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Diversity–function relationship of ammonia-oxidizing bacteria in soils among functional groups of grassland species under climate warming

S. Malchair [a,*], H.J. De Boeck [b], C.M.H.M. Lemmens [b], R. Ceulemans [b], R. Merckx [c], I. Nijs [b], M. Carnol [a]

[a] Laboratory of Plant and Microbial Ecology, University of Liège, Institute of Botany B22, Boulevard du Rectorat 27, 4000 Liège, Belgium
[b] Research Group of Plant and Vegetation Ecology, Department of Biology, Universiteit Antwerpen (Campus Drie Eiken), Universiteitsplein 1, 2610 Wilrijk, Belgium
[c] Division Soil and Water Management, Department Earth and Environmental Sciences, Katholieke Universiteit Leuven, Kasteelpark Arenberg 20-bus 2459, 3001 Leuven/Heverlee, Belgium

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ABSTRACT

Although warming and plant diversity losses have important effects on aboveground ecosystem functioning, their belowground effects remain largely unknown. We studied the impact of a 3 °C warming and of three plant functional groups (forbs, grasses, legumes) on ammonia-oxidizing bacteria (AOB) diversity (polymerase chain reaction-denaturing gradient gel electrophoresis, PCR-DGGE) and their function (potential nitrification) in artificial grasslands. Warming did not influence AOB diversity and function. Sequencing of 16S rRNA gene fragments retrieved from DGGE gel revealed that they were all related to *Nitrosospira*-like sequences. Clustering analysis of DGGE profiles resulted in two nodes, separating AOB community structure under legumes from all other samples. Decreased AOB richness (number of DGGE bands) and concurrent increased potential nitrification were also observed under legumes. We hypothesized that ammonium availability was the driving force regulating the link between aboveground and belowground communities, as well as the AOB diversity and function link. The results document that the physiology of AOB might be an important regulator of AOB community structure and function under plant functional groups. This study highlights the major role of the microbial community composition in soil process responses to changes in the functional composition of plant communities.

1. Introduction

While Charles Darwin hypothesized already in 1859 that plant diversity would influence ecosystem productivity, interest in the diversity–function relationship has only grown substantially in the last few decades, since ecosystems are now facing important environmental changes such as global warming and biodiversity losses. These environmental changes are known to affect aboveground ecosystem functioning (Tilman et al., 2001; De Boeck et al., 2008) and numerous studies have explored the link between aboveground diversity and function (Naeem et al., 1994; Tilman et al., 2001). Previous studies have revealed a positive impact of higher biodiversity on resource use (Tilman et al., 2001), primary productivity (Karaveira, 1996), resistance against environmental disturbances and resilience (Tilman and Downing, 1994). However, other studies have shown no effect of aboveground biodiversity on primary productivity (Hooper and Vitousek, 1997) and no impact of biodiversity on resistance against disturbances (De Boeck et al., 2008). Although microorganisms mediate most soil processes, belowground functioning has often been studied without explicit reference to the microbial populations involved (Balser et al., 2001). With advances in genetic, molecular-based techniques, recent evidence is accumulating that microbial community composition may affect ecosystem functioning (Carney et al., 2004; Hawkes et al., 2005). In studying the role of microbial community composition on ecosystem functioning, Schimel (1995) suggested that more attention should be paid to the 'broadness' of the physiological group of microorganisms carrying out the processes, as microorganisms present greater functional redundancy than macroorganisms. 'Broad' processes are those performed by many redundant microbial populations, whereas 'narrow' processes are carried out by a restricted group of microorganisms. This concept is close to the classification by 'sensitivity groups', corresponding to the sensitivity with respect to losses of organisms or functions (Domsch et al., 1983). According to these theories, loss of species involved in narrow or sensitive processes would have more important consequences for ecosystem functioning than the loss of some species within a group of numerous functionally redundant species (Schimel, 1995). Within the microbial diversity–function research, the study of 'narrow'/'sensitive' processes may therefore be most relevant.

* Corresponding author. Tel.: +32 4 3663817; fax: +32 4 3663817.
E-mail address: S.Malchair@ulg.ac.be (S. Malchair).

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We investigated the effects of warming and of plant functional groups on the nitrogen cycle and specifically on the nitrification process, which can be considered as a narrow process of high sensitivity (Domsch et al., 1983; Schimel, 1995). This key process also influences primary production and controls ecosystem nitrogen retention (Rotthauwe et al., 1997). The first, limiting step of nitrification is carried out by ammonia-oxidizing bacteria (AOB). All AOB enriched or isolated from soils to date belong to the β-*Proteobacteria* (Stephen et al., 1996). Phylogenetic analysis of 16S rDNA genes amplified from environmental samples by PCR with AOB-specific primers indicated the existence of at least ten clusters, five belonging to the genus *Nitrosospira* and five belonging to the genus *Nitrosomonas* (Koops et al., 2003). Recently, some studies (e.g. Leininger et al., 2006; Shen et al., 2008) suggested an important role of non-thermophilic *Crenarchaea* in soil ammonia oxidation due to their high abundances. However, the presence or high abundance of a functional gene does not mean that the function is operating (Prosser and Nicol, 2008). Furthermore, although crenarchaeal cell numbers and transcripts dominated over their bacterial counterparts, the pattern of changing ammonia oxidizer bacterial gene copies and transcriptional levels correlated more closely with measured nitrification rates (Nicol et al., 2008).

Changes in microbial communities in response to environmental constraints will depend on the capacity of the member populations to adapt physiologically (Schimel et al., 2007). The selective pressure may lead to competitive advantage/disadvantage of some populations, resulting in turn in a change of the community structure. Thus, community composition can shift towards organisms better suited to new environmental conditions in response to environmental stress (Balser et al., 2001). Currently, information about the time required for community shifts and whether they would affect the functioning of ecosystems is scarce.

In changing environments, microorganisms are faced with stress resulting from a change in environmental factors not directly necessary for growth (i.e. temperature) and stress resulting from a difference in resource availability (Balser et al., 2001). In our study, warming may affect AOB community structure directly through effects on environmental factors such as moisture or temperature, and indirectly through increased plant production and hence substrate availability for microbial growth (Anderson, 1992). In longer term, another effect of warming on soil microbial community can occur indirectly via a shift in the functional composition of vegetation (Wardle et al., 1998) as climate change is likely to alter species distribution and biodiversity (Davis et al., 1998). As plant functional groups vary in resource acquisition mode (Davies et al., 2007) and as species differ in quality and quantity of their root exudates, litter, dead roots and shoots (Wardle, 2002), they can also affect AOB through their impact on resource availability. Plants can also alter environmental factors like soil pH and moisture, which have recently been shown to alter AOB community structure (Gleeson et al., 2008; Nicol et al., 2008). Adaptation of microbial communities to altered environmental factors and resources regimes may condition their response to perturbations (Balser et al., 2001).

The aims of this study were to investigate (i) the impact of 3 °C warming and three plant functional groups (forbs, grasses, legumes) on AOB diversity (polymerase chain reaction-denaturing gradient gel electrophoresis, PCR-DGGE) and function (potential nitrification), and (ii) the link between AOB diversity and function, in synthesized grasslands. To address these questions, potential nitrification, AOB richness and AOB community structure were characterized under monocultures of forbs, grasses, legumes and in bare soils in unheated and in heated chambers.

2. Materials and methods

2.1. Experimental setup

An experimental platform was established in July 2003 at the Drie Eiken Campus of the University of Antwerp (Belgium, 51°09′N, 04°24′E). The platform consisted of 12 sunlit, climate-controlled chambers (2.25 m^2 ground area) facing south. Half of the chambers were exposed to ambient air temperature (unheated chambers) and half were continuously warmed 3 °C above fluctuating ambient values (heated chambers). Annual mean temperature was 9.6 °C, and mean monthly temperature ranged between 2.2 °C (January) and 17.0 °C (July). Each chamber contained bare soils ($n = 6$) and grassland monocultures ($S = 1$, $n = 9$). The monocultures were established using nine grassland species from three functional groups: grasses (*Dactylis glomerata* L., *Festuca arundinacea* Schreb., *Lolium perenne* L.), legumes (*Trifolium repens* L., *Medicago sativa* L., *Lotus corniculatus* L.), and forbs (*Bellis perennis* L., *Rumex acetosa* L., *Plantago lanceolata* L.). The species were chosen based on their presence in temperate grasslands, perennial life cycle, preference for loam or clay soil and known differences in productivity, temperature and drought resistance. Each monoculture was grown in containers (PVC containers of 24 cm diameter by 60 cm height), filled with sieved, thoroughly mixed soil collected from a 25-year-old corn field in March 2003 and placed at level with the surrounding soil. The soil consisted of 76.3% silt, 14.8% clay and 8.7% sand. Initial soil characteristics were (means ± SE): pH_{H_2O} 6.44 ± 0.08, total C 1.60% ± 0.18, total N 0.15% ± 0.01; the exchange complex was largely dominated by Ca^{2+} (85%), followed by Mg^{2+} (8%) and K^+ (4%). Annual precipitation averaged 776 mm and was distributed evenly throughout the year. The amount of rainfall administrated to the chambers was calculated weekly from the difference between the soil moisture inside and outside the unheated chamber, to create the same soil water content (SWC). From June 2004 onwards, irrigation was based on precipitation data from a nearby weather station (Lint, Belgium) rather than on SWC, and the precipitation data were corrected for the artificial enhancement of evapotranspiration in the chambers. Both heated and unheated chambers received the same amount of water, so lower soil moisture levels were recorded in heated communities (De Boeck et al., 2006). Weeding was performed manually throughout the experiment. Further details on experimental set-up, growth conditions, climate and water supply can be found in Lemmens et al. (2006) and De Boeck et al. (2008).

2.2. Soil sampling

Soils were sampled from containers taken at 4 and 16 months after the beginning of the experiment (in November 2003 and 2004, respectively). At each sampling, containers from two chambers (one unheated and one heated) were destructively harvested. Soils under monocultures ($S = 1$) of forbs, grasses, legumes and bare soils were sampled and separated into two depths: upper (U) 1–5 cm, lower (L) 6–10 cm. We collected bulk soil, which we defined as soil not adhering to roots. Roots, present through the entire profile (De Boeck et al., 2008), were removed manually. The soil was sieved (4 mm mesh) on flame-sterilized sieves and homogenised. A subsample of the soil was immediately freeze-dried for molecular analysis and the remaining soil was stored at 4 °C until analysis (less than 1 week). Unpublished data had shown no effect of the storage under these conditions on the nitrification potential.

2.3. Soil characteristics

The following soil characteristics were determined: extractable NH_4^+-N, pH_{H_2O} and pH_{KCl}. For extractable NH_4^+-N, 20 g of field

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moist soil were shaken for 1 h with 100 ml KCl 1 M (Allen, 1986). The extract was filtered (595 1/2, Schleicher & Schuell, Dassel, Germany) and the filtrate was analyzed colorimetrically for NH_4^+-N with an autoanalyser (Bran-Luebbe, SPX Process Equipment, Norderstedt, Germany). Soil pH_{H_2O} and pH_{KCl} were measured on moist soil in distilled water or KCl 1 M, respectively, using a 1/1 (v/v) soil: liquid ratio.

2.4. Nitrification potential

We determined the nitrification potential, using the shaken soil slurry method (Hart et al., 1994). Briefly, 10 g of fresh soil was shaken in 100 ml of a solution (pH 7.2) containing 1 mM PO_4^{3-} and 15 mM NH_4^+ at 25 °C. These experimental conditions (moisture, pH, oxygen, ammonium and phosphate availability) assure a maximum production rate of nitrate in the absence of N immobilization and denitrification. We took 15 ml subsamples of the slurry at 2, 6, 22, 26 and 30 h after the start of the incubation. The subsamples were filtered (595 1/2, Schleicher & Schuell, Dassel, Germany) and the filtrate was analyzed colorimetrically for NO_3^--N with an autoanalyser (Bran-Luebbe, SPX Process Equipment, Norderstedt, Germany). Nitrification rates were calculated by linear regression of nitrate concentrations over time ($\mu g\ N\ g^{-1}\ d^{-1}$).

2.5. Microbial community analysis

2.5.1. Genomic DNA extraction and PCR amplification

Genomic DNA was extracted from 0.2 g freeze-dried soil using the UltraClean Soil DNA kit (MoBio, Solana Beach, CA, USA), according to manufacturer's instructions with a minor modification. 200 μl $AlNH_4(SO_4)_2$ 100 mM were added before the first step of lyses to remove soil-based inhibitors through chemical flocculation (Braid et al., 2003).

A region of the small subunit (16S) ribosomal gene spanning the V2–V3 variable domains was amplified using polymerase chain reaction (PCR) and the ammonia oxidizer specific primer set: GC-clamped CTO 189f-ABC/CTO 654r (Kowalchuk et al., 1997). The PCR reaction mixture (final volume of 25 μl) consisted of 1 μl genomic DNA extract, 21 pmol of each primer (Invitrogen, Merelbeke, Belgium), 15 nmol dNTPs (Bioline Ltd, London, UK), 2.5 μg bovine serum albumine (Fluka Analytical, St Gallen, Switzerland), 2.5 μl of Accubuffer 10× and 1.25 U Accuzyme polymerase (Bioline, Luckenwalde, Germany). Amplification was performed using an initial denaturation step at 94 °C (2 min), 35 cycles of 92 °C (30 s), 59 °C (1 min), 72 °C (45 s + 1 s $cycle^{-1}$) and a final extension at 72 °C (5 min).

2.5.2. Analysis of PCR products by denaturing gradient gel electrophoresis (DGGE)

DGGE was performed with the DCode universal mutation detection system (BioRad, Hercules, CA, USA). The DGGE gel was performed using a 6% acrylamide gel with a 30–60% denaturant gradient, where 100% denaturant was defined as 7 mM urea plus 40% formamide. The fragments were separated under electrophoresis at a constant temperature of 60 °C for 10 min at 200 V followed by 16 h at 80 V. The gels were stained for 30 min with SybrGold (Molecular Probes, Leiden, The Netherlands) before visualization by a CCD camera under a blue-light transilluminator (Dark Reader, Clare Chemical, Ross on Wye, UK).

2.5.3. Gel normalization and analysis

DGGE patterns were analyzed and compared using GelCompar II (Applied Maths, Kortrijk, Belgium). Samples were run alongside reference markers using cluster controls derived from clones of known sequences (Stephen et al., 1996; Kowalchuk et al., 1997). The reference markers were used as a standard during gel normalization and analysis, to ensure gel-to-gel comparability. The total number of bands ('band richness') in each sample pattern is an indicator of the number of dominant phylotypes, and consequently of AOB species richness. Differences in AOB community structure between samples were assessed using hierarchical clustering analysis, joining similar profiles into groups (Fromin et al., 2002). Similarity matrices were generated from pairwise comparison of banding patterns (presence/absence of bands) of all samples, using the Dice Coefficient (C_D) as follows: $C_D = 2j/(a + b)$, where j = number of bands in common between lanes A and B, a = the total number of bands in lane A, b = the total number of bands in lane B. Samples generating similar banding patterns were clustered by means of the unweighted pair-group method with arithmetic averages (UPGMA), resulting in the construction of dendrograms.

2.5.4. DNA sequencing and data analysis

The middle portion of bands selected for sequencing was excised from the DGGE gel and slices were left to diffuse passively for 24 h at 4 °C in 25 μl TE pH 8 (Bollmann and Laanbroek, 2001). 1 μl of buffer containing the recovered DNA was used as template for PCR amplification under the same conditions as described above. The re-amplification products were evaluated by DGGE to verify purity before sequencing (Genomex, Meylan, France). We used GenBank BLAST (http://www.ncbi.nlm.nih/gov/blast/) to search for best matches with the obtained rDNA sequences retrieved from the DGGE profiles. These sequences have been deposited in GenBank under the accession numbers EU327537–EU327546 and FJ792833–FJ792837.

2.6. Statistical analysis

We used three-way analysis of variance for unbalanced data (general model procedure, GLM) to test the effects of (i) plant functional group (legumes, grasses, forbs) and bare soil, (ii) warming (unheated, heated) and (iii) depth (upper, lower) on potential nitrification, soil characteristics and band richness, at both times of sampling separately. When interactions between the independent variables were significant, two-way or one-way analysis of variance for separate variables were applied (Cody and Smith, 1991). One-way analysis of variance was used to test the effect of time of sampling on band richness. *Post Hoc* Tukey tests were performed to separate multiple means ($P < 0.05$). Relationships between nitrification potential, band richness and/or soil characteristics were analyzed using Pearson's correlation coefficient. All analyses were performed using SAS 9.1. (Sas Institute Inc., Cary, USA).

3. Results

3.1. Influence of plant functional groups and warming on AOB richness

Soil AOB richness (band richness) was not significantly affected by plant functional group but was influenced by the presence of plants (Tables 1 and 2). Four months after sampling, there was no significant interaction between sampling time and depth (Table 1), and data were analyzed by two-way ANOVA. At this sampling, AOB richness was not significantly different under the legumes, forbs and grasses (data not shown). However, it appeared that bare soils were more diverse (band richness = 12) than soils under grasses (band richness = 9) ($P = 0.01$). This difference was only significant for the upper depth when analyzed by separate one-way ANOVA for each depth (Table 2). After 16 months, due to a significant interaction term between functional group and soil depth,

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Table 1
Effects of plant functional group (FG), warming treatment (T°) and soil depth on AOB richness (DGGE bands), potential nitrification, pH_{H_2O}, pH_{KCl} and extractable ammonium (three-way ANOVA).

Time after planting		Band richness		Potential nitrification ($\mu g\ N\text{-}NO_3\ g^{-1}\ h^{-1}$)		pH_{H_2O}		pH_{KCl}		Extractable NH_4^+ ($\mu g\ N\text{-}NH_4\ g^{-1}$)	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
4 months	FG	4.04	**0.0152**	2.59	0.069	6.02	**0.002**	10.29	<**0.0001**	–	–
	T°	1.32	0.2599	2.62	0.115	0.10	0.095	2.15	0.152	–	–
	Depth	0.82	0.4942	3.6	0.067	34.22	<**0.0001**	88.08	<**0.0001**	–	–
	FG × T°	0.47	0.4963	0.69	0.565	0.80	0.501	0.78	0.515	–	–
	FG × depth	0.54	0.6609	0.18	0.908	6.86	**0.001**	7.77	**0.0005**	–	–
	T° × depth	1.32	0.2599	0.92	0.346	0.28	0.602	0.02	0.895	–	–
	FG × T° × depth	0.47	0.7028	0.8	0.505	0.52	0.673	2.33	0.093	–	–
16 months	FG	9.90	<**0.0001**	8.47	**0.0003**	5.58	0.003	5.65	0.003	1.08	0.3707
	T°	1.25	0.2726	0.19	0.664	1.42	0.243	4.74	0.037	5.40	**0.0272**
	Depth	0.30	0.8233	7.93	**0.008**	12.54	0.001	39.09	<0.0001	89.28	<**0.0001**
	FG × T°	0.02	0.9021	1.63	0.203	0.85	0.477	0.39	0.764	1.20	0.3254
	FG × depth	3.34	**0.0314**	2.05	**0.008**	2.21	0.106	3.46	**0.028**	10.89	<**0.0001**
	T° × depth	0.38	0.5395	0.38	0.542	0.03	0.865	2.91	0.098	0.01	0.9668
	FG × T° × depth	0.67	0.5756	0.14	0.935	0.27	0.843	0.32	0.808	0.14	0.4372

one-way ANOVA was performed separately for each depth (Table 2). In the upper depth, AOB richness was significantly ($P < 0.0001$) higher in all planted containers than in bare soils. No significant differences appeared in the lower depth of the 16 months' sampling. Furthermore, band richness decreased significantly with time of experimentation in bare soils (from 12 to 5 bands, $P < 0.0001$), and in soils under legumes (from 10 to 7 bands, $P = 0.001$), forbs (from 11 to 6 bands, $P = 0.0004$) and grasses (from 9 to 7 bands, $P = 0.005$). In contrast, AOB richness was not influenced by warming whatever the time of sampling.

3.2. Influence of plant functional groups and warming on AOB community structure

The DGGE patterns revealed between 4 and 16 detectable bands per sample, situated in a mobility range of approximately 45–57% of denaturant and co-migrating with *Nitrosospira*-like clusters (Fig. 1). Multiple PCR reactions on the same soil DNA extract and PCR realised on different DNA extracts of the same soil yielded identical DGGE profiles (data not shown), suggesting a high reproducibility of the DNA extraction and of the PCR and DGGE procedures used. Furthermore, DGGE bands situated at the same gel position in different patterns were related to the same sequence (SM3 and SM14, SM2 and SM15 for example). All gene sequences retrieved from our DGGE profiles were related to sequences of AOB belonging to the class '*β-Proteobacteria*'. All sequences showed their best matches in the NCNI Genbank database with different uncultured strains from soils related to *Nitrosospira*-like sequences (minimum similarity level: 99%). Within the *Nitrosospira* lineage, we found sequences grouping into clusters 0 (SM4, SM6, SM12 and SM13), 3 (SM1, SM2, SM8, SM9, SM10, SM15 and SM16) and 4 (SM5) (Koops et al., 2003). We also found sequence related to *Nitrosopira* Nsp 65 (SM3, SM7 and SM14), forming an independent branch within the *Nitrosospira* lineage (Koops et al., 2003). No bands/sequences related to AOB clusters 1, 2, 5, 7 and 8 were detected (Fig. 1).

Community structure of AOB, as assessed by clustering analysis, revealed that soils from the lower depth under legumes (except *T. repens* in the unheated chamber) were distinctively grouped from all other samples, 4 months after the beginning of the experiment (Fig. 2a). AOB profiles from heated and unheated chambers, and from the upper and lower depth did not separate in the cluster analysis. The dendrogram generated from samples collected 16 months after planting (Fig. 2b) revealed two main nodes. AOB community structure in soils under legumes, for both depth and temperature treatments, was clearly distinct from all other samples. The second group was further branched, separating AOB community composition for samples under forbs (lower depth, heated) from those under bare soils and under all other forbs and grasses. Within this subgroup, soils under forbs and grasses from the same depth and temperature treatment clustered together. The shift in AOB community structure among the different samples was due to the addition and loss of specific bands related to *Nitrosospira* cluster 3 and *Nitrosopira* Nsp 65. Bands with the same DGGE mobility grouping into cluster 0 (SM4) and 4 (SM5) were present in all samples. Between the two sampling times, bands SM12 (cluster 0) disappeared from all samples. Bands SM7 (related to *Nitrosospira* Nsp 65) and SM8 (cluster 3) disappeared under grasses and forbs, whereas bands SM16 (cluster 3) disappeared under legumes and in bare soils.

Table 2
Effects of plant functional groups on AOB richness (DGGE bands) at each depth and at each time of sampling (one-way ANOVA).

Time after planting	Depth	Bare soils	Forbs	Grasses	Legumes
4 months	Upper	12 (0.6)a	11 (0.6)ab	9 (0.5)b	10 (0.5)ab
	Lower	12 (0.2)a	11 (0.5)a	9 (0.9)a	10 (0.7)a
16 months	Upper	5 (0.2)b	7 (0.6)a	8 (0.6)a	7 (0.2)a
	Lower	6 (0.1)a	8 (0.5)a	6 (0.7)a	7 (0.5)a

3.3. Influence of plant functional groups and warming on potential nitrification and soil characteristics

Potential nitrification, soil pH and extractable NH_4^+-N were not significantly influenced by 3 °C warming (Table 1). Therefore, data from both heated and unheated chambers were grouped for further analysis. After 4 months, the nitrification potential was not significantly affected by functional group (Fig. 3a), with mean values ranging between 0.43 and 0.56 $\mu g\ g^{-1}\ h^{-1}\ NO_3^-$-N. After 16 months, nitrification potential was significantly higher under legumes (1.13 $\mu g\ g^{-1}\ h^{-1}\ NO_3^-$-N) than under other functional groups (0.69 and 0.67 $\mu g\ g^{-1}\ h^{-1}\ NO_3^-$-N under forbs and grasses, respectively) in the upper depth ($P = 0.0007$). Nitrification potential in the bare soil (0.90 $\mu g\ g^{-1}\ h^{-1}\ NO_3^-$-N) was not significantly different from the other samples. The increased nitrification potential under legumes in the upper depth resulted in a significantly higher value, compared to the lower depth ($P = 0.0015$). Active (pH_{H_2O}) and exchangeable (pH_{KCl}) acidity were

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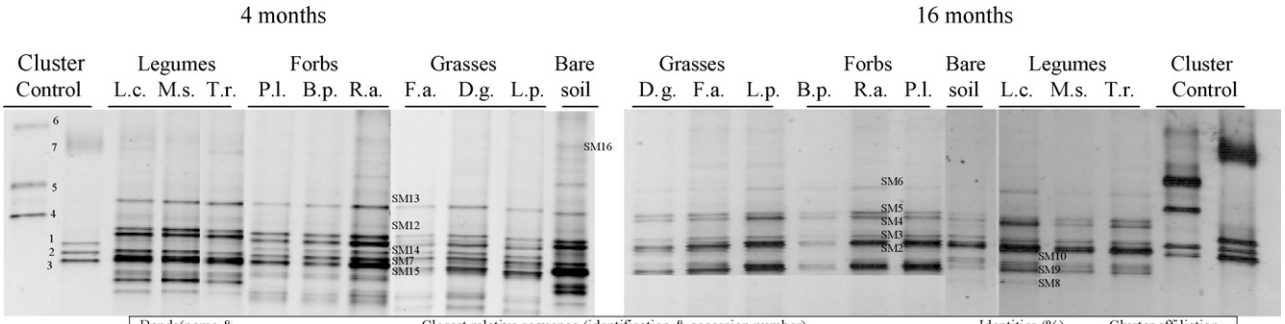

Bands(name & accession number)	Closest relative sequence (identification & accession number)	Identities (%)	Cluster affiliation
SM1 (EU327537)	Uncultured beta proteobacterium 16S rRNA gene, isolate L1-3 (AM410561)	404/405 (99%)	3
SM2 (EU327538)	Uncultured beta proteobacterium clone M82C8-F01 16S rRNA gene, partial sequence (EU070347)	394/394 (100%)	3
SM3 (EU327539)	Uncultured beta proteobacterium 16S rRNA gene, isolate L3-5 (AM410566)	403/403(100%)	-
SM4 (EU327540)	Uncultured beta proteobacterium 16S rRNA gene, clone L6 (AM230794)	465/465 (100%)	0
SM5 (EU327541)	Uncultured *Nitrosospira sp.* isolate DGGE gel band ORC2 16r RNA gene partial sequence (AY773202)	405/405 (100%)	4
SM6 (EU327542)	*Nitrosospira sp.* 16S rRNA gene, isolate D11 (X84660)	405/405 (100%)	0
SM7 (EU327543)	Nitrosospira sp. Nsp65 16S ribosomal RNA gene, partial sequence (AY123813)	334/337 (99%)	-
SM8 (EU327544)	*Nitrosospira sp.* 39-19 partial 16S rRNA gene, isolate 39-19 (AJ298725.1)	403/404 (99%)	3
SM9 (EU327545)	*Nitrospospira sp.* Nsp62 16S rRNA gene, partial sequence (AY123790.1)	402/404 (99%)	3
SM10 (EU327546)	Uncultured beta proteobacterium 16S rRNA gene, isolate L1-3 (AM410561)	404/406 (99%)	3
SM12 (FJ792833)	Nitrosospira sp. Nsp12 16S ribosomal RNA gene, partial sequence (AY123801)	346/346 (100%)	0
SM13 (FJ792834)	*Nitrosospira sp.* 16S rRNA gene, isolate D11 (X84660)	333/335 (99%)	0
SM14 (FJ792835)	Uncultured beta proteobacterium 16S rRNA gene, isolate L3-5 (AM410566)	406/406(100%)	–
SM15 (FJ792836)	Uncultured beta proteobacterium clone M82C8-F01 16S rRNA gene, partial sequence (EU070347)	394/394 (100%)	3
SM16 (FJ792837)	Nitrosospira sp. PM2 16S ribosomal RNA gene, partial sequence (AY856376)	341/341 (100%)	3

Fig. 1. Representative DGGE gel of samples from the upper soil depth in the heated chamber, position of excised bands and phylogenetic affiliations of sequences with environmental sequences of ammonia-oxidizing bacteria. P.l.: *Plantago lanceolata*; B.p.: *Bellis perennis*; R.a.: *Rumex acetosa*; D.g.: *Dactylis glomerata*; F.a.: *Festuca arundinacea*; L.p.: *Lolium perenne*; L.c.: *Lotus corniculatus*, M.s.: *Medicago sativa*; T.r.: *Trifolim repens*; Cluster control *Nitrosospira*-like cluster: cluster 1–4; *Nitrosomonas*-like cluster: cluster 5–7 (Stephen et al., 1996).

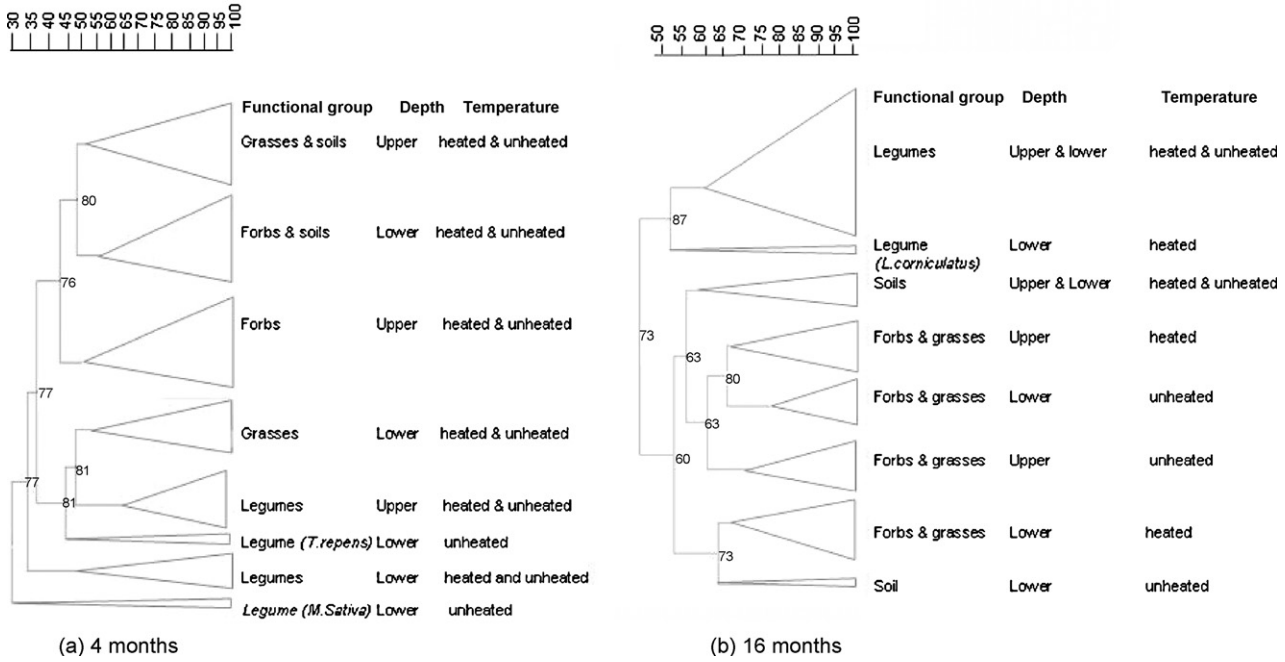

Fig. 2. UPGMA (unweighted pair-group method using arithmetic averages, based on Dice's similarity coefficient) dendrogram generated from AOB DGGE profiles obtained after 4 (a) or 16 (b) months. Treatments were functional groups (bare soils, grasses, forbs, legumes), soil depth (upper, lower) and a 3 °C increase in temperature (unheated, heated).

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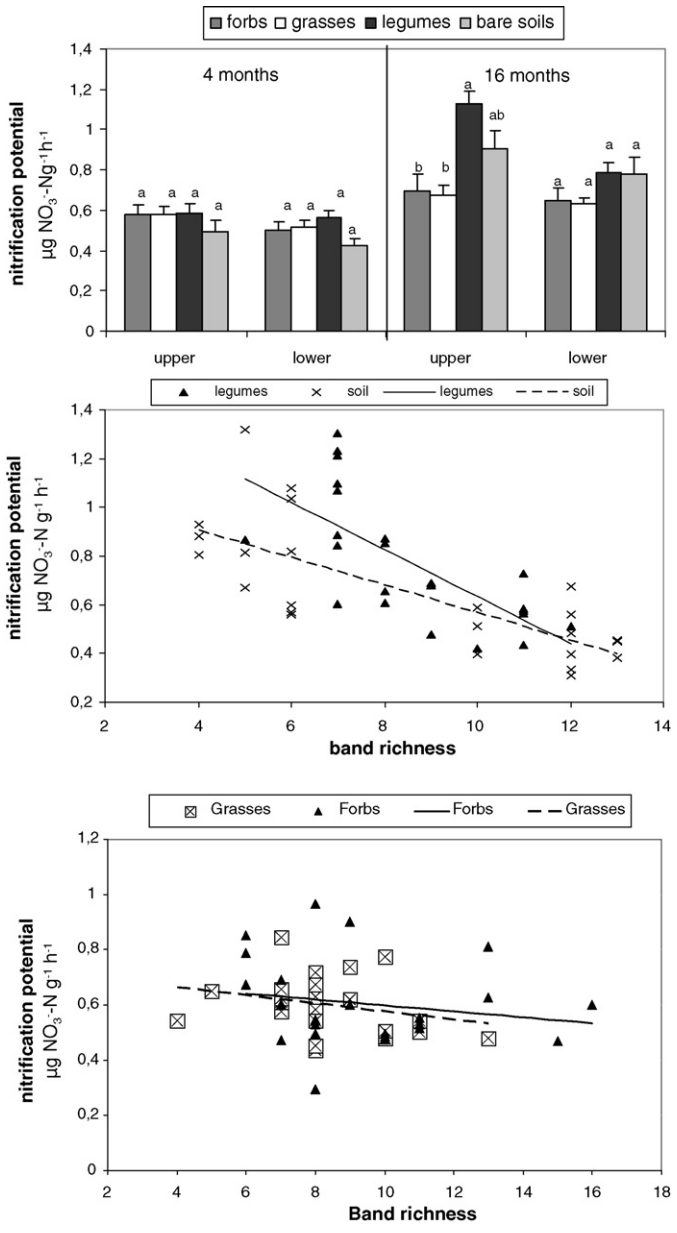

Fig. 3. (a) Mean (± standard error) potential nitrification rates (µg g^{-1} h^{-1}) at the upper and lower depths of bare soils ($n = 12$) and containers planted with forbs ($n = 6$), grasses ($n = 6$), legumes ($n = 6$) at each time of sampling separately. (b) Relationship between potential nitrification and AOB band richness of DGGE profile at both time of sampling and both depth for soils under forbs ($R^2 = 0.03$; $P = 0.38$) and soils under grasses ($R^2 = 0.07$; $P = 0.20$). (c) Relationship between potential nitrification and AOB band richness of DGGE profile at both time of sampling and both depth for bare soils ($R^2 = 0.56$; $P < 0.0001$) and soils under legumes ($R^2 = 0.47$; $P = 0.0002$).

significantly affected by functional groups, but differently according to soil depth (Table 1). After 4 months, pH_{H_2O} and pH_{KCl} in the upper depth, were increased by the presence of plants (pH_{H_2O} 7.3–7.5 (planted containers) vs 6.8 (bare soils); pH_{KCl} 6.4–6.7 (planted containers) vs 6.2 (bare soils)). The pH_{KCl} in the lower depth was lower under bare soils (pH_{KCl} 6.3) and legumes (pH_{KCl} 6.4) than under forbs (pH_{KCl} 6.6) and grasses (pH_{KCl} 6.7). After 16 months, soils under forbs (pH_{H_2O} 7.7–7.9) and grasses (pH_{H_2O} 7.8–8.1) were less acidic compared to bare soils (pH_{H_2O} 7.3–7.6), irrespective of depth. Across all samples, soil pH_{H_2O} and pH_{KCl} were significantly lower in the lower depth. In the upper depth, bare soils contained less extractable NH_4^+-N than planted containers (2.6 µg g^{-1} compared to 6.3–7 µg g^{-1}, $P = 0.002$), but in the lower depth, bare soils contained more NH_4^+-N than planted containers (2.7 µg g^{-1} compared to 0.1–0.5 µg g^{-1}, $P = 0.02$). Across all planted containers, NH_4^+-N was higher at the upper depth ($P < 0.0001$).

3.4. Correlation and AOB diversity–function relationship

Nitrification potential was negatively correlated to soil pH_{H_2O} ($R^2 = 0.15$, $P = 0.0001$) and to pH_{KCl} ($R^2 = 0.16$, $P < 0.0001$). AOB richness was positively correlated with soil pH_{H_2O} ($R^2 = 0.31$, $P < 0.0001$) and with pH_{KCl} ($R^2 = 0.19$, $P = 0.0001$). There was no significant correlation between AOB richness and nitrification potential for soil under forbs ($R^2 = 0.03$; $P = 0.38$) and under grasses ($R^2 = 0.07$; $P = 0.20$) (Fig. 3b). AOB richness was negatively correlated with nitrification potential for soil samples from containers planted with legumes ($R^2 = 0.47$; $P = 0.0002$) and for bare soils ($R^2 = 0.56$; $P < 0.0001$) (Fig. 3c).

4. Discussion

We obtained a higher number of AOB bands (up to 12) in our DGGE patterns compared to those generally reported in other studies (2–4 bands) across different soil types (Wheatley et al., 2003; Webster et al., 2005; Schmidt et al., 2007). Furthermore, although it has been reported that some non-AOB-like sequences have been recovered when using CTO primers (Mahmood et al., 2006), only AOB-like sequences were recovered in our study. The optimisations carried out in this study (DNA extraction, purification, and amplification) for avoiding biases due to poor quality (and quantity) of genomic DNA, preferential amplification, or the presence of PCR inhibitors (Niemi et al., 2001; Drenovsky et al., 2008) were necessary steps for obtaining reproducible and representative DGGE banding patterns of the main members of the AOB community.

DNA-based PCR-DGGE allows the recovery of most dominant bacterial DNA, but in contrary to RNA-based methods does not discriminate between active and dormant populations. However, the two methods generally show similar trends (Hoshino and Matsumoto, 2007). Although we cannot presume that all AOB sequences recovered reflect actively growing AOB populations, appearance or disappearance of AOB bands must be attributed to a change in the number of individuals of AOB phylotypes. This modification of bacterial growth resulted in numbers above or below the detection limit inherent to the method employed. The PCR-DGGE technique revealed to be a useful tool for studying the diversity–function relationship at the microbial level and presents a good compromise between cost, effort and sensitivity.

Our soils were characterized by sequences related to the *Nitrosospira* cluster only, which is in agreement with many other studies of soil AOB (Kowalchuk et al., 1997). The majority of DGGE bands were related to *Nitrosospira* cluster 3, which has been reported to be characteristic of soil amended with inorganic N (Wheatley et al., 2003) and of soil with neutral pH (Stephen et al., 1996). In our experiment, sequences affiliated to *Nitrosospira* cluster 4 were ubiquitous and this cluster has been reported to be typical of temperate soils (Avrahami and Conrad, 2005).

4.1. Influence of warming

A warming of 3 °C (air temperature) did not influence nitrification potential, AOB species richness and community composition. This observation is in accordance with results of Bardgett et al. (1999), who showed that Gram-negative bacteria were largely unaffected by slightly increased temperatures (+2 °C). The 3 °C warming in our study has caused an increase in soil

temperature of 1.57 °C (De Boeck, unpublished data) which is probably within the range of daily/seasonal fluctuations and the temperature optima for soil microorganisms are assumed sufficiently broad to buffer them against such limited changes in soil temperature (Tinker and Ineson, 1990). Furthermore, potential impacts of warming might be masked by other confounding factors, such as decreased soil moisture (De Boeck et al., 2008), decreased stomatal conductance (Lemmens et al., 2006), lower photosynthetic rate (Gielen et al., 2007), and lower above- and belowground biomass (De Boeck et al., 2007), as observed in the present study. The quantities of roots exudates and the available source of N for microorganisms may therefore have been reduced and offset an impact of temperature.

4.2. Influence of plant functional groups

As homogenised soil was used for the construction of containers, initial soils characteristics were identical, and observed differences can be attributed to the influence of the plant functional groups. Plant functional groups influenced potential nitrification and AOB community composition after 16 months, but not after 4 months. As AOB oxidizer are slow growing bacteria (Belser, 1979), and as community composition may be resistant to change (Balser et al., 2001), responses operate at timescales in the order of months to years. For example, in a study of Avrahami et al. (2003), changes in AOB community structure were detected within 4 months of incubation, but not after 4–6 weeks.

Hierarchical cluster analysis revealed that AOB community structure was different under legumes. This was particularly evident 16 months after planting: legumes were clearly separated from all other samples in the dendrogram. This discrimination in AOB community structure could be explained by sequences related to cluster 3 and those affiliated with *Nitrosospira* Nsp 65. Within *Nitrosospira* cluster 3, Webster et al. (2005) indicated the existence of two subgroups, clusters 3a (including i.e. *Nitrosospira* NpAV and En284) and 3b (including i.e. *N. briensis* C-128 and *N. europaea*), respectively sensitive and tolerant to high ammonium concentrations. In our study, a sequence related to cluster 3b (SM8) disappeared under forbs and grasses, whereas a sequence related to cluster 3a (SM16) disappeared under legumes and in bare soils. We hypothesize that these changes could be linked to a difference in ammonium availability. The importance of ammonium availability is further supported by the results of Shen et al. (2008) who showed that N fertilization resulted in a dominance of cluster 3b. Furthermore, Junier et al. (2009) reported that the presence of the legume *Phaesolus vulgaris* promoted the presence of *Nitrosomonas* cluster 8, a cluster including species isolated from ammonium-rich soils. Increased soil N content through the addition of artificial root exudates may also cause a change in the genetic structure of the whole soil microbial community (Baudoin et al., 2003). Although we have detected no differences in extractable NH_4, legumes can, through biological nitrogen fixation and N root diffusion, facilitate the input of significant amounts of N into the rhizosphere (Whitehead, 1995). Furthermore, extractable NH_4 of bulk soil may not reflect NH_4 availability to microorganisms (Davidson and Hackler, 1994). In bare soils, ammonium availability would remain higher due to the absence of plant absorption. Although the basic metabolism is similar within all AOB, physiological differences with regard to NH_3 affinity exist between the distinct species (Koops and Pommerening-Röser, 2001). For example, species within the *Nitrosospira* genera exhibited affinity constants for NH_3 ranging from 6 to 11 μM (Jiang and Bakken, 1999). Moreover, some authors reported a change in AOB community structure following fertilization (Avrahami et al., 2003) and reduced MPN counts under high NH_3 concentrations (Phillips et al., 2000).

4.3. Relationship between AOB diversity and function

Whereas recent studies have shown that ammonia oxidation in soil can also be performed by ammonia-oxidizing archaea (AOA) (Leininger et al., 2006), few results have been published so far on the abundance of AOA in grassland soils and the role of AOA in soil N dynamics is still unclear. In the study of Leininger et al. (2006), higher abundances of AOA compared to AOB have been reported for most soils, except grasslands, where AOA abundances were similar to those of AOB (He et al., 2007; Leroux et al., 2008). The proportion of AOA to AOB is currently assumed to increase with increasing nutrient limitations (Leroux et al., 2008). However, it has been shown that the AOB abundances, and not those of AOA, were correlated with potential nitrification rates (Shen et al., 2008) and that the pattern of changing AOB gene copies and transcriptional levels correlated more closely with measured nitrification rates (Nicol et al., 2008). Furthermore, it is currently assumed that AOB have a higher transformation rate of ammonium to nitrite than AOA (Könneke et al., 2005). We therefore believe that AOB are likely to play an important role in the nitrification process in our nutrient-rich grassland soils and that the role of AOA should be explored in further studies.

The observation of altered soil ammonia-oxidizing communities and increased nitrification potential as a result of the presence of legumes provides a pathway for the effect of functional groups on N cycling in temperate grasslands. The change in AOB community composition and potential nitrification under legumes observed in our study is in accordance with the theory of broad/narrow process suggested by Schimel (1995), stating that a change in characteristics of the populations (involved in narrow processes) could have an observable impact on the process on ecosystem scale. In this study, higher potential nitrification under legumes was observed 16 months after planting, associated with a change in AOB community structure and a decrease in AOB richness. A negative relationship between potential nitrification and AOB richness was also observed in bare soils. Higher nitrification rates under legumes compared to grasses and forbs have also been observed by Hamer and Makeschin (2009). These authors could relate the increases to a rise in total microbial biomass and a change in microbial community structure. Results are also congruent with the observations of Habekost et al. (2008) who revealed a difference in microbial community composition and a higher proportion of Gram-negative bacteria in plots containing legumes compared to those without legumes. Higher ammonium availability, either due to the presence of N-fixing legumes or to the absence of N absorption by plants in bare soils, could be the key factor explaining these results. Increased ammonium availability may influence potential nitrification through two interlinked pathways: an increase in population size (physiological level) and and/or a shift in community structure (Fig. 4). The availability of ammonium is generally recognized as a limiting factor for nitrification (Belser, 1979), so that increased AOB population size as a consequence of higher substrate availability will lead to higher nitrification rates. On the other hand, higher ammonium availability can also operate at the community composition level, supporting the AOB most adapted to these soil conditions. This mechanism may therefore lead to the competitive exclusion of less efficient species and explain higher nitrification rates with a decrease in AOB richness (Fig. 4). This is in accordance with the general ecological theory on diversity–function relationship for grassland species (Tilman et al., 2001) and a study on arbuscular mycorrhizae (Egerton-Warburton and Allen, 2000). These studies reported that an increased resource supply can promote competitive exclusion. These explanations reflect the implications of microbial stress-response physiology for ecosystem function, described by Schimel et al. (2007). In our study, no changes in

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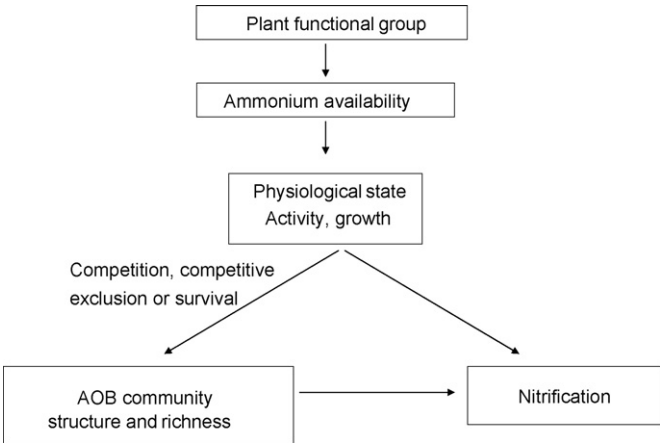

Fig. 4. Hypothetical links between ammonium availability, AOB physiology, AOB community structure and nitrification process in soils (after Schimel et al., 2007).

potential nitrification and AOB community structure were observed after 4 months. After 16 months, the concurrent increased potential nitrification, altered community structure and decreased AOB richness were observed, indicating that community structure may be an important driver of soil processes. This confirms the statements of Schimel et al. (2007) that '*shifts in community composition are likely to regulate soil communities and processes over longer periods*'.

4.4. Ecological consequences

Our results confirm the key role of legumes in ecosystem functioning, often demonstrated by increased aboveground production in the presence of legumes (De Boeck et al., 2008). We measured increased nitrate production under legumes, which could serve as substrate for other plant species (resource complementarity), but also lead to ecosystem N losses through denitrification and leaching. Under legumes, enhanced nitrification was related to lower AOB richness. For aboveground species, decreased richness is generally associated with a decrease in resilience, which could exceed a critical threshold of no-return. The decreased AOB richness under legumes might therefore lead to an increased sensitivity of the nitrification process to additional stresses, impacting the few remaining AOB populations. However, Wertz et al. (2007) showed that a decline of nitrite oxidizer and denitrifier diversity did not influence the resistance of these functional groups following a disturbance in temperature.

5. Conclusions

Results of the present study indicate that AOB function (potential nitrification), AOB richness and community composition were insensitive to a 3 °C increase in air temperature, but were significantly affected by plant functional groups. Our contribution highlights that the soil microbial community composition could be a major pathway of soil process responses to changes in the functional composition of plant communities. We observed modified AOB community structure, decreased AOB richness and concurrent increased potential nitrification under legumes. We hypothesize that the negative correlation between AOB richness and potential nitrification under legumes and in bare soils is driven by the impact of higher ammonium availability on AOB community structure and/or population size. This link could be explained by ammonium acting as a limiting substrate and/or through a selection of AOB populations with different ammonium affinity constants. This indicates that functional attributes of AOB are more important for ecosystem functioning than AOB richness and community composition. Therefore, the physiology of AOB, including so far uncultured organisms, is an important component which must be taken into account when studying AOB community structure and function. Future research could further be directed towards the AOA diversity and their contribution to nitrification in grasslands. This study illustrates that the link between aboveground and belowground diversity might be mediated through the impact of plants on resource availability, affecting soil microbial community structure. Climate warming may therefore indirectly impact soil functions through a change in plant community composition, stressing the necessity of multi-disciplinary research. The linkage between increased nitrification under legumes and its probable positive impact on plant productivity may provide an incentive for grassland managers for considering plant community composition and diversity in their management programs.

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