Amplicon pyrosequencing of fungal communities associated with boreal forest mosses reveals strong host and tissue specificity, and no clear response to nitrogen enrichment

Pyrosekvensering av soppsamfunn assosiert med mosar i boreal skog viser sterk verts- og vevstypespesifisitet, og liten respons på nitrogentilførsel

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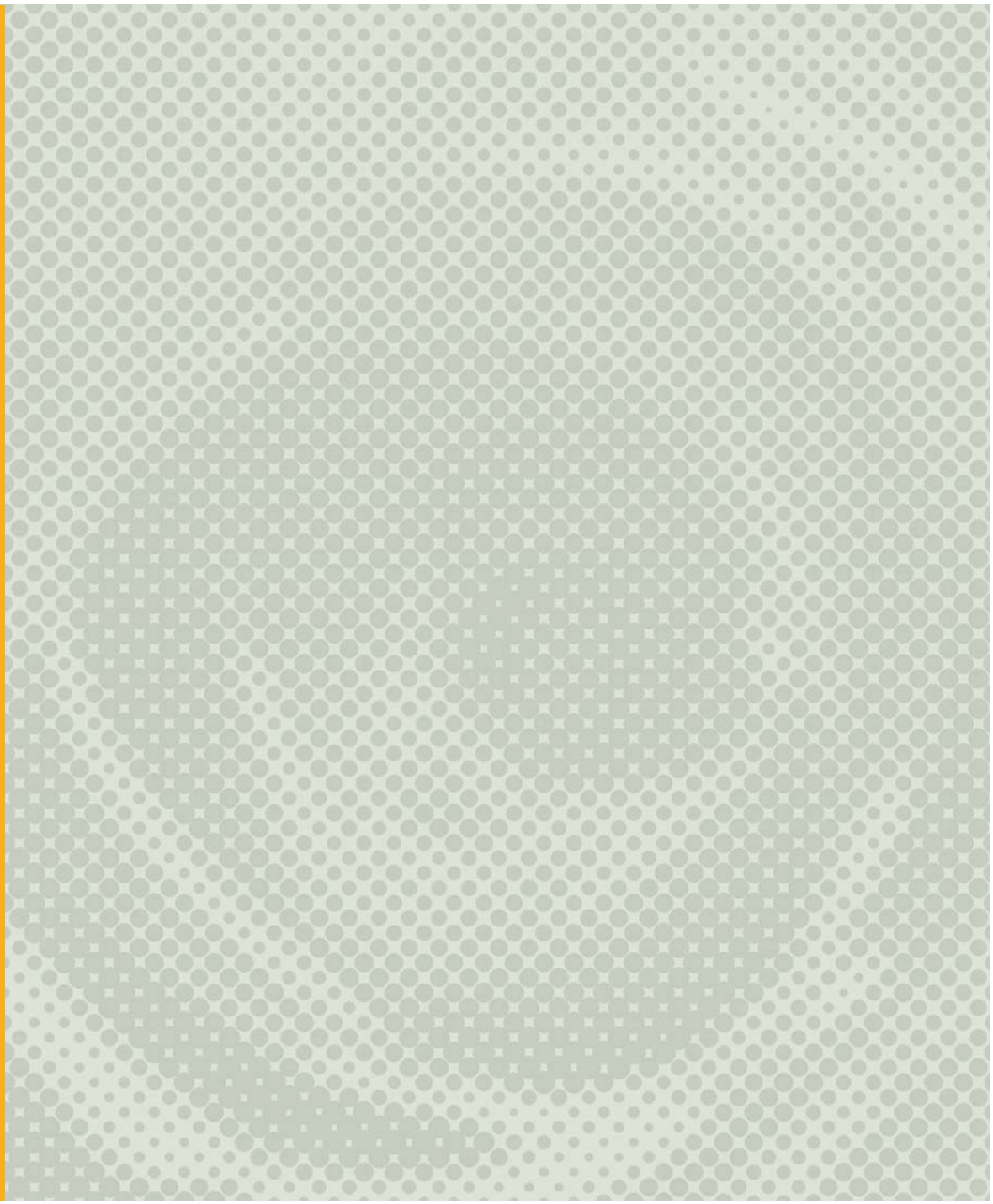

Preface

This work marks the completion of my Master of Science degree in Ecology at Department of Ecology and Natural Resource Management (INA) at the Norwegian University of Life Sciences (UMB). This thesis is a part of the program "There is more to the picture than meets the eye - endophytic fungi in boreal forest bryophytes", funded by the Norwegian Research council.

Firstly I would like to thank my supervisors. Thanks to Dr. Marie Davey for opening the door to the exciting world of fungi, and steady guidance through lab work and analyses. The hours spent at your office have been illuminating and instructive. Thanks to Prof. Mikael Ohlson for funding and helpful comments on the manuscript. I would also like to thank Prof. Rune Halvorsen at Norwegian Natural History Museum for comprehensive answers to the questions of a rookie in gradient analyses and statistical programming.

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Abstract

The fungal community associated with bryophytes is large and diverse. Despite their presumed ecological importance, bryophilous fungi are an understudied group, partly due to their inconspicuous and cryptic nature. The knowledge of fungal community differences between different bryophyte species and between different types of bryophyte tissues is meager. Nitrogen can alter the community structure of the boreal forest and anthropogenic nitrogen deposition over Norway is expected to increase by 10% the next century. The responses of plant communities to nitrogen enrichment are well documented, but the reported effects on fungal symbionts are contradictory, and specific results from bryophilous fungi are lacking.

The aim of this study is to assess the fungal community of two tissue types of three boreal forest mosses and the effect of nitrogen on this community. *Hylocomium splendens*, *Pleurozium schreberi* and *Dicranum scoparium* were collected in a boreal spruce forest in Norway from control and experimental plots established in 2003 that have been fertilized with 150 kg N ha^{-1} per year. 454 amplicon pyrosequencing was used to characterize the fungal communities associated with these mosses and the effects of nitrogen fertilization on community composition and species richness. This also permitted between hosts and tissue comparisons of the fungal associates, using GNMDS ordination and two species richness estimators. Amplicon pyrosequencing can detect rare and inconspicuous species, allowing us to characterize these minute and discreet communities in previously impossible detail.

A great richness of fungi was detected, including 3300 Operational Taxonomical Units (OTUs). The communities were dominated by Ascomycota (approximately 70%), followed by Basdiomycota (approximately 20%). Chytridiomycota, Glomeromycota and Zygomycota were detected, but in very low numbers. There was a clear difference in both species richness and community composition between the hosts. The two tissue types also differed in community composition and to a smaller degree in species richness.

Despite these differences there was no divergence detected at higher taxonomic levels such as phylum and order. There were no clear effects of nitrogen enrichment, as the two richness estimators showed opposite responses and there was no separation in community composition.

Samandrag

Soppsamfunnet i mosar er omfattande og har stor diversitet, men er uanseelege og kryptiske av natur. Dette mangfaldet er lite studert, sjølv om dei er anteke å ha stor betyding for lågarestående plantar. Det er dessutan derfor liten kunnskap om skilnadar på soppsamfunn mellom ulike mosevertar og ulike delar av mosestengelen. Auka nitrogentilførsel kan endra plantesamfunn i boreal skog, og nitrogen frå menneskeskapte kjelder i Noreg er venta å auka med 10% dei neste hundre åra. Plantesamfunnresponsen på auka nitrogentilførsel er godt dokumentert, men rapportar om effekten på soppsymbiontar er motstridande, og det finst ikkje studiar om soppar assosiert med mose.

Målet med denne oppggåva var å fastsetja storleiken og samansetjinga av soppsamfunnet i to vevstypar av tre mosar i boreal skog og undersøkja effekten av nitrogentilførsel på dette samfunnet. *Hylocomium splendens*, *Pleurozium schreberi* og *Dicranum scoparium* vart samla i ein boreal granskog i Noreg, i eit felt med kontroll- og eksperimentplott frå 2003. Desse plotta har årleg vore gjødsla med 150 kg N ha^{-1} årlig. Pyrosekvensering, GNMDS ordinasjon og to ulike estimatorar av artsrikdom vart nytta for å karakterisera soppsamfunnet i og på desse mosane og effekten av nitrogen på samfunnsstruktur og artsrikdom. Dette gjorde det òg mogleg å samanlikna samfunnet i dei ulike moseartane og vevstypane. Denne teknologien kan påvisa sjeldne og uanseelege artar og beskriva soppsamfunn på eit detaljnivå som før var umogleg.

Det vart funne stor artsrikdom av sopp, med 3300 operative taksonomiske einingar (Operational Taxonomical Units, OTU). Ascomycota dominerte (ca. 70%). Basidiomycota utgjorde òg ein stor del (ca. 20%). Chytridiomycota, Glomeromycota og Zygomycota vart funne, men i små kvantum. Det var ein klar skilnad i både artsrikdom og artssamansetning mellom vertsmosane. Dei to ulike vevstypane (brun og grøn) viste skilnad i samansetning, men mindre grad i artsrikdom. Trass i dette var det liten skilnad på høgare taksonomisk nivå, rekke og orden. Resultata viste ingen klar effekt av nitrogentilførsel. Dei to estimatorane gav to ulike svar og ingen skilnad viste seg i artssamansetninga.

Contents

1 Introduction

The symbioses between plants and fungi have a long evolutionary history and may have been crucial for plant colonization of land (Heckman et al., 2001). Plants analogous to bryophytes were the first to colonize land, and these are thought to have had a close association with fungi (Redecker et al., 2000). The shift from water to a terrestrial environment is believed to have happened between 480 to 460 Mya (Heckman et al., 2001) a timespan that allows for coevolution to have occurred between bryophytes and fungi. This is reflected in fungal taxa that are tightly linked to bryophytes, including about 350 species whose host range is restricted to them (Döbbeler, 2002). Despite the fact that feather mosses are dominant in the understory of boreal forests worldwide (Benscoter and Vitt, 2007, DeLuca et al., 2002) and are known to influence the structure of soil microbial communities (Bach et al., 2009), the fungal community of mosses is understudied (Davey and Currah, 2006). Furthermore, studies conducted on mycorrhizae and endophytes of vascular plants may not be transferable to the studies of bryophytes, as e.g. the decomposition rate of bryophytes are slower (Lang et al., 2009), and the fungal biomass present is shown to be higher (Davey et al., 2009).

The leaf surfaces and the total aerial part of a plant are termed the phyllosphere and host a wide range of microorganisms (Whipps et al., 2008). The microorganisms related to this environment can be epiphytic (growing on the host surface) or endophytic (growing inside the host) (e.g. Arnold, 2007, Osono, 2007). As epiphyte fungi live on the surface of the leaf, one can assume that they will be exposed to greater temperature and moisture fluctuations than endophytes, and they may also be less controlled by their host. This difference between the environment outside and inside a leaf has been shown to return a clear difference between epiphytic and endophytic fungal communities (Santamaría and Bayman, 2005), so to assess the whole fungal community associated with the moss phyllosphere, both the epi- and endophytes need to be inspected.

There have been recorded large and diverse epiphytic fungal communities on vascu-

lar plants, but their function is uncertain (Inacio et al., 2002), although they have been suggested as possible biocontrol agents (Buck et al., 1998). Neither is the ecological role of fungal endophytes well understood and the diversity of interactions between fungi and hosts are vast (Rodriguez et al., 2009). A fungal endophyte of vascular plants may have different ecological functions at different life history stages and in different environments. The genotype of the fungi may also play an important role in whether they become parasitic, mutualistic or pathogenetic (Schulz and Boyle, 2005). Their ability to impact host grazing susceptibility makes the endophytes an important factor in competition between plants (Clay, 1996). As in vascular plants, fungi associated with mosses can be pathogens, parasites and mutualists (Davey and Currah, 2006), and they may exhibit similar behavior and have similar implications for their host plants. Nevertheless, according to the differences between bryophytes and vascular plants and the great number of fungal species restricted to mosses, both the species richness and composition of the associated fungal community may be different.

Nitrogen is introduced to terrestrial ecosystems naturally through nitrogen fixation, but during the late 20^{th} century, human production of biologically available nitrogen became far greater than the natural fixation of nitrogen (Galloway and Cowling, 2002). The human sources of nitrogen discharged into natural environments are artificial fertilizer, fossil fuel and legumes planted for their nitrogen fixating properties (Galloway and Cowling, 2002). The total deposition of nitrogen over Norway is expected to increase by about 10% in the next 100 years, with large regional differences (Hole and Engardt, 2008). Most terrestrial ecosystems are nitrogen limited (Nordin et al., 2005, Tamm, 1991), including the boreal forest, making them sensitive to the addition of nitrogen. Results of fertilization experiments in boreal forests have suggested a shift from a shrub to a grass dominated understory (Nordin et al., 2005). The effect of fertilization on bryophytes has been investigated, but the effects on the microbial communities they harbor have not. For example, Skrindo and Økland (2002) found the quantity of mosses to decrease in a nitrogen enriched environment, while others have found the bryophyte community

composition to change while the bryophyte cover did not (Gordon et al., 2001).

The responses of boreal forest fungal communities to increases in nitrogen availability are not well understood. Nitrogen fertilization may reduce species richness of ectomycorrhizal communities (Lilleskov et al., 2002a). However; this change in community structure may also be due to acidification, rather than fertilization. On the other hand; Ishida and Nordin (2010a) found no effect of nitrogen fertilizing on ericoid mycorrhizal fungal communities, suggesting different communities respond differently to nitrogen fertilization. Bryophytes have a high capacity to assimilate inorganic nitrogen through the leaf surface (Nordin et al., 1998), and despite the suspicion of mosses not being limited by nitrogen (Skre and Oechel, 1979), effects of nitrogen enrichment on boreal forest mosses have been recorded (e.g. Nordin et al., 1998, Skrindo and Økland, 2002). The biomass of the microbial community of mosses has been shown to change due to atmospheric pollution including nitrogen dioxide (Meyer et al., 2010a, Meyer et al., 2010b), but the effect on the composition of this community is uncertain.

It has been demonstrated that great fungal diversity exists within the photosynthetic tissues of boreal mosses (Kauserud et al., 2008), and the senescent part of mosses are known to harbor non-mycorrhizal associations with fungi (Carleton and Read, 1991). In vascular plants, differences in diversity and richness between different tissue types are found (e.g. Fisher and Petrini, 1990, Verma et al., 2007), yet the knowledge of differences between fungal communities of tissues of mosses is sparse. There are great structural differences between the two tissue types which can affect the fungi living within. These differences are e.g. related to moisture (Bates, 1998), nutrients (Eckstein and Karlsson, 1999), nutrient leaching (Carleton and Read, 1991), weather exposure and age. There is more fungal biomass in the senescent tissues of mosses than the photosynthetic (Davey et al., 2009) and there is a horizontal difference in fungal colonization between the different depths of the litter layer in forests inhabited by feather mosses (Lindahl et al., 2007), although it is unclear if this difference continues at higher levels. Microscopy studies indicate that there are higher numbers of fungi associated with the brown than the

green tissue of mosses (Parke and Linderman, 1980), but this comparison has not been conducted using molecular tools.

Mosses of different species have different microniches for fungal colonization (Döbbeler, 2002), and harbor different amounts of fungal biomass (Davey et al., 2009). The moss hosts have e.g. different decomposition rates (Lang et al., 2009) and growth form (Bates, 1998) which could indicate the presence of different microniches. The endophytic communities of vascular plants are known to have strong host preferences (Arnold et al., 2001), while the species richness can remain constant (White and Backhouse, 2007). There have been demonstrated great fungal community differences between moss host species when only the green tissue has been analyzed (Kauserud et al., 2008), but comparisons of the community of the whole moss are absent.

The fungi associated with mosses are inconspicuous and cryptic and may be present at life-stages that are not possible to assign to taxa using morphologic criteria. To assess the total fungal community, molecular tools are needed. Other studies using high throughput sequencing to assess fungal communities in soil (Buée et al., 2009) and in the phyllosphere of trees (Jumpponen and Jones, 2009) have revealed an unexpectedly high fungal diversity. This may also apply for the phyllosphere of mosses.

The main aim of this study is to assess the fungal community associated with mosses in boreal Norway spruce forest using pyrosequencing. More specifically my aims are first to compare the fungal community composition and fungal species richness between nitrogen fertilized plots and natural environments and secondly between three different moss host species. I will also make pairwise comparisons of the senescent and photosynthetic part of the same moss shoots.

2 Materials and Methods

2.1 Study area

The sampling was conducted in a Norway spruce (*Picea abies*) forest near Kittilbu in Gausdal Vestfjell, Norway (61°10‘north, 09°90‘east, 816m.a.s.l.). The ground cover was dominated by *Vaccinium myrtillus* and common boreal feather mosses (*Hylocomium splendens, Pleurozium schreberi, Polytrichum commune*, and *Dicranum* spp). Mean annual temperature is -0.1 °C and mean precipitation is 810 mm annually (retrieved from www.met.no). The soil is glacial moraine while the rock is conglomerate and sedimentary breccia (retrieved from www.ngu.no).

2.2 N fertilization experiment and sampling of bryophytes

In a fertilization experiment, nitrogen has been added annually since 2003 to ten 15x15 m experimental plots in the forest at a rate of 150 kg N ha^{-1} per year. The fertilizer used was granulated pellets from Yara, YaraMila™ Fullgjødsel© 25-2-6, with 24.6% N, 2% P, 6% K, and trace elements. In 2010, when the bryophytes for this study were sampled, there was a clear visual difference between nitrogen enriched and control plots (n=10), with a shift towards a more grass dominated forest floor.

The sampling was done in the last week of June 2010. Because bryophytes are known to respond species-specifically to nitrogen treatments (Nordin et al., 1998) three different species were chosen for this study, i.e. *Hylocomium splendens*, *Pleurozium schreberi* and *Dicranum scoparium*. Healthy shoots were selected for analysis. Eight shoots of each species were collected from three nitrogen treated plots and three control plots, giving a total of 144 shoots, which in turn were divided into a senescent and photosynthetic part, resulting in a total of 288 samples. Shoots of each species were collected randomly from five moss colonies in each plot, and kept frozen until DNA extractions were conducted.

The shoots were rinsed in tap water to remove spruce needles and other debris. To remove superficial fungal hyphae and spores adherent to the surface of the moss, they were

washed in 0.1% Triton-X, rinsed thoroughly in distilled water, and then finally rinsed in autoclaved distilled water. The shoots were divided into pairs of green, photosynthetic and brown, senescent fragments and the individual fragments were placed in Eppendorf tubes, while keeping track of the green/brown-pairs. After freeze drying, the shoot fragments were pulverized using a Retcsh ball mill (VERDER Group, Netherlands). DNA was extracted using a modified CTAB-based extraction protocol in accordance to Murray and Thompson (1980) and Gardes and Bruns (1993) and all extracts were cleaned and purified using the Wizard©SV Gel and PCR Clean-Up System (Promega, USA).

2.3 Molecular analyses

The Internal Transcribed Spacer 2 (ITS 2) region of rDNA was amplified for all samples using a nested PCR approach with a 2720 Thermal Cycler from Applied Biosystems. The initial PCR amplified the whole ITS region using the fungal specific primer ITS1-F, and ITS4 (Gardes and Bruns, 1993, White et al., 1990). The second PCR targeted the ITS2 region using the primers ITS3 and ITS4 (White et al., 1990) and added one of 19 unique, 10 bp tags to each sample to allow sample recognition in downstream analyses after pyrosequencing.

The reaction volume in the first PCR was 20 μL containing 0.4 units Phusion polymerase (Finnzymes Oy, Finland), 0.5 μM of each primer, 1.7μM of dNTP, 1x Phusion PCR-buffer (Finnzymes Oy, Finland) and 2 μL template (10x dilution). The PCR conditions in the first run were as follows: Initial heating to 98°C for 30 seconds, denaturation at 98°C for 10 seconds, annealing at 53°C for 20 seconds and extension at 72°C for 10 seconds repeated 30 times, followed by a 7 minute extension at 72°C before storage at 4°C. Conditions in the second PCR were identical to the first, with the exceptions that the reaction volume was 20 μL, including 4 μL of template (a 50x dilution of the PCR product from the first run), and the denaturing-annealing-extension cycle was repeated only 10 times. The products from the final PCR were cleaned with the Wizard© SV Gel and PCR Clean-Up System (Promega, USA) according to the manufacturer's instructions.

All PCR products were quantified using a Quant-It ^{TM} Kit microplate reader with PicoGreen© quantification reagent and equimolar amounts of a subset of samples bearing each of the 19 unique tags were pooled. They were sequenced on a Roche GS FLX Titanium Series 454 sequencing platform at the Norwegian High-Throughput Sequencing Centre (University of Oslo, Oslo, Norway), on a single plate divided into 16 lanes. Of the 288 samples, 16 were sequenced twice to control the validity of the sequencing (from now on termed pyrosequencing controls).

2.4 Bioinformatics

A total of 451 987 sequences generated by the pyrosequencing were processed, clustered and blasted in the CLOTU pipeline (Kumar et al., 2011) (available at www.bioportal.uio.no). No sequencing errors were allowed the tags, but one error was allowed in both forward and reverse primers, and those sequences with additional errors in these regions were discarded. Sequences with incompatible tags were removed to eliminate chimeric sequences, and sequences with one or more ambiguous bases (N's) were removed from the dataset. Sequences shorter than 250 base pairs were also discarded. All homopolymers over 6 base pairs were collapsed to account for imprecise base-calling of long homopolymers. Adaptors and tags were trimmed from the sequences prior to clustering. After the filtering and trimming steps 376 226 sequences (150 224 unique) were clustered into operational taxonomical units (OTUs), using the CD-HIT global algorithm at a level of 75% coverage between sequences and 97% sequence similarity. A total of 6125 OTUs were generated. The longest sequence from each cluster was compared to the NCBI sequence database using the BLAST algorithm. Each OTU was assigned to phylum and order when possible.

Singletons (OTUs containing only one sequence read), non-fungal OTUs (snails, grasses and moss hosts), and the 16 pyrosequencing controls were removed from the dataset. A GLM of the number of reads versus the number of OTUs indicated a strong correlation between reads and OTUs, implying insufficient sampling. Samples with fewer than 800 reads were deemed to have insufficient sampling and excluded, and a GLM of the remain-

ing samples indicated there was no longer a significant correlation between reads and OTUs (p=0.07). The final matrix included 224 of the 288 samples and 3300 OTUs.

2.5 Statistical analysis

The 16 pyrosequencing controls were pair-wise compared with Jaccard similarity index (percentage of species shared), Chao Sørensen Raw Abundance-based similarity index, Chao's estimator for Chao's Sørensen Abundance-based similarity index, Chao Sørensen Raw Incidence-based similarity index and Chao's estimator for Chao Sørensen Incidence-based similarity index. Amend et al (2010) showed that within species comparisons of abundance data from pyrosequencing would be appropriate, but between species comparison should be avoided. However, in this case the within species comparisons of abundance would also be unreliable based on the difference between both the raw similarity indices and the estimator indices (Table 1). Hence, the incidence data set is used for further analyses, with the exception of weighted abundance data used in GNMDS ordination. The indexes were also calculated for species present in more than 66 samples (most common OTUs/core taxa), which resulted in a higher similarity between controls and for the 20 most common OTUs (Table 1).

Table 1: The similarity between the pyrosequencing control pairs for the all taxa, the 66 most common, and the 20 most common taxa. The percentage of species shared between samples (SS), Chao Sørensen Raw Abundance-based similarity index (CSA-raw), Chao's estimator for Chao's Sørensen Abundance-based similarity index (CSA-est), and Chao Sørensen Raw Incidence based similarity index (CSI raw) and Chao's estimator for Chao's Sørensen Incidence-based similarity index (CSI-est) are noted. Control pair is abbrevated to CP.

	All taxa					66 most common taxa					20 most common taxa				
CP	SS (%)	CSA-raw	CSA-est	CSI-raw	CSI-est	SS (%)	CSA-raw	CSA-est	CSI-raw	CSI-est	SS (%)	CSA-raw	CSA-est	CSI-raw	CSI-est
1	31.70	0.866	0.924	0.482	0.598	0.614	0.946	0.953	0.761	0.871	0.875	0.986	0.997	0.933	0.966
2	31.80	0.905	0.943	0.482	1	0.714	0.982	0.996	0.833	0.896	0.714	0.986	1	0.833	0.952
3	32.80	0.906	0.983	0.494	1	0.768	0.979	0.99	0.869	0.869	0.944	0.999	1	0.971	0.971
4	31.00	0.928	0.963	0.473	0.593	0.682	0.986	0.997	0.811	0.811	0.778	0.996	0.997	0.875	0.903
5	46.50	0.96	0.989	0.635	1	0.69	0.977	0.979	0.816	1	0.923	0.948	0.966	0.96	1
6	43.70	0.957	0.99	0.608	0.785	0.758	0.993	1	0.862	0.862	0.857	0.991	1	0.923	0.96
7	31.50	0.885	0.99	0.479	1	0.654	0.977	0.987	0.791	0.907	0.75	0.98	0.988	0.857	0.909
8	36.10	0.929	0.977	0.531	0.684	0.581	0.986	0.992	0.735	0.735	0.6	0.99	0.991	0.75	0.857
9	30.50	0.902	0.952	0.467	0.701	0.625	0.976	1	0.769	0.769	0.8	0.994	0.995	0.889	0.923
10	35.90	0.948	0.975	0.528	1	0.526	0.982	0.989	0.69	0.769	0.4	0.924	0.933	0.571	1
11	40.00	0.922	0.994	0.571	1	0.727	0.989	1	0.842	0.906	0.867	0.996	0.997	0.929	0.929
12	49.40	0.932	0.987	0.661	0.820	0.757	0.985	0.996	0.862	0.933	0.8	0.993	1	0.889	1
13	32.30	0.902	0.928	0.488	1	0.717	0.977	0.987	0.835	1	0.667	0.985	0.985	0.8	0.909
14	34.90	0.958	0.992	0.518	1	0.533	0.961	0.975	0.696	0.842	0.455	0.926	0.952	0.625	1
15	33.30	0.939	0.959	0.5	0.584	0.607	0.969	0.973	0.756	0.756	0.571	0.96	0.961	0.727	0.889
16	41.70	0.932	0.983	0.589	1	0.805	0.983	1	0.892	0.93	0.882	0.995	1	0.727	0.903

2.6 Species richness

Two estimators for species richness were used: Chao2 and the Ugland approach (Ugland et al., 2003). Ugland provides a total species curve (T-S curve), which is the semi-log approximation of the average between several accumulation curves. This curve will not reach asymptote but it will terminate at a chosen size of total area, in this case the total number of moss shoots. The chosen end point is the total species richness. This method takes the heterogeneity between different subareas into account. Both of the estimators were calculated using the OTU richness, which will from here forth be termed 'species richness'. Chao 2 estimate for species richness and species accumulation curves were calculated using EstimateS (Colwell, 2009). The combined species richness was estimated for all samples, and also for the nitrogen plots versus the controls, the three host species, and the brown versus the green part of the shoots. In the Ugland approach the six experimental plots were used as sub-areas, and species richness was calculated for 100 000, 1 000 000, and 10 000 000 shoots for the total and for all the categories. Chi-square tests comparing the nitrogen and control treatments, hosts, and tissue types were calculated in Excel. The average number of OTUs (the raw counts) in the different treatments were calculated and tested with a partially nested ANOVA using the aov function in R (R Development Core Team, 2011).

2.7 Differences in composition

The ordinations were done in R (R Development Core Team, 2011) using the vegan (Oksanen et al., 2011) and MASS (Venables and Ripley, 2002) packages. Global non-metric multidimensional scaling (GNMDS) (Minchin, 1987) and detrended correspondence analysis (DCA) (Hill and Gauch, 1980) were performed in parallel to prevent any disruption of true gradients as recommended by Økland (1996). Correlation analyses were performed between the parallel GNMDS and DCA axes, and between axes found for different types of data. All correlation analyses between ordination axes were run by calculating a Kendalls

Tau value.

DCA ordinations were run in R using the decorana function in the vegan package with default options. GNMDS ordinations were run in R using the vegan and MASS packages, and the functions vegdist, initMDS, isoMDS and postMDS. As the number of reads is a dubious proxy for number of individuals, the ordinations were run on both incidence data and abundance data. The abundance data were transformed by weighting so that the highest number of reads/individuals would be 10 and the lowest would be 1. The transformation allows for the assessment of the effect of species abundance without assuming the number of individuals in the different OTUs is correct. The number of dimensions was to begin with set to two, and the dissimilarity index used was Bray-Curtis (which in the incidence data will be identical to Sørensens index). In order to find the two most similar solutions the number of individual runs was 300 and the process was stopped after 200 iterations and tolerance was set to 10^{-7}. (Procrustes comparison correlation=0.9783, p=0.001). For abundance data the two best solutions were identical (Procrustes comparison correlation=0.997, p=0.001). The best solutions for both ordinations were subjected to varimax rotation by PCA to make the largest part of the variance be on the first axis.

The GNMDS were also run with three and four dimensions to check whether or not these additional axes would give a better ordination of the data. This was done for both incidence and transformed abundance data. Correlation analyses were run between all corresponding GNMDS and DCA axes for both the two, three and four dimensional GNDMS. The higher dimensional GNMDS were only chosen if the additional axes included additional variation. The correlation between incidence base ordinations suggest that two dimensions are appropriate, while for the transformed abundance data three dimensions are more suitable (Table 2).

The DCA showed a pronounced tongue effect (Minchin, 1987) in the second axis when the incidence species matrix was analyzed and a slight tongue effect in the transformed abundance data. The DCA axis 1 and GNMDS axis 1 were highly correlated, and this axis is believed to reveal a true gradient structure. The second axes showed weaker

Table 2: Kendalls Tau for GNMDS versus DCA ($p<0.0001$ for all). Bold numbers indicates the chosen number of dimensions

Incidence data	Two dimensions	Three dimensions	Four dimensions
GNMDS 1 vs DCA 1	**0.8447**	-0.8675	0.8703
GNMDS 2 vs DCA 2	**0.3394**	-0.3786	-0.3945
GNMDS 3 vs DCA 3		-0.3666	-0.3340
GNMDS 4 vs DCA 4			-0.2777
Transformed abundance data			
GNMDS 1 vs DCA 1	0.8443	**0.8657**	0.8776
GNMDS 2 vs DCA 2	-0.3814	**-0.6403**	0.6465
GNMDS 3 vs DCA 3		**0.1568**	-0.1143
GNMDS 4 vs DCA 4			-0.0720

correlation. The GNMDS ordinations were chosen for further analysis because of the tongue structure in DCA and the apparent lack of flaws in GNMDS ordination diagram. The ordinations were repeated with taxa present in more than 66 samples to account for the low similarity between pyrosequencing control samples. Also correlation tests and procrustes correlation test were performed between the ordination axes of the most common taxa and all taxa.

The GNMDS scores were tested in two different ways. A partially nested ANOVA was performed on both GNMDS axes using the aov function in R with nitrogen fertilization, host species, and tissue type as fixed factors and paired samples nested within experimental plots as random factors (where paired samples are nested within experimental plot). Additionally, vectors were fitted to the whole ordination using the function envfit from vegan package in R and tested with 999 permutations.

Both Jaccard and Sørensens similiarity indexes were calculated in EstimateS (Colwell 2009) for each combination of host species and tissue type using the total incidence of species in each category.

3 Results

3.1 Taxonomic results

Of the fungal OTUs recovered 72.09 % were Ascomycetes, 23.39 % were Basidiomycetes, 1.42 % were Chytridiomycetes, 1.15 % were Zygomycetes, 0.18 % were Glomeromycetes, and 1.76 % of them were impossible to assign to any specific phylum (Figure 1). The most common orders were Helotiales (29.43 %), and Agaricales (6.56 %). 90% of the OTUs were present in less than 10 % of the samples. The closest BLAST match to the most abundant OTU was *Catenulifera brachyconia* (Accession no GU727557 in GenBank). It included 27 664 reads and was present in 182 of the samples. The second most abundant OTU was *Lecidea cancriformis* (Accession no DQ534472 in GenBank). This OTU had 24 524 reads recorded and was present in 188 of the samples. On the other hand, the most frequent OTU (present in most samples) had only 5161 reads detected (Accession no HQ335298 in GenBank), and the closest BLAST match was to an ascomycetous fungal endophyte of unknown order (Table 3).

The fungal community associated with the mosses studied was dominated by ascomycetes, especially members of the Helotiales, Lecanorales, Chaetothyriales and Hypocreales. Among the basidiomycetes the most common orders were Agaricales, Tremellales, Atheliales and Sporidiobolales. There were small differences in the taxonomic distribution of OTUs between the nitrogen-fertilized and the unfertilized plots (Figure 2a), between the senescent and photosynthetic tissues (Figure 2b) and between the three host species (Figure 2c). The general taxonomic composition of all of these groups was consistent with that of the whole moss-associated community.

Table 3: The 20 most frequent OTUs and their BLAST results from GenBank. Number of sequences (NR), how many samples they are present in (NS) and the percentage pairwise sequence identity (SI) are noted. No Hit means that no match was found in GenBank.

OTU	Taxonomic affinity	Accession No	NR	NS	% similarity
1	Fungal endophyte (Ascomycota)	HQ335298	5161	196	93%
2	Lecidea cancriformis	DQ534472	24524	188	95%
3	Catenulifera brachyconia	GU727557	27664	182	95%
4	Fungal endophyte (Ascomycota)	HQ335298	1434	169	94%
5	Ascomycota	HM123479	8765	168	94%
6	Cladophialophora sp	EU035413	4417	166	96%
7	Rhodotorula sp	AM922291	4070	166	98%
8	Helotiales sp	FJ196296	6092	161	95%
9	Fungal endophyte (Ascomycota)	HQ335303	5006	152	96%
10	Trichoderma viride	EU871036	3229	147	97%
11	Ascomycota	DQ068342	2520	142	97%
12	Helotiales sp	AY465452	6120	140	97%
13	Helotiales sp	FJ196296	3374	135	94%
14	Lecidea cancriformis	DQ534472	1326	120	96%
15	Ascomycota sp	FJ008678	654	118	97%
16	No hit	NO HIT	9200	117	-
17	Ascomycota sp	FJ008692	389	117	95%
18	Ascomycota	HQ335303	2158	116	98%
19	Tylospora sp	AJ534922	6823	115	98%
20	Cladophialophora sp.	EU139144	3890	114	85%

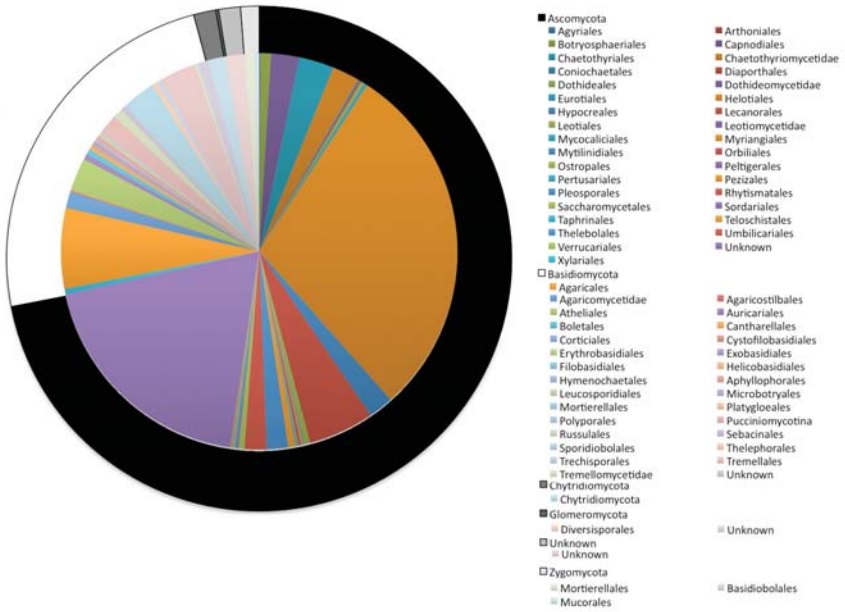


Figure 1: The taxonomic distribution of OTUs from all samples, the inner circle shows the distribution of orders, and the outer ring shows the distribution of phyla.

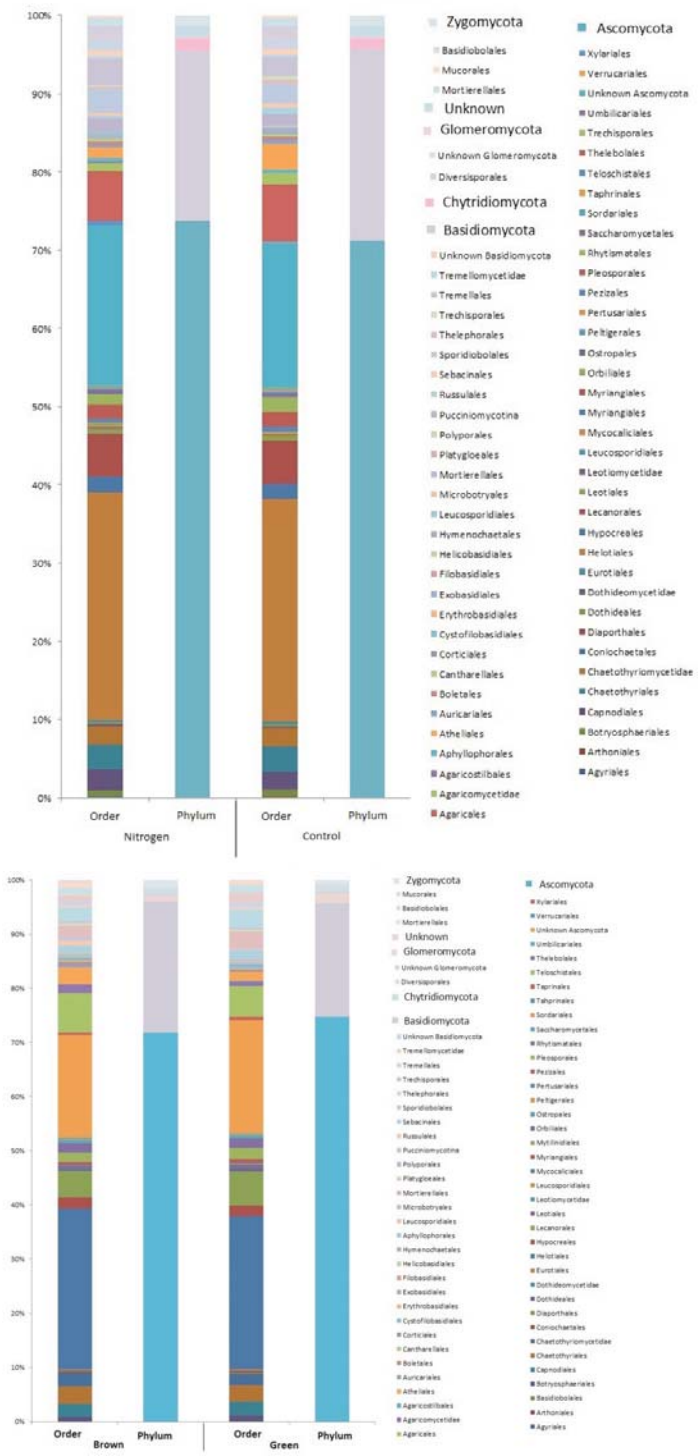
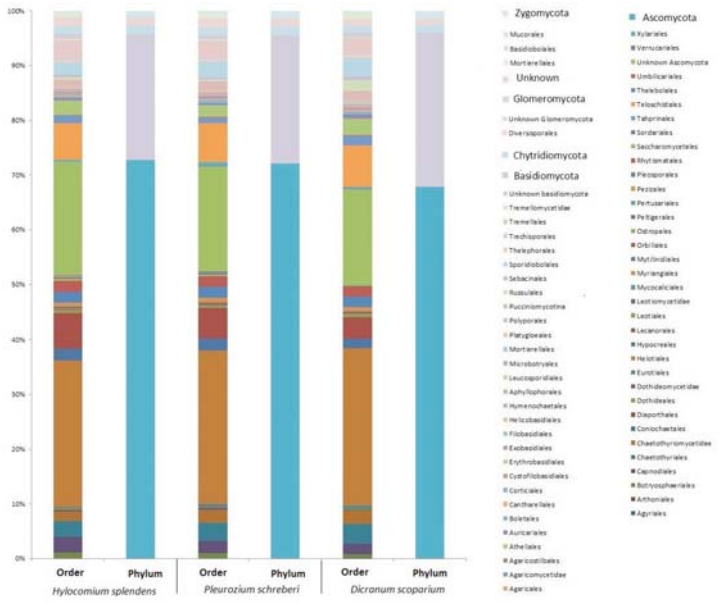


Figure 2: (a): The taxonomic distribution of OTUs present in nitrogen fertilized versus control plots, where the colored bars represent the proportion of taxonomic assignations at order and phylum level. (b): The taxonomic distribution of the OTUs in the photosynthetic and the senescent tissue where the colored bars represent the proportion of taxonomic assignations at order and phylum level.



(c)

Figure 2: (c): The taxonomic distribution of the OTUs in the three host species where the colored bars represent the proportion of taxonomic assignations at order and phylum level.

3.2 Species Richness

The average number of OTUs found in the three different treatments showed some differences, and the difference between nitrogen plots and controls and the three host species were significant (Figure 3 and Table 4). The sampling rarefaction curve for the whole area did not reach asymptote (Figure 4a), nor did the curves for the six experimental plots (Figure 4b). According to the Chao2 species richness estimator, the total species richness was 3600 OTUs (accumulation curve not shown). There was no significant difference in species richness between the fertilized and the non-fertilized experimental plots (Chi-test p=0.326) (Figure 5a). The estimated total species richness was higher in the brown part of the moss shoots than in the green (Chi-test p=0.005) (Figure 5b). There was a significant difference between the estimated species richness in *Dicranum scoparium* and the species richness in *Hylocomium splendens* and *Pleurozium schreberi* (Chi-test p=0.041) (Figure 5c).

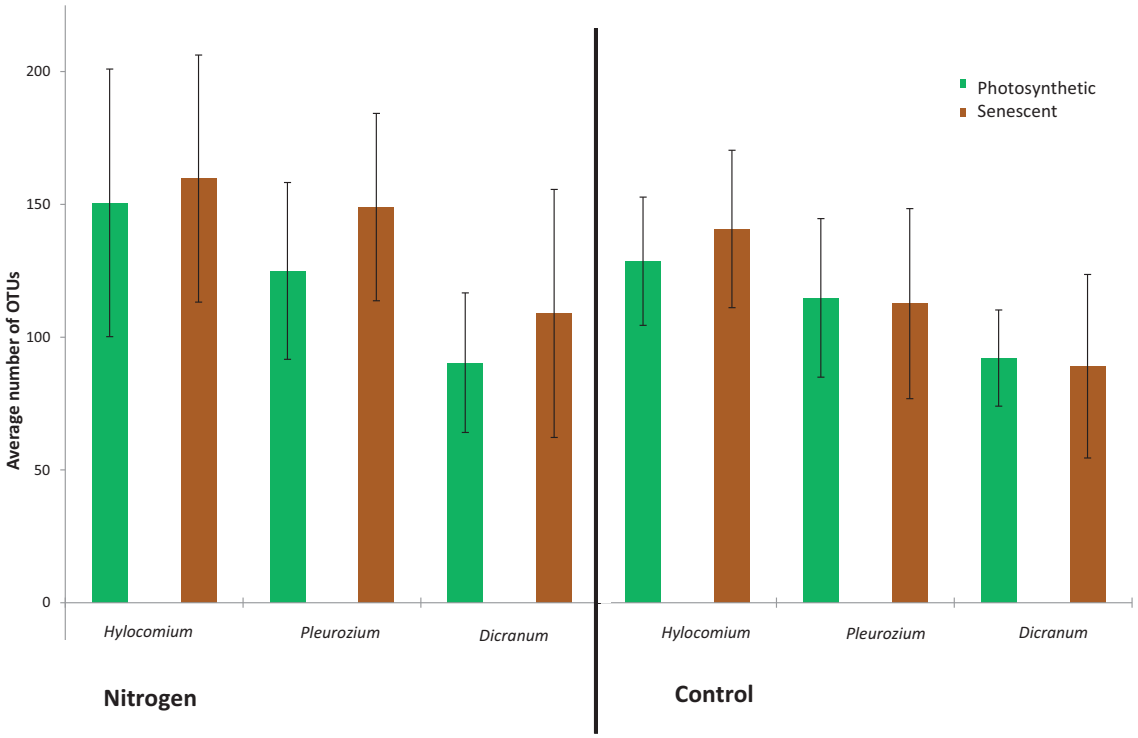
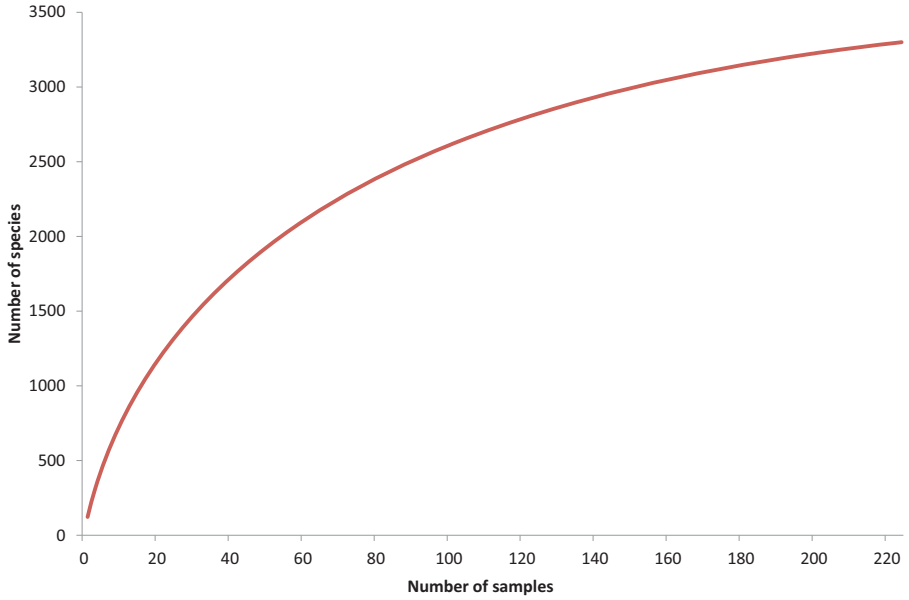


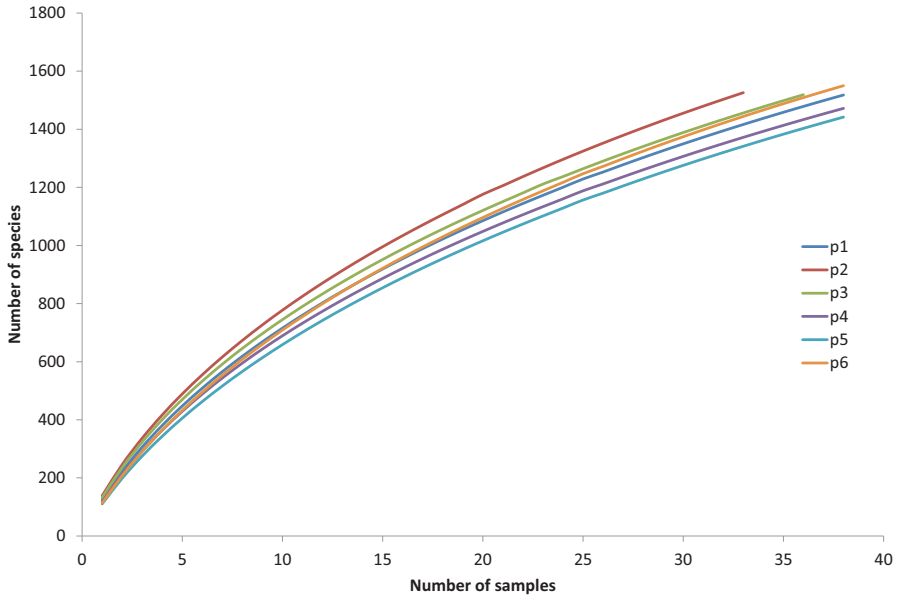
Figure 3: The average number of OTUs detected for each fertilization treatment, host species and tissue type. Standard deviations are marked with error bars.

Table 4: Testing the difference between the average number of raw counts of OTUs using a partially nested ANOVA. Degrees of freedom (Df), Sum of squares (Sum Sq) and Mean sum of squares (Mean Sq) are noted. Significant p-values are in bold.

Response	Factor	Df	Sum Sq	Mean Sq	F value	p-value
Average number of OTU						
	Fertilization	1	19298	19298	13.6044	**0.0003**
	Host	2	89680	44840	31.6108	**<0.0001**
	Tissue	1	877	877	0.6185	0.4330
	Fertilization:Host	2	2398	1199	0.8452	0.4318
	Fertilization:Tissue	1	4226	4226	2.9791	0.0867
	Host:Tissue	2	644	322	0.227	0.7972
	Fertilization:Host:Tissue	2	1294	647	0.456	0.6348
	Residuals	130	184406	1419		

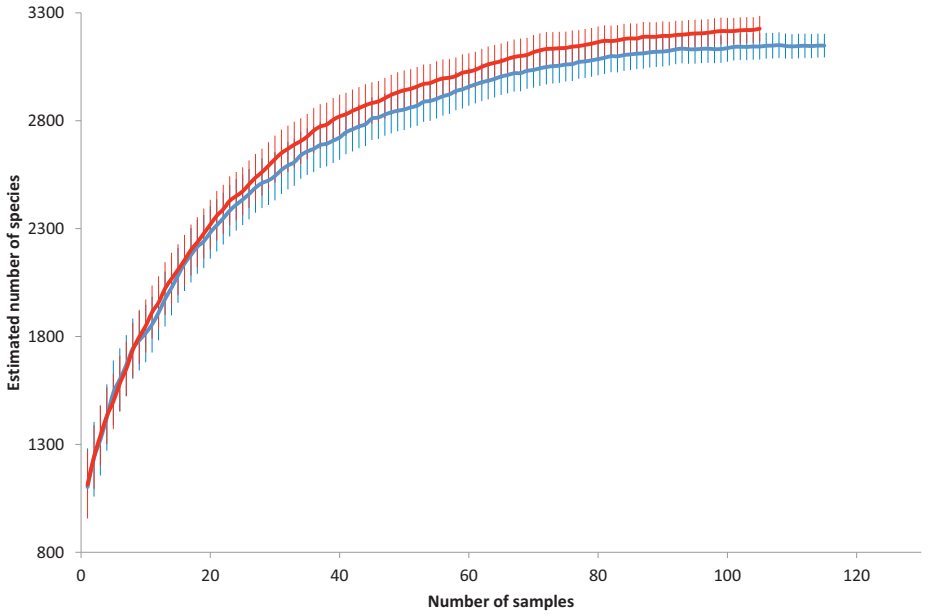


(a)

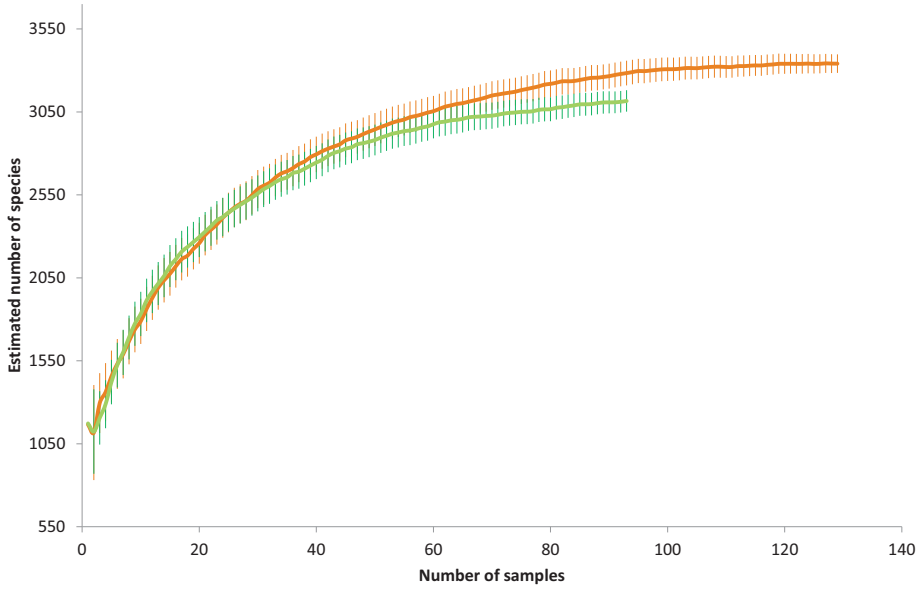


(b)

Figure 4: (a) Rarefaction curve for the whole area and (b) for for the six experimental plots, both calculated by EstimateS.

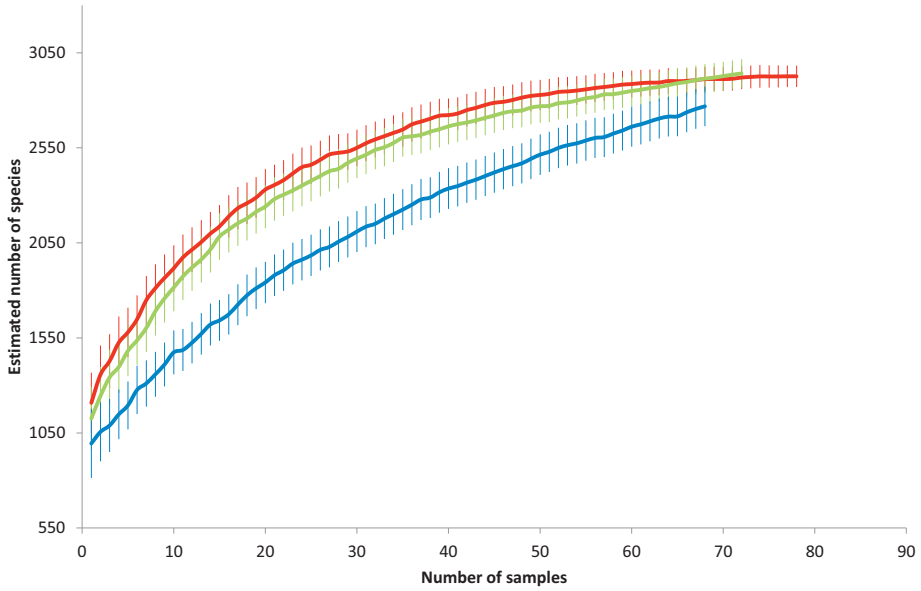


(a)



(b)

Figure 5: (a): Species accumulation curves for the nitrogen fertilized plots (blue) versus the control (red) plots, based on the Chao 2 estimator of species richness. (b): Species accumulation curves for the brown versus the green part of the moss shoots, based on the Chao 2 estimator for species richness. The error bars are standard deviations calculated in EstimateS for both (a) and (b).

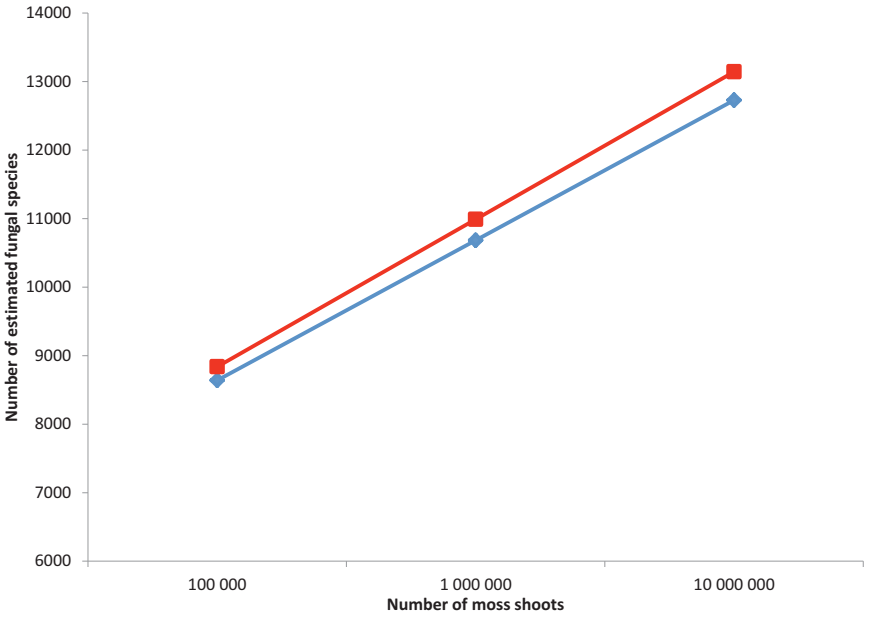


(c)

Figure 5: (c): Species accumulation curves for the three host species, *Hylocomium splendens* (red), *Pleurozium schreber*i (green), and *Dicranum scoparium* (blue), based on the Chao 2 estimator for species richness. The error bars are standard deviations calculated in EstimateS.

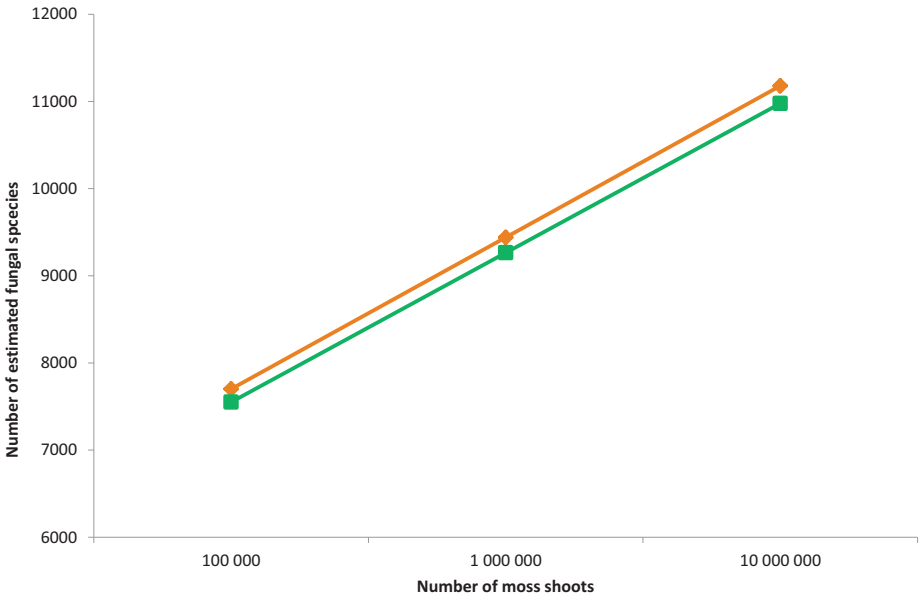
Ugland's method for species richness estimation gave higher species richness estimates than Chao2 for all categories. The total species richness was estimated to 9069 for 100 000 shoots, 11 242 for 1 000 000 shoots, and 13 415 for 10 000 000 shoots. The two estimators show the same differences in species richness between tissue type and host species, but opposite difference between nitrogen fertilized and the control plots. Only between host species did the Ugland approach give a difference of any magnitude. Ugland gave slightly higher species richness in the control plots than in fertilized plots (Control: Y=934.86x - 1925, R^2=0.9994, Fertilized: 888x - 1584.5, R^2=1, (Figure 6a)). There was higher species richness in the brown than in the green part of the moss (Brown: Y=755.28x - 994.09, R^2=0.9897, Green: Y=744.45x - 1021.2, R^2=0.9946, (Figure 6b)), and higher species richness in *Hylocomium splendens* and *Pleurozium schreberi* than in *Dicranum*

scoparium (H. splendens: Y=777.55x - 1129.7, R^2=0.9919, P. schreberi: Y=709.71x - 974.71, R^2=0.9939, D. scoparium: Y=552.44 - 699.17, R^2=0.9885, (Figure 6c)).

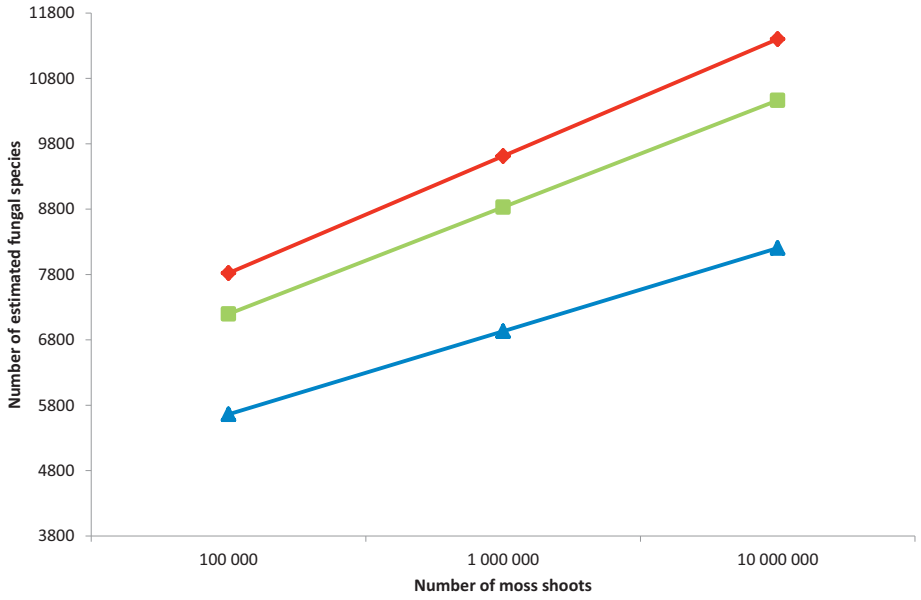


(a)

Figure 6: (a): Estimated species richness for 100 000, 1 000 000, and 10 000 000 moss shoots in the nitrogen (blue) versus the control (red).



(b)



(c)

Figure 6: (b): Estimated species richness for 100 000, 1 000 000, and 10 000 000 moss shoots in the green (green squares) versus the brown part (brown diamonds) of the moss shoots, and (c): for the three moss hosts *Hylocomium splendens* (red), *Pleurozium schreberi* (green), and *Dicranum scoparium* (blue).

3.3 Differences in community composition

The main gradient found in both GNMDS and DCA was separated by host species and tissue type (Figure 7), while the second gradient in GNMDS contained variation related to the two different tissue types. The nitrogen fertilized plots did not segregate on any axes (Figure 8).

The GNMDS ordination was significantly related to the host species (r^2=0.3684, p<0.001, (Figure 7)) and tissue type variables (r^2=0.1651, p<0.001, (Figure 7)) when these parameters were fitted as vectors, while there was no significant relationship between the ordination and the fitted vector of nitrogen treatment (r^2=0.0081, p=0.162, (Figure 8)). A nested ANOVA shows that there is a significant effect of all three main factors, on the firs axis (Table 5), but this has to be regarded in the light of the significant interaction between the effects of fertilization and tissue type and host and tissue (Interaction plot provided in Appendix A).

There was high similarity between the ordination based on the presence-absence species matrix and the ordination based on the transformed abundance of species (ordination diagram not shown) (Axes 1: Tau=0.9146, axes 2: Tau= -0.7414, p<0.0001 for both). Still, there were slight differences when vectors were fitted to the ordination. Both the host species (r^2=0.3935, p<0.001) and tissue type variables (r^2=0.1751, p<0.001) explained significant variation in the data when these were fitted as vectors. However, there was also a marginally significant fit of the fertilization treatment to the ordination (r^2=0.0148, p=0.058). An ANOVA of the abundance data also showed similar structure to the incidence analysis (Table 2 in Appendix A).

On the first axis the ordination of the most common taxa showed high correlation with the ordination of all taxa (Tau= 0.8262, p<0.0001). The second axes showed weak correlation (Tau=-0.3158, p=<0.0001). The procrustes correlation test of the whole ordination was high (0.7283 with p=0.001). When vectors were fitted there were also a significant difference between all factors (Fertilization: r^2=0.0161, p=0.03, Host: r^2=0.3277, p=0.001, Tissue type:r^2=0.1087, p=0.001), and the ANOVA showed a similar structure

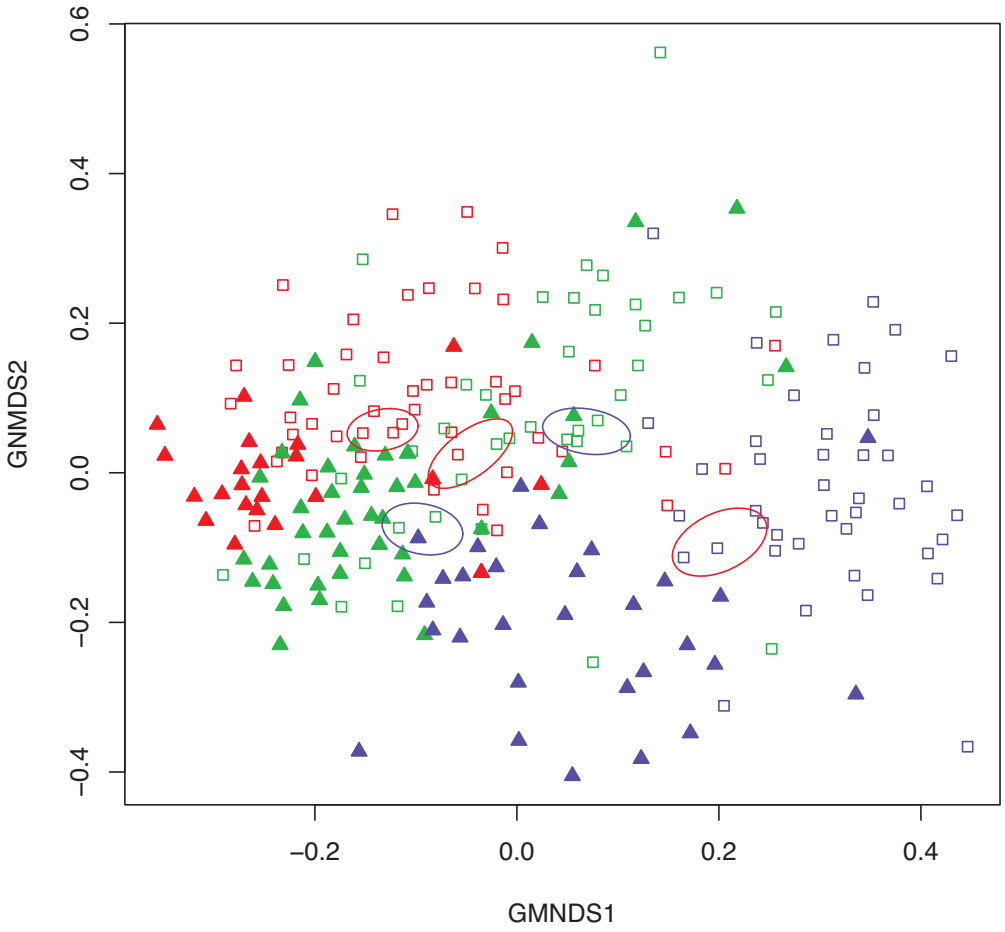


Figure 7: GNMDS diagram for incidence data. The triangles are samples from green shoots and the squares are from brown. The blue symbols are from *Dicranum scoparium*, the green are from *Pleurozium screberi*, and the red are from *Hylocomium splendens*. The ellipses represent the 95 % confidence interval for the focal point of each vector, as calculated with 999 permutations using the envfit function in the vegan package of R. Focal points of host species are in red and tissue type are in blue.

on the first axis, but also found significant differences on fertilization but not on tissue type on the second axis (Anova table, ordination diagrams and Procrustes error diagram provided in Appendix A). This similarity of results was taken as an indication of the full dataset analyses were robust and all further discussion is of the full dataset.

The two similarity indexes showed differences of similar magnitude (Table 6), with the greatest difference being between the green part of *Hylocomium splendens*, and the brown

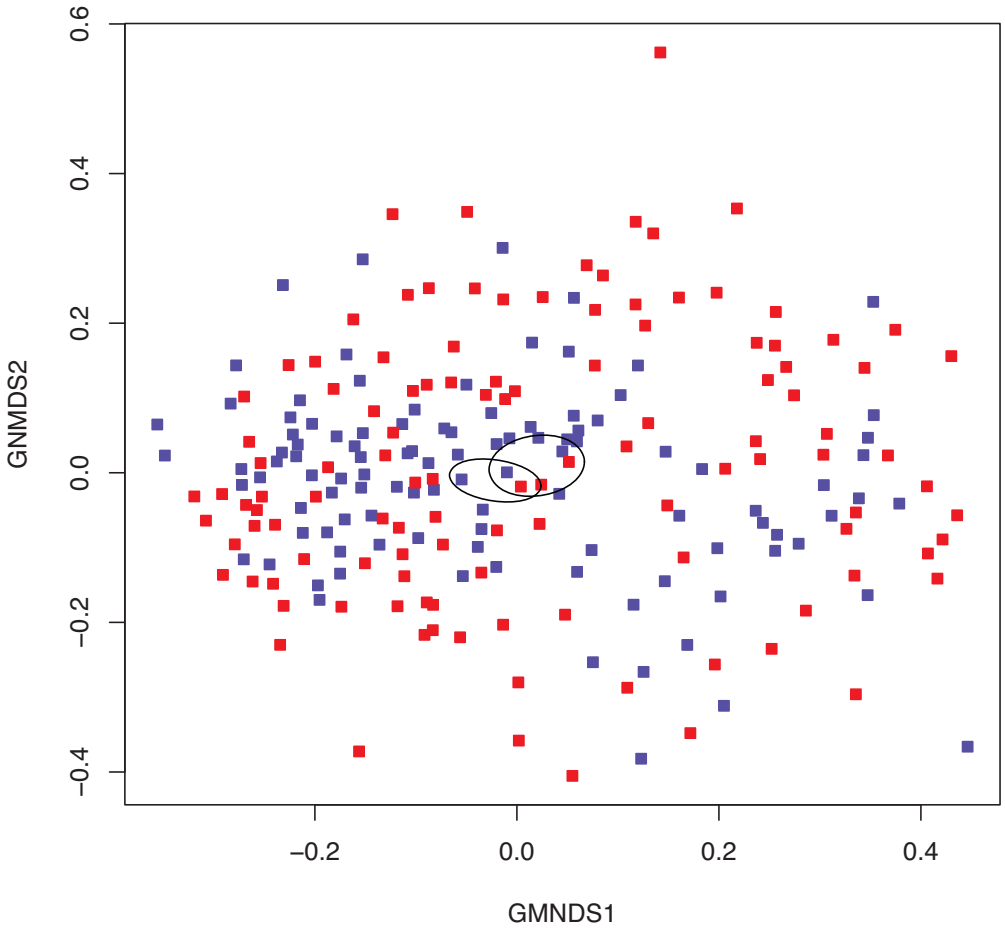


Figure 8: GNMDS diagram for incidence data. The blue squares are shoots from the nitrogen enriched plots and the red are from control plots. The black ellipses represent the 95 % confidence interval for the focal point of the vector, as calculated with 999 permutations using the envfit function in the vegan package of R.

part of *Dicranum scoparium* and the greatest similarity being within the host species. The *D. scoparium* had greatest difference between the brown and the green part of the shoot, and *H. splendens* had least difference between the tissue types.

Table 5: Testing the difference between the incidence ordination results on the two GNMDS axes using a partially nested ANOVA. Degrees of freedom (Df), Sum of squares (Sum Sq) and Mean sum of squares (Mean Sq) are noted. Significant p-values are in bold.

Response	Factor	Df	Sum Sq	Mean Sq	F value	p-value
GNMDS axis 1						
	Fertilization	1	0.0945	0.0945	6.3108	**0.0132**
	Host	2	4.3439	2.17194	145.0502	**<0.0001**
	Tissue	1	0.1974	0.19744	13.1855	**0.0004**
	Fertilization:Host	2	0.035	0.01752	1.17	0.3136
	Fertilization:Tissue	1	0.0748	0.07478	4.9938	**0.0271**
	Host:Tissue	2	0.0949	0.04743	3.1676	**0.0454**
	Fertilization:Host:Tissue	2	0.0242	0.01212	0.8093	0.4474
	Residuals	130	1.9466	0.01497		
GNMDS axis 1						
	Fertilization	1	0.02178	0.02178	1.4286	0.2342
	Host	2	0.92856	0.46428	30.4476	**<0.0001**
	Tissue	1	0.08721	0.08721	5.7194	**0.0182**
	Fertilization:Host	2	0.00248	0.00124	0.0814	0.9217
	Fertilization:Tissue	1	0.01494	0.01494	0.98	0.3240
	Host:Tissue	2	0.02968	0.01484	0.9731	0.3806
	Fertilization:Host:Tissue	2	0.01158	0.00579	0.3797	0.6848
	Residuals	130	1.9823	0.01525		

4 Discussion

4.1 Richness of Fungal Communities

A total of 3300 different fungal OTUs were found in this study (See electronic appendix), which is a large number compared to the roughly 350 that are described as strictly bryophilous species (Döbbeler, 2002). It is probable that a great number of these OTUs are species that are ubiquitously present throughout the plant kingdom, e.g. the Sebacinales have recently been shown to be universally distributed plant-associates (Weiß et al., 2011). Additionally, as a mutually dependent relationship between host and fungi is unlikely in natural environments due to the variability in selective forces (Saikkonen et al., 2004), many of the associated fungi will not be specific to mosses. Many of the fungi detected are probably cosmopolitan species, hence not all will have intimate association

Table 6: Testing the difference between the incidence ordination results on the two GN-MDS axes using a partially nested ANOVA. Degrees of freedom (Df), Sum of squares (Sum Sq) and Mean sum of squares (Mean Sq) are noted. Significant p-values are in bold.

Jaccard index	HS brown	HS green	PS brown	PS green	DS brown	DS green
HS brown	1	-	-	-	-	-
HS green	0.456	1	-	-	-	-
PS brown	0.408	0.352	1	-	-	-
PS green	0.372	0.408	0.395	1	-	-
DS brown	0.312	0.211	0.316	0.226	1	-
DS green	0.309	00.32	0.315	0.337	0.314	1
Sørensen Classic						
HS brown	1	-	-	-	-	-
HS green	0.626	1	-	-	-	-
PS brown	0.579	0.52	1	-	-	-
PS green	0.542	0.58	0.567	1	-	-
DS brown	0.475	0.348	0.48	0.369	1	-
DS green	0.472	0.485	0.479	0.504	0.504	1

with their host. This is exemplified by the 3300 OTUs found in this study versus the 350 species found exclusively on bryophytes (Döbbeler, 2002). The great number of fungal OTUs nevertheless confirms prior claims (Kauserud et al., 2008) that there is a great species richness associated with mosses.

The taxonomic distribution of OTUs within the moss-associated fungal communities is similar to that found by others (Kauserud et al., 2008), with most fungi belonging to Ascomycota. However, members of the Glomeromycota and Chytridiomycota were also found. Glomalean fungi are present as mycorrhizae in most land plants (Read et al., 2000), and they are capable of colonizing bryophytes (Parke and Linderman, 1980, Iqbal et al., 1988). There is, however, no evidence for a relationship of a nutrient transmitting nature between bryophytes and glomalean fungi (Davey and Currah, 2006). One can only speculate what their function is and if they have any positive effect on the moss host. It has been shown that mycorrhizae can transfer leached nutrients from senescent moss tissues to plant roots (Carleton and Read, 1991), although in the present study I can't demonstrate if these fungi are present as random spores or as functional hyphae.

Additionally, Parke and Lidermann (1980) conclude that the relationship between the moss and the glomalean fungi is not mutualistic. The soil chytrid community has been shown to be higher in soils covered by bryophytes than those where bryophytes are absent (Letcher and Powell, 2002), although the potential of bryophytes as hosts for chytrids has not been investigated in any great detail. The close physical proximity between mosses and soil, the increase in Chytridiomycota in soils under bryophyte cover, and the detection of the Chytridiomycota in my study suggests chytrids may represent a previously unknown contributor to the fungal diversity associated with mosses.

The two species richness estimators applied to the dataset, Chao 2 and the Ugland approach, yielded very different estimates. The heterogeneity of fungal communities on small scales has been well documented (Genney et al., 2006) and is accounted for in the Ugland approach (Ugland et al., 2003). But as this method is based on linear regression, the challenge of setting an appropriate endpoint is considerable. The end point is to reflect the total area, or in this case the total number of moss shoots. If the size of a single moss shoot is considered to be about 2.5 cm^2, the moss covers approximately 75 % of a plot size of 225 m^2 the total number of moss shoots in all experimental plots will be about 4 million. To account for the inherent inaccuracy and variation in estimating shoot size and percent coverage, the graphs presented show a range from 100 000 to 10 million shoots. Additionally, local spatial turnover may not reflect turnover on a larger spatial scale (Peay et al., 2008), and the Ugland method was developed for subtidal marine environments (Ugland et al., 2003), and may not be transferable to the microbes of land plants, both factors that may render the Ugland method inappropriate for use with my data. On the other hand, while the Chao 2 estimator is not based on linear calculations and does not require selection of an endpoint, it may greatly underestimate the total species richness, as it does not take the heterogeneity into account. As such, it is difficult to get a truly accurate measure of total species richness, and the estimators generated for our data are best used for comparisons between fertilization treatments, hosts, and tissue types.

Regardless of the extrapolation method employed, the fungal species richness detected in the phyllosphere is greater in mosses than in what has been reported for vascular plants using the same sequencing technology (Jumpponen et al., 2010). Even the sheer numbers of OTUs found are in a different order of magnitude than what has so far been found in vascular plants (e.g. Jumpponen et al., 2010, Jumpponen and Jones, 2010). This pattern also holds true for the fungal biomass (Davey et al., 2009) associated with mosses, which adds to the postulation that the fungal communities of mosses are not only richer, they are also more diverse. Mosses lack a cuticle and roots, have thin leaves, and have a slower active conduction than vascular plants which may make them more vulnerable to the attack of pathogens (Cuming, 2009). Despite their highly active defenses against pathogens (Cuming, 2009), my results suggest mosses host a high number of endo- and epiphytic fungi. Moreover, mosses have a slower decomposition rate than vascular plants (Lang et al., 2009) which suggests the fungal community may be slower growing with a higher rate of species recruitment and accumulation.

4.2 Differences between host species

Both the raw and estimated richness and species composition were significantly different between the three host species, with *Hylocomium splendens* and *Pleurozium schreberi* having similar and higher estimated species richness than *Dicranum scoparium*. The same pattern was also present in the ordination, with the two pleurocarpous mosses being separated from the acrocarpous moss, yet there was also a clear difference between *H. splendens*, and *P. shreberi*. This is consistent with the finding of Kauserud et al. (2008) that very few fungal species were shared between *P. shreberi* and *H. splendens*. On the other hand, the fungal biomass does not differ significantly between those two hosts (Davey et al., 2009), and the overall taxonomic composition of the communities was similar between the three hosts, both on phylum and order levels. As the community differences were detected at the OTU level, community composition will from now on refer to differences on this level.

The moss hosts have different anatomical structures and growth forms, therefore offering different microniches for fungal colonization (Döbbeler, 2002). *H. splendens* and *P. schreberi* are more related and have the same growth form. These two grow in wefts, while *D. scoparium* grows in turfs (Bates, 1998). Moreover, *H. splendens* and *P. schreberi* are the only mosses known to harbor the nitrogen fixating *Nostoc* bacteria (Zackrisson et al., 2009). It is therefore hard to reveal which of these factors, if any, are responsible for the higher similarity in community composition between *H. splendens* and *P. shreberi*.

The rate of litter decomposition is reported to be significantly higher in *P. schreberi*, than in *H. splendens*, while *Dicranum* spp. have intermediate decomposition rates (Lang et al., 2009). A higher decomposition rate of litter could mean that nutrients are more available in *P. schreberi* than in *H. splendens*, or that the higher turnover rate causes greater small scale disturbances in the microhabitats associated with this species. If these assumptions are true, it could explain the higher species richness in *P. schreberi* and the overall differences in fungal species composition in the three hosts. On the other hand, the lack of consistency between the patterns in decomposition (*P. shreberi* $>$ *Dicraum* spp. $>$ *H. splendens*) with the patterns of species richness found in this study (*H. splendens* $>$ *P. shreberi* $>$ *D. scoparium*) suggests that this may be only one of many contributing factors to the differences observed in the fungal communities between the three hosts.

Mosses are found to have species specific responses to nitrogen enrichment experiments (Gordon et al., 2001), with *D. scoparium* decreasing under higher nitrogen contents. Skrindo and Økland (2002) also found most mosses (including *D. scoparium*) to decrease more than *P. schreberi*. It would therefore be expected that the fungal associates would show a similar differences in their responses. However, I found no significant interaction between host and nitrogen addition, in either the ANOVA performed on the ordination results or the average number of OTUs.

4.3 Differences between the two tissue types

Even though there is a physical connection between the green, photosynthetic and the brown, senescent parts of the shoots, there was a clear difference in fungal species composition, and to a smaller degree in estimated species richness, between the two microhabitats. This is also supported by other studies. For example, Parke and Lindermann (1980) found greater numbers of fungi related to the senescent portion of the moss using microscopy. It has also been reported that significantly higher amounts of fungal biomass are in the brown versus the green tissues of mosses (Davey et al., 2009). On the other hand, the raw counts of OTUs showed no significant difference. As with the host species, there was similar overall taxonomic composition between the two tissue types at higher taxonomic levels; differences were only found on the species (OTU) level.

The different parts of a moss shoot may have very different nutrient content (Eckstein and Karlsson, 1999), which may be reflected in the size and structure of the associated fungal community. However, Eckstein and Karlsson (1999), also found the newest shoots to acquire a substantial amount of nitrogen from older part of the moss individual. The lower and senescent parts of the moss shoot are believed to have storage functions (Skre et al., 1983, Eckstein and Karlsson, 1999), and have a different structure than the green part. These differences may be a factor explaining the differences in community structure of the bryophilous fungi. However, despite the nutrient content being a plausible explanation, the effect cannot be construed too strictly, as the nutrient availability also may be dictated in part by the host.

On the other hand, there is also a difference in amount and what sort of nutrition is leached from the different parts of the moss shoot, which is relevant as this study does not discriminate between the endo- end epiphytic communities. The senescent part of moss shoot has been shown to have a greater leaching of nitrogen and glucose than the green part, while the green part leached more sucrose (Carleton and Read, 1991). Carleton and Read (1991) and Parke and Lindermann (1980) found mycorrhizae to colonize the senescent part more than they did the photosynthetic part, probably because of higher

nutrient leaching in this part of the moss.

It is not only the nutrient content and availability that can explain the difference in species richness and species composition. Also the structure of the moss colony can impact the microenvironment. The deep and senescent part of the moss will keep more moisture than the outer photosynthetic part (Bates, 1998). The moist part may be able to sustain a greater fungal activity than the drier and weather exposed outer part. Furthermore, the brown part of the moss is older than the green, so the fungal community has had more time for species recruitment, and may be on a different successional stage, which would give a changed species composition. The brown part of the shoot is in close connection with the soil, and the great fungal inoculum reservoir in the soil may contribute to fungal colonization of the brown part more than it does the green. Similarly, the green parts could to a higher degree be colonized by aerially dispersed spores. The distinct microhabitats and potential for fungal colonization that arise from these factors can be a part of the explanation of the dissimilar fungal communities.

There was a greater difference in community composition between the two tissues of *Dicranum scoparium* than between the other two species, this was evident in both the interaction effects in the ANOVA and in the calculated Jaccard and Sørensens indexes. Growth form could lead to a greater difference between microhabitat in the brown than in the green part of the shoot, e.g. the moisture content in the brown part of a weft may dry up faster than the brown part of a turf, while the moisture in green parts are exposed to dry conditions regardless of growth form. The hosts with greatest taxonomic distance may also differ most in microhabitat between different tissue types. They may also have different levels of host protection of nutrients in the green part of the shoot, while the senescent part will probably be less protected and invariable between hosts.

4.4 Nitrogen effects

The differences between shoots from nitrogen enriched and control plots were small and in some analyses, contradictory. I can therefore not reasonably conclude that there is an

effect of nitrogen enrichment on the fungal community of mosses. The two species richness estimators showed small and opposite differences, and the overall taxonomic composition was similar at all examined taxonomic levels (phylum, order, OTUs). However, the raw counts of OTUs showed significantly higher average number of OTUs in the nitrogen treated plots. The ordination showed no significant difference when the results were tested with vector fitting, while when tested with ANOVA it showed a significant difference on the first ordination axis, but not on the second. However, despite being significant on one axis, this difference is small, making it hard to draw firm conclusions. The opposing results from the two tests of the ordinations may be due to the vector fitting simultaneously taking both axes into account, while the ANOVA tests axis by axis and includes interaction effects. I am therefore unable to conclude that there is a difference in the fungal community between the nitrogen enriched plots and the controls due to the two estimators giving opposite results and the separation in the ordination being small and of varying significance. Nitrogen enrichment can have a dramatic effect on the quantities of boreal forest mosses (Skrindo and Økland, 2002, but see Gordon et al., 2001), so an effect on the fungal community of the mosses could also have been expected. The effect of nitrogen has not been tested on epi- and endophytic communities of mosses, but studies on other fungal symbionts have found different, not to say opposite, effects of nitrogen enrichment (e.g. Ishida and Nordin, 2010b, Lilleskov et al., 2002b), which suggest multiple responses to nitrogen.

There are three possible explanations for the lack of response in the fungal community to the application of nitrogen: there is no effect of nitrogen on the fungal associates, the added nitrogen has not reached the fungi, or there is an effect, but it is masked by other factors. Under the first scenario, the nitrogen enrichment simply may not affect the fungal community. Bryophytes have a great capacity to accumulate applied nitrogen (Gordon et al., 2001, Nordin et al., 1998), which may mean that the fungi residing in and on the mosses are actually not affected by the added nitrogen. The natural fixation of nitrogen of the *Nostoc* bacteria in *H. splendens* and *P. shreberi* decreases when there

is more nitrogen added to the environment (DeLuca et al., 2008). This implies that the nitrogen enrichment could be evened out by the decrease in *Nostoc* nitrogen fixation, so that the overall level of available nitrogen may not change in these two hosts. However, this does not explain the lack of difference in *Dicranum scoparium*.

On the other hand, it is possible that the additional nitrogen has not reached the fungi. The fertilizer may have killed the moss shoots or left them unaffected, and as only healthy moss shoots were collected in this study, the nitrogen enrichment may not have reached the phyllosphere of the sample shoots. The mode of nitrogen application (pellets that are dissolved by rain) could have caused the nitrogen to soak into the soil, where it is easily reached by plant roots but may not be available to mosses. On the other hand, the soil microbes may be affected by this, and that could again affect the brown parts of the moss. About half of the nitrogen applied was ammonium, which is the form that is taken up (for the most part) by mosses (Forsum et al., 2006, Turetsky, 2003), which could explain the lack of a clear effect. Meyer et al (2010a) found no effect on the moss itself, but great effects on the biomass of microbial community associated with them, including fungi. This could lead to the conclusion that the nitrogen applied by pellets actually has not reached the fungi and is not capable of affecting them. Furthermore, Meyer et al (2010a) and fertilized with nitrogen solutes, which adds to the suspicion of the nitrogen applied may not have reached the bryophyte associated fungi.

There is a myriad of effects of nitrogen on the environment, and these may mask or equalize a possible effect on the bryophilous fungi. Nilsson and Wallander (2003) found that the fertilization effect on host trees was the determining factor for mycorrhizal growth, and not the nitrogen working directly on the fungi. The tree growth was probably also responsible for the decrease of quantities of mosses in a fertilization experiment in southern Norway (Skrindo and Økland, 2002). This effect on trees may also be responsible for a possible masking of effects on the fungi associated with the mosses in question. van der Wal et al. (2005) found that for some moss species the competition for light under nitrogen enrichment was more important than the direct effect of nitrogen. Nitrogen

addition can also have an effect on pH (Hallbäcken and Zhang, 1998), and none of these effects are tested in this study. Additionally, Skre and Oechel (1979) claims that water availability and not nutrients are the limiting factor of boreal forest mosses, so the hosts and the communities they support may not respond to the addition of nitrogen. Also, the shift towards grass dominance in nitrogen enriched environments can be attributed to parasitic fungi, and not the direct effect of fertilization (Strengbom et al., 2002) which adds to the complexity of the relationship between fungi and nitrogen.

According to the ANOVA, there were greater differences in OTU composition between the two tissue types in fertilized plots than in control plots. Gordon et al. (2001) found mosses to have more photosynthetic tissue at higher nitrogen levels, which could explain parts of the higher difference. A vigorously growing photosynthesizing tissue under nitrogen enrichment could create a more different environment from the senescent tissue, while a normally growing green tissue could be less different from its corresponding brown tissue. On the contrary, Skre and Oechel (1979) found moss production to be promoted in sites with low nutrients and more restricted by water. One can also assume that in the senescent tissue, the added nutrients can be readily available to the fungi, as the moss host defenses are lowered in dying tissues.

4.5 Methodological considerations

The 454 controls showed a worrying inconsistency in both OTU incidence and abundance. However, when the same comparison were made with only core taxa (OTUs present in more than 66 samples), the percentage of species shared was reaching an acceptable level (between 50 % and 90 % species similarity), and higher levels in Chao Sørensen Raw Incidence based similarity index, suggesting the core-taxa dataset was appropriate for comparative analyses between samples. Subsequent multidimensional analyses of the core taxa dataset showed a high similarity to analyses of the whole dataset. The results generated were the same, except there was no significant effect of nitrogen addition in the core taxa ordination. This suggests the results from the complete dataset are trustworthy

as the ordinations of two data sets were significantly correlated and generated similar results. The difference in significance of nitrogen treatment adds to the conviction that the significant effect of nitrogen found on the first GNMDS axis is to be interpreted cautiously, while the analogous results of tissue type and host species confirms the robustness of this result.

4.6 Conclusions

There is a clear difference in species composition and richness between different moss hosts and the different tissues, while the summarized taxonomic gestalt at higher taxonomic ordinals did not show any difference. Despite that bryophytes are often treated as one functional group, there are differences between species that are reflected in the fungal community. Even within the same shoots, there are large differences that may be conditioned by age and structure of the tissue. While the boreal forest as a whole is nitrogen limited, this may not be the case for the fungal associates of mosses therein as there was no difference between the nitrogen treated and the control plots in taxonomic distribution (at any level), species richness or in ordination of species. The bryophilous fungal species richness is vast and the range of taxonomic affiliations of the detected OTUs is extensive. To date, the question of why the difference between bryophytes and vascular plants should yield such a big difference in abundance is open.

5 References

AMEND, A. S., SEIFERT, K. A. & BRUNS, T. D. 2010. Quantifying microbial communities with 454 pyrosequencing: does read abundance count? *Molecular Ecology*, 19, 5555-5565.

ARNOLD, A. E. 2007. Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. *Fungal Biology Reviews*, 21, 51-66.

ARNOLD, A. E., MAYNARD, Z. & GILBERT, G. S. 2001. Fungal endophytes in dicotyledonous neotropical trees: patterns of abundance and diversity. *Mycological Research*, 105, 1502-1507.

BACH, L. H., FROSTEGÅRD, Å. & OHLSON, M. 2009. Site identity and moss species as determinants of soil microbial community structure in Norway spruce forests across three vegetation zones. *Plant and soil*, 318, 81-91.

BATES, J. 1998. Is 'life-form'a useful concept in bryophyte ecology? *Oikos*, 82, 223-237.

BENSCOTER, B. W. & VITT, D. H. 2007. Evaluating feathermoss growth: a challenge to traditional methods and implications for the boreal carbon budget. *Journal of Ecology*, 95, 151-158.

BUCK, J. W., LACHANCE, M. A. & TRAQUAIR, J. A. 1998. Mycoflora of peach bark: population dynamics and composition. *Canadian Journal of* Botany, 76, 345-354.

BUÉE, M., REICH, M., MURAT, C., MORIN, E., NILSSON, R., UROZ, S. & MARTIN, F. 2009. 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. *New Phytologist*, 184, 449-456.

CARLETON, T. & READ, D. 1991. Ectomycorrhizas and nutrient transfer in conifer-feather moss ecosystems. *Canadian Journal of* Botany, 69, 778-785.

CLAY, K. 1996. Interactions among fungal endophytes, grasses and herbivores. *Researches on Population Ecology*, 38, 191-201.

COLWELL, R. K. 2009. Statistical estimation of species richness and shared species

from samples. Version 8.2. User's Guide and application. http://purl.oclc.org/estimates.

CUMING, A. C. 2009. Plant–pathogen interactions: a view from the evolutionary basement. *New Phytologist*, 183, 237-239.

DAVEY, M. & CURRAH, R. 2006. Interactions between mosses (Bryophyta) and fungi. *Botany*, 84, 1509-1519.

DAVEY, M., NYBAKKEN, L., KAUSERUD, H. & OHLSON, M. 2009. Fungal biomass associated with the phyllosphere of bryophytes and vascular plants. *Mycological Research*, 113, 1254-1260.

DELUCA, T., NILSSON, M. C. & ZACKRISSON, O. 2002. Nitrogen mineralization and phenol accumulation along a fire chronosequence in northern Sweden. *Oecologia*, 133, 206-214.

DELUCA, T. H., ZACKRISSON, O., GUNDALE, M. J. & NILSSON, M. C. 2008. Ecosystem feedbacks and nitrogen fixation in boreal forests. *Science*, 320, 1181.

DÖBBELER, P. 2002. Microniches occupied by bryophilous ascomycetes. *Nova Hedwigia*, 75, 275-306.

ECKSTEIN, R. L. & KARLSSON, P. S. 1999. Recycling of nitrogen among segments of *Hylocomium splendens* as compared with *Polytrichum commune*: implications for clonal integration in an ectohydric bryophyte. *Oikos*, 86, 87-96.

FISHER, P. & PETRINI, O. 1990. A comparative study of fungal endophytes in xylem and bark of *Alnus* species in England and Switzerland. *Mycological Research*, 94, 313-319.

FORSUM, Å., DAHLMAN, L., NÄSHOLM, T. & NORDIN, A. 2006. Nitrogen utilization by *Hylocomium splendens* in a boreal forest fertilization experiment. *Functional Ecology*, 20, 421-426.

GALLOWAY, J. N. & COWLING, E. B. 2002. Reactive nitrogen and the world: 200 years of change. *AMBIO: A Journal of the Human Environment*, 31, 64-71.

GARDES, M. & BRUNS, T. 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. *Molecular Ecology*, 2, 113-118.

GENNEY, D. R., ANDERSON, I. C. & ALEXANDER, I. J. 2006. Fine scale distribution of pine ectomycorrhizas and their extramatrical mycelium. *New Phytologist*, 170, 381-390.

GORDON, C., WYNN, J. & WOODIN, S. 2001. Impacts of increased nitrogen supply on high Arctic heath: the importance of bryophytes and phosphorus availability. *New Phytologist*, 149, 461-471.

HALLBÄCKEN, L. & ZHANG, L. Q. 1998. Effects of experimental acidification, nitrogen addition and liming on ground vegetation in a mature stand of Norway spruce (*Picea abies* (L.) Karst.) in SE Sweden. *Forest Ecology and Management*, 108, 201-213.

HECKMAN, D. S., GEISER, D. M., EIDELL, B. R., STAUFFER, R. L., KARDOS, N. L. & HEDGES, S. B. 2001. Molecular evidence for the early colonization of land by fungi and plants. *Science*, 293, 1129.

HILL, M. O. & GAUCH, H. 1980. Detrended correspondence analysis: an improved ordination technique. *Plant Ecology*, 42, 47-58.

HOLE, L. & ENGARDT, M. 2008. Climate change impact on atmospheric nitrogen deposition in northwestern Europe: a model study. *AMBIO: A Journal of the Human Environment*, 37, 9-17.

INACIO, J., PEREIRA, P., CARVALHO, M., FONSECA, A., AMARAL-COLLACO, M. & SPENCER-MARTINS, I. 2002. Estimation and diversity of phylloplane mycobiota on selected plants in a Mediterranean-type ecosystem in Portugal. *Microbial Ecology*, 44, 344-353.

IQBAL, S., GHAZALA, N. & SHAHJAHAN 1988. Vesicular-Arbuscular Mycorrhizal fungi associated with three mosses (*Sphagnum cumbifolium*, *Polytrichum commune* and *Funaria hygromatricia*). *Biologica Lahore*, 34, 269-274.

ISHIDA, T. & NORDIN, A. 2010a. No evidence that nitrogen enrichment affect fungal communities of *Vaccinium* roots in two contrasting boreal forest types. *Soil Biology and Biochemistry*, 42, 234-243.

ISHIDA, T. A. & NORDIN, A. 2010b. No evidence that nitrogen enrichment affect

fungal communities of *Vaccinium* roots in two contrasting boreal forest types. *Soil Biology and Biochemistry*, 42, 234-243.

JUMPPONEN, A. & JONES, K. 2009. Massively parallel 454 sequencing indicates hyperdiverse fungal communities in temperate *Quercus macrocarpa* phyllosphere. *New Phytologist*, 184, 438-448.

JUMPPONEN, A. & JONES, K. 2010. Seasonally dynamic fungal communities in the *Quercus macrocarpa* phyllosphere differ between urban and nonurban environments. *New Phytologist*, 186, 496-513.

JUMPPONEN, A., JONES, K., DAVID MATTOX, J. & YAEGE, C. 2010. Massively parallel 454 sequencing of fungal communities in *Quercus* spp. ectomycorrhizas indicates seasonal dynamics in urban and rural sites. *Molecular Ecology*, 19, 41-53.

KAUSERUD, H., MATHIESEN, C. & OHLSON, M. 2008. High diversity of fungi associated with living parts of boreal forest bryophytes. *Botany*, 86, 1326-1333.

KUMAR, S., CARLSEN, T., MEVIK, B. H., ENGER, P., BLAALID, R., SHALCHIAN-TABRIZI, K. & KAUSERUD, H. 2011. CLOTU: An online pipeline for processing and clustering of 454 amplicon reads into OTUs followed by taxonomic annotation. *BMC bioinformatics*, 12, 182.

LANG, S. I., CORNELISSEN, J. H. C., KLAHN, T., VAN LOGTESTIJN, R. S. P., BROEKMAN, R., SCHWEIKERT, W. & AERTS, R. 2009. An experimental comparison of chemical traits and litter decomposition rates in a diverse range of subarctic bryophyte, lichen and vascular plant species. *Journal of Ecology*, 97, 886-900.

LETCHER, P. M. & POWELL, M. J. 2002. Frequency and distribution patterns of zoosporic fungi from moss-covered and exposed forest soils. *Mycologia*, 94, 761.

LILLESKOV, E., FAHEY, T., HORTON, T. & LOVETT, G. 2002a. Belowground ectomycorrhizal fungal community change over a nitrogen deposition gradient in Alaska. *Ecology*, 83, 104-115.

LILLESKOV, E. A., FAHEY, T. J., HORTON, T. R. & LOVETT, G. M. 2002b. Belowground ectomycorrhizal fungal community change over a nitrogen deposition gradient

in Alaska. *Ecology*, 83, 104-115.

LINDAHL, B. D., IHRMARK, K., BOBERG, J., TRUMBORE, S. E., HÖGBERG, P., STENLID, J. & FINLAY, R. D. 2007. Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. *New Phytologist*, 173, 611-620.

MEYER, C., BERNARD, N., MOSKURA, M., TOUSSAINT, M., DENAYER, F. & GILBERT, D. 2010a. Effects of urban particulate deposition on microbial communities living in bryophytes: An experimental study. *Ecotoxicology and environmental safety*, 73, 1776-1784.

MEYER, C., GILBERT, D., GAUDRY, A., FRANCHI, M., NGUYEN-VIET, H., FABURE, J. & BERNARD, N. 2010b. Relationship of atmospheric pollution characterized by gas (NO 2) and particles (PM10) to microbial communities living in bryophytes at three differently polluted sites (rural, urban, and industrial). *Microbial Ecology*, 59, 324-334.

MINCHIN, P. R. 1987. An evaluation of the relative robustness of techniques for ecological ordination. *Plant Ecology*, 69, 89-107.

MURRAY, M. & THOMPSON, W. 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research*, 8, 4321.

NILSSON, L. O. & WALLANDER, H. 2003. Production of external mycelium by ectomycorrhizal fungi in a Norway spruce forest was reduced in response to nitrogen fertilization. *New Phytologist*, 158, 409-416.

NORDIN, A., NÄSHOLM, T. & ERICSON, L. 1998. Effects of simulated N deposition on understorey vegetation of a boreal coniferous forest. *Functional Ecology*, 12, 691-699.

NORDIN, A., STRENGBOM, J., WITZELL, J., NÄSHOLM, T. & ERICSON, L. 2005. Nitrogen deposition and the biodiversity of boreal forests: implications for the nitrogen critical load. *AMBIO: A Journal of the Human Environment*, 34, 20-24.

ØKLAND, R. H. 1996. Are ordination and constrained ordination alternative or complementary strategies in general ecological studies? *Journal of Vegetation Science*, 7, 289-292.

OKSANEN, J., BLANCHET, F., KINDT, R., LEGENDRE, P., O'HARA, R., SIMPSON, G., SOLYMOS, M., STEVENS, H. & WAGNER, H. 2011. Vegan: Community Ecology Package. R package version 1.17-8. http://CRAN.R-project.org/package=vegan.

OSONO, T. 2007. Endophytic and epiphytic phyllosphere fungi of red-osier dogwood (Cornus stolonifera) in British Columbia. *MycoScience*, 48, 47-52.

PARKE, J. L. & LINDERMAN, R. 1980. Association of vesicular-arbuscular mycorrhizal fungi with the moss Funaria hygrometrica. *Canadian Journal of Botany*, 58, 1898-1904.

PEAY, K. G., KENNEDY, P. G. & BRUNS, T. D. 2008. Fungal community ecology: a hybrid beast with a molecular master. *BioScience*, 58, 799-810.

READ, D., DUCKETT, J., FRANCIS, R., LIGRONE, R. & RUSSELL, A. 2000. Symbiotic fungal associations in 'lower'land plants. Philosophical Transactions of the Royal Society of London. *Series B: Biological Sciences*, 355, 815-831.

REDECKER, D., KODNER, R. & GRAHAM, L. E. 2000. Glomalean fungi from the Ordovician. *Science*, 289, 1920-1921.

RODRIGUEZ, R., WHITE JR, J., ARNOLD, A. & REDMAN, R. 2009. Fungal endophytes: diversity and functional roles. *New Phytologist*, 182, 314-330.

SAIKKONEN, K., WALI, P., HELANDER, M. & FAETH, S. H. 2004. Evolution of endophyte-plant symbioses. *Trends in Plant Science*, 9, 275-280.

SANTAMARÍA, J. & BAYMAN, P. 2005. Fungal epiphytes and endophytes of coffee leaves (*Coffea Arabica*). *Microbial Ecology*, 50, 1-8.

SCHULZ, B. & BOYLE, C. 2005. The endophytic continuum. *Mycological Research*, 109, 661-686.

SKRE, O. & OECHEL, W. 1979. Moss production in a black spruce (*Picea mariana*) forest with permafrost near Fairbanks, Alaska, as compared with two permafrost free stands. *Ecography*, 2, 249-254.

SKRE, O., OECHEL, W. & MILLER, P. 1983. Patterns of translocation of carbon in four common moss species in a black spruce (*Picea mariana*) dominated forest in interior

Alaska. *Canadian Journal of Forest Research*, 13, 869-878.

SKRINDO, A. & ØKLAND, R. H. 2002. Effects of fertilization on understorey vegetation in a Norwegian Pinus sylvestris forest. *Applied VegetationScience*, 5, 167-172.

STRENGBOM, J., NORDIN, A., NÄSHOLM, T. & ERICSON, L. 2002. Parasitic fungus mediates change in nitrogen exposed boreal forest vegetation. *Journal of Ecology*, 90, 61-67.

TAMM, C. (ed.) 1991. Nitrogen in terrestrial ecosystems (Ecological Studies 81): Springer, Berlin Heidelberg New York.

TEAM, R. D. C. 2011. R: A Language and Environment for Statistical Computing. In: COMPUTING, R. F. F. S. (ed.). Vienna, Austria.

TURETSKY, M. R. 2003. The role of bryophytes in carbon and nitrogen cycling. *The Bryologist*, 106, 395-409.

UGLAND, K., GRAY, J. & ELLINGSEN, K. 2003. The species–accumulation curve and estimation of species richness. *Journal of Animal Ecology*, 72, 888-897.

VAN DER WAL, R., PEARCE, I. S. K. & BROOKER, R. W. 2005. Mosses and the struggle for light in a nitrogen-polluted world. *Oecologia*, 142, 159-168.

VENABLES, W. & RIPLEY, B. (eds.) 2002. Modern Applied Statistics with S. Fourth Edition., New York: Springer.

VERMA, V., GOND, S., KUMAR, A., KHARWAR, R. & STROBEL, G. 2007. The endophytic mycoflora of bark, leaf, and stem tissues of *Azadirachta indica* A. Juss (neem) from Varanasi (India). *Microbial Ecology*, 54, 119-125.

WEIß, M., SÝKOROVÁ, Z., GARNICA, S., RIESS, K., MARTOS, F., KRAUSE, C., OBERWINKLER, F., BAUER, R., REDECKER, D. & VAN DER HEIJDEN, M. 2011. Sebacinales Everywhere: Previously Overlooked Ubiquitous Fungal Endophytes. *PloS one*, 6, e16793.

WHIPPS, J., HAND, P., PINK, D. & BENDING, G. 2008. Phyllosphere microbiology with special reference to diversity and plant genotype. *Journal of applied microbiology*, 105, 1744-1755.

WHITE, I. R. & BACKHOUSE, D. 2007. Comparison of fungal endophyte communities in the invasive panicoid grass *Hyparrhenia hirta* and the native grass *Bothriochloa macra*. *Australian Journal of Botany*, 55, 178-185.

WHITE, T., BRUNS, T., LEE, S. & TAYLOR, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols a guide to methods and applications*, 315-322. Academic Press

WWW.MET.NO. Metreologisk Institutt. Available: http://retro.met.no/observasjoner/oppland/no [Accessed 16.04.2010].

WWW.NGU.NO. Norges geologiske undersøkelser. [Accessed 16.04.2010].

ZACKRISSON, O., DELUCA, T., GENTILI, F., SELLSTEDT, A. & JÄDERLUND, A. 2009. Nitrogen fixation in mixed *Hylocomium splendens* moss communities. *Oecologia*, 160, 309-319.

A Appendix

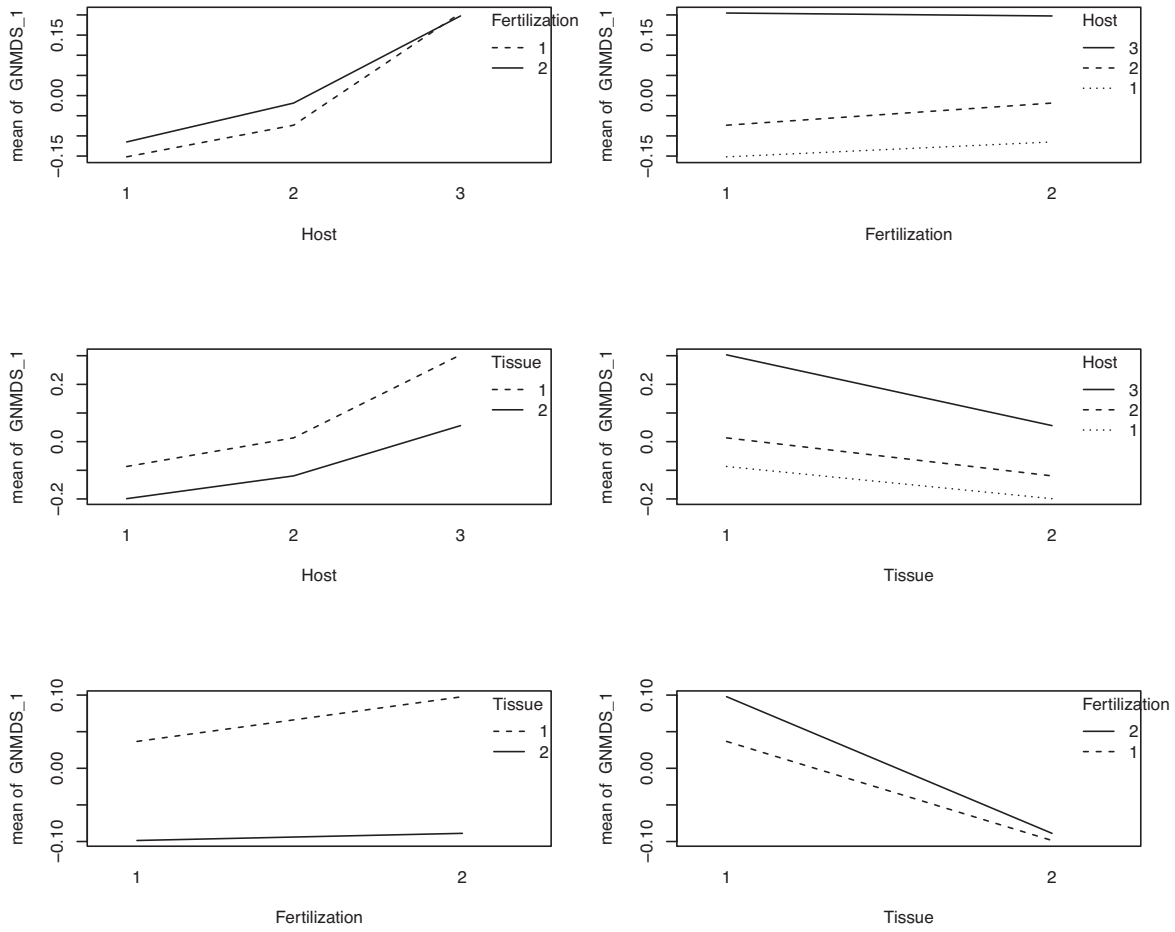


Figure 1: The interaction plot from the ANOVA of GNMDS ordination of incidence data from all OTUs

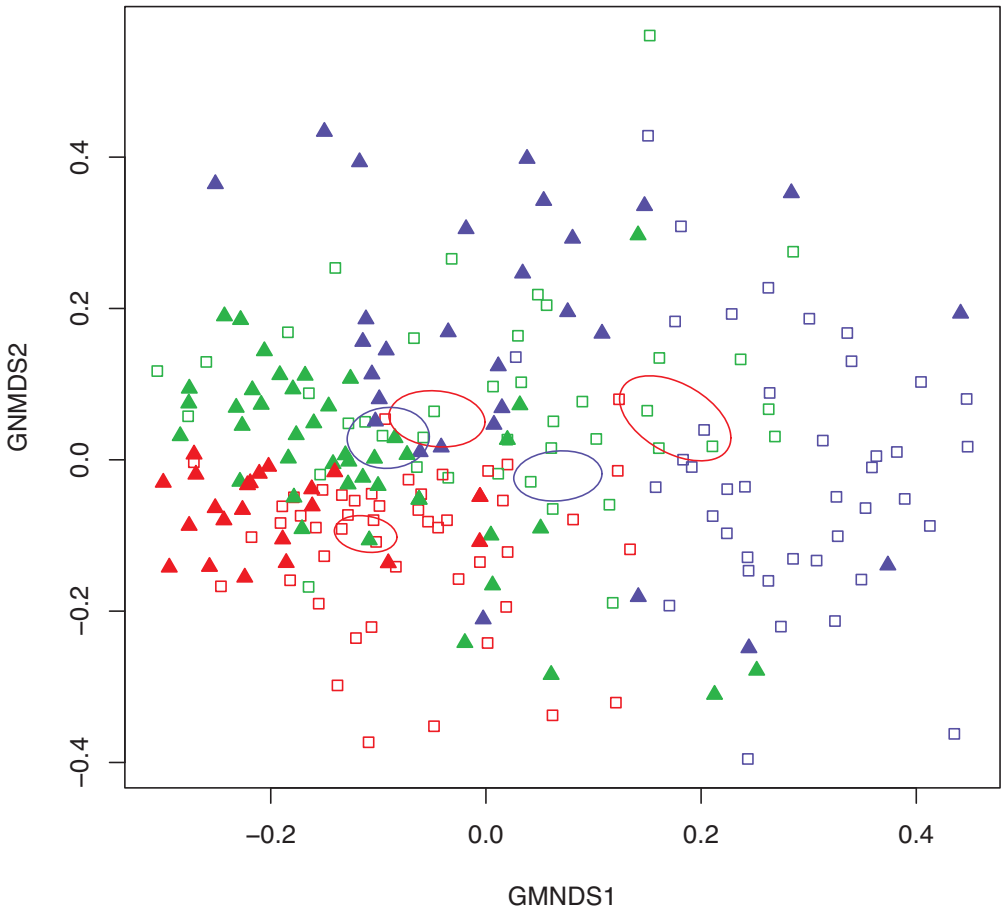


Figure 2: GNMDS diagram for incidence data of the 66 most common OTUs. The triangles are samples from green shoots and the squares are from brown. The blue symbols are from *Dicranum scoparium*, the green are from *Pleurozium screberi*, and the red are from *Hylocomium splendens*. The ellipses represent the 95 % confidence interval for the focal point of each vector, as calculated with 999 permutations using the envfit function in the vegan package of R. Focal points of host species are in red and tissue type are in blue.

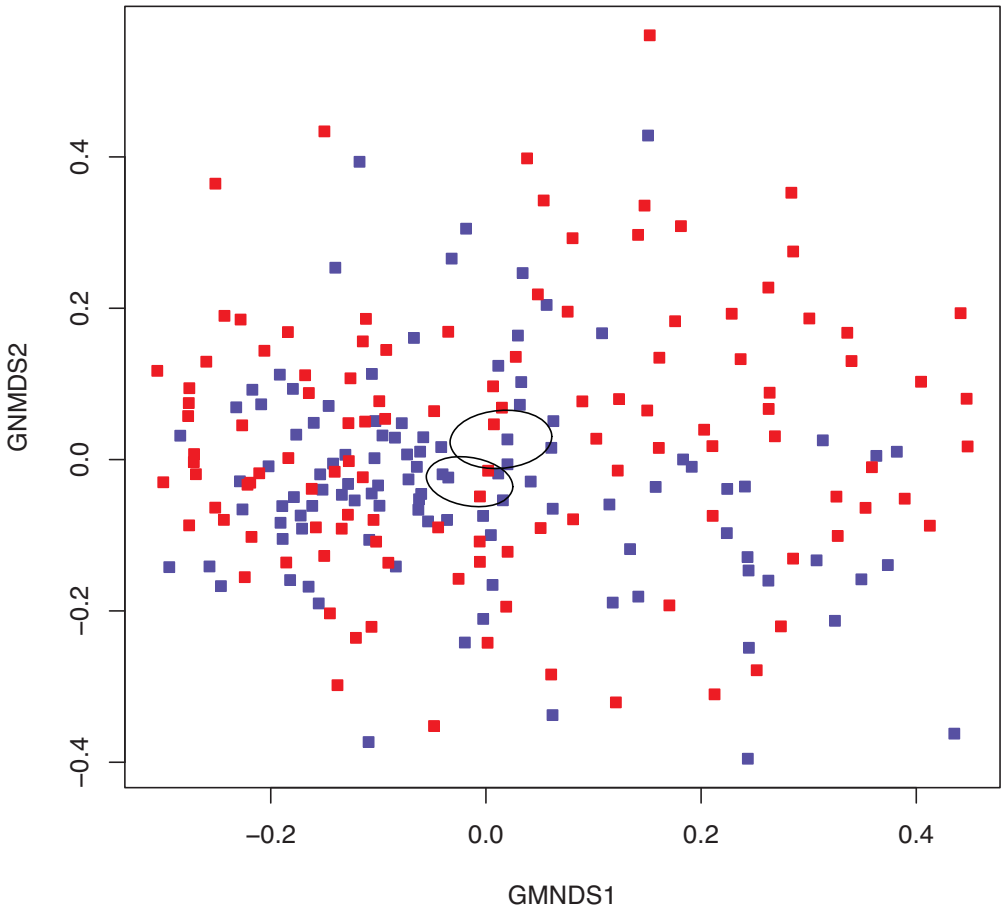


Figure 3: GNMDS diagram for incidence data for the 66 most common OTUs. The blue squares are shoots from the nitrogen enriched plots and the red are from control plots. The black ellipses represent the 95 % confidence interval for the focal point of the vector, as calculated with 999 permutations using the envfit function in the vegan package of R.

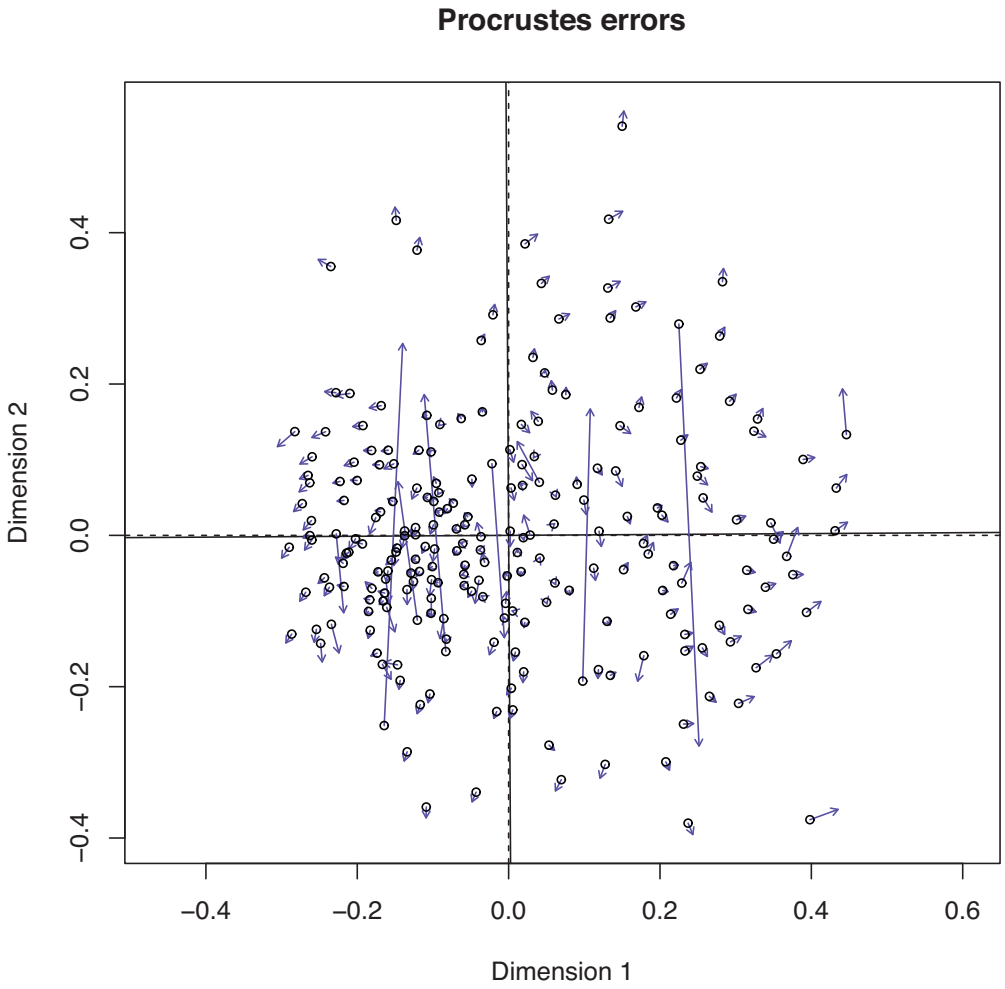


Figure 4: Procrustes diagram for the GNMDS ordination of core species versus all taxa.

Table 1: Testing the difference between the incidence results of the ordination of taxa present in more than 66 samples using a partially nested ANOVA. . Degrees of freedom (Df), Sum of squares (Sum Sq) and Mean sum of squares (Mean Sq) are noted. Significant p-values are in bold.

Response	Factor	Df	Sum Sq	Mean Sq	F value	p-value
GNMDS axis 1						
	Fertilization	1	0.0475	0.04753	2.9724	0.0870741
	Host	2	3.2921	1.64606	102.9304	**<0.0001**
	Tissue	1	0.2059	0.20589	12.8746	**0.0004**
	Fertilization:Host	2	0.0209	0.01043	0.6521	0.5226703
	Fertilization:Tissue	1	0.051	0.05095	3.1861	0.076597
	Host:Tissue	2	0.1016	0.05079	3.176	**0.0450**
	Fertilization:Host:Tissue	2	0.0253	0.01263	0.7895	0.4562
	Residuals	130	2.079	0.01599		
GNMDS axis 1						
	Fertilization	1	0.17464	0.17464	10.6345	**<0.0014**
	Host	2	1.18392	0.59196	36.0476	**<0.0001**
	Tissue	1	0.00844	0.00844	0.5142	0.4746
	Fertilization:Host	2	0.22835	0.11417	6.9527	**<0.0014**
	Fertilization:Tissue	1	0.02951	0.02951	1.7967	0.182446
	Host:Tissue	2	0.10378	0.02951	3.1598	**<0.0457**
	Fertilization:Host:Tissue	2	0.06288	0.03144	1.9146	0.151533
	Residuals	130	2.1348	0.01642		

Table 2: Testing the difference between the transformed abundance ordination results on the two GNMDS axes using a partially nested ANOVA. Degrees of freedom (Df), Sum of squares (Sum Sq) and Mean sum of squares (Mean Sq) are noted. Significant p-values are in bold.

Response	Factor	Df	Sum Sq	Mean Sq	F value	p-value
GNMDS axis 1						
	Fertilization	1	0.0960	0.09605	6.9726	**0.0093**
	Host	2	4.4279	2.21396	160.7201	**<0.0001**
	Tissue	1	0.2158	0.21576	15.6627	**<0.0001**
	Fertilization:Host	2	0.0102	0.00511	0.3709	6909
	Fertilization:Tissue	1	0.053	0.05302	3.8492	**0.0519**
	Host:Tissue	2	0.1095	0.05476	3.9756	**0.0211**
	Fertilization:Host:Tissue	2	0.0173	0.00866	0.6283	0.5351
	Residuals	130	0.0173	0.01378		
GNMDS axis 1						
	Fertilization	1	0.10155	0.10155	8.0204	**0.0054**
	Host	2	0.79816	0.39908	31.5188	**<0.0001**
	Tissue	1	0.09123	0.09123	7.2052	**<0.0082**
	Fertilization:Host	2	0.00636	0.00318	0.2511	0.7783
	Fertilization:Tissue	1	0.03302	0.03302	2.6082	0.1087
	Host:Tissue	2	0.01553	0.00776	0.6133	0.5431
	Fertilization:Host:Tissue	2	0.02146	0.01073	0.8476	0.4308
	Residuals	130	1.64602	0.01266		