



# Nitrogen deposition changes ectomycorrhizal communities in Swiss beech forests



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## HIGHLIGHTS

- In Swiss beech forests high N deposition is the main cause for reduced tree growth.
- Ectomycorrhizal (EMF) communities were analyzed on both root tips and in mesh bags.
- EMF diversity and biomass were strongly reduced with increasing N deposition.
- High base saturation did not diminish the negative N deposition effects on EMF.
- EMF communities were associated with tree nutrients including foliar N and P.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Atmospheric pollution has implications for the health and diversity of temperate forests covering large parts of central Europe. Long-term elevated anthropogenic deposition of nitrogen (N) is driving forest ecosystems from the limitation by N to other nutrients and is found to affect tree health and ectomycorrhizal fungi (EMF), which most trees depend on for nutrient uptake. However, the consequence of EMF community changes for trees remains unclear. Therefore, we investigated changes in EMF communities on root tips and in soil of beech forests along a N deposition gradient ranging between 16 and 33 kg N ha<sup>-1</sup> a<sup>-1</sup>, where high N deposition was found to negatively affect tree growth and nutrient levels. The most important factors significantly explaining variation in root tip and mycelium EMF community composition in both root tips and mesh bags were increased N deposition, base saturation, growing season temperature and precipitation. With increasing N deposition, fine root length, EMF root colonization, EMF diversity on root tips and in soil, and production of extramatrical mycelium decreased significantly. Foliar P and potassium (K) were positively associated with increasing EMF diversity and we found EMF community composition to be associated with foliar P and N:P ratio. The decrease in root colonization, mesh bag ingrowth and abundance of the important species *Cenococcum geophilum* as well as high biomass species with increasing N availability clearly indicate repercussions for belowground carbon allocation, although some indicator species for high N deposition and low foliar P have long mycelia and may reflect a potential optimization of host P uptake. Our study supports the hypothesis that the decrease in nutrient uptake in beech forests across Europe is related to changes in EMF communities and suggests that continued high N deposition changes soil carbon and nutrient cycles, thereby affecting forest ecosystem health.

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## 1. Introduction

Central European forests are negatively affected by anthropogenic impacts, particularly by increased N deposition (Aber et al., 1989; Emmett, 2007; Sutton et al., 2011). N deposition affects nutrient balance in soil and trees, slows soil organic matter decomposition and enhances leaching of base cations leading to soil acidification (Gundersen et al., 1998; Janssens et al., 2010; Matzner and Murach, 1995; Pålsson, 1992; Peñuelas et al., 2013; Schulze, 2000; Vitousek et al., 1997). All together, these changes are increasingly suspected to affect tree growth and nutrient availability for forest trees (Fenn et al., 1998; Jonard et al., 2015; Lang et al., 2016). Indeed, long term monitoring studies of forest tree health in several central European countries find reductions in tree growth and foliar nutrients that can be attributed to the N impact (Gschwantner, 2006; Jonard et al., 2015; Meining et al., 2008; Talkner et al., 2015; Wardle et al., 2004). However, the specific relationship between increased N availability and the declining growth and nutrient trends in trees is not clear yet, therefore, components of the nutrient cycle and their sensitivity to increased N levels need to be investigated.

The ectomycorrhizal symbiosis between trees and fungi is vital to plant uptake of nutrients and water and most temperate forest tree species form associations with EMF to overcome nutrient limitations (Smith and Read, 2008). EMF communities in natural forests were found to respond to increased N availability from local (Dighton et al., 2004; Högberg et al., 2003; Kjeller et al., 2012; Lilleskov et al., 2002; Schirkonyer, 2013; Toljander et al., 2006) to country (Jarvis et al., 2013) and up to continental scales (Cox et al., 2010; Suz et al., 2014; Taylor et al., 2000), as well as in fertilization studies (Avis et al., 2008, 2003; Edwards et al., 2004; Ekblad et al., 2016; Fransson et al., 2000; Kårén and Nylund, 1997; Parrent et al., 2006; Peter et al., 2001). Reported responses to increased N deposition include EMF community shifts, decreased EMF diversity, decreased root colonization and decreased soil mycelial growth. Reductions in belowground C supply to EMF as plant shoot C sink strength increases over roots due to alleviation of N limitation, are likely to reduce the supply of nutrients by EMF to their plant hosts. After alleviation of N limitation, above-ground tree growth can become limited by other nutrients. The observed niche separation of EMF with regards to their nutrient uptake (Buée et al., 2007; Erlandson et al., 2016; Tedersoo et al., 2010), the observed shifts in EMF community composition to increased N availability and increased biomass production in response to N additions (Wallander and Nylund, 1992) may indicate a shift towards an EMF species assemblage better suited to meet the nutrient requirements of the N-replete hosts (Lilleskov, 2005). Thus, the functional significance of N deposition induced changes on EMF for forest health and productivity remain unresolved.

In Central and Eastern Europe beech (*Fagus sylvatica*) is an important broadleaved forest tree that is known to form abundant EMF symbioses with a diversity of fungal species (Buée et al., 2009) and is considered to be highly dependent on EMF for nutrient uptake (Druebert et al., 2009). In numerous beech observation plots from the European ICP Forests network as well as the Swiss intercantonal permanent forest observation program, trends of declining foliar nutrient concentrations over time have been observed (Braun et al., 2010; Jonard et al., 2015; Talkner et al., 2015; Zavišić et al., 2016). According to the limits for P and the N:P ratio for adequate nutrition compiled by Göttlein (2015), 22% of the assessed 79 central European beech forests now have to be regarded as P deficient and 62% have N:P ratios indicating suboptimal nutrition (Talkner et al., 2015). In the Swiss observation plots, where N deposition has proceeded at increased levels for several decades leading to strongly N enriched forest ecosystems (Augustin and Achermann, 2012; Rihm and Achermann, 2016), all observed N:P ratios in mature beech throughout Switzerland are above the ranges for normal nutrition (Braun et al., 2010). A number of reasons, including increased N deposition, climate change and increased masting have been proposed for this negative time trend in P nutrition of trees (Seven, 2014; Talkner et al., 2015). EMF have been shown to play an

important role for the P nutrition of trees (Cairney, 2011; Plassard and Dell, 2010). However, it remains unresolved whether the response of EMF to N deposition is directly affecting P uptake and other nutrient fluxes to trees and is responsible for the nutrient deficiencies observed.

In this study we test the hypotheses that in Swiss beech forests (i) root tip and soil colonization by EMF decrease and (ii) EMF diversity and EMF community composition change across a N deposition gradient. If these two hypotheses are supported, we hypothesize that (iii) the observed nutrient decrease in trees can be related to the observed EMF diversity loss and/or EMF community changes. Additionally, we hypothesize that (iv) we will observe reduced N deposition impacts on soils with high base saturation. Since study scale seems to be important in determining factors that influence biodiversity, studies at larger, regional or continental scales may be preferable to avoid unexpected factor influences (Willis and Whittaker, 2002). Therefore, we assess EMF community diversity and composition on root tips and soil mycelium on a rather large scale and include plots with different soil types and base saturation levels across Switzerland, to examine whether EMF communities in soils with low base saturation were more sensitive to N deposition. Moreover, plots were selected to cover a range of contrasting foliar phosphorus concentrations, in order to look for a relationship between changing EMF communities and tree nutrient status.

## 2. Materials and methods

### 2.1. Forest plots and environmental data

15 beech forest plots were selected on a N deposition gradient across Switzerland ranging from 16 to 33 kg N ha<sup>-1</sup> a<sup>-1</sup> (Table 1; Fig. S1, Supporting Information). These plots belong to a network of permanent forest observation plots (Braun et al., 1999) and cover an area spanning 200 km from north to south and 120 km from east to west. The 15 selected plots are dominated by European beech, which comprises on average 79% of basal area, with a mix of other species including pedunculate and sessile oak (4.5%, 8 plots), European ash (1.4%, 3 plots), Norway spruce (6.6%, 9 plots), silver fir (2.5%, 8 plots), European larch (2.2%, 4 plots), Scots pine (1.3%, 4 plots) and Douglas fir (1.4%, 1 plot). Key environmental, soil and foliar variables are shown in Table 1. For data analysis in this paper, N deposition was modeled on a hectare grid resolution for the year 2010 (Rihm and Achermann, 2016). For a historical comparison of N deposition the modeled data was interpolated according to Braun et al. (2017). Ozone flux values from 2013, the year before soil sample collection, were used (Braun et al., 2014). Meteorological data were interpolated using the “Shepard’s Gravity Interpolation” method (Zelenka et al., 1992) from the nearest eight monitoring stations of the Swiss Meteorological Institute (MeteoSwiss). Temperature was used as a long-term average over the growing season from 1981 to 2015. Drought was expressed either as ratio between actual and potential evapotranspiration (evapotranspiration ratio) or as site water balance, both calculated using the hydrological model Wasim-ETH (Schulla and Jasper, 2013) and averaged over the whole growing season (Braun et al., 2015). Leaf samples from the upper crown were collected end of July 2015 and were analysed for foliar nutrients as described in Braun et al. (2003). Soil type was assessed in soil pits using the classification system of Benzler et al. (1982). Soil samples were collected by horizon in the course of a regular forest plot network assessment in winter 2005/2006 at 6–8 points per plot and combined to one sample per horizon. Total P, soil pH and exchangeable cations (hereinafter referred to as soil K, Ca and Mg) were measured as described in Braun et al., 2003. Organic carbon was determined either using the dichromate procedure according to Walinga et al. (1992) or, in soils with high C content, by ignition loss. Nitrate was measured in 1:1 w/w water extracts of fresh soil samples with previously determined water content (organic soils extract ratio 1:5 w/w) using ion chromatography. Soluble P was extracted with 2% citric acid (Hort et al., 1998). Soil pH, base saturation, soil

**Table 1**

The 15 selected beech forest plots on the N deposition gradient. Soil characteristics pH and base saturation are averaged over the uppermost 40 cm.

Name	Code	Elevation (m)	Total precipitation (mm a <sup>-1</sup> )	Growing season temperature (°C)	Evapo-transpiration ratio (Eta/Etp)	pH	Base saturation (%)	N deposition (kg ha <sup>-1</sup> a <sup>-1</sup> )	Foliar N (mg g <sup>-1</sup> )	Foliar P (mg g <sup>-1</sup> )
Bonfol	BO	450	1114.9	15.27	0.92	4.3	18.1	16.8	18.27	1.08
Rodersdorf	RO	440	1050.6	15.46	0.83	4.1	54.7	19.1	20.97	0.99
Riehen-Maienbühl	RM	470	1012.3	15.54	0.82	5.3	100.0	19.2	20.21	1.21
Rafz	RA	530	982.5	15.31	0.78	4.1	39.4	20.1	18.96	1.37
Allschwil	AL	350	885	16.27	0.84	4.1	36.4	21.1	18.44	1.73
Breitenbach	BB	460	995.4	15.89	0.82	5.2	90.7	21.9	21.15	0.8
Olsberg	OL	380	1013.8	15.72	0.87	4.1	19.8	23.1	19.74	0.91
Pratteln	PR	415	1116.5	15.36	0.87	4.5	100.0	23.7	19.47	1.55
Habsburg	HA	430	1089	15.57	0.84	4.2	16.3	24.7	18.54	1.13
Aarwangen	AW	470	1162.4	15.17	0.89	4	9.6	25.4	18.53	0.9
Frienisberg	FR	725	1218.9	14.13	0.85	3.9	21.1	27.6	21.60	1.39
Bachtel	BA	1030	1882.6	13.08	0.91	3.9	36.4	30.2	22.25	1.33
Vordergeissboden	VG	900	1507	13.06	0.92	3.6	23.2	30.6	17.32	1.01
Muri	MU	490	1133.7	15.38	0.88	4	23.5	32.4	18.30	0.8
Sagno	SA	770	1769.5	15.39	0.79	4.6	81.8	32.7	20.34	0.87

cations and nutrient concentrations were averaged over the uppermost 40 cm. Ratios between C and N or P were calculated for the forest floor or for the uppermost mineral soil horizon if no humic horizon was present (e.g. mull humus forms).

## 2.2. Sampling of root tip mycorrhizas

We applied the standardized sampling method for EMF communities at large spatial scales developed by Cox et al. (2010) during autumn 2013. In the 15 monitoring plots, ten beech trees were randomly selected and a transect was traced to the nearest neighbouring beech tree. Only transects that were a minimum of 10 m from any non-beech trees were used. On each transect, four soil cores were collected at evenly-spaced distances using a 2 cm diameter and 25 cm length soil corer and stored in sealed plastic bags at 4 °C for up to 4 days. Soil samples were rinsed on a 0.5 mm sieve, and roots were collected over a limited time period of 5 min to standardize sampling efforts. To minimize observer bias and maximize sample independence, the three longest roots were selected from each soil sample. One live mycorrhiza (assessed by its turgor and appearance after breaking it apart from the root) was sampled from one end of each root using a stereomicroscope (Wild M3B, Heerbrugg, Switzerland; and Leica camera MC170 HD, Wetzlar, Germany). Accordingly, a total of 120 mycorrhizal root tips were analysed per plot. Photographs and brief descriptions of each morphotype encountered, including the presence of hyphae and rhizomorphs, were recorded. Root colonization by live EMF was assessed on the three roots previously selected applying the gridline-intersect method (Giovannetti and Mosse, 1980). All living EMF root tips intersecting with the grid lines were counted. To measure fine root length per soil core, all roots collected in each soil core were scanned and analysed with WinRhizo version 3.10b (Régent Instruments, Quebec, Canada). The length of the finest roots (<0.2 mm) was related to total root length.

## 2.3. Sampling of soil mycelia with mesh bags

Ingrowth mesh bags were used to both determine the accumulation of extramatrical mycelium by the EMF as a measure of biomass production and to assess the fungal community composition present as extramatrical mycelium (Wallander et al., 2001). Cylindrical nylon mesh bags of 10 cm length, with a diameter of 2 cm and with a mesh size of 50 µm (Sefar, Heiden Switzerland, Nitex 03-50/31) were filled with 50 g acid-washed quartz sand (0.36–2.00 mm, 99.6% SiO<sub>2</sub>, Carlo Bernasconi AG, Bärschwil, Switzerland) and buried in the 40 soil core holes in each plot at the interface between the organic and mineral horizons, where the production of EMF mycelium has been found to be greatest (Lindahl et al., 2006). They were left there for exactly twelve

months, from winter 2013/2014 to winter 2014/2015. After collection, bag contents were freeze dried before visual estimation of the mycelium growth in the sand under a dissecting microscope. The degree of colonization was divided into six classes (similar to Nilsson and Wallander, 2003): (0) no mycelia, (1) few hyphae present, (2) mycelia present but no aggregation of sand particles, (3) mycelia present and some aggregation of sand particles, (4) mycelia abundant and aggregation of sand particles, and (5) mycelia abundant with aggregation of almost all sand particles.

## 2.4. Molecular characterization of EMF communities

Genomic DNA from individual root tip mycorrhizas was extracted using Extract-N-Amp (Sigma-Aldrich, St. Louis, MO, USA), and the internal transcribed spacer (ITS) region of the nuclear rDNA was amplified using ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) primers. Amplicons were purified using ExoSAP-IT (USB, Cleveland, OH, USA) and sequenced bidirectionally using BigDye 3.1 with an ABI 3730 (Applied Biosystems, Foster City, CA, USA). Sequences were assembled and edited with Geneious 7.1.7. (<http://www.geneious.com>; (Kearse et al., 2012). Chimera Checker (Nilsson et al., 2010) implemented in PlutoF (<http://plutof.ut.ee>; Abarenkov et al., 2010) was used to detect potential chimeric sequences. A first identification through BLAST searches via Geneious (Altschul et al., 1990) was carried out assigning them to genus or family to facilitate subsequent alignment. All ITS sequences with <2% ambiguities were aligned by family and clustered with Geneious at a 98% cut-off. A representative DNA sequence of each operational taxonomic unit (OTU) was compared against UNITE (Kõljalg et al., 2005) databases for their taxonomic placement. Sequences presenting over 2% ambiguities, originally discarded from the clustering analyses, but that had similarity to identified taxa in the above database and for which we were able to match their morphotypes with already identified mycorrhizas, were included in further analyses.

Genomic DNA from mesh bag mycelium was obtained using 2 extraction methods. For half of the mesh bags, DNA was extracted from these mycelia with the extract-N-Amp (Sigma-Aldrich, St. Louis, MO, USA) extraction kit after preparation as described by Wallander et al. (2001). For the other half, DNA was extracted in CTAB-SDS extraction buffer according to Rosenstock et al. (2016). The ITS2 region of the mesh bag DNA extracts were amplified using fungal-specific primers ITS7g (Ihrmark et al., 2012) and ITS4 (Gardes and Bruns, 1993). Each primer contained adapters required for the second, Nextera PCR. After the first PCR, the products were subjected to AMPure® purification (AgencourtAMPure XP, Beckman Coulter, Brea, CA, USA), and thereafter a second 8-cycle amplification was performed to attach Nextera sample-specific barcodes for sorting after sequencing. Following the

Nextera amplification, PCR products were subjected to an additional AMPure® purification and quantified using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen™, Carlsbad, CA, USA). Equal amounts of DNA from each sample were then pooled and submitted for Illumina sequencing with paired end (325 bp forward; 275 bp reverse) sequencing on MiSeq sequencer using the MiSeq Reagent Kit v3 chemistry (Illumina Inc., San Diego, CA, USA). After Illumina sequencing sequences were trimmed and filtered using Mothur v1.34 (Schloss et al., 2009). Sequences outside the ITS2 region and chimeric sequences were removed using ITSx extractor v1.5.0 (Bengtsson-Palme et al., 2013). After filtering, a Bayesian clustering was applied to the sequences using the Gaussian Mixture model CROP (Hao et al., 2011) at 97% sequence similarity, and a set of operational taxonomic units (OTUs) was thus obtained. The taxonomic identity was then assigned to the set of clustered sequences using Blastn BLAST 2.2.25 NCBI by searching against a full “UNITE + INSD” dataset (Koljalg et al., 2013), which had been trimmed of all entries lacking taxonomic information (eg. “environmental sequence”, “fungal endophyte”, etc.). Sequences that presented a 96% similarity between the query sequence and top hit, with at least 80% coverage of the query sequence length, were assigned to a taxonomic identity with genus and species. Sequences with values of 94–95% similarity between the query sequence and top hit were assigned a taxonomic identity at the genus level only. All OTUs which represented <10 total reads or occurring in only one sample were removed from the OTU abundance matrix. The community in each sample was rarefied to 10,000 reads using the “rarefy” function of the vegan R package 2.3 (Oksanen et al., 2013). Read abundances for all OTUs for each sample were then summed and expressed as the relative abundance per sample, such that total abundance for each sample was 1. This rarefied relative abundance matrix containing the abundance of OTUs per sample was used to analyze the community composition differences.

Representative sequences for each OTU obtained from root tips were submitted to GenBank (accession numbers KX451142–KX451143, KX885953–KX886201; Table S1, Supporting information), and raw sequence files from Illumina sequencing will be submitted to the NCBI sequence read archive.

OTUs were considered known EMF based on the most current knowledge of the ecology of known close relatives (genera or species) according to Tedersoo et al. (2010). Only ectomycorrhizal taxa were used for analysis of diversity and community composition. Exploration types were assigned to each of the ectomycorrhizal OTUs found on root tips and in mesh bags (Tables S1 and S2, Supporting information) following Agerer (2006, 2001), DEEMY (Information System for Characterization and Determination of Ectomycorrhizae; [www.deemy.de](http://www.deemy.de)) and other published information on ectomycorrhiza colonizing beech trees and exploration types (Lang et al., 2013; Lilleskov et al., 2011; Pena et al., 2013; Suz et al., 2014; Wallander et al., 2013). For taxa for which species-specific information was unavailable, the exploration type was deduced from information on the genus. Ectomycorrhizal taxa were further classified as low biomass (contact, short- and medium-distance smooth exploration types) and high biomass (medium-distance fringe, medium-distance mat and long-distance exploration types), by the presence or absence of rhizomorphs and their hydrophobicity based on Hobbie and Agerer (2010) and Lilleskov et al. (2011).

## 2.5. Statistical analyses

We used R for statistical analyses (version 3.3.1; R Core Team, 2016). Richness and diversity indices were calculated using EstimateS 9.1 (Colwell, 2013): Observed richness (individual-based rarefied species richness, R), estimated richness (abundance-based coverage estimator, ACE; Chao and Lee, 1992), Shannon's diversity index (H), and taxon evenness (Pielou, 1966). To assess the sufficiency of the sampling effort, observed and estimated richness were compared. For root tips, species accumulation curves for EMF communities were constructed using the

*specaccum* function of the vegan R package (version 2.4; Oksanen et al., 2013). The relative abundance of each OTU was calculated by dividing the number of root tips colonized with the same OTU by the total number of root tips in each plot for root tips, and by dividing the number of sequence reads belonging to the same OTU by the total number of sequence reads for mesh bags. Relative abundance was calculated in a similar way for each genus and exploration type. Relationships between environmental factors, soil chemistry, root and foliar nutrient concentrations and abundances of EMF species, genera and exploration types were explored with Spearman's rank correlations and the Benjamini-Hochberg procedure was applied to control the false discovery rate. Regression analysis was used to test whether N deposition or other factors influenced EMF colonization, growth and diversity. Normality of the distribution of residuals was checked by visual inspection of QQ-plots.

For community composition analysis, Bray-Curtis and Euclidean dissimilarity matrices were generated for community and environmental data, respectively. Relative abundance of EMF taxa was square-root transformed and standardized by applying the function *wisconsin* of the vegan R package. Community dissimilarity among plots was visualized by nonmetric multidimensional scaling (NMDS) with the vegan R package. The following environmental variables were fitted to the ordination plots using the ‘*envfit*’ function: latitude, longitude, elevation, mean growing season temperature, total annual precipitation, site water balance, evapotranspiration ratio, N deposition, ozone flux, soil pH, base saturation, soil exchangeable cations K, Ca and Mg, soil N, P, N:P in the uppermost 40 cm of the soil, N, P and C:N in forest floor or Ah, foliar concentrations of N, P, N:P, Mg, K, Ca, Mn, and abundance (percentage of basal area) of co-occurring tree species. NMDS analyses were also applied to the composition of exploration types. Community-level thresholds were assessed by summing all z scores on the gradients. To examine which environmental variables were significantly associated with the EMF community as well as their interactions, we carried out permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) using the *adonis* routine of the vegan R package. Environmental and foliar factors were examined individually, and then, among those significantly associated with the community composition, in combination. Multi-factor models were restricted to factors that were significant in single factor models, and only the best multifactor model with a maximum of three factors is reported. To account for the sensitivity of PERMANOVA analysis to factor ordering, particularly when factors are correlated, multi-factor models were only acceptable if all factors were significantly associated with community composition in all possible factor orderings. Threshold Indicator Taxa Analysis was applied with the TITAN2 R package (version 2.1; Baker and King, 2010) to detect change points in both the occurrence frequency and relative abundance of single taxa across the environmental or tree nutrient gradients and ecological community thresholds. Taxa present in at least three plots for both root tips and mesh bags were tested.

## 3. Results

### 3.1. Environmental, soil and foliar data

N deposition decreased in the last three decades in the selected plots from 25–46 kg N ha<sup>-1</sup> a<sup>-1</sup> in 1980 to ca. 16–33 kg N ha<sup>-1</sup> a<sup>-1</sup> in 2010 (Fig. S2, Supporting information). This deposition exceeds the critical loads for beech forest (10–20 kg N ha<sup>-1</sup> a<sup>-1</sup>; Bobbink and Hettelingh, 2010) and suggests that N availability in soil is rather high and not limiting tree growth. Nitrogen deposition on our plots in 2010 was strongly negatively correlated ( $R^2 = 0.68$ ,  $P < 0.01$ ) with latitude, and strongly positively correlated with longitude ( $R^2 = 0.68$ ,  $P < 0.01$ ) and altitude ( $R^2 = 0.67$ ,  $P < 0.01$ ) and precipitation ( $R^2 = 0.67$ ,  $P < 0.01$ ; Table S1, Supporting information). Soil pH and base saturation were strongly correlated with one another ( $R^2 = 0.81$ ,  $P < 0.001$ ), but not with N

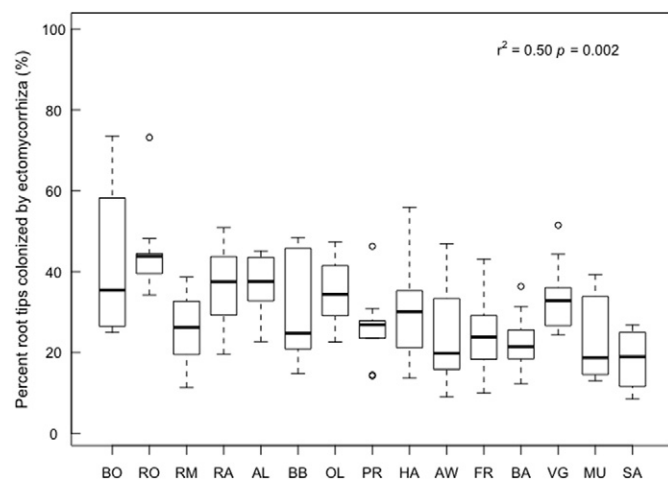
deposition or other N variables. Base saturation was strongly correlated with the soil Ca ( $R^2 = 0.75$ ,  $P < 0.001$ ), soil K ( $R^2 = 0.46$ ,  $P < 0.01$ ) and soil Mg ( $R^2 = 0.69$ ,  $P < 0.001$ ). Foliar K was significantly positively correlated with soil K ( $R^2 = 0.36$ ,  $P = 0.01$ ) and significantly negatively correlated with N deposition ( $R^2 = 0.54$ ,  $P < 0.05$ ). Foliar Ca was significantly positively correlated with soil Ca ( $R^2 = 0.45$ ,  $P = 0.004$ ) and base saturation ( $R^2 = 0.36$ ,  $P = 0.01$ ). Foliar Mg was not correlated with soil Mg and foliar N was only marginally significantly correlated with soil N ( $R^2 = 0.16$ ,  $P = 0.078$ ). All pairwise relationships between key environmental, soil and foliar variables with Pearson's correlations are shown in Table S1 (Supporting information). Foliar nutrient concentrations, ratios to N and deficiency levels are listed in Table S2, Supporting information).

### 3.2. EMF colonization on root tips and in mesh bags

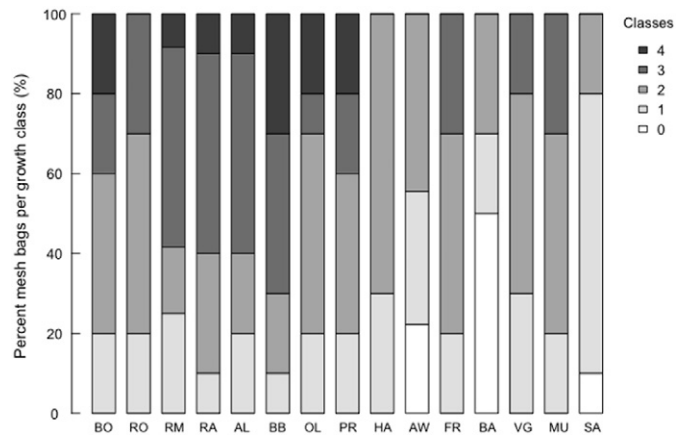
Both relative fine root length ( $R^2 = 0.40$ ,  $P = 0.007$ ) and the relative amount of live EMF colonizing root tips ( $R^2 = 0.50$ ,  $P = 0.002$ , Fig. 1) decreased significantly with increasing N deposition. In plots with  $< 23 \text{ kg N ha}^{-1} \text{ a}^{-1}$  around 40% root tips had live EMF, while this dropped to  $< 20\%$  in plots with  $> 30 \text{ kg N ha}^{-1} \text{ a}^{-1}$  except for plot "Vordergeissboden". The visual estimate of mycelium growth in the mesh bags showed that the colonization after 12 months varied from bags with a considerable mycelial ingrowth with aggregation of sand particles (class 4) in plots with 16 to  $24 \text{ kg N ha}^{-1} \text{ a}^{-1}$  to bags with no mycelial growth at all (class 0) in plots with  $> 25 \text{ kg N ha}^{-1} \text{ a}^{-1}$  (Fig. 2; Page Test,  $X^2 = 2695.9$ ,  $P < 0.001$ ). Accordingly, the average mycelial colonization class per plot decreased significantly with increasing N deposition, from 2.87 in plots with  $< 20 \text{ kg N ha}^{-1} \text{ a}^{-1}$  to 1.97 in plots with  $> 30 \text{ kg N ha}^{-1} \text{ a}^{-1}$  ( $R^2 = 0.32$ ,  $P = 0.016$ ).

### 3.3. EMF diversity on root tips

From the 600 soil cores we examined 1768 ectomycorrhizal root tips morphologically from which we obtained 1519 DNA sequences belonging to 269 fungal taxa. In total 241 taxa were considered to be ectomycorrhizal and were included in the analyses (Table S3, Supporting information). According to the ACE estimator, we detected on average 70% ( $\pm 10\%$ ; range 52%–90%/plot) of the estimated EMF richness across all root tips and all plots. Accumulation curves indicated that the sampling effort for root tips was nearly sufficient to sample expected species richness in plots with relatively high N deposition, but not sufficient to sample total species richness in plots with relatively low N deposition (Fig. S3, Supporting information). Rank abundance



**Fig. 1.** Percent root tips colonized by ectomycorrhiza in the 15 plots ordered according to N deposition (lowest deposition on the left). Two-letter codes refer to plot names as described in Table 1.



**Fig. 2.** Percent mycelial colonization in mesh bags in the 15 plots ordered according to N deposition (lowest deposition on the left). Class explanation: (0) no mycelia; (1) few hyphae present; (2) mycelia present but no aggregation of sand particles; (3) mycelia present and some aggregation of sand particles; and (4) mycelia abundant with aggregation of sand particles. Average mycelial colonization class per plot decreased significantly with increasing N deposition ( $P < 0.001$ ). Two-letter codes refer to plot names as described in Table 1.

curves indicated that communities had two or three abundant and many rare EMF taxa (Fig. S4a, Supporting information). The average observed number of OTUs on root tips per plot was  $38.5 (\pm 9.0)$ ; range 23–55/plot) and Shannons diversity index was on average  $3.19 (\pm 0.34)$ ; Table S4, Supporting information). For root tips, species diversity was strongly negatively correlated with increasing N deposition (Fig. 3; Table 2; Table S6, Supporting information). On the other hand, the number of species was positively correlated with increasing foliar K ( $R^2 = 0.51$ ,  $P = 0.002$ , Fig. 3b) and foliar P ( $R^2 = 0.17$ ,  $P = 0.07$ , Fig. 3c).

### 3.4. EMF diversity in mesh bags

From the 300 mesh bags we recovered 3,557,179 DNA sequence reads belonging to 3114 fungal taxa. From these, 468 taxa (15.1%) were considered ectomycorrhizal and their average fraction of sequence reads in each mesh bag across all mesh bags and monitoring plots was 68% (43–83%). Based on the ACE estimator, on average 82.5% ( $\pm 5.4\%$ ) of the estimated richness across all mesh bags and monitoring plots were recovered, from 70% in Habsburg to 90.9% in Sagno. Rank abundance curves indicated that there were a few abundant and many rare EMF taxa in mesh bags (Fig. S4b, Supporting information). In mesh bags, the average observed number of OTUs per plot was  $187.3 (\pm 24.9)$  and Shannons diversity index was on average  $3.3 (\pm 0.3)$ ; Table S6, Supporting information). As on root tips, species diversity in mesh bags was significantly negatively correlated with increasing N deposition (Table 2b; Fig. S5, Supporting information), and the number of OTUs showed a trend of positive correlation with foliar P concentrations ( $R^2 = 0.14$ ,  $P = 0.09$ ).

### 3.5. Root tip EMF community

Of the EMF taxa detected on the roots tips 78.7% belonged to Basidiomycota and 21.3% to Ascomycota. 25% of the sampled root tips were identified as EMF of the genus *Russula*, 16.5% by *Lactarius*, 8.4% as EMF of the family Gloniaceae (represented exclusively by *Cenococcum geophilum*), and 8.5% by Thelephoraceae (mainly *Tomentella* sp.). Overall, *Lactarius subdulcis* and *C. geophilum* were the most abundant species, they colonized 9.5% (from 0 to 32%) and 8.4% (from 0 to 14%) of all root tips sampled, respectively. *Cenococcum geophilum* was the most abundant species in 5 plots with  $< 22 \text{ kg N ha}^{-1} \text{ a}^{-1}$  and its abundance decreased significantly with increasing N deposition ( $R^2 = 0.21$ ,  $P = 0.05$ ), while it showed a tendency to increase with decreasing

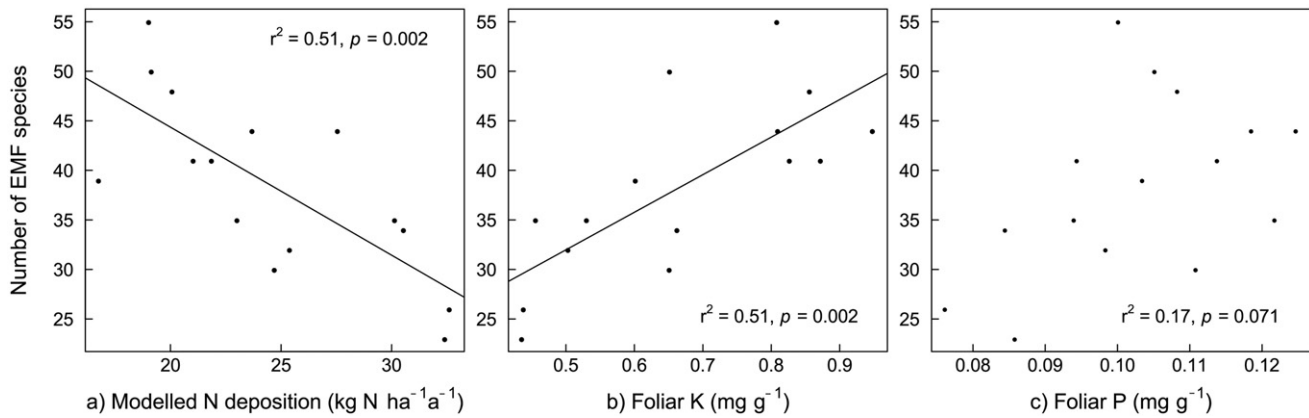


Fig. 3. Relationship between EMF species richness on root tips and a) N deposition, b) foliar K and c) foliar P concentrations.

precipitation ( $R^2 = 0.18$ ,  $P = 0.06$ ). *L. subdulcis* was the most abundant species in 7 plots with  $>21$  kg N ha $^{-1}$  a $^{-1}$ . With increasing base saturation the genera *Toментella* ( $R^2 = 0.38$ ,  $P = 0.009$ ) and *Tricholoma* ( $R^2 = 0.33$ ,  $P = 0.015$ ) increased significantly in abundance, while the genus *Xerocomus* decreased significantly ( $R^2 = 0.36$ ,  $P = 0.01$ ). With increasing foliar P, the genus *Inocybe* increased significantly in abundance ( $R^2 = 0.34$ ,  $P = 0.013$ ).

According to the NMDS ordination, the community composition on root tips was significantly related to latitude, evapotranspiration ratio, soil pH, base saturation, soil Ca, soil K, soil Mg, N deposition, foliar K and foliar Ca (Fig. 4a). Single-factor PERMANOVA analysis revealed that mean growing season temperature, soil pH, base saturation, soil N, soil Ca, soil K, soil Mg and European ash abundance were significantly related to community composition in root tips at  $P < 0.05$ , and N deposition and foliar N were significantly related to community composition at  $P < 0.07$  (Table S7a, Supporting information). The best multi factor model indicated that N deposition, base saturation and temperature were the most important factors driving the community composition patterns we observed (Table 3a). Genus-level abundance analyses yielded similar results in both NMDS and PERMANOVA (Fig. S6a and Table S8a, Supporting information).

Abundance of exploration types and species with high biomass or with rhizomorphs on root tips was significantly associated with elevation, base saturation, soil Ca, soil K, soil Mg, ozone flux, N deposition, foliar N and foliar P according to Spearman's rank correlations (Table 4). With increasing N deposition, EMF species of the medium distance fringe type and species with high biomass decreased significantly ( $P < 0.05$ ). According to NMDS and PERMANOVA, the exploration type composition was significantly related with elevation, base saturation, soil N, soil Mg, foliar N and European ash abundance (Fig. S7a and Table S9a, Supporting information). The best multi factor PERMANOVA indicated that soil N and base saturation were the most important factors driving the exploration types composition on root tips (Table 3c).

We detected indicator species in root tip EMF communities that changed in abundance with increasing N deposition (Fig. 5a), foliar P (Fig. 5c), base saturation and foliar N (Fig. S8a and S9a, Supporting

information). The abundance of several species on root tips was related with both environmental variables and foliar nutrients: *Cenococcum geophilum* decreased with increasing N deposition while it was positively related with foliar P. *Piloderma lanatum* increased with increasing base saturation while it was positively related with foliar N. *Lactarius subdulcis* decreased with both increasing foliar N and foliar P. Peaks in the summed z score curves indicate values along the gradient that produced large amounts of change in community composition and/or structure (data not shown). For the EMF community on root tips, the largest peak for decreasing species on the N deposition gradient was at 24.2 kg N ha $^{-1}$  a $^{-1}$ , for increasing species on the base saturation gradient at 29.9%, for increasing species on the foliar phosphorus gradient at 1.04 mg g $^{-1}$ , and for increasing species on the foliar N gradient at 20.7 mg g $^{-1}$ .

### 3.6. Mesh bag EMF community

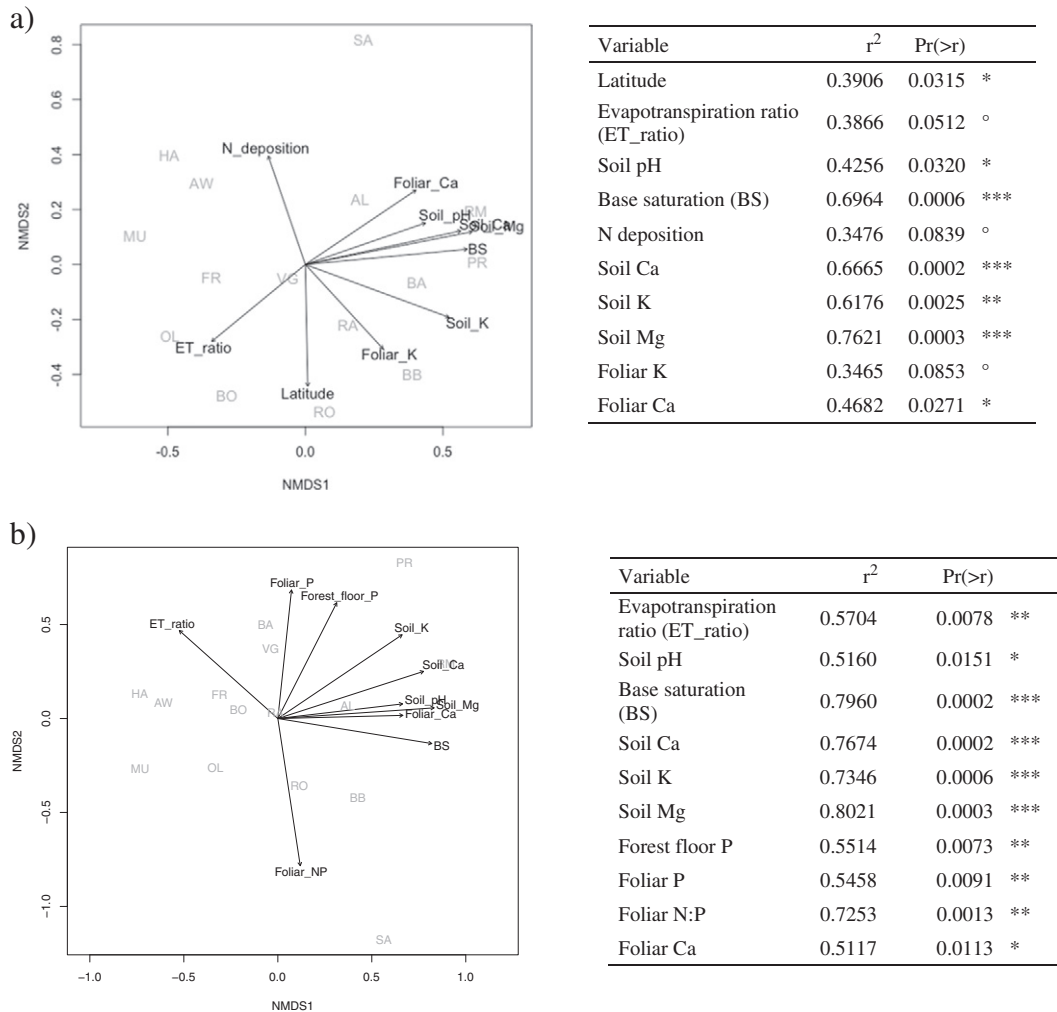
Of the EMF taxa detected in mesh bags 88.7% belonged to Basidiomycota, 11% to Ascomycota, and one taxon belonged to Zygomycota. 19.7% of the sequence reads from the mesh bags were identified as EMF of the family Thelephoraceae (mainly *Toментella* sp.), 13.5% as EMF of the genus *Xerocomus*, 9.9% as *Inocybe*, 8.7% as *Russula*, 6.3% as *Laccaria*. Overall, *Laccaria amethystina* and *Xerocomus pruinosus* were the most abundant species, comprising on average 9.5% (from 0.009 to 14.2%) and 5.8% (from 0.006 to 15.8%) of all mesh bags sequence reads, respectively. *Laccaria amethystina* was the most abundant species in six plots with a N deposition above 20 kg N ha $^{-1}$  a $^{-1}$ . Only 1.63% of the sequence reads in the mesh bags were identified as *C. geophilum* and 0.89% as *L.*

*subdulcis*, the two most common OTUs in the root tip community. With increasing N deposition, the genus *Lactarius* increased in abundance ( $R^2 = 0.28$ ,  $P < 0.026$ ). With increasing base saturation the genera *Toментella* ( $R^2 = 0.31$ ,  $P = 0.018$ ), *Inocybe* ( $R^2 = 0.72$ ,  $P < 0.001$ ) and *Peziza* ( $R^2 = 0.25$ ,  $P = 0.03$ ) increased significantly in abundance, while the genus *Xerocomus* decreased significantly ( $R^2 = 0.29$ ,  $P = 0.02$ ).

Table 2

Relative importance of N deposition for the explanation of EMF diversity on root tips and in mesh bags in the 15 plots as revealed from analysis of variance. Significant values ( $p < 0.05$ ) in bold and significance levels are indicated with  $^{\circ}$ ,  $P = 0.1-0.05$ ;  $^*$ ,  $P = 0.05-0.01$ ;  $^{**}$ ,  $P = 0.01-0.001$ ;  $^{***}$ ,  $P < 0.001$ .

	Number of plots	a) Root tips			b) Mesh bags		
		Coefficient	R <sup>2</sup>	P	Coefficient	R <sup>2</sup>	P
Number of species	15	-1.29	0.51	0.002 <sup>**</sup>	-2.52	0.22	0.046 <sup>*</sup>
ACE (estimated species richness)	15	-2.64	0.47	0.003 <sup>**</sup>	-3.25	0.31	0.018 <sup>*</sup>
R (rarified or expected species richness)	15	-1.02	0.51	0.002 <sup>**</sup>	-4.41	0.39	0.008 <sup>**</sup>
H (Shannon's diversity index)	15	-0.04	0.32	0.02 <sup>*</sup>	-0.02	0.03	0.427
J (community evenness)	15	-0.01	0.01	0.357	-0.01	0.04	0.51



**Fig. 4.** Community composition on root tips (a) and in mesh bags (b) displayed by nonmetric multidimensional scaling (NMDS). Stress of the ordination is 0.16 for root tips and 0.15 for mesh bags. Only significant variables ( $P < 0.1$ ) are shown as arrows whose length is proportional to the strength of the correlation. Significance levels are indicated as follows: °,  $P = 0.1-0.05$ ; \*,  $P = 0.05-0.01$ ; \*\*,  $P = 0.01-0.001$ ; \*\*\*,  $P < 0.001$ . Two-letter codes refer to plot names as described in Table 1.

According to NMDS analyses, community composition in mesh bags was significantly related to evapotranspiration ratio, soil pH, base saturation, soil Ca, soil K, soil Mg, P in the forest floor, foliar P, foliar N:P and foliar Ca (Fig. 4b). Single-factor PERMANOVA indicated, that the community composition in mesh bags was significantly correlated with temperature, evapotranspiration ratio, soil pH, base saturation, soil Ca, soil K, soil Mg, foliar N:P, foliar Ca and European ash abundance at  $P < 0.05$ , and with precipitation, site water balance,

forest floor P, foliar P and K at  $P < 0.1$  (Table S7b, Supporting information). The best multi-factor model indicated that base saturation, temperature and precipitation were the most important factors driving the community composition patterns we observed (Table 3b). Because N deposition was strongly correlated with precipitation, a three-factor PERMANOVA model with temperature, base saturation and N deposition was also significant, but explained slightly less variation. Genus-level abundance analysis yielded similar results in

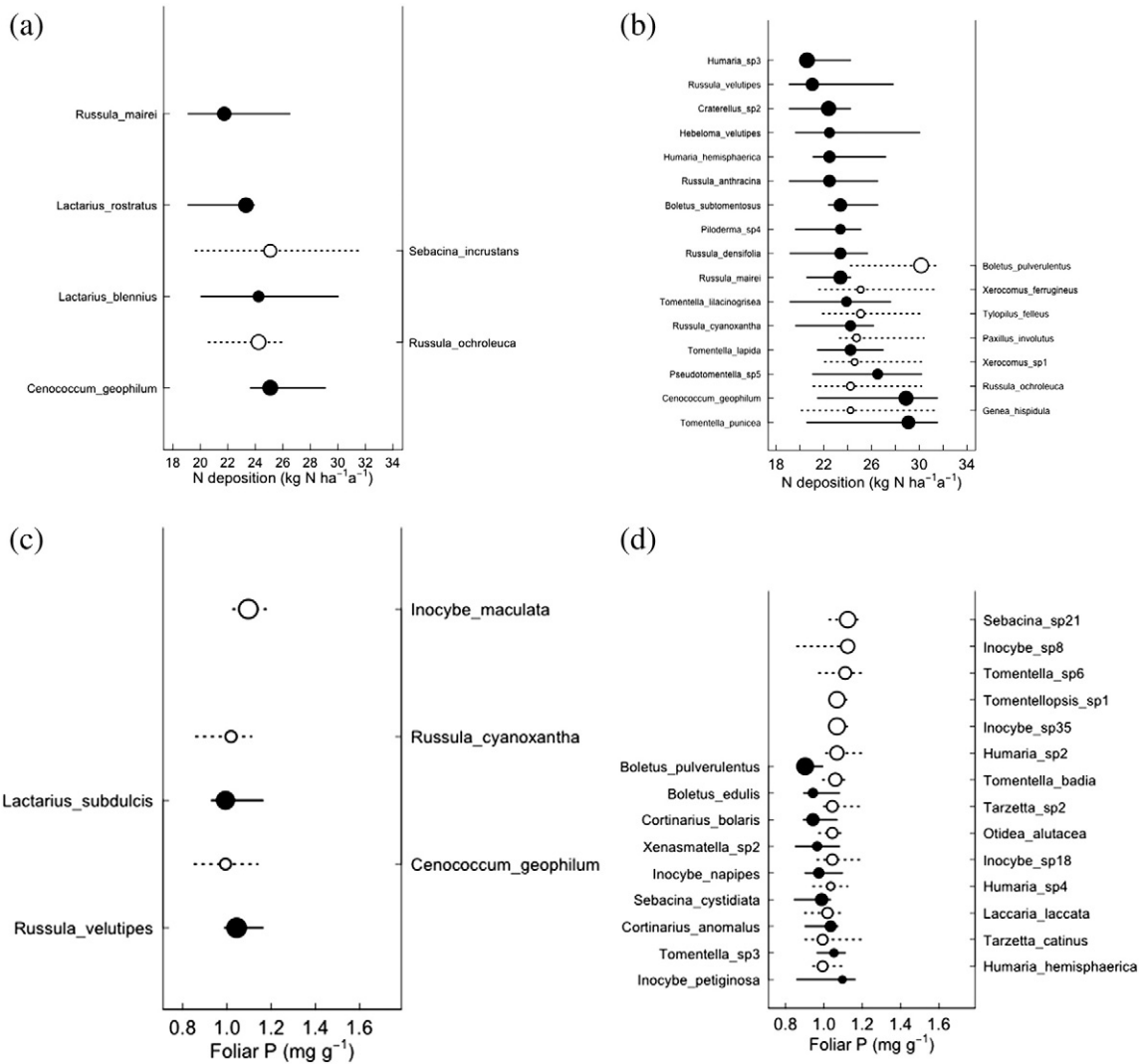
**Table 3**

Test results from multi-factor permutational multivariate analysis of variance (PERMANOVA) with EMF community composition on root tips (a) and in mesh bags (b) and with exploration type composition on root tips (c). These multi-factor models were significant for all factors in all combinations (°,  $P = 0.1-0.05$ ; \*,  $P = 0.05-0.01$ ; \*\*,  $P = 0.01-0.001$ ; \*\*\*,  $P < 0.001$ ). There was no significant multi-factor result for exploration type composition in mesh bags.

Root tips	df	F	R <sup>2</sup>	P	Mesh bags	df	F	R <sup>2</sup>	P
Species composition									
a) Temperature	1	2.517	0.128	0.0004***	b) Temperature	1	1.762	0.103	0.0104**
Base saturation	1	3.213	0.164	0.0003***	Base saturation	1	2.677	0.157	0.0001***
N deposition	1	2.900	0.148	0.0003***	Precipitation	1	1.632	0.096	0.0227*
Residuals	11		0.560		Residuals	11		0.644	
Total	14		1		Total	14		1	
Exploration type composition									
c) Base saturation	1	6.69	0.228	0.0083**					
Soil N	1	10.658	0.363	0.0008***					
Residuals	12		0.409						
Total	14		1						

**Table 4**  
Spearman's rank correlation matrix between EMF exploration types, species with high biomass, species with rhizomorphs, hydrophilic species, environmental and tree variables. Medium-mat types on root tips were excluded from this analysis because of many zeros. Significance levels are indicated with \*,  $P = 0.05-0.01$ ; \*\*,  $P = 0.01-0.001$ ; \*\*\*,  $P < 0.001$ .

	Elevation	Evapotranspiration ratio	Soil pH	Base saturation	Soil Ca	Soil K	Soil Mg	Ozone flux	N deposition	Foliar N	Foliar P
<b>Root tips</b>											
Contact											
Short	+*										
Medium - smooth			-*		-*						-*
Medium - fringe			+*				+*		-*		
Long			-*		-*	-*	-*				
High biomass									-*		
With rhizomorphs								-*			-*
<b>Mesh bags</b>											
Contact			+**	+**	+*		+**				
Short	-*			+**			+*				
Medium - smooth	+***			-***							
Medium - mat											
Medium - fringe											
Long				-**			-*				-**
High biomass											
With rhizomorphs											



**Fig. 5.** Species showing significant changes in response to N deposition and to foliar P concentration on root tips (a, c) and in mesh bags (b, d) according to indicator species analysis. Species increasing in abundance are listed on the right axis (open circles and dashed lines), species decreasing in abundance are listed on the left axis (filled circles and solid lines). Each observed taxon change point is indicated by a circular symbol; the horizontal lines indicate 5–95% quantiles from the bootstrapped change point distribution.



both NMDS and PERMANOVA (Fig. S6b and Table S8b, Supporting information).

Abundance of exploration types in mesh bags was significantly correlated with evapotranspiration, soil pH, base saturation, soil Ca, soil K, soil Mg and foliar N according to Spearman's rank correlations (Table 4). With increasing base saturation, species of the medium-smooth and long distance exploration type decreased significantly ( $P < 0.01$ ), while species of the contact and short distance exploration type increased significantly ( $P < 0.01$ ). According to NMDS and PERMANOVA analysis, the composition of exploration types was significantly related to base saturation, soil pH, evapotranspiration ratio, soil Ca, soil Mg, foliar N, foliar Ca and European ash abundance (Fig. S7b and Table S9b, Supporting information).

We detected indicator species in mesh bag EMF communities that changed in abundance with increasing N deposition (Fig. 5b), foliar P (Fig. 5d), base saturation and foliar N (Fig. S8b and S9b, Supporting Information). The abundance of several species found in mesh bags was related both with environmental variables and foliar nutrients: *Humaria hemisphaerica* decreased in abundance with increasing N deposition, while it increased with increasing foliar P. *Sebacina incrustans* and *Hygrophorus sp1* increased with increasing base saturation and with increasing foliar N. *Humaria hemisphaerica*, *Tarzetta catinus* and *Humaria sp4* increased with increasing base saturation and increased with increasing foliar P. *tomentella lapida* and *H. hemisphaerica* both decreased with increasing N deposition but increased with increasing base saturation. Five of the seven indicator species that we found to increase with greater N deposition in mesh bags (*Boletus pulverulentus*, *Paxillus involutus*, *Tylopilus felleus* and two *Xerocomus* sp.) and five of the seven species that we found to increase in abundance with decreasing foliar P concentrations (two *Boletus* sp., two *Cortinari* sp. and *Xenasmatella* sp2) belong to the long-distance exploration type. For the EMF community in mesh bags the largest peak in the summed z score curves for decreasing species on the N deposition gradient was at 21.2 kg N ha<sup>-1</sup> a<sup>-1</sup>. The largest peak for increasing species on the base saturation gradient was at 68.27%, for increasing species on the foliar phosphorus gradient at 1.04 mg g<sup>-1</sup>, and for increasing species on the foliar N gradient at 2.07 mg g<sup>-1</sup>.

## 4. Discussion

### 4.1. Factors affecting EMF communities

Detailed long-term forest plot monitoring data, national meteorological and pollution data interpolated for the plots at a high spatial resolution enabled us to identify the environmental factors that are related with EMF abundance, diversity and community composition across the gradient. N deposition strongly negatively affected EMF species diversity and abundance on root tips and in soil, while EMF communities were positively associated with foliar P, K and other nutrients. Besides N deposition, base saturation, growing season temperature and precipitation were strong predictors of community composition, with base saturation being the most important predictor for community composition in root tips and mesh bags. Earlier studies based on large-scale N deposition gradients found similar sets of environmental factors to be significantly associated with EMF community composition (Cox et al., 2010; Suz et al., 2014). But they used soil pH and not base saturation, and found parallel effects of increasing N deposition and decreasing soil pH on EMF diversity and community composition. Because of variable bedrock geology in our study, base saturation was not correlated with N deposition, and the fit vectors for N deposition and base saturation were perpendicular to one another (Fig. 4a) indicating different effects of these variables on the community composition. Moreover, our data suggests that some EMF species are more tolerant to base poor soils and that higher base saturation is associated with more short-distance and less long-distance exploration types. Rosenstock et al. (2016) also observed

a strong association between soil mineralogy and EMF community composition across a mineralogical gradient.

The abundance of European ash, present in three sites (RM, BB, PR) with an average basal area proportion of 6.8%, was significantly associated with community composition in both root tips and mesh bags. Earlier research results from a beech forest in southern Germany indicated that European ash leaf litter influenced soil properties and nutrient status in beech trees (Langenbruch et al., 2012), and even the microbial community composition on beech roots (Cesarz et al., 2013; Seven, 2014). However, because European ash was not very abundant and not found near the sampling locations, we suspect the significant association of European ash with EMF community composition to be more of a correlation effect than a causative effect. Indeed the three plots with European ash are those with the highest base saturation, the single strongest explanatory edaphic factor for EMF community composition. European ash is well-known as a “rich-site” species (Ellenberg and Leuschner, 2010) and it is thus not surprising that we find the abundance of European ash to be associated with a distinct fungal community.

### 4.2. Nitrogen deposition effects

In this study we were able to include beech forest plots across Switzerland that cover a wide N deposition gradient ranging from 16 to 33 kg N ha<sup>-1</sup> a<sup>-1</sup>. The lowest N deposition on our gradient falls only 20% below 20 kg N ha<sup>-1</sup> a<sup>-1</sup>, the high mark of current UNECE empirical critical loads of N deposition for deciduous forests in Europe (UNECE, 2016), and 11 of our 15 plots fall above this threshold. The only study that examined the effects of N deposition on EMF in European beech forests utilized a moderate N deposition gradient from 11.6 to 20.1 kg N ha<sup>-1</sup> a<sup>-1</sup> on a north-south transect from Denmark to Italy (Taylor et al., 2000), which was within the critical load of 10–20 kg N ha<sup>-1</sup> a<sup>-1</sup> for EMF in temperate forests (UNECE, 2010). However, in central Europe critical load exceedance of N deposition is common (Rihm and Achermann, 2016; UNECE, 2016) as is the presence of high soil acidity and low base saturation for beech forests (Buée et al., 2005; Dirnböck et al., 2014; Duquesnay et al., 2000; Lang and Polle, 2011; Schirrkonyer, 2013; Talkner et al., 2010). The lack of a relationship between N deposition and foliar N further indicates that these systems are not N limited. Today, low foliar P and high N:P ratios suggest that these areas are likely more P limited (Talkner et al., 2015; Zavišić et al., 2016). In addition, the N deposition in these 15 plots has decreased in the last 3 decades and has likely been over 10 kg N for over 100 years (Augustin and Achermann, 2012; Braun et al., unpublished reports). Thus, it is remarkable that even in these N saturated forests we continue to see a decrease in EMF diversity and abundance as well as changes in community composition across the N deposition gradient. But this is in line with previous studies on these beech forest plots in which modeled N deposition among other N variables was the best predictor for changes in tree growth and tree nutrients (Braun et al., 2017; Braun et al., submitted). This indicates that the observed effect of increased N deposition is not coupled to a continuing alleviation from plant N limitation, but rather due to reduced belowground carbon allocation by host plants, accumulated alterations in soil chemistry and increased limitation by other nutrients.

The observed decrease in EMF diversity with increased N deposition was strong both on root tips and in soil. Compared to plots with a deposition below 20 kg N ha<sup>-1</sup> a<sup>-1</sup>, plots with a deposition of 20–30 kg N ha<sup>-1</sup> a<sup>-1</sup> on average had 20% less and plots with a deposition of 30–40 kg N ha<sup>-1</sup> a<sup>-1</sup> on average had 40% less EMF species on root tips. Similar reductions in species diversity on root tips have been observed in oak (Suz et al., 2014), pine (Cox et al., 2010), and spruce forests (Kjøller et al., 2012; Lilleskov et al., 2002). In a N addition experiment in oak forest with an ambient deposition of about 7 kg N ha<sup>-1</sup> a<sup>-1</sup>, Avis et al. (2008) found a reduction in species richness of 20% with a threefold increase in experimental N deposition. Despite

the decades of relatively high N deposition these sites have experienced, the effects observed on EMF diversity appear to be comparable in scale to the effects of low to moderate N fertilization.

In the two most abundant EMF species on root tips we observed a clear shift with increasing N deposition: *Lactarius subdulcis* did not occur in plots below  $20 \text{ kg N ha}^{-1} \text{ a}^{-1}$  and became relatively more abundant with increasing N deposition, while *C. geophilum* became significantly less abundant with increased N deposition, particularly above  $26 \text{ kg N ha}^{-1} \text{ a}^{-1}$ . A nitrophobic response of *C. geophilum* and nitrophilic response of *Lactarius* sp. has been observed in other studies on broadleaved and coniferous forest using ambient N deposition gradients or applying nitrogen fertilizers (Avis et al., 2008; Cox et al., 2010; Kjølner et al., 2012; Lilleskov et al., 2002; Suz et al., 2014; Toljander et al., 2006). *Cenococcum geophilum*, commonly observed to be highly abundant in a variety of forests across Europe (Horton and Bruns, 2001) has also been associated with enhanced soil carbon sequestration (Fernandez et al., 2013), and its nitrophobicity could have considerable consequences for carbon dynamics in European forest areas. *Cenococcum geophilum* has also been associated with drier soils (Bakker et al., 2006; Suz et al., 2014), which has been linked with the hydrophobicity of its hyphae. Across our study region N deposition was positively associated with precipitation and the abundance of *C. geophilum* tended to decrease with increasing precipitation ( $R^2 = 0.18$ ,  $P = 0.06$ ). However, the abundance of *C. geophilum* was not significantly associated with temperature or evapotranspiration ratio.

We found evidence of decreased belowground carbon inputs as root and mesh bag colonization was generally low compared to other studies on European beech (Lang and Polle, 2011; Pena et al., 2010), and as relative fine root length, root tip colonization, mycelial ingrowth and EMF with large extramatrical mycelium decreased with increasing N deposition. Similar N deposition associated reductions in EMF colonization have been previously observed on both roots (Dighten et al., 2004; Kjølner et al., 2012; Lilleskov et al., 2002) and in soil mycelia of various forest types (Bahr, 2013; Ekblad et al., 2016; Högberg et al., 2010; Kårén and Nylund, 1997; Kjølner et al., 2012; Nilsson and Wallander, 2003; Sims et al., 2007; Wallander et al., 2011). This is in contrast to the responses found by low doses of N fertilizer or small increases in atmospheric N deposition in very N poor systems where EMF colonization or mycelial biomass increased, largely as a result of increased growth by trees (Kou et al., 2015; Terrer et al., 2016; Treseder, 2004, and references therein). In Swiss beech forests an increase in tree growth due to N deposition and deposition levels below  $25 \text{ kg N ha}^{-1} \text{ a}^{-1}$  has been observed, but above  $26 \text{ kg N ha}^{-1} \text{ a}^{-1}$ , N deposition is negatively related with basal area increments (Braun et al., 2017). Our study now supports this N deposition threshold in Swiss beech forest plots, as the greatest reductions in mycelium growth were observed above this threshold and *C. geophilum* became significantly less abundant according to indicator species analysis above  $25 \text{ kg N ha}^{-1} \text{ a}^{-1}$  in both root tips and mesh bags.

One potential explanatory hypothesis for the effects of N deposition on EMF dynamics involves a three-phase system wherein, with increasing N deposition, forests shift from N limitation (and high belowground carbon allocation by plants to an N acquisition specialized EMF community), to relatively high N and P availability (and low belowground carbon allocation and a dominance of EMF that are aggressive colonizers with relatively poor nutrient provision to their hosts), and then to P or cation limitation as the systems become more N saturated (and high belowground carbon allocation to an EMF community adapted to the acquisition of P and other nutrients other than N; Lilleskov, 2005). Our findings of N deposition induced reductions in relative belowground carbon allocation, associated reductions in EMF root colonization and soil mycelial biomass, as well as shifts in EMF community composition do potentially support this hypothesized chain of responses. The very long term (decades) of high N deposition (over  $20 \text{ kg N ha}^{-1} \text{ a}^{-1}$ ) in conjunction with the complete decoupling of foliar N from N deposition or soil N (while foliar N:P is strongly correlated with soil and foliar P) do

seem to indicate that these forests should have transitioned into the P limited state, however, higher N deposition continues to decrease EMF colonization and soil mycelial biomass. Evidence for substantial increase in EMF biomass under P limitation has been found earlier in greenhouse studies on seedlings (Wallander and Nylund, 1992), and very high root to shoot ratios and ectomycorrhizal abundance has been observed in P limited forests (Rosenstock et al., 2016). However, to our knowledge no forest fertilization study has yet observed an increase in proportional belowground carbon allocation or an increase in EMF abundance as a result of N additions pushing a forest into P limitation. Taken together, this would seem to indicate that N remains the dominant control point over belowground carbon allocation in N limited forests well into the transition to P limitation that high amounts of N additions may induce. Alternatively, if Mg and K limitation are concomitantly induced as high N availability induces P limitations, then their reducing effect on belowground carbon allocation found in earlier studies (Ericsson, 1995; Rosenstock et al., 2016) may mask the effects of P demand. Our data do not indicate that N deposition was associated with reductions in foliar Mg, but foliar K was strongly negatively affected by N deposition amounts, and a number of sites (4 sites with N deposition above  $25 \text{ kg ha}^{-1} \text{ a}^{-1}$ ) had foliar K levels defined as deficient (Table S2; Göttelein, 2015).

On the other hand, the observed shifts in EMF community composition may indicate that a dynamic equilibrium exists between the nutrient demands of trees and the nutrient acquisition niches of individual EMF taxa. While our ability to attribute nutrient acquisition niches to particular taxonomic or morphologic groups of EMF remains poor, an increased capacity to release mineral P from soils under laboratory conditions (e.g. by oxalate production) was reported for some medium and long distance exploration type EMF, including *Piloderma* sp., *Suillus* sp., *Cortinarius* sp. and *Paxillus involutus* (Plassard and Dell, 2010). In our study, *Boletus pulverulentus*, *Paxillus involutus*, *Tylopilus felleus* and two *Xerocomus* sp. were identified as indicator species for greater N deposition while two *Boletus* sp., two *Cortinarius* sp. and *Xenasmatella* sp2 were identified as indicator species for low foliar P concentration. Thus, the increased abundance of these species belonging to the long-distance exploration type in high N sites may indicate that adaptation of EMF communities to the P limitation is occurring.

#### 4.3. Other environmental factors

Besides base saturation and N deposition, EMF community composition in both root tips and mesh bags was strongly associated with growing season temperature and evapotranspiration ratio, in mesh bags also with precipitation. Studies that have utilized open top chambers to assess the effects of growing season temperature on EMF communities have in some cases found significant effects of warming on EMF community composition (Geml et al., 2015), while not in other cases (Peltoniemi et al., 2015). In these studies, an interactive effect of warming with soil moisture is commonly reported. In a global meta-analysis, Soudzilovskaia et al. (2015) found that growing season temperature was the most important determinant of arbuscular mycorrhizal abundance, but it was not an important determinant of EMF colonization, for which soil pH and C:N were the major determinants.

Evapotranspiration ratio, site water balance and precipitation were strongly correlated and among these hydrological variables, evapotranspiration ratio best explained the effect on community composition on root tips and mesh bags according to NMDS, while precipitation best explained the significant hydrologic effect on community composition in mesh bags according to PERMANOVA. It was expected that evapotranspiration ratio, a good predictor for drought, and precipitation were significantly associated with EMF community composition because EMF species differ in the way they affect plant water relations based on the extent of their extramatrical mycelium (Lehto and Zwiazek, 2011). Earlier studies have also shown such a relationship (Bakker et al., 2006; Suz et al., 2014). On the other hand, precipitation

was strongly correlated with N deposition in our study plots. When replacing precipitation with N deposition in the multi-factor PERMANOVA model for mesh bags, despite N deposition being non-significant as single factor, the model was also significant. This can be explained by the fact that a large part of atmospheric N is deposited by rain.

In Swiss beech forest plots, a negative relation has been found between ozone flux and foliar P (Braun et al., submitted). Accordingly, enhanced ozone flux was found to be associated with EMF community composition on root tips and with a significant decrease of species with rhizomorphs. But these responses were not strong enough to support the assumption that ozone, like increased N deposition, reduces carbon allocation towards roots and symbionts (Coleman et al., 1995; Lux et al., 1997; Samuelson et al., 1996).

#### 4.4. Tree nutrient status

Foliar N was associated with EMF communities on root tips and one of the most consistent factors correlating with exploration types, increased foliar N being associated with significantly less medium-smooth types on root tips and less long distance types in mesh bags. Yet no relationship between foliar N and N deposition can be seen on the selected forest plots used in this study or across the whole set of beech observation plots (Braun et al., submitted), likely because they have all sustained high levels of N deposition and are no longer N limited. Yet, we still see a response of EMF communities to N deposition. Foliar Ca, K, N:P and P were significantly associated with EMF community composition on root tips and/or mesh bags. Foliar Ca amounts and its association with the EMF community were correlated with variations in soil base saturation, while foliar N:P, P and K, and their associations with EMF communities were strongly correlated with the effect of N deposition. EMF species diversity, which decreased strongly with N deposition, was positively correlated with foliar K and P. And the abundance of several EMF species, including *C. geophilum*, was significantly decreased by increasing N deposition and associated with significantly elevated foliar P. In earlier studies, a hypothesis has been proposed whereby pollutant inputs diminish P uptake by trees by reducing the mycorrhizal colonization of tree roots and the growth of mycelial networks (e.g. Baxter et al., 2002; Jansa et al., 2011; Lang et al., 2016), and our results offer some support for this hypothesis. Recent studies indicate that declining nutrient availability and allocation to mycorrhizal fungi may have contributed to impaired forest nutrient uptake (Rosenstock et al., 2016). The impact of N deposition on nutrient uptake and, in particular, on P uptake is of concern as it can lead to decreased photosynthesis, reduced growth and vitality (Talkner et al., 2015; Yang et al., 2016).

#### 4.5. Root tips vs. mesh bags

As expected, we found significant differences between the EMF diversity and community composition from the root tip and mesh bag samplings. It is well established that EMF mycelium and root tip communities are spatially and temporally stratified (Genney et al., 2006), moreover the substrate in the mesh bags is selective for EMF. Mesh bags are designed to exclude roots, it is thus likely that EMF with a limited extramatrical mycelium (i.e. contact and short distance exploration types) are underrepresented in mesh bags. This may well explain why *L. subdulcis* and *C. geophilum*, the most abundant species in the root tip communities, had comparatively low abundances in the mesh bags, and why *Xerocomus pruinatus* and *Laccaria amethystina*, with long distance and medium distance smooth exploration types, respectively, were the most abundant taxa in the mesh bags and relatively minor components of the root tips community. Nevertheless, the results of both the root tip and mesh bag approach show a similar response on the N deposition gradient and both approaches support our hypotheses.

## 5. Conclusions

Our results show that EMF communities in European beech forests are negatively affected by long-term increased N deposition. Soil pH and base saturation are very strongly associated with EMF community composition. However, our results indicate that N deposition and soil acid-base status act independently and directly on the EMF communities, and that the N effect is not an acidification effect and higher base saturation does not appear to buffer against the effects of N deposition on EMF. We found a substantial decrease in species diversity and fungal biomass with increasing N deposition, as well as a shift from carbon demanding species to species with lower biomass. These changes can be explained by the reduced carbon allocation to the roots and symbionts induced by increased N availability. However, above a N deposition of  $26 \text{ kg N ha}^{-1} \text{ a}^{-1}$  in Swiss beech forest, above-ground tree growth is also reduced indicating that these trees suffer from other nutrient limitations. The further reduction in diversity and abundance, and the shift in community composition detected in forest plots with a N deposition above this level strongly suggests altered nutrient uptake by trees. These results also indicate a significant relationship between foliar nutrient levels and EMF community composition and between foliar P and foliar K levels and EMF diversity. Consequently, our results support the hypothesis that the decrease in nutrient uptake in beech forests across Europe is related to changes in EMF communities. Such investigations linking large scale forest monitoring and environmental pollution modeling with soil microorganisms surveys are demanding and encouraged to address similar hypotheses e.g. in other forest types or in other regions with strong anthropogenic pollutant depositions. The continued decrease in EMF diversity and biomass occurring at high levels of N deposition demonstrates the need for both continued monitoring of the total forest ecosystem, including important soil organisms, and for stronger reductions of anthropogenic N emissions.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scitotenv.2017.06.142>.

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