

The Usual Suspects: Fingerprinting Microbial Communities Involved in Decay of Treated Southern Yellow Pine

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ABSTRACT

Current standards for soil-block testing have long been based on the effectiveness of preservative systems against only a small number of wood decay fungi and even fewer bacteria. Culture-independent molecular methods offer simple, reproducible means to obtain a more holistic view of the microbial communities that colonize wood throughout the decay process. By using a culture-independent PCR-based method called terminal restriction fragment length polymorphism (T-RFLP) analysis, we were able to detect shifts of fungal and bacterial communities in wood treated with sub-lethal concentrations of ACQ-C and CTN. T-RFLP takes into account all species of a taxonomic group and creates a community profile or “fingerprint,” where each peak in the profile represents a unique species. Both compounds appeared to change the patterns of bacterial succession completely, so that beginning and ending communities were significantly different in regard to species composition. Fungal species community structure was initially changed, but became more similar to untreated controls over time, presumably as the preservatives were depleted from samples. Subsequent depletion analysis found >60% depletion of preservatives from treated field stakes after 15 months exposure. Further modification to this process will eventually enable us to accurately identify fungal and bacterial species making up the microbial communities found in treated and decaying wood and offer new insights into the decay process.

Keywords: ACQ-C, CTN, BHT, microbial community analysis, T-RFLP

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INTRODUCTION

Testing standards for evaluating wood preservatives in laboratory settings are largely based on performance of candidate preservative systems against a limited number of fungal isolates. With few exceptions, the industry has typically relied on *Gloeophyllum trabeum*, *Trametes versicolor*, and *Serpula lacrymans* for evaluation of wood preservative treatments. While these fungi are no doubt an integral part of the decay process and do contribute greatly to the durability of wood in service, it discounts the contributions of other groups of fungi that may be present in wood. Culture-independent molecular methods, (i.e., ARISA, T-RFLP, DGGE, and SSCP) can give accurate estimates of community diversity and richness in environmental samples and can also be used to identify fungi to species level (Blackwood et al. 2003). Each of the abovementioned methods has individual drawbacks and limitations; for example, T-RFLP can lead to underestimations of true diversity because of unseen peaks, and DGGE can often lead to poor resolution, making determination of individual species problematic. An increased understanding of the fungal communities that are present in decaying wood would provide more insight into the decay process

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and also identify key events in the process that might be prevented in order to slow the breakdown process. The resultant community profiles can also be used to make comparisons between the different Preservative systems as well as above and below ground exposures. The ultimate goal of this approach is to provide a more holistic approach to characterizing microorganisms that contribute to decay of wood in service. In this study, a molecular method called terminal restriction fragment length polymorphism analysis (T-RFLP) was used to monitor changes in species composition of bacteria and fungi in treated wood. T-RFLP analysis is used to make a community fingerprint, where peaks on a graph represent unique species of interest much like the ridges on a fingerprint. This procedure is often used to monitor changes in microbial communities when an environmental stress is introduced, and in this case we wanted to see what changes occur in bacterial and fungal species composition of early colonizers on treated southern yellow pine (SYP).

MATERIAL AND METHODS

Preservatives Evaluated

Alkaline Copper Quaternary, type C, commonly referred to as ACQ-C, was used as a standard in this study in order to evaluate the efficacy of chlorothalonil (CTN) and butylated hydroxytoluene (BHT) as wood protectants for wood in contact with soil. CTN is a broad spectrum fungicide with multiple modes of action (FRAC 2010). CTN, an approved wood preservative for control of mold and sapstain, is available in a variety of formulations, but it breaks down readily in soil exposure. BHT is an antioxidant and was evaluated as an additive to improve the longevity of CTN in soil contact. The retentions used in this study are much lower than what is typically used to treat SYP, but the lower treatments were necessary to allow microbes to colonize them in a short time frame (<3 years)

Specimen Preparation

Field stakes measuring 1.9 by 1.9 by 100.3 cm (t by r by l) were prepared from defect-free SYP sapwood. The stakes were divided into four treatment sets containing samples from the same boards. Each set contained matching untreated controls. Twenty-four field stakes—six matching sets of four—were treated using a full cell method (95 kPa for 30 min followed by 1,034 kPa pressure for 1 h) with 0.25% and 0.37% alkaline copper quat (ACQ-C), 0.1% and 0.25% chlorothalonil (CTN), 2% butylated hydroxytoluene (BHT), 0.1% and 0.25% CTN in combination with BHT, and controls were left untreated.

After treatment, the ACQ-C samples were bagged for seven days to allow fixation of the preservative and air dried until a constant weight was reached. All other samples were air dried immediately after treatment until a constant weight was reached. An 8.9-cm long sample was cut from the center section of each field stake for initial preservative retentions, leaving two matching 39-cm long field stakes. These matched field stakes were labeled with a specific number and a letter suffix identifying the location of the stake (A is Saucier and B is Dorman Lake).

Test Plots

Test plots were located in the Desoto National Forest in Saucier, Mississippi, and at Dorman Lake on Mississippi State University's John Starr Memorial Forest outside of Starkville, Mississippi. The Dorman Lake test site is designated a Zone 4 decay hazard zone by ASTM standards and the Saucier test is designated as Zone 5 decay hazard. Sites have a similar soil profile; Saucier is a sandy clay and Dorman Lake is a sandy clay but with a slightly more pronounced organic layer. Both of these sites have a long history of use by both Mississippi State University and the USDA Forest Service for testing wood preservatives and termiticides. The experimental design was a completely randomized block design. The stakes were randomized and installed using a gas-powered auger. The 39-cm long stakes were installed by inserting them vertically into the ground to a depth of 19.5 cm.

Sampling Methods

The SYP stakes were sampled every 90 days over a 15-month period. At each sampling interval, a matched set of stakes was removed from the ground and rated using ASTM E1-07 guidelines for both decay and termite damage. Stakes were returned to the laboratory and further processed for DNA analysis. A flame-sterilized wood rasp was used to extract 10 g of sawdust from random points along the above and

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below ground faces of the stakes. Sawdust samples were placed into a -80°F freezer awaiting DNA extraction. An additional 5-g sample was removed from the field stakes for preservative retention analysis.

Laboratory Methods

Sawdust samples were extracted for genomic DNA using Machery-Nagel Nucleospin plant DNA extraction kits (Machery-Nagel, Easton, Pennsylvania) according to manufacturer's specifications. The DNA extraction process basically uses a detergent to dissolve the cell walls and the DNA is collected on a membrane. The membrane is then cleaned by several centrifugal rinses with an ethanol solution and DNA is finally rinsed into solution with an elution buffer.

Isolation of target microbial DNA was done using polymerase chain reaction (PCR) amplification with fluorescently tagged generic primer sets for both fungi (ITS 1F-4NS) and bacteria (16S ForB-16s RevB), which would amplify all DNA from all fungal and bacterial species present in the samples. Additionally, a basidiomycete-specific primer set (ITS 1F-4BS) was used to only look at basidiomycete fungi present in the samples. Additional information on reagent volumes and thermal cycler parameters are presented elsewhere (Kirker 2008).

Amplified DNA was then subjected to digestion enzymes (TaqI for fungi and MspI for bacteria) that cut larger sections of DNA and created the restriction fragments for analysis. Digested DNA was cleaned to remove reagents from PCR and the enzyme digest using a Machery-Nagel Nucleospin® PCR clean-up kit and then loaded into a 96-well plate with size standard and loading buffer before being processed on a Beckman Coulter CEQ 8000XL (Beckman Coulter, Inc., Fullerton, California) capillary electrophoresis analyzer. This machine sizes the fragments as they migrate through a gel matrix and creates the peaks that make up the "community fingerprint." Post processing of T-RFLP cleans up the data by removal of miscalled peaks and manual proofreading of each data run. The final T-RFLP data was then exported as a text file for statistical analysis using SAS v9.2 (Cary, NC) and community analysis using PC-ORD® (MjM Software Design, Gleneden Beach, Oregon) and Estimates® (Storrs, Connecticut).

Analytical Methods

Basic statistical analysis was done in SAS v9.2 using PROC GLM. Time, exposure, location, and preservative were used as main effects at a 0.01 significance level. Additional analysis was necessary to observe the differences in microbial community composition. PC-ORD® uses binary vector data to compare samples based on their overall similarity. This procedure, called non-metric multidimensional scaling, was used to compare treatments, exposure, and time intervals and plots samples based on their similarity on a 2D plot. Samples more similar to each other will cluster closer and more dissimilar samples would cluster further apart. This procedure was also used to look at the progression of communities over time and determine if they became more or less similar over time.

RESULTS AND DISCUSSIONS

Total Microbial Detection

A total of 174 operational taxonomic units (OTUs), which is a representation of total number of species, were detected using the general bacterial primer set. Numbers of bacterial species seemed to fluctuate more in treated samples. Variability from sampling period was much higher in treatments than controls, which remained relatively steady throughout the sampling periods. Table 1 shows least-squares means of mean bacterial richness (number of species) for each treatment in above and below ground exposures.

A total of 92 individual fungal OTUs were detected using the non-specific fungal primers. The overall success rate in extracting T-RFLP was greater in stake with more decay present. In samples decayed more than 10% by ASTM ratings, fungi were detected in 100% of above ground samples and 85% of below ground samples. Detection levels were lower during periods of low precipitation, dropping to 70% success in above ground samples and 59% below ground. The overall trend that was seen in treated samples (with biocides) was that number of fungal species was lower initially and increased over time to become equal to untreated controls. It was concluded that these lower initial numbers are due to suppression of fungal growth by the biocides and fungi recovering once the biocides have depleted to more tolerable levels.

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A total of 53 individual basidiomycete OTUs were detected using the basidiomycete specific primer set. Overall success rate for basidiomycete detection was lower than detection for the general fungi. In sound stakes, basidiomycetes were present in 18% of samples, and 50% of samples decayed more than 10% had basidiomycetes present. The periods of low moisture had drastic impacts on basidiomycete detection at 9 and 12 months, with both controls and treatments showing drastic reductions in basidiomycetes present. Preservative treatment with ACQ-C caused a significant increase in the number of basidiomycete species present and CTN was also significantly higher than untreated controls. A drop in species richness was caused by drought conditions following hurricanes Katrina and Rita in 2005, during which almost no basidiomycetes were detected in any samples.

Bacterial Community Analysis

The bacterial community was significantly altered by preservative treatment; 2D ordinations of these communities based on species composition (Figure 1) shows that untreated controls were different from treated samples and remained different throughout the 15-month exposure. Stakes with only BHT were more similar to untreated controls than the biocide treatments.

Fungal Community Analysis

The fungal community analysis showed that preservative treatment with either CTN or ACQ-C changed the initial species composition of fungi inhabiting the wood stakes, but that these communities eventually changed and became more similar to untreated controls as preservatives were depleted. This also agrees with the general suppression of fungal growth that was observed from the analysis of fungal richness. A 2D ordination showing the progression of fungal communities over 15 months is presented in Figure 2.

Basidiomycete Community Analysis

There were no apparent patterns in the basidiomycete community analysis data. The progression of species was variable and no clustering of treatments was seen. Controls did cluster together to some degree, but outliers were present due to the lack of species present at 9 and 12 months. A 2D ordination showing the progression of basidiomycete communities over 15 months is presented in Figure 3.

Preservative Depletion

ACQ-C retention (0.25 and 0.37%) has not been quantified at this point, but will be presented at a later date. The 0.1% CTN was depleted by 100% after 15 months' soil exposure. The 0.25% CTN was depleted by 73% after 15 months' exposure. The 0.1% CTN with added BHT was depleted by 100% after 15 months exposure. The BHT depletion was the same (100%). The 0.25% CTN with the added BHT was depleted by 63% after 15 month's exposure and the BHT was depleted by 70%. When used alone, BHT was reduced by 67% over the 15-month exposure. The rates used in this study are much lower than standard rates of these compounds and were used in order to speed up the decay process.

CONCLUSIONS

Preservative treatment had noticeable effects on the species composition of both bacteria and fungi. The number of species of bacteria was initially greater in the treated wood, except for BHT. Total number of species did fluctuate over the course of the study and crashed during drought periods between 9 and 12 months, but mean number of species was higher in the treated samples. Mean number of species did increase again once normal rainfall patterns resumed. Bacterial species composition was significantly different in both ACQ-C and CTN treatments, and the communities remained different over time. BHT had no significant effects on bacterial, fungal, or basidiomycete community structure, which reinforces the claim of BHT being "environmentally benign." The general fungal communities were initially suppressed by the preservatives. Presumably the presence of the biocides is preventing colonization, but numbers of species did increase as preservatives were depleted. Subsequent treatment analysis showed >60% depletion of CTN over the course of the study. The co-added BHT did appear to decrease depletion of the CTN, but significant depletion was documented in the CTN+BHT treatments. The sub-lethal concentrations and high surface area of the small stakes no doubt played a role in the abnormally rapid depletion, other studies have

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found CTN+BHT to be very effective as wood preservatives (Schultz et al. 2006), and our results are not an effort to refute those claims. Overall, basidiomycetes made up the smallest percentage of micro-organisms that were detected in the wood stakes and these are similar to findings by Råberg et al. (2007), who found that basidiomycetes made up a small portion of fungi inhabiting above-ground exposed samples. The basidiomycete community was actually stimulated by the presence of biocides. It is hypothesized that the presence of the biocides is reducing the fitness of colonizing species and preventing them from dominating the resource. This could cause more available niches for colonization by less dominant species of basidiomycete fungi. This finding agrees with Tolijander et al. (2006), who found that disturbed environments often have much higher species richness compared with undisturbed environments. It is unknown whether this fragmenting of the microhabitat has any effect on rate of decay and represents an area in need of further investigation. Future research will begin to focus less on changes in community structure and more on identification of individual species within these profiles in order to determine what fungal and bacterial species subsist and sometimes prevail in treated wood.

ACKNOWLEDGEMENTS

The thanks several people without whom this research would have been impossible. Linda Sites at the FPL-MSU was responsible for preparation and treating of the SYP specimens. Juliet Tang at the MSU Life Science Biotechnology Institute (LSBI) offered time and expertise in troubleshooting molecular protocols in preparation of samples for T-RFLP analysis. The student workers in Dr. Diehl's lab were vital to the completion of this project; their hard work is greatly appreciated.

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Table 1—Bacterial species richness, Least-squares means of bacterial species richness (number of species) for each treatment by exposure combination at each sampling period^a

Treatment	Exposure	Time of exposure (months)					
		0	3	6	9	12	15
ACQ-Lo	Above	0	39*	14	33*	16*	25
ACQ-Hi	Above	0	28*	6	19*	7*	23
CTN-Lo	Above	0	11	14	21*	9*	25
CTN-Hi	Above	0	13	18*	16	8*	17
MIX-Lo	Above	0	12	30*	24*	9*	18
MIX-Hi	Above	0	9	7	12	9*	20
BHT	Above	0	7	17*	20*	10*	22
CON	Above	0	12	15	23*	12*	27
ACQ-Lo	Below	0	18	19*	28*	7*	25
ACQ-Hi	Below	0	21	18*	22*	5*	22
CTN-Lo	Below	0	13	13	12	15*	42*
CTN-Hi	Below	0	22	17*	30*	9*	17
MIX-Lo	Below	0	38*	18*	24*	13*	21
MIX-Hi	Below	0	21	16*	20*	13*	18
BHT	Below	0	27	15	26*	16*	31*
CON	Below	0	25	23*	28*	12*	21

^aNote fluctuation of richness values of treated samples compared to controls.

*Denotes significantly highest species richness value (or values) at each sampling interval using 0.01 significance level.

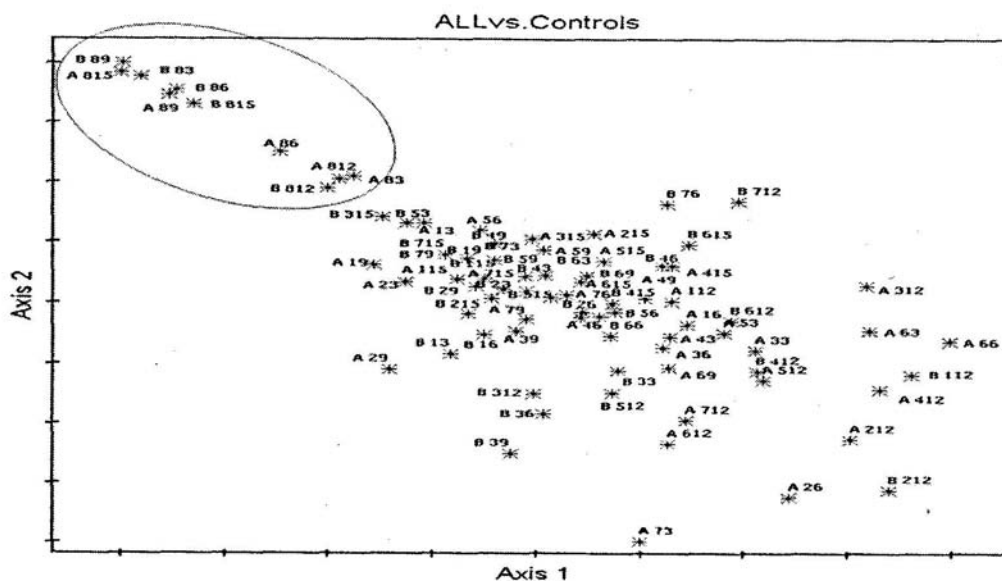


Figure 1. Comparisons of bacterial communities based on Jaccard distance represented in a 2D ordination plot. Samples are numerically coded: 1 is ACQ-C low, 2 is ACQ-C high, 3 is CTN low, 4 is CTN high, 5 is CTN low+BHT, 6 is CTN high+BHT, 7 is BHT alone, and 8 is untreated controls (A is above, B is below). Digits following the treatment indicate time of sampling (0, 3, 6, 9, 12, or 15 months). Clustered controls showing similarity are circled in plot in upper left hand corner.

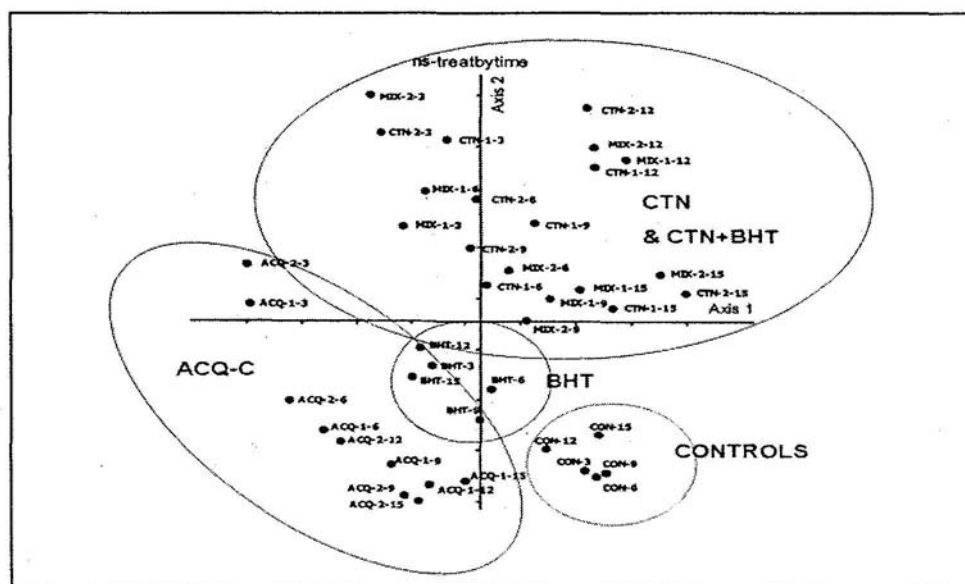


Figure 2. Comparisons of fungal communities based on Jaccard distance represented in a 2D ordination plot. Samples are coded the same as the previous figure (1a). Treated samples initially were different from untreated controls and BHT, but eventually became more similar as preservatives are depleted (note movement of clusters from 3- to 15-month samples).

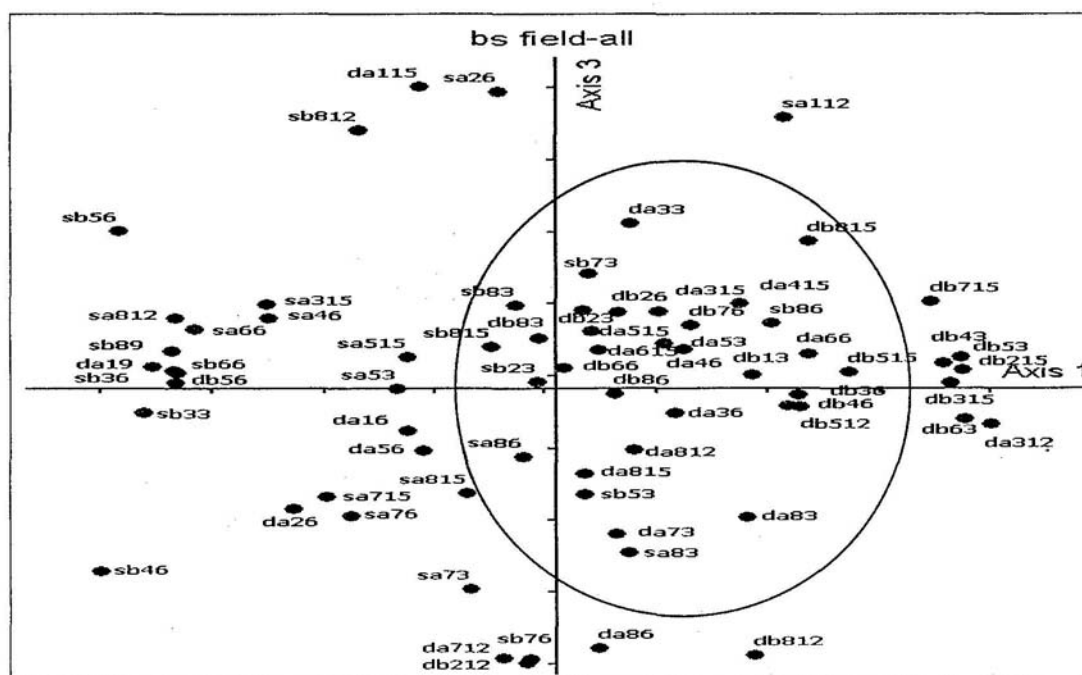


Figure 3. Comparisons of basidiomycete communities based on Jaccard distance represented in a 2D ordination plot. Samples are coded the same as the previous figure (1a). No patterns were found in basidiomycete succession or colonization. Untreated controls clustered somewhat (circled) with outliers found at 9 and 12 months.

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VOLUME 106

AMERICAN WOOD PROTECTION ASSOCIATION
P O BOX 361784 • BIRMINGHAM, ALABAMA 35236-1784 • USA

ISSN 0066-1198

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