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**Section 1** **Biology**

# Microbial Community Analysis of Naturally Durable Wood in an Above Ground Field Test

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# Microbial Community Analysis of Naturally Durable Wood in Above Ground Field Tests

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## ABSTRACT

This paper presents preliminary results of an above ground field test wherein eight naturally durable wood species were exposed concurrently at two sites in North America. Surface samples were taken at regular intervals from non-durable controls and compared to their more durable counterparts. Terminal Restriction Fragment Length Polymorphism was performed to characterize the microbial (bacteria, fungi, and basidiomycetes) communities present. Differences were noted among wood species and seasonal shifts in microbial diversity were noted at both sites. Attempts to correlate diversity indices with decay ratings were unsuccessful, but differences in species richness were noted for several of the naturally durable species. Western red cedar had significantly fewer bacterial species compared to other wood species. Fungal and basidiomycete species richness differed due to site and fungal species richness increased with increased exposure. Clustering of fungal and basidiomycete communities suggests seasonal patterns of colonization at both sites, but was more defined in the more southern site; Saucier, MS (MS). Future analyses will focus on comparison of years to model successional patterns of bacteria, fungi, and basidiomycetes.

**Keywords:** wood decay microbes, T-RFLP, DNA, above ground tests

## 1. INTRODUCTION

Naturally durable wood species are commonly offered as environmentally friendly alternatives to chemically treated wood while service life is unknown and often over estimated due to lack of field test data. In addition, there is limited knowledge of what microbes damage durable woods in service. There have been efforts to identify wood associated fungi with some of the more common naturally durable wood species, but there is a definite lack of biodiversity data for many of the species regarded as durable (Clausen 2010). The goal of this study is to characterize the microbial communities in naturally durable wood species in an above ground test in order to compare the microbial assemblages of bacteria and fungi (which includes molds, stains, yeasts, and wood decay fungi), with emphasis on basidiomycete fungi (which include most of the true wood decay fungi) using Terminal Restriction Fragment Length Polymorhphism (T-RFLP) analysis. T-RFLP is a culture independent molecular method that uses fluorescently labelled primers and restriction enzymes which cut DNA into fluorescent fragments. T-RFLP has been used to characterize microbial communities in a wide range of substrates (Dickie & Fitzjohn 2007), including above ground decay fungi in Germany (Räberg et al. 2009), and fungi in Norway spruce (Allmer et al. 2006). Previous studies using this technique with preservative treated wood found that these preservatives can alter the bacterial (Kirker et al. 2012a), fungal and basidiomycete (Kirker et al. 2012b) communities.

In this study, samples were taken at regular intervals and microbial profiles were compared between naturally durable wood species and non-durable controls over time. This research signifies our first steps in characterizing the microbial communities of these candidate naturally durable species in order to investigate differences in microbial diversity that might correlate with increased durability.

## 2. EXPERIMENTAL METHODS

### 2.1 Test Sites

The two above ground sites were set up by the Forest Products Laboratory for routine evaluation of exposed naturally durable woods. The southern site is located in Saucier, MS in the Desoto National Forest; it is categorized as Zone 5 by the AWPA commodity use map (AWPA 2013) and is in the 80 to 90 range of the Scheffer index (Carll 2009). This site is considered a high biodeterioration zone. The northern site is located at the Valley View test site near Madison, WI. This site is categorized as Zone 2 by the AWPA commodity use map (AWPA 2013) and is in the 40 to 50 range of the Scheffer index (Carll 2009). This site is considered a low biodeterioration zone. The original focus of these study sites was for above ground evaluation of 8 naturally durable wood species for use in repair and rehabilitation of covered bridges for the federal highway administration (Kirker 2013). Samples are exposed laterally on an above ground racks and are evaluated for decay, physical appearance, and dimensional stability (cupping and twisting).

### 2.2 Wood Species Selection

Naturally durable wood (NDW) species were chosen based on previous work indicating some level of natural durability with Southern pine used as a non-durable control. Wood species selected and times of sampling are shown in Table 1.

Table 1: Wood species for above ground evaluation and time points sampled

| Wood Species | Common Name | Abbreviation | Sampling Times* |
|---|---|---|---|
| ***Pinus*** spp. | Southern pine | SP | 1-12 |
| ***Paulownia tomentosa*** | Paulownia | PAW | 1-12 |
| ***Prosopis glandulosa*** | Honey mesquite | HM | 1-12 |
| ***Robinia pseudoacacia*** | Black locust | BL | 1-12 |
| ***Juniperus virginiana*** | Eastern red cedar | ERC | 1-12 |
| ***Catalpa*** **spp.** | Catalpa | CAT | 1-12 |
| ***Callitropsis nootkanensis*** | Alaskan yellow cedar | AYC | 1-12 |
| ***Thuja plicata*** | Western red cedar | WRC | 1-12 |
| ***Juniperus occidentalis*** | Western juniper | WCJ | 1-12 |

*WI was sampled biannually (2X a year), while MS was sampled quarterly (4x a year).

### 2.3 Sample Collection

Samples were collected using a sterilized wood chisel from the surface of each of the naturally durable wood species. Samples were taken in the same area on the wood piece in order to obtain repeated sampling of the same spatial unit for comparison. The surface of the chisel was sterilized with 70% ethanol between samples to prevent cross contamination. Samples were processed at Mississippi State University for T-RFLP analysis.

## 2.4 DNA Preparation

Genomic DNA was extracted from the wood shavings using Promega Wizard genomic DNA extraction kit (Madison, WI) following manufacturer's specifications. The kit uses a CTAB based detergent for cell lysis and DNA is bound to a membrane for subsequent rinses with ethanol and eluted with buffer. Extracted genomic DNA was stored at -30°C until polymerase chain reactions (PCR) were run using non-specific (fungal and bacterial) and specific primers (basidiomycetes). Primers, settings, and source literature are presented in Table 2. . Amplified products were digested with *msp1* for bacteria and *Taq1* for fungi and basidiomycete specific fungi. After appropriate clean-up, the fragments samples were run on a CEQ8000 capillary sequencer.

Table 2: Polymerase chain reaction (PCR) primers for T-RFLP amplification

| Group | Target | Forward Primer (5'-3') | Reverse Primer (5'-3') | ~Size (bp) | Source |
|---|---|---|---|---|---|
| **Bacteria** | 16S rDNA* | AGAGTTTGATCCTGGCTCAG | ACGGGCGGTGTGTRC | 1400 | Liesack and Dunfield 2004 |
| **Fungi** | ITS** | CTTGGTCATTTAGAGGAAGTAA | TCCTCCGCTTATTGATATGC | 600 | White et al. 1990 |
| **Basidiomycetes** | ITS** | CTTGGTCATTTAGAGGAAGTAA | CAGGAGACTTGTACACGGTCCAG | 800 | Gardes and Bruns 1993 |

*Ribosomal DNA, **Internal transcribed spacer region

## 2.5 Data Analysis

T-RFLP fragment data were visually screened using CEQ 8000 software (Beckman Coulter, to remove peaks below threshold and aberrant peaks, and remaining data were exported to create a matrix of binary data corresponding to presence or absence of detectable taxonomic units. Binned T-RFLP data was then exported into PC-ORD v6.0 (Corvallis, OR) for community analysis. Initial diversity values were calculated for each sample for each category, and compared using cluster analysis in PC-ORD v 6.0. Diversity sums were calculated using the software to obtain a diversity index for each sample. The programming is based on the formula for Shannon's diversity index:

**H= -sum (Pi*ln(Pi))**

Shannon's diversity was used as the measure of species diversity and was initially used to make comparisons between the different wood species at the different time intervals. In addition, non-metric multi-dimensional scaling (NMDS) was used to plot the samples based on their species composition on a 2D or 3D axis depending on optimal fit. This procedure was used to generate ordinations based on the species composition of individual samples in order to determine if bacterial and fungal species composition was different for the different naturally durable wood species. Ordinations were calculated using Jaccardian distance as the distance measure (McCune *et al*. 2002), using the following formula:

$$JD_{ih} = \frac{\sum_{j=1}^{p} aij - ahj}{\sum_{j=1}^{p} aij + \sum_{j=1}^{p} ahj + \sum_{j=1}^{p} [aij - ahj]}$$

### 2.6 Statistical Analysis

Statistical analysis was used to compare different NDW species at different time points and between the two sites. The lack of a clear result from analysis of the diversity indices prompted analyses using S (species richness), which is simply a sum of number of species (operational taxonomic units = OTUs) per sample. Bacterial, fungal and basidiomycete richness were fit using Poisson regression models that included factors for year, time period within year, site, NDW, and appropriate interactions, with the models including random effects for dependencies that occurred within the data. Models were fit with and without the decay measurements to better understand the relationships between richness and decay. The models provided estimates of species richness for comparisons of the naturally durable species. The regression models were evaluated for overdispersion, and did not appear to experience significant unexplained variation (Stroup 2013).

## 3. RESULTS

### 3.1 Bacteria

The bacterial data set for the first year revealed a total of 144 OTUs, with highest species richness in AYC (22.2) in Mississippi (MS) and HM (18.1) in Wisconsin (WI) and lowest in WRC (7.3) in MS and ERC (4.9) in WI. Statistical differences in mean species richness were complex with interactions between site and NDW species over years. Based on mean comparisons of species richness in MS in the first year, WRC was statistically lower than ERC, BL, SP and AYC. In WI in the first year, ERC was detected lower than HM and SP. NMDS ordination (Fig. 1) indicated some clustering of sites (MS versus WI), but no clear patterns with regard to NDW species. Wood species HM, WRC, CAT, and ERC were outliers based on their similarity, suggesting that the have a more variable species composition than the others over the course of the first year.

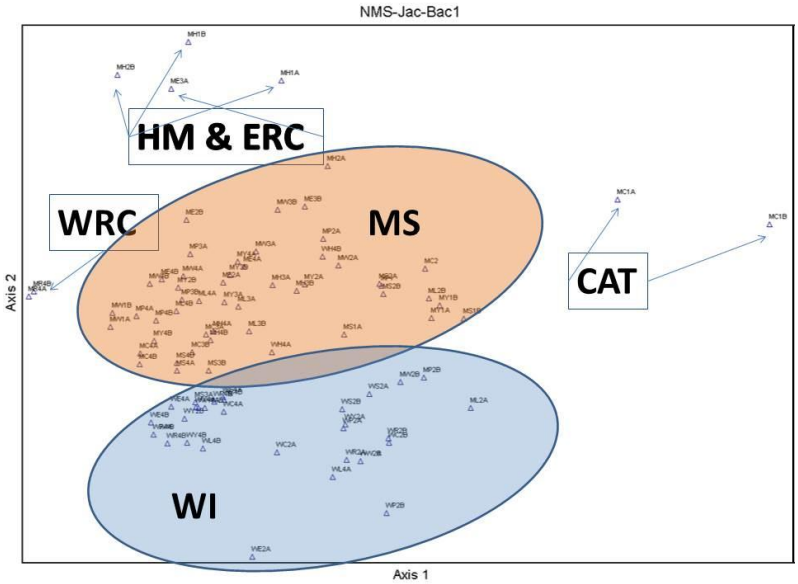


Figure 1: NMDS ordination of bacterial species at both sites (MS-WI) after one year exposure. Sites cluster separately with several noted outliers (HM, ERC, CAT, and WRC).

The second year data set contained a total of 187 OTUs, with highest richness found in BL in MS (28.3) and SP in WI (41.8), with the lowest richness found in WRC in MS (13.1) and ERC in WI (12.3). In MS, WRC was detectably lower in species richness than all other wood species, while in WI, SP tended to have higher species richness than the most other wood species. The

NMDS ordination (Fig. 2) indicated increased dissimilarity between sites. Also, at both sites SP was very dissimilar to the remaining NDW species. In WI, HM and WRC showed a substantial shift in species composition from one sampling to the next, suggesting more seasonal variation in the bacterial community than the remaining NDW species.

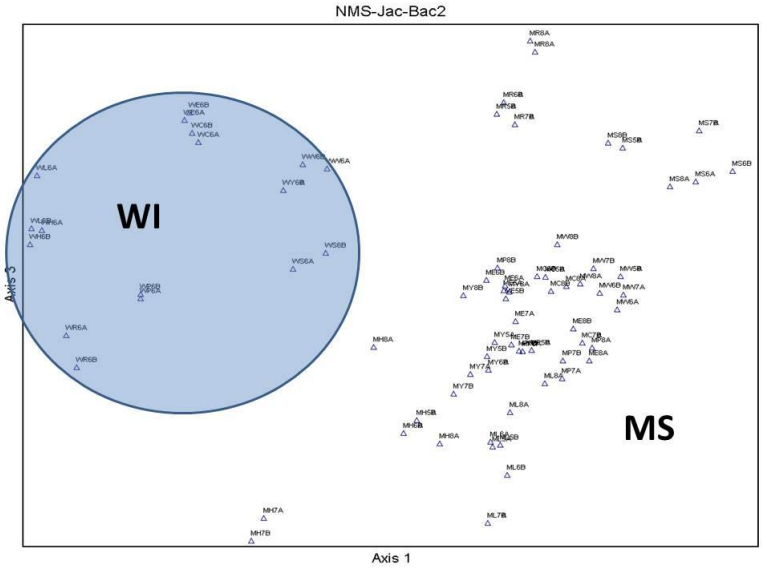


Figure 2: NMDS ordination of bacterial species at both sites (MS-WI) after two years exposure. Note increasing dissimilarity between sites (MS-WI).

The third year bacterial data contained a total of 290 OTUs, with highest richness in CAT in MS (41.3) and in SP in WI (110.9) and lowest in WRC in MS (23.4) and WI (24.1). In MS, species richness in WRC was lower than CAT, HM, and BL; while in WI WRC was lower than CAT, and SP was higher than all the other wood species. NMDS ordination indicated the same pattern of dissimilarity in the two sites (MS and WI). Within the sites, a more defined pattern of seasonal variation was noted, with time points clustering (10, 11, and 12 in Fig. 3).

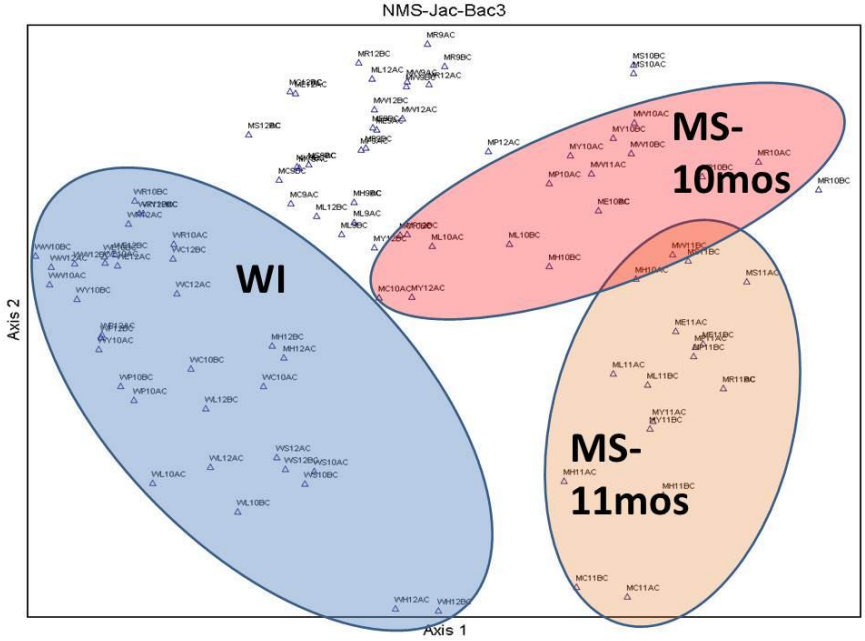


Figure 3: NMDS ordination of bacterial species composition at the two sites after 3 years exposure. Note increasing dissimilarity between sites and clustering of time points indicating seasonal patterns of colonization.

Overall, statistical models showed species richness to increase over the years, and detected interactions between species and time periods. Also, after adjusting for other predictors, as the decay index increased, species richness increased.

### 3.2 Fungal Data

Year one fungal data contained a total of 118 OTUs, with highest mean richness in SP in MS (25.0) and WI (25.1) and lowest in AYC in MS (18.1) and WCJ in WI (15.4). Although the statistical model indicated marginal differences in sites, the NMDS ordination (Fig. 4) indicated a difference between the two sites, as was noted in the bacterial data. There was some clustering of time points, suggesting seasonal shifts, but this was more pronounced in the MS samples. Samples of WRC, PAW, and HM were extreme outliers in WI and WCJ, SP, and PAW were outliers at the MS site.

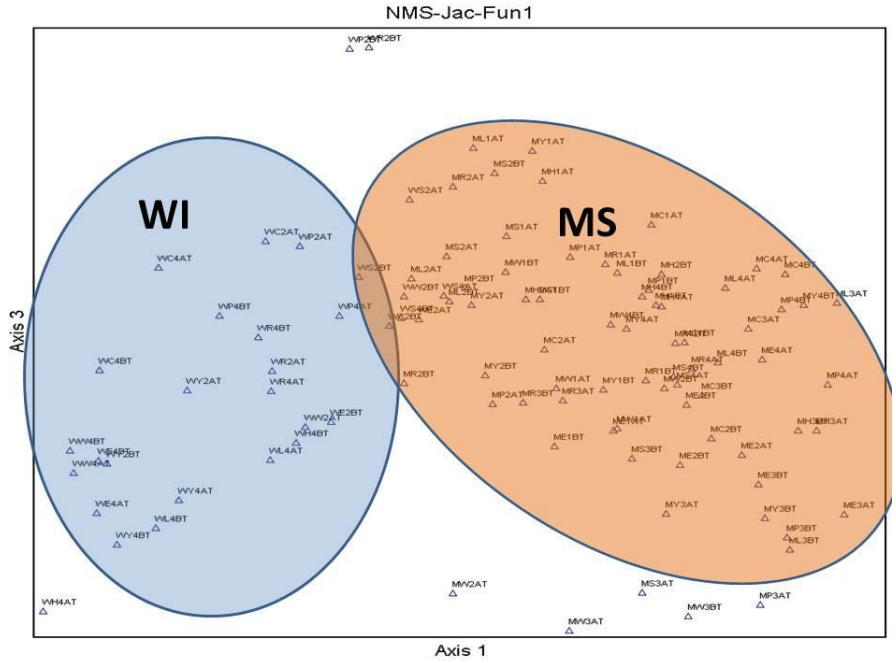


Figure 4: NMDS ordination of year one fungal data. Sites were different and some clustering of time points was noted.

The year two fungal data contained a total of 160 OTUs and indicated extreme dissimilarity between the two sites (Fig. 5), clustering at opposite ends of the ordination. Sampling point 8 in WI was not collected, so seasonal shifts cannot be shown for this data set in WI. Southern pine was an outlier in the WI samples, which had undergone significant decay at this point (rating =7.5). There was some clustering of time points in MS, but it was not consistent throughout. Southern pine in MS also clustered well outside of the other samples (rating=7).

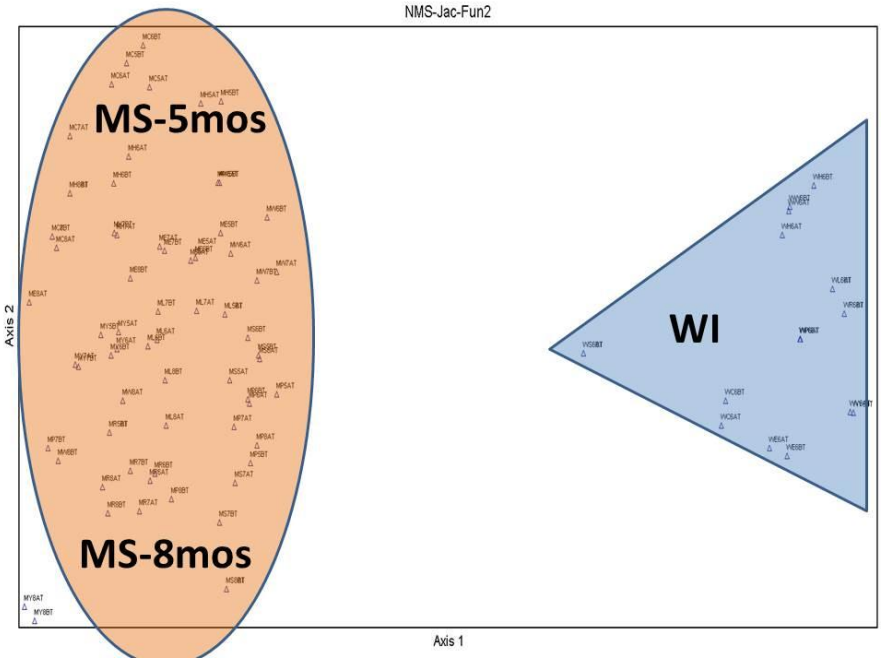


Figure 5: NMDS ordination of fungal species composition of the two sites after two years exposure. Sites are extremely dissimilar and some clustering of time points noted.

The three year fungal data (144 OTUs) showed sites as different clusters and within the sites there was clustering of time points again indicating seasonal fluctuations (Fig. 6). In WI, decayed SP was an outlier from all other samples and was found to be extremely dissimilar when compared with PAW, despite the fact that they are both considered to be non-durable. These patterns suggest that although SP and PAW are both considered non-durable, a different consortium of fungi are involved in their degradation. CAT was also an outlier in this data set, and had undergone moderate degradation at this point.

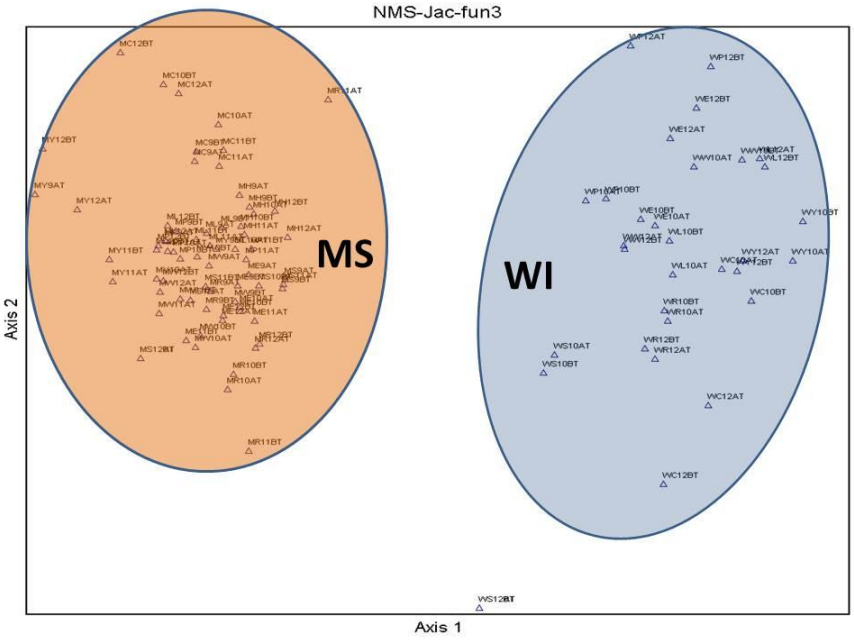


Figure 6: NMDS ordination of fungal species composition after 3 years exposure. Sites still different and time points cluster. Note extreme dissimilarity between PAW and SP (both non-durable) in the MS samples (top and bottom of right hand side).

The statistical models predicted increasing species richness over the years and with significant seasonal patterns within year. Sites were marginally different (P=0.0652) with MS having a slightly higher overall mean species richness (23.5 versus 21.0). If decay is included in the model, it explains some of the remaining random variation causing wood species in site differences to become significant in MS and marginally significant in WI. These differences tended to be primarily related to wood species SP.

### 3.3 Basidiomycete Data

The year one basidiomycete data contained a total of 235 OTUs. In the NMDS analysis, after one year exposure, the basidiomycete community was generally contained within a large cluster of points at the center of the plot (Fig. 7). WRC, SP and CAT were outliers in the WI samples. MS time points 3 and 4 were grouped within the large cluster in the center, but time points 1 and 2 clustered separate from the others, suggesting changing species composition between samplings 1-2 and 3-4.

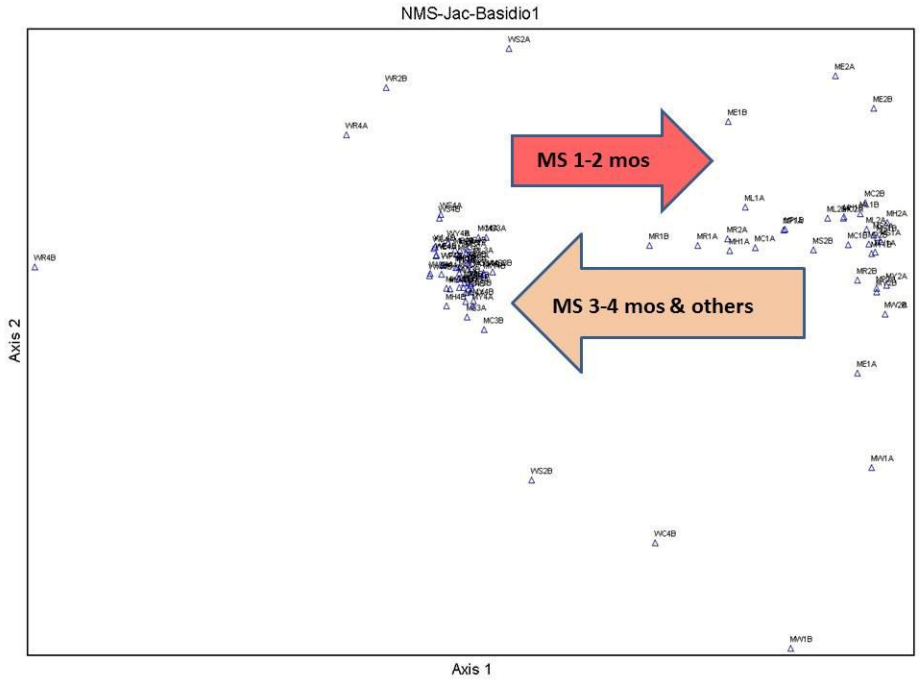


Figure 7: NMDS ordination of year one basidiomycete data; some clustering of time points and CAT, SP, and WRC are outliers.

The second year basidiomycete data (234 OTUs) showed a more pronounced difference between sites. WI clustered entirely in the upper left hand corner (Fig. 8). Time point 8 was not colleted and is missing from the ordination, so again it is not possible to see any seasonal patterns for WI. In the MS samples, time points cluster nicely indicating seasonal shifts of basidiomycete fungi. BL, HM and SP were outliers.

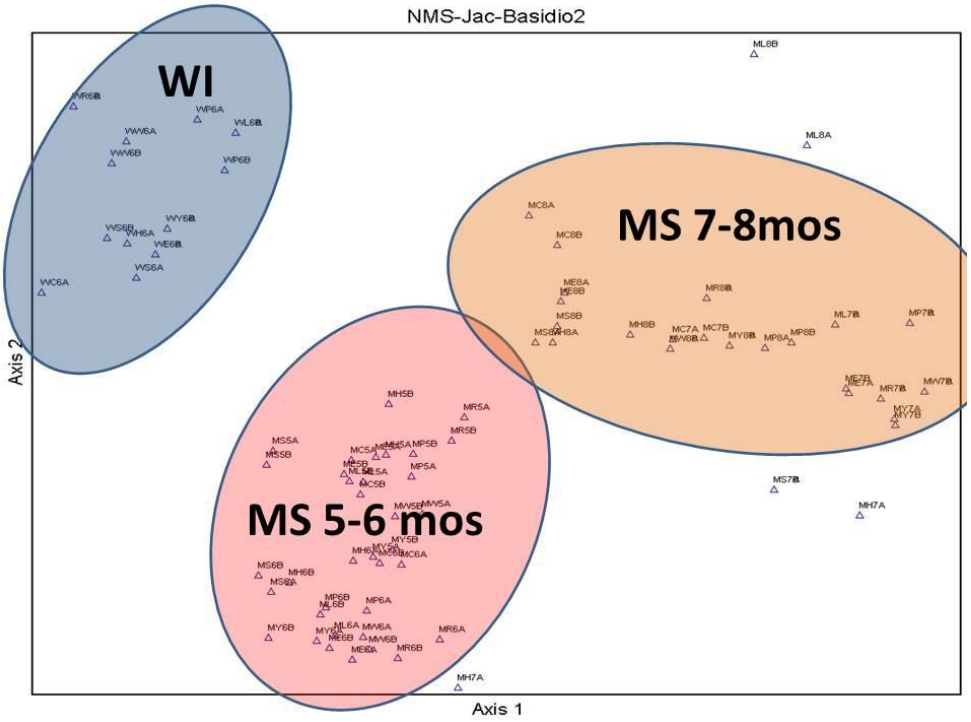


Figure 8: NMDS ordination of year 2 basidiomycete data. Clear differences between site and time points cluster together indicating seasonal patterns of colonization.

The three year basidiomycete data (221 OTUs) also shows clear differences between sites. In the MS samples, there was a pronounced clustering of time points that again denotes seasonal shifts in the basidiomycete community (Fig. 9). The 10 month samples from MS were clustered separately from all the others. This collection corresponds to summer months of the third year and reflects a clear seasonal shift in community composition in MS not seen in WI.

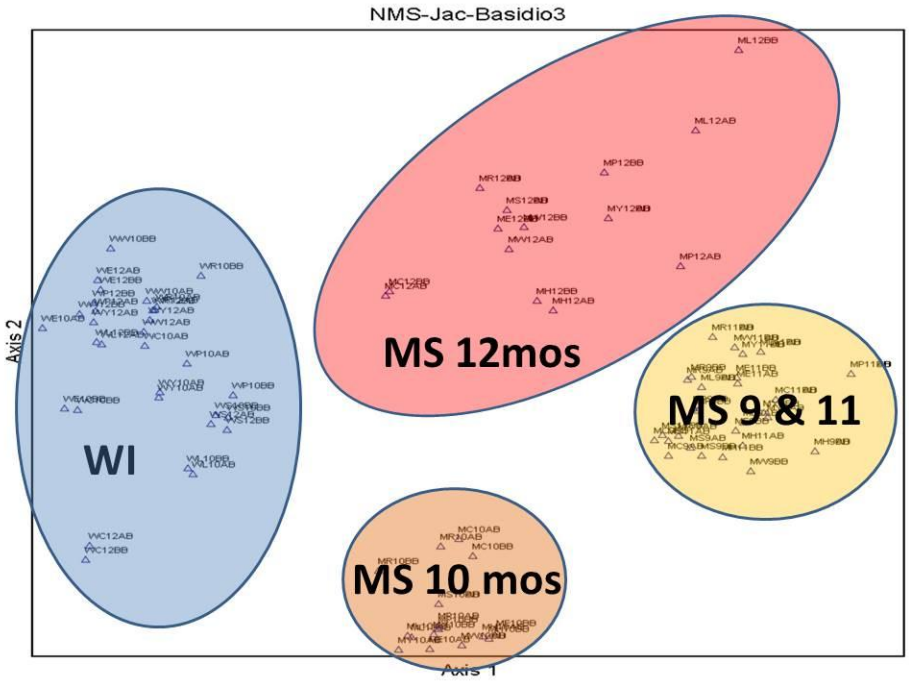


Figure 9: NMDS ordination of year 3 data. Sites are different and time points cluster. Time 10 (summer of year 3) clustering completely separate from all other data points.

In the statistical analysis of species richness for basidiomycetes, year, season and an adjustment to year for species and season appeared significant. Decay did not appear to explain any additional variation in species richness beyond the other predictors.

## 4. DISCUSSION

### 4.1 General Observations

The preliminary results of this study have given us some new insight into the process of evaluating wood in above ground exposure. Differences have been observed between the test sites, with higher microbial diversity at the site with higher decay hazard (i.e. Mississippi). Seasonal shifts were noted in the microbial populations, whereas temperate seasons (spring, fall) were more similar and centralized, with more differences in the winter and summer months. This suggests increased turnover in the microbial populations during extreme seasons.

Some differences were noted among the naturally durable species, but the species did not cluster as expected. Southern pine was noted as an outlier in most of the analyses, being drastically different from all other species in the studies, including PAW, which is now considered non-durable in above ground exposure. The statistical analysis using Shannon's Index (H) yielded very little information so it was decided to use species richness. Modelling species richness with Poisson regression models detected some differences between the naturally durable species; WRC, for example, tended to have significantly less bacterial species present overall while SP tended to have significantly more bacterial species present. Although the regression models indicated some relationship between species richness and decay condition, with the decay condition was significant for bacteria and fungi, but inconclusive for basidiomycete, the direction of the relationship was not expected. Further modelling of data that includes longer exposures will help us to better understand these results.

### 4.2 Bacteria

The bacterial community data indicated sites were different after the first year of exposure. Sites remained different over the three year period. By year three, clustering of time points was noted for each site, with time points 10, 11, and 12 clustering especially at the more extreme decay hazard site (MS). Statistical models tended to detect wood species differences earlier at MS, with WRC having reduced species richness in year one; both sites having differences in year two, with WRC being significantly lower in MS and SP significantly higher in WI; and WI having more differences in year three, again with SP significantly higher.

### 4.3 Fungi

The fungal communities showed a drastic difference in the two sites, becoming more different in the second and third year. The statistical models detected differences over years (species richness increasing with exposure) and seasonal patterns, but interestingly the statistical models were not able to detect the extreme differences found in the NMDS analysis. There could be several reasons for the discrepancy, including more random variability and specific interactions that were not tested.

### 4.4 Basidiomycetes

The basidiomycetes displayed a similar pattern to the fungal communities where populations at sites became more dissimilar over time and some clustering of time points was noted, indicating

seasonal or successional shifts in diversity. The pattern of increased dissimilarity between the two sites was not observed in the first year samples, but did appear in the second and third year of exposure. Several instances of seasonal fluctuations were noted in the second and third year and were most pronounced in the third year. The statistical models for species richness also indicated increasing counts over years with some differences primarily due to season. In these models, sites did not appear to be as much as a factor and wood species had a complex relationship with year and season.

## 5. CONCLUSIONS

The preliminary results obtained from this study represent our first steps in obtaining real-time data of microbial biodiversity as naturally durable wood is colonized and degraded in above ground exposure. The results indicate that the microbial assemblages of bacteria and fungi that attack wood in above ground exposure do differ between the two sites, with higher bacterial and fungal diversity at the more southern site (i.e. Saucier, MS). Seasonal shifts were noted in the fungal and bacterial populations, indicating that the populations are somewhat ephemeral and different groups of microbes colonize at different times of the year, presumably based on rainfall and climatic conditions. Microbial data have been obtained for the fourth year and are currently undergoing analysis. Field ratings will continue annually for up to 10 years and resultant data will be used to generate recommendations on the appropriate use of these wood species in above ground exposure.

Additional analyses are also being conducted on the progression of microbial species that occur during exposure to the environment over four years. Data from all four years of the study are being combined and analysed for differences in species composition over time and between treatments (species). We are also exploring more detailed analyses for total microbial characterization, most likely though next generation sequencing, in order to improve our resolution and focus on what microbes are actively present during the process rather than observing changes in patterns over time.

## 6. ACKNOWLEDGEMENTS

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