Lime amendment to chronically acidified forest soils results in shifts in prokaryotic and fungal communities.

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Abstract

A consequence of past acid rain events has been chronic acidification of both Nova Scotian forests and watersheds, leading to a loss of essential nutrients and subsequently to decreased forest productivity and biodiversity. Liming – supplementing forests with crushed rock (dolomite, limestone, or basalt) – can restore essential nutrients to acidified soils as well as increasing the pH of the soils and the carbon capture by forests by promotion of tree growth. The effectiveness of liming treatments have often been assessed biologically through tree growth measurements, but microorganisms respond rapidly to changes in pH and nutrient availability, and would potentially provide early insights into forest recovery. However, the impact of liming on the soil microbiome is not well understood; understanding the impacts of liming on a micro as well as a macro level will help to determine whether liming is a good remediation strategy for Nova Scotia. A pilot study evaluating liming in acidified forests in Nova Scotia began in 2017. Microbiome analyses (prokaryotic 16S rRNA and fungal ITS2 gene amplicon sequencing) of three different depths (horizons) of soil show significant differences between lime-treated and control soils for the prokaryotic but not fungal communities, particularly in the uppermost soil horizon sampled. Notably, several genera, particularly from the Bacteroidia class, were significantly more abundant in treated than control soils in both upper soil horizons. The impacts of liming treatment were smaller in the deepest soil horizon sampled, suggesting that lime amendment either takes longer to reach these depths, or has little impact on these microbial communities. Future studies that investigate the functional capacity of these microbial communities and longitudinal follow-ups are warranted.

Introduction

Forests are under threats due to anthropogenic-driven global climate change; these include increasing temperatures, drought, increased pests, and increased severity and frequency of fires[1]. Forests are the dominant land cover in Canada and are a key foundation of the Canadian economy. Canadian forests sustain biodiversity and regulate biogeochemical cycling. This ecosystem is habitat to thousands of species and stores of 10–30% of the global terrestrial carbon[2]. There is an increasing need to understand how forests function as well as to develop strategies and practices to increase forest resilience and to increase their use as natural climate solutions[3]. One aspect of forest health that is poorly understood is the role of microbes in forest soil. Bacteria, archaea, fungi, and viruses play important roles in biogeochemical cycling, and their contributions to forest health are becoming increasingly appreciated. Bacteria and fungi break down forest biomass to recycle nutrients and promote soil formation and also directly assist trees in acquiring nutrients[4,5].

Decades of acidic emissions from fossil fuel burning resulted in acid rain deposition over northeastern North America. Legislation, most notably the Clean Air Act Amendments of 1990, drastically reduced industrial emissions and resulted in a rebounding of many of the affected areas[6]. By contrast, Nova Scotia forests remain compromised by the effects of decades of acidification because of its unique geology (slow weathering, limestone-poor and base cation-poor) and extensive wetlands[7,8]. Nova Scotian

soils have now become depleted in essential base cations such as calcium (Ca^{2+}) and magnesium (Mg^{2+}) that are essential for productive forests. In addition, the chronic acidification has resulted in the increased release of toxic forms of aluminum in soils and waterbodies across the province[7,8]. One effective method to restore lost productivity in Nova Scotia forests is to replace the depleted nutrients and alkalinity in a process known as liming. Liming is the application of crushed limestone to soil and has been used to promote forest ecosystem recovery in Scandinavian countries that have also suffered chronic terrestrial acidification[9].

Amendments that are commonly used to treat acidified forests include wood ash, calcite (CaCO3), and dolomite (CaMgCO3). After application, limestone dissolves in soils, releasing bicarbonate and base cations[10]. The bicarbonate raises the pH of soils and base cations become available for uptake by plants and microbes. Raising the pH also causes Al^{3+} to more favorably interact and complex with organic molecules, making it less available for leaching and uptake by plants[11,12]. The effects of liming are most commonly assessed through measurements of soil chemistry[13]. The effects of liming can also be measured through changes in forest health. In most all cases, liming results in an increased productivity of forests. Two recent studies in Canada have shown that liming increases tree health for decades after the treatment has been applied[14].

To examine the effects of liming on five sites in Nova Scotia, we have characterized the soil chemistry and used 16S rRNA gene and Internal Transcribed Spacer (ITS) 2 rRNA gene amplicon sequencing. This approach has allowed us to analyze the effects of liming on alterations in forest soil microbiome composition in the context of a comprehensive analysis of soil chemistry, producing a rich data set. We initially examined the overall community composition of prokaryotes and fungi and then identified specific taxa that are associated with the liming treatment.

Materials and Methods

Study sites

Otter Ponds Demonstration Forest (Mooseland, Nova Scotia) is the site of an experiment to evaluate the effectiveness of helicopter liming (application of crushed dolomite) as a remediation strategy for acidified forests. A total of ten softwood sites (trees predominantly red spruce) were previously chosen based upon tree composition and accessibility. Of the ten sites, five were treatment sites (treated with lime via helicopter) and five untreated controls matched for soil type, forest cover and geologic features. Each circular site had a radius of 10.3 meters and a center point determined with ArcGIS (Table S1). Ten tonnes of crushed dolomitic limestone (Mosher Limestone (8433 Hwy 224, Upper Musquodoboit, NS B0N 2M0)) was applied per hectare to the treatment sites using helicopters in October 2018.

Soil sampling

Three soil horizons were sampled: Upper Forest Floor (~ 1-5cm depth), Lower Forest Floor (~ 5-10cm) and Upper B Horizon(~ 25-35cm) (Fig. 1). Three rounds of soil sampling were completed at the 10 sites. In round one, three replicates of each of the three horizons per site were taken for soil chemistry analysis. In rounds two (October 2020) and three (July 2021), two or three replicates, respectively, of each of the three horizons per site were collected for a total of 150 soil samples. At each site random bearings were used to select the location within the site to collect the first set of replicates. The location of subsequent replicates were chosen by going 180˚ (round two) or 120˚ (round three) from the original random bearing. Approximately 500 g of soil was collected per sample and placed in sterile plastic bags. Bags were transported on ice to Dalhousie University, Nova Scotia. All samples were stored at -80˚C until further analysis.

Soil chemistry measurements

Base cations and available metals were analyzed using inductively coupled plasma mass spectrometry (ICP-MS) by AGAT Laboratories (Dartmouth N.S.) as described by [15]. Soil samples were weighed and digested with hydrochloric acid and nitric acid. After digestion, samples were cooled and brought to volume with double deionized water and analyzed by ICP-MS.

DNA extraction

DNA was isolated from soil samples using the Qiagen PowerSoil DNA Isolation Kit (Lot#PS14K26, Lot#163-118927, Lot#164030916). The manufacturer's protocol was followed except for vortexing being done by hand versus using a vortex adapter as described in the protocol. To ensure sufficient DNA for subsequent analyses, the concentration of the DNA was determined using a NanoDrop spectrophotometer according to the manufacturer's instructions (Thermo Scientific) and the isolated DNA was stored at -20˚C until library preparation.

Library preparation and amplicon sequencing

Extracted DNA samples were sent to the Integrated Microbiome Resource (Dalhousie University) where library preparation and Illumina MiSeq amplicon sequencing (MiSeq Reagent Kit v3) were completed. The V4-V5 regions of the prokaryotic 16S rRNA gene were amplified using primers 515 FB (5'-GTGYCAGCMGCCGCGGTAA) and 926R (5'-CCGYCAATTYMTTTRAGTTT) for bacterial/archaeal identification. The ITS2 region of the fungal ribosomal genes was amplified using primers ITS86(F) (5' – GTGAATCATCGAATCTTTGAA) and ITS4(R) (5' – TCCTCCGCTTATTGATATGC) for fungal identification. Library preparation and amplicon sequencing followed the standard operating procedures set out in Comeau et al. (2017)[16].

Sequencing data processing

Processing of the sequencing data followed the Microbiome Helper workflow[16] utilizing QIIME 2[17]. Briefly, raw reads were imported into QIIME 2 (version 2021.8) and primers were trimmed using Cutadapt[18]. Reads were denoised using the DADA2[19] denoising algorithm (2 errors were allowed for each of the

forward and reverse reads) with trim lengths of 275/180 bp and 260/200 bp for forward/reverse reads from 16S rRNA or ITS2 genes, respectively. The resulting Amplicon Sequence Variants (ASVs) were classified using the SILVA v138 database[20] for 16S rRNA amplicons for bacterial/archaeal identification and the UNITE v8 database[21] for ITS2 amplicons for fungal identification. For 16S rRNA (hereafter 16S) gene amplicons, sequences that were not classified at the phylum level, that belonged to mitochondria or chloroplasts or were present at below 10 total reads were removed from further analysis. A phylogenetic tree was built using SEPP[22] with a reference phylogeny created using the SILVA reference database (version 128)[20] for 16S gene sequences.

Statistical analysis

Downstream analyses were carried out using custom R (version 2021.09.0) and Python (version 3.8.8) scripts (https://github.com/M-Hosmer/NS_LimingTrial_OtterPond). Raw count tables were normalized by conversion to relative abundances, rarefying to the lowest sequencing depth, or conversion to robust centered log ratio [rCLR] (with the package deicode[23]). Alpha diversity was assessed using Faith's phylogenetic diversity for the 16S and Simpson's diversity, Chao 1 richness and Shannon's diversity for both ITS2 and 16S communities with ANOVA with post-hoc Tukey's HSD tests for differences between groups carried out with the Python package bioinfokit[24]. Beta diversity between all samples was assessed using Weighted UniFrac distance[25,26] for 16S and Bray-Curtis dissimilarity[27,28] for ITS2 calculated within the R package phyloseq[29] with visualization using a Principal Coordinate Analysis (PCoA) with the Python package scikit-bio[30]. The associations between microbial composition and helicopter liming as well as soil horizon, site, sample within site and season were assessed using PERMANOVA tests within the R package vegan[31]. The dispersion or variance within groups (i.e treatment or soil horizon) were assessed using "betadisper" function and significance determined with ANOVA (both from the vegan package[31]). In order to assess whether there are any taxa that are differentially abundant between the control and treatment samples we utilized three differential abundance tools: ALDEx2[32], ANCOM-II[33] and MaAsLin 2[34]. These tools were chosen based on the recommendations of Nearing & Douglas et al. (2022)[35]; ALDEx2 and ANCOM-II both minimize the number of false-positive differentially abundant taxa identified, at the cost of reduced sensitivity, while MaAsLin2 may identify more false positives, but is more sensitive. We therefore report on how many, and which, of the tools identify a given taxon as differentially abundant – those with a Benjamini-Hochberg adjusted p-value of ≤ 0.1 in ALDEx2, a False Discovery Rate cut-off of 0.8 in ANCOM-II or a q-value of ≤ 0.1 in MaAsLin 2 – and consider a taxon to be differentially abundant if it is identified by at least two of the three tools. Figures used the additional Python packages matplotlib[36], tidyr[37], numpy[38], scipy[39], pandas[40], Biopython[41] and ete3[42]. Relative abundances were used for alpha and beta diversity analyses, while both relative abundance and rCLR was used to represent abundances when individual taxa are shown. Relative abundances were used for alpha and beta diversity analyses, while both relative abundance and rCLR was used to represent abundances when individual taxa are shown. MaAsLin2 was run with the rarefied abundances while ALDEx2 and ANCOM-II both take raw abundance tables and perform normalisations as part of their workflows.

Data Availability

All raw sequencing data have been deposited under the European Nucleotide Archive BioProject PRJEB58425. All code used for data analysis, performing statistical tests and generating figures is on Github (https://github.com/M-Hosmer/NS_LimingTrial_OtterPond).

Results and Discussion

Liming amendment was effective for altering soil chemistry

Each of five treatment sites had 10 tonnes per hectare of crushed lime dropped from a helicopter. The amount of crushed lime that was measured to have fallen at each site varied between 3.3 and 12.2 tonnes per hectare (means of 3.3, 7.7, 5.43, 12.2 and 5.1 tonnes per hectare were measured for sites 1 to 5, respectively), reflecting heterogeneity in the application method. Soil chemistry measurements (pH, calcium (Ca^{2+}), aluminum (Al^{3+}), magnesium (Mg^{2+}), and manganese (Mn)) were taken one year post liming treatment (10months before the first microbiome samples). No soil chemistry measurements were taken prior to liming treatment. Soil pH, and exchangeable (plant available) Ca^{2+}, Mg^{2+}, and Mn levels were significantly higher while Al^{3+} levels were significantly lower (T-test $p \leq 0.05$) in treatment than control sites in the Upper and Lower Forest Floor horizons (Supp. Figure 1, Fig. 2). The magnitude of the differences between control and treated sites was larger in the Upper Forest Floor (Supp. Figure 1, Fig. 2). In the Upper B Horizon, only Mn levels were significantly higher in the treatment than control samples, with no other significant differences observed between control and treated sites. Taken together, these data demonstrate that the liming treatment was effective for: increasing soil pH, increasing calcium, magnesium, and manganese concentrations, and decreasing aluminum concentrations in the Upper and Lower Forest Floor samples (Supp. Table 1).

Prokaryotic but not fungal alpha diversity is increased by liming treatment

We compared the alpha diversity between our samples by grouping them by their treatment (control or treatment) and their soil horizon (upper forest floor, lower forest floor and upper B horizon) for both the bacterial/archaeal (16S rRNA gene) and fungal data (ITS2 gene). For bacteria/archaea, there is significantly (ANOVA $p \leq 0.05$) higher Faith's phylogenetic diversity in the treatment *vs* control (Fig. 3A) samples, and in the upper forest floor and upper B horizon in comparison to the lower forest floor (Fig. 3B). When examining differences between control and treatment in the different horizons, Faith's phylogenetic diversity was higher in the treatment than control in both the upper and lower forest floor – but was only significant (ANOVA $p \leq 0.05$) in the upper forest floor – while the trend was reversed in the upper B horizon, and this difference was not statistically significant (Fig. 3E). Similar trends were observed using other alpha diversity metrics: number of ASVs, Chao1 richness, Shannon diversity and Simpson's diversity (Supp. Figure 2). In contrast there are no significant differences between control and

treatment samples within the fungal community for any alpha diversity metrics, within any of the soil horizons (Fig. 3C, 3F and Supp. Figure 3). There is significantly higher (ANOVA $p \leq 0.05$) richness (number of ASVs and Chao1 richness) in the upper forest floor than either the lower forest floor or upper B horizon samples, but alpha diversity indices that also incorporate abundance measurements or evenness showed no differences (Fig. 3D and Supp. Figure 3).

Shifts in microbial community composition (beta diversity) are seen with liming amendment in prokaryotic but not fungal communities

We compared the differences in microbial diversity with liming amendment as well as between the different soil horizons, using Weighted UniFrac distance for the prokaryotic community and Bray-Curtis dissimilarity for the fungal community, with PERMANOVA tests that also included site, sample within site, and season (Figs. 3G-3N). For the bacterial/archaeal community, most of the variation between samples was due to the soil horizon ($R^2 = 0.433$, $p = 0.001$), although there were also significant differences with liming treatment ($R^2 = 0.034$, $p = 0.001$) and season ($R^2 = 0.024$, $p = 0.003$), as well as a number of significant interactions between these variables, including between treatment and soil horizon ($R^2 = 0.034$, $p = 0.008$; Fig. 3G and Supp Tables 1 & 2). There is clear clustering of all upper B horizon samples from samples from the other horizons on both the first (36% variation) and second (25% variation) PCoA axes (Fig. 3G), and while there is clustering of the lower forest floor samples away from the upper forest floor samples, this difference is not as large. Within the upper forest floor, there is clearly distinct clustering of the treatment samples from the control (Fig. 3I). By contrast, there is no clear clustering of the treatment from control within either the lower forest floor (Fig. 3J) or upper B horizon (Fig. 3K). The betadispersion analysis indicates that while there are significant differences between groups (see above) there are also significant differences within the treatment (betadisp, ANOVA $p \leq 0.01$) and soil horizon groups (betadisp, ANOVA $p \leq 0.005$).

Within the fungal community there were no significant differences with any of the variables that we included in the PERMANOVA tests (Fig. 3H and Supp. Tables 1 & 2). Unlike for the bacterial/archaeal community, for the fungal community there was no single variable that accounted for a large proportion of the variation between samples (maximum $R^2 = 0.056$ for soil horizon). Furthermore, we note that for the fungal ordination plots only a small amount of variation is shown on the first two axes (7% and 6% for PCoA1 and PcoA2, respectively), indicating that the variation between the samples is not well represented on two axes. We observed some clustering of the upper and lower forest floor samples away from the upper B horizon (Fig. 3H), indicating that – as for the bacteria/archaea – the upper B horizon is slightly distinct from both forest floor horizons. Additionally, we observe minor clustering of the treatment from the control samples within the upper forest floor (Fig. 3L) and lower forest floor (Fig. 3M) that is not seen in the upper B horizon (Fig. 3N). The dispersion of the treatment groups is significantly different between treatment groups (betadisp, ANOVA $p \leq 0.05$) but not between soil horizon groups (betadisp, ANOVA $p \geq 0.05$). Overall, the composition of the fungal communities is not impacted significantly by treatment and horizon.

Taxa associated with liming amendment of chronically acidified soils

We have examined the abundance of taxa using both relative abundance, as this is what most studies to date have used, and rCLR abundance, as this accounts for the compositionality of microbiome data[43]. For the relative abundances, higher values indicate that a larger proportion of the sequences within samples belong to a particular taxon. For the rCLR abundance, a zero value indicates that the abundance of a taxon is equal to the mean $\log_2$ abundance of all taxa, with positive or negative values indicating higher or lower abundances than the mean $\log_2$ abundance, respectively.

In the prokaryotic community, there were seven phyla that were present in all samples (Proteobacteria, Bacteroidota, Acidobacteria, Planctomycetota, Verrucomicrobiota, Actinobacteriota, and RCP2-54) and some of these were also high in relative abundance in a large number of samples: (i) Acidobacteriota dominate in all sample types (42–67% relative abundance); (ii) Proteobacteria are the second most abundant phyla in all sample types (15–29%); (iii) Bacteroidota are abundant in Upper (10%) and Lower (3.4%) Forest Floor Treatment samples; and (iv) Planctomycetota account for ~ 3-5.5% relative abundance in all samples, but are more abundant in Upper Forest Floor samples (Supp. Figure 4).When we look at lower taxonomic levels, there are a large number of ASVs that were not able to be classified; only 8,114 of the 13,684 16S rRNA gene ASVs had genus-level classifications, with a large number of these being made up of genera that are likely not well characterized, *i.e.*, *Candidatus* classifications (431 ASVs), or classifications without Latin scientific names (3,454 ASVs).

The fungal community was dominated by the Basidiomycota (78–90% relative abundance) and the Ascomycota to a lesser extent (8.7–20%); these were the only two phyla that were present in all samples (Supp. Figure 5) and all other phyla were present at < 2% relative abundance on average. The Mortierellomycota were also highly prevalent (present in 95% of Lower Forest Floor Control samples, and 100% of all other sample groups) but were only present at a maximum relative abundance of 1.7%. In contrast to the bacterial/archaeal community, where almost all taxa that were abundant were also highly prevalent, in the fungal community there were very few taxa with genus-level classifications that were present in > 90% of samples within at least one sample grouping; these belonged to the Agaricomycetes (*Galerina*, *Sebacina*, *Amanita*, *Russula* and *Cortinarius*), Mortierellomycetes (*Mortierella*) and Dothideomycetes (*Cenococcum*). For the fungal ASVs there was also a large proportion without a genus-level classification (only 1,210 of 1,785 ASVs had a genus-level classification).

Identifying differentially abundant fungal genera between control and treatment samples

To identify which genera might be driving the differences in community composition seen in the ordination plots (Fig. 3), we carried out differential abundance testing between control and treatment samples separately for each soil horizon. These tests were carried out using ALDEx2, ANCOM-II and MaAsLin2 and we consider a genus to be differentially abundant if two of these three tests identify it (see

Methods section for further details). Overall, seven genera were identified as significantly differentially abundant, predominantly within the upper forest floor horizon, however, most of these were found in very low abundances. If we focus on only the 25 most abundant genera by relative or rCLR abundance (44 total), only three genera were significantly differentially abundant within these (Fig. 4): *Apodus* (more abundant in treatment samples in the upper forest floor horizon), *Galerina* (more abundant in control samples in the upper forest floor horizon) and *Tylospora* (more abundant in control samples in the upper B horizon; these trends were consistent between both relative and rCLR abundance).

Bacterial/archaeal taxa associated with lime amendment.

For the bacterial/archaeal genera we again carried out differential abundance testing between control and treatment samples within each soil horizon using ALDEx2, ANCOM-II and MaAsLin2 and we consider a genus to be differentially abundant if two of these three tests identify it. Overall, 230 genera were identified as being significantly differentially abundant between control and treatment samples in at least one of the three horizons; 185 in the upper forest floor, 13 in the lower forest floor and 8 in the upper B horizon. We focused on those that were also within the most abundant genera, taking the top 25 most abundant genera by relative or rCLR abundance (46 total; Fig. 5). Reflecting the magnitude of alpha and beta diversity shifts in the horizons, 24 or three genera (of the 46 total) were significantly differentially abundant between control and treatment samples in the upper or lower forest floor samples, respectively. Three of these taxa (all unclassified at the genus level; Commomonadaceae, Bacteroidia and Microscillaceae) were identified in both upper and lower forest floor samples, and are all more abundant in treatment than control samples. One genera was significantly differentially abundant in the upper B horizon; *Acidipila* (Acidobacteriae), slightly more abundant in treatment than control samples, 0.41 *vs* 0.18% and − 1.66 *vs* -2.05 for rCLR abundance, respectively. Five genera belonging to the Acidobacteriae class were identified as differentially abundant by at least 2 tests and are more abundant in control vs treatment samples. In contrast, the five genera identified by at least two tests belonging to the Bacteroidia class are more abundant in treatment vs control. Similar trends are not seen within any other classes that have multiple genera identified as differentially abundant.

Discussion

Through the measurement of soil chemistry data and amplicon sequencing data for prokaryotes and fungi, we have evaluated the impact of liming treatment within Nova Scotian forests. We observed large shifts (alpha and beta diversity) in the prokaryotic community between each of the soil horizons, but the largest differences between control and treatment samples were observed in the upper forest floor horizon (Figs. 3 and 5). There were very few differences in the fungal community between either control and treatment samples or the different soil horizons. Our results are similar to two previous reports on softwood forests treated with amendments that observed minimal changes in fungal soil communities upon treatment of softwood forests[44,45].

Currently, many environmental bacteria have not been formally classified or studied, potentially resulting in poor resolution of the taxonomic classifications[46]. Many of the bacteria identified in our study are those from taxa without genus or even family level classifications. Additionally, some of these taxa that do have classifications have not really been characterized as they lack Latin names[46]. As a result, efforts to perform functional predictions are premature. Tools to ascribe bacterial function to communities (e.g. PICRUSt2)[47] need reasonably close matches at the ASV level or predictions will not be valid. We suggest that similar studies in the future should also incorporate metagenomic analysis in order to gain insight of functional capacity without needing previously sequenced representatives of close relatives.

We predict that in future years (as the crushed limestone works its way down to lower forest floor and upper B horizon) there will be shifts in bacterial/archaeal soil communities. Consistent with this notion, Sridhar et al. (2022a)[48] found that fungal taxa became more differentially abundant over longer periods of time (2 years vs 25 years post liming treatment).

Conclusions

Our results suggest that changes in bacterial/archaeal community in the upper forest floor may serve as an early indicator of recovering soil health in response to liming. Future studies should aim to assess microbial community changes longitudinally, both pre- and post-liming amendment, and utilize matched soil chemistry measurements for every microbial community characterization. This would enable the identification of the driving factors of microbial community differences in chronically acidified soils. Furthermore, incorporating metagenomic and transcriptomic studies will help to elucidate changes that are not seen by taxonomic changes alone, particularly when taxonomic annotation is lacking in environmental studies.

Declarations

Ethics approval and consent to participate. Not applicable.

Consent for publication. All authors have consented to the publication of this manuscript.

Availability of data and materials. All raw sequencing data have been deposited under the European Nucleotide Archive BioProject PRJEB58425.

Competing interests. The authors declare no competing interests.

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References

1. Venier LA, Walton R, Brandt JP. Scientific Considerations and Challenges for Addressing Cumulative Effects in Forest Landscapes in Canada. Environ Rev. 2021;29(1):1–22. https://doi.org/10.1139/er-2019-0072.
2. Bradshaw CJA, Warkentin IG. Global Estimates of Boreal Forest Carbon Stocks and Flux. Glob Planet Change. 2015;128:24–30. https://doi.org/10.1016/j.gloplacha.2015.02.004.
3. Watson JEM, Evans TD, Venter O, Maxwell SL. Manage Forests as Protection against Warming. Nature. 2019;567(7748):311–1. https://doi.org/10.1038/d41586-019-00869-5.
4. Lladó S, López-Mondéjar R, Baldrian P. Drivers of Microbial Community Structure in Forest Soils. Appl Microbiol Biotechnol. 2018;102(10):4331–8. https://doi.org/10.1007/s00253-018-8950-4.
5. Uroz S, Buée M, Deveau A, Mieszkin S, Martin F. Ecology of the Forest Microbiome: Highlights of Temperate and Boreal Ecosystems. Soil Biol Biochem. 2016;103:471–88. https://doi.org/10.1016/j.soilbio.2016.09.006.
6. Kahl JS, Stoddard JL, Haeuber R, Paulsen SG, Birnbaum R, Deviney FA, Webb JR, DeWalle DR, Sharpe W, Driscoll CT. Herlihy. Peer Reviewed: Have US Surface Waters Responded to the 1990 Clean Air Act Amendments? Environ Sci Technol. 2004;38(24):484A–90.
7. Clair TA, Dennis IF, Scruton DA, Gilliss M. Freshwater Acidification Research in Atlantic Canada: A Review of Results and Predictions for the Future. Environ Rev. 2007;15(NA):153–67. https://doi.org/10.1139/A07-004.
8. Sterling SM, MacLeod S, Rotteveel L, Hart K, Clair TA, Halfyard EA, O'Brien NL. Ionic Aluminium Concentrations Exceed Thresholds for Aquatic Health in Nova Scotian Rivers, Even during Conditions of High Dissolved Organic Carbon and Low Flow. Hydrol Earth Syst Sci. 2020;24(10):4763–75. https://doi.org/10.5194/hess-24-4763-2020.
9. Johnson DW, Turner J, Kelly JM. The Effects of Acid Rain on Forest Nutrient Status. Water Resour Res. 1982;18(3):449–61. https://doi.org/10.1029/WR018i003p00449.
10. Kreutzer K. Effects of Forest Liming on Soil Processes. *Plant Soil* 1995, *168–169* (1), 447–470. https://doi.org/10.1007/BF00029358.
11. Li W, Johnson CE. Relationships among pH, Aluminum Solubility and Aluminum Complexation with Organic Matter in Acid Forest Soils of the Northeastern United States. Geoderma. 2016;271:234–42.

https://doi.org/10.1016/j.geoderma.2016.02.030.

12. Bolan NS, Adriano DC, Curtin D. Soil Acidification and Liming Interactions with Nutrientand Heavy Metal Transformationand Bioavailability. Advances in Agronomy. Volume 78. Elsevier; 2003. pp. 215–72. https://doi.org/10.1016/S0065-2113(02)78006-1.
13. Lawrence GB, Hazlett PW, Fernandez IJ, Ouimet R, Bailey SW, Shortle WC, Smith KT, Antidormi MR. Declining Acidic Deposition Begins Reversal of Forest-Soil Acidification in the Northeastern U.S. and Eastern Canada. Environ Sci Technol. 2015;49(22):13103–11. https://doi.org/10.1021/acs.est.5b02904.
14. Moore J-D, Ouimet R. Liming Still Positively Influences Sugar Maple Nutrition, Vigor and Growth, 20 Years after a Single Application. For Ecol Manag. 2021;490:119103. https://doi.org/10.1016/j.foreco.2021.119103.
15. Standard Methods Committee of the American Public Health Association., American Water Works Association, and Water Environment Federation. 3125 Metals by Inductively Coupled Plasma-Mass Spectrometry. In *Standard Methods For the Examination of Water and Wastewater.*; WC Lipps, TE Baxter, E Braun-Howland, Eds.; APHA Press: Washington DC.
16. Comeau AM, Douglas GM, Langille MGI. Microbiome Helper: A Custom and Streamlined Workflow for Microbiome Research. *mSystems* 2017, *2* (1), e00127-16. https://doi.org/10.1128/mSystems.00127-16.
17. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ, Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Cope EK, Da Silva R, Diener C, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwardson CF, Ernst M, Estaki M, Fouquier J, Gauglitz JM, Gibbons SM, Gibson DL, Gonzalez A, Gorlick K, Guo J, Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley GA, Janssen S, Jarmusch AK, Jiang L, Kaehler BD, Kang KB, Keefe CR, Keim P, Kelley ST, Knights D, Koester I, Kosciolek T, Kreps J, Langille MGI, Lee J, Ley R, Liu Y-X, Loftfield E, Lozupone C, Maher M, Marotz C, Martin BD, McDonald D, McIver LJ, Melnik AV, Metcalf JL, Morgan SC, Morton JT, Naimey AT, Navas-Molina JA, Nothias LF, Orchanian SB, Pearson T, Peoples SL, Petras D, Preuss ML, Pruesse E, Rasmussen LB, Rivers A, Robeson MS, Rosenthal P, Segata N, Shaffer M, Shiffer A, Sinha R, Song SJ, Spear JR, Swafford AD, Thompson LR, Torres PJ, Trinh P, Tripathi A, Turnbaugh PJ, Ul-Hasan S, van der Hooft JJJ, Vargas F, Vázquez-Baeza Y, Vogtmann E, von Hippel M, Walters W, Wan Y, Wang M, Warren J, Weber KC, Williamson AD, Xu ZZ, Zaneveld JR, Zhang Y, Zhu Q, Knight R. ; Caporaso, J. G. Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2. *Nat. Biotechnol.* 2019, *37* (8), 852–857. https://doi.org/10.1038/s41587-019-0209-9.
18. Martin M. Cutadapt Removes Adapter Sequences from High-Throughput Sequencing Reads. *EMBnet.journal* 2011, *17* (1), 10. https://doi.org/10.14806/ej.17.1.200.
19. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-Resolution Sample Inference from Illumina Amplicon Data. Nat Methods. 2016;13(7):581–3. https://doi.org/10.1038/nmeth.3869.

20. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. The SILVA Ribosomal RNA Gene Database Project: Improved Data Processing and Web-Based Tools. Nucleic Acids Res. 2012;41(D1):D590–6. https://doi.org/10.1093/nar/gks1219.
21. Nilsson RH, Larsson K-H, Taylor AFS, Bengtsson-Palme J, Jeppesen TS, Schigel D, Kennedy P, Picard K, Glöckner FO, Tedersoo L, Saar I, Kõljalg U, Abarenkov K. The UNITE Database for Molecular Identification of Fungi: Handling Dark Taxa and Parallel Taxonomic Classifications. Nucleic Acids Res. 2019;47(D1):D259–64. https://doi.org/10.1093/nar/gky1022.
22. Mirarab S, Nguyen N, Warnow TSEPP. SATé-Enabled Phylogenetic Placement. Biocomputing 2012. Kohala Coast, Hawaii, USA: WORLD SCIENTIFIC; 2011. pp. 247–58. https://doi.org/10.1142/9789814366496_0024.
23. Martino C, Morton JT, Marotz CA, Thompson LR, Tripathi A, Knight R, Zengler K. A Novel Sparse Compositional Technique Reveals Microbial Perturbations. *mSystems* 2019, *4* (1), e00016-19. https://doi.org/10.1128/mSystems.00016-19.
24. Bedre R, Reneshbedre/Bioinfokit. Bioinformatics Data Analysis and Visualization Toolkit, 2020. https://doi.org/10.5281/ZENODO.3965241.
25. Lozupone C, Knight R, UniFrac:. A New Phylogenetic Method for Comparing Microbial Communities. Appl Environ Microbiol. 2005;71(12):8228–35. https://doi.org/10.1128/AEM.71.12.8228-8235.2005.
26. Lozupone CA, Hamady M, Kelley ST, Knight R. Quantitative and Qualitative β Diversity Measures Lead to Different Insights into Factors That Structure Microbial Communities. Appl Environ Microbiol. 2007;73(5):1576–85. https://doi.org/10.1128/AEM.01996-06.
27. Sørensen T. A Method of Establishing Groups of Equal Amplitude in Plant Sociology Based on Similarity of Species Content and Its Application to Analyses of the Vegetation on Danish Commons. Munksgaard: Biologiske skrifter; 1948.
28. Bray JR, Curtis JT. An Ordination of the Upland Forest Communities of Southern Wisconsin. Ecol Monogr. 1957;27(4):325–49. https://doi.org/10.2307/1942268.
29. McMurdie PJ, Holmes S, Phyloseq. An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS ONE. 2013;8(4):e61217. https://doi.org/10.1371/journal.pone.0061217.
30. Pedregosa F, Varoquaux G, Gramfort A, Michel V, Thirion B, Grisel O, Blondel M, Prettenhofer P, Weiss R, Dubourg V, Vanderplas J, Passos A, Cournapeau D, Brucher M, Perrot M, Duchesnay E, Louppe G. Scikit-Learn: Machine Learning in Python. *J. Mach. Learn. Res.* 2012, *12*.
31. Dixon PVEGAN. a Package of R Functions for Community Ecology. J Veg Sci. 2003;14(6):927–30. https://doi.org/10.1111/j.1654-1103.2003.tb02228.x.
32. Fernandes AD, Macklaim JM, Linn TG, Reid G, Gloor GB. ANOVA-Like Differential Expression (ALDEx) Analysis for Mixed Population RNA-Seq. PLoS ONE. 2013;8(7):e67019. https://doi.org/10.1371/journal.pone.0067019.
33. Mandal S, Van Treuren W, White RA, Eggesbø M, Knight R, Peddada SD. Analysis of Composition of Microbiomes: A Novel Method for Studying Microbial Composition. Microb Ecol Health Dis.

2015;26(0). https://doi.org/10.3402/mehd.v26.27663.

34. Mallick H, Rahnavard A, McIver LJ, Ma S, Zhang Y, Nguyen LH, Tickle TL, Weingart G, Ren B, Schwager EH, Chatterjee S, Thompson KN, Wilkinson JE, Subramanian A, Lu Y, Waldron L, Paulson JN, Franzosa EA, Bravo HC, Huttenhower C. Multivariable Association Discovery in Population-Scale Meta-Omics Studies. PLOS Comput Biol. 2021;17(11):e1009442. https://doi.org/10.1371/journal.pcbi.1009442.
35. Nearing JT, Douglas GM, Hayes MG, MacDonald J, Desai DK, Allward N, Jones CMA, Wright RJ, Dhanani AS, Comeau AM, Langille M. G. I. Microbiome Differential Abundance Methods Produce Different Results across 38 Datasets. Nat Commun. 2022;13(1):342. https://doi.org/10.1038/s41467-022-28034-z.
36. Hunter JD, Matplotlib. A 2D Graphics Environment. Comput Sci Eng. 2007;9(3):90–5. https://doi.org/10.1109/MCSE.2007.55.
37. Wickman H, Girlick M, Tidyr. Tidy Messy Data, 2022. https://tidyr.tidyverse.org, https://github.com/tidyverse/tidyr.
38. Harris CR, Millman KJ, Van Der Walt SJ, Gommers R, Virtanen P, Cournapeau D, Wieser E, Taylor J, Berg S, Smith NJ, Kern R, Picus M, Hoyer S, Van Kerkwijk MH, Brett M, Haldane A, Del Río JF, Wiebe M, Peterson P, Gérard-Marchant P, Sheppard K, Reddy T, Weckesser W, Abbasi H, Gohlke C, Oliphant TE. Array Programming with NumPy. Nature. 2020;585(7825):357–62. https://doi.org/10.1038/s41586-020-2649-2.
39. Virtanen P, Gommers R, Oliphant TE, Haberland M, Reddy T, Cournapeau D, Burovski E, Peterson P, Weckesser W, Bright J, Van Der Walt SJ, Brett M, Wilson J, Millman KJ, Mayorov N, Nelson ARJ, Jones E, Kern R, Larson E, Carey CJ, Polat İ, Feng Y, Moore EW, VanderPlas J, Laxalde D, Perktold J, Cimrman R, Henriksen I, Quintero EA, Harris CR, Archibald AM, Ribeiro AH, Pedregosa F, Van Mulbregt P, Vijaykumar A, Bardelli AP, Rothberg A, Hilboll A, Kloeckner A, Scopatz A, Lee A, Rokem A, Woods CN, Fulton C, Masson C, Häggström C, Fitzgerald C, Nicholson DA, Hagen DR, Pasechnik DV, Olivetti E, Martin E, Wieser E, Silva F, Lenders F, Wilhelm F, Young G, Price GA, Ingold G-L, Allen GE, Lee GR, Audren H, Probst I, Dietrich JP, Silterra J, Webber JT, Slavič J, Nothman J, Buchner J, Kulick J, Schönberger JL, De Miranda Cardoso JV, Reimer J, Harrington J, Rodríguez JLC, Nunez-Iglesias J, Kuczynski J, Tritz K, Thoma M, Newville M, Kümmerer M, Bolingbroke M, Tartre M, Pak M, Smith NJ, Nowaczyk N, Shebanov N, Pavlyk O, Brodtkorb PA, Lee P, McGibbon RT, Feldbauer R, Lewis S, Tygier S, Sievert S, Vigna S, Peterson S, More S, Pudlik T, Oshima T, Pingel TJ, Robitaille TP, Spura T, Jones TR, Cera T, Leslie T, Zito T, Krauss T, Upadhyay U, Halchenko. Nat Methods. 2020;17(3):261–72. https://doi.org/10.1038/s41592-019-0686-2. Y. O.; Vázquez-Baeza, Y. SciPy 1.0: Fundamental Algorithms for Scientific Computing in Python.
40. McKinney W. Data Structures for Statistical Computing in Python; Austin, Texas, 2010; pp 56–61. https://doi.org/10.25080/Majora-92bf1922-00a.
41. Cock PJA, Antao T, Chang JT, Chapman BA, Cox CJ, Dalke A, Friedberg I, Hamelryck T, Kauff F, Wilczynski B, De Hoon MJL. Biopython: Freely Available Python Tools for Computational Molecular

Biology and Bioinformatics. Bioinformatics. 2009;25(11):1422–3. https://doi.org/10.1093/bioinformatics/btp163.

42. Huerta-Cepas J, Serra F, Bork PETE. Reconstruction, Analysis, and Visualization of Phylogenomic Data. Mol Biol Evol. 2016;3(6):1635–8. https://doi.org/10.1093/molbev/msw046. *33*.
43. Gloor GB, Wu JR, Pawlowsky-Glahn V, Egozcue JJ. It's All Relative: Analyzing Microbiome Data as Compositions. Ann Epidemiol. 2016;26(5):322–9. https://doi.org/10.1016/j.annepidem.2016.03.003.
44. Cha S, Kim YS, Lee AL, Lee D-H, Koo N. Liming Alters the Soil Microbial Community and Extracellular Enzymatic Activities in Temperate Coniferous Forests. Forests. 2021;12(2):190. https://doi.org/10.3390/f12020190.
45. Cruz-Paredes C, Bang-Andreasen T, Christensen S, Ekelund F, Frøslev TG, Jacobsen CS, Johansen JL, Mortensen LH, Rønn R, Vestergård M, Kjøller R. Bacteria Respond Stronger Than Fungi Across a Steep Wood Ash-Driven pH Gradient. Front For Glob Change. 2021;4:781844. https://doi.org/10.3389/ffgc.2021.781844.
46. Hofer U. The Majority Is Uncultured. Nat Rev Microbiol. 2018;16(12):716–7. https://doi.org/10.1038/s41579-018-0097-x.
47. Douglas GM, Maffei VJ, Zaneveld JR, Yurgel SN, Brown JR, Taylor CM, Huttenhower C, Langille M. G. I. PICRUSt2 for Prediction of Metagenome Functions. Nat Biotechnol. 2020;38(6):685–8. https://doi.org/10.1038/s41587-020-0548-6.
48. Sridhar B, Lawrence GB, Debenport SJ, Fahey TJ, Buckley DH, Wilhelm RC, Goodale CL. Watershed-scale Liming Reveals the Short- and Long-term Effects of pH on the Forest Soil Microbiome and Carbon Cycling. Environ Microbiol. 2022;24(12):6184–99. https://doi.org/10.1111/1462-2920.16119.

Figures

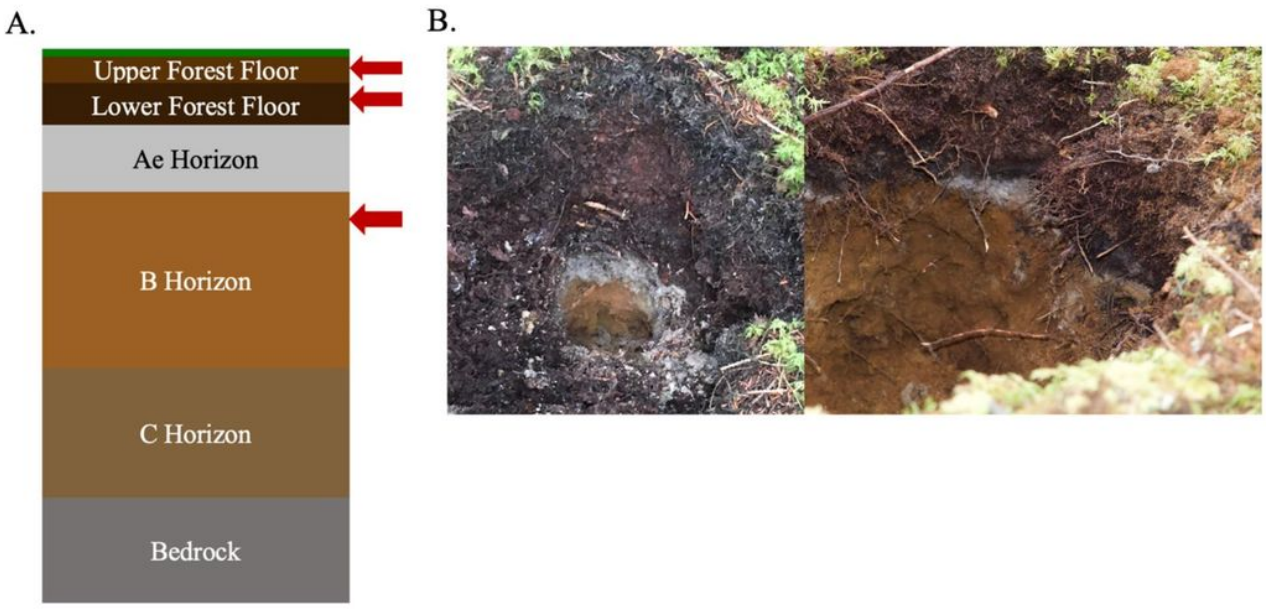

Figure 1

General soil horizon schematic with representative soil photo. (A) General schematic of typical soil horizons. Red arrows indicate the horizons sampled. (B) Representative soil pit showing forest floor and mineral soil horizons.

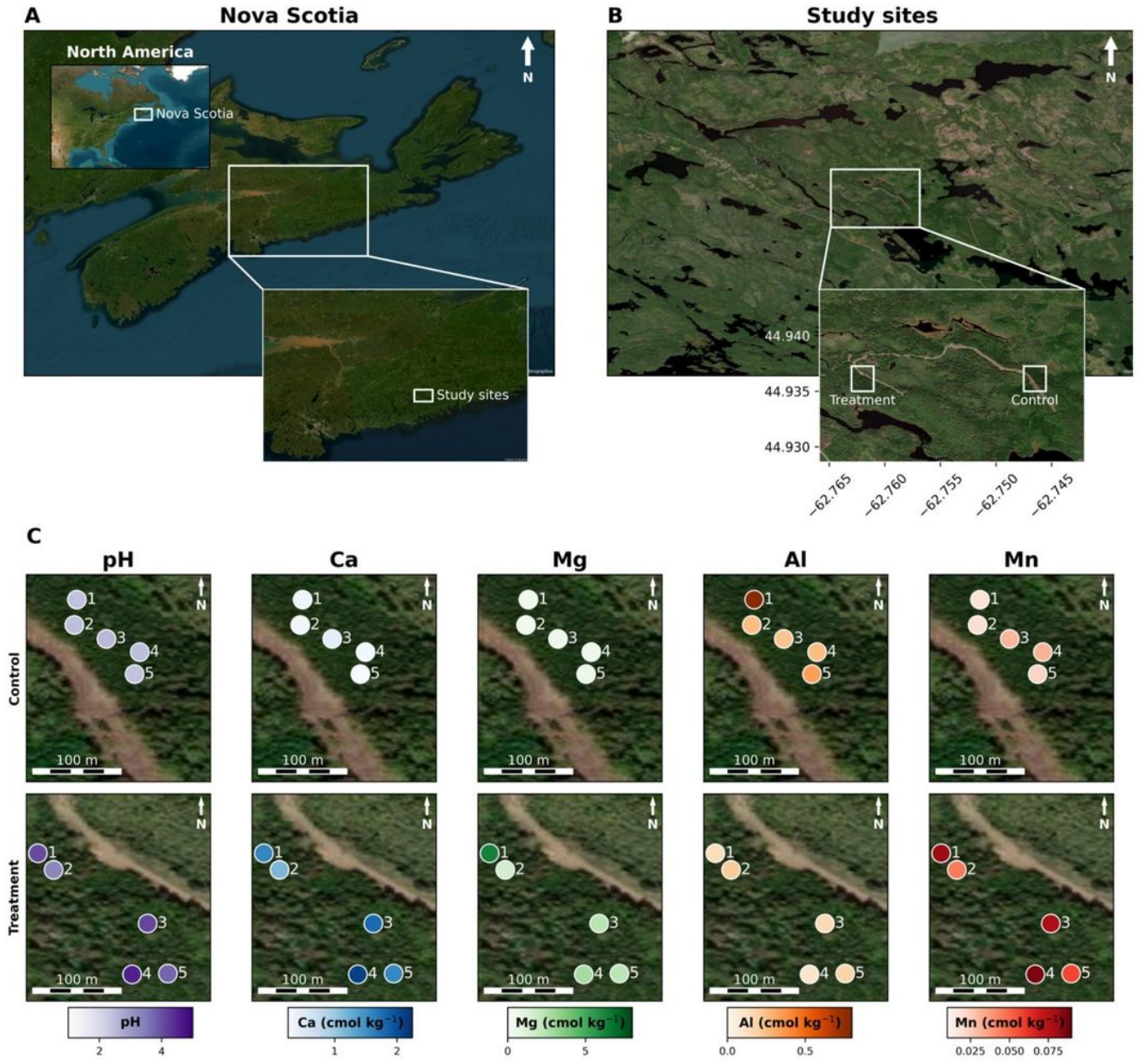

Figure 2

Map of sample sites with soil chemistry data. (**A**) Map showing the study sites within Nova Scotia. (**B**) Map showing the areas used for each of the control (C) and treatment (T) sites. (**C**) Maps of the ten sampling sites are shown in the two treatment and control panels. Each set of two panels shows soil chemistry data (pH, Ca^{2}, Mg^{2+}, Al^{3+} or Mn) for the upper forest floor for five control (top) and five treatment (bottom) sites. Points marking each site are coloured by mean soil chemistry values (see Supp. Table 1 for details). Each control panel shows longitudes -62.7475 to -62.745 (x-axis; left to right) and latitudes 44.9375 to 44.9345 (y-axis; top to bottom). Each treatment panel shows longitudes -62.7630 to -62.7605 (x-axis; left to right) and latitudes 44.9375 to 44.9345 (y-axis; top to bottom).

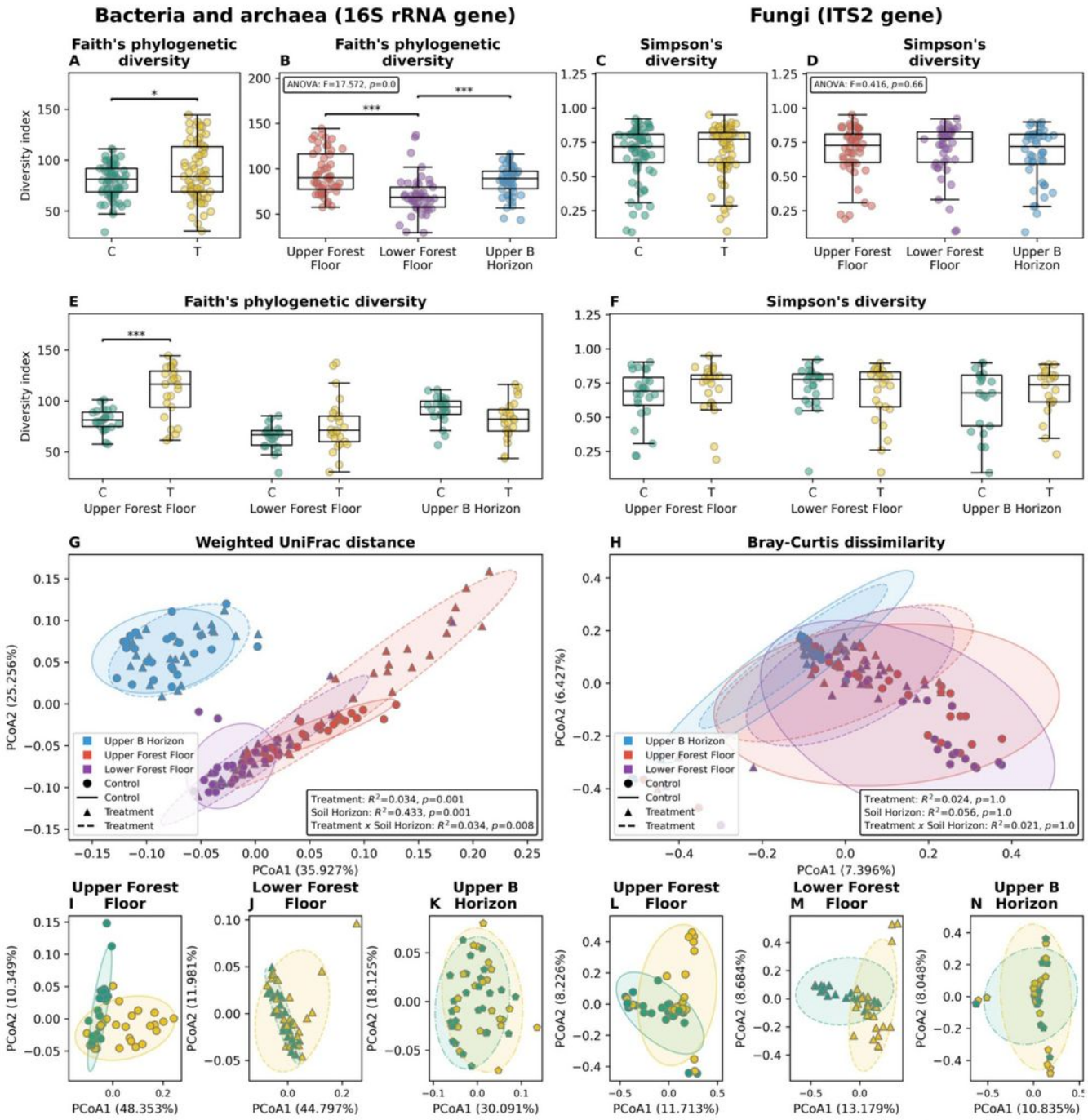

Figure 3

A picture containing text, diagram Description automatically generated

Figure 3: Alpha and beta diversity of microbial community samples. Results for the 16S rRNA gene community (bacteria and archaea) are shown on the left and for the ITS2 gene community (fungi) are shown on the right. (A)-(F) shows alpha diversity within samples grouped by: (A) and (C) control, C, or treatment, T; (B) and (D) by soil horizon; and (E) and (F) by control and treatment within each soil horizon. In each of these plots, each sample is shown as an individual point and boxes show the median, upper and lower quartiles while whiskers show the range of the data (1.5 times the interquartile range).

Significant differences between treatments (ANOVA) are denoted by asterisks, with * denoting that p £ 0.05, ** that p £ 0.01 and *** that p £ 0.005. (G)-(N) Principal Coordinates Analysis (PCoA) and PERMANOVA tests with beta diversity for: (G) and (H) all samples grouped by control and treatment within each soil horizon; (I) and (L) control and treatment samples within the upper forest floor soil horizon; (J) and (M) control and treatment samples within the lower forest floor soil horizon; and (K) and (N) control and treatment samples within the upper B horizon. In each plot, each sample is shown as an individual point and ellipses show the confidence interval (2 standard deviations) for each group. The values shown on each axis label indicate the proportion of sample variation accounted for by that axis. Values shown in the boxes in (G) and (H) are for PERMANOVA R^2 and p-values for treatment, soil horizon and the interaction between treatment and soil horizon. Full details of statistical tests are in the methods section. For the 16S rRNA gene community, a phylogenetic tree was available for insertion of the ASV sequences and we therefore use diversity metrics that take phylogeny into account (Faith's phylogenetic diversity for alpha diversity and Weighted UniFrac distance for beta diversity). We were unable to do the same for the ITS2 gene and therefore use diversity metrics that do not account for phylogeny (Simpson's diversity for alpha diversity and Bray-Curtis dissimilarity for beta diversity).

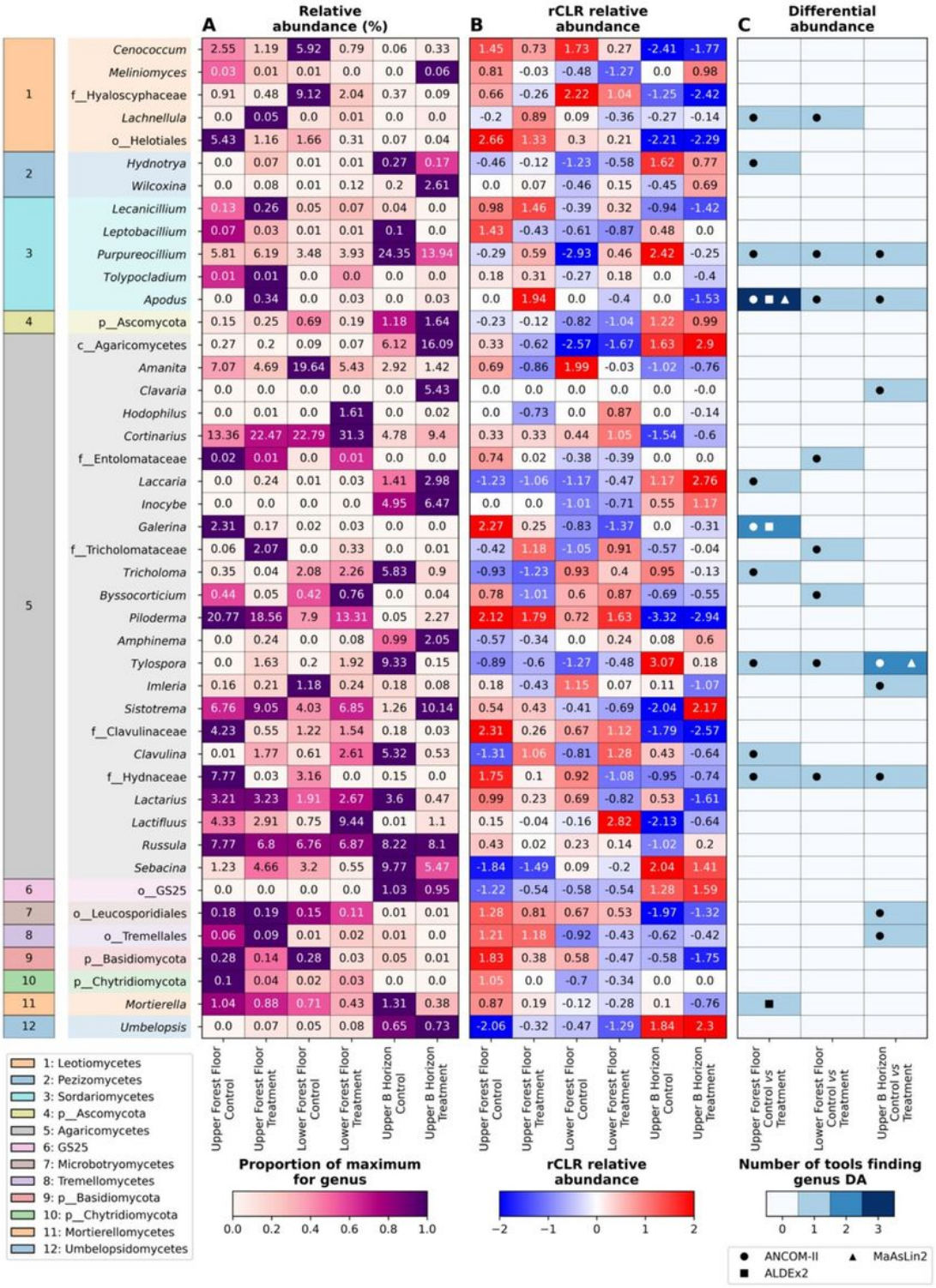

Figure 4

Heatmap of fungal (ITS2) abundance and differential abundance. Abundance and differential abundance of the top 25 most abundant ITS2 gene fungal genera for each of relative and rCLR abundance (44 total genera). Genera (shown on the left) are coloured by the class that they belong to (or the lowest taxonomic level classified). Heatmaps show the relative (A) or rCLR (B) abundance of the top genera within each horizon + treatment group. Values shown in the cells indicate the mean value for

each soil horizon + treatment group. (C) shows which of the differential abundance tests (ANCOM-II, MaAsLin2 or ALDEx2) found a genus to be differentially abundant between control and treatment within each horizon. The boxes are colored by the number of tests that found it differentially abundant and the markers indicate which of the differential abundance tests identified this genus.

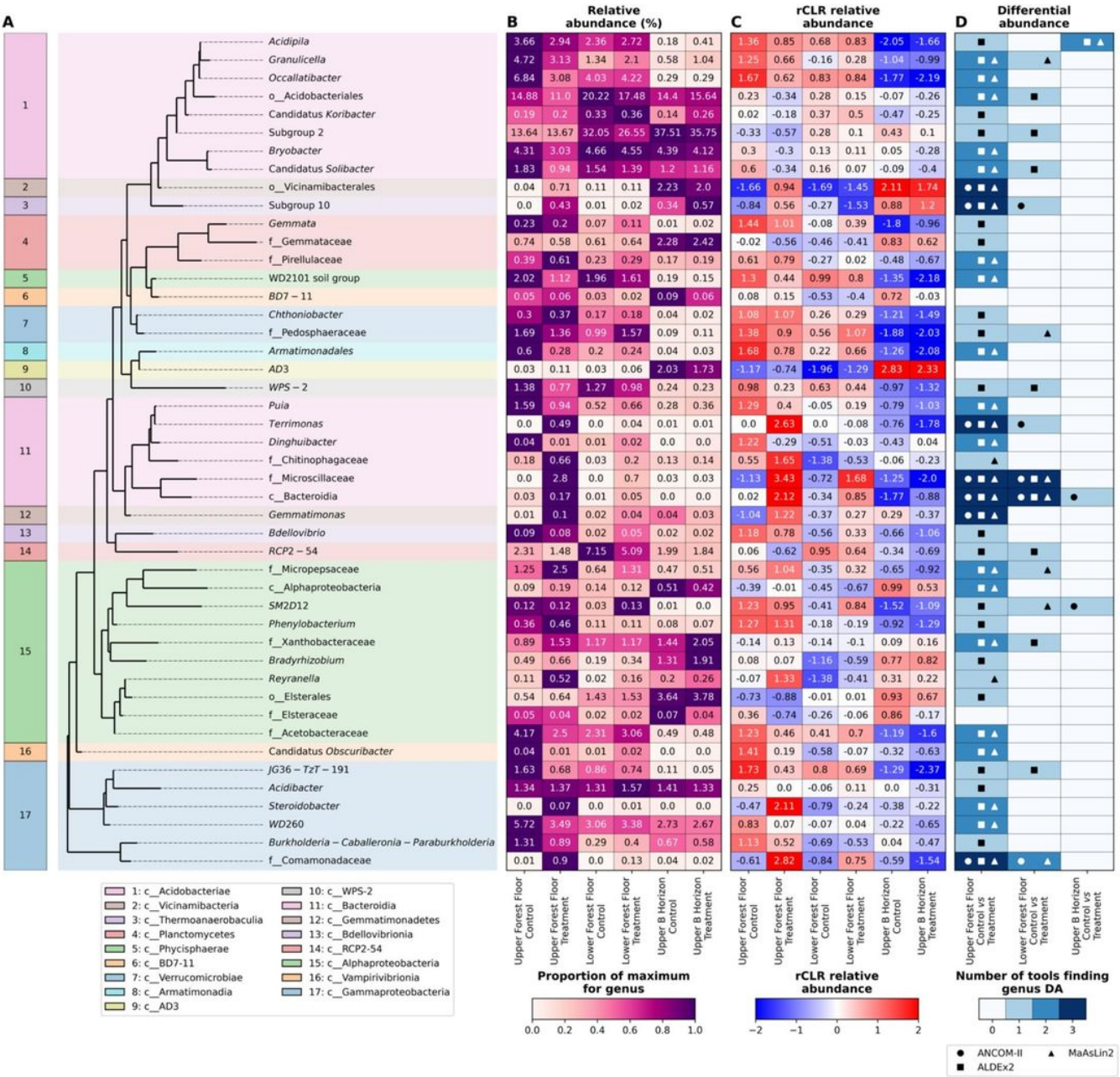

Figure 5

Heatmap of bacterial/archaeal (16S rRNA gene) abundance and differential abundance. Abundance and differential abundance of the top 25 most abundant 16S rRNA gene bacterial/archaeal genera for each of relative and rCLR abundance (44 total genera). (A) phylogenetic tree where genera are coloured by the class that they belong to (or the lowest taxonomic level classified). Heatmaps show the relative (B) or rCLR (C) abundance of the top genera within each horizon + treatment group. Values shown in

the cells indicate the mean value for each soil horizon + treatment group. (D) shows which of the differential abundance tests (ANCOM-II, MaAsLin2 or ALDEx2) found a genus to be differentially abundant between control and treatment within each horizon. The boxes are colored by the number of tests that found it differentially abundant and the markers indicate which of the differential abundance tests identified this genus.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- HosmerWrightSuppFigures.pdf
- SupplementaryTable1.xlsx
- SupplementalTables23.xlsx
- SupplementalTable4.xlsx