SYNERGISTIC DEGRADATION OF LIGNOCELLULOSE BY

FUNGI AND BACTERIA IN BOREAL FOREST SOIL

By

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SYNERGISTIC DEGRADATION OF LIGNOCELLULOSE BY FUNGI AND BACTERIA IN BOREAL FOREST SOIL

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Abstract

Boreal forests contain an estimated 28% of the world's soil carbon, and currently act as a significant global carbon sink. Plant-derived lignocellulose is a major component of soil carbon, and its decomposition is dependent on soil bacteria and fungi. In order to predict the fate of this soil carbon and its potential feedbacks to climate change, the identities, activity, and interactions of soil microbial decomposer communities must be better understood. This study used stable isotope probing (SIP) with ^{13}C-labeled lignocellulose and two of its constituents, cellulose and vanillin, to identify microbes responsible for the processing of lignocellulose-derived carbon and examine the specific roles that they perform. Results indicate that multiple taxa are involved in lignocellulose processing, and that certain taxa target specific portions of the lignocellulose macromolecule; specifically, fungi dominate the degradation of lignocellulose and cellulose macromolecules, while bacteria scavenge aromatic lignocellulose monomers. Major fungal taxa involved in lignocellulose degradation include *Ceratobasidium, Geomyces,* and *Sebacina,* among others. Bacterial taxa processing lignocellulose and cellulose included *Cellvibrio* and *Mesorhizobium* in high abundance relative to other taxa, although *Burkholderia* were the primary vanillin consumers. These results elucidate some of the major players in lignocellulose decomposition and their specific roles in boreal forest soil. This information provides knowledge of small-scale microbial processes that dictate ecosystem-level carbon cycling, and can assist in predictions of the fate of boreal forest carbon stocks.

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This work is dedicated to Stellan and Violet Burgess, the joys of my life,

in the hopes that the effort put forward will help to enrich theirs.

Introduction

Terrestrial forest ecosystems are a large carbon sink, with estimates of carbon inputs up to 2.4 ± 0.4 Pg of carbon per year globally (Pan *et al.*, 2011). Boreal forests comprise roughly one third of terrestrial forests, and an estimated 28% of the world's soil carbon is stored in boreal forest soils (McGuire *et al.*, 1997) not including carbon stored in permafrost. These ecosystems typically exhibit relatively slow decomposition, allowing accumulation of partially-degraded lignocellulosic plant material in surface soils. However, changes in temperature and moisture associated with climate change may alter the decomposition rates of these ecosystems, with uncertain outcomes (Davidson and Janssens, 2006; Natali *et al.*, 2011). For example, a decrease in soil moisture may slow decomposition rates while temperature increases are expected to increase rates of decomposition, reducing the effectiveness of forest soils as a carbon sink and potentially shifting them to a net source (Davidson and Janssens, 2006; Vanhala *et al.*, 2008; Karhu *et al.*, 2010). The decomposition of lignocellulose, and the resulting release of carbon dioxide and methane, is dependent on soil microbes. Therefore, an understanding of small-scale activities and interactions that influence carbon turnover is essential to understanding and predicting feedbacks to climate change (Azam and Malfatti, 2007; Reed and Martiny, 2007; Bardgett *et al.*, 2008; Castro *et al.*, 2010). Microbial community composition and diversity have strong effects on the rate of decomposition of leaf litter (Hattenschwiler *et al.*, 2005; Strickland *et al.*, 2009), demonstrating the importance of understanding the identity, functional activity, and community assemblage of microbes in soil.

It has been hypothesized that specific microbial guilds are responsible for degrading different litter fractions. The most labile substances in litter (e.g., sugars, amino acids, cellulose, hemicellulose) are degraded before the more recalcitrant molecules (e.g., lignin). Several

laboratory experiments have provided evidence in support of this hypothesis in both terrestrial and aquatic environments (Romani *et al.*, 2006; Hanson *et al.*, 2008; Strickland *et al.*, 2009; Fukasawa *et al.*, 2010; Snajdr *et al.*, 2010). However, most of this evidence is indirect or does not identify specific taxa that carry out particular roles. Moorhead and Sinsabaugh (2006) proposed a mathematical model that incorporates three microbial guilds: opportunists, which rapidly consume soluble compounds and intermediate metabolites; decomposers, which degrade lignocellulose and cellulose extracellularly; and miners, which degrade humified organic matter. The structure of lignocellulose is such that lignin often prevents access to cellulose and hemicellulose (Osono, 2007), therefore, microbial taxa that preferentially target cellulose, hemicellulose, and lignin metabolites may rely upon lignin-degrading microbes before they are able to completely degrade these substrates. In other words, microbial consortia degrading lignocellulose may include primary degraders as well as other organisms that benefit from these activities and obtain labile carbon made available as lignin is degraded by extracellular enzymes (Allison, 2005; Schneider *et al.*, 2010).

The current understanding of lignocellulose decomposition suggests that fungi dominate the degradation of lignocellulose while bacteria, though often capable of degrading cellulose, act primarily as secondary scavengers of cellulose and lignin monomers produced by the activity of fungi. Romani *et al.* (2006) found that bacterial decomposers grew poorly when introduced to sterile leaf litter alone compared to when sterile litter was inoculated with both bacteria and fungi, suggesting that bacteria rely on fungal activity to provide carbon and energy sources; these results were mirrored in other studies (Allison, 2005; Schneider *et al.*, 2010; Schneider *et al.*, 2012). However, recent evidence indicates that this hypothesis may not hold in all soil types or fractions or in fresh leaf litter (Eichorst and Kuske, 2012; Šturšová *et al.*, 2012).

Much of the taxonomically explicit knowledge that we currently have about microbial communities and lignocellulose decomposition comes from culture-based laboratory studies or analyses of microbial community structure in decomposing litter. However, less than 1% of microbes are able to grow in culture (Torsvik and Øvreås, 2002), and total community analyses do not reveal which microbes perform certain functions. Stable isotope probing (SIP) is a culture-independent method of studying microbial communities that is able to track stable isotopes of carbon or other elements from specific substrates into microbial DNA or other biomarkers. In this way, SIP is able to link function to individual taxa and microbial community structure (Radajewski *et al.*, 2000; Kreuzer-Martin, 2007; Neufeld *et al.*, 2007; Uhlik *et al.*, 2009). While several studies have performed SIP or similar techniques with plant-based substrates, the majority of these have used cellulose (Haichar *et al.*, 2007; Eichorst and Kuske, 2012; Šturs ová *et al.*, 2012), or ^{13}C-labeled plant litter (Moore-Kucera and Dick, 2008; Lee *et al.*, 2011; Shrestha *et al.*, 2011) which includes a variety of carbon based substrates (e.g., glucose, proteins, amino acids, DNA, etc.) in addition to lignocellulose and its constituents. Other studies have focused exclusively on either fungi (Hanson *et al.*, 2008) or bacteria (Haichar *et al.*, 2007; Lee *et al.*, 2011; Shrestha *et al.*, 2011). These studies have identified major microbial degraders of plant material in various environments at different levels of substrate specificity and taxonomic diversity. While these studies have been invaluable to furthering our understanding of the microbial community responsible for the degradation of plant matter in soils, they are not able to effectively elucidate the complex, interactive roles that bacteria and fungi may play in the degradation of lignocellulose or to identify specific microbes carrying out particular roles in degradation.

The purpose of the current study was to identify specific microbes belonging to particular microbial decomposer guilds in boreal forest soils, and to elucidate some of the interactions between boreal forest microbes involved in lignocellulose degradation. To address these goals, we designed a SIP experiment using ^{13}C-labeled lignocellulose and two of its constituents, cellulose and vanillin, incubated in separate, parallel microcosms containing aliquots of the same boreal forest soil. Microcosms supplied with a particular substrate were harvested over a time course. Microbial opportunists that are able to quickly utilize labile substrates were expected to incorporate the label earlier during cellulose and vanillin incubations, but also at later time points in lower abundance in incubations of lignocellulose due to the slow release of labile compounds made available by primary decomposers. With extended incubation times, carbon flows out into the community, revealing microbes involved indirectly in lignocellulosic carbon processing. SIP results were compared to direct community analyses of bacteria and fungi colonizing buried birch tongue depressors. This work provides insight into the identity and interactions of microbes responsible for carbon turnover in boreal forest soils.

Methods

Soil and birch tongue depressor samples

A single sample (approximately 1 kg) of organic horizon soil was collected from a mid-successional upland boreal forest near Fairbanks, Alaska in the Bonanza Creek Long Term Ecological Research site (BNZ LTER, site designation UP2A, 64.695 N, 148.356 W) on 8 November 2007. The stand was approximately 100 years old and comprised primarily of a mix of white spruce and Alaska paper birch, with scattered trembling aspen and a few balsam poplar. Complete descriptions of the BNZ LTER and site UP2A can be found at

http://www.lter.uaf.edu/bnz_climate.cfm and http://www.lter.uaf.edu/site.cfm?site_pkey=35. The sample was stored at 4 ºC for 4 months before use in the SIP experiment. Sub-samples of this soil were homogenized and used to construct SIP microcosms without removing roots or other organic material.

Birch tongue depressors (BTDs) were buried in the forest floor at multiple locations throughout site UP2A for 12 months as part of a separate study of decomposition rates (Runck, 2008), and were used in our study to compare SIP communities with communities on decomposing wood *in situ*. Adhering soil was scraped from the BTDs at collection. The BTDs were stored at -20 ˚C until sub-samples were taken, and subsamples were stored at -20 ˚C in sterile plastic bags until DNA was extracted.

Stable isotope probing microcosms

^{13}C lignocellulose, cellulose, and vanillin (97 atom% ^{13}C, each) were purchased from ISOTEC (Miamisburg, OH, USA). Soil and ^{13}C compounds were handled using aseptic techniques during measurements and throughout microcosm setup. Lignocellulose SIP microcosms contained 0.025 g of ^{13}C-lignocellulose mixed directly with 2.5 g of soil. Cellulose SIP microcosms contained 0.05 g of ^{13}C-cellulose and 2.5 g soil. Vanillin was dissolved in acetone rather than water to achieve concentrations necessary to ensure that enough vanillin was provided for SIP without over-hydrating the soil. For vanillin SIP microcosms, 7.5 mg of ^{13}C-vanillin was dissolved in 15 mL of acetone, and 0.5 mL of this solution was added to serum bottles containing 2.5 g of sterile sand, for a total of 25 µg ^{13}C-vanillin per microcosm. Serum bottles were left open in a sterile laminar flow PCR hood for a period of 24 hours to allow the acetone to evaporate, leaving the vanillin behind, at which point 5 g of soil were added to the

bottles and mixed with sand by shaking and vortexing. Serum bottles were sealed with a Teflon® stopper and aluminum crimp top. An aliquot of soil without added ^{13}C substrates was frozen at -80 °C to serve as a time zero (T0) sample that served as a background control.

Microcosms were incubated in the dark at 23 ± 2 °C and were destructively harvested over a over a time course of 14, 28, 42, and 56 days for lignocellulose microcosms (samples L14, L28, L42, and L56); 7, 14, 21, and 28 days (C7, C14, C21, and C28) for cellulose microcosms; and 1, 4, 7, and 14 days (V1, V4, V7, V14) for vanillin microcosms. Soils were stored at -80 °C until DNA extraction. Time courses were selected based on predicted rates of degradation for each compound.

Isotopic analyses of headspace gas samples

To confirm substrate utilization, the production of $^{13}CO_2$ at the time of harvest was measured for each microcosm. A 1-mL headspace gas sample was collected through the stopper with a sterile needle and syringe and injected into an airtight 12 mL gas sampling tube previously purged with ambient lab air at the time that microcosms were sealed. Stable isotope analysis was performed by the Alaska Stable Isotope Facility (ASIF) at the Water & Environmental Research Center at the University of Alaska Fairbanks. Stable isotope ratios were reported in δ notation as parts per thousand (‰) deviation from the international standards PDB.

Soil and birch tongue depressor DNA extraction

We extracted DNA from microcosm soils using the Bio101 Fast DNA Spin Kit for soil (MP Biomedicals, Solon, Ohio). Roots, leaf litter, and other debris were not excluded.

The BTD DNA extractions were performed with MoBio PowerSoil DNA extraction kit (MO BIO Laboratories, Inc, Carlsbad, California). Between 0.05 and 0.10 g of BTD clippings were cut into small pieces using a sterile razor blade. DNA was extracted following kit instructions, except that instead of vortexing, BTD clippings with MoBio beads and buffer were more vigorously shaken using a FastPrep Mini-beadbeater instrument (BioSpec Product Inc., Bartlesville, Oklahoma) in order to ensure that the woody material was broken up sufficiently.

^{13}C-DNA isolation

To separate ^{13}C-DNA from unlabeled ^{12}C-DNA, equilibrium (isopycnic) density gradient centrifugation followed by fractionation of the density gradient and precipitation and resuspension of DNA was conducted as previously described (He *et al.*, 2012). In order to determine the distribution of DNA in density gradient fractions, qPCR targeting 16S rRNA genes was performed on every fraction in duplicate as previously described (Leigh *et al.*, 2007). This procedure was also used to target the fungal internal transcribed spacer (ITS) region using primers ITS1-F (Gardes and Bruns, 1993) and 5.8S (Vilgalys and Hester, 1990), with *Saccharomyces cerevisiae* DNA used to generate standard curves. Based on qPCR results, fractions containing ^{13}C-labeled (heavy) DNA spanned the buoyant density range of 1.581-1.620 g/mL (Figure 1). Fractions within this range were pooled for each time point and then used for subsequent analyses. Time zero (T0) heavy samples were also pooled; sequences detected in these samples were treated as background DNA contamination and were omitted from analyses when also detected in ^{13}C-DNA clone libraries or T-RFLP or ARISA profiles.

16S rRNA and ITS clone libraries

Clone libraries were made from PCR products from BTD and total soil community (TC) samples as well as pooled heavy fractions from T0, L28, L42, L56, C14, C28, and all vanillin time points (V1-V14). 16S rRNA gene PCR products were generated as previously described using 27F and 1392R (Leigh *et al.*, 2007) for all samples except cellulose SIP, which were generated with primer 529R (Fields *et al.*, 2006) instead of 1392R. ITS PCR products were generated using forward primer ITS1-F (Gardes and Bruns, 1993) and reverse primer ITS4 (White *et al.*, 1990) with thermal cycler conditions as described by Bent *et al.* (2011). Both 16S rRNA and ITS PCR products were used to generate clone libraries as previously described (Leigh *et al.*, 2007) except the gel purification step was omitted.

Clone libraries were screened to verify the presence and size of insert using PCR with primers M13F and M13R. Clones producing M13 PCR products of the expected size were sequenced. Clone libraries from BTDs, TC and cellulose were shipped to Macrogen, Inc. (Seoul, Korea) for plasmid purification followed by single extension Sanger sequencing with primer 27F for bacteria or ITS1-F for fungi. For vanillin and lignocellulose, M13 PCR products were shipped to Macrogen for direct sequencing using the same primers.

Bacterial 16S rRNA gene sequence analysis

AB1 sequence files were processed using the Ribosomal Database Project II (RDPII) pipeline (www.rdp.cme.msu.edu) with base-calling and masking at Q10 by PHRED, quality trimming and vector removal done by LUCY with cutoffs set at a minimum of 60% bases at Q20 and a minimum sequence length of 200 bp for further analyses. Chimeras were removed using DECIPHER (Wright *et al.*, 2011) with the full sequence setting. Remaining sequences were

clustered into operational taxonomic units (OTUs) with CAP3 (Huang and Madan, 1999) using a 97% identity cutoff. OTUs that were found in T0 heavy fractions were assumed to be background sequences and were omitted from further analysis. The sequences were phylogenetically identified using RDPII Classifier and Sequence Match. Taxonomic assignments scoring less than 80% confidence in RDP Classifier were considered unclassified at that level.

Fungal ITS sequence analysis

Fungal AB1 files were loaded into the program CodonCode Aligner, which was used to call bases and assign PHRED quality scores. Sequences less than 150 bp in length or with less than 25 bases of Q20 or greater were discarded. Remaining sequences and their quality scores were exported. These files were used to mask bases (with N's) of Q<10 using a Perl script on the on the Fungal Metagenomics Project (FMP) website (http://www.borealfungi.uaf.edu/) (Taylor and Houston, 2011). Both ends of the sequences were trimmed using the EMBOSS program TrimSeq (http://imed.med.ucm.es/cgi-bin/emboss.pl?_action=input&_app=trimseq) using a window size of 40 and 3% ambiguity. Sequences with >2% N's and sequences less than 200 bp were removed using Purge on the FMP. Remaining ITS sequences were grouped into OTUs at 97% sequence identity using CAP3 (Huang and Madan, 1999) on the FMP. The longest high quality sequence in each OTU was selected as a representative sequence. To build phylogenetic trees and identify fungi, top BLAST hits with a taxon name were downloaded and aligned with the representative sequence in MUSCLE (Edgar, 2004) before building trees with RAxML (Stamatakis, 2006). Trees were viewed in FigTree v1.4.0 and taxonomic identity of OTUs was

assigned based on the clade in which that sequence was included at a bootstrap threshold of 80% and maximum branch length of 0.03 for species.

Community profiling/Fingerprinting

In order to obtain a more comprehensive view of the degrader communities and verify the relative quantities of cloned sequences, terminal restriction-fragment length polymorphism (T-RFLP) and fungal automated rRNA intergenic spacer analyses (F-ARISA) were performed on heavy DNA fractions. These analyses were performed on PCR products obtained from total soil DNA, including the pooled heavy density gradient fractions from each time point (including T0) and the pooled DNA extracts of ten different BTDs. F-ARISA was performed as previously described (Bent and Taylor, 2010). PCR was performed under the same conditions as cloning, except the forward primers (ITS1-F and 27F) were FAM labeled. For T-RFLP, digestion, precipitation, and analysis were performed as previously described (He *et al.*, 2012).

Results

Isotopic analyses of headspace gas samples

An increase in the $\delta^{13}C$ in all of the microcosms relative to time zero was observed, indicating that carbon from the substrates was being respired and the substrates were being degraded (Table 1).

Table 1 Accumulation of $^{13}CO_2$ in microcosm headspace Soil microcosms were amended with ^{13}C-lignocellulose, ^{13}C-cellulose, and ^{13}C-vanillin. Units are parts per million. Time 0 readings are provided for comparison. NA = not analyzed since microcosms containing different substrates were harvested on different time courses.

Incubation time (d)	δ-^{13}C VPDB (o/oo) of headspace gas during incubation		
	^{13}C-Lignocellulose	^{13}C-Cellulose	^{13}C-Vanillin
0	-12.0	-2.7	-8.6
1	NA	NA	337.6
4	NA	NA	14810.0
7	NA	11179.3	16451.1
14	22297.6	11254.9	7218.7
21	NA	20333.3	NA
28	23164.4	25388.7	NA
42	21188.3	NA	NA
56	19857.7	NA	NA

Quantitative, Real-time PCR (qPCR)

Based on qPCR results, fractions containing ^{13}C-labeled (heavy) DNA spanned the buoyant density range of 1.581-1.620 g/mL (Figure 1). Fractions within this buoyant density range from all incubations (excluding T0 samples) contained bacterial 16S rRNA sequences. Fungal ITS sequences were detected in heavy fractions from both lignocellulose and cellulose incubations. However, heavy fractions from vanillin incubations did not contain detectable concentrations of fungal DNA.

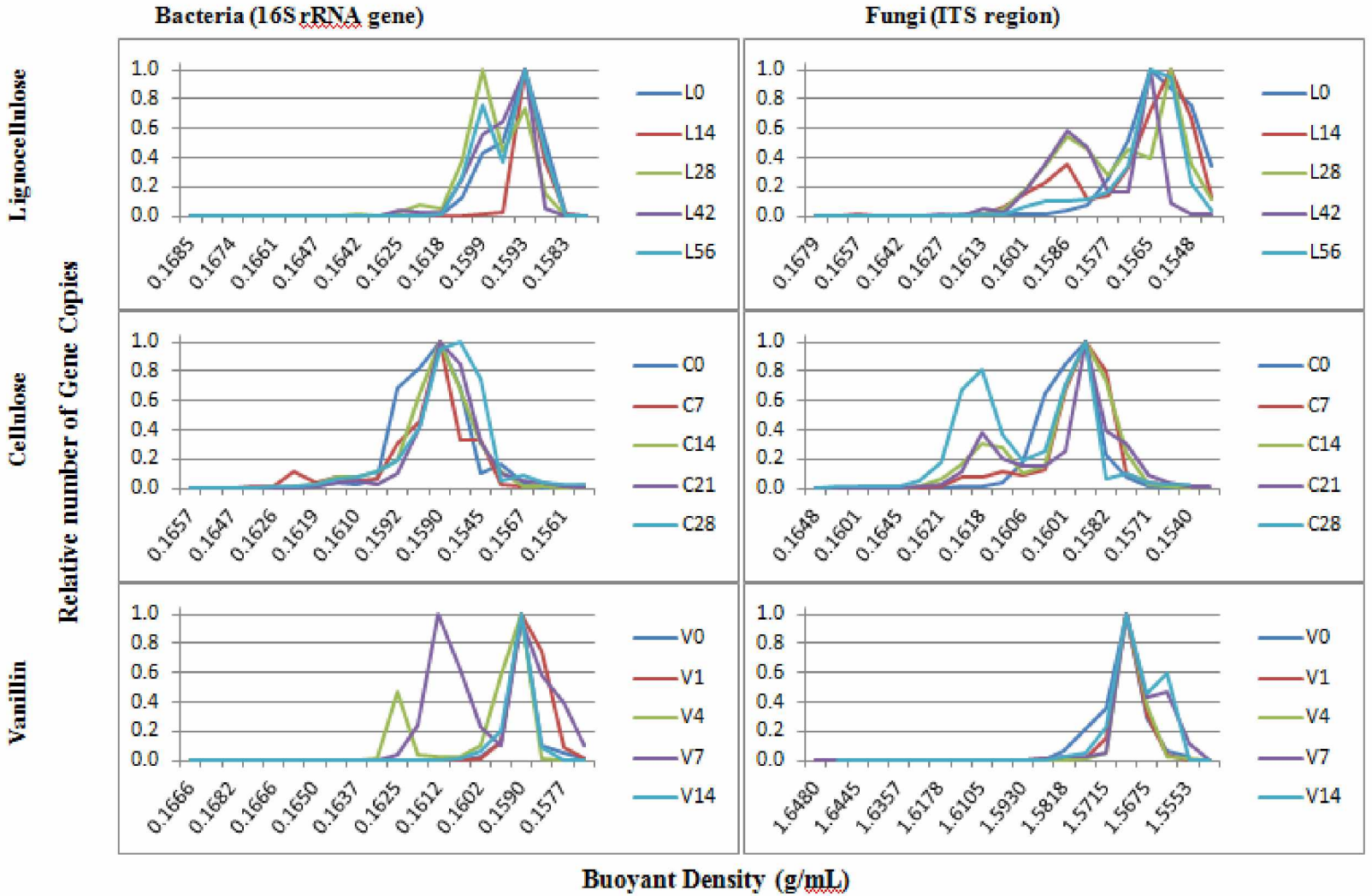

Figure 1 Detection of ^{13}C-labeled DNA with qPCR. Ratio of 16S rRNA (bacterial; left) and ITS (fungal; right) gene copies detected by qPCR in density gradient fractions from stable isotope probing microcosms using lignocellulose constituents as the labeled substrate. Top: Lignocellulose SIP incubations. Middle: Cellulose SIP incubations. Bottom: Vanillin SIP incubations. Numbers indicate the number of days that each incubation ran before harvesting.

Bacterial 16S rRNA sequence analysis

A single bacterial OTU (bOTU) (16S-OTU153) was most abundant in both the lignocellulose and cellulose clone libraries and was present in the earlier time points for both substrates (9.04% and 5.81% of L28 and C14, respectively; Table 2). The sequence was placed in the genus *Cellvibrio*, and the closest SeqMatch hit indicates the species is *Cellvibrio gandavensis* with a similarity score of 0.993 (Table 2).

The two bOTUs in highest abundance in vanillin SIP libraries were 16S-OTU211, comprising 27.5% of all the sequences detected in the V4, 70.3% of all of the sequences detected in V7, and 10% of V14 sequences, and 16S-OTU19, which comprised 24.8% of V4 sequences, 11.6% of V7, and 48.3% of V14 (Table 2). RDP classifier placed both of these bOTUs in *Burkholderia*; the best SeqMatch for 16S-OTU211 was *Burkholderia* sp. SILP5 while 16S-OTU19, was classified as *Burkholderia phytofirmans* (Similarity = 0.993). *B. phytofirmans* was also detected once in L42 (Table 2).

A total of 72 bOTUs were detected in lignocellulose ^{13}C DNA clone libraries (Table 2). Eleven of these were also detected in cellulose libraries, while 9 were detected in vanillin heavy fractions (Table 2).

Seven bOTUs were shared among all 3 substrates. The most abundant of these were 16S-OTU208 and 16S-OTU200. RDP grouped these bOTUs with *Mesorhizobium* and *Dongia*, respectively. In the TC clone library, 3.7% of the sequences belonged to 16S-OTU200 (Table 2).

Six of the bOTUs found in SIP libraries were also detectable in the total community; 5 of these were detected in lignocellulose libraries and 3 were shared by all substrates. A total of 18 bOTUs were present in the BTD clone library. Twelve of these were detected in heavy fractions from SIP clone libraries. Three were also found in the TC library, 2 of which were shared

Table 2 Selected bacterial OTUs detected in ^{13}C-DNA clone libraries. 16S rRNA gene DNA was extracted from stable isotope probing microcosms amended with ^{13}C-lignocellulose, cellulose, or vanillin. Some OTUs were also found in the total community (TC) and/or in DNA extracted from birch tongue depressors (BTD) The percentage of OTU sequences detected in each time point is shown, with the total number of sequences reported below.

			^{13}C-DNA									^{12}C-DNA	
			Lignocellulose			Cellulose		Vanillin				TC	BTD
Class, Family		Genus	L28	L42	L56	C14	C28	V1	V4	V7	V14		
Actinobacteria													
Acidimicrobineae	OTU111	---		0.61%		1.16%		0.87%				1.23%	1.15%
Acidimicrobineae	OTU21	---				1.16%	1.11%	3.48%					
Streptomycetaceae	OTU150	*Streptomyces*	1.60%	0.61%	2.50%								
Streptomycetaceae	OTU148	*Streptomyces*	1.06%	1.21%				0.87%			0.56%		
Proteobacteria (phylum)													
Unclassified	OTU103	---	1.60%			2.33%	1.11%		0.39%				1.15%
Alphaproteobacteria													
Bradyrhizobiaceae	OTU52	*Rhodopseudomonas*	0.53%			2.33%							
Phyllobacteriaceae	OTU208	*Mesorhizobium*	2.13%	0.61%			2.22%	1.74%					
Rhizobiaceae	OTU105	*Rhizobium*	0.53%				1.11%						3.45%
Rhodospirillaceae	OTU200	*Dongia*	1.06%				1.11%				0.56%	3.70%	
Sphingomonadaceae	OTU87	*Sphingomonas*				2.33%	2.22%				1.11%		
Rhizobiales (order)	OTU138	---			1.25%	1.16%		0.87%			0.56%		
Rhizobiales (order)	OTU161	---		0.61%			2.22%	1.74%					
Unclassified	OTU188	---	1.06%		1.25%			0.87%			0.56%	1.23%	
Betaproteobacteria													
Burkholderiaceae	OTU19	*Burkholderia*		0.61%					24.81%	11.61%	48.33%		
Burkholderiaceae	OTU211	*Burkholderia*							27.52%	70.32%	10.00%		
Gammaproteobacteria													
Pseudomonadaceae	OTU153	*Cellvibrio*	9.04%	1.82%		5.81%	2.22%						
Sinobacteraceae	OTU139	*Steroidobacter*		0.61%	1.25%							3.70%	
Xanthomonadaceae	OTU180	*Luteibacter*		1.82%	1.25%		2.22%	0.87%	0.39%			1.23%	1.15%
Opitutae													
Opitutaceae	OTU92	*Opitutus*		1.21%		1.16%	3.33%						
TOTAL # of Sequences in each time point			188	165	80	86	90	115	258	155	180	81	87

between all SIP substrates (Table 2). In summary, several bOTUs were shared among all 3 substrates, while fewer were shared between BTD libraries and substrate libraries.

Two bOTUs were shared among BTDs, TC, and at least one time point from all SIP substrates. The representative sequence for 16S-OTU111 was placed in the Acidimicrobineae family but was unidentifiable at the genus level, and 16S-OTU180 matched with *Lutiebacter rhizovicinus* (Similarity = 0.999; Table 2).

Fungal ITS sequence analysis

A total of 24 fungal OTUs (fOTUs) and 35 singletons were detected in lignocellulose clone libraries, while 18 fOTUs and 29 singletons were detected in cellulose libraries. Of the fOTUs detected, nine were found in both lignocellulose and cellulose libraries. No fungal DNA was amplified from vanillin heavy fractions.

The most abundant fOTU detected in lignocellulose incubations was ITS-OTU48, which was identified as *Ceratobasidium sp*. This fOTU accounted for almost half of the ^{13}C-labeled fungal sequences detected in L56, although it was not detected in the earlier lignocellulose time points. ITS-OTU48 was also detected in C14 and C28 at 2.7% and 9.3%, respectively.

The two most abundant fOTUs detected in cellulose incubations were ITS-OTU42, which comprised 26% of all the ITS sequences detected in C28, and ITS-OTU43, which accounts for 25.3% of the ITS sequences in C28 (Table 3). ITS-OTU43 was also detected in high abundance in C14, L28 and L42 (Table 3). Phylogenetic trees placed ITS-OTU43 in the genus *Sebacina* with 99% bootstrap support. ITS-OTU35 and ITS-OTU21 form a clade with ITS-OTU43 within this genus. ITS-OTU21 was detected in low abundance in L28 and L42, while ITS-OTU35 was the second most abundant fOTU in lignocellulose SIP libraries, comprising 21% of L28, 15% of

Table 3 Selected fungal OTUs detected in ^{13}C-DNA clone libraries. Fungal ITS region DNA was extracted from stable isotope probing microcosms amended with ^{13}C-lignocellulose, cellulose, or vanillin. Some OTUs were also found in the total community (TC) and/or in DNA extracted from birch tongue depressors (BTD). The percentage of OTU sequences detected in each time point is shown, with the total number of sequences reported below.

			^{13}C-DNA					^{12}C-DNA	
			Lignocellulose			Cellulose		TC	BTD
Order, Family		Genus/Species	L28	L42	L56	C14	C28		
	OTU38	*Alatospora aff acuminata*		0.77%		1.33%			
Ceratobasiciaceae	OTU13	Ceratobasidium	10.34%						
	OTU48	Ceratobasidium sp.			46.27%	2.67%	9.21%		
Herpotrichiellaceae	OTU39	*Cladophialophora*		0.77%		1.33%		1.69%	
	OTU20	*Dactylella rhopalota*		19.23%					
	OTU19	*Endogone* sp?		1.54%					
	OTU23	*Endogone* sp?		5.38%					
	OTU24	*Endogone* sp?		1.54%					
Herpotrichiellaceae	OTU28	*Exophiala?*			1.49%	2.67%			
	OTU31	*Geomyces*				1.33%		1.69%	
	OTU33	*Geomyces*				20.00%			
	OTU40	*Geomyces*		0.77%	1.49%	16.00%		5.08%	
	OTU59	*Geomyces*	2.30%	4.62%	1.49%	1.33%		3.39%	
	OTU42	*Geopyxis majalis*					26.32%		
Russulaceae	OTU01	*Lactarius* sp.							33.33%
Russulaceae	OTU06	*Lactarius sp.*							5.56%
Mortierellaceae	OTU36	*Mortierella* sp.				5.33%		1.69%	
	OTU05	*Mycena*							5.56%
	OTU37	*Pochonia bulbillosa*				14.67%	1.32%		
Russulaceae	OTU32	*Russula* sp.				2.67%		6.78%	
Sebacinaceae	OTU21	*Sebacina*	1.15%	0.77%					
Sebacinaceae	OTU35	*Sebacina*	20.69%	14.62%		9.33%	10.53%		
Sebacinaceae	OTU43	*Sebacina*	16.09%	13.85%		6.67%	25.00%		
	OTU44	*Sebacina vermifera*	3.45%	6.15%			6.58%		
	OTU46	Sebacina vermifera					2.63%		
Ceratobasiciaceae	OTU14	unidentified	18.39%						
Total number of cloned seqeunces			87	130	67	75	76	59	36

L42, and 10% each of C14 and C28 ITS sequences. Only one fOTU detected in the BTD clone library was detected in TC or SIP libraries. ITS-OTU51 was detected in BTD, TC, and L42. RAxML placed this fOTU within Herpotrichilliaceae.

Bacterial T-RFLP analyses

Six T-RFs were detected in T0 background DNA (Figure 2). Multiple T-RFs from heavy fractions appeared over the course of the incubations with each substrate that did not appear in background DNA, or that increased in relative abundance over time in (Figure 2). Thirteen total peaks were found in BTD DNA that were also found in most heavy SIP samples. However, there is a large unidentified peak around 260 bp in the BTD profile that was not found in any ^{13}C-SIP sample. Several peaks were found in later time points in lignocellulose incubations that also appeared in earlier time points in either cellulose or vanillin incubations.

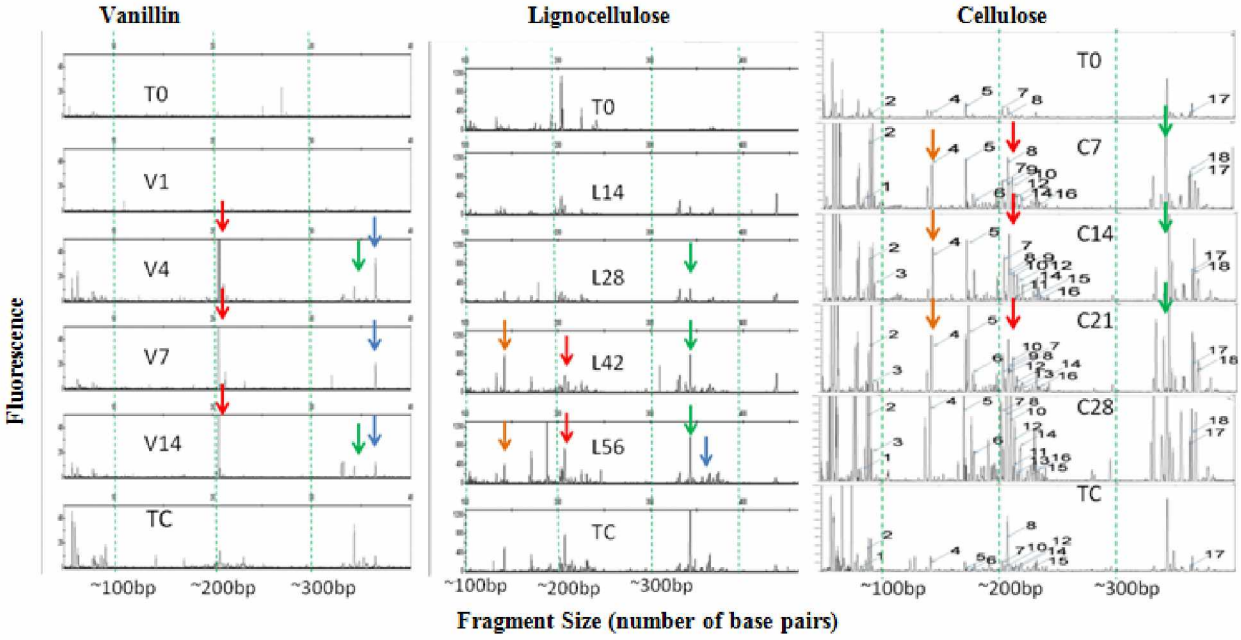

Figure 2 Bacterial 16S rRNA gene T-RFLP profiles from pooled "heavy" DNA. DNA was extracted from stable isotope probing (SIP) microcosms. Profiles from vanillin SIP microcosms are on the left, lignocellulose in the middle, and cellulose on the right. Time points are noted as number of days, with time 0 heavy fractions at the top and total community profiles (unlabeled DNA) on the bottom. Peak height or fluorescence measures of relative abundance. Peaks of the same size in base pairs found in heavy DNA originating from different substrates are indicated with colored arrows. Numbers from cellulose T-RFLP profiles correspond to taxonomic IDs from Stone (2009).

Fungal ARISA analyses

Multiple peaks representing intergenic spacer regions of the fungal ITS region were detected in lignocellulose and cellulose incubations. No peaks were detected in T0 heavy DNA from lignocellulose incubations, however heavy DNA extracted at T0 from soil used for cellulose incubations contained two peaks of relatively high abundance (Figure 3). One of these peaks was present in later time points of both cellulose and lignocellulose heavy fractions, and its relative abundance increased over time in both. Several peaks were detected in both lignocellulose and cellulose incubations; in some cases these peaks were only detected in later lignocellulose time points despite being found in early or all time points from cellulose incubations.

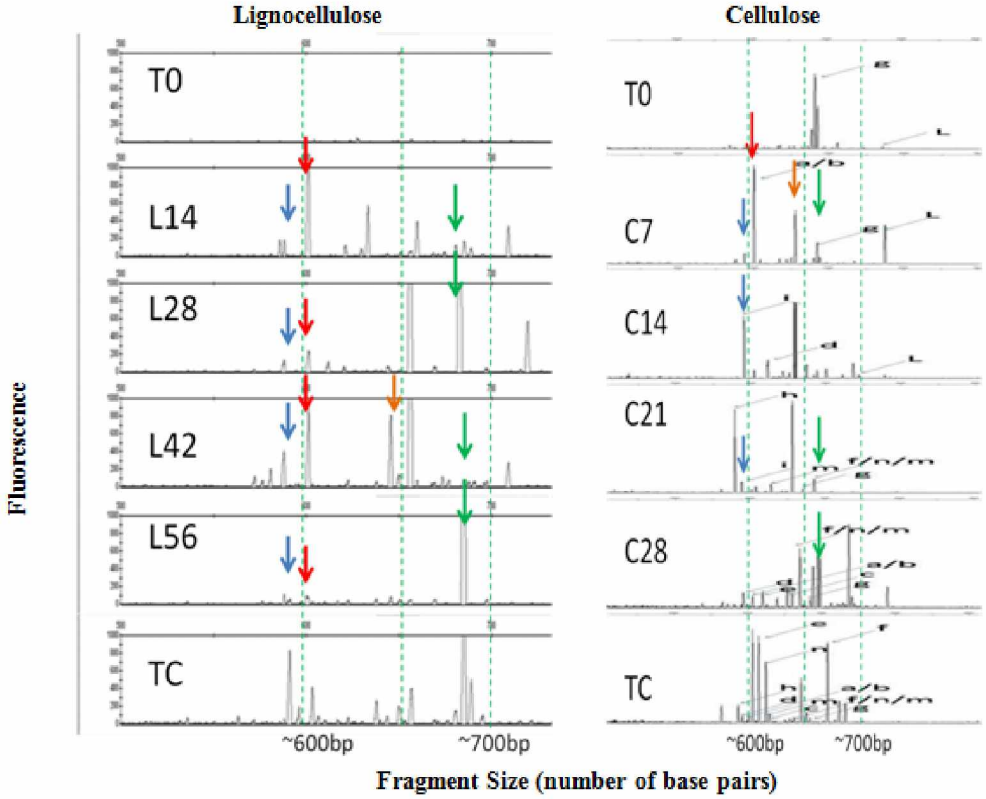

Figure 3. Fungal ITS region ARISA profiles from pooled "heavy" DNA. DNA was extracted from stable isotope probing (SIP) microcosms. Profiles from lignocellulose SIP microcosms are on the left, and cellulose on the right. Time points are noted as number of days, with time 0 heavy fractions at the top and total community profiles (unlabeled DNA) on the bottom. Peak height or fluorescence measures of relative abundance. Peaks of the same number of base pairs originating from different substrates are indicated with colored arrows. Letters on peaks from cellulose ARISA profiles correspond to taxonomic IDs from Stone (2009).

Discussion

The results of this study are generally consistent with the notion that lignocellulose is degraded synergistically in soil. Diverse communities of fungi and bacteria utilizing lignocellulose carbon were detected, indicating that at least 19 bacterial genera and 16 fungal genera are involved in the breakdown of lignocellulose or later processing of lignocellulose-derived carbon in boreal forest soils. The high number of singletons incorporating ^{13}C from the substrates is indicative of an undersampled and rich community of organisms taking part in the processing of lignocellulose-based carbon, and provides evidence that multiple taxa work in concert to degrade plant matter.

Fungal DNA was highly abundant in lignocellulose and cellulose heavy fractions, but was not amplified from vanillin heavy fractions where bacterial DNA was heavily labeled. Very few 16S rRNA sequences were detected in high abundance in any incubations; bOTU153 (*Cellvibrio gandavensis*) was the only bOTU detected at greater than 5% abundance in lignocellulose and cellulose incubations. This pattern and the low proportion of the bacterial community that incorporated the label in these incubations suggest that bacteria do not play a primary role in the degradation of these complex macromolecules. However, the proportionately high bacterial labeling in vanillin incubations suggests that many bacteria are able to efficiently scavenge smaller molecules that are produced during lignin degradation.

The detection of dominant bacterial vanillin utilizers at later time points of lignocellulose incubations provides additional support for the bacterial scavenger hypothesis. For example, *Burkholderia phytofirmans* (bOTU19) was detected in high abundance in vanillin incubations but was detected only once in lignocellulose incubations, and this detection was in the 42-day incubation. Additionally, a Luteibacter species (bOTU 180) was detected in early vanillin

incubations and in days 42 and 56 of lignocellulose incubations (Table 2). At these later time points, it is expected that the extracellular enzymes of primary degraders have degraded a significant amount of lignin, breaking off lignin monomers such as vanillin and making them accessible to other organisms. The degradation of lignin would provide a slow, steady source of vanillin while the addition of vanillin to microcosms would result in a sudden, sharp increase; therefore scavengers would not be expected to be as heavily labeled in lignocellulose microcosms compared to vanillin microcosms. In addition to these two bOTUs, other bacterial taxa were detected in vanillin incubations that were also found in lignocellulose incubations, including the unidentified bOTUs 111, 138, 166, and 188, *Geobacter sp.* (bOTU32), and *Mesorhizobium sp.* (bOTU208)(Table 2). These patterns suggest that bacteria benefit from lignin monomers produced through the energetically expensive activities of primary degraders.

The lack of detectable fungal labeling in vanillin SIP microcosms is inconsistent with the results of Rinnan and Bååth (2009), who detected ^{13}C-labeled fungal phospholipids in high proportions in vanillin incubations relative to incubations with glycine and starch over a seven-day incubation period. However, Rinnan and Bååth (2009) used arctic tundra soils rather than boreal forest soil, added more vanillin per gram of soil compared to the current study, and incubated soils at a colder temperature (15 °C). Another possibility is that the presence and enrichment of certain bacteria that benefited from vanillin addition (or high incubation temperatures) inhibited fungal proliferation in SIP microcosms. The dominant vanillin degrader, *Burkholderia*, has been shown to inhibit fungi (Opelt *et al.*, 2007).

Several bacterial and fungal taxa became ^{13}C-labeled early in cellulose incubations and at later time points in lignocellulose incubations This suggests that some microbes may rely on the activity of lignin degrading fungi in order to gain access to cellulose. Additionally, some fungal

OTUs were detected only in ^{13}C-DNA from cellulose incubations and not in lignocellulose incubations, and some ^{13}C labeled bacteria from cellulose and vanillin incubations were not detected in lignocellulose heavy fractions These patterns suggest that certain microbes preferentially target cellulose or vanillin (or their breakdown products) and may rely on the activity of lignin degraders to gain access to these more desirable substrates. However, it is also possible that these taxa were present but not detected in lignocellulose heavy fractions due to incomplete coverage of microbial sequences.

Communities from BTDs contained a relatively small number of bOTUs that were detected using SIP, and only one fOTU from heavy fractions was also seen in BTD communities. This result may be an effect of low sequencing coverage of the microbial community from the BTDs, which would make it more difficult to detect low abundance degraders that are present in communities associated with BTDs. Differences in laboratory and field conditions or the handling of soil samples could have led to differences in decomposer communities between incubations and BTDs. The disturbance of the soil and its removal from a natural environment is likely to have altered the presence and/or activity of several soil organisms. For example, a lack of living plant roots may result in a lower proportion of mycorrhizal fungi as well as fewer compounds being released from plant roots, which is known to have an effect on microbial community composition. Additionally, BTDs were buried in soils in the field for 12 months, while the longest SIP incubation was 56 days. Taxa colonizing BTDs also may include non-degraders from soil incidentally growing on or into the wood. The lignocellulose in BTDs was likely degraded to a greater extent than the lignocellulose in microcosms, and may have had a higher proportion of more recalcitrant parts of the lignocellulose molecule. This concept is supported by the observation that all of the taxa found in both BTDs and heavy fractions were

present in lignocellulose heavy fractions and the majority were detected with vanillin SIP, suggesting that these taxa use either lignin or its monomers as a carbon source. Overall, the presence of some taxa in both BTDs and in heavy SIP fractions from this study suggests that at least some of the taxa detected with SIP are capable of degrading lignocellulose under natural conditions. Greater sequencing depth may have allowed for the detection of additional taxa shared between these samples.

The identity of lignocellulose-utilizing fungi and bacteria detected in this study are in some ways consistent with those of Štursová *et al.* (2012), who showed that fungi dominate the degradation of cellulose in humic fractions of the organic layer in a cellulose SIP study in Bohemian forest soil. However, there were few genera in common between our study and Štursová *et al.* (2012), the most notable fungi in common being *Geomyces*, *Mortierella, Umbelopsis,* and *Cadophora*. The only bacterial genus found in high abundance in heavy fractions in both studies was *Burkholderia*, which was found by Štursová *et al.* (2012) in ^{13}C-DNA from litter, but not in humic horizons. However, *Burkholderia* was not found in cellulose microcosms in our study, but rather as a dominant vanillin utilizer. *Burkholderia* species are known degraders of aromatic compounds, therefore it is not surprising that they would be capable of degrading vanillin or other lignocellulose monomers in different environments.

Taken together, the findings of Štursová *et al.* (2012) and the current study support the guild decomposition hypothesis. The results of Štursová *et al.* (2012) suggest that bacteria may act as opportunists that preferentially degrade cellulose in fresh litter, while fungal decomposers and miners dominate degradation in older humic soils. This is likely due to the majority of available cellulose being consumed by rapidly proliferating and highly active bacteria. It is also likely that the high amounts of soluble organic compounds in fresh litter stimulate an

opportunistic bacterial community that is better able to compete for available cellulose compared to fungi. Fungal decomposers and miners that are better able to degrade complex molecules such as lignin would be in higher abundance in organic soil layers. Additionally, fungal degraders inhabiting humic soils may have a stronger ability to break down lignin, which allows them to access cellulose that was previously inaccessible. The findings of Štursová *et al.* (2012) indicate that different groups of organisms differ in their ability to compete for cellulose in different soil horizons, while our study indicates that specific taxa differ in the constituents that they target, highlighting one possible mechanism behind this observation.

Additional lignocellulose SIP studies should be performed to further elucidate the mechanisms and interactions of microbes breaking down (and ultimately respiring) lignocellulose. If possible, pure lignin should be included as a substrate to target microbes capable of or preferring to degrade lignin versus cellulose. Other lignocellulose constituents (e.g., hemicellulose or cellobiose) should also be considered to fully reveal the roles of various microbes in the complete mineralization of plant matter. While microcosm experiments like the current study are informative, they likely do not represent field conditions. Methods for *in situ* lignocellulose SIP should be developed to investigate the decomposer communities active in intact soil communities (including intact mycorrhizal fungal hyphae, etc.) under field conditions, similar to studies conducted on in situ doubly-labeled amino acid turnover (Kielland *et al.*, 2007). Additionally, the use of next-generation sequencing technologies that were validated after the initiation of this study would allow for greater sequencing depth, and therefore could provide greater clarity regarding the interactions of microbes involved in the breakdown of complex hydrocarbons.

This study provides a significant step towards our understanding of the complex interactions of microbial decomposer communities in organic soil horizons of ecosystems undergoing rapid climate change. Knowledge of different microbial taxa and how they interact to degrade plant-based carbon is essential to our understanding of how these communities will affect carbon turnover and storage (Azam and Malfatti, 2007; Bardgett *et al.*, 2008; Schimel and Schaeffer, 2012). Our results provide additional support to the microbial guild model, in that they suggest that some microbial taxa preferentially target specific portions of the lignocellulose molecule which become progressively available through the activity of other organisms. These results provide a starting point for further evaluation of the extent and mechanisms of synergistic microbial degradation of lignocellulose, and demonstrate that stable isotope probing is a valuable tool that can be used to evaluate complicated degradative processes involving complex substrates.

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