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Management versus site effects on the abundance of nitrifiers and denitrifiers in European mountain grasslands

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Abstract

It is well established that the abundances of nitrogen (N) transforming microbes are strongly influenced by land-use intensity in lowland grasslands. However, their responses to management change in less productive and less fertilized mountain grasslands are largely unknown. We studied eight mountain grasslands, positioned along gradients of management intensity in Austria, the UK, and France, which differed in their historical management trajectories. We measured the abundance of ammonia-oxidizing bacteria (AOB) and archaea (AOA) as well as nitrite-reducing bacteria using specific marker genes. We found that management affected the abundance of these microbial groups along each transect, though the specific responses differed between sites, due to different management histories and resulting variations in environmental parameters. In Austria, cessation of management caused an increase in *nirK* and *nirS* gene abundances. In the UK, intensification of grassland management led to 10-fold increases in the abundances of AOA and AOB and doubling of *nirK* gene abundance. In France, ploughing of previously mown grassland caused a 20-fold increase in AOA abundance. Across sites the abundance of AOB was most strongly related to soil NO_3^--N availability, and AOA were favoured by higher soil pH. Among the nitrite reducers, *nirS* abundance correlated most strongly with N parameters, such as soil NO_3^--N, microbial N, leachate NH_4^+-N, while the abundance of *nirK*-denitrifiers was affected by soil total N, organic matter (SOM) and water content. We conclude that alteration of soil environmental conditions is the dominant mechanism by which land management practices influence the abundance of each group of ammonia oxidizers and nitrite reducers.

Keywords:

nitrifiers, denitrifiers, land use intensity, mountain grasslands, soil nitrogen cycle

Introduction

Mountain grasslands cover a substantial proportion of the European land surface (Tasser et al., 2005, Quétier et al., 2007). Over the past few decades these marginal grasslands have undergone significant shifts in land management practices, involving both increases and decreases in the intensity of management, as well as complete abandonment. This variety of land-use trajectories is in contrast to lowland grasslands, where management has typically intensified. Changes in land management can have lasting effects on the composition of vegetation and the soil microbiome, with cascading effects on ecosystem processes (Bardgett and Leemans 1995; Robson et al., 2007; Schmitt et al. 2010; Legay et al. 2014; Harris et al. 2018) and ecosystem services (Lavorel et al., 2011; Grigulis et al., 2013). The nitrogen (N) cycle plays a key role in the provisioning of ecosystem services, both directly and indirectly (Galloway et al., 2004). However, the mechanisms by which changes in the soil microbiome are linked to shifts in soil quality and N cycling under different management trajectories in mountain grasslands are poorly understood.

In order to fill this gap, a network of European mountain grasslands was established across three sites in Austria, France and the United Kingdom (UK), each site comprising a gradient of agricultural management intensity representative of the farming practices for the respective region. Management trajectories included agricultural improvement in the UK, land abandonment in Austria, and extensification in France (Lavorel et al., 2017). Previous research using this network of sites indicates that both abiotic soil properties and plant traits influence the microbial processes related to N cycling across the sites (Grigulis et al. 2013; Legay et al. 2014). In the present study we explored the effects of land management changes on key drivers of the grassland N cycle. In particular, we analysed the effects on the abundances of N-transforming microbes that drive nitrification and denitrification, which determine, to a large extent, the availability of ammonia and nitrate in soil. We measured the abundances of ammonia oxidizing bacteria (AOB) and archaea (AOA) as a proxy for nitrifiers using the *amoA* gene as marker, and quantified denitrifiers using the two functional redundant nitrite reductase genes *nirK* and *nirS* as markers.

In lowland grasslands, the abundance of AOB is thought to be regulated primarily by inorganic N availability (Meyer et al., 2013, Di et al., 2009, 2010). AOB abundance has been found to increase with fertilization (Schauss et al., 2009, Hallin et al., 2009), while AOA abundance often showed no such response (Di et al., 2009, Shen et al., 2008, 2011). The abundance of AOA is more strongly influenced by other soil properties, including pH, moisture content and organic matter (Tourna et al., 2011; Stahl and de la Torre, 2012; Zhalnina et al., 2012; Stieglmeier et al., 2014).

Similarly to nitrifiers, population sizes of denitrifiers (*nirK*, *nirS* nitrite reducers) have been shown to respond strongly to environmental factors, such as the soil pH (Nicol et al., 2008; Enwall et al., 2010, Pereira et al., 2012), N concentrations (Prosser and Embley, 2002), plant species identity (Bremer et al., 2007), as well as management practices such as such as tillage (Attard et al., 2010) and grazing (Patra et al., 2005).

Much of the current knowledge on effects of management practices and land-use changes on nitrifiers and denitrifiers stems from lowland grasslands (Stempfhuber et al., 2014; Meyer et al., 2014), where intensification is the key trajectory of land-use change, and where background fertilizer inputs are typically higher than in the less productive mountain grasslands studied here. The aim of our study was to assess the effects of management intensity on the abundances of nitrifiers and denitrifiers for land-use trajectories typical for European mountain grasslands. Furthermore, the range of geographic, edaphic and climatic conditions covered by our study enabled us to explore the role of management versus environmental context on nitrifiers and denitrifiers. We hypothesized that land management change drives the population size of N converting microbes through a direct effect on the size of the inorganic N pools, and that higher inorganic N pools favour AOB abundance more than AOA abundance and increase *nir*-denitrifier abundance. We furthermore tested the hypothesis that soil organic matter content and pH affect the abundances of nitrifiers and denitrifiers, and that higher SOM content is associated with increased abundances of AOA and of *nir*-denitrifiers assorting to a heterotrophic lifestyle.

Methods

Sites and sampling

We studied a total of eight grasslands representing different levels of management intensity in terms of the level of fertilizer application, mowing frequency, and grazing intensity. The selected individual management forms represent typical types of grassland management in the investigated regions. The Austrian research site is located in the Stubai Valley and includes a mountain meadow (M) and an abandoned site (A). The French site is located on south-facing slopes in the Central French Alps and comprises of three fields; a fertilized mown terrace (FMT), an unfertilized terrace (UT) and an unmown meadow (UM). The UK sites are located in the Yorkshire Dales and cover a management gradient including an improved meadow (I), a semi-improved meadow (SI) and an unimproved meadow (UI). For details concerning the management of these sites and for information on their geographic location, climate, geology, soil and vegetation characteristics we refer the reader to Table 1. Photographs of the sites are provided as supplementary materials.

Soils were sampled within three plots (30x30 m) at each of the studied grasslands. In each plot, four replicate quadrats of 50x50 cm were established. At each quadrat four soil cores of the main rooting horizon (0-5 cm depth in A and 0-10 cm depth in F, UK) were sampled at peak vegetation growth phase in July/August 2010. Replicate soil cores (4) sampled within a quadrat were pooled, sieved to 5.6 mm and kept at 4°C for soil chemical analyses, or at -20°C until DNA extraction, respectively. In total 12 samples (3 plots x 4 composite soil samples) were taken for each type of grassland.

Soil chemical and physical properties

Soil abiotic properties including water content, bulk density, porosity, and pH were determined according to Schinner et al. (1996). Soil organic matter content was measured using 5 g dried soil samples (at 70°C for one week) after combustion at 550°C for 4 h; the soil organic fraction was determined as the mass loss during combustion (Schinner et al., 1996). Total C and N contents were determined in sieved soil aliquots, ground to a powder using a ball mill and analysed with a FlashEA

1112 elemental analyser (Fisher Scientific Inc., Waltham, MA, USA). For determining soluble inorganic (NH_4^+, NO_3^-), dissolved organic N (DON), and total organic carbon (TOC), 10 g of fresh soil was extracted with 50 ml of 0.5 M K_2SO_4 and shaken at 150 rpm for 1 h (Jones & Willett, 2006) . Soil TOC, NH_4^+-N, and NO_3^--N concentrations were determined colorimetrically (FS-IV colorimetric chain, OI-Analytical Corp., TX, USA). For measuring total dissolved nitrogen (TDN), extracts were digested with persulfate and subsequently subjected to analyses of NO_3^--N (Ross, 1992; Ameel et al., 1993). N-DON was calculated as the difference between N-TDN and inorganic N ($N\text{-}NO_3^-$ + $N\text{-}NH_4^+$). Inorganic N in leachates was used as a measure of bioavailable N. In each quadrat one extra soil core was taken and placed on a mesh inside a plastic funnel. A defined amount (between 100 and 150 ml) of distilled water was gently poured into the soil core, trickling through until the dripping stopped. Leachates were collected, filtered (Whatmann 42, 2.5 µm) and frozen at -20 °C until analysis of NH_4^+-N and NO_3^--N.

Microbial parameters

Microbial biomass N was analyzed using the chloroform-fumigation method according to Brookes et al. (1985). Microbial community structure was measured using phospholipid fatty acid (PLFA) analysis, as described by Bligh and Dyer (1959), adapted by White et al. (1979) and Bardgett et al. (1996). The fatty acids i150:0, a150:0, 15:0, i16:0, 17:0, i17:0, cy17:0, cis18:1ω7 and cy19:0 were chosen to represent bacterial fatty acids and 18:2ω6 to represent fungal fatty acids, allowing us to calculate the fungal to bacterial PLFA ratio (F:B) which is commonly used as a measure of the composition of microbial communities (Bardgett and McAlister, 1999).

Nitrifiers and denitrifiers were quantified using DNA directly extracted from soil samples, followed by a quantitative real-time PCR (qPCR) approach for marker genes of the respective group. Therefore DNA was extracted from 0.5 g of fresh soil using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Irvine, CA) and a Precellys24 (Bertin Technologies, France). After extraction, DNA was tested in quantity and quality with a spectrophotometer (Nanodrop, PeqLab, Germany). QPCR was carried out

on a 7300 Real-Time PCR System (Applied Biosystems, Germany) using SYBR green as a fluorescent dye. The Power SybrGreen Master Mix was obtained from Applied Biosystems, primers were synthesized by Metabion (Germany), and bovine serum albumin (BSA) and dimethyl sulfoxide (DMSO) were purchased from Sigma (Germany). Compositions of reaction mixes as well as detailed qPCR cycling conditions for the functional marker genes *nirK* (Braker et al., 1998), *nirS* (Michotey et al., 2000), bacterial *amoA* (Rotthauwe et al., 1997) and archaeal *amoA* (Leininger et al., 2006) are provided as supplementary material.

Dilution series of the DNA extracts were tested in a pre-experiment to avoid inhibition of PCR, resulting in an optimal dilution of 1:64 for all samples. Serial plasmid dilutions of the respective genes ranging from 10^1 to 10^6 gene copies μl^{-1} were used as standards. All PCR runs started with an initial step for enzyme activation and pre-denaturation at 95 °C for 10 mins. To confirm the specificity of the amplicons after each PCR run, a melting curve and a 2 % agarose gel stained with ethidium bromide were conducted. The efficiencies (Eff) of the amplification were calculated from the standard curve with the formula Eff = $[10^{(-1/slope)} - 1] * 100$ % and resulted in the following values: AOB 93-95%, AOA 91–98%, *nirS* 99–100%, *nirK* 94–99%.

Data analyses

The effects of grassland management intensity on the abundance of nitrifying and denitrifying microbes were tested by comparing the different degrees of management intensity within each site. As many of these variables did not meet the assumptions of normality and homogeneity of variance required for the use of parametric tests, these analyses were carried out using non parametric tests: The Kruskal-Wallis test (with post hoc pairwise Mann-Whitney U tests with Bonferroni corrections used for testing differences between treatments) was used to compare the three treatments at the French and UK sites, and the Mann-Whitney U (Wilcoxon rank-sum) test was used to compare between the two treatments at the Austrian site. All tests used a significance level of p=0.05. Sites were treated separately to take management gradients within sites into account. Analyses were carried out using the R software package (R Core Team, 2014).

Relationships between the measured variables and ammonia oxidizers (AOA, AOB) and nitrite reducers (*nirK*, *nirS*) were explored using a principal components analysis carried out by using the ade4 package (Dray et al., 2007), with biologically informative axes of variation being retained. Missing values made up less than 6% of the dataset and were replaced by the mean value of that sites variable across all (Peng et al., 2006). Correlations between parameters (Table 3) were calculated by means of multiple-variable analysis (Pearson correlations) at the $p<0.001$ and $p\leq0.05$ level. These analyses extend significantly beyond those of our previous studies (Grigulis et al. 2013, and Legay et al. 2014), which reported on fixed effects retained within multiple variable REML models, but did not report any absolute values nor any potential effects of grassland management.

Results

Abiotic soil properties

At the UK site, total N and C concentrations increased in soil from unimproved to improved and semi-improved grassland (UI<I<SI). In France, soil C and N concentrations did not differ between differently managed grasslands. In Austria, soil C and N concentrations were twice as high in the abandoned grassland compared to the managed grassland (Tab. 2). Soil C/N ratios of the unimproved site in the UK (UI) and the abandoned site in Austria (A) were significantly higher than those of the more intensively managed soils, whereas the opposite trend was observed in France. For Austria and France, soil organic matter content (SOM) was highest at the abandoned and unmown sites (A; UM), which contained up to 15% more SOM than alternative management types (M; UT and FMT). Total soil organic carbon (TOC) concentrations were 2 times higher at the improved (I, SI) compared to the unimproved site in the UK, whereas in Austria the abandoned site contained more TOC than the managed meadow soil.

Soil inorganic N concentrations (NO_3^--N + NH_4^+-N) increased with management intensity at the UK site (UI<SI<I) (Tab. 2). Soil NO_3^--N concentrations were highest at the most intensively managed grassland site (I), while the semi-improved grassland contained most NH_4^+-N (Fig. 1A, 1C). At the

Austrian sites, the abandoned site contained more inorganic N than the fertilized meadow; concentrations of NH_4^+-N were twice as high in the soil of the abandoned site compared to the fertilized meadow site, but NO_3^--N concentrations were distinctly lower (Fig. 1A, 1C). At the French sites, soil NH_4^+-N concentrations differ between the differently managed grasslands and NO_3^--N was highest at the intermediately managed unmown site UT.

The amount of leached NO_3^--N was higher at the more intensively managed sites in France and the UK (Fig. 1D), and amounted to 7% and 13% of the total NO_3^--N measured in the improved grassland (I) of UK and at the more intensively managed sites (UT, FMT) in France, respectively (Tab. 2). In Austria, a higher fraction of NO_3^--N was leached at the abandoned site (25%) as compared to the managed site (4%). The leached NH_4^+-N fraction was highest at the UK sites (Tab. 2), where almost two times more NH_4^+-N was leached from the improved and semi-improved sites (SI and I) compared to the unimproved (UI) site (Fig. 1B). Soil DON concentrations were related to management differences in Austria, where more DON was found in the abandoned as compared to the managed meadow soil, and in the UK, where DON was increased at the more intensively managed site (I).

Soil pH varied from 5.7 to 8.0 across all sites. pH decreased from abandoned to managed grassland in Austria, was greater in previously ploughed grasslands (UT, FMT) than in never ploughed, unmown grassland (UM) in France, and showed no overall management-trend in the UK (Tab. 2). At the Austrian and French site, soil water contents were 1.4- and 2 times higher in the abandoned or unmown soils (A, UM) compared to the more intensively managed soils; however, water holding capacity (WHC) within sites were similar (Tab. 1). At the Austrian site, soil porosity was 9 % lower in the managed compared to the abandoned soil, whereas in the UK and France variation in soil porosity did not vary significantly between the different management regimes.

Microbial biomass and bacteria:fungi ratio

Total fungal and bacterial PLFA were lowest at highest management intensity across all studied transects (Tab. 2). At the semi-improved site in the UK (SI) and the least managed site in France (UM) microbial biomass was 2 and 1.2 times higher compared to the more intensively managed soils,

respectively. At the Austrian site, microbial biomass was three times higher in the abandoned soil compared to the meadow soil.

Microbial biomass N increased with management intensity at the UK and Austrian sites; in the UK, microbial biomass N was more than twice as high in the soil of the improved sites (I and SI), while in Austria the meadow site contained 4 times more microbial biomass N than the abandoned site. The opposite trend was observed in France, where microbial biomass N in formerly ploughed FMT and UT soils was 2 to 4 times lower than in the unploughed soil (UM), which had a distinctly lower pH.

Fungi to bacteria ratios (F:B) decreased by a factor of two along the intensity gradient of management at the UK site (Tab. 2), whereas in France F:B was lowest in unmown grassland (UM) soil. The F:B ratio did not vary with management in Austria.

The abundance of nitrifiers and denitrifiers

Copy numbers of archaeal *amoA* genes were higher in soils of the more intensively managed sites in the UK and France, whereas AOA abundance remained unaffected by management intensity in Austria (Fig. 2). In the UK, AOA abundance increased with increasing management intensity by a factor of approximately 8, and in France AOA abundance was more than 20 times higher in soil of the previously ploughed sites (UT, FMT) relative to the unmown meadow (UM) soil. At the UT and FMT sites in France, AOA abundance was exceptionally high, being an order of magnitude greater compared to the other sites studied (Fig. 2A).

The abundance of AOB was lower and less variable than AOA in the grassland soils investigated. Bacterial *amoA* gene copy numbers increased with management intensity in the UK, were higher at the managed relative to the abandoned site in Austria, but showed no variation according to management in France. AOB abundance was greatest in the soil of the managed (M) than the abandoned (A) site in Austria, and in the most intensively managed site in the UK (Fig. 2B). The AOA/AOB ratio increased with management intensity at the French site, where the AOA/AOB ratio was 10-times higher in the most intensively managed (FMT), compared to the unmown meadow

(UM) (Tab. 2). At the intermediately managed (SI) site in the UK, the AOA/AOB ratio was twice as high compared to the unmanaged (UI) and intensively (I) managed sites.

In the UK, no differences in *nirS* gene copy numbers were observed across the management gradient, whereas at the Austrian site *nirS* gene copy numbers were 2.7 times more abundant in the abandoned compared to the managed soil (Fig. 3). At the French site, *nirS* gene abundance increased with management intensity in the order UM<UT<FMT. Across all sites, *nirK* gene abundance was greater than *nirS* gene abundance (Fig. 3). While along the UK transect, *nirK* gene copy numbers were highest in the more intensively managed soils (SI, I), in Austria and France *nirK* was more abundant at the least intensively managed sites (UM and A). The abandoned soil in Austria and the unmown meadow soil in France harbored 2 and 3 times more *nirK* genes, respectively, than managed soils (M, UT and FMT). The *nirK*/*nirS* ratio was generally highest in the Austrian grasslands, but did not vary with management (Tab. 2). Within the French transect at the unmown meadow (UM), the *nirK*/*nirS* ratio was 20- and 46-times higher than in the more intensively managed grasslands (UT, FMT).

Relationships between soil characteristics, N concentrations and marker genes for nitrifiers and denitrifiers

Amongst all environmental drivers, and across all sites, the abundance of the archaeal *amoA* gene correlated best with soil pH (R = 0.65) (Tab. 3, Fig. 4A), and was negatively correlated to soil water content, leachate NH_4^+-N concentration, SOM and microbial biomass N (Tab. 3). The abundance of the bacterial *amoA* gene was related to soil NO_3^--N concentration and inorganic N (Tab. 3). The abundance of the *nirK* gene correlated best with soil water content, SOM, and soil N and C, while it was negatively related to soil C/N, pH, and inorganic N (NO_3^--N + NH_4^+-N). The *nirS* gene abundance correlated best with soil NO_3^--N, leachate NH_4^+-N, and microbial biomass N, and correlated negatively with the F:B ratio (Tab. 3, Fig. 4B).

Discussion

In the mountain grasslands studied, agricultural management affected a range of soil abiotic properties, including N availability, pH, organic matter content and water availability. However, many soil properties, including total N and C concentrations and soil inorganic N concentrations as well as nitrifier and denitrifier abundances were not clearly related to management intensity. Responses of nitrogen-transforming microbes differed between the management transects in each country.

Drivers for AOA and AOB in managed mountain grasslands

It has been suggested that AOA and AOB differ regarding their preferences for abiotic factors, including pH, nitrogen content and soil organic C. AOB are typically more responsive to fertilizer application compared to AOA (Erguder et al., 2009, Wessen et al., 2011). In our study, AOB abundances increased with management intensity at two of the three studied sites (i.e., UK and Austria). In France, AOB abundance did not change significantly across the management transect, but tended to be higher at the intermediately managed (UT) site, where N in ammonium and especially nitrate tended to be highest. Previous studies identified several drivers for bacterial *amoA* abundance in lowland systems, including soil pH, microbial biomass, nitrate and total nitrogen content (Hayden et al., 2010). Among these factors, N availability related most strongly to the AOB abundance in our study, since the number of bacterial *amoA* genes correlated significantly with NO_3^--N and total inorganic N across all the grasslands investigated. This is in accordance with Che et al. (2018), who also found that soil NO_3^--N and inorganic N concentrations were positively correlated with bacterial *amoA* genes in Tibetan alpine meadows .

AOA abundance was also higher in the more intensively managed grasslands in the UK and France. While Keil et al. (2011) found a higher abundance of AOA in fertilized compared to unfertilized grasslands, which was associated with increased NO_3^- availability, in our study soil inorganic N concentrations were not always related to management intensity; as such, other soil parameters may been the more important drivers of the AOA abundances in the studied grassland

soils. For instance, while soil NO_3^--N concentrations were higher in the managed compared to the abandoned meadow at the Austrian site, concentrations of NH_4^+-N and DON were greater in the abandoned grassland site compared to the fertilized meadow. This latter response is possibly due to the accumulation of plant residue as particulate organic matter over the 29 years since abandonment (Meyer et al. 2012, Zeller et al., 2000), which has increased the amount and C/N ratio of soil organic matter (Table 2), potentially providing negatively charged binding sites for NH_4^+ cation adsorption (Tipping, 2002).

In contrast to AOB, there was no positive relationship between AOA abundance and N concentrations. Additionally, AOA and SOM were negatively correlated. This is in line with the recent finding that in mountain grassland AOA abundance is enhanced under increased soil organic matter contents (Che et al., 2017 and 2018). Along the French transect, the abundance of AOA was much higher at the terraced sites (UT and FMT). The soils of these grasslands were alkaline (pH 8) due to former ploughing and mixing of the soil with alkaline bedrock (Robson et al., 2007 and 2010). In these soils, AOA were between 20- and 529-times more abundant than in all other grassland soils investigated in our study. Thus, AOA abundance appeared to be driven by pH rather than by soil inorganic N availability or organic carbon content.

Until recently, the prevalent paradigm has been that AOA have a preference for low pH environments (Nicol et al., 2008; Erguder et al., 2009; Lu and Jia, 2012; He et al., 2012; Monteiro et al., 2014; Shen et al., 2014; Hu et al., 2013). This general notion is not supported by our findings: AOA abundance clearly increased with soil pH, indicating a potential importance of AOA in alkaline soils. In accordance with our findings, Gubry-Rangin et al. (2011) studied niche specialization of terrestrial AOA and reported archaeal *amoA* diversity and abundance to increase with soil pH. While the extremely high substrate affinity of the archaeal ammonia oxidase enzyme (Könnecke et al. 2014) may explain the advantage of AOA in acidic/oligotrophic environments, the mechanism by which AOA cope with alkaline conditions remains open. These findings imply a niche partitioning for pH within the group of ammonia-oxidizing Archaea (Tripathi et al., 2013). The clear dominance of AOA

under alkaline conditions was underlined by AOA/AOB ratios as high as 57 and 97, which clearly exceed the ratios (between 2 and 16) found previously in managed grasslands (Meyer et al., 2014).

AOA have generally been suggested to be better adapted to hostile conditions such as salinity (Venter et al., 2004), high temperatures (Reigstad et al., 2008), freezing temperature (Nakagawa et al., 2007), sulfidity (Beman and Francis, 2006), acidity (Nicol et al., 2008), as well as alkalinity, as indicated in the present study. This may be partly due to the archaeal lipid membranes which are nearly impermeable to ions and protons (Van de Vossenberg et al., 1998) and may prevent archaeal cells from the invasion of hydroxyl ions under alkaline conditions. Only few mesophilic archaeal ammonia-oxidizers have been successfully cultivated to date (Hatzenpichler, 2012), thus little is known about their biochemistry and about their preference of alkaline conditions.

While higher abundance of AOA does not necessarily signify their functional dominance over AOB (Prosser and Nicol, 2008), increased archaeal *amoA* copy numbers certainly indicate a higher potential for archaeal ammonia oxidation.

Drivers for nitrite reducers in managed mountain grasslands

As with AOA and AOB abundance, the response pattern of *nirK*- versus *nirS*-type nitrite reducers to management was strongly site-specific and driven by management effects on environmental parameters. At the UK site, *nirK* gene abundance was increased under more intensive management, whereas *nirS* gene abundance was unaffected. In contrast, *nirK* gene abundance at the French site was highest in the unmanaged soil, while *nirS* increased with management intensity, which was also reflected in different *nirK*/*nirS* ratios. Similar specific responses of *nirK*- versus *nirS*- type nitrite reducers were reported by Enwall et al. (2010), who hypothesized that they were due to differential habitat selection of the two groups of nitrite reducers. A further study by Keil et al. (2011) on low and high land-use intensity grasslands reported niche partitioning between *nirK*- and *nirS*-type nitrite reducers with pH as a selecting factor. Other driving factors such as copper, or the presence or absence of plants, which were not considered in this study, have been suggested (Hallin et al., 2009; Enwall et al., 2010). However, in our study, soil inorganic N concentration appeared to be a key factor

determining the abundance of *nirS* genes, which was related to NO_3^--N and leachate NH_4^+-N concentrations, as hypothesized. The *nirK* abundance correlated with soil total N content, but also responded to other soil parameters. For example, in Austria and France, *nirK* was most abundant in soils from abandoned or least managed grasslands, which were characterized by highest within-site SOM contents of 34% (A) and of 18.4% (UM) respectively, and at the sampling time both soils had highest within-site water contents at comparable water holding capacities. The enhanced water contents in these highly organic soils may have favored growth of *nirK*-nitrite reducers, which is consistent with the key role of soil water availability and lack of oxygen for denitrification (Firestone 1982; Tiedje 1988; Szukics et al., 2010). Soil water availability may be modulated by grassland management, which can indirectly affect the water balance, e.g. by altering biomass and its functional composition (Obojes et al. 2015) or the thickness of the litter layer (Quétier et al. 2007), which can store water and protects the soil from drying out (Meyer et al. 2012). The importance of these factors determining the availability of soil water was previously highlighted by Fuchslueger et al. (2014), who reported effects of drought and rewetting in Austrian mountain grassland soils. Moreover, we observed a relationship between the *nirK* gene abundance and the SOM content (and soil C), indicating that this group of denitrifiers responds to the availability of organic substrates more than *nirS* nitrite reducers. In a study by Attard et al. (2011), 81% to 92% of the variance observed for potential denitrification in differently managed sites was explained by soil organic carbon, together with water-filled pore space and nitrate. Since denitrification represents a facultative process performed by bacteria alternatively to O_2 respiration, population sizes need to be interpreted cautiously since a vast majority of bacteria is believed to be able to denitrify. Still, in our study the quantification of the respective population sizes indicated that, besides soil N, soil organic carbon and water-filled pore space affected *nirK* abundance, while variation in the *nirS* abundance correlated with N concentrations in mountain grasslands, indicating niche specialization of the two groups of nitrite reducers.

Conclusion

Our study of mountain grassland soils in three European countries has shown that, while both nitrifier and denitrifier abundance is broadly affected by land management intensity, their responses to management are group- and site-specific, and primarily influenced by soil abiotic properties. We found that among ammonia oxidizers, the abundance of AOA was favored by more alkaline conditions, while AOB abundance was preferentially related to soil NO_3^--N concentrations. Across sites, nitrite reducers of the *nirS* type dominated under N rich conditions, while their counterpart (nitrite reducers of the *nirK*–type) was most abundant in moist soils of high organic matter content. We conclude that management practices determine niche specialization of N converting microbes in mountain grasslands through their effects on soil properties and nutrient availability. How the observed changes in nitrifier and denitrifier abundances affect nutrient fluxes and *in situ* transformation rates needs to be studied in further experiments, which also measure activity parameters of the respective functional microbial groups, for example by using specific mRNA as a proxy.

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Legends to Figures

Figure 1. A) Soil NH_4^+-N concentration and B) leachate NH_4^+-N fraction C) soil NO_3^--N and D) leachate NO_3^--N fraction of the meadow (M), abandoned (A), unmown meadow (UM), unmown terrace (UT), fertilized mown terrace (FMT), unimproved (UI), semi-improved (SI) and improved (I) sites in Austria, France and the UK. Different lower case characters represent significant differences between treatments within a country (site), $P<0.05$; n=12 for each treatment. Error bars represent the standard deviation. Statistical tests were performed with the Kruskal-Wallis test (post hoc Bonferroni comparisons) for UK and F and the Mann-Whitney U (Wilcoxon rank-sum) test for AUT.

Figure 2. Functional gene abundances of A) archaeal ammonia oxidizers and B) bacterial ammonia oxidizers on the meadow (M), abandoned (A), unmown meadow (UM), unmown terrace (UT), fertilized mown terrace (FMT), unimproved (UI), semi-improved (SI) and improved (I) sites in Austria, France and the UK. Different lower case characters represent significant differences between treatments within a country (site), $P<0.05$; n=12 for each treatment. Error bars represent the standard deviation. Statistical tests were performed with the Kruskal-Wallis test (post hoc Bonferroni comparisons) for UK and F and the Mann-Whitney U (Wilcoxon rank-sum) test for AUT.

Figure 3. Functional gene abundances of A) type *nirS*-and B) type *nirK*-nitrite reducers on the meadow (M), abandoned (A), unmown meadow (UM), unmown terrace (UT), fertilized mown terrace (FMT), unimproved (UI), semi-improved (SI) and improved (I) sites in Austria, France and the UK. Different lower case characters represent significant differences between treatments within a country (site), $P<0.05$; n=12 for each treatment. Error bars represent the standard deviation. Statistical tests were performed with the Kruskal-Wallis test (post hoc Bonferroni comparisons) for UK and F and the Mann-Whitney U (Wilcoxon rank-sum) test for AUT.

Figure 4. Principal component analyses (PCA) of soil porosity, pH, SOM, soil C, soil N, soil C/N, soil water content, NH_4^+-N, NO_3^--N, inorganic N, leachate NH_4^+-N, leachate NO_3^--N, microbial biomass N, F:B, AOB, AOA, *nirK*, *nirS* including data from all study sites in Austria, France and the UK. A) Axes 1 and 2 represent 26 and 18% respectively of explained variation. Vectors represent parameters associated with the abundance of ammonia oxidisers (bacterial and archaeal *amoA*). B) Axes 1 and 2 represent 29 and 21% respectively of explained variation. Vectors represent parameters associated with the abundance of nitrite reducers (*nirK*, *nirS*); n=96 for each parameter.

Figures and Tables

Table 1. Site characteristics and soil properties of the abandoned (A) and the meadow (M) site, unmown meadow (UM), unmown terrace (UT), fertilized mown terrace (FMT), unimproved (UI), semi-improved (SI) and improved (I) sites in Austria, France and the UK. Abbreviations refer to 'above sea level' (a.s.l.), 'mean annual temperature' (MAT), 'mean annual precipitation' (MAP) and 'water holding capacity of soil samples' (WHC). For further details on the sites see Grigulis et al. (2013) and references therein.

country	**AUT**		**F**			**UK**		
site acronym	**A**	**M**	**UM**	**UT**	**FMT**	**UI**	**SI**	**I**
management type	abandoned	meadow	unmown meadow	unmown terrace	fertilised mown terrace	unimproved	semi-improved	improved
location	Stubai Valley, Kaserstattalm		Villar d'Arène, Central French Alps			Wensleydale Valley, Yorkshire Dales		
geographic coordinates	46° 55' to 47°15' N, 11° 6' to 11° 25' E		45°03' N, 6°24' E			54°18' N, 2°5' W		
elevation (m a.s.l)	1970	1850	2047	1840	1835	220		
MAT (°C)	3		3			7.3		
MAP (mm)	1097		902			1620		
bedrock	Siliceous		Mixture of colluviums dominated by calshists with eolian material			Carboniferous limestone		
soil type	dystric cambisol		brown soils			brown-earth		
WHC (%)	24.5	24.6	48.4	56.3	51.4	94.4	131.1	130.8
clay (%)	33.5	13.5	30.0	28.3	36.8	11.0	13.5	12.9
silt (%)	30.3	44.1	46.6	44.7	45.8	47.6	38.0	32.1
sand (%)	36.1	42.8	23.4	27.0	17.4	41.4	48.5	55.0
vegetation type	*Seslerio Caricetum*	*Trisetum flavescentis*	*Centaureo uniflorae-Festucetum spadiceae*	*Mesobromion erecti / Seslerio caeruleae-Mesobromenion erecti*	*Triseto-Polygion bistortae*	Anthoxanthum – Geranium sylvaticum (MG3)	Lolium perenne – Cynosurus cristatus (MG6)	Lolium perenne – Alopecurus pratensis (MG7)
fraction of legume biomass (%)	1.8	2.4	0.1	14.1	14.6	0.8	1.6	0.6

management details	abandoned since 1983	cut once/season fertilised with manure every 2-3 yrs grazed in late summer	no history of cultivation unmown, summer grazed (1 day of livestock units/ha/yr)	unmown, grazed in spring and autumn (<2 days of livestock units/ha/yr), previously ploughed	fertilised (every 2-3 yrs), mown, previously ploughed	cut annually, low intensity grazing through spring	medium intensity grazing through spring, fertilised with manure every 2 yrs	ploughed, reseeded with *Lolium perenne* cut 1-2 times/yr, high intensity grazing through spring, fertilised with manure once a yr

Table 2. Soil characteristics and microbial parameters of the meadow (M) and the abandoned (A) site, unmown meadow (UM), unmown terrace (UT), fertilized mown terrace (FMT), unimproved (UI), semi-improved (SI) and improved (I) sites in AUT, F and the UK. Different lower case characters represent significant intra-site differences at P<0.05; n=12 for each treatment. Statistical tests were performed with the Kruskal-Wallis test (post hoc Bonferroni comparisons) for UK and F and the Mann-Whitney U (Wilcoxon rank-sum) test for AUT. *na* indicates that data were not available.

	AUT		F			UK		
	A	**M**	**UM**	**UT**	**FMT**	**UI**	**SI**	**I**
Soil characteristics								
Inorganic N (μg g^{-1})	11.7 a	7.1 b	14.5 a	21.1 a	15.1 a	8.4 c	16.6 b	21.7 a
Proportion of leachate NH_4^+-N of total NH_4^+-N (%)	3 a	2 a	3 a	3 a	2 a	8 b	13 ab	14 a
Proportion of leachate NO_3^--N of total NO_3^--N (%)	25 a	4 b	8 a	13 a	13 a	2 a	2 a	7 a
Total soil C (mg g^{-1})	135.2 a	69.0 b	82.5 a	90.0 a	90.1 a	40.7 c	90.0 a	68.8 b
Total soil N (mg g^{-1})	11.7 a	6.5 b	6.8 a	6.6 a	6.1 a	2.8 c	7.0 a	5.5 b
Soil C/N ratio	11.6 a	10.6 b	12.1 b	14.0 a	14.9 a	14.6 a	13.0 b	12.6 b
SOM (%)	34.0 a	18.4 b	18.4 a	14.4 b	13.2 b	11.1 c	26.3 a	18.7 b
Soil TOC (μg g^{-1})	152.7 a	122.2 b	*na*	*na*	*na*	81.5 b	175.1 a	163.0 a
Soil DON (μg g^{-1})	28.4 a	21.3 b	64.6 a	67.2 a	53.6 a	323.5 a	90.1 ab	17.1 b
Soil pH	6.4 a	5.7 b	6.3 b	8.0 a	8.0 a	6.2 b	6.9 a	6.1 b
Soil water content at sampling time (g g^{-1} soil)	0.73 a	0.54 b	0.35 a	0.18 c	0.22 b	0.29 b	0.49 a	0.33 b
Soil porosity (%)	86 a	77 b	78 b	83 a	79 ab	70 b	80 a	68 b
Microbial parameters								
Total PLFA (nmol g^{-1})	101.6 a	32.9 b	86.5 a	73.2 b	60.5 b	47.8 b	82.7 a	38.7 b
Microbial biomass N (μg g^{-1})	8.2 b	115.1 a	338.7 a	176.2 b	96.4 c	290.1 b	780.5 a	666.5 a
F:B ratio	0.09 a	0.09 a	0.07 b	0.1 a	0.09 ab	0.03 a	0.02 a	0.01 b
AOA/AOB ratio	2.8 a	3.1 a	10.6 c	56.6 b	96.8 a	2.4 b	4.9 a	2.3 b
nirK/*nirS* ratio	2211 a	2199 a	2970 a	155 b	136 b	136 a	222 a	179 a

Table 3. Pearson correlations between functional gene abundances and environmental parameters. Numbers given represent R values. Highlighted values are significant at the p<0.001 level, others at the p<0.05 level; n=96 for each parameter.

	AOA	**AOB**	***nirK***	***nirS***
Soil porosity				-0.37
pH	**0.65**		-0.36	
Water content	-0.37		**0.58**	
Inorganic N		0.25	-0.25	0.28
NO_3^--N		0.27		**0.44**
SOM	-0.25		**0.52**	
Soil N			**0.43**	
Soil C			0.25	
Soil C/N			**-0.50**	
Leachate NH_4^+-N	-0.27			**0.60**
Microbial biomass N	-0.31			**0.51**
F:B				**-0.56**

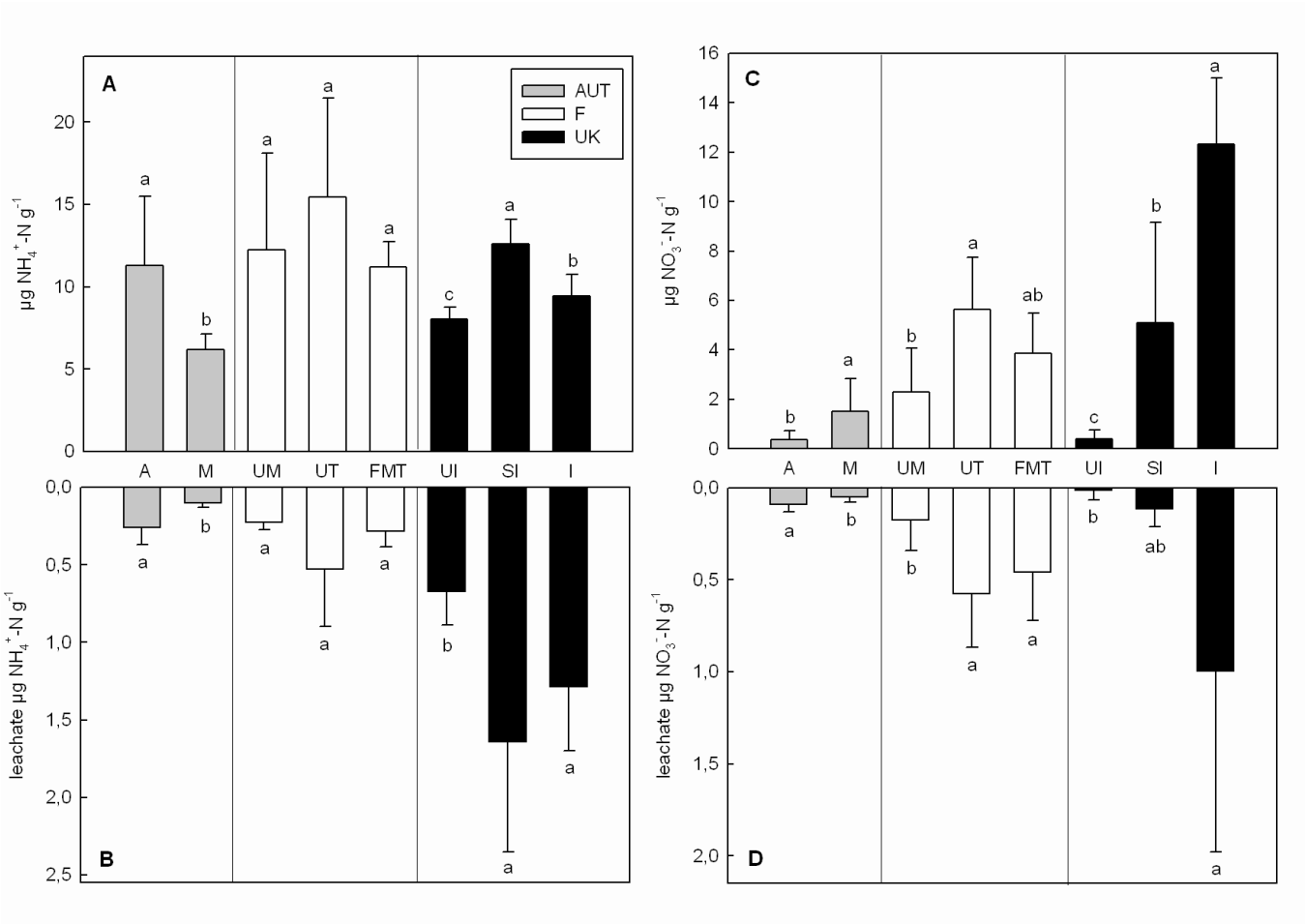


Figure 1

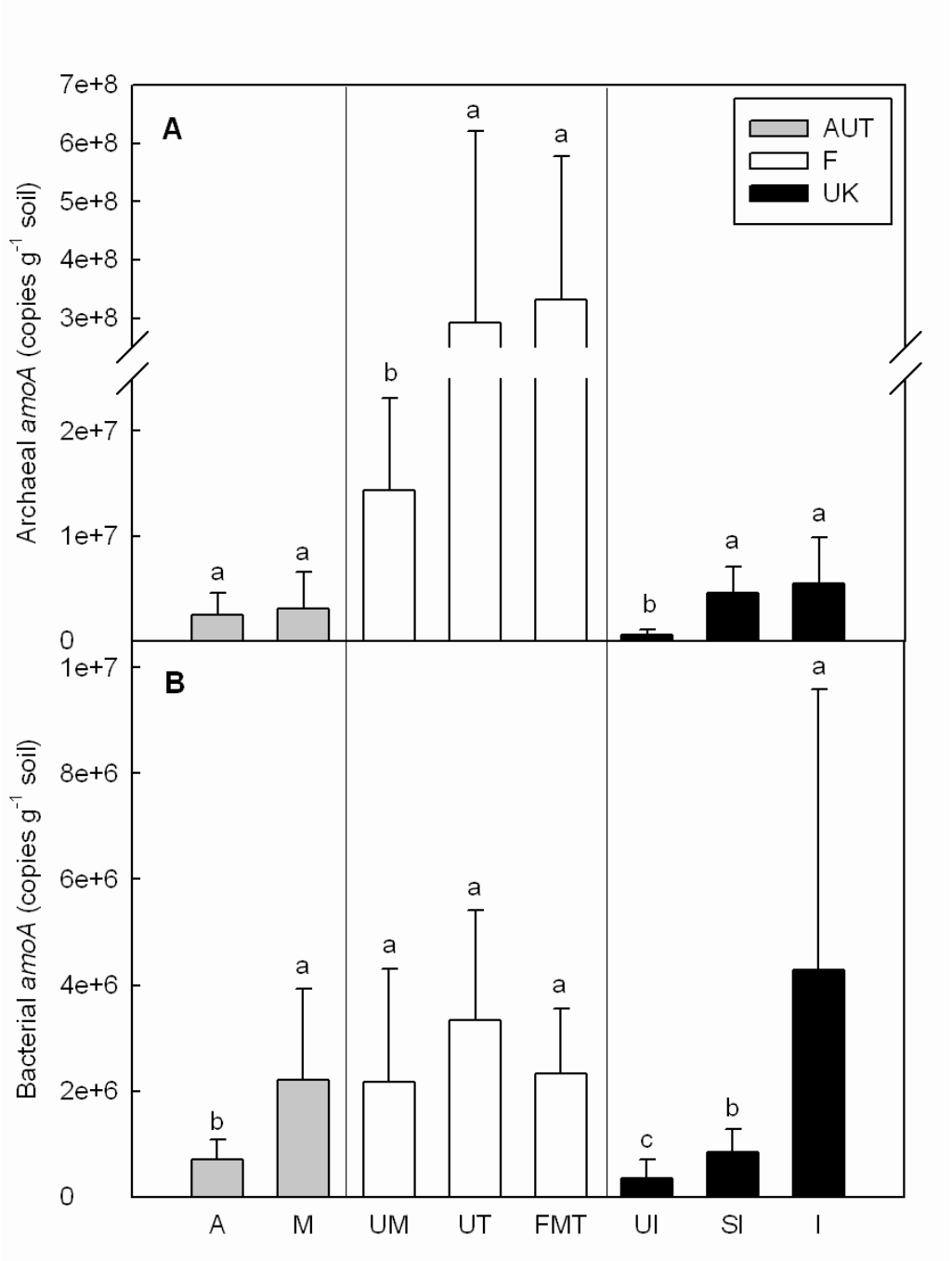


Figure 2

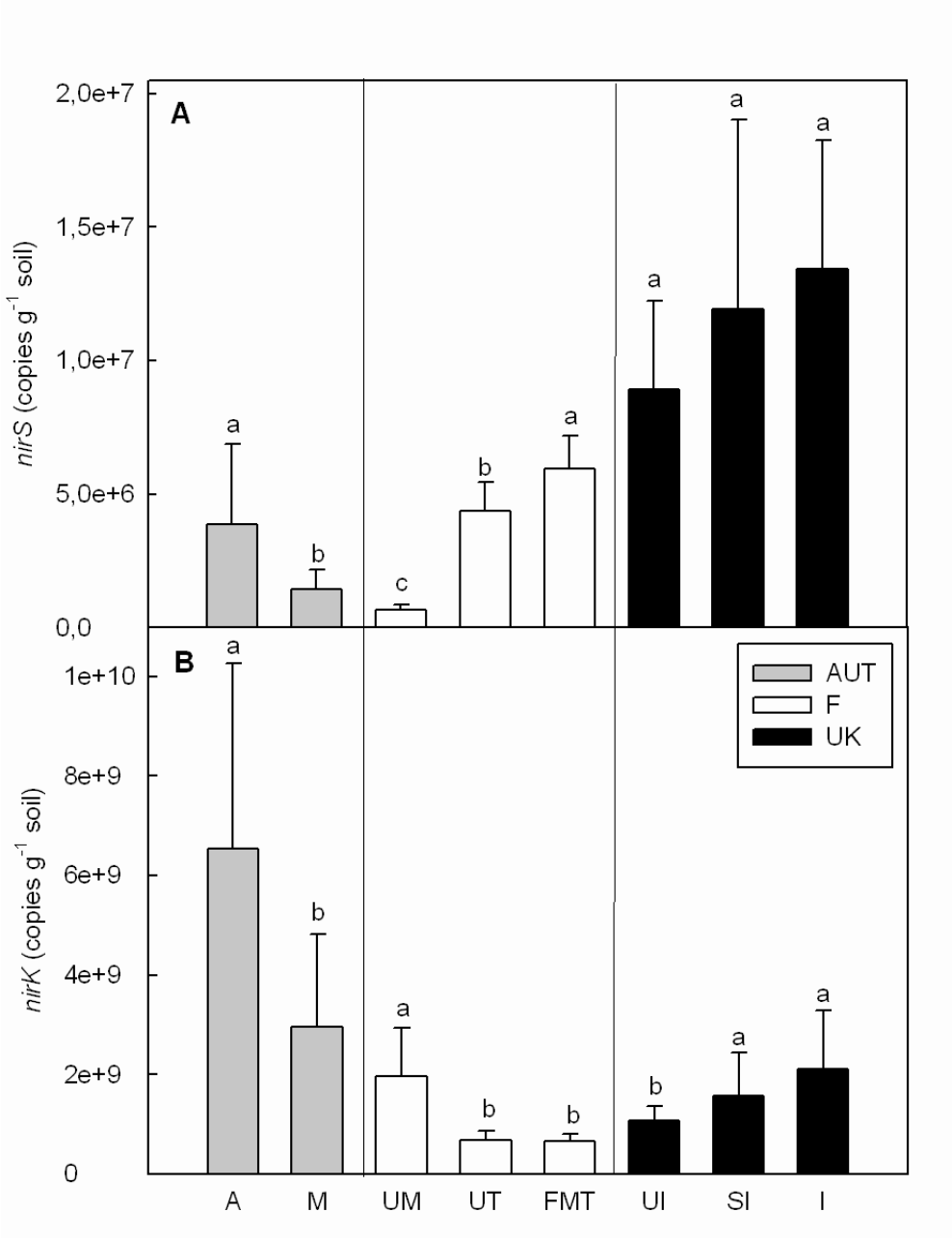


Figure 3

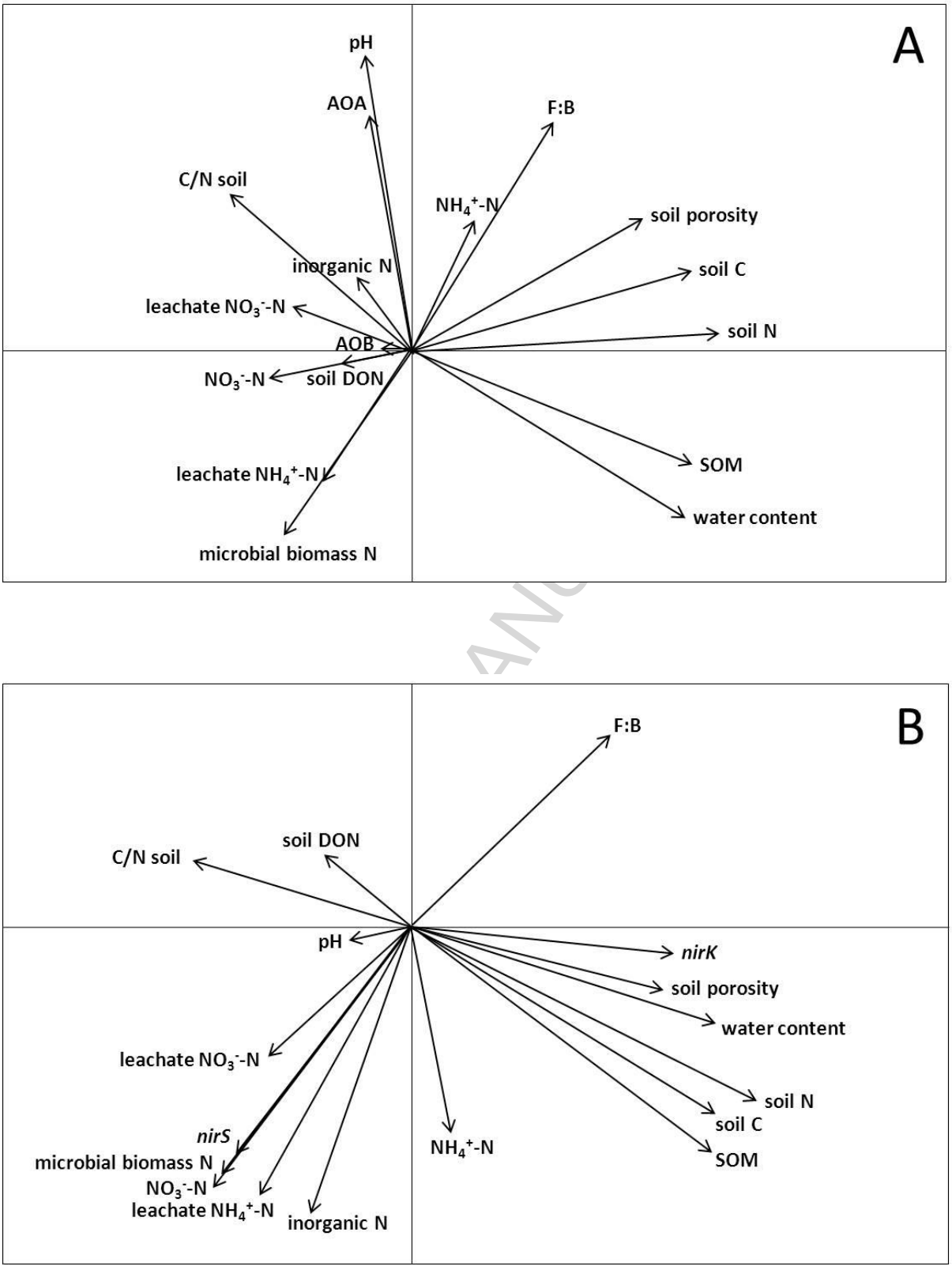
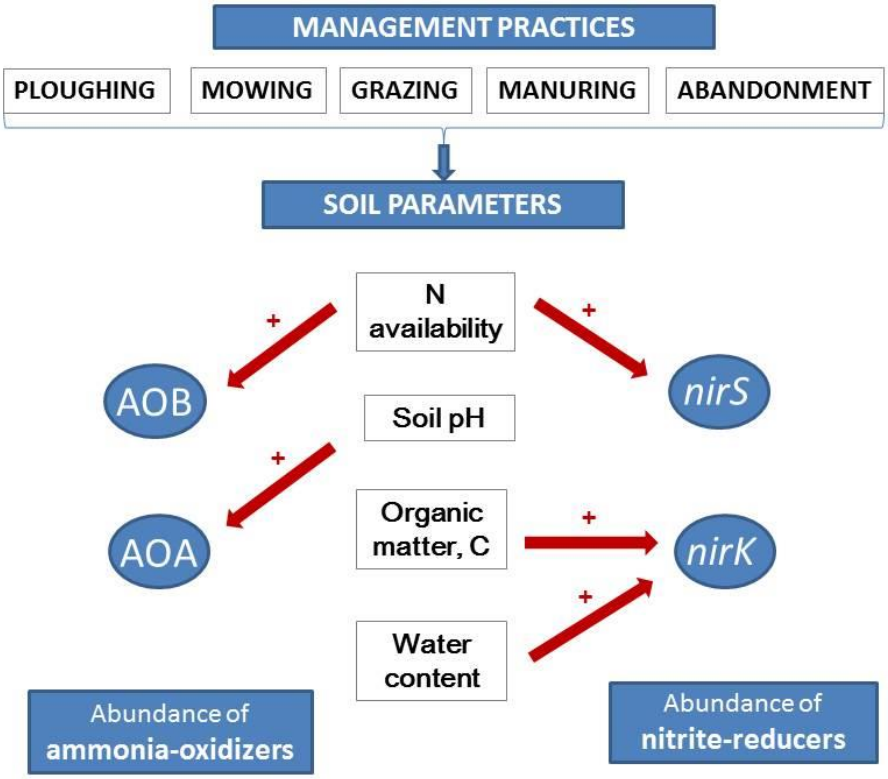


Figure 4

Graphical abstract



Highlights:

- The abundances of nitrifiers and denitrifiers differed across grasslands
- Site-specific conditions and management, but not its intensity, determined abundances
- Management responses within functional groups indicated niche partitioning