_g_JACDDX01 and SFT167_g_UBA4720), while the rest were WOR-enriched. Insignificant correlations were replaced with 0.00 values for clarity.

The Spearman rank order correlation analysis between the 6-year-old orchard (field K74) differentially abundant MAGs relative abundance and POM fraction DRIFTS peak intensities detected significant relationships for 10 out of 25 MAGs (Figure 3.6). Among the 23 WOR-enriched MAGs, several showed robust positive correlations ($\rho > 0.50$) with aliphatic (1470, 2871, 2931 cm^{-1}) and aromatic (1510, 1540, 1600, 1660 cm^{-1}) features, as well as carboxylic acid/ester signatures (1724 cm^{-1}). Specifically, SFT131_g_JAJNBA01, SFT122_f_HRBIN12, SFT203_g_JAUSIF01, and SFT039_g_Illumatobacter exhibited widespread positive associations across the aromatic regions. In contrast, one control-enriched

MAG, SFT175_g__JACDDX01, was the only MAG to display negative correlations with aromatic vibrational peak intensities, specifically 1540 and 1510 cm^{-1} . Several MAGs, including SFT215_g__FEN-1219 and SFT202_g__Bradyrhizobium, showed no significant correlation with any DRIFTS spectral intensities, suggesting their differential abundance may be driven by factors other than the composition of POM.

POM Spearman Correlations (K74 MAGs, p-adj < 0.05)

0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	SFT107_g__VAYN01
0.00	0.00	0.00	0.53	0.00	0.56	0.00	0.00	SFT059_g__JALZDI01
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	SFT204_g__JAUSIF01
0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	SFT198_g__AWTP1-13
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	SFT215_g__FEN-1219
0.00	0.00	0.00	0.00	0.65	0.65	0.51	0.48	SFT203_g__JAUSIF01
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	SFT217_g__CADEED01
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	SFT053_s__Mycobacterium sp900240945
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	SFT202_g__Bradyrhizobium
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	SFT108_g__VRUE01
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	SFT015_g__JADGNW01
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	SFT197_g__AWTP1-13
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	SFT219_g__CADEEJ01
0.00	0.00	0.00	0.00	0.61	0.65	0.51	0.48	SFT039_g__Ilumatobacter
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	SFT065_s__Streptomyces nigra
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	SFT112_g__JAAYBF01
0.00	0.00	0.00	0.00	0.00	0.00	0.48	0.00	SFT124_g__CALGFI01
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	SFT191_g__Dongia_A
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	SFT214_g__FEN-1219
-0.59	-0.64	0.68	0.73	0.81	0.64	0.74	0.67	SFT131_g__JAJNBA01
0.00	0.00	0.00	0.00	0.00	0.00	-0.62	0.00	SFT224_g__Dokdonella_A
-0.52	-0.52	0.55	0.54	0.67	0.74	0.65	0.61	SFT122_f__HRBIN12
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	SFT087_g__JAIBJP01
0.00	0.00	0.00	0.00	0.00	-0.66	-0.55	-0.53	SFT175_g__JACDDX01
0.00	0.00	0.49	0.00	0.00	0.00	0.00	0.00	SFT167_g__UBA4720
2931	2871	1724	1660	1600	1540	1510	1470	

- 1470 cm^{-1}** – d(CH) aliphatic bending
- 1510 cm^{-1}** – v(CH) aromatic C=C-H
- 1540 cm^{-1}** – v(C=C) aromatic C=C stretch
- 1600 cm^{-1}** – v(C=C) phenolic aromatic stretch
- 1660 cm^{-1}** – v(C=C) aromatic C=C stretching
- 1724 cm^{-1}** – v(C=O) carboxylic acids esters
- 2871 cm^{-1}** – v(CH) sym. aliphatic CH₂, CH₃
- 2931 cm^{-1}** – v(CH) asym. aliphatic CH₂, CH₃

Figure 3.6: Heatmap of significant Spearman rank order correlations ($p\text{-adj.} < 0.05$) for the 6-year-old (field K7) differentially abundant bacterial MAGs (Hellinger-transformed relative abundances) and POM spectral subtraction peak intensities (z-score standardized). Wavenumber (cm^{-1}) peak annotations are listed below. As shown in Figure 3.4, MAGs aligned at the bottom were identified as control-enriched (SFT175_g__JACDDX01 and SFT167_g__UBA4720), while the rest were WOR-enriched. Insignificant correlations were replaced with 0.00 values.

Discussion

The combined amplicon (Chapter 1) and metagenomic (Chapter 3) results suggest that WOR does not trigger an immediate community change in soil dwelling bacteria and archaea in the <2 mm soil fraction. The metagenome-level response seems to appear after ~ 4 years, then dissipate within 15 years, depending on orchard age, wood chip application rate, and orchard management. In the 4-year commercial orchard chronosequence, the 16S rRNA gene and ITS1 principal coordinate analyses (PCoAs) showed that sampling time explained more variation in microbial composition than WOR treatment, and the strongest treatment effects were detected in fungi during the first two years after recycling (Chapter 1, Figure 1.2). Notably, the amplicon study was conducted within the same geographical region and fine sandy loam soil type, receiving 40 metric tons of wood chips per acre and annual 1.3 metric tons of winter manure applications (Chapter 1, Figure 1.7). Here, WOR-treated soil metagenomes were not significantly different based on bacterial and archaeal MAGs in the 3- and 15-year-old orchards, in which fine sandy loam soils were amended with 60 and 33 metric tons wood per acre respectively. However, WOR-treated soil metagenomes were significantly different in 4- and 6-year-old orchards, in which fine sandy loam soils were amended with 61 and 85 metric tons of wood chips per acre respectively, as shown by PCoA in Figure 3.2. Although dormant and environmental DNA may have masked and delayed the detection of early microbial community changes associated with WOR treatment, year specific repeated measures PERMANOVAs of 16S rRNA amplicons detected significant shifts in bacterial and archaeal community

composition between sampling months within each of the 4 years immediately following WOR. For fungal ITS amplicons, significant differences in fungal community composition were detected in the first and third years following WOR. Concurrently, cumulative-annual CO₂-C emissions were five and a half times greater in the first year following WOR, with rates rapidly declining to control emissions within 4 years²⁰. Altogether, this pattern suggests a successional response to WOR, in which fungi are more immediately responsive to fresh woody inputs, while bacteria and archaea respond more gradually as the wood-chip material is physically fragmented and biochemically transformed into the <2 mm soil. In the earliest phase after incorporation, much of the woody material may remain inaccessible to many bacteria and archaea, while fungi and other primary decomposers initiate lignocellulose depolymerization. By the later stage, represented by the 15-year-old orchard, the initial pulse of WOR-derived C may have been sufficiently exhausted, such that the microbial communities return to a control composition. This framework of successional decomposition is supported by the double-decay model projections, wherein 49 % of WOR biomass C remains after 4 years and 6.6% remains after 20 years²⁰.

Fungi likely play a central role in the earliest stages of decomposition by colonizing wood chip surfaces and internal cavities^{187,188}, before bacteria and archaea from the surrounding soil matrix contribute to lignocellulose depolymerization. This interpretation is supported by the ITS amplicon results from Chapter 1, in which fungal community composition was significantly shifted by WOR treatment within the first year following wood chip amendment and orchard re-establishment. As seen in Chapter 1, Figure 1.5, WOR-enriched fungal ITS ASVs were primarily Ascomycota and unidentified fungi. Intact wood chips amended by WOR, which can range from greater than 12.7 mm to smaller than 0.2 mm in size¹⁸⁹, likely provide a primary habitat for fungi capable of initiating lignocellulose decay. For instance, white-rot fungi are Basidiomycota

known to produce oxidative enzymes such as laccases and peroxidases that oxidize lignin, exposing cellulose and hemicellulose to further decomposition^{188,190,191}. Brown-rot fungi, which are often Basidiomycota, are known to target cellulose and hemicellulose by modifying lignin¹⁹¹. Soft-rot fungi, which are often soil-inhabiting Ascomycota, can also contribute to lignocellulose decomposition by colonizing wood, consuming cellulose, and demethylating lignin^{188,191}. As for 2 mm sieved soil, incubations have demonstrated moisture induced increases in soil heterotrophic respiration positively associated with the ratio of fungi-to-bacteria¹⁹². Here, the fungal contribution to decomposition of WOR-amended C was not directly resolved, as bacterial and archaeal MAGs were targeted; future analysis of the fungal contribution can be conducted on fungal genes in full co-assembly of all sites or by retrieval of fungal MAGs.

The amplicon and metagenomic results from the 4- and 6-year-old orchards support the successional framework by resolving the taxonomy of WOR-enriched bacteria. In the 4-year-old orchard, the WOR-enriched MAGs were *Alphaproteobacteria*, *Thermoleophila*, *Gammaproteobacteria*, *Blastocatellia*, and *Acidimicrobiia* bacterial classes (Figure 3.3); in the 6-year-old orchard, WOR-enriched MAGs were again dominated *Alphaproteobacteria*, *Gammaproteobacteria*, and *Thermoleophila* bacterial classes, with smaller contributions from *Actinomycetes*, *UBA4738*, *Blastocatellia*, and *Acidimicrobiia* (Figure 3.4). These taxonomic profiles are broadly reflected in the WOR-enriched ASVs from the amplicon study (Chapter 1, Figure 1.5), which were also predominantly enriched in *Alphaproteobacteria* and *Gammaproteobacteria*, with smaller contributions from *Betaproteobacteria*, *Vicinamibacteria*, *Verrucomicrobiota*, *Actinobacteriota*, and *Acidimicrobiia*. Notably, four bacterial ASVs, belonging to the family *Nitrosomonadaceae*, and one archaeal ASV, belonging to the family *Nitrososphaeraceae*, were enriched by WOR in the amplicon study (Chapter 1, Figure 1.5). In

contrast, one archaeal ASV, belonging to the family *Nitrosotenuaceae* (Chapter 1, Figure 1.5), and one archaeal MAG, belonging to the family *Nitrososphaeraceae*, (Figure 3.3) were detected as control enriched. WOR-enriched MAGs that were identified in both 4-year-old (field K92) and 6-year-old (field K74) orchards include SFT217_g__CADEED01, SFT197_g__AWTP1-13, SFT198_g__AWTP1-13, SFT202_g__Bradyrhizobium, SFT204_g__JAUSIF01, SFT039_g__Ilumatobacter, and SFT_g__PALSA-600 (Figures 3.3 and 3.4). These results demonstrate that several of the same broad bacterial and archaeal lineages respond to WOR across both amplicon- and genome-resolved datasets, despite differences in orchard age and wood chip application rate. Importantly, 16S rRNA gene sequencing was utilized for taxonomic profiling of a 4-year chronosequence, potentially capturing a finer-scale temporal response and rare taxa, whereas MAGs were used to assess the functional potential of the most abundant taxa responsive to WOR treatment at 3-, 4, 6-, and 15-year snapshots.

The wood chip C decomposition potential of WOR-enriched bacterial MAGs from the 4- and 6-year-old orchards, as shown in Figures 3.3 and 3.4, largely portrays an opportunistic strategy of scavenging wood chip degradation products, rather than direct action of intact wood polymers by the bacterial community. Although wood chip biomass is composed of lignocellulose, the WOR-enriched bacterial MAGs rarely encode the enzymatic capacity to initiate lignin or cellulose decomposition. Instead, these MAGs are likely to operate as a microbial consortium, as conceptualized in the appendix Figure A3.1, specializing in different substrates released into soil during the decomposition of lignocellulose by adapted fungal taxa. In the 4-year-old orchard (field K92), only two WOR-enriched MAGs (SFT217_g__CADEED01 and SFT013_g__JAJVID01) encoded complete cellulase pathways. For the 6-year-old orchard, SFT217_g__CADEED01 was the only WOR-enriched MAG encoding cellulase out of 23 WOR-

enriched MAGs. By contrast, the absence of cellulase enzymes in most WOR-enriched MAGs suggests limited capacity or need for soil-dwelling bacteria to initiate cellulose depolymerization. In both 4- and 6-year-old orchards, the most prevalent cellulose-related enzyme was β -glucosidase, which cleaves cellobiose and short oligosaccharides into glucose¹⁹³; the widespread occurrence of this enzyme suggests that many WOR-enriched MAGs are positioned to consume cellulose-derived breakdown products rather than intact cellulose fibers. Similarly, WOR-enriched MAGs encoding hemicellulases were not detected. Here enzymes for downstream degradation of pectin, which is made of D-galacturonic acid backbone¹⁹⁴, were more common. For pectin, WOR-enriched MAGs encoded D-galacturonate epimerase, D-galacturonate isomerase, and oligogalacturonide lyase. In the 6-year-old orchard, these pathways were collectively prominent, suggesting that the bacterial community was increasingly specialized for targeting the most downstream lignocellulose decomposition products, such as sugar monomers, which are accessible to after lignin breakdown¹⁸⁸. Starch-active enzymes such as pullulanase and glucoamylase were also detected, especially in the 4-year-old orchard, indicating that some WOR-enriched MAGs can compete for glucose stored as starch, which is often rapidly metabolized by soil microbes^{195,196}. Together, these putative carbohydrate functions suggest that WOR largely favors soil bacteria that cannot initiate lignocellulose depolymerization. This outcome is in alignment with bacterial genome streamlining theory, wherein warmer and drier soil ecosystems have been found to select for smaller bacterial genomes¹⁹⁷. Furthermore, the niche for initiating lignocellulose depolymerization is likely filled by released by wood-inhabiting fungi¹⁹¹ and soil invertebrates¹⁹⁸. Competitive exclusion for cellulose has been well described in agricultural soil, wherein microorganisms that degrade cellulose utilize extracellular

reactions that yield free by-products, which can promote interactions with noncellulolytic organisms¹⁹⁹.

The presence of chitin-related pathways in many WOR-enriched MAGs further elaborates on the succession of bacterial-fungal interactions. As wood chip C is depleted and converted into fungal biomass, chitin is expected to become an increasingly available substrate, initiating a shift toward recycling fungal detritus. Chitinase, diacetylchitobiose deacetylase, and β -N-acetylhexosaminidase are all involved in the degradation of chitin, a structural polymer found in fungal and insect cell walls²⁰⁰. In 4- and 6-year-old orchards, chitinase and diacetylchitobiose deacetylase were most often encoded in WOR-enriched MAGs that did not encode cellulase, suggesting niche partitioning of bacteria that feed on fungal residues and lignocellulose polymers. In bacteria, chitinases primarily function in the catabolism and utilization of chitin as a C and energy source, and they are commonly extracellular or membrane-associated enzymes that degrade fungal cell walls²⁰⁰. Beta-N-acetylhexosaminidase was broadly distributed across both 4- and 6-year-old orchards, reinforcing the idea that many of the enriched genomes are adapted to recycle microbial biomass after the initial wave of wood-chip depolymerization.

The N cycling functional potential of the WOR-enriched MAGs, as shown in Figures 3.3 and 4, depicts widespread potential to perform reductive N transformations, with comparatively fewer MAGs encoding oxidative N functions. Although these N pathway profiles can only be interpreted as the genome-resolved functional potential of a subset of the broader <2mm soil microbial community and therefore cannot be used to infer process rates or active community proportions. The WOR treatment nonetheless significantly increased the relative abundances of bacteria capable of denitrification in the topsoil. Nitrogen fixation was detected in only one

WOR-enriched MAG, SFT202_g__Bradyrhizobium, while urea transport was present in several WOR-enriched MAGs, suggesting their importance as a soil bacterial strategy to balance increased C consumption. More prominently, WOR-enriched MAGs encoded dissimilatory nitrate reduction, DNRA, nitrite reduction, nitric oxide reduction, and nitrous oxide reduction, with nitrite reduction being especially common and present in half of the WOR-enriched MAGs in the 4-year-old orchard and in 12 of 23 WOR-enriched MAGs in the 6-year-old orchard. In contrast, the 16S rRNA gene amplicon results from Chapter 1 showed that WOR-enriched bacterial and archaeal ASVs included recognized nitrifiers from *Nitrosomonadaceae* and *Nitrososphaeraceae*, while a Nitrosotenuaceae ASV and a Nitrososphaeraceae MAG were control enriched. Thus, it cannot be confidently deduced that WOR reduces the proportion of microbial nitrifiers to denitrifiers in the topsoil, though their distributions are likely influenced by wood chip proximity^{161,201}, oxygen availability^{160,201}, and interactions with N fertilizer type²⁰² and application rate¹⁵¹. This interpretation is consistent with a WOR study on 1- and 10-year-old orchards, where WOR significantly affected bacterial *amoA* abundance in a depth-dependent manner after 1 year, with higher *amoA* abundances at 15 – 30 cm depth, while WOR increased *nirK* abundance by 71% after 10 years¹⁷. Together, the 16S rRNA gene amplicon and gene-based studies suggest that the influence of WOR on nitrifying and denitrifying microbes is not uniform across depth and time, while the soil metagenomes indicate that WOR enriches a genome-resolved subset of bacteria with flexible N-scavenging and reductive N capability in 4- and 6-year-old orchards.

Procrustes and Mantel tests indicated that bacterial and archaeal MAG community composition was marginally aligned with DRIFT spectra of POM composition in 4- and 6-year-old orchards, whereas relationships with MAOM composition were weak and insignificant

(Table 2.1). This outcome was expected for orchard soils showing the greatest increases in C content following WOR treatment (Chapter 2, Figure 2.1), in which wood chip C was directly added to the soil as POM. Subsequently, the Spearman rank order correlations performed here provide a basis for identifying specific WOR-enriched bacterial MAGs that respond to changes in specific organic functional groups composing soil POM. As shown in Figure 3.5, most of the WOR-enriched MAGs from the 6-year-old orchard were positively correlated with SOM, TC, POM TC, and especially MAOM TC and TN, suggesting that the abundances of these MAGs increase from inputs of both particulate ($>53\ \mu\text{m}$) and mineral-associated ($<53\ \mu\text{m}$) organic matter. Several of the strongest positive correlations involved SFT215_g__FEN-1219, SFT202_g__Bradyrhizobium, and SFT087_g__JAIBJP01, implying that these taxa are associated with increases in C and N pools of WOR-amended soil fractions. The DRIFTS correlations, as shown in Figure 3.6, reinforce these patterns by showing that several WOR-enriched MAGs were positively associated with aromatic features in the POM fraction, particularly the 1510, 1540, 1600, and 1660 cm^{-1} bands (Chapter 2, Figure 2.3), which have been identified for lignin in wood²⁰³. In contrast, some WOR-enriched MAGs did not show any significant relationships with SOM composition, TC, or TN pools, which suggests that their abundances may be governed by other features of the soil environment, such as ecological interactions²⁰⁴, soil aggregate turnover²⁰⁵, or phosphorus availability²⁰⁶.

Conclusion

In summary, genome-resolved metagenomics revealed that WOR altered bacterial and archaeal community composition primarily in the 4- and 6-year-old orchards, coinciding with the period of greatest C accumulation in the $<2\ \text{mm}$ soil and POM fractions. In alignment with the

increase in TC and SOM contents, observed in Chapter 2 Figure 2.1 and 2.2, the genome-resolved responses to WOR appear to diminish by 15 years. In 4- and 6-year-old orchards, WOR-enriched MAGs were taxonomically dominated by *Alphaproteobacteria*, *Gammaproteobacteria*, *Thermoleophilia*, and other heterotrophic lineages, and their encoded pathways suggest preferential use of partially degraded lignocellulose polymers and reductive N transformations. Collectively, the metagenomic functional profiles of WOR-enriched bacterial MAGS and the immediate response of fungal ASVs, observed in Chapter 1, support a successional decomposition framework, in soil and wood-inhabiting fungi initiate lignocellulose depolymerization, while the most abundant bacteria opportunistically respond to wood chip decomposition products, such as cellobiose and fungal chitin. By integrating the abundances of MAGs with POM composition, this work advances understanding of when and how woody organic amendments influence soil microorganisms.

Chapter 3 Appendix

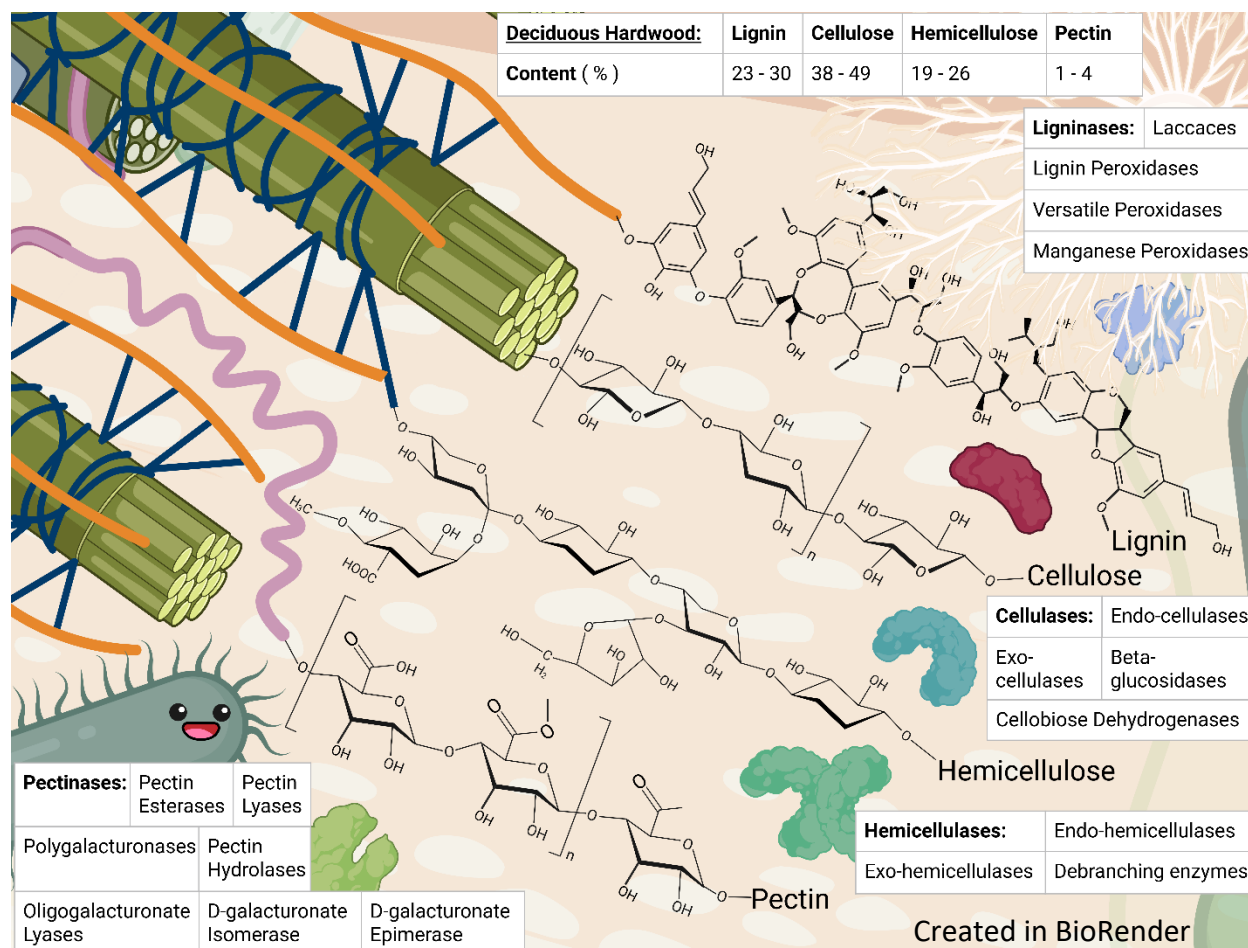


Figure A3.1: Conceptual diagram of lignocellulose polymers and degradation enzymes within a soil matrix, created in BioRender (<https://BioRender.com/dnnr7en>)²⁰⁷. The structure and arrangement of individual lignocellulose units is conceptualized in color on the left, while the molecular structure of each polymer is generalized on the right. The abundance of each polymer in deciduous hardwood is approximated in the table at the top, according to Rowell et al., 2012¹⁵². The enzymes required for decomposition of each lignocellulose polymer and associated byproducts are listed in each of the annotated tables.

Acknowledgment of AI Assistance

I never used AI language models or related tools to interpret my results or create new content for my paragraphs. Perplexity AI was used in this dissertation to suggest minor modifications to my R code, troubleshoot errors, and revise wordy sentences for clarity. All suggestions were reviewed, revised as needed, and incorporated at my discretion in each chapter; I remain solely responsible for the content, analysis, and conclusions of this dissertation.

Any opinions, findings, conclusions, or recommendations expressed in this dissertation are those of the author and should not be construed to represent any official USDA or U.S. Government determination or policy.

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