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Arbuscular mycorrhizal fungi in European grasslands under nutrient pollution

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Title: Arbuscular mycorrhizal fungi in European grasslands under nutrient pollution

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Keywords: arbuscular mycorrhizal fungi, grassland, nutrient pollution, nitrogen, phosphorus, atmospheric deposition, biodiversity

Abstract:

Aim Our aim was to quantify to what extent nutrient pollution explains arbuscular mycorrhizal fungal community richness and composition.

Location Europe

Time period 2014-2016

Major taxa studied Arbuscular mycorrhizal fungi

Methods We sampled soils of calcareous and acidic grasslands and roots of 34 host plant species across a large geographical gradient of atmospheric nitrogen deposition and soil phosphorus availability. Furthermore, we performed an independent pairwise comparison between fertilized and unfertilized grasslands in Belgium and Iceland to compare results.

Results We found that nitrogen deposition was significantly negatively related to arbuscular mycorrhizal fungal richness, with a negative community threshold of 7.7 kg N ha^{-1} $year^{-1}$ corresponding to the highest level of decrease in operational taxonomic units. Additionally, we found that soil phosphorus was strongly negatively related to mycorrhizal fungal richness.

Main conclusions Our results highlight the necessity to revisit the critical loads of atmospheric nitrogen deposition used in European environmental policy currently set at 10-15 kg N ha^{-1} $year^{-1}$. Importantly, our observed threshold of 7.7 kg N ha^{-1} $year^{-1}$ does not correspond to a critical load

below which there is no environmental harm, as the least negative changes in arbuscular mycorrhizal fungal communities were observed below 5 kg N ha^{-1} $year^{-1}$. Therefore, to avoid compromising the policy tenet of no environmental harm with respect to grassland mycorrhizal fungi, areas of zero tolerance to nitrogen pollution should be delimited. Our results also indicate that environmental policy biased towards reducing nitrogen pollution alone will fail to preserve mycorrhizal biodiversity in European grasslands. We advocate increased policy attention to avoid phosphorus enrichment, particularly through agricultural fertilization. Here too, areas of zero phosphorus input, ideally set in the currently unpolluted -or least polluted- areas, seem key for effective environmental policy as elevated levels of soil phosphorus following phosphorus fertilization are known to be extremely persistent.

Main text

Introduction

Mycorrhizal fungi are widely recognized as key below-ground symbionts that sustain primary production, biogeochemical cycling and biodiversity (Wagg et al., 2011; Bardgett & van der Putten, 2014; van der Heijden et al., 2015; Bender et al., 2016; Brundrett & Tedersoo, 2018). Consequently, much ecological research has focused on understanding patterns of mycorrhizal fungal diversity, indicating that mycorrhizal fungal communities show considerable variation that is related to environmental conditions (Leff et al., 2015; Jacquemyn et al., 2016; Partel et al., 2017; Van Geel et al., 2017a; van der Linde et al., 2018). A matter of concern is the numerous observations that anthropogenic disturbances seem to exert a negative impact on mycorrhizal fungal communities, possibly undermining their role in maintaining pivotal ecosystem services (Helgason et al., 1998; Valyi et al., 2015; Partel et al., 2017; Cotton, 2018; Verbruggen et al., 2018). A primary component of environmental change, with potentially far reaching consequences for these symbionts, is nutrient pollution. Primarily driven by anthropogenic combustion processes and intensive agricultural fertilization schemes, nutrient emissions to ecosystems worldwide have increased four- to tenfold compared to pre-industrial levels (Galloway et al., 2004; Peñuelas et al., 2013; Wang et al., 2015). Whereas this excess nutrient input has been consistently linked to above-ground biodiversity loss (Stevens et al., 2004; Clark & Tilman, 2008; Bobbink et al., 2010; Ceulemans et al., 2013; Payne et al., 2017), there is mounting evidence that below-ground biodiversity is also subject to deleterious effects of nutrient enrichment (Ramirez et al., 2012; Leff et al., 2015; Zhang et al., 2018). For instance, diversity of ectomycorrhizal fungi from forest ecosystems has been shown to be adversely affected by nitrogen pollution (Cox et al., 2010; van der Linde et al., 2018; Lilleskov et al., 2019). Similarly, diversity and abundance of arbuscular

mycorrhizal fungi from grassland and particularly agricultural ecosystems have been shown to be negatively affected by nutrient enrichment (De Beenhouwer et al., 2015; Leff et al., 2015; Van Geel et al., 2016; Jiang et al., 2018). Changes in arbuscular mycorrhizal fungal communities can be expected to alter host plant fitness and ecosystem functioning, shifting mycorrhizal symbioses phenotypes from mainly mutualistic forms in nutrient-poor ecosystems, to commensalism and parasitism in increasingly nutrient-rich ecosystems (Wei et al., 2013; Johnson et al., 2015). As a result, the loss of suitable symbiotic partners can alter plant mineral nutrition or plant drought and pathogen resistance, thereby inducing knock-on effects on higher trophic levels in ecosystems and the long-term stability of primary production (van der Heijden et al., 1998; Rillig, 2004; Cahill et al., 2008; van der Heijden et al., 2008; Wagg et al., 2011; Jonard et al., 2015). This also implies that in agricultural practices, tradeoffs may exist between the positive benefits with respect to nutrient uptake and primary production of mutualistic mycorrhizal fungi on the one hand, and fertilization followed by a shift to less mutualistic symbionts on the other (Bender et al., 2016; Van Geel et al., 2017b).

Although our knowledge regarding the deleterious environmental effects of increased nutrient input on mycorrhizal fungal communities is growing, comprehensive empirical support across large geographical scales remains scarce (Rillig, 2004; Leff et al., 2015; van der Linde et al., 2018). Yet, such data are critical to inform environmental policy, since it relies on identifying proper critical loads of nutrient pollution below which there should be no harmful environmental effects. In this context, evidence has been emerging that critical loads are set too high to avoid loss of plant species from grasslands (Payne et al., 2013; Payne et al., 2017) and of ectomycorrhizal fungi from forests (Suz et al., 2014; van der Linde et al., 2018; Lilleskov et al., 2019). Such critical loads, however, are lacking so far for characteristic grassland mycorrhizal fungi.

Here, we investigated arbuscular mycorrhizal fungi in two wide-spread semi-natural grassland types across nine European countries (Fig. 1). We sampled 120 grassland plots located in 21 calcareous and 19 acidic grassland sites along a gradient of atmospheric nitrogen deposition (from 4.11 kg N ha^{-1} $year^{-1}$ to 31.04 kg N ha^{-1} $year^{-1}$) and soil phosphorus (from < 0.01 mg P kg^{-1} to 64.36 mg P kg^{-1}). In a second part of this study, we sampled an additional 24 acidic grasslands under various regimes of agricultural fertilization, together with their adjacent unfertilized counterparts, to assess pairwise changes in community composition and richness of arbuscular mycorrhizal fungi. Our goals were to i) quantify the relationship between richness and community composition of arbuscular mycorrhizal fungi on the one hand and nutrient pollution on the other, ii) disentangle the variability in richness explained by nitrogen and phosphorus and iii) infer community thresholds of mycorrhizal fungi to assess the efficacy of critical loads currently used in European environmental policy.

Materials and methods

Survey along an European gradient of nutrient pollution and sampling protocols

In the first part of this study, we established 120 sampling plots in a total of 40 grassland sites between May and June 2014. Sampling plots were selected across eight European countries (Estonia, Sweden, United Kingdom, The Netherlands, Belgium, Germany, France and Switzerland; Fig. 1) and consisted of 19 grassland sites on acidic parent material and 21 grassland sites on calcareous parent material. The sites were located in areas that had not undergone direct agricultural fertilization or other agricultural practices such as soil tillage or use of biocides for at least 20 years prior to sampling. At each site, we randomly positioned three sampling plots of 1 m × 1 m using a site grid and a random number table, ensuring a homogenous soil surface and vegetation composition by avoiding ditches, soil disturbances or clumped dominance of a single

plant species. Site size varied considerably as we sampled in both more fragmented and degraded landscapes and in larger nature areas (minimum 0.25 ha to maximum 25 ha; mean = 3.1 ha and median = 1.2 ha). However, we sampled our three replicate plots always within a maximum area of 100m x 100m, or across the entire area when the site was smaller. Importantly, site size was unrelated to OTU number (P = 0.347). Per plot, we recorded the presence and abundance of all vascular plant species by visually estimating the relative surface area.

Then, we collected three pooled soil samples (0-10 cm depth, i.e. the topsoil with most plant roots) per plot with an auger of 2 cm diameter, yielding a total of 360 soil samples (120 sampling plots × 3 replicates). Finally, we sampled the roots from 30 plant species with arbuscular mycorrhizae. The plant species identity and number of replicates are indicated in Table S1. The sampled species represented characteristic plant species that are commonly found in both grassland types. Root samples were collected by digging up three separate individuals per species per plot with a small clump of soil and tracing the roots down starting from the above-ground parts of the plant to ensure correct species sampling. Part of the root tips were then clipped, and the clump was then replanted to avoid destructive sampling. Root samples were pooled across three separate individuals per plant species per plot. Both soil samples and root samples were stored in water tight zip lock bags at 4 °C for maximum 4 weeks prior to laboratory analyses.

We obtained data on total atmospheric nitrogen deposition, including wet and dry deposition of oxidized and reduced nitrogen, from the EMEP-based IDEM models of 2016 which is based on 50 km x 50 km grid maps across Europe (Pieterse et al., 2007; downloaded at 25 June 2016 from http://www.emep.int/). To calculate a robust measure of nitrogen deposition over a longer period thereby accounting for possible cumulative effects (Payne et al., 2017), we used the average of the deposition estimates of the years 1990 and every year between 2000 and 2013. To avoid

unrepresentative model estimates, no grassland sites were selected near point sources of nitrogen pollution. For some countries national models estimating nitrogen deposition with greater resolution were available (UK, the Netherlands, Belgium), but the relationships identified in this study did not change when using this data. We then chose to use the EMEP data for all grassland sites, as it is calculated consistently with the same model parameters across all countries in our dataset.

Study site selection and sampling protocols for pairwise comparison of fertilized and unfertilized grasslands

In the second part of this study, we selected an additional 24 acidic grassland sites, of which 20 sites in Belgium under high atmospheric nitrogen deposition (> 20 kg N ha^{-1} $year^{-1}$) and 4 sites in Iceland under low deposition levels (< 3 kg N ha^{-1} $year^{-1}$). Grassland sites in Belgium were sampled between May and June 2015 and grassland sites in Iceland in June 2016. The grassland sites consisted of areas that had undergone various regimes of agricultural fertilization, but no other agricultural practices such as soil tillage or use of biocides that may influence mycorrhizal communities. Each grassland site also contained a part that never underwent (direct) agricultural fertilization. For inclusion in this study, the grassland sites had to adhere to several selection criteria to ensure that the observed differences in mycorrhizal fungal communities could not be attributed to possible factors other than fertilization. Firstly, only sites were selected for which we had access to reliable management records dating back at least 20 years prior to sampling to ensure consistent management history. Next, the sites had to show the same soil characteristics and the same management practices across both the fertilized and unfertilized area. Finally, to avoid possible latency in the effects of fertilization on above- and below-ground communities, we only used sites where fertilization had ceased at least five years prior to sampling. We did not have

access to reliable detailed records on fertilization form, amount and frequency per site, as owners frequently only kept summarily records. To account for these possible differences between sites, we performed pairwise statistical analyses removing variation attributed to site specific characteristics.

For soil and root sampling, we followed the same protocols as in the survey along the gradient of nutrient pollution described above. However, for collection of root samples, the host plant species had to be present in both the fertilized and unfertilized sampling plots. Ultimately, roots of 10 plant species host of arbuscular mycorrhizae were collected, of which 6 plant species in common with the European survey and 4 species only represented in this part of the study (Table S1).

Soil chemical analyses

For each bulked soil sample per plot, soil pH was quantified using a glass electrode in a 1:10 soil/water mixture. As a measure of plant-available soil inorganic nitrogen, ammonium and nitrate were quantified by shaking 10 g of soil in 200 mL of 1 M potassium chloride solution for one hour. Total soil inorganic nitrogen was calculated as the sum of soil ammonium and soil nitrate (called 'soil nitrogen' in the manuscript). As a measure of plant-available soil phosphorus, Olsen P values were quantified by shaking 2 g dry soil for 30 minutes with 0.5 M sodium bicarbonate at pH 8.5 and colorimetric analysis of the extracts using the molybdenum blue method (called 'soil phosphorus' in the manuscript; Robertson et al., 1999). Extracts were analyzed colorimetrically using the Evolution 201 UV-visible Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Community characterization of arbuscular mycorrhizal fungi by high-throughput sequencing

Per root sample, we first mixed the fresh roots with a diameter of 3 mm or less and extracted DNA from 100 mg of this root material using the UltraClean Plant DNA Isolation Kit (MoBio

Laboratories, Solana Beach, CA, USA). Next, we homogenized the soil samples and extracted DNA from 250 mg soil using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA, USA). We performed PCR amplification of all root and soil DNA extracts using the sample-specific barcode-labelled versions of the primers AMV4.5NF and AMDGR (300 bp) (Sato et al., 2005). This primer pair is AMF specific and consistently characterizes AMF communities using 454 pyrosequencing based on the most variable part of the small subunit (SSU) rRNA gene region (Van Geel et al., 2014). We performed PCR on a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, CA, USA) in a reaction volume of 20 µl, containing 0.15 mM of each dNTP, 0.5 µM of each primer, 1x Titanium Taq PCR buffer, 1U Titanium Taq DNA polymerase (Clontech Laboratories, Palo Alto, CA, USA), and 1 µl genomic DNA. To test for contamination when performing PCR amplification, three negative controls were used. We denatured DNA samples at 94°C for 2 min. Next, we ran 35 cycles, consisting of 45 s at 94°C, 45 s at 65°C and 45 s at 72°C, followed by a final elongation of 10 min at 72°C. We separated amplicons by agarose gel electrophoresis, cut out amplicons with the correct length, and purified them using the Qiaquick gel extraction kit (Qiagen, Hamburg, Germany). We quantified purified dsDNA amplicons using the Quant-iT PicoGreen dsDNA Assay Kit and Qubit fluorometer (Invitrogen, Ghent, Belgium), and pooled them in equimolar quantities over ten amplicon libraries. Pyrosequencing of the amplicon libraries was performed by Macrogen (Korea) using the Roche GS-FLX instrument and Titanium chemistry (Roche Applied Science, Mannheim, Germany).

We clustered sequences into operational taxonomic units (OTUs) using the UPARSE algorithm, following the recommended pipeline (Edgar, 2013; USEARCH v7). First, we performed quality filtering of the reads with the fastq_filter command, allowing a maximum expected error of 0.5. To optimize the number and length of retained sequences, we truncated the reads at 222 bp. Next,

we dereplicated the sequences and sorted them by abundance. To improve the accuracy of diversity estimates, we omitted the sequences occurring only once prior to clustering (Brown et al., 2015). Then, we clustered sequences into OTUs defined at 97% sequence similarity, which is commonly used to define SSU-based OTUs in AMF (Öpik et al., 2010; Davison et al., 2015), with the cluster_otus command. In this step, chimeric OTUs predicted by the de novo method built from more abundant reads were discarded as well. We taxonomically identified OTUs by querying the representative sequences (the most abundant sequence in a cluster, as determined by the cluster_otus command) against GenBank using the BLAST algorithm. We discarded non-arbuscular mycorrhizal fungal OTUs or OTUs having a BLAST score lower than 200. To accurately identify the obtained arbuscular mycorrhizal fungi OTUs, we queried representative sequences for each OTU against the MaarjAM database (Öpik et al., 2010); accessed November 13, 2016), a quality-controlled repository for published arbuscular mycorrhizal fungi sequence data.

Bioinformatics and data analyses

To prevent bias owing to a different sequencing depth, rarefaction techniques are frequently used to interpolate to a smaller number of sequences. However, the main disadvantage of rarefaction is that point comparisons force the rarefaction of all samples down to the smallest sample size in the data set (Honnay et al., 2017). For our dataset this would imply a reduction of over 40 % of data points. Instead, we chose to extrapolate (for samples with over 20 sequence reads) or interpolated (for samples with over 200 sequence reads) the OTU richness to 200 sequence reads per DNA sample, to retrieve reliable and comparable measures of arbuscular mycorrhizal fungi OTU richness and OTU diversity. We used the iNEXT command in the iNEXT R package (Hsieh et al., 2016). However, to check whether our results are not an artefact of bioinformatical processing, we

also build a dataset in which we rarefied all samples to 200 arbuscular mycorrhizal fungal sequences per sample and in which all samples with less than 200 arbuscular mycorrhizal fungal sequences were omitted. After reanalyzing using this reduced dataset, we obtained highly similar results (Table S2). Therefore, we are confident that our results are consistent and robust, regardless of using mycorrhizal fungal richness estimates or a reduced rarified dataset.

Next, we built generalized mixed models including grassland site as random factor, to account for the non-independence of plots located in the same grassland site. Then, the identity of the sample (soil or root sample) and the host plant were added as an additional random factor to account for the possible non-independence of mycorrhizal fungal communities across the same host plant species. We did not aim to identify host plant species specific responses as some plant species had too little replicates. However, the mixed modelling approach accounts for possible effects of plant species identity and allows to identify general patterns. Furthermore, we intentionally included extractions directly from soil samples, to corroborate the results and avoid plant species specific bias.

Next, to disentangle variability explained by soil nitrogen and soil phosphorus, we constructed generalized mixed models simultaneously including explanatory variables associated with nitrogen enrichment (soil pH, total inorganic nitrogen, atmospheric nitrogen deposition) and phosphorus enrichment (Olsen phosphorus). Grassland type was used as a categorical variable to account for possible differences in species pools and species composition between the grassland types. We included all first order interactions between the explanatory variables in a first full factorial model. Next, the interaction factors were one by one removed to build a suite of reduced models. Out of the models with all possible combinations of interaction factors, we selected the most parsimonious model using the Akaike Information Criterion (AIC), which were invariably

the fully reduced models containing the five main variables mentioned above (grassland type, soil pH, soil phosphorus, soil nitrogen and atmospheric nitrogen deposition). These statistical analyses were performed by JMP pro 13.0 software (SAS Institute Inc. Cary, NC, USA).

We applied Threshold Indicator Taxon Analysis (TITAN) to identify the occurrence, magnitude and direction of individual arbuscular mycorrhizal OTU responses across the gradient in soil phosphorus and atmospheric nitrogen deposition (Baker & King, 2010). TITAN uses indicator value (IndVal) scores from indicator species analysis to identify change points, i.e. points of rapid change, of individual arbuscular mycorrhizal OTUs across the environmental gradient. Indicator value scores are standardized (z scores) to describe the magnitude of the response relative to each taxon's abundance distribution. TITAN distinguishes between taxa that decrease (z-) and increase (z+) across the environmental gradient. To estimate uncertainty of the change point, TITAN uses bootstrapping (n = 500). For each of the taxa, TITAN also estimates the purity of the change point (proportion of the bootstrap replicates with the same response direction) and the reliability of the change point (proportion of replicates with maximum IndVal reaching a P value of 0.05). Finally, cumulative responses of decreasing [sum(z-)] and increasing [sum(z+)] OTUs are tracked. A sum(z) peak indicates a community-level change point, i.e. a value of the environmental gradient where many taxa exhibit strong directional change. We used the TITAN2 package in R.3.4.1. TITAN analysis was performed on the abundance*OTU dataset.

To explicitly test whether mycorrhizal fungal communities differed between the paired fertilized and unfertilized conditions, we performed canonical redundancy analysis (RDA) using the R-package vegan (Oksanen et al., 2019). Firstly, we removed the variation attributed to the specific grassland site using the Condition statement in the RDA model, and then we explicitly tested for the variable condition (categorical variable with 2 levels: fertilized and unfertilized). We tested for

significance based on a permutation test with 1000 iterations using the anova.cca function (vegan package). As fertilized and unfertilized conditions were paired within grassland site, we restricted permutations of samples only within grassland site (blocks = grassland site) using the how function. Next, to explicitly attribute the differences in mycorrhizal fungal community composition between fertilized and unfertilized grasslands to specific soil variables, we ran a second RDA model, in which the variation owing to grassland site was not removed, but in which we included soil phosphorus, soil nitrogen and soil pH as explanatory variables.

Results

Using 454-pyrosequencing we identified 376 operational taxonomic units (OTUs) of arbuscular mycorrhizal fungi in 670 DNA samples taken both from the roots of 30 different host plant species and directly from grassland soils to avoid possible host plant bias

General mixed modeling revealed no significant differences in richness of arbuscular mycorrhizal fungal OTUs between calcareous grasslands and acidic grasslands ($F_{52.8}$ = 0.35, P = 0.556). Richness in arbuscular mycorrhizal OTUs was negatively related to both atmospheric nitrogen deposition ($F_{34.5}$ = 5.93, P = 0.020) and soil phosphorus ($F_{142.7}$ = 22.24, P < 0.0001; Fig. 2), and positively related to soil acidity ($F_{76.3}$ = 2.22, P = 0.029; Fig. S1). We found no significant relationship with soil nitrogen ($F_{114.1}$ = 3.40, P = 0.068; Fig. S2). Both acidic and calcareous grasslands under higher levels of atmospheric nitrogen deposition showed significantly lower soil pH ($F_{35.8}$ = 4.48, P = 0.0413; Fig. S3).

Redundancy analysis revealed that the community composition of arbuscular mycorrhizal fungi along the gradient of nutrient pollution in acidic grasslands and calcareous grasslands respectively was not only significantly structured by atmospheric nitrogen deposition (F_{316} = 9.70, P < 0.0001; F_{344} = 12.97, P < 0.0001), soil acidity (F_{316} = 8.89, P < 0.0001; F_{344} = 12.50, P < 0.0001) and soil

phosphorus (F_{316} = 8.42, P < 0.0001; F_{344} = 4.62, P < 0.001), but also by soil nitrogen (F_{316} = 2.74, P < 0.001; F_{344} = 5.56, P < 0.001; Fig. S4). Subsequent threshold indicator species analysis along the gradient in atmospheric nitrogen deposition revealed the strongest change in arbuscular mycorrhizal fungal communities at a level of 7.67 kg N ha^{-1} $year^{-1}$. This threshold corresponds with the highest number of significantly decreasing arbuscular mycorrhizal fungal OTUs (Fig. 3). We identified 66 significantly decreasing arbuscular mycorrhizal fungal OTUs (z-) as opposed to 28 increasing OTUs (z+) along the gradient in atmospheric nitrogen deposition. Similarly, more arbuscular mycorrhizal fungal OTUs were lost (82 OTUs) following higher levels of soil phosphorus than gained (36 OTUs). Most decreasing OTUs were found in the range of soil phosphorus below 20 mg P kg^{-1}, revealing an ultimate peak at 7.23 mg P kg^{-1} (Fig. 3).

In the separate pairwise comparison of arbuscular mycorrhizal fungal communities between fertilized and unfertilized grasslands, we identified 286 OTUs of arbuscular mycorrhizal fungi in 154 DNA samples from grassland soils and from the roots of 11 host plant species across 24 grassland sites. When relating pairwise differences in soil nutrient content to pairwise differences in the number of arbuscular mycorrhizal fungal OTUs, lower numbers of arbuscular mycorrhizal fungal OTUs were linked to higher soil phosphorus ($F_{25.2}$ = 9.02, P = 0.006; Fig. 4), but not to higher soil nitrogen ($F_{26.8}$ = 1.89, P = 0.180) or soil acidity ($F_{29.8}$ = 0.07, P = 0.793; Fig. S5). Furthermore, redundancy analysis revealed a clear differentiation in community composition of arbuscular mycorrhizal fungi between fertilized and unfertilized grasslands (F_{129} = 1.57, P = 0.016; Fig 4). These community differences were attributed to differences in soil phosphorus (F_{127} = 2.38, P < 0.001), but not to soil acidity (F_{127} = 0.92, P = 0.597) or soil nitrogen (F_{127} = 0.58, P = 0.998; Fig S5).

Discussion

Our results indicate that atmospheric nitrogen deposition is detrimental for arbuscular mycorrhizal fungi in grasslands. The negative relationship between arbuscular mycorrhizal fungal richness and nitrogen deposition may be explained by indirect deleterious effects of soil acidification. We found that both acidic and calcareous grasslands under higher levels of atmospheric nitrogen deposition showed significantly lower soil pH, corroborating the acidifying effect of nitrogen deposition found in other studies (Stevens et al., 2004; Horswill et al., 2008; Ceulemans et al., 2013). A threshold of nitrogen deposition leading to severe shifts in arbuscular mycorrhizal fungal communities was found at approximately 7.7 kg N ha^{-1} $year^{-1}$. However, current European critical loads for atmospheric nitrogen deposition in grasslands are set at 10 to 15 kg N ha^{-1} $year^{-1}$, nearly twice as high (Payne et al., 2013; Payne et al., 2017). The current critical loads are mainly based on expert estimations and data regarding above-ground plant diversity, and are subject to growing scientific criticism (Payne et al., 2013; Payne et al., 2017; van der Linde et al., 2018). For instance, approximately 60% of negative change points of grassland plant species already occur below or at the established critical loads of nitrogen deposition, seriously questioning their efficacy for biodiversity conservation (Payne et al., 2013; Payne et al., 2017). More recently, a large-scale study of ectomycorrhizal fungal diversity across several European forest types showed a substantial negative shift in ectomycorrhizal fungal communities at 5-6 kg N ha^{-1} $year^{-1}$ (van der Linde et al., 2018). These levels are, same as our results, also at least two times lower than the current critical load for forests set at 10-15 kg N ha^{-1} $year^{-1}$. Furthermore, as pristine areas with the lowest levels of nitrogen deposition are very scarce in Europe (<5 kg N ha^{-1} $year^{-1}$; Pieterse et al., 2007), they may be underrepresented in observational datasets including our own, possibly skewing empirical threshold upwards. Nevertheless, these newly emerging empirical thresholds are highly similar for both forests and grasslands and question the current critical loads of

atmospheric nitrogen deposition for European ecosystems. Following unchanged environmental policy allowing a critical load of 15 kg N ha^{-1} $year^{-1}$, we can estimate based on our linear mixed models results that up to 40 % of richness in arbuscular mycorrhizal fungi in European grasslands may be lost.

Next to relationships with nitrogen pollution indicators, we also identified soil phosphorus as a crucial determinant of arbuscular mycorrhizal fungal richness. We found lower numbers of mycorrhizal fungi in grasslands with high levels of soil phosphorus suggesting a negative effect of soil phosphorus enrichment. This result was corroborated by the pairwise comparison of arbuscular mycorrhizal fungal richness among fertilized and unfertilized grasslands. Our results thus indicate that environmental policy biased towards reducing nitrogen pollution alone will fail to preserve mycorrhizal biodiversity in European grasslands. In this respect, our results are in accordance with several local-scale studies indicating lower numbers of arbuscular mycorrhizal fungi in agricultural ecosystems or phosphorus enriched grasslands and forests (Egerton-Warburton et al., 2007; Camenzind et al., 2014; De Beenhouwer et al., 2015; Van Geel et al., 2016; Jiang et al., 2018). Interestingly, the observed lower richness of mycorrhizal fungi following higher levels of soil phosphorus mirrors previously identified negative relationships between soil phosphorus and grassland plant diversity (Ceulemans et al., 2013; Ceulemans et al., 2014). Furthermore, the peak value of phosphorus leading to negative community changes in arbuscular mycorrhizal fungi was found at 7.23 mg P kg^{-1} and the largest arbuscular mycorrhizal fungal losses in the soil phosphorus range below 20 mg P kg^{-1}. These values correspond remarkably well with soil phosphorus levels that allow for the highest levels of grassland plant diversity (also < 20 mg P kg^{-1}, Ceulemans et al., 2014). This negative relationship of mycorrhizal fungal richness with soil phosphorus cannot be explained by a confounding relationship through higher plant diversity in

this study, as we found no relationship between plant species richness and arbuscular mycorrhizal fungal richness ($F_{130.6}$ = 0.95, P = 0.333; Fig. S6). Nevertheless, experiments have shown that plant species coexistence and subsequent plant diversity is mediated by the diversity of their below-ground mycorrhizal partners (van der Heijden et al., 1998; van der Heijden et al., 2008). It is possible that the lack of relationship between the estimated mycorrhizal fungal richness here, and the observed plant species richness, reflects a time-lagged response of established plant individuals as it is mainly successful germination that is crucially dependent on their symbiotic partner (Jacquemyn et al., 2015). Therefore, it is reasonable to speculate that plant diversity may become jeopardized following the loss of suitable mycorrhizae in grasslands with excess levels of soil phosphorus.

In an era of ever-increasing nutrient pollution, the subsequent loss of mycorrhizal symbionts may constitute a myriad of possible effects with ramifications throughout the entire ecosystem. Our results highlight the necessity to -at least- revisit the current critical loads of atmospheric nitrogen deposition used in European environmental policy. The highest community level change of arbuscular mycorrhizal fungi already occurs at relatively low levels of atmospheric nitrogen deposition (7.7 kg N ha^{-1} $year^{-1}$). Importantly, this level does not correspond to a critical load below which there is no environmental harm, as the least negative changes in arbuscular mycorrhizal fungal communities were observed below 5 kg N ha^{-1} $year^{-1}$ (corresponding to levels of natural background deposition; Fig. 3). Therefore, to avoid compromising the policy tenet of no environmental harm with respect to grassland mycorrhizal fungi, areas of zero tolerance to nitrogen pollution need to be delimited. Furthermore, our results also warrant increased policy attention to avoid phosphorus enrichment, particularly through agricultural fertilization. Here too, areas of zero phosphorus input, ideally set in the currently unpolluted -or least polluted- areas,

seem key for effective environmental policy as elevated levels of soil phosphorus following phosphorus fertilization are known to be extremely persistent (Turner & Haygarth, 2001).

References

Baker, M.E. & King, R.S. (2010) A new method for detecting and interpreting biodiversity and ecological community thresholds. *Methods in Ecology and Evolution*, **1**, 25-37.

Bardgett, R.D. & van der Putten, W.H. (2014) Belowground biodiversity and ecosystem functioning. *Nature*, **515**, 505-511.

Bender, S.F., Wagg, C. & van der Heijden, M.G.A. (2016) An underground revolution: biodiversity and soil ecological engineering for agricultural sustainability. *Trends in Ecology & Evolution*, **31**, 440-452.

Bobbink, R., Hicks, K., Galloway, J., Spranger, T., Alkemade, R., Ashmore, M., . . . De Vries, W. (2010) Global assessment of nitrogen deposition effects on terrestrial plant diversity: a synthesis. *Ecological Applications*, **20**, 30-59.

Brown, S.P., Veach, A.M., Rigdon-Huss, A.R., Grond, K., Lickteig, S.K., Lothamer, K., . . . Jumpponen, A. (2015) Scraping the bottom of the barrel: are rare high throughput sequences artifacts? *Fungal Ecology*, **13**, 221-225.

Brundrett, M.C. & Tedersoo, L. (2018) Evolutionary history of mycorrhizal symbioses and global host plant diversity. *New Phytologist*, **220**, 1108-1115.

Cahill, J.F., Elle, E., Smith, G.R. & Shore, B.H. (2008) Disruption of a belowground mutualism alters interactions between plants and their floral visitors. *Ecology*, **89**, 1791-1801.

Camenzind, T., Hempel, S., Homeier, J., Horn, S., Velescu, A., Wilcke, W. & Rillig, M.C. (2014) Nitrogen and phosphorus additions impact arbuscular mycorrhizal abundance and molecular diversity in a tropical montane forest. *Global Change Biology*, **20**, 3646-3659.

Ceulemans, T., Merckx, R., Hens, M. & Honnay, O. (2013) Plant species loss from European semi-natural grasslands following nutrient enrichment - is it nitrogen or is it phosphorus? *Global Ecology and Biogeography*, **22**, 73-82.

Ceulemans, T., Stevens, C.J., Duchateau, L., Jacquemyn, H., Gowing, D.J.G., Merckx, R., . . . Honnay, O. (2014) Soil phosphorus constrains biodiversity across European grasslands. *Global Change Biology*, **20**, 3814-3822.

Clark, C.M. & Tilman, D. (2008) Loss of plant species after chronic low-level nitrogen deposition to prairie grasslands. *Nature*, **451**, 712-715.

Cotton, T.E.A. (2018) Arbuscular mycorrhizal fungal communities and global change: an uncertain future. *Fems Microbiology Ecology*, **94**

Cox, F., Barsoum, N., Lilleskov, E.A. & Bidartondo, M.I. (2010) Nitrogen availability is a primary determinant of conifer mycorrhizas across complex environmental gradients. *Ecology Letters*, **13**, 1103-1113.

Davison, J., Moora, M., Opik, M., Adholeya, A., Ainsaar, L., Ba, A., . . . Zobel, M. (2015) Global assessment of arbuscular mycorrhizal fungus diversity reveals very low endemism. *Science*, **349**, 970-973.

De Beenhouwer, M., Van Geel, M., Ceulemans, T., Muleta, D., Lievens, B. & Honnay, O. (2015) Changing soil characteristics alter the arbuscular mycorrhizal fungi communities of Arabica coffee (*Coffea arabica*) in Ethiopia across a management intensity gradient. *Soil Biology & Biochemistry*, **91**, 133-139.

Edgar, R. (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature methods*, **10**, 996-998.

Egerton-Warburton, L.M., Johnson, N.C. & Allen, E.B. (2007) Mycorrhizal community dynamics following nitrogen fertilization: a cross-site test in five grasslands. *Ecological Monographs*, **77**, 527-544.

Galloway, J.N., Dentener, F.J., Capone, D.G., Boyer, E.W., Howarth, R.W., Seitzinger, S.P., . . . Vorosmarty, C.J. (2004) Nitrogen cycles: past, present, and future. *Biogeochemistry*, **70**, 153-226.

Helgason, T., Daniell, T., Husband, R., Fitter, A. & Young, J. (1998) Ploughing up the wood-wide web? *Nature*, **394**, 431.

Honnay, O., Helsen, K. & Van Geel, M. (2017) Plant community reassembly on restored semi-natural grasslands lags behind the assembly of the arbuscular mycorrhizal fungal communities. *Biological Conservation*, **212**, 196-208.

Horswill, P., O'Sullivan, O., Phoenix, G.K., Lee, J.A. & Leake, J.R. (2008) Base cation depletion, eutrophication and acidification of species-rich grasslands in response to long-term simulated nitrogen deposition. *Environmental Pollution*, **155**, 336-349.

Hsieh, T.C., Ma, K.H. & Chao, A. (2016) iNEXT: an R package for rarefaction and extrapolation of species diversity (Hill numbers). *Methods in Ecology and Evolution*, **7**, 1451-1456.

Jacquemyn, H., Brys, R., Waud, M., Busschaert, P. & Lievens, B. (2015) Mycorrhizal networks and coexistence in species-rich orchid communities. *New Phytol*, **206**, 1127-34.

Jacquemyn, H., Waud, M., Merckx, V.S.F.T., Brys, R., Tyteca, D., Hedren, M. & Lievens, B. (2016) Habitat-driven variation in mycorrhizal communities in the terrestrial orchid genus Dactylorhiza. *Scientific Reports*, **6**

Jiang, S., Liu, Y., Luo, J., Qin, M., Johnson, N.C., Opik, M., . . . Feng, H. (2018) Dynamics of arbuscular mycorrhizal fungal community structure and functioning along a nitrogen enrichment gradient in an alpine meadow ecosystem. *New Phytologist*, **220**, 1222-1235.

Johnson, N.C., Wilson, G.W.T., Wilson, J.A., Miller, R.M. & Bowker, M.A. (2015) Mycorrhizal phenotypes and the law of the minimum. *New Phytologist*, **205**, 1473-1484.

Jonard, M., Furst, A., Verstraeten, A., Thimonier, A., Timmermann, V., Potocic, N., . . . Rautio, P. (2015) Tree mineral nutrition is deteriorating in Europe. *Global Change Biology*, **21**, 418-430.

Leff, J.W., Jones, S.E., Prober, S.M., Barberan, A., Borer, E.T., Firn, J.L., . . . Fierer, N. (2015) Consistent responses of soil microbial communities to elevated nutrient inputs in grasslands across the globe. *Proceedings of the National Academy of Sciences of the United States of America*, **112**, 10967-10972.

Lilleskov, E.A., Kuyper, T.W., Bidartondo, M.I. & Hobbie, E.A. (2019) Atmospheric nitrogen deposition impacts on the structure and function of forest mycorrhizal communities: A review. *Environmental Pollution*, **246**, 148-162.

Oksanen, J., Blanchet, G., Kindt, R., Legendre, P., Minchin, P., Simpson, G., . . . Wagner, H. (2019) Vegan: Community Ecology Package. R package version 2.5.4: https://CRAN.R-project.org/package=vegan.

Öpik, M., Vanatoa, A., Vanatoa, E., Moora, M., Davison, J., Kalwij, J., . . . Zobel, M. (2010) The online database MaarjAM reveals global and ecosystemic distribution patterns in arbuscular mycorrhizal fungi (Glomeromycota). *New phytologist*, **188**, 223-241.

Partel, M., Opik, M., Moora, M., Tedersoo, L., Szava-Kovats, R., Rosendahl, S., . . . Zobel, M. (2017) Historical biome distribution and recent human disturbance shape the diversity of arbuscular mycorrhizal fungi. *New Phytologist*, **216**, 227-238.

Payne, R.J., Dise, N.B., Stevens, C.J., Gowing, D.J. & Partners, B. (2013) Impact of nitrogen deposition at the species level. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 984-987.

Payne, R.J., Dise, N.B., Field, C.D., Dore, A.J., Caporn, S.J.M. & Stevens, C.J. (2017) Nitrogen deposition and plant biodiversity: past, present, and future. *Frontiers in Ecology and the Environment*, **15**, 431-436.

Peñuelas, J., Poulter, B., Sardans, J., Ciais, P., van der Velde, M., Bopp, L., . . . Janssens, I.A. (2013) Human-induced nitrogen-phosphorus imbalances alter natural and managed ecosystems across the globe. *Nature Communications*, **4**, 1-10.

Pieterse, G., Bleeker, A., Vermeulen, A.T., Wu, Y. & Erisman, J.W. (2007) High resolution modelling of atmosphere-canopy exchange of acidifying and eutrophying components and carbon dioxide for European forests. *Tellus Series B-Chemical and Physical Meteorology*, **59**, 412-424.

Ramirez, K.S., Craine, J.M. & Fierer, N. (2012) Consistent effects of nitrogen amendments on soil microbial communities and processes across biomes. *Global Change Biology*, **18**, 1918-1927.

Rillig, M.C. (2004) Arbuscular mycorrhizae and terrestrial ecosystem processes. *Ecology Letters*, **7**, 740-754.

Robertson, G., Coleman, D., Bledsoe, C. & Sollins, P. (1999) *Standard soil methods for long-term ecological research,*. Oxford University Press, New York, USA.

Sato, K., Suyama, Y., Saito, M. & Sugawara, K. (2005) A new primer for discrimination of arbuscular mycorrhizal fungi with polymerase chain reaction-denature gradient gel electrophoresis. *Grassland Science*, **51**, 179-181.

Stevens, C.J., Dise, N.B., Mountford, J.O. & Gowing, D.J. (2004) Impact of nitrogen deposition on the species richness of grasslands. *Science*, **303**, 1876-1879.

Suz, L.M., Barsoum, N., Benham, S., Dietrich, H.P., Fetzer, K.D., Fischer, R., . . . Bidartondo, M.I. (2014) Environmental drivers of ectomycorrhizal communities in Europe's temperate oak forests. *Molecular Ecology*, **23**, 5628-5644.

Turner, B.L. & Haygarth, P.M. (2001) Biogeochemistry - Phosphorus solubilization in rewetted soils. *Nature*, **411**, 258-258.

Valyi, K., Rillig, M.C. & Hempel, S. (2015) Land-use intensity and host plant identity interactively shape communities of arbuscular mycorrhizal fungi in roots of grassland plants. *New Phytologist*, **205**, 1577-1586.

van der Heijden, M.G., Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., . . . Sanders, I.R. (1998) Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature*, **396**, 69-72.

van der Heijden, M.G., Bardgett, R.D. & van Straalen, N.M. (2008) The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters*, **11**, 296-310.

van der Heijden, M.G., Martin, F.M., Selosse, M.A. & Sanders, I.R. (2015) Mycorrhizal ecology and evolution: the past, the present, and the future. *New Phytologist*, **205**, 1406-1423.

van der Linde, S., Suz, L.M., Orme, D.L., Cox, F., Andreae, H., Asi, E., . . . Bidartondo, M. (2018) Environment and host as large-scale controls of ectomycorrhizal fungi. *Nature*, doi.org/10.1038/s41586-018-0189-9.

Van Geel, M., Busschaert, P., Honnay, O. & Lievens, B. (2014) Evaluation of six primer pairs targeting the nuclear rRNA operon for characterization of arbuscular mycorrhizal fungal

(AMF) communities using 454 pyrosequencing. *Journal of Microbiological Methods*, **106**, 93-100.
Van Geel, M., De Beenhouwer, M., Ceulemans, T., Caes, K., Ceustermans, A., Bylemans, D., . . . Honnay, O. (2016) Application of slow-release phosphorus fertilizers increases arbuscular mycorrhizal fungal diversity in the roots of apple trees. *Plant and Soil*, **402**, 291-301.
Van Geel, M., Jacquemyn, H., Plue, J., Saar, L., Kasari, L., Peeters, G., . . . Ceulemans, T. (2017a) Abiotic rather than biotic filtering shapes the arbuscular mycorrhizal fungal communities of European semi-natural grasslands. *New Phytologist*, **220**, 1262-1272.
Van Geel, M., Verbruggen, E., De Beenhouwer, M., van Rennes, G., Lievens, B. & Honnay, O. (2017b) High soil phosphorus levels overrule the potential benefits of organic farming on arbuscular mycorrhizal diversity in northern vineyards. *Agriculture Ecosystems & Environment*, **248**, 144-152.
Verbruggen, E., Sheldrake, M., Bainard, L.D., Chen, B.D., Ceulemans, T., De Gruyter, J. & Van Geel, M. (2018) Mycorrhizal fungi show regular community compositions in natural ecosystems. *Isme Journal*, **12**, 380-385.
Wagg, C., Jansa, J., Schmid, B. & van der Heijden, M. (2011) Belowground biodiversity effects of plant symbionts support aboveground productivity. *Ecology letters*, **14**, 1001-1009.
Wang, R., Balkanski, Y., Boucher, O., Ciais, P., Penuelas, J. & Tao, S. (2015) Significant contribution of combustion-related emiscions to the atmospheric phosphorus budget. *Nature Geoscience*, **8**, 48-54.
Wei, C.Z., Yu, Q., Bai, E., Lu, X.T., Li, Q., Xia, J.Y., . . . Han, X.G. (2013) Nitrogen deposition weakens plant-microbe interactions in grassland ecosystems. *Global Change Biology*, **19**, 3688-3697.
Zhang, T.A., Chen, H.Y.H. & Ruan, H.H. (2018) Global negative effects of nitrogen deposition on soil microbes. *Isme Journal*, **12**, 1817-1825.

Figures

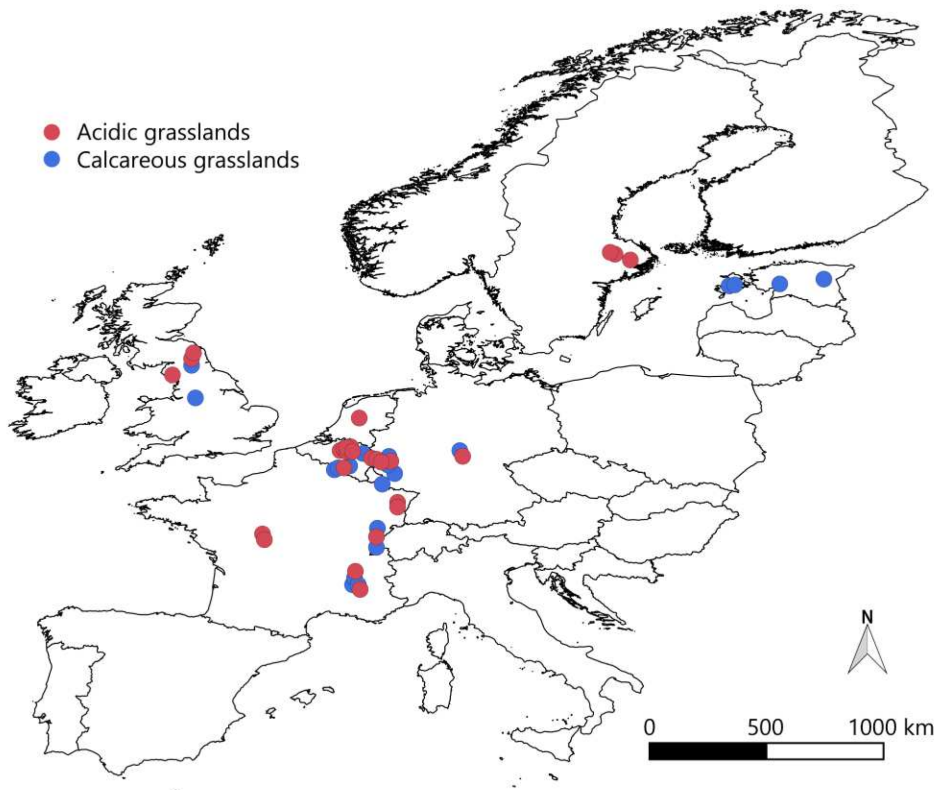

Fig. 1. Map of Europe with the location of the surveyed grassland sites. Acidic grassland sites are shown in red and calcareous grasslands in blue. Markers for unique grassland sites may overlap.

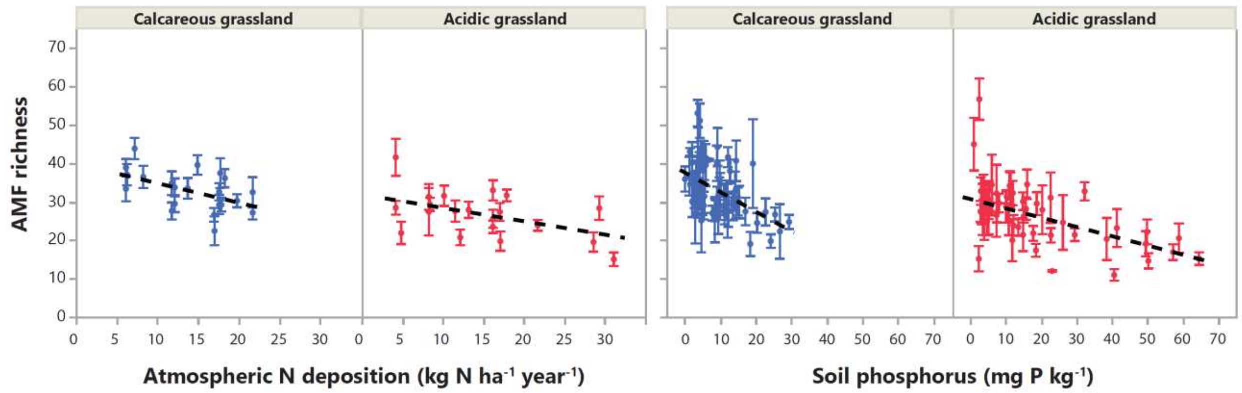

Fig. 2. Richness of arbuscular mycorrhizal fungi across the European gradient of nutrient pollution. Indicators of nutrient pollution are atmospheric nitrogen deposition (main left panel) and Olsen phosphorus (main right panel). Data are mean ± standard error of OTU richness of arbuscular mycorrhizal fungi (AMF) across samples from both host plant roots and grassland soils (N = 670). Samples from calcareous grasslands are plotted in the left panels (blue) and from acidic grasslands in the right panels (red). Significant relationships are illustrated by a dotted trendline.

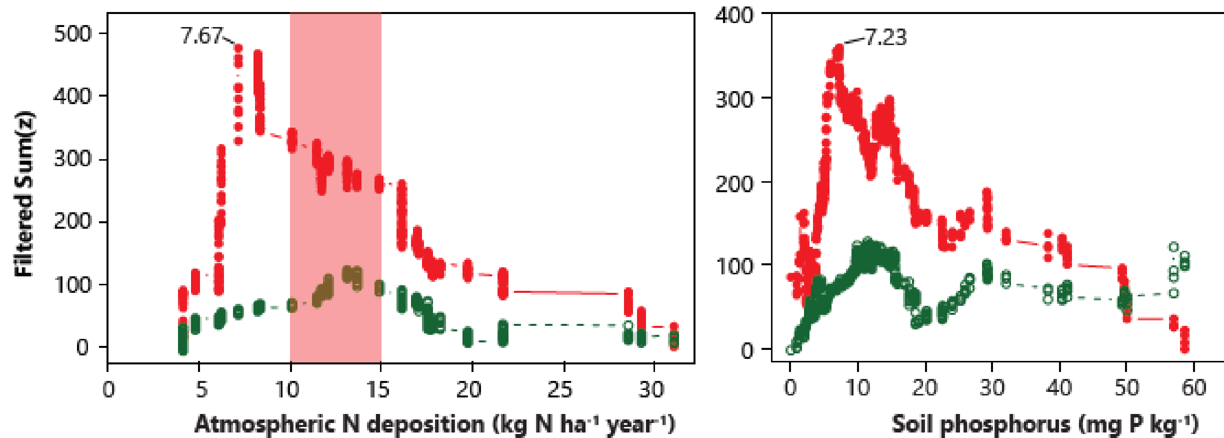

Fig. 3. Results of the threshold indicator taxa analyses across the European gradient of nutrient pollution. Indicators of nutrient pollution are atmospheric nitrogen deposition (left panel) and Olsen phosphorus (right panel). Red symbols show cumulative decreasing arbuscular mycorrhizal fungi OTUs (z-) whereas green symbols show cumulative increasing arbuscular mycorrhizal fungi OTUs (z+), across both calcareous and acidic grasslands. The value of atmospheric nitrogen deposition and soil phosphorus at the sum (z-) peak is depicted, indicating the strongest community-level change point (the value of the environmental gradient where OTUs exhibit the most directional change). Red background shade in the left panel depicts the critical loads of atmospheric nitrogen deposition of 10 to 15 kg N ha^{-1} year^{-1}, currently used in environmental policy for European grasslands.

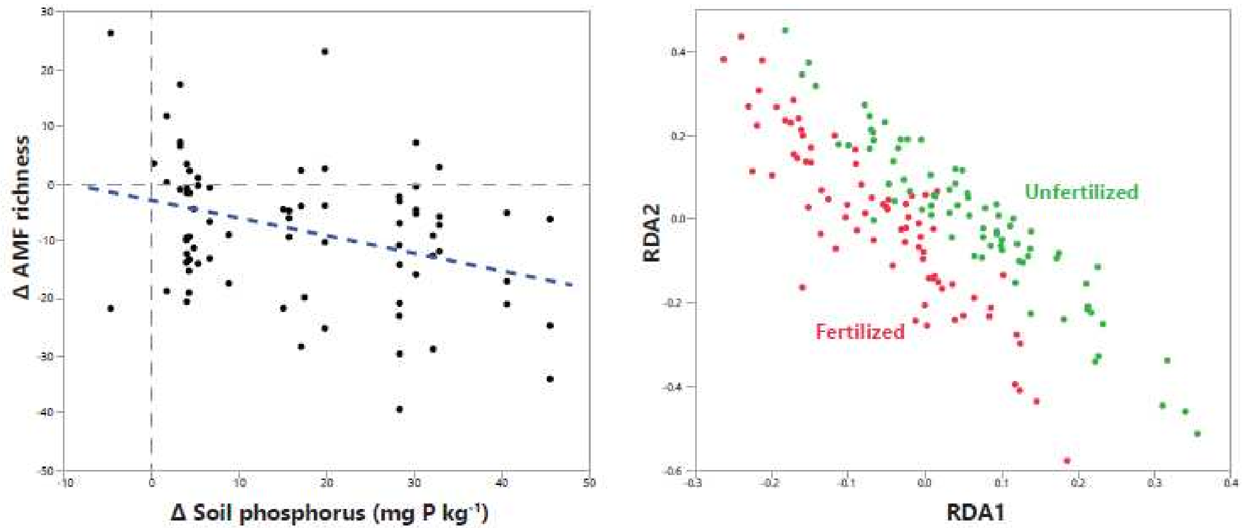

Fig. 4. Pairwise difference in richness and community composition of arbuscular mycorrhizal fungal OTUs between fertilized and unfertilized acidic grasslands. Left panel shows significant relationship with pairwise difference in soil phosphorus. Grey dotted reference lines depict level of no pairwise change. Positive numbers indicate an increase in fertilized grasslands as opposed to unfertilized grasslands, negative numbers a decrease. Right panel shows results of canonical redundancy analysis (RDA) correcting for the variation attributed to a specific grassland site to explicitly test for the effect of fertilization based on a permutation test with 1000 iterations. Significant differences in arbuscular mycorrhizal fungal communities between samples from the paired fertilized (red) and unfertilized grasslands (green) are apparent.

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