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# Bacterial metataxonomic profile and putative functional behavior associated with C and N cycle processes remain altered for decades after forest harvest

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## ABSTRACT

While the impacts of forest disturbance on soil physicochemical parameters and soil microbial ecology have been studied, their effects on microbial biogeochemical function are largely unknown, especially over longer time scales and at deeper soil depths. This study investigates how differing organic matter removal (OMR) intensities associated with timber harvest influence decadal-scale alterations in bacterial community composition and functional potential in the upper 1-m of the soil profile, 18 years post-harvest in a *Pinus taeda* L. forest of the southeastern USA. 16S rRNA amplicon sequencing was used in conjunction with soil chemical analyses to evaluate (i) treatment-induced differences in bacterial community composition, and (ii) potential relationships between those differences and soil biogeochemical properties. Furthermore, functional potential was assessed by using amplicon data to make metagenomic predictions. Results indicate that increasing OMR intensity leads to altered bacterial community composition and the relative abundance of dominant operational taxonomic units (OTUs) annotated to *Burkholderia* and *Aciditerrimonas*; however, no significant differences in dominant phyla were observed. Genes involved in nitrification were significantly lower in the most intensively harvested treatment, most likely as a result of reduced substrate. Additionally, the relative abundance of genes associated with dissimilatory nitrate reduction and denitrification were highest in the most intensively harvested plots, indicating that the volatilization of N was a potential pathway of N loss in that treatment. Genes associated with glycosyltransferases were significantly reduced with increasing harvest intensity while polysaccharide lyases increased. Additionally, when overall differences in N-cycling genes were observed (0–100 cm), they generally occurred at soil depths below 30 cm, indicating the importance of examining deeper soil horizons when assessing the effect of forest disturbance on soil biogeochemical processes.

## 1. Introduction

Forest ecosystems are crucial regulators of global biogeochemical processes and account for roughly 60% of the terrestrial carbon (C) pool (McKinley et al., 2011), with nearly half of that stored in the soil (Pan et al., 2011). Temperate and subtropical forests of the Northern Hemisphere are considered C sinks and have the ability to strongly influence global climate dynamics (Goodale et al., 2002; Pan et al., 2011). Additionally, forest ecosystems are of great economic value and have been estimated to account for \$4.7 trillion annually (Costanza et al., 1997). The coniferous forest region of the southern USA (~93 million hectares) is considered one of the most profitable forest ecosystems in the world, with forest services and products amassing over \$230 billion annually (Butler and Wear., 2013). These natural and plantation forests account for greater than 40% of annual US softwood

and consistently produce 19% and 12% of the worldwide pulpwood and industrial timber, respectively (McNulty et al., 1996; Hodges et al., 2011; Brandeis et al., 2012). Recently, there has been growing interest in the concurrent removal of non-merchantable woody debris, including slash, sawdust, and forest litter during the harvest of primary forest products. These secondary materials are currently being used as substitute feedstocks in industrial processes and for bio-energy production (Janowiak and Webster, 2010). Furthermore, commercial timber corporations and individual landowners are beginning to utilize forest litter for the production of merchantable mulch (Dickens et al., 2012). The removal of the forest floor material following timber harvest may have serious unforeseen consequences on ecosystem processes and the economic sustainability of these forestlands.

Soil microbial communities are vital ecosystem components as they help regulate nutrient availability, influence the formation of soil

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structure, and facilitate energy flow to higher trophic levels (de Boer et al., 2005; Schneider et al., 2012). Intensive OMR associated with timber harvest can significantly modify belowground properties; however, there are knowledge gaps regarding the effect of these practices on soil microbial community composition and their associated functionality. Results from previous studies investigating the effect of OMR on soil microbial communities have led to divergent conclusions with some suggesting that soil microbial consortia possess high resilience to intensive OMR (Li et al., 2004; Busse et al., 2006; Hannam et al., 2006); however, others have challenged this assumption of resilience and shown that intensive OMR can significantly affect microbial communities following harvest (Bader et al., 1995; Hartmann et al., 2012; McGuire et al., 2015). Furthermore, it remains unknown if these disturbance alter deeper (> 30 cm) microbial community structure. Most studies that investigate soil microbial communities have been limited to the top 15–30 cm based on the generalization that organic matter inputs, nutrient stores, and microbial activity are greatest in the uppermost portion of the soil profile (Fierer et al., 2003; Eilers et al., 2012; Stone et al., 2015). Although microbial biomass and nutrient content do tend to decrease significantly below 10 cm, over 50% of forest soil organic C is stored below 30 cm with the majority of this C originating from deep tree roots (Jobbágy and Jackson, 2000; Hartmann et al., 2009; Schutz et al., 2010; Laclau et al., 2013). This suggests that natural and plantation forest systems may harbor microbial taxa of biogeochemical and ecological relevance deeper in the profile, and the disturbance of a forest harvest event may alter microbial composition and functionality at deeper depths due to reduced C and nutrient inputs.

Soil bacteria are functionally diverse and have been shown to act as decomposers, mutualists, pathogens, and chemoautotrophs making them an extremely important component of soil biogeochemical processes that strongly influence the primary productivity in ecosystems. Multiple factors including vegetation composition (Hart et al., 2005; Cong et al., 2015) and soil physicochemical properties (Kaiser et al., 2016) can directly influence bacterial community composition and functionality. Furthermore, natural and anthropogenic disturbances have been shown to alter soil properties (Kurth et al., 2014; Foote et al., 2015; James and Harrison, 2016), resulting in modified soil bacterial community structure and function (Oliver et al., 2015; Shen et al., 2015). Previous studies have shown that forest harvest activities that include the removal of forest floor materials can result in reductions in soil C and nitrogen (N) stocks, which have been shown to last for decades to centuries (Chen et al., 2013; Kellman et al., 2014; Prest et al., 2014; Achat et al., 2015a, 2015b; Dean et al., 2017; Menegale et al., 2016; Mushinski et al., 2017a). Considering that bacterial metabolism relies heavily on soil C and N, it is conceivable that the reduction in these elements could alter community structure and biogeochemical functionality related to C and N cycling. As a case in point, it has been shown that intensive timber harvest has the ability to alter the abundance, composition, and activity of microbes associated with nitrification (Mushinski et al., 2017b), which might suggest that other key bacterial-mediated biogeochemical processes such as nitrogen-fixation, denitrification, dissimilatory- and assimilatory-nitrate reduction, and anammox might be altered by intensive OMR. Additionally, the reduction in soil C has been shown to significantly reduce the abundance of prokaryotic genes associated with C decomposition (Cardenas et al., 2015); however, it is unknown if this trend persists throughout the soil profile. A recent continent-wide study investigating the influence of harvest on the soil bacterial communities of the upper 20 cm of mineral soil showed that the majority of bacterial taxa were unaffected by harvesting (Wilhelm et al., 2017); however, many soils, including those found in the southeastern USA, can span multiple meters in depth, and should be sampled accordingly to determine the full extent of intensive harvest on bacterial community composition and associated biogeochemical functionality.

In this study, we explored the decade-scale response of soil bacterial community composition and its functional attributes related to C and N

cycle processes following intensive OMR in surface and subsurface mineral soils of a coniferous forest in the southern USA. We have previously shown that increasing forest OMR results in reduced soil organic carbon (SOC), total nitrogen (TN), and inorganic nitrogen concentrations throughout the upper 1 m in the soil profile (Foote et al., 2015; Mushinski et al., 2017a), which could significantly alter the relative abundance of taxa that rely on these substrates for C and energy gain. We hypothesized that: (a) the reduced C and nutrient pools in the intensively harvested treatments would lead to reduced bacterial diversity and altered community structure, (b) these changes in bacterial community structure would be more evident in surface rather than subsurface portions of the soil profile due to the strong influence of above- and belowground primary productivity on this portion of the soil profile, and (c) specific biogeochemical functions associated with C and chemoautotrophic activity would be reduced in plots that were subjected to the most severe OMR due to alterations in substrate availability.

## 2. Materials and methods

### 2.1. Study site and sampling design

Research was conducted in Davy Crockett National Forest near Groveton, TX, USA (31° 06' 32.48" N, 95° 09' 59.15" W). The study site is part of the Long-term Soil Productivity (LTSP) network aimed at understanding the effects of forest management practices on soil properties and processes (Powers, 2006; Ponder et al., 2012). The Groveton-LTSP site was established in 1997 and includes differing intensities of organic matter removal associated with timber harvest. These treatments include a low intensity harvest treatment (merchantable bole/stem only; BO), where only the bole of the tree was removed, and a high intensity harvest treatment (whole tree harvest + forest floor removal; WT + FF) where the entire tree (bole, branches, and leaves) was harvested and the forest floor removed by hand-raking. The BO treatment is equivalent to a 40–70% removal of aboveground biomass while the WT + FF removal treatment was a complete removal (100%) of aboveground biomass (Powers, 2006). In addition, the experimental design includes unharvested control stands (tree age = 60–80 yrs). During harvest, trees were hand-felled and lifted off of the plots with a loader to reduce soil compaction. Controls and both harvest treatments were triplicated and each replicate was 0.2 ha. All plots are located within a 1.5 km radius. Plots were harvested in 1996 and then replanted in 1997 with containerized *Pinus taeda* L. (loblolly pine) seedlings of 10-half sib families from the US Forest Service seed orchards using 2.5-m x 2.5-m spacing. Soils across the study area are uniform fine-loamy siliceous, thermic Oxyaquic Glossudalfs in the Kurth series, and the topography is relatively flat (USDA/NRCS, 2003).

Soil samples were collected in April 2015 using a JMC Environmentalist Sub-Soil Probe PLUS, 2.8 cm diameter x 120 cm length coring tube (Clements Associates Inc., Newton, IA, USA). Soil cores were taken at 1.8 m from the base of a randomly selected *P. taeda* L. individual with a diameter at breast height (DBH) between 18 and 24 cm. A three-tree buffer from the outside edge of each plot was not sampled to avoid edge effects. In some of the WT + FF stands, the forest floor had not yet redeveloped; because of this, the organic soil horizon in all other plots (approximate thickness: < 3 cm) was removed prior to coring in order to investigate mineral soil horizons exclusively. Soil sampling followed a stratified random sampling design in which four cores were taken from each plot and pooled by depth increment to increase sample mass and reduce error introduced by environmental heterogeneity. Specifically, each soil core was partitioned into four depth increments in the field (0–10, 10–30, 30–60, and 60–100 cm), pooled together with the other replicated cores, and individual depths were analyzed separately. On the day in which soil cores were taken from the ground, samples were transported at 4 °C from the field to the lab, aseptically mixed by hand, and 5-g subsamples (3 per sample) were

immediately stored at  $-80^{\circ}\text{C}$  for future DNA extraction and analysis. The remaining soil was stored at  $4^{\circ}\text{C}$  for soil characterization.

## 2.2. Soil chemical parameters

Soil was passed through a 2-mm sieve to remove large organic material and roots. A 25-g aliquot of sieved soil was then dried at  $60^{\circ}\text{C}$  for 48 h and finely ground using a TE250 ring pulverizer (Angstrom, Inc., Belleville, MI, USA). Using pulverized soil, total phosphorus (TP) was extracted using the lithium fusion method and concentrations were determined using molybdenum blue colorimetry (Lajtha et al., 1999). Mehlich III extractable phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na), and sulfur (S) were analyzed from pulverized soil with inductively coupled plasma mass spectrometry at the Texas A&M Soil, Water, and Forage Testing Laboratory (Mehlich, 1978). Concentrations of SOC, TN,  $\text{NH}_4^+$ , and  $\text{NO}_2^- + \text{NO}_3^-$  as well as soil pH have been previously reported (Mushinski et al., 2017b). All soil chemical properties are summarized in Supplementary Tables 1 and 2.

## 2.3. DNA extraction

DNA extraction followed the modified version of the International Standard for the extraction of DNA from soil as described by Terrat et al. (2014). Further modification was made to extract DNA from 3 g of soil (dry weight equivalent) rather than the prescribed 1 g. DNA was extracted from 3 analytical replicates per sample and then pooled to increase mass and reduce environmental heterogeneity. DNA library preparation and sequencing of bacterial communities was done by Molecular Research DNA Laboratory ([www.mrdna.com](http://www.mrdna.com), Shallowater, TX, USA). Bacterial sequencing targeted the 16S rRNA coding region using primers 1100F (5'-GGCAACGAGCGMGACCC-3'; Lane et al., 1985; Dorsch and Stackerbrandt, 1992) and 1492R (5'-GGTTACCTTGTTACGACTT-3'; Turner et al., 1999). PCR amplification was accomplished by utilizing the HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA) under the following conditions: an initial denaturation step at  $94^{\circ}\text{C}$  for 3 min, followed by 28 cycles of  $94^{\circ}\text{C}$  for 30 s,  $53^{\circ}\text{C}$  for 40 s and  $72^{\circ}\text{C}$  for 1 min, after which a final elongation step at  $72^{\circ}\text{C}$  for 5 min was performed. PCR products were verified via gel electrophoresis (2% agarose gel). Samples were barcoded and subsequently pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified with calibrated AMPure XP beads (Agencourt Biosciences Co., Brea, CA, USA). The pooled and purified PCR products were then used to prepare an Illumina DNA library for each sample. Synthesis-based sequencing on an Illumina MiSeq followed the manufacturer's guidelines and resulted in single-end reads of  $416 \pm 58$  bp.

## 2.4. Sequencing and bioinformatic analyses

Resulting.fasta and.qual files were demultiplexed, quality filtered, and analyzed using the QIIME 1.9.1 pipeline (Caporaso et al., 2010). Illumina sequences with  $< 200$  and  $> 1000$  bp, barcode or primer sequence errors, and those with homopolymers or ambiguous base calls that exceed six nucleotides were discarded. Raw 16S sequences were deposited in NCBI's sequence read archives under the accession number SRR5218289. Operational taxonomic units (OTUs) were defined by clustering at 97% sequence identity using the QIIME implementation of UCLUST (Edgar, 2010). Subsequent taxonomic annotations, refer to assignments based on  $> 97\%$  sequence identity for species, 95–97% sequence identity for genera, 90–95% for families, and 77–80% for phyla using the Greengenes 16S database (McDonald et al., 2012). Archaeal OTUs were removed following annotation. Bacterial metagenomes were reconstructed using PICRUSt (Langille et al., 2013). Metagenomic inference was done using the *predict\_metagenomes.py* script with a normalized OTU table (rarefied to 1000 16S sequences per sample) as an input. This script generated metagenomic predictions for

each sample based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology (KO) database (Kanehisa et al., 2017). We analyzed the average number of annotated genes in each sample and focused on known genes related nitrogen-cycling processes. N-cycle orthologs analyzed in this study are listed in Supplementary Table 3. Additionally, to assess how intensive timber harvest influenced the abundance of genes involved in biomass degradation, we searched our normalized OTU table against KEGG orthologs associated with gene families listed in the Carbohydrate Active Enzyme Database (CAZy) (Lombard et al., 2014). PICRUSt also calculated the Nearest Sequenced Taxon Index (NSTI), a measure of phylogenetic distances between each empirical OTU and their reference genome match. The accuracy of PICRUSt decreases with increasing NSTI scores, but scores  $\leq 0.17$  can be equated to accurate metagenome predictions (Langille et al., 2013). NSTI scores in this study ranged from 0.07 to 0.16 (Supplementary Table 4).

## 2.5. Statistical analyses

All statistical analyses on bacterial communities were carried out using the sequence count within each OTU as a relative abundance value. All datasets were tested for normality using Shapiro-Wilk's test. When OTU data were not normally distributed, non-parametric statistical tests or  $\log_{10}$  transformations were applied. OTU data generated in QIIME were used to quantify the number of unique OTUs, richness (Menhinick's Index), and diversity (Shannon's Diversity Index). Community metric calculations were analyzed using normalized OTU data set to  $40,814$  reads sample $^{-1}$ . Soil chemical properties, bacterial community metric estimates, OTU relative abundance values, and the relative abundance of predicted genes were analyzed using a linear mixed model ANOVA. Because of the inherent autocorrelation between differing soil depths, a repeated measures experimental design was employed with OMR as the fixed main plot and soil depth as the repeated measure. Replicated plots were nested within harvest treatment and considered a random effect. To explore gene count differences for each depth increment, a one-way mixed-model ANOVA was employed for each depth where OMR was designated as the fixed main variable. When differences were significant, Tukey's honest significant difference (HSD) test was performed to assess post hoc contrasts with significance inferred at  $p < .05$ . Non-metric multidimensional scaling (NMDS) based on a Bray-Curtis dissimilarity matrix was performed on normalized bacterial 16S data. A permutational multivariate analysis of variance (PERMANOVA) using the Bray-Curtis matrix listed above was employed to characterize differences in soil bacterial community composition based on OMR treatment and soil depth. PERMANOVAs were run using 999 permutations. Environmental factors were fitted onto NMDS ordinations using the function *envfit* of the *vegan* package in R (R Development Core Team, 2011). Significance of the associations was determined by 1000 random permutations. Spearman's correlations were calculated using JMP and aggregated across all sample points (SAS Institute, Inc., Cary, NC, USA).

## 3. Results

### 3.1. Bacterial community composition

A total of 2,642,414 high-quality 16S sequences were retained following filtering. Prior to normalization, samples consisted of  $73,400 \pm 3849$  sequences, which did not statistically vary between OMR but did increase with increasing soil depth (0–10 cm:  $58,097 \pm 4574$ ; 10–30 cm:  $64,617 \pm 3323$ ; 30–60 cm:  $77,467 \pm 7014$ ; 60–100 cm:  $93,421 \pm 9329$ ). Following normalization to 40,814 sequences per sample, we observed a diverse bacterial community of  $8727 \pm 221$  unique OTUs per sample. Counts of OTUs did not vary by OMR treatment or soil depth. Coverage estimates were very high ( $0.95 \pm 0.01$ ), indicating an overall excellent OTU coverage

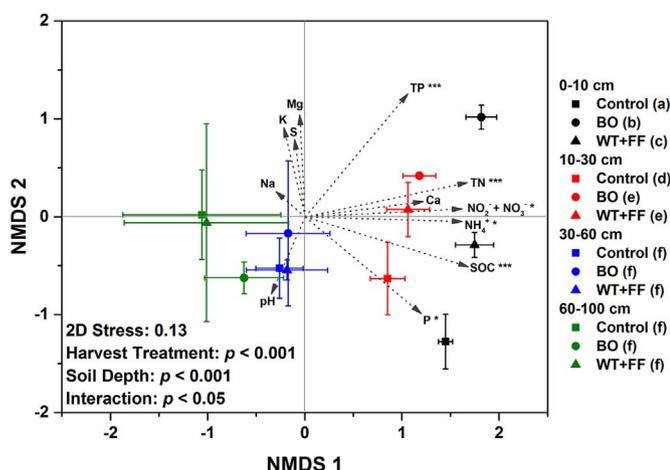


Fig. 1. Nonmetric multidimensional scaling (NMDS) ordinations of soil bacterial communities based upon their OTU composition derived from Bray-Curtis distances matrices. For each point, N = 3. Error bars indicate standard error. Vectors represent the strength/direction of the weight of environmental variables on bacterial communities. SOC: soil organic carbon; TN: total nitrogen; TP: total phosphorus; Mehlich III extractable phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na), and sulfur (S). Significant correlations between ordinations and environmental factors are indicated with asterisks ( $p < .05^*$ ,  $p < .01^{**}$ ,  $p < .001^{***}$ ). Letters following treatment identifications indicate significant differences in community structure.

afforded by the level of sequencing employed in this study, which did not statistically differ amongst OMR or soil depths.

OTU Richness (Menhinick's Index) was not influenced by OMR treatments; however, statistical differences were found between depth increments. Specifically, the 0–10 cm ( $35.6 \pm 0.3$ ) and 10–30 cm ( $37.6 \pm 0.9$ ) increments were significantly higher than 60–100 cm ( $26.7 \pm 2.2$ ) increment, but did not differ from each other (Supplementary Table 5). Richness in the 30–60 cm ( $32.5 \pm 1.3$ ) increment was not statistically different than any other depth (Supplementary Table 5). Similarly, OTU diversity (Simpson's Index) was statistically unaffected by timber harvest intensity, but did vary with soil depth. As was observed for richness, diversity in the 0–10 cm ( $0.96 \pm 0.01$ ) and 10–30 cm ( $0.96 \pm 0.01$ ) increments were significantly higher than in the 60–100 cm ( $0.88 \pm 0.02$ ) increment, but did not differ from each other. Diversity in the 30–60 cm ( $0.91 \pm 0.01$ ) increment was not statistically different than any other depth (Supplementary Table 5).

Non-metric multidimensional scaling (NMDS) plots, based on Bray-Curtis distance matrices, of OTUs resulted in statistical separation for bacterial communities based on harvest treatments (Fig. 1). Post hoc analyses revealed that statistical differences between treatments were observed at 0–10 cm and 10–30 cm (Fig. 1). Furthermore, bacterial community composition was significantly altered by soil depth with unique clustering when soil depth was analyzed independent of OMR treatments; specifically, the 0–10 and 10–30 cm increments were statistically different than the 30–60 and 60–100 cm increments. SOC, TN, TP, Mehlich-III P,  $\text{NH}_4^+$ , and  $\text{NO}_2^- + \text{NO}_3^-$  were significantly linked to bacterial community structure and positively correlated with the more surficial soil depths.

### 3.2. Bacterial taxonomic classification

Looking at the combined influence of all soil depths (0–100 cm), phylum-level OTU characterization indicated that communities were dominated by Acidobacteria ( $43.5 \pm 1.2\%$ ), Proteobacteria ( $20.7 \pm 1.1\%$ ), Verrucomicrobia ( $11.2 \pm 0.8\%$ ), Firmicutes ( $6.8 \pm 0.8\%$ ), Actinobacteria ( $6.3 \pm 0.2\%$ ), Chloroflexi ( $4.4 \pm 0.9\%$ ), and Bacteroidetes ( $4.3 \pm 0.4\%$ ) across all treatments and soil depths (Fig. 2). The remaining 2.7% of sequences were annotated to 22 other

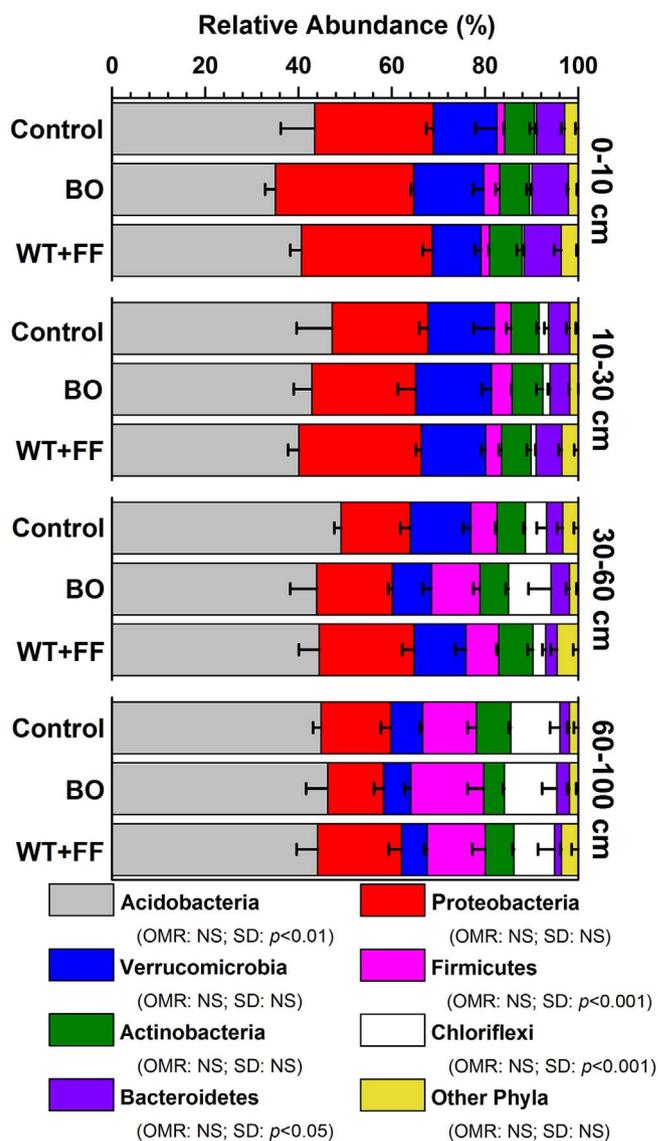


Fig. 2. Distribution and relative abundance of major bacterial phyla for each treatment (Control = unharvested control; BO = bole-only harvest; WT + FF = whole-tree harvest + forest floor removal) nested within soil depth increment. Error bars indicate the standard error of 3 biological replicates. Results of mixed model ANOVA are shown in legend. OMR: organic matter removal; SD: soil depth; NS: not significant.

phyla (Supplementary Table 6). OMR treatment did not statistically alter the relative abundance of any of the phyla analyzed; however, depth did lead to significant differences. Specifically, the relative abundance of Acidobacteria, Firmicutes, Chloroflexi, and Nitrospirae increased with depth while Bacteroidetes, Armatimonadetes, and Saccharibacteria decreased with increasing soil depth.

The three most abundant bacterial genera, *Acidobacterium*, *Holophaga*, and candidate genus *Solibacter* accounted for 38.8% of all 16S sequences and belong to the phylum Acidobacteria (Table 1). The relative abundance of *Acidobacterium*, *Holophaga*, *Ktedonobacter*, *Thermoflavimicrobium*, *Stella*, candidate genus *Koribacter*, *Pseudomonas*, *Aciditerrimonas* increased significantly with depth while the relative abundance of *Prostheco bacter*, *Verrucomicrobium*, *Pseudolabrys*, *Burkholderia*, *Steroidobacter*, and *Bradyrhizobium* decreased significantly with depth (Table 1). Of the bacterial genera that comprised over 1% of all 16S reads, only *Burkholderia* and *Aciditerrimonas* were statistically affected by differences in OMR. Specifically, the relative abundance of *Burkholderia* increased with increasing intensity of organic matter removal while *Aciditerrimonas* decreased with increasing organic matter

**Table 1**  
Summary of genera with relative abundances > 1%, as well as their statistical response to organic matter removal (OMR), soil depth (SD), and their interaction.

Genus	Relative Abundance	OMR	SD	OMR x SD
	(% of all 16S Sequences)			
<i>Acidobacterium</i>	23.75	0.27	44.06*** <sup>a</sup>	1.96
<i>Holophaga</i>	8.82	2.72	6.02*** <sup>a</sup>	1.48
<i>Candidatus Solibacter</i>	6.24	0.09	1.99	1.41
<i>Ktedonobacter</i>	4.48	1.13	48.16*** <sup>a</sup>	0.82
<i>Thermoflavimicrobium</i>	4.07	1.01	90.81*** <sup>a</sup>	0.5
<i>Prostheco bacter</i>	4.04	0.01	3.16* <sup>b</sup>	1.74
<i>Verrucomicrobium</i>	3.60	0.33	9.06*** <sup>b</sup>	1.43
<i>Stella</i>	3.31	3.46	4.57* <sup>a</sup>	0.95
<i>Rhodothermus</i>	2.92	0.64	1.73	0.88
<i>Candidatus Koribacter</i>	2.32	1.07	29.63*** <sup>a</sup>	2.18
<i>Chthoniobacter</i>	1.89	1.68	2.60	1.98
<i>Pseudolabrys</i>	1.82	4.05	10.00*** <sup>b</sup>	1.02
<i>Burkholderia</i>	1.57	7.13* <sup>a</sup>	5.32* <sup>b</sup>	0.48
<i>Pseudomonas</i>	1.54	3.82	5.06* <sup>a</sup>	1.31
<i>Conexibacter</i>	1.50	0.98	2.27	0.77
<i>Steroidobacter</i>	1.39	0.01	21.92*** <sup>b</sup>	0.94
<i>Bradyrhizobium</i>	1.35	0.65	25.83*** <sup>b</sup>	1.01
<i>Aciditerrimonas</i>	1.29	6.67* <sup>b</sup>	7.47*** <sup>a</sup>	1.08
<i>Actinoallomurus</i>	1.10	2.22	2.23	0.97
All Other Genera	23.00	0.35	4.77* <sup>a</sup>	0.07

\**p* < .05.

\*\**p* < .01.

\*\*\**p* < .001.

<sup>a</sup> Relative abundance significantly increased with increasing harvest intensity or soil depth.

<sup>b</sup> Relative abundance significantly decreased with increasing harvest intensity or soil depth.

removal (Table 1). Spearman's correlation analyses revealed that *Burkholderia* was significantly positively correlated to soil pH and Mehlich-III P and negatively correlated to K, Mg, S, and Na (Supplementary Table 7). In contrast, *Aciditerrimonas* was positively correlated to K, Mg, S, and Na and negatively correlated to soil pH, Mehlich-III P, and Ca (Supplementary Table 7). Of the less abundant genera, 56 were significantly affected by timber harvest (Supplementary Table 8).

### 3.3. Nitrogen cycling metagenomic potential

The putative metabolic functions of the microbial communities were predicted using PICRUST, which compares the identified 16S rRNA gene sequences to those of known genome sequenced species, thereby estimating the possible gene contents of the uncultured microbial communities. For nitrogen cycle processes, we quantified the combined abundance of predicted genes involved in nitrogen-fixation (*nifDKH*, *anfG*), dissimilatory nitrate reduction (*narGHI*\*, *napAB*\*, *nirBD*, *nrfAH*), assimilatory nitrate reduction (*narB*, *nR*, *nasAB*, NIT-6, *nirA*), nitrification (*amoCAB*, *hao*, *nxrAB*), denitrification (*narGHI*\*, *napAB*\*, *nirK*, *nirS*, *norBC*, *nosZ*), and anammox (*hzs*, *hdh*) (Supplementary Fig. 1). Genes with asterisks are involved in multiple processes. Increasing harvest intensity significantly affected the number of predicted genes associated with dissimilatory nitrate reduction, denitrification, and nitrification (Fig. 3). Specifically, for dissimilatory nitrate reduction and denitrification, the unharvested control treatment possessed significantly lower cumulative gene counts than the WT + FF treatment (Supplementary Table 9). When we look at the predicted gene counts for each step associated with dissimilatory nitrate reduction and denitrification, we note that the statistical significance is influenced by significant increases with increasing harvest intensity of *narGHI* and *napAB* (data not shown), which are involved in both processes. None of the other genes involved in these two processes were influenced by harvest. Genes involved in nitrification were significantly more abundant in the unharvested control when compared to the WT + FF while

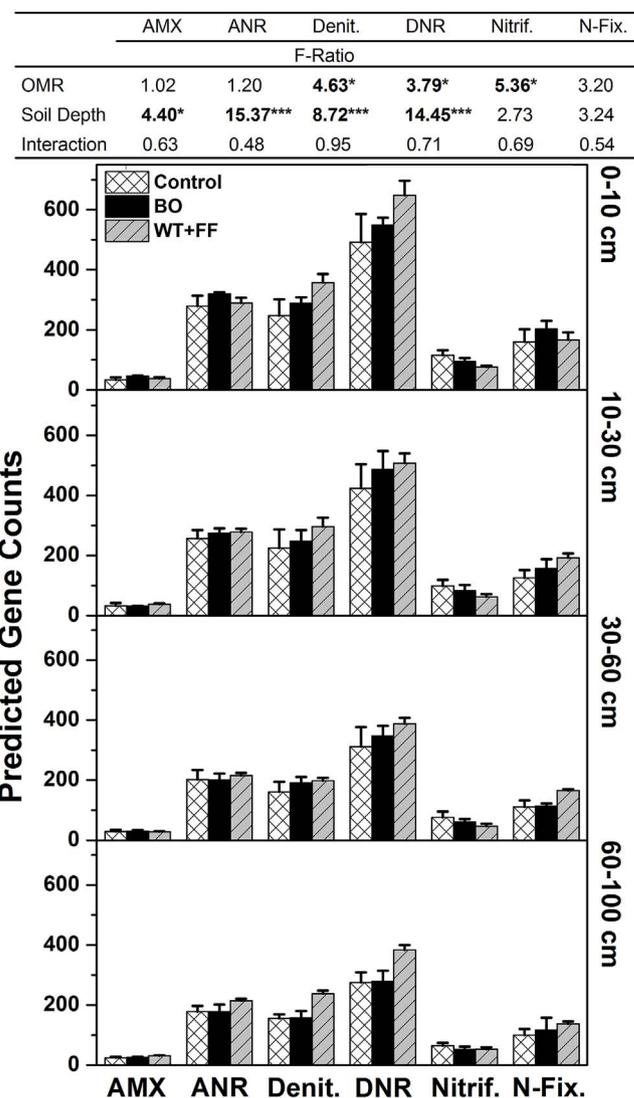


Fig. 3. The number of predicted gene counts associated with nitrogen cycle processes for each treatment within each soil depth. A repeated measures ANOVA table shows the statistical significance of organic matter removal (OMR), soil depth, and their interaction. Data are means  $\pm$  standard error (N = 3). AMX: anammox, ANR: assimilatory nitrate reduction, Denit.: denitrification, DNR: dissimilatory nitrate reduction, Nitrif.: nitrification, N-Fix.: nitrogen fixation. *p* < .05\*, *p* < .01\*\*, *p* < .001\*\*\*.

the BO treatment was not significantly different from either of the other two treatments (Supplementary Table 9). Of the genes involved in nitrification, only *amoCAB* was significantly different between treatments with the control and BO treatments possessing significantly more predicted genes than the WT + FF treatment (data not shown). Soil depth significantly influenced the predicted gene counts of a majority of nitrogen-cycle processes; specifically, we observed that for dissimilatory nitrate reduction, assimilatory nitrate reduction, and denitrification the 0–10 and 10–30 cm increments were significantly higher than the 30–60 and 60–100 cm increments. For anammox, the predicted gene count in the 0–10 cm increment was significantly higher than the 60–100 cm increments but the 10–30 and 30–60 cm increments did not differ from any other depth. When predicted gene counts were analyzed separately for each soil depth, we found that significant harvest-induced differences were only observed for the 30–60 cm and 60–100 cm increments (Table 2). Specifically, differences for N-fixation were found in the 30–60 cm increment with the WT + FF treatment possessing higher gene counts than the unharvested control or BO. Differences in gene counts associated with dissimilatory nitrate reduction, denitrification, and nitrification were found only in the 60–100 cm

**Table 2**

Results from a one-way ANOVA testing the influence of organic matter removal intensity on predicted gene counts associated with specific N-cycle processes or CAZy gene classes within each soil depth.

	Soil Depth (cm)			
	0–10	10–30	30–60	60–100
	F-Ratio			
<b>N-Cycle Processes</b>				
Anammox	1.44	0.28	0.06	1.28
Assimilatory Nitrate Reduction	0.95	0.39	0.19	1.15
Denitrification	2.27	0.87	0.94	<b>6.50**</b>
Dissimilatory Nitrate Reduction	2.76	0.71	0.83	<b>3.73*</b>
Nitrification	3.13	1.51	1.48	<b>3.86*</b>
Nitrogen-Fixation	0.62	2.04	<b>3.98*</b>	0.59
<b>CAZy Gene Classes</b>				
Auxiliary Activity	1.28	0.98	0.62	3.14
Carbohydrate Esterases	1.02	0.53	0.46	1.17
Glycoside Hydrolases	0.88	0.48	0.38	0.85
Glycosyl Transferases	<b>3.97*</b>	0.59	0.83	1.53
Polysaccharide Lyases	2.27	1.42	0.93	1.25

\* $p < .05$ .

\*\* $p < .01$ .

\*\*\* $p < .001$ .

increment with dissimilatory nitrate reduction and denitrification having the highest gene counts in the WT + FF treatment while gene counts associated with nitrification were highest in the unharvested control.

### 3.4. Biomass decomposition potential

To evaluate the influence of harvest treatment and soil depth on the biomass decomposition potential of bacterial communities, we analyzed the relative abundance of gene orthologs associated with the CAZy database. From all samples, we compiled 1,093,632 predicted genes associated with the CAZy family of enzymes of which 46.2% were associated with glycosyltransferases (GT), 44.4% with glycoside hydrolases (GH), 8.14% with carbohydrate esterases (CE), 1.01% with polysaccharide lyases (PL), and 0.231% with auxiliary activity enzymes (AA). Of these 5 main enzyme classes, the relative abundance of associated genes for GT and PL were statistically affected by increasing harvest intensity (Fig. 4). Specifically, the relative abundance of genes grouped into the GT family was significantly higher in the control than the WT + FF treatment (Supplementary Table 10). The BO treatment was not different from any other treatment. Within the GT family, GT1, GT2, GT4, GT21, GT26, and GT83 illustrated significantly higher predicted gene counts in the control relative to the other two treatments. In contrast, the WT + FF treatment had the highest gene count for GT9. In regards to PL, the control was significantly lower than the BO and WT + FF treatments (Supplementary Table 10). Predicted counts of PL01 and PL10 were significantly higher in the WT + FF treatment relative to the control. Although the predicted gene counts for the other 3 enzyme classes were not statistically significant, as a whole, there were three other families (AA01, AA04, and CE15) that decreased significantly with increasing timber harvest intensity. Additionally, increasing soil depth led to significant reductions in the relative abundance of predicted genes associated with CAZy enzymes classes. The CE and GH class possessed significantly higher gene counts in the 0–10 cm and 10–30 cm increments relative to the 30–60 cm and 60–100 cm increments. Additionally, predicted genes associated with the AA, GT, and PL classes illustrated highest gene counts in the 0–10 cm increments which was significantly separated from all other depth increments. When treatment differences were analyzed for each depth, only the 0–10 cm increment of the GT class was statistically affected, with the unharvested control having significantly higher counts than the

	AA	CE	GH	GT	PL
	F-Ratio				
OMR	1.54	1.52	1.15	<b>4.15*</b>	<b>3.53*</b>
Soil Depth	<b>23.92***</b>	<b>22.13***</b>	<b>20.31***</b>	<b>14.93***</b>	<b>18.82***</b>
Interaction	1.04	0.56	0.49	0.61	0.63

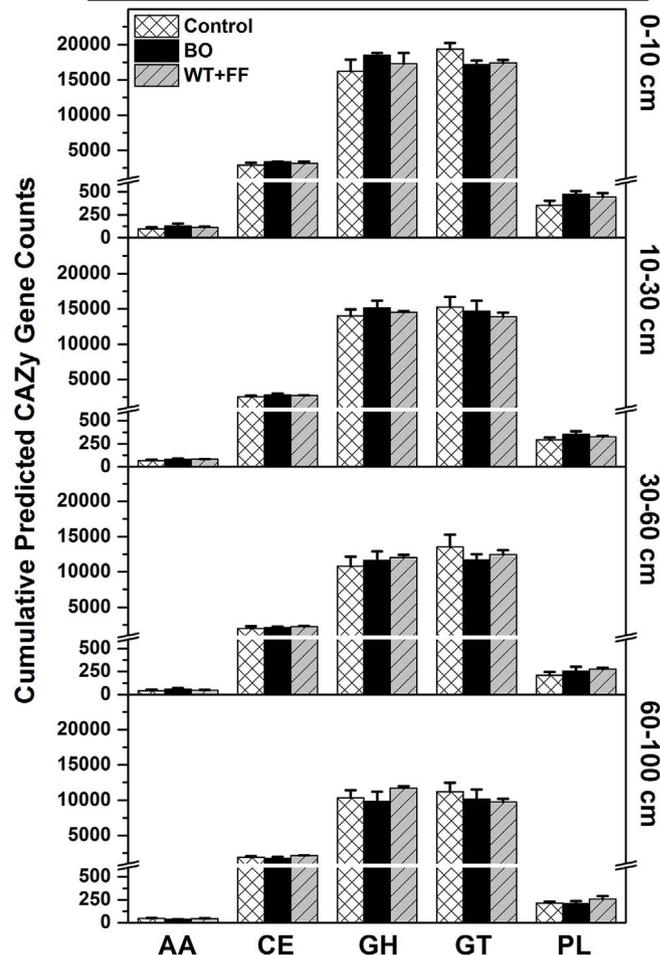


Fig. 4. The number of predicted gene counts associated with CAZy classes. A repeated measures ANOVA table shows the statistical significance of organic matter removal (OMR), soil depth, and their interaction. Data are means  $\pm$  standard error (N = 3). AA: auxiliary activity enzymes, CE: carbohydrate esterases, GH: glycoside hydrolases, GT: glycosyl transferases, PL: polysaccharide lyases.  $p < .05$ ,  $p < .01$ \*\*,  $p < .001$ \*\*\*.

other treatments (Table 2).

## 4. Discussion

### 4.1. Impacts of intensive timber harvest on soil bacterial ecology

Bacterial community structure was statistically affected by harvest treatment with significant separation of the three treatments in the 0–10 and 10–30 cm increments, but not in the two deepest soil depth increments. This indicates that taxa found at 0–30 cm are more susceptible to large changes in plant inputs (such as root turnover and litterfall), and/or changes in the physical environment (temperature, moisture) that occur following timber harvest. Furthermore, soil depth led to significant variations in community structure with the 0–10 cm and 10–30 cm increments being statistically different from each other and from the 30–60 and 60–100 cm increments. This is consistent with previous studies (Killham and Prosser, 2015) as well as with our observation of significant reductions in many N and CAZy gene groupings with depth. The higher abundance in surficial mineral layers indicates that there is potentially increased C and N cycling occurring in horizons

proximal to organic matter sources relative to deeper depths. Shifts in community structure, by both OMR and depth, are most likely due to a gradient of C and nutrients as evidenced by the significant correlations between ordinations and edaphic factors such as SOC, TN, and TP.

The dominant taxonomic group in our study (phylum Acidobacteria) was statistically unaffected by timber harvest intensity. A separate soil microbial survey of this site (Wilhelm et al., 2017) also noted a large abundance of Acidobacterial OTUs. Acidobacteria is one of the most widespread and abundant bacterial groups; however, relatively little is known regarding the actual activities and ecology of member taxa (Kielak et al., 2009; Zhang et al., 2014). From culture-based experiments, Acidobacteria type species have been shown to be heterotrophic, with some subdivisions being quite versatile in carbohydrate utilization (Pankratov et al., 2008). Furthermore, genomic and metagenomic data predict a number of ecologically relevant capabilities for some Acidobacteria, including the ability to persist in environments of varying soil nutrients and pH (Janssen et al., 2002; Quaiser et al., 2003; Lauber et al., 2009; Ward et al., 2009). The relative abundances of sequences similar to *Acidobacterium* were positively correlated to K and Na and negatively correlated to SOC, TN,  $\text{NH}_4^+$ , P, and Ca. This indicates that *Acidobacterium* may be more competitive in low-resource environments, and is consistent with other studies describing the entire phylum as oligotrophic (Kielak et al., 2016).

Of the genera that comprised > 1% of all 16S sequences, only sequences similar to *Burkholderia* (Proteobacteria) and *Aciditerrimonas* (Actinobacteria) were statistically affected by harvest intensity. Specifically, the relative abundance of sequences similar to *Burkholderia* increased with increasing harvest intensity while *Aciditerrimonas* decreased. *Burkholderia* occupy remarkably diverse ecological niches, ranging from contaminated soils to the respiratory tract of humans; furthermore, this genus possesses notable metabolic versatility, and have been exploited for biocontrol, bioremediation, and plant growth promotion purposes (Parke and Gurian-Sherman, 2001; Coenye and Vandamme, 2003). It is possible that their high abundance in the WT + FF is a result of their functional versatility in which this group was better suited to deal with changing C and nutrient availability as opposed to other taxa who may possess more narrow metabolic capabilities. The ecology of *Aciditerrimonas* is much less characterized; however members of this genus exhibit both heterotrophy and autotrophy; specifically, this genus is capable of reducing ferric ions to facilitate autotrophic growth under anaerobic conditions (Itoh et al., 2011). We observed that the relative abundance of *Aciditerrimonas* was negatively correlated to soil pH. In less acidic conditions, as was observed in the WT + FF treatment, it is possible that the availability of  $\text{Fe}^{3+}$  was reduced which may explain the decreased relative abundance of an iron-reducing taxa such as *Aciditerrimonas*. Additionally, the overall reduction in *Aciditerrimonas* with increasing harvest intensity is consistent with previous studies that have implicated some Actinobacterial taxa as being copiotrophic, with higher abundance at higher nutrient concentrations (Fierer et al., 2007, 2012; Ramirez et al., 2012).

#### 4.2. Biogeochemically-relevant predicted genes are altered by intensive harvest

Previous studies at the Groveton-LTSP site showed that increasing harvest intensity led to reductions in SOC, TN,  $\text{NH}_4^+$ , and  $\text{NO}_2^- + \text{NO}_3^-$  as well as alterations in soil pH (Foote et al., 2015; Mushinski et al., 2017a, 2017b). In this study, we show that soil TP was also decreased in the most intensive OMR treatment, probably due to the removal of the litter layer, reduced litterfall and root turnover during forest regrowth, and perhaps greater opportunity for leaching of inorganic P due to lower plant uptake during stand recovery. Contrary to the decrease in TP, we observed an increase in the concentration of Mehlich-III P with increasing harvest intensity. Since pool sizes of TP and Mehlich-III P are controlled by different biological and environmental factors, this differential response is not entirely unexpected.

Although additional studies are needed to identify the underlying mechanisms, we suggest that the increase in this plant-available P fraction following timber harvest may be related to the significant increase in soil pH, which could theoretically reduce the amount of P fixed by soluble iron (Fe), aluminum (Al), and manganese (Mn) as well as by hydrous oxides of Fe, Al, and magnesium (Mg).

Observed increases in soil pH with increasing harvest intensity could be attributed to the removal of forest floor material, which in coniferous forests is generally considered acidifying. It has also been shown that rates of nitrification can alter soil pH, with higher rates resulting in soil acidification (de Boer and Kowalchuk, 2001). Following harvest, rates of nitrification were potentially slowed in the WT + FF treatment as evidenced by the observed reduction in the relative abundance of predicted genes associated with ammonia-oxidation (i.e., *amoCAB*). This observation is remarkably consistent with a previous study at the Groveton-LTSP in which archaeal and bacterial *amoA* were quantified and found to be significantly lower in the WT + FF stands (Mushinski et al., 2017b). Reduced rates of nitrification may also be partially responsible for lower concentrations of  $\text{NO}_2^- + \text{NO}_3^-$  in the intensively harvested treatment. Simultaneous to the reduction in predicted genes involved in ammonia-oxidation, was an increase in genes associated with dissimilatory nitrate reduction and denitrification. Specifically, increases with increasing harvest intensity were observed in *narGHI* and *napAB*, which are both involved in the reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$ . This is somewhat surprising considering the lower concentration of  $\text{NO}_3^-$  in the WT + FF treatments stands; however, lower concentrations of  $\text{NO}_3^-$  may actually be a function of higher rates of  $\text{NO}_3^-$  reduction leading to denitrification. The rate of this process is unknown as well as whether this process is still being selected for, but it is conceivable that changes in biophysical and microclimatic conditions in the intensively harvested treatment (i.e., soil compaction, increased water infiltration, and increased solar radiation reaching the soil surface) would theoretically favor increased rates of anaerobic  $\text{NO}_3^-$  reduction. Furthermore, this pathway of N-loss could partially explain the previously reported reduction in N stocks as well as the enrichment in soil  $\delta^{15}\text{N}$  in the WT + FF stands at the Groveton-LTSP (Mushinski et al., 2017a). Additionally, the influence of OMR on predicted N-cycle genes was significant only at depths > 30 cm. Previous studies have shown that denitrification can occur at several meters below the soil surface (Cannavo et al., 2004; Barkle et al., 2007) due to increased anoxic conditions induced by increases in soil bulk density and soil water content (van Groenigen et al., 2005). We noted previously (Mushinski et al., 2017a) that soil bulk density was statistically unaffected from 0 to 100 cm by timber harvest; however, the 60–100 cm increment was significantly more dense in the WT + FF treatment relative to the control or BO treatment. This increase in bulk density may have favored the development of anaerobic soil zones, which, when combined with the downward movement of  $\text{NO}_3^-$  would create ideal conditions for denitrification to occur. That being said, it is difficult to explain the significant increase in bulk density at depth, and it may simply be a function of environmental heterogeneity. The significantly higher abundance of nitrification genes in the control at 60–100 cm may be related to substrate availability. We previously showed (Mushinski et al., 2017b) that deeper soil (60–100 cm) in the control stands possessed a 30% and 130% greater concentration of  $\text{NH}_4^+$  relative to the BO and WT + FF treatments, respectively. It is conceivable that this difference in  $\text{NH}_4^+$  could lead to shifts in nitrifier community structure at depth. The presumed lack of oxygen at this depth would theoretically inhibit nitrification; however, as the bulk density in control stands was significantly less than WT + FF treatments stands there is potential for greater aerobic conditions at this depth. These findings indicate the importance of analyzing deeper soil depths when attempting to understand the overall effect of disturbance on microbial-mediated N-cycle processes.

Considering the extent of organic matter removal, it is somewhat surprising that we did not observe more differences in gene counts

associated with CAZy families. However, this study was specifically focused on mineral soil horizons and did not investigate O-horizon microbial community composition or genetic potential. This was done in part because of the lack of O-horizon redevelopment in the WT + FF treatment. If this soil layer were investigated, it is possible that our results would have been similar to Cardenas et al. (2015) which found major harvest treatment differences for CAZy predicted genes in the organic matter layer. Nonetheless, we did observe a significant reduction in GT and a significant increase in PL with increasing harvest intensity. GTs are enzymes that catalyze the formation of the glycosidic linkage to form a glycoside. The overall reduction in GTs with increasing harvest intensity could indicate that intensive harvest has the potential to reduce bacterial capacity to form structural cell material. Many of the specific families within the GT class are associated with structural synthases such as cellulose synthase and chitin synthase as found in GT2. Consistent with Cardenas et al. (2015), we observed that the PL class of enzymes increased with increasing harvest and may be linked to an increase in pectin and pectate lyases as observed in PL1 and PL10. Diazotrophic bacteria such as *Bradyrhizobium* and *Rhizobium* have been associated with increased production of pectin-degrading enzymes (Fauvart et al., 2009; Xie et al., 2012), and although we did not observe a statistical difference in the predicted relative abundance of N-fixing genes ( $p = .06$ ), the relative abundance was highest in the WT + FF removal treatment and lowest in the control. The three other families (i.e., AA1, AA4, and CE15) that were statistically affected by timber harvest were all associated with the decomposition of lignin. These families include laccases, ferredoxins, oxidases, and methyltransferases. Considering that lignin is a major component of leaf litter and woody debris, it is expected that the metagenomic capacity to degrade this structural component would be somewhat affected in the intensively harvested treatment.

It must be mentioned that in the context of other LTSP sites, the Groveton-LTSP is considered somewhat of an outlier. Prior to the onset of the OMR treatments, the site had been thinned intermittently and had a history of naturally occurring and anthropogenic fires. As postulated by Wilhelm et al. (2017), these prior perturbations may have selected for soil microbial communities that are more resilient to disturbance when compared to other LTSP soils, which would explain their lack of observed differences in O- and upper A-horizon microbial community composition at the Groveton-LTSP. Contrary to this, we found significant shifts in mineral soil community structure as well as changes in putative biogeochemical functionality. Our results correlate well with reduced mineral C and nutrients in the intensive OMR stands. It should also be noted that second year survival of replanted saplings at the Groveton-LTSP was drastically reduced in WT + FF stands (Scott and Stagg, 2013). Over time, the organic matter lost via forest floor removal coupled with the loss of saplings at year two may have led to the decade-scale reduction in soil C and nutrients as well as the observed changes in microbial community structure and putative functionality in mineral soils.

## 5. Conclusion

We have shown that OMR during forest harvest can result in long-term alterations in soil chemical properties, and can also modify soil bacterial community structure, the relative abundance of dominant genera, and the abundance of biogeochemically-relevant predicted gene groups. Consistent with our hypothesis and previous studies was an overall decrease with increasing harvest intensity in the abundance of predicted genes associated with nitrification (*amoCAB*) as well as significant alterations in some C-degrading gene classes (glycosyltransferases and polysaccharide lyases). Contrary to our initial hypothesis was an overall increase in the predicted gene abundances of dissimilatory nitrate reduction and denitrification with increasing harvest intensity. More specifically, we found increased abundances of *narGHI* and *napAB*, which are both involved in the reduction of  $\text{NO}_3^-$

to  $\text{NO}_2^-$ . Although this observation is counterintuitive due to the reduced concentration of  $\text{NO}_2^- + \text{NO}_3^-$ , it is possible this is an artifact of the processes that led to the loss of nitrogen from the soil system following harvest. Alternatively, the reduction in  $\text{NO}_2^- + \text{NO}_3^-$  could be a function of high rates of denitrifying processes. This study also illustrates the importance of considering deeper soil horizons when attempting to understand the effects of forest disturbance on soil microbial consortia and key biogeochemical transformations. Finally, it is necessary to acknowledge the limitations of inferring biogeochemical functionality from *in silico* predictions. Although our results correlate well with measured soil chemical parameters, the predictive power of these types of analyses may be better utilized for hypothesis generation rather than for offering firm conclusions on actual biogeochemical functionality. Additionally, the inclusion of other major components of the soil microbial community (i.e., fungi and archaea) may lead to profoundly different conclusions regarding microbial biogeochemical cycling following forest harvest.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.soilbio.2018.01.008>.

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