Holzforschung, Vol. 66, pp. 529–535, 2012 • Copyright © by Walter de Gruyter • Berlin • Boston. DOI 10.1515/HF.2011.172

Community analysis of preservative-treated southern pine (*Pinus* spp.) using terminal restriction fragment length polymorphism (T-RFLP) analysis. Part 2: Bacteria field study

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Abstract

The effects of wood preservatives on the bacterial community in southern yellow pine were assessed by the molecular method 'terminal restriction fragment length polymorphism' (T-RFLP). Stakes, treated with 0.25% and 0.37% ammoniacal copper quat (ACQ-C), 0.1% and 0.25% chlorothalonil (CTN), 0.1% and 0.25% CTN with 2% butylated hydroxytoluene (BHT), and 2% BHT were installed with untreated controls in two field test sites in Mississippi and sampled every 90 days. Bacterial DNA was amplified by means of general 16S rDNA primers. Community data were analyzed to determine the effects of test site, exposure (above or below ground), treatment concentrations, and exposure time on the bacterial communities inhabiting the field stakes. Wood preservatives altered the bacterial communities, which fluctuated in numbers and composition over time. Initial exposure to CTN changed the pattern of species that colonized the stakes, and the bacterial communities did not become more similar to controls after CTN depletion. Bacterial communities on untreated controls were the most similar to each other and changed the least over time. Preservative treatment led to greater population turnover and increased diversity by creating a more unstable bacterial environment, which prevented these communities from reaching equilibrium. Although preservative treatment led to changes over time, there were still many shared species within and between the untreated controls and the different preservative treatments.

Keywords: ammoniacal copper quat (ACQ-C); bacteria community ecology; butylated hydroxytoluene (BHT); chlorothalonil (CTN); T-RFLP; wood colonization; wood decay.

Introduction

Bacteria are an integral part of the soil and litter ecosystems and play critical roles in nitrogen fixation and carbon and nitrogen cycling (Reed and Martiny 2007). The colonization of freshly sawn wood with bacteria is a matter of scientific papers (e.g. Mikluscak and Dawson-Andoh 2004). The survival of bacteria on wood is also important from the hygiene point of view (Milling et al. 2005) as well as the retention of *Bacillus cereus* and its toxin in cellulosic fibers (Hoornstra et al. 2006). Wood in ground test was shown to be more accessible to bacteria in the presence of soluble nutrients (Terziev and Nilsson 1999). When bacteria live on wood, they can increase wood permeability as well as, under certain environmental conditions, negatively impacting wood structure and strength. Many bacteria produce pectinases and cellulases that enlarge pits by breaking down the pit membranes and some bacteria can also affect the crystalline structure of cellulose (Schmidt et al. 1987; Schmidt and Liese 1994; Clausen 1996; Burnes et al. 2000; Gelbrich et al. 2008).

These activities increase wood permeability allowing water to penetrate further into the wood cell walls as well as allowing opportunistic fungi to gain greater access. When wood is exposed to very high moisture at low oxygen levels, bacteria may be responsible for the majority of the decomposition, for example of wet archaeological wood (Kim et al. 1996; Björdal et al. 1999; Blanchette 2000; Singh et al. 2003; Hoffmann et al. 2004). Erosion bacteria can attack the secondary cell wall layers depleting the cellulose and hemicelluloses but leaving behind a lignified residue. Tunneling bacteria produce small tunnels that weaken the middle lamella and to a limited extent degrade lignin. Bacteria on wood can also interact with other microorganisms synergistically to enhance wood deterioration or antagonistically produce antibiotics that are detrimental to other microorganisms (Greaves 1971; Payne et al. 2000). Colonization by bacteria can also lead to wood discoloration (Schmidt and Liese 1994), breakdown of wood extractives (Burnes et al. 2000) and breakdown of wood preservatives.

Bacteria also have an extraordinary ability to detoxify complex compounds as demonstrated in decades of bioremediation research to remove environmental pollutants (Alexander 1994). Bacteria can break down extractives in wood that contribute to its natural durability. Burnes et al. (2000) examined the impact of inoculation with cultures of *Pseudomonas fluorescens*, *Pseudomonas* spp., *Xanthomonas campestris* and *Serratia marescens* as they colonized yellow pine chips. Inoculation by these bacteria resulted in a 40% decrease in overall extractives compared to an 11% loss in

controls. Scanning electron micrographs indicated that the bacteria had colonized resin canals, ray parenchyma cells, and fiber tracheids. Tracheids with pit membranes had also been degraded after treatment with *P. fluorescens*.

In addition, bacteria can break down many organic wood preservatives. Numerous bacteria have been isolated that can degrade the first generation organic wood preservatives, such as creosote (Kanaly and Harayama 2000) and pentachlorophenol (McAllister et al. 1996), as well as current preservatives including propiconazole, chlorothalonil, IPBC (3-iodo-2-propynyl butylcarbamate), and quaternary amines (Wallace et al. 2008 and references therein). In many studies, the degradation of the wood preservative was by co-metabolism leading to incomplete degradation. Some bacteria can also leach the copper component of current preservatives from the wood, resulting in as high as 80% copper loss (Crawford and Clausen 1999). Once the preservatives are depleted by bacteria, opportunities exist for invasion by other microorganisms, most importantly decay fungi which might have otherwise been deterred.

Differentiation and identification of bacteria in environmental samples by means of traditional microbiological methods can be difficult and time consuming. Cultural isolation leads to large underestimations of true microbial diversity (Amann et al. 1995). Current and developing molecular methods offer quick, efficient, and effective methods for characterization and identification of bacterial communities in wood, soil, water, and numerous other environments (Smalla et al. 2007). Although a majority of treated lumber in use in the southeastern United States consists of southern yellow pine (SYP), very little is known of the actual bacterial community that colonizes and contributes to its decay. An increased understanding of bacteria through the process of colonization and subsequent decay is needed in order to understand the contributions of bacterial species to wood decay as well as preservative depletion. The objectives of this study are to determine changes in the bacterial communities over time in treated SYP field stakes at two different test sites in Mississippi using "terminal restriction fragment length polymorphism analysis" (T-RFLP), and to determine whether preservative depletion influences bacterial succession. This is the second of a two-part series. The first paper in the series details the fungal field results (Kirker et al. 2012).

Methods and materials

Field sites

The two field sites are described in Kirker et al. (2012) and Schultz et al. (2002). In brief, the Dorman Lake test site (D-site) is classified as a class 4 (high) decay hazard and the Saucier test site (S-site) is classified as a class 5 (severe) decay hazard by AWPA standards.

Preservative treatment

Field stakes, measuring 1.9×1.9×100.3 cm^3 (t×r×l), were cut from defect free SYP sapwood (*Pinus* spp.) and pressure-treated in a full-cell process with 0.25% and 0.37% ammoniacal copper quaternary compound (ACQ-C), 0.1% and 0.25% chlorothalonil (CTN) in toluene, 2% butylated hydroxytoluene (BHT) in toluene, and a combined treatment with 0.1% or 0.25% CTN plus 2% BHT in toluene. The treated stakes were cut into two smaller 1.9×1.9×39 cm^3 (t×r×l) samples (called a matched set), with an 8.9-cm long sample retained from the center-cut for initial preservative retention analysis. ACQ-C treated wood was selected as a positive control to compare the efficacy of the CTN and BHT treatments. CTN is a broad spectrum fungicide applied in the agricultural market and is also registered as a wood preservative. BHT is a common antioxidant in many commercially available consumer products and has shown synergistic effects in field studies against termites and wood decay when used in combination with CTN (Schultz and Nicholas 2002; Schultz et al. 2007). Twenty stakes for each treatment along with forty untreated controls were installed to a depth of 19.5 cm in the soil at each field site in a randomized complete block design. Each field site received samples from each matched set. In addition, four stakes of each treatment and controls were sampled as day 0.

Field stake sampling

Matched sets from each site were sampled every 90 days over a 15-month period. At sampling, four stakes were pulled for each treatment and control per site and visually evaluated for damage due to decay using AWPA Standard E7-01 ratings ranging from 10=sound to 0=destroyed (AWPA 2010). Excess soil was removed from the stakes prior to processing. Random sawdust samples were taken from each stake with a flame-sterilized wood rasp, and approximately 5 g of shavings were separately collected from the above- and below-ground portions of the stake. Sawdust was stored at -20°C prior to processing for microbial DNA. Field stake ratings were analyzed by SAS v9.2 (SAS Institute, Cary, NC, USA) based on the AWPA E7-01 Standard. The decay rating was the response variable to determine the effects of test site, exposure time, and treatment on the extent of decay (AWPA 2010).

DNA extraction, amplification and T-RFLP

Microbial genomic DNA was extracted from 50 mg of sawdust by means of a Machery-Nagel Nucleospin Plant Kit (Machery Nagel, Easton, PA, USA) according to the manufacturer's specifications. Overall DNA extraction efficiency was verified by a nanodrop spectrophotometer. Based on these data, it was possible to establish a lower detection limit for bacterial DNA of 2 ng μl^{-1}.

Bacterial DNA was selectively amplified with a 16S universal primer (Edwards et al. 1989) and all amplified DNA was visually verified on 2% agarose gels. Amplified DNA was digested with *Msp* I and the result was verified visually on 2.5% high resolution agarose gels. Restriction digested samples were purified using a Machery-Nagel PCR cleanup kit (Machery Nagel, Easton, PA, USA) following the manufacturer's specifications. Five µl of sample was analyzed on a Beckman Coulter GeXP Capillary Electrophoresis system (Beckman Coulter, Fullerton, CA, USA) to produce T-RFLP fragment data.

Preservative depletion analysis

After microbial DNA extraction, field stakes were sectioned and ground in a Wiley mill with a 10 mesh screen for chemical extraction of preservative remaining in the stakes. For CTN and BHT, approximately 1 g of milled wood was sonicated for 24 h in 8 ml of toluene and analyzed on an Agilent Series 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA). For ACQ-C, the four

replicates each from D-site above ground and below ground, four replicates each from S-site above ground and below ground were each pooled in order to obtain the 5 g of material needed for the analysis by AWPA Standard Method A9 and A18 (AWPA 2010). Only 0-, 9-, and 15-month samples were analyzed. Field samples were compared with unexposed samples to calculate %-loss of individual preservatives. Preservative depletion data for CTL and BHT were evaluated by means of PROC GLM and PROC CORR in SAS v9.2 for correlations among decay rating, fungal richness, diversity, and preservative depletion. Statistical significance was set at the 0.01 level, and Pearson's correlation coefficient was selected as a measure of co-relatedness. Because replicates were pooled for ACQ analysis, no statistical analysis was possible.

Data analysis

T-RFLP fragment data were visually screened 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 bacterial taxonomic units. α-diversity measures – i.e., species richness (total number of species), and two separate measures of diversity (Simpson's and Shannon's) – were calculated based on binary data with the software PC-ORD v5.0 (McCune and Grace 2002). α-diversity is the measure of diversity within a population (Begon et al. 1990). Richness and diversity data were further analyzed with SAS v9.2 to determine the effects of site, exposure, preservative treatment, and time on richness and diversity values. T-RFLP data were also summed across treatment time combinations to create abundance data. Abundance data were analyzed using non-metric multidimensional scaling to create ordination plots that present a two-dimensional plot of the bacterial communities within a given treatment at a given time. With help of these ordination plots it was possible to determine changes in microbial communities and increasing turnover (change in species composition) as a result of preservative treatment.

β-Diversity measures the similarity in species composition between two populations (Begon et al. 1990). The Chao abundance-based Jaccard distance (Chao et al. 2005) was selected as a measure for overall similarity between communities. Pair-wise comparisons of all treatment by time combinations were calculated and performed by means of the program Estimates® (Colwell 2005).

Results and discussion

Decay ratings

Sites were analyzed separately to eliminate multiple-factor interactions between sites, treatments, and times. Statistical analysis indicates that both treatment and time interacted to affect mean decay ratings at both D-site ($P<0.0001$) and S-site ($P<0.0001$). At the D-site, five treatments showed no decay (rating of 10) over the entire 15-month period, while 0.1% CTN alone, BHT 2.0% alone and untreated controls had mean decay ratings of 9.5 (SD=0.58), 9.0 (SD=0.0), and 7.4 (SD=1.48), respectively. Only untreated control ratings were significantly lower indicating 10–30% decay. At the S-site, the same five treatment groups retained a mean decay rating of 10 for the entire 15-month period, while 0.1% CTN alone, 2.0% BHT alone, and untreated controls had mean decay ratings of 8.25 (SD=0.5), 8.0 (SD=0.0), and 6.0 (SD=3.22), respectively. Once again, only untreated control ratings were significantly lower with 30–50% decay.

Species richness

A total of 16,597 peaks were detected by T-RFLP representing 174 unique bacterial operational taxonomic units (OTUs) or phylotypes (Figure 1). Greater numbers of bacteria were present at the S-site than at the D-site. There were greater numbers of bacteria present below ground than above ground at both field sites.

Bacterial richness and diversity were higher for the ACQ-treated samples at 3 months of exposure compared to untreated controls (Figure 2a). After 3 months, richness levels increased and then decreased following similar trends as the controls. Between 9 and 12 months, there was a sharp decrease in bacterial richness and diversity in all samples, treated and untreated, corresponding to a 6-month period of drought following two major hurricanes. Similar decreases at 9 and 12 months are also seen in the fungi and basidiomycete T-RFLP data (Kirker et al. 2012). Normal rainfall patterns resumed between 12 and 15 months, and bacterial populations increased at a similar rate in preservative-treated samples and untreated controls. These fluctuations resulting in species turnover were less in the below-ground samples compared to the above-ground samples and it is likely that below-ground conditions are less impacted by the climate. Obviously, soil buffers the below-ground wood from rapid moisture changes due to wind or extreme temperature fluctuations lessening the environmental stresses.

BHT treated samples had mean bacterial richness that was similar to untreated controls at all sampling periods, suggesting that BHT has little biological activity towards bacteria (Figure 2a). For CTN-treated samples, bacterial richness was lower than untreated controls at the 0.1% concentration, but increased when 2% BHT was added (Figure 2b). At the 0.25% concentration of CTN, bacterial richness remained similar to control in both numbers and trends (Figure 2b). The addition of BHT to 0.25 CTN decreased the richness and fluctuations between sampling times. Correlation analysis indicates that increasing bacterial richness and diversity are correlated with decreasing residual CTN at the 0.1% retention, meaning that the elevated number of bacterial species enforced proportionally the decrement of preservative concentrations.

Community analysis

The bacterial communities in treated samples were different from controls beginning at the early sampling periods,

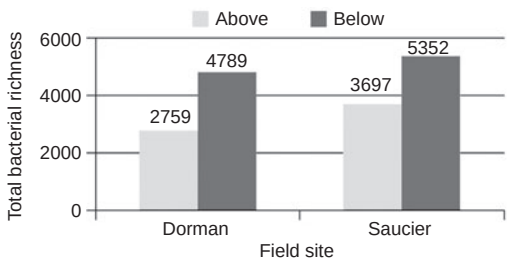

Figure 1 Cumulative bacterial richness at both field sites in above- and below-ground exposure at all sampling times.

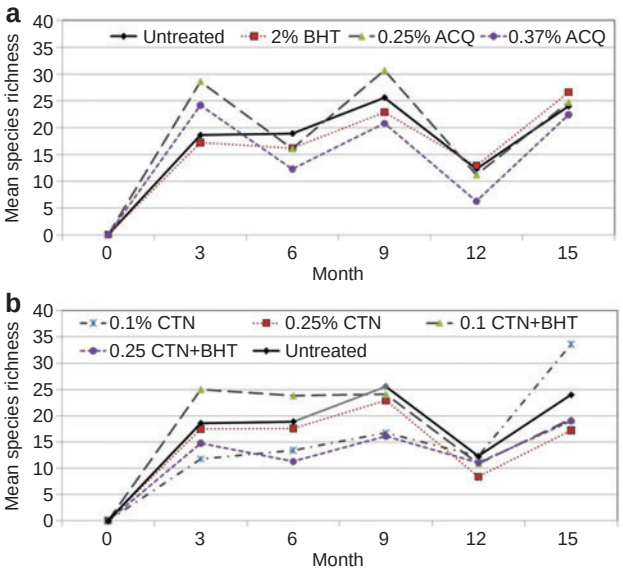

Figure 2 (a) Changes in bacterial species richness over time for ACQ-C treated field stakes and BHT compared to untreated controls. (b) Changes in bacterial species richness over time for chlorothalonil (CTN) treated field stakes compared to untreated controls.

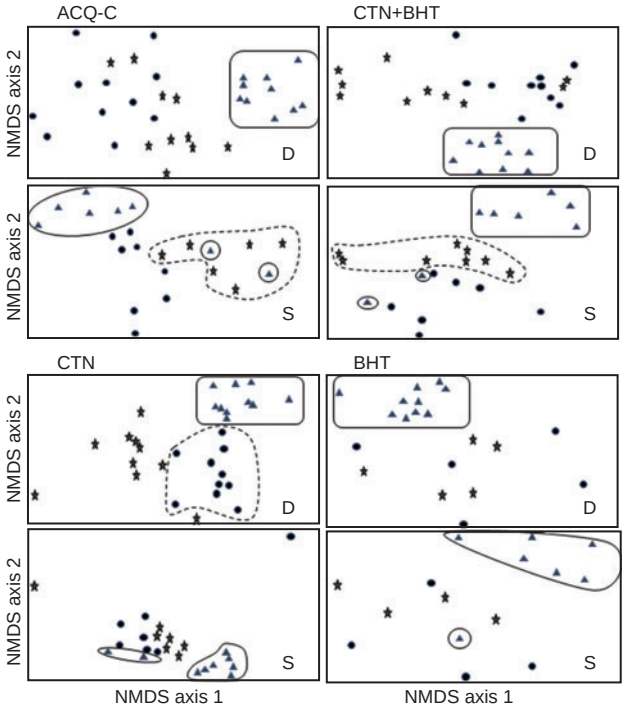

Figure 3 Non-metric multidimensional scaling ordination plots of bacterial communities separated by wood preservative treatment. The (D) Dorman field site is compared to the (S) Saucier field site for each treatment. Each point represents a given bacterial community and the distances between points are approximately proportional to the dissimilarity between communities. The symbols represent the same sample type for each plot with •=above ground samples, ★=below ground samples, and ▲=untreated controls. There was no separation between high and low preservative, when applicable, thus these were not separately identified. Clustering of controls (within the solid line) or above-ground treated samples (within the dotted line) indicates similarity. Ordinations are not to scale.

which caused a shift in the species composition that was present throughout the study. Treated samples remained different even after CTN was depleted indicating that the initial exposure changed the pattern of species that colonized the stakes, and even after the preservative was gone, the bacterial communities did not become more similar to controls. Figure 3 provides a comparison of similarities between bacterial communities from the D-site to the S-site for ACQ-C, CTN, CTN+BHT and BHT alone. Control samples were consistently clustered with a few exceptions, indicating that the microbial community in untreated samples contained shared species throughout the 15-month period (Figure 3). This indicates that there was much less species change over time in the control samples and that these communities always remained distinct from any of the treatments. Control samples from the D-site were more similar to each other than controls from the S-site as evidenced by a few control sample outliers found consistently at the S-site but not at D-site (Figure 3). The treatment, whose bacterial community was closest in similarity to the control communities, was the 2% BHT treatment. BHT is not a biocide, but its antioxidant properties did have some effect on bacterial communities. The ordination data also show that different preservative treatments resulted in different bacterial communities. In a study of Sigler and Turco (2002), it was shown that CTN alters the bacterial communities when applied to soil, although the effect varied with different bacterial populations: two populations were inhibited, four populations were enhanced, and two populations exhibited nonspecific responses.

In some comparisons, the above-ground samples clustered slightly separate from the below-ground samples, but the difference from each other was less than that from the controls. This can be seen for the ACQ-C treated samples from S-site, the CTN-treated samples from D-site, and the CTN+BHT-treated samples from S-site (Figure 3). No distinct separation of bacterial communities could be observed over time between low and high preservative treatments (0.25 ACQ-C vs. 0.37 or 0.1 CTN vs. 0.25 CTN). Greater turnover was found in treated samples, indicating that species composition changed rapidly over time compared to untreated controls. Pair-wise comparisons of all treatment by time combinations indicated that preservative-treated samples were clearly dissimilar to untreated controls during the earlier sampling periods (3–9 months exposure). The most dissimilar of these was 0.37% ACQ-C at 6 months which had the lowest numbers of shared species of all other pair-wise comparisons (data not shown). The bacterial communities on the 0.37% ACQ-C treatment were the most diverse of any treatment or control and had the highest turnover rate. Higher bacterial species richness has been found in disturbed environments (Kennedy and Smith 1995) and as the 0.37% ACQ treatment was likely the most effective preservative in this study, and its presence on the wood was the greatest disturbance. In many ways, the effects of wood preservatives on the bacterial communities

colonizing pine stakes are similar to the effects of keystone predators on the diversity of certain animal communities (Paine 1969).

Influence of preservatives on community profiles

Depletion data for 15-month observation of all treatments separated into above-ground and below-ground values for both field sites are provided in Kirker et al. (2012). There was little to no depletion of ACQ-C at either concentration over the 15-month study at either field site. ACQ did alter the composition of the bacterial communities and these differences remained throughout the study.

For 0.1% and 0.25% CTN treatment (Figure 4), PROC GLM indicates that site, exposure and time interacted to significantly affect mean preservative retention (P=0.0002) and (P=0.0015). Residual treatment from all site and exposure combinations decreased over time. In the below-ground 0.1% CTN samples, CTN was depleted 100% at D-site by 12 months, and depleted by 80% at 15 months at S-site. At the higher 0.25% treatment, below-ground samples at the D-site were depleted by 73%, compared to 33% at S-site at 15 months. Overall, there was less depletion in the above-ground samples, with 58% loss of CTN for the 0.1% samples in D-site above-ground exposure, but 100% in S-site above-ground. For the 0.25% treatment, samples at D-site above-ground exhibited depletion of 39% compared to 28% at S-site. The addition of BHT did not appear to reduce the depletion of CTN at the 0.1% treatment in below-ground applications regardless of site (Figure 4). It did reduce depletion of CTN in above-ground applications, with only 38% depletion at D-site (compared to 58% without BHT) and 22% at S-site (compared to 100% without BHT). When BHT was applied in combination with 0.25% CTN, there was much lower depletion of the CTN for above- and below-ground samples at D-site but not at S-site. This indicates that the antioxidant properties of BHT may hinder some of the redox mechanisms that are contributing to CTN depletion in above-ground applications. Additional discussion and a proposed mechanism of the synergistic effects of CTN and BHT are presented in Schultz et al. (2005). BHT was completely degraded (0 ppm) in field samples by 12 months.

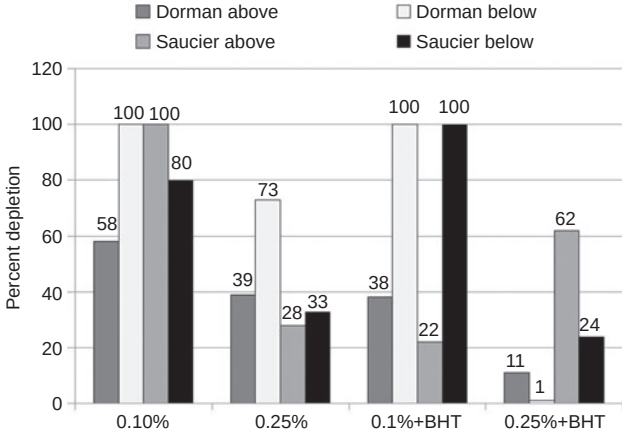

Figure 4 Percent depletion of chlorothalonil over 15 months for the 0.1%, 0.25%, 0.1%+BHT, and 0.25%+BHT treatments. Data are separated into above-ground and below-ground samples from both field sites.

In specimens treated with 0.1% CTN, bacterial diversity indices (both Simpson's and Shannon's) were negatively correlated with preservative retention (-0.35 P=0.0007 and -0.39 P=0.0002) such that higher diversity occurred with more CTN depletion. Similar results were found for 0.25% CTN+BHT (-0.35 P=0.001 and -0.43 P<0.001). No other correlations were found between bacterial diversity and CTN. However, negative correlations were found between BHT retentions and bacterial richness, and Shannon's and Simpson's diversity indices (-0.30 P=0.0052, -0.37 P=0.0004 and 0.40 P=0.0001), again indicating that higher richness and diversity occurred with more BHT depletion. In addition, decay ratings were positively correlated to BHT depletion (0.38 P=0.0004) and preservative retention was positively correlated to BHT concentrations (0.22 P<0.0001). This indicates that the addition of BHT reduced both the decay and the depletion of CTN.

Conclusion and outlook

Bacterial richness and diversity as measures indicate α-diversity, which compares differences within a community. The same samples that were analyzed in this study were submitted to a companion study concerning the changes in the fungal community over time and treatments (Kirker et al. 2012). The α-diversity indices in the quoted study indicate that for fungi, richness and diversity steadily increased between 3 and 9 months, crashing at 12 months due to a drought and recovering at 15 months. In the present study, however, bacteria richness and diversity fluctuated over the time course but only a small non-significant increase, if any, was seen between 3 and 9 months. Fluctuations were less within bacterial populations on the untreated control samples compared to treated samples. But in all cases, preservative treatment altered the bacteria communities. Interestingly, differences in bacterial communities between the different treatments and untreated controls remained throughout the study, unlike what was observed for fungi. Preservative treatment altered the fungal communities initially, but these communities became more similar to the controls as time progressed (Kirker et al. 2012). Bacterial richness was negatively correlated with decay ratings, but it could not be determined whether the increased richness caused more decay or, more likely, led to more preservative breakdown to make the wood more susceptible to decay fungi. There were only two statistically significant correlations to support this supposition, namely those with bacterial richness and breakdown of the 0.1% CTN treatment and the 2% BHT treatment when used in combination with CTN.

β-Diversity facilitates a comparison of differences between groups or populations and was utilized in the present study to compare turnover rates. Non-metric multidimensional scaling (NMDS) ordinations plot the "species space" where

more similar species cluster together and more dissimilar species are mapped further apart. Preservative treatment led to greater population turnover and increased diversity by creating a more unsuitable environment for colonization, which in turn prevented populations from reaching an equilibrium community structure. This is in contrast to the fungal analysis results. For fungi, preservatives did increase species richness and diversity as well as species turnover; however, by the end of the study, the fungal communities were becoming stable as well as more similar to the untreated controls (Kirker et al. 2012).

The Chao-Jaccard analysis also estimates the number of shared species. Although emphasis has been placed on the differences observed between communities, based on these analyses only 11% of the 3160 pair-wise comparisons were <70% similar. Once again, the greatest differences were found in the ACQ-C-treated samples. Although preservative treatments led to changes in species composition within communities and influenced population changes over time, there were still many shared species within and between untreated controls and the different preservative treatments. It is also interesting to note that there were little to no differences between levels of preservative treatment (0.1% CTN compared to 0.25% CTN or 0.25% ACQ compared to 0.37% ACQ) in terms of community similarities, and there was only a moderate level of dissimilarity between above-ground and below-ground bacterial communities within a given treatment.

Based on the gathered community data, it is now possible to focus on assemblages of bacteria that were correlated with either high rates of decay or high rates of preservative depletion with the help of T-RFLP and additional molecular analytical methods. Additional studies will be required to focus on the bacteria present in samples with high rates of CTN depletion to identify possible species linked to biocide depletion. Future research will also begin to focus on common species found in this study and shift our focus to identification and function of species. It is acknowledged that the preservative treatments used in this study are lower than recommended rates for these preservatives. This was done intentionally to shorten the time needed for decay to develop. Additional research will be needed to examine what effects bacteria have on treated stakes when exposed for longer periods of time.

Acknowledgements

The authors would like to acknowledge the assistance of Linda Sites, Katie Jenkins, Justin McDonald, and Yolunda Nabors. Funding was provided by the Wood Utilization Research program and the State of Mississippi. This article has been approved for publication as Journal Article FP612 of the Forest and Wildlife Research Center, Mississippi State University.

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Received May 13, 2011. Accepted October 19, 2011.
Previously published online December 13, 2011.