

Using Metagenomics to Quantify the Bacterial Diversity on Field Stakes Treated with Wood Preservative

Lakshmi Narayanan¹ - Juliet D. Tang² - Grant Kirker³ - Maryam K. Mohammadi-Aragh^{1*} - C. Elizabeth Stokes¹

¹Post-doctoral Associate, Graduate Student, Assistant Professor, Mississippi State University, Mississippi State, MS, USA *Corresponding author
lna45@msstate.edu, mkm276@msstate.edu, ces8@msstate.edu

²Research Forest Products Technologist, USDA Forest Service, Forest Products Laboratory, Starkville, MS, USA
juliet.d.tang@usda.gov

³Research Forest Products Technologist, USDA Forest Service Forest Products Laboratory, Madison, WI, USA
grant.kirker@usda.gov

Abstract

Wood is one of the primary construction materials used in the United States. However, it is susceptible to decay over time, leading to its reduced service life. Development of targeted wood protection methods requires detailed understanding of microbial wood-decay mechanisms. Fungi are the most destructive microorganisms to attack wood and much research effort has been directed towards understanding their mechanisms of decay. The role of bacteria in wood-decay is less studied. The objective of this research was to compare bacterial communities colonizing pressure-treated and untreated wooden field stakes using next-generation sequencing technology at two locations (Saucier, MS and Madison, WI). Micronized copper azole (MCA), copper azole type C (CA-C), chromated copper arsenate (CCA), and untreated (UN) wooden field stakes of size 3/4" H x 3/4" W x 16" L (19 mm x 19 mm x 40.6 cm) were installed at field test sites in Saucier, MS and Madison, WI. Stakes were collected at three different time points within a year of installation. DNA was extracted from sawdust drilled from the A-horizon (within 7.6 cm of the soil line) of the field stakes and then used for 16S metagenomics library preparation. Metagenomic libraries were sequenced using the Illumina MiSeq platform. Data analysis from the three-month collection period revealed sufficient sampling depth and high species diversity. Principal components analysis indicated that the bacterial communities colonizing the CA-C, MCA, and UN field stakes were distinctly different. In addition, the CCA stakes showed some stake-dependent similarities with some MCA stakes. These trends were observed at both field sites. Furthermore, PCA loadings plots showed that the species driving these differences were not the same across two sites. One minor exception was *Luteibacter rhizovicius*. The latter

species was an influencing factor driving the community similarity among some CCA and UN stakes (MS) or some CCA stakes and one MCA stake (MS).

Keywords: pressure-treated wood, wood preservative, metagenomics, bacterial community analysis

Introduction

Constructing buildings with wood has several advantages over alternative construction materials, such as increased environmental sustainability, reduced energy consumption for production, and ease of use. However, wood is biodegradable and approximately 10% of the lumber produced in the United States is used to replace decayed wood (Amburgey et al., 2005). Wood protection aims to lengthen the lifespan of wood products exposed to the environment. One example of wood protection is the impregnation of wood with preservatives that prevent or slow biological degradation. However, over time, microorganisms can decrease the efficacy of wood preservatives through colonization.

Although wood decay fungi are the primary microbiological agents that cause wood decay, bacteria also play a role in wood degradation (Johnston et al., 2016). The role of bacteria in wood decay is less understood than the role of fungi. Research has found that bacteria are early colonizers of wood in the decaying process. Bacteria likely initiate the decay process by increasing the permeability of the wood, hydrolyzing the waxes and pectin in bordered pits, and breaking down wood extractives and preservatives (Clausen, 1996). Bacterial degradation of wood can be of concern because some bacterial species are capable of removing copper, chromium, and arsenic from CCA-treated wood (Blanchette, 2000; Clausen, 2000). In addition, bacteria have been shown to interact with fungi in a decay environment and may house biochemical pathways that can be useful in bioremediation of wood wastes, processes typically carried out by wood decay fungi (Johnston et al., 2016). Therefore, the identification of bacteria involved in the wood decay process, recognizing the environmental conditions that promote their colonization, understanding the interplay among bacterial species, and identifying their mode of attack is critical for developing targeted, more efficient wood preservatives. As next-generation DNA sequencing technologies are becoming more available, it is becoming easier to characterize and compare bacterial species in environmental samples.

The objective of this research is to identify bacterial species that colonize preservative-treated wood that has been in soil contact using next-generation sequencing technologies. This information will provide the baseline data needed to understand how bacteria contribute to the decay of preservative-treated wood and ultimately provide crucial information on more selective strategies to inhibit wood biodegradation.

Materials and Methods

Wooden field stakes of size $\frac{3}{4}$ "H x $\frac{3}{4}$ "W x 18"L were treated with micronized copper azole (MCA), copper azole type C (CA-C), and chromated copper arsenate (CCA). MCA, CCA, and CA-C achieving retentions of 0.075 pcf, 0.2 pcf, and 0.075 pcf, respectively. These retentions

represent ½ of the standardized ground contact retention, and was used to facilitate sufficient bacterial colonization within the scheduled sampling periods. In addition, untreated wood stakes (UN) were included as a control. There were 10 replicates of each treatment (n=40) with seven replicates for bacterial analysis and three replicates for chemical analysis. Two sites were chosen to conduct the field stake test, one in Madison, WI and the other in Saucier, MS. Samples were collected three months after field stake installation at both sites. Samples collected at six and nine months will not be reported here. For sampling, the field stakes were removed from the ground and cleaned of excess soil, individually wrapped in sterilization bags, and transported back to the laboratory on ice. Next, ¼” spade bits were used to drill a series of 6-7 adjacent holes through each stake at the A-horizon, which had been marked approximated three inches from the lowest ground line mark. Sawdust was collected on clean paper sheets, transferred to a labelled sterile 50 mL Falcon tube, and frozen at -70°C prior to being freeze-dried. The dried sawdust was then stored at -70°C until DNA extraction.

Nucleic acids were extracted from each sample by weighing out freeze-dried sawdust (0.8g) and using the PowerSoil DNeasy kit (Qiagen, Germantown, MD). Unwanted RNA was removed by treating the extract with RNase A and then purifying the genomic DNA using the spin column clean up in the DNeasy Blood and Tissue kit (Qiagen, Germantown, MD). Quality of the DNA was determined by agarose gel electrophoresis in Tris-acetate EDTA buffer, pH 8.0, and DNA concentration was measured using a NanoDrop microvolume spectrophotometer (ThermoFisher Scientific, Waltham, MA).

DNA libraries were prepared using the Illumina 16S metagenomics library preparation guide (part # 15044223 Rev. B, Illumina, San Diego, CA). Two successive rounds of polymerase chain reaction (PCR) were performed. In the first round, primers contained an overhang adapter linked to a locus-specific sequence that targeted the hypervariable V3 and V4 regions of the 16S ribosomal RNA gene. In the second round PCR (limited-cycle), Illumina sequencing adapters and indices for multiplexing were included. After each round of PCR, the products were purified using AMPure XP beads (Beckman Coulter, Indianapolis, IN). The final products or libraries were quantified on a Qubit fluorometer (ThermoFisher Scientific, Waltham, MA) with a final electrophoresis quality check done on a 2100 BioAnalyzer system (Agilent Technologies, Santa Clara, CA). A single 20 nM poolplex was made of all libraries and given to the Institute for Genomics, Biocomputing, and Biotechnology at Mississippi State University for sequencing on a MiSeq system using the MiSeq Reagent Kit v3 (600-cycle) (Illumina, San Diego, CA). The returned sequence data was loaded into the 16S Metagenomics application in the Illumina BaseSpace data management system. Taxonomic classifications were made using the Illumina-curated version of the Greengenes 16S ribosomal RNA database.

Results and Discussion

For the 3-month sample collection, MiSeq analysis generated a total of 22,251,898 pass filter (PF) reads. At 3-months, the species richness or number of operational taxonomic units (OTUs) for Saucier, MS and Madison, WS were 1658 and 2184, respectively. Although the dominant phyla between the two sites were similar, their abundances were not (Fig. 1).

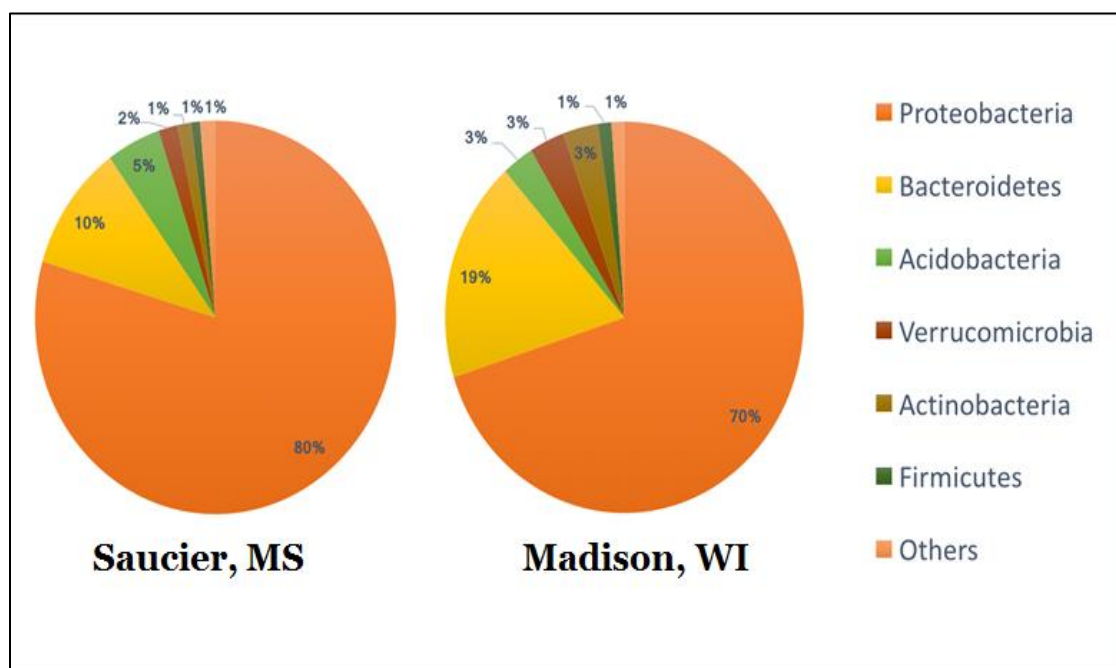


Figure 1: Bacterial phyla abundance at the 3-month field stake collection from Saucier, MS and Madison, WI.

Principle component analysis (PCA) of the bacterial species composition data from the 3-month field stake collection at Madison, WI is shown in Fig. 2. PCA is a statistical method that reduces complex data sets so that strong underlying patterns can emerge. In metagenomics profiling, this means reducing the information from each sample, which may be comprised of abundance data for possibly hundreds of species, down to a single value (per sample) that is plotted in the PCA graph. Spatial separation of two points equates to the degree of similarity between their bacterial communities. In Fig. 2, clustering of the CA-C data points (mislabelled as ACA) indicates that the bacterial species composition in sawdust taken from the CA-C treated field stakes was distinctly different from those communities found in the MCA, CCA, and UN field stakes. There was also a clear separation between the MCA and UN data points, indicating dissimilarity between these two communities. The CCA field stakes, on the other hand, showed some stake-dependent similarities with the MCA or UN stakes.

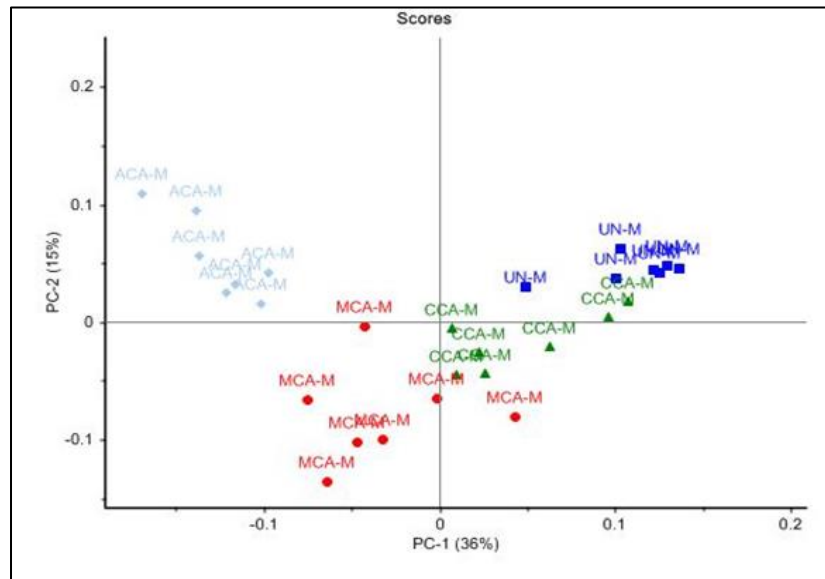


Figure 2: PCA of bacterial species found in the 3-month field stake collection from Madison, WI. Note: CA-C was mislabeled as ACA.

The PCA loadings plot in Fig. 3 illustrates the predominant bacterial species that were driving or influencing each of the principal component axes, PC-1 and PC-2 from Fig. 2. For example, *Sphingobacterium multivorum*, was contributing to the large negative values of PC-1 and large positive values of PC-2 (CA-C points in Fig. 2); *Luteibacter rhizovicius* was driving the large positive values of PC-1 (UN and some CCA points in Fig. 2); *Sphingomonas oligophenolica* and *Pedobacter kwangyangensis* were both strongly influencing the large negative values of PC-2 (MCA points in Fig. 2); while *Sphingobium amiense* was contributing to the moderate negative values of PC-1 (MCA points in Fig. 2) and *Variovorax paradoxus* was contributing to the moderate negative values of PC-1 and moderate positive values of PC-2 (CA-C points in Fig. 2). The angles formed by the vectors drawn from each of these species points back to the origin (0,0; vectors not drawn in Fig. 3) is also meaningful. The acute angle formed by vectors for *S. oligophenolica* and *P. kwangyangensis* indicates a positive correlation for the presence and abundance of these two species. Two vectors that form an angle close to 180° like that found for *L. rhizovicius* and *S. amiense* signifies a negative correlation. An angle of 90° means that the two species are not correlated, e.g. the vectors for *S. oligophenolica* and *V. paradoxus*.

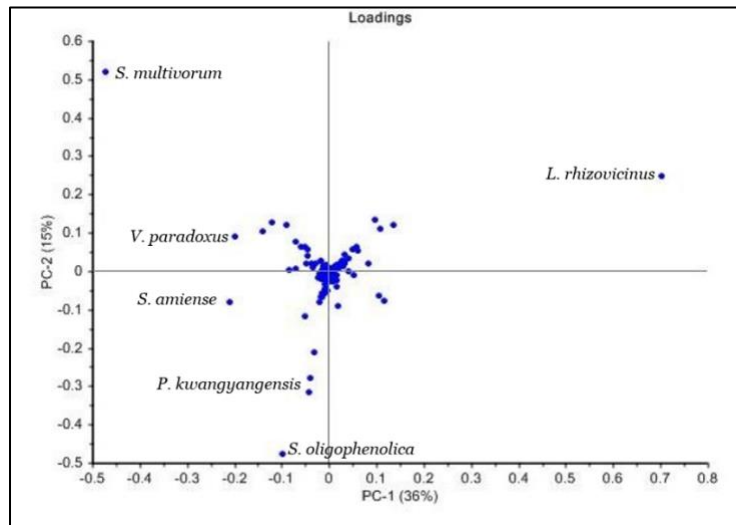


Figure 3: PCA loadings plot for the bacterial species detected in the 3-month field stake collection from Madison, WI. (*Sphingobacterium multivorum*, *Variovorax paradoxus*, *Luteibacter rhizovicius*, *Sphingobium amiense*, *Pedobacter kwangyangensis* and *Sphingomonas oligophenolica*)

The PCA graph of the bacterial species composition data from the 3-month field stake collection at Saucier, MS is shown in Fig. 4. Most of the trends observed were similar between the WI and MS sites. Specifically, like the WI data, the MS data showed that the species composition of the CA-C treated stakes (misabeled as ACA) was, for the most part, different from the bacterial communities identified from the MCA, CCA, and UN field stakes (Fig. 4). Like WI, MS data also showed a clear separation between the MCA and UN data points, indicating their dissimilarity. Another common theme was that some of the bacterial communities found on the CCA stakes resembled the MCA stakes. The only trend that was not shared between the two sites was that in MS there was clear spatial separation between the CCA and UN point clusters, an indication of dissimilarity between these two bacterial communities.

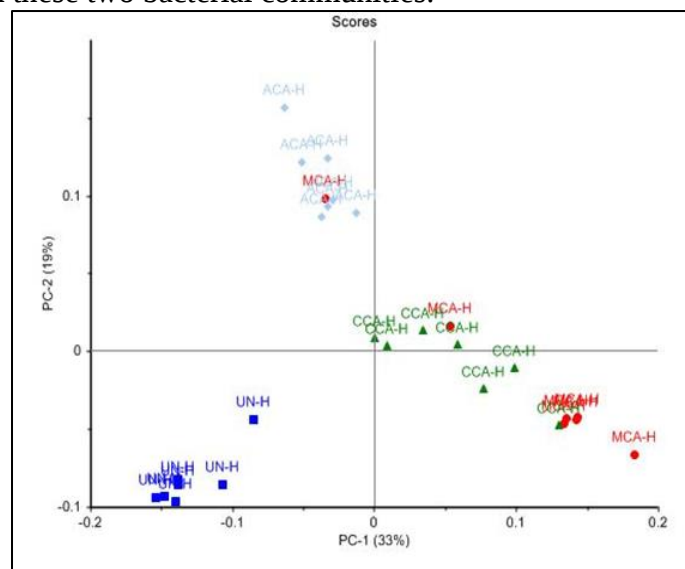


Figure 4: PCA of bacterial species found in the 3-month field stake collection from Saucier, MS. Note: CA-C was mislabeled as ACA.

The PCA loadings graph for the 3-month collection at Saucier, MS is plotted in Fig. 5. Interestingly, despite the shared trends between the two field sites mentioned above, the bacterial species driving the PCA loadings graph for MS (Fig. 5) were different from the species driving the PCA loadings graph for WI (Fig. 3). The one exception was *L. rhizovicinus*. *L. rhizovicinus* was the influencing factor driving the community similarity among some CCA and UN stakes (MS) or some CCA stakes and one MCA stake (MS). Together, these results suggest that there are factors other than copper, such as climate and geographical region, that determine the types of bacteria initially colonizing CA-C, MCA, and CCA treated wood.

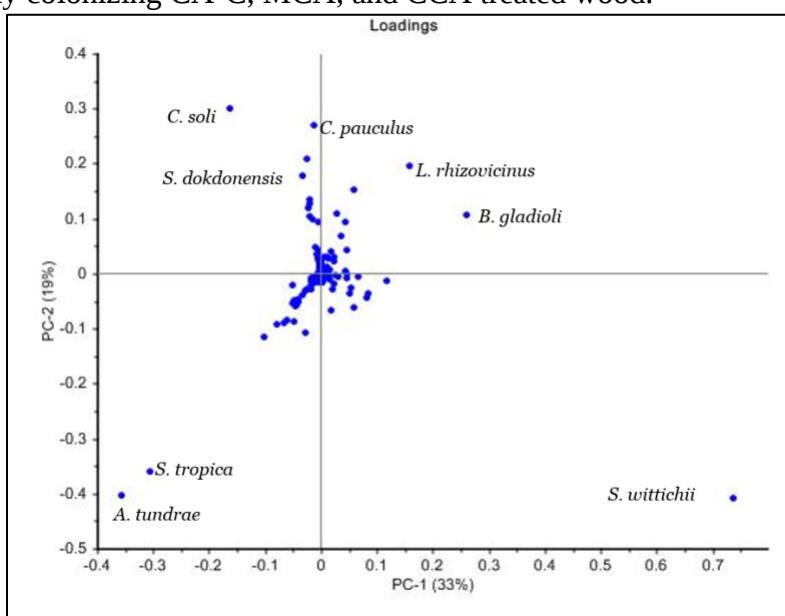


Figure 5: PCA loadings plot for the bacterial species detected in the 3-month field stake collection from Saucier, MS. (*Cellulomonas soli*, *Cupriavidus pauculus*, *Luteibacter rhizovicinus*, *Sphingomonas dokdonensis*, *Burkholderia gladioli*, *Salinispora tropica*, *Acidiosoma tundrae*, and *Sphingomonas wittichii*)

Summary and Conclusions

The procedures used in this work were able to reliably capture the bacteria present in preservative-treated and untreated wooden field stakes. The 3-month PCA plots showed that CA-C, MCA, and UN treatments all had distinctly different bacterial colonizers. Some CCA stakes also showed stake-dependent similarities with some MCA stakes. These trends were observed at both field sites (Madison, WI and Saucier, MS). Moreover, the PCA loadings plots showed that the species driving these differences were not the same across the two sites. Together, these results suggest that there are factors other than copper, such as climate and geographical region, that determine the types of bacteria initially colonizing CA-C, MCA, and CCA treated wood.

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