

Research paper



Long-term mineral fertilization shaped aboveground plant and belowground bacterial and fungal communities more than cutting frequency in a temperate grassland

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ABSTRACT

Agricultural grassland management intensity strongly influences soil microbial community structure in Europe, though the influence of individual practices, such as fertilization and cutting frequency, requires further investigation. Using a 46 year old field experiment in the Swiss Jura region, we examined how both fertilizer input and cutting frequency influence both soil microbial community structure and functional potential. For community structures of soil bacteria and fungi (determined via genetic metabarcoding) and plants (determined via plant species relevés) the effect of fertilization had a stronger effect than that of cutting frequency. The fatty acid biomarker for arbuscular mycorrhizal fungi (AMF, 16:1 ω 5), however, responded more to cutting frequency (relative effect size = 87.71 %) than fertilization (relative effect size = -7.90 %). Among bacteria with genes linked to soil phosphorous (P) and nitrogen (N) mobilization, only the abundance of the *gcd* gene (inorganic P solubilisation) displayed a significant fertilization effect. These findings suggest that long-term mineral fertilizer application is a key driver of differences in microbial community structure in grasslands of contrasting management type, and may influence bacterial P solubilisation capacity. Some microbial groups, such as AMF, appear more sensitive to cutting frequency, possibly due to additional plant re-growth. This study highlights the importance of disentangling agricultural management practices to better predict grassland plant and soil microbial responses to intensification.

1. Introduction

Grasslands are a key component of European agroecosystems, with permanent grasslands covering about 1/3 of the 4.1 million km² of agricultural land in the EU (Guyomand, 2022). Agricultural grasslands provide habitat for many organisms, including soil microorganisms. The soil microbiome plays a vital role in agroecosystem functioning, contributing to soil nutrient cycling, organic matter decomposition and plant productivity (Xiong and Lu, 2022). Research has shown that the soil microbiome is strongly influenced by grassland management intensity, such as through mineral fertilization (typically involving nitrogen (N), phosphorus (P), and potassium (K)) and cutting frequency, at

scales from plot to global (Fox et al., 2021; Hartmann et al., 2015; Leff et al., 2015; Richter et al., 2024). Indeed, agricultural grassland management intensity has been shown to be as strong a driver of soil microbial community structure as continental scale geographic factors (Barreiro et al., 2022; Fox et al., 2021). A deeper understanding, however, of the long-term impacts of individual grassland management practices, and their interactions, on the soil microbial community structure and its functional potential is needed.

Mineral fertilizers directly and indirectly influence the soil microbiome, by altering soil nutrient availability, stoichiometry and pH (Widdig et al., 2020). These changes may also alter above-ground plant community structure (Francioli et al., 2016) as well as nutrient supply

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via N_2 fixation and mycorrhizal P uptake (Reed et al., 2007) and root structure and exudation patterns (Dietz et al., 2020). Cutting, the complete removal of above-ground vegetation, does not return any nutrients to the soil (Rumpel et al., 2015). Its comparative and interactive effect with fertilizer application on the grassland soil microbiome remain unclear. A short-term (~2 year) German study found NPK fertilizer ($180\text{--}30\text{--}100\text{ N-P-K ha}^{-1}\text{ yr}^{-1}$), reduced microbial biomass under one annual cut but not under three. With three annual cuts, however, no difference was observed between fertilized and non-fertilized plots (Lemanski and Scheu, 2015). In contrast, a long-term (~45 years) Irish grassland study, found no significant difference in microbial biomass C among P fertilization rates (0, 15 and $30\text{ kg P ha}^{-1}\text{ yr}^{-1}$) at a cut and remove site (Randall et al., 2019).

Grassland agricultural management also influences soil microbial functioning, with cascading effects on microbial-driven nutrient cycles (van der Heijden et al., 2008). All of the steps of the soil N cycle (fixation, nitrification, denitrification and mineralisation) and soil P turnover (mineralisation, solubilisation and transport into microbial biomass) are mediated by the soil microbiome (Andrade-Linares et al., 2023; Büne-mann et al., 2012), with microbial stoichiometry a key regulator (Buchkowski et al., 2015). Higher soil N suppresses nitrogenase activity, involved in biological N fixation (Dynarski and Houlton, 2018). The response of these genes to P fertilizer application has been contradictory. In grassland soil, the abundance of *phoD*, encoding an alkaline phosphatase involved in hydrolyzing phosphate esters, significantly declined after a single phosphate application (up to 20 kg P ha^{-1} , Ikoyi et al., 2018). In contrast, a long-term arable experiment, showed that moderate P fertilization (16 kg P ha^{-1}) increased the abundance of both *gcd*, which mediates gluconic acid production which may then solubilize inorganic P, and *phoD* (Liu et al., 2024). An increase in the abundance of the *phoD* gene was also observed with long-term P fertilization in grassland (Randall et al., 2019).

In this study, we examined the long-term effects of two common grassland management practices—mineral fertilization (N, P, K) and cutting frequency—and their interaction on soil microbial community structure and function. We focused on (i) soil bacterial, fungal, and plant alpha and beta diversity, and (ii) the abundance of bacterial functional genes involved in N and P cycling. We specifically hypothesized that: 1) Long-term fertilization will reduce species richness and soil microbial alpha-diversity, and shift plant and microbial community structures more strongly than cutting frequency. 2) Long-term higher cutting frequency will reduce bacterial and fungal biomass, including arbuscular mycorrhizal and saprophytic fungi, due to reduced rhizodeposition. 3) Long-term P fertilization without N will increase the abundance of the bacterial marker gene for biological N fixation, while N fertilization will increase those genes involved in nitrification. P fertilization will also elevate the abundance of genes involved in organo-P mineralisation and solubilisation.

2. Materials and methods

2.1. Experimental set-up

This experiment was established in 1972 in the Jura region of Switzerland (930 m, $47^{\circ}19'48.4''\text{N}$ $7^{\circ}39'54.4''\text{E}$, % clay = 42, % sand = 4 and % silt = 54). The 24 plot experiment, included 4 fertilizer treatments and two cutting frequencies: unfertilized (Unfert); PK (35 and 200 kg ha yr^{-1} of P and K, respectively); NPK (PK plus $75\text{ kg N ha yr}^{-1}$); and NNPk (PK plus $150\text{ kg N ha yr}^{-1}$). Thus, there were eight fertilizer by cutting frequency combinations ($n = 3$). The cutting frequency was either 2 cuts per year, with the first in July (late cut), or 3 cuts, with the first in June (early cut) followed by a cut at the beginning of August. The final cut for both cutting frequencies occurred in early October. The P and K fertilizer was applied once annually at the beginning of April. The N fertilizer application was applied either twice (2 cuts treatment) or three times (3 cuts treatment) per year, the first with the PK application in April, the

second after the first cut (July for 2 cuts, June for 3 cuts), and the third after the August cut (3 cuts only). The experimental layout followed a split-plot design with three replicates, arranged in three blocks. In each block the four fertilizer treatments were allocated to main-plots, with two subplots for the two cutting frequency treatments (Fig. S1).

2.2. Soil sampling

On July 16th 2018, eight soil cores (ϕ 2.5 cm, 0–20 cm) were taken in a 'W' pattern across each plot, subsequently combined, homogenized and sieved to 2 mm. Approximately 0.5 g fresh soil was transferred to a 2 ml Eppendorf tube containing 0.5 g of glass beads (ϕ 0.10–0.11 mm), and 1.2 ml extraction buffer (0.2 M Na_3PO_4 of pH 8, 0.1 M NaCl, 50 mM EDTA, 0.2 % CTAB), vortexed and stored at -80°C for later DNA extraction. The remaining soil was stored at 4°C and later used to determine soil characteristics. Plant species % cover was recorded, and Landolt indicators values (T, K, L, F, W, R, N, H and D), were assigned based on their known ecological preferences in Switzerland (Landolt et al., 2010). Total soil carbon (C_{tot}), nitrogen (N_{tot}), soil pH, total soil P (P_{tot}), ammonium acetate extractable P (P_{AAE}) and soil water content (soil WC) were measured as described in Supplementary File 1.

2.3. Molecular and bioinformatic analysis

DNA was extracted from each soil sample in triplicate, using a phenol-chloroform extraction method (Bürgmann et al., 2001), with modifications (Hartmann et al., 2005). Extracts were then diluted to a concentration of $5\text{ ng}/\mu\text{l}$ with sterile dH_2O . The fungal internal transcribed spacer region (ITS2) and the V3-V4 region of the bacterial 16S rRNA gene were PCR amplified, and amplicons were multiplexed and sequenced ($2 \times 300\text{ bp}$) on an illumina Miseq platform. Primer sequences, PCR conditions and extraction protocols are provided in Supplementary File 2.

Sequencing data was processed using DADA2 for quality control and ASV inference, following Longepierre et al. (2021). Taxonomic classification was performed with MOTHUR using the SILVA database (v138) for bacteria and UNITE (v7.2) for fungi. Chloroplast-, mitochondria-, and archaeal-assigned ASVs were excluded. Bacterial and fungal α -diversity metrics were calculated in MOTHUR, with sequencing depth normalized by iterative subsampling to the lowest read counts (21,675 for bacteria; 19,673 for fungi; Fig. S2). Full details of sequence quality filtering, chimera removal, and target verification are provided in Supplementary File 2. Quantitative PCR (qPCR) was used to assess bacterial and archaeal 16S rRNA genes and functional genes involved in P turnover and N cycling. Targeted genes included those for P mineralisation (*phoD*, *phoN*, *phnX*, *appA*), P solubilisation and uptake (*gcd*, *pstS*), N fixation (*nifH*), ammonia oxidation (AOA and AOB), denitrification (*nirS*, *nirK*, *nosZI*), and N mineralisation (*chiA*). Full primer sets, reaction conditions, and thermal profiles are provided in Supplementary File 3 and Table S1.

2.4. Phospholipid fatty acid analysis

PLFA (phospholipid fatty acids) and NLFA (neutral lipid fatty acids) analyses were conducted as described in Supplementary File 4. In total, 26 fatty acids were identified. Of these, 13 were of bacterial origin (i15:0, a15:0, 15:0, i16:0, 16:1 ω 9, 16:1 ω 7t, 17:1 ω 8, i17:0, a17:0, 17:0, cy17:0, 18:1 ω 7, and cy19:0) (Barreiro et al., 2015). The 18:2 ω 6 PLFA served as a saprotrophic fungal (SF) biomarker, since plant contributions are negligible (Kaiser et al., 2010). Twelve detected PLFAs are known to be present in both bacteria and fungi (Frostegård et al., 2011), and were treated as general microbial indicators. For AMF, 16:1 ω 5 NLFA was used (Kundel et al., 2020), as its abundance strongly correlates with AMF spore counts (Frostegård et al., 2011). Relative abundance (%) of all detected PLFAs, including non-specific ones, were used to analyse PLFA patterns.

2.5. Statistical analysis

The effect of factors ‘Fertilization’ and ‘Cutting frequency’, and their interaction, on univariate variables were tested using a linear-mixed effects model. Block was treated as a random effect nested within ‘Fertilization’ to account for the hierarchical structure of the experimental design. When the block effect had an estimated variance of zero, it was omitted from the model.

A Bray-Curtis dissimilarity matrix was constructed from the plant community. Sequencing data was analysed compositionally (Gloor et al., 2017), transformed via centred log ratio and Aitchinson’s distance used to calculate dissimilarities. The effect of ‘Fertilization’, ‘Cutting frequency’ and their interaction on bacterial, fungal and plant matrices was tested using PERMANOVA (9999 permutations), using the ‘adonis’ function in ‘vegan’ (Anderson, 2001), with Block included via ‘strata’. Principle component analysis (PCA) visualized community structure, and centroid distances representing mean structural dissimilarity among treatments were calculated using Euclidian distance (Fox et al., 2021). The percentage of explained variation of community structures were determined using a distance-based multivariate analysis for a linear model (DistLM), only non-correlated, non-constituent univariate variables were included. Differential abundance analysis, with ALDEx2 (version 1.20.0) including posthoc tests, identified differentially abundant ASVs between different fertilizer and cut combinations (Gloor et al., 2017). The top 20 most significant bacterial and fungal ASVs across the 8 treatments were displayed in heatmaps. Indicator plant species were identified with correlation-based analysis in ‘indicspecies’ (‘multiplatt function’, 9999 permutations).

3. Results

3.1. Stronger effect of ‘Fertilization’ than ‘Cutting frequency’ on P_{AAE} and most Landolt values

‘Fertilization’, ‘Cutting frequency’ and their interaction significantly affected P_{AAE} ($p < 0.05$), with ‘Fertilization’ having the stronger influence. All six fertilized treatments (PK/2, PK/3, NPK/2, NPK/3, NNPK/2, NNPK/3) showed higher P_{AAE} (avg. 4.89 mg/g) than unfertilized ones (i. e. 2 and 3 cut, avg. 1.60 mg/g), a relative effect size of 205 %. ‘Cutting frequency’ had a smaller effect (42 %), with higher P_{AAE} in 2 cuts treatments (Unfert/2, PK/2, NPK/2, NNPK/2, avg. 4.77 mg/g) than in the 3 cuts treatments (Unfert/3, PK/3, NPK/3, NNPK/3, avg. 3.37 mg/g, Table S2a). No significant effects of ‘Fertilization’, ‘Cutting frequency’, or their interaction were observed for soil pH, C_{tot} , N_{tot} or P_{tot} (Table S2a).

Four landolt indicators (F, R, N and D) were significantly influenced

by ‘Fertilization’ ($p < 0.01$) but not by ‘Cutting frequency’ or their interaction ($p > 0.05$, Table S2b). Landolt F value (soil moisture) was lowest in unfertilized treatments (avg. 2.74) and highest in NNPK (avg. 3.07). Similar patterns occurred for Landolt N (soil fertility) and D (aeration), with unfertilized lowest (avg. 2.95 and 2.20, respectively) and NNPK highest (avg. 3.80 and 2.60, respectively). By contrast, Landolt indicator R (soil pH) was highest in unfertilized (avg. 3.33) and lowest in NPK (avg. 2.99). Landolt L (light preference) was the only Landolt indicator significantly influenced by ‘Cutting frequency’, with the largest difference between the 2 cuts (3.53) and 3 cuts (3.65) in PK.

3.2. ‘Fertilization’ a stronger driver of soil bacterial and fungal structure than ‘Cutting frequency’, with P_{AAE} being a consistent explanatory variable

A total of 660,173 high-quality partial 16S rRNA gene sequences were obtained (mean Good’s coverage 0.98), yielding 4244 ASVs. In PERMANOVA (Table 1a), both ‘Fertilization’ ($p < 0.05$) and ‘Cutting frequency’ ($p < 0.01$) significantly affected bacterial community structure, with no interaction. ‘Fertilization’ explained more variation in bacterial community structure ($R^2 = 0.293$), than ‘Cutting frequency’ ($R^2 = 0.053$). On the PCA plot (Fig. 1A), unfertilized and fertilized treatments separated clearly, with centroid distances of 53.26–77.59 (Unfert/2 vs. the fertilized treatments) and 51.16–68.89 (Unfert/3 vs. fertilized treatments). Among fertilized treatments, the largest distance was between PK/3 and NNPK/2 (69.25), whilst others ranged from 45.02 to 59.84. In all cases, centroid distances among fertilizer treatments exceeded those between cutting frequencies within each fertilizer treatment (Unfert = 42.79, PK = 41.06, NPK = 42.62 and NNPK = 44.88). DISTLM (Fig. 1A, bottom panel, Table S4a) showed P_{AAE} explained the most variance in soil bacterial community structure ($R^2 = 0.138$, $p < 0.001$), followed by pH ($R^2 = 0.120$, $p < 0.001$). C_{tot} was the only other significant variable ($R^2 = 0.065$, $p = 0.02$), with the Landolt indicator N just missing the significance threshold ($R^2 = 0.053$, $p = 0.06$).

For the fungal community, 734,290 high-quality partial ITS sequences were obtained (mean Good’s coverage = 1), yielding 1416 ASVs. In PERMANOVA (Table 1b), both ‘Fertilization’ and ‘Cutting frequency’ significantly affected fungal community structure ($p < 0.01$), with no significant interaction. As with bacteria, ‘Fertilizer’ explained more variation in fungal community structure ($R^2 = 0.366$) than ‘Cutting frequency’ ($R^2 = 0.054$). PCA plots (Fig. 1B), showed clear separation between the unfertilized and fertilized treatments, but less distinction among fertilized treatments. Centroid distances were 61.01–75.46 (Unfert/2 vs. fertilized), 66.20–82.34 (Unfert/3 vs. fertilized) and 45.08–61.25 (between fertilized treatments). The largest distance between fertilized treatments was comparable to the smallest

Table 1

Results of a PERMANOVA model on the effect of fertilizer treatment (‘Fertilization’), cutting frequency (‘Cutting frequency’) and interaction (‘Fertilization*Cutting frequency’) on the bacterial (a), fungal (b) and plant (c) community structure. Shown is the degrees of freedom (df), sum of squares (SS), F-statistic (F) and the proportion of variance (R^2). Significance code: ‘***’ $p \leq 0.01$ ‘**’ $p \leq 0.05$.

	Factor	df	SS	F	R^2
(a) Bacteria	Fertilization	3	23,249	2.743*	0.293
	Cutting frequency	1	4244	1.502**	0.053
	Fertilization*Cutting frequency	3	6778	0.800	0.085
	Residual	16	45,205		
(b) Fungi	Fertilization	3	27,047	3.939**	0.366
	Cutting frequency	1	3989	1.743**	0.054
	Fertilization*Cutting frequency	3	6311	0.919**	0.085
	Residual	16	36,618		0.495
(c) Plant	Fertilization	3	2.140	17.405*	0.705
	Cutting frequency	1	0.080	1.961*	0.026
	Fertilization*Cutting frequency	3	0.159	1.297*	0.053
	Residual	16	0.656		

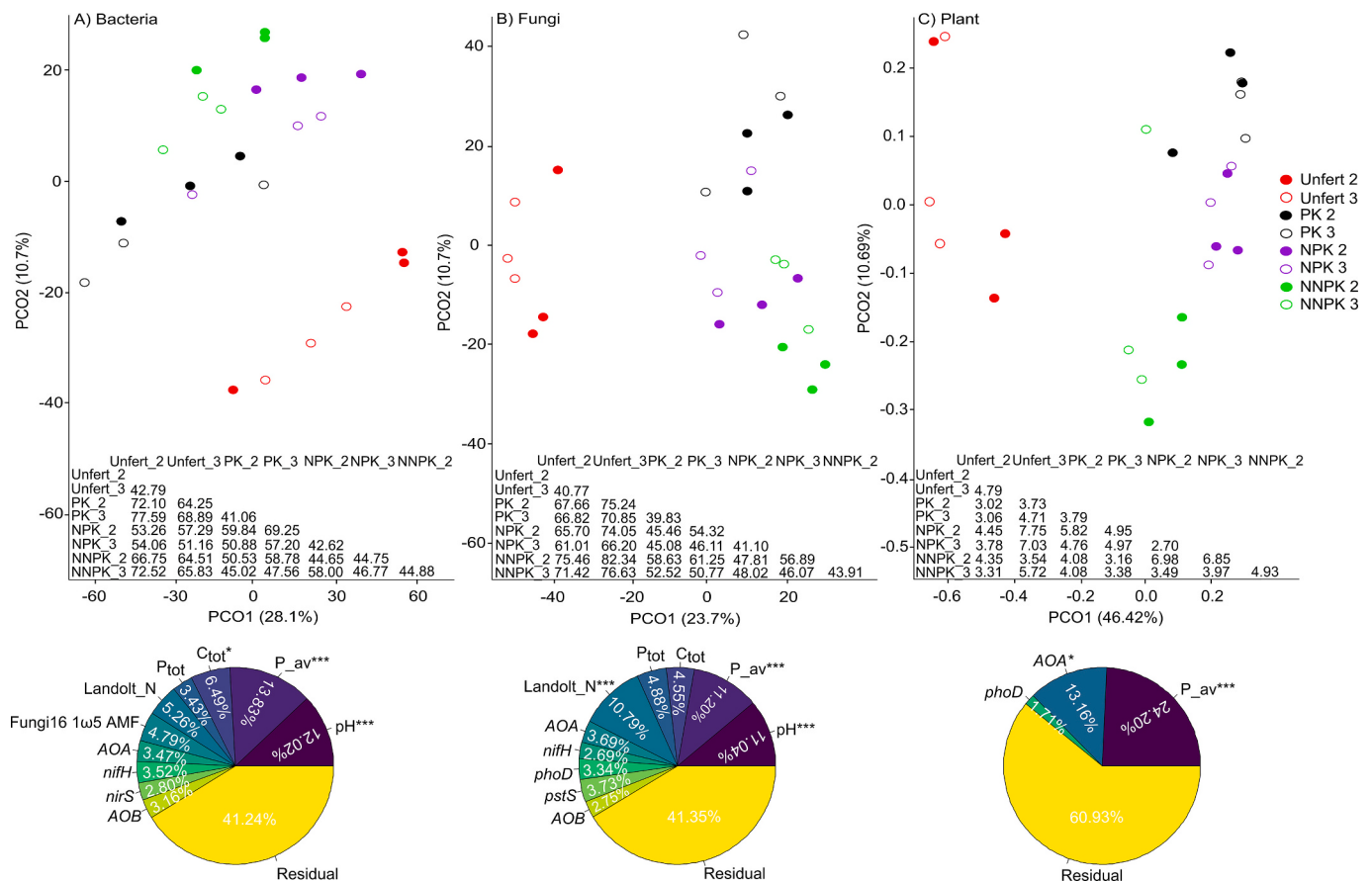


Fig. 1. Top panels: Principle component analysis displaying a) bacterial b) fungal and c) plant community structure across the eight fertilizer (Unfert = red, PK = black, NPK = purple and NNPK = green) and cutting frequency (2 cuts = closed circle, 3 cuts = open circle) treatments. Also displayed in each panel is the community centroid distance (Euclidian distance) between each of the eight fertilizer x cut treatments. Bottom panels: Pie-chart displaying the proportion of variance (R^2) from a distance based linear model of selected environmental variables on bacterial, fungal and plant community structure (chart corresponds to above panel). Further details on the result of the DISTLM model can be found in Table S2. Significance codes: **** $P \leq 0.001$ * $P \leq 0.05$.

unfertilized vs. fertilized distance. The smallest distances occurred between cutting frequencies within treatments: Unfert/2 = 40.77, PK/2 = 39.83, NPK/2 = 41.10 and NNPK/2 = 43.91 (Fig. 1B). DISTLM identified P_{AAE} ($R^2 = 0.112$, $p < 0.001$, Fig. 1B, bottom panel, Table S4b) and pH ($R^2 = 0.11$, $p < 0.001$) as significant explanatory variables. Landolt N also significantly explained fungal community structure ($R^2 = 0.108$, $p < 0.001$), unlike with the bacteria.

For plant community structure, PERMANOVA (Table 1c) showed significant effects of 'Fertilizer', 'Cutting frequency' and their interaction ($P < 0.05$), with 'Fertilizer' explaining by far the most variation ($R^2 = 0.705$). The largest centroid distance was between Cont/3 and NPK/2 (7.75), though some of the fertilized-fertilized distance comparisons were much greater than those seen between the unfertilized and fertilized treatments (e.g., NPK/2 ~ NNPK/2 = 6.98, Fig. 1C, insert). As with bacteria and fungi, DISTLM identified P_{AAE} as a significant explanatory variable of plant community structure ($R^2 = 0.24$, $p < 0.001$, Fig. 2C, bottom panel, Table S4c).

3.3. Stronger effect of 'Cutting frequency' on amount of AMF fatty acid biomarker than 'Fertilization'

The bacterial Inverse Simpson index was significantly influenced by 'Fertilization' ($p < 0.05$), but not by 'Cutting frequency' or their interaction (Table S3a). Values were highest in PK (avg. 154) and lowest in NPK (avg. 116). A significant interaction between 'Fertilization' and 'Cutting frequency' was found for observed and Chao1 bacterial richness (both $p < 0.05$), though no consistent 2 vs. 3 cuts pattern emerged. In

contrast, none of the factors significantly influenced fungal alpha diversity (Table S3b). Both 'Fertilization' and 'Cutting frequency', did however, affect the fungal fatty acid biomarker for saprophytic (18:2 ω 6) and arbuscular mycorrhizal fungi (16:1 ω 5, Table S3c). For the latter, 'Cutting frequency' had a much stronger effect, with a relative effect size of 87.71 % ($p < 0.001$) compared to a negative effect of -7.90 % for 'Fertilization' ($p < 0.05$). This trend was consistent across fertilizer applications, with the 2 cuts treatments (33.21, 44.28, 21.23 and 16.52 nmol g⁻¹ in Unfert, PK, NPK and NNPK, respectively) showing lower values than 3 cuts treatments (55.14, 59.50, 53.51 and 46.49 nmol g⁻¹, respectively).

'Fertilizer' significantly influenced plant species richness ($p < 0.001$), with the unfertilized treatments harbouring the highest counts (33 and 36 in Cuts 2 and 3, respectively), and fertilized treatments lower (range = 18–24). Neither 'Cutting frequency' nor its interaction were significant. 'Fertilizer' also had strong effects on plant Shannon, Simpson and Inverse Simpson Indices ($p < 0.001$), again with no effect of 'Cutting frequency' or interaction ($p > 0.05$). Shannon and Simpson Indices, were highest in unfertilized treatments (avg. 2.89 and 0.93), and lowest in NNPK (avg. 2.35 and 0.89). In contrast, Inverse Simpson values were lowest in Unfert (avg. 1.08) and highest in NNPK (avg. 1.14, Table S3d).

3.4. Nitrogen-fixing bacteria associated with unfertilized treatments, while some fungal pathogens are associated with the NNPK/3 treatment

Aldex analysis of differentially abundant bacterial ASVs (Fig. 2A)

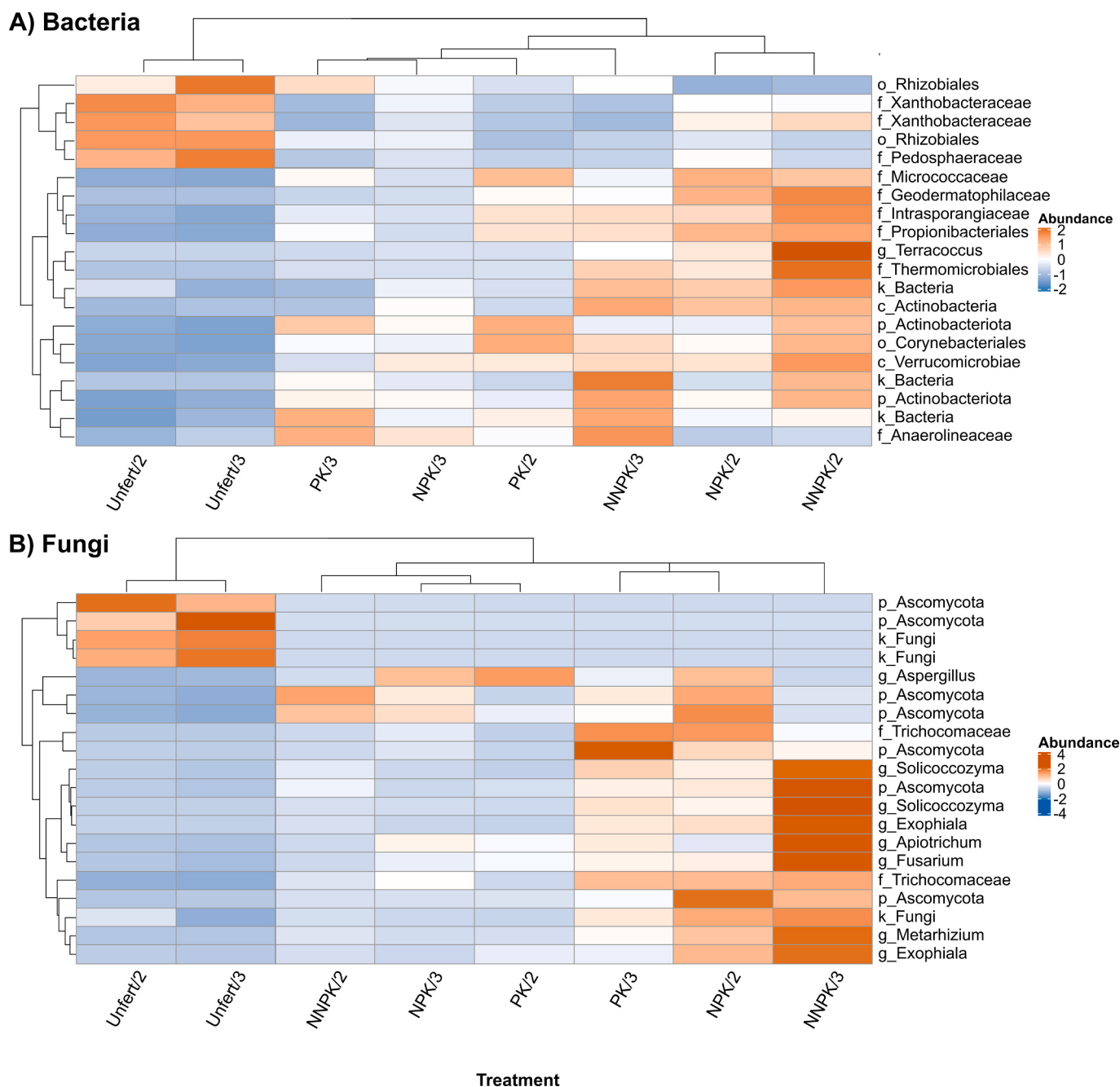


Fig. 2. Heatmap depicting significantly differential abundant bacterial (A) and fungal (B) ASVs across the eight fertilizer (Unfert, PK, NPK and NNP) by cutting frequency (2 and 3 cuts) treatments. Each column represents one sample. Taxonomy of each ASV is given at the lowest assigned taxonomic level: k.: Kingdom, c.: Class, o.: Order, f.: Family, g.: genus.

revealed five ASVs more abundant in unfertilized than fertilized treatments, with four being taxonomically assigned to either the order Rhizobiales or the family Xanthobacteraceae. ASV_36 and ASV_46 (Rhizobiales), were more differentially abundant in Unfert/3 (avg. 172 and 99 ASVs, respectively) compared to fertilized treatments (range = 32–95 and 31–47 ASVs, respectively). Similarly, ASV_10 and ASV_8 (Xanthobacteraceae), were higher in Unfert/3 (avg. 343 and 353 ASVs, respectively) than fertilized treatments (range = 163–237 and 230–294 ASVs, respectively). In contrast, fifteen ASVs were more abundant in fertilized treatments specifically NNP/2. These included ASV_1104 (*Terracoccus*) and ASV_497 (*Thermomicrobiales*), ASV_646 (*Geodermatophilaceae*) and ASV_209 (*Intrasporangiaceae*), with average abundances of 28, 37, 19 and 35 ASVs, respectively in NNP/2,

compared to 0.33, 1.17, 0.33 and 5.3 ASVs in Unfert.

The Aldex analysis revealed, seven fungal ASVs, assigned to genus level, were more abundant in the NNP/3 than in the unfertilized treatments (Fig. 2B). These included ASV_20 and ASV_50 (*Solicozozyma*), ASV_95 and ASV_97 (*Exophiala*), ASV_24 (*Apiotrichum*), ASV_46 (*Fusarium*), ASV_28 (*Metarhizium*), which had higher average abundances in NNP/3; 711, 408, 434, 456, 478, 1186 and 272 ASVs than in unfertilized treatments; 25, 16, 4, 25, 31, 64 and 14 ASVs. Four ASVs, ASV_49, ASV_186, ASV_246 and ASV_25, were more abundant in unfertilized treatments, though these were only taxonomically classified to phylum level or higher.

Several plant species were identified as indicators of unfertilized treatments (Cont/2 and Cont/3, Table S5), including grasses *Briza*

media, *Luzula campestris*, *Carex montana*, *Carex flacca*, *Helictotrichon pubescens*, *Brachypodium pinnatum*, and herbs *Sanguisorba minor*, *Colchicum autumnale* and *Leontodon hispidus* all having an IndVal of at least >0.816 and $p < 0.05$. *Cyrosurus Cristatus* was an indicator for both the control and PK (PK/2 and PK/3, IndVal = 0.866, $p < 0.05$), while *Lathyrus pratensis* was specific to PK (IndVal = 0.906, $p < 0.05$). *Poa trivialis* was the only indicator for N treatments (NPK/2, NPK/3, NNPK/2 and NNPK/3), while *Poa pratensis* was the sole indicator across all fertilized treatments (IndVal, $p < 0.05$).

3.5. No effect of 'Fertilization' on the abundance of *nifH* nor *phoD* communities, but significant effect on *gcd*

No significant effects of 'Fertilization', 'Cutting frequency' or their interaction were found on bacterial or archaeal community marker gene abundance. 'Fertilization', however, did significantly influence the *gcd* gene ($p < 0.05$, Fig. 3C), with the lowest value in the Unfert/2 treatment (avg. 2.21×10^6), and highest NNPK/3 (avg. 7.27×10^6). No effect of 'Cutting frequency' or interaction was observed ($p > 0.05$). A significant interaction ($p < 0.05$, Fig. 3G) was found for the *phnX* gene, involved in organo-P mineralisation, driven by a marked increase in NNPK/3 (avg. 5.45×10^6) compared to other treatments (avg. 2.24×10^6). Finally, AOA (archaeal ammonia-oxidation) gene abundance showed a significant effect of 'Cutting frequency' and its interaction with 'Fertilization' ($p < 0.05$, Fig. J), with markedly higher abundance in NNPK for both 2 (avg. 4.69×10^7) and 3 (avg. 9.00×10^7) cuts than in other treatments (avg. 4.96×10^6).

4. Discussion

4.1. Long-term fertilization significantly alters bacterial, fungal and plant communities, while cutting frequency had a weaker effect

Supporting the 1st hypothesis, long-term mineral fertilization proved a stronger driver of both belowground microbial and aboveground plant community structure than 'Cutting frequency'. Looking first at bacterial community structure, pH, P_{AAE} and C_{tot} were significant correlators. Soil pH is a well-known determinant of bacterial community structure across habitats (Kaiser et al., 2016). P_{AAE} 's significance reflects P fertilization (Randall et al., 2019), while C_{tot} 's significance is surprising as it did not significantly differ between treatments. Small increases in C_{tot} in certain fertilized treatments may be reflective of greater rhizodeposition, due to higher plant biomass, providing more C for microbial utilization (Zang et al., 2017). The higher differential abundance of two Rhizobiales ASVs, a bacterial order well-characterized as biological nitrogen-fixers and phytohormone producers (Garrido-Oter et al., 2018; Ivanova et al., 2000) in the unfertilized treatments, would suggest bacteria with these traits would be selected for in nutrient-poor conditions. Similarly, two *Xanthobacteraceae* ASVs had higher differential abundance in unfertilized treatments, this bacterial family is linked to soil C and N cycling (Oren, 2014) and has been previously reported as enriched in low P_{AAE} soil (Oliverio et al., 2020). By contrast *Terracoccus*, higher differentially abundant in NNPK/2, has been reported to accumulate phosphate within its cell walls, potentially releasing it to plants upon cell death (Prauser et al., 1997), a potential nutrient mobilization strategy in systems with a nutrient imbalance. The higher abundance of *Thermomicrobiales* in NNPK may reflect its known association with soil nutrients and moisture (Li et al., 2021).

Fungal community structure, displayed a stronger response to 'Fertilization' than soil bacteria, and again a lesser effect of 'Cutting frequency' was seen. This aligns with previous research, reporting stronger impacts of grassland management intensity on soil fungal than on soil bacterial community structure across European grasslands (Barreiro et al., 2022; Fox et al., 2021), further indicating that the soil fungal community is more influenced by nutrient availability than the bacterial community. As with soil bacterial community structure, soil

pH and P_{AAE} correlated significantly with fungal community structure. Though in this instance, so did Landolt indicator N, likely reflecting broader nutrient effects, and/or stronger fungal association with specific plant species or community assemblages. Grassland sward composition has previously been shown to shape fungal community structure more than bacteria, even under equal soil conditions (Fox et al., 2020). Many soil fungi have co-evolved symbioses with specific plants (van der Heijden et al., 2006), potentially making them more responsive to changes in plant community structure. The greater relative abundance of fungal ASVs taxonomically assigned to known pathogens suggests that intensified grassland management may create conditions favourable to such fungi. These included two ASVs of the genus *Exophiala*, a known pathotroph previously reported in relatively high abundance in intensively managed grasslands (Fernandez-Gnecco et al., 2022). An ASV of *Fusarium* (Ampt et al., 2019), was also more abundant in this treatment, as was *Metarhizium*, a genus involved in insect pathogenicity (Fernandez-Bravo et al., 2021). Increases in fungal pathogens have been reported in intensified grassland systems, though the effect declined with higher plant species richness (Abrahão et al., 2022). The patterns here may reflect reduced biotic resistance under intensive grassland management.

Unlike belowground communities, plant community structure displayed a significant effect of 'Fertilization' and 'Cutting frequency', with a significant interaction between them. 'Fertilization' explained the largest share of variation, far exceeding that of belowground community structures. All plant indicator species detected for unfertilized treatments are reported to favour nutrient poor conditions (Becker et al., 2012). The indicator for the N-fertilized treatments, *Poa trivialis*, suggests the persistence of 'undesirable' species, as it is considered unpalatable to cattle (Haggar, 1971). Fertilization significantly reduced plant alpha diversity, supporting the study's 1st hypothesis, while 'Cutting frequency' and its interaction showed no effect. This aligns with a long-term grassland study in the Italian Alps, which reported reduced plant species richness after 27 years of mineral fertilization (Scotton and Ziliotto, 2024). Plant community functional composition was highly influenced by 'Fertilizer', with Landolt indicators F, R, N and D responding to this variable but not to 'Cutting frequency'. The only Landolt indicator to be affected by 'Cutting frequency' was L, which is an indicator for light. It is worth noting, however, that the differences between cutting treatments in this study (2 vs. 3 cuts per year) represents a comparatively small management difference, and the lesser effect observed compared to fertilization may in part reflect this.

4.2. Cutting frequency shapes AMF biomass but not total microbial biomass

Contrary to the study's 2nd hypothesis, the extra regrowth period, 2 to 3 cuts in both the unfertilized and fertilized treatments, led to higher amounts of the fatty acid biomarker for arbuscular mycorrhizal fungi (16:1 ω 5), a fungal group capable of forming symbiosis with plants. This fatty acid is a reliable field indicator for the *Glomus* genus (Ngosong et al., 2012). AMF too enhances plant P acquisition, shown previously using four different *Glomus* isolates in simulated calcareous grasslands (van der Heijden et al., 2006), and may also aid in N uptake (Hawkins et al., 2000). The additional re-growth in the 3 cuts treatment may have raised plant physiological demand for nutrients, prompting plant investment in AMF associations. The smaller effect of 'Fertilization' on AMF abundance was unexpected. A 47-year fertilizer experiment in Northern Ireland (NPK application at 200, 32, 160 kg ha⁻¹ yr⁻¹) found no difference in % AMF root colonization (Fornara et al., 2020). The strong 'Cutting frequency' effect on AMF contrasts with a shorter-term Polish study, which found that mowing intensity (low, medium and high) had no significant effect on fungal root colonization, AMF spores or AMF community composition (Zubek et al., 2022). The functional role of AMF may explain why 'Cutting frequency' influenced 16:1 ω 5, more than the saprophytic biomarker (18:2 ω 6).

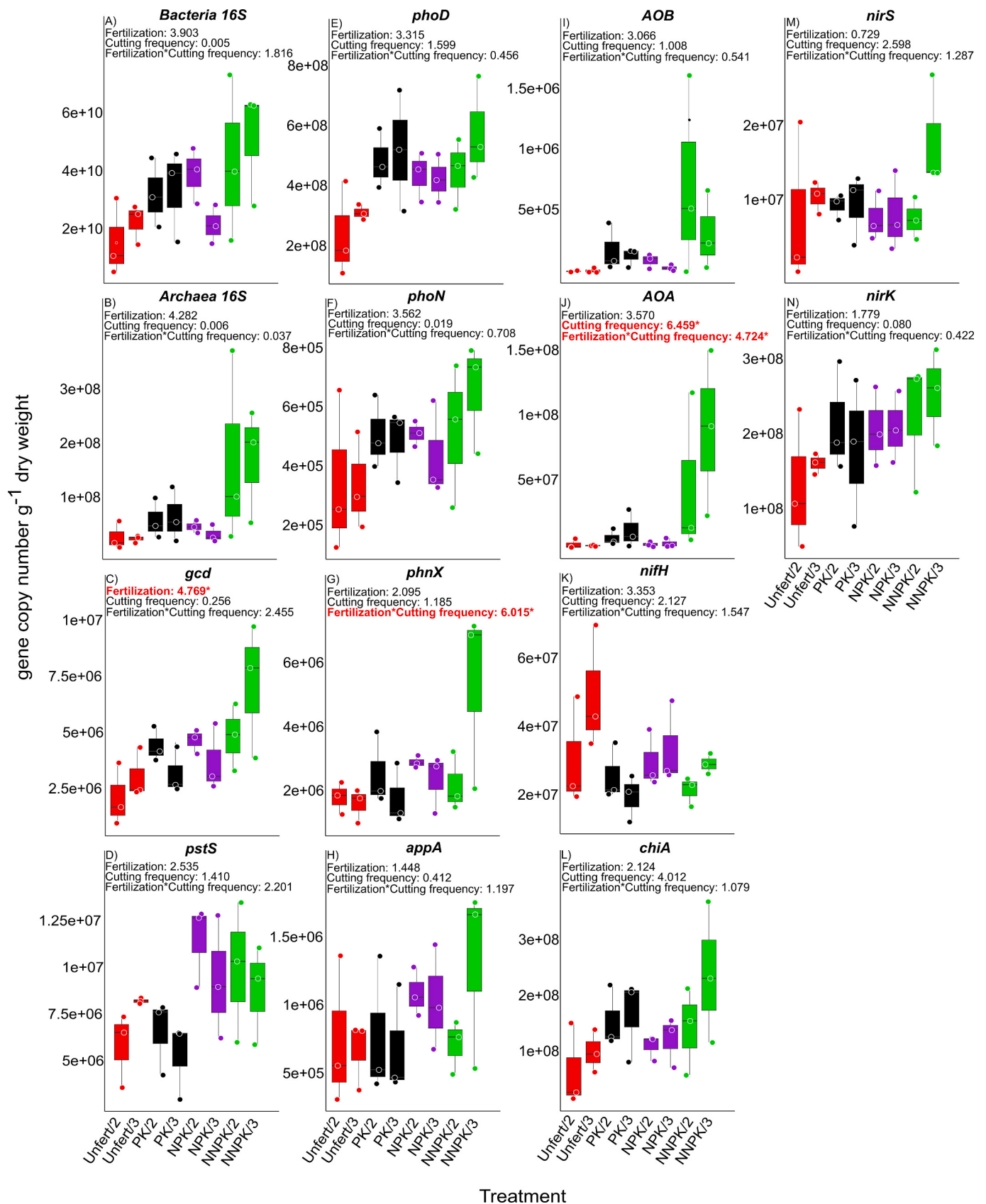


Fig. 3. Boxplot of copies of target genes (per gram soil dry weight) across the eight fertilizer (Unfert, PK, NPK and NNPK) by cutting frequency (2 and 3 cuts) treatments for 16S RNA gene for Bacteria (A), Archaea (B) and six genes involved in soil P turnover *gcd* (C), *pstS* (D), *phoD* (E), *phoN* (F), *phnX* (G) and *appA* (H). Also shown are six genes involved in the soil nitrogen cycle, *AOB* (I), *AOA* (J), *nifH* (K), *chiA* (L), *nirS* (M) and *nirK* (N). Shown is the F – value for each factor and the interaction. Significance codes: “*” $p \leq 0.05$, with significant effect of a factor highlighted in red.

'Fertilization' had a stronger effect on the bacterial Inverse Simpson diversity index, contrasting with a global study of 25 grasslands which found only marginal effects of mineral N and P addition on bacterial alpha diversity (Leff et al., 2015). The stronger effect here may relate to soil pH, a major determinant of bacterial diversity (Kaiser et al., 2016; Mayerhofer et al., 2021). The highest bacterial Inverse Simpson diversity index values occurred in the PK treatments, which had higher, though non-significant, soil pH values. Neither 'Fertilization' or 'Cutting frequency' significantly influenced fungal alpha diversity. This was unexpected, as European studies have reported greater fungal richness in extensively managed grassland (Barreiro et al., 2022; Fox et al., 2021). The belowground biodiversity response to land-use intensity, however, can vary with spatial scale (Le Provost et al., 2021).

4.3. Long-term mineral fertilizer increases *gcd* abundance, with no effect on *nifH* nor *phoD*

Contrary to the 3rd hypotheses, fertilization had no significant effect on genes for P mineralisation (*phoD*) or N fixation (*nifH*). The lack of increase in *nifH* in the unfertilized treatments occurred despite the presence of bacterial indicator ASVs previously characterized for biological nitrogen fixation. The absence of a fertilization effect on *phoD* abundance was surprising, as a long-term P fertilizer experiment in Ireland reported increased *phoD* abundance, though the effect was stronger under grazing than cuts (Randall et al., 2019). A significant 'Fertilization' effect was observed, however, on *gcd* abundance, most notably in the NNPk/3 treatment. This treatment received double the typical N, but a typical P application for over 45 years, likely altering microbial biomass N:P stoichiometry, stimulating soil microbes to solubilize inorganic P. While previous studies suggest P-solubilizing bacteria decline in grasslands with N addition, and that microbes preferentially mineralize organo-P (Widdig et al., 2019), this would not appear to be the case here. Although *phoD* is only one phosphatase marker, other organo-P mineralisation groups may be responding, and P mobilization strategies may vary with site-specific conditions.

Although AOB abundance was higher in NNPk treatments, fertilization had no significant effect on this gene. This finding contrasts with Zhou et al. (2015), who reported increased AOB abundance after 44 years of chronic mineral N addition in grassland. Here, a significant interaction between 'Fertilization' and 'Cutting frequency' was observed on AOA gene abundance, again contrasting with Zhou et al. (2015) who found this gene responded to long-term organic, but not mineral, N additions.

5. Conclusion

This study demonstrated that long-term mineral fertilizer application significantly shaped soil microbial community structure, more so than cutting frequency. A similar pattern was also seen with plant community structure, suggesting fertilization is a key driver of both above and belowground community structure differences across varying management intensities. There were also indications that fertilizer application enhanced the capacity of the soil microbiome to solubilize inorganic P in plots receiving double the typical N application, despite P being applied. Nutrient imbalances likely induce the soil microbiome to mobilize required nutrients. In some instances, however, cutting frequency had a stronger effect than fertilizer application, such as on the abundance of a notable component of the fungal community, the AMF. This would suggest that even in adequately fertilized grassland systems, this fungal group may be involved in nutrient acquisition for plant communities under conditions of multiple re-growth periods. Future work is needed to compare the effect of fertilization on above and belowground alpha and beta diversity across a broader range of grassland management strategies, such as grazing or liming.

CRedit authorship contribution statement

Aaron Fox: Writing – original draft, Methodology, Investigation, Funding acquisition, Data curation. **Stefanie Schulz:** Writing – review & editing, Methodology, Data curation. **Fiona Brennan:** Writing – review & editing, Project administration, Investigation, Funding acquisition. **Florian Gschwend:** Writing – review & editing, Formal analysis. **Ana Barreiro:** Writing – review & editing, Formal analysis, Data curation. **Franco Widmer:** Writing – review & editing, Funding acquisition, Conceptualization. **Michael Schloter:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Olivier Huguenin-Elie:** Writing – review & editing, Investigation, Data curation, Conceptualization. **Andreas Lüscher:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data accessibility

DNA sequences were deposited in the NCBI SRA archive with the project number PRJNA1142777.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.apsoil.2025.106462>.

Data availability

Data will be made available on request.

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