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Stable nitrogen-cycling capacity in relation to fertilization and intercropping in a sub-boreal grassland

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ABSTRACT

Grasslands are important in sub-boreal climate agricultural systems and are managed with various combinations of N fertilization and plant species. Ammonia-oxidizing and denitrifying microorganisms are key players in determining the fate of nitrogen (N) and thereby also the yield in grassland systems and their impact on gaseous N losses and leaching. We established a three-year field study in southern Finland with fertilizer treatment as a main-plot factor, including organic and synthetic fertilizers and plant species and mixtures thereof as the sub-plot factor. We quantified six genes encoding key N-cycling enzymes by quantitative PCR to determine the abundance of the communities involved in N-transformation processes and also included previously published data on crop yield, soil properties and the overall bacterial community composition. With the exception of ammonia oxidizing bacteria (AOB), which were primarily affected by fertilization, the abundances of all other N-cycling communities changed over time with either an increase or decrease from summer to autumn. Differences in gene abundances between plant species treatments and in fertilizer by plant species interactions were detected mainly in the beginning of the cropping season during the first year. The *nirS*-type denitrifiers and *nosZ*II nitrous oxide reducers responded more to changes in soil properties than their functional counterpart *nirK* and *nosZ*I communities. Using structural equation modeling, we show that the overall microbial community composition and diversity played an important role in mediating the management effects on crop yield, genetic potential for N retention and N_2O sink capacity. However, a trade-off between the genetic potential for N retention and N_2O sink capacity was detected, indicating the challenges in managing grasslands in a sustainable way.

1. Introduction

Crop production depends strongly on nitrogen (N) input, but this increases the amounts of reactive N in the environment. The excess of reactive N in soils has major negative environmental impacts, such as pollution of waterways through nitrate (NO_3^-) leaching and climate warming through the emission of nitrous oxide (N_2O), and poses a further threat to biodiversity through acidification, eutrophication and climate change [1]. Synthetic N fertilizers currently contribute to 40–60% of the world's food production [2], but fertilizer production requires large inputs of energy and fixes about 11.5×10^{12} mol N per year [3]. Other options to provide N to crops are organic fertilizers, which also support soil organic matter formation and biodiversity [4,5], and cultivation of symbiotically N-fixing legumes. Nitrogen fixation from fodder legumes adds about 2.4×10^{12} mol N per year into agroecosystems [3,6]. Intercropping with N-fixing legumes combined with organic amendments can thereby replace or reduce the use of synthetic N fertilizers. It has yet to be determined, however, how to optimize such systems to minimize loss of reactive N, and how to manage the underlying mechanisms to avoid compromising crop yield.

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In cropping systems, reactive N in soil is subject to the competition between plants and microorganisms [7] and among microorganisms [8] (Fig. 1). Microorganisms have the capacity to either assimilate and immobilize the reactive N in their cells or to use it in oxidation and reduction reactions relating to their energy metabolism. Nitrogen loss from soils occurs mainly via NO_3^- leaching or denitrification, during which the NO_3^- is transformed stepwise into gaseous N compounds (NO, N_2O and N_2) by enzymes encoded by key N-cycling genes [9]. Since N_2O is a powerful greenhouse gas, the capacity of soil microorganisms to reduce it to N_2 is important [10]. Some denitrifiers carry the *nosZ* gene encoding N_2O reductase, which is the only known biological sink for N_2O. In contrast to denitrification, dissimilatory nitrate reduction to ammonium (DNRA) promotes N retention. The formate-dependent nitrite (NO_2^-) reductase, encoded by the *nrfA* gene, catalyzes the reduction of NO_2^- into NH_4^+ during respiratory ammonification, which is the key step in DNRA [11]. The potential of NO_2^- reduction by DNRA versus denitrification, estimated as the *nrfA/nir* gene abundance ratio, has been suggested as a proxy measure for N retention [12]. However, the effects of N fertilization and plants on soil functional communities can vary with fertilizer type, plant species and functional group analyzed [13–18]. Most of these studies are based on sampling a single time point, so there is limited information on how fertilizer type and legume-grass intercropping management affect the N-cycling microorganisms over time under field conditions.

Short-term grasslands, such as three-year leys with grass and legumes, play an important role in agriculture in cool-temperate regions. For example, in Finland they cover about 30% of the arable land and provide feed for ruminants in terms of silage, hay or grazing [19]. With a three-year field experiment in a sub-boreal grassland, we found that i) the temporal variation in composition of the overall soil bacterial communities was larger than the variation caused by fertilizer and plant species treatments and ii) the major treatment effects on bacterial community diversity and composition were found between fallow and planted treatments and between organic and synthetic fertilizer treatments [20]. However, changes in taxonomic composition do not necessarily reflect changes in the functional capacity of these communities [21]. For the N-cycling microorganisms, the size of the functional groups involved in different processes within the N cycle could reveal effects on specific pathways [22]. In this study, we therefore assessed the effects of fertilizer type and intercropping on the N-cycling communities by using quantitative PCR to investigate several key N-cycling genes. We aimed to disentangle the underlying mechanism of how the agricultural management regimes in these sub-boreal grasslands affect microbial communities and their capacity for N-cycling and how such changes may affect crop yield. Hence, we conducted an integrated analysis, including crop yield, soil properties and the composition of soil bacterial communities previously reported from the same field experiment [20, 23], along with the abundance of several functional groups within the N-cycling communities. We specifically tested the hypotheses that i) the soil microbial communities mediate management effects on crop yield and the potential for both N retention and N_2O sink capacity and ii) an increase in potential for N retention increases crop yield. These are important ecosystem services that ideally should be delivered by grasslands without compromising yield. By identifying the management that can improve crop yield and decrease potential N losses, this study will advance our progress towards sustainable agriculture.

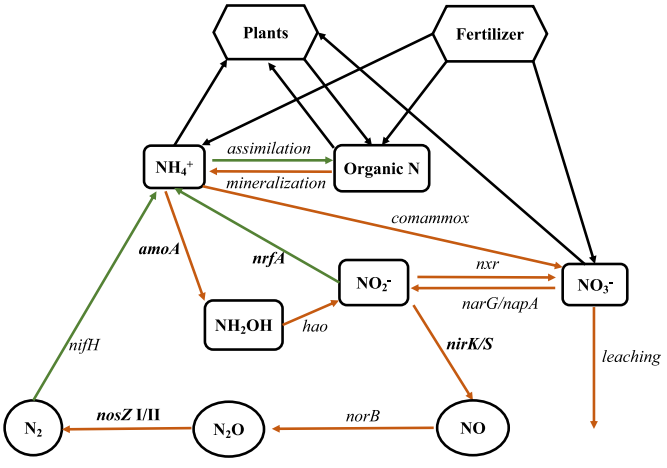

Fig. 1. The N flow among microorganisms, plants and fertilizer in an agricultural soil. Genes encoding the key enzymes catalyzing major biological transformations of inorganic N species [3,24,25] and the major pathways are indicated next to the arrows. Boxes indicate N species as ions or organic compounds and circles as gaseous forms of N. Genes investigated in this study are marked in bold. The processes leading to potential N loss are indicated in orange and those leading to potential N retention in green. *nifH*, nitrogenase; *amoA*, ammonium monooxygenase; *hao*, hydroxylamine oxidoreductase; *nrfA*, nitrite reductase in dissimilatory nitrate reduction to ammonium; *nxr*, nitrite oxidoreductase; *narG/napA*, nitrate reductase; *nirK/S*, nitrite reductase in denitrification; *norB*, nitric oxide reductase; *nosZ* I/II, nitrous oxide reductase. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2. Materials and methods

2.1. Experimental site

A three-year field study was conducted at Viikki Experimental Farm, University of Helsinki, Finland (60.226°N, 25.017°E), from May 2013 to September 2015. The split-plot design comprised four 18 m × 8 m blocks split into three 6 m × 8 m main plots for fertilizer treatments (main-plot factor): a non-fertilized control, organic fertilizer (urine in 2014 and manure slurry in 2015) and synthetic fertilizer calcium nitrate [$Ca(NO_3)_2$]. The main plots were split into four 6 m × 2 m sub-plots for plant species treatment (sub-plot factor): fallow, pure red clover (*Trifolium pratense* L.), pure timothy (*Phleum pratense* L.) and a mixture with 25% red clover and 75% timothy.

According to standard practice for pasture management in this region, fertilizer was applied in spring, the crop was harvested in midsummer, fertilizer was applied a second time in the growing season, and the second harvest was taken in late summer. In 2014, N input was low, with organic fertilizer N applied at 35 kg ha^{-1} and synthetic N at 40 kg ha^{-1} in May and with organic and synthetic N both applied at 20 kg ha^{-1} in July. In 2015, N input was at the normal level, with organic and synthetic both applied at 75 kg ha^{-1} in June and July. Surface soil (0–20 cm) samples were taken in June 2014, September 2014, July 2015 and September 2015, sieved with a 5 mm mesh and preserved at −20 °C.

Soil texture was clay loam, with 32% clay, 36% silt, and 32% sand on average. At the start of the experiment, soil pH was 6.4, electrical conductivity (EC) was 52.3 μS cm^{-1}, NO_3^- content was 5.4 mg kg^{-1}, NH_4^+ content was 4.5 mg kg^{-1}, total carbon content was 25.3 g kg^{-1} and total N content was 1.7 g kg^{-1}. The climate is sub-boreal and the average monthly precipitation and temperature during the field study have been reported previously [23]. Since net N mineralization correlates with air temperature [26], the mean air temperature of the 21 days prior to soil sampling dates was calculated using the database of the Finnish Meteorological Institute (https://en.ilmatieteenlaitos.fi/download-observations). These means show that the summer harvest of 2014 followed a cooler episode than that of 2015 (12.6 °C and 15.7 °C, respectively) whereas the autumn harvests were at similar temperatures (14.2 °C in 2014 and 13.8 °C in 2015). Details of field establishment and management, weather conditions, sampling and analyses are described in Li et al. (2019) [23] and Li et al. (2020) [20] (Table A. 1).

2.2. qPCR for quantification of soil N-cycling communities

DNA isolation was done by using Power Soil DNA Isolation Kit

(MoBio, Carlsbad, CA, United States) and the details were described by Li et al. (2020) [20]. Soil DNA content was measured by using a Qubit fluorometer with the Qubit dsDNA BR assay kit (Life Technologies Corporation, Carlsbad, CA, USA). DNA samples were diluted to 2–5 ng DNA μl^{-1} with low EDTA 1x TE buffer. Quantitative real-time PCR (qPCR) was applied to estimate the genetic potential for ammonia oxidation by quantifying the *amoA* gene from archaea (AOA) and bacteria (AOB), for nitrite reduction (denitrification) by quantifying the *nirK* and *nirS* gene, for nitrous oxide reduction by quantifying the *nosZ*I and *nosZ*II gene, and for respiratory ammonification (dissimilatory nitrate reduction to ammonium, DNRA) by quantifying the *nrfA* gene (Fig. 1). To estimate the abundance of the total bacterial communities, the 16S rRNA gene was quantified.

The duplicate, independent qPCR reactions of each gene was done by using the CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA). The total reaction volume of 15 μl contained iQ™ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 0.1% Bovine Serum Albumin, 4–10 ng of template DNA, and primers (with final concentrations of 0.25 μM for 16S rRNA, 0.5 μM for AOA, AOB and *nrfA*, 0.8 μM for *nirK*, *nirS* and *nosZ*I, and 2 μM for *nosZ*II). Standard curves for quantification were based on serial dilutions of the linearized plasmids containing specific gene fragments. The amplification efficiency was calculated based on the equation: efficiency $= -1 + e^{(-1/\text{slope})}$, where the slope comes from the standard curve. The primers, amplification efficiencies and thermal cycling programs are in Tables A. 2 and A. 3. The quality of the amplicons was estimated based on the melting curves and gel electrophoresis in 1% agarose gel. To determine the potential inhibition in the PCR reactions, we amplified a known amount of the pGEM-T plasmid (Promega, Madison, WI, USA) with M13 forward and reverse primers with and without soil DNA in the reactions. No inhibition was detected with the amount of DNA used.

Examination of the abolute N-cycling gene abundances allowed us to detect specific changes in the N-cycling capacity that were not simply driven by an overall change in the total size of microbial community. To estimate the changes in N-cycling capacity within the microbial community, we examined the gene abundances relative to that of 16S rRNA gene.

2.3. Statistical analysis

All the statistical analyses were performed in RStudio v.1.1.383 [27] based on R v.3.5.0 [28].

Over the four time points, the overall effects between-subjects (fertilizer and plant species) and within-subjects (temporal change) on the size of N-cycling communities were tested using the repeated measures analysis of variance (rmANOVA), where sampling time was the repeated and within-subjects factor, block was a random factor, and fertilizer and plant treatments were fixed factors. The intra-plot correlations (repeated measures correlations) of the genes with soil properties, bacterial community diversity (Shannon index, which was calculated using Mothur v.1.39.5) [29], bacterial community composition (indicated by the first principal coordinate (PCO1) of the bacterial community composition that explained the maximum variance of the composition (data from Li et al., 2020, Table A. 1)) and crop dry matter yield (data from Li et al. 2019, Table A. 1) were calculated using the *rmcorr* v.0.3.0 package [30]. To satisfy the assumptions of linear model, we examined the normality of residuals and transformed the data when necessary.

At each time point, a linear mixed-effects model was used to estimate the main effects of fertilizer, plant species and their interaction on the size of N-cycling communities with the *lme* function in the *nlme* v.3.1–137 package (Pinheiro et al., 2018). Normality and homoscedasticity of the model residuals were examined by plotting the residuals of each model against the theoretical quantiles and against the fitted values. When $P < 0.05$, differences in treatment means were tested with Tukey's test using the *emmeans* and *pairs* functions in the *emmeans* v.1.5.3 package [31]. Correlations between the size of N-cycling communities and soil properties at each time point were visualized with *rcorr* function in *Hmisc* v.4.4–1 package [32] and *corrplot* function in *corrplot* v.0.84 package [33].

To determine the temporal change in and the effects of fertilizer and plant species on the overall N-cycling communities, the functional gene abundances (*amoA* gene from AOA and AOB), *nirK*, *nirS*, *nosZ*I, *nosZ*II, *nrfA*) relative to 16S rRNA gene were included as a compositional data set. To balance out the effects from high and low relative abundance, the abundances relative to 16S rRNA were log or square-root transformed as necessary [34]. Permutational multivariate analysis of variance (PERMANOVA) with 999 permutations of Euclidean distance was performed with the *adonis* function in the *vegan* v.2.5–6 package [35]. When $P < 0.05$, comparisons between two time points or two treatment levels were performed with a spherical test (999 permutations) in *RVAideMemoire* package [36]. The differences in the overall N-cycling communities over time and between treatments were integrated into an ordination analysis with the *rda* function in the vegan v.2.5–6 package [35] and were visualized with principal component analysis (PCA) and redundancy analysis (RDA). To test the direct and indirect correlations of soil properties, overall microbial community composition and N-cycling communities, Mantel and partial Mantel tests were performed with mantel and mantel.partial functions in the vegan v.2.5–6 package [35].

To test the hypothesized mechanism for crop yield, potential N retention (i.e., ratio between genetic potential of DNRA and denitrification) and potential N_2O sink (i.e., *nosZ* abundance), structural equation modelling (Figure A. 6) was performed in the *piecewiseSEM* v.2.2.0 package [37]. The first principal component (PC1) of the bacterial community composition that explained the maximum variance of the composition was used to indicate community composition. Fertilizer and plant species treatments were included as categorical variables in the model.

The parameters of the model were estimated using the maximum likelihood (ML) method. The model fit was evaluated by using Fisher's C test with the null hypothesis that all the missing pathways were not significant and Chi-square test with the null hypothesis that the model-implied covariance matrix was the same as the sample covariance matrix. Thus, $P > 0.05$ indicated that the model fit was acceptable. To detect possible missing pathways in the model, the *dSep* test was performed. The Akaike Information Criterion (AIC) value was used in improving the model, with lower AIC indicating that the model was better supported by the data. To calculate the effect size, *emmeans* and *emtrends* functions were performed with the *emmeans* v.1.5.3 package [30].

3. Results

3.1. Genetic capacity for N-cycling in response to time, fertilization and plant species treatments

The abundance of the 16S rRNA gene, a proxy for the abundance of the total prokaryote community, changed over time ($F_{(3,80)} = 31.85$, $P < 0.001$, Table 1), with an increase in 2014 and a decrease in 2015, but was not affected by either the fertilizer or plant species treatment (Table A. 4).

Across all treatments and time points, the relative abundance of *amoA* from bacteria (AOB) exceeded that of archaea (AOA), *nirS* exceeded *nirK* and *nosZ*II exceeded *nosZ*I (Wilcoxon test, $P < 0.001$, Figure A. 1). With the exception of AOB, which was affected by fertilization, all other gene abundances exclusively changed over time (Table 1). In addition, the temporal change of AOA abundance was different between fertilizer treatments (Table 1), as the increase of AOA in 2015 was greater with synthetic fertilizer than in the non-fertilized or organic fertilizer treatments (Fig. 2). The relative abundances of *nirS* and *nosZ*II increased from summer to autumn, in both 2014 and 2015 ($P < 0.01$), but especially in 2015 (Table A. 4), and the increase in the fertilized plots was more pronounced, especially in those with organic

Table 1
Repeated measures analysis of variance of the absolute abundance of the16S rRNA gene (per g dry weight soil)[a] and the abundance of N-cycling genes relative to the 16S rRNA gene in relation to time (Time), fertilization (Fert), plant species (Plant) and their interaction effects.

Sources	DF[b]	denDF[c]	AOA		AOB		*nirK*		*nirS*	
			F-value	*P*-value	F-value	*P*-value	F-value	*P*-value	F-value	*P*-value
Fert	2	6	2.11	0.20	5.91	< **0.05**	0.07	0.93	0.51	0.62
Plant	3	27	1.32	0.29	0.66	0.58	1.44	0.25	0.15	0.93
Fert:Plant	6	27	0.95	0.48	1.13	0.37	1.41	0.25	1.11	0.38
Time	3	80	6.72	< **0.001**	1.37	0.26	3.57	< **0.05**	17.25	< **0.001**
Time:Fert	6	18	3.46	< **0.05**	2.02	0.11	1.14	0.38	0.72	0.63
Time:Plant	9	80	0.53	0.85	0.92	0.51	1.22	0.29	0.92	0.51
Time:Fert:Plant	18	80	1.07	0.40	1.39	0.16	0.83	0.65	0.93	0.54
	DF	**denDF**	***nosZ*I**		***nosZ*II**		***nrfA***		**16S rRNA**	
			F-value	*P*-value	F-value	P-value	F-value	*P*-value	F-value	*P*-value
Fert	2	6	0.46	0.65	1.54	0.29	0.38	0.70	0.92	0.45
Plant	3	27	0.40	0.75	1.35	0.28	0.52	0.67	0.67	0.58
Fert:Plant	6	27	1.41	0.35	0.70	0.65	0.42	0.86	1.39	0.25
Time	3	80	3.79	< **0.05**	14.71	< **0.001**	11.08	< **0.001**	31.85	< **0.001**
Time:Fert	6	18	0.33	0.91	0.66	0.68	0.34	0.90	0.10	0.46
Time:Plant	9	80	0.54	0.84	0.70	0.70	0.44	0.91	1.19	0.31
Time:Fert:Plant	18	80	1.24	0.25	1.07	0.39	1.45	0.13	1.05	0.42

[a] Data was transformed whenever necessary to satisfy the assumptions of the linear model.
[b] DF, degrees of freedom.
[c] denDF, DF of the denominator.

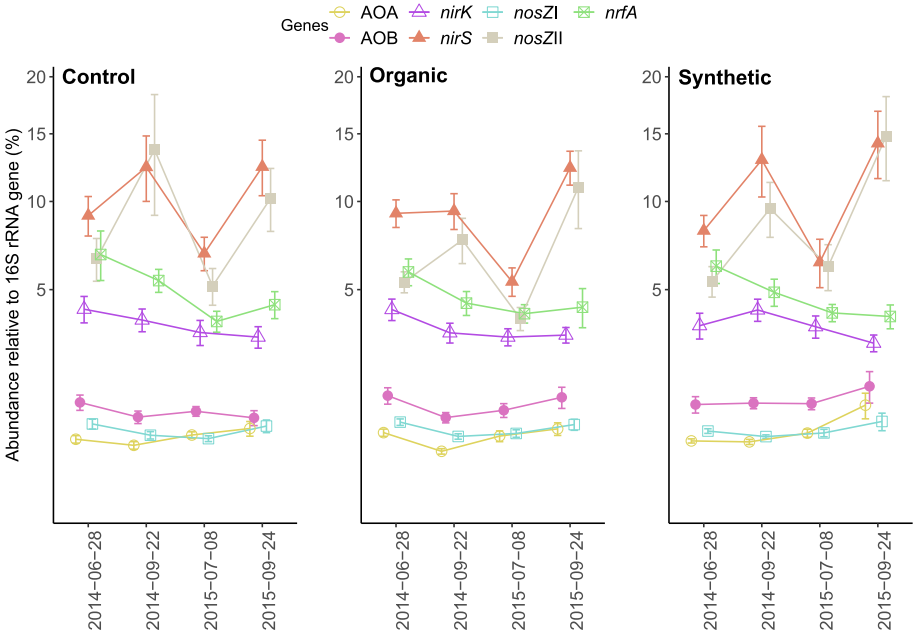

Fig. 2. Temporal changes of the relative abundances of N-cycling genes (mean ± SE, n = 4) in relation to the fertilization treatments with unfertilized control, organic fertilizer and synthetic fertilizer. To visualize the variables with small values better, the y-axis was square-root scaled.

fertilizer (Fig. 2, Table A. 5). In contrast, the relative abundance of *nrfA* decreased from 2014 to 2015 ($P < 0.05$, Fig. 2). The ratio of *nrfA* to the sum of *nirS* and *nirK*, indicating N retention over N loss by denitrification, was <1 at each time point and changed over time ($P < 0.001$, Table A. 4). We observed a consistently lower *nrfA* to *nirS* and *nirK* ratio in autumn than in summer across all treatments and years ($P < 0.01$, Figure A. 2), driven by the increase in *nirS*-type denitrifiers in autumn (Table A. 4). The absolute abundances of *nirK* and *nirS* were affected by the interaction between fertilization and plant species, and between time and plant species, respectively (Table A. 6). The temporal change was greater than the effect of fertilization, plant species or their interactions (comparison between F values) (Table A. 6).

The abundance of AOB was higher in the plots with synthetic fertilizer than in those with organic fertilizer only in September 2014 ($P < 0.05$, Table A. 4). Differences between plant species treatments and from fertilizer by plant species interactions were detected mainly at the first time point in June 2014, with the abundances of *nirK*, *nirS*, *nosZ*I, *nosZ*II and *nrfA* being lower in the pure red clover plots than in the mixture and pure timothy plots, especially when no fertilizer was applied ($P < 0.05$, Table A. 4, Figure A. 3). The absolute abundances of AOB, *nirS* and *nosZ*II differed depending on plant species at several time points (Table A. 7).

3.2. Correlations between genetic capacity for N cycling and soil properties, and between crop yield and microbial communities

The correlations between the gene abundances and soil properties at each time point varied over time (Figure A. 4). However, the intra-plot correlations revealed that *nirS* and *nosZ*II abundances correlated negatively with soil NO_3^- content and positively with soil moisture and total

carbon content ($P < 0.05$, Table 2). The abundance of *nrfA* correlated negatively with soil NH_4^+ content and C/N ratio. The abundances of *nirK* and *nrfA* correlated with the overall microbial community diversity, whereas those of AOB, *nirS* and *nosZ*II along with the *nrfA/nir* ratio correlated with PC1, used as a variable indicating changes in microbial community composition. Crop yield correlated negatively with *nirS* and *nosZ*II and positively with *nrfA/nir* ratio. In non-fertilized treatments, *nirK* abundance correlated with AOB abundance in all planted plots (Table A. 8).

The overall N-cycling communities changed over time (PERMANOVA, $F_{(3,79)} = 9.92$, $P < 0.01$) and these temporal changes correlated with changes in soil moisture, total carbon content, and C/N ratio ($P < 0.05$, Table 2). However, there were no significant differences between September 2014 and September 2015 (pairwise PERMANOVA, $P > 0.05$) (Fig. 3). At individual time points, the overall N-cycling communities did not differ between fertilizer or plant species treatment (Figure A. 5). The microbial community composition correlated with the overall N-cycling communities only in June 2014 ($P < 0.01$ Table 3). In addition, the soil properties indirectly correlated with the overall N-cycling communities through the microbial communities at all time points ($P < 0.05$, Table 3).

3.3. Microbial communities play an important role in mediating grassland functioning

The structural equation model showed that both microbial diversity and composition mediated the management effects on crop yield, potential N retention and N_2O sink capacity, in line with our first hypothesis (Fig. 4). Along with soil moisture, management affected the grassland functioning in terms of crop yield and N-cycling capacity, either directly or indirectly through changing soil pH and NO_3^- content or through the mediation of the soil microbial communities (Fig. 4). The effect of microbial diversity on crop yield varied among fertilizer and plant species treatments, with positive effects being found in the synthetic fertilizer plots of both mixture and timothy (Fig. 4A, Table A. 9A). Crop yield was directly affected by management practices and by soil moisture and NO_3^- content. Soil moisture played a more important role in the plots with synthetic fertilizer than in those with organic fertilizer or without fertilizer (Fig. 4A, Table A. 9A). The microbial diversity was affected by management mainly through soil pH and NO_3^- content, with both of these effects varying among fertilizer treatments, whereas the community composition was mainly affected by soil moisture (Fig. 4, Table A. 9). Soil NO_3^- content affected the potential for N retention indirectly through microbial diversity (Fig. 4). However, N retention did not directly affect crop yield, which negates our second hypothesis (Fig. 4A), rather it was associated with reduced potential N_2O sink capacity (Fig. 4B).

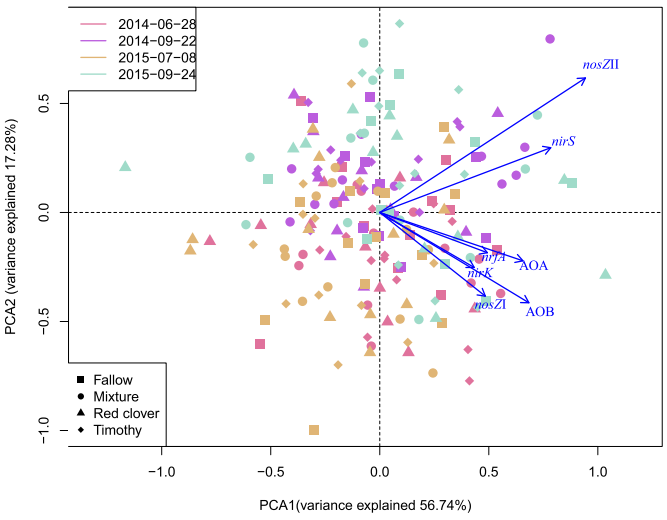

Fig. 3. Principal component analysis (PCA) of the overall N-cycling communities across all treatments and sampling points based on the abundance of each functional group and using Euclidean distance. AOA, ammonia oxidizing archaea; AOB, ammonia oxidizing bacteria; *nrfA*, the gene encoding nitrite reductase in bacteria performing dissimilatory nitrate reduction to ammonium; *nirK*, nitrite reductase (copper based) in denitrifying bacteria; *nirS*, nitrite reductase (cytochrome-cd1) in denitrifying bacteria; *nosZ* I/II, nitrous oxide reductase clade I and II in nitrous oxide-reducing bacteria and archaea. The arrows indicate the species scores of each functional gene that were fitted into the ordination.

4. Discussion

4.1. The abundance of N-cycling communities mainly changed over time

Using a three-year field experiment, we quantified six genes (*amoA*, *nirK*, *nirS*, *nosZ*I, *nosZ*II and *nrfA*) encoding key N-cycling enzymes to track how grassland species and fertilization affect microbial communities and their capacity for soil N-cycling, and how such changes may affect crop yield. Temporal differences in the abundances of the functional groups with genetic potential for cycling of N were more pronounced than management effects in the short-term grasslands. Nevertheless, fertilization affected groups performing the first step of nitrification, the AOB and AOA, which typically respond positively to N input [38–40]. In contrast to the main effect on AOB, the effect on AOA was linked to the increased abundance from summer to autumn, especially noticeable in the plots with high input of synthetic fertilizer in 2015. This coincided with a decrease in soil NH_4^+content [23]. The increased capacity for ammonia oxidation performed by AOA could

Table 2
Intra-correlations (repeated measures correlations) of the gene abundances relative to 16S rRNA gene, soil properties, microbial community diversity and composition, overall N-cycling communities and crop yield. Values in bold indicate $P < 0.05$.

	AOA	AOB	*nirK*	*nirS*	*nosZ*I	*nosZ*II	*nrfA*	*nrfA/nir*	PC1 N-genes
NO_3^-	0.13	0.06	−0.03	**−0.25**	0.12	**−0.18**	−0.02	**0.19**	−0.16
NH_4^+	0.16	−0.03	−0.11	**−0.22**	−0.10	−0.10	**−0.21**	−0.01	−0.15
pH	0.05	−0.03	−0.09	0.06	−0.05	0.03	−0.15	**−0.19**	0.03
EC	−0.03	−0.04	−0.01	**−0.18**	−0.14	−0.02	−0.13	0.02	−0.03
Moisture	0.10	0.03	0.03	**0.30**	**0.25**	**0.19**	0.10	−0.15	**0.20**
TC	0.21	−0.09	−0.16	**0.30**	−0.13	**0.34**	−0.24	**−0.40**	**0.33**
TN	−0.07	0.02	−0.02	0.16	0.02	0.06	0.13	0.03	0.15
C/N	0.17	−0.15	−0.21	0.22	−0.20	**0.41**	**−0.44**	**−0.54**	**0.30**
Shannon	0.13	−0.01	**−0.17**	−0.13	−0.01	−0.11	**−0.27**	−0.13	−0.08
PC1	0.10	**0.22**	0.04	**−0.51**	0.10	**−0.44**	−0.01	**0.44**	**−0.35**
Crop yield	0.16	0.18	0.06	**−0.25**	0.15	**−0.35**	0.12	**0.36**	**−0.33**

NO_3^-, NH_4^+, pH, EC, Moisture, Shannon (microbial diversity), PC1 (first principal component of microbial community composition/N-cycling communities), n = 189; TC (total carbon content), TN (total nitrogen content), C/N ratio (total carbon to total nitrogen ratio), n = 94; Crop yield, n = 140; Data was transformed whenever necessary to satisfy the assumptions of the linear model.

Table 3
Mantel's test for dissimilarity matrices of soil properties, microbial community composition and overall N-cycling communities. Values in bold indicate $P < 0.05$.

Mantel test[a]	June 2014	Sept. 2014	July 2015	Sept. 2015
Soil: microbial community composition	$R =$ 0.45	$R =$ 0.29	$R =$ 0.23	$R =$ 0.35
	$\boldsymbol{P} =$ **0.001**	$\boldsymbol{P} =$ **0.002**	$\boldsymbol{P} =$ **0.002**	$\boldsymbol{P} =$ **0.002**
Soil: overall N-cycling communities	$R =$ 0.11	$R =$ −0.13	$R =$ 0.10	$R =$ 0.09
	$P =$ 0.08	$P = 0.93$	$P =$ 0.15	$P =$ 0.14
Microbial community composition: N-cycling communities	$R =$ 0.25	$R =$ 0.02	$R =$ 0.02	$R =$ 0.05
	$\boldsymbol{P} =$ **0.002**	$P = 0.42$	$P =$ 0.37	$P =$ 0.31
Partial Mantel with all three factors	$R =$ 0.44	$R =$ 0.30	$R =$ 0.23	$R =$ 0.35
	$\boldsymbol{P} =$ **0.001**	$\boldsymbol{P} =$ **0.001**	$\boldsymbol{P} =$ **0.004**	$\boldsymbol{P} =$ **0.001**

[a] The dissimilarity matrices of soil properties, microbial community composition and N-cycling communities were calculated based on Euclidean distance, Bray-Curtis distance and Euclidean distance, respectively.

either indicate their role in ammonia oxidation at the site or a niche preference of AOA, since many AOA prefer low soil NH_4^+ content [41,42]. Nevertheless, AOA abundance and soil NH_4^+ content were not correlated. Despite the differences in the composition of the microbial communities between the fallow and planted plots and between the organic fertilizer plots and synthetic fertilizer plots [20], the abundances of N cycling genes did not respond to the treatments, possibly reflecting functional redundancy. In microbial communities, different taxa may carry the same functional genes and play a redundant role in the functioning of the community. Thus, a shift in taxonomic composition (e.g., loss or gain of taxa) does not necessarily alter the functionality of communities [21]. Similarly, in a Japanese forest slope, the taxonomic composition of ammonia oxidizers changed across ridge and valley without an accompanied change in the abundance of ammonia oxidizers [43], and the pattern of functional communities was conserved despite changes in the taxonomic composition in both a temperate pasture and a subtropical rice farmland [14,44].

The main treatment effect we detected was that of fertilization on the relative abundance of AOB. This is possibly linked to the changes in bacterial community composition caused by fertilization, especially to the changes in relative abundances of *Proteobacteria* OTUs [20]. Of the targeted functional groups, AOB is the most phylogenetically constrained, and this result emphasizes that not all functions are equally redundant. Less redundant functional communities can thus be more easily be affected by changing conditions and loss of diversity [45,46]. Among the denitrifiers, there is yet another level of functional redundancy since similar functions can be supported by different functional groups [10]. The observed decrease in soil NO_3^- content from summer to autumn was associated with an increased abundance of *nirS*-type denitrifiers and *nosZ*II nitrous oxide reducers, whereas their functional counterparts *nirK* and *nosZ*I were less responsive to changes in soil properties. This is consistent with previous studies and supports niche differentiation of *nirK* from *nirS* and of *nosZ*I from *nosZ*II [47–50].

Even though fertilization and plant species did not affect the abundances of most of the N- cycling genes, the observed treatment effects at single time points suggest transient priming effects of previous conditions that promoted specific N-cycling groups, according to the "hot moments" concept for microbial processes [51]. This is exemplified by the higher soil NH_4^+ content in the synthetic than in the organic fertilizer plots in August 2014 [23], which may have caused the difference in AOB abundances between organic and synthetic fertilizer plots. Another example is the lower abundance of *nirK*, *nirS*, *nosZ*I, *nosZ*II and *nrfA* communities in pure red clover plots than in mixture and pure timothy plots in June 2014, especially when no N fertilizer was applied. A similar trend was observed in the soil-associated N-cycling communities in an alfalfa – cocksfoot intercropping experiment [17]. At our site, plant growth is usually fastest in June, so the competition between plants and microorganisms for reactive N would have been at its most intense, particularly when no N fertilizer was provided. This agrees with the use of relative plant growth rate as a predictor for the denitrification process [52]. Considering that soil NH_4^+ and NO_3^- levels are affected by plant growth and development stage, organic matter mineralization and microbial processes as well as fertilization, sampling time is important to consider when seeking to identify hot moments in N-cycling communities.

4.2. *The influence of microbial diversity on crop yield varied among treatments*

With the growing interest in incorporating the role of the microbial communities into ecosystem process models [53–56], we evaluated the direct and indirect effects of management on crop yield, genetic potentials for N retention and mitigation of N_2O emission via bacterial community by using structural equation modelling. As initially hypothesized, soil bacterial communities were involved in mediating the

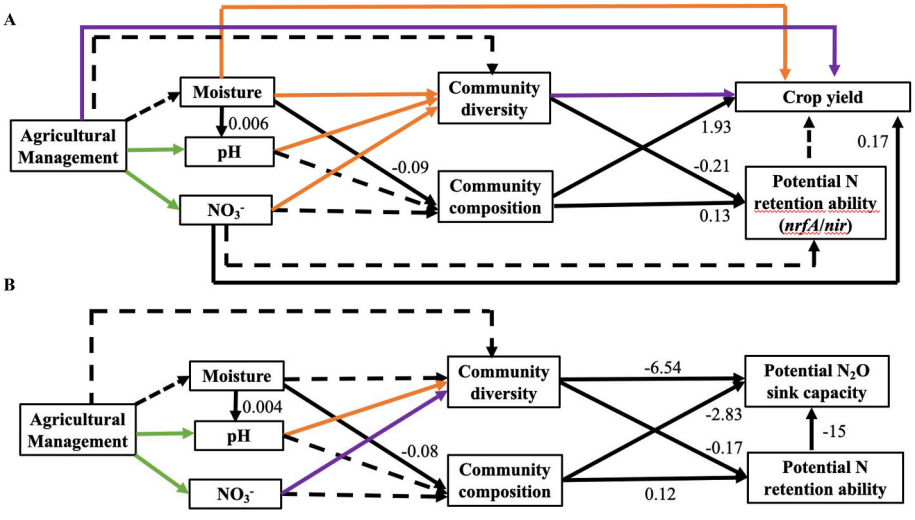

Fig. 4. Structural equation models showing the direct and indirect effects of management on soil properties, microbial community composition and diversity, and grassland functioning in terms of A) crop yield and potential N retention ability (*nrfA/nir*) and B) potential N retention ability and N_2O sink capacity (relative *nosZ* abundance). The lines indicate the relationships, with solid lines $P < 0.05$, and dashed lines $P > 0.05$. The numbers next to the lines are the unstandardized coefficients (absolute effect in terms of the changes per unit by the caused variable). Coefficients affected by fertilizer treatment or plant species treatment are indicated with green lines, by fertilizer treatment with orange lines, by the interaction of fertilizer and plant species treatment with purple lines, and across all treatments with black lines. All the coefficients that varied between treatments in models A and B are in Table A. 9. Results of dSep tests are in Tables A. 10. The à priori models of A and B were presented in Figure A. 6. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

management effects on the specific ecosystem functions. The influence of microbial diversity on crop yield, however, varied among fertilizer and plant species treatments, likely due to the complex competition between crops and microorganisms for available N, which in turn depends on several factors [57]. Despite the potential functional redundancy for N cycling at the microbial community level, the management effects on potential N retention and N_2O sink capacity were largely mediated by the soil microbial communities. This is in line with the finding that change in N-cycling processes follows the loss or change in bacterial diversity or composition [48]. In our model, the mediation of agroecosystem functioning by microbial diversity was mainly affected by the changes in soil pH and NO_3^- content, while the mediation by microbial community composition was mainly affected by soil moisture. These soil properties as well as carbon content and soil structure are often mentioned as the main edaphic drivers of soil microbial diversity and community composition [58–61]. As expected, the potential for soil N retention correlated positively with crop yield, but no direct effect was identified in our model. Instead, these ecosystem services were driven by similar factors. Additionally, we observed a trade-off between N_2O sink and N retention capacity. This suggests that although there is potential for managing agroecosystems towards reducing either N loss or N_2O emission by moderating the major soil properties, it remains challenging to promote both ecosystem functions at once.

5. Conclusion

We determined how management practices in grasslands in a sub-boreal region affected genetic potential for N cycling that underpins important agroecosystem processes and crop yield. Temporal changes were pronounced, whereas only limited treatment effects on the capacity for different N-cycling processes were found. Our results suggest the presence of functional redundancy in the microbial community, particularly for functions that are phylogenetically spread. Moreover, our study suggested that single time measurement of functional gene abundance is insufficient for making reliable conclusions and disclosing the mechanisms. Management could indirectly affect important N-cycling functions as microbial community diversity and composition played important roles in mediating the management effects on crop yield and N-cycling. However, trade-offs between N retention and N_2O sink were detected, indicating the challenges in managing grasslands in a sustainable way.

Author contributions

HL performed the lab work under the supervision of JJ. HL did the statistical analysis. All authors contributed to interpret the results, write and revise the manuscript critically. All authors agreed with the final version of the manuscript.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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