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ORIGINAL ARTICLE

Long-term effects of timber harvesting on hemicellulolytic microbial populations in coniferous forest soils

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Forest ecosystems need to be sustainably managed, as they are major reservoirs of biodiversity, provide important economic resources and modulate global climate. We have a poor knowledge of populations responsible for key biomass degradation processes in forest soils and the effects of forest harvesting on these populations. Here, we investigated the effects of three timber-harvesting methods, varying in the degree of organic matter removal, on putatively hemicellulolytic bacterial and fungal populations 10 or more years after harvesting and replanting. We used stable-isotope probing to identify populations that incorporated ^{13}C from labeled hemicellulose, analyzing ^{13}C-enriched phospholipid fatty acids, bacterial 16 S rRNA genes and fungal ITS regions. In soil microcosms, we identified 104 bacterial and 52 fungal hemicellulolytic operational taxonomic units (OTUs). Several of these OTUs are affiliated with taxa not previously reported to degrade hemicellulose, including the bacterial genera *Methylibium*, *Pelomonas* and *Rhodoferax*, and the fungal genera *Cladosporium*, *Pseudeurotiaceae*, *Capronia*, *Xenopolyscytalum* and *Venturia*. The effect of harvesting on hemicellulolytic populations was evaluated based on *in situ* bacterial and fungal OTUs. Harvesting treatments had significant but modest long-term effects on relative abundances of hemicellulolytic populations, which differed in strength between two ecozones and between soil layers. For soils incubated in microcosms, prior harvesting treatments did not affect the rate of incorporation of hemicellulose carbon into microbial biomass. In six ecozones across North America, distributions of the bacterial hemicellulolytic OTUs were similar, whereas distributions of fungal ones differed. Our work demonstrates that diverse taxa in soil are hemicellulolytic, many of which are differentially affected by the impact of harvesting on environmental conditions. However, the hemicellulolytic capacity of soil communities appears resilient.
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Introduction

Forests remove vast amount of carbon dioxide from the atmosphere annually, equaling ~3 Pg or ~30% of fossil fuel emissions (Canadell and Raupach, 2008). Yet, deforestation results in the annual loss of forestland, the approximate size of the United Kingdom and the release of CO_2 on a scale equivalent to the entire transportation sector, with accompanying loss of habitat and biodiversity (FAO, 2010). There is a need to manage forests to effectively minimize greenhouse gas emissions while maintaining sustainable production. Forest management influences the availability of soil carbon and nitrogen, which are key variables determining soil fertility (Johnson and Curtis, 2001). Soil decomposition processes mediated by microbes have a predominant role in mineralization and cycling nutrients to plants and the atmosphere by breaking down complex biomolecules (Doran, 2002; Van Der Heijden *et al.,* 2008). Improperly managed forests can revert from net carbon sinks to net contributors of greenhouse gas emissions (Kurz *et al.,* 2008). Improved understanding of the relationships among soils, their microbial communities and carbon cycling has great potential to contribute to forest management practices that ensure the long-term productivity while mitigating climate change.

A key question for sustainable forest management is how the removal of biomass during forest harvesting will affect carbon cycling and the long-term productivity of the secondary forest. The long-term soil productivity (LTSP) Study was established to evaluate the consequences of soft-wood lumber harvesting across multiple ecozones in North America (Powers *et al.,* 2005). The LTSP Study has been underway for over two decades and will continue to

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document the long-term effects of pulse soil disturbances, particularly the removal of organic matter (OM) during harvesting. To date, short-term effects of harvesting included altered plant community, nutrient availability, soil structure, microclimate and soil litter composition (Keenan and Kimmins, 1993). However, after 10–15 years, these properties have returned to resemble pre-harvesting conditions (Powers, 1999; Sanchez *et al.,* 2006). Generally, there were no pronounced differences among harvesting treatments with three different levels of OM removal on forest productivity (Powers *et al.,* 2005). Relatively, little is known about the effects of these OM removal treatments on the soil microbial community. A recent study of LTSP sites in BC, Canada found that the removal of OM significantly and persistently altered both bacterial and fungal communities in soil 10–15 years following harvesting (Hartmann *et al.,* 2012). It remains unclear if or how key functional groups within those communities, such as those involved in carbon cycling, were affected and whether such effects are broadly representative of responses in other ecozones.

Recycling photosynthetically derived carbon, roughly half of which is lignocellulose, is an essential aspect of the carbon cycle, releasing 10^{11} tons of monosaccharides annually. Decomposition of plant detritus provides 69–87% of nutrients required for annual forest growth (Swift *et al.,* 1979; Sinsabaugh *et al.,* 1993). Lignocellulosic biomass consists mainly of cellulose (35–50%), hemicellulose (25–30%) and lignin (25–30%), and is recalcitrant to chemical treatments, creating an obstacle in efficient production of lignocellulose biofuels. Hemicelluloses have an integral role stabilizing the components of lignocellulose by forming a robust network of cross-linked polymers, with hemicellulose covalently linked to lignin (Shallom and Shoham, 2003). Unlike cellulose, hemicelluloses are heterogeneous polymers consisting of furanoses, pyranoses and a variety of sugar acids, and they are branched with short lateral chains of varying saccharides. Degradation of hemicellulose is mediated by a consortium of fungi and bacteria using a variety of extra-cellular hydrolytic and oxidative enzymes (Pérez *et al.,* 2002). Although this degradation process is understood, we have a poor knowledge of the microorganisms responsible for hemicellulose degradation in particular environments. In the context of forest management, improved knowledge of hemicellulose degraders in soil communities will advance our understanding of how disturbances affect this functional group, and more broadly, affect carbon cycling. Improved knowledge of hemicellulose degraders may also lead to the discovery of novel biocatalysts, with applications in transforming biomass to valuable chemicals and materials.

Here, we used stable-isotope probing (SIP) of DNA to identify bacterial and fungal populations from forest soil communities that assimilate carbon from hemicellulose. We further used extensive molecular surveys of microbial communities in forest soils to evaluate how these populations were impacted by timber harvesting and to determine their distributions in coniferous forests across North America. These DNA-based approaches were complemented with measures of microbial activity based on ^{13}C incorporation into phospholipid fatty acids. This is the first study to combine these approaches to investigate both bacterial and fungal hemicellulose degraders and to investigate responses of these populations to disturbance in a large-scale field experiment.

Materials and methods

Sample collection

Samples were collected from 18 LTSP Study experimental sites in six different ecozones across North America (Table 1, Supplementary Table S1). The ecozones represent distinct climatic regimes: interior Douglas fir (IDF), cold semi-arid; sub-boreal spruce, western montane; black spruce, boreal moist; jack pine, cool, wet, moist; ponderosa pine (PP), Mediterranean; and loblolly pine, subtropical moist. Each site had controls and timber-harvesting treatment plots, representing three levels of organic matter removal: OM0, unharvested reference; OM1, stem-only harvesting (crown and branches left on site); OM2, whole-tree harvesting; and OM3, whole-tree harvesting plus removal of forest floor (organic soil layer). These harvesting treatments removed the following proportions of above-ground organic matter: OM1, 40–70%; OM2, 70–90%; OM3, ~100% (Powers, 2006). Plots were harvested and replanted from 10 to 15 years prior to sampling for this study. The trees used for replanting correspond to the ecozone names, except that lodgepole pine was planted in the IDF ecozone. Soils were sampled from 9 or 15 randomly selected points in each rectangular treatment plot. The organic layer and top 120 cm of the mineral layer were separately sampled. Sets of three or five corresponding samples were composited to yield triplicate samples from each layer in each treatment plot. Further details of soil sampling are described by Hartmann *et al.* (2012) The OM3 treatments in the IDF, sub-boreal spruce and jack pine ecozones lacked sufficient organic layers for sampling.

SIP soil microcosm conditions

Microcosms used for SIP experiments were prepared in 30-ml serum vials (Wheaton glass) with 0.75 or 1.00 g dry weight of organic or mineral layer soil, respectively. Soil microcosms were prepared with soil samples from the IDF and PP ecozones. All microcosms were in pairs, one with ^{13}C-labeled hemicellulose and one with unlabeled hemicellulose. In SIP-PLFA (phospholipid-derived fatty acids)

Table 1 LTSP Study sites sampled for this study

Ecozone	*Location*	*Site*	*Latitude (°North)*	*Longitude (°West)*	*Elevation (m)*	*Annual precipitation (cm)*	*Precipitation, warmest quarter (mm)*	*Mean annual temperature (°C)*
		Dairy Creek	50.51	102.25	1075			
IDF	British Columbia	O'Connor Lake	50.53	120.21	1180	30–75	NA	1.6–9.5
		Black Pines	50.56	120.17	1150			
		Log Lake	54.35	122.61	785	62		
SBS	British Columbia	Skulow Lake	52.32	121.92	1050	43	146–193	2.2
		Topley	52.32	126.31	1100	53		
		Blodgett	38.88	120.64	1320	165		
PP	California	Brandy City	39.55	121.04	1130	190	51–55	11.2
		Lowell Hill	39.26	120.78	1270	173		
		Fensom 1	89.41	49.07	442			
BS	Ontario	Fensom 2	89.38	49.08	450	61	266	0.4
		Fensom 3	89.39	49.07	442			
		Superior 1	47.58	82.79	458	82		
JP	Ontario	Superior 2	47.58	82.81	461	83	250	1.7
		Superior 3	47.57	83.84	426	85		
		Kurth-1						
LP	Texas	Kurth-2	31.11	95.15	88	109	253	19
		Kurth-3						

Abbreviations: BS, black spruce; IDF, interior Douglas fir; JP, jack pine; LP, loblolly pine; NA, not available; PP, ponderosa pine; SBS, sub-boreal spruce.

experiments, one microcosm was prepared for each triplicate soil sample. In an initial SIP-DNA experiment, one microcosm was prepared for each triplicate soil sample, whereas in later experiments, triplicate soil samples were composited (Supplementary Figure S1). The moisture content was adjusted to 60% of total weight. All microcosms were pre-incubated for 7 days at 20 °C in the dark prior to the addition of substrate. Following pre-incubation, 10 mg of finely ground maize-derived hemicellulose was added, and mixed thoroughly with the soil. Hemicellulose in controls had the natural abundance of ^{13}C (<1.2 atom %; IsoLife; N-10509, Lot:0901-0238); alternatively ^{13}C-labeled hemicellulose was used (97 atom %; IsoLife; U-10509, Lot: 0901-0273). The microcosms were crimp sealed with a butyl rubber stopper and further incubated for 48 h.

SIP-PLFA

PLFAs were extracted as previously described (Bligh and Dyer, 1959; Frostegård *et al.,* 1991) and analyzed with a gas chromatograph isotope-ratio mass spectrometer (UC Davis Stable Isotope Facility; Davis, CA, USA). The combination of C18:2n6, C18:1n9 and C18:3n3 fatty acids was used to determine fungal biomass, whereas terminal branched fatty acids i14:0, i15:0, a15:0, i16:0, i17:0 and a17:0 were used for Gram-positive bacterial biomass, and cyclopropyl and monounsaturated fatty acids cy17:0, c16:1n7 and c16:1n5 were used for Gram-negative bacterial biomass (Oravecz *et al.,* 2004; Moore-Kucera and Dick, 2008).

Cesium chloride gradient ultracentrifugation

Total genomic DNA was extracted from soil microcosms using MPBio FastDNA Spin Kit for Soil, as recommended by the manufacturer. DNA concentrations were quantified using the PicoGreen fluorescent dye assay (Invitrogen, Carlsbad, CA, USA), and 3 µg of DNA was used for each cesium chloride gradient ultracentrifugation. DNA gradients were formed, separated into 20 fractions and purified as previously described (Neufeld *et al.,* 2007). The enrichment of ^{13}C-DNA was measured according to Wilhelm *et al* (2014). In brief, a 5-µl volume of each fraction was hydrolyzed in 100 µl of 88% formic acid at 70 °C for 1 h. Following hydrolysis, fractions were dried in a SpeedVac with an acid trap and suspended in 30 µl of 1% acetic acid (v/v). ^{13}C levels were determined by measuring the isotopic enrichment of individual adenine and guanine nucleotides using an ultra-high-performance liquid chromatograph coupled to a tandem mass spectrometer. Fractions from each gradient containing >50% ^{13}C DNA (average densities 1.725–1.735 g ml^{-1}) were pooled.

Pyrotag sequencing

DNA concentrations were quantified using the PicoGreen fluorescent dye assay. Each set of pooled gradient fractions was diluted to 0.25 ng µl^{-1} DNA prior to PCR amplification. The bacterial V1–V3 hypervariable regions of the 16 S rRNA gene were amplified using universal 27 F and 519 R (Weisburg *et al.,* 1991; Turner *et al.,* 1999) barcoded bacterial primers. The fungal internal transcribed spacer (ITS2) region was amplified using barcoded primers ITS3R and ITS4F (White *et al.,* 1990). PCR reactions were performed in triplicate for each sample, and pooled prior to purification and quantification (Hartmann *et al.,* 2012). Samples were sequenced using the Roche 454 Titanium platform (GS FLX+) at the McGill University and Genome Québec Innovation Centre with a maximum of 40 samples

multiplexed on each quarter plate, yielding an average of 4200 bacterial and 3800 fungal pyrotags per sample.

Sequence processing and analysis
Bacterial sequences were processed using the MOTHUR standard operating procedure (Schloss *et al.,* 2011). This process entails an implementation of PyroNoise to reduce sequencing error, followed by trimming of barcoded sequencing primers, and setting a sequencing score cutoff of 350 flows. Sequences were de-replicated for subsequent steps. Chimeric sequences were filtered out using the uchime software implemented in MOTHUR. Bacterial operational taxonomic units (OTUs) were binned at a distance of 0.03, using the *k*-nearest neighbor algorithm (Schloss and Westcott, 2011). Fungal sequences were binned into OTUs at a Levenshtein distance of 11 using CrunchClust (Hartmann *et al.,* 2012). To assess *in situ* distributions of hemicellulolytic OTUs, the bacterial and fungal sequences were clustered into OTUs with previously constructed pyrotag libraries derived from *in situ* soil community DNA from the six ecozones (Hartmann *et al.,* 2012; VanInsberghe *et al.*, 2015; Short read archive study accession (ERS713483–ERS713552)). Both bacterial and fungal OTU matrices were normalized by subsampling to 1500 sequencing reads per sample. For taxonomy classification, representative OTUs were selected by MOTHUR using the distance method, and the representatives were identified with the SILVA 16 S rRNA v. 102 reference database (Quast *et al.,* 2013) for bacterial pyrotags and the UNITE v. 6 (Kõljalg *et al.,* 2013) database for fungal pyrotags. The representative OTU sequences were deposited in Genbank under accession numbers KP411926 through KP412081 for uncultured bacterial and fungal clones.

Statistical analyses were performed in R and PRIMER 6 with the PERMANOVA+ add-on. All PERMANOVA analyses utilized the permutation of residuals under a reduced model with 999 permutations, using the Bray–Curtis dissimilarity index. The R Boruta package was used for random forest analysis. Species diversity and evenness were calculated using the R Vegan package (Dixon, 2003). A dendrogram was generated using Arb-Silva by a quick-parsimony addition of pyrotag sequences into a small subunit rRNA tree provided by Silva (Quast *et al.,* 2013) and visualized using the web-based Interactive Tree of Life (www.itol.embl.de). The web-based RDP SeqMatch tool (Cole *et al.,* 2009) was used to identify isolate strains most closely related to OTUs for use as reference sequences in the dendrogram.

Results

^{13}C incorporation into biomass
Incorporation of ^{13}C into PLFAs and DNA was consistently observed following incubation of microcosms with ^{13}C-hemicellulose and soils from either the IDF or PP ecozone for 48 h. Incorporation of ^{13}C was greater in organic soil compared with mineral soil (Figure 1). There was a strong correlation between total PLFA carbon, which is proportional to total biomass, and the total amount of ^{13}C incorporated into PLFAs, which approximates hemicellulose assimilation activity. This correlation was positive and linear, but the slope of the line differed substantially between microcosms with soil from the organic versus mineral layer. Based on domain-specific PLFAs, the relative proportion of bacterial and fungal ^{13}C incorporation differed between soils from the IDF ecozone versus the PP ecozone, with a greater proportion of fungal incorporation in the latter (Supplementary Figures S2 & S3). There was a trend of increasing ^{13}C incorporation into PLFAs with increasing degrees of OM removal in the forest-harvesting treatments (Supplementary Figure S4), but incorporation was highly variable and did not differ significantly among treatments.

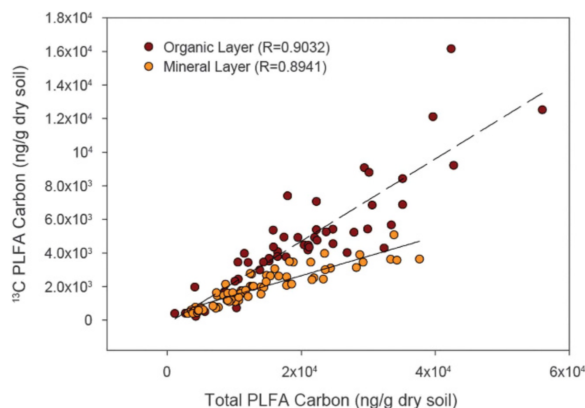

Figure 1 Relationship between the incorporation of hemicellulose and total biomass after microcosm incubations ($n = 52$).

Identification of hemicellulolytic OTUs
Pyrotag libraries derived from the heavy DNA fractions were compared between ^{13}C microcosms and corresponding ^{12}C controls to identify OTUs that assimilated carbon from hemicellulose. Substantial ^{13}C enrichment in DNA from ^{13}C microcosms was found in density gradient fractions between 1.725 and 1.735 g ml^{-1}, corresponding to the first five to six fractions collected (Figure 2a). These fractions were pooled for pyrotag analysis, as were the corresponding fractions from ^{12}C controls. However, owing to the limited amount of DNA in the heavy fractions from ^{12}C controls, 21 of 35 bacterial control samples, and 31 of 35 fungal control samples did not yield sufficient PCR product for sequencing. To identify organisms incorporating ^{13}C from hemicellulose, the OTU matrices from ^{13}C microcosms and ^{12}C controls were separately pooled and compared. This was done separately for each soil layer in each ecozone. Relative abundance profiles of bacterial and fungal OTUs differed significantly ($P < 0.001$,

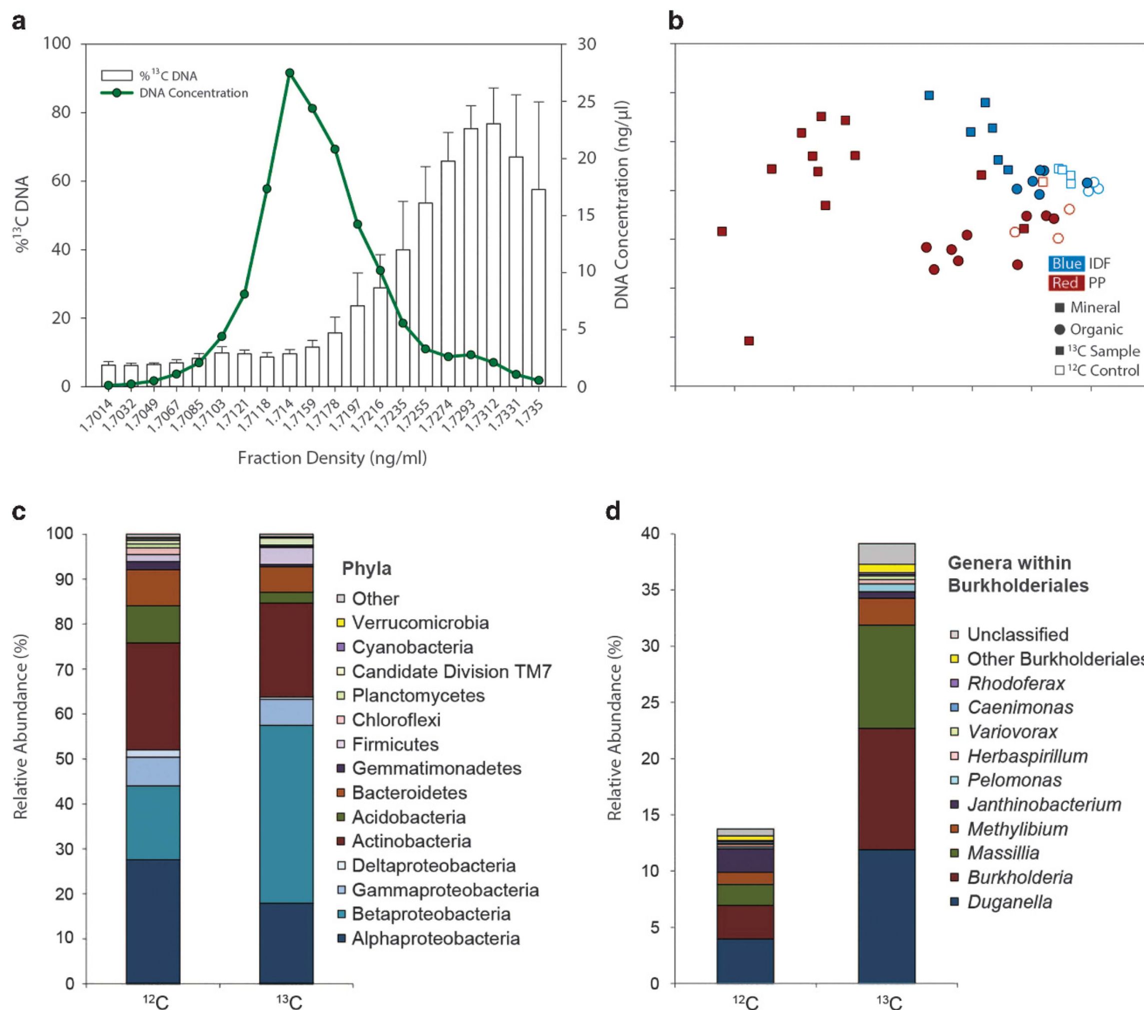

Figure 2 An overview of analysis of pyrotags from heavy DNA fractions showing (**a**) separation of 13C-enriched DNA through CsCl density gradient centrifugation; (**b**) the non-metric multidimensional scaling ordination of pyrotag profiles from ^{13}C microcosms (closed symbols) and ^{12}C controls (open symbols); relative abundances in all ^{13}C microcosms and ^{12}C controls of phyla (**c**) and of genera within the order *Burkholderiales* (**d**).

PERMANOVA) between ^{13}C and ^{12}C microcosms (Figure 2b). OTUs with relative abundances at least fivefold greater in ^{13}C microcosms versus the corresponding ^{12}C controls were identified as ^{13}C-enriched, or putatively hemicellulolytic), OTUs. A total of 104 (Supplementary Table S3) bacterial and 52 fungal (Supplementary Table S4) hemicellulolytic OTUs were identified. The greatest number of ^{13}C-enriched bacterial OTUs were Betaproteobacteria (Figure 2c), with *Burkholderia*, *Duganella* and *Massillia* being the most abundant genera (Figure 2d). Of the hemicellulolytic bacterial OTU sequences, 43 were $<97\%$ identical to those of cultured strains represented in GenBank. Hemicellulolytic fungal OTUs consisted largely of Ascomycota. Of the hemicellulolytic OTUs, 20 of the bacterial ones and eight of the fungal ones were identified in soil from both ecozones. All fungal hemicellulolytic OTUs identified in the mineral layer were also present in the organic layer.

Hemicellulolytic OTUs in situ

In situ relative abundances of the bacterial and fungal hemicellulolytic OTUs were analyzed within a large and highly replicated pyrotag data set representing 691 soil samples from 18 forest sites in six ecozones (Hartmann *et al.,* 2012; VanInsberghe *et al.,* 2015). Hemicellulolytic bacterial OTUs constituted an average of 2.0% of the total bacterial pyrotags from both organic and mineral soil layers across the six ecozones, and were most abundant (5.1%) in the organic layer of the PP ecozone (Figure 3a). Of the 24 hemicellulolytic bacterial OTUs most abundant *in situ*, 18 were *Betaproteobacteria*, including eight *Burkholderia,* and the remainder were *Gammaproteobacteria*, *Firmicutes*, *Bacteroidetes* and *Acidobacteria* (Figure 4). These abundant OTUs generally had similar distributions across ecozones. However, distributions of all 104 hemicellulolytic bacterial OTUs differed between the ecozones IDF and PP,

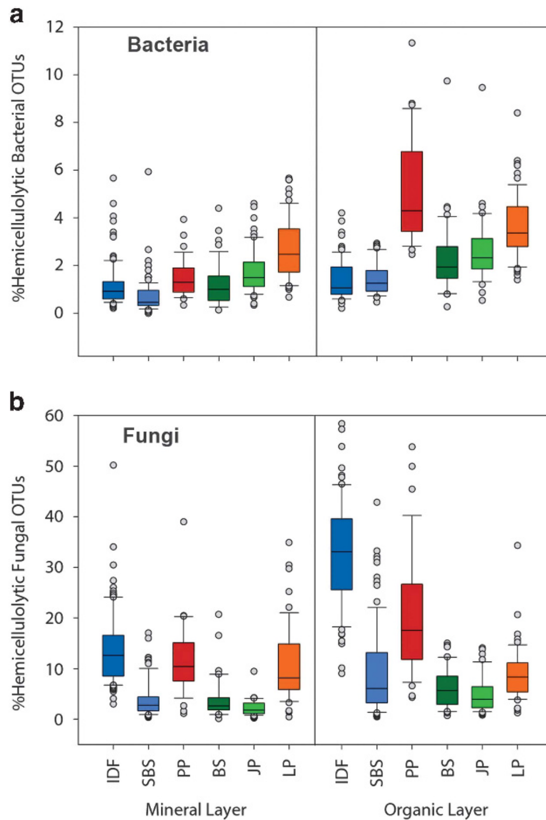

Figure 3 Proportions of hemicellulolytic OTUs in *in situ* pyrotag libraries.

and between soil layers, and PERMANOVA R^2 values indicate that ecozone explains 8% of the variability in the data, whereas soil layer explains 20% (Table 2). A greater proportion of the soil community is hemicellulolytic in the organic layer than that of the mineral layer for both bacterial and fungal populations ($P<0.001$, Wilcoxon signed-rank test).

Hemicellulolytic fungal OTUs were more unevenly distributed across ecozones than bacterial ones. The fungal OTUs constituted an average of 20% of the total fungal pyrotags from both organic and mineral soil layers between IDF and PP, but this value ranged from a high of 33% from the organic layer of the IDF ecozone to a low of 12% from the mineral layer of the PP ecozone (Figure 3b). Even those hemicellulolytic fungal OTUs most abundant overall were of low abundance or absent in several ecozones (Figure 5). With some exceptions, the greatest abundances occurred in the IDF and PP ecozones whose soil was used in the microcosms to identify the hemicellulolytic OTUs. In accordance with their patchy distributions, relative abundance profiles of hemicellulolytic fungal OTUs clustered strongly according to ecozones, and PERMANOVA indicates that ecozone explains 37% of the variability in the fungal OTU abundances, far more than explained by soil layer (Table 2).

Bacterial hemicellulolytic populations overlapped greatly between ecozones, whereas fungal ones did not. The relative abundances of hemicellulolytic bacterial OTUs in the IDF and PP ecozones correlated strongly with each other in both the organic and mineral layers ($R=0.81$ and 0.66, respectively). By contrast, the relative abundances of putatively hemicellulolytic fungal OTUs in those ecozones did not correlate well ($R=0.06$ and 0.07 in the organic and mineral layers, respectively).

Harvesting impacts on hemicellulolytic OTUs

Timber-harvesting treatments had relatively subtle effects on distributions of hemicellulolytic OTUs that varied among the IDF and PP ecozones. Harvesting treatments did not significantly affect the total abundance of hemicellulolytic OTUs (as a proportion of total pyrotags), either in the combined data set or when each soil layer of each ecozone was separately analyzed. And, harvesting treatments had no detectable effect on the evenness of hemicellulolytic OTUs. However, harvesting treatments had a relatively small but statistically significant impact on relative abundance profiles of bacterial and fungal hemicellulolytic OTUs. For both bacterial and fungal OTUs, canonical analysis of principal coordinates ordination revealed distinct clustering of the unharvested reference (OM0) and whole-tree harvesting plus forest floor removal (OM3) treatments, whereas stem-only (OM1) and whole-tree (OM2) harvesting treatments formed a third cluster (Figure 6). Consistent with this clustering, most misclassifications in the canonical analysis of principal coordinates analysis of bacterial populations occurred in the OM1 and OM2 treatments, indicating stronger separation strengths for the OM0 and OM3. In pairwise PERMANOVA comparisons, only bacterial profiles in OM1 and OM2 treatments were not significantly different (Table 2). Fungal profiles in OM1 and OM2 treatments were significantly different, but the effect size, based on the univariate t-statistic, is smaller than those in all other pairwise comparisons of fungal profiles. When profiles for each soil layer in each ecozone were separately examined, the magnitude of harvesting treatment effects on OTU profiles differed greatly, and those effects were not universally significant (Supplementary Figure S5,Supplementary Table S2). Overall, treatment effects tended to be greater for fungal versus bacterial profiles.

Relative abundances of most individual hemicellulolytic OTUs were not consistently affected by harvesting treatments on the basis of the Kruskal–Wallis test (with multiple comparisons). This was the case when the entire data set was analyzed and when each soil layer in each ecozone was separately analyzed. There were notable exceptions, including the hemicellulolytic OTU most abundant overall, OTU13 (*Burkholderia* sp.), whose abundance decreased in response to increasing organic matter

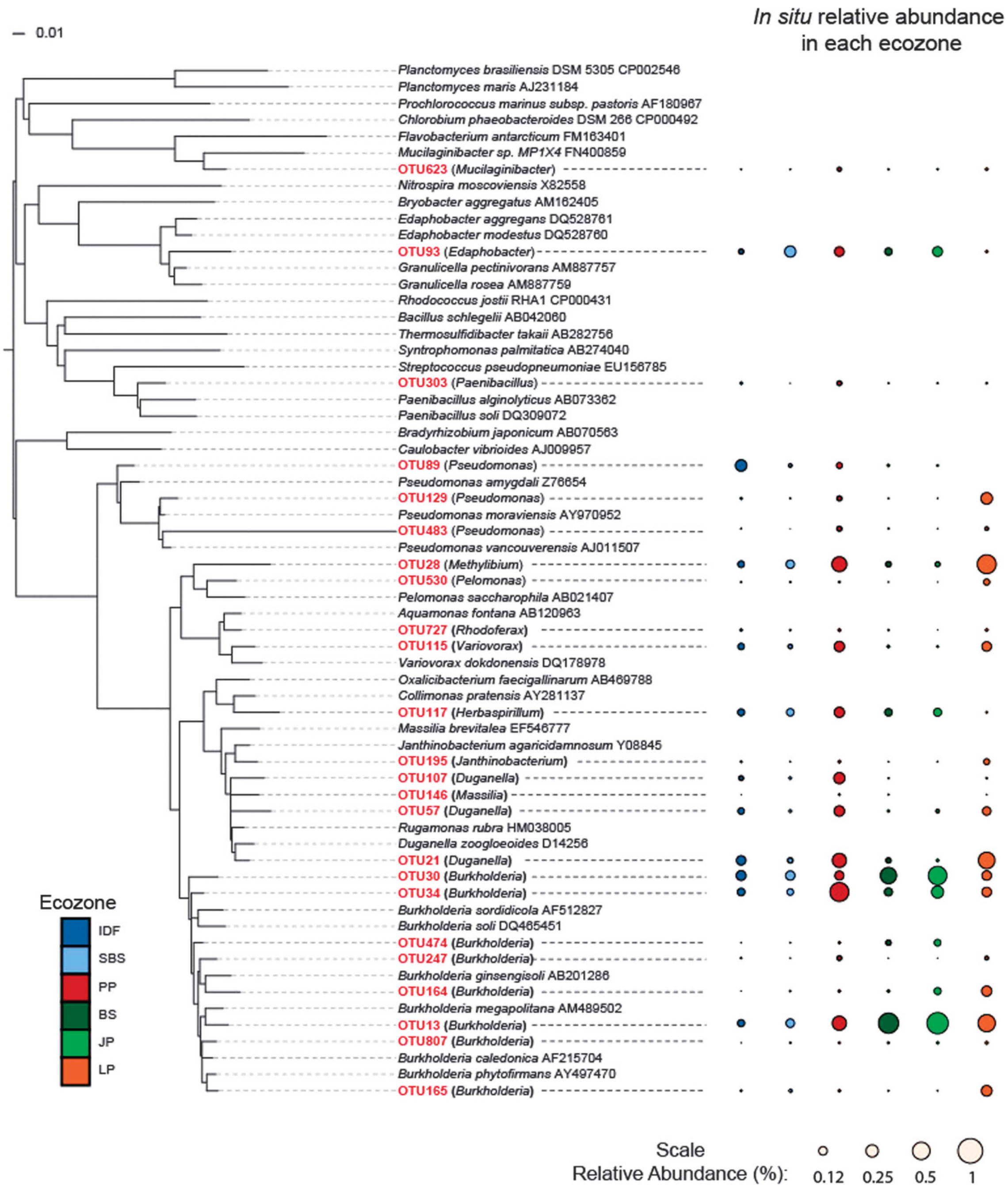

Figure 4 Neighbor-joining tree showing the taxonomic affiliations and *in situ* relative abundances of the predominant hemicellulolytic bacterial OTUs (red) having >0.01% total abundance in the total pyrotag data set. Reference sequences have GenBank accession numbers.

removal in three ecozones (Figure 7). As well, two of the most abundant fungal OTUs, OTU00305 (Unclassified Ascomycota) and OTU00119 (*Penicillium corylophilum*), were affected by harvesting, each in a single ecozone.

Discussion

This investigation identified 104 bacterial and 52 fungal hemicellulolytic populations in coniferous forest soil communities from two very different ecozones in North America. These populations are estimated to account for 2% of the bacterial and 10–30% of the fungal communities *in situ* and likely have important roles in lignocellulose decomposition and carbon cycling. Several lines of evidence support the reliability of the SIP method in identification of these populations. The populations identified were OTUs from highly ^{13}C-enriched DNA and were absent or much less relatively abundant in DNA of the same density from control microcosms with ^{12}C hemicellulose. The direct measurement of ^{13}C-enriched adenine and guanine

nucleotides by ultra-high performance liquid chromatography-tandem mass spectrometry with multiple-reaction monitoring (Wilhelm *et al.,* 2014) was invaluable in efficiently optimizing the microcosm experiments and ensuring high ^{13}C enrichment of DNA prior to sequence analysis. The strict criteria for identifying ^{13}C-enriched OTUs minimized false positives; however, as a consequence, some hemicellulose degraders may have been overlooked if (i) they grew very slowly or not at all in the microcosms, (ii) they were of low relative abundance or (iii) they have high GC content causing their DNA to occur in the heavy fractions of the ^{12}C control DNA. The short, 48-h, incubations limited the movement of label beyond the primary hemicellulose consumers via biomass turnover. However, we cannot exclude the possibility that we identified some 'cheaters' that assimilated hydrolysis products of hemicellulose without producing extracytoplasmic enzymes necessary to hydrolyze hemicellulose. The above caveats are unavoidable in SIP experiments. The correct identification of hemicellulose-degrading populations was directly confirmed in two cases, OTU30 and OTU1367 whose pyrotag sequences match those of strains isolated on xylan, a major constituent of hemicellulose (R. Wilhelm, unpublished data). Finally, many of the hemicellulose-assimilating OTUs belong to taxa previously reported to grow on hemicellulose, as described below.

The hemicellulolytic bacterial populations identified represent diverse taxa from four phyla (Figure 4). However, those populations most abundant *in situ* were mainly Proteobacteria. These include members of *Burkholderia* and *Pseudomonas,* two genera known to degrade a wide variety of organic compounds and previously implicated in lignocellulose degradation (Harazono *et al.,* 2003; Mohana *et al.,* 2008; Cheng and Chang, 2011). Both genera occupy diverse ecological niches, as soil and aquatic heterotrophs, plant mutualists as well as plant and animal opportunistic pathogens (Coenye and Vandamme, 2003; Salles *et al.,* 2004).

Table 2 For selected factors, effect sizes and explained variances on *in situ* in relative abundances of hemicellulolytic OTUs in the IDF and PP ecozones, based on PERMANOVA

Factor	*Bacteria*		*Fungi*	
	F/t	R^2	*F/t*	R^2
Ecozone	13.88***	0.082	74.69***	0.368
Soil Layer	31.06***	0.199	25.01***	0.123
Harvesting treatment	2.38***	0.016	4.00***	0.027
OM0 vs OM1	1.55**		1.44*	
OM0 vs OM2	2.00***		1.75**	
OM0 vs OM3	2.16***		2.19***	
OM1 vs OM2	1.11^{ns}		1.57*	
OM1 vs OM3	1.30^{ns}		2.59***	
OM2 vs OM3	0.86^{ns}		2.06***	
Ec. × layer	13.18***	0.161	5.94***	0.050
Ec. × treatment	1.83**	0.019	1.93**	0.017
Layer × treatment	1.89***	0.022	2.70***	0.033
Ec. × layer × treatment	1.19^{ns}	0.007	1.55^{ns}	0.017

Abbreviations: Ec., ecozone; ns, not significant. *$P<0.05$, **$P<0.01$, and ***$P<0.001$.
Test statistics include pseudo F-ratio (F) for main perMANOVA test, univariate t-statistic (t) for pairwise tests, and estimation of variance components (R^2).

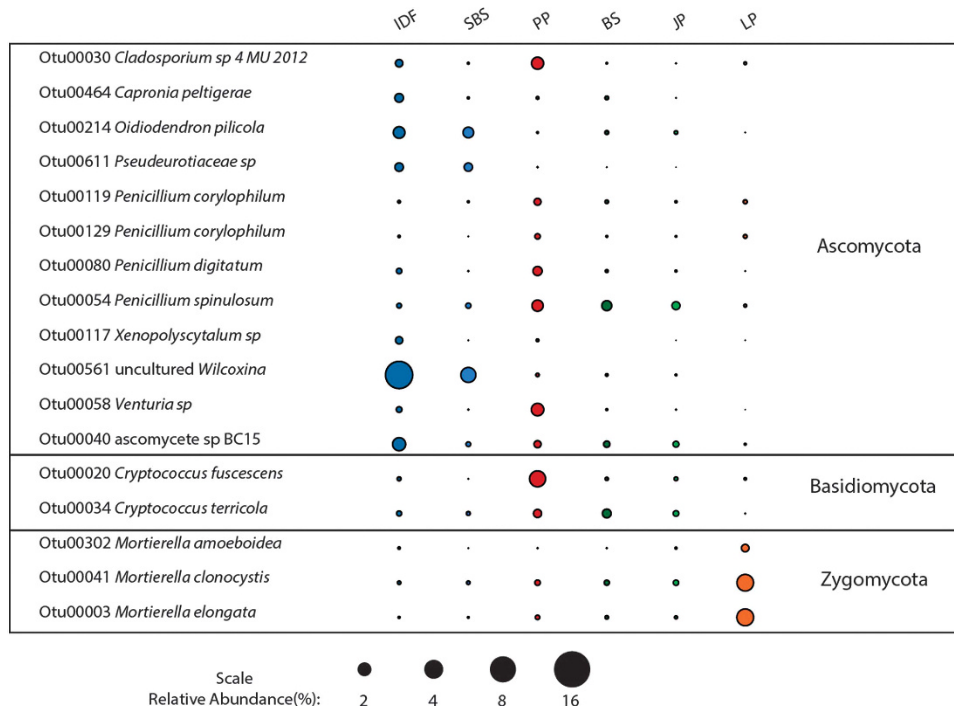

Figure 5 *In situ* relative abundances of hemicellulolytic fungal OTUs having >0.01% relative abundance in the total pyrotag data set.

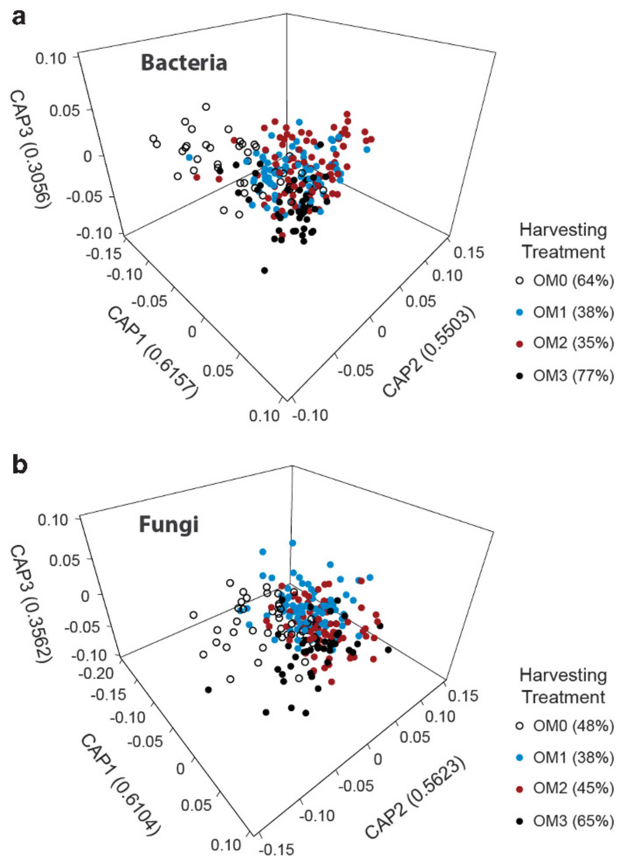

Figure 6 Canonical analysis of principal coordinates ordination based on Bray–Curtis dissimilarity of profiles of hemicellulolytic OTUs within pyrotag libraries from both soil layers of the IDF and PP ecozones. Differences among samples were maximized according to harvesting treatments. Classification success rates for each harvesting treatment are given in parentheses and the canonical correlation of each axis is given in parentheses.

The hemicellulolytic *Burkholderia* identified here are affiliated with plant-associated saprophytes belonging to the Group A *Burkholderia* lineage (Estrada-de los Santos *et al.,* 2013) as determined by RDP SeqMatch (Cole *et al.,* 2009). Additional populations identified belong to *Duganella* and *Variovorax* as well as the non-Proteobacteria, associated with *Mucilaginibacter* (Bacteroidetes) and *Paenibacillus* (Firmicutes). All four of these genera were previously reported to be involved in hemicellulose degradation (Ghio *et al.,* 2012; Maki *et al.,* 2012; Štursová *et al.,* 2012; Talia *et al.,* 2012; Khan *et al.,* 2013a, b). Another population identified is associated with *Granulicella,* a genus within the Acidobacteria, which is abundant in soil but poorly characterized because of a lack of cultured representatives. Members of Granulicella have been reported to grow on xylan (Pankratov and Dedysh, 2010). Other members of the Acidobacteria are reported to grow on hemicellulose (de Castro *et al.,* 2013), and genomic analysis has supported the proposition that polysaccharides are important substrates for Acidobacteria (Ward *et al.,* 2009; Rawat *et al.,* 2014). This is the first report of hemicellulolytic activity in Betaproteobacteria belonging to the genera, *Methylibium, Pelomonas* and *Rhodoferax, Methylibium,* which was abundant in all ecozones (Figure 4), was previously known to include facultative methylotrophs and able to utilize one-carbon substrates and aromatic compounds (Nakatsu *et al.,* 2006; Song and Cho, 2007). Genomes of members of three of these genera, *M. petroleiphilum* and *R. ferrireducens* and *Pelomas sp.,* reveal several enzymes that are predicted to act on hemicellulose (Supplementary Table S5).

The hemicellulolytic fungal populations identified also represent diverse taxa from three fungal phyla (Figure 5). These populations include members of *Mortierella* and *Cryptococcus*, genera previously known to be involved in hemicellulose degradation (Biely *et al.,* 1978; Morosoli *et al.,* 1993; Chávez *et al.,* 2006; Varnaitė and Raudonienė, 2008; Zeng *et al.,* 2013). A third genus identified, *Cladosporium,* is commonly found on both living and dead plant matter, and a xylanse from *Cladosporium cladosporioides* was isolated and characterized (Hong *et al.,* 2011). A fourth genus, *Wilcoxina* is ectendomycorrhizal, penetrating the root cells of its host plant. The previously reported capacity to slowly degrade pectin and cellulose was proposed to facilitate root penetration (Redlak *et al.,* 2001; Trevor *et al.,* 2001), and a hemicellulolytic capacity may further contribute to this. This is the first report of hemicellulolytic activity in Ascomycota belonging to *Venturia*, *Capronia*, *Xenopolyscytalum* and *Pseudeurotiaceae*. A study of 14 *Capronia* spp. found that they were unable to grow on cellulose or lignin and concluded that the group was unable to function as primary degraders of plant biomass (Untereiner and Malloch, 1999); however, our results suggest that this may not be universally true of *Capronia* spp.

The hemicellulolytic bacterial populations identified appear to be similarly distributed between IDF and PP, whereas the fungal populations are not. This appears to be true for the other four ecozones as well. The bacterial populations account for a similar proportion of the overall bacterial communities in all six ecozones, about two percent (Figure 3), and the most abundant of these populations generally have similar abundances across the ecozones (Figure 4). Furthermore, the strains isolated on xylan, which correspond to two hemicellulolytic populations identified by SIP, were isolated from multiple ecozones (jack pine and PP). By contrast, the fungal populations identified are most abundant in the IDF and PP ecozones (Figures 3 and 5), which were the sources of soil for the microcosms. Thus, it appears that distinct hemicellulolytic fungal populations dominate each ecozone. This pattern is consistent with the previously reported patchy distribution of total fungi in IDF and sub-boreal spruce ecozones and soils from other mixed coniferous forests (Kranabetter and Wylie, 1998; Tedersoo *et al.,* 2003; Hartmann *et al.,* 2012). Based on these observations, it might be possible to infer

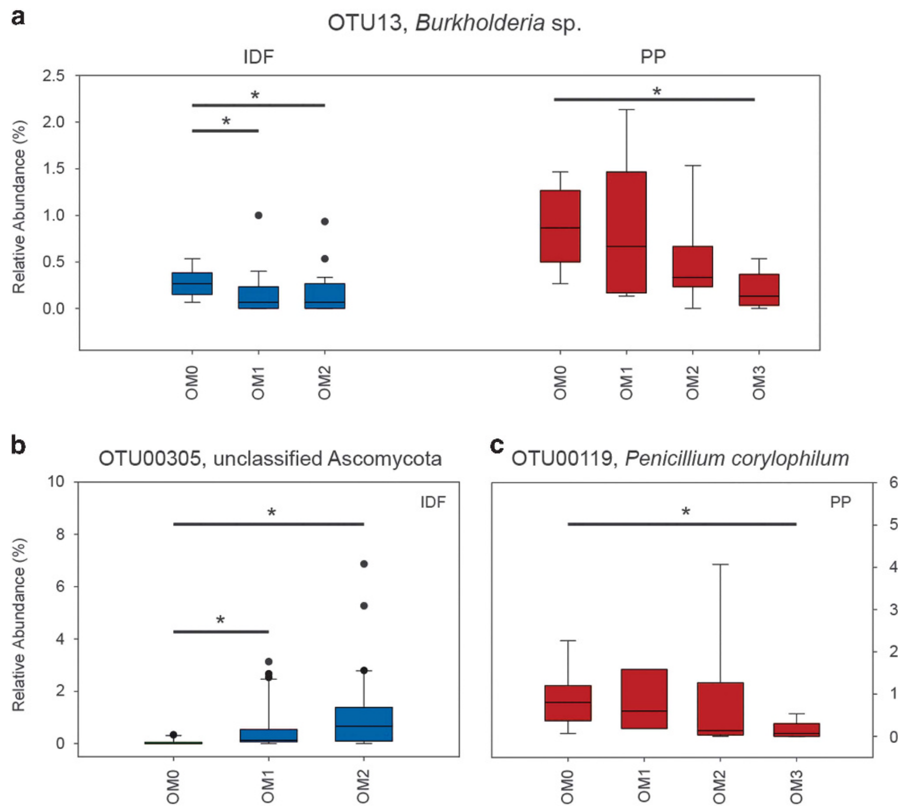

Figure 7 Effects of harvesting treatments on relative abundances of major hemicellulolytic OTUs in the IDF and PP ecozones (*bars denote significant pairwise differences, $P<0.05$).

distributions of hemicellulolytic bacterial populations in all six ecozones on the basis of the *in situ* pyrotag libraries, whereas it is possible to do so for hemicellulolytic fungal populations in only the IDF and PP ecozones.

A greater proportion of the organic versus mineral layer community, both bacterial and fungal populations, is hemicellulolytic in the IDF and PP ecozones. This difference is indicated by *in situ* pyrotag data (Figure 3) and the steeper slope for the organic versus mineral layer in the correlation of hemicellulose assimilation and total biomass (Figure 1). The pyrotag data indicate that bacterial hemicellulytic populations are slightly, but significantly more abundant in the organic layer, whereas fungal populations are almost three-times more abundant in the organic layer. This difference is consistent with the expectation that lignocellulose decomposition occurs to a greater extent in the organic versus mineral layer. However, our SIP-PLFA experiments demonstrate that the communities in the mineral layer are clearly capable of metabolizing hemicellulose. This is consistent with hemicellulose degradation associated with root decomposition in the mineral layer. Our results from microcosms do not necessarily reflect *in situ* rates of hemicellulose degradation, rather they reflect the size and metabolic potential of hemicellulolytic populations *in situ*.

Timber harvesting significantly affected hemicellulolytic populations in the IDF and PP ecozones in a manner that persisted for 10 or more years. However, these effects were limited to subtle shifts in community composition. Relative abundances of only a few populations were clearly affected by harvesting. Most notably, the most abundant bacterial hemicellulolytic population (OTU13), affiliated with *Burkholderia,* exhibited a consistent decrease in relative abundance with harvesting (Figure 7). Harvesting did not alter the proportion of the overall community, that is, hemicellulolytic, nor did it alter the evenness of hemicellulolytic populations. The magnitude of change due to harvesting was much smaller than community differences between soil layers and between ecozones (Table 2). Variable effects of harvesting on soil characteristics and tree regeneration have been observed throughout the LTSP study (Kranabetter *et al.,* 2006). Hemicellulolytic populations were more severely affected by harvesting in PP versus the IDF ecozone (Supplementary Figure S5). Interestingly, this is one of few ecozones in which tree regeneration was affected by harvesting method, being stimulated by increasing levels of OM removal (Fleming *et al.,* 2006; Ponder *et al.*, 2012). Of the six ecozones sampled for this study, the PP ecozone has an extreme precipitation regime, with the highest annual precipitation but the lowest precipitation

during the warmest season (Table 1). Harvesting effects, in terms of variability explained by harvesting treatments, on hemicellulolytic soil populations in the IDF ecozone are of a similar magnitude as previously reported effects on the overall bacterial and fungal community composition in that ecozone (Hartmann *et al.,* 2012). Thus, there is no indication that the hemicellulolytic populations were disproportionately affected by harvesting, as originally hypothesized.

Varying the level of OM removal during harvesting modulated the effect of harvesting on hemicellulolytic soil populations in the IDF and PP ecozones. This conclusion is supported by both canonical analysis of principal coordinates (Figure 6) and PERMANOVA (Table 2). Again, differences among the three harvested treatments amounted to subtle shifts in relative abundances. There were very few populations with consistent and significant responses to varied levels of OM removal, even when each soil layer of each ecozone was separately examined. In particular, differences were minimal between effects of stem-only harvesting (OM1) versus whole-tree harvesting (OM2). Stem-only harvesting is intended to leave some of the harvesting residue to maintain soil fertility and structure (Powers *et al.,* 2005). However, for soil hemicellulolytic populations, those residues did little to offset the disturbance associated with harvesting. Accordingly, previous studies have shown only small differences in the effects of OM1 versus OM2 on overall bacterial and fungal communities (Hartmann *et al.,* 2012) and small or no differences in effects on soil chemistry and tree growth (Ponder *et al.*, 2012). The OM3 treatment, involving removal of whole trees plus forest floor, is a very severe disturbance, which was previously found to impact the overall soil microbial community due to the loss of nutrients and habitat (Simard *et al.,* 2003; Hartmann *et al.,* 2012). This treatment eliminated all above-ground woody biomass and was expected to have an extreme effect on hemicellulolytic populations. Of course, the hemicellulolytic populations in the organic layer were removed by the OM3 treatment, but the effect on those in the mineral layer was comparable to the change associated with OM1 and OM2. In those ecozones where the organic layer had regenerated sufficiently in the OM3 treatment to permit sampling (PP and loblolly pine), hemicellulolytic populations were affected by harvesting to a larger extent than those in OM1 and OM2 treatments (Supplementary Table S2).

Varying the level of OM removal during harvesting did not detectably alter potential rates of hemicellulose degradation and assimilation (Supplementary Figure S4). Thus, the observed differences in hemicellulolytic population structure among harvested treatments did not appear to change the hemicellulolytic potential of the community. In accordance with this, most LTSP Study sites so far lack detectable effects of harvesting treatments on tree regeneration or soil properties (Conlin and Driessche, 2000; Powers *et al.,* 2005, 2012). Timber-harvesting effects on hemicellulose degradation have never been directly investigated, but litter decomposition has previously been reported to be unaffected by harvesting in the IDF and sub-boreal spruce ecozones (Prescott *et al.,* 2000). However, tree regeneration at these sites is in an early phase, and as the trees grow, changing factors will limit that growth. It would be informative to monitor the soil communities over the long term to determine whether the relatively subtle effects of harvesting on the soil community ultimately correspond to effects on forest regeneration. It would be also be informative to determine whether the soil communities in harvested treatments eventually converge with those of the corresponding references.

Overall, this study identified major hemicellulolytic bacterial and fungal populations in forest soils, and showed that the bacterial populations, but not the fungal ones, are similarly distributed across multiple ecozones. Forest harvesting caused long-term changes to the structure of hemicellulolytic populations in the IDF and PP ecozones, but it did not appear to substantially alter the total biomass or metabolic potential of hemicellulolytic microorganisms. It remains to be determined whether changes in community structure due to harvesting eventually have consequences for ecosystem processes that affect forest productivity or resilience to disturbance.

Conflict of Interest

The authors declare no conflict of interest.

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