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DIFFERENCES IN BACTERIAL COMMUNITIES ON DECAYING LEAF LITTER OF DIFFERENT TREES IN RESPONSE TO BURNING AS A FOREST RESTORATION TECHNIQUE

By
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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
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In loving memory of Franklin Dixon, my Papaw Root (1924-2013). Thank you for showing me how to tell a good story, appreciate the outdoors, value hard work, and find joy in every day.

ABSTRACT

Allison Marcum: Differences in Bacterial Communities on Decaying Leaf Litter of Different Trees in Response to Burning as a Forest Restoration Technique

Decomposition is the process by which organic matter gets degraded into basic components to provide energy for decomposer microorganisms and to also make nutrients available for plant uptake. Leaf litter decomposition is an important process and influences the nutrient cycling and the productivity and structure of the entire ecosystem; however, few studies have examined the bacterial communities on decomposing litter, especially how they may vary between tree species or in woodlands subject to ecological restoration. Such restoration has become important, as fire suppression beginning in the 1920s has impacted the structure of forest ecosystems through the process of mesophication, diminishing the abundance of fire-dependent species and favoring more shade-tolerant, fire-intolerant trees. These changes likely influence the composition of the litter microbial community. This study used next generation sequencing of 16S rRNA genes to characterize the bacterial communities on litter from six different tree species, including representatives of upland oak woodlands (oaks) and mesophytic species (non-oaks), in a north Mississippi forest undergoing restoration. Results suggest that the bacterial community on leaf litter changes significantly as decomposition proceeds, and that there are differences in bacterial communities present on litter from oak versus non-oak species. Comparing the bacterial communities on leaf litter collected from a site that experienced a prescribed burn within the collection year, to a non-burned site suggested that fire did affect the bacterial community present on the decaying leaf litter. Together, these results indicate that both ecological restoration (fire) and the effects of restoration

(a change in tree species) are likely to influence the composition of the litter bacterial community.

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Introduction:

Decomposition includes many interrelated processes by which organic matter gets degraded into basic components available for subsequent plant uptake. At a basic level, decomposition includes humification of organic carbon and the mineralization or release of inorganic nutrients such as nitrogen and phosphorus from organic matter. These basic components can then be used by other living organisms within the soil (Waring & Schlesinger, 1985). While not often considered by non-scientists, leaf litter decomposition in terrestrial ecosystems is an important process and influences the nutrient cycling and the productivity and structure of the ecosystem (Nowacki & Abrams, 2008). In forests, the decomposition of leaf litter contributes between 69-87% of the nutrients used by the vegetation each year (Waring & Schlesinger, 1985). Therefore, when considering forest restoration practices, such as prescribed burning and mechanical thinning, it is important to examine factors influencing leaf litter decomposition, as it ultimately influences the entire ecosystem.

One of the most common natural disturbances experienced by forests are fires (Smith et al, 2008). Many physical and chemical changes occur during a fire, which can affect both the soil and litter. Surface temperatures rise dramatically, which can cause changes in the concentrations of nutrients. For example, phosphorus, potassium, magnesium, and ammonium levels are all reported to increase following a fire (DeBano, 1991, Smith et al., 2008), while changes to soil nitrogen levels vary depending on the

duration and severity of the fire. Soil pH typically increases after a fire, which can influence nutrient solubility and availability (Almendros et al., 1990). In many instances, these changes can have beneficial long-term effects on the forest ecosystem (Smith et al., 2008). However, forest ecosystems have been steadily changing since the 1920s when fire suppression became a common practice. It is estimated that prior to this time, fires occurred frequently (every 2-3) years and maintained oak woodlands that contained primarily fire tolerant species. These fires were often caused by natural disturbances, such as lightning, or by burning by Native Americans (Fowler & Konopik, 2007). Relatively frequent fires removed (or reduced in size) fire sensitive species such as red maple (*Acer rubrum*), sweetgum (*Liquidambar styraciflua*), and winged elm (*Ulmus alata*), and allowed fire-tolerant oak woodlands to develop (Nowacki & Abrams, 2008). However, when modern fire suppression began, many fire-intolerant, shade-tolerant species were able to thrive, instead of being removed by fire. As these fire-intolerant species grow and mature the canopy becomes more closed, blocking the passage of sunlight and preventing light from reaching the forest floor. As conditions become more shaded, oak seedlings struggle to grow, allowing more shade-tolerant species to grow in their place. This creates a positive feedback system, which makes shade-tolerant, fire-intolerant species more abundant, thus further closing the canopy and increasing shade (Nowacki & Abrams, 2008).

Changes in the structure of the forest because of fire suppression also results in changes in the characteristics of the leaf litter. As oak leaves dry, they tend to curl and this allows more air into the litter layers (Carreiro et al., 2000). Oak leaves also tend to be thicker and more resistant to decay than other species (Abrams, 1990). In contrast, leaves

of fire-intolerant, shade-tolerant species tend to be thinner and more compacted on the forest floor, trapping more moisture between leaves (Brose et al., 2001). This moisture reduces the chance or impact of fire compared to the drier oak litter, which combined with fire suppression and closing of the forest canopy, propels the success of fire-intolerant, shade-tolerant species while disabling the recruitment and growth of fire-tolerant, shade-intolerant oak seedlings. The term "mesophication" has been coined to describe the process of diminishing abundance of fire-dependent species due to environmental conditions favoring more shade-tolerant, fire-intolerant species (Nowacki & Abrams, 2008).

It is probable that the effects of mesophication result in changes in decomposition and nutrient cycling. In forest ecosystems, various organisms within the litter and soil are important in the decomposition process, although the vast majority of the chemical changes that occur in decomposition are performed by bacteria and fungi (Waring & Schlesinger, 1985). Microorganisms are able to degrade chemical components of leaves, which include lignin and cellulose. Environmental changes that alter the microbiota of the leaf litter may arise from mesophication and are likely to change the rate of decomposition (Elliot et al., 1993). For example, the rate of litter decay is strongly affected by temperature and moisture in the forest ecosystem (Waring & Schlesinger, 1985).

Decomposition can be divided into three phases (Berg, 2000). In phase I, there is a high rate of mass loss as soluble materials, which includes sugars and non-lignified cellulose, seep from the leaf to the soil. Phase II typically lasts much longer. In this phase, lignified carbohydrates and lignin account for the majority of mass lost. Lignin is a

complex polymer of aromatic rings, and can only be broken down by specific microorganisms (Waring & Schlesinger, 1985). The lignin content of leaf litter is an important factor in decomposition rates, as the higher the lignin content in litter, the more slowly it decomposes (Nowacki & Abrams, 2008). The third phase of decomposition occurs when only the most resistant materials remain, and the rate of mass loss is very low (Berg & McClaugherty, 2008).

Microbial communities play a key role in decomposition and nutrient cycling, but also in resilience to disturbances and the regeneration of forests after disturbances (Smith et al., 2008). Fungi have been shown to be the dominant microorganisms in terms of soil biomass (Joergensen & Wichern, 2008), which is suggestive of fungal importance in litter decomposition, and fungal communities on decaying litter have therefore been extensively studied (Osono, 2006, Hobara et al., 2014). Fewer studies have investigated the importance of bacterial communities, in particular the changes in bacterial community structure that occur during the decomposition process, or differences in bacterial communities on different types of leaf litter. As part of a larger project examining the decomposition of mesophytic and upland forest species in restored upland oak forests, leaf litter from six different tree species was collected from forest sites undergoing ecological restoration at Strawberry Plains Audubon Center in Holly Springs, Mississippi. Litter was collected throughout the year and the bacterial community present determined through next generation sequencing of the 16S rRNA gene.

Methods:

Study Site:

This research was performed on leaf litter samples collected from two sites at the Strawberry Plains Audubon Center located in Holly Springs, Mississippi. These samples were originally collected as part of a larger study by Megan Overlander of how forest restoration practices affect leaf litter decomposition rates in mesophytic and upland forest species (M. Overlander, personal communication). There were two collection sites used in this project. One site (Site 1) was treated with a prescribed burn on April 10th, 2014 (week 17 of the larger study). The second site was an untreated, control site (Site 2).

Litter Collection:

Leaves from six different tree species were selected for study: *Ulmus alata* (winged elm), *Carya tomentosa* (mockernut hickory), *Liquidambar styraciflua* (sweetgum), *Quercus stellata* (post oak), *Quercus falcata* (Southern red oak), and *Quercus alba* (white oak). From September to November 2013, falling leaf litter was collected from each tree species within the two study sites by using a polypropylene mesh net within a supporting frame constructed from PVC pipe. Using this collection net, as opposed to collecting fallen litter from the forest floor, ensured that litter was from the

current year only. Litter was air dried in the laboratory for 10 days and then sorted by species.

Litter of each species was placed into individual mesh bags ("litterbags"; approximately 2 g litter per bag) to study decomposition. Litterbags were 25 cm x 25 cm and were made of Phifer fiberglass insect screen (2 mm mesh size). A subsample of air dried litter from each species was weighed, oven-dried at 70 °C for 48 h, and reweighed, to obtain a conversion factor of the mass of air-dried leaves to actual (oven-dried) dry mass. Six sets of litterbags were placed in each site. Each set included one litterbag of each of the six tree species used in the study. These sets of litterbags were placed in close proximity to one another within the site. The litterbags were placed on December 11, 2013. Immediately upon placement, one set of bags were collected and brought to the lab and analyzed to generate baseline data from week 0. Subsequent collections were performed at week 1, week 4, week 16, week 32, and week 52.

In addition to collecting leaves within the litterbags, two "in situ" samples were collected in March 2014 and September 2014. These collections were performed by laying a 50 cm x 50 cm square of PVC pipe in a randomly selected area within 15 m of the litterbag plot, and collecting all of the plant litter within that square. Leaves from two of these randomly selected plots were collected from each site, returned to the lab, sorted by species, and processed for DNA extraction and microbial community analysis.

Laboratory Processing:

Once collected, the mass of leaf litter remaining within each bag was determined. Leaves were then cut using sterile scissors and a known amount (typically 0.25 g) was

transferred to a 50 mL tube and homogenized in a sodium acetate buffer (0.5M, pH 5.0). The remaining leaf material was weighed, then dried at 70 °C for 48 h.

DNA Extraction and Amplification:

DNA extraction was performed using PowerSoil DNA Isolation kit (Mo Bio Laboratories, Inc.) following the protocol provided by the manufacturer. Successful DNA extraction was verified by carrying out gel electrophoresis in a 1.5% agarose gel. Paired-end indexed Illumina sequencing was used to assess bacterial community structure. Polymerase chain reaction (PCR) was performed to amplify the V4 variable region of the 16S rRNA gene in bacteria, using the forward primer 515F and the reverse primer 806R (Kozich et al., 2013). Primers included a unique bar code to enable sample multiplexing. Samples were pooled into a sequencing library, which was sequenced at the Molecular and Genomics Core Facility at the University of Mississippi Medical Center using established Illumina procedures. Raw sequence data (fastq files) were downloaded and processed using the bioinformatics software Mothur (Schloss et al., 2009). Sequences were screened, aligned, and classified using recommended procedures for Illumina data (Schloss et al., 2009; Kozich et al., 2013), which both identified the dominant bacterial types within each sample and enabled the bacterial communities present in each litter sample to be compared. Diversity was measured according to the Shannon index. Additionally, analysis of molecular variance (AMOVA) was used to determine significant differences between groups of samples.

Results:

Bacterial 16S rRNA fragments were successfully amplified from all 72 litterbag samples and 23 *in situ* samples (no hickory litter was found in the second *in situ* sampling). A total of 4,053,585 valid bacterial sequences were obtained across the samples, which were classified into 19,723 OTUs with a mean sample coverage of 97.3%. Diversity was measured according to the Shannon index, with the mean diversity among samples being 4.8 but ranging from 3.0-6.6 (Table 1). Diversity was similar in the early and middle stages of decomposition (weeks 0-16), but increased in the later stages (weeks 32-52). Additionally, samples from the site which experienced a prescribed burn (Site 1) showed greater mean diversity than control samples (Site 2; Table 1).

In terms of community composition, 63 bacterial phyla were identified when all samples were considered, although these phyla varied greatly in their relative abundance (Figure 1). The most abundant phylum or subphylum detected was the Alphaproteobacteria, which accounted for 35% of all sequences. The next two most abundant phyla/subphyla were the Bacteroidetes (accounting for 11% of all sequences) and Betaproteobacteria (10% of sequences). Additional phyla with moderately high relative abundance included Actinobacteria, Acidobacteria, Cyanobacteria, Planctomycetes, Gammaproteobacteria, Verrucomicrobia, and Deltaproteobacteria.

Site 1			**Site 2**		
Time Period	*Group*	*Shannon Index*	*Time Period*	*Group*	*Shannon Index*
Early	Oak	4.5	Early	Oak	4.2
	Non-oak	4.5		Non-oak	4.0
Middle	Oak	4.4	Middle	Oak	4.0
	Non-oak	4.2		Non-oak	4.2
Late	Oak	6.1	Late	Oak	5.3
	Non-oak	6.1		Non-oak	5.6

Table 1: Diversity of bacterial communities on decaying leaf litter at two sites in an upland oak woodland in north Mississippi undergoing ecological restoration. Diversity is expressed as the Shannon index, with scores representing the mean of six samples for the early groups, and nine samples for the middle and late groups.

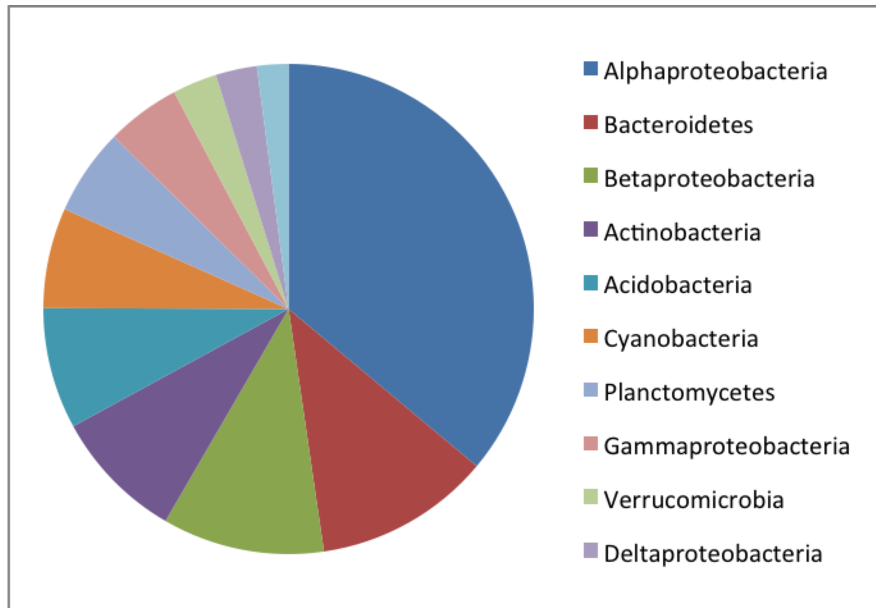

Figure 1: Relative abundance of bacterial phyla in decomposing leaf litter in litterbags in a north Mississippi woodland. Relative abundance was determined upon classification from next generation sequencing of 16S rRNA, and was calculated from total of 3,700,023 bacterial sequences obtained from 95 samples over a one year period. "Other" included 53 different phyla.

(Figure 1). Fifty-three other bacterial phyla combined to account for just 2% of the total sequences recovered

At a finer taxonomic resolution, the most abundant OTUs were OTU26 (classified in the Oxalobacteraceae family of the Betaproteobacteria), which accounted for 3.63% of all sequences, and OTU1 (classified as a species of *Sphingomonas* in the Alphaproteobacteria), which accounted for 3.59% of sequences (Table 2). Of the 20 most abundant OTUs (which accounted for 3,873,096 of the sequences), eight were representatives of the Alphaproteobacteria. Of those eight, four were in the order Rhizobiales, three were in the order Sphingomonadales, and one was unclassified at a finer resolution. All five of the sequence types classified as Betaproteobacteria that were in the 20 most abundant OTUs were members of order Burkholderiales.

Bacterial communities in litter samples were compared across sample dates using AMOVA. Samples from week 0 and week 1 were suggestive of being different, but this was not statistically significantly (AMOVA, *F-statistic(Fs)=2.10, p=0.055*, degrees of freedom (df)=1,22). Samples from week 0 and week 1 were, however, significantly different from each of the subsequent samples (AMOVA, $p<0.001$), with the exception of week 1 not being significantly different from week 4 (AMOVA, *Fs=3.50, p=0.002* [critical p values are lower than the usual 0.05 because of required Bonferroni correction], df=1,22). Samples from week 4, week 16, and *in situ* 1 were not significantly different from one another (AMOVA, $p>0.001$ for all), but were significantly different from the remaining samples (AMOVA, $p<0.001$ for all) with the exception of week 1. Lastly, samples from week 32, week 52, and *in situ* 2 were not significantly different

OTU	Size	Relative Abundance (%)	Phylum	Lowest Taxonomic Classification
Otu000026	134255	3.63	Betaproteobacteria	(f) Oxalobacteraceae
Otu000001	132984	3.59	Alphaproteobacteria	(g) *Sphingomonas*
Otu000023	124174	3.36	Alphaproteobacteria	*Sphingomonas wittichii*
Otu000006	95077	2.57	Alphaproteobacteria	*Pseudomonas viridiflava*
Otu000011	82634	2.23	Gammaproteobacteria	(o) Rhizobiales
Otu000004	82266	2.22	Acidobacteria	(g) *Terriglobus*
Otu000038	74186	2.01	Alphaproteobacteria	*Sphingomonas echinoides*
Otu000010	73357	1.98	Bacteroidetes	*Pedobacter cryoconitis*
Otu000032	73304	1.98	Alphaproteobacteria	(g) *Methylobacterium*
Otu000092	60259	1.63	Alphaproteobacteria	(c) Alphaproteobacteria
Otu000018	54286	1.47	Betaproteobacteria	*Burkholderia glathei*
Otu000014	50354	1.36	Betaproteobacteria	*Burkholderia andropogonis*
Otu000056	49473	1.34	Alphaproteobacteria	(o) Rhizobiales
Otu000005	49391	1.33	Bacteroidetes	(g) *Hymenobacter*
Otu000007	47854	1.29	Actinobacteria	(g) *Salinibacterium*
Otu000003	46190	1.25	Bacteroidetes	(f) Sphingobacteriaceae
Otu000009	45854	1.24	Actinobacteria	(f) Microbacteriaceae
Otu000055	40508	1.09	Alphaproteobacteria	(g) *Rhizobium*
Otu000071	40172	1.09	Betaproteobacteria	(f) Oxalobacteraceae
Otu000002	39772	1.07	Betaproteobacteria	(f) Comamonadaceae

Table 2: Most abundant bacterial OTUs recovered from decaying leaf litter in a north Mississippi woodland. Only the twenty most abundant OTUs in all samples are presented. Size refers to the number of bacterial sequences that classified as the corresponding OTU, with relative abundance showing that number as a percentage of the total number of sequences in the dataset. OTUs are identified to the corresponding phylum and to the lowest taxonomic classification level of each that was possible (c=class, o=order, f=family, g=genus).

from one another (AMOVA, *p>0.001* for all) while they were significantly different from each of the samples from other weeks (AMOVA, *p<0.001* for all).

Patterns in the differences in bacterial community structure between dates suggested that communities could be separated into three distinctive time intervals: "early", "middle", and "late". "Early" included litterbag samples from weeks 0 and week 1. "Middle" included litterbag samples from week 4, week 16, and an *in situ* sample taken between weeks 4 and 16. "Late" included samples from week 32, week 52, and an *in situ* sample taken between those weeks. Differences in relative abundances of phyla found within oak samples and non-oak samples in each stage of decomposition (early, middle, and late) were seen (Figure 2). For example, early samples showed greater relative abundance of Cyanobacteria than middle or late samples. Also, late samples saw a reduction in the relative abundance of Alphaproteobacteria (Figure 2).

NMDS ordinations also supported the concept of three distinct time intervals (early, middle, late; Figure 3). Several OTUs appeared to drive the differences in community structure between time periods, as determined from correlations of NMDS axes scores with OTU representation. OTUs that accounted for > 1% of all sequences were selected for this analysis. In order to account for OTU correlations to both axis 1 and axis 2 scores on NMDS ordinations, OTU importance was assessed by the length of the vector line that would run from the origin to the point of the OTU on the relevant NMDS plot. Longer vector lines indicate OTUs that are more highly correlated to NMDS axes scores, and OTUs with a vector line length of 0.70 were considered significant drivers of community differences (and always corresponded to a statistically significant relationship between that OTUs relative abundance and at least one NMDS axis score).

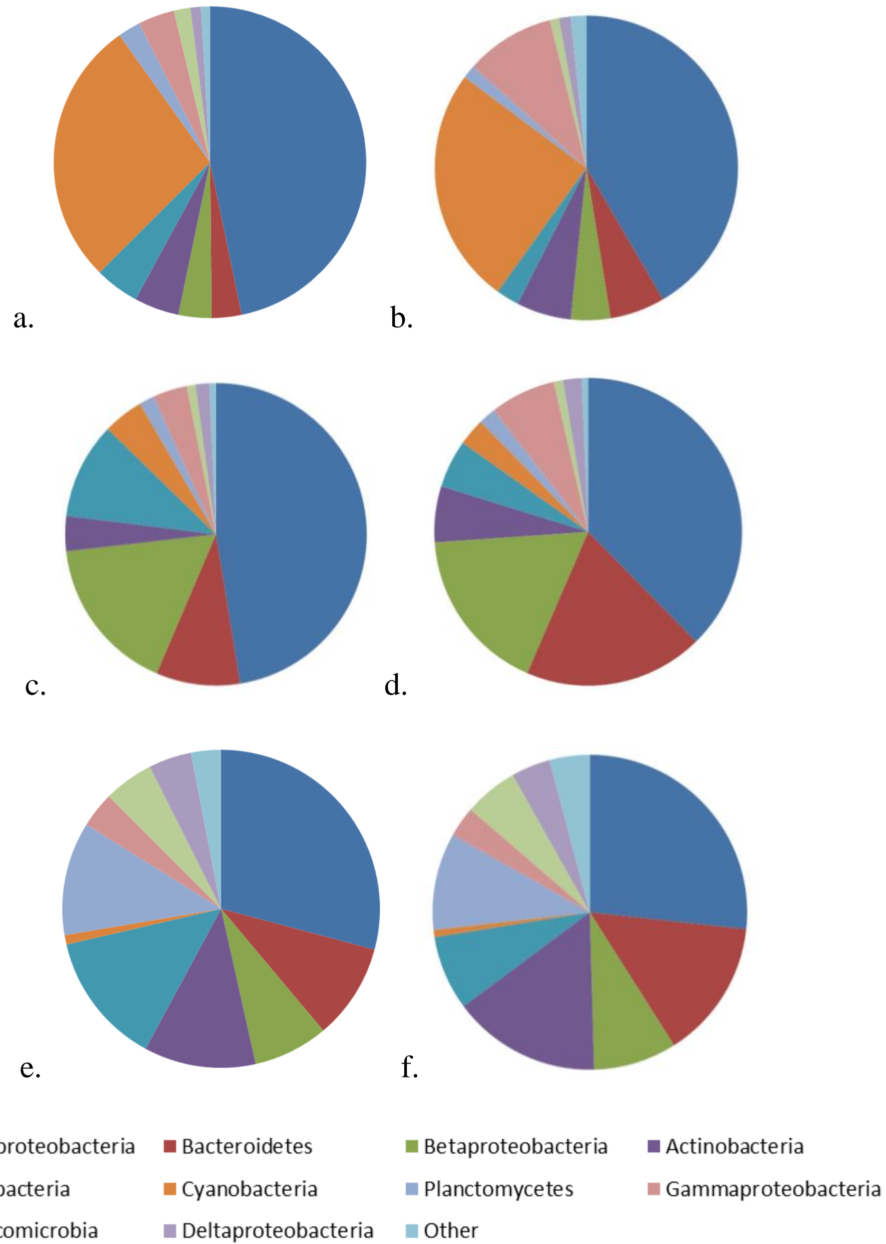

Figure 2: Relative abundance of bacterial phyla on decaying leaves in a north Mississippi woodland throughout early, middle, and late stages of decomposition. Chart a includes sequences from 12 oak samples from the early stages of decomposition (week 0-1). Chart b includes sequences from 12 non-oak samples from the early time period. Charts c and d each include sequences from 18 samples in the middle stage of decomposition (week 4-16), from oak and non-oak samples, respectively. Chart e includes sequences from 18 oak samples from the later stages of decomposition (week 32-52). Chart f represents sequences from 17 non-oak samples from the same time period.

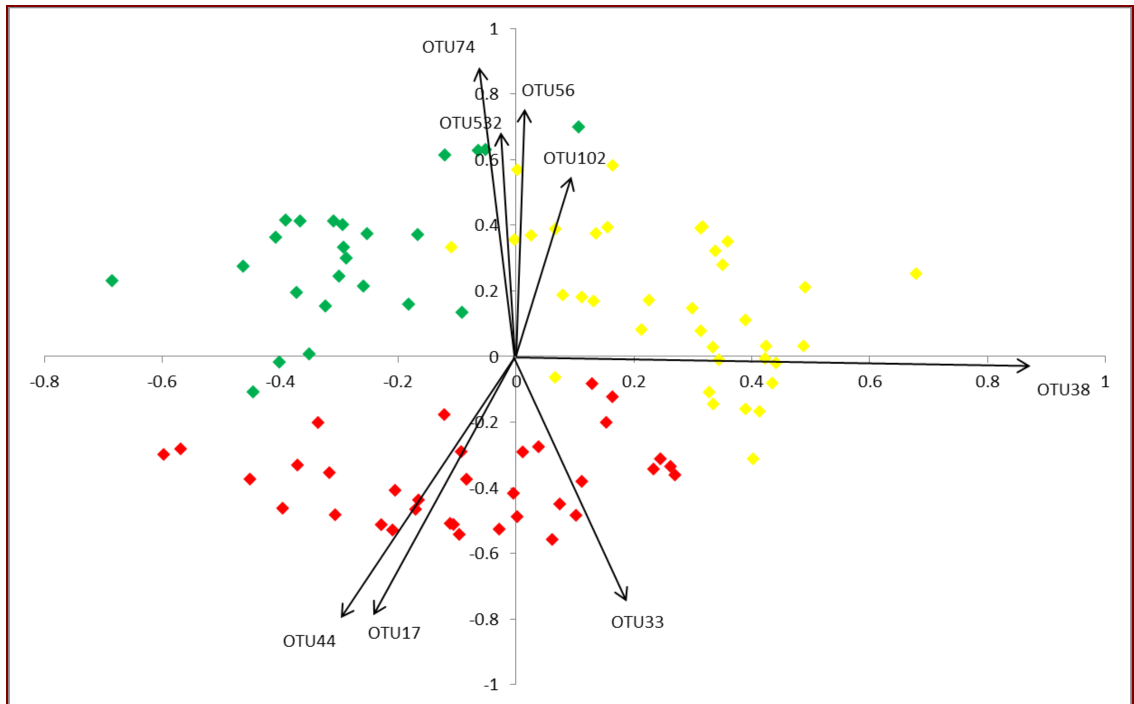

Figure 3: NMDS ordination of bacterial communities sampled throughout decomposition of six different species of litter as determined from next generation sequencing of 16S rRNA genes. Green indicates samples from the "early" time period (weeks 0-1). Yellow represents samples from the "middle" time interval (weeks 4-16). Red indicates samples from the "late" time period (weeks 32-52). Three dimensions were used for NMDS with the lowest stress being 0.17. Distinct grouping of "early", "middle", and "late" points on the NMDS plot suggests that time contributes to differences in bacterial community structures. Arrows indicate selected bacterial OTUs that were driving the bacterial community differences. Arrows that are within a distinct cluster of the same color indicate greater abundance of that OTU within that respective time interval.

There were 101 OTUs which fit this criterion. These included OTU56 (classified as a species of Rhizobiales, Alphaproteobacteria), OTU33 (classified as *Bosea genospecies* a member of Alphaproteobacteria), OTU74 (classified as *Methylobacterium*, a member of Alphaproteobacteria) and OTU44 (a member of the Haliangiaceae family of Deltaproteobacteria). In some cases differences in these OTUs reflected differences over the course of the study; for example, OTU56 accounted for 5% of all sequences in the "early" time period, 2% of all sequences in the "middle" time period, and 0% of all sequences in the "late" time period, and time proved to be a major separator in NMDS plots (Figure 3). Because of this overriding influence of time/sample date, subsequent analyses focused on differences between sites and species type (oak vs. non-oak) within each time interval.

<u>*Early:*</u>

Bacterial communities on litter samples did not differ significantly between burned and control sites (Sites 1 and 2, respectively) for the early time interval (Figure 4; theta-based AMOVA, Fs=0.42, $p=0.926$, $df=1,22$). However, bacterial communities in this time interval were dependent upon the type of litter sampled, and communities on species of oak were significantly different from those on non-oak species (Figure 4; theta-based AMOVA, Fs=6.96, $p<0.001$, $df=1,22$). The distribution of seven major OTUs appeared to be responsible for these differences (Figure 4), although eight additional OTUs also contributed to this pattern. OTU1, classified as *Sphingomonas* (Alphaproteobacteria), and OTU113, classified as a member of the Acetobacteraceae

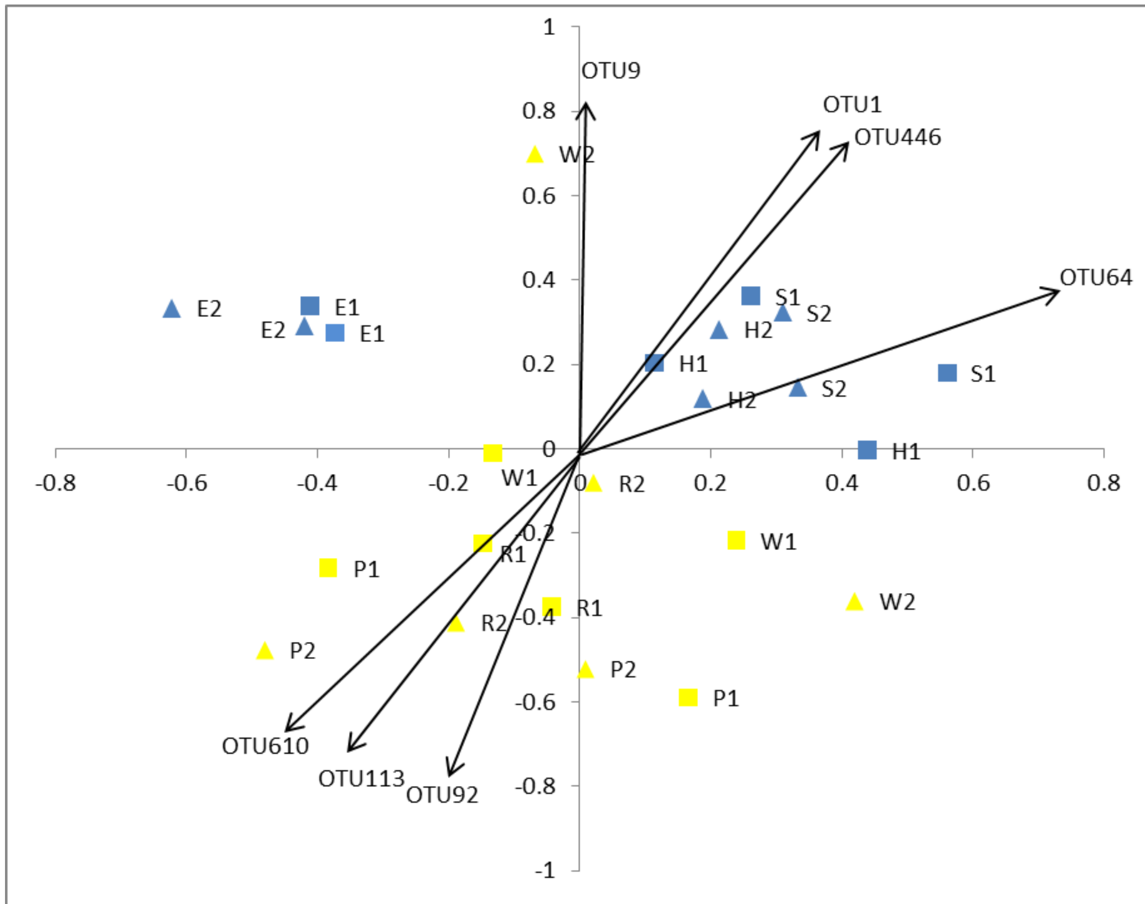

Figure 4: NMDS ordination of bacterial communities sampled in the early stages (0-1 weeks) of decomposition of elm (E), hickory (H), sweetgum (S), post oak (P), red oak (R), and white oak (W) as determined from next generation sequencing of 16S rRNA genes. Squares indicate samples from Site 1, while triangles indicate samples from Site 2. Yellow points are oak samples, and blue points are non-oak samples. Two dimensions were used for NMDS and the lowest stress was 0.23. Arrows indicate selected bacterial OTUs that were potentially driving bacterial community differences. OTUs 610, 113, and 92 were more abundant in oak samples, while OTUs 9, 1, 446, and 64 were more abundant in non-oak samples.

(Alphaproteobacteria) were among those OTUs driving this difference. OTU1, on average, accounted for 3% of the sequences recovered from oak leaves, but 9% of those from non-oak leaves. OTU113, on average, accounted for 6% of sequences from oaks, but < 1% of those from non-oaks.

Middle:

Bacterial communities on litter samples collected in the middle time interval also did not differ significantly between Sites 1 and 2 (Figure 5; theta-based AMOVA, $Fs=0.64$, $p=0.649$, $df=1,34$). As with the early time period, there were significant differences between bacterial communities of oak and non-oak species in the middle time interval (Figure 5; theta-based AMOVA, $Fs=16.95$, $p<0.001$, $df=1,34$). Seven major OTUs appeared to be responsible for these differences along with 31 additional OTUs that were generally less abundant. Similarly to samples from the early time period, OTU1 (*Sphingomonas*) was more abundant in non-oak samples, accounting for 7% of non-oak sequences but only 2% of oak sequences from samples in this time interval. Similarly, OTU26, a member of the Oxalobacteraceae family (Betaproteobacteria) account for 11% of non-oak sequences, yet only 4% of oak sequences. Conversely, OTU23, classified as *Sphingomonas wittichii* (Alphaproteobacteria) accounted for only 3% of non-oak sequences in samples from this time interval, but 10% of the sequences derived from oak samples, and OTU5 (classified as a species of *Hymenobacter* (Bacteroidetes)) accounted for < 1% of non-oak sequences, but 5% of oak sequences.

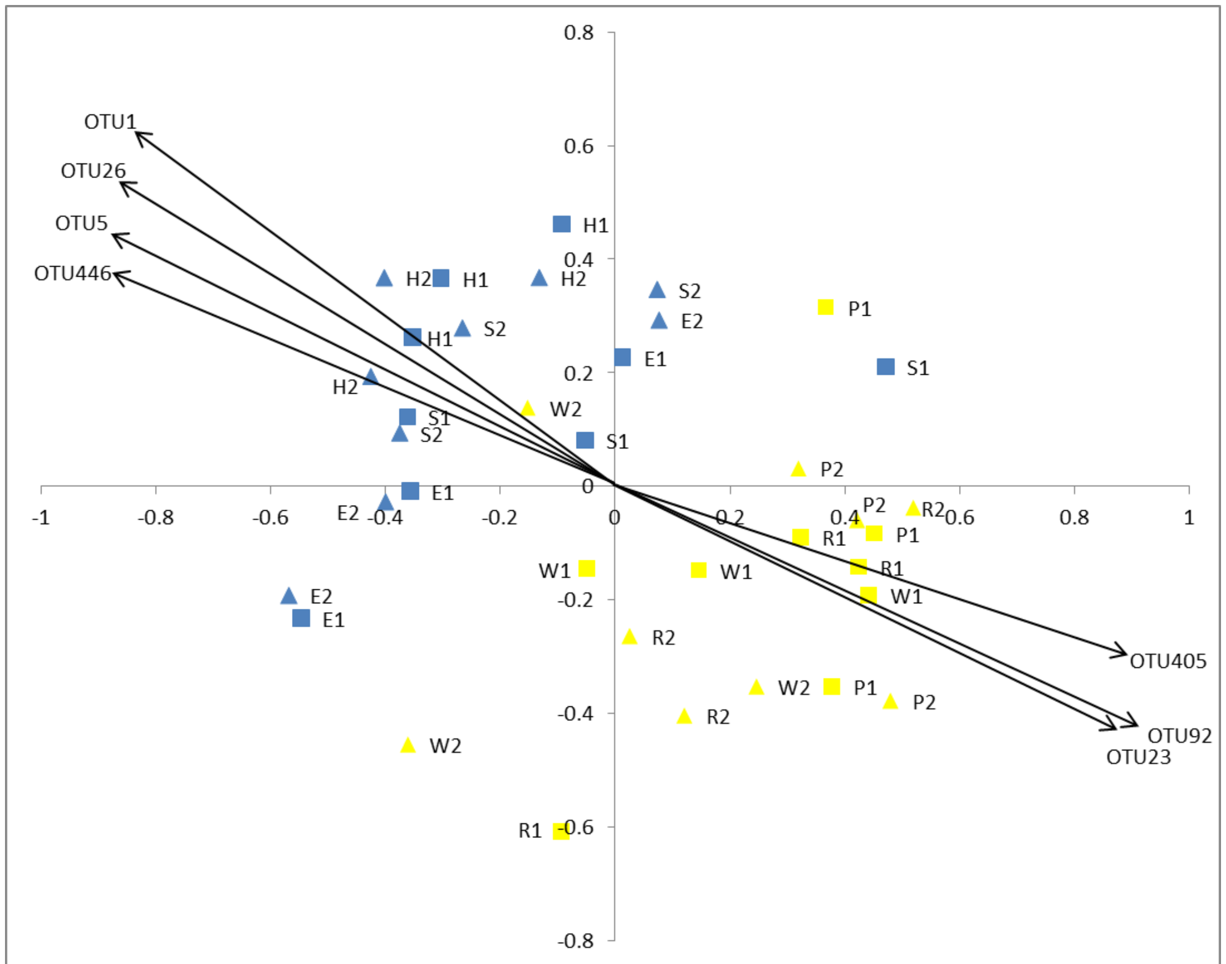

Figure 5: NMDS ordination of bacterial communities sampled in the middle stages (4-16 weeks) of decomposition of elm (E), hickory (H), sweetgum (S), post oak (P), red oak (R), and white oak (W) as determined from next generation sequencing of 16S rRNA genes. Squares indicate samples from Site 1, while triangles indicate samples from Site 2. Yellow points are oak samples, and blue points are non-oak samples. Two dimensions were used in NMDS with the lowest stress being 0.18. Arrows indicate selected bacterial OTUs that were potentially driving bacterial community differences. OTUs 405, 92, and 23 were more abundant in oak samples, while OTUs 1, 26, 5, and 446 were more abundant in non-oak samples.

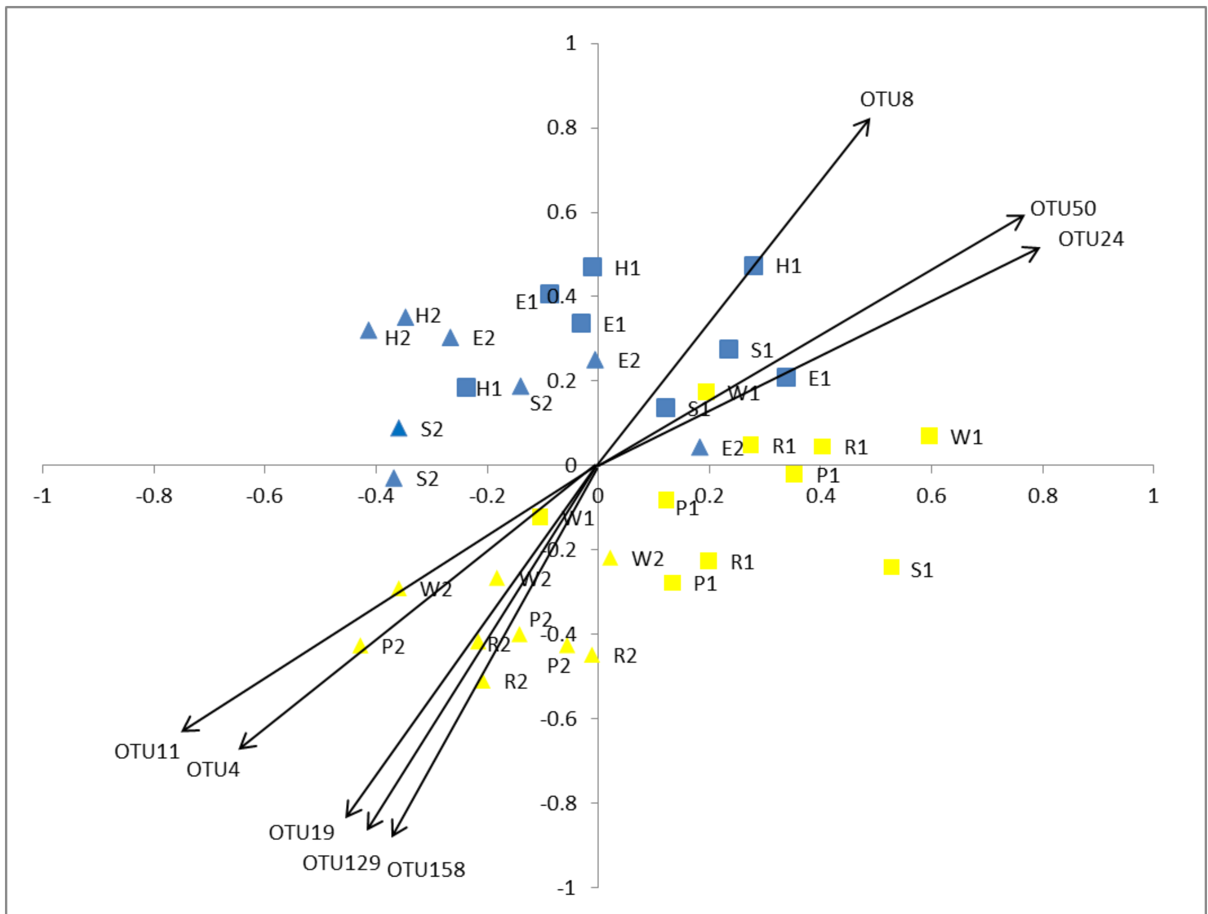

Figure 6: NMDS ordination of bacterial communities sampled in the late stages (32-52 weeks) of decomposition of elm (E), hickory (H), sweetgum (S), post oak (P), red oak (R), and white oak (W) as determined from next generation sequencing of 16S rRNA genes. Squares indicate samples from Site 1, while triangles indicate samples from Site 2. Yellow points are oak samples, and blue points are non-oak samples. Two dimensions were used for NMDS with the lowest stress being 0.18. Arrows indicate selected bacterial OTUs that were among the top drivers of bacterial community difference. These arrows indicate the top drivers of difference in the late samples are mainly separators based on site. OTUs 11, 4, 19, 129, and 158 were more abundant in Site 2 samples, while OTUs 8, 50, and 24 were more abundant in Site 1 samples.

Late:

In contrast to the early and middle time intervals, bacterial communities on litter samples collected in the later time interval (after the prescribed fire at Site 1) showed a significant difference between Sites 1 and 2 (Figure 6; theta-based AMOVA, Fs=8.55, $p<0.001$, $df=1,33$). However, as with the other time intervals, litter samples in the late time interval also showed significant differences between oak species and non-oak species (Figure 6; theta-based AMOVA, Fs=10.41, $p<0.001$, $df=1,33$). Because of the significant site effect on bacterial community structure, sites were examined separately in order to determine driver OTUs responsible for separating oak and non-oak. Within Site 1, a total of 50 OTUs were significantly related to differences between oak and non-oak samples in NMDS plots. However, six of these 50 (Figure 7a) were more dominant either in terms of strength of effect or relative abundance. Two of most important OTUs separating these communities were OTU26 (a member of the Oxalobacteraceae) and OTU23 (*Sphingomonas wittichii*). OTU26 accounted for 1.4% of non-oak sample sequences in this time interval but only 0.4% of oak sequences, while OTU23 accounted for 0.6% of non-oak sample sequences but 1.6% of oak sequences. At Site 2, six OTUs were important (in terms of abundance or strength of effect) in driving differences between oak and non-oak litter (Figure 7b), out of a total of 100 that could be related to community differences. OTUs contributing most to this difference included OTU131 (classified as a member of the Acidobacteriaceae family, in the Acidobacteria) and which accounted for 2.5% of oak sequences from Site 2, but only 0.2% of non-oak sequences. Additionally, OTU33 (classified as *Bosea genospecies*, a member of

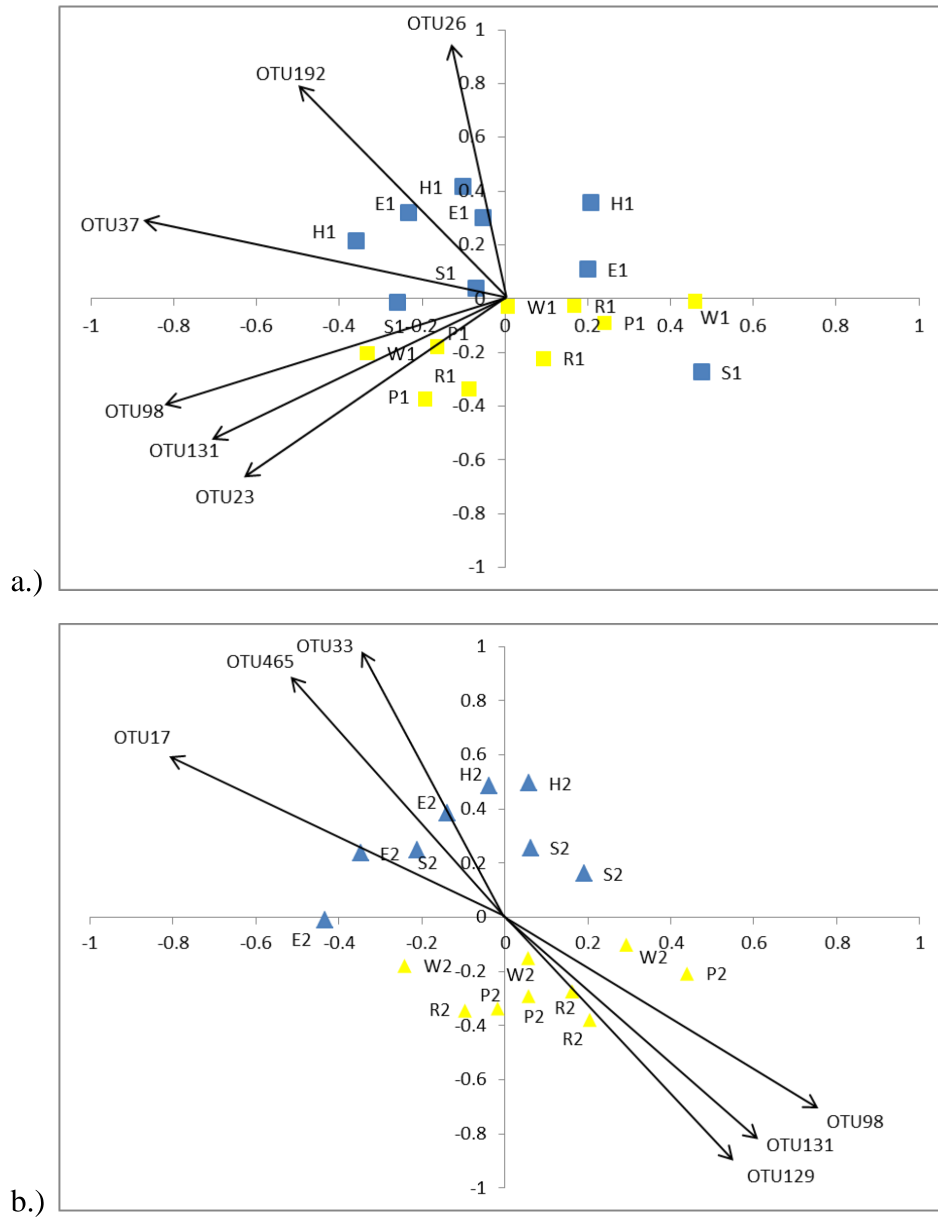

Figure 7: NMDS ordinations of bacterial communities sampled in two different sites during the later stages of decomposition of six species of litter as determined from next generation sequencing of 16S rRNA genes. Oak samples are represented in yellow, while non-oak samples are represented in blue. Two dimensions were used for NMDS. Panel a shows all samples from Site 1 (lowest stress=0.20), while panel b contains all samples from site 2 (lowest stress=0.12) with arrows indicating selected OTUs which separate communities on oak samples from communities on non-oak samples. OTU 98 and OTU 131 are seen in both panels indicating greater abundance in oak litter than non-oak litter at each site.

Alphaproteobacteria) was present in just 0.6% of oak sequences from Site 2, but 2.0% of non-oak sequences. This OTU was also important in separating bacterial communities on oak vs. non-oak litter in the other time intervals. In terms of separating specific sample types by site during this time interval, there were 77 OTUs that separated oak litter associated bacterial communities at Site 1 from those at Site 2. Six appeared to be more important in contributing to this difference (Figure 8a). These included OTU19 (classified as *Granulicella paludicola*, a member of the Acidobacteria), which was present in 1.0% of oak samples from Site 1, but 2.7% of oak samples from Site 2. OTU50 (classified as a species of *Actinoplanes*, a member of Actinobacteria) was present in 1.7% of oak samples from Site 1, but only 0.3% of oak samples from Site 2. In the non-oak litter samples, there were 48 OTUs that were correlated with the differences between Sites 1 and 2. However, five OTUs had a more substantial impact than others based on relative abundance and/or strength of relationship (Figure 8b). Significant OTUs included OTU7 (classified as *Salinibacterium*, a member of Actinobacteria), which accounted for 1.2% of sequences of non-oak litter sequences from Site 1, but 2.7% of those sequences from Site 2. OTU1 (*Sphingomonas*) accounted for 2.4% of the non-oak sequences from Site 1, but 3.9% of the same sample types at Site 2. OTU1 was also a driver of differences in bacterial community in the early and middle stages of decomposition, when it was found in higher relative abundance in non-oak species, as mentioned previously.

Because of the high number of significant driver OTUs, the percentages of each OTU were relatively small, thus making it more challenging to see the specific impact

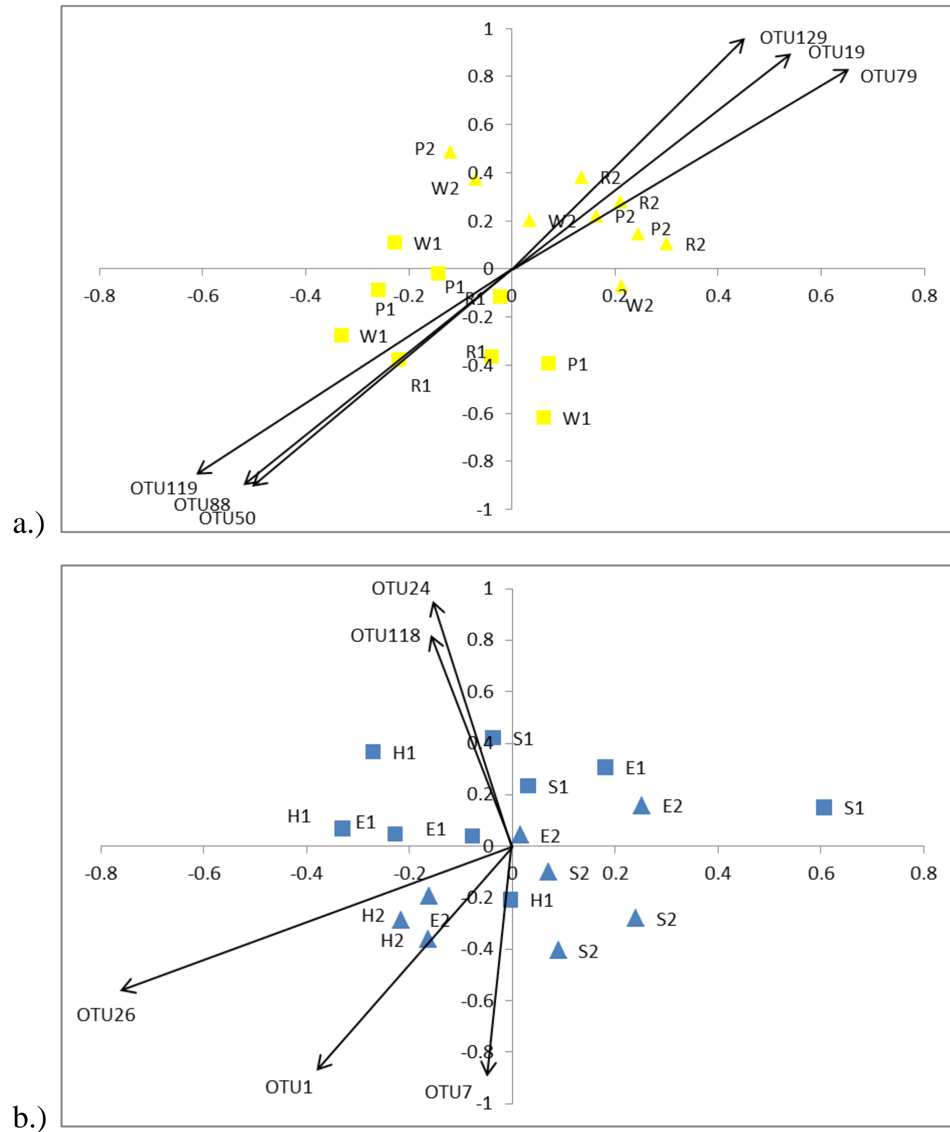

Figure 8: NMDS ordinations of bacterial communities sampled from oak and non-oak litter during the later stages of decomposition of six species of litter determined from next generation sequencing of 16S rRNA genes. Squares indicate samples from Site 1, while triangles indicate samples from Site 2. Two dimensions were used in NMDS. Panel a shows all oak samples (lowest stress=0.15), while panel b shows all non-oak samples (lowest stress=0.18). Arrows indicate selected bacterial OTUs that were potential driving bacterial community differences between Site 1 and Site 2 samples.

each significant OTU is having on community differences. OTU98 (classified as a member of Rhizobiales, an Alphaproteobacteria) and OTU131 (a representative of the Acidobacteriaceae) were found as part of the ten most significant OTUs for community differences distinguishing oak samples from non-oak samples in both Sites 1 and 2. Since these OTUs were found to be significant at both sites, it is suggestive that they are important separators of oak litter samples from non-oak litter samples.

Discussion:

In this study, bacterial community structure and diversity on the leaf litter of six different tree species was assessed. Significant shifts in bacterial community structure occurred throughout the first year of decomposition, and patterns in bacterial community composition suggested that the bacterial communities present on the litter of oak and non-oak trees were different. Furthermore, prescribed burning also affected the composition of bacterial communities present in leaf litter, resulting in differences in bacterial community composition on litter between burned and unburned sites. Previous studies have examined the effects of burning on microbial communities in soil (Staddon, et al., 1997, Hamman et al., 2007), but this project is somewhat novel in that it aimed to determine the impacts of restoration treatments on the bacterial communities that occur in leaf litter, as well as to characterize the influence of tree species on the litter bacterial community.

Previous studies that have examined the effect of fire on bacterial communities in the soils of forest ecosystems have found that microbial diversity increased following a fire (Fontúrbel et al., 2012). In this study, bacterial diversity was generally similar on oak and non-oak litter, but showed evidence of increasing during the decomposition process, especially in the burned plots, potentially confirming the idea that fire increases microbial diversity. The mechanism for this is unclear, but it could be that fire acts as a perturbation to the system, removing (or at least reducing) any dominant bacterial populations and

allowing others to become more prevalent. Fire might also release nutrients, stimulating the overall bacterial community.

At a taxonomic level, Alphaproteobacteria were the most abundant phyla present across all samples, accounting for more than 1/3 of all sequences. The subphylum Alphaproteobacteria is very diverse, but many species are found in symbioses with plants and can be either mutualistic or pathogenic (Williams et al., 2007). Three of the most common OTUs detected in the study were identified as members of the genus *Sphingomonas*, a genus of Alphaproteobacteria that contains many species that are found in association with plants (White et al., 1996). The high frequency of these plant-associated bacteria is not surprising given the origins of the litter material, and it may be that many of the bacterial species associated with decaying leaf litter are also those that are typically associated with living plants.

Based on our analysis, bacterial communities on leaf litter could be divided into three distinct time intervals. However, these time intervals do not necessarily correspond to the three classic phases of decomposition, largely because this study ran for just under one year, a time period that may only correspond to the earliest phase of decomposition (Berg, 2000). Regardless, differences in the litter bacterial communities among the time intervals are likely because of the changing availability of nutrients and other resources during the decomposition process. Nutrient content of the litter was not monitored in this study, but determining the specific nutrients present at different times during decomposition might help relate bacterial community structure to changes in environmental or litter parameters. For example, Cyanobacteria were relatively abundant in the earliest time interval, but significantly decreased in abundance in the middle and

late stages. While generally considered to be independent phototrophs, Cyanobacteria can form symbioses with a broad range of host plants. In some plant-cyanobacterial symbioses, Cyanobacteria supply the host with fixed N_2, while receiving protection from predation and changing environmental conditions from their plant host (Adams et al, 2006). Thus, reductions in the prevalence of Cyanobacteria might reflect changing nutrient availability that makes N_2 fixing organisms less competitive, or simply a loss in potential plant symbionts as the fresh litter became more decayed. OTU56, a member of the order Rhizobiales (Alphaproteobacteria), also decreased in abundance from the early to late stages of litter decomposition, and members of the Rhizobiales are also known to benefit from plant-microbial interactions (Erlacher et al., 2015) by carrying out nitrogen fixation. Thus, reductions in the proportions of Cyanobacteria and Rhizobiales in the bacterial community during litter decomposition could be occurring because of similar reasons (changes in nutrient availability and/or a lessening of beneficial plant-microbial interactions as leaves senesce and decay).

Fresh litter differs chemically from older, partially decomposed litter (Berg, 2000). As stated previously, even though this study ran for almost a year, it really only examined the microbial community present on litter in the earlier stages of the entire decay continuum. Examining the changes in bacterial community structure over a longer period of time could be beneficial, and would assess changes in bacterial community composition over the entire decomposition process. However, such studies could be difficult as litter becomes increasingly fragmented and potentially harder to identify to a specific type. Even within the duration examined here, there was separation of bacterial communities by different time periods, and differences in community structure between

some litter species and between sites undergoing active restoration or not. NMDS ordinations suggested that bacterial communities on some litter types appeared to form distinct clusters; although, there were no significant differences among individual litter species within any time period. However, when species were pooled into oaks vs. non-oaks, these groups of litter types did appear to harbor different bacterial assemblages in the early, middle, and late time intervals, and these patterns were likely driven by differences in the relative proportions of specific bacterial populations.

The driver OTUs that designated non-oak litter in the early time period were identified as belonging to the genera *Sphingomonas*, *Kineococcus*, *Methylobacterium*, and *Hymenobacter*, while many of the OTUs found in greater abundance in early oak samples classified into the family Acetobacteraceae, commonly referred to as acetic acid bacteria. These differed from the OTUs separating oak and non-oak litter in the middle time interval, when OTUs identified as Acidobacteria were proportionally more abundant in oak samples. Acidobacteria are common in soil and an increased abundance of this phylum has been correlated with lower pH values (Jones et al., 2009). The oak rhizosphere tends to be more acidic than surrounding soils (Uroz et al., 2010) and it is possible that oak litter may follow this same pattern, potentially explaining an increased prevalence of Acidobacteria on oak litter as compared to non-oak species. Future studies linking pH differences between litter types with aspects of bacterial community structure would be highly beneficial.

Bacterial communities in the latest time interval were potentially impacted by litter species type, as well as the prescribed burn. Both appeared to have an effect, and as with the other time intervals, there were differences in bacterial community structure

between oak and non-oak species. OTUs found in greater relative abundance in non-oak communities included OTU26, which classified into the Oxalobacteraceae family, and OTU 33, classified as *Bosea genospecies*. OTUs found in greater abundance in the oak samples included OTU23 (*Sphingomonas wittichii*) and OTU131, which classified as a member of the Acidobacteriaceae. As well as often being associated with plants, many species of *Sphingomonas* are known for their ability to degrade natural recalcitrant compounds (Balkwill et al., 2006) and oak leaves are known to be thicker and more recalcitrant that other species (Abrams, 1990). More recalcitrant and potentially more acidic litter from oaks compared to non-oak species may have led to some of the observed differences in bacterial species distributions.

Bacterial community structure in both oak and non-oak litter was markedly affected by the prescribed burn. These differences could not be attributed to differences in the proportions of any particular phylum, but rather were found when looking at specific OTUs. An increase in overall bacterial diversity occurred, which also led to an increase in the number of potential driver OTUs, including OTU19 (*Granulicella paludicola*), OTU50 (*Actinoplanes*), OTU7 (*Salinibacterium*) and OTU1 (*Sphingomonas*). Diversity in microbial communities has been reported to either decrease (Acea & Carballas, 1996, Hamman et al., 2007) or increase (Fontúrbel et al., 2012) following a fire, and this study generally supports the latter. However, changes in bacterial community diversity following a fire are likely to depend upon many factors including burn severity, plant composition, and nutrient availability (Scharenbroch et al., 2012), so there may not be any consistent pattern in the response of overall bacterial diversity to burning.

Regardless of the specific outcome, changes in bacterial community structure following fire suggest the importance of periodic burning to oak woodlands. When fire suppression occurs, the result is mesophication, a gradual change from fire tolerant, shade intolerant species (i.e. oaks) to shade tolerant, fire intolerant species (i.e. non-oak species) (Nowacki & Abrams, 2008). When these shifts in the plant community occur, the leaf litter changes as well. Changes in leaf litter composition in turn result in changes in the litter-associated bacterial community. Large changes in bacterial community structure are a concern because of the important role microorganisms have in nutrient cycling (Williams et al., 2012). Future studies that relate nutrient availability and environmental conditions such as pH to microbial community structure would be beneficial for understanding the presence or absence of particular bacterial populations. Assessing the bacterial communities on greater numbers of litter samples, especially with increased replication of each species and of sites, would also be useful, particularly if the bacterial communities on specific tree species could be compared in more detail, instead of limiting it to oak or non-oak species. These studies would provide us with a greater understanding of the nature of the bacterial communities present on leaf litter, and how they related to patterns in plant species composition and the ecosystem as a whole.

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