

## Research Article

# Responses of Ammonia-Oxidising Bacterial Communities to Nitrogen, Lime, and Plant Species in Upland Grassland Soil

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Agricultural improvement of seminatural grasslands has been shown to result in changes to plant and microbial diversity, with consequences for ecosystem functioning. A microcosm approach was used to elucidate the effects of two key components of agricultural improvement (nitrogen addition and liming) on ammonia-oxidising bacterial (AOB) communities in an upland grassland soil. Plant species characteristic of unimproved and improved pastures (*A. capillaris* and *L. perenne*) were planted in microcosms, and lime, nitrogen (NH<sub>4</sub>NO<sub>3</sub>), or lime plus nitrogen added. The AOB community was profiled using terminal restriction fragment length polymorphism (TRFLP) of the *amoA* gene. AOB community structure was largely altered by NH<sub>4</sub>NO<sub>3</sub> addition, rather than liming, although interactions between nitrogen addition and plant species were also evident. Results indicate that nitrogen addition drives shifts in the structure of key microbial communities in upland grassland soils, and that plant species may play a significant role in determining AOB community structure.

## 1. Introduction

The impact of anthropogenic activities on soil biodiversity is central to our understanding of the links between soil functional diversity, species diversity, and overall ecosystem functioning. Agricultural improvement of natural upland pastures is widespread in NW Europe, with increased fertilisation, liming, and grazing producing a shift in the floristic composition of acidic upland grasslands [1, 2]. Such intensification practices result in a gradual shift from a plant species-rich *Agrostis capillaris* pasture to a species-poor grassland dominated by *Lolium perenne* [3], with concurrent changes in soil physicochemical properties [1, 4, 5], most notably soil nutrient status. Nitrogen pools in particular have been shown to be held in different ratios between unimproved and improved grasslands, with ammonium dominating unimproved pastures, while nitrate is prevalent in improved pastures [5].

Soil bacterial and fungal communities are also understood to undergo changes in response to agricultural management [4–7], so it is likely that specific functional groups, such as ammonia-oxidising bacteria (AOB), may be similarly affected. The initial step in nitrification—the conversion of ammonium to nitrite—is microbially mediated by ammonia oxidisers via the enzyme ammonia monooxygenase (AMO). In recent years, exploitation of the *amoA* gene as a molecular marker and the application of community fingerprinting techniques have revealed considerable AOB diversity [8].

While previous studies have suggested that AOB community structure may be affected by land management regimes [9–11], the impacts of individual components of improvement (i.e., fertiliser addition, liming, floristic composition) and their interactions on AOB communities in grasslands are not well defined. This study investigates the responses of AOB to nitrogen and lime additions in upland grassland microcosms. Previous work has indicated that the general

rhizosphere bacterial community in acidic grasslands is impacted more by chemical treatments, particularly liming, than by plant species [6]. We hypothesise that the AOB community will be similarly unaffected by plant species. Due to the crucial role of AOB in nitrogen cycling, we expect that nitrogen manipulations will have a greater influence than liming.

## 2. Materials and Methods

Soil was collected from an area of unimproved *Nardo-Galium* grassland at Longhill, County Wicklow, Ireland, and used for microcosms as described previously [6]. Pots were filled with 80 g (dry mass) of sieved bulk soil, then planted with 20–25 seeds (Emorsgate Seeds, Kings Lynn, UK) of the chosen grassland species (*Agrostis capillaris* or *Lolium perenne*), and water content was adjusted to 35% (w/w). A set of unplanted pots was also included, and microcosms were harvested 75 days after visible germination of seeds. Both planted and unplanted pots were treated on Day 25 as follows: (1) no treatment (NT); (2) addition of lime equivalent to 5 tons ha<sup>-1</sup> (L); (3) addition of NH<sub>4</sub>NO<sub>3</sub> equivalent to 150 kg N ha<sup>-1</sup> (N); and (4) addition of both lime and NH<sub>4</sub>NO<sub>3</sub> as above (LN). Microcosms were destructively sampled on Day 75. Plant root and shoot material were removed, dried at 70°C for 7 days, and weighed. Due to the small size of the microcosms used and the high root density in pots, all soil was assumed to have been in contact with plant roots and was considered rhizosphere. Soil was sieved to <4 mm and stored at 4°C for less than 7 days for pH, microbial activity, and biomass analysis, and at -20°C for molecular analyses. Soil pH, nitrogen, phosphorus, and potassium were measured as described previously [6]. Total microbial activity was measured as triphenylformazan dehydrogenase activity [12] and was determined based on a modification of the method of Thalmann [13]. Total soil DNA was extracted as described by Brodie et al. [5]. Briefly, soil (0.5 g) was added to tubes containing glass and zirconia beads, to which CTAB (hexadecyltrimethylammonium bromide) extraction buffer was added. After incubation at 70°C for 10 min, phenol:chloroform:isoamylalcohol (25:24:1) was added and tubes were then shaken in a Hybaid Ribolyser at 5.5 m/s for 30 s. Following bead beating, tubes were centrifuged and the aqueous layer was removed and extracted twice with chloroform:isoamylalcohol (24:1). A further purification procedure was performed involving incubation with lysozyme solution (100 mg/mL) for 30 min at 37°C. Tubes were again centrifuged and the aqueous layer removed and further purified using a High Pure PCR Product Clean Up Kit (Roche Diagnostics GmbH, Penzberg, Germany) according to manufacturer's instructions. DNA was eluted in a final volume of 50 µL. Terminal restriction fragment length polymorphism (TRFLP) was carried out using the primer set *amo*-1F and *amo*-2R [14, 15], with the forward primer labelled with fluorescent dye D4. PCR reactions were performed in 50 µL volumes using PCR Master-Mix (Promega), 15 pmol of each primer, and ~10 ng extracted DNA, and subsequently purified using a PCR

product purification kit (Roche). Approximately 50 ng of PCR product was digested using restriction endonuclease *TaqI* (NEB) according to manufacturer's instructions [15]. One microlitre of desalted digests was mixed with 38.75 µL of sample loading solution and 0.25 µL of Beckman Coulter size standard 600. Terminal restriction fragment lengths were determined by electrophoresis using a Beckman Coulter CEQ 8000 automated sequencer, and analysed using the Beckman Coulter fragment analysis package v 8.0. Profiles were generated for each sample based on relative heights (abundance) of peaks. Peak heights for each terminal restriction fragment (TRF) were first converted into proportions of the total peak height of all TRFs for each replicate. TRFs that differed by less than 0.5 bp were considered identical and only fragments occurring in two or more replicates were used [16]. Each fragment was then considered a unique operational taxonomic unit (OTU).

The experimental design consisted of two factors: management type (4 levels, fixed) and plant type (3 levels, fixed) with four replicates. Data sets for soil chemistry, root and shoot biomass, and microbial activity and number of TRFs were analysed by one-way factorial analysis of variance (ANOVA) using Genstat v 6 (significance level:  $P < .05$ ), after being tested for normality. Multivariate statistical analyses were performed on *amoA* TRFLP profiles with Primer 6 package (Primer-E Ltd, UK), using standardised and transformed ( $\log(X + 1)$ ) TRFLP profiles and the Bray-Curtis similarity measure. Principal coordinate analysis (PCO) was used as an unconstrained ordination method to visualise multivariate patterns within each plant treatment. Analysis of similarity (ANOSIM) was used to investigate the effects of treatment and plant species on AOB community structure. ANOSIM was performed on Bray-Curtis similarity matrices. Comparisons of mean distances within treatments were used to calculate the ANOSIM  $R$ -statistic ( $R$ ), with  $R = 1$  indicating the populations were dissimilar, and  $R = 0$  indicating that populations were random. Permutational multivariate analysis of variance (PERMANOVA) was also carried out to investigate the effects of treatment and plant species on AOB community profiles, as this procedure can determine if interactions between treatment and plant species were significant. The SIMPER procedure (similarity percentage analysis) was used to identify those TRFs (OTUs) that characterised each treatment group (NT, L, N, LN) identified by cluster analysis [17].

## 3. Results

The soil used for these microcosms had a starting pH of 4.0–4.5. Results of microcosm soil physicochemical analysis are presented in Table 1 (modified from [6]). Soil pH varied significantly ( $P < .005$ ) with chemical treatment, most notably with a significant increase in soil pH after liming (to approximately a value commonly found in semi-improved grassland [5]), and a decrease in soil pH under N treatment (although more acidic than the original soil, this value was still within the normal range for an unimproved acidic grassland [5]). Addition of nitrogen typically reduces soil

TABLE 1: Soil, plant, and microbial characteristics for grassland microcosms. Nitrogen/Lime treatments,  $n = 12$ ; plant treatments,  $n = 16$ . Different letters indicate values that are significantly different. SED, standard error of the difference. Significance levels: \*\* $P < .01$ ; \*\*\* $P < .001$ . Parts of this table have been previously presented in the work by Kennedy et al. [6].

	pH	Root Biomass (mg per plant)	Shoot Biomass (mg per plant)	Microbial Activity (mg TPF g <sup>-1</sup> soil)	Nitrogen (%)	Phosphorus (mg P kg <sup>-1</sup> )	Potassium (mg K kg <sup>-1</sup> )
Nitrogen/Lime treatments							
No treatment (plant only)	4.23b	1.90a	1.94a	49.2b	0.62a	8.54a	140.7b
Lime	4.92d	1.64a	3.68c	158.0d	0.55a	11.88c	156.3d
NH <sub>4</sub> NO <sub>3</sub>	3.78a	3.81d	2.83b	8.7a	0.62a	9.84b	143.7c
Lime + NH <sub>4</sub> NO <sub>3</sub>	4.55c	2.72c	4.66d	109.9c	0.60a	12.67d	128.2a
SED	0.09	0.41	0.39	7.05	0.04	0.15	2.20
Plant species							
Unplanted soil	4.50b	N/A	N/A	101.9b	0.61b	7.88a	131.2b
<i>A. capillaris</i>	4.13a	0.79a	0.84a	38.3a	0.48a	11.96b	174.2c
<i>L. perenne</i>	4.48b	4.25b	5.73b	104.2b	0.70c	12.36c	121.4a
<i>P</i> -value	**	***	***	***	***	***	***
SED	0.09	0.49	0.48	11.47	0.04	0.13	2.85

pH, as ammonium is converted to nitrate (nitrification) [18]. Amendment with lime plus nitrogen raised pH to nearly the same level as lime alone, indicating that the neutralizing effect of lime overrode the acidifying effect of nitrogen. Plant species also altered pH, with lowest pH recorded in *A. capillaris* pots. Plant biomass varied with chemical treatment, with N resulting in higher root biomass across plant treatments, while liming appeared to have the biggest effect on shoot biomass. Plant biomass was significantly different ( $P < .001$ ) depending on plant species, with *L. perenne* producing significantly greater ( $P < .001$ ), root, and shoot biomass compared to *A. capillaris*. Microbial activity decreased significantly under N treatment, whereas lime application (both lime alone, plus lime with N) appeared to have a positive effect on microbial activity. Within plant treatments, a significant decrease ( $P < .001$ ) in microbial activity was noted in *A. capillaris* pots. Soil nitrogen (%), phosphorus and potassium values are also presented in Table 1.

ANOSIM  $R$ -statistics are presented in Table 2. AOB community structure was significantly affected by both chemical treatment (global  $R = 0.684$ ) and plant species (global  $R = 0.584$ ), with significance level at  $P < .005$ . The larger  $R$  values for chemical treatment indicated that treatment had a greater effect on AOB community structure than plant species type. PERMANOVA pairwise comparisons for AOB community structure showed significant effects ( $P < .001$ ) of all management treatments (L, N, LN), plant species, and their interactions on AOB community structure. In order to elucidate the main drivers of AOB community structure, PCO ordinations (Figure 1) were derived and showed marked separation of samples depending on chemical treatment. For both unplanted and planted soils, there was a general grouping together of NT and L treatments, suggesting a high similarity within TRFLP profiles under NT and L treatments. Figure 1(a) indicated that for unplanted soils, the addition of N selected for markedly different

TABLE 2: ANOSIM  $R$ -statistics for pairwise comparisons of chemical treatments (lime, nitrogen, lime + nitrogen) and plant species (unplanted, *A. capillaris*, *L. perenne*) on ammonia-oxidising bacterial communities. NT, no treatment. All comparisons were significant ( $P < .005$ ).

Treatment/Plant groups	$R$ -statistic
NT, Lime	0.382
NT, Nitrogen	0.819
NT, Lime + Nitrogen	0.708
Lime, Nitrogen	1
Lime, Lime + Nitrogen	0.852
Nitrogen, Lime + Nitrogen	0.535
Unplanted, <i>A. capillaris</i>	0.568
Unplanted, <i>L. perenne</i>	0.477
<i>A. capillaris</i> , <i>L. perenne</i>	0.755

AOB communities in comparison to NT and L treatments, while the LN interaction also resulted in a divergent AOB community, indicating a significant combined effect of N and L within these microcosms. Figure 1(b) showed that in *A. capillaris* planted soils, although the NT and L treatments were grouped together and considerably separated from N and LN, there was no definite difference between N and LN, as was seen in the unplanted pots. Figure 1(c) (*L. perenne*) followed a similar pattern to unplanted, with N and LN separating out from each other on the PCO ordination.

Figure 2 indicated broad treatment-driven shifts in AOB community structure, as determined from relative abundances of dominant AOB OTUs using the SIMPER procedure. The most abundant OTUs in N-treated microcosms were markedly different from NT and L pots, suggesting apparent selection for different AOB species depending on management factor. Interestingly, the four OTUs present in N treatment (Figure 2) were also present in LN treatment,

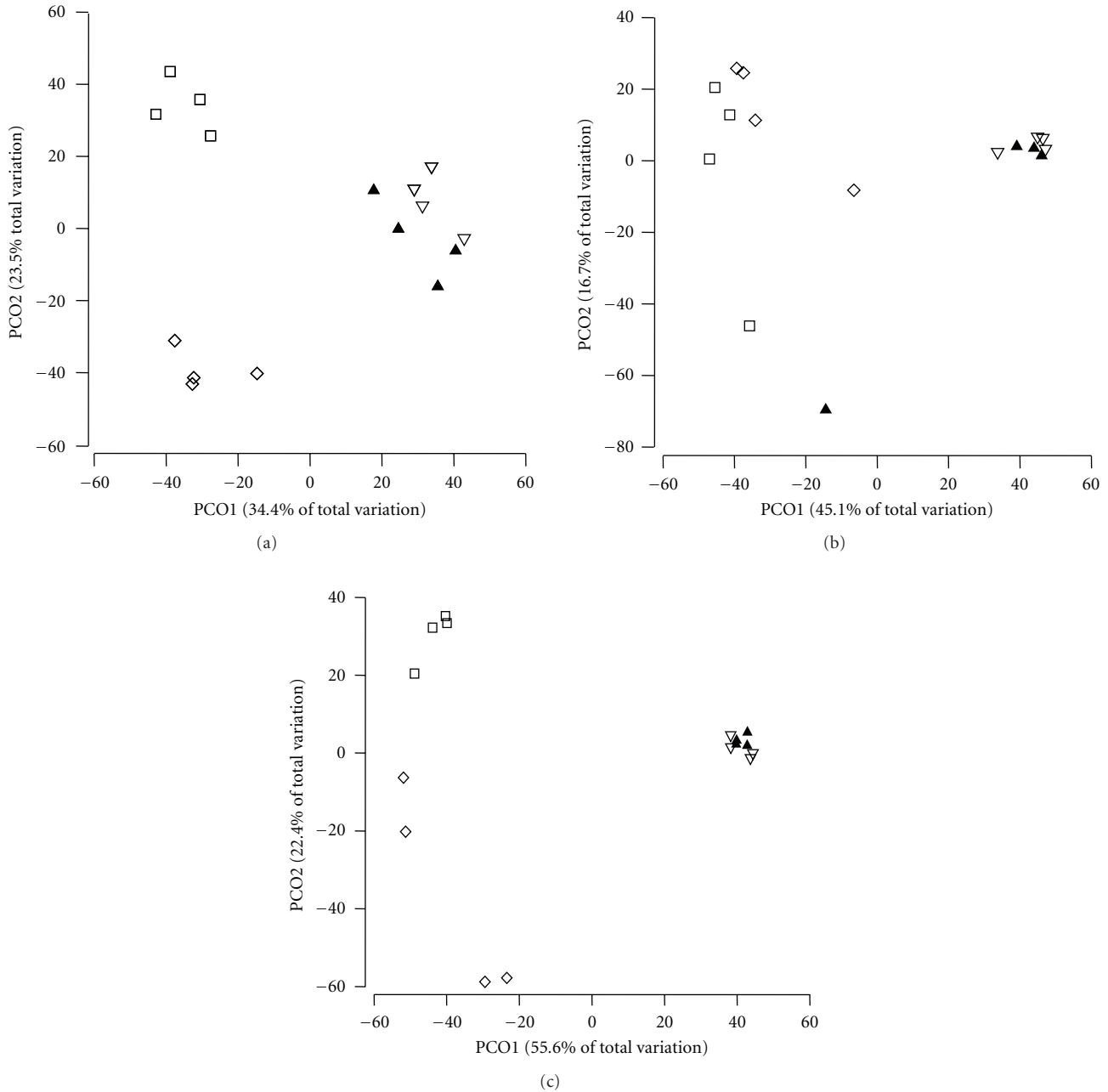


FIGURE 1: Principal coordinate analysis (PCO) plots of ammonia-oxidising bacterial community profiles examining management effects within each plant treatment. (a) unplanted soil, (b) *A. capillaris*, (c) *L. perenne*. Key: closed triangle, no treatment; inverted triangle, lime treatment; square, nitrogen treatment; and diamond, lime plus nitrogen treatment.

suggesting that N was very influential in determining AOB community structure. On the other hand, NT and L treatments were more similar in terms of AOB species composition. These data imply that N was dominant in driving AOB community structure. Table 3 gives the number of TRFs present under each treatment. Under NT, there was a slight but significant decrease in number of TRFs detected. There were no significant differences in number of TRFs between plant treatments in the L treated pots, while L, N, and LN treatments revealed some significant differences in TRF number. The most noticeable difference in number of

TRFs was in the N treatment and LN treatments, where a significant reduction in number of TRFs in *A. capillaris* and *L. perenne* pots, respectively, was found.

#### 4. Discussion

The main finding of this study was that nitrogen addition to upland grassland soil significantly influenced AOB community structure, and that the response was also dependent on which plant species was present. It has been shown

TABLE 3: Number of ammonia oxidiser TRFs (*amoA*) detected under each treatment and plant species. Different letters represent significant differences within a treatment,  $n = 4$ .

	No treatment	Lime	Nitrogen	Lime + Nitrogen
Unplanted	30a	42a	65a	57a
<i>A. capillaris</i>	30a	49a	3c	49a
<i>L. perenne</i>	20b	49a	10b	29b

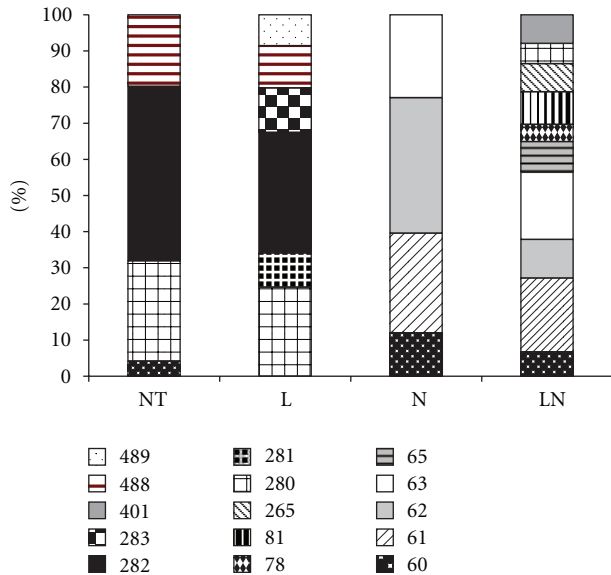


FIGURE 2: Relative percentage abundance of individual ammonia-oxidising bacterial OTUs occurring in the Top 10 most abundant OTUs within treatment. Top 10 most abundant OTUs were determined based on ranked relative abundances of OTUs within each treatment. No treatment (NT), lime (L), nitrogen (N) and lime + nitrogen (LN) treatments. Abundances are illustrated according to the key at bottom, with the corresponding TRF length (bp) listed alongside.

previously that nitrogen manipulations can lead to shifts in AOB community structure [19, 20], but the simultaneous effects of different plant species on AOB community structure have not been characterised. The results of this study present evidence for floristic and chemical interactions in grasslands selecting for distinct AOB populations. The ability of key functional communities in soils to respond to environmental influences suggests that overall functioning, in this case nitrification may persist over a broad range of ecological conditions.

In terms of plant growth responses to nitrogen and lime treatments in this study, *L. perenne* accounted for the majority of variance seen in root and shoot biomass measurements [6, 7], probably due to fundamental differences in growth morphology and root architectures between the two key plant species. *L. perenne* responds positively to N inputs in soil and it has been suggested that the root system of *L. perenne* may be competitively advantageous over other plant species when high concentrations of soil N are present [21]. In addition, high inputs of fertiliser N are

required to maintain the floristic characteristics of improved pastures, in which *L. perenne* dominates. The differences in the fundamental morphologies and physiologies of these two key plant species in response to nitrogen and lime manipulations, may result in concurrent shifts in rhizosphere microbial ecology, including AOB populations.

Liming of grasslands is routinely carried out during agricultural improvement, therefore, AOB community structure may also be influenced by lime-induced pH changes that are concurrent with improvement. Slightly higher soil pH induced by liming may favour certain microbial processes, hence, encouraging greater soil microbial activity. Figure 1 indicates that the presence of *A. capillaris* prevented the LN treatment from substantially altering AOB community structure in these microcosms, when compared to the unplanted control. Additionally, AOB populations under N and LN treatments on the *A. capillaris* ordination plot revealed similar AOB community composition. Interestingly, in both unplanted and *L. perenne* pots, AOB populations in N and LN treatments are markedly different, suggesting differential selection for AOB populations depending on interacting environmental factors. Several studies have indicated that selection for acid/alkaline tolerant AOB species may occur in natural environments in response to shifts in soil pH status [22–24]. As *A. capillaris* is known to have an acidifying effect on its rhizosphere [25], perhaps this phenomenon prevents lime from influencing soil pH in *A. capillaris* pots as compared to unplanted and *L. perenne* pots (Table 1). The results presented herein propose that liming and nitrogen applications to acidic grasslands may alter the structure of resident AOB populations, selecting for those that are better suited to the prevailing environmental conditions, such as variations in N availability (fertilised/unfertilised) or changes in pH, either as a result of liming or via addition of  $\text{NH}_4\text{NO}_3$ , which can acidify soil. In unimproved acidic grasslands, where there is higher floristic and chemical diversity [11], soil heterogeneity could lead to greater grassland AOB diversity. AOB diversity has been shown to be lower in improved grasslands [11, 26], while greater AOB diversity in unimproved soils is likely to reflect natural physical and chemical heterogeneity, emphasising the importance of soil physicochemical parameters in the determination of microbial community structure and functioning in general.

For unplanted and planted (both *A. capillaris* and *L. perenne*) soils, there was a high similarity in TRFLP profiles under NT and L treatments (Figure 1), suggesting that lime application alone did not markedly change the AOB community structure in these microcosms. Conversely, N is proposed as a dominant factor governing AOB community structure in this study, which supports previous studies [19, 20, 27–29]. Mendum and Hirsch [19] further suggested that  $\text{NH}_4\text{NO}_3$  application is specifically required for certain *Nitrosospira* clusters to become the dominant AOB population, perhaps indicating a substrate-driven selection for AOB populations, while Cavagnaro et al. [29] suggested that AOB in an agricultural soil was determined by  $\text{NH}_4^+$  availability, which selected for ammonia oxidizers that rapidly oxidise available  $\text{NH}_4^+$ . Additionally, in our study,  $\text{NH}_4\text{NO}_3$  could alternatively have induced AOB populations to respond

to a change in pH, selecting for AOB populations that have adapted to more acidic conditions, as brought about by  $\text{NH}_4\text{NO}_3$  fertilisation. This is further supported by Figure 2, which showed marked differences in AOB population structure under different treatments. Many recent studies on AOB communities have also found that AOB in terrestrial environments are more likely to be dominated by *Nitrosospira* species [30, 31], rather than *Nitrosomonas*. Extensive phylogenetic analyses of  $\beta$ -proteobacteria AOB from terrestrial environments have revealed the presence of a number of clusters within the AOB community, encompassing both *Nitrosomonas* and *Nitrosospira* genera [22, 32, 33]. It is possible that these phylogenetic clusters are a result of physiological differences in AOB populations that may allow certain populations to become dominant under different environmental conditions [20], but problems with culturing AOB make it difficult to prove this for certain.

While only two plant species were investigated in this study, natural acidic grasslands maintain a higher diversity of plant species, presenting a highly complex system in which it is difficult to generalise the effects of plant species. Additionally, the Crenarchaea have recently been revealed as important contributors to ammonia oxidation in terrestrial systems [33]. Although crenarchaeal ammonia oxidation was not investigated as part of this study, it is likely that AOB is only partially responsible for regulating ammonia oxidation in grassland soils, so caution must be expressed when evaluating the importance of findings such as those presented in this study. Nevertheless, AOB community dynamics are an integral component of overall ammonia oxidation in soil.

## 5. Conclusion

In conclusion, this study has shown that nitrogen status and plant species type, plus their interactions, play major roles in the determination of AOB community structure in upland acidic pastures. While much valuable data have previously been presented on the responses of soil AOB communities to changes in soil N status (e.g., [11, 28, 34–36]), often studies do not consider the combined effects of plant species type an important component of agricultural improvement. The data presented herein suggest that individual plant species may affect AOB communities differently in response to fertiliser treatment, adding to the complexity of grassland microbial ecology. Future work should aim to link ammonia-oxidiser community dynamics with soil properties and functional processes, including those regulated by plants, to gain a more comprehensive insight into the factors regulating ammonia oxidiser structure and nitrification dynamics in agricultural systems.

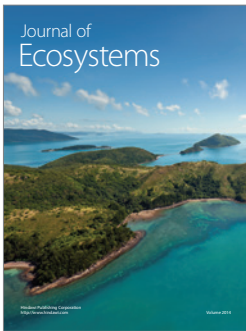
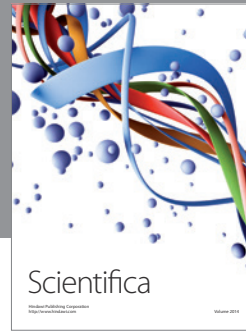
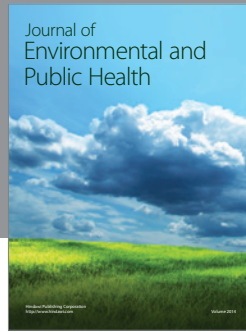
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