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Microbial community response to growing season and plant nutrient optimisation in a boreal Norway spruce forest

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ABSTRACT

Interactions between Norway spruce trees and bacteria and fungi in nutrient limited boreal forests can be beneficial for tree growth and fitness. Tree-level effects of anthropogenic nutrient addition have been well studied, however understanding of the long-term effects on the associated microbiota is limited. Here, we report on the sensitivity of microbial community composition to the growing season and nutrient additions. High-throughput sequencing of the bacterial 16S rRNA gene and fungal ITS1 region was used to characterise changes in the microbial community after application of a complete mineral nutrient mixture for five and 25 years. The experiment was conducted using the Flakaliden forest research site in northern boreal Sweden and included naturally low nutrient control plots. Needle and fine root samples of Norway spruce were sampled in addition to bulk soil during one growing season to provide comprehensive insight into phyllosphere and belowground microbiota community changes. The phyllosphere microbiota was compositionally distinct from the belowground communities and phyllosphere diversity increased significantly over the growing season but was not influenced by the improved nutrient status of the trees. In both root and soil samples, alpha diversity of fungal, in particular ectomycorrhizal fungi (EMF), and bacterial communities increased after long-term nutrient optimisation, and with increasing years of treatment the composition of the fungal and bacterial communities changed toward a community with a higher relative abundance of nitrophilic EMF and bacterial species but did not cause complete loss of nitrophobic species from the ecosystem. From this, we conclude that 25 years of continuous nutrient addition to a boreal spruce stand increased phylotype richness and diversity of the microbiota in the soil, and at the root-soil interface, suggesting that long-term anthropogenic nutrient inputs can have positive effects on belowground biodiversity that may enhance ecosystem robustness. Future studies are needed to assess the impact of these changes to the microbiota on ecosystem carbon storage and nitrogen cycling in boreal forests.

1. Introduction

One third of the world's forests are found in the boreal region (FAO, 2010) where N supply is a primary factor limiting tree growth (Tamm, 1991). Conifers dominate these forests and host diverse fungal and prokaryotic communities, forming a meta-community with complex symbiotic interactions that together form what is termed a holobiont individual (Pillai et al., 2014; Vandenkoornhuyse et al., 2015). Plant associated microbiota contribute to key processes including nutrient uptake by plants and can improve plant growth, fitness and ecosystem productivity (Horton et al., 2014). However, beyond the well-known mycorrhizal associations, little is known about the diversity or function of the communities of bacteria and fungi associated with conifer needles or the considerable diversity of other soil and root-associated microbes. High latitude forests, including the coniferous boreal forests, are key players in the global carbon cycle and are expected to experience the most rapid and extreme increases in temperature due to climate change (IPCC, 2013). Studies of the resiliency of these high latitude forest holobionts to perturbations caused by climate change and anthropogenic N-input are therefore needed to establish whether they will

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continue to serve as carbon sinks in the future.

In boreal forest soils the majority of N is bound in organic forms, which becomes accessible for plant uptake only after decomposition of the organic matter, processes involving both fungi and bacteria (Robinson, 2002; Read and Perez-Moreno, 2003; Schimel and Bennett, 2004; Högberg et al., 2006). More than 95% of the fine roots of conifer trees in boreal forests are colonised by ectomycorrhizal fungi (EMF) that are thought to facilitate tree nutrient uptake (Taylor et al., 2000). Mycorrhizal fungal communities are therefore important drivers of carbon and nutrient cycling at forest and ecosystem scales (Read et al., 2004). Various studies of boreal and other ecosystems have focused on how EMF communities are affected by soil edaphic and climatic factors (Gehring et al., 2006; Kranabetter et al., 2009), forest succession (Nara, 2006; Twieg et al., 2007), host diversity (DeBellis et al., 2006) or litter chemistry (Conn and Dighton, 2000; Dighton et al., 2000). These studies have shown that while EMF species differ in their distribution according to site and/or host characteristics, a species-rich EMF community is typically present on root-tips (Horton and Bruns, 2001) whereas less is known about the composition in soil (Gardes and Bruns, 1996). In addition to enhanced nutrient uptake provided via the EMF communities, boreal forests also likely benefit from a combination of additional functions served by a diverse microbial metacommunity, which can enhance resiliency to environmental stresses and disturbances (Kranabetter, 2004; Swaty et al., 2004).

The role(s) of bacteria in coniferous and boreal forest soil processes has received less attention but it has been shown that soil communities are dominated by *Actinobacteria*, *Proteobacteria* and *Acidobacteria* and in lower abundances by *Bacteroidetes*, *Gemmatimonadetes*, *Firmicutes*, *Verrucomicrobia* and *Planctomycetes* (Hartmann et al., 2009, 2012; Yarwood et al., 2009; Baldrian et al., 2012; Sun et al., 2014). Studies in herbaceous plants, such as *Arabidopsis thaliana* have indicated that bacteria found in association with plant roots can be characteristic of the root, as they represent a specific subset of bacteria found in soils and are essential for growth and survival of the plants in a given habitat (Bulgarelli et al., 2012; Lundberg et al., 2012). Furthermore, for mycorrhizal plants the composition and structure of the bacterial communities differ between bulk soil, ectomycorrhizal root tips and non-mycorrhizal root habitats (Burke et al., 2008; Vik et al., 2013; Nguyen and Bruns, 2015). Thus not only mycorrhiza, but also soil bacterial communities, interact with plant roots and in addition the mycorrhizosphere offers micro-niches for adapted bacteria (Heinonsalo et al., 2001; Warmink et al., 2009).

For the dominant conifers of boreal forests, stand productivity and foliar N concentrations are positively correlated with increasing N availability in N addition studies (Seith et al., 1996; Flückiger and Braun, 1998; Kranabetter et al., 2007; Kranabetter and Simard, 2008). With alleviated N-limitation in the soil and improved N status of the trees, the belowground allocation of C to roots and to symbiotic mycorrhizal fungi decreases (Wallenda and Kottke, 1998). Early observations reported reduced sporocarp production with increasing N-availability in boreal forests (Wallenda and Kottke, 1998; Lilleskov et al., 2001). However, more comprehensive studies showed that increasing soil N availability had less pronounced effects on belowground root-tip colonisation (Arnebrant and Söderström, 1992; Kårén and Nylund, 1997; Jonsson et al., 2000; Taylor et al., 2000), ectomycorrhizal mycelia growth (Nilsson and Wallander, 2003; Nilsson et al., 2005) and species richness (Kranabetter et al., 2009; Högberg et al., 2014) than implied by sporocarp production (Gardes and Bruns, 1996; Jonsson et al., 2000; Dahlberg, 2001). Nevertheless, changes in fungal community composition are commonly observed both above- and belowground, with increasing N-availability (Fransson et al., 2000; Lilleskov et al., 2002; Avis et al., 2003; Cox et al., 2010; Kjøller et al., 2012). EMF differ in their N tolerance, and observations in N-addition experiments have frequently reported that taxa such as *Lactarius*, *Laccaria* and *Paxillus* increase in abundance, referring to them as “nitrophilic”, while “nitrophobic” taxa such as *Cortinarius*, *Suillus*, *Tricholoma* and *Piloderma* decrease in abundance with increasing N availability (Fransson et al., 2000; Lilleskov et al., 2001). However, knowledge of whether EMF communities in boreal forests respond directly to changes in soil N concentrations and pH levels or rather to changes in the tree N status and/or tree C-allocation remains limited, as does understanding of how they respond to nutrients other than N or P. Modern sequencing techniques may provide new insights and overcome reported difficulties in detecting EMF from bulk soil in comparison to colonised root-tips (Högberg et al., 2014), as they allow for higher resolution in semi-quantitative studies of belowground fungal communities (Lindahl et al., 2013; Nguyen and Bruns, 2015).

The response of bacterial communities to nutrient additions has been studied for agricultural, grassland and arctic tundra soils (Campbell et al., 2010; Leff et al., 2015) or with a focus on nitrifying bacteria in boreal forests (Long et al., 2012). While the effects of N-addition on the diversity of bacterial communities are not always clear, including negative or no effects, compositional changes are the norm (Campbell et al., 2010; Ramirez et al., 2010; Koyama et al., 2014; Leff et al., 2015). Bacteria thriving in nutrient rich conditions, including *Actinobacteria*, *Bacteroidetes*, *Alpha-*, *Beta-* and *Gammaproteobacteria* increase in relative abundance, while oligotrophic groups, such as *Acidobacteria*, decrease with higher N availability (Campbell et al., 2010; Ramirez et al., 2010; Leff et al., 2015). N-addition changes the C input quantity and/or quality of the plant roots and either N alone or both N and C changes in combination might be the cause for these compositional shifts in belowground bacterial communities (Ramirez et al., 2010).

Given the manifold interactions of fungi, bacteria and Norway spruce trees in boreal ecosystems, the study of microbial responses to changing environmental conditions, such as long-term changes in nutrient availability, provides valuable knowledge regarding the plasticity of these interactions. Here, we provide a comprehensive description of the microbiota associated with needles and roots of Norway spruce trees from a nutrient limited boreal forest using high-throughput sequencing of fungal and bacterial phylogenetic markers. In contrast to previous studies that focused primarily on the impact of high doses of N or P only or a combination of these two (Nilsson and Wallander, 2003; Treseder, 2004; Treseder et al., 2007; Leff et al., 2015), here we report the effects of a fully optimised macro- and micronutrient mixture on the diversity and composition of the fungal and bacterial communities after five and 25 years of continuous nutrient enrichment. We focus on ectomycorrhizal fungi, which have important functions in soil organic matter decomposition and nutrient mobilisation for tree growth. In addition, we evaluate the effects of tree nutrient status and growing season on microbiota of the phyllosphere, the habitat defined as the surface and internal tissue of the living leaf (Osono, 2006).

2. Materials and methods

2.1. Study site and sampling

Samples were collected at the Flakaliden research site (64°07′N, 19°27′E, altitude 310–320 m) within the Vindeln experimental forest in northern boreal Sweden from a Norway spruce (*Picea abies* L. (Karst.)) stand planted in 1963 (Linder, 1995). The site has previously been characterised as highly N-limited, with poor N-mineralisation rates due to low annual mean temperatures of 1.2 °C and low atmospheric deposition of $\sim 3\ kg\ ha^{-1}$ (Lindberg and Persson, 2004; Demoling et al., 2008). In addition, the ground can be frozen and covered by snow from mid-October to early May, with at least one-third of the mean annual precipitation of 600 mm falling as snow. The short and cold growing season results in low stand productivity of $3.2\ m^2\ ha^{-1}\ yr^{-1}$ (Demoling et al., 2008). In 1987 a long-term experiment to explore the potential yield of Norway spruce under these climatic conditions was started by application of a balanced complete nutrient solution to optimise tree growth (Linder and Flower-Ellis, 1992; Linder, 1995; Bergh et al.,

1999). Addition of macro- and micronutrients, including N, P, K, Mg, Ca, S, Fe, B, Mn, Cu and Zn followed defined target values for an optimal nutrition and was revised based on analysis of the foliar nutrient status every year (Linder, 1995). Plot size was 50 m x 50 and fertiliser was added with irrigation every second day between June and mid-August. In 2007 a second treatment was initiated with liquid fertiliser added to previously irrigated control plots, after it was shown that there was no influence of irrigation on tree growth of control plots at this site (Bergh et al., 1999). Each treatment (long-term 25 years nutrient optimisation, five years nutrient optimisation and non-irrigated control) was replicated with three plots in a randomised block design (Supplementary file 1). N content in the foliage changed markedly after addition of ammonium nitrate amounting to 75–100 kg ha^{-1} $year^{-1}$ in the nutrient mixture (Linder, 1995). All other nutrients were supplied in a fixed proportion to N. On average ~15 kg ha^{-1} P, 42 kg ha^{-1} K, 7 kg ha^{-1} Mg, 10 kg ha^{-1} Ca and 5 kg ha^{-1} S were added annually. After 25 years, this treatment resulted in a significant increase in N, P, S and B concentrations and a decrease in Ca and Zn in the foliage in comparison to untreated stands (Supplementary file 2). Furthermore, only Ca:N and Zn:N decreased significantly, with the abundance of all other elements occurring without significant changes in their proportions relative to N.

For the current study, samples were collected in 2012, representing five or 25 years of nutrient optimisation and from control plots, from the organic F + H layer of the podozolic sandy glacial till, from fine roots (≤1 mm) and one-year-old needles of Norway spruce. Samples were collected four times during the growing season. At the beginning on June 5th during bud break and shoot elongation, in early summer June 24th when starch accumulation and carbohydrate content is at a maximum in the needles, in late summer August 6th when carbohydrate demand is high and starch reserves decline and at the end of the growing season on October 9th when needles show a minimum of carbohydrate content (Linder, 1995). For the soil sampling a metal corer of 10 cm diameter was used and three samples were taken within each plot in order to account for within-plot heterogeneity, but were merged after sequence analysis (see below). The sandy layer and plant material were removed and samples sieved (4 mm) and stored at −80 °C. Fine roots of the mature Norway spruce trees were harvested within the upper 25 cm of the soil and washed with distilled water. Needles of the previous growing season (2011) and roots were sampled in triplicates for each plot and stored at −80 °C. Altogether, for each sample type (needles, roots and soil) 108 samples (3 treatments x 3 replicate plots x 3 samples in each replicate plot x 4 seasonal sampling points) were processed in this experiment.

2.2. Microbial DNA extraction and sequencing

Genomic DNA from soil samples was extracted following the procedure described in Griffiths et al. (2000) and modified after DeAngelis et al. (2010) starting with 0.5 g of frozen soil. DNA from root- and needle-associated microbiota was extracted from 75 mg of tissue using a method summarised in Hanania et al. (2004) with natriumdiethyldithiocarbamat trihydrate (DETC) in the extraction buffer instead of sodium bisulfite and a final DNase and protease-free RNase A (ThermoFisher Scientific, Waltham, MA, USA) treatment. Purity and concentration of nucleotides was assessed using a NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific). DNA was diluted and used for PCR amplification in concentrations ≤50 ng/μl. The amplification protocol was adapted from the Earth Microbiome Projects PCR protocol (http://www.earthmicrobiome.org/emp-standard-protocols/16s/), as described in Caporaso et al. (2011, 2012), for a dual indexing approach as in Kozich et al. (2013). PCR amplification of the 16S V4 region of the bacterial 16S rRNA gene was performed for each sample in triplicate using the 515F/806R primers both containing 12-bp barcodes to allow for dual indexing of the samples. Similarly, primers ITS1-F/ITS2 (White et al., 1990) of the internal transcribed spacer (ITS) region were used for amplification of the fungal ITS1 region. The PCR reaction included 12.5 μl GoTaq® hot start green master mix (Promega, Madison, WI, USA) and 0.5 μg/μl bovine serum albumin (BSA) (Thermo Fisher Scientific) and RT-PCR grade water (ThermoFisher Scientific) for a 25 μl PCR reaction. For bacteria the use of peptide nucleic acids (PNAs) was necessary to arrest elongation of plastid 16S and mitochondrial 18S ribosomal sequences. Anti-mitochondrial PNA (mPNA) and anti-plastid PNA (pPNA) sequences for use with *Arabidopsis thaliana* were described by Lundberg et al. (2013) and ordered from PNA Bio (Newbury Park, CA, USA). For a successful elongation arrest of mitochondrial ribosomal 18S sequences originating from Norway spruce during the PCR reaction a degenerate nucleotide 5′-GGCAAGTGTTMTTCGGA-3′ was implemented in the original mPNA sequence. PNA PCR end concentrations for root samples was 0.2 μM and 0.6 μM with needle samples. Activation of the PNA clamps took place at 78 °C for 10 s before primer annealing during the PCR reaction cycles. Triplicate reactions were pooled and quantified with a Qubit® 2.0 Fluorometer (model 2.0; Invitrogen, Carlsbad, CA, USA) and 50 ng of each sample was combined in one amplicon pool per sample type. AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) were used for cleaning and once again concentrations were measured. Libraries were sequenced on an Illumina MiSeq using the 300 (16S soil) or 500 cycle MiSeq Reagent kit v2 (Illumina, San Diego, CA, USA). For the phyllosphere bacteria study two sequencing runs were performed using the same amplicon pool. A fungal mock community, consisting of equimolar genomic DNA, was constructed from DNA extracted from locally collected sporocarps of 17 fungal species in the *Ascomycota* and *Basidiomycota* (Supplementary file 3). A bacterial mock community representing 20 bacterial strains and containing staggered ribosomal RNA was obtained from BEI Resources (NIAID, NIH as part of the Human Microbiome Project: Genomic DNA from Microbial Mock Community B, v5.2L, HM-783D). Mock communities served as positive controls and were treated the same as the experimental samples and negative water controls in the amplification and sequencing process.

2.3. Sequence analysis

Illumina data were processed using QIIME version 1.9.0 (Caporaso et al., 2010b). Initially, each mapping file was checked with validate_mapping_file.py before adjusting them with a custom Python script ('fix_mappingfile.py' available at: https://github.com/druvus/16S-demultiplexing) for use in demultiplexing the sequence reads by sample index. The raw sequences were quality filtered using the default settings of the split_libraries_fastq.py command. Cutadapt's "-a" option was used to remove primer sequences at the 3′ end of the reads (Version 1.4.1) (Martin, 2011). Paired-end sequences of bacteria were merged using FLASH (Version 1.2.9) (Magoč and Salzberg, 2011) with the settings "–m 20 –M 100 –r 251 –f 253". Analysis of fungi was based on the forward directional sequences only as described in Nguyen et al. (2015). For sample assignment of the reads the split_libraries_fastq.py and another custom Python script split_fastq.py ('split_fastq.py' available at: https://github.com/druvus/16S-demultiplexing) were used. The software ITSx (Version 1.0) (Bengtsson-Palme et al., 2013) was run on the fungal reads with the settings "-t f –preserve T -E 1 -allow_single_domain 1e-5,0" to identify and retain only sequences of the fungal lineage by comparison to HMM-profiles of the HMMER3 software package (Eddy, 2011) incorporated in the ITSx software and to separate the ITS1 subregion from the highly conserved SSU. VSEARCH (Version 1.10.2) (Rognes et al., 2016) was used with default settings to dereplicate the sequences, sort by decreasing abundance in addition to singleton removal "–minsize 2", before *de novo* clustering at ≥ 95% (ITS1), following suggestions in Schmidt et al. (2013), and ≥97% (16S) sequence similarity levels using the "–cluster_size" command. *De novo* chimera detection was performed with the UCHIME algorithm (Edgar et al., 2011) as implemented in VSEARCH, before mapping the raw demultiplexed sequences against the *de novo* created ITS1 and 16S

databases at 95% or 97% sequence identity, respectively, and generation of count tables using a custom Python script ('create_otu_table_from_uc_file.py' available at: https://github.com/leffj/helper-code-for-uparse). Taxonomy was assigned for bacteria using the RDP classifier (Version 2.2) (Cole et al., 2009) or for fungi using the BLAST (Version 2.2.26) (Altschul et al., 1990) implementation of QIIME referenced against the SILVA (Version 119) (Quast et al., 2013) database at 97% similarity or the UNITE (Version, 2016_01_31) (Kõljalg et al., 2005) database at a dynamic level, respectively. Clusters, referred to as operational taxonomic units (OTUs), classified as chloroplast, mitochondria, archaea or unclassified reads were removed from the bacteria dataset before further analysis. The FastTree (Price et al., 2010) algorithm in QIIME was used to build a bacterial phylogeny of the representative sequence after aligning at 60% identity using the PyNAST (Caporaso et al., 2010a) algorithm against the SILVA database. Protist and unclassified sequences were filtered from the fungal dataset. For this study any OTU having ten or fewer sequences was removed from a sample. In addition, a minimum abundance of 0.005% in any of the three sample types was required. This approach was effective in removing low abundance OTUs that represented noise and resulted in 22 OTUs for the fungal mock community (Supplementary file 4) and 54 OTUs for the bacteria mock community (Supplementary file 5). The sequences of the replicates within a plot were combined for downstream analysis. EMF were identified according to Glassman et al. (2015). The raw data has been submitted to the European Nucleotide Archive (ENA, www.ebi.ac.uk/ena) under the accession number PRJEB21692. FASTA file information and corresponding mapping files for demultiplexing are available as supplementary information (Supplementary files 6–12). A guide for the preprocessing steps is available at https://github.com/juliaha/Flakaliden_2012.

2.4. Ecological and statistical analyses

Observed OTU counts were used to estimate phylotype richness for each of the biological replicates after rarefaction of the samples to 39,000 reads for ITS1 or 16,500 reads for 16S, which represented the minimum read counts in a single sample for ITS1 or 16S samples, respectively. Rarefaction plots were examined using the three samples with the lowest and highest read counts for each sample type (soil, roots, needles) of ITS1 or 16S, respectively (Supplementary file 13). The Shannon diversity index was used to calculate the microbial alpha diversity on log-transformed normally distributed non-rarefied OTU count values. The "lme" function of the nlme package (Pinheiro et al., 2016) was used in R (R Development Core Team, 2016) (Version 3.3.1) to build linear mixed effect models testing for significance in diversity between the three sample types of control plots using the log-transformed Shannon diversity index values (Diversity ~ SampleType). Additionally, we tested for differences of nutrient optimisation after five or 25 years within each sample type (Diversity ~ Treatment). Furthermore, the effect of the sampling time point within each treatment and sample type was investigated (Diversity ~ Date). Sampling plot was always treated as a random variable and Tukey honestly significant differences (HSDs) were assigned using the "glht" function in the R package multcomp (Hothorn et al., 2008) when significant differences were detected ($\alpha = 0.05$).

Compositional differences between communities were investigated using the Bray-Curtis beta diversity index, for computing dissimilarity matrices after variance stabilising transformation of the data using the package DESeq2 (Love et al., 2014) and visualised with principal coordinate analysis (PCoA) as implemented in the phyloseq package (McMurdie and Holmes, 2013) in R. Shifts in overall community composition of the belowground samples were tested across sample type, nutrient optimisation and season after performing constrained ordination with the "capscale" function and using permutational multivariate ANOVA (PERMANOVA) as implemented in the vegan package (Dixon, 2003) in R. This was also performed for the needle data set to test for seasonal and treatment effects. Nonsignificant terms or interactions were removed from the final PERMANOVA model. To test for significant differences in the relative abundance of specific OTUs of the fungal data set or at genus level for the bacterial data set between soil and root control samples, between control and nutrient treatment plots or for different seasons in the needle samples, the raw count tables were used to estimate size factors and dispersion before fitting a generalised linear model using DESeq2 in R. Results of pairwise comparisons were included in heatmap visualisations with a Log2 fold change > 2 and an adjusted p value < 0.01. Description of an exemplary analysis in R and the OTU tables used are provided (Supplementary files 14–18).

2.5. Nutrient analysis and soil properties

Total C and N contents of needle, root and soil samples were determined after Werner et al. (1999) using an Elemental Analyser (Flash EA, 2000; Thermo Fisher Scientific, Bremen, Germany) together with an Isotope Ratio Mass Spectrometer (DeltaV, Thermo Fisher Scientific, Bremen, Germany). ICP-AES (Inductively Coupled Plasma-Atomic Emission) analysis of nutrient concentrations (P, K, Ca, Mg, Mn, S, Fe, Cu, Zn, B) per unit mass of needle and soil samples taken in October 2012 were performed with an Avio ICP 200 Optical Emission Spectrometer (PerkinElmer, MA, USA) after wet digestion of 1g dry material in 65% nitric acid, as described in Hellsten et al. (2013). Soil pH was measured in a 1:5 (v:v) dilution in water, as described in Demoling et al. (2008).

3. Results

3.1. Norway spruce needles, roots and boreal forest soil host distinct fungal and bacterial communities

Soil and root samples from the control plots contained 176 common OTUs, representing 59% and 71% of all fungal OTUs found in soil and root samples, respectively (Fig. 1a). Both sample types were dominated by the genera *Piloderma* and *Cortinarius* (> 5%) and by *Archaeorhizomyces* and *Hygrophorus* in lower abundance (Supplementary file 19). *Piloderma* alone accounted for 54 ± 5% and 46 ± 4% (Mean ± standard error) of all fungal ITS reads in the control soil and root samples, respectively. Despite the overall similarities of the belowground communities, there were significant compositional differences between control soil and root samples ($P < 0.001$). OTUs classified as *Venturiaceae* sp, *Cortinarius vibratilis* and *Meliniomyces variabilis* were notably more abundant in root samples, while *Archaeorhizomyces* sp and taxa of the genera *Tylospora* and *Pseudotomentella* were more abundant in the soil. (Supplementary file 20).

The needle associated fungal communities on the control plots were distinct from the belowground fungal communities. Total fungal phylotype (i.e. OTUs) richness was highest in the needle samples and the five most abundant genera were of the phylum *Ascomycota* (Fig. 1a and Supplementary file 19). Alpha diversity was about 36% higher in phyllospheric communities than in root control communities ($P < 0.001$, Fig. 1c) and only the diversity of fungal communities associated with the needles was strongly influenced by the growing season ($P < 0.05$).

The bacterial phylotype richness in the soil samples of the control plots comprised 1503 OTUs, by far the highest of all the sample types studied, and included 77% of the root-associated bacterial taxa (Fig. 1b). In comparison to root and needle samples, soil samples from control plots were also the most diverse, as indicated by alpha diversity (Fig. 1d). The majority of the belowground bacterial taxa were of the phyla *Proteobacteria*, *Acidobacteria* and *Actinobacteria*. In particular, bacteria of the order *Xanthomonadales* within the *Proteobacteria* were highly abundant in belowground samples (Supplementary file 21). Despite great similarities in community structure, composition of the soil and root bacterial communities was distinct ($P < 0.001$),

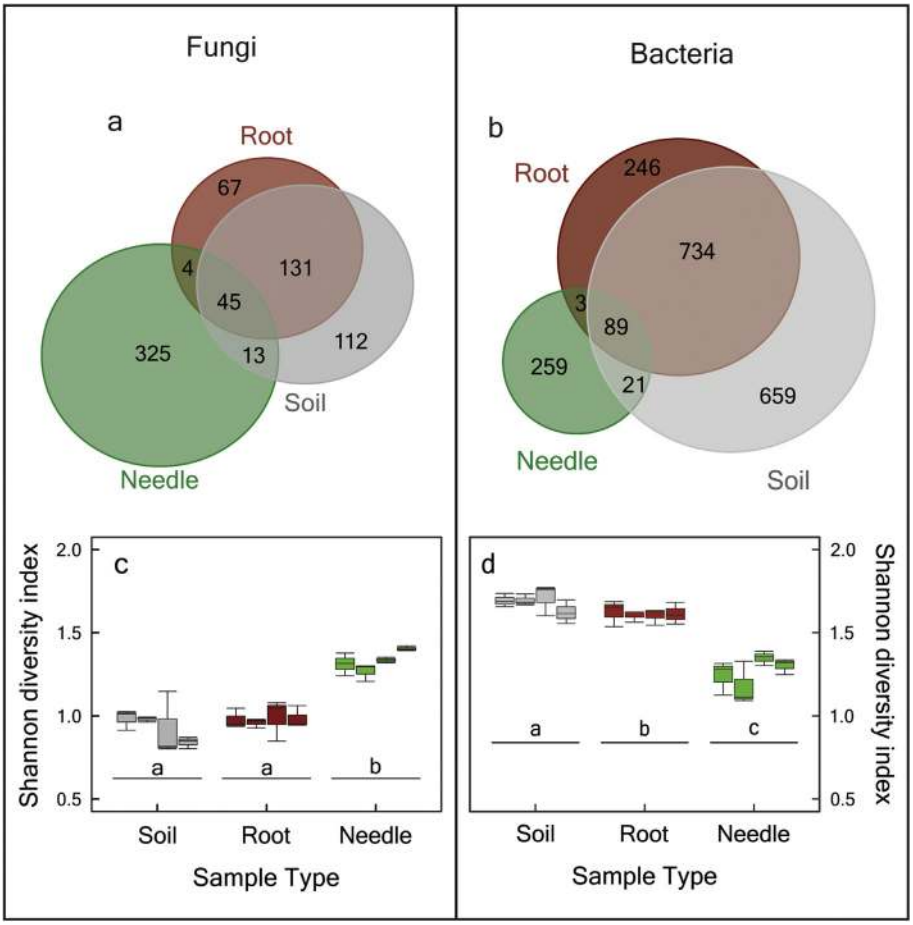


Fig. 1. Fungal and bacterial community diversity of the control treatment.
Venn diagrams comparing fungal (a) and bacterial (b) operational taxonomic units (OTUs) in the forest soil (grey) relative to fungal OTUs in the root (brown) and needle (green) samples of the control plots. OTUs with 10 or less counts and a minimal abundance of 0.005% per sample type were removed and the biological replicates were rarefied to 39.000 or 16.500 sequences, respectively. Boxplots of median fungal (c) and bacterial (d) alpha diversity, using the Shannon diversity index of operational taxonomic units (OTUs), coloured by sample type soil (grey), root (brown) and needle (green) and split by season within each sample type (from left to right: early June, late June, August and October). Shannon diversity index represents log-transformed values. Lowercase letters represent Tukey honestly significant differences (HSDs) at $P < 0.05$, testing the significance of sample type (below boxes) and of season (above boxes). Whiskers represent interquartile range (IQR) x 1.5.

highlighting compositional differences between these communities. Proteobacteria of the genera *Acidocella*, *Burkholderia* and *Aquicella* were more abundant in root than soil samples and bacteria of the order *Rhizobiales* (*Proteobacteria*), *Solirubrobacterales* (*Actinobacteria*) and *Planctomycetales* (*Planctomyces*) were less abundant in root samples than in the soil (Supplementary file 22). The needle samples had the fewest bacterial OTUs, with the majority of those OTUs being unique to the phyllosphere. Similarly, alpha diversity was much lower in the needle than in the root samples on the control plots ($P < 0.001$). The most abundant genus found in the phyllosphere was *Acidiphilium* within the *Proteobacteria*.

3.2. Nutrient optimisation increased alpha diversity of belowground fungi and bacteria

Alpha diversity of both soil and root fungal communities was positively affected after long-term (25 years) nutrient optimisation ($P < 0.05$; Fig. 2a). In particular, greater EMF community diversity was observed after long-term nutrient optimisation (Fig. 3a and b). In contrast, short-term (five year) nutrient optimisation resulted in greater fungal diversity only in the soil. Nutrient optimisation also had a significant effect on belowground fungal community composition ($P < 0.001$; Fig. 2c). Sample type and the interaction term of treatment and sample type were both significant ($P < 0.001$; $P < 0.01$), driven by differences in the composition of soil and root fungi and a dissimilar responsiveness of these two distinct communities to the treatment. Fifteen percent (48) of the root associated and 6% (22) of the soil fungal OTUs responded significantly to nutrient optimisation, including 16 of 149 putative ectomycorrhizal fungal OTUs, (with an adjusted p value < 0.01) (Fig. 4 and Supplementary file 23). After nutrient optimisation, fungi of the genus *Tylospora* became more abundant, especially in association with roots. We also saw the genus *Amphinema* in higher abundance in root samples from the nutrient optimised plots. In general, members of the genus *Pseudotomentella* were found in higher abundance in both root and soil samples after nutrient optimisation. In contrast, members of the genus *Cortinarius* were less abundant in both sample types after nutrient optimisation. Fungi of the genus *Piloderma* were also less abundant after nutrient optimisation but were only significantly reduced in soil samples. In addition, several non-mycorrhizal fungi classified as *Venturiaceae* sp and *Oidiodendron pilicola* declined in abundance in root samples.

In contrast to the clear effects of long-term nutrient optimisation, the growing season had no effect on the alpha diversity of the belowground fungal communities ($P > 0.05$) but did influence the composition of the soil and root fungal communities to some extent ($P < 0.05$). This effect was due only to a seasonal response of fungal OTUs within the nutrient optimised plots. The one root-associated OTU that did respond significantly to both treatment and growing season was the ectomycorrhizal OTU *Pseudotomentella griseopergamacea*. This OTU was in general of low abundance in root samples (< 1%) but was more prevalent in nutrient optimised plots and here more in August than in samples from October.

Alpha diversity, both of soil and root-associated bacterial communities, was significantly higher after long-term (25 years) nutrient optimisation ($P < 0.001$; Fig. 2b). Alpha diversity in the soil was also higher after the short-term (five years) nutrient optimisation treatment ($P < 0.001$), and the root bacterial communities showed a tendency to increase alpha diversity after five years of treatment ($P > 0.05$). Nutrient optimisation also resulted in major changes in the composition of the root- and soil-associated bacterial communities ($P < 0.001$; Fig. 2d). Root and soil communities were distinct in their composition ($P < 0.001$) and nutrient optimisation differed in the effect it had on

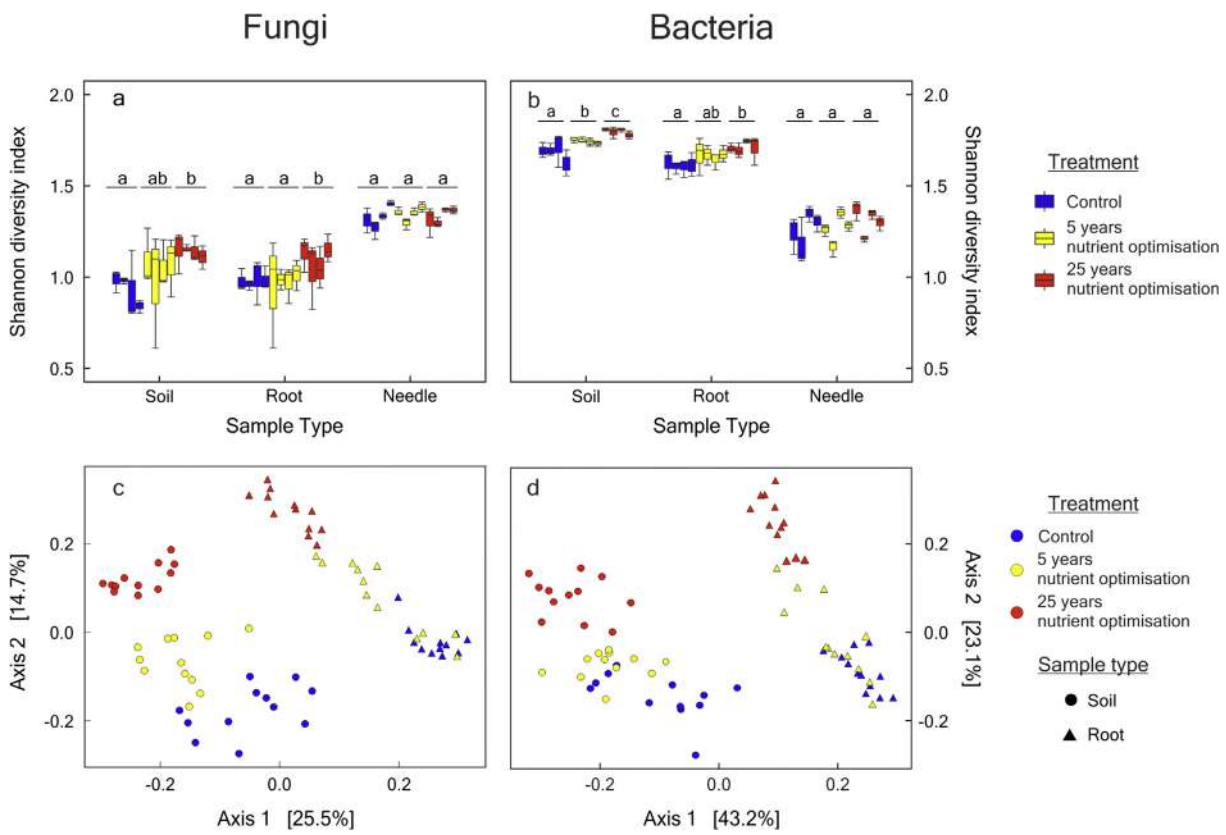


Fig. 2. Fungal and bacterial community diversity in belowground and needle samples after nutrient optimisation.
Boxplots show median fungal (a) and bacterial (b) alpha diversity, using the Shannon diversity index of operational taxonomic units (OTUs), coloured by treatment: Control (blue), five years of nutrient optimisation (yellow) and 25 years of nutrient optimisation (red) and split by season within each treatment (from left to right: early June, late June, August and October). Shannon diversity index represents log-transformed values. Lowercase letters represent Tukey honestly significant differences (HSDs) at $P < 0.05$, testing the effect of treatment within each sample type. Whiskers represent interquartile range (IQR) x 1.5. Principal coordinate analysis (PCoA) of soil and root fungal (c) or bacterial (d) communities across the three treatment levels. After variance stabilising transformation of the OTUs abundance values the Bray-Curtis index was used as an abundance-based metric. Samples are coloured by treatment and shaped corresponding to a sample type.

the established communities of soil and roots ($P < 0.005$). Of the genera associated with roots, 22% (30) responded to nutrient optimisation in comparison to 7% (10) in the soil (Fig. 5 and Supplementary file 24). Several members of the *Acetobacteraceae* family consistently decreased in both soil and root communities with increasing nutrient availability. Improved nutrient status led to a depletion of genera within the phyla *Cyanobacteria* and *Planctomycetes* in the root communities. In contrast, *Actinospica* and *Streptomyces*, both of the phylum *Actinobacteria*, which includes more copiotrophic bacterial groups, increased in relative abundance in root communities of soils where nutrient conditions were higher. In contrast to nutrient optimisation, season had no effect on alpha diversity and community composition of soil and root bacteria.

3.3. Changes in alpha diversity of phyllospheric fungal and bacterial communities follow seasonal dynamics

Alpha diversity of the fungal community of the phyllosphere varied throughout the seasons ($P < 0.001$; Fig. 2a). However, in contrast to the belowground fungal communities, we found there was no effect of nutrient optimisation on the diversity of the phyllospheric fungal community even after 25 years of treatment ($P > 0.5$). Alpha diversity of the phyllospheric fungal community was initially high in early June samples but decreased towards late June before increasing again in August, eventually even exceeding the level of the early June samples (Fig. 2a).

The first two coordinates of the PCoA of the needle samples explained 52% of the variation in community composition between samples (Fig. 6a). Variation in the fungal community composition of the phyllosphere was largely driven by the growing season ($P < 0.001$) but nutrient optimisation also contributed ($P < 0.001$). Fungal OTUs that were more abundant in the phyllosphere in June were classified as *Endoconidioma populi* and *Lophodermium piceae* (Supplementary file 25). Later in the growing season OTUs classified as *Cladosporium exasperatum*, *Cryptococcus victoriae*, *Pectenia plumbea, Dothideomycetes* sp, *Devriesia pseudoamericana* and *Teratosphaeriacea* sp became more prevalent in needle samples. One OTU, *Fusicolla violacea,* was higher in abundance towards the end of the growing season and additionally with increasing nutrient availability. The effect of nutrient optimisation alone was particularly pronounced for an OTU of the order *Rhytismatales*, which was more abundant in nutrient optimised plots at three of four sampling points in June and August.

Similar to fungal communities, alpha diversity of the phyllospheric bacterial communities was strongly influenced by the growing season ($P < 0.001$; Fig. 2b). The effect of growing season was more pronounced on plots where nutrient availability had been increased for five ($P < 0.005$) or 25 years ($P < 0.01$) in comparison to the control condition ($P < 0.1$). However, alpha diversity of bacterial communities of the needle samples was not responsive to increased nutrient availability alone ($P > 0.5$). Diversity of the phyllospheric bacterial communities decreased between early and late June and increased again later in the growing season, with a peak in August (Fig. 2b).

Community composition of phyllosphere bacteria was strongly influenced by the time of growing season ($P < 0.001$) and nutrient optimisation ($P < 0.05$ Fig. 6b). Community shifts due to growing season were observed for several genera. Relative abundance of the genera

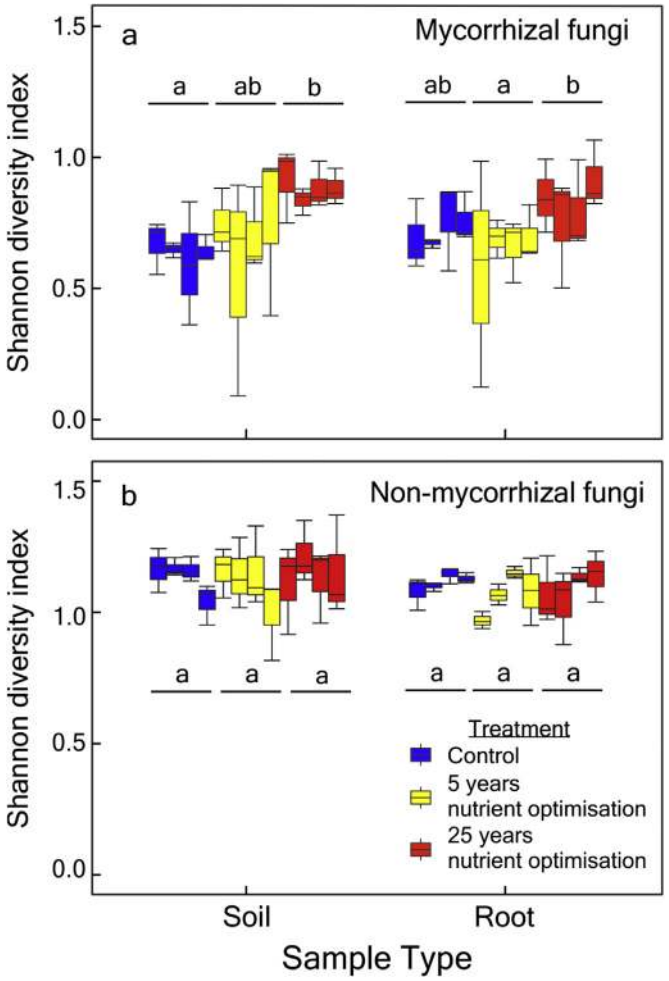


Fig. 3. Alpha diversity of ectomycorrhizal and putative ectomycorrhizal (a) or non-mycorrhizal OTUs (b).
Boxes represent median fungal diversity in soil and root samples and were coloured by treatment: control (blue), five years of nutrient optimisation (yellow) and 25 years of nutrient optimisation (red) and the seasons kept apart within each treatment (from left to right: early June, late June, August and October). Alpha diversity is based on log-transformed Shannon diversity index values. Whiskers represent interquartile range (IQR) x 1.5.

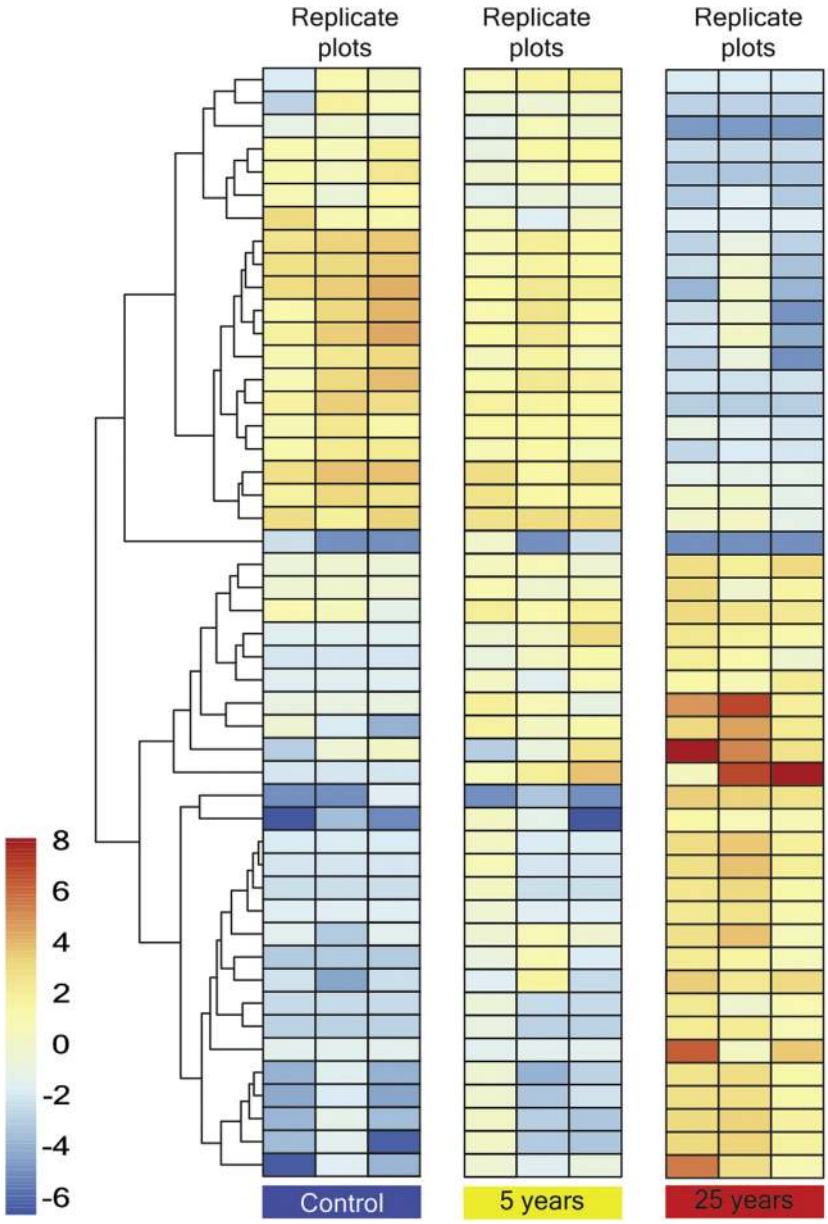
Buchnera and *Rhodanobacter* within the *Proteobacteria* was highest in the beginning of the year. The abundance of *Methylocella, Methylobacterium, Novosphingobium, Bdellovibrio, Legionella* (*Proteobacteria*), *Bryocella* (*Acidobacteria*) and *Hymenobacter* (*Bacteroidetes*) increased from August onward (Supplementary file 26). Bacteria of the genus *Singulisphaera* (*Planctomyces*) also increased late in the season (from August onward) but in addition their abundance was higher on the nutrient optimisation plots, in comparison to the control plots, in late June. In contrast, members of the order *Xanthomonadales* (*Proteobacteria*) were less abundant with nutrient optimisation than in samples from the control in August (Supplementary file 26).

4. Discussion

Fungal and bacterial species, and the complex metacommunities they form, represent key players in carbon and nitrogen cycles in boreal forest ecosystems, however our understanding of the effects of altered nutrient status on the forest microbiota is currently limited. In this study, we report the effects of short (five year) and long-term (25 years) nutrient optimisation on the diversity and composition of fungal and bacterial communities in the soil of a boreal forest and on the microbiota associated with Norway spruce roots and needles. Using amplicon-based metagenomics sequencing of the fungal ITS1 variable region we found that five years of nutrient optimisation increased alpha diversity of fungal communities in the mor layer and significantly increased alpha diversity after 25 years. The observed increase in alpha diversity of the root associated fungal community was only significant after 25 years, suggesting that fungi in association with roots respond more slowly to changes in edaphic conditions. In particular, the diversity of the ectomycorrhizal fungal community increased with nutrient optimisation. This increase in diversity was linked to a shift of the community composition towards more nitrophilic taxa (Fig. 4 and Supplementary file 23), indicating adaptation to higher inorganic nutrient but decreased plant carbon availability, consistent with the observation that C-allocation to small and fine roots was significantly reduced at this site after 12 years of nutrient optimisation (Iivonen et al., 2006). The diversity of belowground bacterial communities also increased significantly after five and 25 years in the soil and after 25 years in root samples, with shifts in community compositions towards putatively copiotrophic taxa occurring under nutrient optimisation (Fig. 5 and Supplementary file 24). The positive effect on diversity we observed suggests that bacterial communities respond to both the higher nutrient and the higher C availability resulting from increased litter input, which is consistent with the observation that total C and N had increased in the mor layer after 12 years (Olsson et al., 2005), and again after 25 years (Supplementary file 27) of nutrient optimisation. The direct response of bacterial communities to nutrient optimisation might also explain their earlier changes in comparison relative to changes in fungal communities, where nutrient optimisation only has an indirect effect on the EMF community, due to reduced C-allocation by the plant host.

In our belowground samples, EMF taxa reported to be nitrophobic and to have abundant extramatrical mycelium of the medium distance exploration type, such as *Cortinarius* and *Piloderma* (Fransson et al., 2000; Agerer, 2001; Lilleskov et al., 2002; Allison et al., 2008), decreased in abundance after nutrient optimisation. On the other hand nitrophilic taxa such as *Tylospora* (Fransson et al., 2000; Lilleskov et al., 2002) and *Pseudotomentella* increased in abundance in response to increased nutrient availability. These are of the relatively short distance exploration types and might have lower need of plant-derived C for the production of mycelia (Colpaert et al., 1992; Wallenda et al., 1996; Nilsson et al., 2005). A study of root morphotypes of EMF at the Flakaliden research site after 10 years of nutrient optimisation indicated similar changes (Fransson et al., 2000), suggesting a dynamic response of the belowground EMF communities to increased nutrient availability. Our data support the conclusion that certain mycorrhizal fungi may partially replace or compensate for other EMFs under conditions of high nutrient availability, and that differences in life-history traits and the tolerance to higher nutrient concentrations influence EMF community composition. Similarly, ericoid mycorrhizal hyphae of the *Leotiomycetes* also decreased in the nutrient optimised plots. These findings are consistent with the hypothesis that trees invest less C into EMF and thus the costs of foraging for nutrients by EMF need to be reduced in systems with higher nutrient availability, resulting in the establishment of more C efficient symbionts or ones that grow better with highly available NO_3^-.

Increases in forest productivity can potentially be achieved by irrigation alone if water availability is a limiting factor for tree growth. At the Flakaliden site, where nutrient levels were naturally very low, irrigation did not have a positive effect on tree growth and biomass and did not result in changes to the composition of microarthropod communities (Bergh et al., 1999; Lindberg and Persson, 2004). It is therefore unlikely that the observed changes in fungal communities on the plots receiving liquid nutrition were a result of changes to soil water status. However, combined effects of irrigation and fertilisation have been reported and were shown to reduce the toxic effects of solid fertiliser addition and acidification on the soil fauna (Lindberg and

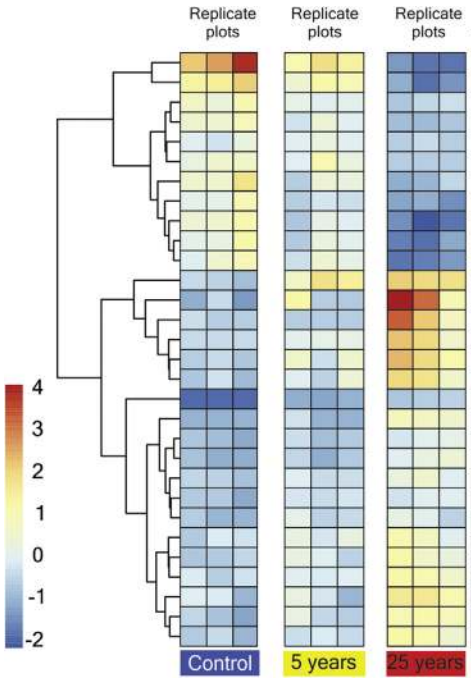


Phylum	Species	OTU_no.
Ascomycota	Helotiales sp	OTU_41
unidentified	Fungi sp	OTU_352
Ascomycota	Penicillium thomii	OTU_14
Zygomycota	Mortierella sp	OTU_1030
Ascomycota	Oidiodendron maius	OTU_996
Basidiomycota	Leucosporidiales sp	OTU_1235
Ascomycota	Chaetothyriales sp	OTU_68
Ascomycota	Oidiodendron pilicola	OTU_4263
Ascomycota	**Oidiodendron pilicola**	**OTU_103**
Ascomycota	Oidiodendron pilicola	OTU_2894
Basidiomycota	Cortinarius alpinus	OTU_9
Basidiomycota	Cortinarius alpinus	OTU_824
Ascomycota	**Helotiales sp**	**OTU_76**
Basidiomycota	Cortinarius alpinus	OTU_4369
Basidiomycota	Sporidiobolales sp	OTU_1029
Ascomycota	Hyaloscyphaceae sp	OTU_22
Ascomycota	**Helotiales sp**	**OTU_17**
Basidiomycota	Sporidiobolales sp	OTU_1020
Ascomycota	Meliniomyces variabilis	OTU_59
Ascomycota	**Venturiaceae sp**	**OTU_222**
Ascomycota	Archaeorhizomyces sp	OTU_6
Ascomycota	Cladophialophora sp	OTU_422
Basidiomycota	Amphinema byssoides	OTU_15
Basidiomycota	Agaricales sp	OTU_443
Ascomycota	Lachnum pulverulentum	OTU_4787
Ascomycota	Archaeorhizomyces sp	OTU_4512
Ascomycota	Ascomycota sp	OTU_204
Ascomycota	Hyaloscyphaceae sp	OTU_1185
Basidiomycota	Pucciniales sp	OTU_223
Basidiomycota	Thelephoraceae sp	OTU_258
Ascomycota	**Helotiales sp**	**OTU_916**
Basidiomycota	Pseudotomentella griseopergamacea	OTU_151
Basidiomycota	Pseudotomentella mucidula	OTU_7
Basidiomycota	Atheliales sp	OTU_116
Basidiomycota	Atheliales sp	OTU_237
Basidiomycota	Atheliales sp	OTU_272
Basidiomycota	Bullera sp	OTU_3212
Ascomycota	Hypocreaceae sp	OTU_124
Zygomycota	Mortierella globulifera	OTU_1182
Basidiomycota	Pseudotomentella tristis	OTU_172
Zygomycota	Mortierella sp	OTU_1110
Basidiomycota	Tylospora fibrillosa	OTU_3074
Ascomycota	Ascomycota sp	OTU_218
Basidiomycota	Tylospora fibrillosa	OTU_4474
Basidiomycota	Atheliaceae sp	OTU_32
Basidiomycota	Tylospora fibrillosa	OTU_282
Basidiomycota	Tylospora fibrillosa	OTU_131
Basidiomycota	Tylospora asterophora	OTU_126

Fig. 4. Heatmap of fungal OTUs differing in abundance in root samples across nutrient levels.
OTUs were filtered to contain > 10 counts and a relative abundance of > 0.005%. Log2 fold change > 2 and an adjusted *p* value of < 0.01 were set as significance cut-offs and 48 OTUs were found to differ significantly. For display, OTU abundances are centred across all samples. Names of the most similar database sequences on species level are shown along with each OTU. OTUs with an abundance of > 1% are indicated in bold letters. Ectomycorrhizal taxa are marked red. The coloured bars at the bottom represent the treatments, control in blue, five-year nutrient optimisation in yellow and 25 years of nutrient optimisation in red.

Persson, 2004). In agreement with this, the effect of increasing fertility, either naturally or by anthropogenic means, on fungal richness and diversity has often been inseparably linked to pH effects (Lilleskov et al., 2002; Frey et al., 2004; Treseder, 2004; Kranabetter et al., 2009; Högberg et al., 2014). Fertilisation and anthropogenic N-deposition are often associated both with decreasing pH and fungal species richness and diversity (Lilleskov et al., 2002; Frey et al., 2004; Treseder, 2004; Högberg et al., 2014), whereas positive correlations of higher nutrient availability on pH and fungal alpha diversity are found along natural fertility gradients (Kranabetter et al., 2009; Högberg et al., 2014). In the Flakaliden experimental forest, the long-term addition of a complete nutrient mixture resulted in no significant change in soil pH (Supplementary file 2) but it did result in a significant increase in fungal diversity. This finding suggests that large increases in nutrient availability can be balanced in this ecosystem if supplied in repeated low-dose and in liquid form, and indicates further that mycorrhizal community diversity can be increased by increasing soil fertility alone, independent of changes to soil pH.

Like fungi, bacteria are influenced by pH as well as N and/or C availability in the soil (Sessitsch et al., 2001; Frey et al., 2004; Burke et al., 2006; Wallenstein et al., 2006). Studies in mixed hardwood stands, loblolly pine plantations, Arctic tundra and grassland soils have all reported either no effect or an increase of bacterial richness and diversity after long-term N-addition (Burke et al., 2006; Ramirez et al., 2010; Turlapati et al., 2013; Koyama et al., 2014). Similarly, in our experiment nutrient optimisation significantly increased the alpha diversity of the bacterial community in the mor layer after five and 25 years. In common with fungal species, the diversity of the root-associated bacterial community increased after five and 25 years but, as with the fungal community, this change was significant only after 25 years, suggesting that bacteria in direct contact with plant roots are also less influenced by changes in nutrient availability. Bacteria are often



Class	Order	Family	Genus
Alphaproteobacteria	**Rhodospirillales**	**Acetobacteraceae**	**Acidocella**
Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acidisoma
Gammaproteobacteria	Legionellales	Legionellaceae	Legionella
Deltaproteobacteria	Myxococcales	0319_6G20	uncultured Syntrophobacteraceae bacterium
Deltaproteobacteria	Myxococcales	0319_6G20	bacterium GLA1
Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Rhodopila
Alphaproteobacteria	**Rhodospirillales**	**Acetobacteraceae**	**uncultured**
Deltaproteobacteria	Bdellovibrionales	Bdellovibrionaceae	Bdellovibrio
Melainabacteria	**Obscuribacterales**	**uncultured bacterium**	**uncultured bacterium**
Alphaproteobacteria	**Rhizobiales**	**alpha I cluster**	**uncultured forest soil bacterium**
Planctomycetacia	**Planctomycetales**	**Planctomycetaceae**	**Isosphaera**
Actinobacteria	**Catenulisporales**	**Actinospicaceae**	**Actinospica**
Actinobacteria	**Streptomycetales**	**Streptomycetaceae**	**Streptomyces**
Actinobacteria	Corynebacteriales	Nocardiaceae	Nocardia
Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Asticcacaulis
Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	Labrys
Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia
Acidobacteria	Subgroup 2	uncultured forest soil bacterium	uncultured forest soil bacterium
Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	uncultured
Thermoleophilia	Solirubrobacterales	YNPFFP1	uncultured bacterium
Acidobacteria	Subgroup2	uncultured bacterium	uncultured bacterium
Thermoleophilia	Gaiellales	uncultured	uncultured Conexibacter sp
Acidobacteria	Acidobacteriales	Acidobacteriaceae (Subgroup 1)	Candidatus Koribacter
Nitrospira	Nitrospirales	4_29	uncultured bacterium
Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Sedimini bacterium
Cytophagia	Cytophagales	Cytophagaceae	Cytophaga
Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Caulobacter
Deltaproteobacteria	Myxococcales	Haliangiaceae	Haliangium
OPB35 soil group	uncultured bacterium	uncultured bacterium	uncultured bacterium
Actinobacteria	Micromonosporales	Micromonosporaceae	Actinoplanes

Fig. 5. Heatmap of bacterial genera differing in abundance in root samples across nutrient levels.
OTUs were filtered to contain at least 10 counts and a relative abundance of more than 0.005 % and were then merged at genus level. Log2 fold change > 2 and an adjusted p value of < 0.01 were set as significance cut-offs and 30 genera were found to differ significantly. For display, genera abundances are centred across all samples. Names of the most similar database sequences on genus level are shown. Genera with an abundance of > 1% were indicated in bold letters. The coloured bars represent the treatments, control in blue, five-year nutrient optimisation in yellow and 25 years of nutrient optimisation in red.

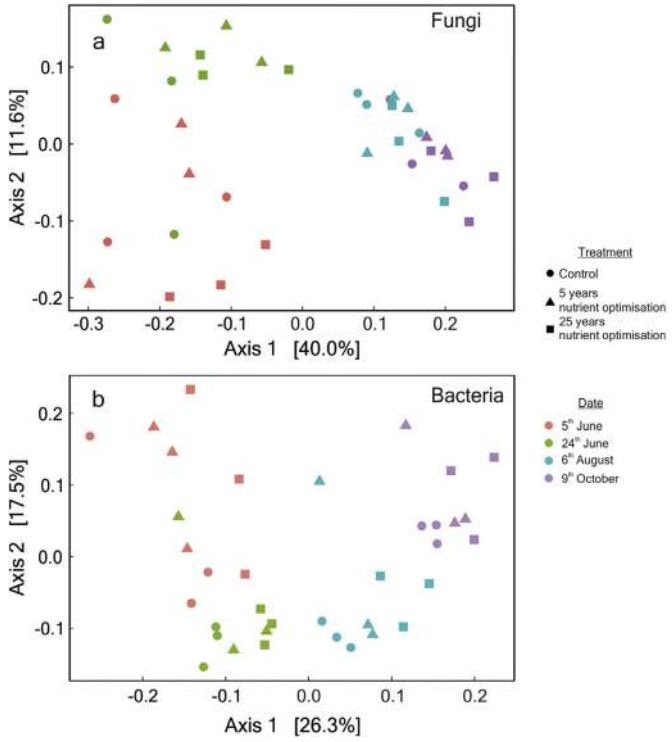


Fig. 6. Principal coordinate analysis (PCoA) of phyllospheric fungal (a) and bacterial (b) communities.
After variance stabilising transformation of the OTUs abundance values the Bray-Curtis index was used as abundance-based metric to display compositional differences across season and treatment. Samples are coloured by sampling time point and shaped corresponding to a treatment.

associated with mycorrhiza (Bonfante and Anca, 2009) and are thought to stimulate mycorrhization (Garbaye, 1994) and also colonise various niches of the mycorrhizosphere (Cairney and Meharg, 2002). For example in a soil-*Quercus petraea-Scleroderma citrinum* continuum, *Burkholderia* dominated the ectomycorrhizosphere (Uroz et al., 2013). At Flakaliden, *Burkholderia* were more abundant in root compared to soil samples in the control plots, suggesting that also here tree-associated mycorrhizal fungi may be important for certain bacteria during colonisation. Furthermore, trees and fungi have been shown to select microbes with complementary functions (Uroz et al., 2013). In our case bacteria with the capacity to fix nitrogen, such as of the family *Acetobacteraceae*, a taxa assigned to the order *Rhizobiales* (within the *Proteobacteria*) or the class *Melainabacteria* (*Cyanobacteria*), decreased in both soil and root samples with long-term nutrient optimisation. On the other hand, in nutrient optimised plots the genus *Streptomyces* became more abundant at the root level, together with other putatively copiotrophic bacteria of the phylum *Actinobacteria*. Strains of *Streptomyces* spp. have been studied in the presence of certain EMF, such as *Amanita muscaria, Suillus bovinus* or *Piloderma croceum,* where they had positive effects on mycorrhization, but could also act as competitors for root colonisation with fungi (Schrey et al., 2005; Kurth et al., 2013). Our findings highlight that complex communities comprising fungal and bacterial species are formed in association with tree fine roots. Bacteria will directly react to concurrent changes in C and/or N inputs across N gradients (Ramirez et al., 2010) in addition to changes in diversity and composition of soil and root-associated fungi. Mycorrhiza can be a source of low molecular weight organic compounds influencing bacterial communities in the soil (Fransson et al., 2016), further emphasising that interactions between mycorrhizal and bacterial communities exist. As such, interpretation of ecosystem observations will benefit from inclusion of both components.

Phyllosphere fungi occupy a broad range of niches on and in living plants, they can be asymptomatic endo- and epiphytes, pathogens or live as litter degrading saprobes on dead plant material and many can

switch between states during their life cycle in response to resource availabilities (Müller et al., 2001; Schoch et al., 2006). Our findings suggest that fungi in the phyllosphere of Norway spruce form highly diverse communities that are more diverse than belowground fungal communities of the same stand. Endophytic leaf or phyllospheric fungal communities of trees are, in general, described as highly diverse (Arnold et al., 2007; Jumpponen and Jones, 2009, 2010; Bálint et al., 2013; Millberg et al., 2015) and endophyte diversity of conifers at higher latitudes was shown to be almost as diverse as that of tropical hosts (Arnold and Lutzoni, 2007). At high latitudes leaf-associated fungal communities of evergreen trees with multi-annual foliage are even more diverse (Millberg et al., 2015) than those of deciduous trees (Bálint et al., 2013). The main effect on diversity and composition of phyllospheric communities in our samples was growing season and not nutrient treatment. The increased N availability in proportion to C, as seen by lower C:N ratios after 25 years of nutrient optimisation treatment (Supplementary file 27), had no significant influence on the diversity of phyllosphere fungi. However we found that phyllosphere fungi, such as *Lophodermium piceae* or different *Dothideomycetes* species, were most abundant either at the beginning or the end of the growing season. This pattern suggests a link to changes in foliar carbohydrates, mainly starch, which account for up to 40% of the needle dry weight early in the growing season but are found at very low concentrations during the rest of the year (Linder, 1995).

In contrast to our observations for fungal diversity, bacterial richness and diversity in the phyllosphere was lower compared to that of belowground communities, matching findings in grapevine, mustard and *Arabidopsis* (Bodenhausen et al., 2013; Zarraonaindia et al., 2015; Wagner et al., 2016). Both the phyllosphere and rhizosphere environments are heterogeneous and dynamic, and they are not exclusive of each other, with microbes able to migrate in both directions (Bodenhausen et al., 2013; Zarraonaindia et al., 2015). Nevertheless, abiotic and biotic factors influence community structures of various sample types differently and only diversity of phyllospheric bacterial communities responded significantly to season in our experiment. Several endophytic bacteria, members of the *Enterobacteriaceae* and *Bdellovibrionaceae* (Rúa et al., 2016), and other *Proteobacteria* from needle samples, such as *Xanthomonadales*, *Legionellales*, as well as *Acidobacteriales* (*Acidobacteria*), *Cytophagales* (*Bacteroidetes*), *Planctomycetales* (*Planctomyces*), *Chthoniobacterales* (*Verrucomicrobia*) varied during the growing season. Similarly, changes in abundance of methylo- and methanotrophic *Proteobacteria* such as *Methylobacterium* and *Methylocella* indicated a seasonal adaptation to shifting resource availability in the leaf habitat. Our work cannot exclude the importance of other factors, such as UV-radiation or humidity, in shaping phyllospheric communities over the growing season but our results suggest a predictable temporal variability for certain members of the phyllosphere habitat that most likely reflects changes in C availability of the needles.

5. Conclusions

Overall, our study demonstrated that nutrient optimisation treatments in boreal forests to maximise tree growth also had significant effects on diversity and composition of belowground soil and root-associated fungal and bacterial communities, which will ultimately affect processes of carbon and nitrogen cycling in the soil. Continuous application of a balanced nutrient solution for five and 25 years increased species richness and alpha diversity, where root-associated fungal and bacterial communities were influenced less after five years than the soil communities. Sequencing of phylogenetic markers showed that the composition shifted towards copiotrophic taxa that have preferences for inorganic N, and that are adapted to decreased plant belowground C allocation. Variation in the diversity and abundance of the phyllospheric fungal and bacterial communities was most likely connected with seasonal variation in needle carbohydrate status, with no pronounced effect of higher nutrient status of the tree. Identification of indicator species and further investigation of the autecology of important fungi will help determine whether the changes in communities are positive or negative for a functioning boreal forest ecosystem. In particular, the shift we identify from an oligotrophic to copiotrophic microbial community could, at least partially, be driven by an altered N supply to the trees, from one dominated by organic N compounds produced through the action of exoenzymes of saprotrophic and mycorrhizal fungi, to one dominated by the supplied inorganic N sources ammonium and nitrate. The extent to which this suppression of N extraction from soil organic matter also leads to a higher rate of soil C accumulation merits further studies.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analysed during the current study were deposited to the European Nucleotide Archive (ENA, www.ebi.ac.uk/ena) and are available under the accession number PRJEB21692.

Conflicts of interest

The authors declare that they have no competing interests.

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Authors ' contributions

VH and TN planned and designed the study. JCH performed the experiments and analysed the data together with NRS, AS and VH. JCH wrote the initial draft of the manuscript. JCH, NRS, AS, NML, MNH, TN, VH edited the manuscript into its final form. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://

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