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Carbon to nitrogen ratios influence microbial diversity in the soil of interior Douglas-fir forests in British Columbia

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SUMMARY In British Columbia, clear-cut logging and subsequent reforestation is practiced, which has previously been shown to cease the flow of carbon and cause changes to the physical soil environment. After logging treatments, forests experience changes in the ratio of carbon to nitrogen levels which can in turn cause changes to soil microbial communities. These changes can ultimately lead to a change of the metabolic profile of the soil, which affects the entire forest soil ecosystem. Wilhelm *et al*. collected soil data from various North American forest sites over ten years after logging using different levels of organic matter removal to analyze the soil microbiomes with amplicon sequencing. Additionally, they collected information on carbon and nitrogen levels. Those data were used to investigate the changes in soil microbial communities that occur at different carbon to nitrogen (C:N) ratios. By investigating the microbial community diversity in the organic layer of interior Douglas-fir forest sites, phylogenetic distance was found to drive the differences in microbiota between low, medium, and high C:N ratio categories in interior Douglas-fir sites. Furthermore, low-abundance taxa unique to each C:N category, as well as bacterial families indicative of low and high C:N ratio soils were identified.

INTRODUCTION

The stoichiometric balance of essential elements in soil influences the community composition and productivity of above- and belowground ecosystems (1). In forests, the ratios of carbon (C) and nitrogen (N) are fundamental for regulating plant and microbial growth and maintaining their ecosystem services. Forests are estimated to store 45% of the planet's C and are major contributors to the global C and N cycles (1–4). In most forests, N is the limiting resource for growth (3).

Ecologically distinct environments have different soil carbon to nitrogen (C:N) ratios (5). Due to the lower amounts of standing biomass, deserts, tundra, and grasslands have a lower C:N ratio relative to deciduous forest, while the ratio is highest in coniferous forests (5). Over the long course of succession from first plant colonizers, grasses, shrubs, and broadleaves to mature stand forests, the C:N ratio fluxes in response to a complex interplay of above- and belowground processes, as dominant plant species influence nutrient availability in the soil (6, 7). Fast-growing, early successional plants produce larger volumes of nitrogen-rich litter that favors bacterial-dominated soils with fast nutrient cycling, whereas a shift towards fungal-dominated communities occurs as the litter quality shifts towards more recalcitrant organic matter (5, 7, 8). These changes in litter quality and composition also lead to acidification, which again affects the soil microbiome (7, 8). Bacteria have a lower C:N ratio in their biomass than fungi (5). Hence, microbial necromass further enforces the feedback loop towards a higher C:N ratio (5). While it is difficult to parse out the causation of these complex relationships, a positive correlation between fungal:bacterial ratio and C:N ratio can be drawn (5, 9).

Clear-cut logging is practiced in British Columbia (BC), Canada. Soon after an area is logged, reforestation begins, which resembles the cycles of forest succession. However, widespread herbicide applications are used to suppress broadleaf vegetation and favor the growth of conifer seedlings (10). Clear-cut logging has immediate and drastic effects on microbial communities by decreasing the flow of C though root exudates from primary producers, and by changes to the physical soil environment (11–15). Furthermore, logging practices vary in intensity of organic matter (OM) removal during logging; from leaving branches on site and stumps in the ground (OM1), to the complete removal of all vegetal

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debris and even the topsoil (OM3) (16). During the first decade after logging, decomposing organic matter is the primary nutrient source for a recovering forest (14). Hence, the severity of OM removal treatments affects the nutrient availability on site in the years after logging (14, 17). Additionally, logging can promote microbial respiration, ultimately leading to a decrease in soil C:N ratios (4). Longitudinal studies in BC forests assessing ecosystems responses to logging are rare and vary in their analyses of diversity: It has been found that plant communities in several BC ecozones had recovered 20 years after logging without diversity loss (18). However, fungal communities remained significantly altered even 10 – 15 years post-harvest (16, 19). Bacterial communities in BC's interior Douglas-fir (IDFBC) and sub-boreal spruce (SBSBC) ecozones were overall less affected by different levels of severity of organic matter removal (16, 19, 20).

Considering the altered OM availability after logging paired with C:N ratio changes during succession and their positive correlation with fungal:bacterial ratios, the question arises whether bacterial diversity is affected by C:N ratios. While fungal associations with plants over the course of succession have received much interest, less is known about how shifts in C:N ratios affect the soil bacterial diversity. Here, the extensive metagenomic data set collected by Wilhelm *et al.* in a long-term soil productivity (LTSP) study is being revisited (21). Wilhelm *et al*. collected 16S rRNA sequence and metadata from soil samples in six different North American ecozones over a decade after these plots were harvested using different levels of OM removal (21). This dataset is a valuable resource in the study of long-term effects of OM removal practices. Focusing on the organic layer in the IDFBC ecozone, three hypotheses were tested: (i) C:N ratios are significantly affected by LTSP treatment, (ii) bacterial community diversity is affected by relative levels of C:N, and (iii) unique indicator taxa exist at relative levels of C:N ratios. It was predicted that OM removal severity significantly affects C:N ratios, and that bacterial diversity is driven by phylogenetic distance based on the assumption that different C:N ratios offer distinct functional niches in the soil environment.

METHODS AND MATERIALS

Sample collection and amplicon sequencing overview. All soil sampling and amplicon sequencing was performed by Wilhelm *et al*. in a previous LTSP study (21). The samples were collected from eighteen different sites in six ecozones across North America. Relevant to this study are the SBSBC and IDFBC ecozones in British Columbia, Canada. SBSBC and IDFBC each contain three sites. Log Lake (LL), Skulow Lake (SL), and Topley (TO) are within SBSBC. Black Pines (BP), Dairy Creek (DC), and O'Connor Lake (OC) are within IDFBC. Each site is comprised of plots with four types of LTSP treatments: unlogged reference plots (REF); logged plots on which only tree trunks were removed, and the remaining branch wood, debris and stumps were left on site (OM1); logged plots on which trunk and branches, but not stumps, were removed (OM2); and logged plots on which entire tree and topsoil were removed (OM3). Tree species common to each respective ecozone were replanted in the plots and no herbicide treatment was applied (16, 21). Soil samples were collected from the organic and mineral layers of soils 11 to 17 years after harvesting. Additional metadata was collected for each sample, including total % C and N content and other abiotic factors. DNA was extracted from soil samples and the V1-V3 regions of the bacterial 16S rRNA gene were used as a template to generate an amplicon library for sequencing. Raw data containing the 16S rRNA gene sequences were deposited in the European Sequencing Archive with study accessions PRJEB8599 and PRJEB12501 (21).

Metadata processing. All steps for processing metadata were performed using R version 4.1.2 (2021-11-01) (22). The original metadata file was imported and filtered to include only the organic layer of the SBSBC and IDFBC ecozones. The organic layer of IDFBC did not include the OM3 treatment due to an insufficient amount of organic layer soil at those sites (21). Moving forward in the analysis of IDFBC specifically, the metadata file was modified to exclude samples without C:N ratio values; hence, all samples from the OC site in IDFBC were removed. In addition, three samples in the BP site with uncharacteristic total % C values (2.5%) were removed due to suspected data entry error. C:N ratio categories were assigned to each sample based on quartiles (Table 1) and this new categorical variable was appended

to the metadata file. This resulted in a sample size of 12 for the low category, 15 for the mid category, and 12 for the high category.

Table 1. Assignment of C:N ratio categories based on quartiles.

low		mid	high	
Minimum	1st quartile	Interquartile range	3rd quartile	Maximum
25.10	29.5	29.50 - 39.20	39.2	50.9

Processing rRNA sequences. Quantitative Insights Into Microbial Ecology (QIIME 2) was used to process the raw sequencing data, perform filtering steps and to calculate diversity metrics (23). First, the raw data was imported and demultiplexed. Inspection of the read qualities resulted in the decision to trim at 400 base pairs (bp), as the quality rapidly declined above 400 bp. The DADA2 software package was then used to denoise the data and assign amplicon sequence variants (ASVs) (24). Taxonomies were assigned to the ASVs by using the Greengenes database (release of gg-13-8-99-nb) (25). Then, filtering steps were performed to remove unwanted chloroplast and mitochondrial DNA sequences. The data was then further sub-set to study the organic layer of either the SBSBC or IDFBC ecozone. Rooted and unrooted phylogenetic trees were generated in QIIME 2 to calculate diversity metrics and rarefaction depths of 2904 and 1086 reads were chosen for SBSBC and IDFBC, respectively (26).

Preliminary statistical analyses. First, to determine whether sites accounted for differences in bacterial community diversity within SBSBC (n=3) and IDFBC (n=2), weighted UniFrac beta diversity was calculated for each ecozone (27). Weighted UniFrac was used based on its broad scope of measuring beta diversity, which takes both evenness and phylogenetic distance into account. The weighted UniFrac principal coordinate analysis (PCoA) plot from QIIME 2 was imported into R and a figure was generated using the ggplot2 and ggpubr packages (28, 29). After this initial analysis, all further analyses were performed for IDFBC only.

Next, to determine whether LTSP treatments accounted for differences in bacterial community diversity within IDFBC, alpha diversity metrics including Faith's phylogenetic diversity and Pielou's evenness (30, 31), and beta diversity metrics, including unweighted and weighted UniFrac, Bray-Curtis, and Jaccard (27, 32, 33, 34) were calculated for IDFBC. A pairwise Kruskal-Wallis test was performed for statistical analysis of each alpha diversity metric. Permutational analysis of variance (PERMANOVA) was performed for statistical analysis of each beta diversity metric. 999 permutations were used for all PERMANOVA tests. All q-values < 0.05 were considered significant.

Correlation analysis. To test the first hypothesis that C:N ratios are significantly affected by LTSP treatments, a box plot comparing the C:N ratio values in relation to LTSP treatments was generated in R. A statistical analysis comparing the C:N ratios between LTSP treatment groups was performed using a one-way analysis of variance (ANOVA) with a p-value significance cut-off of 0.05. In addition, box plots comparing the total % C and the total % N against LTSP treatments were generated to determine whether changes in total C or N in response to LTSP treatments were driving factors for changes in the C:N ratio. One-way ANOVA tests were used to determine whether changes in total C and N in response to LTSP treatments were significant (p<0.05) and a pairwise t-test was used to determine specifically which groups were significantly different (p<0.05) from each other.

Diversity metrics and relative abundance calculations. To test the second hypothesis that bacterial community diversity is affected by relative levels of C:N, the alpha and beta diversity metrics previously calculated for IDFBC in the context of the C:N ratio categories were analyzed. Pairwise Kruskal-Wallis tests for alpha diversity metrics and PERMANOVA tests for beta diversity metrics were performed to determine the statistical significance of differences in bacterial community diversity between C:N ratio categories. 999 permutations were used for all PERMANOVA tests, and q-values < 0.05 were considered significant. To

further analyze the bacterial community composition, the relative abundance of ASVs within C:N ratio categories in IDFBC was calculated in R using the qiime2R, phyloseq, file2meco, and microeco packages (35, 36, 37). A helper function was created to calculate relative abundance, low abundant features (<0.05%) were removed, and a minimum sequencing depth of 500 was set. A Venn diagram was then generated from the relative abundance calculations using the microeco package (37).

Indicator taxa analysis. To test the third hypothesis that unique indicator taxa exist at relative levels of C:N ratios, the indicspecies package in R was used to determine indicator ASVs at the family level for the C:N ratio categories (38). Subsequently, four indicator families were selected and boxplots comparing their relative abundance between the low and high C:N ratio categories were generated in R (28). Their selection was based on (i) resolution to the family level and (ii) availability of information about their characteristics and ecological relevance in the literature. Koribacteraceae and Bradyrhizobiaceae were selected for the high C:N ratio and Methylobacteriaceae and Geodermatophilaceae were selected for the low C:N ratio.

RESULTS

Site accounts for differences in bacterial community composition within sub-boreal spruce (BC) but not interior Douglas-fir (BC). Before testing the hypotheses, the SBSBC and IDFBC data were analyzed to check whether site contributed to significant differences in bacterial community composition within each ecozone. To do so, weighted UniFrac PCoA plots were generated for the organic layer of each ecozone (Fig. S1). Statistical testing using weighted UniFrac PERMANOVAs showed that there were significant differences between all sites in SBSBC (Fig. S1A; LL vs SL: $F_{20,19} = 11.573$, $q = 0.001$; LL vs TO: $F_{20,20} = 5.227$, $q = 0.001$; SL vs TO: $F_{19,20} = 6.982$, $q = 0.001$). Because site drove changes in bacterial community composition in SBSBC and dividing the ecozone into separate sites left too small a sample size, the SBSBC samples were not pursued further in this study. Alternatively, sites were not significantly different in IDFBC (Fig. S1B; Weighted UniFrac PERMANOVA: $F_{17,38} = 0.989$, $q = 0.385$). Therefore, samples in IDFBC could remain grouped to retain a sufficient sample size for further analyses.

LTSP treatment does not affect bacterial community composition in interior Douglas-fir (BC). To confirm that bacterial community composition in the organic layer of IDFBC was not affected by LTSP treatments, pairwise Kruskal-Wallis and PERMANOVA tests were used to analyze differences between the treatments. The Kruskal-Wallis test for the alpha diversity metrics revealed no significant differences between the treatment groups (Table S1). Similarly, the PERMANOVA analysis of the beta diversity metrics showed no significant differences between the treatment groups (Table S2). Because LTSP treatment did not affect the bacterial community composition in IDFBC, the treatments could remain grouped in IDFBC for further C:N ratio analyses.

LTSP treatments do not significantly affect C:N ratios. To test the first hypothesis that C:N ratios are significantly affected by LTSP treatment, a one-way ANOVA was used to compare between the treatments. The C:N ratio appeared to decrease with increasing severity of LTSP treatment (Fig. 1), however, the ANOVA test revealed there was no significant difference between the treatments (One-way ANOVA; $F_{2,36} = 0.896$, $p = 0.417$). Interestingly, additional ANOVA and pairwise t-tests revealed that total % C significantly decreased (Fig. S2; One-way ANOVA: $F_{2,36} = 15.2$, $p < 0.001$) between REF and OM1 (Fig. S2; Pairwise t-test; $p < 0.001$), and between REF and OM2 (Fig. S2; Pairwise t-test: $p < 0.001$), but not between OM1 and OM2 (Fig. S2; Pairwise t-test: $p = 0.136$). Total % N did not significantly change (Fig. S3; One-way ANOVA: $F_{2,36} = 2.513$, $p = 0.952$). Hence, the slight decrease in C:N ratios with increasing severity of OM removal was driven by the decrease of total % C.

Differences in bacterial community diversity between C:N ratio categories in interior Douglas-fir (BC) are driven by phylogenetic distance. To test the second hypothesis that bacterial community diversity is affected by relative levels of C:N ratio, pairwise Kruskal-

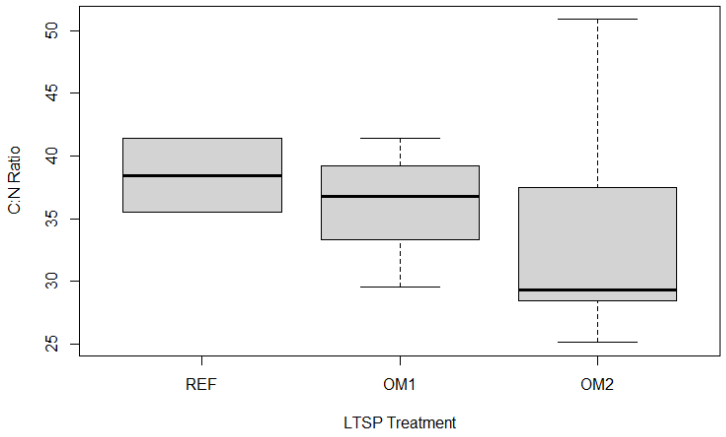

FIG. 1 C:N ratio changes in response to organic matter removal are not significant. Boxplots showing differences in C:N ratio between the different Long-Term Soil Productivity treatments in the organic layer of interior Douglas-fir (BC). One-way ANOVA: $F_{2,36} = 0.896$, $p = 0.417$.

Wallis and PERMANOVA tests were used to analyze differences between the C:N ratio categories. The Kruskal-Wallis test for the alpha diversity metrics revealed no significant differences in bacterial community diversity between the categories (Table S3). The results for the unweighted UniFrac beta diversity showed significant differences in bacterial community diversity between the low and medium C:N ratio categories (Table 2; Unweighted UniFrac PERMANOVA: $F_{11,14} = 1.140$, $q = 0.050$) and between the low and high C:N ratio categories (Table 2; Unweighted UniFrac PERMANOVA: $F_{11,14} = 1.210$, $q = 0.036$). The PERMANOVA results for the remaining beta diversity calculations showed no significant differences between the C:N ratio categories (Table 2). The significance in the unweighted, but not the weighted UniFrac indicated that the differences in community diversity were driven by phylogenetic distance. This suggested that taxonomically distant ASVs existed in the individual C:N ratio categories.

Unique ASVs identified in each C:N ratio category in interior Douglas-fir (BC). To determine if there were unique ASVs between the different C:N ratio categories, the Venn diagram displaying relative abundance was analyzed. The Venn diagram showed that each category did contain unique ASVs as well as shared ASVs between the different categories (Fig. 2). In total, 270 ASVs were identified.

Different taxa are associated with high and low C:N ratio categories in interior Douglas-fir (BC). To test the third hypothesis that unique indicator taxa exist at relative levels of C:N ratios, an indicator taxa analysis was performed at the family level. Indicator taxa were

Table 2. Pairwise PERMANOVA comparisons between interior Douglas-fir (BC) samples in different C:N ratio categories. q <0.05 are bolded and denoted with an asterisk.

Diversity metric	Groups	Sample size	F model	q-value
Bray-Curtis	Low vs. Mid	27	0.641	1.000
	Low vs. High	24	0.604	1.000
	Mid vs. High	27	0.665	1.000
Jaccard	Low vs. Mid	27	0.948	0.999
	Low vs. High	24	0.944	0.999
	Mid vs. High	27	0.949	0.999
Unweighted UniFrac	Low vs. Mid	27	1.140	**0.050** *
	Low vs. High	24	1.210	**0.036** *
	Mid vs. High	27	0.999	0.474
Weighted UniFrac	Low vs. Mid	27	1.092	0.383
	Low vs. High	24	2.231	0.084
	Mid vs. High	27	1.018	0.383

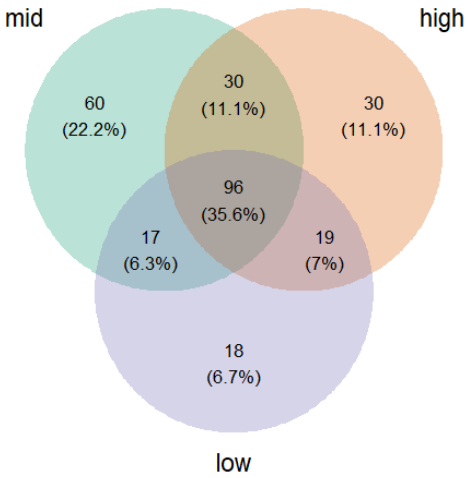

FIG. 2 Unique ASVs are present in each C:N ratio category in interior Douglas-fir (BC). A Venn diagram displaying shared and unique ASVs between C:N ratio categories in the organic layer of interior Douglas-fir (BC). In total, 270 ASVs were identified. The total number of ASVs in each group are shown followed by the percent relative abundance.

identified in the low and high C:N ratio categories (Table 3). Two indicator families from high (Koribacteraceae and Bradyrhizobiaceae) and low (Methylobacteriaceae, Geodermatophilaceae) C:N ratio categories were selected for further relative abundance analysis. Most families selected had low (<2%) relative abundances, except for Bradyrhizobiaceae (Fig. 3).

DISCUSSION

Forest environments are shaped by the intricate flux of nutrients. C and N are required by every life form and their stoichiometric ratios in the soil affect the microbial communities of the forest floor. Since the C:N ratios are affected by clear-cut logging and the following succession, it was hypothesized that C:N ratios decrease with increased severity of OM removal and that microbial communities change in response to varying C:N ratios.

Different OM removal treatments did not significantly change C:N ratios in interior Douglas-fir (BC) soil. It was hypothesized that logging would affect the C:N. This prediction was based on previous literature that suggested there would be reduced C in the soil due to

Table 3. Indicator taxa analysis identified bacterial families indicative of soils with low or high C:N ratios. Specificity is the degree to which a family is found in the only low or high C:N ratio soils. Fidelity is the degree to which a family is found in all samples of the low or high C:N ratio soils. The indicator value is the degree to which a family is an indicator of low or high C:N ratio soils. *p*-value ≤ 0.01 are annotated with '**'. Families highlighted in bold were used for relative abundance analysis (Fig. 3). NA = not assigned.

C:N ratio	Phylum	Class	Order	Family	Specificity	Fidelity	Indicator value	p-value
high	Acidobacteria	Acidobacteriia	Acidobacteriales	**Koribacteraceae**	**0.480**	**1.000**	**0.693**	**0.045**
		Acidimicrobiia	Acidimicrobiales	NA	0.473	1.000	0.687	0.005 **
		Thermoleophilia	Solirubrobacterales	Conexibacteraceae	0.522	0.750	0.626	0.050
	Actinobacteria	NA	NA	NA	0.622	0.667	0.644	0.020
	Gemmatimonadetes	Gemmatimonadetes	N1423WL	NA	0.532	0.833	0.666	0.020
			KD8-87	NA	1.000	0.333	0.577	0.020
	Proteobacteria	Alphaproteobacteria	NA	NA	0.452	1.000	0.673	0.005 **
			Ellin329	NA	0.448	1.000	0.670	0.005 **
			Rhodospirillales	Rhodospirillaceae	0.403	1.000	0.634	0.020
			Rhizobiales	**Bradyrhizobiaceae**	**0.380**	**1.000**	**0.617**	**0.015**
		Betaproteobacteria	Ellin6067	NA	0.727	0.750	0.738	0.010 **
		Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	0.535	1.000	0.732	0.005 **
low	Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	0.767	0.583	0.669	0.020
				Geodermatophilaceae	**0.743**	**0.500**	**0.610**	**0.015**
				Actinosynnemataceae	0.897	0.333	0.547	0.015
		Thermoleophilia	Gaiellales	AK1AB1_02E	0.765	0.500	0.618	0.015
	Proteobacteria	Alphaproteobacteria	Rhizobiales	**Methylobacteriaceae**	**0.767**	**0.750**	**0.758**	**0.005** **
			Sphingomonadales	NA	0.818	0.333	0.522	0.050
			Rhodospirillales	NA	0.814	0.333	0.521	0.045

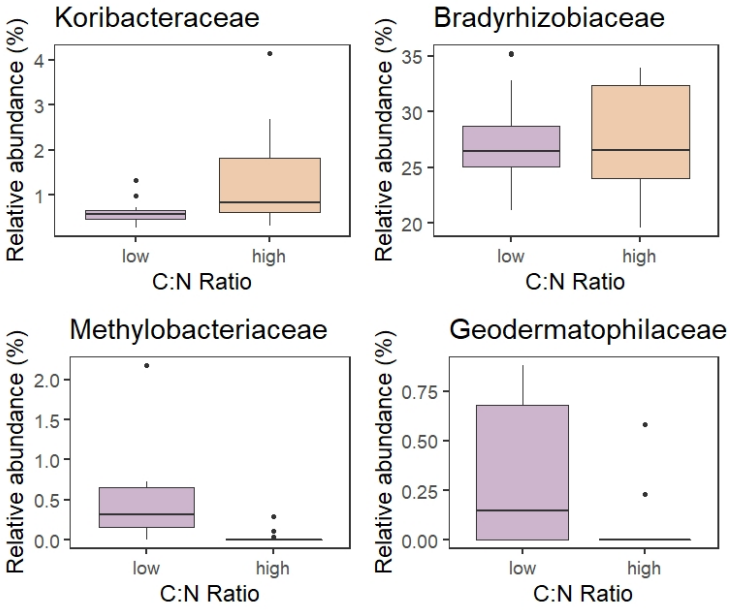

FIG. 3 Indicator families in low and high C:N ratio categories in interior Douglas-fir (BC). Koribacteraceae and Bradyrhizobiaceae are indicative of high C:N ratios. Methylobacteriaceae and Geodermatophilaceae are indicative of low C:N ratios.

reduced input from plants and increased C loss due to microbial respiration (12-15). Moreover, physical changes to the soil environment can have effects on microorganism-mediated processes such as nitrification and the decomposition of C containing organic matter (15).

While a decrease in the median C:N ratio was observed with increasing severity of LTSP treatment, these values were not significant. This lack of significance is likely due to the large degree of variance in C:N ratio values. However, a significant decrease in the % C present in the soil with increasing severity of OM removal was observed. This is in line with results from Hume *et al*. who also observed a significant decrease in C but not N in forest soil after logging, with greater effects correlating with greater logging intensity (39). Other studies made similar observations (40-42). However, repeated logging of reforested sites may lead to a significant decrease of C:N ratios over the time span of a century. Therefore, longer term studies are much needed to inform decision making in silviculture.

It can only be speculated why C:N ratios became more variable as OM removal intensity increased. Logging changes the soil environment in many ways through compaction, mixing of the soil layers, redistribution of organic matter, increased soil exposure to sun and desiccation, which may destabilize the soil environment. Soil conditions can vary drastically even within a small area and some areas may have been more disrupted than others or recovered faster.

It is assumed that the total % C and N data used in this analysis were obtained by means of combustion. This methodology cannot distinguish between labile and recalcitrant forms of C and N. Hence, it is unknown which proportions of total C and N were immediately available to microbes or required complex enzymatic breakdown due to their recalcitrance.

Soils with different C:N ratios provide unique environments for lowly-abundant, taxonomically diverse bacteria in interior Douglas-fir (BC). The performed diversity analyses indicated that the differences in microbial community structure between the different C:N groups are minimal. The data suggests that taxonomically distant, but lowly-abundant taxa are responsible for the differences of microbial communities in low and high C:N ratio soils. This means that low and high C:N ratio soils are uniquely different environments. This enables the presence of niche bacterial taxa with presumably narrow spectrums of metabolic capacity and low tolerance for shifts in C:N ratios. Other studies also identified C:N ratios as an important factor in influencing microbial community composition (43, 44).

Since the relative abundances of all select indicator families except Bradyrhizobiaceae were below 2%, it can be inferred that the overall bacterial community composition in IDFBC soil does not shift dramatically when faced with different C:N ratios. Generally speaking, the IDFBC soil bacterial communities appear to be robustly adapted to C:N ratios ranging from

25 - 50. While a relative abundance of less than 2% may appear low, further studies are needed to conclude their ecological significance. Hermans *et al.*'s analysis of the family Gaiellaceae in response to various C:N ratios concluded significance, although it also showed a very low relative abundance of <0.2%. Therefore, it can tentatively be considered that the results of this study are noteworthy despite the low relative abundances.

C:N ratios affect soil bacteria communities. Bacterial community abundances and composition differ throughout the soil horizons, which can broadly be divided into organic and mineral horizons. The upper organic horizon comprises the first 1-3 centimeters of the soil and is characterized by high levels of organic matter and labile nutrients (8, 10, 45). Therefore, it harbors the most activity of microbes. Throughout diverse ecosystems, the bacteria phyla Acidobacteria, Proteobacteria, and Actinobacteria are the most abundant in the organic soil layers (5, 8). The presence of diverse genes for enzymes needed for plant polysaccharide and chitin breakdown indicate that Acidobacteria and Actinobacteria degrade plant and fungal biomass (8, 46). These enzymes could provide them with a competitive advantage in soils with low concentrations of labile, but high concentrations of recalcitrant C (46). Proteobacteria classes contain symbiotic and free-living N-fixers, as well as methanotrophs which are responsible for the important function of forest soils as methane sinks (8).

Hermans *et al.* found that Proteobacteria, Alphaproteobacteria, Gammaproteobacteria and Acidobacteria relative abundances are positively correlated with C:N ratios, whereas Betaproteobacteria and Actinobacteria negatively correlated (43). Additionally, Hermans *et al.* identified a strong negative correlation between members of the Gaiellales and C:N ratios (43). The identification of Gaiellales as an indicator family of low C:N ratios is therefore in agreement with Hermans *et al* (Table 3).

Due to the lack of culturability, the current knowledge of soil bacteria is still in its infancy. Based on literature availability, two indicator families of each low (Geodermatophilaceae and Methylobacteriaceae) and high (Bradyrhizobiaceae and Koribacteraceae) C:N ratio category were selected for relative abundance analysis and further discussion of their role in the soil ecosystem. This may allow for a better understanding of the changes to the soil environment that may have led to their changed abundances.

Bradyrhizobiaceae is a family that includes 12 genera diverse in their ecological and metabolic traits (47). They cover a range of pathways within the nitrogen cycle, contribute to the sulfur cycle, grow aerobically and/or anaerobically, and in some cases grow photoheterotrophically. *Bradyrhizobium* is a genus within this family well known for its ability to fix nitrogen while symbiotically associated with a leguminous plant host (47). Interestingly, *Bradyrhizobium* was identified as being dominant in microbial communities within coniferous forest soils lacking leguminous plants from the same North American LTSP study ecozones (48). Further genetic investigation revealed that the isolated free-living *Bradyrhizobium* ecotypes lack the *nif* and *nod* gene clusters involved in nitrogen fixation and that they are characterized instead by a collection of aromatic degradation pathways (48). This demonstrates that *Bradyrhizobia* that dominate these coniferous forests are not the typical endosymbiotic nitrogen fixers. Other possible nitrogen fixers from the Bradyrhizobiaceae family include the *Agromonas, Rhodoblastus,* and *Rhodopseudomonas* genera (48).

Koribacteraceae, a family of the acidobacteria, are adapted to low nutrient conditions and capable of nitrate and nitrite reduction (49). Koribacteraceae are acidophilic and possess cellulases, hemicellulases, polysaccharide lyases, and pectin esterases to facilitate degradation of plant-biomass (50). It is hence not surprising to find them to be an indicator of high C:N ratio soils, which are rich in plant organic material and litter. Koribacteraceae have been found to positively correlate with methanogenic taxa (50).

Members of the family Geodermatophilaceae are mainly found in soils and include three genera, *Geodermatophilus*, *Blastococcus*, and *Modestobacter* (51). Some of these bacteria can colonize stone and extremely dry soils and are thus adapted to survive low nutrient conditions, temperature extremes and desiccation (51). Due to their ability to travel long distances on dust particles, Geodermatophilaceae are ubiquitous in most enrvironments (51). In this study, an increased abundance of Geodermatophilaceae in soils with low C:N ratio

were found. Therefore, it is speculated that low C:N soils from logged forests provide a niche habitat for Geodermatophilaceae as these soils are more vulnerable to temperature extremes and desiccation.

Methylobacteriaceae are obligate aerobes and comprise the three genera *Methylobacterium*, *Microvirga*, and *Meganema*. As facultative methanotrophs, they are an indicator for the presence of 1C-compounds such as methane (52). Log landings, which are staging areas in managed forests for the processing and storage of trees before transport, have been implicated with a release of methane (53). Hence, there may be a connection between logging, methanogens and the presence of the methanotrophic Methylobacteriaceae in low C:N ratio soils.

While it is beyond the scope of this work to draw strong ecological inferences from these briefly discussed families, their change in abundance in different C:N ratio soils emphasizes their sensitivity to differences in C:N ratio in the soil. More work is needed to investigate the connections between C:N ratios, the long-term effects of logging and OM removal, and changes of the microbial communities as a result.

Limitations As demonstrated by the SBSBC ecozone, collecting samples from various sites can introduce misleading variables in the data. This affects where and how samples should be obtained for analysis. It would be advantageous to collect a greater number of samples from a singular site.

Overall, a major limitation when analyzing the effects of the C:N ratio on the soil microbiome in BC forests was the available sample size from the Wilhelm *et al.* dataset (21). Many filtering steps were required due to confounding variables which reduced the sample size to 12 for the low category, 15 for the mid category, and 12 for the high category out of a total of 125 original BC samples. In addition to confounding variables, the OC site samples from IDFBC were discarded because no C:N ratio data was recorded for those samples. The 'low', 'mid', and 'high' categories used in this study were determined based on 1st and 3rd quartile calculations from the C:N ratio values specific to IDFBC with the values ranging from 25.1 to 50.9. While this seems to be close to other forests where C:N ratios typically range from 30-40 in the O horizon, it is important to be aware of how the categories were created when compared to other forest sites (45). Furthermore, due to significant differences in bacterial diversity between ecozones, it may be difficult to extrapolate these results obtained in this study to other forests (16). Another limitation is the classification of ASVs. Not all indicator taxa were resolved down to the family level. Lastly, major gaps exist in our knowledge of soil bacteria species at a functional and taxonomic level due to their unculturability. The process of improving the classification of metagenomic sequencing is continuous as more sequence data becomes available and reference packages are updated.

Conclusion This study aimed to investigate whether OM removal from logging practices have long-term effects on the C:N ratio and whether differences in the C:N ratio affect bacterial diversity in the IDFBC ecozone. While there was a decreasing trend in the median C:N ratio with increasing OM removal, it was not significant. Alpha and beta diversity analyses comparing C:N ratio categories within the organic layer of IDFBC were mostly non-significant with the exception of the unweighted UniFrac. Therefore, IDFBC soil microbial communities are generally little affected by changes in the C:N ratios ranging from 25 - 50. However, lowly-abundant, and phylogenetically diverse ASVs were found to be associated with low, medium, and high C:N ratios. This suggests that these taxa have stricter C:N ratio requirements. Relative abundance calculations revealed that 35.5% of ASVs are shared between all three C:N ratio categories and that 6.7%, 22.2%, and 11.1% of ASVs are unique to the low, mid, and high categories respectively. Investigation of indicator taxa at the family level showed that there are 7 families indicative of the low, and 12 families indicative of the high C:N ratios. Relative abundance analysis of 4 selected indicator families showed low overall abundance of less than 2% except for Bradyrhizobiaceae.

Future Directions The concept of sampling site as a confounding variable can serve as a point of consideration and inspiration for future studies. To minimize the effect of site as a confounding variable, future studies on soil microbiomes should make use of samples from

the same sampling site or sites in close proximity. Moreover, datasets should be examined for site as a confounding variable before proceeding with analyses.

The soil microbiome is extremely complex and likely influenced by many factors (elevation, sun exposure, moisture, soil type, nutrients etc.). Given the influence of site on microbial diversity, future research might investigate the different factors that influence microbial diversity to explain the differences between sites in British Columbia.

In addition to collecting bacterial sequencing data in North American soils, Wilhelm *et al*. also collected data on fungal community composition. Investigating this fungal data may be of interest considering that the higher C:N ratios that occur during the succession of forest regrowth are known to favor fungal dominance in soil communities (7). Additionally, there may be associations between fungi and specific plants or bacteria that could be investigated.

Lastly, while indicator taxa analysis was performed in this paper, further investigation into the metabolic functions of these bacteria could be carried out using PICRUSt2 (54). Bacteria living in environments with higher or lower C:N may exhibit different metabolisms as they are adapted to their environments.

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CONTRIBUTIONS

D.G. processed data in QIIME 2, modified the metadata file and performed statistical analysis of C and N in R, wrote the introduction, sections of the results and discussion. T.N. processed data in R, generated the figures, wrote the methods, sections of the results, limitations, discussion, conclusion. R.F. processed data in QIIME 2, wrote the abstract, sections of the methods, results, discussion, and future directions. All authors edited the manuscript and provided intellectual contributions

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