

Community analysis of preservative-treated southern pine (*Pinus* spp.) using terminal restriction fragment length polymorphism (T-RFLP) analysis. Part 1: Fungal field study

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Abstract

The effects of chlorothalonil (CTN), butylated hydroxytoluene (BHT), and ammoniacal copper quat (ACQ-C) on the fungal community on southern yellow pine (SYP) were assessed using terminal restriction fragment length polymorphism (T-RFLP) analysis over 15 months. Field stakes, treated with 0.25 and 0.37% ACQ-C, 0.1 and 0.25% CTN, 2% BHT alone, 0.1 and 0.25% CTN combined with 2% BHT, and untreated controls, were installed in two field sites in Mississippi. Stakes were sampled at 90-day intervals and rated for decay damage. Fungal DNA was extracted and amplified by non-specific (total fungi) and specific (Basidiomycete) primers and processes for T-RFLP. α -Diversity (richness and diversity) and β -diversity (similarity between communities) were calculated by means of T-RFLP data. The presence of wood preservatives slowed the initial colonization of field stakes by total fungi, resulting in lower richness and diversity that increased over time; however, preservatives increased the richness and diversity of Basidiomycetes. The β -diversity of treated samples was less similar in the early stages of exposure (3–9 months), but coalesced over time into equilibrium communities that were similar to communities on untreated controls. Basidiomycete species compositions were different among treated samples while control communities shared more than 75% of their species. Correlations were found between depletion of 0.1% CTN and increasing fungal diversity, but no other significant correlations were found.

Keywords: fungal community ecology; T-RFLP; wood colonization; wood decay.

Introduction

Performance of wood preservatives in field exposure is the industry standard by which candidate preservative treatments are evaluated. While extensive work has studied the

effects of decay fungi on treated wood, little is known about the interacting microbial community that contributes to the decay of wood and degradation of organic biocides, and what alterations occur in the community due to wood preservative treatment.

Past research has indicated a step-wise progression of microbes colonizing wood once it is exposed to the environment. Bacteria are the first to inhabit the wood, gaining access through exposed ray parenchyma and pit membranes in the wood (Clubbe 1980; Daniel 2003). The bacteria produce extracellular enzymes that enlarge pits by breaking down waxes and pectins in the pit aperture, which enables larger opportunistic fungi, such as molds, to gain access (Greaves 1971). Generalist fungi, such as stains and molds, feed on simple sugars located on and in wood fibers. Although many molds have been isolated that produce enzymes capable of breaking down cellulose (Lynd et al. 2002) and hemicelluloses (Biely and Tenkan 1998), these fungi are not major contributors to the decay process. Soft rot fungi are common inhabitants of wood under certain environmental conditions and produce pits within the S² layer that can severely impact the dimensional stability of wood (Daniel 2003). Wood decay fungi, mostly Basidiomycetes, are thought to colonize later, and past studies have shown that these fungi only colonize after primary invaders begin to decline (Boddy 2000). Decay fungi are specialists and compete for space but not food source with primary invaders. In addition to producing decay enzymes that break down lignin and polysaccharides, Basidiomycetes produce chitinolytic enzymes that break down hyphal remnants of primary invaders as a way of obtaining additional nutrients, especially nitrogen, in a nutrient limited substrate (Lindahl and Finlay 2005).

Molecular methods, which rely on selective amplification of DNA by polymerase chain reaction (PCR), have become popular due to their ease and rapid throughput capabilities. PCR-based screening methods provide an effective means to analyze microbial diversity of environmental samples that gives a more accurate representation of the actual community than cultural methods alone. Indeed, it is estimated that as little as 5% of the actual microbial community can be cultivated onto artificial media (Amann et al. 1995). Primer sets have been developed that allow for selective amplification of a wide range of organisms, from phylum- to species-level specificity. These primers involve regions of DNA that exhibit some level of similarity, but also contain enough variation to make comparisons among closely related members of the representative taxonomic group. The development of real time PCR has allowed for not only detecting but also quantifying

fungus species in wood (Pilgård et al. 2010, 2011). Restriction fragment length polymorphism (RFLP) analysis is a DNA-based fingerprinting technology that uses specific primers coupled with restriction enzymes that cut DNA at particular sites to yield fragments that are characteristic of a given species, and these fragments are helpful to identify species (Jasalovich et al. 2000; Råberg et al. 2005). A variation of this technique is terminal RFLP (T-RFLP) analysis that employs fluorescently-labeled primers, with the terminal fragment of each species detected with a capillary detection system. The result is a DNA species fingerprint of a specified taxonomic group within a sample. An excellent review of the history and utility of T-RFLP is presented by Thies (2007). T-RFLP has been used as an identification tool for fungi in above-ground and ground-contact exposed wood (Råberg et al. 2007) to determine seasonal shifts in community structure at the interface of CCA-treated and untreated wood (Noll and Stephan 2010) and determine shifts in bacterial and fungal communities in stored wood chip piles (Noll et al. 2010).

The objectives of this study were to detect changes in fungal species richness and diversity, including both total fungi and Basidiomycetes, in treated southern yellow pine (SYP) field stakes exposed at two field sites for 15 months, by T-RFLP and to correlate these changes with advancing decay and preservative depletion. Changes in the bacterial community were also analyzed by T-RFLP and are reported in Part 2 of this study (Kirker et al. 2012).

Materials and methods

Field sites

The Dorman Lake test site (D-site) is located in central MS at 33.341°N and 88.876°W and is classified as a class 4 (high) decay hazard rating by the American Wood Protection Association (AWPA 2010) decay hazard rating system. Annual rainfall is 142 cm per year and the soil is a Faulkner silty clay loam (Aquic Paleudalf). The Saucier test site (S-site) is located in southeastern MS at 30.631°N and 89.051°W and is classified a class 5 (severe) decay hazard. Annual rainfall is 172 cm per year and the soil is a McLaurin loamy sand (Typic Paleudult) (Schultz et al. 2002).

Preservative treatment

Field stakes, measuring 1.9×1.9×100.3 cm³ (txr×l), were cut from defect free southern yellow pine (SYP) sapwood (*Pinus* spp.) and pressure-treated by 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 of 0.1 or 0.25% CTN with 2% BHT in toluene. The stakes were cut into two smaller 1.9×1.9×39 cm³ (txr×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 served as a positive control in order 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 an antioxidant in many commercially available consumer products and has shown synergistic effects in recent field studies against termites and wood decay when combined with CTN (Schultz et al. 2007). Twenty stakes for each treatment

along with 40 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 one of a matched set. In addition, four stakes of each treatment and controls were sampled on 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 as per 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 Standard E7-01. The decay rating was used as the response variable to determine the effects of site, time, and treatment on decay extent (AWPA 2010).

DNA extraction, amplification and T-RFLP

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

Total fungal DNA was amplified by the universal ITS-1F-4NS primer set (White et al. 1990), while Basidiomycetes were selectively amplified by the ITS 1F-4BS primer set, which uses a different reverse primer that amplifies a region further downstream that is conserved within the Phylum Basidiomycota (Gardes and Bruns 1993). All amplified DNA was visually verified on 2% agarose gels.

Amplified DNA was digested by *Taq* I for total fungi and Basidiomycetes and verified visually on high resolution (2.5%) agarose gels with a 100 bp ladder to obtain sizes of restriction digest fragments. Restriction-digested samples were purified with a PCR cleanup kit (Machery Nagel, Easton, PA, USA) following specifications of the manufacturer. 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 with a Wiley mill with a 10 mesh screen for chemical extraction of preservatives remaining in the stakes. For CTN and BHT, approximately 1 g of milled wood was sonicated for 24 h in 8 ml toluene and analyzed on an Agilent Series 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA). For ACQ-C, the four replicates from D-site above ground, four replicates from D-site below ground, four replicates from S-site above ground and four replicates from S-site 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 percent loss of individual preservatives. Preservative depletion data for CTL and BHT were analyzed by PROC GLM and PROC CORR in SAS v9.2 to determine possible 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 the 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 value and aberrant peaks, and remaining data were exported to create a data matrix of binary data corresponding to presence or absence of detectable fungal taxonomic unit. These binary data were the basis for calculating α -diversity measures: species richness (total number of species), and two separate measures of diversity (Simpson's and Shannon's) with 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 effect of test 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 by non-metric multidimensional scaling to create ordination plots that present a 2D plot of the fungal communities within a given treatment at a given time. With 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 communities (Begon et al. 1990). Chao et al. (2005) suggests applying an abundance-based estimator that takes into account unseen shared species that are inherent in T-RFLP data. The Chao abundance-based Jaccard distance (Chao et al. 2005) was used to measure overall similarity between communities. Calculation and pair-wise comparisons of all treatment by time combinations was done by the program Estimates[®] (Colwell 2005).

Results and discussion

Decay ratings and influence of preservative on community profiles

Sites were analyzed separately to eliminate multiple-factor interactions between sites, treatments, and times. Statistical analysis indicated 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 both the D-site and S-site, five treatments (ACQ-C 0.37%, ACQ-C 0.25%, CTN 0.25%, CTN 0.1%+2.0%BHT, and 0.25%+2.0% BHT) retained a mean decay rating of 10 (no decay) over the entire 15-month period. Treatment with 0.1% CTN alone or 2% BHT alone had mean decay ratings of 9.5 (SD=0.577) and 9.0 (SD=0.0), respectively at the D-site and 8.25 (SD=0.5) and 8.0 (SD=0.0), respectively at the S-site, although these means were not significantly different from the other treatments. Untreated controls had significantly lower decay ratings, with a mean of 7.4 (SD=1.48) indicating 10–30% decay at the D-site and 6.0 (SD=3.22) indicating 30–50% decay at the S-site.

For ACQ, there was little to no depletion at either concentration over the 15-month period (Table 1). Thus, any changes seen in the microbial community could not be correlated to

preservative changes. For 0.1% and 0.25% CTN retention data (Table 1), PROC GLM indicated that site, exposure and time interacted to significantly affect mean preservative retention ($P=0.0002$) and ($P=0.0015$), respectively. The addition of BHT with 0.1% CTN appeared to reduce CTN loss in above-ground exposure, but not in below-ground exposure and a similar trend is seen in the 0.25% CTN treatments. This indicates that the antioxidant properties of BHT may hinder some of the fungal or bacterial redox degradation mechanisms that are contributing to CTN depletion. Proposed mechanisms of the synergistic effects of CTN and BHT are presented in Schultz et al. (2005).

PROC CORR analysis indicated that, for the total fungal community, decay rating was positively correlated with 0.1% CTN preservative retention (P coeff=0.42, $P<0.0001$), while species richness (P coeff=-0.28, $P=0.0074$), Simpson's diversity (P coeff=-0.2823, $P=0.0088$), and Shannon's diversity (P coeff=-0.268, $P=0.0130$) were all negatively correlated with CTN retention. Mean preservative retention was significantly lower in samples with greater species richness and diversity. For the basidiomycete specific samples, decay rating was positively correlated with 0.1% CTN retention (P coeff=0.4297, $P<0.0001$). For both the fungal and basidiomycete community, the samples with high decay ratings had the highest mean CTN retentions. None of the α -diversity measures were correlated with mean CTN retention. For both CTN treatments with BHT, PROC GLM results again indicated that the variables of site, exposure, and time interact to significantly affect CTN and BHT preservative retention. Results from the depletion analysis for the 2.0% BHT alone showed that there was a positive correlation between BHT retention and decay rating. Samples with higher BHT retentions had higher ratings (less decay). PROC GLM results indicated that only time affects mean BHT retention and the retention decreased in a linear manner from 0 to 15 months. Increasing richness and diversity of Basidiomycetes were not correlated with decay ratings or preservative depletion.

Species richness

T-RFLP analysis detected 4000 total fungal peaks representing 92 taxonomic units, and 408 Basidiomycete peaks representing 53 taxonomic units (Figure 1). Numbers of fungi

Table 1 Fifteen month depletion values (%) for CTN, BHT and ACQ for each treatment concentration in field stakes at Dorman and Saucier field sites in above- and below-ground exposure.

Treatment	Dorman above	Dorman below	Saucier above	Saucier below
0.1% CTN	58	100	100	80
0.25% CTN	39	73	28	33
0.1% CTN (with 2% BHT)	38	100	22	100
0.25% CTN (with 2% BHT)	11	1	62	24
2% BHT (with 0.1% CTN)	67	100	75	98
2% BHT (with 0.25% CTN)	1	75	79	86
2% BHT	67	100	75	98
0.25% ACQ	20	0	4	0
0.37% ACQ	2	2	2	1

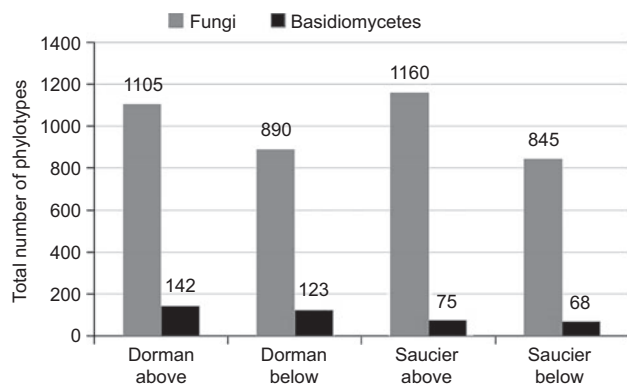


Figure 1 Total fungal phylotypes (different taxonomic units) of general fungi and Basidiomycetes at both field sites in above- and below-ground exposure at all sampling times.

were very similar at both sites, although more fungi were found in above-ground than below-ground exposure. The numbers of Basidiomycetes were considerably lower than total fungi, but D-site had twice as many Basidiomycetes as S-site. Numbers of Basidiomycetes at each test plot were similar for above- and below-ground exposure.

Total fungal richness and diversity were initially suppressed in preservative-treated wood. The richness and diversity values were significantly lower in all treated stakes compared to untreated stakes, with the exception of the BHT treatment which contains no active biocide. After 6 months of exposure, total fungal richness began to increase and, after 9 and 12 months, increased to levels equal to untreated controls (Figure 2). Controls and the BHT treatments are not statistically different (Figure 2).

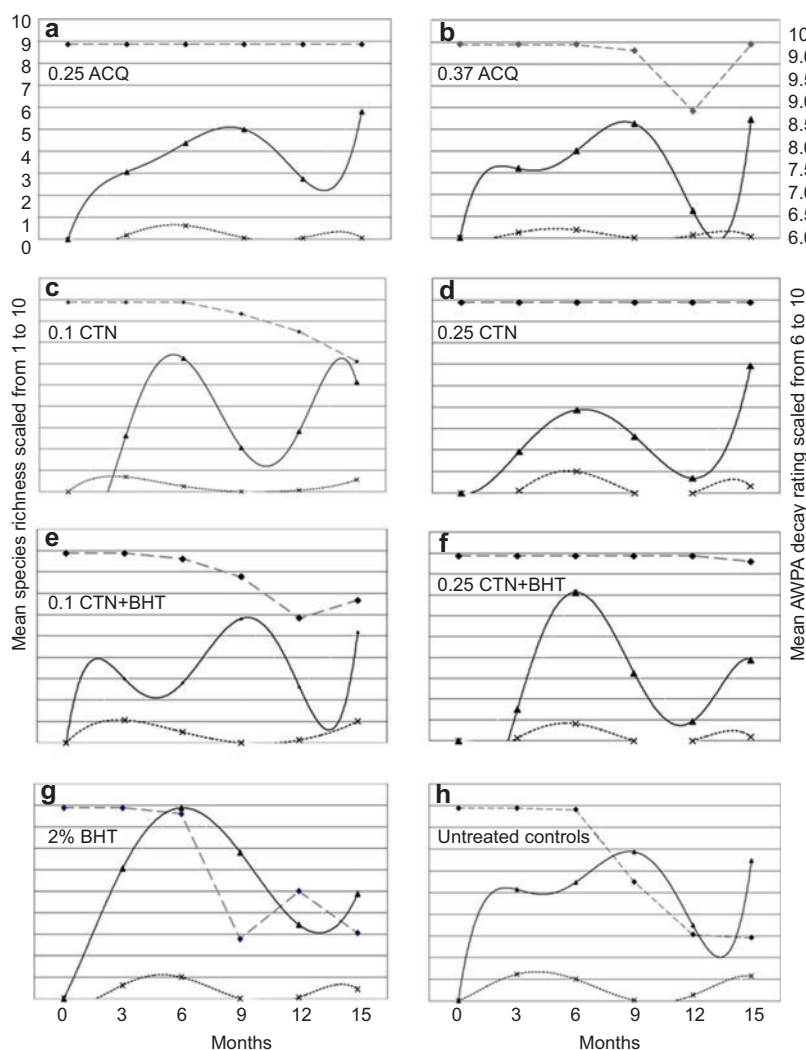


Figure 2 Comparison of mean trends in increasing fungal (solid black line) species richness and mean Basidiomycete (dashed line) species richness for (a) 0.25% ACQ-C, (b) 0.37% ACQ-C, (c) 0.1% CTN, (d) 0.25% CTN, (e) 0.1% CTN with BHT, (f) 0.25% CTN with BHT, (g) 2% BHT alone, and (h) untreated controls. Trends are plotted against mean AWPA decay rating ($\diamond$ -) on the secondary axis to indicate relative efficacy of preservative treatment. All axes for each graph are the same.

Overall, richness and diversity of Basidiomycetes were much lower compared to total fungi. This has also been reported by Råberg et al. (2007) in above- and below-ground exposed panels for fungi using T-RFLP at test sites throughout Germany. They concluded that Basidiomycetes comprised only a small percentage of the actual fungal community in these samples, and that a soft rot ascomycete, *Coniochaetia lignaria*, was encountered in 57% of the samples. These results suggest that while Basidiomycete fungi can be major contributors to the decay process, there are exceptions in which other lignocellulosic degraders can contribute to significant decay in exposed wood in the environment.

Basidiomycete richness was higher in the preservative-treated samples at both low and high retentions compared to untreated controls (Figure 2). These results are consistent with past studies that found higher species richness resulting from a disturbed ecosystem. Toljander et al. (2006) assembled artificial communities of wood decay fungi to observe the effects of a fluctuating temperature. They found higher species richness and persistence under a fluctuating environment indicating that a fluctuating environment permits co-existence of species. In contrast, a non-fluctuating environment resulted in low species persistence and richness indicating intense competition among the wood decay fungi. Wood decomposition was enhanced in fluctuating environments with intermediate species richness. White rot fungi had significantly higher metabolic efficiency than brown rots, but overall results agreed with previous studies that there is an intense competition among wood decay Basidiomycetes resulting in the highest rates of metabolic activity. The authors concluded that niche differentiation was likely important in maintaining species diversity of wood decay fungi, which has been previously characterized as a highly competitive environment.

Community analyses

Comparisons were made between controls and treated samples to determine if preservative treatment changed the patterns of fungal species which inhabited the wood. Species turnover describes the change in species composition over time, either by extinction, replacement, or succession, and can be interpreted by examining the composition of different communities at individual sampling times. The distance between points representing communities indicates the level of species turnover. Clustering of communities in ordinations is often due to some explainable variable, such as similar treatments. The Chao-Jaccard estimate value (Chao et al. 2005), which corrects for unseen shared species, was used to measure overall similarity between communities.

Ordination data indicated that, initially, total fungal communities were different in preservative-treated wood than untreated controls. However, after 6 months' exposure the fungal communities in treated wood began to coalesce and gradually became similar to those in untreated samples. This indicates that preservative treatment does alter the initial fungal community on treated wood, but that the fungal

communities eventually reach a similar species composition. The Chao-Jaccard indices values for total fungal communities gave similar results. The successional trajectory of these communities was basically linear, with the exception of the 12-month data which caused a divergence. Between 9 and 12 months, there was a severe drought at both locations and all microbial communities drastically decreased and then were reestablished between 12 and 15 months. A summary of all collection times and treatments for the total fungal communities is shown in Figure 3. The controls and BHT alone grouped closely together and shifted very little over time. The ACQ treatments clustered together and the 15-month samples moved closer to the controls, while the 3-month samples are furthest from the controls. All CTN treatments, with or without BHT, clustered together.

Because the low numbers of Basidiomycetes occurred in patches, many treatments lacked contiguous communities over time to compare. However, the species composition of untreated controls was clustered, meaning that they were similar in species composition, while biocide treatments (ACQ-C and CTN) also clustered separately (Figure 4). This suggests that both preservatives effected the species composition. The Jaccard estimate values indicated that many of the Basidiomycete communities were different among treated samples, while controls and BHT samples were similar, sharing more than 75% of their species.

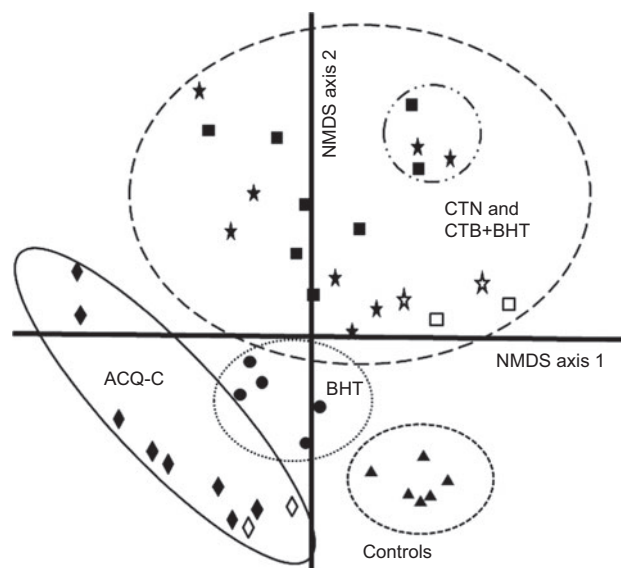


Figure 3 Nonmetric multidimensional scaling ordination of all sites (x) exposures (x) treatments (x) times combinations of total fungi detected in field study. Each point represents a given fungal community and the distances between points are approximately proportional to the dissimilarity between communities. ACQ samples (◆) are grouped in the lower right, CTN with (●) and without (■) BHT grouped in the upper half, BHT samples (●) grouped closest to the untreated controls (▲) grouped in the lower right. Note that in most cases the 15-month samples (unfilled symbols) moved closer to the controls. Also note the clustering of month 12 CTN samples encircled within CTN and CTN+BHT cluster.

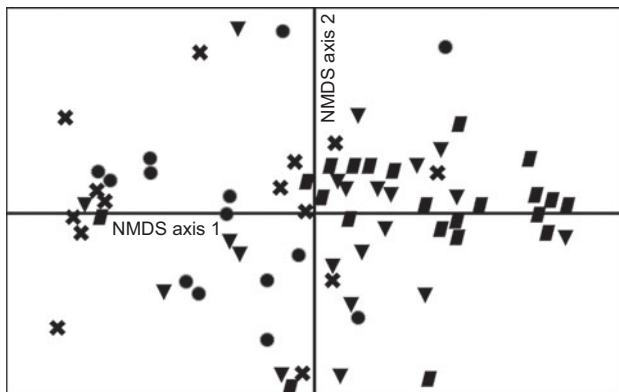


Figure 4 Nonmetric multidimensional scaling ordination of all sites (x) exposures (x) treatments (x) times combinations of Basidiomycetes detected in field study. Each point represents a given Basidiomycete community and the distances between points are approximately proportional to the dissimilarity between communities. Labels are ▼=Dorman above ground, ■=Dorman below ground, ●=Saucier above ground and ×=Saucier below ground.

Conclusions and outlook

The T-RFLP, community, and β -diversity data indicate that wood preservatives influenced total fungal colonization and species composition. The community data showed that initial communities on treated samples were very different based on species composition but eventually coalesced to become similar to untreated controls. Increasing fungal richness and diversity were also correlated with decreasing ratings, but not preservative depletion. This means that as the numbers of fungal species increased, decay ratings decreased accordingly (more decay).

The presence and diversity of Basidiomycetes were sparse in this study, so it is not clear what the contribution of these fungi to the initial process of colonization and eventual decay might be. Although these findings suggest that Basidiomycete fungi are less diverse than all other fungi in the field stakes, T-RFLP only detects the presence of these species and does not give information regarding the metabolic activity of individual species. Probably, a single Basidiomycete species dominated a wood sample and its presence contributed to decreased diversity. Additional research with protein and gene expression would enable more useful characterization of the metabolic activities of the fungal community.

The Basidiomycete data indicated that after some field exposure time, preservative treatment resulted in higher richness and diversity. It is possible that the presence of wood preservatives in this instance prevented the usual species from dominating the substrate and allowed other species to colonize the substrate and create additional niches for colonization. It could also be that preservative treatment slows the metabolic activities of these decay fungi and lengthens the time that the food source is available to be colonized. Higher species turnover was also seen as a result of preservative treatment. An increase in decay due to rising diversity was not visible in the preservative-treated wood, but this may have been due to

differing environmental conditions, the presence of the preservatives themselves or the relatively short exposure time.

With the community data, it is now possible to use T-RFLP and additional molecular analytical methods to focus on assemblages of fungi that were correlated with either high rates of decay or high rates of preservative depletion. An additional component of our field research was to analyze patterns of bacterial community ecology based on T-RFLP. The results of this component of the field study are presented in a companion manuscript, Part 2 (Kirker et al. 2012).

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