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Bacterial, fungal, and mycorrhizal communities in the soil differ between clearcuts and insect outbreaks in the boreal forest 50 years after disturbance

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ABSTRACT

Soil microorganisms influence the functions and processes of forest ecosystems, and their composition is affected by natural and anthropogenic disturbances. Timber harvesting disturbs boreal soil microbiomes, most notably ectomycorrhizal communities which are reportedly less diverse in the first decade following a clearcut. However, the long-term impact of harvesting on forest soil microorganism communities have rarely been investigated nor compared with natural disturbances. Our objective was to compare the composition and diversity of bacterial, fungal, and mycorrhizal communities between boreal old-growth and nearby 50–year–old stands regenerating after either an insect outbreak or a clearcut. Our main hypothesis was that the nature of the stand–replacing disturbance influences the composition of the soil microbiome, and that the effect is still detectable 50 years later. We collected 90 samples from 30 plots across six forest stands dominated by *Abies balsamea*. We sequenced the genome regions 16S rRNA v3v4 for bacteria and ITS1 for fungi and we constructed distance matrices to evaluate changes in community composition with permutational analyses of variance. Results show that 10.2 % to 12.4 % of the variability in community composition can be explained by stand type alone for bacteria, fungi, and mycorrhizae. The composition of soil microbiomes did not vary with soil physicochemical properties. Stands regenerating after a clearcut had a greater alpha diversity (H') of fungi and mycorrhizae than stands regenerating after an insect outbreak, while old–growth stands were intermediate. Our data indicate that soil microbiomes associated with natural disturbance dynamics differ from those of clearcutted stands, although the mechanisms underlying this pattern remain unclear. Therefore, we suggest that forest managers spare the largest possible tracts of unmanaged forests across the harvested landscape, including areas affected by natural disturbances, so that benchmark soil communities remain available for future studies.

1. Introduction

The decline of biodiversity due to human activities is an accelerating global crisis (Johnson et al., 2017). One component of this crisis is associated with the destruction of intact forest landscapes due to the extraction of natural resources within previously unexploited territories (Potapov et al., 2017). Intact forest ecosystems contribute to carbon sequestration, hydrographic regulation, biodiversity conservation, and are of cultural significance (Watson et al., 2018). Furthermore, unmanaged landscapes represent an essential benchmark for sustainable forest management (Venier et al., 2018). Opportunities to conserve and study intact forest landscapes are still available in the boreal biome. Boreal biodiversity is related to forest stands and landscape heterogeneity, which emerges from the accumulation of natural disturbances of variable nature, intensity, and size (Desponts et al., 2002; Kuuluvainen, 2009; Martin et al., 2019). In contrast, clearcut logging simplifies forest structure, and therefore limits the availability of microhabitats and biological legacies to succeeding communities (Swanson et al., 2010; Fedrowitz et al., 2014; Rudolphi et al., 2014).

The soil microbiome influences the functions and processes of

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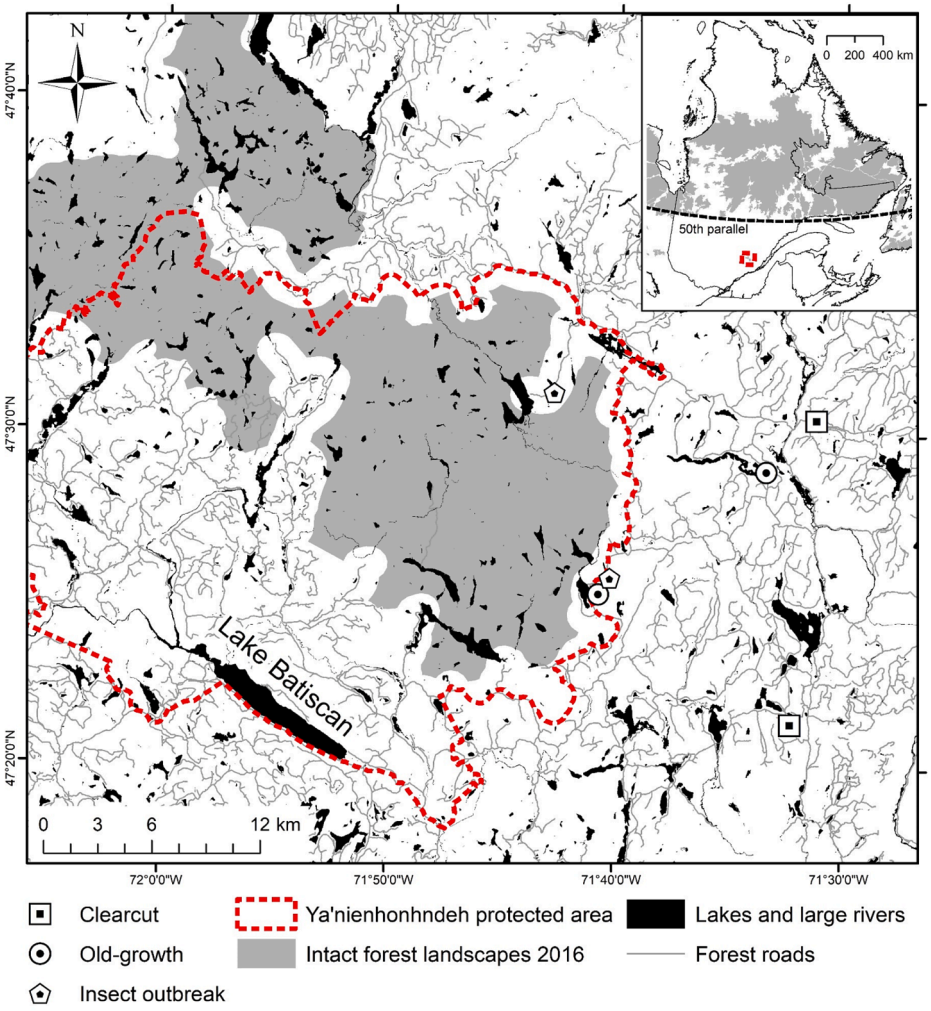

Fig. 1. Location of the stands where soil was sampled. The northernmost three stands belong to one site while the southernmost three stands belong to the other site. The inset map locates the study area in eastern Canada where the dotted line shows the 50th parallel. The data on intact forest landscapes are from Potapov et al. (2017).

natural and managed ecosystems such as carbon sequestration, nitrogen fixation, and methanogenesis (Fierer, 2017). Likewise, forest soil bacterial and fungal communities respond to many factors including climate, vegetation, soil horizon, physicochemistry, and disturbances (Hartmann et al., 2009; Uroz et al., 2016; Tomao et al., 2020). For instance, timber harvesting in boreal forests can reduce the diversity of ectomycorrhizal fungi and the relative abundance of biomass decomposition genes in the soil (Hartmann et al., 2012; Cardenas et al., 2015). Moreover, soil compaction caused by heavy machinery increases the abundance and activity of archaea and bacteria producing methane and nitrous oxide (Frey et al., 2011; Hartmann et al., 2014). However, fungal communities similar to unmanaged stands can be maintained through partial harvesting practices (Tomao et al., 2020; Kim et al., 2021). The impacts of timber harvesting on soil microorganisms have mainly been assessed on the short–term (<15 years) and compared among silvicultural treatments of varying intensity rather than with natural disturbances. To our knowledge, only Varenius et al. (2016) addressed this issue 30 to 50 years following disturbance, suggesting that ectomycorrhizal communities may not return to their initial state between two clearcut rotations. Our study goes further by including all soil fungi and bacteria, in addition to mycorrhizae.

Our objective was to compare the composition and diversity of soil microorganisms 50 years following stand-replacing disturbances of either natural (insect outbreak) or anthropogenic (clearcut) origin. We hypothesized that the nature of the disturbance affects the composition of soil microorganisms, and that the effect is still detectable once the secondary growth is sufficiently mature to be harvested again. We further hypothesized that physicochemical properties influence the composition of the soil microbiome. Finally, because clearcuts create a time-lapse where no mature trees remain at the local scale, we expected mycorrhizal diversity to be lower following a clearcut than after an insect outbreak.

2. Material and methods

2.1. Study area

The study area is located approximately 80 km northwest of Québec City, Canada (47°28′N; 71°47′W), overlapping the Réserve Faunique des Laurentides and the adjacent *Ya'nienhonhndeh* protected area, which includes part of the last intact forest landscape south of the 50th parallel in the province of Québec (Fig. 1). Altitude varies between 500 and 800 m with forest stands largely dominated by balsam fir (*Abies balsamea* (L.) Mill.), mostly co–occurring with white birch (*Betula papyrifera* Marshall) and white spruce (*Picea glauca* (Moench) Voss). Mean annual rainfall and snowfall in the area are 964 mm and 620 cm, respectively, whereas July and January mean temperatures are 14.6 °C and −15.9 °C, respectively (Government of Canada, 2021). Wildfires of natural origin are rare (Couillard et al., 2013) with a natural fire cycle of 750 years (Boucher et al., 2011). The dominant natural disturbance in the region is

the spruce budworm (*Choristoneura fumiferana* Clemens) (Blais, 1965, 1983). In fact, part of the *Ya'nienhonhndeh* territory was spared by the pulp and paper companies because of the severe impacts of spruce budworm outbreaks in the 1940 s and 1970 s (Blouin, 1981).

2.2. Sampling and environmental variables

Soil samples were collected during the second week of July 2019 using a stratified sampling design in two sites across three stand types: **1.** 50 years–old regenerating from a clearcut; **2.** 50 years–old regenerating from a severe insect outbreak; and **3.** > 80 years–old affected by a mild insect outbreak, hereafter referred as old-growth (Fig. 1). Stands were selected based on the 5th decennial forest inventory (Government of Québec, 2021) using ArcGIS 10.8 (ESRI, 2020). Suitability of the stands was validated in the field prior to sampling. Further selection criteria included moderate drainage, slope inferior to 30 %, thick glacial till surface deposit, podzol soil type, and humus mor type. Additionally, a team from the Laurentian Forestry Center (Québec, Canada) carried out three dendrometrical plots in each stand following standard Canadian methodology (National Forest Inventory, 2008), measuring tree, snag, stump, and sapling density, diameter, and composition.

We sampled five plots per stand, hence 10 plots per stand type (Fig. A1), each located between 50 m and 80 m away from each other and from the boundaries of the stands. In each plot, we collected three soil samples, 1 m from each other and spatially distributed along a triangle. Microsites (=exact location where samples were collected) were all mid-slope, well-drained, covered with *Pleurozium schreberi* (Willd. ex Brid.) Mitt., the most common moss in the area, and away from machinery tracks for plots in clearcut stands. Soil was collected a few centimeters below the surface, in the humic layer of the humus (Fig. A2). We disinfected the shovel and nitrile gloves with 90 % ethanol before storing each sample in a 2 ml autoclaved cryotube. Samples were kept on ice until being immersed in liquid nitrogen no later than 4 h after sampling, then stored at −80 °C until DNA extraction. In addition, we collected an extra 100 g of humic humus in each plot. This material was processed by the soil analysing services of the Centre for Forest Research (Québec, Canada) to assess physicochemical properties of the soil. We measured pH (in water), phosphorus concentration (Bray-II), the percent content of humidity, the percent of total carbon (C), nitrogen (N) and sulfur (S), the C/N ratio, and the percent of organic carbon (loss by ignition).

2.3. Molecular manipulations

We transferred 150 mg (±10) of soil from our 90 samples into bead tubes from the NucleoSpin Soil DNA extraction kit (Takara Bio Inc. Shiga, Japan) and followed the protocol provided therein. We used the *SL2* lysis reagent recommended for organic-dominated samples and grinded the soil with a vibro–mill MM400 (Retsch, Éragny, France) for 5 min at 30 Hz. We measured the extracted DNA using a Nanodrop 1000 spectrophotometer (ThermoFisher, Waltham, USA) and reextracted samples with a concentration lower than 10 ng/µl or with ratios of absorbance (nm) (260/280 and 260/230) below 1.5 or above 2.5. Then, we combined in equimolar ratio each trio of DNA extractions from the same plot into a single composite sample to be amplified. Composite samples are used in soil microbiome studies because of the high variability of communities (Song et al., 2014). We ascertained the methodological soundness of using composite samples, by amplifying all three samples from six plots (one per stand) and comparing their individual community composition with that of their corresponding composite sample (Fig. A3).

We amplified DNA through a two-step dual-indexed polymerase chain reaction (PCR) (amplicon sequencing), targeting the genomic regions 16S rRNA v3v4 for bacteria and ITS1 for fungi (Internal Transcribed Spacer), for which we used the primer pairs 341F/805R (Klindworth et al., 2013) and ITS1/ITS2 (White et al., 1990), respectively. In both cases, we used Q5 high-fidelity DNA polymerase (New England Biolabs, Ipswich, USA) and its associated PCR recipe with a reaction volume of 50 µl. We used the following thermocycles (*sec* [°C]): PCR#1 (16S): 120[98], 35x(10[98], 30[60], 30[72]), 120[72], ∞[8]; PCR#1 (ITS1): 60[98], 15x(10[98], 30[65], 30[72]), 120[72], ∞[8]; PCR#2 (16S & ITS1): 120[98], 12x(10[98], 30[60], 30[72], 120 [72], ∞[8]. We included a negative control in each PCR batch, verified the absence of contamination by gel electrophoresis, and processed these controls through sequencing and bioinformatics. We purified PCRs with magnetic microbeads and successive washes in 80 % ethanol on a magnetic rack, then recovered it in a TE elution buffer (10 mM Tris-HCl (pH 8.0) + 0.1 mM EDTA). Final products were combined in equimolar ratio, quantified spectrophotometrically using a Spark 10 M plate reader (Tecan US Inc., Raleigh, USA), then sequenced by the MiSeq system (Illumina Inc., San Diego, USA). These last steps were performed at the Platform of genomic analysis of the Institute of Integrative Biology and Systems at Université Laval (Québec, Canada).

2.4. Bioinformatics and statistical analyses

Analyses were conducted in R software version 4.1.0 (R Core Team, 2021). We used the *dada2* package (Callahan et al., 2016a) assisted by the *cutadapt* plug-in for ITS sequences (Martin, 2011) to denoise raw reads into amplicon sequence variants (ASVs) (Callahan et al., 2017). We assigned taxonomy from the databases SILVA v.138.1 for bacteria (Quast et al., 2013) and UNITE v.8.0 for fungi (Nilsson et al., 2018; Abarenkov et al., 2019). We excluded chimeric, chloroplast and mitochondria sequences, along with those highlighted as external contaminants by the *decontam* package (Davis et al., 2018). Then, we processed our data through *phyloseq* (McMurdie and Holmes, 2013), relying upon its associated workflow (Callahan et al., 2016b). To avoid ASVs with small mean and trivially large coefficient of variation, we only retained taxa that were sequenced at least five times in two separate samples. We constructed heat trees using the *metacoder* package (Foster et al., 2017) to represent the overall composition of communities. Mycorrhizae include genera that were classified as highly probable or probable ectomycorrhizae and ericoid or orchid mycorrhizae, according to FUNGuild (Nguyen et al., 2015). We used the FAPROTAX database (Louca et al., 2016) to assign putative functional groups to bacterial genera.

To compare community composition between stand types, we normalised our data (McMurdie and Holmes, 2014; Weiss et al., 2017) using a negative binomial model of variance stabilisation implemented in the *DESeq2* package (Love et al., 2014). Normalised counts were then used to build dissimilarity matrices based on the weighted UniFrac distance (Lozupone et al., 2007). We assessed the influence of stand type on community composition using permutational analysis of variance (PERMANOVA) implemented in the *vegan* package (Oksanen et al., 2020), after ensuring that the assumption of homogeneous multivariate dispersions was met (Anderson, 2001, 2006). We further investigated taxa associated with specific stand types using indicator species analyses based on normalized counts (De Cáceres et al., 2010). We also carried out distance-based redundancy analyses (Legendre and Anderson, 1999) to evaluate how much of the variation in community composition could be explained by physicochemical properties, independently of site and stand type. The explanatory variables considered in the redundancy analyses were not strongly correlated (Pearson correlation $|r| < 0.5$). We calculated the Shannon Diversity Index (H') of bacterial, fungal, and mycorrhizal communities, as recommended for the study of microbial diversity (Haegeman et al., 2013), and performed two-way ANOVAs to test differences between site and stand type. Annotated scripts are available upon request to the corresponding author. Raw sequence data are available on GenBank (BioProject ID: PRJNA847613).

Physicochemical properties and dendrometrics were compared between stand types and sites using two-way ANOVAs and Tukey HSD *post-hoc* tests. We checked assumptions with residual diagnostics. When

Table 1
Results from PERMANOVA (sequential test of terms) on weighted UniFrac distance matrices of bacterial, fungal, and mycorrhizal communities, each with 10,000 permutations. *P* values in bold are below the 0.05 threshold of significance. Abbreviations: DF, degrees of freedom; SS, sum of squares; MS, mean sum of squares; ASVs, amplicon sequence variants. Multivariate dispersions were homogeneous in all three groups. Degrees of freedom differ between analyses of fungi and bacteria because of two missing data generated during sequencing.

Community (number of ASVs)	Source	*DF*	*SS*	*MS*	*F*	R^2	*P*
All fungi (n = 295)	Stand type	2	4.850	2.425	1.783	0.124	**< 0.001**
	Site	1	1.754	1.754	1.290	0.045	0.149
	Stand type × Site	2	2.698	1.349	0.992	0.069	0.478
	Residuals	22	29.918	1.360			
Mycorrhizae (n = 88)	Stand type	2	8.542	4.271	1.522	0.105	**0.036**
	Site	1	4.275	4.275	1.524	0.052	0.084
	Stand type × Site	2	6.950	3.475	1.239	0.085	0.159
	Residuals	22	61.721	2.806			
Bacteria (n = 1499)	Stand type	2	0.345	0.172	1.555	0.102	**0.022**
	Site	1	0.123	0.123	1.111	0.036	0.269
	Stand type × Site	2	0.268	0.134	1.209	0.079	0.151
	Residuals	24	2.660	0.111			

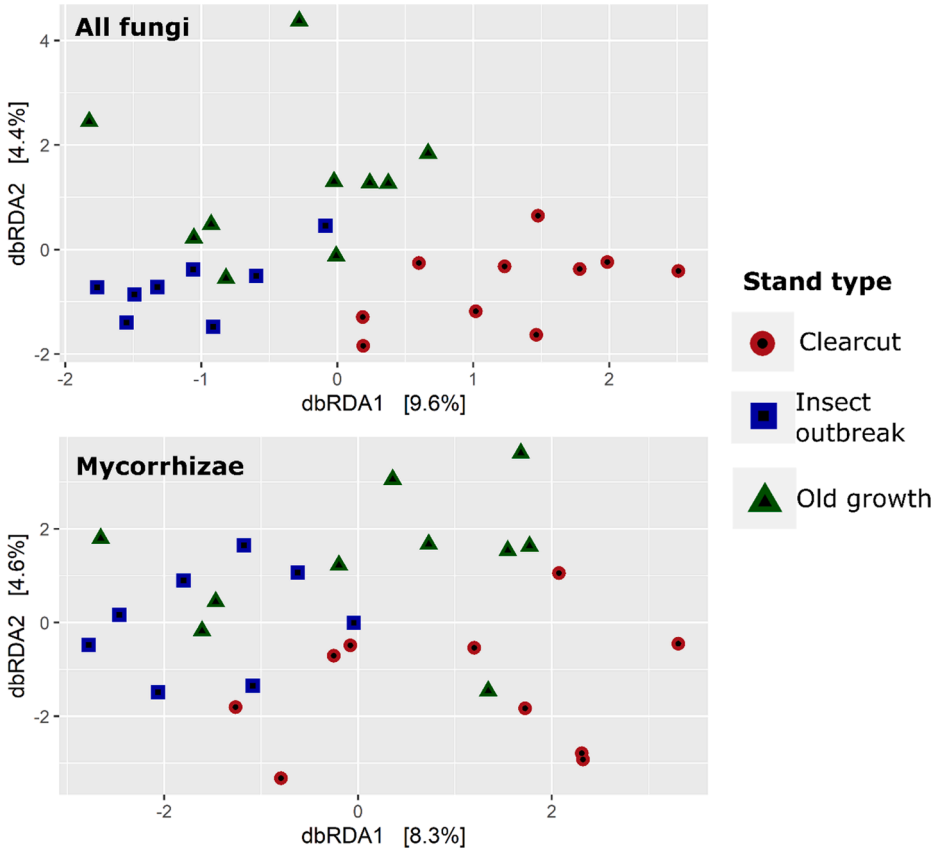

Fig. 2. Distance-based redundancy analysis ordination plots representing the results of the PERMANOVA for fungi and mycorrhizae. In both cases, only the first axis (dbRDA1) was statistically significant ($F > 2.37$; $P < 0.038$). Although the overall model was significant for bacteria, the individual ordination axes for this group were not and are not presented in the figure.

variances were heterogeneous, we log-transformed the response variable, but in some cases, this was not sufficient to meet assumptions of homoscedasticity. In such cases, we used a generalized least square model that included a term to explicitly model heteroscedasticity (Pinheiro and Bates, 2000).

3. Results

Fifty years after disturbance, 10.2 %, 12.4 %, and 10.5 % of the variability in the composition of soil bacterial, fungal, and mycorrhizal communities, respectively, was explained by stand type alone (Table 1). Clearcut and insect outbreak stand hosted the most different communities of fungi and mycorrhizae, while old growth plots were intermediate (Fig. 2). Individual ordination axes for bacteria were not significant even though the overall model was (Table 1). Fungal genera *Tolypocladium* (*IndVal* = 0.760, *P* = 0.007) and *Trichoderma* (*IndVal* = 0.676, *P* = 0.023) were detected as indicators of clearcuts, while *Amphinema* (*IndVal* = 0.849, *P* = 0.036) were associated with intact forests (group '*insect outbreak* + *old-growth*'). Also, the fungal order Tremellales (*IndVal* = 0.853, *P* = 0.007), which includes six ASVs in our dataset, was assigned as an indicator of regenerating forests ('*clearcut* + *insect outbreak*'). Fifteen ASVs of both bacteria and fungi were further designated as indicators of a specific stand type (Table A1, A2). However, for bacteria, no taxonomic rank was detected as an indicator of any

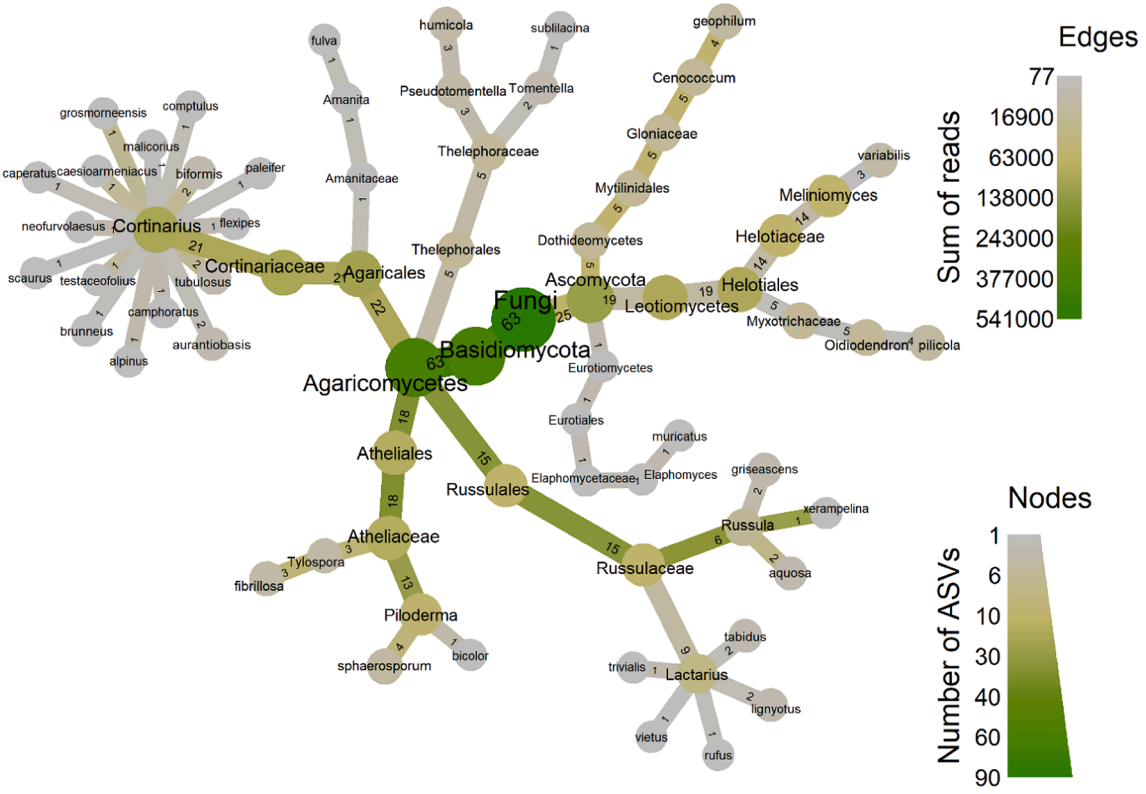

Fig. 3. Heat tree representing the overall community composition of mycorrhizae species. Size and color of nodes show the number of ASVs, whereas size and color of edges represent the sum of sequence reads. Numbers on edges indicate the number of ASVs belonging to the next node.

specific stand type or group of stand types.

No part of the variability observed in the community composition of either group of microorganisms was explained by physicochemistry (PERMANOVA on constrained axes of distance-based redundancy analyses: $F < 1.02$; $P > 0.43$). Indeed, soil physicochemical properties were largely consistent among stand types and sites, except for the percentage of humidity, which was highly variable. The C/N ratio was significantly higher in insect outbreak than in old-growth and clearcut stands, while total sulfur differed between both sites but not among stand types (Table A3, A4). In terms of dendrometry, clearcut and insect outbreak stands were relatively similar, although the latter notably had larger snags, higher sapling density, and greater spruce cover. Moreover, white birch cover was more important in old-growth than in insect outbreak and clearcut stands (Table A5, A6).

In total, 1499 bacterial and 295 fungal ASVs occurred in two samples with at least five reads. Accumulation curves for the number of ASVs reached a plateau in both groups around the 20th plot (Fig. A4). The five richest and most abundant bacterial orders (Acetobacterales, Acidobacteriales, Frankiales, Isosphaerales, and Rhizobiales) represented 36 % of total bacterial ASVs (Fig. A5). Putative functional groups could only be assigned to 19 % of all bacterial ASVs, the vast majority of which belonged to the aerobic chemoheterotrophy group. A few exceptions were not associated to any stand type and belonged to the genera *Bdellovibrio* (n = 2) and *Haliangium* (n = 4), annotated as 'predator or exoparasite', *Xiphinematobacter* (n = 6) as 'animal parasite or symbiont', and *Rhodoplanes* (n = 2) as photoheterotroph. The five dominant fungal

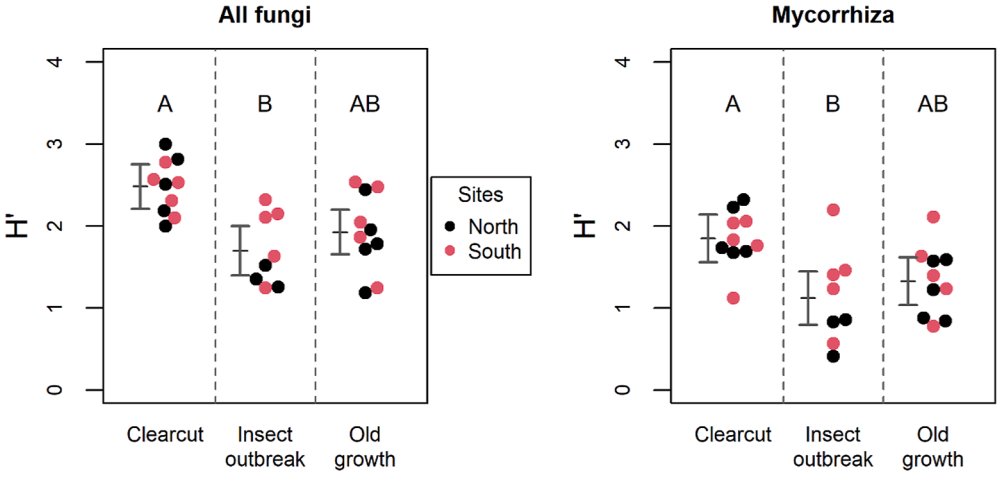

Fig. 4. Shannon diversity index (H') in each plot for all fungi (left) and mycorrhizae only (right) communities according to stand type. Error bars represent the 95% confidence interval around the predicted means for stand types from two-way ANOVA (type I sum of squares). Letters indicate significantly different groups.

orders (Agaricales, Atheliales, Helotiales, Mortierellales, and Russulales) included 54 % of all fungal ASVs (Fig. A6). Thirty percent of fungal ASVs belonged to genera known to be either ectomycorrhizae, ericoid, or orchid mycorrhizae (Fig. 3). Out of 88 mycorrhizal ASVs, 80 were found in clearcut, 77 in old-growth, and 67 in insect outbreak stands. Stand type impacted the Shannon diversity index (H') of fungi ($F = 8.89$; $P = 0.001$) and mycorrhizae ($F = 7.21$; $P = 0.004$), with clearcut stands having a significantly higher predicted mean than insect outbreak, while old-growth was intermediate (Fig. 4). Likewise, stand type did not affect the alpha diversity (H') of bacteria ($F = 1.16$; $P = 0.33$). Moreover, the site and the interaction between stand type and site had no effect on the alpha diversity (H') of any of the three groups of microorganisms ($F < 2.12$; $P > 0.15$).

4. Discussion

We hypothesized that the nature of stand-replacing disturbances and the soil physicochemical properties affect the composition of soil microorganism communities. We observed that stand type explained a small but significant part of the variability in the community composition of all three groups of microorganisms considered (bacteria, fungi, and mycorrhizae). This result may indicate that there is something special about soil microbiomes associated with natural disturbance dynamics compared to clearcuts. Perhaps this is unsurprising if we acknowledge that boreal forests have been regulated by insect outbreaks for centuries (Blais, 1965, 1983; McCarthy and Weetman 2007). Besides, the variability observed in the composition of communities could not be explained by soil physicochemical properties, which reinforces the possibility that the effect of stand type is genuine. Nonetheless, we stress that our data must be interpreted carefully due to the observational nature of the study design.

In terms of ecosystem function, the differences of the mycorrhizae communities between stand types suggest different vascular plant communities, different stand productivity, or a different state of fungal succession. Notably, the indicator genus of intact forests *Amphinema* is a highly probable ectomycorrhizae. Furthermore, *Tolypocladium* spp. and *Trichoderma paraviridescens*, indicators of clearcuts, are annotated as possible animal pathogens or fungal parasites (Nguyen et al., 2015). In contrast, it is difficult to infer ecosystem function from the bacteria data. Indeed, most ASVs could not be putatively assigned to any functional group, or their taxonomic status was unclear because the marker resolution could not be resolved beyond the order. Moreover, the bacteria species linked to a specific function were overwhelmingly aerobic chemoheterotrophs and not associated with any stand type.

We further expected mycorrhizal diversity to be lower following a clearcut than after an insect outbreak. However, we uncovered just the opposite: higher fungal and mycorrhizal alpha diversity in 50–year–old stands regenerating after a clearcut. This could be perceived as an encouraging sign of resilience from boreal ecosystems in the face of major anthropogenic disturbances. Yet, as the composition of communities is different between stand types (beta diversity), it would be inappropriate to conclude that clearcuts are good for fungal diversity per se. Indeed, a large number of edge-tolerant species might cohabit in managed forests while forest-interior species become scarce across the exploited landscape (Pfeifer et al., 2017; Franklin et al., 2021). Furthermore, Hartmann et al. (2012) have shown that in the short term (<15 years), ectomycorrhizae diversity can decline following a clearcut. This suggests that species may go locally extinct in the aftermath of a clearcut, and later must recolonize the regenerating stands once the conditions become adequate again. In the case of most ectomycorrhizal fungi, despite the production of billions of spores, dissemination appears generally limited to less than 1 km (Peay et al., 2012). Thus, source populations (e.g., old-growth) shall be preserved in the vicinity to ensure that communities have a chance to reassemble in–between timber harvesting rotations. Alternatively, the movement of heavy machinery across widely-spaced forest stands may disseminate mycelium and spores, contributing to the increased fungal diversity in regenerating clearcuts. At the same time, this may be disrupting the natural succession of fungal communities.

In conclusion, we highlight that boreal forest soils are an extremely complex environment where thousands of microorganisms cooccur. Indeed, we have observed that 50 years after disturbance, soil microbiome composition in stands reinitialised by an insect outbreak differs from that of comparable clearcutted stands. Therefore, we recommend that forest managers spare the largest possible tracts of unmanaged forest across the exploited landscape, including areas affected by natural disturbances. This cautious approach would provide benchmark soil communities that will remain unaffected by forestry, which are needed to understand gaps between natural and managed forests and to continue the improvement of harvesting practices.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foreco.2022.120493.

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