Principal Component Analysis of Microbial Community Data from an Accelerated Decay Cellar Test

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ABSTRACT

Analysis of microbial communities is a valuable tool for characterization and identification of microbes in a myriad of environments. We are currently using the molecular method terminal restriction fragment length polymorphism (T-RFLP) analysis to characterize changes in bacterial and fungal communities on treated and untreated wood in soil. T-RFLP uses fluorescently labeled primers that bind to DNA and provide a fluorescent tag for each unique organism in a sample. The result is a microbial fingerprint that can be used to characterize the community of organisms present in the sample. This paper highlights the process by which microbial data from T-RFLP is obtained, analyzed, and interpreted. In this study, fungal and bacterial data from an accelerated fungal cellar test was analyzed to determine changes in fungal and bacterial species richness and composition during exposure to treated or untreated wood materials. Principal component analysis was used to analyze the microbial data. Results show differences in bacterial and fungal species composition in treated wood compared to untreated wood. Future uses for this technique are discussed.

Keywords: preservative-treated wood, microbial communities, principal component analysis, T-RFLP

INTRODUCTION

Complete degradation of untreated woody biomass in contact with the soil typically occurs in 1 to 5 years but varies depending on latitude and climatic conditions (Moorhead and Reynolds, 1992). Temperature, pH, available moisture and nutrient availability are all major contributing factors to decomposition and vary greatly from one climate to another. Observed rates of degradation are greatest in tropical and equatorial climates and tend to decrease at higher latitudes. Variable climates were therefore, the premise for development of the hazard and use class systems widely used to evaluate performance of wood preservatives. Prior to a preservatives registration, protective compounds are required to undergo extensive efficacy testing that often takes five or more years in the field. Methods for this type of accelerated decay testing have been adapted to shorten the time required for evaluation of these potentially new wood preservatives (Van Acker et al. 1999; Nicholas and Crawford 2003).

Accelerated test methods are aimed at promoting microbial biodiversity and metabolic activities of wood-decaying microorganisms by elevating moisture, pH, temperature and nutrient availability (Nicholas and Crawford 2003). Frequently, added composted organic matter serves as an inoculum source for wood decay basidiomycetes, soft rot fungi and tunneling bacteria, reducing the amount of time required for these organisms to colonize wood (Daniel et al. 1987; Edlund and Nilsson 1999; Nicholas et al. 2004). In addition to serving as an inoculum source, composted soil also increases the available nutrients and provides a more favorable environment for decay microorganisms.

While accelerated decay tests are not currently used as a stand-alone measure of preservative performance, the results from these tests can be used to make service predictions prior to more long term field-testing and also to compare new preservative systems to older established formulations (Li et al. 2007). While these methods are becoming increasingly popular for screening new wood preservatives, very little is known about the actual successional patterns of micro-organisms that contribute to these accelerated rates of decay or if these patterns mimic what occurs in nature. Traditional cultural methods to evaluate microbial communities are time consuming and often lead to underestimations of true microbial diversity (Amann et al. 1995). Polymerase chain reaction (PCR)-based molecular methods offer a fast, reliable means of characterizing the patterns of microbial colonization and succession. Terminal restriction fragment length polymorphism (T-RFLP) analysis has been used to characterize microbial assemblages in a wide variety of substrates (Lui et al. 1997). This

study evaluates changes in the populations of bacteria and fungi that colonize preservative treated wood over time leading to eventual decay and/or preservative depletion in an accelerated test situation using T-RFLP.

MATERIALS AND METHODS

Accelerated Decay Test Procedures

Test Material

Test sticks were cut from defect-free southern pine sapwood and divided into density groups to provide a uniform sample base for each treatment level. Sticks were treated using a full cell method (94.82 kPa vacuum for 30 minutes followed by 1034.21 kPa for 1 hr). Preservative treatment groups are listed in Table 1.

Table 1: Preservative treatments of CTN and BHT in water used in accelerated decay test (CTN=chlorothalonil, BHT=butylated hydroxytoluene)

Treatment	Soil	Time months	No. of stakes
CTN 0.1%	A*	1--12	36
CTN 0.1%	B**	1--12	36
CTN 0.25%	A	1--12	36
CTN 0.25%	B	1--12	36
CTN 0.1%+2%BHT	A	1--12	36
CTN 0.1%+2%BHT	B	1--12	36
CTN 0.25%+2%BHT	A	1--12	36
CTN 0.25%+2%BHT	B	1--12	36
2% BHT	A	1--12	36
2% BHT	B	1--12	36
Untreated controls	A	1--12	36
Untreated controls	B	1--12	36
		Total Stakes	**432**
*Soil A is a 50/50 mix of soil from Dorman Lake and Saucier test sites.			
**Soil B is a 50/50 mix of soil as above, but amended with quail manure.			

Test Setup

The accelerated decay test chambers were square plastic freezer boxes measuring 80 mm wide and 60 mm deep. A schematic diagram of the cellar decay apparatus is shown in Figure 1. The soils used in this test were collected from Dorman Lake (33.341°N, 88.876°W) and Saucier (30.631°N 89.051°W) test sites in north and south Mississippi respectively. Half of the boxes were filled with 380g of a 1:1 mixture of soil from both test sites, and the other half was filled with 355g of the previously mentioned soil mix amended with 30% compost. The lids were closed tightly before placing the decay chambers into an incubator at 28º C. The individual boxes were removed weekly, weighed, and additional water was added to maintain uniform moisture content for the two soils. Small sticks for monitoring moisture levels were inserted into each box to ensure that the wood moisture remained in the 40-80% range. These samples were untreated sticks (3 x 19 x 19 mm (TxRxL) that were wrapped in a nylon stocking and placed at the same depth as the test samples.

A static bending test was used to determine the initial modulus of elasticity (MOE) for each stake. MOE was calculated from the amount of applied force needed to bend a test sample to a 2mm deflection from its resting state (Li et al. 2007). Reductions in MOE were used as a qualitative measure of decay for test sticks in the accelerated decay test. The bending test was performed after the test sticks became equilibrated in the test decay chambers to a MC above the fiber saturation point in order to obtain initial MOE data.

Laboratory Procedures

Two gram samples of wood shavings were removed aseptically from the test sticks and extracted for whole genomic DNA using commercial extraction kits following manufacturer's protocols (Machery Nagel, Easton, PA). Microbial DNA was selectively amplified by using taxon-specific fluorescently labeled primers for bacteria, fungi and basidiomycetes.

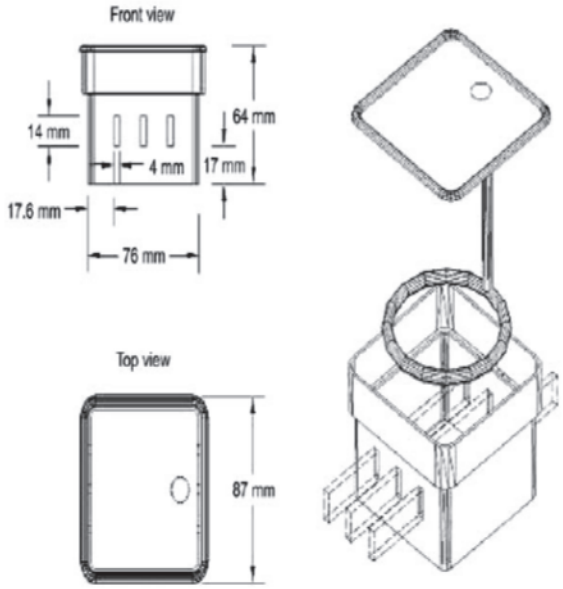

Figure 1: Diagram of accelerated laboratory apparatus

Fluorescent tags are required in order to generate the terminal restriction fragments from each unique species present in the sample. Polymerase chain reaction (PCR) was used to amplify the target regions of the DNA and amplified DNA was verified on 2% agarose gels. Subsequently, DNA was digested with restriction enzymes to produce terminal fragments containing the fluorescent tagged end and fragments were analyzed using a Beckman Coulter CEQ genetic analyzer. The CEQ uses capillary electrophoresis to identify and size fragments by comparing them to commercial size standards. For each wood sample, a fragment profile was created showing the total bacteria, fungi, or basidiomycete species present in each sample. Terminal restriction fragment data was used to calculate diversity indices for bacteria, fungi and basidiomycetes.

Analysis

Basic statistical analysis and analysis of variance was conducted in SAS 9.2 (SAS Institute Inc., Cary, NC) using the PROC GLM function to compare means for logMOE based on the effects of time, treatment, bacterial species richness, fungal species richness and basidiomycete species richness. Due to the presence of several interacting factors, a principal component analysis was conducted using PROC PLS in SAS 9.2 to model interrelated variables and calculate the proportion of variance explained by the individual components. PCA is a multivariate statistical method that transforms interrelated variables into new uncorrelated variables and calculates z-scores which are in turn used to rank the components. The highest scored component is the component that contributes to the highest percent of the variation in mean response variable and is referred to as the first principal component (Quinn and Keough 2002). The components can also be plotted on different axes to visually examine their patterns similar to how one would view a scatterplot. Preliminary analysis with PROC PLS was done with the entire data set and a second analysis was done looking at individual treatment and soil type combinations. Missing values were imputed by PROC PLS using default settings and 5 iterations of imputation.

RESULTS AND DISCUSSION

Analysis 1: PROC GLM

MOE Data

Results from the static bending test were analyzed using PROC GLM in SAS v9.2. Treatment, soil type, time, and microbial diversity measures were used as experimental variables. The effects of treatment, soil type, time, and all possible interactions of these effects were tested with an alpha value of 0.01. Pair wise comparisons of least square means (Lsmeans) tested differences among factorial combinations of interacting effects. Treatment, soil type, time, and all 3 microbial diversity measures (bacteria, fungi, and basidiomycetes) all interacted to effect log MOE ($p=0.005$). Pairwise comparisons did not show any clear patterns that were meaningful. The community data from the bacterial, fungal, and basidiomycete community

analysis were combined and analyzed using PROC CORR in SAS v9.2 in order to determine what effects these variables have on log MOE values. Pearson's coefficient was used as the measure of correlation. An alpha of 0.01 was used to accept or reject the null hypothesis of no correlation using Pearson's correlation coefficient as the measure of correlation. The goal of this analysis is to gain some measure of the relative contributions of microbial richness or diversity to decreasing MOE values due to decay (Table 2).

Table 2: Results of PROC CORR Analysis for logMOE, and species richness for accelerated test

			16S Bacteria			ITS1-4NS General fungi			ITS1-4BS Basidio specific		
		Log MOE	Spp. Rich	Simp Div	Shann Div	Spp. Rich	Simp Div	Shann Div	Spp. Rich	Simp Div	ShannDiv
r*		**1.00**	**-0.16**	**-0.30**	**-0.35**	**-0.30**	**-0.36**	**-0.38**	**-0.22**	**-0.22**	**-0.23**
P-value**	**Log**		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
N***	**MOE**	468.00	394.00	394.00	394.00	250.00	250.00	250.00	409.00	409.00	409.00
		-0.16	**1.00**	**0.86**	**0.58**	**0.35**	**0.45**	**0.49**	**0.37**	**0.40**	**0.39**
	Species	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
	Richness	394.00	394.00	394.00	394.00	229.00	229.00	229.00	362.00	362.00	362.00
16S Bacteria		**-0.30**	**0.86**	**1.00**	**0.89**	**0.53**	**0.67**	**0.74**	**0.52**	**0.58**	**0.62**
	Simpson's	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
	Diversity	394.00	394.00	394.00	394.00	229.00	229.00	229.00	362.00	362.00	362.00
		-0.35	**0.58**	**0.89**	**1.00**	**0.60**	**0.77**	**0.86**	**0.55**	**0.66**	**0.73**
	Shannon's	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
	Diversity	394.00	394.00	394.00	394.00	229.00	229.00	229.00	362.00	362.00	362.00
		-0.30	**0.35**	**0.53**	**0.60**	**1.00**	**0.92**	**0.77**	**0.51**	**0.57**	**0.59**
	Species	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001
	Richness	250.00	229.00	229.00	229.00	250.00	250.00	250.00	230.00	230.00	230.00
ITS1-4NS General fungi		**-0.36**	**0.45**	**0.67**	**0.77**	**0.92**	**1.00**	**0.95**	**0.62**	**0.70**	**0.76**
	Simpson's	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001
	Diversity	250.00	229.00	229.00	229.00	250.00	250.00	250.00	230.00	230.00	230.00
		-0.38	**0.49**	**0.74**	**0.86**	**0.77**	**0.95**	**1.00**	**0.67**	**0.77**	**0.83**
	Shannon's	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001
	Diversity	250.00	229.00	229.00	229.00	250.00	250.00	250.00	230.00	230.00	230.00
		-0.22	**0.37**	**0.52**	**0.55**	**0.51**	**0.62**	**0.67**	**1.00**	**0.95**	**0.82**
	Species	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001
	Richness	409.00	362.00	362.00	362.00	230.00	230.00	230.00	409.00	409.00	409.00
ITS1-4BS Basidio specific		**-0.22**	**0.40**	**0.58**	**0.66**	**0.57**	**0.70**	**0.77**	**0.95**	**1.00**	**0.95**
	Simpson's	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001
	Diversity	409.00	362.00	362.00	362.00	230.00	230.00	230.00	409.00	409.00	409.00
		-0.23	**0.39**	**0.62**	**0.73**	**0.59**	**0.76**	**0.83**	**0.82**	**0.95**	**1.00**
	Shannon's	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	
	Diversity	409.00	362.00	362.00	362.00	230.00	230.00	230.00	409.00	409.00	409.00

* r=coefficient for Pearson's product-moment correlation.

** P-value=Alpha value, cutoff of 0.01 used to reject or accept null hypothesis.

** Number of observations in each variable.

Analysis 2: Principal components analysis using PROC PLS

All Data

The entire data set was initially analyzed using PROC PLS in order to separate the variance of some of the interacting variables. The first step in the process is to validate the model and determine the minimum number of factors that effectively explains the variance. The scree plot that shows R-squared values for each factor leveled off at 6, which indicated the lower limit for explaining the variance. In this data set, the first five components (which is a combination of the reprojected factors) explained 97% of the variation in logMOE. These values are used to compute the components for the analysis and give an indication of the relative weight of each factor. PROC PLS was also used to create correlation loading plots that visually depict the influence of factors. In the two examples below, logMOE is found to be inversely related to diversity measures for bacteria, fungi, and basidiomycetes, as well as time. This result fits our previous assumptions, as the diversity measures go up

(more microbes colonize the substrate), logMOE goes down. These results were in agreement with the results from the previous PROC CORR analysis (Table 2). The same applies to time; as time progresses, logMOE decreases. Treatment was separate from these variables and appears to have some effect on logMOE, but not inversely related as the others. Treatment is a categorical value (1-6) and does not have the same linear structure as the other variables. The influence of soil type and treatment were not clear in the first analysis, so a second analysis was conducted with samples grouped by soil type.

Proc PLS: Data Separated by Treatment and Soil

PROC PLS was used to run a second analysis sorted by treatment and soil type to reduce noise in the scatterplots. There are noticeable differences between treatments variance structure indicated by the scatterplots shown below (Figure 2). Treatment appears to have an effect on logMOE as well as soil type.

In order to better characterize the differences between treatments, correlation loading plots were generated of treatment soil combinations to look at changes in the ordination of variables along the axes. The correlation loading plots below show the differences between treated (0.25% CTN) and untreated samples. The plots show the same inverse correlation between diversity measures and logMOE, but the relative positions of the different groups of organisms trades off from treated to untreated samples. In the CTN treated samples bacteria are promoted to the upper range of the y-axis indicating that they have higher influence on the variance of the data. In the untreated data, basidiomycetes were highest on the y-axis and seemed to have greater influence over fungi and bacteria.

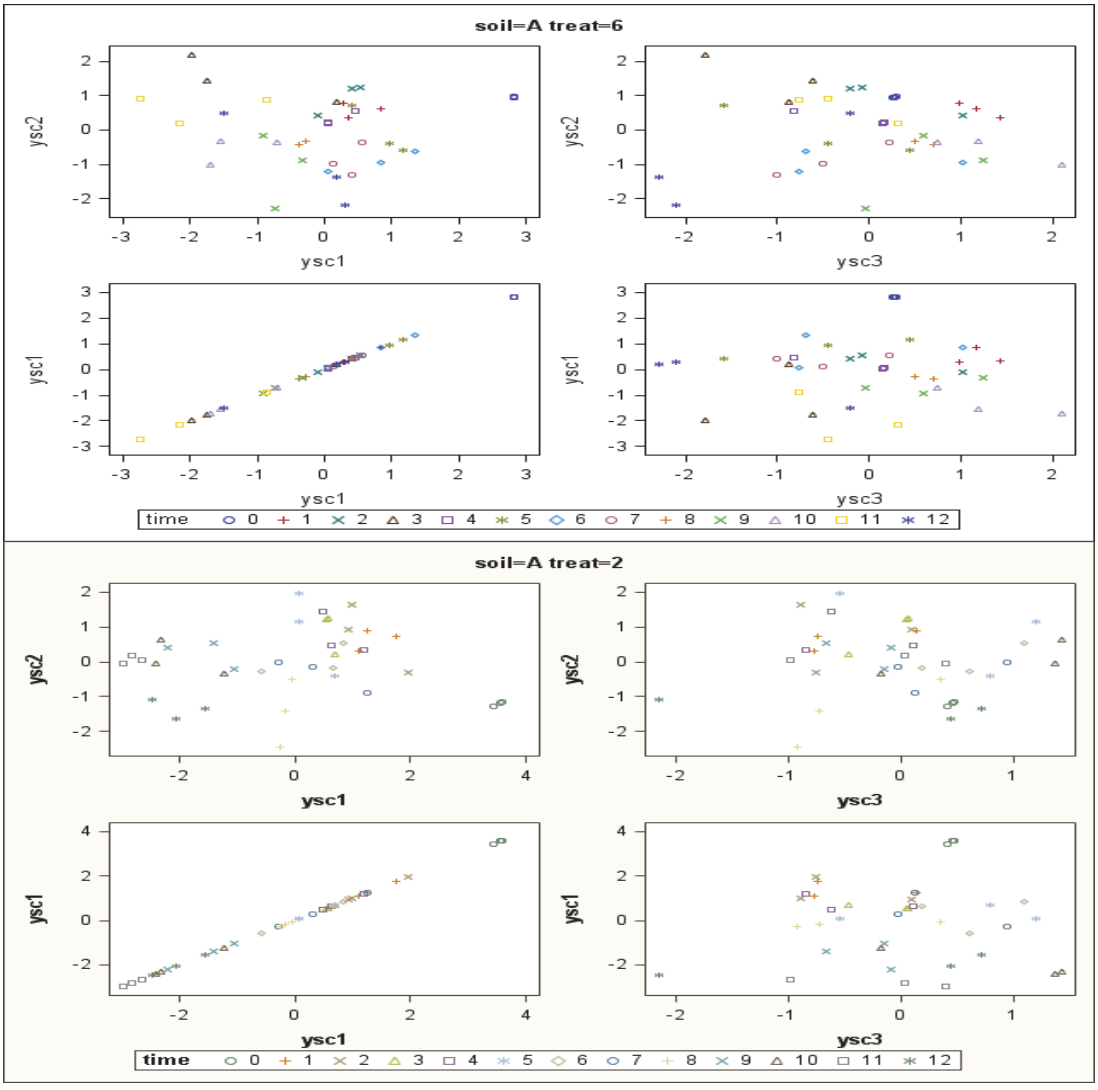

Figure 2: Scatterplots of transformed PCA data for untreated controls (treat=6) and 0.25% CTN (treat=2) showing differences in variance structure between treatments

CONCLUSION

PCA analysis provided some supporting data to strengthen the previous analysis. However, the diversity measures did not provide as much information as was hoped and the end result was similar to what was achieved with PROC GLM and PROC CORR. According to our results, microbial diversity is inversely related to logMOE and the relative contributions of different groups of organisms (bacteria, fungi, basidiomycetes) seem to vary between treatments. When analyzing treatment and soil type combinations separately, the influence of diversity appears to shift from organisms. In treated wood, bacterial richness seems to contribute more to variations in logMOE and basidiomycetes contribute more in untreated samples. The lack of preservative retention data to accompany the MOE data is unfortunate (preservatives were completely leached/ depleted after the 1st month of accelerated exposure) because it would be interesting to see where preservative depletion would plot in this analysis. Future analysis will look at other methods for imputing missing factors, which would allow the use of PROC PRINCOMP for analysis rather than PROC PLS. PROC PLS was chosen for PCA chiefly because it has built in functions for imputing missing values. There are several other analytical methods that we can explore for analysis of this type of data.

This analysis was very exploratory in nature, a better understanding of the application of these methods and careful interpretation of the results will provide more data in the future. Also, further attempts will be made to examine raw data (vectors) prior to calculation of diversity indices. By using this method, we can potentially break out individual sources of variation in response variables that can be attributed to a certain species of bacteria, fungi, or basidiomycetes. Analysis would need to be conducted separately for the 3 groups, but this would be extremely informative when looking at mass loss, preservative retentions, and declining stiffness properties.

REFERENCES

1. Amann, R.I., W. Ludwig, K.H. Schleifer 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59: 143-169.
2. Daniel, G.F., T. Nilsson, A.P. Singh. 1987. Degradation of lignocellulosics by unique tunnel forming bacteria. Can. J. Microbiol. 33: 943-948.
3. Edlund, M.L. and T. Nilsson. 1999. Performance of copper and non-copper based wood preservatives in terrestrial microcosms. Holzforshung 53: 369-375.
4. Li, G., D.D. Nicholas, and T.P. Schultz. 2007. Development of an accelerated soil contact decay test. Holforshung 61(2): 214-218.
5. Liu W.T., T.L. Marsh, H. Cheng, L.J. Forney. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. 63:4516–4522.
6. Moorhead, D.L. and J.F. Reynolds. 1992. Modeling the Contributions of Decomposer Fungi in Nutrient Cycling. Pp. 691-715. *In:* The Fungal Community: Its Organization and Role in the Ecosystem (Mycology Series, Vol. 9). G.C. Carroll and D.T. Wicklow (Eds). 2nd Edition. Dekker Marcel Publishing.
7. Nicholas, D.D. and D. Crawford. 2003. Concepts in the Development of New Accelerated Test Methods for Wood Decay. *In:* Goodell, B., Nicholas, D.D. and T.P. Schultz. Wood Deterioration and Preservation. ACS Symposium series. Oxford University Press.
8. Nicholas, D.D., H. Borazjani, and T.P. Schultz. 2004. Accelerated laboratory soil contact decay test using soil amended with composted wood. Document IRG/WP 04-20284. International Research Group on Wood Protection, Stockholm, Sweden.
9. Quinn, G.P. and M.J. Keough. 2002. Experimental Design and Data Analysis for Biologists. Cambridge University Press. New York, NY. 537p.\
10. Van Acker, J., H. Militz and M. Stevens. 1999. The significance of accelerated laboratory testing methods determining the natural durability of wood. Holzforshung 53 (5): 449-458.

Kirker, G.T. and Lebow, P.K. 2014. Principal component analysis of microbial community data from an accelerated decay cellar test. In: Proceedings of the American Wood Protection Association, Birmingham, AL. 108; 2014