The establishment of spruce plantations in native birch forests causes major changes in the belowground fungal communities

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All best

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Abstract

Trees are among the primary structuring factors in forest ecosystems and have consequential influence on belowground microbial communities. Plantations of Norway spruce have been established well beyond its natural range in many parts of the world, potentially impacting native microbial ecosystems and the processes they mediate. This thesis investigates fungal communities in soils from several plantations of Norway spruce and native birch forests located in western Norway. The aim was to understand which impacts introducing spruce beyond its natural range have on the associated belowground fungal communities in a native birch forest. Soil cores were sampled from neighboring stands of spruce and birch around two lakes to determine differences in fungal communities caused by the differences in vegetation. Using DNA metabarcoding, fungal community composition, diversity and functional guilds were assessed, while ergosterol was used to estimate fungal biomass. In the two investigated layers, litter and humus, fungal communities were strongly affected by the change in dominating tree species regarding fungal community composition, diversity and biomass. The birch stands displayed a significantly higher richness and abundance of saprotrophic fungi, while the spruce stands had significantly higher fungal biomass and abundance of ectomycorrhizal fungi. Establishing plantations on Norway spruce in a native birch forest leads to a loss in diversity but increase in mycelia of ectomycorrhizal fungi, with a potential impact on C sequestration processes.

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1 Introduction

In many parts of the world, plantations of non-native species have been established to provide humanity with a variety of beneficial ecosystem services, such as timber and food production. Trees used for timber production, e.g. Norway spruce (*Picea abies* (L.) Karst.), display traits that make them desirable for the forest industry, such as fast growth and frost hardiness. At the same time, these traits can facilitate naturalisation and invasion of the species beyond their native range, causing them to outcompete other native species. A shift in the dominating tree species may in turn result in an altering of the belowground communities of microorganisms in their diversity, composition and ecological function (Prescott and Grayston 2013). This makes non-native tree invasions a subject of global environmental concern (Richardson and Rejmánek 2011).

In Norway, the forest industry is of huge importance, as 38% of the country's land area is covered by trees. Norwegian forestry has a history of more than a thousand years, with Norway spruce being the cornerstone of the industry (Ministry of Agriculture and Food 2014). Norway spruce's natural range spans from central and eastern Norway across Fennoscandia, the Baltic States, Belarus and Russia as well as an unconnected southern range in central and south-eastern Europe (Aarrestad *et al*. 2014). From the late 19th century, Norway spruce from central European countries has been used in forestation in Norway, and over the course of 100 years, timber extraction has tripled (Ministry of Agriculture and Food 2014). In the temperate zones in western Norway, the forests are to a large extent made up of deciduous trees, such as birch (*Betula* spp.). This area is outside Norway spruce's natural range. However, intensive planting of central European Norway spruce has been conducted here in the last 50 years. These trees are now spreading to new areas in western Norway and will likely become more widespread in the future (Eriksen 2005; Tveite 2006).

It is well-established that aboveground vegetation is tightly linked to the belowground communities of various microorganisms. Introducing coniferous species into deciduous forests will change the environment in many ways, including a reduction of solar radiation through the canopy, higher accumulation of litter and changes in soil moisture and nutrient availability (Smolander *et al*. 2005; Aarrestad *et al*. 2014; Stoutjesdijk and Barkman 2014). Considering trees' strong effect on soil microbial community structure (Bach *et al*. 2010), establishing plantations of Norway spruce in non-native habitats may in turn affect the

diversity of the belowground fungal communities where they are introduced, and the ecosystem processes they mediate.

Fungi play major roles in forest ecosystems as pathogens, nutrient cyclers and crucial symbionts, providing nutrients to their host plants. In forest soils, they are mainly represented by two distinct functional groups; saprotrophic and mycorrhizal fungi. Saprotrophic fungi acquire energy by degrading dead organic matter, acting as the principal decomposers of wood and litter (Rayner and Boddy 1988). Mycorrhizal fungi obtain carbon derived from photosynthesis in exchange for soil minerals and water (Smith and Read 2008). More recent studies on ectomycorrhizal fungi, common in forest ecosystems, has shown that they also have the potential to decompose organic matter, although not as efficiently as saprotrophs (Lindahl and Tunlid 2015).

The temperate and boreal forest soil is characterized by a clear vertical stratification caused by the aboveground accumulation of plant litter and absence of soil mixing (Lindahl *et al*. 2007). It consists of decomposing freshly fallen plant litter in the topmost layer, increasingly decomposed organic matter (humus) underneath, followed by an underlying mineral layer. Fungi with different modes of nutritional uptake, i.e. different functional guilds, are spatially separated in the soil layers; saprotrophic fungi thrive in energy-rich litter, but as the available energy in substrate decreases with soil depth, they are less competitive, and will be replaced by mycorrhizal fungi, which do not rely on litter-derived energy (Rosling *et al*. 2003; Lindahl *et al*. 2007; Smith and Read 2008; Baldrian *et al*. 2012).

Forests are considered important terrestrial C sinks (Myneni *et al*. 2001), and an important ecosystem process mediated by fungi is carbon (C) sequestration. Aboveground accumulation of plant litter exceeding decomposition rates results in high amounts of carbon stored in the soil (Schlesinger and Bernhardt 2013). However, it has been found that mycorrhizal fungi also contribute considerably in carbon sequestration from beneath (Clemmensen *et al*. 2013). Much of the carbon fixed by photosynthesis is directed towards the roots, where mycorrhizal fungi use it to build hyphae, keeping it belowground (Clemmensen *et al*. 2013). Competition for soil nitrogen between mycorrhizal fungi and microbial decomposers, i.e. the ‘Gadgil effect’ (Gadgil and Gadgil 1975), is likely important in slowing down the carbon cycle, reducing the carbon respiration rate from the soil (Averill *et al*. 2014; Averill and Hawkes 2016). Thus, changes in the composition of fungal communities in soil may have global

consequences, as carbon sequestration help offset the release of greenhouse gases to the atmosphere.

Prescott and Grayston (2013) showed that different tree species, being the dominating factor in forest ecosystem structure, determine differences in microbial community composition in soil and litter. These findings are supported by recent studies (e.g. Uroz *et al*. 2016; Bahnmann *et al*. 2018) which show that fungal communities are strongly structured by the aboveground vegetation. Shifts in forest vegetation will likely be reflected in fungal community composition in litter and soil, especially during a shift from deciduous to coniferous forests (Urbanová *et al*. 2015). One study also concludes that host identity is the dominant driver of mycorrhizal fungal community composition (Dickie *et al*. 2015). Yet there is still uncertainties regarding how the establishment of spruce plantations in natural birch forests impacts the soil fungal community.

This study will address how the belowground fungal communities are affected when the aboveground vegetation shifts from native birch to spruce plantations in western Norway. This was done by investigating differences in the fungal community structure between soil from spruce plantations and native birch forests in the same area. More precisely, the study will address these aims;

(i) Understand how the soil fungal communities are affected by the establishment of non-native spruce plantations in native birch forests in terms of diversity, composition and biomass in the two soil layers; plant litter and humus

(ii) Investigate the composition of the fungal functional guilds across the different vegetation types, as well as the soil layers

(iii) Infer which abiotic factors drive the composition of the fungal communities and how these are affected by the introduction of non-native spruce.

2 Materials and methods

2.1 Study site and sampling design

The sampling for this study was conducted in the Voss municipality in October 2016 around two neighboring lakes, Myrkdalsvatnet and Oppheimsvatnet, with an altitude of about 230 m and 330 m, respectively. The area is made up of birch forests with a diverse understory of grass and herbaceous plants, and patches of planted non-native Norway spruce stands with an understory dominated by *Sphagnum* sp., aged around 40-50 years. The sites were chosen because of the alternating pattern of spruce and birch forests along the two lakes, making it possible to sample soil from different types of vegetation in an area with the same conditions (paired-plot approach). From the two main study sites, eight soil cores (3 cm in diameter) were collected from 10 paired plots per site (40 plots and 320 cores in total) and stored in field freezers. Within each stand, the cores were collected from points in an outer and inner circle with a radius of 4 and 2 meters, respectively. This was done to preserve consistency in the sampling.

After thawing the samples, all green plant parts were removed and the cores were split according to layer; litter (topmost layer of freshly fallen litter) and humus (underlying layer of decomposed litter). The remaining lower layers containing mineral soil were discarded. Both layers were pooled according to plot (resulting in 80 samples), freeze dried and then pulverized (30 sec x 3 at 4 m/sec speed) with ceramic beads for complete homogenization.

2.2 Soil analysis

1.5 mL pulverized litter and humus were aliquoted for chemical analyses. At the Department of Biosciences, University of Oslo, C and N-amounts were determined using a flash elemental analyzer (Thermo Finnigan Flash EA 1112, ThermoFisher Scientific, Waltham, USA), P-amounts were determined with a segmented flow analyzer (SEAL AA3 HR AutoAnalyser, SEAL Analytical Ltd, Southampton, UK), and pH was measured from a solution of 10 mL deionized water and 1g litter/humus using LAQUA Twin pH Meter (Spectrum Technologies Inc, Aurora, USA) following the manufacturer's protocol. Ergosterol was measured at the Norwegian University of Life Sciences using a protocol provided as supplementary information (Supplementary methods).

2.3 Molecular work

DNA was extracted from the finely grounded litter and humus samples following a chloroform and CTAB DNA extraction protocol; 1 g of sample was mixed with 10mL CTAB, then 600 µL of the CTAB/soil-sludge was ground using 2 tungsten carbide-beads. After extracting with 600 µL chloroform, the DNA was precipitated using 400 µL cold isopropanol. Following centrifugation, the liquid was drained on paper carefully to not disturb the DNA pellet, which then was cleaned up using the E.Z.N.A. soil kit (Omega Bio-tek, Norcross, USA), following the manufacturer's protocol. DNA quality and concentrations were measured with NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). ITS2 was targeted for PCR amplification with primers gITS7 (forward) and ITS4 (reverse) (Ihrmark *et al*. 2012). Unique 6-mer multiple identifier (MID) tags were attached to both of the primers. For each reaction, 1 µl of DNA extract was added to a master mix containing 14.6 µl dH2O, 2.5 µl Gold buffer, 2.5 µl Gold MgCl2, 0.2 µl dNTPs, 0.2 µl AmpliTaq Gold, 1 µl 20 mg/ml BSA and 1.5 µl 10 µM of each primer (gITS7 and ITS4). The PCR reactions were run with an initial step of 5 minutes at 95°C, followed by 32 cycles of denaturation at 95°C for 30 seconds, annealing primers at 55°C for 30 seconds and elongation at 72°C for 1 minute. After the cycles, a final step of elongation was performed at 72°C for 7 minutes before cooling the reaction chamber to 4°C allowing the PCR product to be stored. The resulting amplicon quality were visualized with gel electrophoresis in a 2% agarose gel, their DNA-concentration measured with Qubit, equimolar pooled in two libraries, purified with Agencourt AMPure (Agencourt Bioscience) before submitted to StarSeq (Mainz, Germany) for paired-end (PE; 300 x 2) sequencing using the Illumina MiSeq platform.

2.4 Bioinformatics

The paired-end forward and reverse sequences received from the two MiSeq runs were submitted to error correction with BayesHammer (Nikolenko *et al*. 2012). Sequences were merged with PEAR (Zhang *et al*. 2014) with a minimum threshold of 30 overlapping bases, a maximum length of 550 base pairs, a minimum length of 200 base pairs and a quality threshold of >30. The merged reads were then subjected to quality filtering using FASTX-Toolkit,/0.0.14 (Gordon and Hannon 2010) keeping reads that had >35 quality score in minimum 90% of the bases. This was followed by VSEARCH/v2.4.3 (Rognes *et al*. 2016) to only keep sequences with maximum 0.5 expected errors. The remaining high-quality

sequences were then demultiplexed to their sample of origin, based on the unique MIDs, using Simple demultiplexing (https://github.com/hildebra/sdm). Read direction was identified with FQGREP/v0.4.4 (https://github.com/indraniel/fqgrep/), and the reverse sequences were reverse complemented and merged with the forward sequences using the FASTX-Toolkit,/0.0.14. MOTHUR/v.1.38.1(Schloss *et al*. 2009) was used to gather the unique sequences before extracting the ITS2-region using ITSx/1.0.11(Bengtsson-Palme *et al*. 2013), followed by deuniquing in MOTHUR. The same program was used for removing smaller reads (<100 bp) to improve the clustering process. VSEARCH was implemented to dereplicate the sequences, remove global singletons, cluster the sequences based on 97% similarity into operational taxonomic units (OTUs), detect and delete possible chimeras, create an OTU table, followed by removal of OTUs with less than 10 reads. OTU taxonomy was assigned using the UNITE database (Abarenkov *et al*. 2010). Identified OTUs were categorized into fungal guilds based on the FUNGuild database (http://funguild.org).

All bioinformatics was performed on the Abel Cluster, owned by the University of Oslo and Uninett/Sigma2, and operated by the Department for Research Computing at USIT, the University of Oslo IT-department (http://www.hpc.uio.no).

Preceding statistical analyses, some samples were removed. Some were from a plot which in retrospect was seen to only contain Sphagnum sp., no litter or humus. One was removed due to an extraction error. The negative control included was shown to contain relative high amounts of DNA, and clustered together with two outliers in ordination analyses. These were subsequently removed, likely being the result of a contamination. Their shared variance is shown in the supplementary ordination (Fig. S1).

2.5 Statistical analyses

All statistical analyses were conducted in R (R Core Team, 2017). I made a generalized set of functional guilds from the FUNGuild annotations by weighting the proportions of the relevant functional guilds from OTUs with multiple annotations. Statistical analyses of the fungal community composition were carried out using a rarefied dataset of 25 000 sequences per sample. Community composition was visualized using global non-metric multidimensional scaling (GNMDS) of a Bray-Curtis distance matrix with the metaMDS function in the R package vegan (Oksanen *et al*. 2017). The envfit function in the vegan package was used to fit

all environmental variables onto ordinations. I used a multiple response permutation procedure (MRPP) with 999 permutations and Bray-Curtis distances to test if there was a significant difference between vegetation types and layers in fungal species composition. Fungal species diversity was assessed by calculating the Shannon index (Shannon, 1948) using the diversity function in vegan (Oksanen *et al*. 2017), and I calculated Pielou's evenness to estimate how numerically equal the fungal communities were. Two-way analysis of variance (ANOVA) followed by a Tukey HSD post hoc test was used to test if there were significant differences between vegetation types and layers for the various measurements in the dataset. I made boxplots with the package ggplot2 (Wickham, 2009) to visualize differences between our sample groups.

3 Results

3.1 Data characteristics

Out of 10 108 861 raw data sequences, 7 428 761 passed quality trimming and removal of singletons and chimeras. Clustering at 97% yielded 6160 OTUs, where 5092 remained after collapsing OTUs with the LULU algorithm to reduce species overestimation. 1093 OTUs received no blast hits and were removed. A following curation of the OTU table removed OTUs assigned to non-target organisms and OTUs with < 80% identity and < 80% coverage to known fungal sequence. The remaining table contained 3626 OTUs with 5 939 338 high quality fungal sequences.

3.2 Fungal community composition

The GNMDS ordination (Fig. 1, Table S1) demonstrated a clear shift in the fungal communities, with vegetation explaining 65% ($r^2 = 0.65$, $p = 0.001$) and soil layers explaining 18% of the variation ($r^2 = 0.18$, $p = 0.001$). Fungal community composition was significantly different between vegetation types ($p = 0.001$), as well as between layers ($p = 0.001$), according to the Multiple Response Permutation Procedure (MRPP) (Table S2). There was no observed site effect ($p = 0.6$). Of the abiotic factors fitted onto the ordination space, pH explained the most of the variation, 60% ($r^2 = 0.6$, $p = 0.001$), C explained 34% ($r^2 = 0.34$, $p = 0.001$), N explained 17% ($r^2 = 0.17$, $p = 0.001$), while C:N ratio explained 53% ($r^2 = 0.53$, $p = 0.001$). OTU richness, total ergosterol and abundances of saprotrophs and ECM were added as vectors in the ordination space to investigate which sample groups they correlated with. OTU richness correlated strongest with birch litter, ergosterol with spruce litter, ectomycorrhizal abundances with spruce humus and saprotrophic abundances with birch litter (Fig. 1).

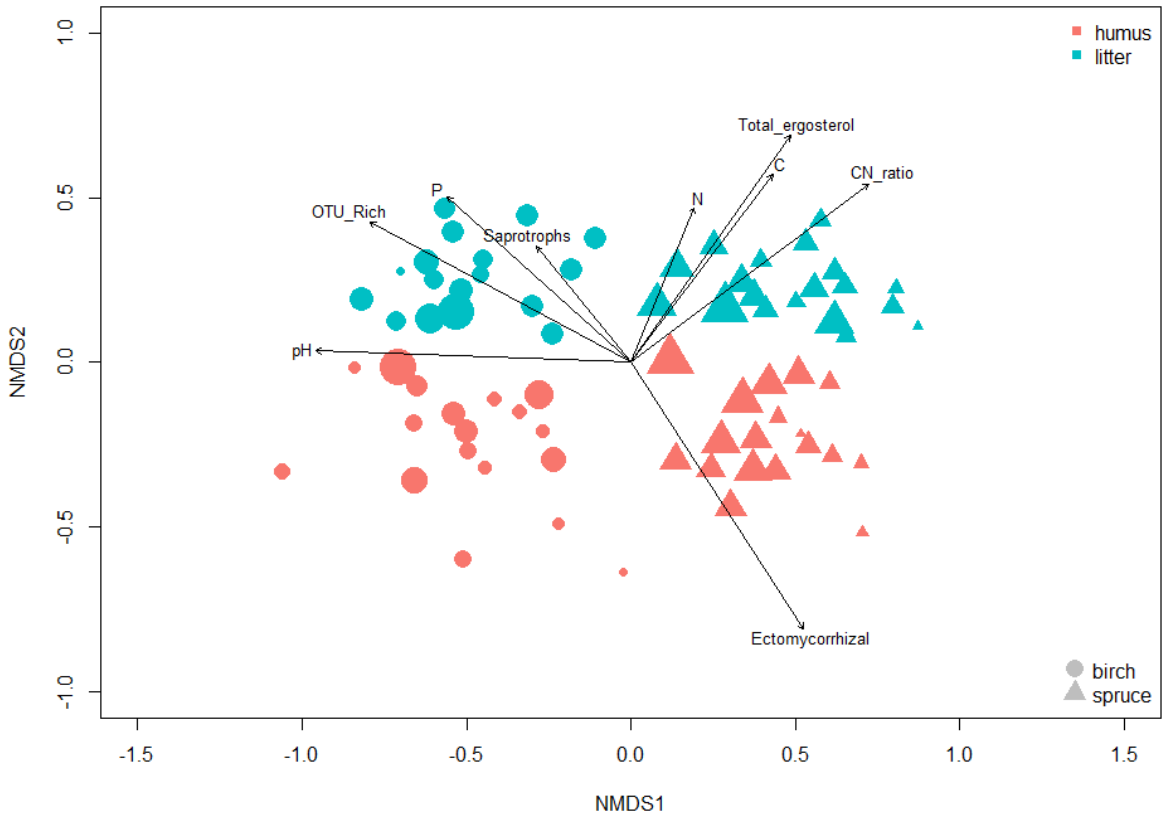

Figure 1. Global multidimensional scaling (GNMDS) ordination biplot. Samples with affinity to the humus layer are shown in red and litter samples in cyan. Triangles represent the spruce samples, circles represent the birch samples. Arrows represent how environmental variables fit to the ordination space. Relative OTU richness within sample groups is shown as different sized points.

3.3 Fungal diversity and biomass

In the overall data, OTU richness, Shannon indices and Pielou's evenness were higher in both layers in the birch forests than in the spruce forests (Fig. 2a, b, c). The OTU richness was significantly different between vegetation types when testing with ANOVA ($p < 0.001$; Table S3) but not between layers, whereas the Shannon indices and Pielou's evenness were significantly different between both vegetation types ($p < 0.001$) and layers ($p < 0.001$). The amount of total ergosterol was significantly higher (almost five times) in the spruce litter samples compared to the other sample types (Fig. 2d).

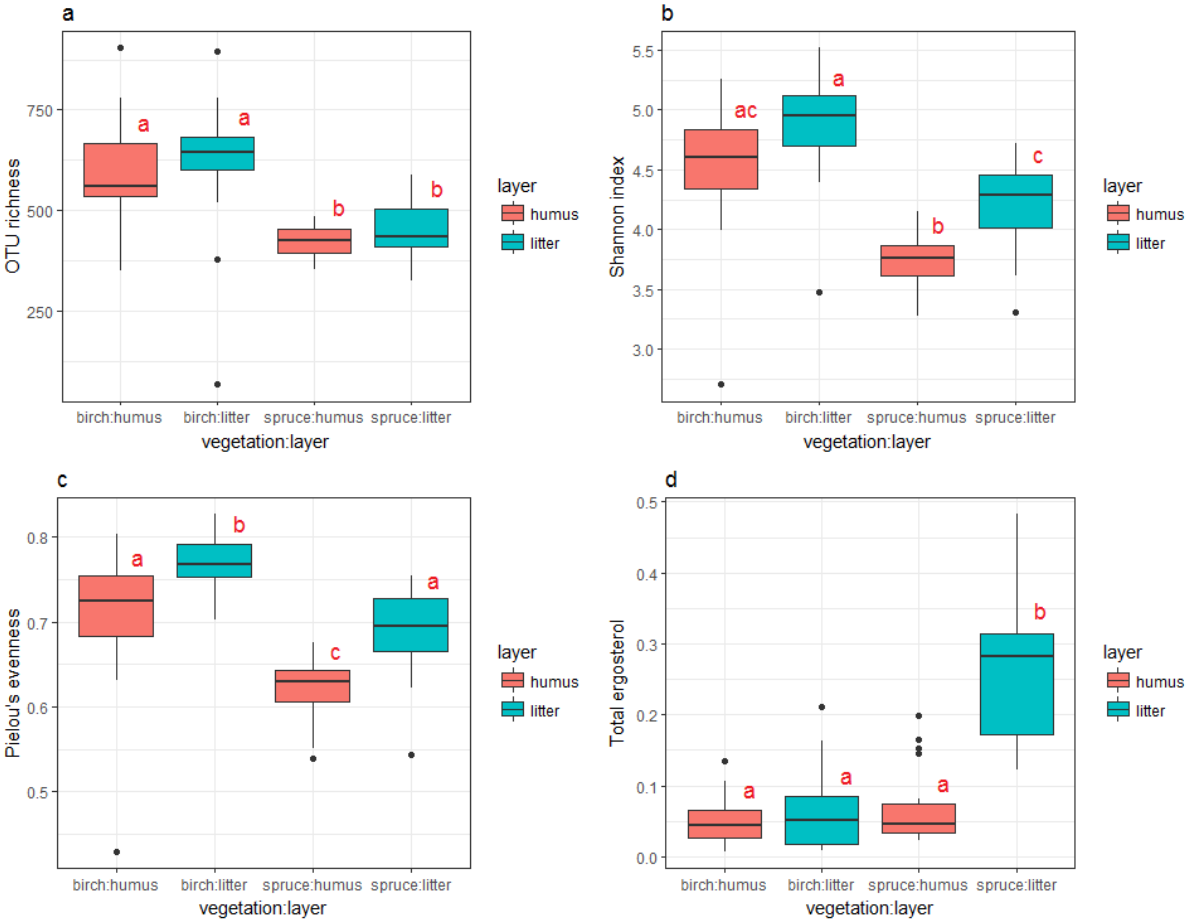

Figure 2. Boxplot of OTU richness (a), Shannon diversity index (b), Pielou's evenness (c) and the amount of total ergosterol (mg/g) (d) in each vegetation type and soil layer. Red letters indicate significant ($p < 0.05$) differences from post hoc Tukey tests.

3.4 Taxonomic composition

In the overall data, the dominating taxa was represented by 62.4% Basidiomycota, 31.3 % Ascomycota, 3.8% Morteriellamycota and 2.5% unidentified fungi (Table 1). The most striking differences between the vegetation types were that the spruce samples had larger amounts Atheliales and Telephorales than birch, while the birch samples had more Agaricales, Filobasidiales and unassigned OTUs than spruce. The most abundant species in the overall data was *Tylospora fibrillosa*, which accounted for 4.51% of the total sequences (Table S4).

Table 1. Taxonomic affinity of OTUs accounting for >0.5% of the total reads, presented as phyla and orders.

Taxonomic affinity	% of reads in birch litter	% of reads in birch humus	% of reads in spruce litter	% of reads in spruce humus	% of reads in total data
Ascomycota	**42.8**	**30.2**	**35.1**	**26.0**	**31.3**
Helotiales	15.2	20.4	16.4	20.8	18.8
Mytilinidales	2.0	3.0	6.5	4.1	4.3
Sordariales	7.8	3.1	0.3	0.3	1.6
Venturiales	17.9	3.7	11.9	0.8	6.5
Basidiomycota	**40.9**	**51.2**	**63.4**	**72.0**	**62.4**
Agaricales	24.1	23.3	10.0	10.8	14.2
Atheliales	1.9	4.5	30.4	37.1	25.7
Filobasidiales	10.1	15.8	2.1	3.6	5.9
Russulales	2.9	6.6	0.1	0.2	1.5
Thelephorales	0.8	0.9	15.0	20.2	13.3
Trechisporales	1.0	0.1	5.7	0.1	1.8
Mortierellomycota	**10.7**	**10.6**	**1.2**	**1.0**	**3.8**
Unidentified	**5.5**	**8.0**	**0.3**	**1.1**	**2.5**

3.5 Soil properties

As visualized in the box plots in figure 3, the pH was higher in the birch samples than the spruce samples (ANOVA, $p < 0.001$; Table S4), but no difference between layers within vegetation types was observed (Fig. 3a), which was further supported by ANOVA ($p = 0.955$; Table S5). The amount of carbon was higher in samples from the spruce litter (Fig. 3b), with significant differences between both vegetation types and soil layers, as well as the interaction between them (invariably $p < 0.001$; Table S5). Post hoc analyses with Tukey's HSD test revealed that this significance was only present when comparing spruce litter samples with the rest (Fig. 3b). This was also the case for nitrogen content, which showed the same pattern as carbon (Fig. 3c). Spruce humus samples had a significantly lower content of phosphorus compared to the birch forest samples (Fig. 3d; ANOVA, $p < 0.001$; Table S5).

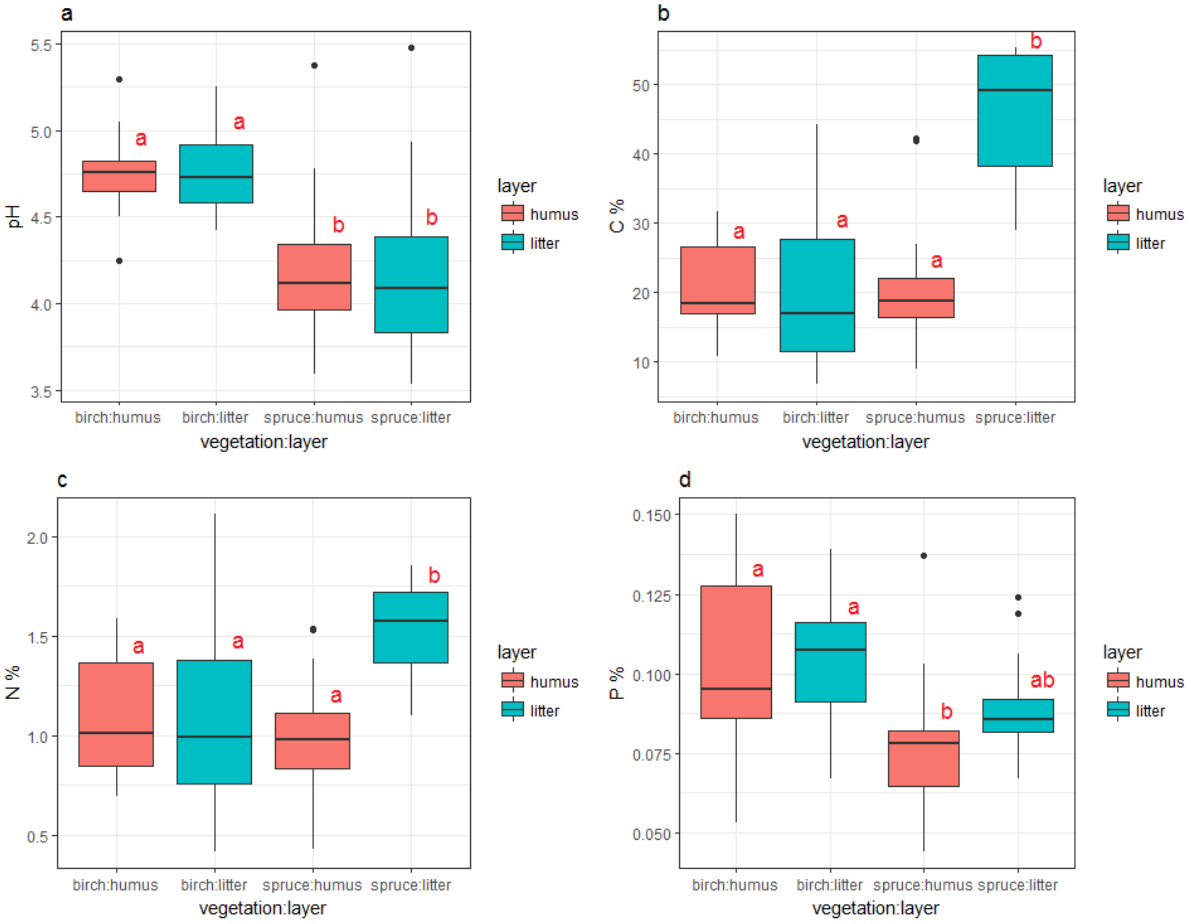

Figure 3. Results of soil chemical analyses shown as box plots of pH-measurements (a) and C-N-P-analyses (b, c, d) in each vegetation type and soil layer. Different red letters indicate significant ($p < 0.05$) differences from post hoc Tukey tests.

3.6 Functional guild composition

Regarding the distribution of functional groups in the overall data, 35% of the OTUs were assigned to be ectomycorrhizal (ECM) and 22% as saprotrophic. Further, 3% were annotated as plant pathogens, while 8% were grouped as 'others', which included arbuscular and ericoid mycorrhiza, parasites, endophytes and lichens. 32% of the OTUs had unknown functions. Based on these annotations, the birch samples contained generally more saprotrophic fungi than the spruce samples, which in turn contained more ectomycorrhizal fungi (Fig. 4a, c; Fig. S1). The birch samples also contained more OTUs with unassigned functions (Fig. S1). As expected, the litter layer included clearly more saprotrophic fungi while the humus layer generally had more ectomycorrhizal fungi (Fig. 4a, c; Fig. S1).

ANOVA revealed that the abundance of ECM fungi, measured as relative sequence abundance, was significantly different between vegetation types ($p < 0.001$) and layers ($p < 0.001$), and that there was a significant interaction effect ($p = 0.01$). The same was the case for the abundance of saprotrophic fungi, excluding the interaction (Table S5). Post hoc Tukey's test further showed significant differences between vegetation types and soil layers, although no significant difference was found between the spruce litter and birch humus samples for the relative abundance of either ECM or saprotrophs (Fig. 4a, c). In addition, ECM richness was only significantly different between the layers in birch, while saprotrophic richness was significantly different between the vegetation types (Table S5; Fig. 4b, d).

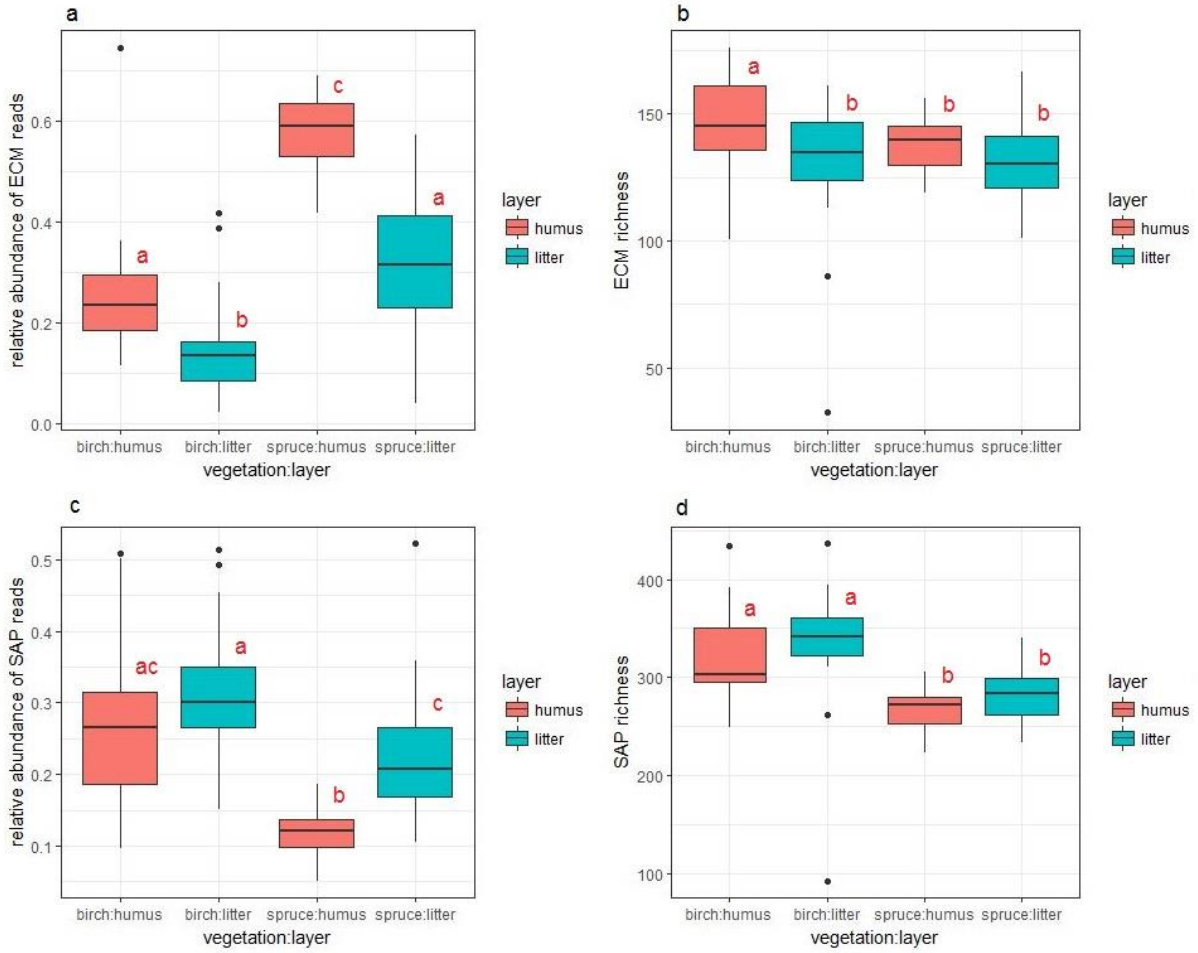

Figure 4. Box plots showing relative abundance of ECM and saprotrophic reads, as well as ECM and saprotrophic richness in the four sample groups. Different red letters indicate significant ($p < 0.05$) differences from post hoc Tukey tests.

4 Discussion

In this study, I investigated changes in the soil fungal communities caused by the establishment of non-native spruce plantations in two geographically separate but otherwise similar birch forests in western Norway. This was done by looking at how the fungal community composition, diversity and biomass in the topmost two soil layers, litter and humus, were affected by the shift in vegetation. I also investigated if the two major ecological groups in the forest soil ecosystems (saprotrophs versus ECM) were affected, by comparing their relative abundance, as well as which abiotic factors are driving the shifts in fungal community structures.

I found substantial differences in the soil fungal community composition between the spruce plantations and birch forest. In many studies, soil depth is found to be the strongest driver of fungal community composition (Rosling *et al*. 2003; Lindahl *et al*. 2007; Baldrian *et al*. 2012). However, these studies look at soil from boreal coniferous forests, not taking in account differences between vegetation types. A recent study by Asplund *et al*. (2018) investigated differences in soil fungal communities between spruce and beech, and still found soil depth as the primary factor structuring fungal community composition. Interestingly, in the present study, vegetation type comes out as the strongest factor in the ordination analysis, accounting for most of the compositional variation. This corroborates the findings of Urbanová *et al*. (2015), where they studied fungi in litter and soil under seven tree species and found that the dominating trees had the strongest effects on the fungal community composition in litter and soil. Such a dramatic shift in the fungal community composition between spruce and birch might be attributed to the altering of soil properties, e.g. C, N, P and pH, that the soil fungi has to cope with. Sterkenburg *et al*. (2015) showed that soil fungal communities varied significantly along their soil fertility gradient which included soil acidity and nutrient availability. The more recent study by Bahnmann *et al*. (2018) found that fungal communities in litter were strongly coupled with dominating tree species, while the soil communities correlated with pH as well as dominating trees. In accordance with these findings, the community composition in the present study was strongly structured by soil pH and nutrient availability. Soil and litter pH was higher in birch, while nutrient availability was higher in spruce, especially in litter. This was also the case in a study by Smolander *et al*. (2005) on soil nutrient transformations between humus from birch (*B. pendula*) and Norway

spruce (*P. abies*), which found soil pH to be lower, and C:N-ratio to be higher in spruce than birch.

The higher fungal richness in the birch forests may be because higher pH litter and humus could contain more of the nutrients not measured in this thesis, such as Ca, Na, Mg. This would provide more favourable conditions for various fungi and thus increase the richness (Prescott and Grayston 2013; Tedersoo *et al*. 2014). Another likely reason could be that the understorey vegetation in birch was visibly more diverse, consisting of grassy and herbaceous species, compared to the *Sphagnum*-dominated spruce understorey, facilitating a more varied community of decomposers and root-associated fungi. Otsing *et al*. (2018) enhances this thought, with their study showing that foliar litter richness determine fungal richness in litter.

The observed amount of total ergosterol was markedly higher in spruce litter compared to the other soil types, paralleled by the higher amounts of C, N and C:N-ratio also in spruce litter. This is in agreement with Sterkenburg *et al*. (2015) who found ergosterol-content to be higher N-rich needle litter. In addition, Wallander *et al*. (2010) found that the EcM biomass production in a chronosequence of managed Norway spruce stands peaked when C allocation belowground was highest, coinciding with canopy closure in the 10-30 years old stands. At this stage, the fungal community was dominated by *Tylospora fibrillosa*, which they argued was a C-strategist adapted to high density populations, effectively converting resources to biomass and dominating ecosystems when resources were abundant. In a recent study by Kyaschenko *et al*. (2017), they investigated a chronosequence of managed *Pinus sylvestris* stands and found that the early and mid-successional stages was dominated by Atheliaceae, a family of which *T. fibrillosa* is a member. *T. fibrillosa* is the most common species in this thesis' data, and the marked increase in Atheliales in the mid-successional spruce plantations further cements its importance in the succession of coniferous forests. Although it is an EcM species, its high abundance may cause it to partly overlap in distribution with saprotrophic fungi in the litter layer, thus explaining the high amount of fungal biomass. An alternative reason to the increase in fungal biomass in spruce litter could be that the turnover rate of fungal biomass is much slower there than elsewhere due to resistant fungal tissue, e.g. melanised hyphae which has been shown to negatively affect decomposition of EcM necromass (Fernandez *et al*. 2016). A heavily melanised species, *Cenococcum geophilum* (Fernandez *et al*. 2013), was one of the 20 most common species in my dataset (Table S4), supporting this idea. However, a more likely explanation is that the high amount of fungal

biomass is a direct effect of the increased levels of C and N, caused by an accumulation of litter in spruce plantations, providing more nutrients to enhance fungal growth of certain species, most likely members of Atheliales and Telephorales.

The vertical distribution of fungal functional groups in this study was as expected and in accordance with earlier studies (Rosling *et al*. 2003; Lindahl *et al*. 2007; Baldrian *et al*. 2012); saprotrophic fungi were most abundant in the litter layer, while ectomycorrhizal fungi were more abundant in the humus layer. The birch forest contained a higher abundance of saprotrophic fungi than spruce, possibly explaining the high amounts of C and N in spruce litter as a result of reduced decomposition. In accordance with Urbanová *et al*. (2015) which found highest EcM abundance in both litter and soil under coniferous trees, the abundance of EcM was highest in the spruce plantations, especially in the humus layer. Here, it was mainly due to an increasing abundance of species from Atheliales and Telephorales. The Shannon index was clearly lowest in spruce humus, which means that the fungal community was both relatively species poor and unevenly composed. Due to the high abundance of EcM fungi found in spruce humus, this community is dominated by a small group of species. Regarding findings on EcM fungi's role in carbon sequestration (Clemmensen *et al*. 2014; Averill and Hawkes 2016), the marked increase in EcM fungi in spruce could possibly lead to a higher C sequestration in the spruce plantations than the in native birch forests. The high abundance of EcM fungi could lead to a stronger "Gadgil-effect", in which EcM fungi compete with saprotrophs for limiting N, suppressing decomposition rates (Gadgil and Gadgil 1975) and thus respiration of CO2 to the atmosphere.

In conclusion, a strong shift in the belowground fungal communities as a result of establishing spruce plantations in birch forests was found. The birch forest displayed a higher fungal richness and abundance of saprotrophic fungi, while the spruce plantations represented an increase in fungal biomass and abundance of EcM. This means that continuous planting of Norway spruce and its spread in native birch forests leads to an altered fungal community structure with a loss in fungal diversity and potentially higher C sequestration.

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5 Supplementary

5.1 Supplementaty methods

Ergosterol was measured at the Norwegian University of Life Sciences, Ås, using a modified version of the protocol of Davey *et al*. (2009). Approximately 200 mg prepared soil sample was mixed with 7 ml 3M KOH in MeOH, vortexed and sonicated in a 70 °C ultrasonic water bath in darkness for 90 min. After being vortexed and centrifuged (c. 16 400 rpm, 15 min), the supernatant was mixed with 2 ml purified water in new tubes. Ergosterol was extracted by adding 5 ml hexane, vortexed vigorously (approx. 1 min), and the hexane phase was collected after the two phases divided. This extraction was repeated twice. Both extractions were collected in the same vial and evaporated using an Eppendorf Concentrator Plus 5301 (Eppendorf, Hamburg, Germany). Dried extractions were re-dissolved in 500 µl MeOH, and the supernatant was analysed for ergosterol content using high performance liquid chromatography (HPLC). The extractions were analysed on an 1100 Series HPLC (Agilent Technologies, Waldbronn, Germany). Ergosterol was separated using a reversed phase ODS ultra sphere column (250 mm × 4.6 mm; particle size 5 µm). MeOH was used as the mobile phase (flow rate 1.5 ml min-1, total analysis time 12 min). Absorption of ergosterol was detected at 280 nm, and identified by comparing retention time, online UV-spectra, and co-chromatography of a commercial standard of ergosterol (Sigma, St. Louis, USA).

5.2 Supplementary results

Table S1. Results from environmental variables fitted onto the ordination space, presented as r2-values of the correlation between the variables and the community matrix, and their supporting p-values.

Variable	r2	p-value
P	0.3608	**0.001***
C	0.3377	**0.001***
N	0.1724	**0.004***
pH	0.6057	**0.001***
OTU richness	0.5256	**0.001***
Ectomycorrhizal abundance	0.6081	**0.001***
Saprotroph abundance	0.1326	**0.007***
Total ergosterol	0.4643	**0.001***
Vegetation	0.6486	**0.001***
Soil layer	0.1774	**0.001***
Site	0.0060	0.631

Table S2. Results from a multiple response permutation procedure (MRPP) with 999 permutations and Bray-Curtis distances.

	vegetation	layer
Chance corrected within-group agreement	0.08508	0.02673
p-value	**0.001***	**0.001***

Table S3. ANOVA results for overall OTU richness, Shannon index and Pielou's evenness responding to vegetation, layer and the interaction between the two. Results are reported as F-statistic/p-value, with significant values presented in bold font.

	vegetation	layer	vegetation:layer
Richness	**44.6414/4.252e-09***	0.4961/0.4835	0.0944/0.7595
Shannon index	**55.533/1.609e-10***	**18.641/4.955e-05***	0.361/0.5498
Pielou's evenness	**41.6002/1.124e-08***	**26.5440/2.164e-06***	0.0993/0.7536
Total ergosterol	**49.01/1.33e-09***	**50.26/9.16e-10***	**34.54/1.34e-07***

Table S4. Summary of the 20 most common species and their related family in the complete dataset.

	#reads	**% of total**	**Family**	**Species**
OTU 1	282766	4.51 %	Atheliaceae	*Tylospora fibrillosa*
OTU 3	202554	3.23 %	Thelephoraceae	*Pseudotomentella mucidula*
OTU 4	138562	2.21 %	Atheliaceae	*Amphinema* sp.
OTU 5	128430	2.05 %	Piskurozymaceae	*Solicoccozyma terricola*
OTU 8	92454	1.47 %	Hygrophoraceae	*Hygrophorus pustulatus*
OTU 6	89349	1.43 %	unidentified	*Helotiales* sp.
OTU 7	81349	1.30 %	Thelephoraceae	*Pseudotomentella* sp.
OTU 13	81183	1.29 %	Mortierellaceae	*Mortierella humilis*
OTU 17	74124	1.18 %	Atheliaceae	*Amphinema* sp.
OTU 19	57855	0.92 %	Helotiales family *Incertae sedis*	*Cadophora finlandica*
OTU 10	56010	0.89 %	Venturiaceae	*Venturiaceae* sp.
OTU 11	54767	0.87 %	Inocybaceae	*Inocybe xanthomelas*
OTU 16	53834	0.86 %	unidentified	unidentified fungi
OTU 12	53598	0.85 %	Atheliaceae	*Amphinema* sp.
OTU 14	52196	0.83 %	unidentified	*Agaricales* sp.
OTU 100	51187	0.82 %	Venturiaceae	*Venturiaceae* sp.
OTU 32	50827	0.81 %	Hyaloscyphaceae	*Hyaloscyphaceae* sp.
OTU 15	47516	0.76 %	Gloniaceae	*Cenococcum geophilum*
OTU 30	46266	0.74 %	Gloniaceae	*Cenococcum geophilum*
OTU 20	40837	0.65 %	Hydnangiaceae	*Laccaria laccata*

Table S5. ANOVA results for the relative abundance and richness of ectomycorrhizal (ECM) and saprotrophic fungi, as well as soil properties across vegetation types and layers. Results are reported as F-statistic/p-value, with significant values presented in bold font.

	vegetation	layer	vegetation:layer
ECM relative abundance	70.169/3.04e-12*	**43.292/6.52e-09***	**7.032/0.00984***
ECM richness	0.590/0.4448	**5.869/0.0179***	1.467/0.2298
Saprotroph relative abundance	**32.825/2.18e-07***	**16.004/0.000152***	2.585/0.112248
Saprotroph richness	**23.851/6.08e-06***	1.897/0.173	0.119/0.731
ECM-Saprotroph ratio	**47.92/1.54e-09***	3**4.38/1.27e-07***	**14.46/0.000298***
pH	**39.143/2.51e-08***	0.003/0.955	0.066/0.798
Carbon	**46.17/2.63e-09***	**42.78/7.67e-09***	**42.38/8.74e-09***
Nitrogen	**5.963/0.017104***	**13.109/0.000547***	**15.136/0.000223***
Phosphorous	**21.685/1.43e-05***	1.633/0.205	1.646/0.204

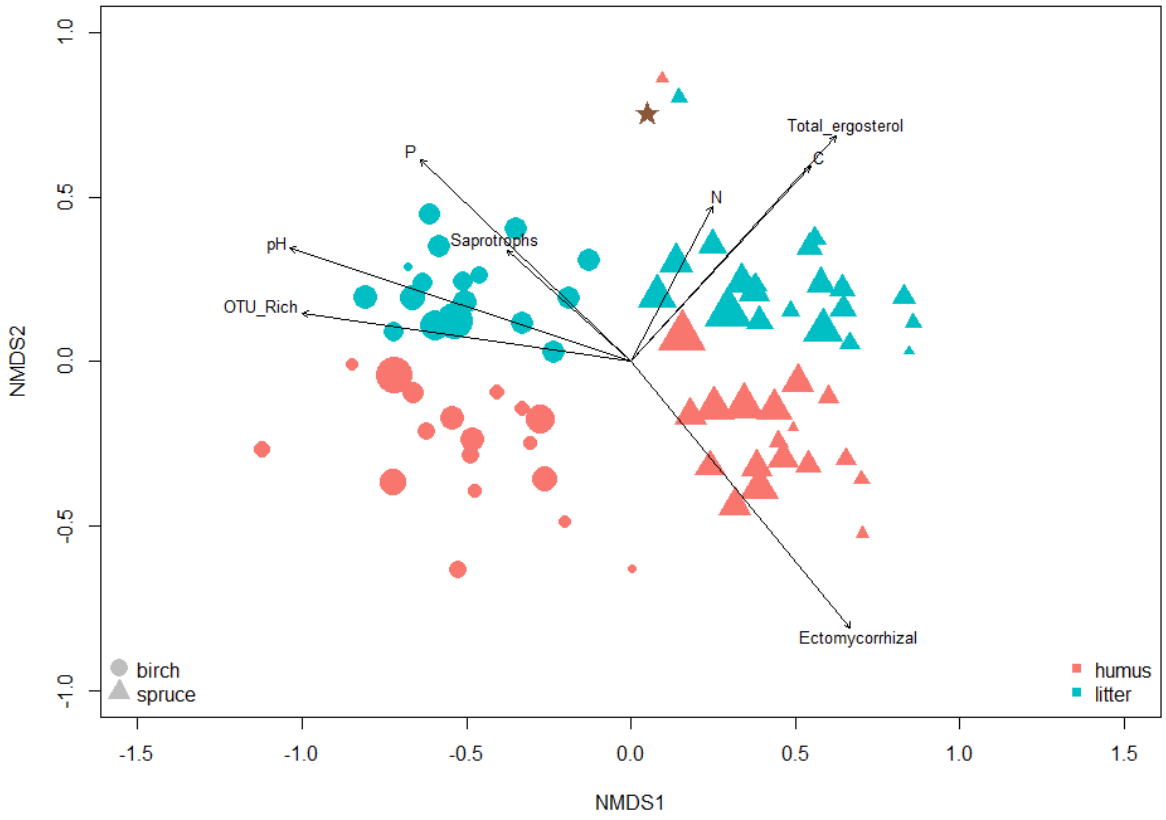

Figure S1: Global multidimensional scaling (GNMDS) ordination biplot, which includes the positive negative (brown star) and two outliers that were excluded from the main analyses (they are likely the result of an extraction error due to their clustering). Samples with affinity to the humus layer are shown in red and litter samples in cyan. Triangles represent the spruce samples, circles represent the birch samples. Arrows represent how environmental variables fit to the ordination space. Relative OTU richness within sample groups is shown as different sized points.

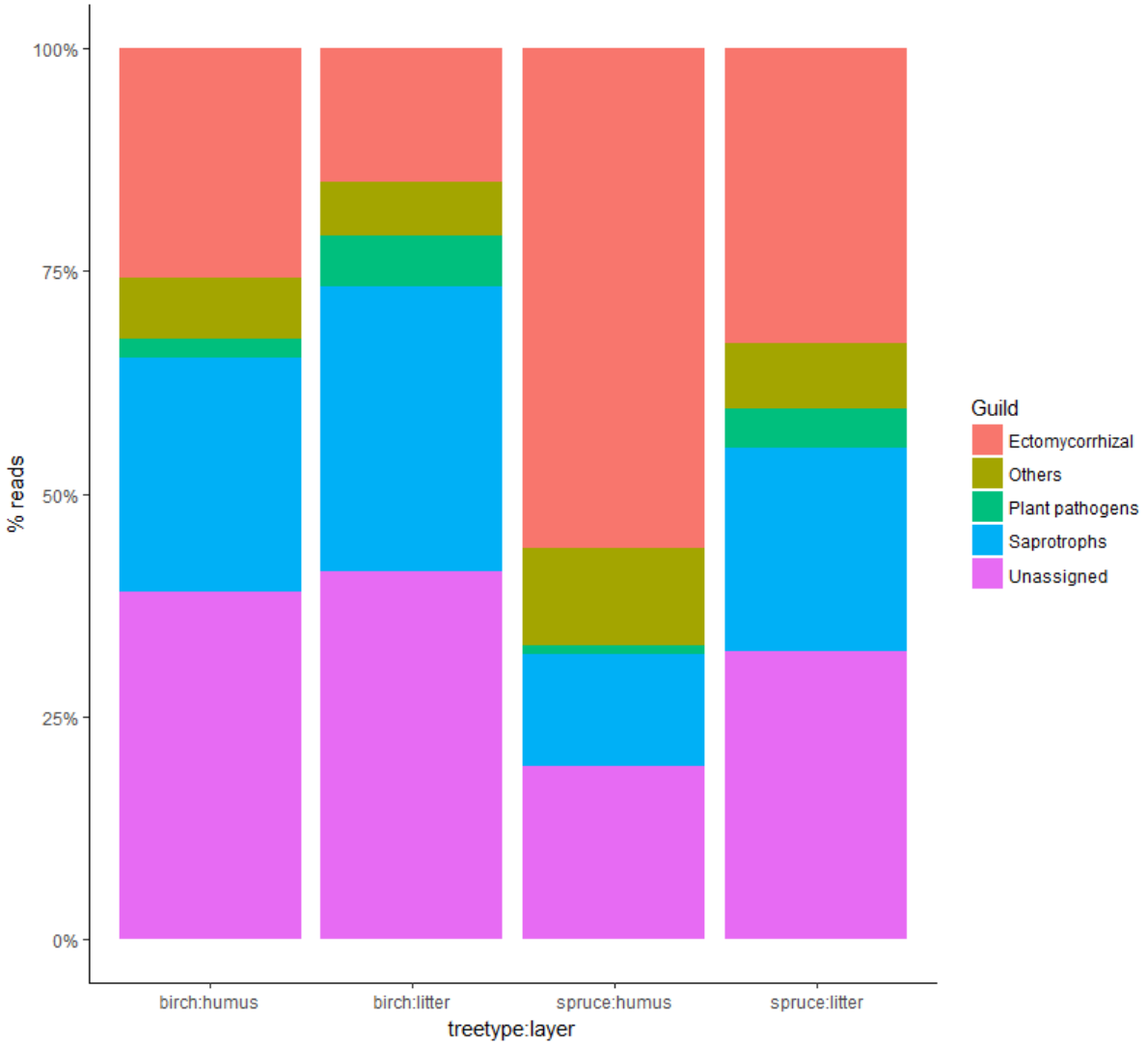

Figure S2. Stacked bar chart showing relative abundance of fungal functional groups shown in each sample category, with abundances estimated as total proportion of fungal reads.