Moss species, sections, and land use type drive moss-associated microbial taxonomy and functions in boreal forests

Sarah Ishak[1*], Maria Faticov[1], Jonathan Rondeau-Leclaire[1], Sébastien Roy[12] and Isabelle Laforest-Lapointe[123]

[1]Département de biologie, Université de Sherbrooke, Sherbrooke, Québec, Canada

[2]Centre Sève, Université de Sherbrooke, Sherbrooke, Québec, Canada

[3]Centre d'Étude de la Forêt, Université du Québec à Montréal, Montréal, Québec, Canada

*Corresponding author: s.isaac1905@protonmail.com

ORCID NUMBERS

Sarah Ishak	(0000-0003-3412-3642)
Maria Faticov	(0000-0001-8206-9332)
Jonathan Rondeau-Leclaire	(0000-0002-4433-3566)
Sébastien Roy	(0000-0001-7183-0691)
Isabelle Laforest-Lapointe	(0000-0003-3630-9475)

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Summary

- Moss microbial communities play important roles for ecosystem processes in boreal forests. Host moss identity and leaf litter presence can affect microbial community structures of mosses. However, the extent to which host-specific characteristics and land use type affect taxonomic and functional profiles of microbial communities of boreal mosses is still poorly understood.

- We used shotgun metagenomic sequencing to characterize microbial taxonomy and metabolic KEGG ortholog (KO) profiles between green and brown sections of five moss species across different natural sites, and two moss species between a natural site and a mine site in Eeyou Istchee, Québec, Canada.

- Our results demonstrate that the abundance of nitrogen metabolic genes differ between moss sections and that mosses from natural and mine environment associate with different microbial taxa and KO profiles. Importantly, conditions at the mine site appear to favor microbial taxa that can tolerate perturbated environments, including taxa that can oxidize sulfur and participate in biofilm formation.

- Overall, our results highlight that moss section, moss species identity, and land-use type are strong drivers of diversity and community structure of moss-associated microbial taxa and metabolic genes. These findings could have major implications for boreal forests facing climate change and anthropogenic pressures.

Key words: bacteria, fungi, boreal forests, KEGG profiles, metagenomics, microbial ecology, mining, mosses, shotgun sequencing.

Introduction

Boreal forests are one of the largest biomes covering 29% of land surfaces (Kayes & Mallik, 2020) and storing at least 30% of the Earth's carbon (Walker *et al.*, 2019). This terrestrial ecosystem is habitat to a diversity of understory plants including lichens, and bryophytes (small non-vascular plants including mosses, liverworts, and hornworts). Ubiquitously distributed in boreal forests, mosses are crucial contributors to two key ecosystem processes: nitrogen fixation and carbon sequestration (Turetsky, 2003). These processes are also facilitated by symbiotic micro-organisms colonizing mosses (Holland-Moritz *et al.*, 2018; Ininbergs *et al.*, 2011). In boreal ecosystems, moss-microbe interactions contribute up to 50% of nitrogen inputs to the forest and play crucial roles in facilitating carbon degradation in peatlands (Lin *et al.*, 2014a; Rousk & Michelsen, 2017). Driven by the pressures of the Anthropocene, boreal ecosystems face a temperature increase of ~2-5ºC before the end of the century which could alter moss-associated processes with major implications for global N and C cycles (Masson-Delmotte *et al.*, 2021). Considering the strong impact of climate change on boreal forests combined with the ever-increasing anthropogenic impacts on land-use (e.g., urbanization and mining), improving our understanding of the drivers and functions of moss-microbe interactions is imperative to identify and mitigate the risks of altering this precious habitat (Jonsson *et al.*, 2015; Royles & Griffiths, 2015).

In the last two decades, substantial molecular evidence have provided insights into the drivers of moss microbial communities (through the genetic material recovered from the microbiota, including dead, dormant, and active cells) and how these moss-microbe interactions can influence ecosystem functions (Stuart *et al.*, 2020a). The sequencing of moss microbial communities allowed for the identification of microbial taxonomy and consequently the inference of their ecological functions, e.g., nitrogen fixation and phosphorus solubilization (Lin *et al.*, 2014a). To date, cyanobacteria are the most commonly-studied microbial group associated with mosses due to their nitrogen-fixing activities (Rousk *et al.*, 2013). Yet, other non-cyanobacterial microbial families such as *Acidobacteraceae, Acetobacteraceae,* and *Bradyrhizobiaceae* have been much less studied, despite their ability to fix nitrogen (Holland-Moritz *et al.*, 2018; Vile *et al.*, 2014). Outside of carbon and nitrogen metabolism, moss-associated micro-organisms likewise show the capacity for (1) phosphorus acquisition (Lin *et al.*, 2014b); (2) antagonism to fungal and bacterial pathogens (Shcherbakov *et al.*, 2013); (3) biofilm formation (Bragina *et al.*, 2014); (4) quorum sensing (Vesty *et al.*, 2020); and

(5) organic matter decomposition (Turetsky *et al.*, 2012). These functions can be attributed not only to bacteria, but fungi as well. In fact, several studies have demonstrated that moss-fungal interactions can influence ecosystem functions, such as nitrogen fixation and utilization (Chen *et al.*, 2019; Davey *et al.*, 2012). Overall, moss-microbe associations are crucial in various biogeochemical processes. Importantly, their interactions are not static. Thus, understanding the determinants of moss-associated microbial community assembly and function could be key to facilitate predictions of their roles in boreal ecosystems in the Anthropocene.

Moss microbial communities can be affected by biotic and abiotic factors in their local ecosystem (Jean *et al.*, 2020; Klarenberg *et al.*, 2021). Moss-associated cyanobacteria can be affected by light exposure and moisture levels (Kox *et al.*, 2020; Liebner *et al.*, 2011). McLaren et al. (2017) demonstrated that the expansion of temperate shrubs into boreal ecosystems in response to higher temperatures results in a higher density of labile shrub litter. This, in turn, alters the assembly of moss bacterial communities. Moss phylogeny is a major driver of moss bacterial community structure (Holland-Moritz *et al.*, 2018). Moss diversity can reach up to as many as 130 species in Canadian boreal forests (Macdonald & Fenniak, 2007). Yet a vast number of studies on moss-microbe interactions have focused on dominant boreal mosses such as the feathermoss *Pleurozium schreberi* (Elzen *et al.*, 2020) and peat mosses of the genus *Sphagnum* sp. (Kostka *et al.*, 2016). Certainly, other mosses such as the acrocarpous mosses *Dicranum undulatum* and *Polytrichum commune*, while less abundant, could have an impact on ecosystem functions through their interactions with microbes (Rousk *et al.*, 2015). Finally, physiological differences between green and brown sections can affect moss microbial communities. Moss tissue can be separated along their vertical gradient into distinct sections: an above-ground living “green” section, and an underlying, decaying “brown” section (Lindo & Gonzalez, 2010). These sections can show distinctions in both bacterial and fungal taxonomic diversity (Arróniz-Crespo *et al.*, 2022; Chen *et al.*, 2019). The green section is thought to be photosynthetically active and is often in focus when studying bacterial communities associated with mosses. The green section is also associated with nitrogen fixing cyanobacteria (Renaudin *et al.*, 2022a). Meanwhile, microbial communities associated with brown sections are much less studied, even though they are hypothesized to be an important source of nutrients for certain bacterial taxa (Shen *et al.*, 2018). To gain a comprehensive perspective of moss-microbe interactions, it appears necessary to characterize how moss sections differ in microbial community assembly and functions.

Up until recently, moss microbial functions have been mostly inferred by looking at microbial taxonomy and associating it with measures of nitrogen fixation (Stuart *et al.*, 2020b; Warshan *et al.*, 2017). Recently, metagenomic analyses have enabled functional profiling of microbial communities beyond the inference of functions from taxonomy (Quince *et al.*, 2017). Instead, this molecular approach focuses on total genomic material present in a sample and allows for the characterization of microbial genes, which in return provides information on microbial metabolic profiles. This improved understanding of metabolic profiles across different moss species could help to predict functional changes occurring as climate change and human activities impact boreal ecosystems. Much remains to be done to provide a global portrait of moss-microbe interactions and functions beyond a few dominant moss species and the sole microbial kingdom of bacteria. Presently, most moss-microbiome studies have been confined to amplicon-based metagenomic analysis of specific microbial kingdoms, such as bacteria or fungi, and do not necessarily explore the functions of the moss microbiome. To gain a comprehensive perspective of moss-microbe interactions, it appears necessary to characterize moss microbial community assembly and functions, especially between different moss sections.

Alongside climate change, boreal ecosystems are also frequently perturbed by human activities, such as mining. Mining can result in one or a combination of consequences – from biologically toxic concentrations of metals in soils to moisture loss and pH imbalances (Vareda *et al.*, 2019; Wong, 2003). Mosses have been found on mine sites (Monaci *et al.*, 2021) and were shown to have positive impacts on degraded soils (Cheng *et al.*, 2021). They possess characteristics including increased cation exchange capacity and weak hydro-phobic barriers that enable their survival in mines (Antreich *et al.*, 2016). Mosses can also increase evaporation rates during rainfall events, lowering the soil moisture content (Merlin *et al.*, 2019) and mitigating desertification (Cao *et al.*, 2020; Pombubpa *et al.*, 2020). However, it remains unclear whether the taxonomy and functions of moss-associated microbial communities are distinct in this highly perturbed and unique environment. In vascular plants naturally growing in mine sites, community structures of bacteria and fungi were found to be driven by plant species and environmental factors, namely pH and humidity (Gagnon *et al.*, 2020a,b; Klarenberg *et al.*, 2023). In moss microbiomes, pathways related to oxidative stress, such as biofilm formation have been previously detected, which can contribute to resilience of moss microbiomes in mine sites (Bragina *et al.*, 2014). Interestingly, a study by Goth et al. (2019)

demonstrated that the ability of moss microbes to fix nitrogen was not inhibited despite heavy metal pollution. Understanding the mechanisms of the moss microbiome in mine sites will be crucial in determining the capacity for mosses to facilitate reclamation of mine sites.

In this study, we quantified the relative influence of multiple drivers of moss microbial community taxonomical and functional profiles in boreal forests. Specifically, our objectives were to (1) provide a comprehensive portrait of moss-associated microbial communities beyond bacteria using shotgun metagenomic sequencing; (2) explore whether microbial taxonomy and functions differ among boreal moss sections as well as moss species; and (3) determine the taxonomical and functional profiles of moss microbial communities in natural and mine sites among multiple moss species. To this end, we investigated moss bacterial and fungal microbial communities in natural stands of boreal forest and at the Whabouchi mine site of the Eeyou-Istchee region of northern Québec, Canada. Supported by previous findings, we hypothesized that (i) location type (natural vs mine) will explain most of the variation in moss microbial community assembly and functions followed by section type (green vs brown) and moss species identity. We further hypothesize that (ii) green sections will harbor a higher relative abundance of cyanobacteria and nitrogen-fixing genes than the brown sections; and (iii) that there will be more functional genes related to biofilm formation in moss-associated microbial communities from the mine site in tolerating the stresses of the mine environment, compared to natural sites.

Materials and Methods

Study area

We conducted this study within the Eeyou-Istchee (James Bay) region of northern Québec (N51.7040721°, W76.0521982°) and the Whabouchi mine site (N51.68694°, W75.83597°; Fig. **1a**) (Nemaska Lithium Inc. *et al.*, 2014). All sampling locations were part of Québec's boreal forest biome which is dominated by Jack pine (*Pinus banksiana*) and black spruce (*Picea mariana*) (Moussaoui *et al.*, 2020). The annual average temperature of this region in 2021 was approximately 8.9°C, in June, and 11.3°C in September (Environment and Climate Change Canada Historical Climate Data, 2021). The soil of these boreal forests is mainly acidic and nutrient poor (Collin *et al.*, 2018). This project focused on five moss species: *Polytrichum juniperinum*, *Polytrichum commune*, *Polytrichum piliferum*, *Dicranum undulatum*, and *Tortella tortuosa* (Fig. **1b**).

Study design

Sampling occurred over two periods in June and September of 2021. June samples were used to compare the taxonomical and functional diversity of moss microbes from naturalized environments and the Whabouchi mine site. September samples were used to characterize taxonomical and functional profiles of moss microbes between green and brown moss sections.

Across the two periods, mosses were sampled from six different locations within the Eeyou-Istchee (James Bay) region of northern Québec (Fig. **1a**). For the September sampling, we randomly selected three microsites 5 to 10 meters apart (Fig. **1a**). In each of the microsites, we further established 3-by-3-inch moss patches (Fig. **1a**). In September, a total of 69 individuals were sampled across *P. juniperinum*, *P. commune*, *P. piliferum*, and *D. undulatum* from five sites in Eeyou Istchee (Table S1). Individual moss samples were further separated into "green" and "brown" sections (determined visually, Fig. **1c**). Meanwhile, there was no microsite sampling for June. Specifically, a total of 10 individuals of *P. juniperinum* and *T. tortuosa* were sampled from one natural and one mine site in June (Table **S2**). Only green parts of the moss were analyzed from these samples. During both sampling periods, mosses were first visually identified to species level in the field and then confirmed with microscopy in the laboratory. Moss samples were placed in 50 mL Falcon tubes and immediately stored at -20°C until back to the laboratory. In the laboratory, samples were then stored at -80°C. A breakdown of the sampling procedure can be viewed in Tables **S1-S2**.

DNA extraction and shotgun metagenomic sequencing

DNA extraction of moss samples were performed using modified protocols by Holland-Moritz, *et al.* (2021) and Klarenberg, *et al.* (2021). In brief, 0.25g of each moss sample were initially weighed out and divided into two sterile 2 mL bead beating tubes with three sterilised 2.3 mm diameter stainless-steel beads (BioSpec Products, Bartlesville, Oklahoma) without extraction buffer. Tubes were then frozen in liquid nitrogen for approximately 30 seconds and moss tissue was broken down using the FisherbrandTM Bead Mill 24 Homogenizer for 30 seconds at 1.75 m/s. DNA was subsequently extracted using the protocol of QIAGEN DNEasy PowerSoil DNA Extraction Kit with minor changes (see *Supplementary Materials* for details). All samples were sent to Génome Québec (Montréal, Canada) for library preparation and metagenomic sequencing. Ten metagenomic libraries were generated from green part of the mosses from June samples, and 131 metagenomic libraries were generated from green and brown sections

from September samples. Seven samples from September were not included in the analysis due to insufficient read quality. Thus, a total of 141 samples were sequenced on a single lane of the Illumina NovaSeq6000 with 2 × 150bp chemistry at Génome Québec.

Assignment of taxonomy and functions

On average, library read numbers ranged from 10.4×10^6 to 22.5×10^6. Reads were quality-filtered using KneadData v0.10.0 paired-end run with default parameters. To remove host DNA, reads mapping to *Homo sapiens* and *Physcomitrella patens* (the closest available full moss genome) were excluded using Bowtie 2.3.5 (Langmead *et al.*, 2019). Filtered reads were input to Kraken 2.1.2 for taxonomical annotation using default parameters. Then, Bracken was used for Bayesian re-estimation of abundance to obtain species tables which were used for statistical analysis (Lu *et al.*, 2017). Next, a taxonomic profile report was generated from KrakenTools, which was used to generate a custom subset of annotated genomes from the ChocoPhlAn 201901b database. Annotated genomes together with the Uniref90 proteins database were used to quantify gene families and metabolic pathways using the HUMAnN 3.0 pipeline (Beghini *et al.*, 2021). Finally, gene annotation step generated KEGG orthologs (KOs) – genes that are homologous across different species, and are identified with K numbers (Kanehisa *et al.*, 2016). KOs were used to explore the metabolic profiles of microbial communities between green and brown moss sections, and among mosses from natural sites and the Whabouchi mine site. To classify KOs into biologically meaningful categories, i.e., genes that are relevant for biogeochemical processes and stress responses in the moss microbiome, we compiled a list from relevant pathways in the KEGG database. The final list included pathways responsible for the nitrogen metabolism, photosynthesis, biofilm formation, heat and cold adaptation, and oxidative stress.

Statistical analyses

All statistical analysis and visualization were performed in R v4.2.1. Each of four datasets (microbial taxonomical and functional data from June and September samples) were handled separately to answer slightly different questions (see Table **S3** for details). For univariate responses, we ran linear models based on Shannon diversity using functions *lmer*, *lm*, and *glm* from R base package. Prior to calculating Shannon diversity of microbial taxa, reads were rarefied to 90% of the lowest sample sum of reads using *rarefy_even_depth* in the *phyloseq* package to retain all samples while accounting for uneven sequencing depth (McMurdie &

Holmes, 2013; Wicaksono *et al.*, 2021). To calculate Shannon diversity of microbial KOs, reads were Hellinger transformed to correct for a double-zero effect (Franzetti *et al.*, 2020). We used Shapiro-Wilk from the base R package and Breusch-Pagan tests from the *bptest* package to determine the normality and homoskedasticity of Shannon values (Zeileis & Hothorn, 2002). Where variables were not normal, we transformed them using the Ordered Quantile Normalisation (ORQ) using the package *bestNormalize* (Peterson & Cavanaugh, 2020; Peterson, 2021). We then used the *Anova* function in the *car* package to assess significance of the models, and *emmeans* to estimate specific contrasts in our models (with Tukey adjustment). For the taxonomical and functional community assembly analyses, we first addressed uneven sequencing depth and heteroskedasticity, by transforming raw taxa counts using variance stabilizing transformations (VST) in the package *DESeq2* (Love *et al.*, 2014). To obtain community assembly matrices, for VST transformed data, we used Jaccard dissimilarity index calculated from presence-absence data (Tian *et al.*, 2019); while for Hellinger transformed data, we used the Euclidean dissimilarity index (Pakgohar *et al.*, 2021). Finally, *adonis2* (with the argument *by = "terms"*) in the vegan package was used to run PERMANOVAs (Oksanen *et al.*, 2022), every time making sure that the order of parameters didn't affect the effect size or significance.

To examine the relationship between microbial taxonomic and KO diversity and moss characteristics, we independently modelled Shannon diversity of microbial taxa (n=131) and microbial functions (n=131) as a function of the fixed effects '*moss section*', '*moss species identity*', and their interaction in September samples. We included "*microsite*" as a random effect to account for variation among sites. To explore the relationship between the taxonomic community assembly, the KEGG functional gene data, and moss characteristics of September samples, we independently modelled multivariate taxonomic (n=131) or KO (n=131) community assembly as a function of fixed effects '*moss section*', '*moss species identity*', and their interaction. We restricted permutations within blocks (i.e., '*microsite*') using the function *how* in the package *permute* (Simpson *et al.*, 2022). June samples (n=10) were used to examine whether mosses growing in natural sites and the Whabouchi mine site differed in their microbial taxonomic and functional diversity. We modelled Shannon diversity of microbial taxa (n=10) and KOs (n=10), as a function of fixed effects '*location*', '*moss species identity*', and their interaction. Multivariate analysis was then used to examine the relationship between multivariate taxonomic community assembly (n=10), KOs potential profiles (n=10) and

'*location*', '*moss species identity*', and their interaction. Finally, to investigate which microbial taxa and KOs differed in relative abundance between moss sections, the natural and mine locations, as well as across moss species, we conducted differential abundance analysis with the *ANCOM-BC* package without structural zeroes (Lin & Peddada, 2020). See Table S3 for details on the data used, statistical model structure, and transformations.

Results

In September, samples collected from green and brown sections contained several relatively abundant taxa. In green sections, the phylum *Proteobacteria* (including the classes *Alpha-*, *Beta-*, *Gamma-*, and *Delta-*) was the most prevalent, making up to 59.5% of the relative abundance of all taxa. Next in order were *Actinobacteria* (27.1%), *Cyanobacteria* (4.7%), *Acidobacteria* (4.6%), *Firmicutes* (1.8%), and *Bacteroidetes* (1.7%). For brown sections, these were also *Proteobacteria (Alpha-, Beta-, Gamma-* and *Delta-*) (52.5%), *Actinobacteria* (27.1%), *Cyanobacteria* (5.5%), *Acidobacteria* (4.7%), *Bacteroidetes* (2.8%), *Firmicutes* (2.2%), and *Planctomycetes* (1.4%). *Ascomycota* was the most prevalent fungal taxa (a relative abundance of 1.3% in both green and brown sections) and *Evosea*, an amoeba, was found to be most relatively abundant eukaryote in green sections (2.6%) and brown sections (1.4%).

When comparing respectively samples from the mine and natural sites in June, the most prevalent phyla included *Proteobacteria* (63.2% vs 65.2%), *Actinobacteria* (19.4% vs 15.6%), *Bacteroidetes* (4.8% vs 1.1%), *Cyanobacteria* (4.4% vs 11.1%), and *Acidobacteria* (3.3% vs 2.8%). Relative abundances of microeukaryotes and fungi were less than 1% overall. Archaea was the least abundant kingdom making up only 0.21% and 0.13% of the relative abundance in September and June samples, respectively.

Effect of moss sections on microbial community assembly and diversity

Overall, moss microbiome taxonomical and functional profiles in natural environments were distinct between green and brown sections. For community composition, section type was the strongest driver of microbial community assembly (R^2 = 21.0%, p = 0.001; Fig. **2a**; Table **S4a**), followed by moss species (R^2 = 17.3%, p = 0.001; Table **S4a**). The effect of moss sections on microbial community assembly also differed among moss species (R^2 = 4.79%, p = 0.001; Table **S4a**). Altogether this model accounted for 43.1% of the variation in taxonomic structure of microbial communities.

Regarding alpha-diversity, we observed lower Shannon diversity of microbial taxa in green sections compared to brown sections for all moss species (Fig. **3a**; Table **S5a**). Shannon diversity of microbial taxa also differed across moss species (Fig. **3a**; Table **S5a**). Overall, our model explained 78.3% of variation in Shannon diversity. We did not find a significant interactive effect between section and moss species on microbial taxonomic diversity (Table **S5a**). Furthermore, pairwise comparisons revealed species-specific differences in Shannon diversity of moss microbial taxa between sections. *D. undulatum* had a significantly higher diversity of microbial taxa in brown section compared to three *Polytrichum* species (Fig. **S1a**; Table **S6a**). However, in brown sections of *Polytrichum* species taxonomical microbial diversity did not change significantly (Fig. **S1a**; Table **S6a**). Similarly, the green sections of *P. juniperinum* and *P. piliferum*, as well as of *D. undulatum* and *P. commune*, did not significantly differ in diversity of microbial taxa (Fig. **S1b**; Table **S6b**). Our results also showed that *D. undulatum* had lower microbial Shannon diversity in green sections than both *P. juniperinum* and *P. piliferum* (Fig. **S1b**; Table **S6b**). Overall, all four species differed in microbial diversity between green and brown sections (Fig. **3a**; Table **S7a**).

The differential abundance analysis identified several bacterial and fungal taxa that changed in relative abundance between green and brown sections. The bacterial families *Verrucomicrobia* ($\log_2$-fold = 0.38), *Planctomycetes* ($\log_2$-fold = 0.37), and the fungal families *Agariomycetes* ($\log_2$-fold = 0.51), and *Leotiomycetes* ($\log_2$-fold = 0.26) were relatively more abundant in brown compared to green sections (all *P*-values <0.001; **Fig. 4a,b**; Table **S8a**). In green compared to brown sections, *Proteobacteria* ($\log_2$-fold = 0.41), *Acidobacteria* ($\log_2$-fold = 0.27), and *Saccharomycetes* ($\log_2$-fold = 0.24) were relatively more abundant (all *P*-values <0.001; Fig. **4a,b**; Table **S8a**).

<u>Effect of moss sections on community assembly and diversity of KO profiles</u>

For community composition, moss section type was a strong primary driver (R^2 = 11.6%, p = 0.001; Fig. **2c**; Table **S4b**), while moss species identity was a strong secondary driver (R^2 = 9.59%, p = 0.001; Fig. **2c**; Table **S4b**) of KO profiles. Interaction between moss section type and moss species identity accounted for 4.94% of the total variation (Table **S4b**). Altogether this model explained 26.1% of the variation in moss microbial KO profiles. Similarly, the Shannon diversity of microbial KOs was lower in green sections compared to brown sections across all moss species except for *D. undulatum* (Fig. **3c**; Table **S7b**). The

univariate model for Shannon diversity of KOs explained 75.5% of variation in September samples. Further exploration of pairwise differences of KO diversity between moss sections and moss species revealed that only *D. undulatum* showed strong differences in alpha-diversity in brown section (Fig. **S1c**; Table **S6b**). In contrast, within green section, the greatest differences in alpha-diversity were observed between *D. undulatum* and *P. piliferum* as well as between *D. undulatum* and *P. juniperinum* (Fig. **S1d**; Table **S6b**).

Using ANCOM-BC, we found differentially more abundant moss-associated nitrogen fixing genes in green sections (Fig. **5a,b**; Table **S8b**). Among them are KOs involved in nitrate/nitrite transportation, K02575 ($\log_2$-fold = 0.06, *P*-value <0.001) nitrogen fixating protein *nifU*, K04488 ($\log_2$-fold = 0.05, *P*-value <0.001; Fig. **5a**, Table **S8b**). Interestingly, KOs that were differentially abundant in moss brown sections were part of gene families involved in nitrogen assimilation rather than nitrogen fixation (Fig. **5a**; Table **S8b**).This list includes KOs involved in amino acid production, for example K00264 ($\log_2$-fold = 0.03, *P*-value <0.001), K01915 ($\log_2$-fold = 0.03, *P*-value = 0.001) and K00266 ($\log_2$-fold = 0.07, *P*-value <0.001), and ammonia production, namely K01725 ($\log_2$-fold = 0.04, *P*-value = 0.001; Fig. **5a**; Table **S8b**). In green sections, several KOs that are part of gene families that make-up chloroplasts and facilitate photosynthesis were shown to increase (Fig. **5b**; Table **S8b**). However, only a single KO of the *atpD* gene involved in photosynthesis was differentially abundant in the brown section (K02112; $\log_2$-fold = 0.05, *P*-value <0.001; Fig. **5b**; Table **S8b**).

<u>Effect of location on community assembly and diversity of microbial taxa</u>

In June samples, variation in moss microbial community assembly was driven primarily by location (R^2 = 33.6%, p = 0.001; Fig. **2b**; Table **S4c**), then by moss species (R^2 = 23.0%, p = 0.004; Fig. **2b**; Table **S4c**) and finally by their interaction (R^2 = 14.5%, p = 0.025; Table **S4c**). Overall, this model accounted for 71.1% of the variation in moss microbial community assembly. Furthermore, we found that moss microbial Shannon diversity (taxonomic level) were lower in mine sites compared to natural sites between two moss species (Fig. **3b**; Table **S5c**). This model explained 79.6% of variation in June samples. Shannon diversity of microbial taxa associated with *T. tortuosa* and with *P. juniperinum* was lower in the mine site compared to natural sites (Fig. **3b**; Table **S6b**).

Based on ANCOM-BC analyses, we noted differential abundant bacteria from the classes *Cytophagia* (log_2-fold = 2.38), *Actinomycetia* (log_2-fold = 0.74), and *Alpha-proteobacteria* (log_2-fold = 0.91) in moss samples from different sites in the mine (all *P*-values <0.001; Fig. 4c; Table **S9a**). Conversely, several cyanobacterial families were found to be differentially abundant in natural sites compared to the mine site including *Scytonemataceae* (log_2-fold = 4.38), *Nostocaceae* (log_2-fold = 3.67), *Calotrichaceae* (log_2-fold = 3.40), and *Aphanizomenonaceae* (log_2-fold = 2.80; all *P*-values <0.001; Fig. **4d**; Table **S9a**).

<u>Effect of location on community assembly and diversity of KO profiles</u>

In June samples, location was a primary driver of KO profiles (R^2 = 20.3%, p = 0.001; Fig. **2d**; Table **S4d**), followed by moss species (R^2 = 19.1%, p = 0.005; Fig. **2d**; Table **S4d**) accounting in total for 33.4% of variation in KO profiles. However, ANOVA results, based on the univariate model of Shannon diversity, showed no significant differences in the KO diversity between sections (Fig. **3c**; Table **S5d**), mine and natural sites nor between moss species (Fig. **3d**; Table **S5d**).

ANCOM-BC showed that there were differential increases of various microbial nitrogen-fixing genes from mosses in natural sites compared to samples from the mine site (Fig. **5c-e**; Table **S9b**). These KOs specifically come from a family of genes that encode for components of the nitrogenase enzyme: *nifH* (K02588, log_2-fold = 0.21, *P*-value <0.001; Fig. **5c**; Table **S9b**), *nifB* (K02585, log_2-fold = 0.13, *P*-value <0.001; Fig. **5c**; Table **S9b**), *nifD* (K02586, log_2-fold = 0.10, *P*-value <0.001; Fig. **5c**; Table **S9b**) and *nifW* (K02595, log_2-fold = 0.12, *P*-value <0.001; Fig. **5c**; Table **S9b**), as well as differential increases of KOs involved in catalysing the oxidation of organic sulfur, such as taurine dioxygenase (K03119, log_2-fold = 0.11, *P*-value = 0.001; Fig. **5d;** Table **S9b**), a sulfur-containing organic compound. There were also differentially more genes associated with sulfur oxidation (K20035 and K00387; log_2-fold = 0.11, *P*-value <0.001 and log_2-fold = 0.15, *P*-value <0.001, respectively; Fig. **5d**; Table **S9b**) of the moss microbial community in the mine site. Stress adaptation KOs that were differentially abundant in the mine site include genes that encode heat shock proteins (K04761, log_2-fold = 0.074; *P*-value <0.001; Fig. **5e**; Table **S9b**), as well as those involved in biofilm formation (K04761 and K01991; log_2-fold = 0.12, *P*-value <0.001 and log_2-fold = 0.15, *P*-value = 0.001, respectively Fig. **5e**; Table **S9b**).

Discussion

By using deep shotgun sequencing over multiple moss species and across contrasting land-use types, this study highlights the importance of moss sections, location, and moss species identity as key ecological drivers of microbial community assembly across multiple kingdoms. In addition, our work goes beyond taxonomy by providing novel evidence that these drivers also impact the potential metabolic profiles and diversity of moss microbial communities. Our results show that specific functional pathways preferentially occur between green and brown sections from the same moss species. In addition, we observed pathways and taxa that were more tolerant of the anthropogenic perturbations at the Whabouchi mine site. Most importantly, given the importance of moss-microbe interactions for boreal forests, these findings can improve our assessment of the impact of anthropogenic activities and climate change on microbe-mediated ecosystem functions of mosses.

Moss microbial community assembly varies strongly between moss sections

Our results show that the physiological and chemical differences between moss sections drive moss microbial community assembly and diversity for both microbial taxonomy and KO profiles. Interestingly, the alpha-diversity of microbial taxa in green sections was consistently lower than in brown sections across all moss species. This contrast could potentially be explained either by (i) a phenomenon of niche dominance of a few taxa in green compartments or by (ii) the proximity of brown sections to soil which is extremely rich in microorganisms. As such, brown sections could behave as an interface in which both soil and moss-associated micro-organisms can thrive. This area is also mostly shaded from UV by the overlying green sections, and is generally more humid, due to the boundary layer effect (i.e., the physical phenomenon in which wind velocity is reduced), minimizing moisture loss (Halpern *et al.*, 2014). In general, green sections lose moisture before brown sections, which leads to higher humidity in brown compared to green sections (Lindo & Gonzalez, 2010). To sum up, our results suggest that brown sections represent a more conducive environment for the proliferation of various micro-organisms whereas green sections are generally dominated by fewer taxa that tolerate harsher conditions.

Regarding individual taxa, the *Proteobacteria*, *Acidobacteria*, and *Saccharomycetes* were found to be differentially abundant in green sections. These groups are closely associated with nitrogen fixation (Rahimlou *et al.*, 2021; Reji & Zhang, 2022). For example,

Saccharomycetes is a soil-dwelling taxon and was previously found to be differentially abundant in mosses growing in soil supplied with extra nitrogen, indicating an opportunistic pathway in obtaining nitrogen (Allison *et al.*, 2008). In line with previous literature, our results suggest that there is an association with nitrogen metabolism and the microbial taxa found in green sections. On the other hand, bacterial families of *Verrucomicrobia* and *Planctomycetes* and the fungal families of *Agariomycetes* and *Leotiomycetes*, which were differentially abundant in brown sections, are often found in boreal forest soils (Hofer, 2016; Ivanova *et al.*, 2016; Sun *et al.*, 2013). These phyla are less associated with nitrogen fixation. Instead, *Planctomycetes* and *Agariomycete*s were shown to efficiently degrade complex polysaccharides found in soil (Dedysh & Ivanova, 2019; Elliott *et al.*, 2015; Wang *et al.*, 2015). Likewise, *Verrucomicrobia* were found in soil organic matter and carbon rich substrates (Nam *et al.*, 2021). Furthermore, many studies on *Verrucomicrobia* (e.g., Methylacidiphilaceae) have demonstrated its ability to consume methane and hydrogen oxidation to obtain energy (Carere *et al.*, 2017). Taken together, these results suggest that moss-associated micro-organisms in brown sections may have broader metabolic activity related to carbon cycling, rather than nitrogen metabolism.

Finally, in contrast with our hypothesis, we detected no significant differences in differential abundances of cyanobacterial taxa between sections. However, cyanobacteria activity may not have necessarily been constant throughout the whole moss gametophyte. Rather, this finding could be a caveat of metagenomic analysis on DNA. Cyanobacterial DNA may linger in brown sections, though the actual cells could be dormant or even dead, not actively fixing nitrogen as shown by in Renaudin *et al.* (2022b), whose work was based on cDNA and *nifh* sequencing. Further quantitative analyses may reveal true differences in activity between different moss sections. Nevertheless, our data suggests that microbial taxa associated with mosses diverge between green and brown sections, a conserved phenomenon repeated across various boreal moss species.

In terms of functional profiles, our results show similar patterns of diversity of KOs to that of taxonomic microbial diversity, where alpha-diversity of KOs from green sections were found to be lower than those of brown sections across all taxa except in *D. undulatum*. This may be related to the capacity of microbes associated with green sections to fix nitrogen. KOs specifically related to nitrogen fixation were indeed found to be differentially abundant in

green sections compared to brown sections, except in *D. undulatum*. Because nitrogen fixation is such a metabolically costly process (Rastetter *et al.*, 2001), the capacity for other microbial functions to occur might have been reduced, thereby reducing overall microbial-associated KO diversity in moss green sections. Differentially abundant KOs in brown sections, on the other hand, were part of gene families that were more often related to nitrogen assimilation than fixation (Carrell *et al.*, 2022; Newsholme *et al.*, 2003). Hence, nitrogen metabolic processes may diverge between the two sections, where nitrogen fixation is more prominent in green sections, and brown sections may be utilizing nitrogen and incorporating it into microbial cellular functions. It remains unclear why this is occurring, although it may be a consequence of photosynthesis mediated nitrogen fixation. Correspondingly, various photosynthetic KOs were differentially abundant in green compared to brown sections. Yet, our results confirm the hypothesis that nitrogen fixation is associated with moss green sections. This work provides unprecedented demonstration that (1) microbial taxa distribution is strongly driven by moss sections and (2) although microbial nitrogen metabolism can occur throughout the entire moss gametophyte, nitrogen-fixation is mostly occurring in green sections.

Moss microbial community assembly differs between mine and natural sites

The comparison of moss microbial communities in mine and natural environments revealed that location type respectively explains 33.6% and 20.3% of taxonomy and functions, suggesting a significant shift in both microbial community assembly and potential metabolic profile. In addition, the mine site samples displayed lower microbial alpha-diversity compared to natural sites. These differences in beta- and alpha-diversity could be explained by the difficult conditions of mine tailings. Environmental factors such as shade, UV (Cardona-Correa *et al.*, 2015), wind exposure, and moisture (Whiteley & Gonzalez, 2016) can influence the survival of various micro-organisms. Our results showed that several cyanobacterial taxa were differentially abundant in natural sites. For example, the *Nostocaceae* were found to have a drastic change in relative abundance – from 74.6% in natural sites to 14.9% in mine site. Most importantly, this sharp change in relative abundance of *Nostocaceae* may have implications for nitrogen fixation, as several studies have previously shown that cyanobacteria were found to be the main nitrogen fixers in mosses (Stewart *et al.*, 2011). Conversely, differential abundance of bacterial taxa such as *Alphaproteobacteria* and *Cytophagia* were observed from the mine site moss samples. *Alphaproteobacteria* is a known contributor to nitrogen fixation and has been previously found in the rhizosphere of vegetation growing in mine sites (Bragina *et al.*,

2012; Gagnon *et al.*, 2020c). Our results also showed that there was a great differential increase of *Cytophagia* at the mine site, a taxa generally known to grow in stressful environments including mine tailings (Gagnon *et al.*, 2020c; Ren *et al.*, 2021). Future work will confirm if conditions at mine sites are unfavorable for cyanobacteria so that other bacterial taxa may be filling the nitrogen-fixing niche. Taken together, our results demonstrate that between the natural sites of Nemaska and the Whabouchi mine site, there is a shift in moss microbial community structure, where bacteria that can survive and thrive in the mine environment are favored over cyanobacteria.

Although there was no difference in alpha-diversity of microbial KO between mosses from natural sites and the mine site, our results suggest that differential abundances of certain KOs can be related to moss microbial community structure. Several KOs in the *nif* family were differentially abundant in natural sites compared to the mine site. This may be explained by the higher relative abundance of cyanobacteria in natural sites. Our study identified the presence of K04761 and K01991, which are involved in the formation of biofilms, among differentially abundant KOs. In addition, K11940, a heat-shock protein was found to be the most differentially abundant KO from mine site mosses. This KO was previously identified to be associated with a *nif* gene island in *Bradyrhizobium*, a genus of *Alphaproteobacteria* (Tao *et al.*, 2021). Although mine tailings at the Whabouchi mine site were found to have low sulfur content, various KOs associated with sulfur metabolism, such as K00387 is a sulfite oxidase, were differentially abundant (Chen *et al.*, 2016; Gao *et al.*, 2020). The KEGG ortholog of dmdC, K20035, which is another enzyme involved in sulfur metabolism found to be differentially abundant in the mine site, plays an important role in breaking down dimethylsulfoniopropionate (DMSP), generating a source of organic sulfur. The production of DMSP by marine micro-organisms is highly correlated with salinity (Rinnan *et al.*, 2014). Conversely, the increased abundance of taurine dioxygenase can be due to the presence of this gene in the genome of *Nostocaceae*, which was relatively abundant in natural sites (Warshan *et al.*, 2018). In summary, the differences in taxonomical structure and functional capacity of the moss microbiome between mine and natural sites could reflect contrasting conditions in mine sites. Future studies will confirm the role of biotic and abiotic conditions in mine sites in driving moss-microbe interactions. Overall, our results suggest that natural sites are more conducive for microbe-associated nitrogen fixation processes and enable the proliferation of various micro-organisms.

Moss species identity is a secondary driver of microbial community assembly

Moss species was consistently found to drive moss microbial taxonomic community structure and functional profiles (KOs). Additionally, moss section and moss species identity interactively explained microbial taxonomic community assembly. Through pairwise dissimilarities analysis, the diversity of moss microbial taxa and KOs of *D. undulatum* were different to that of all *Polytrichum* species in brown sections. Interestingly, there were also no differences in microbial diversity of moss taxa and KOs among *Polytrichum* species. These results point to the important role of moss phylogeny in structuring microbial communities in boreal mosses (Holland-Moritz *et al.*, 2021). For example, moss physiological characteristics such as leaf morphology and phenolic compounds may explain the differences in microbial community assembly of taxa and functional profiles among moss species (Jassey *et al.*, 2013; Renaudin *et al.*, 2022a).

Furthermore, the interaction between moss section and moss species identity may explain the different taxonomical and functional microbial assembly patterns in green sections compared to brown sections among different moss species. For example, in green sections, microbial taxonomic diversity differed between *P. commune* and the two other *Polytrichum* species: *P. piliferum* and *P. juniperinum*. This result may be explained by the fact that *P. piliferum* and *P. juniperinum* are phylogenetically closer to each other than to *P. commune* (Bell & Hyvönen, 2010). Nevertheless, our findings recapitulate previous literature in which moss species was shown to be an important driver of microbial taxonomical and functional community assembly (Kostka *et al.*, 2016; Holland-Moritz *et al.*, 2018; Jean *et al.*, 2020).

Conclusion

Overall, our findings provide a comprehensive portrait of the drivers of moss microbial communities, both taxonomically and functionally, for multiple species and different land use types. Importantly, our results show that mosses from natural sites and the Whabouchi mine site show significant differences in microbial communities, which can have major implications for boreal ecosystem functions facing accelerating climate and anthropogenic pressures. In line with previous studies, our results show that abundances of nitrogen metabolic genes differ between green sections and brown sections of mosses. In the mine site, contrasting conditions could favor microbial taxa that can tolerate the harsh environments, and functions such as sulfur oxidation and stress tolerance processes. Future studies using true abundance measurements of

nitrogen fixation genes and *in situ* nitrogen fixation activity are needed to confirm the correlation with differences in taxonomic and functional profiles. As global change continues to affect ecosystems worldwide but especially boreal and northern ecosystems, it is crucial to understand moss microbe functions and adaptability beyond focusing entirely on bacteria and taxonomical profiles. In this view, our work suggests novel avenues of research to leverage the key actors of nitrogen fixation and carbon sequestration by protecting these pristine environments and by leveraging mosses to restore perturbed sites.

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Competing interests

Authors declare no conflict of interests.

Author contributions

SI, ILL, and SR designed the experiment. SI conducted the sample collection, processing, and molecular work. SI, JRL, and ILL conducted the bioinformatic analyses. SI analyzed the data with support from ILL, MF, and JRL. SI wrote the first draft and ILL, MF, JRL, and SR contributed to the final manuscript.

Data availability

Scripts for statistical analyses, along with taxonomy tables, functional abundance tables, and metadata used for analysis will be available on FigShare. Raw sequence data is being deposited on NCBI.

FIGURES

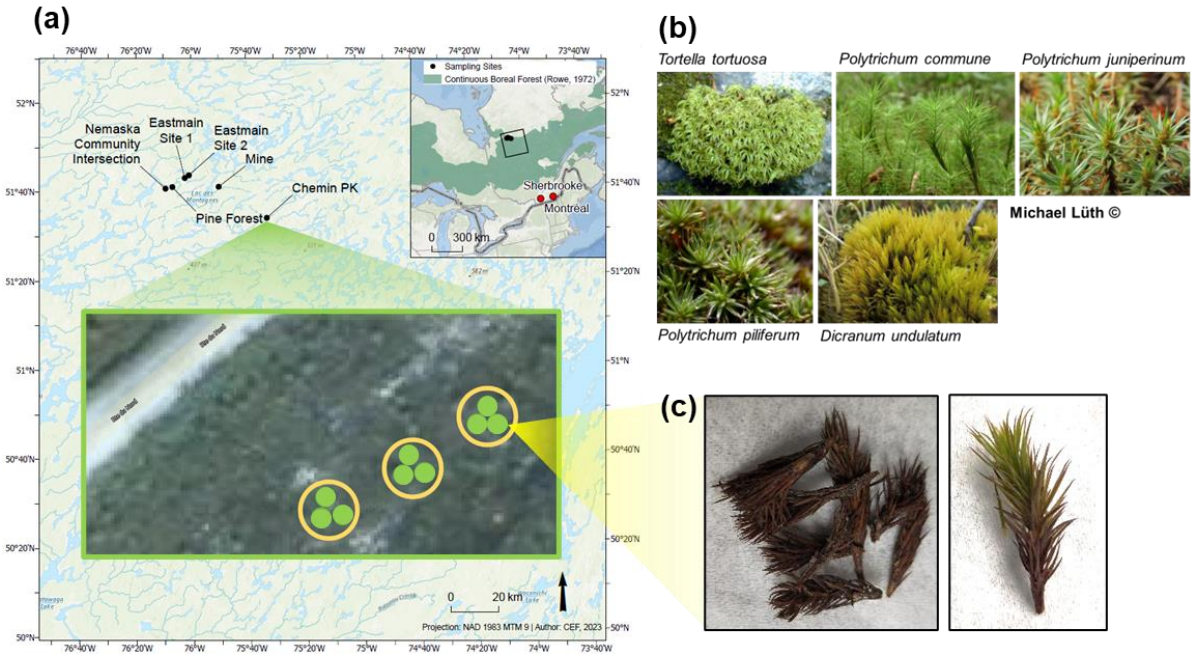

Fig.1. Sampling design: For September sampling, within each location **(a)**, three microsites were randomly selected (yellow circles, inlaid). Three replicates were then sampled within each microsite (green circles). Top right, inlaid in **(a)**: map of Québec showing sampling sites within the continuous boreal forest section in Québec. **(b)** Images of each moss species that were sampled (displayed with permission from Michael Lüth). **(c)** Moss samples separated into green and brown sections.

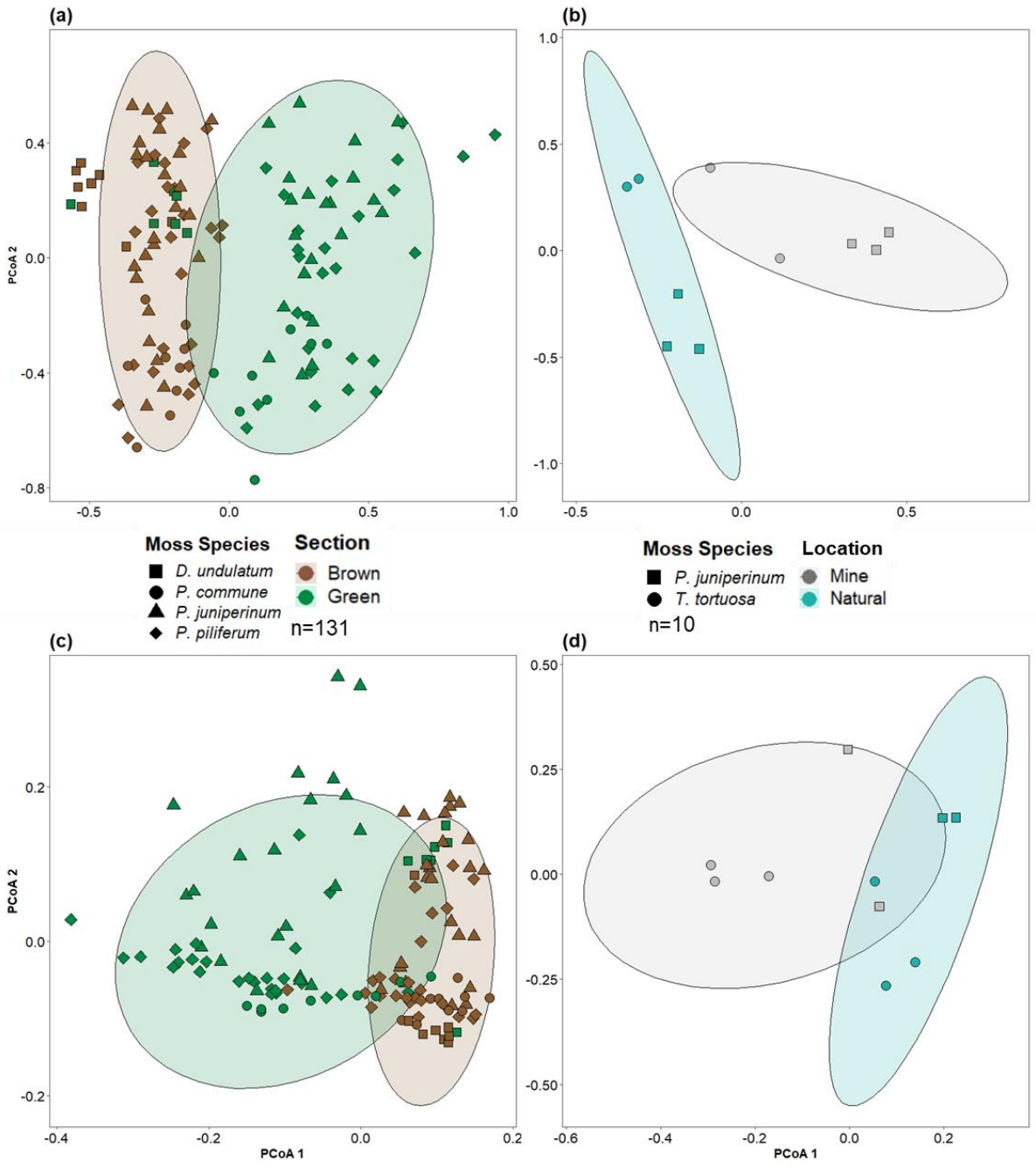

Fig. 2. Two-dimensional principal coordinate analysis (PCoA) of community similarities for **(a, b)** microbial taxonomy based on Jaccard indices of variance-stabilizing transformed taxonomical abundances and **(c, d)** functional gene abundances based on Euclidean distances calculated from Hellinger transformed data. Panels **a** and **c** show samples collected in September, while panels **b** and **d** show samples collected in June. Shapes represent moss species, while colours correspond to different variable types. Each point is one sample. Ellipses indicate a 95% confidence interval around samples from each moss section or location.

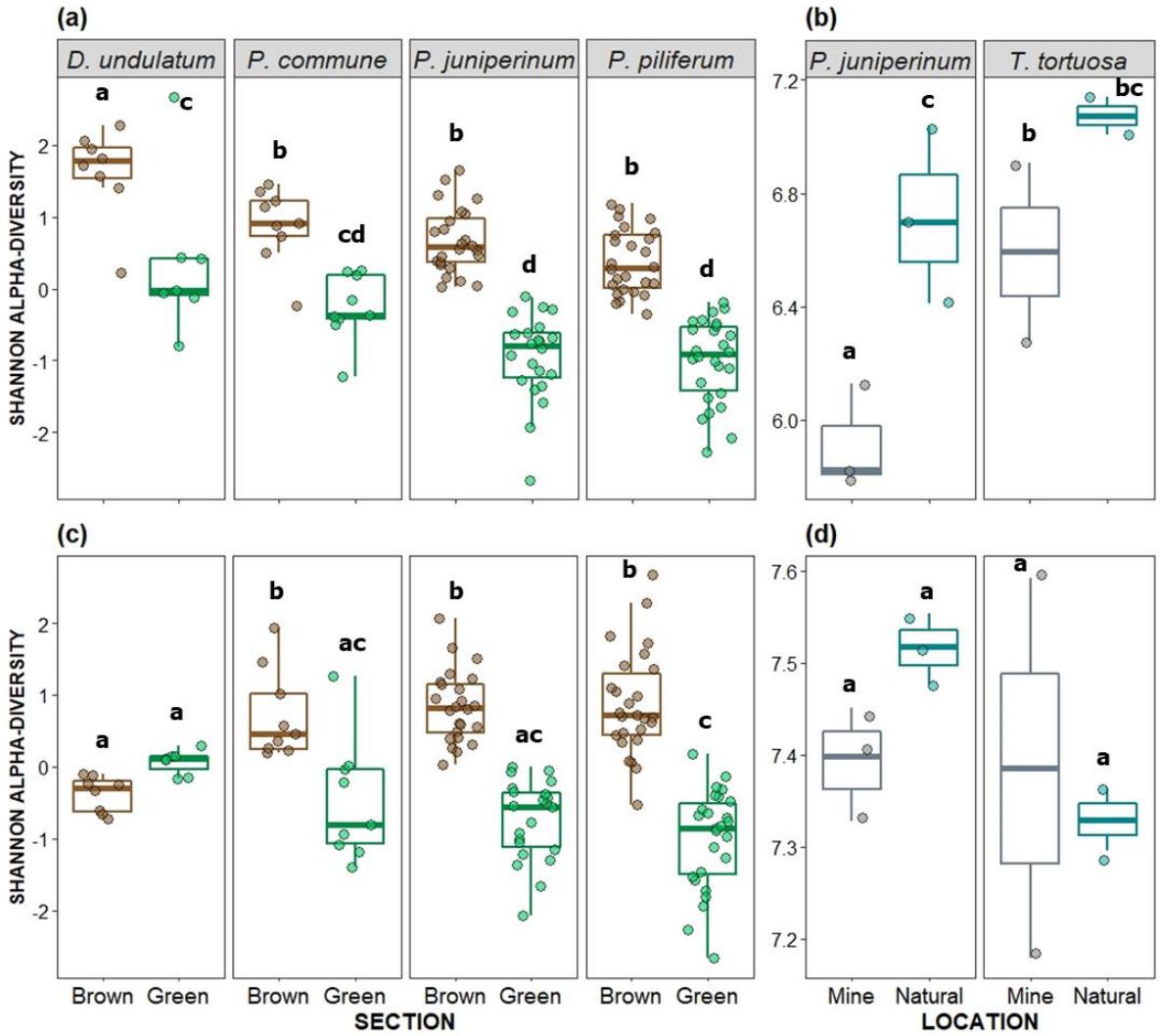

Fig. 3. Differences in alpha-diversity (Shannon diversity) of moss microbial **(a, b)** taxa and **(c, d)** functional genes. Colors indicate different variable types: green and brown for section types (n=131) and teal and grey for natural vs. mine sites (n=10), respectively. Boxplots are faceted according to moss species identity. Letters indicate Tukey post-hoc pairwise comparisons of mean differences.

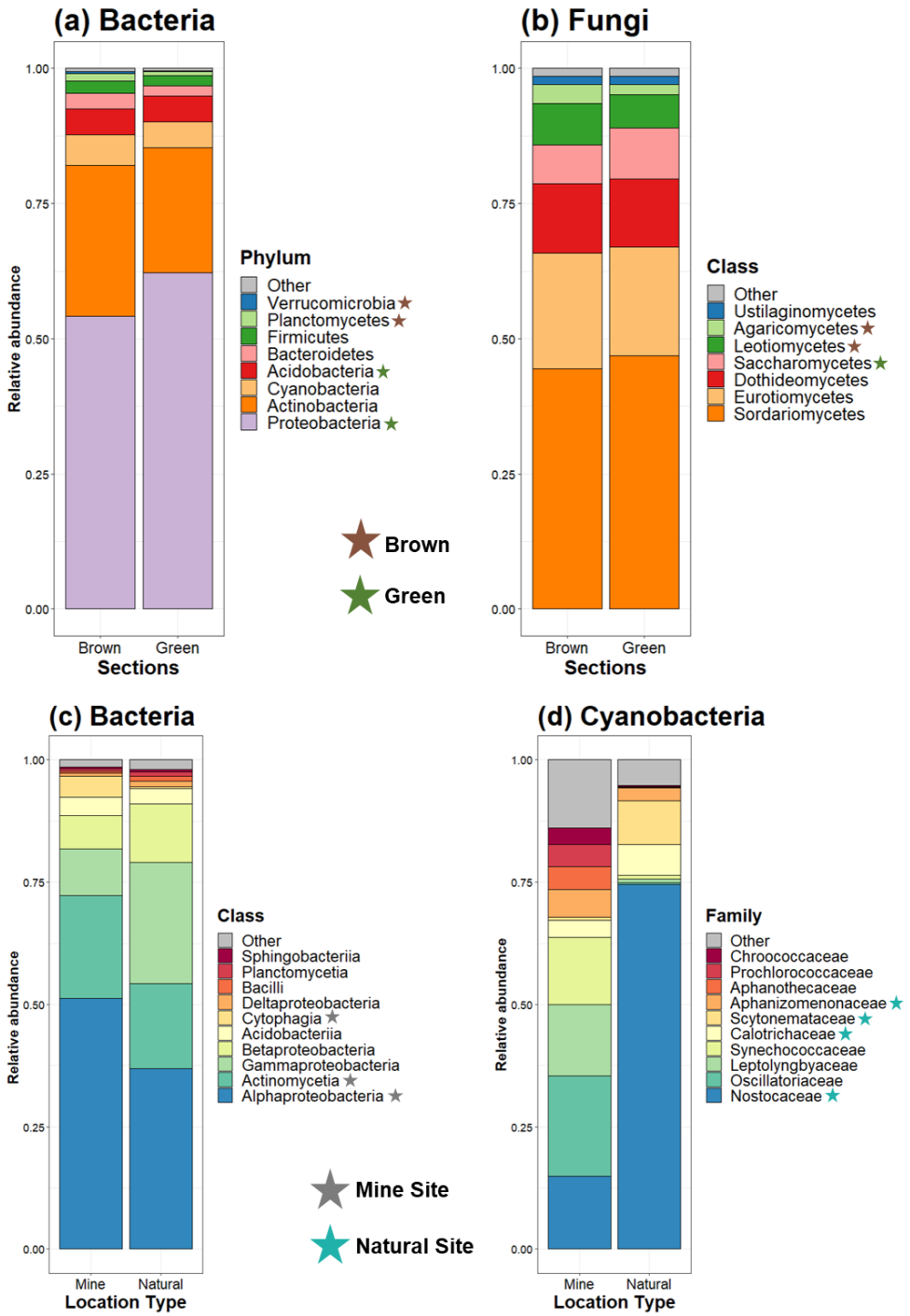

Fig. 4. Microbial taxonomic profiles between moss sections **(a, b)** and locations **(c, d)** highlighting (stars) differentially abundant taxa levels. Bar chart colors represent different microbial taxa. Star colors indicate taxa that are relatively abundant in brown (brown) or green (green) sections **(a, b)** as well as mine (grey) or natural (teal) sites **(c, d)**.

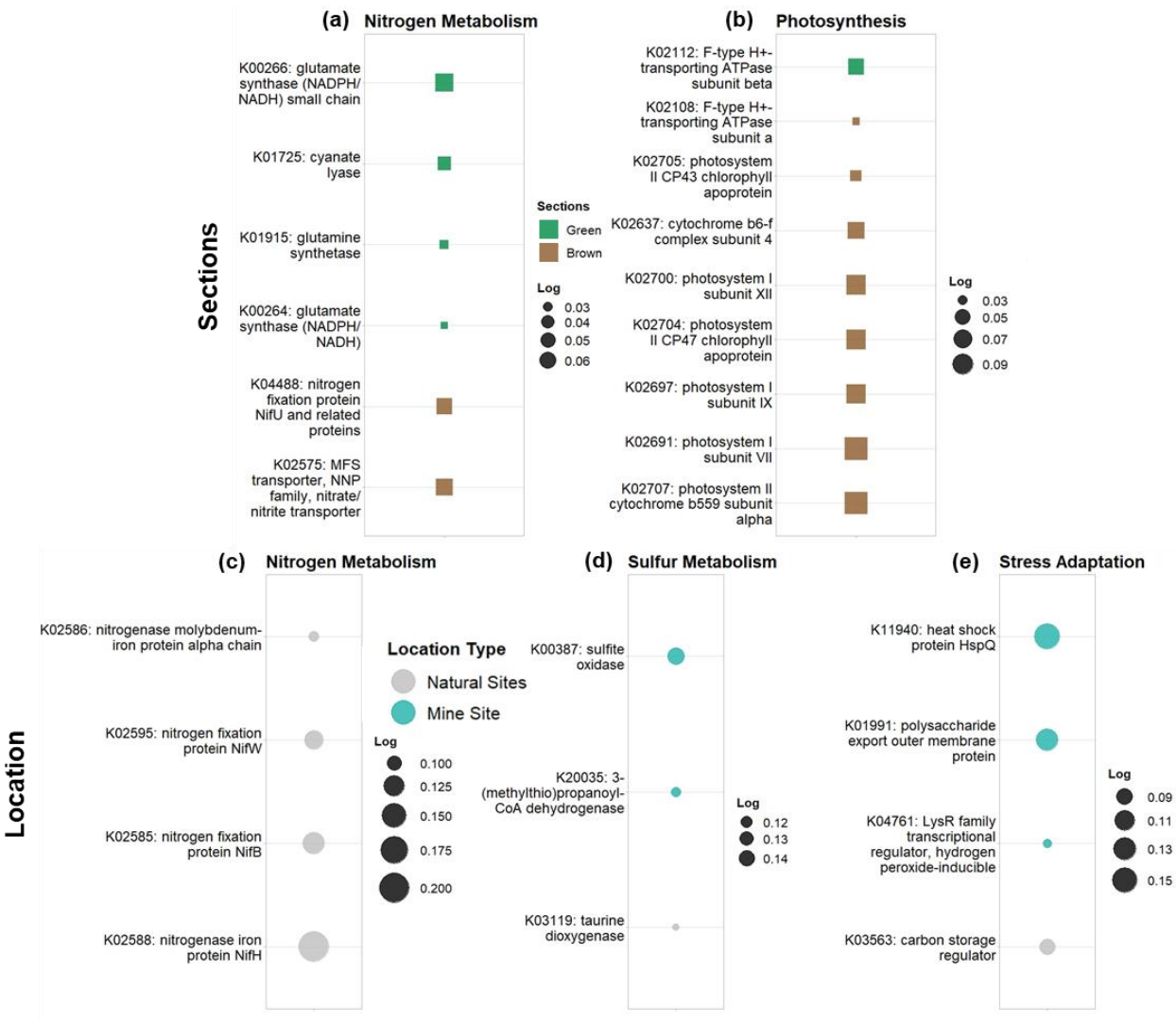

Fig. 5. Differential abundance (Log_2 fold change) of microbial functional genes (KOs) part of various metabolic pathways identified by ANCOM-BC between **(a, b)** moss sections from September sampling and **(c-e)** locations from June sampling. Colours represent variable type according to which KOs were differentially abundant. Circle size shows differential abundance weight at each variable type.

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Supporting Information

Article title: Moss species, sections, and land use type drive moss-associated microbial taxonomy and functions in boreal forests

Authors: Sarah Ishak, Maria Faticov, Jonathan Rondeau-Leclaire, Sébastien Roy and Isabelle Laforest-Lapointe

The following supporting documents are available for this paper:

Methods S1 Detailed description of DNA extraction protocol.

Fig. S1 Heatmap of p-values that show the magnitude of pairwise differences between different moss species, faceted by moss section type.

Table S1 Moss sample counts of June sampling.

Table S2 Moss sample counts of September sampling.

Table S3 Summary of analyses, responses examined, and models according to sampling periods and metagenomic data (microbial taxa or KO profiles).

Table S4 The effect of moss section type, location, and moss species on community composition of taxa and KO profiles of moss microbial communities.

Table S5 The effect of moss section type, location, and moss species on diversity of moss microbial taxa and KO profiles.

Table S6 Pairwise differences of moss microbial functional and taxonomical diversity between different moss species within each section.

Table S7 Pairwise differences of moss microbial functional and taxonomical diversity between different moss sections for each species.

Table S8 Log_2 fold changes of microbial taxa between green and brown sections of the moss microbiome.

Table S9 Log_2 fold changes of microbial taxa between mosses from natural sites and the Whabouchi mine site.